

Research Article

Macroporous Composite Cryogels with Embedded Polystyrene Divinylbenzene Microparticles for the Adsorption of Toxic Metabolites from Blood

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Composite monolithic adsorbents were prepared by the incorporation of neutral polystyrene divinylbenzene (PS-DVB) microparticles into macroporous polymer structures produced by cryogelation of agarose or poly(vinyl alcohol). The composite materials exhibited excellent flow-through properties. Scanning electron microscopy of the composite cryogels revealed that the microparticles were covered by thin films of poly(vinyl alcohol) or agarose and thus were withheld in the monolith structure. Plain PS-DVB microparticles showed efficient adsorption of albumin-bound toxins related to liver failure (bilirubin and cholic acid) and of cytokines (tumor necrosis factor- α and interleukin-6). The rates of adsorption and the amount of adsorbed factors were lower for the embedded microparticles as compared to the parent PS-DVB microparticles, indicating the importance of the accessibility of the adsorbent pores. Still, the macroporous composite materials showed efficient adsorption of albumin-bound toxins related to liver failure as well as efficient binding of cytokines, combined with good blood compatibility. Thus, the incorporation of microparticles into macroporous polymer structures may provide an option for the development of adsorption modules for extracorporeal blood purification.

1. Introduction

Monolithic cryogels are produced in a semi-frozen aqueous state by radical polymerization of water-soluble monomers or by cross-linking of water-soluble polymers [1–3]. Ice crystals grow during freezing and finally occupy about 90% of the volume, while the chemical reactions take place in a highly concentrated unfrozen liquid microphase. After defrosting, voids appear as a result of ice melting and form an interconnected macroporous polymer structure (MPPS) with pore diameters up to 200 to 300 μm , whereas the pore walls are represented by thin and dense sheets of the cross-linked polymer. Cryogel monoliths are typically elastic, spongy, and

highly permeable for aqueous media. Various applications of cryogels in biotechnology and biomedical sciences have been reported [1, 4–7]. In particular, cryogels and hydrogels based on poly(vinyl alcohol) and agarose are low-cost, nontoxic, and biocompatible polymers and have been employed for the production of biomaterials and cell culture scaffolds [8–13]. Both PVA- and agarose-based cryogels are characterized by supermacroporous morphology, but differences in their overall properties exist. Cryogels made of agarose are rigid and brittle, whereas PVA-based cryogels possess a high degree of elasticity and toughness. A series of reactive and functional cryogels was developed [3–5], including cryogels capable of removing toxins from protein solutions [14, 15].

Here, we report on the application of PVA- and agarose-based MPPS to remove toxic metabolites related to liver failure and cytokines from blood plasma.

Liver failure is associated with a diminished detoxification capability of the liver, resulting in the accumulation of hydrophobic toxic metabolites in the blood. Metabolites such as bile acids, bilirubin, tryptophan, and phenolic compounds are albumin-bound, and thus, they cannot be removed by diffusive or convective extracorporeal blood purification systems, but require adsorptive techniques for depletion [16, 17]. Extracorporeal liver support systems apply columns containing polystyrene divinylbenzene (PS-DVB) copolymers to remove toxic metabolites via hydrophobic interactions [18–20]. In particular, neutral PS-DVB copolymers of adequate porosity (8–9 nm pore diameter) are efficient adsorbents for albumin-bound toxic metabolites [21]. In addition to extracorporeal blood purification, the depletion of toxic metabolites from human plasma is also relevant for research purposes. This was demonstrated by *in vitro* studies investigating the effect of the removal of toxic metabolites related to liver failure from plasma on cultured hepatocytes [22]. These and similar research applications require simple adsorbent devices with low resistance to the flow of plasma in combination with the fast adsorption of toxins. The latter can be achieved by using small adsorbent particles, providing a large outer surface area. However, the dense packing of small particles supported by filters of adequate porosity creates significant flow resistance. Therefore, a promising approach to the design of effective adsorbents may be the incorporation of PS-DVB copolymer microparticles into highly permeable monolithic MPPS, which are well compatible with common low-pressure chromatography columns or syringes.

The aim of this study was to produce monolithic composite cryogels based on poly(vinyl alcohol) and agarose with embedded PS-DVB microparticles and to characterize them with respect to flow through, adsorption, mechanical stability and biocompatibility. Since the pore size distribution and the accessibility of the pores is critical for the adsorption characteristics of PS-DVB microparticles, we assessed the influence of the embedding procedure on the accessibility of the adsorbent pores by comparing plain PS-DVB particles and PS-DVB particles embedded into monolithic MPPS with respect to their adsorption characteristics for cytokines and hydrophobic metabolites related to liver failure.

2. Materials and Methods

2.1. Materials. Fresh frozen human plasma was obtained from a local plasma donation center (Retz, Austria). To remove cryoprecipitates, the plasma was centrifuged at 3,600 g for 10 min at room temperature after thawing. Alternatively, blood was drawn from healthy volunteers into tubes containing trisodium citrate (Greiner Bio-One, Kremsmünster, Austria) after informed consent of the donors. To obtain plasma, the blood was centrifuged for 10 min at 3,600 g without brake. Bilirubin, cholic acid, phenol, tryptophan, glutaraldehyde, and sodium cyanoborohydride were purchased from Sigma-Aldrich (Steinheim, Germany).

All other chemicals were obtained from Merck (Darmstadt, Germany). Poly(vinyl alcohol) (PVA, Mowiol 18–88) was purchased from Clariant (Frankfurt am Main, Germany) and agarose was obtained from Fluka Chemie (Buchs, Switzerland).

2.2. Polystyrene Divinylbenzene Copolymers. Polystyrene divinylbenzene copolymer microparticles were provided by Polymerics GmbH, Berlin, Germany [23]. The average pore size of the microparticles was 7.7 nm and the BET surface was 680 m² per g. The total pore volume of the particles was 1.37 mL/g, with a micropore volume (pores below 2 nm) of 0.27 mL/g, a mesopore volume (pores between 2 and 50 nm) of 0.88 mL/g, and a macropore volume (pores above 50 nm) of 0.22 mL/g. Prior to use, the adsorbent particles were washed in ethanol, distilled water, and 0.9% (w/v) saline solution, followed by centrifugation at 3,500 g for 5 min after each washing step. The size distribution was determined using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The average particle size was 85.5 μm.

2.3. Preparation of Cryogels. Monolithic cryogels used in this study are available from Protista (Bjuv, Sweden) under the trademark of MPPS (macroporous polymeric structures). Synthesis of the monolithic MPPS cryogels was carried out using a liquid bath cryostat (Proline RP 1840, Landa-Koenigshofen, Germany) and an air bath cryostat (Arctest Oy, Espoo, Finland). All gels were prepared in 2 mL syringes in order to obtain monoliths with a diameter of 9 mm and a length of 20 mm.

For the preparation of PVA cryogels, 4.5% (w/v) PVA solution in 0.1 M HCl was put into an ice bath and kept at 4°C for 30 min. Glutaraldehyde (50%, w/v) was added to the cooled solution at a final concentration of 1% (w/v) and the mixture was precooled for 60 min in the liquid bath cryostat at –12°C and subsequently transferred to the air bath cryostat where the samples were kept at –12°C, overnight. To prepare PVA-based composite cryogels, 125 mg PS-DVB microparticles (dry weight) were washed twice with 5% (w/v) PVA and once with 4.5% (w/v) PVA in 0.1 M HCl, followed by centrifugation at 6,700 g for 4 min after each washing step. The microparticles were resuspended in acidic 4.5% PVA solution to a final volume of 1.2 mL and the cryogels were produced as described above. After defrosting, the cryogels were washed with distilled water and the residual aldehyde groups were reduced by circulating 0.1 M NaBH₃CN solution in 0.1 M sodium bicarbonate buffer (pH 9.2) through the cryogels at a flow rate of 0.5 mL/min at 4°C for 4 h. Thereafter, the cryogels were extensively washed with distilled water.

To prepare monolithic agarose-based composite cryogels, agarose was dissolved at 3% (w/v) in boiling deionized water and the pH was adjusted to 12.5–13.0 with NaOH. The agarose solution was added to 125 mg PS-DVB microparticles, mixed and centrifuged at 14,100 g for 30–60 sec. The particles were resuspended in the agarose solution to a final volume of 1.2 mL and the suspension was transferred into 2 mL syringes. The cryogels were immediately placed into the liquid bath cryostat (–32°C, 30 min) and thereafter into the air bath

cryostat (-12°C , overnight). To prepare agarose cryogels, the above procedure was performed without the addition of microparticles. After defrosting, the cryogels were extensively washed with distilled water and stored in 20% (v/v) ethanol at 4°C .

2.4. Scanning Electron Microscopy. Discs of 4 mm thickness were cut from the top and bottom of the cryogels and fixed overnight in 2.5% (v/v) glutaraldehyde in 0.12 M sodium phosphate buffer (pH 7.2). Samples were dehydrated with increasing concentrations of ethanol (30, 50, 75, and 99.5% v/v), critical point dried (CPD 030 critical point dryer, BAL-TEC AG, Balzers, Liechtenstein), and coated with gold-palladium (40 : 60) using a Polaron SC7640 High Resolution Sputter Coater (Thermo VG Microtech, West Sussex, Great Britain). The samples were examined on a JEOL JSM-5600 LV scanning electron microscope at an acceleration voltage of 7 kV. The pore sizes of the cryogels were estimated based on these images.

2.5. Flow Characteristics of the Cryogels. The flow rate of water through particle-free and composite cryogels was assessed at different pressures. The hydrostatic pressure was adjusted to 1, 4, 8, and 15 kPa and the time and volume of water flowing through the monoliths were recorded to calculate the flow rate in mL/min.

2.6. Particle Leakage. Particle-free cryogels and composite cryogels were washed with 4-5 column volumes of deionized water. Subsequently, 25 mL of deionized water was passed through plain cryogels and composite cryogels at a flow rate of 1 mL/min. The quantity of particles released from the cryogels was determined in the flow through by light obscuration using a HIAC Royco device (Pacific Scientific Instruments, Oregon, USA).

2.7. Adsorption of Toxins Related to Liver Failure. Human plasma was spiked with $300\ \mu\text{M}$ bilirubin, $100\ \mu\text{M}$ cholic acid, $100\ \mu\text{M}$ tryptophan, and 2 mM phenol. Adsorption of bilirubin and cholic acid to the cryogels was assessed under static and dynamic conditions. For the static setup, plain cryogels and composite cryogels were cut into 4 mm slices, which were incubated with 7.65 mL of spiked plasma at 37°C with gentle shaking. For comparison, the equivalent amount of plain PS-DVB microparticles (125 mg) was incubated with spiked plasma. For the dynamic setup, 13 mL of spiked plasma was recirculated through the plain and composite cryogels at a flow rate of 1 mL/min. For both setups, samples were taken after 0, 15, 30, 60, and 120 min. After immediate centrifugation (18,600 g, 3 min), the supernatants were stored at -20°C until further analysis. Bilirubin, cholic acid, and albumin were quantified using a Hitachi 902 analyzer (Roche Diagnostics, Mannheim, Germany).

2.8. Adsorption of Cytokines. Recombinant human tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) were purchased from R&D Systems, Minneapolis, USA. Human

plasma was spiked with TNF- α and IL-6 at target concentrations of 500 pg/mL and 200 pg/mL, respectively. Particle-free cryogels and composite cryogels containing 125 mg PS-DVB microparticles were cut into slices (4 mm) and incubated at a 10% (v/v) microparticle-to-plasma-ratio at 37°C with gentle shaking. Samples were taken after 0, 15, and 60 min. For comparison, the equivalent amount of plain PS-DVB microparticles (125 mg) was incubated with spiked plasma as described above. Cytokines were quantified by ELISA (R&D Systems Quantikine, Minneapolis, USA).

2.9. Activation of Coagulation. The blood compatibility of PS-DVB microparticles, particle-free cryogels, and composite cryogels was assessed using two global coagulation tests, namely, prothrombin time (PT), which detects coagulation deficiencies in the extrinsic coagulation pathway, and activated partial thromboplastin time (aPTT), which detects coagulation deficiencies in the intrinsic coagulation pathway. Single 4 mm slices of the composite cryogels were incubated with plasma at a 10% (v/v) microparticle-to-plasma-ratio for 60 min at 37°C . Equivalent volumes of plain PS-DVB particles served as controls. Thereafter, the samples were centrifuged for 3 min at 18,600 g. PT and aPTT were determined from the supernatants using a Sysmex CA-500 coagulation analyzer (Sysmex Corporation, Kobe, Japan) and the reagents Dade Innovin and Dade Actin FS (both from Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany) according to the instructions of the manufacturers.

3. Results and Discussion

3.1. Characterization of Plain Cryogels. Scanning electron microscopy (SEM) revealed that the particle-free monolithic PVA cryogels possessed randomly shaped pores (Figures 1(a) and 1(b)). The majority of the pores had a diameter of 100–140 μm . In addition, smaller pores with a diameter of 10–60 μm were observed. Plain PVA cryogels had dense pore walls consisting of thin polymerized layers of PVA (Figure 1(b)). PVA cryogels yielded maximum flow rates of 3.6 mL/min at 8 kPa. At higher pressures, the flow rates did not increase due to mechanical compression of the gels, which was associated with increasing resistance to the flow (Table 1).

In contrast to PVA-based cryogels, agarose-based cryogels showed regular elongated pores with a length of several hundred micrometers and a width of about 60–70 μm . The monolithic agarose-based cryogels had very thin pore walls, from which small irregular protrusions were extending (Figure 2(a)). The surface of the pore walls was smooth and non-porous (Figure 2(b)). Agarose cryogels were less permeable than PVA cryogels. The flow rate through agarose cryogels reached a maximum of 2.9 mL/min at 4 kPa and decreased at a pressure of 15 kPa (Table 1).

3.2. Composite Cryogels. When developing composite cryogels, we aimed to embed significant amounts of PS-DVB microparticles to achieve high adsorption capacities. Suspending 125 mg PS-DVB microparticles in 1.2 mL of PVA solution corresponded to an approximately twofold weight

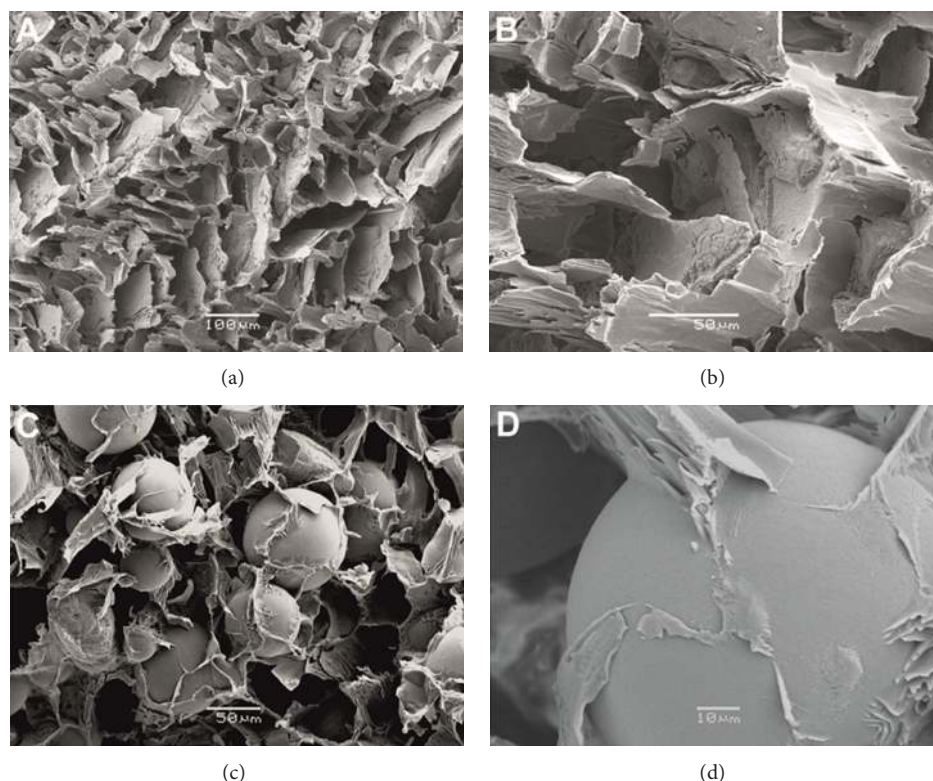


FIGURE 1: Scanning electron micrographs of plain PVA cryogels (a)-(b) and PVA-based composite cryogels loaded with 125 mg PS-DVB microparticles (c)-(d).

TABLE 1: Flow characteristics of particle-free and composite cryogels ($n \geq 4$).

Cryogel matrix	PS-DVB	Flow rate [mL/min]			
		1 kPa	4 kPa	8 kPa	15 kPa
PVA	—	1.0 ± 0.8	3.2 ± 1.2	3.6 ± 2.4	3.2 ± 1.8
	125 mg	0.5 ± 0.7	1.5 ± 1.4	2.4 ± 0.6	4.2 ± 1.6
Agarose	—	0.8 ± 0.2	2.9 ± 1.8	2.8 ± 1.8	1.8 ± 1.2
	125 mg	1.2 ± 0.7	4.0 ± 3.0	5.9 ± 4.8	6.7 ± 6.0

excess of PS-DVB microparticles over PVA. The pores within the resulting composite cryogels were in the range of 10–60 μm , and 40–60 μm pores were the most common (Figure 1(c)). Thus, the pore size was reduced as compared to particle-free PVA cryogels. Overall, the macroporous structure of plain cryogels significantly differed from that of composite cryogels, as embedded microparticles occupied the cryogel macropores.

The flow rates for PVA-based composite cryogels increased in a linear way with increasing pressure. The flow rate for PVA-based composite cryogels loaded with 125 mg microparticles was 4.2 mL/min at 15 kPa. For agarose-based composite cryogels the pore sizes varied between 10 and 150 μm (Figure 2(c)). Agarose-based composite cryogels showed improved mechanical stability in comparison to particle-free cryogels, as they could be tested at a pressure of 15 kPa, yielding flow rates of 6.7 mL/min when loaded with 125 mg of microparticles. In conclusion, the mechanical

strength of both PVA- and agarose-based cryogels was improved due to the integration of PS-DVB microparticles. Therefore, the composite cryogels allowed the testing at higher pressures and higher flow rates could be achieved as compared to plain cryogels. In the case of PVA composite cryogels, the PS-DVB microparticles were covered by PVA layers and showed a stable integration into the cryogel (Figures 1(c) and 1(d)). In contrast, the PS-DVB particles embedded into agarose-based cryogels appeared to be less tightly incorporated into the gel structure but were rather mechanically entrapped inside the pores (Figure 2(c)).

3.3. Particle Leakage. None of the effluents collected from the composite cryogels contained microparticles in the size range of 40–100 μm . Thus, the composite cryogels did not need filters to support the embedded microparticles, which were withheld inside the macroporous polymer structure.

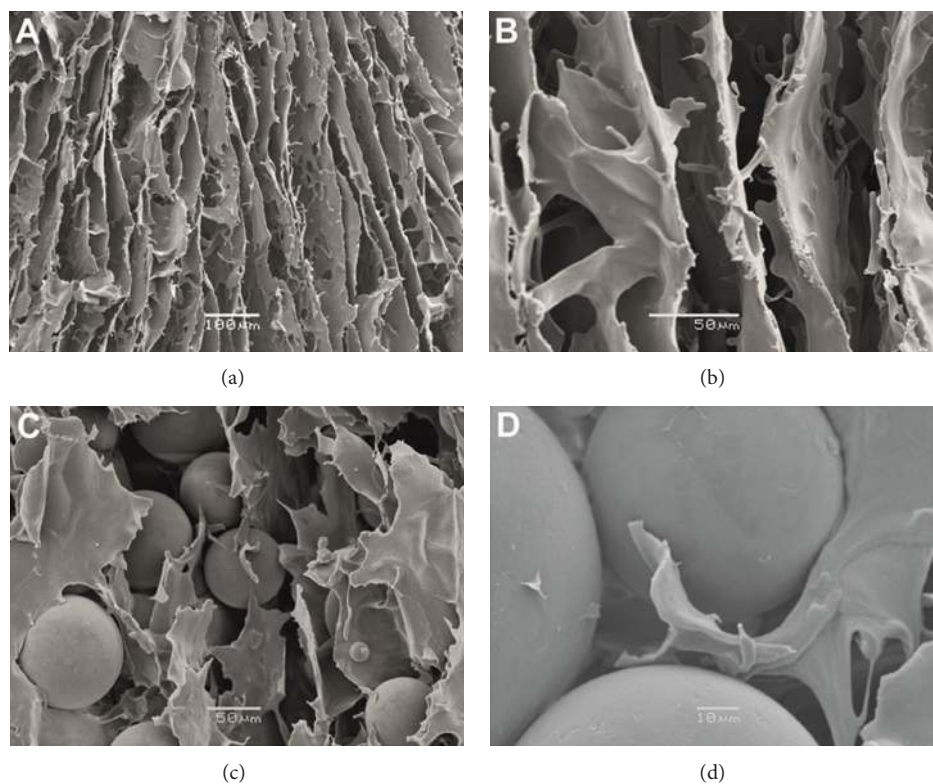


FIGURE 2: Scanning electron micrographs of plain agarose cryogels (a)-(b) and agarose-based composite cryogels loaded with 125 mg PS-DVB microparticles (c)-(d).

TABLE 2: Adsorption of bilirubin under static and dynamic conditions after 15 and 60 min ($n = 3$).

	Static		Dynamic	
	15 min	60 min	15 min	60 min
PS-DVB	3.3 ± 0.2	4.3 ± 0.2	n.a. ^a	n.a. ^a
PVA + 125 mg PS-DVB	0.5 ± 0.4	1.3 ± 0.3	0.6 ± 0.2	2.1 ± 0.7
Agarose + 125 mg PS-DVB	1.2 ± 0.4	2.8 ± 0.9	2.6 ± 1.9	5.0 ± 0.5

^aNot applicable.

3.4. Adsorption of Toxins Related to Liver Failure. The adsorption characteristics of parent PS-DVB microparticles and PS-DVB microparticles embedded into cryogels were investigated under static and dynamic conditions. Under static conditions, the adsorption of bilirubin was reduced by a factor of 3.3 and 1.5, respectively, for PS-DVB microparticles embedded into PVA- and agarose-based cryogels as compared to the parent microparticles (Table 2). Likewise, cholic acid adsorption was reduced by a factor of 4.5 and 3.4, respectively, for PS-DVB microparticles embedded into PVA- and agarose-based cryogels as compared to the parent PS-DVB microparticles (Table 3). Nevertheless, cholic acid was reduced to 10% and 6.6% of the initial concentration for PVA-based and agarose-based cryogels containing 125 mg microparticles, respectively.

The importance of convective mass transfer for the adsorption of toxins was reflected by the results of dynamic experiments where the adsorbed amounts of both, bilirubin,

TABLE 3: Adsorption of cholic acid under static and dynamic conditions after 60 min ($n = 3$).

	Cholic acid adsorbed per $\mu\text{mol/g}$ PS-DVB	
	Static	Dynamic
PS-DVB	$22.2 \pm 0.4^{\text{a}}$	n.a. ^b
PVA + 125 mg PS-DVB	4.9 ± 0.2	9.6 ± 0.5
Agarose + 125 mg PS-DVB	6.5 ± 0.3	9.4 ± 0.5

^aCalculated based on an assay with 1% (v/v) adsorbent-to-plasma-ratio.

^bNot applicable.

and cholic acid were generally higher than under static conditions (Tables 2 and 3). Agarose-based composite cryogels performed superiorly with respect to bilirubin removal than PVA-based composite cryogels ($5.0 \pm 0.5 \mu\text{mol/g}$ versus

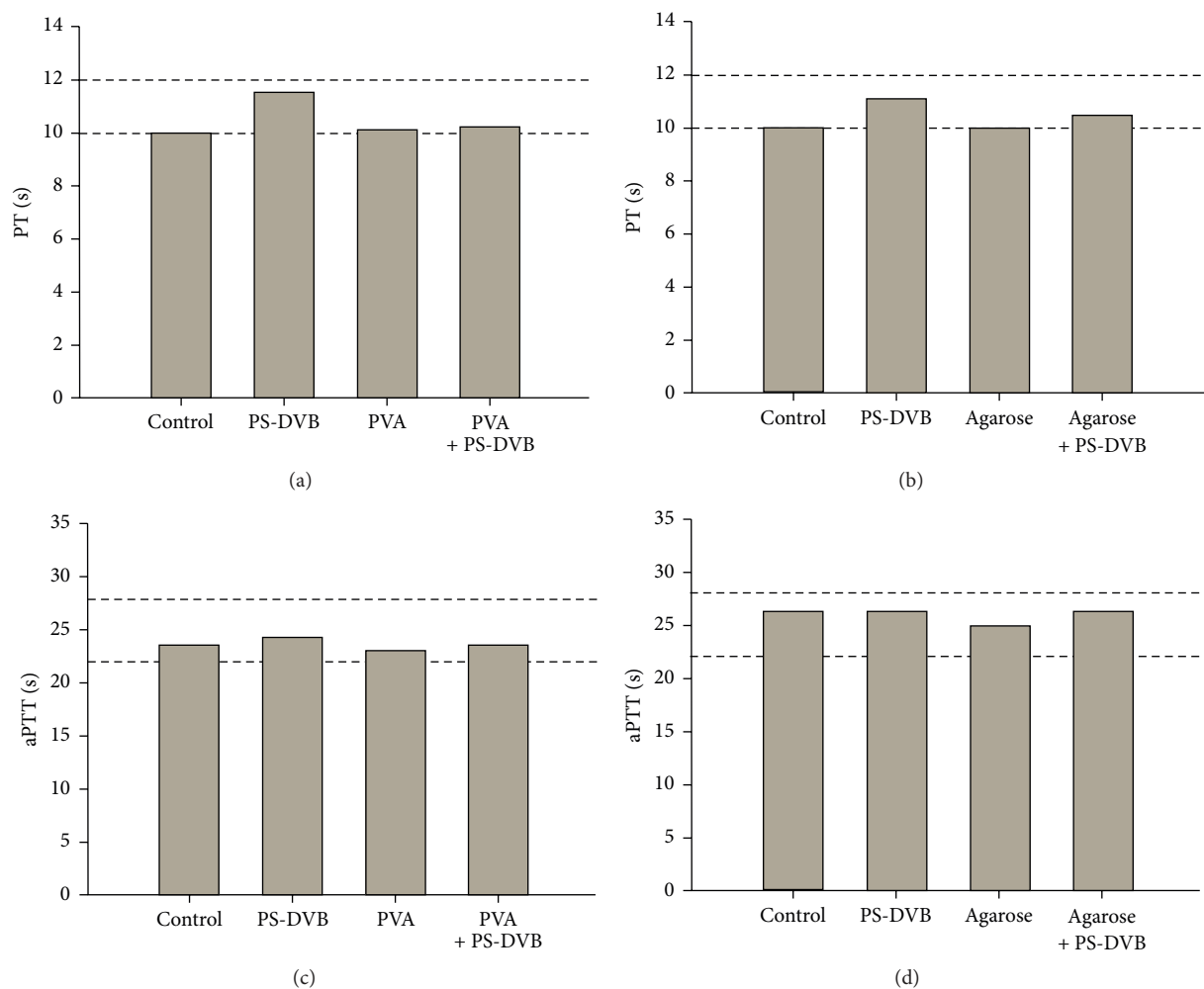


FIGURE 3: Prothrombin time (PT) and activated partial thromboplastin time (aPTT) for PVA- and agarose-based composite cryogels and parent PS-DVB microparticles. The horizontal lines refer to the reference range of the respective coagulation parameter.

$2.1 \pm 0.7 \mu\text{mol/g}$, Table 2), while no difference was observed with respect to cholic acid removal.

The finding that the accessibility of the pores had a greater influence on the adsorption of bilirubin as compared to cholic acid may be explained by the different affinities of cholic acid and bilirubin for albumin: bilirubin is strongly albumin-bound with an association constant of $9.5 \times 10^7 \text{ M}^{-1}$, corresponding to an unbound fraction at the equilibrium of less than 0.1% [24], while the association constant of cholic acid is $0.3 \times 10^4 \text{ M}^{-1}$, corresponding to a free fraction of cholic acid at the equilibrium of about 16% [25]. Thus, the diminished accessibility of the pores due to the embedding of PS-DVB microparticles had a stronger influence on the adsorption of bilirubin than on the binding of cholic acid, because the pores remain accessible to the free fraction of the latter metabolite.

The incorporation of 250 mg PS-DVB microparticles into cryogels resulted in a higher decrease in bilirubin and cholic acid concentrations in comparison to cryogels loaded with 125 mg microparticles. However, when calculating the amount of metabolites adsorbed per gram of microparticles,

the less densely loaded cryogel composites eliminated bilirubin and cholic acid more efficiently, both under static and dynamic conditions (data not shown).

3.5. Adsorption of Cytokines. Cytokines are mediators of inflammation and their removal from the blood may be relevant both in liver failure and as a supportive treatment option for septic patients [26, 27]. Human plasma was spiked with TNF- α and IL-6 to yield concentrations of 535 pg/mL (TNF- α) and 182 pg/mL (IL-6). Cytokine adsorption was compared for the parent microparticles and the composite cryogels containing PS-DVB microparticles (Table 4). Remarkably, TNF- α , which is a 51 kD homotrimer in its bioactive form, was removed by plain PS-DVB microparticles to a greater extent than IL-6 (21 kD). This is in contrast to the hypothesis that adsorption would be more efficient for smaller solutes due to better accessibility of the pores. This finding will be further investigated and might eventually be due to deoligomerization of TNF- α into its subunits.

TABLE 4: Adsorption of TNF- α and IL-6 on PS-DVB microparticles and composite cryogels after 60 min under static conditions ($n = 3$).

	pmol cytokine adsorbed per g PS-DVB	
	TNF- α	IL-6
PS-DVB	0.27 \pm 0.01	0.18 \pm 0.01
PVA + 125 mg PS-DVB	0.10 \pm 0.03	0.06 \pm 0.04
Agarose + 125 mg PS-DVB	0.11 \pm 0.01	0.11 \pm 0.05

Similar to the case of albumin-bound liver toxins, embedding of the PS-DVB microparticles into the cryogels resulted in decreased cytokine adsorption. This may be explained by the formation of a hydrophilic polymer layer on the PS-DVB microparticles during embedment into the cryogels, which reduces hydrophobic interactions between PS-DVB and the target cytokines.

3.6. Blood Compatibility. Incubation of PS-DVB microparticles with plasma did not result in an increase of prothrombin time and activated partial thromboplastin time. Both PT and aPTT also stayed in the normal range for agarose- and PVA-based composite cryogels, indicating good blood compatibility for PS-DVB microparticles as well as for composite cryogels (Figure 3).

4. Conclusions

PS-DVB microparticles with defined porosity are efficient adsorbents for both albumin-bound toxins related to liver failure and cytokines. In this study, PS-DVB microparticles were embedded into monolithic cryogels. The resulting composite cryogels did not require filters to support microparticles and were mechanically stable when perfused with human plasma at flow rates of 4 to 7 mL/min. The composite cryogels efficiently adsorbed albumin-bound hydrophobic metabolites related to liver failure (bilirubin, cholic acid) and cytokines (TNF- α , IL-6). The molecular adsorption characteristics of PS-DVB microparticles were influenced by the embedding procedure, and the accessibility of the pores was diminished due to the formation of a polymer layer on the surface of the particles. This resulted in a decrease of both adsorption capacity and rates of adsorption as compared to the parent microparticles.

The specific adsorption, that is, the amount of target substance bound per g of adsorbent, was higher for composite cryogels containing 125 mg of particles as compared to the more densely packed composite cryogels containing 250 mg of particles, indicating that there is an optimal density of particles within the composite cryogel. The composite cryogels exhibited good blood compatibility, as global coagulation parameters (prothrombin time and activated partial thromboplastin time) remained stable after incubation of the cryogels with plasma. Thus, we conclude that composite cryogels containing PS-DVB microparticles are suitable as adsorption modules to deplete toxic metabolites from plasma for research and production purposes.


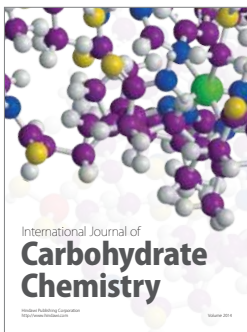
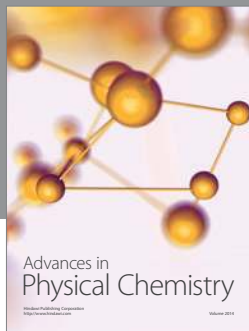
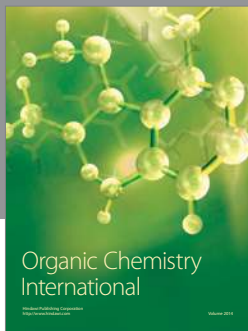
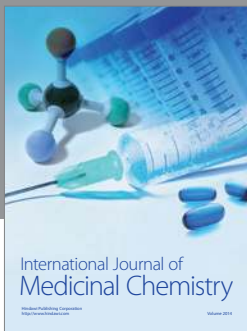
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