NIR Light Activated Hollow Gold Nanoshell Structures Based on Layersome Template

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NIR LIGHT ACTIVATED HOLLOW GOLD NANOSHELL STRUCTURES BASED ON LAYERSOME TEMPLATE

BY

YASER KASHCOOLI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMICAL ENGINEERING

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OF

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ABSTRACT

In biomedical applications, nanocarriers provide specific advantages such as delivery and sensing. These nanocarriers can be made from different sources including biopolymers and lipids. On the other hand, stimuli-responsive nanocarriers provide a safe method for drug delivery applications. These carriers use different triggers (including temperature) to release the drug. Light can be used as an external trigger to increase the temperature and finally releasing the drug. This thesis describes the development of functionalized liposomes via using different polyelectrolyte coatings to template the synthesis of light activated nanocarriers.

In the first part, layer-by-layer coating of liposomes with strong biopolyelectrolytes was examined to have a better understanding of structure-property relationship of polyelectrolytes and layersome behaviors. The stability study of the structures in NaCl solutions with different concentrations demonstrate that the stability behavior depended on the outer layer coating. Samples with positive outer layer show more stability in salt solutions compared to the samples with negative outer layer. For the second part, near-infrared (NIR) active gold nanostructures on hollow spherical soft templates were prepared by using wet chemistry method. Light scattering, spectroscopy and imaging techniques were used to examine morphology and NIR activity of hollow gold nanoshell structures based on layersome template. The results show by using layersome, small hollow gold nanoshell structures with NIR activity could be formed.

All in all, these studies show a method to change the lipid ordering through coating process, alter the stability just by changing the outer layer of liposome and also an easy procedure to make a substrate for stimuli-responsive nanocarriers.
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PREFACE

This thesis was prepared in manuscript format. Chapter 1 with the title of “Layer-by-layer coating of liposomes with strong biopolyelectrolytes yields heterogeneous multilayers“ is currently in preparation for submission to Langmuir journal. In this chapter soft layered structures based on liposome and polyelectrolytes were investigated.

Chapter 2 with the title of “NIR Light Activated Hollow Gold Nanoshell Structures Based on Layersome Template” will be submitted to Biomaterials journal. In the second chapter, NIR active drug delivery vehicles were developed based on the layered template.
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CHAPTER 1

Layer-by-layer coating of liposomes with strong biopolyelectrolytes yields heterogeneous multilayers

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1. Abstract

Layer-by-layer deposition of polyelectrolytes (PEs) onto self-assembled liposomes represents an alternative to PE deposition on solid particles for the formation of hollow nanoscale capsules. PE-coated liposomes (referred herein as layersomes) reported in the literature display the typical charge inversion behavior that accompanies the deposition of sequential oppositely charged PE layers. However, liposomes are soft, dynamic templates that can be distorted or disrupted by adsorbing PEs. In this work we show that sequential deposition of dextran sulfate-sodium salt (DxS\(^-\)) and poly-L-arginine (PA\(^+\)) onto cationic liposomes does yield the expected charge inversion, however, cryogenic transmission electron microscopy (cryo-TEM) results show that the layersomes formed (up to \(\sim\)200 nm), and their PE coatings, were heterogeneous. This was due to the formation of PE complexes (PECs) when an even number of layers were deposited (PA\(^+\) onto DxS\(^-\)). Some of the PECs desorbed from the layersome surfaces, while many remained attached as patches and were coated by the next PE layer forming layersome-PEC clusters. This behavior was confirmed through fluorescence anisotropy measurements of liposome (bilayer) fluidity, where PA\(^+\) counteracted the ordering effects of DxS\(^-\) on the lipid bilayer through charge neutralization and PEC desorption. With increased charge screening, DxS\(^-\) desorbed from the layersomes, while the layersomes terminating in PA\(^+\) retained their PE coatings. To our knowledge this is the first time such layersome structures have been observed with biopolypelectrolytes.
2. Introduction

Layer–by–layer (LbL) deposition is a versatile technique for creating multilayer micro- and nano-structured materials. LbL technique is based on sequential surface deposition of opposite charged macromolecules, typically polyelectrolytes (PEs), on to a charged substrate to create a self-assembled coating [1]. By using particle templates, it is possible to create multifunctional polymeric capsules with tailored surface functionality and barrier properties.[2-7] The advantages of using LbL technique to create capsules include the ability to control the chemical, physical, and mechanical properties of the capsules by using different materials in capsule wall[2]; the ability to tailor the capsule wall charge and morphology by varying the terminal layer or the assembly conditions (for example, temperature, pH, and salt concentration);[8-10] and the ability to encapsulate macromolecules.[11, 12]

Capsules prepared by LbL technique are typically formed by depositing PEs on solid or porous inorganic particles as sacrificial templates that can be dissolved under acidic conditions. An alternative capsule template is liposomes, which have been used extensively in the areas of biomedicine, particularly drug delivery.[13, 14] Liposomes are self-assembled phospholipid vesicles that have a bilayer membrane structure with an internal aqueous phase core. They can encapsulate both hydrophilic and hydrophobic compounds and, when used as capsule templates, liposomes are layered with PEs (the structures are referred to as layersomes) and remain a functional component of the capsule wall.[15-17] This approach has been used to create layersomes for drug delivery.[18, 19] When formed with biologically-based PEs, including naturally derived PEs[20, 21] and polypeptides,[15, 16, 22] layersomes can
provide a nanoscale colloidal capsule that is biocompatible and biodegradable, and are
often more stable than to bare liposome. It is generally observed that adding each PE
layer leads to charge reversal and increases layersome size, capsule wall thickness and
stiffness, and capsule barrier properties that resist spontaneous leakage.[23, 24]
A key aspect to creating layersome capsules and understanding their colloidal stability
is determining how competition between PE–liposome and inter–PE interactions
affects layersome structure. Volodkin et al.[22] investigated the interaction of poly-L-
lysine (PLL) coated dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol
(DPPC/DPPG) liposomes with polyanions of varying pKₐ and charge density
including (poly-(4-styrenesulfonate), PSS; poly-L-glutamic acid, PGA; hyaluronic
acid, HA). PSS with a low pKₐ (below 1) and high charge density led to complete PLL
desorption, and PGA with a high pKₐ (around 5) and a lesser charge density than PSS
led to partial PLL desorption. Partial PLL desorption led to surface charge
heterogeneity and layersome aggregation, which was described based on patch-charge
attraction and was used to create stable clusters of single-layered layersomes in
electrolyte solutions.[25-28] In contrast, HA with a lower charge density yielded
stable layersome capsules because PLL–liposome interactions were stronger than
PLL–HA interactions. The competition between PE–liposome and inter–PE
interactions has not been thoroughly investigated for other PEs or multilayered
structures. An understanding of this competition could be used to predict and control
layersome behavior, and may provide new routes for designing unique layersome
structures.
In this work layersome structures were formed using cationic liposomes composed of dioleoylphosphatidylcholine (DOPC) and dioleoyltrimethylammonium-propane (DOTAP), coated with alternating layers of dextran sulfate (DxS\(^-\)) and poly-L-arginine (PA\(^+\)) (Figure 1-1). DxS\(^-\) and PA\(^+\) were chosen because they are FDA approved and have been used to create capsules for therapeutic applications via LbL deposition on solid particles. DxS\(^-\)/PA\(^+\) microcapsules prepared using calcium carbonate particle templates are reported to be biodegradable and biocompatible *in vivo* \[29\], and are capable of encapsulating proteins. \[12\] They have also been shown to activate pulmonary antigen presenting cells \[30\] and achieve immune-activity by targeting antigens to dendritic cells. \[31\]

![Schematic depicting the formation of layersomes via LbL using DOPC/DOTAP liposomes as the template (not to scale). Dextran sulfate (DxS\(^-\)) is shown in blue and poly-L-arginine (PA\(^+\)) is shown in red.](image)

DxS\(^-\) and PA\(^+\) were also chosen for layersome formation to determine polyelectrolytes influence on layersome structure, and how the terminating PE layer controls layersome stability. The linear charge densities of DxS\(^-\) and PA\(^+\) used in this study were 0.46 Å\(^-1\) \[32\] (2.2 Å charge spacing) and 0.035 Å\(^-1\) (29 Å charge spacing) based on the van der Waals radius of the monomers, the charge per monomer, and complete
dissociation or association of the charged groups, respectively: –OSO \textsubscript{3}Na \rightarrow –OSO \textsubscript{3}^- + Na^+ for DxS\textsuperscript- and =NH + H\textsuperscript+ \rightarrow =NH\textsuperscript3 + for PA\textsuperscript+. In this study, washless method was used to prepare layersome by using dextran sulfate and poly-l-arginine which yield sub-200 nm diameter nanoscale capsules with different morphologies and layer structures, including patchy layersomes with surface-anchored polyelectrolyte complexes (PECs). Changes in layersome structure and colloidal stability were examined as a function of sodium chloride (NaCl) concentration. This approach reflects emerging interest in the ability to control the size, shape, and morphology of PE capsules.[33]

3. Materials and methods

3.1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids, Inc. (Alabama, US). Dextran sulfate sodium salt from *Leuconostoc mesenteroides* (DxS\textsuperscript-, 6,500–10,000 MW), poly-l-arginine hydrochloride (PA\textsuperscript+, 5,000–15,000 MW) and diphenylhexatriene (DPH) were purchased from Sigma-Aldrich Company (Missouri, US). The manufacturer’s specification for the average number of sulfate (SO\textsubscript{3}^-) groups per glucose unit was 2.3. All materials were used as received.

3.2. Liposome preparation

Liposomes were prepared in deionized (DI) water at 10 mM total lipid at a DOPC/DOTAP ratio of 1:1 using a rotary evaporator. The lipids in chloroform were
placed in a round-bottom flask and the chloroform was evaporated at 50 °C at 450 mbar for 30 min, then 300 mbar for 30 min, and finally 200 mbar for 30 min. The flask containing a dry lipid film was placed under vacuum for 8 h to remove any residual solvent. The film was hydrated with deionized water and the formed liposomes were diluted by a factor of 10 (from 10 mM to 1 mM) and extruded through 100 nm track-etched polycarbonate membranes to yield small monodispersed unilamellar liposomes. For the samples which include DPH for fluorescence anisotropy measurements, DPH in tetrahydrofuran was added to DOPC and DOTAP in chloroform at a DPH:lipid molar ratio of 1:500 before placing in the rotary evaporator.

3.3. Layersome preparation

A washless method was used to form the layersomes using polyelectrolyte solutions prepared in DI water at 0.05% w/w. This method has been used previously for solid particles.[34] Layersomes were formed by sequentially titrating the liposomes (layer 1) with an initial lipid concentration of 1 mM, or layersomes (layers 2–4) with the respective polyelectrolyte solutions (Figure 1-1). Each layer was coated under stirring for 5 min at room temperature. For each layer, titrations were first carried out beforehand to determine the correct amount of polyelectrolyte to add at each step that would yield a complete coating, similar to the technique used by Cuomo et al.[21] During each titration, the zeta potential was continuously measured as a function of the polyelectrolyte concentration. The point at which the zeta potential began to plateau was taken as the final polyelectrolyte concentration for layersome formation (Figure 1-8).
3.4. **Dynamic light scattering (DLS)**

Hydrodynamic diameter \( (d_h) \) and zeta potential \( (\zeta) \) were measured using a Malvern Instruments Zetasizer Nano ZS with a backscattering detector angle of \( 173^\circ \) and a 4 mW, 633 nm He-Ne laser. To determine \( d_h \), 1 ml of the sample was placed in an optical grade polystyrene cuvette at 25 °C. The reported hydrodynamic diameters are based on 15 scans measured in triplicate for each sample. During the measurements, correlation function is used to convert the obtained light intensity graph to correlation coefficient graph. Diffusion coefficient \( (D) \) is obtained by fitting the correlation function \( (G(\tau)) \) with a suitable algorithm according to the following formula:

\[
G(\tau) = B + \sum A e^{-2q^2D\tau}
\]

Zeta potential was measured by combined Doppler electrophoretic velocimetry and phase analysis light scattering based on the Smoluchowski theory [35]. Measurements were performed using 1 mL samples at 25 °C. It should be noted that the \( d_h \) and \( \zeta \) interpretations were based on layersome diffusion assuming spherical particles. Layersome shape and the conformation of the PE coatings can strongly affect these measurements by altering the slip plane. Hence, \( d_h \) and \( \zeta \) reflect the average values for equivalent spheres.

3.5. **Cryogenic Transmission Electron Microscopy (cryo-TEM)**

Cryo-TEM samples were prepared at 25 °C using a Vitrobot (FEI Company), which is a PC-controlled robot for sample vitrification. Quantifoil grids were used with 2 µm carbon holes on 200 square mesh copper grids (ElectronMicroscopySciences, Hatfield, PA). To prepare a sample, it was equilibrated within the Vitrobot at 25 °C and 100%
humidity for 30 min. After immersing the grid into the sample, it was then removed, blotted to reduce film thickness, and vitrified in liquid ethane. The sample was then transferred to liquid nitrogen for storage. Imaging was performed in a cooled stage (model 626 DH, Gatan Inc., Pleasanton, CA) at 200 kV using a JEOL JEM-2100 TEM (Peabody, MA).

3.6. Bilayer fluidity measurements

DPH anisotropy of the samples as a function of temperature was measured by using a Perkin Elmer LS 55 fluorescence spectrometer. The excitation wavelength and the detection wavelength were set at $\lambda_{\text{ex}} = 350$ nm and $\lambda_{\text{em}} = 452$ nm, respectively, and the excitation and emission slit widths were set at 10 nm. Anisotropy was calculated from the following equation

$$< r > = \frac{(I_{VV} - I_{VH})}{(I_{VV} + 2I_{VH})}$$

where I is the fluorescence emission intensity, and subscripts V and H represent the vertical and horizontal orientation, respectively, of the excitation and emission polarizers.[36]

4. Results and Discussion

4.1. Layersome formation and characterization

Unilamellar cationic DOPC/DOTAP liposomes were used as the initial template for layersome formation. The layer number for layersome structures is denoted as $L_n$ where $n$ is 1 to 4; $L_1$ and $L_3$ were terminated with $\text{DxS}^-$, and $L_2$ and $L_4$ were terminated with $\text{PA}^+$. The weight percent of layersomes compounds and also charge ratio for the samples are shown in Table 1-1. The liposomes were in their fluid phase at room temperature with a $d_h$ of 133 nm and a $\zeta$ of +53 mV. A low polydispersity
index (PDI = 0.08 ± 0.01) indicated that the liposome templates had a narrow size distribution. It should be noted that the polydispersity index range for this DLS equipment is from 0 to 1 related to mono-disperse to highly-disperse sample, respectively. Liposome $d_h$ and lamellarity was confirmed by cryo-TEM analysis, and the average bilayer thickness was approximately 4 nm (Figure 1-2a).

| Table 1-1. Layersome composition in deionized water. |
|----------------------------------------------------|
| Sample $^a$ | wt% $(10^{-2})^b$ | charge ratio$^{b,c}$ |
|------------|-------------------|-------------------|
| Liposome   | 7.42              | 0                 | 0                 |
| D$x^-$/$L1 | 7.42              | 2.25              | 0                 | 2.84              |
| $P^+/L2$   | 7.41              | 2.25              | 2.00              | 1.25              |
| D$x^-$/$L3 | 7.41              | 4.74              | 2.00              | 1.68              |
| $P^+/L4$   | 7.41              | 4.74              | 4.99              | 1.23              |

$^a$L$n$ refers to the layer number ($n = 1–4$)

$^b$Based on the total amount of lipid, D$x^-$, and $P^+$ in each layersome sample.

$^c$Assumes complete dissociation for the strong polyelectrolytes.

Layersomes were formed in DI water with alternating coatings of D$x^-$ and $P^+$ via electrostatic attraction. A schematic of the process reflecting the structures formed is shown in Figure 1-1. The structures of the layersomes were investigated by cryo-TEM (Figure 1-2). The first layer, D$x^-$/$L1, yielded layersomes with a uniform coating approximately 9 nm thick. Also evident was layersome aggregation (for example, a
layersome “dimer” is shown in Figure 1-2b). Based on previous work for polyelectrolytes with high linear charge density adsorbing to oppositely charged liposomes,[37] it is likely that DxS⁻ adsorption led to lipid exchange where the cationic DOTAP lipids accumulated in the outer liposome leaflet in contact with DxS⁻.

The second layer, PA⁺/L2, led to the formation of patches of DxS⁻:PA⁺ PECs on the layersome surface (Figure 1-2c). Layersome clusters, defined as layersomes linked by or sharing a common PE coating, were also observed. The layersomes were no longer uniformly coated and in some cases the underlying liposome template can be observed. Although the images show that most PECs are attached to liposome surface, but there are also some free (unattached) PECs in the solution (Figure 1-2f). These free PECs can be due to the desorption of formed PECs from liposome surface and/or separate PECs formation in the solution.

The third layer, DxS⁺/L3, coated the structures and there was evidence of the DxS⁺/L3 layer covering the PECs resulting from the second layer, PA⁺/L2 (Figure 1-2d). For the final coating, PA⁺/L4, PEC patches were observed, but we cannot conclude if this was due to the formation of additional PECs or PA⁺ simply coating layersomes that already contained PECs formed with PA⁺/L2 (Figure 1-2e). Also similar to the 2 layer structure (PA⁺/L2), there are free PECs in the solution but for this sample, the number of them is less than PA⁺/L2 sample (Figure 1-2g).
Figure 1-2. (a-g) Representative cryo-TEM images of the liposome and layersome structures formed in deionized water: (a) DOPC/DOTAP liposomes, (b) DxS+/L1 layersomes, (c,f) PA+/L2 layersomes, (d) DxS+/L3 layersomes, and (e,g) PA+/L4 layersomes. Scale bars = 100 nm, (h) Change in layersome diameter over time, and (i) Change in layersome zeta-potential over time.
Additional evidence of patch formation is shown for PA+/L2 layersomes (Figure 1-3; corresponding to Figure 1-2c). As it is shown in Figure 1-3, most of the observed layersomes contain PEC patches, but their structures differ. Layersomes labeled 1 show a single patch with the remaining layersome coated by a PE layer approximately 5-10 nm thick. The layersome labeled 2 shows a PEC patch and since by adding the second polyelectrolytes to system, these structures were observed, we assumed that they have been formed from some of the adsorbed PE and the underlying liposomal bilayer can be seen. The structure labeled 3 show two layersomes bridged by the patches. The patch diameters based on all clearly defined patches in Figures 1-1 and 1-2 ranged from 26 to 56 nm with an average of 36 ±10 nm (standard deviation of 10 patches).
Figure 1-3. Cryo-TEM of layersomes with a PA+/L2 coating containing surface-bound PECs. Labels 1, 2, and 3 show single PECs, a PEC where the layersome bilayer is observed, and a PEC bridging two layersomes, respectively.

Layersome $d_h$ and $\zeta$ were examined over time. Results for $d_h$ show that the layersomes remained dispersed over 30 days (Figure 1-2h). Given that there was evidence of PEC formation and layersome clustering based on cryo-TEM, and that $d_h$ corresponding to the cryo-TEM samples (cryo-TEM samples were prepared 1-2 days after making layersomes) did not increase linearly with layer number, we cannot use $d_h$ results directly to determine layer thickness. Zeta potential results show that each layer led to charge reversal consistent with charge overcompensation (Table 1-1; $n^-/n^+$ and $n^+/n^-$) and that the layersome charge also persisted over 30 days (Figure 1-1d). Based on $|\zeta|$, electrostatic stabilization is likely what prevented aggregation.
4.2. PE-liposome interactions based on lipid bilayer fluidity

Wang et al.[38] have reported that PE multilayers restructure on lipid monolayers to optimize their interactions with the lipids. We extend this concept to layersomes to determine how PE-lipid and inter-PE interactions changed lipid ordering within the liposome template. Lipid ordering was determined based on DPH anisotropy, \(<r>\), where \(<r>\) is inversely proportional to fluidity; a ‘high’ \(<r>\) reflects a ‘high’ degree of lipid ordering or a ‘low’ degree bilayer fluidity, and vice versa. Measured values for \(<r>\) reflect an average membrane fluidity assuming that the distribution of DPH within the liposomes was independent of lipid ordering.[39] Results are shown as the change in anisotropy at 25 °C between the layersomes and the liposome template; \(\Delta <r> = <r>_{\text{layersome}} - <r>_{\text{liposome}}\), where \(<r>_{\text{liposome}} = 0.097\). Based on cryo-TEM results that show that PEC formation leads to regions of ‘bare’ liposome template, changes in \(<r>\) should capture this phenomenon that occurred when PA\(^-\) was deposited on DxS\(^-\) layersomes.

The first DxS\(^-\) layer (L1) led to a two-fold increase in \(<r>\) due to lipid ordering driven by electrostatic attraction between DxS\(^-\) and the lipids, notably DOTAP\(^+\) (Figure 1-4). The deposition of PA\(^+\) (L2) counteracted the effects of DxS\(^-\) on lipid ordering due to charge neutralization which reduced the strength of the DxS\(^-\)-liposome interaction. Also due to PECs desorption from liposome surface, some parts of layered structure do not have coating which leads to lower \(<r>\) value compared to layer 1 structure.

Layers 3 and 4 followed this same pattern where DxS\(^-\) (L3) increased lipid ordering and PA\(^+\) (L4) decreased it. An even number of layers with strong inter–PE interactions coincide with the formation of PEC patches. As shown by cryo-TEM, patch formation
and also PECs desorption from liposome surface exposed regions of uncoated bilayer that would exhibit similar $<r>$ values to bare liposomes. In this case $<r>$ reflected the average lipid ordering of bare regions (‘low’ $<r>$) and regions with bound PECs (‘high’ $<r>$). These results provide further evidence that PECs formed as a result of inter-PE interactions.

![Figure 1-4](image)

Figure 1-4. Changes in the lipid bilayer fluidity of layersomes based on DPH anisotropy as a function of layer number. Error bars represent standard deviation of duplicate samples. Inset: cartoon schematic of DPH within a lipid bilayer.

4.3. Layersome response to increasing ionic strength

To probe layersome stability, the zeta potential and the hydrodynamic diameter of each layersome was examined as a function of NaCl concentration (Figure 1-5). Layersomes terminating in PA$^+$ retained a high $|\zeta|$ compared to DxS$^-$, similar to the liposome template. Layersomes terminating with DxS$^-$ showed a significant reduction
in $\zeta$ despite having a similar initial $\zeta$ as layersomes terminating with PA$^+$ in deionized water (Figure 1-2i). Results for $d_h$ show that the liposomes were stable against aggregation over the entire range of [NaCl]. For the layersomes, aggregation or clustering with increasing [NaCl] was dependent on the terminal PE layer; cationic layersomes terminating in PA$^+$ (L2 and L4) began aggregating at 50 mM NaCl, while anionic layersomes terminating in Dxs$^-$ (L1 and L3) began aggregating at 10 mM NaCl. Above these NaCl concentrations large clusters were observed ranging from approximately 400-3,000 nm with PDIs from 0.4-0.9.
Figure 1-5. Layersome zeta potential (ζ, a) and hydrodynamic diameter (dh, b) and as a function of layer number and NaCl concentration. Cationic liposomes and layersomes are shown in red and anionic layersomes are shown in blue.

...DPH anisotropy was again used to determine the effects of increasing [NaCl] on PE-lipid interactions. Results for 0, 10, and 100 mM [NaCl] are shown in Figure 1-6.

With increasing [NaCl], Δ<r> decreased when DxS⁻ was the terminating layer. This could be attributed to Na⁺ condensation and reduced electrostatic attraction to the liposome (L1) or layersome (L3) and/or desorption of DxS⁻. There was no significant change in <r> when PA⁺ was the terminating layer (L2 and L4) which repelled Na⁺ and prevented the DxS⁻ counterions from diffusing into the PE layers.
Layersomes terminating in DxS\(^-\), L1 and L3, were more prone to surface charge neutralization and aggregation in the presence of NaCl. The significant reduction in $|\zeta|$ up to 10 mM NaCl followed by a plateau with increasing [NaCl] is consistent with counterion (Na\(^+\)) condensation.\cite{32,40} Yamaguchi et al.\cite{41} observed similar behavior for 500k MW DxS\(^-\) alone in water based on electrophoretic mobility measurements. To examine this further we performed DLS on the PEs as a function of [NaCl]. Na\(^+\) condensation reduced the diffusion coefficient of DxS\(^-\) based on the extended time lag in the correlogram (Figure 1-9a). The time lag was significant and independent of NaCl concentration because NaCl was in excess relative to DxS\(^-\). In contrast, the correlogram for PA\(^+\) was less sensitive to NaCl concentration and, compared to DxS\(^-\), a modest shift in the time lag was observed (Figure 1-9b). We can conclude that DxS\(^-\) layers condensed Na\(^+\) and that condensation was responsible for
the decrease in the ζ of the layersomes terminating in DxS− that led to aggregation. DPH anisotropy results further suggest that DxS− desorption may have occurred, which would have also caused the reduction in surface charge that led to aggregation. The schematics of layersome behavior in salt solution are shown in Figure 1-7.

![Figure 1-7. Schematics of layersome behavior in salt solutions.](image)

5. **Conclusions**

We have shown that a washless method can be used to create multilayered layersomes with DxS− and PA+ polyelectrolytes. In deionized water, electrostatic ‘coupling’ between PEs, when PA+ was the terminal layer, led to the formation of nanoscale PECs. Most of these PECs remained anchored to the liposome surface and some of them were desorbed from liposome surface. This appears to be a feature of the strong PE pair and to our knowledge such structures have not been reported for layersomes prepared by LbL deposition. We assumed that due to stronger affinity between the polyelectrolytes compared to liposome-polyelectrolytes, the patchy structures are formed and so some parts of layersomes are without polyelectrolytes so those parts
have bare liposome anisotropy. Also PECs desorption from liposome surface leads to
inhomogeneous liposome surface coating which results in decreasing anisotropy
compared to coated structures.

Fluorescence anisotropy results suggest that PEC formation occurred when the
adsorption of PA\(^+\) weakened the interaction between DxS\(^-\) and the liposome template.
In the presence of NaCl the interactions between the layers and the liposome, and the
stability of the layersomes, was dependent upon the terminating PE layer. Layersomes
terminating in PA\(^+\) exhibited zeta potentials >20 mV, and charge screening in high
[NaCl] likely drove aggregation. For these layersomes, the interaction between the
layers and the liposome was only modestly affected by [NaCl], indicating that the
layers remained intact on the liposome surface. Additional work is needed to
determine if the PEC patches remained present in NaCl. In contrast, for layersomes
terminating in DxS\(^-\), counterion condensation led to very low zeta potentials, probable
DxS\(^-\) desorption, and significant aggregation.

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TEM imaging.
Figure 1-8. Zeta potential as a function of the amount of added polyelectrolyte to oppositely charged liposomes (a, layer 1) or layersomes (b, layer 2; c, layer 3; d, layer 4). The saturation point is denoted on each graph.
Figure 1-9. Normalized correlation coefficients from DLS for the polyelectrolytes (a) DxS- and (b) PA+ in water as a function of [NaCl] (shown in the legend). The PE concentration was 0.05 wt%.
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CHAPTER 2

NIR Light Activated Hollow Gold Nanoshell Structures Based on Layersome Template

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1. Abstract

Plasmonic active gold nanostructures have applications in imaging and cancer heat treatment. The combination of these structures with drug carrier agents for example liposomes, can be used to create controlled release drug delivery systems. In this work, near-infrared (NIR) active gold nanostructures on hollow spherical soft templates were prepared by using wet chemistry method. The resultant structures are approximately 200 nm and have an absorption peak in NIR range (650-900 nm). The prepared structures are suitable vectors for therapeutics and can be used for targeted delivery systems.

2. Introduction

Photothermal therapy refers to the methods that use electromagnetic radiation to treat diseases such as cancer by increasing the temperature of the cancerous cells [1]. Metallic nanostructures have been widely used in the development of external triggered drug delivery systems [2], [3]. Among these nanostructures, plasmon resonant nanostructures have been used for drug release using localized heat via light exposure [4]. Plasmon resonant nanostructures are made of noble metals and show enhanced absorption and scattering due to the collecting oscillation effect. This is a phenomenon that results from the interaction between electric field of a specific wavelength light and the free electron of a metal that leads to metal free electron oscillation and subsequent absorption and scattering [5]. Plasmon resonant gold nanostructures are attractive for a range of biomedical applications due to their biocompatibility, stability and light absorption properties [6]–
The absorption behavior of these nanostructures, which include nanoparticles, nanorods, nanoshells, nanocages, and nanostars, depends on shape, size, aspect ratio, core material, shell thickness and particle diameter [9]. One advantage to the use of plasmon resonant nanostructures in this application is their potential to enable precise heating, thereby avoiding damage to nearby healthy cells.

Liposomes are self-assembled phospholipid vesicles that have a bilayer membrane structure with an internal aqueous core. These structures are suitable vectors for both hydrophilic and hydrophobic entities [10], [11]. The merging of the liposome with the gold nanostructure is an attractive concept for light-triggered drug delivery systems as well as cancer heat treatment systems.

The combination of the NIR active gold nanostructures with liposomes, have attracted much interest for drug delivery applications. The main release mechanisms are liposome phase transition by way of a temperature increase or liposome rupture due to nanobubble formation induced by pulsed laser [12][13]. These two release mechanisms can be used in conjunction. Four different configurations exist for the construction of these types of delivery systems.

2.1. Gold-liposome suspensions

In this configuration, the therapeutic is loaded inside of the liposome. Polymer and non-attached NIR light absorbent gold nanoparticles are also present in suspension. The polymer is attached to liposome surface. Under NIR light irradiation and due to plasmon resonant gold nanoparticles, the temperature of the solution increases and the polymer contracts when the solution temperature reaches a certain value. The polymer contraction imposes mechanical stress on liposome, ruptures the liposome wall and the
therapeutic is released. Beside the polymer action, the temperature can also cause phase transition in liposome and subsequently drug release (Figure 2-1) [14]. Gold nanoparticles are formed separately and then added to the solution and since the gold nanoparticles are not necessarily attached to liposome surface, compared to the situation where the nanoparticles are attached to liposome, for heating up the polymers, more radiation energy is required.

![Figure 2-1. Schematic of gold-liposome suspensions and its release mechanism [14].](image)

### 2.2. Gold nanostructures-liposome composites

In these structures, NIR-sensitive gold nanostructures (gold clusters, nanoshells, nanorods, etc) are attached to the liposome surface. By NIR light irradiation of these structures, the liposome content (payload) will be released (Figure 2-2). Similar to
previous configuration, gold is usually formed through a separate step [15][16]. The main differences between this configuration and the previous one are the absence of temperature sensitive polymer and also direct attachment of gold nanostructures to liposome surface.

![Diagram of gold nanostructures-liposome composites](image.png)

**Figure 2-2. Schematics of gold nanostructures-liposome composites [18].**

### 2.3. Encapsulated gold nanostructures in liposome core

This type of structure is similar to gold nanostructures-liposome composites but with one difference. In this configuration, the NIR-sensitive gold nanostructures, including hydrophilic gold nanorods, gold nanoparticles and hollow gold nanoshells, are inserted into liposome core [17][18]. So in the case of loading inside the liposome core, direct contact between gold and payload is possible.

### 2.4. Gold nanoshell
Despite the other configurations that the gold nanostructures are formed in a separate process, in this configuration, gold is formed directly on liposome surface as a coating. These structures combine optical properties of the coating (plasmon resonant shell) and biodegradability and encapsulation properties of liposome. Due to the fact that the gold is attached to liposome surface, the generated heat by light can be transferred to liposome in an efficient way [19] [20].

Since in all the configurations, formed gold structure is not continuous and they are formed on liposome surface which have leaky nature, the idea of using layersome as a coated liposome to improve leak resistant material for the substrate of gold nanoshell structure was considered [21]–[23].

In this work, by using poly-L-histidine as a FDA-approved polyelectrolyte, the efficacy of using a layersome as a template for NIR light active gold coated hollow soft structures, gold nanoshells, was investigated and the formation of gold nanoshells with NIR absorption capability on two different substrates, liposome and layersome, was evaluated. On the other hand, there are only few studies about in-situ gold nanoshell formation on soft structures, so to have a better understanding of this process and dominant parameters, this study was done. To achieve the goals, three sets of samples (L1, L2 and L3) were considered. For the first series (L1), the main hypothesis was to use PLH affinity for gold through histidine-gold binding to make gold nanoshells. This affinity plus charge attraction between PLH and gold ions help in gold ion attraction to the liposome surface. For the second series (L2), regarding the strong affinity of gold to sulfur, the idea of using PEG-thiol with liposome to absorb gold was considered. It was mentioned that the strong covalent between gold and thiol
group could lead dense gold nanoparticles network formation [33], [34]. PEG-thiol also helps bind gold and improve steric stabilization after formation. For the third series (L3), the idea of applying PEG-thiol in the presence of PLH was considered. The hypothesis behind this idea was to use the charge attraction between PLH and PEG-thiol to have more PEG-thiol on the layersome surface and to determine the synergistic effect of PEG-thiol and PLH on gold shell formation.

3. Materials and methods

3.1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) were purchased from Avanti Polar Lipids, Inc. (Alabama, US). Poly-l-histidine (5,000–25,000 MW) (PLH), Gold(III) chloride trihydrate (HAuCl₄·3H₂O), Ascorbic acid and Poly(ethylene glycol) methyl ether thiol (6,000 MW) (PEG-thiol) were purchased from Sigma-Aldrich Company (Missouri, US). All materials were used as received.

3.2. Liposome preparation

Liposomes were prepared in deionized (DI) water at 10 mM total lipid concentration at a DOPC/DOPG mole ratio of 1:1 via thin film hydration method. The lipids in chloroform were placed in a round-bottom flask and the chloroform was evaporated at 50 °C at 450 mbar for 30 min, then 300 mbar for 30 min, and finally 200 mbar for 30 min using rotovaporator. The flask containing a dry lipid film was placed under vacuum for 8 h to remove any residual solvent. The film was hydrated with deionized water and the formed liposomes were diluted by a factor of 10 (from 10 mM to 1 mM)
and extruded through 100 nm track-etched polycarbonate membranes to yield small monodispersed unilamellar liposomes.

3.3. Layersome preparation

The washless method was used to form the layersome using polyelectrolyte solution (PLH) prepared in DI water at 0.05% w/w [24]. In this method, the required amount of polyelectrolyte to cover the liposome surface was determined through a titration process and determining the plateau point of zeta-potential. After determining the required amount of polyelectrolyte, it was added to liposome solution and mixed for 5 min at room temperature for coating process.

3.4. Gold nanostructure formation

To make gold nanoshell structures (GNSs), three sample series (L1, L2 and L3) were prepared. The main difference between these series is the type of template used to form GNSs. The first template was a liposome with a mixture of DOPC:DOPG (1:1 mole ratio) and the second template was a layersome (PLH layered on DOPC:DOPG liposomes). To make GNSs, different amounts of gold ion solution were added to the substrate solution and mixed using mixing bar for 5 min at room temperature. Then ascorbic acid, as a reducing agent, was added to the above solution and mixed for another 5 min. For samples that included PEG-thiol, before adding gold solution, PEG-thiol was added to liposome or layersome solutions. For all the gold samples, the mole ratio of ascorbic acid to gold ions was constant and equal to 4, but the mole ratios between gold ions and the templates varied between 1 to 7. For control samples (L1-6-control, L2-6-control and L3-6-control), the preparation conditions were the
same as L1-6, L2-6 and L3-6 samples, respectively but without any liposome or layersome. In the other words, there was no substrate for the control samples.

3.5. Dynamic light scattering (DLS)

Hydrodynamic diameter ($d_h$) and zeta potential ($\zeta$) were measured using a Malvern Instruments Zetasizer Nano ZS with a backscattering detector angle of 173° and a 4 mW 633 nm He-Ne laser. To determine $d_h$, 1 ml of the sample was placed in an optical grade polystyrene cuvette at 25 °C. The reported z-averaged intensity-weighted hydrodynamic diameters are based on 15 scans measured in triplicate for each sample. Zeta potential was measured by combined Doppler electrophoretic velocimetry and phase analysis light scattering based on the Smoluchowski theory [35]. Measurements were performed using 1 mL samples which were diluted 10 times at 25 °C over 3 cycles (12 data points per cycle).

3.6. UV/Vis/NIR spectroscopy

Perkin–Elmer Lambda 1050 spectrophotometer with Deuterium and Tungsten Halogen light sources was used for the absorbance spectra of the samples. The spectrophotometer was used in the wavelength range from 500 nm to 1300 nm. Quartz cell was used for this measurement. The samples were diluted 10 times.

3.7. Cryogenic Transmission Electron Microscopy (cryo-TEM)

Cryo-TEM samples were prepared at 25 °C using a Vitrobot (FEI Company), which is a PC-controlled robot for sample vitrification. Quantifoil grids were used with 2 µm carbon holes on 200 square mesh copper grids (ElectronMicroscopySciences, Hatfield, PA). To prepare a sample, it was equilibrated within the Vitrobot at 25 °C and 100% humidity for 30 min. After immersing the grid into the sample, it was then removed,
blotted to reduce film thickness, and vitrified in liquid ethane. The sample was then transferred to liquid nitrogen for storage. Imaging was performed in a cooled stage (model 626 DH, Gatan Inc., Pleasanton, CA) at 200 kV using a JEOL JEM-2100 TEM (Peabody, MA).

3.8. Field Emission Scanning Electron Microscopy (FE-SEM)

To prepare samples for FE-SEM microscopy, 5 µL of 10 times diluted samples was put on a silicon wafer and dried in vacuum oven during night. Imaging was performed using Sigma VP FE-SEM instrument (ZEISS Inc., Thornwood, NY). The Energy Dispersive Spectroscopy (EDS) analysis was performed using Silicon Drift Detector (OXFORD Inc. Concord, MA). Since the samples including gold are conductive, we decided not to use sputter coating, so only the structures with gold are seen.

1. Results and Discussion

As mentioned before, three sets of layersome-templated gold nanoshell samples (L1, L2, and L3) were prepared. For the first sample series (L1), liposomes were coated with PLH. Gold affinity to PLH and electrostatic interaction between PLH and gold ion in solution (AuCl₄⁻) were considered to drive absorption of gold ions on the layersome surface and then by adding ascorbic acid as a reducing agent, metallic gold would be formed on the layersome surface and covered the surface in the form of shell. The schematic of proposed mechanism is shown in Figure 2-3.
DLS data (Table 2-1) show that for all the samples, the average size is smaller than bare layersome (166 nm). Despite layersome, the intensity weighted size distribution graphs for this sample series show multiple peaks in different size ranges which indicates that we have different structures in L1 series. Peaks around 100 nm are gold structures. Peaks around several thousands may relate to the dust in samples.

Similarly, the number size distribution graphs don’t show layersome peak (near 100 nm) (Figure 2-4). One of the possibilities that in gold nanostructure solution, we can not see the peaks for liposomes is due to the fact that we have gold structures and since gold is heavier, it absorbs more signal in DLS measurement and so bare liposome can not be detected by the equipment. To check this possibility, cryo-TEM was used. Regarding the z-potential, for layersome we have a quite large value (+48.8 mV) and other than control sample, all of them have positive surface charge. Since we used ascorbic acid for reduction process, it was expected to have negative zeta potential for samples. This phenomenon can be explained by PLH absorption on gold structures. By increasing the gold to lipid ratio, the absolute value of zeta potential decreases and this is due to the fact that the amount of added ascorbic acid increases but the PLH remained constant.
Table 2-1. DLS data of L1 series. The ratio of AA:Au = 4:1.

| Sample       | Au to lipid (mole ratio) | Average size (nm) | PDI  | Z-potential (mV) | comments                                      |
|--------------|--------------------------|-------------------|------|------------------|-----------------------------------------------|
| Layersome    | 0:1                      | 166               | 0.117| 48.8             | Charge reversal confirms PLH deposition       |
| L1-1         | 1:1                      | 78                | 0.233| 38               |                                               |
| L1-2         | 3:1                      | 85                | 0.586| 34.8             | High polydispersity (PDI > 0.3)               |
| L1-3         | 4:1                      | N/A               | N/A  | 30.5             | Large aggregates observed                     |
| L1-4         | 5:1                      | 109               | 0.652| 29.6             | High polydispersity                           |
| L1-5         | 6:1                      | N/A               | N/A  | 27.6             | Large aggregates observed                     |
| L1-6         | 7:1                      | 98                | 0.368| 19.4             | High polydispersity (PDI > 0.3)               |
| L1-6-Control | --                       | 88                | 0.246| -1.68            | Negative charge reflects coating by AA        |
(a)

(b)
To investigate more about the structure and check the morphology, SEM images of the L1-6 and L1-6-control samples were taken. The L1-6 sample shows either aggregates of small nanoparticles in a spherical form and/or the gold coated layersome (Figure 2-5a). The SEM images of L1-6-control (Figure 2-5b) are similar to L1-6 sample but with bigger clusters. It should be noted that the hollow that is seen around the samples is instrument artifact because of using high magnification.
Figure 2-5. SEM images of (a) L1-6 sample; nanoparticle aggregates and/or gold coated layersomes. (b) L1-6-control sample; clusters of gold nanoparticles.

To check the substrate in which gold was formed on, EDS analysis for L1-6 sample was done (Figure 2-6). Gold mapping is shown in red and phosphorous, as liposome representative, is shown in yellow. Based on the EDS analysis, it is clear that gold is almost everywhere but it is more concentrated on liposome surface (where the phosphorous exists) and close to liposome.
Cryo-TEM images of L1-6 sample are shown in Figure 2-7. The results show that we have three different structures: layersome, gold nanoparticles and gold coated structures or clusters of gold nanoparticles. Most of the formed structures are
layersome or separate gold nanoparticles but there are also some structures which can be gold coated layersome and/or aggregates of gold nanoparticles. These images confirm existence of layersome in solution despite the fact that they were not detected in DLS.

Figure 2-7. cryo-TEM of L1-6 sample. Labels 1, 2 and 3 show layersome, gold nanoparticles and gold coated structures, respectively.
Based on the absorption spectra of this sample series (Figure 2-8), although there is absorption in NIR range (650-900 nm), the wavelengths corresponding to the peak maxima are out of that range. By increasing the Au to lipid ratio, the peak location maxima go to higher wavelength. The obtained absorption spectra is similar to the gold nanoparticles spectra which is another evidence to have gold nanoparticle for L1 series [12], [25]–[28]. There is some absorption in the NIR zone that is due to either clusters of gold nanoparticles and/or gold coated liposomes. The possible reason of having peak out of NIR range is that for this sample series we have more gold nanoparticles and so the absorption of gold nanoparticles is stronger than gold coated liposomes. Although we might have some gold coated liposome in this sample series, but according to DLS data and also absorption spectra, these structures were not dominant so we were trying to modify the process to increase the number of gold coated structures.
Figure 2-8. Absorption data of L1 series. (a) Absorption spectra graphs. The inside graph is magnified absorption spectra in the range of 500–700 nm. (b) Absorption peak wavelength for different samples.
For the second series (L2), the substrate was made by adding 200 μl of 5mM PEG-thiol solution to 1 ml of 1mM liposome solution and mixing for 5 minutes. To check the interaction between PEG-thiol and liposome, size and zeta potential of the obtained substrate was determined. Despite the fact that liposome and PEG-thiol solutions both have negative surface charge (Zeta potential of -60 mV and -12.5 mV for liposome and PEG-thiol, respectively), by adding these solutions together, the absolute value of zeta-potential decreased and average size was increased (from 137 nm for diameter and zeta potential of -60 mV for bare liposome to 147 nm for diameter and -45 mV for zeta potential of PEG-thiol attached liposome) which indicated the PEG-thiol absorption on liposome surface. The schematics of L2 formation process is shown in Figure 2-9.

![Figure 2-9. Schematics of L2 formation process using liposomes as the template with PEG-SH present as a capping agent.](image)

DLS data for this series shows bigger structures than L1 series (Table 2-2). Also the structures are more uniform (lower PDI) and all the samples have single peak in size graphs (Figure 2-10). The zeta potential of this series is all negative which shows that the surface of structures were covered either with ascorbic acid and/or PEG-thiol.
Table 2-2. DLS data of L2 series.

| Sample       | Au to lipid (mole ratio) | Size (nm) | PDI   | Z-potential (mV) |
|--------------|--------------------------|-----------|-------|------------------|
| L2-1         | 0:1                      | 140       | 0.131 | -25.5            |
| L2-2         | 1:1                      | 155       | 0.187 | -18              |
| L2-3         | 3:1                      | 156       | 0.158 | -15.1            |
| L2-4         | 4:1                      | 149       | 0.165 | -12.8            |
| L2-5         | 5:1                      | 142       | 0.147 | -11.2            |
| L2-6         | 6:1                      | 188       | 0.187 | -10.5            |
| L2-6-Control | 7:1                      | 169       | 0.175 | -7.2             |

(a)
Since except L2-6 and L2-6-control, all other samples show similar average sizes to PEG-thiol coated liposome, there is a possibility that we have gold coated liposomes.

Also when we check the number size distribution, only L2-6 and L2-6-control samples
show smaller peaks. For L2-6-control, since we do not have any liposome, we expected this behavior but for L2-6 sample, it seems that we made much more gold nanoparticles compared to gold coated liposomes and that’s why we see a bigger peak in smaller size range. Absorption spectra of this series show peaks in NIR range (Figure 2-11), So based on spectra and DLS data we may have desired shell structures. Besides L2-1 sample, for all the other samples, we have absorption peak in NIR range and this also includes L2-6-control sample. By adding more gold ions to the system (from L2-2 to L2-6), the peak location moves toward lower wavelength which can be due to increasing in shell thickness. For L2-6-control, we have a very high absorption peak that maybe due to the gold nanoparticle aggregates. To have a better understanding and to check the morphology, SEM technique was used.
Figure 2-11. Absorption data of L2 series.

SEM images of L2-6 and L2-6-control samples are shown in Figure 2-12. The agglomerates in control sample are gold nanoparticle aggregates. The structures of L2-6 and L2-6-control samples seem to be similar.

(a)
To check the existence of liposome as a substrate for the formed structures in L2-6 sample, EDS analysis was done (Figure 2-13). The red and yellow dots are gold and liposome representatives respectively. Based on the EDS results, we have gold on liposome surface and also in other places (as gold nanoparticles).
Cryo-TEM images of the samples are shown in Figure 2-14. For this sample we see three different structures including coated structures, bare liposome and gold nanoparticle attached liposome and finally gold nanoparticles.

Figure 2-13. EDS analysis for L2-6 sample.
Figure 2-14. Cryo-TEM images of L2-6 samples (a) coated structures (could be either gold coated liposomes and/or agglomerates of gold nanoparticles), (b) bare liposome and gold nanoparticle attached liposome and (c) gold nanoparticles.
To make the L3 series, layersome was used as the substrate. The hypothesis behind this idea was to use charge attraction between PLH and PEG-thiol to have more PEG-thiol absorption on liposome (substrate) surface and more gold formation on substrate and a denser gold layer. The schematics of L3 formation process is shown in Figure 2-15.

![Schematics of L3 formation process.](image)

**Figure 2-15. Schematics of L3 formation process.**

DLS data (Table 2-3) shows that compared to L2 samples, this series has bigger size and higher PDI but number size distribution size graphs show single peak similar to L2 series (Figure 2-16). This sample series shows positive surface charge that can be due to the existing of extra PLH and coating the final structures.

| Sample     | Au to lipid (mole ratio) | Size (nm) | PDI     | Z-potential (mV) |
|------------|--------------------------|-----------|---------|------------------|
| L3-1       | 0:1                      | 180       | 0.337   | 10.9             |
| L3-2       | 1:1                      | 187       | 0.225   | 16.4             |
| L3-3       | 3:1                      | 202       | 0.223   | 13.7             |
| L3-4       | 4:1                      | 219       | 0.218   | 12.9             |
| L3-5       | 5:1                      | 210       | 0.211   | 10.9             |
| L3-6       | 6:1                      | 239       | 0.216   | 11.7             |
| L3-6-Control | 7:1                  | 172       | 0.184   | -3.9              |
Figure 2-16. (a) Intensity weighted size distribution of L3 series, and (b) number size distribution of L3 sample series.
Similar to L2 samples, the L3 series shows NIR absorption peak but the peaks shift to higher wavelengths (Figure 2-17). As described elsewhere, this can be due to the fact that for this series, layersomes are covered more densely by gold nanoparticles [12]. One of the interesting phenomenon about this samples series is their absorption peak locations. Besides L3-1, for the rest of samples, the absorption peak location is almost the same.
According to SEM images for L3-6 sample (Figure 2-18a), the formed gold nanostructures were in spherical form. Based on the size and shape it seems that these structures were formed on a layer some surface. For control sample (Figure 2-18b), agglomerates of gold nanoparticles can be seen.
Figure 2-18. SEM images of (a) L3-6 sample and (b) L3-6-control.
To check the morphology with another technique, cryo-TEM was used for L3-6 sample (Figure 2-19). These images show bumpy structures which is the result of gold formation on layersome surface and the TEM results are in agreement with obtained SEM images.

Figure 2-19. Cryo-TEM images of L3-6 sample.
2. **Conclusions**

The idea of using layerosome as a substrate for NIR active gold nanoshell formation was investigated. According to this study the electrostatic interaction provided by PLH is not solely sufficient to make desired structures on layerosome substrate. By using PEG-thiol, gold nanoshell can be formed on layerosome substrate through strong covalent bond. On the other hand, and according to formed structures on liposome with negative surface charge, it seems that the covalent bond role is stronger than electrostatic interaction in this process. By using the applied method, small size NIR active gold nanostructures based on layerosome template were formed which have potential application for cancer heat treatment.

3. **Acknowledgements**

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