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Functionalization and Application of Cellulose Microparticles as Adsorbents in Extracorporeal Blood Purification

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Summary: Therapeutic apheresis is established as supportive therapy for various diseases, such as hypercholesterolemia, autoimmune diseases, liver failure, and sepsis. In combined membrane-adsorption systems, the patient's plasma is continuously separated from whole blood by means of a hollow fiber filter, and pathogenic factors are removed from the plasma by selective or specific adsorbents. While adsorbent particles with a size range of 300–800 μm are used in conventional systems, we are currently developing a system based on adsorbent microparticles (1–5 μm), the Microspheres-Based Detoxification System (MDS). The characteristics of the matrix used for immobilization of specific ligands influence the performance of the resulting adsorbents. Desirable matrix characteristics are an open porous structure with an inner surface accessible for target molecules, mechanical stability, narrow particle size distribution, and ease of derivatization. In addition, biocompatibility is a critical issue, since the particles are in direct contact with the patient's plasma. Cellulose represents an ideal support matrix, as it combines all the above-mentioned features, and cellulosic polymers are widely applied in medicine and generally regarded as biocompatible. Cellulose microparticles can be activated using e.g. sodium periodate and functionalized with Polymyxin B or anti-tumor necrosis factor (TNF) antibodies to generate specific adsorbents for endotoxins or TNF. In summary, cellulose microparticles represent an excellent matrix as basis for adsorbent development in blood purification.

Keywords: adsorption; biomaterials; blood purification; cellulose; sepsis

Introduction

Therapeutic apheresis is an extracorporeal blood purification method for the elimination of pathogenic factors, such as toxins, proteins, or cells[1] either directly from blood (haemapheresis) or from plasma after separation of the blood cells (plasmapheresis). Currently, the major clinical applications of therapeutic apheresis include elimination of immunoglobulins or of specific autoantibodies in patients with autoimmune disease, elimination of low-density lipoproteins in patients with hypercholesterolemia, as well as elimination of hydrophobic pathogenic factors in liver failure.[2–5] In addition, the modulation of inflammatory cytokine concentrations is regarded as a promising therapeutic approach for the supportive treatment of sepsis and multiorgan failure.[6–7]

Historically, unselective plasma exchange was the first technology used for therapeutic apheresis.[8–10] However, this method has some drawbacks, such as the limited volume that can be exchanged per session, the elimination of valuable

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plasma components in addition to pathogenic factors and, consequently, the need for substitution fluids with the potential risk of allergic reactions or viral infections. To overcome these limitations, selective plasmapheresis techniques have been developed, which rely on the removal of pathogenic factors from the plasma by precipitation, filtration, or adsorption to biocompatible polymers. Among these methods, combined membrane-adsorption techniques are most widely used.[11]

Microparticle-Based Adsorption Systems for Blood Purification

The standard implementation of membrane-adsorption systems for blood purification is the perfusion of plasma through cartridges containing adsorbent particles with average sizes of 150 to 400 μm. In principle, this set-up can be compared to column chromatography, where the patient's plasma represents the mobile phase, from which certain factors are to be removed. According to a recommendation of the American Association of Blood Banks, the extracorporeal volume should not exceed 15% of the total blood volume of the patient, and even this limit may not be well tolerated by patients with haemodynamic instability. Therefore, the tolerated plasma loss in patients without severe hypotension is less than 1L, and consequently, the extracorporeal volume of adsorbent systems and the adsorbent volume that can be used in such systems is limited. Thus, it is important to optimize the performance of the available adsorbents, which is the underlying idea for the use of smaller adsorbent particles.[12,13] The binding capacity of an adsorbent depends on its surface area and on the accessibility of the surface to the substances that are to be bound. The external surface of a given volume of adsorbent increases with decreasing particle size. Hence, the distance between the fluid and the interior binding sites becomes shorter for smaller particles, and their diffusion time to the binding sites is reduced. Thus, smaller particles exhibit both higher binding capacity and better kinetics of adsorption as compared to large particles.

Based on this concept, blood purification systems that use microparticles have been introduced: the Biologic DT and derived techniques (DTPF and sorbent suspension reactor SSR) and the Microspheres-Based Detoxification System, MDS. Since it is technically not feasible to circulate plasma through columns containing particles with diameters of less than 50 μm due to high back-pressure, adsorbent suspensions are re-circulated at high speed in the filtrate circuit in the MDS. With about 300 mL, the extracorporeal volume of the MDS is much smaller than in column-based systems. A flow scheme of the MDS and examples of adsorbent microparticles are shown in Figure 1.

The MDS is developed as a versatile platform technology with a range of potential applications, depending on the specificity of the adsorbent particles. While selective adsorbents bind groups of factors with similar physico-chemical properties (e.g., hydrophobicity), adsorption of individual factors can be achieved with specific adsorbents, which are mostly based on polymers functionalized with antibodies. Examples of adsorbents developed in our group include styrene divinylbenzene copolymers, which can be applied to selectively remove albumin-bound hydrophobic toxins related to liver failure[14], adsorbents for immunoglobulins consisting of cellulose microparticles coated with S-layer fusion proteins comprising the IgG-binding domain of Protein A from *Staphylococcus aureus* [15], and adsorbents for fibrinogen based on sucrose methacrylates functionalized with specific peptides.[16] A major aim of our current work is the application of the MDS technology to modulate the concentrations of pathogenic mediators (e.g., lipopolysaccharide and cytokines[17]) in patients with sepsis and multi-organ failure. For this application, we plan to utilize specific adsorbents based on cellulose microparticles functionalized by covalently

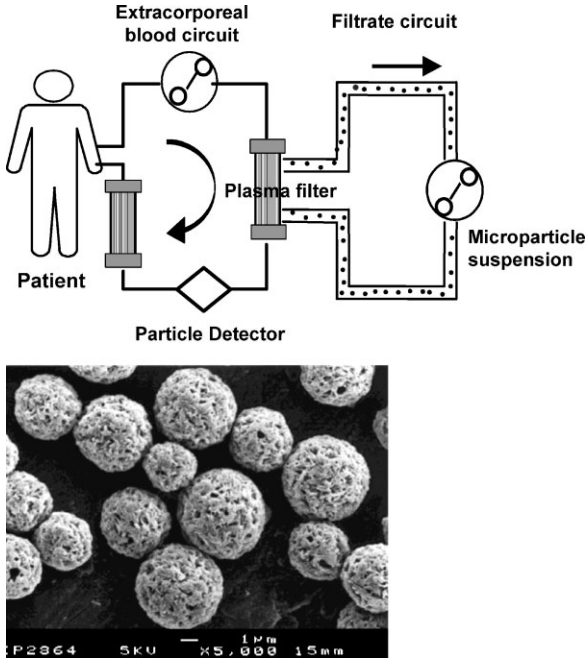

Figure 1.
Flow scheme of the Microspheres-Based Detoxification System and scanning electron micrograph of cellulose microparticles used as carriers for adsorbent development.

bound antibodies, antibody fragments, or other bioactive molecules.

Cellulose as Carrier for Adsorbent Development

Cellulose microparticles combine several desirable characteristics for adsorbent development, such as an open porous structure and mechanical as well as chemical stability. Moreover, cellulose can easily be activated with reagents such as periodate or epichlorohydrine for the covalent binding of ligands. Cellulosic polymers are widely applied in medicine and generally regarded as biocompatible.

Due to its central role in the pathogenesis of sepsis, the pro-inflammatory cytokine tumor necrosis factor-α (TNF) was chosen as a model substance to optimize the parameters of adsorbent development. We prepared specific adsorbents for TNF by covalent binding of a chimeric human-mouse monoclonal anti-TNF antibody (Remicade, Infliximab) to cellulose microparticles activated with sodium periodate (Figure 2). The anti-TNF-antibody binds TNF with high affinity (affinity constant Ka: $10^{10}\,M^{-1}$). The cellulose microparticles used as carriers for adsorbent development were provided by the Institute for Wood- and Plant Chemistry, University of Technology, Dresden, Germany. The particle size range was 1.1–5.1 μm (d10: 1.6 μm; d50: 2.3 μm; d90: 3.5 μm).

The activation of cellulose with sodium periodate was performed by incubation of cellulose with sodium periodate solution (5 g cellulose with 25 mL of 0.05–0.2 mM sodium periodate) for 2 h at 40 °C in the dark. The degree of activation was determined by treatment of the particles with sodium hydroxide solution and titration of sodium hydroxide consumption. Figure 3 shows the correlation between the degree of activation (μmol dialdehyde groups per g cellulose) and the amount of sodium periodate used for activation. For immobilization of the anti-TNF antibodies,

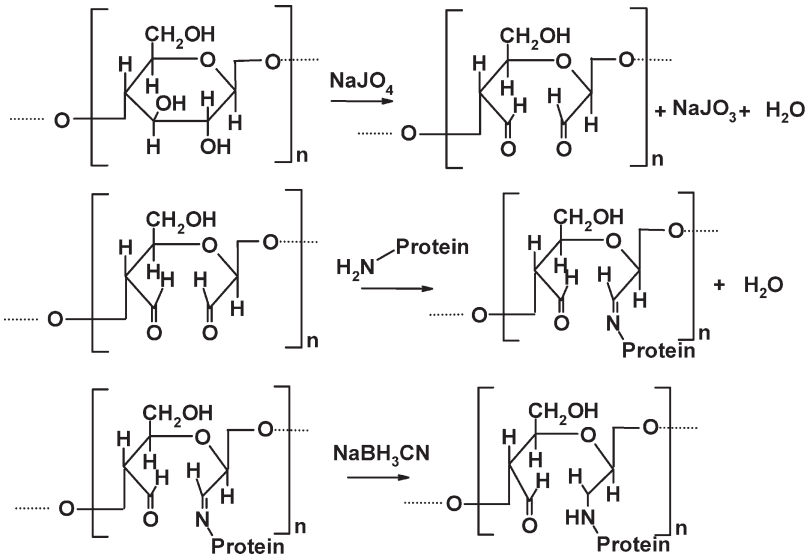

Figure 2.
Activation of cellulose microparticles with sodium periodate and covalent immobilization of proteins. Top: reaction of cellulose with periodate; middle: immobilization of protein; bottom: reduction of Schiff bases with sodium cyanoborohydride.

cellulose microparticles with degrees of activation of 100–400 μmol/g were used. Comparable degrees of activation were previously shown to result in efficient immobilization of anti-TNF antibody for larger cellulose particles.[17] Higher degrees of activation led to micro- and macroscopic changes in the structure of the cellulose beads, most likely resulting from destruction of the cellulose chains by periodate oxidation.

In an *in vitro* set-up of the MDS using a pool of human plasma (1L) spiked with TNF (800 pg/mL) and soluble TNF receptors (sTNFRI and II, 1000 pg/mL each) and 2g of adsorbent, TNF was efficiently removed (Figure 4). The soluble TNF receptors were added to the spiked plasma

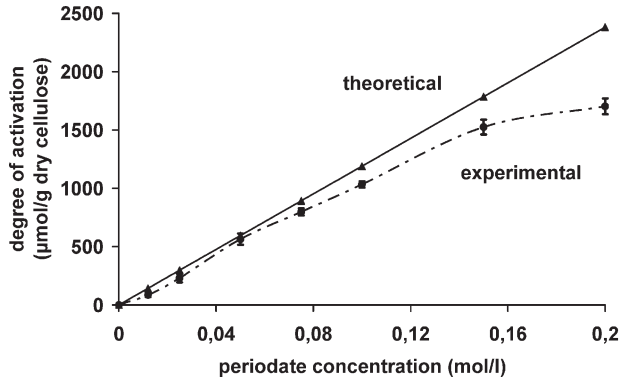

Figure 3.
Degree of activation in correlation to the amount of sodium periodate used for activation. For immobilization of the anti-TNF antibodies, cellulose microparticles with degrees of activation of 100–400 μmol/g were used.

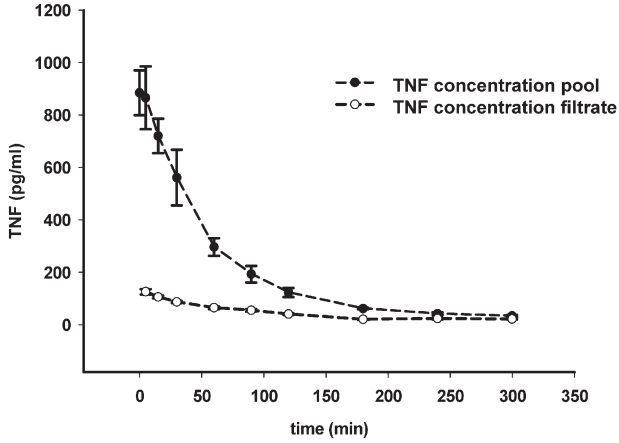

Figure 4.
Removal of TNF from a pool of spiked human plasma with an *in vitro* set-up of the MDS. A pool (1L) of spiked plasma (800 pg/mL TNF) was treated with 2 g of adsorbent. The TNF concentration both in the pool (Albuflow pool) and in the adsorbent circuit (Albuflow filtrate) was quantified by enzyme-linked immunosorbent assay.

since they are present also *in vivo* in the plasma of septic patients; thus it is crucial to verify that they do not interfere with TNF binding to the adsorbent.

Based on this principle, further specific adsorbents for pathogenic mediators of sepsis are currently under development. Examples of interesting targets include other cytokines (e.g. IL-6), or factors of the complement system (e.g. C5a or factor B). In addition, the removal of lipopolysaccharide (endotoxin) can be accomplished with adsorbent particles functionalized with polymyxin B, a cationic amphiphilic antibiotic that binds lipopolysaccharide by both, electrostatic and hydrophobic interactions.

To identify other potential targets for adsorption and to test the effect of adsorption of individual factors or of defined combinations of factors, we have developed a cell culture model for Gram-negative sepsis. The model is based on activation of blood monocytes with lipopolysaccharide. Stimulated monocytes secrete a range of factors (cytokines) to the culture medium, which is harvested and transferred to endothelial cells in culture. The activation of endothelial cells is monitored by different read-out assays (e.g., cytokine secretion, expression of adhesion molecules) and by addition of specific adsorbents at various time points, the effect of adsorption of defined mediators on endothelial activation can be monitored.[18] First results demonstrate that modulation of TNF concentration results in significantly reduced endothelial activation.

Conclusion

Cellulose microparticles are well suited for the immobilization of antibodies or other functional macromolecules to develop specific adsorbents for blood purification. The modulation of inflammatory mediators in the plasma of patients suffering from sepsis may represent a supportive therapy for the treatment of this disease. In the course of sepsis, inflammatory mediators such as lipopolysaccharide, cytokines, or complement factors are released.

Lipopolysaccharide, a component of the outer membrane in Gram-negative bacteria, is the main trigger of Gram-negative sepsis. It can be removed by adsorbents functionalized with Polymyxin B, a cationic amphiphilic antibiotic that binds LPS via electrostatic and ionic interactions. While the therapeutic window for initial LPS removal in sepsis is very short due to its

fast initiation of the septic process, LPS is also released in high amounts at later stages of sepsis due to altered permeability of the gut.

As a prototype proinflammatory cytokine, tumor necrosis factor-α triggers the release of further inflammatory mediators and activates both, the complement and the coagulation system. Thus, lowering of TNF in the circulation is also a promising treatment option for the early stage of sepsis. Furthermore, other inflammatory cytokines, such as interleukin 6 or HMGB-1, a late mediator of sepsis, are interesting as targets for adsorption. In addition, factors of the complement system, above all the anaphylatoxin C5a, which is generated during complement activation, are regarded as potential targets for therapy. However, due to its low molecular mass, C5a can at least be partly removed by high-flux hemodialysis.[19]

Specific adsorbents for each of these factors based on cellulose microparticles functionalized with antibodies can be combined in the Microspheres-Based Detoxification System according to the patient's needs.

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