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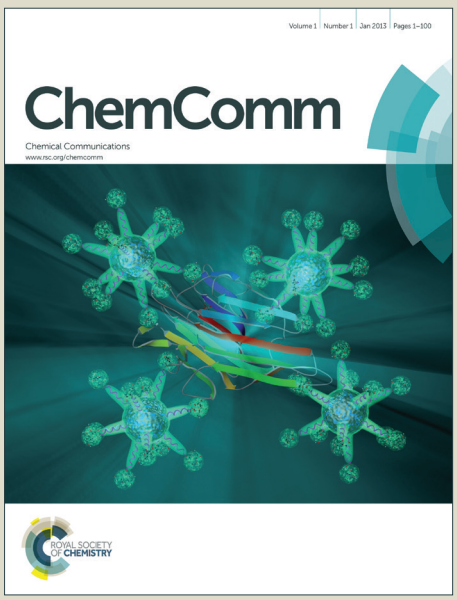

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Ultrastable Core-Shell structured Nanoparticles Directly Made from Zwitterionic Polymers

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Novel strategy was achieved to make ultra-stable core-shell nanoparticles directly from nonfouling zwitterionic polymers through one-step microwave method. The resulting nanoparticle showed superior colloid stability in bio-relevant media and even the freeze-drying conditions

Colloid stability is crucial for nanoparticles (NPs) to remain nano-effects in various functioning media. NPs with hydrophobic core suffered from aggregation problem seriously, especially in bio-related media, which hindered their applications in drug delivery and imaging.[1] Typically nonfouling polymers, capable of resisting nonspecific protein adsorption, were utilized to modify a NP core to prevent it from aggregation.[2] This modification has been done through various methods, e.g., via surface adsorption,[3] chemical conjugation[4] (e.g., the commonly used "graft-to" method to modify the surface with nonfouling materials such as PEG[5]), and particle encapsulation in polymeric micelles,[6] to produce hydrophobic core-nonfouling polymer shell NPs. These methods however either involved complex chemical synthesis procedures[7] or were low in efficiency due to unpredicted NP surface environment.[8] As an alternative method to these complex modification procedures, we explore the feasibility to make the NPs directly from nonfouling polymers with superior colloid stability. To the best of our knowledge, a hydrophobic NP stabilized by nonfouling polymers made purely from the polymer itself has not been reported.

Inspired by the facile one-step microwave method to fabricate carbon nanoparticles,[9] for the first time, we directly made ultrastable NPs from zwitterionic polymers with part of the polymer chain carbonized into the hydrophobic core and the rest of the intact polymer as the hydrophilic stabiliz-ing shell. Zwitterionic polymers were chosen, since they have demonstrated their excellent nonfouling properties in resisting non-specific binding from proteins, cells and microorganisms.[10] NPs previously modified with zwitterionic polymers showed good colloid stability and long-circulation time in blood.[11] The synthesized carbon-core polymer-shell structure was confirmed using NMR and TEM. The directly made NPs were found to be ultra-stable in bio-relevant media and even the harsh freeze-drying conditions (with no cyro-protectant in presence), note that most existing NPs could not survive from such extreme lyophilizing conditions.

The synthetic route to core-shell zwitterionic NPs composed of one single step, microwave heating of zwitterionic polymers (Fig. 1). Typically, Zwitterionic polymers were synthesized through UV initiated polymerization of respective monomers. Then, a beaker containing 100 mg of zwitterionic polymer (Mw 8-10 KDa) in 5 ml DI water was placed at the center of the rotation plate of a 1000 W microwave oven and heated for 5 minutes. As water evaporated, light brown substance was obtained in the bottom of the beaker (for details see ESI). This microwave method is simple, facile and green, however, there are several obstacles to overcome before obtaining the targeted core-shell zwitterionic NPs.

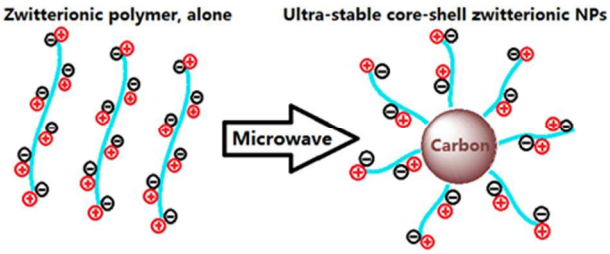


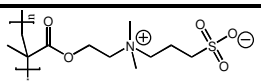
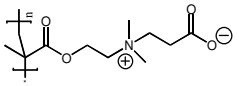
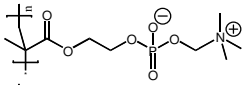
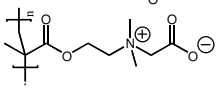
Fig. 1 Illustration of making carbonized core- zwitterionic polymer shell NPs directly from zwitterionic polymers

The primary challenge is that microwave oven heating can't be easily controlled and resulting polymer NPs are contaminated with unreacted polymers and have broad size distribution (PDI>0.6 as measured by dynamic light scattering, DLS). Conventional purification methods such as centrifugation, filtration, or dialysis can't effectively remove the contaminating polymers or obtain homogeneously sized NPs. We found a sucrose gradient centrifugation method can be effective to purify the rough products.[12] NPs migrate under the centrifugation force with their migration pattern retained by sucrose while linear free polymers barely moved and stayed on top of the sucrose column. This was confirmed by control experiment where free polymer solution alone was loaded on top of the sucrose column and DLS results showed no polymer presented in the NPs layer. By exposing the centrifuge tube to UV light, polymer protected NPs can be visualized and retrieved

(fluorescent property will be discussed below). Further removal of sucrose was achieved by using a PD-10 column and highly purified polymer protected NPs (free from unreacted polymers with narrow size distribution, diameter < 30 nm, PDI<0.15) can be obtained (detailed steps see ESI)

The next challenge we met is that zwitterionic polymer chosen should stand high temperature of the microwave heating reaction. While part of the polymer carbonized into the NP core, the other part of the polymer should be thermally intact maintaining the nonfouling zwitterionic structure. Our initial testing with poly(sulfobetaine) (PSB)[13] and poly(carboxybetaine)-2 (PCB-2)[10b] (Table 1), two common zwitterionic polymers we previously worked on, was not successful; the obtained NPs were positively charged as indicated by zeta-potential measurement in water. This could be explained by the thermo-instability of SB and CB-2 units; when heated at high temperature, the quaternary amine group tends to undergo "elimination reaction" splitting into a tertiary amine and an acrylic group.[14] The resulting NPs thus showed positive net charges due to the protonated tertiary amines formed on the protecting polymer shell. By contrast, poly(2-methacry-loyloxyethyl phosphorylcholine) (PMPC)[15] and poly(carboxybetaine)-1 (PCB-1)[16] polymers were found to render resulting NPs with zwitterionic property, as characterized by a slightly negative zeta potential of the resulting NPs. It should be noted that their parent zwitterionic polymers gave similar negative zeta potentials in the control experiment (Table 1). Among four kinds of polymer derived NPs, only PMPC and PCB-1 NPs were chosen for further study due to their zwitterionic shell nature.

Table 1. Structures for zwitterionic polymers involved and zeta-potentials for these free polymers and the resulting NPs

Zwitterionic Polymer Structures		Zeta-potential (mV) Polymer (Parent)	Zeta-potential (mV) NP (Synthesized)
PSB		-3.25±0.56	23.05±1.26 (cationic)
PCB-2		-4.77±0.32	18.73±2.06 (cationic)
PMPC		-2.73±0.13	-4.33±0.80 (Zwitterionic)
PCB-1		-3.22±0.45	-4.75±0.78 (Zwitterionic)

It was hypothesized that the zwitterionic polymer was partially burnt into the carbon core while the remaining part forming a super-hydrophilic shell. The structure of the purified zwitterionic NPs was further examined using transmission electron microscopy (TEM) and nuclear Magnetic resonance H^1 (NMR). Organic molecules are known for their carbonization under microwave heating conditions.[17] Carbon core structure for PCB-1 NPs and PMPC NPs could be detected under the TEM (Fig. 2(a) and Fig. S1). Average core diameters for PMPC NPs and PCB-1 NPs were 12 nm and 11 nm, respectively. NMR results (Fig. 2(c) and Fig. S2) showed a perfect match between the peaks of polymer shell of NPs and the parent free polymers, indicating that the protecting polymer structure remained intact on the NP surface.

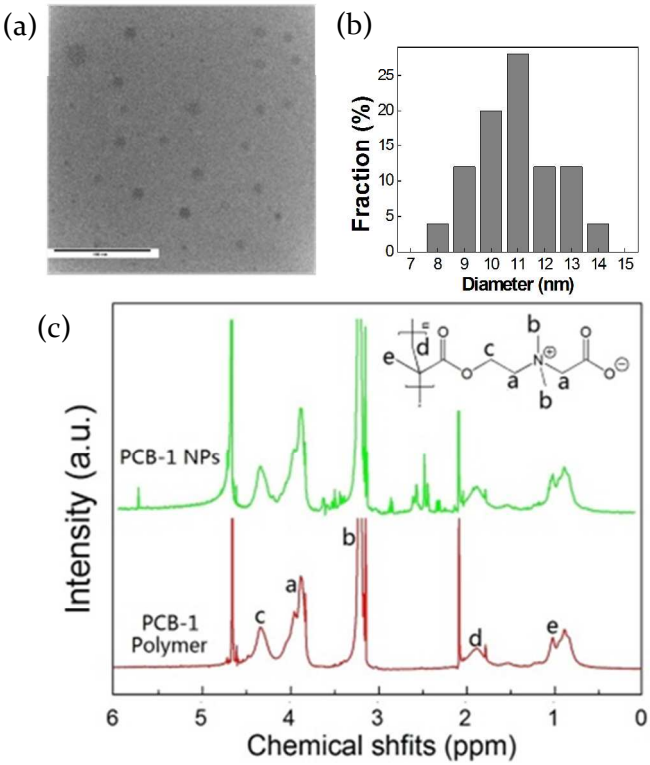


Fig. 2 (a) TEM image of PCB-1 NPs (scale bar: 100 nm) (b) statistic size distribution of PCB-1 NPs (c) NMR spectra of parent CB-1 polymer and synthesized PCB-1 NPs

For potential biological applications, NPs have to be stable in complex environment where bio-elements (e.g., proteins) were present. It is expected that the synthesized zwitterionic NPs are well protectedfrom aggregation by the zwitterionic shell in the bio-related media. As non-zwitterionic polymer NP controls, we synthesized CA NPs, from citric acid and ethylenediamine through the same microwave method (a well-established fluorescent NP system with ultrahigh quantum yield[18]), and purified it via the same sucrose centrifugation procedure. The resulting CA NPs had slightly negative zeta potential (-1.78 mV), similar to zwitterionic polymer stabilized NPs, but lacked zwitterionic polymer shell surface. For colloid stability testing, purified PMPC NPs, PCB-1 NPs and CA CNPs (size<30 nm PDI<0.18) were placed in PBS (phosphate buffered saline, salt concentration 153 mM), PBS solution of 5 wt% bovine serum albumin (BSA) and PBS solution of 5 wt% fibrinogen from bovine plasma. The sizes for NPs were measured as a function of time (Fig. S3, Fig. 3a and Fig. S4). No obvious size increase appeared in any of the zwitterionic polymer NPs groups while CA NPs aggregated drastically. It is a general trend that particles tend to be less stable in high salt concentration solution.[19] Zwitterionic PCB-1 NPs, however, retained its original size in a solution with salt concentration as high as 500 mM in a 40 min study (i.e.). CA NPs by contrast doubled its size overnight even in DI water with zero ionic strength. Long-term stability study (Fig. S5) showed that both PMPC NPs and PCB-1 NPs, after one week incubation in PBS, have no significant size increase while CA NPs continuously aggregated.

Lyophilization, a necessary procedure for NP storage and precise weighing, introduces harsh interaction among individual NPs, causing aggregation that could not be segregated even under sonication. To the best of our knowledge, NPs even protected by nonfouling polymers are very rare to survive from lyophilization induced aggregation.[4] We used lyophilization conditions to further

push the limit of stability performance of the zwitterionic core-shell particles. PMPC NPs, PCB-1 NPs, and CA NPs were lyophilized without adding any cryoprotectant and redissolved in DI water and their sizes were measured using DLS (Fig. 3b). CA NPs aggregated substantially after freeze-drying while zwitterionic NPs survived such harsh condition by maintaining the particle size. We have previously observed that PCB-1 protected PLGA NPs can sustain the original size through lyophilization, while other NP protecting technology (such as PEG-PLGA) can't but require the addition of cyroprotectant (e,g., 10% sucrose).[5b, 20] It was believed that zwitterionic polymers strongly bind water molecules to prevent hydrophobic interactions among carbon cores in this case, and keep them apart even in a highly dehydrated environment.[21] With excellent stability both in bio-relevant media and harsh freeze drying conditions, we demonstrated the effectiveness of our strategy to render NPs ultra-stabile properties.

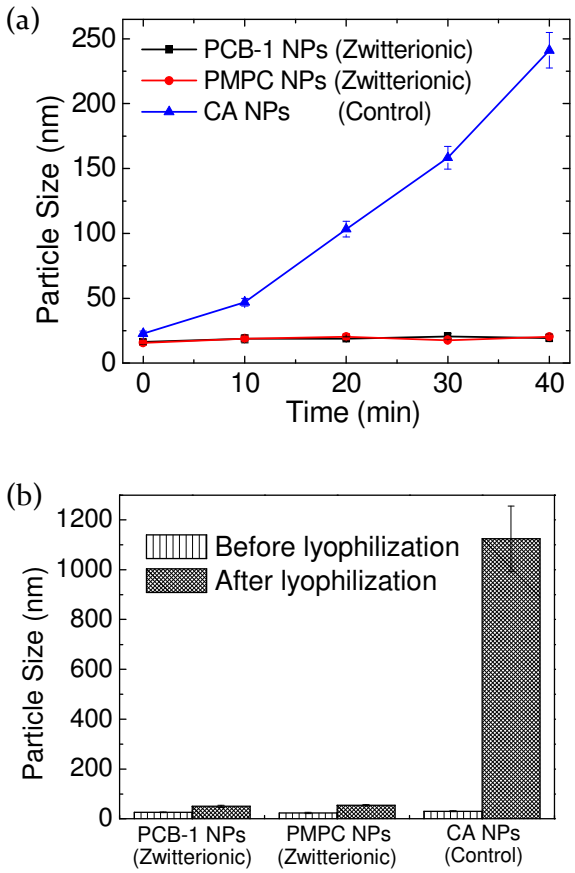


Fig. 3 Colloid stability (a) in BSA/PBS and (b) in freeze-drying condition of PCB-1 NPs, PMPC NPs and CA NPs

To verify that our way towards stable NPs do not cause cytotoxicity, MTT assay was conducted (Fig. S6). NIH/3T3 cells remained more than 85% and 80% viability in PMPC NPs and PCB-1 NPs, respectively, at concentration as high as 10 mg/ml, while CA NPs were toxic to cells (30% viability) at concentration as of 5 mg/ml. It is worth-mentioned that the synthesized NPs had fluorescent property (Fig. S7). Under UV excitation, Both the PCB-1 NPs and PMPC NPs exhibited blue light (detail see ESI). It was hypothesized that the fluorescent property was related to the core-shell structure, which was previously reported as surface-passivation mechanism.[22] The fluorescent property can lead to potential imaging applications[8, 23] of the resulting zwitterionic NPs.

In summary, we have demonstrated a novel strategy to make core-shell NPs with superior stability directly and solely from nonfouling zwitterionic polymers through single step of microwave heating. Only those zwitterionic polymers, that are stable through high temperature microwave heating can produce zwitterionic polymer shell-carbon core NPs, which can be further purified through sucrose gradient centrifugation. It was found that zwitterionic NPs maintained excellent colloid stability in various bio-relevant media, and even under the most harsh lyophilization condition. With undetectable cytotoxicity, the new strategy will lead to ultra-stable NPs for drug delivery and imaging application. We expect this green chemistry in making ultra-stable NPs can inspire future functional NPs to be developed.

Notes and references

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Electronic Supplementary Information (ESI) available: Material, Characterization results, Synthesis detail, Stability data, MTT results and Fluorescent property. See DOI: 10.1039/c000000x/

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