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Review Article

Multifunctional quantum dots and liposome complexes in drug delivery

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Abstract

Incorporating both diagnostic and therapeutic functions into a single nanoscale system is an effective modern drug delivery strategy. Combining liposomes with semiconductor quantum dots (QDs) has great potential to achieve such dual functions, referred to in this review as a liposomal QD hybrid system (L-QD). Here we review the recent literature dealing with the design and application of L-QD for advances in bio-imaging and drug delivery. After a summary of L-QD synthesis processes and evaluation of their properties, we will focus on their multifunctional applications, ranging from *in vitro* cell imaging to theranostic drug delivery approaches.

Keywords: liposomes, quantum dots, nanomedicine, drug delivery

Introduction of liposomal quantum dot hybrid delivery system

The use of nanoparticles has increased in the areas of drug delivery[1–2], cancer detection[3], and therapeutics[1,4–7]. Quantum dots (QDs) are one of the most interesting examples due to their unique optical characteristics that enable them to overcome the limitations of previously used organic fluorophores[8–9]. On the other hand, liposomes are nanoscale spherical vesicles composed of phospholipid bilayers[10]. The more recent proposition to combine liposomes with QDs not only increases biocompatibility of QDs, but also opens opportunities for creating theranostic nanoscale delivery systems, which can combine simultaneous therapeutic and imaging functions[11]. Combining QD with therapeutic agent in a liposomal delivery system allows the bio-distribution of the payload to be monitored *in vivo*, reducing the potential for unintended side effects of drug toxicity in healthy tissues. In addition, the use of such hybrid systems potentially allows clinicians to monitor the progress and efficacy of a therapy throughout the course of treatment[11]. In this review, we have focused on the liposomal QD hybrid system (L-QD), where imaging agent and therapeutic agent can be co-delivered.

Liposome based drug delivery system

Liposomes were discovered by Alec D Bangham in the 1960s at the Babraham Institute, University of Cambridge[12–13]. Since their discovery, liposomes have been widely investigated, and are considered to be one of the most successful drug delivery systems. The first

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liposome formulation was composed solely of natural lipids; now liposomes can be created from cholesterol and natural or synthetic phospholipids with additional modification to prolong their *in vivo* circulation time[10]. In aqueous solution, due to their amphipathic nature, phospholipids have a strong tendency to form membranes[14]. Hydrophobic drugs/QDs can be enclosed in-between membranes, while hydrophilic drugs/QDs can be encapsulated inside central compartment. To improve the delivery of therapeutic molecules, surface modification of liposomes by the inclusion of hydrophilic carbohydrates, glycolipids or polymers, such as polyethylene glycol (PEG) can be used[15–16]. The PEGylation of the liposomes would prolong the blood-circulation time while suppressing the uptake by the reticuloendothelial system (RES)[17–18]. These long-circulating liposomes can passively accumulate in the the tumor site through the porous endothelium present in tumor — the enhanced permeability and retention (EPR) effect[16]. Furthermore, active targeting can be achieved by modification of the PEG terminus with functional molecules e.g. specific ligands or monoclonal antibodies[19–21].

Liposomes can vary from a few nanometres to several micrometres and may have one or more lipid bilayers. The sizing of liposomes is a critical parameter which determines their therapeutic efficiency[22] and helps characterize liposomes. On the basis of their size and lamellarity, liposomes can be classified into one of three categories: multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV)[23]. MLV have an onion like structure with diameters of 1-5 μm. SUV are typically 50 nm in diameter while LUV range from 100 to 250 nm or even larger[24]. There are several methods for the preparation of liposomes. One of the most widely used techniques for liposome synthesis is the thin-film hydration[10], where dry lipid film is hydrated by adding an aqueous buffer solution under agitation at temperature above the lipid transition temperature[25]. During this process, hydrophilic drugs are entrapped by using the aqueous solution of these materials as hydrating fluid, while lipophilic drugs are solubilized in the organic solution of the constitutive lipid. However, using this method, large and nonhomogeneous MLVs have formed and further steps are needed to produce homogeneous SUV. The second method is solvent solvent-injection using either ether or ethanol. This method involves the dissolution of the lipids into either or ethanol, followed by the injection of the lipid solution into aqueous solution[26–27]. The ether injection method has one advantage over ethanol injection method as ether is immiscible with water so it can be removed directly during injection process by using warmed aqueous phases above the boiling point of ether[25]. The third method is reverse-phase evaporation, which is based on the creation of inverted micelles. Briefly, the water-in-oil emulsion is formed by brief sonication of organic solvent containing phospholipids and aqueous buffer. Then the liposomes are shaped when the organic solvent is evaporated during continued rotary evaporation under reduced pressure[28]. Other methods for liposomes preparation include detergent removal[29], spray-drying[30], freeze drying[31], and microfluidisation[32].

Drug loading into liposome can be attained either passively (i.e., the drug is encapsulated during liposome formation) or actively (i.e., after liposome formation). Hydrophobic drugs can be directly combined into liposomes during the formation process. The encapsulation efficiency is dependent on the properties of the lipid such as length and packing density as well as drug-lipid interactions. Passive loading of hydrophilic drugs depends on the ability of liposomes to trap aqueous solution containing a dissolved drug during vesicle formation. Only a small percentage of a hydrophilic drug can be encapsulated by passive loading using the most common thin film hydration method[33]. The highest encapsulation efficiency by means of passive loading (up to 65%) is achieved by the reverse-phase evaporation method. However, this technique uses organic solvents, which will leave probably a small amount of residue in the liposome suspension, and thus is not feasible for commercial production[23,33]. Compared to passive loading, active loading employing pH gradient methods could achieve near 100% encapsulation efficiencies[34–35].

Due to their biocompatibility and biodegradability, liposomes are considered safer drug delivery systems. Both diagnostic and therapeutic agents can be encapsulated into liposomes. The encapsulation of drugs into the liposomes protects them against enzymatic degradation and immunologic inactivation, thus improving their therapeutic activity. Meanwhile, the encapsulation minimizes exposure of healthy tissue to drugs during their circulation in the blood. Therefore, systemic toxicity will be largely reduced compared with free drugs[36]. Currently, several liposome-based drugs are approved for clinical practice such as Doxyl®[37–38] and AmBisome®[39]; many others are in various stages of clinical trials[40–43].

Quantum dots

Among various types of nanomaterials, *i.e.* metals, metal oxides, organic materials or biomaterials, semiconductor nanoparticles (NPs), also referred to as QDs,

has become a major interdisciplinary area of science due to their unique properties[44]. QDs are generally considered to be particles of material which have diameters in the range of 1 to 10 nm[45]. The small dimensions of QDs result in properties differing from those seen in the corresponding bulk material. These unique physical properties give rise to many potential applications in areas such as luminescence, electronics, catalysis, and optoelectronics[46–49]. The size of the particle and surface to volume ratio are two fundamental factors of individual QD, which are responsible for these unique properties. As a particle becomes smaller, the band gap gradually becomes larger because of quantum confinement effect. Also, as the size of the semiconductor material becomes smaller, the ratio of the number of surface atoms to those in the interior increases, which leads to the surface playing an important role in the properties of the material[50]. Thus, both particle size and the synthetic method determine the physical and electronic properties of the QD produced, which gives scientists the unique ability to change the electronic and chemical properties of a QD.

QDs have quite a few advantages over traditional fluorescent organic molecules. They have size- and composition-tuneable fluorescence emission from ultraviolet to infrared wavelengths, narrow spectral line widths, high luminescence quantum efficiency, broad absorption profiles, and stability against photobleaching[9,51–53]. The broad absorption and narrow emission spectra of QDs allow simultaneous detection of multiple colors of QDs upon illumination with a single light source which is very useful for fluorescent multiplexed analysis in biological system[51,54]. Due to their photophysical characteristics, QDs are thought to have potential as novel fluorescent probes for diagnostic purposes[9,52]. However, their biocompatibility and potential toxicity remain critical issues for use in humans and thus limit their application[55–56]. The combination of liposome and QDs to create stable and multifunctional assemblies could overcome the shortfall of QDs and pave the ways for development of novel drug delivery system.

Liposome and QD hybridization

QD are an ideal candidate for fluorescent imaging in both the biomedical and pharmaceutical fields. However, QD are mostly synthesized in non-polar organic solvents; their surface hydrophobicity or poor colloidal stability at physiologic conditions frequently renders them inappropriate for biomedical and clinical applications. Many strategies have been developed to overcome this limitation, such as functionalization of QD with peptides, antibodies, and polymers[57–58]. However, evidence shows that the presence of these ligands could be disadvantageous for their application. Surface modification often quenches QD fluorescence and decreases their photostability[59–60], which for *in vivo* applications requires higher doses of administered QD and therefore increases potential toxicity. Encapsulation of QD within liposomes may avoid chemical functionalisation of QDs, which can lead to enhanced stability in plasma, better control of the pharmacological fate, and an overall improvement in their biocompatibility. Moreover, therapeutic agent, e.g. anticancer drug, could also be added to the QD liposome hybrid to create a device with both therapeutic and diagnostic functions. For example, hydrophobic QDs could be embedded in the lipid bilayer, whereas hydrophilic therapeutic agents can be encapsulated within the internal liposome aqueous core[61–62]. The hybridization of the QDs with liposome generates a novel, interesting class of nano-range delivery devices, which combines the unique optic properties of QD and the lipid functional moiety, thus offering new opportunities in multifunctional drug delivery system development. A summary of the types of hybrid delivery systems that could be used for simultaneous diagnostics and therapeutics of cancer is presented in ***Table 1***.

Formulation of liposomal QD hybrid system

Developing liposome-based drug delivery system with QD-based fluorescence imaging requires the hybridization of QD with liposomes. ***Fig. 1*** depicts three different strategies for QD incorporation. Hydrophobic QDs have been inserted into the hydrophobic interior of the liposomal membrane[61,63–64], and hydrophilic QDs have been encapsulated in the interior aqueous compartment[66,71,74–75] or on the outer membrane of liposomes[69–70]. To achieve these, different methods have been used, depending on properties of QD.

Thin film hydration method is one of the most common techniques used in liposome production. Both hydrophobic and hydrophilic molecules can be encapsulated into liposomes during the synthesis process. Hydrophobic QDs could be easily incorporated into the lipid bilayer when added to the thin lipid film. Vogel and coworkers have incorporated hydrophobic TOPO functionalized CdSe QDs into the bilayer membrane of liposome using thin film hydration[63]. A high yield of LUV along with some MLV was obtained using this approach, and the sizes ranged from 50 nm to 50 μm. The researchers also used electric field aided hydration

Table 1 **Representative liposome QD hybrid systems for simultaneous imaging and therapy of cancer**

Types of QD used	Incorporated therapeutic agent	Structure of hybrid systems	Target (tumor) cells or xenografts [targeting mechanism/species employed]	Ref.
Trioctylphosphine oxide (TOPO)-coated CdSe QDs	N/A	QD inside hydrophobic interior of lipid bilayer	HEK293 human embryonic kidney cells [non-specific internalization]	[63]
TOPO-coated CdSe/ZnS	N/A	QD inside hydrophobic interior of lipid bilayer	A549 human epithelial lung cells and CD-1 nude mice 7 inoculated with C33a human cervical carcinoma cells [non-specific internalization]	[64-65]
TOPO-coated CdSe/ZnS and carboxyl-functionalized QD	N/A	TOPO-QD inside hydrophobic interior of lipid bilayer and carboxyl-QD inside interior aqueous compartment	HuH-7 human hepatocellular carcinoma cells [non-specific internalization]	[66]
Cetrimonium bromide (CTAB) capped CdTe or CdHgTe QDs	N/A	QD inside hydrophobic interior of lipid bilayer	MCF7 human breast carcinoma cells and Kunming mice [non-specific internalization]	[67]
TOPO-coated CdSe/ZnS	Doxorubicin (Dox)	TOPO-QD inside hydrophobic interior of lipid bilayer and Dox inside interior aqueous compartment	N/A	[61-62]
CdSe and CdSe/ZnS QDs (Lumidot®)	Cisplatin	QD inside hydrophobic interior of lipid bilayer and cisplatin inside interior aqueous compartment	Melanoma cells and nude mice [non-specific internalization]	[68]
Dihydroxylipoic acid (DHLA)-capped CdSe/ZnS QDs	N/A	QD on the surface of liposome	B16F10 cells and C57BL/6 mice [tracking extravasation with QD labeled cells]	[69]
Carboxyl CdSe/ZnS QDs	Dox	QD on the surface of liposome and Dox inside interior aqueous compartment	MCF-7/HER2 cells and xenografts [anti-HER2 single chain Fv fragments]	[70]
Streptavidin conjugates CdSe/ZnS QDs and ITK-carboxyl CdSe/ZnS QDs	N/A	streptavidin-QDs on the surface and carboxyl-QD inside interior aqueous compartment	A431 human epidermoid carcinoma and CHO Chinese hamster ovary cell lines [epidermal growth factor (EGF) ligand]	[71]
3-mercaptopropionic acid (MPA)-capped CdTe QDs	N/A	QD inside interior aqueous compartment	Xenografts [non-specific internalization]	[72]
Thiol-capped CdTe QDs	N/A	QD inside interior aqueous compartment	U2OS human osteosarcoma cell, Hela human cervical carcinoma cell and 293T human embryonic kidney cell [non-specific internalization]	[73]

to shorten process time and produced more homogeneous unilamellar structures. However, they didn't show if there was any influence on vesicle size by such process and there weren't any further steps used to reduce liposome size. In another study, Al-Jamal *et al*.[64] encapsulated TOPO capped CdSe/ZnS QDs into zwitterionic and cationic lipid bilayer of SUV using thin film hydration plus sonication. The incorporation of hydrophobic QDs in the lipid bilayer led to significant enhancement of their optical stability during storage and exposure to UV irradiation compared to that of QD alone in toluene. This method was adapted by Tian, *et al*.[61], who not only incorporated QDs into the lipid bilayer, but also encapsulated doxorubicin (Dox) into the core of the liposome. The loading of Dox into L-QD was done by the osmotic gradient technique and achieved more than 97% loading efficiency.

Unlike encapsulating hydrophobic QD, where thin

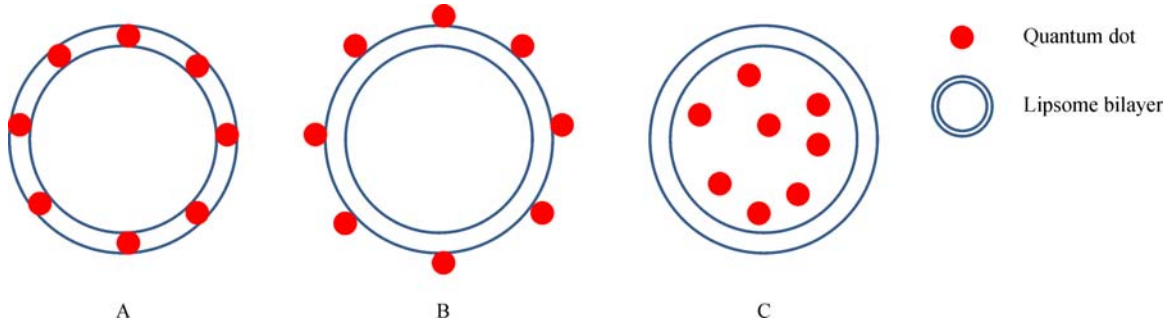

Fig. 1 **Quantum dots incorporation models of hybrid delivery systems.** A-hydrophobic quantum dots encapsulated shell model, B-hydrophilic quantum dots linked shell model, C-hydrophilic quantum dots encapsulated core model.

film hydration is the dominant method[61–62,64,76–78], there are several different methods to incorporate hydrophilic QD into liposomes. First, thin film hydration could also be used for incorporating hydrophilic QD into the aqueous core of liposome[72,74–75]. Al-Jamal, *et al.*[74–75] synthesized COOH-PEG-lipid coated L-QDs by thin film hydration followed by sonication as they did for the hydrophobic CdSe/ZnS QD. The thin lipid film was prepared and was hydrated with QD suspension. SUV were then prepared by further bath sonication. Ye, *et al.*[67] have also incorporated hydrophilic CdTe QDs into the vesicle lipid bilayer using the classic thin film hydration method. CTAB was used to make negatively charged hydrophilic QDs soluble in lipid mixture. Further sonication step was also used to form SUV loaded with QDs. Second, Bothun, *et al.*[66] used a single step reverse phase evaporation method to create a liposomal system that deliver both green hydrophobic and red hydrophilic CdSe/ZnS QDs into carcinoma cells. In the reverse phase evaporation process, an organic solvent phase containing lipids and the hydrophobic QDs was mixed with an aqueous phase containing the hydrophilic QDs to form an emulsion. The organic solvent was then removed by rotary evaporation to collapse the emulsion phase and drive QD incorporation within the liposome. Hansen, *et al.*[79] also used reversed phase evaporation approach and further purified their QD-loaded liposome by ion-exchange method. Third, the detergent dialysis technique was also used to encapsulate carboxyl CdSe/ZnS QD into core of the PEGylated liposome[71]. To do this, the lipid film was hydrated in Hepes buffer saline containing both 1-O-N-octyl-β-D-glucopyranoside detergent and carboxyl CdSe/ZnS QDs. The result solution was immediately dialysed against Hepes buffer saline to form hybrid liposome.

An alternative approach to hybridize hydrophilic QD with liposome is to conjugate the particle onto the surface of liposome. Voura, *et al.*[69] combined DHLA-capped QDs with the commercially available Lipofectamine. Because the liposome composed of cationic lipids are positively charged and the DHLA-QDs are negatively charged, they joined together by electrostatic force. The L-QD was simply formed by incubation of QD with liposome solution. In another report, Weng, *et al.*[70] have developed a multifunctional L-QD with imaging, targeting, and therapeutic modalities. The liposomes were simply prepared by extrusion. The carboxyl CdSe/ZnS QDs were then covalently linked to liposomes through a cross-linker. Furthermore, Dox was also encapsulated into the L-QD by a gradient loading technique either before or after covalent linking of the QDs to liposomes. It is worth to note that lower drug loading efficacy (~30%) was shown when drug loaded into liposomes after QD conjugation, which was probably due to the shielding of QD on liposome surface.

Characterization of liposomal QD hybrid system

Morphology of liposomal QD hybrid system

Morphological characterization of liposomal QD hybrid system helps in understanding the relative structure, position of the QD molecules and assembly of the L-QDs. These characteristics can influence the stability of the L-QD *in vitro* as well as *in vivo* and also can impact bio-distribution. Morphology of QD and liposome complexes can be studied using techniques such as, cryogenic transmission electron microscopy (cryo-TEM), freeze fracture electron microscopy (ff-EM) and atomic force microscopy (AFM).

Using Cryo-TEM Al-Jamal, *et al.*[74] showed clearly that vesicular structures of liposome were formed and that hydrophilic QDs were incorporated into the core of vesicle. It was also consistently observed that the QDs were interacting with the lipid bilayer rather than being simply encapsulated into the vesicle inner aqueous compartment, suggesting some degree of mixing between the bilayer lipids and the functional ligands

on the QD surface. In a later study, the same group showed several Cryo-TEM images which indicated that incorporating hydrophobic QD into lipid bilayer with low lipid:QD ratios (1000:1) resulted in elongated and deformed vesicular structures. Moreover, the lipid bilayer thickened at specific locations, which suggested that the incorporation of QD was taking place in "pockets" rather than being evenly distributed throughout the bilayer. According to their results, the ideal lipid to QD ratio for L-QD formation is 10000 to 1. At this ratio, perfect spherical liposomes were formed, and there was no noticeable physical deformation of the liposomes by hydrophobic QDs[64]. In general, hydrophobic QD containing liposome showed darker rim than the interior of the vesicles because of the presence of the electron-dense QD in the lipid bilayer[64,77] (***Fig. 2A***). Whereas, in case of hydrophilic QD (third model, ***Fig. 1C***), TEM images showed a pale edge and dark center of the obtained L-QD, which suggested that the QDs were incorporated into the core of liposome[72] (***Fig. 2B***). The technique of AFM was used by Tian, *et al.*[61] to investigated the structural elucidation of the hybrid vesicles. Interestingly, in contrast to the liposome control which showed a smooth surface, the image of L-QD showed that the incorporation of QD into the lipid bilayers resulted in a rough surface (***Fig. 2 C & D***). In addition, 3D image analysis indicated that QD associated with the lipid bilayers and distributed throughout the vesicle surface. Furthermore, the cross-section analysis suggested that QD incorporation increased the height of the liposome from 8 nm to almost 20 nm. Weng, *et al.*[70] used ff-EM to characterize the nanoscale structures of both free carboxyl QDs and QDs conjugated to the liposome outer layer. QDs appeared as small, mostly spherical particles with shadowing behind the structure, which was typical for hard-core particles. In contrast to QDs, liposomes displayed convex and concave fracture planes, which was typical for membrane-bound structures. The ff-EM images also showed that a small number of QDs appeared on liposomal fracture planes, which reflected imprints of surface-attached QDs showing through the semifluid, liquid-crystalline bilayers.

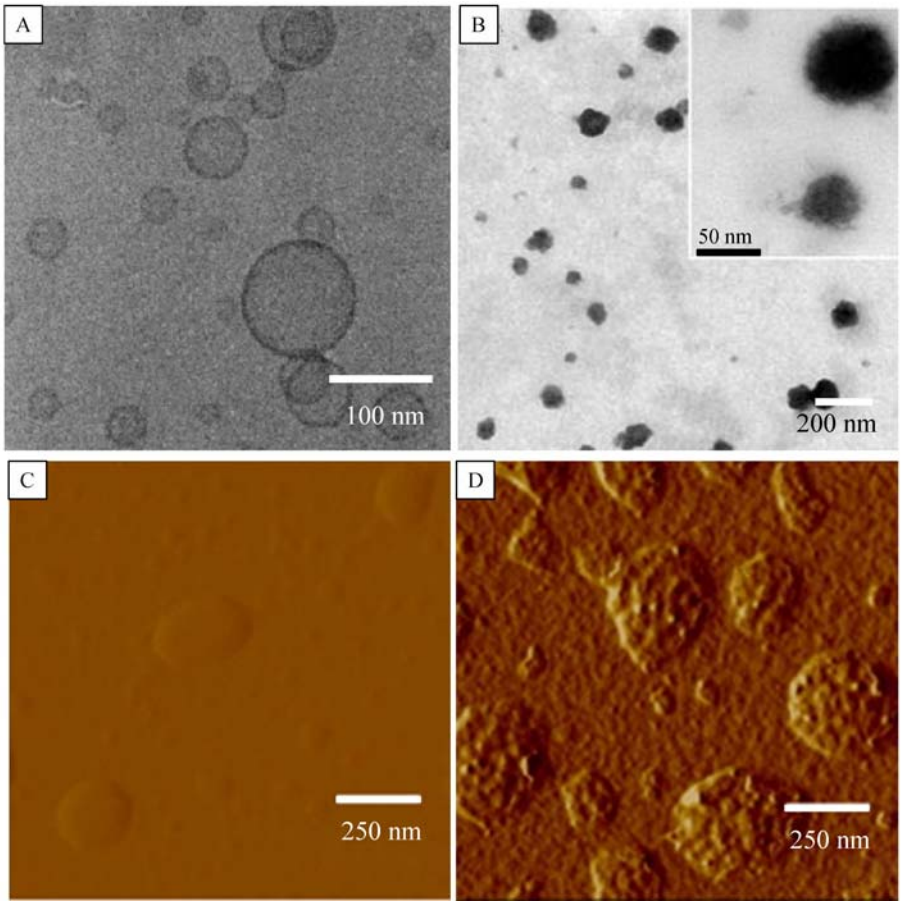

Fig. 2 **Electron microscopy and atomic microscopy images of the L-QD hybrid system.** A: Cryo-TEM images of Liposome-QD hybrid vesicles, scale bar 100 nm. Reproduced with permission from ref. 64. Copyright 2008 American Chemical Society. B: TEM images of the liposome-coated QDs. Reproduced with permission from ref. 72. Copyright 2010 Springer. C: AFM images of empty liposomes. D: AFM images of (DSPC) Liposome-QD hybrid vesicles. Reproduced with permission from ref.61. Copyright 2011 Elsevier.

Particle size and surface charge

Size and size distribution measurements are formulation parameters that indicate homogeneity of the particles in liposomal formulations and are very important for formulation and process optimisation. The poly-dispersity index (PI) of liposome reflects the range of liposome species present around the target average liposome size. In general, uniformly sized liposomes with a lower PI (< 0.2) are preferred for drug delivery. Moreover, changes in the average particle size and PI can be used as indicators of long-term stability. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) are the most common techniques to determine the size of liposomes. Al-Jamal, Al-Jamal, Bomans, Frederik and Kostarelos[74] demonstrated that incorporating of hydrophilic QD into the core of liposome largely increased liposome size as determined by both DLS and cryo-TEM. The size distribution was wide, as expected from thin film hydration preparation method. The surface charge characteristics of the L-QDs were in accordance with the characteristics of the lipid molecules used to form the bilayers. Sigot, *et al*.[71] showed a similar result from TEM measurement that hydrophilic QD and liposome hybrid was larger than empty liposome, and the vesicle size increased with increase in the number of encapsulated QD. In addition, Chu, *et al*.[72] noted that hydrophilic QD and liposome hybrids in water retain their spherical structures, as well as most of QDs remain trapped within the vesicles even after storing at 4°C for 609 days. This suggested that incorporation of QD to the core of liposome not only prevented the QDs being released from the vesicles but also reinforced the vesicle structure. Furthermore, Weng, *et al*.[70] conjugated hydrophilic QD to the surface of liposome, which also resulted in adversely increase of both vesicle size and size distribution. Interestingly, for hydrophobic QD and liposome hybrids, Al-Jamal, *et al*.[64] have shown that incorporating QDs did not significantly change the size and surface charge of hybrids. The mean L-QD diameter and surface charge were in the range of those empty liposomes. Subsequent studies carried out in this group have shown that both the size and the surface charge of hydrophobic QD and liposome hybrid vesicles remained almost the same even after Dox loading, which was consistent with liposome control. This suggested that the process of hydrophilic drug loading does not affect the physicochemical properties of L-QD[62]. The same group also demonstrated that such L-QD had pronounced colloidal stability and no mean vesicle diameter increased at both 4°C and 25°C over three weeks[61]. However, Kethineedi, *et al*.[77] have found that incorporating hydrophobic QD slightly increased the size of L-QD from a mean diameter of 75 nm to 100 nm. Zhang, *et al*.[68] have also shown an increase of liposome size with QD and cisplatin loading. Such changes also happened on surface charge when incorporating cisplatin and QDs into the liposome. The loading of cisplatin or CdSe QDs significantly reduced the negative zeta potential, and dual loading further shielded the electric surface potential. Such differences may result from different formulations and production procedures used among studies.

Photostability of QDs in the hybrid system

Photostability of QD after incorporating to the L-QD could be quantitatively studied by photoluminescence (PL) spectroscopy[61,64,67,72,78]. Interestingly, incorporation of QDs within the lipid bilayer led to enhanced photostability compared to that of bare QDs[64,67]. Al-Jamal, *et al*.[64] showed that the spectral characteristics of TOPO–caped CdSe/ZnS QD in obtained MLV and SUV were similar to those QDs in toluene. Only 30%–40% reduction of the initial fluorescence intensity was observed when the QD were embedded within the MLV and SUV lipid bilayers, compared to more than 70% loss of fluorescence intensity in the case of QD in toluene suspensions. Furthermore, QD in toluene were photochemically unstable when exposed to UV light, witnessed as a sharp reduction in fluorescence intensity and a marked blue shift at 7 days, and a complete loss of fluorescence after 14 days of UV exposure. On the other hand, the hybrid exhibited improved photostability after both 7 and 14 days of UV exposure. Similar results were obtained by Ye, *et al*.[67]. When incorporating CTAB-coated CdTe or CdHgTe QD into the lipid bilayer, fluorescence of QD was preserved and the photostability was enhanced. After 10 hour exposure to UV light, fluorescent intensity of the bare QDs was reduced sharply, whereas, only 20%–30% reduction was observed in the case of encapsulated QD. These results indicated that the L-QD improved the photostability of QD on storage and against UV light exposure, which can be attributed to the tight packing of the QD within the lipid bilayer. The increased photostability of liposome-encapsulated QDs might be expected to improve their performance as fluorescence markers. In another report, photostability of CdSe QD encapsulated in lipid bilayers with different physical state was investigated[78]. Three phospholipids with different melting temperature (Tm) were used. The PL of CdSe QDs changed in a phospholipid-dependent manner when stored under ambient conditions. Their results suggested that the Tm of the lipid membrane controls

optical and chemical properties of embedded QDs and QDs encapsulated within gel-phase lipid bilayer were the most stable. Furthermore, Tian, *et al*.[61] and Zhang, *et al*.[68] demonstrated that the addition of model drugs to the liposomes did not significantly alter the PL of QD, which is quite important for such multifunctional drug delivery system.

Encapsulation efficiency

Encapsulation efficiency (EE) determines the loading of QDs in liposomal hybrid formulations. EE is critical as it can be used to optimise the formulation composition as well as the manufacturing process. EE is calculated using the following formula:

$$\%EE = \frac{Encapsulated\ QD\ concentration}{Initial\ QD\ concentration} \times 100$$

The initial QD concentration is usually a known concentration determined in the formulation. Two techniques have been used to determine EE, namely fluorescence spectroscopy (FS) and thermal lens microscopy (TLM). In the first technique, encapsulated QD concentration is estimated using photoluminescence. Percentage EE is then calculated using the formula. For example, Wang, *et al*.[73] encapsulated water soluble thiol-capped CdTe QDs into liposome vesicles by agglomeration. The PL intensities of obtained L-QD in solutions were measured with a fluorescence spectrophotometer, and compared with that of the initial QDs solution. The PL in L-QD solution totally comes from the loaded QDs and no fluorescence self-quenching of the QDs inside the liposomes was observed, so that the comparison of PL intensities between the L-QD solution and the initial QD solution could estimate the loading content of QDs in L-QD. In this study, 95% EE was achieved with a lipid/QDs molar ratio of 0.15:1. The second technique, TLM, was developed by Batalla, *et al*.[80] which is a useful tool for determining EE when quenching of fluorescence happens after liposome encapsulation of the QDs. Self-quenching of QD is quite rare in hydrophobic loaded QD liposome hybrid; however, it may happen when hydrophilic QD is incorporated to the liposomes. The methodology of TLM consists in measuring the TL signal amplitude as a function of known QDs concentrations in water, *i.e.* the calibration curve. Next, the unknown concentration of encapsulated QDs is determined using the calibration curve. Then, the encapsulation efficiency can be calculated as the ratio of the encapsulated QDs concentration versus the initial concentration of QDs mixed with the empty liposome solution. It was found that the optimal EE for encapsulating carboxyl CdSe/ZnS inside aqueous compartment of the classic soy lecithin/cholesterol liposomes by thin film hydration method was 36% with an initial QDs concentration ranged between 1.25 and 10 nmol/L. The authors suggested that TLM not only determine the encapsulation efficiency but also the optimum quantity of the initial concentration of QDs to be mixed with liposomes.

In vitro and *in vivo* biodistribution of liposomal QD hybrid system

In vitro and *in vivo* biodistribution of L-QD could be studied using flow cytometry and fluorescence microscopy. Flow cytometry studies help to determine the average QD fluorescence intensity inside the cells; thus, they can be used to quantify cellular uptake of QD liposome hybrids. For example, cellular bonding and uptake of free QDs and various liposomal QD constructs were examined in MCF cells with HER2 overexpression 60 minutes post incubation at 37°C[70]. Flow cytometry studies revealed that the uptake of anti-HER2 L-QDs in HER2-overexpressing MCF cells was significantly higher than in normal MCF cells. In contrast, free carboxyl QDs bound non-specifically to all tested cells. Furthermore, anti-HER2 L-QDs showed markedly greater uptake *vs*. free QDs or *vs*. non-targeted L-QDs in HER2-overexpressing MCF7 cells at matching concentrations. Therefore, reduced amounts of QD could be used with anti-HER2 L-QDs to reduce the potential cytotoxicity of QDs. The biodistribution of L-QDs was also visualized using confocal microscopy. Free carboxyl QDs were observed in close association with the cell surface, while anti-HER2 L-QDs accumulated mostly in the perinuclear region of the cells (***Fig. 3A & B***). In addition, intravenous administration of L-QDs resulted fluorescence signals were readily detected at the tumor site 24 hours post-injection. Interestingly, tumor accumulation of both targeted and non-targeted L-QD showed similar results, which suggested that long circulating L-QDs localize in tumors predominantly *via* the EPR effect rather than *via* antibody-mediated targeting[70]. In another study, Kostarelos and coworkers studied intracellular trafficking of cationic and zwitterionic L-QD by confocal microscopy[64–65,74–75]. The L-QD intracellular signal was found to be time and dose dependent for both L-QD types[64,74]. It is evident from the microscopy images obtained that the cationic L-QD were uptaken more efficiently by A549 cells, compared to weak negative or neutral surface charge L-QD[64,74]. Cationic L-QDs were bound to the cell membrane within 1 hour and internalized throughout the cell volume and close to the nucleus after 3 hours incubation presumably through

endosomal uptake[74]. Similar results were demonstrated by Bothun, *et al*.[66], where fluorescence microscopy images showed that cationic L-QDs by HuH-7 was higher than zwitterionic L-QD. These studies suggested that the positive charge is a key parameter responsible for increased cellular uptake of the liposomal QD hybrid system. *In vivo* transportation of L-QD was investigated using a 3D multicellular tumor spheroids (MCS) cultures from melanoma cells (B16F10)[74]. Confocal microscopy images depict strong interaction between cationic L-QDs and the MCS. The L-QD localized 30–50-mm deep within the MCS mass. However, zwitterionic L-QD with a weak negative surface charge were able to diffuse deeper into the spheroids. *In vivo* uptake and retention of L-QD in tumour xenografts were also investigated[64–65,74–75]. Intratumoral injection of cationic L-QD gave much stronger fluorescence signals than zwitterionic L-QD after both 5 min (***Fig. 3C*** & ***D***) and 24 hours[64,74]. It is suggested that zwitterionic vesicles around 100 nm in diameter were leaking from the tumor immediately after intratumoral administration, while cationic delivery vesicles of similar size significantly increased tumor retention[81]. This agreed with the liposomal QD hybrid system, where cationic L-QD vesicles were uptaken by tumor cells and retained within tumor xenografts 24 hours post-injection, in contrast to zwitterionic L-QD vesicles that were drained out of the xenografts within 5 min following administration[64,74]. *In vivo* behavior of L-QD were further investigated following intravenous administration[65,75]. Sharp differences were obtained between the tissue biodistribution of the various L-QD types dependent on their lipid composition. Cationic L-QD exhibited rapid clearance from blood circulation

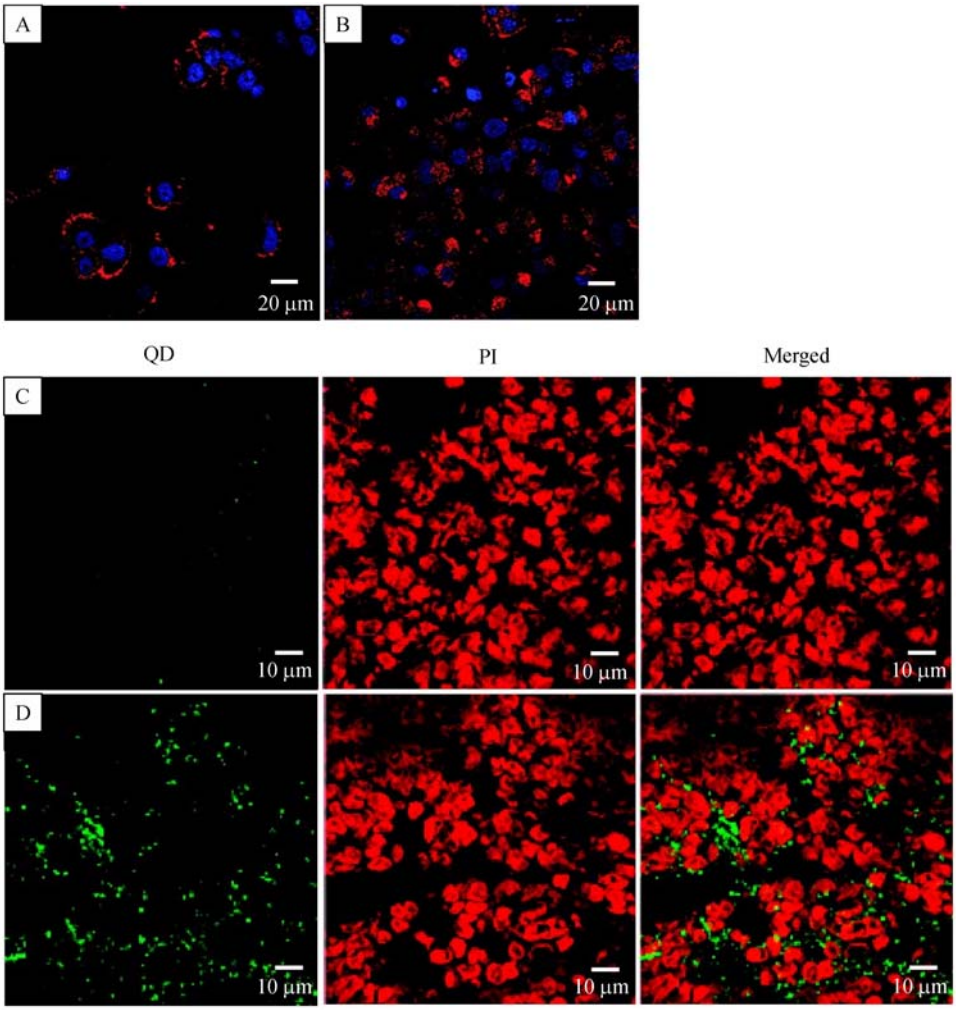

Fig. 3 ***In vitro*** **and** ***in vivo*** **uptake of L-QD illustrated by confocal microscopy.** A: SK-BR-3 cells were treated with free QDs, showing nonspecific association on the cell surface. B: SK-BR-3 cells were treated with QD-conjugated anti-HER2 liposomes (red fluorescence) for 60 min at 37°C. QD-conjugated liposomes were internalized in SK-BR-3 cells. Cellnuclei were stained by DAPI (blue fluorescence). Reproduced with permission from ref. 70. Copyright 2008 American Chemical Society. Panels C-D represent *in vivo* tumor xenograft uptake and retention of L-QD hybrid vesicles. Confocal microscopy images of human cervical carcinoma (C33a) tumors dissected 5 minutes after intratumoral injection with zwitterionic L-QD (C) and cationic L-QD (D). Left panels, L-QDfluorescence; middle panels, PI-stained nuclei; and right panels, the merged green and red channels. Reproduced withpermission from ref. 64. Copyright 2008 American Chemical Society.

due to transient lung accumulation. After 24 hours post-administration, redistribution of the cationic L-QD led to their localization mainly in the liver and spleen is thought to be due to adsorption of negatively charged plasma proteins on the surface of the cationic L-QDs hinders the nonspecific interaction with the pulmonary endothelium[65]. PEGylated L-QD exhibited improved blood circulation compared to cationic L-QD. The gel phase PEGylated L-QD accumulated in the solid tumor very rapidly and could be retained at the tumor site for at least 24 hours[75]. Overall, L-QD offers great potential for tumor imaging applications.

In vivo degeneration of liposomal QD hybrid system

Though the *in vivo* biodistribution of L-QDs is relatively well investigated, there is still a lack of knowledge about the degeneration of such delivery system. Biomedical applications mainly focus on intravenous injection or oral application routes. Upon injection or ingestion, L-QDs come into contact with various complex physiologic environments, leading to formation of a corona made of various biomolecules[82] which cover the L-QDs[83–86]. So for the degeneration process, L-QD should be considered as a system including corona, liposome envelope and QDs. So far the protein corona (PC) has mostly been studied, which can either has a stabilizing effect or has a destabilizing impact[84,87]. The corona complexes are quite stable in plasma; however, once they have been untaken into the liver, corona and L-QD degradation could be triggered[88]. Then, the elimination of liposome envelope takes place in the different ways, such as metabolized by Kupffer cells, splenic macrophages or eliminated by the target tissues after their accumulation[10]. Eventually, the innermost part of QDs may also be degraded, which will be highly dependent on the composition of the QD. For example, QDs made of CdSe are known to corrode, and thus release metal ions[89], while silica QDs can be completely dissolved by hydrolysis[90].

Application of liposomal QD hybrid system

In vitro and *in vivo* bio-imaging

QDs have been used as imaging agents to overcome many of the limitations of conventional contrast agents (i.e. organic dyes). The liposomal QD hybrid system provides further improvement of photostability and biocompatibility of QD, which could be used in bio-imaging applications *in vitro* and *in vivo*. Quite a few reports have appeared describing the use of such hybrid liposomal devices. For example, Voura, *et al*.[69] injected the tail veins of C57BL/6 mice with L-QD labeled B16F10 cells. The author demonstrated that QD labeling by L-QD has no detectable toxicity to the labeled cells or the host animal. The QD signal was observed in B16F10 cells seeded organs, which suggested that metastatic tumor cell extravasation could be tracked using L-QD. In addition, the use of a set of L-QDs with different emission spectra in conjunction with multiphoton and emission-scanning microscopy provides the opportunity to simultaneously identify and study the interactions of different populations of tumor cells and normal tissue cells within the same animal. In another study, Chu, *et al*.[72] used three different sizes of L-QDs for sentinel lymph node (SLN) mapping. The encapsulated CdTe QD had near-infrared (NIR) emission band which is ideal for *in vivo* imaging. The NIR light offers great advantages for deep tissue imaging applications, because the NIR light could penetrate deeply in living tissues, as well as autofluorescence and absorbance from tissue-intrinsic chromophores reach their minima in this range. After intradermally injection of L-QDs, a bright red fluorescent spot appeared rapidly in the axillary location of a nude mouse. The fluorescent signal was retained for 24-hour post-injection. Ye, *et al*.[67] also labeled MCF7 cells with CdTe or CdHgTe QDs encapsulated in liposomes. It was found that L-QDs were efficiently internalized by MCF7 cells in a time-dependent manner (***Fig. 4A-C***). Meanwhile, no cytotoxic effects were observed when cells exposed to the L-QD during the whole incubation period. All these findings suggested that L-QD increased biocompatibility and stability of QDs, thus improving the imaging effects for cancer cell labeling. Their *in vivo* imaging study using L-QD containing CdHgTe QDs also demonstrated the capability of L-QD for imaging in living animals (***Fig. 4D-G***). In the imaging process, strong fluorescence signal throughout the entire body was observed immediately after L-QD injection. Within 20 min of injection, fluorescence signal increased strongly in the liver and spleen and decreased sharply from the rest of the body, which suggested that L-QDs were taken up mainly in the liver and spleen due to RES effect. In addition, the authors showed that the injection of L-QD did not appear to induce significant toxicity in living animals. All these findings suggested that the L-QD is a promising tool for *in vitro* and *in vivo* bio-imaging study.

Multifunctional (therapeutic and diagnostic) delivery devices

One of the key advantages offered by the QD liposome hybrid system is the versatility of potential structural and surface characteristics by selection of different lipid components with minimal manipulation

of the QD. Also, they offer the possibility to simultaneously encapsulate therapeutic agents for the construction of multifunctional (therapeutic and diagnostic) delivery devices (***Table 1***). The first QD liposome theranostic systems was developed by Kostarelos and co-authors, who loaded L-QD with Dox using the osmotic gradient technique[61]. The PL spectra of Dox-loaded L-QD showed that both Dox and QD could be simultaneously detected, indicating the coexistence of both QD and Dox in the hybrid vesicle population[62]. The process of Dox loading into hybrid vesicles was further investigated at various loading concentrations compared with liposome control. The highest loading efficiency in hybrid vesicles was 97% compared with liposome control (99%), which indicated that QD incorporation into the lipid bilayer does not affect Dox loading through the lipid membrane of liposomes using the pH-gradient technique. It was also found that high Dox loading efficiency was achieved at a certain Dox concentration (> 0.5 mmol/L) when Dox crystal-like structures were formed. Moreover, the following release study showed that the fastest Dox release was observed from Egg phosphocholine (EPC)-QD vesicles in serum with 50% of Dox released over 6 hours compared with that from DSPC-QD vesicles (< 10%), which indicated that Dox release profile from L-QD could be modulated simply by changing lipid compositions. In another attempt, Weng *et al.*[70] have covalently conjugated hydrophilic QD at the outer surface of HER2-targeted liposomes (monoclonal antibody fragments of HER2 conjugated to liposomes) and then loaded Dox into the aqueous core of these vesicles through ammonium sulfate gradient method. While drug loading into liposomes prior to QD conjugation was highly efficient (> 90%), drug loading after QD conjugation was largely reduced with ~30% loading efficiency. The obtained L-QDs were relatively stable with 15-30% Dox lost after cold storage in buffer solution for 2 months. The anticancer activity of the Dox-loaded hybrids was evaluated in HER2-overexpressing SK-BR-3 cells. The Dox-loaded hybrids showed potent cytotoxicity against SK-BR-3 cells (IC50 ~ 0.5 μg/mL), which was comparable to Dox-loaded liposome without QD (IC50 ~ 0.7 μg/mL). This result suggested that intracellular Dox delivery of the hybrid system was as efficient as direct permeation of free Dox *in vitro* and also indicated that the HER-2 targeting effect was not compromised by QD conjuga-

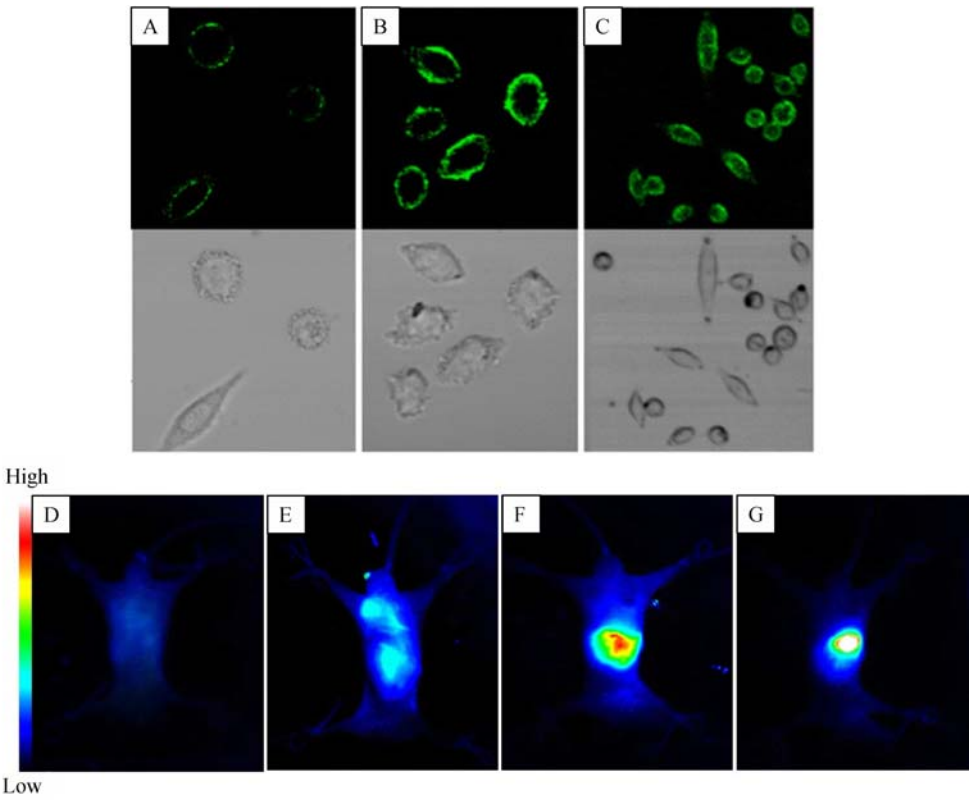

Fig. 4 **Temporal internalization of L-QD by confocal microscopy and NIR images.** Confocal microscopy images of MCF-7 cells incubation with lipid-QD after different time, top panel-fluorescencechannel, bottom panel-bright-field. A: lipid-CdTe 30 minutes. B: lipid-CdTe 60minutes. C: lipid-CdTe 120 minutes. D-G represent NIR images of the denuded mouse after lipid-CdHgTe was injected *via* tail vein for 10 seconds, 5, 10, and 20 minutes, respectively. Reproduced with permission from ref. 67. Copyright 2013 Springer.

tion. Furthermore, L-QD without dox showed minimal cytotoxicity, confirming that anticancer activity was due to Dox and not from the QD liposome carrier system. In addition, *in vivo* properties of the L-QD delivery system were studied in nude mice. The L-QD exhibited moderately prolonged circulation time, which was greatly prolonged over that of free QDs. No weight loss or obvious signs of toxicity were observed on mice during the study period (3 months). After 24 hours, following intravenous injection, in nude mice bearing HER2-overexpressing MCF-7/HER2 xenografts, fluorescence signals were readily detected at the tumor site as well as in mononuclear phagocytic system (MPS) organs known to mediate liposome clearance (***Fig. 5A***). Tumour fluorescence reached a plateau after 24 hours, with up to 18±5% of total body fluorescence localizing to the tumor region. Tissue sections of tumors collected 48 h post-injection showed that L-QD extensive accumulation within tumor tissue and intracellularly within tumor cells (***Fig. 5B***). In another report, Zheng *et al*.[68] have encapsulated both cisplatin and CdSe/ZnS into liposomes using thin film hydration method. The loading of cisplatin is approximately 50% with or without QDs. This suggested that incorporation of QDs did not affect the drug entrapment efficiency, which is agreed with previous studies. *In vitro* cytotoxicity assay on melanoma cells demonstrated that the L-QD without the drug had no cytotoxic effect on melanoma cells, while cisplatin-loaded L-QD showed greater cytotoxicity (22% viability). *In vivo* bio-imaging study showed the possibility to track such theranostic systems by IVIS imaging system after intravenous injection. The QD activity was primarily found in the brain and skin, which was also confirmed by *ex vivo* imaging of organs (***Fig. 5C-E***). The biodistribution of cisplatin also showed that the drug accumulation in brain and skin was significantly increased after loading into QD liposome vesicles as compared to that in the free control. Meanwhile, liver and spleen uptake of both QD and cisplatin was significantly reduced after encapsulation. These results suggested that entrapment of QDs and the drug into

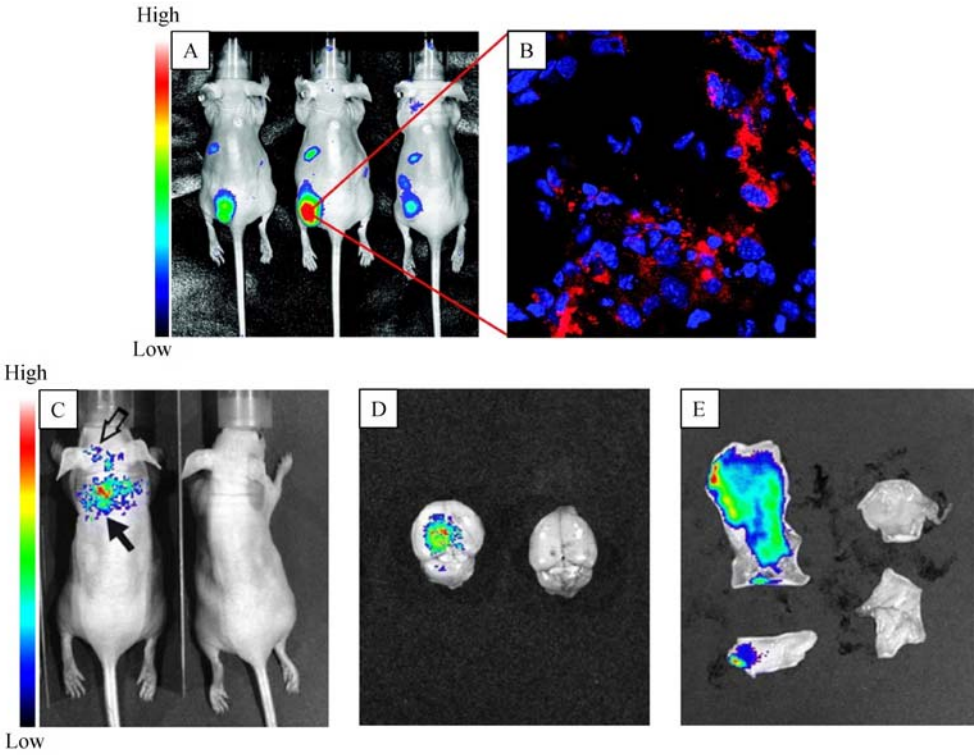

Fig. 5 **Fluorescence imaging of L-QD localized inside tumors.** A: *In vivo* fluorescence imaging of three nude mice bearing MCF-7/HER2 xenografts implanted in the lower back 30 hours after i.v.injection with anti-HER2 QD-ILs. Imaging showed that QD-ILs had localized prominently in tumors as well as in MPS organs. Units: efficiency (the fractional ratio of fluorescence emitted per incident photon). B: A 5 mm section cut from frozen tumor tissues harvested at 48-hour postinjection and examined by confocal microscopy. The tumor section was examined in two-color scanning mode for nuclei stained by DAPI(blue) and QD-ILs (red). Reproduced with permission from ref. 70. Copyright 2008 American Chemical Society. (C-E) represent fluorescenceimaging of representative nude mice detected with the IVIS system following an intravenous injection of CdSe/ZnS QD-loaded liposomes for 2 hours. The negative control (normal saline treatment) mice are represented by the animals placed on the right side of each image. C: whole-bodyimaging *in vivo* (open arrow indicates the possible site of the brain; close arrow indicates the possible site of the skin). D: *ex vivo* imaging of thebrain. E: *ex vivo* imaging of the skin. The scale bar is 1 cm. Reproduced with permission from ref. 68. Copyright 2012 Springer.

liposomes could protect them against degradation, reduce the RES uptake and enhance their delivery to the specific tissue site. All these studies demonstrated that the potential of the QD liposome hybrid delivery system could be used as a platform for synchronous therapeutic and diagnostic modalities.

Conclusion and perspectives

Liposomal QD hybrid systems have great potential for future clinical use since they are designed to integrate the well-established physicochemical and pharmacodynamic properties of liposomes with unique photochemical properties of QDs[11]. QDs can be loaded into the inner space of a liposome, insertion into the liposomal membrane, or attached onto the liposome surface. Careful attention should be paid to stability of such complex systems since incorporation of QDs and/or therapeutic agents in a liposome may cause payload leakage or a decrease *in vivo* stability of the liposomal nanostructure. Physical deformation of the liposomes could be eliminated by optimisation lipids and QD ratio in preparation formulation. In addition, high drug loading could be achieved by gradient method after liposome formation (with hydrophobic QD) or before hydrophilic QD attached onto liposome surface. For *in vitro* bio-imaging, the L-QD were shown to be efficiently uptaken by living cells in the absence of cell death. For *in vivo* applications, surface charge and lipid composition are important factors for blood circulation and tumor accumulation/retention of L-QDs. For example, cationic L-QD were retained much longer than zwitterionic L-QD when administered intratumorally. Whereas, PEGylated gel phase L-QD showed prolonged blood circulation than cationic L-QD when administered intravenously. Much more effort should be focused on the *in vivo* behavior of such hybrid delivery systems. Further investigations are needed to further prolong the L-QD blood circulation time, increase their accumulation in tumors and reduce their uptake in the liver and spleen. Overall, L-QD offers great potential for tumor imaging applications and could be easily adapted to construct theranostic device which achieve both diagnostic and therapeutic functions.

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