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Research Article

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Polymyxin-Coated Nanostructured Materials: An Option for Sepsis Treatment

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Abstract

Objective: Endotoxins (lipopolysaccharides, LPS) are among the main targets in extracorporeal therapies. LPS, which is the major component of the outer cell wall of Gram-negative bacteria, strongly induces inflammatory responses in humans at concentrations lower than 1 ng/kg body weight. Although the elimination of LPS is promising for the supportive therapy of sepsis and liver failure, endotoxin neutralization using endotoxin adsorbents is controversial. Earlier studies show that Polymyxin B (PMB) could be applied for endotoxin inactivation in blood. Aim of this study was to establish an adsorbent-based PMB release system which ensures a constant PMB level in plasma during extracorporeal therapies.

Methods: A polystyrene-divinylbenzene based cytokine adsorbent (CG161c) with nanostructured pores was coated with a defined amount of PMB by hydrophobic interactions. The PMB release by the PMB coated adsorbent was studied in plasma and fractionated plasma.

Results: In plasma or blood, an equilibration between the free and bound form of PMB leads to a constant PMB level in plasma. The PMB release was influenced by the adsorbent inner surface area and the protein concentration of the plasma. In fractionated plasma where the protein concentration is lower, the PMB release was much less than in whole plasma. Additionally we could show that the PMB coating doesn´t influence the cytokine removal of the CG161c adsorbent.

Conclusion: Our *in vitro* model shows that the combination of cytokine removal and controlled PMB release by the same adsorbent could be an option for Gram-negative sepsis treatment.

Keywords

Endotoxin; Nanostructured Materials; Polymyxin; Multidrug resistant

Introduction

Endotoxins are lipopolysaccharides (LPS) which form the outer cell wall of Gram-negative bacteria and are released by cell lysis and during proliferation. Lipopolysaccharides are pyrogenic substances and can cause strong inflammatory response in human. Endotoxin accumulation in the blood circulation can result in uncontrolled activation of leucocytes and can lead to a disorder of the coagulation system [1,2]. In further consequence this can cause sepsis, severe sepsis or septic shock where the mortality rate, as a function of the severity of the disease, is between 30-60%. Endotoxemia can also occur in patients with impaired immune defense which can be a consequent of chemotherapy or liver failure [3,4]. For sepsis treatment, antibiotics against infection and corticosteroids to restore cardiovascular homeostasis and terminate systemic inflammation are used beside conventionally applied intensive care. Polymyxin B (PMB) is a peptide based antibiotic especially applied to treat infections caused by multidrug resistant Gram-negative bacteria. According to the LPS type, PMB build a very stable complex, which has much lower endotoxin activity compared to free LPS. The association constant (K_a) of the PMB-LPS complex depends of the LPS type and has a value between 1.8×10^{-6} and 2.3×10^{-6} M [5]. By establishing this stable complex, PMB is able to inactivate endotoxins very effectively. The dose for LPS-inactivation in human blood is much lower than the Minimal Inhibitory Concentration (MIC) of this antibiotic which is in the range of 2 to 8 mg/L [6]. Because high PMB serum levels can cause nephrotoxic effects, intravenous administration was avoided the last 50 years and consequently knowledge about pharmacokinetics and pharmacodynamics of Polymyxins is very limited. Although some clinical studies were focused on endotoxin inactivation, the applied PMB concentration was as high as normally used in order to kill bacteria [7,8]. Recently we could show that the optimal PMB concentration in human plasma is between 50 and 200 ng/ml [9]. However, a big challenge is not only to reach and hold the desired PMB level by intravenous administration, but also the lack of a quick and solid clinical test for PMB quantification in blood.

Endotoxins are among the main targets in extracorporeal blood purification therapies. There are two endotoxin adsorbents for clinical use available, the Toraymyxin and the Alteco endotoxin adsorbent cartridge. In recently published *in vitro* experiments we could show that the efficiency for LPS removal for these adsorbents is limited [10]. Since endotoxins are negatively charged molecules, anion exchange resins (e.g. DEAE or PEI groups bound to cellulose) are able to bind LPS by ionic interactions to the phosphate groups of the Lipid A domain of the LPS [11]. The reason why anion exchangers are not established in blood purification systems is due the unwanted side effects. By removal of anionic charged proteins like some coagulation factors (protein C, protein S and fibrinogen) they can cause coagulation, consequently, anion exchangers lack the needed biocompatibility for blood purification systems.

Aim of this study was to establish an adsorbent-based PMB release system which ensures a constant PMB level in plasma during extracorporeal therapies. A polystyrene-divinylbenzene based cytokine adsorbent with nanostructured pores [12] was coated with a defined amount of PMB by hydrophobic interactions. In plasma or blood, an equilibration between the free and bound form of PMB will lead to a constant PMB level in plasma.

Materials and Methods

Materials

The polystyrene-divenylbenzene based adsorbents used in this study were obtained from Dow Chemical (former Rohm and Haas, Philadelphia, Pa, USA). Polymyxin B and lipopolysaccharide (LPS)

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from *Escherichia coli* O55:B5 was purchased from Sigma-Aldrich (Vienna, Austria). Human plasma we got from a local plasma donation centre (fresh-frozen plasma) and Cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) were purchased from R&D Systems, (Minneapolis, MN).

Adsorbent characterization

The size distributions of the used adsorbents were measured by using a laser light scattering particle size analyser (Mastersizer 2000, Malvern Instruments, UK). Therefore 500 µl of adsorbents were suspended in 100 ml distilled water and stirred during sonication to avoid agglomeration. The particle size distribution results are volume based and expressed in terms of equivalent spheres. The structural features of each adsorbent were determined by scanning electron microscopy (SEM). SEM images were obtained by washing the adsorbent particles with pure ethanol and dry these in a heating cabinet at 100°C for 12 hours. The particles were then sputtered with gold (Q150R ES, QUORUM) and imaged by SEM (TM-1000, Tabletop Microscope, Hitachi).

PMB coating of the adsorbent material

Before the experiments were carried out, the adsorbents were washed with ethanol, water and 0.9% saline solution for 60 min using 4 ml washing volume per ml adsorbent for each washing step. PMB coating of the adsorbent was done by incubation of 3 ml of adsorbent with different amounts of PMB solution containing 10 mg PMB/ml (Table 1). The coating was performed for 12 hours on a roller mixer at room temperature. Thereafter, the coated adsorbents were washed twice with physiological saline solution and were finally stored in a 50% (w/v) suspension at 4°C before they were used for adsorption experiments. To determine if the whole PMB is hydrophobically bound on the adsorbent, PMB quantification of the coating and washing solutions was conducted by an HPLC based method.

PMB-quantification

The concentration of PMB in aqueous solutions was determined by a high performance liquid chromatographic method [13] with minor changes as described earlier [10]: Two millilitre samples (washing solution) were transferred to a solid-phase extraction C18 cartridge (Sep-Pak, Waters). After the cartridge was washed with 500 µl of carbonate buffer (1%, w/w, pH 10), 110 µl of 9-fluorenylmethyl chloroformate (FMOC-Cl, Sigma) solution (containing 30 µl of 100 mM FMOC-Cl in acetonitrile and 80 µl of methanol) was added. Following 10 min of reaction in the dark, the PMB derivatives were eluted with 900 µl of acetone. The eluate was mixed with 600 µl of boric acid (0.20 M) and 500 µl of acetonitrile. After vortexing, 20 µl of the eluate was injected onto the HPLC column (50 × 4.6 mm Onyx Monolithic C18 column coupled with a 4 × 3.0 mm C18 guard column, Phenomenex). The mobile phase with a flow rate of 1 ml/min was acetonitrile-tetrahydrofuran-water (50:25:25) and the run time was 10 min. Fluorescence detection was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm. The concentrations of PMB were calculated on the basis of the sum of the chromatographic peak areas of Polymyxin B1 and B2 in the HPLC assay. The limit of quantification was 0.025 mg/l. The quantification of PMB in blood, plasma and serum was measured using a competitive enzyme immunoassay kit for the analysis of Polymyxin B and E (Kwinbon Biotech, Beijing, China) with a detection limit of 1 ng/ml. To obtain a signal in the standard range between 0 and 81 mg/ml, the samples were diluted with the dilution reagent which was provided with the ELISA kit.

Table 1: PMB coating procedure of the PS-DVB based adsorbents.

Adsorbent [ml]	PMB solution 10 mg/ml [ml]	0.9 % NaCl solution [ml]	PMB/g adsorbent [mg]
3	0.00	10.00	0.0
3	0.30	9.70	1.0
3	0.75	9.25	2.5
3	1.50	8.50	5.0
3	4.5	5.5	15
3	7.5	2.5	25

PMB desorption experiments in plasma vs fractionated plasma

To illustrate that the amount of PMB desorption depends on the protein composition in the medium, desorption experiments of the PMB coated CG161c were carried out in plasma and fractionated plasma. Fractionated plasma was produced using the Alfuflow® filter (Fresenius Medical Care, Germany) which is a plasma filter with a sieving coefficient for albumin of ≥ 60% and a molecular weight cut-off of about 250 kDa [14]. This specially designed albumin-permeable filter is used in the Prometheus® system which is an extracorporeal liver support system where the detoxification of the blood takes place in a secondary circuit (fractionated plasma) by two adsorbent cartridges. For these PMB desorption experiments, one ml adsorbent coated with different amounts of PMB was incubated in 9 ml plasma or fractionated plasma for 60 min at 37°C on a roller mixer. Thereafter, the samples were centrifuged and the supernatants were stored at -80°C until PMB quantification was conducted using the HPLC method and the ELISA kit.

PMB desorption experiments as a function of the adsorbent surface area

To demonstrate that the desorption of PMB is not only effected by the adsorbent material but also by the available surface of the adsorbent, adsorbents with different porosity and surface areas were used for the desorption experiments. The CG161c with 900 m^2/g and the HPR10 with 600 m^2/g inner surface were coated with different amounts of PMB (0, 5, 10, 15, 20 and 25 mg PMB/ml adsorbent) using the coating protocol described above. Desorption experiments in plasma were conducted by incubation of 1 ml PMB coated adsorbent in 9 ml plasma for 60 min at 37°C. Thereafter, PMB in the supernatant was quantified.

Endotoxin inactivation experiments

To determine the PMB level which is needed for endotoxin inactivation, fresh human heparinized plasma spiked with 5 ng/ml LPS from *E. coli* was incubated with different PMB concentrations (0, 10, 50, 100, 500, 1000 and 1000 ng/ml) for 60 min at 37°C. The experiments were performed in pyrogen-free 3 ml glass vials and the endotoxin activity was measured using the Limulus Amoebocyte Lysate (LAL) test (Charles River, Oxford, UK). To determine the LPS inactivation by PMB which is released from the PMB coated adsorbent, batch tests using PMB coated CG161c adsorbent were performed. Therefor, 0.5 ml of different PMB coated adsorbent (0, 2.5, 5, 10, 15, 20 and 25 mg PMB/ml adsorbent) were incubated in 4.5 ml plasma spiked with 5 ng/ml LPS. The incubation was carried out on a roller mixer for 60 min and thereafter the LPS activity was measured in the supernatant using the LAL test.

Cytokine removal experiments

To check if the PMB coating of the CG161c adsorbent

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influences the cytokine adsorption, cytokine removal batch tests were performed. Plasma was spiked with selected cytokines to achieve a concentration between 250 and 500 pg/ml for each (Table 2). One ml PMB coated adsorbent was incubated in cytokine spiked plasma for 60 min at 37°C on a roller mixer. Samples were centrifuged and the supernatants were collected and stored at -80°C until cytokine quantification using the Bio-Plex cytokine array (Biorad, Vienna, Austria).

Results and Discussion

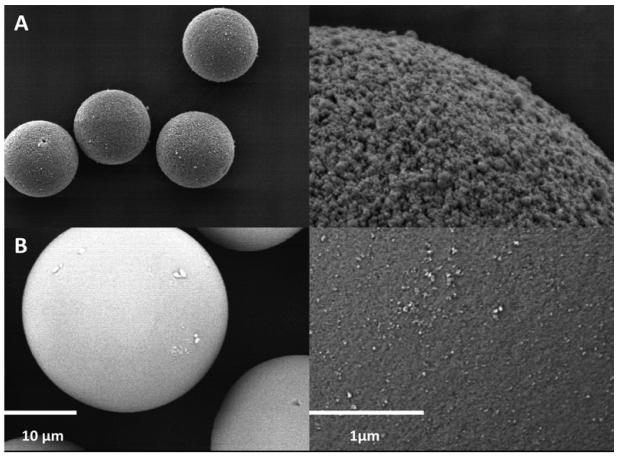
The SEM images (Figure 1) show the pore structures of the adsorbents surfaces. The pictures clearly show that the HPR10 adsorbent has a rougher pore structure than the CG161c adsorbent. These findings confirm the manufacturers which quote 30 nm pores and an inner surface of 600 m^2/g for the HPR10 adsorbent and 15 nm pores and an inner surface of 900 m^2/g for the CG161c adsorbent. Adsorbents with very high inner surface have to be loaded with higher amounts of PMB to obtain the desired PMB concentration in plasma. Consequently, the CG161c adsorbent can maintain the desired PMB concentration much longer.

The adsorption by adsorbent based extracorporeal blood purification systems can take place in blood, plasma or fractionated plasma. Fractionated plasma can be produced by specially designed plasma filters (e.g. Albuflow®) which have membranes with lower molecular weight cut-off and are for example used in the Prometheus® system. Due to the fact that this filter excludes the high molecular weight plasma fraction, the plasma composition and also the hydrophobicity of fractionated plasma is totally different to whole plasma. In our experiments we compared the PMB release of the CG161c adsorbents in plasma to fractionated plasma. The results clearly show that in fractionated plasma, where the hydrophobicity is lower, the PMB release is strongly reduced (Figure 2). To give an example: After incubation of PMB coated CG161c (25 mg PMB/ml CG161c) the PMB level in plasma was 7594 ng/ml whereas in fractionated plasma only 165 ng PMB/ml was measured. The results of these experiments suggest that the PMB release into plasma depends on the protein concentration. It makes a big difference whether the PMB coated adsorbent is used in plasma or in fractionated plasma where the hydrophobicity is much lower.

Because the equilibrium between adsorption and desorption of PMB depends not only on the material composition but also on the material property and especially on the available surface for the adsorbate we compared the PMB release between the CG161c with 900 m^2/g surface and the HPR10 with 600 m^2/g. Although both adsorbents are polystyrene-divinylbenzene based, the CG161c shows strongly reduced PMB release into plasma after coating compared to the HPR10 (Figure 3). The results clearly show that the adsorption and desorption is a function of the ratio of PMB concentration to adsorbent surface. The PMB coating will basically work with all hydrophobic based adsorbent material, but to get a desired PMB concentration in plasma, only the PMB loading per gram adsorbent material differs as a function of the material and the available inner surface of the adsorbent.

Table 2: Plasma concentrations of selected cytokines for the cytokine removal experiment.

Cytokine	Plasma concentration [pg/ml]
TNF-α	500
IL-1β	250
IL-6	200
IL-8	200
IL-10	300



Adsorbent	*Polymer type*	*mean diameter [µm]*	*d_{pore} [nm]**	*surface area [m^2/g]**
A HPR10	PS-DVB	10	30	600
B (CG161c)	PS-DVB	120	15	900

* Manufacturer data

Figure 1: SEM images at 200 and 5000 x magnification and particle size distribution using laser-light scattering of the HPR10 (A) and CG161c (B) adsorbents.

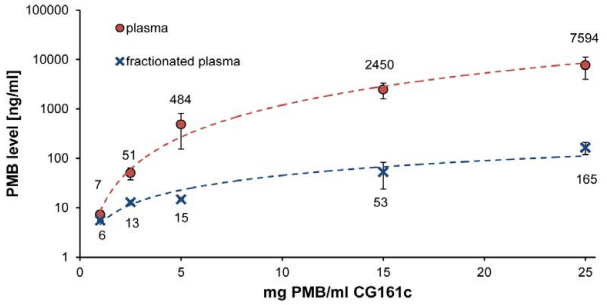


Figure 2: Comparison of PMB desorption from PMB coated CG161c adsorbent into plasma and fractionated plasma. The results are shown in mean ± SD (n=3).

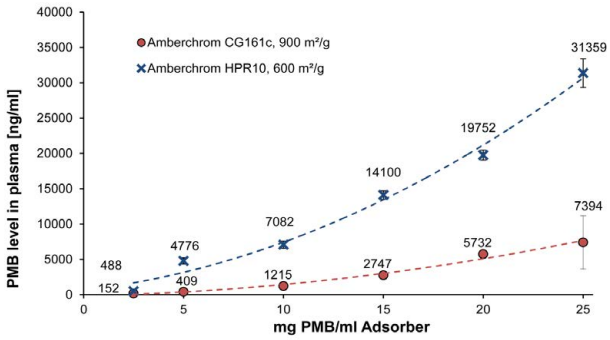


Figure 3: Comparison of PMB release into plasma between the CG161c adsorbent with an inner surface of 900 m^2/g and the HPR10 adsorbent with 600 m^2/g. Both adsorbent were coated with different PMB amounts and incubated in plasma. After incubation PMB quantification was done. The results are shown in mean ± SD (n=3).

Because, when using a PMB coated adsorbent, the constant PMB level in plasma is maintained by a permanent balancing act between adsorption and desorption of PMB on and from the adsorbent surface, a question arises: Does the permanent ad- and desorption of PMB influence the LPS inactivation? To answer this open question we compared the LPS inactivation between released and infused PMB. The results show that the presence of adsorbents does not influence the LPS inactivation by the released PMB (Figure 4).

To verify if the PMB coating influences the cytokine removal of the CG161c adsorbent, we compared PMB-coated and uncoated adsorbent regarding cytokine removal in plasma. The experiments suggest that the PMB coating of the CG161c adsorbent doesn´t influence the cytokine removal which can take place in parallel (Figure 5).

In general, for safe and optimal endotoxin inactivation therapy by intravenous PMB administration, further systematic investigations concerning pharmacokinetic studies and drug monitoring should be performed. Because of the fact that Polymyxins are small molecules, they are removed by hemodialysis [7] and even by adsorption. Consequently the clearance of the dialyzer and/or the adsorption unit has to be taken into account when PMB is applied simultaneously with extracorporeal blood purification. Unlike to intravenous PMB treatment, a PMB desorption model for endotoxin inactivation during extracorporeal blood purification can be realized in different systems and guarantee the maintenance of the desired PMB level during treatment. One example is shown in Figure 6 where a PMB coated adsorbent is used in a closed plasma circuit system. This PMB release system is not limited for adsorbents used in plasma. For example the

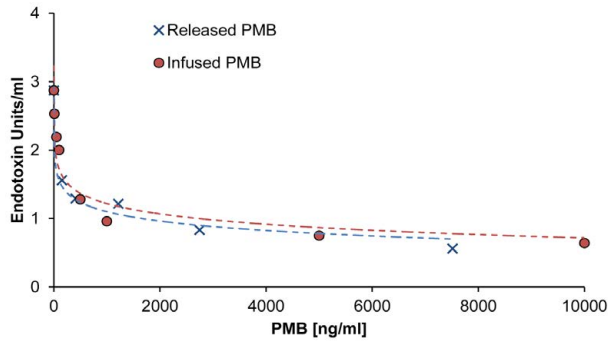


Figure 4: Endotoxin inactivation in plasma was compared between added PMB and the PMB coated adsorbent. The results are shown in mean (n=3).

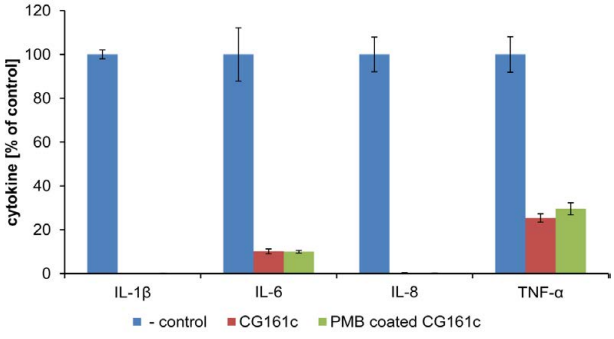


Figure 5: To test if PMB coating influences the cytokine adsorption, the CG161c and the PMB coated CG161c where compared regarding cytokine removal from plasma. The results are shown in mean ± SD (n=3).

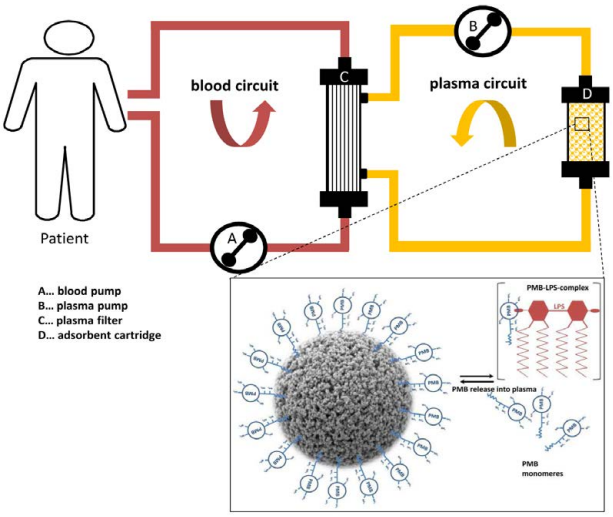


Figure 6: Setup of a blood purification system combining cytokine removal by adsorption and inactivation of lipopolysaccharides by released Polymyxin B. The system consists of a blood pump (A), a plasma pump (B), a plasma filter (C) and an adsorbent cartridge (D) containing the PMB-coated adsorbent.

clinically proofed Cytosorb®, which is a polystyrene-divinylbenzene based cytokine adsorbent and used in hemoperfusion, can also be coated with PMB. *In vitro* tests will be necessary to validate how much PMB should be bound per ml adsorbent to get the desired PMB level in blood for endotoxin inactivation. Other possibilities to maintain equilibrium of PMB during extracorporeal sepsis treatment could be the use of PMB containing dialysate. Because of the nephrotoxicity of PMB, other LPS inactivating antimicrobial peptides (AMP) can be used for such a release system. Basic requirements for AMP are the binding on the used adsorbent by hydrophobic interactions. Promising AMP for future applications could be alkylated lactoferrin derived peptides [15,16]. Our *in vitro* model shows that the combination of cytokine removal and controlled PMB release for endotoxin inactivation by the same adsorbent is possible. This combination may be an option for Gram-negative sepsis treatment.

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