Giant vesicle formation through self-assembly of chitooligosaccharide-based graft copolymers†

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A simple approach is described for the preparation of chitooligosaccharide-based giant vesicles with variable size by simply tuning the water content in the water–dioxane mixture, by which reactive vesicles with diameters in the range of 0.5–400 μm were easily prepared.

Giant vesicles (GVs), usually having diameters larger than 5 μm, refer to a special type of spherical lamellar assemblies of amphiphilic molecules. Since the structure and dynamic behaviour of GVs are similar to those of biological cell membranes, such vesicles have been attracting much attention as plausible models for artificial cells.[1] While the literature is rich in GV formation from small amphiphiles such as lipids (liposomes)[2] and surfactants,[3] amphiphilic copolymers are seldom employed in the study of GV formation, although polymer vesicles possess unique properties such as good stability and broader range of accessible solvents.[4] Discher and his coworkers[5] first succeeded in swelling vesicles from polyethyleneoxide-*block*-polyethylethylene (PEO-*b*-PEE) by electroformation, an effective method used to prepared GVs from phospholipids. Rotello *et al.*[6] discovered GVs, by chance, *via* the self-assembly of complementary random copolymers through specific interchain hydrogen-bonding in chloroform. Richtering *et al.*[7] reported the shear-induced formation of block copolymer vesicles. Recently, Yan and Zhou developed a new type of polymer GVs,[8] which were generated from the molecular self-assembly of an amphiphilic multiarm copolymer with a poly(3-ethyl-3-oxetanemethanol) core and many poly(ethylene oxide) arms (HBPO-*star*-PEO) in water. To the best of our knowledge, the GV formation from the self-assembly of amphiphilic graft copolymers has not yet been reported in spite of the advantages of providing integration of considerable functionality onto the polymer backbone that can be addressed chemically after the assembly process.[9]

Using chitooligosaccharides (COS) for the backbone and polycaprolactones (PCL) for the branches, we recently succeeded in producing a novel type of amphiphilic graft copolymer with a large amount of free amino and hydroxyl groups remaining on the COS backbones.[10] Aggregation of these graft copolymers (COS-*g*-PCL) with PCL branches of various lengths in tetrahydrofuran–water mixture gave rise to multiple morphologies that were similar to those from linear block copolymers. In the present study, a simple approach was developed to enable self-assembly of COS-*g*-PCL copolymers into GVs (Fig. 1). Two new kinds of COS-*g*-PCL graft copolymers, **g1** and **g2** (Fig. 1), with different hydrophobic PCL branches were synthesized based on our modified procedure. Scheme S1, Fig. S1–S2 and Table S1 in the Supporting Information display the detailed synthesis and structure characterization of these molecules.†

To prepare the GV aggregates, COS-*g*-PCL copolymers with different lengths of PCL branches (**g1** and **g2**) were initially dissolved in 1,4-dioxane at 0.5 wt%. Deionized water was added dropwise at a rate of 0.2% per minute to the COS-*g*-PCL solution under vigorous stirring until a predetermined water content was reached, and then the morphology evolution of the aggregates was monitored with phase-contrast microscopy (BX-51, Olympus) or transmission electron microscopy (TEM).

The optical and TEM images of the self-assembled aggregates from **g1** at various water contents are shown in Fig. 2. At the water content of *ca.* 20 wt%, the diameters of the aggregates obtained are less than 1 μm. The low resolution of microscopy limits direct imaging of the aggregates (Fig. 2a). TEM was used, instead, to observe the aggregates (Fig. 2b). When the water content was increased to *ca.* 35%, the vesicles grew to bigger ones (Fig. 2c), forming GVs with diameters larger than 5 μm. At 50 wt% water content, the size of the GVs became even larger and a variety of morphologies were observed, including GVs with diameters up to 400 μm

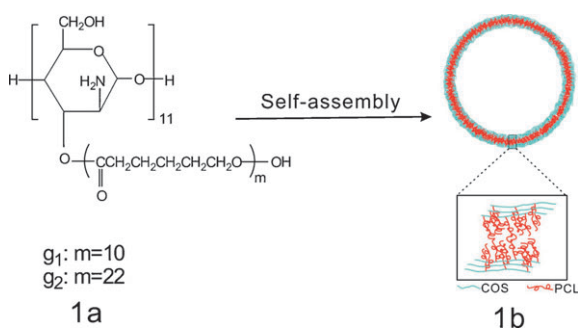

Fig. 1 Chemical structure of COS-*g*-PCLs (1a) and the GV formed (1b), wherein *m* represents the degree of polymerization of the PCL branch.

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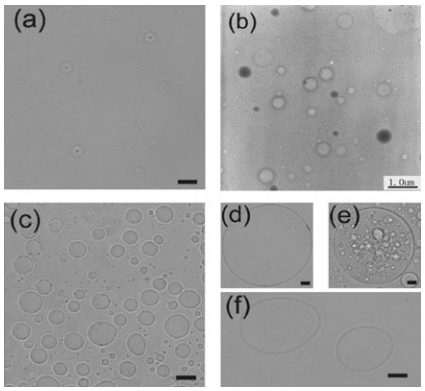

Fig. 2 Phase contrast micrographs (a, c, d, e, f) and TEM image (b) from copolymer **g1** in dioxane–water mixtures at different water content. The initial copolymer concentration in dioxane is 0.5 wt%. (a), (b) vesicles at 20 wt% water, (c) vesicles at 35 wt% water, (d) vesicles with diameter up to 400 μm at 50 wt% water, (e) vesicle-in-vesicle at 50 wt% water, (f) ellipse-shaped vesicles at 50 wt% water. The samples for TEM measurement were negatively stained with 2% aqueous phosphorous tungstenic acid solution. The scale bars are 40 μm in (a), (c), (d), (e), (f) and 1.0 μm in (b).

(Fig. 2d), vesicle-in-vesicle (Fig. 2e) and ellipse-shaped GVs (Fig. 2f). The size distribution of COS-*g*-PCL vesicles obtained from various content in water–dioxane is displayed in Fig. 3. The size of the vesicles was measured from the staff gauge in the microscopy studies, and results were based on an analysis of 100 vesicles. The results indicate that the size of the generated vesicles increases and the size distribution becomes broader as the water content increases. These results are consistent with the observation of Discher *et al.*[5] who mentioned that vesicle size could be tuned by adjusting the osmotic pressure external to freely suspended polysomes. However, in our case the observation that the membrane of the prepared GVs is flexible enough to expand from 0.5 to 400 μm is very remarkable, and, to the best of our knowledge, is reported for the first time. Stepwise dilution of the external osmolarity would lead to water permeation and swelling of vesicles at the expense of small vesicles (through the fusion of smaller vesicles). Indeed, due to the fusion, a decrease in the overall number of vesicles was observed during the course of adding water. When the water content exceeded 50 wt%, the GVs self-assembled from **g1** in dispersion became unstable and most of them coalesced and settled at the bottom of the container (not shown). However, the vesicles in dispersion with water content less than 50 wt% appeared very stable and they could be stored for at least three months at room temperature, and even at enhanced temperature (< 50 °C, Fig. S3 in the Supporting Information†).

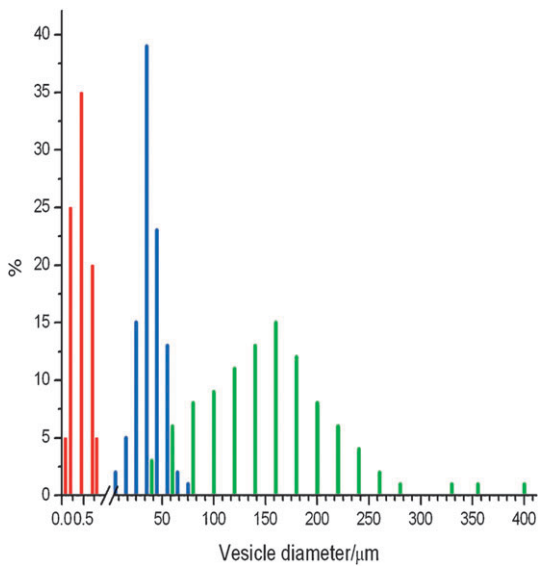

Fig. 3 Statistic size distribution of GVs formed from the copolymer **g1** in dioxane–water mixtures at different water content, based on an analysis of 100 vesicles. The red, blue, and green bars represent GVs prepared at 20 wt%, 35 wt%, and 50 wt% water content, respectively.

Surprisingly, for polymer **g2** which has a longer PCL branch, the diameter of the formed vesicles decreases with increasing water content in the dioxane–water mixture (Fig. 4a–c), suggesting that vesicle fission probably occurs. A great amount of literature on GV fission has been published, but the GV fission was usually induced by adding amphiphilic additives,[11] glucose,[8] or metal ions[12] into preformed vesicles. The "spontaneous curvature" and "area difference elasticity" models were developed and have been successfully used to forecast, explain, and analyze the fission of lipid vesicles.[13] The GV fission induced by adding water alone to GV dispersion, however, has never been reported.

The different response of **g1** and **g2** to water content can probably be attributed to the different permeability of water into the interior of the vesicles. The graft copolymer **g1** possesses shorter hydrophobic PCL branches and the degree of crystallinity of the vesicle membrane is lower. Stepwise dilution of the external solution of vesicles provides water with enough time to permeate into the interior of vesicles. In contrast, graft copolymer **g2** has longer hydrophobic branches and the vesicle membrane has a high degree of crystallinity (Fig. S4†). Therefore, in this system, it would be difficult for water to penetrate instantly across the membrane of vesicles during the course of increasing water content, resulting in significant osmotic imbalance between the inner and the outer solutions. The osmotic imbalance becomes the driving force for vesicle fission. However, we cannot exclude another possible mechanism, namely, dioxane

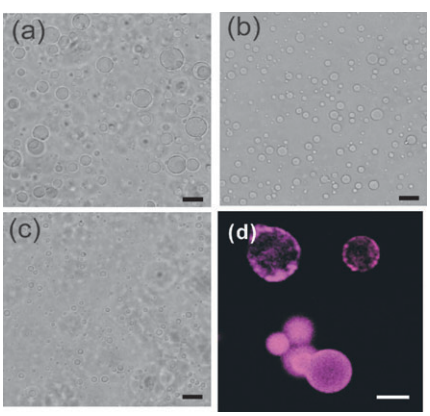

Fig. 4 Phase contrast micrographs (a, b, c) and LSCM image (d) from copolymer **g2** in dioxane–water mixtures at different water content. Vesicles at (a) 20 wt%, (b) 35 wt%, (c) 50 wt%, and (d) 20 wt% water. The scale bars represent 40 μm in (a), (b), (c) and (d).

should also be able to penetrate the vesicle wall. For the thin **g1** wall, maybe water diffuses through the wall faster than dioxane, giving the growth as observed. And in the **g2** vesicles where water penetration is strongly suppressed, dioxane may go through faster, out from the vesicles and thus result in shrinking. Therefore, to design experiments to test the various mechanisms is a question for our future work.

Proof of the vesicular nature of the assemblies formed from **g2** was obtained using laser scan confocal microscopy (LSCM) and TEM. The LSCM micrographs of the assemblies showed that the vesicles consisted of bright edges with less fluorescent central regions (Fig. 4d), indicative of a hollow interior.[14] The TEM image clearly revealed the vesicular structure with a thickness of 23 nm, which is good agreement with a double-layer structure (Fig. S5†).

Taking into account the nature of the structure of COS-*g*-PCL, the self-assembly mechanism should include two driving forces: one is the hydrophobic interaction which is attributed to the amphiphilic features of COS-*g*-PCL consisting of the hydrophilic COS backbone and hydrophobic PCL branches, and the other is the formation of hydrogen bonds among the amino and hydroxyl groups remaining on the COS backbone. The hydrogen bonding plays a key role for the formation of GVs. When the amino and hydroxyl groups were protected by trimethylsilylation, the formation of GVs could not be observed, only ill-defined precipitation was detected (Fig. S6†). The importance of hydrogen bonding has also been confirmed by variable-temperature FTIR spectroscopy (Fig. S7†). The molecule packing in the vesicle wall was investigated by ^{1}H NMR (Fig. S8†), indicating that the PCL segments form the middle layer of the vesicle wall, and the COS segments form the outer layers, which remain in a solvated state. For our system, the graft copolymers can form large vesicles (on micrometre scale) spontaneously. This may be due to the tendency for the COS backbones to form parallel arrays driven by intra- and intermolecular hydrogen bonds originating from the –OH and –NH_2 groups. In addition, the wall thickness of vesicles from the graft copolymers of **g1** and **g2**, measured by TEM, is about 12 nm and 23 nm, respectively (Fig. S5†). The average length of PCL branches in **g1** and **g2** molecules is 13.8 nm and 22.5 nm, respectively (calculated by the MM2 method at minimizing energy, available from Chem3D Pro software). Consideration of the **g1** and **g2** molecule size and the vesicle wall thickness leads us to suggest that COS-*g*-PCL molecules pack into an interdigitated structure, as shown in Fig. 1. A similar packing model was reported by Srinivas *et al.*[15] and Ryan and Battaglia,[16] suggesting that hydrophobic polymer coils with a higher molecular weight are more likely to interdigitate and become entangled in vesicle walls.

After the formation of GVs, the free amino groups remaining on COS backbones are still chemically addressable and exhibit chemical reactivity. Upon the addition of glutaric dialdehyde to a GV-dispersive solution, Schiff bases formed, and cross-linked stable encapsulates were obtained (Fig. 5a). The crosslinked vesicles exhibit good resistance in water, dioxane or alcohol (Fig. S9†). Quantitative information about the release of prepared vesicles in the course of adding water into vesicle suspensions was also evaluated (Fig. S10†). We found that a relatively low release of calcein occurred in the course of adding water into the vesicle suspension, suggesting a high stability of the prepared vesicles. More interestingly, these amino groups can serve as ligands to chelate different metal ions and to produce novel hybrid GVs. Our preliminary work shows that well-defined GVs containing various quantum dots (ZnS, PbS, CdS) or metal clusters (Au, Cu, Ag) inside the membrane are easily realizable (Fig. 5b).

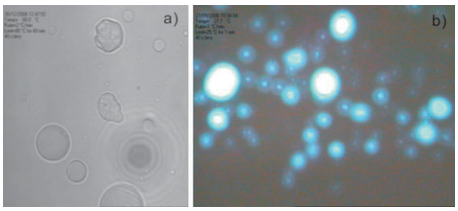

Fig. 5 (a) Phase contrast micrograph of cross-linked GVs by using glutaric dialdehyde, which are stable at high temperature (80 °C); (b) fluorescence image of the hybrid GVs containing ZnS quantum dots inside the membrane.

In summary, we successfully obtained a new type of polymer GVs from the self-assembly of chitooligosaccharide-based graft copolymers in water–1,4-dioxane mixtures. Unlike the GV formation reported in earlier literature, the work in this communication presents a simple approach for the preparation of GVs with variable size by adjusting the water content in water–dioxane mixtures. With this system, reactive vesicles with diameters in the range of 0.5–400 μm were easily prepared.

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