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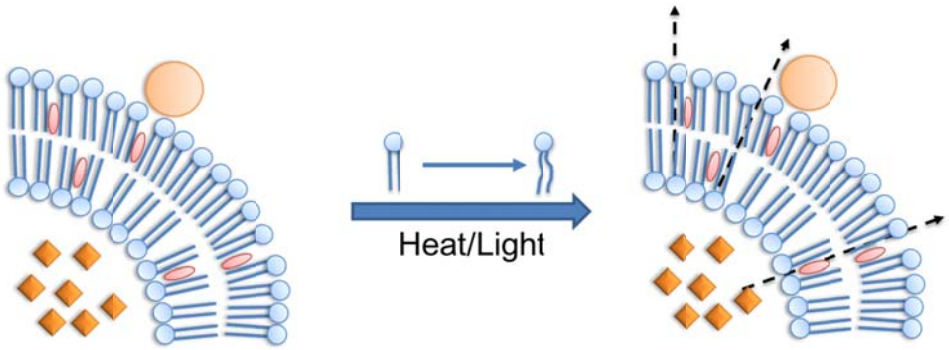

GNP-DPPC, gold nanoparticles decorated DPPC liposomes, can release encapsulated dyes upon heating or illumination. GNP-DPPC also has faster thermal response and higher critical leakage temperature than liposomes.

Construction of thermal- and light-responsive liposomes with noncon-valently decorated gold nanoparticles†

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Liposome-nanoparticle assemblies (LNAs) are promising drug carriers that can easily encapsulate drugs and probes and can manifest smart response to the environment. Here in this work we constructed a GNP-liposome complex, called GNP-DPPC, by decorating GNPs onto the surface of the thermal-responsive DPPC liposomes using nonconvalent interaction. We found that the introduction of GNP not only makes GNP-DPPC have a higher CLT (critical leakage temperature), but also makes GNP-DPPC have a faster leakage near its CLT. Furthermore, GNP-DPPC could release content under the illumination of 532 nm laser beam, which is relatively a mild condition. Moreover, GNP-DPPC is stable in cell medium and can be absorbed into cells. Such a light-induced content release behavior and the fast thermal response as well as its cellular behavior would make it to be further used in drug delivery in the living cells.

1 Introduction

The last decade has witnessed the rapid development of nanoparticles in targeted imaging and/or therapeutic agents. Among all nanostructured materials, the liposome-nanoparticle assemblies (LNAs) have attracted particular interest[1,2]. It is because that LNAs have the advantages of both liposomes and nanoparticles. On one hand, the liposomes in these LNAs can encapsulate both hydrophobic and hydrophilic drugs and probes. On the other hand, the nanoparticles enable LNAs to manifest smart responses and imaging properties, as well as stabilization of liposomes[3–6].

Among all the nanoparticles in LNAs, gold nanoparticles (GNPs)[7] are the most frequently investigated due to their typical local surface plasma resonance (LSPR) effects[8,9], which bring GNPs the ability of photothermal conversion. GNPs can be decorated onto membrane surface (*d*-LNAs)[10–17], incorporated into lipid bilayer (*b*-LNAs)[18–25] or encapsulated into core interior of liposomes (*e*-LNAs)[22,26,27]. These subclasses of LNAs have different properties due to their structural differences.

In *d*-LNAs, the GNPs on the membrane surface could form either a unified gold shell[13,14], from which drug could be released under the illumination of near infrared ray, or discrete gold coating[15–17], which has a similar photothermal conversion reaction with better biodegradability. It is because that the discrete coating of GNPs could be destroyed under the disruption of liposomes. In *b*-LNAs the incorporated GNPs are usually very small (2 nm to 5 nm), therefore the illumination of UV could induce the leakage due to the non-resonant photothermal conversion of such small incorporated GNPs[22–25]. For *e*-LNAs, since the GNPs are far from membrane (compared with *d*-LNAs and *b*-LNAs), during the photothermal conversion the disturbance to the liposomes is weakened and thus the content release is reduced[22]. To have a satisfactory release efficiency high energy laser source like 0.5 ns laser pulse[28], or femtosecond laser[27] could be used (the mechanism of the latter is not photothermal but photoacoustic conversion). Moreover, in some cases GNPs could crosslink several liposomes to form higher hierarchy aggregates, which are called *c*-LNAs[29].

These subclasses of LNAs are not always suitable for further application *in vivo*. Firstly, *b*-LNA is not safe to cells, since its use involves UV light, which is harmful to cells. Secondly, *e*-LNA is not efficient in content release, since it can only release contents efficiently after illumination of high energy pulse-laser. Thirdly, *c*-LNA is too large to be used *in vivo*, since it consists of several liposomes and is large than 200 nm. Finally, *d*-LNA does not have the problems as other subclasses of LNA, but is safe to cells, efficient in content release and proper in size. Therefore, *d*-LNA is most suitable for further application *in vivo*.

So far, most *d*-LNA is synthesized by reducing $HAuCl_4$ to GNPs or gold shell on the membrane surface chemical-

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ly. However, the study of constructing *d*-LNA by noncovalent bonds is very few[30]. The noncovalently bonded *d*-LNA is concise in structure, easy to prepare and modify and environment-friendly.

In this paper we constructed a GNP-liposome complex, called GNP-DPPC, by decorating GNPs onto the surface of the thermal-responsive DPPC liposomes. GNP-DPPC is a *d*-LNA in which GNPs and DPPC liposomes are bonded noncovalently. We found that the introduction of GNP will enhance the stability of liposomes, by increasing the critical leakage temperature (CLT) of DPPC liposomes by 1 °C. Moreover, GNP-DPPC has a more sensitive thermal response and faster leakage near its CLT. Furthermore, compared with the control DPPC liposome which has no response to the light exposure, GNP-DPPC could release content under the illumination of 532 nm laser beam, which is relatively a mild condition. Moreover, we investigated the the stability of GNP-DPPC in cell medium and its cellular uptake. Therefore, such a light-induced content release behavior and the fast thermal response as well as its cellular behavior would make it to be further used in drug delivery in the living cells.

2 Materials and Methods

2.1 Materials

The dry powder of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, $\geq$ 99%), cholesterol (Chol, $\geq$ 99%), Sephadex G-50 and the aqueous solution of gold nanoparticles (20 nm, 0.1 mM PBS) were purchased from Sigma. The chloroform solution of 16:0 Liss Rhod PE was purchased from Avanti Polar Lipids. Glucose ($C_6H_{12}O_6{\cdot}H_2O$, $>$ 99%) was obtained from Sinopharm Chemical Reagent Co., Ltd, China. The powder of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Hepes, 99%) was purchased from Amresco, US. Calcein, Triton X-100 (99%), ammonium thiocyanate (97.5%), Iron(III) chloride hexahydrate (97%) and phosphotungstic acid were purchased from J&K Chemical Ltd, China. Wahaha water (Wahaha Company, China) was used as the pure water.

2.2 Preparation of liposomes

DPPC/chol 8/2 (molar ratio) liposomes encapsulating calcein were prepared by reverse phase evaporation method[31] followed by extrusion. Briefly, the chloroform solution of lipid mixture was added to a 50 mL pear-shaped flask, and then the solvent was removed under reduced pressure at 50 °C by a rotary evaporator. The lipid film was then redissolved in 3 mL of mixed organic solution of chloroform and ether (1/1, v/v). The organic solution was further mixed with 1 mL of Buffer A (50 mM calcein, 0.9% glucose solution, 20 mM Hepes, 0.1 mM EDTA, pH = 7.3) and then sonicated for 1-2 min in 0 °C water bath. After that, the organic solvent was removed from the mixed solution under reduced pressure at 37 °C and the multilamellar liposomes were obtained. The multilamellar liposomes were then extruded through 100 nm polycarbonate membrane using mini-extruder for 21 times. The obtained unilamellar liposomes were then passed through a Sephadex G-50 column to remove nonencapsulated materials and residual organic solvent. The elution buffer was Buffer B (5% glucose with 20 mM Hepes, 0.1 mM EDTA, pH = 7.3). The concentration of DPPC in the purified liposomes was determined to be 1.8 mM by Stewart's method[32].

For cell studies, DPPC/chol 8/2 (molar ratio) labeled with 1% fluorescent 16:0 Liss Rhod PE (named DPPC-Rh) was prepared in the same way and the concentration of DPPC in the liposomes was determined to be 6.4 mM.

2.3 Preparation of GNP-DPPC

The GNP solution is prepared as follows: first, 9 mL of the commercial GNP solution was mixed with 1 mL of Hepes 10$\times$ (100 mM Hepes, 1 mM EDTA, pH = 7.3, 1 mL) to maintain the pH, and then 0.55 g glucose powder was added to adjust the osmatic pressure, which equals to the osmatic pressure of the liposomes solution.

After the optimal mixing ratio was obtained (See supporting information for more details), we prepared the GNP-DPPC complex as follows: first, GNPs at 6.5×10^{11} particle/mL were titrated into equal volume of DPPC (DPPC/chol 8:2, DPPC 0.18 mM) under agitation. Next, the solution was kept stirring for 30 min. After that, the excess unadsorbed GNPs were removed by centrifugation (Eppendorf Centrifuge 5810R) at 12000 r/min for 30 min. Finally, the supernatant was collected for further investigation. In the control group, the same volume of Buffer B instead of GNP was added into the DPPC solution, stirred and centrifuged under the same condition and was collected (named DPPC-Lipo) for further investigation.

GNP-DPPC-R complex was prepared using the optimal mixing ratio in the same way.

2.4 Transmission electron microscopy

Transmission electron microscope measurements were performed with H-600 (Hitachi) operated at 75 kV accelerating voltage. 50 μL of the dispersions (0.18 mM DPPC) was placed on formvar/carbon coated copper grids and stayed for 18 min. Then the excess dispersions were removed with filter paper. After that, 50 μL 3% phosphotungstic acid was dropped on the copper grids and stayed for 4 min. Then the excess of liquid was removed with filter paper.

2.5 Thermal leakage measurement

The thermal induced leakages of GNP-DPPC and DPPC-Lipo were investigated under both program-increasing temperature condition and constant temperature condition. The measurements were performed on an F7000 Fluorescence Spectrometer equipped with a RE5 Refrigerated Circulating Bath (Froilabo). For program-increasing temperature measurement, the solutions were heated from 30 °C to 50 °C with the interval of 1 °C. The solution was allowed to stay for 5 min to reach equilibrium before each measurement was taken. The constant temperature measurement was taken under 37 °C and 42 °C, and the time interval was 5 min.

The leakage of GNP-DPPC and DPPC-Lipo are measured according to the fluorescence of the leaked calcein. The percentage of leakage was calculated as:

$$L\% = \frac{(F_x - F_0)}{(F_t - F_0)} \times 100\% \quad (1)$$

where F_x is fluorescence of GNP-DPPC or DPPC-Lipo solution at each measurement, F_0 is the initial fluorescence of GNP-DPPC or DPPC-Lipo before heating, and F_t is the fluorescence after the addition of 50 μL Triton X-100.

2.6 Light-induced leakage measurement

For the light-induced leakage measurement, the sample solution (GNP-DPPC or DPPC-Lipo) was put into a square quartz cell which is transparent to the laser. A 200 mW laser (DD532-200-5, Huanic Corporation, Xi'an, China) at 532 nm with a diameter of 5 mm was used to induce the leakage. Then the solution was exposed under the laser beam at room temperature and the measurements were taken at the time points 10 min, 30 min and 70 min. At each time point, the fluorescence was measured and the leakage of GNP-DPPC or DPPC-Lipo was calculated according to Equation (1).

2.7 Stability of GNP-DPPC in cell media

The stability of GNP-DPPC in cell media is estimated by measuring the size and encapsulation efficiency of GNP-DPPC in 10% FBS (fetal bovine serum) daily for a week. The dispersion of GNP-DPPC was prepared as follows: 0.3 mL of FBS was added into 2.7 mL of GNP-DPPC under stirring and the mixture was kept stirring for 20 min at 25 °C.

The measurement of size of GNP-DPPC in 10% FBS is similar to those of GNP-DPPC in Buffer B. The encapsulation efficiency of calcein in the GNP-DPPC dispersion(in 10% FBS) was determined according to Paasonen's work[22]. Briefly, fluorescence of 1 mL GNP-DPPC dispersion. Then the solution before and after adding 50 μL 10% Triton X-100 was measured by F7000 (HITACHI). The encapsulation efficiency of calcein (EE) was calculated by:

$$EE\% = 100\% - \frac{(F_i - F_b)}{(F_t - F_{bt})} \times 100\% \quad (2)$$

where F_i and F_t are the fluorescence of GNP-DPPC dispersion before and after the addition of 50 μL Triton X-100; F_b and F_{bt} are the fluorescence of 10% FBS before and after the addition of 50 μL Triton X-100.

The encapsulation efficiency of calcein in GNP-DPPC dispersion(in Buffer B) and that of liposomes were measured in the same way.

2.8 Cellular uptake of GNP-DPPC-Rh

Measurement of cellular uptake of GNP-DPPC-Rh is performed using U87 cells. U87 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% Penicillin/Streptomycin. For cellular uptake studies, GNP-DPPC-Rh (final concentration of DPPC is 0.66 μM) was introduced to U87 cells (3.5×10^4 cells/mL) and incubated for 4 hours. Meanwhile, U87 cells without GNP-DPPC-Rh were set as control. After the incubation, cell medium containing free GNP-DPPC-Rh was dropped out and the remaining cells on the bottom were washed with PBS and then supplemented with cell medium before observation under fluorescent microscopy (DMI 4000B, LEICA).

3 Results and Discussion

3.1 Size of GNP-DPPC

Dynamic Light Scattering (DLS) was used to measure the size distribution of GNP, liposomes and GNP-DPPC dispersion. As shown in Figure 1A, GNP in buffer B has a unimodal distribution with mean diameter of 24 nm, while GNP in 0.1 mM PBS has a diameter of 20 nm, indicating that glucose in Buffer B will not cause the aggregation of GNPs and Buffer B is suitable as the solvent in this work. Then, liposomes in Buffer B have narrow distribution (PDI = 0.182) with diameter of 168 nm; GNP-DPPC also has narrow distribution (PDI =0.083) with diameter of 173 nm. Meanwhile, the zeta potential of GNP, liposomes and GNP-DPPC were measured. The values are -30.4±15.6 mV, -7.0±6.7 mV and -19.0±12.8 mV, respectively. Seeing that GNP-DPPC is a litter larger than liposomes and carries weak negative charge, the proportion of GNP to liposomes must be relatively small. Also, GNPs are negatively charged and liposomes are weakly negatively charged, their interaction could only be charge-dipole interaction, namely, the interaction between charge on GNP surface and dipole of liposome surface. Furthermore, Figure 1B shows the morphology of GNP-DPPC. Only one GNP is attached to one liposome at most, meaning that the non-convalent interaction between GNP and liposome is relatively weak.

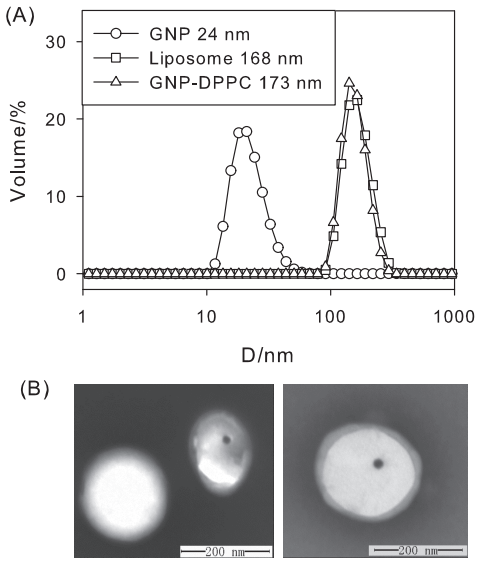

Fig. 1 (A) Size distribution of GNP, liposomes and GNP-DPPC in 5% gluocose with Hepes buffer; (B) TEM images of GNP-DPPC.

3.2 Thermal response of GNP-DPPC

In the liposomes, 20% cholesterol is incorporated to increase the stability of liposomes and do not affect major transition temperature (T_k) of liposomes. This is because that the incorporation of less than 30% cholesterol in DPPC liposomes will not affect its major transition temperature, which is 41 °C, according to Binder[33]. Although the proportion of attached GNPs on liposomes is relatively small, the attached GNPs should have effect on thermal response of liposomes. Figure 2 shows the content leakage of DPPCLipo and GNP-DPPC under program-heating from 30 °C to 50 °C. For DPPCLipo, the leakage process can be divided into three stages: first, when the temperature is lower than 34°C, no obvious leakage was observed; second, between 35 °C and 37 °C, a fast leakage was observed: the leakage increased sharply from 17% to 55%; finally, after 38 °C the curve of leakage became slow again. Based on the analysis of the leakage process, it can be determined that the CLT of DPPCLipo is 35 °C. Notice that the pretransition temperature (T_p) and major transition temperature T_k of DPPC liposomes are 41 °C and 35 °C, respectively[34], we can conclude that such leakage is subject to the pretransition of liposomes. For the GNP-DPPC in which the DPPC liposomes were decorated by GNPs, a similar three-stage leakage curve was obtained. However, the CLT shifted up 1 °C and the fast leakage started at 36 °C (14 %)and ended at 38 °C (42%). This indicates that GNPs have a stabilizing effect on liposomes. Since the lateral movement of lipids is essential for pretransition of liposomes, in which the phase

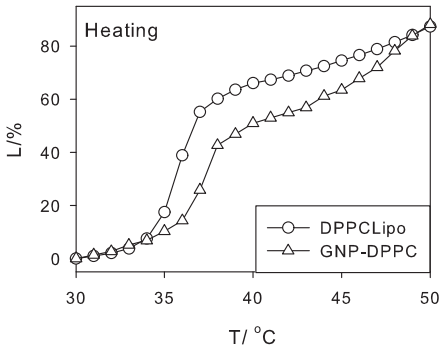

Fig. 2 Thermal leakage from GNP-DPPC and DPPCLipo solution under program-heating condition from 30 °C to 50 °C with a interval of 1 °C.

of the liposomes changes from solid phase to ripple phase[33], we conjecture that GNPs stabilize DPPC by immobilizing the lipids. The slow leakage in the final stage is probably due to the the lower concentration gradient of calcein between the inside and outside of liposomes.

To further investigate the thermal response of GNP-DPPC and DPPCLipo, we monitored the kinetics of calcein release under constant temperature condition. Since the CLTs for GNP-DPPC and DPPCLipo are 35 °C and 36 °C, respectively, we chose temperatures above 36 °C, namely, 37 °C and 42 °C, to ensure both of them have thermal responses. Figure 3A shows the calcein release of GNP-DPPC and DPPCLipo at 37 °C. Here the initial calcein leakage of GNP-DPPC is not equal to zero (3%), because the leakage has already started when the sample is incubated in the bath before the zero time point. As can be seen from Figure 3A, the release of calcein in GNP-DPPC is much faster than that in DPPCLipo at 37 °C in the whole time range. The maximum difference is up to 26% which occurred at 20 min. Figure 3B shows the kinetics of calcein release from GNP-DPPC and DPPCLipo at 42 °C. Due to the same reason, the initial calcein leakages of GNP-DPPC and DPPCLipo are 20% and 3%, respectively. But after 5 min, their leakages are almost the same.

The faster leakage in GNP-DPPC could also be attributed to the immobilization effect of GNPs on the lipids. Since the normal pretransition temperature of DPPC liposomes without GNP is 35 °C, under the experimental condition the lipids are just in ripple phase. However, the introduction of GNPs will stabilize lipids in some regions, where the pretransition temperatures are slightly increased. Therefore under the experimental condition 37 °C these regions will stay in solid phase while other regions are in ripple phase. This phase separation will enhance the leakage, since calcein could be released from the phase boundaries. However, when the experimental temperature is 42 °C which is greatly over T_p, the stabiliza-

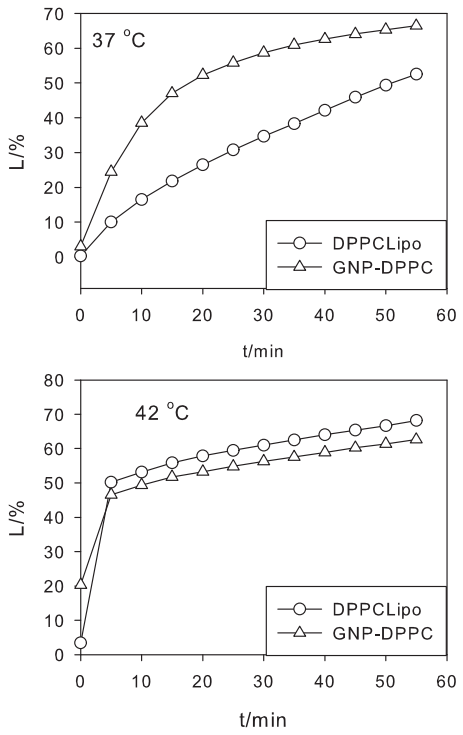

Fig. 3 Thermal leakage from GNP-DPPC and DPPCLipo solution under constant temperature condition at 37 °C (A) and 42 °C (B).

tion effect of GNP is overcome and such phase separation no longer exists. In such a case the leakage of GNP-DPPC and DPPCLipo are more or less the same.

By combining the thermal leakage of GNP-DPPC in program-heating condition and constant temperature condition, we can find that GNPs have dual effects on liposomes. On one hand, GNPs can stabilize DPPC liposomes by increasing the CLT by 1 °C; on the other hand, GNPs can also make DPPC liposomes more sensitive near the pretransition temperature. Both effects can be attributed to the immobilizing effect of GNPs on DPPC lipids.

3.3 Light response of GNP-DPPC

GNPs are well-known for their typical photothermal effects, while a lot of researches have demonstrated that GNP-based LNAs can be triggered to release the contents by exposing to suitable laser beam[7–9]. Since the resonant wavelength of GNPs in this work is 520 nm (data not shown), we use a continuous laser of 532 nm to illuminate the GNP-DPPC to trigger the content release. Figure 4 shows the light-induced calcein leakage of GNP-DPPC and DPPCLipo, while the latter is used as control group. After 10 min of illumination, 5% calcein leaked from GNP-DPPC, while in the control DPPCLipo the

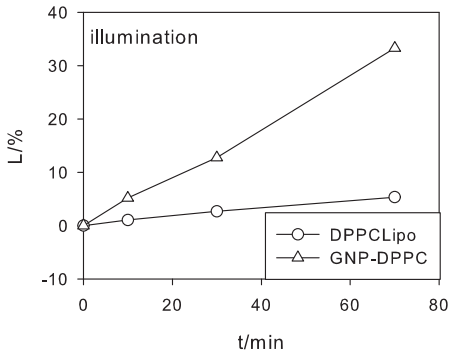

Fig. 4 The leakage of GNP-DPPC and DPPCLipo solution after illumination by 532 nm laser.

calcein leakage is only 1% under the same condition. After 30 min of illumination, the leakage in GNP-DPPC increased to 12%. Then after another 40 min exposure (70 min in all), the leakage rose to 33%. However, in the control DPPCLipo the leakage was 3% at 30 min and 5% at 70 min. Since the 5% leakage of DPPCLipo in this experiment is rather small, it indicates that 532 nm laser has no significant thermal effect on the phase behavior nor the structure of DPPC liposomes. Therefore, the 33% leakage in GNP-DPPC can only be attributed to the presence of adsorbed GNPs. A possible mechanism is that under the illumination of the laser, GNPs absorb the photon energy and the nearby lipids are heated to reach the pretransition temperature. Therefore a partial phase transition from solid phase to ripple phase occurs on the liposome. Such a phase separation triggers the content release. Therefore, it can be concluded that GNP-DPPC has light response to laser based on the photo-thermal transition of GNPs on liposomes.

3.4 Stability of GNP-DPPC in cell medium

To investigate the stability of GNP-DPPC in cell medium, the size and encapsulation efficiency (EE) of GNP-DPPC in 10% FBS were monitored for a week. As shown in Figure 5A, on the zeroth day, the day when FBS was added into original GNP-DPPC dispersion in Buffer B, the diameter was 168 nm, close to the original value (173 nm). This indicates that the GNP-DPPC didn't aggregate in the presence of FBS. Then, the diameter stayed in the range of 164-186 nm in a whole week, which implies that the structure of GNP-DPPC is stable in 10% FBS. Furthermore, Figure 5B shows the change in encapsulation efficiency of GNP-DPPC 10% FBS as well as the original GNP-DPPC dispersion and DPPC liposomes. In a week, the decreases in EE of GNP-DPPC in 10% FBS, original GNP-DPPC dispersion and DPPC liposomes are 1.1%, 2.0% and 2.7%, respectively. Therefore, GNP-DPPC in 10% FBS is most stable, followed by the original GNP-DPPC dis-

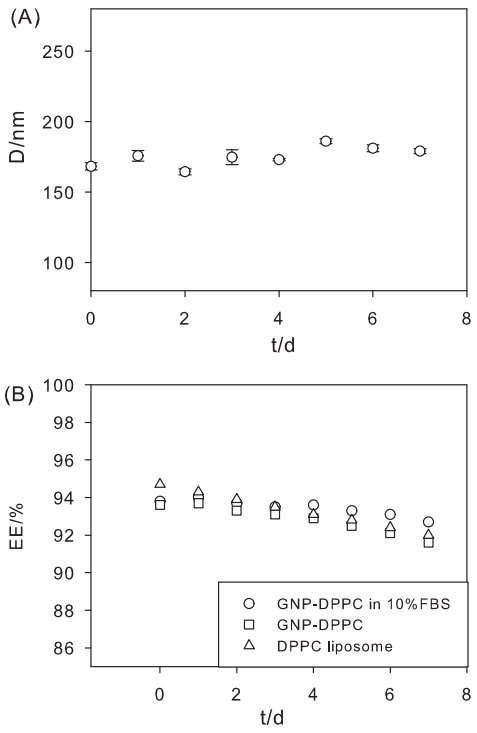

Fig. 5 (A) Change in size of GNP-DPPC in 10% FBS in a week; (B) Change in the encapsulation efficiency of GNP-DPPC in 10% FBS as well as that of GNP-DPPC in Buffer B and DPPC liposomes.

persion, and then the DPPC liposomes. The results further demonstrate that adsorbed GNPs on liposome surface is effective in increase the stability of liposomes. The FBS futher increases the stability of GNP-DPPC. This is probably due to the adsorption of serum proteins on liposome surface.

3.5 Cellular uptake of GNP-DPPC-Rh

To observe cellular uptake of GNP decorated liposomes, GNP-DPPC-Rh (labeled with 16:0 Liss Rhod PE) is used instead of GNP-DPPC. The diameter and zeta potential of GNP-DPPC-Rh is 140 nm (Figure S5)and 0±8.2 mV. As shown in Figure 6A and A', the cells themselves are visible under light microscopy, but not under fluorescent microscopy. However, after the cells incubated with GNP-DPPC-Rh at 37 °C for 4 h, almost all the cells are visible both under light microscopy and under fluorescent microscopy (Figure 6B and B'). The fluorescence in the cells must come from the absorbed fluorescent GNP-DPPC-Rh. Therefore, the GNP-DPPC-Rh can be further be used in cell studies.

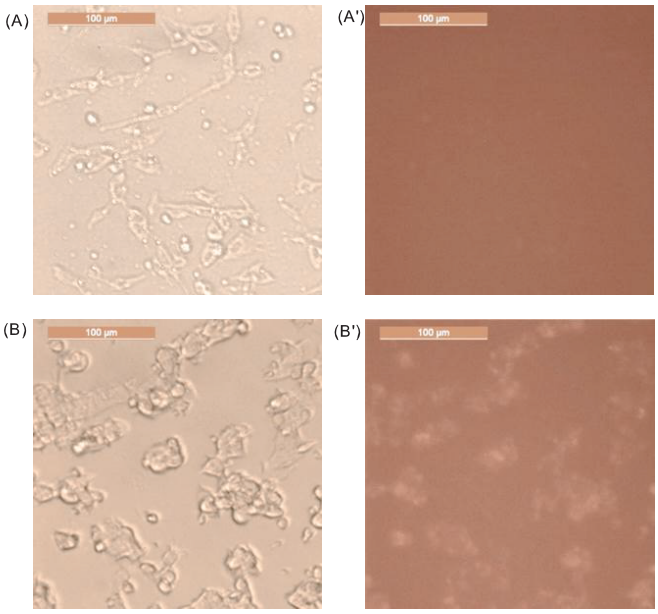

Fig. 6 (A-A')The light microscopy and fluorescent images of controlled cells; (B-B') The light microscopy and fluorescent images of cellular uptake after incubation with GNP-DPPC for 4 h at 37 °C.

4 Conclusion

In conclusion, we synthesized a GNP-liposome complex called GNP-DPPC, in which GNPs are decorated onto the surface of DPPC liposomes by noncovalent interaction. The synthesis is convenient and environment-friendly. The resulting GNP-DPPC complex has both thermal- and light-responsive properties. The introduction of GNPs enhance the stability of DPPC liposomes by increasing its CLT of 1 °C. Moreover, GNP-DPPC has a more sensitive thermal response and faster leakage near its CLT. Furthermore, GNP-DPPC has a light response, i.e., content leakage, under mild laser exposure. Moreover, GNP-DPPC is stable in cell medium and can be absorbed by cells. Therefore, Such sensitive thermal- and light-induced content release behaviors would make GNP-DPPC a potential drug carrier to be used in the living cells.

Acknowledgement

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