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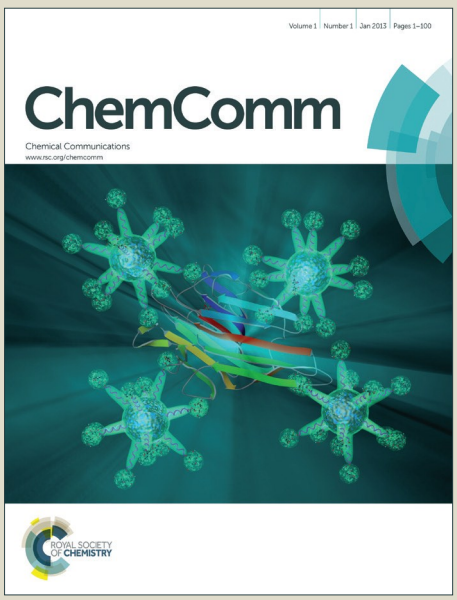

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Water-triggered Self-assembly Polycondensation for the One-Pot Synthesis of Cyclomatrix Polyphosphazene Nanoparticles from Amino Acid Ester†

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Water-triggered self-assembly polycondensation was proposed for preparation of cyclomatrix polyphosphazene nanoparticle from amino acid esters, and found a critical solubility parameter to determine whether the nanoparticles were formed. Based on this rule, we also investigated the control of the size of its nanoparticles.

Polyphosphazenes are a class of inorganic–organic hybrid polymers that consist of an inorganic main chain (-P=N-) with two of the same or different organic side chains attached to each phosphorus atom.[1] These polymers are very designable and exhibit versatile characteristics depending on their substituents.[2] Of these polymers, poly(amino acid ester)phosphazenes derived from amino acid ester substituting chlorine atoms on polydichlorophosphazene (-$P(Cl)_2$=N-) have shown significant potential in the field of tissue engineering,[3-4] and as carriers for drug and gene delivery,[5,6] due to their excellent biocompatibility, tunable biodegradation and nontoxic degradation products.[7,8] However, preparation of the linear polyphosphazenes is difficult, the conventional ring opening polymerization route from hexachlorocyclotriphosphazene (HCCP) requires harsh synthesis conditions (250°C in vacuum), and the obtained polymer possesses a wide molecular weight distribution (PDIs≈10).[9-11] Despite intensive efforts in pioneering studies,[12-14] mass preparation of the linear polyphosphazenes has not been achieved. Recently, Tang et al developed a new class of polyphosphazene, i.e. cyclomatrix polyphosphazene, which can be obtained by precipitation polycondensation based on nucleophilic substitution from HCCP and aromatic organic monomers with dual-nucleophilic groups under ambient conditions.[15] This new polymerization reaction offers an approach for the large-scale production of polyphosphazene in the form of particles.[16,17] Since the introduction of this method, several cyclomatrix polyphosphazenes (CMPPZs) have been successfully fabricated using aromatic monomers, such as 4,4-sulfonyldiphenol,[18] 4,4-(hexa-fluoroisopylidene) diphenol,[19] benzidine,[20] and phloroglucinol.[21] However, the polyphosphazene nanoparticles are distinctly unsuitable for bioapplication due to the potential safety risks of the monomers used. Amino acid esters with diamine are ideal alternatives for the aromatic monomers. However, the successful synthesis of CMPPZs from amino acid esters has not been reported, and the reason for this is believed to be due to the lack of rigid structure in amino acid esters, or the polymerization is stayed at the oligomer stage due to its hard-to-nucleation in common solvents. To determine whether this is true we investigated and tracked the polymerization of HCCP with an amino acid ester under different conditions and proposed a new method of water-triggered self-assembly and polymerization, which results in CMPPZ nanoparticles with amino acid ester segments. This new method not only extends the range of CMPPZs from aromatic monomers to flexible aliphatic monomers, but also helps to understand the mechanism of precipitation polycondensation of CMPPZs. The controlled growth of CMPPZs particles was also systematically studied.

L-cystine is an important amino acid and nutritional supplement, which is widely used in medicine and the food industry. L-cystine is mainly extracted from hair, therefore, no adverse effects on the human body have been found.[22] In this work, L-cystine methyl ester (CysM) was selected as the nucleophilic monomer. The esterification of L-cystine was designed to promote its dissolvability in the reaction solvent (acetonitrile). A schematic diagram of the preparation of cyclomatrix poly(amino acid ester)phosphazene was shown in Fig. 1a. According to a previous approach,[23,24] triethylamine (TEA) as the catalyst was added to an acetonitrile solution of HCCP and di-nucleophilic monomer. After stirring for several hours, the solution became a light-blue suspension, indicating the formation of CMPPZ. However, no change in the appearance of HCCP and CysM was found, even when the reaction solution was stored for 2 weeks. Interestingly, a light-blue suspension occurred when a certain amount of water was added to the solution under stirring, A white solid product was collected by centrifugation and successively washed three times using deionized water and alcohol. The resultant precipitate showed a nano-spheric structure under electron microscopy. The FT-IR spectra (Fig. 1b)

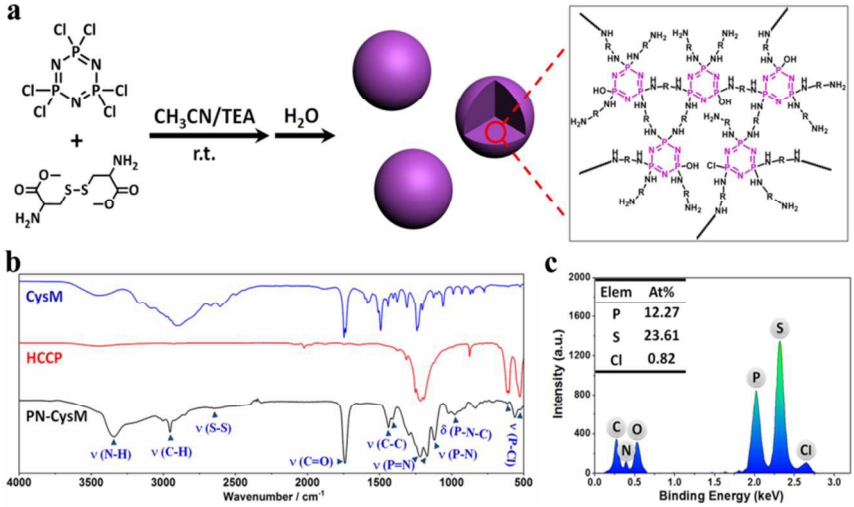

Figure 1. The synthesis of cyclomatrix polyphosphazene and conformation of its structure. (a) The brief synthesis of PN-CysM nanoparticles, and its calculated molecular structure. (b) The FT-IR spectra of CysM·2HCl (blue), HCCP (red), PN-CysM (black), the former two are reactants, the last one is the product. (c) EDS data of the PN-CysM nanoparticles, and its content of phosphorus, sulphur, and chlorine.

indicated that the precipitate consisted of cross-linked cyclotriphosphazene group (1218 cm^{-1} and 1170 cm^{-1}), CysM group (1740 cm^{-1}) and crosslinking bond of P-N-C (976 cm^{-1}). The precipitate had no glass-transition temperature (Fig. S1a), and had an obvious increase of the onset of the thermal-degradation (T_d) temperature (Fig. S1b), also implying that the polymer particles consisted of crosslinked structure. Energy dispersive specrometry (EDS) was employed as a simple semi-quantitative analysis mothed to confirm the change in P, S, Cl elements. According to EDS data (Fig. 1c) and the calculated result from the FT-IR standard curve (Fig. S2), approximate 96.7% of chlorine on the phosphorus was substituted, of which 83.3% was by CysM, 13.4% by hydroxyl groups, and 3.3% remained unsubstituted. The molar ratio of P to S (approximately 1:2) showed that the majority of phosphorus atoms were connected with two CysM segments. These results demonstrate the successful preparation of poly(amino acid ester)phosphazene nanoparticles, this polymer was termed PN-CysM.

In order to explore the mechanism of the polymerization process, the reaction solution (for 48 h) of CysM and HCCP was analyzed before water was added. Gel permeation chromatography (GPC), with DMF as the eluent and HCCP as the control, showed two new broad peaks (Mn = 2655 and Mn = 1341, red curve) (Fig. 2a), indicating that the size of the HCCP molecule was enlarged due to the replacement of its chlorines by CysM, and the product was a mixture of oligomers composed of substituted monomers and dimers (with one and two cyclotriphosphazene groups, respectively). The EDS analysis (Fig. 2b) showed that there was still 33.3% of unreacted chlorine on HCCP in the oligomers. ^{31}P NMR results also confirmed that the product was a mixture with unsubstituted chlorine on P atoms (Fig. S3). The valence states of phosphorous in oligomers are shown in Fig. 2c (without considering stereoisomer difference).

The long-term stability of the oligomers can be attributed to two factors. Firstly, the substituted linear side chains increased the conformational numbers of the oligomers, which macroscopically increased its stability in the reaction system. Secondly, the oligomer end-capped by CysM segments had good solubility in acetonitrile. When the oligomer solution was concentrated by solvent evaporation, a gum-like oligomer aggregation was formed (Fig. S4a), which showed excellent solvent resistance, even under high temperature (70 °C) for days (Fig. S4b). This indicated that solvent evaporation

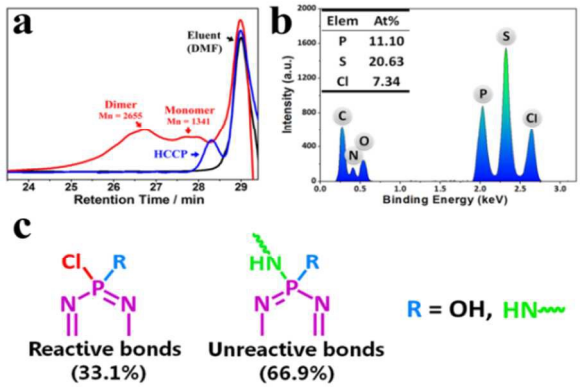

Figure 2. Molecular structure of the oligomer. (a) GPC analysis shows the oligomers consist of trimer (Mw = 3226), dimer (Mw = 2153) and monomer (Mw = 988). (b) EDS data of the oligomer, and its content of phosphorus, sulphur, and chlorine. (c) The possible molecular formula of trimer oligomer.

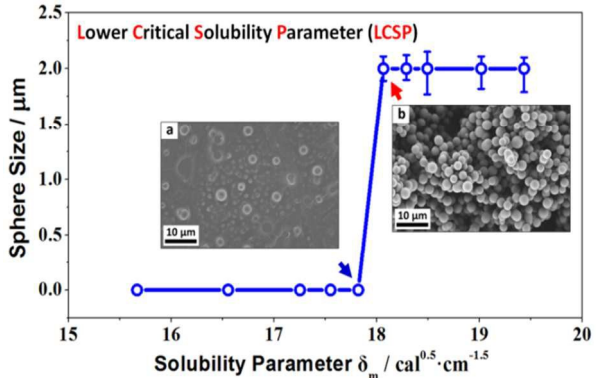

Figure 3. The Hildebrand solubility parameter of the mixed solvent (δ_m) and its relationship with the size of PN-CysM spheres. Sphere growth did not occur at a δ_m value lower than 17.8 $cal^{0.5}\cdot cm^{-1.5}$ (point a, water/acetonitrile is 11/10). However, spheres were formed when the δ_m value was bigger than 18.2 $cal^{0.5}\cdot cm^{-1.5}$ (point b, water/acetonitrile is 12/10). The greater the δ_m value, the quicker spheres grew, however, sphere size was little affected.

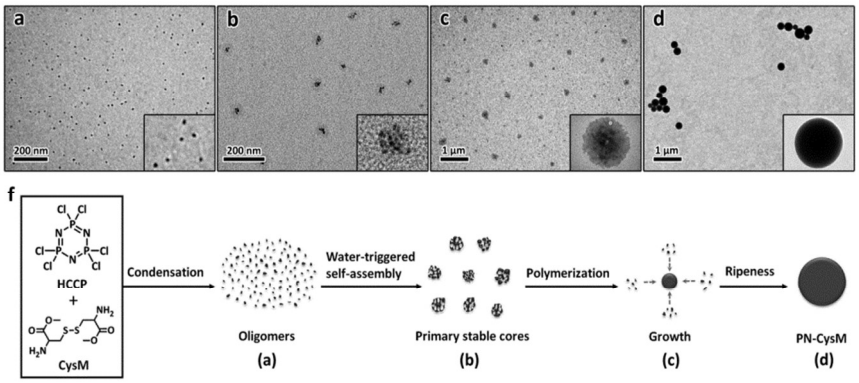

Figure 4. Tracking PN-CysM sphere growth by TEM and the formation mechanism. (a) Tiny particles (about 10~20 nm) obtained from oligomer aggregation after evaporating the solvent. (b) After adding water to the oligomer solution to reach the LCSP, the oligomer particles begin to aggregate into clusters, which are considered stable primary cores. (c) The primary stable cores start to grow by absorbing oligomers, and their density increased. (d) Inside the particles, further crosslinking takes place and finally cross-linked polymeric nanoparticles are formed. (f) The scheme of PN-CysM sphere growth process, in which each corresponding stage is marked, respectively.

resulted in the oligomers being close to each other and further polymerized via nucleophilic substitution, thereby a cross-linked polymer was formed.

Inspired by the above results, a certain amount of deionized water (poor solvent) was added dropwise to the oligomer solution to induce self-assembly of the oligomers and cause further polycondensation. As expected, the oligomer solution became a light-blue suspension, and a white precipitate was formed after centrifugation. Solid-state ^{31}P NMR of the precipitate (Fig. S5) showed that although some hydrolysis took place (≡POH(NHR) at -3.91 ppm),[25] the main peak at 5.47 ppm corresponded to the ≡P(NHR)$_2$ structure,[26] and the small peak at 17.35 ppm corresponded to the ≡PCl(NHR) structure,[27] indicating that the oligomer was further cross-linked with CysM and cyclomatrix polyphosphazene was formed (see Fig. 1). Next, the effect of the amount of water added on the formation of particles was investigated. Here, the solubility parameter (δ) was employed to quantify the compatibility of the mixed solvent.[28] The solubility parameter of the mixed solution (δ_m) ($\delta_m = \sum \phi_i \delta_i$, i=1,2) and the corresponding volume ratio (ϕ) are shown in Table S1. The mean diameter of PN-CysM nanoparticles as a function of the solubility parameter is plotted in Fig. 3. It can be seen that the value of 18.2 cal$^{0.5}$·cm$^{-1.5}$ is the lower critical solubility parameter (LCSP), i.e., no polymeric nanoparticles were formed when the solubility parameter was below the LCSP, and nanoparticles were formed when the solubility parameter reached the LCSP. In addition, it can be seen that the diameter of the particles did not vary with the change in solubility parameter when the solubility parameter was higher than the LCSP. The inset pictures are SEM images of the nanoparticles, in which the solubility parameters of the solutions were very close, but slightly lower or higher than the LCSP, respectively. We found that the LCSP also existed in other cyclomatrix polyphosphazenes derived from amino acid ester monomers. In addition, water triggers the self-assembly of phosphazene oligomer also could occur in other solvent systems such as acetone, alcohol, tetrahydrofuran, and so on.

In order to understand the evolution of the polymeric nanoparticles, the formation and growth of sphere particles were tracked by transmission electron microscopy (TEM) and dynamic light scattering (DLS, Fig. S6). The oligomer solution was dropped onto a copper grid for TEM observations, and tiny particles with a diameter of 10~20 nm were found, which was the result of the aggregation of oligomers (Fig. 4a). After adding water to the oligomer solution, the solution was sampled at regular intervals and analyzed by TEM. As shown in Fig. 4f, when water was added, the solubility of the oligomer decreased in the mixed solvent system, resulting in the aggregation of oligomers to form clusters (Fig. 4b, 30 min), which can be considered the primary stable cores in the solution system. When collision and incorporation between oligomers and clusters or between clusters and clusters took place, the volumes of the primary particles continued to grow, and their density successively increased (Fig. 4c, 1 hour).[29,30] Inside the particles, crosslinking took place and cross-linked polymeric nanoparticles were finally formed (Fig. 4d, 2 hours). During this process, water changed the solubility parameter of the solvent system, triggering the self-assembly of oligomers and crosslinking inside the assemblies. Therefore, we named this process water-triggered self-assembly and polycondensation and the corresponding production yield reached 78.6%. The mechanism of cyclomatrix poly(amino acid ester)phosphazene nanoparticles production is shown in Fig. 4f. The previous method of polyphosphazene polymerization from rigid monomers can be considered a special case in terms of this mechanism. Due to poor solubility of the

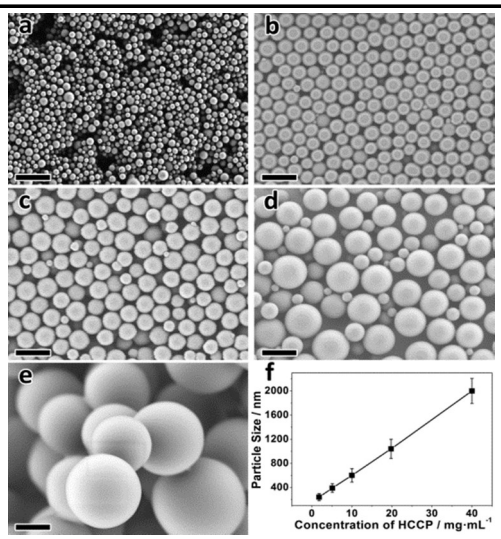

Figure 5. PN-CysM spheres with a mean diameter of (a) 250 nm, (b) 400 nm, (c) 600 nm, (d) 1 μm, and (e) 2 μm, and (f) The relationship between monomer concentration and sphere diameter.

oligomers in acetonitrile, the oligomers directly self-assemble, cross-link and form nanoparticles without water.

The amount of water added only determined whether polymeric nanoparticles were formed, but did not determine the diameter of the nanoparticles (Fig. 3). However, we found that the mean diameter of PN-CysM spheres can be precisely tuned by adjusting the concentration of monomer HCCP with the molecular ratio of HCCP and CysM fixed at 1:3. As shown in Fig. 5, the diameters of the prepared spheres were 250 nm, 400 nm, 600 nm, 1 μm, and 2 μm, when the concentrations of monomer HCCP were 2 $g \cdot L^{-1}$, 5 $g \cdot L^{-1}$, 10 $g \cdot L^{-1}$, 20 $g \cdot L^{-1}$, and 40 $g \cdot L^{-1}$, respectively. With increased monomer concentration, the increase in sphere diameter obeyed a well-defined linear growth pattern (Fig. 5f). Controllable particle size is another important advantage of cyclomatrix polyphosphazenes in biological applications.

Conclusions

We developed the new approach of water-triggered self-assembly and polycondensation for the synthesis of cyclomatrix polyphosphazenes, which not only expands the range of the precipitation polycondensation from aromatic monomers to flexible alicyclic monomers, but also reveals the mechanism of the formation of cyclomatrix polyphosphazene nanoparticles. The LCSP was proposed, which decided the solvent conditions in which cyclomatrix polyphosphazene nanoparticles were formed. PN-CysM particles were successfully synthesized when the solubility parameter of the solution exceeded 18.2 $cal^{0.5} \cdot cm^{-1.5}$, and the size of the PN-CysM spheres were precisely controlled from 250 nm to 2 μm by adjusting the monomer concentration. These spheres will have significant potential in biomedical applications, for instance drug delivery, gene transfection, and as embolic agents.

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Notes and references

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