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Device for continuous extracorporeal blood purification using target-specific metal nanomagnets

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Abstract

Background. The present work illustrates how magnetic separation-based blood purification using ultra-strong iron nanomagnets can be implemented into an extracorporeal blood purification circuit. By this promising technique, today's blood purification may be extended to specifically filter high-molecular compounds without being limited by filter cut-offs or column surface saturation.

Methods. Blood spiked with digoxin (small molecule drug) and interleukin-1β (inflammatory protein) was circulated *ex vivo* through a device composed of approved blood transfusion lines. Target-specific nanomagnets were continuously injected and subsequently recovered with the aid of a magnetic separator before recirculating the blood.

Results. Magnetic blood purification was successfully carried out under flow conditions: already in single-pass experiments, removal efficiencies reached values of 75 and 40% for digoxin and interleukin-1β, respectively. Circulating 0.5 L of digoxin-intoxicated blood in a closed loop, digoxin concentration was decreased from initially toxic to therapeutic concentrations within 30 min and purification extents of 90% were achieved after 1.5 h.

Conclusions. Magnetic separation can be successfully implemented into an extracorporeal blood purification device. Simultaneous and specific filtering of high-molecular compounds may offer promising new therapeutic tools for the future treatment of complex diseases, such as sepsis and autoimmune disorders.

Keywords: detoxification; dialysis; magnetic separation; nanoparticle; selective

Introduction

New mechanistic insights into complex diseases (sepsis, autoimmune disorders) allow for the identification of potential new therapeutic targets and make high demands on today's blood purification techniques [1, 2]. Despite remarkable achievements of current blood purification systems, target-specific filtering of multiple compounds remains a major issue, particularly for molecules differing in physicochemical character (i.e. mass, size, charge) and high-molecular weight compounds, such as proteins. Where diffusion is slow (large target molecules) and filtration is not appropriate (e.g. due to filter cut-offs), patients require (centrifugal) plasma exchange (loss of plasma, risk of transfusion reactions) or blood purification through adsorbents. New technologies, such as antibody-coated adsorbents [3] or microsphere-based detoxification systems (MDS) [4, 5] are now being introduced to specifically aim at therapeutic targets. The use of spherical nonporous particles as adsorbents has been shown to benefit from the intrinsically better surface accessibility (no pore diffusion) due to higher external surface areas and shorter diffusion distances compared to membranes [6]. Most recently, we introduced the concept of magnetic blood purification using target-specific metal nanomagnets to selectively and efficiently remove toxins from whole blood in small volume batches [7]. In this approach, ligands (e.g. antibodies) immobilized on tiny free-flowing magnetic particles specifically capture noxious compounds. By applying external magnetic gradient fields, the toxin-loaded nanomagnets can be immediately separated and removed from the blood.

To bring this promising technology one step closer to clinical application, a first implementation into a continuously operating extracorporeal circuit was developed. Target-specific (e.g. antibody functionalized) metal nanomagnets are continuously injected into a continuous blood flow where they specifically capture the desired compounds. Subsequent removal of the pathogen-loaded nanomagnets from the bloodstream by continuous magnetic separation allows recirculation of the purified blood. In order to

demonstrate the potential of this new approach to remove molecules completely differing in character, digoxin and interleukin-1β (IL-1β) were used as model compounds [7]. Digoxin (MW 780 g/mol) is a typical representative of a small molecule drug with a narrow therapeutic window. In contrast, the much larger interleukin-1β (MW 17 000 g/mol) is a pro-inflammatory protein. Among other cytokines, it plays a crucial role in the orchestration of inflammatory processes, such as infections [8]. Digoxin and IL-1β antibodies were anchored on the carbon surface of highly magnetic carbon-coated metal nanomagnets and applied as capturing moieties. Using these two model compounds, we demonstrate how magnetic separation can be successfully implemented into an extracorporeal blood purification device allowing for fast and efficient clearance rates. This raises attractive opportunities for future therapeutic strategies, where we remove a noxious compound from a patient's blood rather than attempting a treatment through addition of another compound (i.e. drug) to counteract the negative effect of the pathogen.

Materials and methods

Synthesis of target-specific nanomagnets

Carbon-coated iron nanoparticles of core/shell geometry (C/Fe, mean diameter 30 nm, saturation magnetization 150 emu/g, specific surface area 30 m^2/g) were manufactured and washed in acid solution (1 week in HCl, 24 vol%) prior to use in order to dissolve incompletely coated particles following previously reported procedures [6, 7]. The nanomagnets were employed to magnetically tag digoxin antibody fragments (Beacon Pharmaceuticals Ltd, Kent, UK) and human interleukin-1β (IL-1β) antibodies (eBioscience, Inc. San Diego, CA).

Anti-digoxin-functionalized nanomagnets (C/Fe-anti-digoxin) were obtained via a stepwise procedure as previously reported [7]: briefly, anti-digoxin FAB fragments (antigen binding fragments) were covalently linked to an amine-reactive crosslinker (MAL-dPEG$_{24}$-NHS ester; Quanta Biodesign, Powell, OH) on the NHS end. Subsequently, the FAB-crosslinker complex was covalently bound to thiol-functionalized nanomagnets.

For the synthesis of anti-interleukin-1β nanomagnets (C/Fe-anti-IL-1β), interleukin-1β antibody was tagged by the aid of a third-party compound: streptavidin (Leinco Technologies, Inc. St. Louis, MO) was first physisorbed on the nanomagnets' outer shells. Biotinylated anti-human IL-1β was then anchored onto the streptavidin-covered nanomagnets.

Magnetically tagged agents were stocked in hydroxyethyl starch (Tetraspan 6%; B. Braun Medical AG, Sempach, Switzerland, containing hydroxyethyl starch 6%) and ultrasonicated for short periods (<3 min) before usage. Good dispersion stability was achieved up to nanomagnet concentrations of 5 mg/mL (see Supplementary Materials). Nanomagnet concentrations in the used dispersions were kept as high as possible in order to minimize the volume added to the blood.

Blood

Blood for experiments was withdrawn from healthy volunteers and stabilized with sodium heparin (25 000 U, 5 mL, B. Braun Medical AG) at 250 U/mL. Blood used in continuous experiments (500 mL) was purchased from Blutspende Zürich (Schlieren, Switzerland) as separate bags of freshly frozen plasma (FFP, A+) and erythrocyte concentrate (EC, A+) and then reconstituted.

Construction of a device for continuous magnetic blood purification

An extracorporeal circuit was assembled of approved transfusion sets (Dispomed, Gelnhausen, Germany) as follows: a line equipped with a silicon injection port and a 10-mL chamber (art. 51800) was connected to extension lines (art. 51130), all lines having inner diameters of 3 mm. To drive fluid flow, two different peristaltic pumps were used: REGLO Digital (Ismatec SA, Glattbrugg, Switzerland) for pumping the particle dispersion and a haemofiltration-approved pump head supplied by Infomed SA (Geneva, Switzerland) for the blood flow. During experiments, the nanoparticle dispersion container was shook continuously while being pumped. Dosing was kept constant over time in order to grant a constant concentration of nanomagnets in the device.

A multiline injection system was developed in order to inject the nanoparticle dispersion. Tygon standard tubing (R-3607, 0.27 mm; Ismatec SA) was slipped onto stainless steel cannulas (Ismatec SA, art.ISM 581), which were then used as injection nozzles collocating them into the injection port by perforation.

For the magnetic separation, the blood purification line was passed through a magnetic separator (average field intensity ~0.5 T). High-field gradients within the tubing were achieved by using an assembly of magnetized steel wires as initially proposed by Bockenfeld *et al*. [9] (detailed description in Supplementary Materials). Nanomagnets were subject to intense gradient fields which made them gradually deviate from the blood flow trajectory and eventually stick to tubing walls.

Sample collection

Single-pass pathogen removal efficiencies were determined by collecting freshly purified blood via a three-way connector (Dispomed Witt oHG, art. 70017) after the magnetic separator (open loop configuration). Samples for the continuous blood purification experiments were withdrawn from the mixed blood reservoir, where the contaminant concentration only gradually lowered over time (closed loop configuration).

Chemical analyses

Digoxin concentration was measured by the clinical chemistry laboratory in the University Hospital Zurich. The lower limit of detection was 0.2 nmol/L. Interleukin-1β concentrations were measured in duplicate by commercial enzyme-linked immunosorbent assays (BD Biosciencies, Allschwil, Switzerland) at a lower limit of detection of 1 pg/mL.

Theoretical estimate for clearance efficiency

The theoretical pathogen removal efficiency was calculated assuming constant per-pass removal efficiency and an ideally mixed blood reservoir (homogenous toxin concentration in the tank). The corresponding equation describing the evolution of the toxin concentration in the blood reservoir is given below. The concentration is denoted as C and C_0 is the initial concentration of the noxious compound in the tank:

$$C = C_0 \cdot e^{-\frac{V_R \cdot x \cdot t}{V_T \cdot \tau}}$$

and t is the time elapsed from the beginning and τ denotes the cycle time. The ratio t/τ equals the number of cycle runs through the purification unit. V_R and V_T are the extracorporeal and the total volume, respectively, and x is a fixed per-pass removal (toxin concentration in the blood leaving the extracorporeal device relative to the toxin concentration entering the device). A brief derivation of the clearance equation can be found in the Supplementary Materials.

Results

Assembly of a magnetic blood purification device

A first prototype device of magnetic separation-based continuous extracorporeal blood purification was assembled from three parts: (i) an injection port for nanomagnet administration into the bloodstream, (ii) a contact chamber where nanomagnets bind to the target molecules and (iii) a separator unit using magnetic field gradients to remove the pathogen-loaded nanomagnets from the blood before recirculation (Figure 1).

Continuous injection of nanomagnets into the bloodstream

To overcome limitations of surface saturation that impair frequent changes of sorbent cartridges in today's blood purification, a continuous administration of nanomagnets

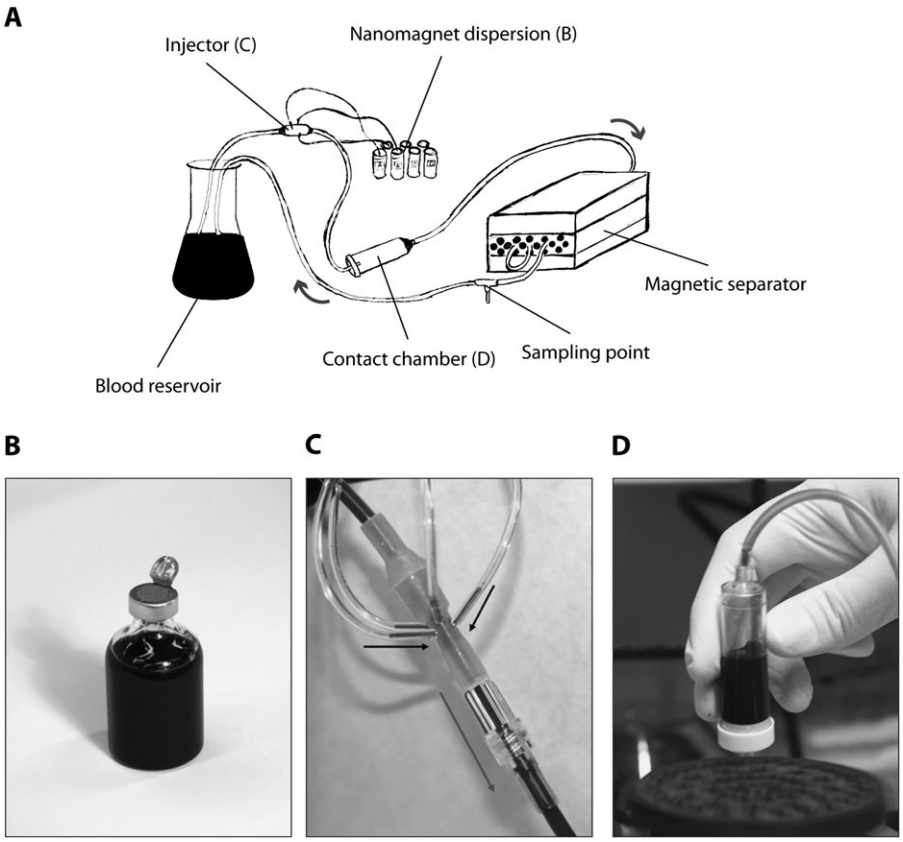

Fig. 1. Magnetic separation implemented in an extracorporeal blood purification device (**A**). Nanomagnets dispersed in hydroxyethyl starch (**B**) are continuously injected into the bloodstream (**C**). After capturing the toxins by selective capturing moieties immobilized on the nanomagnet surface, the toxin-loaded particles are magnetically separated from the bloodstream before the blood is recirculated into the blood reservoir. Ten-milliliter drip chambers were used to increase the contact time between nanomagnets and blood and to ensure homogenous concentration of nanomagnets (**D**).

with vacant binding sites is crucial. To address this issue, an injector having multiple injection ports for continuous administration of fresh nanomagnets was developed. Multiple nanomagnet dispersions with different target specificity can be simultaneously connected to the injector and fed into the blood flow at different rates (Figure 1).

Magnetic nanoparticles with a metallic core were used to achieve optimal particle separation after treatment. The use of metal nanomagnets gives access to superior magnetic properties (3–10 times higher saturation magnetization than traditional iron oxide-based nanoparticles) but entrains increased agglomeration due to magnetic dipole–dipole interactions. To prolong dispersion stability, various (protein or biopolymer containing) approved products for intravenous injection were evaluated for the dispersion of the nanomagnets. Among the polymer-based physiological solutions, hydroxyethyl starch turned out to be most effective to ensure stable dispersions ($\leq$5 mg nanomagnets/mL) while maintaining binding site accessibility (Figure 1).

Nanomagnet blood contact chamber

After injection of nanomagnets into the extracorporeal blood circuit, blood contact (area, time) is crucial as it is immediately linked to the extraction efficiency. In order to ensure a homogenous concentration of nanomagnets in the blood and to increase nanomagnet blood contact time (and thus time for binding), drip chambers of the transfusion sets with a volume of 10 mL each were employed as contact chamber(s) (Figure 1). The number of contact chambers connected allowed for controlling the exact contact time between particles and blood.

Magnetic separator—continuous separation of nanomagnets from the bloodstream

Finally, a high-efficiency magnetic separator assembled according to a concept initially described by Bockenfeld *et al*. [9] was used as a continuously operating magnetic separation unit (Figure 2). The extracorporeal circuit was passed through the separation unit multiple times (4×) in order to ensure complete particle separation before blood recirculation (see Figure 2).

Ex vivo blood circulation setting

For continuous blood purification experiments, blood was filled into a reservoir (tank with a volume of 1 L, Figure 1) and circulated through the device using an approved peristaltic pump at 15 mL/min, which has earlier been described to be a feasible flow rate in an *in vitro* haemodialysis model [10]. Particle sedimentation was minimal at this

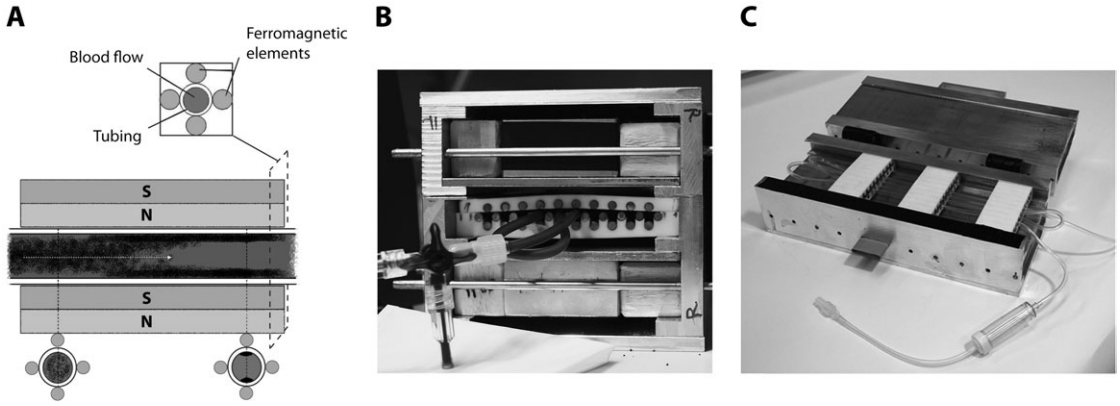

Fig. 2. Magnetic separator. A schematic drawing of the distribution of nanomagnets in the blood flow during recollection in the magnetic separator (**A**). A magnetic separator operating under continous conditions (**B**). The blood purification device is passed multiple times through the separation unit to achieve complete separation of nanomagnets from blood (**C**).

flow rate with still reasonably high nanomagnet-blood contact time. Approved transfusion lines were assembled into an extracorporeal circuit with a total volume of 50 mL (disposable parts) and volume between nanomagnet injection point and magnetic separator of 30 mL. With the overall flow rate of 15 mL/min, the corresponding blood-nanomagnet contact time in the extracorporeal circuit was 2 min. Based on the results obtained in batch experiments (see Supplementary Materials and an earlier study [7]), 0.5 mg of nanoparticles/mL of blood were applied for both digoxin and IL-1β removal. To demonstrate continuous magnetic blood purification, *in vitro* blood purification runs were performed using both open- (Figure 3) and closed-loop circuits (Figure 4).

Single-pass blood purification (ex vivo)

Figure 3 reports results of single passes through the magnetic blood purification device ('open-loop configuration', blood passes the device only once). The removal efficiency was determined in samples withdrawn directly after the separator unit. Significant purification efficiencies were achieved under continuous conditions: 66 and 38% for digoxin and IL-1β, respectively. For digoxin, a blood sample (60 mL) was collected after a first pass purification and subjected to a second purification cycle (readdition of fresh nanomagnets with vacant binding sites). Again, purification efficiencies of 70% were achieved using the same nanoparticle dose (0.5 mg/mL) (Figure 3).

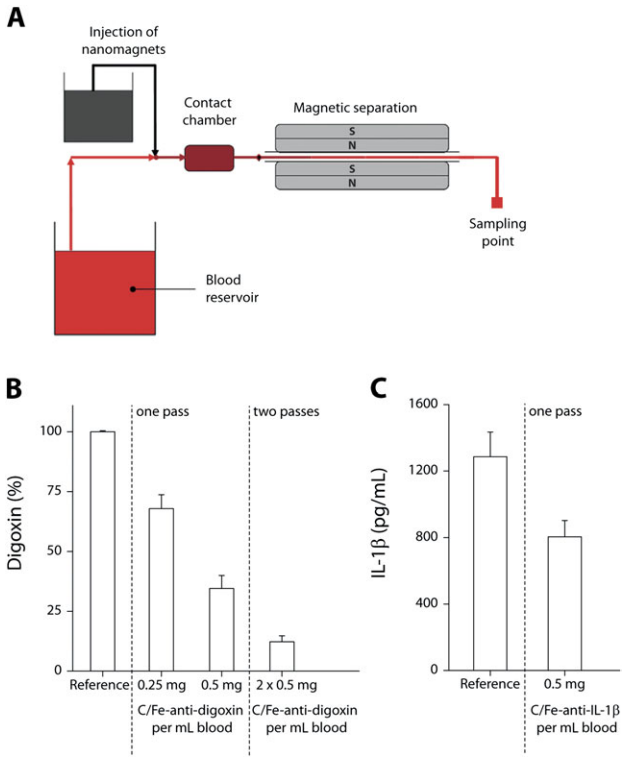

Fig. 3. Magnetic blood purification under continuous conditions (**A**). Samples collected after the separator (open loop configuration) show nanomagnet concentration-dependent extraction of digoxin (**B**) and interleukin-1β (**C**). Subjecting the purified blood to a second purification cycle shows again a significant decrease in digoxin level (**B**).

Continuous ex vivo blood purification

In order to simulate a more realistic scenario, blood purification was operated under continuous mode where the purified blood was recirculated into the blood reservoir after leaving the separation unit (closed-loop configuration). Continuous treatment was conducted on 500 mL of digoxin-spiked blood (5.7 nmol/L). One additional mixing chamber was added to the circuit in order to prolong effective contact time to 2.5 min while maintaining a standard flow rate of 15 mL/min. With respect to the imposed dilution constraint ($\leq$20%), overall 100 mL of dispersion of FeC-anti-digoxin (5 mg/mL) was allowed to be injected over a time span of 90 min (steady concentration of 0.38 mg nanoparticles/mL). Samples withdrawn after the separator after the first 2 min of operation allowed for evaluation of an initial single-pass removal efficiency of 75%. In the following, samples were taken every 4 min from the blood reservoir (Figure 4). After 30 min, digoxin concentration was halved and reduced below toxic levels. One hour later, the treatment was ended at a final purification extent of 89%.

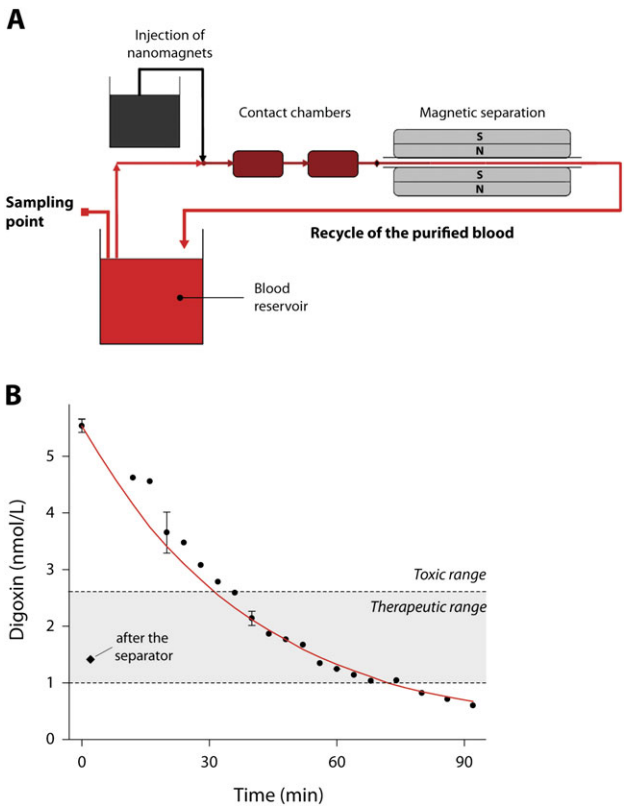

Fig. 4. Continuous purification of 500 mL digoxin-spiked blood using a closed-loop configuration where the purified blood is recirculated to the blood reservoir after the separator. A sample withdrawn directly after the separator at $t = 2$ min (closed triangles) shows the digoxin concentration in freshly purified blood. All other experimental points (closed circles) refer to samples taken from the blood reservoir where the purified blood has mixed with the intoxicated one, as it would happen in a patient. The red curve represents calculated clearance dynamics assuming an ideally mixed blood reservoir and constant removal efficiency.

Theoretical clearance efficiency

The theoretical purification level was calculated based on the assumptions of an ideally mixed blood reservoir (homogenous concentration) and a constant fraction of digoxin being removed in every pass (constant single-pass removal efficiency). The calculated removal efficiency as a function of time is shown in Figure 4 as a red line and compared to the experimental data.

Discussion

We present a first implementation of magnetic separation-based blood purification [7] into a continuously operating extracorporeal blood purification device. The feasibility of magnetic blood purification using functionalized metal core nanomagnets is proven in *ex vivo* experiments demonstrating remarkably high removal efficiencies which are very promising regarding future treatment in clinically critical situations. The prototype device for magnetic blood purification consists of a disposable unit (conventional approved transfusion set) where the blood circulates just as in standard blood purification systems. Magnetic separation-specific items, such as the nanomagnet injector and the magnetic separation unit, were designed as reusable units.

The miniaturized multiline injection port system where dispersed nanomagnets are injected into the extracorporeal circuit allows for simultaneous injection of nanomagnets with different target specificity. This is possible via independent regulation of every injection line flow rate. Every line can be connected to a different stock of functionalized nanomagnets. Such flexibility offers the possibility to perform tailored patient-specific substance removal adjusted to different clinical settings. Using target-specific nanomagnets, we claim target selectivity of these magnetically tagged ligands so far achieved only via MDS (simultaneous and selective) [11] or FAB-coated columns (Glycosorb) [12, 13]. Moreover, nano-sized particles show comparable contact surface per weight with respect to membranes or adsorptive matrixes. No pore diffusion hinders surface accessibility in the case of free-flowing particles. Thus, the whole surface is readily accessible and enables uniquely fast ligand binding.

The better purification efficiency achieved for digoxin can be explained by different binding characteristics (binding constant, binding kinetics and compound concentration range) and different capacity of FeC-anti-digoxin and FeC-anti-IL-1β nanomagnets. The removal efficiency could be further optimized by increasing the number of binding sites per particle (achievable via optimal synthesis) and the use of antibodies with an even higher binding constant. Theoretical clearance was estimated assuming the reservoir as an ideally mixed tank (homogenous toxin concentration distribution) and the circuit tract as a converter (a fixed fraction of the incoming substance is constantly converted). Although the model is rudimental, the theoretical values (red line) are in good agreement with the experimentally determined clearance dynamics.

A further fundamental improvement in the proposed approach is represented by the continuity in supplying fresh adsorptive surface carrying vacant binding sites. This overcomes saturation, an inevitable event for any non-regenerating sorbent. For example, if nonspecific adsorption takes place, media capture desired and undesired compounds, e.g. as observed by De Vriese *et al*. [14, 15] on the adsorbent Hopsal AN69: anti-inflammatory compounds (i.e. IL-1 receptor antagonist) were subject to adsorption as much as pro-inflammatory agents were. This unfavourable fact accelerates the saturation process (loss of target-binding sites on the adsorbent) and thus decreases adsorption rates besides removing a wider class of compounds than what may be targeted.

In the continuous *ex vivo* purification setting used here, the major assumption, also previously made by Uchino *et al*. [16], is that a blood container is able to replicate a human body. This limited model cannot take into account that the human body dynamically interacts with changes caused by extracorporeal factors, i.e. septic patients continuously produce cytokines (i.e. *de novo* synthesis) while undergoing therapy [2].

The experimental conditions used in the investigated *ex vivo* blood purification experiments can be compared to real clinical dimensions [17] with potential for scale up. It is expected that the use of multiple lines in parallel would allow analogous extraction efficiencies for a patient's whole blood volume (4.5 L) using similar conditions as in the

ex vivo experiments shown here. Although the transfusion sets used consisted of clinically approved materials, toxicity of the injected nanomagnets needs to be thoroughly investigated before the magnetic blood purification technology can be evaluated in first *in vivo* animal trials.

For clinical application, biocompatibility and/or complete removal of the nanomagnets from blood before recirculation are critical and ultimately determine the clinical applicability of the proposed approach. Various settings for high-gradient field magnetic separation units for efficient capturing of magnetic particles under flow conditions have been developed and are now being tested [9, 18, 19]. Compared to commonly used polymer/metal oxide beads, metal nanomagnets benefit from their higher magnetic moments that facilitate magnetic recovery and separation [20]. Matrix effects of blood (high iron background) and the high chemical stability render quantification of carbon encapsulated iron nanomagnets complex. First experiments using water–glycerol mixtures with blood-like viscosity and the above-mentioned operating conditions (concentrations of particles before the separator 500 ppm, flow rate of 15 mL/min) showed that <4.5 ppm iron nanomagnets were detectable after the separator (as determined by atomic absorption spectroscopy, see Supplementary Materials), corresponding to a removal efficiency at least 99%.

Although first haemocompatibility tests of carbon-coated nanomagnets have shown promising results (normal haemolysis and iron metabolism parameters, unaffected blood coagulation) [7], biocompatibility—with particular focus on possible interactions with the vascular compartment—needs to be thoroughly investigated in order to ensure a safe implementation of this technology (e.g. exclude the danger of embolization). Compatibility of the nanomagnets with the human body poses a major limitation of the presented blood purification device at this stage as a small amount of nanomagnets remains in the blood after treatment ($<1\%$). In a next step toward clinical application, *in vivo* performance and tolerance of magnetic blood purification needs to be evaluated in animal models (e.g. rodent model of severe intoxication and gram-negative sepsis) to prove feasibility in a living organism and to gain insight into dynamic interactions of the organism with the blood purification device (e.g. activation of coagulation cascade or the complement cascade, *de novo* synthesis of cytokines).

Conclusions

Successful implementation of magnetic blood purification into a continuously operating extracorporeal device proves the feasibility of using magnetic separation for blood purification. Results were achieved in a first implementation of such a device already showing a promising capacity for treating a real patient's blood volume in clinically relevant time when scaled up. It combines many favorable features that had been claimed in the past: high clearance rate in relatively short time frame, customization and potentially high selectivity toward targets and no fluid or beneficial substance loss [16, 21–25]. In clinically complex situations, e.g. sepsis and systemic inflammatory response syndrome [26], the system proposed here may offer a new tool to be added to the actual ones. Numerous clinically most attractive hypotheses, such as cytokine removal in sepsis and its effect on mortality, now remain to be proved in first animal trials. Detailed *in vivo* tests are necessary to bring magnetic separation-based blood purification closer to daily clinical use.

Supplementary data

Supplementary Materials are available online at http://ndt.oxfordjournals.org.

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