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Novel hybrid vesicles co-assembled from a cationic lipid and PAAc-*g*-mPEG with pH-triggered transmembrane channels for controlled drug release†

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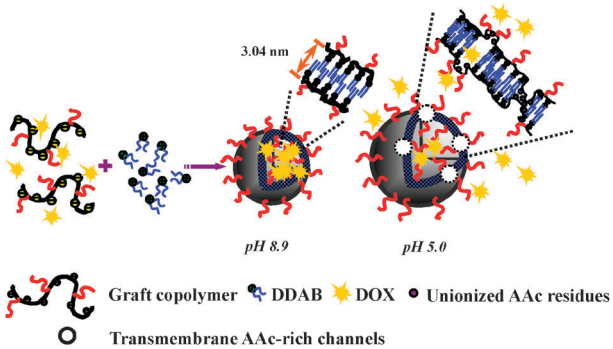
This work presents an important example of novel hybrid vesicles with pH-triggered transmembrane channels prepared by co-assembly of poly(acrylic acid)-*g*-poly(monomethoxy ethylene glycol) (PAAc-*g*-mPEG) with a cationic lipid, didodecyldimethylammonium bromide (DDAB), *via* electrostatic interaction for effective doxorubicin (DOX) release.

Various complex assemblies such as micelles and vesicles from co-association of ionic block or graft copolymers with oppositely charged lipids or surfactants *via* cooperative electrostatic interactions have received great attention due to their potential applications in drug delivery and biological fields.[1,2] Park *et al.* proposed a drug delivery system of polyelectrolyte complex micelles composed of PEG-conjugated oligonucleotide and a cationic lipid, lipofectamine, to promote the antisense cellular uptake and resistance to enzymatic degradation.[2] For promoting practical applications of complex assemblies in drug delivery, it is essential to endow them with responsive capability to environmental stimuli (*e.g.*, temperature, pH, electromagnetic field and/or light). Recently, a new concept of fabricating complex vesicles comprising an azobenzene-carrying surfactant and a double-hydrophilic block copolymer, PEG-*b*-PAAc, as an effective photosensitive drug delivery carrier was proposed.[3]

In this study, the lipid/PEG-containing complex vesicles with pH-triggered AAc-rich transmembrane channels were obtained by spontaneous co-assembly of PAAc-*g*-mPEG with DDAB in an aqueous phase of pH 8.9 *via* cooperative electrostatic interactions. The pH-induced morphological changes in the membrane structure of hybrid vesicles and the corresponding release profiles of the encapsulated DOX therein strongly illustrate that the vesicles show great potential as an intracellular drug delivery carrier, as shown in Scheme 1.



Scheme 1 pH-triggered vesicle morphologic transition and DOX release *via* the transmembrane AAc-rich channels of complex vesicles.

By partial aminolysis of poly(*N*-acryloxysuccinimide) (PNAS) with mPEG-NH_2 (M_n = 2000 g mol^{-1}) in DMF at 60 °C, followed by the full hydrolysis of unreacted NAS moieties in the graft copolymer into AAc residues by addition of tris buffer solution of pH 8.5, the PAAc-*g*-mPEG (M_w = 6.4×10^4 g mol^{-1}, polydispersity = *ca.* 1.77) used in this work was obtained.[4] The detailed synthetic route is shown in Fig. S1 (ESI†). The copolymer composition was determined by ^{1}H-NMR (AAc/mPEG = 92.1/7.9 (mol/mol)). Fig. S2† shows the ^{1}H-NMR spectrum of the copolymer in DMSO-d_6. To prepare copolymer/DDAB complex assemblies, the aqueous solution of the copolymer (2.0 mg mL^{-1}) at pH 8.9 (I = 0.01 M), where AAc units were completely dissociated as corroborated by potentiometric titration (Fig. S3†), was added into a vial deposited with a dry DDAB film at a preset molar ratio ($Z_{+/-}$ = 1.0) of DDAB to AAc residues and the solution was stirred at 20 °C for 24 h.

In agreement with the fluorescence characterization (Table S1†), the dynamic light scattering (DLS) data by the cumulant program indicate that, distinct from the well-dissolved fully ionized copolymer with an extremely low light scattering intensity (3.2 kcps) that is well beyond the DLS detection limit and typical DDAB-constituting multilamellar vesicles at equilibrium characterized by rather broad particle

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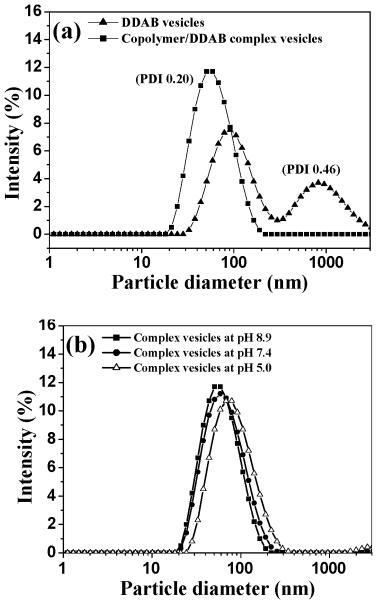

Fig. 1 DLS particle size distribution profiles of (a) DDAB vesicles and copolymer/DDAB complex vesicles at pH 8.9, and of (b) complex vesicles at pH 8.9, 7.4 and 5.0, respectively.

size distribution (PSD),[5] copolymer/DDAB complex assemblies at pH 8.9 possess not only a mean hydrodynamic particle diameter (D_h) of *ca.* 57.5 nm and a mono-modal PSD (polydispersity index = 0.20) (Fig. 1a), but also a zeta potential (ξ) of 0 mV as a result of the complementary ionic complexes between ionized AAc residues and DDAB molecules. The TEM image (Fig. 2a) and a linear relationship between the relaxation frequency (Γ) and the square of the scattering vector (q^2) observed in the angle-dependent DLS data (Fig. S4a†)[6] further confirm the assembly morphology in a spherical form.

To gain an insight into the molecular packing architecture of complex assemblies in aqueous solution of pH 8.9, their mean hydrodynamic radius (R_h) and root-mean-square radius of gyration (R_g) were evaluated by DLS and static light scattering (SLS), respectively, and the R_g/R_h ratio that is sensitive to assembly morphology[7] was thus determined. The R_g/R_h ratio of *ca.* 1.02 (Table 1 and Fig. S4a†) in agreement with the theoretical value (1.0) for thin-layer hollow microspheres[7] clearly demonstrates that hybrid assemblies formed in vesicle structure, in which the inner aqueous compartment is enclosed by a thin hydrophobic PAAc/DDAB membrane encircled by the hydrophilic mPEG coronas (Scheme 1). Note that, with the effective steric stabilization provided by mPEG coronas, these complex vesicles in aqueous solution (pH 8.9) maintain superior colloidal stability for at least one month. As revealed in the ^{1}H-NMR spectrum (Fig. S5†), the chemical

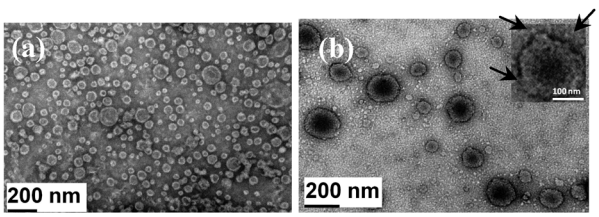

Fig. 2 TEM images of complex vesicles at (a) pH 8.9 and (b) 5.0.

Table 1 pH-evolved DLS and SLS data for the complex vesicles in aqueous solutions at 20 °C

pH	Intensity/kcps	R_g/nm	R_h[a]/nm	R_g/R_h
8.9	131.4	49.8	48.7	1.02
7.4	133.7	54.1	50.3	1.08
5.0	119.0	66.5	57.5	1.16

[a] Determined by DLS (Brookhaven BI-200SM) at a scattering angle of 90° using the CONTIN analysis method.

shifts and shape of the detected feature signals of DDAB, particularly from the tail end methyl groups, within complex vesicles comparable to regular dimyristoylphosphatidylcholine-based small unilamellar vesicles (SUV) in D_2O as reported previously[8] demonstrate that the complex vesicles exhibit a unilamellar architecture. Furthermore, based on the appearance of a characteristic diffraction peak at $2\theta = 2.93°$ in the wide-angle X-ray diffraction (WXRD) profile of vesicles (Fig. S6†) corresponding to a lattice spacing (d_2) of 3.04 nm (calculated by the Bragg equation) that is slightly shorter than the double extended length of DDAB (3.53 nm),[9] the unilamellar AAc/DDAB membranes are further characterized by forming a long-range interdigitated structure.

As shown in Table 1, with the medium pH being decreased from 8.9 to the target pH 5.0, an appreciable reduction in light scattering intensity and increases in R_h, R_g and R_g/R_h ratio (from 1.02 (pH 8.9) to 1.16 (5.0)) of complex vesicles were observed, a strong indication of the swelling of particles. This is primarily due to the reduced ionization of AAc residues and concomitant partial disruption of electrostatic interaction between DDAB and AAc moieties and, as a consequence, the decreased hydrophobic association originally elicited by electrostatic pairings. This then renders the vesicular membrane more hydrated and permeable for water influx into the inner aqueous compartment at pH 5.0. More importantly, compared to R_h, R_g that is mainly related to the mean distance of individual atoms (groups) constituting the assembly of target to its mass center is increased remarkably with increasing pH. This illustrates that, in addition to the spatially enlarged inner aqueous space of complex vesicles caused by water influx, the vesicular membranes are also transformed in part into the loose and swollen structure. Nevertheless, the virtually unchanged diffraction peak at $2\theta = 2.93°$ in the WXRD pattern with pH (Fig. S6†) suggests that the ordered interpenetrated layer morphology is still retained and the structural transition only occurs in rather localized regions. It is therefore postulated that the local unionized AAc residues are re-orientated into the membrane wall in such a manner that transmembrane channels form at pH 5.0 (Scheme 1). On the other hand, the uncomplexed DDAB molecules arising from the reduced pH tend to reside within the membranes *via* hydrophobic packing of lipid tails, thereby leading to a slight increase in ξ (*ca.* 3.6 mV) at the expense of the PEG shielding effect. These unionized AAc-rich transmembrane channels are created exclusively near the mPEG coronas because the intersegmental entanglements therein are largely reduced by extremely hydrated and mobile mPEG chain segments extending into the aqueous phase. Owing to the reduced integrity of the vesicular membrane in the presence of channels, slight

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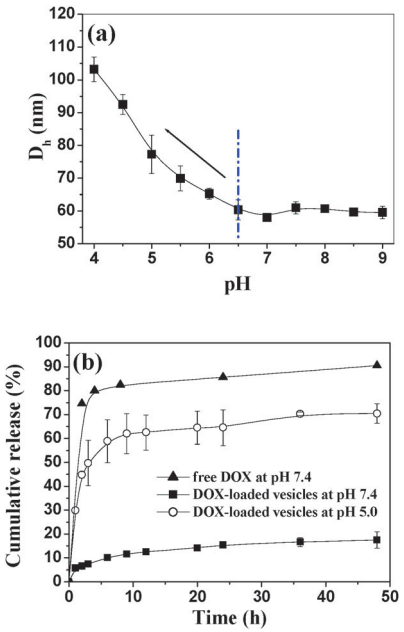

Fig. 3 (a) D_h *vs.* pH for copolymer/DDAB complex vesicles at 20 °C. (b) Cumulative DOX release from drug-encapsulated complex vesicles at pH 5.0 and 7.4 at 37 °C, using the dialysis technique. For comparison, transport of free DOX across the dialysis membrane at pH 7.4 is included.

deformation of vesicles accompanied with an appreciable increase in the particle size (though partly induced by water evaporation during the TEM sample preparation) is demonstrated in Fig. 1b and 2. Indeed, several cracks observed within the vesicular membranes (see the inset of Fig. 2b) strongly support the above hypothesis. While experiencing pH-triggered structural transition, vesicles in aqueous solution still maintain the mono-modal PSD (Fig. 1b).

In contrast, in the absence of mPEG, complex vesicles assembled from homoPAAc (M_w = 1.5 × 10^4 g mol^{-1}) and DDAB at pH 8.9 have a rather large size (*ca.* 260 nm in D_h) and a multilamellar architecture detected by DLS and small angle X-ray scattering (SAXS), respectively (data not shown here). The resultant homoPAAc/DDAB vesicles display very weak pH-responsive capability in terms of pH-evolved variations in both particle size and light scattering intensity (Table S2†). Such significant differences in the morphology of these two types of vesicles and their pH-responsive behavior strongly indicate that the mPEG grafts of copolymer not only facilitate the formation of unilamellar complex vesicles but also play a crucial role in the development of the pH-triggered highly permeable transmembrane channels.

As shown in Fig 3a, D_h gradually increases with the pH being decreased beyond 6.5 due to the formation of rather loose transmembrane channels with appreciably enhanced water permeability. Such complex vesicles undergo structural variation at rather mild acidic medium compared to physiological conditions (pH 7.4). To evaluate the potential of hybrid vesicles as pH-responsive anticancer drug delivery vehicles, DOX was chosen as a model drug. While the cumulative release of DOX from complex vesicles (with a drug loading efficiency of 30.8%) is limited to only 12% at pH 7.4 over a period of 10 h, a significantly increased drug release at pH 5.0 can be attained (60%, see Fig. 3b). This strongly suggests that the transmembrane channels formed at pH 5.0 facilitate the release of encapsulated DOX in comparison with the severely retarded drug release at pH 7.4 with the intact membrane structure. The average channel pore diameter is thus at least equivalent to the DOX size and the channel length should be close to the bilayer thickness (3.04 nm). The operation window for the formulation parameter $Z_{+/-}$ to successfully prepare such hybrid vesicles implemented with the pH-evolved channels fell in the range *ca.* 0.9–1.0. Such a pH-triggered change in the membrane morphology of this kind of hybrid vesicles has been long desired for controlled intracellular drug release within acidic endosomal and lysosomal compartments upon the cellular uptake *via* the endocytic pathway.[10]

In this study, a facile approach to prepare novel hybrid vesicles *via* co-assembly of a cationic lipid with PAAc-*g*-PEG was developed. Although further studies on the *in vitro* cell uptake and cytotoxicity and the *in vivo* biological performance as a drug delivery system are required, the novel vesicles show superiority in controlling intracellular drug release by the prominent pH-triggered membrane permeability.

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