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A biomimetic nanosponge that absorbs pore-forming toxins

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Abstract

Detoxification treatments such as toxin-targeted anti-virulence therapy^{1, 2} offer ways to cleanse the body of virulence factors that are caused by bacterial infections, venomous injuries, and biological weaponry. Because existing detoxification platforms such as antisera³, monoclonal antibodies⁴, small-molecule inhibitors^{5, 6}, and molecularly imprinted polymers⁷ act by targeting the molecular structures of the toxins, customized treatments are required for different diseases. Here we show a biomimetic toxin nanosponge that functions as a toxin decoy *in vivo*. The nanosponge, which consists of a polymeric nanoparticle core surrounded by red blood cell membranes, absorbs membrane-damaging toxins and diverts them away from their cellular targets. In a mouse model, the nanosponges markedly reduce the toxicity of staphylococcal alphahemolysin (α -toxin) and thus improve the survival rate of toxin-challenged mice. This biologically inspired toxin nanosponge presents a detoxification treatment that can potentially treat a variety of injuries and diseases caused by pore-forming toxins.

Pore-forming toxins (PFTs) are one of the most common protein toxins found in nature^{8, 9}. These toxins disrupt cells by forming pores in cellular membranes and altering their permeability. In bacterial infections, the attack by PFTs constitutes a major virulence mechanism⁹. It has been demonstrated that the inhibition of the pore-forming α-toxin can reduce the severity of *Staphylococcus aureus* infections¹⁰, and similar PFT-targeted strategies have shown therapeutic potential against other pathogens including *Escherichia coli*¹¹, *Listeria monocytogenes*¹², *Bacillus anthracis*¹³, and *Streptococcus pneumoniae*¹⁴. Aside from their roles in bacterial pathogenesis, PFTs are commonly employed in venomous attacks by animals including sea anemones, scorpions, and snakes¹⁵. Over 80 families of PFTs have been identified, displaying diverse molecular structures and distinctive epitopic targets¹⁶. Despite these differences, the functional similarity among these toxins in

Competing financial interests

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Author contributions

L.Z. conceived and designed the experiments with C-M.H., R.F. and J.C.; C-M.H., R.F. J.C. and B.L. performed all the experiments. All authors analyzed and discussed the data; L.Z., C-M.H. and R. F. wrote the paper.

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perforating cellular membranes provides the design cue for an action mechanism-targeted detoxification platform with a broad applicability.

In this study, a toxin nanosponge is constructed with a polymeric core wrapped in natural RBC bilayer membranes (Fig. 1a). The RBC membrane shell provides an ideal mimicry to absorb a wide range of PFTs regardless of their molecular structures. Meanwhile, the inner polymeric core stabilizes the RBC membrane shell to enable prolonged systemic circulation essential for absorbing toxins in the bloodstream. The nanosponges were prepared by fusing RBC membrane vesicles onto poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles through an extrusion approach (Fig. S1)¹⁷. Under transmission electron microscopy, the resulting nanosponges exhibited a core-shell structure approximately 85 nm in diameter (Fig. 1b).

To test the nanosponges' ability to neutralize PFTs, α -toxin was mixed with the nanosponges and then added to purified mouse RBCs. Equivalent amounts of PLGA nanoparticles (coated with PEG for stability), liposomes (coated with PEG for stability), and RBC membrane vesicles of comparable particle sizes were tested in parallel as controls. As shown in Fig. 2a, the nanosponge sample was noticeably different from the other samples, exhibiting a clear supernatant that indicated the RBCs were undamaged. The degree of hemolysis was quantified by measuring the absorbance of the released hemoglobin in the supernatant at 540 nm (Fig. 2b). A positive control sample containing anti- α -toxin antibodies verified that the observed hemolysis was toxin-specific. The capability of the nanosponges to absorb toxins was further examined by measuring the RBC hemolysis at varying amounts of α -toxin with fixed nanosponge content (Fig. 2c). Experiments with streptolysin-O (a pore-forming exotoxin produced by *Streptococcus pyogenes*¹⁸) and melittin (a membrane-disrupting peptide in bee venom¹⁹) showed similar patterns of reduced RBC hemolysis by the nanosponges (Fig. S2), demonstrating the platform's applicability against different types of membrane-targeted toxins.

Next the nanoformulation/ α -toxin mixtures were filtered through a column to separate out free-floating, unbound toxin. Given α -toxin's tendency to spontaneously incorporate into erythrocyte membranes²⁰, the nanosponges and the RBC membrane vesicles were expected to absorb and retain the toxin after being run through the filtration column. Following SDS-PAGE analysis, it was found that the nanosponges and the RBC membrane vesicles retained 90.2% and 95.3% of the α -toxin, respectively (Fig. 2d and Fig. S3). In comparison, the toxin protein band was almost nonexistent in the PLGA nanoparticle and liposome samples, which suggested that their PEG coating precluded protein interactions. The purified α -toxin-bound nanosponges and RBC membrane vesicles were subsequently examined for their hemolytic activities. It was found that the nanosponges showed no hemolytic activity whereas the RBC membrane vesicles went on to lyse the RBCs (Fig. S4).

The fact that the RBC membrane vesicles were able to absorb a-toxin but failed to reduce its hemolytic activity highlights the role of the polymeric cores in the nanosponges. A cellular uptake study was conducted to better understand the disparity between their neutralization capabilities. Fluorescence microscopy of the nanoformulations with fluorescently doped RBC membranes portrayed their different fates upon incubation with human umbilical vein endothelial cells (HUVECs) (Fig. 2e). In the sample with RBC membrane vesicles, broadly

To examine whether the nanosponges can detoxify α -toxin and render it harmless to cellular targets, cellular cytotoxicity was studied using HUVECs. It was shown that α -toxin's toxicity against the cells was significantly reduced upon both pre-mixing with nanosponges (Fig. 2f) and conjointly mixing with nanosponges (Fig. S5). Similar detoxification properties of the nanosponges were observed with other PFT types including streptolysin-O and melittin (Fig. S6). The virulence neutralization by the nanosponges was likely due to both toxin diversion from cellular membranes and enhanced endolysosomal digestion of the absorbed toxin protein following the endocytic uptake observed in Fig. 2e. Based upon the pre-incubation experimental cytotoxicity results and the physicochemical characteristics of the nanosponges and the toxins, it was estimated that each nanosponge was able to neutralize approximately 85 α -toxin, 30 streptolysin-O, or 850 melittin monomers (supplementary discussion).

nanosponges, however, were able to not only arrest but also lock in the toxins to keep them

away from other RBC membranes.

The ability of the nanosponges to neutralize α -toxin was further demonstrated *in vivo* by subcutaneous injection of α -toxin or α -toxin/nanosponge mixture beneath the right flank skin of mice. 72 hr after the injection of 150 µL of free α -toxin (12 µg/mL in PBS), severe skin lesions were induced with demonstrable edema and inflammation (Fig. 3a) and closer examination of the skin tissue showed necrosis, apoptosis, and inflammatory infiltrate of neutrophils with dermal edema (Fig. 3b). Moreover, the toxin damaged the underlying muscle tissue as evidenced by interfibril edema, tears on muscles fibers, and a significant number of extravasating neutrophils from the surrounding vasculature (Fig. 3c). However, mixing 100 µg of the nanosponges with the injected amount of α -toxin (toxin-to-nanosponge ratio \approx 70:1) appeared to neutralize the toxin, as there was no observable damage on the mice (Fig. 3d). The tissue samples showed normal epithelial structures in skin histology and intact fibrous structures with no visible infiltrate in the muscle histology (Fig. 3e,f). In contrast, PEG-PLGA nanoparticles and RBC membrane vesicles failed to prevent the toxin damage in the skin (Fig. S7).

Finally, the nanosponges were assessed for their systemic detoxification efficacy against α -toxin, which in circulation can inflict serious complications by causing blood coagulation, systemic inflammation, and endothelial dysfunction²³. To examine the nanosponges' potential in reducing toxin burden *in vivo*, a bolus lethal dose of α -toxin (75 µg/kg), known to induce acute death in mice²⁴, was injected into mice through the tail vein. In the two experimental settings, 80 mg/kg of the nanosponges was injected either 2 min before or 2 min after the toxin injection. Note that a separate study verified that such a nanosponge dose was well tolerated by mice (Fig. S8). Fig. 4a and b show that mice without any treatments had a 100% mortality rate within 6 hr following the α -toxin injection. In the group treated

with nanosponge pre-inoculation, the mortality rate was reduced markedly to 11% (p<0.0001, n=9). In contrast, pre-inoculation with PEG-PLGA nanoparticles and RBC membrane vesicles failed to improve the survival rate of the toxin-challenged mice (Fig. 4a). In the post-inoculation treatment groups, the nanosponge injection remained beneficial to the overall survival, yielding a 56% mortality rate (p=0.0091, n=9), whereas the control formulations showed no survival advantage (Fig. 4b). It should be noted that in both of the nanosponge treatment groups, no additional death occurred past the 6 hr mark, suggesting that the absorbed toxin was detoxified rather than merely having its toxicity delayed.

To elucidate the *in vivo* fate of the nanosponge-sequestered toxin, the biodistribution of the toxin-bound nanosponges was studied, which revealed that they accumulated primarily in the liver (Fig. 4c). Liver biopsies on day 3 and day 7 following the intravenous injection of the toxin-bound nanosponges were performed to investigate the potential effect of the sequestered toxin upon liver accumulation. Examination of the liver sections revealed normal hepatocytes supplied by blood vessels with no inclusion of Kupffer cells in the sinusoids (Fig. 4d). The lack of liver tissue damage suggests that the sequestered toxin was safely metabolized, likely through ingestion by hepatic macrophages.

Based on a rational nanostructure design, a broadly applicable, biodegradable and biocompatible detoxification platform against PFTs was developed. Consisting of nanoparticle-stabilized RBC membranes, the nanosponges are capable of absorbing and diverting the membrane-damaging virulence of PFTs. The RBC membranes, upon translocation to nanoparticle surfaces, retain their toxin affinity. A comparison study of nanosponges prepared from human and mouse RBCs showed differing propensities in toxin interaction that mirrored the toxin-binding characteristics of the source RBCs. Whereas streptolysin-O and melittin were detoxified to similar degrees by the two nanosponges, stronger interactions between a-toxin and mouse-RBC-derived nanosponges reflected the mouse RBCs' higher susceptibility to the particular toxin as compared to human RBCs²³ (Fig. S9). Such membrane-toxin affinity is an important factor to consider toward future applications, and strategies to improve membrane-toxin interactions^{25, 26} may be applied to further enhance the platform's efficacy. While the roles and actions of PFTs are subjects of clinical investigation concerning many bacterial infections, the nanosponges have tremendous therapeutic implications given that membrane perforation exists as one of the most common virulence mechanisms. The absorption mechanism of the nanosponges can be distinguished from the current paradigm of detoxification treatments, where toxin antagonists rely primarily on structure-specific epitopic binding. By targeting a common mechanism shared by a broad range of toxins, the nanosponges introduce a unique strategy for the use of injectable nanocarriers for biodetoxification²⁷.

Methods

A summary of Methods is provided below and a detailed description of Methods is included in the Supplementary Information.

The toxin nanosponges were prepared by fusing RBC membrane vesicles on preformed poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles through an established extrusion

process¹⁷. The size of the nanosponges was obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer. The morphology of the nanosponges after absorbing toxins was measured by transmission electron microscopy (TEM). For preparation of human RBC nanosponges, the RBCs were collected from whole human blood (Bioreclamation) and the characterization results were shown in Fig. S9. For lyophilization, nanosponges were prepared in 5% sucrose solution. Reconstitution of the lyophilized samples was performed by solubilizing the samples in water and the characterization results were included in Fig. S10.

The *in vitro* toxin neutralization ability of the nanosponges was examined by mixing 3 μ g of α -toxin with 200 μ L of 1 mg/mL nanosponges for 30 min, followed by adding into 1.8 mL of 5% purified mouse RBCs. The released hemoglobin was then quantified to determine the degree of RBC lysis. The retention of α -toxin by the nanosponges was measured using SDS-PAGE. The *in vitro* toxin absorption capacity of the nanosponges was determined through titrating α -toxin to a fixed amount of nanosponges. The interaction of the nanosponges with cells was examined by a scanning fluorescence microscopy by incubating fluorescent nanosponges and RBC membrane vesicles with human umbilical vein endothelial cells (HUVEC). The *in vitro* cellular cytotoxicity of nanosponge-sequestered toxins was examined by incubating nanosponges of different concentrations with varied amounts of α -toxin, streptolysin-O, and melitin for 30 min, followed by adding to HUVECs for 24 hr. Then the cell viability was assayed using an MTT assay.

The *in vivo* toxin neutralization ability of the nanosponges was tested through subcutaneous injection of the nanosponge/toxin mixture to the flank region of nude mice, followed by histological analyses. On-site neutralization of α -toxin by the nanosponges was conducted by subcutaneously injecting 50 µL of 36 µg/mL of α -toxin solution, immediately followed by a 100 µL injection of 2 mg/mL nanosponges. The mice were imaged 3 days later for visualization of skin lesion formation (Fig S11). The *in vivo* detoxification efficacy was tested through intravenous injection of nanosponges before or after administration of a lethal dose of α -toxin to ICR mice, followed by monitoring the survival rate of the mice. For the *in vivo* hepatotoxicity study, one group of mice was sacrificed on day 3 following the injection of the toxin-bound nanosponges and another group was sacrificed on day 7. The livers were collected, sectioned, and stained with H&E for histological analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic and actual structures

(a) Schematic structure of toxin nanosponges and their mechanism of neutralizing poreforming toxins (PFTs). The nanosponges consist of substrate-supported RBC bilayer membranes into which PFTs can incorporate. After being absorbed and arrested by the nanosponges, the PFTs are diverted away from their cellular targets, thereby avoiding target cells and preventing toxin-mediated hemolysis. (b) TEM visualization of nanosponges mixed with α -toxin (scale bar = 80 nm) and the zoomed-in view of a single toxin-absorbed nanosponge (scale bar = 20 nm). The sample was negatively stained with uranyl acetate prior to TEM imaging.



Figure 2. In vitro characterizations

(a) Centrifuged RBCs after incubation with α -toxin mixed in PBS, PLGA nanoparticles, liposomes, RBC membrane vesicles, or nanosponges. (b) Hemolysis quantification of samples in (a), with anti- α -toxin as a positive control and nanoformulations alone as negative controls. Errors bars represent SD (n = 3). (c) Hemolytic activity of α -toxin alone or mixing with 200 µg nanosponges. (d) α -toxin absorption by different nanoformulations. (e) Uptake of RBC membrane vesicles (left) and nanosponges (right) by cells (scale bar = 5 µm). (f) Dose-dependent α -toxin neutralization by nanosponges against HUVECs. Errors bars represent SD (n = 6).

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Figure 3. In vivo toxin neutralization

Mice injected with α -toxin: (a) skin lesions occurred 3 days following the injection; (b) H&E stained histological sections revealed inflammatory infiltrate, apoptosis, necrosis and edema in the epidermis (scale bar = 80 µm); (c) tears on muscle fibers, interfibril edema, and extravasation of neutrophils from surrounding vasculature indicated muscular damage (scale bar = 20 µm). Mice injected with α -toxin/nanosponge: (d) no skin lesion occurred; (e) no abnormality was observed in the epidermis (scale bar = 80 µm); (f) normal muscle structure was observed (scale bar = 20 µm). (n = 6 for each group).



Figure 4. In vivo detoxification

Survival rates of mice over 15 days following an intravenous injection of 75 μ g/kg α -toxin; 80 mg/kg of nanosponges, RBC vesicles, or PEG-PLGA nanoparticles were administered intravenously 2 min either before (**a**) or after (**b**) the toxin injection. All injections were performed via tail vein (n = 9). (**c**) Biodistribution of α -toxin-bound nanosponges 24 hr after intravenous injection (n = 6). (**d**) H&E stained liver histology showed no tissue damage on day 3 (left) and day 7 (right) following α -toxin-bound nanosponge injections. Each image was representative of 5 examined sections (scale bar = 100 μ m).