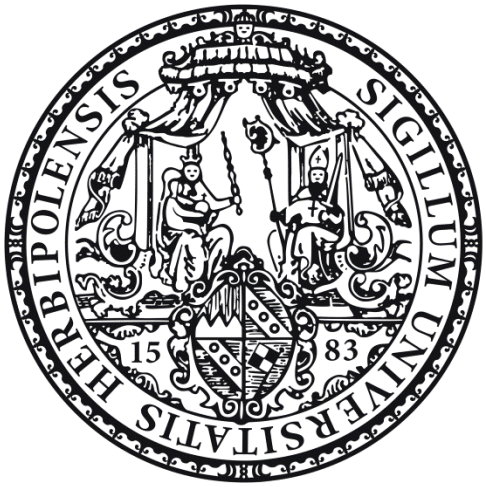
Development of diagnostic systems targeting the human tongue as a 24/7 available detector

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der

Julius-Maximilians-Universität Würzburg



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Doktorurkunde ausgehändigt am

„Eine der Lektionen, die ich in den verschiedenen Phasen meiner Karriere gelernt habe, ist, dass Wissenschaft nicht alleine gemacht wird. Fortschritte werden erst durch Gespräche und den Austausch mit anderen erzielt.“

Carol W. Greider (Molekularbiologin)

Die vorliegende Arbeit wurde in der Zeit von Oktober 2015 bis März 2019 am Institut für Pharmazie und Lebensmittelchemie der Bayerischen Julius-Maximilians-Universität Würzburg unter der Anleitung von Herrn Prof. Dr. Dr. Lorenz Meinel angefertigt.

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1. SUMMARY

To diagnose diseases correctly requires not only trained and skilled personnel, but also cost-intensive and complex equipment. Rapid tests can help with the initial evaluation, but result generation can also take up to several hours, depending on the test system. At this point, novel bioresponsive diagnostic systems are used, responding to the disease related shift of biological processes. They monitor changes in the biological environment and can react to them e.g. with the release of substances. This can be used in drug delivery formulations but can also help to diagnose diseases occurring in the oral cavity and inform patients of their state of health. The tongue is herein used as a 24/7 available detector.

In **section I** of this work, the foundation for the development of these diagnostic systems was laid. A suitable flavoring agent was found, which is stable, can be coupled to the N-terminus of peptides and has a strongly conceivable taste. For the optimization of the protease-sensitive linker (PSL), an analytical system was established (PICS assay), which determines protease-specific cleavable amino acid sequences. In order to replace the PMMA particles previously required, an acetyl protecting group was introduced N-terminally as it protects peptides and proteins in the human body from degradation by human aminopeptidase. The new synthesized flavor was examined with a NIH cell line for cytotoxicity and with an electronic tongue setup for its bitterness.

Section II deals with the structure of a system which detects severe inflammations in the oral cavity, e.g. PA. The established PICS assay was used to confirm the previously used PSL sequence in its application. Using solid phase peptide synthesis, 3 linkers were synthesized which respond to the elevated MMP concentrations present in inflammation. The resulting peptides were acetylated and coupled with HATU/DIPEA to the modified denatonium. Cutting experiments with MMPs over different concentration and time ranges confirmed the response of the diagnostic sensor to these enzymes. The obtained construct was examined for cell toxicity by WST assay. The masked bitterness of the sensors was confirmed by an electronic tongue setup.

To address non-human proteases (and thereby infections), **section III** focuses on the establishment of detection system of a cysteine protease SpeB expressed by *Streptococcus pyogenes*. The in-house expression of SpeB using *E. coli* cells was established for this purpose. An analysis of the SpeB cleavage sites was performed using a PICS assay setup. Four constructs with different PSL were synthesized analogous to section II. Cleavage experiments with the expressed and purified SpeB showed a response of two constructs to the

protease. In addition, a system was established to quantify the concentration of SpeB in human saliva using western blot technique with subsequent quantification.

In **section IV** a compound was synthesized which can now be coupled to a flavor. The final coupled construct is able to detect present NA activity specifically from influenza A and B. The market for existing influenza diagnostics was explored to determine the need for such a system. A neuraminic acid was modified in positions 4 and 7 and protected in such a way that subsequent coupling via the hydroxy-group in position 2 was selectively possible.

In summary, this results in a diagnostic platform that can be used anywhere, by anyone and at any time. This represents a new dimension in the rapid diagnosis of inflammations and bacterial or viral infections.

For additional information about this work, scan the QR-code below and view the “chewing-gum movie”.

2. ZUSAMMENFASSUNG

Krankheiten korrekt zu diagnostizieren erfordert nicht nur geschultes und ausgebildetes Personal, sondern zudem auch kostenintensive und komplexe Geräte. Schnelltests helfen bei der ersten Auswertung, können aber je nach Testsystem dennoch bis zu einigen Stunden in Anspruch nehmen, bevor ein Ergebnis vorliegt. An dieser Stelle werden neuartige, sogenannte bioresponsive diagnostische Systeme eingesetzt. Sie überwachen Ihre biologische Umgebung und können auf Veränderung dieser z.B. mit der Freisetzung von Substanzen reagieren. Durch das in dieser Arbeit entwickelte diagnostische System können Veränderungen im Mundraum erkannt und Patienten über ihren Gesundheitszustand in Kenntnis gesetzt werden. Hierbei wird die Zunge als 24/7 verfügbarer Detektor genutzt.

Im **Abschnitt I** wurde das Fundament zur Entwicklung dieser diagnostischen Systeme gelegt. Ein geeigneter Geschmacksstoff wurde gefunden, welcher stabil, koppelbar an Peptide und geschmacklich gut wahrnehmbar ist um ein positives Testresultat anzuzeigen. Für die Optimierung des Protease-sensitiven Linkers (PSL) wurde ein System etabliert (PICS-Assay), welches in der Lage ist eine Protease-spezifische Aminosäure-Schneidsequenz zu bestimmen. Um den im vorherigen System benötigten PMMA-Partikel zu ersetzen, wurde eine Acetylschutzgruppe am N-terminus eingeführt, welche die gleiche schützende Funktion wie ein solcher Partikel gegen den Abbau von Peptiden und Proteinen durch die körpereigene Aminopeptidase besitzt. Das gesamte Konstrukt wurde mit einer NIH-Zelllinie auf toxikologische Aspekte hin untersucht und die Maskierung des Geschmacks im ungeschnittenen Zustand mittels elektronischer Zunge überprüft.

Abschnitt II handelt vom Aufbau eines Systems, welches schwerwiegende Entzündungen im Mundraum, wie sie z.B. bei einer Parodontitis vorliegen detektiert. Der etablierte PICS-Assay wurde genutzt, die vorher verwendete PSL-Sequenz in ihrer Anwendung zu bestätigen. Mittels Festphasen-Peptidsynthese wurden drei Linker synthetisiert, welche auf die erhöhten MMP-Konzentrationen, welche bei Entzündungen vorliegen ansprechen. Die erhaltenen Peptide wurden acetyliert und mit HATU/DIPEA an das modifizierte Denatonium gekoppelt. Schneidversuche dieser Modelsysteme mit MMP‘s über verschiedene Konzentrations- und Zeitbereiche bestätigten das Ansprechen des diagnostischen Sensors auf diese Enzyme. Das erhaltene Konstrukt wurde mittels WST-Assay auf Zelltoxizität hin untersucht.

Um auch non-humane Proteasen, welche auf Infektionen hinweisen, adressieren zu können konzentriert sich **Abschnitt III** auf die Etablierung eines Nachweis-Systems der Cystein-Protease SpeB, welches von *Streptococcus pyogenes* exprimiert wird. Hierzu wurde die hauseigene Exprimierung von SpeB mittels *E. Coli* Zellen etabliert. Vom gewonnenen SpeB

wurde eine Analyse der Schnittstelle mittels PICS-Assay durchgeführt. Vier Konstrukte mit verschiedenen PSL wurden analog zu Abschnitt II synthetisiert. Schneidversuche mit dem exprimierten SpeB zeigten ein Ansprechen von zwei Konstrukten auf die Protease. Zudem wurde ein System etabliert um mittels Western Blot die Konzentration von SpeB im menschlichen Speichel zu quantifizieren.

Im **Abschnitt IV** wurde eine Verbindung synthetisiert, welche an einen Geschmacksstoff gekoppelt werden kann. Das gesamte diagnostische System ist im Stande Influenza-Viren nachzuweisen. Der Markt zur bestehenden Influenza Diagnostik wurde exploriert um die Notwendigkeit eines solchen Systems zu ermitteln. Eine Neuraminsäure wurde in Position 4 und 7 modifiziert und so geschützt, das nachfolgendes Koppeln über die Hydroxygruppe in Position 2 selektiv möglich wurde.

Zusammengefasst ergibt sich eine diagnostische Plattform, welche überall, von jedem und jederzeit angewandt werden. Dies stellt eine neue Dimension der Schnelldiagnostik von Entzündungen und bakteriellen oder viralen Infektionen dar.

Um mehr über die Arbeit zu erfahren, scannen Sie den QR-Code um den „Kaugummi Film“ zu sehen.

3. STATE OF KNOWLEDGE

3.1. Rapid oral test systems

3.1.1. History

The correct diagnosis of diseases is the first step in a successful treatment chain. The first reported diagnosis goes back to the years 2630-2611 BC. The Edwin Smith Papyrus proves medical diagnosis already at this time in ancient Egypt.[1]

Since then, a lot has happened in the diagnosis of diseases. Nowadays, diagnoses are no longer made only by questioning the patient about their symptoms and medical history, as well as conducting a physical exam of the patient. Diagnostic Tests, such as laboratory markers and imaging techniques have long found their way into modern medicine, for detecting, differentiating, and monitoring the patients state of health. Blood or urine are often examined diagnostic matrices with their qualitative and quantitative composition determined.

With a successful monitoring of diseases, especially in early stages, severe impact on a patient's health is prevented and/or succeeding complications delayed.[2] By gaining knowledge about the state of health of the patient, this can be utilized in selecting a therapy that could cure the disease, or alleviate the symptoms. The time needed for a correct diagnosis is a critical issue, because infections may lead to sepsis with a mortality rate close to 50%.[3]

In the last decades, interest in saliva as a diagnostic medium has increased exponentially.[4] This is mainly due to two advantages over blood and urine, which are still the most commonly used diagnostic body fluids.[5] The non-invasive extraction that can be done even by untrained medical personnel and the cost-effectiveness, which makes it an ideal screening tool for large populations. Children, anxious or handicapped people, for whom blood collection is a major obstacle, can be more easily tested for diseases. The risk of infection by saliva is much lower than the handling of blood. In addition, saliva is less complex than serum and is subject to less variability.[2, 6]

3.1.2. Saliva as a diagnostic medium

By definition, saliva is the secretion of the major salivary glands: glandula parotis (located opposite the maxillary first molars), glandula submandibularis, glandula sublingualis (both located in the floor of the mouth) as well as the 700 to 1000 minor salivary glands of the oral cavity and the oropharynx.[7] Each of these glands produces differently composed saliva resulting in a clear, slightly acidic liquid.[8, 9] In Diagnostics, the whole saliva (mixed saliva) is often used as a medium. It comprises gland specific saliva and gingival crevicular fluid (GCF),

expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris (**Figure 1**).[10] Of this "whole saliva", the healthy adult produces about 1.5 L/d at a rate of about 0.5 mL/min.[9]

Saliva is of crucial importance in the maintenance of a balanced oral flora, the predigestion of carbohydrates and fats by amylases and lipases, modification of food consistency for swallowing, lubrication of the oral cavity, defense against pathogens (bacteria, viruses and fungi), neutralization of toxins, buffering of food acids, the protection and repair of the oral mucosa and for this work most important, normal taste perception (**Figure 2**).[9]

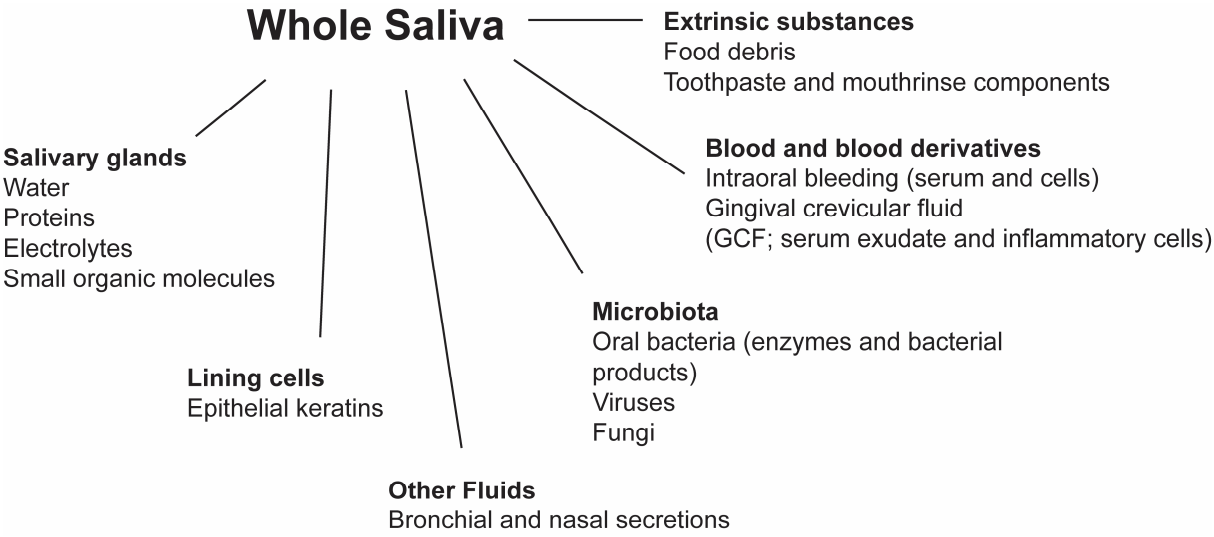

Figure 1. Different components of whole saliva. Adapted from Kaufman & Lamster, 2000.

Saliva is a complex matrix containing a broad spectrum of proteins and peptides, nucleic acids, electrolytes and hormones.[10, 11] However, the concentrations in which these biomolecules are present are much lower (about 1000 times) than in serum.[12] A transferability of saliva concentrations to serum blood concentrations is often not given. Recent proteomics analyses have identified more than 1000 salivary proteins and peptides, most of which can also be found in plasma. However, in some cases no correlation with plasma concentrations is possible.[13] Saliva secretion is also subject to a strong circadian rhythm and continues to depend on age, hereditary influences of oral hygiene and physical exercise.[14-16] The total amount of saliva as well as the qualitative composition is also influenced by physiological changes (depression, menopause, stress situations) and medication (e.g. psychotropic drugs, antihistamines, diuretics, cytostatic drugs).[17]

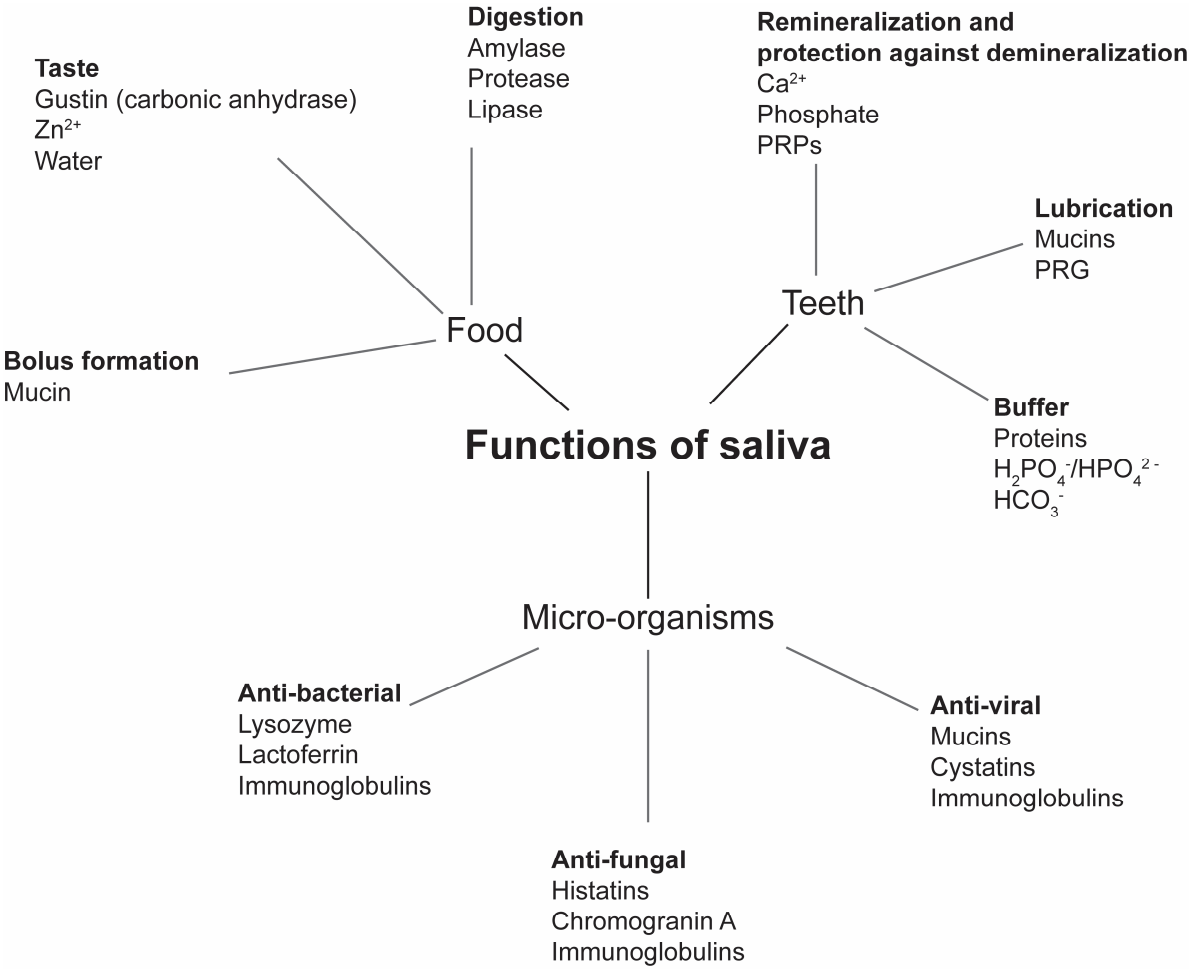

Figure 2. Physiological functions of saliva in relation to its constituents. Modified from Humphrey and Williamson, 2001.

The understanding of which diseases cause which changes in the parameters is, however, not yet given in every case. In recent years, however, intensive research has provided a great increase in information. By salivary-omics (genomics, transcriptomics, proteomics, metabolomics and metagenomics) disease-specific biomarkers were found. The pharmacokinetics of several drugs could also be traced in saliva.

Based on these results, numerous point-of-care tests (POCT), which use saliva as a diagnostic medium have been established on the market, which can be used for diagnosis of diseases, both oral and systemic (**Table 1**).

Table 1. Established rapid tests using saliva as diagnostic body fluid.

Product (company details)	Condition	Analyte	Principle	References
Strand Germline Cancer Test	Cancer risk assessment	DNA	Targets entire coding regions of the *BRCA1*, *BRCA2* and *TP53* genes for breast and ovarian cancer predisposition	[18]
Oral Fluid NanoSensor Test (OFNASET)	Determination of various diseases	Nucleic acids and protein	Polymer Micropatterned electrodes Electric-induced deposition	[19]
Ora Quick ADVANCE Rapid HIV-1/2 Antibody Test (OraSure)	HIV (Screening and risk assessment)	Protein	Detects antibodies to HIV-1 and HIV-2	[20]
Vigilant's OncAlert	Oral cancer screening	Hyaluronic acid, hyaluronidase and CD44	Detects specific protein markers	[21]
PerioSafe	Periodontal disease	aMMP-8	PoC/chair-side aMMP-8 lateral-flow immunotest	[22]
MyPerioPath®	Periodontal disease	Bacteria	Identifies type and concentration of specific perio-pathogenic bacteria that are known to cause periodontal disease	[23]
OraRisk® HPV	Oral Human Papillomavirus	Viral	Identifies the type(s) of oral HPV	[24]

3.1.3. Gustatory Perception

Gustatory perception is triggered by chemical components when they come into contact with taste receptor cells located on taste buds in the oral cavity.[25] These taste buds are a collection of 50-100 specialized epithelial cells. Taste receptor mechanisms can be divided into 2 general classes: the ion channel receptors, mediating salt and sour taste and the G protein–coupled receptors (GPCRs), implicated in sweet, bitter, and umami taste.[26-28] The family of GPCRs responsible for mediating bitter taste, the TAS2R family, comprises about 25 different taste receptors.[29] Molecules mediating sweet, bitter or umami taste bind to that receptor on the apical membranes specific for taste quality and trigger different intracellular transduction processes leading to depolarization. If a certain threshold is reached by the depolarizing sensor

potential, a transmitter is released that changes the action potential frequency in the afferent nerve fiber. However, taste results not from the receptors themselves but from downstream neural consequences of the activation of these receptors.

Through gustatory perception, we check whether our food contains indigestible or poisonous substances.[30] Natural bitter compounds are often known to be toxic, which is why the gustatory threshold of perception is very low, sometimes in the nanomolar concentration range. Quinine, which is the basis for the fixed bitter value, is already tasted by adults in concentrations of 8 µmol/l.[31] A large number of molecules are known for their bitter taste. These include organic molecules (e.g. polyphenols, flavonoids or methylxanthines), peptides, ions and salts.[32] In the case of bitter substances, no uniform basic structure can be identified; the molecular structures show a high degree of variability.

So far, only a few possible partial structures indicating bitterness have been found. Another difficulty is that there is a large number of bitter receptors, but little is known about which molecules associate with which receptors.[29, 33]

The alarming effect that bitter substances have on us is used in the household goods industry. By adding Denatonium (Den) benzoate (brand name Bitrex), an intensely bitter and non-toxic substance, the accidental ingestion of liquids (shampoos, detergents, soaps ...) by small children is prevented.[34, 35] Since rats do not have such a pronounced taste for Den, it is also added in rat poison. Chemically, Den is a quaternary ammonium compound and a derivative of the local anaesthetic lidocaine, which was quaternized with a benzyl group on the amine nitrogen. The human tongue can taste Den down to concentrations of 10 nM.[36]

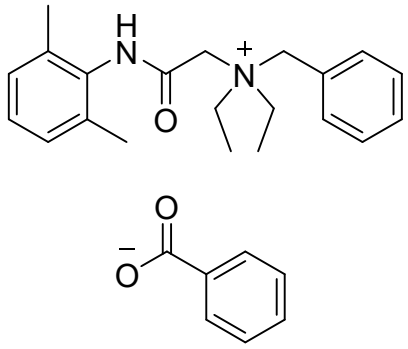

Figure 3. Chemical structure of Denatonium benzoate.

3.1.4. Bioresponsive systems in diagnostics

In recent years, bioresponsive systems have established themselves as the basis for rapid tests, i.e. systems that sense disturbances of homeostasis in their biological environment. They monitor changes caused by infections by viruses, bacteria, fungi, and parasites, tumors or other harmful artificial or natural agents, and traumatic damage and react to them by releasing detectable substances. Such existing systems are currently mainly used in oncology. Since cancer cells have a different milieu than healthy cells (pH, enzymes ...), Drug Delivery System (DDS) are designed that release cytostatic drugs only in the affected tissue and cause

few systemic side effects.[37] A DDS should avoid interactions with the biological environment in order to minimize the influence of possible side reactions.[38]

Numerous strategies for nanoparticle-based protease-responsive assays have been described. In most examples, the cleavage of certain peptides induces particle aggregation. A colorimetric assay for thrombin (a serine protease) was developed, using a peptide that crosslinked unmodified gold nanoparticles unless first cleaved by the protease.[39] Another approach was described, where Matrix metalloproteinase 2 and 7, both of which are associated with tumorigenesis, were detected by aggregation of nanoparticles.[40, 41]

The principle of bioresponsive release can also be exploited outside the systemic cycle. Negative influences such as inflammations or diseases of the mouth and throat caused by pathogens change the balance of the oral flora and release detectable substances.

3.1.5. Proteases

Proteases (also called proteolytic enzymes, peptidases or proteinases) constitute a large number of structurally and catalytically diverse enzymes that hydrolytically cleave the amide bond of proteins or peptides.[42, 43] Proteases alone account for about 2% of the human genome and their essential role in regulatory pathways makes them useful as prognostic indicators.[44] Because they play key roles in disease development they are further important targets for a large number of existing drugs as well as drugs that are still under development. According to the Enzyme Commission (EC) numbering, a numerical classification scheme for enzymes, they belong to main class 3 (hydrolases), subclass 4 (substrate: peptide bonds).[45, 46]

Table 2. Classification of proteases according to their catalytic mechanism.[47, 48]

Type	Functional group in the active center	Examples
Serine proteases	Serine	Thrombin, Plasmin, Trypsin
Aspartyl proteases	Aspartate/H_2O	Pepsin, Cathepsin
Cysteine proteases	Cysteine	Caspases, Streptopain
Metalloproteinases	Mostly Zn^{2+}/H_2O	Collagenases, Gelatinases, Carboxypeptidase A
Threonine proteases	Threonine/H_2O	Proteasome

This enzyme category can be subdivided into two major groups namely exopeptidases and

endopeptidases, based on the position of the cleaved peptide bond.[49] Exopeptidases are only active at the end of a polypeptide chain and typically remove a single amino acid (AA), but also di- or tripeptides from the end of the chain. Bonds within a protein cannot be cleaved by them. These include aminopeptidases (AP), for example.[50] Endopeptidases cleave peptide bonds preferably within a polypeptide chain. Depending on the catalytic mechanism, they are further subdivided into serine proteases, aspartate proteinases, cysteine proteinases, threonine proteinases and metalloproteinases according to their mechanism of action (**Table 2**).[47, 48]

In order to describe the substrate specificity and the binding of the substrate to the protease, Schechter and Berger introduced a nomenclature which describes the AA residues of the substrate with Pi, ..., P3, P2, P1, P1', P2', P3', ..., Pi', where the cleavage of the substrate takes place between P1 and P1'. Similarly, the corresponding binding sites on the enzyme are designated Si, ..., S3, S2, S1, S1', S2', S3', ..., Si' (**Figure 4**).[51]

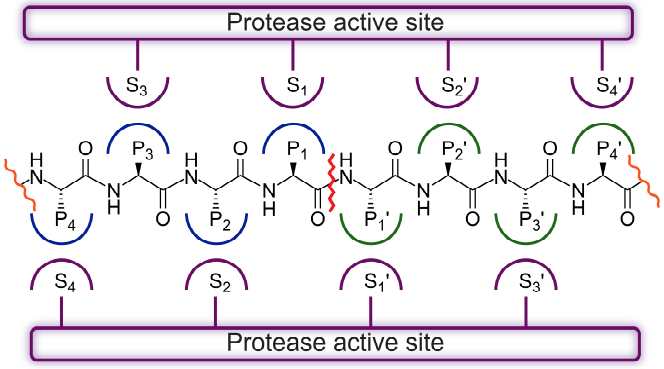

Figure 4. Schematic representation of the substrate binding sites. S4 - S4' of the protease and the AA residues P4 - P4' of the protein or peptide substrate according to Schechter and Berger. Cleavage of the substrate takes place between P1 and P1'.

Most proteases act as processing enzymes that carry out highly selective cleavage of specific substrates and influence cell behavior, survival and death.[52] Their specificity is dictated by the AA sequence of the polypeptide to be cleaved. Intracellular proteases degrade damaged or superfluous peptides in the cell and are essential for the transport of peptides to their site of action (cleavage of signal peptides by membrane proteases in membrane proteins or exported proteins).[53]

More important for drug delivery systems and bioresponsive diagnostics are proteases, which also exert their biological (catalytic) activity outside the cells. These proteases ensure the digestion of food or the activation of the blood coagulation system, the complement system and the fibrinolytic system. The secretion can be constitutive, i.e. continuous and constant, or regulated. This happens via the same mechanisms as for other proteins, i.e. with the participation of so-called signal or pro-sequences, which are usually 16-20 AA residues long.[54] During membrane passage, they are cleaved by signal peptidases, whereby the proteases are usually still present as inactive precursors (zymogens). The proteases reach their active form only after cleavage of the pro-sequence, which can act as a chaperone during folding[55]; and

as an inhibitor[56]. This proteolytic cleavage can take place autocatalytically or by other proteases.

Technological applications for proteases are found in industry, mainly in detergents, the baking industry, the dairy and beverage industry and also in the food industry. In the medical field proteases also find important direct applications, e.g. in the treatment of cardiovascular disease, sepsis, inflammation, cystic fibrosis, retinal disorders, digestive disorders, psoriasis and other diseases.[57] Indirect use is made of proteases in the production of therapeutics, e.g. for the C-terminal amidation of bioactive peptide precursors. They are also an important tool for research, often used in the analysis (biochemical characterization of proteins) and production of proteins.[58]

3.2. Periodontal disease

Periodontal disease is the most common chronic inflammation worldwide. Periodontal diseases prevalence varies in different populations and is further influenced by the employed methodology, including case definitions of periodontal diseases and recording protocols.[59, 60] Chronic periodontitis (PA) affects about 50% of the Europeans, and over 10% may suffer from an aggressive form, increasing up to 70–85% at 60–65 years of age.[61] It is expected that these numbers are increasing during the next decade in Europe.[62] Therefore, periodontal diseases are considered one of the most common diseases.[63] Chronic PA is a major clinical health problem due to its prevalence and consequences. Advanced periodontal disease may lead to root caries, compromised esthetics, impaired eating and socialization. It also may increase patients' risk of developing systemic diseases such as cardiovascular disease, rheumatoid arthritis, lung disease, diabetes mellitus and stroke.[64-67]

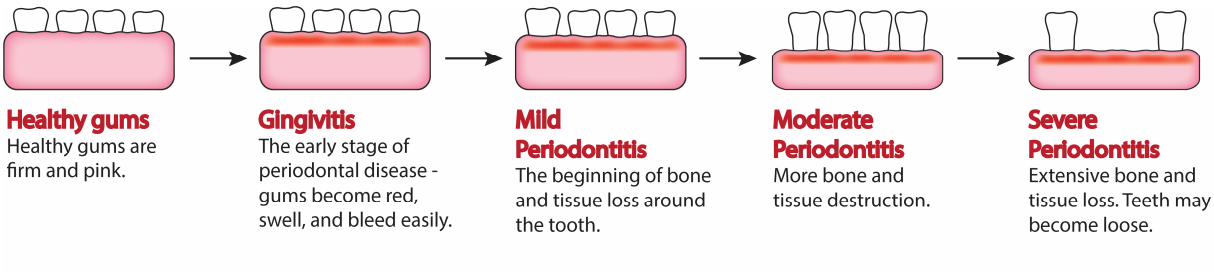

Figure 5. Stages of PA from healthy status to severe PA. Inflammation of the gums can progress affecting the bone that surrounds and supports the teeth. If not treated this can result in bone and tissue destruction and teeth loss.

PA is a polymicrobial infection-induced inflammatory process in the tissues around the teeth; this site is known as the periodontium and compromises all of its components. It is characterized by a progressive destruction of the tooth-supporting apparatus. Its primary

features include the loss of periodontal tissue support, manifested through clinical attachment loss and alveolar bone loss, presence of periodontal pocketing and gingival bleeding (**Figure 5**).[68] The severity of the PA is determined by various clinical measurements and indices including pocket probing depths, bleeding on probing, and assessment of clinical attachment level together with radiographic findings. Additional information such as medical, hereditary, specific features and the amount of dental plaque are also evaluated.[22, 69, 70] The diagnosis of a PA is therefore carried out by professional dentists, usually during routine checks. In the last 25 years, however, the use of biochemical parameters for the diagnosis of diseases has increasingly been started in order to expand the spectrum of classical diagnostics and to diagnose periodontal diseases more easily.

3.2.1. Current diagnostic approaches of periodontal disease

There are established tests for estimating the activity of parodontopathogenic bacteria (BANA test)[71] or for determination of the bacterial spectrum of the subgingival biofilm. On the host side, besides tests for the detection of a genetic risk (interleukin-1-2 polymorphism), tests that provide direct information about the inflammatory process on a local level - e.g. in the sulcus fluid - are of particular interest, be it as metabolic products (e.g. cytokines, degradation products of the matrix) or enzyme activities (e.g. myeloperoxidase, non-specific proteases, etc. enzymes).

The tissue loss associated with PA or peri-implantitis mainly affects the extracellular matrix and alveolar bone.[72] In both cases, the degradation of collagen plays a decisive role - manifested by periodontal attachment loss and in the case of bone degradation the destruction of the collagen-containing protective layer enabling access by osteoclasts.

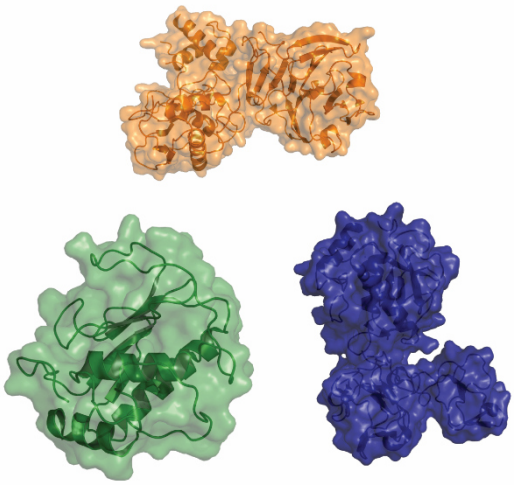

Figure 6. 3D structures of MMP-1 (green), MMP-8 (orange) and MMP-9 (blue).

Collagen forms an important class of insoluble fibrillar proteins. These are essential for the formation of connective tissue and the extracellular matrix. Under physiological conditions, there is a finely balance between tissue-building -degrading processes. An increase in concentration and activity of Matrix metalloproteinases (MMP) as degrading enzymes inevitably leads to tissue loss.

Permanent inflammatory stimuli induce the expression of a number of hydrolytic enzymes in epithelial cells, stromal cells and cells of

hematopoietic lineage. MMPs represent a structurally related, but genetically distinct, superfamily of proteases.[73, 74] They play a key role not only in physiological development and tissue remodeling but also in pathological inflammatory and malignant tissue destruction.[75-77] MMPs can be divided into five major groups: collagenases (MMP-1, MMP-8 and MMP-13); gelatinases (MMP-2 and MMP-9); stromelysins (MMP-3, MMP-10 and MMP-11); membrane-type MMP (MMP-14, MMP-15, MMP-16 and MMP-17); and others.[73, 78] Collectively, MMP can degrade nearly all components of the extracellular matrix and basement membrane, leading to periodontal tissue destruction.[79, 80]

Subantimicrobial doses of tetracycline derivatives, in particular doxycycline, are used in Great Britain and the USA for periodontal therapy (Periostat®, Alliance Pharmaceuticals Limited, Chippenham, Great Britain).[81, 82] Chemically modified tetracyclines are being developed to eliminate antibiotic activity while maintaining or even improving the inhibitory effect on MMPs.[81, 83, 84]

An immense number of publications have demonstrated that activity and expression of matrix metalloproteinases, are significantly increased in PA -affected gingival tissue and subsequently in gingival crevicular fluid samples, mouthrinse and salivary samples.[85-91] Based on these biochemical or immunological findings, an early diagnosis of periodontal diseases is possible by determining the MMP level in the oral cavity. This can be utilized for monitoring periodontal and peri-implant diseases via chair-side or POCT.[92, 93]

Recently, two lateral-flow POCT named PerioSafe (periodontal disease) and ImplantSafe (peri-implantitis), discovered in Finland and further developed in Germany[94, 95] have been developed based on earlier described technologies and monoclonal antibodies towards active MMP-8.[94-96] The tests, and reader for quantitative determination (ORALyser) have been developed and manufactured by Medix Biochemica Ltd (Espoo, Finland) and dentognostics GmbH (Jena, Germany) and are commercially available from dentognostics GmbH (Jena, Germany). In fact, these POCT resemble the classical pregnancy and/or recently described HIV-POCT.[22, 97]

Another POCT system developed for periodontal disease is based on diagnostic chewing gum, deploying the human tongue as a detector. The first proof of principle of this platform technology was for detecting inflammations in the mouth and throat, such as those present in peri-implantitis. The chewing gum contains sensors, which are *per se* tasteless. These sensors contains an engineered peptide sequence cut by a target protease, which is present in saliva in inflammation. This initial step kick starts further degradation of the sensor, resulting in the formation of bitter tasting substances alarming the patient. Thus this radical design replaces expensive detectors by a detector at no charge – our 24/7 available human tongue.

3.3. Infectious diseases of the respiratory tract

3.3.1. Type A influenza virus (IAV)

IAV belongs to the Orthomyxoviridae family of negative-sense, single-stranded, enveloped ribonucleic acid (RNA) viruses with a segmented genome.[98, 99] The Orthomyxoviridae family is divided into six other genera, which include influenza types A (IAV), B and C, Isavirus, Quaranfilvirus and Thogotoviruses, of which, however, only genera A and B are clinically relevant for humans.[100] IAV are subdivided according to molecular and serological characteristics of the nucleoprotein (NP), matrix protein 1 (M1) and matrix protein 2 (M2). The surface proteins haemagglutinin (HA) and neuraminidase (NA), differ greatly in their antigenic properties, classify IAV into further subtypes. To date, 18 HA (H1-18) and 11 NA subtypes (N1-11) have been identified. The nomenclature system is composed of the virus genus/host of origin (for a human isolate, often no information is given about the species)/geographical location of the first isolate/strain number/year of isolation and the virus subtype in brackets (e.g. human isolate A/California/7/2009 (H1N1)).[101, 102]

3.3.1.1. Replication cycle of IAV

The replication cycle of IAV describes the proliferation of virus particles in a host cell. This takes place in several steps (**Figure 7**).[103]

First, the virus binds via its HA to the sialic acid found on glycoproteins or glycolipid receptors of the host cell and is subsequently taken up into the cell interior via receptor-mediated endocytosis. This is followed by membrane fusion, unpacking and nuclear import of the viral ribonucleoproteins (vRNPs). Subsequently the viral genome is transcribed, replicated and necessary viral proteins for replication are synthesized. The last part of the replication cycle consists of the composition of the virus and the budding of newly formed virus particles.[101, 104] The replication cycle of the virus is only possible through various interactions with cellular processes and host proteins, which are also increasingly being investigated in order to develop novel therapeutic approaches.[105-107] Since this work focuses on the diagnosis of influenza by detection of NA, we will take a closer look on this step in virus replication.

Accumulation of viral surface proteins on the inside of the cell membrane causes the membrane to bulge out and budding of progeny virions occurs. Newly formed vRNPs are transported to the cell membrane in order to be integrated into the forming virions. The membrane budding is further extended, a new virus particle is formed which is still connected to the host cell. [108] In viruses, the viral envelope of the progeny virus always consists of the double lipid membrane of the host cell. The enzymatic activity of the NA of the progeny virus separates the bound sialic acids and thus releases the virus.[109] This separation of the sialic

acids prevents cross-linking, aggregation and binding of the progeny viruses to the already infected host cell. The replication cycle is completed with this step; the newly formed virus can now infect new host cells.[101, 110]

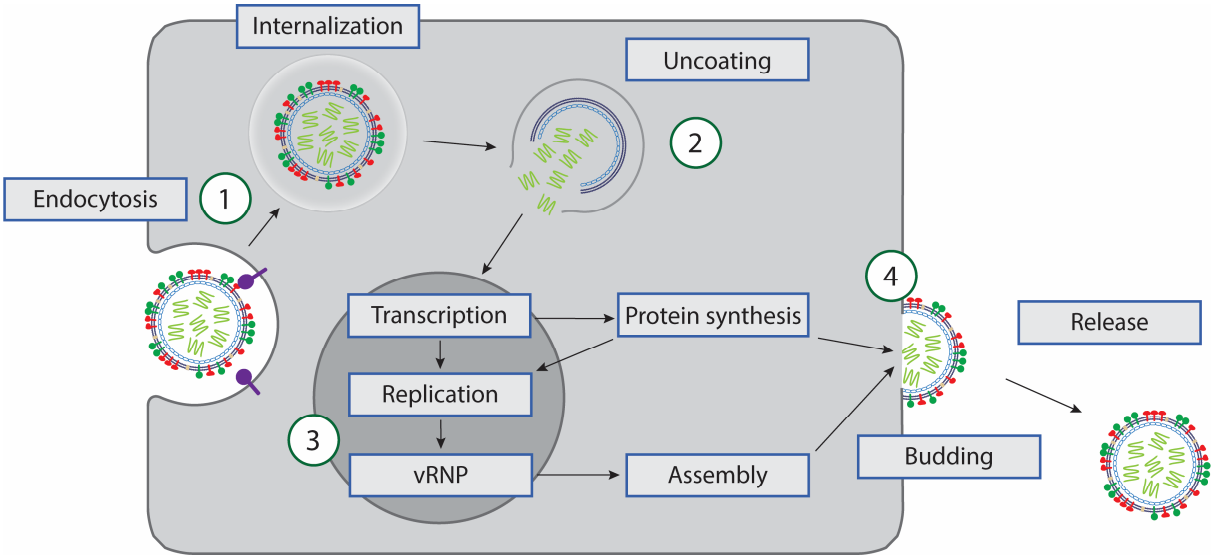

Figure 7. IAV replication cycle. The first step consists of adsorption [**1**] and receptor-mediated endocytosis for uptake of the virus into the host cell. The virus is then unpacked [**2**] and the vRNPs are transported into the cell nucleus. [**3**] This is followed by transcription, translation, replication and protein synthesis of the viral proteins and the assembly of progeny viruses [**4**] and their budding from the host cell (modified according to Shi *et al.,* 2014[103]).

3.3.1.2. Pathogenesis in humans

IAV are highly infectious and can be transmitted through aerosols, large droplets, or direct contact with secretions (or fomites), with large-droplet transmission being the predominant mode by which influenza virus infection is acquired.[111-114] By binding to the HA protein, IAV usually first infect epithelial cells in the upper respiratory tract (nose, throat and bronchi and rarely also the lungs), but infection via the ocular route is also possible.[115] Depending on the virus subtype, the viruses spread from there mainly in the lower respiratory tract, with average incubation times between 24 and 96 h.[116, 117] An IAV is commonly called “the flu” in humans. Typical symptoms of this genuine viral flu are a sudden onset of illness with headaches, severe muscle and limb pain, high fever and chills. These symptoms are triggered by the cellular infiltration of proinflammatory cytokines. As the infection progresses, Fatigue (tiredness), loss of appetite and body temperatures of up to 41 °C are observed. Symptoms like Diarrhea, nausea and vomiting during IAV infections were also documented, though this is more common in children than adults.[118, 119] Inflammatory reactions and the subsequent lysis of infected epithelial cells in the upper respiratory tree and trachea leads to typical flu symptoms like mucus formation, oedema, sore throat and dry cough.[120] In children, middle ear infections were

often observed in connection with IAV infections.[121] Normally, humans with IAV infections will recover in a few days to less than two weeks, usually resolving inconsequentially.[116, 119]

In rare cases, primary viral, interstitial and partial hemorrhagic pneumonia may develop during IAV infection. Such severe disease variants are usually triggered by infection with highly pathogenic avian influenza virus (HPAIV) or an epidemic or pandemic version of the IAV, patients with chronic pulmonary or cardiac disease, or diabetes mellitus or other persons belonging to the IAV risk groups.[101, 116, 122] Pulmonary capillary permeability increases due to destruction of the epithelium, resulting in pulmonary haemorrhages and oedema.[123] This often results in a hypoxemia, which if untreated can end lethal after a few days.[124-126] If IAV penetrates the bloodstream, there is also a risk of viremia.[127, 128] During infections with pandemic H1N1 viruses and HPAIV of the H5 subtype, cytokine storms (an potentially lethal overreaction of the immune system) were observed due to necrosis and pyroptosis of host cells.[104, 129] These cell death pathways allow for the rapid release of intracellular contents, including any viral components, from the infected host cell. This promotes host inflammatory responses and the formation of a cytokine storm which causes host tissue damage leading to multiple organ failure.[130, 131] Another risk during IAV infection is a secondary bacterial infection, which can contribute significantly to the development of pneumonia and further increases morbidity and mortality of influenza infection. *Streptococcus pneumoniae*, *Staphylococcus aureus* (*S. aureus*) and *Haemophylus influenza eden* can coinfect already virus-infected organisms. IAV aids secondary bacterial infection in a number of ways, including unveiling/providing more sites for adhesion, decreases immune responses and causing cell and tissue destruction allowing for the spread of bacteria and development of an invasive infection.[132, 133] In addition, HPAIV are able to infect the body systemically, resulting in influenza-associated neurological complications like acute encephalopathy and encephalitis.[134, 135] Conjunctivitis, myositis, myocarditis and Reye's syndrome also occurred during some severe courses of IAV.[136-139]

Only by laboratory diagnosis, an infection with IAV can reliably be confirmed, by the detection of IAV antigen, virus isolation or isolation of viral RNA.

3.3.1.3. Prevention and therapy of influenza A virus infections

Vaccines offer the possibility of averting an IAV infection from the immune system already in the initial phase. By accumulating AA changes, the HA protein is said to “drift” from one form, recognizable by host antibodies, to another antigenically different form which is less recognizable and more successful at infecting vaccinated and unvaccinated hosts. This process is therefore called antigenic drift.[140] This is why various vaccines against IAV exist, whereby the vaccine is generated from embryonic chicken eggs or in cell cultures and consists

of inactivated influenza A and B viruses. The EU recommendations for 2019/2020 seasonal flu vaccine composition contained the following virus strains: A/Brisbane/02/2018 (H1N1)pdm09-like virus; A/Kansas/14/2017 (H3N2)-like virus; B/Colorado/06/2017-like virus (B/Victoria/2/87 lineage).[141, 142] To develop new vaccine formulations, currently circulating IAVs are genotyped in reference laboratories worldwide, such as the Robert Koch Institute (Berlin, Germany). These genotyping data are collected worldwide by the World Health Organization (WHO). Twice a year, the WHO discuss the epidemiological data collected on circulating IAV to propose new recommendations for vaccines formulations for each northern and southern hemisphere influenza season annual vaccination.[143] In the event of a pandemic, a new suitable vaccine based on the current IAV sequences is generated as quickly as possible.[144]

Although vaccination is the primary strategy for the prevention of IAV infection, there are scenarios for which vaccination is inadequate and effective antiviral agents are of utmost importance.[145] Currently, only a few drugs are available for the treatment and prophylaxis of IAV infections. One class of drugs are M2 ion channel inhibitors of adamantane derivatives called amantadine, rimantadine and memantine.[146] In chemical terms, those compounds are tricyclic primary amines.[147] These three substances inhibit the release of the viral genome into the host cell by interfering with the ion channel activity of the matrix (M)-2 protein and/or indirectly with the pH-dependent conformational change of the hemagglutinin (HA), which is necessary for fusion of the endosomal and viral membranes. This subsequently interrupts the replication cycle of IAV.[148, 149] However, IAV develops resistance very rapidly during a therapy with these adamantane derivatives through mutations in the M-2 protein (between AA positions 27 and 31), which is why these substances are usually only used very selectively.[149]

Two available NA inhibitors oseltamivir (Tamiflu®) and zanamivir (Relenza®) represent the newest class of drugs effective against IAV. Oseltamivir and zanamivir should be administered within the first 48 hours of infection. Their mechanism of action is based on the potent and specific inhibition of influenza NA activity, which is essential for virus budding from the infected host cell.[150] Zanamivir is approved for influenza treatment and prophylaxis in adults and children of over 5–7 years of age (varying internationally). Resistance in vitro or in vivo can either be NA-dependent or NA-independent. If NA-dependent, viruses become resistant due to mutations in the NA, decreasing inhibitor-binding affinity. These mutations have been observed to involve AA substitutions in previously conserved structural or catalytic residues of the NA active site.[151, 152] Unexpectedly, the majority of the NA inhibitor-resistant mutants selected in vitro involve mutations in the HA, a mechanism known as NA-independent resistance.[153-155] Such mutations occur in or close to the HA receptor binding site, making the virus less dependent on the activity of NA for virion release.[156]

Therefore, a large number of other new drugs against IAV infections have been developed in recent years. Most of the clinical phases of these drugs will continue for several years. One drug that has not yet been sold in Germany, only been licensed in Japan is peramivir, which is known in Japan under the trade name Rapiacta. It is a NA inhibitor effective against IAV and Influenza B viruses.[157-159] Another drug with a new mechanism of action is favipiravir, also called T-705. The mechanism of action of this drug is not yet fully understood, although it is suspected that the polymerase of IAV is inhibited by T-705.[160-163] The successful development of further classes of drugs directed against IAV infections would be a major step in the fight against influenza. Despite the availability of vaccines and drugs, 3-5 million severe cases are reported in patients with IAV infections in an inter-pandemic year, of which between 250,000 and 500,000 die.[164] These high infection and mortality rates in people infected with IAV make it urgent to continue improving vaccines and drugs and generate new, more effective vaccines and drugs.

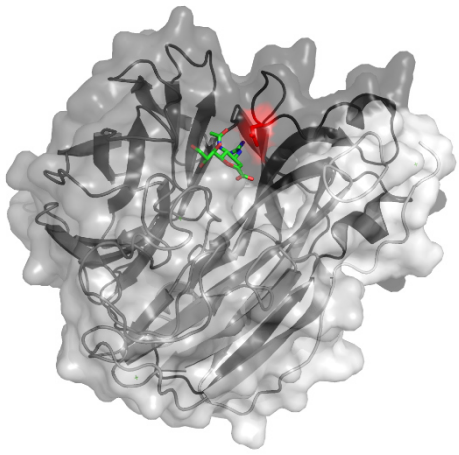

Figure 8. 3D structure of neuraminidase (NA). The active center is colored in red.

3.3.1.4. Neuraminidase (NA)

Eleven antigen subtypes of NA from IAV have been identified so far (N1 to N11).[165, 166] It is composed of four identical monomers (homotetramer) with circular 4-fold symmetry. The polypeptide chain folds into six, topologically identical, four-stranded, antiparallel β-sheets which are arranged as if on the blades of a propeller.[165] An approximate 6-fold symmetry axis passes through the center of the subunit, relating the six β-sheets with the active center of the enzyme, which lies close this pseudosymmetry axis.[167] Each monomer consists of a globular head region connected to a fibrous stalk domain, but the function of this remains unknown.[168] NA is associated with integral type II membrane glycoproteins, which are anchored in the membrane via a hydrophobic sequence of 29 AA near the N-terminus.[169] The 1413 nucleotide long gene encodes a protein comprising 454 AA which has five potential glycosylation sites.[170] Three types of NA are phylogenetically distinguished: NA of IAV, influenza B viruses and influenza C viruses. The latter have no clinical relevance. The sialic acid hydrolyzing subtypes N1-N9 of the NA of influenza A viruses can be divided into two groups. Group one (1-NA) comprises the subtypes N1, N4, N5 and N8, group two (2-NA) comprises the subtypes N2, N3, N6, N7 and N9.[171]

The main structural feature of group 1 NA is an extra cavity in the active site near the binding site of the ligand, the 150-cavity.[172] The NA of the pandemic virus of 2009A(H1N1)pdm09 occupies an intermediate position. Phylogenetically it evolved from group 1, but structurally resembling the NA of group 2, without the 150-cavity.[172] Recently, a tenth and eleventh subtype of NA-like proteins (N10 and N11) have been discovered in IAV in bats in South America. It differs structurally and functionally strongly from those of groups 1 and 2 and shows only weak up to no NA activity in vitro.[166, 173, 174] They could form a third group within type A NA. The NA of influenza B viruses form a separate group (B-NA).

The active site of NA (R118, D151, R152, R224, E276, R292, R371 and Y406) and the AAs framing and stabilizing the catalytic center (E119, R156, W178,S179, D/N198, I222, E227, H274, E277, N294 and E425) are highly conserved both between the different subtypes of influenza virus A and between type A and B.[175] The NA of influenza viruses recognizes α2,3- or α2,6-glycosidically linked terminal N-acetylneuraminic acids, and cleaves them by hydrolysis.[176] This is essential for the excretion of virus particles from the host cell as wells as for the prevention of superinfection by other sialic acids receptors using viruses. NA also cleaves sialic acids present in mucus and therefore avoids adherence of virus particles to non-target cells. To what extent it is involved in viral membrane fusion by haemagglutinin remains unclear.

3.3.2. Group A *streptococcal* (GAS) diseases

3.3.2.1. *Streptococcus pyogenes*

Streptococcus pyogenes (*S. pyogenes*) is a human-specific pathogen that is spread worldwide. The exact number of Streptococcal diseases per year is unknown. However, it is estimated that the prevalence of severe GAS diseases is at least 18.1 million cases, with 1.78 million new cases each year. Due to this numbers, severe GAS infections are responsible for about 517,000 deaths annually.[177-179] Typical colonization sites for this ubiquitous bacterium human infections are skin and the upper respiratory tract, where it is commonly causing mild infection (mostly local, purulent, non-invasive infections such as pharyngitis, tonsillitis, impetigo or erysipelas).[180] In rare cases, infections with certain serotypes and more frequently also in patients with predisposing underlying diseases lead to generalized infections of tissues with subsequent life threatening necrotizing fasciitis or sepsis.[181] In general, infections of humans with *S. pyogenes* are divided into three different forms[182]: 1. Local, surface-associated infections of skin and mucous membranes such as impetigo, pharyngitis or tonsillitis. 2. systemically invasive infections such as necrotizing fasciitis, scarlet fever or streptococcal toxic shock syndrome. 3. asymptomatic colonization associated with recurrent local or systemic infections. Irrespective of antibiotic therapy, asymptomatic colonization may occur as

a result of local infection.[183, 184] Untreated group A β-hemolytic streptococcal pharyngitis typically lasts 8 to 10 days. Patients are infectious during the period of acute illness up to approximately 1 week after. Antibiotic treatment decreases severity of symptoms, reduces their duration by approximately 1 day and the risk of transmission to others after 24 hours of treatment. It also reduces the likelihood of suppurative complications and rheumatic fever.[185] *S. pyogenes* produces numerous virulence factors responsible for its infectivity, including secreted toxic superantigens and proteases.[186]

3.3.2.2. Streptopain (SpeB)

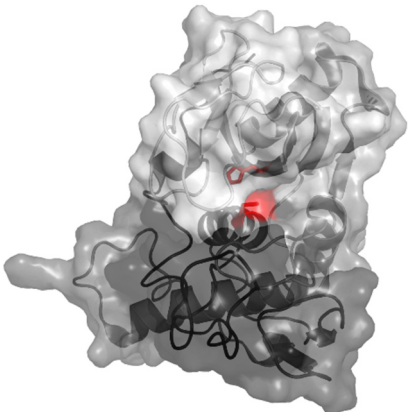

Figure 9. 3D structure of SpeB. The active center is colored in red.

SpeB, the first cysteine protease that was isolated from a prokaryote, was also characterized in the year 1945.[187] It is present in virtually all strains of *S. pyogenes* isolated and is often the predominant extracellular protein, accounting for about 95% of total secreted protein.[188, 189] Other names for SpeB in literature are Streptococcal pyrogenic exotoxin B (SpeB), streptococcal cysteine protease (*SCP*) or streptococcal peptidase A (*SPP*). SpeB produced is released extracellularly in a zymogen form (proSpeB) with a molecular weight of 40 kDa (371 residues). Conversion of proSpeB to the 28 kDa active SpeB (253 AA) can be achieved by exogenous proteases (i.e. trypsin and subtilisin) as well as by autoprocessing.[190] Its gene is encoded in 398 AA: the residues 1-27 is signal peptide; the residues 28-145 is the prodomain; and the residues 146 – 398 is the protease domain. The highly conserved *SpeB* gene is located on the bacterial chromosomal deoxyribonucleic acid (DNA).

SpeB degrades extracellular matrix proteins fibronectin and vitronectin and increases the bacterial attachment to host cells. To increase bacterial dissemination, it cleaves (and activates) MMP-2 and MMP-9 and human interleukin 1β precursor to generate fully active mature interleukin 1β, suggesting a role in inflammation and shock. It also cleaves kininogens into biological active kinins.

3.3.3. Distinction between viral and bacterial sore throat

Respiratory infectious diseases are usually caused by both, viral and bacterial pathogens. In this case, a fast and reliable diagnosis is essential for selecting an appropriate treatment, e.g. antimicrobial therapy. Pneumonia is the most severe of these and is characterized by acute inflammation of the alveolar lung parenchyma.[191] It is still one of the most frequent causes of

death in children and adults worldwide (WHO, 2014). Sore throat is usually caused by direct infection of the pharyngeal tissue (pharyngitis). Sore throat can also be caused by conditions such as gastroesophageal reflux disease, acute thyroiditis, persistent cough, and postnasal drainage due to allergic rhinitis or sinusitis. However, reliable estimates for the likelihood of these conditions among patients with sore throat are not available.[192]

These high prescription rates must be viewed very critically, especially for antibiotics, since inappropriate or frequent use induces resistance. This results in the fact that in life-threatening cases some of these drugs cannot develop their efficacy. It is therefore important to further develop diagnostics in this area so that antibiotics are only given in cases where they are actually necessary.

In a survey of 63 physicians in or around Wuerzburg, 57 (90.48%) evaluated a chewing gum-based rapid test to differentiate between viral and bacterial diseases as potentially helpful in clinical everyday life (**Figure 10**). Especially pediatricians (11/11) favorite such a test. It could make patients understand the use of antibiotics, or even serve as a means of persuading them not to use them. About half of the doctors (31 votes (49%)) believe that the pressure to prescribe antibiotics could be reduced by such a simple to understand rapid test.

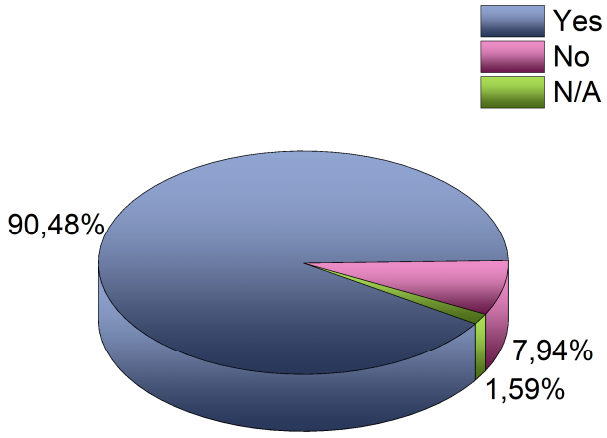

Figure 10. Pie chart of answers to the question "Is there a need for a rapid test on influenza".

To be accepted by doctor and patient it should take between 2-5 min (36 votes (57%)) until the result is achieved, whereby up to 10 min are still appropriate (19 votes (30%)). A diagnostic rapid test set should best consist of an influenza test (24 votes (38%)) or a combination of one influenza test and a test for *S. pyogenes* (24 votes (38%)). According to doctors, these tests should cost 5 euros for the individual test and 10 euros for a combination set of tests.

Asked about self-diagnosis, the acceptance of physicians is rather low. Thus, 34 of the 63 physicians (54%) would not advise their patients to self-diagnose with this chewing gum.

4. INSERTION - PERSPECTIVE

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Perspective

Frugal Innovation for Point-of-Care Diagnostics Controlling Outbreaks and Epidemics

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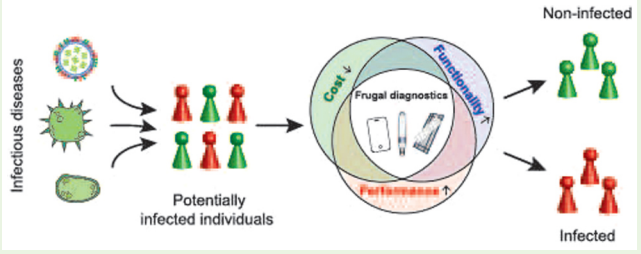
ABSTRACT: Today epidemics of infectious diseases occur more often and spread both faster and further due to globalization and changes in our lifestyle. One way to meet these biological threats are so-called "Frugal Innovations", which focus on the development of affordable, rapid, and easy-to-use diagnostics with widespread use. In this context, point-of-care-tests (POCTs), performed at the patient's bedside, reduce extensive waiting times and unnecessary treatments and enable effective containment measures. This Perspective covers advances in POCT diagnostics on the basis of frugal innovation characteristics that will enable a faster, less expensive, and more convenient reaction to upcoming epidemics. Established POCT systems on the health care market, as well as currently evolving technological advancements in that sector are discussed. Progress in POCT technology and insights on how to most effectively use them allows the handling of more patients in a shorter time frame and consequently improves clinical outcomes at lower cost.



KEYWORDS: *Frugal Innovation, Epidemics, Emerging Markets, Point-of-Care-Tests*

INTRODUCTION

Infectious diseases benefit from globalization and faster transportation that accelerate pathogen distribution. Some of these so-called epidemics have become sadly famous such as the outbreaks of Ebola, HIV, and some forms of influenza.[1] Especially developing and emerging countries face challenges in establishing functioning health care systems to tackle fast spreading diseases. Therefore, epidemics have proven to expand quickly, due to structural poverty, poor infrastructure and sanitary conditions, malnutrition, and armed conflicts.[2,3] Due to the close link between the gross domestic product (GPD) and health care expenditures,[4−7] emerging market countries with their large number of people and increasing economic capital and prosperity are especially interesting when studying health care innovations. Oftentimes existing technologies that could significantly improve health care service in emerging markets does not reach the end user. This is because most diagnostic devices are not handy and robust enough for the existing infrastructure. Additionally, they are prohibitively expensive or require operation by trained medical staff that is not available. These constraints are a major driver to rethink the innovation paradigm of industrialized western industries and come up with ideas that are better adapted to constrained contexts. So-called frugal innovations aim to approach upcoming epidemics by reaching more people and faster in adverse geographical regions.[8,9]

There is also a need for new and cost-effective methods in the health care services of industrialized countries. Value-based reimbursement instituted by the Affordable Care Act of the Obama administration in 2010 has initiated this trend. The shortage of doctors and medical supplies in rural areas, an aging population, and increasing expenditures of health insurances require a focus on the cost-performance ratio of diagnostics and treatment measures.[10−13] In Germany, an alarming number of one in three hospitals is in the red with its accounts.[14] This situation calls for a fundamental rethinking of how medical services are provided. This void can be filled with frugal developments that balance high-quality requirements and affordability.

Bringing the analysis from the specialized lab to the patients themselves to self-monitor one's own state of health could increase prevention and treatment effectiveness. In frugal innovation, diagnostic products and processes are optimized, reduced to their essentials, and adapted to their specific application context. This way the tests carry diagnostics even

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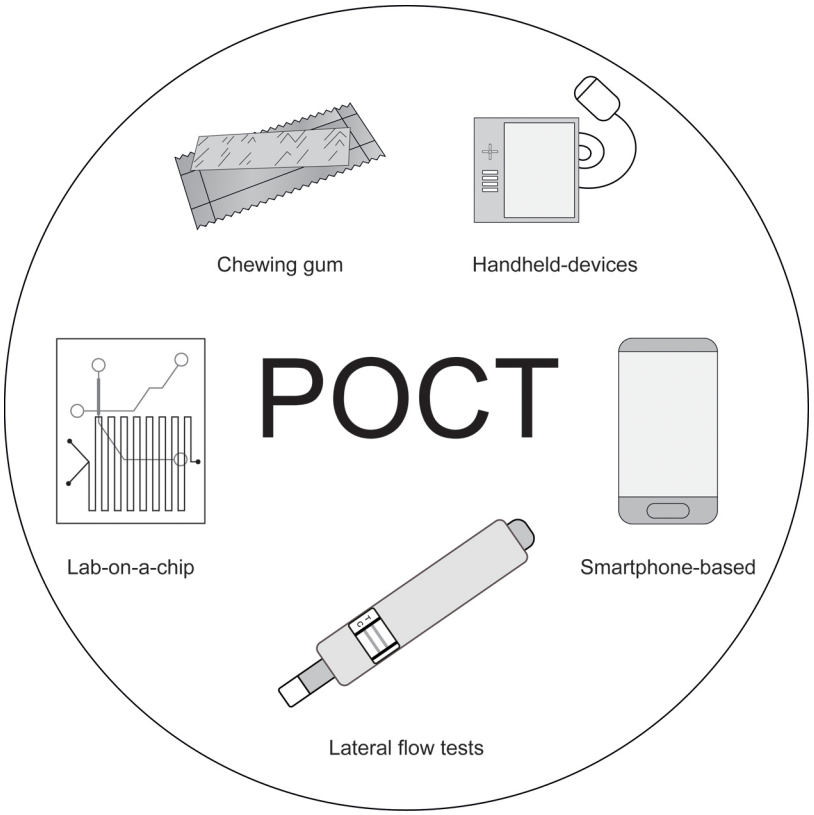

Figure 1. Overview of the POCTs described in this Perspective.

Table 1. Criteria of Frugal Innovation[a]

	Criteria			
dimension	Weyrauch et al. 2017[23]	Agarwal et al. 2017[21]	Wimschneider et al., 2020[24]	Rao, 2017[33]
cost/price	Substantial cost or price reduction (reduction of about 1/3 or more compared to the prevalent or nonfrugal product version)	cost-effectiveness	value-based pricing (from customer perspective), cost-effectiveness (from company perspective)	low-cost
functionality	concentration on core functionalities	ease-of-use	ease-of-use	
performance	optimized performance level	contextual factor (sustainable, ecological, social, etc.)	service, branding, product positioning	
technology				use of advanced technology and science from scratch by involving technologically trained staff

[a]As defined in Weyrauch and Herstatt (2017),[23] Agarwal et al. (2017),[21] and Wimschneider et al. (forthcoming).[24]

to the most remote areas and alleviate challenges in clinic settings.

This Perspective focuses on the concept of frugal innovation in general and specifically in the field of diagnostics. Subsequently, a review of different forms of frugal point-of-care tests (POCTs) will be given and analyzed according to their technological complexity and usability (Figure 1). By deriving the current advantages but also shortcomings of frugal POCTs, we will be able to provide an expanded picture of the possibilities that exist and evolve in hospital and nonhospital setups for both developed and emerging market contexts. Recommendations on the advancement and application of frugal POCTs are given to physicians and developers of diagnostics supplies and equipment. The ultimate goal of this Perspective is to stimulate a broader discussion on new and modified approaches in diagnostics for an improvement of global health care, specifically concerning epidemics.

THEORETICAL BACKGROUND

Frugal Innovation. New approaches to address modern health care issues are decisive steps to remedy the increasing need for global medical coverage. However, financing and providing for advanced health care services on a worldwide scale turns into a challenge. Especially less developed regions but also remote areas in industrialized countries are characterized by a number of conditions that massively hinder access to world-class medicine for millions of people. The most significant barriers are poor infrastructures of transport and electricity, medical equipment standards that are inadequate for developing countries' conditions, a lack of trained personnel, and societal objections against western conventional medicine.[15−20]

Against this background, a stimulus has been created to think of innovation differently. Instead of raising a newly developed product to the next level with ever more functionality and complexity, innovations should, first and

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foremost, solve a problem. This need-based approach is adapted to the specific circumstances, making the innovation robust, flexible, easy-to-use, and accessible. The shift away from the belief that an innovation needs to be more expensive than its comparable ancestor is especially important. Balancing cost and price with the provided value has become the guideline. With increasing attention and diffusion of these new innovation patterns, the concept has been given the name "frugal innovation".

In recent years, the phenomenon of frugal innovation has been increasingly examined especially in the health care and pharmaceutical industries.[19,21] As such, some criteria addressing the cost, functionality, and performance domain have prevailed that qualify a product as frugal (Table 1).

While Weyrauch and Herstatt[23] provide a rather exact indication with respect to the cost dimension, Wimschneider et al.[24] point toward the signaling function of prices in frugal innovation. Quality and attractiveness are often communicated to the consumer through a certain price level. A low price is not always the gold standard of frugal innovation as it can well be misinterpreted as lack of quality or second-best option. Reaching the optimal price/value ratio of these products is therefore an important and challenging aspect.[22] It is agreed that the focus should be on key functionalities, skipping superfluous ones for easier handling of the product. Depending on the application context, the frugal product's ultimate performance is crucial. Performance, in this connection, can refer to a variety of aspects. Whereas the cost and the functionality dimensions distinguish a frugal innovation from the conventional product, the performance dimension shall close this gap again. The frugal product has to perform equally to or better than the traditional offering in the particular context for which it has been designed. This unique selling proposition can be achieved by features that fulfill the core purpose of the product extraordinarily well, enable its sustainable, social or large-scale application, correspond to cultural preferences, or show an attractive brand positioning.[23]

The relevance of the single frugal product characteristics can differ between players of the same industry. A recent study conducted in the Indian health care sector showed that practitioners (hospital physicians) and medical equipment developers (product managers) do not entirely share the same view on what they consider as important in a frugal medical product, for example, concerning low or no maintenance (Figure 2). Although these results stem from a specific context in an emerging market environment and may only be generalizable to a limited extent, they offer some initial orientation when developing a frugal medical product. In order to ensure a successful dissemination and customer satisfaction of medical products, it is crucial to consider and integrate heterogeneous requirements.[34]

Frugal innovation has long been considered as inherent to emerging market firms and their customers. That is because the concept has its origin in typical problem-solving strategies of nonprofessionals and entrepreneurs in resource-scarce, depressed regions, for example, in India known as Jugaad.[25−29] The frugal innovation, as we know it today, is a derived management approach that complies to high quality standards, certifications, and organizational product development requirements.[30] Moreover, an increasing number of frugal innovations formerly associated with low-tech or no-tech are now high-tech.[9,24,31,32] As such, it is no longer limited to the innovation context of emerging markets. The focus on cutting-edge

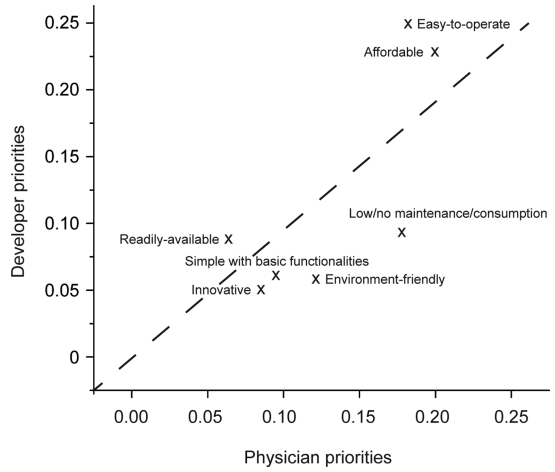

Figure 2. Two-dimensional plot of physician and developer priorities of diagnostics. Adapted with permission from ref 31. Copyright 2016 European Union.

technologies and latest scientific knowledge as the crucial enabler of frugality has made it a global approach. Rao acknowledged this by introducing a new category to the field known as Advanced Frugal Innovations (AFIs).[33] AFIs are designed on the basis of newly developed technologies for retinal imaging, digital X-rays, nanotechnology, or biometric systems. These developments allow for portability, resource-efficiency, user-friendliness, and value for money.[9,21,24] The technological excellence involved in frugal innovations opens the door for established Western market firms to participate and lead this field with their expertise, technology leadership and highly trained employees. Technology could be the reason why established Western market firms see value in frugal innovation and take up these principles.

AFIs in health care offer advantages as they allow for affordable access to medical services through remote telemedical products and systems. In developed countries, they open new fields of application that improve and extend medical care. A prominent example that illustrates this opportunity is GE's portable low-cost ultrasound machine. The device was originally developed for rural areas in India and China to bring such a service to these unserved regions for the first time. Subsequently, it became a great success in the US, where it was used in ambulances and emergency units and thus defined a new application context of ultrasound, which prevalent ultrasound machines could not offer.

In summary, a frugal innovation in health care optimally balances and combines the cost, functionality, and performance dimensions on the basis of the applied technology. Frugal innovation in health care provides (1) a service in thus far unserved areas, (2) a better or more suitable service to thus far poorly served areas, or (3) an additional service to already well-served areas. Whereas the first two scenarios rather take place in developing countries and rural or remote areas of developed countries, the last scenario is seen when a frugal innovation competes with the conventional solution in developed regions.

Frugal Innovation in the Field of Diagnostics. The simplification of products and processes through cost-effectiveness, optimal functionality, and performance significantly improves medical coverage and treatment in upcoming epidemics. In particularly, due to reduced time for diagnosis

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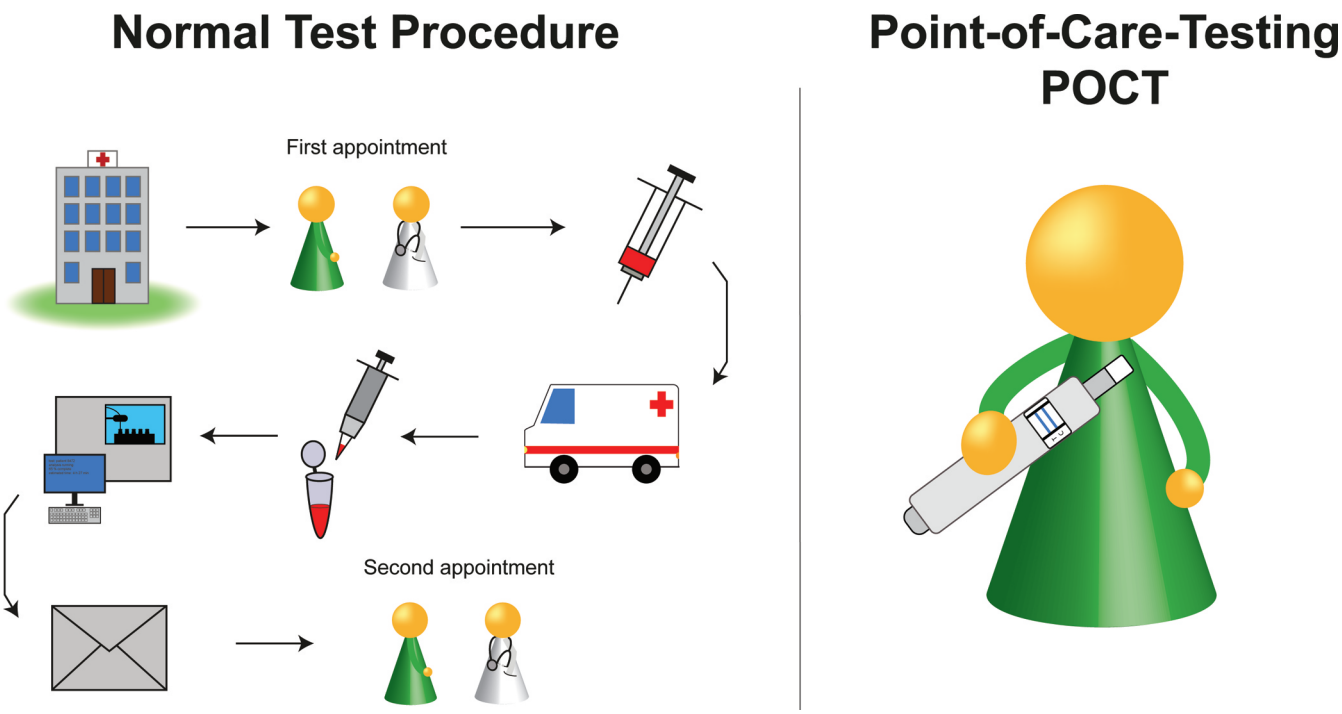

Figure 3. Conventional test procedure vs POCT. Time-consuming procedures such as transportation, processing, and aliquoting processes are reduced or even eliminated by bringing every step (sample acquisition, preparation, analysis) directly to the patient's bedside, thereby creating a more streamlined and faster workflow.

generation. Indeed, time saving is most critical in fighting rapidly developing infections for the individual and for the larger population.

The Dictionary of Epidemiology defines an epidemic as "the occurrence in a community or region of cases of an illness, specific health-related behavior, or other health-related events clearly in excess of normal expectancy".[35] This definition specifies neither a minimum number of cases, the area covered, nor a specific time frame. Thus an epidemic may be limited to a small area or extend to a global occurrence, lasting from a few hours to many years.

Epidemics or pandemics therefore are not necessarily of infectious nature. The rapid increase in the number of cases of diabetes or metabolic syndrome is also referred to as an epidemic. However, within this Perspective, we focus on epidemics of infectious diseases.

The frugal wave of health care innovation has reached diagnostics, especially in the field of POCTs. These are medical diagnostic tests performed in nonlaboratory settings at the patient's bedside, directly at the point-of-care. POCTs can be performed on an easily accessible sample, generating results in a timely manner, as significantly fewer steps from the access into the health care system to the results of the test are necessary. Therefore, costs should be automatically reduced (Figure 3).[36]

The POCT diagnostic history began in 1962 with a method for simple blood glucose measurement[37] and with the advent of the pregnancy test in the 1970s.[38] Measurements that used to take days to be evaluated and needed trained personnel were then performed by the patients themselves in just a few minutes. These tests are not only suitable for the broad market in developed countries but also vital for resource-limited countries with low supply infrastructure. These advantages have made the POCTs highly profitable products. According to a recent research study published by Global Market Insights, Inc., the market size of POCTs is set to exceed US $36 billion by 2025, compared to US $22 billion in 2018.[39]

The WHO Sexually Transmitted Diseases Diagnostics Initiative (SDI) has set the following standards for POCT systems. The so-called ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users)[40] decide whether the tests can be utilized to efficiently diagnose diseases in remote areas. These criteria are certainly useful for diagnostics of sexually transmitted infections (STIs) due to their fast spread but can generally serve as a requirement for rapid tests for a multitude of diseases. Although a bit more specific about diagnostic assets, the ASSURED criteria of the SDI coincide clearly with the criteria of frugal innovation (Table 1). This match indicates that the overall purpose and goal of innovative POCT systems and frugal innovations point in the same direction. Therefore, it is valuable to review typical applications in this field and show how frugal product development can improve diagnostics of epidemics.

Besides their already mentioned obvious strengths and opportunities, the weaknesses of POCTs and threats in their environment show potential disadvantages. POCTs are a challenging market due to high costs and efforts for their development and registration but low sales prices afterward. Also infrastructure barriers and societal reluctance to use them are obstacles. These prevailing disadvantages need to be addressed in future development of POCTs (Tables 1 and 2).

This SWOT analysis shows the current state of the POCT market. Compensating the weaknesses and limiting the threats of the POCTs available on the market are challenges, which are addressed by different techniques and approaches. These are presented in the following. Subsequently the positive effects on fighting epidemics with the help of POCTs are

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Table 2. SWOT Analysis of POCT

strengths	fast (in diagnosis itself and subsequent earlier beginning of treatment)
	low costs of the test
	low sample quantity required
	ease-of-use, no clinical staff required
	no sample pretreatment required
	mass production of test kits possible
	robust to environmental influences
weaknesses	objections by medical practitioners
	lower specificity and sensitivity than lab-methods
	distribution channels are often not fully developed
opportunities	growing consumer market in both emerging and developed countries (demographic shift, globalization, and increase of epidemics)
	new-on-site fields of applications and technologies (e.g., lab-on-a-chip) for remote areas or mobile use
	multiplexing capabilities
threats	strict regulations and laws in the health care sector
	precision tests with higher sensitivity and specificity (GC/LC-MS, PCR)
	pressure of low prices on the POCT market
	mishandling and error rates through testing performed by nonlaboratory personnel

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evaluated, to give recommendations for physicians and developers.

THE STATUS QUO: ESTABLISHED POCTs FOR INFECTIOUS DISEASES

The Diagnostic Chewing Gum. In modern POCTs, smartphones and the human eye are often used as the detector component to evaluate POCT results.[42] In the case of the diagnostic chewing gum, the human tongue is used as a detector.[43] The chewing gum contains sensors, which are in themselves tasteless. These sensors contain an engineered peptide sequence cut by a target protease, which is present in saliva (Figure 4A). This initial step kick-starts further degradation of the sensor (through human aminopeptidases), resulting in the formation of a bitter tasting substance recognized by and alerting the patient. Other tastes are also imaginable by choosing a different flavor component that is coupled to the peptide sequence. However, the bitter taste sensation is well perceived by humans as it often warns of toxic substances in nature.[44]

Two cases can be distinguished in the development of a new sensor. If the protease of the pathogen is already well characterized in its cleavage profile, the linker sequence can be found in literature. With the natural cleavage sites, the construct can be synthesized and analyzed in cleavage studies

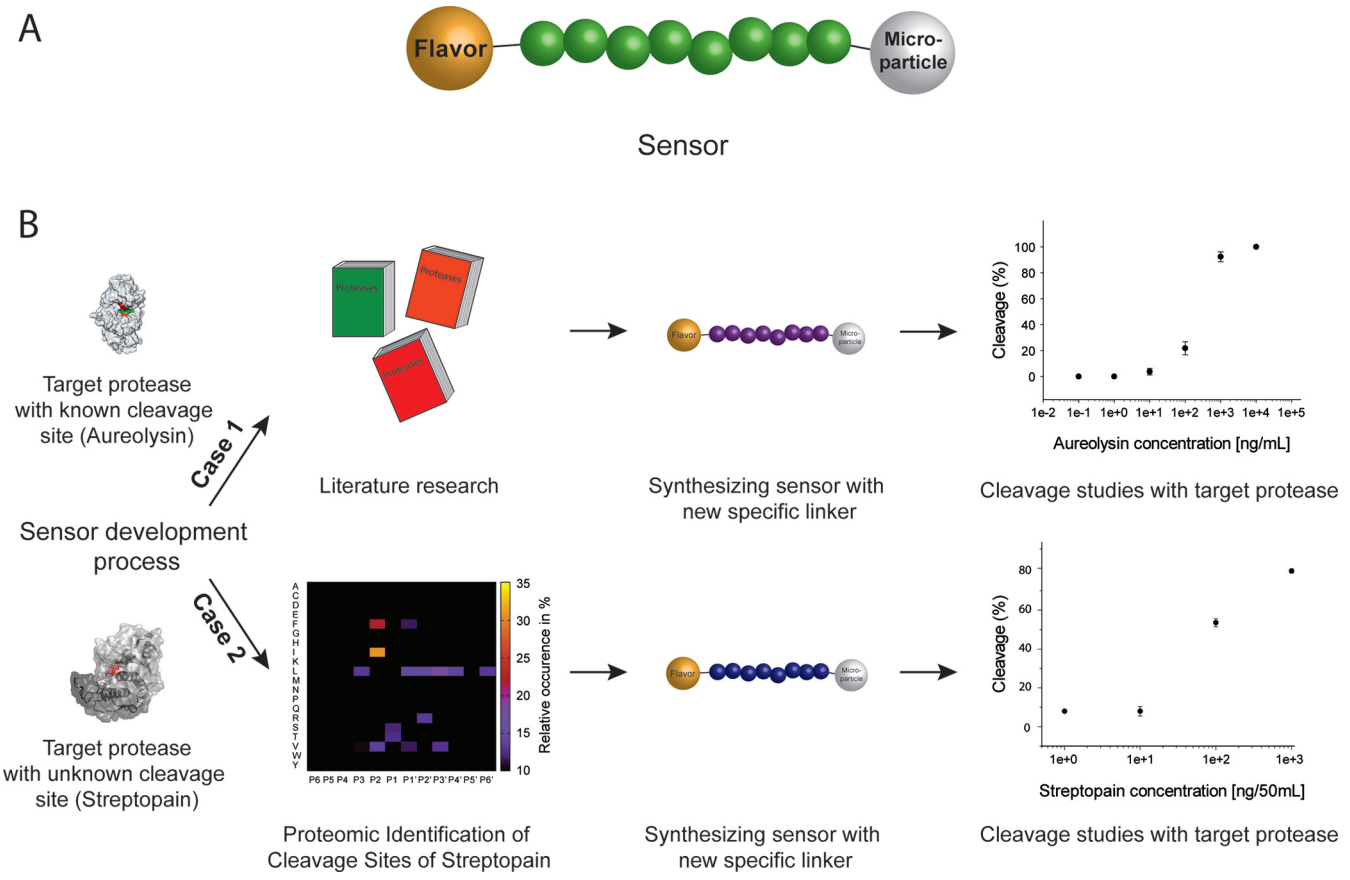

Figure 4. Development of a sensor used in a diagnostic chewing gum. (A) Diagnostic construct, consisting of a flavor molecule, a protease sensitive peptide linker (PSL), and a microparticle. (B) Development of diagnostic linkers can be carried out in two ways. Case 1, with a well-studied target protease like aureolysin, the natural cleavage sequences can be used as PSLs. PSLs are synthesized and analyzed for their cleavage characteristics. Case 2, if no natural cleavage sites are known from literature (e.g., for streptopain), these can be determined by biotechnological methods, for example, through proteomic identification of cleavage sites (PICS).[45] The rest of the procedure is the same as in case 1. Further details are available from the corresponding authors upon request.

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(Figure 4B1). Contrarily, if the protease is not well characterized, cleavable sequences can be determined via proteomic methods (e.g., PICS assay). Afterward, sensors with newly identified cleavage sites can be synthesized and similarly analyzed in cleavage studies (Figure 4B2).

This radically simplistic design replaces expensive detectors with a natural one at no charge: the 24/7 available human tongue.[43,46] Because pathogens also rely on very specific proteases, this platform holds promise to screen entire populations in the event of an upcoming epidemic even in environments characterized by constraints and limitations. Possible detectable diseases with their target protease occurring in saliva are periodontitis (matrix metalloproteinases), caries (proteases of *Streptococcus mutans*) or group A streptococcal infections (proteases of *Streptococcus pyogenes*).[47−49] The innovativeness here lies in its simplicity as it can be performed by everyone, at any place, and at any time without electrical power supply. Drawbacks that need to be overcome are the variability in taste perception and the concentration and activity of target proteases.

The clinical value of this radically simplistic and novel platform needs to be demonstrated in the future.

Lateral Flow Immunoassays. Nondigital rapid tests, such as the lateral flow immunoassay (LFIA), have become indispensable in today's modern diagnostic routine. They are often used because of their simplicity in execution and analysis, the fast generation of results and their enormous cost advantage compared to conventional diagnostic procedures.[50] Moreover, they serve multiple diagnostic needs. Such rapid tests are available, for example, in the fields of pregnancy testing and detection of toxicologically active substances but also for infectious diseases such as influenza, ebola, malaria, tuberculosis, or HIV.[51−55]

In LFIAs, the liquid sample containing the analyte of interest moves along a strip of polymeric material thereby passing various zones. In these zones, molecules (often antibodies) have been attached that exert more or less specific interactions with the analyte. A typical LFIA format consists of a surface layer carrying the sample from the sample application pad to the conjugate release pad along the strip encountering the visible detection zone and finally to the absorbent pad.[41]

Infections often manifest in unspecific symptoms such as fever or sore throat. As soon as typical signs of an infection occur, the more common diseases (e.g., malaria) are treated prophylactically in developing countries.[56] Screening tests have done much to improve the situation in this field in recent years. They helped to distribute scarce resources, such as medicine, physicians, and bed capacity, more efficiently, and in developed countries their potential is mainly seen in relieving the burden on health insurance funds.[31] An example is the currently developed antibody-based POCT that can discriminate between Zika and other flaviviruses such as Dengue in around 15 min.[57] It is made from paper- or plastic-based materials, costing about just US $2.

The annual influenza epidemic is both unpredictable and inevitable, with existing drugs for the treatment being effective within the first 48 h of infection. This has led to the development of several rapid tests in LFIA format for influenza that are commercially available. Concerns over low sensitivity of LFIAs have been raised, due to the low concentration of antigens in clinical samples. Compared to laboratory testing methods with reverse transcription-PCR (RT-PCR) or viral culture, a negative test result of commonly used POCTs should be interpreted with caution.[58] A newly introduced modern rapid test uses a variation on isothermal DNA amplification technology, detecting RNA gene targets from influenza A and B viruses with a sensitivity of 97.8% and 91.8%, respectively.[59]

POCT systems furthermore exist for group A streptococci, which cause symptoms often resembling those of influenza. This has led to a measurable reduction of antibiotic prescription of about 50−60% if POCTs are used.[60,61] In the US alone, the overprescription of antibiotics and the following antimicrobial resistance costs billions of US dollars every year in additional treatments and loss of productivity.[62]

Following the unprecedented scope of the West African Ebola epidemic, three rapid diagnostic antigen detection tests have received Emergency Use Authorization.[52] Rapid tests for Ebola with a sensitivity near 100% are currently developed. The collection of test results and geographical data was enabled by a LFIA with a smartphone application. As this disease is highly contagious and infected patients develop rapidly critical conditions, the gathered information can be of utmost importance.[63]

To address malaria infections, LFIAs detecting various *Plasmodium* sp. protein antigens are now among the most heavily used laboratory diagnostics worldwide.[53,64,65] Rapid tests on malaria are also valuable in nonhospital setups due to the ability to provide 24/7 first-line triage results. Positive test results can later be confirmed by a diagnostic expert via microscopy during standard business hours.[66]

Even though tuberculosis (TB) is the world's leading infectious cause of death (claiming about 1.3 million deaths every year), only 64% of cases are diagnosed.[54,67,68] In the past decade, impressive progress has been made in the development of a TB POCT. A cartridge-based integrated miniature PCR system was developed, obtaining test results from unprocessed sputum within 90 min.[69]

Screening tests are also being developed for STIs. These infections are particularly dangerous because no or indefinable symptoms occur for a long time and unknowing transmission is therefore very likely. By detecting HIV-1 and HIV-2 antibodies, very specific POCT diagnostics were commercially established.[55] The fourth generation of commercially available POCTs for HIV is currently on the market, reliably detecting the virus already 1 month after the person has contracted it.[70] The fact that drugs against HIV are reportedly more effective in the early than in the late stage further supports the importance of a fast and accessible POCT. Despite many advantages of a HIV POCT that can be performed at home, studies also found limitations to the use of oral fluids. Some studies found that a POCT using oral specimens demonstrated lower sensitivity than the test's sensitivity with blood specimens.[71,72] Some POCTs for HIV antibodies may fail to identify acute early HIV-1 infection. This is especially problematic as due to the high viral load and the lack of awareness, the risk of HIV-1 transmission is much higher in this stage than with an established infection.[73,74] POCT devices also exist for detection of syphilis, gonorrhea, and chlamydia (current time to result, 1−2 days).[75,76]

The Smartphone as a Tool for Epidemic Control. In POCTs, the human eye is often used as a tool for evaluating the color or intensity of a band. Nowadays, smartphones are also increasingly used to optimize the evaluation of nondigital POCT devices. Smartphone cameras are a convenient tool to optically read-out clinical specimens or band intensities that

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the human eye would not be able to adequately evaluate.[77] Through advances in mobile imaging algorithms, the development of new external hardware applications, and specifically designed lens arrangements, smartphones are applicable in a broad range of diagnostic assets.[78]

An example is the combination of a lateral flow test strip for avian influenza detection with a lightweight smartphone-based fluorescence detector powered through the smartphone's micro-USB port.[79] Data communication with a central database collecting human throat swab samples was realized via SMS. A different publication reported a paper-based device with smartphone read-out to develop an at-home testing system for urinary tract infections and gonorrhea.[80]

Via Bluetooth, it is possible to connect the smartphone wirelessly to other "wearables", such as smartwatches and fitness trackers, to measure a variety of human body functions. Through flexibly printed electronics and advances in material sciences, temporary tattoo sensors have been invented, analyzing body fluids on a molecular level.[81,82] Analyzing the heart rate and the composition of human sweat, which changes in the event of an infection, can give first hints in diagnosing diseases.

Mobile applications are becoming more popular and technologically sophisticated. This field of eHealth is known as mHealth (mobile health).[83] Currently, around 165 000 smartphone apps with data storage or health monitoring functions are available.[84] To assist personal health care, several applications for multiple devices are now available, such as apps for information gathering, time management, health record maintenance and access, communication, consulting, reference, patient management and monitoring, clinical decision-making, and further for medical education purposes and medical training.[85]

Due to their mobility, data sharing capabilities, and easy access, smartphones and POCT apps become an increasingly important epidemic control tool in both developed and emerging countries. By creating suitable applications, unique solutions for the diverse medical situations in different countries can be designed. In addition, results can be shared worldwide in seconds and treatment proposals can be generated (known as telemedicine). In the case of an upcoming epidemic, this can facilitate self-diagnosis, support self-therapy, and disseminate information on disease prevention.

It should not be neglected that, unfortunately, mHealth is subject to severe data protection and privacy concerns. A recent study found that the overwhelming majority of the studied apps listed as the most popular ones concerning health and fitness available on GooglePlay, do not deal in a sensitive and confidential way with health related data. On the contrary, data regulations are regularly breached by giving data access to third parties or inappropriate installment of encryption mechanisms, to name but a few. Unsurprisingly, user trust in these devices is low.[86]

New Frugality-Enabling Technologies in Diagnostics. The goal of globally applicable POCTs to efficiently tackle mass infections in unfavorable environments is to bring complex processes into miniature format using already accessible and available supplies. This is what happened with a cellphone-sized real-time polymerase chain reaction device that was developed to detect the Ebola virus RNA. It works with a sample volume of 100 nlL, produces results in under 40 min, and is powered by a car battery.[87] Another paper-based 3D microfluidic POCT biochip for the detection of whooping cough was developed at the University of Texas at El Paso. This device uses isothermal DNA amplification technology to detect bacteria from nasal swabs in under one hour and costs only a few dollars, having comparable results to PCR.[88]

POCT diagnostics represent the main segment (about 57%) of the current biosensor market, which is expected to reach US $22.68 billion by 2020.[89] As the biosensor is directly responsible for the bioanalytical performance, it is the most critical component in the diagnostic setup. An approach that combines electronic devices and biochemical testing is the lab-on-a-chip (LOC) platform. Recent developments in nanotechnology, microfluidics, and 3D-printing show potential for miniaturization and multiplexing of biosensors, leading to cost-effective, portable, and easy-to use POCT diagnostics. Through miniaturization, signal enhancement, and process automation, LOC platforms feature several advantages such as reduced sample consumption, massive parallelization, and high sensitivity due to different functional units giving responses in real-time.[90] As a read-out interface, again, the smartphone can be utilized since it possesses the required components.[91] Researchers from Stanford University developed a LOC with production costs of just 1 cent capable of separating cells based on their intrinsic electrical properties. No clean room or trained personnel are needed to create this combination of microfluidics, electronics, and inkjet printing technology.[92]

POCTs with multiplexing capabilities could be one of the game changing technologies in fighting epidemics. As mentioned above, in some areas the most often occurring disease (e.g., malaria) is treated without a proper preceding diagnosis. This behavior is wasting resources and exposes patients to unnecessary medical treatment. Testing several diseases at once could make treatment processes in those settings more efficient.

The most common method for multiplexed POCT systems (xPOCT) is the lab-on-a-disc format.[93] One example of an already commercialized product is the Gyrolab Bioaffy CDs of Gyros Protein Technologies AB from Sweden.[94] Here, the fluid regulation is implemented by a combination of capillary and centrifugal forces. Also, it contains hydrophobic stopping barriers. Different cancer biomarkers (e.g., interleukin-6, α-fetoprotein) were simultaneously detected to demonstrate its multiplexing performance. The Quanterix Simoa Disc is yet another example, containing over 200 000 femtoliter-sized wells and integrated microfluidics. It is capable of the automated detection of single molecules for *in vitro* diagnostics applications.[95] As a platform technology it offers great flexibility in assay design but has to be further improved in cost-effectiveness to be considered as frugal. One further example for xPOCT systems is microfluidic large-scale integration (mLSI). The proof of concept for multianalyte measurements on various analytes was demonstrated (proteomics,[96] cellomics,[97] and genomics[98]). It is not yet commercialized due to system complexity and bulky instrumentation for pneumatic control.[99]

With its Triage platform, Alere Inc. (USA) introduced a lateral flow xPOCT with the ability to screen up to 20 different analytes at once.[100] It combines LFIAs with quantitative multianalyte immunoassays and a portable fluorometer. Now owned by Quidel, it provides xPOCT systems for cardiac biomarkers and drug analytes but could be used in the future as a diagnostic platform for multiple infectious diseases.

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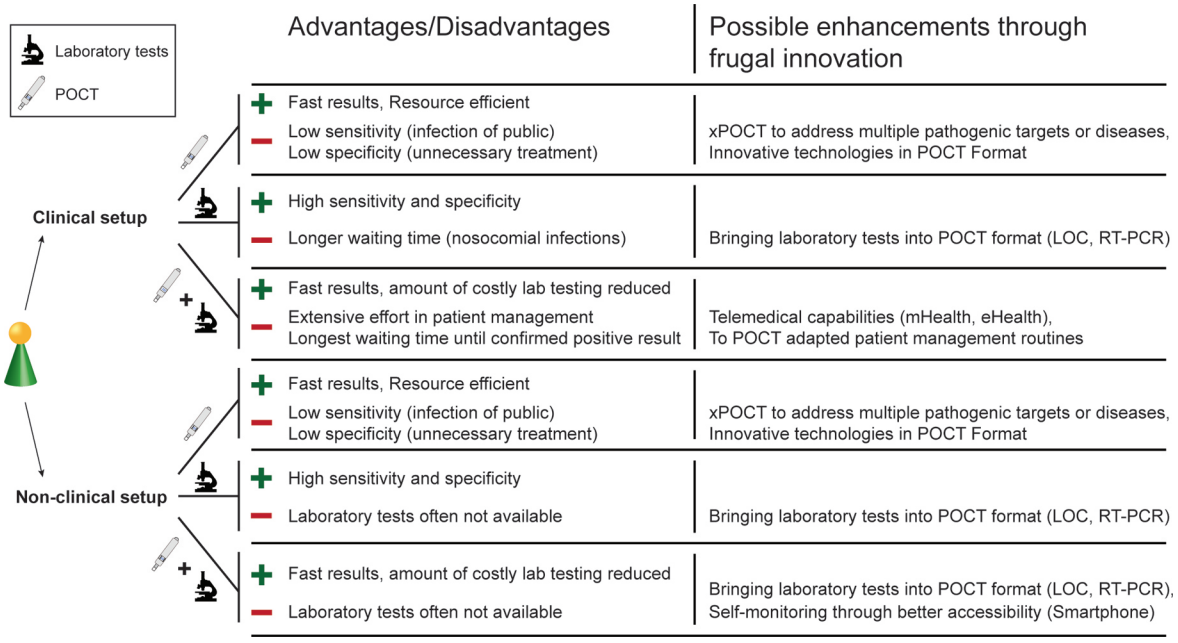

Figure 5. Overview of advantages and disadvantages of laboratory tests and POCTs in different health care settings and possible solutions provided through frugal innovation.

Diagnostics in Hospital versus Nonhospital Setups. The use of POCTs could make the medical support of patients more efficient. Implementation success depends not only on technological advances but also on the optimization of the use of POCTs. The clinical utilization of POCTs differs substantially from their use in environments with few medically trained personnel, little medication, and few appropriately equipped facilities. Depending on the specific settings, POCTs must be applied differently to ensure the best possible results in patient care.

In a hospital or well-equipped medical facility, a high density of physicians and other medically trained personnel is available. Also, resources for the treatment of the most common diseases are present in adequate quantities. The essential problem is, that medical care at a hospital cannot be guaranteed or retained in case of an epidemic.

The Ebola epidemic, for instance, brought hospitals in Africa to their limit in a short amount of time concerning bed capacity.[101] Due to this situation, diagnosis and subsequent medical treatment of infected patients were dramatically slowed down. As a result, the hospitals in Sierra Leone needed up to a week to diagnose the disease.[102] This led to patients spending longer waiting times in hospitals, occupying the limited bed capacity. Another problem was that during this time period when infected and noninfected patients were accommodated together in a small area, the risk of nosocomial infections increased.[103]

POCTs could significantly remedy such situations. First and foremost, POCTs lead to resource savings in many cases.

In a **hospital setup**, POCTs should be combined with laboratory diagnostic tests and not be used solely. On the one hand, the results from POCTs often provide lower specificity (more false positives), which identifies noninfected persons as infected ones, exposing them to the risk of nosocomial infections until the correct result of the laboratory examination becomes known. On the other hand, there is imperfect sensitivity, which fails to detect infected persons who then spread the disease further among the population. A high sensitivity of rapid tests is therefore always comparatively more important than the level of specificity. The exclusive use of POCTs is justified when resources are scarce. This mainly refers to a shortage of medical supplies but also to the available equipment such as the bed capacity.

This is more often the case in **nonhospital setups** where the supply of already limited resources needs to be ensured and access to laboratory testing is often not available around the clock. Through POCTs, the distribution of medical resources can be controlled and the spread of epidemics slowed down or even prevented by adequate containment measures. High sensitivity of the rapid tests is more important than their specificity. In remote regions, the mere accessibility to diagnostic tests has a greater impact on health care than sensitivity and specificity,[104] which can be confirmed by a model of the public health impact of new theoretical malaria diagnostics where diagnostic accessibility was found to be a more influential parameter on total lives saved than diagnostic sensitivity or specificity.[105]

This way, even remote areas can be reached, and infected patients can later be transferred to centralized facilities if necessary.

Screening for infectious diseases via POCTs could also be carried out by nonmedical personnel, without the need for a physician to be present in crowded places, such as airports or refugee camps, or at major event sites. This could stop the further distribution of the pathogen around the globe. In 2015, one passenger infected with Middle East Respiratory Syndrome returning to the Republic of Korea caused an outbreak of the disease with 36 deaths and an outbreak-related loss of approximately 8 billion US dollars.[106]

The use of body fluids other than blood as test media offers a further decisive advantage in POCTs. Saliva and urine have gained increased importance as test materials, because they are immediately available and can be obtained noninvasively, which in turn reduces the risk of infection, and depending on

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the disease, increased concentrations of markers can be measured.[107,108]

Figure 5 gives a summary about the current advantages and disadvantages of conventional lab tests and POCTs and their separate or joint use in different settings. It provides solutions for the disadvantages of POCTs through the already presented developments in the POCT market.

ADVANTAGES AND SHORTCOMINGS OF FRUGAL POCTs: THE GAPS TO THE OPTIMAL DIAGNOSTIC DEVICE

The above-described cases and application scenarios of advanced POCTs contain characteristics of the frugal innovation concept and are undoubtedly moving toward a health care system designed for emerging markets or constraint-based contexts. Putting emphasis on time and cost efficiency and on nonhospital usage options, as well as for highly complex examinations, marks a turnaround in diagnostics that offers an abundance of starting points for new development, examinations, and treatments. The following paragraphs contrast the main achievements of frugal POCTs with its ongoing shortcomings against the background of frugal and ASSURED criteria (Table 1). Finally, evidence from frugal innovation literature is given on how to solve identified deficits.

Cost Dimension. POCTs demonstrate enormous cost advantages on several levels. For the examined patient, they display high and direct affordability due to their low purchase price. Indirectly they lead to savings in travel costs when tests are performed at the patient's home or remote medical site. For the health care providers, the biggest cost advantage results from the use of alternative detectors in frugal POCTs. As the detector is the biggest cost driver, its substitution with less expensive options such as human biosensors (e.g., the tongue) or smartphone-based technologies is very helpful. The health care providers experience indirect cost savings through more efficient resource allocation in affected areas because data on the context of the disease and the patients is available faster. Direct benefits from POCT systems compared to conventional lab equipment can also be expected for test manufacturers as soon as new POCTs are authorized by regulatory authorities and can be produced on a large scale. Insurance companies and public health care should especially bear in mind that lengthy treatment costs and the usage of partly ineffective medication can be avoided through early detection and the fast administration of drugs that are only effective within a limited time frame.

The time aspect is a further influencing factor on the overall costs of POCT for all involved parties. A timely diagnostic result leads in the follow-up to several cost advantages in further treatment and medication and in avoiding the spread of the infection.

The upside potential of frugal POCTs rises especially for xPOCTs. They offer a great advantage concerning savings in materials and resources as well as examination time and costs as they can detect several analytes from one single specimen at the same time. Yet, these most promising approaches on the basis of cutting-edge technologies need to become more cost-effective in themselves to become a true frugal solution for mass application in epidemic scenarios. A typical approach in frugal innovation to solve this problem is collaborating with established market players to promote the diffusion and commercialization of the system. But also working together with nontraditional market players (community workers, nonmedical operators of the system, etc.) is crucial for the system's success as it enables a strong integration in the local context, increases user trust, and ensures its adaptation to the specific requirements.[109−111] In order to downsize costs, not only the final offering should be taken into consideration. It is, indeed, vital to show a frugal engineering mindset, where already in each development step of the innovation process of the new POCT frugality is practiced. This can happen through the localization of the R&D to the test country and the sourcing of local material and employees to increase proximity to the application context.[9,20,112,113]

A further shortcoming is that more efficient and effective POCTs do not solve the issue of how and who will finance the subsequent, oftentimes long and uncertain treatment and containment of infectious epidemic disease patients. Affordability may not stop after diagnostics; otherwise the purpose of frugal innovations as need-based solution is obsolete. The establishment of a comprehensive frugal health care system instead of mere nonscalable on−off solutions needs to be the focus. On the one hand, this might reduce the objections of medical practitioners against POCTs; on the other hand a multitude of new business possibilities will arise for established health care market firms to participate in this frugal movement, diminishing fears of cannibalization.[32,114,115]

Functionality Dimension. This point responds to the usability aspects observed in frugal POCTs. Summarizing the above outlined cases, it becomes clear that priority needs to be given to accessibility before sensitivity and sensitivity, in turn, before specificity in order to achieve comprehensive and effective diagnostics for infectious diseases on a worldwide scale. Accessibility is reached by, for example, downsizing and miniaturizing for the POCT to be portable and mobile.

Compared to conventional lab-based testing equipment, POCTs only require low sample consumption (e.g., blood) or work with easily accessible, noninvasive body fluids other than blood as test materials. This makes testing safe and easy and allows for the employment of nonmedical staff. Also the use of new or unconventional technologies as described above (substitution of expensive parts (e.g., the detector) through inexpensive alternatives (human senses of taste or vision) or related technologies (smartphone camera)) broadens the application field of POCTs and makes them more user-friendly. If POCTs are operated through smartphone devices or fitness trackers as well as by the patient him- or herself as in the case of the diagnostic chewing gum the limits to where medicine can be practiced have started to become blurry and are brought to a new level.

In the medium term, also sensitivity and specificity need to be improved in frugal POCTs. Their so-far rather unsatisfying performance levels compared to lab-tests constitute a major disadvantage in the ultimate usability of POCTs. Here again, the miniaturization of typical lab tests such as PCR systems (see POCT for TB diagnostics) or the use of technologies from different areas (e.g., car battery), as well as multiplexing capabilities, can lead to considerable progress in this aspect.

Ultimately, connecting POCT devices to databases and larger networks allows for new functionalities such as the sharing of health care data and the evolution of powerful data pools, from which researchers and practitioners can learn and improve their activities in order to preempt certain epidemics in the future.

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Performance Dimension. The optimized performance level, a frugal criterion that indicates the importance of quality in frugal products and thus distinguishes them clearly from mere low-cost, "cheap" solutions, is given through POCT clinical evaluation and approval. It is significant that despite extensive simplification and miniaturization as well as multiplexing, the quality of POCT results is appropriate and reliable. This finding makes frugal POCTs a true globally applicable system.

As mentioned above, the time aspect is a critical cost factor, but it also affects performance. In Germany, POCTs are often referred to as "Schnelltests", which directly translates to "fast test". Rapid turnaround times (TATs) are expected from both physicians and patients. The TAT has to be balanced with sensitivity and specificity of the product, as they often mutually influence each other. For successful commercialization of a new POCT, the TAT mostly needs to be put into the foreground. In order to clearly stand out from competitors, the TAT is a necessary unique selling proposition.

Modern POCTs also have to be robust, reliable, and give reproducible results for on-site applications in emergency settings worldwide. Sensitive chemicals and electronic compounds have to be stored safely for transportation. Especially in nonhospital settings, humidity and dust resistance is of utmost importance. It has to be ensured by stress tests that POCTs work reliably under these extreme situations.

As Wimschneider et al.[24] found in their study of frugal innovations in Brazil, just because a product is affordable and appropriately solves a need does not automatically mean that the product is adopted by the users. Social norms and the need for status and prestige are factors that should not be underestimated. They decide the perception and consumption of specific products, as well as the use of certain services. This is especially the case for unknown, unconventional offerings as in frugal innovations. Even though POCTs are already widely used in diagnostics such as in pregnancy or as blood glucose meters, their adoption as an inexpensive frugal service could be viewed with greater skepticism if it is about a more critical or stigmatized disease. Making use of a POCT in these cases might be interpreted as a sign of financial incapacity or even might lead to the risk of social exclusion. Such an image could have severe consequences for the success of a POCT. A solution or precautionary measure to avoid such a reaction toward POCTs is education and awareness building. It could well be that the trend to use POCTs based on smartphone technology and together with health and fitness perceived as lifestyle activity could automatically lead to higher acceptance and awareness among patients.

Furthermore, pricing is a key factor, especially in countries where health care costs are not covered by insurances but assumed directly by the patient. Instead of offering the lowest price possible and thus signaling low-quality or labeling a frugal POCT as a second-class service for the poor, POCT providers should come up with different affordable payment models, designed for different customer segments and necessities. This could include buying and leasing models on the B2B side as well as pay-per-test strips and insurance-based models on the B2C side.

Performance of frugal POCTs (Figure 6) is particularly superior compared to conventional diagnostics if time and capacity are critical. A fast diagnosis and decision on hospitalization or discharge significantly contains the further spread of the epidemic, and resources can be distributed more effectively to those in need. Also, for diseases that are subject to stigmatization in some cultures such as HIV, a fast, discrete test at home by the patients themselves corresponds better to the cultural circumstances than a clinical test.

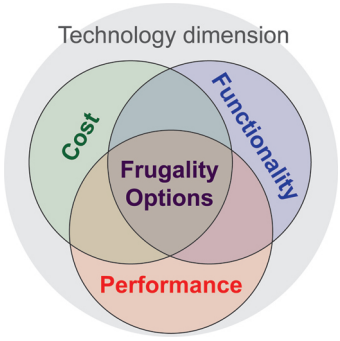

Figure 6. Diagnostic frugality approach portrayed as a Venn diagram. Technology dimension: high technological impact advances frugality. Cost: direct cost advantages through purchase price, application of alternative technologies, efficient usage, and savings of material; indirect cost advantages through faster and more effective subsequent treatment, simplified access to diagnostics. Functionality: high accessibility (easy-to-use, portable and mobile), low sample consumption (blood) or working with easily accessible (noninvasive) body fluids, rich health care data (e.g., through smartphone connectivity of POCTs) will lead to more efficient and specific diagnostics. Performance: ability to perform state-of-the-art testing methods (multiplexing), optimal adaptation to unfavorable circumstances, especially concerning time and capacity, ability to circumvent social and cultural barriers. Frugality Options: (1) New service to former unserved areas. Self-monitoring of diseases, medical coverage of rural and remote areas, telemedical sharing of test results. Especially in (rural) emerging market regions. (2) Better service to formerly poorly served areas. More specific treatment options, more efficient containment measures. Especially in emerging market regions and rural developed market areas. (3) Additional service to already well served areas. Advanced patient management in hospitals, more accurate epidemic forecasting models through data analysis. Especially in developed market regions.

Technology Dimension. Technology plays a key role in many frugal innovations, and so it does in POCTs.[9,24,32] In sum, it can be argued that frugality options are first and foremost enabled and increased through new technologies and advances in disciplines such as material sciences, (bio)sensor technology, or the rediscovery of the natural diagnostic capabilities of the human senses. This said, frugal POCTs can be regarded as Advanced Frugal Innovations.[33] Therefore, the development of frugal POCTs is a promising business activity even more so for providers of established and expensive care. They possess the technological capabilities, resources, and infrastructure to be at the forefront of their development. Agarwal et al.[34] further found that the most important product requirement concerning health care innovations for physicians in emerging markets is low or no maintenance of the equipment. However, it seems that this has been a neglected aspect so far for Western health care equipment providers. Well-functioning, robust POCTs represent low-maintenance devices; therefore this sector could be a good point for companies active in health care to start their frugal activities.

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IMPLICATIONS FROM FRUGAL POCTs

In order to overcome the outlined key challenges, some key measures need to be taken.

Easy-to-Operate, Simple with Basic Functionalities, and Readily Available. These three characteristics form the cornerstones of a successful frugal diagnostic. But even if easy-to-operate, necessary compromises for these cost-effective variants may not affect the quality of the diagnostics, so that they hold up to scientific and clinic evaluations before widespread utilization and in order not to violate frugal innovations' dictum of optimal performance.[116] As POCT systems are supposed to be utilized by nonmedically trained patients, the diagnostic outcome is largely operator dependent. Incorrect operation, poorly controlled environments with the possibility of interference with endogenous substances, and degradation of reagents and controls can negatively affect test results.[117] High analytical precision and reproducibility for analyte quantification in large numbers of patient samples have to be guaranteed. According to the latest legislation, the distributor must assess the risk of misuse and prevent it through constant market observation. Training and continued education programs need to be established along the commercialization of POCTs in order to constantly ensure proper and effective handling and to stay connected with the local context conditions. Also, sociocultural challenges of a market might stand in the way of its establishment. For instance, diagnoses of infections such as HIV might be subject to stigmatization and thus make the handling of POCTs more complex. Due to their newness and simplicity in functionality and price, frugal products sometimes can invoke negative social judgment and skepticism, which in case of stigmatized diseases can increase the effect.[118]

Diagnostics without sufficient access to treatment options is another challenge. Without established medical care, POCTs merely increase the quality of containment measurements to prevent further spread of the disease but not its cure. As already mentioned, if treatment is available (even in limited amount), frugal innovation can help to distribute scarce resources and manage medical material. Due to POCT diagnostics, health care delivery becomes increasingly decentralized. Healthcare professionals as well as patients have to adapt their workflows and behavior to fit into this new situation. Some clinics address this challenge by appointing point-of-care coordinators responsible for correct documentation of results, regulatory compliance, consistent procedures, and end user assistance.[119]

Nevertheless, one must not forget that the infrastructure and thus distribution channels of services are the limiting factor in many places. For this reason, despite the simple applicability of POCTs, their dissemination and access to patients may be restricted and delayed. Functioning distribution channels that can keep up with innovative services (e.g., AFIs) depend on a solid health care system and continuous investment in infrastructure. This is still a shortcoming of POCTs, where health care providers, policy makers, and government agencies must work closely together. In many cases, the market entry of a POCT could be faster if downstream activities were ready. In this regard, emerging markets also have some advantages to offer. For instance, data from Sub-Saharan Africa shows that the vast majority of the population is connected to the Internet via mobile networks.[120] For Latin America, forecasts expect a smartphone adoption rate of 77% in 2020, as such outperforming the global average of 66%.[121] This strong penetration of smartphone usage and expansion of mobile networks could be beneficial for the diffusion of advanced POCTs and easy handling options via mobile applications and read-outs in these regions. For ensuring the ease-of-use of POCTs, it is strongly recommended to developers and physicians to familiarize themselves with the local technological and infrastructure circumstances and develop POCTs accordingly.

Innovative. As shown in Figure 7, the innovativeness of diagnostics is not the priority for physicians or for developers.

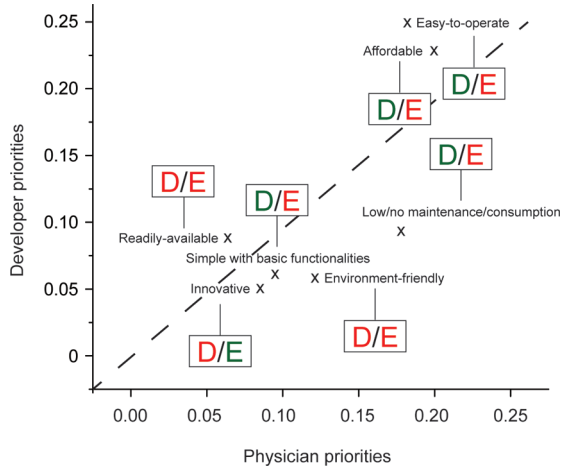

Figure 7. Two-dimensional plot of physician and developer priorities. In addition to Figure 2, the focus of emerging and developed countries on these criteria is illustrated (D = developed countries, E = emerging countries, red = highly important, green = lower importance).

But it is precisely the transfer of innovative technologies into POCTs that will increase the performance of these diagnostic tests in the medium to long term. Open communication between scientists and experienced clinicians helps to determine if an emerging technology is translatable into the POCT format.[122] This aspect is certainly valued more highly in the developed countries than in the developing countries, since it is often the case that basic services first have to be guaranteed and approved there. As mentioned above, a higher GDP is linked to an increase in medical care. Nevertheless, the higher usage rate of mobile devices in emerging markets could also lead to leapfrogging situations, where innovative mobile technologies are first applied in emerging market contexts and then in developed areas.[21]

The widespread use and communication of electronic data have raised concerns about data security and privacy issues. Especially in the medical sector with critical personal health records, this is of utmost importance to patients and data protection agencies. The implementation of using Google Glass to gather and share patient information has so far been unsuccessful, due to data privacy issues.[123] Telemedicine using cloud computing and mHealth has to be secured by advanced encryption technologies and international data storage standards.[117] There has to be a compromise between advancing medical diagnosis via data analyses and protecting patients' data. This might be especially challenging for regions where data privacy is traditionally handled more seriously and strictly and its protection is considered the responsibility of each individual, such as in Europe.[124] With respect to data security

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there is no general, clear-cut distinction between emerging and developed market practices. It is advisable to integrate data protection mechanisms into the POCT systems' functionalities as early as possible and comply with stricter policies in order to ensure applicability in a high number of countries.

Affordable. The economic feasibility is a fundamental aspect for thriving commercialization of POCT devices. Especially in developing countries, POCT systems will be utilized only if accessible to a critical mass of people. Therefore, consultation and involvement of the industry sector in early stages of development is of highest importance. Health insurance companies will only be attracted by increased affordability of diagnostic test routines. Another obstacle in establishing new POCT systems could be the supremacy of large diagnostic companies. Dominant institutional forces tend to fight simpler alternatives because these supposedly threaten their profits.[125] Furthermore, the high monetary hurdles in the POCT approval process can often only be tackled by the big players in the health care sector or by joint collaboration and alliances of many different (local) market players and stakeholders.[110]

Whereas the approval process is of most significant importance for POCTs to be successful in countries where health insurance generally covers the costs for medical services, in emerging markets it might be the different payment models offered. Innovative solutions, such as "pay-as-you-go" options, financial self-help groups,[126] or package prices for large project-based diagnostics, might be some ideas for both developers and physicians to offer their service.

Decrees such as the EU Regulation 2017/746 of the European Parliament and of the Council on in vitro diagnostic medical devices (IVDR) prescribe new requirements for POCTs. On the one hand, the more stringent approval procedure and obligation for recertification will lead to higher performance and safety outcomes in POCTs and thus might raise their reputation and scope of application. On the other hand, this can also pose a high challenge for smaller, financially constrained developers of POCTs to enter and remain in the market.[127,128]

Low or No Maintenance and Consumption. Direct maintenance costs are not incurred for most POCTs compared to complex diagnostic systems. However, the functionality of the tests must be ensured centrally during the manufacturing process. Especially in developing countries, this can be an advantage, as fewer experts have to be available on site. The expertise is thus transferred from the periphery to the headquarters. Particularly in the event of an epidemic, however, the supply routes for POCTs must effectively function and be established resiliently, and also enough test systems must be available in stock. In emergency situations, infrastructure is often overloaded, resulting in supply bottlenecks and long delivery times.

Figure 7 shows that this point is considered particularly important by physicians but is often neglected by developers. This is probably due to physicians' greater practical experience on site. Especially complex POCT evaluation systems, which are used several times, can certainly be optimized concerning this point. Consequently, there is still great potential for developers to develop low or no maintenance POCTs with low resource consumption. Advances in this direction might increase the penetration of POCT usage especially in emerging market regions.

Environmentally Friendly. Climate change and the accelerated public discourse and growing consciousness of it make the environmental impact of POCTs an important commercialization factor. POCTs are most often single-use tests, and the amount of generated waste is very high. Correct disposal must be guaranteed here. As POCTs are often units of several components, the entire unit must be disposed of in accordance to the regulations for the most toxic component in the system. Contamination with blood or other body fluids must also be taken into account. Due to, on average, more advanced recycling systems and possibilities in developed countries, this challenge might be more prominent in emerging countries. It is the responsibility of developers to support these countries with the task and offer environmentally friendly products and recycling solutions.

Thorough market exploration by developers is important to increase the accessibility of POCTs. This is one of the first key steps for successful frugal innovation.[129] While highly developed countries serve virtually all levels of POCT complexity, the situation in developing countries is often different. The lower the country's level of development, the easier and the more adapted to local challenges the performance and evaluation of POCTs should be (Figure 8).

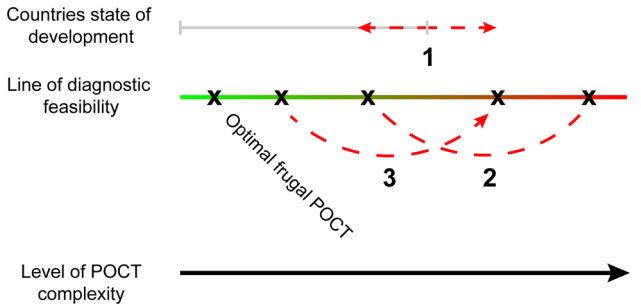

Figure 8. Changes in POCT feasibility between two time points. POCTs are portrayed as ×, placed on the "Line of feasibility" according to their complexity: (1) increase or decrease of the level of development of the targeted country; (2) decrease of technological requirements (smartphone evaluation, more-user-friendly design); (3) increase of technological requirements to gain new features (quantification, multiplexing capability).

In Figure 8, the developed POCT can be placed on the "Line of diagnostic feasibility" according to its complexity of performance and evaluation. If the point lies within the affordability of a country, the market launch is expected to be successful. In order to reach everyone, everywhere, the POCT should be as close as possible to the point of optimal feasibility. However, the simplification of the POCT often goes hand in hand with a reduction in performance, so a reasonable balance has to be found.

This state of development is highly dynamic as Figure 8 indicates. For example, previously underdeveloped countries can improve their medical infrastructure to address more products following the "Line of diagnostic feasibility". However, this can also be the opposite (e.g., war, collapse of states). But also product requirements can change. Thus, a decrease of technological requirements leads to a shift of the product on the "line of feasibility" downward. If new features are implemented, an increase in complexity is to be expected, which can no longer be provided by every country. An exact

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Table 3. Recommendations for POCT Developers and Physicians

developers	physicians
analyze the current state of development of the targeted country and if the developed POCT is usable within the existing medical infrastructure → adapt POCT accordingly	consider context-dependent operation of POCT (hospital/nonhospital)
look out for multiplexing opportunities	train nonclinical staff on epidemic relevant places (e.g., airports, train stations)
search for and evaluate new technologies for their POCT potential	implement state-of-the-art patient and diagnostic management routines for upcoming epidemics (eHealth, mHealth)
process test result with the help of telemedicine and state-of-the-art data security	collect and share data to establish better epidemic forecasting models

evaluation of product and target country is therefore obligatory prior to market launch.

Based on the examples discussed, Table 3 summarizes practical advice for the development and establishment of POCT by developers and physicians, allowing the full potential of POCTs to be exploited.

CONCLUSION

An ever faster changing world requires diagnostics that can keep pace with specific local conditions and challenges and still be capable of efficiently generating test results. POCTs are a promising way to realize this. Rethinking conventional development approaches outside the box and taking on new perspectives has led to extremely successful frugal innovations in this field. While the majority of rapid tests are based on chemical and biotechnological reactions, digitization is pushing heavily into this market.[130] Results are read out in electronic form and monitored via smartphone applications. eHealth, mHealth, and telemedicine are already buzzwords leading to futuristic smart devices enabling affordable personalized health care. By focusing on resources nearly everyone possesses (a smartphone, taste, visual read-out) and low-cost POCT kits, the utilization of POCT diagnostics in every area worldwide is possible. Properly applied, they could limit the spread of epidemics and improve the distribution of medical supplies. Especially in remote areas, where medicines have to be deployed sparingly, they could relieve the burden on health systems.

Further development of POCT diagnostics positively impacts health systems around the globe.[131] In 2004, the Bill & Melinda Gates Foundation organized a Global Health Diagnostics Forum on the attributable benefits of improved global POCT systems.[131] The conclusion of this symposium was that investment in increasing access to POCTs would have a greater impact than enhancements of test performance. This statement is a true advocate for the support of frugal innovations. But not only the POCT market is facing exciting transformations. The global health care market in general has to adapt to this new situation. POCT systems meeting the requirements of frugal innovations (affordable, rapid, and easy-to-use) can make health care solutions more personalized and efficient in epidemic situations, so that low-cost comes hand in hand with high-quality.

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#T.M. and C.W. contributed equally to the work.

Notes

The authors declare the following competing financial interest(s): L.M. owns shares of 3a diagnostics, a company developing chewing gum based diagnostics. The chewing gum is mentioned in this Perspective.

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5. AIM OF THESIS

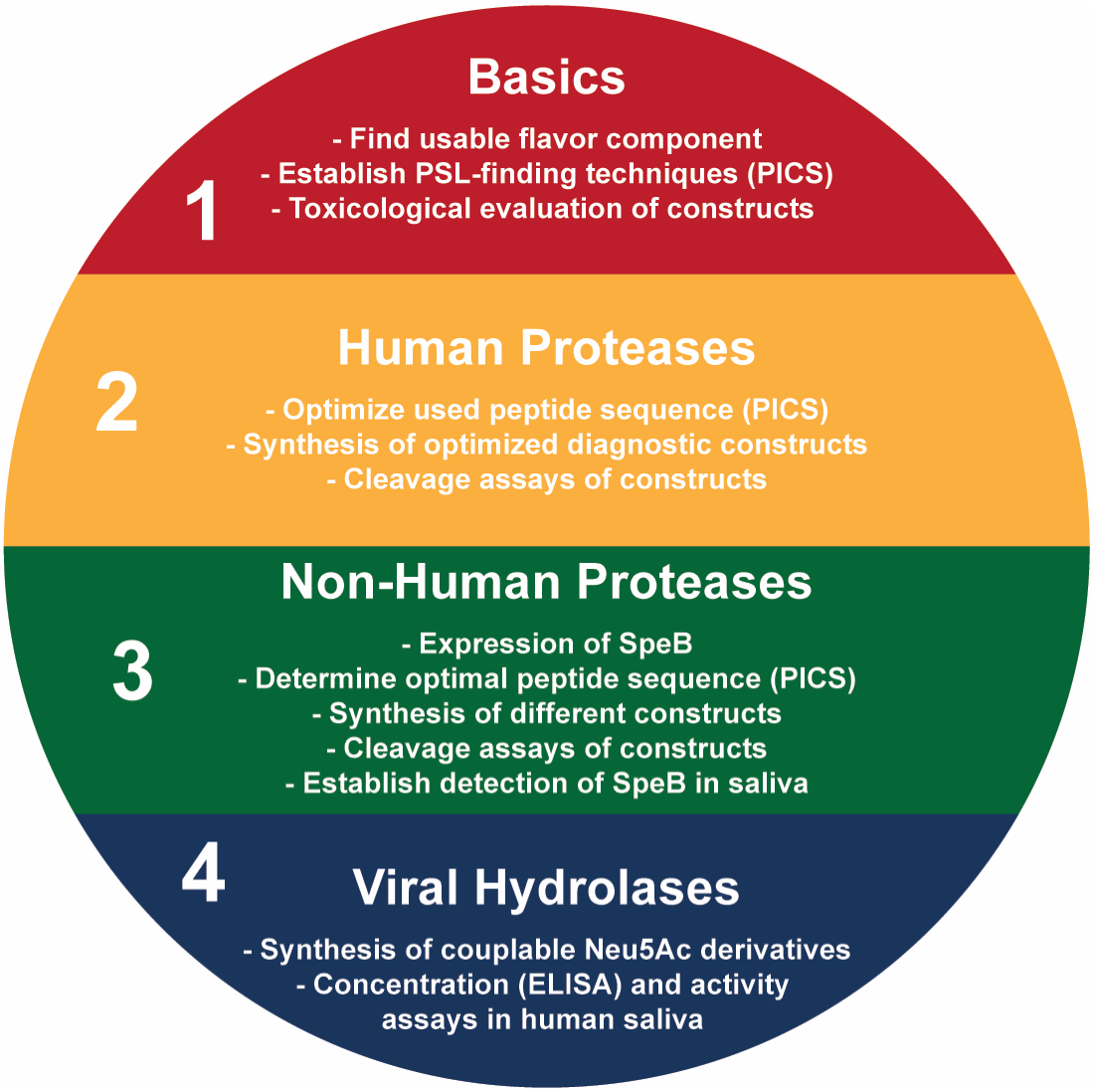
The aim of this work was to further develop an existing diagnostic system, that can only be cleaved by specific enzymes. After cleavage by the target protease and subsequent degradation by human aminopeptidase a flavor is released which can be tasted by the patient. Integrated in a chewing gum, the diagnostic system should indicate inflammations and infections in the oral cavity.

So far, the denatonium-peptide constructs were coupled to PMMA particles in order to mask the bitter taste and to block the degradation of the peptide by the body's own aminopeptidases. Previous studies showed, that the gustatory perception of denatonium is already completely blocked by several AAs, if the denatonium compound is linked to the C-terminus of the peptide via an amine function. Therefore, compared to the prior art, PMMA particles should be replaced by smaller molecules, to streamline the diagnostic system.

The peptide sequence that constitutes the PSL was designed according to the addressed target protease. A method to determine new cleavage sites, addressing new indications was implemented.

Denatonium has been modified by introducing a carboxyl group so that it was coupled to the peptide chain via lysine. This not only led to very small coupling yields, but also left an AA on the denatonium after degradation by human AP, which reduced its taste intensity. This problem was to be solved by using a new denatonium with an amino function coupled to the C-terminus of the PSL. The new flavor was evaluated, regarding its gustatory perception, the coupling efficacy to peptides and its toxicological profile.

To develop a diagnostic system for the detection of viral infections, a modified neuraminidase was synthesized. Since position 4 in sialic acids plays an essential role in the interaction between enzyme and substrate, this position was modified with known methods.


1
Basics
- Find usable flavor component
- Establish PSL-finding techniques (PICS)
- Toxicological evaluation of constructs
2
Human Proteases
- Optimize used peptide sequence (PICS)
- Synthesis of optimized diagnostic constructs
- Cleavage assays of constructs
3
Non-Human Proteases
- Expression of SpeB
- Determine optimal peptide sequence (PICS)
- Synthesis of different constructs
- Cleavage assays of constructs
- Establish detection of SpeB in saliva
4
Viral Hydrolases
- Synthesis of couplable Neu5Ac derivatives
- Concentration (ELISA) and activity assays in human saliva

6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Equipment and Devices

Device	Type	Manufacturer
Range XR 105	AT 21 Comparator	Mettler Toledo
	Delta Range XP 105	Mettler Toledo
	PB3002 DeltaRange®	Mettler Toledo
Centrifuge	Allegra ® X-15R + SX4750A rotor	Beckman Coulter
	Varifuge 3.ORS	Heraeus
	miniSpin 2416 centrifuge	Eppendorf
	Sprout™	Biozym
Dialysis Membrane	SpectraPor MWCO 6000-8000	SPECTRUM LABORATORIES INC
Electrophoresis Power	Model 3000Xi	Bio-Rad
Evaporator	TurboVap® LV Evaporator	Zymark
Freeze-drier	Alpha 1-4	Christ
	Benchtop K	VirTis
Hemocytometer	Neubauer	LO Laboroptik GmbH
Incubator	TH15 - KS15 Control	Edmund Bühler GmbH
	MIR 220 shaking incubator	Sanyo Gallenkamp
Mass spectrometry	Shimadzu 2020	Shimadzu Scientific instruments
	Autoflex II LRF (MALDI)	Bruker Daltonics
	micOTOF-focus (ESI)	Bruker Daltonics
Sample incubation	Thermomixer Thermostat 5320	Eppendorf
	HX Mini	peqlab
UV/Vis spectrophotometer	Genesys 10S UV-Vis + Tray Cell (HellmaAnalytics)	Thermo Scientific
Microscope		VWR
Microwave	NN-E209W	Panasonic

Device	Type	Manufacturer
NMR	Avance 400 Spectrometer	Bruker BioSpin MRI GmbH
Rollermixer	Roller mixer - SRT1	Stuart
Vortex Mixer	VV3	VWR
Geldocumentation	FluorChem® FC2	Alpha Innotech
Peptide Synthesizer	SP Wave LibertyBLUE	Biotage CEM
pH Meter	pHenomenal™ pH 1000 L + pHenomenal 221	VWR
Pipette controller	Accurpette	VWR
Platereader	SpectraMax 250 Gene flash doku system	Molecular Devices Syngene
Protein and construct purification system	ÄKTApure ÄKTApurifier	GE GE
Safety Cabinets	SAFE 2020	Thermo Scientific
Shaker	KS 130basic Duomax 1030 (Gel stain/destain)	IKA Heidolph
Ultracentrifuge	L8-60M	Beckman-Coulter
UPLC	Elite LaChrome Ultra 1260 Infinity II	VWR Agilent
Water bath	ED thermostate	Julabo

6.1.2. Substances

Solid Phase Peptide Synthesis

Name	Manufacturer
Acetic anhydride (Ac_2O)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Fmoc-protected amino-acids	Novabiochem Merck-Millipore (Darmstadt, Germany)
Chlorotrityl chloride resin (CTC)	Chem-Impex Wood (Dale, IL, USA)
Dichloromethane (DCM)	Fisher Scientific (Schwerte, Germany)
Diethyl ether	Sigma-Aldrich Chemie (Schnelldorf, Germany)
N,N′-Diisopropylcarbodiimide (DIC)	Fluka (Buchs, Switzerland)
N,N-Diisopropylethylamine (DIPEA)	Carl-Roth GmbH (Karlsruhe, Germany)
Dimethylformamide (DMF)	Fisher Scientific (Schwerte, Germany)
1-Hydroxybenzotriazole hydrate (HOBt)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Methanol (MeOH)	Fisher Scientific (Schwerte, Germany)
Piperidine	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Trifluoroacetic acid (TFA)	VWR (Radnor, Pennsylvania, USA)

Chromatographic characterization and purification of peptides and constructs

Name	Manufacturer
Acetonitrile (ACN) (HPLC grade)	VWR (Radnor, Pennsylvania, USA)
Formic acid	Sigma-Aldrich Chemie (Schnelldorf, Germany)

Name	Manufacturer
Methanol (HPLC grade)	VWR (Radnor, Pennsylvania, USA)
Trifluoroacetic acid (TFA)	VWR (Radnor, Pennsylvania, USA)
Ultra-pure water for chromatography	Prepared by a water purification system (Merck Millipore (Darmstadt, Germany))

Cleavage assays and protein expression

Name	Manufacturer
Acrylamide/ N,N'-methylenebisacrylamide	Carl-Roth GmbH (Karlsruhe, Germany)
Bromophenol blue	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$CaCl_2$	Sigma-Aldrich Chemie (Schnelldorf, Germany)
D/L-dithiothreitol (DTT)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Glacial acetic acid	Sigma-Aldrich Chemie (Schnelldorf, Germany)
α-D-Glucose	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Glycerol	VWR (Radnor, Pennsylvania, USA)
Glycine	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Kanamycin	Sigma-Aldrich Chemie (Schnelldorf, Germany)
KCl	Grüssing GmbH (Filsum, Germany)
KH_2PO_4	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Lactose	Sigma-Aldrich Chemie (Schnelldorf, Germany)

Name	Manufacturer
MeOH	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$MgCl_2$	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$MgSO_4$*7H_2O	Sigma-Aldrich Chemie (Schnelldorf, Germany)
NaCl	Sigma-Aldrich Chemie (Schnelldorf, Germany)
NaH_2PO_4	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Na_2HPO_4	Sigma-Aldrich Chemie (Schnelldorf, Germany)
NaOAc	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Sodium dodecyl sulfate	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Tetramethylethylendiamin (TEMED)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Tris base	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Tryptone	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Tween-20	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Yeast-extract	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$ZnCl_2$	Sigma-Aldrich Chemie (Schnelldorf, Germany)

Purchased solutions and media

Name	Manufacturer
Dulbecco's modified eagle’s medium high glucose (DMEM) (D5796)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Penicillin/Streptomycin solution (P/S sol.) (P4333)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Fetal calf serum (FCS)	Sigma-Aldrich Chemie (Schnelldorf, Germany)

Name	Manufacturer
Roti®-Block	Carl-Roth GmbH (Karlsruhe, Germany)

Carbohydrate synthesis

Name	Manufacturer
Acetone	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Ac_2O	Sigma-Aldrich Chemie (Schnelldorf, Germany)
BaO	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$Ba(OH)_2$	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Camphorsulfonic acid	Sigma-Aldrich Chemie (Schnelldorf, Germany)
DCM	Sigma-Aldrich Chemie (Schnelldorf, Germany)
4-(Dimethylamino)pyridine (DMAP)	Sigma-Aldrich Chemie (Schnelldorf, Germany)
DMF	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Ethyl acetate	Sigma-Aldrich Chemie (Schnelldorf, Germany)
HCl	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Methyl iodide	Sigma-Aldrich Chemie (Schnelldorf, Germany)
MeOH	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Mg_2SO_4	Sigma-Aldrich Chemie (Schnelldorf, Germany)
NaCl	Sigma-Aldrich Chemie (Schnelldorf, Germany)
N-acetylneuraminic acid	Sigma-Aldrich Chemie (Schnelldorf, Germany)
NaOH	Sigma-Aldrich Chemie (Schnelldorf, Germany)
$Na_2S_2O_3$	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Nafion NR50	Sigma-Aldrich Chemie (Schnelldorf, Germany)
Neu5Ac	Carbosynth (Compton, Newbury, UK)

Name	Manufacturer
Triethylamine (TEA)	Sigma-Aldrich Chemie (Schnelldorf, Germany)

Cell culture

Organisms and cell lines

E. Coli	**genotype**
DH5α	F-ϕ80 *lac*ZΔM15D (*lac*ZYA- *arg*F) U169 *deo*R *rec*A1 *end*A1*hsd*R17 (rk-,mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 l-
BL21 (DE3)	*E. coli* str. B F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)

Plasmids used for protein expression

Name	**Used abbreviation**	**Antibiotic resistance**	**Manufacturer**	**Expressed protein**
***SpeB*-M3C3**	*SpeB*-M3C3	kanamycin	Burkhard Seelig	*SpeB*

Used enzymes

Enzymes	**Manufacturer**	**Used for**
MMP-1	EMD Millipore Corp.	MMP cleavage assay
MMP-8	EMD Millipore Corp.	MMP cleavage assay
MMP-9	EMD Millipore Corp.	MMP cleavage assay
Leucine AP	Sigma Aldrich	AP cleavage assay
Trypsin	Promega	PICS Assay

Enzymes	Manufacturer	Used for
***Staphylococcus aureus* Protease V8 (GluC)**	Promega	PICS Assay

Used markers

Gene Ruler 1kb Plus, as well as Page Ruler prestained protein 10-180 kDa ladder were bought from Thermo Fisher Scientific

Used equipment for chromatography (analytical and preparative)

Name	Manufacturer	Used for
HiTrap HP XL 5 mL	GE Healthcare	Cation exchange chromatography
Hiload™ 16/600 Superdex™ 75 pg	GE Healthcare	Size exclusion chromatography

Used antibodies

Name	Order	Type	Origin species	Manufacturer	Used for
***SpeB* Ab**	1st Ab	polyclonal	rabbit	Biozol	Western blot
Anti-rabbit IgG	2nd Ab	polyclonal	goat	Thermo Fisher Scientific	Western blot

Solutions used for SDS-PAGE and Western blotting

Name		
Acrylamide/Bis-acrylamide solution 30%/0.8%	30% (w/v) 0.8% (w/v)	Acrylamide N,N‘-methylenebisacrylamide

Name		
4x stacking gel buffer	1.38 mM	Sodium dodecyl sulfate (SDS)
	375 mM	1.5 M Tris-CL solution pH 8.8
4x separating gel buffer	1.38 mM	Sodium dodecyl sulfate (SDS)
	125 mM	0.5 M Tris-CL solution pH 6.8
1x SDS-PAGE buffer pH 8.3	30 mM	Tris base
	19.1 mM	Glycine
	3.4 mM	Sodium dodecyl sulfate (SDS)
6x SDS sample buffer	714 mM	0.5 M Tris-Cl solution pH 6.8
	4.1 M	Glycerol (30% (v/v))
	3.4 mM	Sodium dodecyl sulfate (SDS)
	602 mM	D/L-dithiothreitol
	180 µM	Bromophenol blue
Coomassie stain solution	0.1% (w/v)	Coomassie Brilliant Blue
	10% (v/v)	Glacial acetic acid
	50% (v/v)	MeOH
Coomassie destain solution	10% (v/v)	Glacial acetic acid
	20% (v/v)	MeOH
1x Western blot buffer	37.5 mM	Tris base
	28.8 mM	Glycine
	20% (v/v)	MeOH
	80% (v/v)	ddH_2O
Tris-buffered saline-Tween 20 (TBS-T)	154 mM	NaCl
	0.5% (v/v)	Tween-20
	99.5% (v/v)	0.1 M Tris-CL solution pH 7.5
Blocking solution	10% (v/v)	10x Roti®-Block
	90% (v/v)	TBS-T

Media used for protein expression and purification

Name			
LB medium	1%	(w/v)	Tryptone
	0.5%	(w/v)	NaCl
	0.5%	(w/v)	Yeast-extract
	0.241%	(w/v)	$MgSO_4$
	0.1%	(w/v)	α-D-Glucose
TB medium	13.3%	(w/v)	Tryptone
	26.6%	(w/v)	Yeast-extract
	4.44%	(w/v)	Glycerol
10x PBS	1.37	M	NaCl
	26.92	mM	KCl
	100	mM	Na_2HPO_4
	18	mM	KH_2PO_4
SOC medium	2%	(w/v)	Tryptone
	0.5%	(w/v)	Yeast extract
	10	mM	NaCl
	2.5	mM	KCl
	10	mM	$MgCl_2$
	10	mM	$MgSO_4*7H_2O$
	20	mM	Glucose
Autinduction medium	50	mM	Na_2PO_4
	50	mM	KH_2PO_4
	2	mM	$MgSO_4$
	36	µg/mL	Kanamycin
	2%	(w/v)	Tryptone
	0.5%	(w/v)	Yeast extract
	0.5%	(w/v)	NaCl
	60%	(w/v)	Glycerol
	10%	(w/v)	Glucose
	8%	(w/v)	Lactose

Name			
Culture Medium			DMEM high-glucose
	10%	(v/v)	FCS
	1%	(v/v)	P/S

Buffers

Name			
Human Matrix Metalloproteinase buffer pH 7.4	200	mM	NaCl
	50	mM	Tris-HCl
	5	mM	$CaCl_2$
	1	µM	$ZnCl_2$
SpeB buffer pH 7.0	50	mM	Sodium phosphate
	2	mM	DTT
Leucine aminopeptidase buffer pH 7.0	50	mM	Tris-HCl
	1	mM	$CaCl_2$
	150	mM	NaCl
Cation exchange			
SpeB buffer A (pH 5.0)	20	mM	NaOAc
SpeB buffer B (pH 5.0)	50	mM	NaCl
	20	mM	NaOAc
	200	mM	NaCl
SEC Buffer pH 7.4	25	mM	Sodium phosphate
	150	mM	NaCl

Used Kits

Name	Manufacturer	Used for
Plasmid DNA Mini Kit I	Omega bio-tek	Plasmid DNA purification

Name	Manufacturer	Used for
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Sigma Aldrich	Immunodetection
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	PICS Assay
Bradford Assay	Sigma Aldrich	PICS Assay

6.2. Methods

Lyophilization

For long-time storage, Den derivatives, peptides and final constructs were lyophilized and stored at -80 °C.

Primary lyophilization of purified constructs was carried out utilizing a laboratory freezing dryer (Alpha 1-4, Christ). After purification by fast protein liquid chromatography (FPLC), constructs were put under a nitrogen atmosphere prior to lyophilization in order to remove containing acetonitrile (ACN). Aliquots of 2 - 5 mL volume were frozen at -80 °C for at least 2 h or with liquid nitrogen for 10 min. Lyophilized samples were stored at -80 °C.

Final lyophilization was achieved by dissolving the lyophilized sample in ultra-pure water and lyophilize it for a second time (Benchtop K, VirTis). A second lyophilization provides the constructs as a white powder, which can easily be weighed in the correct amount.

Preparative peptide and construct purification

After confirmed synthesis, the purification of the Den derivatives, peptides and final constructs was performed by preparative HPLC (GE Äkta Pure 25). A preparative reversed phase C18 column (Phenomenex Jupiter 15 µm C18; 250 x 21.2 mm; particle size 800 Å) served as the stationary phase. The mobile phase consisted of eluent A (0.1% (v/v) TFA in water) and eluent B (0.1% (v/v) TFA in ACN).

The unpurified compounds were prepared for purification in differently concentrated ACN solutions (ACN in ultra-pure water and 0.1% (v/v) TFA). The preparative column was equilibrated with 5 CV in the same concentration ACN as in the peptide solution. After that, the column was loaded with the sample solution either manually or via a superloop (GE Healthcare). The column was washed with 2 CV and the peptides were then eluted from the column by a linear gradient of the mobile phase (up to 95% (v/v) eluent B). Fractions were collected by a fraction collector F9R in 6 mL fractions. The column was washed with 95% phase B and afterwards properly equilibrated for the following sample. The purity of the fractions was subsequently analyzed via HPLC, MALDI-TOF-MS or LC-MS.

Analytical high performance liquid chromatography (HPLC)

HPLC analysis was performed using a VWR Hitachi Elite LaChrom HPLC (Autosampler L-2200; Pump L-2130; Column Oven L-2300; UV detector L-2400) as well as an Hitachi LaChrom Ultra UHPLC system (Autosampler L-2200U; Pump L-2160U; Column Oven L-2300; Diode Array Detector L-2455U) (both VWR International GmbH, Darmstadt, Germany). A ZORBAX Eclipse XDB-C18 column (4.6 mm internal diameter, 150 mm length) (Agilent, Santa Clara,

CA) was utilized; the mobile phase consisted of eluent A (0.1% (v/v) TFA in ultra-pure water) and eluent B (0.1% (v/v) TFA in ACN). Column oven temperature was kept at 25 °C and the absorbance was monitored at a wavelength of λ = 214 nm, unless specified otherwise.

To test the correct function of used columns, 10 µL of a “HPLC Peptide-Standard-Mix” obtained from Sigma Aldrich were injected and analyzed after the run, using the proposed parameters.

Liquid chromatography-mass spectrometry LC-MS

The LC-MS system from Shimadzu contained a DGU-20A3R degassing unit, a LC20AB liquid chromatograph, and a SPD-20A UV/Vis detector (Shimadzu Scientific Instruments, Columbia, MD, USA). Mass spectra were obtained with an LC-MS 2020. A Synergi 4 µm fusion-RP column (150 x 4.6 mm) (Phenomenex Inc., Torrance, CA) was used, as well as eluent A (0.1% (v/v) formic acid (FA) in ultra-pure water) and eluent B (0.1% (v/v) FA in MeOH) as mobile phases. The detection range was set to 60 - 1000 (m/z). A wavelength of λ = 214 nm was used for detection after loading 20 µL of the respective sample. The gradient was set as (i) linear gradient from 5 to 90% eluent B in 8 min, (ii) flushing at 90% eluent B for 5 min, (iii) linear gradient to 5% eluent B in 1 min, and (iv) equilibrating the column with 5% of eluent B for 4 min.

For the measurement of the ESI, the spectrometer micOTOF-focus was used. The spectra were measured at a capillary temperature of 210 °C and a voltage of 3.5 kV with N_2 as an inert carrier gas.

1H-NMR measurement

Spectras were recorded on an Avance 400 Spectrometer (Bruker BioSpin MRI GmbH (Ettlingen, Germany). For calibration of spectras, remaining protons of deuterized solvents (DMSO-d6 = 2.5 ppm) were used. The stated coupling constants n*J* are in Hz and stated chemical shifts in ppm. To describe signal multiplicities the abbreviations d for duplet, t for triplet, q for quartet and m for multiplet were used.

The following parameters were used for spectra recording and processing:

Table 3. Parameters of a standard 400.132 MHz ^{1}H-NMR measurement

Parameters	400.132 MHz ^{1}H-NMR-spectra
Recording	
sample head	BBO BB-1H/D19F Z-GRD
pulse program	zg30
data points	64k
solvent	DMSO-d6
temperature	300 K
rotation	20 Hz
number of scans	16
spectral width	20.55
ray frequency	6.175
recording time	3.984 s
digital resolution	0.251 Hz/point
relaxation delay D1	1 s
Processing	
data points	64k
window function	exponentially
LB-factor	0.30 Hz

Table 4. Parameters of a standard 100.132 MHz ^{13}C-NMR measurement

Parameters	100.623 MHz ^{13}C-NMR-spectra
Recording	
sample head	BBO BB-1H/D19F Z-GRD
pulse program	Zgpg30
data points	64k
solvent	DMSO-d6
temperature	300 K
rotation	20 Hz
number of scans	512
spectral width	238.90
ray frequency	99.991
recording time	1.361 s
digital resolution	0.733 Hz/point
relaxation delay D1	2 s
Processing	
data points	64k
window function	exponentially
LB-factor	1 Hz

Cleavage assays

Cleavage assays by AP

The substrates were incubated using leucine AP from porcine kidney at 37 °C. An AP stock solution was prepared by diluting 4.7 mg of purified lyophilized peptide in 1 mL ultra-pure water, resulting in a final concentration of 4.7 mg/mL. To evaluate the cleavage of AAs from Den, AP was added in concentration of 940 ng/mL and the samples were incubated at 37 °C for 15 min, 60 min and 24 h. Enzymatic activity was stopped by heating to 95 °C for 15 min. The cleavage percent was analyzed by comparing the area under the curve (AUC) of the main peak from peptide incubated with AP and in absence of AP.

The gradient was set as (i) equilibration of the column at 15% eluent B for 1 min, (ii) linear gradient from 15 to 40% eluent B in 7 min, (iii) linear gradient to 95% eluent B within 0.5 min, (iv) flushing at 95% eluent B for 1 min, (v) linear gradient to 15% eluent B within 1 min and (vi) re-equilibration at initial conditions.

Cleavage assays by human proteases

Pro-MMPs (MMP-8, MMP-1, or MMP-9; adapted to 0.1 mg/mL and > 100.0 mU/mg based on the manufacturer certificate if applicable) were activated with 4-aminophenylmercuric acetate (APMA) as described in the manufacturer instructions.

The substrates were incubated with activated MMPs at 37 °C. Enzymatic activity was stopped by adding 1 µL of a 100 mM EDTA solution and heating to 95 °C for 5 - 10 min. The relative decrease of the constructs main peak was analyzed by RP-HPLC as mentioned before. The gradient was set as (i) equilibration of the column at 15% eluent B for 1 min, (ii) linear gradient from 15 to 40% eluent B in 7 min, (iii) linear gradient to 95% eluent B within 0.5 min, (iv) flushing at 95% eluent B for 1 min, (v) linear gradient to 15% eluent B within 1 min and (vi) re-equilibration at initial conditions.

Cleavage assays by SpeB

1 mg of purified lyophilized peptide was diluted into 2 mL of SpeB buffer, resulting in a final concentration of 0.5 mg/mL of the substrate. To analyze the impact of SpeB concentration on construct cleavage, a logarithmic screening of SpeB concentration from 0.1 to 1000 ng per 50 µL was performed. The samples were incubated with SpeB under agitation for 5 to 7 min at 37 °C. Protease activity was interrupted by incubating the sample at 95 °C for 15 min. The relative decrease of the PCL main peak was analyzed by RP-HPLC, applying a determination wavelength of λ = 214 nm and loading 20 µL of the sample. The gradient was set as (i) equilibration of the column at 5% eluent B for 4 min, (ii) linear gradient from 5 to 95% eluent B

in 30 min, (iii) flushing at 95% eluent B for 8 min, (iv) linear gradient to 5% eluent B within 4 min. and (v) re-equilibration at initial conditions.

Statistical analysis

All data are reported as mean and standard deviation of at least five independent measurements, unless specified otherwise. Statistical significance was calculated by one-way ANOVA with post-hoc Tukey HSD with an overall significance level of 0.05 using Origin 2018. Outliers were excluded after testing using the grubbs-test.

Proteomic Identification of Cleavage Sites (PICS) Assay

PICS Assay – Generating the peptide library

HEK293 cells were grown to confluency in 150-175 cm^2 cell culture flask (each with a yield of 2 - 6 mg of final, purified peptide library). Cells of 3 cell culture flasks were detached using protease-free detachment methods like phosphate-buffered saline (PBS) supplemented with 0.2% (w/v) EDTA. Used detachment buffer was removed by centrifuging cells at 400 xg at 4 °C for 5 min. Afterwards, supernatant was decanted and the cell pellet kept. Cell pellets were resuspended in hypotonic lysis buffer. The lysate was centrifuged at 20,000 xg for 20 min.

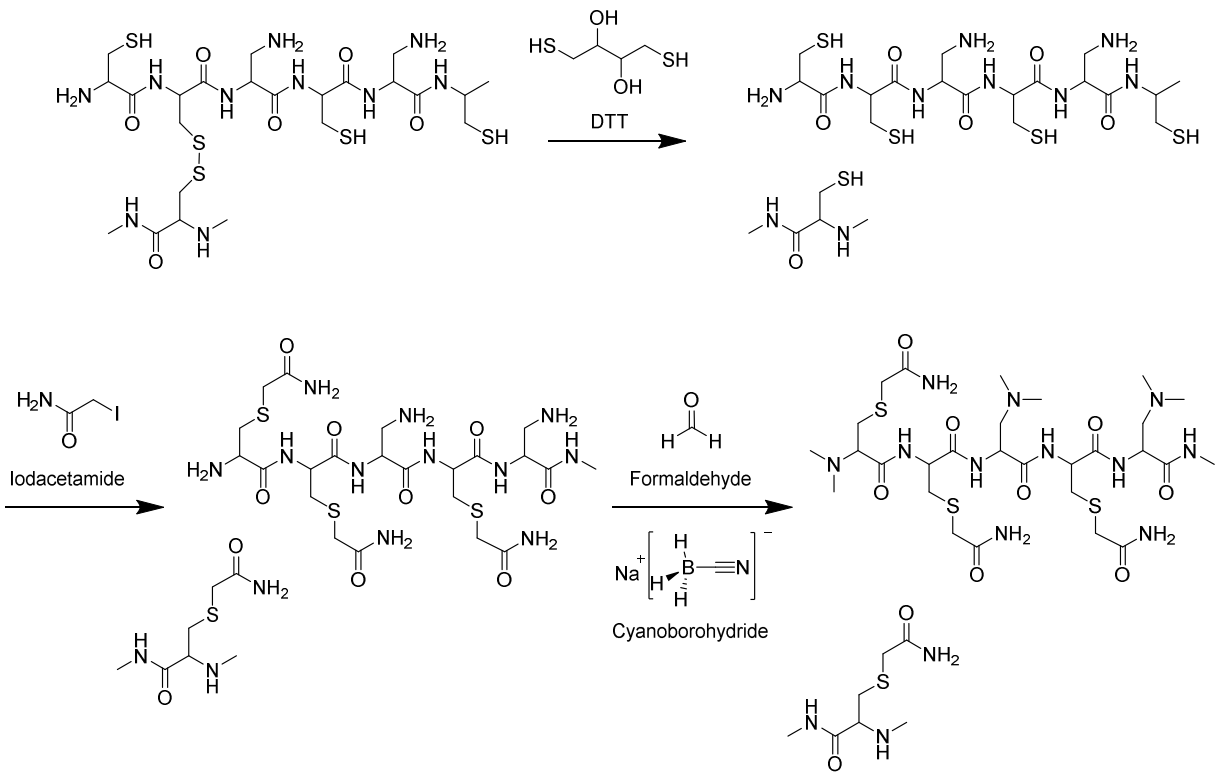
Protein concentration of the supernatant was determined using the Bradford assay method and adjusted to 100 mM HEPES, pH 7.5. The solution was incubated with 5 mM DTT for 60 min at 25 °C, following an incubation with 20 mM iodoacetamide (final concentration) in the dark for 60 min at 25 °C. After this time, another 5 mM DTT were added and incubated for 5 min at 25 °C for 1 h to quench excess iodoacetamide. The sample was adjusted to 15% (v/v) trichloroacetic acid and incubated on ice for 1 h. After this time, it was centrifuged at 20,000 xg at 4 °C for 10 min.

The pellet was washed twice with small amounts of -20 °C cold methanol. If pellet loosens, it was briefly centrifuged. The pellet was briefly air-dryed after the second wash step, where after the pellet was overlaid with ice-cold 20 mM NaOH. Based on the total protein amount determined before, (assumed all protein was precipitated and will entirely redissolve) the protein concentration was adjusted to 2.0 mg/mL and to 200 mM HEPES, pH 7.5.

After centrifugation at 20,000 xg for 10 min the protein concentration and total protein amount was again determined using the Bradford assay method. Hence, it was digested with either trypsin or *S. aureus* Protease *V8* (GluC), utilizing a protease to proteome ratio of 1:100 (w/w). The incubation was performed at 37 °C for 16 h. After the protease digest, samples were incubated at 70 °C for 20 min to stop digestion. 1 mM PMSF was added to entirely abolish activity of the digestion protease.

Afterwards, 1.0 M guanidine hydrochloride was added and centrifuged at 20,000 xg at 4 °C for 10 min. 5 mM DTT was added to the supernatant and incubated at 37 °C for 1 h. Subsequently, 40 mM iodoacetamide was added and the mixed solution incubated at 37 °C for 1.5 h. Again, 15 mM DTT was added and incubated at 37 °C for 10 min. 30 mM formaldehyde and 30 mM sodium cyanoborohydride were added and incubated at 25 °C for 2 h. After this time, 30 mM formaldehyde and 30 mM sodium cyanoborohydride was given into the solution and incubated at 25 °C for 16 h. A graphical representation of the reactions is provided in **Scheme 1**.

On the next day, 100 mM glycine was added and incubated at 25 °C for 30 min. The solution was acidified with TFA and purified by C18 solid phase extraction. After the elution, peptide concentration was determined by the bicinchoninic acid assay. The generated peptide libraries were stored in aliquots of 200-300 µg at -80 °C at a peptide library concentration of approximately 1 mg/mL.



Scheme 1. Reactions for the preparation of a suitable peptide library.

PICS Assay – Digestion with the test protease

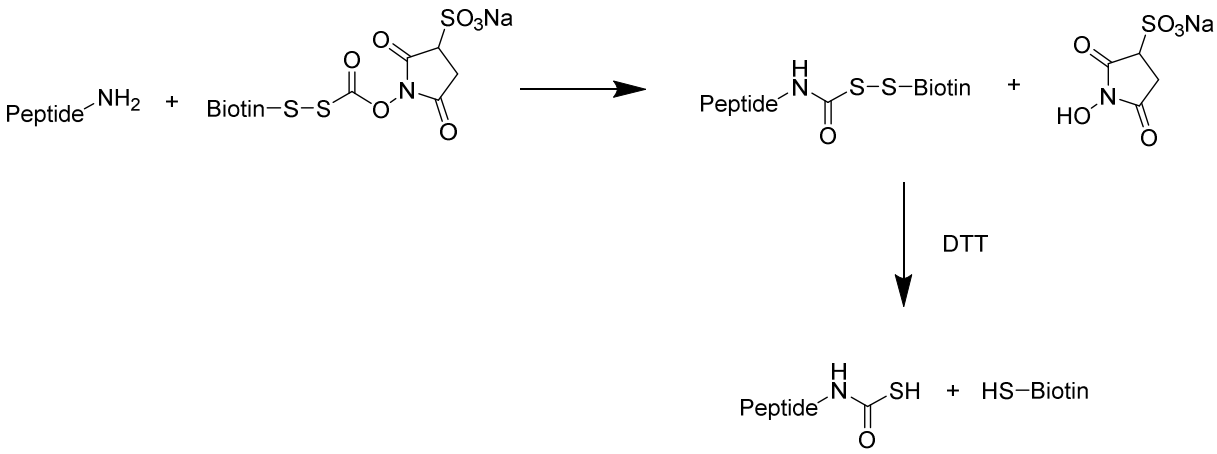
200 µg of peptide library were thawed per protease and appropriate buffer conditions for the test protease adjusted (pH, type of buffer, reducing agents, co-factors). Active test protease in a ratio of protease to peptide library 1:100 (w/w) was added and incubated for 2 h (SpeB) or 16 h (MMP-9) at a 37 °C. After incubation, the test protease was inactivated at 70 °C for 30 min and checked that the pH is between 7 and 8.

To biotinylate cleavage products (**Scheme 2**), the PICS assay was incubated with 0.5 mM sulfo-NHS-SS-biotin (prepared immediately prior to mixing with the PICS assay in DMSO) for 2 h at 25 °C. High-capacity streptavidin sepharose was equilibrated (as high-capacity streptavidin sepharose is typically supplied as a diluted slurry) by repeated centrifugation and resuspension in 50 mM HEPES, 150 mM NaCl, pH 7.5. The buffer-equilibrated streptavidin sepharose was added to the biotinylated PICS assay and incubate for 0.5 h at 22 °C. Mild agitation was applied to keep the slurry mix in suspension. Subsequently, the slurry was poured into a spin column of sufficient volume with a filter of about 10 µm pore size.

The column was centrifuged at a centrifugation speed that is sufficiently high to pass the PICS assay solution through the column without letting the column run completely dry. After centrifugation, the resin should still be wet but without supernatant.

First flow-through was re-applied to the streptavidin sepharose resin and centrifuged for a second time. Subsequently, the column was washed 10 times by applying 500 µL of washing buffer by centrifugation. 500 µL of freshly prepared elution buffer was applied and incubated for 2 h at 25°C with mild agitation. After centrifugation, the eluate was kept.

500 µL of elution buffer was applied for a second time and centrifuge with an estimated eluate amount of about 1-5% of library amount (2-10 µg for 200 µg library) depending on biotinylation efficiency as well as protease activity and specificity. The samples were lyophilized and can be stored at -80 °C for several weeks, before analysis.



Scheme 2. Biotinylation and cleavage of Peptides using Sulfo-NHS-SS-Biotin. In a first step, the sulfo-NHS ester react spontaneously with amines to form an amide bond. After binding to high-capacity streptavidin sepharose and intense washing, elution was achieved with reductive agents like DTT.

Bradford assay

The Bradford Assay was conducted to determine the protein concentration in several steps of the PICS protocol.

The Protein Assay solution with dye reagent concentrate (Bio-Rad) and bovine serum albumin (BSA) were added to a 96-well microtiter plate. The BSA standard concentrations used were 50, 125, 250, 500, 750 and 1000 µg/mL. Bradford Reagent solution totaling 245 µL was incubated with either 5 µL of standard or protein sample at room temperature for 5-10 min. The absorbance was measured at 595 nm using a SpectraMAX 250 well plate reader from Molecular Devices, with Soft Max R Pro 4.7.1 software. The equation of the best fitting line of the standard curve generated by Microsoft Excel was used to calculate the protein concentration.

BCA Assay

The BCA assay was conducted to determine the protein concentration in several steps of the PICS protocol and the expression of SpeB.

BCA Assay reagent was prepared according to the proposed protocol. 25 µL of each standard (analogue to Bradford) or unknown sample replicate were pipetted into a 96-well microtiter plate. Afterwards, 200 µL of the BCA reagent was added to each well and mixed thoroughly in the plate reader (same as Bradford) for 30 seconds. The plate was covered and incubated at 37 °C for 30 min and the absorbance measured at 562 nm on a plate reader.

Cytotoxicity experiments

Cell culture

NIH 3T3 fibroblast (CRL-1658; ATCC, Manassas, VA) were harvested from exponentially growing sub confluent monolayer. The cells were maintained in 75 cm² culture flasks in growth medium (DMEM high-glucose containing heat-inactivated FCS (10%), penicillin G (100 U/mL) and streptomycin (100 µg/µL)) at 37 °C under 5% CO_2.

WST-1 Assay

Toxicity of Den-CH_2-NH_2, Den-CH_2-NH-Ac and Ac-GPQGIAGQ-Den was determined in NIH 3T3 fibroblasts as previously described. Briefly, $5*10^3$ NIH 3T3 fibroblasts in 100 µL assay medium (5000 cells/well) were seeded into the wells of a 96 wells tissue culture plate and in growth medium overnight at 37 °C under 5% CO_2. 24 h after seeding, dilution series of a stock solution were prepared in assay medium and 100 µL of each dilution was added to the cells in a 96-well plate and incubated for 48 h at 37 °C, 5% CO_2. Pre-warmed WST-1 solution was mixed with assay medium in equal parts and 20 µL of this mixture was gently added into the wells. After an incubation time of 4 h at 37 °C and 5% CO_2, the well-plate was protected from light and 100 µL of the supernatant was transferred to a clear 96-well flat bottom microtiter plate. The soluble formazan product was quantified at a wavelength of 450 nm as well as background noise at 630 nm was determined using a plate reader system.

SpeB expression and characterization

Transformation of competent *E. coli* cells

The SpeB-containing plasmid pET24a-SpeB-M3C3 was generously donated by Dr. Burckhard Seelig.

For chemical transformation, aliquots of competent cells (*E. coli* DH5α and BL21DE) were thawed on ice before adding 1 µL (30 ng) of plasmid DNA. The transformation mix was incubated on ice for 30 min, subjected to a heat shock at 42 °C for 60 s in a Thermoshaker followed by a second incubation on ice for 2 min. Afterwards, 500 µL SOC-medium were added to induce cell recovery and the cells were shaken at 750 rpm and 37 °C on a Thermoshaker for 1 h. For positive selection, 100 µL of cells were plated on pre-warmed LB-agar plates supplemented with the kanamycin and incubated at 37 °C overnight.

A single colony of *E. coli* DH5α and *E. coli* BL21DE was inoculated into 5 mL LB-medium supplemented with kanamycin, grown at 37 °C overnight and harvested by centrifugation for 1 min at 10,000 xg.

The obtained pellet was resuspended in 50% glycerol/50% TB medium. Small volumes (50-100 µL) of cell aliquots were frozen in liquid nitrogen and stored at -80 °C.

Sequencing of colonies

Sequencing of colonies was performed by Eurofins Genomics. Samples were prepared according to the proposed protocol.

Overexpression of *SpeB* by autoinduction in *E. coli*

A single colony of *E. coli* DH5α was picked with a pipette tip, inoculated in 150 mL of LB medium containing kanamycin and incubated at 37 °C overnight in a sterile environment.

At the following day, 400 µL of the overnight culture was used to inoculate 200 mL of auto-induction media. Cultures were grown at 37 °C for ~5 - 6 h until the OD_{600} reached 0.3 - 0.4 and then moved to 25 °C for an additional 24 h. After centrifugation, the cleared supernatant was divided into 50 mL aliquots and frozen at -80 °C for storage.

Matrix assisted laser desorption ionization (MALDI)-MS

According to manufacturer's instructions, 20 µL of a collected SpeB fraction was desalted using ZipTip® pipette tips (C18 resin, Millipore, Billerica, MA). 5 µL of the eluate was embedded in a matrix consisting of equal part of sinapinic acid and ACN + 0.1% TFA in water (1:4). MALDI-MS spectra were acquired in linear positive mode by using an Autoflex II LRF instrument (Bruker Daltonics, Billerica, USA). Mass spectra were calibrated externally using a peptide standard (Bruker Daltonics, Billerica, USA). Theoretical masses of wild-type proteins were calculated (http://web.expasy.orgp/eptide mass).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins used were analyzed by standard tris-glycine SDS-polyacrylamide gel electrophoresis. To ensure a successful separation of proteins, 12% polyacrylamide gels were used.

Approximately 15 µL of each protein elution sample was thoroughly mixed with 6x SDS buffer in a 5:1 ratio. The solution was heated at 95 ºC for 5 min, loaded onto a 12% SDS-PAGE gel, and separated by electrophoresis at 120 volts for 90 min.

Gels were stained with Coomassie solution followed by destaining or the protein was detected via western blotting.

Table 5. Protocol to prepare stacking and separating gel for SDS-PAGE.

Solution	Stacking gel (3.9%)	Separating gel (12%)
Acrylamid/Bis-acrylamide 30:0.8	0.65 mL	6 mL
4x Tris-Cl/SDS pH 8.8	-	3.75 mL
4x Tris-Cl/SDS pH 6.8	1.25 mL	-
ddH_2O	3.05 mL	5.25 mL
10% (w/v) ammoniumpersulfate solution	25 µL	50 µL
Tetramethylethylendiamin (TEMED)	5 µL	10 µL

Gel staining

Gels were stained with Coomassie brilliant blue (CBB) G-250 (80 mg/L CBB G-250, 3 mL/L HCl 37%) overnight at RT. The stain was aspirated and replaced with destain solution. The gel was destained for 2 h under gentle agitation at RT. The destain solution was removed with water and the gel was analyzed using a FluorChem FC2 imaging system.

Western Blotting

Through Western blotting, proteins separated by gel electrophoresis are transferred to a nitrocellulose membrane and afterwards detected by incubation with specific antibodies.

A nitrocellulose membrane was cut to gel size and pre-wetted with blotting buffer. The stacking gel was removed and placed onto the equilibrated nitrocellulose membrane which was placed on an also pre-wetted Whatman paper. The gel was covered with Whatman paper and two pre-wetted sponges were placed on top and below this arrangement. Potential air bubbles were removed by gently applying pressure onto the stack from the middle towards the border. Electroblotting was performed using 80 V for 1 ½ h, pulling the negatively charged proteins from the SDS gel onto the nitrocellulose membrane towards the positively charged electrode of the blotting chamber.

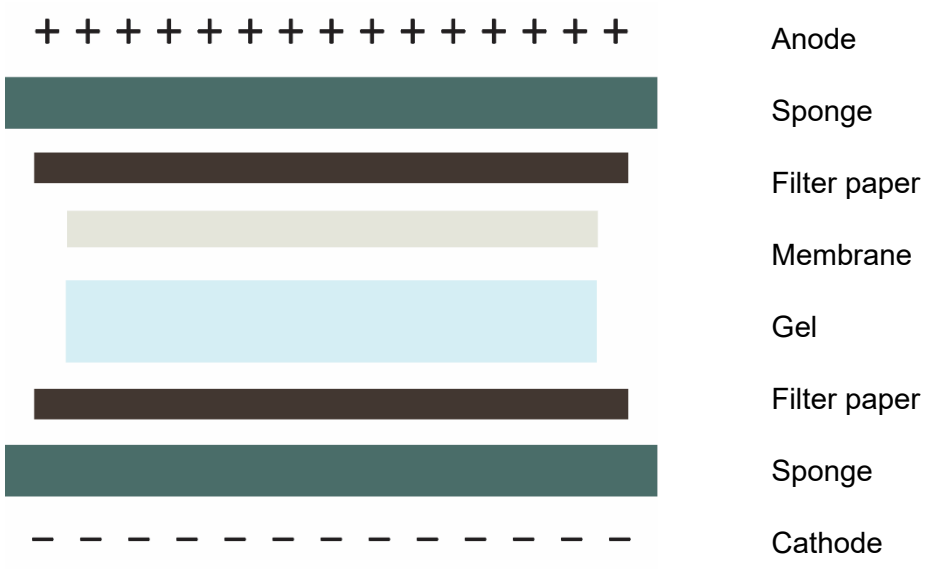


Figure 11. Western blotting device assembly.

Between all following steps, the membrane was washed three times for 15 min in TBS-T. To prevent unspecific antibody (Ab) binding during following steps, membranes were blocked in Roti®-Block solution in TBS-T. Incubation was carried out under gentle agitation for 60 min at RT. Samples were then incubated with a 1:3,000 dilution of the primary Ab in TBS-T at 4 °C overnight on a rolling mixer. On the next day, the washing steps were performed and the membrane was incubated for 1 h in a 1:4,000 dilution of the second Ab in TBS-T.

The secondary Ab is linked to a reporter enzyme named horseradish peroxidase which under cleavage of a chemiluminescent agent produces a luminescent signal. This can be detected by exposure to a special camera. Hence, the membrane was analyzed using a SuperSignal™ West Pico PLUS Chemiluminescent Substrate on a Fluor Chem FC2 machine.

Western blotting quantification

ImageJ is an open source program for image processing that has been developed at the National Institute of Health.

Profile blots showed the relative density of the respective lanes, with lighter signals resulting in more intensity then darker. The obtained intensity for the measured areas showed only arbitrary units and could therefore only be compared within the context of one single blot. To calculate absolute amounts of specific proteins the dilution series of purified SpeB was loaded on each gel. During this quantification process a standard curve was established using the arbitrary units obtained from the signals of the dilution series. Afterwards, the absolute amount

of a specific protein on a western blot was calculated by referring its arbitrary units to the appropriate standard curve. Hence, it is possible to quantify the amount of protein.

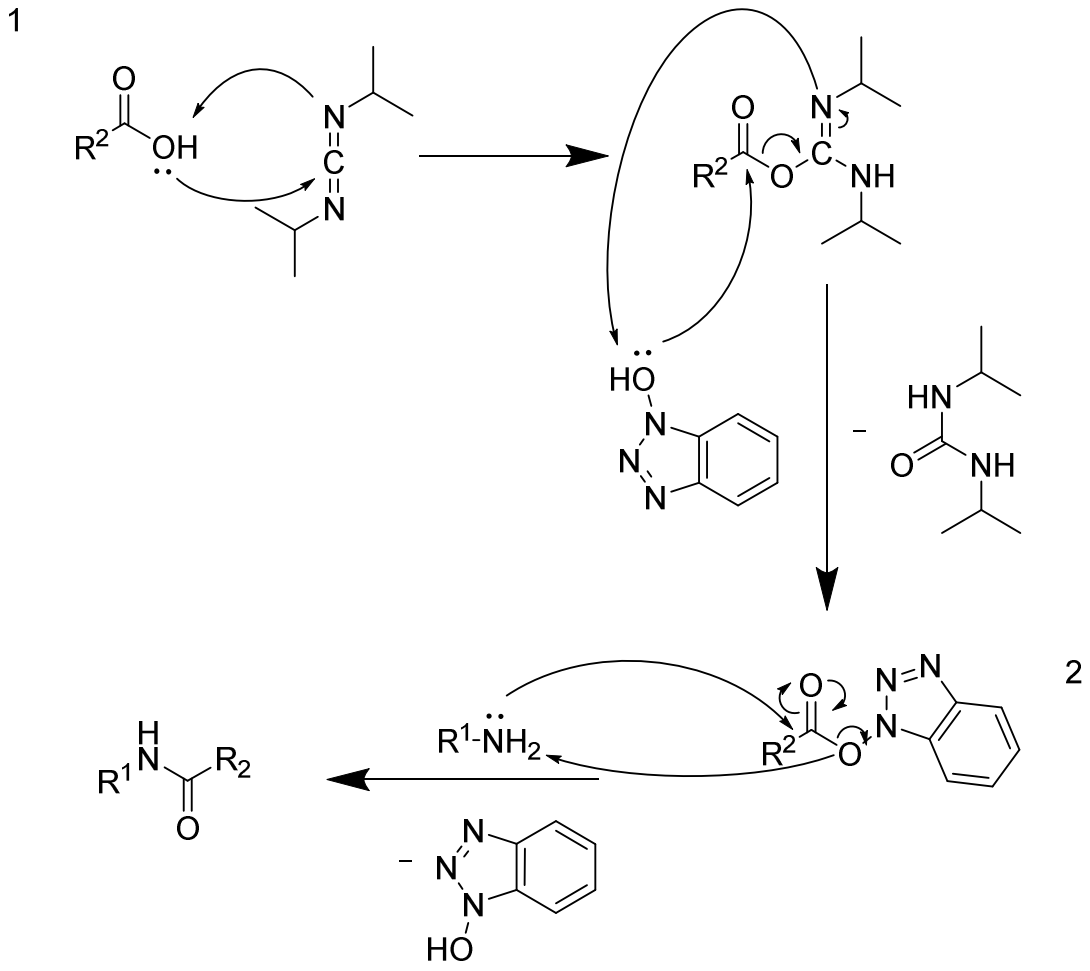
6.3. Synthesis

6.3.1. Peptide synthesis

6.3.1.1. Synthesis of protease sensitive linker (PSL) sequences

All PSL sequences were manually synthesized in-house by solid phase peptide synthesis (SPPS) using Fmoc AA coupling strategy (**Scheme 3**). Since the peptide chain remains covalently attached to the resin throughout the synthesis process, excess reagents and by-products can be removed by excessive washing and filtration.

R^1 = Resin-Peptide
R^2 = New amino acid



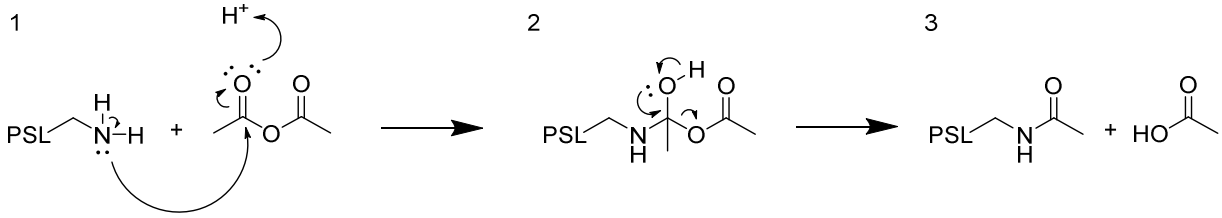
Scheme 3. Synthesis mechanism of peptides via SPPS. (**1**) The carbodiimide reacts with the deprotonated carboxylic acid of the next AA to form the O-acylisourea mixed anhydride (**2**) This intermediate can then react with the amine to the desired amide and an urea side product.

Fmoc-protected AAs were coupled to an acid labile CTC resin (loading 1.0 meq/g) in a 5-fold molar excess compared to the resins' loading capacity. The CTC resin was loaded into in a polypropylene-reactor. Subsequently, free bindings sites were blocked by adding methanol in a final concentration of 0.1 mL/g and incubation for 25 min under agitation. The resin was washed three times with DCM and another three times using DMF.

For coupling of the subsequent AA, the respective AA was dissolved in 2.5 mL of 0.5 M HOBt in DMF, followed by 80 µL DIC and 88 µL DIPEA. The resulting solution was used in 5-fold molar excess with respect to the AA as compared to the loading of the resin. Coupling was carried out for 5 min at 75 °C using a microwave. Removal of the protecting Fmoc group was done by incubating with 20% (v/v) piperidine in DMF for 5 min at 75 °C using a microwave. Between steps, the resin was washed four times with DMF.

6.3.1.2. Synthesis of the N-acetylated peptides

An in-house established acetylation protocol was used, for end capping of the N-terminus through an acetyl protection group (**Scheme 4**).



Scheme 4. Acetylation of the synthesized peptide. (**1**) Nucleophilic attack of the free electron pair of the primary amin to a carbonyl oxygen of the anhydride. (**2**) Proton migration of an H of the amino group to the bridge oxygen of the anhydride. (**3**) Loss of acetic acid results in the formation of the amide.

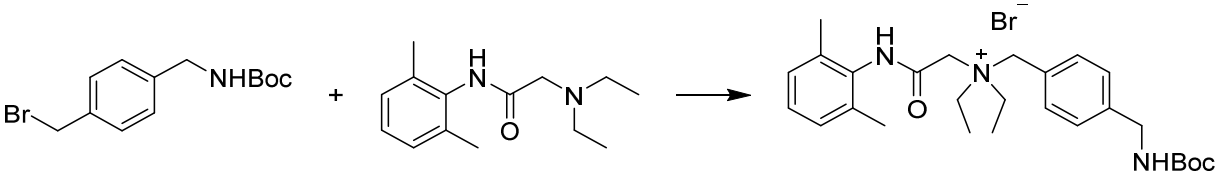
If necessary, acetylation of the N terminal amino group was achieved by incubation with 50 µL acetic anhydride and 90 µL DIPEA in 2 mL DMF for 30 min. Final peptide cleavage from the resins was attained with 3% TFA (w protection groups) or 95% TFA (w/o protection groups) in DCM for 1 hour followed by precipitation with diethyl ether at -20 °C. The suspension was centrifuged for 5 min (3000 xg, 5/min), the supernatant was discarded and washed twice with diethyl ether to dry the resin completely. As SPPS provides peptides with a purity of >90%, synthesized PSL were directly coupled to the modified Den.

6.3.2. Synthesis of flavor components

6.3.2.1. Synthesis of Den-CH_2-NH-Boc

Several synthesis routes starting from carboxylated or carboxyalkyl-substituted benzyl halides and lidocaine are known for the preparation of carboxylic acid derivatives of Den. The solvent-free synthesis at room temperature, in which the desired derivative is obtained in high yields after a short reaction time, is particularly promising.

In a nucleophilic substitution with subsequent deprotection, the commercially acquired products lidocaine and tert-butyl-4-(bromomethyl)benzylcarbamate react to the desired Den derivate (**Scheme 5**).

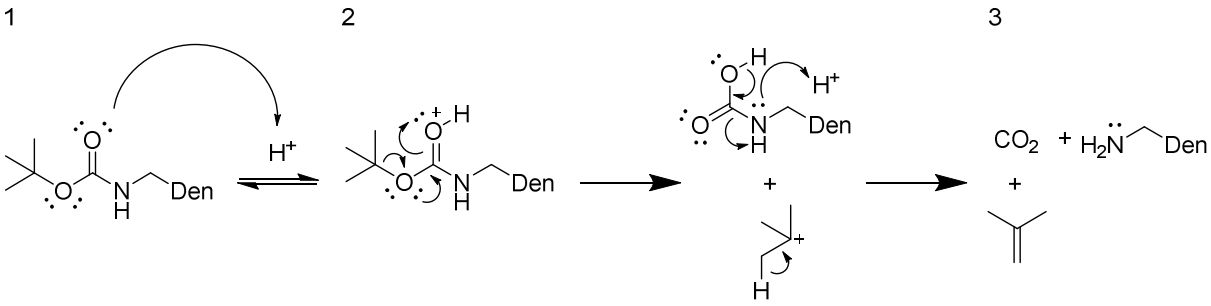


Scheme 5. Synthesis of Den-CH_2-NH-Boc. The reaction mechanism is a nucleophilic substitution.

Therefore, 1.5 to 1.6 equivalents of lidocaine and 1 equivalent of tert-butyl-4-(bromomethyl)benzylcarbamate were heated under stirring to 80 to 100 °C until the formation of a molten mixture which becomes highly viscous, up to solid. After a resting period of 5 to 15 min at 80 to 100 °C, the obtained yellow solid was treated with of a mixture of ethyl acetate and n-hexane (1:1) to remove yellow impurities. A white solid was obtained, with yields of approximately 90%.

6.3.2.2. Synthesis of Denatonium-CH_2-NH_2 (Den-CH_2-NH_2)

For this deprotection reaction, a method to remove protecting groups (i.e. Boc) utilizing an acidic milieu from synthesized peptides was chosen (**Scheme 6**).

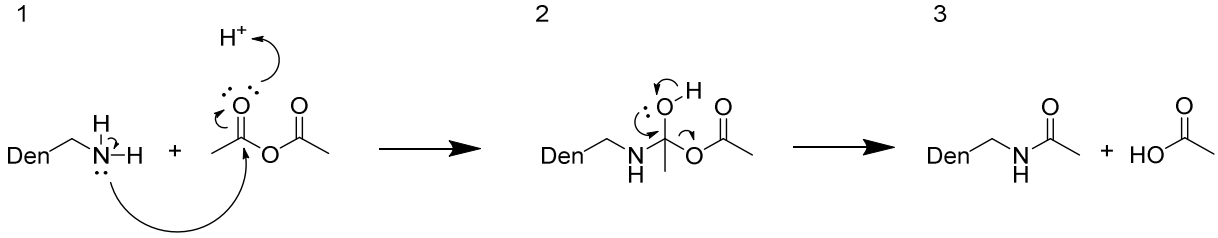


Scheme 6. Mechanism of Boc-deprotection. (**1**) The tert-butyl carbamate becomes protonated by TFA. (**2**) Loss of the tert-butyl cation results in a carbamic acid. (**3**) Decarboxylation of the carbamic acid results in the free amine.

The product obtained from Example 1 was dissolved in pure TFA and shaken at room temperature for 1 h, to quantitively remove the protection group. The raw product was precipitated with diethyl ether at -20 °C. After 1 h at -20 °C, the suspension was centrifuged, the supernatant was discarded and the precipitate was washed another two times with diethyl ether to remove rests of TFA. The product was further purified by chromatography**.** In this procedure, a double TFA-salt of Den was formed. After purification, a yield of about 75% was obtained.

6.3.2.3. Synthesis of Denatonium-CH_2-NH-Ac (Den-CH_2-NH-Ac)

A classical acetylation reaction was chosen for this reaction, which is used in peptide synthesis for capping of the N-terminus (**Scheme 7**).



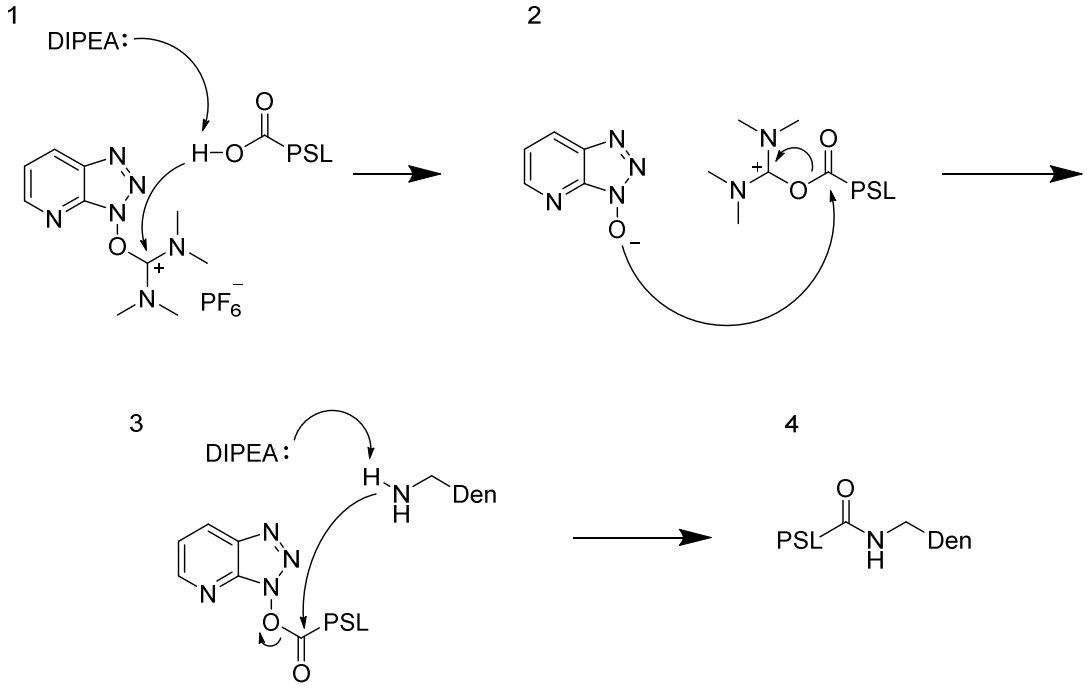
Scheme 7. Acetylation of Den-CH_2-NH_2 by acetic anhydride. (**1**) Nucleophilic attack of the free electron pair of the primary amin to a carbonyl oxygen of the anhydride. (**2**) Proton migration of an H of the amino group to the bridge oxygen of the anhydride. (**3**) Loss of acetic acid results in the formation of the amide.

Den-CH_2-NH_2 was therefore dissolved in dry DMF. DIPEA and acetic anhydride were added and the reaction mixture was shaken at room temperature for 1 h. The raw product was precipitated in diethyl ether and filtered off. The product was purified by chromatography.

6.3.3. Synthesis of final constructs

6.3.3.1. Coupling of Peptides with Den-CH_2-NH_2

As both components of the diagnostic construct, are successfully synthesized, they have to be combined. Several strategies were tried to couple the two connections. EDC/NHS coupling, coupling using HOBt/DIPEA and HATU/DIPEA. The coupling by HATU/DIPEA was used for the further coupling of PSL and **Den-CH_2-NH_2** due to high yields and better purity after reaction (**Scheme 8**).



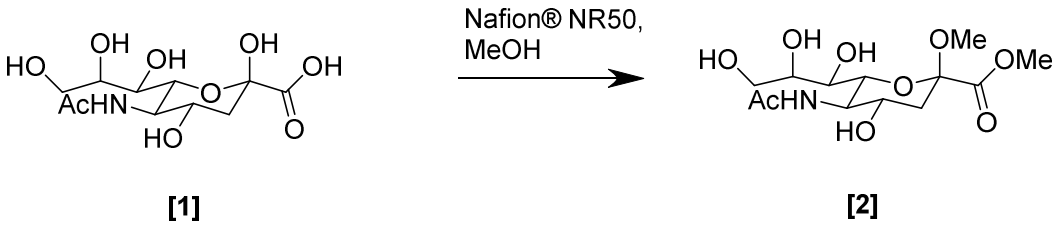
Scheme 8. HATU coupling of the PSL with modified Den. (**1**) DIPEA deprotonates the carboxylic acid. The resulting carboxylate anion attacks the electron deficient carbon atom of HATU. (**2**) The resulting HOAt anion reacts with the newly formed activated carboxylic acid derived intermediate to form an OAt activated ester. (**3**) The amine reacts with the OAt activated ester to form the amide (**4**).

2 equivalents of **Den-CH_2-NH_2** salt, 1 equivalent of the chosen peptide and 3 equivalents of HATU were dissolved in dry DMF. 4 equivalents of DIPEA were added and the reaction was shaken under the exclusion of light at room temperature overnight. After one day, the same amount of HATU and DIPEA was added and the reaction was again shaken under the exclusion of light at room temperature overnight. The resulting raw product was precipitated in diethyl ether and filtered off. The product was purified by liquid chromatography.

6.3.4. Carbohydrate synthesis

6.3.4.1. Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-D-glycero- β-D-galacto-2-nonulopyranosidonate)

In a first step, compound **[1]** is converted into a dimethyl derivative (**Scheme 9**). This can be done in one step or with higher yields in a two part reaction. As the methoxylation reaction of C1 produces water, it is removed by evaporation before methylation of the C2 to decrease the rate of hydrolysis. Both reaction are executed by treatment with methanol under conditions of acid catalysis. As an effective acid catalyst, the protonated form of Nafion NR 50, a resin with perfluorosulfonic acid groups, was used.



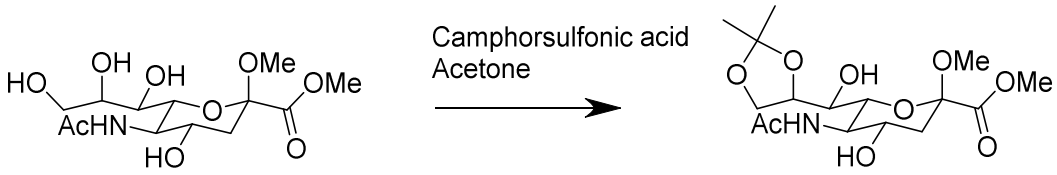
[1] **[2]**

Scheme 9. Dimethylation of position 1 and 2 under acid catalysis conditions.

A suspension of Neu5Ac **[1]** in dry methanol was treated with Nafion NR50 resin and stirred until it becomes a clear solution. This reaction transforms the carboxylic acid into a methyl ester. The solution was evaporated to dryness, treated again with dry methanol and refluxed with vigorous stirring for 72 h at 65 °C. The resin was filtered off and washed with methanol. Afterwards, the filtrate was evaporated to dryness and crystallized in dry methanol at -20 °C overnight. The crystals were collected and washed with cold ethyl acetate:methanol (6:1) to provide white powder of Neu5Ac methyl ester methyl ketoside **[2]**.

6.3.4.2. Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-β- D-galacto-2-nonulopyranosidonate)

To protect the vicinal diol group in position 8 and 9, another protection group was introduced in this position. In a preferred embodiment, the ketal is provided by reaction with dry acetone under acid catalysis (**Scheme 10**). This single step reaction, affords the ketal in essentially quantitative yield.



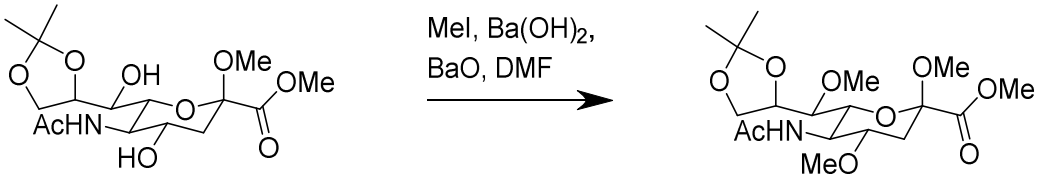
[2] **[3]**

Scheme 10. Protection of position 8 and 9 of Neu5Ac by a ketal group.

A mixture of **[2]** and camphorsulfonic acid in dry acetone was stirred at room temperature for 3 h, then neutralized with TEA to pH = 6-7. The solution was evaporated to dryness. The solid was triturated and washed with ether, then purified by chromatography to afford 8,9-isopropylidene Neu5Ac methyl ester methyl ketoside **[3]** as a white powder.

6.3.4.3. Synthesis of methyl (methyl 5-acetamido-3,5-dideoxy-4,7-di-O-methyl-8,9-O- isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosidonate)

The following dialkylation was achieved by reaction with an alkyl halide in the presence of a basic substance (**Scheme 11**). The reagent employed in this step is methyl iodide with barium hydroxide as the basic compound. A mix of barium hydroxide and barium oxide is used to keep the reaction dry.



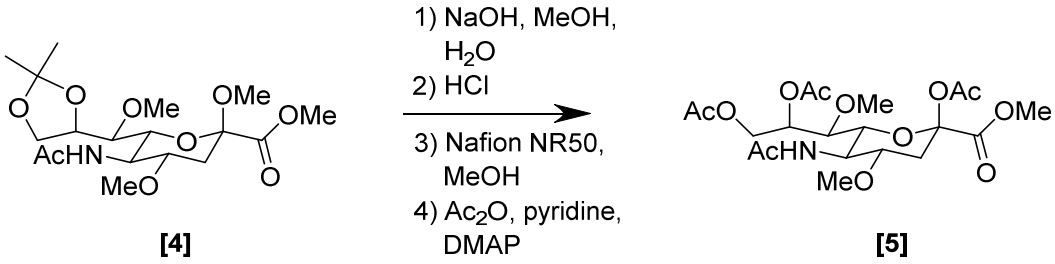
[3] **[4]**

Scheme 11. Dialkylation of derivatized Neu5Ac in position 4 and 7.

A mixture of 8,9-isopropylidene Neu5Ac methyl ester methyl ketoside **[3]** was treated with BaO and $Ba(OH)_2 \cdot 8H_2O$ in dry DMF. After 20 min, CH_3I was added. The reaction suspension was kept stirring for 45 h at room temperature. The solids were filtered off and washed exhaustively with ethyl acetate. The filtrate was concentrated by evaporation under diminished pressure, ethyl acetate was added and washed with 5% NaCl solution and 5% $Na_2S_2O_3$ solution. The organic phase was separated and the aqueous phase was extracted with ethyl acetate again. The collected organic phase was dried over anhydrous Mg_2SO_4, then evaporated under reduced pressure and kept at -20 °C with ether overnight to give the product 4,7-Dimethoxy 8,9-isopropylidene Neu5Ac methyl ester methyl ketoside **[4]** as a white powder.

6.3.4.4. Synthesis of methyl (5-acetamido-2,8,9-tri-O-acetyl-3,5-dideoxy-4,7-di-O-methyl- D-glycero-α,β-D-galacto-2-nonulopyranosidonate)

The 8,9-protected ketoside **[4]** is next deprotected and derivatized at the 8,9 position by acetylation. In the same set of reactions, the methyl ketoside group in C2 is also deprotected and acetylated (**Scheme 12**). A series of steps is undertaken in which purification is only carried out on the last step. The obtained triacetylated derivative is purified.



Scheme 12. Deprotection of introduction of new protection groups.

The dimethylated product **[4]** was suspended in a methanol/water. After addition of 1 M NaOH, the reaction mixture was stirred for 3 h. Subsequently, the reaction was neutralized by adding Nafion NR50. The resin was filtered off, washed with methanol and the washing was evaporated to dryness. HCl was added to remove the introduced protection groups as well as Bio-Rad resin, then the resulting mixture was heated at 70 °C overnight. On the next day, the resin was filtered off and washed with water. After evaporation of the solvent, the residue was dissolved in dry methanol. Nafion NR50 resin was added and the reaction mixture was stirred for 5 h at room temperature. The resin was filtered off and the methanol was evaporated.

The residue was dried under high vacuum overnight. It was then treated with Ac_2O, pyridine and DMAP for overnight at room temperature. The liquids were evaporated off and the residue was purified by chromatography on silica gel to afford the triacylated compound **[5]** as a white powder.

7. RESULTS & DISCUSSION

7.1. Section I – Fundamentals

7.1.1. Development of a couplable denatonium (Den) derivate

To this day, the coupling of Den to the protease sensitive linker (PSL) has been carried out by an introduced carboxy group (**Figure 12**). This was coupled either to the N-terminus of the PSL or to the last AA (lysine) of the C-terminus using an EDC/NHS strategy. On the other end, the Den-peptide construct was coupled to Poly(methyl methacrylate) (PMMA) particles. The PMMA particle masks the bitter taste of this molecule due to its size and insolubility in water. Only dissolved compounds can be perceived by the gustatory perception.

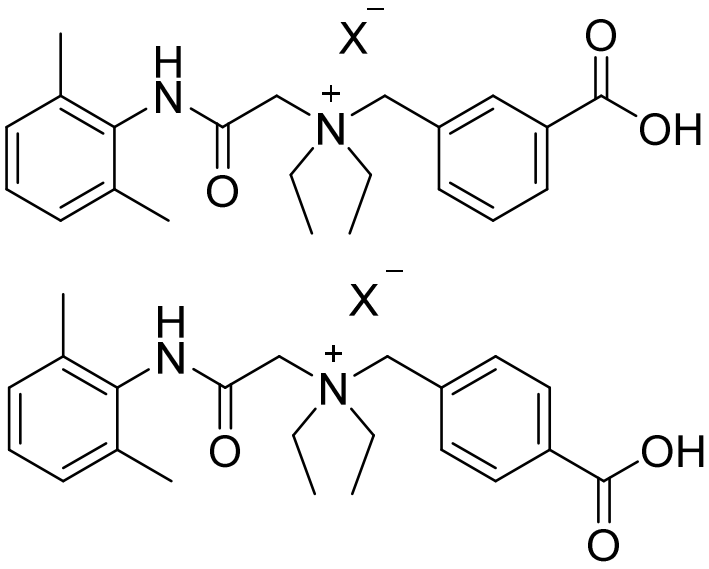


Figure 12. Chemical structures of carboxylated versions of Den. They have so far been used for the diagnostic construct for coupling to the N-terminus of the PSL. X are negatively charged counterions like TFA, Br, benzoate.

When this bioresponsive system is incubated with proteases like MMP-8 (the target protease in the diagnosis of peri-implantitis), it is quickly and specifically cleaved into two segments with four remaining AAs at the Den-COOH.

A disadvantage of this method, however, was the limited degradation by human AP. If Den was linked to the N-terminus, there was no degradation because AP attack from the N-terminus and this was prohibited. When coupled to a lysine as the last AA of the C-terminus, this residue could not be cleaved off. This results in a weakened bitter perception of the flavoring.

A Den derivate that can be coupled to the C-terminus of a PSL therefore requires a primary amine. Attempts to attach this directly to the aromatic benzyl ring, however, resulted in a blackish-oily liquid through decomposition. As soon as the Boc protection group on the primary amine was removed, this decomposition process began. Thus, this compound cannot be used for coupling to peptides and proteins. Fast decomposition can also lead to the formation of toxic or harmful products, that would make an application impossible. An analysis of this product by LC-MS showed a decomposition of the Den via electron shift to compounds shown in **Figure 13**.

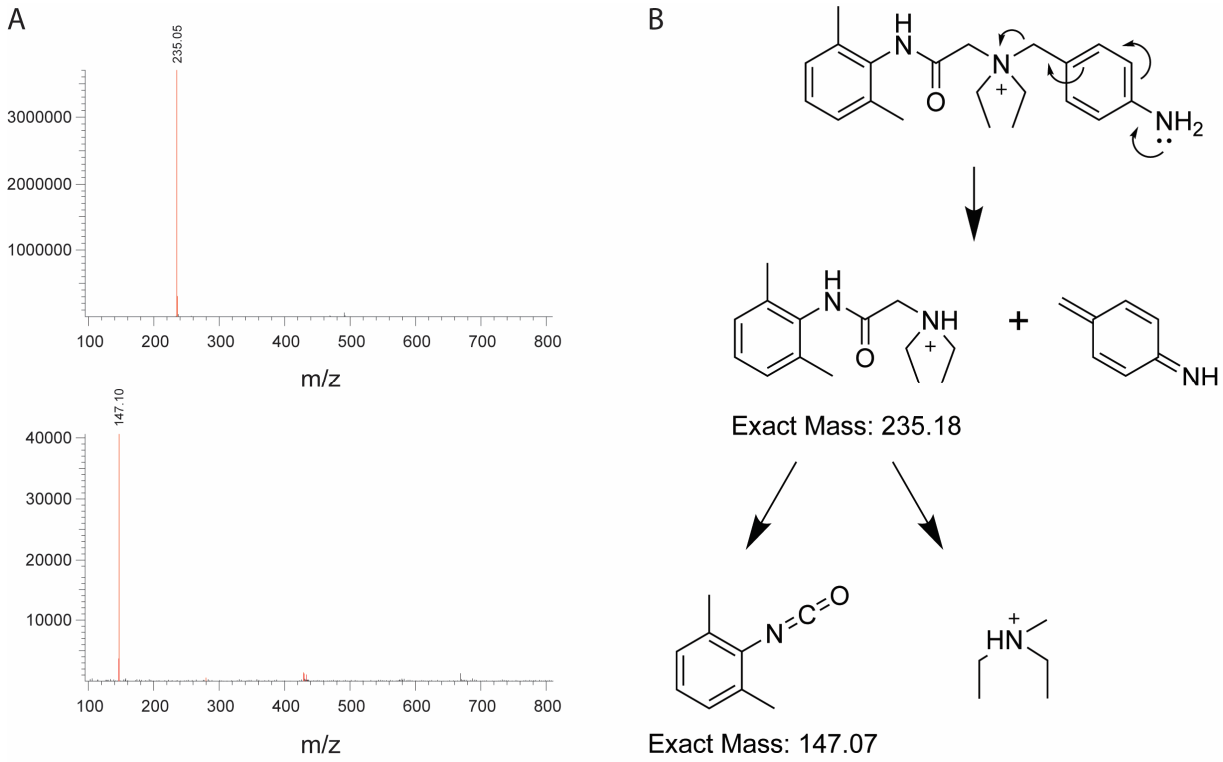


Figure 13. Mass spectrometry analysis of the decomposition product. (**A**) LC/MS analysis revealed 2 former unknown masses of 235.05 m/z and 147.10 m/z. (**B**) Postulated decomposition process leading to both found unknown masses.

Thus the amine could not be coupled directly to the aromatic system. However, this degradation mechanism can be prevented by an additional methyl group between benzyl and amine. With the methyl group blocking the electron shift, the compound should be stable for long term storage.

The synthesis of **Den-CH_2-NH-Boc** was carried out analogously to the previous Den synthesis in the melt. The reaction in melt, meaning solvent free, has the advantage of short reaction times.

To obtain the couplable product **Den-CH_2-NH_2**, the in the first step produced derivate **Den-CH_2-NH-Boc** must further be deprotected (**Figure 14**).

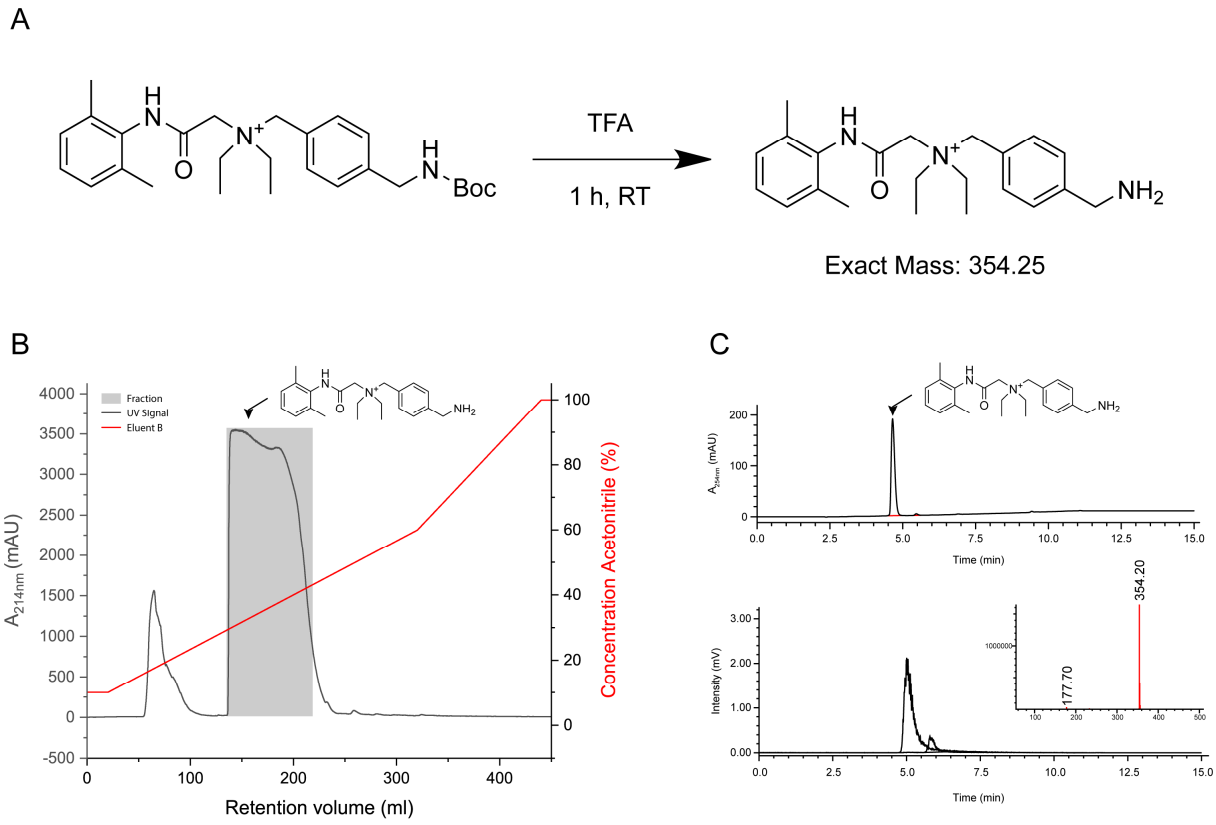


Figure 14. Synthesis, purification and LC/MS analysis of Den-CH_2-NH_2. (**A**) Synthesis of Den-CH_2-NH_2 from Den-CH_2-NH-Boc. (**B**) Purification via FPLC with UV detection at 214 nm. (**C**) HPLC chromatogram and mass spectrum. It is possible to identify the single charged mass of 354.20 m/z and the double charged mass of 177.7 m/z.

7.1.2. Measuring the bitterness of modified Den

The modified Den was made couplable by the newly inserted CH_2-NH_2 group. However, this chemical modification also changes the gustatory perception of the compound.

However, since it is a completely new substance which has not yet been toxicologically evaluated, taste testing by humans is not practicable. Nevertheless, in order to check the extent to which the modification affects bitterness, the newly synthesized Den derivative was measured on an electronic tongue. This method includes determining the electronic potentials of the substance in aqueous solution in a defined concentration.

Amino groups impact the potentiometric read-out, which is why this group was masked by acetylation. In order to be measured by the electronic tongue, **Den-CH_2-NH_2** had to be converted from a primary amine into a non-reactive compound (**Figure 15**). Since a Boc protective group is sterically demanding, a smaller protective group was chosen which does not mask the bitterness of the molecule and at the same time does not reduce the solubility. Since we can only measure substances in aqueous solution with the electronic tongue, this aspect should not be neglected.

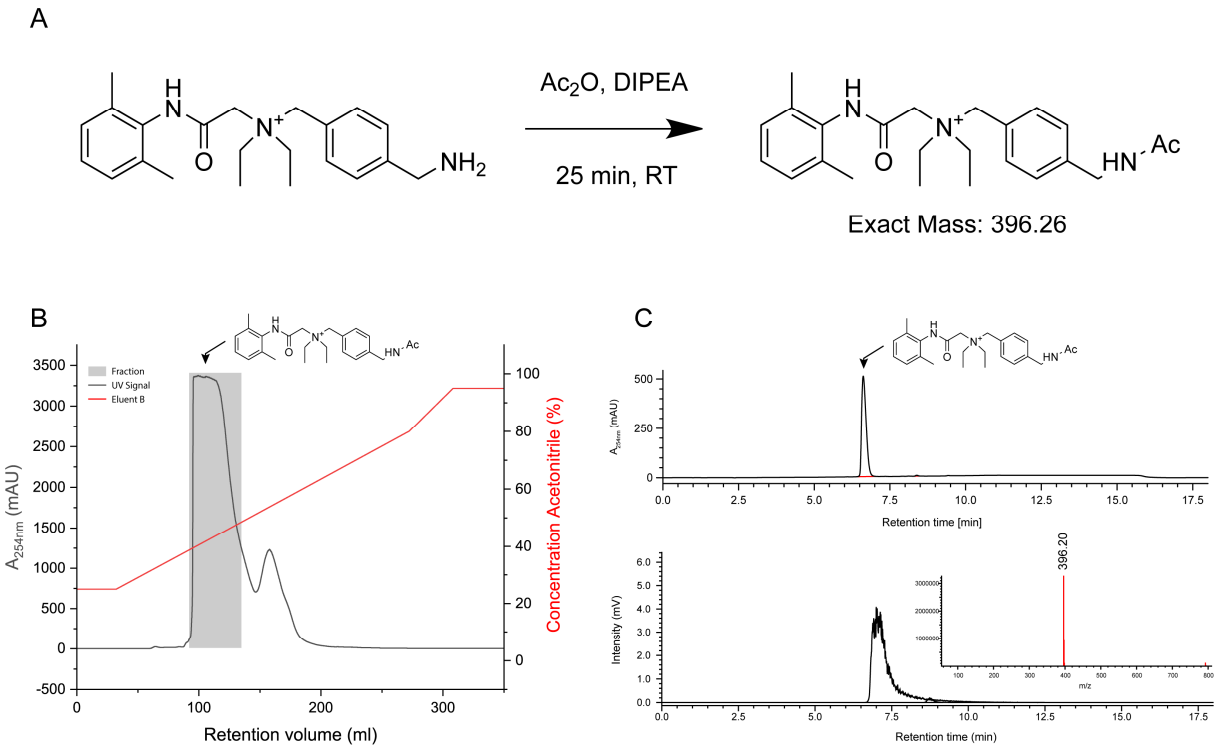


Figure 15. Synthesis, purification and LC/MS analysis of Den-CH_2-NH-Ac. (**A**) Synthesis of Den-CH_2-NH-Ac via an established acetylation protocol. (**B**) Purification via FPLC with UV detection at 254 nm. (**C**) HPLC chromatogram and mass spectrum. It is possible to identify the single charged mass of 396.20 m/z.

7.1.2.1. Electronic tongue measurements

The potentials of the Den derivative were measured according to previous experiments. For comparison, Den benzoate was used. All samples were measured by an electronic tongue in the range of 0.1 mM to 5 mM (**Table 6**). A 5 mM stock solution of both compounds was prepared and diluted with PBS to 1, 0.5 and 0.1 mM. SB2AC0, which is dedicated to detect bitter cationic substances and SB2AN0 which is dedicated to bitter cationic and neutral substances are used to measure the potentiometric signal. A third bitterness-detecting sensor is not used in this experimental approach, as it is for the measurement of bitter anionic substances. Sensor checks were carried out before every measurement to ensure the sensors

were working in the correct mV range. Each sample was measured four times. Between samples, the sensors were cleaned by an implemented cleaning protocol. Data are presented as mean ± standard deviation (SD). A graphical illustration of these results is provided in **Figure 16**.

Table 6. Electronic tongue measurements

Sensor SB2AC0				
Concentration	**Sensor response Den-CH_2-NH-Ac (mV)**	**SD (mV)**	**Den benzoate (mV)**	**SD (mV)**
0.1 mM	-57.69667	2.06108	-56.12667	1.18056
0.5 mM	-57.69667	2.28456	-45.32	2.27535
1 mM	-53.65667	2.23844	-35.76333	0.62979
5 mM	-36.89333	1.72929	10.92333	0.66365
Sensor SB2AN0				
Concentration	**Sensor response Den-CH_2-NH-Ac (mV)**	**SD (mV)**	**Den benzoate (mV)**	**SD (mV)**
0.1 mM	-72.87	1.28736	-71.37667	0.52501
0.5 mM	-67.9	1.05399	-58.80667	2.31716
1 mM	-64.56667	1.00451	-45.91	0.55245
5 mM	--38.94667	1.08487	11.92	0.63174

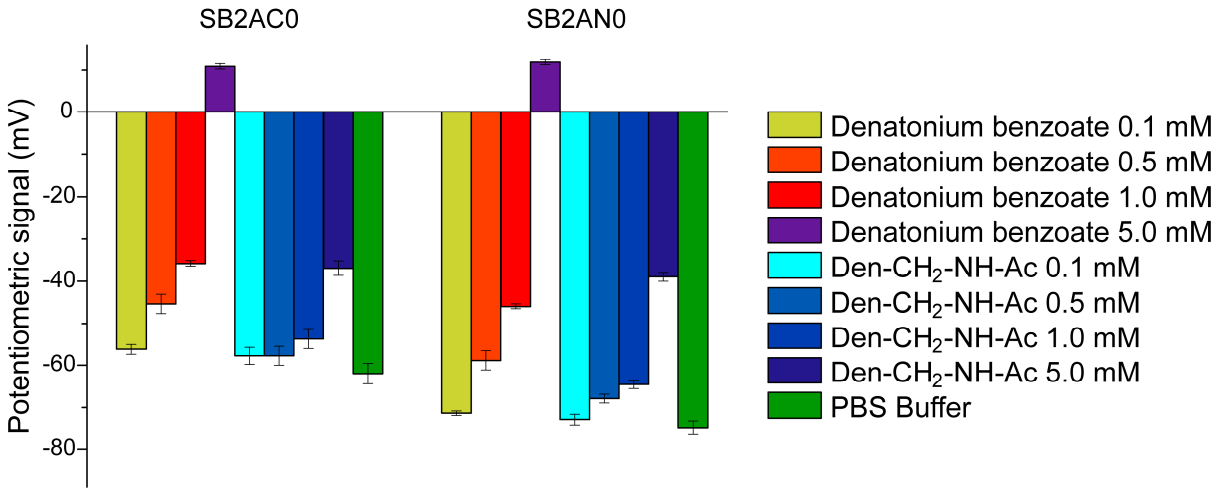


Figure 16. Graphical representation of the electronic tongue data. Data are presented as mean ± standard deviation (SD).

Based on the data of the present example, it can be concluded that **Den-CH_2-NH-Ac** is perceived similarly as Den benzoate by the electronic tongue, despite the functionalization by the CH_2-NH_2-group. With both substances, the mV values increase with higher concentration of the compound. The change in the potentiometric signal goes faster at the increase of the Den benzoate concentration. At the concentration step of 0.1 to 0.5 mM, the Den derivative shows no change in signal at the SB2AC0 sensor, and just a small change at the SB2AN0 sensor. From 0.5 mM to 1.0 mM, both sensors detected an increase in potentiometric signal. At a concentration of 5.0 mM, the effect of the modified Den is clearly observable. An immense increase in potentiometric signal was detected.

Comparing both compounds, the potentiometric read-out of the Den derivate was approximately $1/5^{th}$ of the changes of signals of Den benzoate. This is best to see, as the signals from 5 mM Den-CH_2-NH-Ac and 1 mM Den benzoate have a fairly equivalent value. Even one fifth of the bitterness would generate a strong gustatory perception, as Den benzoate is currently the bitterest compound known to human.

The change of potentials in both sensors allows the conclusion that **Den-CH_2-NH_2** is also perceived as bitter in taste by humans. It is therefore a usable compound in the synthesis of the diagnostic constructs. In the event of cleavage by the target protease and the further degradation by AP, a bitter gustatory perception would be generated.

7.1.3. Generating a Proteomic Identification of Cleavage Sites Assay Library

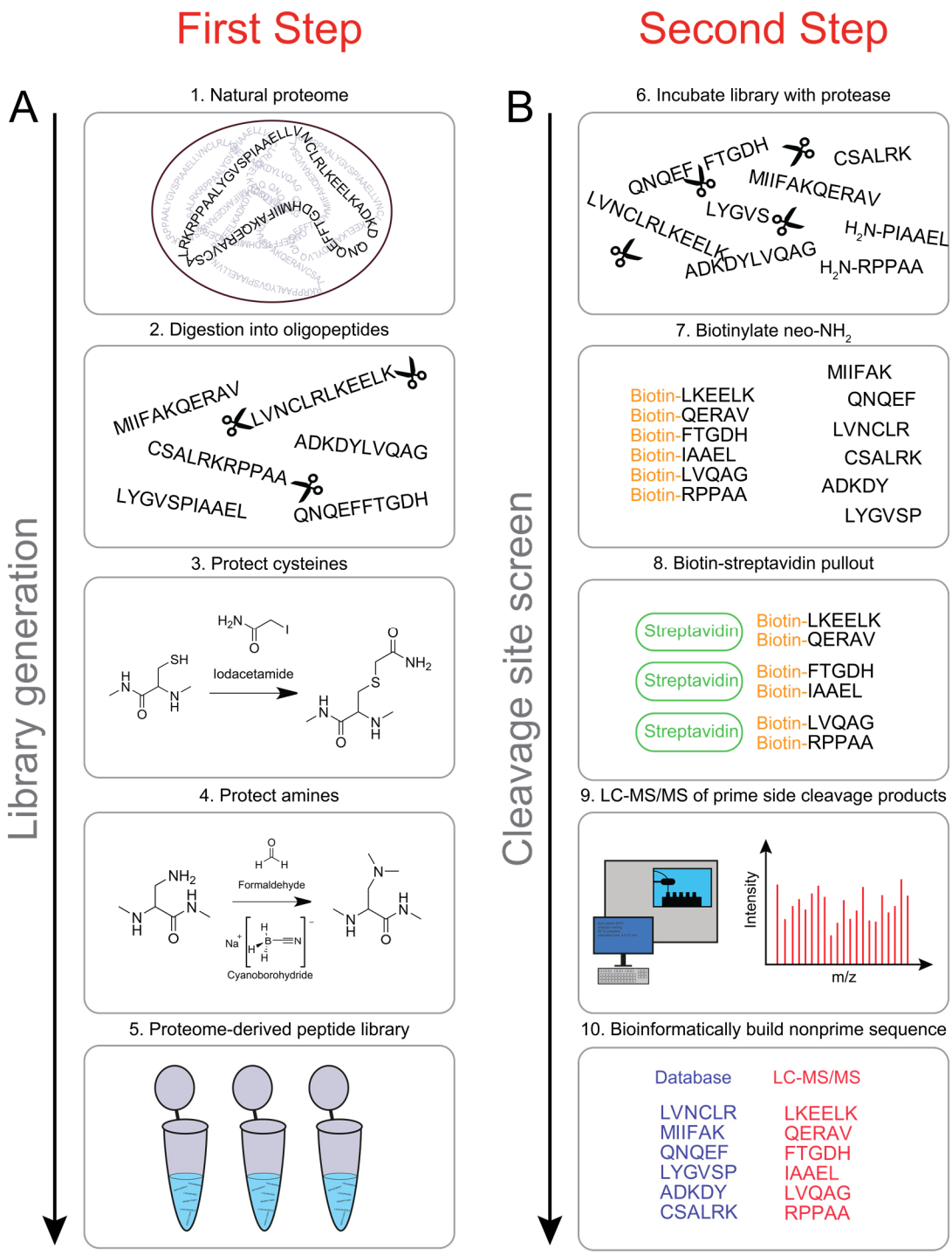


Figure 17. PICS peptide library generation and cleavage site screen. (**A**) Library generation. Proteome derived peptide libraries are generated by digesting the proteome with trypsin, GluC or chymotrypsin, to convert proteins into multiple oligopeptides. Subsequently, cysteines and amine groups are masked with protecting groups. (**B**) Cleavage site screen. The peptide library is incubated with the protease of interest. Prime-side cleavage products have neo-amino termini that are conjugated to biotin through a cleavable disulfide bond. After isolation by immobilized streptavidin followed by mild elution conditions, the sequences of the primeside cleavage products are determined by LC-MS/MS (shown in red). The prime-side sequence is used to bioinformatically reconstruct the nonprime sequence of the cleaved peptide up to the first occurrence of a cleavage site of the protease used for library generation.

Determining the optimal PSL sequence for our diagnostic systems, requires the identification of the complete repertoire of the target proteases natural substrates and corresponding substrate cleavage sites. The specificity of proteases can vary significantly, depending on the protease and the active sites. Some protease cleavage site selectivity ranging from preferences for limited and specific AAs at specific positions, while others have more general preferences with little discrimination. Existing literature, like the MEROPS database (http://merops.sanger.ac.uk) can give first information for some prominent proteases.

Current experimental approaches for proteolytic cleavage characterization include one-dimensional and two-dimensional gel-based methods (used for identifying the substrates), N-terminal peptide identification methods (for identifying both substrates and cleavage sites), and methods using mass spectrometry.

The PICS workflow is graphically summarized in **Figure 17**. To generate peptide libraries, proteome samples from HEK293 cells were digested with trypsin (cleavage after R or K in P1) or GluC (cleavage after E or D in P1). Before incubation, disulfide bridges were cleaved with DTT, to achieve better cleavage efficiency. After inactivation of the digestion protease, peptide sulfhydryl groups were carboxyamidomethylated using iodacetamide and amino termini and lysine ε-amino groups were dimethylated using formaldehyde.

Before the measurement the peptide library was purified by FPLC. The resulting peptide library was analyzed by liquid chromatography tandem mass spectrometry (**Figure 18**). To perform spectrum-to-sequence assignment searches with the program Mascot were performed. Mascot enables identification, characterization and quantitation of proteins using mass spectrometry data. The following parameters had to be chosen as modifications, due to former implemented protection reactions (**Table 7**).

Table 7. Static modifications in spectrum-to-sequence assignments

Name	Delta
Carbamidomethyl (C)	+57.021464
Acetyl (Protein N-term)	+42.010565
Gln→pyro-Glu (N-term Q)	-17.026549
Oxidation (M)	+15.994915
Dimethyl (K)	+28.0313
Dimethyl (N-term)	+28.0313

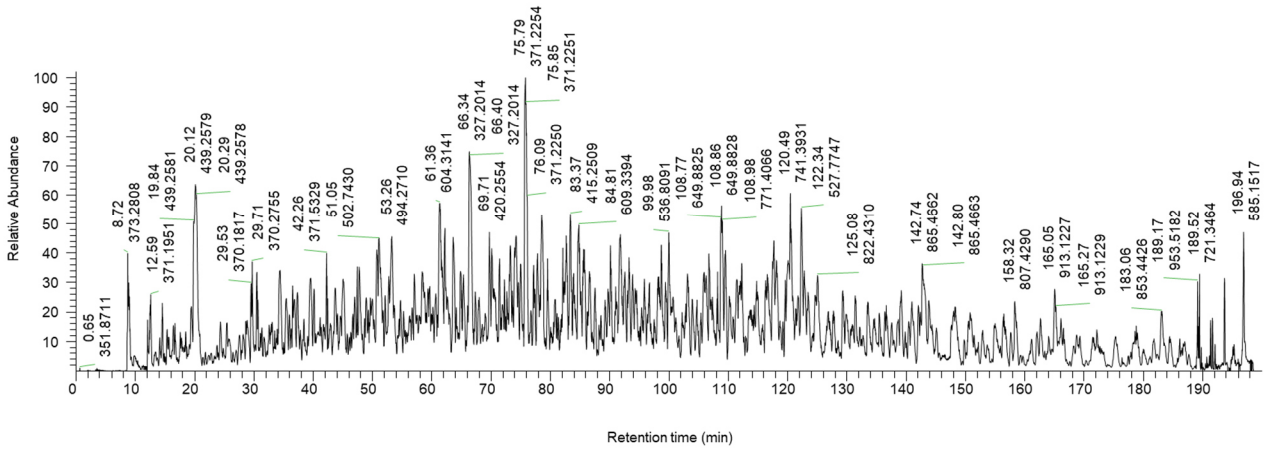


Figure 18. Base peak chromatogram of the generated peptide library (trypsin).

95106 peptide sequences could be assigned to this mass spectrum by Mascot search. The reaction with iodoacetamide and reductive methylation has thus produced sufficient evaluable sequences. 200 µg of each peptide library were frozen at -80 °C to be incubated with the proteases of interest.

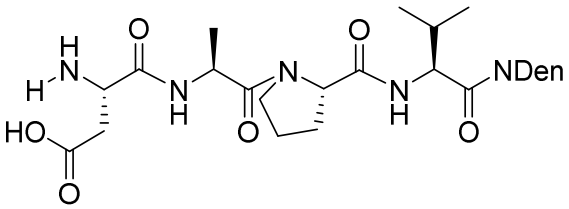
7.1.4. Coupling of PSL and Den-CH_2-NH_2

Formulated in chewing gum, the construct is designed to be cleaved only and efficiently by the target protease, leaving four AAs remaining on the flavoring. These mask the produced taste sensation.[193] To trigger maximum gustatory perception, all AAs must be removed from the modified Den by a human enzyme, called amino peptidase (AP). APs are not supposed to cleave the whole construct, as the N-terminal is protected through an acetyl protection group.

AP, are often zinc-containing exopeptidase, and selectively catalyze the hydrolysis of the N-terminus in the residues of proteins, peptides, or other substrates. They are widely distributed throughout plant and animal kingdom and abundantly present in all humans.[194]

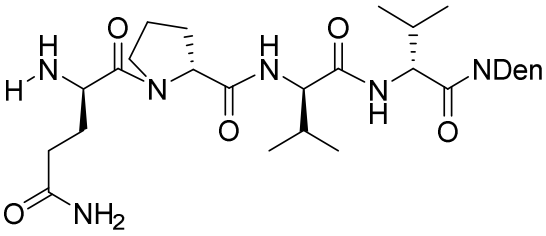
The degradation was analyzed with two constructs which were synthesized for this purpose (**Figure 19**). Several strategies were tried to couple the two compounds e.g. EDC/NHS coupling, coupling using HOBt/DIPEA and HATU/DIPEA, due to high yields and better purity after reaction the coupling by HATU/DIPEA was used for the further coupling of PSL and **Den-CH_2-NH_2**.

A



[DAPV-Den]

B



[QPVV-Den]

Figure 19. Peptide Sequences of the synthesized PSL. The modified Den is coupled via the primary amine to the C-terminus of the PSL. (**A**) Peptide QPVV (**B**) Peptide DAPV.

7.1.5. Cleavage of QPVV-Den and DAPV-Den

Both synthesis routes as well as the mass spectra of the final products (**Figure 19**) can be found in the **Appendix**.

The synthesized constructs have no acetyl residue at the N-terminus as this blocks the degradation of the peptide. Since AP as exoproteases always degrade one AA after the other, different intermediate fragments can be found using LC/MS (**Table 8**).

Table 8. Intermediate AA – Den fragments

Construct	Calculated molecular mass (Da)	Construct	Calculated molecular mass (Da)
QPVV-Den	777,50	**DAPV-Den**	736,44
PVV-Den	649,44	**APV-Den**	621,41
VV-Den	552,39	**PV-Den**	550,38
V-Den	453,32	**V-Den**	453,32
Den	354,25	**Den**	354,25

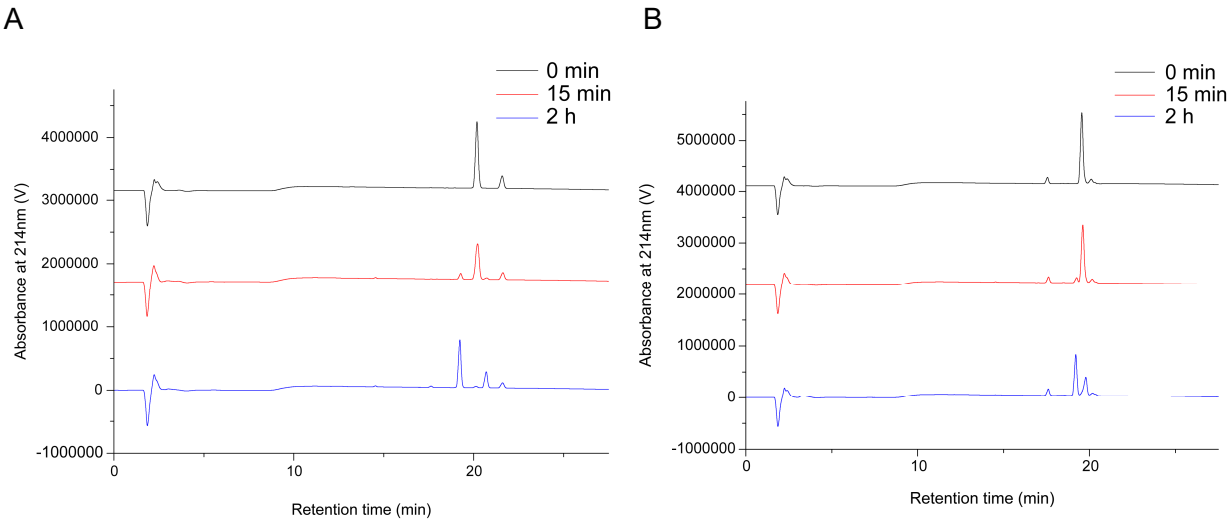


Figure 20. HPLC chromatogram of the two compounds after 0 min, 15 min and 2 h incubation with AP. (**A**) QPVV-Den (**B**) DAPV-Den.

When a four AA residue is obtained after cleavage of an acetylated construct, it is now possible for the AP to attack the free N-terminus so that further degradation occurs. Optimally, the four AAs are cleaved off one by one, until the Den derivate is completely released.

The degradation of both compounds was monitored by HPLC (**Figure 20**) and progressed visibly after 15 min and 2 h. In order to be able to follow the degradation more closely, both constructs were incubated with AP for 24 h and the result were analyzed by LC/MS (**Figure 21**).

This showed that after this time of incubation, the last AA remained on the constructs. When using QPVV-Den, the degradation product PVV-Den was also detectable after 24 h. Thus, the cleavage of proline in this position, also determines the reaction speed.

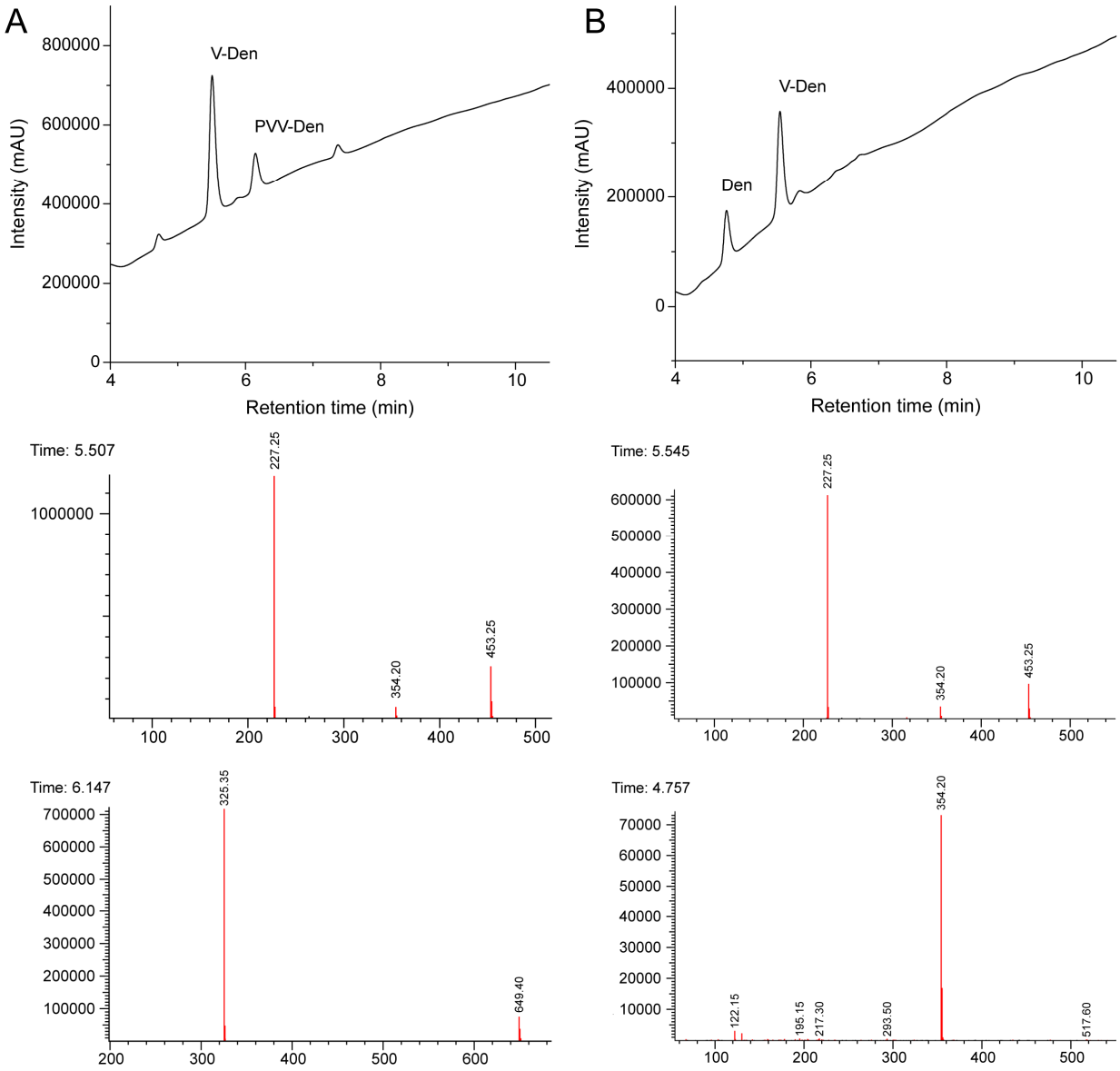
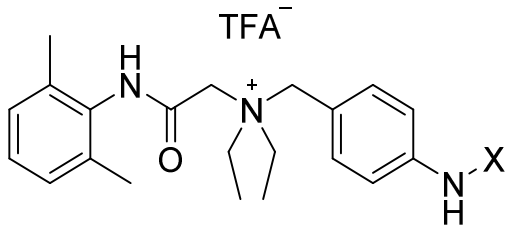


Figure 21. Chromatograms and mass spectra of QPVV-Den and DAPV-Den after 24h AP incubation. (**A**) QPVV-Den: PVV-Den and V-Den can still be detected after 24 h of incubation. (**B**) DAPV-Den: V-Den can be detected as the main product after incubation.

A complete degradation therefore does not take place. The remain of an AA can drastically reduce the taste perception. In this example, valine cannot be attacked by the AP. This is probably due to steric reasons or to the continuous charge of the modified Den, which shields the molecule from the protease.

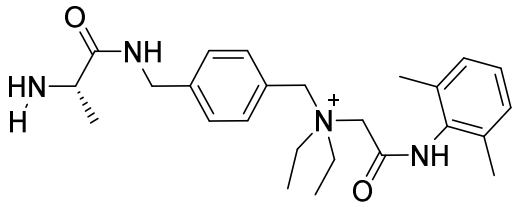
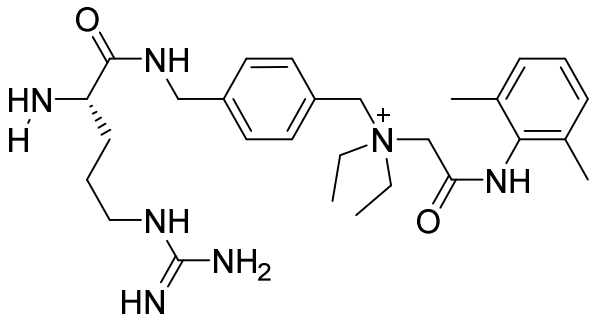
7.1.6. AA-Den-CH_2-NH_2 constructs

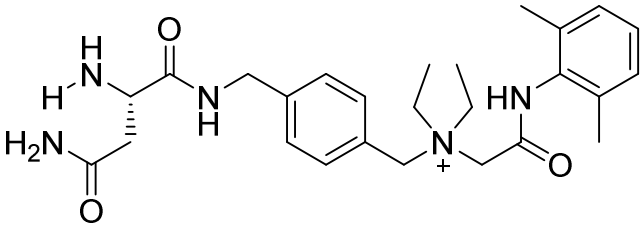
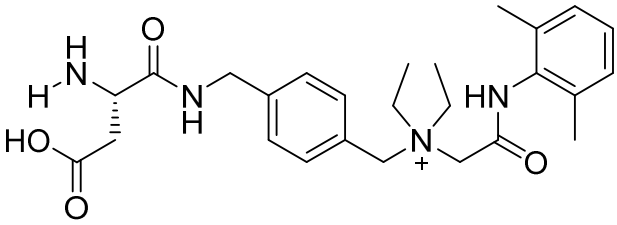
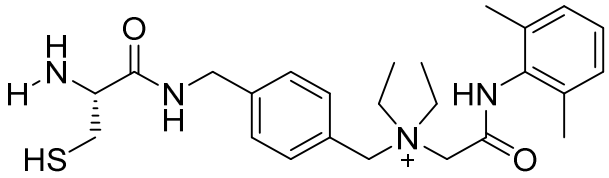
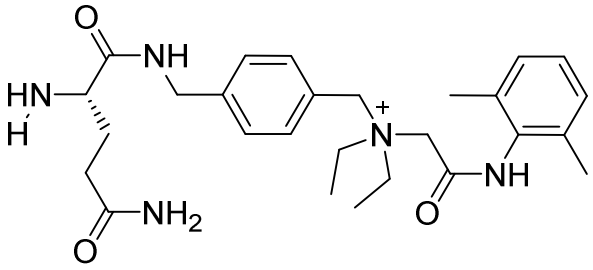
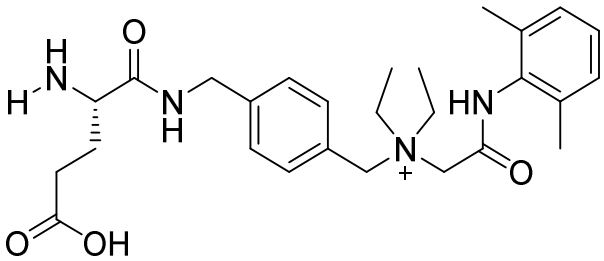
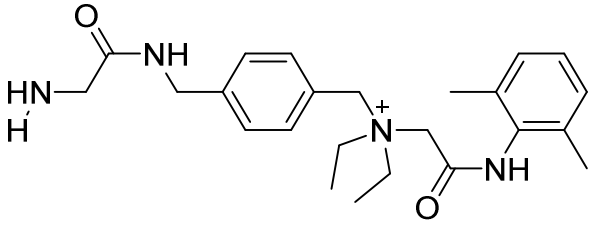
So we can assume, that not all AAs are equally well suited for this purpose. The perfect AA should be easily couplable in high yields to the modified Den, as well as quickly released by human AP. To evaluate the cleavage efficiency of AP towards the last residue, 20 constructs consisting of one proteinogenic AA coupled to **Den-CH_2-NH_2** were synthesized (**Table 9**), thereby obtaining compound **[Den-X]**, wherein X is an AA:

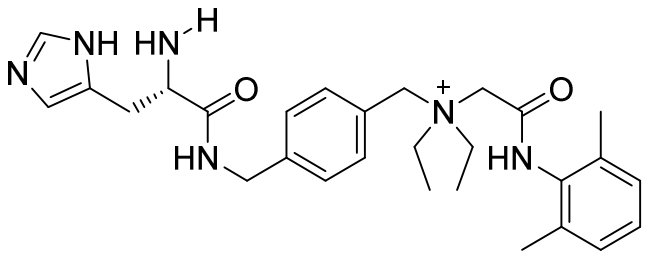
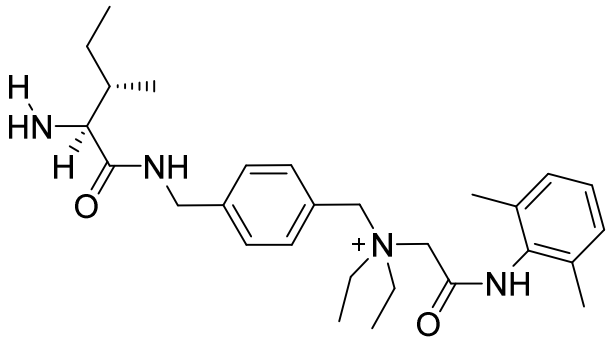
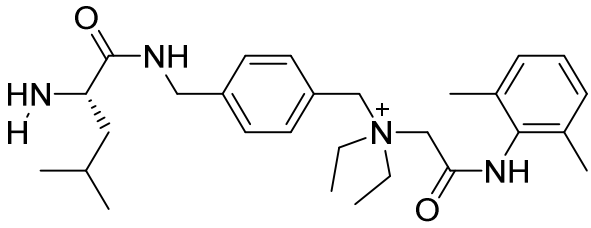
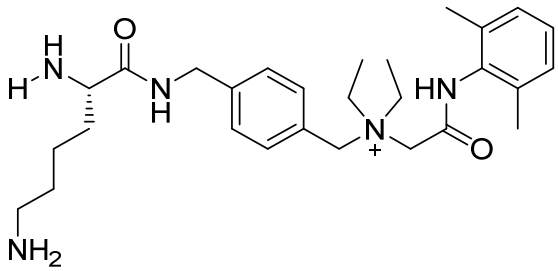
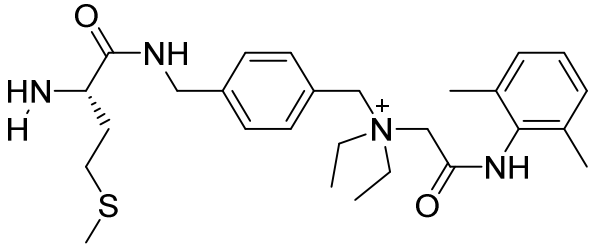


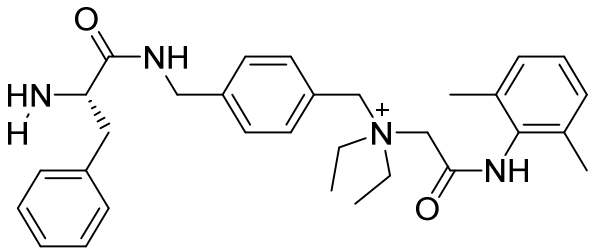
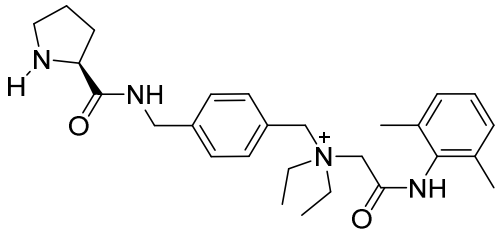
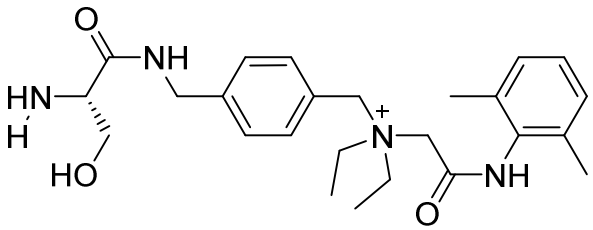
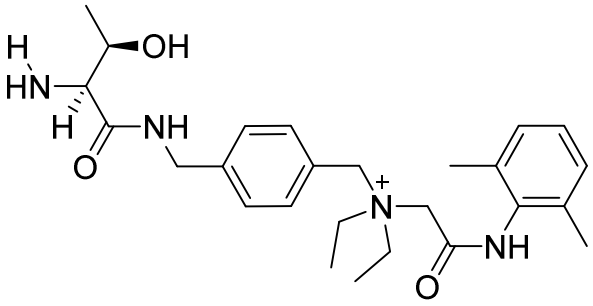
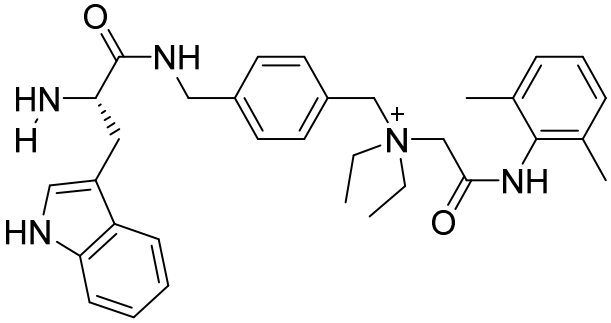
[Den-X]

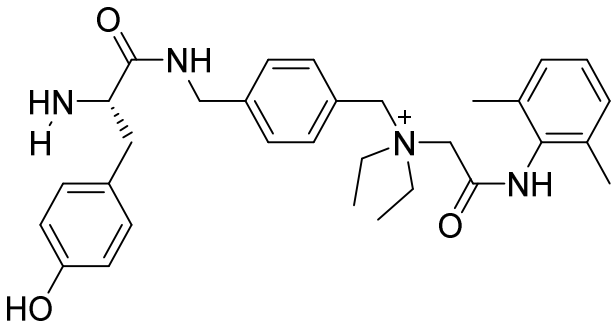
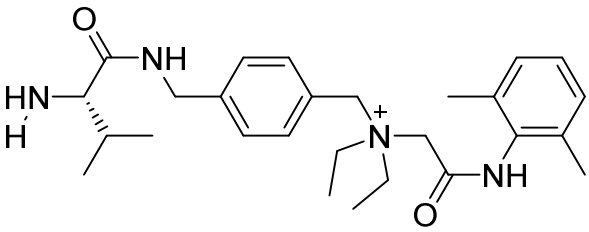
Table 9. Synthesized Den-X constructs.

AA coupled to Den-CH_2-NH_2	Chemical structure	Theoretical mass (Da)	Measure Mass (Da)
Alanine (A)		425.29	425.30
Arginine (R)		510.36	510.30

AA coupled to Den-CH_2-NH_2	Chemical structure	Theoretical mass (Da)	Measure Mass (Da)
Asparagine (N)		468.30	469.25
Aspartic acid (D)		469.28	469.30
Cysteine (C)		457.26	457.25
Glutamine (Q)		482.31	482.35
Glutamic acid (E)		483.30	483.25
Glycine (G)		411.28	411.30

AA coupled to Den-CH_2-NH_2	Chemical structure	Theoretical mass (Da)	Measure Mass (Da)
Histidine (H)		491.31	491.30
Isoleucine (I)		467.34	467.30
Leucine (L)		467.34	467.35
Lysine (K)		482.35	482.30
Methionine (M)		485.29	485.30

AA coupled to Den-CH_2-NH_2	Chemical structure	Theoretical mass (Da)	Measure Mass (Da)
Phenylalanine (F)		501.32	501.30
Proline (P)		451.31	451.30
Serine (S)		441.29	441.25
Threonine (T)		455.30	455.30
Tryptophane (W)		540.33	540.30

AA coupled to Den-CH_2-NH_2	Chemical structure	Theoretical mass (Da)	Measure Mass (Da)
Tyrosine (Y)		517.32	517.30
Valine (V)		453.32	453.30

7.1.7. Cleavage of Den-X by AP

To better understand the degradation process and to find an optimal coupling candidate, three time points were observed more closely. Incubation times of 15 min, 60 min and 24 h were applied, and afterwards samples were inactivated by heat. HPLC measurements were obtained to analyze cleavage, which was determined by decrease of the peaks in relation to their negative control peaks (**Figure 22**). Only 16 out of 20 constructs were used, as lysine, asparagine, cysteine, and histidine did not produce significant synthesis yields, which also would interfere with peptide to flavor coupling. PSL containing these AAs at the C-Terminus should be modified towards better couplable residues.

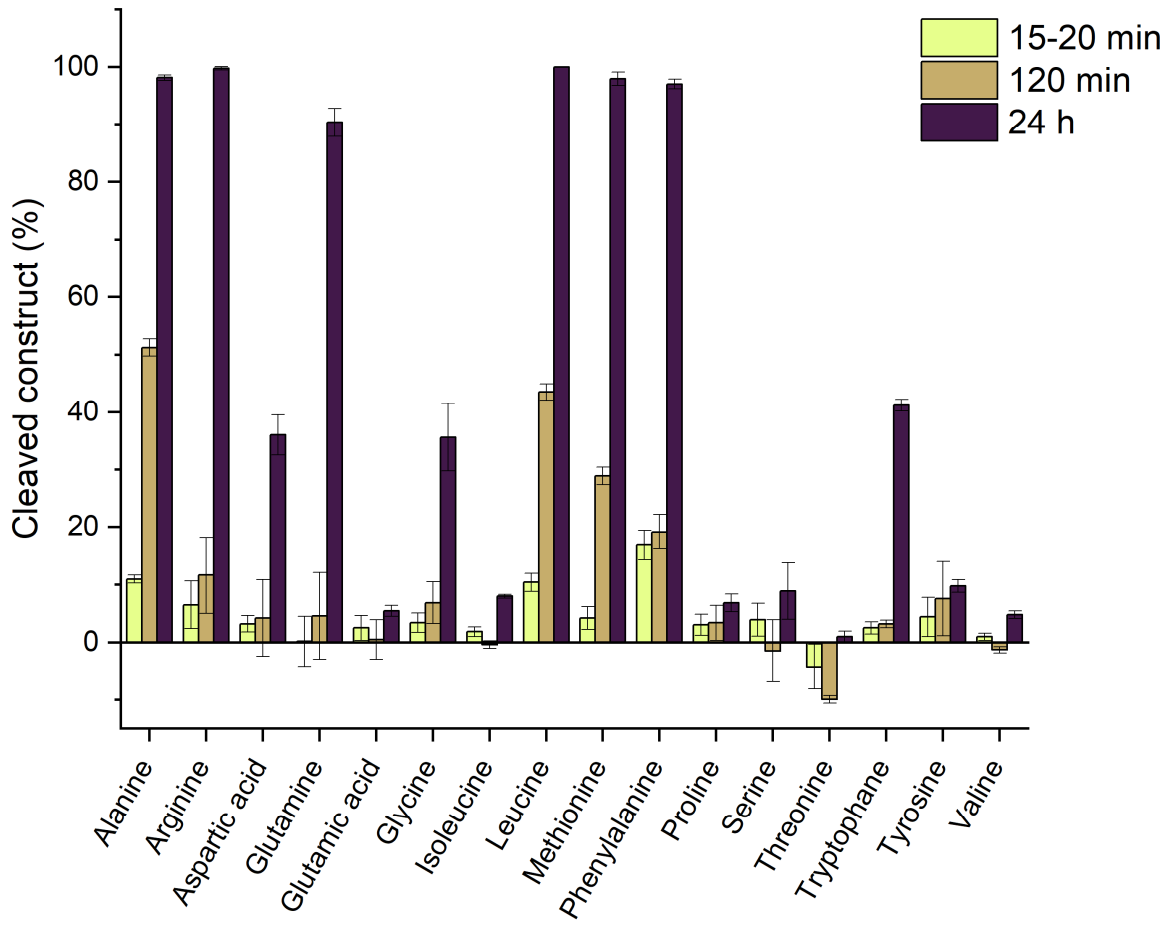


Figure 22. Cleavage of [Den-X] after 15-20 min, 60 min and 24 h. Data are presented as mean ± SD.

Out of the 16 constructs **[Den-X],** Den-A and Den-L showed the most promising results. After 15-20 min 10% and after 120 min 50% were cleaved off the modified Den. Also both AAs are chemically inert, and therefore not interfering with possibly used proteases. Arginine, methionine and phenylalanine showed also good cleavage rates, resulting in a full cleavage after 24 h. These three AAs are however more difficult to handle than alanine and leucine and can interfere with the active centers of proteases. New synthesized PSL should contain these AAs at the C-terminus for optimal performance.

7.2. Section II - Matrix metalloproteinases

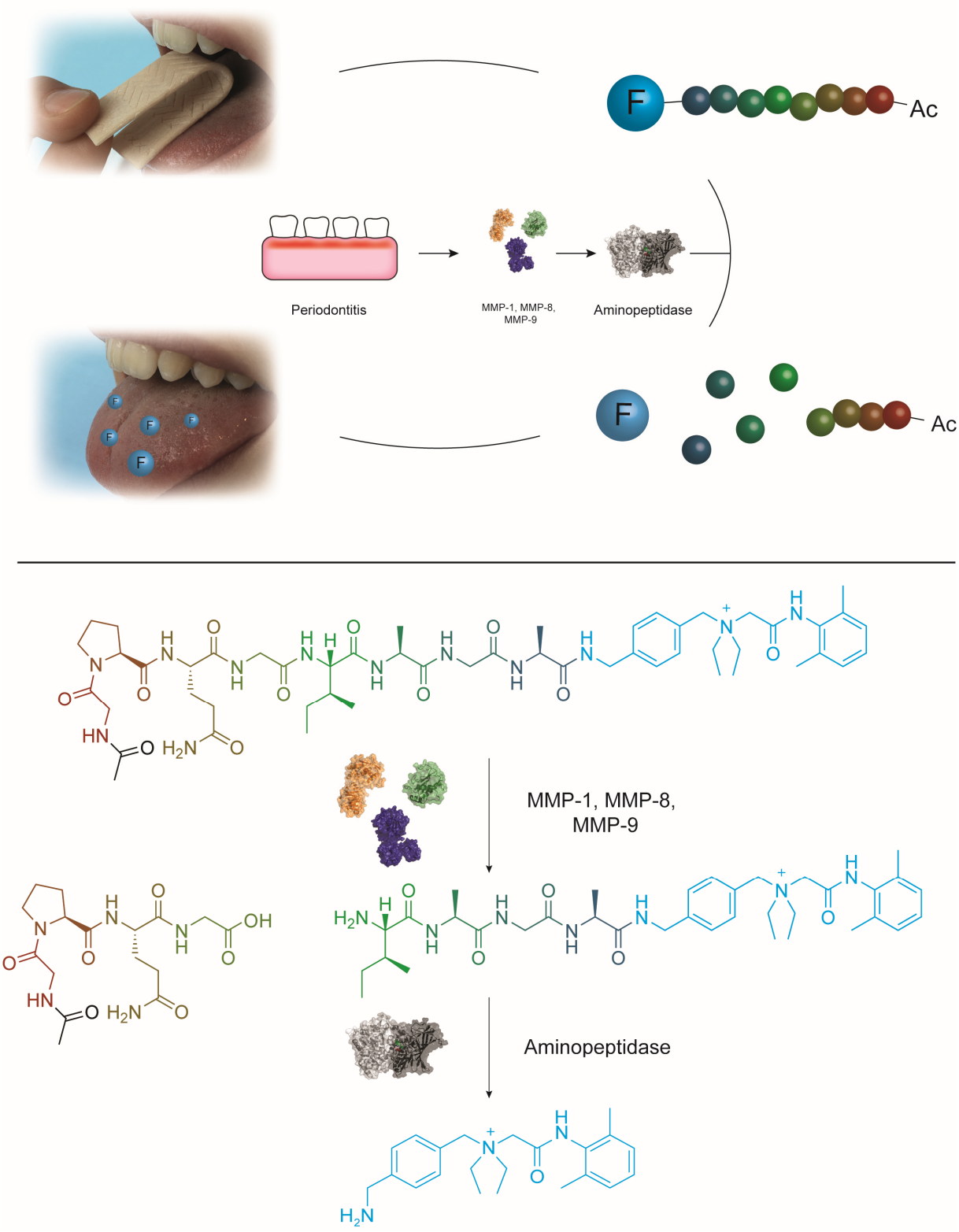


Figure 23. Schematic representation of the working principle of the PA sensing construct. (Upper panel) Formulated in a chewing gum, the diagnostic system is released into the oral cavity. If PA is present, matrix metalloproteases are released which cleaves the construct. The new free N-terminus makes the system vulnerable to AP. The flavour is released and the patient is informed about the state of health. (Lower panel) Intermediate step of 2 to 3 with chemical formulae.

7.2.1. PICS Assay of MMP-9

Until now, the sequences of PSL for the diagnostic constructs have mainly been based on literature sequences. The cleavable sequence (GPQGIAGQ) previously used for the system is derived from the MMP cleavage site in collagen.[195] In order to be able to determine cleavable sequences of MMPs and other target proteases in the future and to verify the established PICS assay with already known results, an analysis was performed with active MMP-9.

The peptide library produced was incubated with the protease of interest, the test protease afterwards deactivated and the system incubated with sulfo-NHS-SS biotin. This links the neo-amino terms created by cleavage to biotin (**Figure 24**).

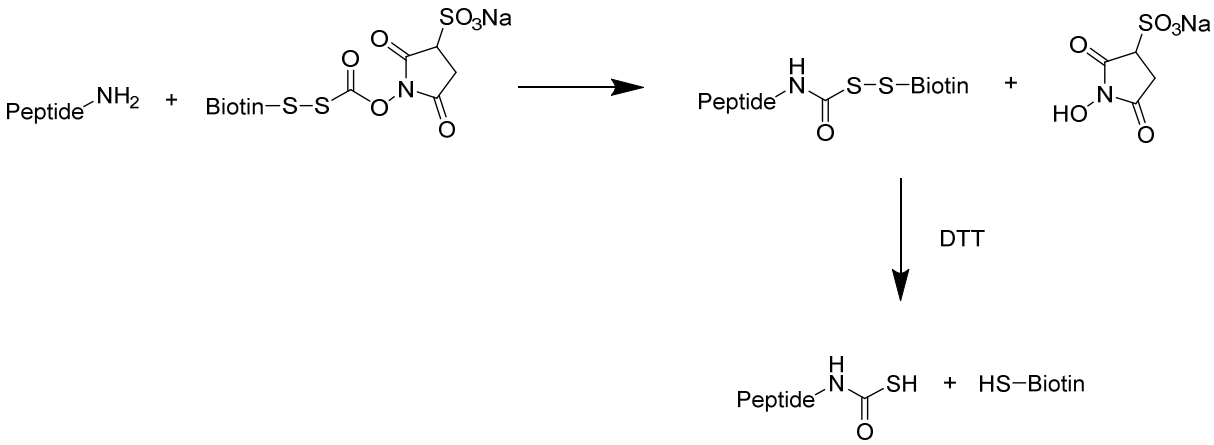


Figure 24. Chemical reactions of biotinylation and elution with DTT. N-hydroxysuccinimide (NHS) esters readily form stable bonds with primary amines. The biotinylated peptides can now be purified via a streptavidin column. Biotin can be cleaved from the peptides by reductive agents like DTT, however a thioacyl-residue remains.

Thus the cleaved peptides can be purified. When they are eluted from the column again with DTT, a thioacyl residue remains, which is used in subsequent mass spectrometry to find the cleaved peptides (**Figure 25**). The previously biotinylated peptide fragments show a mass difference of 87.998285 Da (**Table 10**).

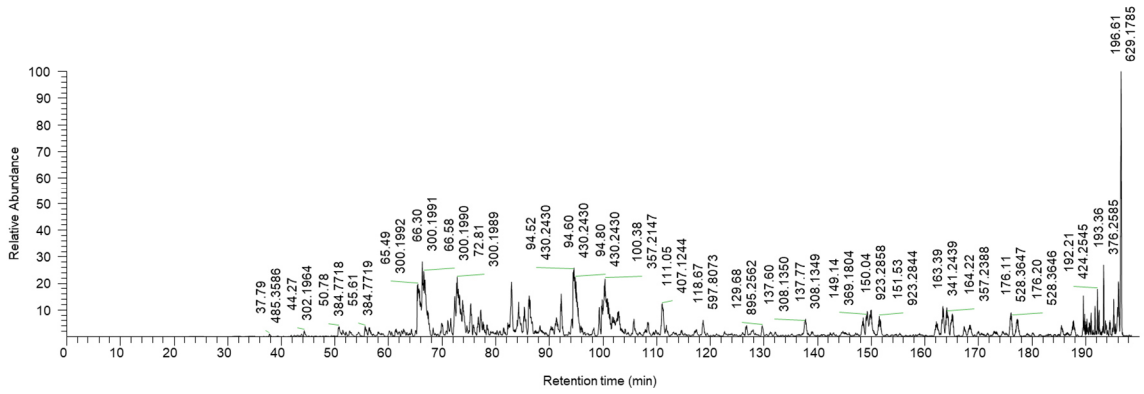
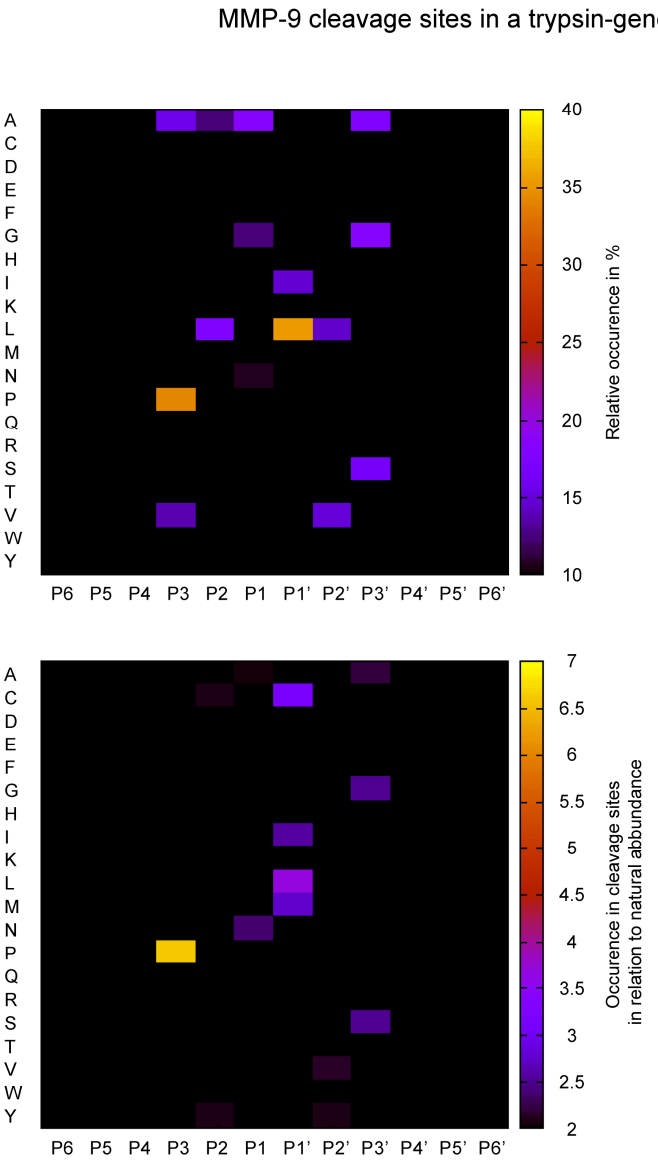


Figure 25. Base peak chromatogram of the PICS assay (trypsin library) incubated with MMP-9.

Table 10. Static modifications in spectrum-to-sequence assignments

Name	Delta
Carbamidomethyl (C)	57.021464
Acetyl (Protein N-term)	42.010565
Gln→pyro-Glu (N-term Q)	-17.026549
Oxidation (M)	15.994915
Dimethyl (K)	28.0313
Dimethyl (N-term)	28.0313
Thioacyl (N-term)	87.998285

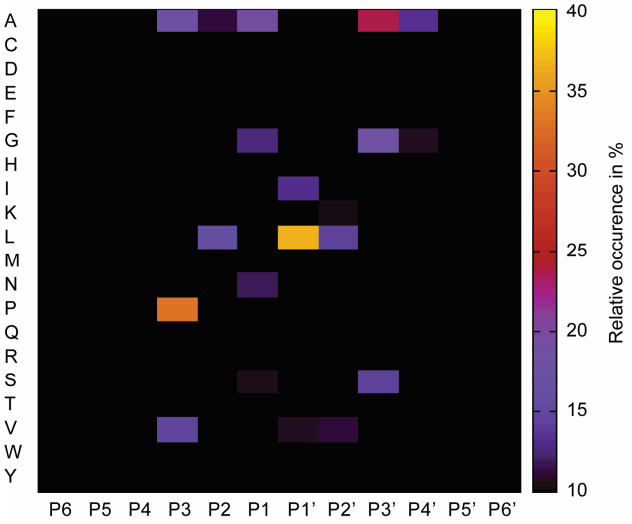


	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	5.2	7.1	6.4	15.5	12.4	18.2	3.4	5.9	17.5	9.3	9.1	8.2
C	1.1	1.1	1.6	1.7	2.9	1.9	4.5	2.2	1.3	2.1	1.4	1.1
D	4.2	2.4	3.1	0.4	1.2	7.5	0	0.7	3.8	7.7	6.8	7.8
E	4.7	3.1	5	1.5	4.9	8.1	0.9	2.1	4	8.4	7.3	7.9
F	2.5	2.9	4.3	1.5	7.1	2.6	4.3	6.4	2.6	3.9	3.1	2.1
G	4.3	5.7	6.3	2.6	4.6	12.4	1.5	2.6	18.2	8.8	7.2	6.8
H	1.3	1.7	1.5	1	2.2	2.2	0.9	4.7	2.3	3.8	2.9	2.7
I	2.2	4.5	4.3	6.5	3.5	1	14.6	8.7	2.6	4.1	6.1	5.2
K	0.1	0.1	3.5	0	0.3	0	0.4	1.3	0.8	1.3	2.6	4.6
L	5.6	8.5	6.8	5.1	17.6	2.7	35.3	14.4	3.6	6.4	9.5	8.2
M	1.1	1.4	1.8	0.6	3.6	1.9	7.1	4	1.5	2.1	2.1	1.3
N	2	2.6	2.6	1.5	2	10.5	0.7	2.6	4.2	5.4	3.5	5.3
P	3.2	4.5	4.7	34.2	1.5	6.2	0.7	0.7	0.6	5.7	6	6.9
Q	2.4	3.9	3.5	0.8	4.6	3.7	5	5.2	3.3	6	4.4	5.4
R	0.3	0.2	2.1	0	0.6	0	0.1	1.5	0.3	0.9	1.5	2.4
S	4.1	4.5	4.8	2.3	7.8	8.5	3.4	6.1	16.6	7.7	7.4	5.6
T	2.9	4	5.9	2	2.1	3.5	2.1	8	5.2	4.6	7.6	7.4
V	4.3	6	6.3	13.7	7.1	2.7	8.1	14.9	8.1	7.7	8	7.2
W	0.6	0.7	0.8	0.4	2.3	1.9	0.9	1.4	0.5	0.4	0.6	0.1
Y	2.3	1.5	2.9	1.3	6.5	2	6.1	6.5	2.9	3.6	2.9	3.3

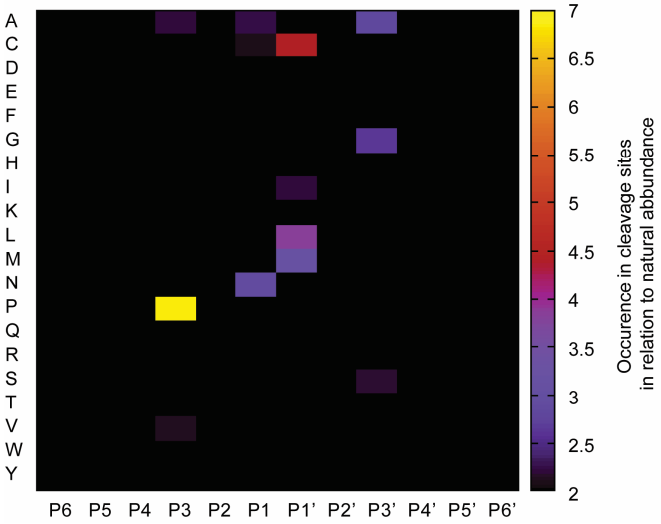
	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	0.6	0.8	0.8	1.8	1.5	2	0.4	0.7	2.2	1.1	1.1	1.1
C	1	0.9	1.2	1.2	2.1	1.4	3.2	1.5	0.9	1.3	1	0.9
D	0.8	0.4	0.6	0.1	0.3	1.3	0	0.1	0.7	1.4	1.2	1.4
E	0.7	0.5	0.8	0.3	0.8	1.3	0.2	0.3	0.6	1.2	1.1	1.2
F	0.6	0.8	1.1	0.4	1.8	0.8	1	1.6	0.6	1	0.7	0.6
G	0.6	0.8	0.8	0.4	0.6	1.7	0.2	0.4	2.5	1.2	1.1	1
H	0.5	0.8	0.8	0.5	1.1	1	0.4	2	0.9	1.7	1.1	1.2
I	0.4	0.7	0.7	1.1	0.6	0.3	2.6	1.6	0.5	0.8	1	0.9
K	0	0	0.5	0	0.1	0	0.2	0.3	0.2	0.4	0.7	0.9
L	0.6	0.9	0.7	0.5	1.7	0.4	3.7	1.6	0.4	0.7	1	0.9
M	0.4	0.6	0.8	0.2	1.4	0.9	2.7	1.5	0.7	0.7	0.7	0.5
N	0.5	0.6	0.6	0.4	0.6	2.3	0.2	0.7	1	1.3	0.9	1.2
P	0.6	0.9	1	6.6	0.5	1.3	0.2	0.2	0.1	1.2	1.2	1.4
Q	0.6	1	0.8	0.3	1.1	0.9	1.3	1.3	0.7	1.5	1.1	1.3
R	0.1	0	0.3	0	0.1	0	0	0.3	0.1	0.2	0.3	0.5
S	0.6	0.7	0.8	0.4	1.2	1.2	0.5	0.9	2.5	1.1	1.1	0.8
T	0.5	0.7	1.1	0.5	0.5	0.7	0.3	1.5	0.9	0.9	1.4	1.3
V	0.6	0.8	0.9	1.8	1	0.5	1.1	2.1	1.1	1.1	1.2	1
W	0.5	0.6	0.7	0.4	1.9	1.7	0.7	1.1	0.6	0.4	0.6	0.1
Y	0.7	0.5	1	0.5	2.1	0.8	1.8	2.1	0.9	1.1	1	1

Figure 26. PICS sequence specificity profiles of MMP-9 using trypsin-generated human peptide libraries. Identified cleavage sites (n values shown) are summarized as heat maps showing relative occurrence (upper left panel) and the more incisive fold-change over the natural abundance of AAs (lower left panel) P6 to P6′ subsite positions are shown on the x axes; plotted AAs are indicated on the y axes with one-letter codes. On the right, the corresponding numerical representation of PICS specificity profiles.

MMP-9 cleavage sites in a GluC-generated peptide library (n=416)



	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	7.9	6	7.2	18.1	11.3	18.5	3	6.7	23.1	13.9	7.9	7.6
C	1.9	1.4	2.1	0.9	2.5	2.8	6	0.7	0	2.5	0.7	2.3
D	3.9	4.2	4.4	0.5	2.5	5.3	0	1.2	4.2	7.2	8.8	9.5
E	0	0	1.2	0.2	0	0	0.2	1.4	1.4	3.7	3.9	4.6
F	0.9	4.6	1.6	1.6	6.7	3.7	3	4.6	2.5	3.5	3.2	1.6
G	3.7	6	6.5	0.7	3	12.3	0.5	1.6	18.5	10.9	6.5	6.2
H	2.1	3	1.6	1.9	2.1	2.1	0.5	0.7	0.2	3.7	2.3	2.5
I	4.4	2.8	4.6	5.6	2.3	0.7	13.2	9.7	1.4	3.5	5.1	6.9
K	5.6	3.5	8.3	0.9	5.1	3.5	1.2	9.7	6.9	6.2	8.6	7.6
L	6.7	5.8	7.2	4.4	15.3	4.4	37	15	3.9	6.7	7.6	7.6
M	1.2	2.1	0.9	0.5	3.9	2.3	7.6	4.6	0.5	2.1	1.6	2.1
N	2.8	3.7	2.3	0.2	2.1	11.6	0.5	2.3	5.8	5.1	5.1	5.1
P	4.4	4.2	2.8	32.2	1.4	4.6	0.7	1.2	0.2	5.8	3.5	5.6
Q	3.2	5.1	3.7	1.2	5.3	4.2	6	3	2.8	4.2	6.2	5.3
R	4.2	4.2	6.2	1.2	5.8	3	0.7	6.7	0.7	3.2	4.4	5.3
S	5.3	3.7	3.9	1.4	6.9	10	2.8	7.2	14.1	4.2	7.2	6.2
T	1.9	5.6	6	4.4	3	3.9	1.4	8.1	4.4	3.5	7.6	5.3
V	3.5	4.2	8.1	14.4	7.9	1.4	10.6	10.6	6.9	7.4	7.4	6
W	0.2	0.5	0.7	0.2	1.6	0.9	0.9	0.9	0.7	0.5	0	0
Y	0.9	3.2	2.3	0.9	4.9	2.3	4.2	3.9	1.6	2.3	2.3	2.3



	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	1	0.7	0.9	2.2	1.4	2.2	0.4	0.8	2.8	1.7	1	0.9
C	1.4	1	1.5	0.7	1.8	2.1	4.4	0.5	0	1.8	0.5	1.7
D	0.7	0.8	0.8	0.1	0.5	1	0	0.2	0.8	1.3	1.6	1.7
E	0	0	0.2	0	0	0	0	0.2	0.2	0.5	0.6	0.7
F	0.2	1.2	0.4	0.4	1.7	1	0.8	1.2	0.6	0.9	0.8	0.4
G	0.5	0.8	0.9	0.1	0.4	1.7	0.1	0.2	2.6	1.5	0.9	0.9
H	0.9	1.3	0.7	0.8	0.9	0.9	0.2	0.3	0.1	1.6	1	1.1
I	0.7	0.5	0.8	0.9	0.4	0.1	2.2	1.6	0.2	0.6	0.9	1.2
K	1	0.6	1.4	0.2	0.9	0.6	0.2	1.7	1.2	1.1	1.5	1.3
L	0.7	0.6	0.7	0.5	1.6	0.5	3.8	1.6	0.4	0.7	0.8	0.8
M	0.5	0.9	0.4	0.2	1.6	1	3.1	1.9	0.2	0.9	0.7	0.9
N	0.7	0.9	0.6	0	0.5	2.9	0.1	0.6	1.4	1.3	1.3	1.3
P	0.9	0.9	0.6	6.9	0.3	1	0.1	0.3	0	1.2	0.7	1.2
Q	0.8	1.3	0.9	0.3	1.3	1.1	1.5	0.8	0.7	1.1	1.6	1.3
R	0.8	0.8	1.1	0.2	1	0.5	0.1	1.2	0.1	0.6	0.8	1
S	0.8	0.6	0.6	0.2	1.1	1.5	0.4	1.1	2.2	0.6	1.1	1
T	0.4	1.1	1.1	0.8	0.6	0.7	0.3	1.5	0.8	0.7	1.4	1
V	0.5	0.6	1.2	2.1	1.1	0.2	1.5	1.5	1	1.1	1.1	0.9
W	0.2	0.5	0.6	0.2	1.5	0.8	0.8	0.8	0.6	0.5	0	0
Y	0.3	1.1	0.8	0.3	1.7	0.8	1.4	1.3	0.5	0.8	0.8	0.8

Figure 27. PICS sequence specificity profiles of MMP-9 using GluC-generated human peptide libraries. Identified cleavage sites (n values shown) are summarized as heat maps showing relative occurrence (upper left panel) and the more incisive fold-change over the natural abundance of AAs (lower left panel) P6 to P6′ subsite positions are shown on the x axes; plotted AAs are indicated on the y axes with one-letter codes. On the right, the corresponding numerical representation of PICS specificity profiles.

7.2.1.1. MMP-9 subsite analysis

Subsite P3

The P3 preference for proline is a hallmark feature of the MMP family. MMP-9 display a high prevalence of P3 proline (34.2% (trypsin)/ 32.2% (GluC)). MMP-9 also favors small to large aliphatic residues in P3, such as alanine and valine.

Subsite P2

Specificity profiles obtained were fairly diverse at P2. The presence of acidic residues in P2 co-occurred with an increased presence of proline in P3. Aside from small residues (alanine, leucine, valine, serine), MMP-9 preferred positively charged residues (arginine, lysine). The selectivity of MMP-9 towards basic residues in P2 is linked to an aspartic residue (Asp410) in proximity to S2.[196]

Subsite P1

Preferences of P1 included small AAs such as glycine, alanine or serine, whereas aromatic and larger aliphatic residues were barely observed in P1. Another AA found to be often in P1 position is asparagine.

Subsite P1′

P1′ leucine clearly constituted the primary specificity determinant for all MMPs. Together with isoleucine, and to a lesser extent valine and methionine, a combined occurrence of 68% was observed. MMP-9 shows the lowest frequency of leucine (37%) in the MMP family. MMP-9 displayed a noticeable preference for carbamidomethylated cysteine introduced during PICS library preparation, most likely due to its aliphatic property. Due to this protection group, it resembles the AA glutamine, to which MMP-9 has a weak preference.[197]

Subsite P2′

In P2′, MMP specificity was dominated by a preference for leucine, isoleucine and valine (average occurrence of 38% (trypsin)/ 35% (GluC)) together with a preference for positively charged AAs (6% (trypsin)/ 16% (GluC)). The latter feature was detected mostly in GluC libraries since tryptic libraries have lysine and arginine only at the C-terminus of the peptide. In accordance to previous studies, MMP-9 shows a comparably low occurrence of basic residues in the GluC profile. This was counter-balanced by a notable overrepresentation of histidine in the tryptic profile. The preference for medium-sized aliphatic residues such as valine and isoleucine was more evident with tryptic PICS libraries, most likely as this feature is superseded by the pronounced MMP preference for basic residues in GluC libraries, illustrating

the benefit of using more than one peptide library type for comprehensive active site specificity profiling.

Subsite P3′

In P3′, MMP-9 showed a marked preference for small AA such as glycine and alanine, with a combined occurrence of 36% (trypsin)/ 42% (GluC). The selectivity of this position appeared to be as inconsistent as that of P2 across the MMP family and thus could be of similar importance as P2 for the specific targeting of individual family members as previously suggested.[198]

Subsite P4-P6 and P4’-P6’

No preference for specific AA in this region were observed. Small and hydrophobic AAs like alanine, valine, glycine and proline occurred slightly more often.

7.2.1.2. MMP-9 PICS dependency analysis

The PICS Assay displays the AA that occur most frequently in a certain position. However, a sequence that can be cleaved cannot necessarily be determined from the residues with the highest probability. Depending on the AA in a certain location, the probability of another AA occurring in another location increases and decreases. However, the PICS Assay and the determined cleavage sequences can clarify these dependencies.

Cooperativity with P3 Proline

Proline in position P3 of the substrate is an essential feature in the entire MMP family. It is therefore highly important to understand the cooperativity of proline with other residues. Using MMP-9, leucine also occurs in position P1' with an extremely high probability (37% (trypsin)/ 37% (GluC)). Both AAs are therefore analyzed in their cooperativity (**Figure 28**). If one of the two AAs is present, however, the probability decreases drastically that the other position is occupied by the otherwise frequently occurring AA. The frequency of finding valine at this point, however, increases.

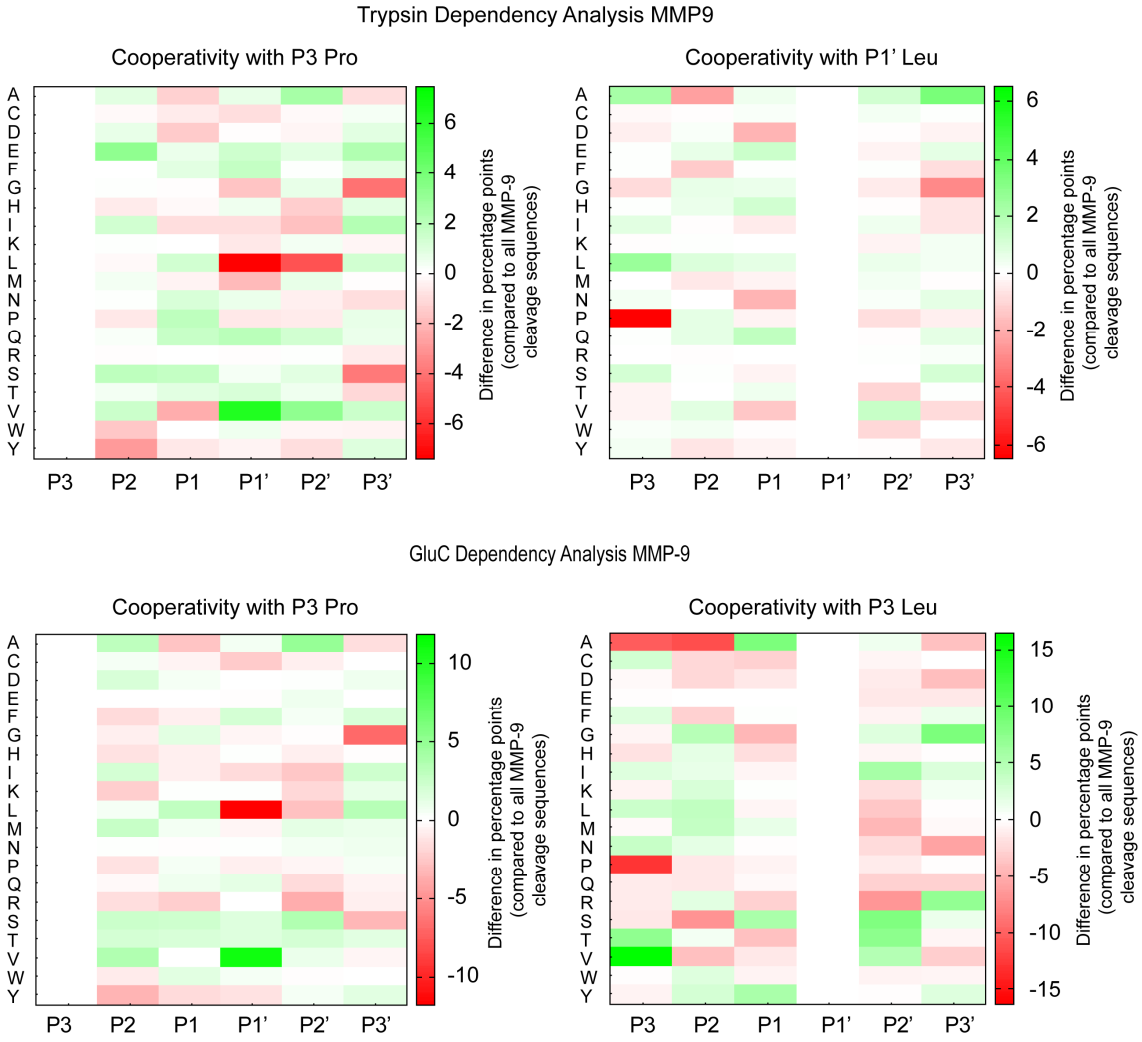


Figure 28. Subsite cooperativity from selected residues. The web-based PICS analysis enables to set proline as a fixed residue in P3 (left panel) and leucine in position P1' (right panel), thus visualizing those AAs that stay in cooperative relation. Upper panel shows results from trypsin library, lower from GluC library. Single letter code for AA residues plotted is on the y axes. P and P′ subsite positions are as shown on the x axes.

7.2.2. Cleavage experiments with final constructs

As mentioned above, the sequence GPQGIAGQ was used in former experiments to diagnose peri-implantitis by detection of increased MMP activity in the oral cavity. This peptide sequence was logically used as the first PSL sequence to synthesize as a diagnostic system with the modified Den. The synthesis was analogous to the above described constructs QPVV-Den and DAPV-Den (88). First, the peptide was synthesized via SPPS on CTC resin and N-terminal acetylated. After this, the peptide was cleaved from the resin and precipitated into diethyl ether. The white residue was coupled to **Den-CH_2-NH_2** with HATU/DIPEA in dry DMF and again precipitated into diethyl ether after reaction. After centrifugation at 4 °C, the yellowish, slightly oily residue was purified with preparative HPLC via a C18 column. The fractions containing the construct were lyophilized. The resulting white powder was treated with 100% TFA to remove residual protection groups from the peptide. After precipitation in diethyl ether, the residue was purified again using preparative HPLC and the fractions containing the construct were double lyophilized. The obtained white powder was analyzed for purity by HPLC and the molecular mass was determined by ESI-MS (**Figure 29**). As the purity was over 95%, it could be used in the following cleavage assays.

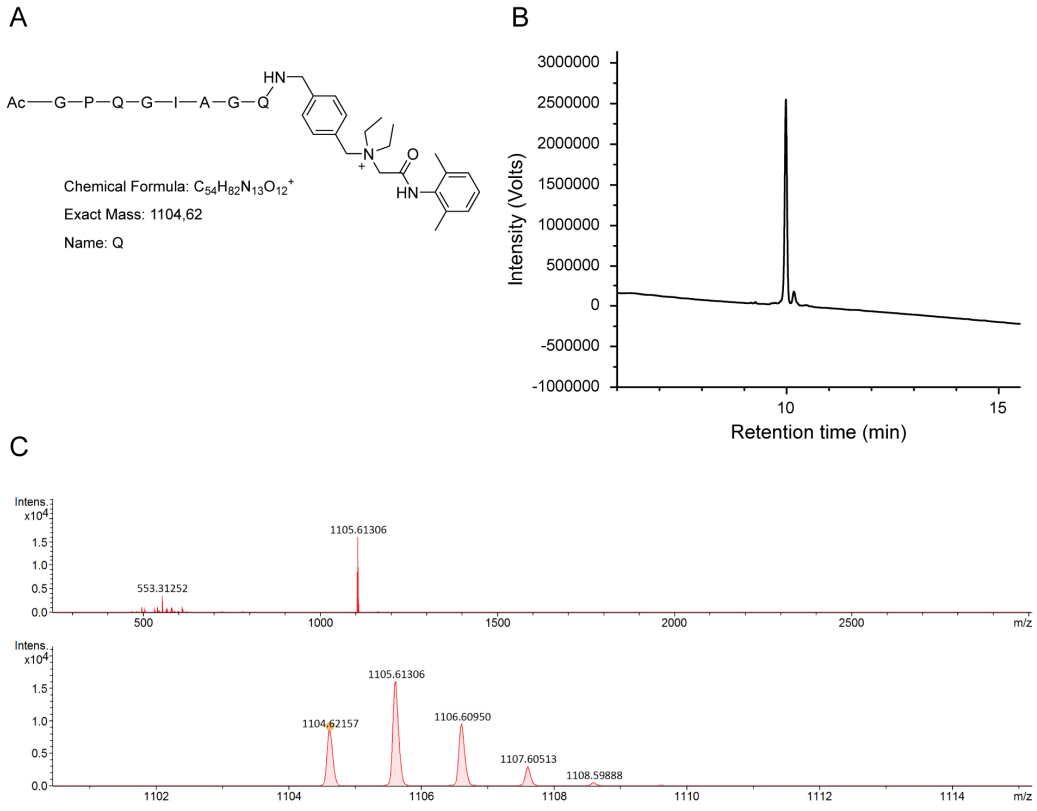


Figure 29. Structure, HPLC chromatogram and ESI spectrum of Ac-GPQGIAGQ-Den. (**A**) Chemical structure with AAs in one letter code. The construct is referred to as Q in the following. (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 95%. (**C**) Positive ion mass spectrum. It is possible to identify the single charged mass of 1104.62157 m/z.

In a first cleavage experiment, construct Q was incubated with an equimolar mixture of MMP-1, MMP-8 and MMP-9 at 37 °C in MMP buffer, to evaluate if it is cleaved by the target protease and can be used as a diagnostic system for e.g. peri-implantitis.

For comparison, the comparative construct A(N_3)-GPQGIAGQK-Den, according to the prior art[193] was incubated to compare the cleaving efficiency of the new system to that of the old one. In this construct, Den is connected via its carboxylic acid group to the lysine of a protease-sensitive peptide, as mentioned above.

The concentration of the two compounds was 0.1 mM. The cleavage efficiency was measured using a HPLC system with a ZORBAX Eclipse XDB-C18 column. Eluent A (0.1% TFA in water, (v/v)) and eluent B (0.1% TFA in ACN (v/v)) with a gradient of 5 to 95% eluent B over 55 min with UV absorption measured at λ = 214 nm.

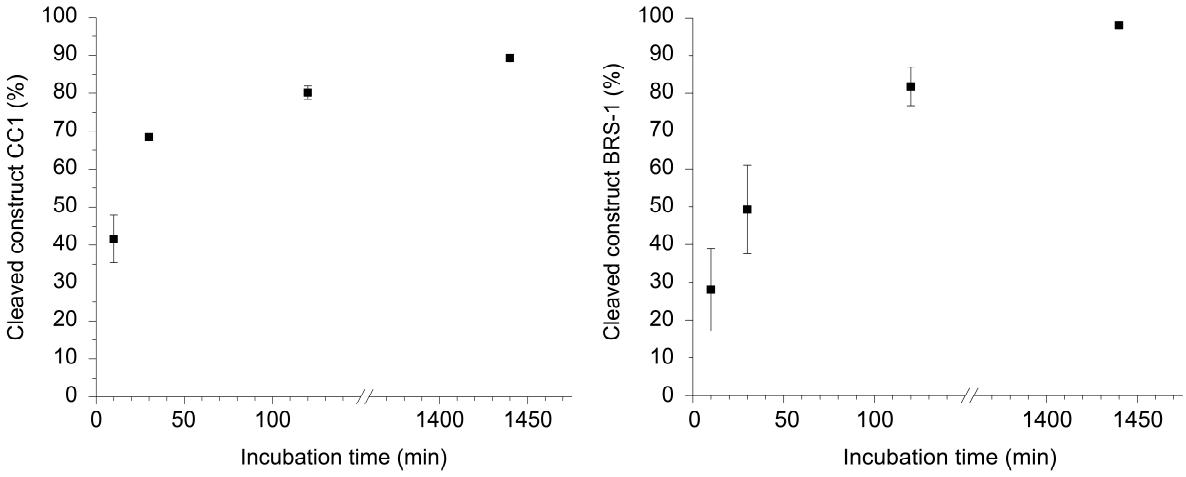


Figure 30. Time dependent cleavage of A(N_3)-GPQGIAGQK-Den (CC1) and Q (BRS-1). Cleavage (%) of the sensor by a MMP-Mix containing 400 ng/mL of MMP-1, MMP-8 and MMP-9. Data are presented as mean ± SD (n=3).

A comparable cleavage efficiency of both systems was observed (**Figure 30**). The slightly better performance of A(N_3)-GPQGIAGQK-Den is caused by the fact that it was not the final construct that was analyzed here. The overall system is further enlarged by coupling it to spatially demanding PMMA particles leading to less susceptibility towards MMPs. These data are already published in patent 2, which can be found in the **Appendix**.

7.2.2.1. Synthesis of optimized diagnostic constructs

In the sequence GPQGIAGQ, formerly used as a PSL in the detection of peri-implantitis, the N-terminal glutamine is coupled to Den. As mentioned above, glutamine is not the optimal choice as last AA in a PSL system. It cannot be cleaved by the AP, decreasing the gustatory perception. To avoid this difficulty, two constructs were synthesized, having alanine as the N-terminal AA, the modified Den is coupled to. Alanine was both, good couplable to **Den-CH_2-NH_2** and also quickly cleaved by the AP. As we do not know the impact of the glutamine in P4' on the cleavage efficiency towards MMPs, glutamine is exchanged with alanine in one construct (Q→A) and in the other alanine is inserted between glutamine and **Den-CH_2-NH_2** (Q+A).

The synthesis route was analogue to the synthesis of the construct Q. Purity and mass were also determined using the same methods (**Figure 31** and **Figure 32**).

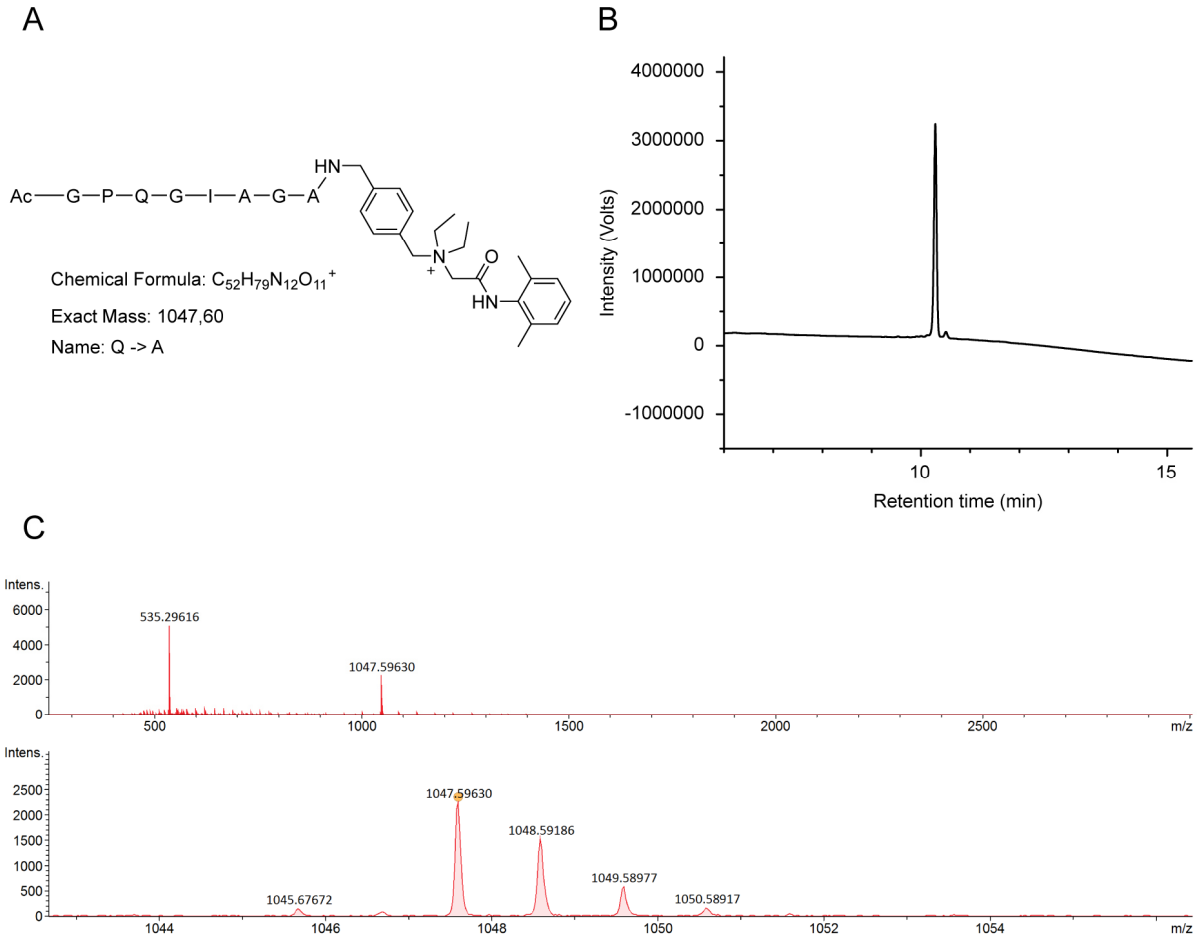


Figure 31. Structure, HPLC chromatogram and ESI spectrum of Ac-GPQGIAGA-Den. (**A**) Chemical structure with AAs in one letter code. The construct is referred to as Q→A in the following. (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 95%. (**C**) Positive ion mass spectrum. It is possible to identify the single charged mass of 1047.5963 m/z.

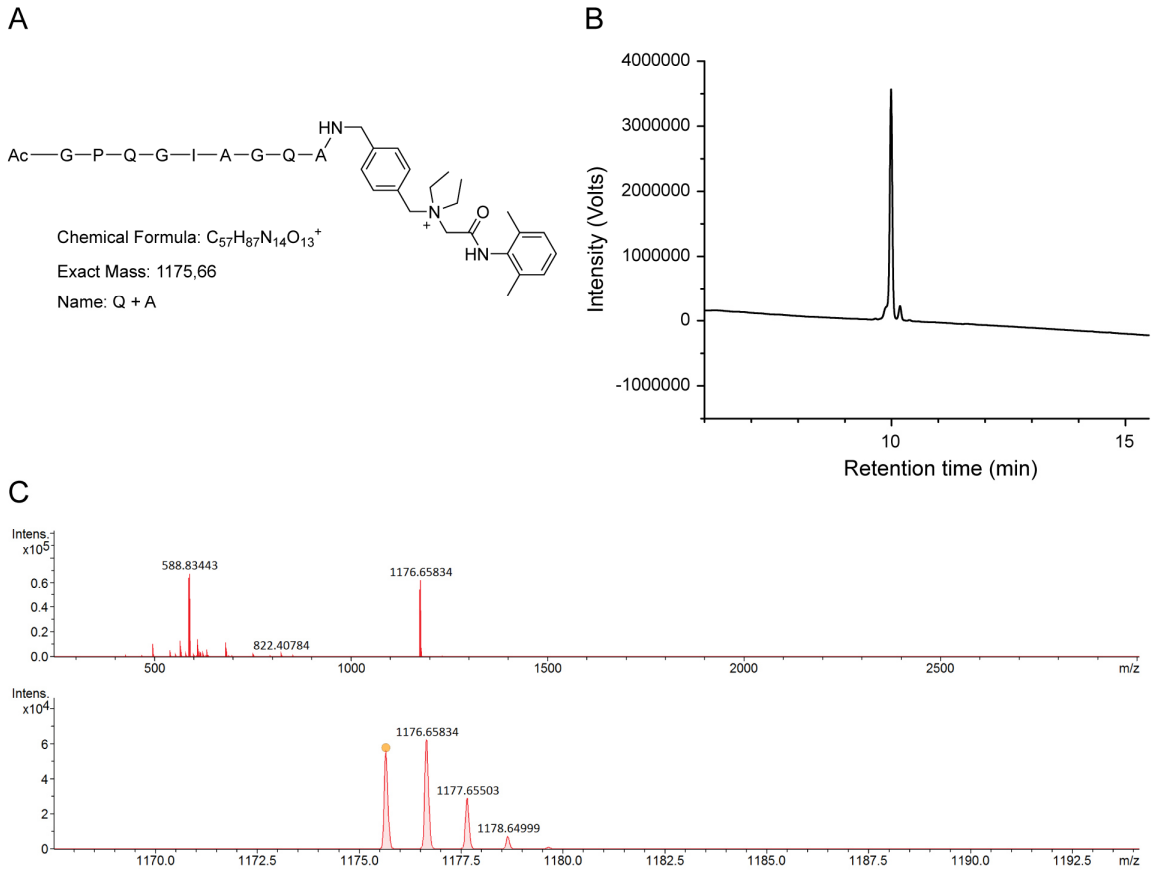


Figure 32. Structure, HPLC chromatogram and ESI spectrum of Ac-GPQGIAGQA-Den. (**A**) Chemical structure with AAs in one letter code. The construct is referred to as Q+A in the following (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 95%. (**C**) Positive ion mass spectrum. It is possible to identify the single charged mass of 1176.65834 m/z.

All three constructs now had to be examined in their cleavage efficiency towards MMPs. As in previous investigations by Ritzer *et al*., a 400 ng/mL mix of MMP-1, MMP-8 and MMP-9 was used for this purpose.

As in the cleavage experiment above, the concentration of the three constructs was 0.1 mM. The cleavage efficiency was measured using a Hitachi Elite LaChrom HPLC system (VWR, Darmstadt, Germany) with a ZORBAX Eclipse XDB-C18 column (4.6 mm internal diameter, 150 mm length (Agilent, Santa Clara, CA)), eluent A (0.1% TFA in water (v/v)) and eluent B (0.1% TFA in ACN (v/v)) with a gradient of 15 to 60% eluent B over 15 min with UV absorption measured at λ = 214 nm.

7.2.3. Comparison of final constructs Q, Q→A and Q+A

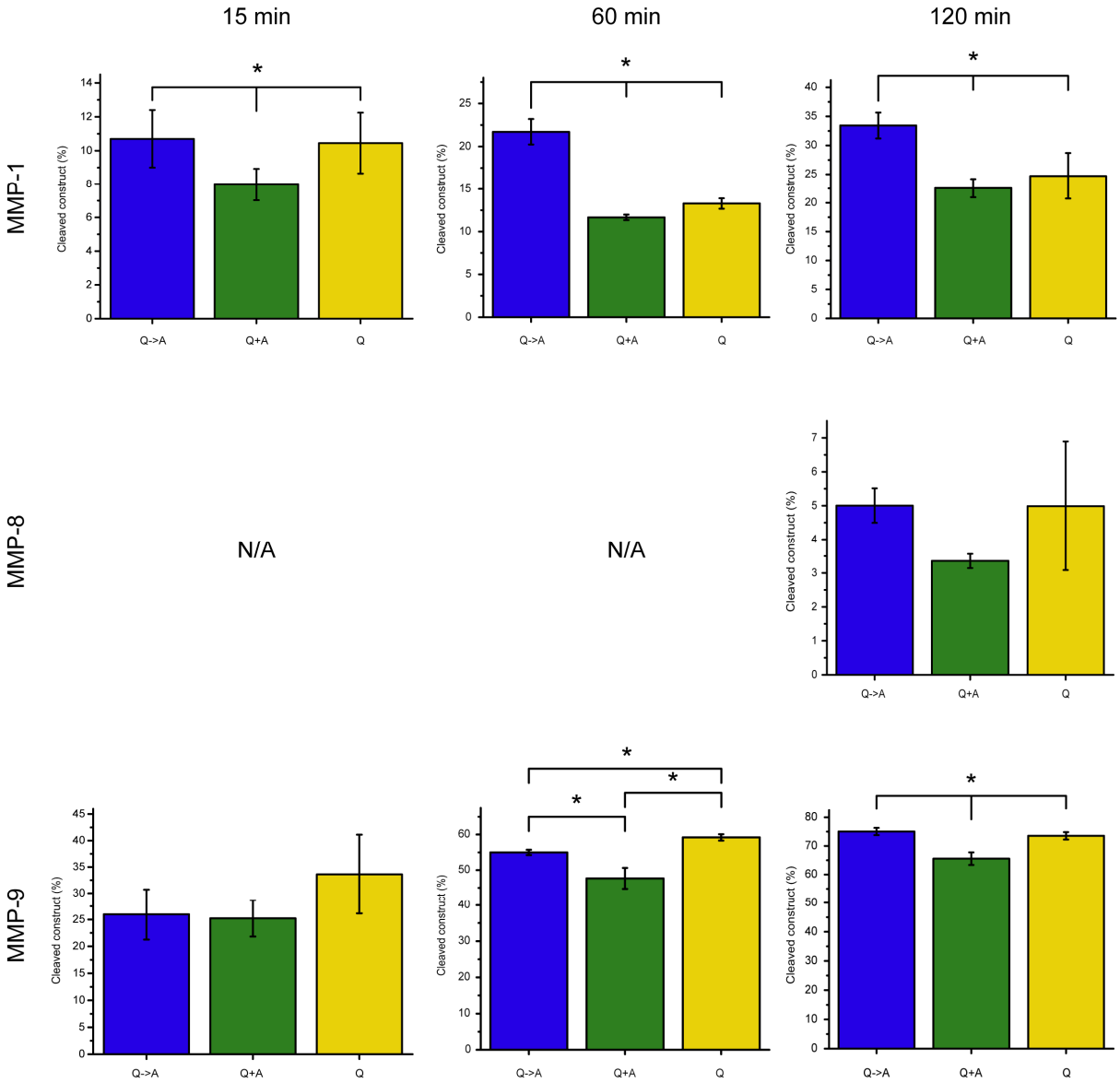


Figure 33. Comparison of Q, Q→A and Q+A in their cleavage by MMP-1, MMP-8 and MMP-9. (**A**) Cleavage (%) of the sensor by MMP-1 after 15, 60 and 120 min. (**B**) Cleavage (%) of the sensor by MMP-8 after 120 min (**C**) Cleavage (%) of the sensor by MMP-9 after 15, 60 and 120 min.

All three synthesized constructs were incubated with 400 ng/mL of MMP-1, MMP-8 and MMP-9 for 15, 60 and 120 min. With this experimental setup, the response of the constructs to the MMPs was to be investigated. The modified AA sequence of the construct could have an effect on the cleavage efficiency of MMP towards the substrate. Significant differences in cleavage are determined using one way ANOVA with a significance level of 0.05.

7.2.3.1. Incubation with MMP-1

After 15 min incubation with MMP-1, the original construct Q (10.4% ± 1.80%) as well as the construct Q→A (10.7% ± 1.70%) performed equal in terms of cleavage. Q+A (7.98% ± 0.95%) was cleaved significant less, than the both other constructs. After 60 min, Q→A was cleaved significantly better (21.7% ± 1.48%) than both other constructs (Q: 13.3% ± 0.61%, Q+A: 11.7% ± 0.33%). This trend was also visible after 120 min, with Q→A (33.4% ± 2.23%) being cleaved in larger amounts than both of the other constructs (Q: 24.7% ± 3.98%, Q+A: 22.6% ± 1.62%).

7.2.3.2. Incubation with MMP-8

Due to the very slow cleavage by MMP-8, just the cleavage after 120 min is analyzed. No significant difference in cleavage efficiency of all the three constructs could be determined (Q→A: 5.00% ± 0.51%, Q: 4.99% ± 1.90%, Q+A: 3.35% ± 0.21%).

7.2.3.3. Incubation with MMP-9

Cleavage with MMP-9 for 15 min did not reveal differences in cleavage efficiency (Q→A: 25.97% ± 4.73%, Q: 33.6% ± 7.48%, Q+A: 25.2% ± 3.39%). Whereas after 60 min, the cleavage rates of all three constructs are different (Q→A: 55.0% ± 0.76%, Q: 52.2% ± 0.88%, Q+A: 47.7% ± 2.94%). This can be explained by the small standard deviation in this experiment, so that even a small difference of Q→A and Q (4%) is significant. This changes, so that after 120 min no significant difference between Q→A (75.0% ± 1.25%) and Q (73.5% ± 1.28%) can be detected. As in the MMP-1 cleavage assay, Q+A (65.6% ± 2.17%) is cleaved in the least amount.

7.2.3.4. Overall

That the exchange in position P4' of the PSL does not change the recognition by the MMPs much was to be assumed by the PICS results. As explained above, there was no preference of MMP-9 for a particular AA in this position of the peptide chain. A preference of e.g. glutamine in this position could have indicated an important binding site with the active center of the enzymes.

Since Q→A is now at least equivalent in cleavage efficiency to Q, this construct can be used for further testing instead of Q. Thus, an effective cleavage of the construct occurs, as well as an immediate degradation to the last AA. This means maximizing the patient's gustatory perception.

Q+A was not used any further, as it was cleaved like Q by the MMPs and alanine can also be cleaved from the AP of Den, but after cleavage 5 AAs remain on the Den. This means that in contrast to Q→A, one more AA would have to be cleaved off the flavor.

7.2.4. Cleavage sensitivity of Q→A to MMP-1, MMP-8 and MMP-9

In the next step, the Q→A construct was examined for its response to a specific MMP. Different concentrations of the respective MMPs were incubated with 0.1 mM of the construct for 15 min and after time the enzymes were deactivated by EDTA and heat. The cleavage efficiency was determined by HPLC. As in the other experiments, the cleaved construct refers to the relation to the main peak.

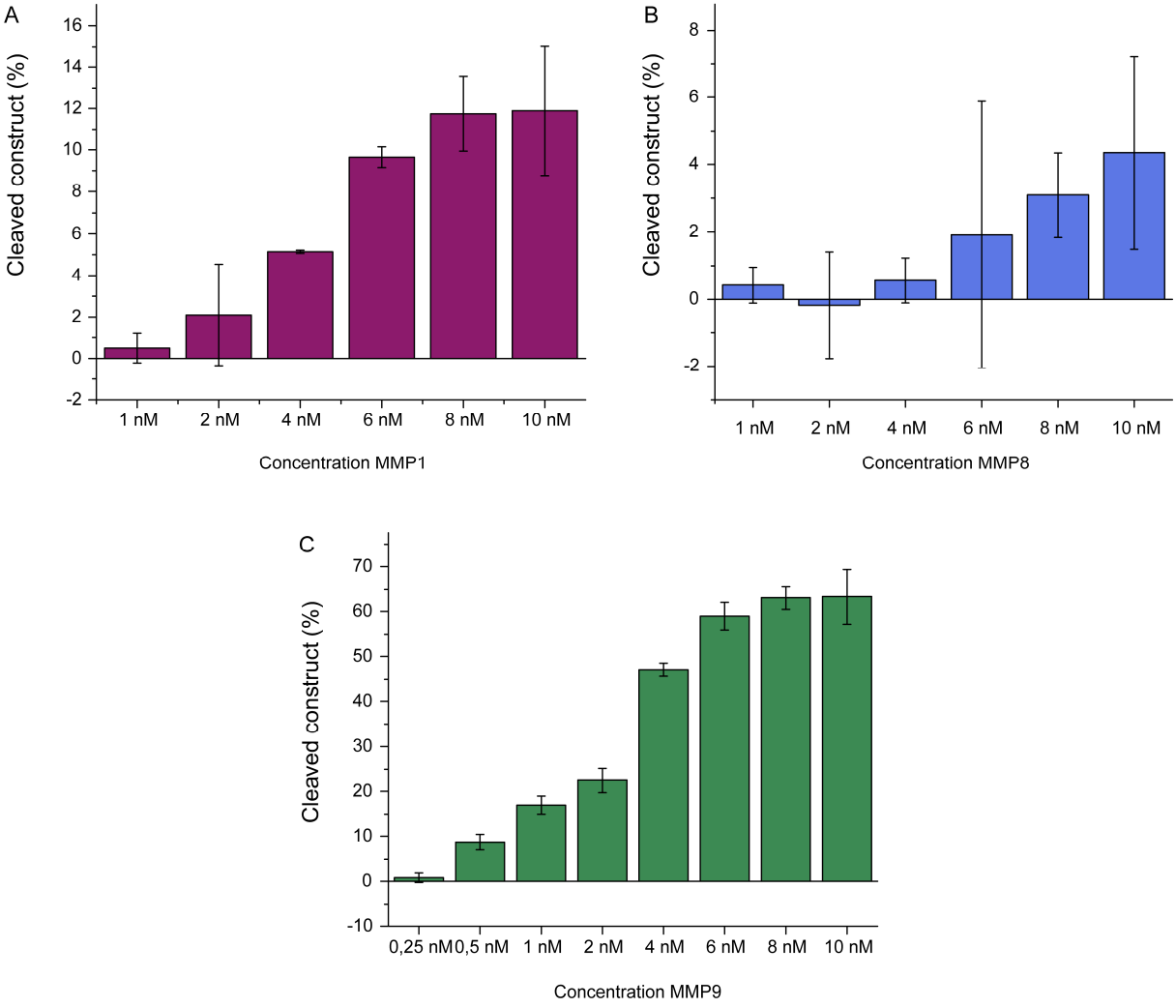


Figure 34. Concentration dependent cleavage of Q→A. (**A**) Cleavage (%) of the sensor by MMP-1 $n = 6$, (**B**) Cleavage (%) of the sensor by MMP-8 $n = 6$ (**C**) Cleavage (%) of the sensor by MMP-9 $n = 6$, The construct was most sensitive to MMP-9 and to a lesser extent to MMP-1, which in return cleaved the sensor better than MMP-8. Data are presented as mean ± SD.

In the cleavage assays of individual MMPs showed a preference of the construct of MMP-9 to be cleaved. While after 15 min incubation with 4 nM MMP-1 6% of the construct, and with MMP-8 less than 1% were cleaved, with the same concentration MMP-9 half of the construct was cleaved.

7.2.5. Time-dependent cleavage of Q→A

Finally, the time-dependent cleavage of the construct was checked. Therefore, the construct Q→A was incubated with 2 nM and 4 nM of the MMP-Mix (containing MMP-1, MMP-8 and MMP-9) for 1, 2, 5, 10 20, 30, 60, 120 and 360 min at 37 °C (**Figure 35**).

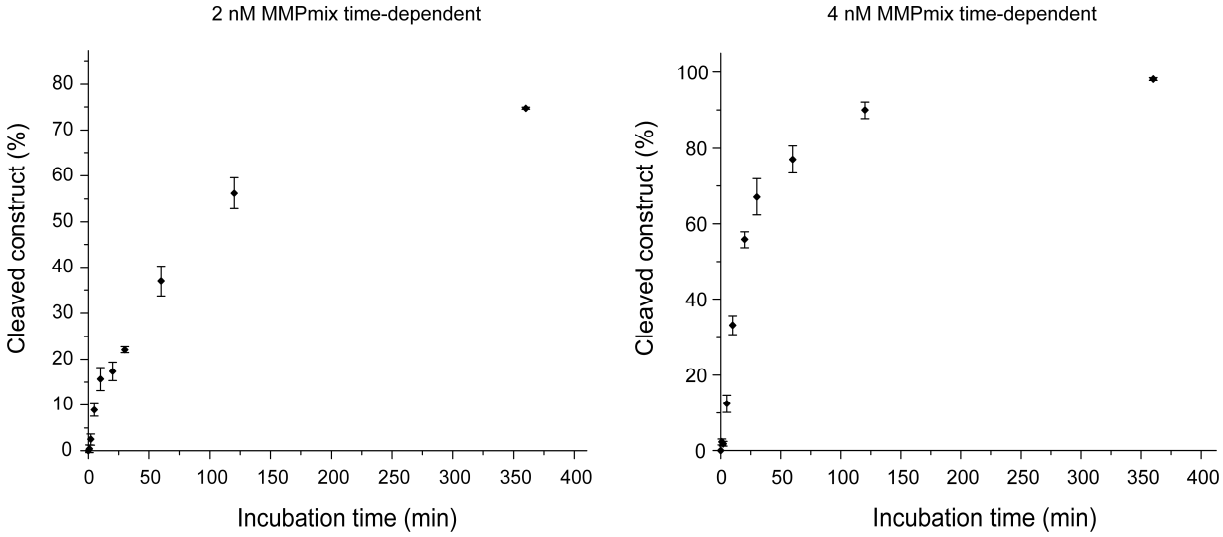


Figure 35. Time-dependent cleavage of Q→A by a 2 nM and a 4 nM MMP-mix. (**A**) Cleavage profile (%) of the sensor Q→A by a 2 nM MMP-mix. (**B**) Cleavage profile (%) of the sensor Q→A by a 4 nM MMP-mix.

The equations of both curves can be found in the **Appendix**.

7.2.6. Measurement of Q→A on the electronic tongue

That the taste perception of Den derivatives decreases with the number of AAs coupled to them has already been shown in earlier publications.[199] To show that the bitterness of **Den-CH_2-NH_2** is masked by coupling to a PSL, the construct is also analyzed with the electronic tongue setup. Only when the PSL is cleaved by the target protease (in this case MMP) and the remaining AAs are degraded by the AP is it possible to taste the bitterness. A taste perception of the intact construct would lead to false positive results.

Three concentrations in the range of 0.1 - 1 mM were tested for their bitterness in an electronic tongue method. The experimental setup and the test performance were the same, as described in former experiments. Both compounds are also tested for bitterness using the two sensors SB2AC0 and SB2AN0. The results are displayed numerically (**Table 11**) and graphically (**Figure 36**).

Table 11. Electronic tongue measurements

Sensor SB2AC0				
Concentration	**Sensor response Ac-GPQGIAGA-Den (mV)**	**SD (mV)**	**Sensor response Den benzoate (mV)**	**SD (mV)**
0.1 mM	-113,10333	0,55029	-106,8	0,29878
0.5 mM	-113,78667	0,25197	-88,63	0,3021
1 mM	-124,68667	0,58801	-73,18667	0,38274

Sensor SB2AN0				
Concentration	**Sensor response Ac-GPQGIAGA-Den (mV)**	**SD (mV)**	**Sensor response Den benzoate (mV)**	**SD (mV)**
0.1 mM	-127,46333	1,11048	-120,32333	0,65045
0.5 mM	-134,91333	1,18506	-93,62333	0,76839
1 mM	-156,1	0,68998	-74,51	0,98887

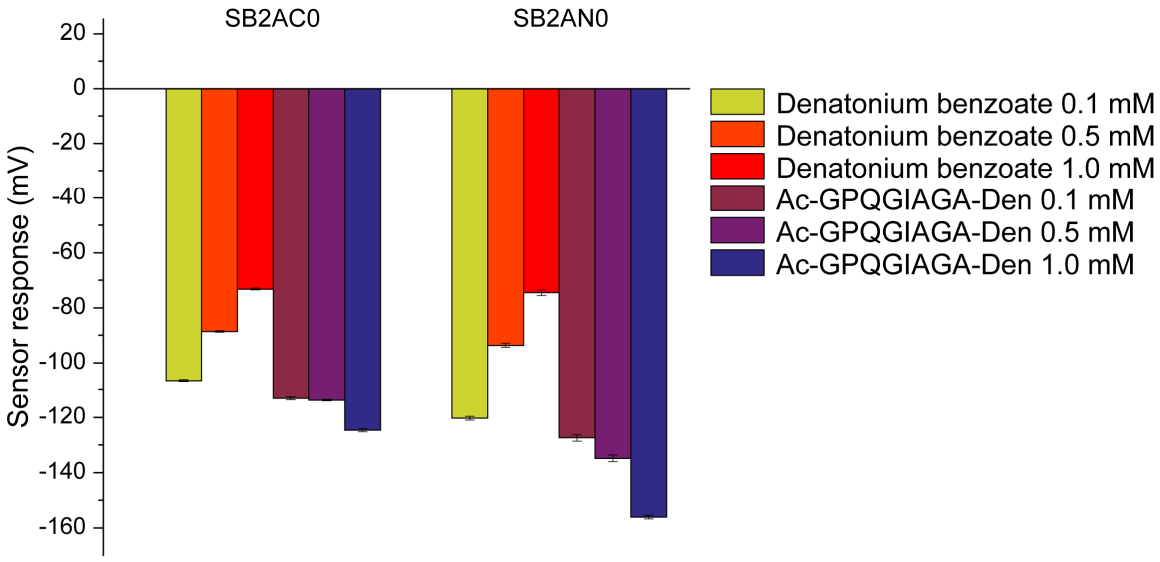


Figure 36. Graphical representation of the electronic tongue data. Data are presented as mean ± SD.

The data of **Table 11** and **Figure 36** shows that for Den benzoate, the mV values increase with higher concentration of the compound. This indicates that the perception of the bitter taste of the compound increases at higher concentration. This was already be seen in **Figure 16**, when **Den-CH_2-NH-Ac** was compared to Den benzoate.

In contrast thereto, for the Ac-GPQGIAGA-Den, the mV values decrease with higher concentration. This indicates that the intact diagnostic construct is not perceived as a bitter compound. Coupling to a PSL successfully masks the bitter gustatory perception. The decrease of the mV value can be explained with a higher concentration of the construct. This effect is stronger to observe at the SB2AN0 sensor than the SB2AC0 sensor. The construct is somehow interfering with the potentiometric sensors, in case of bitterness the value should increase though.

The bitter taste is only perceivable when the coupling product is cleaved and **Den-CH_2-NH_2** is released. Therefore, the construct can be used in diagnostic setups.

7.2.7. Cytotoxicity of Den-CH_2-NH_2, Den-CH_2-NH-Ac and Ac-GPQGIAGA-Den

The WST-1 assay is a colorimetric assays for measuring the activity of enzymes that reduce WST to formazan dye, giving an orange yellow. It can be utilized to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would inhibit cell viability and growth. A decrease in cell proliferation (and therefore signal intensity) can indicate the toxic effects of compounds.

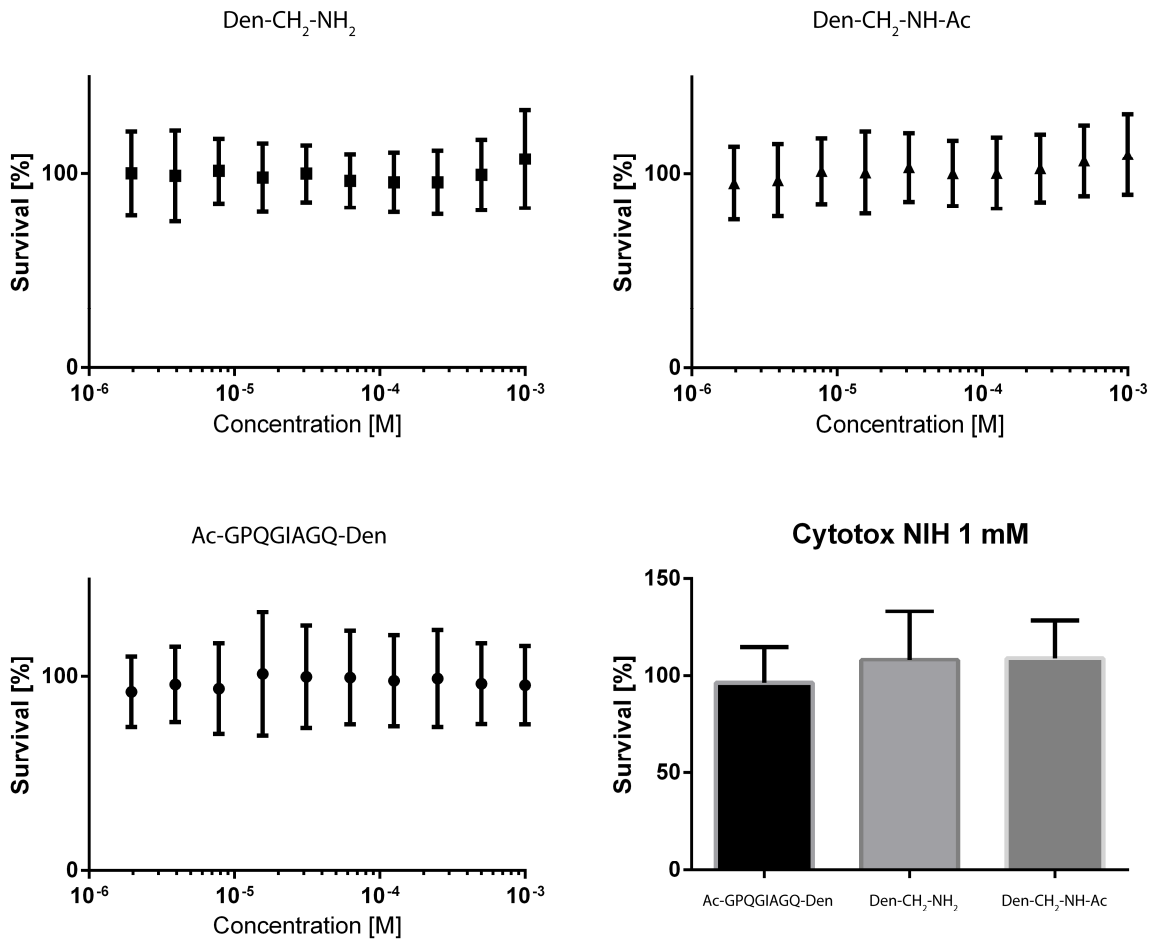


Figure 37. WST cytotoxicity assay results of Den-CH_2-NH_3, Den-CH_2-NH-Ac and ACGPQGIAGQ-Den (Q→A). No cytotoxic effects were observed. Data are presented as mean ± SD.

Even in highest concentrations, none of the three compounds showed any influence to the NIH cells (**Figure 37**). A full cytotoxicity profile has to be done but this experiment gives first hints that all three compounds are non-toxic.

7.3. Section III - S. pyogenes

7.3.1. Expression and purification of SpeB

A plasmid for the expression of SpeB described earlier (**Figure 38**), was generously donated by Dr. Burckhard Seelig.

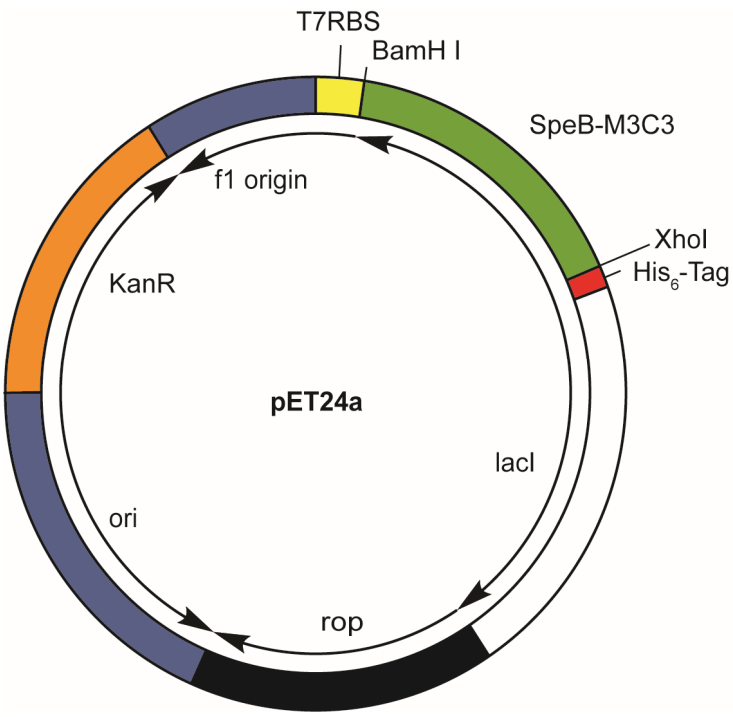


Figure 38. Schematic representation of *SpeB*-M3C3 containing plasmid.

The plasmid was transformed into competent *E. coli* BL21(DE3) and DH5α cells and the positive colonies tested on their correctness by sequencing (data not shown).

For the production of the recombinant protein, standard LB medium with the addition of Kanamycin was inoculated with a single colony and grown overnight. This overnight culture was used to inoculate the auto-induction media. Cultures were grown at 37 °C for around 3 - 4 h until the OD_{600} reached about 0.3 - 0.4 and then transferred to 25 °C for an additional 24 h. On the next day, cells were divided into 50 mL aliquots, collected by centrifugation, and frozen at -80 °C. The supernatant was also stored in 50 mL aliquots at -80 °C.

Dialysis and purification of the supernatant was performed as described above. Purification was carried out in two steps. In a first purification step the protein was purified using CEX. The elution profile is shown in **Figure 39A**. SpeB was eluted at NaCl concentrations ranging from approximately 100 to 140 mM. Due to the autoinduction approach, the ~40 kDa protease zymogen was cleaved into its ~28 kDa active form and a 12 kDa propeptide. The fractions were also analyzed by SDS-Page to prove that the correct protein was purified. After the first purification step both, the active protease and the propeptide, can still be observed in the SDS-gel of the collected fraction (**Figure 39B**).

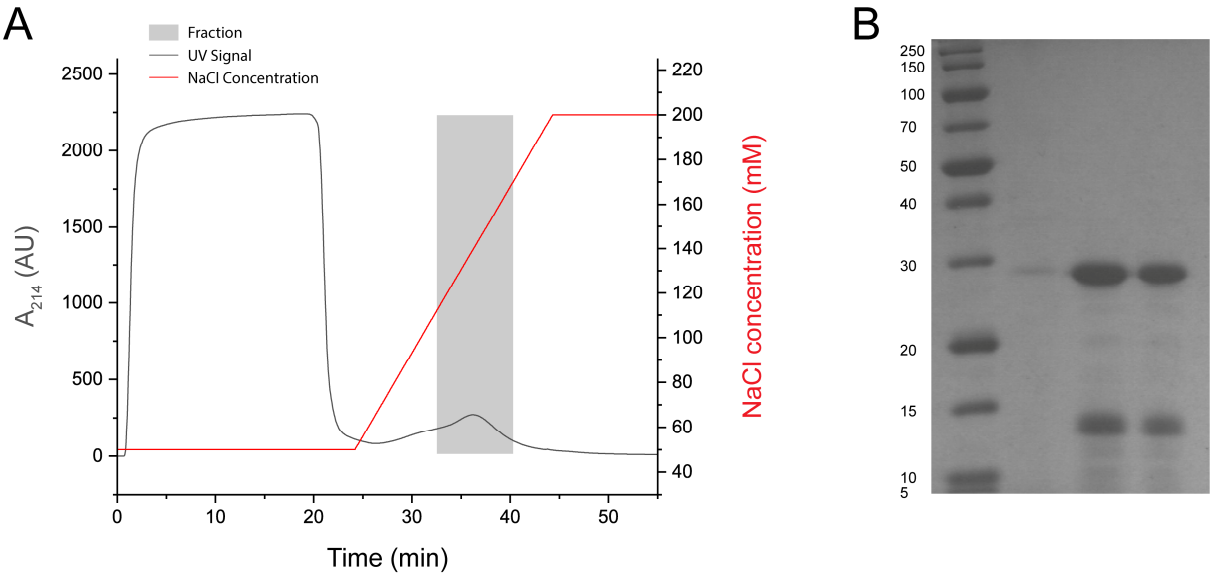


Figure 39. Chromatogram profile of ion exchange chromatography for SpeB and SDS-PAGE analysis on eluted protein. (**A**) Chromatogram of CIEX for expressed SpeB; (**B**) SDS-PAGE of the CIEX purification o SpeB. Lane M, standard protein marker; Lane 1, 20 µl of collected fraction; Lane 2, 10 µl of collected fraction.

Therefore a second purification step was implemented, using a SEC to separate both proteins due to their different hydrodynamic radii. Using this method, the 28 kDa active form of SpeB can be obtained, with a purity of >95% (**Figure 40A** and **B**). To confirm the expressed protein, the molecular mass was determined by MALDI-MS (**Figure 41**).

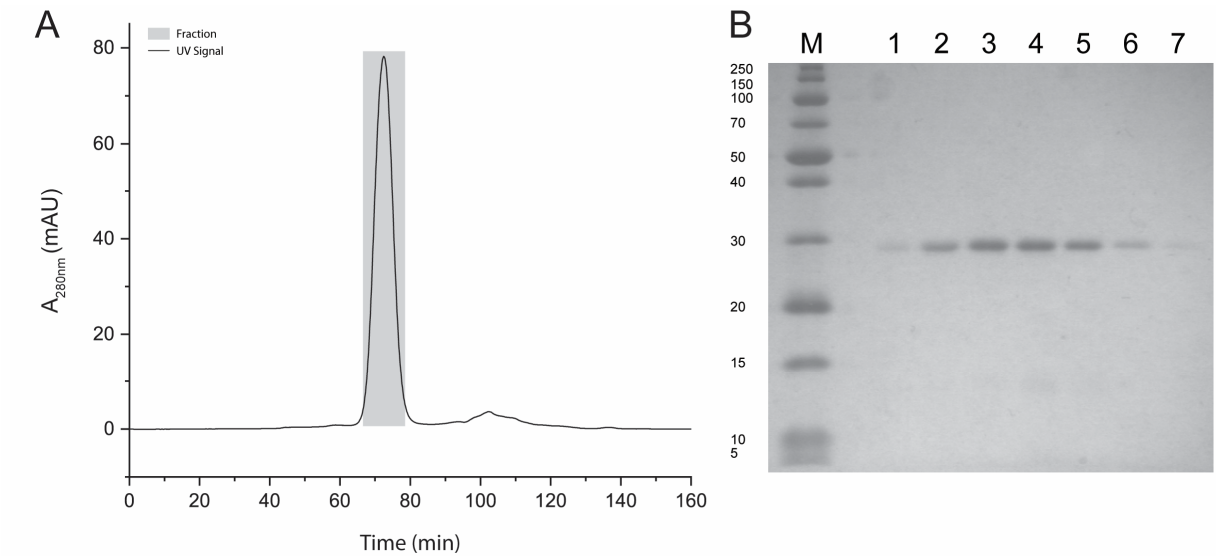


Figure 40. Chromatogram of the SEC of SpeB, SDS-PAGE analysis of eluted protein. (**A**) SEC chromatogram, (**B**) SDS-PAGE of the SEC purification of SpeB. Lane M, standard protein marker; Lane 1-7 collected fractions of the chromatogram peak**.**

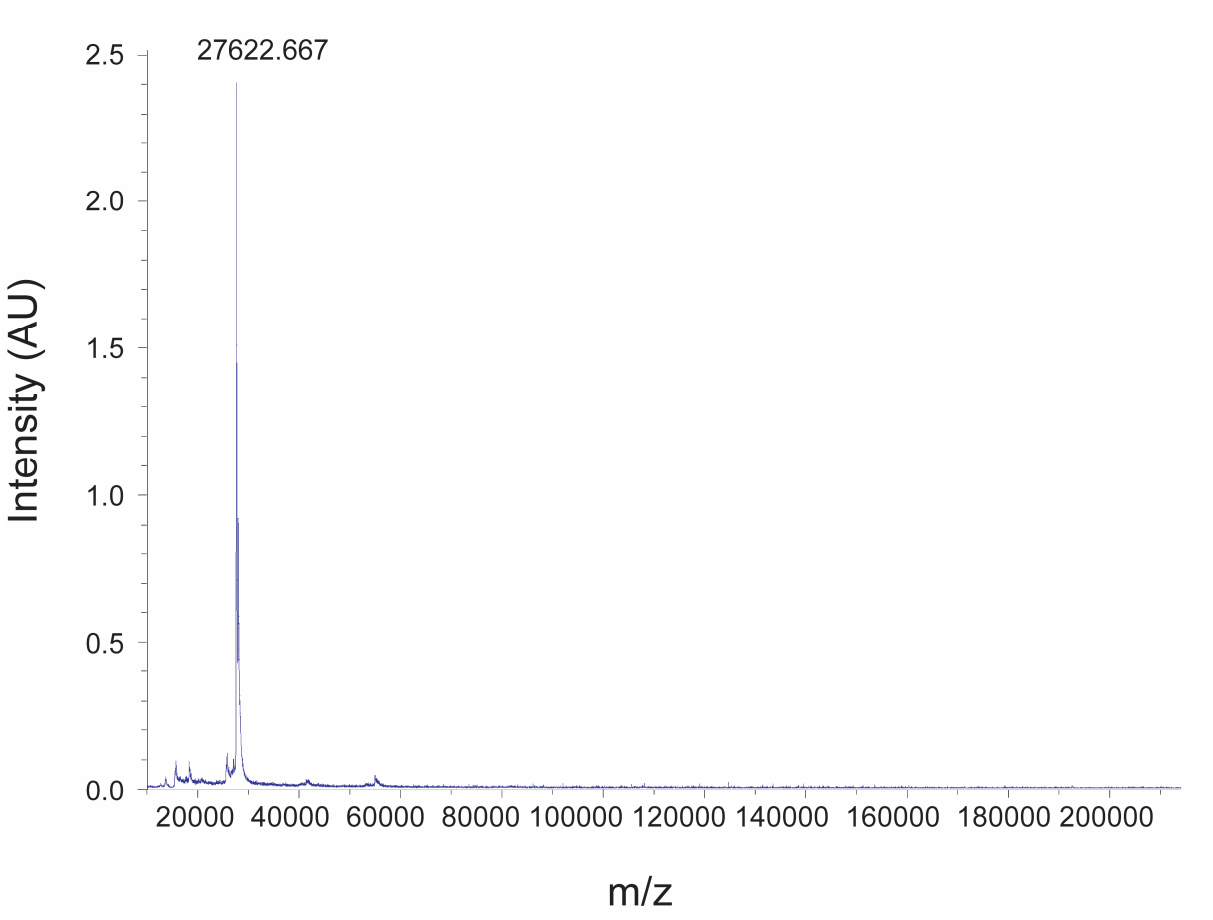


Figure 41. Confirmation of SpeB by MALDI-MS. In the MALDI-TOF Mass spectrum **i**t is possible to identify the single charged mass of 27622.667 m/z (m/z calc. SpeB [M+K]+: 27622,5003).

7.3.2. PICS analysis of SpeB

In order to be able to develop own constructs on SpeB in the future, the protease was tested for cleavage sites using the PICS assay. The procedure was analogous to the PICS assay on MMP-9. 2 µg of the expressed SpeB were incubated with a 200 µg peptide library for 1 h. After purification of the cleaved library this was evaluated by means of mass (**Figure 42**).

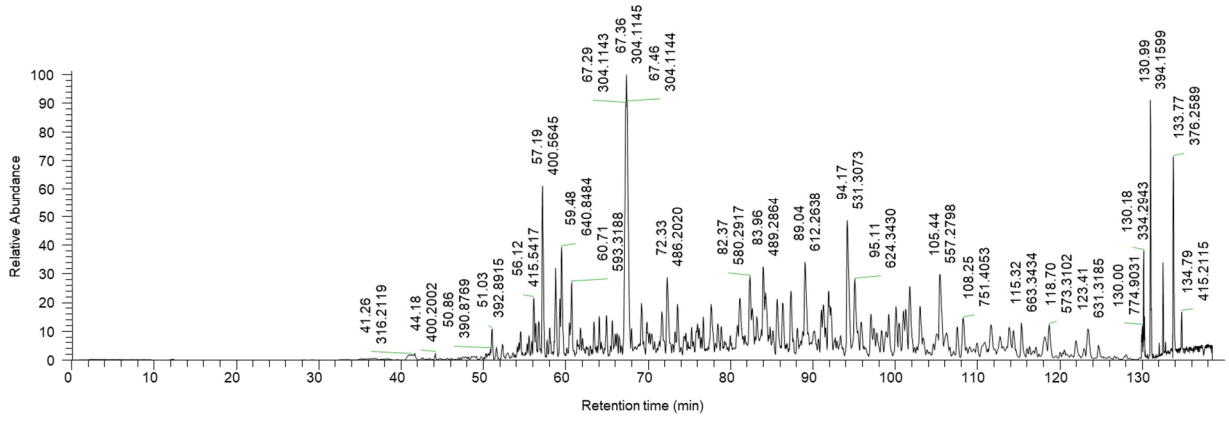
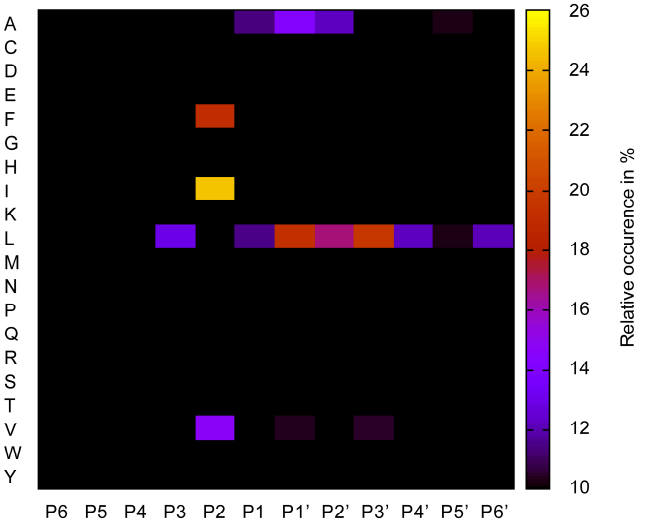
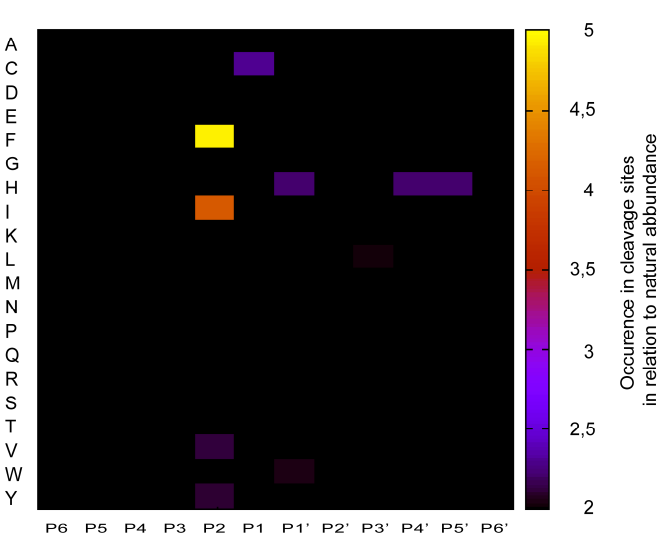


Figure 42. Base peak chromatogram of the PICS Assay (trypsin library) incubated with SpeB.

SpeB cleavage sites in a trypsin-generated peptide library (n=1765)

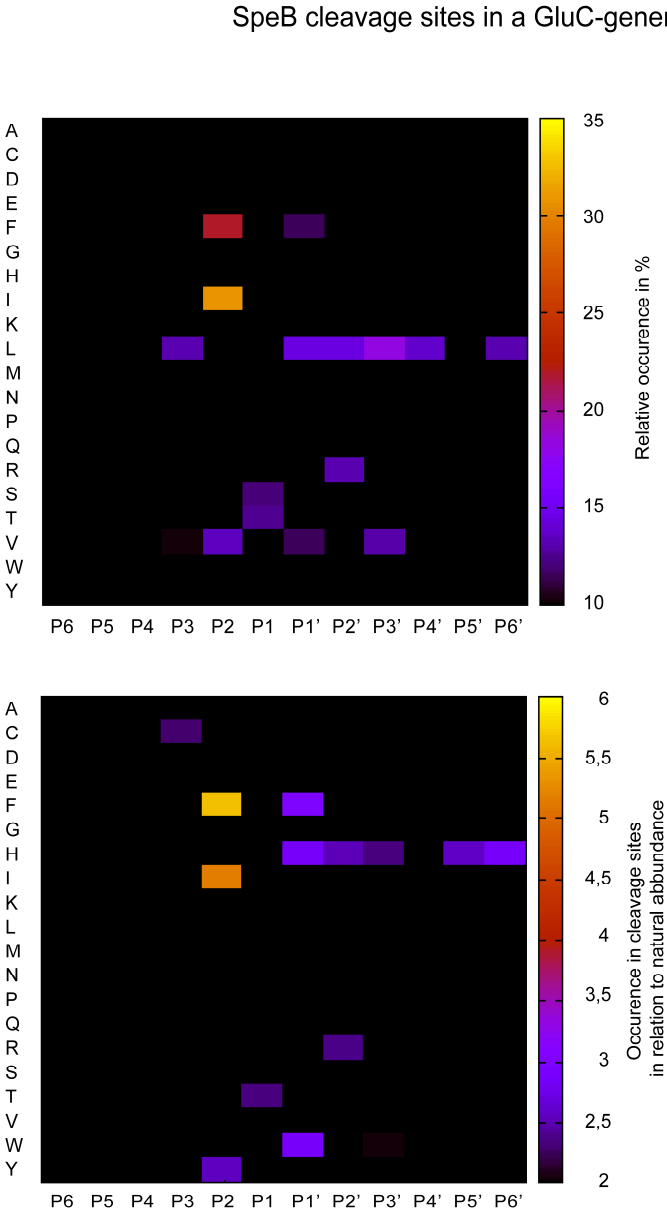


	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	5.3	7	6.7	7.6	6.2	11.3	14.2	12.2	9.7	8.8	10.2	7.6
C	1	1.4	1.7	1.4	0.5	3.1	1.4	1.8	1.7	1.7	1.4	1.4
D	5.6	6.3	5	4.1	1	3.7	0.4	2	2.5	5.6	5.2	5.2
F	5.2	6.8	6.2	5.4	1.8	7.6	1	0.7	2.2	5.6	7.6	6.1
F	1.9	3	3.1	4	19.1	4.8	7.5	4.9	3.8	3.5	3.4	2.3
G	4.4	5.6	6.1	5.1	1.4	7.4	4.4	5.8	8.4	7.5	7.5	8.3
H	1.8	1.5	2.8	2	1.2	3.1	5	4.5	4.5	5	5	3.9
I	2.9	3.5	4.6	5.2	24.6	1.9	8.1	7.4	8.9	4.8	6.3	5.6
K	0.3	0.3	0.2	0.1	0.1	0	0.1	0.3	0.3	0.7	1.5	1.5
L	6.6	6	9.1	12.9	5.4	11.4	19.3	16.8	19.6	12.2	10.2	12.1
M	1.5	1.8	1.6	2.2	4.1	2.8	2.8	2	1.6	1.4	1.4	1.8
N	2.2	4.3	5	4.8	0.8	5	3.2	5.6	3.6	5.4	5.3	4.3
P	3.9	5	5.8	3.9	2.4	1.3	0.2	4.5	6.1	8.4	4.5	6
Q	2.5	3.4	5	5	0.9	7	2.2	5.3	2.8	5.3	5	4.6
R	0.2	0.2	0.2	0.1	0.1	0	0.7	1.4	0.9	1.4	3.2	7.5
S	4.8	3.3	5.1	6	1.6	9.5	7	6.6	4.9	6.2	5	5.7
T	3.6	4	4.6	6.3	1.5	9.8	6.9	5.6	4	6	6.3	5.2
V	4.9	5.4	6.2	9.3	14.6	2.4	10.3	7.5	10.4	7.6	7.9	7.6
W	0.5	0.5	0.6	1.4	0.2	0.6	2.2	1.6	0.9	0.7	1.1	0.7
Y	1.4	1.5	3.3	4.2	6.1	4.7	3.3	3.5	3.1	2	1.9	2



	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	0.6	0.8	0.8	0.9	0.7	1.4	1.7	1.5	1.2	1.1	1.2	0.9
C	0.7	1	1.2	1	0.4	2.3	1	1.3	1.2	1.2	1	1
D	1	1.2	0.9	0.8	0.2	0.7	0.1	0.4	0.5	1	1	1
E	0.8	1	0.9	0.8	0.3	1.1	0.1	0.1	0.3	0.8	1.1	0.9
F	0.5	0.8	0.8	1	4.9	1.2	1.9	1.3	1	0.9	0.9	0.6
G	0.6	0.8	0.9	0.7	0.2	1	0.6	0.8	1.2	1.1	1.1	1.2
H	0.8	0.7	1.2	0.9	0.5	1.4	2.2	2	2	2.2	2.2	1.7
I	0.5	0.6	0.8	0.9	4.1	0.3	1.4	1.2	1.5	0.8	1.1	0.9
K	0.1	0.1	0	0	0	0	0	0.1	0.1	0.1	0.3	0.3
L	0.7	0.6	0.9	1.3	0.6	1.2	2	1.7	2	1.3	1.1	1.3
M	0.6	0.7	0.7	0.9	1.7	1.2	1.2	0.8	0.7	0.6	0.6	0.7
N	0.5	1.1	1.2	1.2	0.2	1.2	0.8	1.4	0.9	1.3	1.3	1.1
P	0.8	1.1	1.2	0.8	0.5	0.3	0	1	1.3	1.8	1	1.3
Q	0.6	0.9	1.3	1.3	0.2	1.8	0.6	1.3	0.7	1.3	1.3	1.2
R	0	0	0	0	0	0	0.1	0.3	0.2	0.3	0.6	1.4
S	0.7	0.5	0.8	0.9	0.2	1.5	1.1	1	0.8	1	0.8	0.9
T	0.7	0.8	0.9	1.2	0.3	1.8	1.3	1.1	0.8	1.1	1.2	1
V	0.7	0.8	0.9	1.4	2.1	0.3	1.5	1.1	1.5	1.1	1.1	1.1
W	0.5	0.5	0.6	1.3	0.2	0.6	2	1.5	0.8	0.6	1	0.6
Y	0.5	0.5	1.1	1.4	2.1	1.6	1.1	1.2	1.1	0.7	0.7	0.7

Figure 43. PICS sequence specificity profiles of SpeB using trypsin-generated human peptide libraries. Identified cleavage sites (n values shown) are summarized as heat maps showing relative occurrence. (upper left panel) and the more incisive fold-change over the natural abundance of AAs. (lower left panel) P6 to P6′ subsite positions are shown on the x axes; plotted AAs are indicated on the y axes with one-letter codes. On the right, the corresponding numerical representation of PICS specificity profiles.



	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	4.6	7.4	6.7	5.3	3.6	8.8	9.5	7.7	7.4	8.4	7.6	7.2
C	1	1.7	2.2	3.1	0.2	2.2	1.2	1.9	0.7	0.3	0.7	1.5
D	7.2	9.8	5.8	3.6	1.4	2.9	0	0.7	0.9	4.5	5.5	7.9
E	0.9	0.7	0.9	0	0	0	0	0.2	0.9	1.9	1.5	3.6
F	3.8	3.6	3.1	4.3	21.8	5.5	11.5	6.7	6	3.6	4.1	3.3
G	5.2	8.1	6.7	5.5	0.7	5.7	2.4	4.6	8.1	7.6	8.6	9.1
H	0.9	0.7	3.6	1.5	0.5	2.6	6.5	5.7	5.3	4.1	5.8	6.5
I	2.2	3.1	5.2	5.7	30.9	1.5	6.2	7	8.8	6.7	5.2	5.3
K	4.5	4.3	3.4	4.5	0.3	6	0.3	0.5	0.5	0.3	1.9	0.5
L	4.6	5.5	6.2	13.1	4.3	8.9	14.4	14.3	18.2	13.7	9.6	13.1
M	2.4	2.7	1.5	1.5	4.8	4.3	3.1	2.1	1.4	1	1.5	1.5
N	2.4	3.8	4.8	3.8	1.2	4.5	3.8	6.2	3.1	5.5	6.9	4.6
P	5.3	4.8	7.6	5	1.4	0.7	0.3	3.8	5.7	7.9	8.4	5.3
Q	2.6	2.7	5.8	4	0.3	7.6	1.2	3.6	3.4	3.6	6.2	4.8
R	2.6	1.5	2.1	3.4	0.3	4.1	5.2	13.1	4.8	7	7.6	6.2
S	5.3	4.6	5.8	5.8	0.3	12	7.7	7.2	3.8	5.8	4.5	5.3
T	3.6	2.9	1.4	5.7	1.7	12.4	8.2	3.4	2.2	6.7	5.2	4.3
V	5.5	5.2	7.4	10.1	13.4	1.7	11.5	6	12.9	8.8	5.7	5.8
W	0.5	0.3	1	0.7	0.2	0.5	3.1	1.7	2.2	0.5	0.9	0.7
Y	1.4	2.6	2.9	3.6	7.4	5.5	3.8	3.6	3.8	1.9	2.7	2.9

	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
A	0.60	0.90	0.80	0.60	0.40	1.10	1.10	0.90	0.90	1.00	0.90	0.90
C	0.70	1.20	1.60	2.30	0.10	1.60	0.90	1.40	0.50	0.20	0.50	1.10
D	1.30	1.80	1.10	0.70	0.30	0.50	0.00	0.10	0.20	0.80	1.00	1.40
E	0.10	0.10	0.10	0.00	0.00	0.00	0.00	0.00	0.10	0.30	0.20	0.50
F	1.00	0.90	0.80	1.10	5.60	1.40	3.00	1.70	1.60	0.90	1.10	0.90
G	0.70	1.10	0.90	0.80	0.10	0.80	0.30	0.60	1.10	1.10	1.20	1.30
H	0.40	0.30	1.60	0.70	0.20	1.10	2.90	2.50	2.30	1.80	2.60	2.90
I	0.40	0.50	0.90	1.00	5.20	0.30	1.00	1.20	1.50	1.10	0.90	0.90
K	0.80	0.70	0.60	0.80	0.10	1.00	0.10	0.10	0.10	0.10	0.30	0.10
L	0.50	0.60	0.60	1.40	0.40	0.90	1.50	1.50	1.90	1.40	1.00	1.40
M	1.00	1.10	0.60	0.60	2.00	1.80	1.30	0.90	0.60	0.40	0.60	0.60
N	0.60	0.90	1.20	0.90	0.30	1.10	0.90	1.50	0.80	1.40	1.70	1.10
P	1.10	1.00	1.60	1.10	0.30	0.10	0.10	0.80	1.20	1.70	1.80	1.10
Q	0.70	0.70	1.50	1.00	0.10	1.90	0.30	0.90	0.90	0.90	1.60	1.20
R	0.50	0.30	0.40	0.60	0.10	0.70	0.90	2.40	0.90	1.30	1.40	1.10
S	0.80	0.70	0.90	0.90	0.00	1.80	1.20	1.10	0.60	0.90	0.70	0.80
T	0.70	0.50	0.30	1.10	0.30	2.30	1.50	0.60	0.40	1.30	1.00	0.80
V	0.80	0.80	1.10	1.50	2.00	0.20	1.70	0.90	1.90	1.30	0.80	0.80
W	0.50	0.30	0.90	0.60	0.20	0.50	2.90	1.60	2.00	0.50	0.80	0.60
Y	0.50	0.90	1.00	1.20	2.50	1.90	1.30	1.20	1.30	0.70	0.90	1.00

Figure 44. PICS sequence specificity profiles SpeB using GluC-generated human peptide libraries. Identified cleavage sites (n values shown) are summarized as heat maps showing relative occurrence. (upper left panel) and the more incisive fold-change over the natural abundance of AAs. (lower left panel) P6 to P6′ subsite positions are shown on the x axes; plotted AAs are indicated on the y axes with one-letter codes. On the right, the corresponding numerical representation of PICS specificity profiles.

7.3.2.1. Subsite analysis of SpeB

Subsite P3

SpeB favors small to large aliphatic residues in P3, such as leucine and valine, and with a slight less percentage of occurrence alanine, glycine and isoleucine.

Subsite P2

In position P2, the substrate specificity of SpeB was similar to the substrate preference of the papain-like family. SpeB specificity was dominated by a preference for hydrophobic AAs, namely isoleucine, phenylalanine and valine, with a combined occurrence of 58% (trypsin)/66% (GluC). A preferential cleavage with hydrophobic residues from P2-P1' is described in literature.

Subsite P1

Preferences of P1 include small hydrophobic residues like leucine and alanine, and AAs with polar uncharged side chains such as serine and threonine. The latter feature was detected mostly in GluC libraries.

Subsite P1′

As stated above, P1' is part of the SpeB hydrophobic bond, ranging from P2-P1'. Hence, phenylalanine, leucine, valine and alanine are dominant residues in this position (combined occurrence of 51% (trypsin)/47% (GluC)). Interestingly, occurrence in cleavage sites in relation to natural abundance of histidine and tryptophan is quite high in this position. Also polar uncharged residues like serine and threonine display a noticeable occurrence.

Subsite P2′

In P2′, SpeB specificity was dominated by a preference for leucine, isoleucine, alanine and valine (combined occurrence of 44% (trypsin)/35% (GluC)) together with a preference for positively charged AAs in the GluC profile (19%). Especially arginine (13.1%), but also histidine (5.7%) were above average occurrence. As explained earlier, this was detected mostly in GluC libraries since tryptic libraries have lysine and arginine only at the C-terminus of the peptide. This illustrates once more the benefit of using more than one peptide library type for comprehensive active site specificity profiling.

Subsite P3′

In P3′, SpeB showed a marked preference for small to large hydrophobic AAs such as glycine, alanine leucine and isoleucine, with a combined occurrence of 36%(trypsin)/42%(GluC).

Subsite P4-P6 and P4'-P6'

No preference for specific AAs in this region were observed. Small and hydrophobic AAs like alanine, valine, glycine and proline occurred slightly more often.

The upper left panel of **Figure 43** was already published in the Perspective "Frugal Innovation for Point-of-Care Diagnostics Controlling Outbreaks and Epidemics".[200] In this "Perspective", SpeB represents the case of a protease with widely unknown cleavage sites. In this example, the PICS assay is utilized to determine cleavable PSL sequences which can be used to synthesize diagnostic constructs.

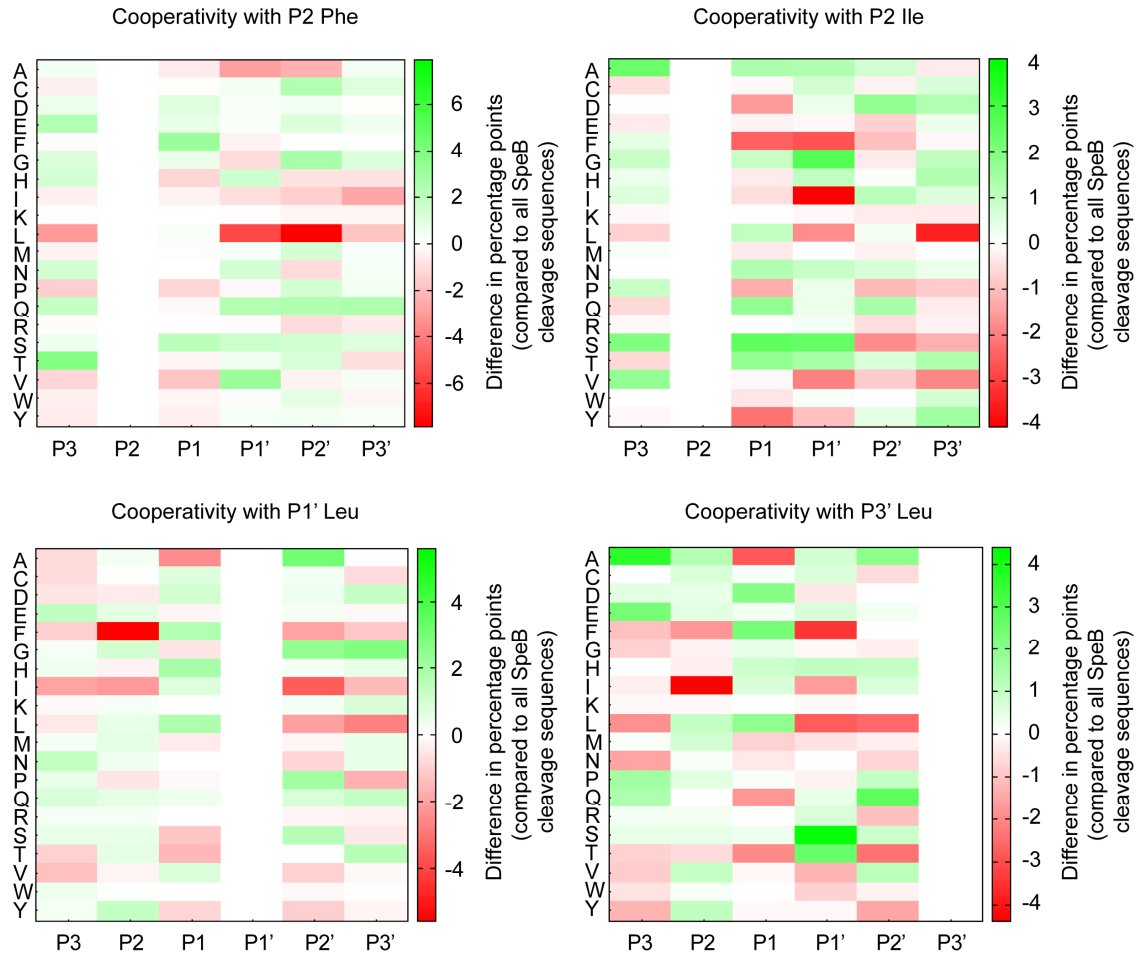


Figure 45. Subsite cooperativity from selected residues. The web-based PICS analysis enables to set phenylalanine as a fixed residue in P2 (upper left panel), isoleucine in position P2 (upper right panel), leucine in position P1' (lower left panel) and leucine in position P3' (lower right panel), thus visualizing those AAs that stay in cooperative relation. Single letter code for AA residues plotted is on the y axes. P and P′ subsite positions are as shown on the x axes. Results are generated with the trypsin library.

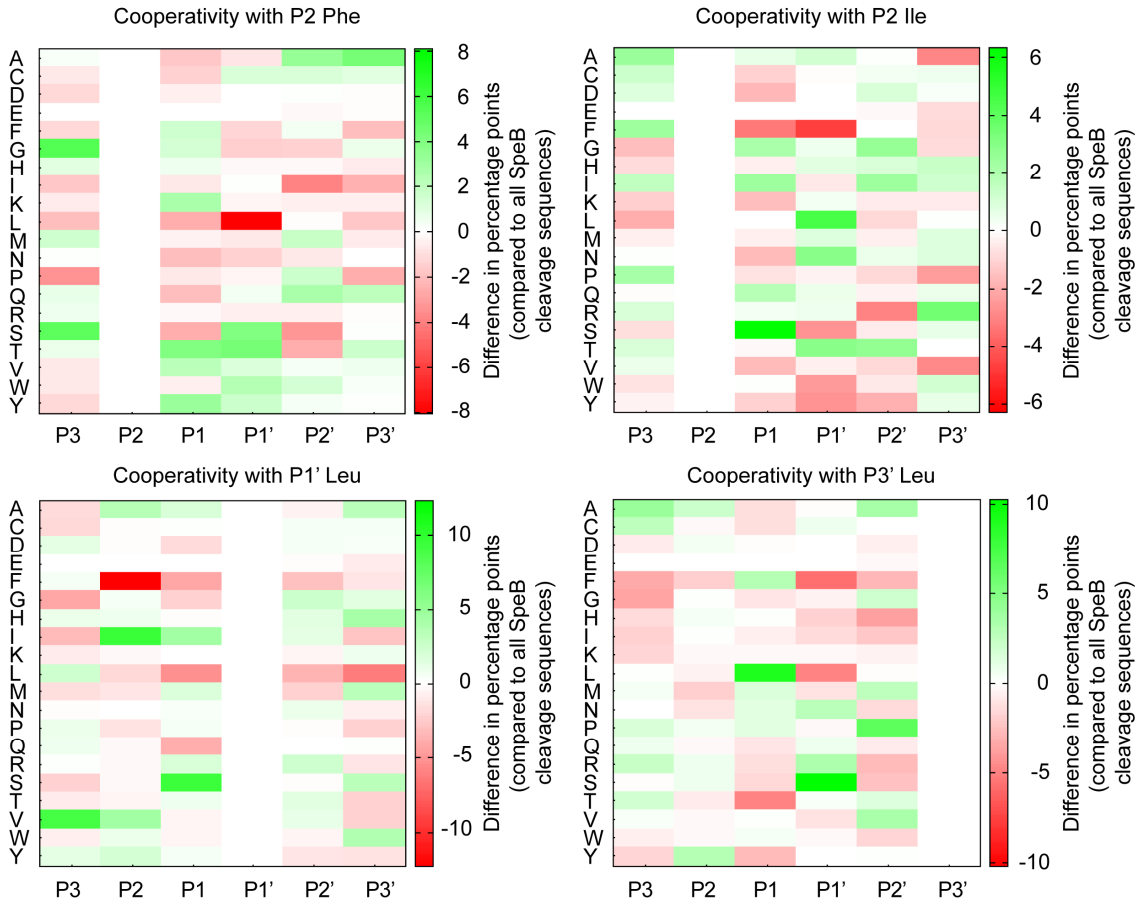


Figure 46. Subsite cooperativity from selected residues. The web-based PICS analysis enables to set phenylalanine as a fixed residue in P2 (upper left panel), isoleucine in position P2 (upper right panel), leucine in position P1' (lower left panel) and leucine in position P3' (lower right panel), thus visualizing those AA that stay in cooperative relation. Single letter code for AA residues plotted is on the y axes. P and P′ subsite positions are as shown on the x axes. Results are generated with the GluC library.

7.3.2.2. SpeB dependency analysis

As isoleucine and phenylalanine are the most often occurring AA in position P2, both were observed in their influence towards other residues (**Figure 45** and **Figure 46**). If P2 is phenylalanine, an isoleucine and leucine occurrence in the other positions becomes less likely. Hydroxy-group containing residues like serine, threonine and tyrosine occur more often at the cleavage site (P1 and P1'). This effect was more dominant in the GluC library.

7.3.3. Literature sequences and constructs

In order to design constructs, which are cleaved by SpeB, the sequences known in literature were used. These were taken from the protein literature database MEROPS (https://www.ebi.ac.uk/merops/) (**Table 12**).

Table 12. Substrates of SpeB from literature.

Construct-Name	Substrate	Organism (Substrate)	Cut-site	Reference
Ac-AEIKQPVV-Den	SpeB Pyrogenic exotoxin B	Streptococcus pyogenes	AEIK-QPVV 145-146	[201]
Ac-AYVHDAPV-Den	IL1β Interleukin 1, beta proprotein	Homo sapiens	AYVH-DAPV 115-116	[202]
Ac-QAPAKTAD-Den	scpA C5A peptidase precursor	Streptococcus pyogenes	QAPA-KTAD 89-90	[203]
Ac-TVMLKDKS-Den	endoS endo-beta-N-acetylglucosaminidase F2 precursor	Streptococcus pyogenes	TVML-KDKS 463-464	[204]

The sequences were synthesized, using methods explained above. After confirmation of the right PSL by LC/MS, they were coupled to **Den-CH_2-NH_2**. The yield of the fourth sequence in synthesis and coupling was very low, so the first three sequences were used as PSL for constructs. Purity and molecular mass were determined via HPLC and LC/MS (**Appendix**) before cleavage assays with expressed SpeB.

7.3.3.1. Cleavage of literature sequences

The constructs thus obtained, which react to the presence of SpeB, were incubated with different amounts of SpeB for 5 - 7 min to test their response to the protease (**Figure 47** and **Figure 48**). Even though the sequences are natural cleavage sites of SpeB, the presence of Den can prevent cleavage due to its size and permanent positive charge.

In order to sensitively detect SpeB in saliva from infected patients, the construct should be cleaved at minimal concentrations of the enzyme.

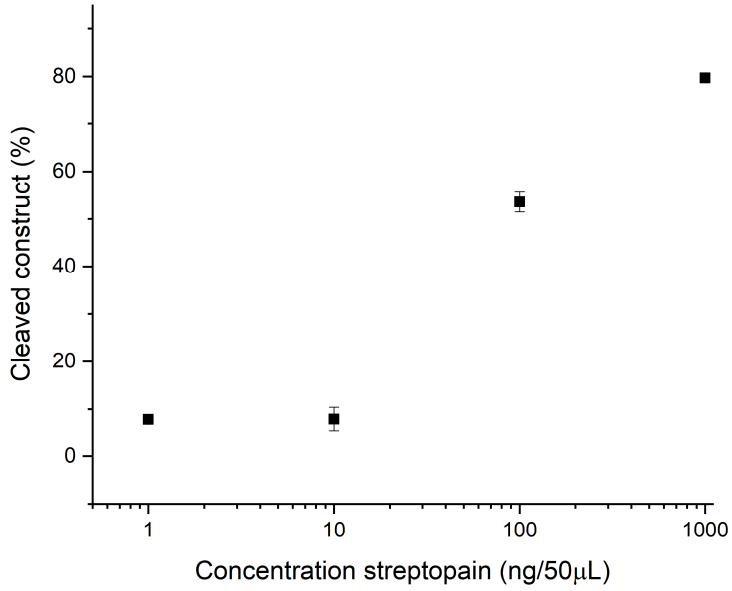


Figure 47. Concentration dependent cleavage of Ac-AEIKQPVV-Den. No cleavage occurred under a concentration of 10 ng SpeB per 50 µL. Using 100 ng per 50 µL, cleavage after 5 - 7 min was about 60%. Cleavage was determined by analyzing the relative decrease of the main peak as compared to the negative control. Data are presented as mean ± SD (n=3).

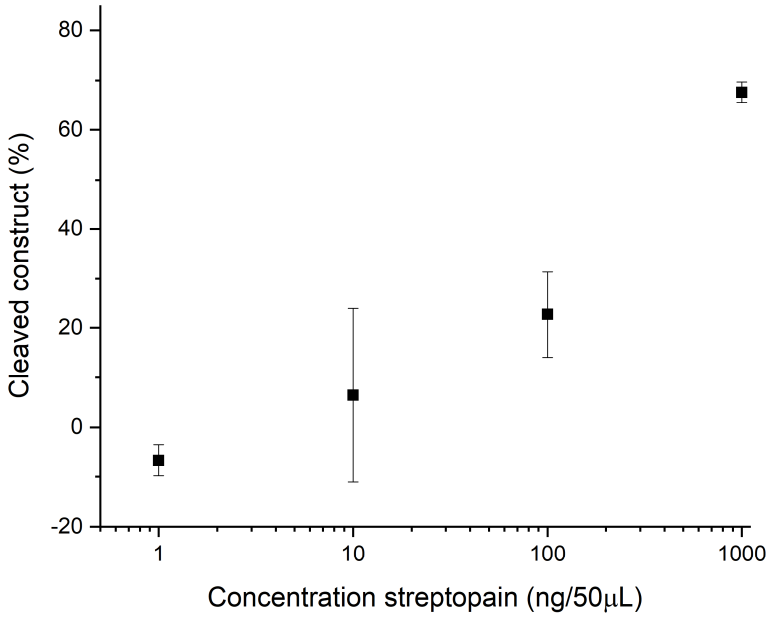


Figure 48. Concentration dependent cleavage Ac-AYVHDAPV-Den. No cleavage occurred under a concentration of 10 ng SpeB per 50 µL. Using 100 ng per 50 µL, cleavage after 5 - 7 min was 20%. Cleavage was determined by analyzing the relative decrease of the main peak as compared to the negative control. Data are presented as mean ± SD (n=3).

Two of the three synthesized constructs were cleaved by the expressed SpeB. Only construct No. 3 (Ac-QAPAKTAD-Den) was intact even after incubation with the highest SpeB concentration. Thus, this construct cannot be used as a diagnostic system. The other two systems were cleaved at concentrations between 10 and 100 ng per 50 µL SpeB. The first construct (Ac-AEIKQPVV-Den) is more sensitive to SpeB, as 60% are cleaved after incubation with 100 ng, as opposed to 40% for construct No. 2 (Ac-AYVHDAPV-Den).

The cleavage data shown in **Figure 47** were already published in the former noted review "Frugal Innovation for Point-of-Care Diagnostics Controlling Outbreaks and Epidemics".[200] In this "Perspective", SpeB represents the case of a protease with widely unknown cleavage sites. Herein, cleavage assays are carried out to determine the efficiency of protease cleavage towards different synthesized constructs.

7.3.4. Quantification of SpeB via western blot

In order to establish a method for quantifying SpeB, different concentrations of SpeB in saliva were determined. A standard curve is to be established using SpeB so that concentrations in clinical samples can be measured (**Figure 49**).

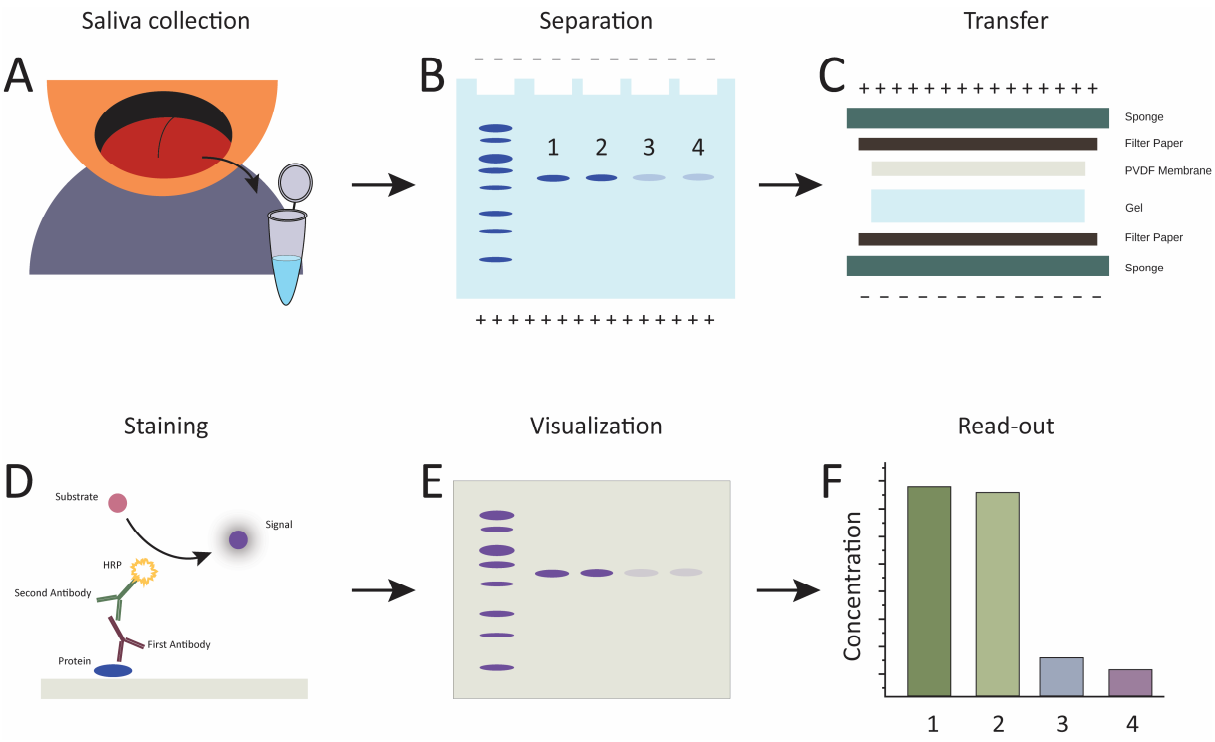


Figure 49. Proposed quantification scheme for clinical samples. (**A**) Saliva is collected from healthy and infected patients. (**B**) The clinical specimen is separated by SDS-Page (**C**) The separated protein fractions in gel are transferred onto a nitrocellulose membrane. (**D**) The membrane is treated with first Ab (SpeB Ab) and subsequently with second Ab (Anti-rabbit IgG). The cleavage of a chemiluminescent agent produces a luminescent signal. (**E**) Visualization is achieved (**F**) Signals can be quantified in their intensity by ImageJ.

Expressed SpeB was inserted into the pockets a 12% acrylamide gel in amounts of 15.6 to 125 ng (diluted in PBS and human saliva) and separated by gel electrophoresis. After treatment utilizing western blot protocol and the recording of the final blot, intensity of the bands was analyzed.

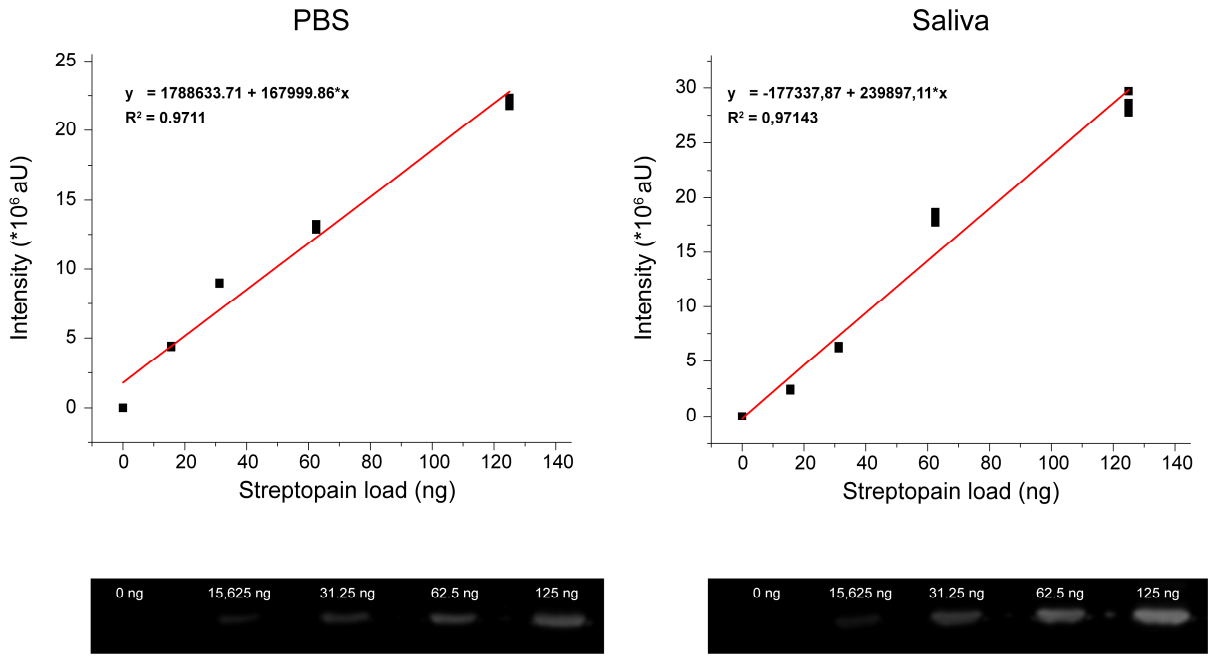


Figure 50. Standard curve of SpeB in PBS and saliva. Added amounts of SpeB in PBS (left) and human saliva (right) showed comparable signal intensity.

The proportional increase of the signal in PBS as well as in human saliva could be proven (**Figure 50**). At higher concentrations of SpeB, the linearity was not given and no linear increase of the measured intensity was observed. This makes the system suitable for quantifying the enzyme in saliva samples in the concentration range specified.

7.4. Section IV - Influenza

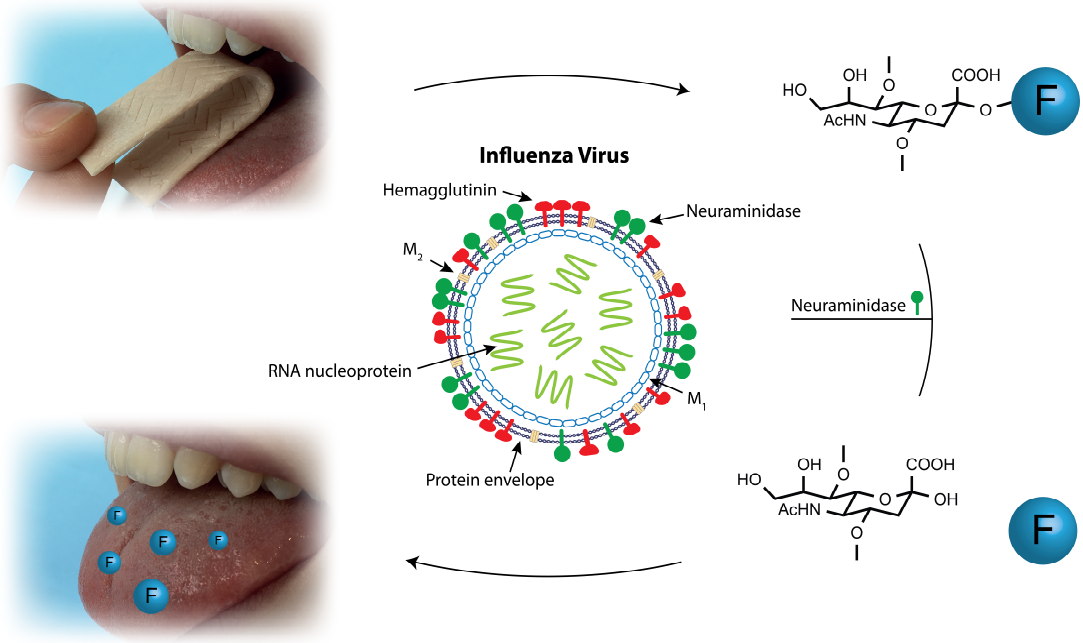


Figure 51. Schematic representation outlining the working principle of the influenza sensing diagnostic construct. First, the patient applies the diagnostic chewing gum, where the tasteless sensor comes in contact with saliva. In the event of an infection, neuraminidase from influenza cleaves the flavor, and releases it. The patient can now taste the flavoring molecule and is therefore warned about the infection.

This project provides the synthesis of a compound, which can be coupled to a flavor via a hydroxy-group (**Figure 52**).

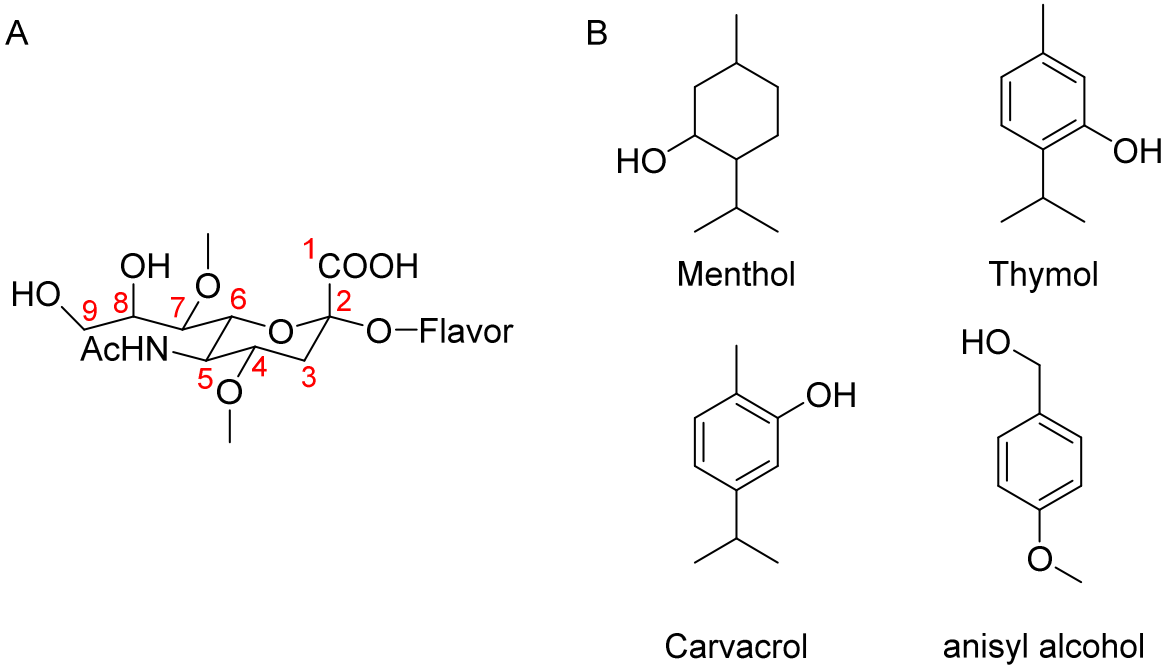


Figure 52. Graphical representation of the final construct and possible flavor components. (**A**) the final compound with numbered C-atoms (**B**) Possible flavor candidates with a couplable hydroxy-function.

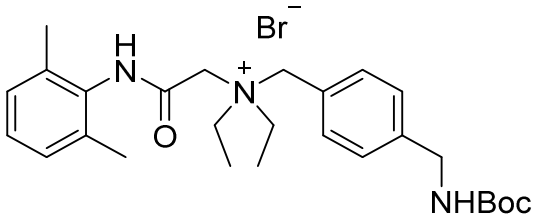
The coupled product functions as a diagnostic system, as it releases a flavor in the presence of NA. Formulated into a chewing gum, it can be used as a POCT for influenza virus types A and B. As mentioned in the introduction, NA cleaves the terminal saccharide residues of many complex carbohydrate side chains of cell surface glycoproteins. The glycosidic linkage, over the anomeric C2 position, bonding Neu5Ac with the penultimate saccharide is the substrate of the NA activity. Due to the alkylation of the construct at the hydroxy-groups in position 4 and 7, the system is selectively cleaved by a NA on influenza virus, but not the NA s found on other viruses or bacteria. The following steps provide the synthesis of a couplable, di-methylated version of Neu5Ac.

NMR of all steps are known to literature and therefore not provided. All synthesized steps are analyzed via ^{1}H-NMR.

7.5. Synthesis of Flavor components

7.5.1. Synthesis of Den-CH_2-NH-Boc

The synthesis was carried out analogously to the previous Denatonium modifications in the melt. The reaction in melt, meaning solvent free, has the advantage of short reaction times.



[Den-CH_2-NH-Boc]

1.2 g lidocaine (5.2 mmol) and 1.0 g tert-butyl-4-(bromomethyl)benzylcarbamate (3.4 mmol) were heated to 80 °C until the formation of a yellow melt. The melt then became highly viscous, up to solid, and subsequently, after a resting period of 10 min at 80 °C, the obtained yellow solid was treated with 40 mL of a mixture of ethyl acetate and n-hexane (1:1). To obtain the protected product, the yellow solid was stirred for 10 min at 80 °C in the mixture. The resulting white residue was filtered off and washed with a mixture of ethyl acetate and hexane (1:1). 1.6 g of the protected product **Den-CH_2-NH-Boc** were obtained.

Sum formula: $C_{27}H_{40}N_3O_3^+$ Br^-

Molecular mass: 534.5 g/mol

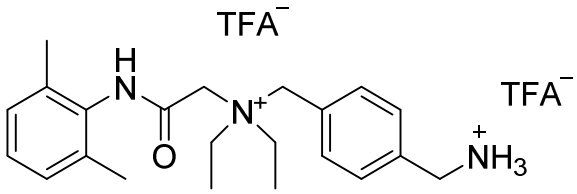
Yield: 1.6 g (91%).

^{1}H-NMR (400 MHz, DMSO-d6): δ = 10.0 (s, 1H), 9.64 (s, 1H), 7.61-7.59 (d,$^3J_{H,H}$ = 8.6, 2H), 7.46-7.44 (d, $^3J_{H,H}$ = 8.6, 2H), 7.16-7.10 (m, 3H), 4.71 (s, 2H), 4.11 (s, 2H), 4.13 (s, 2H), 3.50 - 3.45 (m, 4H), 2.19 (s, 6H), 1.48 (s, 9H), 1.40 (t, $^3J_{H,H}$ = 7.1, 6H).

^{13}C-NMR (100 MHz, DMSO-d6): δ = 162.3 (s, 1C), 155.9 (s, 1C), 142.8 (s, 1C), 135.0 (s, 2C), 133.3 (s, 1C), 132.8 (s. 2C), 127.9 (s, 2C), 127.4 (s, 2C), 127.2 (s, 1C), 125.8 (s, 1C), 77.9 (s, 1C), 61.2 (s, 1C), 55.3 (s, 1C), 54.2 (s, 2C), 42.9 (s, 1C), 28.2 (s, 3C), 18.1 (s, 2C), 7.9 (s, 2C).

7.5.2. Synthesis of Den-CH_2-NH_2

To obtain the couplable product **Den-CH_2-NH_2**, **Den-CH_2-NH-Boc** was deprotected.



[Den-CH_2-NH_2]

For the subsequent deprotection, 228 mg of **Den-CH_2-NH-Boc** (0.4 mmol) were dissolved in 1 mL of trifluoroacetic acid and shaken at room temperature for 1 h. The crude product was precipitated in diethyl ether and filtered off. The product was purified by chromatography.

Sum formula: $C_{22}H_{33}N_3O^{2+}$ 2 $C_2F_3O_2^-$

Molecular mass: 354 g/mol (Double TFA-salt: 581.6 g/mol)

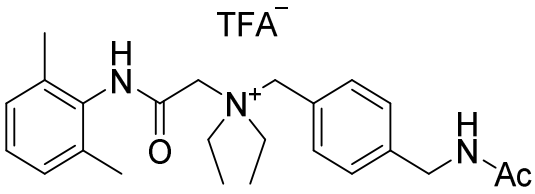
Yield: 175 mg (75%).

^{1}H-NMR (400 MHz, DMSO-d6): δ = 10.14 (s, 1H), 8.30 (s, 3H), 7.62 (m, 4H), 7.20-7.10 (m, 3H), 4.84 (s. 2H), 4.18 (s, 2H), 4.12 (q. $^3J_{H,H}$ = 5.8, 2H), 3.54-3.48 (m, 4H), 2.20 (s, 6H), 1.42 (t, $^3J_{H,H}$ = 7.1, 6H).

^{13}C-NMR (100 MHz, DMSO-d6): δ = 162.7 (s, 1C), 136.8 (s, 1C), 135.5 (s, 2C), 133.7 (s, 1C), 133.6 (s. 2C), 129.9 (s, 2C), 128.5 (s, 2C), 128.2 (s, 1C), 127.7 (s, 1C), 61.6 (s, 1C), 56.0 (s, 1C), 54.9 (s, 2C), 42.2 (s, 1C), 18.6 (s, 2C), 8.3 (s, 2C).

7.5.3. Synthesis of Den-CH_2-NH-Ac

A classical acetylation reaction was chosen for this reaction, which is used in peptide synthesis for capping of the N-terminus.



[Den-CH_2-NH-Ac]

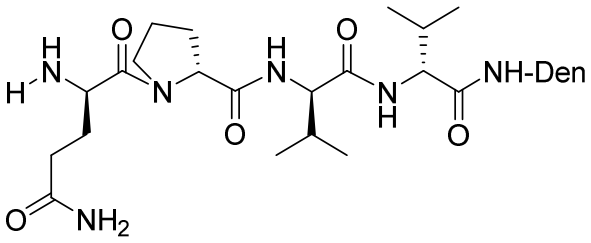
1.2 g **Den-CH_2-NH_2***2TFA (2.1 mmol) were dissolved in 5 mL of dry DMF. 600 µL acetic anhydride (6.35 mmol) and 1.2 mL DIPEA (6.9 mmol) were added and the reaction was shaken at room temperature for 25 min, until acetylation was quantitively acchieved. The raw product was precipitated with 35 mL diethyl ether at -20 °C. After 1 h at -20 °C, the suspension was centrifuged, the supernatant was discarded and the precipitate was washed another two times with 15 mL diethyl ether. After purification and lyophilization, **Den-CH_2-NH-Ac** was obtained as a white powder.

Sum formula:	$C_{24}H_{34}N_3O_2^+ C_2F_3O_2^-$
Molecular mass:	396.26 g/mol (TFA-salt: 509.25 g/mol)
Yield:	1,0 g (94%).

^{1}H-NMR (400 MHz, DMSO-d6): δ = 10.12 (s, 1H), 8.45 (t, $^3J_{H,H}$ = 6.0, 1H), 7.41-7.39 (d, $^3J_{H,H}$ = 8.1, 2H), 7.54-7.52 (d, $^3J_{H,H}$ = 8.1, 2H), 7.16-7.10 (m, 3H), 4.79 (s. 2H), 4.32 (d, $^3J_{H,H}$ = 6.0, 2H), 4.16 (s, 2H), 3.52-3.47 (q, $^3J_{H,H}$ = 7.1, 4H), 2.19 (s, 6H), 1.89 (s, 3H), 1.41 (t, $^3J_{H,H}$ = 7.1, 6H).

^{13}C-NMR (100 MHz, DMSO-d6): δ = 169.3 (s, 1C), 162.2 (s, 1C), 142.3 (s, 1C), 135.0 (s, 2C), 133.3 (s, 1C), 132.8 (s. 2C), 127.9 (s, 2C), 127.7 (s, 2C), 127.2 (s, 1C), 125.8 (s, 1C), 61.2 (s, 1C), 55.3 (s, 1C), 54.2 (s, 2C), 41.6 (s, 1C), 22.5 (s, 1C), 18.0 (s, 2C), 7.8 (s, 2C).

7.5.3.1. Synthesis of QPVV-Den



[QPVV-Den]

42 mg of **Den-CH_2-NH_2***2 TFA (72 µmol), 27 mg of Q(Trt)PVV (33 µmol) and 22.5 mg of HATU (60 µmol) were dissolved in dry DMF. 20 µL of DIPEA were added and the reaction was shaken under the exclusion of light at room temperature overnight. After one day, the same amount of HATU and DIPEA was added and the reaction was again shaken under the exclusion of light at room temperature overnight. The resulting crude product was precipitated in diethyl ether and filtered off. The product was purified by liquid chromatography and lyophilized.

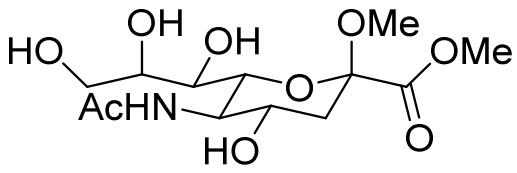
For deprotection the obtained product was treated with 100% TFA for 1 h under agitation. The resulting product was precipitated in diethyl ether and filtered off. The product was purified by liquid chromatography and lyophilized for two times.

Sum formula: $C_{20}H_{34}N_5O_5$

Molecular mass: 777.4 g/mol

Yield: 11,3 mg (44%).

7.5.4. Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-D-glycero- β-D-galacto-2-nonulopyranosidonate)

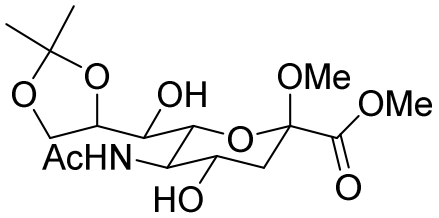


[2]

A suspension of N-acetylneuraminic acid (15.0 g, 48.5 mmol) was treated with Nafion NR50 resin (7.5 g) in methanol (300 mL) at RT until a clear solution was formed. This solution was evaporated to dryness, filled with fresh dry methanol and refluxed with vigorous stirring for at least 72 h at 65 °C. The resin was filtered off and washed with methanol. Afterwards, dry methanol was filled in and the mixture kept at -20 °C overnight for crystallization. The crystals were collected and washed with an ice cold mixture of ethyl acetate:methanol (6:1) to provide white powder of Neu5Ac methyl ester methyl ketoside **[2]**.

Sum formula:	$C_{13}H_{23}NO_9$
Molecular mass:	337,33 g/mol
Yield:	4.03 g (25%).

7.5.5. Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosidonate)



[3]

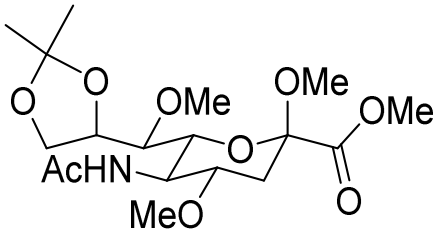
A mixture of **[2]** (4.2 g, 11.1 mmol) and camphorsulfonic acid (0.14 g, 0.603 mmol) in dry anhydrous acetone (100 mL) was stirred at room temperature for 3 h, then neutralized with TEA to pH = 6 - 7. The solution was evaporated to dryness. The solid was purified by chromatography (DCM:MeOH:TEA = 95:5:0.1) to afford **[3]** as a white powder.

Sum formula: $C_{16}H_{27}NO_9$

Molecular mass: 377.17 g/mol

Yield: 3.55 g (76%).

7.5.6. Synthesis of methyl (methyl 5-acetamido-3,5-dideoxy-4,7-di-O-methyl-8,9-O-isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosidonate)

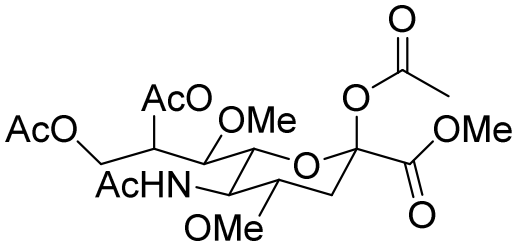


[4]

A mixture of **[3]** (3.4 g, 8.4 mmol) was treated with BaO (4.77 g, 31 mmol) and $Ba(OH)_2$ (0.578 g, 3.4 mmol) in dry DMF (36 mL). After 20 min, CH_3I (10 mL, 197.3 mmol) was added. The reaction suspension was kept stirring for 45 h at room temperature. The solids were filtered off and washed exhaustively with ethyl acetate. Under diminished pressure, the filtrate was concentrated to about 30 mL by evaporation, further extracted with ethyl acetate (60 mL) and 5% Na_2SO_3 (30 mL) followed by 5% NaCl solution (30 mL). The collected aqueous phase was extracted with ethyl acetate (60 mL) again. The collected organic phase was dried over anhydrous Mg_2SO_4, then evaporated to 10 mL under reduced pressure and kept at -20 °C with 20 mL ether overnight to give the product **[4]** as a white powder.

Sum formula:	$C_{18}H_{31}NO_9$
Molecular mass:	405.20 g/mol
Yield:	900 mg (26%).

7.5.7. Synthesis of methyl (5-acetamido-2,8,9-tri-O-acetyl-3,5-dideoxy-4,7-di-O-methyl-D-glycero-α,β-D-galacto-2-nonulopyranosidonate)



[5]

The product **[4]** (306 mg, 0.76 mmol) was suspended in mixture of methanol/water (1:1, 6 mL). 1 M NaOH (3 mL) was added and the reaction mixture was stirred for 3 h, the reaction was neutralized by adding Amberlite (H+) resin to pH = 7.0. The resin was filtered off, washed with methanol and the washing was evaporated to dryness. HCl (30 mM, 5 mL) was added to residue as well as Bio-Rad resin (2.0 g), then the resulting mixture was heated at 70 °C for 16 h. The resin was filtered off and washed with water. The solvent was evaporated off and the residue was dissolved in dry methanol (26 mL). Bio-Rad resin (1.7 g) was added and the reaction mixture was stirred for 5 h at room temperature. The resin was filtered off and the methanol was evaporated. It was then treated with Ac_2O (1 mL, 10.6 mmol), pyridine (1 mL) and DMAP (0.041 g) for 15 h at room temperature. The liquids were evaporated off and the residue was purified by chromatography on silica gel. The column was washed with mixtures of hexane and ethyl acetate in the ratio 2:1 and 1:1 respectively, and then the product was eluted with ethyl acetate to afford **[5]** as a white powder.

Sum formula: $C_{20}H_{31}NO_{12}$

Molecular mass: 477.18 g/mol

Yield: 120 mg (33%).

8. CONCLUSION AND OUTLOOK

8.1. Conclusion

In this work, diagnostic systems responding to the presence of a specific hydrolase by releasing a flavoring substance in the oral cavity were synthesized. In case the addressed enzyme is a protease, the basis of the diagnostic system comprises a protease-sensitive peptide sequence that combines a bitter flavoring agent with a protection group. In the case of influenza, the targeted enzyme is NA, so the construct comprises a modified sialic acid coupled to a flavor.

The systems thereby respond to increased enzyme concentrations in the oral cavity and informs the patient of his state of health. The human tongue is exploited as a 24/7 available detector, allowing diagnosis of several diseases. In order to come into sufficient contact with the enzymes present in human saliva and to establish a simple form of application, a chewing gum was chosen as the formulation, as this form of application is easy for everyone to use and the sensor stays in the mouth for a sufficiently long time.

In order to generate a taste sensation only in the case of a present inflammation, two design requirements are essential. On the one hand, the (unsplit) intact sensor is designed in such a way that it is tasteless. On the other hand, in the presence of inflammation or infection, it is transformed into a compound that can be easily perceived in terms of taste. The technology provides diagnostics with a revolutionary though always present instrument – the tongue.

The result is a diagnostic system that is completely independent of access to energy sources, does not require complex equipment, and can be carried out by specially trained personnel alone. It should be ready for use everywhere and at any time and generate the desired information in the shortest possible time (< 5 min).

8.2. Section I - Fundamentals

This part of the work concentrated on enhancing the performance of the individual components of the peptide-based diagnostic construct,

The performance of the flavor component was optimized by designing a compound that was both couplable to the C-terminus of the PSL and simultaneously has a strongly bitter gustatory perception. The flavoring compound denatonium was therefore derivatized, to meet this criteria. The determination of the bitterness by an electronic tongue showed, that the denatonium derivate is similarly perceived as the industrial used denatonium benzoate. Due to the newly introduced amine group, no AA remains at the denatonium after cleavage and

subsequent degradation. Hence, the full taste can be generated. In the cytotoxicity assay with NIH cells, none of the compounds showed an effect on cell viability. A full toxicity evaluation has to be carried out in the future, but these results give first hints, that this newly synthesized flavoring agent is non-toxic.

In the future, further flavors could be utilized functioning as the taste component of this diagnostic system. A bitter tasting component is bad for adherence of the patients, especially for children. So new flavors, which generate a more pleasant taste sensation could be coupled to the diagnostic system. Generally, all flavor components with a primary amine function can be tested in suitability of usage. As not many flavors with this functional group exist, new compounds have to be synthesized and evaluated.

With the now fully established PICS technique, new sequences for PSL can be developed, by incubating the peptide library with the protease of interest. For proteases with known substrates, the optimal sequence in terms of specificity and cleavage efficiency can be determined. This would decrease the time for result generation and increase sensitivity and specificity of the diagnostic construct. On the other hand, new indications can be opened up by analyzing the cleavage site of other target proteases. This results in a lot more indications, which can be detected by this diagnostic platform technology.

The best strategy of coupling the carboxy group of the peptides to the primary amine of the denatonium derivate was determined using different established protocols. HATU/DIPEA showed best results in terms of yield and purity after reaction. It was therefore used for all coupling reactions of modified denatonium and peptides in this work. With the limitation of some uncouplable AAs, satisfying yields were achieved.

The coupling as well as the cleavage by an AP of all natural occurring AAs was investigated. Alanine seems to be the perfect coupling candidate in both synthesis and cleavage efficiency.

The final construct was a simplified version of the previous used diagnostic system for peri-implantitis. It is therefore more efficient in production. After the determination of cleavage efficiency in human saliva samples, the next step would be to optimize the performance in clinical studies. For this, the construct and the final formulation has to be carried out under good manufacturing process (GMP) conditions.

8.3. Section II - Matrix metalloproteinases

The dental health market shows great potential for these kind of diagnostic products. The project originally derived from a dental indication (peri-implantitis). As the teeth are an important part of the oral cavity and constantly in contact with human saliva, diagnostic tests on dental diseases focus naturally on this body fluid.

A diagnostic system, reacting to the presence of MMPs was developed and evaluated in terms of cleavage efficiency, taste sensation and toxicity.

Starting with the last, no cytotoxicity was observed with used NIH cells. The construct showed no effect on cell viability and proliferation. As stated above, an approved toxicity evaluation has to be carried out, to prove the construct to be non-toxic. Only with this information, clinical studies on patients will be approved.

The taste sensation was evaluated using the electronic tongue setup and denatonium benzoate as a reference substance. No interaction with the sensors for bitterness lead to the assumption that the final construct is not perceived as bitter by the gustatory perception. After a full toxicity evaluation of the construct, a determination of its bitterness by trained test subjects is possible.

Due to the demographic shift and lifestyle changes, dental health problems occur more often nowadays, especially in the industrialized countries. A fast and easy instrument for self-diagnosis could be a game changer in fighting dental diseases, costing the health care systems billions each year. In Europe, dental health expenditures make up to 82% of the health care expenditures. Diagnosing could been carried out more efficient in terms of cost and time. The market is additionally characterized by a high percentage of private spending.

As the next indication in the field of dental health, the diagnostic system is tested as a PA screening tool. It responds to the presence of PA, by releasing the flavoring agent in the presence of increased activity of MMP.

8.4. Section III - *Streptococcus pyogenes*

Before this project, only human proteases were used as targets, to design diagnostic systems for the oral cavity. However, the technique can also be transferred to pathogenic, non-human enzymes, which also cleave their recognition sequences extremely efficiently and specifically. For instance, pathogens utilize diverse strategies to protect against an attack by the human complement system as a way to facilitate survival inside the host organism and effectively increase virulence. One of these strategies is the complement neutralization via degradation or inactivation of complement components by proteases.[205]

In this work, 4 peptide sequences were synthesized and coupled to the modified denatonium. Used peptides should not only be optimized in terms of cleavability but also in terms of synthesis yield and coupling efficacy. From these 4 sequences, one couldn't be used because of problems with manufacturing. A replacement of the AA at the C-terminus could be a solution, as seen in the MMP-project. However, after changing the PSL, a new cleavage evaluation has to be done afterwards. From the resulting 3 constructs in this work, only two were cleavable by the target protease. This could be a consequence of the coupling with denatonium, as the sequence, but not the whole construct, is recognized by SpeB.

From the two found cleavable constructs, the first (Ac-AEIKQPVV-Den) was the most sensitive to SpeB. It detects SpeB in concentrations of a few nanograms per µL. Both cleavable constructs are coupled to denatonium via a valine, which cannot be degraded by the AP. Future constructs should search for cleavable systems, coupled to denatonium via a alanine or leucine, to trigger maximal gustatory perception.

To develop a most efficient diagnostic tool for infectious diseases, the optimal peptide sequences in terms of specificity and cleavage efficiency have to be determined. In this work, new cleavage sites of SpeB, an extremely important extracellular protease of *S. pyogenes* were determined, using the newly established in-house PICS assay. With the generated peptide libraries, 1765 (trypsin) and 582 (GluC) cleavage sites are discovered. The found cleavage sites could be synthesized and incubated with SpeB, or better with the saliva of infected patients, to identify next generation PSL sequences.

Constructs with the most promising sequences then have to be synthesized and cleavage experiments repeated. The positive charge and steric hindrance of the denatonium derivate could slow cleavage by the target protease down if they interfere with the active center. If the construct shows promising results, it can be utilized to discriminate between saliva of infected and healthy patients.

Current POCT on *S. Pyogenes* are dominated by throat swab tests. A throat sample can't be obtained by the patient themselves, so professional personnel is needed. This increases the risk of infection and is an unpleasant experience for both, the physician and the patient. Additional processing of the sample is needed, which results in longer waiting times.

In contrast to tests on the market, the gum reacts to enzymes that indicate activity rather than presence of a pathogen. Many bacteria that can cause serious diseases are naturally present in the human oral cavity, so simply proving presence is not enough to diagnose a specific disease.

8.5. Section IV - Influenza

In addition to the diagnostic systems reacting to the presence of proteases, a sensor for the NA activity is in development. As the other constructs, it can be brought directly into the oral cavity, releasing a flavor substance in the presence of NA from influenza A and B.

The couplable compound was synthesized, which can now be linked to diverse flavoring agents. In the next steps, the molecule is coupled with a library of flavors, and analyzed if the taste sensation is still present as a construct. If not, the taste sensation is only generated in the presence of NA from Influenza A and B. Two methyl-groups were successfully introduced in the positions 4 and 7. Through this modification, the construct is only cleaved by viral NA.

Almost everyone can be assumed to be the target group of this annual occurring epidemic. Practicing physicians play a central role in the prescription of antibiotics. They are the first port of call for people suffering from influenza. Since our chewing gum is intended to be used in this field, knowledge about the behavior of physicians in this type of patient is of utmost importance for further development.

Especially in the discrimination between a viral and a bacterial infection, rapid test diagnostics are of interest, as their use could minimize the uncontrolled use of antibiotics. Both infectious diseases (infection with GAS and Influenza) resemble in their clinical symptoms. With this system, we aim to develop diagnostics that can detect relevant concentrations of influenza viruses and Streptococcus pyogenes bacteria, therefore differentiate between those both infections. With regard to the antibiotic crisis, diagnostics can reduce the prescription rate of antibiotics, thereby avoiding bacterial resistance to this enormously important class of drugs.

8.6. Outlook

First generations of the newly designed diagnostic systems were successfully synthesized and tested for specificity and selectivity towards several target proteases. The sensors are currently investigated for their bitterness before and after cleavage in comparison to unmodified denatonium and will be used to discriminate saliva collected from patients. Therefore saliva samples of healthy and infected patients will be acquired to test the developed system ex vivo and to optimize screening performance.

The POCT Market will increase rapidly in size of spending, both by health care systems and the private sector. An increase in patient numbers leads subsequently to a decrease of time that is spent on single patients. In consequence, there is a growing need for fast reliable diagnosis. Its simple structure allows the technology to be applicable to any disease that appears in the oral cavity and by design it has some critical advantages to established POCT

technology. The oral health market shows even more potential. The market is characterized by a high individual willingness to pay and is driven by demographic change.

Due to the new synthesis route and formulation, it is not only cheap in production. By providing medical professionals and patients with a tool to quickly diagnose a specific disease in an uncomplicated way it increases the hands-on-time in medical facilities and empowers the patient to self-diagnose whether a visit at the doctor's is necessary or not.

Furthermore disease can be detected in an early stage allowing early treatment reducing the severity of consequential damages. This is beneficial for the patient and reduces the costs of a treatment. It gives the patient the opportunity to avoid late complications and the associated unwanted visits to the doctor or dentist, as well as expensive treatments.

Also oromucosal films which comprise mucoadhesive buccal films and orodispersible films, could be used as the galenic formulation. The first one remains longer in the buccal cavity, dispensing the diagnostic construct for a longer time frame. The second formulation would instantly dissolve when it comes in contact with saliva, releasing the diagnostic construct into the oral cavity. The formulation as a spray is also imaginable, to apply the diagnostic system. These new drug formulation approaches would lead to an increased target group. As one of the requirements to use the diagnostic system in a chewing gum is the ability to chew. Old people as well as very young ones, do have problems with this. The diagnostic system however has to be optimized for this kind of application. Swallowing of the dissolved construct could lead to false negatives, so the process of cleavage of the construct has to be accelerated. The established PICS assay could help, finding the best cleavable sequence in terms of specificity and cleavage rate.

Also unnatural AAs or peptidomimetic linkers could be used as the foundation of new PSL, to develop more specific targets for proteases. In the last decades, a lot has been done in this area. In addition, those linkers would be more stable and robust to environmental influences.

The innovative approach is also very applicable to developing and emerging countries, as no medical staff or facilities are needed to make a first diagnosis. Screening could be carried out anywhere, due to the robustness and transportability of the dried construct. As the medical coverage (e.g. physicians per capita) and health care infrastructure is weak, a diagnostic tool is needed to spare the scarce resources effectively. Also, through this system, the management of occurring epidemics and pandemics could be improved. The formulation and the simplified construct reduces costs and make the process safer and faster for all parties involved, as no clinical sample has to be obtained by another person. It can also potentially empower patients to self-test in the privacy and comfort of their homes.

In developed countries it could relieve the health systems of financial burdens. An ageing population means more patients for the same number of doctors and therefore increased costs for health insurance systems. Also chronic disease become more and more costly, especially due to resistant bacteria. As a result healthcare delivery is moving to increasingly decentralized settings such as rapid clinics and the home, driven by POCT that provide accurate and directional results. The market shows growth rates of 22% in the segment of infection disease undermining the observable trend towards an informed patient that is empowered to self-diagnose whether the consultation of a professional is necessary or not.

The bacterium *S. aureus* has been detected in the nasopharynx and is also dependent on extracellular proteases which could be detected by this system. In case of positive testing of clinically otherwise inconspicuous patients, preemptive hygiene measures could be taken and further diagnostics with high-precision methods (genotyping) could be carried out. A cost efficient improvement of hospital hygiene would be possible in order to counter the threat of multi resistant *S. aureus*.

The industry's desire for a diagnostic system for peri-implantitis has led to the development of a platform technology for the detection of a variety of diseases. There is a significant demand for new diagnostic tests to move closer to the patient, whether in the hospital, physician‘s office or at home, effectively decreasing the time to result generation, and subsequently helping patients and doctors to make better informed and individual treatment decisions regarding the patient‘s health. Furthermore as patient numbers are increasing, markets show immense growth rates. It is therefore crucial for doctors to decrease the hands-on-time for a single patient.

Only a fraction of the possibilities offered by this technology has been explored so far. It represents a novelty in the rapid test diagnostics of bacterial or viral diseases, whereby indication and application can still be expanded. Since the basic research of the system at the University of Würzburg will soon be completed, the upscaling of the production as well as clinical studies are the next logical steps.

With our findings, we created a validated basis from which further steps can be planned and implemented.

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10. APPENDIX

10.1. Introduction – Survey of physicians

Question 1:

Is there a need for a rapid test on influenza, which could be used in patients with pharyngitis and unclear symptoms?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Yes	36	10	11	57	90
No	3	2	0	5	8
N/A	1	0	0	1	2

Question 2:

In which time period, a chewing gum-based test in pharyngitis should be able to differentiate between viral and bacterial infections in order to be relevant in clinical routine?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Up to 2 min	4	2	0	6	10
2-5 min	22	6	8	36	57
5-20 min	13	4	2	19	30
N/A	1	0	1	2	3

APPENDIX

Question 3:

Which diagnostic test would you prefer?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Influenza	13	4	7	24	38
S. pyogenes	0	0	0	0	0
Combination: 1 Influenza + 1 *S. pyogenes*	16	5	3	24	38
Combination: 1 Influenza + multiple *S. pyogenes*	5	3	1	9	14

Question 4:

Are you prescribing antibiotics because of suspected future superinfections?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Yes	5	2	2	9	14
No	32	10	7	49	78
N/A	3	0	2	5	8

Question 5:

How high do you rate the benefit of the patient using a diagnostic chewing gum in the days following the diagnosis, presenting again if necessary?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Very high	4	1	0	5	8
high	9	6	2	17	27
low	21	5	8	34	54
N/A	6	0	1	7	11

Question 6:

Would you recommend your patients to purchase these chewing gums independently in the future in order to obtain initial evidence of a possible disease?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Yes	14	6	1	21	33
No	19	5	10	34	54
N/A	7	1	0	8	13

Question 7:

What do you think a single test for influenza detection or a combination set with a single test for influenza and S. pyogenes could cost?

Test version	Most frequent answer (€)	Range (€)
Single test	5	2-25
Combination	10	4.5-45

Question 8:

Some colleagues feel "prescription pressure" for colds due to the expectations of the patients. Do you think a POCT will help you to explain the chosen therapy option better to patients, so that this "pressure" could decrease?

Answer	General practitioner	Otolaryngologist	Pediatrician	Total	Percentage (%)
Very high	21	7	3	31	49
high	4	1	2	7	11
low	10	3	3	16	25
N/A	5	2	3	7	15

10.2. NMR Spectra

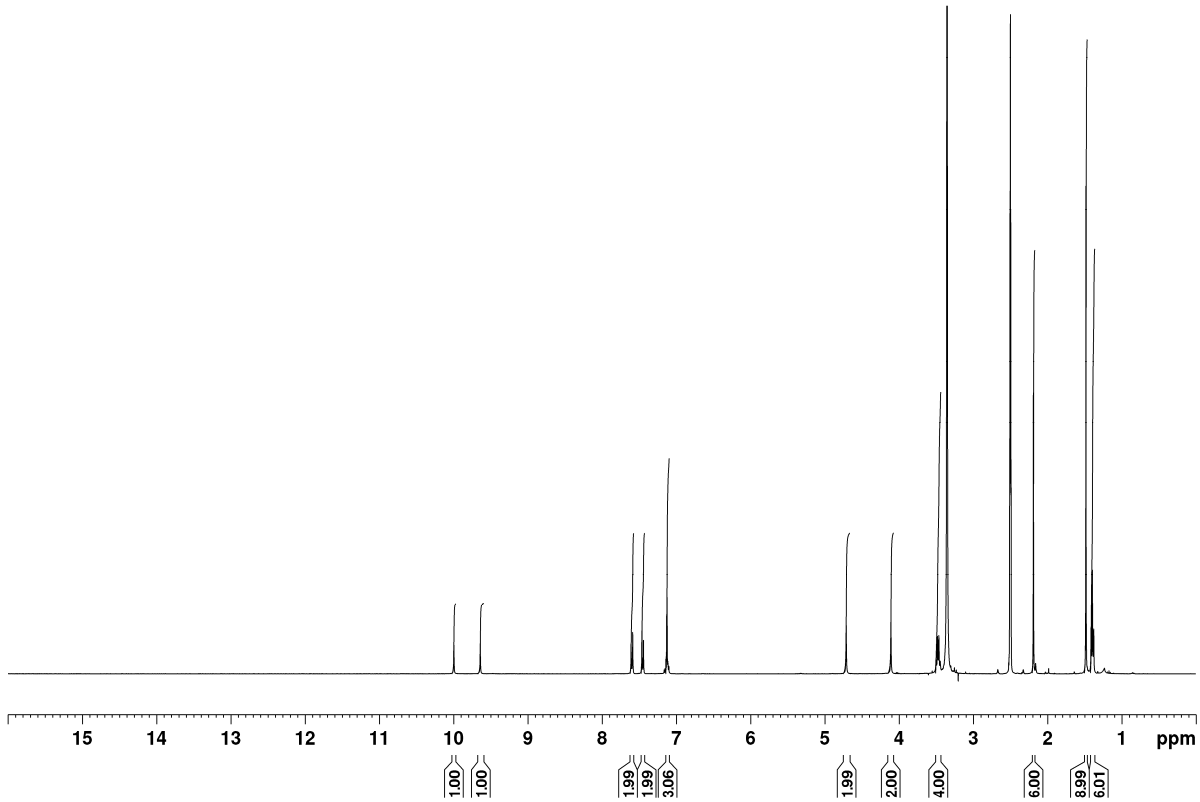

Figure 53. ^{1}H NMR spectrum of Den-CH_2-NH-Boc in DMSO-d6.

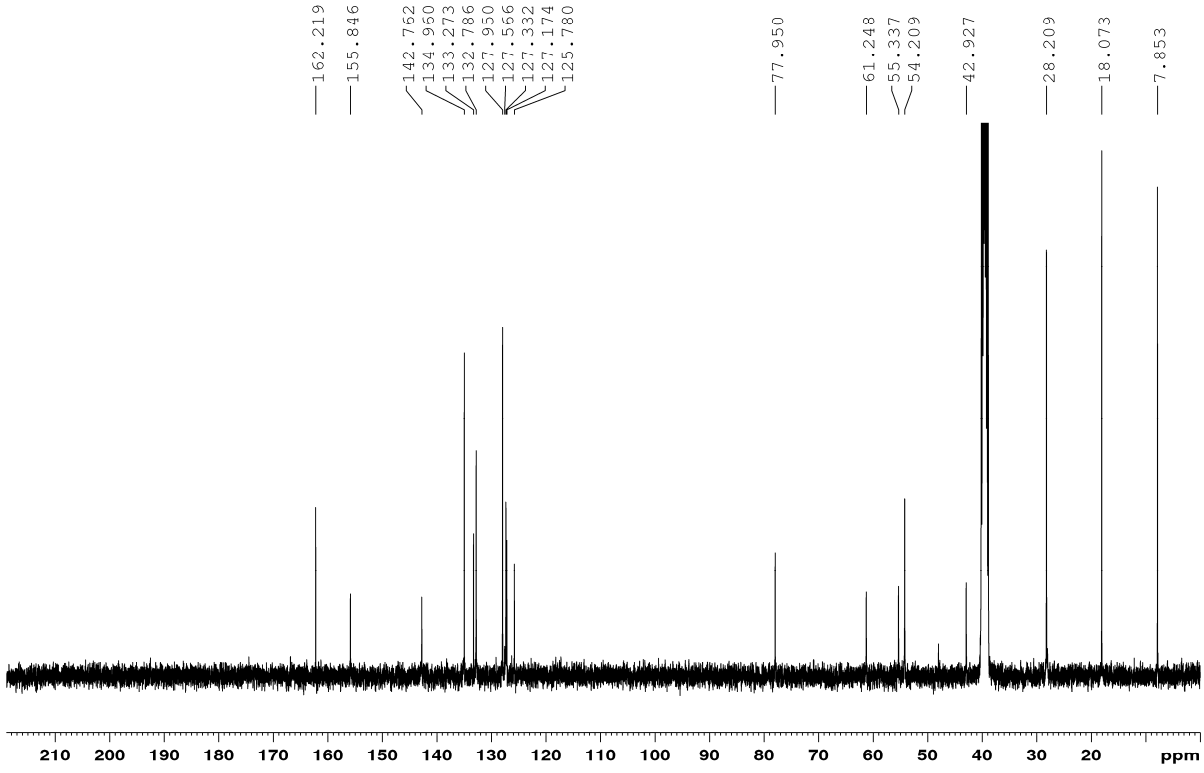

Figure 54. ^{13}C NMR spectrum of Den-CH_2-NH-Boc in DMSO-d6.

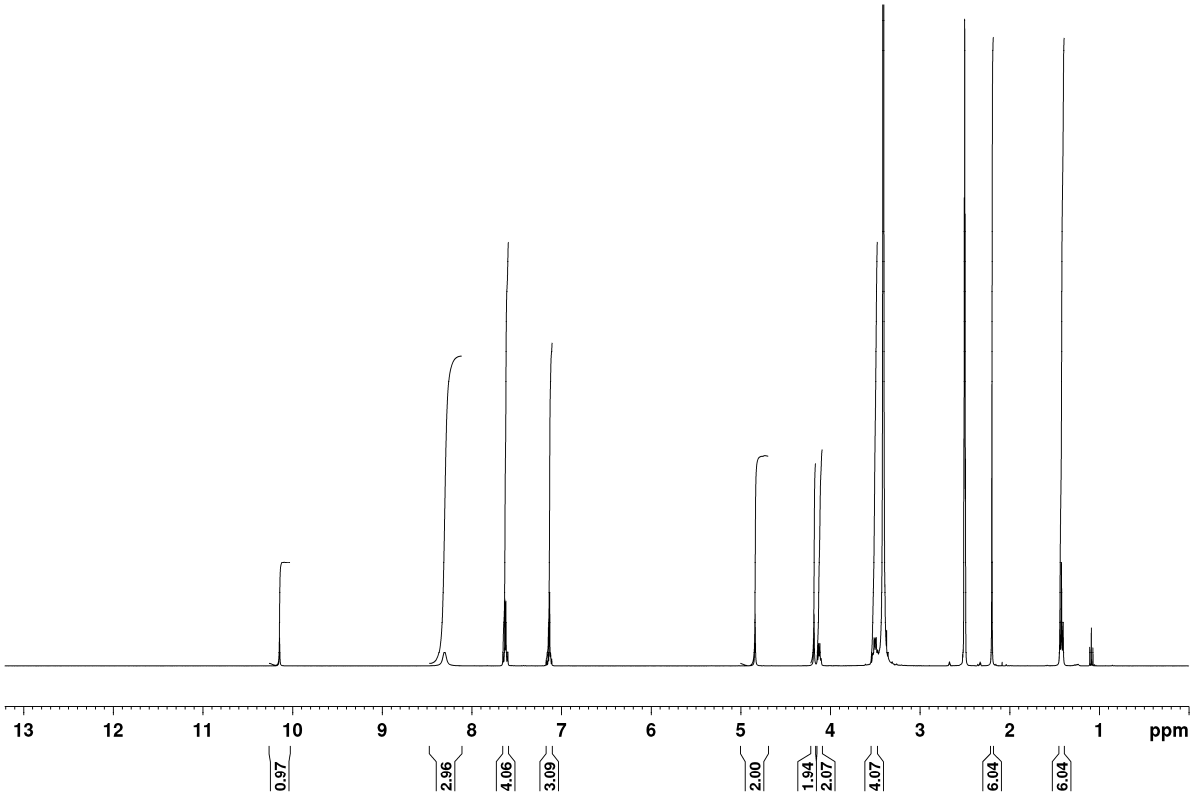

Figure 55. ^{1}H NMR spectrum of Den-CH_2-NH_2 in DMSO-d6.

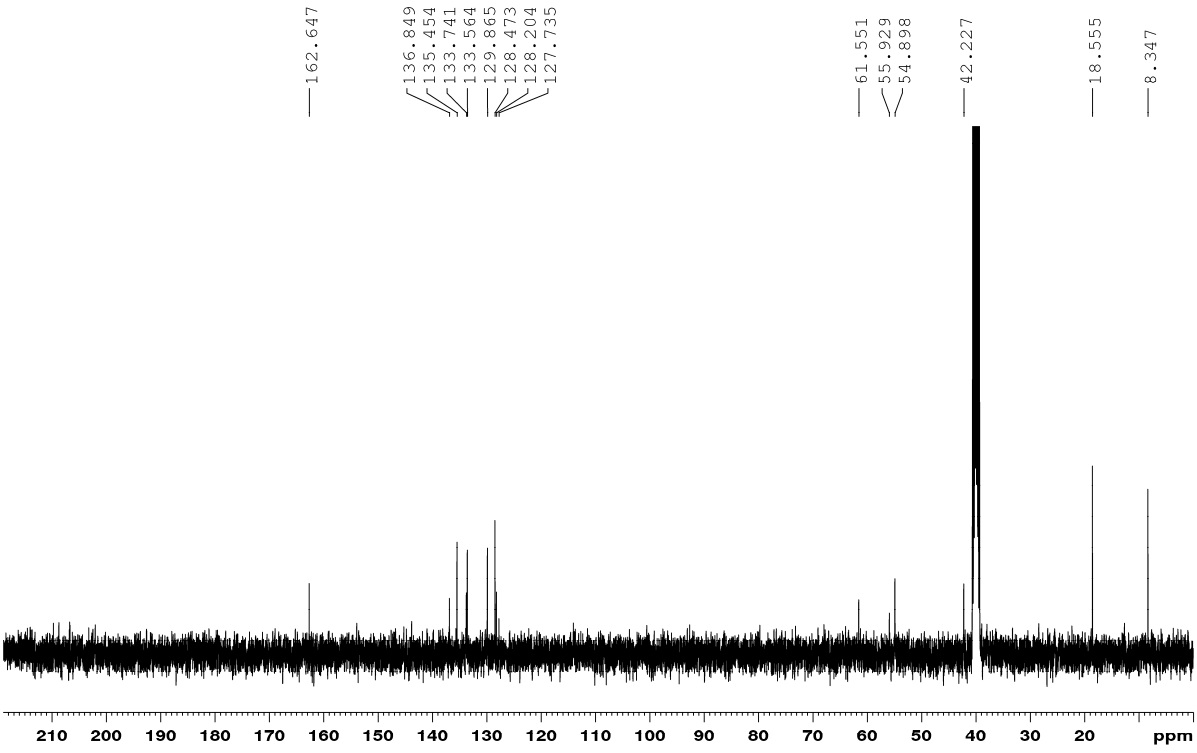

Figure 56. ^{13}C NMR spectrum of Den-CH_2-NH_2 in DMSO-d6.

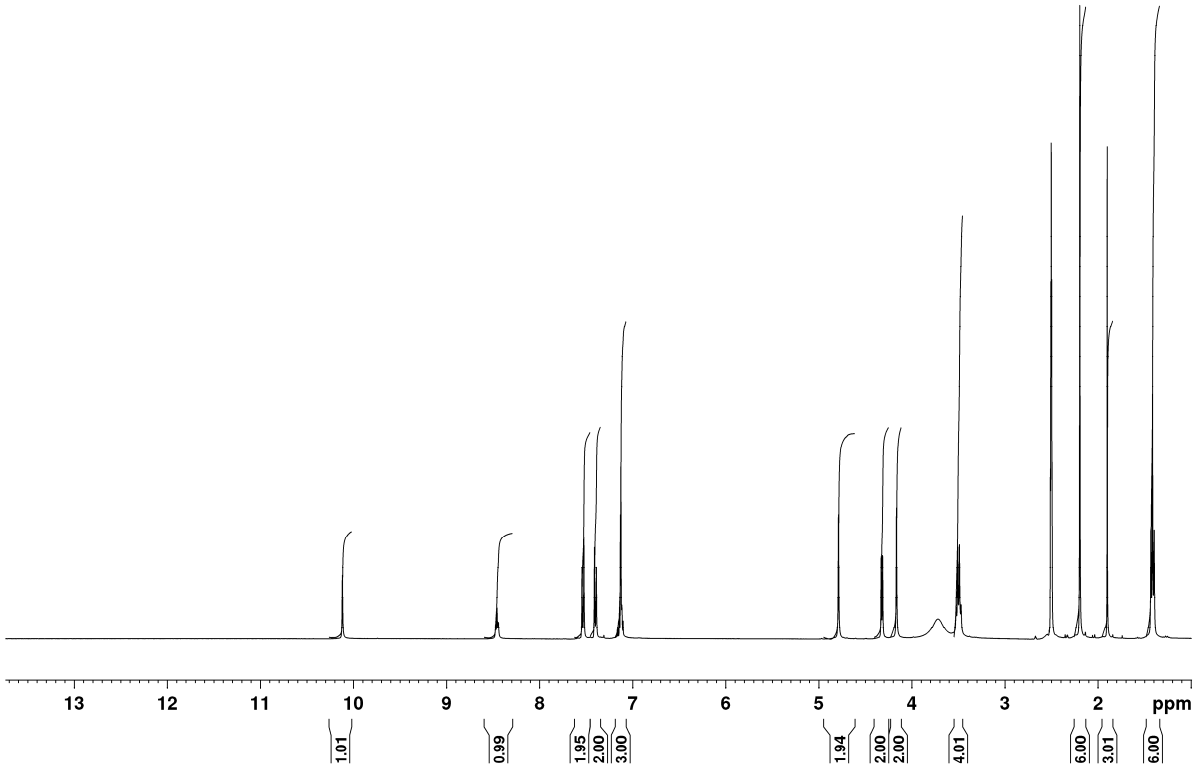

Figure 57. ^{1}H NMR spectrum of Den-CH_2-NH-Ac in DMSO-d6.

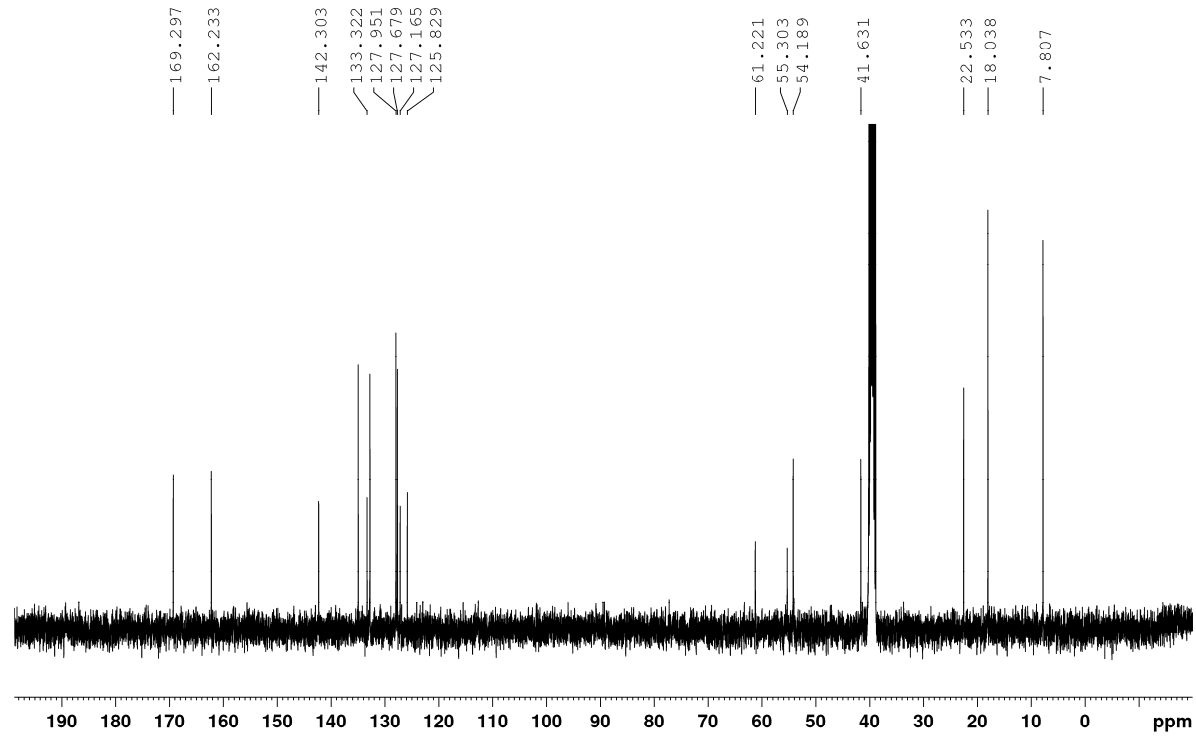

Figure 58. ^{13}C NMR spectrum of Den-CH_2-NH-Ac in DMSO-d6.

10.3. QPVV-Den and DAPV-Den

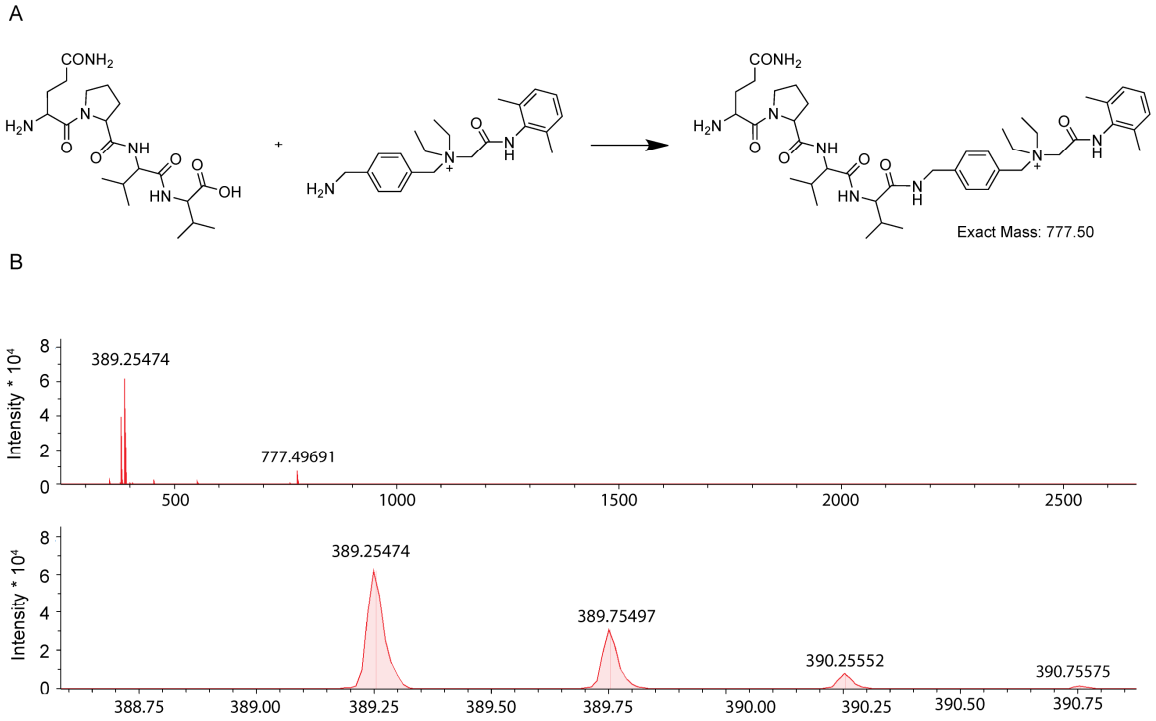

Figure 59. Synthesis procedure and MS data of QPVV-Den. (**A**) Chemical synthesis (**B**) Positive ion mass spectrum. It is possible to identify the single charged mass of 777.49691 m/z and the double charged mass of 389.25474 m/z.

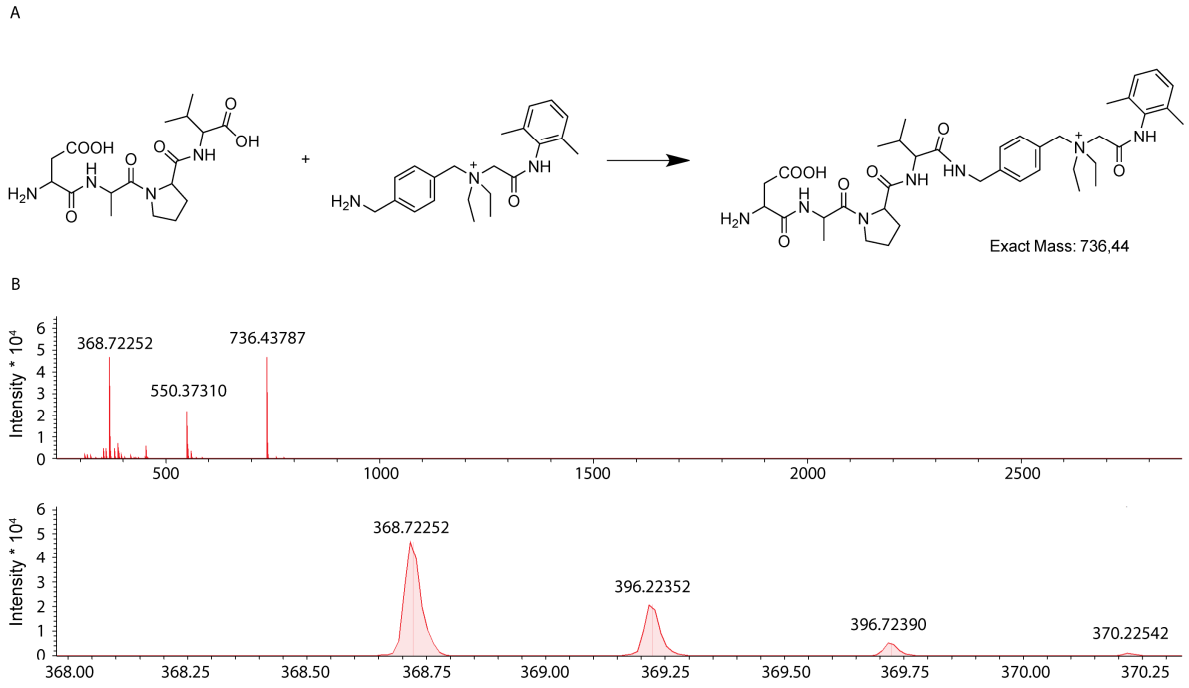

Figure 60. Synthesis procedure and MS data of DAPV-Den. (**A**) Chemical synthesis (**B**) Positive ion mass spectrum. It is possible to identify the single charged mass of 736.43787 m/z and the double charged mass of 368.72252 m/z.

10.4. Used DNA sequences for expressed SpeB

Plasmid SpeB-M3C3

```
ATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCCATCATCATCATCATCATGATCAAAACTTTGCTCGTAACGAAAAAGAAGCAAAAGATA  < 100
M  A  S  M  T  G  G  Q  Q  M  G  R  G  S  H  H  H  H  H  H  D  Q  N  F  A  R  N  E  K  E  A  K  D  S
         10        20        30        40        50        60        70        80        90
GCGCTATCACATTTATCCAAAAATCAGCAGCTATCAAAGCAGGTGCACGAAGCGCAGAAGATATTAAGCTTGACAAAGTTAACTTAGGTGGAGAACTTTC  < 200
  A  I  T  F  I  Q  K  S  A  A  I  K  A  G  A  R  S  A  E  D  I  K  L  D  K  V  N  L  G  G  E  L  S
         110       120       130       140       150       160       170       180       190
TGGCTCTAATATGTATGTTTACAATATTTCTACTGGAGGATTTGTTATCGTTTCAGGAGATAAACGTTCTCCAGAAATTCTAGGATACTCTACCAGCGGA  < 300
 G  S  N  M  Y  V  Y  N  I  S  T  G  G  F  V  I  V  S  G  D  K  R  S  P  E  I  L  G  Y  S  T  S  G
         210       220       230       240       250       260       270       280       290
TCATTTGACGCTAACGGTAAAGAAAACATTGCTTCCTTCATGGAAAGTTATGTCGAACAAATCAAAGAAAACAAAAAATTAGACACTACTTATGCTGGTA  < 400
S  F  D  A  N  G  K  E  N  I  A  S  F  M  E  S  Y  V  E  Q  I  K  E  N  K  K  L  D  T  T  Y  A  G  T
         310       320       330       340       350       360       370       380       390
CCGCTGAGATTAAACAACCAGTTGTTAAATCTCTCCTTGATTCAAAAGGCATTCATTACAACCAAGGTAACCCTTACAACCTATTGACACCTGTTATTGA  < 500
  A  E  I  K  Q  P  V  V  K  S  L  L  D  S  K  G  I  H  Y  N  Q  G  N  P  Y  N  L  L  T  P  V  I  E
         410       420       430       440       450       460       470       480       490
AAAAGTAAAACCAGGTGAACAATCTTTTGTAGGTCAACATGCAGCTACAGGATGTGTTGCTACTGCAACTGCTCAAATTATGAAATATCATAATTACCCT  < 600
 K  V  K  P  G  E  Q  S  F  V  G  Q  H  A  A  T  G  C  V  A  T  A  T  A  Q  I  M  K  Y  H  N  Y  P
         510       520       530       540       550       560       570       580       590
AACAAAGGGTTGAAAGACTACACTTACACACTAAGCTCAAATAACCCATATTTCAACCATCCTAAGAACTTGTTTGCAGCTATCTCTACTAGACAATACA  < 700
N  K  G  L  K  D  Y  T  Y  T  L  S  S  N  N  P  Y  F  N  H  P  K  N  L  F  A  A  I  S  T  R  Q  Y  N
         610       620       630       640       650       660       670       680       690
ACTGGAACAACATCCTACCTACTTATAGCGGAAGAGAATCTAACGTTCAAAAAATGGCGATTTCAGAATTGATGGCTGATGTTGGTATTTCAGTAGACAT  < 800
  W  N  N  N  I  L  P  T  Y  S  G  R  E  S  N  V  Q  K  M  A  I  S  E  L  M  A  D  V  G  I  S  V  D  M
         710       720       730       740       750       760       770       780       790
GGATTATGGTCCATCTAGTGGTTCTGCAGGTAGCTCTCGTGTTCAAAGAGCCTTGAAAGAAAACTTTGGCTACAACCAATCTGTTCACCAAATTAACCGT  < 900
 D  Y  G  P  S  S  G  S  A  G  S  S  R  V  Q  R  A  L  K  E  N  F  G  Y  N  Q  S  V  H  Q  I  N  R
         810       820       830       840       850       860       870       880       890
AGCGACTTTAGCAAACAAGATTGGGAAGCACAAATTGACAAAGAATTATCTCAAAACCAACCAGTATACTACCAAGGTGTCGGTAAAGTAGGCGGACATG  < 1000
S  D  F  S  K  Q  D  W  E  A  Q  I  D  K  E  L  S  Q  N  Q  P  V  Y  Y  Q  G  V  G  K  V  G  G  H  A
         910       920       930       940       950       960       970       980       990
CCTTTGTTATCGATGGTGCTGACGGACGTAACTTCTACCATGTTAACTGGGGTTGGGGTGGAGTCTCTGACGGCTTCTTCCGTCTTGACGCACTAAACCC  < 1100
  F  V  I  D  G  A  D  G  R  N  F  Y  H  V  N  W  G  W  G  G  V  S  D  G  F  F  R  L  D  A  L  N  P
         1010      1020      1030      1040      1050      1060      1070      1080      1090
TTCAGCTCTTGGTACTGGTGGCGGCGCAGGCGGCTTCAACGGTTACCAAAGTGCTGTTGTAGGCATCAAACCTTAG  < 1176
 S  A  L  G  T  G  G  G  A  G  G  F  N  G  Y  Q  S  A  V  V  G  I  K  P  *
         1110      1120      1130      1140      1150      1160      1170
```

10.5. PSL sensitive to SpeB

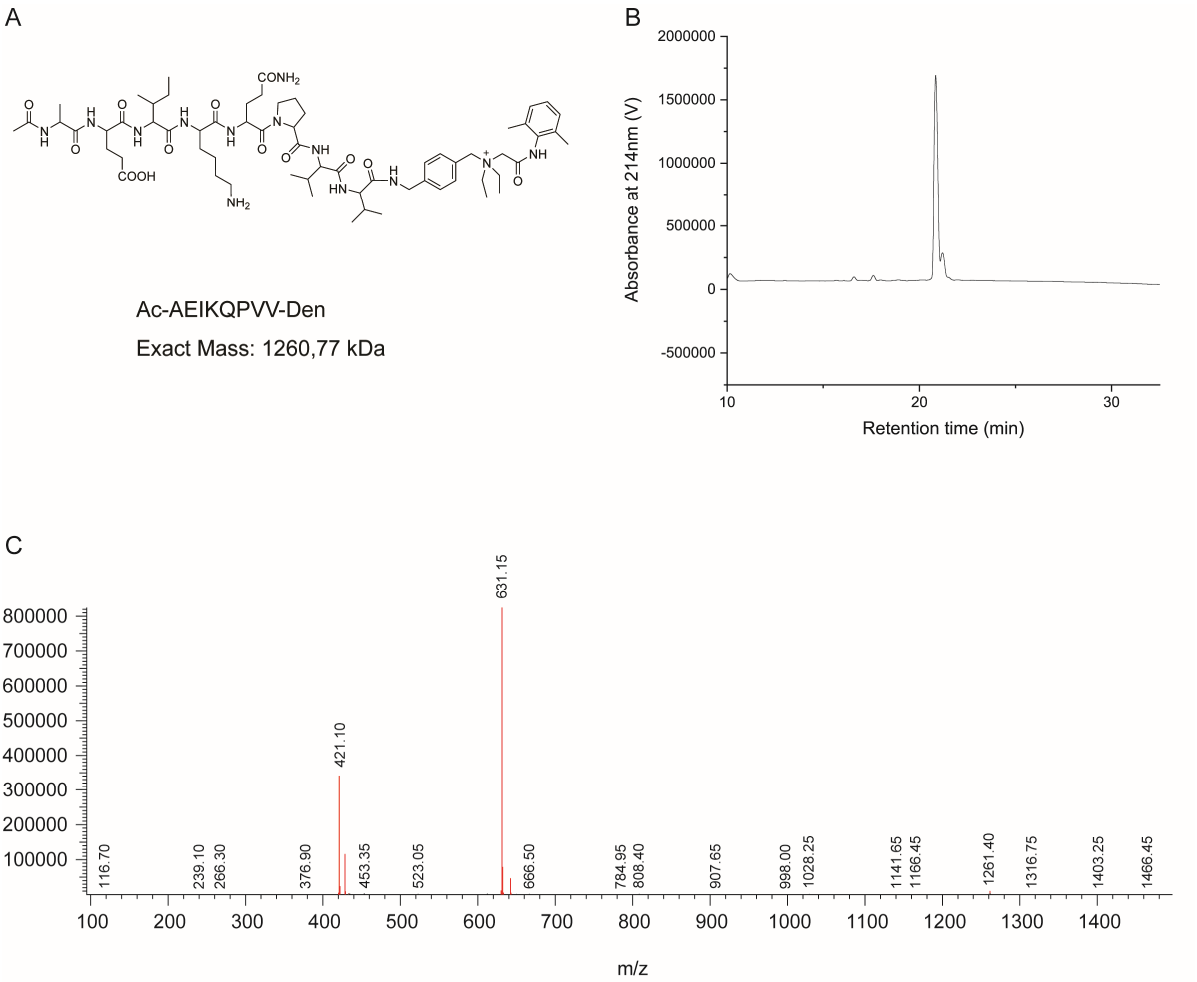

Figure 61. Structure, HPLC chromatogram and MS data of Ac-AEIKQPVV-Den. (**A**) Chemical structure with AAs in one letter code. (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 90%. (**C**) Positive ion mass spectrum. It is possible to identify the double charged mass of 631.15 m/z.

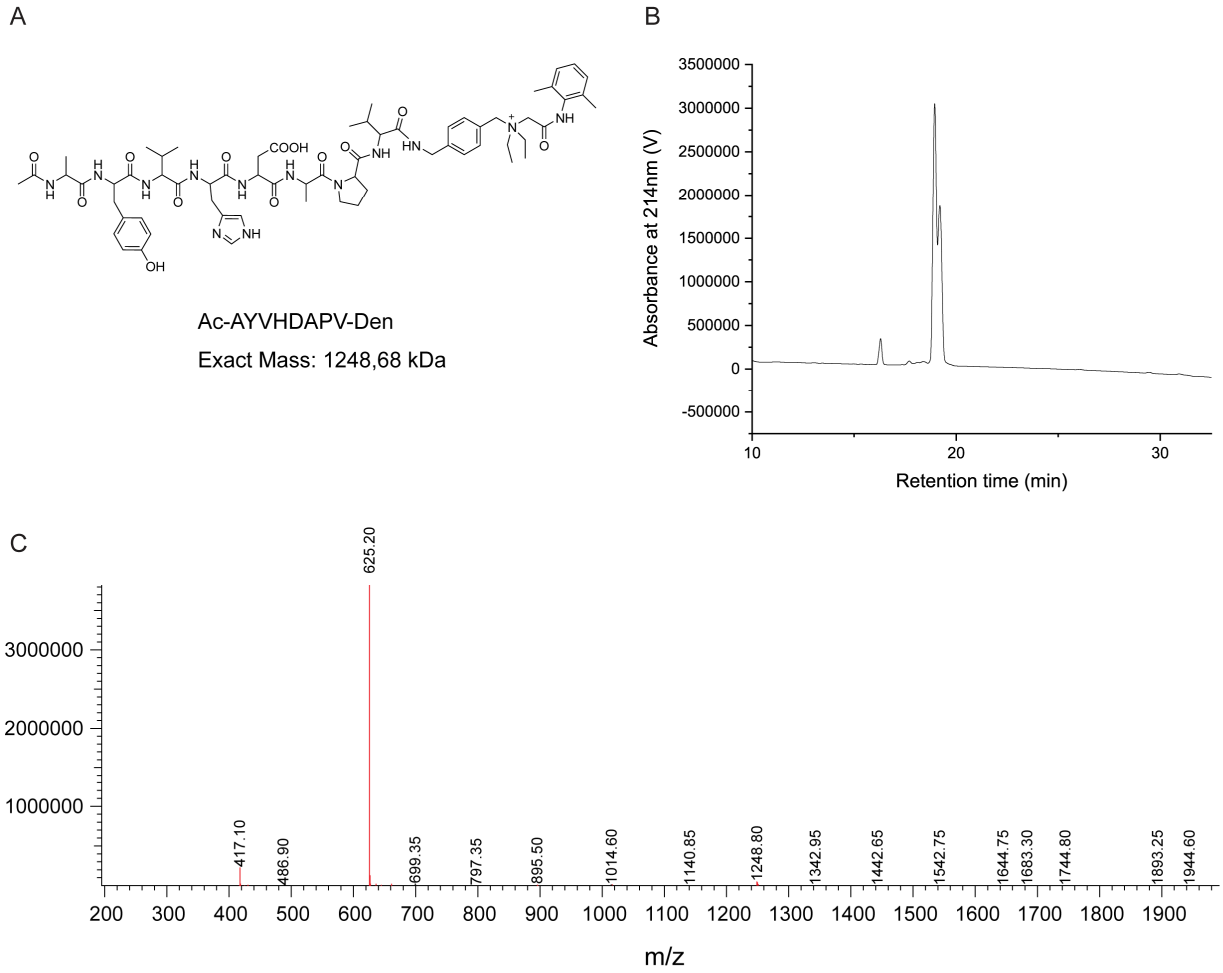

Figure 62. Structure, HPLC chromatogram and MS data of Ac-AYVHDAPV-Den. (**A**) Chemical structure with AAs in one letter code. (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 90%. (**C**) Positive ion mass spectrum. It is possible to identify the double charged mass of 625.20 m/z.

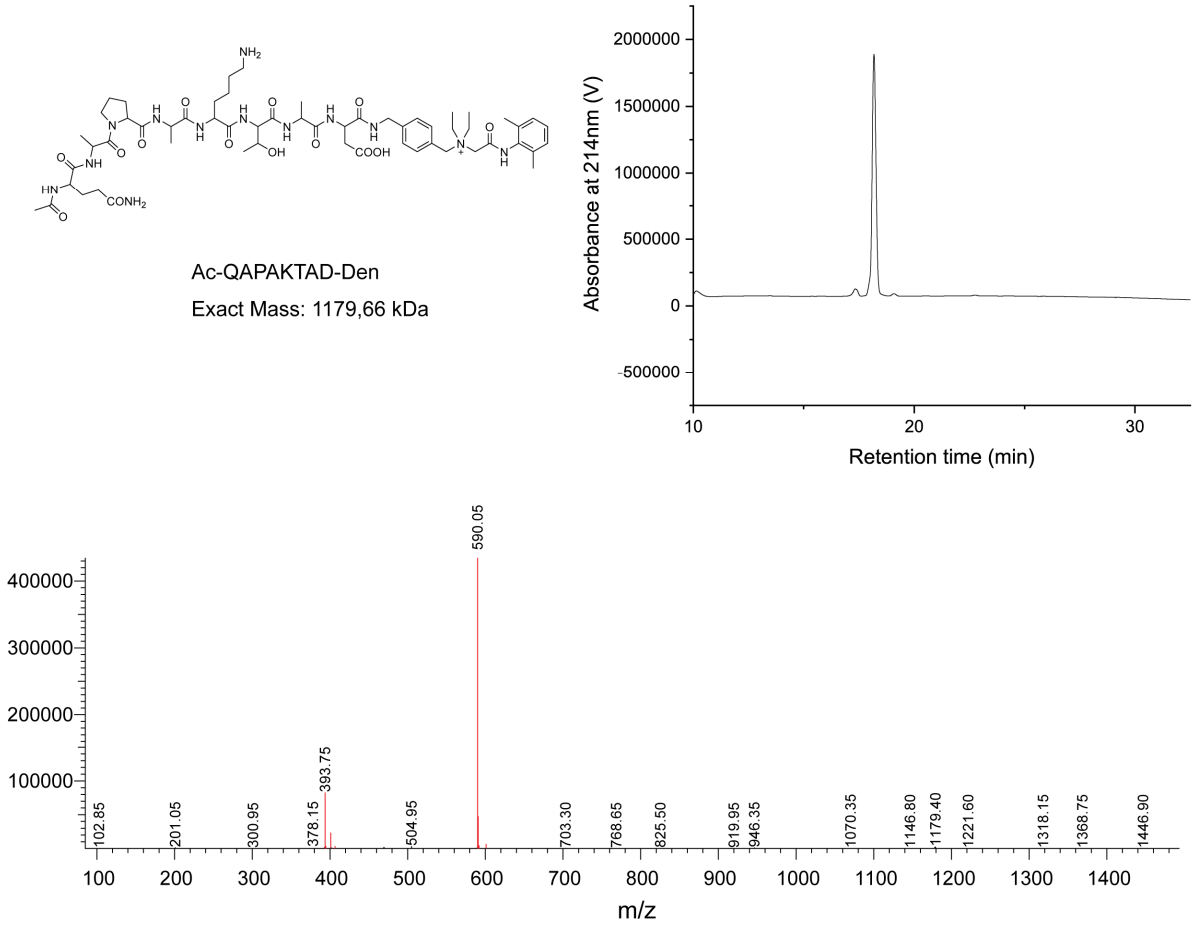

Figure 63. Structure, HPLC chromatogram and MS data of Ac-QAPAKTAD-Den. (**A**) Chemical structure with AAs in one letter code. (**B**) HPLC chromatogram of the synthesized and purified construct, certifying a purity of more than 90%. (**C**) Positive ion mass spectrum. It is possible to identify the double charged mass of 590.05 m/z.

10.6. Construct cleavage by SpeB

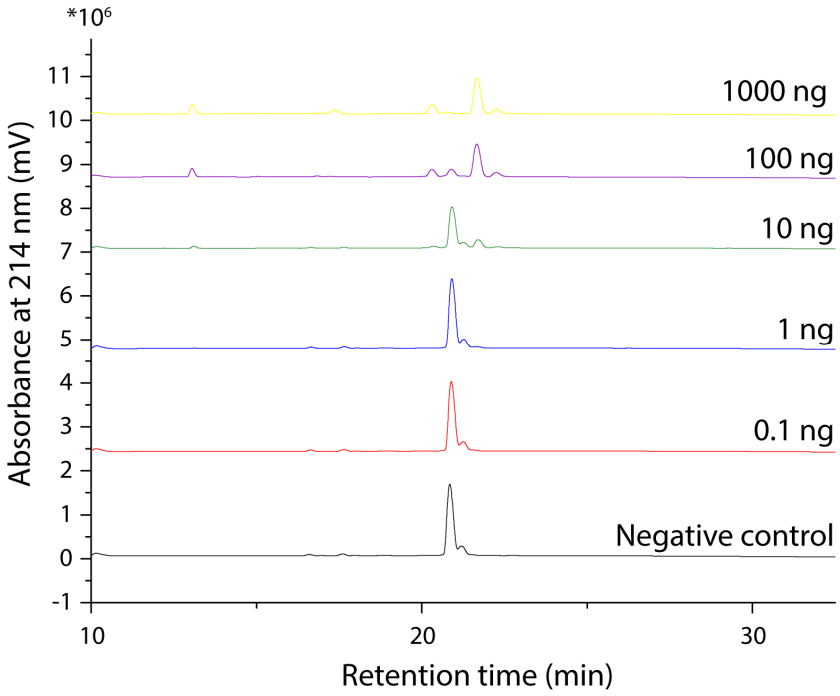

Figure 64. Overlay of HPLC chromatograms of Ac-AEIKQPVV-Den incubated with different concentrations of SpeB. A first cleavage is observable at a concentration of 10 ng per 50 µL. Using 100 ng and 1000 ng per 50 µL most of the construct was cleaved.

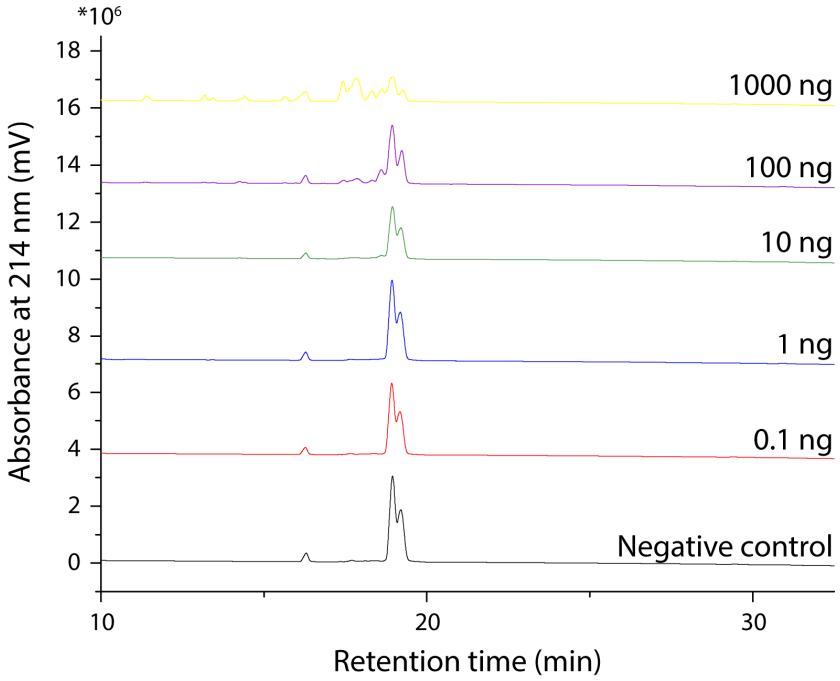

Figure 65. Overlay of HPLC chromatograms of Ac-AYVHDAPV-Den incubated with different concentrations of SpeB. A first cleavage is observable at a concentration of 10 ng per 50 µL. Using 100 ng per 50 µL, further cleavage can be detected.

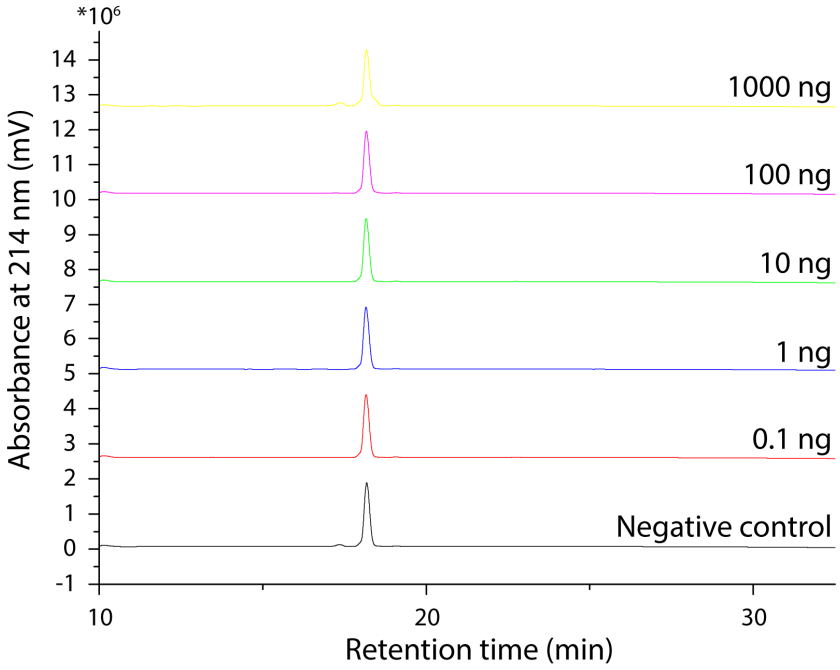

Figure 66. Overlay of HPLC chromatograms of Ac-QAPAKTAD-Den incubated with different concentrations of SpeB. No cleavage can be observed with the used concentrations of SpeB.

10.7. NonLinear Regression of time dependent MMPmix cleavage of Q→A

2 nM MMPmix

Equation: Exponential Rise to Maximum; Single, 2 Parameter

$$f = a*(1-\exp(-b*x))$$

Number of Observations = 10

Parameter Estimates:

	Coefficient	Std. Error	t	P
a	74.7475	3.3484	22.3237	<0.0001
b	0.0122	0.0013	9.4595	<0.0001

Global Goodness of Fit:

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9931	0.9862	0.9845	3.1387

Analysis of Variance:

	DF	SS	MS
Regression	2	11150.8769	5575.4384
Residual	8	78.8091	9.8511
Total	10	11229.6860	1122.9686

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	5638.6132	5638.6132	572.3818	<0.0001
Residual	8	78.8091	9.8511		
Total	9	5717.4223	635.2691		

4 nM MMPmix

Equation: Exponential Rise to Maximum; Single, 2 Parameter

$$f = a*(1-\exp(-b*x))$$

Number of Observations = 10

Parameter Estimates:

	Coefficient	Std. Error	t	P
a	92.6631	3.1471	29.4443	<0.0001
b	0.0405	0.0041	9.7612	<0.0001

Global Goodness of Fit:

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9936	0.9872	0.9855	4.6391

Analysis of Variance:

	DF	SS	MS
Regression	2	32376.3839	16188.1920
Residual	8	172.1733	21.5217
Total	10	32548.5572	3254.8557

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	13227.7644	13227.7644	614.6257	<0.0001
Residual	8	172.1733	21.5217		
Total	9	13399.9377	1488.8820		

10.8. List of Figures

APPENDIX

10.9. List of Schemes

10.10. List of Tables

10.11. INSERTION – PATENT 1

Applicant

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Inventors

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Classifications

IPC

A61K31/00; A61K49/00; C12Q1/04; C12Q1/34;

CPC

A61K49/0004 (EP,US); C07H5/06 (EP,US); C12Q1/04 (EP); C12Q1/34 (EP);

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Application

EP2017071572W·2017-08-28

Publication

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Published as

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US2019209710A1

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(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2019/0209710 A1**

MEINEL et al. (43) **Pub. Date: Jul. 11, 2019**

(54) **DIAGNOSTIC SENSOR AND CHEWING GUM COMPRISING SUCH A DIAGNOSTIC SENSOR FOR THE TASTE-BASED DETECTION OF VIRUSES**

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(73) Assignee: **Julius-Maximilians-Universitaet Wuerzburg**, Wuerzburg (DE)

(21) Appl. No.: **16/334,293**

(22) PCT Filed: **Aug. 28, 2017**

(86) PCT No.: **PCT/EP2017/071572**

§ 371 (c)(1),
(2) Date: **Mar. 18, 2019**

(30) **Foreign Application Priority Data**

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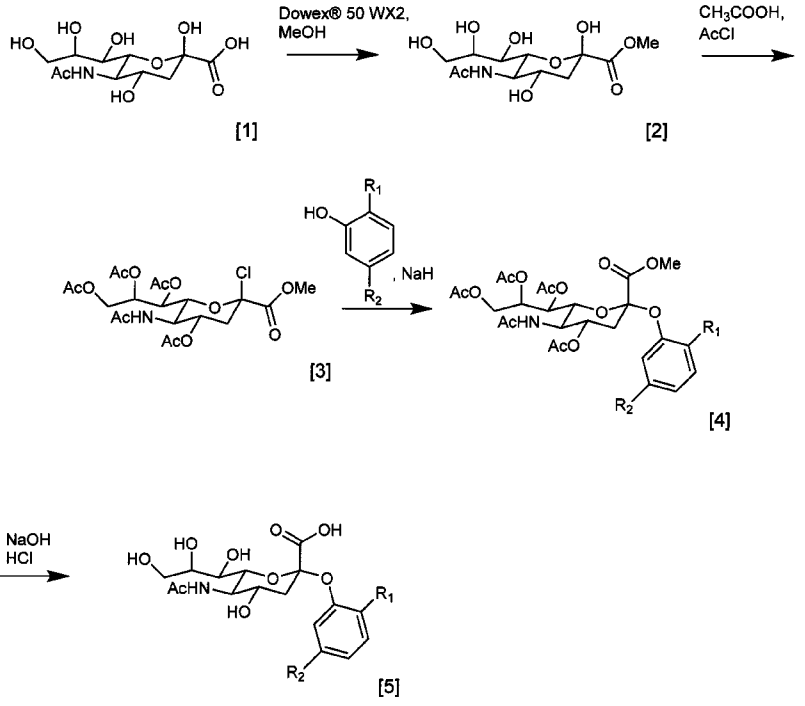
Publication Classification

(51) **Int. Cl.**
A61K 49/00 (2006.01)
C07H 5/06 (2006.01)

(52) **U.S. Cl.**
CPC ***A61K 49/0004*** (2013.01); ***C07H 5/06*** (2013.01)

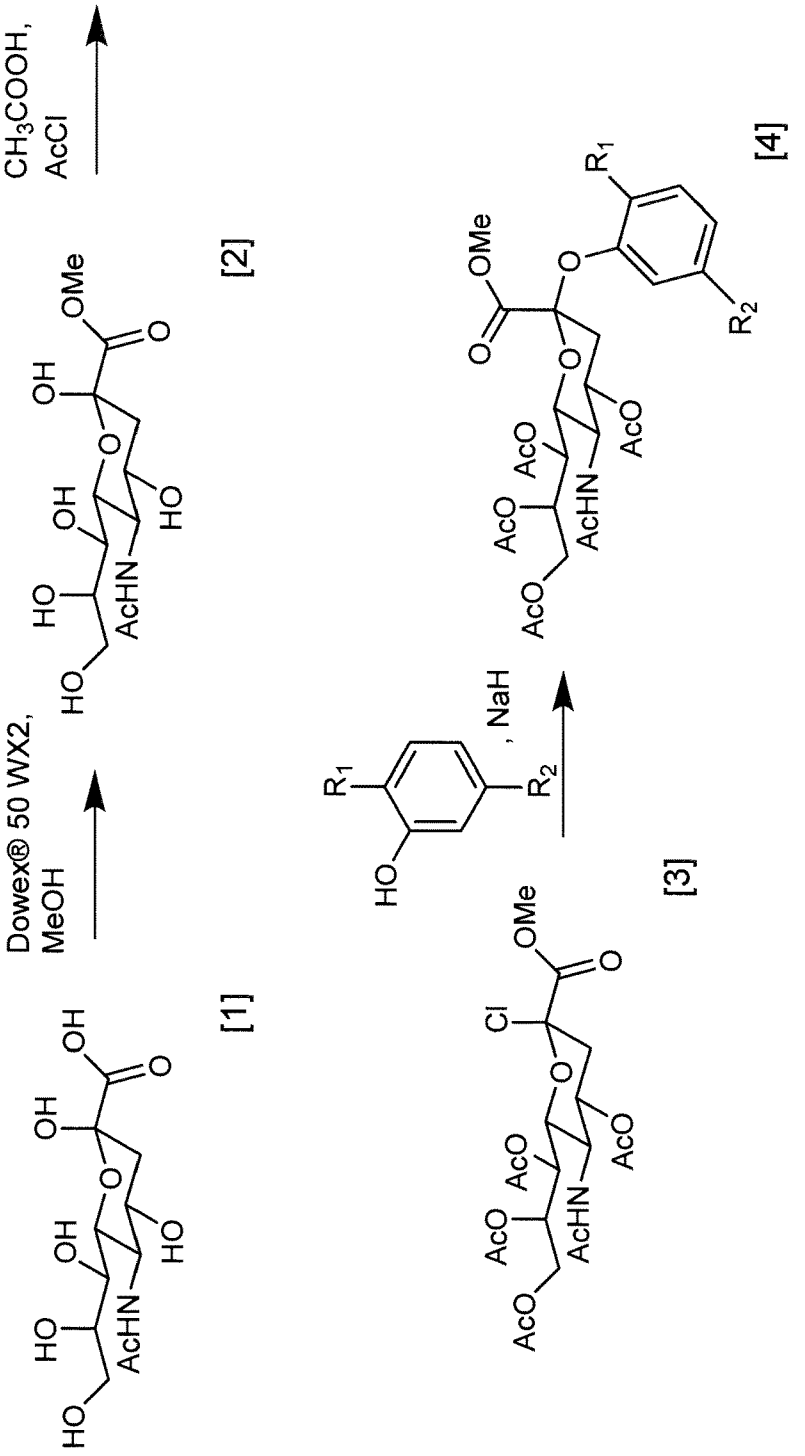
(57) **ABSTRACT**

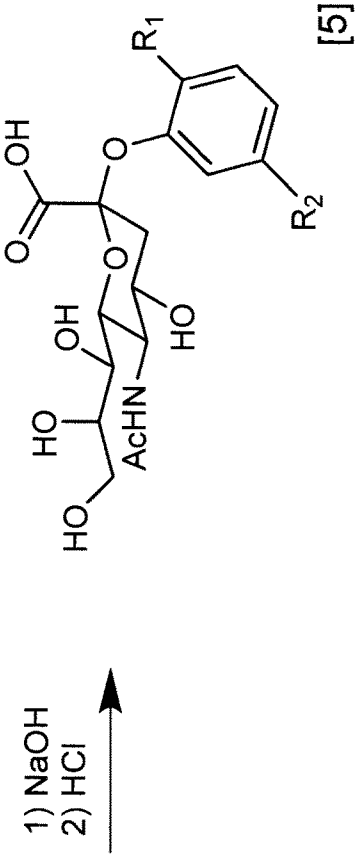
The subject matter of the invention is a diagnostic sensor that can be formulated into a diagnostic chewing gum that detects relevant concentrations of influenza viruses in the oral cavity. The technical application is the initial diagnosis by those affected themselves, e.g. sore throats manifesting in them. By the detection of the influenza viruses a selective therapy of the infection is possible and employment of antibiotics that only are effective with bacterial diseases can be reduced.



	R_1	R_2
[4a]	$C(CH_3)_2$	CH_3
[4b]	CH_3	$C(CH_3)_2$
[5a]	$C(CH_3)_2$	CH_3
[5b]	CH_3	$C(CH_3)_2$

Fig. 1a:





[5]

	R_1	R_2
[4a]	$C(CH_3)_2$	CH_3
[4b]	CH_3	$C(CH_3)_2$
[5a]	$C(CH_3)_2$	CH_3
[5b]	CH_3	$C(CH_3)_2$

Fig. 1b:

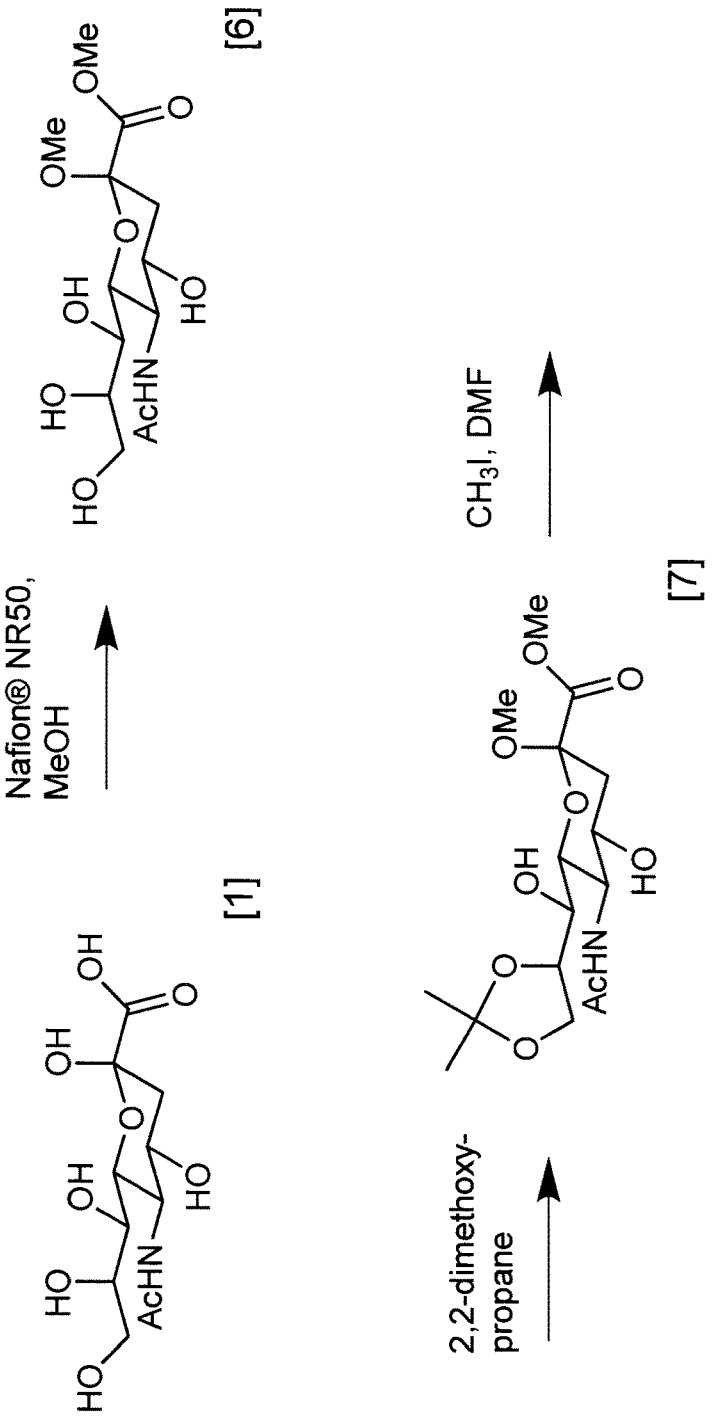

[1]
Nafion® NR50,
MeOH
[6]
CH3I, DMF
[7]
2,2-dimethoxy-
propane
OH
HO
AcHN
OMe
O

Fig. 2a:

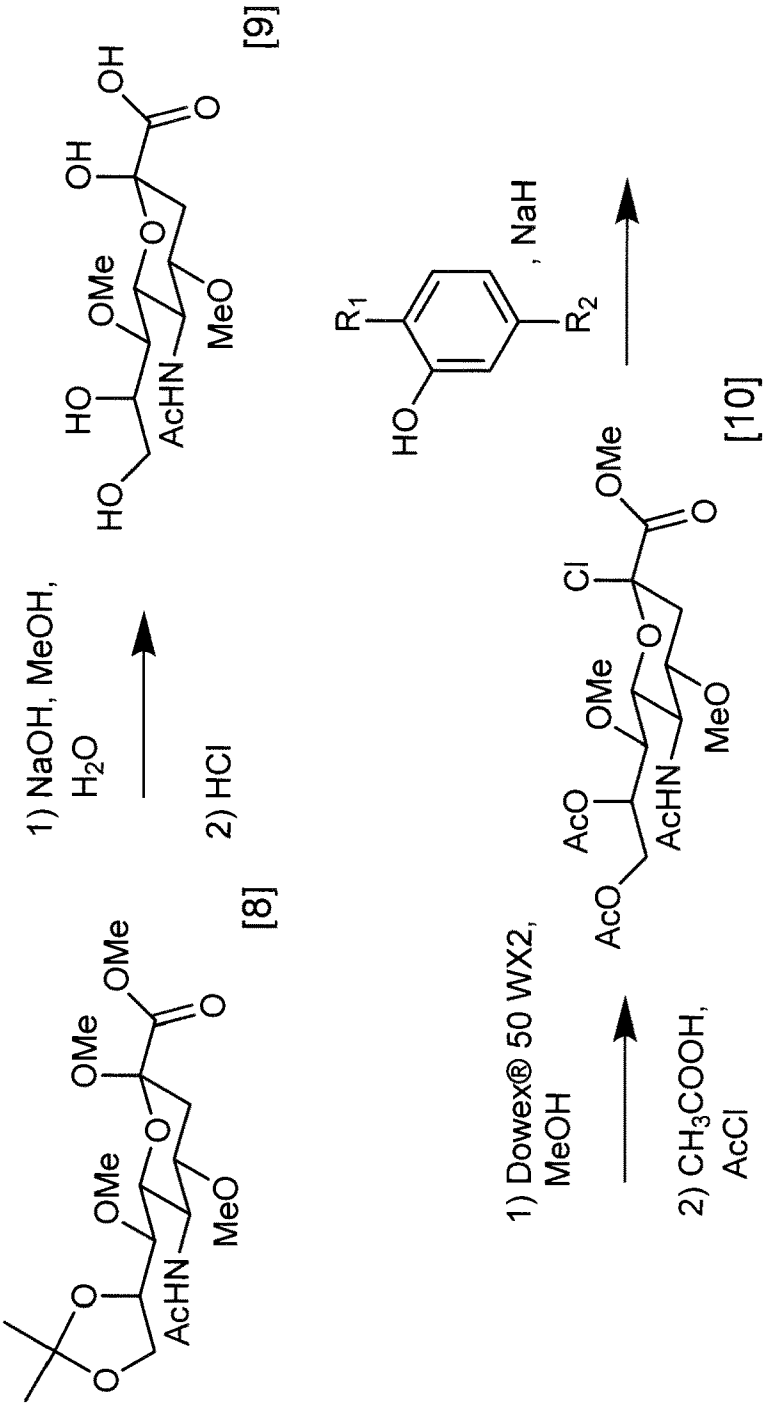
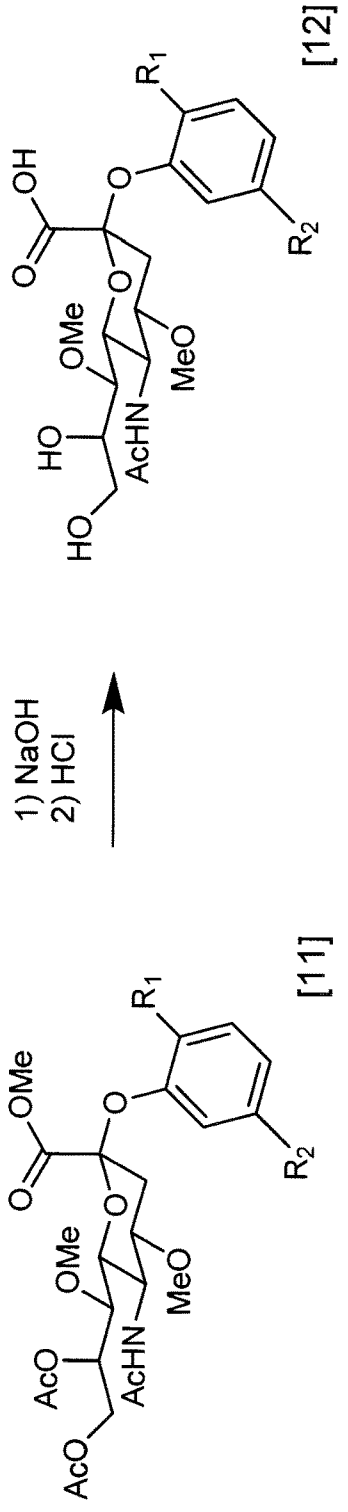

1) NaOH, MeOH, H2O
2) HCl
[8]
[9]
1) Dowex® 50 WX2, MeOH
2) CH3COOH, AcCl
, NaH
[10]
AcHN
MeO
OMe
AcO
Cl
HO
R1
R2

Fig. 2b:

Fig. 2c:



	R_1	R_2
[11a]	$C(CH_3)_2$	CH_3
[11b]	CH_3	$C(CH_3)_2$
[12a]	$C(CH_3)_2$	CH_3
[12b]	CH_3	$C(CH_3)_2$

DIAGNOSTIC SENSOR AND CHEWING GUM COMPRISING SUCH A DIAGNOSTIC SENSOR FOR THE TASTE-BASED DETECTION OF VIRUSES

BACKGROUND OF THE INVENTION

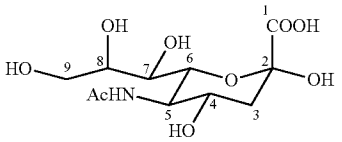
[0001] Viral infections cause a number of diseases that burden large parts of the population and the health care system every year to a considerable extent. Above all, infections with the influenza A and B viruses in this regard concern the general public in a large scale. According to the Robert Koch-Institut two to ten millions of people fall ill with influenza every year only in Germany, wherein deaths in 2013 are estimated at 20,600 (Robert Koch-Institut, Epidemiologisches Bulletin Nr. 3 of Jan. 19, 2015). However, the mortality rates are often even higher, since many people do not directly die of the infection, but of an insufficient healing and the weakening of the immune system. Because of the non-specific symptoms such as fever, aching head and limbs, cold, cough, nausea or vomiting it is often very difficult to correctly diagnose the disease. Moreover, frequent mutations of the virus stems contribute to the fact that virulence and intensity of the symptoms often and rapidly change.

[0002] A reliable option of diagnosis contributes to a rapid and specific detection of the infection with the virus. For that, antibody-based immunoassays are often applied that identify particular epitopes of the viruses by specific binding. Moreover, a PCR-based method is available with which characteristic regions in the genotype of the virus can be detected. Both methods are disadvantageous in that they are very expensive, time-consuming, and complicated in handling, so that they are only of a small effectiveness. Moreover, immunoassays are of disadvantage in that the surfaces of influenza viruses that are partially altered by frequent mutations are no longer recognized by the assays.

[0003] Enzymatically active proteins that are specific for viruses—these are located on their envelopes—that are essential for their replication may also be used to carry out a virus detection. In that way, the influenza virus expresses a virus-specific neuraminidase that is released outward.

[0004] In nature N-acetylneuraminic acid (Neu5Ac) [1] (here indicated with numbered C atoms) is present in the glycocalyx of higher organisms terminally coupled to glycan chains and is cleaved off therefrom by neuraminidase in the process of virus release.

[1]



[0005] Here, position 2 of [1] is the anomeric carbon atom on which the glycosidic bond to the sugar chains is. This bond is hydrolytically cleaved by neuraminidase in the course of the virus release. That is, it is logic to attach a compound that can be detected after cleavage at this site.

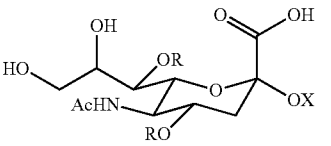
[0006] A method how the detection of the presence of influenza viruses can be carried out by means of the reaction of this enzyme is described in U.S. Pat. No. 5,252,458. To achieve a selective detection of only virus-related diseases the N-acetylneuraminic acid is slightly modified. In sialic acids position 4 plays an important role in the interaction between the enzyme and the substrate. It has been shown that 4-methoxy-Neu5Ac is not cut by bacterial sialidases at all, but very quickly by viral sialidases (Beau et al., Eur. J. Biochem., 106 (1980) 531-540). This differentiation is additionally enhanced by the alkylation of the hydroxyl group on position 7, so that a distinction can also be made between different virus neuraminidases. In U.S. Pat. No. 5,719,020 it is shown that 4,7-modified Neu5Ac bound to a chromogenic component is able to differentiate between influenza A and B and at the same time to differentiate bacterial from viral neuraminidases.

[0007] A similar sensor that releases a detectable dye when contacting neuraminic acid has already been marketed (ZymeTx, ZstatFlu™ Test for Influenza Types A and B). However, a disadvantage of this system is the complicated handling that like the other mentioned methods has to be performed by medical specialists. So far, only optically active systems have been connected to neuraminic acid for detection purposes.

[0008] Thus, the object of the present invention is to provide a means or method that enables the patients to detect a viral infection themselves and that enables the physician to simply differentiate a viral infection from a bacterial infection and thus, prevent the needless use of antibiotics.

[0009] According to the invention, this object is solved by a diagnostic sensor for the detection of viruses in the form of a compound of the following general formula [A]

[A]



wherein the residues R independent of each other represent hydrogen or C1-C6 alkyl, preferably methyl, and the residue —OX is derived from a flavoring agent or a dye having the general formula HOX, wherein OH is a hydroxyl group and X is an aliphatic, aromatic, or heterocyclic residue.

[0010] Further, the object is solved by a diagnostic chewing gum comprising the diagnostic sensor according to the invention.

[0011] Coupling a flavoring agent is a novelty. Based on the ZstatFlu™ test we have now extended the optical application of this system in that now recognition can be by sensation of taste or odor, respectively. That is, detection is not performed outside the human body, but directly in the oral cavity by using the human tongue (sense of taste) and the nose (sense of smell) as detectors, wherein the detection in particular is by the sense of taste. When the synthesized compound contacts the neuraminidase of one of these types of viruses a flavoring agent is released that now can be perceived gustatory or olfactory, respectively.

[0012] If a dye is coupled to N-acetylneuraminic acid or a derivative thereof instead of a flavoring agent, then the detection of the viruses can also be performed in the oral cavity in case of cleavage of the coupling product by neuraminidase on the basis of a discoloration of the human tongue, i.e. by optical perception.

[0013] The diagnostic sensor according to the invention is formulated in a chewing gum and thus, touches the saliva containing neuraminidase long enough to be able to react.

[0014] Thus, the patients are able to easily diagnose themselves whether the ambiguous symptoms such as e.g. "fever" come from a bacterial or a viral infection. Such information is of importance in the subsequent treatment strategy also for the physician. By an established diagnosis of the influenza that often is wrongly treated with antibiotics it is also possible to effectively reduce the prescription of antibiotics without relevant benefit.

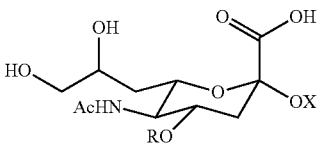
[0015] Additionally, the present invention relates to a method for the preparation of the diagnostic sensor and the diagnostic chewing gum according to the invention.

SUMMARY OF THE INVENTION

[0016] In summary, the present invention relates to:

[0017] [1] A diagnostic sensor for the detection of viruses in the form of a compound of the following general formula [A]

[A]

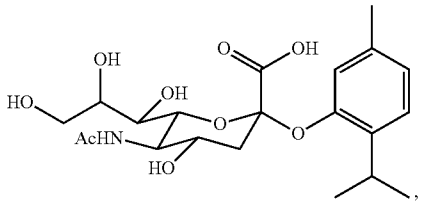


[0018] wherein the residues R independent of each other represent hydrogen or C1C6 alkyl, preferably methyl, and the residue —OX is derived from a flavoring agent having the general formula HOX, wherein OH is a hydroxyl group and X is an aliphatic, aromatic, or heterocyclic residue.

[0019] [2] A diagnostic sensor according to item [1], wherein the flavoring agent is a phenolic flavoring agent, preferably thymol or carvacrol.

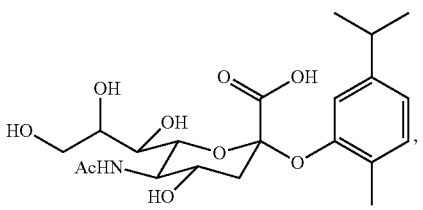
[0020] [3] A diagnostic sensor according to item [1] or [2], wherein the compound of formula A is Neu5Ac-thymol having the following formula [5a]

[5a]



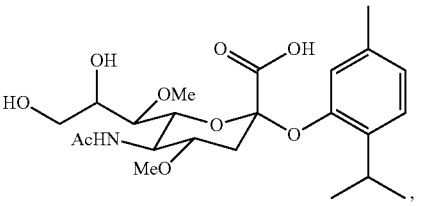
[0021] Neu5Ac-carvacrol having the following formula [5b]

[5b]



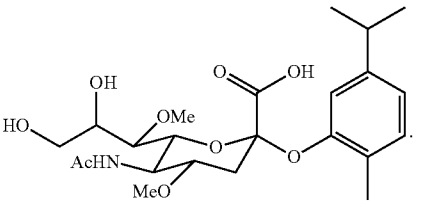
[0022] 4,7-di-O-methyl-Neu5Ac-thymol having the following formula [12a]

[12a]



[0023] or 4,7-di-O-methyl-Neu5Ac-carvacrol having the following formula [12b]

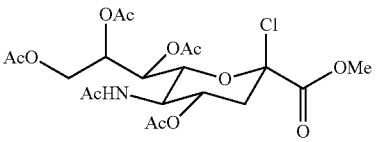
[12b]



[0024] [4] A method for the preparation of a diagnostic sensor according to any of items [1] to [3] comprising:

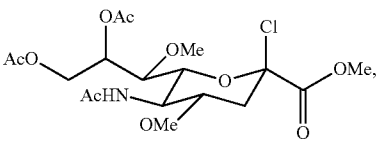
[0025] (a) coupling a flavoring agent having the general formula HOX, preferably a phenolic flavoring agent, particularly preferred thymol or carvacrol, with a compound of the following formula [3]

[3]



[0026] or a compound of the following formula [10]

[10]



[0027] (b) deprotecting the coupling product obtained in step (a).

[0028] [5] A method according to item [4], wherein the flavoring agent in step (a) is deprotonated by a base and coupling is by nucleophilic substitution.

[0029] [6] A diagnostic chewing gum comprising the diagnostic sensor according to one of items [1] to [3].

[0030] [7] A diagnostic chewing gum according to item [6] for use in a method for the detection of viruses in a human patient.

[0031] [8] A diagnostic chewing gum for use according to item [7], wherein the detection of the viruses is in the oral cavity of the patient by optical, gustatory or olfactory perception after cleavage of the compound according to formula A and release of the flavoring agent, wherein the cleavage of compound A is by viral neuraminidase contained in the saliva of the patient.

[0032] [9] A diagnostic chewing gum for use according to item [8], wherein the viruses are influenza viruses.

[0033] [10] A diagnostic chewing gum for use according to one of items [8] and [9], wherein the detection of the viruses is by the patients themselves by means of perception of taste.

[0034] [11] A method for the preparation of a diagnostic chewing gum according to one of items [6] to [10], wherein the method comprises formulating a chewing gum with a diagnostic sensor as defined in one of items [1] to [3] or as obtained according to a method as defined in items [4] and [5].

DETAILED DESCRIPTION OF THE INVENTION

[0035] As used in the context of the present invention, a "flavoring agent" is a substance that causes a taste-based (gustatory) or smell-based (olfactory) perception upon release in the oral cavity of a person, preferably a patient with a virus infection. Especially, thereby a taste-based perception occurs, so that the flavoring agent in particular is a tastant. The flavoring agents used in the present invention have the suitability to be used in foodstuff.

[0036] The flavoring agents used in the present invention are flavoring agents having the general formula HOX, wherein OH is a hydroxyl group and X is an aliphatic, aromatic, or heterocyclic residue.

[0037] The flavoring agents that can be used in the present invention are for example and not exhaustive acetovanillone, [alpha]-amylcinnamyl alcohol, anisyl alcohol, carveol, 4-carvomenthenol, carvacrol, cinnamyl alcohol, citronellol, dihydrocarveol, [alpha], [alpha]-dimethylphenethyl alcohol, dimethylbenzylcarbinol, 4-ethyl guaiacol, farnesol, fenchyl alcohol, 1,3,3-trimethyl-2-norbornanol, guaiacol, hydroxycitronellol, [alpha]-isobutyl phenethyl alcohol, isoeugenol, cuminyl alcohol, isopulegol, menthadienol, 2-methoxy-4-methylphenol, 2-methoxy-4-vinylphenol, methyl-5heptene-2-ol, nerol, nerolidol, 2,6-nonadiene-1-ol, phenethyl alcohol, 4-phenyl-2butanol, 4-phenyl-3-butene-2-ol, 1-phenyl-3-methyl-3-pentanol, 1-phenyl-1-propanol, 3-phenyl-1-propanol, pinocarveol, propenylguaethol, [alpha]-propylphenethyl alcohol, l-citronellol, [alpha]-terpineol, [beta]-terpineol, thymol, or verbenol.

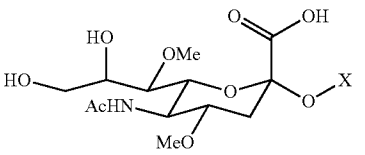
[0038] Phenolic flavoring agents are preferred, carvacrol and thymol are particularly preferred.

[0039] In the present invention, for sake of simplicity, coupling products derived from Nacetylneuraminic acid, or a derivative thereof, are referred to as "Neu5Ac flavoring agent" or "derivative of Neu5Ac flavoring agent". That is, a coupling product for example derived from N-acetylneuraminic acid (Neu5Ac) and thymol, in the present invention is referred to as "Neu5Ac thymol".

[0040] The invention shows a synthesis route with which N-acetylneuraminic acid can be coupled to various flavoring agents in a quantitative yield. In combination with Nacetylneuraminic acid or its 4,7-C1-C6 alkylated derivative the flavoring agent is not or hardly gustatory perceivable by the patient. However, if the system contacts viral neuraminidase the compound is cleaved and Neu5Ac (or 4,7-(C1-C6) alkoxy-Neu5Ac) and the flavoring agent in its alcoholic OH form are formed. Now, the free flavoring agent again is able to cause gustatory and olfactory stimuli to a significant extent. Thus, a perception by the patient becomes possible, so that by the appearance of taste and smell of the selected coupled compound or by a discoloration of the tongue an influenza caused by influenza viruses can be detected.

[0041] The structure of the system that was synthesized by way of example can be characterized by the following formula [B]:

[B]



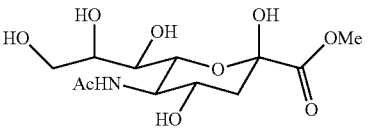
wherein —OX is derived from thymol or carvacrol. Thymol or carvacrol upon contact with the enzyme neuraminidase are cleaved off from 4,7-dimethoxy-N-acetyl-neuraminic acid and cause in the free form HO—X a sensation of taste or smell. Here, the 2-ketoside is coupled to the flavoring agent in the β-anomeric confirmation.

[0042] The compounds thymol and carvacrol are phenolic systems that sufficiently stabilize the deprotonated hydroxyl group that is required in the coupling for the nucleophilic attack on the anomeric C2 of the Neu5Ac. By our synthesis strategy it is now possible to couple the mentioned compounds to Neu5Ac or its derivatives.

[0043] N-acetylneuraminic acid bought from CarboSynth (Compton—Berkshire—UK) served as the starting material.

[0044] In the first step of the synthesis this is converted to the methyl ester. Thereby, the carboxy group in position 1 is methylated by treatment with methanol under conditions of an acidic catalysis to form product [2].

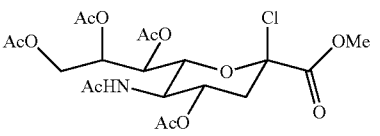
[2]



[0045] In this step yields of 80-85% are obtained.

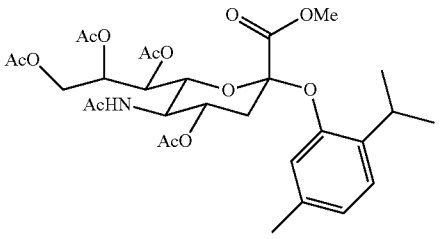
[0046] Subsequently, the methyl ester is treated in acetic acid with acetylchloride (AcCl), whereby on the one hand free hydroxyl groups are acetylated, but on the other hand the hydroxyl group in position 2 is converted to a chloride due to its particular reactivity as a hemiacetal. Due to the reactivity and instability processing of the obtained product [3] of this reaction is continued immediately.

[3]



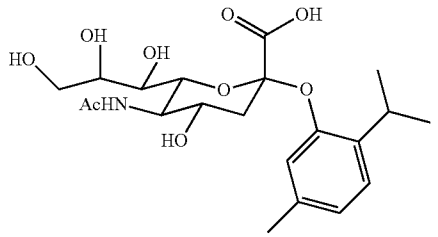
[0047] Now, the compound to be coupled is deprotonated with sodium hydride (NaH), so that it can nucleophilically attack on the activated C2 of the compound. In the absence of atmospheric oxygen (with a protective gas N_2) now the coupling reaction of N-acetyl-neuraminic acid and the selected flavoring agent can proceed. Under these conditions, coupling with thymol resulted in yields of ca. 30%. Thereby, the following product [4a] was formed:

[4a]



[0048] After cleavage of the protective groups in positions 4, 7, 8, 9 by alkaline treatment of the compound, e.g. by aqueous NaOH, and saponification of the methyl ester the following final product [5a] is formed:

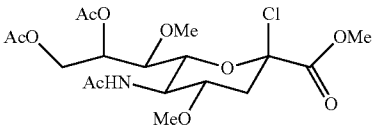
[5a]



[0049] In the present case, Neu5Ac was coupled to thymol. A further possible coupling component is the isomer of the thymol carvacrol that also is well perceptible in taste and in which the isopropylidene group and the methyl group are exchanged compared to thymol. Because in this the steric hindrance is lower, but otherwise reactivity is similar, this compound can also be coupled with the synthesis scheme used with thymol. The synthesis strategy of Neu5Ac-thymol and Neu5Ac-carvacrol is summarized in FIG. **1**.

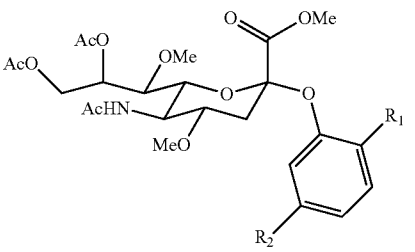
[0050] In the coupling of the flavoring agent with the 4,7-di-O-methylated derivative first the methyl groups have to be inserted at the Neu5Ac before both components can be coupled. In U.S. Pat. No. 6,303,764 B1 it is dealt in detail with the synthesis of a compound suitable for coupling and it is carried out as described there. Only the last step, the coupling with the now 4,7-di-O-methylated Neu5Ac, is changed, so that our compounds can be coupled. The synthesis strategy is schematically set forth in FIG. **2**, wherein component [10] described in the publications is coupled with the flavoring agents we used. In previous publications and patents the unchanged and mono- and di-methylated Neu5Ac has been coupled to a number of fluorogenic and chromogenic components. There were no significant differences among the various Neu5Ac derivatives as to the reactivity in the coupling. In order to achieve a selective coupling the Koenigs-Knorr method is applied to the di-methylated compound. Here, the phenolic group of the flavoring agent is coupled to the Neu5Ac derivative by means of silver carbonate. Thereby, first a glycosyl chloride [10] is formed by reaction with acetyl chloride as with the non-methylated variation, wherein at the same time the remaining hydroxyl groups are again provided with acetyl protective groups.

[10]



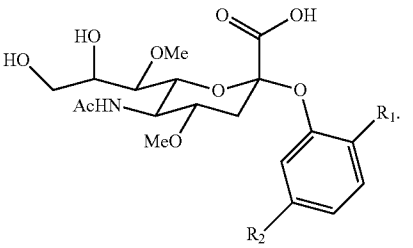
[0051] By reacting the glycosyl chloride with silver carbonate a dioxolanium ion is finally formed that can be attacked by an alcoholic group. Thereby, the following product [11] is formed:

[11]



[0052] By deprotection with catalytic amounts of sodium methanolate in methanol the non-protected final product [12] is formed:

[12]



[0053] Thus, we request patent protection not only for compounds Neu5Ac-thymol [5a] and Neu5Ac-carvacrol [5b], but also for compounds 4,7-di-O-methyl-Neu5Ac-thymol [12a] and 4,7-di-O-methyl-Neu5Ac-carvacrol [12b] derived therefrom.

[0054] The invention is illustrated by the following examples.

Example 1: Synthesis of Neu5Ac-methyl ester [2]

[0055] 1.563 g of Neu5Ac [1] (CarboSynth) are dispersed in 50 ml of anhydrous methanol. 2 g of Dowex 50 W×2 (washed with dry methanol) are added and the batch is stirred at room temperature for 5 hours. Thereafter, the ion exchanger is filtered off and washed several times with

methanol. After having removed the methanol by distillation product [2] is purified with silica gel (eluent:methanol) to obtain 1.354 g (83%) of [2].

Example 2: Synthesis of Neu5Ac-thymol [5a]

[0056] Acetyl chloride (10 ml) is added to a solution of the methyl ester of Neu5Ac [2] (603 mg, 1.95 mmol) in acetic acid (10 ml) and the batch is left sealed at room temperature for 18 hrs under stirring. Subsequently, the solution is evaporated under vacuum and dried by adding toluene to obtain [3] as a white residue. The product is used without further purification due to the instability of the chloride.

[0057] A solution of thymol (510 mg, 3.40 mmol) in dimethylformamide (DMF) (10 ml) is slowly added dropwise to a suspension of NaH (60% in mineral oil, 82 mg, 3.429 mmol) in tetrahydrofurane (THF) at 0° C. and the reaction mixture is stirred for 10 min. To the blend is added the solution of the chloride [3] in THF within 15 minutes. After complete addition the mixture is stirred for 19 hours at room temperature. Thereafter, the mixture is diluted with 40 ml of ethyl acetate and transferred to a separatory funnel. Now, the organic phase is washed with water (2×30 ml) and saturated sodium chloride solution (2×30 ml), dried over Na_2SO_4, and the solvent is removed under reduced pressure.

[0058] The residue (containing [4a]) is dissolved in methanol (8 ml), 0.1M of NaOH (2 ml) is added, and stirred for one hour at room temperature. The reaction batch is brought to pH=4.5-5 with 3M HCl and extracted with dichloromethane (DCM) (3×15 ml). The aqueous phase is purified with a FPLC system (Äkta purifier, GE Healthcare) using reversed phase chromatography (RPC) on a C18 column (Phenomenex®) (eluent A: 0.1% trifluoroacetic acid (TFA) in water, eluent B: 0.1% TFA in acetonitrile). Subsequent freeze drying results in N-acetylneuraminic acid-thymol [5a] as a white powder. The total yield is 253 mg (29.4%).

Example 3: Synthesis of 4,7-di-O-methyl-Neu5Ac-thymol [12a]

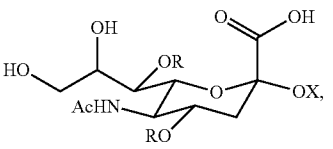
[0059] Acetyl chloride (3 ml) is added to a solution of the methyl ester of 4,7-di-O-methyl-Neu5Ac [9a] (33 mg, 0.094 mmol) in acetic acid (3 ml) and the batch is left sealed at room temperature for 18 hrs under stirring. Subsequently, the solution is evaporated under vacuum and dried by adding toluene to obtain [3] as a white residue. The product is used without any further purification due to the instability of the chloride.

[0060] The beta-silyl chloride [10a] is dissolved in 10 ml of anhydrous DCM. In a further flask, 586 mg of thymol (3.9 mmol), 95 mg of silver carbonate (0.345 mmol), and 300 mg of molecular sieve 4 Å are dissolved in 10 ml anhydrous DCM in the absence of light and oxygen. The first solution with the educt is slowly added to the second one and stirred for 72 h at room temperature.

[0061] Thereafter, the mixture is diluted with 40 ml of ethyl acetate and transferred to a separatory funnel. Now, the organic phase is washed with water (2×30 ml) and saturated sodium chloride solution (2×30 ml), dried over Na_2SO_4, and the solvent is removed under reduced pressure. The residue (containing [11a]) is dissolved in methanol (8 ml), 0.1M sodium methanolate is added up to a pH of 8-9, and stirred for one hour at room temperature. The reaction batch is neutralized with 1M HCl, filtered, and extracted with dichloromethane (DCM) (3×15 ml). The aqueous phase is purified with a FPLC system (Äkta purifier, GE Healthcare) using reversed phase chromatography (RPC) on a C18 column (Phenomenex®) (eluent A: 0.1% trifluoroacetic acid (TFA) in water, eluent B: 0.1% TFA in acetonitrile). Subsequent freeze drying results in 4,7-di-O-methyl-Neu5Ac-thymol [12a] as a white powder. The total yield is 10.2 mg (23.1%).

1. A diagnostic sensor for the detection of viruses in the form of a compound of the following general formula [A]

[A]



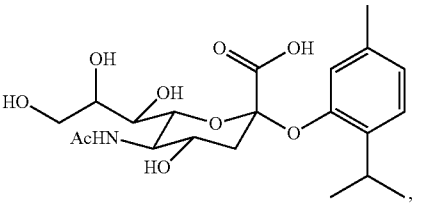
wherein each R residues is independently a hydrogen or a C1-C6 alkyl, and the residue —OX is derived from a flavoring agent having the general formula HOX, wherein OH is a hydroxyl group and X is an aliphatic, aromatic, or heterocyclic residue.

2. The diagnostic sensor according to claim **1**, wherein the flavoring agent is a phenolic flavoring agent.

3. The diagnostic sensor according to claim **1**, wherein the compound of formula A is selected from the group consisting of:

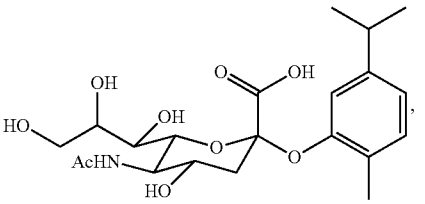
Neu5Ac-thymol having the following formula [5a]

[5a]



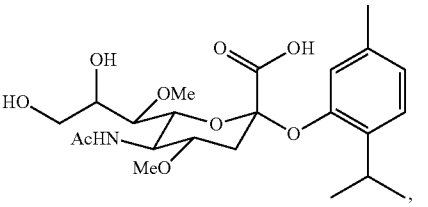
Neu5Ac-carvacrol having the following formula [5b]

[5b]



4,7-di-O-methyl-Neu5Ac-thymol having the following formula [12a]

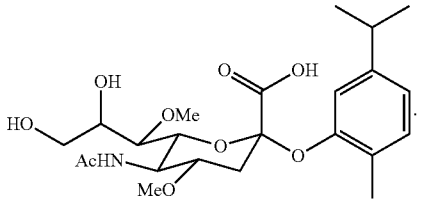
[12a]



and

4,7-di-O-methyl-Neu5Ac-carvacrol having the following formula [12b]

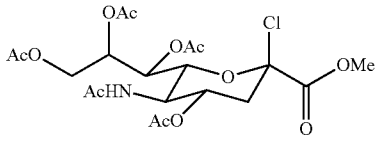
[12b]



4. A method for the preparation of a diagnostic sensor according to claim **1**, said method comprising the steps of:

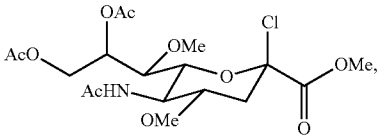
(a) coupling a flavoring agent having the general formula HOX with a compound of the following formula [3]

[3]



or a compound of the following formula [10]

[10]



and

(b) deprotecting the coupling product obtained in step (a).

5. A diagnostic chewing gum comprising the diagnostic sensor according to claim **1**.

6. A method for the detection of viruses in a human patient comprising the step of administering a diagnostic chewing gum comprising the diagnostic sensor according to claim **1** to said patient and using said diagnostic sensor to detect the presence of viruses in said patient.

7. The method according to claim **6**, wherein the viruses are detected in the oral cavity of the patient by gustatory or olfactory perception after cleavage of the compound according to formula A and release of the flavoring agent, wherein the cleavage of compound A is by viral neuraminidase contained in the saliva of the patient.

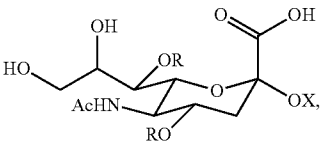
8. The method according to claim **7**, wherein the viruses are influenza viruses.

9. The method according to claim **8**, wherein the detection of the viruses is by the patients themselves by means of perception of taste.

10. A method for the preparation of a diagnostic chewing gum comprising the diagnostic sensor according to claim **1**, wherein the method comprises the step of formulating a chewing gum with a diagnostic sensor as defined in claim **1**.

11. A method for the preparation of a diagnostic chewing gum comprising a diagnostic sensor for the detection of viruses in the form of a compound of the following general formula [A]

[A]



wherein each R residue is independently a hydrogen or a C1-C6 alkyl, and the residue —OX is derived from a flavoring agent having the general formula HOX,

wherein OH is a hydroxyl group and X is an aliphatic, aromatic, or heterocyclic residue,

wherein the method comprises the step of formulating a chewing gum with a diagnostic sensor obtained according to a method as defined in claim **4**.

12. The diagnostic sensor according to claim **1**, wherein each R is methyl.

13. The diagnostic sensor according to claim **2**, wherein the phenolic flavouring agent is either thymol or carvacrol.

14. The method according to claim **4**, wherein the flavoring agent having the general formula HOX is a phenolic flavouring agent.

15. The method according to claim **14**, wherein the phenolic flavouring agent is either thymol or carvacrol.

* * * * *

10.12. INSERTION – PATENT 2

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COMPOUND AND DIAGNOSTIC SYSTEM COMPRISING SAID COMPOUND FOR THE GUSTATORY DETECTION OF INFLAMMATIONS IN THE ORAL CAVITY

Background of the Invention

Inflammations in the oral cavity often occur in the form of swelling and redness, and can be extremely painful. Sometimes it comes to bleeding of the inflamed area. However, especially at an early stage, these inflammations often remain unrecognized by the patients or the severe consequences, if not treated, are underestimated.

If the gums (gingiva) are affected by an inflammation such as periodontitis and peri-implantitis, for example, this can lead to a loosening of the teeth followed by tooth loss in the longer term. Periodontal disease is a bacterial inflammation, which manifests itself in a largely irreversible destruction of the periodontium. It is associated with a loss of holding due to degradation of the periodontium. Peri-implantitis is a destructive inflammatory process affecting the soft and hard tissues surrounding dental implants. The soft tissues become inflamed whereas the alveolar bone (hard tissue), which surrounds the implant for the purposes of retention, is lost overtime. Pain when swallowing, increased salivation, bad breath and an unpleasant taste in the mouth are possible accompanying symptoms. Sometimes associated with fever.

Medical advice is often consulted only when the symptoms become manifest and it is too late for timely treatment. Sometimes, these inflammations are only diagnosed by chance, for example when a patient visits a dentist due to pain because of another condition such as tooth decay. Accordingly, there is always the risk that inflammations in the oral cavity are missed in patients who for some reason do not go to see a dentist on a regular basis.

Nevertheless, early detection and treatment of these diseases is particularly important as they are suspected of affecting the course of other diseases such as diabetes or chronic ischemic cardiovascular disease for the patient adversely or even cause other complications of the general health themselves.

It is therefore desired to find a reliable, specific, uncomplicated and rapid way of diagnosing these diseases at the earliest possible stage, preferably by the patient himself without having to consult a dentist. Suitable solutions include easy to use products for daily use. New rapid

tests based on bio-responsive systems reduce costs, time and complexity and can therefore be used for a first classification.

Bioresponsive systems react to changes in the environment with the release of substances. This principle is used on the one hand therapeutically, but can also be used in the diagnosis of diseases. In case of a disease otherwise stable parameters of the organism change, which is noticed by these systems and as a result of which an easily detectable indicator substance is released.

In this context, US. Pat. App. Pub. No. US 2011/0081673 discloses a diagnostic chewing gum for diabetes screening. The patent application teaches a diagnostic chewing gum for screening a medical condition such as diabetes due to a detectable change in color based on whether the patient is healthy or not. Thereby, the intensity of the color change may indicate a degree of seriousness of a medical condition or a degree of risk for a medical condition.

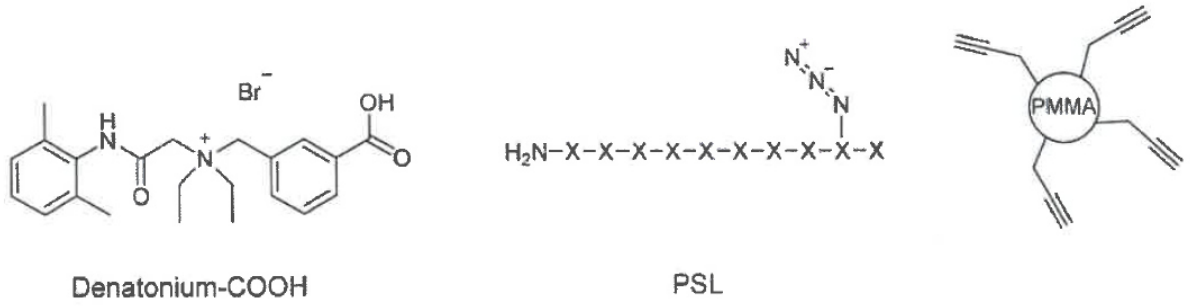
Another disclosure PCT Pat. Pub. No. WO 2013/131993 relates to a device for the diagnosis of inflammatory tissues in dental applications. Specifically, said disclosure teaches a diagnostic chewing gum for identifying the presence of inflammatory tissues in the mouth, in particular in or adjacent to the mandible, the maxilla, an implant or the teeth of a user. Also, US Pat. No. 9,526,803 provides an approach for the direct detection of pathogens (viruses, bacteria, fungi and combinations thereof) in the mouth and adjacent tissues. This is done by means of identifying the presence of the pathogens via the mouth with the chewing gum.

In both PCT Pat. Pub. No. WO 2013/131993 and US Pat. No. 9,526,803 a poly(methylmethacrylate) (PMMA) substrate and/or anchor for attachment of the flavoring or colorant substance via a linker is required.

A similar sensor for the diagnosis of periodontal disease and peri-implantitis is explained by Ritzer, J et al. (Nat. Commun. Diagnosing peri-implant disease using the tongue as a 24/7 detector. volume 8, Article number: 264 (2017)). Therein, it is reported on sensory chewing gums as a bioresponsive system for the detection of periodontal disease and peri-implantitis. The bioresponsive system contains peptide sensors consisting of a protease-sensitive peptide linker ((PSL), also referred to as protease cleavage linker (PCL)) between a bitter flavoring substance such as a denatonium compound and a PMMA microparticle.

Denatonium is the bitterest compound currently known. Due to its extremely bitter taste, it is used in cosmetics and household products as an additive to prevent inadvertent ingestion. A medical use of the bitter substance is the addition in nail polish against nail biting.

Scheme A is a graphical representation of the diagnostic system according to Ritzer, J et al. Therein, the flavoring compound (denatonium-COOH) is connected to the N-terminus of the PSL via the carboxyl group of the denatonium-COOH. Subsequently, the product is connected to the PMMA particle via the PSL's azide group.

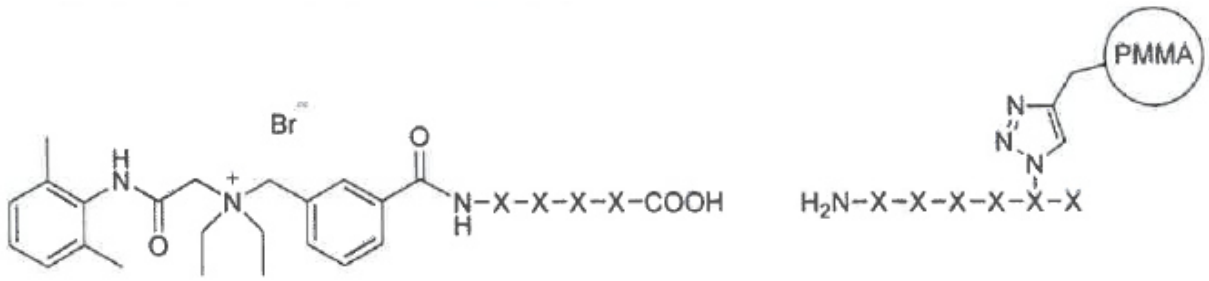


Denatonium-COOH PSL

[Scheme A]

In a healthy patient, the large (uncleaved) intact bioresponsive sensor is water insoluble and tasteless. If a disease is present, the sensor is specifically cleaved by disease-induced matrix metalloproteinases (MMP) in the patient saliva. This cleavage results in low molecular weight, water soluble and bitter substances. It is for this bitterness that the patient is alarmed by recognizing a strong taste. Comprised in a chewing gum, the system may be used for the diagnosis of e.g. periodontal disease and peri-implantitis.

When the bioresponsive system is incubated with MMP, it is cleaved quickly and specifically at a peptide function of the PSL into two segments with four remaining amino acids at the denatonium-COOH, as it is exemplified in Scheme B.



[Scheme B]

Subsequently also the other amino acids are cleaved off. Finally, only the denatonium-COOH remains, which can be detected by the tongue in low concentrations due to its bitterness.

So far, the denatonium-peptide constructs were coupled to PMMA particles in order to mask the bitter taste. This is because the known denatonium-peptide constructs were gustatory

detectable, unless linked to a PMMA particle. The PMMA particle prevented the gustatory detection due to its size and insolubility in water.

The inventors of the present invention have surprisingly found that the gustatory detection of the denatonium compound is already effectively blocked by a four-amino acid peptide, if the denatonium compound is linked to the C-terminus of the peptide via an amine function. Therefore, compared to the prior art, no PMMA particles are required anymore for the bioresponsive sensors. Accordingly, the inventive bioresponsive sensor can be prepared faster, cheaper and more easily. It was further found by the inventors that it is difficult to provide stable amino-functionalized denatonium derivatives that can be coupled to peptides via their C-terminal carboxyl group.

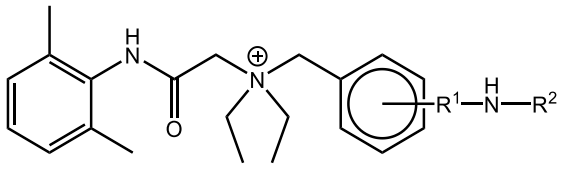
Modified denatonium compounds are provided that can be attached to the C-terminus of a protease-sensitive peptide so that a bioresponsive sensor can be prepared that enables the patient to diagnose inflammations in the oral cavity such as periodontal disease and/or peri-implantitis by himself.

Summary of the Invention

In summary, the present invention is directed to the following items [1] to [41]:

[1] A compound having the structure Den–R^1-NH-R^2, or a salt thereof, wherein
Den is denatonium,
R^1 is an optionally substituted C_{1-3} alkylene group
R^2 is a protease-sensitive peptide,
wherein the C-terminus of the protease-sensitive peptide forms an amide bond with the group -NH-.

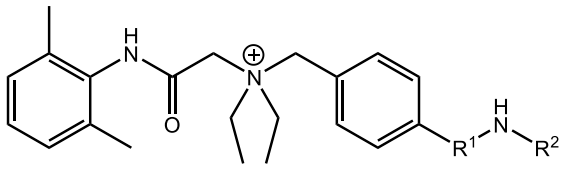
[2] The compound or salt of item [1] having the formula [I]:



[I]

wherein
R^1 and R^2 are as defined in item [1].

[3] The compound or salt of item [1] or [2], having the formula [Ia]:



[Ia]

wherein

R^1 and R^2 are as defined in item [1].

[4] The compound or salt of any one of items [1] to [3], wherein R^1 is $-CH_2-$.

[5] The compound or salt of any one of items [1] to [4], wherein the protease-sensitive peptide comprises or consists of at least 4 amino acids, preferably 4 to 15 amino acids, more preferably 5 to 12, even more preferably 6 to 10, yet even more preferably 7 to 9, most preferably 8 amino acids.

[6] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is selected from the group consisting of alanine, arginine, glutamine, leucine, methionine and phenylalanine.

[7] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is not alanine or valine.

[8] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is glutamine.

[9] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is alanine.

[10] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is arginine.

[11] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is tyrosine.

[12] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is glutamic acid.

[13] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is leucine.

[14] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is serine.

[15] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the protease-sensitive peptide is methionine.

[16] The compound or salt of any one of items [1] to [5], wherein the C-terminal amino acid of the peptide is phenylalanine.

[17] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide is not QPVV, DAPV or GPQGIAGA.

[18] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide is susceptible to cleavage by a matrix metalloproteinase.

[19] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide is susceptible to cleavage by a matrix metalloproteinase-8 or activated matrix metalloproteinase-8.

[20] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide is susceptible to cleavage by a pathogen-specific protease.

[21] The compound or salt of item [20], wherein the pathogen is a bacterial pathogen.

[21] The compound or salt of item [20], wherein the pathogen is a viral pathogen.

[22] The compound or salt of any one of the preceding items, wherein the protease is an endopeptidase

[23] The compound or salt of any one of the preceding items, wherein the compound is not susceptible to cleavage by an aminopeptidase.

[24] The compound or salt of any one of the preceding items, wherein the compound is not susceptible to cleavage by an exopeptidase.

[25] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide has a length of 5 to 20 amino acids.

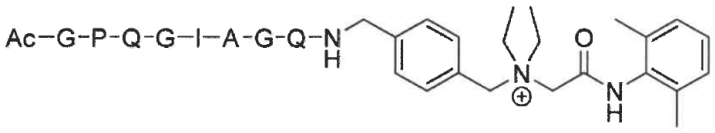
[26] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide has a length of 6 to 18 amino acids.

[27] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide has a length of 7 to 16 amino acids.

[28] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide has a length of 8 to 14 amino acids.

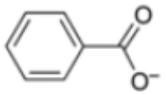
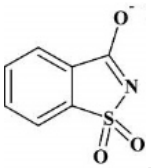
[29] The compound or salt of any one of the preceding items, wherein the protease-sensitive peptide has a length of 9 to 12 amino acids.

[30] The compound or salt of any of the preceding items, having the formula [BRS-1]:



[BRS-1].

[31] The salt of any one of the preceding items, further comprising a counter ion selected from the group consisting of

TFA^-, F^-, Cl^-, Br^-,  , and  .

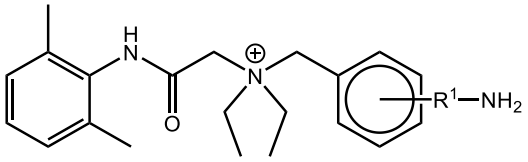
[32] A bioresponsive sensor comprising the compound or salt of any one of the preceding items.

[33] The compound or salt according to any one of items [1] to [31], or the bioresponsive sensor according to item [32] for use in a method of detecting an inflammation in the oral cavity of a human patient.

[34] The compound or salt for use according to item [33], or the bioresponsive sensor for use according to item [33], wherein the inflammation is detected by gustatory perception by the patient.

[35] The compound or salt for use according to item [33] or [34], or the bioresponsive sensor for use according to item [33] or [34], wherein the inflammation is detected upon cleavage of the compound, thereby releasing the denatonium.

[36] The compound or salt for use according to any one of items [33] to [35], or the bioresponsive sensor for use according to item [33] to [35], wherein, upon cleavage of the compound, a compound having the formula [II]:

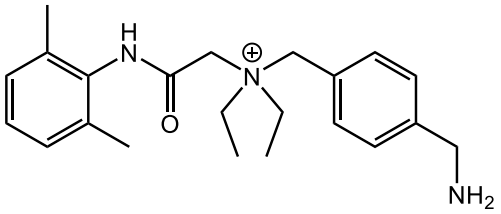


[II]

is released,

wherein R^1 is an optionally substituted C_{1-3} alkylene group.

[37] The compound or salt for use according to item [36], or the bioresponsive sensor for use according to item [36], wherein the compound of formula [II] is a compound having the formula [IIa-1]:



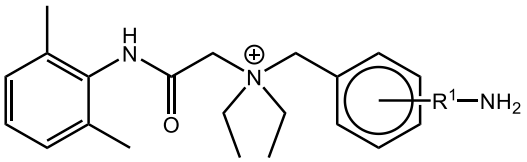
[IIa-1].

[38] A method for the preparation of the bioresponsive sensor, comprising the step of linking an amino-modified denatonium compound to the C-terminus of a protease-sensitive peptide, wherein the amino-modified denatonium compound is preferably a compound of formula [II] or formula [IIa-1].

[39] The method of item [38], further comprising, prior to the step of linking:
- preparing a protease-sensitive peptide by solid phase peptide synthesis, and/or
- protecting the N-terminus of the protease-sensitive peptide with a protecting group, preferably acetic anhydride.

[40] A diagnostic chewing gum or a diagnostic confectionary, comprising the compound or salt of any one of items [1] to [31].

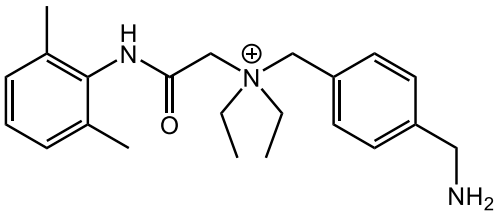
[41] A compound of formula [II]:



[II]

wherein R^1 is an optionally substituted C_{1-3} alkylene group;

with the proviso that the compound of formula [IIa-1]



[IIa-1]

is excluded.

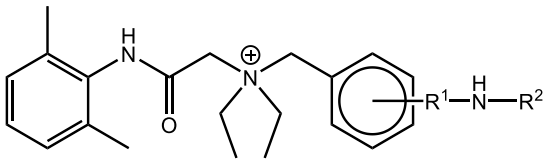
Description of the Figures

Figure 1 shows as graphical representation of the cleavage rate of [BRS-1] and [CC1] accompanied by the standard deviations.

Figure 2 is a graphical representation of the cleavage rate of [IIa-1] + AA of 16 of the 20 proteinogenic amino acids.

Detailed Description of the Invention

In a first aspect, the present invention relates to a compound comprising denatonium linked to the C-terminus of a protease-sensitive peptide; or a salt thereof. Preferably, the compound is a compound according to formula [I]:



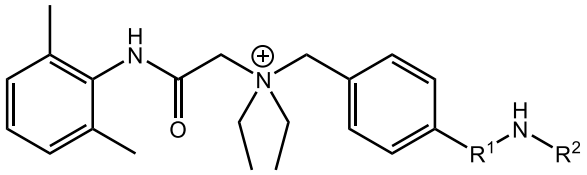
[I]

wherein

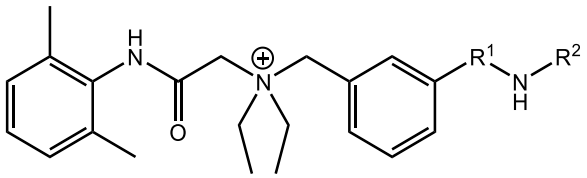
R^1 is an optionally substituted C_{1-3} alkylene group, and

R^2 is the protease-sensitive peptide; or a salt thereof. Preferably R^1 is an unsubstituted C_{1-3} alkylene group. More preferably R^1 is methylene, ethylene or propylene. Most preferably, R^1 is methylene.

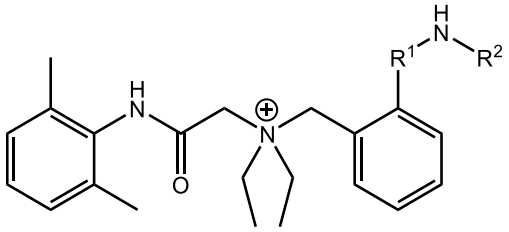
Preferred embodiments of the compound according to formula [I] are selected from the following group consisting of [Ia] to [Ic], wherein [Ia] is particularly preferred:



[Ia]



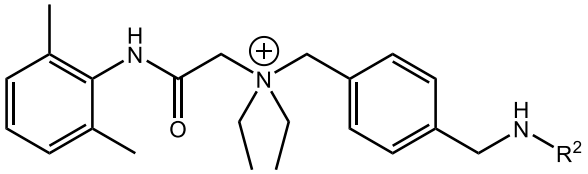
[Ib]



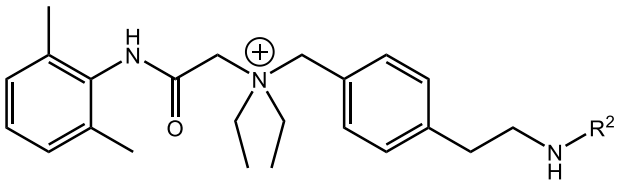
[Ic]

wherein R^1 and R^2 are as defined above.

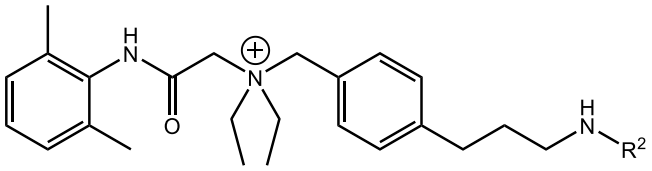
In one embodiment, the compound according to formula [Ia] is selected from the group consisting of [Ia-1] to [Ia-3], wherein formula [Ia-1] is preferred:



[Ia-1]



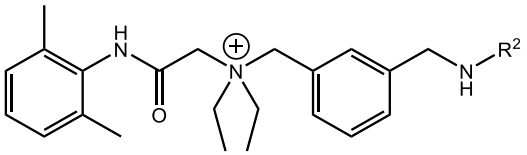
[Ia-2]



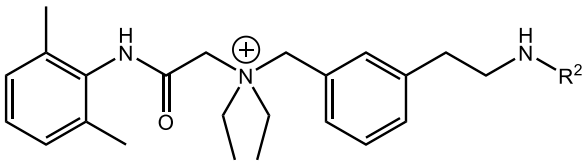
[Ia-3]

wherein R^2 is the protease-sensitive peptide.

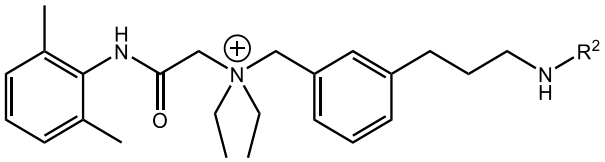
In another embodiment, the compound according to formula [Ib] is selected from the group consisting of [Ib-1] to [Ib-3]:



[Ib-1]



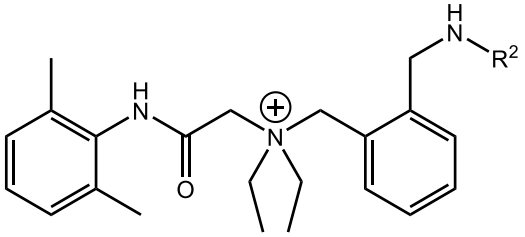
[Ib-2]



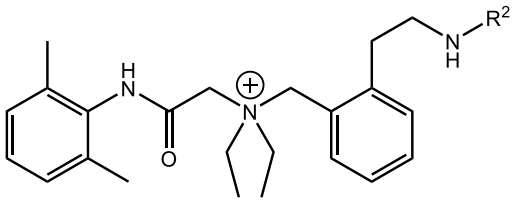
[Ib-3]

wherein R^2 is the protease-sensitive peptide.

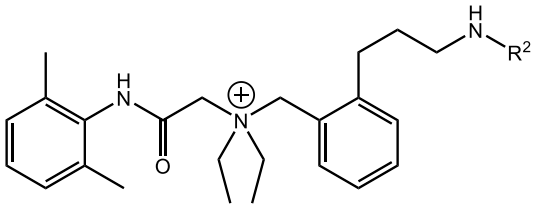
In another embodiment, the bioresponsive sensor according to formula [Ic] is selected from the group consisting of [Ic-1] to [Ic-3]:



[Ic-1]



[Ic-2]



[Ic-3]

wherein R^2 is the protease-sensitive peptide.

The amino acid sequence of the protease-sensitive peptide is not particularly limited as long as it is susceptible to cleavage by an endopeptidease. Preferably the protease-sensitive peptide has a length of at least 4 amino acids, preferably it has a length of 4 to 15, more preferably 5 to 12, even more preferably 6 to 10, yet even more preferably 7 to 9, most preferably 8 amino acids.

The amino acids of the protease-sensitive peptide are independently selected from the group consisting of alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y) and valine (V).

In one embodiment, the amino acids are independently selected from the group consisting of alanine (A), arginine (R), aspartic acid (D), glutamine (Q), glutamic acid (E), glycine (G), isoleucine (I), leucine (L), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y) and valine (V).

In another embodiment, the amino acids are independently selected from the group consisting of alanine (A), glutamine (Q), glycine (G), isoleucine (I), proline (P).

To obtain maximum gustatory perception, the amino acid directly attached to the nitrogen atom of the amino-modified denatonium, i.e. the C-terminal amino acid of the protease-sensitive peptide, is preferably selected from the group consisting of alanine (A), arginine (R), aspartic acid (D), glutamine (Q), glutamic acid (E), glycine (G), isoleucine (I), leucine (L), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y) and valine (V).

More preferably, the C-terminal amino acid of the protease-sensitive peptide is selected from the group consisting of alanine (A), arginine (R), aspartic acid (D), glutamine (Q), glutamic acid (E), glycine (G), isoleucine (I), leucine (L), methionine (M), phenylalanine (F), serine (S), tryptophan (W) and tyrosine (Y).

More preferably, the C-terminal amino acid of the protease-sensitive peptide is selected from the group consisting of alanine (A), arginine (R), glutamine (Q), leucine (L), methionine (M) and phenylalanine (F).

In another preferred embodiment, the C-terminal amino acid of the protease-sensitive peptide is selected from the group consisting of alanine (A), arginine (R), glutamic acid (E), leucine (L), methionine (M) and phenylalanine (F).

In one embodiment, the protease is a pathogen-specific protease. The pathogen may be selected from the group consisting of virus, bacterium, protozoa, prion, fungus and combinations thereof. According to a preferred embodiment of the present invention, the protease or proteolytic enzyme is released or, in case of a virus or a prion, upregulated, by pathogens, preferably by bacteria, viruses, protozoa or fungi, more preferably the following class, order, genera, family of species of herpes, varicella, parvovirus, papillomavirus, polyomavirus, adenovirus, hepadnavirus, variolavirus, picornavirus, aso-and caliciavirus, human cytomegalovirus, hepatitis-A-virus, hepatitis-C-virus, hepatitis-E-virus, togavirus, flavivirus, coronavirus, retrovirus, HIV, reovirus, orthomyxovirus, bunyavirusarenavirus, human rhinovirus, dengue virus, varicella-zoster virus, paramyxovirus, rubulavoris, morbillivirus, west nile virus, yellow fever virus, pneimovirus, non classified paramyxovirus, rhabdovirus, folovirus, viroids and prions, staphylococcus, streptococcus and enterococcus, bacillus, listeria, erysipelothrix, garderella, corynebacterium, actinomyces, mycobacterium, nocardia, neisseria, acinetobacter and moraxella, enterbacteriacea including salmoneslla shigella, yersinia, E. coli and vibrio, aeromonas, plesiomonas, haemophilus, pasteurella, campyhlobacter, heliobacter, spirillum, pseudomonas, stenotropomonas, burkholderia, legionella, brucella, bordetella francisella, bacteriodaceae ioncluding trepponema, borrelia peptospira rickettsia, coxiella, orientia, ehrlichia, baronella afipia, chlamydia, mycoplasma and histoplasma, coccidioides, blasomyces, paracoccidioides, candida, aspergillus, Cryptococcus, mucor, absidia,rhizopus, phaeohyphomycetes, hyalohyphomycetes, penicillium, pneumocystis, tyrpanoma, leishmania, giradia, trichomonas, entamoeba, naegleria, toxoplasma isspora, cyclospora, sarcocystis, cryptosporidium, plasmodium, babesia, microsporida, and balantidium.

The protease may be selected from the group consisting of: KSHV-, HSV-, HAV-; HCV-, HIV-, human cytomegalovirus-, Yellow fever, CMV-, HRV14-, HRV2a-, Malaria aspartyl-, Sars protease, proteases of the S1, S2, S6, S8, S9, S33, S11, S12, S26, S18 family, streptomyces trans- and carboxypeptiidases, signal peptidase I, Omtpin and Clp, C10C11, C15, C25 cysteine proteases, porphyromonas gingivalis cyxteine proteases, sortase, metalloproteases of the thermolysin family (m4), Metalloproteases of the M9 family inclusive of vibrio and clostridium collagenases, Serralysin and related M10 Proteases and proteases of the M12 family, bacterial metallo exopeptidases, proteases of the M19, M20, M22, M23, and M26 families, tetanus and botulinum beurotoxins as being part of a group of bacterial metalloproteases, anthrax toxin lethal factor, lysostaphin and aureolysin, and AAA proteases.

In a preferred embodiment the pathogen is a pathogen listed in Table 1 of WO 2013/132058 A1, the content of which is incorporated herein in its entirety.

The protease-sensitive peptide may be selected from SEQ ID NOs:1-141 disclosed in WO 2013/132058 A1. The amino acid sequences of SEQ ID NOs:1-141 disclosed in WO 2013/132058 A1 are incorporated herein by reference.

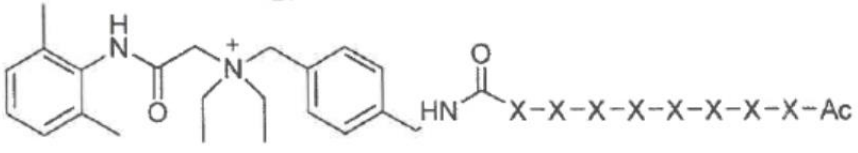
In a particular embodiment, the protease-sensitive peptide comprises or consists of the amino acid sequence GPQGIAGQ.

In another embodiment the protease-sensitive peptide is not GPQGIAGA, DAPV or QPVV.

In yet another embodiment, the C-terminal amino acid of the protease-sensitive peptide is not alanine or valine.

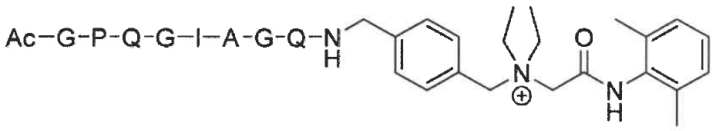
In another embodiment the compound or salt of the invention does not comprise trifluoracetate (TFA). In another embodiment the salt of the invention comprises trifluoracetate (TFA).

In one embodiment, the compound of the invention is a compound of formula [BRS-X], wherein X stands for an amino acid:



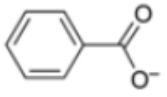
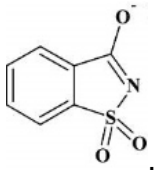
[BRS-X]

In a particular preferred embodiment, the compound of the invention is a compound of formula [BRS-1]:



[BRS-1].

In another aspect the present invention relates to a salt of a compound of formula [I] as defined hereinabove. The salt typically comprises a nutritionally or pharmaceutically acceptable counter ion. The counter ion is preferably selected from the group consisting of

F^-, Cl^-, Br^-,  , and  .

In yet another aspect the invention relates to a bioresponsive sensor comprising the compound of the present invention or the salt of the present invention. In a preferred embodiment the bioresponsive sensor does not comprise a base material or particles embedded and/or attached to said base material. In another preferred embodiment the bioresponsive sensor does not comprise particles. In another preferred embodiment the bioresponsive sensor substantially consists of the compound or salt of the invention.

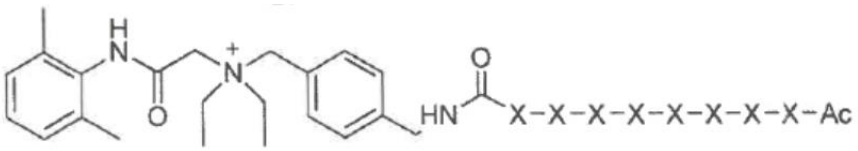
In yet another aspect the invention relates to the compound, salt or bioresponsive sensor of the present invention for use in a method of detecting an inflammation in the oral cavity of a human patient. In accordance with the invention, the detection occurs directly in the oral cavity of the patient by the use of the human tongue (sense of taste) and the nose (sense of smell) as a detector, in particular by the sense of taste. If the patient suffers from an inflammation of the oral cavity, the bioresponsive diagnostic sensor comprised e.g. in a chewing gum comes into contact with the saliva containing the matrix metalloproteinases (MMP) long enough to react. Accordingly, it is possible for the patient himself to detect an inflammation in the oral cavity, e.g. periodontal disease and/or peri-implantitis by gustatory detection of a bitter taste. Therefore, a person using the inventive chewing gum knows when

detecting a bitter taste, that an inflammation in the oral cavity, e.g. periodontal disease and/or peri-implantitis is present.

In another aspect the present invention provides a diagnostic chewing gum comprising the compound, salt or bioresponsive sensor of the present invention. The diagnostic chewing gum is typically used for detecting an inflammation, e.g. periodontal disease and/or peri-implantitis.

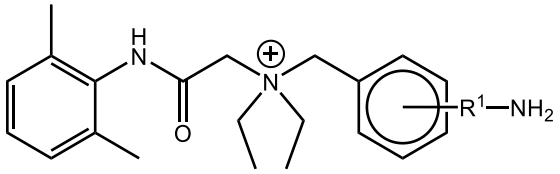
The present invention is further directed to a method of providing the inventive compound, salt or bioresponsive sensor. It is further directed to a method of providing a chewing gum comprising the inventive compound, salt or bioresponsive sensor.

According to the method of the invention for the preparation of a bioresponsive sensor, the protease-sensitive peptide is typically synthesized by solid-phase peptide synthesis (SPPS). Since sensory measurements with an electronic tongue showed that already four amino acids (AA) effectively mask the bitter taste of denatonium, no poly(methylmethacrylate) PMMA particles are needed anymore. The N-terminus of the protease-sensitive peptide is acetylated after the solid-phase peptide synthesis (SPPS) with acetic anhydride to prevent attack of the aminopeptidase (AP), and to prevent side reactions in further synthetic steps. Cleaved from the resin, the acetylated protease-sensitive peptide is now linked to Den-CH_2-NH_2 via 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU). Thereby, a bioresponsive sensor with the general formula [BRS-x] is obtained, wherein X is selected from the group of the 20 proteinogenic amino acids independently from each other:



[BRS-x]

In another aspect, the present invention relates to a compound of formula [II]



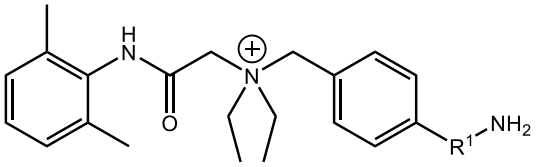
[II]

wherein

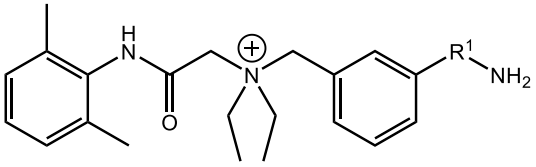
R^1 is an optionally substituted C_{1-3} alkylene group. The preferred embodiments of R^1 in formula [II] correspond to the preferred embodiments of R^1 as defined above for formula [I].

The compound according to formula [II] can be coupled to a protease-sensitive peptide to provide the compound of formula [I]. When the compound according to formula [I] comes into contact with the saliva of a person having a disease of the oral cavity, e.g. periodontitis or peri-implantitis, the compound according to formula [II] is released.

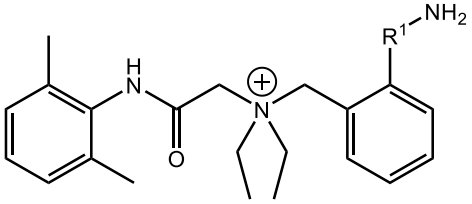
Preferred embodiments of the compound according to formula [II] are selected from the following group consisting of [IIa] to [IIc], wherein [IIa] is particularly preferred:



[IIa]



[IIb]

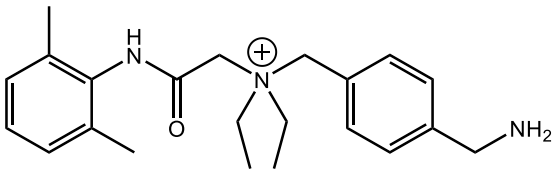


[IIc]

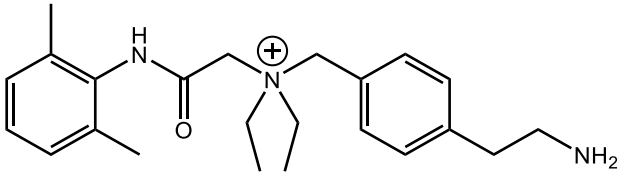
wherein

R^1 is an optionally substituted C_{1-3} alkylene group.

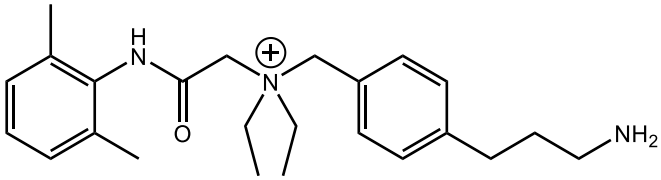
In one embodiment, the compound of formula [IIa] is selected from the group consisting of [IIa-1] to [IIa-3], wherein formula [IIa-1] is particularly preferred:



[IIa-1]

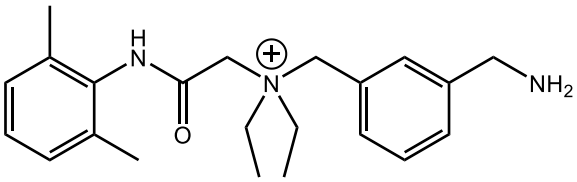


[IIa-2]

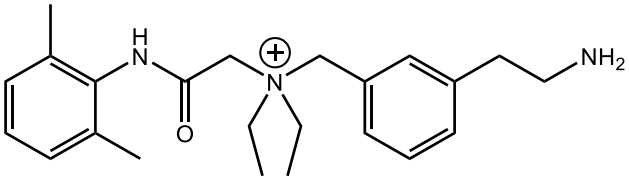


[IIa-3]

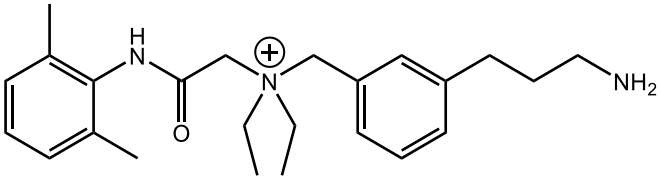
In another embodiment, the bioresponsive sensor according to formula [IIb] is selected from the group consisting of [IIb-1] to [IIb-3]:



[IIb-1]

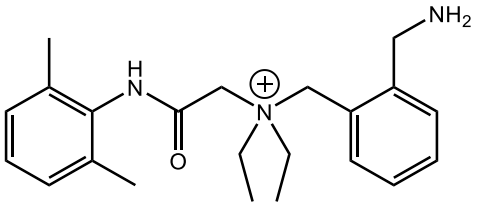


[IIb-2]

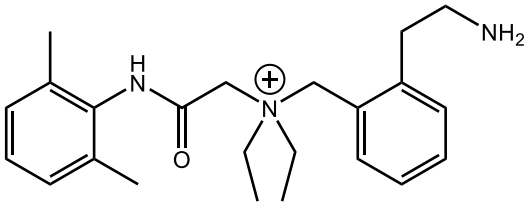


[IIb-3]

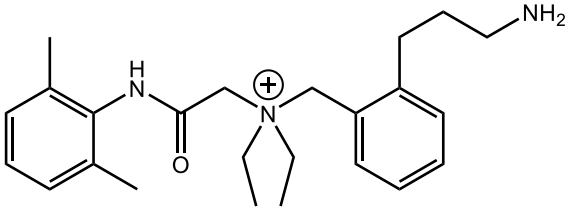
In another embodiment, the bioresponsive sensor according to formula [IIc] is selected from the group consisting of [IIc-1] to [IIc-3]:



[IIc-1]

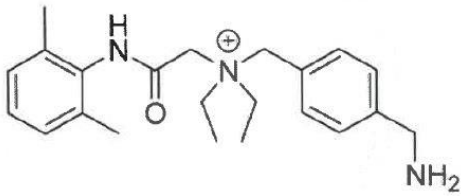


[IIc-2]



[IIc-3]

In a preferred embodiment the invention relates to a compound having the formula [IIa-1]:



[IIa-1]

The compound is a stable Den-CH_2-NH_2 compound which comprises a methylamine group with which it can be attached to the peptide chain of a protease-sensitive peptide.

Examples

Example 1: Synthesis of denatonium-CH_2-NHBoc

1.2 g lidocain (5.2 mmol) and 1.0 g *tert*-butyl-4-(bromomethyl)benzylcarbamate (3.4 mmol) were heated to 80 °C until the formation of a yellow melt. The melt then became highly viscous, up to solid, and subsequently, after a resting period of 10 minutes at 80 °C, the obtained yellow solid was treated with 40 mL of a mixture of ethyl acetate and *n*-hexane

(1:1). To obtain the protected product, the yellow solid was stirred for 10 minutes at 80 °C in the mixture. The resulting white residue was filtered off and washed with a mixture of ethyl acetate and hexane (1:1). 1.6 g of the protected product were obtained.

Sum formula:	$C_{27}H_{40}N_3O_3^+$ Br^-
Molecular mass:	534.5 g/mol
Yield:	1.6 g (91 %).

Example 2: Synthesis of a denatonium-CH_2-NH_2 salt

For the subsequent deprotection, 228 mg of the product (0.4 mmol) obtained from Example 1 was dissolved in 1 mL of trifluoroacetic acid and shaken at room temperature for 1 hour. The raw product was precipitated in diethylether and filtered off. The product was purified by chromatography.

Sum formula:	$C_{22}H_{33}N_3O^{2+}$ 2 $C_2F_3O_2^-$
Molecular mass:	581.6 g/mol
Yield:	175 mg (75 %).

^{1}H-NMR (DMSO, δ [ppm], *J* [Hz]): δ 10.14 (s, 1H), 8.30 (s, 3H), 7.62 (m, 4H), 7.20-7.10 (m, 3H), 4.84 (s. 2H), 4.18 (s, 2H), 4.12 (q. $^3J_{H,H}$ = 5.8, 2H), 3.54-3.48 (m, 4H), 2.20 (s, 6H), 1.42 (t, $^3J_{H,H}$ = 7.1, 6H).

^{13}C-NMR (DMSO, δ [ppm], *J* [Hz]): δ 162.7 (s, 1C), 136.8 (s, 1C), 135.5 (s, 2C), 133.7 (s, 1C), 133.6 (s. 2C), 129.9 (s, 2C), 128.5 (s, 2C), 128.2 (s, 1C), 127.7 (s, 1C), 61.6 (s, 1C), 56.0 (s, 1C), 54.9 (s, 2C), 42.2 (s, 1C), 18.6 (s, 2C), 8.3 (s, 2C).

Example 3:

The protease-sensitive peptide was synthesized using solid-phase peptide synthesis to 2-chlorotrityl chloride resin. For the proof-of-concept of the system, the amino acid sequence GPQGIAGQ was prepared.

H–G–P–Q–G–I–A–G–Q–OH

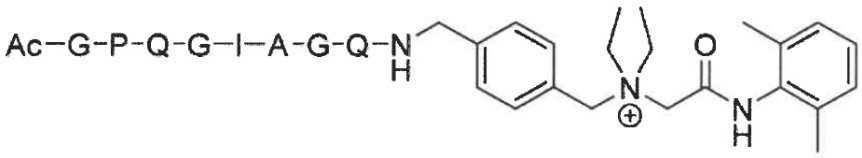
[PSP-1] (m/z = 726.37)

This sequence was acetylated at the N-terminus according to a method known *per se*, so that a protected protease-sensitive peptide 2 [PSP-2] was obtained:

Ac–G–P–Q–G–I–A–G–Q–OH

[PSP-2] (m/z = 768.38)

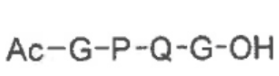
Using HATU / DIPEA, [PSP-2] was then linked to compound [IIa-1]. Thereby the bioresponsive sensor 1 [BRS-1] was obtained.



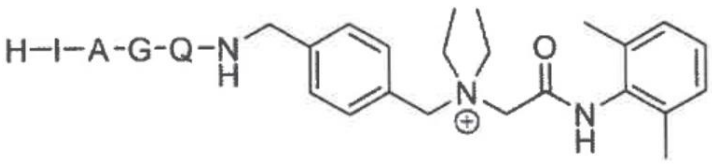
[BRS-1] (m/z = 1104.62)

Example 4: Cleavage Experiments

After incubation of the construct with MMP 1, 8 and 9, the following cleavage products CP1 and CP2 were obtained.



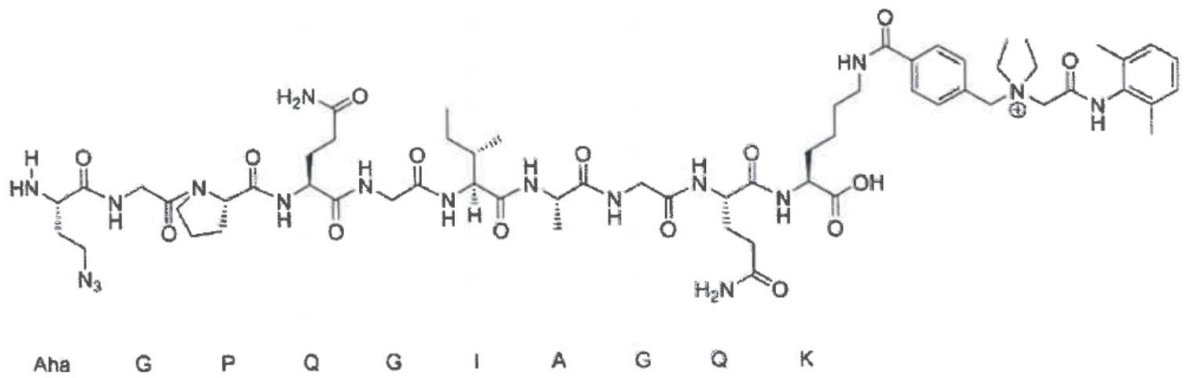
CP1 (m/z = 399.18)



CP2 (m/z = 723.46)

In the cleavage experiments, compound [BRS-1] was incubated with an equimolar mixture of MMP 1, 8 and 9 at 37 °C in MMP buffer (200 mM NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.05% Brij 35, pH 7.0).

For comparison, a comparative construct [CC1], according to the prior art (Ritzer, J et al. Nat. Commun. volume 8, Article number: 264 (2017)) was incubated to evaluate the cleaving efficiency of the new system. In [CC1] denatonium is connected via a carboxylic acid group to the N-terminus of a protease-sensitive peptide, as given below:



[CC1] (m/z = 1331.72)

The concentration of the two compounds was 0.1 mM. The cleavage efficiency was measured using a Hitachi Elite LaChrom HPLC system (VWR, Darmstadt, Germany) with a ZORBAX Eclipse XDB-C18 column (4.6 mm internal diameter, 150 mm length (Agilent, Santa Clara, CA)), eluent A (0.1%). TFA in water, (v/v)) and eluent B (0.1% trifluoroacetic acid (TFA) in acetonitrile (v/v)) with a gradient of 5 to 95% eluent B over 55 min. The UV absorption was measured at l = 214 nm (Table 1, Fig. 1).

Table 1: cleavage rate (%) of [BRS-1] and [CC1] accompanied by the standard deviations.

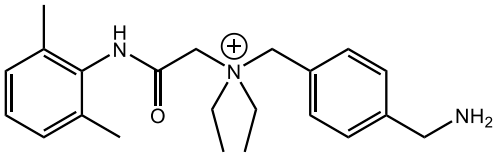
	[BRS-1]	[CC1]
Cleaved construct after 10 min	28.0 % ± 10.9 %	41.6 % ± 6.29 %
Cleaved construct after 30 min	49.3 % ± 11.7 %	68.4 % ± 0.587 %
Cleaved construct after 120 min	81.8 % ± 5.09 %	80.2 % ± 1.78 %
Cleaved construct after 120 min	98.0 % ± 0.0583 %	89.2 % ± 0.888 %

The cleavage efficiency of both systems was comparable. The slightly better cleavage rate of [CC1] is caused by the fact that it was not the final construct that was analyzed here. Due to the lack of coupling with a spatially demanding PMMA particle, the overall system is further

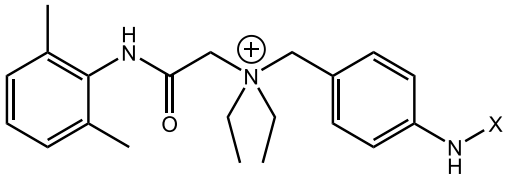
enlarged and thus less susceptible to MMP. On the other side, [BRS-1] is the final construct used as bioresponsive sensor in the detection of inflammations in the oral cavity.

When [CP2] is obtained after cleavage of [BRS-1] it is now possible for the aminopeptidase to attack the free N-terminus of [CP2], so that further cleavage to [IIa-1] occurs. Previously, in the full construct [BRS-1] this further cleavage process is prevented by the acetyl protecting group. Therefore, no degradation occurs in [BRS-1], when no MMP is present as it is the case in healthy individuals. [CC1] is cleaved by aminopeptidase analogously. However, in the case of [CC1] an amino acid (in this case lysine) remains on the denatonium-COOH, since it is attached to the ε-position. This significantly reduces the gustatory perception compared to free denatonium.

To trigger maximum gustatory perception, the last amino acid from the modified denatonium must also be removed by the aminopeptidase. Not all amino acids are equally well suited for this purpose. To evaluate the cleavage efficiency, 20 constructs consisting of one proteinogenic amino acid coupled to compound [IIa-1] were synthesized, thereby obtaining compound [Ia-X], wherein X is an amino acid:



[IIa-1] (m/z = 354.25)



[Ia-X]

The resulting constructs were incubated with AP for 24 h and analyzed by LC / MS for complete degradation (Table 2).

Table 2: Cleavage Experiments of different compounds with AP und their detectability after 24 h. + stands for detectability, - stands for full degradation to [IIa-1].

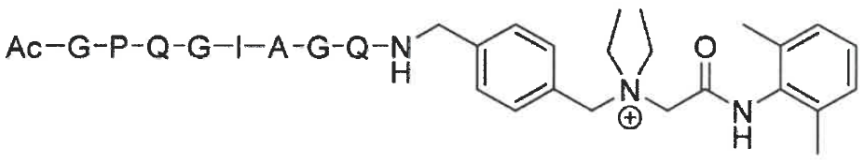
[Ia-X] with X =	**Detectable after 24 h incubation with AP**
Alanine	-
Arginine	-
Asparagine	+
Aspartic acid	+

[Ia-X] with X =	**Detectable after 24 h incubation with AP**
Cysteine	-
Glutamine	+
Glutamic acid	-
Glycine	+
Histidine	+
Isoleucine	+
Leucine	-
Lysine	-
Methionine	-
Phenylalanine	-
Proline	+
Serine	-
Threonine	+
Tryptophan	+
Tyrosine	-
Valine	+

Subsequently, cleavage experiments were performed for 20 min, 120 min and 24 h with an aminopeptidase concentration of 940 ng/ml of compound Ia-X. Only 16 out of 20 constructs were used, as lysine, asparagine, cysteine, and histidine did not produce significant synthesis yields, which also would interfere with subsequent peptide coupling.

Example 5: Synthesis Example

The invention is further exemplified by the following example of the synthesis of [BRS-1]:



[BRS-1]

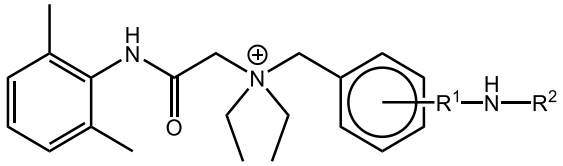
[PSP-2] (71.3 mg, 0.0928 mmol) was dissolved with HATU (34.0 mg, 0.0894 mmol) in anhydrous dimethylformamide (DMF) (2 mL). Subsequently, [IIa-1] (63.0 mg, 0.178 mmol) was added and after complete dissolution DIPEA (31.9 µL) was added. The mixture was stirred for 18 h protected from light at room temperature. Then ice-cold diethyl ether (20 mL) was added. After centrifugation and decantation of the supernatant, the residue was dried overnight.

The residue was purified using a FPLC system (Äkta purifier, GE Healthcare) using reversed phase chromatography (RPC) on a C18 column (Phenomenex®) (eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile). After subsequent lyophilization, [BRS-1] was obtained as a colorless (white) powder. The overall yield of high purity [BRS-1] was 5.32 mg (5.19%).

Claims

1. A compound having the structure Den–R^1-NH-R^2, or a salt thereof, wherein
Den is denatonium,
R^1 is an optionally substituted C_{1-3} alkylene group
R^2 is a protease-sensitive peptide,
wherein the C-terminus of the protease-sensitive peptide forms an amide bond with the group -NH-.

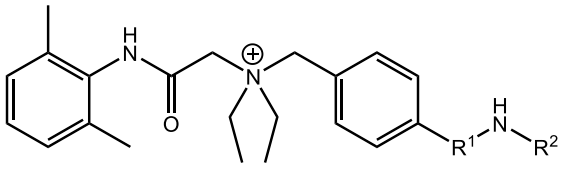
2. The compound or salt of claim 1, having the formula [I]:



[I]

wherein
R^1 and R^2 are as defined in claim 1.

3. The compound or salt of claim 1 or 2, wherein the compound has the formula [Ia]:



[Ia]

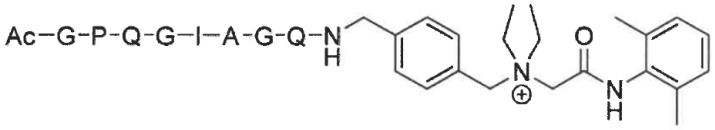
wherein
R^1 and R^2 are as defined in claim 1.

4. The compound or salt of any one of claims 1 to 3, wherein R^1 is -CH_2-.

5. The compound or salt of any one of claims 1 to 4, wherein the protease-sensitive peptide comprises or consists of at least 4 amino acids, preferably 4 to 15 amino acids, more preferably 5 to 12, even more preferably 6 to 10, yet even more preferably 7 to 9, most preferably 8 amino acids.

6. The compound or salt of any one of claims 1 to 5, wherein the C-terminal amino acid of the protease-sensitive peptide is selected from the group consisting of alanine, arginine, glutamine, leucine, methionine and phenylalanine.

7. The compound or salt of any one of claims 1 to 6, wherein the compound has the formula [BRS-1]:

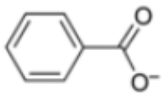
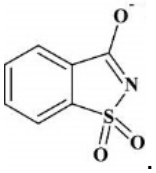


[BRS-1].

8. The compound or salt of any one of the preceding claims, wherein the protease is a pathogen-specific protease.

9. The compound or salt of any one of the preceding claims, wherein the protease-sensitive peptide is not QPVV, DAPV or GPQGIAGA.

10. The salt of any one of claims 1 to 9, comprising a counter ion selected from the group consisting of

F^-, Cl^-, Br^-,  , and  .

11. A bioresponsive sensor comprising the compound or salt of any one of claims 1 to 10.

12. The compound or salt according to any one of claims 1 to 10, or the bioresponsive sensor according to claim 11, for use in a method of detecting an inflammation in the oral cavity of a human patient.

13. A method for the preparation of the bioresponsive sensor, comprising the step of linking an amino-modified denatonium compound to the C-terminus of a protease-sensitive peptide, wherein the amino-modified denatonium compound is preferably a compound of formula [II] or formula [IIa-1].

14. The method of claim 13, further comprising, prior to the step of linking:
- preparing a protease-sensitive peptide by solid phase peptide synthesis, and/or

- protecting the N-terminus of the protease-sensitive peptide with a protecting group, preferably acetic anhydride.

15. A diagnostic chewing gum or a diagnostic confectionary, comprising the compound or salt of any one of claims 1 to 10.

Abstract

The present invention relates to compounds comprising denatonium linked to the C-terminus of a protease-sensitive peptide; or a salt thereof. The compounds are useful in the diagnosis of inflammatory conditions of the oral cavity.

Figure 1

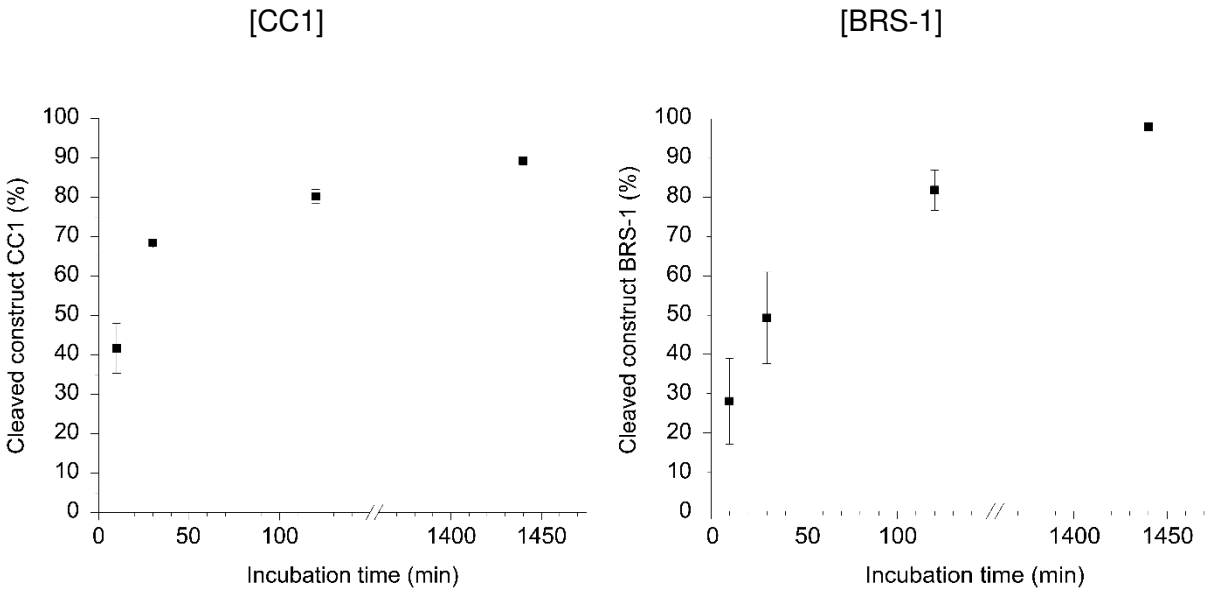
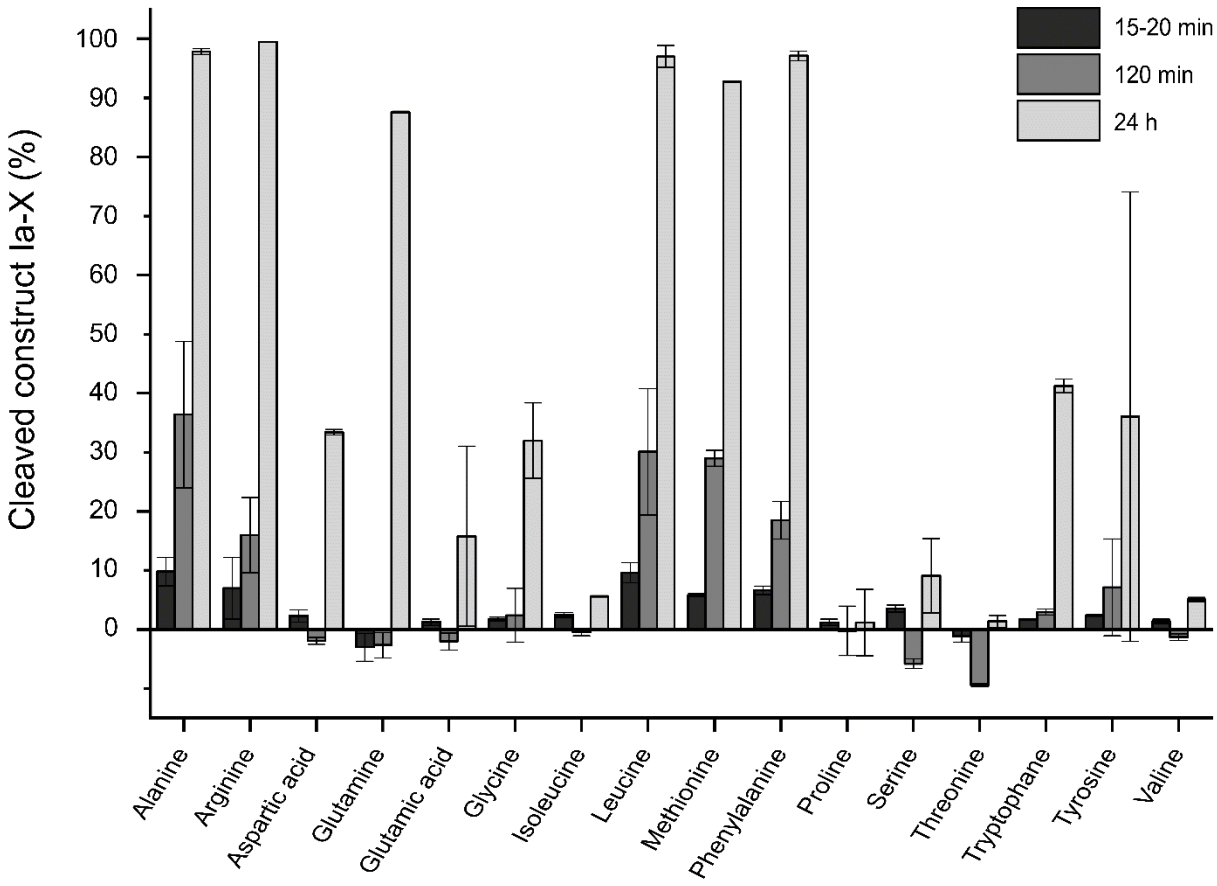

[CC1]
[BRS-1]
Cleaved construct CC1 (%)
Cleaved construct BRS-1 (%)
Incubation time (min)
Incubation time (min)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
1400
1450

Figure 2


15-20 min
120 min
24 h
Cleaved construct Ia-X (%)
100
90
80
70
60
50
40
30
20
10
0
Alanine
Arginine
Aspartic acid
Glutamine
Glutamic acid
Glycine
Isoleucine
Leucine
Methionine
Phenylalanine
Proline
Serine
Threonine
Tryptophane
Tyrosine
Valine

10.1. Documentation of Authorship

Section 1	**Miesler T, Raschig M, Ritzer J, Meinel L**			
	1	**2**	**3**	**4**
Synthesis of Den-CH_2-NH-Boc		x		
Characterization of Den-CH_2-NH-Boc		x		
Synthesis of Den-CH_2-NH_2 and Den-CH_2-NH-Ac	x	x		
Characterization of Den-CH_2-NH_2 and Den-CH_2-NH-Ac	x	x		
Synthesis of Den-X Constructs	x			
Synthesis of QPVV-Den and DAPV-Den	x			
Cleavage of QPVV-Den and DAPV-Den by AP	x			
Manufacturing of the PICS Library	x		x	
Electronic tongue measurements	x	x		
Cleavage of Den-X by AP	x			
Data analysis and interpretation	x		x	x
Design of the graphics	x			
Supervision of Tobias Miesler			x	x

Section 2	**Miesler T, Raschig M, Ritzer J, Meinel L**			
	1	**2**	**3**	**4**
PICS Assay of MMP-9	x		x	
Synthesis of Q, Q→A and Q+A	x			
Characterization of Q, Q→A and Q+A	x			
Cleavage experiments of Q, Q→A and Q+A	x			
Study design/concept development	x		x	x
Data analysis and interpretation	x		x	x
Supervision of Tobias Miesler			x	x

Section 3	**Miesler T, Raschig M, Ritzer J, Meinel L**			
	1	**2**	**3**	**4**
Expression and purification of SpeB	x		x	
PICS analysis of SpeB	x			
Synthesis of SpeB sensitive constructs	x			
Quantification of SpeB via western blot	x			
Study design/concept development	x		x	x
Data analysis and interpretation	x		x	x
Supervision of Tobias Miesler				x

Section 4	**Miesler T, Raschig M, Seibel J, Meinel L**			
	1	**2**	**3**	**4**
Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-D-glycero- β-D-galacto-2-nonulopyranosidonate)	x	x		
Synthesis of methyl(methyl 5-acetamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosidonate)	x	x		
Synthesis of methyl (methyl 5-acetamido-3,5-dideoxy-4,7-di-O-methyl-8,9-O- isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosidonate)	x	x		
Synthesis of methyl (5-acetamido-2,8,9-tri-O-acetyl-3,5-dideoxy-4,7-di-O-methyl- D-glycero-α,β-D-galacto-2-nonulopyranosidonate)	x	x		
Supervision of Tobias Miesler			x	x

Perspective	**Miesler T, Wimschneider C, Brem A, Meinel L**			
	1	**2**	**3**	**4**
Manuscript planning	x	x		x
Manuscript writing	x	x		
Design of the graphics	x			
Correction of manuscript	x	x	x	x
Supervision of Tobias Miesler and Christine Wimschneider			x	x

Patent 1	**Miesler T, Seibel J, Meinel L**		
	1	**2**	**3**
Concept developement	x	x	x
Performance of experiments	x		
Manuscript writing	x	x	x
Design of the graphics	x		
Correction of manuscript	x	x	x
Supervision of Tobias Miesler		x	x

Patent 2	**Miesler T, Ritzer J, Meinel L**		
	1	**2**	**3**
Concept developement	x	x	x
Performance of experiments	x		
Manuscript writing	x	x	x
Design of the graphics	x		
Correction of manuscript	x	x	x
Supervision of Tobias Miesler		x	x

ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AA	amino acid
Ab	antibody
ACN	acetonitrile
AUC	area under the curve
AP	aminopeptidase
BCA	bicinchoninic acid
Boc	butoxycarbonyl
BSA	bovine serum albumin
CBB	Coomassie brilliant blue
CEX	cation exchange chromatography
CTC	chlorotrityl chloride resin
CV	column volumes
DCM	dichloromethane
DDS	drug delivery system
Den	denatonium
DIC	N,N′-Diisopropylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMEM	Dulbecco's modified eagle’s medium high glucose
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	D/L-dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FA	formic acid
FCS	fetal calf serum
FPLC	fast protein liquid chromatography

ABBREVIATIONS AND SYMBOLS

GAS	Group A *Streptococcus*
GCF	gingival crevicular fluid
GluC	Staphylococcus aureus *Protease* V8
GPCRs	G protein–coupled receptors
HA	haemagglutinin
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-Hydroxybenzotriazole hydrate
HPAIV	highly pathogenic avian influenza virus
HPLC	high pressure liquid chromatography
IAV	Influenza A virus
LB	Lysogeny broth
LC	liquid chromatography
M1	matrix protein 1
M2	matrix protein 2
MALDI	matrix-assisted laser desorption/ionization
MeOH	methanol
MMP	matrix metalloproteinases
MS	mass spectrometry
NA	neuraminidase
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
NP	nucleoprotein
OD	optical density
PA	periodontitis
PBS	phosphate-buffered saline
PICS	proteomic identification of cleavage sites
PMMA	poly(methyl methacrylate)
POCT	point-of-care tests
PSL	protease sensitive linker
RNA	ribonucleic acid
SD	standard deviation

SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SpeB	streptococcal pyrogenic exotoxin B
SPPS	solid phase peptide synthesis
TBST	tris-buffered saline-Tween
TEA	triethylamine
TFA	trifluoroacetic acid
TEMED	tetramethylethylendiamin
UV	ultraviolet
vRNPs	viral ribonucleoproteins
WHO	World Health Organization
WST	water soluble tetrazolium

SYMBOLS

3D	three dimensional
°C	degree Celsius
C	centi, 10^{-2}
(k)Da	(kilo)Dalton
Λ	wavelength
eq	equivalent
(k)g	(kilo)gram
h	hour
Hz	Hertz
K	Kelvin
L	liter
m	meter
m	milli, 10^{-3}
M	molar
µ	micro, 10^{-6}
min	minute
ppm	parts per million
s	second
t	time
V	volt

ACKNOWLEDGMENTS

I would like to take this opportunity to thank all those who have contributed to the success of this work.

My special thanks go to my doctoral father Prof. Dr. Dr. Lorenz Meinel for giving me the opportunity to join his research group and to perform my PhD thesis in this fascinating field. During these eventful years, I have gratefully appreciated his guidance through each stage of the process, helpful advice, as well as providing excellent equipment conditions.

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The whole Gym Guys Group (Björn ter Mors, Matthias Beudert) for work-related as well as non-work-related discussions and exciting trips to Leipzig, Munich, Berlin as well as destinations in and around Würzburg.

Furthermore, I would like to thank Christine Schneider and Sascha Zügner for their helpfulness in organizational matters.

It was a pleasure for me to meet you all and I hope that you learned as much from me as I did from you.

Last but not least, I would like to express my very profound gratitude to my family for their support during this scientific work. Finally, there are my outside-university friends, who were of great support in providing happy distractions to rest my mind outside of my research.

Again: Thank you all.

Affidavit

I hereby confirm that my thesis entitled “Development of diagnostic systems targeting the human tongue as a 24/7 available detector” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Hiermit erkläre ich an Eides statt, die Dissertation „Development of diagnostic systems targeting the human tongue as a 24/7 available detector“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift