The Pennsylvania State University
The Graduate School
College of Engineering

IMMUNE CELL-MEDIATED BIODEGRADABLE THERANOSTIC NANO PARTICLES
FOR MELANOMA TARGETING AND DRUG DELIVERY

A Thesis in
Bioengineering
by
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ABSTRACT

Although tremendous efforts have been made on targeted drug delivery systems, current therapy outcomes still suffer from low circulating time and limited targeting efficiency. The integration of cell-mediated drug delivery and theranostic nanomedicine can potentially improve cancer management in both therapeutic and diagnostic aspects. Herein, by taking advantage of innate immune cell’s ability to target tumor cells, we developed a novel drug delivery system by using macrophages as both nanoparticle-carriers and navigators to achieve cancer-specific drug delivery. Firstly, we fabricated theranostic nanoparticles from a unique biodegradable photoluminescent poly (lactic acid) (BPLP-PLA), which inherently possesses strong tunable fluorescence, biodegradability, and cytocompatibility. In order to minimize the damage of cancer drugs to carrier immune cells and other healthy cells, a BRAF V600E mutant melanoma specific drug (PLX4032) was loaded into BPLP-PLA nanoparticles. Muramyl tripeptide (MTP) was also conjugated onto the nanoparticles in order to improve the nanoparticle loading efficiency. The resulting nanoparticles were internalized within macrophages, which were tracked via the intrinsic fluorescence of BPLP-PLA to demonstrate potential in imaging-based diagnosis and therapy. Nanoparticle-carrying macrophages were able to bind with and deliver drugs to melanoma cells under both static incubation and dynamic shear-flow conditions. Finally, pharmacological studies indicated that the delivered PLX4032 loaded nanoparticles did not show cytotoxicity against macrophages, while effectively killing melanoma cells. Our “smart” immune cell-mediated drug delivery system demonstrates the advancement of intrinsically traceable and targeted cancer nanomedicine.
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Chapter 1

Introduction

Drug delivery systems (DDS) have been extensively studied in past several decades as a powerful tool to achieve improved therapeutic efficacy and diagnostic effects by remarkably improving the pharmacokinetics and pharmacodynamics. Recently, a great deal of effort has been made to design novel nano-scale DDS with controlled therapeutic outcomes, targeted modalities towards specific diseases, and minimized side effects. However, traditional DDS, including injection formulations, particles, liposomes, and hydrogels, cannot meet the ever-growing requirements of modern medicine, such as truly targeted therapies and personalized medicine. Thus, cell-mediated drug delivery has emerged as a new frontier in medicine.

Conventional DDS and their challenges

Current DDS typically employ vehicles to carry therapeutics in order to improve the drug solubility, reduce toxicity, prolong circulating time, limit bio-distribution, achieve specific targeting, control drug release, and diminish immunogenicity. Ideal drug delivery vehicles should be biocompatible, biodegradable, easy to modify, and targeted towards specific diseases. A wide range of vehicles have been employed as controlled DDS, e.g. liposomes⁷, nanoparticles (NPs)², micelles³, hydrogels⁴,⁵, fibers⁶,⁷, and films⁸. Particularly, nano- and micro-materials decorated with targeting ligands or molecules, such as peptides, antibodies, aptamers, and proteins that are specific to the receptors expressed or overexpressed on aberrant cells and their surrounding microenvironments have been widely studied in targeted drug delivery⁹,¹⁰. Unfortunately, very
few of these nanomedicines have shown clinical efficacy due to significant challenges associated with the trafficking and targeting \textit{in vivo}.

Current targeting strategies can be categorized into passive and active targeting. Passive targeting is typically dependent on enhanced permeability and the retention (EPR) effect caused by leaky vasculature and poor lymphatic drainage. The EPR effect is a unique phenomenon, by which macromolecules and nanoparticles (NPs) escape from the blood flow and preferentially accumulate more in tumors rather than in normal tissues. It occurs frequently in solid tumors, in which blood vessels commonly have defects. Additionally, defective lymphatic drainage leads to the loss of the ability to clear infiltrating substances. These distinguishing anatomical and pathophysiological characteristics of solid tumors have been generally utilized in tumor targeting. However, passive targeting is largely chance-dependent. Therefore, active targeting, especially molecular targeting, has been the focus of drug delivery research in recent years. Until now, numerous surface markers have been found on abnormal cells or in their surrounding microenvironment. Integrins, folate, growth factors, and cytokines offer possibility for counter-ligand functionalized drug vehicles to recognize cells and tissue targets\textsuperscript{11, 12}. With the capability to bind to specific surface markers, circulating drug carriers may have a higher tendency to attach and accumulate at the sites of diseases such as cancers. In addition, a number of cell-penetrating molecules have also been discovered that can further improve the drug delivery efficiency\textsuperscript{13}. Certainly, the combination of passive and active targeting for drug delivery may yield amplified results.

Despite tremendous efforts made towards discovering novel materials and biomolecule markers for targeted DDS, very few of them are truly specific after intravenous injection and the targeting still remains chance-dependent. Both active and passive targeting approaches require exogenous drug vehicles to disperse and voyage in circulation for a long time to pass through the leaky vasculature or detect the surface markers. However, the circulating environment, in which
many drug vehicles cannot have a long enough circulating time to achieve targeted binding, is extremely complicated\textsuperscript{14,15}. In addition, the human body has an innate defense mechanism for invasion. For example, the reticuloendothelial system (RES) rapidly recognizes foreign bodies and destroys them via a series of biological processes. The RES, also called the mononuclear phagocyte system, is comprised primarily of bone marrow progenitors, blood monocytes, and tissue macrophages\textsuperscript{16}. Furthermore, the EPR effect is somehow heterogeneous in the tumor microenvironments and varies among patients\textsuperscript{17}. For example, hypoxic regions of solid tumor generally do not even exhibit EPR effects due to poor angiogenesis\textsuperscript{17,18}. Considering the complexity and sophistication of in vivo conditions, conventional passive and active targeting strategies still remain inadequate. Hence, developing novel DDS with truly specific targeting is a formidable challenge for modern medicine and nanotechnology.

**Cell-mediated DDS**

Recently, cell-mediated DDS have emerged as a promising strategy that is poised to address the above challenges. This novel strategy takes advantage of cell properties, such as long circulation time, abundant surface ligands, flexible morphology, cellular signaling, and metabolism, to offer a unique opportunity to maximize therapeutic outcomes as well as minimizing side effects. The increasing attention towards this field can be seen from the increasing numbers of publications according to Web of Science\textsuperscript{TM} (Figure 1-1).
Figure 1-1. The numbers of publications searched with the keywords of "Cell Mediated" and "Drug Delivery". Source: Web of Science™.

Circulating Cells

Circulating cells can serve as ideal drug delivery carriers for a number of reasons. They are highly mobile and able to travel through blood flow without immunogenicity. Circulating cells are involved in various disease processes, including infection, inflammation, and cancers development, so they can offer multiple advantages for disease targeting. For instance, leukocytes have the innate ability to cross the blood brain barrier (BBB) to access tumor cells in the brain\textsuperscript{19}. Inflammatory responses and wound healing of many diseases are involved in cell homing processes that spontaneously attract circulating cells to disease sites. Furthermore, using circulating cells as delivery vehicles is advantageous as it significantly reduces immune clearance and prolongs the biological half-time for drug delivery. Candidate cells to mediate drug delivery
include erythrocytes, leukocytes, platelets, and stem cells, whose properties are summarized in Table 1-1.

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Leukocytes</th>
<th>Stem Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount</strong> (/ml blood)</td>
<td>4.2-6.0×10⁶</td>
<td>4×10⁸⁻¹⁻¹×10³</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Diameter (µm)</strong></td>
<td>~7</td>
<td>7-15</td>
<td>30-40</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td>Blood circulation</td>
<td>Circulation, tissues and organs</td>
<td>Circulation, tissues and organs</td>
</tr>
<tr>
<td><strong>Life Span</strong></td>
<td>~120 days</td>
<td>A few days; years for memory lymphocytes</td>
<td>Depending on cell types</td>
</tr>
<tr>
<td><strong>Functions</strong></td>
<td>Transport oxygen</td>
<td>Immune defense</td>
<td>Repair tissues and organs</td>
</tr>
<tr>
<td><strong>Pros as DDS</strong></td>
<td>Large encapsulating volume; large surface areas; longer life span; reversible deformation; RES targeting.</td>
<td>Capable of cross biological membrane; tumor-homing; immune response.</td>
<td>Potency; homing to injured cells, tissues and organs.</td>
</tr>
<tr>
<td><strong>Cons as DDS</strong></td>
<td>Rapid elimination; RES hard to handle.</td>
<td>Short life-span; hard to handle.</td>
<td>Hard to maintain potency in vitro.</td>
</tr>
</tbody>
</table>
Red Blood Cells

Erythrocytes, or red blood cells (RBCs), make up the largest population of blood cells (>99%). Approximately 2 million new erythrocytes are continuously produced per second in the human body. RBCs are non-nuclear biconcave discs that average ~7 µm in diameter and ~2.5 µm in thickness, and have a large internal capacity volume of 185-191 µm³. Utilizing hemoglobin as an iron-containing protein, RBCs transport oxygen from the respiratory organs to the rest of body. In addition to oxygen, RBCs can carry a range of valuable payloads from therapeutics to imaging contrast agents.

RBC-based drug delivery has attracted increasing attention for many reasons. RBCs can be easily isolated, frozen, and stored for an extended period of at least ten years. RBCs in the blood circulation have a life span of 120 days and thus may act as a reservoir for sustained drug release. The biconcave shape and non-nuclear architecture allow RBCs to encapsulate a large amount of drugs. The membranes of RBCs have reversible deformability, making them capable for taking up payloads via physical methods. Additionally, RBCs are completely biodegradable without producing toxic byproducts, as the RES recognizes old and incompatible RBCs and rapidly removes them. The clearance pathways of RBCs have been widely employed in targeting the RES of the liver, spleen and bone marrow.

The use of RBCs as drug delivery vehicles, however, still encounters several challenges. RBC carriers can be rapidly eliminated by the RES after drug encapsulation or cellular modification due to potential morphological and functional alterations. The RES rapidly
recognizes and eliminates modified RBCs before they reach disease sites, which makes non-RES targeting particularly challenging. Drugs may also be released from destructed cells and result in cytotoxicity. The abundance of RBCs could be a double-edged sword that prevents efficient delivery of therapeutics to disease sites. In addition, RBCs are generally not as selective as other circulating cells in terms of targeting disease sites and promoting the healing process.

**Leukocytes**

Leukocytes, or white blood cells (WBCs,) play significant roles in the immune system, by cleaning cellular debris and defending the body against infections and diseases. Normal human blood contains 4-11x10^9 leukocytes per mL. Leukocytes are found in five major types, neutrophils (40% - 75%), eosinophils (1% - 6%), basophils (less than 1%), monocytes (2%-10%), and lymphocytes (20%-45%). Although the lifespan of leukocytes (up to 20 days) is typically shorter than that of RBCs, their specialized functions make them attractive as drug delivery carriers because leukocytes are involved in various immune responses, cellular interactions, cell adhesion, and are capable of penetrating through biological barriers into non-vascular areas. Neutrophils, monocytes/macrophages, and lymphocytes are all potential candidates for delivering therapeutics to treat various diseases.

Neutrophils, also known as polymorphonuclear granulocytes (PMNs) that contain distinctive cytoplasmic granules, are the most abundant leukocyte found in the human body. They are the first cells that arrive at the sites of infection or inflammation, produce cytokines to recruit other cells, and are cleared after a few days. Neutrophils also engulf invading microorganisms or foreign substances and consequently eliminate the invaders using digestive enzymes (e.g. lysozyme, hydrolytic enzymes and myeloperoxidase) or respiratory burst. Unfortunately, neutrophils have the average lifespan of 5.4 days in circulation and only a few hours after their
isolation from blood\textsuperscript{25}. The short life span of neutrophils limits their applications in DDS, but their ability to immediately migrate and transport make them attractive as drug carrier candidates.

Monocytes are mononuclear leukocytes with kidney-shaped nuclei and clear cytoplasm. They are produced from stem cell precursors in the bone marrow. Monocytes circulate in the bloodstream and migrate to tissues, particularly the liver, lymph nodes, and lungs. Meanwhile, monocytes continuously migrate to and accumulate at disease sites in association with infection or inflammation\textsuperscript{26, 27}. Once leaving the blood flow, monocytes differentiate into macrophages in response to various stimulations. Otherwise, they return to the bone marrow without activation. Macrophages play versatile roles in inflammation, cell recruitment, secretion of cytokines and growth factors, and bacteria/cellular debris clearance. Emerging studies also indicate that macrophages are the major players in disease microenvironments and disease progression, such as in cancer invasion\textsuperscript{28, 29}. Additionally, monocytes/macrophages present phagocytic capability that enables the spontaneous encapsulation of drugs/particles.

Lymphocytes are identified by their large nucleus surrounded by a thin layer of cytoplasm. The average diameter of lymphocytes is 7-15 \(\mu m\). Lymphocytes are primarily found in the circulation and central lymphoid organs such as the spleen, tonsils, and lymph nodes\textsuperscript{30}. T cells and B cells are the major types of lymphocytes and are responsible for the adaptive immune system. T cells mature in the thymus and play a critical role in cell-mediated immunity and can be broadly divided into helper T cells, cytotoxic T cells, and regulatory T cells\textsuperscript{31}. When an antigen appears, antigen-presenting cells (APCs) recognize and present the antigen to T cells. Then, helper T cells secrete various cytokines, which stimulate toxic T cells to directly kill abnormal cells. Regulatory T cells are also activated to suppress immune response in order to maintain immunological tolerance. B-cells are produced in the bone marrow and involved in humoral immunity. B cells make antibodies against antigens and can be characterized by the presence of immunoglobulin on their surface\textsuperscript{32}. Meanwhile, B cells can differentiate into memory B cells,
which respond rapidly when exposed to the same antigen. Thus, both lymphocytes present multiple functions in human immunity and are involved in numerous diseases: detecting antigens, infiltrating disease sites, and attacking abnormal cells. Clearly, lymphocytes could serve as a potential platform to deliver drugs specifically.

Overall, leukocytes have a rapid response and intrinsic homing properties with respect to infections, inflammations, and tumors. Such sensitive detections and biological barriers infiltration abilities give rise to opportunities for leukocytes-mediated drug delivery. However, vulnerable leukocytes are difficult to harvest and handle with relative short life spans, which hinder the manipulation processes for loading drugs and NPs.

**Stem Cells**

Stem cells are self-renewable with a potential to differentiate into various cell types and therefore essential in tissue repair and regeneration. They are broadly classified into two categories according to their source and plasticity: embryonic stem cells and adult stem cells. Embryonic stem cells originate from the inner cell mass of a blastocyst in an early-stage embryo, which are pluripotent that can generate all cell types in the body. Adult stem cells are tissue-specific stem cells that can differentiate into limited specialized cells. Stem cell therapy has been significantly explored in tissue engineering, regenerative medicine, and even translated into clinical trials. Utilizing stem cells as drug carriers can be particularly beneficial since it may add another dimension into existing stem cell therapy. Current stem cell therapies including those for neurological disease, heart disease, and cancer can all be improved by incorporating therapeutics into stem cells. Interestingly, stem cells are inherently tumor homing, which is mediated by inflammatory factors, tumor-derived factors, and tumor-specific receptors. Generally, stem cells take 2-4 days to migrate to tumor sites. Thus, stem cells also have natural
living targeting capabilities that respond to diseases and tissue regeneration. Additionally, stem cells are relatively easy to harvest, culture in vitro, and can be induced to differentiate into specialized cells under certain conditions. These properties make stem cells promising candidates for targeted drug delivery.

**Encapsulation/Conjugation of Drugs/Particles to Circulating Cells**

In order to obtain cell-mediated drug delivery, drugs or drug-loaded particles should be able to attach onto or internalized into cells to form drug-cell or particle-cell complexes. The basic requirements for drugs and drug-loaded particles in cell-mediated delivery are no or low toxicity to cell carriers, particle degradability, and controlled release of drugs. For drug/particle-cell complexes, strong binding between payloads and cell carriers, long circulating time, and low immunogenicity are desired. The use of cell carriers should not only transport drugs in high specificity to target tissues, but also regulate pharmacokinetics and pharmacodynamics via altering the properties of biomaterials that are used to encapsulate therapeutics. To date, drug vehicles can be added to cells by biological pathways, physical approaches or chemical modifications (Figure 1-2).
Figure 1-2. Strategies of encapsulation and conjugation of drugs/particles onto/into circulating cells, including red blood cells (RBCs), leukocytes, and stem cells, by biological, physical and chemical means.
**Cellular uptake and binding by biological pathways.**

The most straightforward way to load drugs with circulating cells is using unmodified cells, which possesses natural biological pathways that can be utilized. Biological pathways represent a series of actions that regulate cell behavior and fate at the molecular level. Various cells are able to load drugs via different pathways. In these cases, their morphology and functional integrity can be largely preserved. In this section, we will review the pathways that facilitate the encapsulation of drugs or genes within cells and/or conjugating nanoparticles onto the surface of cells. Loading therapeutics via physical and chemical modifications of cells will be reviewed in later two sections, respectively.

*Endocytosis.*

Endocytosis is a fundamental cellular process, by which eukaryotic cells engulf fluid, molecules, and even other cells\textsuperscript{39}. Most substances, especially large and polar molecules, cannot directly pass through the hydrophobic cell membranes. Endocytosis provides a pathway to internalize these substances via plasma membrane deformation. During this process, plasma membranes invaginate and form intracellular vesicles around the substances to be internalized, followed by the membrane fusion. Endocytosis is dominated by several parameters such as sizes, shapes, charges, and mechanical properties of the foreign substances\textsuperscript{40}. It offers an opportunity to regulate the encapsulation of drug vehicles by an endocytosis-dependent pathway.

Pinocytosis is a mode of endocytosis that can non-specifically “drink” the surrounding liquid and molecules. Eukaryotic cells continuously conduct pinocytosis and are prone to engulf relatively small molecules. This plasma membrane homeostasis process has been utilized encapsulation bare drugs into erythrocytes in early attempts. Drugs, such as hydrocortisone,
primaquine, vinblastine, and chlorpromazine, can directly induce erythrocyte membrane internalization by pinocytosis.\textsuperscript{41-43} The formation of vacuoles is age-related\textsuperscript{44}. Pinocytosis is adenosine triphosphate (ATP)-dependent and influenced by the drug concentration, temperature, pH, and the balance between the concentrations of magnesium and calcium in the cell membrane.\textsuperscript{43, 45} These drug-loading methods are based on simple absorption; however, they tend to induce potential cytotoxicity.

In contrast to pinocytosis, phagocytosis sporadically occurs in specialized cells (e.g. neutrophils, macrophages, lymphocytes, and dendritic cells). Stimulated by immune responses, these white blood cells can internalize particles in nano or micro sizes via an actin-dependent mechanism associated with receptor reorganizations such as Fc receptors, complements, and mannoses\textsuperscript{46}. The sizes of drug vehicles, including NPs, micelles, and liposomes, commonly fall in this range. Then, the engulfed particles fuse with lysosomes that are later broken down by abundant digestive enzymes in lysosomes. Since phagocytosis is a type of immune defense procedures against foreign invaders, including drugs or drug-encapsulated particles, it gains tremendous interest in cell-mediated DDS design. Drug-laden particles are susceptible to rapid RES clearance in vivo, which means immune cells are the first cells to sense and catch particles during circulation. Instead of designing particles to overcome rapid RES elimination, phagocytic cells can be rationally utilized as delivery carriers. Further, phagocytosis can be significantly enhanced by opsonization, by which particles are coated with proteins and/or antibodies to enable the rapid reorganization of particles by the mononuclear phagocytic system\textsuperscript{47}. For example, immunoglobulin and albumin were coated on superparamagnetic iron oxide nanoparticles (SPIO NPs) to facilitate the monocyte-macrophage uptake\textsuperscript{48}. Compared with pristine SPIO NPs, a larger amount of opsonized-SPIO NPs accumulated in monocytes nuclear periphery\textsuperscript{48}.

Numerous nanomaterials, including inorganic NPs, polymeric NPs, and liposomes, have been successfully loaded into phagocytic cells and delivered to target various diseases (Figure 1-3
One important understanding in the application of phagocytic cell carriers is that the material geometry plays a significant role in regulating cell internalization. Specifically, particle size is crucial in this process, since phagocytosis takes place at the nano/microscale level\textsuperscript{54, 55}. IgG-opsonized and non-opsonized polystyrene (PS) microspheres with an average diameter of 2-3 µm exhibited the highest internalization in a rat alveolar macrophages phagocytosis model.\textsuperscript{54, 55} Material geometry also affects phagocytosis. Champion et al. studied phagocytosis of alveolar macrophages and cultured the cells with PS particles with various sizes and shapes\textsuperscript{56}. Results suggested that macrophage internalization largely depends on the local particle shape at point of initial contact. The local particle shape is defined as the angle (Ω) between the normal of initial cell/particle contact point and a vector from the initial contact point to the center point of the particle contour. Thus, Ω represents the local curvature. There is a critical point, Ω = 45°, where the internalization cannot be observed. Phagocytosis does not occur any more where is Ω > 45°.\textsuperscript{56} The relationship between local particle shape and phagocytosis leads to so-called shape engineering, which is important in cell-mediated drug delivery. Additionally, macrophage phagocytosis is regulated by the mechanical properties of the substrates, as macrophages show a strong preference to engulf rigid objects.\textsuperscript{58} For example, macrophages internalized about 6 times IgG opsonized polyacrylamide micro-beads with a higher crosslinking rate (stiff beads) than those soft beads with 4 times lower crosslinking rate.\textsuperscript{57} The effect of surface charge on phagocytosis, however, raises a controversy. Ari et al. coated drugs with poloxamer and/or phospholipid surfactants to form NPs with various surface charges. They found that positively charged NPs accumulate more in macrophages than negatively charged NPs.\textsuperscript{50} But liposomes with negative charges on surfaces were uptaken more by macrophages through the scavenger receptor recognition.\textsuperscript{58, 59} These paradoxical observations might be due to various phagocytic pathways adopted in the cellular uptake of different materials.
Certainly, it is also important to prevent drug release within phagocytic cells before reaching the target disease site, since it may compromise the therapeutic effects and also damage cell carriers. In some cases, it is necessary to inhibit phagocytosis since cells may rapidly degrade the internalized particles. Thus, the design of drug-loaded particles is critical to achieve ideal particle location. Generally, it is considered that surface anchored particles would be ideal. For example, worm-like particles with very high aspect ratios (>20) were designed to partially inhibit macrophage phagocytosis in order to protect the drug during the circulating and targeting processes. \(^6\)

Therefore, endocytosis is a powerful and tricky pathway to load drugs and/or NPs. However, due to its complexity involving diverse receptors and a series of fusion and fission events, precisely controlling cell internalization is still a significant challenge. New techniques to fine-tune particle properties and endocytosis processes are still needed to improve endocytic cell-mediated nanoparticle delivery.

**Ligand-receptor interactions.**

Ligand-receptor interactions are one of the most basic ways for cells to communicate with each other and the environment. Cellular receptors are generally embedded within plasma membrane surfaces, which contain specific sites for ligand binding and vice versa. Ligand-receptor interactions have been utilized by currently active nanoparticle targeting strategies due to its selectivity and specificity. Thus, this approach also can be used to load particles onto/into circulating cells. Considering that circulating cells are relatively rich in receptors on the surface, a variety of drug carriers have been linked to them via ligand-receptor interactions.

As an example, CD44 markers are involved in cell-adhesion and extensively expressed on a large number of circulating cells, e.g. leukocytes and stem cells. CD44 is also a receptor for
hyaluronic acid (HA), which is a glycosaminoglycan that can be found in the extracellular matrix. HA is a promising drug delivery material that is known for its biocompatibility and intrinsic binding capability to CD44. HA coated polyelectrolyte multilayer (PEM) cell patches were developed to attach on the surfaces of B and T lymphocyte by binding to the CD44 on surfaces (Figure 1-3B)\textsuperscript{61, 62}. The cell-PEM-HA complexes retained strong retention that can be controlled by varying the size of the patches and the ratio of cells to patches\textsuperscript{63}. The binding efficiency between PEM-HA and B cells increased at lower pH and higher NaCl concentrations\textsuperscript{61}. This phenomenon may result from the change of HA conformation that exposed more binding sites to CD44 receptor\textsuperscript{61}. Compared to conventional spherical particles of equal volume, the low curvature particles significantly decreased cell uptake. Interestingly, flat PEM-based disks with ~6 µm average diameters that exhibited low phagocytosis were able to tightly attach on cell surfaces\textsuperscript{64}. This so called cellular backpack is an alternative approach for cell-mediated drug delivery by attaching drug-loaded particles on cell surfaces, maximizing protection for therapeutic payloads and cell integrity. The cellular backpack achieved controlled release of drugs since the drugs were encapsulated in biodegradable poly(lactic-co-glycolic acid)\textsuperscript{64}. These approaches are promising in preventing drug release off-site, avoiding RES elimination, and enhancing the retention time of NPs in blood flow. Most recently, self-assembled thermo-sensitive poly(ethylene glycol) methacrylate-HA nanogels were found rapidly phagocytized by macrophages and accumulated in the liver and spleen 13 minutes after intravenous injection in mice. This study also suggested that HA-CD44 binding was effective for attaching nanocarriers on macrophages\textsuperscript{65}. These macrophage/nanogel complexes have shown potential in drug delivery for macrophage-associated diseases.

The cell-particle encapsulation/conjugation also can be achieved by antibody–antigen interactions. Silica nanorattles, namely hollow silica spheres with moving cores, were bioconjugated with monoclonal antibody to recognize membrane proteins of CD73 and CD90 on
mesenchymal stem cells (MSCs)\textsuperscript{66}. After antibody–antigen recognition, antibody-modified nanorattles were internalized within MSCs, suggesting that multiple pathways were involved in the process\textsuperscript{66}. Each MSC can retain up to 1,500 nanorattles for at least 48 hours, which is sufficient for MSCs to migrate to tumor sites\textsuperscript{66}.

Additionally, some receptors only exist in particular cell types, granting opportunities for cells taking up particles in the circulation instead of assembling the cell-drug complex in vitro. For instance, complement receptor type 1 (CR1) is primarily expressed on RBCs. Monoclonal antibody (mAb) against CR1 (Anti-CR1) was conjugated on a tissue-type plasminogen (tPA) activator in order to couple with RBC carriers\textsuperscript{67}. Anti-CR1/tPA can rapidly bind to RBCs to prolong circulation time\textsuperscript{68}. Glycophorin A is another erythrocyte specific surface marker. ERY1 is a 12 amino acid peptide that is able to specifically bind to the RBC surface via glycophorin A\textsuperscript{68}. Antigen ovalbumin conjugated with ERY1 spontaneously recognized RBCs within 30 minutes and remained tightly bind for at least 72 hours after intravenous injection in mice (Figure 1-3C)\textsuperscript{69}. However, to the best of our knowledge, no drug-loaded particle has been conjugated to circulating cells through these specific interactions.

Thus, ligand-receptor binding is a powerful method to attach particles to circulating cells. Advances in cellular engineering with discoveries of new surface markers and binding ligands are beneficial for ligand-based drug carrier design. Improving the binding efficiency on particular cell types is still remaining as a challenge. It is also desired to accomplish in vivo specific binding within the blood circulation.
Figure 1-3. Examples of drugs/NPs internalized into or conjugated onto circulating cells via innate cellular uptake/binding. (A): Endocytosis pathway. Fluorescence microscopy images of phagocytosis of gold silica nanoshells (red) by macrophages. Reprinted with permission from Ref 53. Copyright © 2012, PLOS ONE. (B): Ligand-receptor interaction. Confocal microscopy images of HA functionalized PEM-based cellular patches (green) attached on the surface of a T cell (B1) and a B cell (B2) via CD44 reorganization. Scale bar, 10 μm. Reprinted with permission from Ref 62. Copyright © 2008, American Chemical Society. (C): Specific ligand-receptor interaction that exclusively occurs in certain cells. Confocal microscopy images of OVA (red) conjugated ERY1 peptide, binding to a mouse RBC (labeled with anti-mouse glycophorin-A, green) through glycophorin-A, C1; instead, no free-OVA observed on RBC, C2. Scale bar, 5 μm. Reprinted with permission from Ref 69. Copyright © National Academy of Sciences USA. (D): Covalent conjugation. Confocal microscopy images of D1: Maleimide-functionalized liposome-coated NPs (purple) conjugated on T cells (green) via thiol groups; D2: NPs relocated during migration. Scale bar, 2 μm. Reprinted with permission from Ref 70. Copyright © 2012 Elsevier Ltd.

**Gene modification and delivery.**

Great efforts have been made to insert therapeutic plasmid DNA, mRNA, and siRNA into living cells to build novel gene or drug delivery systems. Transduction and transfection, both of which are powerful ways to obtain a large number of functional cells in vitro and in vivo, can introduce foreign genes, including therapeutic genes, into host cells.

Cell-mediated viral delivery to tumors has been well studied. Oncolytic virus (OV) is an anti-cancer agent that can selectively induce cell death in cancer cells, while leaving normal cells healthy. Transduction can effectively introduce OVs into circulating cells based on a virus-mediated DNA transfer mechanism. Furthermore, OVs can be transported with cell carriers to
tumors, followed by local replication and amplification to suppress tumor growth. A variety of OVNs, such as adenovirus, herpes simplex virus, poxvirus, vesicular stomatitis virus, measles virus, Newcastle disease virus, influenza virus, and reovirus, has been combined with different cell-based delivery systems. For example, mesenchymal progenitor cells (MPCs) have served as vehicles to deliver oncolytic adenoviruses. To enhance transduction efficiency, oncolytic adenoviruses were first genetically modified with cellular integrin-binding motifs. Transfused MPCs maintained their intrinsic capacity of sensing tumors and further improved the therapeutic effects in an ovarian carcinoma model. Additionally, human activated T lymphocytes infected with oncolytic measles viruses were able to transfer viruses to tumor cells via the heterologous cell fusion, and subsequently deliver viruses to myeloma.

Virus therapy is widely studied, but its clinical safety is still a concern due to rapid proliferation of viruses, off-target cytotoxicity, and body antiviral immunity. Nonviral transfection is an alternative strategy to introduce therapeutic nucleic acids into cellular carriers. Hu et al. developed nonviral vehicles, based on low molecular weight polyethylenimine-co-β-cyclodextrins (β-CD), to transfec tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAILs) into circulating MSCs. MSC-TRAILs efficiently killed tumor cells in a lung metastasis model. In order to achieve non-invasive drug delivery and MRI contrast capabilities, light sensitive, gold-coated SPIO core-shell magnetic NPs (SPIO@AuNPs) were transfected into cell adipose-derived MSCs. Adipose-derived -MSCs retained high cell viability, osteogenic differentiation, and disease homing ability after transfection. Interestingly, SPIO@AuNPs stimulated cell growth at a certain concentration (<10 μg/mL) and did not induce pro-inflammation, which is typically promoted by other SPIO NPs.

With the help of genetic modifications, cell carriers are capable of constantly expressing desired therapeutic or imaging molecules. Since transduction/transfection permanently alters cell
properties, it inevitably gives rise to safety concerns and even ethical issues. Researchers should take them into account when applying genetic modifications.

**Loading therapeutics to cells by physical methods.**

RBCs possess various reversible transformation capabilities that allow for drug loading simply with external physical treatments such as hypotonic haemolysis and electroporation (Figure 1-2). These approaches are relatively easier to handle *in vitro* and eliminate the use of chemical reagents to reduce possible alterations of RBCs. RBCs can recover to their normal morphology and maintain the role of transporting therapeutics and particles to targeted sites.

**Hypotonic haemolysis.**

Hypotonic haemolysis has been widely applied in RBC encapsulation. RBCs behave like osmometers that shrink in hypertonic conditions and swell in hypotonic conditions. Pores open on cell membranes, when the critical haemolytic volume is reached. The increased cell membrane permeability in hypotonic solution enables the entrapment of drugs, biomacromolecules, and NPs in RBCs by a passive mechanism\(^80,81\). The principle of reversible swelling is based on the fact that the absence of a superfluous membrane allows cells to accommodate additional volume by changing the shape from biconcave to spherical. Several hypotonic haemolysis techniques have been generated, such as hypotonic dialysis, hypotonic dilution, and hypotonic pre-swelling\(^82,83\). Hypotonic dialysis is predominantly applied in encapsulating enzymes, proteins, and contrast agents due to its relative ease of use, ability to preserve cell characteristics and high encapsulation rate. In the process, erythrocytes with a hematocrit value of 70-80% are prepared in a dialysis tube and immersed in a hypotonic buffer for a few hours under gentle stirring. The techniques
have been applied in encapsulating drugs, enzymes, and proteins since 1970s. Peptide nucleic acids (PNAs), as therapeutic agents, were loaded into RBCs via hypotonic dialysis and achieved 82% encapsulation after 18 hours incubation. PNAs-laden RBCs underwent opsonization with ZnCl₂ and bis-sulfosuccinimidil-suberate treatment, and then specifically targeted macrophages. PNAs were effectively delivered and resulted in reduced production of nitric oxide and inhibited protein expression of the enzyme nitric oxide synthase.

**Electroporation.**

Electroporation is a physical method that uses an electrical pulse to create temporary pores on cell surfaces. Cells are typically suspended in a conductive solution and exposed to a high-intensity external electrical field. The disturbance of the phospholipid bilayer induces temporary dysfunctions of the cell membranes, resulting in the encapsulation of exogenous molecules in cells. During the process, cells keep their morphology when engulfed molecules are larger than the size of electropores. However, cells may swell and the cell membranes may rupture if the substances are smaller than the size of electropores. Various compounds such as drugs, enzymes, and genes have been encapsulated within RBCs accomplished with sustained release. The drawbacks of electroporation are obvious. The electrical force causes irreversible deterioration and the recovery rate is commonly low. Thus, it is necessary to optimize the applied voltage to keep cell integrity in electroporation.

**Loading/conjugating therapeutics into/onto cells by chemical modifications.**

Compared to biological and physical methods to load drugs into cells, chemical modification may fix drugs and drug vehicles onto/into circulating cells and avoid the metabolic
degradation of materials and drugs within the carrier cells. The surface of circulating cells is a highly heterogeneous structure composed of different proteins, lipids, and carbohydrates with various functional groups for chemical modification opportunities as mentioned previously\textsuperscript{95, 96}. Furthermore, chemical modification is not solely dependent on the naturally available cell surface molecules. The complexity of the cell membrane offers alternatives to introduce additional functional groups for a number of conjugating reactions. Compared to pristine cells, modified cells enable convenient conjugations with therapeutics and particles via versatile chemistry tools (Figure 1-2).

\textit{Covalent conjugation onto surface markers.}

A number of functional groups have been discovered on different cell surfaces that allow bioconjugation with molecules or particles. Among the available functional groups, primary amine and thiol groups are mostly used for covalent reactions due to the ease of labeling and low toxicity of the reactions. N-hydroxysuccinimide (NHS) ester-based crosslinkers are most popular for primary amines modification via carbodiimide reaction. Rossi et al. grafted hyperbranched polyglycerol (HPG) onto the surface of RBCs to generate immunocamouflaged cells\textsuperscript{97}. HPG is a hemocompatible polymer with abundant hydroxyl groups that are ideal for surface modification. HPG was functionalized with succinimidy succinate (SS) groups by initially reacting with succinic anhydride and then with NHS\textsuperscript{98}. SS-HPG was eventually grafted with primary amine groups presented on the RBC surfaces. Also, the thiol groups in cysteine containing membrane proteins are available for coupling via maleimide-thiol conjugation. Maleimide-functionalized liposome-coated NPs were conjugated on the surface of lymphocytes and MSCs\textsuperscript{98}. The attachment of NPs did not activate lymphocytes or alter stem cell tumor-homing ability\textsuperscript{98}.\textsuperscript{99}
Stephan et al. also found maleimide-functionalized NPs rapidly relocated to T-cell/tumor cell contact zone following antigen recognition (Figure 1-3D) \(^70\).

Covalent coupling typically exerts stronger binding than ligand-receptor interactions to avoid drug vehicle detachment during cell migration. Versatile conjugation chemistry enables exclusively covalent reactions with amino or thiol groups that are naturally expressed on cells. These covalent bindings help anchoring drug carriers on the surfaces of cells, which have advantages over engulfing inside cells in terms of cellular safety and drug protection.

**Biotinylation.**

Biotin is a water-soluble vitamin that has a high affinity for avidin and streptavidin proteins\(^99\). Biotinylated cells can be conjugated with various proteins, enzymes, and NPs without compromising their viability and biological activities. NHS ester-activated biotins are widely-accepted biotinylation reagents that can introduce biotins on living cell membranes that contain primary amino groups\(^100,101\). NHS esters form stable amide bonds with primary amino groups in buffers within a few hours at room temperature, which can be tolerated by most cells. NeutrAvidin-coated NPs were anchored on the biotinylated membranes of human mesenchymal stem cells (hMSCs) for up to 2 days, while a certain amount of NPs were observed within the cytoplasm of hMSCs possibly resulting from the membrane recognition of NPs\(^102\). hMSCs maintained the tumor-homing capability after nanoparticle attachment\(^102\). RBCs were also coupled with a thrombolytics agent on their surface via avidin-biotin bridges, serving as Trojan horses for prophylactic fibrinolysis\(^103,104\). With increasing attention to cell surface biotinylation, additional biotinylation reagents have been developed to target different functional groups such as sulfhydryls, carboxyls and carbohydrates. This diversity provides multiple choices in terms of surface modification of circulating cells to bind and load drugs or NPs.
Click chemistry.

“Click” chemistry represents a rapid, selective, and high-yielding bio-orthogonal reaction that is also capable of immobilizing materials on cell surfaces. Numerous click chemistry strategies have been recently developed for living cells, including copper(I)-catalyzed azide-alkyne (CuAAC), strain-promoted azide–alkyne cycloaddition (SPAAC), thiol-ene, Diels–Alder, and Pseudo-Click reactions. Unlike many traditional synthetic routes, click reactions take place in water solution at physiological pH (6-8) and temperature (37°C) without toxic by-products, which are ideal platforms for loading drug vehicles onto circulating cells.

To improve the binding efficiency, abiotic functional groups are initially introduced onto cells allowing counter groups on drugs and particles to undergo click chemistry. To date, CuAAC, SPAAC, and thiol-ene reactions have been applied to click living cells.

CuAAC and SPAAC are [3+2] azide-alkyne cycloaddition in the context of Staudinger Ligation. Azide groups, which are naturally absent on mammalian cell surfaces, need to be introduced to enable click reactions between cells and therapeutics via biosynthesis. Cell glycosylation, which is also called carbohydrate reaction, provides accessibility to incorporate azido sugars onto membranes through sialic acid. Generally, azido sugars can be metabolically inserted into the cell membranes after 24-48 hours of incubation. The azide-functionalized cells subsequently undergo reaction with drugs or NPs containing activated alkyne groups. The reaction, however, is thermodynamically favorable at high temperature or pressure, which is not applicable in living systems. Thus, CuAAC utilizes Cu+ catalyst to accelerate the reaction within 30 minutes at physiological conditions. Unfortunately, the cytotoxic Cu+ catalyst limits the in vitro and in vivo applications. Importantly, studies have indicated that alkyne activation can be achieved by introducing ring strain, namely SPAAC, which is a Cu-free click chemistry for selective biomolecule labeling in living organisms based on different glycosylation degrees.
between abnormal and normal cells\textsuperscript{107, 111}. Xu et al. immobilized highly branched polyamidoamine dendrimers on azido-modified macrophage surfaces by SPAAC copper-free click chemistry\textsuperscript{112}. Cell motility and its common stress-activated signaling pathways, including AKT, p65, and p38, have no significant alteration during the cell-nanoparticle hybridization process. An alternative approach to employ click chemistry is the thiol-ene reaction. Briefly, methacryloyl groups can be incorporated onto cell surfaces through a glycosylation pathway and then clicked with a thiol-terminal polymer. The thiol-ene reaction was explored on clicking cancer cells\textsuperscript{113}. Despite few developments on clicking circulating cells, click chemistry has shown potential in delivering therapeutics and imaging agents. A shortcoming of linking cell and therapeutics by click chemistry is that relatively complicated surface chemistry is required.

**Targeting Strategies of Cell-mediated Drug Delivery**

Once drug vehicles are assembled with circulating cells either in vitro or in vivo, the complexes can travel and diffuse within the blood circulation and further navigate to the desired targets (Table 1-2). By taking advantages of carrier cells’ intrinsic capabilities, controlled drug/particle delivery to specific disease sites can be achieved (Figure 1-4). In this section, we will review the existing circulating cell-based targeting strategies for various objectives.

Table 1-2. Summary of current drug vehicle encapsulation/conjugation methods for different cell-mediated drug delivery and their targeted applications.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Mechanism</th>
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<th>Drug/Particles</th>
<th>Target</th>
<th>Ref.</th>
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</table>
Figure 1-4. Illustration of circulating cell-mediated targeting and drug delivery pathways. #1: living targeting to disease sites via leukocytes and stem cells. #2: RES targeting via RBCs. Passive targeting: RES/non-RES targeting.

The first rational living cell-based targeted drug delivery strategy is passive targeting, including RES and non-RES effects. For example, as RBCs age, they are recognized by the RES and subsequently undergo cell membrane lysis by lysosomal enzymes, hemoglobin degradation, and eventually lose their flexibility, integrity, and functionality\textsuperscript{114, 115}. Taking advantage of the metabolic pathways in physiologic conditions, RBCs-based drug/particle complexes can easily and efficiently target RES-related regions such as liver, spleen and bone marrow. RES clearance can be accelerated by altering the properties of RBCs during drug-loading processes in order to
enhance the targeting efficiency. This strategy has been demonstrated in drug delivery for over 30 years. Its applications range from RES organ targeting to targeting specific cell types, including hepatic Kupffer cells, alveolar macrophages, peritoneal macrophages and peripheral blood monocytes, for delivering drugs to treat diverse diseases such as infections, inflammations, leukemia and cancers.

However, rapid clearance by the RES significantly limits the employment of RBC carriers in non-RES targeting. To address this problem, several approaches have been developed to permit the survival of heterologous or even xenogeneic RBCs in the circulation. A straightforward strategy to modify RBCs is by conjugating with nonimmunogenic materials onto the surfaces of RBCs in order to generate immunocamouflaged cells. Polyethylene glycol (PEG) is a bioinert and hydrophilic polymer that is widely used for cell stealth purposes. Using the same technique, methoxy(polyethylene glycol) (mPEG) was attached on the surface of RBCs and the immunogenity of modified RBCs was adjusted by varying the surface density and chain size of the coating polymer. The mPEG-modified RBCs still retained normal morphology, osmotic fragility, oxygen transportation, and high cell viability, but became resistant to RES clearance with prolonged circulation time. An alternative polymer, HPG, has similar antigen “mask” effects. HPG is a hyperbranched polymer with the capability of further modification to achieve controlled release. Furthermore, stimuli-responsive RBCs, such as ultrasound-responsive RBCs and magnetic-responsive RBCs, can be directed to disease sites, providing a platform for non-RES targeting. However, cell-based passive targeting strategies are still similar to the passive targeting of bare NPs, which typically cannot reach the desired targeting and drug delivery efficiency. Additionally, RBCs are mostly used in passive targeting pathways, which are less favorable for many diseases.
Live cell-mediated targeting strategies.

Many circulating cells such as neutrophils, monocytes/macrophages, and stem cells present targeting capabilities to many diseases, including cancer, wounds, and ischemic tissues. For example, tumor-homing, one of the most studied targeting mechanisms of living cells, suggests that various cells can not only sense solid tumors, but can also track circulating cancer cells in the blood flow and reach the primary and metastatic tumor sites. Targeting solid tumors, tumor microenvironments, and cancer metastases will be reviewed, respectively here.

Leukocytes are the cells involved in immune response so they are essential for inflammation, disease development, regulation, and healing processes. The general paradigm of neutrophil and monocyte extravasation includes tethering, rolling, adhesion, crawling, and eventually transmigration\textsuperscript{131-132}. For many diseases, an injured endothelium is activated directly by pathogens or indirectly by pro-inflammation\textsuperscript{133-134}. Neutrophils are well recognized as the first cells to be recruited by chemoattractants (e.g. platelet-activating factor, interleukin 8, interleukin 17, formyl-methionyl-leucyl-phenylalanine, and complement component 5a), cytokines (e.g. TNF-\(\alpha\)), growth factors (e.g. granulocyte-macrophage colony-stimulating factor), and bacterial products (e.g. formylated peptides and lipopolysaccharide)\textsuperscript{135-136}. With the increasing expression of adhesion molecules, P-selectin and E-selectin in particular, neutrophils tether and slowly roll onto the surface of the endothelium\textsuperscript{137-139}. Mediated by chemokines and adhesion molecules, neutrophils firmly attach on the endothelium and subsequently crawl towards endothelial adjunctions\textsuperscript{140-143}. Finally, neutrophils cross the endothelium, basement membrane, pericytes, and reach disease sites\textsuperscript{144}. Monocytes are also recruited by similar chemokines, cytokines and growth factors\textsuperscript{132, 145}. Afterwards, monocytes differentiate into highly specialized macrophages to participate in the following immune responses\textsuperscript{146}. Similarly, lymphocytes can infiltrate tumor sites and display a series of immune responses\textsuperscript{147,148}. Therefore, the tumor-homing mechanisms of
leukocytes provide active and “living” approaches for cell-mediated drug delivery. Doxorubicin (Dox) encapsulated liposome-carrying macrophages accumulated in lung tumors and released Dox after 24 hours of administration, but a smaller amount of Dox was observed without using macrophage carries (Figure 1-5A)\textsuperscript{50}. Adhesion molecule-associated extravasation also provides targeting options. PEM functionalized T cells can migrate to ICAM-coated surface\textsuperscript{62}. A recent study has demonstrated that RGD modified single-walled carbon nanotubes can target monocytes and enhance the delivery efficiency of monocyte carriers via integrin mediation (Figure 1-5B)\textsuperscript{149}.

A particularly important application of leukocyte-mediated drug/nanoparticle delivery is the brain drug delivery across the BBB. The BBB, which is a tight assembly of endothelium cells and astrocytes, separates the brain from the circulatory system\textsuperscript{118}. The barrier is therefore highly selective and only allows the passage of essential nutrition such as water, soluble lipid molecules, amino acids, and glucose while blocking other substances. The BBB protects the brain from harmful substances; however, it also establishes a huge obstacle for delivering therapeutic agents to the brain. Interestingly, monocytes/macrophages are able to transmigrate across the BBB. Therefore, they can potentially serve as carriers for sheathed brain drug delivery. Bone-marrow-derived macrophages were found to be able to infiltrate into the brain and deliver nanozyme in a Parkinson's disease model\textsuperscript{150}. MRI contrast agent-labeled murine monocytes/macrophages also targeted and accumulated in rat brain tumors\textsuperscript{151}. A similar concept was applied in FluoSpheres® NeutrAvidin® labeled microspheres delivered to brain metastasis of breast cancer via monocytes/macrophages (Figure 1-5C)\textsuperscript{54}.

The tumor microenvironment, which involves a vast amount of cells, vessels, and stroma, is another potential target for circulating cell-mediated therapeutic delivery. Solid tumors generally contain hypoxic regions with low oxygen levels due to poor angiogenesis. Hypoxia is associated with necrosis and facilitates tumor mutation\textsuperscript{152}. Due to the lack of blood vessels, hypoxic cells are resistant to traditional drug delivery approaches. Promisingly, monocytes,
tumor-associated macrophages, and tumor infiltrating lymphocytes are frequently found within tumor microenvironments including hypoxic and necrotic regions, suggesting that leucocytes could access in the depth of tumors. Choi et al. utilized monocytes/macrophages as “Trojan Horses” to deliver gold nanoshells to the hypoxic regions of cancer and their results suggested that malignant cells were completely killed within these areas, where free nanoshells could not access\textsuperscript{53}.

Stem cells can also naturally migrate towards injured tissues as part of the healing process. In addition, stem cells display tumor-homing and BBB-infiltrating properties\textsuperscript{153}. Emerging evidences suggest that various types of stem cells have exclusive tropism for tumors and can thus mediate drug delivery. Previous attempts have emphasized on producing a large number of therapeutic agents in situ via genetically-modified stem cells. For example, interferon-\(\beta\) gene transduced/transfected hMSCs preferentially accumulated in gliomas, melanomas, and pulmonary metastases, then locally released interferon-\(\beta\), and induced tumor regression\textsuperscript{154-156}. In another study, neural stem cells migrated toward intracranial gliomas and delivered the oncolytic drug, 5-fluorouracil, to reduce about 80% of the tumor mass within 2 weeks\textsuperscript{38}. Although stem cell-mediated gene therapy showed great outcomes, recent studies have put more attention on developing drug/biomaterial-laden cell carriers to reduce the potential risk of gene modification. NeutrAvidin nanoparticle-coated hMSCs were found be able to sense liver tumor spheroids \textit{in vitro} (Figure 1-5D)\textsuperscript{103}. MSCs surface-modified with Dox-loaded silica nanorattles also successfully tracked glioma cells and released Dox to kill cancer cells\textsuperscript{67}. Recently, multi-functional theranostic NPs, such as SPIO-loaded gold NPs and photosensitizer silica NPs, were successfully delivered to different tumors via MSC-mediated approaches\textsuperscript{157-158}.

Cancer cells are particularly lethal when they reach the metastatic state. Unfortunately, delivering therapeutics to metastatic circulating tumor cells (CTCs) has been a significant challenge for decades due to their scarcity in the blood circulation. CTCs are capable of leaving a
primary tumor, seeding metastases in distant sites. Effective therapies that directly remove CTCs from the blood circulation have not been found yet since CTCs circulate in the bloodstream with a low concentration (average 8 cells/ml) and are difficult to detect\textsuperscript{159}. Interestingly, studies have indicated that tumor cells exhibit interactions with leukocytes and endothelial cells via adhesion molecules, which provide a platform for targeting CTCs through leukocytes. Inspired by selectin-dependent rolling along the vessel, human E-selectin, and cancer-specific TRAIL functionalized leukocytes were used to target CTCs, which displayed sialylated carbohydrate ligands for selectin under blood flow resulting in effective tumor killing outcome\textsuperscript{160}. Studies have also found that tumor cells express adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1, also known as CD54) that can bind to lymphocyte function-associated antigen (LFA-1, also known as CD11a) and macrophage-1 antigen (MAC-1, also known as CD11b) in the circulation\textsuperscript{11,161}.

Therefore, circulating cells have shown promise as a powerful drug delivery system with an innate ability to detect diseases, cross biological barriers, and release drugs to disease sites. Compared to conventional drug delivery and nanomedicine, utilizing the natural ability of different cells is a completely different targeting mechanism, in which living cells act as guiders and drivers for delivering therapeutics and NPs. This strategy has also opened up new avenues for treatments of diseases that cannot be easily reached by traditional DDS.
Figure 1-5. Cell-mediated targeting approaches to different disease sites via leukocytes and stem cells that retain their intrinsic capacities of crossing biological barriers and homing to tumor after nanoparticle assembly\textsuperscript{49}. (A): Bioluminescence images of macrophages carrying liposome-doxorubicin, which accumulated at lung cancer metastasis sites. Reprinted with permission from
Engineered targeting/binding.

To maximize the targeting efficiency, engineered unnatural targeting/binding strategies have been developed for living cell-mediated DDS, allowing flexibility and diversity in binding pathways. Antibody decoration is one of the most popular techniques, by which cell carriers spontaneously search for specific antigens and even trigger the clearance of certain cells. Lipophilic anti-CD45 and anti-CD20 (Rituximab) surface painted RBCs effectively depleted CD45 positive leukocytes and CD19+/CD20+/CD45+ human lymphoma cells.

An alternative approach is to use an external magnetic field to enhance the accumulation of cell carriers in targets. Magnetic nanomaterials can enable remote control of the location of drug vehicles including circulating cells by an external magnetic field. SPION NPs have been widely explored due to their biocompatibility, ease of handling, and good MRI contrast. SPIO NPs loaded RBCs successfully targeted to tumor cells under magnetic stimulation, further reducing the RES aggregation. The combination of molecular binding and cell-mediated drug delivery is a new and emerging field. The techniques in cellular engineering and biomaterial modifications offer significant opportunities for targeted drug and nanoparticle delivery.

Controlled Drug Release
The controlled release of therapeutics is desired in medicine\textsuperscript{164}. It is even more critical for circulating cell-mediated drug delivery, since it requires not only maximum treatment effects, but also limited drug release in carrier cells. Generally, the encapsulation or conjugation of free drugs in cell carriers is not ideal due to the cytotoxicity of drugs and drug-cell interactions. Drugs are thus protected in vehicles made with various materials forming new complexes to ultimately control the payload release. In this section, strategies for controlled drug release from circulating cells will be reviewed.

**Degradation controlled drug release.**

Biodegradable polymers are the most widely used biomaterials for controlled drug release, since the release can be controlled by degradation mechanisms. Hydrolytic and enzymatic degradations are typical approaches to control drug release rates after being uptaken by cells. Abundant enzymes are located in the cytoplasm of phagocytic cells and able to facilitate the breakdown of engulfed substances. The degradation can be fine-tuned to obtain minimal release in circulating cells and release at desired disease sites. Liposomes are popular drug vehicles that have been used in many commercialized pharmaceutical formulations. Through elegant engineering designs, the desired release of drugs from liposomes can also be achieved after being delivered by living cells. For example, when liposome-Dox-loaded macrophages arrived at the tumor sites, drugs were released via macrophage death cause by leaky Dox from liposome degradation\textsuperscript{50}. Biodegradable polymeric particles, for instance, polylactic acid (PLA) NPs, have manageable degradation profiles that are ideal for controlled drug release\textsuperscript{165}. The slow degradation may ensure safety to circulating carrier cells and programmed release upon reaching disease sites. However, degradation controlled release may not achieve the ultimate safety and therapeutic effects due to the complicity of the $in \ vivo$ environment and the fact that drug
diffusion is also somewhat independent from material degradation. Thus, stimuli-triggered release from a smart drug delivery system can be advantageous.

**Stimulated drug release by cells and local microenvironment.**

Environmental responsive/smart drug release has gained much attention in recent decades in the field of drug delivery, as payloads can be discharged by responding the local cellular/physiological changes, which may have resulted from disease development or inflammation\textsuperscript{166-167}. It is also promising to combine the cell-mediated "live" targeting mechanism with a "smart" releasing mechanism. For instance, glutathione can reduce disulfide bonds and is available for responsive drug release within cytoplasm. An acute lymphoblastic leukemia drug, L-asparaginase, was covalently conjugated with cell-penetrating peptides (CPP) via disulfide linkages, followed by the encapsulation by RBCs without any membrane alteration\textsuperscript{168}. The disulfide linkage disassociation occurred within the cytoplasm of RBCs, which contains glutathione, and resulted in the release of L-asparaginase release. RBCs spontaneously triggered drug release in the absence of external interventions, but underwent apoptosis to release the drug out of the cells\textsuperscript{168}.

Another pathway to facilitate drug release from cell carriers is discharging the drug-loaded nanoparticle as a whole. Generally, cells retain their equilibrium via constantly engulfing foreign substances and liberating engulfed particles. In contrast to endocytosis, exocytosis allows secreting drug vehicles out of cells, as a potential approach for nanoparticle delivery. In a recent study, bone-marrow-derived macrophages internalized catalase/polyethyleneimine-PEG nanozymes within 40–60 min and sustained released the cargos for 4-5 days\textsuperscript{150}. And the release rate can be enhanced by phorbol myristate acetate treatment\textsuperscript{52}. While the integrity of cell carriers
can be maintained after the drug is released by taking advantages of exocytosis, the hemostasis may occur and the chances of off-site targeting may rise.

**External stimulation triggered drug release.**

External stimulation is an attractive alternative to trigger the localized release of therapeutic agents after reaching the disease sites, if the local microenvironmental change is not available or not strong enough. Particularly, stimuli-responsive polymers have unique characteristics that respond to small changes in the microenvironment and are therefore being widely used in smart drug delivery\textsuperscript{166,169}. Drugs are securely protected by polymeric vehicles until external stimulation, which ensures rapid, transient and precise drug release is applied. Again, combining external stimuli-responsive release with living cell-mediated targeting is a potential area to be explored.

Until now, numerous smart drug vehicles have been developed that respond to external stimuli, such as temperature, light, pressure, light, or ultrasound. In some cases, drugs can be released by multiple stimulations to ensure an on-demand administration. For example, Swiston \textit{et al.} functionalized lymphocytes with PEM coatings by combining both pH-sensitive and temperature-sensitive polymers. And these lymphocytes were able to regulate drug release by dual factors\textsuperscript{62}. Overall, stimuli-responsive drug release can be crucial for a cell based DDS, even more significant than for the conventional DDS.

**Comparison of cell-mediated DDS and conventional DDS**

Circulating cell-mediated drug delivery has emerged as a new concept that focuses on the targeted delivery of therapeutics by living cells instead of passive factors or surface markers. A
summary of comparison between conventional DDS and cell-mediated DDS is shown in Table 1-3. As we have discussed, various cell types in the circulation, including RBCs, leukocytes, and stem cells, are available for carrying drugs and particles to targeted diseases. Generally, three steps are considered in terms of design strategies: drugs/particles loading by cell carriers, cell carriers targeted to desired locations, and controlled drug release/treatment. Compared to traditional targeted drug delivery and nanomedicine strategies, additional factors must be considered for cell-mediated drug delivery. The drugs/particles must not be toxic to the cell carrier and should prevent cell alteration after drug loading. Thus, more attention has been paid to anchoring therapeutics on the surface of living cells. Certainly, high drug/particle loading or binding efficiency is favorable. We have elaborated on the feasibility and variety of cell targeting pathways to target different diseases. Rational selection of appropriate cells is necessary to achieve high targeting efficiency for new cell-mediated drug delivery system. Finally, it is imperative to employ a controlled drug releasing mechanism to ensure safety and maximize therapeutic effects. New treatment methods, such as photothermal and magnetic hyperthermia therapies, can also benefit from living cell-based delivery systems. The development of suitable biomaterials and biomedical engineering techniques is particularly important in creating new and effective cell-mediated drug delivery systems.

Table 1-3. A comparison of conventional DDS and cell-mediated DDS.
Immune Cell-mediated Drug delivery to Melanoma cells

In recent years, circulating cells such as immune cells have gained interest as novel "living" delivery vehicles\textsuperscript{124, 125}. Research has shown that leukocytes are capable of homing into tumor sites and regulating metastasis by adhesion molecule-mediated interactions with circulating tumor
cells (CTCs)^{126}. Such evidence points to the rational design of using immune cells as “smart” vehicles for cancer targeting and therapeutics delivery. For example, Choi et al. demonstrated that macrophages could be manipulated as "Trojan Horses" to deliver gold nanoparticles to breast tumors and brain metastases^{127, 128}. A similar concept was used to guide gold nanoshells to brain gliomas for photothermal therapy via macrophages^{129}. These studies, however, only focused on using macrophages as delivery carriers to reach tumor sites. As such, the potential of leukocytes targeting circulating cancer cells under dynamic blood stream conditions remained relatively unknown. Recently, leukocytes were functionalized with tumor necrosis factor-related apoptosis-inducing ligands (TRAIL)/E-selectin adhesion receptors to kill cancer cells in the circulation^{130}, providing insight into immune cell-mediated targeting. These studies suggest that utilizing immune cells as carriers to mediate therapeutic nanoparticles is an attractive approach to targeted drug delivery.

Herein, our goal is to develop biodegradable photoluminescent poly(lactic acid) (BPLP-PLA) nanoparticles to delivery drugs via immune cell-mediated targeting to melanomas. Considering the natural recognition of leukocytes and tumor cells, we aim to establish in vitro model to investigate the potential of using live circulating cells to target tumor and deliver drugs. THP-1 macrophage was selected as live circulating cell because it is a well-established native monocyte-derived macrophage model involved in series of immune responses including melanoma development^{131}. The design of nanoparticles and immune-mediated targeting to melanomas is illustrated in Figure 1-6. The main objectives and hypotheses are listed below.

Aim 1: fabricate tumor-specific drug-laden BPLP-PLA-PLX4032 nanoparticles to serve as smart drug delivery vehicle.

Hypothesis 1: BPLP-PLA polymer can be fabricated into nanoparticles that can encapsulate melanoma-specific drug PLX-4032 (also known as Vemurafenib; a drug specifically
designed for treating BRAF V600E mutated melanomas). BPLP-PLA-PLX4032 nanoparticles DDS platform presents good biocompatibility, controlled degradability and drug release, high quantum yields, tunable fluorescence emission (up to 700nm), and multiple functional groups for chemical modification. The intrinsic fluorescence of nanoparticles enables in vitro and in vivo visualization without secondary conjugation/encapsulation with imaging agents. Additionally, the therapeutic agent PLX4032 minimizes the adverse effects on leukocytes and maximizes the antitumor effects on cancer cells.

Objective 2: modify BPLP-PLA-PLX4032 nanoparticles with muramyl tripeptide (MTP) and develop THP-1 macrophage/MTP-BPLP-PLA-PLX4032 nanoparticles complex to form live circulating cell vehicles.

Hypothesis 2: THP-1 macrophages uptake BPLP-PLA-PLX4032 nanoparticles without compromising cell viability and cellular functions. MTP conjugated BPLP-PLA-PLX4032 nanoparticles further improve the THP-1 cellular uptake efficiency of nanoparticles.

Objective 3: THP-1 macrophages mediate nanoparticle delivery and release drug to melanoma cells.

Hypothesis 3: THP-1 macrophages loaded with MTP-BPLP-PLA-PLX4032 nanoparticles target to melanoma cells in vitro. MTP-BPLP-PLA-PLX4032 nanoparticles are transported from THP-1 macrophages to melanoma cells. PLX4032 is controlled released from nanoparticles and killed melanoma cells.
Figure 1-6. Schematic illustration of the immune cell-mediated nanoparticle delivery to cancer strategy. Specifically, MTP conjugated BLP-PLA-PLX4032 nanoparticles target to THP-1 macrophages, then deliver to melanoma cells via THP-1 macrophages/melanomas interactions, eventually release PLX4032 drugs to kill cancer cells.

Chapter 2

Fabrication of Biodegradable Fluorescent Nanoparticles

Today, significant leaps are being made in nanomedicine by integrating controlled drug delivery with imaging-based diagnosis, resulting in the new, emerging field of theranostic nanomedicine. Advances in fluorescence imaging techniques have enabled early detection of cancer and real-time monitoring of the drug delivery processes. However, living cell-mediated theranostic drug delivery has not been widely investigated, partly because fluorescence imaging agents such as organic dyes, green fluorescence proteins, and quantum dots generally suffer from poor stability, solubility, and potential cellular toxicity. Due to these major drawbacks, traditional fluorescence imaging
materials cannot be used as drug delivery carriers alone, but must undergo conjugation or encapsulation steps, contributing to unfavorable drug release profiles that are difficult to control.

To overcome these unmet challenges, we herein report the development of theranostic immune cell-mediated biodegradable polymeric nanoparticles with intrinsic fluorescence in order to enable carrier cell imaging with improved photostability and cytotoxicity, as well as better control over cancer-specific drug release. This novel polymeric nanoparticle is synthesized from biodegradable photoluminescent poly (lactic acid) (BPLP-PLA), which is a fully degradable polymer with tunable intrinsic fluorescence as we reported previously\textsuperscript{133}. BPLP-PLA possesses strong and stable fluorescence for optical imaging, along with good cytocompatibility on par with the widely used PLA in many FDA approved devices \textsuperscript{133}. The degradation rates of BPLP-PLA can also be easily tuned to achieve controlled drug delivery, making BPLP-PLA an ideal biomaterial for theranostic drug delivery. To the best of our knowledge, no study has reported controlled delivery of biodegradable polymeric nanoparticles by using immune cells for cancer treatment.

**Fabrication of BPLP-PLA Nanoparticles**

BPLP was synthesized according to our previous protocol with a simple polycondensation of reacting citric acid, 1,8-octanediol, and L-serine at 140°C\textsuperscript{134}. Next, BPLP-PLA was synthesized via a ring-opening polymerization according to our previously reported method\textsuperscript{133}. The feeding molar ratio of BPLP to L-lactide monomers
was 1:50. The characterization of BPLP-PLA can be found in our previous reports. BPLP-PLA nanoparticles were prepared by a single emulsion method. Briefly, 50 mg BPLP-PLA polymer was dissolved in 2 mL chloroform solution, which was added drop-wise into 20 mL 5wt% poly(vinyl alcohol) (87% hydrolyzed, Mw of 87 kDa) solution during sonication. The solution was stirred vigorously overnight for solvent evaporation. Resulting nanoparticles were centrifuged and washed with DI water for three times before lyophilized. PLX4032 drug loaded nanoparticles (BPLP-PLA-PLX4032) was prepared by dissolving 20 wt% (to BPLP-PLA) of PLX4032 in 200 μL DMSO and mixing with the polymer solution, followed by the same single emulsion and washing procedure to obtain drug loaded nanoparticles.

**Modification of BPLP-PLA Nanoparticles**

MTP was conjugated to nanoparticles by carbodiimide chemistry, according to an established protocol. Specifically, 40 mg nanoparticles were dispersed in 20 mL MES buffer (pH 4.5) by sonication. 20 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 20 mg N-hydroxysuccinimide (NHS) were added sequentially to activate the carboxyl groups of BPLP-PLA nanoparticles under stirring for one hour each at room temperature. Then, 100 µg MTP was then added into the mixture and stirred for four hours. MTP-conjugated nanoparticles were washed by DI water for three times before lyophilized as well.
Characterization of BPLP-PLA based nanoparticles

The particle size, size distribution, and zeta potential of various nanoparticles were measured by dynamic light scattering (DLS, Malvern Zetasizer ZS). The chemical structures and morphology of nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR, Bruker Vertex V70) and scanning electron microscopy (SEM, FEI Nova NanoSEM 630). Fluorescence spectra were measured by a fluorescence spectroscopy (Horiba FMax-4) with a slit size of 2 nm by 2 nm. The concentration of nanoparticles was 20 µg/mL in Dulbecco's Phosphate-Buffered Saline (DPBS). The drug loading efficiency was measured by using a high-performance liquid chromatography (Shimadzu) equipped with a photodiode array detector (Shimadzu) and a Phenomenex Kinetex C18 column. The mobile phase was a mixture of 40% acetonitrile and 60% DI water, and the flow rate was 1 ml/min. PLX4032 concentration was determined by reading the absorbance at 270 nm, and a calibration curve was built based on the same conditions. For drug release tests, 50 mg PLX4032 loaded nanoparticles were dispersed in 5 ml of 50 mM PBS in a tube and shaken at 37°C. At each time point, the nanoparticles suspension was centrifuged and then 0.5 ml of release solution was removed for HPLC measurement. Afterwards, 0.5 ml of fresh PBS was added into the tube and followed by re-dispersion.

Results and Discussions

Biodegradable BPLP-PLA was used in this study for immune cell-mediated delivery to overcome the limitations of existing immune cell-mediated nanoparticle delivery strategies that utilize inorganic nanoparticles, as well as of liposomes that lack theranostic capabilities and a controlled drug release mechanism\textsuperscript{127, 128, 130}. BPLP-PLA copolymer with intrinsic fluorescence was synthesized as we reported previously\textsuperscript{133, 134}. As mentioned above, varying the BPLP to L-
lactide molar ratio allows control over the degradation rate.\textsuperscript{133} A BPLP to L-lactide ratio of 1:50 was used in this study to ensure minimal degradation and drug release in the initial 24 hours, which is the time window for immune cells to uptake nanoparticles and further bind to melanoma cells. BPLP-PLA nanoparticles were fabricated by a single emulsion method\textsuperscript{133, 134}. In order to increase THP-1 cell targeting efficiency, we further modified drug-laden nanoparticles with MTP, which has macrophage immuno-potentiating effects without significant cytotoxicity\textsuperscript{136-138}. MTP was successfully conjugated with BPLP-PLA and BPLP-PLA-PLX4032 nanoparticles by carbodiimide chemistry, as confirmed by FTIR (Figure 2-1). The increments of \textendash\text{NH} stretching and \textendash\text{(C=O)}N- stretching indicate the presence of peptides on BPLP-PLA nanoparticles.

Next, PLX4032 was selected as the drug for encapsulation as it is specific for the treatment of BRAF (V600E) mutation melanoma\textsuperscript{139}. PLX4032-encapsulated BPLP-PLA nanoparticles were fabricated by the same single emulsion method by mixing PLX4032 into BPLP-PLA solution with a ratio of 1:5 w/w. The drug loading efficiency was 54% as determined by HPLC (Figure 2-2). Scanning electron microscopy (SEM) images of PLX4032-loaded nanoparticles (BPLP-PLA-PLX4032) and MTP-modified BPLP-PLA-PLX4032 nanoparticles (MTP-BPLP-PLA-PLX4032) were shown as Figure 2-3. The average diameters of BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles as determined by dynamic light scattering (DLS) were 217.2 nm and 209.1 nm (Figure 2-4), respectively. The results were consistent with those from SEM images. Zeta potentials of our nanoparticles were -30.3 mV and -36.2 mV, suggesting that they can be stable in physiological solutions\textsuperscript{140}.

Finally, we examined the effects of nanoparticle fabrication on the fluorescence properties of BPLP-PLA. In our previous work, we found that BPLP-PLA exhibits intrinsic fluorescence and band shifting emission with different excitation wavelengths\textsuperscript{133, 134}. Here, our nanoparticles maintained strong fluorescence emission, tunable up to 700 nm by varying the excitation wavelength (Figure 2-5). The intrinsic fluorescence of nanoparticles enables \textit{in vitro}
visualization without secondary labeling with traditional imaging agents such as organic dyes and quantum dots that often demonstrate significant toxicity. BPLP-PLA also possesses excellent photostability, which is desirable for cell tracking applications\textsuperscript{133,141}.

Figure 2-1. FTIR spectra of BPLP-PLA nanoparticles and MTP-BPLP-PLA-PLX4032 nanoparticles
Figure 2-2. Calibration curve of PLX4032 as determined by HPLC. The absorbance of PLX4032 was read at 270 nm at retention time of 5.8 minutes.
Figure 2-3. SEM images of BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles.

Size Distribution

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<tr>
<th>Nanoparticles</th>
<th>Average Size(nm)</th>
<th>PDI</th>
<th>Zeta Potential(mV)</th>
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<tr>
<td>BPLP-PLA-PLX4032</td>
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<td>0.147</td>
<td>-30.3</td>
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<td>MTP-BPLP-PLA-PLX4032</td>
<td>209.1</td>
<td>0.121</td>
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Figure 2-4. DLS characterization of average size of BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles.
Chapter 3

Loading Drug-laden Nanoparticles to Immune Cells

Nanotechnology plays a crucial role in modern medicine, especially in targeted cancer drug delivery and imaging. Nanocarriers provide protection for chemotherapeutics, genes, and imaging agents against the harsh environment encountered in systemic circulation. Compared to conventional systematic delivery and passive targeting, active targeted delivery of nanoparticles improves the therapeutic index and reduces side effects as well as minimizing immunogenicity. Most existing active targeting strategies utilize surface molecules such as antibodies, proteins, aptamers, peptides, or small molecules to recognize receptors that are
expressed or overexpressed in cancer cells or cancer microenvironments. However, despite tremendous efforts towards discovering surface markers and targeting molecules, traditional nanomedicine still fails to meet the expectations of efficient delivery of therapeutics to specific tumors via intravenous injection. The major limitations are low circulation time and poor target selectivity for a specific disease or cancer. The complexity of living systems makes specific recognition chance-dependent, which compromises the effectiveness of drug delivery systems. Thus there is an urgent need to develop novel efficient targeting strategies for cancer nanomedicine. Herein, THP-1 cells were selected as the nanoparticle carrier in order to model monocyte/macrophage systems, while the theranostic nanoparticles were equipped with melanoma-specific drugs to target melanoma cells.

**THP-1 Uptake Drug-laden Nanoparticles**

**Cell Culture**

THP-1 cells were maintained with RPMI-1640 medium (Life Technologies) with 10% FBS (Atlanta Biologicals) and 0.05 mM 2-mercaptoethanol (Life Technologies) at 37°C under 5% CO₂. Before THP-1 uptake, cell binding and pharmacological studies, THP-1 cells were differentiated by 200nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) in RPMI-1640 medium for 3 days followed by 1 day in PMA-free medium. Then, 1µg/ml lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich) was applied to stimulate differentiated THP-1 for 24 h.
THP-1 Uptake

Differentiated THP-1 cells were lifted by cell dissociation buffer and used for further studies. THP-1 uptake studies were carried out by incubating $1 \times 10^6$ differentiated THP-1 cells and 200 μg/ml BPLP-PLA and MTP-BPLP-PLA nanoparticles in 1 ml DPBS at 37°C for 2 hours on a rocker, respectively. Afterwards, THP-1 cells were washed gently by DPBS for three times, and then subjected for characterization and further studies.

Immunofluorescence Staining.

To prevent non-specific binding, THP-1 cells were blocked by 1% BSA for 1 hr at room temperature and incubated with 2 μg/ml CD11b rat anti-mouse mAb overnight at 4 °C. Cells were then stained with Alexa Fluor® 647 Goat anti-rat IgG (H+L) (2 μg/ml) for 1 hr at room temperature. THP-1 cells incubated with secondary antibodies served as controls. The cells were fixed by 4% paraformaldehyde at room temperature for 30 minutes and subjected to flow cytometry and confocal microscopy. For confocal microscopy, DAPI was used to stain the nuclei of THP-1 cells.

Results and Discussions

THP-1 Uptake

As a well-established native monocyte-derived macrophage model, THP-1 cell was chosen for this study to demonstrate macrophage uptake of drug-laden nanoparticles. THP-1 cells were differentiated by PMA, which is commonly used chemicals to induce THP-1 monocyte
differentiating to macrophage by activating protein kinase C\(^{149}\). THP-1 differentiation into tissue macrophage is also associated with the acquisition of phagocytic capabilities\(^{150}\). Then BPLP-PLA-PLX4032 or MTP-conjugated nanoparticles were incubated with THP-1 cells for two hours on a rocker, followed by washing steps to remove unbound nanoparticles. Preliminary confocal microscopy studies suggested that our nanoparticles were internalized by THP-1 cells, and that cellular fluorescence could be detected in both FITC and PE-Texas Red channels due to the intrinsic variable fluorescence of BPLP-PLA nanoparticles (Figure 3-1). Indeed, flow cytometry confirmed that both FITC and PE-Texas Red intensities increased from nanoparticle incubation (Figure 3-2A). These results demonstrate the versatility and effectiveness of BPLP-PLA nanoparticles in cellular imaging and tracking, since the band shifting behavior from different excitation wavelengths enables a wide range of detection channels, even to red fluorescence.

Next, since MTP is a macrophage activator known to enhance nanoparticle uptake\(^{151}\), MTP was conjugated to BPLP-PLA-PLX4032 to correlate enhancement of FITC and PE-Texas Red signals to nanoparticle internalization. It is clear that both FITC and PE-Texas Red fluorescence increased with MTP-conjugated nanoparticles from a greater extent of nanoparticle internalization by THP-1 cells (Figure 3-2B). Quantitatively, the average FITC and PE-Texas Red intensities of THP-1 cells treated with MTP-BPLP-PLA-PLX4032 nanoparticles were stronger than those with just BPLP-PLA-PLX4032 nanoparticles, suggesting that MTP conjugation increased the nanoparticle loading efficiency of THP-1 cells. The nanoparticle targeting efficiency was determined by the percentage of cells in Q1, Q2, and Q3 that had increased fluorescence. About 96% of the THP-1 cells were labeled by MTP-conjugated BPLP-PLA-PLX4032 nanoparticles, whereas 61% of the THP-1 cells were marked by pristine BPLP-PLA-PLX4032 nanoparticles (Figure 3-2C). Thus MTP conjugation was shown to improve nanoparticle binding to THP-1 cells.
Further, we studied CD11b (namely MAC-1 or Integrin alpha M) expression to determine whether nanoparticle internalization affects THP-1's macrophage-like functionality. Studies have indicated that polymorphonuclear neutrophils (PMNs) bind to melanoma cells in the blood circulation through β 2 integrins (e.g. CD11b/Mac-1) of PMNs and intercellular adhesion molecule-1 (ICAM-1) of melanoma cells\textsuperscript{152}. We hypothesized that macrophages such as THP-1, upon PMA-induced CD11b expression, have this same melanoma binding ability through ICAM-1\textsuperscript{153, 154}. Thus, the maintenance of CD11b would be critical in binding melanoma. First, we showed that macrophage-like phenotype from differentiated THP-1 cells (induced by PMA) could be identified by CD11b expression (from the fluorescence of Alexa647) (Figure 3-3). Next, confocal microscopy and 3D flow cytometry plots showed strong immunofluorescence from Alexa647 (accounting for FITC and PE-Texas Red fluorescence signals from the nanoparticles), indicating that THP-1 cells still expressed CD11b after uptaking nanoparticles (Figure 3-1 and 34) thereby suggesting that the encapsulation of nanoparticles did not alter THP-1's functionality as a macrophage-like cell. These results showed that BPLP-PLA-PLX4032 nanoparticles were internalized by THP-1 cells while MTP conjugation increased loading efficiency without affecting THP-A macrophage-like functionality. Based on these results, MTP-BPLP-PLA-PLX4032 nanoparticles were deemed suitable for follow-up experiments on melanoma binding and therapeutic studies.
Figure 3-1. Confocal images of MTP-BPLP-PLA-PLX4032 nanoparticles internalized by THP-1 cells. Nuclei were stained by DAPI; nanoparticles were shown in the FITC and PE-Texas Red channels, CD11b was immunostained by Alexa647 (pseudo color in pink).
Figure 3-2. Flow cytometry results of THP-1 uptaking nanoparticles. (A) FACS analysis of THP-1 cells before and after treated with BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles (B) FITC and PE-Texas Red average fluorescence intensity of THP-1 cells, THP-1 cells with BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles. *, p<0.01 compare to the THP-1 control. (C) THP-1 cell binding efficiency as the percentage of THP-1 cells that were labeled by BPLP-PLA-PLX4032 and MTP-BPLP-PLA-PLX4032 nanoparticles. *, p<0.01.

Figure 3-3. CD11b expressions of differentiated THP-1 cells as the fluorescence intensity of Alexa647 measured by flow cytometry.
Figure 3-4. CD11b expressions of MTP-BPLP-PLA-PLX4032 nanoparticles loaded THP-1 cells. Bare THP-1 and nanoparticle loaded THP-1 were immune stained Alexa647; while for control samples, no primary antibody (anti-CD11b) was added. Fluorescence in FITC and PE-Texas Red channels is from nanoparticles. Z-axis (heat intensity) is the fluorescence of Alexa647, which indicates the expression of CD11b.
Chapter 4

Immune-cell Mediated Targeting

Drug delivery systems, particularly nanomaterial-based drug delivery systems, possess a tremendous amount of potential to improve diagnostic and therapeutic effects of drugs. Controlled drug delivery targeted to a specific disease is designed to significantly improve the pharmaceutical effects of drugs and reduce their side effects. Unfortunately, only a few targeted drug delivery systems can achieve high targeting efficiency after intravenous injection, even with the development of numerous surface markers and targeting modalities. Thus, alternative drug and nanomedicine targeting approaches are desired. Circulating cells, such as erythrocytes, leukocytes, and stem cells, present innate disease sensing and homing properties. Hence, using living cells as drug delivery carriers has gained increasing interest in recent years.

**THP-1/ Melanoma Cells Binding and Nanoparticles Delivery**

*Cell culture*

1205Lu and GFP-tag 1205Lu were cultured in 2% Tumor Medium containing a 4:1 mixture of MCDB 153 medium (Sigma-Aldrich) with 1.18 g/L sodium bicarbonate and Leibovitz's L-15 medium (Life Technologies) with 2 mM L-glutamine supplemented with 0.005 mg/ml bovine insulin, 1.68 mM CaCl$_2$, and 2% fetal bovine serum at 37°C under 5% CO$_2$. WM35 melanoma cells were maintained with RPMI-1640 medium with 10% FBS and 0.05 mM 2-mercaptopethanol at 37°C under 5% CO$_2$. 
Cell Binding Studies under Static Conditions.

Two melanoma cell lines, 1205Lu (high metastatic) and WM35 (low metastatic) cells, were selected as BRAF mutant melanomas. One million nanoparticle-loaded THP-1 cells were incubated with one million GFP-tagged 1205Lu cells in 1 ml DPBS at 37°C for two hours on a rocker. The resulting cells were gently washed with DPBS for three times and fixed with 4% paraformaldehyde at room temperature for 30 minutes. Cell binding was analyzed by using a BD Fortessa LSRII flow cytometry and FACS analysis was performed by using FlowJo 10. Confocal microscopy was performed in inverted mode on an Olympus Fluorview 100 confocal microscope. For both flow cytometry and microscopy, the FITC channel was set to detect the fluorescence from GFP-tagged 1205Lu cells and the Texas Red channel was used to detect the fluorescence from BPLP-PLA nanoparticles. For binding studies between THP1 and WM35 cells, the WM35 cells were stained by CellTrace™ CFSE (Life Technologies) to obtain green fluorescence.

Cell Binding Studies under Flow Conditions.

To simulate the shear-flow conditions of the blood flow, cell binding studies were performed in a uniform shear flow by using a cone-plate viscometer (Thermo Scientific). One million nanoparticle-loaded THP-1 cells were mixed with GFP-tagged 1205Lu cells at 1:1 ratio in 1 ml DPBS. The cell mixtures were immediately added into the cone-plate viscometer and exposed to shear flows at a range of shear rates varied from 50 s⁻¹ to 200 s⁻¹ at room temperature for one hour. Then, the cells were removed from the cone-plate viscometer and washed twice by DPBS. The cells were fixed by 4% paraformaldehyde at
room temperature for 30 minutes and subjected for confocal and flow cytometry studies as described in the above section. Results and Discussions

**Results and Discussions**

To demonstrate such value in immune cell-mediated theranostic drug delivery, the next step was to assess the effectiveness of THP-1 cell binding and MTP-BPLP-PLA-PLX4032 nanoparticle delivery to melanoma cells. 1205Lu cells and WM35 cells were chosen as models for high and low metastatic melanoma cells respectively, and co-culture studies were performed under both static and dynamic conditions to model laminar and shear flow conditions. First, bare THP-1 cells without nanoparticles were co-cultured with either type of melanoma cells for two hours under static conditions (on a rocker) to demonstrate that THP-1 cells are able to attach to melanoma cells (Figure 4-1). Next, THP-1 cells loaded with MTP-BPLP-PLA-PLX4032 nanoparticles (with a two hour incubation and washing steps) were co-cultured with either type of melanoma cells for two hours under static or dynamic conditions.

Nanoparticle-laden THP-1 cells were able to successfully bind to GFP-1205Lu cells as determined by confocal microscopy (Figure 4-2). To better show THP-1/melanoma cell binding for confocal microscopy and quantitative flow cytometry analysis, 1205LU cells were tagged with Green Fluorescent Protein (GFP-1205Lu) and WM35 cells were stained with CellTrace™ CFSE. Since GFP intensity is much greater than BPLP-PLA nanoparticle intensity at the FITC channel, nanoparticle-labeled THP-1 cells emit minimal fluorescence at the FITC channel at an exposure time suitable for
GFP-labeled melanoma cells. Likewise, GFP-labeled melanoma cells emit minimal red fluorescence compared to nanoparticle-labeled THP-1. Thus, intensity along the FITC channel indicated melanoma cells, whereas intensity along the PE-Texas Red channel indicated BPLP-PLA nanoparticles, thereby enabling qualitative and quantitative discriminatory analysis. Next, flow cytometer analysis was performed to quantitatively evaluate this binding. Compared to untreated GFP-1205Lu cells which shows no fluorescence in the PE-Texas Red channel, co-culturing with nanoparticle-bearing THP-1 cells induced the population of GFP-1205Lu cells to shift into the Q2 region with a greater degree of red fluorescence (Figure 4-3A). This enhanced red fluorescence indicates binding of GFP-1205Lu cells with nanoparticle-bearing THP-1 cells to form leukocyte/nanoparticle/melanoma complexes, or transference of nanoparticles from THP-1 to GFP-1205Lu upon binding (Figure 4-3B). In fact, confocal microscopy images showed trace red fluorescence within GFP-1205Lu cells, supporting nanoparticles released from THP-1 cells to GFP-1205Lu cells It is likely that nanoparticles were released via exocytosis, owing to the equilibrium of engulfing foreign substances and liberating engulfed particles, particularly since BPLP-PLA nanoparticles are constructed by a polyester copolymer with minimal charges. Previous studies suggested that neutrally charged nanoparticles were able to transport out of macrophages significantly faster than cationic nanoparticles.

In order to mimic the dynamic shear-flow environments of the bloodstream, GFP-1205Lu cells were exposed to nanoparticle-carrying THP-1 cells on a cone-plate viscometer. As shown in Figure 4-2 and 4-3, confocal microscopy and flow cytometry results showed that THP-1 cells were bound to GFP-1205Lu cells even under shear flow.
Lastly, FACS analysis was performed to quantify the binding efficiency of THP-1 and melanoma cells, which is defined as the ratio of the cells in Q2 to the cells in both Q1 and Q2. Over 90% of 1205Lu cells were bound to nanoparticle-laden THP-1 cells or received nanoparticles via exocytosis when shear rates ranged from 50 s$^{-1}$ to 200 s$^{-1}$. These numbers suggest nanoparticle-carrying THP-1 cells to adhere to melanoma cells. This macrophage-mediated delivery strategy is also verified by a low metastatic melanoma cell line, WM35. Similar results were observed, indicating THP-1/WM35 binding and nanoparticle transportation to WM35, as shown in Figure 4-4 and Figure 4-5. Thus, $in$ $vitro$ binding was demonstrated between THP-1 cells and melanoma cells, as well as THP-1 cell-mediated nanoparticle delivery.
Figure 4-1. Bare THP-1 cells without nanoparticles bind to WM35 (green, CFSE stained) and 1205Lu (green, GFP-tagged) cells after two hours of static incubation.

Figure 4-2. Confocal images of THP-1/GFP-1205Lu binding and nanoparticles (PE-Texas Red) delivery, scale bar: 20 µm.
Figure 4-3. FACS of THP-1/1205Lu binding under static and dynamic environments. (A) FACS analysis of THP-1 control, GFP-1205Lu control, THP-1/nanoparticle/1205Lu complexes after static incubation and dynamic binding. (B) Average fluorescence intensity of PE-Texas Red and FITC within the Q1 and Q2 areas of Figure 4A. *, p<0.01.
Figure 4-4. Confocal microscopy images of nanoparticle-laden THP-1 (PE-TEXAS RED) binding to WM35 (FITC) under static and dynamic conditions.

Figure 4-5. FACS plots of cell binding in same static and dynamic conditions.
Chapter 5

Cell-mediated Drug Release

Since we have verified the binding and nanoparticle delivery capabilities of THP-1 cells to melanoma cells, the final step was to examine the safety of our immune cell-mediated nanoparticle delivery system and its pharmacological effects on cancer cells. In order to minimize the potential damage to immune cells and normal tissues, a cancer-specific drug, PLX4032, was used as a chemotherapeutic that specifically inhibits the BRAF V600E oncogene of V600E-positive melanomas\textsuperscript{157,158}.

Pharmacological Studies

Two melanoma cell lines, 1205Lu (high metastasis) and WM35 (low metastasis) were applied to cytotoxicity studies. First, toxicity and selectivity of PLX4032 were confirmed by adding PLX4032 solutions at different concentrations into 96 well plates with THP-1, 1205Lu and WM35 cells separately (cell seeding density = 5,000 cells per well). After 24 hours of incubation, the cells were washed with PBS twice and supplemented with 10 $\mu$l CCK-8 in 100 $\mu$l RPMI-1640 medium in each well. After two hours of incubation, the absorbance at 450 nm of each well was measured by a micro-plate reader (TECAN, infinite M200 PRO) and converted to the cell viability by normalized to the control (tissue culture plates). Next, MTP-BPLP-PLA nanoparticles with and without PLX4032 were dispersed in RPMI-1640 medium at various concentrations, followed by incubation with THP-1, 1205Lu, and WM35 cells (2,000 cells per well) separately in same conditions for seven days. At last, MTP-BPLP-PLA-PLX4032 loaded
THP-1 cells and pristine THP-1 cells were seeded with 1205Lu or WM35 cells (2,000 cells per well) together with different ratios for seven days, respectively. The cell viabilities were tested by CCK-8 assays as well. All data was recorded as mean ± standard error, unless otherwise stated. All statistical analyses were performed via one-way ANOVA on GraphPad Prism 6.0. For all studies, n equals to 6, unless specifically stated.

**Results and Discussions**

We investigated two melanoma cell lines, 1205Lu (high metastatic) and WM35 (low metastatic), which are both BRAF mutant with V600E expression. First, we found that free PLX4032 itself selectively killed 1205Lu and WM35 at concentrations of 50 ng/mL (Figure 5-1). With PLX4032 concentration above 5 µg/mL, almost 100% death of melanomas was achieved. However, no significant reduction in viability of THP-1 cells was observed even concentrations as high as 100 µg/mL. Thus, PLX4032 was determined to be an ideal drug for immune cell-mediated drug delivery to melanoma cells, presenting minimal toxicity to the carrier immune cells. Second, *in vitro* drug release studies showed sustained release of PLX4032 from our nanoparticles (Figure 5-2). No clear burst release was observed in the release curve, which is important in minimizing side effects and achieving high therapeutic outcomes. Third, we tested the effects of drug-free MTP-BPLP-PLA nanoparticles and PLX4032-loaded MTP-BPLP-PLA nanoparticles on THP-1 and melanoma cells. As shown in Figure 5-3, pristine BPLP-PLA nanoparticles lacking the drug did not significantly reduce the THP-1 cell viability at nanoparticle concentrations as high as 1000 µg/mL. For 1205Lu and WM35 melanomas, however, pristine nanoparticles exhibited toxicity at 500 µg/mL. After 7 days of incubation, PLX4032 loaded nanoparticles killed far more melanomas than pristine nanoparticles, especially for WM35, which
indicated significant difference at 50 μg/mL of nanoparticles. The cell viability of both 1205Lu and WM35 melanoma cells were significantly reduced to around 30% at 1000 μg/mL MTP-BPLP-PLA-PLX4032 nanoparticle concentrations after seven days of incubation, suggesting that the drug released from nanoparticles were effective in killing melanomas. Finally, we assessed the effectiveness of the complete THP-1-mediated MTP-BPLP-PLA-PLX4032 nanoparticle delivery system. THP-1 cells were treated with PLX4032 loaded nanoparticles, and after removal of free nanoparticles, THP-1 cells were co-cultured with 1205Lu or WM35 cells (at 2000 cells per well each) with different THP-1 cell to melanoma cell ratios for seven days. Bare THP-1 cells lacking nanoparticles served as the control. Compared with pristine THP-1 cells, nanoparticle-bearing THP-1 significantly decreased the viability of both 1205Lu and WM35 cells; even at the lowest THP-1 number (5,000 cells per well), as shown in Figure 5-4. With increasing number of THP-1 cells added, more melanoma cells were killed, likely due to activated macrophages releasing tumor necrosis factor that kill cancer cells159. Again, THP-1 cells that engulfed PLX4032-loaded nanoparticles further reduced both 1205Lu and WM35 cells viabilities than that of pristine THP-1 cells even at higher ratios. Since we have previously noted that nanoparticles can be transported from THP-1 cells into melanoma cells (Figure 4-2), it is likely that PLX4032 was released to effectively treat melanomas. Noticeably, THP-1 cell-mediated drug delivery was more effective in killing WM35 cells than 1205Lu cells, probably because PLX4032 is more effective on WM35 cells139. In conclusion, our immune cell-mediated nanoparticle delivery strategy is an effective approach to transport therapeutics to melanoma cells.
Figure 5-1. Pharmacological studies of free drugs, nanoparticles and nanoparticle-bearing THP-1 cells on melanoma cells (1205Lu and WM35 cells). Cell viability tests were conducted by CCK-8 assay (n=6). Toxicity of PLX4032 to THP-1, WM35 and 1205Lu cells after 24 hours of incubation. #, p<0.01 compared to controls.
Figure 5-2. *In vitro* PLX4032 drug release profile in PBS at 37°C.
Figure 5-3. Cytotoxicity of MTP-BPLP-PLA and MTP-BPLP-PLA-PLX4032 nanoparticles to THP-1, WM35 and 1205Lu cells after seven days of incubation (n=6). #, p<0.01 compared to controls; *, p<0.01 between two groups.
Figure 5-4. THP-1 mediated nanoparticles delivery and drug release effects on melanoma cells with different THP-1 to melanoma cell (2,000 cells per well) ratios after seven days of incubation. #, p<0.01 compared to controls; *, p<0.01 between two groups.
Chapter 6

Conclusions

In this paper, we developed a novel targeted nanomedicine strategy based on a “living” nanoparticle delivery, mediated by immune cells such as macrophages. Our novel biodegradable fluorescent polymeric nanoparticles encapsulated melanoma specific therapeutics, PLX4032, to provide cell tracking capabilities, safe protection toward macrophages, and controlled release of drugs to cancer cells. High macrophage uptake of nanoparticles was achieved by modifying particles with MTP peptides. The active binding of macrophages to melanoma cells was confirmed with and without the presence of nanoparticles. Guided by THP-1 cells, nanoparticles were delivered to melanoma cells and consequently released chemotherapeutics to kill cancer cells. In summary, our immune cell-mediated nanoparticle targeting strategy could be a new pathway for targeted cancer drug delivery by taking advantage of the nature of interactions between immune cells and tumors. This work may potentially innovate immune cell-mediated system not only for cancer drug delivery and imaging, but also for other diseases that involve innate immune responses.

Circulating cell-mediated drug delivery has emerged as a new concept that focuses on the targeted delivery of therapeutics by living cells instead of passive factors or surface markers. A summary of comparison between conventional DDS and cell-mediated DDS is shown in Table 6-1. As we have discussed, various cell types in the circulation, including RBCs, leukocytes, and stem cells, are available for carrying drugs and particles to targeted diseases. Generally, three steps are considered in terms of design strategies: drugs/particles loading by cell carriers, cell carriers targeted to desired locations, and controlled drug release/treatment. Compared to
traditional targeted drug delivery and nanomedicine strategies, additional factors must be considered for cell-mediated drug delivery. The drugs/particles must not be toxic to the cell carrier and should prevent cell alteration after drug loading. Thus, more attention has been paid to anchoring therapeutics on the surface of living cells. Certainly, high drug/particle loading or binding efficiency is favorable. We have elaborated on the feasibility and variety of cell targeting pathways to target different diseases. Rational selection of appropriate cells is necessary to achieve high targeting efficiency for new cell-mediated drug delivery system. Finally, it is imperative to employ a controlled drug releasing mechanism to ensure safety and maximize therapeutic effects. New treatment methods, such as photothermal and magnetic hyperthermia therapies, can also benefit from living cell-based delivery systems. The development of suitable biomaterials and biomedical engineering techniques is particularly important in creating new and effective cell-mediated drug delivery systems.

Although novel cell-mediated drug/nanomedicine delivery holds promises in improving diagnosis and therapeutic effects, it is debatable whether this strategy is safe enough for clinical practices. Many challenges still remain for this state-of-art approach. Generally, candidate cells are isolated and functionalized in vitro, followed by re-injection into the circulation or disease sites. Although current techniques allow effective isolation and expansion of certain cells in vitro, cell-carriers are susceptible to contamination and other risks. Alternatively, drugs and drug-engulfed vehicles can be directly injected into the circulation to bind with cell carriers in vivo. However, the drug loading efficiency may be compromised due to immune surveillance and drug vehicles’ limited binding ability. Potential therapeutics leaking from the carrying cells is another concern. Additionally, a wide spectrum of cells playing multiple roles in disease development and the healing process may participate in the drug delivery procedure. Introducing cell carriers may interrupt the balance of natural physiological conditions. Foreign cells with drug complexes may exacerbate pathological conditions since the mechanism and development of many diseases are
not well understood. In the meantime, more evidence is needed to verify the applicability of cell-mediated DDS in various diseases, including, but not limited to, cancers, wound healing, brain diseases, and cardiovascular diseases.

Regardless of these challenges, cell-mediated drug delivery holds great potential to revolutionize current diagnostic and medical techniques. Advances in biomaterials may open up the door to a new paradigm of DDS designs. Engineering tools for manipulating living cells and fabricating drug vehicles, such as NPs, will create efficient and intelligent targeting, releasing, and imaging strategies. Combining living circulating cells and nanotechnology will be the new direction for controlled drug delivery, while offering a powerful technique to improve overall diagnostic and therapeutic outcomes.
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