Drug delivery of therapeutic gases – strategies for controlled and local delivery of carbon monoxide

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der

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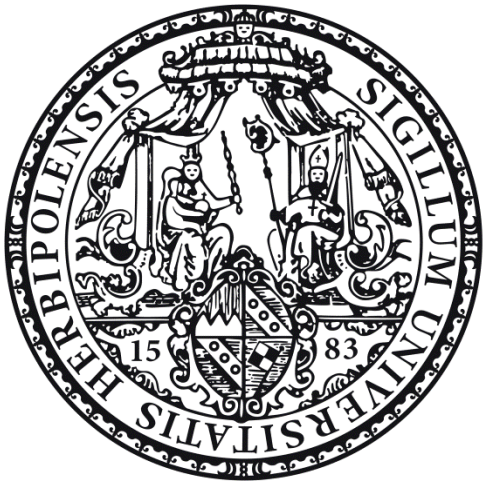

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Summary

The isoenzyme heme oxygenase 1 (HO-1) is a key element for maintaining cellular homeostasis. Upregulated in response to cellular stress, the HO-1 degrades heme into carbon monoxide (CO), biliverdin, and Fe^{2+}. By means of a local cell-protective feedback loop the enzyme triggers numerous effects including anti-oxidative, anti-apoptotic, and anti-inflammatory events associated with complex signalling patterns which are largely orchestrated by CO. Various approaches to mimic this physiological HO-1 / CO system aiming for a treatment of medical conditions have been described [1]. These preclinical studies commonly applied CO systemically via (i) inhalation or (ii) using CO-Releasing Molecules (CORMs) [2]. The clinical use of these approaches, however, is challenged by a lack of practicability and substantial safety issues associated with the toxicity of high systemic doses of CO that are required for triggering therapeutic effects. Therefore, one rational of this thesis is to describe and evaluate strategies for the local delivery of CO aiming for safe and effective CO therapeutics of tomorrow.

In the first chapter the current concepts for CO delivery are discussed and evaluated based on existing approaches while suggesting possible solutions to current application hurdles. With a particular focus on the physiology of the HO-1 / CO system, challenges and potential pitfalls as well as opportunities are analysed. Based on current delivery modes including CO inhalation and CORMs, the chapter highlights novel approaches designed to shuttle CO precisely to the site of the disease thereby mimicking the local effects of the HO-1 / CO system. The chapter is submitted for publication.

One of these approaches is described in chapter two and addresses the previously detailed therapeutic potential of CO in gastrointestinal (GI) diseases including ulcerative colitis, postoperative ileus, and diabetic gastroparesis. The chapter describes an oral gas delivery system referred to as Oral Carbon Monoxide Release System (OCORS) designed for controlled and local delivery of CO for the treatment of GI diseases. CO release from the OCORS is driven by sulfite induced CO generation from the ruthenium based CO-Releasing Molecule 2 (CORM-2). Formulation strategies were performed to ensure independence of environmental pH on CO generating reaction kinetics thereby enabling uniform CO release in simulated GI fluids representing different parts of the GI tract. With the CO generating reaction being controlled by GI fluids permeating into the system following oral application, the release pattern of OCORS was tuneable by modifying the permeability of the cellulose

acetate coating covering the release system. The chapter was published in the Journal of Controlled Release [3].

Chapter three describes a downsized modification of the OCORS, namely the micro scale OCORS (M-OCORS), which was designed for applicability in small animal models. As compared to the OCORS, further formulation strategies were performed to increase the controllability of CO release kinetics from the M-OCORS with patterns lasting from several minutes up to almost one day. This profile was designed for delivering CO to different parts of the murine GI tract thereby locally triggering therapeutic effects. *In-vivo* pharmacokinetic studies in mice analysing the CO-haemoglobin (CO-Hb) formation following oral application confirmed the *in-vitro* release profiles. The therapeutic potential was profiled in a chemically induced murine colitis model with an M-OCORS formulation providing a release profile designed for intestinal CO delivery. Therefore, two independent studies were performed, demonstrating efficacy by means of a significant reduction of a colon damage score as well as a downregulation of colitis biomarkers as compared to the control. Aiming for a possible future clinical translation of the OCORS platform, the safety profile of the ruthenium based system was improved by introducing a novel iron based modification (ET-OCORS). The chapter was published in the Journal of Controlled Release [4].

Chapter four describes another local delivery approach based on numerous studies on the therapeutic efficacy of CO in treating ischemia reperfusion injury (IRI). IRI is a major factor limiting the ability to successfully transplant organs. A promising approach addressing IRI is the enrichment of organ perfusion solutions with CO gas or CORMs, respectively. The translation of this approach into a clinical setting, however, is challenged by safety issues associated with handling complexity of CO gas in high, toxic, concentrations or exposing the transplant to potentially toxic CORMs, respectively. Addressing these challenges, the chapter describes a Therapeutic Gas Releasing System (TGRS) for the controlled release of gases including CO and hydrogen sulfide (H2S). The system comprises a gas releasing compound (e.g. CORM) within a membrane selectively releasing the therapeutic gas but retaining all other constituents (e.g. CORM). Consequently, the perfusion solution can be enriched with therapeutic gas but no other potentially toxic constituent can leave the system (e.g. CORM). In addition, varying the thickness of the membrane allows for tailoring the gas release profile of the system. The therapeutic potential of this concept was profiled in an ischemic rat liver transplant model.

As compared to the control group, cytosolic HMGB1 - an important biomarker for IRI - was significantly downregulated in liver tissues treated with perfusion solutions which were enriched with CO using TGRS. The chapter was published in the European Journal of Pharmaceutics and Biopharmaceutics [5].

Chapter five describes an anti-counterfeiting strategy for solid dosage forms. Drug counterfeiting is a global medical problem with growing significance. Although various anti-counterfeiting strategies have been introduced - including watermarks or RFID chips - most approaches are limited to serializing the packaging of the drug. The approach described in chapter five, however, is designed to directly tag the dosage form by using FDA approved excipients for unambiguous serialization and easy implementation. Therefore, we tagged tablets with a unique code “written” with monodisperse polyethylene glycols (PEG) of different degrees of polymerization. The individual mixture was spiked into the coating solutions of the tablets in nanomolar concentrations. The code was subsequently identified using LC-MS/MS analysis allowing for unambiguous recovery of the individual dosage form / batch. This approach can exemplary be integrated in OCORS (chapter 2) and M-OCORS (chapter 3), respectively. The chapter was published in the European Journal of Pharmaceutics and Biopharmaceutics [6].

In summary, this thesis describes and evaluates novel strategies for the targeted local delivery of CO. Two gas delivery platforms were developed and characterized *in-vitro*. The therapeutic potential of these approaches was subsequently demonstrated *in-vivo*. The systems allow local, reliable, and controlled release of CO, thereby removing safety concerns of current approaches and thus positively impacting the benefit / risk profile of therapeutic CO in GI and transplant applications. In addition, a novel anti-counterfeiting approach was introduced with FDA approved excipients being functional for uniquely serializing the individual dosage form.

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Zusammenfassung

Das Isoenzym Hämoxygenase 1 (HO-1) ist ein zentraler Bestandteil in der Aufrechterhaltung der zellulären Homöostase. Es wird durch zellulären Stress induziert und baut daraufhin Häm zu Kohlenstoffmonoxid (CO), Biliverdin und Fe^{2+} ab. Im Sinne eines lokalen Rückkopplungsmechanismus stößt es damit eine Vielzahl physiologischer Mechanismen mit anti-oxidativen, anti-apoptotischen und anti-inflammatorischen Effekten an, welche zumeist durch CO reguliert und durch ein komplexes Netzwerk aus Signaltransduktionsprozessen vermittelt werden. Es wurden zahlreiche Versuche unternommen, diesen als HO-1 / CO System bezeichneten Mechanismus nachzuahmen, um dadurch eine Behandlung von verschiedenen Krankheitszuständen zu ermöglichen [1]. In diesen präklinischen Studien wurde CO regelmäßig systemisch (i) per Inhalation oder (ii) in Form von CO freisetzenden Verbindungen (CO-Releasing Molecules - CORM) verabreicht [2]. Die klinische Anwendung dieser Strategien ist jedoch durch Sicherheitsrisiken erheblich erschwert, insbesondere durch die Toxizität der notwendigen hohen systemischen Dosen von CO. Entsprechend beschäftigt sich diese Dissertation unter anderem mit der Beschreibung und Evaluation von Strategien zur lokalen Verabreichung von CO, mit dem Ziel sichere und effektive Konzepte zu dessen Anwendung zu entwickeln.

In Kapitel eins werden gängige Konzepte zur Applikation von CO diskutiert und im Kontext bisheriger medizinisch-translationaler Ansätze bewertet. Dabei werden Anforderungen, Risiken, wie auch Chancen insbesondere mit Bezug auf die Physiologie des HO-1 / CO Systems analysiert. Ausgehend von gängigen Konzepten wie der pulmonalen Applikation von CO oder der Anwendung von CORMs beschreibt dieses Kapitel neuartige Konzepte, die mit dem Ziel entwickelt wurden CO direkt am Wirkort und damit identisch zur Physiologie des HO-1 / CO-Systems freizusetzen. Das Kapitel ist zur Publikation eingereicht.

Eines dieser Konzepte wird in Kapitel zwei dargelegt und zielt auf das eingängig beschriebene therapeutische Potential von CO bei einer Reihe gastrointestinaler Erkrankungen, insbesondere der Colitis Ulcerosa, dem Postoperativen Ileus sowie der Diabetischen Gastroparese ab. Das Kapitel beschreibt ein als Oral Carbon Monoxide Release System (OCORS) bezeichnetes Gasfreisetzungssystem, welches mit dem Ziel entwickelt wurde, die lokale und steuerbare Freisetzung von CO zur Behandlung gastrointestinaler Erkrankungen zu ermöglichen. Das Prinzip von OCORS basiert auf der Sulfit-induzierten

Generierung von CO durch rutheniumhaltiges CORM-2 (CO-Releasing Molecule 2). Das System wurde so formuliert, dass die darin ablaufende CO generierende Reaktion unabhängig vom pH-Wert des Mediums ist, welches das System umgibt. Dies ermöglichte es, identische Freisetzungsverläufe in biorelevanten Medien zu gewährleisten, welche verschiedene Abschnitte des gastrointestinalen Systems widerspiegeln. Die Sulfit-induzierte Generierung von CO wird durch gastrointestinale Flüssigkeiten ausgelöst, welche nach oraler Applikation in das System permeieren. Entsprechend konnte die Freisetzungskinetik durch Modifikationen der Permeabilität der das System umgebenden Hülle aus Celluloseacetat gesteuert werden. Das Kapitel wurde im Journal of Controlled Release veröffentlicht [3].

Kapitel drei beschreibt eine verkleinerte Modifikation dieses Systems, die als micro scale OCORS (M-OCORS) bezeichnet wird, und zur Applikation im Kleintiermodell entwickelt wurde. Durch die Anwendung weiterer Formulierungsstrategien konnte die Freisetzungskinetik im Vergleich zu OCORS in bedeutend weiteren Grenzen gesteuert werden und ermöglichte damit Freisetzungsverläufe, welche von wenigen Minuten bis zu beinahe einem Tag variiert werden konnten. Diese Modifikationen wurden vorgenommen, um im Mausmodell CO gezielt in verschiedenen Teilen des gastrointestinalen Systems freizusetzen und dort therapeutische Effekte lokal zu erzeugen. Pharmakokinetische *in-vivo* Untersuchungen, welche den Carboxyhämoglobin (CO-Hb) Verlauf nach oraler Applikation analysierten, bestätigten die entsprechenden Freisetzungsverläufe *in-vitro*. Das therapeutische Potential dieses Ansatzes wurde sodann in einem murinen Colitismodell mit einer Formulierung evaluiert, deren Freisetzungsprofil zur lokalen Exposition des Darms mit CO entwickelt wurde. Zwei unabhängig voneinander durchgeführte Versuchsreihen zur Prävention von chemisch-induzierter Colitis konnten die Effektivität durch eine signifikante Reduktion des angewendeten Scores zur Quantifizierung des Gewebsschadens im Colon, sowie einer verminderten Hochregulation von colitisassoziierten Biomarkern bestätigen. Mit dem Ziel eines zukünftigen klinischen Einsatzes des OCORS, wurde in der Folge das Sicherheitsprofil des rutheniumbasierten Systems durch die Entwicklung einer auf Eisen basierenden Modifikation (ET-OCORS) verbessert. Das Kapitel wurde im Journal of Controlled Release veröffentlicht [4].

Das vierte Kapitel beschreibt ein weiteres lokales Freisetzungssystem, welches ausgehend von vielfältigen Untersuchungen über den therapeutischen Effekt von CO zur Behandlung des Ischämie-Reperfusionsschadens (IRS) entwickelt wurde. IRS ist ein bedeutender Faktor welcher die Transplantierbarkeit von Organen negativ beeinflusst. Entsprechend wurde die Anreicherung von CO oder CORMs in Organkonservierungslösungen erfolgreich dazu verwendet, IRS zu adressieren und damit die Qualität von transplantierten Organen zu verbessern. Klinisch ist dieses Verfahren jedoch wegen Sicherheitsrisiken schwer anwendbar, da entweder CO Gas im Klinikalltag in toxischen Konzentrationen gehandhabt werden müsste oder potentiell toxische CORMs das Transplantat belasteten würden. Das Kapitel beschreibt daher eine Möglichkeit, diese Hindernisse technisch zu beseitigen. Das Freisetzungssystem für therapeutische Gase (Therapeutic Gas Releasing System - TRGS) ermöglicht die kontrollierte Freisetzung von CO und Schwefelwasserstoff (H_2S). Im System befindet sich eine Gas freisetzende Verbindung (z.B. CORM) innerhalb einer Membran, durch welche lediglich das therapeutische Gas, nicht aber andere Stoffe austreten können (wie etwa CORMs). Dadurch kann die Konservierungslösung mit Gas angereichert werden, es können aber keine weiteren Komponenten das TGRS verlassen und das Transplantat kontaminieren. Die Gasfreisetzung aus dem TGRS war außerdem in Abhängigkeit der Dicke der verwendeten gaspermeablen Membranen steuerbar. Das therapeutische Potential dieses Konzeptes wurde anhand von ischämischen Rattenlebertransplantaten evaluiert. Im Vergleich zur Kontrollgruppe wurde dabei cytosolisches HMGB1 - ein wichtiger Biomarker für IRS - in jenem Lebergewebe signifikant herunterreguliert, welches mit einer Perfusionslösung behandelt wurde, die mittels TGRS mit CO angereichert wurde. Das Kapitel wurde im European Journal of Pharmaceutics and Biopharmaceutics veröffentlicht [5].

Medikamentenfälschungen sind ein globales medizinisches Problem, welches zunehmend an Bedeutung gewinnt. Obwohl eine Vielzahl von Fälschungssicherungssystemen wie etwa Wasserzeichen oder RFID Chips entwickelt wurden, sind diese meist darauf beschränkt, die Medikamentenverpackung mit einem eindeutigen seriellen Code zu markieren. Im Gegensatz dazu wurde die in Kapitel fünf beschriebene Strategie dazu entwickelt, die Arzneiform unmittelbar mit einem Muster aus Hilfsstoffen zu kennzeichnen, welches aufgrund bestehender FDA-Zulassung einfach in die Arzneiform implementiert werden kann. Dazu markierten wir Tabletten mit einem unverwechselbaren Code aus monodispersen Polyethylenglycolen (PEG) unterschiedlicher Kettenlänge, welche in nanomolarer

Konzentration zu Überzugslösungen hinzugefügt wurden. Das Muster wurde im Anschluss mittels LC-MS/MS Analyse identifiziert und erlaubt dadurch die unverwechselbare Wiederfindung der Arzneiform bzw. der Charge. Das Prinzip kann beispielsweise für OCORS (Kapital 2) und M-OCORS (Kapitel 3) Anwendung finden. Das Kapitel wurde im European Journal of Pharmaceutics and Biopharmaceutics veröffentlicht [6].

Zusammengefasst beschreibt und evaluiert diese Arbeit Strategien zur gezielten lokalen Verabreichung von CO. Entsprechend wurden zwei Freisetzungssysteme für therapeutische Gase entwickelt und *in-vitro* charakterisiert. Das therapeutische Potential dieser Ansätze wurde anschließend *in-vivo* gezeigt. Die Systeme ermöglichen eine lokale, zuverlässige und steuerbare Freisetzung von CO und bieten damit eine Möglichkeit die Sicherheitsbedenken derzeitiger Ansätze adäquat zu adressieren. Entsprechend tragen sie zu einem verbesserten Nutzen-/ Risiko-Profil bei, um das therapeutische Potential von CO für gastrointestinale Erkrankungen und die Transplantationsmedizin verfügbar zu machen. Außerdem beschreibt diese Arbeit ein neues Fälschungssicherungssystem für feste Arzneiformen.

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Chapter 1: Localized delivery of carbon monoxide

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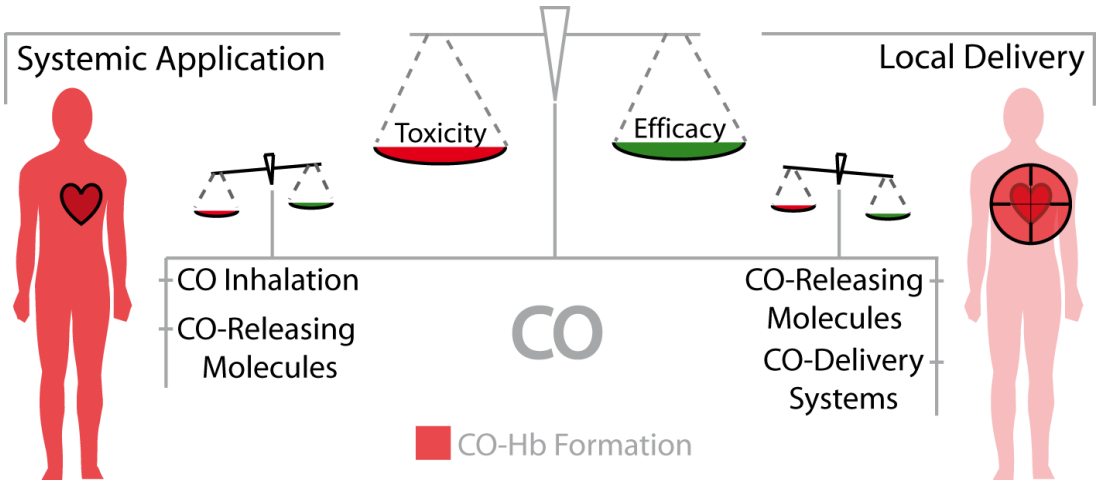

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Abstract

The heme oxygenase (HO) / carbon monoxide (CO) system is a physiological feedback loop orchestrating various cell-protective effects in response to cellular stress. The therapeutic use of CO is impeded by safety challenges as a result of high CO-Hemoglobin formation following non-targeted, systemic administration as well as the use of CO-Releasing Molecules containing critical transition metals. An emerging number of local delivery approaches addressing these issues have recently been introduced and provide exciting new starting points for translating the fascinating preclinical potential of CO into a clinical setting. This review will discuss these approaches and link to future delivery strategies aiming at establishing CO as a safe and effective medication of tomorrow.

Introduction

The heme oxygenase (HO) / carbon monoxide (CO) system is an important cellular feedback-loop towards oxidative and inflammatory insults in man (**Figure 1**) [3]. Central to this system is the inducible enzyme HO-1, catalyzing heme degradation into CO, biliverdin, and Fe^{2+}. Comprehensive reports on cell-protective, anti-oxidative, anti-apoptotic, and anti-inflammatory effects of the HO-1 and its downstream effectors (in particular CO) have been previously detailed [3]. Polymorphisms impairing upregulation of this enzyme have been linked to various pathophysiological conditions including coronary artery disease [4], diabetes [5], or the outcome of organ transplantations [6]. The disease modifying impact of CO was demonstrated in disease models mainly by systemic application either directly applying CO mostly through inhalation or CO-Releasing Molecules (CORM) [3]. Both modalities face substantial challenges driving the need for novel approaches as discussed below. Inhaled CO leads to one principal challenge of this (and other systemic) administration routes as of the rather exhaustive binding of CO to hemoglobin (Hb), e.g. following diffusion through the alveolar membrane when pulmonary administered. Therefore, systemic trafficking of pulmonary delivered CO is mainly in a tightly bound form as the carboxyhemoglobin (CO-Hb) complex and very little 'free' CO is available to act pharmacologically (**Figure 2**). Therefore, the extraordinary stability of CO-Hb requires quite high CO-Hb plasma concentrations such that therapeutically relevant levels of free CO are achieved – critically narrowing the therapeutic window [7].

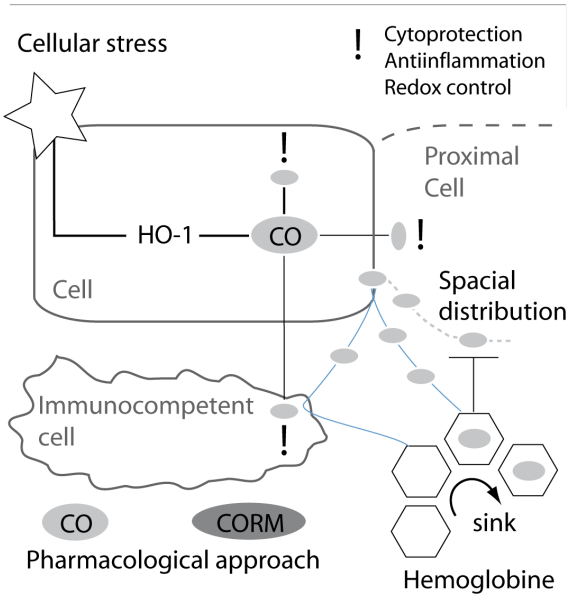

Figure 1: The heme oxygenase (HO) / carbon monoxide (CO) system as an important cellular feedback-loop towards local oxidative and inflammatory insults. Following cellular stress the HO-1 is induced catalyzing heme degradation into CO, biliverdin, and Fe^{2+} (latter two are not shown for simplification) thereby orchestrating various cellular response mechanisms and triggering auto- as well as paracrine effects. Counterbalancing this effect by hampering the unhindered special distribution of CO is hemoglobin constantly removing CO with the blood flow. Approaches for mimicking this feedback loop include the inhalation of CO as well as the application of Carbon Monoxide Releasing Molecules (CORMs).

This conundrum has to be solved for any systemic CO delivery attempt, as very high, perhaps toxic CO-Hb levels have to be condoned in order to achieve therapeutically relevant levels of free CO in the target tissues (**Figure 2**) [8]. This disadvantageous prerequisite is also true for the delivery of CO using systemically administered CORMs. On top, these CORMs aggravate the development risk in that they further hold potentially toxic transition metals required for binding CO (**Figure 2**) [3, 9].

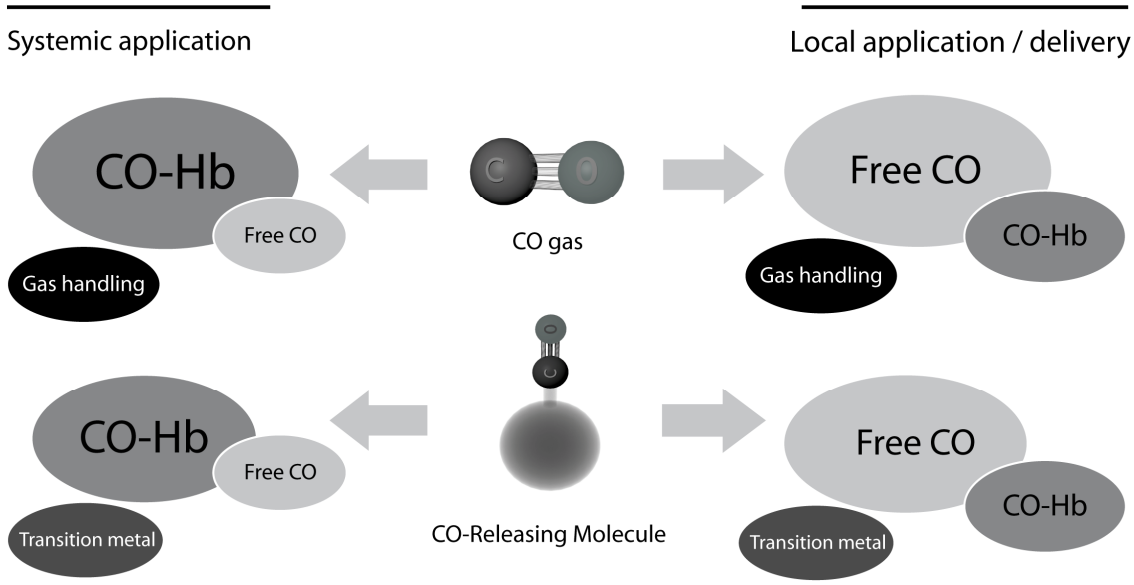

Figure 2: Principle dilemma of CO therapies: The systemic application (left site) of CO or CORMs is challenged by the instantaneous formation of Carboxyhemoglobin (CO-Hb) limiting the availability of free (unbound) CO. On top increasing CO-Hb formation hampers the oxygen transportation thereby provoking systemic toxicity. This can be addressed by the local application of CO or CORMs (right site) with this delivery mode being limited by the complicated handling of gaseous CO as well as safety concerns associated with transition metal of metal based CORMs, respectively.

Therefore, and in spite of the exciting therapeutic potential of CO, current systemic application strategies resulted in an uphill battle against both, the CO-Hb to free CO ratio and potential systemic safety concerns associated with the molecular scaffolds most CORMs deploy. Consequently, novel developments point to local delivery and targeting approaches addressing these concerns. These include CORMs that decarbonylate in diseased hepatic tissue (thereby, the CO is carried into the liver before it releases from the CORM – therapeutically relevant free CO is increased while CO-Hb percentages can be kept low) as well as Drug Delivery Systems (DDS) that locally control CO release in the gastrointestinal (GI) tract (thereby further reducing unfavorable CO-Hb binding while maximizing exposure of the target tissue to free CO) [10, 11]. This review will start off past and current CO delivery concepts and therapeutic evidence, define desirable pharmaceutical specifications

turning CO into a druggable molecule and finally link to future delivery strategies aiming at translating CO into effective medication of tomorrow.

Nature's equivalent for the therapeutic approaches discussed here within is HO-1 being central to the local feedback loop of the HO / CO system (**Figure 1**). Technologies designed for mimicking this loop, therefore, preferably simulate (i) signaling and (ii) the special spatiotemporal characteristics of this system (*vide infra*). Resembling the function as a cellular sentinel, the enzyme is induced by an array of diverse imbalances including inflammation, radiation, and hypoxia [12]. Multiple transcriptional factors including activator protein 1 (AP-1), NF-κB, nuclear factor like 2 (NRF-2), cAMP response element-binding protein (CREB), and enhancer box (E-Box) induce HO-1 expression and are themselves controlled through a multitude of pathways including extracellular-signal regulated kinases (ERK), p38 mitogen-activated protein kinases (p38 MAPK), as well as c-Jun N-terminal kinases (JNK) activation [12]. Thereby, HO-1 orchestrates a network of para- and autocrine events providing a protective response (**Figure 1**). Autocrine effects are mainly attributed to the high diffusivity and stability of CO, a special characteristic that differentiates CO from other signaling molecules [13]. Hemoglobin is tightly controlling this spatial distribution by instantaneously scavenging CO following passage to the capillary networks (**Figure 1**) [14].

The mobility of CO as well as its ability to induce HO-1 has been discussed as important features for effective exogenous application of CO and CORMs [13, 15]. Likewise, genuine HO-1 inducers including cobalt protoporphyrin and hemin have been used as almost equivalent lab stage alternative [3, 16]. Induction is also observed in response to various other compounds including drugs like curcumin [17], acetylsalicylic acid [18], resveratrol [19], lansoprazole [20, 21], and 5-aminosalic acid [22]. The lack of selectivity of this mode of action leading into numerous downstream effects, however, challenged the development of HO-1 inducers for therapeutic purposes [23, 24]. Until today, induction of HO-1 in man was solely demonstrated for hemin [25], which failed in a trial profiling for gastroparesis both in prolonged HO-1 induction (> 7 days) and ameliorating the disease activity [26] contrasting previous preclinical trials [27, 28]. Furthermore, hemin is a fairly unselective molecule that besides the induction of the HO-1 represses δ-aminolevulinic acid synthase, the rate-limiting enzyme for synthesis of tetrapyrrols [12, 29]. Therefore, novel effectors of the HO-1 pathways are required and CO is a promising option discussed here within.

Systemic application of CO

Transferability of preclinical results

The systemic application of CO by inhalation demonstrated efficacy in numerous preclinical models [3] while failing in clinical trials: CO had no effect on endotoxemia in volunteers inhaling 500 ppm CO for one hour resulting in 7 % CO-Hb [30] contrasting previous preclinical reports [31, 32]. Likewise, a safety and tolerability study using inhaled CO in kidney transplant patients motivated by promising preclinical results [33-35] was withdrawn for unknown reason [36]. Other trials focused on local exposure of the lungs through inhaled CO for the treatment of pulmonary diseases. One study on chronic obstructive pulmonary disease (COPD) demonstrated a trend to disease modification following inhalation of 100 ppm CO (resulting CO-Hb was 2.6 % of total Hb) [37]. Three further clinical trials assessing the potential in arterial hypertension, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis following CO inhalation are ongoing [38-40].

The fundamental mechanism of this lack of transferability from animal model systems to humans is not well understood, however, dissimilarities in quantities applied as well as pharmacokinetic differences among species in the specific context of therapeutic gas delivery had been suggested as major translational hurdles [1, 10, 41]. Systemic CO levels can be compared among preclinical and clinical studies using the amount of CO-Hb formation [42]. Due to toxicity challenges (*vide infra*) clinically applied CO was limited to doses equivalent to CO-Hb levels of < 10 %. In contrast the majority of preclinical trials have been conducted with 250 to 500 ppm CO gas [3] resulting in CO-Hb levels above 20 % [43-47]. A solid data package identifying the minimal CO-Hb associated with an anti-inflammatory and cell protective state is not available and potentially tissue and site specific (*vide infra*).

Tissue distribution and free concentration of CO

In-vivo CO is exhaustively bound to hemoglobin (80 %) or cellular proteins including myoglobin (15 %) with less than 1 % being available in free form (free CO, **Figure 2**) [48]. This translates in free physiological tissue concentrations in the nanomolar range [49] being available for activating therapeutic pathways and translating into therapeutic value. The signaling of CO is at least in part linked to reactive oxygen species (ROS) as of inhibiting the cytochrome-c-oxidase [50, 51]. Consequently, linking the quantity of CO-Hb formation with ROS signaling can provide theoretic insights about the minimal CO-Hb level translating into therapeutic effects [51]. Superoxide dismutase (SOD) serving as a surrogate for ROS was increased in bovine pulmonary artery endothelia starting from a minimal effective concentration of 10 ppm CO (corresponding to 11 nM at a pressure of 0.008 mmHg; aqueous solubility of CO is 1.1 µmol/L/mmHg)) [52]. The respective free CO concentration is equivalent to a CO-Hb level of 10 % [48]. Therefore, systemic administration of CO (including application through the lung) would roughly require (already critical) CO-Hb levels of 10 %. Comparing this level to systemic toxicity levels at 16 % CO-Hb (*vide infra*), outlines the narrow therapeutic window for this administration route.

Toxicity of CO

Contrasting the dramatic acute toxicity of high doses [53, 54], CO is safe in low doses [55-59]. In fact CO exposure is physiological in low concentrations in tissues as a result of heme catabolism [60]. Therefore, CO toxicity is closely linked to CO-Hb levels and balancing locally required therapeutic CO levels against CO-Hb by means of effective drug delivery or CORM targeting is instrumental in opening the therapeutic window of this drug to an extent rendering an exciting treatment modality. With respect to acute CO-Hb levels, neurological effects such as headache start from 16 % CO-Hb, leading into severe effects including irreversible neurotoxicity starting from 20 % CO-Hb. Levels from 67 % CO-Hb are lethal [61]. Chronic toxicity of CO is less clear with even physiological concentration of 0.4–0.96 % CO-Hb being discussed to predispose for cardiovascular diseases [55-59]. CO's Lowest Observed Adverse Effect Level (LOAEL) is 2 % CO-Hb and No Observable Adverse Effect Level (NOAEL) at 1.1–1.3 % CO-Hb [62] with both parameters based on controlled inhalation studies detailing ST-segment changes in patients with well-established coronary artery disease [63, 64]. The **National Institute for Occupational Safety and Health** defines a recommended exposure limit (REL – the highest acceptable concentration

at a workplace) of 35 ppm – equivalent to a steady state concentration of 5 % CO-Hb [65, 66]. In spite of these toxicities linked to CO-Hb levels, CO generates no signals of genotoxicity, reproductive toxicity, or carcinogenicity [67-69]. Therefore, acute exposure may be discussed as safe up to levels of 10 % CO-Hb.

CO-Releasing Molecules

Metal carbonyl complexes for CO delivery were introduced in 2002 [70]. Starting off these initial studies, various classes of CORMs have been developed with the ultimate goal to combine efficacy and safety as fundamental requirements for drug approvals (**Figure 3**). Novel targeting approaches including CORMs for hepatic CO delivery significantly reduce safety issues associated with systemic CO toxicity. Metal toxicity, however, is a golden threat running through all metal based CORMs and has been considered the Achilles tendon of this technology due to presumed safety challenges when applied to humans [1]. In fact, most of the CORMs developed until today are carboxygenated organometallic compounds with central atoms including ruthenium, manganese, or molybdenum [71]. In particular, rare metal-based CORMs lack proper safety profiling as their chemistry and toxicity during systemic trafficking is poorly understood [1, 9, 70, 72]. Future studies and alternative development strategies such as metal free CORMs (*vide infra*) are needed to rigorously address these challenges and to strengthen the available safety assessments today.

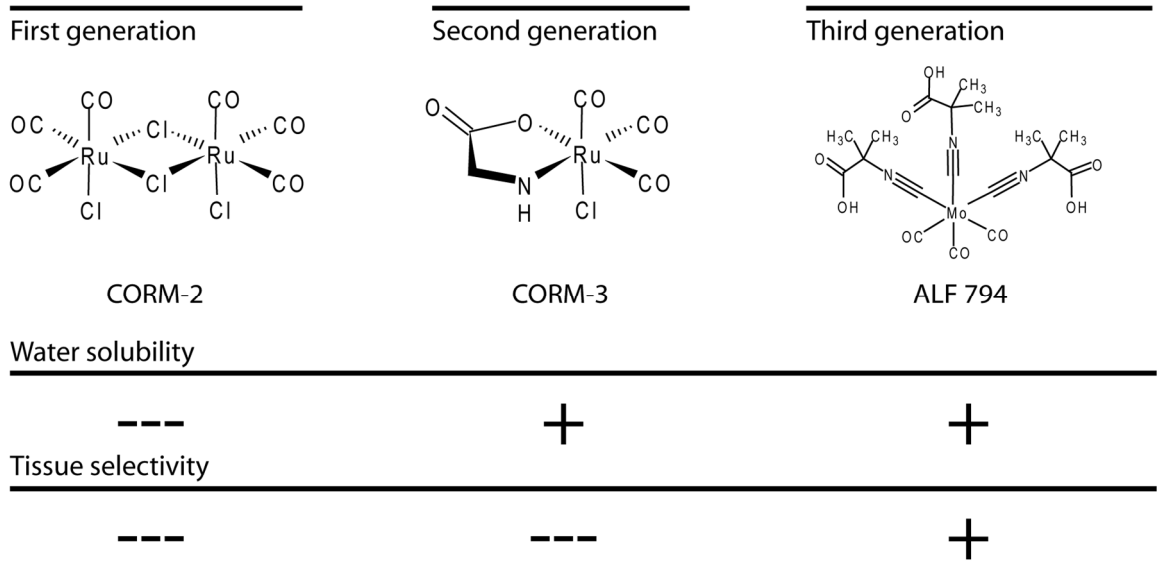

Figure 3: Development stages of CORMs: Starting off water insoluble metal carbonyls (first generation) CORMs were improved with respect to their water solubility with the efforts resulting in water soluble second generation derivatives. The third generation was designed for tissue specific delivery addressing the risk-benefit profile of the systemic CO application.

First and second generation CORMs

The first generation of CORMs started of common metal carbonyls including Tricarbonyldichlororuthenium(II) dimer (later named CORM-2, first generation, **Figure 3**) with unfavorable low water solubility [73]. Subsequent optimization strategies, therefore, aimed at improving the water-solubility of these compounds resulting in water soluble derivatives (CORM-3, second generation, **Figure 3**) [74]. Surprisingly, recent results suggested that early and widely used ruthenium based CORMs such as CORM-2 and CORM-3 specifically release CO in response to sulfite species including intracellular sulfur moieties, thereby facilitating CO release within cells and providing novel targeting modalities [70, 71, 75-78]. Also CO release from the extensively used metal free, boron based CORM-A1 and derivatives thereof is pH dependent, potentially opening the possibility for site specific CO release at ischemic sites with low pH [79, 80]. Metal free CORMs such as these boroncarboxylates [80] have been developed for many years aiming for an improved safety profile of the CORM technology by moving away from transition metals. In contrast to most metal based CORMs releasing CO through a ligand transfer reaction in physiological media, CO release from most organic compounds requires non physiological triggers including high temperature or UV light [81]. Addressing this issue, an emerging number of recent developments provide a novel starting point for metal free approaches [81]. An interesting prototype concept was recently introduced, within which CO is generated through a reaction of two organic educts (Diels Alder Reaction) [82]. The approach was referred to as ‘click and release’ reflecting the ability of generating CO by mixing both educts under physiological conditions. Aiming for the translation of this concept into a druggable molecules featuring local delivery novel derivatives are currently being developed casting this bimolecular concept into a unimolecular system comprising both organic educts in one molecule [81]. To achieve this goal stability during storage and systemic trafficking has to be ensured by separating both reactive moieties using a linker sequence (preferably a side specific cleavable linker, *vide infra*). Ultimately the approach therefore aims at developing a molecule releasing CO only when the desired location is reached at which the linker will be cleaved (e.g. by a selective enzyme), and the intramolecular CO generating reaction is initiated accordingly [81].

Third generation CORMs

The third generation addresses safety concerns of the preceding CORM generations improving the risk benefit profile for the therapeutic use of CO [1, 2, 10, 11, 83, 84].

Molybdenum compounds for liver targeting

Carlos Romao and his team pioneered in designing CORMs with tissue specify and drug like properties. In 2012 they introduced their concept for CORMs, which divides the molecule in a "coordination sphere and a drug sphere [1]. The "coordination sphere" is the central element of the CORM and functional for hosting all ligands including CO (**Figure 4A**). This sphere is surrounded by the "drug sphere", providing structural modifications tailoring the pharmacokinetic profile of the CORM.

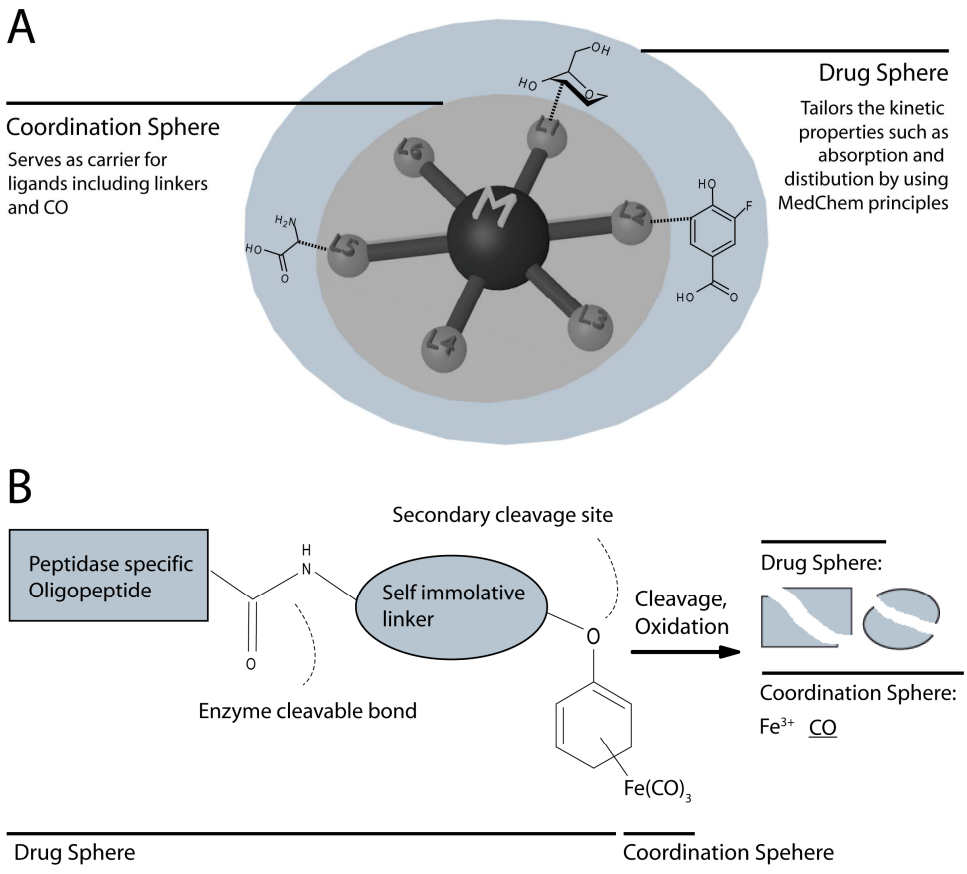

Figure 4: Structural concepts of third generation CORMs (adapted from [1, 2], with modifications):
A) Conceptual model of third generation CORM: The "coordination sphere" is the central element of the CORM and functional for hosting all ligands including CO. This sphere is surrounded by the "drug sphere", providing structural modifications tailoring the pharmacokinetic profile of the CORM. B) Protease Triggered CO-Releasing Molecules. A protease specific peptide is via a self-immolative linker coupled to a CO releasing basic structure (acyloxy-diene-$Fe(CO)_3$ complex). CO release is initiated exclusively in the event that a specific protease cleaves the peptide sequence. A cascade like process thereafter results in CO release.

Starting off this design scheme, various series of organometallic compounds were introduced leading to specific CO delivery into hepatic tissue (third generation, **Figure 3**) [10]. This targeting modality of CO (site specific release of CO in the liver) allowed for addressing several hepatotoxic conditions (**Table 1**) [85, 86]. For example, acetaminophen induced acute liver failure (ALF) [87] was previously alleviated in response to inhaled CO in rodents [88]. Based on these pilot studies, molybdenum CORMs were tailored for stability and safety leading into a drug accumulating in the liver with CO release being triggered by liver (microsomal) enzymes [88]. This exciting strategy of localized CO generation in hepatic tissues (but to a lesser extent in off-liver tissues) proofed effective in ALF mice and resulted in uncritical CO-Hb formation (< 7.6 % CO-Hb) even when dosed at 100 times the effective single dose. These studies demonstrated the advantage of targeted delivery of CORMS and site directed generation of CO within the target tissue.

Table 1: Preclinical status of the hepatoprotective potential of CO and HO-1 inducers

Model	**Therapeutic agent/route of application**	**Reference**
Ethanol induced liver injury, mouse	CoPP and CORM-A1, i.p.	[89]
Hemorrhagic shock, rat	CO enriched red blood cells, i.v.	[90]
LPS cholestasis, rat	CO, inhalative	[91]
Caecal ligation and puncture induced septis, mouse	CORM-2, i.v.	[92]
LPS induced liver injury, mouse	CoPP, i.p.; methylene chloride, oral	[93]
Cytokine and CD 95 mediated apoptotic liver damage, mouse	CoPP, i.p.	[94]
TNF-alpha/D-gal–induced hepatitis, mouse	CO, inhalative	[88]
Acetaminophen liver failure, mouse	Various molybdenum CORMs, i.v.; CO, inhalative	[10] [88]
Hepatic ischemia reperfusion injury, rat and mouse	CO, inhalative; CO or CORM-3 applied to the graft storage and perfusion solution; HO-1 adenovirus transfection; hemin, i.p. ; CoPP, i.p.	[38, 40, 95-103]

ET-CORMs

Based on enzyme responsive drug delivery, the Enzyme Triggered CORM (ET-CORM) concept was introduced for site specific delivery of CO in 2011 (**Figure 4B**) [104]. Enzyme responsive delivery typically deploys a cleavable linker sequence functional for the local release of a conjugated drug [105]. These studies are based on an excitingly broad platform of site specific linkers, ranging from organ specific trypsin cleavable linkers addressing pancreatic tissue, to linkers susceptible to specific metalloproteinases present in inflamed tissue [106]. A second generation applying further structural modifications was developed for tailoring release kinetics, accordingly [107]. Recently the concept was adapted to proteases by introducing protease specific oligopeptides connected to a self-immolative linker (**Figure 4B**) [108]. These compounds feature gradients of index proteases in diseased tissues as stimulus leading to CO release. Tissue or disease-state specific drug delivery has - to our knowledge - not been reported to date.

Pharmaceutical formulations of CORMs

Within the last years an emerging number of controlled drug delivery systems (DDS) have been introduced for CORMs. Some of these DDS feature first generation CORMS for stimulus driven CO generation, thereby forming an alternative approach to the exciting achievements reported for third generation CORMs (*vide supra*). Successes were reported for systemic as well as non-invasive use [78, 84].

Dermal delivery

CO has been attributed with bactericidal activity and consequently been discussed as a new bactericidal agent for multi drug resistant bacteria, a growing and inadequately addressed problem [109, 110]. CO releasing antimicrobial hybrid non-wovens were used for dermal applications [111, 112]. These electrospun polymers contained photo-responsive CORMs releasing CO in response to UV-irradiation (‘photoCORM’). Formulating the CORM into a wound dressing allowed for local CO delivery (reducing CO-Hb formation by minimizing systemic exposure) by activating the release system with light within the wound dressing.

Gastrointestinal delivery

CO is a basic regulating factor in GI motility, and inflammation [113] and has therapeutic potential for GI diseases including ulcerative colitis, postoperative ileus, and diabetic gastroparesis (**Table 2**) [114, 115]. Addressing safety challenges of systemic applications CORM-2 was formulated into a styrene-maleic acid copolymer leading to a sustained CO release (21.2 hours) compared to CORM-2 alone (0.6 hours). Orally applied, the controlled release system proved efficacy in a colitis model [84]. In an effort to develop a CO releasing system providing controlled and local GI delivery the Oral Carbon Monoxide Releasing System (OCORS) was developed [11]. OCORS is a tablet based release system. It comprises a CORM (e.g. iron or ruthenium based [116]) as well as a second compound triggering CO release once the two molecules are contacted in solution. The kinetic of this contacting reaction was precisely controlled by the tailorable permeation rate of GI fluids into the system, controlled by the hydrophobicity of the coatings covering the system. The approach allowed for the precise clocking of CO release from minutes to days thereby allowing controlled and local delivery of CO to the GI tract. This feature was also confirmed in murine *in-vivo* studies [116]. Likewise an OCORS formulation providing extended release kinetics for intestinal delivery proved to be successful in chemically induced colitis in mice (TNBS) while resulting in minimal CO-Hb formation (< 1.4 % CO-Hb) [116].

Table 2: Preclinical status of the therapeutic potential of CO and HO-1 inducers in the gastrointestinal tract

Model	Therapeutic agent	Reference
Diabetic gastroparesis, mouse	CO, inhalative; Hemin, i.p.	[27, 117]
Postoperative ileus, mouse	CO, inhalative; CORM-3 i.p.; CO saturated ringer's solution, i.p.	[118-121]
Inflammatory bowel disease: IL-10 (-/-)-,TCR-α (-/-)-, TNBS-, and DSS colitis mouse model, TNBS rat model	CO inhalative; 5-ASA intracolonic; ALF-186 CORM-2, and CoPP i.p.	[22, 122-126]

Extracorporal delivery approaches - CO in transplant medicine

Ischemia-reperfusion injury (IRI) is a major limiting factor in the transplantation of solid organs [127]. The pathophysiology is a complex interplay resulting from inflammation, cell damage, and apoptosis, thereby limiting organ quality following transplantation [127, 128]. Moreover, adequate protection may allow harvesting organs from borderline donors. Successfully addressing this challenge, therefore, holds promise to directly expand the donor pool as the pace making step in organ transplantation. With currently no pharmacological approach available [99, 129] the potential of CO and HO-1 inducers in IRI was intensively studied in various preclinical and clinical settings [128, 130]. CO is associated with protective effects towards vascular endothelial cells during ischemia, as well as anti-inflammatory and vasodilatative effects during reperfusion, thereby reducing the stress to the transplant. Interestingly, the aforementioned challenge as of CO-Hb formation is less of a concern within the transplants, as these are exsanguinated before transport [40, 128, 131]. The translation of CO gas, however, is challenged by inevitable occupational safety concerns during solution preparation for medical personnel [83]. This roadblock was recently addressed by a system referred to as Carbon Monoxide Releasing System for Transplants (CORST) [83]. The system is based on a CO permeable silicone membranes encasing a CORM as well as a cartridge (with a predetermined breaking point) within which an aqueous solution with the CO release trigger is held (resembling the design of a glow stick). CO release from the system can be initiated by breaking the container at the predetermined breaking point, thereby contacting both compounds and triggering CO release. The striking advantage of CORST is that all by- and degradation products are strictly retained within the system (only CO is released) hence transplant exposure is limited to CO only and patient exposure to by- and degradation products following transplantation is avoided.

Conclusion

The therapeutic potential of local CO delivery is enormous as physiological mechanisms have been elucidated and linked to various preclinical mechanistic as well as efficacy studies. Likewise, numerous approaches for the delivery of this challenging gaseous drug have been introduced, thereby providing a versatile toolbox for future translational approaches. Inspired by these, an emerging number of local and target delivery approaches have been introduced addressing the major issue of systemic CO toxicity as of rather exhaustive CO-Hb binding. Future developments have to proceed further in this direction and on top rigorously address toxicity of metal based CORMs. Overcoming these hurdles holds great promise to ultimately result in a clinical tool utilizing the HO / CO system as a central physiological mechanism to address an array of currently untreatable diseases.

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Chapter 2: Oral drug delivery of therapeutic gases - carbon monoxide release for gastrointestinal diseases

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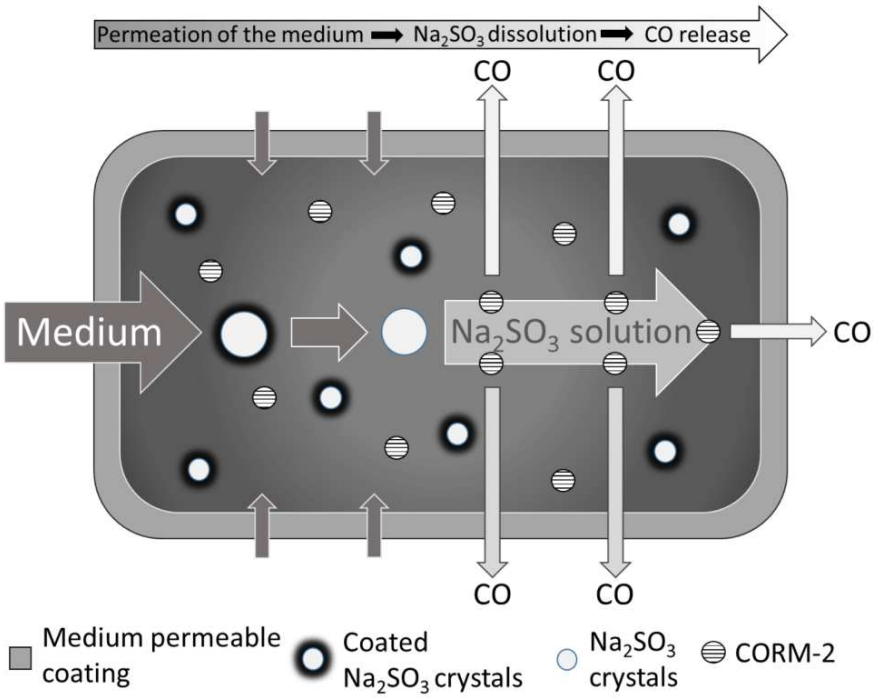

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Abstract

Deploying the therapeutic potential of carbon monoxide (CO) in various gastrointestinal diseases is challenged by inappropriate oral delivery modes. It is for this challenge, that we developed an easy to use tablet referred to as oral carbon monoxide release system (OCORS) providing precise, controlled, tunable and targeted CO delivery for the treatment of sequelae of gastrointestinal diseases. OCORS is an oral tablet based on sulfite induced CO release from the CO releasing molecule 2 (CORM-2). OCORS performance was detailed as a function of the presence of buffer within the tablet core and the composition of a semipermeable cellulose acetate coating, shielding the tablet core. OCORS delivered CO for up to 10 hours. This controlled release system delivered the therapeutic gas independent of environmental pH for reliable CO generation at gastric, intestinal or colonic sites. *In vivo* experiments and toxicological assessments of OCORS are required to demonstrate the pharmacokinetics and clinical potential of this oral delivery platform for therapeutic gases.

Introduction

The traditional perception of carbon monoxide (CO) as a harmful gas is shifting towards deploying this exciting physiological transmitter for therapeutic purposes[1]. CO is derived from heme oxigenase (HO) activity, degrading heme to CO, iron and biliverdin [2]. Two HO isoforms have physiological relevance, with HO-1 being rapidly responding to various stimuli, including CO [3], chemical or physical stress [4]. In contrast, HO-2 is constitutively expressed in various tissues, including neurons, liver, kidney and the vascular endothelium [5]. CO has beneficial impact on inflammatory conditions including down-regulation of pro-inflammatory proteins such as interleukin 1β or tumor necrosis factor-α, a process which is regulated by mitogen-activated protein kinase (MKK-3)/p38 through the mitogen-activated protein kinase pathway[6]. Evidence for disease modifying impact of CO in the gastrointestinal arena including inflammation and repair of injury has been collected for diabetic gastroparesis, post-operative ileus, organ transplantation, inflammatory bowel disease and sepsis in both, relevant animal model systems and in humans for some but not all of the mentioned indications (**Table 1**) [1, 7-9]. In spite of the promising therapeutic potential, a readily available, reliable, easy to use, and safe oral CO delivery system targeting gastrointestinal sites is unavailable and contrasting the medical need [1]. Most studies deliver CO gas through the lungs [10-13], a mode of administration challenged by a lack of targeting to the affected tissues. Local CO delivery was realized by taking advantage of the gases strong affinity to transition metals, leading to the development of carbon monoxide releasing molecules (CORM) [14-16]. CO release from CORM has been induced by light [17-19] or by administration of sulfur compounds [20-22], limiting the application to conditions within which easy accessibility of these exogenous stimuli to the CORM is feasible or within which environmentally present molecules such as cysteines trigger release in a bioresponsive fashion [20]. Therefore, the accessible routes of administration for gastrointestinal diseases are surprisingly limited to either direct inhalation, intestinal insufflation of CO gas or an inconvenient, complicated and risky intraperitoneal administration of CORMs rendering clinical use rather challenging (**Table 1**). It is this pharmaceutical gap which in our view critically challenges the rigorous exploitation of CO for gastrointestinal diseases.

Consequently, we addressed the challenge by developing OCORS, a CO releasing tablet for oral use. The system is designed to shuttle CO to the sites of gastrointestinal lesion or inflammation through controlled delivery by the oral route.

Table 1: Routes of administration of CO-based therapies for gastrointestinal diseases

Indication		Route of administration	Species	Reference
Inflammatory bowel disease	CO	Inhalation	Mouse	[10-13]
	CO	Colonic insufflation	Rat	[23]
	CORM	intraperitoneal	Mouse	[14-15]
Diabetic gastroparesis	CO	Inhalation	Mouse	[24]
Postoperative ileus	CO	Inhalation	Mouse	[25]
	CORM	intraperitoneal	Mouse	[26]
Gastric ulcer	HO-1 inducer	intraperitoneal	Mouse	[27]
	HO-1 inducer	intragastrical	Mouse	[48]

Experimental Details

Materials

Carbon monoxide releasing molecule 2 (CORM-2); Tricarbonyldichlororuthenium(II) dimer; $[Ru(CO)_3Cl_2]$[16]), CORM-A1 (sodium boranocarbonate; $Na_2[H_3BCO_2]$), and absolute ethanol were purchased from Sigma Aldrich Chemie (Schnelldorf, Germany). Citric acid was from Jäkle Chemie (Nürnberg, Germany). Na_2SO_3 was purchased from Grüssing (Filsum, Germany). Eudragit E PO was from Evonik Industries (Essen, Germany). Tableting mixture ("Tabletiermischung, technisch") consisting of 88 % lactose, 9 % cellulose, 2 % aluminium oxide and 1 % magnesium stearate was from Meggle (Wasserburg am Inn, Germany). Potassium phosphate was from Acros Organics (Nidderau, Germany). Renex™ PEG 400 was from CRODA (Nettertal, Germany). Sam specracol erythrosine lk was from Sensient (Geesthacht, Germany). 100 ppm calibration gas (diluted in air) was from Real Gas (Martinsried,Germany). Eastman cellulose acetate CA398-10NF/EP was from Gustav Parmentier GmbH (Frankfurt am Main, Germany). All other reagents were from Sigma Aldrich (Schnelldorf, Germany) and at least of pharmaceutical grade unless otherwise noted.

Amperometric detection system

We used an Erlenmeyer flask purchased from Gebr. Rettberg (Göttingen, Germany) as reaction space. For insertion of the CO sensor and to seal the reaction space, a conically tapered joint from Schott Medica (Wertheim, Germany) was equipped with a glass guide tube for an electric wire. As a prerequisite for cross referencing with calibration gas, a single-bore stopcock was connected to the Erlenmeyer flask as well as to the conically tapered joint. The integrated CO sensor of the "Ei207D" CO detector (Ei Electronics, Shannon, Ireland) was removed and

externally connected using a "Wire Wrap" 0.404 mm² wire (Kabeltronik, Denkendorf, Germany) linked with a 1.3 mm accessory shoe (Vogt AG, Lostorf, Switzerland). The wire was glued into the guide tube with "UHU plus endfest" epoxide resin (UHU, Bühl, Germany) to seal the system. On-line videos were collected monitoring the detector with USB webcams with the Eyeline video surveillance software (NCH Software, Canberra, Australia).

Measurements of CO release

CORM-2 suspension

The CO release from CORM-2 was triggered by Na_2SO_3 and amperometrically detected. For that, 4.3 mg CORM-2 were placed in the Erlenmeyer flask filled with 15 ml Millipore water and stirred at 130 rpm (Variomag Telesystem, Thermo Scientific, MA). After 25 minutes, 10 mg Na_2SO_3 were added to the reaction space. CO release in ppm as read from the detector of each system was calibrated with 100 ppm CO calibration gas (diluted in air) and normalized, accordingly (see Figure 1 for results).

Calibration of CO release from CORM-2

A concentration series of CO was generated by transferring different amounts of CORM-2 into 25 mL of a stirred (130 rpm) 333 mg/L aqueous Na_2SO_3 solution. CO release [ppm] after 60-80 minutes was plotted against the amount of CORM-2 [mg] placed into the system. Following this procedure, CO release [ppm] is expressed as amount of CORM-2 [mg], with one CORM-2 equivalent being defined as the amount of CO released per milligram CORM-2 after 60 – 80 minutes and when exposed to the experimental conditions described in this section (see Figure 2B for results). CO release from CORM-2 expressed in CORM-2 equivalents [mg] followed a linear pattern ($y = 0.0192 + 1.0199x$; $r^2 > 0.99$; n = 1 for 14 independent measurements).

Calibration of CO release from CORM-A1

The calibration of CO release using CORM-A1 was modified from previous reports [28]. In brief, a concentration series of CO was generated by diluting different amounts of CORM-A1 in ice water and transferring this solution to 25 mL of a stirred (130 rpm) citric acid buffered solution (pH 5.5) at room temperature. Release of CO expressed as CORM-2 equivalents [mg] (*vide supra*) was recorded as a function of CO release from different amounts of CORM-A1 [µmol]. Thereby, CO release from CORM-A1 was linked to CO release from CORM-2 and,

consequently, CO release from CORM-A1 could now be expressed in CORM-2 equivalents [mg] by means of linear regression ($y = -0.0776 + 0.6996x$; $r^2 > 0.99$; (n = 3).

Data correction

CO data reported within the manuscript is calculated from the corresponding release from CORM-2 equivalents and if reported for CORM-A1, calculated using linear regression. The sensor is destructive to CO (oxidizes CO to CO_2) and hence consumes the analyte. Therefore, CO loss by and from the system was quantified and used as basis for data correction. For the correction, the loss by and from the system was recorded and added to the data as read from the detector (*vide infra*). For assessment of CO loss and data correction, CO was generated from CORM-2 in presence of Na_2SO_3 with stirring at 130 rpm, until reaching a value of approximately 800 ppm. At this point the stirring was stopped. CO loss [ppm] was recorded when a value of 720 ppm was reached and followed an exponential decline ($y = 717.5 * e^{-0.145x}$; $r^2 > 0.99$; n = 5; see Figure 3 for results). This regression was used for data correction and the respective computer sheet for calculation is provided (Supplementary information, available online).

Na_2SO_3 crystal collection and crystal coating

Na_2SO_3 crystals of appropriate size were collected using an AS 200 Retsch analytical sieve tower (Haan, Germany) and the 250 – 500 µm fraction was used. These crystals were coated using solutions consisting of 8.6 g Eudragit E PO, 0.9 g sodium dodecyl sulfate, 1.3 g stearic acid, 4.3 g talcum, 50 mL of distilled water and 50 mL of absolute ethanol. The dye Sam specracol erythrosine lk was added in minute amounts to visualize the coating with the overall recipe following the manufacturer's instructions [29]. The preparation was homogenized for 20 min at 13'000 rpm using a Silent Crusher M (Heidolph, Schwabach, Germany) and sieved through a mesh with 375 µm aperture size for removal of disruptive agglomerates. 60 g Na_2SO_3 crystals were coated with a Mini-Coater (Glatt, Binzen, Germany) used in top-spray configuration at a temperature of 45 °C, an atomizing pressure of 0.86 bar. The coating solution was pumped into the coater by a Flocon 1003 flexible tube pump (Roto-Consulta, Lucerne, Switzerland) at 0.7 mL / min. Coating lasted for about 2 hours and the fluidized bed was maintained for another 10 minutes, thereafter. Na_2SO_3 content of the coated crystals was 86 % as quantified by the compendial method for iodometric determination [30] . Scanning electron microscopy (SEM) was used to assess the homogeneity of the coating of Na_2SO_3 crystals and the distribution of Na_2SO_3 crystals within the tablet. Samples were sputter coated with palladium/gold prior for evaluation on a JEOL JSM 7500F scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 5 kV using lower secondary electron signals.

Development of the oral carbon monoxide release system (OCORS)

Preparation of the tablets

Tablets were prepared from a blend of 72 mg pulverized citric acid H_2O, 128 mg pulverized trisodium citrate 2 H_2O (pH of the buffer system: 4.5), 200 mg coated Na_2SO_3 (*vide supra*), 60 mg CORM-2 and 1.54 g tableting mixture (Meggle), mixed for 30 minutes in a Turbula T2F mixer (WAB AG, Muttenz, Switzerland). The resulting blend was transferred into an eccentric tableting machine model FE136SRC from Korsch (Berlin, Germany) using a 7 mm tablet punch from Korsch (Berlin, Germany) resulting in average tablets weights of 120 mg. Non-buffered tablets were prepared by replacing the amount of citric acid buffer mentioned above by the tableting mixture. A tablet coating solution was prepared from 0.9 g PEG 400 as pore former in 100 mL acetone (1 x pore former PEG 400; *vide infra*)into which 5.8 g cellulose acetate was slowly added under stirring at 130 rpm. In another set of experiments, 9 g of PEG

400 was introduced (10 x pore former PEG 400; *vide infra*). The tablet cores were dip coated in the cellulose acetate coating. For that, the cores were completely immersed into the coating solution and subsequently air dried using an air gun at about 60 °C for 1 min. Thereafter, the pre-dried sample was transferred into a desiccator and left in an ED 53 drying chamber (Binder, Tuttlingen, Germany) at 50 °C for 30 min. Tablet cores were coated once (10 x pore former PEG 400 used), four or eight times (1 x pore former PEG 400 used). Although OCORS was handcrafted with an overall production time of roughly four hours for a batch of 20 tablets the applied techniques can easily be upscaled for an industrial production process. The structure of the tablet core and the coating were assessed following palladium/gold sputter coating and by a JEOL JSM 7500F scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 5 kV using lower secondary electron signals.

CO release from the oral carbon monoxide release system (OCORS)

Coated tablets were transferred into 25 mL of distilled water, compendial simulated gastric fluid without enzymes (prepared as described in USP 37 [31]) or simulated intestinal fluid without enzymes (prepared as described in USP 37 [31]). Experiments assessing the impact of the release medium on CO release (see Figure 6A, B) were done in comparison to control experiments, within which 12 mg coated Na_2SO_3 crystals were measured in simulated gastric fluid without enzymes to control for possible detector interference with sulfites (interference was not observed with these experimental conditions; data not shown). Experiments assessing the impact of the coating procedure on CO release were done in comparison to control experiments, within which 3 mg CORM-2 was suspended in 25 mL of 0.333 g/L Na_2SO_3 solution (see Figure 7 for results). In another set of control experiments, the impact of the ionic strength of the release medium and the impact of the salt with which the ionic strength was set (using sodium chloride solutions to set ionic strenghts of 0.88 mol/L and 1.8 mol/L, respectively, and magnesium chloride to set an ionic strengths of 1.26 mol/L) on CO release was studied using CORM-2 suspensions. For that, CORM-2 was exposed to 25 mL of 0.333 g/L Na_2SO_3 in the appropriate release medium (for results, see Supplementary Figure 2).

Ruthenium release

CORM-2 contains ruthenium as a metal. Consequently, ruthenium release from tablets coated four times in water was monitored in 25 mL water for 13 hours. Samples collected at the end of the study were filtered using a glass filter (porosity 3; nominal maximum pore size is 14 – 40 µm; from Winzer, Wertheim, Germany) and measured by inductively coupled plasma optical emission spectrometry (ICP-OES) with triple measurement of each data point at 240.3 nm, 245.6 nm, 245.7 nm, 267.9 nm (ICP-OES Vista Pro Radial, Agilent Technologies, Santa Clara, CA). The result was referenced to a 10 mg/L ruthenium standard. An uncoated tablet core was used as positive control and processed as described for the coated tablets (see Figure 8 for results).

Statistics

All data were reported as mean ± standard deviation unless specified otherwise. Statistical significance for ruthenium analytics was calculated by Student's t-test for pairwise comparison and linear regression was made using conventional software packages (Sigma Plot, Systat Software Inc., CA). One-Way ANOVA was used for determination of release pattern and Tukey test for *post-hoc* comparison (Minitab-statistics, Coventry, UK). $p < 0.05$ was considered statistically significant.

Results

Sulfite triggered release from CORM-2

CO release of CORM-2 in the presence of water was studied. CORM-2 suspended in water demonstrated no detectable CO release (**Figure 1)**. After 25 min, Na_2SO_3 was added triggering instantaneous CO release, which was completed within 25 minutes, and plateauing at 550 ppm after 40 minutes (**Figure 1**).

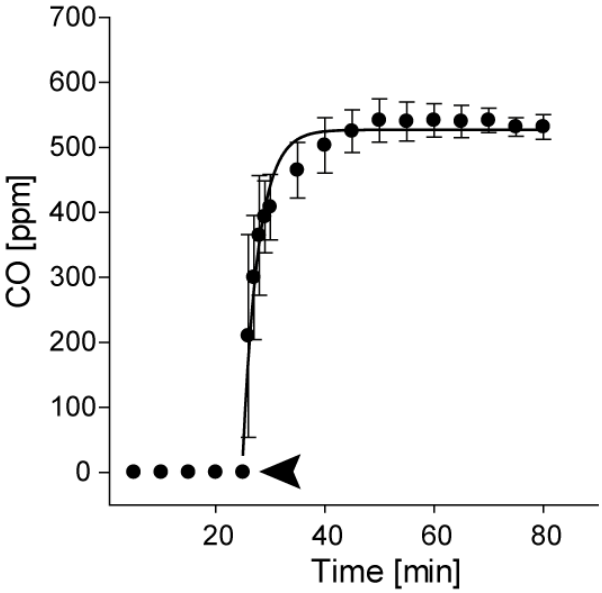

Figure 1: CO release [ppm] over time [minutes] from aqueous CORM-2 suspension. Na_2SO_3 solution was added as indicated by the arrowhead (n = 3).

Calibration procedure

The amperometric sensor was connected to the CO detector and continuously monitored the headspace within a sealed Erlenmeyer flask, within which the CO release experiments were conducted (**Figure 2A**). A schematic drawing of the setup is provided (**Supplementary Figure 1**). CO release from CORMs was expressed in CORM-2 equivalents, with one CORM-2 equivalent being defined as the amount of CO released per milligram CORM-2 and when exposed to 25 mL of a stirred (130 rpm), 333 mg/L aqueous Na_2SO_3 solution for 60 – 80 minutes. The time frame of 60 – 80 minutes was sufficient to ensure > 90 % release (**Figures 1, 3**). CO release from different amounts of CORM-2 directly correlated to the CORM-2 equivalents, indicating the suitability of the amperometric platform and the normalization method deployed here within for the assessment of CO release profiles (**Figure 2B**). This calibration procedure was instrumental for normalizing the three amperometric systems used in parallel for the experiments. The regression of the CO released from different amounts of CORM-A1 against CORM-2 equivalents indicated a linear relationship with a slope of approximately 0.7 (**Figure 2C**).

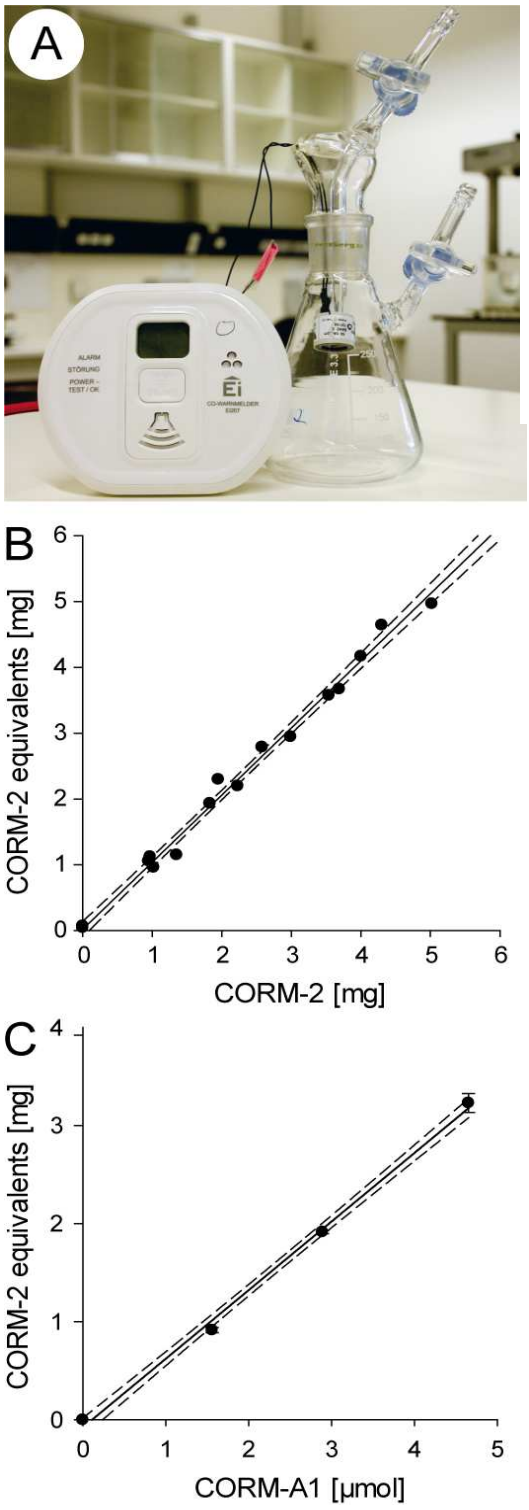

Figure 2: (A) Amperometric detection system used to study the (B) calibration of CORM-2 (n = 1 for 14 independent measurements), expressed as CORM-2 equivalents [mg] (one CORM-2 equivalent represents the amount of CO [ppm] released per milligram CORM-2 when exposed to 25 mL in a 333 mg/L aqueous Na_2SO_3 solution at room temperature). (C) Calibration of CORM-A1; n = 3; dashed lines indicate the 95% confidence interval.

Data correction was necessary to further refine the data and reflecting the destruction of CO by the sensor (oxidizes CO to CO_2), and hence consumption of the analyte or possible escape from the system. For that, the actual released amount was calculated by correcting the data as read from the display by the CO loss curve (**Figure 3A**). CO loss over time started at 720 ppm and declined to 150 ppm within 10.5 hours, following an exponential decline (**Figure 3A)**. The impact of data correction was outlined by control experiments using sulfite triggered CO release from CORM-2 (**Figure 3B**). Without correction, CO release peaked at approximately 60 - 80 min followed by a subsequent decline reflecting CO consumption by the sensor or loss of CO from system, ultimately declining to a third of its peak value after 8.5 hours. Applying the correction function (**Figure 3A**) plateauing at approximately 140 minutes was observed and no decline, thereafter (**Figure 3B**). These calculations are detailed in the appendix (**supplementary information –** available online).

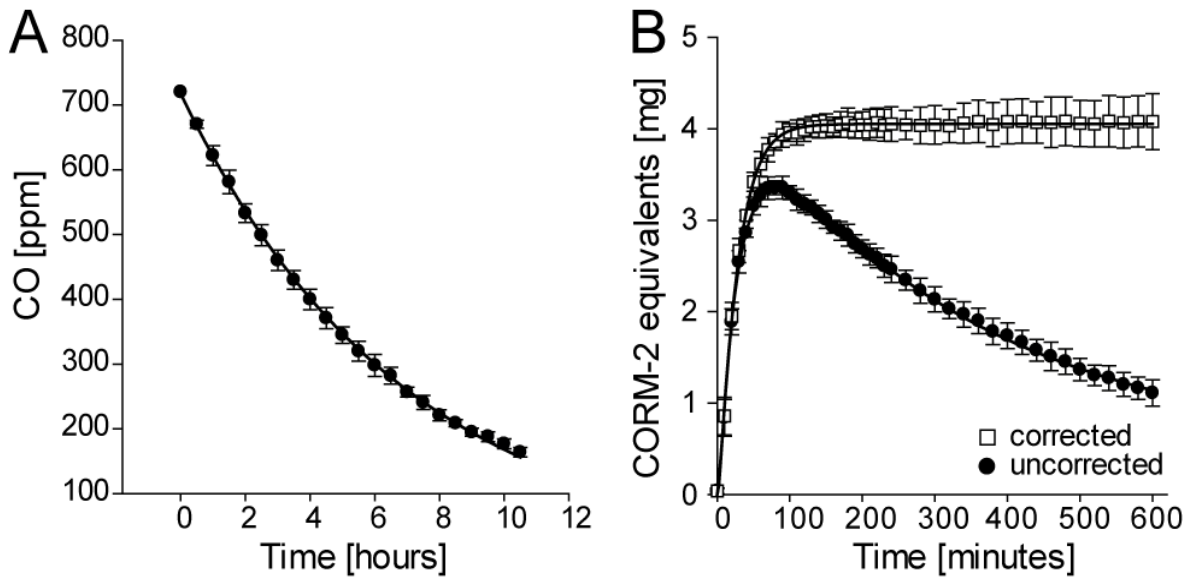

Figure 3: (A) CO loss [ppm] in the amperometric detection system over time [hours] following complete release of CORM-2 in 25 mL Na_2SO_3 solution (n = 5). (B) CO release profile using CORM-2 in 25 mL Na_2SO_3 solution plotted as uncorrected, raw data (circles) and corrected data (squares) over time [minutes]. Corrected data was calculated using the CO loss function as shown in A; (n = 3).

Development of the oral carbon monoxide release system (OCORS)

OCORS is a CO release system, controlled by permeation of a medium (water) through the semipermeable cellulose acetate shell. The permeating medium causes swelling of the coating around the coated sodium sulfite crystals within the tablet core and rapid dissolution, thereof. Thereby, dissolved sodium sulfite gets access to the CORM-2, sparking CO release. Cellulose acetate coating was used to control the flux of the medium into the system (**Figure 4**).

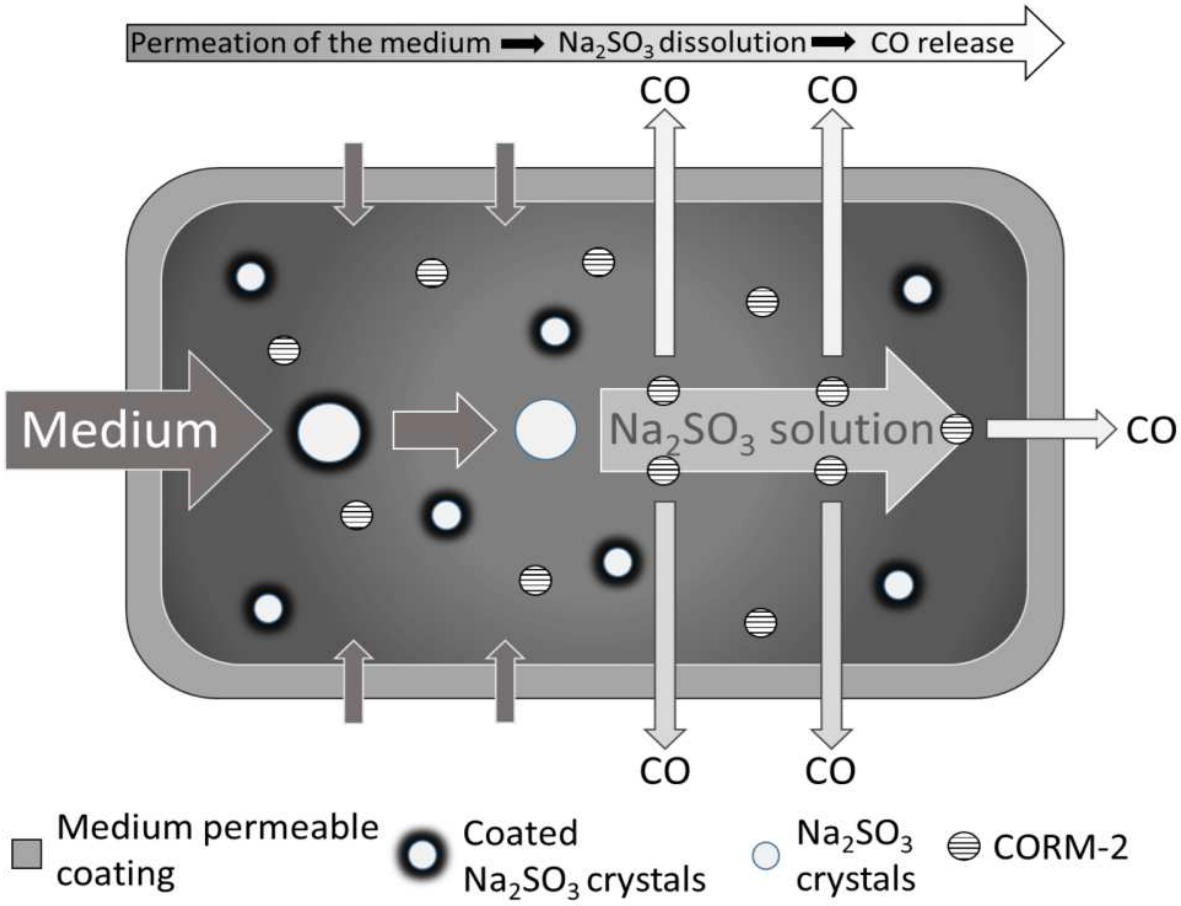

Figure 4: Schematic drawing of the oral carbon monoxide release system (OCORS). Left part of the cartoon: Medium permeates the semi-permeable cellulose acetate shell, dissolving the swelling coating layer around the sodium sulfite crystals which readily dissolve. Right part of the cartoon: The Na_2SO_3 interacts with CORM-2, thereby causing CO release.

The coating around the sodium sulfite crystals was instrumental in separating it from CORM-2 in the solid state and as a prerequisite to allow sustained storage of OCORS without CO release. The coating of the sodium sulfite crystals resulted in smooth films covering the entire crystal surface (**Figure 5A, B**) and coated crystals were homogenously distributed throughout OCORS (**Figure 5C**). OCORS's shell consisted of a smooth cellulose acetate coating (**Figure 5D**).

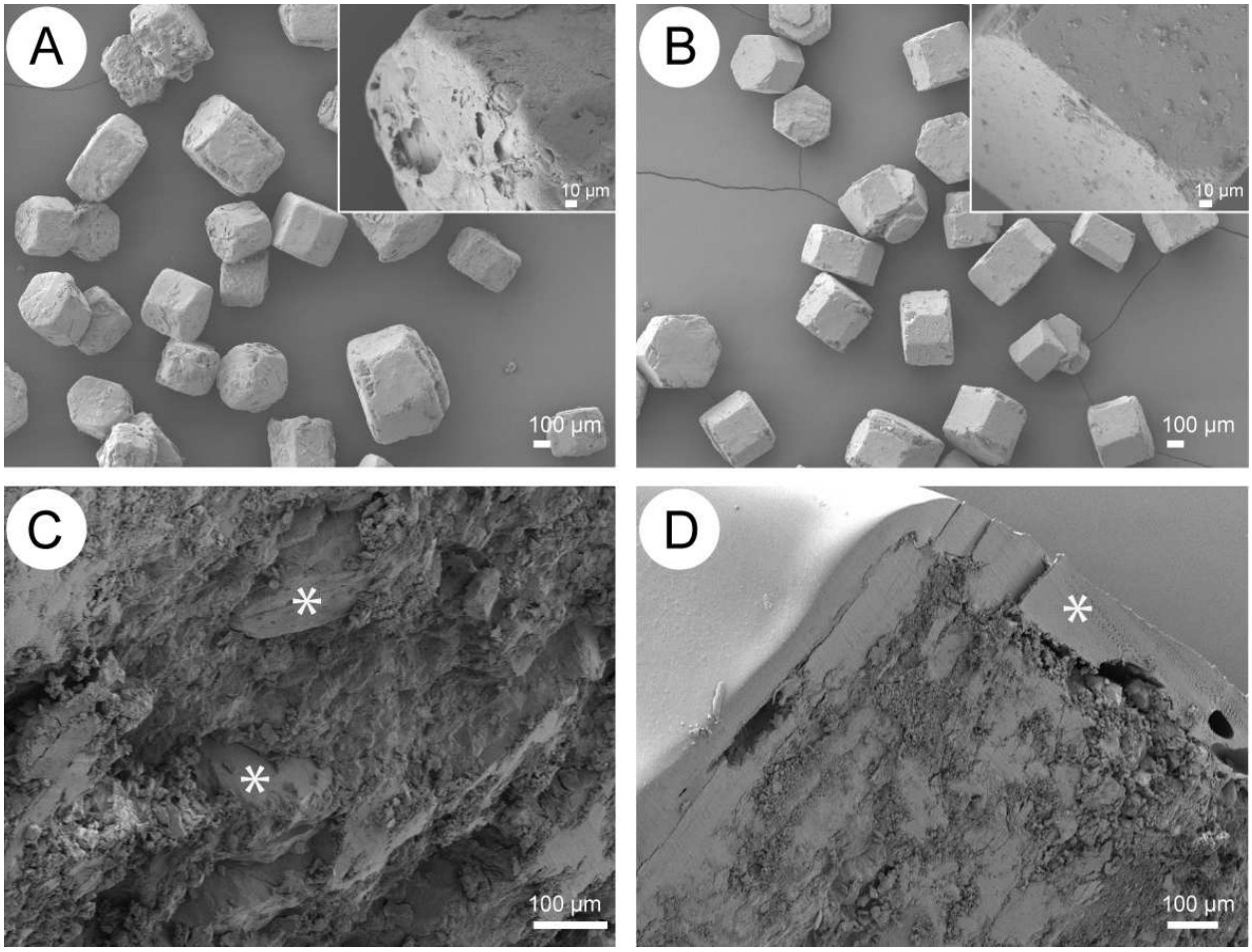

Figure 5: SEM images of (A) coated Na_2SO_3 crystals (B) uncoated Na_2SO_3 crystals (C) coated Na_2SO_3 crystals within the bulk of the tablet core (asterisks) and the (D) cellulose acetate coating of the oral carbon monoxide release system OCORS (asterisks). Inserts are magnifications of (A) coated crystals and (B) uncoated Na_2SO_3 crystals.

When OCORS was prepared from powder blends with no citric acid buffer, CO release was affected by the release media, with exposure to simulated gastric fluids (pH 1.2) resulting in a trend to higher CO release as compared to water or simulated intestinal fluid (pH 6.8; **Figure 6A**). The sensitivity to environmental parameters was addressed by blending citric acid buffer into the powder blend before tableting, yielding an overall higher CO release as compared to tablets prepared without buffer and indistinguishable release profiles in all three selected release media (**Figure 6B**). The OCORS release pattern was in three distinct consecutive phases, with phase (i) up to 30, phase (ii) 30 to 240, and phase (iii) exceeding 240 minutes, respectively. The second phase was characterized by a linear relationship of CO release and time ($y = 0.017 * t^{0.98}$; $r^2 = 0.9$), whereas the third phase deviated from the linear relationship and asymptotically plateaued at about a total CO release of 4 CORM-2 equivalents.

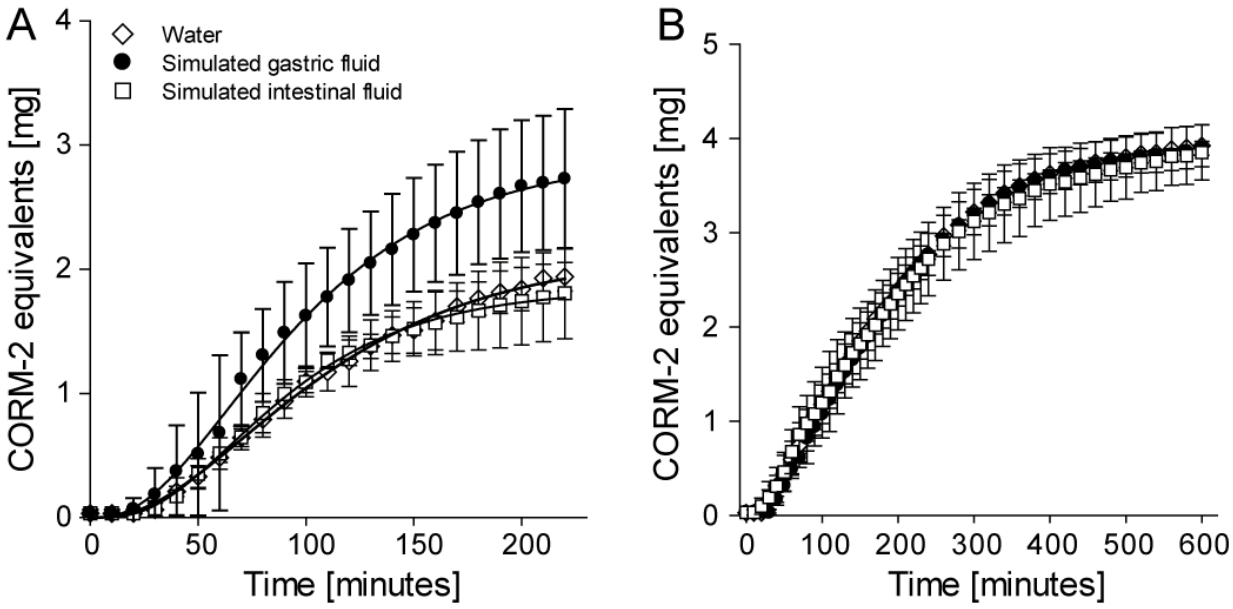

Figure 6: CO release from the oral carbon monoxide release system OCORS in biorelevant media. Tablets were prepared from blends (A) without citric acid buffer or (B) containing buffer. CO release was monitored in water (diamonds), simulated gastric fluid (circles) and simulated intestinal fluid (squares).
The data was corrected; (n = 3).

CO release from CORM-2 suspensions was impacted by ionic strength but not by the salt with which the ionic strength was set (**Supplementary Figure 2**). Increasing the ionic strength from 0.008 mol/L in Na_2SO_4 solution to 0.88 mol/L using NaCl reduced CO release by 26 ± 9 % (n = 3). An ionic strength of 1.26 mol/L set with $MgCl_2$ significantly reduced the CO release by 50 ± 3 % (n = 3). Likewise an ionic strength of 1.8 mol/L set with NaCl significantly reduced the CO release by 53 ± 6 % (n = 3; **Supplementary Figure 2**).

In order to further control the CO release rate, the permeability through OCOR's shell was modified by (i) varying the concentration of pore former and (ii) the coating thickness **(Figure 7)**. CORM-2 suspension used for control demonstrated half maximal release after 20 minutes. Likewise half maximal release was reached within 175 ± 33 minutes when 8 times dip coated, 157 ± 16 minutes when 4 times dip coated and 103 ± 15 minutes when 1 time dip coated (the solution used for 1 time dip had tenfold concentrations of the pore former PEG 400 in the coating solution as compared to the solutions used for the 4 times and 8 times dip coating, respectively). Results were statistically significant for the difference between 8 and 1 time coating and between the 4 and 1 time coating, respectively ($p < 0.001$; n = 6). CO release was completed from all coated systems within approximately 10 hours **(Figure 7)**.

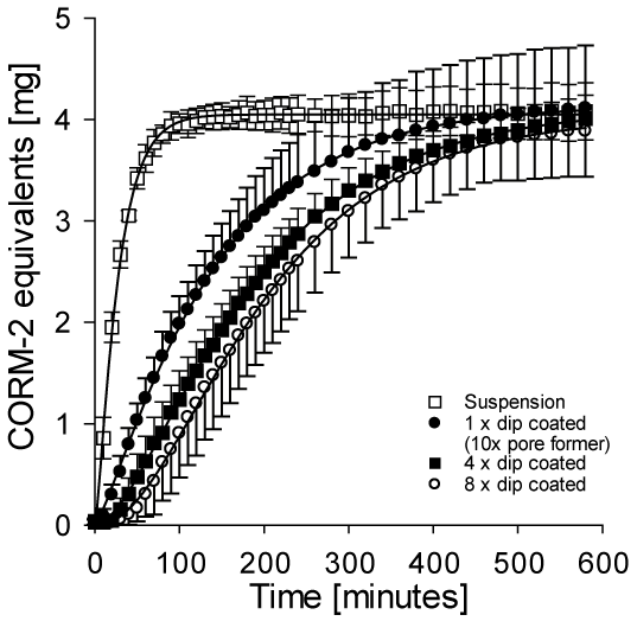

Figure 7: CO release from the oral carbon monoxide release system OCORS as a function of tablet shell porosity and number of coated layers in comparison to CORM-2 suspension (open squares; n = 3). The oral system was dip coated once (with tenfold pore former; filled circles), fourfold (filled squares) or eightfold (open circles) and the release was monitored and data was corrected for system related CO loss (n = 6).

The dimensions and appearance of OCORS remained unchanged throughout the study (not shown). In spite of visual integrity of OCORS, approximately 90% of the ruthenium load was released after 13 hours (**Figure 8**). The difference in ruthenium release between coated and uncoated systems was statistically significant ($p < 0.05$; $n = 3$).

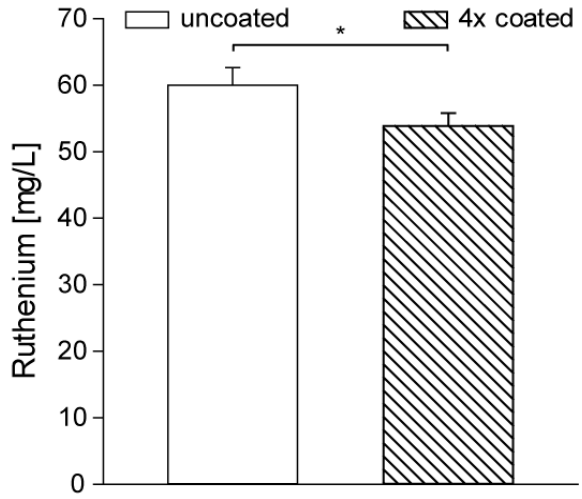

Figure 8: Ruthenium release into water for uncoated and fourfold coated oral carbon monoxide release system OCORS as analyzed by ICP-OES
(* $p < 0.05$; $n = 3$).

Discussion

Ruthenium containing CORM typically release CO in response to sulfur compounds, including dithionite or sulfite [20, 21]. Previous studies on CO release deploy the myoglobin (Mb) assay for spectrophotometric assessment of CO binding to Mb leading to the conversion of deoxy-Mb to carbonmonoxy-Mb, accordingly [16, 32, 33]. Hence, the myoglobin assay protocol typically includes the use of sodium dithionite for reducing myoglobin to deoxy-Mb. It is sodium dithionite impacting CO release from CORM-2 - as sulfur compounds may act as coordinated ligands to ruthenium complexes and, thereby, liberate CO [16] - thereby challenging the precision of the myoglobin assay for the purpose outlined here within [21]. Therefore, we developed a system for amperometric CO detection, modifying previously presented systems and measuring CO in the headspace [20, 28, 34, 35].

The data as read from the system in ppm was calibrated using certified calibration gas, and CORM-2 or CORM-A1, the latter being an established calibration standard by virtue of its complete CO release [28]. We linked CORM-2 to CORM-A1 (**Figure 2C**) which fully decomposes to CO [36] allowing determination of molar ratios [28]. One finding was, that the molar ratio of CO released from CORM-2 over CORM-A1 was approximately 0.7 to 0.8 corroborating previous findings on CO release from CORM-2 [22]. Control experiments demonstrated that the sensor was insensitive to sulfur compounds (i.e. sulfites or SO_2) within the range relevant for the experiments reported here (data not shown).

Correction of CO loss was obligatory for the assessment of the long-term release process of OCORS. CO loss described in this study is a combination of CO consumption by the system and loss from the system. The impact of the CO consumption could have been reduced by increasing the space of the system and thereby the amount of CO, however this adjustment would negatively impact the distribution of CO within the system. We are currently designing a second generation of a CO detection system applying a more sensitive sensor in a larger and completely air tight reaction space with a feature which allows constant air circulation.”OCORS was designed to function autonomously upon oral administration and composed of elements allowing for tailored CO release profiles. Blending the tablet core with citrate buffer (**Figure 4**) was instrumental in introducing insensitivity to the surrounding medium as required for reliable function at gastric, duodenal, jejunal or colonic sites (**Figure 6**) and adaptation of the porosity and number of coating layers of the semipermeable tablet

shell impacted CO release (**Figure 7**). The citric acid buffer was assumed to address a dual function, i.e. (i) control of pH within the tablet core and (ii) control of ionic strengths by complexation, which impacted CO release from CORM-2 (**Supplementary Figure 2**). The CO release correlated nearly linearly with time between approximately 30 to 240 minutes, suggesting zero order release kinetics. These profiles suggested, that the release of CO was controlled by OCORS, i.e. OCORS functionality included the slowest and rate limiting kinetic step in the chain of events leading to CO release. We hypothesize that this rate limitation in OCORS is by means of controlled water permeation through the semipermeable tablet shell and by virtue of (i) OCORS ability to sustain CO release as a function of the tablet shell thickness (**Figure 7**), (ii) relatively unhindered gas permeability including CO through cellulose acetate membranes [37, 38] (iii) immediate disintegration of the coating around the Na_2SO_3 crystals suggesting spontaneous Na_2SO_3 dissolution upon contact with water (not shown) and (iv) previous report with a similar systems suggesting water flux across the cellulose acetate membrane as rate limiting [39]. OCORS is providing CO coverage by linear release for up to 3 hours before the kinetics deviated from linearity at which point the kinetic control from the water permeation through the shell was lost and dictated by the amount of unreacted components within the tablet core, thereafter. These profiles can be adapted. Similar pharmaceutical platforms for the release of non-gaseous therapeutics [40] allow for activation of the dosage forms within minutes upon oral uptake in targeting gastric sites [41] up to hours or even days when colonic sites are of interest [42]. In these dose dispensing systems an osmotic pressure is utilized to release non-gaseous therapeutics through small holes within the coating. In contrast CO autonomously penetrates the coating of OCORS and evidently, the system is independent of osmotic pressure. Future studies need to translate these advantageous targeting patterns to OCORS.

OCORS released therapeutically relevant CO doses. Previous studies by means of b.i.d. intestinal bolus insufflation of 200 ppm had an ameliorating impact on colitis [43] with other studies dosing at 20-1000 ppm [8]. Fundamental assessment of the risk-benefit profile associated with any CO therapy including assessments for acute or chronic use have been discussed elsewhere [1, 44-46]. Challenges for OCORS remain to be addressed. Following CO release, the resulting ruthenium complex iCORM-2 is a water soluble molecule and ruthenium diffusion from OCORS into the release medium was nearly quantitative (**Figure 8**). Both, CORM-2 and iCORM-2 have been flagged for cellular toxicity [22, 47] although at concentrations which are far beyond of what is relevant for OCORS. Also, these previous

studies profiled iCORM-2 as a result of CORM-2 exposure to DMSO and not to Na_2SO_3 as used here within, possibly resulting in different degradation products [22, 47]. Hence, extrapolation of the toxicological evidence from these previous reports may not be extrapolated to the conditions outlined here within. We are currently exploring the toxicity of the iCORM presented here as well as deploying other CORM scaffolds yielding water insoluble reaction products, which arguably will be retained by the OCORS shell and in an effort to limit ruthenium release from OCORS. Thereby, future studies will help to further detail and improve the risk benefit profile of this dosage form.

Conclusion

OCORS is an oral tablet for sustained CO delivery to gastric and / or intestinal sites and its functionality is independent of environmental fluids, thereby providing a reliable, easy to use pharmaceutical platform serving various indications of gastrointestinal injury and inflammation. CO release was precisely controlled by modification of the tablet shell. Future studies aim at profiling OCORS for the treatment of gastrointestinal diseases.

Acknowledgement

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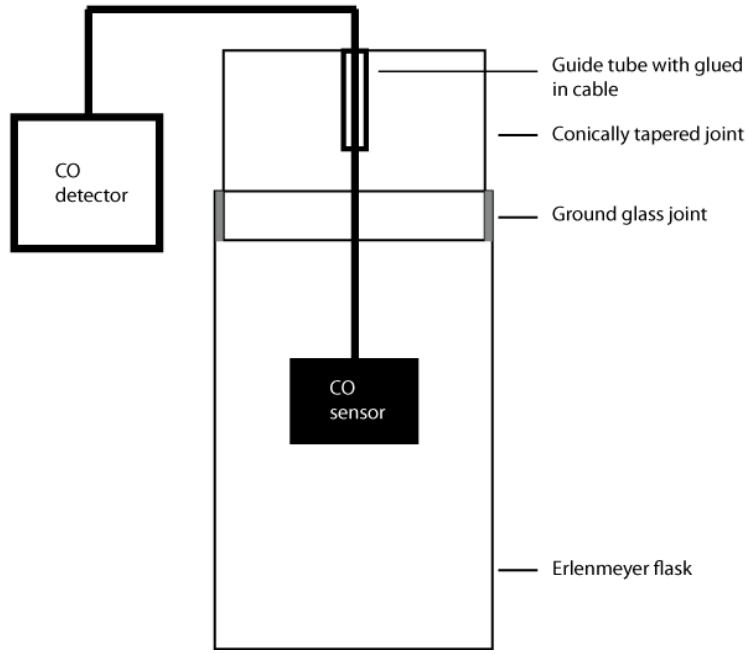
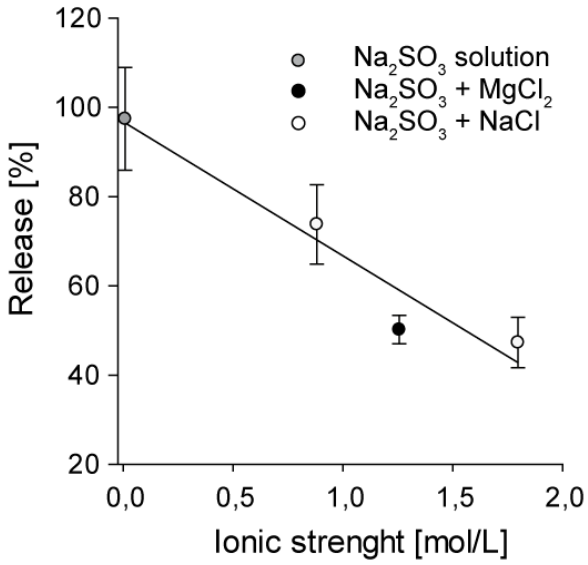
Supplementary Figures



Supplementary Figure 1: Schematic drawing of the amperometric CO detection system.



Supplementary Figure 2: CO release from CORM-2 suspension [% of total CORM-2 release] was measured in 25 mL of a 333 mg/L aqueous Na_2SO_3 solution at room temperature as a function of ionic strength. The ionic strength was increased with magnesium chloride (black filled circle; n = 3) and sodium chloride (open circle; n = 3) in comparison to no addition of any salt (grey filled circle, n = 9).

Chapter 3: Prevention of ulcerative colitis by controlled oral drug delivery of carbon monoxide

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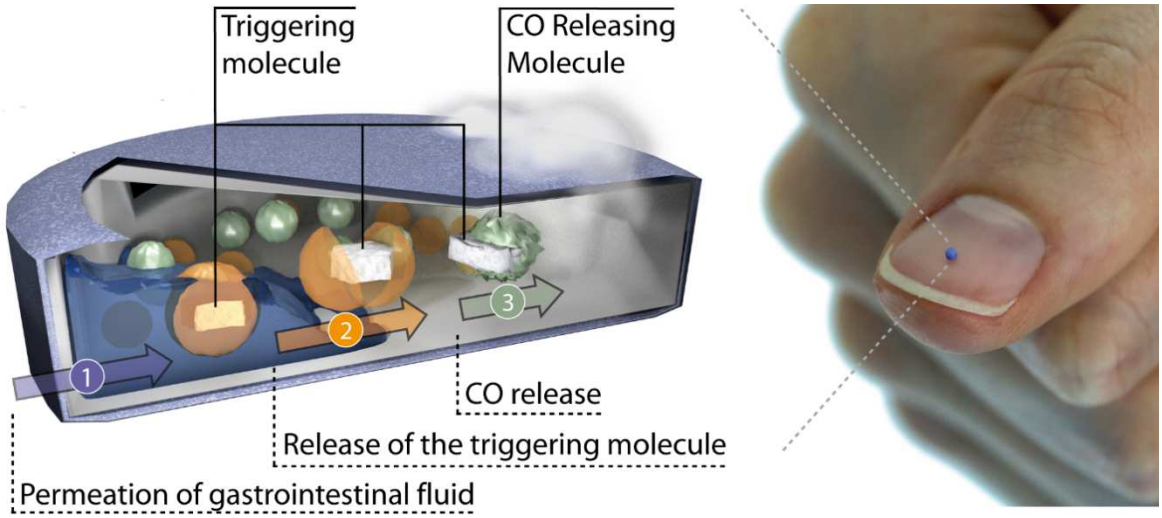

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Abstract

Carbon monoxide (CO) is an endogenous signal transmitter involved in numerous physiological processes including the gastrointestinal (GI) homeostasis. CO has been recognized as potential new therapeutic agent for motility related and inflammatory disorders of the GI tract. A therapeutic use, however, is challenged by inappropriate drug delivery modes. Here we describe a micro scale Oral Carbon Monoxide Release System (M-OCORS) designed for localized and controlled exposure of the GI tract with *in situ* generated CO. M-OCORS allowed for controlled release profiles lasting for several minutes or up to almost one day. These *in vitro* release profiles translated into a large pharmacokinetic design space following oral administration in mice and measured as CO-hemoglobin (CO-Hb) formation. M-OCORS with a release profile featuring exposure of the intestine was profiled in two independently performed studies demonstrating preventive effects in chemically induced colitis. M-OCORS significantly reduced damage scores and prevented upregulation of colitis biomarkers.

Introduction

The prevalence of ulcerative colitis (UC) is 24.3 and 19.2 per 100,000 individuals for Europe and North America, respectively [1]. Annual costs per patient in the United States have been approximated at 15,000 USD driving the need for better and more affordable treatments [2]. The heme oxygenase (HO)/carbon monoxide (CO) system constitutes an important cellular feedback-loop responding to oxidative and inflammatory insult in the gastrointestinal tract (GIT) [3]. Central to this is the inducible HO-1 catalyzing heme degradation into CO, biliverdin, and iron thereby triggering cell-protective, anti-oxidative, anti-apoptotic, and anti-inflammatory effects, with a particularly interesting role for CO [3]. It is for CO's role in regulating gastrointestinal (GI) motility and inflammation that this gas has been suggested as a potential novel therapy for inflammatory GI diseases including ulcerative colitis, gastric ulcers, postoperative ileus, and diabetic gastroparesis [4-7]. Earlier studies also provide indirect epidemiological evidence showing that smoking reduces the risk of developing UC as compared to non-smokers or former smokers [6, 8, 9]. Later studies linked the mechanism of action of 5-aminosalicic acid – which is used as first line UC therapy – to HO, catalyzing the endogenous production of CO [10, 11]. Based on these insights, the therapeutic effect of CO has now been established in numerous colitis models using various administration modalities for this therapeutic gas [12, 13]. Two principle options are currently available for the administration of CO being (i) systemic application of Carbon Monoxide Releasing Molecules (CORM) and (ii) inhalation of the gas [3, 14, 15]. A quite striking disadvantage challenging the risk-benefit ratio of many otherwise useful CORMs are molecule-centered transition metals (ruthenium, cobalt, molybdenum, etc.) required for CO binding in solid state [3, 16]. Implementation of inhalation into clinical practice is challenged by general handling complexity and safety issues posing medical personnel and the patient at risk e.g. in case of accidental release. Furthermore, both modalities, the administration of CORMs or the inhalation of CO, result in nearly exhaustive CO binding to hemoglobin (Hb). From a pharmacokinetic perspective, the patient is exposed to a considerably high amount of CO (bound to the Hb) of which very little is available for the tissues in need [17]. With the conventional methods (*vide supra*), however, high systemic concentration of CO are a prerequisite to get therapeutically relevant levels at the target site [18, 19]. It is for these and other reasons, that the gases benefits cannot be exploited by specialists or family doctors, calling for simple, convenient products such as a capsule or a tablet generating the gas within or close to the tissue in need, rather than homing it through the Hb sink. Addressing this

need, we previously reported the technical development of an Oral Carbon Monoxide Release System (OCORS) with dimensions preventing use in rodent model systems (7 mm) [20]. We now report the first pharmacokinetic (PK) and pharmacodynamic (PD) data set of this concept by using a novel micro scale OCORS (M-OCORS) for the prevention of 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice. Furthermore, the ruthenium based OCORS is compared to an iron based, Enzyme Triggered Oral Carbon Monoxide Release System (E-OCORS) in terms of pharmaceutical as well as cell-toxicity performances, respectively.

Experimental Details

Materials

Carbon Monoxide Releasing Molecule 2 (CORM-2; $[Ru(CO)_3Cl_2]$), dibutyl phthalate, absolute ethanol, sodium hydrogen phosphate dibasic dodecahydrate, Pig Liver Esterase (PLE ≥15 units/mg), sodium citrate tribasic dihydrate, sodium dodecyl sulfate, ruthenium (III) chloride hydrate, Dulbecco's Modified Eagle Medium (DMEM), fluorescein diacetate (FDA), propidium iodide (PI), and randomly methylated β-cyclodextrin (RAMEB) were purchased from Sigma Aldrich Chemie (Schnelldorf, Germany). Citric acid monohyrdrate was from Jäkle Chemie (Nuernberg, Germany). Talcum was from Caelo (Hilden, Germany). Sodium sulfite was purchased from Gruessing (Filsum, Germany). Eudragit E PO and Aerosil were acquired from Evonik Industries (Essen, Germany). Penicillin G and streptomycin solution were purchased from Biochrom (Berlin, Germany). Fetal bovine serum (FBS) was from Gibco (Darmstadt, Germany). Tabletose 80 and premixed tableting powders ("Tablettiermischung, technisch") consisting of lactose, cellulose, aluminum oxide and magnesium stearate were from Meggle (Wasserburg am Inn, Germany). Sam specracol erythrosine lk was purchased from Sensient (Geesthacht, Germany). 100 ppm CO calibration gas (diluted in air) was from Linde (Munich, Germany). Eastman cellulose acetate (CA398-10NF/EP) and cellulose acetate butyrate (CAB-171-15NF) was from Gustav Parmentier (Frankfurt am Main, Germany). 2,4,6-Trinitrobenzenesulfonic acid (TNBS), tetraborate pH standard solution (pH 9.18), and 3,3′,5,5′-tetramethylbenzidine were obtained from Wako Pure Chemicals (Osaka, Japan). Hexadecyltrimethylammonium bromide, hematoxylin, ferricyanide, and eosin were from Sigma Aldrich (Tokyo, Japan). M-MLV reverse transcriptase was from Promega (Madison, WI) and oligo (dT) 15 primer from (Takara Bio Inc. Shiga, Japan). All other reagents were purchased from Sigma Aldrich (Schnelldorf, Germany) and at least of pharmaceutical grade unless noted otherwise.

Development of the micro scale Oral Carbon Monoxide Release System (M-OCORS)

Three M-OCORS formulations providing different release kinetics (formulation 1 (F1) with a fast release, formulation 2 (F2) with an intermediate release, and formulation 3 (F3) with an extended release, (see **Table 1)**) were prepared as previously described with modifications [20]. For that, a 100–125 µm fraction of sodium sulfite crystals was collected using an AS 200 analytical sieve tower from Retsch (Haan, Germany). For F1 and F2, 60 g of the 100–125 µm fraction was coated with a solution consisting of 8.6 g Eudragit E PO, 0.9 g sodium dodecyl sulfate, 1.3 g magnesium stearate, 4.3 g talcum, 50 mL distilled water, and 50 mL absolute ethanol using a Mini-Coater from Glatt (Binzen, Germany). The coating solution was pumped into the Mini-Coater with 187 mL/hour using a Pumpdrive 5001 from Heidolph (Schwabach, Germany). For the F3, 51 g of the 100–125 µm fraction was coated with a suspension consisting of 4 g talcum, 4 g magnesium stearate, 11.6 g cellulose acetate butyrate, 2.32 g dibutylphthalate and 460 mL acetone using the Mini-Coater in top-spray configuration at 21 °C, a fluid bed pressure of 0.6 bar, and an atomizing pressure of 0.76 bar. Cellulose acetate butyrate was slowly added to the coating solution while stirring at 130 rpm (Variomag Telesystem, Thermo Scientific, MA). The coating process was performed twice. The tablet cores were prepared from a blend of 64 mg pulverized citric acid monohydrate, 128 mg pulverized trisodium citrate, 75 mg coated sodium sulfite (coated with different compositions (see **Table 1**), 75 mg CORM-2, 5 mg magnesium stearate, 2.5 mg Aerosil and 143 mg tableting mixture, blended for 30 min in a Turbula T2Fmixer (WAB AG, Muttenz, Switzerland). The resulting blend was transferred into an FE136SRC eccentric tableting machine from Korsch (Berlin, Germany) equipped with a custom made 1 mm tablet punch (Franz-Oberthuer School for mechanical engineering (Wuerzburg, Germany) and was equipped with an interchangeable 1 mm ejector pin (Z 41/1 X 40) from Hasco (Luedenscheid, Germany) to allow for immediate replacement in case of break. The tableting process resulted in average tablet weights of 0.80 ± 0.05 mg (F1, n = 10), 0.64 ± 0.01 (F2, n = 10), and 0.80 ± 0.04 mg (F3, n = 10) respectively. The tablet cores were fluid bed coated using the Mini-Coater in top-spray configuration as described above. 30 g sieved Tabletose 80 was used as filling material accordingly. For the F1, the coating solution was prepared from 1.8 g PEG 400, 4 g talcum in 460 mL acetone into which 11.6 g cellulose acetate was slowly added while stirring at 130 rpm (Telesystem). The coating process resulted in systems with a diameter of 1.07 ± 0.00 mm (n = 3), a height of 0.49 ± 0.03 mm (n = 3), and a weight

of 0.75 ± 0.03 mg (n = 10). F2 was coated (Mini-Coater) with a solution containing 2.32 g dibutyl phthalate, 4 g talcum and 460 mL acetone into which 11.6 g cellulose acetate butyrate was slowly added while stirring at 130 rpm (Telesystem). F3 was coated with the identical procedure as for the F2 resulting in a diameter of 1.08 ± 0.02 mm (n = 3), a height of 0.48 ± 0.02 mm (n = 3), and a weight of 0.85 ± 0.03 mg.

For the control formulations CORM-2 was replaced with ruthenium (III) chloride following otherwise identical procedures leading to the inactive M-OCORS (i-M-OCORS) formulation serving as controls for F1, F2, and F3, respectively. The integrity of the external coating was assessed using a SZ11 microscope from Olympus (Tokyo, Japan). All formulations were stored and shipped in closed 1,5 mL Eppendorf tubes from Eppendorf (Hamburg, Germany) under ambient conditions. Shipping studies (samples shipped to the lab in Japan and back to Germany for control) indicated proper stability (data not shown).

Preparation of the Enzyme Triggered Oral Carbon Monoxide Release System (E-OCORS)

The *rac*-**1** (see **Figure 5A**) and a randomly methylated β-cyclodextrin (RAMEB) inclusion complex thereof were prepared as previously described [21-23]. In brief, 135 mg RAMEB and 50 mg *rac*-**1** were dissolved in 10 mL water and treated with a Sonorex Super RK 255H ultrasonic bath from Bandelin (Berlin, Germany) at 80 °C for 30 minutes. Residual water was evaporated using a Hei-VAP Precision rotary evaporator (Heidolph, Schwabach, Germany). Light exposure was prevented at all time by using amber glass round-bottomed flask (Duran Group, Wertheim, Germany). The spatial separation of *rac*-**1** and the Pig Liver Esterase (PLE) within E-OCORS was achieved by layer-by-layer coating of disodium hydrogen phosphate crystals using Eudragit E PO. The first layer comprised Eudragit E PO and PLE, the second layer comprised Eudragit E PO without PLE. Therefore the 100–125 µm fraction of disodium hydrogen phosphate crystals was collected (AS 200 analytical sieve tower) and 30 g coated at 45 °C with an atomizing air pressure of 0.8 bar, and a microclimate of 0.5 bar (Mini-Coater) with the overall procedure following the manufacturer's instructions [24]. The first coating solution consisted of 1.1 g PLE, 8.6 g Eudragit E PO, 0.9 g sodium lauryl sulfate, 1.3 g stearic acid, 4.3 g talcum, and 85 g water. The procedure was repeated without PLE using otherwise identical procedures. Tablet cores were prepared from a blend of 1724 mg tableting mixture, 14 mg citric acid, 248 mg sodium citrate tribasic, 1000 mg coated PLE particles, and 18 mg RAMEB ET-CORM. This blend was mixed for 45 min

(T2F mixer), and pressed into tablet cores (FE136SRC tableting machine) using a 9 mm tablet punch from Korsch leading to an average weight of 260 ± 5 mg (n = 3). The tablet cores were dip coated 5 times in a cellulose acetate coating consisting of 0.9 g PEG 400, and 5.8 g cellulose acetate as previously described [20].

Amperometric detection system

The CO release pattern of all formulations was assessed as previously described [20, 25]. In brief, the pattern was recorded in a reaction chamber consisting of two sealed DN 40 flanges (Rettberg, Goettingen, Germany) using a X-am 5000 CO sensor from Draeger (Luebeck, Germany) at room temperature (ambient conditions), not stirred. The device was filled with 15 mL water prior to the release assessment. Likewise, the release of F1 was studied in different bio-relevant media. Therefore, compendial simulated gastric fluid without enzymes [26] or simulated intestinal fluid without enzymes [26] was used following the otherwise identical analytic protocol (*vide supra*). The sensor was calibrated with 100 ppm CO calibration gas with sulfite triggering CO release from CORM-2 for comparability tests among and calibration of the different detectors, respectively. Data correction for CO loss was performed as described before [20]. Curve fitting of the CO release patterns was preformed using the Box Lucas model of Origin PRO 9.1 from the Origin Lab Corporation (Northampton, MA).

In vivo Pharmacokinetics

Carboxyhemoglobin (CO-Hb) formation patterns following the application of F1 and F3 were assessed. For that, arterial blood (50 µl) was collected from the abdominal aorta with a heparinized capillary tube (Drummond Scientific, Broomall, PA), and transferred to a 13 mL glass vial. Subsequently, 0.5 mL of a saturated potassium ferricyanide and 1 ml tetraborate pH standard solution (pH 9.18) were added to this glass vial and tightly closed thereafter, as previously described [27]. The solution was then vigorously vortexed for 30 s and left standing for 5 min. 1 mL of the gas phase was withdrawn with a 1 ml syringe and injected into a TRIlyzer mBA-3000 machine (TAIYO instruments, Osaka, Japan) for the quantification of the CO concentration accordingly. The blood hemoglobin concentration was assessed using a Hemoglobin B test kit from Wako Pure Chemical Industries (Tokyo, Japan) and used for the calculation of the CO-Hb formation accordingly. For pharmacokinetic modelling and fitting see **Supplementary Information**. The PK was profiled with n = 4 animals in each group.

2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice

Animals

Seven-week-old male C57BL/6 mice were obtained from SHIMIZU Laboratory Supplies (Kyoto, Japan). The mice were caged individually in a room kept at 18–24 °C and 40–70 % relative humidity, with a 12 hour light/dark cycle, and access to food and drinking water *ad libitum*. The mice were fed rodent diet CE-2 (Nihon-Clea, Tokyo, Japan) during their 1-week of acclimatization. The animals were maintained and the experimental procedures carried out in accordance with the US National Research Council guidelines for the use of experimental animals [28]. All experimental protocols were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan). Both studies, the initial study and the confirmatory study, comprised 6 animals in each group.

Induction of colitis

Using a KN-348 catheter from Natsume Seisakusho (Tokyo, Japan) colitis was induced with 200 mg/kg TNBS dissolved in 30 % ethanol administered intrarectally to ketamine/xylazine anesthetized mice [29, 30]. The control group was treated with the overall identical procedure without TNBS. Using the KN-348 catheter featuring a manually widened tip, 2 x F3 or 2 x i-F3 were administered orally three days before and two days after TNBS administration. Therefore the M-OCORS was inserted into the tip of the catheter and following oral application released by flushing with 0.1 mL water. Three days after TNBS administration the animals were sacrificed, their colons were removed, and examined accordingly. The severity of colonic damage was graded as previously described [29, 30]: The colon damage score included the presence of visible damage, serosal adhesions, diarrhea, strictures, and bowel wall thickening (see **Supplementary Table 2** for further details). The scoring was performed observer-blinded. For histologic evaluation, specimens of the distal colon were fixed in 10 % neutral buffered formalin. After fixation, the specimens were embedded in paraffin, divided into 7 mm sections, and stained with hematoxylin and eosin. An independent repetition of the protocol using a new batch of F3 was performed accordingly and for independent corroboration of the results from the first trial.

Measurement of Myeloperoxidase (MPO) Activity

Tissue-associated myeloperoxidase (MPO) activity was determined as previously described, with modifications [31, 32]. In brief 2 mL of mucosal homogenates were centrifuged at 20,000 x g for 15 min at 4 °C to pellet the insoluble cellular debris. The pellet was then re-homogenized in an equivalent volume of 50 mmol potassium phosphate buffer (pH 5.4) containing 0.5 % hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000 x g for 15 min at 4 °C and the supernatants retrieved. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidin. The enzyme activity was assessed accordingly with one unit being defined as the amount of MPO that caused a change in absorbance of 1.0 /min at 655 nm and 25 °C.

RNA Analysis

Total RNA was isolated with acidic guanidinium phenol chloroform using an Isogen Kit from Nippon Gene Co. Ltd. (Tokyo, Japan) and stored at -70 °C. 1 µg of the extracted RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using a Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase and an oligo (dT) 15 primer. Real-time PCR for tumor necrosis factor alpha (TNF- α) and β-actin (housekeeping) was performed using a 7300 real-time PCR system from Applied Biosystems (Foster City, CA) applying the DNA-binding dye SYBER Green I for the detection of PCR products. The primers were designed using the 'Primer Express' software (version: 3.0.1, Applied Biosystems) and ordered at Takara Bio Inc. (Shiga, Japan). The primers had the following sequences: TNF- α (accession number: X02611): Sense 5′- ATCCGCGAC GTGGAACTG -3′ and antisense 5′–ACCGCCTGGAGTTCTGGAA-3′. β-actin (accession number: X03672): Sense 5′–GAGCAAACATCCCCCAAAGTT-3′ and antisense 5′–GCCGTGGATACTTGGAGTGACT-3′. The quantification of the TNF- α gene expression level was calculated relative to β-actin.

Cytotoxicity

Cytotoxicity was evaluated using a fibroblast cell line to ensure comparability with official guidelines [33]. NIH 3T3 fibroblasts (CRL-1658; ATCC, Manassas, VA) were maintained in 100 mm culture dishes in growth medium (DMEM containing heat-inactivated FBS (10 %), penicillin G (100 U mL^{-1}) and streptomycin (100 µg $µL^{-1}$)) at 37 °C under CO_2 (5 %). Before use, the cells were seeded in quadruplets at a final cell density of 2.5 x 10^4 cells mL^{-1} (1 mL per well) in 24-well plates in growth medium and were grown for 24 hours at 37 °C under CO_2 (5 %). Abrogated CORM-2 (a-CORM-2) was prepared by dissolving 10 mg CORM-2 and 10 mg sodium sulfite in 2 ml growth medium allowing to react at room temperature for 12 hours. The cells were then treated with increasing concentrations of a-CORM-2 including 0.125, 0.25, 0.5, and 1 mM for 24 hours. Untreated cells were used as control. Cell viability was assessed using fluorescein diacetate (FDA) and propidium iodide (PI). The medium was gently aspirated and the cells were washed twice with PBS. After washing with PBS, the cells were detached using an aqueous 0.005 % trypsin [m/V] and 0.0025 % EDTA [m/V] solution. Trypsin/EDTA activity was stopped by the addition of growth medium. The cells were re-suspended in PBS accordingly, counted, allocated into equal parts, and stained either with (0.01 µg / 10^4 cells) FDA or (0.003µg / 10^4 cells) PI dissolved in PBS for 3 minutes at room temperature. The cells were subsequently analyzed by flow cytometry on a FACS Calibur system from BD Bioscience (San Jose, CA). For detection a 488 nm laser was used with the emission channel FL1 (530 nm / ± 15 nm) for FDA (λ_{ex}= 492 nm λ_{em}= 517 nm) and emission channel FL2 (585 nm / ± 21 nm) for PI (λ_{ex}= 540 nm λ_{em}= 608 nm) as previously described [34-36]. A number of 5000 events were counted with BD CellQuest Pro (BD Biosciences) and the geometric mean fluorescence intensity was determined for each condition using Flow Software (version 2.5.1; Turku Bioimaging, Turku, Finland).

Statistics

Minitab 16 (Minitab, Coventry, UK) was used for all statistic tests. Differences between the analyzed groups were assessed by a 2-tailed t-test assuming normality. $p < 0.05$ was considered statistically significant. All data were reported as mean ± standard deviation unless specified otherwise.

Results

Micro scale Oral Carbon Monoxide Release System (M-OCORS) for oral applicability in mice

M-OCORS was designed for (i) oral applicability in mice while (ii) maintaining control of CO generation and release kinetics for spatial delivery of CO within the murine GIT. Therefore 1 mm tablet cores containing CORM-2 and coated sodium sulfite were functionalized through different coating procedures (**Figure 1**). M-OCORS formulation 1 (F1) was coated with cellulose acetate. Half maximal release was within 6.6 ± 1.2 minutes (0.11 ± 0.02 hours) and the pattern plateaued after 20 minutes (0.33 hours) at 26.3 ± 3.3 ppm (plateau is defined here within as ≥ 90 % of the maximal release, n = 6, **Figure 2A, Table 1**). The pattern followed first order kinetics ($y\ [ppm] = 31 * \left(1 - e^{(-5 * x\ [h])}\right)$, $r^2 = 0.98$, with y being the amount of CO released [ppm] and x being the time [hours]), with CO release being controlled by water/medium permeation through the semipermeable cellulose acetate coating thereby dissolving sodium sulfite which in return drives CO generation from the CORM-2 (**Figure 1**).

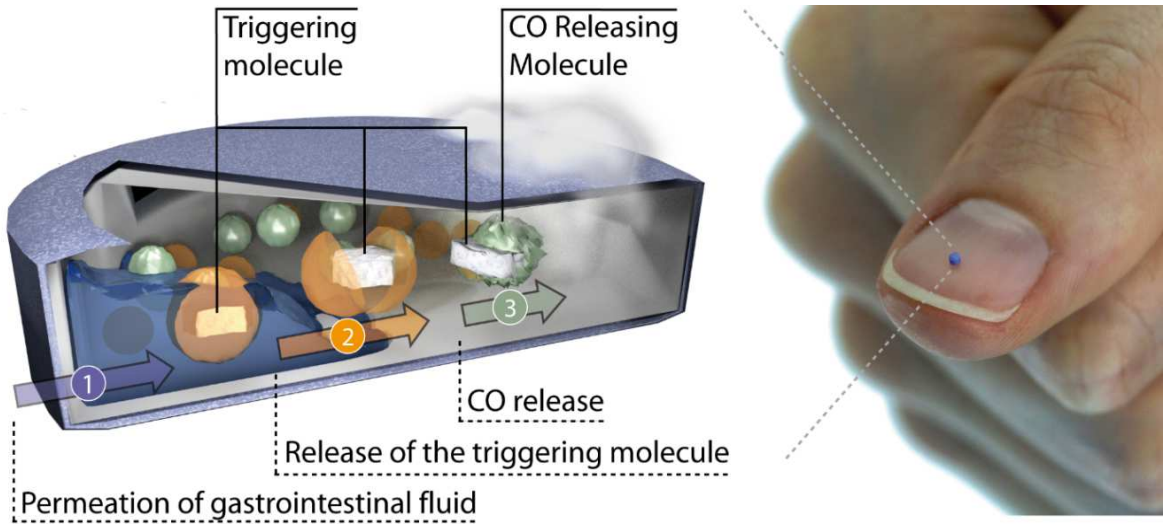

Figure 1: Cartoon outlining the function of the micro scale Oral Carbon Monoxide Release System (M-OCORS). The CO release rate of OCORS is tailorable as a function of the hydrophobicity and nature of coatings controlling two consecutive rate controlling steps, (1) the permeation of the water/medium (e.g. intestinal fluid) into M-OCORS is controlled by the type and hydrophobicity of the cellulose acetate shell (purple); (2) the coating (orange) around the triggering molecule controls the lag phase of reaction and the reaction rate between CORM and the triggering molecule (3).

Further coating was tested to tailor the CO generation and release rate. For that, the hydrophobicity of the coating was increased (water influx across the coating into the tablet core is expected to decrease) by using cellulose acetate butyrate as coating polymer for F2, replacing cellulose acetate as used for F1 (**Table 1**). Indeed, a sustained CO generation and release profile was recorded leading to half maximal release after 1.71 ± 0.37 hours (n = 6) and plateauing after 4.1 hours at 41.3 ± 6.8 ppm (**Supplementary Figure 1, Table 1**). The pattern followed first order kinetics (y [ppm] $= 50 * \left(1 - e^{(-0.3 * \text{x [h]})}\right)$, r^2 = 0.97, **Supplementary Figure 1)**. Following the demonstration of controlling CO generation and release through coating of the tablet core, we approached further control leverages by modifying the coating around the sodium sulfite crystals from Eudragit E PO to cellulose acetate butyrate (leading to formulation F3) under otherwise identical parameters as used for F2 (*vide supra*). CO release from F3 was further sustained leading to a lag time of release as of 3.32 ± 0.81 hours, half-maximal release after 9.1 ± 0.8 hours, and plateauing after 16.6 hours at 39.9 ± 5.7 ppm (n = 5, **Figure 2B, Table 1**). With commencing CO generation and release (i.e. after the lag time lapsed) the release pattern followed first order kinetics (y [ppm] $= 62 * \left(1 - e^{(-0.07 * \text{x [h]})}\right)$, r^2 =0.98). F3 was stable when stored for 9.5 months under ambient conditions as indicated by unchanged CO generation and release patterns as compared to tablets tested right after manufacture (**Supplementary Figure 2**). CO release from F1 was identical in three different bio-relevant media, namely simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and water (**Supplementary Figure 3**).

Table 1: CO generation and release data of the M-OCORS formulations.

Formulation	F1	F2	F3
Coating of the tablet core	Cellulose acetate	Cellulose acetate butyrate	
Coating of sodium sulfite crystals	Eudragit E PO		Cellulose acetate butyrate
Lag time [hours]	0	0	3.32 ± 0.81
Time to half-maximal release [hours]	0.11 ± 0.02	1.71 ± 0.37	9.1 ± 0.8

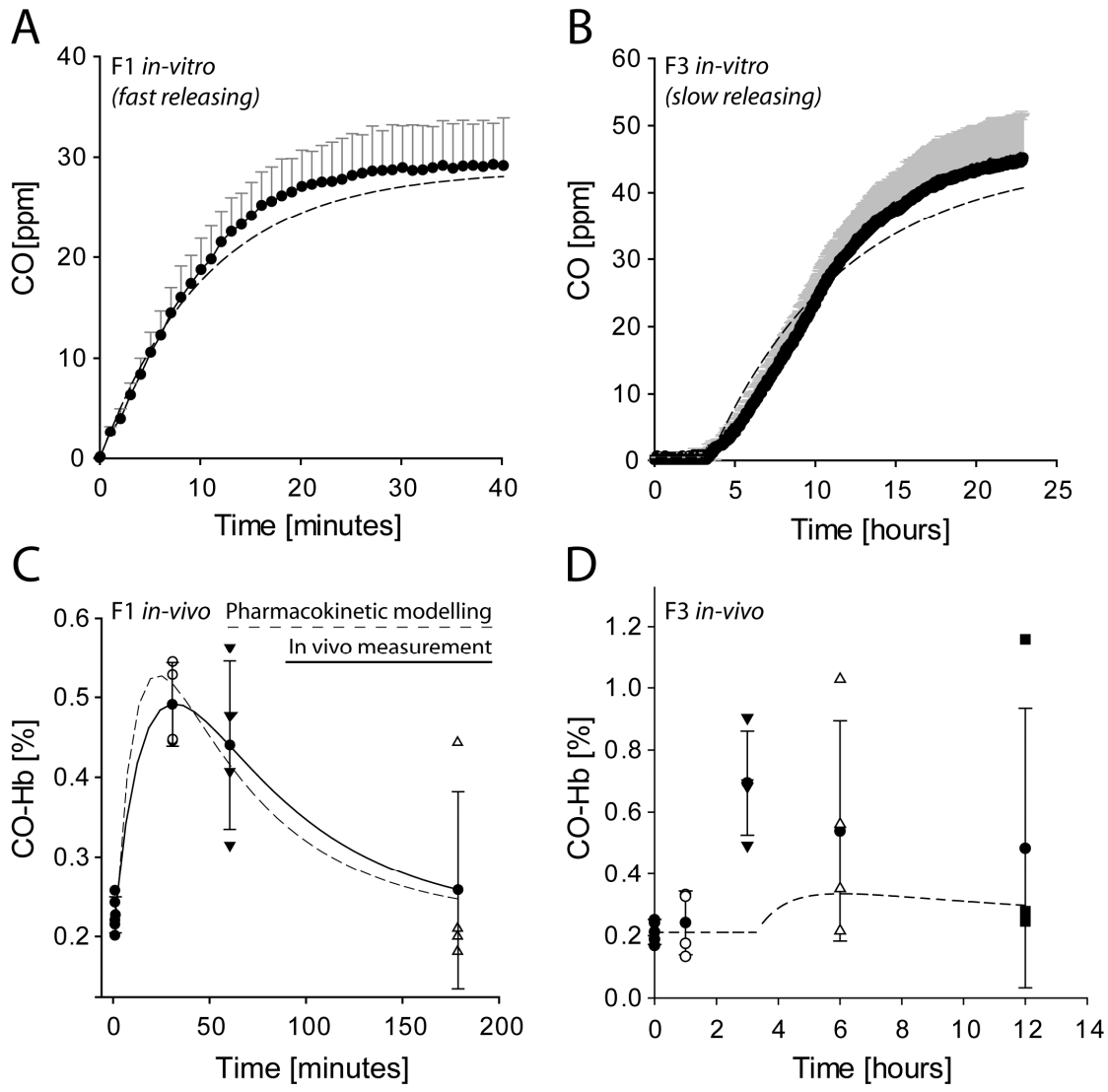

Figure 2: Pharmacokinetic profile of two M-OCORS formulations (F1 and F3) providing modified release kinetics. (A) *In-vitro* CO release profile of the fast releasing F1 (n = 6) following first order kinetics (dashed line) in comparison to (C) *in-vivo* pharmacokinetic data detailing the hemoglobin binding of CO (CO-Hb; n = 4, black pattern). The *in-vivo* data was fitted (solid line) and modelled (details provided in text). (B) *In-vitro* CO release profile of the slow releasing F3 (n = 5) following first order kinetics (dashed line) in comparison to (D) the modelled (dashed line) and *in-vivo* (n = 4, black pattern) CO-Hb formation. All data are expressed as mean ± SD.

CO pharmacokinetics (PK) in mice suggest tailorable generation and release profiles

CO-Hb formation as a function of time was assessed for F1 and F3. Basal CO-Hb level serving as control was 0.23 ± 0.03 % CO-Hb (n = 8, pooled data from both PK profiles). CO-Hb following oral application of F1 peaked after 30 minutes at 0.51 ± 0.05 % and declining to the basal level (0.27 ± 0.12 %) within 3 hours (n = 4, **Figure 2C**). Oral administration of F3 resulted in CO-Hb detection exceeding the basal level within 1-3 hours. The overall pattern varied among animals with evidence for lasting CO-Hb formation for at least 12 hours in one animal (n = 4, **Figure 2D**). Peak mean CO-Hb levels of 0.69 ± 0.14 % were recorded after three hours.

M-OCORS ameliorates TNBS induced colitis in mice

F3 was selected for efficacy testing in preventing TNBS induced colitis assuming that the benefit from a sustained CO generation and release profile as observed from the PK study (F3) is more appropriate than rapid PK profiles as observed for F1 (**Figure 2C**). Simultaneous administration of 2 tablets of F3 or 2 tablets of inactive F3 loaded with ruthenium (III) chloride (i-F3; not releasing CO and serving as negative control) were administered for three days prior (- 3 days) and two days after TNBS exposure (day of TNBS exposure is day 0). The mice were sacrificed on day 3. In contrast to the simultaneous administration of three tablets of F3, the simultaneous administration of 2 tablets was practically feasible when using our custom made gavage. F3 administration resulted in protection from colitis as compared to i-F3, resulting in a significant reduction of the colon damage score (**Figure 3A**). Furthermore, F3 exposure significantly decreased TNF-α expression levels ($p < 0.05$, **Figure 3B**) and insignificantly MPO activity ($p = 0.086$, **Figure 3C**) in mucosal colonic tissue. The study was repeated for confirmation (**Supplementary Figure 4**), resulting in significant reduction of the colon damage score for F3 over i-F3 ($p < 0.01$, **Supplementary Figure 4A**), insignificantly decreased TNF-α expression levels (p = 1.121, **Supplementary Figure 4C**) and significantly decreased MPO activity (p < 0.01, **Supplementary Figure 4D**).

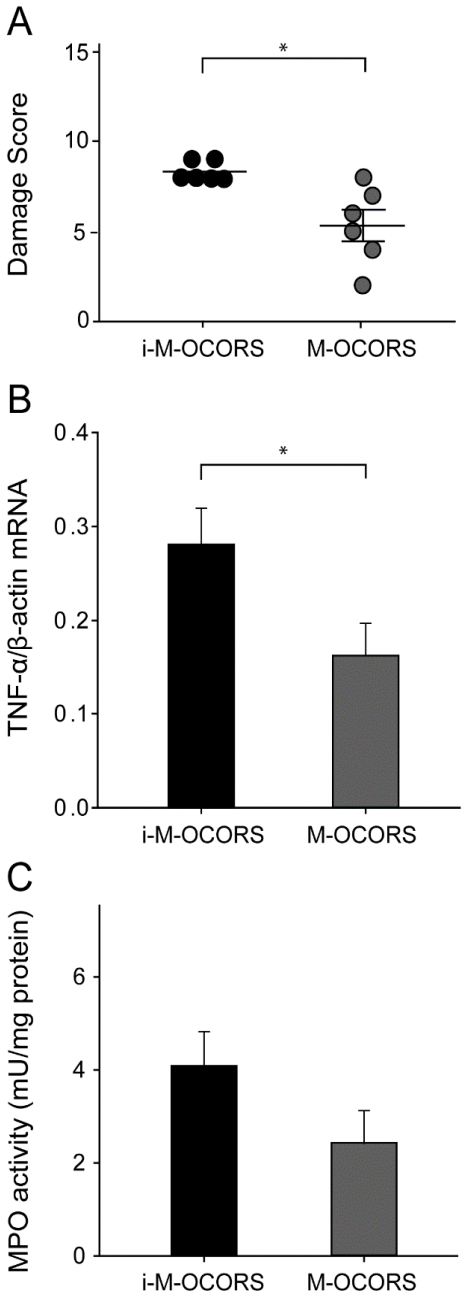

Figure 3: Pharmacodynamic, preventive effect of M-OCORS formulation 3 (F3) versus inactive i-F3 (control) on 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice as detailed by (A) colon damage score (n = 6) (B) TNF-α expression (n = 4), and (C) MPO activity (n = 4). Data are expressed as mean ± SE and asterisks indicate statistical difference at $p < 0.05$. An independent repetition of the animal study is detailed in Supplementary Figure 4.

Cytotoxicity

We assessed the cytotoxicity of the products resulting from the reaction of CORM-2 and sodium sulfite in aqueous solution (abrogated CORM-2 (a-CORM-2)). Therefore NIH 3T3 fibroblasts were exposed to 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM a-CORM-2 for 24 hours. Cell membrane integrity as well as intracellular esterase activity was studied using FDA and PI staining followed by flow cytometry accordingly. 24 hour exposure resulted in a significant reduction of cell survival following 0.25 mM, 0.5 mM, and 1 mM a-CORM-2 following FDA or PI staining ($p < 0.05$, **Figure 4A, B**). No significant effect was recorded for 0.125 mM a-CORM-2.

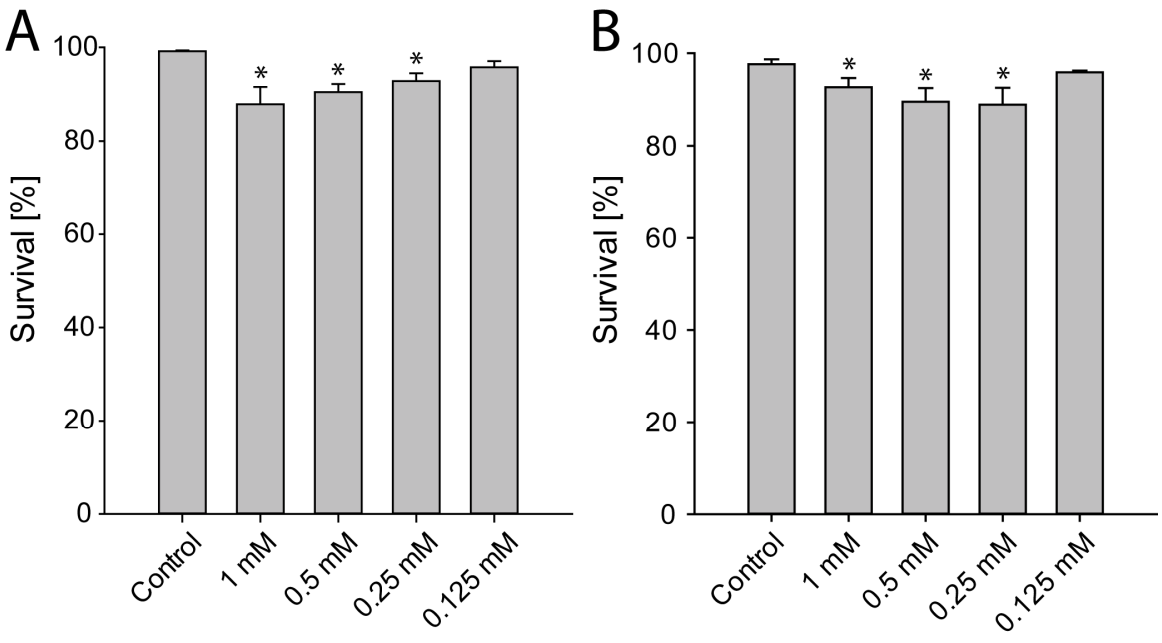

Figure 4: Cell survival of NIH 3T3 fibroblasts exposed to 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM abrogated CORM-2 for 24 hours using (A) flouresceindiacetae (FDA) and (B) propidiumiodide (PI) staining. All data are expressed as mean ± SD and asterisks indicate statistical difference at $p < 0.05$ as compared to 0 mM (control).

Enzyme Triggered Oral Carbon Monoxide Release System (E-OCORS)

In an effort to replace ruthenium (CORM-2) we modified OCORS by loading it with a complex consisting of (i) a relatively benign central element leading to (ii) precisely defined degradation products upon CO release as featured by *rac*-**1** - an iron containing CORM (**Figure 5A**) [22]. The system required adaptation as CO release from *rac*-**1** is not triggered through sulfite but through esterases. We hence used Pig Liver Esterase (PLE) as release trigger for this modified system, namely the Esterase-Triggered OCORS (E-OCORS). Spatial separation of *rac*-**1** and the PLE was by layer-by-layer coating of disodium hydrogen phosphate crystals serving as scaffolds, followed by coating with Eudragit E PO. The first layer of Eudragit E PO contained PLE, the second did not (to seal the PLE from *rac*-**1** during storage). The release pattern of E-OCORS was tailored to approach CO generation characteristics as observed for F3 with similar dimensions as E-OCORS and as previously reported [20]. Release had a lag time of 3.52 ± 1.35 hours followed by zero order kinetics for 13.32 hours (y [ppm] = 6.5 * x [h] – 29, r^2 = 0.98; **Figure 5B**).

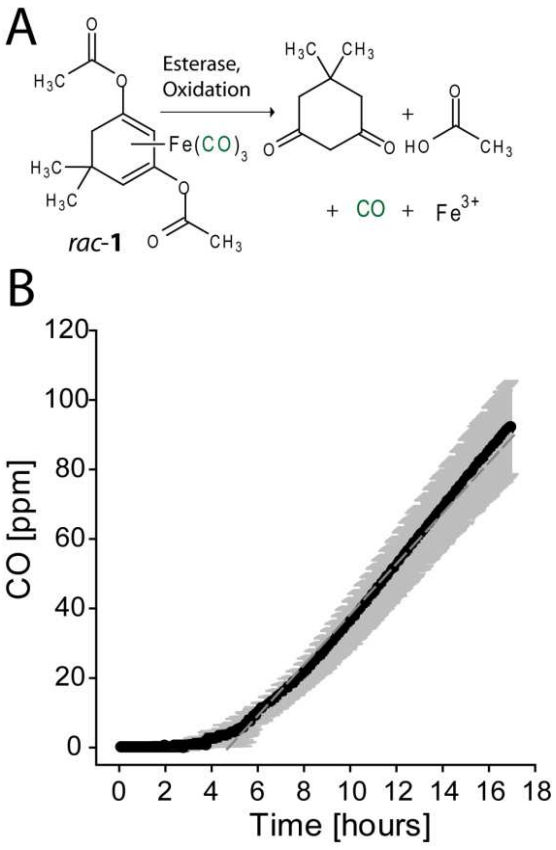

Figure 5: Esterase Triggered Oral Carbon Monoxide Release System (E-OCORS). (A) Scheme of the esterase triggered release of CO from *rac*-**1**. (B) CO release profile of the E-OCORS following zero order kinetics (dashed line; n = 6). Data is expressed as mean ± SD

Discussion

Two major roadblocks prevent the general use of CO for therapy, the absence of convenient application systems allowing safe, widespread use by specialists or family doctors and the high and potentially toxic CO-Hb formation following systemic administration (e.g. inhalation) [19]. M-OCORS was designed to overcome both limitations. Mechanistically, M-OCORS links CO generation and release rates to controlled water/medium influx across the tablet shell (**Figure 1**), thereby locally generating high concentrations within the target tissues while overall patient exposure is marginal. It is for the effective homing of CO to tissues in need through oral delivery that CO-Hb levels are far below safety thresholds of 5-10 % CO-Hb (*vide infra*) [3, 19]. To illustrate these further, relevant previous reports on CO induced amelioration of murine colitis required inhalation of 200–250 ppm CO over a period of at least three hours for a day. This translates into an equivalent of 22 % CO-Hb (see **Table 2**) with CO-Hb levels > 20 % being associated with irreversible- and > 16 % with reversible neurotoxicity (in humans) [19, 37].

Table 2: Overview of selected preclinical studies in colitis models using inhalation for administration. The steady state CO-Hb level was calculated combining outcome from five reports applying 250 ppm CO over more than three hours (steady state) in mice.

Model	CO [ppm]	Exposure [hours/day]	Reference	Steady state CO-Hb [%]	Reference
DSS, mouse	250	3-4	[38, 39]	21.76 ± 6.16 (n = 5)	[40-44]
TNBS, mouse	200	24	[45]		
IL-10-deficient, mouse	250	24	[6]		
TCR-α-deficient, mouse	250	24	[46]		

In contrast F3 resulted in CO-Hb levels in line with official occupational safety limits (maximal level observed was 1.4 %, **Figure 2D** - please note that two F3 tablets were used in the TNBS colitis study at each administration, whereas the PK study was performed with one F3 tablet per administration) – with the US National Institute for Occupational Safety and Health recommending an exposure limit of 35 ppm – equivalent to a steady state concentration of 5 % CO-Hb [47, 48]. The underlying assumption of our extrapolating is that the CO-Hb formation is comparable among species [49]. The impact of further dose escalation was not detailed here within, although it would have been interesting to evaluate efficacy at tolerated levels of 5 % CO-Hb.

Theoretically, concentrations up to 5 % (*vide supra,* 3.5 times the currently used dose) for chronic settings and up to 10 % CO-Hb (7 times the currently used dose) for acute settings seem to be justifiable [3, 19]. We are currently planning a large animal study to evaluate this in detail and with a higher translational significance using GI release systems with higher payload.

M-OCORS addresses the aforementioned challenges of existing treatment options while opening convenient, wide-spread and safe use for future treatment opportunities. All constituents other than CORM-2 and ruthenium (III) chloride (control) are FDA approved or generally recognized as safe (GRAS, FDA). The use of ruthenium based CORM-2 is undesirable and replacement is warranted. In spite of irrelevant cytotoxicity effects (relevance as being defined as 50 % decrease in cell viability after 24 hours of exposure) of ruthenium (III) – serving as a surrogate for the impact of ruthenium on cell survival [50-54] – the metal-center should be replaced by other, relatively safe metals. Our initial results using a modified OCORS featuring the iron based *rac*-**1** in concert with OCORS-controlled exposure to esterase for CO generation and release (E-OCORS) point to better safety profiles (**Figure 5A**). Future *in vivo* studies are required to assess the performances of E-OCORS and possible toxicities. *Rac*-**1** within E-OCORS was originally inspired by bio-responsive drug delivery systems featuring cleavable linkers for targeted delivery. In a proof of concept study *rac*-**1** selectively released CO in response to esterase activity, thereby setting the scene for targeting approaches [22]. Since then the concept has been significantly expanded by introducing various linkers cleavable by enzymes that have been associated with inflammation [55, 56]. Future modifications of E-OCORS might, therefore, adopt this approach and aim for an E-OCORS that selectively releases CO in response to inflammations in the GI tract **[57]**. Besides of the aforementioned issues, CORM-2 is also challenging from a technical perspective, in that it results in various degradation products upon CO generation [20, 58-60]. These products are hard to specify and some have been associated with toxicities [16]. Slightly higher quantities of CO released from F3 compared to F1 (**Figure 2A and B**) might reflect different reaction/degradation kinetics of these products. In light of these insights, the efficacy of F3 was compared to a formulation comprising ruthenium (III) chloride as widely used physiologically inactive and chemically clearly defined control for ruthenium CORMs [50-54]. Confirming these earlier reports the colon damage score following treatment with the control formulation (**Figure 3A**) was identically compared to

two disease only controls from frontrunner studies independently performed in our laboratory [32, 45].

Fitting the *in vitro* CO generation kinetics for F1 (**Figure 2A**) provided the absorption constant (k_a) for subsequent modelling of the F1 PK data (**Figure 2C,** the elimination constant (k_e) was reported previously for mice [61]). Assuming first order absorption and elimination (Bateman equation, **Supplementary Information**) this modelling approach resulted in satisfying correlation with the PK data *in-vivo* (**Figure 2C)**. The data was further fitted assuming first order absorption and elimination using these and the bioavailability (BAV, Bateman equation) as variables (in contrast to the modeling approach, within which k_a and k_e were from *in vitro* observation and literature and only the BAV was fitted; **Supplementary Information**). This comparison provided evidence, that the kinetics of tissue exposure can be tightly controlled through the pharmaceutical parameters of M-OCORS and not through physiological constraints such as biological barriers. Noteworthy the *in-vitro* release pattern of M-OCORS (F1) is independent of different bio-relevant (**Supplementary Figure 3**) media suggesting suitability for in vivo applications and confirming our previous technical development study [20]. Prolonged exposure is thereby dictated by the pharmaceutical design parameters of M-OCORS and only restricted by gastric emptying (for targeting the stomach) and/or regular propulsion through the gut (for targeting e.g. UC). It should be noted, that the modeling and curve fitting for F1 (**Figure 2C**) failed for F3 (**Figure 2D**) when applying the same assumptions (first order). The design of the releases system for intestinal delivery – on-set of the CO release from F3 occurred after a lag time – translated temporal profiles of murine gastric emptying time of particles with comparable size [62]. Furthermore, the CO release profile (release up to 12 hours *in-vivo*, **Figure 2D**) was tailored to match the murine gastrointestinal transit times of comparable molds with approximately 70 % having left the small intestine 6 hours after oral application [63]). Noteworthy, cellulose acetate and derivatives are FDA approved coatings for clinical GI delivery applications providing sufficient evidence for coating stability during GI passage (*vide supra*). In summary, the release pattern allows for sustained and local CO release along the intestinal tract *in toto*. Although both inflamed- as well as uninflamed tissues are exposed to CO the striking advantage over systemic approaches (**Table 2**) is that CO reaches the affected tissue in the free (unbound) form and not via the blood stream tightly bound to hemoglobin thereby providing both efficacy and safety. Future modifications using

possible bio-responsive targeting functionalities of the E-OCORS (*vide supra*) might allow CO delivery precisely to the inflamed part of the GI tract.

The TNBS model represents only aspects of UC and is dominated by a Th1-mediated immune response [64]. Consequently, the data set provided here within is insufficient to conclude therapeutic efficacy in preclinical models of UC. However, through our interpretation it provides credible evidence for bridging efficacies by CO inhalation to oral administration of M-OCORS although a head-to-head comparison (inhalation *vs*. oral administration of M-OCORS) would have been more powerful). Arguably, we also believe that the successful bridging through the TNBS model provides evidence for M-OCORS efficacy in UC models beyond TNBS for which inhalation proved to be successful, including the DSS model, studies in IL-10 deficient mice or TCR-α deficient mice (**Table 2**).

Conclusion

M-OCORS provides a convenient treatment modality for the therapeutic gas carbon monoxide (CO). Effective tissue permeation across epithelial barriers allows for tight control of exposure kinetics through pharmaceutical design of the CO generating platform. Efficacy was demonstrated in one model of ulcerative colitis and performed *at par* with CO inhalation protocols while removing the inacceptable safety barrier inherent to inhalation as of excessive CO-Hb formation. An alternative to the ruthenium based M-OCORS was demonstrated featuring iron in E-OCORS instead thereby facilitating clinical translation. Further preclinical efficacy and toxicity profiling studies are required before one can embark into first in man studies with this easy to use CO generating device suitable for wide spread application.

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Supplementary Tables

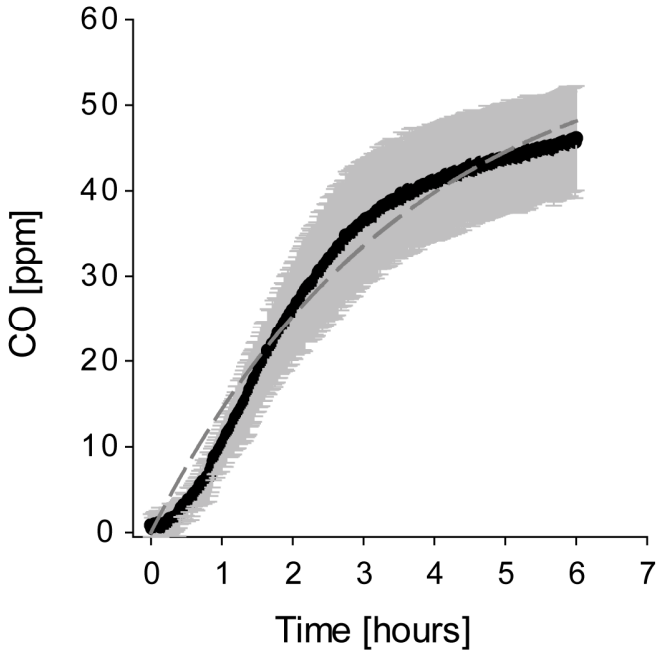
Supplementary Table 1: Constant input parameters and modelled variables used for modelling of CO-Hb formation patterns following the application of F1 and F3 (assuming first order absorption and elimination, Bateman equation).

CO – Hb formation pattern	**Constant input parameters**					**Modelled variables**
	Parameters described in the literature		**Measured parameters**			
			In-vivo	***In-vitro***		
	k_e [1/h] ($t_{e_{1/2}}$ [h])	**D [µmol]**	**B [µmol]**	**t_l[h]**	**k_a [1/h] ($t_{a_{1/2}}$ [h])**	**f [%]**
Modelled F1	1.04 (0.67) [61]	0.169 [20]	7.85 *(blood volume: 1 mL [65]; mean hemoglobin concentration :0.126 g/mL)*	0 (Figure *2A)*	5.04 (0.14) *(Figure 2A)*	22
Modelled F3				3.32 *(Figure 2B)*	0.073 (9.5) *(Figure 2B)*	100

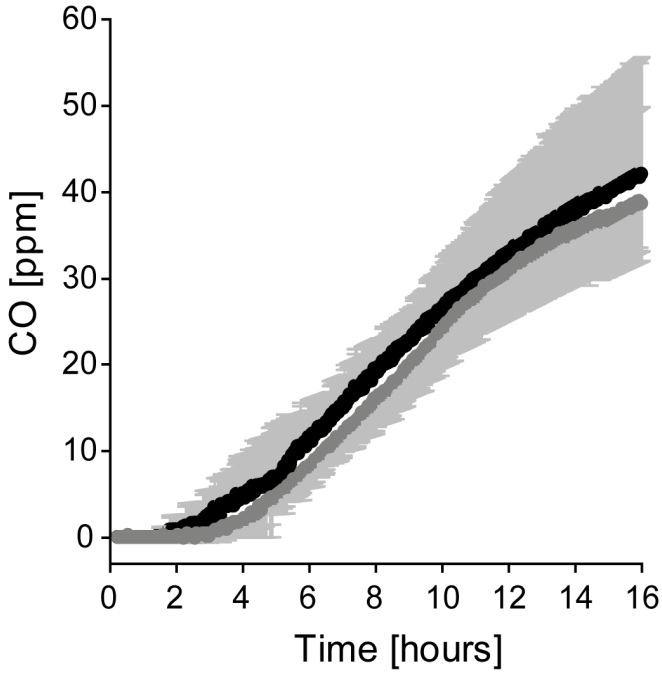
Supplementary Table 2: Criteria used for the assessment of the damage score in 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice (the table was adopted from [29], with modifications).

Score	**Parameter**
0	No damage
1	Hyperaemia without ulcers
2	Hyperaemia and thickening of bowel wall without ulcers
3	One site of ulceration without bowel wall thickness
4	Two or more sites of ulceration or inflammation
5	0.5 cm of inflammation and major damage
6-10	1 cm of major damage. The score is increased by 1 for every 0.5 cm of damage observed to a maximum of 10
+ 0 or 1	Absence or presence of diarrhoea
+ 0 or 1	Absence or presence of stricture
+ 0,1, or 2	Absence or presence (mild or severe) of adhesions

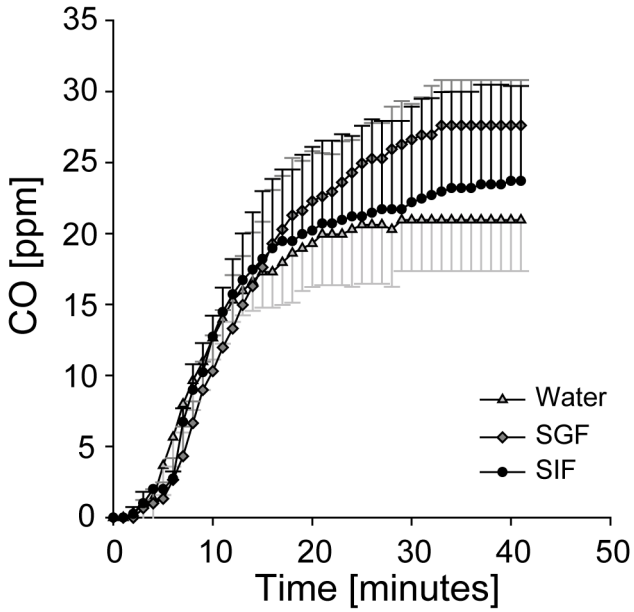
Supplementary Figures



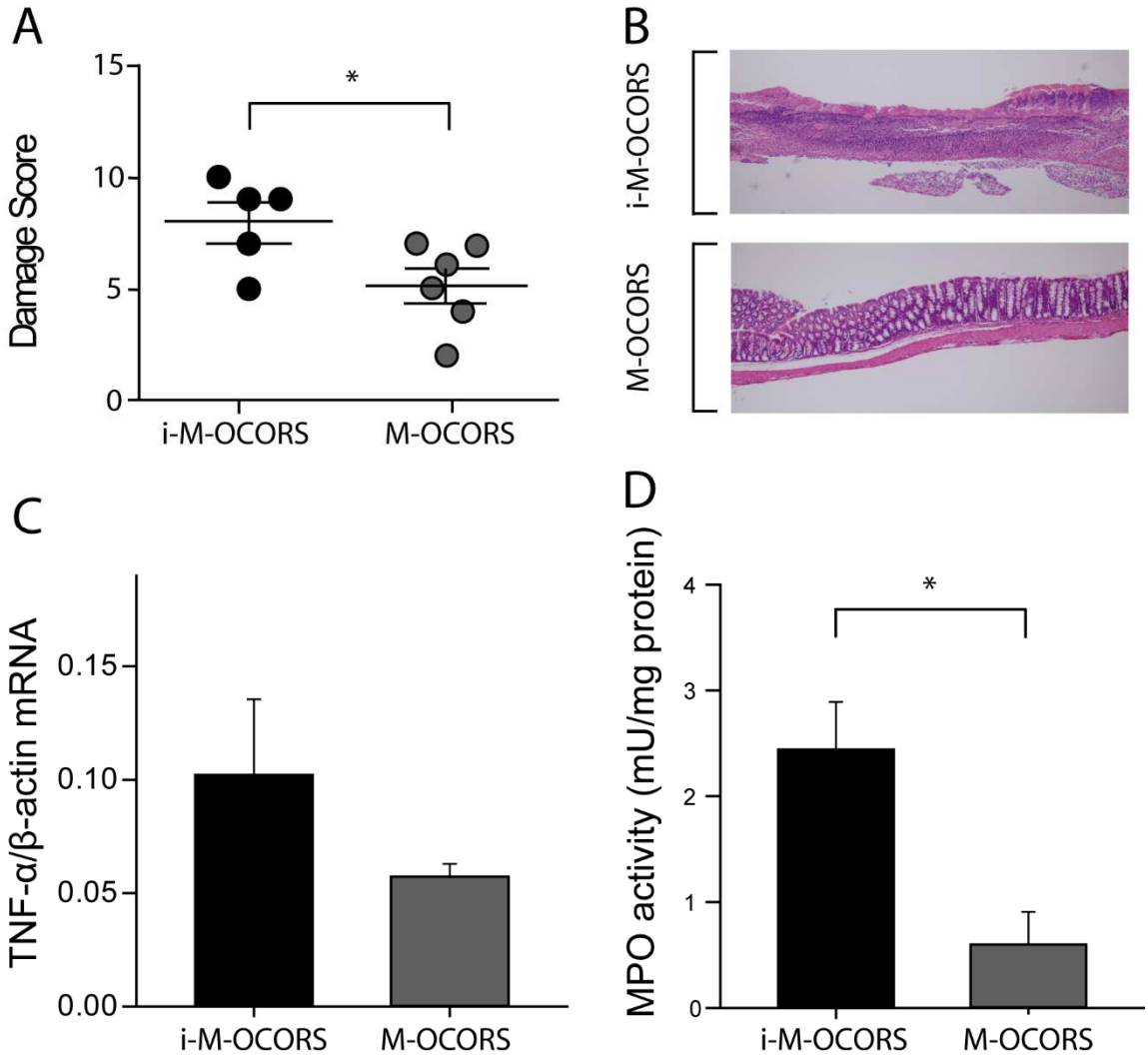
Supplementary Figure 1: CO release profile of F2 following first order kinetic (dashed line; n = 6).



Supplementary Figure 2: CO release profile of F3 at day 0 (black) and when stored for 9.5 months under ambient conditions (grey) (n = 6).



Supplementary Figure 3: Identical CO release profile of F1 in three different bio-relevant media including water (triangle), simulated gastric fluid (SGF, diamond), and simulated intestinal fluid (SIF, circles) (n = 3).



Supplementary Figure 4: Repetition of the animal trial in the TBNS mouse model for confirmation using F3 as assessed by (A) colon damage score (n = 7) (b) qualitative observation of histological changes (H&E stained), (C) TNF-α expression (n = 6) and (D) MPO activity (n = 7) in mucosal colonic tissue. Data are expressed as mean ± SE and asterisks indicate statistical difference at $p < 0.05$.

Supplementary Information

Pharmacokinetic modelling

The pharmacokinetic modelling, and curve fitting of CO-Hb formation patterns was performed using the Bateman equation assuming first order kinetics for both, absorption and elimination [66]:

$$c\,(t) = BAV * \frac{D}{B} * \frac{k_a}{k_a - k_e} * (e^{-k_e * t} - e^{-k_a * t}) \quad (1)$$

Where BAV is the bioavailability, *D* is the applied dose, B is the molar amount of available binding sites for CO on hemoglobin, k_a and k_e are the constant rates of absorption and elimination.

For modelling B (< 100 %) was considered as variable. For curve fitting k_a, k_e, and *f* were considered as variable. With all other parameters assumed as constant (for all parameters see **Supplementary Table 1**) the generalized reduced gradient (GRG) algorithm from EXCEL 2013 (Microsoft, Redmont, WA) was used to compute a Bateman equation (**Equation 1**) resulting in a minimal deviation factor f_d:

$$f_d = \sqrt{(\sum CO - Hb_m - \sum CO - Hb_s)^2} \quad (2)$$

Where $CO\text{-}Hb_m$ are sample values obtained *in vivo* and $CO\text{-}Hb_s$ are calculated values using the Bateman equation (**Equation 1**). The basal CO-Hb level before treatment (0.23 ± 0.03 (n = 8)) was used as the baseline for all calculations.

Chapter 4: Controlled therapeutic gas delivery systems for quality-improved transplants

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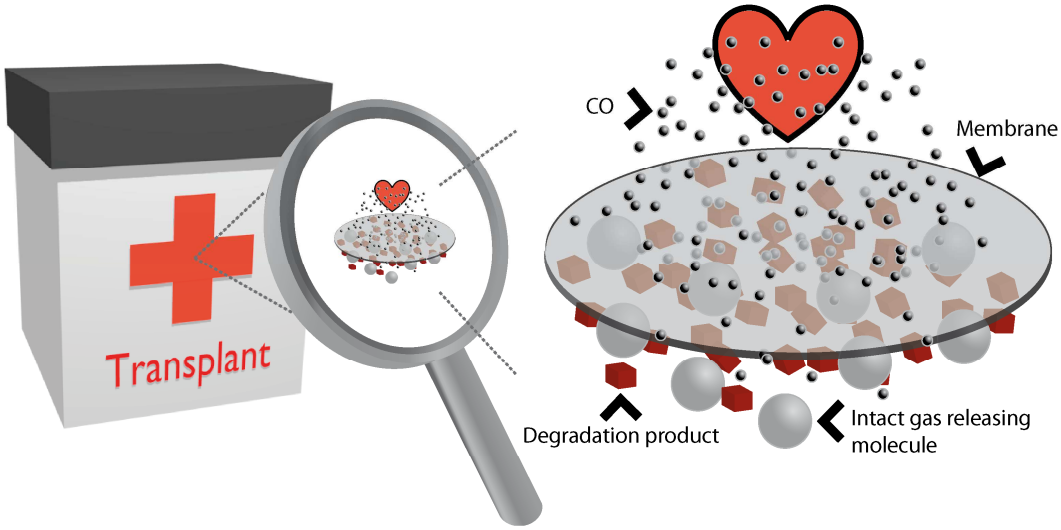

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Abstract

Therapeutic gases enriched into perfusion solutions have been effectively used for the improvement of organ transplant quality. At present, the enrichment of perfusion solutions with gases requires complex machinery/containers and handling precautions. Alternatively, the gas is generated within the perfusion solution by supplemented carbonylated transition metal complexes with associated toxicological concerns when these metals contact the transplant. Therefore, we developed therapeutic gas releasing systems (TGRS) allowing for the controlled generation and release of therapeutic gases (carbon monoxide and hydrogen sulfide) from otherwise hermetically sealed containers, such that the perfusion solution for the transplant is saturated with the gas but no other components from the TGRS are liberated in the solution. The release from the TGRS into the perfusion solution can be tailored as a function of the number and thickness of gas permeable membranes leading to release patterns having been linked to therapeutic success in previous trials. Furthermore, the surrogate biomarker HMGB1 was significantly downregulated in ischemic rat liver transplants perfused with enriched CO solution as compared to control. In conclusion, the TGRS allows for easy, reliable, and controlled generation and release of therapeutic gases while removing safety concerns of current approaches, thereby positively impacting the risk benefit profile of using therapeutic gases for transplant quality improvement in the future.

Introduction

We have an organ shortage crisis [1]. In 2013, the US had a ratio of approximately 9 : 1 for patients in need of a transplant per available donor [2]. Waiting may lead to death in those with vital transplant failure, those with non-vital transplant failure loose life time and need medical attention. One study suggested, that the cost of dialysis in kidney patients is about three times the cost of transplantation over a 4-year period [1]. It is for this and other challenges that several centers started to use transplants from extended criteria donors (ECD), including non-heart beating, elderly, or diabetic, etc. Subjects receiving transplants from ECD have an increased risk for impaired treatment outcome such as delayed graft function (DGF) [2-4]. DGF increases the risk of graft loss by 41 % in kidney transplantation [5] and is strongly associated with ischemia and blood flow constraints of the transplant leading to Ischemia-Reperfusion Injury (IRI) [6]. Therapeutic prevention or reduction of IRI is “a holy grail” in transplantation medicine [7]. In spite of the urgent need, pharmacological intervention allowing a broader use of ECD transplants to address challenges by the recipient waiting list is still in early development. A suite of studies demonstrated a decrease of DGF and IRI when using therapeutic gases, including carbon monoxide (CO), hydrogen sulfide (H_2S), and nitric oxide (NO) [8-10]. For example, the efficacy of CO was demonstrated in various preclinical studies either exposing the donor and/or recipient to CO or by perfusing the transplant with saturated CO solutions (**Table 1**). These perfusion solutions were either prepared with CO gas (leading to logistical and staff safety challenges in routine use) or from molecules generating CO *in situ*, referred to as CO releasing molecules (CORM). CORMs are typically carbonylated complexes coordinating around a central transition metal (e.g. ruthenium, manganese, iron, etc.) from which decarbonylation is possible. It is particularly for these metal ions and their associated potential toxicity, why CORMs failed to move forward in clinical development of improved transplants [11, 12].

Therefore, the challenge to solve was to develop safe and easy to use therapeutic gas delivery systems (TGRS) reliably generating and delivering therapeutic gases into solutions for transplant perfusion while preventing exposure of the transplants to toxic components at any stage.

Table 1: Preclinical status of CO in transplant medicine limited to ex vivo graft- CO therapy during storage.

Transplant	Gas source	Model	Effect	Reference
Kidney	CORM-3 [50–100 µM]	Swine, rat	Ameliorated Ischemia Reperfusion Injury	[13] [14]
	CORM-3,CORM-A1 [50 µM]	Rabbit		[15]
Liver	Blood supplemented with 300 ppm CO	Rat		[16]
	5 % saturated CO solution [41 µM]	Rat		[17]
	CORM-3 [50 µM]	Rat		[12]
Lung	5 % saturated CO solution [41 µM]	Rat		[18]
Heart	1 % saturated CO solution	Rat		[19]

Materials and Methods

Materials

Carbon monoxide releasing molecule 2 (CORM-2) - (tricarbonyldichlororuthenium(II) dimer, [$Ru(CO)_3Cl_2$]), sodium chloride, trisodium citrate monohydrate, phenylmethanesulfonyl fluoride (PMSF), sodium orthorvanadate, 1,4 dithioerythritol (DTT) and methyleneblue were purchased from Sigma Aldrich (Schnelldorf, Germany). Sodium sulfite was from Grüssing (Filsum, Germany), sodium sulfide nonahydrate was from Acros Transplantics (Geel, Belgium). 4 % CO gas in nitrogen , CO 100- and 300 ppm in synthetic air were from Linde (Munich, Germany). Sicovit Tartrazinee 85 E 102 was from BASF (Ludwigshafen, Germany). Anti-HMGB1 antibodies were from Abcam (Cambridge, UK). Citric acid monohydrate was from Jäkle Chemie. PageRuler Prestained Protein ladder was from Life Sciences (St. Petersburg, FL). The Super Signal West Pico Luminescent Substrate was purchased from Thermo Scientific (Waltham, MA). The Quick Start Bradford 1x Dye Reagent was purchased by Bio Rad (Hercules, CA). Custodiol solution was from Dr. Franz Köhler Chemie (Bensheim, Germany). All other reagents were from Sigma Aldrich (Schnelldorf, Germany) and at least of pharmaceutical grade unless otherwise noted.

Methods

Electrochemical headspace gas detection for release systems

Electrochemical gas detection was performed as previously described [20], with modification. In brief, we used a desiccator (DN 100) DURAN (Wertheim, Germany) as gas release detection space tightly sealed with a desiccator lid (**Figure 1**). For headspace gas detection a lid having an opening to a flange (DN 40) Rettberg (Göttingen; Germany) was attached and the setup was clamped with a three-point flange clamp (Rettberg) to another flange equipped with an XXS LC sensor for the detection of the therapeutic gas from Draeger (Luebeck, Germany). The sensor was connected to an X-am 5000 gas detector (Draeger). The cable was glued into a guide tube using a hot meld adhesive 'Pattex Heißklebesticks' from Henkel (Düsseldorf, Germany) consisting of copolymer of ethylene with vinyl acetate. In a modification of the system the lid was closed with a perforated plastic stopper instead of attaching the flange to the lid. The cable connecting the sensor for headspace gas detection to the detector outside of the desiccator was glued in the plastic stopper with hot melt adhesive "Pattex Heißklebesticks" (Henkel). The setup of the detection system is shown in (**Figure 1**).

The sensors were ether calibrated by the manufacturer or using certified calibration gas for CO (100 ppm). All devices were additionally calibrated as previously described, with modification [20]. Briefly a concentration series of CO was generated by transferring increasing amounts of CORM-2 into 600 mL of a stirred (600 rpm) 0.333 g/L aqueous sodium sulfite solution. Maximal CO release [ppm] was plotted against the amount of CORM-2 [mg] transferred to the system. Linear regression was applied to determine the linear correlation between applied amount of CORM-2 and corresponding concentrations of CO (n = 3) (forced to intercept at zero). The slope of the regression line was used for normalization of the detectors. Leakiness was assessed with 300 ppm CO gas enclosed in the system for 1 hour.

Therapeutic Gas Release System

A Therapeutic Gas Releasing System (TGRS) (**Figure 1A**, **Supplementary Figure 1A, B**) with a reaction space and a lid were manufactured from a stainless steel cylinders by our tool shop. For gas release, the reaction space (volume 4.5 mL) has one, the lid two openings. The gas release from the reaction space is controlled either by one or two membranes clamped between the lid and the reaction space. The membrane was sealed towards the steel part by two gasket rings (20 x 2 mm, Schwarz, Wuerzburg, Germany). The reaction space and the lid were pressed together with a flange clamp (Varian, Palo Alto, CA) sealing the device.

CO release from TGRS

In a first setting CO release from the TGRS was controlled by one 0.01″ SSP-M823 polydimethylsiloxane membrane from SSP (New York, NY) clamped between the lid and the reaction space (**Supplementary Figure 1A**).

12 mg CORM-2 and 6 mg sodium sulfite were weighted into the reaction space with an AJ 100 from Mettler Toledo (Greifensee, Switzerland) and 3.0 mL water were added. The device was immediately closed using a flange clamp. The desiccator was filled with 600 mL water stirred at 600 rpm (Variomag Telesystem, Thermo Scientific, MA). The gas releasing device was immersed into the water in the desiccator which was closed subsequently. The measurement of the therapeutic gas was performed in the headspace of the desiccator.

In a modification the CO release from the TGRS was controlled by two 0.01″ or - in another setting - two 0.04″ SSP-M823 polydimethylsiloxane membranes clamped between the lid and the reaction space (**Figure 1A, Supplementary Figure 1B**) in an otherwise analogous setting as outlined above. The membranes were separated by a 2 mm thick PTFE ring (Schwarz, Wuerzburg, Germany) creating a distinct gas phase between the TGRS and the outer solution. For the setup using SSP-M823 0.01″ silicone membranes, aliquots of the medium were collected for ruthenium analysis (*vide infra*).

The TGRS setup for the release of H_2S was similar to the above mentioned silicon based TGRS but different amounts of substances and liquids were used. 1 mL of a 0.7 mg/mL sodium sulfide solution was injected into the reaction space (**Figure 1A, Supplementary Figure 1B**). A 250 µL Eppendorf vial without lid was filled with 100 µL 0.1 M HCl and also transferred to the reaction space in upright position. In this setting we applied two SSP-M823 0.01″ silicone membranes and filled the desiccator with 600 mL water stirred at 600

rpm. Before transferring the device to the desiccator the compounds were mixed by shaking the device manually such that the HCl solution got mixed with the sodium sulfide solution thereby generating H_2S. H_2S measurement was performed in the headspace of the desiccator and stopped when gas bubbles accumulated at the membrane of the TGRS.

Inductively coupled plasma optical emission spectrometry

Ruthenium content of the medium exposed to CO releasing TGRS equipped with two 0.01″ silicone membranes (*vide supra*) was measured by inductively coupled plasma optical emission spectrometry ICP-OES Vista Pro Radial from Agilent Technologies (Santa Clara, CA) as previously described [20]. The positive control was a solution prepared from 12 mg of a CORM-2 and 150 mg sodium sulfite in 450 mL water.

Leakage tests with a dye

To further test for leakage of nongaseous compound from the TGRS, the system was filled with 150 mg methyleneblue dissolved in 4 mL water. The TGRS equipped with two 0.005″ SSP-M823 silicone membranes was enclosed using a flange clamp. The desiccator was filled with 600 mL water stirred at 600 rpm for 12 hours using a Variomag Telesystem. Another integrity stress test was performed using a TGRS filled with 150 mg methyleneblue in 4 mL water and equipped with one 0.005″ silicone membrane with challenge at 70 °C in a WB 1041 water bath from GFL (Burgwedel, Germany) for 6 hours. Coloration of the medium was assessed visually and using a Genesys 10S UV-VIS spectrometer (Thermo Scientific).

Animal studies

Transplants were harvested from nine male Sprague Dawley rats (mean weight: 332 ± 0.2 g), which were obtained from Janvier Labs (Saint Berthevin Cedex, France). Animals were kept conform to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* at a 12 hours day and night cycle. Standard chow and tap water were provided *ad libitum*.

Animals were sacrificed by an overdose of isoflurane 5 % (v/v) from Abbott (Wiesbaden, Germany) and nitrous oxide 50 % (v/v) in a rodent anesthesia induction chamber. During a quick procedure the common hepatic artery and portal vein were cannulated. Therefore a median laparotomy was performed and the right liver lobe was gently elevated to access the gastroduodenal artery. This vessel was cannulated using a primed PE-25 catheter. The

catheter was then advanced into the hepatic artery proper [21]. Afterwards, the hepatic portal vein cannulation was performed with a shortened 18 Gauge plastic i.v. catheter and advanced proximally to the branching segmental veins. The thoracic cavity was opened to cut the inferior vena cava cranially to the diaphragm. Perfusion of the liver was performed with either 100 mL of carbon monoxide (CO) enriched (*vide infra*) or conventional Custodiol solution for 12 minutes. 20 % of the perfusion solution was infused via the arterial system. The liver was harvested and incubated in a jar of the respective perfusion solution for 24 hours at +6° C.

The CO enriched solution was prepared from a 600 mL Custodiol solution in a desiccator using a TGRS equipped with two SSP-M823 0.005″ silicone membranes from SSP. CO was released from a suspension of 25 mg CORM-2 with 12.5 mg sodium sulfite in 25 mg citric acid in 3 mL water stirred at 600 rpm on a Variomag Telesystem. CO release was stopped by removing the TGRS from the desiccator when a threshold of a CO concentration in the headspace was 1400–1600 ppm. The solution was stored in the closed desiccator and used within 1 hour. After 24 hours incubating the transplant in the CO enriched or conventional Custodiol solution, respectively, central and peripheral tissue samples were collected from the right and left liver lobe and snap frozen in liquid nitrogen and stored at -80 °C for western blot analysis.

Western blot

Deep-frozen samples were pulverized under liquid nitrogen using a mortar and pestle at 4 °C room temperature. 10 mg was incubated with cytosolic extraction buffer (Hepes 10 mM pH 7.9, $MgSO_4$ 1.5 mM, KCl 10 mM, 0.5 % Triton X-100, with fresh added 0.2 mM PMSF, 0.5 mM DTT and 0.1 mM Na_3VO_4). Samples were homogenized using a Mini-Beadbeater-1 and 2.3 mm stainless steel beads (both from Biospec Products (Bartlesville, OK)) at 4800 rpm for 30 seconds. The supernatant containing the cytosolic fraction was collected after centrifugation (12000 x g) using a Sigma 3K12 centrifuge from Sigma Laborzentrifugen (Osterode am Harz, Germany) and snap frozen using liquid nitrogen. The protein concentration of the supernatant was determined using a Quick Start Bradford following the manufacturer's instructions. 50 µg of the supernatant was processed using SDS-PAGE and Western blotting as previously described, with modification [22]. The blotted nitrocellulose membrane was cut at the height of the 35 kDa PageRuler Prestained Protein ladder for the separate antibody labelling of HMGB1 (~25 kDa) and ß-actin (~52 kDa). For detection of

HMGB1, an anti-HMGB1 antibody (1: 10000 in Tris-buffered saline, containing 0.1 % (w/w) Tween 20) was used on the lower part of the blot (1–35 kDa). ß-actin was detected on the upper part of the blot (35–170 kDa) using an anti-ß-actin antibody (1: 10000 in Tris-buffered saline, containing 0.1 % (w/w) Tween 20). After incubation with a peroxidase conjugated secondary antibody, the signal intensity was assessed using a Super Signal West Pico Luminescent Substrate and a FluorChem FC2 imaging system from Protein Simple (Santa Clara, CA).

CO release kinetics - bridging to previous protocols flushing perfusion solutions with CO gas

For this a minimized CO-detection system was used. It was constructed as such that the desiccator described above was replaced with a sealed flange (DN 40) from Rettberg to reduce the headspace volume enabling CO headspace detection in equilibrium state (**Supplementary Figure 3**). Calibration was performed using CORM-2 as described above. Isotonic NaCl solution was enriched with CO using the TGRS following the preclinical protocol at room temperature until reaching 1400 – 1600 ppm CO in the headspace. Following protocols of previously described efficacy studies 300 mL isotonic NaCl was treated with 4 % CO gas for 5 minutes at 4 °C [17, 18]. 40 mL of each solution was analyzed at 37 °C at a KS-15 control from Bühler (Hechingen, Germany) using the minimized CO detection system (*vide supra*). Due to technical constrain we used 4 % instead of 5 % CO gas used in above mentioned reports.

CO release stick

A 10 cm silicon tube (ID: 7 mm, OD: 10 mm) from Haeberle (Lonsee-Ettlenschieß, Germany) was used as release controlling membrane within which an enclosed Duran glass NMR tube (178 x 4.95 mm; Duran, Wertheim, Germany) was used for water tight entrapment of CORM-2 and sodium sulfite. The NMR tube was shortened to 6 cm and filled with 15 mg CORM-2 and 20 mg sodium sulfite using a XP105 balance from Mettler Toledo (Greifensee, Switzerland) before the open end was fused in a gas flame. The silicone tube was filled with the enclosed NMR tube, 100 mg tartrazine, and 2 mL water before it was closed with polypropylene plugs sealed with vacuum grease from Dow Corning (Midland, MI). CO release was initiated by breaking the NMR tube within the stick which was manually shaken for 10 seconds, thereafter. CO release was assessed while the air in the

desiccator was ventilated by stirring at 400 rpm with a Variomag Telesystem. For integrity testing the stick was loaded with 100 mg tartrazine in 2 mL water. Coloration of the liquid within which the stick was placed (300 mL) was assessed after 20 hours visually and using a Genesys 10S UV-VIS spectrometer.

Cartridge system for gas enrichment of a perfusion solution

A first system for rapid CO generation and release was prepared using CORM-A1. For that, a tube in tube cartridge system was designed (length: 92 cm - **Figure 5B, Supplementary Figure 4**). The inner tube was a silicone tube "SIK8649" (ID: 2 mm, OD: 2.6 mm) from Raumedic (Helmbrechts, Germany). The outer tube was a PVC tube (ID: 4 mm, OD: 7 mm) from VWR (Darmstadt, Germany). On both ends the inner and outer tube were connected with a polylactide valve which was custom made by 3D printing by the computing center of our university (**Supplementary Figure 4** - white box). The inlet for the inner tube was replaced with a PTFE HPLC tube (OD: 2.4 mm). The valve was then casted in hot meld adhesive. A three way stop cock was attached and used to perfuse the inner tube with 2 mL of a 0.5 mg/mL CORM-A1 citric acid buffer solution (pH 2.5) prepared right before the perfusion. The outer tube was perfused with water at a flow rate of 3.5 mL/min using a flexible tube pump Pumpdrive 5001 from Heidolph (Schwabach, Germany) with discarding the residual gas in the outer tube using a three way cock from B. Braun (Melsungen, Germany). For online gas quantification the CO enriched solution was then pumped through an ePTFE tube (Aeos ePTFE: ID 2.1 mm, OD 3 mm) from Zeus (Orangeburg, NC) directly attached to a XXS CO sensor (*vide supra*) using a heat-shrink tube and sealed with hot melt adhesive (**Supplementary Figure 4**). The heat shrink tube was perforated laterally to the ePTFE tube for gas measurement under sink conditions. The CO sensor readouts were corrected to the exact amount of CORM-A1 used in the respective experiment (ranging from 0.9–1.2 mg). Leak tightness was tested following release experiments by infusing a tartrazine solution (10 mg/mL) for 10 minutes into the inner tube and testing possible leakage into the outer tube filled with water visually and photometrically. In another experiment, the inner tube was loaded with a methyleneblue solution (10 mg/mL). 5 cm of the tube were immersed for 12 hours in 30 mL water at 50 °C in an "ED 53" drying chamber from Binder (Tuttlingen, Germany). Evaporated water was refilled accordingly.

A second system was developed to allow precise control of the CO enrichment kinetics within the perfusion solution. The setup was analogous to the one outlined in the previous

paragraph with respect to the assembly of the inner and outer tube, but some adaptations were necessary to precisely control the influx of the sodium sulfite solution into the circle containing the inner tube (**Figure 6A**). In this setting applying the tube in tube cartridge system both ends of the inner tube were connected to a Flocon 1003 flexible tube pump at 35 rpm (flow rate of 7.4 mL/min) from Roto-Consulta (Luzern, Switzerland), thereby constantly circulating a suspension of 19.15 mg CORM-2 in 2 mL water (**Figure 6A**). CO generation and release was triggered by injecting aliquots of a sodium sulfite solution (0.5 mg/mL) into the inner tube via an "Acrodisc" 25 mm syringe filter with a 5 µm "Versapore" membrane from Life Sciences using the three way valve and a 50 mL perfusor syringe from B. Braun as described above. The increasing pressure in the inner tube due to the injection was released via an outlet equipped with a syringe filter. Firstly, the sodium sulfite was gently injected manually until CO was detectable at the online detector (*vide supra*). At any time that readouts decreased to 25 ppm, sodium sulfite was injected using a "Perfusor Space" syringe pump from B. Braun at a rate of 33.33 mL/h. Injection was stopped when readouts reached 35 ppm. The outer tube was perfused with water at a flow rate of 7.4 mL/min using a Flocon F1003 flexible tube pump. Leak tightness was tested following release experiments with tartrazine were conducted as described above for the CORM-A1 based system.

The system was further modified for the generation and release of H_2S. For that, the identical setting was used as outlined in the previous paragraph, but the tubes were filled with different suspensions/solutions (**Supplementary Figure 5B, C**). The inner tube was filled with a citric acid solution (pH 2.5). H_2S release was triggered by injecting aliquots of a sodium sulfide solution (0.44 mg/mL) into the inner tube following the exact procedure described above for the injection of sodium sulfite. Leak tightness was tested with tartrazine solution as described above.

Statistics

All data were reported as mean ± standard deviation unless specified otherwise. One-tailed t-test was applied using Minitab 16 from Minitab (Coventry, UK) for western blot analysis. $p < 0.05$ was considered statistically significant.

Results

Design of the therapeutic gas releasing system prototype

The therapeutic gas releasing system (TGRS) prototype is designed to allow controlled exposure of the transplant to the *in situ* generated therapeutic gas wile separating the transplant from impurities, the gas releasing molecule itself, or by- and degradation products, thereof. For that, TGRS was profiled to generate the desired therapeutic gas *in situ,* and to allow controlled release of the therapeutic gas through a gas permeable membrane which is impermeable to other molecules. Thereby, the controlled release of the therapeutic gas from TGRS into the perfusion/storage solution of the transplant was achieved. That CO loaded perfusion solution carries the therapeutic gas into and around the transplant, respectively (**Figure 1A**).

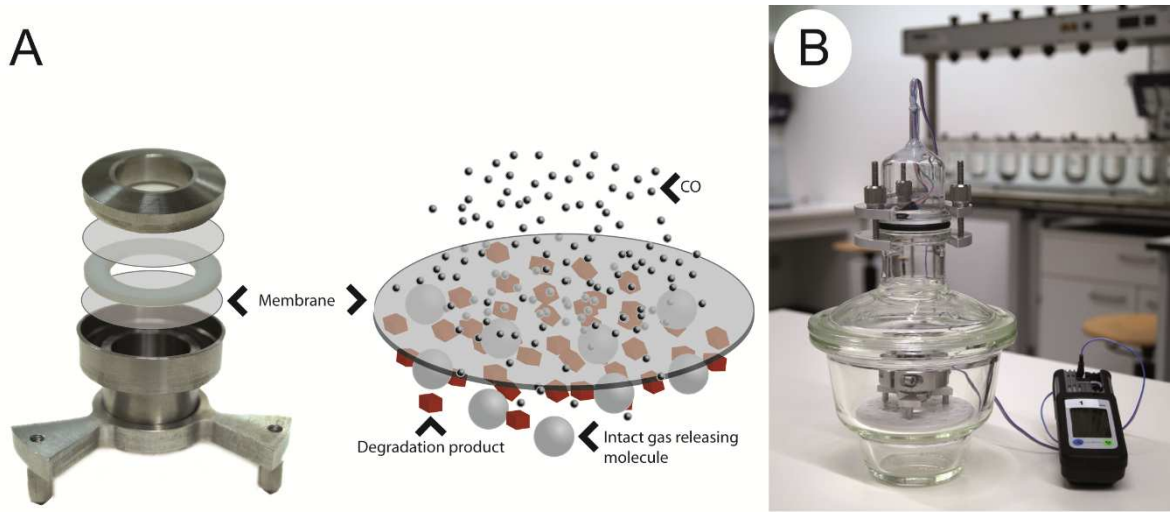

Figure 1: (A) Therapeutic Gas Release System (TGRS) in the two membrane setup. The gas releasing molecule (grey) disintegrates when in contact with the trigger molecule (not shown) as soon as water is added. Thereby, the therapeutic gas is produced (black) freely diffusing through the membrane. All impurities, by- and degradation products are retained within the TGRS and do not penetrate through the membrane thereby restricting exposure of the transplant to the therapeutic gas only. (B) Therapeutic gas detection system with amperometric measurement in the headspace and equipped with a TGRS in the desiccator.

The TGRS prototype was designed in a one membrane setup and two membrane setup (**Figure 1A**; **Supplementary Figure 1**). For that, a reaction space was designed within which the gas releasing molecule and a trigger molecule – the trigger molecule is required to initiate the replacement of the gas from the transition metal center of the gas releasing molecule (decarbonylation) in the moment water is added to the system – were loaded [20]. The reaction space was separated from the perfusion solution by one membrane or two membranes. The two membrane setup was chosen to introduce an additional safety layer for in case of membrane rupture. This prototype required the manual addition of water to the reaction space whereas later systems were designed to provide all necessary components required for gas generation within the TGRS (*vide infra*). Therapeutic gas generation and release from the TGRS was measured in an amperometric setup within which released therapeutic gas was measured in the headspace (**Figure 1B**) [20]. The analytic setup was tightly sealed (leakage of CO gas was ≤ 1 % per hour (n = 3, data not shown)).

In vitro therapeutic gas release

Controlled, therapeutic gas release was demonstrated for CO and H_2S (**Figure 2, Supplementary Figure 5**). The TGRS was first profiled with one 0.01" silicone membrane. (**Figure 2A**). CO generation and release from this TGRS was at a rate of 1.17 ppm CO per minute in the linear section of the release pattern (150–550 minutes) with zero order kinetics lasting for 6.67 hours after a lag time of 108 minutes (**Figure 2A**). The TGRS was further equipped with two 0.01" silicone therapeutic gas permeable membranes and a gas space in between the membranes (**Figure 2B**). In the linear section of the release pattern (300–720 minutes) CO generation and release from the two 0.01" silicone membrane based TGRS was at a rate of 0.42 ppm CO per minute with zero order kinetics lasting for 7 hours and a lag time of about 85 minutes (n = 3; **Figure 2B**). Furthermore, the thickness of the membranes was modulated in an effort to further modulate the therapeutic gas release from the TGRS (**Figure 2B**). Using two 0.04" silicone membranes reduced the release to a rate of 0.24 ppm CO per minute in the linear section of the release pattern (240–720 minutes) with zero order kinetics lasting for 8 hours and a lag time of about 146 minutes (n = 3; **Figure 2B**). This demonstrated, that the CO release from the silicone membrane based TGRS can be adapted by proper choice of the membrane. In an effort to extend the nature of the therapeutic gases generation and production by the TGRS prototype, a H_2S releasing TGRS was developed (n = 3; **Supplementary Figure 5A**). Using two 0.01" silicone membranes the H_2S release rate was 0.04 ppm per minute with zero order kinetics lasting for 100 minutes with no lag time.

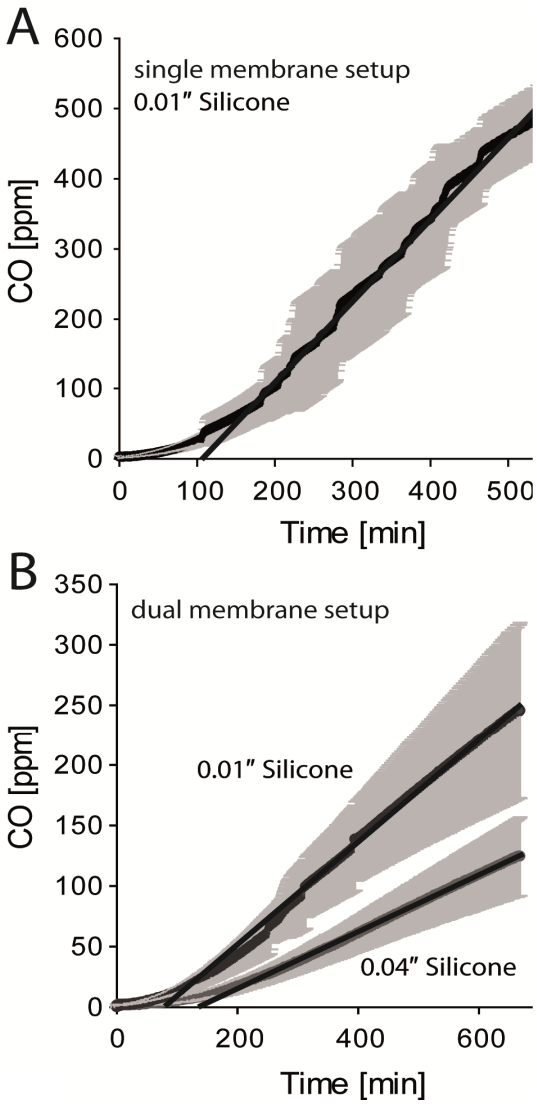

Figure 2: (A) CO release pattern of the Therapeutic Gas Release System (TGRS) equipped with one 0.01″ or (B) two silicone membranes with thicknesses of 0.01″ silicone or 0.04″ silicone. All data are expressed as mean ± SD with n = 3. The linear line indicates the linear section of the release pattern.

Integrity test of the therapeutic gas releasing system

Integrity tests were performed for the TGRS equipped with 0.005" or 0.01" silicone membranes with the single and dual membrane setup, respectively (**Table 2** is providing an overview of the experiments). No methyleneblue diffusion from the TGRS was observed when using the single 0.005" membrane setup and exposing the system to 70 °C for 6 hours or when using the 0.005" dual membrane setup at room temperature for 12 hours to mimic the subsequent *ex vivo* experiments with liver transplants (*vide infra*). The TGRS was challenged for ruthenium release from the TGRS when placed into a solution for 12 hours at ambient conditions using the 0.01" dual membrane setup (ruthenium is the transition metal cation in the CO releasing molecule 2 (CORM-2)). For control, a suspension of equimolar amounts of CORM-2 as compared to what was placed in the TGRS resulted in 9.61 ± 0.21 mg/L ruthenium in a sodium sulfite solution during 12 hours.

Table 2: Used CO release systems and leakage tests.

CO release system		**Integrity test**	**Method**	**Figure**
Therapeutic Gas Releasing System	One silicone membrane	Dye (methyleneblue) 6 h exposure (70 °C) 0.005″ membrane	Visual / UV-metric	Figure 2A
	Two silicone membranes	Ruthenium 12 h exposure (21 °C) 0.01″ membranes	ICP-OES	Figure 2B
		Dye (methyleneblue) 12 h exposure (21 °C) 0.005″ membranes	Visual / UV-metric	
CO release stick		Dye (tartrazine) 20 h exposure (21 °C)	Visual / UV metric	Figure 4
Cartridge gas release system	Cartridge system for immediate release	*Systems:* Dye (tartrazine) following perfusion for 10 min (21 °C) *Inner tube:* Dye (methyleneblue) 12 h exposure (50 °C)	Visual / UV metric	Figure 5 B/C Supplementary Figure 4
	Cartridge system for controlled release			Figure 6

Bridging study

Some relevant previous preclinical efficacy studies on transplant preservation demonstrated the efficacy of CO when using *a priori* equilibrated CO solutions. Some of these studies flushed perfusion solutions with CO gas for equilibration (**Table 1**). Therefore, we bridged the CO generation and release from the CORM based TGRS prototype to perfusion solutions saturated with CO gas in analogy to the perfusion solutions used in these previous efficacy studies (**Figure 3A**). The experiments were conducted in isotonic NaCl solution having the same colligative properties as typical perfusion solution used clinically, including the "University of Wisconsin" solution or Custodiol solution which we used for the *in vivo* experiments [23-25]. CO headspace kinetics from CO gas enriched solutions was comparable to solutions enriched by using the TGRS but more precise for the TGRS as indicated by the substantially lower standard deviation (**Figure 3A**).

We assessed efficacy of the TGRS saturated perfusion solutions on liver explants using HMGB1 as a well described early surrogate marker for ischemic injury. Rat livers perfused and stored in TGRS treated CO-Custodiol solution - CO enrichment resulted in 1400–1600 ppm CO after 115.8 ± 19 minutes as measured above the transplant in the CO-Custodiol solution (n = 4, data not shown) - had significantly lower cytoplasmic HMGB1 compared to CO free Custodiol solution as determined after 24 hours of storage both in the central and peripheral sections of the liver, respectively, and in analogy to previous trials exposing transplants to CO enriched perfusion solutions (**Figure 3B, C; Table 1**).

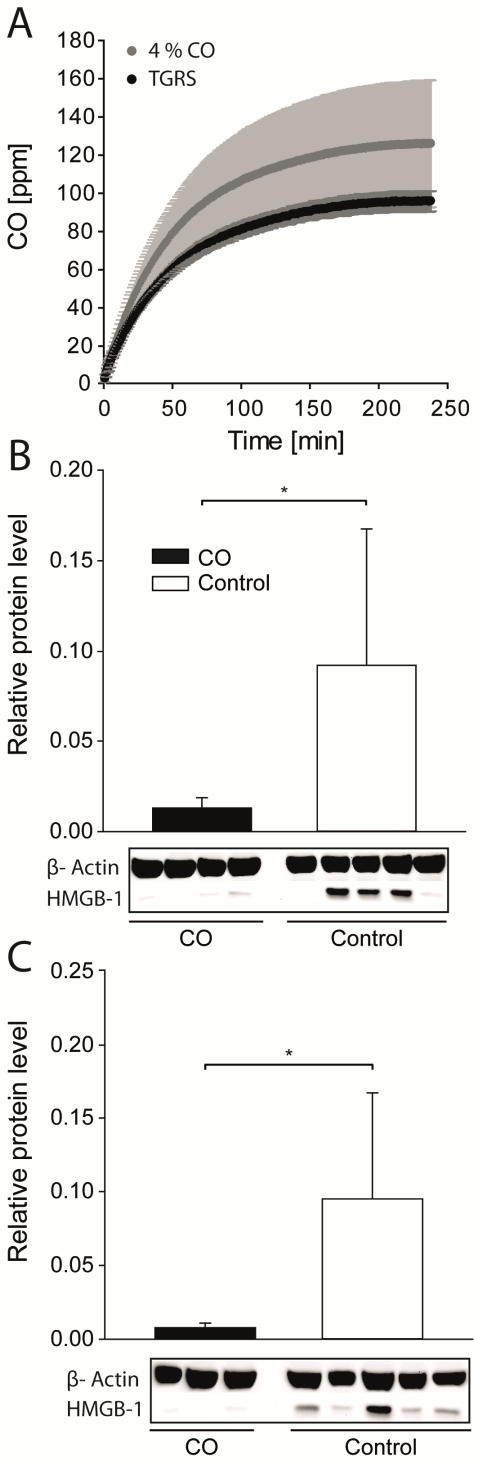

Figure 3: (A) Headspace CO release profile in isotonic NaCl using solutions flushed with 4 % CO prior to measurement or CO saturated using the TGRS (n = 3). Western blot analysis of cytosolic HMGB1 from (B) central or C) peripheral sections of rat liver transplants perfused with and stored in Custodiol solution for 24 hours with and without TGRS enriched CO solutions (* $p < 0.05$). All data are expressed as mean ± SD.

Development of the all-in-one TGRS

We identified three major necessary improvements of the TGRS prototype to meet possible demands of future clinical settings namely (i) the need for a closed system containing all necessary components within the TGRS, an (ii) easy and safe trigger mechanism to initiate gas generation and release upon activation by the user, and (iii) the ability for controlled CO generation and release.

The first setup was developed to allow easy therapeutic gas generation and release independent of complex facilities (such as in an ambulance car, small hospitals without special transplant equipment, etc., **Figure 4A**). The resulting stick setup is an all in one, self-contained system which upon bending breaks the CORM containing inner cylinder thereby exposing the CORM to the surrounding solution all of which being hermetically sealed (other than for gas release) within the stick. Thereby, instantaneous CO generation commences within the stick. The generated CO (but no other by and degradation products or the CORM itself) permeates through the outer and gas-permeable membrane of the stick, thereby saturating a solution with CO in which the stick is given or within which it was activated by bending (**Figure 4A**). Upon activation, the CO release from the stick cannot be turned off and it is designed for single use. After release initiation through bending, CO release was 2 ppm per minute for 100 minutes after which the release levelled off plateauing at 400 ppm after 1000 minutes (**Figure 4B**). For integrity testing of the outer and gas-permeable membrane of the stick, a dye solution was included into the stick. No dye was found in the medium into which the stick was placed throughout 20 hours (the experiment was finished after 20 hours; data not shown).

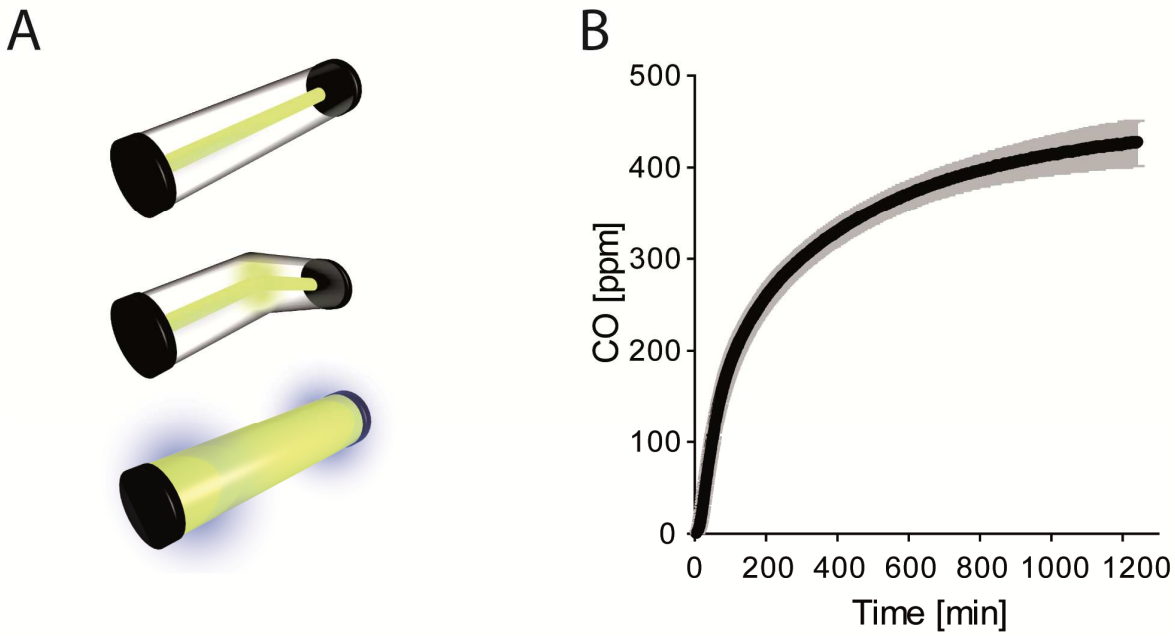

Figure 4: (A) Schematic drawing of the stick system before, during, and after activation from top to bottom, respectively. The CORM and the trigger molecule are within the inner breakable cylinder for water tight entrapment and in solid form. This inner cylinder is surrounded by water, within a container allowing gas permeation through its surface by means of a silicone membrane. (B) CO release pattern of a CO from an activated release stick prototype (n = 3, mean ± SD).

The second setup was designed for integration into existing transplant perfusion systems providing continuous perfusion of the transplant throughout storage/transport to be used in more sophisticated environments and with the goal to achieve very rapid enrichment of the perfusion solutions to minimize the waiting time between CO generation and release into the circulating perfusion solution and the initiation of the perfusion of the transplant. Therefore, and inspired by membrane oxygenators, we developed a tube in tube cartridge system for the immediate online enrichment of a perfusion solution (**Figure 5A, B** and **Supplementary Figure 4**). A thin walled inner silicone tube was functional for gas release from CORM-A1, and the generated CO gas diffused through the membrane and into the outer perfusion solution. CO was generated from CORM-A1 by injection of citric acid buffered solution (pH 2.5) given into the inner tube, thereby instantaneously releasing CO as detected by system integrated detectors. The solution was pumped through a highly gas permeable ePTFE tube directly attached to a CO sensor. This setting is functional for (i) protecting the sensor from liquids and (ii) assessing the amount of CO which freely permeated through the ePTFE membrane (data not shown). The sensor was attached as such that CO gas once detected by the sensor can freely passage out of the system thereby generating sink conditions. CO release was detected within seconds with the pulse peaking after approximately 3 minutes and declining gradually, thereafter (**Figure 5C**). The tube in tube cartridge system was tested for integrity by infusing a dye into the inner tube and no diffusion through the membrane and into the perfusion solution was detected (data not shown).

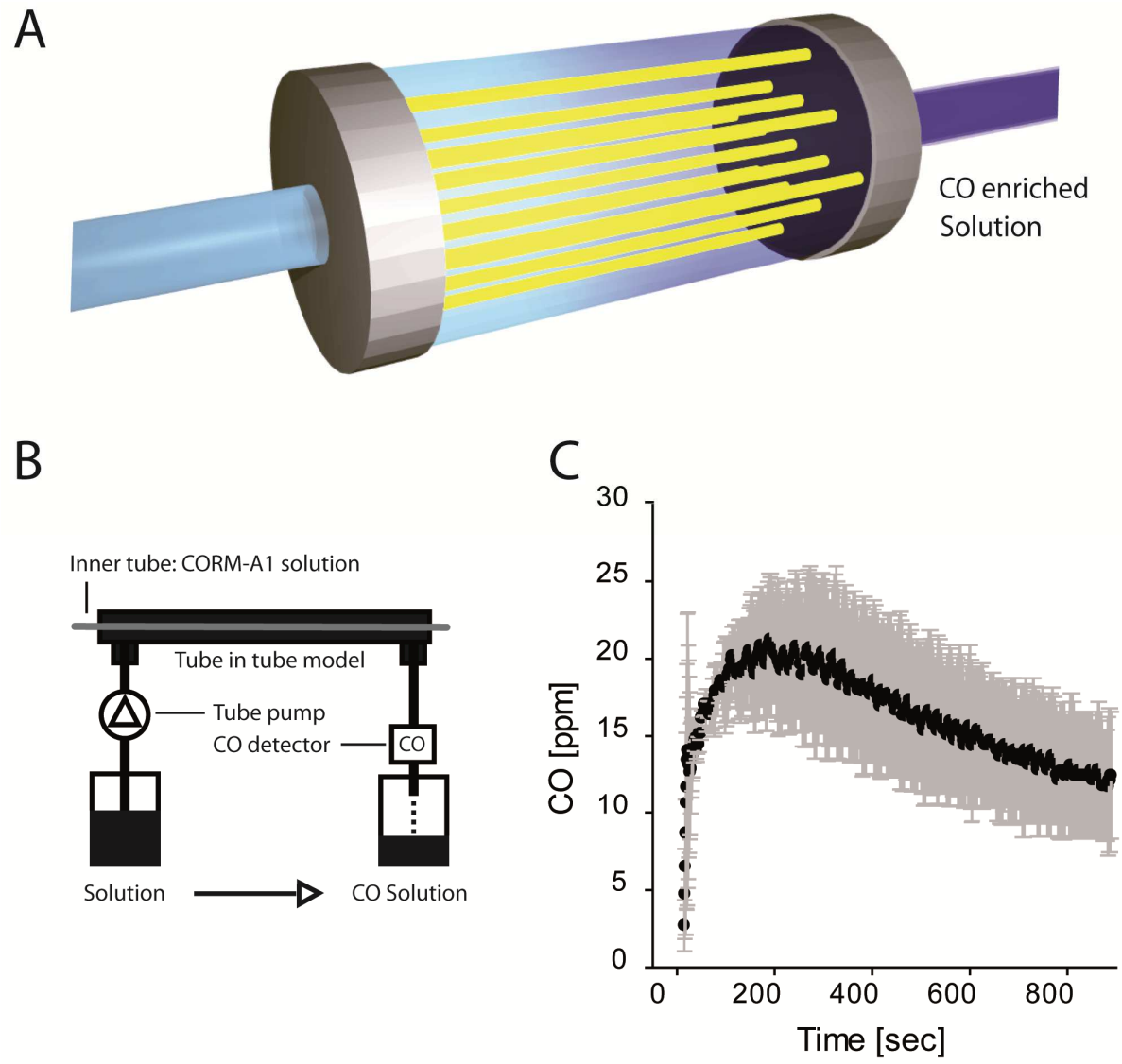

Figure 5: (A) Cartridge tube model for the online enrichment of a perfusion solution with CO. The inner tube is a hydrophobic membrane perfused with a CORM-A1 solution (yellow). Upon acidification the solution releases CO diffusing through the tubing into the surrounding perfusion solution, which is circling through the transplant. (B) Schematic drawing of the single tube in tube cartridge system for immediate enrichment of a perfusion solution using CORM-A1 (C) CO release pattern of water enriched with CO gas using the single tube in tube cartridge system for online enrichment of perfusion solutions (n = 3, mean ± SD).

The tube in tube cartridge system was further refined to allow controlled transplant perfusion with pulses of one or more than one therapeutic gases when integrated in transplant perfusion systems (**Figure 6**). CORM-2 was used in this setup, responding with CO release when exposed to sodium sulfite. For that, the inner tube was loaded with a CORM-2 suspension (**Figure 6A**) and injections of sodium sulfite resulted in instantaneous CO generation and release as measured in the perfusion solution circulating outside the inner tube. Typical release patterns reached t_{max} within 3 minutes leading to c_{max} of about 40–50 ppm with a linear decline, thereafter reaching the pre-injection status within 20 minutes. Repeated cycles of CO generation through sodium sulfite injection resulted in declining c_{max} values, possibly reflecting the beginning exhaustion of the CORM upon repeated challenge (**Figure 6B**). The identical setup was used for the generation of H_2S (**Supplementary Figure 5B**). In contrast to the CO releasing tube in tube cartridge system, the trigger for therapeutic gas generation and release - a citric acid solution (pH 2.5) - was circulated within the inner tube and sodium sulfide was injected into the inner tube for gas generation. Repeated H_2S pulses similar to the CO pulses were generated (**Supplementary Figure 5C**). The tube in tube cartridge system was tested for integrity by infusing a dye into the inner tube and no diffusion through the membrane and into the perfusion solution was detected (data not shown). We additionally stressed the integrity of the inner tube by loading it with methylene blue and exposing it to 50 °C for 12 hours providing evidence for the tightness of the approach in that the escape of other molecules than the therapeutic gas is prevented (data not shown).

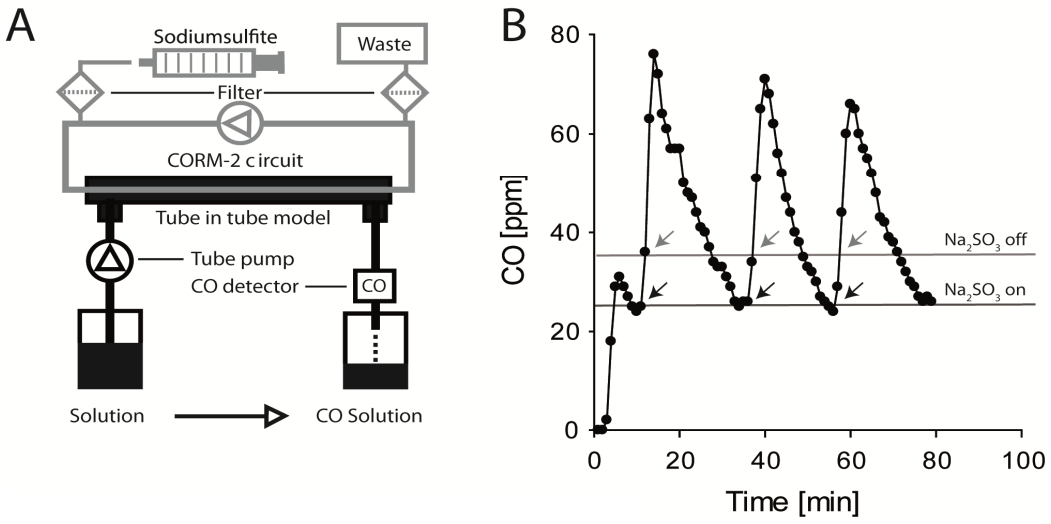

Figure 6: (A) Schematic drawing of the cartridge tube model for controlled enrichment of a perfusion solution with CO. (B) Online CO release pattern of water enriched with CO. Release was triggered by repeated injection of sodium sulfite solution when readouts dropped below 25 ppm (arrows) and stopped when readouts reached 35 ppm (arrows).

Discussion

The TGRS systems presented here allow for a release of different therapeutic gases for the enrichment of transplant perfusion solutions, with *in situ* generation and release kinetics being controlled by the choice of the number and thickness of the membranes through which the gas penetrated (**Figure 2**). Other systems were developed for generating therapeutic gases without the need of complex logistical setups (**Figure 4**), or for implementation into existing, advanced transplant storage systems allowing for easy to implement, controlled and tailored enrichment of perfusion media for transplants during storage and shipment. These were either designed for instantaneous availability of CO (**Figure 5**), and/or pulsatile generation and delivery of therapeutic gas spikes into perfusion solutions as demonstrated for both, CO and H_2S (**Figure 6, Supplementary Figure 5**). All presented systems were thoroughly checked for tightness in an effort to restrict transplant exposure to the generated therapeutic gas while keeping all other molecules (the gas releasing molecules, by- and degradation products, thereof) tightly sealed within the TGRS. Within this context, we selected silicone membranes due to their non-microporous, physiologically inert, and biocompatible features leading to safe and effective gas transfer characteristics [26].

Numerous preclinical studies indicated the benefit in terms of reducing ischemia reperfusion injury by exposing the donor and/or recipient or the transplant itself to therapeutic gases, particularly to CO (**Table 1**). In order to bridge the gas generation by the TGRS to the modalities of gas generation in these previous efficacy trials, we matched the respective CO profiles. For that, perfusion solutions were either CO saturated by flushing a perfusion solution with CO gas (as done in relevant previous trials [17, 18]) or the enrichment of the perfusion solution was performed with the TGRS with the resulting overlay of the profiles indicating the equivalence of both approaches (**Figure 3A**). The TGRS was sealed for all components other than the generated gas based on evidence from the surrounding medium, within which neither ruthenium (the metal center of the CORM) or supplemented dyes placed within the TGRS for integrity assessment were detected upon use. Obviously, deploying the TGRS rather than flushing solutions with CO for the enrichment of perfusion solutions is removing a logistical burden, and supports handling hygiene. For example, the stick system TGRS (**Figure 4**) can be conveniently placed into the transplant transport bag by the manufacturer under aseptic conditions and the user can activate it easily through the bag by breaking the inner chamber thereby minimizing the risk for contamination. Staff

safety is of no concern in this setting, as no handling with larger amounts of gases is required. We consider the matching CO profiles in the perfusion solutions prepared in analogy to previous reports on efficacy and as generated by the TGRS as ample evidence that the TGRS can replace the current complex procedures in the future.

Some novel approaches for transplant transport move away from the classical cold graft storage in a bag to constant hypo- and normothermic machine based perfusion systems [27]. These more sophisticated systems have demonstrated advantage over conventional systems [27] and the TGRS developed for integration in these machines may provide further advantage by the easy to implement cartridge systems presented here (**Figure 6**). Ongoing studies address the ability to generate long lasting spike series of gases including spikes generated with different therapeutic gases (e.g. CO and/or H_2S) from one cartridge (**Figures 5, 6, Supplementary Figure 5**). Thereby, optimal dose-response profiles for quality improved transplants can be generated and treatment may proceed with different gases.

In addition, we confirmed the impact of CO on transplants as previously described [17, 18] (**Table 1**) using High mobility group box-1 (HMGB1) [28], an early key mediator of Ischemia Reperfusion Injury (IRI) in various tissues as demonstrated for liver, kidney, and heart models [29-35] (**Figure 5B, C**). CO gas, CORMs, and inducers of heme oxygenase 1 (HO-1) are potent suppressors of pathological HMGB1 release [22, 36-45] and have been described to ameliorate IRI in various transplantation models, including heart [19, 46-48], liver [12, 16, 17, 49-55], lung [18, 56-61], kidney [13-15, 22, 62-69], and gastrointestinal [70-74] models, an effect that is reversible by the application of rHMGB1[22]. Our studies confirmed that CO saturated perfusion solutions significantly reduce cytoplasmic HMGB1 release in ischemic rat liver model as compared to plain perfusion solutions (**Figure 3**). Building off previous protocols this proof of concept study provides first evidence for feasibility of membrane based gas delivery in transplant medicine. Further preclinical studies are necessary to evaluate the potential of this approach for future clinical development.

Conclusion

CO is effectively improving the quality of transplants. The TGRS is removing the road block of complex machinery and safety measures as currently required, thereby opening transplants to intervention with therapeutic gases. The TGRS outlined here generated therapeutic gases in a controlled way, including kinetic profiles leading to efficacious outcome as described in previous studies.

Acknowledgement

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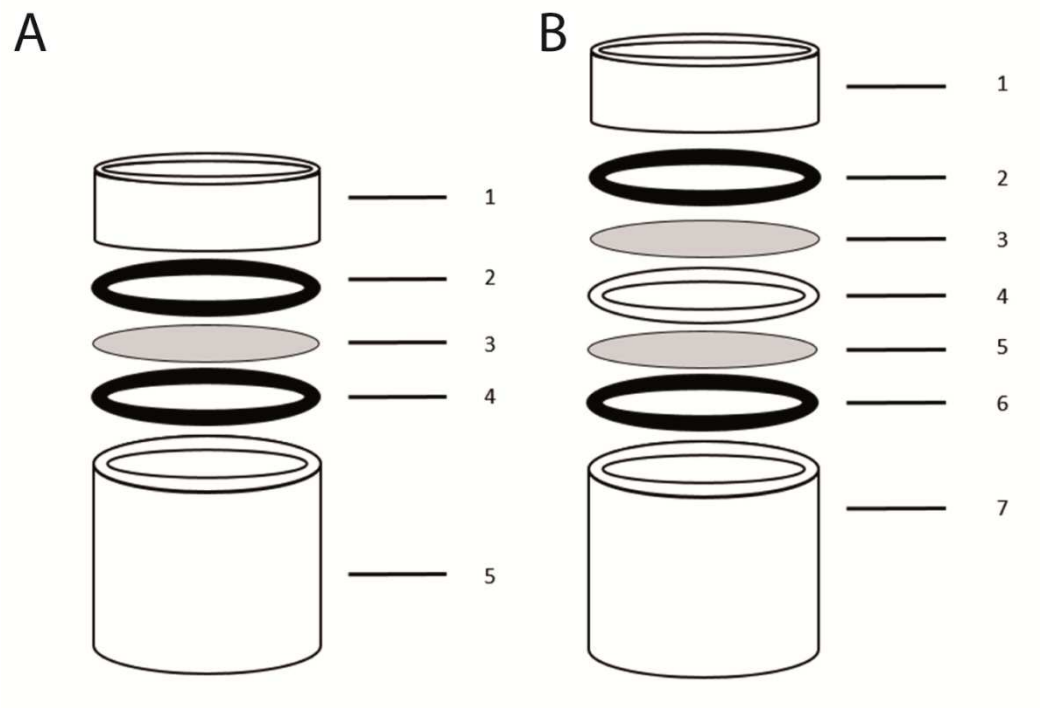
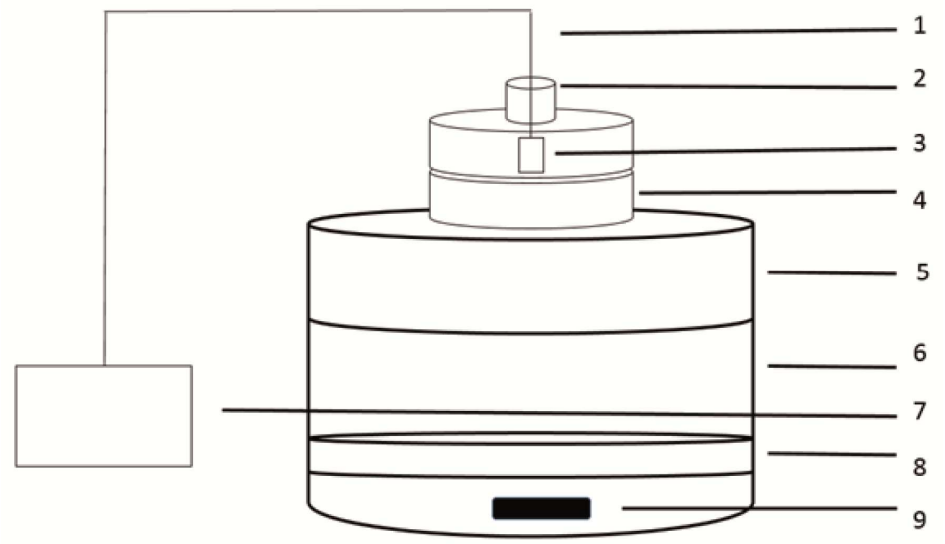
Supplementary Figures

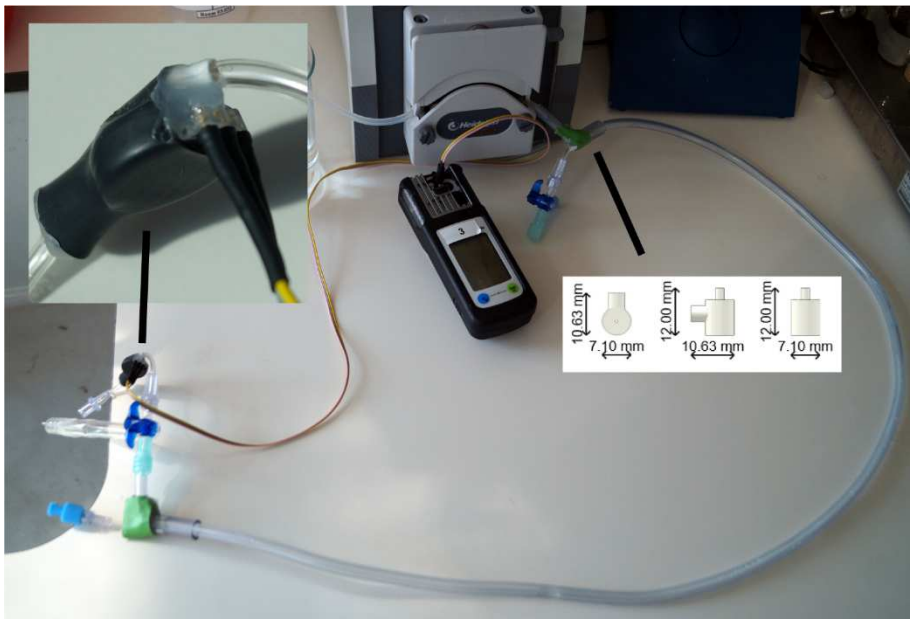


Supplementary Figure 1: (A) Schematic drawing of the therapeutic gas release system (TGRS) A) One membrane based: 1. Stainless steel lid, 2. gasket ring, 3. membrane, 4. gasket ring, 5. stainless steel reaction space. (B) Two membrane based TGRS: 1. Stainless steel lid, 2. gasket ring, 3. membrane, 4. teflon lining disc, 5. membrane, 6. gasket ring, 7. stainless steel reaction space.

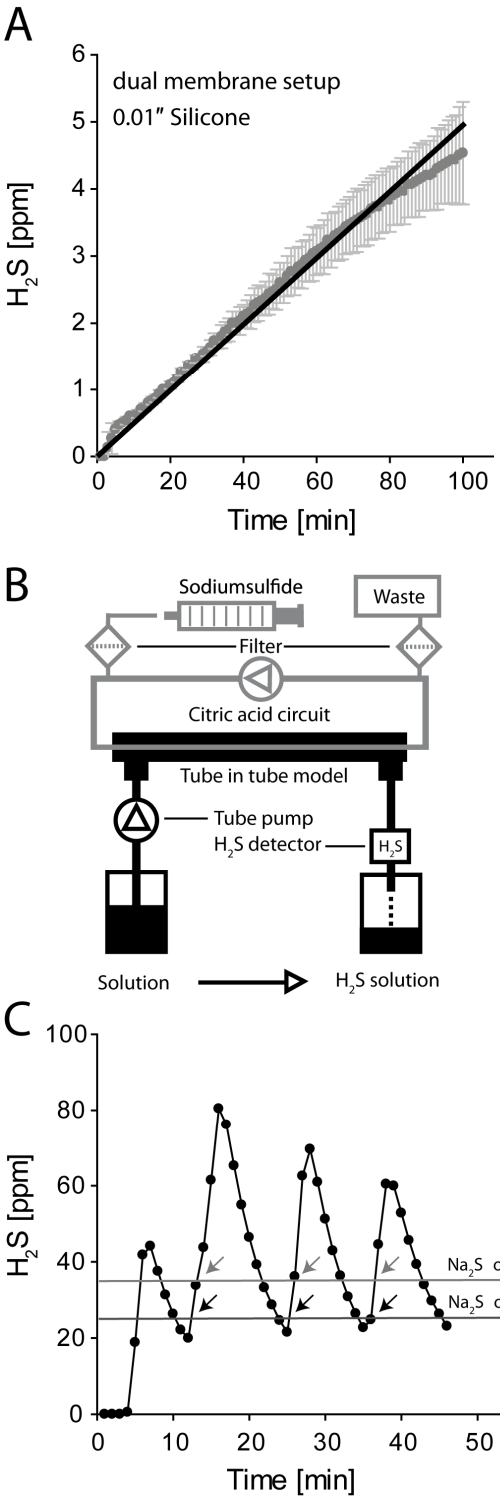


Supplementary Figure 2: Amperometric CO detection system. 1. cable, 2. guide tube with glued cable, 3. XXS LC sensor,4. flange, 5. desiccator lid, 6. desiccator, 7. X-am 5000, 8. tile, 9. stirring bar.

Supplementary Figure 3: Amperometric detection system for carbon monoxide.



Supplementary Figure 4: Tube in tube cartridge system for the online enrichment of a solution with CO. The inner tube was perfused with a CO donor solution (CORM-A1) in citric acid buffer as trigger. The outer tube was constantly perfused with a perfusion solution (water) using a tube pump. CO content in the acceptor solution was assessed online using an amperometric detection system. A highly gas permeable ePTFE tube (white tube - magnification) is directly attached to a CO sensor. This setting is functional for (i) protecting the sensor from liquids and (ii) assessing the amount of CO which freely permeates through the ePTFE membrane (magnification). The inner and outer tube were connected on both ends using a 3d printed valve as shown in the schematic drawing/white box.



Supplementary Figure 5: (A) H_2S release pattern of TGRS with sodium sulfide solution as H_2S donor. The reaction is initiated with HCl and controlled by two 0.01″ silicone membranes (in analogy to CO releasing TGRS (Figure 2B)). The linear line highlights the linear section of the release pattern. The data is expressed as mean ± SD with n = 3. (B) Schematic drawing of the single tube in tube cartridge system for controlled enrichment of a perfusion solution with H_2S (in analogy to Figure 6). (C) Online H_2S release pattern of water enriched with H_2S gas using the single tube in tube cartridge system with sodium sulfide solution as H_2S donor and citric acid solution as trigger. The trigger was added in the beginning and as soon as values dropped below 25 ppm (arrows) and stopped when readouts reached 35 ppm (arrows).

Chapter 5: Tamper-proof tablets for distinction between counterfeit and originator drugs through PEG coding

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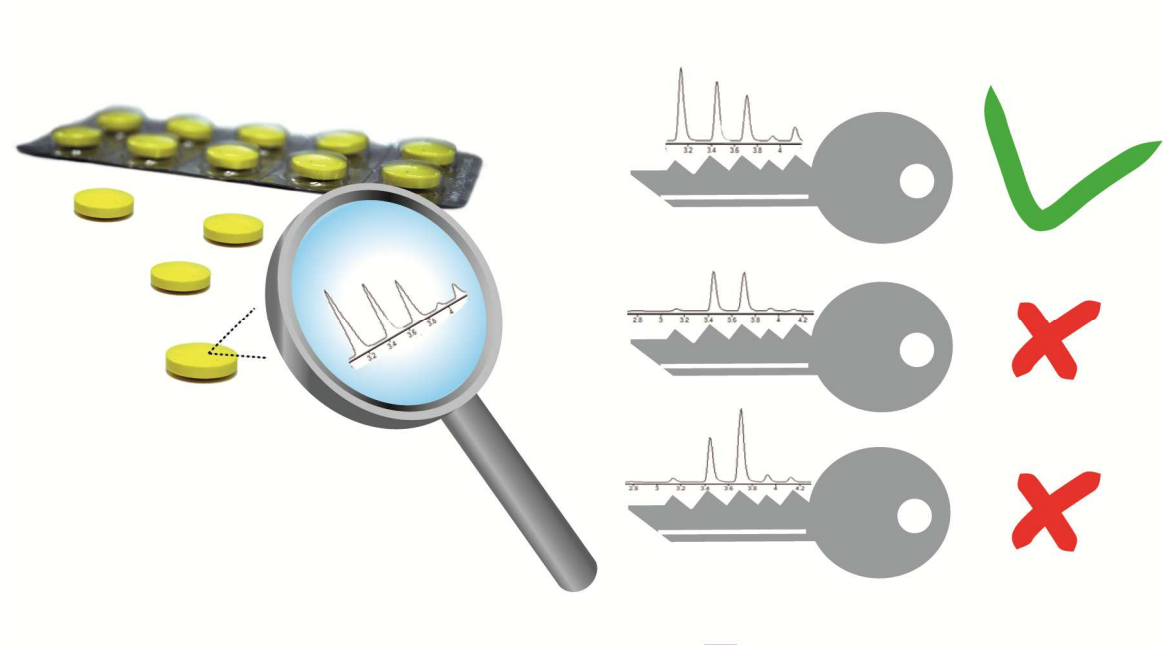

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Abstract

Counterfeit drugs are a major threat to public health. Current efforts focus on serialization of the secondary packaging which do not allow to trace to the individual unit. As a proof of concept, we intended to mark each tablet for its unambiguous recognition. Spiking monodisperse PEGs into tablet coating solutions at concentrations as low as 3 ppm was instrumental to “write” a code into each tablet film which was readily read upon isolation and LC-MS/MS analysis. Different qualities and amounts of monodisperse polyethylene glycols can be used for coding solid drug products. The approach is limited to cases in which PEGs are not present for formulation purposes as excipients, as coding against this background was unfeasible.

Introduction

Counterfeit drugs are a substantial challenge of increasing relevance, posing an immediate health threat to public health. In the framework of the IMPACT study the WHO assessed that up to 30% of the drugs used in developing countries are counterfeits [1]. While socioeconomic factors including improper or absent regulatory control are a major reason for the desastrous situation in the third world today, the outlook of gigantic margins have been ruthlessly pushing the fakes onto the global market [2]. Counterfeiting is also a threat to industrialized countries. For instance, packages of Avastin were released to the US market without active ingredient in 2012 [3]. In 2014, Herceptin vials were stolen in Italy and reintroduced to the EU market with either diluted content or no active ingredient [4]. With Sutent capsules containing no drug substance, another expensive anti-cancer drug was found in 2013 [5]. Counterfeiting today is a growing billion dollar business, and typically presenting as medicines (i) being mislabeled, (ii) containing the correct active ingredient in a wrong dose (too high or substandard), (iii) containing another than the declared active ingredient, (iv) having no active ingredient, and (v) being contaminated with impurity-burdened drug substances [6]. Governmental bodies together with the pharmaceutical industry are addressing these challenges with the development of quite complex anti-counterfeiting approaches, including end-to-end or aggregate solutions resulting in problems to the supply chain and tracking thereof [7]. These enormous efforts reflect the huge push on governments and companies alike by counterfeited medication. One approach is labeling a secondary package (e.g. the medication packages) with holograms, digital watermarks, or serialisation through bar codes or RFID (Radio Frequency Identity) chips for end-to-end solutions [8, 9]. These valuable options are expected to translate into quite substantial improvements in terms of medication safety.

However, ideally each tablet, capsule, liquid or other formulation could be directly traced down to its origin (the production batch or campaign) and not only through its packaging. Following this approach, the differentiation into fake or authentic is made based on the individual dosage form (the drug product) and not its packaging. In fact, incorporating “forensic markers” leading to a “chemical barcode” including stable isotopes [10] unique fluorescent patterns [11], saccharides [12], or scents [13] have been presented to meet this goal. However, these interesting approaches frequently rely on supplements which are not

listed in the FDA's "generally recognized as safe database" thereby increasing the developmental and regulatory complexity and costs to an extent, that proper implementation becomes unlikely.

We address this challenge by using an established excipient, polyethylene glycol (PEG). Typically, PEGs are polydisperse mixtures. These polydisperse PEGs have been used for the correct assignment in doping controls. Urine probes from athletes who ingested the polydisperse PEG were traced back by virtue of PEG found in the body fluid [14, 15]. We used highly purified PEG fractions with known monodisperse characteristics. Several batches of these PEGs, each batch being monodispers with respect to its molecular weight, served as the basis for coding and specific codes were "written" into the coating of the drug product itself.

Materials and Methods

Materials

Eudragit E PO was purchased from Evonik (Essen, Germany). Tableting mixture ("Tabletiermischung, technisch") consisting of 88% (all % in m/m unless noted otherwise) lactose, 9% cellulose, 2% aluminium oxide and 1% magnesium stearate was acquired from Meggle (Wasserburg am Inn, Germany), talkum was from Caelo (Hilden, Germany), microcrystalline cellulose "MC-101 SP" from Lehmann & Voss (Hamburg, Germany). PEG 300 and 600 as well as all used monodisperse polyethylene glycols were from Ruma (Cologne, Germany). The selection included monodisperse PEG n = 8, 9, 10, 12. Renex PEG 400 was from Croda (Nettetal, Germany), gradient grade acetonitrile (Chromasolv), absolute ethanol, and formic acid from Sigma Aldrich (Schnelldorf, Germany), and water for LC-MS (HiPerSolv Chromanorm) from VWR (Darmstadt, Germany). All other reagents were obtained from Sigma Aldrich and were at least of pharmaceutical grade unless otherwise stated.

Instrumentation

Measurements were carried out on an Agilent 1200 LC system (Waldbronn, Germany) equipped with a binary pump, an online degasser and a thermostated column compartment coupled to an Agilent 6460 triple-quadrupole mass spectrometer equipped with an

electrospray ionization (ESI) source. A Microfuge 22R was used for centrifugation (Beckman Coulter, Krefeld, Germany).

Preparation of the tablets

A PH 100 rotary press equipped with 12 mm tableting stamps from Korsch (Berlin, Germany) was used to produce biconvex placebo tablets. The tablets (weight: 571 ± 8 mg (n = 3)) consisted of 80 % tableting mixture and 20 % microcrystalline cellulose. The coating solution for the tablets contained of 8 g Eudragit E PO, 6 g talcum in 100 mL ethanol, and different compositions of the monodisperse PEG fractions according to the respective code for each batch. The selection was from monodisperse PEG n = 8, 9, 10, 12, as well as PEG 300 and 600. The composition of each code within the respective coating solution (**Table I**) or for the coated tablets is detailed in (**Table II**). The tablets were coated like previously described, with modifications [16]. In brief the tablets were dip coated five times with a solution of Eudragit E PO containing a selection of monodisperse polyethylene glycols (coating solution) according to the code and air-dried with an air gun at about 60 °C for 1 minute. The amount of coating applied per tablet was approximately 30 mg (MXX-123, Denver Instruments, New York, NY).

LC-MS/MS analysis

Each tablet was disintegrated in 10.0 mL water under sonication for 30 min. 1 mL of this solution was centrifuged for 15 min (4 °C, 18000 g). The supernatant was collected and analyzed by LC-MS/MS. The separation was carried out on a C_{18} analytical column (100 x 3.0 mm, 3.5 µm particle size; Zorbax SB-C18, Agilent, Waldbronn, Germany) at 20 °C. A linear gradient was employed at a flow rate of 0.5 mL/min with 0.1 % formic acid in water as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. The proportion of mobile phase B was changed from 10 to 40 % B within the first 7 min. The column was then flushed with 95 % B for 1 min and reequilibrated at 10 % B for 3 min. The injection volume was 5 µL. Using the internal switching valve, the mobile phase was directed to the mass detector in the time range of 1.5 to 5.0 min. At all other times, it was directed to the waste. The mass spectrometer was operated in ESI positive mode. Dry gas temperature and flow were 300 °C and 12 L/min. The gas pressure of the nebulizer was set to 45 psi. The sheath gas flow and temperature were 12 L/min and 400 °C. The capillary voltage was +2500 V. The analytes were detected using multiple reaction monitoring (MRM) and the

transitions are provided (**Supplementary Table I**). Collision energy was 5 or 10 V, respectively, depending on the analyte. Solely commercially available PEGs as detailed in the materials section were used for LC-MS/MS analysis.

Results

Analytical method development

After extraction of the PEGs from the tablets, a reversed phase HPLC method with MS/MS detection was used. PEGs with five to twelve polyethylene glycole units (abbreviated as PEG n = 8, PEG n = 9, PEG n =10, and PEG n = 12 for n= 8, 9, 10 and n = 12 polyethylene glycole units, respectively) were included in the method development, which were separated on a C_{18} column as previously described [17, 18]. The molecular weights of these monodisperse PEGs are 370.4, 414.5, 458.3, and 546.3 u for n =8, 9, 10, and 12, respectively. The polydisperse PEG used here have an average molecular weight of 300, and 600 u. PEG 300 - a polydisperse PEG consisting mainly of a mixture of the above mentioned PEG homologues - was readily separated into its homologues within 5 minutes (data not shown). The washing step (95% mobile phase B) after the separation run was obligatory to remove PEGs of higher molecular weight or possible other substances that might be extracted

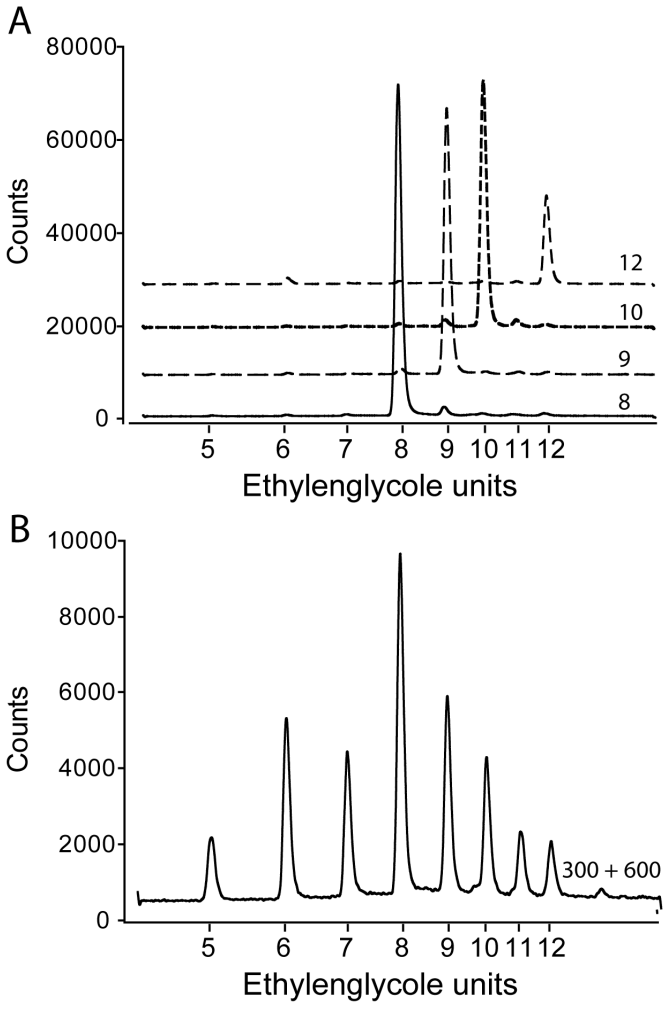

Figure 1: Chromatographic patterns of (A) monodisperse PEG with n = 8, 9, 10, or n = 12 ethyleneglycole units in water, respectively, and of (B) mixture of polydisperse PEGs 300 and 600 (2:1, m/m). Total ion current (TIC) chromatograms in MRM-mode are shown. Chromatographic conditions and MS parameters are given in chapter "*LC-MS/MS analysis*"

from the tablet before the next run commenced (data not shown). Peaks were identified via mass-to-charge ratio m/z and assigned by spiking PEG 300 with monodisperse PEGs with n = 8, 9, 10 or n = 12 ethyleneglycole units. The monodisperse character of these fractions was confirmed (**Figure 1A**).

The triple quadrupole mass spectrometer was used in the MRM mode for selective and sensitive detection. For PEGs with $n \leq 7$ polyethylene glycole units, *m/z* of the precursor ion in the first quadrupole (Q1) was the molecular ion ($[M+H]^+$) and *m/z* of the product ion after fragmentation in the third quadrupole (Q3) was 89.0. In cases in which the number of polyethylene glycol units exceeded 7, the PEGs formed the adducts $[M+18]^+$ representing the signal with the highest intensity. For these species, Q1 and Q3 were changed to $[M+18]^+$ and $[M+H]^+$, respectively, to achieve comparable sensitivity (**Supplementary Table I**). For validation, the repeatability was tested by six individual injections of PEG 300 (1 µg/mL). The relative standard deviation (RSD) values were between 2.02 and 4.62 %. Based on a signal-to-noise ratio of 10 the limits of quantification (LOQ) were 0.2, 0.4, 3.0, 2.4, 6.9, 3.4, 1.6, and 2.5 ng/mL for polyethylene glycol chain lengths of 5, 6, 7, 8, 9, 10, 11, and 12, respectively. were 0.2, 0.4, 3.0, 2.4, 6.9, 3.4, 1.6, and 2.5 ng/mL for polyethylene glycol chain lengths of 5, 6, 7, 8, 9, 10, 11, and 12, respectively. Thus, the method provides sufficient sensitivity for the determination of low amounts of marker substance spiked to the tablet coating. Linearity was confirmed from 1 ng/mL or the LOQ (whichever was higher) to 90 ng/mL. The coefficients of determination (R^2) exceeded 0.99 for all monodisperse fractions. Furthermore, mixtures of PEG300 and PEG600 (polydisperse) were characterized (**Figure 1B**).

Writing the code into the coating solution

The code was written with monodisperse fractions of PEG n = 8, PEG n = 9, and PEG n = 12 in different combinations and together (**Figure 2A**; compositions of the formulations are provided in **Table I**). The code was further modified by repeating these experiments in presence of PEG300/600, providing an additional signature on top of the monodisperse PEGs by the polydisperse PEG300/600 (**Figure 2B**). The binary code (monodisperse PEG is present or not) was further modulated by spiking PEG n = 9 in different amounts in a solution with constant amounts of PEG n = 8 and n = 12 (**Figure 2C**). This resulted in a continuous decrease for the PEG n = 9 response from high to low PEG n = 9 spiking concentrations. The experiment was repeated in presence of PEG300/600 to assess the suitability of the approach in presence of the PEG300/600 signature.

Table I: Relative concentrations (m/m) of PEGs in the coating solutions and detailing Figure 2.

Figure 2	**Monodisperse PEG**				**Polydisperse PEGs**	**Total PEG (ng/mL)**
	n = 8	**n = 9**	**n = 10**	**n = 12**	**300+600**	
2A	1	1	0	0	0	40
	1	0	0	1	0	40
	0	1	0	1	0	40
	1	1	0	1	0	60
2B	1	1	0	0	1	60
	1	0	0	1	1	60
	0	1	0	1	1	60
	1	1	0	1	1	80
2C	1	1	0	1	0	60
	1	2	0	1	0	80
	1	3	0	1	0	100
	1	4	0	1	0	120
2D	1	1	0	1	1	80
	1	2	0	1	1	100
	1	3	0	1	1	120
	1	4	0	1	1	140

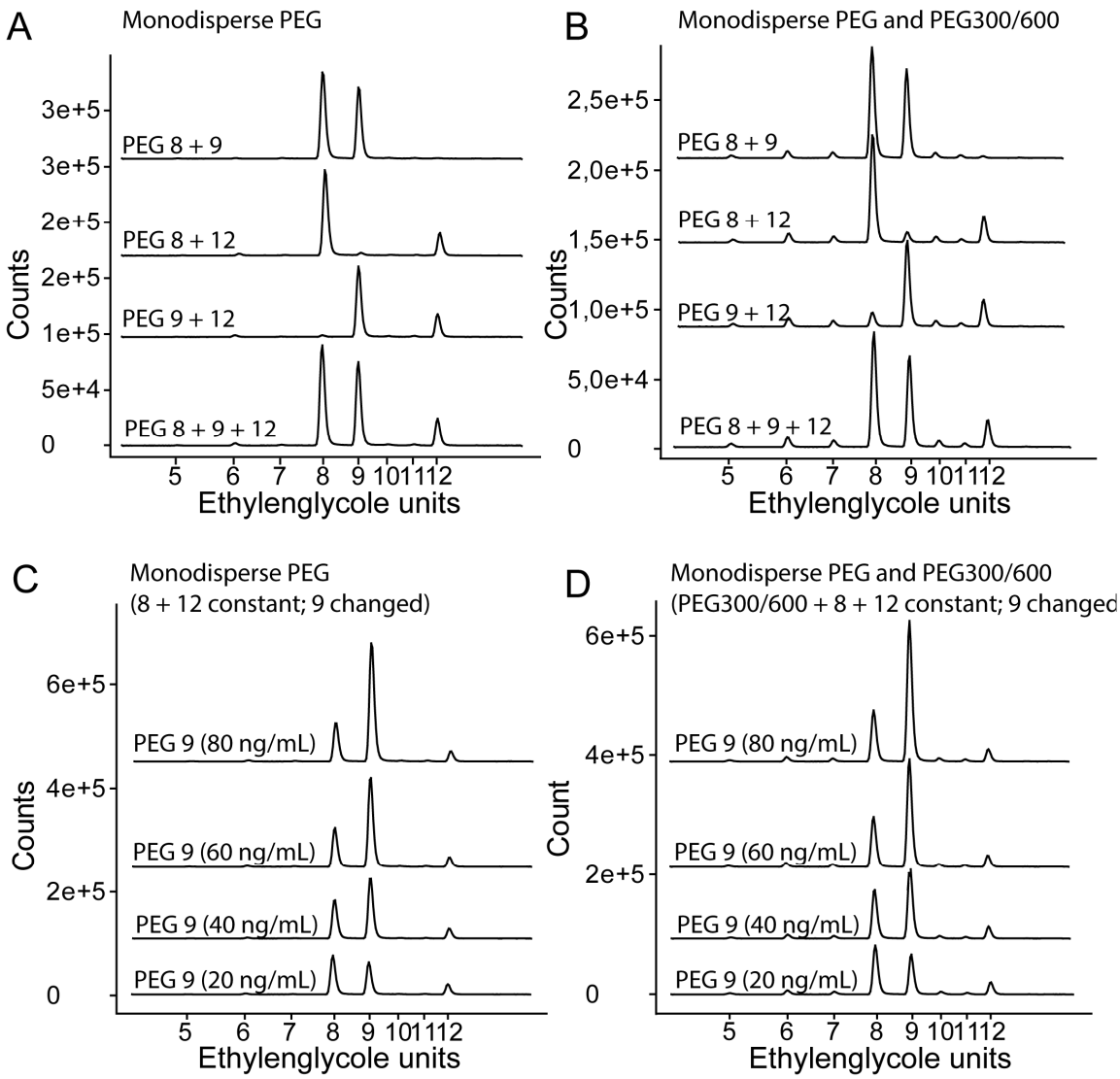

Figure 2: (A) Spiking experiments with monodisperse PEGs n = 8, 9, and n = 12; (B) same pattern of monodisperse PEGs as in A in presence of PEG 300/600; (C) Aqueous solution of PEGs n = 8 and n =12 with different amounts of PEG 9; (D) same pattern of monodisperse PEGs as in C in presence of PEG 300/600. Details of the formulations are provided in **Table I**. Total ion current (TIC) chromatograms in MRM-mode are shown.
Chromatographic conditions and MS parameters are given in chapter "*LC-MS/MS analysis*".

Coding of tablets

A coated control tablet devoid of PEG showed no peaks interfering with our PEG analytes (**Figure 3A;** formulation a; compositions of the formulations are provided in **Table II**). Tablets were coated with combinations of the monodisperse PEGs for coding by directly spiking these into the coating solution (writing a binary code, i.e. the respective PEG monodisperse fraction is present or not; **Figure 3A**; Formulation b). The presence of low amounts of PEG 300 and PEG 600 introduced a specific distribution pattern characterized by small peaks and supplementation with additional monodisperse PEGs increased the respective peaks, i.e. for peaks representing polyethylene glycols with n = 8, 9, 10, and n = 12 units (**Figure 3A**; Formulation c). A 100 fold dilution was necessary for the analysis of the samples isolated from formulations a-c and the data indicated that another logarithmic dilution step was possible. Therefore, one formulation was prepared to assess the feasibility of using PEGs in 1000 fold lower concentrations for coding and resulting peaks could be identified (**Figure 3A**; Formulation d). However, the approach of using PEGs in this diluted form for coding was unfeasible in cases in which PEG 400 was supplemented to a coating solution amounts relevant for manufacture (**Figure 3B**, formulation c versus e).

Table II: Relative concentrations (m/m) of PEGs in the tablet coating and detailing Figure 3.

Figure 3	Monodisperse PEG				Polydisperse PEG		Total PEG[1]
	n = 8	n = 9	n = 10	n = 12	300	600	
a	0	0	0	0	0	0	0
b	1	1	1	1	0	0	200 mg
c	1	1	1	1	1	1	300 mg
d	1	1	1	1	1	1	300 µg
e	1	1	1	1	1	1	300 µg + 1 g PEG[2]

[1]Total PEG is the overall amount of PEG added to 14 g (solids) of the basic coating solution;
[2] Formulation d is identical to formulation e other than 1 g PEG 400 was additionally added to the coating solution.

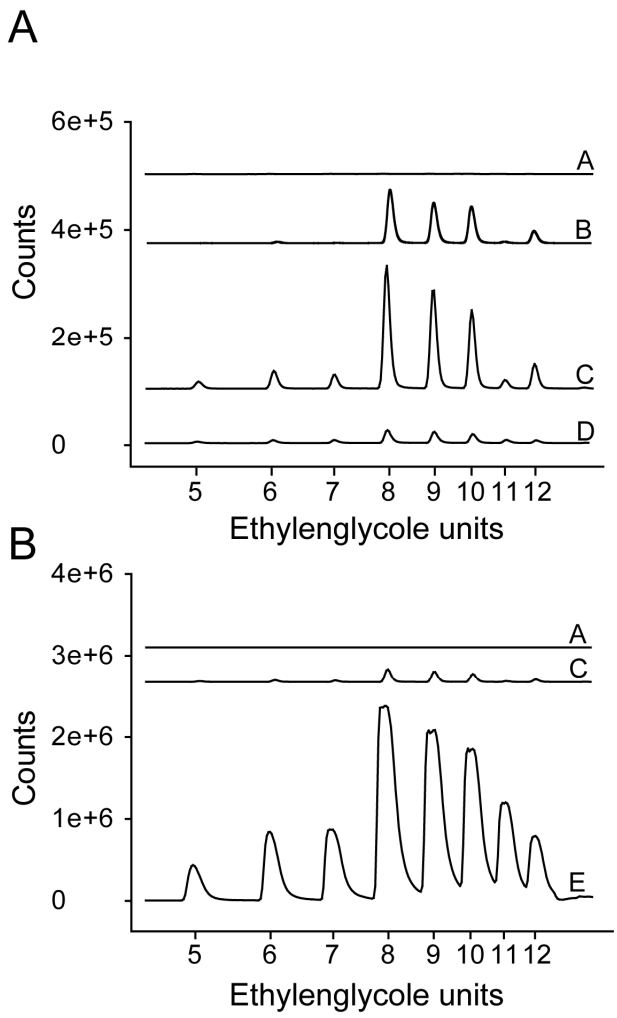

Figure 3: Total ion current (TIC) chromatograms in MRM-mode of the different tablets coded with monodisperse and polydisperse PEG. Formulation a: tablet without any PEG; Formulation b: tablet spiked with monodisperse PEGs n = 8, 9, 10 and n = 12 (diluted 1:100 prior to analysis); Formulation c: tablet spiked with monodisperse PEGs n = 8, 9, 10 and n = 12 and polydisperse PEGs 300 and 600 (diluted 1:100 prior to analysis); Formulation d: tablet with the same PEGs as in Formulation c in a 1000 fold lower concentration; Formulation e: tablet with the same PEGs as in Formulation d with an excess of PEG 400 (The detailed composition of the tablet coatings is detailed in **Table II)**. Please note, that Formulations a and c are shown in both panels A and B. Chromatographic conditions and MS parameters are given in chapter "*LC-MS/MS analysis*".

Discussion

Monodisperse PEG fractions can be efficiently identified (**Figure 2A**). The signature of the code can be further modulated by spiking polydisperse PEGs into the solution (**Figure 2B**). This binary code (monodisperse PEG present or not) can be modulated by changing the amount of one or more monodisperse PEG fractions (**Figure 2C**) and this is also feasible in presence of polydisperse PEGs. Thereby, an additional signature is provided to further complicate the code (**Figure 2C, D**). Analogous coating solutions were deployed to prepare coded and coated tablets (**Figure 3A**; formulation b).

(Polydisperse) PEGs are described in the major pharmacopeias and use of the existing release specifications for the monodisperse fractions is expected to facilitate the regulatory challenge of implementing this anti-counterfeiting strategy. In the extreme scenario (**Figure 3**; Formulation d), PEGs were spiked in 1000 fold dilution as compared to Formulations b and c, demonstrating that a concentration of about 3 ppm was sufficient for coding of this drug product. For implementation of this approach one must demonstrate that PEG supplementation to the coating solution has no functional consequences (low risk due to the low amount of required PEG), and that appropriate release specifications have been met (low risk, as polydisperse PEG specifications are in the pharmacopeias). The approach is further supported by the fact that PEGs are generally recognized as safe (GRAS) [19, 20]. Therefore, we assume that the approach delinated here may provide a rather uncomplicated way to code drug products currently in development or those with an existing marketing-authorization. The versatility of the code is quite broad and can be written based on the (i) selected monodisperse PEGs (number and type), (ii) the variation of the amount of one or more monodisperse fraction(s), or (iii) by the presence of a signature of a polydisperse PEG. As the selected binary code is only known to the marketing authorization holder (MAH), each individual tablet or other coated solid dosage form becomes fully traceable, down to the campaign or even the batch level, respectively. For example, the MAH may decide to use monodisperse PEGs for building his code. There are 2^n -1 options for coding, with n being the number of available monodisperse PEG fractions. Currently, one supplier is offering 15 monodisperse PEG fractions, providing the opportunity to the MAH to write one out of 32,767 possible codes.

Thereby, the hurdle for counterfeited versions of a medication is substantially increased. In addition, the MAH gets a tool with which a reliably link of each tablet independent of the

secondary packaging can be build to a certain production and release date, respectively. This information may be valuable to the MAH in cases she sees the necessity to proof that a tablet was used beyond its expiration date or in cases in which she desires to link a drug product to a specific production batch - a demand which may occasionally help to clarify situations during litigation or in other settings. The implementation of this approach into marketed products is quite uncomplicated and (very) probably by a type-II variation only. This optimism is by virtue of the small amounts of PEG required for writing the code, its known compendial status, and its generally recognized as safe status. Obviously, proper consultation with the relevant health authorities is mandatory before implementing this strategy.

The results indicated the limits. The approach is questionable or unfeasible in cases in which PEGs are used as excipients for drug product manufacture (i.e. PEG is added for formulation purposes). The inability for identification of the code against the huge background jeopardizes the approach (**Figure 3B**, Formulation e)- estimated 20 % of all capsules and pills contain PEG as excipients [21] and are thus not suitable for this concept. PEG coding is applicable in traces (formulation d (**Figure 3**) exemplary contains a calculated overall PEG content of 643 ng / tablet ≙ 1 ppm (m/m)) and therefore, doesn't distort most analytical methods. E.g. conventional Near-infrared Spectroscopy (established method for the identification of counterfeits) is used for concentrations greater than 0.1 % and is not designed to detect traces [22, 23]. The interference of PEGs with highly sensitive analytical methods is dependent on the limit of detection of the respective method and needs to be addressed by further studies if necessary. Like every security system the concept can be outwitted. The effort to crack this coding approach with a serialized system with variations among individual batches however would be tremendous. Each original product would have to be analysed and reproduced by a well-equipped laboratory in a elaborate process. This effort would have no impact on the direkt market access and therefore is highly uneconomic and unlikely.

Although the applied PEGs are "practically involatile" [24] and according to the manufacturer stable in terms of the implementation into a pharmaceutical product the approach delineated here is not generic but requires adaptation or confirmation for each new formulation.

Conclusion

Monodisperse PEG fractions can be used for coding of solid drug products. These fractions can be spiked into coating solutions for tablets in small amounts, such that pharmaceutical performance characteristics of the films are likely unaffected. PEGs are compendially described and generally recognized as safe, such that rapid translation of this anti-counterfeiting approach appears feasible for existing medications or drug products in development. The so-tamper-proofed tablets allow the distinction between drugs produced by counterfeiters and “originators”.

Acknowledgement

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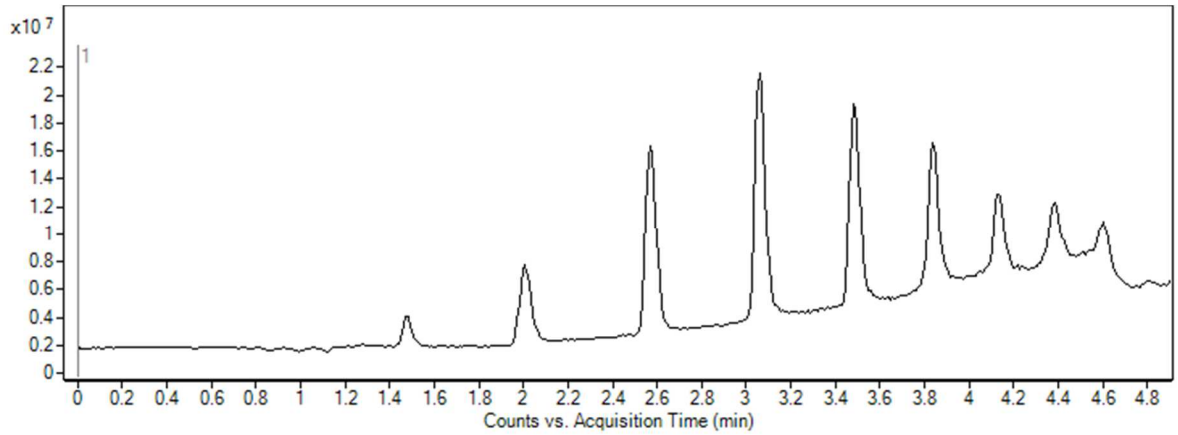
Supplementary Table

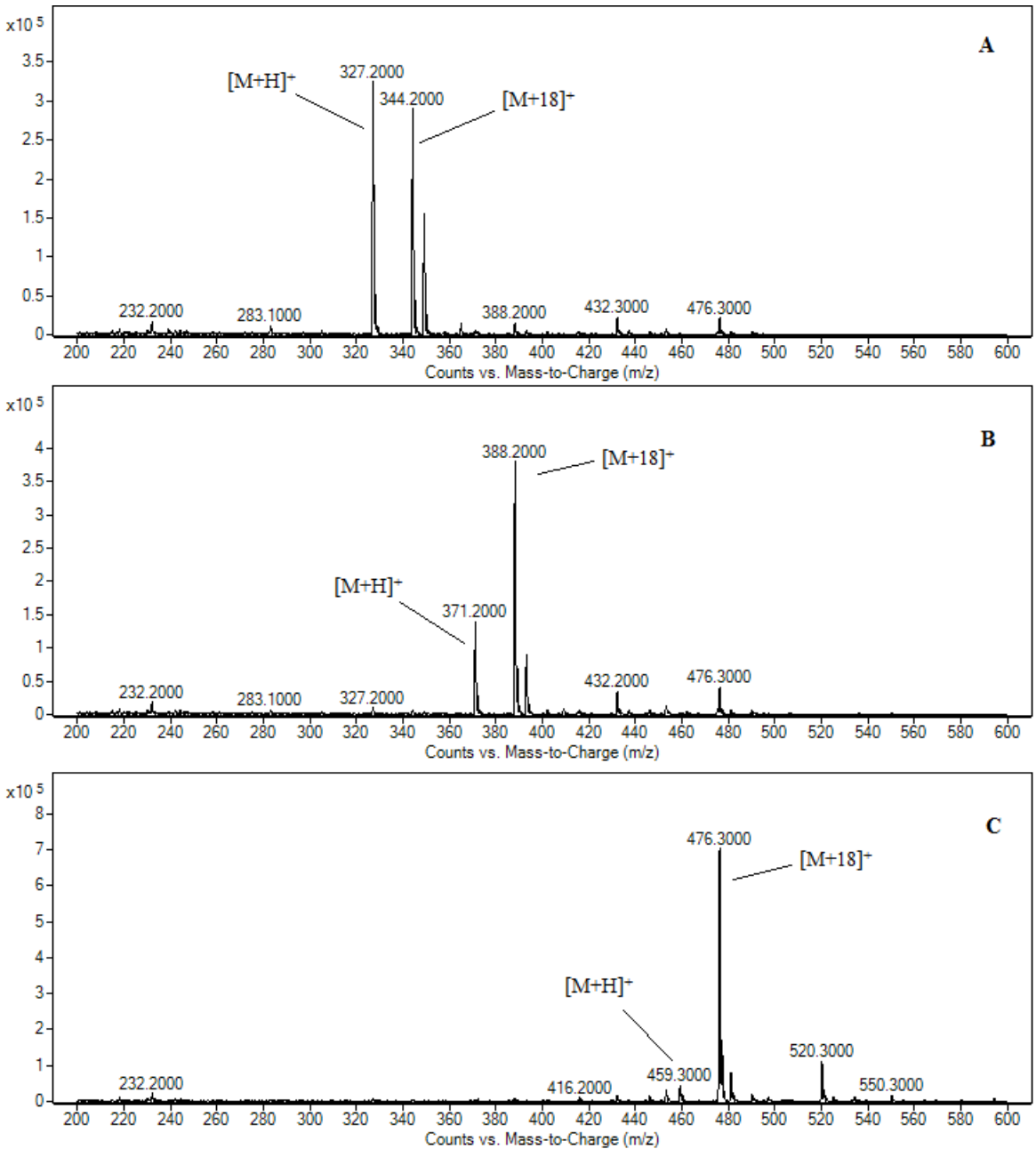
Supplementary table 1: The monoisotopic masses and m/z values of of precursor (Q1) and product ion (Q3) for the respective PEG species.

PEG, n =	monoisotopic mass [u]	Q1 [*m/z*]	Q3 [*m/z*]
5	238.1416	239	89
6	282.1679	283	89
7	326.1941	327	89
8	370.2203	388	371
9	414.2465	432	415
10	458.2727	476	459
11	502.2989	520	503
12	546.3251	564	547

Supplementary Figures



Supplemental figure 1: Total ion chromatogram (TIC) in full-scan mode of the separation of 1 µg/mL PEG 300 in water.



Supplementary figure 2: Average mass spectra of the peaks due to PEG with n = 7 (A), 8 (B) and 10 (C).

Conclusion and outlook

The translation of the therapeutic potential of CO into clinical settings is impeded by a lack of practicability of current approaches and substantial safety issues associated with high CO doses required for triggering therapeutic effects [1]. The release systems described here within address these limitations and offer easy applicability as well as local delivery, thereby demonstrating a practicable way for improving the benefit / risk profile of therapeutic CO in GI and transplant applications.

The Oral Carbon Monoxide Release System (OCORS) was introduced as the first GI delivery system for therapeutic gases and designed to address various inflammatory GI diseases. CO release was independent of simulated GI fluids and was precisely tunable by modifying the coating covering the OCORS.
In-vivo, the performance of OCORS was detailed using the downsized micro scale OCORS (M-OCORS) designed for preclinical mouse models. The release profile of M-OCORS lasting for minutes up to almost one day was tailored to adequately reflect the GI transit time of the system thereby allowing for local GI delivery of CO. Efficacy was demonstrated in chemically induced colitis in mice. In contrast to earlier studies applying CO doses that have been associated with toxic CO-Hb levels in man, M-OCORS demonstrated both efficacy and safety by means of negligible CO-Hb formation. Consequently, CO delivery from M-OCORS allows for a distinct improvement of the safety profile of CO in GI applications. Currently two further ongoing studies are assessing the potential of M-OCORS in diabetic gastroparesis and postoperative ileus, two motility related diseases that are not adequately treatable at present [2]. The OCORS might therefore not only serve as a delivery system for ulcerative colitis, but also for other inflammatory GI diseases.

The OCORS is manufactured by standard procedures for solid dosage forms. Consequently, the technology can easily be implemented into established manufacturing protocols thereby facilitating possible future clinical development phases and ultimately, mass production. A significant hurdle for any of these approaches are multiple technical challenges associated with the CORM technology. The first generation of OCORS was impeded by the complicated solution chemistry of ruthenium based CORM-2 resulting in complex degradation products leaking out of OCORS [3-6]. Other challenging features of CORMs include solubility, toxicity, as well as CO-payload issues [1,7]. These are general impediments of the CORM technology and are currently being addressed by various groups

with numerous approaches including strategies for CO delivery from organic compounds [8, 9]. Consequently, future CORMs might complement the OCORS technology thereby improving the safety profile of this delivery concept. ET-OCORS featuring iron CORMs with a relatively benign safety profile was described in this thesis as one possible translational approach. Further validation studies focusing on the stability and implementability of esterases deployed as triggering compound in this modification are necessary. A series of promising alternative CORMs including preliminary data on degradation products and toxicity is on file. These include a series of molybdenum compounds degrading into polymolybdates [10] as well as CORM-A1, a boron based CORM degrading into boric acid [11]. Consequently, OCORS can serve as blueprint for further modifications using other CORMs aiming for an approved safety profile for clinical testing as compared to current CORMs. In addition, future modifications might comprise molecules releasing other therapeutic gases, including hydrogen sulfite and nitric oxide.

Evidently, the ideal GI delivery system for therapeutic gases provides the performance of OCORS, meaning easy applicability as well as local and controlled release, and on top exclusively releases therapeutic gas without harmful (by-) products. Future delivery modalities might therefore comprise the OCORS machinery in a gas-permeable capsule, thereby allowing CO delivery through a gas permeable membrane while entrapping all other potentially challenging components in these capsules. Delivery systems moving in this direction are currently being developed in our lab. To this end we designed a membrane based delivery systems discussed in chapter four, which was originally introduced as a tool for transplant applications. Used in the transplant setting, the approach was effective in ameliorating ischemia reperfusion injury in a rat liver model. The membrane was functional for (i) controlling the release rate of the therapeutic gas and (ii) entrapping potentially harmful non-gaseous degradation products of gas releasing compounds. The concept had a “glow-stick”-like design for easy applicability in medical settings. Thereby, the approach removed the need for complex machinery and safety measures currently required for CO delivery in transplant medicine. Graft transportability and ischemia reperfusion injury are considered to be the limiting factors for the transplantation of marginal organs from Expanded Criteria Donors (ECD). Therefore, future applications based on this approach might be a promising strategy for expanding the global organ pool, and becoming a lifesaving technology.

Although transition metal based toxicity – forming a major translational hurdle for CO delivery – can be adequately addressed by the membrane based delivery concept, transfer into industrial settings might be challenged by introducing these novel manufacturing concepts into existing production lines. In contrast, OCORS was designed to fit into routine development and production settings. Noteworthy, however, some silicone based delivery systems, such as the contraceptive "Jadelle®" (Bayer®) [12], are established tools in clinical practise [13]. Consequently, comparable development and upscaling protocols are on file and might facilitate possible future translational developments.

Membrane based gas delivery systems described in this thesis mainly comprised challenging ruthenium based CORM-2 (*vide supra*). Hence, either intensive analytical- and toxicological evaluation or replacement with less challenging compounds is warranted as possible dose dumping (unintentional burst release) should be adequately reflected in a possible future clinical development strategy. These include compounds listed as potential CORM alternatives for novel modifications of OCORS including CORM-A1 or molybdenum based CORMs (*vide supra*). Implementation into the membrane based delivery concept appears trivial, however, possible permeability through silicone membranes will have to be adequately assessed for any of these compounds. Manually activating the CO release by the "glow-stick"-like approach with CO generation being initiated following manual disruption of a compartment storing the trigger solution (as detailed in chapter three) adequately integrates into clinical routine. The device is designed for convenient on-site activation by patients or doctors. Future clinical modifications should comprise a compartment storing the trigger solution in that convenient breakage is possible as well as storage stability is achieved. Adequate shelf live will have to be addressed by using highly water impermeable polymers such as cyclic olefin copolymer, an established polymer in pharmaceutical packaging [14]. Thereby, latent premature CO release triggered by solution leaking though the walls of the storing compartment must be prevented, accordingly.

Findings of this thesis identified two promising modifications of the membrane based gas delivery concept being (i) a stick-like development for transplant applications and (ii) a capsule-like development for GI applications. Transplant applications provide an easy access to the clinical proof of concept as *ex-vivo* applications are targeted. Also, a comprehensive dataset on the therapeutic efficacy of CO enriched organ storage solutions is on file [15] and discarded organs from marginal donors are available facilitating early phase clinical research

[16]. The clinical access of systems modified for GI delivery appears to be more challenging from both technological as well as safety aspects, but holds promise to emerge to a new therapeutic approach addressing multiple GI diseases including diabetic gastroparesis, gastric ulcers, postoperative ileus, and colitis [2]. Therefore, prototype designs will have to be tested in large animal models (e.g. pig, dog) to reliably predict system characteristics such as GI transit time, *in-vivo* CO release profile, and therapeutic efficacy. These models allow for (i) better comparability to clinical settings and (ii) an increased design space for PK studies including x-ray visualization of GI transit [17] as well as online CO quantification using an intestinal gas sensors (e.g. as previously described [18]). This preclinical development / research strategy will provide a reliable basis for possible future clinical studies.

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Abbreviations

ANOVA	Analysis of variance
a-CORM	Abrogated Carbon Monoxide Releasing Molecule
BAV	Bioavailability
CO	Carbon monoxide
CO-Hb	Carboxyhemoglobin
CORM	Carbon Monoxide Releasing Molecule
CORST	Carbon Monoxide Release System for Transplants
DMEM	Dulbecco's Modified Eagle Medium
DGF	Delayed graft function
DSS	Dextran sulfate sodium
ECD	Expanded criteria donors
E-OCORS	Enzyme Triggered Oral Carbon Monoxide Releasing System
ePTFE	Expanded polytetrafluorethylen
ET-CORM	Enzyme Triggered Carbon Monoxide Releasing Molecule
F	Formulation
FDA	Fluorescein diacetate
GI	Gastrointestinal
GRAS	Generally Rrecognized as Safe
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HMGB1	High mobility group box 1
HO	Heme oxygenase
HPLC	High performance liquid chromatography
H_2S	Hydrogen sulfide
i-CORM	Inactive Carbon Monoxide Releasing Molecules
MAH	Marketing authorization holder
M-OCORS	Micro-sized Oral Carbon Monoxide Releasing System
MPO	Myeloperoxidase
NO	Nitric oxide
NMR	Nuclear magnetic resonance
OCORS	Oral Carbon Monoxide Releasing System
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Propidiumiodide
PK	Pharmacokinetic
Ppm	Parts per million
PTFE	Polytetrafluorethylene
PLE	Pig liver esterase
RAMEB	Randomly Methylated β-Cyclodextrins
RFID	Radio-frequency identification
RSD	Relative standard deviation
SEM	Scanning electron microscope
SD	Standard deviation
SE	Standard error
TH	T-helper cell
TIC	Total ion chromatogram
TGRS	Therapeutic Gas Releasing System
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
USP	United States Pharmacopeia
UC	Ulcerative colitis
WHO	World Health Organization

Curriculum vitae

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Publications

Steiger, C., et al. (2016). "Localized delivery of carbon monoxide", review article, submitted to the Eur J Pharm Biopharm.

Steiger, C., et al. (2016). ", Prevention of colitis by controlled oral drug delivery of carbon monoxide ", J. Control Release (accepted).

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Patents

Meinel L, Steiger C, Wunder C (2015). "Gas delivery device", PCT/EP2016/050146

Meinel L, Steiger C (2014). "Therapeutic gas delivery system", PCT/EP2015/001187

Abstracts / Posters

Steiger C, et al. (2016). "Controlled oral delivery of therapeutic gases - local carbon monoxide for ulcerative colitis", abstract, 9th International Conference on Heme Oxygenases, Prague, Czech Republic. (accepted)

Steiger C, et al. (2015). "Therapeutic Gas Delivery Systems", abstract, DPHG Jahrestagung, Frankfurt, Germany

Steiger C, et al. (2015). "Gastrointestinal delivery of carbon monoxide", poster presentation, Joint Symposium, Hoshi University, Tokyo, Japan

Steiger C, et al. (2014). "Oral drug delivery of therapeutic gases - carbon monoxide release for gastrointestinal diseases", poster presentation Frauenhofer IGB, Stuttgart, Germany

Steiger C, et al. (2014). "Oral drug delivery of therapeutic gases - carbon monoxide release for gastrointestinal diseases", poster presentation DPHG Jahrestagung, Frankfurt, Germany

Oral presentations

„Therapeutic Gas Delivery Systems" (2016). Massachusetts Institute of Technology, Boston, MA (invited)

"Oral Carbon Monoxide Release System" (2015). Mayo Clinic, Rochester, MN (invited)

"Gas Delivery System" (2015). University of Ulsan, Ulsan, South Korea (invited)

"The therapeutic potential of carbon monoxide" (2014). Anatomic Colloquium, Institute for Anatomy, Erlangen, Germany (invited)

"Das therapeutische Potential von Kohlenstoffmonoxid" (2014). Drägerwerk AG & Co. KGaA, Luebeck, Germany (invited)

“Oral drug delivery of therapeutic gases - carbon monoxide release for gastrointestinal diseases” (2014). COST meeting on Gasotransmitters, Gent, Belgium (invited)

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Documentation of authorship

This section contains a list of the individual contribution for each author to the publications reprinted in this thesis. Unpublished manuscripts are handled, accordingly.

P1	**Steiger C, Hermann C, Meinel L** (2016) Localized delivery of carbon monoxide												
Author	**1**	**2**	**3**										
Study design/concept development	x		x										
Literature analysis and interpretation	x		x										
Manuscript planning	x		x										
Manuscript writing	x	x											
Correction of manuscript	x		x										
Supervision of Christoph Steiger			x										

P2	**Steiger C, Luhmann T, Meinel L** (2014) Oral drug delivery of therapeutic gases – carbon monoxide release for gastrointestinal diseases.												
Author	**1**	**2**	**3**										
Amperometric detection system	x												
Measurement of CO release	x												
Data Correction	x												
Na_2SO_3 crystal collection and crystal coating	x	x											
Development of the oral carbon monoxide release system (OCORS)	x												
CO release from the oral carbon monoxide release system (OCORS)	x												
Ruthenium release *	x												
Statistics	x												
Study design/concept development	x		x										
Data analysis and interpretation	x		x										
Manuscript planning	x		x										
Manuscript writing	x												
Correction of manuscript	x	x	x										
Supervision of Christoph Steiger			x										

* Analytic procedure performed by Frauenhofer ISC, Würzburg

P3		**Steiger C, Uchiyama K, Takagi T, Mizushima K, Higashimura Y, Gutmann M, Hermann C, Botov S, Schmalz H, Naito Y, Meinel L** (2016) Prevention of colitis by controlled oral drug delivery of carbon monoxide

Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**			
Development of the micro scale Oral Carbon Monoxide Release System (M-OCORS)	x													
Preparation of the Enzyme Triggered Oral Carbon Monoxide Release System (E-OCORS)	x							x						
Amperometric detection system	x						x							
In vivo Pharmacokinetics	x	x	x	x	x									
2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice		x	x	x	x					x				
Cytotoxicity	x					x								
Statistics	x													
Supplementary Information: Pharmacokinetic modelling	x													
Study design/concept development	x	x							x	x	x			
Data analysis and interpretation	x	x									x			
Manuscript planning	x										x			
Manuscript writing	x													
Correction of manuscript	x	x									x			
Supervision of Christoph Steiger											x			

P4	**Steiger C, Wollborn J, Gutmann M, Zehe M, Wunder C, Meinel L** (2015) Controlled therapeutic gas delivery systems for quality-improved transplants.

Author	**1**	**2**	**3**	**4**	**5**	**6**							
Electrochemical headspace gas detection for release systems	x												
Therapeutic Gas Release System	x												
CO release from TGRS	x												
Inductively coupled plasma optical emission spectrometry *	x												
Leakage tests with a dye	x												
Animal studies	x	x											
Western blot	x		x										
CO release kinetics - bridging to previous protocols flushing perfusion solutions with CO gas	x												
CO release stick	x												
Cartridge system for gas enrichment of a perfusion solution	x			x									
Statistics	x												
Study design/concept development	x				x	x							
Data analysis and interpretation	x					x							
Manuscript planning	x					x							
Manuscript writing	x												
Correction of the manuscript	x				x	x							
Supervision of Christoph Steiger						x							

* Analytic procedure performed by Frauenhofer ISC, Würzburg

P5	**Ilko D, Steiger C, Keller R, Holzgrabe U, Meinel L** (2016) Tamper-proof tablets for distinction between counterfeit and originator drugs through PEG coding

Author	**1**	**2**	**3**	**4**	**5**								
Preparation of the tablets		x											
LC-MS/MS analysis	x												
Study design/concept development	x	x	x	x	x								
Data analysis and interpretation	x	x	x	x	x								
Manuscript planning	x	x	x	x	x								
Manuscript writing	x	x											
Correction of the manuscript	x	x	x	x	x								
Supervision of Christoph Steiger					x								

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Prof. Dr. Dr. Lorenz Meinel	Unterschrift
Christoph Steiger	Unterschrift