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The engineering of doxorubicin-loaded liposome-quantum dot hybrids for cancer theranostics*

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Many studies have recently attempted to develop multifunctional nanoconstructs by integrating the superior fluorescence properties of quantum dots (QD) with therapeutic capabilities into a single vesicle for cancer theranostics. Liposome-quantum dot (L-QD) hybrid vesicles have shown promising potential for the construction of multifunctional nanoconstructs for cancer imaging and therapy. To fulfil such a potential, we report here the further functionalization of L-QD hybrid vesicles with therapeutic capabilities by loading anticancer drug doxorubicin (Dox) into their aqueous core. L-QD hybrid vesicles are first engineered by the incorporation of TOPO-capped, CdSe/ZnS QD into the lipid bilayers of DSPC:Chol:DSPE-PEG_{2000}, followed by Dox loading using the pH-gradient technique. The loading efficiency of Dox into L-QD hybrid vesicles is achieved up to 97%, comparable to liposome control. All these evidences prove that the incorporation of QD into the lipid bilayer does not affect Dox loading through the lipid membrane of liposomes using the pH-gradient technique. Moreover, the release study shows that Dox release profile can be modulated simply by changing lipid composition. In conclusion, the Dox-loaded L-QD hybrid vesicles presented here constitute a promising multifunctional nanoconstruct capable of transporting combinations of therapeutic and diagnostic modalities.

Keywords: nanomedicine, theranostic, multimodality, nanotechnology

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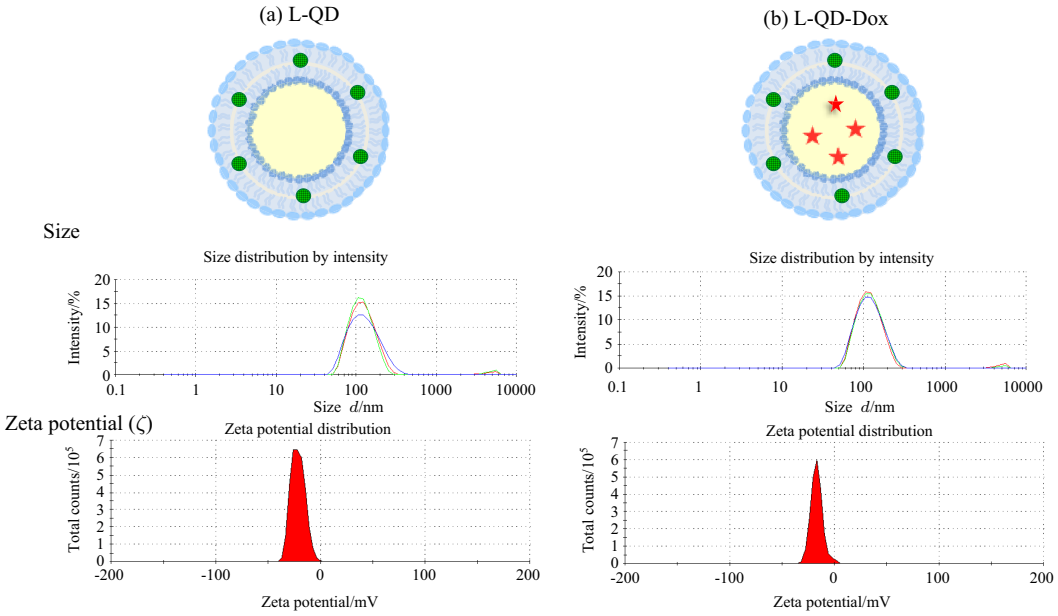
1. Introduction

Quantum dots (QD) are fluorescent semiconductor nanocrystals with superior fluorescence properties which have been extensively explored as fluorescent probes for various biological investigations.[1–7] Previously, we have engineered L-QD hybrid vesicles by incorporating hydrophobic QD (core size of 2 nm in diameter) into the hydrophobic domain of lipid bilayer of liposomes as an initial attempt to improve the dispersibility of hydrophobic QD in water.[8] This is a straightforward method of transforming hydrophobic nanoparticles (around 4 nm in diameter) directly into hydrophilic environments. Subsequent studies carried out in our group have shown that L-QD hybrid vesicles made of lipid formulation (DSPC:Chol:DSPE-PEG_{2000}) exhibited pronounced colloidal stability with a hydrodynamic diameter of 100 nm.[9] Such L-QD hybrid vesicles could be further explored as theranostic systems that both imaging (QD) and therapeutic (Dox) agents can be loaded in their lipid bilayer and internal aqueous phase simultaneously. In this study, we investigate Dox loading into L-QD hybrid vesicles by the established pH-gradient technique, and characterize Dox-loaded L-QD hybrid vesicles using dynamic light scattering (DLS) and monitor Dox release in both HEPEs buffer and serum.

2. Results and discussion

L-QD hybrid vesicles are first prepared following the thin lipid film hydration protocol as previously described.[8,9] Briefly, 1.68×10^{15} p/ml QD are mixed with 8 μmole of DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2) phospholipid molecules in chloroform, and the organic solvent is evaporated using the rotovaporator. L-QD hybrid vesicles are formed by hydrating the lipid film in 1 ml of ammonium sulphate (pH8.5) and bath sonication for 10 min at 60 °C. The L-QD hybrid vesicles exhibit 120-nm average diameter and negative surface charge (−21 mV) (Fig. 1(a)), similar to liposome control. To further equip L-QD with therapeutic capabilities, Dox is loaded into L-QD hybrid vesicles using the pH-gradient technique of ammonium sulphate.[10–14] Both Dox-loaded liposomes and L-QD hybrid vesicles are studied by DLS. Figure 1 shows that both the size and the surface charge of L-QD hybrid vesicles remain almost the same after Dox loading, which is consistent with liposome control. Dox-loaded L-QD hybrid vesicles show a size of 129 nm in diameter and surface charge of −21.8 mV, compared with L-QD (129.5 nm in diameter, −18.6 mV). The DLS study indicates that the process of Dox loading does not affect the physicochemical properties of L-QD hybrid vesicles

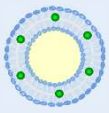
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Nanoparticle type	Mean diameter/nm	Polydispersity index	Surface charge/mV
L	129.2±2.14	0.203±0.023	-21.8±1.13
L-Dox	129.6±4.76	0.125±0.042	-18.6±1.55
L-QD	115.5±1.48	0.161±0.039	-21.6±4.84
L-QD-Dox	114.2±3.75	0.114±0.057	-17.6±0.16

Fig. 1. (color online) Dox-loaded L-QD hybrid vesicles characterized by DLS. Dox was loaded into L-QD using the pH-gradient technique at the lipid to Dox ratio of 5:1 molar ratio.

Table 1. (color online) Doxorubicin loading into L-QD and liposomes using the pH-gradient technique of ammonium sulphate, pH 8.5.

Type	Lipid Con./mM	Dox Con./mM	QD/(particle/ml)	Loading efficiency/%
Liposome 	8.0	1.6	-	99.88 ± 3.24
	5.0	1.0	-	98.20 ± 2.91
	2.5	0.5	-	91.32 ± 2.65
	1.0	0.2	-	75.72± 6.25
L-QD 	8.0	1.6	1.68×10^{15}	97.70 ± 2.24
	5.0	1.0	1.05×10^{15}	95.12 ± 3.66
	2.5	0.5	0.53×10^{15}	87.67 ± 4.38
	1.0	0.2	0.21×10^{15}	74.84± 7.31

Dox is loaded into liposomes and L-QD at 1:5 molar ratio. Dox loading efficiency is quantified by measuring UV absorbance at 480 nm after lysis of liposomes by 1% Triton, pH 7.4.L: liposomes DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2 molar ratio). L-QD:8 mM DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2 molar ratio) with 1.68×10^{15} p/ml of QD in HEPES buffer.

The process of Dox loading into L-QD hybrid vesicles is further investigated at various loading concentrations using the pH-gradient technique compared with liposome control. After the removal of free Dox, Dox loading efficiency is quantified by measuring UV absorbance after the lysis of liposomes. Table 1 shows that Dox can be loaded into L-QD hybrid vesicles using the pH-gradient technique at all the Dox concentrations used. The highest loading efficiency in L-QD hybrid vesicles achieved is 97% compared with liposome control (99%). The comparable Dox loading efficiency between L-QD hybrid vesicles and liposome control indicates that QD incorporation into the lipid bilayer does not affect Dox loading through the lipid membrane of liposomes using the pH-gradient technique. Despite loading Dox at a fixed lipid to Dox ratio (5:1 molar ratio), it is found that high Dox loading efficiency is achieved at a certain Dox concentration (> 0.5 mM). The Dox loading efficiency at Dox concentration of 0.5 mM ($> 90\%$) is greatly different from that of 0.2 mM (75%) in both liposome and L-

QD hybrid vesicles. This is deemed to be due to the fact that Dox crystal-like structures are formed at high Dox concentration (> 0.5 mM) as reported in Ref. [12].

After the successful loading of Dox into L-QD hybrid vesicles, Dox release is monitored in HEPEs buffer (Fig. 2(a)) and 50% serum (Fig. 2(b)) for 6 hours. Dox release study is monitored from L-QD hybrid vesicles made of two types of lipids, DSPC and EPC. High Dox loading efficiency (>95%) is achieved in both DSPC-QD and EPC-QD hybrid vesicles. Figure 2 shows that fastest Dox release is observed from EPC-QD in serum with 50% of Dox released over 6 hours compared with that from DSPC-QD ($< 10\%$). This release study indicates that Dox release profile from L-QD hybrid vesicles can be modulated simply by changing lipid compositions. Further, Dox releases from both EPC-QD and DSPC-QD are faster than that in HEPEs buffer. This suggests that the presence of serum interacting with the lipid bilayer[15] may destabilize the lipid bilayer of L-QD hybrid vesicles, thus further facilitating Dox release.

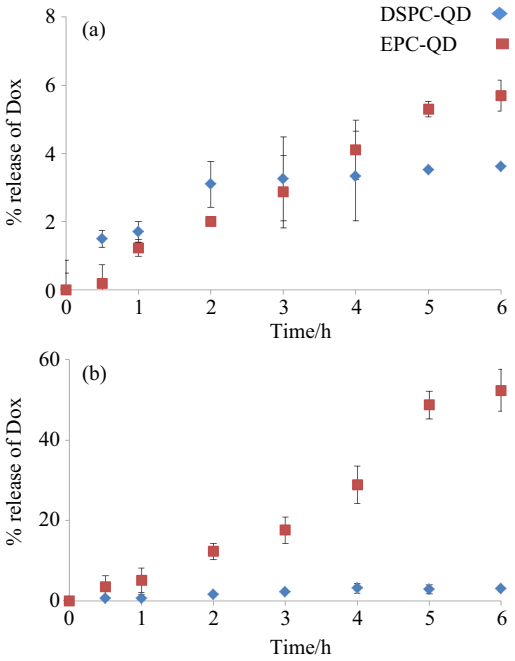


Fig. 2. (color online) Dox release from L-QD hybrid vesicles in HEPES buffer (a) and 50% serum (b) at 37 °C. Two lipid formulations use DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2 molar ratio) and EPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2 molar ratio).

Many studies have recently attempted to develop QD-based nanoconstructs for cancer theranostics by integrating the superior fluorescence properties of QD with therapeutic capabilities into a single vesicle, for sensitive and specific diagnosis and therapy *in vitro* and *in vivo*.[16–18] Bagalkot *et al.* (2007) covalently conjugated aptamers to the surface of hydrophilic QD and loaded such an aptamer-conjugated QD with Dox through intercalation with the RNA aptamers (1 to1 molar ratio),[17] as a first attempt to construct theranostic modalities against cancer. However, Dox loading by intercalation with the aptamers may suffer low drug loading efficiency (10 Dox per QD). Alternatively, Weng *et al.* (2009) covalently conjugated hydrophilic QD to the surface of liposomes and took advantage of liposome structure for Dox loading into the aqueous core of liposomes.[19] Such a design greatly improved the drug loading efficiency, prolonged blood circulation half-life and improved the therapeutic effect compared with the case of conjugating directly to QD surface; however conjugating hydrophilic QD to the liposome surface size adversely increasing from 112 nm to 212 nm. Comparing the previously reported liposome-QD hybrid system,[20] we show in this study that the incorporation of hydrophobic QD into the lipid bilayer of liposomes allows Dox loading above 95% (Table 1) using the established pH-gradient technique without causing adverse size increase (Fig. 1). Further, Dox release profile from L-QD hybrid vesicles can be modulated simply by changing lipid compositions (Fig. 2). Especially, the fast release of EPC-QD observed in serums indicates that the incorporation of small hydrophobic nanoparticles could offer a feasible way to modulate the release of cargo in a controlled manner for cancer therapy. Further, the Dox-loaded L-QD hybrid vesicles also allow the surface conjugation to various targeting ligands (e.g. antibody and antibody fragments) for specific targeting receptors overexpressed by cancer cells.[21–24]

3. Conclusion

The engineering of Dox-loaded, PEGylated L-QD hybrid vesicles demonstrates that the physicochemical versatility of liposomes, QD optical properties and Dox therapeutic activity can be successfully integrated into one nanoconstruct (about 100 nm in diameter) that could be potentially used for cancer theranostics. The design of liposome-nanoparticle hybrids offers a rich toolbox for the fabrication of various multifunctional modalities with modulated release profile of cargo possible

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Appendix A

A1 Matherials and methods

CdSe/ZnS core/shell quantum dots (QD) are obtained from Evident Technologies (USA), with a reported CdSe core

size of 2 nm in diameter and an overall QD diameter of 4 nm–5 nm. QD has an absorption peak at 502 nm and an emission peak at 517 nm.

Distearoylphosphocholine (DSPC) and Egg phosphocholine (EPC) are obtained from lipoid Gmbh (Germany). MPEG2000-disterroylphosphatidyl-ethanolamine (DSPE-PEG_{2000}) and cholesterol (Chol) are purchased from Avanti polar lipids. Triton-X 100, chloroform, methanol and doxorubicin from Sigma (UK); toluene (low in sulfur) from Fisher Scientific (UK). Deionized water (dH_2O) is used in all preparations.

A2 Lipid-quantum dot hybrid vesicle preparation

L-QD is prepared following a previously published protocol.[8,9] The final QD concentration in all L-QD preparation is 1.68×10^{15} particle/ml.

A3 Nanoparticle sizing by dynamic light scattering (DLS)

All measurements are made using the ZetasizerNano ZS (Malvern Instruments, Malvern, UK). Default instrument settings and automatic analysis are used for all measurements.

A4 Doxorubicin loading using pH-gradient technique

A dried lipid-quantum dot film is hydrated with 250-mM ammonium sulphate (pH 8.5), followed by desalting using Sephadex G-50 column eluted with HEPES buffer. The sample suspension is then mixed with a doxorubicin saline solution (5:1 lipid:Dox molar ratio), followed by incubation in 60-°C water bath for 1 hour. The loading efficiency is determined after removing unloaded doxorubicin using a spin column of Sephadex G-50. The loading efficiency of doxorubicin is quantified by the UV absorbance at 480 nm after lysis of liposome using 1% Triton X-100.

A5 Doxorubicin release study

To monitor doxorubicin (Dox) release from liposomes and L-QD, samples are diluted to a final lipid concentration of 0.5 mM using HEPE buffer (25-mM HEPES, 150-mM NaCl, pH 7.4) or 50% serum (mouse CD1). The diluted samples (1 ml) are then loaded into a snakeskin dialysis tubing (10000 WMCO) and the tubing was immersed into a 250-ml beaker filled with HEPEs buffer. At designated time points, 1-ml sample is collected from the beaker for measurement and then returned to the beaker afterwards in order to remain the volume of the buffer unchanged. At 0 time point, fluorescence intensity of the sample is monitored twice before (f) and after addition of Triton X-100 (F); at other time points, sample is measured once (f_t). The release of Dox calculated from the following equation:

$$\text{Dox release } (\%) = (f_t - f)/(F - f).$$

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