Articles

Magnetic Fluorescent Microparticles as Markers for Particle Transfer in Extracorporeal Blood Purification

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The microspheres-based detoxification system (MDS) is a combined membrane-adsorption system for extracorporeal blood purification in which adsorbent microparticles are recirculated in an extracorporeal filtrate circuit. Because the plasma filter represents the only barrier between the adsorbents and the patient's blood, there is the potential risk of particle entrance into the patient in case of a membrane rupture. To guarantee first fault safety of the system required for clinical application, magnetic fluorescent microparticles are added as markers to the adsorbent circuit. Detection of these particles in the venous blood line results in immediate shutdown of the pumps. Magnetic beads were functionalized with cresyl violet and tested with an in vitro setup of the particle detector to assess the detection limit in different matrices (water versus blood) as well as the influence of flow rate and particle size on the signal. In addition, biocompatibility and influence of sterilization on the performance of the particles were assessed. Functionalization of the magnetic particles with cresyl violet yielded fluorescent particles that were stable at 4 °C for at least 12 months. No leakage of dye was detectable, and the particles were neither cytotoxic nor mutagenic. The particles could be steam sterilized without significant loss in fluorescence intensity. With an in vitro setup of the particle detector, 0.1 mg and 5 mg of particles were reproducibly detectable in water and blood, respectively.

Introduction

Combined membrane-adsorption systems are clinically applied for the supportive treatment of various diseases such as liver failure, autoimmune diseases, hypercholesterolemia, or sepsis.[1–3] In these systems, the patient's blood is separated into a cell and a plasma fraction by means of a hollow fiber filter. The plasma passes cartridges containing adsorbent particles with a size range of 300– 500 μm to remove pathogenic substances.[4,5]

A new technology, the microspheres-based detoxification system (MDS), which is currently under development in our group, differs from conventional blood purification systems mainly with respect to particle size.[6–8] In the MDS, adsorbent microparticles (size range: 1–10 μm) are recirculated in an extracorporeal plasma circuit (Figure 1).

The main advantages of the MDS are high adsorption capacity due to high surface-to-volume ratio, excellent kinetics, as well as high flexibility because adsorbents of different specificities can be added to the plasma circuit. However, there is the potential risk of microparticle release into the blood stream of the patient in case of a rupture of the plasma filter, which represents the only barrier between the adsorbent microparticles and the patient's blood. Because clinical application of a medical device requires first fault safety (i.e., the system must remain safe when one fault occurs), a particle detector consisting of a magnetic trap and an optical detection system was developed.[9,10] Fluorescently labeled magnetic particles are added to the adsorbent suspension. In case of membrane rupture, these marker particles are detected in the venous blood line, which results in an immediate shut-down of the pumps and thus prevents particle transfer into the patient.

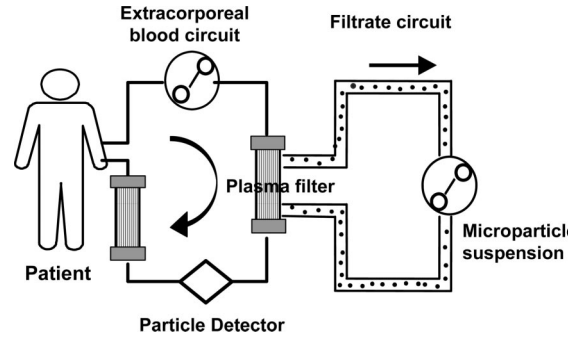

Figure 1. Schematic drawing of the MDS. The primary circuit containing the patient's whole blood is separated from the secondary circuit by a plasma filter. In the secondary circuit, plasma is recirculated and kept in suspension together with the adsorbent particles. The purified plasma is filtered back to the primary circuit, and whole blood is reinfused into the patient.

Here, we describe the development of fluorescently labeled magnetic particles as markers for particle transfer in the MDS. In addition to high fluorescence intensity and sufficient magnetic properties, biocompatibility as well as storage stability are required for clinical application. Commercially available magnetic particles (Dynabeads M-280 tosylactivated) were functionalized with the fluorescent dye cresyl violet, and the resulting particles were characterized with respect to biocompatibility and

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tested with an in vitro setup of the detector both in water and in whole blood. We demonstrate that the resulting functionalized particles are highly efficient markers for particle transfer in the MDS.

Materials and Methods

Particles and Chemicals. Dynabeads M-280 and M-450 tosyl activated were purchased from Invitrogen/Dynal Biotech. Dynabeads M-280 and M-450 are superparamagnetic polystyrene beads with a diameter of 2.8 and 4.5 μm, respectively. They are coated with a polyurethane layer and activated with *p*-toluenesulfonyl chloride, which allows for covalent binding of ligands via amino or sulfhydryl groups. The iron content is 12% for M-280 and 20% for M-450.[11] The degree of activation as given by the manufacturer is 100–200 μmol tosyl groups per g. Cresyl violet (5,9-diaminobenzophenoxazonium perchlorate) was purchased from Radiant Dyes (Wermelskirchen, Germany). This fluorescent dye has an absorption maximum of 601 nm and an emission maximum of 632 nm in ethanol.

Plasma and Blood. Fresh-frozen human plasma was obtained from a local plasma donation center. The plasma was passed over a hollow fiber filter (Albuflow AF01, Fresenius Medical Care, Bad Homburg, Germany). The Albuflow filter was designed for the removal of toxins in liver failure and has a sieving coefficient of 0.65 for albumin. Substances of higher relative molecular mass are retained by the filter (sieving coefficient for fibrinogen $\leq$0.1).[12] Human whole blood was drawn from healthy volunteers.

Fluorescent Labeling of the Magnetic Particles. Dynabeads M-280 or M-450 were washed with 0.1 M sodium phosphate (pH 7.4). The beads were incubated with cresyl violet (200 μM in 50% ethanol, 50% 0.1 M sodium borate, pH 9.5) for 24 h at 37 °C in the dark at a ratio of 10, 100, and 1000 nmol cresyl violet per mg beads. On the basis of a degree of activation (tosyl group content of the particles) of 100 nmol/mg, this corresponds to a ratio of dye to tosyl groups of 1:10, 1:1, and 10:1, respectively.

After the coupling step, the beads were washed extensively with a solution of 50% ethanol, 50% 0.1 M sodium borate (pH 9.5) to remove unbound dye. The washed particles were resuspended in phosphate-buffered saline (PBS) and stored at 4 °C.

To determine the amount of bound dye, the concentration of cresyl violet in the supernatant before and after the coupling step was determined fluorimetrically (Perkin-Elmer LS 55 fluorescence spectrometer) using a standard curve of cresyl violet in 50% ethanol, 50% 0.1 M sodium borate, pH 9.5. The amount of bound dye was calculated from the difference in dye concentration in the supernatant before and after the coupling step.

Testing of the Particles with an in vitro Set-Up of the Particle Detector. A detailed description of the particle detector and its working principles is given elsewhere.[9]

Briefly, the detector, which is placed in the venous blood line of the MDS, consists of a magnetic trap and an optical detection system. The trap (two neodymium-iron-boron magnets) collects the magnetic fluorescent particles that would enter the blood stream in case of a membrane rupture in the focus of the excitation beam of the optical detection system (excitation wavelength 595 nm). The light emitted by the particles (emission wavelength 620 nm) is detected by a photomultiplier.

If not otherwise stated, Dynabeads M-280 functionalized with cresyl violet were used. All experiments were performed at least in triplicate. Varying quantities (see below) of fluorescently labeled Dynabeads were added to a 250 mL water or blood pool, respectively. After mixing for 60 s, the pool was recirculated through the detector with a roller pump at a flow rate of 250 mL/min. The fluorescence intensity of the particles collected in the magnetic trap was recorded. For testing in water, 0.1, 0.05, and 0.01 mg of particles were added to the pool without pretreatment. Prior to testing in blood, particles (1, 3, and 5 mg, respectively) were incubated in 5 mL of human plasma in a glass tube for 30 min at 37 °C with gentle shaking. The plasma was prefractionated through an Albuflow filter as described in Materials and Methods to simulate the situation in clinical application where the particles are recirculated in the filtrate circuit of the MDS. To assess the influence of the flow rate on particle detection, an additional series of experiments was run using flow rates of 150 and 300 mL/min.

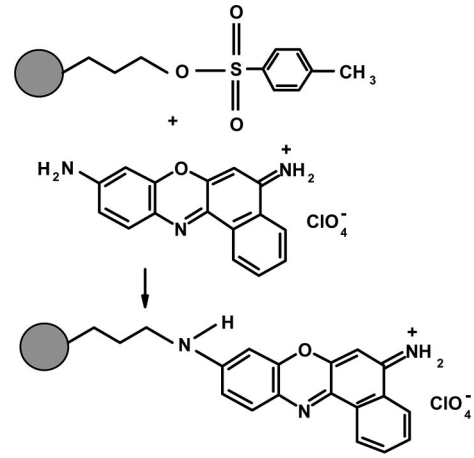

Figure 2. Covalent binding of cresyl violet to particles activated with tosyl chloride.

Sterilization of the Marker Particles. The fluorescently labeled marker particles were steam sterilized (121 °C, 2 bar, 40 min) and tested with the particle detector as described above. Testing was performed for the sterilized marker particles alone, for a mixture of markers and adsorbent particles after separate sterilization, and for a mixture of markers and adsorbent particles, which had been sterilized together. Neutral polystyrene divinylbenzene copolymers with a size range of 1–20 μm were used as "model" adsorbents. A ratio of 1% of marker particles related to adsorbent particle weight was used for all experiments.

Biocompatibility. The marker particles were tested for cytotoxicity with the EZ4U cell proliferation and cytotoxicity test (MTT test, Biomedica, Vienna, Austria). This test is based on the ability of living cells to reduce uncolored tetrazolium salts (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into colored formazan derivatives. For the test, 10% (v/v) suspensions of the particles in pyrogen-free 0.9% NaCl were incubated at 37 °C overnight. The particles were removed by centrifugation, and 20 μL of the supernatant was added to L929 mouse fibroblasts grown in a 96 well plate. Each well contained 2×10^5 cells in 200 μL of culture medium. Samples were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. The MTT assay was performed according to the instructions of the manufacturer.

To test for mutagenicity, particles were extracted for 72 h at 37 °C either with saline solution or with dimethylsulfoxide using 1 mL of solvent per 6 cm^2 of particle surface area. The extracts were subjected to a *Salmonella typhimurium* reverse mutation assay (Ames test according to ISO 10993-3; the test was carried out by Bioservice Scientific Laboratories, Planegg, Germany).

Results and Discussion

Fluorescent Labeling of Dynabeads. The free amino group of cresyl violet allows for the covalent attachment of the dye to the Dynabeads via the tosyl groups at their surface (Figure 2).

After immobilization of cresyl violet, the particles exhibited high fluorescence intensity, which remained stable for at least 12 months at 4 °C. No fluorescence was detectable in supernatants of the particles by fluorescence spectrometry, indicating that there was no leakage of the fluorescent dye.

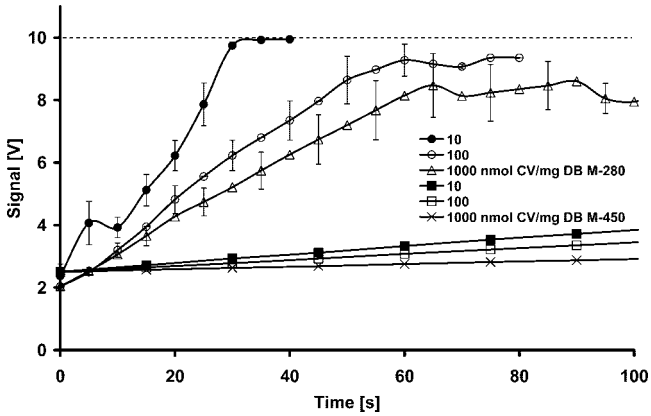

Figure 3. Testing of Dynabeads M-280 and M-450 functionalized with cresyl violet in an experimental setup of the particle detector. The graph shows the detector output voltage over time after addition of 0.1 mg of particles functionalized with increasing amounts of cresyl violet to a 250 mL water pool (flow rate: 250 mL/min). CV, cresyl violet; DB, Dynabeads.

Dynabeads M-280 functionalized with 10, 100, and 1000 nmol cresyl violet per mg of particles (corresponding to a ratio of dye and tosyl groups of 1:10, 1:1, and 10:1) bound 5.2 ± 0.2, 53.2 ± 2.3, and 525.5 ± 21.8 nmol cresyl violet per mg of particles ($n = 4$). Dynabeads M-450 functionalized under the same conditions bound 4.5, 65.3, and 648 nmol Cresyl violet per mg, respectively. On the basis of the degree of activation given by the manufacturer, the theoretical maximum binding capacity of the particles (tosyl group content) is 100 nmol per mg. As shown above, the amount of bound dye exceeded this theoretical binding capacity for the particles functionalized with the highest amount of dye, which bound 525.5 and 648 nmol cresyl violet per mg. This can be explained with unspecific adsorption of dye to the particles at high dye concentrations. However, the dye could not be released by extensive washing of the particles with ethanol, indicating that it is strongly adsorbed on the particle surface. On the basis of these data, strong nonspecific binding might also be the predominant mechanism at low dye concentration, which implies that this might even be the method of choice for attachment of the dye. Further experiments with nonfunctionalized beads will be performed to clarify this issue.

Testing of the Fluorescently Labeled Particles in an Experimental Set-Up of the Particle Detector: Water versus Blood. When tested in water, the sensitivity was highest for Dynabeads M-280 functionalized with 10 nmol Cresyl violet per mg, i.e., with the particles containing the lowest amount of dye. As shown in Figure 3, the maximum output voltage of the detector was rapidly reached with 0.1 mg of these particles. The comparatively lower sensitivity of particles loaded with higher amounts of dye may be due to fluorescence quenching effects. Notably, Dynabeads M-450 which were functionalized and tested under the same experimental conditions as Dynabeads M-280 and contained similar amounts of fluorescent dye as shown above exhibited a significantly slower increase of the detector signal over time and did not reach the maximum output voltage. This might be due to a higher viscous drag for the larger particles. Therefore, Dynabeads M-280 functionalized with 10 nmol cresyl violet per mg were used for all further studies.

Prior to testing in blood, the particles were preincubated in fractionated plasma as described in Materials and Methods to mimic the situation in the MDS where the particles are in permanent contact with the plasma filtrate. Under these conditions, 5 mg of marker particles were required in blood to reach the maximum output signal of 10 V. Several factors may contribute to the higher detection limit in blood as compared to water: upon contact with plasma, a protein film forms both on the particles and on the tubing system. In addition, the higher viscosity of blood as well as interaction and/or collisions of the particles with blood cells, which are in a similar size range as the particles, may result in a weaker signal as compared to water.

Influence of the Flow Rate. In a series of experiments in blood, particles were recirculated through the detector at different flow rates (150, 250, and 350 mL/min, respectively). There was no significant difference in sensitivity for the different flow rates (data not shown).

Testing of Marker Particles after Sterilization. The marker particles were steam sterilized as described in Materials and Methods and tested with the particle detector in blood. Testing was performed for the sterilized marker particles alone for a mixture of markers and "model" adsorbent particles after separate sterilization and for a mixture of markers and adsorbents that had been sterilized together. The ratio of markers to adsorbent was 1:99 relating to particle weight. As shown in Figure 4 (panel a), the maximum output signal of the detector was reproducibly reached within 10 s using 5 mg of sterilized marker particles under the conditions described in Materials and Methods. The signal obtained for a mixture of markers and adsorbent after separate sterilization (Figure 4, panel b) showed a lower increase over time as compared to markers alone. The difference remained statistically significant for 10 s ($p = 0.031$ and 0.004 at 5 and 10 s, respectively). There was no statistically significant difference after 15 s, where the maximum output voltage was reproducibly reached for both conditions. For markers and adsorbents that were sterilized in mixture (Figure 4, panel c), the slope of the signal remained significantly lower as compared to separate sterilization over the whole course of the experiment. This finding points to an interaction of marker particles and the model adsorbent polymer used in this study. Because there was no indication for macroscopic or microscopic aggregation of particles during the sterilization procedure, this issue remains under investigation.

Biocompatibility. Extracts of the marker particles in 0.9% saline solution were incubated with mouse fibroblasts and the number of living cells was determined by their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT test).[13] As shown in Figure 5, cells cultured with extracts of the marker particles did not exhibit reduced viability as compared to the control. Thus, it can be concluded that the particles do not release cytotoxic substances. In addition, extracts of the particles in in inorganic (saline solution) or organic (dimethylsulfoxide) solvents did not lead to an increased mutation rate in a *Salmonella typhimurium* reverse mutation assay (data not shown), which indicates that no mutagenic substances are released from the particles.

Conclusions

Covalent immobilization of the fluorescent dye cresyl violet to tosyl Dynabeads M-280 activated with tosyl chloride yielded fluorescent magnetic microparticles that could be detected with high sensitivity (detection limit in water and blood: 0.1 and 5 mg, respectively). The particles were developed as markers for particle transfer to the patient to provide first fault safety in the extracorporeal blood purification system MDS. They were shown to be biocompatible and stable upon steam sterilization.

Literature data concerning the biological effects and tolerable amount microparticles are rare. Published data from studies on

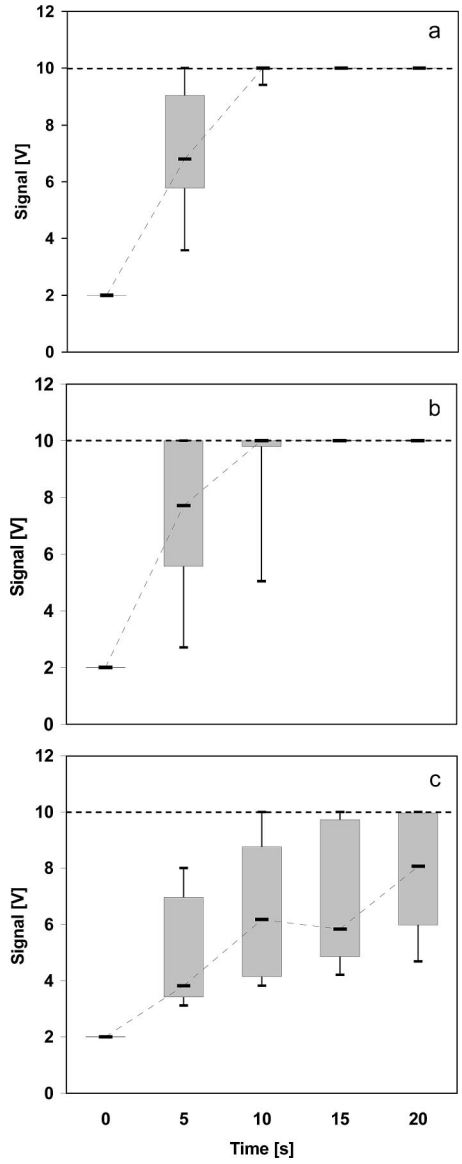

Figure 4. Box plots showing the detector signal over time obtained by addition of fluorescently labeled Dynabeads M-280 to a 250 mL blood pool (flow rate: 250 mL/min). The following amounts of particles were added to the pool: (a) 5 mg of labeled beads, steam-sterilized; (b) 5 mg of labeled beads plus 495 mg of "model" adsorbent after separate sterilization; (c) 5 mg of labeled beads plus 495 mg of "model" adsorbent after sterilization in mixture. The median for each time point is represented by the black bar in the box; the whiskers represent minimal and maximal values; $n = 9$ for each time point.

beagle dogs show that the distribution pattern of intravenously administered particles is size-dependent: particles smaller than 4 μm pass through the pulmonary circulation and are cleared by the reticulo-endothelial system of liver and spleen by phagocytic processes, while particles larger than 7 μm are retained in the lungs. Bolus intravenous injections of 1g of

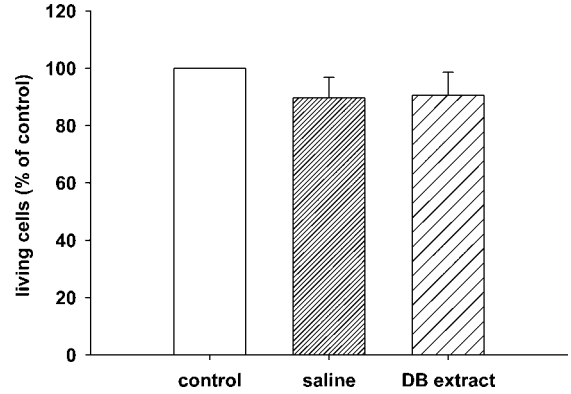

Figure 5. Viability of mouse fibroblasts after addition of extracts from fluorescently labeled Dynabeads and controls (MTT test; $n = 3$).

microparticles could be administered to dogs (13–15 kg) without serious consequences.[14] Our own animal studies regarding biological effects and tolerable amounts of microparticles are underway.

In summary, the combination of fluorescent and magnetic labeling of the marker particles used in this study provides the basis for minimization of particle transfer into the patient in the very unlikely case of a membrane rupture.

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