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# Engineered biomimetic nanoabsorbent for cellular detoxification of chemotherapeutics

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## Supporting Information

**Materials and methods.**

**Chemicals and reagents.** 50:50 Poly(D,L-lactide-co-glycolide) carboxylate end group (0.55-0.75 dL/g) was purchased from DURECT Corporation (Birmingham, AL, USA). L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg-Transphosphatidylated, Chicken) (Egg Liss Rhod PE) and L-α-Phosphatidylethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (Ammonium Salt) (Egg-Transphosphatidylated, Chicken) (Egg NBD PE) were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, USA). Doxorubicin hydrochloride salt was purchased from LC Laboratories (Woburn, MA, USA). Methotrexate was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Mouse Melanoma Cell line B16-F10 (CRL-6475™) was purchased from ATCC and maintained according to the manufacturer's recommendation. All other chemicals and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used as received.

**Red blood cell membrane extraction.**

Red blood cell (RBC) membrane was extracted from whole blood using hypotonic treatment.[1,2] In brief, 500µL of whole blood was centrifuged to remove any buffy coat and plasma and mixed with 500µL of Mili-Q water following freezing and thawing cycle to disrupt RBC. Further, the aliquot was sonicated briefly using bath sonication for 2-3min to hemolysis RBCs. The blood suspension was then centrifuged at 5,000 rpm for 5min to separate RBC membrane from the hemoglobin, as the supernatant was removed carefully. The RBC membrane pellet was washed several times to remove hemoglobin completely. Further, the purified RBC membrane was lyophilized and stored for further use.

**Preparation of PLGA nanoparticles and nanoabsorbents.**

PLGA nanoparticles (NPs) were prepared by nanoprecipitation technique. In brief, 100µL of PLGA in acetonitrile stock (10mg/mL) was added drop wise to 1mL Milli-Q water under magnetic stirring condition. The mixture was stirred continuously overnight to facilitate the formation of nanoparticles and evaporation of organic solvent. The PLGA nanoparticles were further purified using Amicon Ultra-4 centrifugal filter (Millipore, MA) with a molecular weight cut-off of 10kDA. For nanoabsorbent preparation, 1mg/mL of purified PLGA NPs was fused with 100µL of purified RBC membrane suspension (1mg/mL), and the mixture was stirred at 1200 rpm for 5min, followed

by the extrusion through 100nm polycarbonate porous membrane using Avanti mini extruder. The purified NAbs were stored in 4°C for further use. Rhodamine dye labelled NAbs were prepared by mixing 10µg of L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg Liss Rhod PE) with RBC membrane vesicles prior to extrusion with PLGA nanoparticles. Green fluorescent dye labeled NAbs were prepared by using RBC membrane with 10µg L-α-Phosphatidylethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (Ammonium Salt) (Egg-Transphosphatidylated, Chicken) (Egg NBD PE) before performing extrusion with PLGA NPs.

**Characterization of PLGA NPs and nanoabsorbents.**

The hydrodynamic size and zeta potential measurements of the prepared PLGA NPs and NAbs were analyzed by Dynamic light scattering (DLS) using a Zeta sizer Nano ZSP apparatus (Malvern, Worcestershire, UK). The Smoluchowski model was used to calculate the zeta potential value. All data represents the average of triplicate measurements of samples prepared in different preparations. The morphology of the prepared NAbs was further analyzed using Transmission Electron Microscope (TEM, Tecnia G2, Spirit Bio TWIN). TEM samples were prepared by drop casting and evaporation technique using fomvar coated cupper grid (400 mesh). TEM images were analyzed by GATAN digital imaging system (GATAN, Inc.). The serum stability of the prepared PLGA NPs and NAbs were carried out as reported.[2] Specifically, 100µL of 1mg/mL nanoparticles were incubated with 100µL of Fetal Bovine Serum at 37°C and measure its change in absorbance at 560nm kinetically every 5s over a period of 1h, double-orbital shaking with slow speed was applied prior to each measurement using Microplate reader (BioTek, Synergy H1 hybrid reader).

**Membrane protein retention study using SDS-PAGE.**

The RBC membrane integrity and surface protein retention on the nanoabsorbent was investigated using SDS-PAGE analysis. In brief, equivalent protein concentration (5µg/mL) of RBC membrane coated Nanoabsorbent and pure RBC were lysed and prepared in SDS Laemmli buffer using standard protocol. The samples were run in Bio-Rad mini Protean TGX 7.5% ready gels at 200V for 30min and the gels were stained in Coomassie brilliant blue overnight and de-stained before visualization.

**In vitro chemotherapeutics absorption study.**

Different concentration of PLGA NPs and NAbs (50, 100, 250 and 500 µg/mL) was incubated with 1.5µg/mL of DOX (2.6 µM) and 2.9µg/mL of MTX (6.4 µM) for 30 min. After incubation, samples were centrifuged at 10,000 rpm for 10 min and both pellet and supernatant were used to quantify the amount of absorbed and unabsorbed chemotherapeutics by measuring the DOX fluorescence ($\lambda ex$ = 490 nm, $\lambda em$ = 580 nm) and MTX absorbance ($\lambda max$ = 300 nm). In order to evaluate the role of PLGA core in absorption, the surface charge properties of PLGA NPs and NAbs were also investigated by tracking the changes of Zeta Potential of samples before and after incubation with chemotherapeutics. Briefly, two different concentrations 50 and 100 µg/mL of PLGA NPs and NAbs were incubated with different concentration of DOX (2.6, 3.4, 4.3 and 5µM) and MTX (2.9µg/mL) for 30 min. Samples were then used to measure surface zeta potential.

**In vitro cytotoxicity of NAbs**

The in-vitro cytotoxicity of NAbs was conducted on mouse melanoma cell line B16-F10 using MTT assay. In brief, 2 x $10^4$ cells per well in DMEM medium were seeded in a 96-well plate and incubated for 24h. After incubation, the media were replaced with different NAbs concentration (10, 25, 50, 100, 150 and 200µg/mL) and incubated for additional 24h. Control cells were also maintained without any NAbs treatment (n=6). After the completion of incubation, MTT was added to each well and further incubated for 3h according to the manufacturer recommendation. The insoluble formazan crystals were solubilized using DMSO and their absorbance was recorded at 570 nm using a microplate reader (BioTek, Synergy H1 hybrid reader).

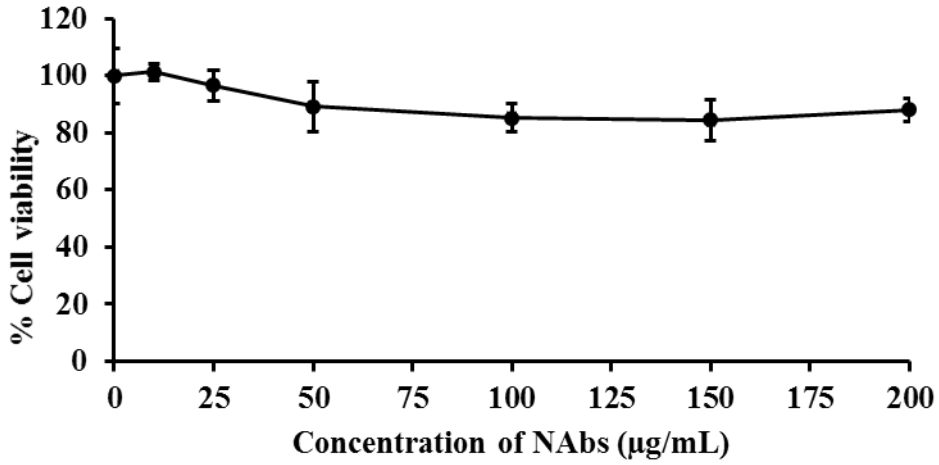
**Cellular uptake study.**

In order to verify cellular uptake efficiency of NAbs, the RhB labelled NAbs was used in mouse melanoma cells (B16-F10). In brief, cells were seeded in Poly-D-lysine coated 8 chamber slide at a density of 50,000 cells per well and incubated for 24h. Then, the cells were treated with 50µg/mL RhB-labeled NAbs suspension prepared in complete DMEM and incubated for 4h. After incubation, treated cells were washed twice with 1X PBS (pH 7.4), fixed with 4% paraformaldehyde for 30 min at room temperature, stained with DAPI for additional 10 min and imaged under a Confocal Laser Scanning Microscope (Carl Ziess, LSM-700). In addition to cellular internalization, the structural integrity of the NAbs was also assayed by labelling core and

shell of NAbs with different color dyes. In brief, lipid-NBD conjugate was used to label RBC vesicles simply by hydrating lipid-NBD film with RBC membrane and extruded to produce NBD labelled vesicles. These vesicles were further used to prepare NAbs following aforementioned protocol. NBD labelled NAbs were finally treated with aqueous solution of DOX (1.5 µg/mL), which got absorbed into the core of NAbs and acted as a red fluorescent dye. The colocalization of NBD and DOX fluorescent was studied as mentioned above using B16-F10 cells and fluorescent images were acquired with Confocal Laser Scanning Microscope.

**Cellular detoxification study.**

The cellular detoxification ability of NAbs was performed on Mouse melanoma cell line B16-F10 using MTT assay by treating cells with DOX in the presence and absence of NAbs. In brief, $2x10^4$ cells per well in DMEM medium were seeded in 96-well plates and incubated for 24h. Then the media was replaced with fresh media containing different concentration of NAbs (25, 50 and 100 µg/mL) and incubated for 4h. After 4 h of NAbs incubation, the cells were treated with different concentration of DOX (0.01, 0.1, 0.5, 1.5, 2, 3 and 5µM) and incubated for additional 24h. Control cells were also maintained without any drug or NAbs treatment (n=6). After 24h of incubation, 5µg/mL of MTT dye was added to each well and further incubated for 3h. The insoluble formazan crystals were solubilized using DMSO and the absorbance was recorded at 570 nm using microplate reader (BioTek, Synergy H1 hybrid reader).




Supplement Fig. 1. Concentration dependent In vitro cytotoxicity of NAbs.

## References

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