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Liposome-Supported Enzymatic Peritoneal Dialysis for Alcohol Detoxification

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ANNA PRATSINIS

Master of Science ETH in Pharmaceutical Sciences, ETH Zurich

born on 21.07.1987
citizen of Switzerland and USA

accepted on the recommendation of

Prof. Dr. Jean-Christophe Leroux, examiner
Prof. Dr. Robert Grass, co-examiner

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Summary

Peritoneal dialysis (PD) is a modality in which the blood is dialyzed intra-corporeally against the peritoneum. It is a less invasive, simple and cost-effective dialysis modality and is most widely used during the management of end-stage renal disease. This detoxification procedure may also be employed during the management of non-renal indications such as acute alcohol poisoning, it is however not the modality of choice owing to its modest clearance capacity and extracorporeal dialysis prevails for such indications. Previous efforts from our research group have demonstrated that the extraction capacity of PD for ionizable endogenous and exogenous toxins can be clearly augmented by supplementing conventional dialysate solutions with transmembrane pH-gradient liposomes. Capitalizing on this, the objective of this PhD thesis was to investigate the feasibility of improved PD functionality *via* enzymatic metabolism by introducing enzyme-loaded liposomes (E-Liposomes) into conventional PD solutions for the model indication acute alcohol poisoning.

Chapter 1 provides a general overview of the prevalence, symptoms and recommended treatment measures for acute alcohol poisoning. Previous approaches to raise the ethanol clearance by enzymatic metabolism are described as well as the accompanying limitations, herewith formulating the major objectives of this PhD thesis.

In **Chapter 2** the current status of PD's non-renal indications (*e.g.* poisoning and hyperammonemia) as well as potential future indications (*e.g.* stroke and oxygen therapy) are reviewed. A special focus on the underlying dialysate formulation and strategies to increment the functionality of PD are given wherever relevant.

Chapter 3 describes the successful optimization and characterization of E-Liposomes with the enzymes, alcohol oxidase (AO) and catalase (CAT). Ethanol is metabolized by AO under the consumption of oxygen to acetaldehyde and hydrogen peroxide. The latter is then further

degraded by CAT to oxygen and water, thereby recycling oxygen to a certain extent. For the effective preparation of E-Liposomes, the enzymes were hydrophobically modified and subsequently loaded onto liposomes by the detergent dialysis procedure. *In vitro* stability measurements demonstrated increased thermo-stability for AO-Liposomes at 37 °C. The improved stability of the E-Liposomes subsequently enabled enhanced *in vitro* enzymatic metabolism of ethanol. By adding hydrogen peroxide which was subsequently degraded to oxygen by CAT, the stoichiometric oxygen dependency for ethanol metabolism by this enzyme pair was highlighted.

In **Chapter 4** the *in vivo* characterization of E-Liposomes supplemented to conventional PD, termed liposome-supported enzymatic peritoneal dialysis (LSEPD) is described. As surmised from the *in vitro* experiments, liposome loading enabled maintained activity in the peritoneal cavity for AO-Liposomes in contrast to free AO. By anchoring enzymes onto a lipid scaffold their systemic exposure, measured in the blood circulation and major organs could be reduced appreciably. In a rodent model of acute ethanol poisoning, LSEPD was able to clearly enhance the enzymatic metabolism of ethanol when compared to control PD. This was manifested by the markedly increased levels of ethanol's primary metabolite, acetaldehyde, as well as the reduced ethanol concentrations in the dialysate. The action of LSEPD was however insufficient to detectably lower the systemic ethanol levels.

The key findings of this PhD thesis are concluded in **Chapter 5**. Strategies to address the limitations of this current formulation either by increasing the oxygen availability or employing alternative ethanol metabolizing enzymes are discussed, and a general outlook on potential further applications of LSEPD is provided.

Zusammenfassung

Die Peritonealdialyse (PD) ist eine Methode bei der das Blut direkt im Körper gegen das Peritoneum dialysiert wird. Das Verfahren ist wenig invasiv, einfach und kostengünstig und wird meist für die Therapie von Urämie beziehungsweise bei Nierenversagen eingesetzt. Diese Detoxifikationsmethode kann auch bei nicht-renalen Indikationen zum Einsatz kommen, beispielsweise bei der akuten Alkohol Vergiftung. Die PD ist aber in solchen Fällen wegen der bescheidenen Ausscheidungskapazität nicht die Therapie erster Wahl. Bei solchen Fällen werden überwiegend extrakorporale Dialysemethoden eingesetzt. Vorangehende Bemühungen aus unserer Arbeitsgruppe haben gezeigt, dass die Extraktionskapazität der PD für ionisierbare endogene und exogene Toxine deutlich verstärkt werden kann, wenn Liposomen mit einem transmembranen pH-Gradienten zur konventionellen Dialyseflüssigkeit hinzugefügt werden. Darauf aufbauend lag das Ziel dieser Doktorarbeit darin die Durchführbarkeit einer enzymatisch gestützten PD für eine Beispielsindikation, nämlich die akute Alkohol Vergiftung, zu untersuchen. Um dies zu erforschen wurden mit Enzym beladene Liposomen (E-Liposomen) formuliert.

Kapitel 1 verschafft einen generellen Überblick über die Prävalenz, Symptome und Behandlungsansätze im Falle einer akuten Alkohol Vergiftung. Es werden vorangehende Ansätze beschrieben, welche die Ethanol Auscheidung mittels enzymatischem Metabolismus zu erhöhen versuchen, sowie deren miteinhergehenden Einschränkungen. Die Hauptziele dieser Doktorarbeit werden dargelegt.

In **Kapitel 2** wird der derzeitige Status von den nicht-renalen Indikationen der PD (z.B. Vergiftung und Hyperammonämie) als auch potentielle zukünftige Indikationen (z.B. Gehirnschlag und Sauerstoff Anreicherung) besprochen. Wenn möglich wird ein spezieller

Fokus auf die Dialyse Formulierung und Möglichkeiten zur die Funktionalitätssteigerung der PD gesetzt.

Kapitel 3 beschreibt die erfolgreiche Optimierung und Charakterisierung von E-Liposomen mit den Enzymen Alkohol Oxidase (AO) und Katalase (CAT). Ethanol wird durch AO unter Verbrauch von Sauerstoff zu Acetaldehyd und Wasserstoffperoxid metabolisiert. Letzteres wird dann durch CAT weiter zu Sauerstoff und Wasser abgebaut, wodurch der Sauerstoff zu einem gewissen Grad wiederverwertet werden kann. Für eine effektive Zubereitung von E-Liposomen wurden die Enzyme hydrophobisch modifiziert und anschliessend mittels Detergenz-Dialyse auf Liposomen geladen. *In vitro* Stabilitätsmessungen wiesen eine erhöhte Thermostabilität für AO-Liposomen bei 37 °C auf. Folglich, ermöglichte die verbesserte Stabilität von den E-Liposomen einen erhöhten enzymatischen Abbau von Ethanol *in vitro*. Unter Zugabe von Wasserstoffperoxid, welches darauffolgend durch CAT zu Sauerstoff abgebaut wird, wird eine stöchiometrische Sauerstoff-Abhängigkeit für den Ethanol Abbau mit diesem Enzympaar nachgewiesen.

In **Kapitel 4** wird die *in vivo* Charakterisierung von E-Liposomen, die zu konventionellen PD Lösungen hinzugefügt werden, kurz als Liposomen unterstützte enzymatische Peritonealdialyse (LUEPD) bezeichnet, erläutert. Wie bereits in den *in vitro* Versuchen vermutet, ermöglichte die Liposomenbeladung die Aufrechterhaltung der enzymatischen Aktivität von AO-Liposomen innerhalb der Bauchhöhle im Gegensatz zu freier applizierter AO. Durch das Beladen von Enzymen auf einem Lipid Gerüst konnte deren systemische Verteilung, gemessen im Blut und den Hauptorganen, merkbar gesenkt werden. In einem Nagetier Model mit akuter Alkohol Vergiftung konnte die LUEPD Therapie einen offensichtlich erhöhten enzymatischen Ethanol Metabolismus im Vergleich zu einer PD Kontrolle aufweisen. Dies machte sich bemerkbar durch die ausgesprochen hohen Spiegel an Acetaldehyd, dem primären Metabolit von Ethanol, als auch durch die reduzierten Ethanol Konzentrationen im Dialysat. Die Aktivität von LUEPD war jedoch nicht ausreichend um den systemischen Ethanol Spiegel zu senken.

Kapitel 5 diskutiert abschliessend die Haupterkenntnisse dieser Doktorarbeit. Es werden Ansätze besprochen, die Einschränkungen der derzeitigen Formulierung zu überwinden versuchen mittels erhöhter Sauerstoff Verfügbarkeit oder unter Verwendung von alternativen Alkohol metabolisierenden Enzymen. Schliesslich wird ein genereller Ausblick gegeben und mögliche weitere Applikationen von LUEPD werden aufgezeigt.

Chapter 1

Background and Purpose

1.1. Prevalence of acute alcohol intoxication

Alcohol, most notably ethanol, is a well-known psychoactive and dependence-producing substance that has been consumed by mankind for millennia [1,2]. Heavy drinking is a major contributing factor to the global burden of disease and according to the World Health Organization (WHO), in 2012 approximately 3.3 million deaths were attributed globally to the harmful use of alcohol [3]. This corresponds to 5.9% of yearly global deaths, with men crediting for 7.6% and women for 4.0% [1]. **Figure 1.1** provides a global overview of the alcohol consumption per capita, which is defined as the total amount of alcohol in liters consumed per individual aged 15 or older per year, indicating that higher income countries are most affected.

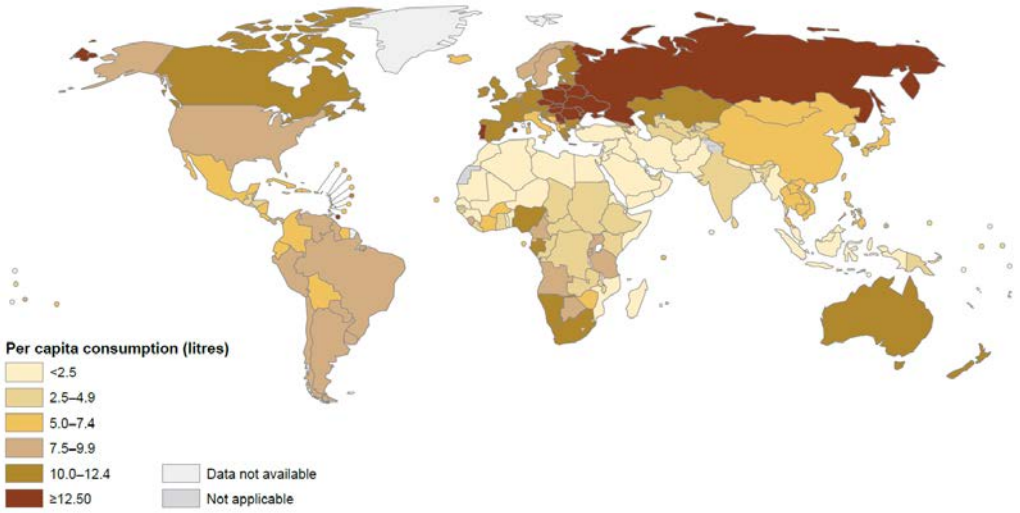

Figure 1.1: **Global schematic of the alcohol consumption per nation**. The per capita consumption of alcohol is defined as the total annual volume of alcohol consumed per person older than 15 and is expressed in liters. The here presented values were calculated from years 2008 – 2010 [1].

Switzerland is no exception to the use of alcohol as a common recreational substance. According to the Federal office of public health, alcohol abuse causes approximately 1'600 annual deaths, while every 12th death is related to alcohol [4]. On weekend nights, every other traffic accident ending fatally or with severe injuries is related to alcohol [4]. It has been described that 30 – 40% of adolescents and young adults consume risky amounts of alcohol at least once per month [4]. The total number of adolescents and young adults aged 10 – 23, that were hospitalized due to acute alcohol poisoning over the years 2001 – 2010 is shown in **Figure 1.2a**. Interestingly, when the hospitalization rate (number of hospitalizations/1'000 inhabitants) is plotted for specific age groups, adolescents aged 14 – 15 exhibit the highest hospitalization rate amongst the youth (**Figure 1.2b**) [5].

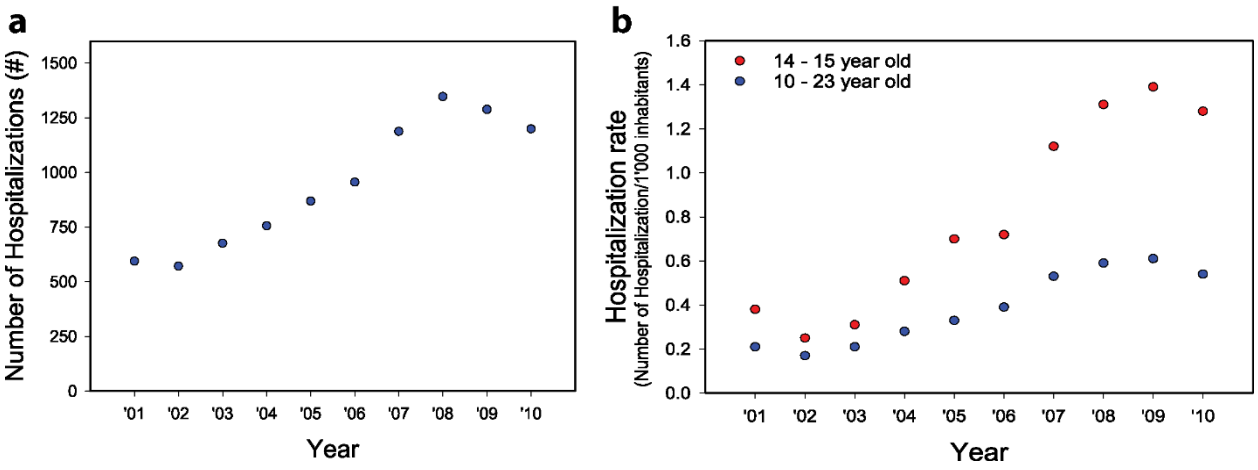

Figure 1.2: **Hospitalization in Switzerland due to acute alcohol poisoning (main and adventitious cause). (a)** The total number of hospitalizations in Switzerland concerning adolescents and young adults aged 10 – 23, shown for years 2001 – 2010. **(b)** The rate of hospitalization calculated per 1'000 inhabitants for adolescents aged 14 – 15 (red) and total adolescents and young adults aged 10 – 23 (blue). Data were extracted from [5].

1.2. Alcohol poisoning amongst the youth

In comparison to adults, the younger population tends to drink alcohol less frequently but in larger volumes [6,7]. This phenomenon is derived from heavy episodic drinking, also known as binge drinking, which is defined as having more than 4 to 5 drinks on a single occasion leading to intoxication [3,7–9]. The main driving force for binge drinking is said to be peer pressure as well as tension reduction [6]. Binge drinking is more frequent in males than in females and habits in the youth often reflect the drinking culture of the adult population, where a higher prevalence of binge drinking is observed in northern and central Europe compared to southern Europe [6,10].

As depicted in **Figure 1.2**, the prevalence of acute alcohol poisoning in Switzerland has doubled from 2001 – 2007 and seems to have levelled off in the recent years. A similar increase in prevalence of acute alcohol poisoning among the youth was observed in a retrospective study conducted in a children's hospital from 1998 – 2004 [11]. The patients admitted for alcohol poisoning were on average 14.5 years old and showed a mean blood ethanol concentration of 177 mg/dL, while more than one third of these children were unconscious [11]. This is particularly alarming, as lower blood alcohol concentrations are required to elicit toxicity in young teenagers than in adults [12].

This behavior not only results in short term consequences, such as acute intoxication (see below for acute symptoms) and accidental injuries, but also bears long term effects [13]. In a single-blinded study where healthy volunteers received either a high alcohol dose or placebo, memory retrieval was found to be significantly impaired in the hangover phase of the alcohol group [14]. Furthermore, retrospective analyses show that an early initiation of alcohol use (< 14 years) is associated with increased risk of alcohol dependence and abuse [15]. The alarming severity combined with the increased prevalence of alcohol intoxication among young people has set the focus of multiple studies to assess the underlying cause of binge-drinking in the youth [10,16]. The role of family has been proposed to be an effective preventive measure and contrariwise, early onset of alcohol use has been linked to a family history of drinking [6,17]. Nonetheless, multiple alcohol policy programs have been initiated on the national and

international level, such as the National Program Alcohol by the Swiss Federation (2008 – 2016).

Besides the widely spread binge drinking in adolescents, alcohol poisoning has also been reported in infants and young children [18]. This typically occurs by accidental ingestion, although involuntary intoxication has also been described [19,20].

1.3. Adverse effects of acute ethanol poisoning

Consumption of low doses of ethanol can lead to initial euphoria, increase in self-confidence and sociability [21]. Upon ingestion of higher doses, various metabolic, gastro-intestinal, behavioral, neurological, cardiac and pulmonary disorders can arise [22,23]. The well-known clinical image of acute ethanol poisoning involves slurred speech, incoordination and reduced awareness [22,24,25]. Hypothermia can result from cardiovascular manifestations such as tachycardia, peripheral vasodilation and volume depletion [22]. Severe ethanol poisoning can lead to death, mainly by respiratory depression, hypothermia or hypoglycemia, notably in the pediatric population [12,21,26]. **Table 1.1** gives an overview of the various stages of acute alcoholic intoxication in relation to blood ethanol concentration ranges, which is however subject to inter-individual variability depending on past exposure to ethanol, genetic predisposition, age and gender [21,22,26]. Death from acute ethanol poisoning is suggested to occur at blood ethanol levels greater than 450 – 500 mg/dL [21,22]; however, deaths at much lower levels [19] and survival at significantly higher concentrations have been reported [27]. In a retrospective forensic analysis of 693 deaths due to acute ethanol poisoning, the mean and median blood ethanol concentration was found to be 360 mg/dL, which is within the above suggested concentration [28].

Table 1.1: Simplified overview of stages of acute ethanol intoxication, adapted from [21].

BEC (mg/dL)	Stage of ethanol influence	Clinical signs/symptoms
10 – 50	Sobriety	Behavior nearly normal by ordinary observation
30 – 120	Euphoria	Mild euphoria, sociability, increased self-confidence, decreased inhibition
90 – 250	Excitement	Loss of critical judgement, Impairment of memory and comprehension, increased reaction time
180 – 300	Confusion	Disorientation, impaired balance, dizziness, decreased pain sense, slurred speech
270 – 400	Stupor	Apathy, marked muscular incoordination, vomiting, impaired consciousness
350 – 500	Coma	Complete unconsciousness, subnormal temperature, depressed abolished reflexes
> 450	Death	Death from respiratory paralysis

BEC: blood ethanol concentration

1.4. Ethanol pharmacokinetics and metabolism

Upon ingestion, ethanol is absorbed mainly from the proximal intestinal tract, chiefly the duodenum and jejunum [29,30]. Absorption from the stomach is comparatively slow, therefore the rate of ethanol absorption following oral application is dependent on gastric emptying, which can be influenced by the fed state [31,32]. This explains the general recommendation not to drink alcohol on an empty stomach [33]. Ethanol absorption from the gastrointestinal tract can be altered by diseases or pharmacological intervention (*e.g.* domperidone), which affect the gastric motility. Furthermore, higher ethanol concentrations (> 30 w/v%) of the ingested beverage may slow down absorption due to irritation of the gastric mucosa [29,30,33].

Ethanol is readily miscible with water and practically insoluble in fats and oils, however, like water it can easily permeate membranes (*e.g.* cell membrane, blood brain barrier) [24,33]. The ethanol tissue accumulation depends on the total water content and perfusion of the corresponding tissue and organs [30,33]. The volume of distribution of ethanol depends on the proportion of fat to lean tissue and therefore differs for men and women [34].

Ethanol is mainly metabolized in the liver by alcohol dehydrogenase (ADH) [33,35]. Gastric ADH can account for approximately 10% of the alcohol metabolism, though the extent is subject to high variability depending on the aforementioned gastric emptying [22]. Ethanol is oxidized to acetaldehyde by ADH and subsequently to acetate by aldehyde dehydrogenase (ALDH) under the consumption of nicotinamide adenine dinucleotide (NAD) [36], as depicted below in **Figure 1.3**. Acetate is eventually metabolized to CO_2 and water in peripheral tissue [34,37]. In cases of severe ethanol poisoning, increased ethanol metabolism can cause a shortage of NAD, impairing other vital functions such as gluconeogenesis and leading to hypoglycemia [24]. Given that most ADH isoforms have a low K_M (2 – 10 mg/dL) for ethanol, its metabolism follows zero-order kinetics at blood ethanol concentrations greater than 20 mg/dL [33,34]. This means that at higher concentrations, the rate of metabolism is constant and independent of the substrate concentration. For moderate drinkers, an average elimination rate of 15 mg/100 mL/h can be assumed [34].

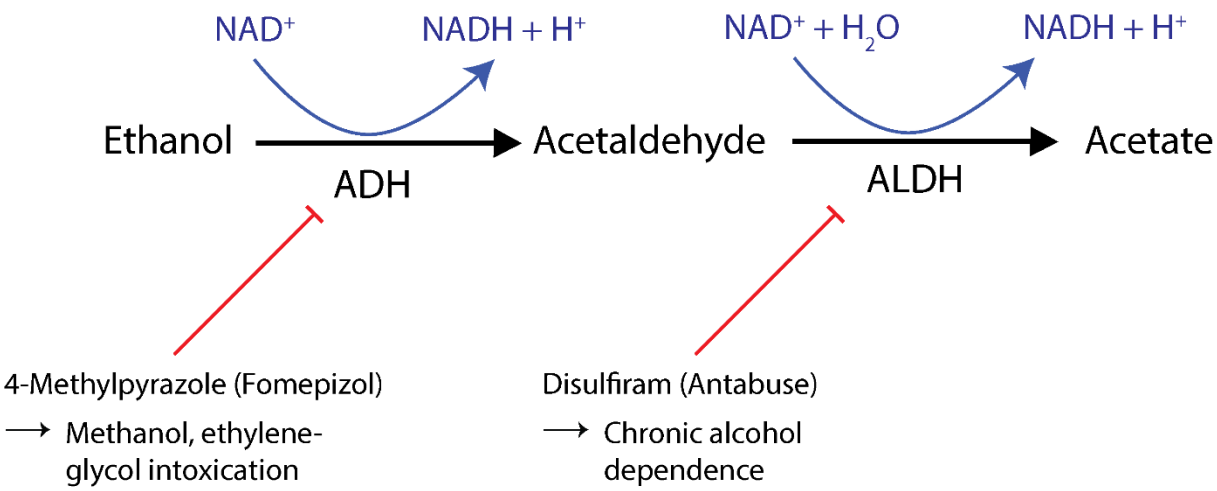

Figure 1.3: Schematic of alcohol metabolism reaction by ADH, ALDH. Ethanol is metabolized under consumption of NAD. 4-Methylpyrazole has a high affinity for ADH and can be used to saturate ADH enzymes during methanol and ethylene glycol poisoning to prevent buildup of toxic metabolites. Disulfiram blocks ALDH metabolism, leading to a buildup of acetaldehyde. As acetaldehyde is associated with the unpleasant symptoms of ethanol, disulfiram can be used as a deterrent to alcohol consumption to support abstinence during the management of alcohol dependence.

In addition to ADH, the microsomal ethanol oxidizing system (MEOS), can also contribute to the elimination of ethanol [33]. Of the MEOS, the cytochrome P450 enzyme, CYP2E1 displays the highest ethanol oxidizing activity. The K_M of CYP2E1 is approximately 60 – 80 mg/100 mL, which can lead to increased ethanol metabolism at higher concentrations [33,34]. Furthermore, cytochrome P450 enzymes can be induced by their substrates and CYP2E1 levels have been shown to be augmented by chronic alcohol consumption, which may be an important factor in metabolic tolerance [32,38].

While ethanol and acetaldehyde synergistically contribute to the neurochemical and behavioral effects of alcohol consumption [39,40], acetaldehyde is regarded as more toxic and carcinogenic [41]. Flushing symptoms such as face flushing, nausea, vomiting, headache and tachycardia are largely attributed to acetaldehyde [40,42]. Individuals lacking functional ALDH2 enzymes, due to a single point mutation predominantly affecting the South East Asian

population, exhibit the prominent face flushing and other distressing symptoms upon consumption of low doses of ethanol due to build-up of acetaldehyde [33,43]. Concurrently, by blocking ALDH with disulfiram, also known as Antabuse, alcohol withdrawal treatment exploits the unpleasant manifestations of acetaldehyde as a means to support abstinence during the management of alcohol dependence [44,45] (**Figure 1.3**).

Additional pharmacological interventions of the alcohol metabolizing enzymes involves the saturation of ADH by 4-methylpyrazole also known as Fomepizol (**Figure 1.3**). While ethanol presents the most frequent cause of alcohol poisoning, other alcohols such as methanol, ethylene glycol, diethylene glycol, propylene glycol and isopropanol can also cause poisoning. Such intoxications can occur following accidental ingestion of certain household products (*e.g.* anti-freeze, windshield-washer fluids and fuel additives). Though rare, they are typically associated with a more severe clinical profile requiring immediate therapeutic intervention [46,47]. With the exception of isopropanol, the primary aldehydes of these alcohols are highly toxic and the most effective treatment strategy involves prevention of their metabolism by saturation of ADH with 4-methylpyrazole, which has a higher affinity for ADH [46,47]. In the absence of 4-methylpyrazole ethanol can be administered, as it too possesses a higher binding capacity for ADH [46].

1.5. Management of acute ethanol poisoning

Primary treatment of patients admitted to the emergency center and suspected of acute ethanol poisoning involves conservative measures in the first instance and clinical examination to rule out the possibility of poisoning by more toxic alcohols [23,24]. Supportive measures typically comprise of intravenous administration of glucose and thiamine as well as airway stabilization and cardiovascular support [48]. Warming blankets can also be provided to prevent hypothermia [26]. Blood should be analyzed to assess the ethanol concentration, glucose, electrolytes and osmolality [24]. A simplified overview of the recommended sequential measures to manage severe ethanol poisoning is given in **Figure 1.4**.

Various methods exist to determine the blood ethanol concentration, mainly involving colori- or fluorimetric enzyme-based assays or gas chromatography. In the absence of such methods, osmolality measurements by freezing point depression can be employed to estimate whether alcohol is present in the blood [46]. Headspace gas chromatography presents the gold standard for alcohol analysis because of its simple analysis, ability to discern different alcohols, sensitivity and precision, and is thus widely used within the forensics community [49]. However, such instruments are expensive, require special training and are not always available in clinical laboratories [50]. Therefore, easy-to-use enzyme-based methods employing either ADH or alcohol oxidase (AO) are frequently used in the clinic [50].

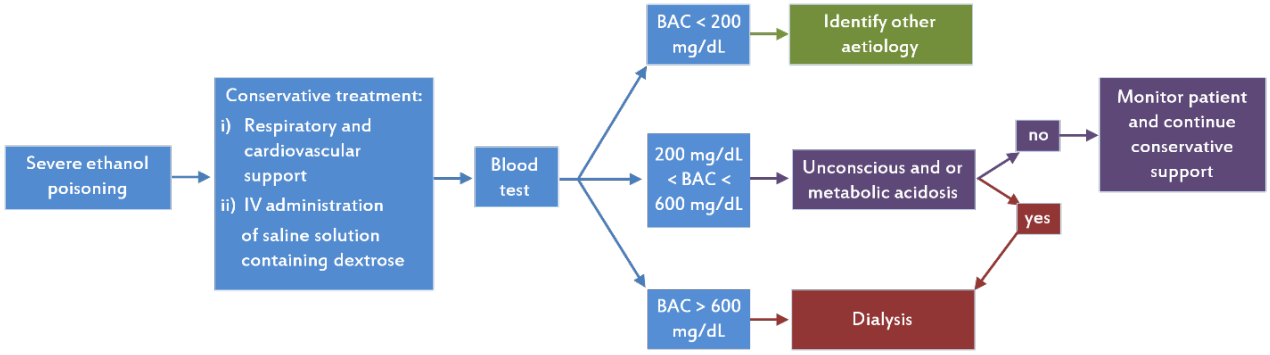

Figure 1.4: Sequence of the recommended measures to manage severe ethanol intoxication. When a patient suspected of such poisoning is admitted to the hospital, he/she is immediately given conservative treatment to prevent respiratory depression. In addition, a saline solution containing dextrose and thiamine is administered to prevent metabolic disorders, and the blood ethanol and electrolyte levels are regularly assayed. Adapted from [24].

If high blood levels are detected (> 600 mg/dL) or if the patient is in a comatose state, ethanol-lowering measures should be considered [24,48], which is most effectively achieved by dialysis owing to the small size and high water solubility of ethanol [51]. Various case reports have described the efficacious use of hemodialysis (HD) during the management of severe ethanol poisoning [27,48,52–54]. HD is however highly invasive and only offered in specialized centers. In addition, it is rarely the modality of choice in the pediatric population (*e.g.* neonates), owing to the reduced vascular catheter access and their limited toleration to large circuit volumes [55,56]. Therefore, an alternative dialysis modality called peritoneal dialysis (PD) has

been employed to increase the ethanol removal in this patient population, where poisoning may occur by accidental or forced ingestion [20,57]. Though less invasive and more suited for this patient population, PD is associated with a 2- to 4-fold lower clearance capacity than HD [20], indicating that efforts should be made towards increasing the functionality of this easily accessible therapy.

1.6. Peritoneal dialysis

In comparison to HD where patients are connected to a machine and blood is filtered extra-corporeally, PD poses an intra-corporeal dialysis modality where blood is dialyzed against an intraperitoneally instilled dialysis fluid, which can either be left to dwell or be rapidly exchanged [58,59]. A schematic of PD is shown in **Figure 1.5**. The clearance capacity of PD in the poisoned patient has been described to be less efficacious than HD on multiple accounts [59–61].

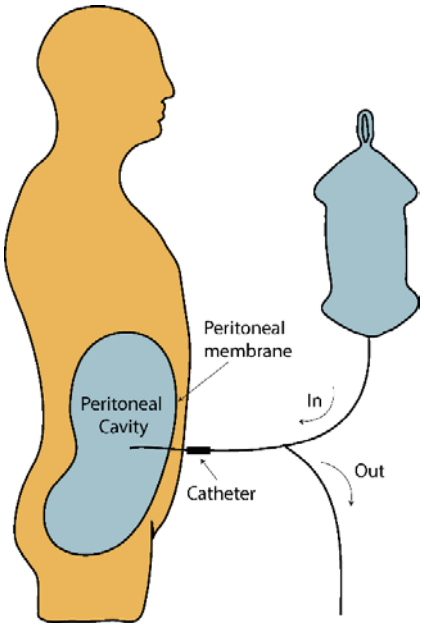

Figure 1.5: Schematic overview of PD procedure. Dialysis is initiated by instilling a fresh dialysate solution, into the peritoneal cavity *via* a catheter. The waste fluid is drained at the end of the dialysis session.

An osmotic agent present in the dialysate fluid enables fluid removal, commonly referred to as ultrafiltration, as well as solute exchange across the peritoneal membrane by diffusion [62]. Thereby, during the management of acute ethanol poisoning by PD, ethanol enters the peritoneal cavity by diffusion along the concentration gradient. Most commercial PD fluids utilize glucose as an osmotic agent to enable fluid influx into the peritoneal cavity. However, owing to its small size, glucose can escape the peritoneal cavity, slowly annihilating the osmotic gradient and precluding prolonged dialysis sessions. Moreover, the local exposure of glucose and its degradation products can cause structural damage to the peritoneal membrane [63]. The most recent development in the field is thus a high molecular weight glucose polymer called icodextrin, which owing to its higher peritoneal retention enables prolonged dialysis (*e.g.* overnight sessions) [63]. Current formulations under investigation involve the development of hyperbranched glucose polymers [64–66] and supplementation of glucose-based dialysis formulations with a cytoprotective dipepetide, AlaGlu, under development by Zytoprotec in Austria [67,68]. While the increased fluid and waste removal with hyperbranched glucose-based PD fluids has only been shown in preclinical settings, the reduced risk of peritoneal membrane failure and peritonitis with AlaGlu-supplemented PD fluid has been shown in first clinical trials.

Most recent efforts from our group to increase the modest clearance of PD for endogenous and exogenous toxins involve the development of liposome-supported peritoneal dialysis (LSPD). This technology exploits the ability of transmembrane pH-gradient liposomes to concentrate ionizable compounds within their core by ionization following diffusion across the lipid bilayer in their neutral form [69,70]. Commercial, icodextrin-PD solutions supplemented with transmembrane pH-gradient liposomes demonstrated a significantly enhanced extraction of the endogenous toxin ammonia as well as exogenous, overdosed ionizable drugs [71]. Furthermore, LSPD was able to reverse the hypotensive action of the calcium channel blocker verapamil significantly more rapidly than the control receiving an icodextrin-PD. The hypotensive recovery with LSPD was markedly enhanced over what had been observed in a previous study following the intravenous application of such liposomes [70,71]. These results provided the cornerstone for the foundation of Versantis, a spin-off company from our research group focused on developing an LSPD formulation for the primary indication

hyperammonemia (HA). Since this initial study, the formulation and its production have been optimized and its efficacy established in a rodent model of HA to enable its clinical translation [72]. These findings demonstrated that the functionality of PD could be augmented by introducing nano- to micrometer-sized vesicles into the peritoneal cavity and suggested that under adequate optimization the functionality of PD for other non-ionizable xenobiotics, such as ethanol, may be increased.

1.7. Enzyme-based preclinical approaches to enhance ethanol clearance

The idea to treat alcohol intoxication by enhanced enzymatic metabolism has been previously described. To the best of our knowledge, the first published report involved the encapsulation of the methylotrophic yeast enzyme alcohol oxidase (AO) into human and murine erythrocytes, and was proposed as treatment for acute methanol poisoning [73] as AO has a higher affinity for methanol [74]. When injected intravenously in mice, the blood methanol concentration was reduced by 50%, which presents a significant reduction in the systemically exposed methanol [73]. However as this alcohol mediates its toxicity *via* its primary aldehyde metabolite, formaldehyde, such an approach would not be viable owing to the increased production of toxic metabolites [46,47]. Subsequent development of ADH- and ALDH-loaded erythrocytes for ethanol detoxification demonstrated a 43% reduction of blood ethanol following intravenous application [75]. Though promising, no further developments of this platform have been described since it was reported in 2001, possibly owing to the high cost and potential risk associated with the delivery of human erythrocytes.

Approximately half a year before this thesis was started, an innovative approach to treat severe ethanol poisoning by intravenous administration of polymeric nanocomplexes of AO and catalase (CAT) was published in *Nature Nanotechnology* [76]. The confinement of AO and CAT within the same polymeric scaffold with an inter-enzyme distance smaller than 10 nm was shown to enhance the clearance of ethanol in a murine model of alcohol poisoning and reduce

the exposure of the intermediate hydrogen peroxide (H_2O_2). The metabolism of ethanol by AO and CAT is shown within **Figure 1.6.** The current set-up was considered to be particularly appealing as the cofactor of AO was already included in the protein structure, unlike for ADH/ALDH where NAD needs to be additionally supplied. Even though this publication has been cited over 80 times (Web of Science, accessed August 2017), to the best of our knowledge, the *in vivo* ethanol clearance demonstrated with this enzyme pair has yet to be reproduced.

Finally, in 2015 an alternative approach was proposed to enhance the ethanol and acetaldehyde clearance in mice with an ALDH2 loss of function mutation, by recruiting an alternative ALDH isoform ALDH3A1. This was mediated by administering a pharmacologic agent, which resulted in a temporarily increased elimination of ethanol and acetaldehyde [77]. Though exogenous enzymes were not administered, increased metabolite clearance was accomplished by induction of endogenous metabolism.

1.8. The scope of this thesis

Capitalizing on the substantially enhanced scavenging capacity of LSPD for ionizable toxins *via extraction*, the aim of this thesis was to further extend the functionality of PD by investigating the feasibility of supporting PD *via* enhanced *metabolism*. As alcohol readily crosses membranes and is dialyzable, acute alcohol poisoning was selected as a model indication. The enzymes AO and CAT were employed here, as this enzyme pair had been shown to synergistically increase ethanol clearance in a mouse model [76]. The catalytic activity of AO is inversely proportional to the alcohol chain length [78], with the highest activity for methanol. The focus of this application was however set to ethanol owing to the aforementioned toxicity associated with methanol's primary metabolite and the existence of an efficacious antidote for methanol, 4-methylpyrazole [46,47]. Since a liposome size-dependent peritoneal leakage into the circulation has been shown [71,79], enzymes were anchored onto a lipid support to reduce the systemic exposure following intraperitoneal administration. The platform consisted of enzyme-loaded liposomes (E-Liposomes) supplemented to conventional PD fluids and was

termed liposome-supported enzymatic peritoneal dialysis (LSEPD) and is outlined below in **Figure 1.6**.

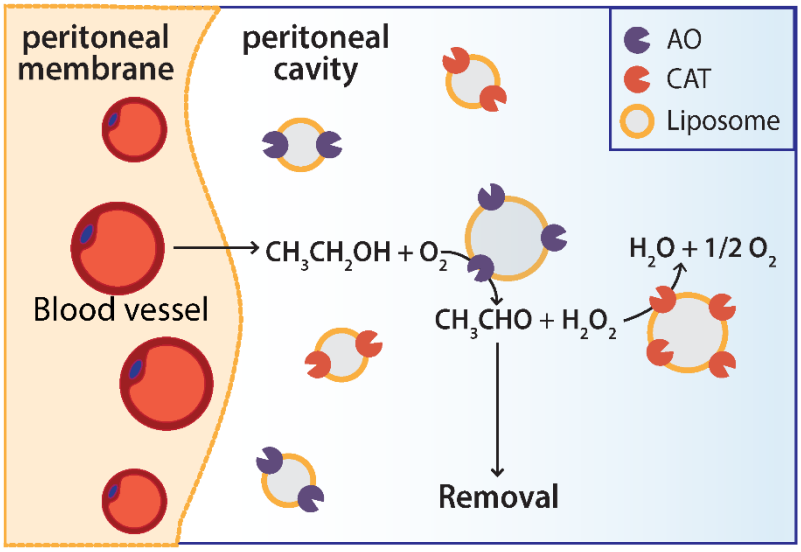

Figure 1.6: **Concept of LSEPD**. During PD, circulating ethanol diffuses from the blood into the peritoneal cavity, where it is metabolized to acetaldehyde and H_2O_2 under the consumption of O_2 by AO anchored onto a lipid support. The H_2O_2 is then further degraded by liposome-anchored CAT to water and O_2. The PD suspension containing E-Liposomes and waste products is removed after 3 h.

In the following section (**Chapter 2**), a review of the non-renal indications of PD is presented. The indications were classified as historically or currently relevant. Potential future applications and adaptations of classical PD, where currently only preclinical data is available were discussed. Wherever possible, special focus was placed on the applied formulations. In **Chapter 3**, the optimization of the hydrophobic enzyme modification required for increased liposome loading is described. Furthermore, the preparation of E-Liposomes and their subsequent characterization in terms of stability and *in vitro* ethanol metabolism is presented. In **Chapter 4**, the *in vivo* characterization of the E-Liposomes is described. Here, the E-Liposomes were compared to the free enzymes with respect to their *in vivo* activity and peritoneal leakage to the circulation and major organs. In a final step, the capability of LSEPD

to support the physiological metabolism of ethanol was examined in comparison to conventional PD. Finally, the main findings of this thesis are summarized in **Chapter 5**, where an outlook towards the further development of LSEPD and strategies to extend the functionality of PD are also given.

Chapter 2

Peritoneal Dialysis beyond Kidney Failure?

2.1. Introduction

PD is an intra-corporeal, life-sustaining therapy by which blood circulating in the peritoneal membrane is dialyzed against a large fluid volume ("the dialysate") that is instilled into the peritoneal cavity and left to dwell. Effective exchange of fluid and solutes is enabled by the large peritoneal exchange area of the highly vascularized peritoneal membrane [80], exposed to the dialysate containing an osmotic agent (most often, glucose) [62,81,82]. The PD solutions are categorized as drugs by health regulatory agencies [83] and therefore as for any drug, the development process is long and costly.

The first human application of PD was described by Georg Ganter in 1923 for the treatment of uremia, at the same time as the early reports of today's widely applied HD [84,85]. Unlike HD where patients are connected to an extracorporeal circuit and dialysis machine, PD does not require sophisticated equipment and specialized centers, permitting simple and easy to implement daily home dialysis and treatment in less endowed environments [83,86]. A number of technical innovations, have led to major developments in the clinical use of PD for the treatment of end-stage renal disease (ESRD). In a most recent outcome study, these improvements were found to result in a significant reduction in the mortality risk for patients starting with PD and a similar survival rate as patients subjected to in-center HD [87]. The less invasive and more flexible nature of PD are important arguments for a positive selection of this dialysis modality. Furthermore, as PD is less invasive, it is the preferred dialysis modality in the pediatric population given the large extracorporeal circuit volumes implied in HD [55,88]. Recent evidence pointing to the lower societal costs and relative underutilization of PD [86] has prompted various governmental policies to encourage its use [89,90].

Traditional dialysate formulations employ glucose as a crystalloid osmotic agent. However, over time on dialysis, glucose and its degradation products can cause structural changes in the peritoneal membrane by local exposure [63,91,92]. Furthermore, the rapid absorption of glucose leads to an early disemination of the osmotic gradient and can additionally cause adverse metabolic and cardiovascular effects, thereby limiting long-term PD [63,93]. The rapid absorption of glucose may be countered by shorter dwells using a cycling machine, as in

automated PD [94]. The use of icodextrin, a high molecular weight glucose polymer, has improved PD formulations notably, as this colloidal osmotic agent allows prolonged dwell time with sustained ultrafiltration (water removal) given its enhanced peritoneal retention [63,95]. Recent PD fluids under investigation include a hyperbranched polyglycerol-based solution [64] and a glucose-based solution supplemented with the cytoprotective dipeptide AlaGlu [67]. Solutions employing hyperbranched poly(glycerol) as an osmotic agent demonstrated more effective fluid and waste removal, as well as superior peritoneal membrane preservation compared to a glucose-based dialysate solution in rodents [64–66], but their clinical characterization is still pending. AlaGlu-supplemented PD is currently being investigated by Zytoprotec under the trade-name PD-protec®, with which a first-in-man trial (NCT01353638) and Phase II study (EudraCT 2013-000400-42) have been successfully completed, demonstrating a reduced risk of peritoneal membrane failure and peritonitis [68,96].

Although PD is mainly indicated for the management of patients with ESRD, this technique has also been explored for the primary treatment of other conditions [97,98]. This review will provide an overview of PD's relevance during the management of non-renal indications with an emphasis on the administered dialysate formulations and strategies to increase the functionality of PD whenever pertinent (**Figure 2.1**). The encompassed indications tested for PD treatment have been classified as either historically relevant, currently relevant or as potential future applications. The use of PD in ESRD as well as the peritoneal route for the administration of drugs have been discussed in depth in recent reviews [87,99–101] and will not be covered here.

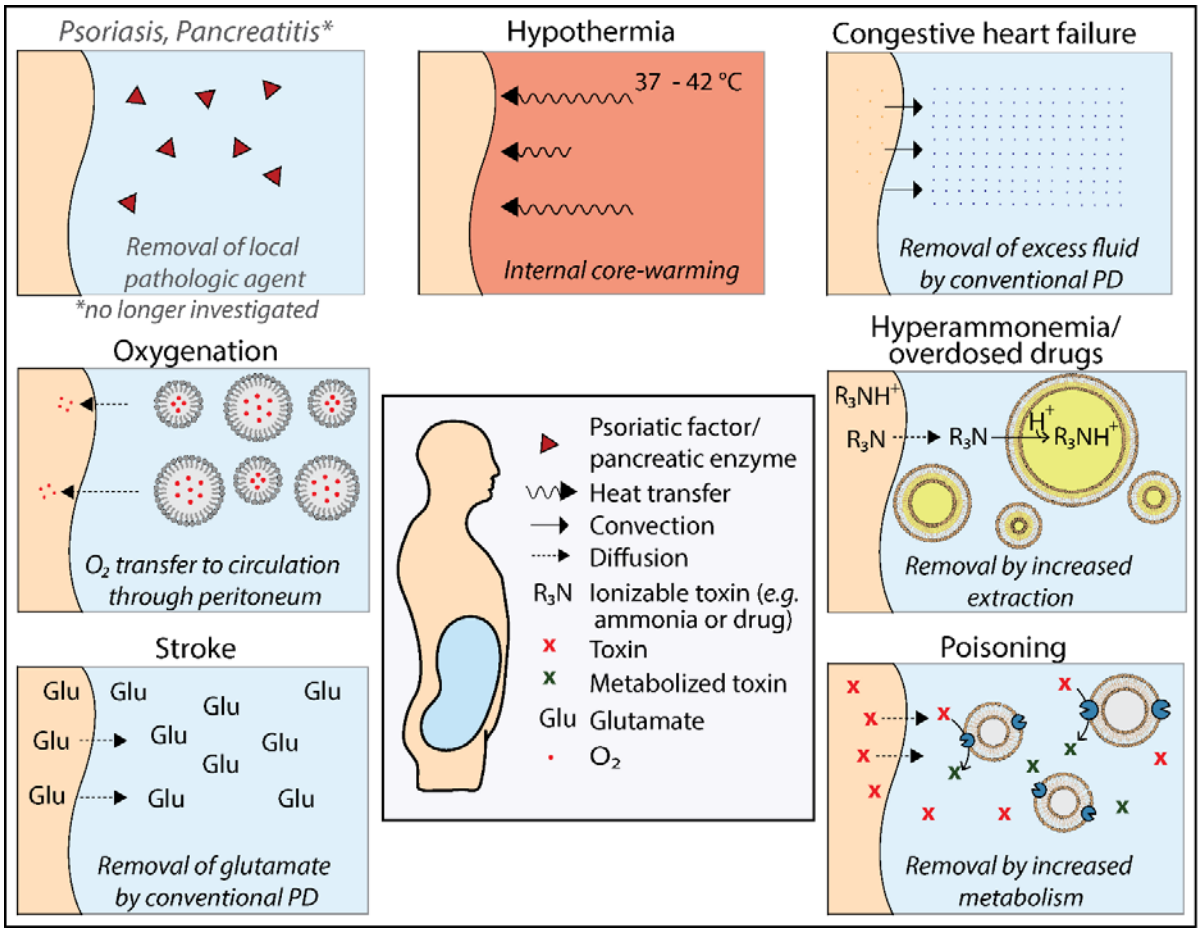

Figure 2.1. Overview of PD's role during the management of non-renal indications. Even though PD is no longer utilized as a therapeutic measure for psoriasis and pancreatitis, it had been suggested to act by clearing so-called psoriatic factors or trans-/exudated pancreatic enzymes from the peritoneal cavity. In cases of severe hypothermia dialysate fluids warmed to 37 – 42 °C can be administered for internal core-warming. During the management of congestive heart failure fluid overload is combated by removing excess fluid by convection driven by an osmotic force. HA and poisoning can be managed by conventional PD where endogenous and exogenous toxins are removed by diffusion. The functionality of these formulations can be augmented by supplementing conventional PD fluids with transmembrane pH-gradient liposomes to concentrate ionizable compounds within the liposomal core, as is shown in the HA/overdosed drugs panel. Alternatively, enhanced toxin metabolism can be achieved by supplementing PD fluids with liposomes loaded with specific toxin metabolizing enzymes, as is depicted in the poisoning panel. Conventional glucose-based PD solutions have been shown to effectively reduce the infarct volume by increasing the brain-to-blood efflux of glutamate. The peritoneal cavity can be used as an oxygenation organ by administering dialysate solutions constituting of OMBs.

2.2. Indications historically tested for treatment with conventional PD

2.2.1. Psoriasis

The therapeutic benefit of dialysis during the management of psoriasis was first described in the late 1970s and 1980s following the spontaneous improvement of psoriatic lesions in comorbid patients undergoing dialysis [102–104]. These observations prompted the initiation of clinical studies to further elucidate the underlying mechanism. A higher therapeutic benefit was accredited to PD compared to HD [105,106], which was further improved with multiple dialysate exchanges [103]. The underlying mode of action was hypothesized to involve the clearance of a psoriatic factor by dialysis (**Figure 2.1**) [107].

Glinski *et al.* suggested the dependence of psoriatic lesions clearance on the removal of peritoneal polymorphonuclear leukocytes (PMNL), which were found to carry high levels of serine proteases capable of destructing the stratum corneum [107,108]. A follow-up study by the same group confirmed the regression of psoriatic lesions by PD and leukopheresis, a procedure by which leukocytes are separated from the blood, supporting the involvement of cleared PMNLs [109]. However, when performed by another group, only moderate improvements of psoriatic lesions could be detected with leukopheresis [110]. Conversely, recent reports have described the emergence of psoriasis in patients undergoing PD for renal insufficiency [111]. To date, the connection between psoriasis and PD remains elusive, as well as the mechanism of action underlying the potential beneficial effects, hindering the use of PD as a standard modality of care for this indication [112].

2.2.2. Acute pancreatitis

Pancreatitis is an acute inflammation of the pancreas leading to the release and activation of pancreatic enzymes such as trypsin, which can enter the peritoneal cavity by trans- or exudation [113,114]. The accumulation of such compounds in the peritoneal fluid could potentially be counteracted by PD (**Figure 2.1**) [113]. Indeed, improvement of pancreatitis was documented

in comorbid patients undergoing PD as early as 1965 [115]. This initial report was followed by anecdotal case reports covering small groups of patients [116,117], and by animal studies backing the use of PD for the management of acute pancreatitis [118,119].

Table 2.1 provides an overview of the prospective randomized trials performed in the 1980s and 1990s with divergent results. Only two studies favored treatment of pancreatitis with PD [120,121], while the majority demonstrated a lack of clinical improvement [122–127], even when a trypsin inhibitor aprotinin was included in the dialysate [113,128]. Various meta-analyses and systematic reviews have concluded that PD is not associated with significant clinical benefit in this indication [129–131], as reflected in some guidelines [132]. However, opposite views have emerged recently, based on a specific animal model [133] or on clinical studies comparing clinical variables pre- and post- PD treatment, without an appropriate control group [134,135].

Finally, in a recent 10-year national cohort study, PD was shown to be associated with an increased risk for acute pancreatitis [136]. This was based on the fact that its occurrence was observed more frequently during PD than HD [137–139], with a yearly incidence of 148 or 32 per 100'000 [138], respectively. Although the link remains to be established, it has been suggested that acute pancreatitis may be a rare but serious complication of icodextrin-based PD [140].

Table 2.1. Clinical trials evaluating the influence of PD on morbidity and mortality rate in patients with acute pancreatitis.

Publication	No. of centers	Intervention, duration, fluid exchange rate	Control	Mortality, intervention group, (%)	Mortality, control group, (%)
Ranson *et al.*, 1978 [141]	2	PD, 2 – 4 days, 2L/h	Supportive measure or Operative procedures	7/24, (29)	10/61, (16) or 12/18, (67)
Stone et al., 1980 [120]	1	PD, > 1 days, 1 L/2 h	Supportive measures	5/34, (15)	9/36, (25)
Cooper *et al.*, 1982 [123]	3	PD, 3 days, 2 L/h	Supportive measures	3/9, (33)	7/14, (50)
Balldin *et al.*, 1983 [113]	1	PD[1], 2.5 days (mean), 2 L/2 h	PD, 2.7 days (mean), 2 L every 2 h	0/26, (0)	3/29, (10)
Kivilaakso *et al.*, 1984 [122]	1	PD, 7 – 12 days, 1 L/h	Pancreatic resection	8/17, (47)	14/18, (78)
Mayer et al., 1985 [124]	3	PD, 3 days, 2 L/h	Supportive measures	12/45, (27)	13/46, (28)
Ihse *et al.*, 1986 [125]	1	PD, 4 days, 1 L/h	Supportive measures	4/19, (21)	1/20, (5)
Teerenhovi *et al.,* 1989 [126]	1	PD, 4 – 12 days, 1 L/h	Drainage, Supportive measures	4/12, (33)	2/12, (17)
Ranson *et al.*, 1990 [127]	2	PD, 7 days, 2 L/h	PD, 2 days, 2 L/h	2/14, (14)	3/15, (20)
Schröder *et al.*, 1991 [121]	1	PD, 8 – 12 days, 1 L/h	Pancreatic resection	1/10, (10)	3/11, (27)
Berling *et al.*, 1998 [128]	4	PD[1], 1.3 days, 7 L/30 h	PD, 1.3 days, 7 L/30 h	4/26, (15)	4/22, (18)

[1] Aprotinin included in PD fluid

2.3. Indications currently being investigated for treatment with conventional PD

2.3.1. Hypothermia

Hypothermia is defined as a condition with abnormally low core temperatures (≤ 36°C) [142] and is caused by prolonged exposure to low temperatures and/or drug poisoning. Patients suffering from hypothermia are typically managed by passive techniques, such as removal of wet clothes and covering with warm blankets [142]. Active, internal core-warming is only indicated in severe cases [142]. PD-based internal core-warming was first reported by Lash *et al.* in 1967 [143], and was followed by multiple reports describing body temperature increase by a rapid exchange of warmed PD fluids (37 – 42 °C, **Figure 2.1**) [144–149]. As water and solutes do not need to be extracted for hypothermia treatment, the inclusion of an osmotic agent in dialysate formulations is not essential. This is exemplified by the fact that besides glucose-based solutions [145,150,151], saline or isotonic fluids have been frequently applied [146,147,152,153]. The application of PD for the management of hypothermia is longstanding; however, reports are anecdotal and its functionality has yet to be validated in randomized, controlled clinical trials. Although the enhanced benefit of PD-mediated over external warming has been demonstrated in dogs [154], the effectiveness of PD in humans compared to spontaneous rewarming has to the best of our knowledge only been investigated in one report, where a patient hospitalized twice for hypothermia showed similar recovery times with either treatment modality [147].

Systemically applied internal core-warming techniques such as HD are said to be more effective than PD [151]; however, the latter is preferred in hemodynamically instable patients, implying that the management of severe hypothermia should be assessed individually and be adapted to the patient's hemodynamic status [155]. Furthermore, when comparing the efficacy of dialysis, the rate of dialysate exchange and temperature should be factored. Whilst the number of reports describing the use of PD for core rewarming has declined since the beginning of the millennium, PD may still be relevant for this indication as it continues to be listed as an active internal warming technique in the 2010 European guidelines [156].

2.3.2. Congestive heart failure

Congestive heart failure (CHF) is a condition in which the heart is incapable of maintaining an adequate blood flow. Resulting fluid overload is typically well-managed by diuretics, with the exception of a small subset of patients resistant to medication [157–159]. Refractory CHF is often accompanied by renal dysfunction, which can cause salt and water overload as well as azotemia [157,158]. Over the past decade, reports have emerged describing supportive treatment of refractory CHF patients with PD or HD as a means to remove the excess fluid. During PD, water and solutes cross the peritoneal membrane either by diffusion along the concentration gradient or by convection which is driven by osmotic force (**Figure 2.1**) [158,160,161].

Patients suffering from refractory CHF are known to benefit from fluid removal which can ultimately lead to improved diuretic responsiveness and quality of life [158,162]. Two recent systematic reviews [163,164] of the literature comprising a total of 471 or 673 refractory CHF patients treated with PD and documented through 2003 – 2013 or 1954 – 2014, respectively, pointed to a clear improvement of symptoms linked to fluid overload, quality of life and decline in hospital days. The effect on survival, however, could not be extracted [163,164]. Comparisons of dialysis modalities suggest that HD is more effective in alleviating acute volume overload in severe cases, albeit PD may still present a viable ambulatory treatment as a bridge- or palliative therapy as it is also associated with less hemodynamic variations [158,162]. At the same time, the way a modality (*e.g.* high or low flux HD and continuous ambulatory or automated PD) is performed is said to be more important than the selected modality [165]. To date, large volume prospective randomized controlled trials are still lacking to assess the clinical effectiveness of PD for this patient population [159,163,164].

2.3.3. Hyperammonemia

HA is a rare condition manifested by pathologically elevated ammonia concentrations in the blood and can result from either inborn metabolic disorders or acute liver failure [166,167]. Primary and secondary HA are distinguished depending on whether urea cycle proteins are

directly affected as in primary HA or inhibited by metabolite accumulation or substrate deficiencies, caused by dysfunctional proteins outside the urea cycle as in secondary HA [168]. Ammonia is highly membrane-permeable and acute HA can lead to severe neurological impairment such as encephalopathy; therefore, upon detection of elevated ammonia levels (> 200 µM) protein catabolism should be counteracted by discontinuation of protein intake and initiation of glucose administration to provide caloric intake [169,170]. At ammonia concentrations greater than 300 – 400 µM, the time of intervention is crucial and dialysis should be initiated promptly to limit ammonia's neurotoxic effects and correct metabolic disturbances [168,170,171]. It is widely appreciated that PD provides less efficient ammonia removal than extracorporeal modalities such as HD [171–175] and as summarized in a recent in-depth review by Gupta *et al.* [167], larger cohorts generally point to a superior functionality with extracorporeal modalities [171,176]. The lower clearance capacity associated with PD is likely attributed to the architecture of the peritoneal membrane, which is composed of a multilayer of cells and tissue [71,177]. This poses a considerable distance for toxins to diffuse across, in contrast to the thin and highly porous filters applied during HD [71]. Current guidelines therefore recommend the use of extracorporeal modalities for the management of acute HA [169,170,167].

However, PD may be indicated as a bridge therapy until extracorporeal modalities become available [167] and may be employed in the absence of HD facilities in developing countries [178] or due to facilitated vascular access in neonates [56]. The latter is of particular importance considering that metabolic disorders are hereditary diseases typically noticed soon after birth. The ammonia removal by PD in both neonates with inborn metabolic diseases (such as urea cycle disorders or acidemia [178–180]) and cirrhotic adults with acute liver failure [181] has been demonstrated in single cases and retrospective studies when HD was either unavailable or not applicable. In such cases, the administered dialysate formulations mostly consisted of 1.5% dextrose [180] or 1.36% glucose buffered with lactate [178].

To address the low clearance capacity of PD, our group has recently developed PD fluids supplemented with transmembrane pH-gradient liposomes (850 nm – 10 µm), termed LSPD,

and has demonstrated its potential in a pre-clinical setting to scavenge small ionizable compounds such as ammonia within the dialysate [71]. Peritoneally administered liposomes exhibited a size-dependent exposure to the blood circulation (**Figure 2.2a**) [71,182]. Therefore, to prolong the residence in the peritoneal cavity, relatively large vesicles were required for this engineered formulation. Furthermore, the transmembrane pH-gradient allowed trapping of the compounds within the liposome core by ionization upon diffusion across the lipid bilayer (**Figure 2.1**) [69,183,184]. Capitalizing on the enhanced ammonia scavenging capacity of LSPD, the manufacturing process was subsequently optimized to allow larger scale production and the impact of the different LSPD constituents was closely examined to verify a dose-dependent ammonia extraction in healthy rats (**Figure 2.2b**) [72]. Efficacy was demonstrated in a rodent model of advanced cirrhosis with HA as manifested by a 10-fold enhanced dialysate ammonia removal compared to conventional PD, significantly reduced plasma ammonia levels and attenuated brain edema [72]. Furthermore, the absence of a hypersensitive reaction upon injection in pigs provided the first evidence for a safe application of LSPD in a pre-clinical setting [72]. Although further testing is required to fully assess its safety, these findings support the continued clinical development of LSPD.

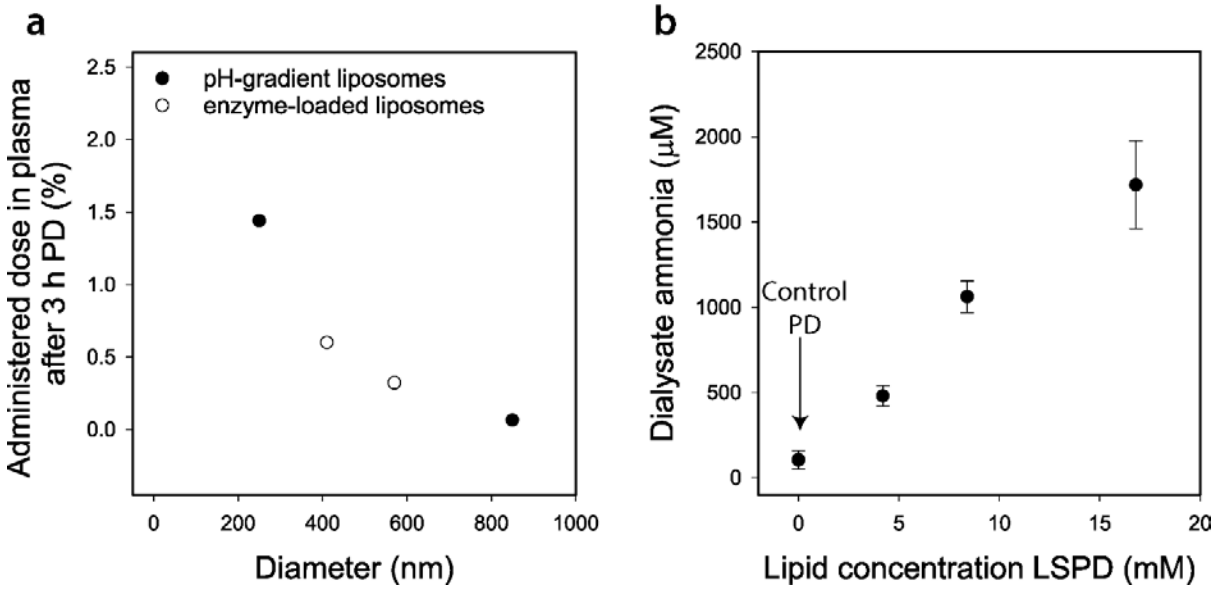

Figure 2.2. Characterization of liposome-based PD. (a) The exposure of liposomes in the blood circulation measured 3 h after PD initiation and expressed as the percentage of total administered dose is liposome-size dependent. Peritoneally leaked transmembrane pH-gradient liposomes were detected by fluorescently-labelled lipids and the enzyme-loaded liposomes by fluorescently-labelled enzymes. Adapted from Forster *et al.* [71] and Pratsinis *et al.* [182]. **(b)** The *in vivo* ammonia uptake of transmembrane pH-gradient liposomes, measured 4 h after PD initiation, correlates well with applied lipid dose. Adapted from Agostoni *et al.* [182].

2.3.4. Poisoning

In the context of drug overdose, certain xenobiotics and their metabolites can be removed from the body by extracorporeal modalities. This primarily applies to low-molecular weight, hydrophilic compounds that can reach sufficient concentrations in the blood to produce an adequate concentration gradient between the blood and dialysate [185]. Lithium, alcohols (*e.g.* ethylene glycol, methanol, ethanol), salicylates and anticonvulsants are the most common compounds cleared by HD [60,185]. The use of PD during the management of poisoning with xenobiotics is well-described [186–188] but it is not broadly indicated due to its lower clearance capacity compared to HD [60,188,189]. The clearance of ethanol and theophylline has been reported to be nearly 4- and 10-fold lower with PD than HD [20,190]. However, definitive clearance differences depend on the pharmacokinetic properties of the drug in question, the applied dialysate exchange volume and rate, and the patient's hemodynamic state [59]. The

reduced clearance capacity of PD has been largely acknowledged, leading to a decline of reported cases and prompting its removal from the National Poison Data System in 1993 [60]. As a result, the use of PD for poison management is currently only advocated in patients already undergoing PD for kidney failure treatment, in neonates and in cases where the effect of poisoning is of marginal severity and/or more efficient modalities are unavailable [59,61,189].

Having recognized the potential of PD for poison management and the advantage of administering antidotes *via* this route with reduced systemic exposure [191], recent efforts have focused on increasing PD performance by introducing lipid-based emulsions or functionalized liposomes into conventional dialysate solutions. Almost a decade ago Harvey *et al.* demonstrated the potential of lipid-based emulsions included in dialysate formulations, where a nearly 10-fold enhanced clomipramine extraction was achieved compared to saline-based PD [192]. More recently, our group included enzyme-loaded liposomes in a peritoneal dialysate formulation for the model indication acute alcohol poisoning, resulting in significantly elevated local ethanol metabolism in rats (**Figure 2.1**) [182]. This demonstrated that the peritoneal cavity could be utilized to perform chemical reactions, and future efforts should focus on increasing the systemic alcohol clearance of this enzyme-based formulation. The scavenging capacity of transmembrane pH-gradient liposomes described (**Figure 2.1**) in section 2.3.3 was also examined for ionizable drugs such as verapamil, propranolol, amitriptyline, haloperidol and pentobarbital [71]. As with ammonia, a significantly elevated extraction of drugs was evidenced with LSPD, resulting in a 1.5- to 130-fold increased uptake over conventional icodextrin PD [71]. Furthermore, the hypotensive action of verapamil in a rodent model of overdose was reversed more rapidly with LSPD with a recovery time of 6.5 h in contrast to 21 h with conventional icodextrin PD [71]. While this platform is currently being advanced for the primary indication of HA, future developments may broaden its application to the management of poisoning in a clinical setting.

2.4. Potential future utilization of conventional PD

2.4.1. Stroke

The proposed utilization of PD for the management of acute stroke is possibly one of the most exciting developments in the field of PD [193]. Glutamate is an abundant neurotransmitter that plays a key role during the course of acute stroke and while its intracellular concentration is relatively high (10 mM), its extracellular level in the brain is kept in the low micromolar range (< 2 μM) [194]. Even if plasma glutamate concentration lies between 30 and 100 μM [194,195], influx from the circulation is prevented by the blood-brain-barrier [196]. The concentration gradient is enabled by the confined compartmentalization and is maintained by energy-dependent transporters [196]. In the event of ischemic stroke, the energy shortage disrupts glutamate homeostasis [196]. The uncontrolled release of glutamate leads to excitotoxicity, causing apoptotic death of neurons and generation of reactive oxygen species, ultimately leading to irreversible brain damage. Extracellular glutamate is then transported to brain endothelial cells and when its concentration there exceeds that of the blood, it is released into the bloodstream [195].

Current management of acute ischemic stroke includes thrombolysis by administration of fibrinolytic recombinant tissue plasminogen activator and/or endovascular thrombectomy [193,197,198]. Plasma glutamate concentrations can be lowered by blood-resident glutamate scavenging enzymes. By administering their co-substrates oxaloacetate or pyruvate in excess, it is possible to augment the rate of brain-to-blood efflux and reduce cerebral extracellular glutamate concentrations [199]. Effective glutamate scavenging has actually been observed during PD in patients undergoing treatment for chronic kidney failure [200,201]. Patients exhibited a 20 to 45% reduction in blood glutamate after 1 h [200] to 4 h [201] with a concomitant increase of glutamate in the dialysate over time (**Figure 2.3a**), supporting the rapid and efficient exchange between the blood and dialysate.

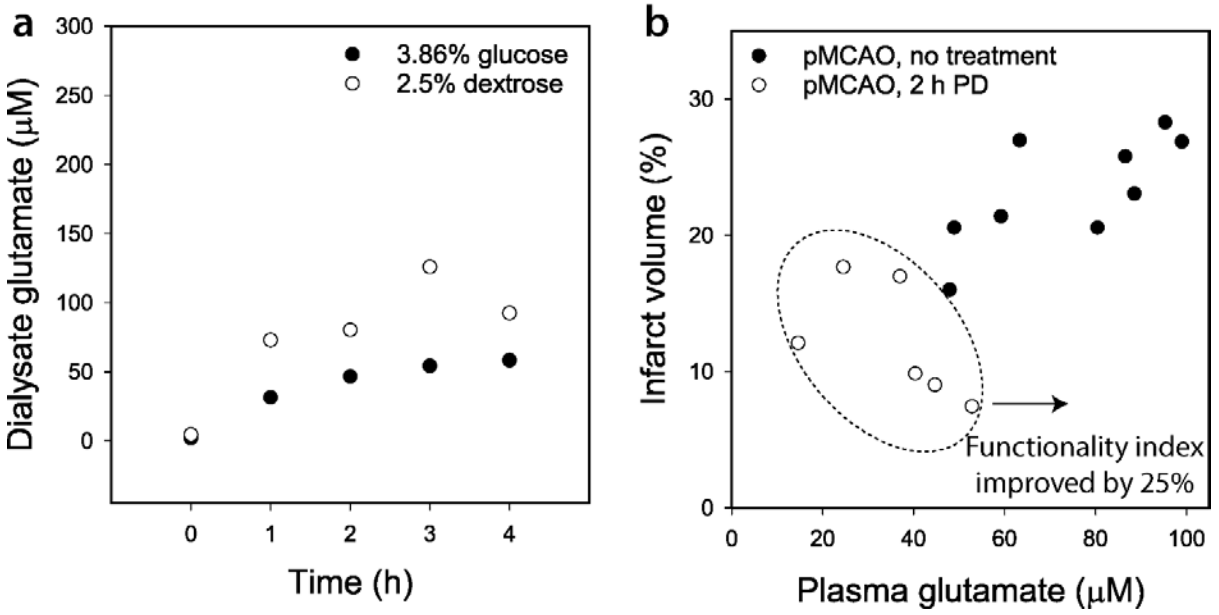

Figure 2.3. Removal of glutamate by PD during acute stroke. (a) The dialysate glutamate concentration measured in chronic kidney failure patients receiving Physioneal™ (3.86% glucose [201]) or Dialine™ (2.5% dextrose [200]) demonstrates effective glutamate removal by PD in two independent studies. Adapted from del Carmen Godino *et al.* [201] and Rogachev *et al.* [200]. **(b)** Rats subjected to acute stroke induced by pMCAO and treated with conventional PD demonstrated reduced plasma glutamate, which correlated with lowered infarct volume. This ultimately improved the functionality index by 25% compared to the non-treated control. Adapted from del Carmen Godino *et al.* [201].

A study by del Carmen Godino *et al.* demonstrated the potential of conventional PD therapy to minimize brain damage following ischemia in rats, by lowering the extracellular glutamate level *via* enhanced brain-to-blood efflux (**Figure 2.1**) [201]. Ischemia was induced by permanent middle cerebral artery occlusion (pMCAO), after which plasma glutamate levels steadily increased to a concentration 2-fold greater than the basal levels during the first 4.5 h. Dialysate administered intraperitoneally either 2.5 or 5 h following pMCAO, in alignment with typical emergency scenarios, effectively reduced the infarct volume. Furthermore, initiation of PD 2.5 h after pMCAO significantly lowered the plasma glutamate levels, which ultimately correlated with the infarct volume (**Figure 2.3b**) [201]. Interestingly, when the PD fluid was supplemented with 400 μM glutamate the reduction of plasma glutamate and infarct volume was abrogated, yielding comparable values to those of untreated control animals. The functionality of the salvaged tissue was verified 14 days later by functional magnetic resonance

imaging and limb-use asymmetry test for behavioral testing, both of which indicated that functional loss provoked by ischemia can be partially prevented by PD. This study was welcomed by the PD community, as evidenced by a number of commentaries and short reviews [193,195,196,202,203].

As for PD, reduced plasma glutamate has also been observed in patients with chronic kidney failure undergoing HD [204]; however, the haemodynamic instability and concomitant use of anticoagulants during HD may limit its application in ischemic stroke [193]. On the other hand, the hemodynamic stability of PD is an advantage and PD may be applicable to both ischemic and haemorrhagic stroke, as blood glutamate levels are elevated in both conditions. In comparison to glutamate scavengers, dialysis provided longer-lasting reduction and possibly also a higher safety profile [195]. Currently, a Phase II clinical trial (EuraCT 2012-000791-42) investigating the application of PD in acute stroke is in progress [205].

2.4.2. Oxygenation

Extracorporeal membrane oxygenation is achieved by means of a blood gas exchange device [206] and serves as an oxygenation modality for patients suffering from severe respiratory failure, but given its high invasiveness and association with several complications, it is only used as a last resort [207,208]. One of the common complications is thrombosis, and while its occurrence can be prevented by administering anticoagulants, the use of the latter is associated with increased risk of intracranial hemorrhage [207].

Alternatively, peritoneal oxygenation has been suggested as a less invasive therapeutic modality that would spare the need for anticoagulants. Indeed, the peritoneal cavity has long been suggested as an oxygenation organ [209], owing to its relatively large peritoneal exchange area, *i.e.* the density of perfused capillaries in the peritoneal membrane, lining the peritoneal cavity [80,160]. The most straightforward oxygen (O_2) delivery approach is peritoneal ventilation, which consists in mechanical ventilation of the peritoneal cavity with gaseous O_2. It was first successfully demonstrated in dogs in the late 1920s [209] but its efficacy has remained controversial to date [210–212]. The technique has been used more recently in a

rabbit model of asphyxia, yet the difference was marginal in terms of mean survival time (6.5 min with peritoneal ventilation compared to 5.0 min in the no treatment control group) [213]. In an attempt to increase the efficacy of peritoneal O_2 delivery, the administration of oxygenated perfluorocarbons [214–217] and red blood cells or liposome-encapsulated hemoglobin [206,218] has been investigated in animal models. While the arterial O_2 tension was increased in rabbits by administration of oxygenated perfluorocarbon emulsions (20% Fluosol-DA or FC_{43}) [214,215], contradictory results were seen in dogs [216,217]. In a more recent study performed in hypoxic pigs, oxygenated perfluorocarbons yielded clinically relevant increases in arterial O_2 tension. However this increase was distinctly lower than what is typically achieved with extracorporeal membrane oxygenation [219].

The most recent development in the field of peritoneal O_2 delivery involves peritoneal administration of O_2 microbubbles (OMB), which are micrometer-sized pure O_2 bubbles stabilized by a lipid monolayer composed of 1,2-distearoyl-sn-glycero-3-phosphocholine and polyoxyethylene-40 stearate at a 9:1 molar ratio (**Figure 2.1**) [220,221]. In a rat model of lung injury induced by right pneumothorax, the peritoneal oxygenated saline-treated animals died within 30 min, whereas the animals treated with peritoneal OMBs survived for at least 2 h, presenting an impressive 6.5-fold increased survival time (**Figure 2.4a**) [221]. However, when this system was investigated in a rabbit model of asphyxia, the survival time of the OMB treated group was 12.2 min, yielding a merely 1.7-fold increase in survival time compared to the saline-control (solid lines, **Figure 2.4b**) [222].

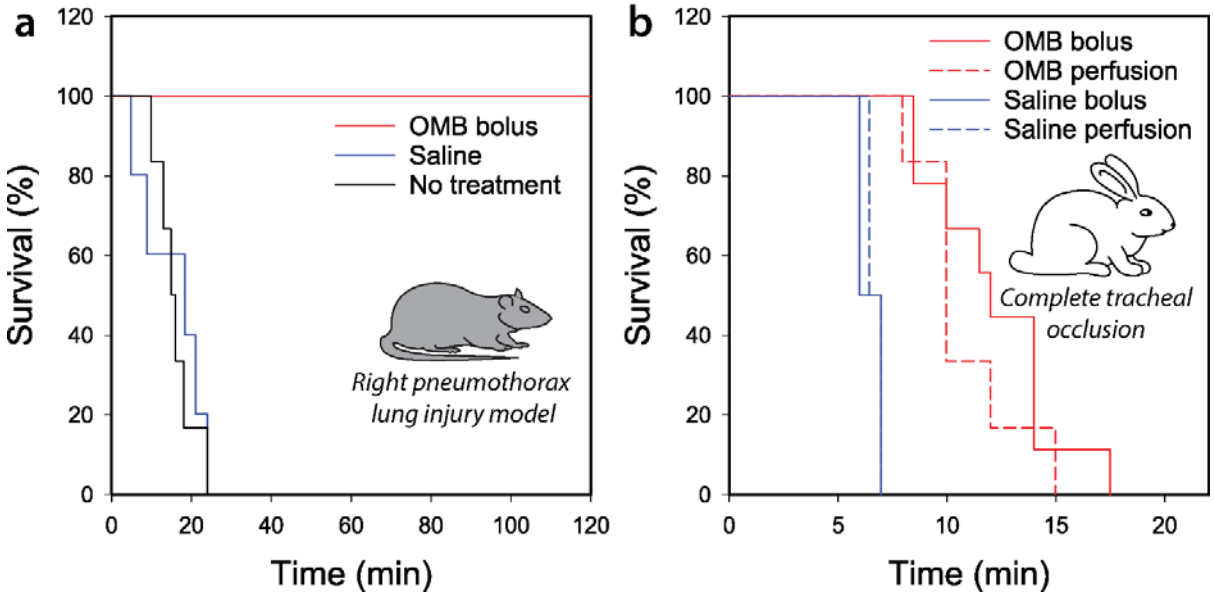

Figure 2.4. Efficacy of peritoneal oxygenation using OMBs. (a) Rats subjected to lung injury by right pneumothorax and treated with OMBs survived for at least 2 h whereas the saline-treated control died within the first 30 min. Adapted from Feshitan *et al.* [221]. **(b)** Peritoneally administered OMBs, investigated in a rabbit model of asphyxia induced by complete tracheal occlusion, prolonged survival by 1.7-fold. The survival was comparable when formulations were administered by an intraperitoneal bolus injection (solid lines) or by continuous application (perfusion, broken lines). Adapted from Legband *et al.* [222].

In the above experiments, the OMBs were administered by an intraperitoneal bolus injection [221,222]. In an attempt to increase the efficacy of OMBs by continuous application, termed perusion, fresh OMBs were continuously administered to the peritoneal cavity and subsequently removed through a scavenging port during the treatment, however prolonged survival could not be achieved (broken lines, **Figure 2.4b**) [222]. The reduced oxygenation efficacy detected in this follow-up study was largely ascribed to the complete asphyxia as well as the scale-up from rats to rabbits, where the blood flow through the splanchnic circuit is lower, yet more comparable to humans [222]. Given the exploratory status of this research, current reports are limited to pre-clinical testing. In view of the above listed constraints, it seems to be of utmost importance to validate such delivery systems in animal models with comparable ratios of peritoneal surface area-to-blood volume.

It is worth noting that the dialysate formulations investigated for peritoneal oxygenation were administered at a comparatively high volume, ranging from 200 to 300 mL/kg [206,221,222]. Furthermore, these fluids typically did not contain specific osmotic agents, as the fluid exchange for removal of noxious agents is in this case irrelevant. These deviations in dialysate formulation reflect the primary intention of the employed dialysate fluids as a means of O_2 delivery rather than removal of toxins or fluids, as is typically the case in most applications of PD.

2.5. Conclusion

Peritoneal dialysis is a well-established and longstanding technique mostly applied as renal replacement therapy in patients with ESRD. In the past years, PD has been exploited for various non-renal indications with contrasting results. For some indications, such as acute pancreatitis and psoriasis, PD could not be associated with a clear benefit and, in view of the emergence of potent drugs or modalities, its role for such indications has faded. On the other hand, PD appears to be of potential therapeutic value in specific conditions such as hypothermia, HA, drug poisoning and congestive heart failure. However, even for these indications, a clear assessment of PD's efficacy through prospective randomized trials is lacking, and extracorporeal modalities still prevail in acute settings. The emergence of innovative nano/micromedicine-based technologies (*e.g.* LSPD and OMBs) has fueled the rejuvenation of PD in the recent years. By supplementing conventional PD solutions with engineered liposomes the extraction capacity for a variety of ionizable endogenous and exogenous toxins could be enhanced substantially. At the same time, dialysate fluids consisting of OMBs have demonstrated relevant O_2 delivery. These advances open new possibilities to broaden the application scope of PD. Interestingly, recent preclinical evidence suggests that conventional PD may be a promising strategy to minimize brain damage during acute stroke. Still the clinical safety of conventional PD for novel indications and adapted nanomedicine-based PD formulations has yet to be established. Given that currently approved dialysis solutions can be employed for the management of acute stroke, this development has the potential for a rapid

translation to the clinic. Nonetheless, detailed clinical studies are still required to determine whether PD can provide superior efficacy over currently employed pharmacologic interventions.

Future developments may involve clinical investigation of catheters coated for reduced bacterial adhesion or with antimicrobial activity, enabling improved functionality with a reduced risk of peritonitis [223,224]. Adaptations of PD may also include mechanical effects on the peritoneal cavity (*e.g.* shaking or vibration), thereby enhancing exchange of small solutes for maximal efficacy [225,226]. Furthermore, the administration of specific antidotes in the peritoneal dialysate formulation may be further investigated. This is particularly appealing as the large volumes administered intraperitoneally during PD enable a relatively high dosing and the peritoneal confinement reduces systemic exposure micrometer-sized carriers, which overall facilitate the removal of antidotes [71,182,191]. This may for example involve cell membrane-mimetic particles to decoy intraperitoneal bacterial pathogenic toxins [227,228]. As exogenous chemical reactions can be carried out within the peritoneal cavity following administration of enzyme-loaded liposomes [182], a similar scaffold bearing engineered butyrylcholinesterase to enhance the cocaine metabolism [229] may be envisioned. While the bio-distribution of such nano- and microcarriers has been investigated as a function of size and composition during the peritoneal dwell [71,79,230], more in-depth studies are required to investigate the fate of the non-recovered carriers following dialysate removal as well as their efficacy following multiple administrations and potential immune response. Another crucial aspect lacking comprehensive studies is the integrity of such colloidal carriers after intraperitoneal administration. A thorough understanding of these parameters should be acquired to optimize and simplify the development of such formulations for increased translational potential.

Chapter 3

Liposome-supported enzymatic peritoneal dialysis, optimization and *in vitro* characterization

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3.1. Introduction

The abuse of alcohol is one of the leading risk factors for premature deaths or disabilities worldwide [1]. Typical symptoms of ethanol poisoning include metabolic, gastro-intestinal, behavioral, cardiac and pulmonary disorders [22]. If left untreated, severe intoxication can lead to death mainly due to respiratory depression [22,24,26] or hypoglycemia, especially in young children [231]. Ethanol is most effectively removed by dialysis [46] owing to its size and high water solubility [51]. Patients presented with severe coma or elevated blood alcohol levels are treated with HD, where the blood is dialyzed extra-corporeally against a semipermeable membrane [48,189]. This is, however, highly invasive and generally only offered in specialized centers. PD is a fairly simple, inexpensive and more convenient alternative to HD, though the latter is the method of choice due to its higher clearance capacity, with a roughly 2- to 4-fold enhanced ethanol removal compared to PD [20].

Ethanol is metabolized mainly in the liver by ADH to its primary, toxic metabolite acetaldehyde, which is further metabolized by ALDH under the reduction of NAD. Previous attempts to augment the ethanol metabolism during acute alcohol poisoning included the induction of endogenous enzymes with safrole [77] or intravenous enzyme injection [76]. The administration of safrole demonstrated improved ethanol and acetaldehyde clearance in an ALDH2-deficient mouse model by recruiting another ALDH enzyme to metabolize acetaldehyde [77]. Though safrole is known to be carcinogenic [232]. Recently, polymeric nanocomplexes of the alcohol metabolizing enzymes, AO and CAT, were assembled *via* an inhibitor-DNA scaffold to maximize the detoxification reaction by the close proximity of AO to CAT [76]. These sophisticated polymeric nanocomplexes were designed for intravenous injection, which prevents enzyme removal following detoxification, potentially increasing the risk of adverse reactions. While only changes in blood ethanol levels were reported for the nanocomplexes [76], it can be expected that acetaldehyde was accordingly elevated in the blood during the detoxification reaction.

Inspired by the AO and CAT nanocomplexes, we hypothesized that E-Liposomes administered as a PD suspension could locally enhance ethanol metabolism, limiting the enzymatic exposure and consequently that of acetaldehyde by allowing its removal from the

peritoneal cavity. As illustrated in **Figure 1.6**, circulating ethanol diffuses from the blood across the peritoneal membrane and into the peritoneal cavity. There, it is oxidized by AO (anchored onto a liposome support) to acetaldehyde and H_2O_2. The latter is then further degraded by liposome-anchored CAT to water and O_2. This chapter describes the optimization and subsequent characterization of E-Liposomes, loaded with either AO or CAT. These enzymes were hydrophobically modified to facilitate liposome anchoring. The resulting E-liposomes were compared to free enzymes in respect to *in vitro* stability and ethanol metabolism.

3.2. Materials and Methods

3.2.1. Preparation of E-Liposomes

N-(Succinimidyloxy-glutaryl)-L-α-dioleoylphosphatidylethanolamine (DOPE-NHS, NOF, Osaka, Japan) was dissolved in chloroform, dried under nitrogen flow and subsequently kept under vacuum for over 12 h, forming a thin lipid film. The film was solubilized in 1 – 2% m/v β-octylglucoside (Apollo Scientific, Cheshire, UK), 100 mM potassium phosphate (KPi), pH 7.0 to a final DOPE-NHS concentration of 8 mM. The DOPE-NHS was added to AO from *P. Pastoris* (10 – 40 U/mg) or CAT from bovine liver (≥ 30,000 U/mg) (Sigma-Aldrich, Buchs, Switzerland) at a final concentration of 3.5 g/L enzyme, 0.7 – 1% m/v β-octylglucoside, 100 mM KPi, pH 8.0 at a phospholipid:protein molar ratio of 316:1 or 100:1, respectively. Solutions were kept at 4 °C under constant rotation for 4 – 16 h, yielding hydrophobically modified AO and CAT (HmAO, HmCAT). Please see below for characterization by activity, reverse phase HPLC, amine titration and circular dichroism.

The HmAO and HmCAT in the detergent based solution were added separately to lipid films composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[azido(polyethylene glycol)-2000] (DSPE-PEG, both from LIPOID, Ludwigshafen, Germany) (98:2 mol%), prepared as described above. The ratio of initially added proteins to lipid remained constant at a mass ratio of 3:12.4, where the enzyme concentration was varied from 1 – 3 g/L and the lipid from 5 – 15 mM. For the preparation of AO-CAT-Liposomes, HmAO and HmCAT were added at an initial 3:1 mass ratio. The buffer

composition was 0.2 – 0.9% m/v β-octylglucoside, 100 mM KPi. Enzyme lipid mixtures were subjected to three freeze-thaw cycles and were dialyzed for 36 h against 100 mM KPi, pH 7.4 using 1,000 kDa MWCO Float-A-Lyzers (Spectrum Labs, Breda, Netherlands) with three changes of buffer to remove unbound enzymes and detergent, generating E-Liposomes. Forty mM sodium chloride (NaCl) were added to E-liposomes intended for *in vivo* administration. A scheme of the AO- and CAT-Liposomes is shown in **Figure 3.1**. Please see below for characterization by residual activity, discontinuous sucrose density gradient and dynamic light scattering.

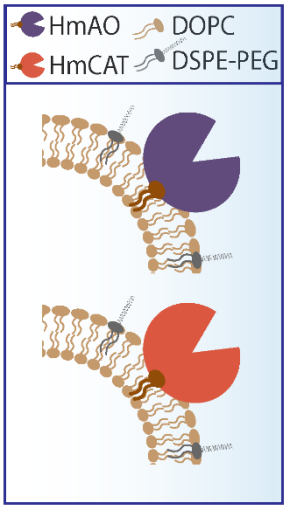

Figure 3.1: Scheme of E-Liposome constituents. E-Liposomes are composed of DOPC:DSPE-PEG (98:2 molar ratio) and loaded with either AO or CAT. The enzymes were hydrophobically modified with DOPE-NHS yielding HmAO and HmCAT.

3.2.2. Characterization of hydrophobic modification

3.2.2.1. Activity

The activity of AO was measured by incubating the enzyme (0.01 µg/mL) at room temperature with 100 mM methanol, 100 µM Amplex Ultra Red (AUR, $\lambda_{Ex/Em}$: 490/585 nm, Thermo Fisher Scientific, Waltham, MA) and 1 U/mL horseradish peroxidase (≥ 225 U/mg, Sigma-Aldrich) in 100 mM KPi, pH 7.4. The H_2O_2 production was monitored over a period of

5 min by AUR fluorescence, using an M200 infinite plate reader (Tecan, Männedorf, Switzerland) and the specific activity quantitated using the following equation (1).

$$Specific\ activity\ (U/mg) = \frac{\Delta H_2O_2\ (\mu M) * volume\ (L)}{time\ (min) * amount\ (mg)} \quad (1)$$

The activity of CAT was determined by incubating CAT (0.05 µg/mL) with 10 mM H_2O_2 in 100 mM KPi (pH 7.4) at room temperature and the H_2O_2 decomposition monitored by the change in absorbance at 240 nm over a period of 1 min using an M200 infinite plate reader (Tecan). Equation 1 was applied to quantify the specific activity. The specific activity of the hydrophobically modified enzymes was normalized to that of the native, non-modified enzymes, set to 100%.

3.2.2.2. Reverse phase HPLC

The elution profile of HmAO and HmCAT was assayed by HPLC (Hitachi LaChrom Elite, Tokyo, Japan) equipped with a POROS R1/10 column (Thermo Fisher Scientific) using a method adapted from a previous publication [233]. Buffer A consisted of H_2O, 0.05% trifluoroacetic acid (TFA, Thermo Fisher Scientific) and buffer B of 90% acetonitrile (Sigma-Aldrich), 0.045% TFA. Proteins were eluted in a gradient from 20 to 90% buffer B in 8 min with a flow rate of 0.5 mL/min. The sample absorption was recorded at 220 nm and the column kept at 30 °C. Samples were injected within the linear range of 1 – 10 µg.

3.2.2.3. Amine titration

The degree of modification was determined by titration of the enzyme's primary amine groups using *o*-phthalaldehyde (Sigma-Aldrich). The *o*-phthalaldehyde reagent was freshly prepared as previously described [234] and 200 µL were transferred to 20 µL AO or CAT at a concentration of 200 µg/mL prepared in 50 mM carbonate (Merck, Darmstadt, Germany), pH 10 and incubated for 2 min prior to recording the fluorescence ($\lambda_{Ex/Em}$: 330/455 nm) at room

temperature, using an M200 infinite plate reader (Tecan). The number of modified amine groups was derived from the percentage of free amines normalized to the native enzyme, taking the total number of primary amines into consideration.

3.2.2.4. Circular dichroism

Circular dichroism measurements were performed at room temperature on a Chirascan spectrometer (Applied Photophysics, Surrey, UK) using a 1-mm path length quartz cuvette. Far UV scans (190 – 260 nm) were carried out with 1-nm step size and 1-nm bandwidth with a recording time of 0.5 s per step. Enzymes were buffer-exchanged into 10 mM KPi, pH 7.4 by gel filtration (Sephadex G-100, Sigma-Aldrich) and assayed at 0.11 mg/mL. The molar ellipticity [θ] [235] was calculated from the machine units (θ), path length of cell (L) and molar concentration (C) using equation 2. Scans of each native, non-modified and modified enzyme were averaged and the buffer background signal subtracted.

$$molar\ ellipticity\ [\theta]\ (deg * cm^2 * dmol^{-1}) = \frac{\theta(mdeg)}{L(mm)*C(M)} \quad (2)$$

3.2.3. Characterization of E-Liposomes

3.2.3.1. Recovery of E-Liposomes

Enzymatic activity of AO- and CAT-Liposomes, assayed within the linear range of H_2O_2, was determined as described above and normalized to the specific activity of free enzymes to quantify the protein loading. The activity was additionally measured in 1% w/v cholic acid (ABCR, Karlsruhe, Germany), 100 mM KPi, pH 7.4.

3.2.3.2. Discontinuous sucrose density gradient

The HmAO and HmCAT, purified using Sephadex G-100 gel filtration columns, and E-Liposomes were subjected to discontinuous sucrose density gradients, adapted from a previous protocol [236]. In brief, samples were diluted in a 60% w/v sucrose solution yielding a 45% w/v sucrose concentration (1 mL). This was overlaid with 1 mL of a 35% w/v-, 1.5 mL of a 25% w/v-

and 1.4 mL of a 15% w/v sucrose solution and finally covered with 100 µL of buffer. Gradients were centrifuged at 4 °C for either 4 h at 200,000 *x g* or overnight at 160,000 *x g* in a SW55ti rotor connected to an Ultima XE-90 instrument (Beckman Coulter, Brea, CA), and finally fractioned manually from the top. Protein abundance per fraction was assayed by normalizing the enzymatic activity per fraction over the total activity measured, set to 100%.

3.2.3.3. Dynamic light scattering

The hydrodynamic diameter was measured by dynamic light scattering (DelsaNanoC, Beckman Coulter) and derived from the cumulant method.

3.2.3.4. Estimation of interenzyme distance on liposomes

The interenzyme distance for AO and CAT loaded liposomes was estimated as described below from the number of enzymes per liposome and average liposome diameter [237], while assuming unilamellar spherical vesicles [238].

Following the purification of separately prepared E-Liposomes with HmAO and HmCAT, 31.4% AO activity (intact state, **Figure 3.6a**) and 45.9% CAT activity (disrupted state, **Figure 3.6a**) was observed when 3 mg/mL enzyme were added to 15 mM lipid. Taking the molecular weight of AO and CAT (675 kDa and 250 kDa, **Figures 3.2a, b**) into consideration, a molar protein:phospholipid (PL) ratio of 1:11,000 for AO and 1:2,700 for CAT was achieved. Based on the average hydrodynamic diameter ($d_{liposome}$, **Table 3.2**) and assuming a bilayer thickness of 3.75 nm [239], the total surface area (SA) of AO-Liposomes was 2,000,000 nm^2 and that of CAT-Liposomes 1,000,000 nm^2 (equation 3).

$$total\ SA\ = 4 * \pi * \left(\frac{d_{liposome}}{2}\right)^2 + 4 * \pi * \left(\frac{d_{liposome}}{2} - 3.75\right)^2 \quad (3)$$

Considering each PL occupies 0.67 nm^2, each vesicle contains 3,000,000 PL for AO-Liposomes and 1,600,000 PL for CAT-Liposomes (equation 4).[240]

$$\frac{total\ SA}{lipid\ area} = \frac{PL}{vesicle} \quad (4)$$

Multiplying the number of PL per vesicle by the protein:PL ratio gives the amount of protein per vesicle (equation 5), which is 280 for AO and 570 for CAT.

$$\frac{protein}{vesicle} = \frac{PL}{vesicle} * \frac{protein}{PL} \tag{5}$$

On the basis of the enzymatic activity of E-Liposomes measured in the intact and disrupted state (**Figure 3.6a**), AO is only located on the exterior of the liposome, indicated by the unchanged activity of AO-Liposomes when measured in both states. CAT-Liposomes on the other hand demonstrated increased activity upon liposome disruption, suggesting presence of CAT on both the interior and exterior of the liposomes. Therefore, when calculating the average SA allocated per enzyme, one bilayer is considered for AO (equation 6) and two bilayers are considered for CAT (equation 7), yielding 3,600 nm^2 and 1,800 nm^2 allocated per enzyme, respectively.

$$\frac{SA}{protein} = \frac{4*\pi*\left(\frac{d_{liposome}}{2}\right)^2}{\frac{protein}{vesicle}} \tag{6}$$

$$\frac{SA}{protein} = \frac{4*\pi*\left(\frac{d_{liposome}}{2}\right)^2 + 4*\pi*\left(\frac{d_{liposome}}{2}-3.75\right)^2}{\frac{protein}{vesicle}} \tag{7}$$

The free SA is determined by subtracting the SA per protein using the proteins' estimated diameter ($d_{protein}$, **Figures 3.2a, b**, equation 8), yielding 3,500 nm^2 for AO and 1,800 nm^2 for CAT.

$$Free\ SA = \frac{SA}{protein} - \pi * \left(\frac{d_{protein}}{2}\right)^2 \tag{8}$$

Finally the interenzyme distance is calculated by the square root of the free SA divided by π and multiplied by 2 (equation 9). The calculated interenzyme distance is approximately 67 nm for AO and 47 nm for CAT on separately prepared E-Liposomes.

$$interenzyme\ distance = \sqrt{\frac{Free\ SA}{\pi}} * 2 \tag{9}$$

3.2.4. *In vitro* stability

The AO, HmAO, CAT, AO-Liposomes and CAT-Liposomes (0.1 mg/mL of enzymes) in 100 mM KPi, pH 7.4 were kept at 37 °C for 3 h under gentle agitation. The measured residual enzymatic activity was normalized to samples from the same preparation kept on ice. The activity of AO and AO-Liposomes (0.005 µg/mL) in 100 mM KPi, pH 7.4 at room temperature was continuously measured in the presence of substrate, amplex ultra red and horseradish peroxidase over a period of 1 h in 5 min intervals. Activity was normalized to the initial activity recorded in the first 5 min.

3.2.5. *In vitro* metabolism of ethanol

Free enzymes or E-liposomes with a nearly equimolar ratio of AO (0.15 mg/mL) and CAT (0.05 mg/mL) were incubated with 40 mM ethanol in open tubes, to maximize O_2 delivery and maintained for 6 h at 37 °C in 100 mM KPi, pH 7.4, under gentle agitation. The ethanol metabolism of E-liposomes was additionally assayed in the presence of 40 mM H_2O_2. A control vial devoid of enzymes was included to assess the degree of evaporation. Samples were prepared at a total volume of 1 mL, and 40 µL aliquots were taken after 1, 2, 3, 4, and 6 h, and immediately heated to 95 °C for 10 min to stop the reaction. Samples were thereafter cooled on ice prior to centrifugation (10 min, 10,000 *x g*) and the supernatant collected for ethanol quantification by gas chromatography allowing adequate metabolite separation. Please see below for details on gas chromatography.

3.2.6. Gas chromatography

Ethanol and acetaldehyde concentrations were determined by gas chromatography using a flame ionization detector, as the metabolites could be readily separated (Please see appendix, **Figure A3.1**). This allowed accurate concentration determination. Samples were assayed by liquid split injection (GC-2014, Shimadzu, Kyoto, Japan or Focus GC, Thermo Fisher Scientific) coupled with a DB-5 (30 m x 0.25 mm x 0.25 µm, Agilent, Santa Clara, CA) or an Rtx-BAC1 column (30 m x 0.32 mm x 1.6 µm, Restek, Bellefonte, PA). Helium (Pangas, Dagmarsellen,

Switzerland) was selected as a carrier gas throughout all measurements. Ethanol metabolites and internal standards (1-butanol or isopropanol) were either separated using a temperature gradient (30 – 100 °C) or by isothermal elution (40 °C), depending on the selected column. Detailed information regarding injection- and detection temperature, split ratio and flow rate is summarized in **Table A3.1** of the appendix.

Samples were diluted 1:4 with an internal standard. Ethanol and acetaldehyde quality controls, derived from commercially available analytical standards (20, 100 and 300 mg/dL ethanol and 1 mg/mL acetaldehyde, all from Sigma-Aldrich) were included in each sample sequence to ensure accuracy and precision values were within specifications for chromatographic methods, according to FDA guidelines on bioanalytical method validation [241].

Calibration curves were based on the relative response of ethanol and acetaldehyde to the internal standard. Limit of detection (LOD) and quantitation (LOQ) were derived either from the standard deviation of the analyte response (Sy) and the slope (m) or from the baseline height (BL). The LOD and LOQ are defined as 3.3 x Sy/m and 10 x Sy/m or 3 x BL and 10 x BL. The LOD and LOQ values for ethanol and acetaldehyde are summarized in **Table A3.1**.

3.2.7. Statistical analysis

Statistical analysis was performed with Sigma Plot 13.0 (Systat Software, Chicago, IL) using a one way ANOVA followed by a Tukey test for pairwise comparison. A p-value smaller than 0.05 was considered significant.

3.3 Results and Discussion

3.3.1. Characterization of E-Liposomes

To assist the incorporation of AO and CAT (**Figures 3.2a, b**) onto liposomes, the enzymes were hydrophobically modified using N-hydroxysuccinimide-activated phospholipids solubilized in a buffered β-octylglucoside solution. The HmAO and HmCAT were subsequently loaded onto liposomes by detergent dialysis. For the current application, large liposomes (> 250 nm) [71] were required to allow their peritoneal retention. The detergent β-octylglucoside was selected over cholic derivatives, as β-octylglucoside allows large unilamellar vesicles to form and proteins are believed to be less susceptible to denaturation [242].

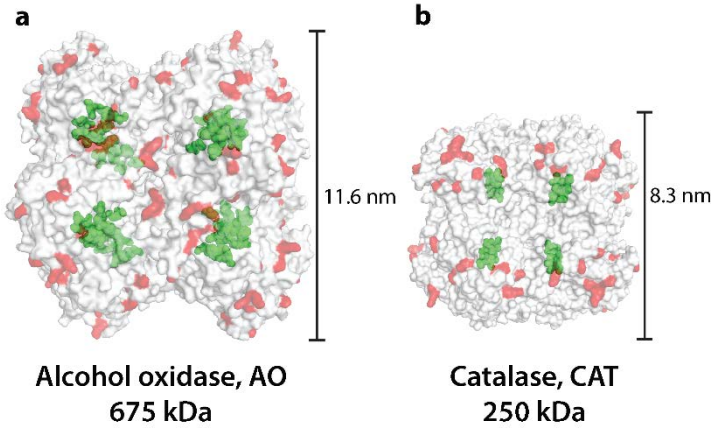

Figure 3.2: Three-dimensional structure of homooctameric AO (PDB 5HSA) and homotetrameric CAT (PDB 1TGU) [78,243]**, respectively**. Catalytic sites (green) are buried well within the protein core. Lysine residues (red), potential sites for covalent modification by NHS-activated lipids, are evenly distributed on the surface of the proteins. Enzymes are scaled to estimated diameter [244].

The optimal molar conjugation ratio of DOPE-NHS to enzyme was set to 316:1 for AO and 100:1 for CAT. This corresponds to a mass ratio of 0.46:1 for AO and 0.41:1 for CAT, which is within the range of previous acylation protocols ranging from 0.09 to 0.44:1 [245,246]. Effective hydrophobic enzyme modification was confirmed by reverse phase HPLC, as done typically for antibody drug conjugates [247], where most extensively modified subunits eluted later due to increased interaction with the solid phase of the column (green box in **Figure 3.3**). The degree

of modification was assessed by amine titration, with 80 ± 9% and 94 ± 3% residual unmodified primary amine groups following hydrophobic modification, resulting in 62 ± 9 and 7 ± 4 covalently modified amines per HmAO and HmCAT, respectively (**Figures 3.4a, b**). Despite the apparent difference, the conjugation efficacy was in fact comparable for both enzymes if the molecular weight and conjugation ratio are considered. A deviation from a stoichiometric conjugation could be explained by the rapid hydrolysis of the NHS ester in aqueous solutions [248], inhomogeneous solubilisation of DOPE-NHS in mixed micelles [249], as well as steric hindrance preventing accessibility of all primary amine groups.

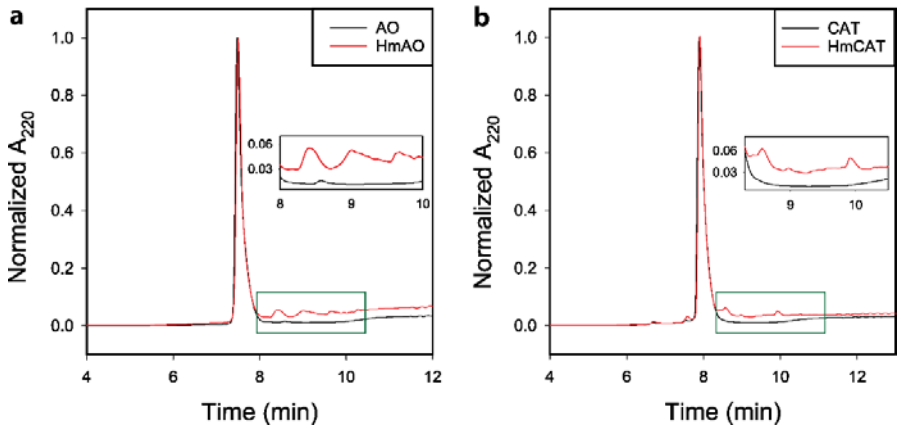

Figure 3.3: Separation of hydrophobically modified subunits by reverse phase HPLC for AO (a) and CAT (b). HmAO and HmCAT show additional peaks, highlighted with a green box, eluting at a higher retention time due to increased interaction of the acylated subunits with the reverse phase column. This region is expanded in the inset. The AO and CAT were conjugated at a DOPE-NHS to protein molar conjugation ratio of 316:1 and 100:1, respectively. A representative elution profile is shown for each enzyme.

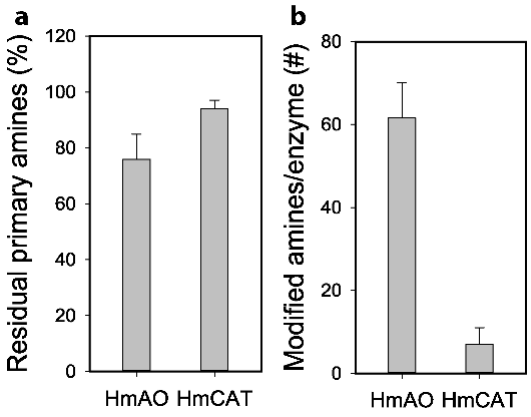

Figure 3.4: Quantification of hydrophobic modification. (a, b) The degree of modification was additionally assessed by amine titration using o-phthalaldehyde as DOPE-NHS reacts with primary amines. At a molar conjugation ratio of DOPE-NHS to enzyme of 316:1 for AO and 100:1 for CAT, 62 ± 9 and 7 ± 4 amine residues are coupled, respectively. Mean + SD (n = 3).

Figure 3.5a shows that the activity of modified enzymes (grey bars) was hardly impacted by the lipid coupling, as confirmed by the absence of significant differences and as previously reported in literature [250], where covalent linkage of CAT did not lead to any noticeable loss in activity. This is further supported by the observation that the circular dichroism spectra of the modified enzymes (broken lines) matched those of the non-modified (solid lines) for each enzyme, indicating that the secondary protein structure was unaffected by covalent modification (**Figure 3.5b**). In addition, the $\theta_{223\ nm}/\theta_{209\ nm}$ ratio from these UV spectra (**Table 3.1**) remained unaltered, quantitatively confirming the spectral observations. Conservation of the enzymatic activity and secondary structure following covalent modification may be explained by the abundance of surface-exposed lysine residues and the fact that active sites are well buried within the three-dimensional structure, protecting the catalytic activity (red residues, **Figures 3.2a, b**).

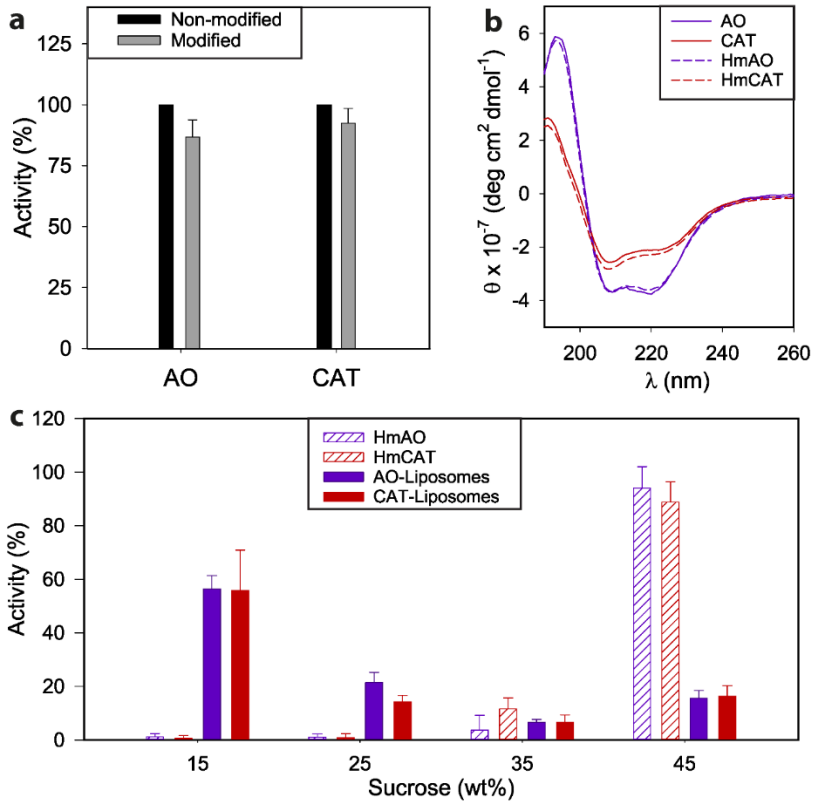

Figure 3.5: Characterization of hydrophobically modified enzymes and E-Liposomes. Enzymes were hydrophobically modified by DOPE-NHS amine coupling at a molar lipid to enzyme conjugation ratio of 316:1 for AO and 100:1 for CAT, yielding HmAO and HmCAT, and loaded onto DOPC:DSPE-PEG (98:2 mol%) liposomes by detergent dialysis. **(a)** Activity of HmAO and HmCAT (grey bars) and of non-modified enzymes (black bars). Mean + SD (n = 6). **(b)** Molar ellipticity of the non-modified (solid line) and hydrophobically modified enzyme (broken line) recorded by circular dichroism spectroscopy. Mean (n = 3). **(c)** Discontinuous sucrose density gradient analysis of hydrophobically modified enzymes with lipid bilayer (E-Liposomes, filled bars) compared to free HmAO and HmCAT (lined bars). Mean + SD (n = 4 – 5).

Table 3.1: Comparison of $\theta_{223\ nm}/\theta_{209\ nm}$ ratio between the non-modified and modified enzyme confirmed the secondary structure was not affected by hydrophobic modification. Mean ± SD (n = 3).

	$\theta_{223\ nm}/\theta_{209\ nm}$	
	AO	CAT
Non-modified	0.95 ± 0.07	0.82 ± 0.01
Modified	0.93 ± 0.03	0.81 ± 0.01

Hydrophobically modified enzymes solubilized by β-octylglucoside were added to lipid films composed of DOPC and DSPE-PEG at a molar ratio of 98:2, forming mixed micelles. Unbound enzymes and β-octylglucoside were removed by detergent dialysis, yielding E-Liposomes with a residual activity of roughly 30% of the originally added enzymes (**Figure 3.6a**). Lipid bilayer disruption in the presence of cholic acid increased the enzymatic activity of CAT-Liposomes to 45% (**Figure 3.6a**). As H_2O_2 does not readily penetrate the lipid bilayer [251], the increased activity upon bilayer disruption suggests that a portion of enzymes was located in the interior of the liposomes. On the other hand, the AO activity was not further increased by liposome disruption, possibly owing to the enzyme's larger size. Therefore, active CAT is presumably located on both the interior and exterior of the liposomes, while active AO is predominantly located on the surface. Physical association of the enzymes with the lipid bilayer was confirmed by discontinuous sucrose density gradient centrifugation (**Figure 3.5c**). The degree of unbound active enzyme in E-Liposomes prepared with HmAO and HmCAT was estimated to be lower than 20% (**Figure 3.5c**). When compared to E-Liposomes prepared with native AO and CAT, it becomes evident that hydrophobic modification is required for enhanced loading (**Figures 3.6a, b**). The vesicle size was relatively heterogeneous with an average diameter ranging from 410 to 570 nm (**Table 3.2**). Successful hydrophobic modification was demonstrated, allowing effective enzyme liposome loading for subsequent *in vitro* and *in vivo* examination.

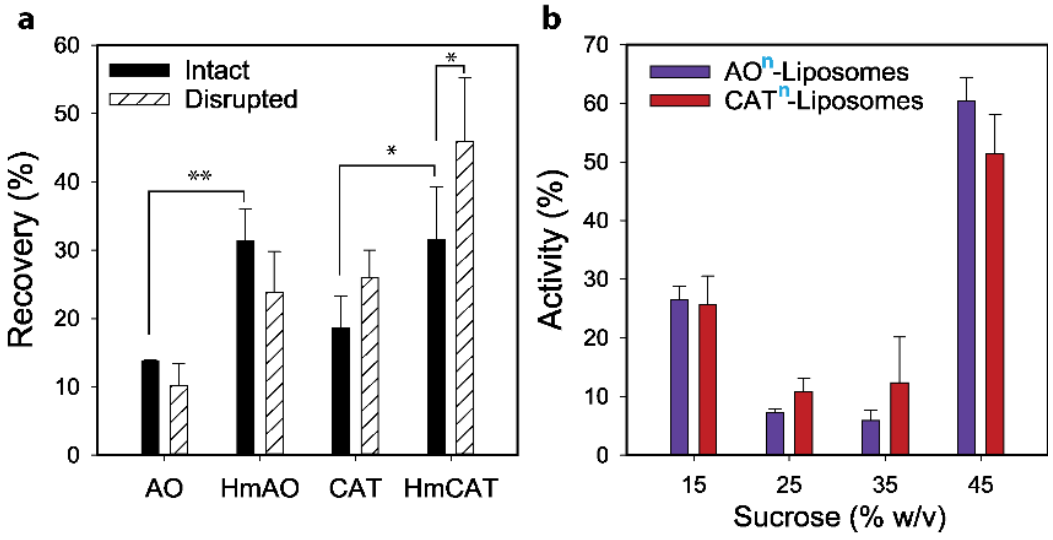

Figure 3.6: Improved liposome loading by hydrophobic modification. (a) The enzymatic recovery of AO- and CAT-Liposomes is shown for preparation with HmAO, HmCAT and native AO and CAT, prepared by detergent dialysis. The residual activity was measured in 100 mM KPi, pH 7.4 (intact, filled bars) and in 1% w/v cholic acid, 100 mM KPi, pH 7.4 (disrupted, lined bars). Mean + SD (n = 3 – 5). * $p \leq 0.05$ and ** $p \leq 0.01$. **(b)** Discontinuous sucrose density gradient analysis of E-Liposomes prepared with native enzymes, without hydrophobic modification and hence marked by a superscript n (blue). The degree of unbound enzyme with E-Liposomes prepared with HmCAT can vary, and should therefore be reevaluated when new reagents are employed. Mean + SD (n = 4 – 5).

Table 3.2: Size distribution of E-Liposomes as measured by dynamic light scattering was relatively heterogeneous. These E-Liposomes were prepared with hydrophobically modified enzymes. Mean ± SD (n = 3).

	Hydrodynamic diameter (nm)	PDI
AO-Liposomes	570 ± 110	0.30 ± 0.02
CAT-Liposomes	410 ± 30	0.33 ± 0.03

3.3.2. *In vitro* stability and metabolism

The enzymatic stability and ethanol metabolism of E-Liposomes was investigated *in vitro*. **Figure 3.7a** compares the activity of free native enzymes (lined bars) to that of the E-Liposomes (filled bars) following a 3 h incubation at 37 °C, which corresponds to the average duration of

the dialysis session in the current experimental set-up. While AO lost roughly 40% of its activity, CAT and CAT-Liposomes were hardly affected. The activity of AO-Liposomes on the other hand was increased by roughly 50%. Control experiments examining the stability of HmAO showed a 45% loss in activity and a good overlap with data obtained for free AO, indicating that the improved stability of the AO-Liposomes was not mediated by the hydrophobic modification of the enzyme. The superior stability of the AO-Liposomes was further confirmed by monitoring AO activity at room temperature (**Figure 3.8**), which showed that AO is less susceptible to unspecific interactions and denaturation when immobilized on a lipid support [252]. These findings are consistent with reports of enhanced activity and stability of AO when encapsulated within giant vesicles [253] or coupled onto enzyme nanocomplexes [76]. More recently, AO exhibited improved stability in the presence of protein destabilizing agents when embedded within a DNA-protein hybrid hydrogel [254].

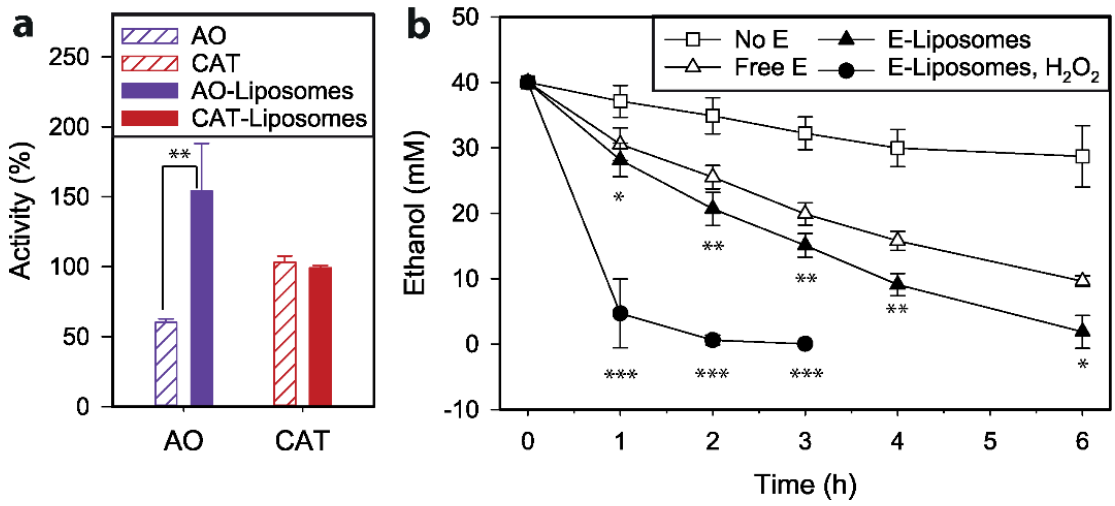

Figure 3.7: *In vitro* stability and metabolism of E-Liposomes. (a) Residual activity of free enzymes (lined bars) and E-Liposomes (filled bars) at 0.1 mg/mL following 3 h incubation at 37 °C. Mean + SD (n = 3). ** $p \leq 0.01$ AO vs. AO-Liposomes. **(b)** Metabolism of ethanol (37 °C) with free enzymes (Free E, open triangles), E-Liposomes (filled triangles) and E-Liposomes with 40 mM H_2O_2 (filled circles), containing 0.15 mg/mL AO and 0.05 mg/mL CAT for roughly equimolar ratio, compared to no enzyme control (no E, open squares). Mean ± SD (n = 6 – 9). * $p \leq 0.01$ E-Liposomes vs. No E; ** $p \leq 0.01$ E-Liposomes vs. Free E and No E; *** $p \leq 0.001$ E-Liposomes with H_2O_2 vs. all conditions.

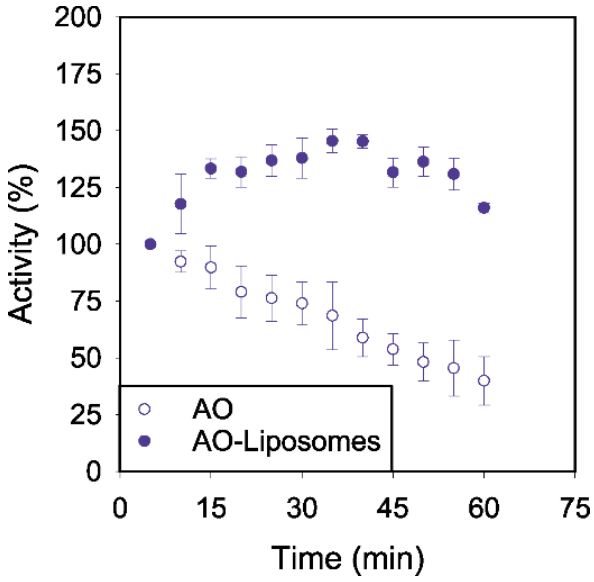

Figure 3.8: Activity of AO at room temperature. AO-Liposomes (filled circles) exhibited improved stability compared to free AO (open circles) at 0.005 µg/mL maintained at room temperature, with a slight increase of maximally 40%. Mean ± SD (n = 3).

Figure 3.7b shows the enzymatic metabolism of 40 mM ethanol by AO and CAT maintained for up to 6 h at 37 °C. Both types of samples, free enzymes (open triangles) and E-Liposomes (filled triangles), contained AO and CAT at a mass ratio of 3:1 to reach a nearly equimolar ratio considering the difference in molecular weight. A substantial decline in ethanol concentrations was observed for free enzymes and E-Liposomes at all measured time points compared to control vials void of enzymes (open squares), in which the ethanol level decreased only slightly and by means of evaporation. This shows that ethanol can be effectively metabolized in these conditions, as was also evidenced by elevated acetaldehyde levels (**Figure 3.9a**). The E-Liposomes (filled triangles, **Figure 3.7b**) oxidized ethanol more readily than free enzymes (open triangles), significantly reducing ethanol levels 2 h after the start of the experiment. Taking the ethanol evaporation into account, the E-Liposomes metabolized ethanol roughly 50% more effectively than free enzymes, which could be ascribed to the improved stability of AO-Liposomes (**Figure 3.7a**).

Nonetheless, ethanol metabolism by E-Liposomes was remarkably enhanced when H_2O_2 (filled circles, **Figure 3.7b**) was added, which served as a direct O_2 supply for ethanol

metabolism by AO through H_2O_2 degradation by CAT (**Figure 1.6**). Increased metabolism was further evidenced by elevated acetaldehyde measured after 1 h (**Figure 3.9a**). With an O_2 solubility of 1.07 mM in aqueous solutions at 37 °C and atmospheric pressure [255], the addition of 40 mM H_2O_2 can provide up to 40 mM O_2. This highlights the importance of O_2 with the currently employed enzymes and suggests a possible co-substrate limitation in aqueous solutions.

The purpose of CAT during ethanol metabolism is therefore two-fold: it prevents AO inhibition by precluding build-up of H_2O_2 [74], while regenerating O_2 to support substrate oxidation [76]. To exploit this, various oxidase enzymes including AO and uricase, have been co-administered with CAT or CAT-mimetic materials [76,256]. Reports on tandem reactions have shown increased enzymatic activity upon co-localization onto the same scaffold, allowing immediate vicinity of the enzymes [76,256–259]. A study examining the interenzyme dependency of glucose oxidase and horseradish peroxidase over a range of 10 – 65 nm using spatially addressable DNA nanostructures, demonstrated maximal activity at 10 nm [257]. In the current set-up, co-loading AO and CAT onto the same liposomes, termed AO-CAT-Liposomes, did not lead to a further improvement in metabolism (**Figure 3.9b**). This could be explained by the fact that in our system under the maximal loading capacity, the minimal interenzyme distance lies in the range of 47 - 67 nm for separately prepared liposomes, as estimated from the enzyme loading (equation 3.3 – 3.9). It is unlikely that an interenzyme distance of 10 nm, required for maximal synergistic activity, would be achieved for co-loaded liposomes with the current preparation. To the best of our knowledge, such an interprotein distance has never been described for proteoliposomes. Considering that liposomes loaded with only AO or CAT are easier to characterize than the co-loaded ones, separate populations of liposomes were employed for subsequent experiments.

Taken together, liposome loading overcame the thermal instability of AO and improved the *in vitro* metabolism of ethanol, which was further enhanced by O_2 delivery through H_2O_2 degradation by CAT, highlighting the O_2 dependence of AO.

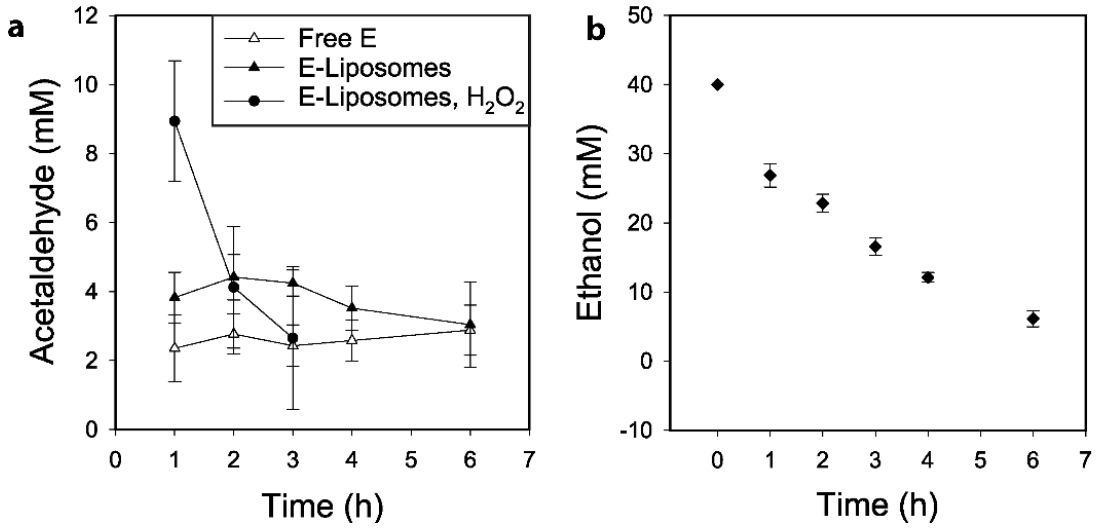

Figure 3.9: *In vitro* metabolism of E-Liposomes. (a) The acetaldehyde concentrations during *in vitro* metabolism of ethanol, same groups as in **Figure 3.7b**. The acetaldehyde was detected in all samples where ethanol was enzymatically metabolized. Mean ± SD (n = 6 – 9). **(b)** Enzymatic metabolism of ethanol *in vitro* with co-loaded liposomes (AO-CAT-Liposomes) at 37 °C. The AO-CAT-Liposomes were prepared at 3:1 mass ratio (AO:CAT), and a total enzyme concentration of 0.2 mg/mL was employed, as determined by enzymatic recovery based on residual fluorescence. Ethanol metabolism was not improved by co-loading enzymes (see **Figure 3.7b**, filled triangles). Mean ± SD (n = 3).

3.4. Conclusion

In this chapter, we have described the optimization and *in vitro* analysis of E-Liposomes, thereby establishing the fundamentals for subsequent *in vivo* characterization of LSEPD. The hydrophobic modification of AO and CAT, required for maximal enzyme-loading onto liposomes, was optimized to preserve the enzymatic activity. Phospholipids were coupled to surface-exposed amine residues by conventional NHS-chemistry. The effective covalent enzyme-acylation was verified by reverse-phase HPLC and quantified by amine titration. Aligned with the maintained catalytic activity, the conservation of the secondary structure of the modified enzymes was confirmed by circular dichroism. Subsequently, hydrophobically modified enzymes were effectively anchored onto liposomes by detergent dialysis, as affirmed by discontinuous sucrose density gradient. This close characterization corroborated the validity of the here applied production protocol. The E-Liposomes effectively improved the enzymatic

metabolism of ethanol *in vitro*, most likely due to improved stability under physiological conditions compared to free enzymes. Ethanol removal was further accelerated by the addition of H_2O_2, which was rapidly degraded to O_2 by CAT. This highlights the O_2 dependence of AO for ethanol metabolism, which quite likely can be extended to other oxidase enzymes. The effective liposome loading as well as the superior *in vitro* stability and metabolism herewith provide the framework for the subsequent *in vivo* characterization of E-Liposomes.

Chapter 4

Liposome-supported enzymatic peritoneal dialysis, *in vivo* characterization

A part of this chapter is published in:

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4.1. Introduction

While PD is considered a less-invasive, convenient and cost-effective corporeal dialysis modality compared to HD, the latter is the modality of choice owing to its higher clearance capacity [20,86,89,174,260]. In the pediatric population PD is the preferred dialysis modality [88], as children often cannot tolerate the large extracorporeal circuit volumes present in HD [55]. Recent evidence has suggested an underutilization of PD [86], which has triggered various governmental policies in developed countries to effectively encourage its use [89,90]. The main application of PD lies in the management of end-stage renal disease, though it can also be used for the detoxification of endogenous or exogenous toxins.

To address the lower clearance capacity of PD, conventional PD solutions have recently been supplemented with transmembrane pH-gradient liposomes that can concentrate overdosed protonizable drugs and some toxic endogenous metabolites in the peritoneal space, yielding for example a 20-fold increased extraction of ammonia within 3 h [70,71]. In a rat model of cirrhosis, this platform termed LSPD was shown to reduce plasmatic ammonia levels and attenuate the brain edema associated to the hyperammonemic status [72]. The absence of a hypersensitive reaction when injected in pigs confirmed the safety of LSPD in a preclinical setting [72].

The idea to dialyze blood against enzymes using an artificial kidney was first proposed approximately 50 years ago, as a means for enzyme replacement therapy in genetic disorders [261]. Since then there have been sporadic reports discussing the inclusion of enzymes in PD solutions for the treatment of cerebral infarction [262] or pancreatitis [263]. The aim of this chapter was to clearly establish that exogenous enzymatic reactions can take place in the peritoneal space and to minimize the systemic exposure of these enzymes and their metabolites by anchoring the enzymes onto sub-micrometer vesicles. This contribution brings the PD procedure one step further by endowing it with enzymatic function. Dialysate withdrawal from the peritoneal cavity following enzymatic metabolism renders this modality particularly appealing from a detoxification perspective [191].

Capitalizing on the superior *in vitro* stability and ethanol metabolism properties of E-Liposomes compared to free enzymes described in **Chapter 3**, the objective of this chapter was to characterize the *in vivo* enzymatic stability and peritoneal retention within the peritoneal space. In a final step, the *in vivo* enzymatic metabolism was studied in rats exposed to an overdose of ethanol.

4.2. Materials and Methods

4.2.1. Fluorescent labelling

Commercial dyes, IRDye 800CW NHS Ester and IRDye 680LT NHS Ester (both from Li-Cor, Lincoln, NE) were dissolved in anhydrous DMSO to 10 mg/mL. The enzymes AO and CAT (7 mg/mL) were incubated with the respective dyes in 50 mM NaCl, 100 mM KPi, pH 8.0 for 4 - 16 h at 4 °C under constant rotation, yielding AO800 and CAT680. The molar dye to protein conjugation ratio was 15.8:1 for AO and 15:1 for CAT. Free dye was removed with Dye Removal - or Zeba Spin Desalting Columns (Thermo Fisher Scientific).

Covalent coupling was investigated by reverse phase HPLC using the TFA-based separation method as described above, additionally recording IRDye 680 by fluorescence at $\lambda_{Ex/Em}$: 676/694 nm and IRDye 800 by absorbance at 774 nm. Unbound dye was detected by tetrabutylammonium bromide (TBAB) based reverse phase HPLC, adapted for proteins using a POROS R1/10 column, from a previous protocol [264]. Buffer A consisted of water, 10 mM TBAB and buffer B of 90% acetonitrile, 10 mM TBAB. Samples were injected at a total enzyme amount of 10 µg and eluted in a gradient of 10 to 50% buffer B in 10 min, maintained for 5 min at 50% before further eluting the samples in a gradient from 50 to 90% Buffer B in 10 min. Free IRDye 680LT was detected by fluorescence at $\lambda_{Ex/Em}$: 676/694 nm and free IRDye 800CW by absorbance at 774 nm. The degree of impurity was assessed by normalizing the peak area of residual dye after purification over that of the free dye prior to purification, set to 100%.

Dye coupling was measured in triplicate by absorbance at 676 and 774 nm for IRDye 680 and -800, respectively, using a Nanophotometer P330 (Implen, Munich, Germany).

Fluorescently labelled enzymes were hydrophobically modified for incorporation onto liposomes, as described above. Final enzyme loading was assessed based on residual fluorescence of the IRDye 680 ($\lambda_{Ex/Em}$: 670/709 nm) and IRDye 800 ($\lambda_{Ex/Em}$: 774/805), using an M200 infinite plate reader (Tecan).

4.2.2. Gas chromatography

Plasma and dialysate samples were probed by headspace (HS) injection (TurboMatrix HS 40, Perkin Elmer, Waltham, MA) and measured by HP6890 (Hewlett Packard, Palo Alto, CA) equipped with a TR-FFAP column (50 m x 0.32 mm x 0.5 µm, Thermo Fisher Scientific), where samples were heated for 20 min at 65 °C and the HS needle and transfer line were set to 90 and 120 °C, respectively. Samples were injected for 0.2 min at a total flow of 4.2 mL/min using a split flow of 0.3 mL/min. A prolonged sampling time was applied to increase sensitivity. Helium (Pangas, Dagmarsellen, Switzerland) was selected as a carrier gas throughout all measurements. Ethanol metabolites and the internal standard (1-butanol) were separated using a temperature gradient (55 – 130 °C). Detailed information regarding injection- and detection temperature, split ratio and flow rate is summarized in **Table A3.1**.

Handling of quality controls is as described in section 3.2.5 of **Chapter 3**. Samples were prepared by adding 1 mL of 5 mM 1-butanol to 100 µL plasma or dialysate, of which 1 mL was transferred to a 20 mL ND20 HS Crimp Vial (BGB Analytik AG, Boeckten, Switzerland) which was sealed with a 20 mm Crimp Top Aluminum Silver Cap with PTFE/Silicone Septa (Perkin Elmer). If less than 100 µL sample was available, chromatography water was added, and the metabolite concentration was corrected based on sample volume. Standard series were based on the analyte response. Calculation of LOD and LOQ is as described under section 3.2.7 of **Chapter 3**.

4.2.3. Animal experiments

All animal experiments were performed in accordance with procedures approved by the cantonal veterinary office of Zurich (ZH031/15). Male Sprague Dawley rats (weighing roughly 300 g, Charles River, Sulzfeld, Germany) were given at least 7 days to acclimate to their surroundings, if not otherwise stated had access to food and water *ad libitum*, and followed a 12 h light/dark cycle. Dialysate fluid pre-warmed to 37 °C containing enzymes or purified E-Liposomes diluted in Physioneal 35 glucose 1.36 wt% (Baxter, Opfikon, Switzerland) or Physioneal alone, were administered to the peritoneal cavity (60 mL/kg) at a total enzyme dose of 2.5 – 20 mg/kg. Co-administered enzymes were dosed at a 3:1 (AO to CAT) mass ratio. Rats were discontinuously anesthetized (1.5 – 2.5% v/v isoflurane, 0.8 – 2 mL/min O_2 flow) for dialysate infusion, sampling, removal as well as for imaging. Rats were returned to their cages between time points. Blood (200 µL) was sampled from the tail vein or artery and collected in lithium heparin-coated MiniCollect Tubes (Greiner Bio-One, Kremsmünster, Austria) and immediately placed on ice. Plasma was separated by centrifugation (10 min, 1,500 *x g*) within 2 h of sampling for ethanol and metabolite analysis or 4 h for characterization of fluorescently labelled enzymes. Peritoneal fluids (500 µL) were sampled after 1 and 2 h in ethanol dosing and enzymatic activity experiments and removed after 3 h in all experiments with an Angiocath silicon catheter (Becton Dickinson, BD, Waltham, MA) perforated at its tip, and immediately placed on ice. The peritoneal cavity was subsequently rinsed with 5 mL 0.9 wt% NaCl. For ethanol dialysate kinetics, enzymes were heat-inactivated (10 min, 95 °C) within 3 h after sampling, centrifuged (10 min, 10,000 *x g*) and the supernatant collected. Plasma and dialysate samples were either directly subjected to spectrofluorimetry and activity (dialysate only) measurements or stored at -20 °C for ethanol and acetaldehyde quantitation by headspace gas chromatography (for details see supporting information). At the end of each experiment, rats were euthanized by carbon dioxide asphyxia followed by a thoracotomy, if not otherwise stated.

4.2.3.1. *In vivo* imaging

A PD fluid containing co-administered fluorescently labelled free enzymes or E-Liposomes (10 mg/kg) was instilled to healthy rats. The initial fluorescence signal of both enzymes (AO800

and CAT680) was comparable between groups injected with free enzymes and E-Liposomes, with a percentage deviation lower than 10% in each experiment. Animals were placed in the *in vivo* imaging system (IVIS, Caliper, Mainz, Germany), equipped with a heated platform (37 °C), prior to injection, 1, 2 and 3 h after and following dialysate removal and rinse, for white light and fluorescence imaging ($\lambda_{Ex/Em}$: 745/800, binning 8, exposure time 1 s). Plasma and dialysate samples were measured in triplicate by spectrofluorimetry using an M200 infinite plate reader (Tecan) for enzyme quantification. An estimated rat plasma volume of 4.22 mL per 100 g bodyweight was applied when calculating the fraction of total administered dose present in plasma [265].

Following dialysate removal and rinse, rats were euthanized by cardiac perfusion (with 0.9 wt% NaCl) under deep controlled anesthesia (5% isoflurane, 2 mL/min O_2 flow). Rats were given buprenorphine (0.1 mg/kg, *s.c.*) 30 min prior to cardiac perfusion. Liver, kidneys, lungs and spleen were harvested and briefly rinsed with 0.9 wt% NaCl. The organs were imaged as described above, additionally at $\lambda_{Ex/Em}$: 640/700, with the exception that the liver, kidneys and lungs were imaged with a 5 s exposure time. Images were processed with the Living Image software (Caliper Life Science, Waltham, MA). A region of interest surrounding each organ was selected, and the average fluorescence intensity was normalized to the value of corresponding control organs harvested from animals not subjected to fluorescently labelled enzymes. A Grubbs outlier test identified one outlier ($p < 0.05$) in the data set comprising lungs of E-Liposomes ($\lambda_{Ex/Em}$: 640/700), which was subsequently removed.

4.2.3.2. *In vivo* ethanol metabolism

Rats, fasted for 8 – 10 h, were dosed with 2 g/kg ethanol (25.3 vol%, 10 mL/kg) by gavage using a metallic curved button-head rat gavage needle. The syringe was weighed prior to administration to confirm correct ethanol dose was administered, employing a density of 0.97 g/mL [266]. A PD fluid was injected 1 h after, comprised of either Physioneal (control treatment) or 20 mg/kg LSEPD and removed after 3 h. Blood was sampled over a period of 6 h, after which time the animals were euthanized.

4.2.3.3. *In vivo* activity

The PD media containing AO-Liposomes, AO or CAT were separately injected in healthy rats at a final AO or CAT concentration of 7.5 mg/kg or 2.5 mg/kg, respectively. Activity profile of CAT-Liposomes was obtained from a group dosed with ethanol and treated with 10 mg/kg LSEPD. Activity of sampled dialysate fluid was normalized to the value of a reference sample maintained at 4 °C during the experiment.

4.2.4. Statistical analysis

Statistical analysis was performed with Sigma Plot 13.0 (Systat Software, Chicago, IL) using a one way ANOVA followed by a Tukey test for pairwise comparison. A p-value smaller than 0.05 was considered significant.

4.3. Results and Discussion

4.3.1. Biodistribution of fluorescently labelled enzymes

To study the systemic exposure of free enzymes compared to E-Liposomes, the enzymes were coupled to near-infrared fluorophores enabling deep tissue imaging with minimized tissue auto fluorescence [267]. Effective fluorescent labelling of AO and CAT is demonstrated in **Figure 4.1**. The immobilization of the labelled enzymes on the liposomes should minimize systemic exposure by reduced transfer to the lymphatic system [268]. To investigate this, healthy rats were subdivided into two groups receiving PD fluids (60 mL/kg) containing either fluorescently labelled E-Liposomes or free enzymes (10 mg/kg). The AO and CAT were administered at a 3:1 mass ratio, and PD was maintained for 3 h after which an overall enzyme content of 80% was recovered, as assessed by the total collected dialysate volume and residual fluorescence. The dialysate recovery is dependent on the intraperitoneal pressure [62,269] and is in line with previously reported ones ranging from 70 to 99% [270]. However, as this was not sensitive enough to distinguish the two groups, the systemic exposure of AO was monitored in the whole body of living animals and AO and CAT were quantitated in plasma at selected time points. The relative organ deposition was also assayed following dialysate removal.

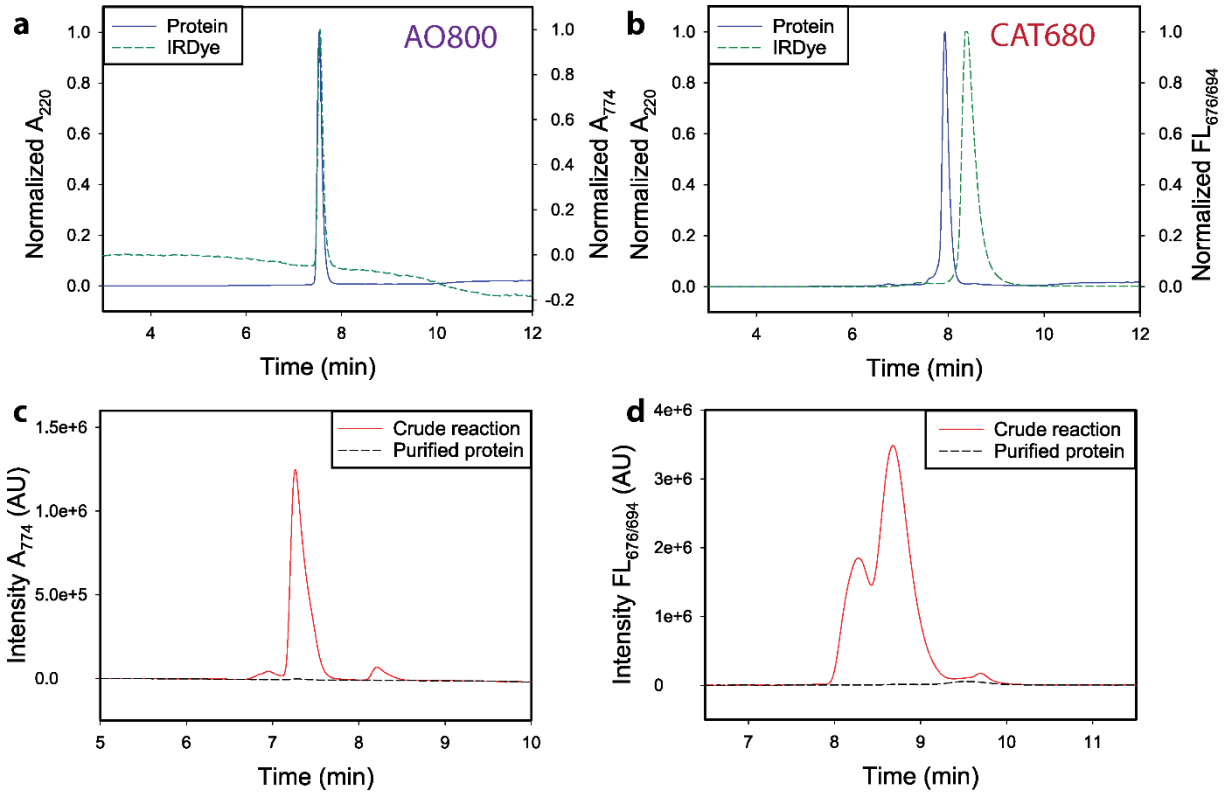


Figure 4.1: Characterization of fluorescently labelled proteins by reverse phase HPLC. (a, b) Fluorescent coupling was confirmed using a TFA-based separation method. Proteins were detected at 220 nm (blue solid line) and fluorophores either by absorbance at 774 nm for AO800 (green broken line, **a**) or by fluorescence $\lambda_{Ex/Em}$: 676/694 nm for CAT680 (green broken line, **b**). Effective labelling is obvious for AO800 where the elution profile of the protein and fluorophore overlapped, as both were detected by absorbance. Fluorescent labelling of CAT680 was successful, despite the slightly later retention times witnessed with the fluorophore, though this is expected, as samples are first detected by spectrophotometry and second by fluorimetry with the here employed HPLC instrument. On average, 3 – 4 IRDye 800 and 1 IRDye 680 molecules were coupled to AO and CAT, respectively. **(c, d)** Uncoupled dyes were detected by TBAB based reverse phase separation. The elution profiles of the crude reaction (red solid line), AO and IRDye800CW NHS ester at 774 nm in **(c)** and CAT and IRDye680LT NHS ester at $\lambda_{Ex/Em}$: 676/694 nm in **(d)** and purified (black broken line) AO800 **(c)** and CAT680 **(d)** are shown. The peak area of the purified dye was on average lower than 1% of that prior to purification. A representative elution profile is shown for each sample.

Figure 4.2 shows the evolution of fluorescence signal of AO800 ($\lambda_{Ex/Em}$: 745/800) over time using an IVIS for free enzymes (**Figures 4.2a – e**) and E-Liposomes (**Figures 4.2f – j**). Upon

injection, the labelled enzymes were locally confined to the peritoneal cavity for both groups (**Figures 4.2a, f**). However, after 3 h the animals receiving free enzymes exhibited fluorescence signal outside the peritoneal cavity (**Figure 4.2d**, arrowheads), for example in the paw region, a highly vascularized tissue free of animal hair [271] enabling fluorophore detection in systemic circulation. On the other hand, the E-Liposomes seemed to be well retained within the peritoneal cavity for the duration of dialysis (**Figure 4.2i**). In addition, dialysate removal and rinsing of the peritoneal cavity was visibly improved with the E-Liposomes, as the fluorescence signal was less spread out and limited to the dialysate removal site (**Figure 4.2j**). In contrast, in the animals receiving free enzymes the fluorescence signal remained dispersed in the peritoneal cavity (**Figure 4.2e**), and systemic exposure was possibly even further increased, especially in the superior part of the body (arrowheads).

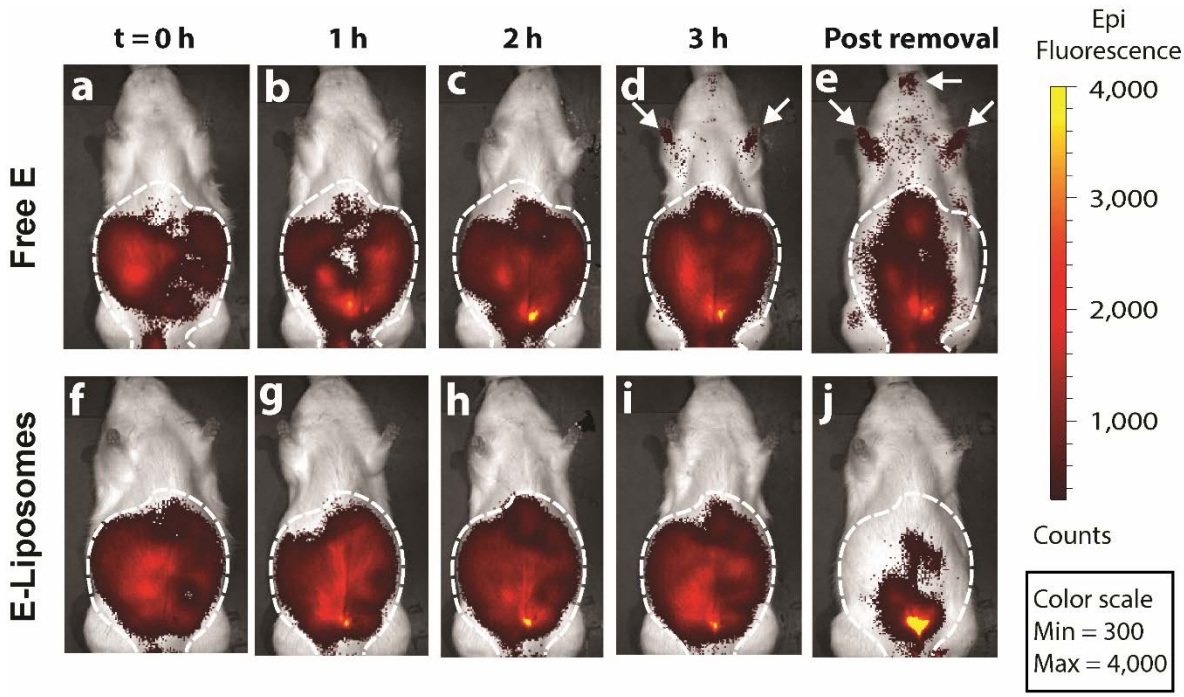


Figure 4.2: *In vivo* fluorescence imaging during PD. Live imaging ($\lambda_{Ex/Em}$: 745/800 nm) of rats injected with PD fluids containing AO800 and CAT680. Evolution of the fluorescent signal immediately after injection, and after 1, 2, and 3 h and following dialysate removal and rinse (post removal) for free enzymes **(a – e)** and E-Liposomes **(f – j)**. For each group a representative animal is shown. (n = 5) Color scales are identical for all pictures.

During the course of the same experiment, blood was sampled hourly to quantify enzymes that leaked into systemic circulation from the peritoneal cavity. **Figure 4.3a** shows the plasma concentration of AO (purple circles) and CAT (red triangles) over time for free enzymes (open symbols) and for E-Liposomes (filled symbols). Exposure of AO-Liposomes was significantly reduced compared to free AO after 2 and 3 h, whereas significantly lowered CAT plasma level by liposome loading was only observed after 3 h. The systemic exposure after 3 h was reduced roughly 7-fold for AO and 2-fold for CAT. The observed higher AO plasma concentration is likely due to the 3-fold higher dose administered.

Rats were euthanized following dialysate removal and the organs were harvested. The fluorescence of kidneys, liver, lungs and spleen was recorded (Please see appendix, **Figures A4.1a – h**) and normalized to the respective control organs, not exposed to fluorescently labelled enzymes. **Figure 4.3b** shows the relative AO and CAT signal (purple and red bars, respectively) for animals receiving free enzymes (lined bars) and E-Liposomes (filled bars). Fluorescently labelled enzymes could be detected in nearly all organs, characterized by a normalized signal greater than 1 (blue dashed line). In line with the dialysate leakage into blood circulation, the administration of free enzymes led to a higher distribution in organs than the E-Liposomes, with statistically significant differences in the kidneys, liver and lungs. Indeed, a reduction of roughly 4-fold for AO and 2- to 3-fold for CAT was observed for the E-Liposomes in the liver and kidneys (highly vascularized organs with fenestrated capillaries) [272], while in the lungs no fluorescence could be detected. This could indicate that liposome loading either prevents lung targeting or reduces exposure to a level below the detection limit.

The exposure of dialysate proteins in blood circulation and subsequently in organs is mediated by their absorption to the surrounding interstitial tissue, with a slow uptake into the lymphatic vasculature by which they are conveyed into the blood circulation [62]. Particulate size has been described to be a key determinant in reducing systemic exposure of vesicles injected intraperitoneally by minimized diffusion of large vesicles [71,79,230]. While the here presented platform results in lower systemic exposure, low quantities of systemically exposed enzymes may still lead to side-reactions such as systemic immunogenicity or hypersensitivity response (both can also be triggered by the enzymes present in the peritoneal cavity). These

aspects would have to be studied in detail in future development studies, and the occurrence of immune reactions could be reduced by modifying the enzyme structure, optimizing the PEGylation procedure and/or by grafting non-linear polymers onto enzymes as we have shown in a previous study [273]. Still the lower systemic exposure observed with E-Liposomes, combined with the ability of PD to recover the vast majority of the injected enzymes, renders this approach particularly appealing from a safety point of view and indicates that it may provide an advantage over intravenously administered enzymes.

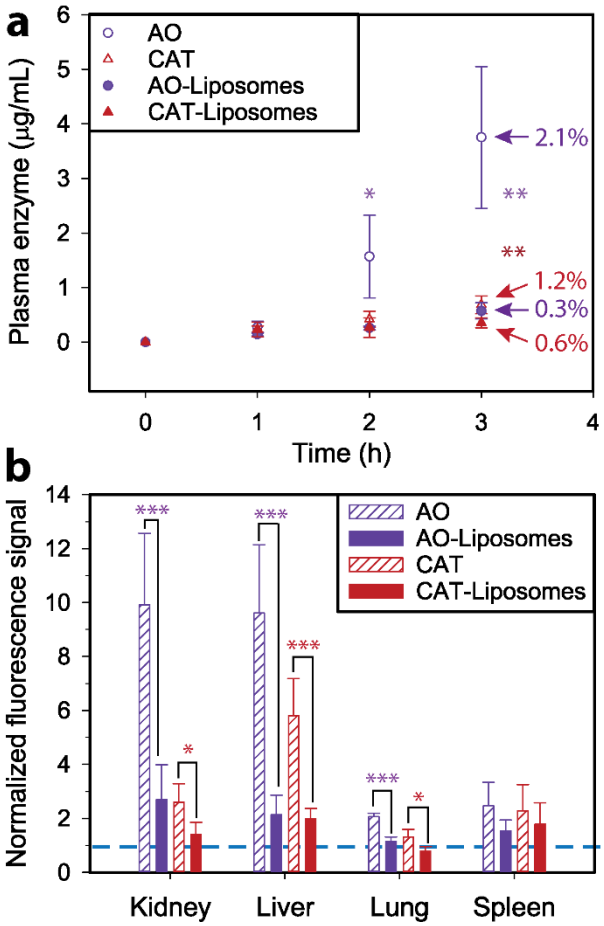


Figure 4.3: Systemic exposure of LSEPD. Detection of enzymes in plasma during PD and in organs after PD removal in rats of **Figure 4.2**. **(a)** Exposure of AO (purple circles) and CAT (red triangles) in plasma during PD in rats administered with free enzymes (open symbols) or E-Liposomes (filled symbols). The fraction of total administered enzyme dose detected in plasma after 3 h PD is shown. Mean ± SD (n = 4 –5). **(b)** Relative AO (purple) and CAT (red) organ distribution is shown for E-Liposomes (filled bars) compared with free E (lined bars). A normalized signal greater than one (blue dashed line) indicates fluorescence detection in organs. Mean + SD (n = 4 – 5). Asterisk in purple or red denotes statistical significance of AO vs. AO-Liposomes or CAT vs. CAT-Liposomes, respectively, with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

4.3.2. *In vivo* stability and metabolism

To investigate the *in vivo* stability, AO (7.5 mg/kg, purple circles) and CAT (2.5 mg/kg, red triangles) were injected intraperitoneally, either as free enzymes (open symbols) or as E-Liposomes (filled symbols), and the enzymatic activity was measured during PD as shown in **Figure 4.4**.

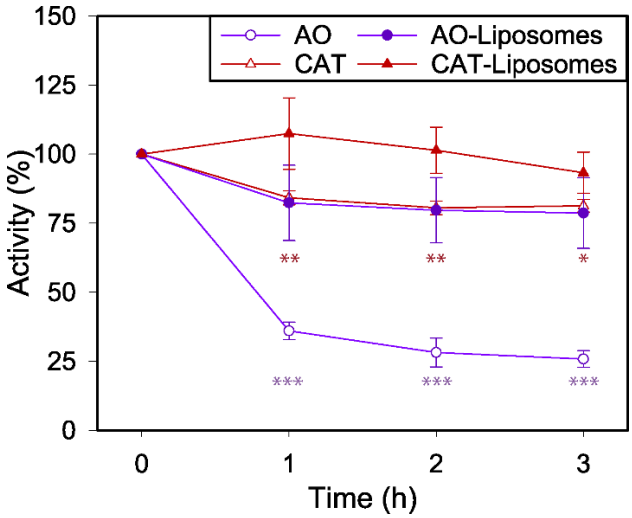


Figure 4.4: *In vivo* activity. Normalized activity of AO (purple circles) and CAT (red triangles) administered either as free enzymes (open symbols) or E-Liposomes (filled symbols), measured during PD. Mean ± SD (n = 4 – 7). Asterisk in purple or red denotes statistical significance of AO vs. AO-Liposomes or CAT vs. CAT-Liposomes, respectively, with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

The E-Liposomes and CAT retained their activity with maximally 20% loss, which may be attributed to dilution by fluid influx due to the osmotic gradient [270], rather than actual inactivation. Free AO, on the other hand, was rapidly inactivated with a 65% loss within the first hour, which was gradually reduced to a residual activity of 25% at the end of dialysis. While activity was maintained under the here examined conditions, stability under harsher conditions (*e.g.* prolonged dialysis or inflammation) would require further examinations. The *in vivo* activity profile was comparable to the enzymatic stability at 37 °C (**Figure 3.7a**), where only free AO was susceptible to partial inactivation, although the increase in activity observed *in vitro* for AO-Liposomes was not observed in the animals. Furthermore, compared to the *in vitro* measurements an apparent slightly higher stability of CAT-Liposomes vs. free CAT was

observed *in vivo*. These deviations could result from unknown agents in the peritoneal cavity altering substrate binding, conformational stability of the enzyme or fluidity of the liposome membrane.

Finally, the *in vivo* ethanol metabolism of LSEPD was examined in rats. The ethanol metabolism with free enzymes was not investigated in this experiment to minimize the interference that would be caused by systemically exposed enzymes. **Figure 4.5a** gives a schematic representation of the applied experimental procedure. One hour following oral ethanol dosing (2 g/kg), the rats were injected with either LSEPD suspension (20 mg/kg enzymes) or control PD solution (Physioneal), which was removed after a 3 h dwell time. Blood samples were taken over a period of 6 h as well as dialysate samples during PD. Rats dosed with ethanol exhibited mild forms of ataxia and piloerection within the first hour, lasting up to 4 h. Intoxication was otherwise relatively asymptomatic.

Figures 4.5b, c show the ethanol and acetaldehyde concentration profile for LSEPD (triangles) and Physioneal (circles) in plasma (filled symbols) and dialysate (open symbols). The PD duration is highlighted in green. While no noticeable difference in plasma ethanol clearance could be detected between the groups (**Figure 4.5b**), plasma acetaldehyde demonstrated an immediate rise in LSEPD-treated animals following dialysate administration, with a peak concentration just under 0.5 mM (**Figure 4.5c**). In contrast, acetaldehyde was hardly detectable in Physioneal-treated animals. Acetaldehyde is known to be toxic and is considered to contribute to the side effects of ethanol including nausea, vomiting, face flushing and headache [40,41]. Therefore, future developments of LSEPD for acute alcohol poisoning should address the elevated acetaldehyde levels, for instance by co-administering acetaldehyde scavengers (*e.g.* L-cysteine or D-penicillamine) or aldehyde-metabolizing enzymes (*e.g.* ALDH or aldehyde oxidase) [274,275].

Similarly, the dialysate acetaldehyde levels for LSEPD-treated groups were substantially elevated with an average peak concentration around 4 mM (**Figure 4.5c**), indicating enhanced ethanol metabolism. Furthermore, dialysate ethanol levels were appreciably lower in this group than in the control group, demonstrating that ethanol elimination was improved locally (**Figure 4.5b**).

The ratio of ethanol and acetaldehyde dialysate over plasma sampled 2 h after ethanol dosing was quantified for each animal separately as it is not affected by the variability in absorbed ethanol [276–278] (**Figure 4.5d**). A ratio of nearly 1 for ethanol in Physioneal-treated animals (lined bar) confirmed the straightforward dialyzability and rapid diffusion of ethanol into the peritoneal cavity. On the other hand, the ethanol ratio for the LSEPD group (black bars) was significantly reduced compared to that of the Physioneal group. This corroborates the improved ethanol removal with LSEPD, as surmised from **Figure 4.5b**. The measured acetaldehyde was predominantly located in the peritoneal cavity of LSEPD-treated animals, yielding an acetaldehyde ratio of 8 in this group. Plasmatic acetaldehyde most likely originated from the peritoneal cavity by diffusion, due to its small size and water solubility.

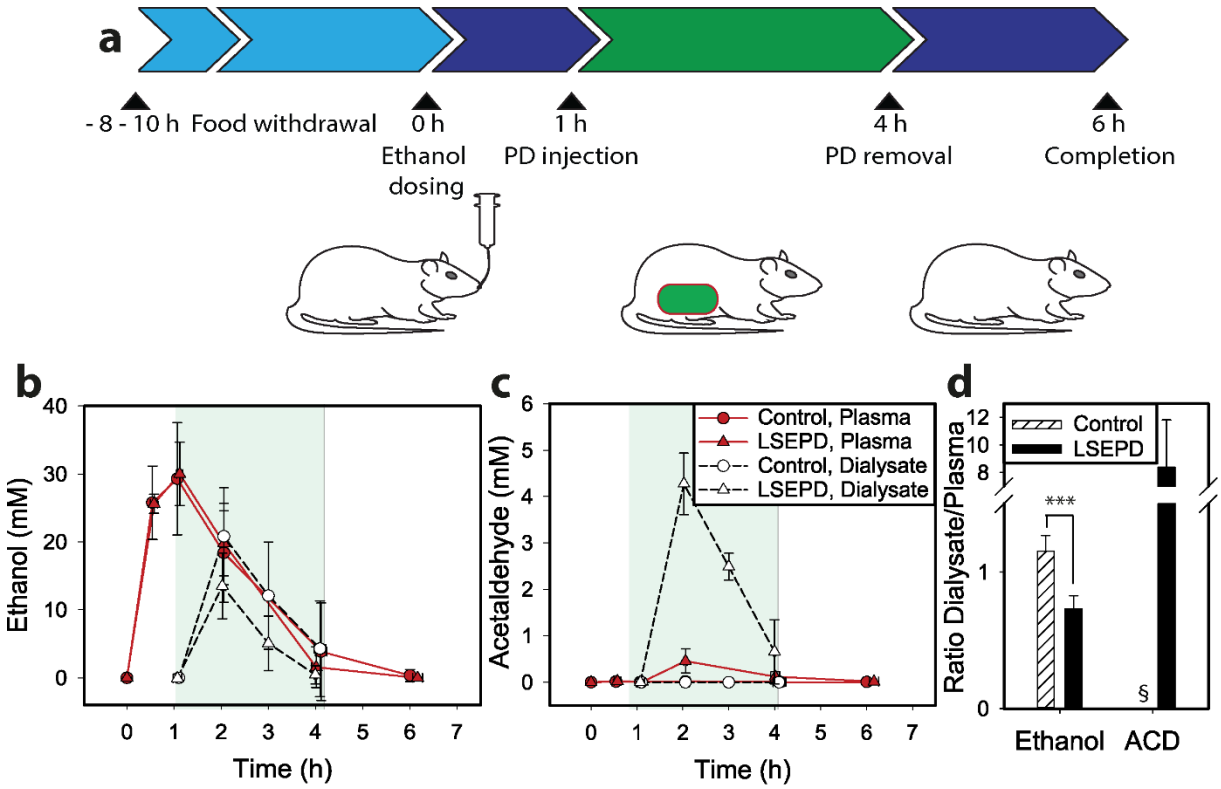


Figure 4.5: *In vivo* ethanol metabolism. (a) Schematic representation of experimental timeline applied in ethanol intoxication experiments. Rats were fasted for 8 – 10 h prior to oral administration of ethanol (2 g/kg). The PD (60 mL/kg) was initiated after 1 h and lasted for 3 h after which it was removed. Blood samples were taken over a period of 6 h and dialysate samples during PD. **(b, c)** Ethanol **(b)** and acetaldehyde **(c)** plasma (filled symbols) and dialysate (open symbols) concentration profile for LSEPD (20 mg/kg, triangles) and Physioneal (circles). Dwell time of PD is highlighted in green. Mean ± SD (n = 6). **(d)** Ratio of dialysate metabolite concentrations (ethanol and acetaldehyde) over plasma concentration for both Physioneal (lined bar) and LSEPD (filled bars), sampled 2 h after ethanol dosing. §: plasma and dialysate acetaldehyde below LOD. ACD: Acetaldehyde. Mean + SD (n = 6). *** $p \leq 0.001$ Physioneal vs. LSEPD.

While LSEPD treatment effectively lowered the ethanol content in the peritoneal cavity, this difference could not be extended to the systemic level. Ethanol is metabolized by AO by consumption of O_2, which given its inherently low solubility in water compared to blood carrying erythrocytes with hemoglobin, needs to be constantly supplied to the peritoneal cavity from the circulation by diffusion. This may have rendered O_2 the limiting factor and

considerably slowed down the reaction. While systemic reduction of alcohol levels has been previously described for AO when encapsulated within erythrocytes [73] or co-entrapped with CAT within polymeric nanocomplexes [76], the implied delivery vehicles had been injected intravenously, where they were in close contact with erythrocytes. Enhanced O_2 availability may be accomplished by co-administration of perfluorocarbon emulsions or O_2 microbubbles [217,221]. The relatively high ethanol dose required to elicit toxicity may have additionally hindered improved ethanol clearance within the blood circulation. Systemically improved toxin removal with enzyme- or lipid-based antidotes has been demonstrated for dichlorvos (10 mg/kg [279]), verapamil (50 mg/kg [71]), cocaine (100 mg/kg [280]) and most recently hyperuricemia, where an initial plasma uric acid concentration of 600 µM was observed [256]. These values are roughly 20- to 200-fold lower than the ethanol dose applied in this study. The high ethanol dose administered, together with its high endogenous metabolism, may have outweighed the metabolism of LSEPD, thereby likely masking the systemically improved clearance. Indeed, the dialysate ethanol was clearly reduced, and plasma and dialysate acetaldehyde were elevated with LSEPD.

4.4. Conclusion

In this chapter we have presented the functionalization and detailed characterization of conventional PD-fluids supplemented with E-Liposomes, termed LSEPD, to extend the functionality of this easily accessible and less-invasive modality. The superior enzyme stability of E-Liposomes observed *in vitro* (**Chapter 3**) was confirmed *in vivo,* and reduced systemic exposure was demonstrated by live imaging and blood sampling, which ultimately lowered organ distribution. Finally, enhanced metabolism of ethanol was observed with LSEPD, manifested by the increased acetaldehyde production, thereby improving peritoneal ethanol clearance. Plasmatic acetaldehyde was markedly lower than in the dialysate, demonstrating local therapeutic action of E-Liposomes and highlighting how the confinement of antidotes to the peritoneal cavity can reduce the exposure of toxic metabolites such as acetaldehyde to the systemic circulation. While additional studies are required to better understand the diffusion of

O_2 into the peritoneal cavity and how this can be improved, our data reveal that LSEPD can temporarily enhance enzymatic ethanol metabolism, in view of the improved enzyme stability and lower systemic exposure. The here established protocols could be extended to further studies by employing other relevant enzymes.

Chapter 5

Conclusion and Outlook

The widest application of PD lies in the management of kidney failure, though less frequently it can also be employed for poisoning management. Owing to its modest clearance capacity, it is however generally not recommended as a first line treatment for any poisoning scenario and treatment with more invasive, extra-corporeal modalities prevails. This also applies for severe, acute alcohol intoxication for which a specific antidote is still lacking. Capitalizing on the enhanced extraction capacity for ionizable toxins achieved by supplementing conventional PD fluids with transmembrane pH-gradient liposomes, the objective of this current thesis was to increase the functionality of PD for the management of acute alcohol poisoning by increased metabolism.

In **Chapter 2** the benefit of PD for the management of various, non-renal indications was reviewed. It was established that despite the flurry of clinical studies in the second half of the last century, PD does not confer a clear advantage as a therapeutic measure for psoriasis and pancreatitis. Currently, in the absence of more efficacious therapeutic modalities PD is still indicated in cases of severe hypothermia, refractory CHF, HA and poisoning. The typically employed dialysis solutions and application scheme differ somewhat amongst one another, where for example during the treatment of hypothermia, dialysis fluids are warmed slightly above the physiological temperature.

Novel dialysate formulations and applications, currently in the preclinical or early clinical development phase were presented, demonstrating the potential to increase the clearance capacity for indications currently treated with PD (*e.g.* LSPD for HA). Furthermore strategies to widen the scope of PD to the management of stroke or as an oxygenation support, by

including O_2 carriers within the PD fluid in the event of respiratory failure were discussed. Of the mentioned novel developments, we believe the use of PD for stroke may have the highest potential for a rapid clinical translation, as it involves conventional, already marketed PD fluids. On the other hand, the development of nano- and micro-particle based formulations involving liposomes or O_2 carriers still requires further comprehensive clinical investigation on their toxicity and efficacy. Nonetheless, these advances clearly demonstrate how this simple modality may be manipulated to widen its application to further indications and improve its functionality for existing ones.

In **Chapter 3** the optimization of E-Liposomes was presented as well as their subsequent in-depth *in vitro* characterization. Hydrophobic modification of the alcohol metabolizing enzymes, AO and CAT was fine-tuned to maximize enzyme-loading onto liposomes while maintaining catalytic activity. The effective covalent modification was confirmed and quantitated by reverse-phase HPLC and amine titration, respectively. The conservation of the secondary structure of the modified enzymes was attested by circular dichroism and their subsequent effective liposome loading following detergent dialysis was verified by discontinuous sucrose density gradient. This detailed characterization confirmed the validity of the here applied production protocol. Liposome loading led to improved thermo-stability, which presumably accounted for the increased *in vitro* ethanol metabolism observed when contrasted to the free enzymes. The ethanol metabolism by this enzyme pair was substantially increased by the addition of H_2O_2, which was rapidly degraded to O_2 by CAT. The hereby generated O_2 substantially elevated its availability to fuel the ethanol oxidation, confirming the O_2 dependence for ethanol metabolism using this enzyme pair.

Chapter 4 of this thesis covers the *in vivo* characterization of E-Liposomes supplemented to conventional PD, termed LSEPD. The ulterior motive to load enzymes onto liposomes was to reduce their peritoneal leakage into the blood circulation. To test this hypothesis, enzymes were fluorescently labelled with near infrared dyes and their systemic exposure monitored during dialysis by live-imaging and blood sampling as well as after dialysis by fluorescence analysis of the major organs. Indeed, liposome-loading resulted in lower systemic exposure, when measured in the blood and major organs. This difference in systemic exposure, however,

could not be evidenced in the collected dialysis fluid, where an approximate enzyme recovery of 80% was attained with both E-Liposomes and free enzymes, suggesting an incomplete washing. The in *vivo* stability profile of free enzymes and E-Liposomes administered intraperitoneally followed a similar trend as the corresponding *in vitro* measurements, where only free AO was partially inactivated during the 3 h dwell time. As enzymes are predominantly located on the liposome surface, maintained stability during dialysis suggests that the peritoneal cavity, investigated in its current state (*i.e.* non-inflamed tissue and 3 h dwell), may present mild conditions favoring enzymatic reactions without requiring further protection. In a final step, the capability of LSEPD to enhance the ethanol metabolism was compared to a control dialysate solution (Physioneal) in a rodent model of acute ethanol poisoning. While no difference in plasma ethanol levels could be observed amongst the two groups, an immediate rise in plasma and dialysate acetaldehyde, ethanol's primary metabolite, was detected following LSEPD administration. Furthermore, dialysate ethanol levels were decreased in the LSEPD group compared to the control group, strongly suggesting that the ethanol metabolism was locally enhanced.

Previous reports describing the use and modification of AO and CAT typically employed a 100 mM potassium phosphate buffer [74,76]. This buffer was therefore also selected for the here described optimizations and preparations and enabled adequate comparison of our *in vitro* results to the literature. However, given the high potassium content of this buffer, which exceeds the physiological levels 25-fold, we would recommend to perform the final dialysis exchange step during the purification of E-Liposomes in a buffer more suitable for peritoneal application. Please see **Table A5.1** in the appendix for an overview of the maximally recommended dialysate constituents [281]. The LSEPD formulation in its current form was typically diluted 5 to 10-fold in Physioneal prior to its administration, which may not have dramatically increased the potassium levels. Such an adaptation of the preparation procedure could however enable administration of higher E-Liposome doses potentially increasing the ethanol metabolism in future optimizations. Furthermore, given the superior ultrafiltration properties enabling longer peritoneal dwell times with Extraneal (7.5% icodextrin) we initially considered to dilute the E-Liposomes within this dialysis medium. Nevertheless, preliminary tests revealed that the AO

activity was reduced to approximately 40% in Extraneal, while it was practically unaffected in Physioneal (1.36% glucose) (data not shown). The latter solution was therefore selected for subsequent *in vivo* experiments. The underlying reason for this partial inactivation was however not further investigated.

The here described E-Liposomes were prepared by the detergent dialysis procedure as detergents were required to solubilize the NHS-activated phospholipids for coupling and to prevent aggregation of the hydrophobically modified enzymes. This preparation method, however, resulted in a relatively heterogeneous size distribution and on average, smaller liposomes than what is obtained by conventional lipid film hydration. In nanomedicine a smaller particle size is typically advantageous where a general optimal size of 20 – 100 nm pertains [282]. However, for this current application larger liposomes were sought, as increased particle size had been shown to increase the peritoneal residence time [71,79]. The present approach served as a starting point for enzyme-based PD, however further developments of this platform may benefit from a larger particle size, mediated either by alternative scaffolds or a variation of the liposome preparation procedure. Such an adaption could involve the manufacturing of E-Liposomes by coupling enzymes onto preformed liposomes [236,283,284], in this case, the liposome preparation needs to be quick to prevent deactivation of the NHS-moiety and allow subsequent enzyme coupling. Liposomes are generally regarded to have a good safety profile owing to their biodegradability [191], however the use of micrometer-sized silica or even magnetic nanoparticles could be envisioned as an enzyme support. The latter technology could additionally confer improved recovery by applying a magnetic field, as indeed only very few scaffolds escape the peritoneal cavity during the dialysis and a substantial proportion which constituted nearly 20% in this application, remained in the peritoneal cavity. Nonetheless, the toxicity of such materials should be considered as relatively large doses can be administered through this route and a certain fraction can still reach the systemic circulation.

Finally, despite the clearly increased enzymatic metabolism achieved with LSEPD, the metabolizing capacity was insufficient to lower the systemic ethanol concentrations. This could be attributed to multiple reasons. To start with, if the ethanol metabolizing capacity extracted from *in vitro* measurements is calculated, the administered enzyme formulation could only

account for 3.9 – 5 % of the total administered ethanol dose, depending on whether the total ingested or bioavailable dose is factored [285]. A maximally 5% contribution would unlikely be detected with the applied sample size and inter-individual variation. In addition, by adding H_2O_2 to the here investigated enzyme pair *in vitro,* we were able to demonstrate the stoichiometric dependency of O_2 in the current reaction. While the intraperitoneal route is attractive for multiple reasons including reduced systemic exposure of the antidote and the toxic metabolites, it may not be ideal for detoxification using oxidase enzymes such as AO. In comparison to the blood where erythrocytes carry O_2 binding hemoglobin thereby offering a higher O_2 availability, the peritoneal cavity is filled with an aqueous solution which consequently provides a lower O_2 availability than blood. This could explain the reduced efficacy of the current system compared with previous reports where a lower dose of intravenously injected enzymes (3.7 mg/kg) was able to detectably lower ethanol concentrations in the blood [76].

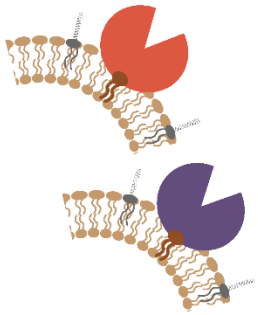
Strategies to increase the ethanol clearance of LSEPD could include administration of a higher enzyme dose, though with the current production protocol a maximal application of 50 mg/kg enzyme could be achieved. An alternative strategy could involve the co-administration of O_2 solubilizing/dispersing scaffolds such as perfluorocarbon emulsions or O_2 microbubbles. However, preliminary *in vitro* ethanol metabolism tests only showed a modest improvement and likely a better understanding of the preparation of such O_2 solubilizing/dispersing scaffolds and O_2 delivery would be required to significantly impact the ethanol metabolism. Also, the enzymes ADH and ALDH could be considered as in this case O_2 does not participate in the ethanol metabolism, and their smaller molecular weight may additionally simplify the liposome loading procedure. While this adaptation would also bear the advantage of reduced acetaldehyde toxicity [40] by its further metabolism to acetate by ALDH, strategies to maintain sufficient amounts of NAD, the cofactor required ADH and ALDH metabolism, within the peritoneal cavity would have to be developed.

Accordingly, there seems to be significant hurdles remaining to achieve an increased ethanol clearance by LSEPD and further optimization of the system is required. Still, we believe that the presented platform may be used as a detoxification strategy for other indications if the

appropriate enzymes are employed. Having previously demonstrated prophylactic *in vivo* efficacy following intravenous administration [229,286,287], butyrylcholinesterase (BChE) or organophosphate hydrolases could be used to combat exposure to neurotoxic organophosphates, and BChE could additionally be employed for cocaine detoxification. The use of BChE for organophosphate scavenging may be limited by the underlying stoichiometric binding thereby requiring high enzyme doses [191,288]. Cocaine detoxification on the other hand could provide a more viable approach as recent studies have shown high efficacy with engineered BChE capable of protecting mice from a lethal dose of cocaine [229]. Alternatively, this technology may also be used as an enzyme replacement therapy with the use of uricase for hyperuricemia or phenylalanine ammonia lyase for phenylalanine hydrolase deficiency. Even though uricase is an oxidase, the O_2 availability would likely be less problematic as pathologic uric acid concentrations are in the sub-millimolar range. Nonetheless, a close examination of the enzyme's functionality and the clinical safety would have to be investigated since the repetitive application during enzyme replacement therapy could lead to immunogenicity. All in all, for an effective adoption of LSEPD the substrate or toxin should demonstrate adequate membrane permeability and at the same time be sufficiently water-soluble to end up in the dialysate fluid in adequate concentrations.

In conclusion, a detailed *in vitro* (**Chapter 3**) and *in vivo* (**Chapter 4**) characterization of LSEPD was presented, and the main results extracted from this thesis are summarized in **Table 5.1**. The key findings entail the successful immobilization of enzymes onto lipid bilayers, which increased their thermo-stability (in particular of AO) and reduced their systemic exposure and organ deposition without compromising their catalytic activity. A rodent model to investigate PD for acute alcohol poisoning was established and the increased ethanol metabolism with LSEPD witnessed *in vivo*. This contribution signifies that enzymatic reactions can take place within the peritoneal cavity and illustrates how conventional PD can be engineered to endow this modality with increased metabolism.

Table 5.1: Overview of the key findings of this thesis grouped by *in vitro* and *in vivo* implications.

	In vitro	*In vivo*
 Free enzymes	• CAT is stable, AO susceptible to activity loss at 37 °C. • O_2 dependent metabolism highlighted.	• CAT is stable, AO susceptible to activity loss in peritoneal cavity. • Higher systemic exposure, and organ deposition. • *Metabolism not investigated, to minimize interference caused by systemically exposed enzymes.*
 E-Liposomes	• Improved thermostability with AO-Liposomes at 37 °C. • Metabolism significantly enhanced over free enzymes.	• Maintained activity of AO-Liposomes during PD. • Reduced systemic exposure (0.3 - 0.6% after 3 h), and organ deposition. • Ethanol metabolism increased, though systemic ethanol unchanged.

Appendix

Chapter 3

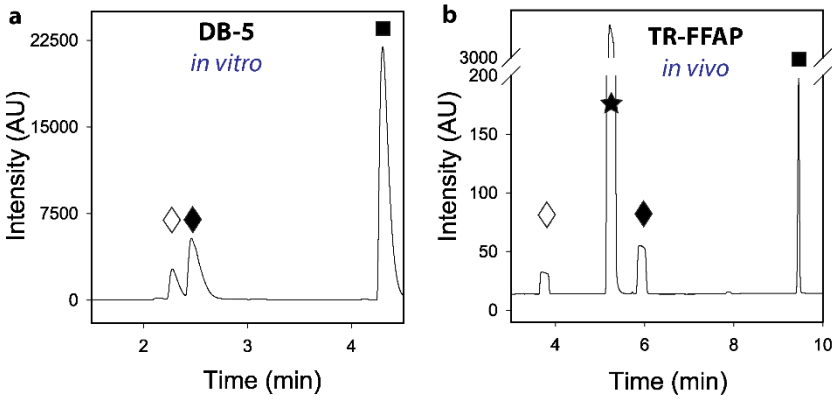


Figure A3.1: Detection of volatile metabolites by gas chromatography. The separation of acetaldehyde (open diamond), ethanol (filled diamond), isoflurane (star) and 1-butanol (filled square) is shown for the **(a)** DB-5 column and **(b)** TR-FFAP column. Metabolites were clearly separated, allowing accurate measurement. A representative elution profile is shown for each column.

Table A3.1: Overview of gas chromatography instrumentation and elution methods applied for ethanol and acetaldehyde quantitation.

Instrument	Sample type	Column	Oven T (°C)	Injection T (°C)	Split ratio	Detection T (°C)	Internal standard	Flow (mL/ min)	LOD, LOQ Ethanol (mM)	LOD, LOQ ACD (mM)
GC-2014	*In vitro*	DB-5	30 - 100	220	19	300	10 mM 1-butanol	1.85	1.5, 4.5	0.52, 1.58
Focus-GC	*In vitro*	Rtx-BAC1	40	200	19	220	10 mM isopropanol	1	1.3, 3.9	0.02, 0.05
HP6890	*In vivo*	TR-FFAP	55 - 130	120	0.1:1 (*split-less*)	250	5 mM 1-butanol	2	0.6, 1.7	0.05, 0.17

T: temperature, ACD: acetaldehyde

Chapter 4

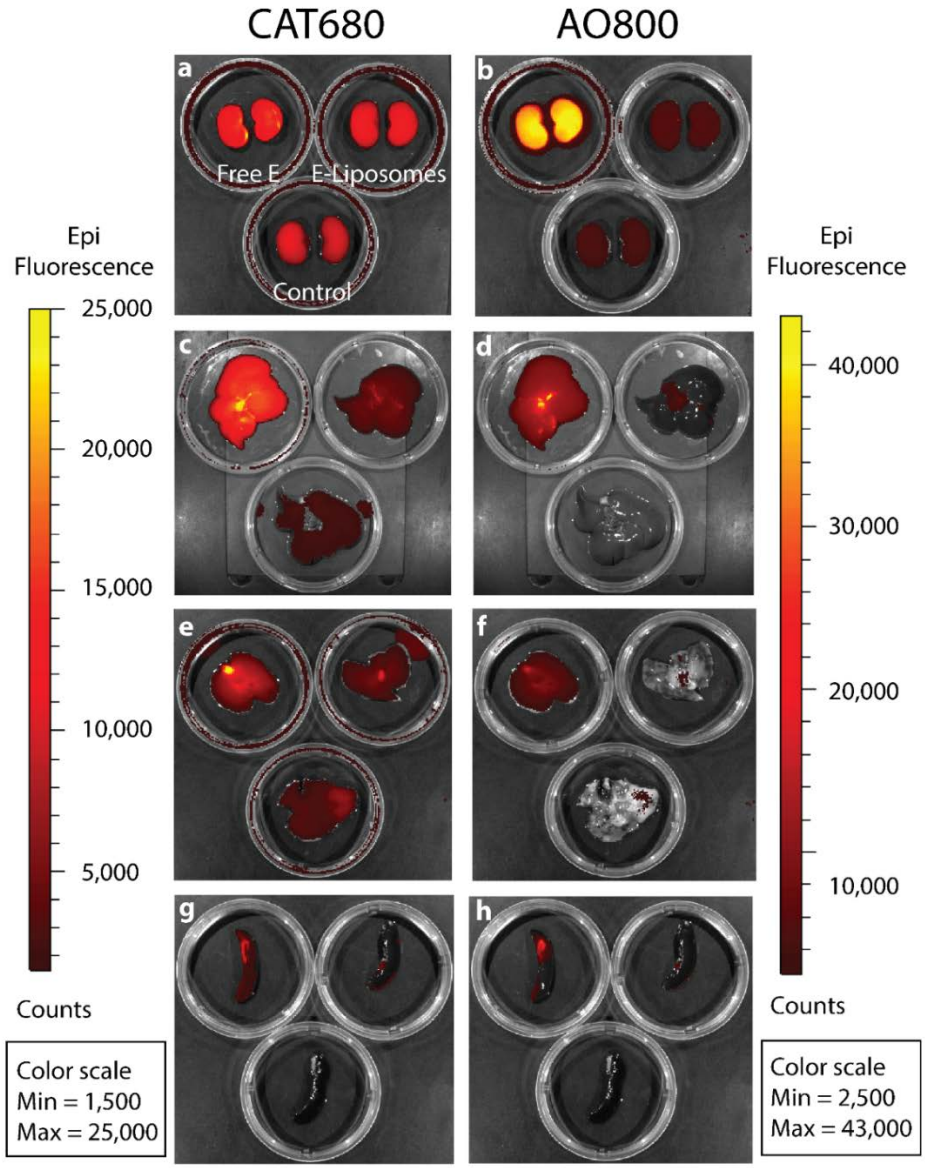


Figure A4.1: Fluorescence imaging of harvested organs. Same animals as in Figure 4 and 5. Kidneys **(a, b)**, liver **(c, d)**, lungs **(e, f)** and spleen **(g, h)** harvested from animals injected with peritoneal dialysate containing fluorescently labelled free enzymes (Free E) and E-Liposomes are contrasted to organs harvested from rats injected with PD fluids free of fluorescently labelled enzymes. Organs from Free E are shown on the upper left corner of each image, E-Liposomes on the upper right and control organs below, as indicated in **(a)**. Each organ was imaged twice, first at $\lambda_{Ex/Em}$: 640/700 to detect CAT680 (left panel) and second at $\lambda_{Ex/Em}$: 745/800 to detect AO800 (right panel). Organs from animals injected with Free E exhibit visibly more fluorescence than those injected with E-Liposomes. For each group a representative set of organs is shown. Color scale on the left refers to images on the left panel (CAT680) and color scale on the right pertains to images on the right panel (AO800), and is identical for all images recorded at the same wavelength.

Chapter 5

Table A5.1: Overview of recommended PD component concentration range as adapted from the European Pharmacopoiea [281].

	Concentration (mM)
Sodium	125 – 150
Potassium	0 – 4.5
Calcium	0 – 2.5
Magnesium	0.25 – 1.5
Acetate and/or lactate and/or hydrogen carbonate	30 – 60
Chloride	90 – 120
Glucose	25 – 250

List of Abbreviations

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AO	Alcohol oxidase
BEC	Blood ethanol concentration
CAT	Catalase
CHF	Congestive heart failure
CO_2	Carbon dioxide
CYP	Cytochrome P450
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE-NHS	N-(Succinimidyloxy-glutaryl)-L-α-dioleoylphosphatidylethanolamine
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[azido(polyethylene glycol)-2000]
E-Liposomes	Enzyme-loaded liposomes
ESRD	End-stage renal disease
Free E	Free enzymes
HA	Hyperammonemia
HD	Hemodialysis
Hm	Hydrophobic modification
H_2O_2	Hydrogen Peroxide
HPLC	high-performance liquid chromatography
IVIS	*In vivo* imaging system
K_M	Michaelis-Menten constant

KPi	Potassium Phosphate
LOD	Limit of detection
LOQ	Limit of quantitation
LSEPD	Liposome-supported enzymatic peritoneal dialysis
LSPD	Liposome-supported peritoneal dialysis
MEOS	Microsomal ethanol oxidizing system
NaCl	Sodium Chloride
NAD	Nicotinamide adenine dinucleotide
NHS	N-Hydroxysuccinimide
O_2	Oxygen
OMB	O_2 microbubbles
PD	Peritoneal dialysis
pMCAO	permanent middle cerebral artery occlusion
PMNL	polymorphonuclear leukocytes
TBAB	tetrabutylammonium bromide
TFA	Trifluoroacetic acid
WHO	World Health Organization

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