Amphiphilic Gold Nanoparticle-Based Cytosolic Drug Delivery Platform for Cancer and Infections

by

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Submitted to the Department of Materials Science and Engineering in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Materials Science and Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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ABSTRACT

Cancer progression relies on deregulation of normal immune cell functions. One of the signature strategies utilized by tumor cells is immunosuppression. Deregulation of cytosolic kinases negatively alters immune cell signaling, proliferation, differentiation, and migration. Small molecule kinase inhibitors offer unique opportunities to enhance anti-tumor immunity by inhibition of immunosuppressive kinases. However, potent inhibitors are often poorly soluble in physiological conditions and suffer from poor pharmacokinetics in vivo. In this thesis we designed a small molecule cytosolic delivery platform based on cell-penetrating amphiphilic nanoparticles (amph-NPs), which are capable of embedding within and subsequently penetrating cell membranes without toxicity. Amph-NPs have dual properties of entrapping concentrated hydrophobic small molecules in their ligand shells and preferentially targeting lymph nodes post subcutaneous injection. Successful reversal of immunosuppression in T cells via diacylglycerol kinase inhibitors (DGKi) was achieved via amph-NP delivery, while DGKi delivered in its soluble form was ineffective. A hydrophobic small molecule antimicrobial ciprofloxacin loaded in amph-NPs resulted in early clearance of local bacterial infection, while ciprofloxacin delivered freely was significantly less effective. This novel cytosolic delivery platform is broadly applicable to a variety of hydrophobic small molecules because drug loading solely relies on physical adsorptions. Ongoing work focusing on delivering small molecule immunosuppression-reverting drugs to the cytosol of autologous T cells prior to adoptive transfer therapies may effectively protect T cells in the immunosuppressive tumor microenvironment and significantly improve anti-tumor immunity. In conclusion, this effective cytosolic delivery platform may accelerate accurate evaluation of new drugs that target cytosolic signaling pathways and ultimately the development of new translational therapeutics.

Thesis Supervisor:
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1 Introduction

Modulation of host immunity has proven a powerful strategy to treat cancer and infectious diseases. Cancer development relies on deregulation of normal immune cell functions, leading to cancer progression and metastases. One of the conditions triggered by cancer cells to evade normal immune constraints is immunosuppression. For example, transforming growth factor β (TGF β) is one of the general immune-suppressive cytokines found in cancer patients, ranging from breast cancer, gastric cancer, colorectal cancer, non-small-cell lung cancer, malignant melanoma to renal cancer. Approaches currently used in the clinic for cancer immunotherapy can be categorized into two types based on their structures: proteins and small molecules. Immunotherapy based on monoclonal antibodies (mAb) that block protein-protein interactions between checkpoint receptors and their ligands have recently demonstrated breakthrough clinical outcomes in cancer treatments.

While protein-based therapies may be efficacious in some settings, there are many immune-oncology pathways that can solely be targeted by small molecule drugs. For example, small molecules are uniquely suited to modulate intracellular targets owing to their size advantage. Deregulation of intracellular kinases in the immune cells have been found associated in cancer patients, leading to decreased immune surveillance and benefited tumor progression. Small molecule kinase inhibitors may overcome tumor-associated immunosuppression and facilitate cancer treatments via the reversal of immune tolerance. Many small molecule inhibitors bind to the allosteric site of an enzyme to inhibit its activity, and these interactions are mostly non-covalent and reversible. Allosteric sites on enzymes or other “hydrophobic pockets” on enzymes are hydrophobic regions that are sterically hard to reach by larger molecules such as proteins. Therefore it is obvious that small molecule kinase inhibitors are mostly somewhat hydrophobic. Although in vitro identification of drug targets may be achieved, verification of a small molecule’s in vivo potency is complicated by many factors such as unpredictable pharmacokinetics and poor cellular infiltration efficiencies.

Nanoparticle-based therapeutics have evolved considerably in the past two
decades and are now applied clinically to treat cancer and infectious diseases. Compared to soluble therapeutics they can concentrate drugs in tumors or other target cells while reducing systemic toxicity. In this thesis, we aim to engineer a nanoparticle-based delivery platform that enables efficient transport of small molecules in vitro and in vivo. A general platform that enhances hydrophobic molecules' solubility in physiological condition and enables transport of bioactive drugs to their cytoplasmic protein targets may broaden many aspects of current disease treatments from the insights on basic cytosolic signaling pathways to applications on drug delivery in the clinic.

This thesis focuses on the design and applications of small amphiphilic gold nanoparticles (amph-NPs) of 2-4 nm core diameters that have the capability to entrap a high concentration of hydrophobic small molecules in their inner hydrophobic spaces between ligands. In the next section, we discuss current limitations of small molecule therapeutics and cytosolic delivery strategies. Gold nanoparticles used in the biomedical field will also be discussed. Finally, building on these motivations, our efforts to design a generally viable platform for targeted delivery of small molecules will be demonstrated throughout the thesis, focusing on cancer immunomodulation and blockade of bacterial infection.

1.1 Background

1.1.1 Small molecule therapeutics

Small molecules are significantly smaller than protein therapeutics, and thus have advantages such as crossing biological barriers— for example, TRAIL inducing compound (TIC 10), a small molecule that up-regulates endogenous anti-tumor activities by transcriptionally inducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), was reported to be a potent brain cancer treatment owing to its ability to cross the blood brain barrier. Vemurafenib, a clinically used small molecule drug, is a potent BRAF kinase inhibitor that blocks tumor proliferation. These examples represent a portion of small molecule drugs that are highly potent and deliverable in vivo. However,
researchers in the small molecule drug discovery field identified many potentially potent small molecule inhibitors that are hydrophobic and poorly soluble at concentrations required for therapeutic intervention. As mentioned earlier, many drug targets are located in the cytoplasm, thus ways to improve cytosolic delivery efficiency may strongly impact therapeutic outcomes.

Cell membranes are natural biological barriers composed of phospholipid bilayers. Membranes prevent macromolecules and other foreign substances from entering cells. While some small molecules may traverse bilayers via passive diffusion, the diffusion rate is much lower as membranes are 100-1000 times more viscous than water.\(^{13}\) The rate-limiting step in the passive diffusion of molecules across cell membranes occurs in the hydrophobic area of lipids. Small uncharged polar molecules such as \(\text{H}_2\text{O}\) readily cross membrane barriers, whereas larger uncharged polar molecules such as glucose cannot (Figure 1-1). Charged molecules are unlikely to cross cell membranes via passive diffusion regardless of size—charged ions such as \(\text{Na}^+\) cannot cross membranes via passive diffusion. It requires ion pumps for intracellular and extracellular ion exchange. Strategies on getting substances across cell membranes thus became a field of intense investigation.

![Figure 1-1 Passive diffusion across a lipid bilayer. Adapted from [The Cell: A Molecular Approach. 2nd edition. Cooper GM.]](image-url)
1.1.2 Current cytosolic delivery strategies

Most cells internalize foreign molecules by endocytosis, a process by which substances are engulfed into intracellular vesicles such as endosomes and lysosomes. Endocytotic pathways can be categorized into the following—clathrin-mediated endocytosis, caveolar-mediated endocytosis, macropinocytosis and phagocytosis (Figure 1-2). Drugs confined in endosomes tend to degrade due to low pH in late endosomes, resulting in low drug efficacy.

Figure 1-2 Modes of cellular internalization of nanoparticles and respective size limitations. Internalization of large particles is facilitated by phagocytosis (a). Nonspecific internalization of smaller particles (>1 μm) can occur through
macropinocytosis (b). Smaller nanoparticles can be internalized through several pathways, including caveolar-mediated endocytosis (c), clathrin-mediated endocytosis (d) and clathrin-independent and caveolin-independent endocytosis (e), with each being subject to slightly different size constraints. Nanoparticles are represented by blue circles (> 1 μm), blue stars (about 120 nm), red stars (about 90 nm) and yellow rods (about 60 nm). Adapted from reference [10]

Considerable literature exists which seeks to improve drug efficacy via endosomal disruption. Proposed means to accomplish this include positive charge induced-endosomal membrane destabilization, proton sponge effect-induced endosomal burst, and cell-penetrating peptide-induced transient membrane pore generation. Among these strategies, “proton sponge effect” induced endosomal burst” is the most common (Figure 1-3), although its molecular mechanism of action has not yet been conclusively resolved. Proton sponge polymers such as PEI have a high buffer capacity, and protonation of PEI in acidic lysosomes may result in protons (and counter ions) being pumped into lysosomes, causing osmotic pressure sufficient to burst the vesicles. Burst of endosomes/lysosomes releases drugs into subcellular components in the cytosol. Most endosomal disruption strategies involve cationic materials or fusogenic peptides that are generally toxic to cells or cell-penetrating peptides that target specific cell types.
To date, the development of safe and efficient drug delivery remains a challenging topic. Drug delivery to the immune system is even more challenging because most adaptive immune cells are non-phagocytic. Passive diffusion of small molecules into plasma membranes is inefficient and inefficacious; therefore, new methodologies for drug delivery to the immune cell network are in need.

1.1.3 Gold nanoparticles in biomedical fields

The first laboratory gold nanoparticle synthesis can be dated back to the 1850s. In 1857, Michael Faraday synthesized the first colloidal gold nanoparticles (Au NPs) by the reduction of gold chloride with phosphorous. In the mid 20th century, the first TEM with 100k magnification was developed. In 1951, Turkevich et al. conducted the first structural analyses of gold nanoparticles via TEM. Since this time, Au NP synthesis and
characterization have been refined, with the Brust method—based on sodium borohydride reduced H(AuCl₄⁻) and alkanethiol-stabilized Au NPs—being the first size controllable method developed. These Au NPs can be reversibly precipitated and redissolved in solvents without causing aggregation.

Recent advances in surface functionalization have allowed Au NPs to serve as vehicles for antibody, peptide, nucleic acid, and small molecule drug delivery. Au NP vectors such as AuroShell and CYT-6091 have entered clinical trials to treat cancer. There are several advantages of using NPs as carriers—delivery of insoluble/membrane impermeable drugs, and targeted delivery. Comprehensive in vitro and in vivo studies have shown that cell-nanoparticle interactions are dependent on the size, charge and surface composition of the particles. NPs smaller than 100 nm are of interest in biomedical applications due to their ability to circulate in the body. Particles modified with cationic groups such as the cell-penetrating peptide TAT have been shown to enter cells more efficiently due to their high affinity to anionic cell membranes, however, they may cause severe toxicity due to pore formation on cell outer membranes. In general, increasing NP surface positive charge density enhances cellular uptake but also increases toxicity. Mixed charged zwitterionic AuNPs were reported to be stable in the presence of high salinity and serum proteins, giving them excellent biocompatibility in vivo. Liu et al. reported that in comparison to traditional PEGylated AuNPs, zwitterionic AuNPs were superior at minimizing nonspecific cellular uptake in vitro. However, reduced cellular uptake also limits their ability to efficiently deliver therapeutic cargos into cells. Hydrophobic AuNPs of 2 nm core size coated with dodecanethiols were reported to stably embed within lipid bilayers without disrupting the bilayer structures. However, these particles are not applicable in biomedical applications due to their poor solubility in physiological conditions. Increased NP surface hydrophobicity favors membrane insertion, while increased hydrophilicity tends to enhance cell surface adsorption due to electrostatic interactions. Therefore, a balance between charge and hydrophobicity may maximize NP interaction with cell membranes while minimizing toxicities. In this thesis, we seek to develop a cytosolic drug delivery platform based on highly water-soluble, anionic, amphiphilic gold nanoparticles that are able to efficiently penetrate cell membranes with minimal toxicity.
1.2 Scope of the thesis

This thesis seeks to develop a broadly applicable cytosolic delivery platform that enables efficient and efficacious therapeutic delivery for cancer and infectious disease treatments. We used small gold nanoparticles with amphiphilic monolayer coatings as a drug delivery platform. The amphiphilic ligands are critical to nanoparticle’s high solubility in both aqueous environments and within lipid bilayers, allowing particles to penetrate cellular membranes without causing membrane rupture and toxicity.

In chapter 2, the synthesis and characterization of nanoparticles are demonstrated. Their membrane-penetration property was demonstrated in model cell membranes and a broad cytosolic dispersion was demonstrated in a murine cancer cell line. A novel method to entrap hydrophobic small molecules (immunomodulators, chemotherapy drugs, and antibiotics) into the hydrophobic ligand interstitial spaces was developed. We further demonstrated that membrane-penetrating nanoparticles significantly enhanced cytosolic delivery of TGF-β inhibitors (a small molecule immunomodulator) at least by 30-fold compared to its soluble form.

A detailed study focusing on the investigation of membrane-interaction properties and their intracellular distribution is shown in chapter 3. We first investigated the effect of nanoparticle’s size and ligand compositions on the cytosolic distribution in various murine cell lines. The effect of cell surface glycocalyx thickness on nanoparticle’s intracellular distribution and membrane penetration capacity was further elucidated.

In chapter 4, we evaluate the biodistribution of amphiphilic gold nanoparticles (amph-NPs) post intravenous (i.v.) injection and subcutaneous (s.c.) injection in vivo in mice. A striking phenomenon was observed: a high percentage of amph-NPs preferentially accumulated in the lymph nodes post s.c. injection. To investigate if this intrinsic lymph node targeting was associated with specific subsets of immune cells, we performed single cell analysis using cytometer time-of-flight (CyTOF). For the first time, CyTOF was demonstrated to be extremely useful for the understanding inorganic nanoparticle’s fate in vivo. Using this method, we identified a subset of cells in the lymph node that preferentially capture amph-NPs—myeloid-derived dendritic cells (Ly6G CD11b⁺ CD11c⁺ F4/80⁺). Using this newly demonstrated label-free in vivo
tracking of nanoparticles method, we further elucidated nanoparticle’s biodistribution in the lungs post intratracheal injection and in the spleens post intravenous injection. Finally, we demonstrated the intrinsic properties of lymph node tropism and dendritic cell targeting resulted in effective vaccine delivery.

Motivated by the results of enhanced cytosolic delivery of immunomodulators shown in chapter 2, the evidence of cytosolic dispersion shown via TEM in chapter 3, and the intrinsic lymph node tropism demonstrated in chapter 4, amph-NP may efficiently deliver therapeutics to the targeted organ, cell type, and finally the intracellular targets. As a proof of concept, we tested if a poorly water-soluble T cell kinase inhibitor (DGKi) can reverse immunosuppression in vitro. As discussed in chapter 5, DGKi delivered by amph-NPs recovered T cell function, while soluble DGKi was completely ineffective. To enhance in vivo cell targeting specificity, a method to conjugate amph-NPs to antibodies (Ab-NP) was developed. As demonstrated in chapter 6, anti-CD8 antibody conjugation enhanced CD8+ T cell targeting, although the fate of Ab-NP in vivo was a function of nanoparticle surface ligands and antibody moieties. In chapter 7, we demonstrate another aspect of amph-NP-based cytosolic delivery platform in the context of treating pseudomonas skin infection. Two approaches were tested: (1) immunomodulation of innate immune cells as an attempt to boost the host anti-microbial immunity. (2) Small molecule antimicrobial delivery for efficient eradication of local infections. In both methods, we aimed to use lymph node targeted amph-NPs to efficiently deliver therapeutics to the lymph nodes for early eradication of lymph-borne bacteria, potentially preventing lethal systemic spread. Finally comprehensive conclusions from the work done in this thesis and promising future directions will be discussed in chapter 8.
2 Amphiphilic Membrane Embedding Gold Nanoparticles

2.1 Introduction

Two of the major challenges in drug delivery are for therapeutics to reach target tissues and cells following administration and then on a subcellular level, to escape from endosomes and reach their sites of action in the cytosol. In this thesis, we aimed to tackle the cytosolic delivery problem by using MUS/OT amphiphilic gold nanoparticles (amph-NPs) of 2-5 nm core diameter as cytosolic drug carriers. Amph-NPs are coated with binary mixtures of a completely hydrophobic ligand with a slightly longer hydrophobic ligand terminated with a hydrophilic group, which have the unique property of being both highly water soluble and highly membrane-interactive.\textsuperscript{34} Previously, our experimental data and simulation results suggested that these highly water-soluble amph-NPs penetrate and embed in lipid bilayers without causing membrane ruptures.\textsuperscript{35,36} In this chapter, we will review MUS/OT amph-NPs’ membrane embedding/penetration properties, and introduce their capacity to absorb small molecules into their hydrophobic ligand vacancies. Finally, we demonstrate how these dual properties of membrane-embedding and drug loading facilitate efficient small molecule drug delivery into mammalian cells.

2.2 Experimental Methods

**Nanoparticle synthesis.** 11-mercaptoundecane sulfonate ligand (MUS) was synthesized following previously reported methods.\textsuperscript{34} All other chemicals were purchased from Sigma-Aldrich and used without further purification. 0.9 mmol gold(III) chloride trihydrate (99.9%) was dissolved in 150 mL of ethanol and 0.75 mmol of ligands (MUSOT: MUS and 1-octanethiol; MPSA: Sodium 3-mercapto-1-propanesulfonate) with a desired molar ratio were added to the solution. After 15 minutes of stirring at 900 rpm, an ethanolic solution of sodium borohydride (NaBH\textsubscript{4} - 10 times molar excess of gold salt in 150 ml ethanol) was added dropwise to the solution at 25°C. Black precipitates were almost immediately observed. The final solution was stirred for an additional
hours to ensure reduction of the gold salts. The reaction was quenched by removing the solvent with centrifugation. To remove unreacted chemicals, additional washes with acetone and ethanol were carried out. Finally, water-soluble salts and any residual free ligands were removed using a centrifugal dialysis membrane (Amicon, MWCO 30kDa). PEGylated gold nanoparticles coated by thiol-terminated poly(ethylene glycol) (molecular weight 282.35 g/mole) were purchased from NanoPartz™.

Transmission electron microscopy. To analyze size distributions of gold nanoparticles, 10 μL of nanoparticle solution at 2 mg/mL was deposited on a carbon coated copper grid, and allowed for 10 min deposition. Residual solution was blotted away using a filter paper and the grid was air-dried. Images were taken under JEOL 2010 FEG Analytical Electron Microscope at 200 kV.

Synthesis of BODIPY-C₁₁-SH. Fluorescent dye BODIPY® 650/665-X NHS Ester (Invitrogen) and thiol linker (11-mercaptoundecyl amine hydrochloride) (Prochimia, Poland) were mixed as follows: 4.2 umole of BODIPY dye and 4.6 umole of thiol linkers were dissolved in 1 mL of argon-purged amine-free dimethyl formamide and stirred for 6 hours at 60 °C in the dark.

Conjugation of BODIPY-C₁₁-SH to amph-NPs. An optimized protocol to conjugate an average of 0.5 dye per NP was as follows: 5 μL of BODIPY-C₁₁-SH (4.2 mM in 95% dimethylformamide) was added to 1 mg of gold nanoparticles (100 μL of 10 mg/mL) in water. The solution was covered with foil and agitated at speed of 750 rpm on a shaker for 3 days at 25°C to permit ligand exchange. Unconjugated BODIPY-C₁₁-SH was removed by topping up the eppendorf with acetone and centrifuging at 14Kxg for 2 minutes (repeated four times). Excess acetone was evaporated in the vacuum oven overnight. Finally, vacuum dried nanoparticles were weighed and re-solubilize in the desired solvent. Concentrations of NPs were determined by TEM, UV-vis and ICP-AES.

Quantification of number of dyes conjugated per NP. Dye-conjugated NPs (1-1.5 mg) were etched in 200 μL of 15 mg/mL Cyanide in deuterated methanol on a sonicator
until black precipitates occurred. Supernatant containing stripped ligands and dye molecules were quantified using absorbance or H1 NMR.

**GMV synthesis.** Giant multilamellar vesicles (GMVs) were synthesized as model membranes in order to study how membrane composition, curvature, and fluidity affect the penetration ability of striped gold nanoparticles. The synthesis process is as follows: 6 mmoles of desired lipids in chloroform were dried under nitrogen flow, and the residual chloroform was evaporated in a chemical hood overnight. Dried lipids in glass vials were kept cap off and hydrated in a 70°C water bath for 2-6 hours. 2 mL of 50 mM sucrose in PBS or water was then added to rehydrate the lipids in a 70°C water bath for formation of GMVs overnight. GMVs were harvested and stored at 4°C.

**Cell Culture.** B16F10 cells were seeded in an 8-well chamber (Fisher Scientific Lab-TekTM II Chambered Coverglass) at 60,000 cells per well in 500μL cell culture medium. The cells were allowed to grow overnight to about 80% confluence, and then treated with prepared solutions (typical dosage: 0.6mg/ml gold nanoparticles or 0.1mg/ml lipids) for 3 hours or 18 hours. Cells were washed twice with PBS and subsequently imaged in RPMI (phenol red free) medium. Confocal laser scanning microscopy was performed on a Zeiss LSM 510 using a 63X oil lens, with excitation wavelengths being 488nm, 543nm, and 633 nm.

**Drug loading and quantification.** Hydrophobic small molecules were dissolved in ethanol or DMSO at a concentration of 5-20 mg/mL, and mixed with an aqueous suspension of amph-NPs (10 mg/mL in water) at a molar ratio of 500 drug molecules per NP. The mixture was placed in a dialysis cassette with 100-500 Da MWCO against stirring water for at least 24 hours. Pores on dialysis membranes allow ethanol to permeate while retaining most small molecules, leading to partitioning of drug molecules into the hydrophobic pockets of the amph-NPs. To quantify drug loading efficiency, the drug-NP mixture was retrieved from dialysis cassettes 24 later, and free unloaded small
molecules were removed using 30k amicon centrifugal tubes at 14 k x g for 10 minutes. The process was repeated twice. The concentrated suspension of drug loaded amph-NPs (~ 30 uL) was collected, and a portion of the batch was used for quantification. To extract loaded small molecules from gold core, 0.1 mg of drug-loaded NP (~ 5 uL) solution was mixed with 10 uL of beta-mercaptoethanol (BME, 14.3 M from Sigma) and 165 uL of ethanol or DMSO for 24 hours on a shaker. Clear supernatant (without gold precipitates) was collected next day and analyzed by UV-vis or HPLC. For drugs with a strong absorbance at wavelength > 350 nm, UV-vis was used to quantify loading efficiency, otherwise HPLC (reserve-phase C18 column) was used for quantification.
2.3 Synthesis and characterization amph-NPs

Amph-NPs have a gold core of tunable size (typically 2-5 nm), surrounded by an organic ligand shell comprised of amphiphilic ligands (MUS, 11-mercaptoundecane sulfonate) and optionally hydrophobic ligands (OT, octanethiol) as shown in Figure 2-1.

![Figure 2-1 MUSOT amph-NP. (a) Surface ligand composition and (b) representative TEM image.](image)

Synthesis of these particles by the Stucky method yielded NPs of ~5nm core size; whereas the Brust method synthesized NPs with a polydisperse core size distribution ranging from 2 to 8 nm (Figure 2-2). For the purpose of studying NP’s size effect on membrane interactions and drug loading, we fractionated amph-NPs using a sucrose density gradient column to acquire more mono-dispersed fractions. As illustrated in Figure 2-3 a, as-synthesized NPs were loaded to the surface of 20-60% w/w sucrose gradient columns, and ultracentrifuged to reach equilibrium where different sized-NPs were settled in different regions of the gradient. Three fractions of NPs were collected, and their size distributions were analyzed using TEM and ImageJ. The gold core diameter of MUS/OT amph-NP fraction A was roughly 2.6 ± 0.5 nm; fraction B 2.8 ± 0.9 nm; fraction C 3.0 ± 0.6 nm. (Figure 2-3 b) The core diameter of MUS amph-NPs fraction A was roughly 3.0 ± 0.6 nm; fraction B 4.0 ± 0.6 nm; fraction C 4.3 ± 1.1 nm (Figure 2-3 c). MUS NPs consistently exhibited better fractionation quality than MUSOT particles. This is likely due to another parameter that influences the sedimentation
coefficient of MUSOT amph-NPs: the MUS:OT ligand ratio, which can vary from particle to particle.

Figure 2-2 ImageJ size analysis of as-synthesized amph-NPs by Brust method or Stucky method.

Figure 2-3 Fractionation of amph-NPs. (a) Illustration of sucrose density gradient based nanoparticle size fractionation processes. (b) Size distribution of fractionated MUSOT NPs and (c) MUS NPs.
To characterize the ratio of MUS to OT on mixed ligand coated amph-NPs, ligands on amph-NPs were dissolved in iodine containing deuterated methanol, and analyzed using H\(^1\) NMR. An example NMR spectrum of MUS:OT 2:1 NPs is shown in Figure 2-4. Hydrogens immediately next to sulfonate groups, thiol groups and methyl groups can be identified and calculated to obtain the MUS:OT ligand ratio. In addition, the mass ratio of organic ligands to gold core per NP can be experimentally measured using thermogravimetric analysis (TGA). The boiling point of MUS and OT ligands are 550 °C and 200 °C respectively, therefore, the mass difference of NPs before and after TGA process (heated up to 800 °C) is approximately the mass of organic ligands present. The ligand mass of MUSOT or MUS amph-NPs usually falls in 10-20% of total NP mass (data not shown).

![H\(^1\) NMR spectrum of iodine-quenched MUS/OT NPs.](image)

Figure 2-4 H\(^1\) NMR spectrum of iodine-quenched MUS/OT NPs. Up-field peak (centered ~0.9 ppm) represents three hydrogens from the end group of OT ligands. Two low-field peaks (centered ~ 2.7 and 2.8 ppm) represent 2H neighboring sulfonate groups, 2H neighboring SH of MUS and OT ligands respectively.
2.4 Membrane embedding property of amph-NPs

One of the most interesting and unique properties of amph-NP is their ability to embed and penetrate through lipid bilayers. To track amph-NPs via fluorescence-based microscopy, NPs were ligand exchanged with thiolated BODIPY 650/665. We showed experimentally that amph-NPs embed in the bilayers of synthetic lipid vesicles (Figure 2-5 a). To evaluate if amph-NP can transit between bilayers, we incubated BODIPY-tagged amph-NP with giant multilamellar vesicles (GMVs) for an hour and imaged their interaction via confocal microscopy. Results shown in Figure 2-5 b suggested that amph-NP were able to transit from outer lipid membranes to inner membranes without rupturing bilayers. Interestingly, NPs free in solution did not fluoresce. We hypothesize that in solution, hydrophobic BODIPY dyes bent into the hydrophobic ligand shells of amph-NPs to reduce the solvent accessible surface area (SASA), thus decreasing the distance of dyes from the gold core and resulting in quenched/undetectable fluorescent signal. By contrast, on embedding in a lipid bilayer, the nonpolar dye can extend away from the gold particle surface, unquenching its fluorescence signal. If membrane embedding and penetration properties of amph-NPs remain true in mammalian cells, NPs are expected to reach a broad distribution in the cytosol rather than remaining punctate spots in endosomes. We tested this by incubating amph-NPs with B16F10 melanoma cells at 37°C for 18 hours. The confocal image shown in Figure 2-5 c shows evidence of broadly distributed NPs throughout the cytosol, in agreement with our hypothesis.

Figure 2-5 Amph-NPs interaction with lipid membranes. (a) TEM images of multilamellar lipid vesicles loaded with amph-NPs (~ 3 nm). (b) Confocal images of
amph-NP (~ 3 nm) embedded in GMVs. (c) Confocal images of amph-NPs’ (2.18 nm) broad cytosolic distribution in B16F10 cells.

To understand the mechanism by which anionic amph-NP utilize to embed into lipid bilayers, we collaborated with Professor Alexander-Katz’s group. They used computer simulations to calculate the free energy change of nanoparticles moving from aqueous solution into lipid bilayers, and showed that the free energy decreased when amph-NPs are embedded in bilayers. The results suggested that the key to membrane embedding is the flexibility of MUS and OT ligands. Anionic groups “snorkel” out to aqueous solution to minimize unfavorable charge insertion into lipid bilayers, while hydrophobic alkane chains maximize physical contacts with hydrophobic lipid tails, which reduced the energetically unfavorable SASA (Figure 2-6 a). Simulation results also demonstrated that the free volume available to the organic ligands enable the ligands to substantially reorganize in response to the external environment.

Next, the effect of NP ligand compositions, ligand morphologies and core size on membrane penetration was evaluated. Simulation results shown in Figure 2-6 b suggested that the organization of MUS OT ligands on the particle surface (striped, random, or mixed) does not affect the free energy change for membrane embedding. However, increased hydrophobic contact surface area between NP ligands and bilayers directly correlates with the magnitude of driving force for NP to embed within bilayers. Thus, particles coated with MUS:OT at a 1:1 molar ratio with a greater fraction of hydrophobic uncharged ligands resulted in a higher free energy change compared to MUSOT 2:1 or all-MUS NPs (Figure 2-6 b). The particle’s core size also plays a role in its affinity to membrane insertion, with simulation results predicting that 2.5 nm all MUS, 3.5 nm MUSOT 2:1, and 4.5 nm MUSOT 1:1 have the greatest free energy change on membrane insertion. All simulation work was performed by Dr. Reid Van Lehn (a former graduate student in Professor Alfredo Alexander-Katz’s group).
Figure 2-6 Flexible ligands are key to membrane embedding. Adapted from reference [36] (a) Modeling snapshot of amph-NP ligand flexibility while embedded in lipid bilayers or free in aqueous environments. (b) Effects of ligand composition and morphology on total free energy change of NP embedded in bilayer v.s. free in aqueous solution.

2.5 Small molecule drug loading

Gold nanoparticles are widely used as drug delivery vehicles, owing to the ease of surface attachment of payloads on AuNPs using gold-thiol chemical bonds. However, replacing ligands in the organic layer in our system with anchored drug cargos could lead to an alteration in the particles’ membrane-embedding properties, which might lessen their effectiveness for intracellular drug delivery. We thus sought to employ an alternative strategy to load drugs that involves no ligand exchange—a method to load hydrophobic small molecules into the hydrophobic pockets of amph-NP ligand shells, using hydrophobic-hydrophobic interactions instead of covalent bonds.7

Two key features of amph-NPs include: the presence of free volume between ligands and their ligand flexibility. Here, we hypothesized that the available free volume between alkane ligands (so called “hydrophobic pockets”) may provide sufficient space to entrap hydrophobic small molecules. To test this idea, a loading method was developed as illustrated in Scheme 1. Amph-NPs solubilized in water were mixed with a small volume of small molecule drug cargo in pure organic solvent (ethanol or DMSO), the mixture was loaded into a 100-500 Da MWCO dialysis cassette, and dialyzed against
water to allow slow removal of the organic solvent from local environment, forcing small molecules to sequester into hydrophobic pockets of amph-NPs. This process is driven by the reduction of SASA of the system where water or hydrophobic pockets are the only two choices for hydrophobic small molecules to reside in. For example, as ethanol gradually removed from NP drug mixture, hydrophobic small molecules are driven into hydrophobic ligand shells of NPs to minimize unfavorable interactions with water.

Figure 2-7 Illustration of small-molecule loading into NP organic ligand shell.

We found that this method is applicable to a variety of hydrophobic drugs (shown in Figure 2-8 a,b), ranging from small molecule immunomodulators (DGKi, a diacyl glycerol kinase inhibitor; TGFbi, a TGF-β inhibitor; R848, a TLR7/8 agonist; Diacerein, an IL-1β inhibitor; VX-765, a caspase-1 inhibitor; wiskostatin, a selective inhibitor of N-WASP) to chemo drugs (doxorubicin) and antibiotics (ciprofloxacin). Drugs in organic solvent were mixed with an aqueous suspension of amph-NPs and dialyzed to remove the organic solvent, leading to partitioning of the drug into the hydrophobic pockets of the amph-NP ligand shell. This approach allowed us to load drugs that are nearly insoluble in aqueous solutions to high degrees of “solubility” via incorporation into MUS or MUSOT amph-NPs. Loading efficiency of drug molecules appeared to be a function of drug hydrophobicity, polarity as well as NP’s size/ligand ratio (Figure 2-8 b,c).

To determine what factors regulate this mode of drug loading, we focused on one particular drug of interest, DGKi, and evaluated its loading as a function of gold core diameter and ligand composition. Amph-NPs with either all-MUS ligand shells or mixed MUSOT ligand shells were synthesized and fractionated into samples with distinct size distributions (Figure 2-8 c). We expected the potential for non-monotonic drug loading behavior with gold core size, as larger-diameter particles have more ligand “pockets” to load drugs, but as particle size increases the free volume available in the ligand shell
decreases with decreasing particle surface curvature. This effect was observed experimentally: The number of drug molecules loaded per NP increased significantly from MUS (3.2 nm) to MUS (4 nm) and from MUSOT (2.6 nm) to MUSOT (2.9 nm), with a maximum drug loading of ~150 DGKi molecules per nanoparticle achieved with 4 nm diam. core all-MUS amph-NPs. However, loading capacity decreased with further increases in core size for both ligand shell chemistries, suggesting that ligand packing density and curvature both play important roles in drug loading. Besides DGK inhibitors, TGF-β inhibitors was also tested. MUS NPs with a 4 nm core diam. had optimal loading for DGKi, while 3.2 nm MUS NPs showed optimal loading capacity for TGFβi.

Figure 2-8 Hydrophobic drug loading to amph-NPs. (a) Chemical structures of small molecules tested. (b) Quantification of loaded drug to gold mass ratio. The
results were pooled from several experiments, with nanoparticle's size and composition ranging from MUS to MUSOT (2 - 4 nm in core diameter) (c) NP's size effect on drug loading. A molar ratio of 500:1 (drug: NP) was mixed in water (with 10-20% organic solvent) for 24 hours in a 100-500 Da MWCO dialysis tube against water.

2.5.1 Effect of ligand chemistry on small molecule drug loading

To evaluate whether the observed small molecule loading is unique to the amph-NP ligand composition, we compared the effect of ligand length (i.e. available hydrophobic surface area) on drug loading, assuming that long hydrophobic ligands are crucial for efficient drug loading. We used DGKi as a model drug in this experiment. Figure 2-9 showed that short hydrophobic ligand (MPSA)-coated gold NPs with only three hydrocarbons have 10-fold decreased loading capacity compared to MUSOT amph-NPs. PEGylated gold NPs with a hydrophilic poly(ethylene glycol) ligand shell also resulted in low drug loading, as expected for the proposed mechanism of hydrophobic drug sequestration. Thus, optimally-sized MUSOT or MUS amph-NPs provide a means to solubilize hydrophobic drugs that are otherwise insoluble as concentrated aqueous solutions, with high levels of drug loading per particle.

Figure 2-9 Ligand's effect on hydrophobic drug loading. (a) Schematics of three types of AuNP ligand structures. (b) Number of DGKi loaded per MPSA (3.79±1.1 nm), MUSOT (2.78±0.9 nm) or PEG NP (2.61±1.0 nm).
2.5.2 Drug release kinetics

The hydrophobic regions in the NP ligand shells allow for an energetically favorable temporary storage location for small hydrophobic molecules. The stability of drug-loaded nanoparticles is an important factor to consider for *in vivo* applications. We used doxorubicin as a model drug to analyze the release kinetics of loaded drug in amph-NP ligand shells. The solubility of doxorubicin is pH sensitive, with a higher solubility in lower pH environments. Dox-loaded amph-NP or free amph-NP (without drug) were incubated in PBS (pH= 5.5), PBS (pH=7.4) or 10% serum containing RPMI over a period of 24 hours, and supernatants containing released drug were measured at various time points to determine the drug release kinetics. As shown in Figure 2-10 a, supernatant without dox showed no peak above 330 nm (orange curve), whereas dox-containing supernatants showed a characteristic peak around 480 nm. Using UV absorbance, drug release at various time points were quantified. Interestingly, only 5% of drug released to supernatant throughout the time course of 24 hours when NP were incubated in physiological conditions (PBS pH7.4 or 10% serum RPMI), suggesting that 95% of loaded drug remained stable in the ligand shell of NPs. Decreased pH in the environment accelerated drug release from NP ligand shells (Figure 2-10 b). This is likely due to decreased free energy change for drug to reside in ligand shell v.s. free in aqueous solution. In summary, this highly stable drug loaded state is desirable for the development of translatable drug delivery vectors.

![Figure 2-10](image)

**Figure 2-10** Doxorubicin release kinetics from MUSOT (2.58±0.7 nm) amph-NPs. (a) Representative UV-vis spectrum of supernatant with or without DOX. (b) DOX
release kinetics in PBS (pH=5.5), PBS (pH=7.4), or RPMI 10% serum. Each test tube consisted of 50 ug/mL NP and 12.5 ug/mL DOX. The lower detection limit of DOX is approximately 0.6 ug/mL.

2.6 Enhanced hydrophobic small molecule delivery via amph-NPs

We hypothesized that hydrophobic small molecule loaded amph-NPs might provide a means to enhance small molecule drug delivery to cells. Our hypothesis is illustrated in Figure 2-11. As apposed to passive diffusion of free small molecules into cells, which mainly relies on Brownian motion, amph-NPs may actively carry concentrated small molecules (50-200 molecules per NP) due to their high affinity to embed within and transit cell membranes. Upon delivery to the target cells, trans-membrane passage of NPs results in disturbance of the ligand shell and release of some cargo drugs.

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**Passive diffusion V.S.**

**Active NP mediated cytosolic delivery**

Cytosol

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Figure 2-11 Proposed mechanism of amph-NP mediated delivery of small molecules into the cytosol.
To validate this hypothesis, we delivered a TGFβ inhibitor, a potent small molecule that can reverse immunosuppression in the tumor microenvironment, either in its soluble form or loaded in amph-NPs to murine CD8+ T cells in vitro. The process is illustrated in Figure 2-12. The concentration of drug per cell was determined by HPLC following cell membrane permeation and small molecule extraction in ethanol. Strikingly, amph-NPs enhanced small molecule delivery into T cells by at least 15-fold compared to free drug added to cells at the same concentration. Further, soluble drug delivered at a 10-fold higher dose resulted in 50% lower drug concentration in CD8+ T cells compared to drug delivered by amph-NPs. This result showed that the dual properties of membrane-embedding and small molecule entrapment of amph-NPs allows for enhanced drug delivery efficiency even in vitro. Given this promising result, applications such as loading adoptively transferred T cells with high concentration of TGFβ inhibitors before infusing T cells back to tumor-bearing recipients to improve cancer treatment are under current investigation.

Figure 2-12 Enhanced drug delivery to CD8+ T cells by amph-NP mediated delivery in vitro. Naïve CD8+ T Cells were isolated from C57BL/6 splenocytes and incubated...
with TGFb-i loaded NPs (5 ug/mL) or soluble TGFbi (5 ug/mL, 25 ug/mL, 50 ug/mL) for 4 hours at 37 °C in complete T cell media.

2.7 Conclusions

Many small molecules are under intensive investigation as new pharmaceuticals for cancer and infection treatments. They are potent, have well-defined structures and are often cost effective. However, many of them have very limited solubility in water and have intolerable off-target toxicity. If a hydrophobic molecule cannot traverse the environments and membranes it encounters en route to its cytosolic target, then the drug cannot be effective. In this chapter, a new method using amph-NPs to solubilize hydrophobic small molecules in aqueous solution was demonstrated. We showed that this method is generally applicable to a variety of different small molecules: hydrophobic immunomodulatory drugs, antibiotics, and chemo drugs. We further showed that utilizing the membrane-embedding property of amph-NPs, small molecule delivery to CD8+ T cells was significantly improved in vitro. Therapeutic effect of an immunomodulator delivered in its free soluble form or with nanoparticles will be discussed in chapter 5.

While in vitro testing of various new hydrophobic small molecules can demonstrate desirable therapeutic effects, the same molecules generally suffer from exceptionally rapid clearance in vivo. Doses required to achieve the observed in vitro effects often cause systemic toxicity. Strategies to deliver concentrated small molecules via NPs to targeted cells in vivo will be discussed in chapter 6.
3 Amph-NP interactions with mammalian cells

3.1 Introduction

If amph-NP’s membrane embedding property observed in synthetic membranes and simulation snapshots is applicable to real cell membranes, then this feature becomes attractive for enabling the delivery of otherwise undeliverable therapeutics (e.g. hydrophobic small molecules, peptides, proteins, oligonucleotides). In giant multilamellar vesicle (GMV) model membrane studies, we showed that BODIPY-labeled amph-NPs embed in outer membranes and transit into inner membranes, absorbing within the whole membrane structure without rupturing membranes (Figure 2-5). In this chapter, we investigated the cytosolic distribution of amph-NPs in various cell lines, ranging from cancer cells such as B16F10 melanoma, 4T1 breast cancer, LLC Lewis lung carcinoma, and MC38 colon carcinoma to leukocytes (splenocytes and RAW-blue macrophage cell lines) and erythrocytes. If amph-NP can pass the energy barrier posed by electrostatic repulsion with anionic cell membranes and proceed to embed within membranes, then according to our simulation results, it reaches a thermodynamically stable form with a decreased total free energy. To simplify our testing to solely membrane interactions and rule out the complexities that often come with live cells such as endocytosis, we chose red blood cells (RBCs) as the first model, because endocytosis does not occur in RBCs. We further investigated amph-NP interactions with splenocytes, which include various immune cells relevant to immunomodulation. Finally, to test whether membrane transit property remains true in live cells, we packed amph-NPs in multilamellar lipid nanocapsules (Au-NCs), and asked if they transit from their originally embedded membrane to other intracellular membranes/hydrophobic protein domains. Finally, we investigated the influence of glycocalyx on the ability of amph-NP to disperse in the cytosol, and showed that cell membrane composition regulates amph-NP uptake and cytosolic distribution.38

In addition to membrane-embedding MUSOT amph-NPs, we also included non-embedding NPs as control particles. AuNPs (2-4 nm) coated with monolayers of pure
MPSA (short ligand terminated with sulfonate group) or a 1:1 mixture of MPSA and OT (longer hydrophobic ligand) NPs were included as controls. Simulation results suggested that MUS and MUSOT amph-NPs (of different sizes and different ligand ratio) are capable of membrane embedding (Figure 3-1). Therefore, in this chapter, we focused on MUSOT amph-NPs (2.18 ±0.47 nm) as delegates for membrane-embedding NPs in most cell types unless stated otherwise. The size distribution of this batch is shown in Figure 3-2.

Figure 3-1 Illustration of NP's size and composition effect on membrane insertion. Adapted from Reid Van Lehn's MIT doctoral thesis.

Figure 3-2 Size distribution of MUSOT amph-NPs (2.18 ±0.47 nm).
3.2 Experimental Methods

**Thin-sectioned biological samples for TEM.** Thin-sectioned electron microscopy was conducted to more precisely determine the location of the amphiphilic gold nanoparticles in or on affected cells. After 5 hours of incubation of 0.6 mg/mL 1:1 MUS:OT gold nanoparticles with *E. Coli* (5 million cells/mL) in the presence of antibiotics (cefotaxime) at 37°C, the cells were washed 2X with phosphate-buffered saline pH 7.4 (PBS) to remove unbound nanoparticles. The cells were fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4) for 1 hr at 4°C, pelleted, and post fixed in 1% OsO4 in veronal-acetate buffer. The cell pellet was stained in block overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH 6.0), then dehydrated and embedded in Embed-812 resin. Sections were cut on a Reichert Ultracut E microtome with a Diatome diamond knife at a thickness setting of 50 nm, stained with uranyl acetate, and lead citrate. Thin-sectioned TEM sample grids were prepared by Nicki Watson at the Whitehead Institute.

**Synthesis and characterization of Au-NCs.** Lipids in chloroform (1.26 μmol total lipid, 1:1 molar ratio of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB PE), all lipids from Avanti Polar Lipids, Alabaster, AL) were dispensed to glass vials, and the organic solvents were evaporated under vacuum overnight to prepare dried thin lipid films. Lipid films were rehydrated in 10 mM bis-tris propane buffer (pH 7.0) containing amph-NPs and ovalbumin (Life Technologies) at indicated concentrations for 1 h with rigorous vortexing every 10 min, and then sonicated in alternating power cycles of 6W and 3W in 30 s intervals for 5 min on ice (Misonix Microson XL probe tip sonicator, Farmingdale, NY). The liposomes formed in this first step were induced to undergo fusion by addition of CaCl2 at a final concentration of 10 mM. The resulting multilamellar vesicles were incubated with 1.5 mM DTT (maleimide:DTT molar ratio of 2:1) for 1 h at 37 °C to conjugate opposing bilayers of maleimide-functionalized lipids and form crosslinked nanocapsules; the resulting vesicles were recovered by
centrifugation at 14,000g for 4 min, and washed twice with deionized water. Finally, the Au-NCs (0.3 mg/mL lipids) were incubated with 2 kDa PEG-SH (Laysan Bio, Arab, AL) in a 3-fold molar excess of PEG-SH to maleimide groups for 1 h at 37 °C to PEGylate the particle surfaces. As-synthesized Au-NCs were diluted 2 times and passed through a sterile filter twice (200 nm membrane Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Filters) to remove aggregates. The resulting particles were centrifuged and washed 3 times with deionized water, then stored in phosphate-buffered saline (PBS) pH 7.4 at 4 °C. Particle sizes were determined by dynamic light scattering (DLS, Brookhaven 90Plus Particle Size Analyzer). Gold:lipid mass ratios as a function of Au-NC diameter were measured using DLS (for size), UV-vis (for gold concentration) and infrared spectrometry (Direct Detect, EMD Millipore, for lipid concentration) after collecting 4 different size fractions of Au-NCs by CL4B gravity columns. Briefly, 200 uL of as-synthesized Au-NCs in PBS were added to CL4B gravity columns and manually collected four different fractions. Au-NC size distribution in each fraction was determined by DLS. Au-NCs was then disrupted with 0.1% triton-x 100, and gold mass in each size fraction was determined by UV-vis absorption at 520 nm, while lipid mass was determined by Direct Detect lipid concentration measurement.

Confocal imaging and colocalization determination. Cells were seeded in an 8-well chamber (Fisher Scientific Lab-Tek™ II Chambered Coverglass) at 60,000 cells per well in 500μL cell culture medium. The cells were allowed to grow overnight to about 80% confluence, and then treated with prepared solutions (typical dosage: 250 nM amph-NPs) for 3 hours or 24 hours. Cells were washed twice with PBS and subsequently imaged in RPMI (phenol red free) medium. Confocal laser scanning microscopy was performed on a Zeiss LSM 510 using a 63X oil lens, with excitation wavelengths being 488nm, 543nm, and 633 nm. Colocalization indices and scatter plots of red (OVA) and green (gold) channels were determined by ImageJ software using the FIJI plugin. Representative confocal images (N=3 per cell type) were imported to FIJI and regions of interest containing 3-4 cells were defined prior to Pearson’s correlation coefficients (PCC) determination. Statistical significance of PCC obtained was determined by the Costes test.
3.3 Amph-NP interaction with cells

We first investigated whether amph-NP’s membrane embedding property observed in GMV model membranes (80% DOPC and 20% DOPG) translates into embedding within a real cell membrane. Red blood cell membranes are constituted of 30% zwitterionic choline-containing phospholipids and 25% sphingomyelin on the outer layer, and zwitterionic phospholipids like phosphatidyl ethanolamine (28%) and phosphatidyl serine (14%). To cope with stress coming from passage through narrow capillaries while they circulate in the blood stream, RBC membranes are highly flexible and mechanically stable (Figure 3-3). RBC cytosolic proteins form structures that connect with outer membranes, rendering the entire cell flexible and resistant to rapid deformation.

![Red blood cell membrane structure and composition. Adapted from David Stoke’s lab webpage, NYU school of medicine.](image-url)
Human RBCs were incubated with MUSOT amph-NPs for 16 hours at 37 °C and thin-sectioned, negatively stained cells were imaged via transmission electron microscopy. Compared to larger amph-NPs (5-6 nm), smaller amph-NPs (2-3 nm) were found associated with RBC membranes more densely and closely (Figure 3-4). No particles were found in the cytosol of RBCs.

![Figure 3-4 TEM images of red blood cells treated with amph-NPs of different core sizes. (a) Small MUSOT (2-3 nm gold core diam.) were more tightly associated with RBC membranes than (b) larger MUSOT (5-6 nm gold core diam.). RBCs were incubated with 0.3 mg/mL amph-NPs at 37 °C for 16 h, and free NPs were removed by centrifugation prior to fixation.](image)

Next we evaluated the interaction of these particles with erythrocytes via confocal microscopy. RBCs were incubated with BODIPY-labeled MUSOT amph-NPs (3 nm core diam.) for 3 hours at 37 °C and samples were directly imaged via confocal
microscope without removal of free amph-NPs. Confocal results shown in Figure 3-5 a suggested that amph-NPs do interact strongly with RBCs. However, given the resolution of confocal images, it is reasonable to expect that the observed level of fluorescence should represent more than “sparse” NP decoration on RBC membranes shown in the TEM images (Figure 3-4). It is plausible that some hydrophobic BODIPY dissociated from gold surface, leading to increased signal intensity from dyes that do not represent NPs. It could be that amph-NP association with RBC membrane is meta-stable, and that numerous centrifugation and dehydration steps performed prior to TEM sample preparation removed most of NPs from the membranes.

One of the biomedical applications for amph-NPs is to serve as drug carriers for immunity modulation. Therefore, it is crucial to characterize their interaction with immune cells. As shown in Figure 3-5 b amph-NPs were internalized by splenocytes, however, given the size of NPs and the resolution of confocal microscopy, imaging tools that provide better resolution are required. Thin-sectioned electron microscopy was conducted to more precisely determine the location of the amph-NPs in cells. Among these leukocytes, we first investigated amph-NPs' interaction with macrophages, a class of phagocytes that are important in antigen presentation.

![Confocal Images](image)

**Figure 3-5** Confocal Images of (a) splenocytes and (b) red blood cells incubated with BODIPY-labeled amph-NPs. Cells were incubated with NPs at a concentration of 0.35 mg/mL at 37 °C for 3 h.
RAW-blue cell lines are derived from RAW 267.7 macrophages. RAW-blue macrophages were incubated with 0.38 mg/mL of BODIPY-MUSOT amph-NPs for 6 hours at 37 °C in serum containing DMEM media and unbound NPs were removed prior to TEM sample preparation or confocal imaging. Confocal results (Figure 3-6 a) showed obvious punctate spots, suggesting that a high number of amph-NPs were confined in the endosomes. In agreement with confocal results, TEM images showed that endosomes (highlighted with red arrows in Figure 3-6 b) were densely packed with amph-NPs, providing greater contrast even at low magnification due to significantly higher electron density of gold compared to organic substances. Although some NPs were found in non-endosome-like structures, the majority were found confined in endosomes. It appears that amph-NP were associated with endosomal membranes/endosomal proteins shown in gray contrast (Figure 3-6 c).
Figure 3-6 MUSOT amph-NPs associated with endosomal membranes/proteins in RAW-blue macrophages. (a) Confocal and (b-c) TEM images of cells incubated with 0.38 mg/mL of BODIPY-labeled amph-NPs for 6 hours. (b) Representative TEM image of cells with gold-loaded endosomes (highlighted with red arrows) at low magnification. (b) High mag TEM image showing the association of NPs with gray contrast area (likely proteins and membranes) in endosomes.
Another possible application is to use amph-NP as chemotherapy drug (e.g., doxorubicin) carriers for tumor targeted therapies. To investigate the cytosolic distribution of amph-NPs in cancer cells, we treated B16F10 melanoma and 4T1 breast cancer cells with BODIPY-labeled amph-NPs or control particles (MPSA NPs and MPSA/OT NPs). MPSA NPs were assumed to be non-membrane embedding due to their insufficient hydrophobic surface area. MPSA/OT NPs were included as another control to investigate the effect of increased hydrophobicity.

Following incubation with 4T1 cells for 3 hours, punctate spots shown in Figure 3-7 a revealed that amph-NP were likely confined in endosomes. Twenty four hours post incubation, some cells appeared to be rounded and the cytosolic distribution remained punctate with a diffuse fluorescence background. In contrast to 4T1, amph-NP achieved broad cytosolic dispersion without obvious punctate spots in B16F10 cells (Figure 3-7 b). At 24h post incubation, cytosolic fluorescent signals increased, suggesting increased uptake of amph-NP with time. Attempts to test control particles using confocal were unsuccessful due to technical difficulties for dye conjugation. However, TEM could overcome this problem, and results are discussed in the next paragraph.

![Confocal images showing amph-NP distribution in two different cancer cell lines. (a) 4T1 breast cancer and (b) B16F10 melanoma cells were incubated](image-url)
with 0.2 mg/mL of BODIPY-labeled MUSOT amph-NPs for 3h or 24h in cell culture medium at 37 °C and then imaged by confocal microscopy.

TEM images revealed quite distinct distributions of the amph-NPs in 4T1 cells. Free amph-NPs were detected associated with both the plasma membranes and cytosolic cellular membranes of these cells, as shown in Figure 3-8. A majority of amph-NPs were associated with multivesicular bodies (MVBs) that are known for the clearance of protein aggregates. Interestingly, significantly less MVBs were found in 4T1 cells without NP treatment, suggesting that the observed prevalent MVBs in amph-NP-treated 4T1 cells were associated with the presence of amph-NPs.

![Figure 3-8 Thin-sectioned TEM images of 4T1 breast cancer cells incubated with free amph-NPs (2-3 nm) for 24 hr (scale bars (a-b) 100 nm, (c) 500 nm). Arrows highlight amph-NPs dispersed among endosomal and intracellular membranes.](image)

In B16F10 cells, TEM showed that the majority of amph-NPs were found both dispersed in the cytosol (block arrows, Figure 3-9 a) and associated with endosomal structures (line arrows, Figure 3-9 a). Notably, amph-NPs trapped in endosomes of these cells appeared to be associated with/surrounded by electron-dense material, suggesting association with lipid or proteins within the endosomes, as highlighted by the arrows in Figure 3-9 b. Larger MUSOT amph-NPs (5 nm) showed a similar cytosolic distribution...
pattern in B16F10 cells (Figure 3-9 c). Comparing the size and quantity of endosomes, B16F10 appeared to have more but smaller (100-150 nm)-sized endosomes, while 4T1 appeared to have fewer but large (500-700 nm) MVBs. This explains the confocal results where punctate spots were observed only in 4T1 but not B16F10 cells.

Figure 3-9 Thin-sectioned TEM images of B16F10 melanoma cells incubated with free amph-NPs for 24 hr. (a,b) Small amph-NPs (2-3 nm) localized in the cytosolic space and endosomes. Line arrows in (a and b) highlight amph-NPs in endosomes; Block arrows in (a) highlight amph-NPs dispersed in the cytosol. (c) Larger amph-NPs (5 nm) showed a similar cytosolic distribution. Scale bars = 250 nm (a), 100 nm (b), 200 nm (c).

To investigate if ligand chemistry alters cytosolic distribution, B16F10 and 4T1 cells were incubated with control non-embedding MPSA NPs or MPSA/OT NPs for 24 hours at 37 °C. In contrast of MUSOT or MPSA/OT treatment, B16F10 cells treated with MPSA NPs showed significantly reduced intracellular concentrations of particles (Figure
3-10 a)—no NPs were visible in low magnification mode of TEM, suggesting lack of contrast due to low concentration in any specific regions. A high magnification image (Figure 3-10 b) showed that MPSA NPs colocalized with grey regions (likely proteins or membranes) in endosomes. As shown in Figure 3-10 c, low magnification images showed many intracellular regions packed up with MPSA/OT NPs, suggesting that increasing hydrophobic ligands on NPs significantly enhanced NP uptake in B16F10 cells.

Figure 3-10 Control MPSA NPs (a-b) concentration in B16F10 cells was significantly lower than MPSA/OT NPs (c-d) 24h post incubation at 37 °C. (a) Low magnification image of cells treated with 0.2 mg/mL MPSA NPs for 24h. (b) High magnification image showing MPSA NPs confined in endosomes. (c) Low
magnification image of cells treated with MPSA/OT NPs. (d) MPSA/OT NPs aggregated in the entire endosomes.

Next, we evaluated the effect of changing the ligand chemistry from MUS to MPSA in the 4T1 breast tumor cells. Strikingly, a high fraction of MPSA NPs surrounded the entire 4T1 outer membrane even 24h post incubation, and some NP packed membranes were observed being internalized likely via macro-pinocytosis (Figure 3-11 a). It is plausible that MPSA NPs were immobilized in the thick glycocalyx layer present on the outer membrane of 4T1 cells, forming ~150 nm thick NP laces (Figure 3-11 b). More studies focusing on the influence of glycocalyx on NP uptake will be discussed in the next sub-chapter. Finally in this chapter we tested if the effect of NP hydrophobicity on increased cellular uptake (shown in MPSA/OT treated B16F10) is applicable to other cell types. In contrast B16F10 cells, densely packed endosomes was not observed in MPSA/OT NPs treated 4T1 cells (Figure 3-11 c). Moreover, MPSA/OT association with multivesicular bodies (MVBs) in 4T1 cells (Figure 3-11 a) was very different from what was observed in MUSOT (Figure 3-8 c). MPSA/OT selectively aggregated in certain region of MVBs while MUSOT tightly outlined membranes on each individual vesicle inside MVBs.
Figure 3-11 Striking surface packing of MPSA NPs (a-b) observed on 4T1 cell membranes. Significantly lower concentration of MPSA/OT NPs (c-d) was observed in 4T1 cells. All NPs were treated at concentration of 0.2 mg/mL for 24h at 37 °C.

(a) Low magnification image of 4T1 cells treated with MPSA NPs. (b) High mag image showing MPSA NPs densely packed in the outer membrane of 4T1. (c) Low mag image of cells treated with MPSA/OT NPs. (d) MPSA/OT NPs aggregated in specific endosomal regions.
3.4 Influence of the glycocalyx on inter-membrane transitions

The TEM results of MUS vs. MPSA NPs interacting with different cell lines (the 4T1 breast carcinoma cells in particular) suggest that the particles may interact with cell surface glycoproteins in a manner dependent on ligand chemistry. To qualitatively and quantitatively measure the ability of amph-NPs to transit from their originally embedded membrane to other intracellular compartments of cells, we loaded amph-NPs into interbilayer cross linked multilamellar vesicles (ICMVs) and investigated the colocalization index of amph-NPs with ICMV membranes in cells 24 h post incubation. ICMV nanocapsules are formed by fusion of anionic, maleimide-functionalized lipid vesicles with divalent cations, followed by addition of a membrane-permeable crosslinker (dithiothreitol (DTT)) that diffuses between the membranes and covalently crosslinks maleimide lipids of adjacent bilayers forming the capsule wall. To load gold nanoparticles in the capsule walls, we added amph-NPs to the aqueous buffer during rehydration of dried lipids, with the goal of forming precursor liposomes with amph-NPs embedded in the vesicle bilayers (Figure 3-12). We also included fluorescent ovalbumin (OVA) protein in the aqueous phase during lipid hydration as a model co-delivered drug cargo and tracer to label the aqueous core of the ICMVs. Fusion of the gold-loaded precursor vesicles and crosslinking the bilayers with DTT led to gold-loaded ICMV nanocapsules (Au-NCs). Remaining maleimide groups at the surfaces of the particles were quenched by capping with PEG-thiol to enhance the colloidal stability of the nanocapsules.
To examine gold particle entrapment efficiency, we loaded 2.2±0.5 nm diam. core MUSOT amph-NPs into ICMVs (Figure 3-13). The MUS and OT ligands protecting the gold core span approximately 1.6 nm, resulting in a total hydrodynamic diameter of 5.4 nm that is similar to the thickness of a lipid bilayer (4-5 nm). Post-PEG capping, the Au-NCs were spun down and the supernatant was collected; absorbance measurements on
these supernatants showed no detectable unentrapped gold remaining in the aqueous phase. Quantitative analysis of the loading efficiency of 2.2 nm core amph-NPs into ICMV capsules was performed via UV-vis and infrared spectrometry. As shown in Table 3-1, gold loading increased with increased concentrations of added amph-NPs, while the tracer OVA protein encapsulation decreased with increased Au loading.

Figure 3-13 TEM and Cryo-TEM micrographs of Au-NCs with encapsulated OVA. (a-b) Au-NCs were dried on grids followed by negative staining with phosphotungstic acid and TEM imaging. Scale bars 200 nm (a), 100 nm (b). (c) Cryo-TEM of Au-NCs in water (scale bar 100 nm). (d) Gold to lipid mass ratios as a function of NC diameter measured for a preparation of nanocapsules divided into 4 different size fractions. Error bars show the std. dev. of each size fraction. Adapted from reference [38]
Table 3-1 Quantitative analysis of gold and protein loading in ICMVs as a function of cargo concentration. Adapted from reference [38]

<table>
<thead>
<tr>
<th>amph-NP/OVA cargos added to synthesis</th>
<th>lipid capsule size* (nm)</th>
<th>OVA encapsulated (µg/ mg lipid)</th>
<th>amph-NPs encapsulated (mg/ mg lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.28 mg/mL amph-NPs 300 µg/mL OVA</td>
<td>242 ± 151</td>
<td>24.26</td>
<td>0.31 ± 0.024</td>
</tr>
<tr>
<td>0.7 mg/mL amph-NPs 300 µg/mL OVA</td>
<td>236 ± 32</td>
<td>20.45</td>
<td>0.35 ± 0.012</td>
</tr>
<tr>
<td>1.13 mg/mL amph-NPs 300 µg/mL OVA</td>
<td>254 ± 39</td>
<td>13.41</td>
<td>0.59 ± 0.021</td>
</tr>
<tr>
<td>No amph-NPs 300 µg/mL OVA</td>
<td>207 ± 67</td>
<td>67.27</td>
<td>N/A</td>
</tr>
<tr>
<td>No amph-NPs, No OVA</td>
<td>160 ± 23</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Measured by dynamic light scattering.

To examine the interaction of Au-NCs with tumor cells, we employed four cell lines: B16F10 melanoma, 4T1 breast cancer, MC38 colon carcinoma and LLC lung carcinoma cell lines. First, viability of both cell types 24 hours post-treatment with free amph-NPs, empty ICMV nanocapsules, or Au-NCs was evaluated by flow cytometry. The cells were transduced with GFP for the ease of determination of the live cell population; dying or dead cells were stained with DAPI. As shown in Figure 3-14, all treatments at doses as high as 250 nM amph-NPs (0.11 mM lipids and 250 nM amph-NPs for Au-NCs) yielded >80% cell viability. Thus, the amph-NPs and lipid carriers show little toxicity per se to these tumor lines, consistent with prior toxicity measurements made on a variety of other cell types with these particles.
Figure 3-14 Toxicity of ICMV, amph-NP, and Au-NC treatments. (a) Gating scheme for analysis of cell viability showing gates for live (DAPI low) and dead (DAPI high) cells. (b) Cell viability of B16F10 and 4T1 cells left untreated or incubated with empty ICMV nanocapsules (0.11 mM NCs), amph-NPs (250 nM), or Au-NCs (250 nM amph-NPs) for 24 hr at 37°C. Adapted from reference [38]

We next characterized the internalization and intracellular distribution of fluorescently-labeled amph-NP-loaded ICMV lipid nanocapsules. Confocal microscopy showed a homogeneous distribution of free amph-NPs within the cytoplasm of B16F10 cells (but excluded from cell nuclei) at both 3 hr and 24 hr time points (Figure 3-15). By contrast, amph-NPs delivered via lipid capsules were initially confined in endosomes with obvious punctate spots after 3 hours of incubation with cells. Amph-NPs were always co-localized with OVA fluorescence, suggesting that they remained associated with the lipid nanocapsules at this early time-point. However, after 24 hours, amph-NPs were distributing through the cytoplasm of cells, while OVA signals remained punctate (presumably confined to endosomes, Figure 3-15). This data suggests that amph-NPs can pass out of the ICMV nanocapsules into the cytosol or intracellular membranes of cells without overt disruption of endosomes, as endosomal rupture would have led OVA to also disperse into the cytosol. Strikingly, when 4T1 cells were incubated with Au-NCs, the protein and amph-NPs signals remained co-localized in punctate structures in the cells through 24 hr (Figure 3-16), suggesting that in these tumor cells, the majority of amph-NPs remained trapped in endosomes, at least through this time point.
Figure 3-15 Uptake of amph-NPs and Au-NCs by B16F10 melanoma cells. B16F10 cells were incubated with 250 nM Au (green) loaded in ICMV nanocapsules and imaged after 3 or 24 hr by confocal microscopy. ICMVs were co-labeled by encapsulated fluorescent ovalbumin protein (red). (Scale bars 20 um). Reproduced from reference [38]

Figure 3-16 Uptake of amph-NPs and Au-NCs by 4T1 breast cancer cells. 4T1 cells were incubated with 250 nM Au (green) loaded in ICMV nanocapsules and imaged after 3hr or 24 hr by confocal microscopy. ICMVs were co-labeled by encapsulated fluorescent ovalbumin protein (red). (Scale bars 20 um). Reproduced from reference [38]
To assess the subcellular distribution of amph-NPs delivered into each of the tumor cell lines, we also carried out TEM imaging on thin sections of treated cells. B16F10 cells incubated for 3 hr with Au-NCs showed apparent unraveling ICMV capsules within endosomes, and amph-NPs were still confined in these endosomes at this time-point, in agreement with the confocal data (not shown). However, after 24 hr, the vast majority of amph-NPs were found delocalized away from ICMV capsule fragments—amph-NPs were found dispersed into the cytosol and associated with distant intracellular membranes (Figure 3-17 b). Amph-NPs were not observed to associate with mitochondrial or nuclear membranes.

In 4T1 cells, TEM images revealed quite distinct distributions of the amph-NPs. Free amph-NPs were detected associated with both the plasma membranes and cytosolic cellular membranes of these cells. However, the majority of amph-NPs were localized within in multi-vesicular endosomes as shown in Figure 3-17 a. In agreement with the confocal imaging results, 4T1 cells that internalized Au-NCs showed little detectable amph-NP dispersion outside of large endosomes where the ICMV lipid capsules were trapped even after 24 hr. Thus, the fate of the membrane-penetrating amph-NPs at the subcellular level was dependent on the tumor cell type.

Figure 3-17 Thin-sectioned TEM images of (a) 4T1 and (b) B16F10 cells incubated with Au-NCs (equivalent to 250 nM Au) for 24 hr at 37 °C. Arrows highlight amph-NPs dispersed among endosomal and intracellular membranes. Reproduced from reference [38]
We hypothesized that the distinct behavior of amph-NPs in the two tumor cell types may reflect a more substantial glyocalyx present on the plasma membrane and interior membranes of endosomes of epithelium-derived 4T1 cancer cells compared to the melanocyte-derived B16 tumor cells. To confirm that this is a key distinction between the cell surface chemistry of B16F10 and 4T1 cells, we labeled the cells with fluorophore-conjugated wheat germ agglutinin (WGA), a lectin that binds sugars of the glyocalyx. As shown in Figure 3-18, WGA staining confirmed that 4T1 cells have a much more substantial glyocalyx lining the plasma membrane, which may form a barrier to amph-NP penetration through the cell surface or endosomal membranes. To determine whether this trend holds in other cell types, we also assessed the intracellular distribution of Au-NCs in two other tumor cell lines—MC38 (an epithelial colon adenocarcinoma) and LLC (an epithelial Lewis lung adenocarcinoma). As shown in Figure 3-18 a-b, the glyocalyx density of these two other tumor cells was intermediate between 4T1 and B16F10, and the intracellular distribution of gold particles was also intermediate between the complete cytosolic dissemination observed with B16F10 melanoma cells and the complete endosomal entrapment seen with 4T1 cells; LLC tumor cells that had higher WGA staining showed more endosomal entrapment of amph-NPs. We quantified the degree of colocalization between fluorophore-conjugated OVA protein delivered into endosomes by ICMVs vs. the co-delivered amph-NPs by measuring a colocalization index (Pearson’s correlation coefficient) for intensity of OVA signal vs. amph-NP signal at a given pixel in a set of confocal microscopy images for each cell type. As shown in Figure 3-18 c-d, there was a clear correlation between the strength of glyocalyx staining and the degree of endosomal entrapment (OVA colocalization) for amph-NPs delivered by the lipid nanocapsules. Thus, the ability of amph-NPs to escape ICMVs nanocapsules and disseminate to the cytosol appears to be regulated by the glyocalyx density on tumor cells. It is worth pointing out that there may be other governing factors involved in the observed cytosolic distribution, as glyocalyx is only one factor among numerous others that is distinct between four tested cell lines.
Figure 3-18 Association of glycocalyx thickness and colocalization index. (a) Confocal images of the glycocalyx of B16F10, 4T1, LLC and MC38 cells labeled with AF555-Wheat Germ Agglutinin (WGA). (b) Flow cytometry quantification of WGA staining of glycocalyx on 4T1, LLC, MC38 and B16F10 cells. ***P<0.0001, **P<0.01, *P<0.05 by ANOVA. (c) Colocalization of OVA and gold NP fluorescence in different tumor cells following Au-NC uptake. B16F10, MC38, LLC, and 4T1 tumor cells were incubated with Au-NCs carrying labeled amph-NPs and OVA as in supplemental Figure 3, and imaged by confocal microscopy. (a) Colocalization index (Pearson’s correlation coefficient) of fluorescence from gold and OVA in nanocapsules imaged in confocal microscopy as a function of glycocalyx density on
3.5 Conclusions

We demonstrated membrane interactions of MUSOT amph-NPs with erythrocytes by both confocal microscopy and transmission electron microscopy. Smaller amph-NPs (2-3 nm) were observed embedded within RBC membranes; and larger amph-NPs (5-6 nm) were found loosely bound to RBC surfaces rather than embedding. Cytosolic distribution of amph-NPs in 4T1 and B16F10 cancer cells was well characterized by confocal and TEM. In B16F10 cells, amph-NPs were found both freely in the cytosol and confined in endosomal membranes/proteins regardless of their sizes (2-3 nm or 5-6 nm). In 4T1 cells, increased formation of multivesicular bodies (MVBs) was observed in amph-NPs treated cells compared to untreated cells. Amph-NPs were found embedded within membranes of individual vesicles in MVBs. Control short ligand, non-embedding MPSA NPs formed ~150 nm thick layers throughout the entire 4T1 outer membrane; notably this was not observed in B16F10 cells which have a significantly thinner glycocalyx layer. In MPSA/OT NPs treated cases, high numbers of concentrated NP-packed endosomes were observed in B16F10 cells but not 4T1 cells. Finally, among the four cancer cell lines tested: B16F10, 4T1, LLC and MC38, we observed a correlation between increased glycocalyx thickness and decreased cytosolic dispersion, suggesting that membrane compositions play a role in tuning the membrane embedding property of amph-NPs. Interaction of amph-NP with RAW-blue macrophage cell line suggested most NPs were confined in endosomes 6h post incubation. More studies on amph-NP’s interaction with non-phagocytes such as T cells will be discussed in chapter 5.
4 Biodistribution of Amph-NPs

4.1 Introduction

Nanoparticles' size, ligand chemistry, shape and charge all play important roles in their biodistribution and clearance. One major concern of introducing inorganic NPs to the clinic is their long-term accumulation in tissues. It is reported that solid NPs smaller than 5 nm are cleared via the kidneys efficiently and may be of interest as safe delivery vectors for clinical application. Many strategies have been investigated to achieve targeted delivery. Experimentation with PEGylated nanoparticles given intravenously for systemic delivery has demonstrated reduced non-specific protein adsorption, which increases blood circulation half-life. However, administered subcutaneously to target lymphatic tissues, they may not accumulate in lymph nodes efficiently.

In this chapter, we investigated amph-NPs' biodistribution post intravenous (i.v.) injection and subcutaneous (s.c.) injection. Conventional tools such as inductively coupled plasma atomic emission spectroscopy (ICP-AES), histology and flow cytometry were used. ICP-AES provides insight on bulk organ distribution; histology and flow cytometry provide cellular distribution data based on fluorescent dyes that are conjugated on amph-NPs. However, there are limitations to fluorescence-based tracking. For example, fluorescent dyes can be degraded or otherwise lost over time in vivo. ICP-AES is a very powerful, sensitive technique to detect gold in whole tissue samples but does not provide single-cell-level uptake information. To overcome these issues, we developed a novel label-free strategy based on the use of single-cell mass cytometry as a means to track gold nanoparticle uptake in vitro and in vivo. We employed a commercial Cytometry by time-of-flight (CyTOF) for this approach, a hybrid instrument combining flow cytometry with mass spectrometry. In CyTOF measurements, cells are labeled via antibodies conjugated with metal isotopes, and labeled cells are nebulized into a single cell stream that is vaporized by an inductively-coupled plasma, followed by quadrupole mass spectrometry analysis of the identities of the cloud of resulting ions (Figure 4-1). In this way, the device can directly count the number of metal ions associated with each cell, providing a quantitative readout. Current CyTOF instruments detect isotopes with
atomic weights ranging from 75 to 209 with 1 Da mass resolution. CyTOF permits up to 50 distinct metal isotope labels on a single cell to be detected and quantified simultaneously with minimal spectral overlap, permitting deep cellular phenotyping.\textsuperscript{45,46} Since its invention, CyTOF has offered a new means to unravel the complexity of biology, in applications ranging from deep phenotyping of tumors to immune system signaling pathways.\textsuperscript{47,48}

**Figure 4-1 CyTOF operation flowchart. Adapted from reference [44]**

Here we show that CyTOF is also ideally suited as a label-free method to track and quantify inorganic nanoparticles in tandem with highly multivariate cellular phenotyping, enabling quantitative analysis of the in vivo fate of inorganic nanomedicines at the single cell level. Using gold NPs as a representative inorganic nanomaterial with relevance for diverse biomedical applications\textsuperscript{49–54} (and which has already entered clinical trials as a drug carrier\textsuperscript{24}), we demonstrate the use of CyTOF to enumerate gold
nanoparticles in individual cells, with a sensitivity orders of magnitude greater than flow cytometry. We show that CyTOF enables precise quantification of NP concentrations in a variety of cell types, and overcomes challenges in fluorescence-based analysis of highly autofluorescent tissue cells. Using biodistribution insights provided by CyTOF, we demonstrate how the intrinsic dendritic cell-targeting properties of amph-NPs can be used to improve prophylactic cancer vaccine therapeutic outcomes.

4.2 Experimental Methods

**ICP-AES.** Cells or tissue samples were dissolved in 1 mL freshly prepared aqua regia for three days to digest AuNPs. Digested solution was then diluted in 3-4 mL of 2% nitric acid immediately prior to ICP-AES analysis on a Horiba Activa.

**Histology.** Inguinal lymph nodes were harvested 24h post subcutaneous injection of 0.3 mg AuNPs or saline. Tissues were embedded in OCT and frozen in liquid nitrogen. Frozen tissues were sectioned at 10 um thickness and placed on glass slides. Tissue sections were permeablized and fixed for 10 minutes in cold acetone stored in -20 ºC followed by a rinse in PBS for 5 minutes. Fixed tissue sections were incubated in 100 uL of 1:100 Antibody cocktail diluted in 1% BSA in PBS for 1 hour at 25 ºC. Tissue slides were rinsed in PBS, vectashed on glass cover slips, and imaged using a confocal microscope (Zeiss LSM 510) using a 63x oil lens with excitation wavelengths being 488, 543, and 633 nm.

**Cell culture and in vitro treatment conditions.** RAW-Blue™ cells derived from RAW 264.7 macrophages were purchased from InvivoGen and cultured in DMEM-based cell culture media. BODIPY-conjugated MUSOT NPs were dissolved in cell culture media at 100 µg/mL and diluted to various concentrations indicated in Figure 4-9 and Figure 4-10. One and a half million cells per well were seeded over night and the next day cells were treated with NPs for 6 hours at 37 ºC with 5% CO₂. Six hours later, excess NP solution was removed and cells were washed in PBS three times. Cells were collected
and split into three tubes for three separate analyses: flow cytometry, CyTOF, and ICP-AES.

**CyTOF cell isolation and antibody staining.** Lymph node cells were isolated by enzyme digestion. Briefly, fresh enzyme mix was prepared by dissolving 0.8 mg/mL of collagenase/dispase (Roche Diagnostics) and 0.1 mg/mL DNase I (Roche Diagnostics) in RPMI-1640 medium. Each lymph node was pierced by a forcep and incubated in enzyme mix at 37 °C on a shaker for 30 minutes. Cells and tissue fragments in enzyme mix were mixed vigorously with a 1 mL syringe (without needle) for 30 seconds and quenched by adding 10 mL of ice-cold PBS with 1% BSA and centrifuged at 1700 rpm for 5 minutes. Cell pellets were resuspended in staining buffer followed by antibody staining and fixing: cells were incubated with a selected antibody cocktail (Anti-Mouse CD45 (30-F11) -147Sm; Anti-Mouse CD3e (145-2C11)-152Sm; Anti-Mouse CD8a (53-6.7)-168Er; Anti-Mouse CD4 (RM4-5) -172Yb; Anti-Mouse CD45R/ B220(RA36B2)-176Yb; Anti-Mouse CD11b (M1/70)-148Nd; Anti-Mouse Ly-6G (Gr-1) (RB6-8C5)-174Yb; Anti-Mouse CD11c (N418)-142Nd; Anti-Mouse F4/80 (BM8)-159Tb; Anti-Mouse NK1.1 (PK136) -170Er; Anti-Mouse CD64 (X54-5/7.1)-151Eu; Anti-Mouse CD326 [EpCAM] (G8.8)-165Ho) at 25 °C for 30 minutes, excess antibodies were removed by centrifugation, and cells were stained with cell-ID™ Intercalator-Ir in fix and perm solution (detailed protocol available from Fluidigm website. https://www.fluidigm.com/productsupport/cytof-helios). Prior to CyTOF analysis, fixed cells were washed in MaxPar staining buffer twice and MaxPar water once to remove excess iridium. Cells were resuspended at 0.5-1 million per mL in 1:10 calibration beads (EQ™ Four Element Calibration Beads, Fluidigm) in water and 250 uL samples were analyzed by a Fluidigm CyTOF2 at a flow rate of 0.045 mL/min.

**Calculation of number of nanoparticles per cell.** Mean Au intensity in a cell population of interest measure by CyTOF is called “dual counts”. This value is proportional to the number of gold ions per cell—and it is the product of the integral over time of detector intensity multiplied by the “dual count coefficient.” Number of Au atoms per cell is calculated as the mean dual count per cell divided by the transmission coefficient of Ir 193. The transmission coefficient is calculated by dual counts of Ir in
tuning solution divided by the natural abundance of Ir 193, and further divided by the number of ions in 0.5 ppb Ir tuning solution.

**Peptide conjugation and quantification.** SIINFEKL peptide constructs were custom synthesized by LifeTein with the following structure: (N terminus) FITC-aminohexanoic acid (Ahx)-SIINFEKL-Ahx-cysteamide (C terminus), with purity > 95%. Lyophilized peptide was dissolved in DMF at 1 mg/ mL. A molar ratio of gold:peptide of 1:4 in DMF was mixed in a glass vial and placed on a shaker to allow coupling reaction for 4 days. To remove uncoupled peptide, the MUS/OT-peptide solution was first diluted in water (< 5% DMF) and spun at 3500 rpm for 15 minutes in an amicon 10kDa MWCO centrifugal tube. The above-mentioned washing step was performed repeatedly for a total of four times. To quantify peptide conjugation efficiency, 20 uL beta-mercaptoethanol (14.3M stock solution) and 20 uL DMF were added to an aliquot (0.1 mg in 60 uL H₂O) of purified MUSOT-peptide conjugates and allowed to react for 48 hours on a shaker at 25°C. Peptide conjugation efficiency was determined by fluorescence readout of FITC at excitation of 488 nm and emission of 520 nm using a standard curve made using uncoupled MUSOT particles doped with known amounts of peptide construct subjected to the same reaction conditions. The mass ratio of conjugated peptide to gold was determined to be ~ 51 µg peptide per mg gold, which corresponds to ~ 9 peptide constructs per NP.

**Vaccine studies.** Eight week old female C57BL/6 mice were immunized (primed on day 1, boosted on day 14) with 8 µg of CpG (ODN 1826 VacciGrade, InvivoGen) mixed with SIINFEKL peptide (10 µg peptide conjugated-AuNP, 10 µg free peptide, 50 µg free peptide or 10 µg free peptide construct). Vaccines were formulated in 100 µL sterile saline with half of the volume injected subcutaneously on either side of the tail base. To monitor antigen-specific T-cells, mice were bled, and blood samples were processed as follows: 100 µL of blood was incubated with 500 µL ACK lysis buffer at 25°C for 5 minutes followed by centrifugation, then this process was repeated for a second round of lysis. Cells were incubated in tetramer staining buffer (PBS, 1% BSA, 5mM EDTA, 50 nM dasatinib), Fc block, and OVA tetramer (iTAG Tetramer/PE - H-2Kb OVA, MBL) in
the dark for 45 minutes at 25°C. Anti-CD8a (53-6.7) APC antibody (1:200) was added to cell solutions and incubated for an additional 15 minutes at 4 °C. Cells were washed twice in flow cytometry buffer containing 100 nM DAPI, and run on a BD FACS LSR Fortessa. Data was analyzed using FlowJo.

**Tumor challenge.** B16-OVA cells were a kind gift from Dr. Glenn Dranoff at the Dana-Farber Cancer Institute. B16-OVA cells were cultured in complete DMEM (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 4 mM L-alanyl-L-glutamine), maintained at 37°C and 5% CO₂, and passaged when 70-80% confluent. A challenge of 2.5x10⁵ B16-OVA cells was injected subcutaneously on the right flank of previously immunized mice in 50 µL of sterile saline. Tumor size was measured (longest dimension x perpendicular dimension) three times weekly, and an area was calculated by multiplying these dimensions. Mice were euthanized when tumor area exceeded 100mm². All animal work was conducted under the approval of the Massachusetts Institute of Technology (MIT) Division of Comparative Medicine in accordance with federal, state, and local guidelines.

**Intracellular cytokine staining.** PBMCs were isolated from immunized mice and cultured in RPMI supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 4 mM L-alanyl-L-glutamine with 10µg/mL SIINFEKL peptide. After 2 hours, Brefeldin A (1/1000, eBiosciences) was added to inhibit cytokine secretion. After 6 hours total incubation with peptide, cells were washed, stained extracellularly with anti-CD8a (53-6.7, eBioscience), fixed and permeabilized (BD Cytofix/Cytoperm), and stained intracellularly with anti-IFN-γ (XMG1.2, eBioscience) and anti-TNF-α (MP6-XT22, eBioscience). Cells were run on a BD FACS LSR Fortessa and data was analyzed using FlowJo.
4.3 Intrinsic lymph node targeting property of amph-NPs

Efficient NP delivery to lymph nodes may significantly enhance vaccine delivery and immunotherapy outcomes. We tested nanoparticles of equivalent core sizes (2-4 nm) for lymphatic transport and lymph node accumulation as a function of ligand chemistry (Figure 4-2). Four types of organic ligand shells were tested: Lipid membrane embedding amph-NPs composed of MUS or mixed MUS and OT ligands; PEG(4CH) NPs capped by tetra-ethylene glycols with carboxylic acid end groups; and PEG3k NPs coated by 3 KDa polyethylene glycols. In the following sub-sections, we demonstrate detailed studies of biodistribution—bulk organ distribution, histology, and single cell analysis at extended time periods post i.v. or s.c. injections.

**Figure 4-2** Ligand chemistry of four types of AuNPs tested in biodistribution studies.

4.3.1 Bulk analysis: ICP-AES and histology

In order for amph-NPs to impact immunosuppression-related diseases, they would ideally be delivered to lymphoid organs (lymph nodes and spleen) where lymphocytes and other leukocytes are concentrated. As a first step toward understanding the tissue targeting of
these amphiphilic particles in vivo, we analyzed the biodistribution of amph-NPs injected i.v. or s.c. in C57Bl/6 mice, and then quantified the amount of gold accumulating in different organs by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Strikingly, amph-NPs accumulated in lymph nodes to ~8-fold higher levels than all other tissues collected (spleen, liver, kidney, lung, blood, and bladder) in terms of mass amph-NPs per mg tissue. Notably, ~8% of the total injection dose was found in the lymph nodes (axillary and inguinal) 24 hours following s.c. injection (Figure 4-3). MUSOT amph-NPs accumulated in LNs 12-fold more than control PEG(4CH) NPs, providing evidence that surface chemistry of AuNPs impacts their LN targeting efficiency.

Figure 4-3  Biodistribution of MUSOT and PEG(4CH) AuNPs post s.c. and i.v. injection. (a-g) Percentage of total injection dose in organs of interest (LNs, blood,
spleen, livers, lung, kidneys, and bladder) as determined by ICP-AES. (h) Summary graph of NP distribution in all organs collected 24h post s.c. injection. All C57Bl/6 mice (n=3/group) received 300 ug of AuNPs (MUSOT or PEG4CH) either via tail base s.c. injection or tail vein i.v. injection.

To characterize the extent of lymph node targeting achieved by s.c. nanoparticle injection as a function of ligand shell chemistry, we injected groups of C57Bl/6 mice with MUSOT, MUS, PEG₃₉, or PEG(4CH) coated gold particles of similar core size (~3 nm) on one site subcutaneously near the tail base, and collected local and distal LNs from the left and right flanks of the animals 24 hr later (Figure 4-4).

Figure 4-4 Illustration and photographs of LNs in the single site s.c. injection experiment. MUSOT or PEG(4CH) AuNPs were injected on the left site of tail base (0.3 mg NPs), and the following LNs (both left and right sides) were collected: lumbar, inguinal, axillary and cervical LNs. Representitive photographs shown here were inguinal and axillary LNs 24h post s.c. injection.
ICP-AES quantification of gold in the tissues showed that amph-NPs (MUS or MUSOT) not only dispersed to the local (left flank) inguinal, lumbar, and axillary lymph nodes, but also reached the contralateral (right flank) nodes (Figure 4-5). Interestingly, no detectable NPs reached cervical LNs. Summing the uptake across all of these nodes, MUS amph-NPs showed the greatest accumulation with 10% of the injected dose accumulated at 24 hr. By contrast, control PEG(4CH) NPs injected into a single site accumulated only at the nearest draining LN (L-lumbar) with minimal dispersion to either distal or contralateral sites, and (PEG)_{3k} NPs had less than 0.5% of total injection in the local site, and an undetectably low number of NPs in all other LNs. Altogether amph-NPs accumulated in LNs 12-fold more than control PEG-NPs, providing evidence of the amphiphilic ligand surface chemistry’s importance for this LN targeting effect. It is plausible that the observed result is due to the amph-NPs superior tissue diffusivity and membrane embedding ability. Previously we found that tumor cells with a dense glycocalyx show a low uptake of amph-NPs (chapter 3), and we hypothesize that this efficient trafficking through tissues and lymphatic vessels to reach lymph nodes reflects the particles being blocked from entering lymphatic endothelial cells by their glycocalyx layer, providing a means of specific targeting to lymphoid tissues. Although more studies need to be carried out to better understand the mechanism of LN-targeting, we have demonstrated a possibility of the development of a single-dosed local treatment for drug delivery to the entire lymphatic system using amph-NPs.
Figure 4-5  ICP-AES quantification of Au NPs in lymph nodes 24h post single site s.c. tail base injection. Au NPs solubilized in PBS and 50 μL of 6 mg/mL was injected subcutaneously on the left side of tail base. Lymph nodes were collected 24h post injection and analyzed by ICP-AES.

Next, the nanoparticles’ distributions in lymph node tissue sections were investigated. Lymph nodes from mice dosed with 300 μg of NPs or 50 μL of PBS were excised 24 post s.c. injection. Tissue sections were imaged under confocal microscopy. Unstained lymph node sections showed concentrated amph-NPs in tissues, whereas PEG4CH were not visible in tissues macroscopically (Figure 4-6 a). To identify cell types that have high levels of amph-NP accumulation, tissues were immunohistochemically stained with antibodies against CD3, B220, F4/80 and CD11c. An example confocal image of a LN section from PBS treated mice stained with F4/80-FITC and CD11c-PE is shown in Figure 4-6 b. LNs from MUSOT injected mice showed colocalization of high amph-NP concentrations in F4/80+ macrophages (Figure 4-6 c, seen as black stains highlighted with red arrows in the brightfield/fluorescence overlay). Attempts to visualize amph-NP distribution in lymph nodes using BODIBY-labeled amph-NPs were unsuccessful. This is likely due to dye degradation and loss of fluorescence at extended time periods in vivo. To address this issue, we labeled amph-NPs with FITC-C_{11}-SH dyes prior to s.c. injection, and stained FITC-labeled amph-NPs with APC labeled anti-FITC antibodies for confocal imaging. APC signal (representing NPs) was observed in lymph node
sections (Figure 4-6 d), with similar accumulation patterns shown in dark spots in Figure 4-6 c. Notably, dimmer APC signal was observed throughout the interior compartments in LNs, revealing that some portion of amph-NPs were also taken up by other cell types. In the next sections, we will cover a more detailed study that revealed NP distribution in LNs at the single cell level.

Figure 4-6 Histology of LN sections. (a) Bright field images of unstained LN tissue sections. (b) LN section stained with FITC-F4/80 and CD11c-PE. (c) LN section from MUSOT amph-NP (300 ug) treated mice 24 post injection. Tissues were stained with B220-PE/Cy7, CD3-FITC, and F4/80-PE. Regions of cells containing high concentration of amph-NPs were highlighted with red arrows. (d) LN section from FITC-labeled MUSOT amph-NP (300 ug) treated mice 24 post injection. Tissues were stained with antiFITC-APC and F4/80-PE.
4.3.2 Amph-NP cellular distribution analysis using conventional flow cytometry

A nanoparticle’s fate in vivo is often very different from in vitro results. To compare the differences of amph-NP uptake in cell types in vitro and in vivo, we incubated cells isolated from LNs with BODIPY-MUSOT for 8 hours at 37 °C and analyzed NP uptake via flow cytometry. As shown in Figure 4-7 upper panel, all cell types tested contained similar concentration of NPs (MFI ~ 5x10^5). However, results from in vivo experiments showed distinctive differences of uptake in phagocytes vs. non-phagocytes. We assessed which cells in lymph nodes were acquiring amph-NPs by flow cytometry analysis of cells from inguinal LNs 4 hours following s.c. injection of BODIPY(650/665) dye-labeled amph-NPs. As shown in Figure 4-7 lower panel, all of the common lymphocyte populations in LNs showed some uptake of particles above background, with highest levels of particle uptake in the phagocytic cells- dendritic cells, macrophages (MΦ), and neutrophils.

In vitro or early time points in vivo, cellular distribution of BODIPY-labeled amph-NPs can be analyzed using flow cytometry; however, dyes conjugated via gold-thiol chemistry
were likely to degrade or exchange with other free thiols such as glutathione and cysteine that are present \textit{in vivo}, leading to undetectable fluorescent signals at extended time periods. In the next section, we demonstrate for the first time a precise, label free quantitation method to track amph-NP biodistribution at single cell level using mass cytometry.

4.3.3 Label-free Quantitation of NPs on the single-cell level via Cytometer by Time of Flight (CyTOF)

CyTOF enables label-free metal ion quantitation in a variety of cell types. We sought to test whether the biodistribution of gold nanoparticles of different ligands could be precisely mapped out using CyTOF (without the need of fluorophore conjugation). We synthesized gold NPs (AuNPs) with comparable inorganic core diameters but 3 different surface chemistries, which we have shown in the previous chapter to have distinct biodistributions and cellular uptake \textit{in vivo} (Figure 4-8 a): 3-mercapto-1-propanesulfonate (MPSA) NPs, coated by a dense layer of short sulfonate-terminated ligands that strongly interact with water; 11-mercapto-1-undecanesulphonate/1-octanethiol (MUSOT) NPs bearing an amphiphilic mixed ligand shell, which strongly interact with cell membranes; and poly(ethylene glycol) NPs sterically stabilized by PEG to reduce opsonization by serum components. The particles were relatively monodisperse with similar mean gold core diameters 2.5-4 nm and negative zeta potentials (Figure 4-8 b-d).
Our pilot experiments established that gold was readily detected by CyTOF analysis of cultured cells incubated with AuNPs. We first sought to compare the sensitivity of CyTOF and flow cytometry for detection of NP uptake, using MUSOT NPs conjugated with a BODIPY-alkanethiol fluorophore. RAW macrophages were incubated with titrated concentrations of BODIPY-MUSOT NPs for 6 hr, followed by washing and analysis by flow cytometry or CyTOF. Gold uptake in the cells was clearly detectable by CyTOF across this entire concentration range, whereas NPs at concentrations of 0.1 µg/mL or lower were not detected above background using flow cytometry (Figure 4-9 a-b). Calibration of the CyTOF detector (see Methods) enables a direct enumeration of
Gold ions, and thereby mean numbers of nanoparticles accumulated in individual cells. Gold detection saturated the CyTOF detector in cells incubated with 100 µg/mL particles, corresponding to an upper detection limit of ~1±10^6 particles per cell. Using the traditional bulk analysis method of inductively coupled plasma atomic emission spectrometry (ICP-AES) to independently validate the CyTOF measurements, we found that the CyTOF-determined count of gold nanoparticles per cell (averaged from 16,000 single cells) was in close agreement with the average gold content calculated from ICP-AES analysis of 5±10^6 cells (Figure 4-9 c).

Figure 4-9 CyTOF enables sensitive detection of gold nanoparticles in single cells with a wide dynamic range. RAW macrophages were incubated with BODIPY-labeled MUSOT NPs at 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, or left untreated for 6 hours at 37°C, washed 3 times, then analyzed by CyTOF or flow cytometry (n=3 samples/group). (a) Histogram of AuNP levels detected in cells treated at five different concentrations. (b) Median fluorescence intensity (MFI) of cells treated at five different concentrations as assessed by flow cytometry (n=3 samples/group). (c) Quantitative analysis of number of AuNPs per cell at five
different treatment concentrations acquired by CyTOF compared to parallel bulk measurements of AuNP uptake by ICP-AES. Shown are means±s.e.m.

Strikingly, the lower detection limit for CyTOF extrapolated to 10 NPs per cell (Figure 4-10). In order to detect such low concentrations of particles per cell via ICP-AES, a 10-fold greater number of cells is required. CyTOF was 2,400 times more sensitive than flow cytometry in detecting AuNP uptake, and provided a direct quantification of total gold particles per cell (rather than a relative fluorescence value).

![Graph and Table]

Figure 4-10 CyTOF can detect as few as ~10 gold nanoparticles (~3 nm in diameter) in macrophages. RAW blue macrophages were treated with titrated concentrations of MUS/OT-BODIPY NPs: 0.1, 0.01 0.005 0.0025, or 0.00125 µg/ml for 6 hours at 37°C. (a) Representative CyTOF gold histograms in RAW cells treated with various concentrations of MUS/OT-BODIPY AuNPs. (b) Mean number of NPs per cell determined from CyTOF data. (c) Regression with error shown by dashed lines (n=3 samples per concentration). * P < 0.05; *** P < 0.0001 by Bonferroni's Multiple Comparison Test compared to zero NPs per cell. Data points show mean ± s.d.
4.3.4 Amph-NP biodistribution in spleens post intravenous injection

Due to dye degradation, quenching, or physical detachment from NPs, flow cytometry was unable to detect any trace of fluorescence signals in all cells 24h post injection (dose at high as 20 mg/kg), even though ICP-AES showed high NP uptake in the organs of interest. Given our results shown in the last section, NP's distribution across many cell types of interest in vivo at extended time points may now be analyzed via CyTOF. To demonstrate this, BODIPY-labeled MUSOT amph-NPs were injected i.v. and spleens were excised 24 hours post injection. Isolated splenocytes were stained with metal-chelated antibodies or fluorophore-tagged antibodies followed by CyTOF or flow cytometry analysis. An example CyTOF gating strategy is shown in Figure 4-11 a. Across all cell types, increasing the dose of injected particles led to increased number of NPs per cell (Figure 4-11 b). In contrast, NP containing splenocytes analyzed by flow cytometer showed no signal difference compared to PBS treated group (Figure 4-11 b). Doses as low as 0.05 nmole per mouse led to NP accumulation in spleen cells quantifiable in CyTOF, as illustrated by histograms of NP accumulation in CD3+CD8+ T cells (Figure 4-11 c); whereas flow cytometry was unable to detect fluorescence above the background autofluorescence of the cells (which differed from cell type to cell type) for any injected NP dose (Figure 4-11 d). As shown in Figure 4-11 b, gold NP uptake in all cell subsets was relatively low at the 0.05 nmol dose, but at 0.15nmole, significantly increased accumulation is seen in phagocytic CD11b+CD11c+ dendritic cells and macrophages. At the 0.25nmol dose, a significant increase in particle accumulation is observed in all of the leukocyte subsets analyzed.

To evaluate the impact of NP surface chemistry on spleen cell subset accumulation, particles with each of the three different ligand chemistries shown in Figure 4-12 were injected intravenously into C57BL/6 mice, and splenocytes were analyzed 24 hr later by CyTOF. MUSOT NPs in splenocytes was significantly higher than that of PEG or MPSA NPs, with a preferential uptake observed in CD11b+CD11c+ dendritic cells. PEG NPs and MPSA NP had comparable biodistributions, with numbers
of NPs being highest in dendritic cells, F4/80+ macrophages, and CD11b+CD11c- cells.

Figure 4-11 Biodistribution of MUSOT amph-NPs in spleens 24h post i.v. injection via CyTOF. (a) Example CyTOF gating plots of mouse splenocytes isolated and stained with metal-chelated antibodies. (b) Analysis of number of NPs per cell across ten different cell types in vivo. Three different doses of BODIPY-MUSOT NPs were given intravenously: 10 µg, 30 µg or 50 µg, and 24 hours post injection spleens were collected and cells isolated stained followed by CyTOF or flow cytometry analysis. (c-d) Representative histogram of Au (c) or BODIPY (d) signal intensity on CD8+ T cells isolated from mouse spleens 24 hours post injection of PBS, 10 µg or 50 µg of BODIPY-MUSOT NPs.
Figure 4-12 Surface ligand’s effect on in vivo cellular distribution in mouse spleens. Biodistribution of in spleens 24h post i.v. injection. Three different NPs (MUSOT, PEG(4CH) and MPSA) or PBS were injected i.v., and spleens were collected 24 hours post injection followed by CyTOF analysis.

4.3.5 Amph-NP biodistribution in lung tissues post intratracheal injection

We next compared CyTOF and flow cytometry for detection of AuNPs taken up by cells in vivo. BODIPY-labeled MUS/OT particles, which we have previously shown exhibit cell penetrating properties by dispersing through cell membranes, were administered intratracheally into the lungs of mice. Lung tissues were collected at 2 hrs post injection, stained with antibodies to CD326 (conjugated with $^{165}$Ho or FITC for detection in CyTOF and flow cytometry, respectively, identifying airway epithelial cells), and then analyzed by the two methods in parallel. A significant autofluorescence signal from the digested tissue cells was observed in the BODIPY channel, a common issue in flow cytometry. However, following administration of MUS/OT particles, AuNP uptake was clearly detected in a fraction of both epithelial cells (CD326') and CD326 cells, accounting for approximately 13% of all lung cells (black gates in Figure 4-13 a-b). A very different
picture emerged from CyTOF analysis of replicate tissues treated in the same manner: relative to the minimal background signal observed in control saline-treated lungs, MUS/OT particle-dosed lungs showed that by 2 hr particles were detectable in virtually all of the cells in the tissue (Figure 4-13 a). While distinct AuNP⁺ CD326⁺ and CD326⁻ populations were observed corresponding to the AuNP⁺ populations detected by flow cytometry in terms of frequencies (black gates, Figure 4-13 a), the majority of the remaining epithelial and other lung cells were also clearly positive for MUS/OT particles (Figure 4-13 a-b). To verify that the discrepancy between CyTOF and flow cytometry was due to a failure of flow to detect low level BODIPY-NP signals above the background autofluorescence of the cells, we flow-sorted 5x10⁶ CD326⁻&BODIPY cells from lung tissues (red gate, Figure 4-13 a) and analyzed their gold content via traditional ICP-AES. The AuNP level in this cell population was non-trivial—38,000 particles per cell on average (Figure 4-13 c)–a value that was not statistically different from the mean AuNP content determined by CyTOF in the CD326 AuNP⁺ population (red gates in Figure 4-13 a and d).

The capacity to simultaneously label cells with multiple antibodies for phenotypic analysis provided additional insights: We intratracheally administered a low dose of MUS/OT NPs (1 μg), recovered lung tissues 24 hr later, and stained with a panel of 9 different metal-chelated antibodies to leukocyte cell surface markers for CyTOF analysis. Gating separately cells that were “Au low” vs. “Au high” (Figure 4-13 e), CyTOF revealed a CD45⁻CD11b lymphocyte population present only among the “Au low” cells, which included AuNP⁺ B-cells, CD4⁺ T-cells, and CD8⁺ T-cells (Figure 4-13 f-h). Alveolar macrophages (AMs), an important target for antimicrobial drug delivery were located in the “Au high” population (Figure 4-13 i-k), and these cells contained 8-fold more nanoparticles than dendritic cells (DCs) and 18-fold more gold than B/T-cells (Figure 4-13 l). Notably, at 24 hr no BODIPY signal was detectable in any cell population by flow cytometry, suggesting either degradation or loss of the fluorophore by this time point (data not shown). These results demonstrate that multiple issues associated with fluorescence detection of nanoparticles can be overcome through CyTOF analysis.
Figure 4-13 MUS/OT AuNPs are detected by CyTOF in all lung cell populations following pulmonary administration. (a-d) BODIPY-MUS/OT AuNPs (50 µg in PBS) or saline were administered intratracheally to C57Bl/6 mice (n=4/group,
pooled from two separate experiments). Two hours post administration, lung tissues were digested and stained with labeled antibodies followed by CyTOF or flow cytometry analysis. (a) Parallel CyTOF and flow cytometry analysis of lung cells stained with metal (165Ho)-chelated or FITC-labeled CD326 antibodies. (b) Mean percentages of epithelial and non-epithelial cells detected as AuNP-positive by flow cytometer vs. CyTOF analysis. (c) Five million cells defined as CD326 - BODIPY+ in flow cytometry (red gate in 3a upper right panel) for AuNP-treated or saline control lung tissues were sorted for bulk ICP-AES quantification of AuNPs per cell. (d) Number of NPs per cell determined from direct Au analysis in the CyTOF CD326 Au+ population (red gate in 3a lower right panel). (e-k) Representative CyTOF gating analysis of cells isolated from lungs 24 hours post i.t. injection of 1 µg MUS/OT NPs. (l) Histogram of gold intensity in B-cells (CD45+ B220+ CD3-), T-cells (CD45+ B220- CD3+), dendritic cells (DCs, CD45+CD11c+CD11b+CD64-), and alveolar macrophages (AMs, CD45+CD11c+ CD11b+CD64+).

4.4 Amph-NP based Vaccines

We finally tested the utility of CyTOF for guiding the design of novel gold nanoparticle-based therapeutics. We observed using bulk ICP-AES analysis of excised tissues that MUS/OT NPs injected subcutaneously in mice exhibited striking accumulation in draining inguinal and axillary lymph nodes (LNs), 13-fold higher than an injection of the same quantity of PEG NPs at 24 hrs post injection (Figure 4-14 a). To evaluate the cellular biodistribution of these particles in lymph nodes, AuNPs were injected s.c. and 24 hrs later LNs were excised, digested, and single cell suspensions stained with antibodies for CyTOF analysis. As shown by the example phenotypic panel gating and inset gold NP histograms, CyTOF detected MUS/OT particles in B220+ B-cells, CD4+ and CD8+ T-cells, CD11b+/CD11c+ dendritic cells, as well as neutrophils and F4/80+ macrophages (Figure 4-14 b). However, the greatest particle accumulation (~2-fold greater than CD11b+/CD11c+ DCs or T-cells) was detected in CD11b+/CD11c+ myeloid dendritic cells (Figure 4-14 c). Both PEGylated NPs and MPSA NPs that have the same
sulfonate functional groups on the ligand shell but much shorter alkyl chains showed much lower accumulation in all cell types analyzed (Figure 4-14 c).

Myeloid dendritic cells are an important antigen presenting cell population in the immune response. The strong lymph node tropism and preferential accumulation of MUS/OT particles in these DCs revealed by CyTOF prompted us to test these particles as a vaccine carrier. The following vaccine and tumor studies were performed by Kelly Moynihan (a graduate student in our lab). A model fluorophore-labeled peptide derived from ovalbumin (SIINFEKL) was conjugated to MUS/OT particles through an N-terminal cysteine, linking ~9 peptides per particle (Figure 4-14 d). C57Bl/6 mice were then vaccinated with free peptide or peptide-MUS/OT NPs mixed with CpG DNA (as adjuvant), with a boost of the same formulations administered on day 14. As shown in Figure 4-14 e-f, MUS/OT-mediated peptide delivery greatly increased the potency of the peptide vaccination, eliciting at peak ~6-fold more CD8+ T-cells than the equivalent dose of free SIINFEKL peptide, which was also not matched by a 5-fold higher dose of free peptide. This enhanced response did not simply reflect an effect of the linker sequence on the stability or antigen processing of the peptide, because immunization with the same FITC-SIINFEKL-linker construct that was conjugated to the particles elicited even weaker T-cell priming than neat SIINFEKL peptide (Figure 4-14 f). Vaccinated mice were challenged with ovalbumin-expressing B16F1O melanoma tumor cells at day 150. Fourteen-fold higher levels of cytokine-producing CD8+ T-cells were detected in the peripheral blood of MUS/OT-peptide vaccinated mice compared to free peptide-immunized animals 6 days after tumor challenge (Figure 4-14 g-h), and these animals were fully protected from tumor outgrowth (Figure 4-14 i). While much more should be done to fully understand the mechanisms of this striking enhancement in peptide vaccination, this example illustrates the power of single-cell inorganic NP analysis coupled with multiparameter phenotyping to develop novel nanomedicines.

In summary, single cell mass cytometry by time of flight allows straightforward quantification of heavy metal nanoparticle biodistributions in conjunction with highly multivariate phenotypic analysis. The broad elemental sensitivity of CyTOF makes this approach relevant for diverse inorganic nanomaterials. A limitation of this approach (shared by most bulk measurement techniques) is the inability of CyTOF to distinguish
the precise physical state of nanomaterials (aggregation state, potential for reductive dissolution in cells, etc.); like any other measurement approach complementary analytical techniques should be employed to obtain a complete picture of nanomaterials’ fate in vivo. However, the ability to track the cellular distribution of a wide range of inorganic nanomaterials should be of great interest for understanding the toxicology of nanomaterials, and will facilitate the design of potent and safe imaging agents, diagnostics, and therapeutics.
Figure 4-14  Preferential dendritic cell uptake of MUSOT nanoparticles detected by CyTOF correlates with effective vaccine delivery by peptide-conjugated AuNPs. (a, b, c) C57Bl/6 mice (n=3/group) were injected s.c. with 20 or 100 µg of MUS/OT, MPSA, or PEG AuNPs, and lymph nodes were excised, digested, and stained for CyTOF analysis 24 hr later. Shown are representative Au histograms showing AuNP levels in various leukocyte populations (a) and mean number of NPs per cell for lymph node cell populations (b-c). (d) Schematic structure of SIINFEKL peptide construct and coupling to MUS/OT NP surface. (e-i) C57Bl/6 mice (n=5/group) were immunized s.c. on days 1 and 14 with 8 µg CpG mixed with SIINFEKL-conjugated MUS/OT NPs (10 µg peptide), 50 µg SIINFEKL peptide, 10 µg SIINFEKL peptide, or 10 µg SIINFEKL peptide construct. Animals were then challenged with 2.5x10^5 B16-OVA tumor cells s.c. in the flank on day 150. Shown are representative flow cytometry plots of SIINFEKL tetramer staining (e) and mean SIINFEKL tetramer^+ CD8^+ T-cells in blood on day 29 (f), representative intracellular cytokine staining flow cytometry (g) and mean percentages of cytokine^+ T-cells (h) 6 days after tumor challenge, and tumor size measurements (i) and survival curves (j). Shown are means±s.e.m. * P < 0.05; ** P < 0.01, ***P < 0.001 by one-way ANOVA with Bonferroni post tests.
4.5 Conclusions

We report results showing dramatic lymph node targeting achieved by MUS or MUSOT amph-NPs in vivo, which when loaded with drugs, will facilitate immunomodulation in these sites where immune responses are orchestrated. This intrinsic lymph node targeting property was not observed in other Au NPs with similar core size but different ligand chemistry. Yet the mechanism of the observed high lymph node uptake and trafficking is still unclear, possible factors include anionic charges from sulfonate groups on MUS ligands, membrane-embedding property of long hydrophobic ligands, influence of glycocalyx layers and stability of NPs in vivo. Other control particles such as replacing sulfonate groups with zwitterionic groups on MUS ligands will help to elucidate possible mechanisms to design and optimize nanoparticle’s lymph node targeting efficiency.

Besides lymph node targeting, we further identified an antigen presenting cell type (CD11b+ CD11c+ cells) to which amph-NPs preferentially target in lymph nodes. Building on these findings, we conjugated thiolated SIINFEKL peptides onto amph-NPs using gold-thiol chemical bonds, and showed enhanced antigen delivery to dendritic cells in lymph nodes resulted in long-lasting anti-tumor immunity in T cells.

In conclusion, the lymph node tropic accumulation property of amph-NPs demonstrated promising results for vaccine delivery. This interesting biodistribution may also facilitate immunomodulatory drug delivery. Efforts on exploring small molecule immunosuppression-reverting drug DGKi delivered by amph-NPs will be discussed in the next chapter. Another application using amph-NPs’ lymph-node tropism to combat lymph-borne bacterial infections will be discussed in chapter 7.
5 Reversing PD-1 associated T cell Immunosuppression by Amph-NP Enabled Diacylglycerol Kinase Inhibition

5.1 Introduction

Effector CD8+ cytotoxic T lymphocytes (CTLs) are crucial mediators of anti-tumor immunity. They use a combination of lytic granule and receptor-mediated mechanisms to trigger apoptotic death of malignant cells. CTLs also employ nonlytic effector mechanisms such as the production of cytokines to orchestrate anti-cancer immunity. CTLs' high sensitivity and selectivity to directly or indirectly eradicate tumor cells render them attractive mediators for anti-tumor immunity. However, an important feature of developing tumors is active suppression of the host immune system. For example, in cancer patients, CD8+ T cells express the negative regulatory receptor PD-1, which is engaged by PD-L1 on tumor cells or immunosuppressive macrophages, resulting in impaired CD8+ T cell function. These dysfunctional CD8+ T cells became unresponsive to CD3 and CD28 co-stimulation. Diacylglycerol kinase (DGK), which inhibits TCR signaling pathways by catalyzing the conversion of diacylglycerol (DAG) to phosphatidic acid (PA), was reported to be elevated in the immunosuppressive CD8+ T cells. Although it is unclear whether DGK elevation is implicated in the immunosuppressive action of PD-1 signaling, it is known that the termination of DAG signaling and initiation of PA signaling pathway is responsible for the dampening effect of T cell activation (Figure 5-1).

Figure 5-1 Phosphorylation of DAG leads to the production of PA. Adapted from reference [64]
Notably, this DAG signaling inhibition phenomenon is reversible, and cell functionality can be restored by inhibition of DGK. There are two isoforms of DGK: DGKα and DGKζ; DGKα is particularly relevant in promotion of T cell anergy. To enable DGK modulation as a therapeutic strategy to enhance anti-tumor immunity, it is critical to provide cytosolic access of DGK inhibitors to T cells, because DGK is located in the cytosol (Figure 5-2).

Figure 5-2 DGKs are mediators for impaired T cell function. High levels of DGKs catalyze the phosphorylation of DAG and production of phosphatidic acid (PA), blunting signaling pathways such as Ras, ERK and AP1. Down regulation of IL-2 is one of the defects that resulted in impaired proliferative capacity in T cells. Adapted from reference [69]

Like many other small molecule inhibitors, DGKi is hydrophobic which renders it poorly soluble in physiological conditions. We have shown previously that DGKi partitioned
efficiently into the hydrophobic ligand shells of amph-NPs, in a manner dependent on particle size and ligand composition (Figure 2-8 and Figure 2-9). In this chapter, we explored amph-NPs as targeted cytosolic delivery vectors to deliver small molecule DGK inhibitors to T cells. Amph-NP’s interaction with T cells was characterized by TEM and confocal microscopy at various points. To optimize drug-NP incubation time for treatments, NP uptake kinetics in T cells in vitro were quantified by ICP-AES. Finally, we developed an in vitro model to study therapeutic effects of DGKi on PD-L1 mediated immunosuppressed T cells. The effect of DGKi on immunosuppressed naïve T cells as well as activated T cells was investigated.

5.2 Experimental methods

Confocal imaging. Naïve CD8+ T cells were isolated from the spleens of C57Bl/6 female mice, and labeled with CFSE. Cells were incubated with BODIPY-labeled MUSOT amph-NPs for 3 hours at 37 °C. Free NPs were removed by centrifugation and cells were imaged under confocal microscope (Zeiss LSM 510) using 63x oil lens with excitation wavelengths being 488, 543, and 633 nm.

TEM. Naïve CD8+ T cells were isolated from the spleens of C57Bl/6 female mice and 2 million cells were seeded on a 6-well TC plate coated with IgG isotype and αCD3 and soluble αCD28 were added to keep cells alive. NPs were incubated with cells (5 million cells per well) at a concentration of 0.08 or 0.25 mg/mL in complete T cell media (RPMI, 10% FBS, 1% penicillin-streptomycin, 1% Sodium Pyruvate, 1% MEM NEAA, 0.1% 2-mercaptoethanol) at 37 °C for 30 min, 3.5h, 18h or 24h. Free NPs were removed from cell pellets by centrifugation at 1700 rpm for 4 minutes in PBS. The washing step was performed twice. Cells were fixed in TEM cell fixative provided by Nicki Watson at the Whitehead institute. TEM thin-sectioning process is the same as described in chapter 3.

Kinetic studies. Spleens from pmel-1 female mice were collected and CD8+ T cells were activated by adding ConA and IL-7 and co-cultured with splenocytes for 3 days. Activated CD8+ T cells were isolated on day 4 and 10 millions of cells were used per
condition. Cells were incubated with 0.1 mg/mL of MUSOT amph-NPs at 37 °C for 15 min, 30 min, 4h, 6h, and 24h (triplicate wells per time point) in complete T cell media in the presence of coated αCD3 antibodies and soluble αCD28. Free NPs were removed by centrifugation for three times in PBS, and cell pellets were dissociated by the addition of 200 uL aqua regia. Samples were dissolved in aqua regia for two days at 25 °C. Five standards composed of known concentration of MUSOT amph-NPs were also dissolved in aqua regia. Prior to ICP-AES analysis, samples were diluted in 2% nitric acid to a total of 4 mL per tube.

**Naïve T cell in vitro experiment.** A day prior to T cell isolation, non-TC coated 24 well plate was pre-coated with antibody cocktails (total volume 300 uL per well): 1.2 ug/mL αCD3 + 8 ug/mL IgG isotype (or PD-L1) at 4 °C overnight. Next day, antibody-coated plates were rinsed with 1 mL PBS to remove unbound antibodies. CD8+ T cells were isolated from spleens of C57Bl/6 female 8 weeks old mice, and stained with CFSE as a proliferation tracer. A half million of cells were seeded on Ab-coated plates per well (triplicate per condition). Anti-CD28 was added to each well at a final concentration of 0.1 ug/ mL. Treatment solutions (free DGKi, DGKi-NP, free NP or PBS) were added and the final volume per well was brought up to 1 mL using T cell complete media. Four days post incubation at 37 °C, cells were centrifuged at 1700 rpm for 6 minutes twice and stained with αCD8-PE and DAPI prior to FACS analysis.

**Activated T cell in vitro experiment.** Spleens from pmel-1 female mice were excised and isolated splenocytes were treated with ConA (2 ug/mL) and IL-7 (1 ng/mL) for T cell activation. Three days post activation T cells were isolated using EasySep isolation kit per their protocol (Cat# 19753) and expanded by the addition of IL-2 (10 ng/mL). Non-TC coated 96 well plate was pre-coated with antibody cocktails (total volume 60 uL per well): 8 ug/mL IgG isotype (or PD-L1) and optionally 1.2 ug/mL αCD3 at 4 °C overnight. Next day, antibody-coated plates were rinsed with 200 uL PBS to remove unbound antibodies. Activated CD8+ T cells were stained with CFSE as a proliferation tracer. One hundred thousand cells were seeded on Ab-coated plates per well (triplicate per condition). αCD28 was added to each well at a final concentration of 0.1ug/ mL.
Treatment solutions (free DGKi, DGKi-NP, free NP or PBS) were added and the final volume per well was brought up to 200 uL using T cell complete media. Three days post incubation at 37 °C, cells were centrifuged at 1700 rpm for 6 minutes twice and stained with αCD8-PE and DAPI prior to FACS analysis.

5.3 Amph-NP interaction with CD8⁺ T cells

To reverse the immunosuppression that accompanies cancer or infection-driven inflammation, we tested the ability of amph-NPs to target suppression-reverting drugs to CD8⁺ T cells. Confocal images of CFSE (green)-labeled CD8⁺ T cells after 3 hours of incubation with BODIPY (red)-labeled amph-NPs showed that the NPs are avidly taken up by T-cells (Figure 5-3 a), with the majority of particles clustering in one sub-region of the cell. Flow cytometry analysis showed that high levels of particle uptake were uniformly observed in the entire T cell population (Figure 5-3 b). To further investigate the interaction of amph-NPs with T cell membranes and their uptake with subcellular resolution, we imaged T cells incubated with amph-NPs at various times via TEM. At 3 hr, amph-NPs were observed binding to the cell surface/plasma membrane of T-cells (Figure 5-3 c, left) and beginning to concentrate in endosomes as seen by confocal microscopy. By 18 hr, amph-NPs were observed both within multivesicular bodies and disseminating to other internal membranes of the T-cell (Figure 5-3 c, right). Thus, despite the low level of endocytosis displayed by T-cells in the steady state, amph-NPs efficiently entered lymphocytes to high levels.
Figure 5-3 Amph-NPs avidly enter CD8\(^{+}\) T cells. (a) Confocal images of CFSE labeled CD8 T cells (green) incubated with BODIPY- labeled MUS:OT amph-NPs (red) for 3 hours at 37°C. (Scale bars 20 µm) (b) Flow cytometry quantification of control CD8\(^{+}\) T cells and amph-NP treated CD8\(^{+}\) T cells. (c) Representative TEM images of CD8\(^{+}\) T cells incubated with amph-NPs (0.25 mg/mL) for 3 hours and 18 hours at 37 °C. (Scale bars 100 nm).

One of the factors important to nanomedicine is to understand the time required for sufficient amount of NPs to enter cells. This is especially useful for adoptive T cell therapy where T cells are boosted with supporting drugs \textit{ex vivo} and infused back into patients. A kinetic study of MUSOT amph-NP uptake in activated pmel CD8\(^{+}\) T cells suggested that throughout 24 hours of incubation, NP concentration in T cells reaches a plateau—10\% NP of total incubated dose was delivered into T cells at 4h post incubation (Figure 5-4).
Figure 5-4 Kinetics of MUSOT amph-NP uptake in CD8+T cells in vitro measured by ICP-AES. MUSOT amph-NPs (final concentration 0.1 mg/mL) were incubated with 4 million T cells at 37 °C for 10 min, 30 min, 4h, 6h and 24h. N=3 per time point.

Some of the small molecule hydrophobic drugs we tested have better loading efficiency in MUS than MUSOT NPs. Therefore, we also investigated the interaction of MUS NP (2.18 nm) with T cells via TEM. NPs were found in close proximity with T cell outer membranes 30 min post incubation at 37 °C (Figure 5-5 a). Surprisingly, a broad cytosolic distribution (rather than high dose in endosome-like compartments) was also observed in CD8+ T cells 30 minutes post incubation (Figure 5-5 b). At extended time points when endocytosis is likely occur, NPs were located in both endosomes and free in the cytosol (Figure 5-5 c). This distinctive result was likely due to the core size advantage of small MUS nanoparticles (2.18 nm in diameter) not the ligand chemistry.
Figure 5-5 TEM images of microtome-sectioned CD8+ T cells treated with all MUS amph-NPs (2.18 nm gold core diam.) at 0.08 mg/mL for 30 min or 24h at 37 °C. (a) NPs were associated with outer lipid membrane 30 min post incubation. (b) NPs were also found broadly distributed in the cytosol of T cells 30 min post incubation. Some NPs are highlighted with red circles. (c) NPs were found both inside and outside of endosomal compartments 24h post incubation.

To investigate whether the observed cytosolic dispersion of all-MUS and MUSOT NPs in T cells is unique to the membrane-embedding property of NP ligands, control non-
membrane embedding PEG(4CH) AuNPs were incubated with CD8+ T cells at 37 °C for 24h and imaged via TEM. Interestingly, as shown in Figure 5-6 a and b, NPs were found clustering into branch structures and loosely associated with outer cell membranes (unlike the tight membrane interaction observed in allMUS and MUSOT amph-NPs). These NP clusters/aggregates were wrapped up by membranes via endocytosis (Figure 5-6 c), and remained in the aggregated state in endo/lysosomes, but did not appear to access the cytosol (Figure 5-6 d). This result suggested that both ligand composition and size of amph-NPs are important factors to consider for optimal distributions in a particular cell type.
Figure 5-6 TEM images of microtome-sectioned CD8+ T cells (5 millions) treated with PEG(4CH) AuNPs (~3 nm) at 0.08 mg/mL for 24h at 37 °C. (a) Branched structures of clustered NPs formed outside of cell membranes. (b) These clustered NPs were not in immediate proximity to the membrane. (c) Clustered NPs were taken up by endocytosis (~150 nm vesicle formation). (d) NPs remain aggregated in endo/lysosomes. No free NPs were found in non-endosomal cytosolic space.
5.4 Effect of DGKi on naïve CD8\(^+\) T cells \textit{in vitro}

Studies of T-cell activation pathways have shown that diacylglycerol is an essential signaling intermediate in normal T-cell receptor (TCR) signaling.\(^{67,68,69}\) Diacylglycerol kinase (DGK), a cytosolic enzyme, inhibits TCR signaling by converting dialyglycerol (DAG) to phosphatidic acid (PA). Recently, studies in cancer immunotherapy have demonstrated that immunosuppression induced by tumors acts in part through elevated DGK activity, and small molecule inhibitors of DGK \(\alpha\) and \(\zeta\) have shown promise in boosting anti-tumor T-cell function \textit{in vitro} and \textit{in vivo}.\(^{70,71}\) In addition, elevated DGK activity is induced by a number of immunosuppressive T-cell surface receptors. Therefore, we sought to test the capacity of amph-NPs to deliver a DGK inhibitor (R59002, also referred to as DGKi in this chapter) into T cells as an immunosuppression-reverting drug. R59002 was first reported in 1985.\(^{72}\) The paper stated that it has an IC\(_{50}\) of 2.8 \(\mu\)M in inhibition of DGK in red blood cell membranes while other kinase activities remained unaffected. It was later reported that R59002 strongly inhibited type I DGK\(\alpha\) and moderately attenuated type III DGK\(\epsilon\) and type V DGK\(\theta\).\(^{73}\) We hypothesized that DGKi would reverse suppression induced through the receptor Programmed Death-1 (PD-1) expressed by activated T-cells, which has recently been shown to be a critical suppressive pathway during infection-induced immunosuppression (\textbf{Figure 5-7}).
Figure 5-7 PD-L1 mediated T cell immunosuppression is common in cancer, severe burns and infections. High cytosolic concentration of diacyl glycerol kinase is associated with immunosuppressed T cells. Inhibition of functional cytosolic DGK may reverse immunosuppression in T cells.

We loaded ~160 DGKi molecules per MUSOT amph-NP (3.15 nm) by a solvent exchange method, sequestering the drug within the hydrophobic inner core of the organic ligand shell. To compare the therapeutic effect of DGKi delivered in solution or via amph-NP carriers, CD8+ T cells were isolated from naïve C57BL/6 mouse spleens, and labeled with CFSE dye. Labeled T-cells were then cultured on TC plates coated with recombinant PD-L1, the ligand for PD-1 to mimic engagement of this suppressive pathway during infection. These suppressed (or control) T cells were then left untreated or co-cultured in the presence of 5 ug/mL of free DGKi, DGKi loaded in amph-NPs, or control amph-NPs lacking drug for 4 days, and T-cell proliferation was tracked by flow cytometry analysis of CFSE dilution. As shown in Figure 5-8 a, control T cells in each group cultured without PD-L1 suppression underwent substantial cell division, while cells cultured in the presence of PD-L1 suppression with no added drug showed completely blocked proliferation. Strikingly, while the free DGKi drug had no effect at this dose, delivery into the T-cells via amph-NPs led to ~50% recovery of the T-cell expansion (Figure 5-8 b). We tested whether the effectiveness of free DGKi was dose-dependent. Free DGKi (without NPs) was administered to T cells at various doses.
ranging from 2.5 ug/mL, 5 ug/mL, 10 ug/mL to 20 ug/mL. Free DGKi was completely ineffective in reversing PD-L1 mediated immunosuppression in naïve CD8+ T cells regardless of dose concentrations (Figure 5-8 c). At the highest dose tested (20 ug/mL), the viability of T cells (regardless of IgG or PD-L1 TC plate coating) was nearly zero.

Figure 5-8 MUSOT amph-NPs effectively deliver DGKi into naïve CD8+ T cells and reverse immunosuppression in vitro. (a) Structure of DGKi and (b) In vitro CD8+ T cell proliferation assay in the presence of immunosuppressing PD-L1 ligand with 5 ug DGKi treatment delivered in solution or via amph-NP carriers. (c) In vitro CD8+ T cell proliferation assay in the presence of immunosuppressing PD-L1 ligand with free DGKi treatment of 2.5 ug/mL, 5 ug/mL, 10 ug/mL and 20 ug/mL.

One of the possible reasons for the observed therapeutic effect achieved by NP delivery is that amph-NPs may embed within T cell membranes, and act as drug depot to sustainably release DGKi at the “sweet spot”, because DGKα is a cytosolic enzyme that
must relocate to the lipid membranes in response to exercise its function. Other possible reasons include improved solubility of DGKi and enhanced cytosolic delivery efficiency due to active membrane embedding rather than passive diffusion. All together, these promising in vitro data suggest that amph-NPs may serve as effective cytosolic delivery vehicles for reversion of immunosuppression, and more generally, for the delivery of many potential poorly water-soluble drugs to lymphocytes.

5.5 Effect of DGKi on activated CD8 T cells in vitro

As a step prior to investigation of DGKi’s effect on a tumor model in vivo, we tested its effect on activated T cells (rather than naïve T cells) to better mimic adoptive cell therapy protocols where activated T cells are administered to tumor bearing animals. The experimental set-up was similar to the one described in previous section for naïve T cells, except that cells were pre-pulsed with treatments (free DGKi, DGKi-MUSOT, or PBS) for 6 hours prior to seeding onto pre-coated PD-L1 plates (or control IgG plates). We pretreated activated T cells with DGKi prior to exposure to PD-L1 (rather than continuous treatment for 3-4 days) to mimic the scenario used in adoptive T cell transfer therapies. For in vivo adoptive transfer therapies, activated T cells will be isolated, expanded and pre-treated with DGKi prior to being infused back to tumor-bearing recipients. To test if DGKi can reverse immunosuppression on activated T cells, splenocytes were isolated from pmel-1 female mice, and T cells were activated in the presence of other leukocytes plus ConA and IL-7 for 3 days. Primary CD8⁺ T cells were isolated on day 4, stained with CFSE dyes and proceed to treatments. Activated CD8⁺ T cells were pulsed with free DGKi (5 ug/mL), DGKi loaded in MUSOT amph-NPs (equivalent to 5 ug/mL) or PBS for 6 hours at 37 °C followed by removal of free drugs/free NPs by centrifugation. These pre-treated T cells were seeded on a TC plate pre-coated with PD-L1 or control IgG, and cells were incubated at 37 °C for 3-5 days with or without αCD3 / αCD28 stimulation. An aliquot of T cells stained with CFSE were measured on day 1, as shown in Figure 5-9 a (purple peak). All T cells without αCD3 / αCD28 stimulation (regardless of the presence of PDL1 or control IgG) resulted in
minimal proliferative ability and low viability ( > 90% DAPI') on day 3 (Figure 5-9 a, orange peak). With αCD3 / αCD28 stimulation, PD-L1 negatively impact the proliferation capacity of T cells compared to control IgG group (Figure 5-9 a). Surprisingly, T cells pretreated with DGKi (with or without NP) prior to incubation with PD-L1 + αCD3 / αCD28 stimulation were unable to proliferate better compared to untreated cells (Figure 5-9 b). Pretreatment of T cells with three fold higher dose of DGKi (15 ug/mL) did not change the result (data not shown). Measurement of T cell proliferation at later time points such as five days post cell seeding also showed no difference in terms of therapeutic efficacy. Although the reason DGKi was ineffective on activated T cells in vitro in this setting remains unclear, many studies have shown the correlation of elevated DGKα in anergic T cells and unresponsiveness of T cells. More studies should be carried out to understand the role of DGKi on PD-L1 mediated T cell immunosuppression.

Figure 5-9  DGK inhibition (regardless of delivery method) is not effective on reversing immunosuppression in activated pmel-1 CD8+ T cells. CFSE-stained CD8+ T cells were pretreated with 5 ug/mL of DGKi (with or without MUSOT amph-NPs) for 6 hours at 37°C prior to being plated on IgG or PD-L1 antibody-coated TC plates. Note: “No stim.” denotes conditions where cells without αCD3/αCD28 stimulation.
5.6 Conclusions

CTLs play important roles in adaptive immunity in cancer and infections, rendering them desirable targets for therapeutic interventions. Immunity in cancer patients are compromised due to immunosuppressive factors produced by tumor cells. We sought to explore an immunosuppression-reverting drug DGKi's effect on reversal of immunosuppression in CTLs in vitro. In order to define therapeutic efficacy of such hydrophobic small molecules, two requirements must be met. First, a meaningful dose of DGKi must be delivered to cells. Second, delivered drug molecules must remain active and reach the cytosol where their target proteins (DGK) located in. Our solution to deliver meaningful dose of hydrophobic polar molecules to the cytosol of T cell is to use drug-loaded, membrane-penetrating amph-NPs as carriers. The question is: could amph-NPs transport concentrated drugs to T cells via membrane embedding and penetration? First, we studied amph-NP's interaction with T cells via TEM and confocal imaging. Clusters of MUSOT amph-NPs were found bound on or embedded with membranes of primary T cells, and at later time points they were mostly in embedded in endosomal membranes. Interestingly, allMUS amph-NPs (2.18 nm) were found freely dispersed in cytosolic compartments besides some found in endosomal membranes. PEG(4CH) AuNPs formed aggregates extracellularly and remained in their aggregation form in endosomes. Amph-NPs uptake in T cells in vitro reached a plateau at 4h post incubation—10% of total NPs were internalized by T cells throughout the course of 24 hours.

We demonstrated that DGKi delivered via amph-NPs recovered the proliferative capacity of PD-L1-mediated immunosuppressed naïve CD8+ T cells in vitro, whereas free DGKi drug had no therapeutic effect. Although DGKi was not effective in activated T cells, this chapter provides a proof of concept that amph-NPs enabled cytosolic delivery of small molecule inhibitors and the down regulation of immunosuppressive protein kinase resulted in the resistance to T cell anergy in vitro. Notably, free DGKi (without amph-NP delivery) had no therapeutic effect even at 4-fold higher dose (which led to severe toxicity and cell death). In a broader context, once a potential drug is
selected, amph-NP may transport and disperse useful dose of drugs to the cytosol for enhanced therapeutic outcomes.

Using the developed delivery platform, we are currently investigating the effect of a TGFB inhibitor, another small molecule immunosuppression-reverting drug, on protecting adoptively transferred T cells from failing in immunosuppressive tumor microenvironments.

In the previous chapter, we showed that amph-NPs achieved broad lymphatic distribution following a single site s.c. injection, where NPs were found in the both local and distant lymph nodes. Because most T cells reside in the lymph nodes, lymph node-targeted amph-NPs may enable efficient small molecule immunomodulator delivery in vivo. Combining amph-NP's lymph node tropism, drug loading and cytosolic delivery efficacy, we will focus on the development of antibody-conjugated amph-NPs for improved CD8+ T cell targeting in vivo.
6 In vivo Cytotoxic T cell Targeting via Antibody Conjugation

6.1 Introduction

In the previous chapters, we showed that amph-NPs have the dual property of absorbing hydrophobic drug molecules and an ability to disperse in cells via direct membrane penetration or following endocytic uptake by non-disruptively penetrating endosomal membranes. In vitro, amph-NPs allowed a diacylglycerol kinase (DGK) inhibitor, R59002, to be delivered efficiently into CD8\(^+\) T-cells, reversing PD-1-mediated inhibition of T-cell proliferation.

In this chapter, we engineered an antibody nanoparticle drug carrier conjugate. Conventional antibody-targeted NPs have a NP significantly larger than antibody, and employ many antibodies per particle. In contrast to the conventional design, we have a NP (2-3 nm) smaller than an antibody (~15-20 nm), lowering the chance of reticuloendothelial system (RES)-mediated clearance. This design (Figure 6-1) closely resembles current clinical antibody drug conjugates (ADCs)\(^ {74,75} \), but has the capacity to improve the number of drug per antibody by 50-fold compared to current ADCs. Current ADCs are limited to carrying only 4 or less molecules per antibody due to stability issues. In addition to significantly enhanced drug payloads, amph-NPs may further enhance drug cytosolic delivery efficiency using their membrane penetration capability.

In this chapter, we will discuss an approach to conjugate and quantify antibody nanoparticle conjugation efficiency, and demonstrate enhanced CD8\(^+\) T cell targeting efficiency in vitro and in vivo.
6.2 Experimental methods

**Antibody conjugation.** MUS or MUSOT amph-NPs were mixed with a 120-fold molar excess of 11-Amino-1-undecanethiol hydrochloride (Sigma) at a final NP concentration of 10 mg/mL NPs in pure water and placed on a shaker for 1 hour at 25°C. NPs were washed in water two times to remove excess unbound ligands by centrifugation at 14K x g for 10 minutes using Amicon 30 KDa MWCO filters. A third wash was performed in PBS (pH 7.4). NMR results confirmed that 14% of total ligands on amph-NPs were exchanged by amine ligands. To introduce maleimide groups to amino-functionalized NPs, amino-NPs were mixed with a 40-fold molar excess of sulfo-MBS linkers (Life Technologies) at a final NP concentration of 10 mg/mL in PBS (pH 7.4) and placed on a shaker for 1 hour at 25°C. NPs were washed in PBS (pH 7.4) two times to remove excess unbound linkers by centrifugation at 14K x g for 10 minutes using Amicon 30KDa MWCO filters. A third wash was performed in PBS (pH 7.2). The concentration of maleimide-functionalized amph-NPs was measured by UV-vis (Absorbance at 520 nm) using a known extinction coefficient and applying Beer's law A = e c l. Antibody solution (Anti-Mouse CD8a Purified Clone 53-6.7; Rat IgG2a K Isotype Control Purified
purchased from eBioscience) was concentrated to a final concentration of 4 mg/mL and mildly reduced by addition of 25-fold molar excess dithiothreitol (DTT) in the presence of 10 mM EDTA for 20 minutes at 25 °C. Excess DTT was removed via centrifugation using 7KDa MWCO desalting columns. Mildly reduced antibodies (1 mg/mL) were immediately coupled with maleimide-functionalized NPs (5 mg/mL) at a mass ratio of 4:1 NP:Ab in PBS (pH 7.2) overnight. Uncoupled free antibodies were removed by airfuge.

**SDS PAGE.** The conjugation efficiency of antibody-NP products was quantified by SDS PAGE (NuPAGE™ Novex™ 4-12% Bis-tris protein gels purchased from ThermoFisher Scientific). The concentration of airfuge-purified Ab-NPs was determined by UV-vis (Absorbance at 520 nm) using a known extinction coefficient and applying Beer’s law $A = \varepsilon \cdot c \cdot l$. Ab-NPs (15-30 µg) were reduced in β-mercaptoethanol (βME) for 1-2 days to completely strip off ligands on NPs. Supernatant containing 15-30 µg NPs (15 µL) was mixed with 5 µL of 4x SDS PAGE sample loading buffer and heated at 80°C for 10 minutes. Denatured samples were loaded into gel wells and run for 40 minutes at 100kV. Gels were stained with Coomassie blue, imaged under ImageQuant gel imager and analyzed by software ImageJ.

**Native PAGE.** The percentage of free NP remaining in the Ab-NP conjugation product was qualitatively measured by Native PAGE (NativePAGE™ Novex™ 4-16% Bis-Tris Protein Gels). Purified Ab-NPs or unmodified NPs (25 µg) and free Ab (4 µg) were mixed with 4x sample loading buffer (without denaturing or reducing samples) and run in Novex™ 4-16% Bis-Tris Protein Gels for an hour at 150 kV. Gels were destained 8% acetic acid overnight and visualized using an ImageQuant gel imager.
6.3 Antibody gold construct

While the lymph node-tropic biodistribution behavior of amph-NPs discussed in chapter 4 is very promising for delivery of immunomodulatory drugs, targeted delivery of drug-loaded nanoparticles to specific cell types could further increase our ability to tailor the immune response and minimize off-target toxicities. To deliver drugs selectively and exclusively to CD8+ T cells \textit{in vivo}, we conjugated anti-CD8 antibodies to amph-NPs. The surface modification steps are shown in Figure 6-2 a: Primary amines were introduced on the surface of amph-NPs by ligand place exchange with an amine-terminated alkanethiol ligand (11-Amino-1-undecanethiol hydrochloride), followed by coupling of an NHS-maleimide crosslinker, and finally conjugation of maleimide-bearing amph-NPs to anti-CD8 antibodies with a reduced hinge region disulfide. As shown in Figure 6-2 b and c, when particles were dissolved in β-mercaptoethanol to release attached protein and analyzed by SDS-PAGE, control NP without maleimide linkers (sample #2) resulted in 20 ug antibody per mg gold. In contrast, nanoparticles functionalized with linkers (sample #1) resulted in 220 ug antibody per mg gold. These reaction conditions led to ~1 Ab attached per gold particle. To determine whether the anti-CD8 gold conjugate retains its functionality, we tested the binding specificity \textit{in vitro} using ELISA (recombinant CD8 as a capture agent) for anti-CD8-functionalized particles. As shown in Figure 6-2 d, αCD8 and αCD8-MUSOT NP bound specifically to CD8, whereas control IgG-MUSOT NP and unmodified MUSOT NP showed no binding over background.
Figure 6-2 Design, quantification and functionality test of antibody conjugated amph-NPs. (a) Illustration of amph-NPs surface modification chemistry: Amph-NPs were modified with 11-Amino-1-undecanethiol hydrochloride by ligand place exchange. Free amines on NPs were then reacted with NHS on sulfo-MBS and finally coupled to hinge thiols of mildly reduced antibodies. (b-c) SDS-PAGE quantification of mass ratio of antibody to nanoparticles (220 ug Ab per mg NP). (d) ELISA showed anti-CD8 bound to amph-NPs retains binding specificity for the CD8 antigen.
The homogeneity of Ab-NP samples is essential for defining the antibody’s effect on therapeutic efficacy. If Ab-NP samples contain an unknown mixture of Ab-NP and free NP (without antibody conjugated), then it becomes challenging to quantitatively define the contribution of antibody compared to free NP. Methods to remove free NPs from Ab-NPs are yet to be developed, however, techniques to quantify the percentage of free NP out of total “Ab-NP” mixture were established as shown in Figure 6-3. Samples (antibodies, amph-NPs, amine-functionalized amph NPs, maleimide-functionalized NPs, Ab-NPs) in their intact functional form (un-denatured) were run in a native protein gel. A combination of charge and size determines the position of samples. An example native PAGE image (Figure 6-3 a) shows that free MUSOT amph-NPs (2.6 nm) synthesized by the Brust method localized in a very distinctive position compared to free Ab. A fraction of Ab-NP (Figure 6-3 a, sample “B3”, gold detected by visible absorbance) showed up in the similar vertical position as free antibodies (Figure 6-3 a, sample Ab), indicating that those NPs were bound to antibodies. Another fraction of Ab-NP (Figure 6-3 a, sample “B3”) showed up in proximity to the vertical position of free NPs (Figure 6-3 a, sample “Brust NP”), revealing that those NPs were not conjugated. Amine-functionalized NPs (Figure 6-3 a, B2) and maleimide-functionalized NPs (Figure 6-3 a, B2) showed up in the same vertical position as free unmodified NPs (Figure 6-3 a, sample “Brust NP”). Unmodified Stucky MUSOT amph-NPs (5.5 nm) overlapped with free Ab, rendering the Ab-conjugated fraction indistinguishable from free NPs. In addition, antibody conjugation to Stucky particles caused some NP aggregation, rendering them immobilized in the gel loading well (Figure 6-3 a, S3). An optimized protocol was establish to conjugate ~220 ug antibody/mg (Brust) NPs, which contains 85% antibody-conjugated NPs and 15% free NPs (Figure 6-3 b-c, sample #1). Both MUSOT and MUS amph-NPs of 2-3 nm achieved comparable quantitative conjugation efficiencies, whereas larger amph-NPs ( > 4 nm) resulted in NP aggregation.
Figure 6-3  Native PAGE analysis of non-denatured samples showing NP position at each surface medication step compared to free NP and free anti-CD8 antibody. (a) “B” denotes MUSOT amph-NPs synthesized by the Brust method. “S” denotes MUSOT synthesized by the Stucky method. “1” denotes amine-functionalized NPs; “2” denotes maleimide functionalized NPs; “3” denotes antibody-conjugated NPs. (b) Optimized Ab-NP conjugation showing minimal free NPs remained post antibody conjugation. “#1” Antibody conjugated MUSOT amph-NPs; “#2” unmodified MUSOT amph-NPs mixed with antibodies; “#3” free unmodified MUSOT amph-NPs. (c) Example plot analyzed by ImageJ quantifying the amount of free NPs (unbound to antibodies) in each sample.

Our ultimate goal is to improve the safety and efficacy of small molecule drugs via antibody targeting. In theory an antibody conjugated amph-NP can entrap tens to hundreds of small molecules in its hydrophobic ligand shell vacancies. We showed experimentally that an immunosuppression-reverting drug TGFβ inhibitor (TGFβ-i) could be stably partitioned into the hydrophobic shell of antibody-conjugated amph-NPs. Antibody conjugated on NPs did not sterically inhibit drug loading efficiency compared to unmodified amph-NPs (Figure 6-4).
6.4 CD8⁺ T cell targeting *in vitro*

An initial experiment was established to test the targeting efficiency of anti-CD8 conjugated amph-NPs (abbreviated “aCD8-NPs”) to CD8⁺ T cells *in vitro*. Splenocytes were isolated and incubated with aCD8-NPs or NPs without antibody at 37 °C for one hour. Cells stained with metal-tagged antibodies and analyzed by CyTOF. Splenic CD8⁺ T cell marker intensity decreased in the group where cells were pretreated with anti-CD8 antibody conjugated NPs, suggesting that receptor-mediated NP delivery occurred (Figure 6-5 a, upper panel). CD4 cell marker intensity was unaffected regardless of treatment groups (aCD8-NPs or free NPs), but the NP concentration per cell decreased in aCD8-NP treated cells (Figure 6-5 a, lower panel). As shown in Figure 6-5 b, antibody conjugation increased NP delivery to the target cell by 9.2-fold, and decreased NP uptake in off-target cell types.
Figure 6-5 Quantification of number of NPs per cell using Cytometer by Time-of-Flight (CyTOF) following incubation of splenocytes with anti-CD8 Ab-NPs or free NPs. Splenocytes from C57Bl/6 mice were incubated for 1 hr at 37°C with aCD8-NPs or control MUS NPs (2.39 ± 0.9 nm gold core diam.; 10 ug/mL), then analyzed by CyTOF. (a) Splenic CD8+ T cell marker intensity decreased in the group which cells were pretreated with anti-CD8 antibody conjugated NPs, suggesting either receptor-mediated NP delivery occurred or staining antibodies were blocked by targeting antibodies. (b) Ab-NP concentration was lower than free NP in CD4+ T cells, while CD4+ cell marker signal remained the same. (c) CD8+ T cells internalized 9.2-fold more NPs than NP delivered freely without Ab.

Endocytosis is a cellular internalization mechanism responsible for nutrient uptake, signaling transmission, cell activation, protein degradation, etc. When anti-CD8 antibody binds to CD8 receptor on the cell surface, internalization signals in the cytoplasmic tail is triggered and the antibody will be endocytosed. In the “antibody-conjugated MUSOT/MUS amph-NP” design, the mechanism of antibody internalization could be altered due to the membrane-penetration capability of amph-NPs. Do amph-NPs
facilitate the transport of antibodies to the cytosol via membrane-penetration? In another scenario, if antibodies dominate the internalization processes, will amph-NPs be cleaved off and transit intracellular membranes to reach the cytoplasm post endocytosis? If the latter is true, small molecule therapeutics loaded in Ab-NPs may reach their cytosolic targets to trigger meaningful therapeutic effect. To answer these questions, we used TEM to visualize amph-NPs’ intracellular distribution. Naïve CD8+ T cells were treated with anti-CD8 antibody-conjugated MUSOT amph-NPs (aCD8-NPs) for 0.5h or 24h at 37 °C. At 0.5h post incubation, most NPs were bound to cell surface relatively uniformly throughout a large cell surface area, implying that anti-CD8 antibodies were bound to CD8 antigen (Figure 6-6 a). It is worth noting that although free amph-NPs (without antibody) were observed bound to specific regions of CD8+ cell surfaces (Figure 5-5), the degree to which the cell surface was labeled and the homogeneity were both significantly higher in aCD8-NP treated cells. One day post incubation, NPs were found both free in the cytoplasm and associated with endo/lysosomal membranes (Figure 6-6 b). This suggests that antibody conjugated NPs were able to transit through intracellular membranes, even though they were initially chemically linked to antibodies. Cleavage of amph-NPs from antibody fragments is likely attributed to a combination of membrane-penetration and proteolysis that occurs in endosome/lysosomes, cleaving linkages between the NPs and antibodies. This result strengthens our motivation to use Ab-NPs as carriers to cross biological barriers for specific cell and sub-cellular targeted drug delivery.

Next, we evaluated the cytosolic distribution of control IgG isotype conjugated amph-NPs (IgG-NPs) in CD8+ T cells. Cells were treated at the same NP concentration, and TEM images were taken 0.5h or 24h post incubation. A low concentration of IgG-NPs was observed confined in endosomes 0.5 h post incubation (Figure 6-7 a). Very few IgG-NPs were found on cell surface at this time point, suggesting that IgG antibodies dominated membrane interactions. One day post incubation, cytosolic NP concentration was relatively low compared to 0.5h, implying that a majority of NPs may have been exocytosed (Figure 6-7 b). Although more work should be done to conclude the mechanisms by which aCD8-NP and IgG-NP utilized to interact with T cells, TEM results were in agreement with CyTOF results: aCD8-NP enhanced NP delivery to CD8+
T cells. In addition to that, TEM results provided insights on NP’s fate 24h post incubation, showing that amph-NPs dispersed widely in the cytosol rather than confined with antibodies in the endosomes.

Figure 6-6 TEM images revealing cytosolic distribution of anti-CD8 antibody conjugated MUSOT (aCD8-NP). CD8+ T cells were treated with 0.08 mg/mL of aCD8-NP for 0.5h (a) or 24h (b) at 37 °C. Red arrows in (b) highlighted NPs dispersed in the cytosol and confined in endosomes 24h post incubation.
Figure 6-7 TEM images revealing cytosolic distribution of isotype control IgG2b, x antibody conjugated MUSOT (IgG-NP). CD8+ T cells were treated with 0.08 mg/mL of IgG-NP for 0.5h (a) or 24h (b) at 37 °C. Red arrows highlighted regions where NPs were found.
6.5 CD8+ T cell targeting \textit{in vivo}

Given that MUSOT/ MUS amph-NP’s biodistribution post intravenous injection is relatively typical compared to other small gold nanoparticles (\textasciitilde{} 40\% accumulation in the liver and 5\% in the spleen 24h post injection), methods to target them to specific cell types is desirable. Here, we tested whether amph-NPs conjugated with anti-CD8 antibodies can enhance CD8+ T cell targeting \textit{in vivo}. In a pilot study, a low dose of NPs (aCD8-MUSOT, IgG-MUSOT or free MUSOT) were injected i.v. and spleens were excised 24h later to analyze NP concentration across a variety of lymphocytes and innate immune cells. Results shown in Figure 6-8\textbf{a} suggested that aCD8 antibodies enhanced the percentage of CD8+ T cells that contained NPs by 3-fold compared to control IgG conjugated NPs or free NPs. Anti-CD8 antibodies also increased the on-target/off target ratio; for example, the ratio of NP containing CD8+T cells to macrophages is “1” for aCD8-NP whereas it is “0.25” for IgG-NP or free NP (Figure 6-8\textbf{b}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6-8.png}
\caption{Anti-CD8 antibody conjugated MUSOT NPs increased the percentage of CD8+ T cells that contained amph-NPs in the spleen by 3-fold compared to control IgG-NP or free-NP. Anti-CD8 or control IgG Ab-NPs (32 ug NPs and 8 ug antibodies) or free NPs (32 ug) were injected i.v. and spleens were excised 24h later for CyTOF analysis. NP core diameter = 2.66\pm0.7 nm.}
\end{figure}
Next we tested antibody’s effect on number of NPs per CD8\(^+\) T cell. Amph-NPs or aCD8-NPs (equivalent dose of free amph-NPs) were injected in C57BL/6 mice via the tail vein, and spleens and PBMCs were isolated 24 hours post injection. In this experiment, a significantly higher dose of NPs (150 ug NPs) was used. At this dose, all cells in the spleen reached \(\sim\)100% NP-positive 24h post injection, meaning that all of the cells analyzed contained some nanoparticles. Anti-CD8 antibody conjugation enhanced NP delivery to CD8\(^+\) T cells in the spleen by 2-fold; and in the blood by 35-fold (Figure 6-9 a and b). As shown in Figure 6-9 c and d, while antibody conjugation increased uptake by phagocytes compared to bare NPs (potentially due to Fc receptor mediated uptake), aCD8-NP resulted in dramatically reduced ratio of NP concentration in non-targeted phagocytes to targeted cells in the blood (e.g. ratio of NP concentration in macrophages v.s. CD8\(^+\) T cells is “1” for aCD8-NPs while the ratio for free NPs is “36”). This resulted in an overall significantly improved on-target to off-target ratio.

It is essential to understand the components that are driving non-specific uptake in phagocytes in order to improve the targeting efficiency of our design. To test whether amphiphilicity or anionic sulfonate groups on MUS ligands are the major cause of enhanced non-specific phagocyte uptake (especially the CD11\(^+\)CD11c\(^+\) population), we evaluated the cellular distribution of NPs coated with different surface ligands: PEG(4CH), PEG3k, MUSOT, MUS and MPSA (Figure 4-2). A high dose of NPs (150 ug) was injected i.v. and spleens and blood were analyzed 24h post injection. The overall concentration of PEG-coated NPs in cells was higher than other NPs in the blood (Figure 6-10 a); the ratio of NP concentration in CD11\(^+\)CD11c\(^+\) cells v.s. CD8\(^+\) T cells was \(\sim\)3. In the spleen (Figure 6-10 b), the concentration of PEG3k NPs was low in all cell types except for CD11\(^+\)CD11c\(^+\) population, with the ratio of NP concentration in CD11\(^+\)CD11c\(^+\) cells v.s. CD8\(^+\) T cells being \(\sim\)4; in contrast the concentration of MPSA NPs was low in all cell types. Interestingly, MUSOT and MUS NPs share similar distribution patterns—the ratio of NP concentration in CD11\(^+\)CD11c\(^+\) cells vs. CD8\(^+\) T cells was “20” (Figure 6-10 c) in the blood, while this ratio dramatically decreased to “4” in the spleen (Figure 6-10 d). Thus, we can conclude that preferential uptake of NPs in CD11\(^+\)CD11c\(^+\) population is likely the result of ligand amphiphilicity, because this intrinsic high uptake ratio was only observed in PEG(4CH), MUSOT, and MUS NPs, which are all
amphiphilic, although the effect on PEG(4CH) NPs was not as profound as MUSOT and MUS NPs. Hydrophilic MPSA and PEG3k NPs did not have such high uptake ratio in CD11+CD11c+ cell subset.

Figure 6-9 Anti-CD8 antibody conjugated MUSOT amph-NPs enhanced NP delivery to CD8+ T cells in the blood (a) and spleen (b) 24h post i.v. injection. NP concentration in other leukocytes in the blood (c) and spleen (d) was analyzed. A higher dosage of Ab-NPs (150 ug NP; 32 ug Ab) was injected in this experiment. N=3 per group. NP core diameter = 2.66±0.7 nm.
Our present design of antibody conjugated amph-NPs increased CD8$^+$ T cell targeting efficiency in vitro, while targeting efficiency was also enhanced in vivo, more studies is warranted in order to significantly reduce off-target absorption in CD11b$^+$ CD11c$^+$
subset. Meanwhile, the intrinsic CD11b' CD11c+ "specific targeting" property of amph-NPs could be of interest to deliver drugs to modulate this subset in some disease settings.

6.6 Conclusions

Small molecule drugs that modulate signaling pathways in lymphocytes could represent a powerful strategy for enhancing anti-cancer immunity, but often have pleiotropic effects on other cells, leading to toxicities. We showed in previous chapters that amph-NPs have the capacity to transport high doses of small molecules into the cytoplasm and cause therapeutic effects in vitro. To achieve optimal therapeutic index in vivo, it is essential to add another component to our current design: a targeting moiety for in vivo specificity.

In this chapter, we introduced a strategy to achieve this goal via antibody-conjugated amphiphilic nanoparticles. We showed that anti-CD8-functionalized amph-NPs increased uptake of the particles by CD8+ T-cells in vitro and in vivo. These antibody conjugates are an exciting new type of nanoparticle delivery vector, given that unlike most targeted drug delivery systems, here the particles themselves are actually smaller than the antibody—potentially enabling the antibody to dominate the biodistribution behavior of the conjugate. In vivo data showed that current design enhanced NP concentration in CD8+ T cell by 35-fold in the blood and 2-fold in the spleen 24h post i.v. injection. However, off-target effects remain to be minimized—a high number of NPs were taken up by CD11b+ CD11c+ cells regardless of anti-CD8+ antibody conjugation. This phenomenon may be linked to recognition of the nanoparticle sulfonate ligands through scavenger receptors, or other pathways of opsonization. Modifications on the current design such as shielding NPs with cleavable dense PEG layers may solve this issue, because PEG3k AuNPs did not achieve high uptake in CD11b+ CD11c+ cells compared to other cell types. Another possible solution to resolve off-target effects is to conjugate multiple mini-antibodies (e.g. fragmented antibody with only variable regions) to amph-NPs, shielding amphiphilic ligands from being recognized and internalized by phagocytes systemically.

Although targeting issues and therapeutic effect of drug loaded Ab-NPs remain to be tested, possible mechanisms of drug payload release by Ab-NPs are illustrated in
Figure 6-11: (1) Drug-loaded Ab-NPs bind to the target cell surface. (2) Internalization of drug-loaded Ab-NPs via receptor mediated endocytosis. Amph-NPs are cleaved off from antibody due to reducing environment in endosomes that destabilize maleimide-thiol bonds. (3) Drug loaded amph-NPs embed in endosomal membranes and/or transit through intracellular membranes, resulting in ligand shell fluctuation which then allow drugs to be released into the cytosol.

Successfully engineered drug carriers that target at both the intercellular and intracellular levels in vivo may prove valuable in clinical settings for the delivery of immunosuppression-reverting drugs into the cytosol of CD8+ T cells in vivo.

Figure 6-11 Illustration of possible drug release mechanisms once Ab-NPs reached target cell.
7 Lymphoid tissue targeted amph-NPs as drug carriers to combat lymph-borne bacterial infection in vivo

7.1 Introduction

Innate immune cells are professional phagocytes responsible for the frontline host defense to combat bacterial infections. Macrophages (MΦ) have many microbicidal features, for example, they can ingest and destroy bacterial pathogens by phagocytosis; MΦ also secrete pro-inflammatory cytokines to elicit immune responses in addition to presenting bacteria-derived antigens to T cells. Recently, it has further been shown that macrophages in the lymph nodes (LNs) play important roles in the blockade of bacterial dissemination. Kastenmueller et al. showed that spatial distribution of functional macrophages in the LNs is crucial for the blockade of systemic dissemination of subcutaneously-inoculated *P. aeruginosa* Figure 7-1 a). Depletion of macrophages in LNs resulted in a 10-fold increase in bacterial load locally and systemically (Figure 7-1 a and b). Bacteria are thus proposed to migrate to LNs as a strategy disseminate systemically. MΦ in LNs thus coordinate to capture and kill invading microbes, preventing them from disseminating into the blood stream and causing life-threatening systemic infections.

Figure 7-1 Lymph node macrophages prevent systemic spread of pathogens. (a and b) Confocal IF of a draining LN (a) and bacterial counts of blood and of LN homogenates (b) 8 hr after s.c. (footpad) infection with PA-GFP. Mice were
pretreated 7 days before infection by s.c. (calf) injection of control or clodronate-containing liposomes. Adapted from reference [76]

In this chapter, we seek to develop strategies to combat bacterial infections in two different ways: 1. Promoting and boosting innate immune cells to clear local bacterial infection by immunomodulator delivery. 2. Concentrating antibiotics to local infection sites for controlled release to suppress infection progression. The rationale is: Using lymph node targeted amph-NPs that entrap high concentration of poorly soluble small molecule drugs (immunomodulators or antibiotics), we may be able to control infections in an early stage before systemic spread occurs.

First, we developed a mouse model with *Pseudomonas aeruginosa* chronic skin infection to model bacteria disseminating to local LNs from a local infection site (e.g., infected wound or burn site). *Pseudomonas aeruginosa* is the most common bacteria responsible for nosocomial infection, and can range from external infections to life-threatening infections. Individuals with compromised immune systems (e.g., HIV patients, hospitalized patients, transplant recipients, seniors, and young children) are more likely to be infected. However, healthy adults can develop serious infections if open wounds were to occur.

We postulate that efficient delivery of amph-NPs to lymphoid organs where lymphocytes and other innate immune cells are concentrated would be an effective strategy for antimicrobial immunomodulator delivery. Type I interferons (IFNs) have proven effectiveness in protecting the host from viral infections, and recently their beneficial effects on some bacterial infections were reported (Figure 7-2). Resiquimod (R848, a TLR7/8 agonist) is an immune-stimulant known for eliciting type I interferons production in dendritic cells and macrophages, which may result in innate immune cell activation and the promotion of bacterial clearance. We tested R848's immunological effect on healthy mice prior to testing its therapeutic effect on controlling chronic *p. aeruginosa* skin infection in a mouse model.
Multi-drug resistant bacterial strains are emerging worldwide. Increased doses and diversity of antibiotics are thus being used in the clinic, which exacerbates the development of multi-drug resistant microbes. Many antibiotics are poorly soluble in physiological conditions, rendering unavoidable increases in the treatment frequency and dose. One means to reduce the dose and frequency required for effective eradication of pathogens is to increase the accessibility of drug molecules to targeted infection sites. In the last section of this chapter, we will demonstrate a new method to effectively deliver ciprofloxacin (an effective antibiotic for *P. aeruginosa* that is poorly soluble in water) using amph-NPs—a promising new type of antimicrobial therapeutic that may significantly change current dosing frequency requirements and minimize systemic side effects.
7.2 Experimental methods

**Bacterial culture.** A strain of bioluminescent *Pseudomonas aeruginosa* (Xen 5) was purchased from PerkinElmer. Streaks of bacteria from a frozen vial were plated on an agar plate (containing 60 μg/mL tetracycline) overnight, and a single colony was sub-cultured in Luria Bertani (LB) medium at 37 °C under ambient aeration overnight. One mL of bacterial culture was added to 5 mL of fresh LB and cultured at at 37 °C under ambient aeration until the bacterial concentration reaches OD600=1 (measured by UV-vis). An absorbance measurement at 600 nm of 1 (against a LB blank) is roughly 7.5×10^8 cfu/mL of *pseudomonas aeruginosa*.

*Pseudomonas aeruginosa* infection animal model. One mL of freshly prepared *p. aeruginosa* at 7.5×10^8 cfu/mL were pelleted and resuspended in sterile 1 mL PBS, and further diluted to obtain a final concentration of 4×10^7 cfu/mL. Eight week old albino C57Bl/6 female mice (purchased from Jackson laboratory) were randomly picked to form five mice per group. A single dose of 8×10^5 cfu (20 μL of 4×10^7 cfu/mL) of *p. aeruginosa* was subcutaneously injected into the left tail base, followed by IVIS imaging to track their bacterial load. A group of clean mice (n=5) without infection were imaged to determine IVIS background signals. Mice were closely monitored 2h, 6h, 24h post infection, and then at least once per day afterwards.

Cytometric bead array (CBA). BD™ CBA Mouse Inflammation Kit was to quantitatively measure Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in serum samples. Eight week old albino C57Bl/6 female mice (n=3 per group) were s.c. injected with R848, R848-MUS(fracB), free MUS(fracB) or PBS and were bled 1h post injection. Serum samples (25 μL per mouse) were prepared following the manual provided by BD Biosciences. Samples were analyzed on a Fortessa flow cytometer.
**R848's activation effect in lymph nodes.** Eight week old albino C57Bl/6 female mice C57Bl/6 (n=3 per group) were s.c. injected with R848, R848-MUS(fracB), free MUS(fracB) or PBS and their inguinal LNs were excised 24h post injection and digested using an enzyme mix (collagenase and DNase). Samples were Fc blocked for 10 minutes and split into two tubes for two antibody staining settings. Setting 1: CD11b PE-Cy7; CD8a-PE; B220-APC; CD3e-FITC; CD86-eFluor 450; MHC II (I-A/I-E)-AF 700; CD69-PE-TxR. Setting 2: CD11b PE-Cy7; CD3e-PE; B220-APC; CD11c-FITC; CD86-eFluor 450; MHC II (I-A/I-E)-AF 700; CD69-PE-TxR. Samples were stained with antibody cocktails for 40 minutes at 4 °C and washed with FACS buffer for 2 times prior to live/dead eFluor 506 staining. Samples were analyzed on a Fortessa flow cytometer.

### 7.3 Pseudomonas aeruginosa skin infection model

As a first step, we developed a model that mimics bacterial dissemination to local LNs from a local infection (e.g., burn or wound) site. We introduced $10^6$, $10^7$ or $10^8$ cfu bioluminescent *Pseudomonas aeruginosa* to C57Bl/6 albino mice via s.c. tail base injection (50 uL on the left tail base and 50 ul on the right tail base), and monitored bacterial migration via IVIS whole-tissue imaging. As shown in Figure 7-3 a, *Pseudomonas* disseminated to lumbar LNs 8 hours following injection, and spread to inguinal LNs 24 hour post injection. Bacterial loads were found to increase from 8h to 24 h in the lumbar LNs, resulting in 100% mortality at 24 h at this high challenge dose (Figure 7-3 b and c). A decreased dose of $10^6$ cfu resulted in 50% mortality (Figure 7-3 c). Interestingly, among all of the $10^6$ cfu infected mice (n=14), post-mortem study showed that 5 mice that died at 44h post infection all have detectable bacterial load in their inguinal LNs; in contrast, lymph node excised from a survivor ($10^6$ cfu infected) at 44h were swollen but with undetectable bacterial load (Figure 7-3 d). This result implied that high bacterial load in LNs may be associated with lethality.
Figure 7-3 Pseudomonas skin infection model. Bacteria migrated into lymph nodes and the concentration in LNs increased with time. (a) Lymph nodes excised from mice receiving \(10^8\) cfu subcutaneously 8h or 24h post infection. (b) Bacterial load progression monitored by IVIS. Mice shown here were infected with \(10^8\) cfu \(p.\) aeruginosa expressing the lux operon for luminescence imaging. Three mice were used per group and one mouse was an uninfected control. (c) Survival rate of mice treated with \(10^8, 10^7\) or \(10^6\) cfu s.c. (100 uL total volume split into 50 uL left and 50 uL right). (d) Post mortem analysis of bacterial load in inguinal LNs 44h post infection of \(10^6\) cfu per mouse. LNs from a PBS treated uninfected mouse and a \(10^6\) cfu infected mouse (survivor) are shown on the right.

To develop a chronic infection model, titrated doses of \(10^6, 8\times10^5, 4\times10^5\) and \(10^5\) cfu \(p.\) aeruginosa were tested with n=7-8 per group. The injection site was limited to the left tail base subcutaneous space instead of injections on both left and right sides. Injection volume was reduced from 50 uL to 20 uL. As shown in Figure 7-4 a, this method yielded survival rates of nearly 100\% in all doses (except for \(8\times10^5\) cfu group in which 1 mouse out of 7 mice died 72h post infection). Infection progressed quickly in the first 24h post infection, and slowly declined with time but still remained chronic for at least one to two weeks (Figure 7-4 b).
In next section, we will demonstrate how amph-NP delivery of R848 impact bacterial loads and survival rates using this developed \( p. \) aeuruginosa chronic skin infection model.

![Figure 7-4 Chronic skin infection model of \( p. \) aeuruginosa. (a) Survival rates of 8-weeks old female albino C56Bl/6 mice infected with \( 10^6 \), \( 8 \times 10^5 \), \( 4 \times 10^5 \) and \( 10^5 \) cfu bacteria on the left subcutaneous space near the tail base (20 uL). (b) Bacterial load monitored by IVIS.](image)

7.4 Immunomodulator delivery for lymph-borne infection blockade

It has been reported that some bacteria enter lymphatic vessels and invade lymph nodes as a strategy to systemically disseminate from a local site of entry into tissues. We showed in the previous section that \( \textit{pseudomonas aeruginosa} \) is one of those bacteria that invade LNs. It is reported that innate immune cells in lymph nodes coordinate to capture and kill such invading microbes. We expect that our lymph node-targeting amph-NPs provide a unique opportunity to both provide immunomodulatory drugs at a primary infection site but also target the tissue-draining LNs to block this pathway of microbial dissemination. To test this idea, the immunotherapeutic small molecule adjuvants R848 were loaded into amph-NPs using our previously described method. We first tested R848's immunological effect on healthy uninfected C57BL/6 mice. Doses of 1 ug, 5 ug
and 10 ug of free R848 or R848 loaded in amph-NP (R848-MUS 2.39±0.9 nm gold core diam.) were injected subcutaneously in C57BL/6 mice; free NPs without drugs and PBS were included as control groups. Serum inflammatory cytokines were tested one hour post injection given the short half-life of small molecules. As shown in Figure 7-5 a-b, 5 ug of R848 delivered freely induced systemic cytokine production, which is considered undesirable systemic toxicity.

Importantly, doses above 5 ug activated DCs, B and T cells regardless of delivery method 24h post s.c. injection (Figure 7-6). Although a dose of 1 ug R848 did not trigger systemic cytokine secretion, it also resulted in minimal immune cell activation. Free NPs and PBS treatment had no effect on systemic toxicity or immune cell activation. All together, this experiment demonstrated an application to use amph-NPs to deliver small molecule R848 that have comparable therapeutic outcomes but with minimal systemic toxicity. Overall, this result suggested that amph-NPs were able to limit systemic spread of small molecules while effectively activating local immune cells in the lymph nodes. The dose of 5 ug R848-NP appeared to have the best therapeutic index; thus we chose this dose for p. aeruginosa chronic infection interventions.
Figure 7-5 R848-induced systemic toxicity in serum was significantly reduced amph-NP delivery platform. Serum (n=6 C57Bl/6 mice) was collected 1 hour post s.c. injection of 1 µg, 5 µg, or 10 µg R848 freely or loaded in MUS amph-NPs. (a) Representative flow cytometry plot of cytometric bead array assay. (b) Quantitative analysis of serum cytokines.
Next, we tested if stimulation of type I interferon production via R848 delivery can facilitate microbe clearance in a chronic pseudomonas skin infection model. Three separate treatments (5 ug free R848, R848-NP, or PBS) were given subcutaneously at the tail base of infection site at 2h, 6h, and 23h post 8×10^5 cfu *pseudomonas* infection. Unexpectedly, R848 sensitized bacterial infection progression regardless of its delivery method (free soluble form or with nanoparticles). Twenty-four hours post infection pseudomonas progression surged in the groups that had received three treatments of R848 or R848-NP (Figure 7-7 a). This progression is associated with mortality—100% of R848 and R848-NP treated mice succumbed 48h post infection (Figure 7-7 b). Post-mortem study revealed that lymph nodes of R848 and R848-NP treated mice contained high concentration of bacteria (Figure 7-7 c). Our data clearly indicated that type I interferon is likely pro-bacterial progression at least in a chronic *pseudomonas* infection model.
The effect of cytokines may be beneficial for certain infections but detrimental to others, depending on the route of injection, the type of bacteria, and the infected host organ. The evidence that such detrimental effects occur during such early stage (24h) of *p. aeruginosa* bacterial infections implies that innate immunity was likely suppressed by the cytokines orchestrated by R848. Such an anti-inflammatory effect may be of useful for controlling chronic inflammation in some infection settings.

Evidences of the pro-bacterial role for type I IFNs had been shown for infections of *Listeria monocytogenes* and *Mycobacterium tuberculosis*. To initiate cell-mediated immune responses, low levels of type I IFNs may be required at an early stage of infection. However, it is reported that high level of type I IFNs may trigger production of immunosuppressive molecules and reduce macrophage responsiveness to activation by IFNγ.

Combatting bacterial infection via immunomodulators is an attractive idea that may solve current issues of fast emerging drug resistance strains. However, pathogens interfere with normal cell functions to disable and sabotage the host defenses. For example, *pseudomonas aeruginosa* uses a type III secretion system to inject proteins to host cells, and this helps killing of phagocytes, promotes tissue damages and retards wound healing, enabling *p. aeruginosa* to invade other tissues or cause septic shock. More studies on the pathogenesis of *pseudomonas* infections will help select suitable immunomodulators that may help boosting the pathogen clearance features in the innate immunity.
7.5 Concentrating poorly water soluble antibiotics to local infection sites

Antibiotic resistance has been one of the most pressing health problems worldwide. To date, antibiotic development continues to be outpaced by emergent resistance. The emergence of resistant strains is partly a combinational result from overuse and misuse of antibiotics since their discovery in the 1940s. Antibiotic discovery was originally based on screening of soil-derived actinomycetes, which benefited countless patients who suffered from bacterial infection. Owing to the prevalent usage of antibiotics, bacteria develop antibiotic resistant genes, diminishing the efficacy of antibiotics by a combination of efflux pumps and antibiotic degrading enzymes. Efforts to synthesize new molecules have been slow to produce new drug candidates, partly due to the lack of efficiency to penetrate bacterial envelopes.

In this sub-chapter, we aim to improve antibiotic delivery using amphiphilic gold nanoparticles. Amph-NPs intrinsically target lymph nodes and may effectively transport concentrated antibiotics originally partitioned in their ligand shells to infection sites that could not be reached easily by free drugs. If targeted delivery to critical infection sites such as LNs (where bacteria utilize to disseminate systemically) could be achieved, dosing frequency and concentration required to achieve therapeutic efficacy may be reduced.

Ciprofloxacin is a fluoroquinolone antibiotic that targets the related DNA gyrase and DNA topoisomerase of a wide spectrum of bacteria. It is poorly soluble in physiological conditions thus require high dosing concentration and frequency when used in the clinic, leading to the promotion of antibiotic resistance. For example, to treat acute urinary tract infections in humans, 500 mg of ciprofloxacin was orally dosed twice per day for 7 consecutive days, and that reduced frequency (single dose per day) but same total dose affected the treatment outcome. 81 In a murine model, ciprofloxacin was dosed at 80 mg/kg and treated twice daily for 4 consecutive days in a systemic methicillin-susceptible S. aureus infection. 82,83 It is plausible that soluble ciprofloxacin aggregates and becomes inactive in vivo, increasing the efficacious dose required. We first tested loading ciprofloxacin to MUS amph-NPs (2.18 ±0.7 nm), and resulted shown in Figure
7-8 a-b suggested that 142 ug cipro was loaded into 1 mg NPs, which corresponds to ~30 molecules per NP. NP of 2.18 nm diameter core size are estimated to have ~60 ligands on their surfaces. The UV spectrum of cipro-loaded NPs (Cipro-NPs) shown in Figure 7-8 c suggested that NPs remained colloidal without aggregation, because characteristic peak of NPs (~510 nm) did not shift compared to blank NPs. Cipro-NP also exhibited ciprofloxacin characteristic peaks (282 nm and 336 nm). Quantification results using UV absorbance of undigested cipro-NPs and standards composed of known concentration of NPs and known concentration of cipro (Figure 7-8 b) were in agreement with HPLC results (data not shown).

Figure 7-8 Ciprofloxacin were efficiently partitioned in the ligand shells of MUS amph-NPs (2.18 nm). Loading efficiency was ~14% which resulted in 142 ug cipro per mg NP. (a) Schematic of ciprofloxacin loaded in amph-NPs. (b) UV-vis spectrum of Cipro loaded NPs, free NPs, and standards containing known concentration of cipro and fixed concentration of NPs. (c) Full spectrum of cipro loaded in NP, free cipro and free NP.
Next, we evaluated ciprofloxacin’s *in vitro* inhibition effect on *pseudomonas aeruginosa*. Bacteria cultured in LB were grown to an absorbance of ~0.1 at 600 nm, and treated with cipro loaded in MUS (cipro-MUS) or free cipro at various concentrations (n=6 samples per concentration) on a 96 well plate. Free NPs were also tested. As shown in Figure 7-9 a, MUSOT NPs (2.08±0.9 nm gold core diam.) without antibiotics appeared to have an inhibitory effect at the highest concentration 0.5 mg/mL. While MUS (2.18±0.7 nm gold core diam., approximately the same core size as MUSOT tested) did not show an inhibitory effect on *pseudomonas* even at 1 mg/mL (Figure 7-9 b). Free cipro or Cipro-NP (2.18 nm MUS) showed overlapping IC<sub>50</sub> values at 60 ng/mL (Figure 7-9 c-d).

![Figure 7-9](https://example.com/figure79.png)

Figure 7-9 Minimum inhibitory concentration (MIC) test for *pseudomonas aeruginosa* suggested that ciprofloxacin IC<sub>50</sub> was ~60 ng/mL and the value was independent of delivery method *in vitro*. N=6 samples per concentration. (a) Free MUSOT NPs, (b) free MUS NPs, (c) free cipro, and (d) cipro-loaded MUS NPs (142 ug cipro/ mg NP) at various concentrations were tested *in vitro* in LB broth at 37 °C for 16-20 hours as indicated in each plot.
A beneficial effect of nanoparticle delivery was not observed *in vitro*, possibly due to the extremely high ciprofloxacin sensitivity of *pseudomonas*. However, delivery *in vivo* is complicated by many other factors such as short half-life, stability with serum proteins and tissues as well as systemic side effects. Ciprofloxacin comes with severe side effects such as tendinitis, anxiety, diarrhea, and in some cases peripheral neuropathy. Technologies that enable targeted delivery of a concentrated dose to infection sites may be able to solve these issues. We thus tested if chronic *pseudomonas* skin infection could be controlled at an early stage before inflammatory skin damage or systemic spread occurs. To test this, *pseudomonas* chronic skin infections were induced on the left s.c. tail base (8*10^5 cfu). A single dose of PBS, 3 ug free cipro, or cipro-NP (equivalent to 3 ug cipro) was given 6 hours post infection on the local infection site subcutaneously.
Figure 7-10 IVIS images of $8\times10^5$ cfu pseudomonas infected mice with different treatment groups immediately post infection, 1 day and 2 days post infection.

In this experiment, bacterial load progressed aggressively in the group of mice treated with PBS (Figure 7-10 left panel). Infection progression was significantly suppressed in the group treated with free cipro (Figure 7-10 middle panel). Strikingly, cipro delivered with amph-NPs cleared infections completely within 24h and remained effective for 2 days, before recurrence happened in one (out of five) mice (Figure 7-10 right panel and Figure 7-11 a). Overall, nanoparticle mediated cipro delivery facilitated early control of infections; with only a single dose, infection was controlled for 48 hours before a low, controlled chronic infection reoccurred in 3 out of 5 mice throughout a course of 14 days (Figure 7-11 b). In contrast, infection remained chronic in 5 out of 5 mice for 4 days in the group of mice administered with free cipro (without nanoparticles). Control and
clearance of the infection at an early stage achieved via ciproNP may be critical for the elimination of inflammation-associated tissue damage. Two mice in the free cipro treated group developed dermatitis on the tail base starting day 3 post infection, and one of them developed ulcerative dermatitis on day 7, and reached euthanasia criteria on day 14 (Figure 7-11 c and Figure 7-12 a). Two days post infection, three mice out of five succumbed in the group without antibiotic treatment (Figure 7-12 b). Mice that received a single dose of free cipro or ciproNP achieved 80% and 100% survival respectively. Although the mechanisms by which cipro-NP clear infections in this model remain to be elucidated, possible explanations include: sustained release of concentrated small molecules at the infection site, prevention of lymph-borne bacterial infection development and progression, etc. Future experimental plans such as separating treatment site from infection site, or initiating treatment at a later time point post infection (12h or 24h) may prove NP valuable for infection control.
Figure 7-11 Cipro delivered via amph-NPs effectively eradicated local infection with minimal recurrence. (a) Bacterial infection progression in C57Bl/6 mice tracked by IVIS within 24h post infection of 8x10^5 cfu with PA. Treatments were dosed at 6h post infection: 3 µg free cipro, cipro-NP (equivalent to 3 µg cipro), and PBS. N=5 animals per group. (b) Plot represents means of total flux per treatment condition (n=5) (c) Plot represents total flux tracking of individual mouse and representative image of dermatitis developed in two out of five free cipro treated mice.
Conclusions

Amphiphilic nanoparticles of 2-3 nm core diameters have the capacity to entrap tens to hundreds of small molecules. Combining their drug loading capacity with their intrinsic lymph node (LN) targeting property, we demonstrated the delivery of an immune-stimulant R848 and an antibiotic ciprofloxacin in the context of *pseudomonas* skin chronic infection.

R848 is a TLR7/8 agonist that induces high levels of inflammatory cytokine secretion such as type I interferon (IFN). We hypothesized that R848 may be able to
boost natural innate immune network in the LN to promote bacterial clearance. We demonstrated in healthy mice that R848 delivered via amph-NPs activated leukocytes in the lymph nodes while induced minimal systemic inflammatory cytokine production—an indicator of systemic toxicity. The role of type I interferon in bacterial infections is complicated by the type of infection, the site of infection, and the stage of infection. We showed that R848 sensitized pseudomonas skin infection, and resulted in 100% mortality in two days; while 80% infected mice without R848 treatment were long-term survivors. This result suggested that type I IFN is exacerbated pseudomonas skin infection. Literature findings reported that pseudomonas utilizes type III secretion system to inject proteins that damages the host immune cells as a way to progress to deeper tissues. The role of type I IFN in causing immunosuppression in macrophages (such as macrophage unresponsiveness to IFNγ activation) could partly explain the pro-bacteria effect of R848 observed in our study.

While immunomodulation represents a powerful strategy to combat infection, another approach to control infection is antibiotic treatments. If an effective dose of antibiotic could be targeted to infection sites and sites where bacteria attack for dissemination, then bacteria resistance may be dampened. This is supported by the theory that systemic delivery of antibiotic kills good bacteria that exist in the host and allows resistant strain to replicate and dominate due to the lack of competition from healthy bacteria. We showed that ciprofloxacin delivered with or without amph-NP were highly efficacious in inhibition of pseudomonas growth in vitro. In vivo, amph-NP delivery facilitated bacterial clearance—an order of magnitude difference in bacterial load was observed compared to mice that received free cipro. Amph-NP delivery also accelerated infection clearance—mice that received cipro-NP treatment cleared infection to an undetectable concentration one day post infection, whereas free cipro treated group remained moderately infected until day 4. Prolonged infection in free cipro treated group was associated with the development of inflammation-driven dermatitis. More studies will be carried out to elucidate the association of LN targeting with early stage infection blockade. We expect that LN-targeted amph-NPs will greatly increase the efficacy of antimicrobial therapy of these disseminating infections.
8 Summary and Outlook

In this thesis, we developed a cytosolic drug delivery platform based on amph-NPs that are highly water-soluble, membrane-interactive, and home to lymph nodes efficiently. We focused on one class of therapeutics—small molecule drugs that are potent, cost-effective but often have short half-life, poor solubility in physiological conditions and require repetitive/frequent dosing. Amph-NPs have the capacity to entrap \( \sim 50-200 \) small molecules per NP, depending on the drug molecule’s hydrophobicity and polarity. We aimed to enhance cytosolic delivery of immune-relevant drugs to enhance host immunity in cancer and chronic bacterial infections.

Targeting immunonodulators to lymph nodes where most immune cells reside in could strongly enhance therapeutic efficiency and efficacy. We showed that MUSOT and MUS amph-NPs target lymph nodes and accumulated in multiple distant lymph nodes post one single local subcutaneous (s.c.) injection. This intrinsic lymph node tropism was not observed in other gold nanoparticles with the same core size but different ligand compositions. To test whether this phenomenon was associated with a specific cell type, fluorescent dye labeled amph-NPs were injected s.c. and single cell analyses of lymph node cells were performed using traditional flow cytometer. Fluorescent-based method was unsuccessful due to quenching, dye degradation and unstable linkage to nanoparticles in vivo. To overcome this problem, we developed a label-free detection method using existing single cell mass cytometry (CyTOF). We showed for the first time that CyTOF could be used to quantitatively measure gold nanoparticle concentration in a single cell in a high-throughput manner. Using this method, we further identified cell types that amph-NPs intrinsically target: dendritic cells and macrophages in the lymph nodes. Dendritic cells are important antigen presenting cells. We demonstrated that efficient cytosolic delivery of peptide antigens using amph-NPs enhanced prophylactic cancer vaccine outcomes. Macrophages play an important role in the clearance of pathogens in bacterial infection. We developed a mouse model of lymph-borne bacterial infection, in which mortality is associated with heavy bacterial loads in lymph nodes. We showed that ciprofloxacin, a small molecule hydrophobic antimicrobial, when delivered by amph-NP achieved early infection clearance in a
**pseudomonas** skin infected mouse model. The understanding of key mechanisms that lymph node targeted nanoparticle utilized to achieve such effective antimicrobial delivery will facilitate the design and optimization new translational therapeutics for lethal bacterial infections.

In addition to vaccine delivery and antimicrobial delivery, lymph node targeted amphiphilic gold nanoparticles show promise as a versatile delivery vector for modulation of the host immune response in cancer. To reverse immunosuppression that accompanies cancer or other infection-driven inflammation, we aimed to target DGKi, a small molecule immunosuppression-reverting drug, to lymphocytes and innate immune cells. We tested the interaction of amph-NPs with CD8⁺ T cells, an important adaptive immune cell that aids in clearance of intracellular infections. We showed that amph-NPs were avidly taken up by T-cells, allowing DGKi loaded into the particles to restore ~50% of T-cell to a proliferative state activity in an *in vitro* immunosuppression assay. The development of drug-loaded amph-NPs provides a strategy to target a diverse range of small molecule drugs to lymphocytes or other target cells for direct modulation of cytosolic signaling pathways. In ongoing studies, we are testing the capacity of this approach to target other important kinase inhibitors and modulators of key suppressive pathways to T-cells to enhance endogenous anti-tumor immunity.

To extend the development of immunomodulation of T cells *in vivo*, we developed a method to couple amph-NPs (2-3 nm) to anti-CD8 antibodies (10-20 nm), forming an Ab-NP conjugate that closely resembles the structure of current antibody drug conjugates (ADCs). ADCs are effective therapeutics that can maximize cytotoxin delivery to tumor cells while reducing off-target toxicities. Today there are two FDA-approved ADCs used in the clinic: Brentuximab vedotin and Trastuzumab emtansine. However, more than 30 ADCs are currently under clinical trials. Due to antibody stability concerns, current ADCs usually have one to four drug molecules per antibody. Given that, drugs that are suitable for ADC developments are required to be highly potent. By attachment of a drug nanocarrier to an antibody, we may enable 100-fold enhanced drug payload per antibody. We showed that antibody conjugation enhanced nanoparticle CD8⁺ T cell targeting in the blood and spleen post i.v. injection. Off-target effect of Ab-NPs was
observed in phagocytes. Two possible factors that might have caused off-target uptake in phagocytes include Fc receptors and scavenger receptors. Future experiments focusing on replacing whole antibodies with antibody fragments such as Fab, ScFv, or VHH may solve this issue. Surface modification of cleavable PEG layers on amph-NPs may further decrease off-target effects. We envision that the present invention of Ab-NPs may provide a means to deliver small molecule immunomodulators or cytotoxins to enhance anti-cancer immunity, reverse autoimmune conditions, and combat infection.
References


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