9-11-2015

Interaction between Au25 Monolayer Protected Clusters with Lipid Nanodiscs

Yue Bao

University of Connecticut - Storrs, nkby@outlook.com

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Interaction between Au$_{25}$ Monolayer Protected Clusters with Lipid Nanodiscs

Yue Bao

B.S., Nankai University, 2013

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

At the

University of Connecticut

2015
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2015
APPROVAL PAGE

Masters of Science Thesis

Interaction between Au25 Monolayer Protected Clusters with Lipid Nanodiscs

Presented by
Yue Bao, B.S.

Major Advisor________________________________________________
Dr. Mu-Ping Nieh

Associate Advisor_______________________________________________
Dr. Elena Dormidontova

Associate Advisor______________________________________________
Dr. Kazunori Hoshino

University of Connecticut
2015
Acknowledgements

This work could not have been accomplished without the help of many talented people. I want to thank my major advisor, Dr. Mu-Ping Nieh for the training process of both thinking and experiment, which are of huge benefit to my future research career. I really appreciate the patient guidance from Dr. Nieh and I learnt a lot. Thanks to our collaborators Dr. Flavio Maran for supplying us the gold nanoparticles, Dr. Vijay T. John for providing Cryo-TEM support. Thanks to Dr. Elena Dormidontova for the molecular dynamic simulation results and also being my thesis defense committee member. Thanks to Dr. Kazunori Hoshino for spending time on my defense and also the thesis writing. I am really appreciate that. Many thanks to my labmates, Ying Liu for helping me on TEM results and thesis preparation, Yan Xia for teaching me almost everything in the laboratory, Armin Rad for keeping encouraging me until the end. And Thanks to Justin Letendre for revising the thesis.

Thanks to my family for the unyielding support and kindness. I could not achieve the results today without your help. I am so lucky being with all my good friends at UConn for two years. All precious memories will keep me moving forward till the end.
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Abstract

In spite of considerable achievements in the field of cancer research over the past years, the early detection and treatment of cancer is still desirable. Advanced biodiagnostic nanotechnology has been developed rapidly recently, nevertheless there remains great room for improvement through a better design of nanoparticles (NPs). Gold nanoparticles (AuNPs) have been actively studied in biomedical applications for their advantages of low toxicity, high contrast in imaging, and capability of absorbing the infrared which has high penetration to tissues. However, several challenges need to be addressed including enhancement of the signal and maximization of the infrared absorption. Bicelles are model membranes made of long-chain and short-chain phospholipids. The microscopic morphology and structural diagram have been well studied. Since the self-assembled bicelles provide good analogue to the bio-membrane fragment, they have been commonly used in the study of membrane associated peptides. Another potential application of bicelles is to serve as carriers for hydrophobic drug or imaging contrast agent for treating or detecting cancer. In this study, bicelles made of long-chain 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), short-chain 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) and negative charged 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG) demonstrate capability of entrapping monolayer thiolate-ligand protected Au25 Clusters (MPC) in the bilayer. The encapsulation is investigated by using UV-visible spectroscopy, dynamic light scattering (DLS), small-angle x-ray scattering (SAXS), transmission electron microscopy (TEM) and Cryogenic TEM. Our result shows that the encapsulated amount of the MPC into bilayer increases with the increased MPCs concentration and thiolate chain length. Meanwhile, result also showed that the morphology of bicelles is subjected to change after encapsulation. At least two different particle distributions were observed after bicelles encapsulate MPCs: the smaller-sized nanodiscs (compared to the bare bicelles) and larger irregular particles.
1. Chapter 1: Introduction

1. **Introduction to Nanomaterials and Nanotechnology**

Nanomaterials are materials size in nanoscale (1-100 nm) for at least one dimension\(^1\), including nano-objects and nanostructured materials. Nanomaterials are widely existed in nature, such as bio-macromolecule including DNA, protein and lipid structures as the building blocks of our membrane system, or metal oxides which existed widely in the earth. Nanotechnology is defined as manipulating and controlling matters in the nanoscale to make use of size- and structure-dependent properties. Since the first time nanotechnology was introduced by Richard Feynman’s lecture titled “There’s Plenty of Room at the Bottom” at the American Physical Society meeting in 1959, various types of nanomaterials have been synthesized, together with the development of nanotechnology. Artificial nanomaterials, such as carbon nanotubes, metallic nanoparticles, model lipid bilayer structures and porous graphene film become more and more popular in both research fields because of the potential they have. The development of nanoscience has been also applied to the manufacturing process of integrated circuits in personal computers or smartphones and has achieved to the level of 10 nm. By the advanced technology, manufacturers could make their computers more integrated and more power efficient.

The nanomaterial and nanotechnology has been applied in many different aspects in daily life. Thanks to the development in nanotechnology, (1) digital contents could be recorded in CD/DVDs, (2) the plastic storage bins could be used to transport food which often lined with silver nanoparticles that kill bacteria from the previously stored food, (3) carpets and clothing are becoming stain-resistant because of the nanotextiles that prevent the absorption of liquids, coffee, wine and other substances that might they can bead up and be wiped away, or (4) flame retardant formed by coating the foam used in furniture with carbon nanofibers. Generally, nanotechnology has played an important role
behind the scene for a long period of time, although few people realize the existence of the nanoscale world.

Nanotechnology also has significant impact on energy and medicine fields, which are critical to our society. Solar energy, clean and abundant, is a very promising replacement to fossil energy. However, in solar cell system designation and fabrication, there is a trade-off between the efficiency and the cost, which is due to the contradiction between the material purity and device performance. In a typical planar solar cell, the charge carriers are collected in the same direction as light is absorbed. The minimum thickness of the cell is determined by the thickness of material required to absorb most of the incident sunlight. However, there is also a constrain on the required purity of the material due to the required thickness, since the excited charge carriers must have a sufficient lifetime to arrival the electrical junction, where they could be separated to produce electrical current flow through the metallic contacts to the cell. High aspect ratio nanorods could provide a long dimension for light absorption while only requiring the carriers moving along the short dimension of the nanorods rapidly. This conception could become true by applying CdTe tetrapods or organic conducting polymers, which could increase the efficiency of solar cells and reduce the manufacturing cost in terms of utility-scale electrical power generation.

When it comes to improving public health, nanotechnology also brings people great benefit in solving obscure problems such as cancer diagnosis and therapeutics, environment pollution control, food safety issue and so on. For example, recently study has shown that NaCl salt could be filtered from water by single-layer freestanding graphene with nanoscale pores in appropriate size. The membrane has the ability of allowing water flow while preventing ions passing through. Meanwhile, the hydroxyl on the edge of the pores could double the water flux. The water permeability of the nanoporous graphene membrane is several orders higher than conventional reverse osmosis membranes, which could be very promising in water purification. There are also some examples of
nanotechnology applied in oil-water separation⁴, which could become a promising way of dealing with oil spills in ocean.

Polymer nanocomposites (PNCs) are latest research findings aimed at solving the problems encountered in protecting food from dirt, oxygen, light, pathogenic microorganisms and other harmful substance during the time of being kept or transported. PNCs, created by dispersing an inert nanoscale filler throughout a polymeric matrix, are stronger, more flammable-resistant, better in thermos properties, lower in cost and more flexible in dealing with different food protection demands compared with conventional polymer protectors⁵. Besides, silver nanoparticles are used as potent antimicrobial agents. Nanosensors and nanomaterial-based assays could be used for food-relevant analytes. For example, gold nanoparticles functionalized with cyanuric acid groups could be used to detect melamine, an adulterant used to artificially inflate the measured protein content of pet foods and infant formulas⁶. Photo-activated indicator ink based upon TiO₂ or SnO₂ nanoparticles and a redox-active dye (methylene blue) has been applied in in-package oxygen detection⁷. Functionalized, magnetic iron oxide nanoparticles could be used to efficiently separate the target bacteria from contaminated milk, followed by a real-time PCR detection. This is achieved by attachment of antibodies selective for L. monocytogenes onto the metallic oxide nanoparticles⁸.

Another popular area of nanotechnology is nanomedicine, including the application in disease diagnosis and therapeutics, imaging and regenerative medicine⁹. Compared with conventional medical treatments, new technologies with participation of nanomaterials could (1) increase accuracy of disease diagnosis and therapeutics, as well as inception, (2) avoid damage on patient organism caused by inevitable extreme physical or chemical conditions in traditional treatments, (3) be more specific and target-oriented, (4) develop early and continuous treatments against fatal diseases. Here are several examples illustrating how nanomaterials have been or could be used in medical techniques improvement. Combination of hollow iron oxide nanoparticles and polymer nanobeads could be used as contrast agent in MRI. The new nanocomplex exhibited an increased effective magnetic anisotropy,
as well as smaller $r_1$ and higher $r_2$ compared to individual nanoparticles, which make such nanobeads excellent candidates as selective negative contrast enhancing agents, with a broad range of potential applications in medical MRI\textsuperscript{10}. Besides, semiconductor quantum dots represent another promising platform for cancer imaging\textsuperscript{11}. Gold nanorods are also confirmed to be a good candidate in cancer cell imaging\textsuperscript{12}. Furthermore, it could be used as a photothermal therapy candidate in near infrared region (NIR) due to the strong absorption and scattering of electromagnetic radiation of rod shape gold nanoparticles (AuNPs) with suitable aspect ratio. Moreover, the nanoparticles could be modified by other molecules or groups in order to be target-oriented. In the research referred above, gold nanorods are conjugated to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies. Combined with the fact of strong scattering of AuNPs, the specific malignant cells are clearly visualized and diagnosed from the nonmalignant cells under microscope. Meanwhile, malignant cells need only half the laser energy to be destroyed than the nonmalignant cells in photothermal way\textsuperscript{12}. In addition to be photothermal activated, the AuNPs could be conjugated with drugs and target aptamers simultaneously for disease treatment. One research shows AuNPs are synthesized and modified by different terminal end groups such as dicarboxylates or thiols. The anticancer drugs, such as cisplatin and multinuclear drugs, are attached to the AuNPs. Besides, the drugs also have different attachments such as folate, estrogen, prostate or leukemia targeting aptamers, lung cancer targeting peptides, and B-cell lymphoma targeting antibodies. The platinum-tethered nanoparticles demonstrated significantly better cytotoxicity than oxaliplatin alone in all of the cell lines tested and remarkable penetrating ability into lung cancer cells nucleus\textsuperscript{13}. Tissue engineering is another field that nanomaterials have huge potential in\textsuperscript{14}. For example, carbon nanotubes/carbon nanofibers could act as scaffolds for bone regeneration. People found that CNTs/CFTs could promote osteoblast adhesion and decrease competitive cell adhesion which is desirable in orthopedic implants since it could lead to faster bone integration to the implant surface in vivo\textsuperscript{15}. Research also shows that CNFs/CNTs have nanostructured features highly similar to natural neural tissue and exceptional electrical, mechanical and biocompatible properties, which are excellent candidates for neural tissue repairing or
regeneration\textsuperscript{16} \textsuperscript{17} \textsuperscript{18}. Interestingly, CNTs and CNFs also have functions in drug and gene delivery\textsuperscript{19} \textsuperscript{20}. Generally speaking, nanomaterials and nanotechnology are playing an important role in different medical aspects including disease screening, diagnosis, treatment and monitoring, etc.\textsuperscript{21}.

2. Introduction to Gold nanoparticles in Biomedical Fields

Among the numerous kinds of nanomaterials which are widely used in different areas, AuNP is one of the most shining members. AuNPs have a number of advantages that make them appealing for multiple applications in medical area\textsuperscript{22}. For example, the absorbance and fluorescence of AuNPs is stronger than bulk gold, which could be tuned from visible to near-infrared (NIR) region by changing size and morphology\textsuperscript{23} \textsuperscript{24} \textsuperscript{25}. Electromagnetic field enhancement by sharp edges of AuNPs, such as stars or nanorods shape, is another feature which could be used in medical imaging, such as surface enhanced Raman spectroscopy (SERS) imaging\textsuperscript{26} \textsuperscript{27} \textsuperscript{28} \textsuperscript{29}. The feature of absorption and scattering of electromagnetic radiation enhancement also provides the potential of novel optically active reagents for photothermal cancer therapy\textsuperscript{12} \textsuperscript{30} \textsuperscript{31} \textsuperscript{32} \textsuperscript{33} \textsuperscript{34} \textsuperscript{35} \textsuperscript{36}. Besides, AuNPs have emerged as an attractive candidate for targeted-delivery of various payloads, such as small drug molecules or large biomolecules\textsuperscript{37} \textsuperscript{38} \textsuperscript{39} \textsuperscript{40} \textsuperscript{41} \textsuperscript{42} \textsuperscript{43} \textsuperscript{44}, and targeted-release triggered by internal\textsuperscript{45} \textsuperscript{46} or external\textsuperscript{47} stimuli. Another reason why AuNPs are widely studied in medical field is their low-cytotoxic, low-immunogenicity and good biocompatibility features\textsuperscript{48} \textsuperscript{49} \textsuperscript{50} \textsuperscript{51} \textsuperscript{52} \textsuperscript{53} \textsuperscript{54} \textsuperscript{55}, which made the AuNPs better than QDs or metallic or polymer nanoparticles. Besides, other features such as straightforward synthesis\textsuperscript{56}, stability\textsuperscript{57} and the facile ability to incorporate secondary tags such as peptides targeted to specific cell types to provide selectivity\textsuperscript{58} \textsuperscript{59} \textsuperscript{60} which made the AuNPs a very promising candidate for biomedical applications.

Although AuNPs are much safer than QDs or other nanoparticles, toxicity is still observed at high concentration in AuNPs modified by cationic side chains, which may threaten body health. Meanwhile, AuNPs with anionic ligands are quite non-toxic. Other kinds of ligands such as tiopronin monolayer, are also toxic at higher concentration\textsuperscript{51} \textsuperscript{61}. Furthermore, the solubility of AuNPs are
different depending on the way of modification\textsuperscript{36,62}. Therefore, usually it is not appropriate to use AuNPs directly in order to achieve highly sensitive detection or highly efficient delivery.

In order to improve the delivery efficiency and avoid the toxicity of AuNPs, people have designed a variety of carriers to enhance the biocompatibility. Phospholipid-coated AuNPs has been studied in different aspects. By the presence of lipid structure, the biocompatibility of AuNPs become much higher, while the toxicity become much lower than using AuNPs alone. For example, Li et al. have used dimethyldioctadecylammonium bromide (DODAB), a commercial cationic lipid bilayer with function as gene carriers, to coat onto AuNPs which could transfect 2 kinds of plasmid DNA into human embryonic kidney cells (HEK 293) efficiently. By the presence of AuNPs, the interaction between DNA and DODAB becomes more stable, which lead to a higher transfection efficiency\textsuperscript{63}. Zhang et al. have successfully synthesized didodecyldimethylammonium bromide (DDAB) lipid bilayer-protected AuNPs, which is water soluble\textsuperscript{64}. Tatur et al. found that AuNPs functionalized with cationic head groups could penetrate into the hydrophobic moiety of floating lipid bilayers, while AuNPs with anionic head groups could not, which indicate that the encapsulation might be driven by charge state of the AuNPs\textsuperscript{65}. Furthermore, there are also studies focusing on the cellular uptake and intracellular fate of lipid-coated AuNPs. Wang et al. have worked on their interaction with A549 cell, a human lung adenocarcinoma cell line which is a good in vitro model for the type II epithelial cells of the lung. They found that the lipid-coated gold nanoparticles could promote the formation of lamellar bodies, the secretory organelles found in type II pneumocytes which could fuse with the cell membrane and release pulmonary surfactant into the extracellular space\textsuperscript{66}.

3. Introduction to Model Lipid Membrane Structures

Lipid bilayers, or model lipid membranes, have been widely studied for decades of years. Amphiphilic phospholipid molecules could form bilayer structures simultaneously under simple conditions. The artificial lipid bilayers are used to study the properties of membrane system in organisms. For example, single channel recording apparatus was combined with an artificial lipid
bilayer and a fluorescence microscope to detect single fluorescent molecules. The novel apparatus was a very powerful tool for the study of ion channels, which could measure electrical and spectroscopic parameters of the channel simultaneously. Peptides or protein could also participate the formation of lipid bilayers by binding or insertion. Sometimes the peptides could affect the stabilization of bilayer structure and the mechanism has been revealed in some cases. Among all applications of lipid bilayer in biomedical area, the drug delivery function of liposomes was the most successfully commercialized one, especially for cancer treatment. Currently, several kinds of cancer drugs have been applied to this lipid-based system. Liposomal formulations of the anthracyclines doxorubicin (Doxil, Myocet) have been approved for the treatment of metastatic breast cancer. Moreover, cholesterol liposome and DNA complex could enhance the expression of chloramphenicol acetyltransferase gene in a number of tissues, which showed the potential of liposomes in genetic therapies.

All ongoing and potential applications of lipid bilayers are attribute to several unique features of phospholipid molecules – the fundamental elements of lipid bilayers. The phospholipid molecules are amphiphilic containing polar head group and two hydrophobic hydrocarbon tails. The tails are usually fatty acids with different length and saturation. These are very important because they influence the ability of phospholipid molecules packing against one another, then affecting the fluidity of the lipid bilayer. The shape and amphiphilicity of the lipid molecules cause bilayers spontaneous formation of bilayer structure in aqueous environment. Hydrophilic molecules could dissolve in water easily because they contain charged or polar groups that could form either electrostatic interactions or hydrogen bonds with water molecules, while hydrophobic molecules are insoluble because their uncharged and nonpolar groups cannot form energetically favorable interactions with water molecules. When lipid molecules are dispersed in water, they force the adjacent water molecules to reorganize into cages that surround the hydrophobic molecule. Since the cage structures are more ordered than the surrounding water, their formation increases the free energy.
This free energy cost could be minimized if the hydrophobic molecules or the hydrophobic portions cluster together so that the number of water molecules being affected reaches minimum. Therefore, lipid molecules spontaneously aggregate to bury hydrophobic tails in the interior and expose hydrophilic heads to water. They can assemble in two ways: (1) they can form spherical micelles, with the tails inward, (2) they can form bimolecular sheets or bilayers with the hydrophobic tails sandwiched between the hydrophilic head groups.

Mixture of short chain and long chain phospholipid molecules could form into aggregates in a variety of shapes under different conditions, such as liposomes, ribbons or nanodiscs, etc. (as shown in Figure 1.1). In this thesis, nanodiscs that consist of long chain lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG) and short chain lipid 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) were used as the carrier as shown in Figure 1.2.

![Figure 1.1 Phase diagrams adapted from Nieh et al. (2002) of DMPG and Tm<sup>+</sup> doped DMPC/DHPC](image)

\[
\frac{[\text{DMPC}]}{[\text{DHPC}]/[\text{DMPG}]} = 3.2/1.0/0.21
\]
Figure 1.2. (A) Negative staining transmission electron microscopic (TEM) image of nanodiscs composed of long-chain (DMPC) and short-chain (DHPC) lipids (taken by Ying Liu). (B) Sketch of DMPC-rich planar bilayer and highly-curved DHPC-rich rim.

The lipid mixtures form nanodiscs when the environmental temperature is lower than the melting transition temperature ($T_m$) of the long chain lipid molecule DPPC. This is because the tails of long chain lipid are in gel phase, while the tails of short chain lipid are in liquid crystalline phase. Therefore they separate from each other and the long chain lipids form the planar region, while the short chain lipids with higher curvature locate at the rim. When the temperature is raised above the $T_m$ of the long chain lipids undergo gel-to-liquid crystalline transition. The membrane structure becomes more flexible and eventually the nanodisc will transform into vesicles\textsuperscript{77,78}.

4. Motivation and Thesis Outline

Based on knowledge of lipid aggregates and previous studies on interaction between lipid bilayers and AuNPs, a new nanocomplex of lipid-based nanodiscs with Au-MPCs in the bilayer was presented in this project, which could possibly improve the efficiency of cellular uptake of AuNPs. Monolayer Protected Clusters (MPCs) were gold nanoparticles with 25 Au atoms and 18 thiolate hydrocarbon chains, which have high affinity to the hydrophobic moiety of nanodiscs. This novel nanocomplex could be used as a simple platform for SERS effect in medical imaging system if there
are enough AuNPs encapsulated and the Au-Au distances are uniform. Therefore, there are several key issues need to be addressed to characterize the nanocomplex including (1) the evidence of encapsulation, (2) the encapsulation efficiency, (3) effect of MPCs on nanodisc morphology.

The outline of the thesis is: Chapter 2 basically reviews all sample preparation and characterization procedures. Chapter 3 discusses about the evidence of encapsulation, the encapsulation efficiency of MPCs and the morphology alternation after having MPCs within nanodiscs. Finally conclusions and discussion are in Chapter 4.
2. Chapter 2: Material and Method

1. Material and instrument

(1) Phospholipid: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0 PC, DMPC), 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol), (14:0 PG, DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0 PC, DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (16:0 PG, DPPG) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (06:0 PC, DHPC).

(2) Au Clusters: Au$_{25}$(SC$_n$H$_{2n+1}$)$_{18}$, n=2, 4, 8, 12 and 16.

(3) Solvent: de-ionized water, dichloromethane, chloroform, benzene, and methanol.

(4) Other chemicals: uranium acetate, NaCl, toluene, ethanol, acetone.

(5) Instruments: Static/Dynamic Light Scattering (ALV CGS-3 MD), Cary 5000 UV-Vis-NIR System (Agilent), Small Angle X-Ray Scattering, SAXS (Bruker, Nanostar), Tecnai T12 S/TEM (FEI).

(6) Glassware and tools: 4ml glass vial, 1.5ml Eppendorf centrifuge tube, glass vial for DLS, Quartz capillary, bruker cell, spatula, beaker, syringe and needle, etc.

(7) Utilities and others: balance, vortex, sonication, pump, vacuum oven, fume hood, dry ice, nitrogen flow, etc.

2. Determination of nanodisc to AuNPs ratio

There are several different ratios between AuNPs and lipid molecules used in this project to investigate effects of different AuNPs amount on encapsulation and morphology. The ratio was under one assumption that there are 5000 lipid molecules in one well-defined nanodisc. As the result suggests there might be no more than half of the initial amount of AuNPs encapsulated by lipid bicelles, we set up 2 ratios in DPPC case. One is 500:1 (lipids: MPCs, molar ratio) in which small
amount of AuNPs that might be encapsulated totally. The other is 200:3 where lipid is still overwhelmed.

To determine how much each component should be added at a specific molar ratio, the nanodiscs amount of given lipid weight, the molar ratio between total long-chain lipid and total short-chain lipid (Q) and the molar ratio between total charged lipid and total long chain lipid (R), as well as the concentration in a solution shall be known.

In this project, the total lipid weight is 10mg, Q=3 and R=0.01. So the amount (n) of DMPC, DMPG and DHPC is $1.1939 \times 10^{-5}$ (mol), $1.20596 \times 10^{-7}$ and $4.01985 \times 10^{-6}$ (mol), respectively. The number (N) of lipids is $7.18725 \times 10^{18}$, $7.25985 \times 10^{16}$ and $2.41995 \times 10^{18}$. The average size of bicelles (nanodiscs) was determined based on previous DLS and SANS results. DLS could determine the equivalent hydrodynamic radius. In the case of nanodiscs, the hydrodynamic radius can be transformed into actual dimension by a series of equations:

\[
\begin{align*}
a_j &= \left[\frac{1}{3a_\parallel(r_d)} + \frac{2}{3a_\perp(r_d)}\right]^{-1}, \\
a_\parallel(r_d) &= \frac{8r_d}{3} \left[\frac{2\lambda}{1-\lambda^2} + \frac{(2-4\lambda^2)tan^{-1}\left(\sqrt{(1-\lambda^2/\lambda)}\right)}{(1-\lambda^2)^{3/2}}\right]^{-1}, \\
a_\perp(r_d) &= \frac{8r_d}{3} \left[\frac{-\lambda}{1-\lambda^2} + \frac{(2\lambda^2-3)sin^{-1}\left(\sqrt{(1-\lambda^2)}\right)}{(1-\lambda^2)^{3/2}}\right]^{-1},
\end{align*}
\]

in which $a_j$ is apparent hydrodynamic radius obtained by DLS. $a_\parallel$ and $a_\perp$ are perpendicular and parallel contribution of the hydrodynamic radius of nanodisc. $r_d$ is the disc radius and $\lambda$ is the disc thickness.
to-diameter aspect ratio. In this case, $a_j$ is 14nm, so $r_d$ is 20.427nm and thickness is 5nm. The total surface area of a nanodisc is about 3263nm$^2$. The number of lipid molecules used in a nanodisc is about 5021. Therefore the number of nanodisc in 10mg lipid system is:

$$N(\text{nanodisc}) = \frac{N(\text{DMPC}) + N(\text{DMPG}) + N(\text{DHPC})}{5021} = 1.93 \times 10^{15},(4)$$

Then the amount of AuNPs could be known by a specific ratio given.

3. Synthesis of lipid-based bicelles with AuNPs.

   (1) Calculation of lipid and AuNPs: based on Q and R value, as well as the bicelle/AuNPs ratio desired,

   (2) Sample weighting: weight DMPC (DPPC), DHPC in a vial, AuNPs in another vial. Also weighting DMPG (DPPG) in another vial since the amount is small.

   (3) Sample dissolving: dissolve DMPG (DPPG) by a mixed solvent composed of CHCl$_3$ and CH$_3$OH by 65:35 (volume ratio). Dissolve AuNPs in benzene and then use sonication for 30 minutes. Mix DMPC (DPPC), DMPG solution (as the amount required), AuNPs solution and some CHCl$_3$ together to make the final solution transparent.

   (4) Solvent evaporation: After all components are mixed well, put the vial in heat block and add nitrogen flow to help the solvent evaporating process. When the solution turns into high viscosity film at the bottom, put it into vacuum oven at 50°C for overnight to ensure solvent evaporation.

   (5) Sample hydration: take the vial out and measure the weight of lipid with AuNPs. Hydrate the sample using filtered deionized water to 10wt%, followed by temperature cycling for several times (heating the sample up to a temperature that is higher than the melting transition temperature (Tm), and cool it down to a temperature lower than the Tm).

   (6) Final dilution: dilute the sample to a relatively lower concentration for the following tests (1wt% or lower).
4. Dynamic light scattering:

The dynamic light scattering measurement is measured by ALV/CGS3MD Compact Goniometer System with a fixed angle of 90°, which could measure the autocorrelation function of the scattered intensity fluctuation. The experiment steps are listed as follows:

(1) Prepare samples by diluting stock lipid solution from 10wt% to 0.1wt% using filtered deionized water and keep the sample at room temperature (keep DMPC samples under 10°C).

(2) Start the ALV/CGS3 Compact Goniometer and ALV-7004 Multiple Tau Digital Real Time Correlator. Setup scattering angle, measurement program and auto save in the software.

(3) Put the sample in a clean glass vial and close the lid. Rinse the external wall of the vial by toluene. Inset the vial in the sample hold and start the measurement.

(4) After all runs are finished, fit the correlation function into size distribution function and the hydrodynamic radius is obtained.

5. Small-angle X-ray Scattering:

(1) Sample loading: the sample is loaded by Bruker cell or by quartz capillary sealed by torch. It depends on the time length and temperature of the test since Bruker cell could not hold for very long time especially under a relatively high temperature. Empty capillary is background when quartz capillary is used to load sample. It turns out to be water as background when Bruker cell is used. There is a little difference on arbitrary intensity between using capillary and using Bruker cell, but the peak position is not changing. First, take some 1wt% sample using a needle and syringe and gently inject into the bottom of the capillary to a reasonable height. Then use a torch to melt and seal the open end. When it comes to Bruker cell, clean all cell parts by ethanol and deionized water, and then assemble it back. Next, inject about
60µl sample gently into the cell. Finally close the cap and test the cell in vacuum in case leaking might happen.

(2) Instrument preparation: the sample chamber of SAXS should always keep evaculated. The X-ray generator shall be on all the time as well. Calibration is need prior to any sample measurement. Close the vacuum valve and open air valve to make vacuum collapsed. Then push open door button and open the chamber gate. Install the temperature stage inside the chamber, adjust the stage to a proper position. Insert an AgBeh standard into the sample holder in the chamber. Close the gate, take a measurement for at least 60 seconds followed by data unwrapping to obtain enough data point for calibration. Then click ‘process’ -> ‘calibration’, make sure the file ‘AgBeh.std’ is used and auto mode is checked. Then press ‘Enter’. After the auto calibration stops, press ‘Enter’ again and update the sample-to-detector-distance (SDD). Repeat the calibration for several times until the SDD doesn't change anymore. After calibration, load the project configuration file and update the SDD to current project. To obtain a reliable scattering result, the beam stop shall be at the center of the frame.

(3) Data collecting: first, a background shall be measured prior to the sample. As mentioned before, use bruker cell filled with water or use empty capillary as background. A 3600 seconds measurement is need for background. The sample measurement may last for 3600 seconds or 7200 seconds depends on the instrument mode (high flux or high resolution), as well as the scattering intensity of sample.

(4) Transmission measurement: Transmission factor could be calculated by the following steps. First, move the beam stop away from the path, scan the sample for 60 seconds and record the count number \(i(S)\) at the beam center (\(\theta\) from 0’ to 0.1’). Adjust the stage, let the beam go through an empty pinhole and record the count number \(i(E)\) again. Next, insert the background into the sample holder, obtain count number of background sample \(i(B)\) and also \(i(E)\). Then the transmission factor (T) of one sample is: \(\frac{i(S)/i(E)}{i(B)/i(E)}\).
(5) Data analysis: after sample measurement finished, the 2D image data could be convert into 1D wave data (q versus intensity). Start SAXS offline application, load project, and then load sample raw file and subtract background file by applying the transmission factor as well as time factor. For example, if the sample measurement is 2 hours and background is 1 hour with a transmission factor of 0.9, type -1.8 in the scale factor box, then click ‘ok’ to do the subtraction. Then the data image with background excluded appears. Then click ‘peaks’ -> ‘integrate’ -> ‘chi’, set a proper ‘2theta’ and ‘chi’ value that could include most area of the frame and click ‘OK’ to finish the integration. The final scattering data is exported into a txt file with 3 columns: q, arbitrary intensity and intensity standard error. They could be plotted into an X-Y coordinate.

6. UV-visible Spectroscopy:

The instrument is Cary 5000 UV-Vis-NIR made by Agilent. The absorbance of lipid sample and gold nanoparticle alone for pre-calibrated curve was conducted by scan mode. The procedures of performing a scan with baseline correction are listed as follows:

(1) Start the UV-vis spectrophotometer 15 minutes prior to the experiment.

(2) Start ‘Scan’ app, click the ‘Setup’ button, and select Baseline Correction to perform a baseline correction on each sample data point.

(3) Close ‘Setup’, click Zero to perform a zero correction, make sure that there is no sample in the chamber.

(4) Click ‘Baseline’ to measure a baseline. Insert the blank sample (benzene solution) into the holder, and click ‘OK’. After the baseline is collected, the word ‘baseline’ will be displayed in red in the ordinate status box, indicating that you are in baseline correction mode and have a valid baseline file for the correction.

(5) Insert a sample, click ‘start’ to perform a scan for the sample, type in sample name.

(6) When all samples are finished, click ‘save spectra’ to export all data into one excel file.
(7) Plot data: Absorbance vs. wavelength, use the absorbance at around 400nm to make the pre-calibrated curve and to measure concentration of unknown lipid with AuNPs samples.
3. Chapter 3: Result

The interaction between lipid bilayers and monolayer protected gold clusters (MPCs) were characterized in 3 aspects: (1) the encapsulation of MPCs within lipid bilayers, (2) the encapsulation efficiency and (3) the disruption of bilayer structure by accommodating MPCs. To illustrate these 3 major issues, several techniques were applied for the interaction characterization. UV-vis spectroscopy was used to calculate the encapsulation efficiency. Monodispersity of MPCs was also examined by the absorbance curve at the same time. Dynamic Light Scattering (DLS) was used to discover the size change of lipid-based nanodiscs after having MPCs within the hydrophobic moiety of the bilayer, as well as under different temperature. Small-Angle X-ray-Scattering was utilized to investigate the morphology of nanodisc with/without MPCs encapsulation at room/high temperature. Finally, Cryo-TEM gave the high resolution image containing single or multiple nanodiscs under different treatment as referred above, which could verify the MPC distribution within lipid bilayer and the morphology alternation caused by the encapsulation. In addition, the interaction between lipid bilayers and MPCs were also simulated by Prof. Dormidontova and her group, which could supply evidence of encapsulation and reveal possible process dynamically.

Sample preparation was based on the calculation prior to the experiment as explained in material and method section. 3 molar ratios of MPC to lipid were set in order to find out the difference between low or high MPC concentration. However, the morphology of nanodisc might change during the MPC encapsulation and temperature rising. Besides, the MPCs could not be distributed in each nanodisc evenly, so the weight ratio between lipid and MPCs were used instead of molar ratio after encapsulation, which was more reasonable. The molar ratio used in the following sections will be referred as “initial molar ratio”.

1. Monodispersity evaluation of MPCs in organic solvent

The Au atoms are coated by hydrocarbon chains which play a role in displaying functional groups for potential applications\(^1\)\(^-\)\(^2\), which has been discussed in introduction above. Meanwhile, the monolayers also act as the capping shields protecting the Au cores from aggregation\(^3\)\(^-\)\(^4\). The MPCs used in this study are stable gold clusters consist of 25 Au atoms protected by 18 alkanethiolate with different chain length (\(\text{Au}_{25}(\text{SC}_n\text{H}_{2n+1})_{18}\), \(n=2, 4, 6, 8 \ldots\))\(^3\)\(^-\)\(^5\). The sample was synthesized and characterized by Prof. Flavio Maran and his group members from University of Padova, Italy. Although the thiolate ligands on the surface of Au clusters could prevent from aggregation theoretically, the MPCs benzene solution was sonicated for 30 minutes as suggested. SAXS was applied after the sonication, which could resolve the size of monodispersed MPCs, making sure the MPCs were monodispersed before being encapsulated into lipid bilayers. Figure 3.1 showed SAXS data of \(\text{Au}_{25}\text{C}_{12}\) benzene solution on a Guinier plot\(^8\). The logarithm of scattering intensity (I) was plotted as a function of \(q^2\) and the slope of the trend line was \(<R_G^2>/3\), where \(<R_G^2>\) is the root mean square radius of gyration. The obtained best fitted \(<R_G^2>^{1/2}\) was 0.369 nm. Under the assumption of solid spheres, \(R_G^2= (3*R^2)/5\), where R is the radius of the sphere, yielding 0.477nm for the radius of MPC. It has been reported that the Au25 core was 1-2nm approximately in diameter\(^3\)\(^-\)\(^6\), which is consistent with our experimental data, indicating complete dispersion of MPCs after a 30-minute sonication.
2. Encapsulation of hydrophobic MPCs in lipid-based nanodisc

1. Appearance of nanodisc-MPCs supernatant

It has been reported that Gold nanoparticles could interact with model lipid membranes by penetrating into the bilayer.65 90. Because liposomes are biocompatible in biomedical applications such as drug delivery73 91 92 93 94, there were several studies focusing on liposomes encapsulating AuNPs44 95 96 and cell uptake of the nanocomplex97. The AuNP/lipid nanocomplex also has the functions such as triggering content release98 99, improving transfection efficiency100 or providing phototherapy101. In this study, lipid-based nanodisc synthesized via self-assembly is used as the carrier, which has higher nonspecific uptake rate than liposomes due to a larger aspect ratio102 103 104. Meanwhile, the cost of nanodisc synthesis could be lower than that of liposome. The AuNPs used are monodispersed hydrophobic MPCs as introduced above. The lipid components are DPPC, DPPG and DHPC with Q=3 and R=0.01. The mixed organic solution containing phospholipid and MPCs was dried and re-dispersed by water to 1wt%. The precipitation was taken out after centrifuge (1500 g, 5 minutes). As was shown in Figure 3.2, all samples in the presence of Au-MPC have dark color, compared with colorless nanodisc in the absence of AuMPC in Figure 3.2 (A). The 4 types of

![Figure 3.1. SAXS data of the Au25C12 in benzene with best Guinier fit, q2 from 0.002 to 0.01 (Å^-2).](image)
AuMPCs with different chain lengths and 2 lipid/AuMPC ratios render different levels of darkness of supernatants. It is apparent that the color differences of lipid supernatant (after centrifugation) were presumably originated from AuMPCs, since the same color was observed when the pure AuMPCs were dissolved in benzene as shown in Figure 3.3. Besides, it was interesting that the appearance of all 4 AuMPC solutions was almost identical to each other at the same concentration. It should be noted that the AuMPCs were highly hydrophobic due to the external lipophilic molecules and were not soluble in the aqueous phase. A simple experiment was conducted by loading 30µl AuMPC benzene solution in contact with 30ul water in a quartz capillary. As the organic solvent evaporated, the AuMPCs completely precipitated out of the water phase and finally settled on the wall of the capillary in presence of water which remained colorless and transparent. Therefore this provides an indirect indication that the color of AuMPC/lipid supernatants was evidence of encapsulation.

Experiments on DMPC and DMPG, instead of DPPC and DPPG, bicelles also indicated encapsulation of AuMPCs in nanodiscs. The initial molar ratio of lipid: MPCs was 500:1. In Figure 3.4, the supernatant also showed similar color as in Figure 3.2 and 3.3.
Figure 3.2. Supernatant of DPPC, DPPG and DHPC with MPCs at 1wt%. (A) DPPC nanodisc without any MPCs. (B) lipid with Au$_{25}$C$_4$ at 500:1. (C) Lipid with Au$_{25}$C$_4$ at 200:3. (D) Lipid with Au$_{25}$C$_{16}$ at 500:1. (E) Lipid with Au$_{25}$C$_{16}$ at 200:3. (F) Lipid with Au$_{25}$C$_8$ at 500:1. (G) Lipid with Au$_{25}$C$_8$ at 200:3. (H) Lipid with Au$_{25}$C$_{12}$ at 500:1. (I) Lipid with Au$_{25}$C$_{12}$ at 500:1. All supernatants were took out from 1wt% solution after centrifuge for 5mins.

In DMPC case, the sample of Au$_{25}$C$_2$/lipid complex illustrated hardly any color in the supernatant indicative of little or none encapsulation of AuMPCs. This was also consistent with the UV-vis absorption spectrum. Presumably, the protected ligands of Au$_{25}$C$_2$ was too short (not lipophilic enough) to be entrapped by bilayer.
Figure 3.3. Appearance of MPCs benzene solution. (A) Au$_{25}$C$_4$, (B) Au$_{25}$C$_6$, (C) Au$_{25}$C$_{12}$, (D) Au$_{25}$C$_{16}$. All samples were sonicated for 30 minutes. The concentration was 1mg/mL for all 4 samples. The color was almost identical in all of the samples.

Figure 3.4. Supernatant of DMPC, DMPG and DHPC with MPCs at 1wt% with original molar ratio of 50:1 (Lipid: MPCs).

(A) Nanodisc with Au$_{25}$C$_2$. (B) Nanodisc with Au$_{25}$C$_4$. (C) Nanodisc with Au$_{25}$C$_8$. (D) Nanodisc with Au$_{25}$C$_{16}$. 
2. Encapsulation of MPCs confirmed by Cryo-TEM

The encapsulation of MPCs within lipid bilayers was also confirmed by Cryo-TEM, which was conducted at Prof. Vijay John’s group at Tulane University. The AuMPC nanoparticles spanning in the planar area of nanodiscs was found in the lipid/Au$_{25}$C$_{16}$ complex (200:3) as shown in Figure 3.5. The dark rod-like objects implied that the MPCs might be localized at the rim side of nanodiscs or induce cylindrical lipid nanoparticles.

![Cryo-TEM result of nanodisc with Au$_{25}$C$_{16}$ at 200:3.](image)

Figure 3.5. Cryo-TEM result of nanodisc with Au$_{25}$C$_{16}$ at 200:3.

3. Molecular dynamic simulation of the encapsulation of MPCs in nanodisc

Molecular dynamic simulation was applied to further understand the encapsulation process of AuMPCs in lipid-nanodiscs as shown in Figure 3.6. Figure 3.6 (A), and (B) illustrate the schematics of Au$_{25}$C$_8$ and Au$_{25}$C$_{16}$ which are simulated with coarse-grain model where the Au cluster core (with 25 atoms), sulfur and 4 methylene (butyl) groups are represented in brown, yellow and green units, respectively. Figure 3.6 (C) shows a simulated snapshot of a single nanodisc with the diameter of 17.3nm and the thickness of 4.6nm at Q =3. Figure 3.6 (D) shows the aggregation of hydrophobic MPCs in water.
Figure 3.6. Schematic diagrams of nanodiscs and MPCs for molecular dynamic simulation. (A) Au$_{25}$C$_8$. (B) Au$_{25}$C$_{16}$. (C) Nanodiscs, DPPC: DHPC=3:1. (D) Behavior of MPCs aggregating in the aqueous phase.

Figure 3.7 was a series of snapshots of nanocomplex from the molecular simulation animation along the time. Initially, the nanodiscs and the Au$_{25}$C$_{16}$ MPCs were separated as shown in Figure 3.7 (A). Then the MPCs were starting to be entrapped in the bilayer of the nanodiscs [Figure 3.7 (B) and (C)]. After being encapsulated within the nanodiscs, the MPCs move inside the bilayered disk randomly. After a period of time, the Au-MPCs were mostly distributed at the rim of the nanodiscs (Figure 3.7 (D-F)), consistent with what have been found in Cryo-TEM images in Figure 3.5. The molecular simulation results further confirms the conclusion that lipid based nanodiscs were capable of encapsulating MPCs.
Figure 3.7. Molecular dynamic simulation revealed the process of MPC encapsulated within lipid nanodiscs. (A) Initiating interaction between MPCs and nanodisc. (B)-(F) MPCs penetrated into nanodiscs and Brownian motion of MPCs within the lipid boundary. Time interval: (A) before penetration, (B) 0 ns, (C) 31.25 ns, (D) 62.5 ns, (E) 93.75 ns, (F) 125 ns.

4. Evidence of MPCs encapsulation from SAXS

The low-\(q\) SAXS scattering intensity of Au-MPC encapsulated samples increased in comparison with that of nanodiscs in the absence of AuMPCs as shown in Figure 3.8. This is expected because the electron density of AuMPC is much higher than those of phosphor, sulfur, carbon and hydrogen atoms. The contrast was significantly enhanced as the Au-MPC aggregation takes place in the bilayer.
3. Efficiency of nanodiscs encapsulating MPCs: chain-length dependent.

A higher loading capacity of Au-MPCs in the lipid carriers is desired in order to achieve higher transfection efficiency for drug or biomolecule delivery (i.e., accumulating nanoparticles in cells, tumors, and organs maximally). Moreover, stronger infrared absorbance was another benefit of more efficient MPCs loading for biomedical applications such as medical imaging, cancer diagnosis and phototherapy. Surface-enhanced Raman spectroscopy (SERS), a surface-sensitive technique that enhances Raman scattering by molecules adsorbed on rough metal surface, has been applied in single-molecule detection\textsuperscript{105} and diagnosis\textsuperscript{106} since it could enhance the infrared absorption or emission by a factor of $10^5$, up to $10^{11}$\textsuperscript{107} $10^{10}$. Factors including size, shape of the AuNPs as well as the particle distance are crucial for the enhancement of SERS effect. The Au-Au distance could be estimated in the Au-MPC/lipid nanocomplex if sufficient MPCs accommodated in the lipid bilayers, since it is controlled by the closest distance between 2 Au-MPC molecules. The encapsulation also increases the solubility of Au-MPCs in water, beneficial to the in vivo applications of Au-MPCs.

From the appearance of supernatant (Figure 3.2), the TEM images (Figure 3.5) and the simulation results (Figure 3.7), it strongly implies that all 4 types of Au-MPCs (Au$_{25}$C$_4$, Au$_{25}$C$_8$, Au$_{25}$C$_{12}$, and Au$_{25}$C$_{16}$).
Au$_{25}$C$_{12}$ and Au$_{25}$C$_{16}$) were encapsulated in the lipid complex. The differences on the appearance of supernatants seemed to suggest that the encapsulation efficiency might be different among the 4 Au-MPCs. However, the exact efficiency of each kind of MPCs encapsulated within nanodiscs and the influencing factors remained to be characterized quantitatively. UV-vis absorption spectroscopy was used to address this issue. Absorbance curves of 4 kinds of MPCs in benzene solution were shown in Figure 3.9. All curves shared similar shape and spectral features. The absorbance curve showed a sharp peak at 400nm, a shoulder at 450nm and a broad peak at 690nm$^{83}$. The peak at 400nm was selected for the pre-calibrated curves of Au-MPC concentrations in order to calculate encapsulation efficiency (Figure 3.10).

![Absorbance spectrum](image)

**Figure 3.9.** Comparison of the UV-vis absorption spectra of Au$_{25}$(SC$n$)$_{16}$, $n=4, 8, 12, 16$. The measurements were carried out in benzene at 25°C. A 30-minute sonication was conducted before each measurement. For clarity, the spectra have been vertically shifted.

The supernatant of centrifuged samples containing Au-MPC/lipid complex was dried for 24hrs and re-dispersed in benzene, followed by sonication for 30 minutes. The absorbance of MPC-benzene solution was measured by UV-vis spectroscopy. The amount of MPCs in supernatant was
calculated using pre-calibrated curves shown in Figure 3.10. Each Pre-calibration curve contained 5 absorbance data points from 0 to a certain concentration. According to the Beer-Lambert Law,

\[ A = \log \frac{I}{I_0}, \]

\[ \log \frac{I}{I_0} = \varepsilon \cdot l \cdot c, \]

in which A: absorbance, I₀: intensity of the light passing through the reference cell, I: intensity of the light passing through the sample cell, ε: extinction coefficient, l: length of light path, c: concentration of sample solution.

The absorbance is proportional to the concentrations of the attenuating species in the material sample. It is valid only for low concentrated solutions\(^{109}\). Therefore, a linear correlation function was derived from the 5 data points, served as the pre-calibration function.

![Figure 3.10. UV-vis spectroscopy pre-calibrated curves. (A) Au_{25}C_4 at 399nm. (B) Au_{25}C_8 at 398nm. (C) Au_{25}C_{12} at 400nm. (D) Au_{25}C_{16} at 398nm. The function of pre-calibrated curve was: y=b*x+a (y=concentration, x=absorbance). (A) a=-0.0002662 ± 0.00018, b=0.3419 ± 0.000441. (B) a=-0.00022642 ± 0.00017, b=0.38227 ± 0.000523. (C) C12: a=-0.0019831 ± 0.00111, b=0.33889 ± 0.003. C16: a=-0.0016847 ± 0.00201, b=0.53861 ± 0.00465.](image)
The absorbance of each sample was measured by UV-vis spectrophotometer at the peak position located around 400nm. Then the amount of Au-MPCs in whole supernatant was estimated based on the pre-calibrated curve. The encapsulation efficiency results were collected in Table 1.

From Table 1, a correlation was found between the chain-length of the ligands and the encapsulation efficiency. First of all, the high initial MPC concentration (lipid: MPC = 200:3) samples have a higher encapsulation amount than the low initial MPC concentration (lipid: MPC = 500:1) samples. In both low (500:1) and high (200:3) concentration groups, a similar efficiency trend was found. The encapsulated amount of Au$_{25}$C$_4$ was the lowest. In C12 and C16 samples the encapsulation efficiency kept at a high level but there was a difference between the low initial concentration and high initial concentration. The 500:1 samples, encapsulation efficiency increases with the chain lengths, while for 200:3 samples, the encapsulation efficiency saturates at the chain length of C8 (i.e., the outcomes from C8, C12 and C16 are similar). This was also consistent with the appearance of supernatant (Figure 3.2): the more encapsulation the darker color. A similar trend of efficiency was found in DMPC case shown in Table 2. The encapsulation efficiency increased rapidly before the ligand length reached C8, while the efficiency of C8 was almost the same as C16. There was hardly any encapsulation in Au$_{25}$C$_2$ sample. Generally, the encapsulation efficiency of MPCs within nanodiscs was affected by both initial amount and the thiol-chain length. For low initial MPC concentration, the encapsulation efficiency kept increasing continuously, while it had a “saturation point” near the chain length of C8 in high initial concentration group.
<table>
<thead>
<tr>
<th>Sample name</th>
<th>Initial nanodisc : Au</th>
<th>Encapsulated (mg)</th>
<th>Total input (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanodisc (ND)</td>
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<td>0</td>
</tr>
<tr>
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<td>0.09</td>
<td>0.22</td>
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<td>ND with Au$_{25}$C$_8$</td>
<td>200:3</td>
<td>0.98</td>
<td>1.70</td>
</tr>
<tr>
<td>ND with Au$<em>{25}$C$</em>{12}$</td>
<td>500:1</td>
<td>0.11</td>
<td>0.25</td>
</tr>
<tr>
<td>ND with Au$<em>{25}$C$</em>{12}$</td>
<td>200:3</td>
<td>0.92</td>
<td>1.95</td>
</tr>
<tr>
<td>ND with Au$<em>{25}$C$</em>{16}$</td>
<td>500:1</td>
<td>0.14</td>
<td>0.30</td>
</tr>
<tr>
<td>ND with Au$<em>{25}$C$</em>{16}$</td>
<td>200:3</td>
<td>1.18</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Table 1. Encapsulation efficiency of MPCs in DPPC nanodisc.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Initial nanodisc : Au</th>
<th>Encapsulated (mg)</th>
<th>Total input (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanodisc (ND)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ND with Au$_{25}$C$_2$</td>
<td>500:1</td>
<td>0</td>
<td>0.92</td>
</tr>
<tr>
<td>ND with Au$_{25}$C$_4$</td>
<td>500:1</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>ND with Au$_{25}$C$_8$</td>
<td>500:1</td>
<td>0.39</td>
<td>1.2</td>
</tr>
<tr>
<td>ND with Au$<em>{25}$C$</em>{16}$</td>
<td>500:1</td>
<td>0.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 2. Encapsulation efficiency of MPCs in DMPC nanodisc.
Although the UV-vis spectroscopy was an easy-to-use way to calculate the encapsulation efficiency of MPCs within nanodiscs, there was a noteworthy phenomenon according to the absorbance curve itself. The nanodisc-MPC sample was dried and re-dispersed into benzene followed by a 30-minute sonication. Then the absorbance of benzene solution was measured. The absorbance curves (Figure 3.11) were slightly different compared with the pure MPC-benzene curves shown in Figure 3.9. For example, for all initial 500:1 samples, the curve of Au\textsubscript{25}C\textsubscript{4} and Au\textsubscript{25}C\textsubscript{8} had the peak at around 370nm, compared with Au\textsubscript{25}C\textsubscript{12} and Au\textsubscript{25}C\textsubscript{16} at around 400nm. In initial 200:3 samples the scene was similar. Moreover, the peak shift more and more towards left together with the length of ligand coated on the Au core increasing.
Figure 3.11. UV-vis spectroscopy results of nanodisc with MPCs. (A) Initial molar ratio between lipid and MPCs was 500:1. (B) Initial molar ratio was 200:3. All samples were dried and re-dispersed in benzene followed by a 30-minute sonication. The spectra have been vertically shifted for clarity.
4. Disturbance of nanodisc morphology by encapsulating MPCs

The dependence of encapsulation efficiency on chain length of the ligand showed that Au$_{25}$C$_8$, Au$_{25}$C$_{12}$ and Au$_{25}$C$_{16}$ were better incorporated with lipid bilayer than Au$_{25}$C$_2$ and Au$_{25}$C$_4$ do. However, there were other important issues that may affect the cell uptake process of this novel nanocomplex: morphology, including size and shape$^{43,102,110,111,112,113,114,115}$. For example, our previous result showed that the nanodiscs have a higher cell uptake rate than vesicles do. Therefore, it is equivalently important to understand the morphological variation when Au-MPCs are incorporated.

Previous studies on the nanoparticles uptake within lipid bilayers has suggested that the structure is disturbed by accommodating nanoparticles in the lipid bilayers$^{65,98,116,117}$. The encapsulation of MPCs may cause morphological changes, since the comparable dimension of MPCs to the lipid bilayer thickness (~5 nm) has strong effect on packing parameter of the complex. Likewise, the interaction of hydrocarbon chains associated with Au clusters can interact with the hydrophobic tails of the phospholipids, affecting the formation of nanodiscs. Therefore, it is important to reveal the morphology variation of nanodiscs before and after the MPCs uptake. To address this issue, DLS was used for the measurement of the size of nanocomplex. Small angle X-ray Scattering was able to resolve the morphology parameters such as disc radius and bilayer thickness$^{118}$. Finally, Cryo-TEM provided direct evidence of if the morphology disturbance. Besides, molecular dynamic simulation was also used to mimic the scenario of encapsulation.

1. Size study of nanodiscs and nanovesicles before and after MPCs encapsulation

Morphology is the one of the primary parameters in the characterization since it is important to cellular uptake efficiency. In this project, dynamic light scattering (DLS) was used to measure the size of DPPC nanodiscs with MPCs globally. Previously, a disk-to-vesicle structural transformation upon the elevation of temperature above the melting transition temperature of the long chain lipid in bicellar mixtures has been reported$^{76,119}$ in literature. Here, the structure of the Au-MPC/lipid complex is also evaluated at a higher temperature (50°C) to investigate the effect of Au-MPCs on the
vesiculation. Figure 3.12 shows the representative number-weighted size distribution outcome. All size data was listed in Table 3.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
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<td>7.62nm</td>
<td>13.8nm</td>
<td>13.8nm</td>
</tr>
<tr>
<td>Au_{25}C_4</td>
<td>5.55nm</td>
<td>5.76nm</td>
<td>11.56nm</td>
<td>11.49nm</td>
</tr>
<tr>
<td>Au_{25}C_8</td>
<td>5.72nm</td>
<td>6.52nm</td>
<td>13.57nm</td>
<td>11.29nm</td>
</tr>
<tr>
<td>Au_{25}C_{12}</td>
<td>5.26nm</td>
<td>6.18nm</td>
<td>12.94nm</td>
<td>12.46nm</td>
</tr>
<tr>
<td>Au_{25}C_{16}</td>
<td>5.8nm</td>
<td>6.48nm</td>
<td>12.62nm</td>
<td>12.36nm</td>
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</tbody>
</table>

Table 3. Number-weighted DLS results of lipid with MPCs at 25°C and 50°C. The ratio 500:1 and 200:3 were initial theoretical molar ratio between lipid and MPCs.

Figure 3.12 and Table 3 evidently showed that the size distribution of lipid nanoparticles was influenced in the presence of Au-MPCs at both 25°C and 50°C. At 25°C, the addition of MPCs induces the decrease of nanodisc radius, which was consistent with the Cryo-TEM data. At high temperature (50°C), the DPPC nanodiscs in the absence of Au-MPC were transformed into vesicles. Although Figure 3.12 (C) and (D) indicated that the Au-MPC/lipid complexes (presumably nanodiscs) were enlarged, the vesiculation might not take places since Cryo-TEM images did not show a high population of vesicles. Most likely, these are enlarged nanodiscs or elongated micelles. Further study is needed to confirm the structure. The larger aggregates seem to be less sensitive to the elevation of temperature, indicating they are not strongly affected by the melting of long-chain lipid, DPPC.
Figure 3.12. Number-weighted DLS results of lipid with MPCs at room temperature (A, B, 25°C) and high temperature (C, D, 50°C). The legend at the bottom is generic from (A) to (D). (A) Lipids with MPCs with 500:1 initial molar ratio. (B) Lipids with MPCs at 200:3 initial molar ratio. (C) Lipids with MPCs at 500:1 initial molar ratio. (D) Lipids with MPCs at initial 200:3 molar ratio.
2. Variation of morphological behavior induced by gold MPCs

From DLS results it was clear that the size of nanodiscs were changed. Most of nanodiscs were squeezed a little after letting MPCs in, while some of them formed into large aggregations with sizes above 100nm. However, DLS could only provide the average size information based on spherical assumption. It was still not clear how Au-MPCs affect local structure of the bilayer, e.g., the thickness of nanodiscs, or if they remained in disc-shape. Small Angle X-ray Scattering (SAXS) could resolve the morphology of nanodisc based on well-defined models such as “core-shell-cylinder model” which could mostly describe nanodisc. Information could be obtained such as the size and form of particle, inter- and intra-particle structures by model fitting or by observation. Lipid bilayer can be considered as a lipophilic core (hydrocarbon chains) being sandwiched by two shells (hydrophilic phosphatidylcholine headgroups). Since phosphate group has the highest electron density in the system (in the absence of Au-MPCs) – higher than those of hydrocarbon tails and water, the electron density profile across the bilayer (i.e., water-headgroup shell-hydrocarbon core-headgroup shell-water) can be approximated by a “square well”. As a result, the SAXS pattern leads to a broad peak corresponding to the correlation length of headgroup-headgroup distance as shown in Figure 3.13 (A). Moreover, the slope at low q region could also infer possible morphology of the lipid aggregates. All SAXS results (at both room and high temperature) with best fitting at high q region after appropriate background subtraction were shown in Figure 3.13. The red markers were reduced SAXS data after transmission correction, the blue curves were best fitted Gaussian peaks. In some cases such as Figure 3.13 (G) (I) (P) (R), one cumulative peak is not sufficient to describe the curves. We will apply 2 additive peaks (in red and green) to fit the SAXS patterns.

Figure 3.13 (A-I) showed SAXS results of nanodisc (A) and nanodisc with Au-MPCs (B-I) at room temperature. A transition of slope from $q^2$ to $q^4$ was found indicating that the lipid aggregates were presumably in planar bilayered structure for all cases. One or two high $q$ peaks were found: one of them corresponding to the distance between phosphate headgroups, the other one corresponding to
the Au-Au distances. The peak positions were listed in Table 4. For significant high electron density of headgroup over those of hydrocarbon core and water, the distances between phosphate headgroups can be estimated by the peak position, \( q_{\text{peak}} \). That is:

\[
\lambda = d \cdot \sin \left( \frac{\theta}{2} \right)
\]

\[
q_{\text{peak}} = 4 \cdot \pi \cdot \sin \left( \frac{\theta}{2} \right) / \lambda
\]

So

\[
d = 2 \cdot \pi / q_{\text{peak}}
\]

All estimated distances were also listed in Table 4. After encapsulation, the peaks were shift to right generally, implying the spacing between the headgroups of the bilayer seemingly decreases in the presence of MPCs. Moreover, the distances of 500:1 samples were found larger than those of the 200:3 samples (within the same series), which showed higher encapsulation efficiency based on UV-vis absorption spectroscopic data. For 500:1 samples, the thickness of bilayer seems to decrease with increased encapsulation from \( \text{Au}_{25}\text{C}_4 \) to \( \text{Au}_{25}\text{C}_{16} \). All these observations are apparently against the previous evidence of encapsulation of Au-MPC in the bilayer, thus requiring further analysis on the shift of the peak.

In 200:3 samples, the peak position of lipid/\( \text{Au}_{25}\text{C}_{12} \) was the highest, while all the others were still higher than that of the bare nanodiscs. Furthermore, the SAXS patterns of 200:3 samples containing \( \text{Au}_{25}\text{C}_{12} \) and \( \text{Au}_{25}\text{C}_{16} \) contain sharp peaks significantly different from the broad lipid peaks in shape and position. Those sharp peaks were attributed from the spacing between Au-MPCs consistent with the pure Au-MPC results (Figure 3.14). This result shows that the distance between
Au-MPCs is invariant after being encapsulated by nanodiscs. Such Au-Au peaks may not be significant in all cases depending on the peak locations and compactness of the Au-MPCs in the bilayer.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>LIPID PEAK (q)</th>
<th>NOMINAL DISTANCE (Å)</th>
<th>GOLD PEAK (q)</th>
<th>NOMINAL DISTANCE (Å)</th>
</tr>
</thead>
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<tr>
<td>NANDOISC</td>
<td>0.12067</td>
<td>52.06916</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
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<td>ND_C4_500:1</td>
<td>0.12301</td>
<td>51.07865</td>
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<td>n.a.</td>
</tr>
<tr>
<td>ND_C4_200:3</td>
<td>0.12877</td>
<td>48.79386</td>
<td>n.a.</td>
<td>n.a.</td>
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<td>ND_C8_500:1</td>
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<td>50.48763</td>
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<tr>
<td>ND_C12_500:1</td>
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<tr>
<td>ND_C12_200:3</td>
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<td>37.16981</td>
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<tr>
<td>ND_C16_500:1</td>
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<td>V_C4_500:1</td>
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<td>0.13158</td>
<td>47.75182632</td>
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</table>

Table 4. Peak position of SAXS results on nanodiscs and vesicles with MPCs. ND stands for nanodisc, V stands for Vesicle.
Figure 3.13. SAXS results of lipid with MPCs with fitting at high q region at 25°C (A-I) and 50°C (J-R). Red markers (rural SAXS data after appropriate background subtraction) and blue curves (best fitting of SAXS data at high q region) were shown in each Figure. Two guide lines: \( q^2 \) and \( q^4 \) slope were shown in each figure. (A) DPPC nanodisc. (B) Nanodisc with \( \text{Au}_{25} \text{C}_4 \) at 500:1 initial molar ratio. (C) Nanodisc with \( \text{Au}_{25} \text{C}_4 \) at 200:3. (D) Nanodisc with \( \text{Au}_{25} \text{C}_8 \) at 500:1. (E) Nanodisc with \( \text{Au}_{25} \text{C}_8 \) at 200:3. (F) Nanodisc with \( \text{Au}_{25} \text{C}_{12} \) at 500:1. (G) Nanodisc with \( \text{Au}_{25} \text{C}_{12} \) at 200:3. (H) Nanodisc with \( \text{Au}_{25} \text{C}_{16} \) at 500:1. (I) Nanodisc with \( \text{Au}_{25} \text{C}_{16} \) at 200:3. (J) DPPC vesicle (K) Vesicle with \( \text{Au}_{25} \text{C}_4 \) at 500:1 initial molar ratio. (L) Vesicle with \( \text{Au}_{25} \text{C}_4 \) at 200:3. (M) Vesicle with \( \text{Au}_{25} \text{C}_8 \) at 500:1. (N) Vesicle with \( \text{Au}_{25} \text{C}_8 \) at 200:3. (O) Vesicle with \( \text{Au}_{25} \text{C}_{12} \)
at 500:1. (P) Vesicle with Au_{25}C_{12} at 200:3. (Q) Vesicle with Au_{25}C_{16} at 500:1. (R) Vesicle with Au_{25}C_{16} at 200:3. The red and green curves in (G) (I) (P) (R) were peak of lipid and gold, respectively.

To verify the assumption of sharp peaks coming from Au-MPCs, SAXS was conducted on pure MPC with water. Result showed peaks which located at similar position compared with the curves of lipid with MPCs (Figure 3.14). In the case of nanodisc with Au_{25}C_{12} and Au_{25}C_{16} at 200:3 where sharp peaks were spotted in SAXS curves, similar peaks were also found at same position for MPCs in benzene solution without any lipid, confirming that the sharp peaks originate from MPCs.

Figure 3.14. SAXS Peak verification. (A) Position of Au_{25}C_{12}. (B) Position of Au_{25}C_{16}
At 50°C where vesiculation would happen for bare nanodiscs, SAXS were also conducted in order to investigate how MPCs would affect the transformation from nanodisc to vesicles. The results were shown in Figure 3.13 (J-R). The slope of curve in Figure 3.13 (J) was $q^{-2}$ which could prove that the particles in the absence of Au-MPCs were vesicles composed of bilayer, confirmed by Cryo-TEM micrographs. Uniform round hollow lipid aggregates were observed throughout the image (Figure 3.15). However, for lipid/Au-MPC complex, the vesiculation was not clearly found as in the bare nanodiscs. The slopes of SAXS curves of lipid with MPCs samples were significantly different from that of bare vesicles. Instead, their SAXS patterns were more similar to those of nanodiscs with a slope transition from $q^{-2}$ to $q^{-4}$ (Figure 3.13), implying that the Au-MPCs did significantly affect the vesiculation and most nanodiscs (or elongated micelles) remained their morphology. The phenomenon was also confirmed by the TEM micrographs. For instance, in Figure 3.16 and 17, although the samples had been incubated at 50 °C for overnight, there were still elongated shapes of nanoparticles found in the image. To verify the morphology, several images were taken with the TEM stage rotating from -30° to 30° as shown in Fig 3.18. The object changed from round to rod, indicating that the specific elongated shape was indeed a disk. This may apply to other nanoparticles with elongated shapes.
Figure 3.15. Cryo-TEM result of vesicles at 50°C.

Figure 3.16. Cryo-TEM result of lipid with Au25C4 at 500:1 at 50°C.
Figure 3.17. Cryo-TEM result of lipid with Au$_{25}$C$_{16}$ at 200:3 at 50°C. There were not many hollow round lipid aggregates appeared in the image, which meant the vesiculation was interfered by the appearance of AuMPCs.

Figure 3.18. Stage-tilting cryoTEM of nanodisc with Au$_{25}$C$_{16}$ at high temperature: (a) -30°, (b) 0°, and (c) 30°. The marked object changed from round to rod shape indicated that the object was a nanodisc.
Chapter 4: Discussion and Conclusion

1. Discussion

The Au-MPCs were dissolved in benzene and the solutions were sonicated for half an hour for better dispersion. Then SAXS was used to resolve the size of particles in solution. The result showed that the Au nanoparticles were about 1nm in diameter, which was keeping consistency with both the result coming from the characterization of the sample provider and the molecular dynamic simulation result. It indicated that the 30-minute-sonication achieve best monodispersity of MPCs.

In size characterization section, the size of lipid aggregates with MPCs was measured by DLS. In Figure 3.12, there were usually 2 peaks (sometimes 3) in each of the curves. One is about 10nm and the other is about 100nm. After encapsulation the peaks at 10nm were shifted towards left a little, and the peaks at 100nm became higher. Since the principle of DLS is Rayleigh scattering, the scattered intensity is proportional to the sixth power of the particle size, which is biased immensely to the large particles. In this project usually there are 2 kinds of particles in one system, one is around 10nm and the other is about 100nm in hydrodynamic radius. There are more 100nm particles than 10nm ones at first glance (Figure 3.12). However, the scattering intensity of 10nm particles is about \(1/10^6\) to those 100nm particles. DLS would emphasize the large particles while the small particles around 10nm are still the majority in terms of number. Therefore, the nanodiscs were presumably smaller after encapsulation. Some of the nanodiscs aggregate together and form larger particles.

It is illogical that the distance between phosphate head groups would have been shortened after having MPCs within lipid bilayers. The fact that the right shift of the phosphate-phosphate peak occurs in all samples at both low and high compositions of MPCs needs further explanation. We have found the higher encapsulation of MPCs the more right shift of the peaks. One possible reason would be that the electron density contrast might also influence the peak position when MPCs was encapsulated by lipid bilayer. To verify this assumption, the pure nanodisc scattering pattern was
fitted by PolyCoreShellCylinder model which containing a hydrocarbon core and a phosphate shell. The scattering signal is derived from the average electron density contrast between the solvent and the solute (shell to water, core to water), or between different portions of the solute (core to shell).

Figure 4.1 (A) showed the best fit of the SAXS data of nanodiscs in the absence of MPCs. Since the SAXS data were not corrected with absolute scale, the scale and absolute values of $\rho_{\text{shell}}$ and $\rho_{\text{core}}$ are not supposed to be accurate. Nevertheless, the ratio of \(\frac{(\rho_{\text{shell}}-\rho_{\text{solvent}})}{(\rho_{\text{core}}-\rho_{\text{solvent}})}\) should be accurate. The best fitting parameters also demonstrate a correct relative relationship: $\rho_{\text{shell}} > \rho_{\text{solvent}} > \rho_{\text{core}}$ because the high electron density phosphate group and low volumetric density of acyl chains\textsuperscript{120}. It should be noted that the best fitting bilayer thickness was consistent with the nominal bilayer thickness listed in Table 4, further providing the confidence of our best fitting result. A test of the effect of contrast variation in the core of bilayer on the SAXS pattern were performed by increase the value of $\rho_{\text{core}}$ as expected when MPCs are incorporated in the bilayer. Figure 4.1 (B) showed that the calculated SAXS pattern when the electron density of the bilayer core was increased to similar level of $\rho_{\text{solvent}}$ with all the other parameters remaining the same values. The broad peak corresponding to phosphate-phosphate distance shifted towards right simply by increasing $\rho_{\text{core}}$. Such observation provides further evidence of the right shift possibility originates from the contrast instead of the decrease of the bilayer thickness.
Figure 4.1. Contrast effect on peak position of SAXS scattering curve. (A) Best fitting of nanodisc SAXS pattern. (B) Fitting result of nanodisc SAXS pattern when electron density of core was increased.

2. Conclusion

Since gold nanoparticles are very promising candidates in variety of biomedical applications due to their advantages such as high contrast, easily being modified and being conjugated, low toxicity and some photothermal properties, the utilizing of gold nanoparticles has become a more and more popular research topic. Although it is safer to organism than quantum dots or other polymer particles, the cytotoxicity is still a potential problem since it is related to the dosage\textsuperscript{121} or the way of surface modification\textsuperscript{51,122}. Model lipid membrane becomes a good carrier since it could help the delivery and improve the in vivo stability of gold nanoparticles\textsuperscript{63,64,65,66}. The cell uptake efficiency are relying on variety of features including size, shape, surface charge state and modification. This project focuses on the encapsulation of MPCs by nanodiscs and the change in size and shape of the carrier. The results showed that the MPCs were encapsulated in the lipid aggregates indeed, by the appearance of lipid supernatant as well as the TEM pictures. Furthermore, the encapsulation efficiency was proved to be correlated with the length of thiolate chain coated on the surface of gold clusters. The longer chain length was, the higher encapsulation efficiency was. However, it seems that the encapsulation
efficiency approached to a saturation value at high MPCs concentration. The efficiency of initial molar ratio samples were similar within Au$_{25}$C$_8$, AU$_{25}$C$_{12}$ and Au$_{25}$C$_{16}$ but higher than Au$_{25}$C$_4$ and Au$_{25}$C$_2$ samples. The characterization of morphology was done by DLS and SAXS, and the results were confirmed by Cryo-TEM. After encapsulating MPCs, most of nanodiscs remained their disc shape or changed to cylinder micelles, while their sizes were smaller than the size of bare nanodiscs, although some of large aggregates were also observed. The temperature-induced vesiculation of nanodiscs was not clearly observed in the case of loaded nanodiscs indicating that the encapsulation of MPCs hampered the vesiculation. The SAXS result indicates possible significant change in the electron density of the bilayer hydrocarbon core further confirm the encapsulation of the Au-MPCs. Moreover, the traditional wisdom of estimation of bilayer thickness from $q_{\text{peak}}$ breaks down due to the variation of contrast in the bilayer. A detailed model fitting is needed for obtaining the structural information of bilayer thickness.
Reference


