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Cell Membrane-Coated Nanoparticles: Bridging Natural and Synthetic Nanomaterials for Novel Therapeutics

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Cell Membrane-Coated Nanoparticles: Bridging Natural and Synthetic Nanomaterials for Novel Therapeutics

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

NanoEngineering

by

Ronnie Hongbo Fang

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2014
The Dissertation of Ronnie Hongbo Fang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2014
DEDICATION

This dissertation is dedicated to my loving family: George Fang, Shirley Fang, and Dennis Fang. Their unconditional support has helped me through the most trying of times, and I am forever grateful to have them in my life.
EPIGRAPH

“Beautiful things don’t ask for attention.”

Sean O’Connell in The Secret Life of Walter Mitty
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ABSTRACT OF THE DISSERTATION

Cell Membrane-Coated Nanoparticles: Bridging Natural and Synthetic Nanomaterials for Novel Therapeutics

by

Ronnie Hongbo Fang

Doctor of Philosophy in NanoEngineering

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Professor Liangfang Zhang, Chair
Professor Shaochen Chen, Co-Chair

The advent of nanomedicine has had a tremendous impact on patient outcomes in the clinic. At the nanoscale, drug-loaded particles exhibit unique attributes that allow them to be more efficacious compared to free drug formulations, leading to
better standard of care as well as increased patient compliance. Since the approval of Doxil, a liposomal formulation of the chemotherapy drug doxorubicin, two decades ago, researchers have been focused on leveraging nanotechnology to solve many of the hurdles in modern medicine. Despite the tremendous progress that has been made on this front, there is still much room for improvement. One major challenge in nanomedicine exists at the interface between synthetic nanomaterials and natural biological systems. My work has focused on a novel strategy for addressing the challenge of effective biointerfacing. This involves using natural membrane derived from the surface of cells to camouflage synthetic nanomaterials for the design of more effective and novel therapeutic modalities.

The first portion of this thesis will focus on the design of long-circulating drug delivery carriers by using a red blood cell membrane cloaking strategy to prolong systemic half-life. Building upon an initially reported red blood cell membrane-coated nanoparticle platform, drug-loaded nanocarriers are designed that can be actively targeted to tumors. Not only does natural membrane cloaking enable prolonged circulation for more effective drug delivery, but the presence of the natural membrane coating also facilitates the development of entirely new therapeutic modalities. The second part of this thesis will focus on the use of membrane-coated nanoparticles for the clearance of pathologic moieties. This is demonstrated for both pore-forming toxins that are secreted by bacteria and autoimmune antibodies targeting red blood cells. The final part of this thesis will outline the use of membrane-coated particles for specifically modulating the immune system. The concept is demonstrated for
vaccination against both pathogen-derived toxins as well as against autologous cancer cells. Ultimately, the cell membrane-coated nanoparticle platform has the potential to greatly change the landscape of nanomedicine. From more effective drug delivery carriers to novel applications that have yet to be discovered, there are many avenues still waiting to be fully explored.
Chapter 1

Introduction
Introduction

The advent of nanoparticle-based drug delivery systems has made a significant impact in the clinic [1]. In the last two decades, a plethora of nanoparticle-based therapeutic and diagnostic agents have been developed for the treatment of cancer, diabetes, pain, bacterial infections, asthma, etc. [2, 3]. Among the different nanotherapeutics, polymeric nanoparticles represent a unique class of nanocarriers that promises increased efficacy through controlled drug delivery to diseased sites. Well recognized advantages and features of polymeric nanoparticles include sustained drug release, delivery of poorly soluble drugs, multi-drug co-delivery, and the ability to be functionalized with targeting ligands for targeted delivery [4-9]. In addition, many biocompatible and biodegradable polymers, such as poly(D,L-lactide-co-glicolide) (PLGA), poly(lactic acid) (PLA), poly(glutamic acid) (PGA), poly(caprolactone) (PCL), N-(2-hydroxypropyl)-methacrylate copolymers (HPMA), and poly(amine acids) [10, 11], provide safe and non-toxic nanoparticle building blocks primed for in vivo administration. Extensive research efforts in nanoparticle design have enabled advanced functionalities including disease targeting [2, 12-15], stimuli-responsive triggers [16-18], combinatorial drug encapsulation [19-21], and temporally controlled drug release [22, 23]. Aided by recent developments in polymer engineering and preparation methods, polymeric nanoparticles can now be reliably manufactured and fine-tuned toward optimal performance [24-27]. These improved manufacturing techniques significantly benefit the bench-to-bedside translation of polymeric
nanotherapeutics, giving rise to a growing number of nanoformulations in clinical use or test. The increasing number of nanoparticles in clinical and preclinical studies also helps fuel continuing research interest in the various interactions between biological components and synthetic nanomaterials; much effort is devoted to developing novel immune-evasive platforms that promise enhanced pharmacokinetic profiles and therapeutic efficacy. In this article, we highlight recent clinical development regarding polymeric nanotherapeutics and review emerging stealth technologies aimed at passivating nanoparticles against the multi-faceted nature of immune clearance.

1.1 Clinical Development of Polymeric Nanotherapeutics

The advent of “stealth” nanoparticles has made great impact on nanoparticle drug delivery, particularly toward cancer treatment. First introduced in 1994 in a landmark paper by Langer and his colleagues [28], polymeric nanoparticles grafted with polyethylene glycol (PEG) can circulate in blood for an extended period of time owing to the PEG’s passivation effect. By providing a hydration layer and steric barrier surrounding the polymeric core, PEG grafting can reduce non-specific binding of serum proteins to the particles, thereby reducing their clearance by cells of the mononuclear phagocytic system (MPS) [29, 30]. These long-circulating nanoparticles have been shown to benefit drug delivery to the tumor microenvironment owing to the enhanced permeation and retention (EPR) phenomenon [31-33]. A number of formulations based on PEGylated polymeric nanoparticles are currently undergoing clinical trials (Table 1.1). In general, these drug-loaded nanoparticles exhibit
prolonged systemic circulation lifetime, sustained drug release kinetics, and better tumor accumulation as compared to small-molecule drugs [34-37]. Several notable polymeric nanoformulations in clinical trial are reviewed herein.

Genexol-PM was the first commercialized polymeric nanotherapeutic and it is currently approved in South Korea for the treatment of metastatic cancer, non-small cell lung cancer, and ovarian cancer. Comprising of paclitaxel and monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (mPEG-PLA) [38], the ~25 nm micellar formulation highlights the advantage of polymeric nanocarriers in delivering poorly soluble chemotherapy drugs. Compared to Taxol®, a paclitaxel formulation solubilized in castor oil Cremophor EL, Genexol-PM has also demonstrated stronger effectiveness as a radiosensitizer for the treatment of non-small cell lung cancer while decreasing the exposure of paclitaxel in normal tissues [39]. These benefits can be attributed to the physicochemical properties of the nanocarriers, which allow them to exploit the EPR phenomenon in tumors. In addition, Genexol-PM allows for a higher maximal tolerated dose (MTD) of paclitaxel as compared to Taxol [40], which contains a solvent that may cause allergic reactions. The formulation has undergone Phase II clinical trials in the United States and is undergoing several other trials involving combinations with other chemotherapeutics [41, 42].

CRLX101 is another example of an anti-cancer drug-loaded polymeric nanoparticle currently undergoing human clinical trials. The formulation is comprised of cyclodextrin-poly(ethylene glycol) copolymer chemically conjugated to camptothecin (CPT), a potent anticancer drug that inhibits topoisomerase 1. The
cyclodextrin on CRLX101 is capable of forming inclusion complexes with hydrophobic small molecules, which helps address the poor water solubility issue of CPT. CRLX101 containing ~10wt% CPT are prepared from the self assembly of CPT-conjugated copolymers, which forms nanoparticles 20 to 60 nm in size [43]. The morphological and stealth features of the nanoparticles significantly prolong their residence time in the bloodstream, with a circulation half-life of approximately 1 day in rodents [44]. Tail vein injection of CRLX101 to mice bearing tumor xenografts has revealed that the nanoparticle can localize at the tumoral sites. The formulation has also shown increased anti-tumor activity in murine models compared to FDA-approved CPT analogues [45, 46]. CRLX101 is currently in a Phase II clinical trial and recent publications on the platform show strong evidence that results from animal studies are translatable to humans [47].

Also comprising of cyclodextrin-containing polymer is CALAA-01, which is a nanoformulation carrying siRNA for targeted RNA interference (RNAi) therapy. These nanoparticles are prepared via the self-assembly of cyclodextrin-containing polymers in the presence of nucleic acids, which yields colloidal particles approximately 70 nm in diameter [48]. The particles are also stabilized with PEG and functionalized with a targeting ligand, transferrin, through the host-guest inclusion of adamantane tethers and cyclodextrins. The platform highlights the application of nanoengineering design to overcome the many physiological delivery barriers and facilitate effective delivery of fragile therapeutic cargoes. By providing a protective barrier that precludes siRNA from plasma degradation and renal clearance, CALAA-
01 enables the nucleic acid-based therapeutic to be systemically administered. In addition, the targeting ligands are incorporated to promote the intracellular uptake of CALAA-01 [49], and the nanocarriers readily undergo particle disassembly in acidic environments to promote endosomal escape. These design rationales serve to address the many challenges of siRNA therapeutics, including poor stability and inadequate transfection efficiency. CALAA-01 is currently undergoing a Phase Ib clinical trial against melanoma; it has demonstrated evidence of successful RNAi in humans [50].

BIND-014 is another polymeric nanoformulation consisting of PEGylated PLA co-polymer loaded with docetaxel, an anti-mitotic chemotherapeutic. Particularly notable in BIND-014 is the incorporation of targeting ligands specific for prostate-specific membrane antigen (PSMA), a surface marker highly expressed in prostate cancer cells and the neovasculature of other solid malignant tumors. The development of BIND-014 involved the screening of a combinatorial library of hundreds of nanoparticle formulations, and an optimal formulation was identified among particles with varying sizes, surface hydrophilicity, drug loading, and drug release kinetics. This rigorous screening process resulted in a robust formulation that balances the stealth properties of the PEG coating and the targeting capabilities of surface ligands. As a result, BIND-014 was shown to increase the concentration of docetaxel in mouse xenografts by up to an order of magnitude compared to conventional docetaxel formulations, resulting in a significant improvement in the drug’s anti-tumor activity without increasing its toxicity. Thorough testing of the BIND-014 formulation was carried out in other animals as well, such as rats and nonhuman primates, prior to
clinical translation. BIND-014 demonstrated differentiated efficacy in preclinical models of both prostate and non-prostate solid tumors. In Phase I clinical studies, the formulation was well-tolerated and demonstrated predictable and manageable toxicities [27]. There are currently two Phase II clinical trials to evaluate the therapeutic activity of BIND-014 in non-small cell lung carcinoma and metastatic, castration-resistant prostate cancer. Other examples of polymeric nanotherapeutics can be found in Table 1.1.

Table 1.1 PEGylated polymeric NPs in clinical trials.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Formulation</th>
<th>Company</th>
<th>Indication</th>
<th>Status</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1049C</td>
<td>PEGylated glycoprotein micelle of doxorubicin</td>
<td>Supratek Pharma Inc.</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[51]</td>
</tr>
<tr>
<td>CRLX101</td>
<td>PEG-cyclodextrin camptothecin +</td>
<td>Cerulean Pharma</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[47, 52]</td>
</tr>
<tr>
<td>NC-6004</td>
<td>PEG-poly(amino acid) + cisplatin</td>
<td>NanoCarrier Co.</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[53]</td>
</tr>
<tr>
<td>NK105</td>
<td>PEG-poly(aspartate) + paclitaxel</td>
<td>Nippon Kayaku Co., Ltd.</td>
<td>Various cancers</td>
<td>Phase III</td>
<td>[54, 55]</td>
</tr>
<tr>
<td>NK911</td>
<td>PEG-poly(aspartate) + doxorubicin</td>
<td>Nippon Kayaku Co., Ltd.</td>
<td>Various cancers</td>
<td>Phase III</td>
<td>[56]</td>
</tr>
<tr>
<td>NK012</td>
<td>Polymeric micelle SN-38</td>
<td>Nippon Kayaku Co., Ltd.</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[57]</td>
</tr>
<tr>
<td>BIND-014</td>
<td>PEG-PLGA + docetaxel</td>
<td>BIND Bioscience</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[27]</td>
</tr>
<tr>
<td>CALAA-01</td>
<td>Transferrin-targeted PEG-cyclodextrin + siRNA</td>
<td>Calando Pharmaceuticals</td>
<td>Solid tumors</td>
<td>Phase I</td>
<td>[50]</td>
</tr>
<tr>
<td>Genexol-PM</td>
<td>PEG-PLA + paclitaxel</td>
<td>Sorrento Therapeutics</td>
<td>Various cancers</td>
<td>Phase II</td>
<td>[41, 42, 58]</td>
</tr>
</tbody>
</table>

1.2 Development of Stealth Functionalization Strategies

A common feature behind the many polymeric nanotherapeutics in clinical development is their stealth functionality, which enables prolonged pharmacokinetics and improved biodistribution of the particles. Currently, PEG remains the gold standard for stealth functionalization in clinics, and optimization of PEG coating on
polymeric nanoparticles has been extensively studied. Stealth functionality imparted by PEG to nanoparticles is found to be strongly influenced by its length and surface density on nanoparticle surfaces. PEG can adopt either a mushroom or a brush-like conformation depending on its length and density; scarcely distributed long PEG favors mushroom conformation and densely packed, short PEG favors brush-like conformation. Generally speaking, higher surface coverage of PEG increases the circulation time of nanoparticles, and PEG with a molecular weight of 5000 Da provides the ideal surface coating for stealth functionalization.

While PEGylation remains the most widely accepted standard for stealth functionalization, recent observations of anti-PEG immunological response have prompted much scrutiny over the immunological implications of PEG. There has been conflicting evidence in literature in regards to the induction of PEG-specific antibodies, and it has been proposed that repeated administration of PEGylated nanoparticles leads to the production of anti-PEG antibodies, which subsequently increases phagocytosis by the reticuloendothelial system (RES) and results in an accelerated blood clearance phenomenon. In addition, complement activation through both the classical and alternative pathways by PEG has been reported. While fine-tuning of PEG length and density has shown much promise in attenuating PEG-associated immune responses, researchers have also begun to explore alternative long-circulation strategies in aim of developing nanoparticles with better compatibility and improved performance. Alternative polymer-based stealth functionalization strategies for emerging polymeric
nanotherapeutics are summarized in Figure 1.1 and described in the following sections.

![Figure 1.1 Alternative hydrophilic polymers for nanoparticle stealth functionalization.](image)

### 1.2.1 Synthetic Polymers for Stealth Functionalization

Poly [N-(2-hydroxypropyl) methacrylamide] (HPMA) is a hydrophilic polymer with multiple functionalization sites. Upon conjugation with hydrophobic drug molecules, unimolecular micelles can form via a self-assembly process. Such HPMA-drug conjugates have been extensively studied for drug delivery applications [76-80]. In contrast to PEG, the multiple functionalization sites on HPMA allow for multiple therapeutic compounds to be covalently attached. Drug-loaded unimolecular HPMA has shown significantly improved pharmacokinetic profiles as compared to free drugs [77, 81] and have entered various stages of clinical trials [82-84]. Owing to HPMA’s hydrophilicity, biocompatibility, and lack of immunogenicity [80, 85], the polymer has also drawn much research interest as a stealth functionalization candidate in the
development of polymeric nanocarriers. HPMA has been applied in place of PEG to stabilize polycation/DNA micellar complexes [86, 87]. Neutral hydrophilic HPMA shells have been grafted onto gene delivery vesicles with the aim to minimize plasma protein interactions and prolong circulation time [88]. Core-shell structured nanoparticles with a biodegradable polymeric core and a hydrophilic HPMA corona have also been prepared through the self-assembly of block copolymers. Nanoparticles consisting of HPMA/poly(caprolactone)(PCL) and of HPMA/poly(D,L-lactide) (PLA) have been prepared using both A-B-A triblock copolymers and star-shaped block copolymers [89-91]. For both HPMA/PCL and HPMA/PLA copolymers, particles approximately 100-150 nm in size have been prepared. Encapsulation of hydrophobic therapeutic cargoes has also been demonstrated in these HPMA grafted nanocarriers using indomethacin and paclitaxel as model drugs.

Another type of synthetic polymer that has emerged as a compelling alternative to PEG is betaine-based polymers, which are zwitterionic materials with ultralow-fouling properties. Similar to many hydrophilic polymers, the zwitterionic material achieves its low fouling property through its hydration effect. However, in contrast to most hydrophilic polymers that bind water through hydrogen bonding, the zwitterionic material possesses a stronger hydration effect attributable to electrostatic interactions [92, 93]. Polybetaine functionalized surfaces have shown significantly reduced non-specific protein adsorption [94-97] and biofilm formation [98-100]. Therapeutic materials coated in polybetaines also display unique advantages over those decorated in other hydrophilic polymers. Notably, therapeutic proteins stabilized with
poly(carboxybetaine) (PCB) display higher substrate binding affinity as compared to those conjugated with PEG. Such enhanced bioactivity is attributed to the superhydrophilic nature of PCB in contrast to the inherent amphiphilicity in PEG [101]. Implant materials containing PCB also elicit minimal foreign-body reaction owing to the polymer’s ultralow-fouling properties [102]. Numerous nanoparticle platforms, including gold [103], iron oxide [104], silica [103], liposomes [105], and hydrogels [105-107], have been successfully functionalized with PCB, which helps improve the particles’ colloidal stability in protein solutions. Regarding polymeric nanoparticles, PCB-grafted PLGA nanoparticles have been prepared using PCB-PLGA block copolymers [108]. The sharp polarity contrast between the PCB and PLGA blocks allows for efficient nanoparticle self-assembly. Uniquely, the nanoparticles retain their stability even under lyophilization conditions in the absence of cryoprotectant, which can likely be attributed to PCB’s strong hydration. A biodistribution study of PCB-grafted and PEG-grafted poly-(acrylic acid)-b-poly(lactide) nanoparticles also reveals comparable in vivo survival in the bloodstream [109], affirming PCB’s potential as a stealth coating.

Many other synthetic polymers have been explored as alternative stealth coatings for nanocarriers. Examples of these polymers include poly(vinyl alcohol) (PVA) [110, 111], poly(oxazoline) [112], poly(4-acyrloylmorpholine) (PAcM) [113, 114], poly(N,N-dimethylacrylamide) (PDAAm) [114, 115], and poly(N-vinyl-2-pyrrolidone) (PVP) [113-115]. In a pharmacokinetic study using a rat model, Ishihara et al. examined the circulation half-lives of polymeric nanoparticles grafted with PVP,
PAcM, and PDAAm and compared the results to that of PEGylated nanoparticles [114]. All four polymers successfully extended the nanoparticle residence time in the circulation, and PEG yielded the longest half-life among the group. Interestingly, however, upon repeated injections, PEG-grafted nanoparticles exhibited accelerated blood clearance phenomenon whereas PVP-, PAcM-, and PDAAm-grafted particles did not. The accelerated clearance was found to correlate with the production of IgM antibodies, which was elicited by PEG but not by PVP. The study highlights potential alternative materials for developing stealth nanocarriers with improved performance.

1.2.2 Biopolymers for Stealth Functionalization

Poly(amino acid)s (PAAs) are a major class of biopolymers that have been explored as stealth coating for nanocarriers. As compared to synthetic polymers, PAAs are susceptible to degradation by proteases, which helps reduce the risks of in vivo accumulation [116]. Deierkauf et al., first examined the anti-phagocytic property of poly(amino acid) by grafting polystyrene latex beads with polyglutamic acid. The amino acid coating proved to be an effective inhibitor of particle uptake, which was ascribed to the electrostatic repulsion between the amino acids and the external surface charge on the polymorphonuclear leukocytes [117]. The potential of PAAs in improving nanoparticles’ pharmacokinetic profiles was further demonstrated on liposomes by Romberg et al., who showed that liposomes coated with poly(hydroxyethyl-L-asparagine) (PHEA) can outlast PEG-coated liposomes in the circulation [118]. Upon intravenous administration in rat at less than 0.25 µmol/kg,
PHEA-coated liposomes showed superior survival in the blood as compared to PEG-coated liposomes. In addition, upon repeated injections, PHEA-coated liposomes exhibited a less pronounced accelerated blood clearance phenomenon. The stealth functionality and the excellent biodegradability of PAAs motivated the development of PAA-grafted polymeric nanoparticles. Block copolymers consisting of biodegradable polymers and PAAs have been prepared with different types of amino acids, including poly(aspartic acid) [119, 120], poly(glutamic acid) [121-123], poly(cysteine) [124], and poly(lysine) [125]. Generally, a hydrophilic peptide sequence is conjugated with a hydrophobic polymer such as PLA for the self-assembly of PAA-grafted nanoparticles.

Polysaccharides are another class of hydrophilic biopolymers that are frequently used to coat nanoparticles. These polymers are a desirable coating material for nanoparticle development owing to their excellent biodegradability and low immunogenicity [126-128]. These biopolymers provide a hydrated surface that can be likened to the dense, carbohydrate-rich glycocalyx on cellular surfaces. In examining the effect of polysaccharides on the blood circulation half-life of polymeric nanoparticles, Passirani et al. demonstrated that the brush-like structure of heparin and dextran served to protect the nanoparticles from in vivo clearance. Covalent conjugation of heparin and dextran to a poly(methyl methacrylate)-based nanoparticle significantly prolonged the circulation half-life of the particles from 3 min to several hours. Sheng et al. also showed that functionalization with water-soluble chitosan reduced the phagocytic uptake efficiency and retarded the blood clearance of PLA
nanoparticles. An impressive finding was the fact that nanoparticle functionalization with both PEG and chitosan yielded a circulation half-life of 63 hr, which was much longer than that of the PEGylated particles reported in the study. The extended circulation time was attributed in part to the charge neutralization between PEG and chitosan and demonstrated the stealth potential of polysaccharides. Among other carbohydrate-based biopolymers, poly(sialic acid) (PSA) has also drawn significant interest as a stealth moiety because the human body has no specific receptors for the compound [129]. Adoption of PSA by foreign pathogens and metastatic cancer cells for antigen masking and *in vivo* survival [130-132] also suggests PSA’s stealth potential. Conjugation with PSA has successfully prolonged the pharmacokinetic profiles of several protein therapeutics [133, 134] as well as quantum dot nanoparticles [135]. Sialic acid has been applied to PLGA nanoparticles for drug delivery applications and has been shown to prolong particle residence within the brain [136, 137].

### 1.2.3 Biologically Inspired Stealth Strategies

In the engineering of stealth nanocarriers that are capable of evading immune clearance, researchers have also taken design cues from biology by exploiting the surface mechanisms behind blood cells’ long circulation. Early work on liposomal carriers demonstrated that incorporation of sialylated glycolipids and glycoproteins, which are major components on erythrocyte surfaces, significantly prolonged the liposomes’ *in vivo* survival. While Surolia et al. first introduced asialogangliosides to
liposome as a biomimetic approach to improve hepatic drug delivery [138], later studies by Allen et al. showed that the incorporation of the ganglioside GM1 greatly prolonged the liposome’s residence time in the blood. The stealth effect of GM1 was observed across several different liposomal formulations. For instance, addition of a 0.07 molar ratio of GM1 to a sphingomyelin liposome increased the blood-to-RES ratio from 0.02 to 5.7 2 hr post-injection [139]. This enhanced blood residence was attributed to GM1’s dense surface carbohydrates that mimic cellular membranes. In addition, the role of terminal sialyl groups on these glycolipids was also highlighted as incorporation of asialogangliosides showed a less pronounced stealth effect. Optimization of the biologically inspired stealth liposome by Allen et al. resulted in a liposomal composition that mimicked the outer leaflet of red blood cell (RBC) membranes, consisting of egg phosphatidylcholine/sphingomyelin/cholesterol/ganglioside GM1 at a 1:1:1:0.14 molar ratio [140]. Liposomal formulations containing other sialic acid-containing gangliosides [141-143], ganglioside derivatives [144-146], and glycoproteins [147] also showed extended circulation time in the blood. The stealth effect of these glycosylated complexes has been ascribed to their ability to fend off complement activation and macrophage uptake [141, 148].

Despite the early success of biologically inspired stealthy liposomes, the utilization of biomolecules for stealth functionalization remained relatively unexplored in the development of polymeric nanocarriers. This was in part due to the engineering challenge in functionalizing nanoscale substrates. To bestow nanocarriers with biomimetic functionalities, biomolecules such as proteins and glycans need to be
attached to the particles in a non-disruptive and regio-selective manner. With the advances in molecular biology and nanoengineering techniques, biologically inspired stealth polymeric nanoparticles have recently emerged. Possessing active biological components on their surfaces, these nanoparticles are unlike conventional stealth particles that rely on hydration coronas for in vivo survival. Currently, techniques to prepare biologically inspired polymeric nanoparticles can be classified into a bottom-up approach based on protein conjugations and a top-down approach based on cell membrane coating.

1.2.3.1 Polymeric NPs Tagged with Self Markers

Among the many surface proteins and molecules present on mammalian membranes, an integrin-associated protein CD47 has gained much research interest as a “marker-of-self” that can protect cells against macrophage uptake through an inhibitory action via SIRPα binding [149]. The protein and its analogues have been identified on cancer cells and viruses as immune-evasive masking [150, 151]. Such anti-phagocytic properties have motivated researchers to apply the protein to mask foreign materials from immune recognition [152, 153]. Recently, CD47 and its derivative peptide have been attached to polystyrene nanoparticles to enhance their delivery and inhibit phagocytic clearance (Figure 1.2). To enable controlled CD47 functionalization on nanoparticles, Rodriguez et al. mixed 160 nm streptavidin-coated polystyrene beads with a recombinant CD47 protein expressing regioselective biotinylation. The streptavidin/biotin coupling approach allowed CD47 to be coated in
the proper orientation at a controllable density. In addition to CD47-functionalized beads, the authors also identified a “minimal” self peptide sequence (GNYTCEVTELTREGETIIELK), which was also applied for nanoparticle camouflage. Studies on the CD47- and self peptide-functionalized nanobeads showed that they could inhibit myosin-II recruitment at the phagocytic synapse, thereby suppressing the ensuing phagocytosis. The anti-phagocytic effect of CD47 functionalization was shown to be highly potent, requiring approximately 1 molecule per 45,000 nm² (equivalent to the lowest CD47 densities reported for human RBCs), suggesting that a nanoparticle 60 nm in radius needs only one CD47 molecule to achieve the active stealth effect [154].

Figure 1.2 Schematic of a “self” nanoparticle prepared by immobilizing CD47 or its peptide variant on the nanoparticle surface. Signaling between CD47 or a minimal “self” peptide and their receptor, SIRPα, inhibits phagocytic activation. Reproduced from Ref. 154 with permission from the American Association for the Advancement of Science

The difference between the active stealth approach behind the “self” nanoparticles and the passive stealth approach behind conventional PEGylated nanoparticles was tellingly demonstrated in in vivo studies conducted with pre-opsonized nanoparticles. Recognizing the challenge in maintaining stealth
functionalization following nanoparticle opsonization, Rodriguez et al. showed that the “self” nanoparticles retained their anti-phagocytic properties \textit{in vivo} despite controlled opsonization with IgG. In contrast, PEGylated nanoparticles were completely stripped of their stealth properties upon opsonization, exhibiting equivalent blood residence time to non-functionalized polystyrene nanobeads. The authors also demonstrated enhanced tumoral uptake and increased treatment efficacy using A549 lung adenocarcinoma cells and paclitaxel-loaded polystyrene nanobeads, validating the biologically inspired stealth approach to improve nanoparticle drug delivery [154].

1.2.3.2 Polymeric NPs Camouflaged in Cellular Membranes

In contrast to the bottom-up approach in attaching “marker-of-self” molecules to nanoparticles, a top-down approach, which exploits cellular membranes in their entirety to coat and camouflage polymeric nanoparticles from immune clearance, was first reported by Hu \textit{et al.} Using a co-extrusion process, the investigators demonstrated successful coating of sub-100 nm PLGA nanoparticles with natural membranes derived from RBCs [155]. The resulting RBC-membrane-coated nanoparticles (RBC-NPs) exhibited a core-shell structure, in which the RBC membrane formed a single bilayer around the polymeric core (Figure 1.3). Interestingly, the cell membrane-coating approach adopts the same immune-evasive strategy adopted by many viruses in nature. It was recently discovered that hepatitis A and hepatitis C viruses are capable of “hijacking” cellular membranes, enclosing themselves in these non-immunogenic coatings to escape from neutralizing antibodies.
The cell-membrane coating technique presents a unique biomimetic approach in nanoparticle stealth functionalization [156, 157].

From a nanoengineering perspective, the membrane coating approach provides unprecedented control in enabling biomimetic surface functionalization on nanoscale polymeric substrates. By translocating cellular membranes in their entirety to nanoparticles, the complex biochemistry on cellular surfaces can be faithfully translocated as well [155]. Careful studies of CD47 on the surface of RBC-NPs demonstrated that the particles possess the same density of the biomarker as its RBC source [158]. More importantly, the proteins were shown to be oriented almost exclusively in the right-side-out fashion with the extracellular portion displayed on the particle surfaces (Figure 1.3). This right-side-out orientation was ascribed in part to the electrostatic repulsion between the negatively charged PLGA nanoparticles and the negatively charged sialyl moieties residing on the exoplasmic side of the membranes. In addition, the stabilization effect by the exoplasmic glycans likely contributed to the observed membrane orientation. As a result of this oriented membrane coating, macrophage uptake of the RBC-NPs was significantly impeded in vitro. RBC-NPs also remained stable in phosphate buffered solution and serum unlike bare PLGA nanoparticles, suggesting that the glycan-rich membranes conferred stability to the polymeric cores. An important finding is that the RBC-NPs possessed longer blood survival as compared to an analogous PEGylated formulation. These results highlight the strength of the biomembrane-coated nanoparticles, whose self-camouflage presents a comprehensive evasion strategy against the multi-faceted nature of immune
clearance [159, 160].

Much development on cellular-membrane coated nanoparticles has been made since RBC-NPs were first reported. Anti-phagocytic properties have been demonstrated with RBC membrane-coated gold nanoparticles [161] and white blood cell membrane-coated silica particles [162], demonstrating the broad applicability of the approach. Toward drug delivery, RBC-NPs have been successfully loaded with doxorubicin and showed treatment efficacy against a leukemia cell line [163]. The membrane bilayer coating was shown to enable a more sustained drug release profile by serving as a diffusion barrier. Incorporation of targeting ligands, such as folate and aptamers, into RBC-NPs has also been made possible through the insertion of lipid-tethered ligands into the RBC membrane bilayer, which demonstrated the platform’s potential for targeted drug delivery [164]. Perhaps most exciting is the recent demonstration of the RBC-NPs as a toxin nanosponge against pore-forming toxins. Owing to the particle’s structural stability and biomimetic exterior, the RBC-NPs readily interact with the many pore-forming toxins that typically target and damage cellular membranes. Absorbed toxins remain locked-in by the nanoparticles and therefore can be safely metabolized in vivo [165]. The toxin nanosponge application highlights the unique strengths of the membrane-camouflage stealth strategy, which allows nanoparticle interaction with other biomolecules as opposed to inhibiting such interactions using non-fouling polymers. This biologically inspired approach provides the venue for novel therapeutic interventions against pathogenic factors that interact with cellular surfaces.
1.3 Conclusions

Polymeric nanoparticles offer a robust and versatile platform for designing novel and more efficacious therapeutics. The impact of polymeric nanotherapeutics is evidenced by the many nanoformulations currently undergoing clinical trials. Ongoing research efforts continue to explore stealth strategies with the aim of developing nanocarriers that have better immunocompatibility and improved pharmacokinetic profiles. Such efforts are highlighted by a variety of hydrophilic polymers employed...
for stealth functionalization.

Notably, development of biologically inspired stealth nanoparticles highlights increased understanding of the complex immune clearance mechanisms and of the many biomolecules that contribute to immune evasion. Advancement in nanoengineering techniques has enabled nanoparticle surface functionalization with immunologically relevant components (i.e. CD47 and cellular membranes). These biologically inspired nanoparticles present a new paradigm in nanoparticle functionalization. Rather than hiding foreign particles behind a hydration layer, drug carriers can be camouflaged as “self”, readily evading immune clearance despite direct interaction with biological components in circulation. While the field of “self” nanoparticles remains in its nascent stage, the growing number of cell-derived nanocarriers in literature provides a glimpse of the drug delivery potential behind biological functionalizations [166-170]. Ongoing research in biologically inspired nanoparticles promises unique carrier functionalities that combine the intricacies of natural biomolecules with the flexibility and robustness of synthetic nanomaterials.

Chapter 1, in full, is a reprint of the material as it appears in *Nanoscale*, 2014, Che-Ming Hu, Ronnie Fang, Brian Luk and Liangfang Zhang. The dissertation author was a major contributor and co-author of this paper. The remainder of this dissertation will focus on the development of novel biomimetic nanotherapeutics using a cellular membrane cloaking strategy to disguise polymeric nanoparticles. This strategy is generalized to several different types of membrane coatings and enables new applications that extend beyond the traditional drug delivery paradigm.
1.4 References


Chapter 2
Development of Red Blood Cell Membrane-Coated Nanoparticles for Targeted Drug Delivery
2.1 “Marker-of-Self” Functionalization

2.1.1 Introduction

Enabling active immune evasion through biomimetic surface functionalization presents an emerging stealth strategy for developing long-circulating delivery vehicles [1, 2]. The identification of CD47, a transmembrane protein that serves as a universal molecular ‘marker-of-self’, has led to its utilization in the growing development of bio-inspired, immune-evasive devices. Capable of inhibiting phagocytosis and conferring anti-inflammatory properties through interactions with signal regulatory protein alpha (SIRPα) expressed by macrophages, CD47 and its analogs have been found to contribute to the in vivo survival of red blood cells (RBCs) [3], cancer cells [4], and viruses [5]. Application of CD47 to modulate the immune responses against synthetic devices was first demonstrated with macrophages treated by purified recombinant, soluble CD47, which showed reduced uptake of colloidal emulsions [6]. Synthetic materials covalently conjugated with recombinant CD47 further advanced this biomimetic stealth approach, yielding polymeric microspheres [7] and implant surfaces with reduced affinity to inflammatory cells [8, 9]. On nanoscale particles, however, interfacing with native biological components through chemical conjugation of immunomodulatory proteins to particle surfaces can be difficult to manipulate. In particular, inconsistent protein surface density and randomized ligand orientations are notable issues that can greatly undermine the performance of the resulting nanocarriers.
Toward engineering nanocarriers that can actively suppress immune attack by macrophages, herein we demonstrate a robust ‘top-down’ approach to functionalizing nanoscale particles with native CD47 by cloaking sub-100 nm nanoparticles with cellular membranes derived directly from natural RBCs (Figure 2.1.1). The uniqueness of this membrane coating approach lies in its ability to functionalize nanoparticles with native immunomodulatory proteins including CD47 at an equivalent density to that on natural RBCs. In this study, we show direct evidence that the ‘marker-of-self’ proteins are transferred to the particle surfaces and present in the right-side-out orientation. A macrophage uptake study confirms the stealth functionality conferred

![Figure 2.1.1 Controlled CD47 functionalization enabled by RBC membrane coating. The resulting RBC membrane-coated nanoparticle (RBC-NP) is expected to have a CD47 density equivalent to that on a natural RBC.](image-url)
by the immunomodulatory proteins. Since cellular membranes anchor the many molecular tags that define cellular identities, attaching these membranes to nanoparticle surfaces provides unparalleled control over the functionalization of synthetic nanocarriers toward biomimicry.

2.1.2 Experimental Methods

2.1.2.1 Preparation of RBC Membrane-Coated NPs

RBCs were collected from 10 week-old male ICR mice (Charles River Laboratories) by centrifuging the whole blood at 2000 x g for 5 min, following which the supernatant and buffy coat were removed. Collected RBCs were then subject to hypotonic treatment to remove interior contents. The resulting RBC ghosts were extruded through 100 nm polycarbonate porous membranes using an extruder (Avanti Polar Lipids) to prepare RBC membrane-derived vesicles with a diameter of approximately 120 nm. Poly(lactic-co-glycolic acid) (PLGA) polymeric cores were prepared using 0.67 dL/g carboxy-terminated 50:50 PLGA polymer (LACTEL Absorbable Polymers) through a solvent displacement process, during which 1 mg of PLGA was dissolved in 200 µL of acetone and added drop-wise to 3 mL of water. Following solvent evaporation for 2 hr, the particles were washed using 10 kDa molecular weight cutoff (MWCO) Amicon Ultra-4 Centrifugal Filters (Millipore). The RBC-NPs were then prepared by fusing the RBC membrane-derived vesicles onto the PLGA particles by extruding the particles with the RBC membrane-derived vesicles through 100 nm polycarbonate porous membranes. The size and the zeta potential of
the resulting RBC-NPs were obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer, which showed an average hydrodynamic diameter of 70 and 85 nm before and after the extrusion process, respectively. The particle morphology was characterized using scanning electron microscopy (SEM). Samples for SEM imaging were prepared by dropping 5 µL of the RBC-NP solution onto a polished silicon wafer. After drying the droplet at room temperature overnight, the sample was coated with chromium and then imaged by SEM.

2.1.2.2 Identification of Membrane Proteins and CD47

RBC-NPs were isolated from free RBC membrane materials by ultracentrifugation at 14000 x g for 30 min. The resulting RBC-NPs were lyophilized, prepared in lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen), and separated on a 4-12% Bis-Tris 10-well minigel in MOPS running buffer using a Novex® Xcell SureLock Electrophoresis System (Invitrogen). For membrane protein identification, the gel was stained using SimplyBlue™ SafeStain solution (Life Technologies) following the manufacturer’s instructions and imaged using a gel imager. For CD47 identification, the resulting gel was transferred to a nitrocellulose membrane. The membrane was then stained with a primary rat anti-mouse CD47 antibody (BD Biosciences) and a secondary goat anti-rat IgG HRP conjugate (Millipore). The membrane was then subject to ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks).
2.1.2.3 Quantification of CD47 on RBC-NPs

The amount of CD47 retained on the RBC-NPs was quantified by comparing the CD47 protein intensity to protein standards prepared on the same western blotting membrane. The protein standards were prepared from predetermined volumes of blood, which yielded a positive linear correlation between the CD47 band intensity and the blood volume following western blotting. The band intensities were quantified through an image analysis with Adobe Photoshop software. A CD47 standard curve was then established by converting the blood volumes to their corresponding CD47 quantity, using an estimated concentration of mouse RBCs in the blood \((10^{10}/\text{mL})\) [10] and an average number of CD47 per mouse RBC \((16,500 \text{ copies/cell})\) [11].

2.1.2.4 Transmission Electron Microscopy

A drop of the RBC-NP or bare PLGA nanoparticle (bare NP) solution \((1 \text{ mg/mL})\) was deposited onto a glow-discharged carbon-coated grid. For immunostaining, the sample droplet was washed with 3 drops of 0.5 mg/mL rat anti-mouse CD47 antibody solution. For staining the intracellular sequence of CD47, an intracellular sequence specific rabbit anti-CD47 antibody (GeneTex). Following 30 sec of incubation, the sample was rinsed with 3 drops of either a goat anti-rat IgG gold conjugate (5 nm) solution (Canemco, Inc.) or a anti-rabbit IgG gold conjugate (5 nm) solution (Sigma-Aldrich) and then washed with 10 drops of distilled water. For negative staining, the particle sample droplet was washed with 10 drops of distilled water and stained with 1% uranyl acetate. These samples were then imaged using an
2.1.2.5 Macrophage Uptake Study

PLGA nanoparticles encapsulating 0.05% (w/w) 1,1'-dioctadecyl-3,3,3',3' -tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) dye (Life Technologies) were prepared for fluorescence quantification using flow cytometry. CD47-blocked RBC-NPs were prepared by incubating 1 mg of the DiD-loaded RBC-NPs with 400 µg of rat anti-mouse CD47 antibodies (BD Biosciences) for 1 hr. For the macrophage uptake study, J774 murine macrophage cells were cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) and plated at a density of $10^5$ cells/well on 12-well plates (BD Biosciences). On the day of the experiment, the cells were washed and cultured in fresh culture media. Bare PLGA nanoparticles (bare NPs), RBC-NPs, and CD47-blocked RBC-NPs were incubated at a concentration of 25 µg/mL with the macrophage cells at 37°C for 10 min. Non-internalized nanoparticles were washed away with PBS. The macrophage cells were then scraped off the plates and analyzed using flow cytometry. All flow cytometry studies were conducted on a FACSCanto II flow cytometer (BD Biosciences) and the resulting data was analyzed using FlowJo software from Tree Star. Thirty thousand events were collected per sample and gated using control cells that were not incubated with any nanoparticles. Histograms were plotted with fluorescence intensity as the x-axis using a biexponential scale. The mean fluorescence was plotted in a bar chart with error bars representing the standard error. Statistical analysis was performed based on
a two-tailed, unpaired t-test.

### 2.1.3 Results and Discussion

With five membrane-spanning regions, CD47 is an integral membrane protein firmly embedded in RBC membranes, exhibiting an IgV-like extracellular domain that helps maintain the RBCs’ survival in the circulation [12]. While it was previously shown that RBC membrane coating associated nanoparticles with the majority of the membrane materials [13], it remained to be investigated whether these RBC membrane-coated nanoparticles (RBC-NPs) properly present the CD47 for immunomodulation. Verification of the protein, its density, and its orientation on the RBC-NP surfaces demands a molecular examination of these RBC-mimicking nanocarriers. To investigate the functionalization of native CD47 on RBC-NPs, 70 nm poly(lactic-co-glycolic acid) (PLGA) particles were first extruded with RBC membrane-derived vesicles following a previously described protocol [13]. Through scanning electron microscopy (SEM) visualization, a spherical morphology was observed for the resulting RBC-NPs (Figure 2.1.2A), and dynamic light scattering measurements showed a mean particle diameter of 85 ± 2 nm. The purified particles were then solubilized in a lithium dodecyl sulphate (LDS) sample loading buffer, following which the protein contents stripped from the nanoparticles were separated by SDS-PAGE. The resulting protein gel was subsequently subjected to western blotting using anti-CD47 antibody as the primary immunostain. The presence of CD47 on the RBC-NPs was confirmed by a distinct, single band at 50 kDa (Figure 2.1.2B),
which is the characteristic molecular weight of the CD47 protein self-marker [12].

To further examine the extent of CD47 protein on the particle surfaces, the RBC-NPs prepared with different RBC membrane to polymeric particle ratios were collected and analysed for retained CD47 contents. An ultracentrifugation process was

Figure 2.1.2 Characterization and quantification of CD47 on the RBC-NPs. (A) A representative scanning electron microscopy (SEM) image shows the spherical structure and morphology of the prepared RBC-NPs (scale bar = 250 nm). (B) Coomassie staining (left) and CD47 western blot (right) of the RBC-NPs’ protein contents following SDS-PAGE separation. (C) Comparison of CD47 contents on the RBC-NPs prepared from different RBC membrane to polymer ratios. (D) Quantitative analysis of CD47 density on the RBC-NPs prepared from different RBC membrane to polymer ratios (n=5).

To further examine the extent of CD47 protein on the particle surfaces, the RBC-NPs prepared with different RBC membrane to polymeric particle ratios were collected and analysed for retained CD47 contents. An ultracentrifugation process was
applied to isolate the resulting RBC-NPs from free RBC membranes, following which protein contents on the nanoparticles was processed through SDS-PAGE and examined by western blotting analysis. Figure 2.1.2C shows the relative CD47 retention on the different particle formulations. As the RBC membrane to polymeric particle ratio increased from 25 to 150 µL of blood per mg of polymer, a corresponding increase in the CD47 intensity was observed. This positive correlation reflects the increasing particle functionalization by the increasing RBC membrane inputs, as more CD47 could be identified in the isolated nanoparticle samples. Saturation in CD47 band intensity was observed upon further raising the RBC membrane to polymer ratio above 150 µL/mg, which reflected the upper limit of CD47 functionalization achievable by the RBC membrane coating. To quantitatively analyze the protein density on the RBC-NPs, CD47 standards were prepared from predetermined volumes of blood, from which CD47 content was estimated based on the average CD47 number on a mouse RBC (16,500 copies per cell) [11] and the RBC concentration in mouse blood (10^{10} cells per mL of blood) [10]. Comparing the CD47 retention from the different RBC-NP formulations to the protein standards showed that the saturation level corresponded to approximately 2\times10^{13} copies of CD47 per mg of polymeric particles (Figure 2.1.2D), yielding on average ~5 copies of CD47 per RBC-NP (Supplement discussion). To put the CD47 density into perspective, the surface area of the 85 nm RBC-NPs was calculated (~1\times10^{11} µm^{2}/mg, Supplement discussion), from which a surface density of ~200 molecules of CD47 per µm^{2} at saturation on the RBC-NPs can be derived. Given that natural RBCs possess 200–250
copies of CD47 per µm²[11, 14], the close match in the CD47 density on the RBC-NPs suggests that the membrane coating brought nearly all of RBCs’ CD47 content onto the sub-100 nm particles. The result reflects the robustness of the membrane functionalization technique, as most of the membrane proteins were retained within the cellular membranes throughout the particle preparation process.

It should also be noted that the RBC membrane to polymer ratio corresponding to the onset of CD47 saturation was in close match to the theoretical ratio for complete unilamellar particle coating. Based on surface area estimations, approximately 125 µL of blood is required to completely cover the surfaces of 1 mg of the 70 nm PLGA particles (Supplement discussion). Experimental observations showed that above the ratio of ~130 µL of blood/mg PLGA polymer, additional RBC membrane materials did not further functionalize the particles with CD47. As additional membrane materials in excess of complete unilamellar particle coverage were removed during the isolation of RBC-NPs, it can be inferred that the RBC membrane coating precluded further membrane interactions and that multilamellar membrane coating on the nanoparticles was unfavorable. To further investigate the RBC-NP formation under excessive RBC membrane to polymer ratios, RBC-NPs prepared with 250 µL of blood per mg of polymer were visualized under TEM. It was found that despite the availability of excess membrane materials in the samples, the nanoparticles were covered by a single, unilamellar coating of lipid membranes with a thickness of 6~8 nm, which is in agreement with the characteristic membrane thickness of RBCs [15]. Excess membranes remained in vesicular forms, which helped to explain the CD47
saturation on the RBC-NPs. In contrast to the unfavored multilamellar coating, unilamellar membrane coating on the RBC-NPs appeared to be highly efficient. By converting the RBC membrane input in Figure 2.1.2D to its corresponding CD47 content, the resulting correlation showed that, below the CD47 saturation, approximately 92% of the input membrane proteins were utilized for particle functionalization. This observation suggests that the RBC membrane coating to the PLGA particle surfaces was a favorable process that readily took place. The high efficiency in translocating CD47 onto nanoparticle surfaces confers a unique advantage to the RBC membrane coating approach.

To verify that the CD47 functionalized RBC-NPs possessed the properly oriented self-markers for molecular interactions, the particle surfaces were examined for the presence of CD47’s extracellular domains. Rat anti-CD47 antibodies specific to the CD47’s extracellular region were applied to the RBC-NPs on a glow-discharged carbon-coated grid. Following 1 min of incubation, the sample was washed and subsequently incubated with anti-rat IgG gold conjugate, which labelled the anti-CD47 antibodies that were retained on the grid. The immunogold-labelled sample was then rinsed with water prior to visualization by transmission electron microscopy (TEM). Figure 2.1.3A shows the attachment of the electron-dense gold particles to multiple gray circular patterns 60-80 nm in diameter, which confirmed that the gold conjugates were attached to the RBC-NPs. A negative control prepared in the absence of the primary stain showed that the gold labelling was specific to the anti-CD47 antibodies. Together, these TEM results confirm the presence of right-side-out CD47 on the RBC-
NPs. To further examine the presence of inside-out CD47 on the RBC-NPs, an antibody that specifically targets an intracellular sequence of CD47 was used. Curiously, while the antibody bound to the CD47 on RBC-NPs in western blotting, it did not yield observable immune-gold staining on either RBC-NPs or bare nanoparticles under TEM. This result indicates the relative absence of intracellular CD47 sequences on the particle surfaces. Even though the immunostaining experiment provides a qualitative rather than a quantitative measure of the membrane sidedness on the RBC-NPs, it suggests that the right-side-out membrane orientation was dominant on the RBC-NPs. Given that the extracellular side of RBC membranes possess a strong negative charge owing to high abundance of sialiated moieties [16], it is likely that electrostatic effects favored the interactions between negatively charged polymeric cores and the less negatively charged intracellular side of the RBC membranes, giving rise to a right-side-out orientation bias. This orientation bias also helps explain the unilamellar coating on the RBC-NPs, which could result from the rich surface glycan content that precludes membrane-membrane interactions. Figure 2.1.3B provides a juxtaposition of magnified images of RBC-NPs and the corresponding bare PLGA nanoparticles under negative staining or immunostaining. It can be observed that, following the RBC membrane coating, the particles were bestowed with a unilamellar membrane shell containing CD47 that predominantly exposes their extracellular domains. The proper CD47 orientation is crucial for their molecular interactions.
Lastly, the immunomodulatory effect of the CD47 functionalized RBC-NPs was studied. Bare PLGA nanoparticles and RBC-NPs loaded with hydrophobic DiD fluorophores (excitation/emission = 644 nm/655 nm) were first incubated with J774 murine macrophage cells and examined for particle internalization. Following 10 min
of incubation, the macrophage cells were washed and examined using flow cytometry, which revealed that the RBC membrane coating rendered the particles less prone to the macrophage uptake, resulting in a 64% reduction in particle internalization (Figure 2.1.4). The reduced susceptibility to macrophage engulfment confirmed the translocation of immune-evasive functionality from RBCs to RBC-NPs and helped to explain the long in vivo circulation previously observed for the RBC-NPs [13]. To identify CD47’s contribution to RBC-NPs’ immune-evasive property, saturating amounts of anti-CD47 antibodies were applied to the RBC-NPs to block the right-side-out CD47 proteins. The antibody blocking was previously demonstrated to disrupt SIRPα signalling and increased macrophage engulfment of RBCs [17]. Similarly, depriving the particles of the molecular protection from phagocytosis resulted in an increase in particle internalization by 20%, which confirmed the

![Figure 2.1.4](image.png)

**Figure 2.1.4** Inhibition of macrophage uptake. (A) Flow cytometry analysis of particle internalization by murine macrophage cells. The blue, green, and orange lines represent the bare PLGA nanoparticles (bare NPs), RBC-NPs, and CD47-blocked RBC-NPs, respectively. (B) Mean fluorescence intensity reflecting the overall particle uptake by the macrophage cells.
immunomodulatory functionality conferred by the particle-bound CD47. Curiously, the CD47-blocked RBC-NPs remained significantly more “stealthy” than the bare PLGA nanoparticles. Given that RBCs have a variety of proteins and glycans on their surface, many of which have been identified to modulate their immunological properties [18, 19], other surface moieties in addition to CD47 on the RBC-NPs likely functioned collectively to inhibit the macrophage activity. Future studies are warranted to verify these other membrane moieties and to examine their implications in nanodevice functionalization.

2.1.4 Conclusions

In summary, RBC membrane coating was demonstrated to functionalize sub-100 nm substrates with native CD47, yielding nanoparticles with equivalent CD47 surface density to natural RBCs. Right-side-out CD47 proteins were identified on the particle surfaces, readily exposing their extracellular domain for molecular interactions. The immune-evasive property of the RBC-NPs, as indicated by their reduced susceptibility to macrophage uptake, further verified the presence of functional immunomodulatory proteins on the particle surfaces. These biomimetic nanocarriers have tremendous potential in drug delivery applications, as they provide the opportunity to actively inhibit the immune clearance of their therapeutic cargo, thereby improving drug pharmacokinetics and therapeutic efficacy. The in-depth examination of the RBC-NPs also provides an up-close look at the fusion process between RBC membranes and PLGA polymeric particles, which appears to favour the
formation of unilamellar membrane coated particles with the right-side-out membrane orientation. From synthesis and fabrication perspectives, the membrane coating technique contrasts with bottom-up functionalization schemes, which often employ chemical conjugation methods that can alter proteins’ innate structures. The non-disruptive protein functionalization through the coating of natural cellular membranes presents a robust and versatile approach in interfacing synthetic materials with biological components, offering a compelling technique for the development of bio-inspired and biomimetic nanodevices.

2.1.5 References


2.2 Drug Loading

2.2.1 Introduction

In recent decades, advances in engineering materials at the nanometer scale have resulted in a myriad of nanoparticle (NP)-based drug-delivery systems for clinical applications [1, 2]. The unique advantages of these nanomedicines, particularly their improvement on existing therapeutic agents through altered pharmacokinetics and biodistribution profiles, hinge on their ability to circulate in the bloodstream for a prolonged period of time [3, 4]. As a result, considerable research interest has focused on the search for novel materials, both natural and synthetically made, that allow NPs to bypass macrophage uptake and systemic clearance [5, 6]. Meanwhile, strategies aimed at extending particle residence time *in vivo* through modifying the physicochemical properties of NPs (including size, shape, deformity and surface characteristics) have also been extensively explored [7, 8].

In this regard, the authors recently developed a red blood cell membrane (RBCm)-cloaked NP drug-delivery system with the combined advantages of a long circulation lifetime (from RBCs), and controlled drug retention and release (from polymeric particles) [9]. The authors top-down approach, based on the extrusion of polymeric particles mixed with preformed RBCm-derived vesicles, translocated the entire RBCm with preserved membrane proteins to the surface of sub 100-nm polymeric cores, resulting in NPs cloaked by the erythrocyte exterior for longer systemic circulation. This cell-mimicking strategy provides a cellular membrane
medium surrounding polymeric cores for transmembrane protein anchorage, hence avoiding chemical modifications in conventional NP surface fictionalizations that could compromise the integrity and functionalities of the proteins.

As part of continuing efforts to further develop this cell-mimicking NP platform for advanced drug-delivery applications, the authors report formulation strategies for loading small-molecule chemotherapy drugs such as doxorubicin (DOX), a model anticancer drug, into NPs and study drug-release kinetics with an emphasis on the role played by RBCm cloaks in drug retention. Specifically, to load DOX molecules into the NP core, two distinct strategies were explored: physically encapsulating drug molecules into the polymer matrix, and chemically conjugating drug molecules to the polymer backbones. It was demonstrated that they result in distinct drug-loading yields and release kinetics. NPs were further formulated with the same polymer cores as RBCm-cloaked NPs, but coated with polyethylene glycol (PEG) rather than RBCm (PEGylated NPs). Comparison of the drug-release profiles for the two delivery systems demonstrated that the RBCm cloak provides a barrier, retarding the outward diffusion of encapsulated drug molecules, and can therefore potentially be exploited to better control drug release. Additionally, in an attempt to examine the therapeutic potential of the RBCm-cloaked NPs, an acute myeloid leukemia (AML) Kasumi-1 cell line was chosen, and it was demonstrated that DOX-loaded RBCm-cloaked NPs exhibited higher toxicity by comparison with the same amount of free DOX.

2.2.2 Experimental Methods
2.2.2.1 RBC Ghost Derivation

RBC ghosts devoid of cytoplasmic contents were prepared following previously published protocols [9, 10]. Briefly, whole blood, withdrawn from male (imprinting control region)mice (6–8 weeks old, Charles River Laboratories) through cardiac puncture with a syringe containing a drop of heparin solution (Cole-Parmer), was centrifuged (800 × g for 5 min at 4°C) to remove serum and buffy coat. The packed RBCs were washed in ice-cold 1 × phosphate-buffered solution (PBS), treated by hypotonic medium for hemolysis, and then suspended in 0.25 × PBS in an ice bath for 20 min. The hemoglobin was removed by centrifuging the suspension at 800 × g for 5 min. RBC ghosts in the form of a pink pellet were collected.

2.2.2.2 Preparation of RBCm-Derived Vesicles

The collected RBC ghosts were sonicated in a capped glass vial for 5 min using a FS30D bath sonicator (Fisher Scientific) at a frequency of 42 kHz and power of 100 W. The resulting vesicles were subsequently extruded repeatedly through 400-nm and then 200-nm polycarbonate porous membranes using an Avanti mini extruder (Avanti Polar Lipids). After each extrusion, the size of the RBCm-derived vesicles was monitored using dynamic light scattering (Nano-ZS, model ZEN3600).

2.2.2.3 Ring-Opening Polymerization of L-lactide

DOX–poly(lactide acid) (PLA) conjugates were synthesized based on a previously published protocol [11, 12]. Briefly, ring-opening polymerization of L-
lactide (Sigma-Aldrich, USA) was catalyzed by an alkoxy complex (BDI)ZnN(SiMe$_3$)$_2$ in a glovebox filled with argon at room temperature. (BDI)ZnN(SiMe$_3$)$_2$ (6.4 mg, 0.01 mmol) and DOX (Jinan Wedo Co. Ltd, China; 5.4 mg, 0.01 mmol) were mixed in anhydrous tetrahydrofuran (0.5 ml), where l-lactide (101 mg, 0.7 mmol) dissolved in 2 ml of anhydrous THF was added dropwise.

After the l-lactide was completely consumed as indicated by proton nuclear magnetic resonance spectroscopy (Varian Mercury 400 MHz spectrometer), the crude product was precipitated in cold diethyl ether and purified by multiple dissolution–precipitation cycles. The conjugation was confirmed by proton nuclear magnetic resonance spectroscopy and conjugates had a molecular weight (MW) of approximately 10,000 g/mol determined by gel permeation chromatography (Viscotek, USA).

2.2.2.4 Preparation of NP Core & Loading of DOX

The DOX–PLA conjugate was first dissolved in acetonitrile to form 1 mg/ml solution. A total of 1 ml of such solution was added dropwise to 3 ml of water. The mixture was then stirred in open air for 2 h, allowing the acetonitrile to evaporate. The resulting solution of NP cores was washed using Amicon Ultra-4 Centrifugal Filters (Millipore, 10 kDa cut-off) to completely remove organic solvent residues. The particles were then re-suspended in 1 ml distilled water. To physically encapsulate DOX, 1 mg poly(lactic-co-glycolic acid [PLGA]; 0.67 dl/g, carboxy- terminated, LACTEL Absorbable Polymers) was first dissolved into 1 ml acetonitrile, followed by
the addition of DOX predissolved in 25 µl dimethyl sulfoxide. Similar procedures as described above were followed to generate suspensions containing NP cores.

2.2.2.5 Fusion of RBCm-Derived Vesicles with NP Cores

To fuse the RBCm-derived vesicles with the aforementioned NP cores, a suspension containing 1 mg of NP cores was first mixed with RBCm-derived vesicles prepared from 1 ml of whole blood. The mixture was then extruded 11 times through a 100-nm polycarbonate porous membrane with an Avanti mini extruder. To fully coat 1 mg of NP cores, an excess of blood was used to compensate for the membrane loss during RBC ghost derivation and extrusion [9].

2.2.2.6 Preparation of PEGylated NPs

The DOX–PLA conjugate and PLA-PEG-COOH (MW = 10 kDa, polydispersity index = 1.12; PEG = 3.5 kDa, PLA = 6.5 kDa) [13] at a weight ration of 1:1 were first dissolved in acetonitrile at a concentration of 1 mg/ml, followed by the same procedures as described above to produce NP suspensions. To physically encapsulate DOX into PEGylated NPs, 1 mg PLGA (0.67 dl/g, MW = 40 kDa, carboxy-terminated, LACTEL Absorbable Polymers) was first dissolved into 1 ml acetonitrile, followed by the addition of 100 µg DOX dissolved in 25 µl dimethyl sulfoxide. The procedures described above were used to produce NP suspensions.

2.2.2.7 NP Stability Studies

NP stability in PBS was assessed by monitoring particle size using dynamic
light scattering. Specifically, 500 µg of the NPs were suspended in 1 ml 1 × PBS and the sizes were measured in triplicate at room temperature every 24 h over a period of 1 week. Between measurements, samples were incubated at 37°C with gentle shaking. NP serum stability was evaluated by monitoring the UV-absorbance at a wavelength of 560 nm. Specifically, NPs were first concentrated to 2 mg/ml in PBS, followed by the addition of 2 × fetal bovine serum (Hyclone) of equal volume. The absorbance was measured using an Infinite M200 multiplate reader at 37°C approximately every 1 min over a period of 2 h. The morphology and particle size were further characterized using scanning electron microscopy (SEM). Samples for SEM were prepared by dropping 5 µl of the NP solution onto a polished silicon wafer. After drying the droplet at room temperature overnight, the sample was coated with chromium and then imaged using SEM.

### 2.2.2.8 Measurement of Drug-Loading Yield & Release

The concentration of DOX in a solution was determined by measuring florescence intensities at 580 nm with an excitation wavelength of 480 nm. To determine the DOX-loading yield of the NPs, the above fluorescence measurement was carried out after incubating 100 µl NP solution with 100 µl 0.1 M HCl in acetonitrile for 24 h. All fluorescent measurements were performed under the same conditions with 0.1 M HCl. To plot DOX release profiles, 200 µl NP solution (1 mg/ml) was loaded into a Slide-A-Lyzer MINI dialysis microtube (Pierce, IL, USA; MW cutoff: 3.5 kDa) then dialyzed against 2 l of PBS (pH = 7.4) at 37°C. PBS buffer
was changed every 12 h throughout the whole dialysis process. At each predetermined
time point, NP solutions from three mini dialysis units were collected and DOX
concentration was measured. Note that the hydrophobic DOX–PLA conjugates are
unlikely to be released from the hydrophobic particles to aqueous solutions, as that is
an energetically unfavorable process. It is expected that only drug molecules that have
detached from the polymer chains will be released.

2.2.2.9 Cell Viability Assay

Cytotoxicity of free DOX and DOX-loaded NPs was assessed against Kasumi-
1 cell line established from the peripheral blood of an AML patient using MTT assay
(Promega Corporation, WI, USA). Cells were first seeded (~5 × 10^3 per well) in 96-well
plates and incubated for 24 h. After the addition of free DOX or DOX-loaded
NPs, the cells were incubated for an additional 72 h. Cell viability was then
determined by using MTT assay following a protocol provided by the manufacturer.

2.2.3 Results and Discussion

The preparation process of RBCm-cloaked NPs was based on a previously
published protocol and is schematically illustrated in Figure 2.2.1 [9]. Briefly, purified
RBCs first underwent membrane rupture in a hypotonic environment to remove their
intracellular contents. Next, the emptied RBCs (~2 µm in diameter) were washed and
extruded through 100-nm porous membranes to create RBC-membrane derived
vesicles (~200 nm in diameter). Meanwhile, polymeric cores (~70 nm in diameter),
such as those made from PLA or PLGA, were prepared by using a solvent displacement method. The resulting polymeric cores were subsequently mixed with RBC-membrane derived vesicles and the mixture was physically extruded through 100-nm pores, where the two components fused under the mechanical force and formed RBCm-cloaked NPs (~90 nm in diameter).

**Figure 2.2.1** Preparation process of red blood cell membrane-cloaked nanoparticles. The hydrodynamic sizes of RBC ghosts, RBCm-derived vesicles, polymeric cores and RBCm-cloaked NPs were measured using dynamic light scattering.

NP: Nanoparticle; RBC: Red blood cell; RBCm: Red blood cell membrane.

In this study, two distinct methods to load DOX as a model drug into the RBCm-cloaked NPs were examined: physical encapsulation and chemical conjugation. Physical encapsulation is achieved by first mixing DOX and the polymers in acetonitrile, followed by precipitation into water. In this case, the drug-
loading yield can be varied through different formulation parameters. For example, when varying initial DOX to PLGA weight ratio from 5 to 20%, the loading yield increases from 0.9 to 1.8% (Figure 2).

Alternatively, DOX molecules can be loaded into NP cores by covalently conjugating drug molecules to polymer backbones. Intuitively, DOX molecules can be directly conjugated to carboxyl-terminated PLA chains through hydroxyl groups; however, this approach causes heterogeneities for polymer–drug conjugates, owing largely to the polydispersity of the polymer chains, the lack of control over the regio- and chemo-selective conjugation of the DOX molecules containing multiple hydroxyl groups, and the lack of control over the conjugation efficiency. Therefore, an alternative approach was adopted, where the hydroxyl groups of the DOX, in the presence of using 1-lactide monomer and (BDI)ZnN(SiMe₃)₂ as a catalyst, were utilized to initiate the ring-opening polymerization and led to the formation of PLA–DOX conjugates [11, 12]. In this approach, as the polymerization reaction is initiated by the drug molecule itself, a conjugation efficiency of near 100% can be achieved. In addition, the metal amido catalyst (BDI)ZnN(SiMe₃)₂ preferentially allows for PLA propagation at C14-OH position of DOX, instead of its more sterically hindered C4’- and C9-OH positions. After the reaction was terminated, products were purified using repeated dissolution–precipitation cycles and then characterized using 1H–NMR spectroscopy. Proton resonance peaks corresponding to both DOX molecules and PLA backbones are present, including the aromatic protons of DOX between d = 7.5 and 8.0 ppm, protons of -CH₃ group of PLA at d = 1.5 ppm, and -CH group of PLA at d =
5.2 ppm, hence confirming the formation of PLA–DOX conjugates [11]. In contrast to physical encapsulation, where the drug-loading yield primarily depends on formulation parameters, in chemical conjugation, the drug-loading yield is dictated by polymer chain length, which is in turn determined by polymerization conditions such as initiator (DOX)-to-monomer ratio. For example, the PLA–DOX conjugates synthesized in this study were found to have a molecular weight of 10 kDa and a narrow polydispersity index of 1.16, corresponding to an approximately 5% loading yield of DOX after the conjugates were formulated into the NPs (Figure 2.2.2).

![Figure 2.2.2](image)

**Figure 2.2.2** Doxorubicin loading yields in the red blood cell membrane-cloaked nanoparticles at various initial drug inputs. Drug molecules were loaded into the nanoparticles through two distinct loading mechanisms: physical encapsulation and chemical conjugation.

Next, the stability of DOX-loaded RBCm- cloaked NPs in physiologically relevant buffer solutions was studied. In PBS, NP stability is monitored by measuring NP sizes at different time points, as unstable particles tend to aggregate and their sizes
increase. In this study (Figure 2.2.3A), NPs loaded with DOX molecules by using both physical encapsulation and chemical conjugation showed similar initial diameters of approximately 90 nm; without significant size increase over the duration of 1 week. Similarly, only a slight change in the polydispersity index of the NPs was observed over the same time period, indicating a high stability of DOX-loaded RBCm-cloaked NPs in PBS. NP stability was further examined in serum by monitoring UV absorbance at 560 nm, a characteristic wavelength reflecting the extent of particle aggregation [14, 15]. RBCm-cloaked NPs, loaded with DOX molecules by either physical encapsulation or chemical conjugation, showed a near constant absorbance at 560 nm over a time period of 2 h (Figure 2.2.3B), suggesting that the NPs are highly stable in 100% fetal bovine serum. The morphological measurements of these RBCm-cloaked NPs by SEM showed spherical structures with an average size of approximately 75 nm (Figure 2.2.3C). By contrast, absorbance of bare polymeric cores made from PLGA or PLA–DOX conjugates without RBCm cloaks immediately increased upon addition into fetal bovine serum, therefore the authors were unable to monitor their long-term stability in either PBS or serum. The results of this study showed that the RBCm cloak played a significant role in stabilizing NPs in both buffer solutions and serum. From a practical perspective, the fast aggregation of uncoated polymeric particles in buffer solutions provided a means of selective precipitation and removal of uncoated particles from RBCm-cloaked NPs after their preparation.

Following the formulation of stable DOX- loaded RBCm-cloaked NPs, their DOX release kinetics were investigated (Figure 2.2.4). The authors first examined
how different drug-loading mechanisms would affect DOX releases from RBCm-cloaked NPs. The results demonstrated that, when DOX molecules were physically
encapsulated into the polymer matrix, the drug-release rate was significantly faster, as 20% of DOX molecules were released within the first 2 h from the RBCm-cloaked NPs. By contrast, when formulations of chemical conjugation were examined, within the first 2 h only 5% of DOX molecules were released. Such difference has been attributed to the fact that covalent bonding of DOX molecules to the polymer backbone requires drug molecules to first be hydrolyzed from the polymer by bulk erosion before they can diffuse out of the polymeric matrix for release [11, 12, 16]. A more sustained-release profile resulted from drug-polymer covalent conjugation also suggests that chemical linkers responsive to environmental triggers can achieve better controlled drug releases when developing RBCm-cloaked NPs for advanced drug-delivery applications [13, 17]. It is expected that acidic pH conditions will increase the drug-release rate of the polymeric cores because the pH drop will accelerate the degradation rate of the polymer backbone and facilitate the cleavage of the ester linkage between the drugs and the polymers [11].

In order to gain a better understanding of the role played by the RBCm cloak in drug retention, an established procedure for generating NPs by blending PLA-PEG diblock copolymers was followed. PEGylated NPs, where NP cores were coated and stabilized by a PEG layer instead of RBCm cloak [18]. It was hypothesized that if two formulations have similar NP cores, the difference in drug release will be primarily caused by the different abilities of the RBCm cloak and surface PEG coating in drug retention. By comparing DOX release from RBCm-cloaked NPs to that from PEGylated NPs, it was found that the release rate of the RBCm-cloaked NPs was
lower; approximately 20% of DOX was released within the first 72 h in the RBCm-cloaked NPs, whereas 40% of DOX was released from the PEGylated NPs over the same time span. In fact, by using NPs formulated by PLGA–PEG diblock copolymers,

![Image](image_url)

**Figure 2.2.4** Drug release profiles and kinetics of the different doxorubicin-loaded nanoparticles. (A) Doxorubicin release profiles of RBCm-cloaked nanoparticles and PEG-coated nanoparticles. For these release studies, initial doxorubicin concentration inside the nanoparticles was 5 wt% for chemical conjugation and 1.8 wt% for physical encapsulation, respectively. (B) For the physical encapsulation systems, the drug release percentage was plotted against the square root of time, which yielded linear fittings using a diffusion-dominant Higuchi model. 
NP: Nanoparticle; RBCm: Red blood cell membrane.
surface PEG molecules have been found to hinder drug release from NP cores [19]. Hence, the observation that DOX is released at a higher rate from PEG-coated NPs compared with RBCm-cloaked NPs, indicates that RBCm indeed acts as a diffusion barrier for DOX release. This observation is in accordance with previous studies demonstrating that phospholipid coating can act as a barrier to drug diffusion [20]. Such a role played by the RBCm cloak further suggests that strategies aimed at engineering lipid membrane coatings may allow for responsive drug releases from RBCm-cloaked NPs under certain environmental cues in addition to those achieved by chemical conjugations embedded in polymer cores [21].

To gain a quantitative understanding of the membrane-coating effect on drug retention, the drug-release profiles were analyzed using mathematical models established in previous particle drug-release studies. Since the degradation of PLGA is in the order of weeks [22, 23], markedly slower than the observed drug release for the physically loaded systems, a diffusion-dominant Higuchi model was applied to both RBCm-coated and PEGylated NPs containing physically encapsulated DOX. Plotting the drug-release percentage against the square root of time yielded linear fittings with $R^2 = 0.98$ and 0.96 for the RBCm- cloaked and the PEGylated NPs, respectively (Figure 2.2.4B). The goodness of the fit implies a diffusion-controlled drug-release mechanism and further allows for the derivation of the diffusion coefficient through the following Higuchi equations 1 & 2 [24, 25]:

$$M_t = Kt^{1/2} \quad (1)$$

$$K = A(2C_{ini}DC_s)^{1/2} \quad (2)$$
Where $M_t$ is drug release at time $t$ in hours, $K$ is the Higuchi constant, $C_{ini}$ is the initial drug concentration, $C_s$ is the drug solubility, $A$ is the total surface area of the particles and $D$ is the diffusion coefficient. Given the particle dimensions, the drug-loading yield, the solubility of DOX in water (1.18 g/l), and the drug-release data, the diffusion coefficients were determined to be $6.6 \times 10^{-16}$ cm$^2$/s and $8.2 \times 10^{-16}$ cm$^2$/s for the RBCm-clocked and PEGylated NPs, respectively, which is consistent with previously reported drug diffusivities from PLGA/PLA NPs [26]. In this study, the bilayered membrane coating reduced the drug diffusivity by 1.2-times. It is expected that this retardation effect by the RBCm cloak would likely vary with different particle sizes, polymer types and therapeutic cargo.

On the other hand, applying zero order, first order and Higuchi models to the drug-release profiles of chemically conjugated DOX yielded poor fittings (data not shown), indicating complex release kinetics when additional drug cleavage is coupled with drug diffusion out of the polymer matrix. Precise modeling of the retardation effect imposed by the RBCm cloak on the chemically conjugated DOX is beyond the scope of this study. Nevertheless, as identical particle cores are present in both RBCm-cloaked and PEGylated NPs, herein, it is hypothesized that polymer matrix relaxation and hydrolytic cleavage of the linkage are not dominant factors contributing to the difference observed in DOX release profiles. Instead, the authors attribute the slower release rate of the RBCm-cloaked NPs to two diffusion-dominated components: the diffusion of water into the polymer matrix; and the diffusion of the cleaved drugs outward across the polymer matrix [27]. As the membrane coating was shown to
decrease the drug diffusivity in the physical entrapment system, it likely affected both the influx of water and the efflux of cleaved drugs in the covalent conjugate system, thereby resulting in a more sustained drug-release profile.

Finally, the therapeutic potential of the DOX-loaded RBCm-cloaked NPs against an AML Kasumi-1 cell line was examined. AML, an illness characterized by uncontrolled growth and accumulation of leukemia blasts in the bloodstream, was chosen as a disease target because of the long circulation lifetime of the RBCm-cloaked NPs in the blood stream, and their sustained drug-release profiles. The current standard of care for AML is high-dose anthracyclines, which raises serious concerns for cardiac toxicity [28]. Long-circulating NPs releasing therapeutic compounds in a sustained manner offer the opportunity to reduce the required dosing and improve upon the treatment efficacy. RBCm-cloaked NPs, where DOX was either physically loaded or covalently conjugated, exhibited higher toxicity by comparison to free DOX over a 72-h incubation period (Figure 2.2.5). In a previous study, it was demonstrated that these RBCm-cloaked NPs can be taken up by cancer cells in a tissue culture and the NPs remain in an intact core–shell structure after cellular internalization [9]. Therefore, the observed enhancement in efficacy can be likely attributed to endocytic uptake of NPs, which enables a high payload of drugs to enter the intracellular region [29]. Several previous reports have shown enhanced cytotoxicity of DOX through NP-based delivery of DOX [30, 31]. The free DOX, by contrast, relies on passive membrane diffusion for cellular entry, which is less efficient and susceptible to membrane-bound drug efflux pumps [32–34]. AML cells, including the Kasumi-1 cell
NPs can with a prolonged circulation lifetime, sustained drug release and improved cell internalization in AML research [36, 37]. In particular, the Kasumi-1 cell line has been used previously as a P-glycoprotein-positive and anthracycline-resistant cell line in AML research [36, 37]. The current study suggests that RBCm-cloaked NPs, with a prolonged circulation lifetime, sustained drug release and improved cell internalization, can become a promising platform toward the treatment of blood cancer. Further studies are warranted to investigate the therapeutic potential of these NPs in vivo.

Figure 2.2.5 Cytotoxicity study against the Kasumi-1 cell line established from the peripheral blood of an acute myeloid leukemia patient. Circles represent RBCm-cloaked NPs with chemically conjugated DOX, squares represent RBCm-cloaked NPs with physically encapsulated DOX and triangles represent free DOX. All samples were incubated with Kasumi-1 cells for 72 h prior to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (n = 4).

DOX: Doxorubicin; NP: Nanoparticle; RBCm: Red blood cell membrane.
2.2.4 Conclusions

In summary, herein, we examined two strategies for loading drugs into an RBCm-cloaked NP delivery system were examined: physical encapsulation and chemical conjugation. Release studies suggest that the chemical conjugation strategy results in a more sustained drug-release profile. We further formulated PEGylated NPs that had the same NP cores but different surface coatings compared with RBCm-cloaked NPs. By comparing drug-release profiles of these two delivery systems, it was demonstrated that RBCm cloaks provide a barrier, slowing down the outward diffusion of encapsulated drug molecules. These results suggest that chemical modifications to the drug–polymer linkage in the NP core and engineering the NP surface coatings can both be explored to gain better control over drug releases of RBCm-cloaked NPs. In a following efficacy study by using the AML Kasumi-1 cell line, RBCm-cloaked NPs exhibited higher toxicity in comparison with free DOX. The previously observed long systemic circulation lifetime in the blood stream and the sustained drug-release kinetics reported indicate that this biomimetic drug-delivery system may hold great promise for systemic delivery of payloads for the treatment of various diseases such as blood cancers. With further development, these RBCm-cloaked NPs are expected to become a robust drug-delivery system that combines the advantages of both synthetic polymers and natural cellular membranes.

RBCm-cloaked NPs represent a novel class of NP formulations bringing together both the long circulation lifetime of RBC, and the controlled drug retention and release of synthetic polymers. After gaining a deeper understanding of the roles
played by the RBCm shell and the polymeric core, this NP formulation can be further tailored by engineering both parts to improve systemic delivery of therapeutic payloads. We believe that with continuing effort, this formulation will result in a robust delivery platform and make significant impact on both biomedical applications and nanotechnology research.

2.2.5 References


30. Ayen, W.Y., K. Garkhal, and N. Kumar, *Doxorubicin-loaded (PEG)3–PLA nanoparticlesomes: effect of solvents and process parameters on formulation*


2.3 Targeting Ligand Functionalization

2.3.1 Introduction

A major goal in engineering nanocarriers for systemic drug delivery is to achieve long circulation half-lives [1], as increased residence time in the bloodstream can improve the pharmacokinetic profile of therapeutic cargoes and allow a greater chance for the nanocarriers to reach the desired location through either passive [2, 3] or active [4-6] targeting mechanisms. Ongoing search for new and effective ways to construct long-circulating nanoparticles has introduced numerous stealth functionalization strategies. While the use of synthetic polymers represents the current golden standard for prolonging particle residence time in circulation, recent efforts have drawn inspiration from nature to bestow immune-evasive properties on nanoparticles [7, 8]. Bridging the complex surface biochemistry of nature’s carriers, RBCs, with the versatile cargo-carrying capacity of polymeric nanoparticles, an RBC membrane-cloaked nanoparticle (RBC-NP) platform represents a new class of bio-inspired nanocarriers with long-circulating capability [9, 10]. In this system, a top-down approach is used to coat polymeric nanoparticles with natural RBC membranes, which possess a litany of immunomodulatory proteins responsible for RBCs’ extraordinarily long survival in circulation [11-14]. This membrane cloak acts to camouflage the nanoparticle in vivo to evade immune attacks [9]. Consisting entirely of biocompatible and biodegradable materials, the RBC-NPs have significant potential for drug delivery applications.
In applying RBC-NPs for disease treatments, particularly against cancers, target-selectivity is a desirable feature that promises minimization of off-target side effects [15-19]. Cancer targeting has been made possible by carrier functionalization with ligands that target overexpressed tumor antigens, and numerous chemical conjugation techniques employing carboxyl-, amine-, or sulfhydryl-based chemistry have been used to decorate synthetic nanocarriers with targeting ligands [20-26]. In the case of biologically derived carriers such as RBC-NPs, however, the presence of biological components on the particle surfaces demands a non-disruptive functionalization strategy since the immune evasion capabilities of cellular membranes is predicated upon having fully functional proteins. In order to address the issue of chemical denaturation, we report a lipid-insertion approach to functionalizing RBC-NPs that exploits the fluidity of bilayered RBC lipid membranes and precludes RBC membrane exposure to chemical reactions. As shown in Figure 2.3.1, targeting moieties are incorporated onto RBC membranes through the aid of lipid tethers. The physical insertion of ligand-linker-lipid conjugates into the RBC membranes produces

**Figure 2.3.1** Schematic of the preparation of RBC-NPs with targeting ability. Ligand-linker-lipid conjugates are synthesized and then inserted into RBC membrane ghosts. The resulting ligand-functionalized RBC membranes are used to coat polymeric cores to form targeted RBC-NPs.
functionalized RBC membranes without damaging the existing surface proteins. Using two differently sized ligands, a small molecule folate (MW ~ 441 Da) and a nucleolin-targeting aptamer AS1411 (MW ~ 9,000 Da), we demonstrate that the lipid-insertion technique can be applied to targeting ligands of different length scales. Following preparation of RBC-NPs with the functionalized RBC membranes, their receptor-specific targeting ability is verified in model cancer cell lines in vitro.

2.3.2 Experimental Methods

2.3.2.1 Synthesis of Ligand-Linker-Lipid Conjugates

1,2-distearoyl-sn-glycero-3-phosphoethanol amine conjugated to polyethylene glycol 2000 with fluorescein isothiocyanate (FITC-PEG-lipid) was purchased from Nanocs (New York, NY). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (folate-PEG-lipid) was purchased from Avanti Polar Lipids (Alabaster, AL). AS1411 aptamer, with the sequence of GGT GGT GGT GGT TGT GGT GGT GG, was custom synthesized by Integrated DNA Technologies (San Diego, CA) with a 3’ thiol modifier. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (maleimide-PEG-lipid) was purchased from Avanti Polar Lipids. For the synthesis of AS1411-PEG-lipid, the aptamer was suspended at 1 mg/mL in a buffer consisting of 10 mM Tris pH 8.0 (Cellgro) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (USB Corporation) with a 100× molar excess of tris[2-carboxyethyl] phosphine (TCEP) (Millipore) to reduce the disulfide bond of the thiol modifier. The solution was allowed to incubate
for 4 hr at room temperature before washing the aptamer in the same reducing solution 3 times using a 10 kDa molecular weight cutoff Amicon Ultra-4 centrifuge filter (Millipore). The reduced aptamer was then incubated overnight with an equimolar amount of maleimide-PEG-lipid before washing again 3 times in water.

2.3.2.2 Preparation of Ligand-Inserted RBC Ghosts

Whole blood was obtained from 10 week-old male ICR mice (Charles River Laboratories) via cardiac puncture using syringes preconditioned with heparin (Sigma Aldrich) and EDTA. All animal experiments were reviewed, approved and performed under the regulatory supervision of The University of California, San Diego’s institutional biosafety program and the Institutional Animal Care and Use Committee (IACUC). RBCs were extracted from the collected blood by centrifuging at 800 × g for 5 min at 4°C and washed 3 times in cold phosphate buffered solution (PBS) (Invitrogen). The buffy coat was removed in the process. The washed RBCs were then lysed in 0.25× PBS, and the ghosts were separated by collecting the pink pellet after centrifuging at 800 × g for 5 min at 4°C. The pellet was then resuspended in water and incubated with ligand-PEG-lipid (ligand = FITC, folate, or AS1411) for 30 min to form ligand-inserted RBC ghosts. All samples were then washed by pelleting at 800 × g for 5 min at 4°C before further use.

2.3.2.3 Characterization of Ligand-Inserted RBC Ghosts

To quantify ligand incorporation onto RBC ghosts, different amounts of FITC-PEG-lipid were incubated with RBC ghosts derived from 1 mL of mouse blood for 30
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min. The total fluorescence of the solution was first quantified to obtain a value for the initial input. The ghosts were then washed 3 times and resuspended to the original volume. Another fluorescence reading was taken to determine the amount of ligand retained on the RBC ghosts relative to the initial input. For flow cytometry measurements, 40 µg of FITC-PEG-lipid was added into RBC ghosts derived from 1 mL of blood while plain RBC ghosts were used as a negative control. The samples were run on a FACSCanto II flow cytometer from Becton, Dickinson, and Company (Franklin Lakes, NJ) and the resulting data was analyzed using FlowJo software from Tree Star (Ashland, OR). Fluorescence imaging studies were conducted by dropping the FITC-modified RBC ghosts on a poly-L-lysine coated slide from Polysciences (Warrington, PA) and imaged using a 60X oil immersion objective on a DeltaVision Deconvolution Scanning Fluorescence Microscope from Applied Precision (Issaquah, WA).

2.3.2.4 Synthesis of Ligand-Functionalized RBC-NPs

The synthesis of ligand-functionalized RBC-NPs was adapted from a previously published protocol [9]. Briefly, RBC ghosts derived from 1 mL of blood that were functionalized with 40 µg of FITC-PEG-lipid were extruded serially through a 400 nm and then 100 nm polycarbonate membranes using a mini extruder (Avanti Polar Lipids). Polymeric cores of about 70 nm in diameter were prepared via the nanoprecipitation of 0.67dL/g carboxyl-terminated poly(D,L-lactic-co-glycolic acid) (PLGA) (LACTEL Absorbable Polymers). The ligand-inserted membrane vesicles and
the polymeric cores were then extruded together at a ratio of 1 mL of blood worth of vesicles to 1 mg of cores through a 100 nm polycarbonate membrane to form the final RBC-NPs. To measure average particle size and zeta potential, functionalized and non-functionalized RBC-NPs were suspended at 1 mg/mL in clear disposable capillary cells (DTS1061) from Malvern and measured by DLS using a Zetasizer (ZEN3600) from Malvern (United Kingdom). All measurements were taken at a backscattering angle of 173° and were performed in triplicate. The morphology of the particles were imaged by scanning electron microscopy (SEM) using an XL30 ESEM from FEI/Phillips (Hillsboro, OR) at a beam intensity of 20 kV. Samples were prepared at 1 µg/mL and 5 µL drops were dried onto silicon wafers. The samples were then coated with iridium at 85 mA for 7 sec on a K575X Sputter Coater from Emitech (Fall River, MA) followed by SEM imaging.

2.3.2.5 Colocalization Studies of Functionalized RBC-NPs

KB cells (ATCC: #CCL-17) were maintained in folate free RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (Gibco). Before the experiment, cultures were detached with 0.25% trypsin-EDTA (Gibco) and plated onto 8-chamber Labtek II slides (Nunclon) at 80% confluency. After allowing the cells to adhere overnight, they were incubated with RBC-NPs, of which the polymeric core was loaded with 0.05 wt% 1,1′-dioctadecyl-3,3,3′,3′-tetrachloroindocarbocyanine (DiD) (Invitrogen) and the RBC membrane shell contained FITC (40 µg FITC-PEG-lipid per 1 mL of blood worth of RBC
ghosts). The cells were incubated with the RBC-NPs at a concentration of 0.25 mg/mL for 2 hr. The cells were then washed with media and allowed to incubate for another 4 hr before fixing with 10% formalin (Millipore) and mounted with DAPI-containing Vectashield® (Invitrogen). Imaging was done using a 60X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope.

2.3.2.6 Cellular Uptake Studies

All flow cytometry studies were conducted on a FACSCanto II flow cytometer (Becton Dickinson) and the resulting data was analyzed using FlowJo software (Tree Star). 10,000 events were collected per sample and gated using control cells with no RBC-NP incubation. Histograms were plotted with fluorescence intensity on the x-axis using a biexponential scale. All imaging studies were conducted using a 20X objective on a DeltaVision Deconvolution Scanning Fluorescence Microscope from Applied Precision.

Both KB Cells (ATCC: #CCL-17) and A549 cells (ATCC: #CCL-185) were maintained as described above in supplemented folate-free RPMI (Gibco). Folate-functionalized particles with 0.05 wt% DiD loaded into the polymeric cores were prepared using folate-PEG-lipid at a ratio of 20 µg folate-PEG-lipid per 1 mL of blood worth of RBC ghosts. Cells were plated at a density of $10^5$ cells per well on 12-well plates (Becton Dickinson) for flow cytometry or at 80% confluency on 8-chamber Labtek II slides (Nuncelon) for fluorescence imaging and were allowed to adhere overnight before use. Folate-functionalized and non-targeted particles were incubated
at a concentration of 250 µg per 1 mL of media. For samples with free folate, the concentration was adjusted to 1 mM using a 100 mM stock solution of folate in PBS (Gibco) 10 min before incubation with nanoparticles. All samples were incubated for 30 min, washed 3 times with media, and incubated in fresh media for another 30 minutes. For flow cytometry, cells were detached with trypsin EDTA (Gibco), washed with PBS (Gibco), and taken for analysis. For fluorescence imaging, the chambers were gently washed with PBS, fixed with 10% formalin (Millipore), and mounted with DAPI-containing Vectashield® (Invitrogen).

MCF-7 Cells (ATCC: #HTB-22) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin (Gibco). AS1411-functionalized particles with 0.05 wt% DiD loaded into the polymeric cores were prepared using AS1411-PEG-lipid at a ratio of 100 µg AS1411-PEG-lipid per 1 mL of blood worth of RBC ghosts. Cells were plated at a density of 10^5 cells per well on 12-well plates (Becton Dickinson) for flow cytometry or at 80% confluency on 8-chamber Labtek II slides (Nunclon) for fluorescence imaging and were allowed to adhere overnight before use. AS1411-functionalized and non-targeted particles were incubated at a concentration of 250 µg per 1 mL of media. For the sample with free AS1411, 100 µg AS1411 in 10 µL PBS (Gibco) was added 10 min before incubation with nanoparticles. All samples were incubated for 30 min and then washed 3 times with media followed by incubating for another 30 minutes. For flow cytometry, cells were detached with trypsin EDTA (Gibco), washed with PBS (Gibco), and taken for analysis. For fluorescence imaging, the chambers were gently
washed with PBS, fixed with 10% formalin (Millipore), and mounted with DAPI-containing Vectashield® (Invitrogen).

2.3.2.7 Cytotoxicity Study

Human umbilical vein endothelial cells (HUVECs) (Cell Applications) were maintained in endothelial cell growth medium (Cell Applications). Before the experiment, the cells were detached and plated at a density of 5000 cells per well in a 96-well plate and allowed to attach overnight. Both unmodified and folate-functionalized RBC-NPs were prepared as previously described and serially diluted 3X starting from a concentration of 2 mg/mL. The samples were added to an equal volume of media in each well and allowed to incubate for 16 hr. Each sample was done in triplicate. The wells were then washed with media and allowed to incubate for another 48 hr. To conduct the cytotoxicity assay, all solution was removed from the wells and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) in PBS was added to each well at a concentration of 0.5 mg/mL. After a 3 hr incubation at 37°C, the solution was removed and the crystals were solubilized with 100 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich) and the plate was allowed to incubate for another 20 m. The data was analyzed after reading the absorbance at 540 nm.

2.2.3 Results and Discussion

To demonstrate that lipid-tethered ligands can be spontaneously incorporated
onto RBC membranes, a FITC-linker-lipid (excitation/emission = 495/519 nm) conjugate was used for a proof-of-concept test, where FITC was linked to a lipid molecule through a short polyethylene glycol chain (MW ~ 2,000 Da). In the study, 40 μg of FITC-PEG-lipid was first incubated with emptied red blood cells (RBC ghosts) collected from 1 mL of whole mouse blood for 30 min. The RBC ghosts were then centrifuged, washed with PBS, reconstituted, and examined using flow cytometric analysis (Figure 2.3.2A). Compared to unmodified RBC ghosts, modified membrane ghosts had significantly higher signal under the FITC channel. Visualization by fluorescence microscopy further confirmed the localization of the lipid-tethered FITC on the RBC membranes, as the microscopy image displays strong FITC signals outlining the exterior of the RBC ghosts (Figure 2.3.2B). To characterize the lipid-insertion efficiency and saturation, varying amounts of FITC-PEG-lipid were incubated with the membrane ghosts followed by membrane purification through centrifugation. Fluorescence quantification showed that the retained FITC fluorescence increased with the initial FITC-PEG-lipid input (Figure 2.3.2C). Saturation was observed as the retained FITC-PEG-lipid approached a plateau at approximately 40 μg per mL of RBCs. Based on the RBC concentration and the molecular weight of the ligand, it is estimated that each RBC ghost contains about 800,000 FITC-PEG-lipid. Figure 2.3.2C also demonstrates adjustability of ligand density on the membranes by controlling the lipid-tethered ligand input.

Upon confirming the incorporation of FITC onto the RBC ghosts, the membrane materials were then used to prepare FITC-modified RBC-NPs. Following a
previously reported protocol [9], the FITC-modified RBC membrane ghosts were extruded to form ~100 nm vesicles, which were then mixed and extruded with 70 nm PLGA particles to generate FITC-modified RBC-NPs. For comparison, RBC-NPs
coated with unmodified RBC membranes were also prepared. Characterization of the two particles by dynamic light scattering (DLS) showed similar physicochemical properties between the FITC-modified and unmodified RBC-NPs (Figure 2.3.2D). Both particles were approximately 80 nm in mean diameter and under -25 mV in zeta potential. Scanning electron microscopy (SEM) further demonstrated the similarity between the two particle types (Figure 2.3.2E), both of which were spherical in morphology and exhibited monodisperse population distributions. To confirm the colocalization of lipid-tethered ligands with the polymeric cores, an in vitro fluorescence colocalization study was conducted by loading DiD dye (excitation/emission = 644/663 nm) into the polymeric cores. Following cellular uptake, significant overlap was observed between the DiD-specific red punctates and the FITC-specific green punctates (Figure 2.3.2F). The colocalization pattern confirms the presence of lipid-tethered FITC on the surface of the polymeric cores, demonstrating successful preparation of ligand-modified RBC-NPs.

After validating the lipid-insertion method for RBC-NP functionalization using lipid-tethered fluorescent probes, particle modification with cancer-targeting ligands was explored. A small molecule ligand, folate (MW ~ 441 Da), which has similar molecular weight as FITC, was first examined. Folate-functionalized nanocarriers have broad applicability as folate receptors are overexpressed on several types of cancers [27]. Upon receptor-mediated binding, folate-functionalized nanocarriers can deliver their cargoes intracellularly through an endocytic uptake pathway. The benefit of folate-induced cancer targeting has been demonstrated on several nanocarrier
platforms [28, 29], and thus its incorporation onto RBC-NPs can improve the particles’ utility in cancer drug delivery.

To prepare folate-functionalized RBC-NPs, a commercially available folate-PEG-lipid conjugate was used (Figure 2.3.3A). Folate-functionalized RBC-NPs were prepared using RBC ghosts inserted with folate-PEG-lipid. Since the targeting ability of folate-functionalized nanoparticles has already been well-established, the KB cell line, a model cancer cell line overexpressing the folate receptor that is commonly used to evaluate folate targeting [30-32], was used to confirm successful functionalization of the RBC-NPs. To assess for folate-mediated differential uptake, the cells were cultured in folate-free media and incubated with folate-functionalized RBC-NPs, non-targeted RBC-NPs, or folate-functionalized RBC-NPs together with 1 mM of free folate. The cells from each sample were then detached, washed, and analyzed using flow cytometry (Figure 2.3.3B,C). Compared to the non-targeted RBC-NPs, the particles functionalized with folate ligand resulted in an 8-fold increase in cellular uptake. Conjoint incubation with folate-functionalized RBC-NPs and 1 mM of free folate yielded a similar level of cellular uptake as compared to non-targeted RBC-NPs, which indicates that the increased uptake of folate-functionalized RBC-NPs was receptor-specific. Fluorescence microscopy visualization of particle uptake further confirmed the results observed from flow cytometry. As shown in Figure 2.3.3D, fluorescence from the DiD dye encapsulated inside the particles was only observed in cells incubated with folate-functionalized RBC-NPs in the absence of free folate molecules. To demonstrate that the targeting effects were exclusive to cells
overexpressing the folate receptor, a negative cancer cell line [33], A549, was incubated with either unmodified or folate-functionalized RBC-NPs. No increased uptake was observed for the targeted nanoparticles compared to the unmodified nanoparticles using both flow cytometry and fluorescence imaging. Important to note also is that no cytotoxicity was observed for the RBC-NPs when incubated with human umbilical vein endothelial cells (HUVECs), a normal cell line, at the concentrations used in these studies. Overall, the results confirm the receptor-specific

**Figure 2.3.3** Lipid-insertion enables targeting functionalization with folate. (A) Schematic representation of folate-linker-lipid. (B) Flow cytometry histograms of KB cells alone (black) and the cells incubated with folate-functionalized RBC-NPs (red), non-targeted RBC-NPs (blue), and folate-functionalized RBC-NPs together with free folate (orange). (C) Quantification of the mean fluorescence intensity of the histograms in (B). (D) Fluorescence microscopy of KB cells incubated with folate-functionalized RBC-NPs, non-targeted RBC-NPs, and folate-functionalized RBC-NPs together with free folate. A fluorescent probe DiD was loaded inside the RBC-NPs for visualization (red) and cellular nuclei were stained with DAPI (blue). Scale bars = 25 µm.
targeting capability of folate-functionalized RBC-NPs.

To demonstrate that the lipid-insertion method can be applied to targeting ligands of different length scales, a nucleolin-targeting oligonucleotide, AS1411 aptamer (MW ~ 9,000 Da), was also tested for particle functionalization. Oligonucleotide-based targeting agents, or aptamers, are a versatile class of ligands that can be customized against specific receptors through affinity screening [34]. AS1411, a 26-mer DNA aptamer with the sequence GGT GGT GGT GGT TGT GGT GGT GGT GG, has shown targeting capability against several cancer cell types owing to frequent overexpression of surface nucleolin on cancerous cells [35-37]. The ligand has also been applied for the preparation of cancer-targeted nanoparticles [38, 39], thus its integration onto the RBC-NPs would greatly benefit the utility of the platform.

To incorporate aptamers onto RBC-NPs via lipid-insertion, a lipid-tethered AS1411 was first prepared. AS1411 aptamers containing a 3' thiol modifier was reduced using tris[2-carboxyethyl]phosphine (TCEP) and conjugated to lipid-PEG-maleimide via maleimide-sulphhydryl chemistry (Figure 2.3.4A). After purification, the AS1411-PEG-lipid conjugates were used to prepare RBC-NPs following the aforementioned procedures. In a cellular uptake study using a surface nucleolin expressing breast cancer cell line, MCF-7 [35], differential targeting was observed. Flow cytometry analysis revealed that the targeted nanoparticles induced a two-fold increase in cellular uptake as compared to the non-targeted RBC-NPs (Figure 2.3.4B,C). The uptake enhancement by the targeted nanoparticles was also confirmed to be receptor-specific, as blocking by free AS1411 reduced the particle uptake to the level of the non-targeted
nanoparticles. Fluorescence imaging corroborated the flow cytometry results with the AS1411-functionalized RBC-NP showing much greater uptake than the non-targeted and blocked samples (Figure 2.3.4D). The results demonstrate that the lipid-insertion method can be applied to relatively large targeting ligands (e.g., MW ~ 9,000 Da) with a molecular weight larger than that of the lipid anchor (MW ~ 748 Da).

![Figure 2.3.4](image)

**Figure 2.3.4** Lipid-insertion enables targeting functionalization with AS1411 aptamer. (A) Schematic representation of AS1411-linker-lipid. (B) Flow cytometry histograms of MCF-7 cells alone (black) and the cells incubated with AS1411-functionalized RBC-NPs (red), non-targeted RBC-NPs (blue), and AS1411-functionalized RBC-NPs together with free AS1411 aptamer (orange). (C) Quantification of the mean fluorescence intensity of the histograms in (B). (D) Fluorescence microscopy of MCF-7 cells incubated with AS1411-functionalized RBC-NPs, non-targeted RBC-NPs, and AS1411-functionalized RBC-NPs together with free AS1411. A fluorescent probe DiD was loaded inside the RBC-NPs for visualization (red) and cellular nuclei were stained with DAPI (blue). Scale bars = 25 µm.
2.3.4 Conclusions

In summary, by employing a lipid-insertion technique for the functionalization of biological membrane, targeted RBC-NPs were successfully prepared with two different types of targeting ligands. Through the aid of lipid tethers and the dynamic conformation of membrane bilayers, targeting ligands can be spontaneously incorporated onto the RBC-NP platform without exposing the biological membranes to chemical reactions. The robustness and simplicity of this functionalization scheme can enable a wide array of functionalized RBC-NPs for specific disease treatments. In addition, the technique can be generalized to help improve the applicability of emerging biologically inspired nanocarriers possessing complex surface chemistry. The capability to control and adjust ligand density through the lipid-insertion technique also provides versatility for platform optimization. Future studies are warranted to examine the in vivo implications of ligand functionalization on RBC-NPs. The targeted RBC-NPs reported in the present work possess significant potential for cancer treatments as they integrate nature’s immune-evasive moieties with cancer-binding ligands.

Chapter 2, in full, is a reprint of the material as it appears in Nanoscale, 2013, Che-Ming Hu, Ronnie Fang, Brian Luk, Kevin Chen, Cody Carpenter, Weiwei Gao, Kang Zhang, and Liangfang Zhang, Nanomedicine, 2013, Santosh Aryal, Che-Ming Hu, Ronnie Fang, Diana Dehaini, Cody Carpenter, Dong-Er Zhang, and Liangfang Zhang, and Nanoscale, 2013, Ronnie Fang, Che-Ming Hu, Kevin Chen, Brian Luk, Cody Carpenter, Weiwe Gao, Shulin Li, Dong-Er Zhang, Weiyue Lu, and Liangfang
Zhang. The dissertation author was either the primary investigator or a major contributor and co-author of these papers.

**2.3.5 References**


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Chapter 3

Red Blood Cell Membrane-Coated Nanoparticles as “Nanosponges” for the Clearance of Pathologic Moieties
3.1 Clearance of Pore-Forming Toxins

3.1.1 Introduction

Detoxification treatments offer the potential to cleanse the body of virulence factors that underlie numerous health threats including bacterial infections, venomous injuries, and biological weaponry. Particularly, toxin-targeted anti-virulence therapy is emerging as a compelling strategy against infectious diseases [1, 2]. Existing detoxification platforms such as anti-sera [3], monoclonal antibodies [4], small-molecule inhibitors [5, 6], and molecularly imprinted polymers [7] target toxins’ molecular structures and thereby require toxin-specific custom synthesis for different disease treatments. Here we show a biomimetic toxin nanosponge that targets the membrane-disrupting mechanism of pore-forming toxins (PFTs) and functions as a toxin decoy in vivo. Consisting of polymeric nanoparticle-supported RBC membranes, these easily fabricated nanosponges readily take in the membrane-damaging toxins and divert them away from their cellular targets. In a mouse model, the nanosponges markedly reduce the toxicity of staphylococcal alpha-hemolysin (α-toxin) and thus improve the survival rate of toxin-challenged mice. This biologically inspired toxin nanosponge presents a new paradigm in detoxification treatments that has the potential to treat a variety of PFT-induced injuries and diseases.

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
It has been demonstrated that the inhibition of the pore-forming α-toxin can reduce the severity of *Staphylococcus aureus* infections [10], and similar PFT-targeted strategies have shown therapeutic potential against other pathogens including *Escherichia coli* [11], *Listeria monocytogenes* [12], *Bacillus anthracis* [13], and *Streptococcus pneumonia* [14]. Aside from their roles in bacterial pathogenesis, PFTs are commonly employed in venomous attacks by animals including sea anemones, scorpions, and snakes [15]. Over 80 families of PFTs have been identified, displaying diverse molecular structures and distinctive epitopic targets [16]. Despite these differences, the functional similarity among these toxins in perforating cellular membranes provides the design cue for an action mechanism-targeted detoxification platform with a broad applicability.

### 3.1.2 Experimental Methods

#### 3.1.2.1 Preparation of Toxin Nanospheres

RBC membrane vesicles were prepared through an extrusion approach using fresh RBCs collected from 6 week-old male ICR mice (Charles River Laboratories). PLGA polymeric cores were prepared using 0.67 dL/g carboxy-terminated 50:50 PLGA polymer (LACTEL Absorbable Polymers) through a solvent displacement process. The nanospheres were then prepared by fusing the RBC membrane vesicles onto the PLGA nanoparticles following a published protocol. The size of the nanospheres was 85 nm as obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer. The morphology of the toxin-
bound nanosponges was visualized using transmission electron microscopy (TEM). Briefly, 100 µg of nanosponges was incubated with 3 µg of *Staphylococcus aureus* α-toxin (Sigma Aldrich) for 15 min. A drop of the nanosponge/toxin solution (1 mg/mL) was deposited onto a glow-discharged carbon-coated grid, followed by washing with 10 drops of distilled water and staining with 1% uranyl acetate. The sample was then imaged using an FEI Sphera Microscope at 200kV. For preparing human RBC nanosponges, the RBCs were collected from whole human blood (Bioreclamation).

For lyophilization, nanosponges were prepared in 5% sucrose solution. The samples were subsequently frozen in a -80°C freezer. The frozen samples were then freeze-dried in a benchtop freeze dryer (Laboconco) overnight. Reconstitution of the lyophilized samples was performed by solubilizing the samples in water. Following reconstitution, the nanosponges were characterized for particle size, particle morphology, and neutralization capacity using DLS, TEM, and an RBC hemolysis assay, respectively.

### 3.1.2.2 Preparation of Control Nanoparticles

The PLGA nanoparticles, coated with polyethylene glycol (PEG, $M_w=2000$), were prepared via a nanoprecipitaton method. The liposomes consisting of 80 wt% of Egg PC (Avanti Polar Lipids) and 20 wt% of DSPE-PEG (2000)-carboxylic acid (Avanti Polar Lipids) were prepared via an extrusion method. The RBC membrane vesicles were prepared following the aforementioned protocol. Measured by DLS, the hydrodynamic diameters of the resulting PLGA nanoparticles, liposomes, and RBC
membrane vesicles were 90, 105, and 120 nm, respectively.

3.1.2.3 *In Vitro* Toxin Neutralization and Retention

200 µL of PBS (1X, pH = 7.2) solution containing 1 mg/mL of nanosponges, PLGA nanoparticles, liposomes, RBC membrane vesicles, or 250 µg/mL of sheep anti-α-toxin polyclonal antibodies (abcam) was prepared. 3 µg of α-toxin was then incubated with the respective formulations for 30 min, followed by addition into 1.8 mL of 5% purified mouse RBCs in PBS. After an additional 30 min of incubation, each sample was spun down at 14,000 rpm in a Beckman Coulter Microfuge® 22R Centrifuge for 10 min. The absorbance of hemoglobin in the supernatant was measured at 540 nm using a Tecan Infinite M200 Multiplate Reader to determine the degree of RBC lysis. All experiments were performed in triplicate. For the toxin retention study, the samples containing the nanoformulations and the α-toxin were filtered through a Sepharose® CL-4B size-exclusion column to remove unbound toxin, lyophilized and prepared in SDS sample buffer (Invitrogen), and then separated on a 4-12% Bis-Tris 10-well minigel in MOPS running buffer using a Novex® XCell SureLock Electrophoresis System (Invitrogen). The samples were run at 200 V for 50 min, and the polyacrylamide gel was then stained in SimplyBlue (Invitrogen) overnight for visualization. To quantify the retention of α-toxin, the band intensity at 34 kDa was analyzed by ImageJ and compared to a standard curve consisting of 0.3, 1, 3, and 9 µg of pure α-toxin.

3.1.2.4 *In Vitro* Toxin Absorption Study
200 µL of 1 mg/mL nanosponges in PBS was incubated with different amounts of α-toxin, streptolysin-O, and melittin for 30 min followed by incubation with 1.8 mL of 5% purified mouse RBCs for 30 min. Each sample was then spun down at 14,000 rpm for 10 min. The absorbance of the hemoglobin in the supernatant was measured at 540 nm to determine the degree of RBC lysis. As controls, solutions containing the same concentrations of the respective toxins without the nanosponges were tested in parallel. All experiments were performed in triplicate.

3.1.2.5 Cellular Uptake of the Nanosponges

10 µg of DMPE-rhodamine B (Avanti Polar Lipids) was incorporated into RBC ghosts collected from 1 mL of whole blood prior to preparing RBC membrane vesicles and the nanosponges. For the fluorescence imaging studies, the fluorescent nanosponges and RBC membrane vesicles were incubated with human umbilical vein endothelial cells (HUVEC) (ATCC #CRL-1730) at a concentration of 300 µg/mL in Medium 199 (Gibco) for 1 hr. The media was then aspirated and the cells were incubated in fresh media for additional 1 hr. Following the second incubation period, the cells were washed with PBS, fixed with 10% formalin (Millipore), mounted with DAPI-containing Vectashield® (Invitrogen), and imaged using a 60X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope.

3.1.2.6 Cellular Cytotoxicity of Sequestered Toxins

Human umbilical vein endothelial cells (HUVECs) were seeded in a 96-well
plate in Medium 199 at a density of $2 \times 10^4$ cells per well. To prepare the samples, nanosponges of different concentrations were incubated with varied amounts of $\alpha$-toxin, streptolysin-O, and melittin for 30 min. The samples were then added to the cells for 24 hr, following which the cells were washed and incubated in fresh media for 48 hr. Cell viability was then assayed using an MTT kit (Invitrogen) following the manufacturer’s instruction. In the conjoint mixture study, corresponding amounts of nanosponges were first added to HUVECs and $\alpha$-toxin was subsequently added to the cultures, followed by a cell viability test ($n=6$).

3.1.2.7 *In Vivo* Toxin Neutralization – Subcutaneous Route

12 $\mu$g/mL of $\alpha$-toxin was incubated with 0.67 mg/mL of nanosponges, PEG-PLGA nanoparticles, or RBC membrane vesicles in PBS for 15 min. A volume of 150 $\mu$L of the mixture was then injected subcutaneously into the flank region of 6 week-old female nu/nu nude mice (Charles River Laboratories). At day 3 after the injections the mice were imaged. Skin and muscles samples were cut at 5 $\mu$m and stained with hematoxylin and eosin (H&E) for histological analyses. On-site neutralization of $\alpha$-toxin by the nanosponges was conducted by subcutaneously injecting 50 $\mu$L of 36 $\mu$g/mL of $\alpha$-toxin solution, which was immediately followed by a 100 $\mu$L injection of 2 mg/mL nanosponges. The mice were imaged 3 days later for visualization of skin lesion formation.

3.1.2.8 *In Vivo* Detoxification Efficacy – Systemic Route

Nanosponges, RBC membrane vesicles, and PEG-PLGA nanoparticles at a
concentration 20 mg/mL and α-toxin at a concentration of 60 µg/mL were prepared beforehand in deionized water. For the pre-inoculation studies, 6 week-old male ICR mice were injected intravenously through the tail vein with 80 mg/kg (dose per body weight) of the nanoformulation followed by a 75 µg/kg injection of α-toxin 2 min later. For the post-inoculation studies, the mice were injected first with 75 µg/kg of α-toxin followed by 80 mg/kg of the nanoformulation 2 min later. The no treatment group was injected with 75 µg/kg of α-toxin solution only. The sample size for each group was 9 mice. The statistic $p$ values were obtained using the log-rank test.

3.1.2.9 Biodistribution of Nanosponge/α-Toxin Complex

200 µg of nanospones containing 0.05% (w/w) DiD probe (Invitrogen) in the polymeric cores was prepared and incubated with 3 µg of α-toxin. The mixture was subsequently filtered in a Sepharose® CL-4B column to purify out the unbound toxin. For the biodistribution study, 6 week-old male ICR mice were sacrificed 24 hr after intravenous injection of the fluorescent nanosponge/toxin complex via the tail vein. The liver, spleen, kidneys, lung, heart and blood were collected and homogenized. The fluorescence of the homogenate at 670 nm with an excitation wavelength of 630 nm was read using a Tecan Infinite M200 Multiplate Reader. The resulting signal was then multiplied by the corresponding organ weight to obtain the total organ fluorescence and the relative distribution of the nanosponge/toxin complex in each organ was calculated ($n = 6$). For the hepatotoxicity study, one group of mice was sacrificed on day 3 following the injection of the toxin-bound nanospomes and
another group was sacrificed on day 7. The livers were collected, sectioned, and stained with H&E for histological analyses.

### 3.1.3 Results and Discussion

In this study, a toxin nanosponge is constructed with a polymeric core wrapped in natural RBC bilayer membranes (Figure 3.1.1a). The RBC membrane shell provides an ideal mimicry to absorb a wide range of PFTs regardless of their molecular

![Figure 3.1.1 Nanosponge schematic and actual structures. (a) Schematic structure of toxin nanosponges and their mechanism of neutralizing pore-forming 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 nanosponge is comprised of a PLGA polymeric core and an RBC membrane shell. The sample was negatively stained with uranyl acetate prior to TEM imaging.](image-url)
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 [17]. Under transmission electron microscopy, the resulting nanosponges exhibited a core-shell structure approximately 85 nm in diameter (Figure 3.1.1b).

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 Figure 3.1.2a, 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 (Figure 3.1.2b). 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 and fixed nanosponge content (Figure 3.1.2c). Experiments with streptolysin-O (a pore-forming exotoxin produced by Streptococcus pyogenes [18]) and melittin (a membrane-disrupting peptide in bee venom [19]) showed similar patterns of reduced RBC hemolysis by the nanosponges, 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 membranes [20], the nanosponges and the RBC membrane vesicles were expected to absorb and retain the toxin after being run.

**Figure 3.1.2 In vitro characterizations.** (a) Centrifuged RBCs after incubation with α-toxin mixed in PBS, PLGA nanoparticle, liposome, RBC membrane vesicle, or nanosponge solution. (b) Quantification of the RBC hemolysis of samples in (a) by measuring the absorbance of the hemoglobin in the supernatant. Anti-α-toxin was used as a positive control. Each sample was examined in the absence of α-toxin to verify that the formulations did not induce hemolysis themselves. (c) Hemolytic activity of varying amounts of α-toxin with or without mixing with 200 μg of nanosponges. (d) SDS-PAGE to examine toxin absorption and retention by the nanoformulations. The same amount of free α-toxin was used as a reference. (e) Cellular uptake of RBC membrane vesicles (left) and toxin nanosponges (right) by cells (scale bar = 5 μm). (f) Dose-dependent neutralization of α-toxin’s toxicity against HUVECs by nanosponges.
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 $\alpha$-toxin respectively (Figure 3.1.2d). 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 $\alpha$-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.

The fact that the RBC membrane vesicles were able to absorb $\alpha$-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 membranes portrayed their different fates upon incubation with human umbilical vein endothelial cells (HUVECs) (Figure 3.1.2e). In the sample with RBC membrane vesicles, broadly distributed fluorescence was cast over the entire cellular area, which can be explained by the fusion of these nanoscale, unstable RBC vesicles with the cellular membranes [21]. In contrast, the nanosponges showed up within the intracellular region as distinct punctates similar to those often seen in the endocytosis of nanoparticles [22]. These findings help to justify the observed hemolysis results; the RBC membrane vesicles with bound $\alpha$-toxin likely fused with RBCs and thus failed to deter the toxin’s hemolytic activity. The nanosponges, however, were able to not only arrest but also lock in the toxins to keep
them away from other RBC membranes.

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 premixing with nanosponges (Figure 3.1.2f) and conjointly mixing with nanosponges. Similar detoxification properties of the nanosponges were observed with other PFT types including streptolysin-O and melittin. 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 Figure 3.1.2e. Based upon these 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.

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 (Figure 3.1.3a) and closer examination of the skin tissue showed necrosis, apoptosis, and inflammatory infiltrate of neutrophils with dermal edema (Figure 3.1.3b). 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 (Figure 3.1.3c). However,
mixing 100 µg of the nanosponges with the injected amount of α-toxin (toxin-to-nanosponge ratio ≈ 70:1) appeared to neutralize the toxin, as there was no observable damage on the mice (Figure 3.1.3d). The tissue samples showed normal epithelial structures in skin histology and intact fibrous structures with no visible infiltrate in the muscle histology (Figure 3.1.3e,f). In contrast, PEG-PLGA nanoparticles and RBC membrane vesicles failed to prevent the toxin damage in the skin.

**Figure 3.1.3 In vivo toxin neutralization capacities.** Mice injected with α-toxin alone: (a) skin lesions occurred 3 days following the injection; (b) histological sectioning with hematoxylin and eosin staining 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 damage of the muscle (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 fiber structures and the lack of inflammatory signs indicated no muscle damage (scale bar = 20 µm).

Finally, the systemic detoxification efficacy of the nanosponges was investigated. A bolus lethal dose of α-toxin (75 µg/kg), known to induce acute death in mice [23], was injected into mice through a 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. Figure 3.1.4a and 3.1.4b 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 (Figure 3.1.4a). In the post-inoculation treatment groups, the nanosponge injection

![Graph showing survival rates](image)

**Figure 3.1.4** *in vivo* detoxification efficacies. Survival rates of mice over a 15-day period following an intravenous injection of 75 µg/kg α-toxin: 80 mg/kg of nanospheres, RBC vesicles, or PEG-PLGA nanoparticles were administered intravenously 2 min either before (a) or after (b) the toxin injection. All injections were performed through the intravenous route via the tail vein (n = 9). (c) Biodistribution of α-toxin-bound nanospheres 24 hr after intravenous injection into mice (n = 6). (d) Liver histology with hematoxylin and eosin staining showing normal hepatocytes with no tissue damage on day 3 (left) and day 7 (right) following the injection of α-toxin-bound nanospheres.
remained beneficial to the overall survival, yielding a 56% mortality rate (p=0.0091, n=9), whereas the control formulations showed no survival advantage (Figure 3.1.4b). 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 (Figure 3.1.4c). 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 (Figure 3.1.4d). The lack of liver tissue damage suggests that the sequestered toxin was safely metabolized, likely through ingestion by hepatic macrophages.

3.1.4 Conclusions

Through the use of a rationally designed nanostructure, detoxification of α-toxin was achieved in this study. As α-toxin in the circulation can promote blood coagulation, systemic inflammation, and endothelial dysfunction by attacking platelets [24], monocytes [25], and endothelial cells [26], the nanosponges offer the potential to reduce toxin burden and relieve disease symptoms associated with the toxin [27]. The function of these nanosponges as an in vivo toxin decoy can be distinguished from the
current paradigm of detoxification treatments, where toxin antagonists rely primarily on structure-specific epitopic binding. The nanosponges address a common membrane-disrupting mechanism shared by a broad range of PFT proteins and thus have the potential to treat a variety of PFT-induced injuries and diseases. Moreover, the platform poses little risk of complication upon administration, as it is comprised entirely of biocompatible and biodegradable materials. The nanosponge platform introduces a unique strategy in injectable nanocarriers for biodetoxification [28], and as PFTs are one of the most common forms of toxin, the nanosponges have tremendous therapeutic implications in clinics.

3.1.5 References


3.2 Clearance of Autoantibodies

3.2.1 Introduction

Type-II immune hypersensitivities are driven by pathologic antibodies targeting self-antigens, either naturally occurring or due to exposure to an exogenous substance present on the cellular exterior or extracellular matrix. This disease type makes up many of the most prevalent autoimmune diseases including pernicious anemia, Grave’s disease, myasthenia gravis as well as autoimmune hemolytic anemia and immune thrombocytopenia [1-4]. In addition, they may occur after the administration of a new drug or following certain infections. Currently, therapies for these immune-mediated diseases remain relatively nonspecific via broad immune suppression [5]. For instance, comprehensive immune suppression through systemic glucocorticoids (i.e. prednisone, methylprednisolone), cytotoxic drugs (i.e. cyclophosphamide, methotrexate, azothioprine), and monoclonal antibodies (i.e. rituximab, belimumab, infliximab), dominate treatment regimens to prevent further tissue destruction [6-8]. Although this approach to therapy is effective for some patients in achieving remission, its efficacy remains variable and there is a well-established risk of adverse side effects, highlighting the need for better tailored therapies [9, 10].

The development of nanoparticle therapeutics has sparked new hope for the treatment of various important human diseases. Herein, we demonstrate the application of a biomimetic nanoparticle for the clearance of pathologic antibodies...
using an established murine model of antibody-induced anemia [11]. This disease may be idiopathic, called autoimmune hemolytic anemia (AIHA), or drug induced, called drug-induced anemia (DIA). In both cases, however, auto-antibodies attack surface antigens present on red blood cells (RBCs). Therapy for AIHA is relatively standardized with patients starting on systemic steroids and escalating to cytotoxic drugs and B-cell depleting monoclonal antibodies, and then possibly splenectomy based on patient response to therapy [12, 13]. The shortcoming of suppressing the immune system with drug-based therapies is the considerable iatrogenic risk associated with non-specific therapy and heightened susceptibility to severe infections following spleen removal [9, 10, 14]. DIA, which can be the result of drug-hapten antibodies or drug-independent auto-antibodies, is treated much the same way with the discontinuance of the offending drug and, much more often than in AIHA, performing blood transfusions [15, 16]. A subsequent limitation of repeated transfusions of packed RBCs, is that, although they revive tissue perfusion, they carry the risks of hemolytic transfusion reactions, the formation of alloantibodies, and iron toxicity [17-19].

It has previously been shown that mammalian cellular membrane, from both nucleated and non-nucleated cells, can be fused onto polymeric nanoparticle substrates to form stable core-shell structures [20, 21]. These particles have been shown to retain and present natural cell membrane and surface antigens [22], which bare the target epitopes involved in antibody-mediated cellular clearance found in AIHA and DIA. To demonstrate the interception of pathologic antibodies, we used RBC membrane-
cloaked nanoparticles, herein denoted RBC antibody nanosponges (RBC-ANS), to serve as alternative targets for anti-RBC antibodies and preserve circulating RBCs (Figure 3.2.1). Unlike conventional immune therapy, these biomimetic nanoparticles have no drug payload to suppress normal lymphocytes or immune effector cells. Additionally, unlike blood transfusions, which serve as a replacement therapy, the RBC-ANS serve to deplete circulating antibody levels, without contributing further toxic metabolites due to the hemolysis of transfused cells. Moreover, it has been demonstrated in animal models of autoimmune diseases that the primary target antigens can vary and shift over the course of the diseases [23]. Exploiting target cell

![Figure 3.2.1](image)

**Figure 3.2.1** Schematic representation of RBC antibody nanosponges (RBC-ANS) neutralizing anti-RBC antibodies (anti-RBC). (A) Anti-RBC opsonize RBCs for extravascular hemolysis, via phagocytosis, as observed in autoimmune hemolytic anemia and drug-induced anemia. (B) RBC-ANS absorb and neutralize anti-RBC, thereby protecting RBCs from phagocytosis.
membranes in their entirety overcomes the varying antigen specificities and presents a novel approach in intercepting the auto-reactive antibody mechanism of type-II immune hypersensitivity reactions.

3.2.2 Experimental Methods

3.2.2.1 Preparation of RBC Antibody Nanosponges

RBC-ANS were prepared following previously described methods [21]. Briefly, ~100 nm PLGA polymeric cores were prepared using 0.67dL/g carboxy-terminated 50:50 poly(DL-lactide-co-glycolide) (LACTEL Absorbable Polymers, Cupertino, CA) in a nanoprecipitation process. The PLGA polymer was first dissolved in acetone at a 10 mg/mL concentration. 1 mL of the solution was then added rapidly to 3 mL of water. For fluorescently labeled formulations, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodocarbocyanine perchlorate (DiD, excitation/emission = 644/665 nm, Life Technologies) was loaded into the polymeric cores at 0.1 wt%. The mixture was then stirred in open air for 1 hr and placed in vacuum for another 3 hr. The resulting nanoparticle solution was filtered using Amicon Ultra-4 Centrifugal Filters with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA). RBC membrane coating was then completed by fusing RBC membrane vesicles with PLGA particles via sonication using an FS30D bath sonicator at a frequency of 42 kHz and a power of 100 W for 2 min. The size and the zeta potential of the resulting RBC-ANS were obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer, which showed an average hydrodynamic diameter of ~100 and
~115 nm before and after the membrane coating process, respectively. The structure of RBC-ANS was examined with transmission electron microscopy (TEM). A drop of the RBC-ANS solution at 100 µg/mL was deposited onto a glow-discharged carbon-coated grid for 10 sec and then rinsed with 10 drops of distilled water. A drop of 1 wt% uranyl acetate stain was added to the grid. The sample was then imaged using an FEI 200 kV Sphera microscope. PEGylated PLGA nanoparticle (PEG-NP) was prepared using poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (PEG-PLGA) (Sigma Aldrich, St. Louis, MO). The PEG-PLGA polymer was dissolved in acetone at 10 mg/mL and 1 mL solution was added to 3 mL of water. For fluorescently labeled formulations, DiD was loaded into the polymeric cores at 0.1 wt%. The mixture was then stirred in open air for 1 hr and subsequently placed in vacuum for another 3 hr.

3.2.2.2 Binding Capacity and Specificity

Antibodies were first labeled with fluorescein isothiocyanate (FITC). Specifically, 100 µL of polyclonal rabbit anti-mouse RBC IgG (anti-RBC) (Rockland Antibodies and Assays, Gilbertsville, PA) at 10 mg/mL was mixed with 3.0 µL of 10 mg/mL FITC (Thermo Scientific, Rockford, IL) in dimethyl sulfoxide (DMSO). The mixture was incubated at room temperature in the dark for 1 hr and then run through a Sephadex® G-25 column (Sigma-Aldrich, St. Louis, MO) with de-ionized water to purify conjugated FITC-anti-RBC for subsequent experiments. For the antibody retention study, 250 µg of DiD-loaded RBC-ANS were combined with 6 serial
dilutions (500 µg, 250 µg, 125 µg, 31.25 µg, 7.81 µg, 1.95 µg) of FITC-labeled antibody in triplicate in a Costar® 96 well plate (Corning Unlimited, Corning, NY). Prior to incubation, the samples’ fluorescence intensities were measured using a Tecan Infinite® M200 reader (TeCan, Switzerland) to determine 100% signal of FITC (515 nm) and DiD (670 nm). Solutions were then incubated for 30 min at 37°C, followed by spinning down in a Legend 21R Microcentrifuge (Thermo Scientific, Rockford, IL) at 21,200 rpm for 5 min to collect pelleted RBC-ANS/anti-RBC complex. Samples were then washed 3 times in 1 mL water and their fluorescence intensity was re-measured to determine signal intensity of FITC in relation to DiD. All DiD signals were greater than 90% original ensuring minimal loss during washing steps. These steps were repeated at optimum concentrations of 250 µg DiD-loaded RBC-ANS or 250 µg DiD-loaded PEG-NP combined with 7.8 µg FITC-anti-RBC and 7.8 µg FITC-conjugated goat anti-mouse Fc IgG (anti-Fc) (Rockland Antibodies and Assays, Gilbertsville, PA) to determine specificity of RBC-ANS against anti-RBC as compared to control samples. To compare binding kinetics, serially diluted concentrations of FITC-anti-RBC (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL and 0.031 mg/mL) were incubated with a constant substrate concentration (0.25 mg/mL RBC-ANS or equivalent amount of RBC ghosts). Final values were normalized to the maximum binding observed at saturation. Binding capacity was expressed as a ratio of the fluorescent signals at saturation. To test binding capacity in serum, RBC-ANS was incubated with a saturated amount of FITC-anti-RBC in PBS or in the presence of 25 vol% fetal bovine serum (Thermo Scientific, Rockford, IL).
Values were expressed as a ratio of the fluorescent signals.

3.2.2.3 Competitive Binding Studies

RBC-ANS were prepared at 1 mg/mL in 1x Dulbecco’s phosphate buffered saline (PBS) (Gibco®, Grand Island, NY) and serially diluted to make 5 solutions (1 mg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 50 µg/mL) with 1x PBS as control. For the pre-incubation study, these solutions were combined with 50 µg anti-RBC and incubated for 2 min at 37°C before the addition of 1 mL of washed 5% mouse RBC solution. For the competitive co-incubation study, RBC-ANS and anti-RBC were added simultaneously to 1 mL of 5% RBC solution. Each experiment was done in triplicate. Samples were allowed to incubate for 10 min at 37°C and then washed three times in 1x PBS to thoroughly remove supernatant and collect RBC pellet. Flow cytometry was used to measure the FITC signal of the collected RBC population using a Becton Dickinson FACSCanto II. Flow cytometry data was analyzed using Flowjo software from Treestar.

3.2.2.4 RBC Agglutination Titration

The experiment was carried out per manufacturer (Rockport Antibodies and Assays) instructions. Briefly, 100 µL of anti-RBC (primary antibody) at 156 µg/mL was added to 100 µL of 5% washed RBC in 1x PBS along with 62.5 µL of RBC-ANS (250 µg, 100 µg, 50 µg, 25 µg or 0 µg) and incubated for 45 min at 37°C. The RBC solution was then washed three times by centrifuging the sample at 3,500 rpm for 1 min and exchanging the supernatant with 1x PBS each time. 100 µL of anti-Fc
(agglutinating secondary antibody) at 156 µg/mL was added to each sample, which was vortexed at 625 rpm for 5 min and then spun down at 3,500 rpm for 20 sec. The sample was then re-suspended using a pipette to disrupt the pellet. For the negative control, 100 µL of 6% BSA was used in lieu of secondary antibody. All samples were then viewed via light microscope at 10x magnification and imaged via mounted camera.

3.2.2.5 In Vivo Stability and Anti-RBC Binding

Following induction of anemia via intraperitoneal injection of anti-RBC, we randomly assorted 12 ICR mice into two groups of 6. Treatment group received 500 µg anti-RBC incubated with 5 mg RBC-ANS for 5 min at 37°C prior to injection and anti-RBC only mice received anti-RBC incubated in 1x PBS for 5 min. A control group of mice received injections of PBS only. A few drops of blood were collected from each mouse prior to injections on day 0 to establish starting blood counts and repeated on each day of the experiment. Samples were stored in Potassium-EDTA Microvette® tubes (Sarstedt, Newton, NC) and vigorously mixed to prevent clotting. Samples were then run the same day in a Drew Scientific Hemavet 950 (Erba® Diagnostics, Waterbury, CT) and RBC count, hemoglobin and hematocrit were recorded daily.

3.2.2.6 In Vivo Neutralization of Circulating Anti-RBC

Using the established intraperitoneal model for antibody delivery, 30 mice were randomized to three groups of 10. Each group of mice received a 100 µL
intraperitoneal injection of 2 mg/mL anti-RBC on days 0, 1, 2 and 3. The treatment group also received a tail vein intravenous injection of 200 µL of RBC-ANS (10 mg/mL) in 1x PBS within 30 min of intraperitoneal antibody delivery. The PEG-NP group received an equivalent intravenous dose of PEG-NP and the anti-RBC only group received 200 µL PBS via intravenous injection. RBC count, hemoglobin and hematocrit of each sample were recorded on days 0, 1, 2, 3, and 4.

3.2.2.7 Anti-RBC Autoimmune Study

Six weeks after the in vivo neutralization studies, serum was collected from 12 mice, 6 in each group, and a standard ELISA was performed. Washed ICR mouse RBCs were plated at 1 x 10⁶ RBCs per well onto a Costar® 96 well plate. 100 µL of collected serum was added in a sequence of six 1:5 dilutions. Horseradish peroxidase-conjugated goat anti-mouse antibody IgG (Biolegend, San Diego, CA) was used to probe for bound antibodies. The plate was developed using TMB (3,3’, 5, 5’-tetramethylbenzidine) substrate and 1 M HCl was used to stop the reaction. Absorbance was measured at 450 nm.

3.2.3 Results and Discussion

We constructed RBC-ANS following a previously reported protocol [21], in which purified mouse RBC membrane was mechanically extruded with 100 nm poly(lactic-co-glycolic acid) (PLGA) polymeric cores. The resulting nanoparticles revealed a core-shell structure under transmission electron microscopy (TEM) that
corresponds to unilamellar membrane coatings over the nanoparticle cores (Figure 3.2.2A). Physicochemical characterizations showed that upon RBC membrane coating, the nanoparticles had a ~20 nm increase in diameter and a 10 mV increase in surface zeta potential (Figure 3.2.2B), which were consistent with addition of RBC membrane to the particle surface [24]. Mixture of RBC-ANS with rabbit anti-mouse RBC IgG antibodies (anti-RBC) resulted in a diameter increase of ~26 nm, which can be attributed to the association of the IgG with the RBC-ANS. Such association also resulted in surface charge shielding as was evidenced by the 10 mV increase in the particle zeta potential (Figure 3.2.2B). To better investigate the binding capacity of RBC-ANS for anti-RBC, 250 µg of RBC-ANS was incubated with fluorescently labeled anti-RBC ranging from 1.75 µg to 500 µg. This titration assay demonstrated a plateau in particle-bound antibody fluorescent signal, or binding maximum, corresponding with an antibody mass of ~27 µg yielding a particle-to-antibody mass ratio of approximately 9:1 (Figure 3.2.2C). To evaluate the specificity of antibody-antigen binding, RBC-ANS were incubated with fluorescently labeled anti-RBC or goat anti-mouse Fc IgG (anti-Fc, as a negative control) for 10 min at 37°C. Figure 3.2.2D shows that significantly higher binding signal was observed between RBC-ANS and anti-RBC with very little nonspecific binding with anti-Fc. PEGylated PLGA nanoparticle (PEG-NP) incubated with anti-RBC served as a negative control and showed little retention of the antibody. Further, binding affinity of anti-RBC to RBC-ANS was near identical to that of an equivalent amount of RBC ghosts (Figure 3.2.2E). In the presence of serum proteins, RBC-ANS still retained greater than 60%
Figure 3.2.2 In vitro characterization of RBC-ANS. (A) TEM image demonstrated the core-shell structure of RBC-ANS (scale bar = 150 nm). (B) Size and surface zeta-potential of pure PLGA cores, RBC-ANS, and RBC-ANS bound with anti-RBC. (C) 250 µg of RBC-ANS incubated with 5 serial dilutions of fluorescent anti-RBC demonstrated particle saturation at ~ 27 µg of antibody, corresponding to ~9:1 particle/antibody mass ratio. (D) Equivalent amounts of RBC-ANS incubated with anti-RBC or anti-Fc demonstrated significantly greater specific binding as compared to nonspecific binding. The corresponding PEGylated PLGA nanoparticle (PEG-NP) incubated with anti-RBC served as a negative control. (E) Comparison of anti-RBC binding kinetics to a fixed amount of RBC-ANS or RBC ghosts. Inset represents relative binding capacity of RBC-ANS versus RBC ghosts at saturation. (F) Relative binding capacity of RBC-ANS in PBS versus in serum at saturation.

of their anti-RBC binding capacity compared with when the incubation was performed in buffer alone (Figure 3.2.2F). These results are indicative of relatively low nonspecific antibody-nanoparticle binding interactions and demonstrate the necessity
for antigen-antibody concordance to achieve neutralization.

To further characterize the binding stability and competitive binding capacity, we varied the amounts of RBC-ANS mixed with a constant amount of fluorescent anti-RBC in 5% RBC solution. To assess *in vitro* binding stability, RBC-ANS were pre-incubated with anti-RBC before mixing with 5% RBC solution (Figure 3.2.3A,B) and to test competitive binding capacity, RBC-ANS was added simultaneously with anti-RBC to 5% RBC solution (Figure 3.2.3C,D). After separating the RBCs from any unbound antibodies and RBC-ANS, we measured fluorescent signal associated with the RBCs using flow cytometric analysis. Both pre-incubation and co-incubation studies showed dose-dependent antibody neutralization. High binding ability and stability of RBC-ANS to anti-RBC was shown in the pre-incubation neutralization experiment, which demonstrated a ~60% reduction in RBC-bound antibodies with 100 µg of RBC-ANS and ~95% reduction with 1 mg RBC-ANS as compared to the negative control. Competitive co-incubation showed a reduction of RBC bound antibody signal by ~40% and ~80% at equivalent RBC-ANS doses, respectively. To correlate dose-dependence to clinically relevant diagnostic parameters, we completed an immunoglobulin agglutination test, which is equivalent to the qualitative Direct Antiglobulin Test that is a gold standard laboratory diagnostic often used in the diagnoses of AIHA [25, 26]. By varying the dose of RBC-ANS from 0 µg to 250 µg we demonstrated a dose-dependent neutralization of anti-RBC (primary antibody) as evidenced by the progressive decrease of RBC agglutination upon addition of an agglutinating secondary antibody (Figure 3.2.3E to I).
Figure 3.2.3 *In vitro* dose-dependent neutralization and stability of RBC-ANS/anti-RBC binding. (A) Flow-cytometry histograms of RBC-ANS (from left to right: 1000, 500, 250, 100, 50 and 0 µg) pre-incubated with 50 µg FITC-anti-RBC prior to mixing with 5% RBC solution demonstrated dose-dependent neutralization of anti-RBC. (B) Mean fluorescence intensity of samples in (A). (C) Flow-cytometry histograms of RBC-ANS (from left to right: 1000, 500, 250, 100, 50 and 0 µg) co-incubated with 50 µg FITC-anti-RBC and 5% RBC solution demonstrated dose-dependent, competitive neutralization of anti-RBC. (D) Mean fluorescence intensity of samples in (C). (E-I) Varying amounts of RBC-ANS (from E to I: 0, 25, 50, 100, and 250 µg) were co-incubated with 15.6 µg anti-RBC (primary antibody) and 5% RBC solution, followed by adding equivalent dose of anti-Fc (agglutinating secondary antibody). The samples were then imaged by light microscope at 10x magnification, demonstrating dose related inhibition of RBC agglutination by RBC-ANS.

After confirming *in vitro* that RBC-ANS could selectively bind anti-RBC, we next assessed the ability of the particles to durably retain antibodies *in vivo*. A previously described anemia disease model, induced through intraperitoneal injection of anti-RBC, was used in the study [11]. 500 µg of anti-RBC, a sufficient amount to induce acute anemia, was injected intraperitoneally into mice in the control group. Following the injection, the antibodies could diffuse across the peritoneal membrane, bind to circulating RBCs, and induce their clearance. Mice in the treatment group received the same dose of anti-RBC incubated beforehand for 5 min at 37°C with 5 mg of RBC-ANS. The relevant clinical parameters used for monitoring anemia
responses, including RBC count, hemoglobin level, and hematocrit, of each group were then assessed daily for 4 days. Comparison of the hematological parameters between the control and treatment groups showed that anti-RBC pre-incubated with RBC-ANS was less effective in inducing an anemic response (Figure 3.2.4). Mice in the treatment group possessed higher RBC count, hemoglobin content, and hematocrit throughout the duration of the study. All parameters were consistent with control mice that had not been challenged with anti-RBC but had their blood drawn daily. This result suggests that the anti-RBC was trapped by the RBC-ANS and was precluded from binding to circulating RBCs. The experiment demonstrates the feasibility of using target-cell mimicking nanoparticles to neutralize pathologic antibodies.

![Figure 3.2.4](image)

**Figure 3.2.4** *In vivo* binding stability of RBC-ANS and anti-RBC. ICR mice (n = 6) were intraperitoneally injected with 500 µg of anti-RBC pre-incubated with 5 mg RBC-ANS (red), 500 µg anti-RBC alone (blue) or PBS (black). Blood was collected daily to monitor (A) RBC count (million/µL), (B) hemoglobin (g/dL) and (C) hematocrit (%) of the mice.

To further validate the clinical relevance of the RBC-ANS, we administered daily injections of low-dose anti-RBC intraperitoneally to maintain a sustained level of the antibodies for anemia progression. RBC-ANS was injected intravenously with the aim of neutralizing the circulating antibodies and retarding anemia development. PEG-NPs of analogous size were also administered as a control. Mice were divided into
RBC-ANS plus anti-RBC, PEG-NP plus anti-RBC, and PBS plus anti-RBC groups. All mice received 100 µg of anti-RBC daily, through intraperitoneal injection, followed by an intravenous injection of 2 mg of either RBC-ANS, PEG-NP, or PBS daily for 4 days. Blood was obtained daily for the duration of the experiment to assess RBC count, hemoglobin and hematocrit. Starting from day 2, significant benefit in anemia related parameters was observed in the RBC-ANS-treated group as compared to PEG-NP control mice and vehicle only mice (Figure 3.2.5). The inability for PEG-NP to prevent anemia further supports the antigen-specific clearance of anti-RBC mediated by RBC-ANS as opposed to the preservation of RBCs via saturation of the mononuclear phagocyte system [27, 28]. To help assess the safety of the RBC-ANS approach, we also examined the autologous anti-RBC serum titers in mice 6 weeks following RBC-ANS treatment. ELISA assessment of autoantibodies against mouse RBCs showed no observable elevation of autologous anti-RBC responses in mice receiving RBC-ANS treatment as compared to the controls. The result confirms that the RBC-ANS/anti-RBC complex does not potentiate a humoral immune response against particle associated membrane antigens (Figure 3.2.6).

**Figure 3.2.5 In vivo neutralization of anti-RBC by RBC-ANS.** ICR mice (n = 10) were intraperitoneally injected with 200 µg anti-RBC on day 0, 1, 2 and 3. After each dose of the antibody, the mice received 2 mg RBC-ANS (red), PEG-NP (black) or PBS (blue) via tail vein intravenous injection. Blood was collected daily to monitor (A) RBC count (million/µL), (B) hemoglobin (g/dL) and (C) hematocrit (%) of the mice.
Autoimmune diseases, which include type-II, type-III, and type-IV immune hypersensitivity reactions, are known to attack almost every body tissue, make up over 50 diseases, and contribute to over $65 billion in healthcare costs annually [29]. AIHA was attributed to an autoantibody in 1904 by Donath and Landsteiner and the mechanism of extravascular hemolysis described by Metchinkoff in 1905, making it the first disease known to be caused by this mechanism [30]. Although the etiology is often idiopathic, it can be induced by drugs (cephalosporins, chemotherapies, quinines) as well as malignancies and viral infections [25, 26, 30]. Despite the differences in etiology, the final common disease pathway is the generation of antibodies against RBC membrane components, typically rhesus group and glycophorins, by a B-lymphocyte population that has lost self-tolerance to RBC surface-antigen(s) [31]. Most commonly, the pathologic mechanism is IgG-mediated attack that leads to the opsonization of RBCs for extravascular destruction by phagocytes. Alternatively, AIHA can also be induced by IgM-mediated attack on
RBCs, which causes RBC intravascular hemolysis via activation of the complement system [26, 30, 32, 33]. Even though autoantibodies have long been recognized to play a significant role in the disease, to our knowledge, therapies specifically directed at these pathologic antibodies were not previously explored. Existing AIHA therapy continues to target upstream disease mechanisms through reliance on broad immune suppression, blood transfusions, or splenectomy for refractory cases [12, 15]. This treatment paradigm holds true for other type-II immune hypersensitivities which are also managed with broad immune suppression such as using systemic glucocorticoids or cytotoxic drugs [3, 34].

Although efficacious for many patients, systemic steroids carry some of the highest risks of iatrogenic illness. Adverse effects of therapy include steroid myopathy, nosocomial infection, aseptic bone necrosis, accelerated osteoporosis, weight gain, metabolic derangements and Cushinoid appearance [35, 36]. In addition to these side effects, if steroid therapy fails, a patient may need to undergo surgery or systemic B-cell depletion with monoclonal antibodies or cytotoxic drugs with side effects of severe infection, antibody transfusion reactions and even the development of malignancies [10, 37]. Given this landscape, it is meaningful to continue development of innovative therapeutic strategies to manage disease burden while minimizing iatrogenic risk. Nanoparticles have already shown promise in reducing the risk of systemic toxicity of chemotherapy while increasing efficacy both in emerging literature and clinically [38-40]. We demonstrated that nanoparticles can be engineered to intercept binding between pathologic antibodies and their target cells to
favorably impact disease status. The particular approach offers a novel therapeutic intervention for type-II immune hypersensitivity reactions by targeting a final pathologic mechanism and presents an attractive alternative to broad-spectrum immune suppression.

Through the stabilization of biological membrane on a polymeric nanoparticle substrate, we unveiled the ability of cell membrane-coated nanoparticles to favorably serve as an antibody-decoy to improve disease parameters. Our results indicate the ability of RBC-ANS to effectively bind to anti-RBC and preclude their interaction with RBCs. The therapeutic potential of the proposed approach was validated in vivo with separate administrations of anti-RBC and RBC-ANS via intraperitoneal and intravenous route respectively. While the RBC-ANS reduced the antibody-mediated anemic response, equivalent doses of PEG-NP of analogous physicochemical properties failed to moderate the effect of the anti-RBC. The outcome of the in vivo study further indicates that the improved hematological status upon RBC-ANS treatment was mediated by specific antibody-antigen interaction, rather than particle-mediated saturation of phagocytic cells [27, 28]. We also established a lack of humoral response against the RBC membrane antigens following administrations of RBC-ANS and anti-RBC, which validates the safety of the approach as the RBC-ANS, in the presence of anti-RBC, did not potentiate an RBC autoantibody immune response. It has been previously reported that RBC membrane-coated nanoparticles are primarily metabolized in the liver [21, 41], where particulate metabolism generally promotes a tolerogenic immune response [42, 43]. In addition, several reports have shown that
antigen-laden polymeric nanoparticles, in the absence of immune adjuvants, are also immune-tolerizing [44-46]. While rigorous immunological studies in more faithful AIHA animal models are warranted, the present study exhibits the feasibility of applying cell membrane-coated nanoparticles for clearing pathologic antibodies. Adding promise to the approach is the demonstration of both nucleated and non-nucleated mammalian cell membranes that have been successfully stabilized by nanoparticle cores [20, 21]. This capacity to functionalize particles, with a variety of multi-antigen membranes, offers a new platform for the development of a robust line of therapies against additional type-II immune hypersensitivities.

3.2.4 Conclusions

Currently, the paradigm in targeted nanomedicine revolves around high-throughput screening for ligand-receptor recognition and the subsequent nanoparticle functionalization with specific targeting molecules [47, 48]. With regards to type-II immune hypersensitivity reactions, such a functionalization process could prove limited owing to the varying antigen specificities among pathologic antibodies from patient to patient such as in the case of AIHA [11, 23, 45]. Through the appropriate application of biological membranes, which possess the diversity of surface antigens susceptible to pathologic antibodies, biomimetic nanoparticles can be prepared in a facile manner for selective immunomodulation. Further, drug-loaded cores or those made from different materials, such as metallic or inorganic nanoparticles, can be employed to create multifunctional formulations. We believe the demonstration of
pathophysiological-inspired nanoengineering serves as a valuable prototype for additional therapeutic advances, offering the opportunity for selective disease intervention while minimizing iatrogenic risks associated with many traditional drug-based therapies.

Chapter 3, in full, is a reprint of the material as it appears in *Nature Nanotechnology*, 2013, Che-Ming Hu, Ronnie Fang, Jonathan Copp, Brian Luk, and Liangfang Zhang, and *Proceedings of the National Academy of Sciences USA*, 2014, Jonathan Copp, Ronnie Fang, Brian Luk, Che-Ming Hu, Weiwei Gao, Kang Zhang and Liangfang Zhang. The dissertation author was a major contributor and co-author of these papers.

### 3.2.5 References


Chapter 4
Cell Membrane-Coated Nanoparticles for Immune Modulation
4.1 Anti-Toxin Vaccination

4.1.1 Introduction

Toxoid vaccines—vaccines based on inactivated bacterial toxins—are routinely used to promote antitoxin immunity for the treatment and prevention of bacterial infections [1-4]. Following chemical or heat denaturation, inactivated toxins can be administered to mount toxin-specific immune responses. However, retaining faithful antigenic presentation while removing toxin virulence remains a major challenge and presents a trade-off between efficacy and safety in toxoid development. Here we show a nanoparticle-based toxin-detainment strategy that safely delivers non-disrupted pore-forming toxins for immune processing. Using erythrocyte membrane-coated nanoparticles and staphylococcal α-hemolysin, we demonstrate effective virulence neutralization via spontaneous particle entrapment. As compared to vaccination with heat-denatured toxin, mice vaccinated with the nanoparticle-detained toxin showed superior protective immunity against toxin adverse effects. We find that the non-disruptive detoxification approach benefited the immunogenicity and efficacy of toxoid vaccines. We anticipate the reported study to open new possibilities in the preparation of antitoxin vaccines against the many virulence factors that threaten public health.

Immunization against bacterial pore-forming toxins (PFTs) has much clinical relevance as these membrane-damaging proteins underlie the virulence mechanisms in numerous public health threats, including infections by pathogenic *Escherichia coli,*
Helicobacter pylori, and Staphylococcus aureus [5-7]. Toward maximizing PFT vaccine efficacy, a major challenge lies in establishing non-toxic toxoids that preserve the antigenic epitopes of the toxin proteins. Conventional toxoid preparation via protein denaturation possesses significant shortfalls that can lead to inadequate vaccine potency and poor quality control [8]. Chemical- and heat-mediated detoxification processes are difficult to fine-tune, and they are known to disrupt a protein’s tertiary structure, causing altered antigenic presentation and compromised immunogenicity [9, 10]. Although immunostimulatory adjuvants have been applied to raise the potency of denatured antigens, risk of reactogenicity and other adverse effects may occur and thus render the option less desirable [11].

Efforts to improve vaccine potency and safety have given rise to alternative toxin-inactivation strategies that subvert a toxin’s virulence while preserving its native structure. For instance, non-virulent toxin mutants, prepared from recombinant protein engineering, have shown strong therapeutic efficacy in animal models and have entered human clinical trials [12-15]. These encouraging results suggest that toxoid preparation may benefit from minimally disruptive detoxification methods that better preserve a toxin’s epitopic expression.

4.1.2 Experimental Methods

4.1.2.1 Preparation of RBC Membrane-Coated NPs

RBCs were collected from 6 week-old male ICR mice (Charles River Laboratories) by centrifuging the whole blood at 800 x g for 5 min followed by
hypotonic treatment to remove interior contents. The RBC ghosts were extruded through 100 nm polycarbonate porous membranes using an extruder (Avanti Polar Lipids) to prepare RBC membrane vesicles with a diameter of approximately 100 nm. Poly(lactic-co-glycolic acid) (PLGA) polymeric cores were prepared using 0.67 dL/g carboxy-terminated 50:50 PLGA polymer (LACTEL Absorbable Polymers) through a solvent displacement process. The RBC-membrane-coated nanoparticles were then prepared by fusing the RBC membrane vesicles onto the PLGA nanoparticles through an extrusion process (1 mg PLGA per 1 mL blood). The size and the surface zeta potential of the resulting particle vectors were obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer. To examine the core-shell structure of the particle vectors by transmission electron microscopy (TEM), a drop of the particle solution (1 mg/mL) was deposited onto a glow-discharged carbon-coated grid. After 1 min, it was washed with 10 drops of distilled water, stained with 1% uranyl acetate, and then imaged using an FEI Sphera Microscope at 200 kV.

4.1.2.2 Preparation of Nanotoxoid(Hla)

3 µg of Hla (Sigma-Aldrich) was incubated with particle vectors ranging from 0 to 200 µg in 100 µL of aqueous solution for 15 min. The mixtures were then filtered through a Sephacryl® 200-HR size-exclusion column (Sigma-Aldrich) to remove the unbound Hla. For protein analysis, nanotoxoid(Hla) solution was lyophilized, prepared in lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen), and separated on
a 4-12% Bis-Tris 10-well minigel in MOPS running buffer using a Novex® Xcell SureLock Electrophoresis System (Invitrogen). The resulting gel was transferred to a nitrocellulose membrane, stained with a primary sheep anti-Hla polyclonal antibody (Abcam) and a secondary rabbit anti-sheep IgG HRP conjugate (Millipore). The membrane was then subjected to ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks). 3 µg of free Hla was prepared alongside the nanotoxoid(Hla) solution as a standard for comparison. Subsequent characterizations were carried out using a ratio of 3 µg Hla to 200 µg particle vectors.

For the release kinetics study, 1 mL of samples at 1 mg/mL was dialyzed against PBS buffer using a Float-A-Lyzer G2 device with a molecular weight cut-off of 100 kDa (Spectrum Laboratories). At 0, 1, 2, 6, 12, 24, and 48 h time points, 20 µL of retentate was collected and analyzed by western blot analysis using known amounts of Hla as standards. Endotoxin analysis was performed using an LAL Chromogenic Endotoxin Quantitation Kit (Pierce) following manufacturer’s instructions. Samples were suspended at 1 mg/mL and run alongside the provided endotoxin standards.

For immunogold staining, nanotoxoid(Hla) solution (1 mg/mL) was deposited onto a glow-discharged grid. After 1 min, the grid was washed with 10 drops of 2% BSA (Sigma-Aldrich), incubated with one drop of polyclonal rabbit anti-Hla antibody (Sigma-Aldrich) for 1 min, washed with 2% BSA, incubated with one drop of gold-labeled anti-rabbit IgG (Sigma-Aldrich) for 1 min, and then washed with distilled water. Imaging was conducted using an FEI Sphera Microscope at 200 kV.
4.1.2.3 Cellular Uptake

100 µg of Hla was labeled with Alexa 488 dye using the Alexa Fluor® 488 Microscale Protein Labeling Kit (Life Technologies) following the manufacturer’s instructions. The fluorescently labeled Hla was then used to prepare nanotoxoid(Hla). The resulting nanotoxoid(Hla) was incubated with mouse dendritic cells derived from the bone marrow of ICR mice at a concentration of 400 µg/mL in culture media. For fluorescent imaging, the cellular membranes were stained with 4 µg of DMPE-rhodamine B (Avanti Polar Lipids). Following 1 h of incubation, the media was aspirated and the cells were washed with PBS, fixed with 10% formalin (Millipore), mounted with DAPI-containing Vectashield® (Invitrogen), and imaged using a 60X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope.

4.1.2.4 Live Whole-Body Imaging

Nanotoxoid(Hla) was prepared with PLGA cores labeled fluorescently with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (Invitrogen). 150 µL of PBS solution containing 200 µg of fluorescent nanotoxoid(Hla) loaded with 3 µg of Hla was injected subcutaneously to the neck region of mice (n=6). At the designated time points, mice were anesthetized with isoflurane (Piramal Healthcare) and imaged using a Xenogen IVIS 200 system. Fluorescent signal intensities were normalized across time points and heat maps were overlaid on bright field images.
4.1.2.5 Skin Damages by Different Hla Preparations

For heat-treated Hla, 20 µg/mL of Hla in PBS in an eppendorf tube was submerged in a water bath equilibrated at 70°C. Both 30 and 60 min of heating were performed to inactivate the toxin. For nanotoxoid(Hla) preparation, 20 µg/mL of Hla was incubated with 1.33 mg/mL of RBC-membrane-coated nanoparticles in PBS for 15 min. An untreated free Hla solution at 20 µg/mL in PBS was prepared as control. For the skin damage study, ICR mice were shaved to remove the hair on their back. 150 µL each of untreated Hla, 30 min heat-treated Hla, 60 min heat-treated Hla, and nanotoxoid(Hla) formulations were then injected into the superficial dorsal skin of mice. For the apoptosis assay, the skin was removed from the injection site 24 h following the injection for tissue processing. O.C.T.-embedded slides were stained with ApopTag® Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore) for microscopy. Histological analyses were performed with skin removed 24 h following the injections; the skin sections were stained with hematoxylin and eosin (H&E) and visualized under a microscope.

4.1.2.6 Cellular Viability and Apoptosis Studies

The cytotoxicity of Hla, nanotoxoid(Hla), heat-treated Hla (60 min treatment), and the blank particle vectors (denoted as nanotoxoid(-)) were assessed against mouse dendritic cells with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega). 24 h prior to the toxicity study, the cells were seeded (2 x 10^4) in 96-well plates. The culture medium was then replaced with 200 µL of fresh
medium containing 3 µg of Hla, 200 µg of nanotoxoid(Hla) detaining 3 µg of Hla, 3 µg of heat-treated Hla, or 200 µg of nanotoxoid(-). Following 48 h of incubation, MTT reagent was applied to the cells following a protocol provided by the manufacturer. PBS buffer was used as a negative control.

To detect for induction of cellular apoptosis, dendritic cells were incubated with nanotoxoid(Hla) at a final concentration of 4 mg/mL particle vector and 60 µg/mL Hla in media. Two sets of experiments were conducted, one for 24 h and another for 72 h. At 2 h before each experimental endpoint, a set of untreated cells was incubated with 5% hydrogen peroxide (Sigma-Aldrich) as a positive control. All cells were collected using Accutase solution (eBioscience), washed in media containing divalent cations, and then stained with propidium iodide (PI) (Biolegend) and FITC-labeled Annexin V (Biolegend) following manufacturer’s instructions. Cells were then washed again in media and analyzed by flow cytometry using a Becton Dickinson FACSCanto II. Annexin V versus propidium iodide plots were gated based on untreated cells, with the PI^{low}/Annexin V^{low} quadrant representing viable cells, the PI^{low}/Annexin V^{high} quadrant representing early apoptotic cells, and the PI^{high}/Annexin V^{high} quadrant representing late apoptotic or dead cells.

4.1.2.7 Nanotoxoid(Hla) Vaccination in Mice

Vaccination was conducted through subcutaneous injections at the neck of the mice following two different schedules: a prime only on day 0, and a prime plus two booster vaccinations on day 7 and day 14. For the nanotoxoid(Hla) vaccination, 150
µL of PBS solution containing 200 µg of nanotoxoid(Hla) loaded with 3 µg of Hla was administered. For the heat-treated Hla vaccination, 150 µL of PBS solution containing 3 µg of Hla was administered, in which the Hla was pre-incubated at 70°C for 60 min to denature the toxin proteins. For alum formulations, 75 µL of PBS containing 3 µg of heat-denatured Hla (60 min) was allowed to adsorb to 75 µL alum (Pierce) for 1 h before injection.

4.1.2.8 Hla-Specific Antibody Response Studies

The cocktail plasma collected on day 21 from 8 mice vaccinated with prime-boost nanotoxoid(Hla) were used as the primary immunostaining against the protein content of Hla and nanotoxoid(-) separated by SDS-PAGE. Goat anti-mouse IgG HRP conjugate (Millipore) was used as the secondary staining. The stained nitrocellulose membrane was subjected to ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks), followed by western blotting analysis. Quantitative anti-Hla IgG titers were analyzed by ELISA using Hla-coated plates. To obtain anti-Hla IgG titer values, the lowest plasma dilution at which the optical density reading ≥ 0.5 was used. For avidity determination, samples were incubated with 6M urea for 10 min to remove weakly bound IgG prior to addition of the secondary antibody. Statistical analysis was performed with GraphPad Prism using an unpaired two-tailed t-test. To evaluate the functionalities of the anti-Hla IgG titers, 25 µL of the plasma was incubated with 1 µg of Hla for 10 min. The mixture was subsequently mixed with 200 µL of 5% purified mouse RBCs. After 30 min of
incubation, the sample mixtures were spun down at 14,000 rpm in a Beckman Coulter
Microfuge® 22R Centrifuge for 10 min. The absorbance of hemoglobin in the
supernatant was measured at 540 nm using a Tecan Infinite M200 Multiplate Reader
to determine the degree of RBC lysis.

4.1.2.9 Autoimmune Studies

Mice were immunized with nanotoxoid(Hla) on the prime-boost schedule as
described above. Serum was collected on day 6 and 21 after initial injection,
corresponding to the peak production of IgM, and IgG/IgA antibody isotypes,
respectively. Autoimmune titers were analyzed using RBC-coated plates. Goat anti-
mouse IgM-HRP, IgA-HRP, or IgG-HRP (Southern Biotech) was used as the
secondary antibody for detecting the presence of auto-antibodies against RBCs. For
RBC counts, whole blood collected from mice on day 6 or day 21 was analyzed using
a Drew Scientific Hemavet.

4.1.2.10 Protective Immunity via Systemic Challenge

Hla at a concentration of 60 μg/mL was prepared in PBS. Mice from the
different vaccination groups were injected intravenously through the tail vein with 120
μg/kg of Hla. Following the injections, the mice were monitored for survival for 15
days. The sample size for each group was 10 mice. Statistical analysis was performed
with GraphPad Prism using a log-rank significance test.

4.1.2.11 Protective Immunity via Subcutaneous Challenge
Hla at a concentration of 100 µg/mL was prepared in PBS. Mice from the
different vaccination groups were injected subcutaneously on their back with 50 µL of
the Hla solution. Following the injections, the mice were monitored for skin lesions
for 14 days. The sample size for each group was 6 mice.

4.1.3 Results and Discussion

To inactivate PFTs without protein denaturation, we neutralise toxins' membrane-damaging activity using a red blood cell (RBC) membrane-coated nanoparticle system [16, 17]. The particle-stabilized biomembranes serve to anchor PFTs without compromising the toxins’ structural integrity (Figure 4.1.1a,b). Using staphylococcal α-hemolysin (Hla) as a model toxin and mixing it with preformed RBC membrane-coated nanoparticles, we first demonstrated the facile preparation of the Hla-loaded nanotoxoids, denoted as nanotoxoid(Hla). Upon removal of the unbound toxin, Hla retention within the nanotoxoids was examined using western blotting (Figure 4.1.1c). The results indicate that 200 µg of the particle vectors was sufficient to absorb 3 µg of Hla, translating to an Hla-to-particle ratio ≈ 40:1. At this toxin loading level no observable changes in the particle’s size, structure, and zeta potential were detected and endotoxin was determined to be undetectable. A release kinetics study further demonstrated that no subsequent toxin release from the nanotoxoid(Hla) occurred over a period of 48 h (Figure 4.1.1d), indicating that the Hla was safely locked into the particle vector. By labelling Hla with a fluorescent dye, it was observed that the nanotoxoid(Hla) enabled uptake of toxins by immune cells. Upon
incubation with mouse dendritic cells, fluorescence microscopy revealed the nanotoxoid(Hla) as distinct features within the cells (Figure 4.1.1e), which is

Figure 4.1.1 Schematic and in vitro characterizations. (a) Schematic preparation of nanoparticle-detained toxins, denoted as nanotoxoid, consisting of substrate-supported RBC membranes into which pore-forming toxins (PFTs) can spontaneously incorporate. (b) TEM visualization of the particle vectors with uranyl acetate staining (scale bar = 80 nm). (c) Western blotting results to verify the retention of 3 µg of staphylococcal α-hemolysin (Hla) by varying amounts of the particle vectors using 3 µg of free Hla as a standard (SD). (d) Release of toxin from the Hla-loaded nanotoxoids, denoted as nanotoxoid(Hla), over time in PBS buffer. Red circles indicate nanotoxoid(Hla) and black squares indicate free Hla. Error bars represent standard errors of the mean. (e) Uptake of nanotoxoid(Hla) by a mouse dendritic cell (scale bar = 10 µm). The cell is membrane stained with DMPE-rhodamine B (red) and nuclei stained with DAPI (blue). FITC-labelled Hla (green) was used to monitor the toxin uptake. (f) Live, whole-body fluorescent imaging of nanotoxoid(Hla) at 1 h after subcutaneous administration.
consistent with the pattern of endocytic uptake frequently observed with nanoparticle vectors [18, 19]. Through direct engulfment of Hla into the digestive endolysosomal compartments, the nanoparticle-facilitated cellular endocytosis precludes the toxin’s perforating attack on cellular membranes and thus allows non-disrupted toxin to be delivered for immune processing. Subcutaneous injection of the nanotoxoid(Hla) to mice showed lymphatic drainage of the particles over time, suggesting the ability of the particle vector to deliver Hla efficiently to the immune system \textit{in vivo} (Figure 4.1.1f).

To assess the toxin inactivation in the nanotoxoid, 200 µg of nanotoxoid(Hla) detaining 3 µg of Hla was injected into the superficial dorsal skin of mice. Untreated free Hla, Hla heated at 70°C for 30 min, and Hla heated at 70°C for 60 min were tested in parallel at an equivalent Hla dose. 24 h following the injections, the skin was sectioned to evaluate the toxicity of the different formulations using both \textit{TUNEL} assay and haematoxylin and eosin (H&E) assay (Figure 4.1.2a). It was revealed that untreated Hla caused a significant level of cellular apoptosis and observable lesions in the skin. Toxin neutralization by heat was shown to be time-dependent, as Hla heated for 30 min remained damaging to the skin, whereas 60 min of heating removed the toxin virulence. For the skin injected with the nanotoxoid(Hla), the epithelial structure remained intact and no cellular apoptosis was observed outside of hair follicles. Visual examination of mice subcutaneously administered with the nanotoxoid(Hla) also showed no observable lesions 48 h following the injections. This lack of toxin damage was observed consistently in 10 mice per test group. \textit{In vivo} imaging of
nanotoxoid(Hla) showed that the particles were eventually cleared over time as there was no trace of the particles after 2 weeks. To further confirm that the nanotoxoid can safely present the toxin antigens to antigen-presenting cells, an *in vitro* cytotoxicity

![Figure 4.1.2](image_url)

**Figure 4.1.2** Hla virulence neutralization. (a) Free Hla, heat-treated Hla (30 min), heat-treated Hla (60 min), and nanotoxoid(Hla) were injected into the superficial dorsal skin of mice. 24 h following the injections, the skin was removed and examined for apoptosis using a TUNEL assay. Histological analyses were performed with H&E stained skin 48 h following the injections (Scale bar = 400 µm). (b) Toxicity of different Hla formulations against dendritic cells derived from mice. The cells were incubated for 48 h with Hla, heat-treated Hla (60 min) and nanotoxoid(Hla) at 15 µg/mL Hla concentration. Cellular viability was assessed using an MTT assay (n=6). (c) Induction of dendritic cell apoptosis by nanotoxoid(Hla) at 60 µg/mL Hla concentration 72 h after initial incubation. Propidium iodide and Annexin V staining were analysed by flow cytometry (n=6). All errors bars represent standard errors of the mean.
test was conducted on mouse dendritic cells. Upon 48 h of incubation in 15 μg/mL of Hla content, untreated Hla resulted in 70% decrease in cell viability, whereas both heat-denatured Hla (60 min treatment) and nanotoxoid(Hla) showed no reduction (Figure 4.1.2b). Flow cytometric analysis showed that the nanotoxoid(Hla) did not induce any additional underlying cellular apoptosis compared to untreated cells over a 72 h period (Figure 4.1.2c). These results confirm the safety and reliability of the nanotoxoid-based toxin inactivation, which allows non-denatured toxin antigens to interact with tissues and immune cells with the same level of safety as those treated with extended heating.

Next, immunization studies were conducted to examine the vaccine potential of the nanotoxoid(Hla). An emphasis was placed on the elicitation of neutralizing antibodies, which are the hallmark of antitoxin immunity. Two vaccination schedules were performed: a prime only on day 0, and a prime on day 0 followed by two booster vaccinations on day 7 and day 14. To verify that the nanotoxoid(Hla) could indeed elicit Hla-specific antibodies, the plasma from 8 mice immunized with nanotoxoid(Hla) on a prime-boost schedule was pooled together and used as the primary immunostain for western blotting analysis. The results confirmed the presence of anti-Hla immunoglobulin G (IgG) (Figure 4.1.3a). It is important to note that this cocktail plasma showed no detectable cross-reactivity with the protein content on the blank particle vectors, denoted as nanotoxoid(-). To further confirm the lack of an autoimmune response, we demonstrated that mice immunized with nanotoxoid(Hla) exhibited no serum immunoglobulin M, immunoglobulin A, or immunoglobulin G
antibodies against RBC proteins. RBC counts were at a similar level as compared to unvaccinated mice, indicating no induction of autoimmune anaemia (Figure 4.1.3b). The results indicate that the RBC membranes provide a non-immunogenic substrate for toxin-detainment, enabling the cargo antigens to be processed selectively without raising potential complications associated with anti-vector immunity [20, 21].

The ability of the nanotoxoid(Hla) to elicit anti-Hla antibodies was then quantified. Determination of antibody responses on day 21 showed that the nanotoxoid(Hla) induced significantly higher Hla-specific antibody titres as compared to the heat-treated Hla (60 min treatment). Enhancements by 7- and 15-fold (geometric mean) under the prime only (p = 0.0951, n=7) and prime-boost vaccinations (p=0.0077, n=7), respectively (Figure 4.1.3c). The increased titre level of nanotoxoid(Hla) was sustainable in a time course study over 150 days (Figure 4.1.3d). Because previous report [22] and our own study have indicated that adjuvants do not significantly boost titre responses for immunizations using denatured Hla, no adjuvant was used in this study in order to best highlight the nanotoxoid(Hla) platform as an effective mode of attenuating toxicity while preserving immunogenicity of toxins. The nanotoxoid(-) vector alone did not induce a detectable antibody response (Figure 4.1.3c), and the vector mixture with heat-treated Hla showed negligible enhancement in titre levels as compared to heat-treated Hla alone. These results highlight the challenge in raising immunogenicity of denatured toxins and demonstrate the benefit of using detained, but undenatured antigens for vaccination.
Figure 4.1.3 Antibody responses. (a) Hla-specific antibody responses were verified in the nanotoxoid(Hla)-vaccinated mice through coomassie staining (left panel) and western blotting (right panel). Blank particle vector, denoted as nanotoxoid(-), was used as a control. (b) RBC counts of mice immunized with nanotoxoid(Hla) (n=6). (c) Anti-Hla IgG titres at day 21 (n=7). Black lines indicate geometric means. Anti-Hla titres from mice vaccinated with non-toxin loaded particle vectors (nanotoxoid(-)) were monitored as controls (triangle). (d) Time course of anti-Hla IgG titres in unvaccinated mice (black triangle) and mice immunized with nanotoxoid(Hla) (prime + boost; red circle) or nanotoxoid(Hla) (prime only; blue circle) (n=7). (e) Avidity index of the anti-sera from immunized mice binding to Hla toxin was quantified (n=7). (f) An RBC haemolysis assay was performed to verify the presence of functional titres (n=7). All error bars represent standard errors of the mean.
The nanotoxoid(Hla) also helped to improve antibody affinity to the targeted toxin, as was evidenced by the increased avidity of the antibody titres (Figure 4.1.3e). To confirm that the antibody titres were capable of neutralizing Hla, an RBC haemolysis assay was conducted. Hla was mixed with the plasma from vaccinated mice and then incubated with purified mouse RBCs. The plasma from unvaccinated mice was used as a control and the haemolytic activity of Hla was determined by measuring the amount of released haemoglobin. The results showed that plasma from nanotoxoid(Hla)-vaccinated mice was more potent at neutralizing the toxin. For the prime-boost nanotoxoid(Hla) vaccination group, 25 µL of plasma was sufficient to completely inhibit the haemolytic activity of 1 µg of Hla (Figure 4.1.3f). We speculate that the improved titre responses from nanotoxoid(Hla) were due to epitopic preservation of the undenatured toxin as well as to well documented benefits of particulate antigen vectors, which can enhance antigen uptake and processing by immune cells [23, 24].

Finally, the protective immunity bestowed by the nanotoxoid(Hla) vaccine was evaluated by subjecting the vaccinated mice to both systemic and subcutaneous toxin administration. 21 days following the prime vaccination, the mice received a lethal bolus dose of Hla at 120 µg/kg through intravenous tail vein injection. This toxin dose resulted in 100% mortality within 2 h in the unvaccinated group. For the mice receiving the prime vaccination only, the benefit of the nanotoxoid(Hla) over the heat-treated Hla was evident as the survival rate increased from 10% to 50% (n=10). Moreover, nanotoxoid(Hla) boosters further improved the survival rate to 100% while
a 90% survival rate was achieved by the heat-treated Hla vaccine with boosters (n=10) (Figure 4.1.4a). Since Hla has also been identified as a key factor in necrotizing skin infections [25], a subcutaneous toxin administration was also conducted to further evaluate the vaccine’s effectiveness in mounting immunity in skin tissue. Observation of skin damage following a 50 µL subcutaneous injection of 100 µg/mL Hla on the back region showed reduced lesion areas in all vaccinated mice, reflecting the presence of extravascular IgG that diminished the subcutaneous toxin threat. With the prime only vaccination, the nanotoxoid(Hla) vaccine conferred modestly stronger protection than the heat-treated Hla. Following booster vaccinations, however, the nanotoxoid(Hla) resulted in complete eradication of the toxin damage. In contrast, the mice vaccinated with the heat-treated Hla boosters remained susceptible to the necrotizing toxin (Figure 4.1.4b).

Figure 4.1.4 Protective immunity. Unvaccinated mice (black) and mice vaccinated with heat-treated Hla (prime; blue square), nanotoxoid(Hla) (prime; blue circle), heat-treated Hla (prime + boost; red square), or nanotoxoid(Hla) (prime + boost; red circle) received intravenous or subcutaneous administration of Hla. (a) Survival rates of mice over a 15-day period following intravenous injections of 120 µg/kg Hla on day 21 via the tail vein (n=10). (b) Skin lesion size comparison following subcutaneous injections of 5 µg of Hla on day 21. The lesion size was measured for 14 days following the administration. Error bars represent standard errors of the mean (n=6).
4.1.4 Conclusions

Compared to the commonly used protein denaturation approach to achieve toxin vaccination, the non-disruptive preparation described here yielded a nanotoxoid with stronger immunogenicity and superior efficacy. Refinement of this approach can benefit from the unique strengths of nanoparticle-based immunoengineering [26, 27], in which vaccine targeting to lymphoid organs and processing by antigen-presenting cells can be enhanced through nanovector designs [24, 28, 29]. Moving forward, this nanotoxoid platform can be generalized for other types of cellular membrane-coated particles [30] and for the neutralization and delivery of other potent toxins to create a broad range of safe and effective antitoxin vaccines. For clinical test and use in humans, it can be applied either on the basis of patients’ blood types following a cross-match test or using donor blood from type O- individuals, as in the case of blood transfusion.

4.1.5 References


4.2 Anticancer Vaccination

4.2.1 Introduction

Nanoparticle technology has enabled a wide array of improvements in the treatment of cancer, ranging from improved efficacy in cancer drug delivery [1, 2] to enhanced immunogenicity of cancer vaccines [3]. More recently, there has been interest in leveraging the increased understanding of biological systems to make nanoparticles with new and enhanced functionalities [4-6]. The inspiration behind this pursuit lies in the fact that natural components have evolved very specific functions over time, and these are difficult to fully recreate with synthetic materials. The unique properties displayed by different cells types can, in large part, be attributed to the complex antigenic profile present on their membranes. Identification of individual membrane factors has enabled researchers to enhance synthetic platforms with biomimetic features for specific applications such as advanced drug delivery [7, 8]. Membrane-bound tumor antigens have also been used to train the immune system to recognize and fight cancers [9], and cancer-mimicking particulate vectors decorated with these surface antigens have been prepared to improve vaccine potency [10, 11]. These examples demonstrate the vast potential of biomolecule functionalized nanoparticles, the design and development of which continue to benefit from increased understanding of cell surface markers.

Among the different bio-inspired strategies, utilization of cellular membrane material for nanoparticle preparation presents a unique top-down approach that offers
the advantage of being able to completely replicate the surface antigenic diversity of source cells [12, 13]. This approach, which involves fashioning cellular membranes directly into nanoparticle form, circumvents the labor-intensive proteomics [14] and the engineering hurdles behind multivalent nanoparticle functionalization [15]. Using this emerging approach, researchers have successfully created nanoparticles possessing many desirable features. Examples include RBC membrane-cloaked nanoparticles with long-circulating properties [12], stem cell-derived ‘nanoghosts’ with cancer targeting capabilities [16], and leukocyte membrane-coated silica microparticles with the ability to traverse endothelium [17]. This strategy has also given rise to novel applications that transcend traditional therapeutic motifs, such as in the case of toxin nanosponges that exploit particle-stabilized RBC membranes to neutralize pore-forming virulence factors [18, 19]. The work done thus far in this field has provided a glimpse of the new possibilities enabled by cell membrane-derived nanoformulations.

In this study, we functionalize biodegradable polymeric nanoparticles with cancer cell membrane and demonstrate that the resulting particles possess an antigenic exterior closely resembling that of the source cancer cells. These cancer cell membrane-coated nanoparticles (CCNPs) provide a platform that can be used towards the development of two distinct anticancer modalities (Figure 4.2.1). We demonstrate that these CCNPs allow membrane-bound tumor-associated antigens, together with immunological adjuvants, to be efficiently delivered to professional antigen presenting cells to promote anticancer immune responses. In addition, as the CCNPs possess the
same cell adhesion molecules as their source cells, they are shown to exhibit source cell-specific targeting that reflects the homotypic binding mechanism frequently observed in cancers [20, 21]. Given the polymeric core’s capacity to encapsulate therapeutic payloads [22], the platform presents an innately targeted nanocarrier for cancer drug delivery. Ultimately, the studies presented demonstrate the broad applicability of the cell membrane coating approach for nanoparticle functionalization, which bridges the properties of natural membrane components with those of synthetic nanomaterials.

**Figure 4.2.1** The cancer cell membrane-coated nanoparticle (CCNP). Schematic representation of CCNP fabrication and two potential applications. Cancer cell membrane along with its associated antigens is collected from source cancer cells and coated onto polymeric nanoparticle cores made of poly(lactic-co-glycolic acid) (PLGA) polymer. The resulting CCNPs can then be used to deliver tumor-associated antigens to antigen presenting cells or to homotypically target the source cancer cells.
4.2.2 Experimental Methods

4.2.2.1 Cancer Cell Membrane Derivation

B16-F10 mouse melanoma cells (ATCC: CRL-6475) and MDA-MB-435 human melanoma cells (provided by Dr. Erkki Ruoslahti) were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (Invitrogen). To harvest membrane, cells were grown in T-175 culture flasks to full confluency and detached with 2 mM ethylenediaminetetraacetic acid (EDTA, USB Corporation) in phosphate buffered saline (PBS, Invitrogen) and washed in PBS three times by centrifuging at 500 × g. The cells were suspended in a hypotonic lysing buffer consisting of 20 mM Tris-HCl pH = 7.5 (Mediatech), 10 mM KCl (Sigma Aldrich), 2 mM MgCl₂ (Sigma Aldrich), and 1 EDTA-free mini protease inhibitor tablet (Pierce) per 10 mL of solution and disrupted using a dounce homogenizer with a tight-fitting pestle. The entire solution was subjected to 20 passes before spinning down at 3,200 × g for 5 min. The supernatant was saved while the pellet was resuspended in hypotonic lysing buffer and subjected to another 20 passes and spun down again. The supernatants were pooled and centrifuged at 20,000 × g for 20 min, after which the pellet was discarded and the supernatant was centrifuged again at 100,000 × g. The pellet containing the plasma membrane material was then washed once in 10 mM Tris-HCl pH = 7.5 and 1 mM EDTA. The final pellet was collected and used as purified cancer cell membrane for subsequent experiments.
4.2.2.2 CCNP Synthesis and Characterization

To synthesize CCNPs, a previously reported extrusion approach was used. Briefly, poly(lactic-co-glycolic acid) (PLGA, Lactel Absorbable Polymers) cores approximately 80-90 nm in size were prepared using a nanoprecipitation method. To prepare cancer cell membrane vesicles, membrane material derived as described above was physically extruded through a 400 nm polycarbonate membrane for 11 passes. The resulting vesicles were then coated onto PLGA cores by co-extruding vesicles and cores through a 200 nm polycarbonate membrane. A ZEN 3600 Zetasizer (Malvern) was used to take dynamic light scattering (DLS) measurements for the characterization of particle size and zeta potential. Samples were suspended in water at 1 mg mL\(^{-1}\). All measurements were done in triplicate at room temperature. Transmission electron microscopy (TEM) imaging was carried out by first glow discharging carbon-coated 400 square mesh copper grids (Electron Microscopy Sciences). Particles at 1 mg mL\(^{-1}\) were left on the grid for 1 min before being washed off with 10 drops of water. Grids were then negatively stained with 3 drops of 1% uranyl acetate (Sigma Aldrich). Excess solution was wicked away with absorbent paper and the samples were imaged using a Tecnai G2 Sphera (FEI) microscope at 200 kV.

4.2.2.3 Cancer Cell Membrane Protein Characterization

For protein characterization by SDS-PAGE, all samples were prepared at a final protein concentration of 1 mg mL\(^{-1}\) in lithium dodecyl sulfate (LDS) loading buffer (Invitrogen) as measured by a BCA assay (Pierce). CCNPs were purified by
centrifugation at 18,000 × g to pellet the coated particles but not free vesicles or protein. Samples were heated to 70°C for 10 min and 20 µL of sample was loaded into each well of a NuPAGE Novex 4-12% Bis-Tris 10-well minigel (Invitrogen) in MOPS running buffer (Invitrogen) in an XCell SureLock Electrophoresis System (Invitrogen) based on the manufacturer’s instructions. Protein staining was accomplished using SimplyBlue (Invitrogen) and destained in water overnight before imaging. For western blot analysis, protein was transferred to Protran nitrocellulose membranes (Whatman) using an XCell II Blot Module (Invitrogen) in NuPAGE transfer buffer (Invitrogen) per manufacturer’s instructions. Membranes were probed using antibodies against pan-cadherin (C3678, Sigma Aldrich), Na⁺/K⁺-ATPase (A01483, GenScript), gp100 (EP4863(2), Abcam), histone H3 (Poly6019, Biolegend), cytochrome c oxidase (A01396, GenScript), or glyceraldehyde 3-phosphate dehydrogenase (GT239, GeneTex) along with either horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Poly4053, Biolegend) or anti-rabbit IgG (Poly4064, Biolegend). Films were developed using ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks).

4.2.2.4 CCNP Optimization and Stability

To optimize the membrane ratio, CCNPs were synthesized at membrane-to-core weight ratios ranging from 0.125 to 4 mg of protein per 1 mg of PLGA. PLGA cores with no membrane were used as a control. Particle sizes were measured in triplicate by DLS right after synthesis, after adjusting the solution to 1X PBS using a
2X PBS stock, and over time for a period of 15 days in 1X PBS. To obtain dendritic cells for the fluorescent co-localization study, cells from the femur bone marrow of 6-week old female C56BL/6 mice (Charles River Laboratories) were derived and plated on non-treated 100 mm petri dishes at $1 \times 10^6$ cells per plate in Iscove’s Modified Dulbecco’s Media (IMDM, Mediatech) supplemented with 10% FBS, 2 mM L-Glutamine (Invitrogen), 50 µM β-mercaptoethanol (Invitrogen), penicillin-streptomycin, and 20 ng mL$^{-1}$ recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF, Biolegend). On days 3, 6, and 8 after plating, half of the media was replaced with fresh media. On day 10 the cells were collected by scraping, washed in PBS, and plated at 50% confluency on 8-well Lab-Tek II CC2 chamber slides (Nunclon). CCNPs were synthesized using membrane labeled with NHS-fluorescein (Pierce). For the labeling, 10 µL of 10 mg mL$^{-1}$ NHS-fluorescein in dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to 500 µg of cancer cell membrane vesicles. After 1 h, the membrane was coated onto PLGA cores loaded with 0.1 wt% 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine (DiD, Invitrogen) and the resulting CCNPs were purified using a Sepharose CL-4B (Sigma Aldrich) size exclusion column. CCNPs were incubated at a final concentration of 100 µg mL$^{-1}$ with the cells for 2 h. Afterwards, the cells were washed 3 times with PBS, fixed with 10% formalin (Millipore) and mounted with DAPI-containing Vectashield (Invitrogen). Imaging was done using a 100X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope, and software deconvolution was done post-acquisition.
4.2.2.5 Dendritic Cell Maturation and T-Cell Activation

To test dendritic cell maturation, bone marrow-derived dendritic cells were cultured as described above and plated at 50% confluency in tissue culture-treated 6-well plates. CCNP samples were incubated in triplicate at a final concentration of 80 µg (polymer weight) per 1 mL media at a membrane-to-core ratio of 0.5 mg protein per 1 mg PLGA. Monophosphoryl lipid A (MPLA, Sigma) was incorporated with the CCNPs at 1 wt% of the polymer weight by gently agitating the sample in a Fisher Scientific FS30D bath sonicator. Samples were allowed to incubate for 48 h before the cells were collected by scraping and washed 3 times using PBS with 2% bovine serum albumin (BSA, Sigma Aldrich). The cells were then immunostained with FITC-conjugated anti-CD11c (N418, Biolegend) and Alexa-647-conjugated anti-CD40 (HM40-3, Biolegend), anti-CD80 (16-10A1, Biolegend), or anti-CD86 (GL-1, Biolegend) for 20 min on ice in the dark. Cells were washed twice more in PBS with 2% BSA before analysis. Flow cytometry data was collected using a Becton Dickinson FACSCanto II and analyzed using Flowjo software from Treestar.

To test for T-cell stimulation, bone marrow-derived dendritic cells were first plated at 75% confluency in 24-well plates. Dendritic cells were pulsed with CCNP samples in triplicate for 24 h at a concentration of 80 µg (polymer weight) per 1 mL media. Splenocytes were derived from a 10-week old female transgenic pmel-1 mouse (The Jackson Laboratory) and plated with the pulsed dendritic cells at a density of $2 \times 10^6$ cells per well. Cells were co-cultured for 3 days. At each 24 h timepoint, a small sample of media was taken from the supernatant and replaced with fresh media.
Interferon gamma (IFNγ) concentration was assayed using an IFNγ ELISA Ready-SET-Go! kit (eBioscience) per the manufacturer’s instructions. At the conclusion of the study, the cells were gently washed with PBS and fixed in 10% formalin. Brightfield phase contrast imaging was performed using a Nikon TE300 inverted microscope.

4.2.2.6 Homotypic Targeting Studies

For flow cytometric analysis, MDA-MB-435 or HFF-1 (ATCC: SCRC-1041) human foreskin fibroblast cells were plated at 50% confluency in 6-well plates. RBCNPs were synthesized as previously reported[1]. All samples contained PLGA cores fluorescently labeled with 0.1 wt% DiD. CCNPs, RBCNPs, or bare PLGA cores were incubated with the cells in triplicate at a final concentration of 150 µg (polymer weight) per 1 mL media for 30 min. Afterwards, the wells were washed 3 times with PBS before incubating for 1 h in cell culture media. The cells were then detached with trypsin-EDTA solution (Invitrogen), washed twice with media, and data was collected using a Becton Dickinson FACSCanto II. Analysis was done using Flowjo software from Treestar.

For fluorescent imaging, MDA-MB-435 cells were plated at 75% confluency on 8-well Lab-Tek II CC2 chamber slides. DiD-labeled CCNPs, RBCNPs, or bare PLGA cores were incubated with the cells at a final concentration of 150 µg (polymer weight) per 1 mL media for 30 min. Afterwards, the wells were washed 3 times with PBS before incubating for 1 h in cell culture media. The wells were then washed 3 times with PBS, fixed with 10% formalin and mounted with DAPI-containing
Vectashield. Imaging was done using a 20X objective on a Keyence BZ-9000 Fluorescence Microscope.

4.2.3 Results and Discussion

In order to fabricate CCNPs, purified cancer cell membrane was first collected. Using B16-F10 mouse melanoma cells as a model cancer cell line, membrane derivation was achieved by emptying harvested cells of their intracellular contents using a combination of hypotonic lysing, mechanical membrane disruption, and differential centrifugation. With the collected membrane, cancer cell membrane vesicles were then formed by physical extrusion through a 400 nm porous polycarbonate membrane. Concurrently, poly(lactic-co-glycolic acid) (PLGA), an FDA-approved polymer, was used to prepare cores through a well-studied nanoprecipitation process [23]. Briefly, PLGA dissolved in acetone was added into water as an anti-solvent, resulting in the spontaneous formation of polymeric nanoparticle cores. The acetone was then allowed to evaporate. In order to coat the PLGA cores with cancer cell membrane, the two components were co-extruded through a 200 nm porous polycarbonate membrane. As measured by dynamic light scattering, the membrane vesicles were under 300 nm in hydrodynamic diameter. Upon fusion of the membrane vesicles with the polymeric cores, the final CCNPs were approximately 110 nm in size (Figure 4.2.a,b). Zeta potential measurements suggested successful coating, as the surface charge of the PLGA cores increased to approximately the level of the membrane vesicles after being coated (Figure 4.2.c).
Membrane coating around the polymeric core was visualized using transmission electron microscopy (TEM), and the final CCNPs were spherical in shape and exhibited a core-shell structure upon negative staining with uranyl acetate (Figure 4.2.2d). It is believed that this coating occurs as the faithful translocation of the membrane bilayer structure onto the nanoparticle surface, resulting in a right-side-out conformation that allows the membrane to retain its ability to interact with the environment while providing stability [13]. From the TEM micrographs, the membrane coating also appeared to be consistent as there were few uncoated PLGA cores.

![Figure 4.2.2 Physicochemical characterization.](image)

(a) Size intensity curves of PLGA cores, cancer cell membrane vesicles, and CCNPs measured by dynamic light scattering (DLS). (b) Hydrodynamic size of PLGA cores, cancer cell membrane vesicles, and CCNPs. Bars represent means ± SD (n=3). (c) Surface zeta potential of PLGA cores, cancer cell membrane vesicles, and CCNPs. Bars represent means ± SD (n=3). (d) Transmission electron micrographs of (i) a PLGA core, (ii) a cancer cell membrane vesicle, (iii) a CCNP, and (iv) multiple CCNPs. Samples were negatively stained with uranyl acetate. All scale bars = 100 nm.
Analysis of the protein content on the CCNPs was carried out to confirm successful functionalization of the nanoparticles with cancer cell membrane antigens. Gel electrophoresis followed by protein staining showed modulation of the protein profile when comparing the purified membrane material to raw cell lysate (Figure 4.2.3a). CCNPs were purified by centrifugation in order to separate out the coated particles from free vesicles, and their protein profile matched closely with that of the purified membrane. To confirm the presence of specific antigens on the CCNPs, western blotting analysis was conducted on a series of membrane and intracellular protein markers (Figure 4.2.3b-g). There was a significant enrichment of cadherins and Na⁺/K⁺-ATPase, both plasma membrane-specific markers, in the final CCNP formulation. Glycoprotein 100 (gp100), a widely reported transmembrane protein that is a tumor-associated antigen for melanoma [9], was also present on the purified CCNPs. Conversely, protein markers for the nucleus, mitochondria, and cytosol were lowly present on the final nanoparticles, demonstrating preferential retention of membrane antigens through the fabrication process.

In order to optimize the membrane coating, CCNPs were synthesized at membrane-to-core weight ratios ranging from 0.125 to 4 mg of membrane protein per 1 mg of PLGA particles (Figure 4.2.4a). At lower membrane-to-core ratios, a significant increase in the hydrodynamic diameter was observed when the particles were transferred to 1X PBS. This suggested incomplete coverage, which exposes the surfaces of the cores to charge screening [24], resulting in low stability in ionic buffers. This effect was even more pronounced after two weeks of storage, as samples
with membrane coverage lower than 0.25 mg of protein per 1 mg of PLGA aggregated significantly. The lowest membrane-to-core ratio at which the particles maintained size stability over time was around 1 mg of protein per 1 mg of PLGA. At this ratio,

Figure 4.2.3 Membrane antigen characterization. (a) SDS-PAGE protein analysis of cancer cell lysate, cancer cell membrane vesicles, and CCNPs. Samples were run at equal protein concentration and stained with Coomassie Blue. (b-g) Western blotting analysis for membrane-specific and intracellular protein markers. Samples were run at equal protein concentration and immunostained against membrane markers including (b) pan-cadherin, (c) Na⁺/K⁺-ATPase, and (d) gp100, and intracellular markers including (e) histone H3 (a nuclear marker), (f) cytochrome c oxidase (a mitochondrial marker), and (g) glyceraldehyde 3-phosphate dehydrogenase (a cytosolic marker).
there was minimal size increase throughout the two weeks of observation (Figure 4.2.4b). To further test for the long-term storage capacity of the CCNPs, the particles were lyophilized in 5 wt% sucrose solution. Upon reconstitution in water, the particles exhibited a hydrodynamic size consistent with that prior to freeze-drying.

Figure 4.2.4 Optimization and stability. (a) Hydrodynamic size as measured by DLS of CCNPs at varying membrane protein to PLGA weight ratios right after synthesis, after adjusting to 1X PBS, and after storage for 15 days in PBS. Bars represent means ± SD (n=3). (b) Stability of CCNPs made at a membrane-to-core ratio of 1 mg protein per 1 mg PLGA versus bare PLGA cores over time. * = particles before adjusting to 1X PBS. Symbols represent means ± SD (n=3). (c) Co-localization of PLGA cores and cancer cell membrane upon cellular uptake. CCNPs were fabricated with PLGA cores loaded with DiD (red channel) and membrane labeled with FITC (green channel). The nucleus was stained with DAPI (blue channel). All channels were deconvolved by software to eliminate out of focus fluorescent signal. Yellow color represents co-localization of the core and the membrane signals. Scale bar = 10 µm.

Integrity of the membrane coating around the polymeric cores was studied by observing the CCNPs upon cellular uptake. PLGA cores were loaded with a far-red fluorescent dye while the membrane proteins were tagged with a green fluorescent
dye. *In vitro* imaging studies demonstrated that the CCNPs were efficiently taken up by bone marrow-derived mouse dendritic cells, allowing for the intracellular delivery of membrane protein antigens. This suggests a stabilization of the relatively fusogenic membrane material upon coating onto the nanoparticle substrate [18], which facilitates uptake through endocytic pathways [24, 25]. Upon uptake, the fluorescent signals from the cores and the membrane exhibited a high degree of co-localization (Figure 4.2.4c). The overlapping fluorescent signals suggest that the core-shell structure of the CCNPs was stable and remained intact upon cellular endocytosis.

The ability of the nanoparticles to deliver tumor antigens and induce dendritic cell maturation was tested by incubating CCNPs made using B16-F10 membrane with dendritic cells derived from C57BL/6 mice. Monophosphoryl lipid A (MPLA), an FDA-approved lipopolysaccharide derivative that binds to toll-like receptor 4 (TLR-4) [26], was incorporated with the CCNPs as an adjuvant to boost the immune response against the lowly immunogenic antigens found on the cancer membrane coating and had little effect on the final physicochemical characteristics of the particles. When incubated with dendritic cells, nanoparticle uptake was visualized by the darkening of the cells as observed under phase contrast microscopy. The CCNPs by themselves, which were derived from a cell line that originates from the exact same mouse strain as the dendritic cells, did not induce additional maturation compared with blank controls (Figure 4.2.5a-c). This lack of response indicates limited immunogenicity of the syngeneic cancer cell membrane material despite being formulated into nanoparticle form, which is consistent with the fact that the B16-F10 cell line has been
reported to be lowly immunogenic in C57BL/6 mice [27]. Only upon incorporation of MPLA with the CCNPs was a significant response observed, with upregulation of the maturation markers CD40, CD80, and CD86 in the dendritic cells.

![Image](image_url)

**Figure 4.2.5** Delivery of tumor-associated antigens. (a-c) Maturation of dendritic cells. Dendritic cells were pulsed with blank solution, CCNPs derived from B16-F10 cells, or CCNPs with MPLA as an adjuvant for 48 h. Afterwards the cells were immunostained with antibodies against CD11c as a dendritic cell marker and (a) CD40, (b) CD80, or (c) CD86 as a maturation marker and analyzed by flow cytometry. For the analysis, CD11c+ cells were gated first before gating on the maturation markers. (d-e) Antigen-specific T-lymphocyte stimulation. Dendritic cells pulsed with blank solution, CCNPs, or CCNPs with MPLA for 24 h were then co-cultured with splenocytes derived from pmel-1 transgenic mice. (d) Phase contrast microscopy image of cells 72 h after co-culture. T-lymphocytes can be seen clustering around dendritic cells. Scale bar = 25 µm. (e) Specific response against the presentation of a melanoma-associated gp100 antigen was assayed using an ELISA for IFNγ at 24, 48 and 72 h after co-culturing. UD = undetectable by the ELISA (bars were made visible to distinguish samples). Bars represent means ± SD (n=3).

To confirm that the observed maturation was also coupled with the presentation of tumor antigen-specific epitopes, pulsed dendritic cells were co-cultured with splenocytes derived from transgenic pmel-1 mice. T-cell receptors in
pmel-1 mice have been genetically engineered to be specific towards a gp100 epitope [28], and consequently their cytotoxic T-lymphocytes can only be stimulated by dendritic cells that properly present the tumor-associated antigen. Phase contrast microscopy showed significant crowding of T-lymphocytes around dendritic cells pulsed with CCNPs incorporated with MPLA (Figure 4.2.5d), which reflects the correct presentation of gp100 antigen fragments on the surface of the antigen presenting cells [29]. Quantification of the cytokine interferon-gamma (IFNγ), an indicator of cytotoxic T-lymphocyte stimulation, further demonstrated that CCNPs with MPLA were able to successfully elicit an antigen-specific response (Figure 4.2.5e). Taken together, the data indicates that the adjuvanted CCNPs were able to correctly deliver tumor-associated antigens to dendritic cells for immune processing, which allowed for the subsequent stimulation of tumor antigen-specific T-cells.

To demonstrate the ability of CCNPs to homotypically target cancer cells for drug delivery applications, membrane from the human cell line MDA-MB-435, which has been extensively studied for its homotypic aggregation properties [20, 21], was collected and coated onto fluorescent dye-loaded PLGA cores. The membrane and particles were suspended in divalent cation chelator-containing solution throughout the process in order to prevent Ca²⁺-dependent homotypic binding between particles [30, 31]. Using fluorescence microscopy, it was demonstrated that incubation of these CCNPs with cultured MDA-MB-435 cells in vitro resulted in significantly increased uptake as compared to both bare PLGA cores and red blood cell membrane-coated nanoparticles (RBCNPs) (Figure 4.2.6a). The decreased uptake of the RBCNPs
compared to bare PLGA cores was consistent with previously reported findings using macrophages [13] and reflects the modulation of cell-to-particle interactions upon membrane functionalization. In order to quantify the difference in uptake, flow cytometric analysis was carried out, and the results indicated that the MDA-MB-435 membrane coating enabled approximately 40-fold and 20-fold increases in uptake.

![Figure 4.2.6](image)

**Figure 4.2.6** Homotypically-targeted delivery vehicle. (a) Fluorescent imaging of MDA-MB-435 cells incubated with PLGA cores, RBC membrane-coated PLGA nanoparticles (RBCNPs), or CCNPs coated with membrane derived from MDA-MB-435 cells. All samples were loaded with DiD (red channel). After 30 min incubation, particles were washed away and the cells were incubated for another 1 h in fresh media before imaging. Nuclei were stained with DAPI (blue channel). All images were taken with the same exposure time for all channels and subjected to the same post acquisition normalization. Scale bar = 50 µm. (b) Flow cytometric analysis of MDA-MB-435 cells incubated with blank solution, PLGA cores, RBCNPs or CCNPs. All particles were loaded with DiD fluorescent dye and subject to the same incubation conditions as in (a). At the conclusion, cells were detached with trypsin-EDTA for analysis. Histograms are representative of experiments done in triplicate. (c) Quantification of the mean fluorescence intensities of the histograms in (b). Bars represent means ± SD (n=3).
compared with RBCNPs and bare PLGA cores, respectively (Figure 4.2.6b,c). To further demonstrate that the binding effect was specific to the membrane coating, a heterotypic human foreskin fibroblast cell line was targeted as a negative control, and it was observed that MDA-MB-435 CCNPs exhibited little increased uptake compared to the bare PLGA cores. These results indicate that coating of nanoparticles with MDA-MB-435 membrane can preferentially increase the affinity of the particles to the source cancer cells, a functionality that can be attributed to the transference of cell adhesion molecules with homotypic binding properties.

The coating of cellular membrane onto nanoparticles as described in this work allows for particle functionalization with an exceptionally high concentration of antigenic material relevant for the replication of specific biological functions. The concomitant removal of nuclear components from the final formulation helps to alleviate the safety concerns regarding genetic material, especially in the case of sourcing membrane from tumorigenic cancer cells. As was observed with previously reported RBCNPs, the nanoparticle core and cancer cell membrane mutually benefit each other when combined into a core-shell structure [12, 18]. The PLGA cores, which can be used to load a wide array of cargoes, are unstable in physiological buffer, but can be stabilized when coated with sufficient membrane. The membrane, which is unstable when unsupported, becomes stabilized upon coating onto a nanoparticle substrate. For CCNPs, the enhanced stability of the two components together can be taken advantage of to promote efficient delivery and internalization of
either antigenic or therapeutic material, enabling their use for the design of new anticancer nanotherapeutics.

Therapeutic cancer vaccines represent an emerging anticancer regimen that utilizes tumor-associated antigens to promote anti-tumor immune responses. One example is Provenge™, the first FDA-approved cancer vaccine, which is a treatment based on the *ex vivo* pulsing of autologous dendritic cells with prostatic acid phosphatase, an antigen associated with a subset of prostate cancers [32]. Application of the gp100 or MART-1 tumor-associated antigens, combined with immunological adjuvants, has also shown promise in treating melanomas [9, 33]. While encouraging, vaccination approaches based on a single tumor-associated antigen can be inadequate when facing the high heterogeneity and mutation rate of cancer cells [34]. Much progress in multi-antigen-based vaccination has also been made using cell lysates to prime the immune system against the complete antigenic profile of tumors [34, 35]. In these formulations, however, treatment efficacy can be compromised by the large presence of intracellular, housekeeping proteins that divert focus away from the relevant antigens, which compose a small percentage of the total protein [36]. The present CCNP formulation was shown to be inherently enriched in membrane components, allowing for the delivery of tumor-associated antigens while bypassing the labor-intensive protocols required for individual antigen identification [37, 38]. Also of note is that stabilization of the cancer cell membrane on a nanoparticle substrate facilitated cellular uptake of membrane proteins and likely contributed to enhanced antigen processing by dendritic cells, an important consideration in vaccine
design. Such antigen stabilization by nanoparticles was also previously reported to benefit antigen processing *in vivo*, as the size of the particles can be tuned for enhanced localization to immune organs such as the lymph nodes [39].

The CCNP platform also allows facile coupling of cancer membrane antigens with immunological adjuvants. In the present study, a TLR-4 activator, MPLA, was used as a model adjuvant to raise the immunogenicity of the CCNP formulation. The correct priming of dendritic cells was demonstrated using gp100-specific spleen-derived lymphocytes, as dendritic cells pulsed with adjuvant-incorporated CCNPs showed visibly higher interactions with the lymphocytes and yielded significantly higher secretion of IFN$\gamma$ (Figure 4.2.5d,e), a hallmark of antigen-specific immunity development [40]. The cargo-loading capacity of the PLGA polymeric core can also be applied to carry other adjuvants such as CpG oligodeoxynucleotides and poly(I:C) to further enhance vaccine potency via multivalent TLR activation [41]. By enabling co-localization and co-delivery of multivalent tumor antigens with immunological adjuvant, the CCNP platform can exploit the many unique properties of particulate vaccines to enhance immune responses [42].

Regarding drug delivery, CCNPs present a cancer-targeting strategy based on the intrinsic self-adhesive properties of cancer cell membranes. Currently, cancer targeting is achieved primarily via receptor-ligand interactions aimed at overexpressed surface antigens on cancer cells [1, 43]. Numerous targeting ligands have been successfully conjugated to nanoparticles, including antibodies, peptides, aptamers, and small molecules [43]. These targeted formulations have demonstrated increased
tumoral accumulation [44], and encouraging treatment efficacy has been observed in clinical trials [45]. In developing novel targeting approaches, the inherent homotypic adhesion property of cancer cells has yet to be considered. It has been demonstrated that many cancer cells express surface antigens with homophilic adhesion domains, which are responsible for multicellular aggregate formation in tumors. For instance, carcinoembryonic antigen and galectin-3 have been identified as homophilic binding proteins frequently overexpressed on cancer cell surfaces for intercellular adhesion [46, 47]. By coating nanocarriers in cancer cell membranes, it is possible to take advantage of this cell-to-cell adhesion for cancer targeting.

In the present study, MDA-MB-435, a tumor cell line with a well-established homotypic binding mechanism that displays homotypic aggregation in vivo [21, 48], was used to demonstrate the cancer targeting potential of CCNPs. Nanoparticles coated in MDA-MB-435 membrane showed significantly increased cellular adhesion to the source cells as compared to bare nanoparticles. It should be noted that structurally analogous RBCNPs showed reduced particle binding to MDA-MB-435 cells, indicating that homotypic cell membrane was responsible for the enhanced particle-to-cell adhesion (Figure 4.2.6). Additionally, the MDA-MB-435 CCNPs were shown to have little increase in affinity compared to bare PLGA cores when incubated with human foreskin fibroblasts, which further reflects the cancer cell-specific affinity of the nanoparticles. As CCNPs leverage the adhesive tendencies of cancer cells for targeting, they also have the potential to target distant body sites that are susceptible to cancer metastasis via heterotypic binding mechanisms to subsets of endothelial cells.
[21]. It has been found that surface adhesion molecules are an important factor that dictates cancer cell dissemination and determines their metastatic propensities [20, 21]. Thus, by using cancer cell membrane for particle functionalization, it becomes possible to prepare nanocarriers with cancer-mimicking binding properties [49]. Such a platform can be applied to localize therapeutics directly to cancer cells or to distant sites in the body that are susceptible to metastases for the treatment of aggressive malignancies.

4.2.4 Conclusions

To conclude, coating polymeric nanoparticles with cancer cell membrane presents an effective method for introducing multiple membrane antigens and surface functionalities that are desirable but hard to achieve using traditional synthetic techniques. We have demonstrated successful cancer cell membrane isolation and particle functionalization, and the resulting CCNPs can be used for different modes of anticancer therapy. For cancer immunotherapy, the platform enables co-localization of multiple antigens together with immunological adjuvants in a stabilized particulate form, which facilitates the uptake of membrane-bound tumor antigens for efficient presentation and downstream immune activation. For anticancer drug delivery, the membrane coating can be applied to target the source cancer cells via a homotypic binding mechanism. Toward future translation, it is possible to derive cancer cell membranes from primary tumors to develop personalized CCNPs for anticancer treatments. The present study also demonstrates the robustness and versatility of the
cell membrane coating approach for nanoparticle functionalization, which provides a feasible method to develop novel, nature-inspired nanotherapeutics with complex antigenic information and surface properties. It can be envisioned that the membrane coating technology presented herein can be further expanded to other cell types for different biomedical applications.

Chapter 4, in full, is a reprint of the material as it appears in *Nature Nanotechnology*, 2013, Che-Ming Hu, Ronnie Fang, Brian Luk, and Liangfang Zhang, and *Nano Letters*, 2014, Ronnie Fang, Che-Ming Hu, Brian Luk, Weiwei Gao, Jonathan Copp, Yiyin Tai, Derek O’Connor, and Liangfang Zhang. The dissertation author was either the primary investigator or co-primary investigator and co-author of these papers.

### 4.2.5 References


Chapter 5

Conclusions
5.1 RBC Membrane-Coated Nanoparticles for Targeted Drug Delivery

Red blood cell membrane-coated nanoparticles (RBC-NPs) represent a promising drug delivery platform due to the ability of the particles to circulate for extended periods of time within the blood stream. This long circulation property is a result of the faithful translocation of membrane elements from the original red blood cells directly onto the particle surface. It has been demonstrated that the self-marker CD47 is both present at its natural density and retains its functionality on the surface of the RBC-NPs. Further, it has been shown that the polymeric core can easily be loaded with a model chemotherapy drug, doxorubicin, using both physical encapsulation and chemical conjugation techniques. The method of encapsulation determines the drug release kinetics, which can be fine-tuned for the desired application. Finally, the particle surface can be modified with active targeting ligands that serve to augment the passive targeting capability that is inherent to nanoparticle platforms. This is accomplished through a lipid-tethering technique that circumvents the need for the particles to come into contact with harsh solvents and reagents that are normally required for conjugation techniques. The technique can easily be generalized to a variety of targeting ligands across different size scales, ranging from small molecules to much larger biomacromolecules.
5.2 RBC Membrane-Coated Nanoparticles as “Nanosponges”

The inclusion of natural cell membrane on the surface of the red blood cell membrane-coated nanoparticles opens up a new range of applications that would otherwise be extremely difficult for synthetic platforms to achieve. One such use is to serve as a decoy for harmful molecules within the body. For example, pore-forming toxins attack membrane structures, and by directly using a toxin’s natural substrate as a coating material, it is possible to target the working mechanism of the toxin in a manner that doesn’t require specifically tailoring the nanoparticle to the toxin. It was shown that RBC-NPs are capable of absorbing and neutralizing α-hemolysin, the main hemolytic toxin secreted by methicillin-resistant Staphylococcus aureus. The nanoparticles are able to successfully divert the toxins away from their intended targets, leaving healthy cells intact. Once it complexes with the nanoparticles, the toxin no longer exhibits any toxicity, even when injected in vivo. In both a preventative and therapeutic setting, RBC nanosponges are able to significantly improve survival following the administration of a normally lethal dose of toxin. Further, this concept was demonstrated with autoantibodies, which are implicated in a number of autoimmune diseases. Using a model of autoimmune hemolytic anemia, it was demonstrated that the nanoparticles are able to divert anti-RBC antibodies away from healthy red blood cells, thereby reducing the presence of associated symptoms.
5.3 Cell Membrane-Coated Nanoparticles for Immune Modulation

Finally, it has been demonstrated that cell membrane-coated nanoparticles can be used for effective vaccine design. In terms of vaccinating to treat infections, it is possible to use nanosponge-toxin complexes to generate immunity against pore-forming virulence factors. Using α-hemolysin, it was shown that the nanotoxoid formulation was completely safe, yet was able to generate significantly higher antibody titers when compared to a heat denatured control. The titers were also very long lasting, with little drop in magnitude over the course of a 150-day observation period. Finally, the titers generated were able to nearly mitigate all adverse effects associated with the administration of α-hemolysin in both an intravenous and subcutaneous setting. In addition to using cell membrane-coated particles to deliver foreign antigens, it is also possible to coat the particles with cancer cell membrane to deliver autologous tumor antigens. Using a model murine cell line, it was confirmed that well-known tumor antigens were transferred to the particle surface. Further, the particles can be incorporated with an immunological adjuvant, which is essential for the generation of a response against lowly immunogenic antigens. These adjuvanted particles were successfully delivered to dendritic cells, which could then further initiate downstream immunity.