Utilization of nanobiotechnology in haemodialysis: mock-dialysis experiments on homocysteine

Dimosthenis Stamopoulos¹, Penelope Bouziotis², Dimitra Benaki³, Constantinos Kotsovassilis⁴ and Panagiotis N. Zirogiannis⁵

¹Institute of Materials Science, ²Institute of Radioisotopes and Radiodiagnostic Products, ³Institute of Biology, NCSR ‘Demokritos’, Aghia Paraskevi 153-10, ⁴Department of Clinical Biochemistry and ⁵Department of Nephrology, General Hospital ‘G. Gennimatas’, National Health System, Athens 115-27, Greece

Abstract

Background. The utilization of modern achievements from nanobiotechnology has resulted in novel modalities for renal replacement therapy. For conventional intermittent haemodialysis (HD), sophisticated membranes are currently being manufactured that guarantee selective removal of target toxins. These membranes have a narrow pore-size distribution that is focused around a mean value at the nanometre level. For continuous HD, novel artificial renal devices are currently being designed and evaluated in vitro experiments that will be both implantable and have continuous function.

Methods. We present mock-dialysis experiments using magnetically assisted HD (MAHD) that we very recently introduced for the selective removal of target toxins. MAHD is based on the preparation of conjugates (Cs) made up of biocompatible ferromagnetic nanoparticles (FNs) and a specifically designed targeted binding substance that must have a high affinity for a specific target toxin substance. The FN–targeted binding substance Cs should be administered to the patient prior to MAHD to allow for binding with the target toxin substance in the bloodstream. The complex FN–targeted binding substance–target toxin substance will then be removed by a ‘magnetic dialyzer’ that is installed in the dialysis machine in series to the conventional dialyzer. In the present work, we compared the in vitro efficiency of MAHD to conventional HD for the removal of homocysteine (Hcy) during mock-dialysis experiments.

Results. These mock-dialysis experiments performed on Hcy revealed that both the removal rate and the overall removal efficiency of MAHD were significantly greater than conventional HD.

Conclusions. MAHD appears to be a promising method that can be employed for the selective and more efficient extraction of toxins that are not adequately removed by conventional HD.

Keywords: haemodialysis; homocysteine; magnetic nanoparticles; magnetically assisted haemodialysis; mock-dialysis

Introduction

Despite great progress, haemodialysis (HD) is still limited by complications resulting from the inability of current low- and high-flux dialyzers to adequately filter and excrete low- and middle-molecular weight toxins that have a high affinity for serum proteins [1–3]. For example, hyperhomocysteinaemia [4–8] and amyloidosis [9–11] are two dialysis-related disorders that are not adequately treated, leading to serious health complications mainly related to cardiovascular disease (CVD), which is the leading cause of death in end-stage renal disease (ESRD) patients.

To overcome the existing inefficiencies, modern trends are focusing on the utilization of nanobiotechnology in HD. For the disposable portions of the extracorporeal circuit, sophisticated dialysis membranes have been manufactured that aim to remove target toxins having specific molecular weights [12,13]. These membranes have a narrow pore-size distribution focused around a mean value that is accurately controlled at the nanometre level [12–15].

Very recently, we introduced the concept of magnetically assisted HD (MAHD), which can selectively and efficiently remove toxins that are not adequately removed by conventional dialyzers [16]. MAHD is based on the utilization of ferromagnetic nanoparticles (FNs) combined with conventional HD. Similar biocompatible FNs have been successfully utilized in many in vitro and in vivo diagnostic and therapeutic biomedical applications, such as magnetic resonance imaging and targeted destruction of tumour tissues [17–20]. For use with MAHD, FNs should form conjugates (Cs) with a targeted binding substance that must have a high affinity for a specific target toxin substance. Ideally, target toxin substance antibodies should serve as the targeted binding substance constituent of the Cs, since they...
guarantee absolute specificity and maximum biochemical reactivity for the respective target toxin substance. The FN–targeted binding substance Cs should be administered to the patient at a specific time prior to the MAHD session to allow for selective binding with the specific target toxin substance in the bloodstream [16]. The complex FN–targeted binding substance–target toxin substance is removed during the MAHD session by means of a magnetic dialyzer (MD), which is a magnetic-field device installed in the dialysis machine in series with the conventional dialyzer. In our device, the MD comprises an array of permanent magnets placed along the extracorporeal blood circulation line (vide infra).

MAHD is based on three main requirements [16]. First, the FN–targeted binding substance Cs should be absolutely biocompatible and perfectly soluble so that both immune system and thrombotic complications are avoided. Second, the host carrier FNs should be highly magnetic and the applied magnetic field should be highly inhomogeneous so that the complex FN–targeted binding substance–target toxin substance is efficiently extracted by the MD. Third, the host carrier FNs should be fully covered by the desired targeted binding substance so that the maximum binding capacity for the respective target toxin substance is achieved. We should stress that it will be very challenging to design biocompatible Cs that are safe for infusion into the bloodstream. This is a cornerstone requirement for the MAHD concept.

In this work, we investigated the in vitro applicability of MAHD in mock-dialysis experiments. Although target toxin substance antibodies are the ideal targeted binding substance, we selected bovine serum albumin (BSA) at this early stage of experimentation since many low- and middle-molecular weight toxins have high protein-binding affinities [1–3]. In addition, we selected Fe₃O₄ as the host carrier FNs because of its significantly higher biocompatibility compared with other candidates (see [17–20] and references therein). We evaluated the magnetic extraction efficiency of the Fe₃O₄–BSA Cs during standard dialysis conditions by using a simple MD consisting of an array of permanent magnets placed along the extracorporeal circulation line. Both the removal rate and the overall removal efficiency of MAHD were compared to conventional HD by using homocysteine (Hcy) as a model target toxin substance.

Materials and methods

Materials and preparation of samples

All chemicals were reagent grade. Iron (II) chloride tetrahydrate (FeCl₂ · 4H₂O, ReagentPlus, 99%), iron (III) chloride (FeCl₃, reagent grade, 97%), and bovine serum albumin (> 95% (titration)) were purchased from Aldrich (St Louis, MO, USA). BSA, Fraction V (minimum 96%, lyophilized powder) was purchased from SIGMA (St Louis, MO, USA). Analytical grade NH₄OH was purchased from Analytical-Cals (Carlo Erba, Milano, Italy). Water was purified using analytical-grade water purification systems (Millipore). Sodium chloride for injection (Fresenius, 0.9% w/v) was purchased from a local pharmacy. For the preparation of Fe₃O₄–BSA Cs, BSA was first dissolved in saline. Anhydrous FeCl₃ and FeCl₂ · 4H₂O were then added in appropriate amounts, and finally NH₄OH was added and vortex stirred so that the preparation of Fe₃O₄–BSA Cs was accomplished by chemical co-precipitation. Additional details can be found elsewhere [16].

Methods

In the present mock-dialysis experiments, we employed standard Hemophan low-flux membranes, because they exhibit inefficient removal of low- and middle-molecular weight toxins that have high affinity for blood-serum proteins. The employed dialyzate was standard bicarbonate that is routinely used in HD (Na 138 mmol/L, HCO₃ 35 mmol/L, K 1.5 mmol/L, Ca 1.25 mmol/L, Mg 0.75 mmol/L).

The magnetic characterization of both bare FNs and FN-targeted binding substance Cs was performed by means of a superconducting quantum interference device (SQUID) magnetometer (Quantum Design, San Diego, CA, USA). Atomic force microscopy images were obtained by means of a NT-MDT Solver PRO scanning probe microscope. A circular dichroism (CD) spectropolarimeter [Jasco J715 (190–900 nm)] and an UV–Vis spectrophotometer [Shimadzu UV2100 (200–900 nm)] were used for evaluating the conjugation between FNs and targeted binding substance proteins. A standard fluorescence polarization immunoassay (FPIA) method (Abbott AxSYM platform) was employed for estimating the binding capacity of the FN–targeted binding substance Cs for Hcy. Additional details can be found elsewhere [16].

Results

To evaluate the in vitro applicability of MAHD in the present mock-dialysis experiments, we employed Fe₃O₄ and BSA as the FNs and the targeted binding substance constituents, respectively, since they are both highly biocompatible. Figure 1a shows a representative atomic force microscopy image of bare Fe₃O₄ FNs, while Figure 1b schematically presents the FN–targeted binding substance–Hcy complex. The atomic force microscopy data revealed that depending on the preparation conditions, the size of Fe₃O₄ FNs ranged between 50 and 150 nm. Thus, the extremely small size of the FN–targeted binding substance Cs will guarantee easy circulation in the bloodstream and will facilitate their utilization in future in vivo applications. Figure 1c shows the dialysis machine and the simple MD employed in the mock-dialysis experiments, which was made up of an array of permanent magnets placed along the extracorporeal circulation line. Figure 1d shows a few of the permanent magnets to illustrate the extracted FN–targeted binding substance–Hcy complex (Figure 1 appears in colour in the Supplementary data online).

Details of the conjugation capacity of Fe₃O₄ FNs for BSA and the magnetic properties of the prepared Fe₃O₄–BSA Cs have been previously reported [16]. Because of the interdisciplinary character of this project, representative results are presented to elucidate key issues of the newly proposed
MAHD concept. The conjugation between Fe$_3$O$_4$ FNs and BSA was evaluated by means of both CD spectropolarimetry and UV–Vis spectrophotometry. We performed systematic measurements using both techniques from supernatant samples drawn from formed Fe$_3$O$_4$–BSA Cs that had been magnetically extracted. These data were compared with reference values obtained from plain BSA solutions. Figure 2a shows representative CD results from the supernatant containing Fe$_3$O$_4$–BSA Cs (drawn under magnetic extraction) prepared at a BSA concentration of 2 mg/mL (solid circles). Respective data from a reference BSA solution are also shown (open circles). We found that the saturation magnetization of Fe$_3$O$_4$–BSA Cs was lower compared to the bare Fe$_3$O$_4$ FNs. As a general trend, higher BSA coverage of Fe$_3$O$_4$ FNs was associated with lower saturation magnetization. As a consequence, the magnetic extraction efficiency of the Fe$_3$O$_4$–BSA Cs, which was a key prerequisite of MAHD, was reduced (for more details on the magnetic properties of Fe$_3$O$_4$–BSA Cs consult [16]). In the mock-dialysis experiments described below, we employed either bare Fe$_3$O$_4$ or Fe$_3$O$_4$–BSA Cs prepared under the relatively low BSA content of 2 mg/mL to produce highly magnetic Cs.

**Evaluation of the magnetic extraction efficiency of bare Fe$_3$O$_4$ FNs and Fe$_3$O$_4$–BSA Cs during mock-dialysis experiments**

We carefully evaluated the magnetic extraction efficiency of both bare Fe$_3$O$_4$ FNs and Fe$_3$O$_4$–BSA Cs under conditions...
that are routinely used in HD practice. To do this, we added either bare Fe$_3$O$_4$ FNs or Fe$_3$O$_4$–BSA Cs at a concentration of 100 mg/L to saline and performed sequential circulations at a saline flow rate of 80–250 mL/min and a dialyzate flow rate of 500 mL/min. For the MD, we employed an array of 10–15 permanent magnets placed along the extracorporeal blood circulation line of the dialysis machine as shown in Figure 1c. Figure 1d shows a portion of the magnet array after completion of two circulations. Magnetic field gradients that exist at the edges of these disc-shaped magnets intensively extract these highly magnetic Cs. We observed that at high flow rates of 200–250 mL/min, the Fe$_3$O$_4$–BSA Cs were not entirely removed, while lower flow rates of 80–150 mL/min allowed for a complete extraction.

**Evaluation of removal rate and overall removal efficiency of MAHD compared with conventional HD**

We selected Hcy as a model target toxin substance to demonstrate the in vitro applicability of MAHD because of its significant biological impact on ESRD patients, and because conventional dialyzers are unable to efficiently remove this toxin due to its protein-binding affinity. Hcy was also chosen because of technical reasons described in [16] and the references therein. Of these, the most important is that Hcy exhibits intense reactivity and rapid chemical dynamics with transition metals due to its free thiol group.

In these MAHD mock-dialysis experiments, the Fe$_3$O$_4$ FNs and Fe$_3$O$_4$–BSA Cs were separately dispersed in 1 L saline. Hcy was added at a desired concentration (50–150 µmol/L) followed by mild stirring for few of minutes without additional treatment. We performed sequential dialysis rounds with sampling at the end of each round. The same procedure was followed in our reference conventional mock-dialysis experiments, which were based on a conventional low-flux dialyzer. In both experiments, we used the same saline flow rate of 220 mL/min and the standard dialyzate flow rate of 500 mL/min. The concentration of Hcy that remained in the saline at the end of each round was determined using standard FPIA methods routinely used in clinical practice [21, 22]. Figure 3a and b shows comparative data for these experiments. The upper panel shows raw data of round-to-round variations in remaining Hcy concentrations normalized to the initial Hcy concentration from each experiment. In the reference HD experiment, the initial Hcy concentration was $C_{\text{Hcy}}^{n=0} = 62$ µmol/L, while for MAHD the respective value was $C_{\text{Hcy}}^{n=0} = 100$ µmol/L. The specific concentration of Cs employed in MAHD was $C_{\text{BSA}} = 0.05$ mmol/L. The figure also shows least-squared fittings of the raw data according to an exponential law. The lower panel presents the respective percentage difference of the remaining Hcy concentration between the reference HD and the MAHD experiments, as was accurately determined from least-squared fitting curves. These data provide clear evidence that during the first three rounds, MAHD produced significantly higher Hcy removal rates compared to reference HD. Even though both MAHD and HD successfully removed the entire Hcy content, after seven circulations, MAHD showed a significantly higher overall removal efficiency since the initial Hcy concentration was approximately 60% higher than that in the reference HD experiment.

**Discussion**

Serum total Hcy (tHcy) exerts an important biological impact on patients, because even mild hyperhomocysteinemia is associated with premature atherosclerosis and CVD [9–11, 23–25]. However, recent studies in ESRD patients reported that high blood-serum tHcy levels are not a CVD risk factor but instead may predict improved survival. While examining this ‘reverse epidemiology of tHcy’ paradox, three recent studies indicated that increased serum levels of tHcy may in fact be causal for CVD. First, Suliman et al. [6] in a careful analysis of 317 ESRD patients found that, although a first evaluation suggested no association between increased tHcy levels and CVD, a reanalysis taking into account the overall nutritional and inflammation status restored the idea that increased tHcy concentrations are related to increased CVD and mortality. In a second study, examining 180 ESRD patients having
coronary artery disease, Pizzolo et al. [7] reported that as tHcy levels increase, a lower number of traditional risk factors are required to produce the same degree of coronary atherosclerosis. Finally, although Ducloux et al. [8] reported the ‘reverse epidemiology of tHcy’ in ESRD patients with inflammation-wasting syndrome, they also found that increased tHcy was directly related to increased all-cause mortality in ESRD patients without the syndrome. However, whether Hcy is a risk factor or simply a marker for CVD is not crucial for the present experiment; we chose Hcy as a model target toxin substance among other candidates for evaluating the in vitro applicability of MAHD. Ultimately, MAHD may be employed for the selective removal of other target toxin substances, such as β2-microglobulin, which is very likely related to dialysis-related amyloidosis [9–11].

We used a nominal Fe₃O₄ concentration in these mock-dialysis experiments that equalled to 0.05 mmol/L (11.6 mg/L). This value is within the well-established safety levels used during the treatment of iron deficiency anaemia. For example, Venofer®, which is an iron agent commonly used in clinical practice, is given as an aqueous iron (III) hydroxide sucrose complex [26]. One 5-mL vial of Venofer® provides 100 mg of iron, which is the well-established recommended dose given to ESRD patients during each HD session for the treatment of iron deficiency anaemia [27]. Thus, the effective blood concentration of iron after the administration of a 5-mL vial of Venofer® amounts to 16 mg/L, assuming that the blood makes up ~8% of total body weight in an 80-kg patient. This concentration is far below the well-accepted safety dose, and possible side effects are therefore negligible [26,27]. Thus, the Fe₃O₄ concentration used in our mock-dialysis experiments was within well-established safety levels, and this should allow for future in vivo application of biocompatible FNs during MAHD evaluation in animal models.

Despite these encouraging in vitro results, there is a major drawback for the use of BSA as the targeted binding substance. The present results were obtained from free Hcy dissolved in saline. In blood, however, this target toxin substance is primarily bound to proteins, mainly human serum albumin (HSA), via a disulfide bond [16]. Once the complex Hcy–HSA is formed, the chemically active site of Hcy is occupied. This will prohibit the binding of Hcy–HSA with the FN–targeted binding substance Cs, because the targeted binding substance constituent of the latter (namely BSA) has almost equivalent chemical reactivity with HSA. Thus, in order to attain the advantages offered by MAHD, it will be necessary to select more sophisticated targeted binding substances that exhibit high affinity for their respective target toxin substances. Antibodies provide ideal targeted binding substances since they can target chemically active sites on the target toxin substances that are different from sites already occupied by HSA, and because they can at times replace HSA due to their relatively higher chemical reactivity.

In summary, we report the first mock-dialysis experiments for the evaluation of MAHD during standard conditions that are used in HD. In these experiments, we used an array of permanent magnets placed along the extracorporeal blood circulation line as a simple MD, and Hcy was chosen as a model target toxin substance. The results obtained with Hcy indicate that both the removal rate and the overall removal efficiency of MAHD are significantly greater compared to conventional HD. We believe that MAHD may be employed for the selective removal of many specific toxins of high biological importance, so that conventional HD may evolve into a modality that can be tailored to the exact needs of each ESRD patient.

Acknowledgements. The nephrologist K. Papadopoulos and nurse D. Michalopoulos are acknowledged for their enlightening discussions. Nurse V. Galani is warmly acknowledged for valuable assistance during the mock-dialysis experiments.

Conflict of interest statement. None declared.

References


25. van Guldener, C. Why is homocysteine elevated in renal failure and what can be expected from homocysteine-lowering? *Nephrol Dial Transplant* 2006; 21: 1161–1166


Received for publication: 2.10.07
Accepted in revised form: 11.3.08