Stimuli-Responsive Liposomes for Controlled Drug Delivery

Dissertation by

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ABSTRACT

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Wengang Li

Liposomes are promising drug delivery vesicles due to their biodegradibility, large volume and biocompatibility towards both hydrophilic and hydrophobic drugs. They suffer, however, from poor stability which limits their use in controlled delivery applications. Herein, a novel method was devised for modification of liposomes with small molecules, polymers or nanoparticles to afford stimuli responsive systems that release on demand and stay relatively stable in the absence of the trigger. This dissertation discusses thermosensitive, pH sensitive, light sensitive and magnetically triggered liposomes that have been prepared for controlled drug delivery application. RAFT polymerization was utilized for the preparation of thermosensitive liposomes (Cholesterol-PNIPAm) and acid-labile liposomes (DOPE-PAA). With low Mw Cholesterol-PNIPAm, the thermosensitive liposomes proved to be effective for controlled release and decreased the cytotoxicity of PNIPAm by eliciting the polymer doses. By crosslinking the DOPE-PAA on liposome surface with acid-labile diamine linkers, DOPE-PAA liposomes were verified to be sensitive at low pH. The effects of polymer structures (linear or hyperbranched) have also been studied for the stability and release properties of liposomes. Finally, a dual-responsive Au@SPIO embedded liposome hybrid (ALHs) was prepared with light-induced “on-and-off” function by photo-thermal process (visible light) and instant release properties triggered by alternating magnetic field,
respectively. The ALH system would be further applied into the cellular imaging field as MRI contrast agent.
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Wanyu--my love, I would always treasure the time we are together, as we promised, forever!
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<td>4’,6-diamidino-2-phenyldione</td>
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<td>4-Dimethylaminopyridine</td>
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<td>Au@SPIO-liposome hybrid</td>
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</tr>
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<td>Chain transfer agent</td>
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<td>Confocal laser scanning microscope</td>
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<td>Critical packing parameter</td>
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<tr>
<td>Dichloromethane</td>
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<td>Ethyl acetate</td>
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<td>GPC</td>
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<td>Gold nanoparticle</td>
<td>AuNP</td>
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<td>Main-chain NIPAm oligomers</td>
<td>MCNOs</td>
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<td>Multilamellar Vesicles</td>
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<td>Rh-PE</td>
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<tr>
<td>N-hydroxysuccinimide</td>
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<td>NIPAm oligomer</td>
<td>NO</td>
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<td>Term</td>
<td>Abbreviation</td>
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<td>----------------------------------------------</td>
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</tr>
<tr>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>Phosphatidylacid</td>
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<td>Phosphatidylethanolamine</td>
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<td>Phosphatidylserine</td>
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<td>PC</td>
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<td>Poly acrylic acid</td>
<td>PAA</td>
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<tr>
<td>Poly dispersity index</td>
<td>PDI</td>
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<tr>
<td>Poly(N-isopropylacrylamide)</td>
<td>PNIPAm</td>
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<tr>
<td>Reversible addition-fragmentation chain transfer</td>
<td>RAFT</td>
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<tr>
<td>Room temperature</td>
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<tr>
<td>Side-chain NIPAm oligomers</td>
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<tr>
<td>Small unilamellar vesicles</td>
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<tr>
<td>Superparamagnetic Iron Oxide</td>
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<tr>
<td>Surface enhanced Raman Scattering</td>
<td>SERS</td>
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<tr>
<td>Tetraethylene glycol</td>
<td>TEG</td>
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<td>Transmission electron microscope</td>
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<tr>
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1 Introduction

1.1 Cancer and Chemotherapy

Cancer\(^1\), a group of diseases where abnormal cells divide without control and are able to spread to other issues, has been one of the most death-causing diseases in clinic practices. Reported by World Health Organization, there was 8.2 million people died in 2012 from cancer, and 14 million new cancer cases occurred in the same year\(^1\). Originated from the gene damage or change during the cell dividing, cancer cells undergo unregulated growth and would often form a mass (tumor tissue). Lung, liver, stomach, colorectal and breast cancers are causing the most cancer deaths, where tobacco is the most important risk factor for cancer (20% of total cancer death and 70% of total lung cancer deaths).

Figure 1-1. Schematic illustration of cancer series\(^1\).
To overcome the gradually increasing cancer threatening, many treatment methods for cancer are developed in clinic practices, such as surgery, chemotherapy, radiation therapy etc. Surgery is one of the primary methods of cancer treatment for most isolated solid cancers, which could also help to prevent the spread of cancer and prolong the life of patients. Radiation therapy is to use ionizing radiation to cure cancers by damaging the DNA of cancer cells leading to cellular death. Radiation therapy is often used in addition to surgery or chemotherapy. Both surgery and radiation therapy are local treatments focus on cancer in certain areas.

Chemotherapy, a systematic treatment to cancer, is to treat cancer with one or more cytotoxic anticancer drugs. And its history could be dated from 1940s, when Goodman and Gilman used nitrogen mustard, which is a derivative of sulfur mustard, to treat a man with advanced lymphoma, leading to a brief cancer regressing\(^2\). Chemotherapy is aimed to cure cancer and to reduce the symptoms. There are several different ways for chemotherapy, such as injection into bloodstream, drip into bloodstream, tablet, capsule and so on.

The objective of chemotherapy is total cell kill resulting in total cure\(^3\). To achieve a high cell death, current treatment usually requires high doses of anti-cancer drugs by intravenous injection or infusion\(^4\). However, to reach a dose that would eliminate the malignant cells was problematic by leading to patient mortality\(^5\). One other disadvantage for chemotherapy lies in the solubility of anti-cancer drugs. As most of the anti-cancer drugs are water insoluble and need pharmaceutical solvents for medical applications; however these solvents may cause life-threatening effects\(^6\). In one word, conventional chemotherapy has a major drawback on practical cancer treatment.
1.2 Nanoparticles in Chemotherapy

To overcome the drawbacks of traditional chemotherapy, nanotechnology has been applied to develop novel nanoscale carriers for targeted and controllable drug delivery in chemotherapy.

![Schematic illustration of a general drug delivery carrier](image)

Figure 1-2. Schematic illustration of a general drug delivery carrier.

Nanoparticles are promising in addressing and remedying some of the significant limitations of traditional chemotherapy due to the colloidal state with nano scale sizes ($10^{-9}$ m). The small size, various composition, surface functionalization and mechanical stability are attractive properties for drug delivery researches. Moreover, the potential nano-carrier for drug delivery should be one which is biodegradable, injectable, biocompatible, targetable and controllable on the release of cargos. Shown in Figure 1-2, an ideal nanoparticle carrier would have the following properties: to carry one or more therapeutic agents, to target cancer tissues through one or more conjugated molecule or antibodies, to image diseased tissue and to prolong the circulation time by perverting systemic clearance.
The controlled release of therapeutics has been one of the most attractive research areas for scientists in the past decades, as with a precise stimuli-responsive nanomaterial, it is possible to control the release of cargo to a certain location, at a specific time and with an accurate amount. Triggers could be internal within or near to targeted cancer tissues, such as temperature, pH, redox, and over-expressed enzymes, or external such as light radiation and oscillating magnetic field (Figure 1-3).

Figure 1-3. “Caged cargo molecules” that are released by specific triggers.
1.3 Common Architectures for Drug Delivery

The most common architectures for drug delivery system are polymeric micelles, dendrimers, nanospheres, nanocapsules and liposomes (Figure 1-4). Each delivering system has its own advantages and challenges, but recent advances have illustrated the medical efficacy of each system using in vitro and in vivo models.
1.3.1 Polymeric Micelles

A polymeric micelle is a nanoparticle composed with amphiphilic macromolecules self-assembling into a structure with a hydrophobic core and a hydrophilic extension\textsuperscript{10}. The diameter of micelles is usually between 5 and 100 nm. Hydrophobic drugs can be incorporated into the micelle either by covalent bond to the hydrophobic segment or by non-covalent attachment to the hydrophobic core. The hydrophilic surface of micelles consists of hydrophilic polymers, such as PEG, L-lysine, phosphatidylethanolamine and D, L- lactic acid. The surface of micelles could also further functionalized with cancer targeting moieties, antibodies for example, to enhance the drug delivery efficiency\textsuperscript{5}.

1.3.2 Dendrimers

Dendrimers are highly branched macromolecules which has a high degree of monodispersity and well defined structure\textsuperscript{11}. Drug could be encapsulated into the dendrimers core or attached to the exterior of the dendrimers. The surface of dendrimers could also be modified with targeting ligand such as folic acid\textsuperscript{12}.

1.3.3 Nanospheres and Nanocapsules

Nanospheres are the spherical polymeric matrixes loaded with drugs\textsuperscript{13}. One typical pathway for drug to release from nanospheres is by diffusion. Nanocapsules consist of polymeric hollow nanoparticles with chemotherapy agents loaded inside the core\textsuperscript{14}. And nanocapsules are usually regarded as reservoir systems for drug delivery due to the large volume.
1.3.4 Liposomes

A liposome, first introduced by British hematologist Dr. Alec Bangham\textsuperscript{15} in 1961, is a hollow vesicle composed of a lipid bilayer, and is usually constituted of some natural phospholipids, which is also the composition of cell membrane. It may also contain some other lipid chains or molecules like cholesterol. Lipid in liposomes is mainly a phospholipid, and such molecules can be defined as hydrophobic or amphiphilic molecules with a hydrophilic head and two, most commonly, hydrophobic tails. Liposomes have been studied for their non-toxicity\textsuperscript{16}, large volume; and their biocompatibility to both hydrophilic and hydrophobic drugs. Liposomes have also been used for loading DNA or RNA for cancer therapy\textsuperscript{17}.

1.4 Components of Liposomes

Liposomes are composed with lipid bilayers, where phospholipid is the main components of liposomes. And there are several main types of phospholipids in natural world such as PC, PE, PI, PS and cholesterol (Figure 1-5).

Phosphatidylcholine (PC) is one of the major neutral constituents of cell membrane, and its primary role is to provide a structural framework and maintain the permeability barrier. Phosphatidylethanolamine (PE), another common neutral phospholipid, is a phospholipid most found in nervous tissue. PE has a smaller headgroup than PC, and it is easy to form hydrogen bonding with surrounded groups. Phosphatidylserine (PS) is a negatively charged phospholipid component of cell and blood platelet membranes. Some other examples of negatively charged phospholipid are phosphatidylinositol (PI),
phosphatidylglycerol (PG) and phosphatidylacid (PA), and these components can change the thermoproperties of membranes. Lyso-phospholipid is the lipid with only one tail looking like a cone, and most unsaturated phospholipids prefer to be in a state of trapezoidal; these two shapes play a key role in the formation of liposomes. Cholesterol (Chol), a waxy steroid of fat, is one other important component of lipid which helps to stabilize the biomembranes.

Figure 1-5. Structures of cholesterol and phospholipids
The packing adaptations of lipids are explained by ‘critical packing parameter’ (CPP), which is the ratio of effective volume \( v \), head group area \( S_0 \) and chain length \( l_c \) (CPP = \( v/S_0 l_c \)). The CPP determines the preferred association structures assumed for each molecular shape. When CPP < 1/3, lipids trend to form sphere; when 1/3 < CPP < ½, rods are preferred; when ½ < CPP < 1, lamellar or vesicles are preferred; when CPP ≈ 1, lamellar or planar bilayers are formed; when CCP > 1, hexagonal structures will be formed.

![Figure 1-6. Scheme of formation of lipid bilayers](image)

### 1.5 Classification of Liposomes

Most commonly, liposomes are divided into several types with the volumes and number of rooms (Figure 1-7), namely the multilamellar vesicles (MLVs), the small unilamellar vesicles (SUVs), and the large unilamellar vesicles (LUVs). Due to the properties of different types of liposomes, people developed several methods for preparing liposome, such as Bangham method, sonication method, freezing-thawing method, reverse evaporating method and extrusion method.\(^{18}\)
1.6 Preparation of Liposomes

Bangham method is the most common way to prepare liposomes, named after Dr. Alec Bangham, which is also the way to prepare MLVs. Disruption of MLVs with sonication method usually leads to the formation of SUVs (15-50 nm). However, the overheating during sonication always causes the oxidation and degradation of phospholipids. And that is the reason why the sonication is always accomplished in ice bath. Due to the high curvature of membrane, SUVs are inherently unstable and fuse to form larger particles spontaneously when stored at 4 °C for overnight. Extrusion is the technique by which MLV suspension is forced through a polycarbonate filter with a uniform pore size (usually 100 or 200 nm) to yield liposomes with a uniform diameter near the pore size. This method can improve the homogeneity of particles and reduce the membrane fouling.
1.7 Phase Transition and Main Phase Transition Temperature ($T_m$)

The basic structure of liposome and other biomembrane is a bilayer made of lipid molecules. At a given temperature a lipid bilayer can exist in either a liquid crystal or an ordered gel phase. And all lipids have their own characteristic temperature above which they will turn from ordered gel phase into disordered liquid crystalline phase, which is defined the phase transition temperature. In gel phase, lipid molecules are arranged in order, and constrained to lateral movement with no flip-flop; while in liquid crystalline phase, lipids trend to be less ordered, and can exchange positions with other molecules around freely (Figure 1-9). This random flow allows lipids to diffuse and walk across the surface of the membrane.
There are several factors that will affect the phase transition temperature, such as unsaturation, charge, headgroup structures and the length of hydrocarbon tails. Mainly decided by Van de Waals’ force between adjacent molecules, interaction between lipids could be enforced by increasing the length of hydrocarbon tails, and it will lead to an increase on the phase transition temperature. Meanwhile, when it comes to unsaturated lipids, the double bond will disrupt the lipid packing, reduce the interaction among molecules, and finally decrease $T_m$. Moreover, when the lipids are charged, especially on the headgroup, the repulsion between lipids will get larger, helping to minimum the interaction, and finally reduce $T_m$.

Figure 1-9. A typical DSC trace of phosphatidylcholine\textsuperscript{33}
1.8 Lipid Rafts and Biomembrane Model

The movement patterns of lipids have become the physical basis of “fluid mosaic model” proposed by S.J. Singer and Garth L. Nicholson in 1972, which is used to describe the interactions of lipids and proteins in biological membranes, also used to describe the complex structure of biomembrane. Therefore, $T_m$ and the lateral diffusion of lipid molecules into liposomes become the basis of all the nature of liposomes, such as leakage, fusion, fission, and the nature of the biomembrane, such as endocytosis, exocytosis, etc.

Lipid rafts are specialized membrane domains enriched in certain lipid, cholesterol or protein. And the existence of lipid rafts was first hypothesized by Simons and van Meer in 1988. The hydrocarbon chains of lipid molecules within the rafts tend to be tightly packed, and floating like a raft in the sea of lipids. Such a modulate will cause a defect between raft and the surrounding lipids, which, if big enough, will cause a leaking or release of drugs loaded in liposomes.
Theoretically, when below $T_m$, it is possible to form a perfect liposome vehicle, which is orderly arranged and has no leading for anything loaded inside. However, in practical application, we cannot find such a perfect particle, because during the preparation process of liposomes, there will be some damages occurred with a result of ‘damaging rafts’. Leaking usually happens around these defects. Release is always one of the most important terms in drug delivery researches. As temperature goes above $T_m$, phase transition starts; and with the increase of degree of disorder, leaking turns into release.
1.9 Liposomes in Drug Delivery

Nanoparticles have been studied for the past two decades in drug delivery with numerous materials as drug delivery vesicles, such as mesoporous silica\textsuperscript{27}, carbon nanotubes\textsuperscript{28}, graphene sheets\textsuperscript{29}, liposomes\textsuperscript{30}, micelles\textsuperscript{31}, dendrimers\textsuperscript{32}, polymer-drug self-assemblies\textsuperscript{33}, etc. Various controllable drug delivery systems have been developed with either the initiative properties of the vesicles or proper modifications with other molecules or nanoparticles. However, many of the studied nanoparticles are faced with the same problem of cytotoxicity, as it is still controversial for researchers that no adequate clinical evidence could be provided confirming the cell viability of most of the nano-size vesicles.

Liposome, approved to be safe in clinic practices by FDA, has been regarded as one of the most promising and practical approaches to controllable drug delivery in medical applications, especially in chemotherapy for cancer treatment. Due to the biocompatibility, biodegradability, low toxicity and immunogenicity, liposomes have attracted intensive attention in the past decades in the field of drug delivery.\textsuperscript{34} However, with liposomes encapsulation, chemotherapy drugs are easily to be up take by cells, leading to a short cycle time in human bodies, which limited the practical applications in chemotherapy. To improve the cycle time in human bodies, stealth liposome was developed; with the coating of PEG on the surface, stealth liposomes could be less likely to be identified and up take by cells and have a longer circulation time for cycling through the whole body. And the covering of PEG polymers has also improved the liposomal stability in the meantime. Liposomal doxorubicin decorated with PEG (such as Doxil®) is now available with an enhanced circulation time and up to six times more effective than free doxorubicin.\textsuperscript{35} To further improve the drug delivery efficiency, stealth
liposomes were modified with targeting ligands, such as folic acid\textsuperscript{36} and antibodies\textsuperscript{37}, by which liposomes can both target to the cancer cell and retain at the target site.\textsuperscript{34} In recent years, ‘smart’ liposomes with different stimuli responsibility were further developed for more controllable drug delivery (Figure 1-11).

Figure 1-11. A. Early stage plain liposomes with water soluble/insoluble drugs; B. Long circulating liposomes grafted with PEG or other protective polymers; C. Targeted liposomes modified with antibodies; D. Long circulating liposomes with protective polymers and targeting antibodies; E. Long circulating liposomes loaded with stimuli responsive cargos leading to more controllable system with protective polymers and targeting antibodies\textsuperscript{34}.

1.10 Stimuli-Responsive Liposomes

1.10.1 Ligands for Stimuli-Responsive Liposomes

The stability of lipid bilayers can be affected by the morphology of different lipids and other molecules embedded in the bilayer. People took cholesterol, hydrophilic polymers to form hydrogen bonding at the headgroup position to stabilize liposomes; and in the meantime, with the same properties, people were seeking to find specific stimuli (temperature, pH, \textit{etc.}) to trigger the release of drugs loaded inside liposomes. There are
two main sorts of stimuli-responsive liposomes: to use polymer-functionalized lipids or to encapsulate stimuli responsive nanoparticles.

1.10.1.1 Lipid Polymer Modified Stimuli-Responsive Liposomes

The first approach, in most cases, takes the advantages of polymers to make the system stimuli responsive. Polymer-lipid conjugates have been studied for years for improving the clinical potential of many drugs. And one of the common examples is the long-circulating liposomes, first discussed in 1992. A hydrophilic polymer, such as PEG, is reacted with lipids to form a flexible chain that occupies the space immediately adjacent to the liposome surface. And such polymers will strongly reduce the MPS uptake and extend the blood circulation time of liposomes, which is called stealth liposomes.

Figure 1-12. Reversibility of structural rearrangements in the membrane of small negative liquid liposome upon electrostatic P2 and PL adsorption/desorption
Other than PEG, some other polymers have also been used to improve the properties of liposomes, such as PAA\textsuperscript{40-41}, PNIPAm\textsuperscript{42}, etc. With these polymers, some triggers have been introduced to the liposomal system in order to make them more applicable. In 1991, Ise designed a pH sensitive liposome with lipophilic \textit{azo} radical initiated polymerization.\textsuperscript{43} In 1998, Toru from Osaka Prefecture University developed a liposome system coated with thermosensitive polymers to make it release at around human body temperature.\textsuperscript{38a} In 1999, Winnik developed PNIPAm-Py-Gly copolymer modified liposomes with a thermo-responsivity.\textsuperscript{44} More recently, Concheiro took UV-absorbance advantages of \textit{azo}benzene to form a light sensitive liposomal vehicle.\textsuperscript{45} Ultrasound triggered release was reported by Kazuaki and his co-workers\textsuperscript{46} by modifying liposomes with poly (NIPMAM-co-NIPAM). Kenji and his co-workers reported in 2010 that the copolymer of copoly (EOEOVE-block-octadecyl vinyl ether) could be anchored into liposome surface to fulfill the high temperature sensitivity.\textsuperscript{47}

Figure 1-13. Design of temperature-sensitive liposomes composed of thermosensitive poly (EOEOVE)-OD4 (a), membrane-forming EYPC (b), membrane-stabilizing cholesterol (c) and
highly hydrophilic and non-toxic PEG-lipid (d). Heat-triggered release of DOX from liposomes was illustrated\textsuperscript{47}.

1.10.1.2 Nanoparticle Modified Stimuli-Responsive Liposomes

Encapsulating nanoparticles in liposomes is another pathway to trigger the release from liposomes with an external stimulus. It is known that liposomes are suitable for encapsulating both hydrophilic and hydrophobic cargos, due to the special phospholipid bilayers. In 2007, Lauri, \textit{et al.} have reported three different loading types for gold nanoparticles in liposomes, which is also applicable to other nanoparticles.\textsuperscript{48}

![Figure 1-14. Schematic representation of the nanoparticle-functionalized liposomes: A) hydrophobic particles embedded in the lipid bilayer, B) anionic hydrophilic nanoparticles in the liposome core and C) gold nanoparticles at the lipid bilayer-water interface.\textsuperscript{48}](image)
In recent years, nanoparticle loaded liposomes have been attracting more and more interest, as they can not only bring more triggers to the vesicle, but also induce more properties like optical imaging, MRI, etc. In 2008, Kostas and his co-workers reported that Quantum Dots-liposomes hybrids were prepared to be cellular imaging agents for cancer treatment. Silica nanoparticles and calcium phosphate nanoparticles were proved to be able to stabilize the liposomes as the coating layers.

Figure 1-15. Scheme of the experimental strategy used to prepare insulin-loaded SNCL.

Other than Kenji’s effort on gold nanoparticles induced photo-thermal triggered release, gold nanoparticle-liposomes hybrids have also been studied with peptide release triggered by phospholipase. Superparamagnetic iron oxide (SPIO) nanoparticles, known to be highly effective in hyperthermia, were encapsulated in liposomes due to both the magnetic hyperthermia property and MRI potential for medical applications. Sylviane and his co-workers reported in 2005 that SPIO loaded liposomes could serve well as MRI contrast agents for in vivo imaging. It is reported by Tina and her co-workers in 2011.
that magnetic hydroxyapatite (HA)-coated liposome were capable of being triggered with ultrasound.54

![TEM images of samples: (a) HA-liposome, (b) HA-liposome-S1, (c) HR-TEM image of the semi-crystalline HA matrix around SPIO nanodots observed for sample HA-liposome-S1 (d) TEM photograph of an HA-liposome-S10 sample54.](image)

1.10.2 Common Stimuli-Responsive Liposomes

Stimuli-responsive liposomes are designed for controlled drug delivery based on that certain stimuli intrinsically characteristic of a pathological zone, or when applied externally, could trigger an enhanced or controlled drug release. Typical stimuli could be
applied either within the tissue (pH, temperature, the redox potential, etc.) or from external triggers (ultrasound, light, alternating magnetic field, etc.).

1.10.2.1 Thermosensitive Liposomes

Thermo-responsive drug delivery is one of the most investigated stimuli-responsive strategies. Ideally, a thermosensitive nanocarrier should be stable at body temperature, and release the loading cargo fast when locally heated to a temperature around 40 or 42 °C. For thermosensitive liposomes, the release usually results from a phase transition of the continuous lipids and the associated conformational variations in the lipid bilayers.

Thermosensitive liposome was first reported by Yatvin, et al., who suggested that liposomes would be stable at normal body temperature while leaky at higher temperature. There are now main types of thermosensitive liposomes either consisting of thermosensitive lipids/lipid polymers or modified with thermosensitive polymers on the surface. Recently thermosensitive bubble-generating liposomes turn out to be one of the promising novel drug delivery systems. Such liposome would generate carbon dioxide bubbles with mild hyperthermia (~42 °C) through the decomposition of ammonium bicarbonate; the generated carbon dioxide bubbles can both accelerate the drug release and serve as ultrasound imaging in cancer tissues.
Figure 1-17. Schematic illustrations of the structure and functions of the thermoresponsive, bubble-generating liposomes as well as the mechanism of localized extracellular thermosensitive drug release\textsuperscript{57}

1.10.2.2 pH Sensitive Liposomes

Subtle pH variations between cancer tissue and normal one or between intracellular compartments (endosomes or lysosomes, etc.) could be used to control the release of chemotherapy agents for drug delivery. pH sensitive liposomes containing pH sensitive lipids, have been studied ever since 1980s by Yatvin et al.\textsuperscript{58}. 
Developed for decades, there are now several different types of pH sensitive liposomes with various mechanisms, such as (1). Charge changing through protonation or deprotonation of polymers or peptides modified on the surface of liposomes to induce phase transition in lipid bilayers; (2). Acid sensitive cleavage of polymers on the headgroup or hydrophobic tails, which leads to either structure defects in the bilayer or the release of drugs loaded.
1.10.2.3 Redox Potential Sensitive Liposomes

The redox potential differences between the reducing intracellular space and oxidizing extracellular area could be applied for stimuli responsive controlled drug delivery. Disulfide bonds are one of the most commonly used redox agents as they could be oxidized by glutathione rapidly. Redox sensitive liposomes have been studied by introducing small amount of special lipids whose head and tails are linked with disulfide bonds to the normal phospholipid liposomes.
Figure 1-20. Schematic illustration of the self-assembly, accumulation at tumor tissue and intracellular trafficking of redox-sensitive HA-ss-DOCA micelles. The intracellular trafficking includes steps of receptor-mediated cellular internalization, endo/lysosomal escape, reduction triggered disassembly, and drug release\(^ {64}\).

Except for the redox sensitive lipids, a PEG modified lipid polymer was also prepared via S-S bonds by Kirpotin, et al.\(^ {65}\) The cleavage of PEG coverage could help to release the contents loaded from liposomes and lead to a rapid clearance of liposomes from circulation by de-protection.

### 1.10.2.4 Light Sensitive Liposomes

Light triggered release has been studied in the past few years due to the non-invasiveness and the possibility of remote control. Different wavelength ranges (ultraviolet, visible or
near-infrared) have been reported to be effective in distinct systems. The light sensitivity is usually achieved by either photosensitive molecules (azobenzene\textsuperscript{66}, \textit{etc.}) or light responsive molecules/nanoparticles (gold nanoparticles\textsuperscript{48}, \textit{etc.}).

![Diagram of Phototiggerable Formulations Strategies](image)

Figure 1-21. Phototiggerable formulation strategies of liposomes\textsuperscript{67}.

Light sensitive liposomes have been explored since 1980s, developing from light sensitive lipids. There are many types of photosensitive liposomes: photocleavable liposomes, photopolymerizable liposomes, \textit{etc.} The general mechanism for light responsive liposomes is to introduce defects in the lipid bilayer with light radiation. In 1990s, Thompson et al. developed new types of light sensitive liposomes by testing the reactivity of vinyl groups in the lipids with UV light\textsuperscript{68}. Photolabile liposomes were
designed by Zaiguo et al. in 2003, with a Corey-Seebach dithiane-aldehyde adducts as photolabile groups.\textsuperscript{69}

Gold nanoparticle encapsulated liposomes have been developed since 1980s, originally aimed for probes of liposome-cell interactions. Kenji, \textit{et al.} in 2007 reported that gold nanoparticle could be used as a photothermal trigger for controlled drug release with different loading pathways (between lipid bilayer, inside hydrophilic core and attached on the surface of liposomes).\textsuperscript{48} Gold nanoparticle encapsulated liposomes have also been studied in the field of cellular imaging.

\textbf{Figure 1-22.} Schematic illustration of mechanism of photolabile linkers.\textsuperscript{69}
Figure 1-23. Multifunctional gold coated liposome nanoparticle for imaging, drug delivery and photothermal therapy\textsuperscript{71}.

### 1.10.2.5 Magnetism Sensitive Liposomes

Magnetic liposomes, also named as magnetoliposomes, are liposomes with magnetic materials (SPIO, \textit{etc.}) either encapsulated inside the hydrophilic core\textsuperscript{72} or embedded between lipid bilayers\textsuperscript{73}. The first reported magnetoliposomes could be dated back to 1986 by Kiwada, \textit{et al}\textsuperscript{74}. Such liposomes could be used to target specific site in therapeutic applications when exposed to a magnetic field.
Due to the superparamagnetic properties and small size, SPIOs have drawn significant interest from scientists in biomedical areas, especially for liposomes loaded SPIOs. Magnetism sensitive liposomes usually take advantage of magnetic thermal properties of the nanoparticles by causing defects in lipid bilayer. One other possible pathway is to kill the cancer cells with increased local temperature by hyperthermia. Moreover, magnetoliposomes have also been proved to be effective in magnetic resonance imaging of cancer cells.\textsuperscript{75}

\textbf{1.11 Conclusion}

Ever since the first observation, liposomes were taken as promising drug delivery carriers due to the non-toxicity and considerable volume. Approved by FDA, Doxil\textsuperscript{®} has been one of the commercially available nano-sized chemotherapy agents which have been verified to be effective and safe to human bodies. With further modification of polymers and nanoparticles, stimuli responsive liposomes were developed for both controllable release and \textit{in vitro/in vivo} imaging however, their poor stability still prevented wider applications of these systems.
2 Preparation and Characterization of Liposomes

2.1 Preparation of Liposomes

In this work, all the liposomes were prepared with sonication method as follows: phospholipids were first dissolved in CHCl₃ (10 mg/mL, 1% CH₃CH₂OH added to avoid the oxidation of phospholipids). Certain amount of phospholipid solutions were mixed together in a small vial (5 mL), and then the mixtures were dried with either rotation evaporation or by N₂ flow to form lipid film on the bottom of the vial. The film was dried under high vacuum for several hours. The film was then rehydrated with HEPES buffer solution (pH=7.4) or calcein solution (50 mmol/L) or doxorubicin solution (1 mg/mL) at room temperature for one hour, and then mixed vigorously by vortex. After the freezing-thawing cycle for five times with liquid nitrogen and water, the residue was sonicated with probe-sonicator in ice-water blend for 20 min. Centrifuged at a low speed of 1000 rpm for 1 min, the residue from the probe and large volume liposomes were removed. The liposomes were kept at room temperature for several hours and then in fridge at 4 ºC for overnight. The liposomes were used for further characterizations.

2.2 Characterization of Liposomes

2.2.1 Encapsulation and Release

Drug encapsulation is one of the most important topics in liposomes. Generally speaking, drug encapsulation of liposomes can be divided into two types: passive trapping and active trapping. Passive trapping of hydrophilic molecules is to use the solution of drugs when hydrolyzing lipids, where the efficiency is constricted by the trapping volume of the vesicle system and the drug concentration. While active trapping, which mainly
depends on the ionic osmotic pressure difference between lipid bilayers (H\(^+\), NH\(_4^+\), etc), may have a better trapping efficiency, and serve more drug candidates.\(^1^8\)

To determine the encapsulation efficiency, a known amount of liposomes was centrifuged at 14000 rpm for 30 min. The supernatant and the original dox solution were analyzed by UV-Vis to determine the encapsulation percentage. The entrapment efficiency of liposomes was calculated by the following equation: EE (\%) = (T-C)/T \times 100, where T is the total amount of drug that is detected in the original dox solution, and C is the amount of drug detected only in the supernatant.\(^7^6\)

The drug release in liposomes is determined with fluorescence spectrum. Triton X-100 is often used as the surfactant to decompose the liposome and get all the loading released. The largest absorbance wavelength is taken for the measurement, and the formula is as follows (2-1).\(^1^8\) (In the formula, \(F_t\) is the fluorescence of drug loaded liposomes at time \(t\), \(F_0\) is the fluorescence of liposome loaded with drugs before release, \(F_\infty\) is the fluorescence of which Triton-X 100 added.)

\[
\text{release } \% = \frac{F_t - F_0}{F_\infty - F_0} \times 100\%, \hspace{1cm} (2-1)
\]

2.2.2 Imaging and Physical Characterization

Transmission electronic microscope (TEM) and atomic force microscope (AFM) are the most common characterization methods for the morphology of liposomes. Negatively stained TEM (Figure 2-1) with U (AcO)_2 is one of the easiest way for dried liposomes; however, in some cases, cryo-TEM is applied to get a convincing structure of liposomes or even dual-layer lipids.
The size of liposomes was estimated with DLS from Malvern NanoZS, and with the same machine, zeta potential data could also be obtained for the surface charging changes before and after modification of liposomes.

![Negatively stained TEM image of liposomes prepared with water-bath sonication method.](image)

Figure 2-1. Negatively stained TEM image of liposomes prepared with water-bath sonication method.

### 2.2.3 *In Vitro Characterization*

Confocal laser scanning microscope (CLSM) is the common way to determine the localization of liposomes in tumor cells. Labeled with fluorescence molecules, we can easily tell both whether the liposomes are up taken by the cells or not and how much of the loaded drugs are released (Figure 2-2). In some cases, TEM is also applied to get a more intuitive image of the cellular uptake.
2.2.3.1 CLSM image preparation

HeLa cells were cultured in EMEM medium containing 10% FBS and 0.1% penicillin-streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. After cell attachment, the medium was replaced by medium containing cargo liposomes, followed by incubation for 1 hr. The cells were then washed twice with DPBS and fixed with a 4% paraformaldehyde solution. For nuclear staining, the cells were incubated with DAPI for 10 min at room temperature, following 3 washes in PBS. Then the cells were observed under confocal laser scanning microscopy (CLSM, Zeiss) and intracellular release and trafficking of DOX were examined.

Figure 2-2. CLSM images of NBD labeled liposomes incubated with HeLa cells for 1 hr.
2.2.3.2 Cell Viability and MTT Assay.

MTT assays are widely used in *in vitro* testing for either the cell viability of the vesicles or the *IC*<sub>50</sub> for drug loaded liposomes. In this thesis, MTT assays are widely used for the cell viabilities of different liposomal systems. HeLa cells were seeded at a density of 5 × 10<sup>3</sup> cells per well in 96-well flat bottom plates and incubated for 12 hrs. Cells were washed by DPBS buffer and incubated in the culture media with the liposomes (at different concentrations, 0.1 mg/mL~10<sup>-9</sup> mg/mL) for 24 hrs at 37 °C. Cell viability was evaluated by the MTT colorimetric procedure.
3 “Nail” and “Comb” Effects of Cholesterol Modified NIPAm Oligomers on Cancer Targeting Liposomes

3.1 Introduction

Liposomes, first described in 1960s, are self-assembled vesicles constructed with lipid bilayers. Due to their biocompatibility, high drug loading and easy preparation process, liposomes have attracted attention for drug delivery ever since their discovery. Liposomes encapsulated doxorubicin (Doxil®) has been approved to largely reduce the cardiac toxicity associated with free doxorubicin. In the past two decades, stimuli-sensitive liposomes have been studied for both controllable and enhanced release. Thermosensitive liposomes were proven to be a promising approach especially combined with local hyperthermia. When the liposomes reach the heated tumor tissue area, the drug will be released due to changes in lipid membrane permeability.

Poly(N-isopropylacrylamide) modified liposome is one of the most typical thermosensitive liposomes. Due to the sharp coil-to-globule transition at the lower critical solution temperature (LCST) of the polymer chain, PNIPAm anchored into liposomes can lead to lateral phase separation of lipid bilayers that consequently allows cargo release through membrane defects. It was reported as early as 2005 that PNIPAm with higher molecular weight (Mw) causes faster release than low Mw PNIPAm. Although proven non-cytotoxic, high Mw PNIPAm is hard to biodegrade and has significant barriers of chronic bioaccumulation in medical applications. On the other hand, NIPAm oligomers (Mw<2,500) can be directly broken down and can have a slower
release rate both of which are more desirable in the area of controlled and sustainable release.

Scheme. 3-1 Schematic illustrations for the release of NIPAm oligomer (NO) liposomes at 37 °C:

A. nail effect: main-chain NIPAm oligomers (MCNOs); B. comb effect: side-chain NIPAm oligomers (SCNOs)

Cholesterol (chol), which takes up 30% of cell membranes, is able to decrease the fluidity of cell membranes due to its hydrogen bonding with lipid and flake structure. Many cholesterol derivatives were reported in the modification of liposome surfaces. Owing to the biocompatibility and “anchor-ability” of cholesterol, it is feasible to functionalize liposomes with either cholesterol derivatives or cholesterol modified polymers. Modifying liposomes with targeting moieties such as folic acid\textsuperscript{36}, biotin\textsuperscript{83}, saccharide\textsuperscript{84}, peptides\textsuperscript{85}, etc. have been heavily studied during the past years. Biotin, known as Vitamin B7, which is highly needed for most cancer cell activities, has been proved to be
promising cancer targeting ligand for many anti-cancer drugs. Biotin-conjugated micelles\textsuperscript{83a,83c} and dendrimers\textsuperscript{83b} have been reported to be effective cancer-targeting drug delivery systems\textsuperscript{86} however, until now uptake of modified biotin carriers was improved without targeting a specific cell compartment.

In this chapter, cholesterol modified NIPAm oligomers with two polymer architectures (main chain, MCNOs and side chain, SCNOs) were prepared by reversible addition-fragmentation chain transfer (RAFT) polymerization and radical polymerization for a thermosensitive DPPC/Chol liposomes system. Cholesterol molecules were used as the “anchor” groups for the oligomers. Biotinylated cholesterol was also synthesized to enhance cellular uptake. Two mechanisms are proposed to understand the influence of the architecture of the thermosensitive component on the overall release process (MCNOs “nail” and SCNOs, “comb”) (Scheme. 3-1). Both MCNOs and SCNOs proved biocompatible and showed thermosensitive driven release. Moreover, biotinylation of MCNOs and SCNOs allowed for excellent nuclei targetability in HeLa cells.

### 3.2 Experimental and Materials

#### 3.2.1 Materials

Pyridine, p-toluenesulfonylchloride, dichloromethane (DCM), chloroform, n-hexanes, ethyl acetate (EtOAc), tetraethyleneglycol (TEG), dioxane, methanol (MeOH), triethylamine (TEA), methacryloyl chloride, N-isopropylacrylamide (NIPAm), N, N’-dimethylformide (DMF), azobisisobutyronitrile (AIBN), doxorubicin (DOX), tetrahydrofuran (THF), isopropanol, 1- Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), 1-dodecanethiol, acetone,
tricaprylylmethylammounium chloride, sodium hydroxide, hydrochloride (HCl), carbon disulfide and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were bought from Sigma-Aldrich; biotin was bought from Fluka; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (chol), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine- N-(7-nitro-2,1,3-benoxadiazo-4-yl) (ammonium salt) (NBD-DPPE) were bought from Avantipolar lipid. All solutions were prepared by deionized water (Milli-Q, 18.2 MΩ cm, 25 °C).

3.2.2 Characterization

All $^1$H NMR were obtained on a Bruker AV-III 600MHz NMR spectrometer. GPC data were obtained on an Agilent 1200 GPC system. TEM images were acquired on FEI Tecnai T12 transmission electron microscope at 120 keV. FTIR spectra were acquired on Thermo Nicolet 6700 FT-IR system. Particle size distribution data were taken on Malvern NanoZS. DSC data were obtained from Netzsch DSC 204 HP Phoenix differential scanning calorimeter. TGA data were acquired from Netzsch TG 209 F1 Iris FTIR Coupling. Fluorescence spectrum was acquired on Varian Eclipse fluorescence spectrometer. CLSM images were obtained from Zeiss LSM 710 inverted microscope.

3.2.3 Methods

Synthesis of cholesterol-OTs (3-1).

To a stirred solution of cholesterol (10 g, 25.86 mmol) in anhydrous pyridine (100 mL) p-toluenesulfonylchloride (10 g, 52.47 mmol) was added at room temperature. After that, water (25 mL) was added to the reaction mixture and the resulting solution was extracted
with DCM (50 mL, 3 times). The combined organic extracts were washed with brine and dried over anhydrous sodium sulfate and evaporated under reduced pressure. The oily material loaded onto a silica column eluting with CHCl3/EtOAc (9:1) to give cholesterol-OTs as a white powder (87% yield).

Scheme 3-2. Schematic illustrations of the synthesized process of A. SCNOs, B. MCNOs, C. biotinylated cholesterol

AIBN: Azobisisobutyronitrile; PTC: Phase transfer catalyst; EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS: N-Hydroxysuccinimide.
**Synthesis of cholesterol-TEG (3-2).**

A mixture of tetraethyleneglycol (4.00 g, 20.6 mmol) and compound 1 (2.00 g, 36 mmol) in 1, 4-dioxane (50 mL) was stirred at 110 °C for 24 hrs. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was chromatographed on silica gel eluting with CHCl₃/EtOAc/MeOH (8:1:1) to end up with a colourless syrup (80% yield).

**Synthesis of cholesterol monomer (3-3).**

Compound 2 (562 mg, 1 mmol) was dissolved in 10 mL dry DCM (10 mL), and triethylamine (0.2 mL) was added and stirred in an ice bath. Methacryloyl chloride (0.2 mL, 2 mmol) was then added drop wise during 3 minutes. The mixture was stirred under nitrogen atmosphere overnight. Then the reaction was quenched by water, and washed by water and brine, dried over anhydrous sodium sulfate, filtrated, and evaporated under vacuum, which yielded a white solid (90% yield). 1H NMR (600 MHz, CDCl₃) δ 6.30 – 6.18 (m, 1H), 5.83 (d, J = 22.6 Hz, 1H), 5.40 – 5.29 (m, 1H), 4.36 – 4.22 (m, 1H), 3.79 – 3.55 (m, 12H), 3.36 (d, J = 13.8 Hz, 2H), 3.07 (ddd, J = 16.2, 12.4, 6.3 Hz, 1H), 2.63 – 2.08 (m, 3H), 2.08 – 1.74 (m, 12H), 1.68 (dd, J = 18.1, 8.6 Hz, 3H), 1.63 – 1.19 (m, 15H), 1.17 – 0.94 (m, 12H), 0.91 (d, J = 6.5 Hz, 3H), 0.86 (d, J = 6.6 Hz, 6H), 0.67 (s, 3H).

**Preparation of SCNOs (3-4).**

Recrystallized NIPAm (1130 mg, 10 mmol) and compound 3 (126 mg, 0.1 mmol) were dissolved in 1 mL DMF. And then Azobisisobutyronitrile (AIBN) solution (10 mg/mL in THF, 16.4 µL) was added. The solution was heated at 75 °C for 7 hrs then dropped into n-
hexanes with vigorous stirring. After filtration a white solid was recovered and dried under vacuum at 50°C overnight. The final product was a white solid (92% yield).

**S-1-dodecyl-S-(a,a’-dimethyl-a”-acetic acid) trithiocarbonate (3-5).**

1-dodecanethiol (8.07 g, 0.04 mol), acetone (19.24 g, 0.33 mol), and tetramethylammonium bromide (0.25 g, 1.6 mmol) were mixed in a jacketed reactor, and cooled in an ice bath under nitrogen atmosphere. Stirred for 20 min, sodium hydroxide solution (50%, 3.35g, 0.042 mol) was added. Reacted for another 15 min, carbon disulfide (3.04 g, 0.04 mol) in acetone (4.04 g, 0.069 mol) was added over 20 min. After 10 min, chloroform (7.12g, 0.06 mol) was added into the reaction mixture, followed by sodium hydroxide solution (50%, 16g, 0.2 mol) added drop wise. The reaction was then left to stir overnight. MilliQ water (60 mL) was added, with 10 mL concentrated HCl to acidify the solution. Nitrogen was purged through the solution to get rid of extra acetone. The solid was dissolved again in 100 mL isopropanol, filtrated, and dried under vacuum. The resulting solid was recrystallized in hexanes twice to get yellow crystals (30% yield).

**Synthesis of Cholesterol modified chain transfer agent (3-6).**

Recrystallized compound 2-5 (387 mg, 1 mmol) and cholesterol (364 mg, 1 mmol) were dissolved in 10 mL DCM; stirred for 10 min, NHS (210 mg, 1.1 mmol) and EDC (192 uL, 1.1 mmol) were added, and then stirred overnight at room temperature. The product was purified by flash column yielding a yellow solid. (85 % yield). 1H NMR (600 MHz, CDCl3): δ 5.32 (m, 1H), 3.55–3.75 (m, 16H), 3.42 (t, 2H), 3.17–3.26 (m, 1H), 1.75 (s, 6H), 1.75–2.50 (m, 7H), 0.87–1.67 (m, 56H), 0.69 (s, 3H).
Preparation of MCNOs (3-7).

Recrystallized NIPAm (1.13 g, 10 mmol) and compound 2-6 (73.2 mg, 0.1 mmol) were both dissolved in 1 mL DMF with nitrogen purged for 30 min. AIBN solution (10 mg/mL in DMF, 33µL, 0.002 mmol) was then added. After freezing by liquid nitrogen under vacuum to remove extra oxygen in solvent, the reactor was heated at 75 °C with stirring for 8 hrs, and then the residue was poured into 30 mL of diethyl ether. Several drops of hexamine were added and stirred for 24 hrs to remove extra CTA. The final product was a white solid (80% yield).

Preparation of NOs modified liposomes.

DPPC solution (CHCl₃, 10 mg/mL, 500 µL), cholesterol solution (CHCl₃, 10 mg/mL, 125 µL), cholesterol-biotin solution (CHCl₃, 10 mg/mL, 11 µL) and NBD-DOPE solution (CHCl₃, 0.2 mg/mL, 11 µL) were mixed in a 3 mL vial. NOs solution (CHCl₃, 10 mg/mL, 41 µL) was added. The solvents were evaporated slowly under low vacuum. The vials were then kept under high vacuum for two hours. The phospholipids were after rehydrated with HEPES buffer solution (pH=7.4, 500 µL) or calcein solution (50 mmol/L, 500 µL) or DOX solution (1 mg/mL, 500 µL) at 46 °C by vortex and short time water bath sonication. After the freezing-thawing cycle (five times with liquid nitrogen and water), the residue was sonicated with probe-sonicator in ice-water blend for 30 min. After kept in 46 °C water bath for 5 min, the liposomes were centrifuged at a low speed of 1000 rpm for 1 min so that the residue from the probe and large volume liposomes were removed. The liposomes were left in fridge at 4 °C for overnight. For drug (calcein/DOX) loaded liposomes, further purification was needed to remove the free drug
in the solution. Thus Sephadex G-50 column (2 mL, 0.9% NaCl) was used as a first-step purification, and then the liposomes residue was dialyzed with a dialysis bag (Mw=8,000) for one day. The as-prepared liposomes were used for further characterizations.

**Negatively stained TEM**

A drop of liposomes at room temperature was dropped on the grid, and then the grid was washed with deionized water 3 times. The grid was after merged into U(OAc)\textsubscript{2} solution (2%) for 3 min for staining. The pre-heated liposomes (37 °C) were stained in the same way.

**Load and release.**

The release test was accomplished with calcein loaded liposomes. The load of calcein was fulfilled by hydrating lipid monolayers with calcein solution (C\textsubscript{calcein}=50 mmol/L).

In order to test the release of NO liposomes, the calcein loaded liposomes were diluted with HEPES buffer by 1000 times and then time-dependent release profiles were acquired with fluorescence spectrometer in 120 min at 25, 37 and 46 °C. Loading efficiency of calcein/DOX is 3-5% w/w.

The biological tests were finished with DOX loaded liposomes. The fluorescence setup for DOX is: $\lambda_{ex.}=490$ nm, $\lambda_{em.}=580$ nm.

**Cell viability and MTT assay.**

The cytotoxicity of pristine, MCNO and SCNO liposomes were evaluated using the MTT assay. HeLa cells were seeded at a density of $5 \times 10^3$ cells per well in 96-well flat bottom plates and incubated for 12 hrs. Cells were washed by DPBS buffer and incubated in the
culture media with the liposomes for 24 hrs at 37 °C. Cell viability was evaluated by the MTT colorimetric procedure.

The cytotoxicity of DOX loaded liposomes was also evaluated by MTT assay. The same procedure as cell viability was followed, but after washing with DPBS buffer, the cells were incubated in the culture media with liposomes for 2 hrs at 37 °C.

**Localization and cellular uptake of liposomes.**

HeLa and HepG2 cells were cultured in EMEM medium containing 10 % FBS and 0.1 % penicillin-streptomycin at 37 °C in a humidified 5 % CO₂ atmosphere. After cell attachment, the medium was replaced by medium containing pristine, MCNO and SCNO liposomes, followed by incubation for 1 hr. The cells were then washed twice with DPBS and fixed with a 4% paraformaldehyde solution. For nuclear staining, the cells were incubated with DAPI for 10 min at room temperature, following 3 washes in PBS. Then the cells were observed under confocal laser scanning microscopy (CLSM, Zeiss) and intracellular release and trafficking of DOX were examined.

The difference between biotinylated liposomes and non-biotinylated liposomes were investigated following the same procedure with HeLa cells. And the incubation time by liposomes containing medium was 5 min and 30 min.

### 3.3 Results and Discussion

SCNOs were prepared via radical polymerization while MCNOs were prepared via RAFT polymerization (Scheme 3-2). Biotinylated cholesterol used for cell targeting was synthesized through a condensation reaction (3-8, Scheme 3-2). The chain transfer agent
(CTA) was prepared following literature\textsuperscript{87}. Cholesterol substituted monomers (3-3, Scheme 3-2, for SCNOs) and modified CTA (3-7, Scheme 3-2, for MCNOs) were synthesized as described in Scheme 3-2. Tetraethyleneglycol (TEG) bridges can minimize the steric effect between DPPC molecules and the biotinylated or polymeric cholesterols. \(^1\)H NMR spectra are shown in appendix. The \(M_n\) of SCNOs and MCNOs are determined by GPC (Figure 3-1), and both are around 2,200.

![Figure 3-1. GPC for SCNO (left) and MCNO (right). GPC data was obtained by Agilent 1200 GPC in THF, \(M_n\text{MCNO}=2270\), DPI=1.26; \(M_n\text{SCNO}=2130\), DPI=1.24.](image-url)
Figure 3-2. TEM images (negatively stained) of different liposomal samples both at room temperature and heated at 37°C for 1 hr: A. pristine liposomes-RT; B. MCNO liposomes-RT; C. SCNO liposomes-RT; D. pristine liposomes-37°C; E. MCNO liposomes-37°C; F. SCNO liposomes-37°C; and G. size distribution results of different liposomes at RT. (Scale bar is 200 nm (A-C) and 100 nm (D-F)).
In this study, liposomes (DPPC: cholesterol: NOs= 60: 40: 1, in mol) are prepared by a modified Bangham method. HEPES buffer or drug (calcein: 50 mmol/L, DOX: 1 mg/mL) solutions are used as cargo. For the drug loaded liposomes, Sephadex G50 mini-column was used to remove the extra free drugs. TEM images, negatively stained by Uranium acetate, were taken to for the morphology of the pristine, SCNO and MCNO liposomes (Figure 3-2). Size distribution data was also obtained for the liposomes (Figure 3-2-G). The results of both TEM and size distribution show that the liposomes were uniform spheres with a diameter around 150 nm. (A. dpristine= 153.6 nm, PDI=1.279; B. dMCNO= 124.2 nm, PDI=1.281; C. dSCNO= 140.7 nm, PDI=1.197).

The LCST (Figure 3-3) for the two NIPAm oligomers were obtained by turbidity method with UV-Vis spectrometer, and both are around 31 °C (TSCNO= 31.8 °C; TMCSNO= 30.8 °C). Hence, 37 °C was chosen as a temperature for the release test, because it is the normal body temperature and is above LCST of the NOs. On the other hand, for pure DPPC liposomes, when temperature goes above the main transfer temperature of lipid molecules (Tm-DPPC= 42 °C), lipid bilayers will turn from gel phase to liquid crystal phase, and the drug loaded inside liposomes will be able to pass through the membrane easily. However, in our case, as the cholesterol takes 40% of total lipid, the lipid bilayer is in a fluid phase over a wide range of temperatures without an exact Tm. One temperature above Tm-DPPC (46 °C) was also chosen for release test as comparison.
Figure 3-3. LCST data determined with turbidity method of A. MCNOs (30.8 °C) and B. SCNOs (31.8 °C).

In order to test the thermosensitivity of the NO modified liposomes, a release profile (Figure 3-4) was obtained. At 25 °C, pristine, MCNO, and SCNO liposomes are stable during the 120 minutes test period. The liposomes displayed a clear time-dependent calcein release profile at both 37 and 46 °C. A rapid release was observed after 10 min heating for all liposomes at 46 °C and the release of both NO modified liposomes was notably higher than the pristine one.
Figure 3-4. Release profiles of liposomes at different temperatures: A. Pristine and MCNO and B. Pristine and SCNO.

When incubated at 37 °C, pristine liposomes show a comparable release rate, which is due to the thermally-activated barrier density fluctuations in the lipid bilayer. However both NO modified liposomes tend to release faster than pristine. This is due to the lateral phase separation that occurs when the temperature is raised above the LCST of the NIPAm chains causing their consequent aggregation.
From the release scheme of Figure 3-4, the difference between MCNO and SCNO liposomes’ release at 37 °C is mainly the different aggregation forms of the oligomers. For MCNO when NIPAm backbone shrinks, it will form a hydrophobic bolus on top of the cholesterol “anchor”, thus the oligomer will tend to go inside the lipid bilayers where the environment is hydrophobic. Such behaviour is just like pressing a nail into a wall (nail effect). On the other hand for SCNO, more cholesterol molecules are inserted into the lipid bilayer thus when the side chains get aggregated, it will look like a comb inserted into hair (comb effect). When temperature goes higher than the LCST of NOs, hydrophobic bolus were formed and inserted into lipid bilayers to cause defects for drug to release in both cases. The main difference between “nail” and “comb” effects is that the backbone shrink of the latter can produce intramolecular lateral forces accelerating the formation of defects so as to promote the drug release, and “comb” effect will bring in more release than “nail”. Due to the limited chain length used in this study, the difference in release was not excessive. In fact, this is another advantage of NIPAm oligomers as the speed of the release can be further controlled by the oligomer’s chain length ie. longer chains will lead to faster release (Figure 3-5).
Figure 3-5. Release profile of MCNO and SCNO' (long chain) liposomes at 25 and 37 °C.

To further verify the thermosensitivity of NO liposomes intuitively, negatively stained TEM images (Figure 3-2, D-F) of both the pristine and NO liposomes were obtained at 37 °C (incubation for one hour). Liposomes kept at room temperature look almost like isolated spheres, due to the NO chain that prohibit the contact between liposomes (Figure 3-2, A-C). Highly shrunk vesicles were observed in NO liposomes as a result of heating-induced phase transition (Figure 3-2, E, F (red arrow)). Fusion-like liposomes, which are another possible mechanism of release, could also be observed in the TEM of NO liposomes at 37 °C. For better understanding the release, Tb$^{3+}$/DPA assay$^{89}$ (Figure 3-6, A-C) and NBD/RhB assay$^{90}$ (Figure 3-6, D-F) were tested followed literature. Slight lipid mixing was observed indicating that some aggregation occurred when the liposomes were heated. However, no fusion was obtained from the Tb$^{3+}$/DPA assays, which indicated that
the defects caused by NOs shrunk were the main trigger of the release. The visualized results clearly confirm the thermosensitivity of both NO systems.

The in vitro cytotoxicity of liposomes was assessed by MTT assay with HeLa cell line at different concentrations of liposomes ($10^{-7}$~1 mg/ml, total lipids). Figure 3-7 (A-C) shows that liposomes are safe at low concentrations ($C_{lipid} < 10^{-3}$ mg/ml). For all further in vitro tests, the total lipid concentration used was lower than $10^{-3}$ mg/ml to minimize the cytotoxicity of liposomes themselves. MTT assays for both NO liposomes (DOX loaded) are also available in Figure 3-7-D, where no obvious difference between the two systems is noted.
Figure 3-6. Tb$^{3+}$/DPA assays (Ext: 276 nm; Ems: 545 nm) of liposomes at 37 °C for 300 s: A. pristine, B. SCNO and C. MCNO. And NBD/RhB assays of liposomes at RT and heated at 37 °C for 5 min (NBD/RhB-labeled liposomes: pure liposomes=1:9): D pristine, E. MCNO and F. SCNO.
Figure 3-7. In vitro cytotoxicity of pristine and modified liposomes (A. pristine liposomes, B. SCNO liposomes, C. MCNO liposomes) incubated with HeLa cells for 24 hrs (37 °C) and (D) MTT assays for DOX loaded SCNO and MCNO liposomes incubated with HeLa cells for 2 hrs (37 °C).

The cellular localization of DOX-loaded liposomes was tested by Zeiss LSM 710 inverted confocal microscope (Figure 3-8). All the tests were done with the same setup. The NBD labelled liposomes (NBD-DPPE: DPPC: Chol: NOs= 1: 60: 40: 1, in mol) with DOX loaded were prepared and then diluted with cell culture medium, and after incubation with HeLa cells at 37 °C, the cellular uptake for DOX loaded liposomes was monitored. After one hour incubation, the liposomes uptake was observed due to NBD fluorescence spreading inside the cytoplasm, and a nuclear uptake of DOX was also
visualized in all samples. According to fluorescence intensity, almost all the DOX released was taken up by the nuclei, with SCNO liposomes releasing the most DOX which is in agreement with the release profiles. The same test was repeated with HepG2 cells (Figure 3-9), and similar results were obtained.

![CLSM images](image)

Figure 3-8. CLSM images of DOX loaded liposomes incubated with HeLa cells at 37°C for 1 hour (from left to right, DAPI, NBD, DOX, merged): A. pristine liposomes, B. MCNO liposomes, C. SCNO liposomes.
To achieve better targeting, biotinylated cholesterol was used to enhance the cell uptake and a time-dependent liposomes uptake test was evaluated by CLSM. Biotinylated and pristine liposomes with NBD labelled (biotin-cholesterol: total lipid=5% in mole; no doxorubicin loaded) were prepared, and then were incubated with HeLa cells ($10^{-3}$ mg/ml, 5, 30 min) to see the localization of liposomes. The cellular uptake could be observed after 5 min for both samples (Figure 3-10). An enhancement was detected for biotinylated liposomes after 30 min treatment, while for the pristine liposomes no increase was observed. Moreover, a Z-stack confocal microscopic image (Figure 3-11, biotinylated liposomes, 30 min) shows an intense fluorescence both in the cytoplasm and around the nuclei area, which indicates that the biotinylated liposomes got aggregated around the
nuclei membrane. From the in vitro studies, biotinylation could significantly enhance the cellular uptake of liposomes, which indicated that the biotinylated liposomes would largely enhance the drug efficiency.

Figure 3-10. CLSM images of the cellular uptake of biotinylated and non-biotinylated liposomes in HeLa (from left to right, DAPI, NBD, merged): A1. Biotinylated liposomes incubated with HeLa for 5 min; A2. Biotinylated liposomes incubated with HeLa for 30 min; B1. Pristine liposomes incubated with HeLa for 5 min; B2. Pristine liposomes incubated with HeLa for 30 min.
Figure 3-11. Z-stack CLSM image for Biotinylated liposomes incubated with HeLa cells for 30 min.

3.4 Conclusions

In this study, we prepared liposomes modified with two types of low $M_w$ cholesterol-NIPAm oligomers instead of high $M_w$ industrial PNIPAm copolymers. The NO liposomes were proved to be efficient in thermosensitive release. Mechanisms of the thermosensitive release from NO liposomes were proved by release amount and named with “nail” and “comb” effects for MCNO and SCNO liposomes. “Comb” effect led a faster release due to the lateral phase separation caused by the shrinking of PNIPAm polymers on the surface. Both MCNO and SCNO liposomes were proved to trigger DOX release at 37 °C, which were not as fast as the high $M_w$ NO liposomes; but this relatively slower release benefits us with a lower dose of PNIPAm, cytotoxic itself to human bodies, which help to increase the biocompatibility of our system. Biotinylation is further
verified to be effective in cancer targeting leading to an enhancement of cellular uptake efficiency. Our liposomal systems modified with low $M_w$ NOs could serve as promising carriers for either drug or gene delivery with potential high transfer efficiency.
4 Poly (Acrylic Acid)-DOPE “Drop-In” Liposomes with Acid-Labile Property and Liposomes-Doxorubicin Complex

4.1 Introduction

Liposomes, especially stimuli-sensitive liposomes\textsuperscript{47, 78}, have been regarded as promising nano-scale drug carriers with various drug candidates like doxorubicin, protein and peptides drugs\textsuperscript{77}. pH sensitive liposomes\textsuperscript{58, 91}, which take advantage of the extracellular environment of solid tumor and the acidic condition in endosomes after liposomes endocytosis, show a promising potential in cytoplasm drug delivery\textsuperscript{91a}. Most commonly, pH sensitive liposomes are composed of DOPE and a pH sensitive amphiphile (\textit{e.g.} oleic acid\textsuperscript{92}, cholesterol hemisuccinate (CHEMS)\textsuperscript{93}, \textit{etc.}), in which case, the amphiphilic molecules stabilized the unstable liposomes by preventing DOPE forming a hexagonal structure to stabilize the bilayers in aqueous solution\textsuperscript{94}. In some other cases, the pH sensitive liposomes can also contain pH sensitive molecules (\textit{e.g.} fusion peptides\textsuperscript{95}, synthetic polymers\textsuperscript{91c, 96}, \textit{etc.}) for modification. Nguyen and his co-workers reported in 2007 that cholesterol terminated PAA modified liposomes could be stabilized with a 50\% cross-linking of total carboxylic groups to form a polymer-caged vesicle\textsuperscript{97}. Recently the same group reported that the PAA modified polymer-caged vesicle could also be used for siRNA delivery with acid-labile properties\textsuperscript{98}.

Polymer based pH sensitive liposomes have been approached with acid-degradable polymers or acid-labile cross-linkers (\textit{e.g.} ortho esters\textsuperscript{99}, poly (ketalts)\textsuperscript{100}, poly (acetals)\textsuperscript{101}, \textit{etc.}). These acid-labile liposomes\textsuperscript{62, 102}, taking advantages of the weakly acidic environment in tumor area, have been widely investigated by applying acid-labile lipid-
polymers such as vinyl ether PEG-DOP\textsuperscript{102b, 102e} and gene\textsuperscript{102d, 103} delivery. Such acid-labile liposomes are designed to be stable at pH 7.4, which is the pH value of physiological condition, and to be unstable in acidic environments nearby the tumor tissues, triggering the instant release of loaded therapeutic agents.

Poly (acrylic acid), due to its water solubility, low price and easy functionalization, has been widely used in the preparation of amphiphilic copolymers\textsuperscript{104} or lipid-polymer\textsuperscript{41a, 97} for drug delivery. Chitosan/PAA copolymers have been prepared to form nanoparticles since 2002 for a controlled drug delivery.\textsuperscript{105} In 2007, chitosan/PAA hollow nanospheres were reported with the same group.\textsuperscript{106} PNIPAm/PAA copolymers modified liposomes were approved to be thermosensitive for trigger release of doxorubicin.\textsuperscript{107} DSPE-PAA lipid polymer has been approved to be able to stabilize liposomes.\textsuperscript{41a} And the interaction between PAA and doxorubicin has also been studied.\textsuperscript{108}

Lipid polymers (lipid-PEG\textsuperscript{102b}, lipid-PNIPAm\textsuperscript{42d, 109}, lipid-PAA\textsuperscript{41a, 98}, etc.) have been reported to stabilize or functionalize the liposomes. In most cases, the polymer chain is anchored into the lipid bilayers with one hydrophobic “lipid tail”, while its hydrophilic part extended into the aqueous solution. Such linear lipid polymer is usually prepared by linking lipid molecules to the polymer with a reactive group (-OH, -NH\textsubscript{2}, -COOH, etc.) at least at one end. Suffering with the low yield limited by the length of polymer chain, researchers are always seeking for better methods for the preparation of lipid polymers. In fact, some advanced procedures, where the lipid modified initiators were synthesized as initiator for either free radical polymerization\textsuperscript{110} or controlled radical polymerization\textsuperscript{111}, have been proved to obtain high yields of desired lipid polymers. In 2008, Maël et al first reported the preparation of well-defined lipid polymer by RAFT polymerization with
lipid-chain transfer agent (CTA)\textsuperscript{112}. With this similar method, we have recently prepared cholesterol-PNIPAm lipid polymers\textsuperscript{42d}.

Herein, we are reporting an acid-triggered drug delivery system of lipid-polymer modified DPPC-Cholesterol liposomes with acid-labile cross-linkers\textsuperscript{101}. Based on the design of cholesterol terminated polymer\textsuperscript{97}, we have prepared novel lipid-polymer (DOPE-PAA) by RAFT polymerization with two distinct polymer architectures, namely linear DOPE-PAA (LDP) and hyperbranched DOPE-PAA (HDP) respectively. Further modification of the liposome surface with an acid-labile cross-linker at a low ratio (~10\% of carboxylic groups), shows an instant acid-triggered release. With highly cross-linked PAA backbones, HDP liposomes are more stable than LDP; while LDP liposomes have higher DOX release efficiency. Moreover, PAA liposomes show high DOX loading efficiency (40-50\%). The liposomes are further modified with folic acid for cancer targeting.
4.2 Experimental and Materials

4.2.1 Materials

Benzene, \( p \)-toluenesulfonic acid, chloroform, dichloromethane (DCM), \( n \)-hexanes, ethyl acetate (EtOAc), methanol (MeOH), dimethylsulfoxide (DMSO), triethylamine (TEA), methoxypentene, \( N \)- (2- hydroxyethyl) phthalimide, azobisisobutyronitrile (AIBN), doxorubicin (DOX), tetrahydrofuran (THF), 1-haxaneamine, acrylic acid, bis-acrylamide, isopropanol, \( N, N' \)-dicyclohexylcarbodiimide (DCC), 1-Ethyl-3-(3-dimethylaminopropyl)
carbodiimide (EDC), N-hydroxysuccinimide (NHS), folic acid, ethylenediamine, 1-dodecanethiol, acetone, tricaprylylmethylammounium chloride, sodium hydroxide, sodium citrate, citric acid, sodium chloride (NaCl), hydrochloride (HCl), carbon disulphide, Sephadex G-50 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were bought from Sigma-Aldrich; 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1, 2-dipalmitoyl-sn-glycero-3-phosphoethanolamine- N-(7-nitro-2-1, 3-benzoazadiazol-4-yl) (ammonium salt) (NBD-DPPE) were bought from Avanti polar lipid. All aqueous solutions were prepared by deionized water (Milli-Q, 18.2MΩ cm, 25 °C).

4.2.2 Characterization

All ¹H NMR spectra were obtained on a Bruker AV-III 600MHz NMR spectrometer. TEM images were acquired on FEI Tecnai T12 transmission electron microscope at 120 keV. Cryo-TEM images were obtained with FEI Titan Cryo Twin transmission electron microscope at 300 keV. Sonication was done with Hielscher Up100H probe ultrasonicator. Particle size distribution and zeta potential data were taken on Malvern NanoZS. Fluorescence spectrum was acquired on Varian Eclipse fluorescence spectrometer. UV-Vis data were obtained on Varian UV-Vis Cary 5000 spectrometer. CLSM images were got from Zeiss LSM 710 inverted microscope.
Scheme 4-2 Schematic illustrations of the synthesis process of cross-linker (4-2), linear and hyperbranched DOPE-PAA lipid-polymers (4-6, 7) and NH2-folate (4-8).

4.2.3 Methods

2, 2’-(propane-2, 2-diylbis(oxy))bis(ethan-1-amine) (4-2).

The acid labile cross-linker (4-2) was prepared according to the procedure according to the literature\textsuperscript{101}, with a yield of 65%.

S- 1- dodecyl- S-(α, α’-dimethyl- α″-acetic acid) trithiocarbonate (Chain transfer agent, 4-3)
Chain transfer agent (CTA) was prepared following literature. And the product residue was recrystallized for twice to get the pure CTA (30% yield).

**DOPE-CTA (4-5).**

Recrystallized CTA (364 mg, 1 mmol) and NHS (138 mg, 1.2 mmol) was dissolved in 10 mL DCM and stirred for 10 min. EDC (523 µL, 3 mmol) was then added drop wise. The reaction residue was stirred for overnight at room temperature. The product was purified by a flash column to get yellow solid. (Compound 4-4, 85 % yield). $^1$H NMR (600 MHz, CDCl$_3$) $^1$H NMR (600 MHz, CDCl$_3$) δ 3.21 (t, $J = 7.4$ Hz, 1H), 2.71 (s, 2H), 1.77 (s, 3H), 1.62 – 1.54 (m, 1H), 1.44 (s, 2H), 1.28 (dd, $J = 14.3$, 6.7 Hz, 1H), 1.17 (d, $J = 19.5$ Hz, 8H), 0.78 (t, $J = 6.7$ Hz, 1H).

Compound 4-4 (60 mg, 0.135 mmol) and DOPE (744 mg, 0.134 mmol) were dissolved in 7 mL CHCl$_3$. The mixture was heated at 30 °C with stirring, and TEA (1 mL, 27 mg/mL, CHCl$_3$) was added for 3 times every 20 min. The reaction was stirred for overnight. And then the product was purified with a flash column with 50% hexane/EtOAc and then 100% methanol to give a yellow residue (95% yield). $^1$H NMR (600 MHz, CDCl$_3$) δ 5.29 (d, $J = 67.2$ Hz, 1H), 4.39 (d, $J = 11.8$ Hz, 1H), 4.17 (d, $J = 6.2$ Hz, 1H), 3.96 (d, $J = 17.3$ Hz, 1H), 3.48 (s, 1H), 3.28 (dt, $J = 13.6$, 7.2 Hz, 1H), 3.06 (s, 1H), 2.81 (s, 1H), 2.30 (d, $J = 7.4$ Hz, 1H), 2.01 (d, $J = 4.9$ Hz, 2H), 1.94 (d, $J = 12.4$ Hz, 1H), 1.87 (s, 1H), 1.85 – 1.50 (m, 5H), 1.45 – 1.18 (m, 17H), 0.88 (t, $J = 6.2$ Hz, 2H).

**Linear DOPE-PAA (4-6).**

AIBN solution (820 µL, 10 mg/mL in THF, 0.05 mmol) was added to a dry schlenk flask, and then dried with N$_2$. Recrystallized acrylic acid (720 mg, 10 mmol) and DOPE-CTA
(103 mg, 0.1 mmol) were dissolved in 2 mL dry DMF with nitrogen purged for 30 min. After freezing-thawing cycle for 5 times to remove extra oxygen, the reactor was heated at 75°C with stirring for 8 hrs. The product was precipitated with over mount of diethyl ether. And then several drops of 1-hexanamine was added and stirred for 24 hrs to remove extra CTA. The final product was white solid. (90 % yield).

Hyperbranched DOPE-PAA (4-7).

Hyperbranched DOPE-PAA was synthesized with similar procedure. The difference is that bis-acrylamide (3.08 mg, 0.02 mmol) was added to the mixture in the beginning. The product was also white solid. (93% yield)

NH₂-folate (4-8).

The amination of folic acid was achieved following literature¹¹³.

Preparation of DOPE-PAA liposomes.

DOPE-PAA liposomes were prepared with a modified Bangham method. DPPC solution (CHCl₃, 10 mg/mL, 1000 μL), cholesterol solution (CHCl₃, 10 mg/mL, 250 μL) and DOPE-PAA solution (MeOH, 10 mg/mL, 40 μL) were mixed in a 3 mL vial. Evaporate the solvents slowly with nitrogen flow. The vials were then kept under high vacuum for two hours. Rehydrate the phospholipids film with HEPES buffer solution (pH=7.4, 1000 μL) or doxorubicin solution (1 mg/mL, 1000 μL) at room temperature for 1 hour, and then the residue was mixed by vortex. After the freezing-thawing cycle for five times with liquid nitrogen and water, the residue was sonicated with probe-sonicator in ice-water blend for 20 min (cycle: 80%, amplitude: 80%). Centrifuged at a low speed of 1000
rpm for 1 min, the residue from the probe and large volume liposomes were removed. For the DOX loaded liposomes, Sephadex G-50 column (2 mL column for 800 µL liposomes, 0.9% NaCl) was used to remove the free drug molecules in the solution. The liposomes were left in fridge at 4 °C for overnight.

Preparation of liposomes with folate targeting.

Compound 4-8 (48.3 µL, 10 mg/mL, DMSO) was and NHS (2.3 µL, 100 mg/mL) were added to 500 µL as-prepared liposomes with stirring. EDC (2 µL, 0.5 g/mL) was then added drop wise. The mixture was stirred for overnight, and then dialyzed for one day to remove the unreacted molecules/side products.

Preparation of cross-linked liposomes.

The liposomes residue was divided into two parts for both acid labile and non-acid labile cross link for comparison. Compound 2 (8.1 µL, 20 mg/mL, 5% cross-linking) and NHS (2.3 µL, 100 mg/mL) were added to the as-prepared liposomes suspend (500 µL) with stirring. After 10 min, EDC (2 µL, 0.5 g/mL) was added to the reaction mixture, and the reaction was stirred for overnight. Then the liposomes residue was dialyzed with dialysis bag ($M_w=8,000$) for one day to remove the side products and un-reacted molecules. As a control, non-cross-linked liposomes were prepared by the same process with 1, 6-hexanediame (5.8 µL, 20 mg/mL, 10% cross-linking) instead of compound 2. The as-prepared acid labile liposomes were used for further characterizations.

Load and release.

The load of DOX was fulfilled by hydrating lipid monolayers with DOX solution
The loading efficiency is calculated by the intensity of fluorescence of drugs ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 590$ nm).

In order to test the release of liposomes, the DOX loaded liposomes were diluted with citrate/citric acid buffer by 100 times and then time-dependent release profiles were acquired with fluorescence spectrometer in 60 min at different pH values (pH= 4 ~8).

The DOX loading efficiency of linear PAA liposomes is 40~45% and the hyperbranched is 45-50%.

**Cell viability and MTT assay for DOX loaded liposomes.**

MTT assay was used to measure the *in vitro* cytotoxicity of HDP-LA, HDP-LN, LDP-LA and HDP-LN. Hela cells were seeded in 96-well plates at a density of 5000 cells per well. Cells were incubated at 37 °C under 5% CO$_2$ for 24 hrs. After that, the four types of liposome samples of various concentrations (0.00001-100 µg/mL) were separately added to the cells wells. Cells were incubated further for 24 hrs. MTT solution were added to the wells and incubated in darkness for another 4hrs. Then, the culture medium was discarded and 150 µL of DMSO were added to each well to dissolve the formazan crystals. The absorbance of the resultant solution was measured at 490 nm using a microplate spectrophotometer.

The cytotoxicity of DOX loaded liposomes was also evaluated by MTT assay. The same procedure as cell viability was followed, but after washed by DPBS buffer, the cells were incubated in the culture media with liposomes for 24 hrs at 37 °C.

**Cellular uptake**
Hella cells were grown in MEM alpha and incubated for 24 hrs at 37°C under 5% CO₂ before being transferred to a Lab-Tek chambered spitted to 8 wells. 35,000 cells per well were added and incubated for 24 hrs. The solution of four liposomes (HDP-LA, HDP-LN, LDP-LA and HDP-LN) was separately added to Hela cells at the concentration of 50 µg/mL and incubated for different periods (5 min, 15 min, 30 min and 60 min). After incubation, cells were washed by PBS, stained by Hoechst dye, and incubated for 10 min. After fixation, cells were washed again by PBS and tested by confocal microscopy and TEM.

**HeLa cell targeting**

HeLa cells were grown for 24 hrs at 37 °C under 5% CO₂. Folate-liposomes/non-folate liposomes were added separately to HeLa cells at the same condition and time series as the cellular uptake testing. After washing, staining and fixation process, the cells were ready for confocal microscopy. MTT assays were also applied for cell targeting as described above.

**4.3 Results and Discussions**

In this study, both the linear DOPE-PAA (LDP) and hyperbranched DOPE-PAA (HDP) lipid polymers were prepared with RAFT polymerization. The chain transfer agent (4-3, Scheme 4-1) was synthesized following literature. And the lipid-CTA was prepared with a two-step reaction: (1) CTA reacted with NHS with the presence of EDC; (2) metathesis reaction between CTA-NHS and DOPE. 1H NMR data were shown in appendix. The molecular weights of the lipid polymers were determined by NMR ($M_w-LDP=\sim7k$, $M_w-HDP=\sim24k$).
Liposomes were prepared with a modified Bangham method (DPPC: Chol: LDP/HDP=100: 25: 4, in weight). Empty liposomes were prepared with HEPES buffer (pH=7.4) and DOX loaded liposomes with DOX solution (1 mg/mL, water). Negatively stained TEM images (Figure 4-1) of HDP and LDP liposomes were obtained from EFI Tecnai T12 electron-microscope. From the TEM images, the morphology of the cross-linked liposomes was determined as uniform spheres. And the diameters for all the liposomes were 180~220 nm.

![TEM images](image)

Figure 4-1. Negatively stained TEM images of A. LDP and B. HDP liposomes. Both images show good and relatively uniform tomography of liposomes.

As reported in literature, 50% cross-linking of PAA modified liposomes will lead to stable polymer-caged liposomes. Our liposomal systems were designed to be sensitive to acid trigger with a fast release; Hence, the cross-linking was done after keeping the liposomes dispersion in fridge for overnight with 5% of total carboxylic groups (~10% of outside surface COOH). The acid-labile cross-linking was fulfilled by applying compound 4-2 and EDC/NHS for a condensation reaction to form the acid-
labile liposomes. Moreover, to make the result more convincing, the non-acid-labile liposomes with 1, 6- hexanediame as cross-linker were prepared for comparison. Cryo-TEM images were shown in Figure 4-2, A-D. The sizes of the liposomes were almost the same either before or after cross-linking. And after cross-linked with acid-labile linkers, the stability of both LDP and HDP liposomes were tested with a long-term size distribution test (Figure 4-3), and both were found to be stable after 30 days’ storage at 4°C under N₂ protection.
Figure 4-2. Cryo-TEM images of LDP and HDP liposomes cross-linked with either AL or NL cross-linkers at pH 7.4 (A-D) and 5 (E-H): A, E. LDP-AL, B, F. LDP-NL, C, G. HDP-AL and D, H. HDP. (Scale bar=200 nm)
The DOX loading efficiency was tested by UV-Vis spectrum as described. What shocked us is that the DOX loading efficiency of both LDP and HDP liposomes are surprisingly high, 40~50%. For comparison, the efficiency of liposomes without DOPE-PAA was tested and the result was around 6~7%. As the only difference between the two types of liposomes is with/without DOPE-PAA, PAA is supposed to be the reason of the largely-enhanced DOX loading efficiency. Yaroslavov, et al. reported in 2004 that doxorubicin and poly (acrylic acid) will form complexes via electrostatic and stacking interactions\cite{108b}, and from which we could indicate that in our case, similar DOX/PAA complexes were formed. To verify the interaction, zeta potential was tested (Figure 4-4) for LDP and HDP liposomes with and without doxorubicin loaded ($\zeta_{\text{HDP}} = -27.8 \text{ mV}$, $\zeta_{\text{HDP-DOX}} = -16.3 \text{ mV}$, $\zeta_{\text{LDP}} = -39.1 \text{ mV}$, $\zeta_{\text{LDP-DOX}} = -22.7 \text{ mV}$).
The surfaces of the LDP and HDP liposomes are both negatively charged due to the ionization of proton in PAA. Therefore the increase of zeta potential indicates that adsorption of DOX occurred on the surface of liposomes. To better explain the surficial adsorption of DOX and the consequential zeta potential change, a new test was performed where DPPC-Chol, LDP or HDP liposomes in HEPES buffer were directly mixed with DOX solution (final concentration is 1 mg/mL). Zeta potential was tested again to check the difference between the three samples (Figure 4-5). For DPPC-Chol liposomes (Figure 4-5, A), no obvious change of zeta potential was observed before and after mixed with DOX solution, while for the other two the value increased with almost the same amount as is shown in Figure 4-5. We can indicate from the two tests that the enhancement of zeta potential in LDP and HDP liposomes are because of the adding of
DOPE-PAA, and that electrostatic interaction between PAA and DOX occurred to form a complex. Moreover, LDP liposomes are even more negative than HDP, which can be attributed to that the architecture of hyperbranched backbone limited the ionization efficiency. Therefore the architecture of the two types of DOPE-PAA polymer may contribute to different formations of complexes. The linear PAA chain is flexible and easy to get folded to form the complex on the surface of either one vesicle or between vesicles. In contrast, for the hyperbranched PAA, the backbone is network and uneasy to get folded for forming the complex on the surface of one liposome, in which case complex would prefer to form between vesicles. The proof of inter-vesicle complex is that slight sediment was observed for both DOX-loaded liposomes after kept in the fridge for overnight; and after cross-linking no precipitation could be observed any more. The encapsulation efficiency of HDP and LDP liposomes are slightly different: LDP: 40~45%, HDP: 45~50%. What caused the divergence lies mainly in the backbone structure: HDP has a network backbone which could help to further stabilize the vesicles, which reduce the leaking of DOX; while LDP does not. In view of this difference, we also expected that there would be some difference in the acid-triggered release.
Figure 4-5. Zeta potential data of HDP liposomes and LDP liposomes with/without DOX loaded.

Stability is always one of the most essential points for liposomes. To test the stability of the acid-labile liposomes, size distribution data was taken for both HDP-AL and LDP-AL liposomes in one month (Figure 4-3). The size tended to be around the same during one month, indicating that the acid-labile liposomes are stable enough. Due to the deprotonation of PAA at low pH value, PAA chains tend to be hydrophobic and aggregating with hydrophobic lipid tails, which is the acid-induced shrink property of
DOPE-PAA (poly[(AAc)-co-(AAm)]-like copolymers). The cross-linked liposomes were expected to be shrunk at low pH values, as was shown in Figure 4-3 that the average diameter of LDP-AL liposomes turned from 180 to 140 nm (for HDP-AL, from 220 nm to 190 nm) after dealt with pH 5 buffers. Cryo-TEM images were obtained for acid-treated (pH=5) liposomes of both AL and NL cross-linking (Figure 4-2, E-H) for further verify the shrink property. Highly shrunk liposomes were observed in all the samples; however, in NL samples many integral liposomes could still be found, though not as much as AL.

The kinetics of DOX release (Figure 4-6, A-D) from the DOPE-PAA liposomes were obtained with fluorescence meter in citrate/citric acid buffers at different pH values (pH=4, 5, 6, 7 and 8). As expected, NL liposomes are quite stable at most pH values (pH= 5~8) with the release of at most 20% DOX after one hour incubation. AL samples are showing a fast and pH-depend release in acidic conditions: 69% and 78% DOX were release at pH 5 after half-an-hour incubation from LDP-AL and HDP-AL samples separately; while at pH 6, the release in the same period went down to around 38% and 62%, respectively. In contrast, the release of DOX was extremely limited to around 20% in neutral and slight basic aqueous solution, which is the same level of NL liposomes. The TEM images of liposomes at pH 5 intuitively demonstrate how the vesicles get damaged, suggesting that the acceleration of DOX release should be attributed to the vesicle degradation. When pH got down to 4, most DOX were release in both AL and NL samples, which is the result of the well-known “shrink” property of DOPE-PAA. Unlike the slow release rate in literature, in our case, all the release process were completed within 30 min, leading to a fast and efficient controlled DOX release. The pH
depended release profile was also shown in Figure 4-6, E. We can easily tell that all liposomes are stable at pH 7 and 8, and that the release of DOX was accelerated from AL liposomes in weak acidic solution.

Comparing with HDP-AL liposomes, LDP-AL liposomes have a higher release efficiency of 78% in slightly acidic condition, which suggests that HDP-AL liposomes are more stable than the linear ones. According to the structural properties of polymer backbones, it is easy to tell that HDP-AL liposomes have higher “actual” cross-linking rate than LDP. And such feature would help both keep more DOX in the vesicles (HDP liposomes have a better loading efficiency than LDP) and slow down the release rate. At pH 5, even if all the AL cross-linkers have been degraded, HDP could still serve as network shelters to stabilize the vesicles.
Figure 4-6. Time dependent release profiles of LDP and HDP liposomes cross-linked with either AL or NL cross-linkers: A. HDP-NL, B. LDP-NL, C. HDP-AL, D. LDP-AL, and E. pH dependent release profiles of DOPE-PAA liposomes

Cell viability was evaluated with MTT assays. Figure 4-7 shows that the liposomes were safe at even high concentration (1 mg/mL), indicating a promising biocompatibility. And
for all the following in vitro tests, we were using the liposome-medium mixture with a total lipid concentration at $10^{-3}$ mg/ml.

Figure 4-7. Cell viability of LDP and HDP liposomes cross-linked with either acid-labile or non-acid-labile cross-linkers incubated with HeLa cells at 37 °C for 24 hrs: A. HDP-NL, B. LDP-NL, C. HDP-AL, D. LDP-AL. The results indicate that both HDP and LDP liposomes are safe to HeLa cells at even high concentration (1 mg/mL).

The cellular localization of DOX-loaded LDP and HDP liposomes was tested by Zeiss LSM 710 inverted confocal microscope (Figure 4-8, 11). All the tests were done with the same setup. The NBD labelled liposomes (NBD-DOPE: DPPC: Chol: DOPE-PAA= 0.2: 100: 25: 4, in weight) with DOX loaded were prepared and then diluted with cell culture medium. Incubated with HeLa cells at 37 °C for different time series, the cellular uptake
for DOX loaded liposomes was monitored. For all the samples, when incubated for 60 min (Figure 4-8, 11, A4-B4) with HeLa cells, the DOX were all transferred into nuclei, according to the DOX fluorescence intensity. And when incubated for 5 min (Figure 4-8, 9, A1-B1), no obvious release was observed in any sample. However, after incubated for 15 min, difference appeared between HDP-AL (Figure 4-9, A2) and HDP-NL (Figure 4-9, B2) that more DOX was released in HDP-AL, which revealed that a faster release occurred for the acid-labile liposomes due to the low pH environment of the lysosomes formed during the cellular uptake of liposomes. Similar difference could also be observed in the following 30 min figures (Figure 4-8, A3 and B3). Also for LDP liposomes, similar discrimination could be discovered when incubated for 15 min (Figure 4-8, A2 and B2) and 30 min (Figure 4-8, A3 and B3). The visualized data intuitively support the pH dependent release results.
Figure 4-8. CLSM images (Hoechst, NBD, DOX, IC and merge) of LDP-AL (A1-A4) and LDP-NL (B1-B4) cross-linkers incubated with HeLa cells at 37 °C for different time series (1-4: 5 min, 15 min, 30 min and 1 hr) (Scale bar= 100 μm).
Figure 4-9. CLSM images (Hoechst, NBD, DOX, IC and merge) of HDP-AL (A1-A4), HDP-NL (B1-B4) cross-linkers incubated with HeLa cells at 37 °C for different time series (1-4: 5 min, 15 min, 30 min and 1 hr) (Scale bar= 100 μm).
Figure 4-10. TEM images (negatively stained of cellular uptake of DOPE-PAA liposomes incubated with HeLa cells for 1 hr: A-C, HDP-AL liposomes; D-F, LDP-AL liposomes (Scale bar: A, C. 1 μm, B, E. 500 nm, C, F. 200 nm).

The detailed localization of cellular uptake was obtained by TEM measurement with high resolution. HDP-AL (Figure 4-10, A-C) and LDP-AL (Figure 4-10, D-F) liposomes were incubated with HeLa cells for 12 hours to get them fully taken up by the cells. As depicted in Figure 4-10, in both HDP-AL and LDP-AL samples multiple pseudopodia of plasma membrane were formed for the uptake of liposomes, indicating that the vesicles might enter cell via endocytosis rather than fusion with cell membrane. NBD/RhB assays (Figure 4-11) were also applied to further certify the interaction between liposomes and cell membrane. And the result indicated that no membrane fusion occurred, reconfirm the
observation that endocytosis is the pathway for liposomes to get into HeLa cells. Moreover, liposomes were also found in the lysosomes (Figure 4-10, A, E and F), where the AL liposomes can function as expected to release the payload, killing the cancer cells effectively.

Figure 4-11. RhB/NBD assays of LDP-AL (A) and HDP-AL (B) liposomes at pH 7.4 and 5 incubated at 37 °C for 10 min (NBD/RhB-labeled liposomes: pure liposomes=1:9).
To further verify the release difference, *in vitro* cytotoxicity of DOX loaded liposomes was also evaluated by MTT assay. The same procedure as cell viability was followed, but after washing by DPBS buffer, the cells were incubated in the culture media with liposomes for 2 hrs at 37 °C. \( IC_{50} \) of the four liposomes were calculated (Figure 4-12):

\[
\begin{align*}
IC_{50-DOX} &= 12.36 \mu g/mL, \\
IC_{50-LDP-AL} &= 2.04 \mu g/mL, \\
IC_{50-LDP-NL} &= 42.23 \mu g/mL, \\
IC_{50-HDP-AL} &= 36.96 \mu g/mL, \\
IC_{50-HDP-NL} &= 74.08 \mu g/mL.
\end{align*}
\]

The \( IC_{50} \) values of various liposomes, indicated the toxicity order of LDP-AL < DOX < HDP-AL < LDP-NL < HDP-NL. The results showed that AL liposomes have an \( IC_{50} \) much lower than NL liposomes, which is due to the highly effective drug delivery in AL liposomes. Meanwhile, the difference between LDP-AL and HDP-AL verifies further the different releasing rates as mentioned in Figure 4-12.

Figure 4-12. \( IC_{50} \) data of DOX and DOX loaded DOPE-PAA liposomes incubated with HeLa cells for 24 hrs at 37 °C.
The PAA-liposomes were further modified with folic acid to improve targeting ability, and a set-time cellular uptake (5 min) test was evaluated with CLSM. NBD labelled PAA-liposomes was first modified with NH₂-folate by condensation reaction with EDC and NHS, and then cross-linked with AL cross-linkers. After purification with dialysis for one day, the folate modified liposomes were incubated with HeLa cells (10⁻³ mg/mL) for 5 min to verify the localization of liposomes. From Figure 4-13, the cell number of both folic acid modified and naked liposomes was close; however, more liposomes could be found in Figure 4-13-B taken up by the cells than in Figure 4-13-E, which means that folic acid modified liposomes have a significant cancer targeting property. For further verification, the $IC_{50}$ of folate LDP-AL liposomes was also tested with MTT assays (Figure 4-12, $IC_{50}$-LDP-AL-F=1.51 µg/mL). With a lower $IC_{50}$ than PAA liposomes, folate liposomes were quantitatively verified to be cancer targeting.

Figure 4-13. CLSM images (Hoechst, NBD and merged) of liposomes incubated with HeLa cells for 5 min: A-C. folated LDP-AL liposomes; D-F. non-folated LDP-AL liposomes.
4.4 Conclusions

In this study, acid-labile liposomes have been prepared with two types of DOPE-PAA lipid polymers (linear and hyperbranched) by condensation reactions with AL cross-linkers. Prepared with passive loading, the DOPE-PAA liposomes have a high loading efficiency (≈50%) of doxorubicin, which is due to the complex formed between DOX and PAA polymers on liposomal surface. Comparing with literature (≈50% crosslinking of total COOH), our DOPE-PAA liposomes (≈5% crosslinking of total COOH) showed a fast release within 15 min in acidic aqueous environment. HDP liposomes are more stable than LDP at pH 6 and 5, which is due to the difference in the two polymer backbone structures. Hyperbranched PAA has a higher crosslinking degree than linear PAA, which helps to stabilize the liposomal structures. Moreover, the PAA branches on liposomal surface were further modified with folic acid for cancer targeting property. Therefore, our acid-labile PAA-liposomes are promising effective drug carriers, especially for cationic drugs, in clinical cancer treatment.
5 A Novel Dual Triggered Programmable Drug Delivery System: Au@SPIO Core-shell Nanoparticles Embedded Liposome Hybrids

5.1 Introduction

Liposome hybrids (NLHs) are very attractive for controlled drug delivery, biosensing and medical imaging. Moreover, compared to regular liposomes, NLHs inherit the responsive properties of the nanoparticles, such as imaging contrast or strong fluorescence label for real time tracking in vivo.

Baring a hydrophobic lipid bilayer shell (~5 nm) and hydrophilic core (usually around 100 nm), liposomes are one of the most investigated vesicles for drug delivery, not only due to the biocompatibility and ease of functionalization, but also because of their capability to delivery both hydrophilic cargo in the core and hydrophobic cargo between the lipid bilayers. Drugs can be released from liposomes above main transition temperature \( T_m \), around which the permeability of lipid bilayers gets intensively enhanced.

To circumvent the stimuli-triggered release, liposomes have been encapsulated with responsive nanoparticles such as AuNPs, SPIO, etc.. Such NLH systems are attractive mainly due to the increased biocompatibility of the hydrophobic nanoparticles and their potential use in triggered release. Gold nanoparticles (AuNPs) encapsulated liposomes have attraction great attention due to the shape-dependent and size-dependent
properties of AuNPs, especially for the photo-thermal effect.\textsuperscript{115b, 118} Christopher\textsuperscript{119} and his co-workers reported in 2012 the structural and thermal effects of AuNPs on the liposomes; in 2013, the concentration effect of AuNPs on the photo-triggered release was reported by Yinyan and his co-workers\textsuperscript{115b} in a reversible light-sensitive AuNPs-liposome hybrid system. Magnetoliposomes (MLs), first introduced in 1988\textsuperscript{120}, were the first nanoparticle-liposomes hybrids, and proved to be efficacious in magnetically triggered release.\textsuperscript{73} In 2010, Geoffrey and his co-workers\textsuperscript{121} reported in detail the concentration effects of SPIOs on the release. Due to the potential applications of SPIO in MRI, magnetoliposomes were considered as a promising MRI reagent.\textsuperscript{122}

Au@SPIO core-shell nanoparticles, which are also called magnetoplasmonic assembly\textsuperscript{123}, were reported to combine the advantages of both AuNPs (plasmonic properties: SERS)\textsuperscript{115c, 124} and SPIO nanoparticles (magnetic properties)\textsuperscript{125}. Such core-shell nanoparticles are particularly attractive mainly because of the potential plasmonic and MRI\textsuperscript{123a} imaging ability. District approaches have been reported in the recent years, and the nanoparticles’ size was ranged from 6 nm to 100 nm\textsuperscript{126}. However, no previous record has been reported to embed the Au@SPIO core-shell nanoparticles into liposomes for stimuli responsive drug delivery.

In this study, Au@SPIO core-shell nanoparticles were prepared with different Au/SPIO ratios, and Au@SPIO nanoparticles were embedded into lipid bilayers of liposomes to form ALHs (Scheme 5-1). The ALHs were proved to be both light and magnetic field responsive for drug delivery. Moreover, an ‘on-off’ type of release was found for light triggered release and an instant release was found in the magnetic field triggered release.
Having both plasmonic and magnetic properties of Au@SPIO, the system was showed great potential for both controllable drug delivery and cellular imaging.

Scheme 5-1. Schematic illustration of the preparation and the release process of NLHs

5.2 Experimental and Materials

5.2.1 Materials

1, 2-hexadecanediol, oleic acid, oleylamine, phenyl ether, ethanol, gold chloride, tetraoctylammonium bromide, iron (III) acetylacetonate, dodecanethiol, sodium borohydride, toluene, hexane, doxorubicin (DOX), chloroform are from Sigma-Aldrich. L-α-phosphatidylcholine (Egg-PC), 1,2-dipalmitoyl- sn-glycero-3-phosphoethanolamine- N- (7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-DPPE) are from Avanti lipid. All solutions were prepared by deionized water (Milli-Q, 18.2 MΩ cm, 25 °C).

5.2.2 Characterization

FTIR data were obtained on Thermo Nicolet 6700 FT-IR system. TEM images were acquired on FEI Tecnai T12 transmission electron microscope at 120 keV And FEI Titan
Cryo Twin transmission electron microscope at 300 keV. Liposomes were prepared with Hielscher Up100H probe ultrasonicator and Avanti Mini-Extruder (1000 μL). Light triggered release was completed with UVP Blak-Ray B-100YP UV inspection lamp. Fluorescence spectrum was acquired on Varian Eclipse fluorescence spectrometer. UV-Vis data were obtained on Varian UV-Vis Cary 5000 spectrometer. CLSM images were got from Zeiss LSM 710 inverted microscope.

5.2.3 Methods

**SPIO-4 nm**

Fe(acac)₃ (1 mmol), 1,2-hexadecanediol (5 mmol), oleic acid (3 mmol), oleylamine (3 mmol) and phenyl ether (10 mL) were mixed and stirred under a flow of nitrogen. The mixture was heated, with N₂ flow, to 200 °C with sand bath for 30 min, and then heated to reflux (300 °C) for another 30 min. After cooling to room temperature, ethanol (20 mL) was poured to the mixture for precipitation. The precipitate was then collected with centrifugation and then dissolved in hexane in the presence of oleic acid (~0.03 mL) and oleylamine (~0.03 mL). Centrifugation (4000 rpm, 5 min) was applied to remove any undispersed residue. SPIO was then precipitated with ethanol, centrifuged (4000 rpm, 5 min) to remove the solvent, and redispersed into hexane.

**AuNPs**

An aqueous solution of HAuCl₄ (30 ml, 30 mmol/L) was mixed with a tetraoctylammonium bromide solution (in toluene, 80 ml, 50 mmol/L). The two-phase mixture was vigorously stirred for 30 min until all the tetrachloroaurate was transferred into the organic layer. Meanwhile, NaBH₄ solution (25 mL, 0.4 mol/L) was prepared with ice bath. The organic layer was separated and then mixed with dodecanethiol (200 μL). The freshly prepared NaBH₄ solution was added to the mixture with stirring. After
stirring for 3 hrs the organic phase was separated and evaporated to 10 ml in a rotation evaporator. Mixed with 400 ml ethanol to remove excess thiol, the residue was then kept at -18 °C for overnight and the dark brown precipitate was then filtered off and washed with ethanol. Dissolved with toluene, the product was precipitated again with 400 ml ethanol and filtered. The final product was redispersed with toluene.

**SPIO/Au core-shell nanoparticles**\(^{129}\). 1 mL of AuNPs and SPIO nanoparticles in toluene with various ratios was placed in a reaction tube. The tube was then placed in a preheated oil bath at 149 °C for 1 hr. Temperature variation from this set point was limited to 1.5 °C. After the 1 hr thermal treatment, the reaction tube was allowed to cool down, and the particles were re-dispersed in chloroform.

**Au@SPIO-liposome hybrids.** ALHs were prepared with a modified Bangham method. Egg-PC solution (CHCl\(_3\), 10 mg/mL, 1000 µL) and Au@SPIO solution (CHCl\(_3\), 10 mg/mL) were mixed with different ratio in a 3 mL vial. The solvent was evaporated slowly with nitrogen flow. And then keep the vials under high vacuum for two hrs. Rehydrate the phospholipid/nanoparticle film with HEPES buffer solution (pH=7.4, 1000 µL) or doxorubicin solution (1 mg/mL, 1000 µL) at room temperature for 1 hr, and then the residue was mixed by vortex. The residue was then sonicated with probe-sonicator at 0 °C for 20 min (cycle: 80%, amplitude: 80%). Centrifuged at a low speed of 1000 rpm for 1 min, the residue from the probe and large volume liposomes were removed. The liposome suspension was then extruded with 200 nm membrane with Avanti Mini-extruder for 10 times and then with 100 nm membrane for 20 times. For the DOX loaded liposomes, Sephadex G-50 column (1 mL column for 200 µL liposomes, 0.9% NaCl) was used to remove the free drug molecules in the solution. The liposomes were left in fridge
at 4 °C for overnight.

5.3 Results and Discussions

![FTIR images of AuNPs, SPIO and Au@SPIO](image)

Figure 5-1. FTIR images of AuNPs, SPIO and Au@SPIO
Characterization of Au@SPIO and ALHs

AuNPs, SPIO and Au@SPIO were prepared following reported methods. The prepared nanoparticles were tested with FTIR (Figure 5-1) to analyze the possible changes on the particles before and after the thermal treatments, and Au@SPIO have the characteristic peak of both precursors. UV-Vis spectrum (Figure 5-2) was also applied to check the plasmatic properties of Au@SPIO. From the spectrum, Au@SPIO, whatever Au/Fe ratio it is, has good absorption peak at around 520 nm, which indicated that it is possible to have a thermal-photo property with a light around 500 nm. Due to the limited size range for nanoparticles to be embedded into lipid bilayers, the size distribution of AuNPs, SPIO
and Au@SPIO is very important in this study. The morphology of all the nanoparticles was observed with TEM (Figure 5-3). The particle sizes were from 3 to 6 nm, which is fit for the embedment. Telling from HR-TEM and EDX (Figure 5-4), we could re-confirm the morphology and the formation of Au@SPIO nanoparticles.

Figure 5-3. TEM images of A. AuNPs; B. SPIO and C. Au@SPIO

According to the UV-Vis spectrum, Au@SPIO (SPIO:Au=1:10, in weight) was used in ALHs preparations. ALHs were prepared with modified extrusion methods as described.
After being stored in the fridge overnight, the product was taken for negatively stained TEM (Figure 5-5), from which the diameter of ALHs could be easily measured (~100 nm). DOX loaded ALHs were further used for both light and magnetic field triggered release test.

Figure 5-4. HR-TEM and EDX of Au@SPIO
The light triggered release was fulfilled with UVP Black-Ray B-100YP UV inspection lamp, which has a wavelength of 543, 574 and 576 nm, very close to the wavelength of Au@SPIO absorption. DOX loaded ALHs were diluted with HEPES buffer (pH=7.2) in a fluorescence cell (3 mL). The covered cell was then placed under UV lamp for certain time exposure. Fluorescence spectrum ($\lambda_{ex}$=495 nm, $\lambda_{em}$=590 nm) was taken right after irradiation. ALHs with different nanoparticle loading efficiency (2, 5 and 10%) was applied in the release testing, and as expected, all of the three samples showed significant DOX release with 1 min or 5 min exposure to green light (Figure 5-6). For comparison,
10% loaded ALHs have the highest release efficiency than the other two, due to the high particle loading rate.

Figure 5-6. Light triggered DOX release of ALHs with different loading amount of Au@SPIO

Meanwhile, magnetic field triggered release was also tested with hyperthermia machine by fluorescence spectrum (Figure 5-7). ALHs (2, 5 and 10% Au@SPIO loaded) were diluted with the same ratio as described above, and then put into the center of the coil of hyperthermia for heating. The heating last for 30, 60 or 90 seconds each time, separately, in accord with the high heating efficiency of hyperthermia. After a total heating time of 360s, the release of DOX was almost completed for all three samples. And the high
loading efficiency of Au@SPIO also leads to a faster release, which is the same with light triggered release. However, due to the high energy of hyperthermia, the release time for magnetism triggered release is much shorter than that of light, which indicated that we can easily control the release rate for distinct case with different triggers.

Figure 5-7. Magnetism triggered DOX release of ALHs with different loading amount of Au@SPIO nanoparticles

From literature\textsuperscript{115b}, we got the news that AuNPs embedded liposomes could show an “on-and-off” type release, which makes the system more controllable. It will be interesting that ALHs could bear with the same advantage. Hence, we repeated the light triggered release with minor modification. After test the fluorescence spectrum of each exposure to
light, we waited the same time and then tested the fluorescence again. Here we got the “on-and-off” release results (Figure 5-8). After each heating and cooling cycle, the DOX release remained almost in the same level, which means that the photothermal process interrupts the lipid bilayer rather than destroy the liposome structure. Comparing with the current UV irradiated release of liposomes, the green light triggered ALHs release could penetrate deeper into the skin and be much safer for human beings.

Figure 5-8. “On-and-off” DOX release of ALHs with different loading amount of Au@SPIO with light trigger.

Benefited with the improvement of releasing test, the magnetism triggered release was also looked over. Due the high energy transforming efficiency of hyperthermia, the
possibility that liposomes were destroyed with the heating was considered. And the testing was repeated in a different way. In this case, after heated for 1 min, the sample was placed to the fluorescence spectrum meter for time dependent scanning. An instant release was revealed from the results (Figure 5-9); as after heated for 1 min, constant release was observed for all the samples lasting for half an hour, which implies that irreversible structure change of the liposomes occurred during the one-minute heating. And such instant release style supplied us with another potential controllable drug delivery pathway for ALH systems.

Figure 5-9. Instant DOX release of ALHs with different loading amount of Au@SPIO with magnetism trigger.
Cell viability was assessed by MTT assays by incubating HeLa cells with ALHs at different concentrations ($10^{-5}$ ~100 μg/mL). As indicated in Figure 5-10, ALHs are non-toxic at a concentration lower than 1μg/mL. For all the following cellular testing with HeLa cells, the concentration of ALHs was controlled below 1 μg/mL.

![Graph showing cell viability](image)

Figure 5-10. Cell viability of ALHs incubated with HeLa cells for 24 hrs.

In vitro light triggered DOX release of ALHs with HeLa cells was tested with Zeiss LSM 710 inverted confocal microscope (Figure 5-11). Egg-PC liposomes and ALHs were decorated with NBD-PE (1%, total lipid) and loaded with DOX. After incubating ALHs with HeLa cells for 1 hr, the plates were placed under the UV lamp and exposed to green light for 3 min. As shown in Figure 5-11, for both Egg-PC liposomes (A-D) and ALHs
(E-F), liposomes were taken up by cells after 1 hr incubation and DOX was released and transferred into nuclei due to the fluorescence label. However, compared with Egg-PC liposomes, ALHs has a more intensive DOX fluorescence, which means that more DOX was released after being exposed to light. These results re-confirm the light triggered DOX release of ALHs.

Figure 5-11. CLSM images (left to right: DAPI, NBD, DOX and merged) of A-D. DOX loaded Egg-PC liposomes incubated with HeLa cells for 1hrs with 5 min light exposure and E-H. DOX loaded ALHs incubated with HeLa cells for 1hrs with 3 min light exposure.

5.4 Conclusions

A novel dual responsive drug delivery vesicle was prepared by embedding hydrophobic Au@SPIO core-shell nanoparticles into Egg-PC liposomes with Avanti mini extruder. This ALH system has both light triggered due to the presence of gold nanoshell and magnetically triggered release caused by magnetic thermal functions originated from SPIOs. The range of the photo thermal process is within visible light (~ 520 nm) rather
than the reported UV light, which makes our system much safer to the human body. “On-and-oOff” release and instant release have been proven to occur in the system for the light and magnetism triggered release, respectively. *In vitro* data showed that ALHs were non-cytotoxic to cells, which indicated a significant potential in the cancer treatment applications for this dual-control drug delivery system.

Figure 5-12. MRI image for ALHs with control of water and liposomes.

**5.5 Future Plans**

As is described in the introduction, Au@SPIO has great potential in cellular imaging for both living cell SERS and magnetic resonance imaging. We can deduce that ALHs, which are much more biocompatible, would bear the same cellular imaging properties. In the future, it will be interesting to adjust the ratio between AuNPs and SPIO in ALHs for
a good MRI property, and try to find a proper loading concentration of Au@SPIO in ALHs for the SERS.
6 Conclusions

Nanocarriers for drug delivery have been developed for decades, such as micelles, dendrimers, liposomes, nanospheres and nanocapsules. Since the first formation of artificial lipid vesicles, liposomes have been the most common and well investigated. However, instability and lack of controllability have been regarded as the major drawbacks of liposomes. This work is aimed at (1). Enhancing the stability of liposomes via lipid/cholesterol polymer protection on liposomal surface; (2). Controlling the release of liposomal contents with either stimuli sensitive lipid polymer modification or loading responsive nanoparticles. Thermosensitive, acid-labile and dual responsive (light and magnetism triggered) liposomes have been prepared for controllable drug delivery, and different stimuli-responsive ligands were induced for various projects.

In chapter 3 and 4, RAFT polymerization was successfully utilized for preparing lipid polymers (Chol-PNIPAm and DOPE-PAA), and lipid-chain transfer agents (Chol-CTA and DOPE-CTA) were proved to be effective in the preparation process. The success of preparing different lipid polymers indicate that RAFT polymerization with different lipid-CTAs could be applied into the preparation of more stimuli responsive lipid polymers for modifying liposomes.

In chapter 3, we design cholesterol-PNIPAm lipid polymer for thermosensitive liposomes. However, considering the structure of cholesterol, the –OH group will be occupied after modification which can lead to the weakening of H-bonds between cholesterol polymer and lipid molecules, bringing instability to liposomes vesicles. To enhance the stability of liposomes, cholesterol was pre-modified with TEG so that the strength of H-bonds
between cholesterol polymer and lipid molecules is not affected a lot. Thermosensitive liposomes was prepared with Chol-PNIPAm oligomers ($M_w \sim 2,000$) and verified to be effective. In this project, the aggregation of low $M_w$ PNIPAm was approved to be strong enough to trigger release in liposomes, conducing to cut down the dose of PNIPAm, which is originally cytotoxic to human bodies. Comparing with chol-PNIPAm with different backbone structures (main-chain/side-chain), distinct release mechanisms were proposed and proved as “nail effect” and “comb effect”, separately.

One the other hand, in chapter 4, intermolecular cross-linking was applied to stabilize the vesicle structure via condensation reaction between diamine and PAA chains following the idea of Sonbinh, *et al*. However, when we look through their design, the OH group on cholesterol was replaced by O-C following with phenyl group, which could cause instability of liposomes (by weakening H-bonds) after cholesterol-PAA was anchored. To improve it, we have designed a novel DOPE-PAA lipid polymer with a phosphate linkage by which the lipid polymer could stay stable in lipid bilayer. Moreover, a low crosslinking ratio (~5% of total COOH) was applied in liposomal surface modification, which led to a faster release (15 min) than that reported in literature (50% crosslinking, 30 hrs release). Comparing to linear DOPE-PAA, hyperbranched lipid polymers with the same crosslinking ration could further stabilize liposomes in acidic conditions. Finally we could conclude that liposomal stability improves with cross-linking ratio. PAA-DOX complex was also proved to be formed by electrostatic interaction with zeta potential testing, which significantly improves the loading efficiency of DOX from 10% to 50%. The formation of DOX-PAA suggests a potential pathway for improving encapsulation efficiency of ionized chemotherapy drugs.
Liposomes could be loaded with both hydrophilic and hydrophobic cargos, inside the aqueous core or between the lipid bilayers, respectively. In chapter 5, we developed a novel dual responsive system loaded with both hydrophilic drugs and hydrophobic stimuli-responsive nanoparticles, taking advantages of the large hydrophilic volume and the direct interaction between nanoparticle and lipids. Au@SPIO core-shell nanoparticles was embedded into lipid bilayers so as to induced light triggered contributed with photothermal process (visible light) by gold nanoshell and magnetism triggered release caused by magnetic thermal functions originated from SPIOs. “On-and-off” release (photothermal) and instant release (magnetic thermal) were further approved to occur in the system for the light and magnetism triggered release, respectively, which made the system more controllable. Moreover, MRI (SPIO) and SERS (Au) will be further studied for both in vitro and in vivo imaging.
REFERENCES


75. Liao, Z.; Wang, H.; Lv, R.; Zhao, P.; Sun, X.; Wang, S.; Su, W.; Niu, R.; Chang, J., Polymeric Liposomes-Coated Superparamagnetic Iron Oxide Nanoparticles as


Stabilization of Lipid/Polymer Particle Assemblies. *Macromolecules* **2008**, *41* (22), 8346-8353.


122. (a) Zhang, L.; Zhou, H.; Belzile, O.; Thorpe, P.; Zhao, D., Phosphatidylserine-targeted bimodal liposomal nanoparticles for in vivo imaging of breast cancer in mice. *Journal of Controlled Release* 2014, 183 (0), 114-123; (b) Fan, C.-H.; Ting, C.-Y.; Lin,


126. (a) Xuan, S.; Wang, Y.-X. J.; Yu, J. C.; Leung, K. C.-F., Preparation, Characterization, and Catalytic Activity of Core/Shell Fe3O4@Polyaniline@Au


Figure A-1. NMR of cholesterol-OTs
Figure A-2. NMR of cholesterol-TEG
Figure A-3. NMR of cholesterol monomer.
Figure A-4. NMR of cholesterol-CTA.
Figure A-5. NMR of cholesterol-biotin.
Figure A-6. NMR of chain transfer agent.
Figure A-7. NMR of MCNO.
Figure A-8. NMR of SCNO.
Figure A-9. NMR of MCNO-hw.
Figure A-10. NMR of acid-labile cross-linker-step 1.
Figure A-11: NMR of acid-labile cross-linker.
Figure A-12. NMR of CTA-NHS.
Figure A-13. NMR of CTA-DOPE.
Figure A-14. NMR of linear DOPE-PAA.
Figure A-15. NMR of hyperbranched DOPE-PAA.