A quantitative cryoTEM study on crosslinked nanocapsule morphology in RAFT-based vesicle polymerization

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1. Introduction

There exist many applications of encapsulation and controlled release technology, for example in the fields of coatings, paints, cosmetics and nanocapsules [1–5]. These nanocapsules are for example prepared by approaches like sacrificial and vesicle templating [6–14]. Controlling the thickness of the polymer shell and the functionality of the nanocapsules is important for controlled release rates [15,16] and efficiency in example human nanotherapeutics or agricultural applications [17,18]. This becomes even more important in the multi-functional and/or anisotropic nanocarriers [19,20].

A few years ago, we reported on the preparation of temperature and pH-responsive nanocapsules by applying a trihexylcarbonate-containing low molecular weight polyelectrolyte adsorbed onto the surface of cationic dimethylidioctadecylammonium bromide (DODAB) vesicles, prepared using 200 nm membrane-extrusion. The oligomer was subsequently chain extended via the reversible addition fragmentation chain transfer (RAFT) mechanism in a starved-emulsion polymerization process (scheme 1) [21]. CryoTEM characterization showed that the applied procedure does not always result in a uniform shell and depends on the used type of functional low molecular weight polyelectrolyte (i.e., RAFT co-oligomer) and reaction conditions [7]. It was found that the use of crosslinkers to fix the polymer shell around the vesicle often leads to the formation of an inhomogeneous shell [21]. Since inhomogeneities in the polymer shell lead to (either desired or undesired) differences in release profiles it is clear that understanding the effect of crosslinker on shell homogeneity is of utmost importance.

The effect of crosslinking on the growing shell of a vesicle-templated nanocapsule was therefore studied in greater detail using methyl methacrylate (MMA) and tert-butyl acrylate (t-BA) as the monomer feed and diacrylates and dimethacrylates as crosslinker in the system, i.e., compositions that, after post modification, ultimately lead to pH-responsive nanocapsules [21].

2. Experimental section

2.1. Materials and methods

Acrylic acid (AA, Fluka, 99%), butyl acrylate (BA, Aldrich, 99%), Triton X-100 (TX-100, Sigma-Aldrich, 99%),...
dimethyldioctadecylammonium bromide (DODAB, Acros, greater than 99%), methyl methacrylate (MMA, Aldrich, 99%), tert-butyl acrylate (t-BA, Aldrich, 98%), ethylene glycol dimethacrylate (EGDMA, Sigma-Aldrich, 98%), ethylene glycol diacylate (EGDA, Sigma-Aldrich, 98%), poly(ethylene glycol) diacrylate ((EG)xDA, Sigma-Aldrich, M₉₀ ≈ 550 g·mol⁻¹ (x = 9)) and poly(ethylene glycol) diacrylate (EG(x)DA, Sigma-Aldrich, M₉₀ ≈ 575 g·mol⁻¹ (x = 10)) were used after inhibitor removal. The RAFT agent dibenzyl trithiocarbonate (DBTTC, Dioxane (Merck) and dimethyl sulfoxide-d₆ (Campro scientific, Sigma-Aldrich, 98%) was used as received. Distilled deionized (DDI) water was used throughout the experiments.

2.2. Preparation of DODAB vesicles

Large polydisperse vesicles with diameters of about 800 nm were made by mixing 1 mmol DODAB in 100 ml DDI water for 2 days at 60 °C, using a magnetic stirrer bar at 250 rpm. The dispersion was then extruded through 200 nm polycarbonate filters for 5 consecutive runs at the same temperature under 7 bar N₂ atmosphere. The resulting DODAB vesicles were analyzed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument (ζ ≈ 7.38 (aromatic H)). Initiator N,N'-azobis(isobutyronitrile) (AIBN, Fluka, 98%) was recrystallized from methanol and the water-soluble azo initiator 4,4'-azobis-4-cyanovaleric acid (V-501, Fluka, 98%) was used as received. Dioxane (Merck) and dimethyl sulfoxide-d₆ (Campro scientific) were used as received. Distilled deionized (DDI) water was used throughout the experiments.

2.3. Synthesis of RAFT Co-oligomer

Random RAFT co-oligomers with an average composition of BA₉₋₁₈ co-AA₉₀ were synthesized using a monomer mixture of 11.9 g (0.093 mol) BA and 10.1 g (0.144 mol) AA. This monomer mixture was dissolved in 25 ml dioxane and purged with argon before being added to a 100 ml three-neck flask containing a magnetic stirrer bar. To this mixture 4.1 g (0.015 mol) of the RAFT agent DBTTC and 0.21 g (1.2 × 10⁻³ mol) of the initiator AIBN were added and the polymerization was performed in a batch reaction for 5 h at 70 °C, as previously described by our group [21]. Molecular weight distributions (MWD), number average molecular weights (Mₙ), and the polydispersity indices (D) of the RAFT co-oligomers were measured by size exclusion chromatography (SEC), using a Waters SEC equipped with a Waters model 510 pump and a model 410 differential refractometer. Tetrahydrofuran was used as the eluent in two mixed bed columns (Mixed-C, Polymer Laboratories, 30 cm, 40 °C) and the system was calibrated using polystyrene standards (range = 580–7.5 × 10⁴ g/mol). Average composition and degree of polymerization were determined via ¹H NMR spectroscopy on a Varian 400 MHz spectrometer (Fig. S1 in SI), using dimethyl sulfoxide-d₆ as solvent. Characteristics: Mₙ,SEC = 1.8 × 10⁴ g/mol, D = 1.2, DPn = 15, AA/BA = 9/6 mol/mol.

2.4. Preparation of RAFT-Vesicle templates

A vesicle stock solution (A) was prepared by adding 0.652 g (1.03 mmol) DODAB to 100 ml DDI water and processed as explained earlier (Dₙ ≈ 200 nm, ζ = + 50 mV). A RAFT-copolymer solution (B) was prepared by adding 1.713 g (0.98 mmol) BA₉₋₁₈ co-AA₉₀ (M₉₀ = 1.8 × 10⁵ g/mol) to 100 ml DDI water kept at pH = 7.3 using potassium hydroxide. Finally, a RAFT-vesicle dispersion (C) was then produced by drop-wise addition of 12.5 ml of vesicle stock solution A into 5 ml of RAFT-oligomer solution B under continuous stirring. The amounts of the RAFT co-oligomer and vesicles are chosen in such a way that the ratio is kept at 2.5 (mol RAFT/mol DODAB) and is sufficiently far removed from the isoelectric point to avoid agglomeration as described in our previous study [7]. The particle size distribution and ζ potential of the RAFT-vesicle dispersion C were determined at 20 °C by dynamic light scattering (DLS), using a Malvern Zetasizer Nano ZS instrument (Dₙ ≈ 200 nm, Poly = 0.2, ζ = − 63 mV). It should be noted here that RAFT-vesicle dispersion C was always used immediately in further steps as some instability over time was found upon storage of the dispersion.

2.5. Synthesis of nanocapsules

17.5 ml of freshly prepared RAFT-vesicle dispersion C was transferred into a 50 ml three-neck flask equipped with a magnetic stirrer bar and a heating bath and diluted by 8 ml DDI water. The reaction mixture was then purged with argon for 30 min at 70 °C under continuous stirring at 350 rpm. After addition of 7 mg (2.5 × 10⁻³ mol) V-501 initiator, nanocapsules were synthesized by starved-feed emulsion copolymerization of MMA and t-BA (33 mol% of MMA) using varying amounts of crosslinker. The level of RAFT control in solution experiments was assessed by monitoring the evolution of the molecular weight distribution with conversion (Fig. 1). It was also confirmed that higher BA contents in the reaction mixture lead to better RAFT control (Fig. S2 in SI).

In a typical experiment, 0.23 g (1.9 × 10⁻³ mol) of monomer mixture of MMA/t-BA/EGDMA (using the following mole fractions of EGDMA: fEGDMA = 0, 0.015, 0.03, 0.05, 0.07 and 0.10, while always maintaining fₙBA = 2 × fMMA and fMMA + fₙBA + fEGDMA = 1) were fed at a rate of 0.01 ml min⁻¹ using a Dosimat auto titrator [21]. After the feeding, the reactor was kept stirring at 70 °C for 2 more hours to ensure that the polymerization is carried out to (essentially) full monomer conversion.

2.6. CryoTEM characterization and 3D tomography

Cryogenic transmission electron microscopy (cryoTEM) measurements were performed on a FEI Tecnai 20, type Sphera TEM instrument equipped with a LaB₆ filament operating at 200 kV. Samples were prepared on 200 mesh Cu TEM grids (Lacey carbon film for 3D imaging). In general, 3 μl of the dispersion (mixed with colloidal gold for 3D imaging) was applied to the hydrophilized TEM grid, blotted and vitrified in an automated vitrification robot (FEI VitrobotTM Mark III) by plunging into liquid ethane. Tomographic tilt series acquisition was done with Inspect3D software (FEI Company). Alignment and reconstruction of the series were performed using IMOD [22] and de-noised before visualization. Segments were completed using the 3D analysis package Avizo Fire [22].
3. Results and discussion

3.1. Synthesis of nanocapsules

First, a vesicle dispersion was prepared by extrusion of a DODAB dispersion. It was found that after five extrusion steps no further narrowing of the vesicle size distribution occurred, as shown in Fig. 2A. The extruded vesicles display some sharp corners (Fig. 2B), consistent with previous reports [7,23]. RAFT-vesicles were then prepared by dropwise addition of the DODAB vesicle dispersion to a BA₆-co-AA₉ RAFT co-oligomer solution. As can be seen in Fig. 2C and D, the resulting RAFT-vesicle dispersion displayed a particle size distribution almost identical to that of the original vesicle dispersion, but the sharp corners of the original vesicles have disappeared in the RAFT-vesicles. This latter observation may be caused by a different molecular arrangement in the RAFT vesicles as compared to the original DODAB vesicles [12,24,25].

Finally, it can be seen from Fig. 2B and D, that the wall thickness increases from ~3–4 nm (in accordance with earlier reports) [26–28] to ~10 nm upon adsorption of the RAFT co-oligomers. The resulting structures, called RAFT-vesicles in the remaining part of this paper, were then used in a starved-feed emulsion polymerization process for...
the synthesis of the polymer shell of the nanocapsules. The locus of polymerization in these RAFT-mediated vesicle encapsulations can be on the outside of the vesicle, in the bilayer (similar to the work of Jung et al., leading to parachute-like structures [24]) and on the inside of the vesicle (leading to inward growth and potentially to solid particles). Most likely we have all three loci of polymerization operating simultaneously, with potentially different resulting morphologies.

Before considering any potential morphologies, we first confirmed that polymerization is indeed controlled by the RAFT process and to this end the molecular weight evolution was monitored during the polymerization of MMA/t-BA ([MMA]/[t-BA] = 1/2) without any crosslinker. Fig. 3A and B show the evolution of the entire MWD and $M_n$, respectively, as a function of the overall monomer conversion. The total amount of monomer mixture added during encapsulation was 0.23 g (corresponding to $[M_0]/[RAFT_0] = 31$), which is similar to what was used in previous studies [7,21]. The results clearly show a shift of the molecular weight distribution to higher $M$, which is indeed indicative of RAFT-control of the polymerization [29–32]. Nanocapsule formation was confirmed via cryoTEM measurements (Fig. 3C), and a typical shell thickness of ~30–40 nm was observed.

It is clear from these results that nanocapsules are formed, in agreement with our previous report [7]. However, in contrast to our previous report, DLS measurements show a decrease, rather than an increase, in z-average diameter (from ~270 nm to ~210 nm with $Poly = 0.2$) during polymerization, which suggests that shrinkage occurs. It is not completely clear why we observe something different to what we reported in our previous study [7,21], but the differences may be caused by the fact that the current system is slightly different in that extrusion of the vesicles now took place through 200 nm filters and a feed ratio of MMA/t-BA = 1/2 was used as compared to 100 nm filters and a feed ratio of MMA/BA = 10/1 in the previous study [7]. Below, we will discuss that the observed discrepancy may actually be a result of the different RAFT-vesicle sizes.

In order to better understand the evolution of the morphology in further polymerization, a few additional reactions were performed using a larger amount of monomer but the same feeding rate; the same feeding rate was used so that the current experiment can be considered as a continuation of the polymerization relating to Fig. 3. The results of these experiments are shown in Fig. 4.

In Fig. 4A we first observe a decrease in $D_s$ in all three systems similar to what was mentioned before relating to the experiments shown in Fig. 3. Upon further addition of monomer in Fig. 4A, however, $D_s$ starts to increase, and this is similar to what was reported in our previous study [7]. CryoTEM imaging of the final sample of the series shown in Fig. 4A, now reveals a large population of solid spheres and hardly any capsules (see Fig. 4B). Although further experiments would be necessary to provide absolute proof, these results clearly suggest that the RAFT-vesicles first grow inwards (resulting in shrinkage and a decreasing inner diameter of the capsule) and after becoming full solid particles start to grow in size as in a conventional emulsion polymerization. Returning now to the observed difference in particle size evolution in our previous study [7], we may speculate about two possible reasons for this difference: (a) the smaller vesicles with $D_s \approx 100$ nm in our previous study could have become solid more quickly and only the size increase of the solid particles was measured, or (b) the higher MMA content and therefore more hydrophilic monomer composition of the previous study indeed resulted in outward growth (and hence an increase in surface area). A further investigation of these effects, although clearly of great interest, lies outside the scope of the current paper.

When not using the high amounts of monomer as used in the experiments corresponding to Fig. 4, we mainly observed hollow capsules. However, as already stated above, we did not always observe homogeneous shell thicknesses depending on the amount of crosslinker used in the monomer feed. In what follows we will investigate this in more detail by using a wider range of compositions and (semi-)quantifying the observed morphologies.

Using the general encapsulation procedure as described above, we varied the fraction of EGDMA between 0 and 10 mol%, relative to the overall monomer content. Several different morphologies were observed in cryoTEM imaging and representative examples are shown in Fig. 5. As can be seen in Fig. 5A, some had a gradually thickening shell by more than 10 nm on one side, while others had a thick shell protruding on one spot (Fig. 5B), and again others (Fig. 5C) appeared to consist of a thin vesicle-like shell and a segregated polymer part (similar to the previously reported “parachute-like” structures [12]). For the sake of simplicity, we call all structures as shown in Fig. 5A–C protrusion structures. This is to differentiate them from the capsules (Fig. 5D–F) and solid particles (Fig. 5G–I). Within the “capsule” or “class” of capsules we observed non-spherical capsules (Fig. 5D), and spherical capsules with thick (Fig. 5E) and thin (Fig. 5F) shells. A few large, possibly solid, latex particles with dark color (Fig. 5G) or gradually changing color (Fig. 5H) were observed which were normally larger than 40 nm and easily distinguished from ice contaminations in cryoTEM images (Fig. 5I).

For our quantitative morphology study described below, we categorized the mentioned particles in three major groups: protrusions, capsules and large solid spheres. Specifically for the protrusion structures, it is important to establish whether the growth of polymer on the RAFT-vesicles is a heterogeneous polymer growth of the capsule shell or growth of a solid particle on one side from the beginning (like the growth of the parachute morphology [12]). Shell thicknesses have been seen from a few nanometers to above 60 nm around up to the seemingly solid particles.

In Fig. 6A a cryoTEM image of a typical nanocapsule synthesized using 7% EGDMA crosslinker is shown. It has a lighter left side and a darker right side which is indicative of different amounts of polymer on the two sides of the nanocapsule. High resolution 3D tomography
(Fig. 6B) of this protrusion structure shows clearly a thicker part of polymer on one side and confirms the combined structure of the protrusion and the capsule made by 7% EGDMA crosslinker. The reconstructed structure shows that the protrusion is an integral part of the shell with thickness of over 30 nm; the other parts have a thickness of ~10 nm. The tomographic view [34] shows that the closed round

Fig. 4. (A) z-Average nanocapsule diameter as a function of formed polymer (●) under the same conditions of Fig. 3, (□, ▲) with 1.8 × amount of monomer used in the experiments shown in Fig. 3 using the same feeding rate (0.01 ml/min) (PDI = 0.1 and decreases with increasing conversion) (B) cryo-TEM image of the final structure of nanocapsules in reaction (■) (PDI = 0.06).

Fig. 5. Representative cryoTEM images of different morphologies observed after the polymerization of RAFT-vesicles: protruded capsules (A, B, C), uniform capsules (D, E, F), and large seemingly solid spherical particles (G, H, I).
structure has an inner diameter of at least 50 nm. From these results it is safe to conclude that the protrusion structure is indeed a true nano capsule with a polymer protrusion that is an integral part of the shell.

It is interesting to note that the clear existence of two distinctly different domains in the protrusion structures may now open up a route to a two-compartment nanocarrier which can hold, for example, a hydrophilic and a hydrophobic compound, being a potential precursor to true nanobottles [35].

3.2. The effect of EGDMA on nanocapsule morphology

In order to understand the effect of crosslinking on the obtained morphologies, we varied the amount of EGDMA in the system and studied the resulting dispersions using cryoTEM. Several different images were taken for each sample (see, for example, Fig. 7A), and the number of occurrences of a particular morphology (i.e., capsule, protrusion, solid particle) was counted. These results are shown in Fig. 7B.

The results in Fig. 7B clearly show that in the polymerizations using no or small amounts of crosslinker capsules are the main structure produced and that the fraction of protrusions increases with increasing crosslinker concentration; at a crosslinker content of 10%, the fraction of protruded capsules is \( \sim 90\% \), with only \( \sim 8\% \) of the observed structures being “normal” spherical capsules. This increase is conceivably caused by a decreasing chain mobility with increasing crosslinker concentration, leading to a “fixation” of the locus of polymerization. What can also be seen from the graph is that a significant proportion (\( \sim 25\% \)) of the observed morphologies in the uncrosslinked system is protruded. This result means that although an increasing crosslinker concentration leads to an increase in the fraction of protrusion structures, the crosslinking is not necessarily the origin of the protrusion. We speculate that a possible origin of the protrusion is the specific adsorption of the RAFT co-oligomer on the vesicle; experiments to test this hypothesis are underway and are the subject of a future publication.

3.3. The effect of crosslinker type on nanocapsule Morphology.

We subsequently investigated the effect of the crosslinker type on the resulting morphologies and used two crosslinkers that are more hydrophilic than EGDMA: EGDA and \((\text{EG})_{10}\text{DA}\). Varying amounts of crosslinker were used and cryoTEM imaging of the samples allowed for counting the occurring morphologies. The results of this process are
It is immediately clear from the results in Fig. 8 that EGDMA leads to a larger proportion of protrusions. In fact, the 1.5 and 3% containing samples show very similar morphology-distributions as the sample without crosslinker. The encapsulation without crosslinker is composed of roughly 23% protrusion structures and 72% capsules (see Fig. 7B).

It is also clear that the use of EGDA leads to the smallest proportion of protruded structures and that the (EG)_{10}DA crosslinker containing systems are somewhat in between with respect to the protrusions, but that these systems also contain a significant fraction of multi-compartment structures (connected capsules and protrusions). A striking difference between EGDMA and EGDA is that an increase in crosslinker content clearly leads to an increase of the fraction of protrusions in the former case, but to a reduction in the latter. The fairly high fraction of solid spheres in the EGDA systems is probably caused by high polymer contents in some of the experiments which leads to a transformation of capsules to solid spheres. It is clear, however, that increasing the EGDA content does not lead to an increase in protrusion structures as is clearly the case for EGDMA.

We also varied the nature of the polymerizing groups (diacrylates - DA - and dimethacrylates - DMA) simultaneously with the spacer length between the two polymerization group (1 vs 9-10 ethylene oxide units) and the results for polymerizations using 10% crosslinker content are shown in Fig. 9.

From Fig. 9 it is clear that for a given spacer length, more uniformly thick capsules are produced when changing from dimethacrylate to acrylate. There does not seem to be an obvious change when changing only the spacer length. (EG)_{10}DMA results in mostly the same morphologies as EGDMA and in going from EGDA to (EG)_{10}DA the proportion of protrusions increases, but these protrusion structures contain fewer sharp features than those in the EGDMA or (EG)_{10}DMA containing samples. Overall these results suggest that EGDMA as the most hydrophobic crosslinker leads to a hydrophobic domain in or on the vesicle bilayer, which then acts as a seed in further polymerization, ultimately leading to protrusion growth.
4. Conclusions

Nanocapsules synthesized by vesicle-templated RAFT polymerization may result in several different structures, with true spherical capsules and protruded capsules being the main products under the used reaction conditions. It is clearly shown that the different monomer compositions of BA (or t-BA) and MMA used in previous studies from our group [7,21] result in different levels of RAFT control. The protrusion structures were observed to have different structures of having thin (like in parasite-like structures reported by Jung et al. [12]) or thick shells together with a significantly protruding part. The cryoTEM study provided a view of growing polymer inside the hollow particle which cannot be easily inferred just from dynamic light scattering studies, as were done in the previous vesicle encapsulation studies [7,21,33]. It was also found that, although crosslinking affects the amount of protrusion morphologies in the system [21], crosslinking is not the prime reason for the formation of protrusion morphologies in the system; EGDMA was found to lead to an increase and EGDA to a decrease in protrusion structures as compared to the uncrosslinked system. Finally, the protrusions were clearly shown to be an integral part of the polymer shell and not separate polymer particles attached to a vesicle structure as reported for the parasite-like morphologies [12]. This may open up a novel route to multi-domain and therefore multifunctional nanocapsules.

5. Notes

The authors declare no competing financial interest.

6. Data availability

Available from the authors on request.

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Appendix A. Supplementary material

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