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# In vitro studies of functionalized magnetic nanospheres for selective removal of a simulant biotoxin

Carol J. Mertz<sup>a,\*</sup>, Michael D. Kaminski<sup>a</sup>, Yumei Xie<sup>b</sup>, Martha R. Finck<sup>a</sup>,  
Sandra Guy<sup>b</sup>, Axel J. Rosengart<sup>b</sup>

<sup>a</sup>Argonne National Laboratory, Chemical Engineering Division, Nanoscale Engineering Group, 9700 S. Cass Ave,  
CMT-205, Argonne, IL 60439, USA

<sup>b</sup>Neurocritical Care and Acute Stroke Program, Departments of Neurology and Neurosurgery,  
The University of Chicago and Pritzker School of Medicine, Chicago, IL, USA

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## Abstract

In this study, the simulant biotoxin (biotinylated horseradish peroxidase) was efficiently removed from simple electrolyte solutions and blood using a highly selective complexation reaction (biotin–avidin). Sequestration of the biotoxin is realized with streptavidin-functionalized magnetic nanoparticles that selectively capture the biotoxin. Quantitative removal of the model toxin is achieved using an external magnetic field to trap the toxin-bound particles.  
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## 1. Introduction

The use of functionalized nanoparticles offers many potential, biomedical applications due to the versatility of applied receptors or encapsulation of drugs for targeted delivery [1–3]. The exploitation of the range of applications is being realized and research for their application is being advanced [4]. In order for the nanoparticles to be used in the

body, the particles must have attributes that allow them to flow freely through the circulatory system [5,6]. The particles must be of the proper size (100–2000 nm), be hidden to the immune system (PEGylation has been shown to be effective for vascular survival), and possess magnetic components and receptors (allowing targeted delivery of drugs or recovery of toxins).

A novel approach for detoxification of blood-borne toxins from humans is being advanced through the use of functionalized, magnetic nanospheres [7]. The nanospheres are envisioned for intravascular injection and circulation through the

\*Corresponding author. Tel.: +1 630 724 9165;  
fax: +1 630 972 4428.

E-mail address: [mertz@cmt.anl.gov](mailto:mertz@cmt.anl.gov) (C.J. Mertz).

blood stream, thus capturing and selectively binding to the toxins via receptors on the particle surface. Subsequent removal of the toxin-bound particles is achieved using a magnetic filter unit allowing purified blood to return to the body. The magnetic nanospheres can be tailored with various receptors (conjugated to the surface of the particles) as applicable, to treat chemical, biological, or radiological agents.

The proof-of-concept for this technology is presented here and employs a highly selective, complexation reaction. The biotin–avidin interaction is one of the strongest known biological interactions between protein and ligand with a  $K_a$  of  $10^{15} \text{ M}^{-1}$ . In this study, streptavidin is attached to the magnetic particles and acts as the receptor for the model toxin, biotinylated horseradish peroxidase (bHRP). Streptavidin is a biotin-binding protein similar to avidin and the biotin is conjugated to horseradish peroxidase (HRP) allowing for protein detection and quantification.

The biotin binding or loading capacity of the particles was determined using an Enzyme-Linked ImmunoSorbant Assay (ELISA). ELISAs are designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen is immobilized onto a solid surface. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product.

We employed a water soluble, colorimetric substrate for HRP, and a modified procedure for particle loading based upon Dorgan [8]. The HRP substrate or chromogen is ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) and yields a green product upon reaction with peroxidase [9]. Results are presented for the removal of the toxin (bHRP) using streptavidin-functionalized magnetic nanoparticles from simple electrolyte solutions and blood.

Current methods of blood detoxification have severe drawbacks and this technology offers numerous advantages. The current treatments (hemodialysis, plasmapheresis, immunoabsorbant detoxification methods) are plagued with many problems such as long treatment times (at least

several hours), requiring large volumes of blood to be filtered extracorporeally, which is risky for the patient, the possibility of causing serious side effects due to the introduction of the chelator with high concentration to the patient [7]. The use of functionalized nanoparticles for detoxification would eliminate and address all of the disadvantages of the current treatment technologies.

## 2. Experimental method

bHRP (Pierce Biotechnology, Inc., ImmunoPure, “bHRP,” used as the model “toxin”), 1Step ABTS (Pierce Biotechnology, Inc., 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt, “HRP substrate”), buffer solution (Pierce Biotechnology, Inc., BupH phosphate-buffered saline, 0.1 M  $\text{Na}_3\text{PO}_4$ , 0.15 M NaCl, pH 7.2; “PBS”), STOP solution (Aldrich, 1% sodium dodecyl sulfate, “SDS”), Tween-20 (Pierce Biotechnology, Inc., Surfact-Amps 20), saline solution (Abbott, 0.9% NaCl), and uncoated and streptavidin-coated microtiter plates (Pierce Biotechnology, Inc., Reacti-Bind Streptavidin Coated Polystyrene Plate, 96 wells, Blocked with SuperBlock Blocking Buffer) were used as obtained or prepared according to the manufacturer's instructions. The solutions were prepared using single distilled reverse osmosis water. Rat blood was taken from breeder rats and used as supplied by The University of Chicago and Pritzker School of Medicine. Magnetic, latex nanoparticles (custom ordered, Latex-M PEG-2000streptavidin, 400 nm diameter, 10 mg/mL) were purchased from Micro-mod (Germany). Biodegradable microparticles composed of poly(lactic acid)–poly(ethylene glycol), and biotin were synthesized in our lab according to the procedure by Salem et al. [10]. A magnet, NdFeB, 0.4 T at surface, was used for separating the magnetic particles.

Characterization of the particle properties was performed using dynamic light scattering or scanning electron microscopy (for particle size distribution information) and zeta potential measurements (for surface charge information). Dynamic light scattering measurements were performed on a modified, Malvern 4700c PCS

unit (Malvern, UK). A Uniphase, argon ion laser (515 nm, 3–75 mW) and associated optics were added to upgrade the Malvern unit. The samples were run at 3 mW and placed in 8 mm OD vials for in situ size distribution measurements. Zeta potential measurements were made on a Brookhaven ZetaPlus Analyzer. Solutions for zeta potential measurements were adjusted for pH using 0.1 M nitric acid or sodium hydroxide. The ELISAs were performed on a Wallac Victor 1420 Multilabel Counter at 405 nm.

The ELISA developed for biotin binding to streptavidin-coated particles in blood is summarized next, but was modified from initial experiments using simple electrolyte solutions (saline). The typical ELISA protocol involves several steps when using antibody or antigen-coated microplates. First, add the sample to be tested (50–100  $\mu\text{L}$ /well) and incubate for 1 or 2 h. Next, wash the plate using PBS with 0.05% Tween 20. Then, add the enzyme–antibody conjugate (100–200  $\mu\text{L}$ /well) and incubate for 1 h. Finally, add the substrate and detect.

The procedure developed in this study for the biotin binding to streptavidin-coated particles in blood follows. A schematic of the streptavidin-coated magnetic particles with bHRP experiments is shown in Fig. 1. A calibration curve for bHRP

in blood was prepared over a range of concentrations from 0.008 to 0.375  $\mu\text{g}/\text{mL}$ . The particle concentrations used for the in vitro experiments was 0.25 mg/mL. All experiments were performed in at least triplicate. For quantification of biotin binding to streptavidin-coated magnetic particles, the particles were first washed three times to remove preservatives. The particle stock solutions were washed after dispersion in 2 mL of buffer (PBS), then the particles were separated from the supernatant using a 0.4 T magnet and the supernatant was discarded.

The particles were then washed with saline prior to contacting the particles with the toxin-contaminated blood (0.375 mg bHRP/mL blood). The particles were incubated with gentle mixing for 15 to 180 min. When the incubation time was complete, the particles were separated from the blood using the magnet. The blood (supernatant) was reserved for analysis. The particles were rinsed at least three times with saline to remove any unbound bHRP (following the wash procedure from above). Next, the solutions containing unbound bHRP were analyzed using the streptavidin-coated plates. The plates were first washed three times with buffer (200  $\mu\text{L}$  per well) prior to adding the sample aliquots to remove preservatives. A 10  $\mu\text{L}$  aliquot of the blood sample solution

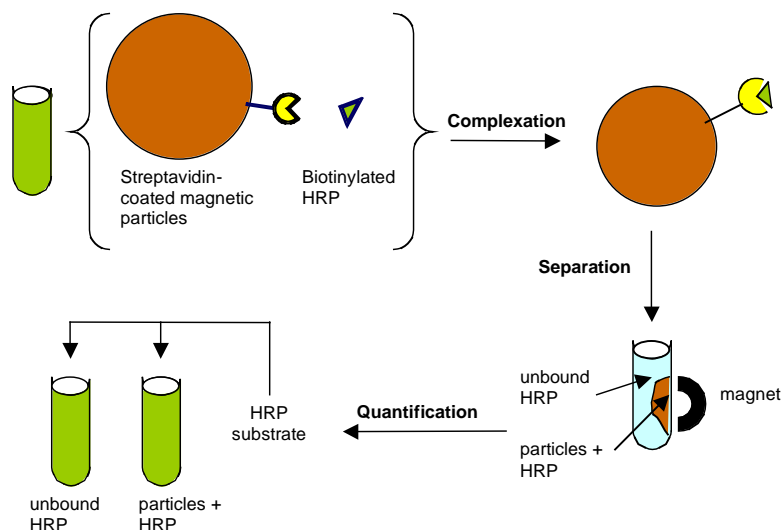


Fig. 1. A schematic of the experiment used to quantify biotin binding to streptavidin-coated magnetic particles using bHRP as the simulant toxin.

was added to microtiter wells in triplicate, then 100  $\mu\text{L}$  of the buffer was added. The plate was incubated for 1 h with gentle stirring. When the incubation time was complete, the wells were washed with buffer (200  $\mu\text{L}$  per well) until residual blood was removed. The HRP substrate was then added to the wells (100  $\mu\text{L}$  of 1Step ABTS) and reacted under gentle mixing for  $\sim 10$  min. The absorbance of the plates was read at 405 nm.

### 3. Discussion

Tests studying the kinetics of bHRP removal by streptavidin-coated nanoparticles are being developed for the removal of blood-borne biotoxins. Initial developmental work for the nanoparticles showed significant differences between the predicted and measured absorbances of the control and initial bHRP concentration in saline. These values should have been the same, however, the initial absorbance of bHRP (prior to contact with the particles) were significantly lower (by  $\sim 80\%$ ) than the control (data not shown). Differences between the tests were noted in the materials of the test vials contacting the bHRP solution. Control tests (those with only bHRP) were run in polypropylene (PP), however, the bHRP test samples were first contacted with PP, then placed in glass vials with the magnetic particles, and aliquots were removed and put in polyethylene (PE). Additional tests were run to examine the effect of absorbance of bHRP in the two different plastics (PP and PE). Results from contacting the bHRP with PP and PE for 60 min are shown in Fig. 2. The PE substrate absorbs bHRP approximately 30% more than the PP substrate for bHRP concentrations spanning the range of 0.008–0.4 ppm. These tests represent the importance of running tests in the same type of material and the same number of contacts with various materials for both samples and the control. Pretreatment of the surface substrate may be another way of minimizing sorption of the test toxin.

In order to evaluate when steady state (equilibrium) had been reached for the binding of bHRP to streptavidin, bHRP (over a range of concentrations from 0.008 to 0.4 ppm) was incubated for 1 and 3 h with streptavidin-coated microtiter wells.

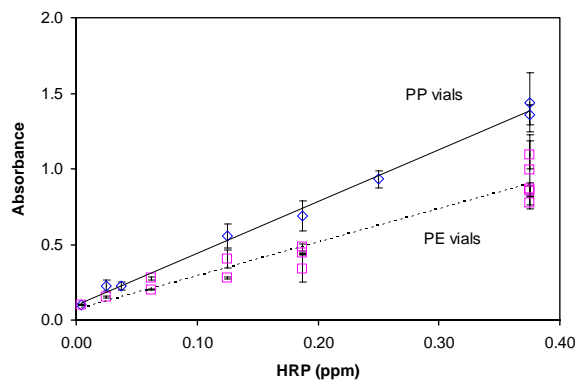


Fig. 2. Absorbance readings for bHRP solutions after contact with PP and PE vials for 60 min.

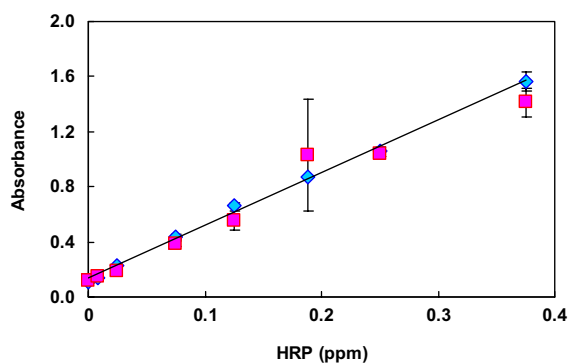


Fig. 3. Absorbance readings for binding bHRP to streptavidin-coated microtiter wells (over a range of bHRP concentrations). The bHRP was incubated for 1 and 3 h (squares and diamonds, respectively) with the wells.

No difference was noted between 1 and 3 h incubation kinetics for bHRP binding to the streptavidin-coated wells (refer to Fig. 3). Thus, 1 h incubation of bHRP samples with the streptavidin-coated wells is sufficient time to reach equilibrium over the range of bHRP concentrations expected in these experiments.

In vitro assays for bHRP with blood have shown the importance of removing residual blood prior to analysis of the bHRP. The absorption spectrum of blood [11] shows a significant contribution due to blood at 405 nm, the wavelength for detection of bHRP quantities. Table 1 shows absorbance readings for blood, saline, and

Table 1

Absorbance readings for saline, water, and blood at 405 nm as a function of rinsing the microtiter plate wells. The interference due to blood absorbance is shown

Sample description	Absorbance	# Rinses
Blood (undiluted)	0.84	3
Blood (diluted 1:2)	0.78	3
Blood (diluted 1:4)	0.42	3
Blood (diluted 1:6)	0.11	3
Saline	0.09	3
Water	0.09	3
Blood (undiluted)	0.10	10
Blood (undiluted)	0.09	15

water (no bHRP present) as a function of well rinses at the HRP substrate wavelength maximum (405 nm). Background readings are represented by the absorbance for saline and water after three rinses. When the background absorbance values for saline and water media are compared to those of undiluted blood that was rinsed only three times, the absorbance at 405 nm was almost a factor of 10 too high. The high background absorbance readings due to presence of blood were eliminated by sufficient rinsing of microtiter plate wells or dilution of blood samples prior to adding aliquots to the wells. At least 10 rinses of the wells for the undiluted blood samples or a six-fold dilution of the blood prior to sampling for the wells was required to eliminate the interference due to blood.

Magnetic, latex nanoparticles (400 nm streptavidin-coated particles) were incubated with the simulant toxin, bHRP in saline, and blood to determine toxin-loading capacities for the nanoparticles (refer to Fig. 4). For saline media, toxin loadings of ~70% were achieved with a single contact of the nanoparticles in 15 and 60 min. The kinetics of the particle loading in saline were very rapid (no difference noted between the 15 and 60 min incubations). However, the bHRP loading for a single contact onto the nanoparticles in blood exhibited a time-dependent response. It took 180 min to reach the maximum loading of ~70% in blood. The mass transfer of the toxin in blood may be the limiting factor for the nanoparticle loading. Vortexing the blood and particles (instead

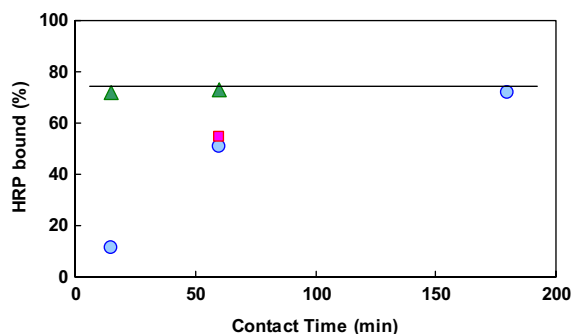


Fig. 4. Toxin (bHRP) loading onto streptavidin-coated magnetic, latex nanoparticles (400 nm) as a function of incubation time in saline and blood (triangles, saline media, gentle mixing; circles, blood media, gentle mixing; square, blood media, vortexed).

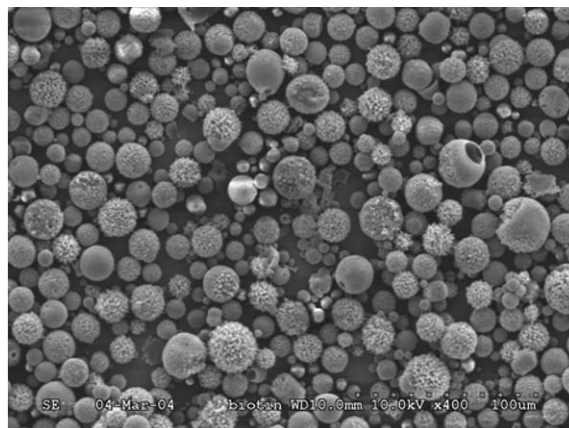


Fig. 5. Micrograph of biodegradable, b-PEG-PLA polymer microparticles.

of gentle mixing) during the 60 min incubation did not appear to affect the loading efficiency (~50% for both gentle mixing and vortexing).

Biodegradable microparticles of biotinylated poly(ethylene glycol)–poly(lactic acid) (b-PEG-PLA) were synthesized in our lab. Initial syntheses of the b-PEG-PLA, biodegradable particles have yielded polydisperse microparticles (refer to micrograph, Fig. 5). Additional syntheses are ongoing to optimize production of monodisperse nanoparticles for effective and complete coverage of PEG and biotin receptors. The b-PEG-PLA particles were incubated at 37 °C for 20 min with

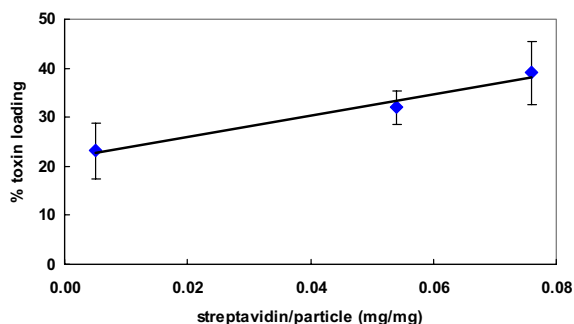


Fig. 6. Toxin (bHRP) loading onto streptavidin-coated b-PEG-PLA microparticles in saline media with single particle contacts and 1 h incubation of the particles with the toxin.

varying amounts of streptavidin according to procedure of Salem et al. [10]. The streptavidin acts as the receptor for the toxin (bHRP) loading onto the particles. The loading efficiencies for the toxin (bHRP) onto the streptavidin-coated particles increased with streptavidin loading. Up to 40% bHRP loading onto the particles was obtained for saline media with single particle contacts and 1 h incubation of the particles with the toxin (Fig. 6). Once particle syntheses are optimized, higher toxin loadings can be expected for initial contacts with the particles.

#### 4. Summary

Results are presented that show efficient removal of the toxin from simple electrolyte solutions (saline) and blood. Toxin loadings of 70% have been achieved using 400 nm latex streptavidin-coated magnetic nanoparticles with a single contact. Biodegradable nanospheres have also been synthesized in our lab for fulfilling the in vivo requirements of physiologically stealth nanoparticles. Toxin loadings of 40% were achieved

with the biodegradable particles synthesized from initial trials (streptavidin-coated, biotinylated poly(ethylene glycol)–poly(lactic acid) polymer microparticles). Optimization of the biodegradable nanoparticle synthesis is ongoing.

Challenges overcome in the development of the enzyme linked immunosorbant assay for the quantitation of the streptavidin-functionalized magnetic nanoparticles were presented. These challenges involved quantitation of enzyme sorption onto surfaces other than the nanoparticles and removal of background effects due to blood in the enzyme analyses. The detection and quantitation of the biotoxin-bound particles developed from these in vitro studies will be extended to future in vivo studies. Results from these in vitro studies support concept feasibility for detoxification of human subjects.

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