RSC Advances

PAPER

Cite this: *RSC Adv.*, 2016, **6**, 42081

Core cross-linked micelles of polyphosphoester containing amphiphilic block copolymers as drug nanocarriers†

Z. Ergul Yilmaz,[ab] S. Vanslambrouck,[ac] S. Cajot,[a] J. Thiry,[a] A. Debuigne,[a] P. Lecomte,[ac] C. Jérôme*[a] and R. Riva[a]

Received 21st March 2016
Accepted 30th March 2016

DOI: 10.1039/c6ra07422g

www.rsc.org/advances

Poly(ethylene oxide)-*b*-polyphosphoester amphiphilic block copolymers are known to self-assemble into polymer micelles when dissolved in water. This work aims at reporting on the improvement of the stability of the micelles at high dilution by crosslinking the hydrophobic polyphosphoester micellar core. Typically, an unsaturated alkene side-chain was introduced onto the cyclic phosphate monomer according to a one-step reaction followed by its organocatalyzed polymerization initiated by a poly(ethylene oxide) macroinitiator. This strategy avoids the use of any organometallic compounds in order to facilitate the purification and meet the stringent requirements of biomedical applications. After self-assembly in water, the micelles were cross-linked by simple UV irradiation. These cross-linked micelles have then been loaded with doxorubicin to evaluate their potential as drug nanocarriers and monitor the impact of crosslinking on the release profile.

Introduction

Polymer micelles have been widely studied as potential drug delivery systems, due to their unique capabilities to accumulate within tumor sites *via* an enhanced permeation and retention effect (EPR) and for loading high amounts of poorly soluble drugs. An important issue of such dynamic micellar systems is their instability, due to the equilibrium between unimers and micelles. Actually, below the critical micellar concentration (CMC), only unimers persist and any pre-encapsulated drug is released. A way to prevent this premature release occurring upon injection in the bloodstream is to cross-link either the shell or the core of the micelles prior injection. Several strategies have been reported to prepare core or shell cross-linked micelles.[1–4]

Needless to say that the polymer constituents of such nanocarriers, must be biocompatible and biodegradable or bioeliminable. For nanocarriers to comply with the stealthiness, the hydrophilic block of amphiphilic block copolymers is usually a biocompatible and bioeliminable short length poly(ethylene oxide) (PEO). Beside, a biocompatible and biodegradable aliphatic polyester, such as poly(ε-caprolactone) (PCL) and polylactides (PLA) is mostly used as the hydrophobic block.[5] As a consequence, PEO-*b*-aliphatic polyester copolymers are the family of amphiphilic block copolymers among the most studied worldwide for the elaboration of biodegradable micelles based-nanocarriers.[6–8] Therefore, functional aliphatic polyesters modified by reactive side-groups have been synthesized to build cross-linked micelles that can be efficiently used as drug nanocarriers.[9,10]

Nevertheless, the hydrolytic degradation of PCL and PLA has an impact on the local pH, which locally decreases due to the degradation products accumulation.[11–13] This acidification may have undesirable side-effect on living tissues. Recently, a new class of biodegradable polymers, *i.e.* polyphosphoesters (PPE),[14–16] is emerging as a valuable alternative towards polyesters. Like polyesters, polyphosphoesters are attractive for biomedical applications such as drug delivery,[17–19] gene delivery,[20–22] tissue engineering[23–25] and dental applications[23] because of their biocompatibility and biodegradability and their structural similarities to nucleic and teichoic acids.[26–28] Their degradation does not change significantly the local pH.[14,29,30] Similarly to aliphatic polyesters, they are easily synthesized by organocatalyzed ring-opening polymerization (ROP) of cyclic phospholane monomers.[26,31–33] Moreover, their functionalities and properties can be controlled by changing the pendant group on the pentavalent phosphorus atom of the cyclic phospholane monomer precursors.

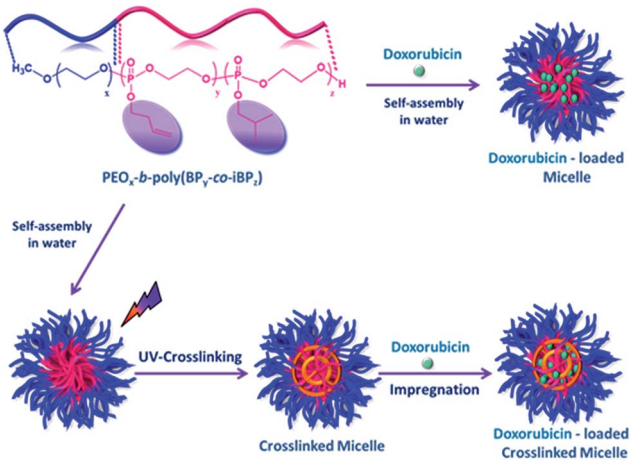
Pioneer works of Wooley *et al.* demonstrated that PEO-*b*-PPE-based drug conjugates are able to form micelles with a high drug loading capacity.[15] Besides, they also showed that the

[a]*Center for Education and Research on Macromolecules (CERM), University of Liège, Chemistry Department, building B6a Sart-Tilman, B-4000 Liège, Belgium. E-mail: c.jerome@ulg.ac.be*

[b]*INSERM U1066, Micro et Nanomédecines Biomimétiques, IBS, University of Angers, 4 rue Larrey, Cedex 9, Angers, 49933, France*

[c]*BioMIMedics, Interreg EMR IV-A Consortium: Lead Partner Maastricht University, Universiteitssingel 50, 6229ER Maastricht, The Netherlands*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ra07422g



Scheme 1 Schematic representation of micelle construction *via* self-assembly of the amphiphilic block copolymers in water and cross-linking by UV irradiation, followed by loading with doxorubicin.

PEBP-*b*-PBYP-*g*-PEO amphiphilic block terpolymer was capable of carrying silver thanks to the presence of alkyne unsaturations on the polyphosphoester block.[16] Therefore, inspired by these researches, we have designed and synthesized novel PEO-*b*-PPE amphiphilic block copolymers bearing unsaturations on the polyphosphoester block, in order to develop core cross-linked micelles as drug carrier of the second generation (Scheme 1).

Firstly, the homopolymerization of butenylphosphoester (BP) and the copolymerization of isobutylphosphoester (iBP) and BP initiated from monomethoxy-PEO (MPEO–OH) by using organocatalysis will be investigated for the first time. Organocatalysis has been especially selected for the ROP to avoid metal residues difficult to extract from the copolymer as a biomedical application is foreseen. Secondly, micellization of the copolymer and the crosslinking by UV irradiation will be studied by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Especially, the stability of the cross-linked nanocarriers will be demonstrated by using good solvent of both blocks. Finally, the effect of the crosslinking of the micelles on the drug loading and release profile will be evaluated by using doxorubicin,[34,35] a typical drug used in cancer therapy.

Results and discussion

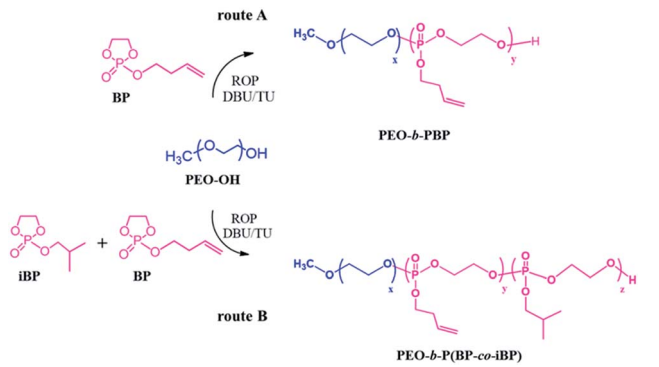
Synthesis of amphiphilic block copolymers

2-(But-3-en-1-yloxy)-1,3,2 dioxaphospholane 2-oxide (BP) monomer was synthesized as reported in the literature.[36] Typically, an excess of 2-chloro-1,3,2-dioxaphospholane-2-oxide was reacted with 3-buten-1-ol in the presence of a stoichiometric amount of triethylamine. After purification by fractionated distillation under reduced pressure, the yield was equal to 60%, which is in good agreement with the results reported in the literature.[26] Stability of BP towards hydrolysis was investigated by ^{1}H NMR analysis by dissolution of BP in D_2O. After only 30 min in D_2O, BP monomer was completely hydrolyzed in phosphoric acid as appeared by the complete disappearance of the signal of BP in favor of new signals characteristic of 3-buten-1-ol and ethylene glycol in the ^{1}H NMR spectrum, and the only signal at 0 ppm in ^{31}P NMR analysis typical of the phosphoric acid. Thus, this very high sensitivity of BP towards hydrolysis makes mandatory the storage and handling of the monomer under dry conditions.

The PEO-*b*-polyBP amphiphilic copolymer was synthesized by initiation of the BP ROP starting from a MPEO–OH macroinitiator (M_n = 5000 g mol^{-1}) as shown in Scheme 2 (route A). The latter was performed 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 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 catalysts for the polymerization of cyclic phospholanes on the polymerization kinetics and control.[26] As a matter of fact, DBU and TU system minimized the intra- and inter-molecular transesterification side-reactions and appears as the most efficient catalytic method for this type of monomers. Following a procedure adapted from Clément *et al.*,[26] the polymerization of BP was studied using MPEO–OH as an initiator. This macroinitiator being not very soluble in toluene at 0 °C, the ROP of BP was studied here in dichloromethane at 0 °C with both catalyst and co-catalyst.

Amphiphilic block copolymers with a hydrophilic-lipophilic balance (HLB) around 15 are usually recommended when the parent micelles are intended for drug delivery applications.[6] Indeed, when the molar mass of the hydrophilic PEO block is higher than the hydrophobic poly(BP) block (HLB>10), amphiphilic copolymers are prone to form spherical micelles. Therefore, because the molar mass of the MPEO–OH macroinitiator used in this study was equal to 5000 g mol^{-1}, the BP/MPEO–OH molar ratio was adjusted to 12 corresponding to a molar mass of the polyphosphoester sequence of 1200 g mol^{-1} (HLB = 15). The conversion of BP monomer reached 87% when the polymerization was stopped after 20 minutes (Table 1).

Size exclusion chromatography (SEC) (Fig. 1A) shows the shift of the SEC trace towards higher molar masses after BP



Scheme 2 General strategy for the synthesis of amphiphilic block copolymers with PEO as first block and poly(BP) (route A) or poly(BP) and poly(iBP) (route B) as second block.

Table 1 Synthetic results of the cross-linkable PEO-*b*-poly(BP) amphiphilic block copolymers synthesis

Monomer	Catalyst	BP (mmol)	MPEO–OH (mmol)	Catalyst (mmol)	Time (min)	Conv. (%)	DP PPE block[a] ^{1}H NMR	M_n PPE block[b] ^{1}H NMR (g mol^{-1})	$Đ^c$ (SEC)	HLB [d]
BP	DBU/TU	30	1	5/2	30	98	25	4500	1.1	5
BP	DBU/TU	12	1	5/2.5	20	87	7	1200	1.2	15

[a] Degree of polymerization for the polyphosphoester block determined by ^{1}H NMR according to the following equation: DP = integral$_{1H\ polyphosphate\ block}$/integral$_{1H\ PEO\ block}$ × 110. [b] Average molar mass of the polyphosphoester block determined according to the following equation: M_n = DP$_{BP}$ × 178. [c] Molar mass distribution determined by SEC. [d] HLB calculated by the Griffin equation 20 × [1 − (M_n,PPE block/M_n,total)].

polymerization, which proved the successful chain extension by ROP from the MPEO–OH macroinitiator and the formation of the expected PEO-*b*-polyBP amphiphilic block copolymer. Broadening of the main peak was observed but an additional small peak was detected at lower elution volume compared to the major population. This higher molar mass peak results from the polymerization of BP initiated from traces of poly(ethylene oxide) having alcohol functions at both ends of the chain (HO-PEO–OH) which contaminates the commercial MPEO–OH as previously evidenced by the MALDI-TOF spectrum.[31] Nevertheless, well-defined PEO$_{110}$-*b*-polyBP$_7$ (M_n SEC = 4900 g mol^{-1}, $Đ$ = 1.1) was obtained. The actual molar mass and composition of the copolymer was determined by ^{1}H NMR (Fig. 2).

Considering the relative intensities of the PEO peak at 3.6 ppm and the signal corresponding to the protons of the unsaturated pendant group (peak b, Fig. 2) of the BP units at 2.5 ppm, the average molar mass of the polyBP block was evaluated to 1200 g mol^{-1}, which represents an average degree of polymerization (DP) of 7. Let us mentioned that to exemplify this diblock copolymerization, a second composition was tested starting from a BP/MPEO–OH molar ratio to 30 (Table 1), for which similar observations and conclusions can be drawn, *i.e.*; a fast polymerization leading to well-defined diblock copolymer with a composition close to the theoretical value and a narrow distribution of the molar masses. In that case, the HLB of the obtained diblock copolymer being only 5, this copolymer was not used further for micellization.

It is also worth to mention that if the cyclic monomer is very sensitive to water traces, the diblock copolymer can be kept in water without significant degradation over a period of several days as demonstrated by ^{1}HNMR in D_2O with time (Fig. S1†). This confirms that the copolymers can sustain purification dialysis without hydrolytic degradation at neutral pH.

In order to modulate the crosslinking density of the micelles and investigate its impact on the drug loading and release kinetics, amphiphilic block copolymers presenting a lower number of unsaturation along the polyphosphate backbone were synthesized (Scheme 2, route B). Typically, a part of the BP monomer was substituted by iBP monomer. In the conditions developed for the PEO-*b*-polyBP (DBU/TU catalytic system in CH_2Cl_2 at 0 °C), the ring-opening copolymerization of BP and iBP mixtures was performed with monomer ratios (iBP/BP) of 7/3, 5/5 and 3/7 (Table 2). The diblock copolymers were analysed by SEC (Fig. 1B, iBP/BP = 5/5), ^{1}H and ^{31}P NMR. The molar mass and the composition of the PEO$_{110}$-*b*-poly(BP$_5$-*co*-iBP$_5$) block copolymers were measured by ^{1}H NMR as illustrated for the iBP/BP ratio of 5/5 in Fig. 3.

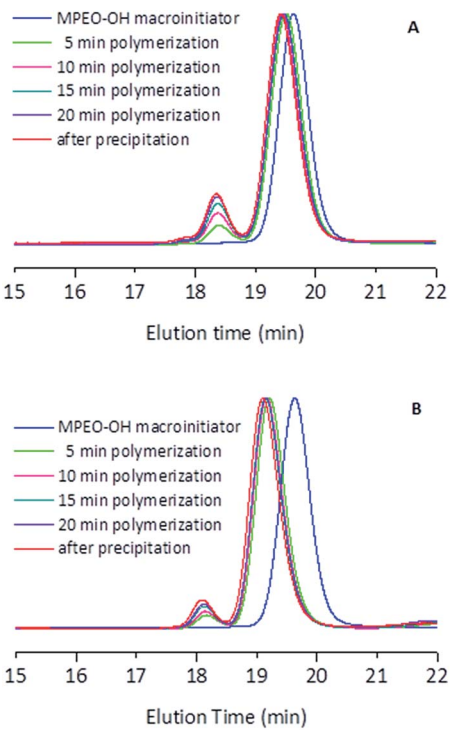


Fig. 1 SEC traces in THF of the (A) MPEO$_{110}$-*b*-polyBP$_7$ and MPEO–OH, (B) PEO$_{110}$-*b*-poly(iBP$_5$-*co*-BP$_5$) and MPEO–OH.

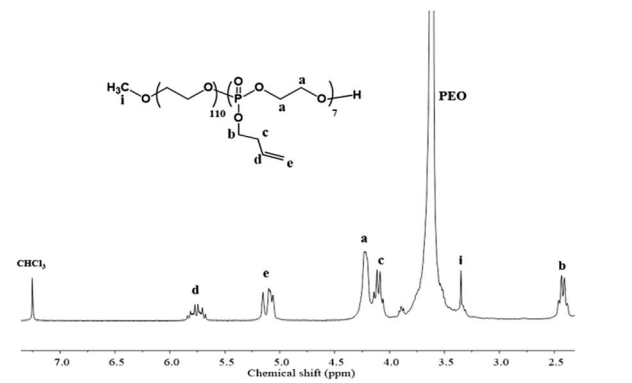


Fig. 2 ^{1}H NMR spectrum of PEO$_{110}$-*b*-polyBP$_7$ copolymer.

Table 2 Synthetic results of the cross-linkable PEO-*b*-poly(BP-*co*-iBP) amphiphilic block copolymers synthesis

Monomer	Catalyst	$[iBP]_0/[BP]_0$[a] (%)	MPEO–OH (mmol)	Catalyst (mmol)	Time (min)	Conv. (%)	DP PPE block[b]	Number of vinyl group/chain[c]	$[iBP]_0/[BP]_0$[d] (%)	M_n PPE block[e] ^{1}H NMR (g mol^{-1})	$Đ$[f] (SEC)
iBP/BP	DBU/TU	70/30	1	5/2.5	20	92	10	3	66/34	1800	1.1
iBP/BP	DBU/TU	50/50	1	5/2.5	20	94	10	5	46/54	1800	1.1
iBP/BP	DBU/TU	30/70	1	5/2.5	20	91	7	5	30/70	1200	1.1

[a] Monomer ratio in the comonomer feed. [b] Degree of polymerization for the polyphosphoester block determined by ^{1}H NMR according to the following equation: DP = $\text{integral}_{1H\ \text{polyphosphate block}}/\text{integral}_{1H\ \text{PEO block}} \times 110$. [c] Number of vinylic function on the polyphosphoester block. [d] Composition of the polyphosphoester block determined by ^{1}H NMR. [e] Average molar mass of the polyphosphoester block determined according to the following equation: $M_n = DP_{BP} \times 178 + DP_{iBP} \times 178$. [f] Molar mass distribution determined by SEC.

Micelle formation and crosslinking

Micellization of the amphiphilic PEO-*b*-polyBP and PEO-*b*-poly(BP-*co*-iBP) block copolymers were carried out by a traditional nanoprecipitation method, *i.e.* the copolymer is first dissolved in 5 mL DMF, a good solvent for both blocks and then micelles are formed upon the addition of 20 mL of Milli-Q water under vigorous stirring. This process was performed in presence of benzophenone, added to the DMF solution, to be used as photocatalyst for further micellar crosslinking. After 2 h of stirring, DMF was removed by dialysis. Excepted for a small part kept as a reference, the micellar solution was placed under UV irradiation with the purpose to cross-link the hydrophobic core as result of the reaction of the unsaturated side-groups.

The average size (D_h,app) of the self-assembled micelles in water was about 100 nm (PDI = 0.2), thus a comparable size (126 nm) of PEO_{114}-*b*-poly(butyl phosphate)$_9$ micelles of comparable composition and DP.[33] This apparent hydrodynamic diameter decreased by 10 nm after crosslinking (PDI = 0.2), which suggests that the crosslinking reaction is responsible for a higher compactness of the polyBP chains in the micelles core. As expected, a spherical morphology of the cross-linked micelles was observed by TEM in agreement with the relative length of the blocks (Fig. 4).

The phosphorus atoms in the hydrophobic core provided it with a high contrast, whereas the PEO corona was collapsed and is unobserved.

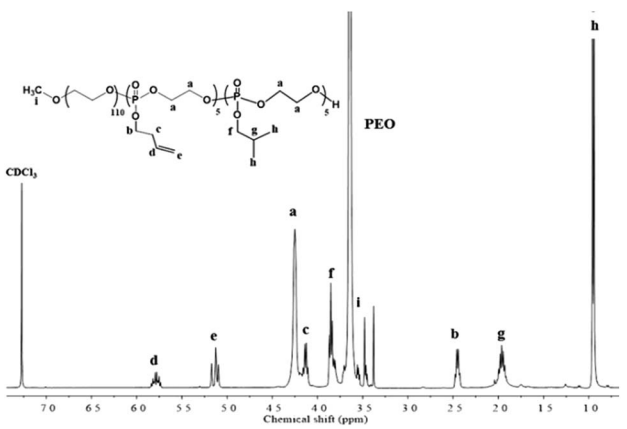


Fig. 3 ^{1}H NMR spectrum of PEO_{110}-*b*-poly(BP_5-*co*-iBP_5) copolymer (BP/iBP = 5/5).

The efficiency of the core crosslinking reaction was also assessed by the resistance of the cross-linked micelles against solubilization in DMF, a good solvent for both PEO and polyBP blocks. After a lyophilization step, the cross-linked micelles were dispersed into DMF. A substantial increase in size, from 100 nm in water up to 230 nm in DMF was observed by DLS (Fig. 5), which can be at least partly explained by the swelling of the polyphosphate core by DMF. The same analysis was also performed on the micelles irradiated but without addition of benzophenone. In that case, no nanoparticles the complete dissolution of the copolymer was observed in DMF evidencing the absence of crosslinking in these conditions.

The key advantage of polymer micelles relies on their ability to encapsulate a hydrophobic drug inside their core. Doxorubicin base (DOX) was physically entrapped into the hydrophobic inner core of the micelles by hydrophobic interactions with the pendent group of the polyphosphotriester block.[34,37] Advantageously, doxorubicin was easily quantified by UV-Vis absorption. Generally, drug loaded micelles are prepared by dissolution of the drug and the amphiphilic block copolymer into a common organic solvent followed by the rapid addition of water leading to the micelle formation concomitant to the encapsulation of the hydrophobic drug inside. This process is usually referred as nanoprecipitation process. In case of non-cross-linked micelles, such procedure could be applied to all the copolymers. Nevertheless, the cross-linked micelles led us to follow a drug impregnation process to load the DOX into the cross-linked systems. Typically, after UV crosslinking of the self-assembled micelles in water, the cross-linked micelles were

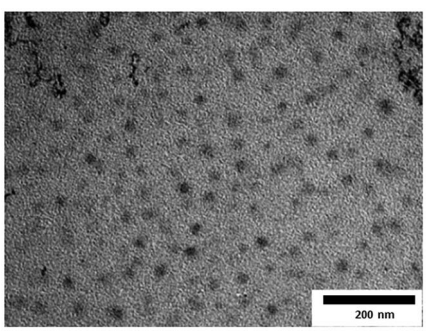


Fig. 4 TEM picture of the cross-linked micelles of PEO_{110}-*b*-$polyBP_7$.

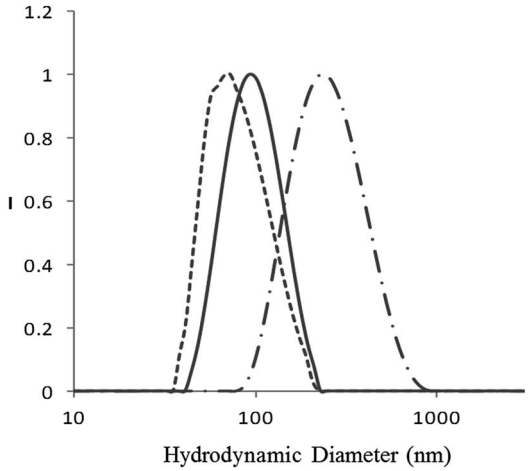


Fig. 5 Size distribution of the PEO-*b*-polyBP self-assembled micelles in H_2O (——), the cross-linked PEO-*b*-poly(BP) micelles in H_2O (– –) and in DMF (▪ —).

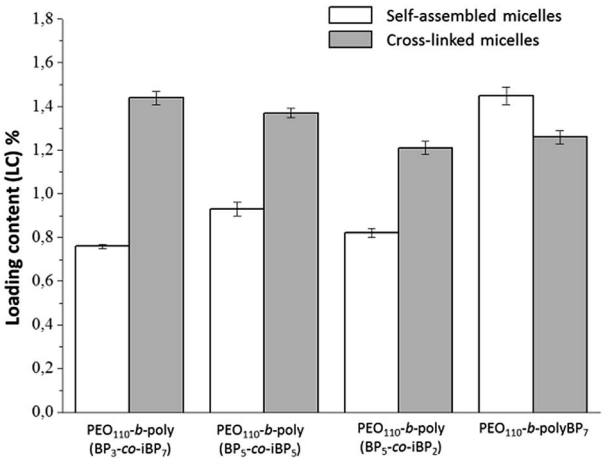


Fig. 6 Loading content (LC)% of doxorubicin in self-assembled and cross-linked micelles.

collected by lyophilization before to be immersed into a doxorubicin solution in DMF. This process allows to avoid possible degradation of the drug during UV curing.

Looking at the loading data for micelles before crosslinking (Table 3), we can conclude that the number of vinyl functions on the polyphosphate backbone has a marked influence on the LC of doxorubicin. Indeed, for an identical polyphosphate molar mass (Table 3, entries A and B), an increase of the LC with the number of vinyl function was observed. This observation was confirmed when comparing entries C and D of Table 3, where a significant increase of the LC is observed when the PPE block is exclusively constituted of polyBP. Secondary interactions of doxorubicin with the unsaturation present on the polyphosphate side-groups may be at the origin of this phenomenon. Nevertheless, this advantage is limited in case of cross-linked micelles since the double bonds disappeared upon the crosslinking reaction. Nevertheless, in case of cross-linked systems, the LC appears less dependent of the micelles composition and remarkably, the impregnation process leads to higher loading than the nanoprecipitation process that has to be applied for the non-cross-linked micelles (Fig. 6).

Interestingly, the doxorubicin release rate was slightly affected by the crosslinking of the core excepted for the PEO_{110}-*b*-poly(BP_3-*co*-iBP_7) based cross-linked micelles, for which a slower release rate was observed after crosslinking (Fig. 7A). The origin of this particular behaviour may come from the lowest crosslinking density allowing a better swelling of the cross-linked core during impregnation leading to a deeper diffusion of doxorubicin in the cross-linked micelles. After elimination of the DMF by dialysis, the cross-linked core collapsed leading to an efficient trapping of the doxorubicin. Nevertheless, in each case, a rather slightly slower release rate was obtained after crosslinking. Crosslinking thus allows improving the drug loading thanks to impregnation process while preserving a controlled drug delivery of the entrapped drug.

Experimental

Materials

2-Chloro-1,3,2-dioxaphospholane-2-oxide (COP, 95% Acros), monomethoxy poly(ethylene oxide) (MPEO–OH, M_n = 5000 g mol^{-1}, Aldrich), triethylamine (NEt_3, Aldrich), 3-buten-1-ol (Aldrich), 2-methyl-1-propanol (Aldrich), benzophenone (Aldrich), doxorubicin (Aldrich), tetrahydrofuran (THF, Chem-lab), dimethylformamide (DMF, Chem-lab), diethyl ether (Chem-lab), dichloromethane (CH_2Cl_2, Chem-lab), toluene (Chem-lab) and phosphate buffer solution (PBS, Aldrich) were

Table 3 DLS data for the doxorubicin loaded self-assembled micelles and cross-linked micelles of PEO-*b*-poly(BP) and PEO-*b*-poly(BP-*co*-iBP) in water

			Self-assembled micelles			Cross-linked micelles		
	Samples	HLB[a]	D_h[b] (nm)	PDI[c]	LC[d] (%)	D_h[b] (nm)	PDI[c]	LC[d] (%)
A	PEO_{110}-*b*-poly(BP_3-*co*-iBP_7)	15.1	120 ± 2	0.173	0.76 ± 0.01	147 ± 3	0.177	1.44 ± 0.03
B	PEO_{110}-*b*-poly(BP_5-*co*-iBP_5)	15.1	110 ± 4	0.209	0.93 ± 0.03	113 ± 11	0.509	1.37 ± 0.02
C	PEO_{110}-*b*-poly(BP_5-*co*-iBP_2)	16.4	110 ± 5	0.268	0.82 ± 0.02	173 ± 7	0.273	1.21 ± 0.03
D	PEO_{110}-*b*-poly(BP_7)	16.4	100 ± 4	0.273	1.45 ± 0.04	92 ± 10	0.568	1.26 ± 0.03

[a] HLB calculated by the Griffin equation 20 × [1 − M_n,hydrophobic block/M_n,total]. [b] Apparent hydrodynamic diameter measured by DLS. [c] Polydispersity index determined by DLS. [d] Loading content determined by UV spectroscopy.

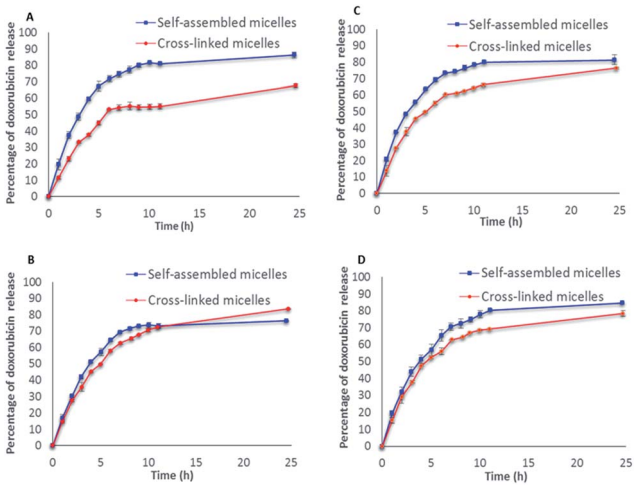


Fig. 7 Release profiles of doxorubicin from (A) PEO_{110}-*b*-poly(BP_3-*co*-iBP_7), (B) PEO_{110}-*b*-poly(BP_5-*co*-iBP_5), (C) PEO_{110}-*b*-poly(BP_5-*co*-iBP_2) and (D) PEO_{110}-*b*-polyBP_7 self-assembled and cross-linked micelles in PBS at room temperature.

used as received. The solvents used for the polymerization were passed beforehand over molecular sieves in order to eliminate water. 1,8-Diazobicyclo[5.4.0]undec-7-ene (DBU) (≥99%, Aldrich) were dried over calcium hydride at room temperature, followed by distillation under reduced pressure just before use. Thiourea (TU) was synthesized according to a method described elsewhere[27] and dried overnight under vacuum just before use. Nanopure water (18 MΩ cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. (St. Charles, MO). Dialysis membrane (Spectra/Por® 7) was purchased from Spectrum-laboratory.

Methods

Synthesis of 2-butenoxy-2-oxo-1,3,2 dioxaphospholane (BP). 5.3 mL of 3-buten-ol (55 mmol) and 8.3 mL of triethylamine (55 mmol) were dissolved in 50 mL of anhydrous THF in a flame dried glass reactor of 250 mL under nitrogen at 0 °C. 5.2 mL of 2-chloro-1,3,2-dioxaphospholane 2-oxide (61 mmol) were dissolved in 50 mL of anhydrous THF and added dropwise to the previous solution under stirring over a period of 3 h. After 1 h of stirring at room temperature, triethylamine hydrochloride was filtered out, and the solvent was evaporated under reduced pressure. The final product was purified by fractionated distillation under reduced pressure (bp = 75 °C, 0.1 Torr) and kept at −20 °C under nitrogen.

^{1}H NMR ($CDCl_3$) 5.8 ppm (m, 1H, CH_2=CH–CH_2), 5.1 ppm (m, 2H, CH_2=CH–CH_2), 4.4 ppm (m, 4H, O–CH_2–CH_2–O), 4.1 ppm (m, 2H, –O–CH_2–CH_2), 2.4 ppm (m, 2H, CH_2–CH_2–CH=CH_2).

^{31}P NMR ($CDCl_3$): 17.44 ppm.

Synthesis of 2-isobutoxy-2-oxo-1,3,2-dioxaphospholane (iBP). 5.1 mL of 2-methyl-1-propanol (55 mmol) and 8.3 mL of triethylamine (55 mmol) were dissolved in 50 mL of anhydrous THF in a flame dried glass reactor of 250 mL under nitrogen at 0 °C. 5.2 mL of 2-chloro-1,3,2-dioxaphospholane 2-oxide (61 mmol) were dissolved in 50 mL of anhydrous THF and added dropwise to the previous solution under stirring over a period of 3 h. After 1 h of stirring at room temperature, triethylamine hydrochloride was filtered out, and the solvent was evaporated under reduced pressure. The final product was purified by fractionated distillation under reduced pressure (bp = 75 °C, 0.1 Torr) and kept at −20 °C under nitrogen.

^{1}H NMR ($CDCl_3$): 4.4 ppm (m, 4H, O–CH_2–CH_2–O), 3.9 ppm (m, 2H, O–CH_2–CH), 1.9 ppm (m, 1H, O–CH_2–CH), 1.1 ppm (m, 6H, CH–$(CH_3)_2$).

^{31}P NMR ($CDCl_3$): 17.52 ppm.

Synthesis of PEO-*b*-poly(BP). 0.462 g of TU (1.25 mmol) was placed in a round bottom glass reactor and dried by three azeotropic distillations with anhydrous toluene. 1 g of BP (5.5 mmol) and 2.5 g of MPEO–OH (M_n = 5000 g mol^{-1}, 0.50 mmol of hydroxyl group) were introduced in a second glass reactor under inert atmosphere, dried by three azeotropic distillations with anhydrous toluene, solubilized in anhydrous CH_2Cl_2 (5 mL) before to be transferred into the reactor containing the TU. 0.38 mL of freshly distilled DBU (2.5 mmol) was finally added to the solution ($[BP]_0/[MPEO–OH]_0/[DBU]_0/[TU]_0$ = 12/1/5/2.5). The reaction medium was stirred at 0 °C for 20 minutes. After evaporation of the solvent under vacuum, the copolymer was purified by precipitation in cold diethylether. The collected amphiphilic copolymer was dissolved in methanol and dialyzed against methanol overnight in order to remove DBU and TU residues. After evaporation of methanol under vacuum, PEO-*b*-polyBP block copolymer was collected and characterized by SEC and NMR.

^{1}H NMR ($CDCl_3$) 5.7 ppm (m, 7H, O–CH_2–CH_2–CH=CH_2), 5.1 ppm (m, 14H, O–CH_2–CH_2–CH=CH_2), 4.3 ppm (m, 28H, P–O–CH_2–CH_2–O), 4.1 ppm (m, 14H, O–CH_2–CH_2–CH=CH_2), 3.6 ppm (m, 440H, O–CH_2–CH_2–O (PEO)), 3.4 ppm (s, 3H, CH_2–CH_2–O–CH_3), 2.5 ppm (m, 14H, O–CH_2–CH_2–CH=CH_2).

^{31}P NMR ($CDCl_3$): −1.37 ppm.

M_n ^{1}H NMR = 1200 g mol^{-1}, M_n SEC = 4900 g mol^{-1}, $Đ$ = 1.2.

Synthesis of PEO-*b*-poly(BP-*co*-iBP). A typical procedure for the copolymerization is as follows: 0.463 g of TU (1.25 mmol) was placed in a glass reactor and dried by three azeotropic distillations with anhydrous toluene. 0.5 g of iBP (3 mmol), 0.5 g of BP (3 mmol) and 2.5 g of MPEO–OH (M_n = 5000 g mol^{-1}, 0.50 mmol) were introduced in a second glass reactor under inert atmosphere, dried by three azeotropic distillations with anhydrous toluene, solubilized in anhydrous CH_2Cl_2 (5 mL) and transferred into the glass reactor containing TU. 0.38 mL of freshly distilled DBU (2.5 mmol) was then added to the solution ($[iBP]_0/[BP]_0/[MPEO–OH]_0/[DBU]_0/[TU]_0$ = 6/6/1/5/2.5). The reaction medium was stirred at 0 °C for 20 minutes. The monomers conversion was evaluated to 94% based on the ^{31}P NMR spectrum (before purification). After evaporation of the solvent under vacuum, the obtained copolymer was purified by precipitation in cold diethylether. The polymer collected was dissolved in methanol and dialyzed against methanol overnight in order to remove DBU and TU residues. After evaporation of methanol under vacuum, PEO-*b*-poly(iBP-*co*-BP) copolymer was characterized by SEC and NMR.

^{1}H NMR ($CDCl_3$): 5.6 ppm (m, 5H, O–CH_2–CH_2–CH=CH_2), 5.1 ppm (m, 10H, O–CH_2–CH_2–CH=CH_2), 4.4–4.0 ppm (m, 20H, O–CH_2–CH_2–O, 10H, O–CH_2–CH_2–C), 3.9 ppm (m, 10H, O–CH_2–CH–$(CH_3)_2$), 3.6 ppm (m, 440H, O–CH_2–CH_2–O), 3.4 ppm (s, 3H, CH_2–CH_2–O–CH_3), 2.4 ppm (m, 10H, O–CH_2–CH_2–CH=CH_2), 2 ppm (m, 5H, O–CH_2–CH–$(CH_3)_2$), 1 ppm (m, 30H, O–CH_2–CH–$(CH_3)_2$).

^{31}P NMR ($CDCl_3$): 1.37 ppm.

M_n ^{1}H NMR = 1800 g mol^{-1}, M_n SEC = 10 000 g mol^{-1}, $Đ$ = 1.1.

Characterization

Size exclusion chromatography (SEC) was carried out in THF at 45 °C at a flow rate of 1 mL min^{-1} with a with Viscotek 305 TDA liquid chromatograph. The PL gel, 5 μm, (10^4 Å, 10^3 Å and 100 Å) columns were calibrated with polystyrene standards. ^{1}H and ^{31}P nuclear magnetic resonance (NMR) analyses were performed on a Bruker Advance 400 spectrometer (MHz) in deuterated chloroform ($CDCl_3$) and deuterium oxide (D_2O) at 25 °C in the FT mode. Dynamic light scattering (DLS) measurements were performed using a Beckman Coulter Delsa Nano C Particle analyzer and the data were treated by the Delsa Nano UI 2.21 software. The average size distribution of aqueous micellar solutions was determined based on the CONTIN method. All the measurements were carried out at 25 °C at a measuring angle of 165°. Aqueous micellar solutions were filtered with a microfilter having an average pore size of 0.45 μm. All DLS measurements were repeated five times in order to check their reproducibility. The samples for transmission electron microscopy (TEM) were prepared by slow evaporation of the solutions after DLS analysis on a formvar-coated copper grid. The excess solution was removed with a filter paper. The samples were analyzed with a Philips CM100 microscope equipped with an Olympus camera and transferred to a computer equipped with the Megaview system. UV measurements were performed on a Hitachi U-3300 UV-Visible spectrophotometer.

Micellization

Micelles of the amphiphilic PEO-*b*-poly(BP) and PEO-*b*-poly(iBP-*co*-BP) block copolymers were prepared by a co-solvent process. Typically, 20 mL of Milli-Q water were added to 5 mL of copolymer solution (100 mg of copolymer in 5 mL of DMF) under vigorous stirring for 2 h. Micelles were purified by dialysis overnight against 1 L of water with a cellulose dialysis membrane (MWCO = 2 kDa).

Crosslinking procedure

Under vigorous stirring, 20 mL of Milli-Q water were added to 5 mL of copolymer solution (200 mg of copolymer in 5 mL of DMF) containing 1 weight% of benzophenone and stirred for 2 h. Micelles were purified by dialysis overnight against 1 L of water with a cellulose dialysis membrane. The crosslinking reaction proceeded under UV irradiation (OmniCure Series 2000, 200 W, 365 nm) of a degassed micellar solution for 2 h. After reaction, the cross-linked micelles were collected by freeze-drying before to be dispersed into DMF.

Preparation of doxorubicin loaded micelles

Nanoprecipitation. 50 mg of copolymer and 5 mg of doxorubicin were dissolved into 5 mL of DMF before adding 1.4 mL of NEt_3. 20 mL of Milli-Q water was then added dropwise to the DMF solution under vigorous stirring. After complete addition, the solution was stirred for 2 hours before being dialyzed (MWCO = 3.5 kDa) against Milli-Q water for 24 hours. Water was changed after 2, 4, 6 and 22 hours of dialysis. After filtration on 0.45 μm acrodisc® filter, the total volume of the solution was precisely measured and 10 mL of the solution was equally spread into 2 vials before being lyophilized. 5 mL of DMF was then added into one of the vial and the solution was analysed with by UV spectrometry at 485 nm in order to measure the weight of doxorubicin in solution according to a calibration curve. The loading content (LC) was calculated according the following equation.

$$\text{LC (\%)} = \frac{\text{weight of loaded doxorubicin (mg)}}{\text{weight of copolymer (mg)}} \times 100$$

Impregnation. 50 mg of cross-linked micelles were suspended into a 5 mL DMF solution containing 5 mg of doxorubicin and 1.4 mL of NEt_3. The suspension was stirred for 30 min before adding 20 mL of Milli-Q water dropwise under vigorous stirring. After complete addition, the solution was dialyzed (MWCO = 3.5 kDa) against Milli-Q water for 24 hours. Water was changed after 2, 4, 6 and 22 hours of dialysis. After filtration on 0.45 μm acrodisc® filter, the total volume of the solution was precisely measured and 10 mL of the solution was spread into 2 vials before being lyophilized. 5 mL of DMF was then added into one of the vial and the solution was analysed with by UV spectrometry at 485 nm in order to measure the loaded doxorubicin according to a calibration curve.

Drug release procedure. 5 mL of Milli-Q water was added into the second vial collected after lyophilization of the drug-loaded micelle solution. The solution was then transferred into a dialysis membrane (MWCO = 3.5 kDa) and placed into 200 mL of PBS solution. At predetermined frequencies, 3 mL of the solution were taken out of the dialysis membrane in order to be analysed by UV spectroscopy at 485 nm. After the analysis, the solution was transferred back into the dialysis membrane.

Conclusions

PEO-*b*-polyphosphate amphiphilic block copolymers bearing unsaturations along the polyphosphate block were successfully synthesized using metal-free strategy. These copolymers were able to self-assemble into spherical micelles when placed in water. In order to demonstrate the potential of these micelles as drug nanocarriers, doxorubicin, a drug used in cancer therapy, was loaded in the micelles core. Interestingly, we demonstrated that the doxorubicin loading increased with the number of double bonds on the polyphosphate block of non-cross-linked micelles prepared by nanoprecipitation method. Besides, these unsaturations have been successfully used to cross-link the micelles core improving their stability. This diblock

amphiphilic copolymer bearing pendant unsaturations appears thus particularly promising candidate to build micellar drug delivery systems for intravenous injection.

Acknowledgements

The authors are grateful for the European financial support in the frame of the NanoFar program, an Erasmus Mundus Joint Doctorate (EMJD) program in nanomedicine and pharmaceutical innovation. They also thank the "National Funds for Scientific Research" (FRS-FNRS) and the Belgian Science Policy for financial support in the frame of the Interuniversity Attraction Poles Program (P7/05)-Functional Supramolecular Systems (FS2) for supporting this research. The authors thank the Interreg Euregio MeuseRhin IV-A consortium BioMIMedics.

Notes and references

1 J. Hu, J. He, D. Cao, M. Zhang and P. Ni, *Polym. Chem.*, 2015, **6**, 3205–3216.
2 Y. Li, Y. Shen, S. Wang, D. Zhu, B. Du and J. Jiang, *RSC Adv.*, 2015, **5**, 30380–30388.
3 S.-Y. Lee, S.-J. Choi, S.-J. Seo and H.-W. Kim, *RSC Adv.*, 2014, **4**, 57702–57708.
4 S. Cajot, N. Lautram, C. Passirani and C. Jérôme, *J. Controlled Release*, 2011, **152**, 30–36.
5 T. K. Dash and V. B. Konkimalla, *J. Controlled Release*, 2012, **158**, 15–33.
6 S. Cajot, R. Riva, L. Billiet, F. Du Prez, M. Alexandre, P. Lecomte and C. Jérôme, *Macromol. Symp.*, 2011, **309–310**, 111–122.
7 Y. Ohya, A. Takahashi and K. Nagahama, *Adv. Polym. Sci.*, 2012, **247**, 65–114.
8 L. I. N. Sun, L. Shen, M. Zhu, C. Dong and Y. E. N. Wei, *J. Polym. Sci., Part A: Polym. Chem.*, 2010, **48**, 4583–4593.
9 Y. Yu, J. Zou and C. Cheng, *Polym. Chem.*, 2014, **5**, 5854–5872.
10 B. Surnar, P. P. Subash and M. Jayakannan, *Z. Anorg. Allg. Chem.*, 2014, **640**, 1119–1126.
11 X. J. Loh, *J. Appl. Polym. Sci.*, 2013, **127**, 2046–2056.
12 Z. Song, B. Shi, J. Ding, X. Zhuang, X. Zhang, C. Fu and X. Chen, *Sci. China: Chem.*, 2015, **58**, 1159–1168.
13 K. Dong, W. Xu, C. Y. You, J. F. Xing, Y. J. Zhang, Y. Gao and K. Wang, *Int. J. Polym. Mater.*, 2013, **62**, 726–732.
14 F. Zhang, S. Zhang, S. F. Pollack, R. Li, A. M. Gonzalez, J. Fan, J. Zou, S. E. Leininger, A. Pavía-Sanders, R. Johnson, L. D. Nelson, J. E. Raymond, M. Elsabahy, D. M. P. Hughes, M. W. Lenox, T. P. Gustafson and K. L. Wooley, *J. Am. Chem. Soc.*, 2015, **137**, 2056–2066.
15 S. Zhang, J. Zou, M. Elsabahy, A. Karwa, A. Li, D. A. Moore, R. B. Dorshow and K. L. Wooley, *Chem. Sci.*, 2013, **4**, 2122–2126.
16 F. Zhang, J. A. Smolen, S. Zhang, R. Li, P. N. Shah, S. Cho, H. Wang, J. E. Raymond, C. L. Cannon and K. L. Wooley, *Nanoscale*, 2015, **7**, 2265–2270.
17 M.-H. Xiong, Y. Bao, X.-Z. Yang, Y.-C. Wang, B. Sun and J. Wang, *J. Am. Chem. Soc.*, 2012, **134**, 4355–4362.
18 Y.-C. Wang, X.-Q. Liu, T.-M. Sun, M.-H. Xiong and J. Wang, *J. Controlled Release*, 2008, **128**, 32–40.
19 Y. Li, F. Wang, T. Sun, J. Du, X. Yang and J. Wang, *Sci. China: Chem.*, 2014, **57**, 579–585.
20 Z. Zhao, J. Wang, H. Mao and K. W. Leong, *Adv. Drug Delivery Rev.*, 2003, **55**, 483–499.
21 Y.-C. Wang, Y.-Y. Yuan, J.-Z. Du, X.-Z. Yang and J. Wang, *Macromol. Biosci.*, 2009, **9**, 1154–1164.
22 J. Wen, H.-Q. Mao, W. Li, K. Y. Lin and K. W. Leong, *J. Pharm. Sci.*, 2004, **93**, 2142–2157.
23 S. Monge, B. Canniccioni, A. Graillot and J.-J. Robin, *Biomacromolecules*, 2011, **12**, 1973–1982.
24 Q. Li, J. Wang, S. Shahani, D. D. N. Sun, B. Sharma, J. H. Elisseeff and K. W. Leong, *Biomaterials*, 2006, **27**, 1027–1034.
25 J.-J. Qiu, C.-M. Liu, F. Hu, X.-D. Guo and Q.-X. Zheng, *J. Appl. Polym. Sci.*, 2006, **102**, 3095–3101.
26 B. Clément, B. Grignard, L. Koole, C. Jérôme and P. Lecomte, *Macromolecules*, 2012, **45**, 4476–4486.
27 J. Liu, Y. Pang, W. Huang, X. Zhai, X. Zhu, Y. Zhou and D. Yan, *Macromolecules*, 2010, **43**, 8416–8423.
28 W.-J. Song, J.-Z. Du, N.-J. Liu, S. Dou, J. Cheng and J. Wang, *Macromolecules*, 2008, **41**, 6935–6941.
29 X. Liu, P. Ni, J. He and M. Zhang, *Macromolecules*, 2010, **43**, 4771–4781.
30 X. Yang, Y. Wang, L. Tang, H. A. I. Xia and J. U. N. Wang, *J. Polym. Sci., Part A: Polym. Chem.*, 2008, **46**, 6425–6434.
31 Z. Ergul Yilmaz, A. Debuigne, B. Calvignac, F. Boury and C. Jerome, *J. Mater. Chem. B*, 2015, **3**, 7227–7236.
32 S. Zhang, J. Zou, F. Zhang, M. Elsabahy, S. E. Felder, J. Zhu, D. J. Pochan and K. L. Wooley, *J. Am. Chem. Soc.*, 2012, **134**, 18467–18474.
33 S. Vanslambrouck, B. Clément, R. Riva, L. H. Koole, D. G. M. Molin, G. Broze, P. Lecomte and C. Jérôme, *RSC Adv.*, 2015, **5**, 27330–27337.
34 J. Xu, Q. Zhao, Y. Jin and L. Qiu, *Nanomedicine*, 2014, **10**, 349–358.
35 N.-V. Cuong, J.-L. Jiang, Y.-L. Li, J.-R. Chen, S.-C. Jwo and M.-F. Hsieh, *Cancers*, 2010, **3**, 61–78.
36 Y. Iwasaki and K. Akiyoshi, *Macromolecules*, 2004, **37**, 7637–7642.
37 S. Cammas, T. Matsumoto, T. Okano, Y. Sakurai and K. Kataoka, *Mater. Sci. Eng., C*, 1997, **4**, 241–247.

