Double hydrophilic polyphosphoester containing copolymers as efficient templating agents for calcium carbonate microparticles†

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The use of calcium carbonate (CaCO₃) microparticles is becoming more and more attractive in many fields especially in biomedical applications in which the fine tuning of the size, morphology and crystalline form of the CaCO₃ particles is crucial. Although some structuring compounds, like hyaluronic acid, give satisfying results, the control of the particle structure still has to be improved. To this end, we evaluated the CaCO₃ structuring capacity of novel well-defined double hydrophilic block copolymers composed of poly(ethylene oxide) and a polyphosphoester segment with an affinity for calcium like poly(phosphotriester)s bearing pendent carboxylic acids or poly(phosphodiester)s with a negatively charged oxygen atom on each repeating monomer unit. These copolymers were synthesized by a combination of organocatalyzed ring opening polymerization, thiol–yne click chemistry and protection/deprotection methods. The formulation of CaCO₃ particles was then performed in the presence of these block copolymers (i) by the classical chemical pathway involving CaCl₂ and Na₂CO₃ and (ii) by a process based on supercritical carbon dioxide (scCO₂) technology in which CO₃²⁻/C₀ ions are generated in aqueous media and react with Ca²⁺ ions. Porous CaCO₃ microspheres composed of vaterite nanocrystals were obtained. Moreover, a clear dependence of the particle size on the structure of the templating agent was emphasized. In this work, we show that the use of the supercritical process and the substitution of hyaluronic acid for a carboxylic acid containing copolymer decreases the size of the CaCO₃ particles by a factor of 6 (≈1.5 μm) while preventing their aggregation.

Introduction

Nowadays, sustained drug delivery systems (DDS) involving purposely designed particles are gaining increasing importance in human therapy against, for example, cancer,12 bowel diseases,3,4 infectious diseases (tuberculosis)5 and skin diseases,6 and for regenerative medicine used in bone cartilage,7 the central nervous system,8 Parkinson’s disease,9–11 and Huntington’s disease.12 The latter often requires biodegradable and biocompatible materials that allow the safe retention as well as controlled delivery of the drug, a better bioavailability and a reduction of adverse effects. In this respect, calcium carbonate particles are safe, biocompatible and biodegradable microcontainers that can fulfill such functions. These inorganic carriers have excellent properties such as low density, high specific surface area and porosity allowing efficient drug encapsulation and release.13–16 Moreover, their ease of preparation and low price make CaCO₃ particles very attractive for protein,17–22 drug,16,23–27 and gene28,29 delivery.

CaCO₃ particles exist in three anhydrous polymorphs: calcite, aragonite and vaterite.30–33 By far, the last crystalline form is the most interesting for drug delivery applications because it exhibits a porous structure favourable to the encapsulation of therapeutic compounds.23,34 Vaterite can easily be obtained in water by mixing aqueous calcium salt and carbonate solutions18,26 (Fig. 1, upper part). The formulation of CaCO₃ particles often requires a templating agent able to control the particles’ size, shape and porosity. Double hydrophilic block copolymers (DHBCs) are a very efficient class of templating agents for controlling the crystallization of CaCO₃ particles.36–44 They are composed of one hydrophilic segment which binds the calcium ions, provides sites of nucleation and controls the CaCO₃ crystal growth, associated to a second hydrophobic block which ensures the steric stabilization of the growing crystals under high-ionic-strength conditions. While PEO is almost invariably chosen as the stabilizing block, several segments with alkaline earth ion binding capacities...
have been tested including poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), polyanionic phosphate functionalized blocks, and polyglycidol segments converted into ionic blocks containing carboxylic, sulfonic, or phosphoric acid groups. As a result, CaCO₃ particles have been produced with a wide variety of sizes and morphologies including spherical, hollow shapes and many others. Interestingly, the templating capacity of phosphoric acid containing copolymers was found to be superior to their carboxylic and sulfonic counterparts.

Recently, some of us have demonstrated the benefit of using supercritical carbon dioxide (scCO₂) for the synthesis of CaCO₃ particles in the vaterite form (Fig. 1, lower part). ScCO₂ is well adapted to the formulation of materials dedicated to biomedical applications and in particular to the synthesis of CaCO₃ carriers, because this supercritical fluid is a non-toxic, non-flammable and environmentally benign solvent. In addition, its quite low critical conditions (T = 31.1°C and P = 73.8 bar) enable the handling of sensitive compounds such as drugs and therapeutic proteins. Hyaluronic acid (HA), i.e. a biodegradable polysaccharide bearing carboxylic acid pendant groups, was used as a templating agent for the synthesis of CaCO₃ particles in scCO₂ and was found to be essential for the production of well-defined micrometer sized porous CaCO₃. Nevertheless, the search for new efficient templating agents able to adjust and further decrease the size of the CaCO₃ particles is still relevant today.

In this study, we designed and explored the potential of a new class of biodegradable copolymers to template CaCO₃ particles. In particular, we focused on poly(phosphoester)s (PPEs) which share the advantages of HA in being biodegradable and biocompatible materials. In contrast to HA, these synthetic polymers have structural similarities to nucleic and teichoic acids and can be obtained with various and well-defined copolymer architectures and compositions. As a consequence, PPEs are nowadays already involved in many fields such as drug delivery, gene delivery, dental applications and tissue engineering. Above all, PPE derivatives might be excellent candidates for templating CaCO₃ particles because their phosphate degradation products could associate with calcium ions from the inorganic carrier and favour some reconstruction processes like bone regeneration. In particular,

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**Fig. 1** Illustration of the “chemical” and the “scCO₂” pathways for the synthesis of CaCO₃ vaterite particles.

**Scheme 1** General strategy for the synthesis of double hydrophilic copolymers with PEO as the first block and poly(phosphotriester) with carboxylic acid pendant groups (route A) or anionic poly(phosphodiester)s (route B) as the second block.
we targeted double hydrophilic block copolymers composed of poly(ethylene oxide) (PEO) and a PPE segment likely to have an affinity for calcium ions like poly(phosphatiestrer)s bearing pendant carboxylic acids (Scheme 1, structure 3) or poly(phosphodiester)s having a negatively charged oxygen atom on each repeating monomer unit (Scheme 1, structure 6). Organocatalyzed ring opening polymerization (ROP) of cyclic phospholane monomers,\textsuperscript{74,68–71} thiol-yne click chemistry\textsuperscript{70–73} and protection/deprotection methods were combined to prepare the desired well-defined block copolymers. Next, we evaluated the potential of these PPE containing copolymers for templating CaCO\textsubscript{3} particles in water and also in a water/scCO\textsubscript{2} mixture. The morphology of the CaCO\textsubscript{3} particles was studied using scanning electron microscopy and X-ray analyses and compared to that of particles produced with HA, the only previously reported polymer used for controlling the growth of CaCO\textsubscript{3} in scCO\textsubscript{2}.

**Experimental part**

**Materials**

2-Chloro-2-oxo-1,3,2-dioxaphospholane (COP) (\(\geq 95\%\), Aldrich), dichloromethane (CH\textsubscript{2}Cl\textsubscript{2}) (Chem-lab), toluene (Chem-lab), tetrahydrofuran (THF) (Chem-lab), methanol (Sigma-Aldrich), n-pentane (extra pure, Acros), acetic acid (Fisher Scientific), diethyl ether (Et\textsubscript{2}O) (Chem-lab), dimethylformamide (DMF) (Chem-lab), sodium thiophenolate (90–95\%, Aldrich), 2,2-dimethoxy-2-phenylacetophenone (DMPA) (99\%, Sigma-Aldrich), 3-mercaptopropionic acid (\(\geq 99\%\), Aldrich), poly(ethylene glycol) methyl ether (PEO-\textsubscript{OH})(Aldrich), glycine (\(\geq 99\%\), Sigma), dehydrated calcium chloride (CaCl\textsubscript{2}, H\textsubscript{2}O) (\(\geq 99\%\), Sigma-Aldrich), hyaluronic acid (\textit{Streptococcus equi}.) (Sigma), sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}) (\(\geq 99.5\%\), Sigma-Aldrich), sodium chloride (NaCl) (\(\geq 97\%\), Fluka), sodium hydroxide (NaOH) (Fisher Chem), calcium hydride (CaH\textsubscript{2}) (90–95\%, Aldrich), 3-butyn-1-ol (97\%, Aldrich), 1-propen-3-ol (\(\geq 99\%\), Aldrich), and triethylamine (TEA) (99\%, Sigma-Aldrich) were used as received. 1,8-Diazobicyclo[5.4.0]undec-7-ene (DBU) (99\%), Aldrich) was dried over calcium hydride at room temperature, followed by distillation under reduced pressure just before use. Thiourea (TU) was synthesized according to the method described\textsuperscript{2} and dried overnight under vacuum just before use. Ultrapure water (18 M\textsubscript{2}O cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. (St. Charles, MO). The photo-irradiation was carried out using a UV light source from Omnihil Cure Series 2000 (200 W, 365 nm).

**Characterization**

\textsuperscript{1}H and \textsuperscript{31}P nuclear magnetic resonance (NMR) analyses were performed using a Bruker Advance 250 and 400 spectrometer (MHz) in deuterated chloroform (CDCl\textsubscript{3}) and deuterium oxide (D\textsubscript{2}O) at 25 °C in the FT mode. The MALDI-TOF spectrum was recorded using an UltraflXtreme spectrometer (Bruker Daltonics, Germany) using 2,5-dihydropyrimidoic acid as a matrix and no additional cationizing agent. Size exclusion chromatography (SEC) was carried out in DMF (flow rate 1 mL min\textsuperscript{-1}) at 40 °C using a water 600 autosampler liquid chromatograph equipped with a differential refractometer index detector. Waters gel 5 μm (105, 104, 500, and 100 Å) columns were calibrated with polystyrene standards. Infrared spectra were recorded using a Perkin-Elmer FT-IR instrument (KBr). The size and morphology of the CaCO\textsubscript{3} microparticles were studied by scanning electron microscopy (SEM). After pyrolysis, samples were sputtered with gold using a high vacuum metal evaporation coater MED 020 (Bal-Tec, Balzers, Lichtenstein) and observed using a scanning electron microscope (SEM) (Jeol 6301F) at an operating voltage of 3 kV. The size and size distribution of CaCO\textsubscript{3} microparticles were determined by light scattering using a particle size analyzer (PSA) in light medium (Malvern Mastersizer withhydro 2000S small volume sample dispersion unit, France).

Microspheres were dispersed in a phosphate buffer solution (PBS, pH: 7.4) and vortexed prior to every measurement. The laser diffusion intensity was recorded as a function of the angle of diffusion, and then application of the Fraunhofer diffraction and Mie scattering theories allowed the size of the particles and the number distribution to be obtained. All measurements were repeated three times with a stirring rate of 3500 rpm with no ultrasound. A Zetasizer 2000 (Malvern Instruments) operating at 150 mV and at room temperature was used to assess the zeta potential of the microparticles. The zeta cell was washed with ultrapure water between every measurement. The crystal structures of the CaCO\textsubscript{3} microspheres were characterized by X-ray diffraction. XRD analysis was carried out using an X-pert diffractometer (CuK\textsubscript{α}1/2 doublet, \(\lambda = 1.54056\) Å, from 2\(\theta\) = 10 to 70° in continuous mode with a step size of 0.07°).

**Synthesis of butynyl phospholane (BYP) (monomer 1, Scheme 1).** A mixture of 3-butyn-1-ol (12.29 g, 176 mmol) and triethylamine (19.4 g, 192 mmol) in dry THF (150 mL) was cooled at 0 °C. Then, a solution of 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) (25 g, 176 mmol) in dry THF (50 mL) was added dropwise under stirring to the reaction mixture ([COP]/[TEA]/[3-butyn-1-ol] = 1/1.1/1). After complete addition, stirring was continued at 0 °C for 12 h. The resulting triethylamine hydrochloride salt was removed by filtration and the filtrate was concentrated by evaporation of the solvent. The residue was then purified by vacuum distillation to obtain a faint yellow and viscous liquid (CuK\textsubscript{α}1/2 doublet, \(\lambda = 1.54056\) Å, from 2\(\theta\) = 10 to 70° in continuous mode with a step size of 0.07°).

**Synthesis of PEO-b-PBPY (copolymer 2, Scheme 1) by ROP.**

A mixture of 3-butyn-1-ol (12.29 g, 176 mmol) and triethylamine (19.4 g, 192 mmol) in dry THF (150 mL) was cooled at 0 °C. Then, a solution of 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) (25 g, 176 mmol) in dry THF (50 mL) was added dropwise under stirring to the reaction mixture ([COP]/[TEA]/[3-butyn-1-ol] = 1/1.1/1). After complete addition, stirring was continued at 0 °C for 12 h. The resulting triethylamine hydrochloride salt was removed by filtration and the filtrate was concentrated by evaporation of the solvent. The residue was then purified by vacuum distillation to obtain a faint yellow and viscous liquid (110–120 °C, 10 \textsuperscript{-2} Torr) with a yield of 24%. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 250 MHz): 4.51–4.25 ppm (m, 4H, O–CH\textsubscript{2}–CH\textsubscript{2}–O), 4.29–4.00 ppm (m, 2H, O–CH\textsubscript{2}–CH\textsubscript{2}–C), 2.51 ppm (t, 2H, O–CH\textsubscript{2}–CH\textsubscript{2}–O), 1.99 ppm (s, 2H, O–CH\textsubscript{2}–CH\textsubscript{2}–C=CH\textsubscript{2}). \textsuperscript{31}P NMR (CDCl\textsubscript{3}, 250 MHz): 17.8 ppm.
vacuum, the obtained copolymer was purified by precipitation in Et2O. The obtained polymer was dissolved in methanol and dialyzed against methanol overnight in order to remove DBU and TU residues. After the evaporation of methanol and drying under vacuum, PEO$_{1.5k}$-b-PBYPCOOH copolymer was collected and characterized by SEC, NMR and IR. $^1$H NMR (CDCl$_3$, 250 MHz): 2.18–2.05 ppm (m, H, –O–CH$_2$–CH$_2$–C–CH$_3$), 2.73–2.53 ppm (m, 2H, O–CH$_2$–C–CH$_3$), 3.38 ppm (s, 3H, CH$_3$–O–CH$_2$–O–CH$_3$), 3.75–3.40 ppm (m, 8H, C–H–O–CH$_2$–O–CH$_2$–), 4.7–4.41 ppm (m, 4H, O–CH$_2$–CH$_2$–O, 4H, O–CH$_2$–CH$_2$–C). $^{31}$P NMR (CDCl$_3$, 250 MHz): $-$1.76 ppm. $M_n$ (NMR: PH$_2$) = 2300 g mol$^{-1}$. $M_n$ (SEC: PS) = 13 000 g mol$^{-1}$. $D = 1.1$. IR peak = 3463, 3290, 2888, 1637, 1466, 1343, 963, 810 cm$^{-1}$.

**Synthesis of PEO-b-PBYPCOOH (copolymer 3, Scheme 1) by thiol-yne reaction.** PEO$_{1.5k}$-b-PBP$_{2.5k}$ (0.30 g, 1.68 mmol of alkynes), 3-mercaptopropionic acid (1.76 g, 16.6 mmol) and DMPA (65.9 mg, 0.255 mmol) were dissolved in 10.0 mL of DMF, and the solution was then irradiated for 2 hours under UV (365 nm) at room temperature. The polymer was collected by precipitation into a pentane/diethyl ether mixture (3:1 ratio). After solubilisation in methanol, the copolymer was purified by dialysis (type of membrane cut off) against methanol overnight in order to remove the triethylamine residues and the photoinitiator by-products. After drying under vacuum, the PEO-b-PBYPCOOH copolymer 3 was characterized by $^1$H and $^{31}$P. $^1$H NMR (D$_2$O, 250 MHz): 3.37 ppm (s, 3H, CH$_2$–O–CH$_2$–C–CH$_3$), 3.83–3.63 ppm (m, 8H, CH$_2$–O–CH$_2$–C–CH$_3$), 1.80–2.05 ppm (m, 2H, O–CH$_2$–CH$_2$–CSH–S–CH$_2$), 2.30–2.60 ppm (m, 2H, O–CH$_2$–COOH, 5H, CH$_2$–S–CH═CH–S–CH$_2$), 4.53–4.20 ppm (m, 2H, O–CH$_2$–CH$_2$–CH$_2$–O–, 4H, O–CH$_2$–CH$_2$–O–). $^{31}$P NMR (D$_2$O, ppm, 250 MHz): $-$1.32 ppm. IR peak = 3550–3200, 2888, 1726, 1635, 1466, 1358, 1243, 1061, 924, 802 cm$^{-1}$.

**Synthesis of allyl phospholane (AlIP) (copolymer 4, Scheme 1).** 1-Propan-3-ol (10.16 g, 0.175 mol) and triethylamine (17.71 g, 0.175 mol) were dissolved in dry THF (50 mL) and cooled to 0°C. A solution of COP (25 g, 0.175 mol) in dry THF (50 mL) was added dropwise under stirring ([COP]$_0$/[TEA]$_0$/[1-propan-3-ol]$_0$) = 1/1/1). After complete addition, the resulting mixture was stirred at 0°C for 5 h. The triethylamine hydrochloride salt was removed by filtration and the filtrate was concentrated. The AlIP was then purified by distillation under reduced pressure (80–90°C, 10$^{-3}$ Torr) giving a yield of 27%. $^1$H NMR (CDCl$_3$, 250 MHz): 4.97–4.46 ppm (m, 4H, O–CH$_2$–CH$_2$–O, 2H, O–CH$_2$–CH═CH$_2$–), 5.76–5.42 ppm (m, 2H, CH═CH$_2$), 6.37–6.07 ppm (m, 1H, CH═CH$_2$). $^{31}$P NMR (CDCl$_3$, 250 MHz): 17.3 ppm.

**Synthesis of PEO-b-PAILP (copolymer 5, Scheme 1) by ROP.** TU (740 mg, 2 mmol), AlIP 4 (163 g mol$^{-1}$, 2 g, 12.2 mmol) and PEO-OH ($M_n$ ~ 5000 g mol$^{-1}$, 2.0 g, 0.40 mmol) were introduced into a flask under an inert atmosphere, dried by three azeotropic distillations with toluene and solubilized in dry and degassed toluene (8 mL). Freshly distilled DBU (0.3 mL, 2 mmol) was then added to the solution ([AlIP]$_0$/[PEO-OH]$_0$/[DBU]$_0$/[Tu]$_0$) = 61/2/10/10, $M_{n,\text{th, AlIP}}$ = 5000 g mol$^{-1}$). The reaction medium was stirred at 0°C for 5 minutes. The monomer conversion was evaluated to be 80% based on the $^{31}$P NMR spectrum. After removing the residual solvent under vacuum, the obtained copolymer was purified by precipitation in cold Et$_2$O. The obtained polymer was dissolved in methanol and dialyzed against methanol overnight in order to remove DBU and TU residues. After the evaporation of methanol and drying under vacuum, PEO$_{1.5k}$-b-PAILP$_{2.5k}$ copolymer was collected and characterized by SEC and NMR. $^1$H NMR (CDCl$_3$, 250 MHz): 4.44–4.09 ppm (m, 4H, O–CH$_2$–CH$_2$–O), 4.74–4.45 ppm (m, 2H, O–CH$_2$–CH═CH$_2$–), 5.52–5.13 ppm (m, 2H, CH═CH$_2$–), 6.17–5.70 ppm (m, 1H, CH═CH$_2$–), 3.38 ppm (s, 3H, CH$_3$–O–CH$_2$–O–CH$_2$–C). $^{31}$P NMR (CDCl$_3$, 250 MHz): $-$1.36 ppm. $M_n$ (NMR: PH$_2$) = 2500 g mol$^{-1}$. $M_n$ (SEC: PS) = 10 800 g mol$^{-1}$. $D = 1.2$.

**Synthesis of PEO-b-PDDO (copolymer 6, Scheme 1) by deprotection of 5.** PEO$_{1.5k}$-b-PAILP$_{2.5k}$ (0.50 g, 3 mmol) was stirred with 1.5 eq. of sodium thiophenolate (C$_6$H$_5$SnNa) (0.6 g, 4.5 mmol) in a DMF/H$_2$O (50/50 v/v) mixture at room temperature for 3 h. The polymer was then collected by precipitation in cold diethyl ether. After solubilisation in DMF, the copolymer was purified by dialysis (MWCO: 1 kDa) against Milli-Q water overnight. The PEO-b-PDDO 6 was recovered by freeze-drying and was characterized by $^1$H and $^{31}$P NMR in D$_2$O. $^1$H NMR (D$_2$O, 250 MHz): 4.19–3.94 ppm (m, 4H, O–CH$_2$–CH$_2$–O), $^{31}$P NMR (D$_2$O, 400 MHz): 0.4 ppm.

**Preparation of CaCO$_3$ particles by the classical chemical route.** According to a previously reported procedure, $^{14,15,39}$ calcium chloride (CaCl$_2$) (1.6% w/v) was added to glycerine buffer (0.62 M NaCl and 0.62 M glycerine), and then the pH was adjusted to 10. Sodium carbonate (Na$_2$CO$_3$) (1.6% w/v) was added to the glycerine buffer. Lastly, as an anionic organic template, HA (0.1% w/v) was added to the CaCl$_2$ solution. Precipitation of CaCO$_3$ was carried out by mixing an equal volume of calcium containing solution (CaCl$_2$) and carbonate containing solution (Na$_2$CO$_3$). After stirring for 5 min at room temperature, the obtained suspension was centrifuged for 10 minutes at 4000 rpm and washed twice when the particles were unloaded. Finally CaCO$_3$ microparticles were recovered by freeze-drying.

The same procedure was repeated with PEO-b-PBPYCOOH 3 or PEO-b-PDDO 6 instead of HA (0.1% w/v of copolymer in the calcium chloride solution).

**Preparation of CaCO$_3$ particles by the scCO$_2$ process.** The synthesis method used in this study was patented by Boury et al.$^{75}$ Calcium chloride (CaCl$_2$) (1.6% w/v) was added to glycerine buffer (0.62 M NaCl and 0.62 M glycerine), and then the pH was adjusted to 10. HA (0.1% w/v) was added to the CaCl$_2$ solution. A stainless steel autoclave with a capacity of 500 mL (Separex, Champignelles, France) was heated to 40.0 ± 0.1 °C, and pressurized with CO$_2$ at 200 ± 1 bar. Liquid CO$_2$ was pumped using a high-pressure membrane pump at 1 kg h$^{-1}$ (Milton Roy Europe, Pont Saint-Pierre-France) and preheated using a heat exchanger before feeding into the autoclave. The stirring speed was set at 1200 rpm, with a Teflon coated stirrer (Top-industrie, Vaux le Penil, France). Once the equilibrium was reached (i.e. stable temperature and pressure), the previously prepared calcium aqueous solution (1.6% w/v) was injected using an HPLC pump (Model 307, Gilson, Villiers le Bel, France) with a flow rate of 10 mL min$^{-1}$ and a nozzle with an inner diameter.
of 1 mm. Once injection was achieved, the final pressure was 240 ± 5 bar and the stirring was maintained at 1200 rpm for 5 min. Then, the stirring was stopped and the autoclave was depressurized at a rate of 40–50 bar min⁻¹ prior to the lyophilization of the CaCO₃ microspheres.

The same procedure was repeated with PEO-b-PBYPCOOH 3 or PEO-b-PPDO 6 instead of HA at (0.1% w/v of copolymer in the calcium chloride solution).

Results and discussion

Synthesis of the CaCO₃ templating agents

Two types of double hydrophilic copolymers were considered in this study for templating calcium carbonate particles. The first candidate consists of a diblock copolymer made of a PEO sequence associated to a poly(phosphotriester) block bearing carboxylic pendant groups which are known for their high capacity to complex calcium ions. In contrast to other previously reported PEO-b-PPE derivatives, this copolymer with acid groups cannot be produced by the direct polymerization of the corresponding acid containing cyclic phospholane monomer. Indeed, such a monomer is extremely unstable and undergoes rapid degradation via a ring opening reaction catalyzed by the carboxylic groups. For this reason, we considered introducing the acid moieties along the polyphosphate backbone by the post-modification of a PEO-b-PPE precursor. This two-step strategy is shown in Scheme 1 (route A). It consists of the ring opening polymerization (ROP) of butynyl phosphate monomer (BYP) 1 initiated by a PEO–OH monomethyl ether followed by addition of mercaptopropionic acid onto the pendant alkyne groups of the PEO-b-polbutynyl phosphate copolymer (PEO-b-PBYP) 2 by a photochemical thiol–yne click reaction inspired by a procedure by Wooley et al. The synthesis of the desired PEO-b-PBYPCOOH 3 is presented and discussed hereafter.

Monomer 1 substituted by an alkynyl group (BYP) was obtained by the coupling of 3-butyln-1-ol with 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) according to a well-established synthetic pathway for cyclic phospholane ester monomers. The structure of BYP 1 was confirmed by 1H NMR spectrum (Fig. 2). Indeed, the chemical shifts and the relative intensities of the signals were in agreement with the values reported previously for this compound. Furthermore, only one strong resonance appeared at 17.32 ppm in the 31P NMR spectrum of BYP (Fig. 2), which is typical of cyclic phospholane monomers and confirms the structure and purity of the BYP monomer.

Polymerization of 1 was then initiated from a PEO–OH macro initiator (M₈ = 5000 g mol⁻¹) in order to produce PEO-b-PBYP 2. The latter proceeded by organocatalyzed-ROP in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1-1-[3,5-bis(trifluoromethyl)phenyl]-3-cyclohexyl-2-thiourea (TU) ([DBU]₀/[TU]₀ = 2). Substitution of organic compounds for metallic catalysts, like tin octoate, in the ROP prevents contamination of the final polymer by any metal traces that are incompatible with biomedical applications. Moreover, Clément et al. demonstrated the beneficial effect of using a DBU/TU mixture as a catalyst for the polymerization of cyclic phospholanes on the polymerization kinetics and control. As a matter of fact, the DBU and TU system minimized the intra- and inter-molecular transesterification side reactions and is the most efficient catalytic method for this type of monomer. Here, the ROP of BYP was carried out with both co-catalysts in dichloromethane at 0 °C. The BYP/PEO–OH molar ratio was adjusted to 17 in order to target a PBYP sequence of 3000 g mol⁻¹. The conversion of 1 reached 83% after 10 minutes and the polymerization was stopped. After purification by precipitation in diethylether, the copolymer was analyzed using size exclusion chromatography (SEC). As shown in Fig. S1 (ESI†), a clear shift of the SEC peak towards higher molar masses was observed, which proved the successful chain extension by ROP of BYP from the PEO–OH macroinitiator and the formation of the targeted PEO-b-PBYP. No residual PEO peak was left but a small peak was detected at a lower elution volume compared to the major population. This higher molar mass peak might result from the polymerization of BYP initiated from traces of poly(ethylene oxide) having alcohol functions at both extremities of the chain (HO–PEO–OH) which contaminates the commercial PEO–OH as evidenced by the MALDI-TOF spectrum (Fig. S2, ESI†). Nevertheless, well-defined PEO-b-PBYP (M₈,SEC,DMF = 13 100 g mol⁻¹, D = 1.1) was obtained. The actual molar mass and composition of the copolymer 2 was measured using 1H NMR (Fig. 2) considering the relative intensities of the PEO peak at 3.6 ppm and the signal corresponding to the propargylic protons c of the BYP units at 2.65 ppm. The molar mass of the PBYP block was evaluated to be 2300 g mol⁻¹, which is close to the theoretical prediction at 83% conversion (M₈,th = 2500 g mol⁻¹) and represents an average degree of polymerization (DP) of 13.

The post-modification of the pendant alkyne moieties of PEOₙ-b-PBYP₂ₙ was then performed by the thiol–yne reaction in order to introduce carboxylic acids along the polyphosphate chains. Following a procedure adapted from Wooley et al. 71
mercapto propionic acid (10 eq.) was reacted with alkyne in methanol under UV irradiation in the presence of catalytic amounts of 2,2-dimethoxy-2-phenylacetophenone (DMPA). After two hours, the irradiation was stopped and the copolymer was analyzed by IR (Fig. S3, ESI†) and 1H NMR (Fig. 2). The disappearance of the C–H stretching band of the terminal alkyne at 3290 cm\(^{-1}\) and the apparition of an intense band at 1726 cm\(^{-1}\) typical of a carbonyl stretch C==O of a carboxylic acid confirmed the successful functionalization of the copolymer (Fig. S3, ESI†). In contrast to the starting PEO\(_{5k}\)-b-PBYP\(_{2.3k}\) 2 that contains a hydrophobic PBYP block, the resulting PEO-b-PBYP-COOH 3 copolymer could easily be solubilized in water, which is another indication of the modification of the polymer, and in D\(_2\)O for 1H NMR characterization (Fig. 2). 1H NMR provided evidence for the full consumption of the alkyne groups of 2 and the insertion of two carboxylic acid functions per BYP unit in the copolymer 3. Considering the near quantitative functionalization, the molar mass of 3 was calculated ([PEO–OH]/[DBU]/[TU]/[Sodium benzenethiolate (C\(_6\)H\(_5\)SNa) (Scheme 1, route B). The block copolymerization and formation of PEO-b-poly(phosphodiester) sequence bearing negatively charged oxygen atoms like to complex calcium ions (copolymer 6 in Scheme 1, PEO-b-PPDO). The general synthetic strategy (Scheme 1, route B) relies on the ROP of 2-(prop-2-en-1-oyloxy)-1,3,2-dioxaphospholane 2-oxide 4, a cyclic phospholane monomer with an allylic moiety as side chain (AlIP), followed by the nucleophilic deprotection of the allyl group of PEO-b-poly(allyl phospholane) 5. Compared to other strategies reported for the synthesis of poly(phosphodiester)s,\(^{68}\) the deprotection of the allyl group can be achieved under non acidic conditions, which prevents premature degradation of the poly(phosphate) chain.

First, prop-2-en-1-ol was esterified with 2-chloro-1,3,2-dioxaphospholane-2-oxide (COP) in the presence of triethylamine (TEA) leading to 4, whose purity and structure were confirmed by 1H NMR (Fig. 3). Next, the metal free ROP of 4 was initiated from PEO–OH (5000 g mol\(^{-1}\)) using the above mentioned DBU/TU catalytic system in toluene at 0 °C ([4]/[PEO–OH]/[DBU]/[TU] = 30/1/5/5). The monomer conversion, calculated based on the relative intensities of the monomer and polymer signals in the 31P NMR spectrum (Fig. 3), reached over 80% within 10 minutes. SEC analysis proved the successful block copolymerization and formation of PEO-b-PAllP 5 (\(M_n\)SEC = 10 800 g mol\(^{-1}\), \(D < 1.2\)) (Fig. S4, ESI†). Again, the copolymer is contaminated by about 10% of a higher molar mass copolymer probably due to the presence of traces of bifunctional HO–PEO–OH in the commercial monomethylether PEO. The composition and average molar mass of 5 were determined by 1H NMR (Fig. 3) by comparison of the intensity of the signal corresponding to PEO at 3.65 ppm with the peak assigned to the allylic protons b of the PAlIP block at 5.2–6.1 ppm and 4.58 ppm, respectively. At the same time, the signal corresponding to the protons of the –O–PAllP = 2500 g mol\(^{-1}\), \(M_n\)NMR,PAlIP = 2500 g mol\(^{-1}\), \(D_{PAlIP} = 16\)).

In the last step, deprotection of PEO\(_{5k}\)-b-poly(allyl phospholane)\(_{2.5k}\) to give the negatively charged PEO-b-PPDO\(^{-}\) 6 was carried out at room temperature in a DMF/water mixture with sodium benzenethiolate (C\(_6\)H\(_5\)SNa) (Scheme 1, route B). The 1H NMR analysis in D\(_2\)O shown in Fig. 3 demonstrates the quantitative removal of the allyl protective groups of 5. Indeed, no signals of vinylic and allylic protons were found in the spectrum of 6 at 5.2–6.1 ppm and 4.58 ppm, respectively. At the same time, the signal corresponding to the protons of the –O–CH\(_2\)–CH\(_2\)–O– of the poly(phosphate) backbone were shifted from 4.3 ppm to 4.0 ppm compared to the native polymer. The 31P NMR of the PEO-b-PPDO\(^{-}\) 6 shows only one single peak at 0.40 ppm (Fig. 3), which proves that the deprotected poly(phosphodiester)-based copolymer is not contaminated by other phosphorous impurities and that no hydrolysis occurs during the deprotection step. Therefore, PEO\(_{5k}\)-b-PPDO\(_{1.9k}\) having an average number of 16 negatively charged oxygen atoms per chain was successfully synthesized.

**Calcium carbonate microparticle formation**

The control of the size, the shape, and the crystal structure of the calcium carbonate particles is very important for the tuning of their properties for specific applications. Among the “crystal engineering” methods, macromolecules able to interact with inorganic salts were used as templating agents during the nucleation and growth of crystals.\(^{39}\) For example, mixing calcium salt (CaCl\(_2\)) and carbonate (Na\(_2\)CO\(_3\)) aqueous solutions (called here the “chemical route”) is a very popular pathway for the formation CaCO\(_3\) particles.\(^{19,22,39,82–84}\) In addition to glycine,\(^{85,86}\) the use of hyaluronic acid (HA), an anionic biopolymer, in the particles’ formulation allows the directing of the polymorphism of CaCO\(_3\) into the vaterite form.\(^{15,18,87}\) Under these conditions, spherical particles are formed with an average diameter of 1.5 μm as shown by Fig. 4A. Nevertheless, the SEM image reveals rather important particle size dispersity and a tendency towards aggregation.
Recently, some of us\textsuperscript{14,15,18} developed a novel method for the production of CaCO\textsubscript{3} particles involving HA as templating agent and supercritical carbon dioxide which serves as a source of carbonate ions (called here “scCO\textsubscript{2} process”). Typically, a CaCl\textsubscript{2} aqueous solution containing HA and buffered by glycine is injected into a reactor pressurized with CO\textsubscript{2} at 200 ± 1 bar at 40 ± 0.1 °C followed by depressurization at 40–50 bar min\textsuperscript{-1} leading to CaCO\textsubscript{3} microparticle formation. After the injection of the basic solution into the autoclave, the fast diffusion of CO\textsubscript{2} molecules into the salt solution leads to the formation of ionic species such as HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{3}\textsuperscript{2-}. The latter species react with Ca\textsuperscript{2+} ions and form the CaCO\textsubscript{3} particles with a spherical shape. In our set of experiments, we also obtained spherical microparticles with an average diameter of 8.5 μm (Fig. 4D). It is shown that the particles formed in scCO\textsubscript{2} media are bigger but less aggregated. It has been shown previously that the zeta potential of the CaCO\textsubscript{3} particles produced with HA was more negative when formulated by the scCO\textsubscript{2} route than the chemical route.\textsuperscript{18} This could lead to better electrostatic repulsion and explain a beneficial effect on the level of aggregation of the particles (compare Fig. 4A and D).

With these references in hand, the double hydrophilic copolymers PEO-b-PBYP\textsubscript{COOH} 3 and PEO-b-PPDO-b-PPDO \textsuperscript{6} were tested as a substitute of HA for the preparation of CaCO\textsubscript{3} microparticles via the chemical route and the scCO\textsubscript{2} process. In this case, we expect that the PEO block will only slightly interact with the dissolved ions and ensure the water solubility whereas the charged polyphosphoester segment (PBYP\textsubscript{COOH} or PPDO\textsuperscript{-}) will strongly interact with the inorganic salts and control the nucleation and growth of the crystal.

When mixing CaCl\textsubscript{2} and Na\textsubscript{2}CO\textsubscript{3} according to the above mentioned chemical route in the presence of 3 and 6 in the place of HA, micron-sized particles were formed and the SEM analyses emphasized the crucial impact of the templating agent on the morphology of CaCO\textsubscript{3} particles (Fig. 4B and C). Indeed, particles obtained in the presence of 6 are bigger (4 μm), poorly defined and largely aggregated. In contrast, using PEO-b-PBYP\textsubscript{COOH} 3 in the formulation decreases the size of the particles down to 0.8 μm, which is significantly smaller than those obtained with HA. Unfortunately, in the latter case, aggregation of particles was also pronounced.

Then, formation of calcium carbonate by the scCO\textsubscript{2} process was tested with copolymers 3 and 6. Particles formed in the presence of PEO-b-PPDO \textsuperscript{6} in scCO\textsubscript{2} are clearly better isolated from each other but also much more regular (Fig. 4F) than those formed by the chemical route (Fig. 4C). The average size of the particles was larger after the scCO\textsubscript{2} process (6.7 μm, Fig. 4F), as was the case for HA (compare Fig. 4A and D). Overall, rather similar particles are formed with HA and PEO-b-PBYP\textsubscript{COOH} 3 leads to much smaller particles (1.5 μm) in scCO\textsubscript{2} than HA (compare Fig. 4E and D) while preventing aggregation of the particles. These size and size distribution evolutions were also confirmed by light scattering (LS) measurements (Fig. S5 and Table S1, ESI†) on the particle samples dispersed in phosphate buffer.

These observations clearly demonstrate the possibility to tune and reduce the size of the CaCO\textsubscript{3} particles by a factor of 6 when using copolymer 3 instead of HA. Given the wide difference of structure between 3 and HA, it is difficult to pinpoint one specific structural parameter as responsible for the difference in the CaCO\textsubscript{3} particle size. However, this size effect could be related to the presence of the neutral PEO segment on 3 which could limit the growth of the microparticles. In addition, the high carboxylic acid density of compound 3 as compared to HA might also favor the interaction of 3 with CaCO\textsubscript{3} nuclei and slow down their growth. Moreover, PEO-b-PBYP\textsubscript{COOH} 3 is much more efficient than PEO-b-PPDO \textsuperscript{6} for templating the calcium carbonate particles, most probably due to a higher calcium ion complexation ability and

![Fig. 4 SEM observations of CaCO\textsubscript{3} particles prepared by the chemical route (A–C) and the scCO\textsubscript{2} process (D–F) in the presence of hyaluronic acid (A and D), PEO-b-PBYP\textsubscript{COOH} 3 (B and E) or PEO-b-PPDO \textsuperscript{6} (C and F).]
interaction with the inorganic surfaces of carboxylic groups compared to the negatively charged phosphodiester moiety. The zeta potential of all the particles (Table S1, ESI†) is clearly negative indicating the presence of the stabilizing copolymers at their surface. The lower values obtained for the block-copolymers as compared to HA could reflect the presence of the neutral PEO segment partially screening the charges.

XRD analysis of the particles prepared in CO2 with copolymers 3 and 6 indicated that CaCO3 is in the vaterite form (Fig. 5) as was the case for HA. Based on the analysis of the broadening of the XRD peaks using the Debye–Sherrer equation, the size of the vaterite crystals that compose both types of microspheres is approximately 25 ± 5 nm. The crystal size appears quite similar to those obtained with HA showing that the templating polymer has few, if any, influence on the vaterite nanograin size. This observation is in line with the fact that the formation of vaterite nanocrystals is mainly governed by the ionic strength, pressure and temperature of the medium that are comparable in all experiments.88,89

Close examination of the cleaved microspheres by SEM evidenced a clear effect of the templating polymer on the internal morphology of the produced calcium carbonate particles (Fig. 6A and B). Particles prepared in the presence of PEO-b-PBYPCOOH are composed of individual aggregated and spherical nanograins and with a less compact structure than in the presence of HA. This demonstrates the key role of the templating polymer on the packing of the vaterite nanograins to form the microsphere. Remarkably, the porous structure of the CaCO3 particles prepared in scCO2 with 3 exhibiting a central cavity (Fig. 6B) could be of particular interest for applications involving particle loading like drug delivery systems.

**Conclusion**

The formulation of CaCO3 particles was performed in the presence of novel well-defined double hydrophilic copolymers containing a PEO segment associated to a poly(phosphotriester) block with pendant carboxylic moieties or to a negatively charged poly(phosphodiester). Two sets of conditions were evaluated, i.e. the classical chemical route involving CaCl2 and Na2CO3 and a recently reported process based on supercritical carbon dioxide technology using CO2 as the source of carbonate. Spherical CaCO3 particles in the vaterite form were obtained in all cases but a dependence of the particle size on the structure of the templating agent was observed by SEM. Particles obtained with the acid containing copolymer PEO-b-PBYPCOOH 3 exhibit a low size dispersity and were 6 times smaller (1.5 μm) than those produced by HA. To the best of our knowledge, these are the smallest vaterite particles ever formulated in scCO2. In contrast, rather similar particles were collected when using the poly(phosphodiester) derivative (PEO-b-PPDO) 6 and HA. The high density of carboxylic acids and the stronger calcium affinity of acid moieties compared to the negatively charged poly(phosphodiester) units most probably account for the excellent templating capacity of the PBYPCOOH containing copolymer. It is also worth noting that the level of particle aggregation was much lower with the scCO2 formulation process compared to the classical procedure based on CaCl2 and Na2CO3. The internal structure of the particles was also proved to be porous with an internal cavity in their center, which is of particular interest for encapsulation of biomolecules and delivery applications. These new poly(phosphate)-based templating agents notably pave the way to the design of inorganic drug carriers with a tunable size and morphology, pointing to the possibility of adjusting and optimizing their loading capacity and release profile.

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Notes and references
