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ABSTRACT

Thermoresponsive polymers, undergoing a reversible chemical or physical change using temperature as stimulus, attract increasing interest in particular as adaptable biomaterials. Except for zwitterionic polymers, fully charged polymers require the presence of specific ions to exhibit an upper critical solution temperature (UCST) in water. Herein, we report the discovery of an UCST in pure water for fully cationic comb polymers based on oligoarginine pendent grafts. These polymers were prepared using an original strategy based on solid-phase peptide synthesis of pentaarginine methacrylate-based macromonomer and its polymerization through reversible addition-fragmentation chain transfer. Despite their cationic nature, guanidinium groups from the arginine have the ability to self-associate at low temperature through hydrophobic interactions into stacked pair configuration defying the expected Coulomb interactions. These results pave the way to biomedical applications such as antimicrobial materials and drug delivery systems through the tuning of the polymer structure.

KEYWORDS. Thermoresponsive polymer, arginine, upper critical solution temperature, Coulomb-defying interaction, guanidinium self-association.

1. INTRODUCTION

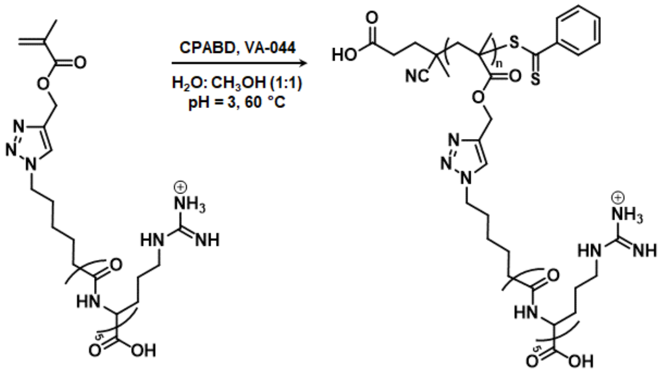
Polymers with lower critical solution temperature (LCST) or upper critical solution temperature (UCST) undergo drastic changes in solubility with phase separation on heating or cooling, respectively [1, 2]. While water-soluble polymers with a LCST have been widely studied [3-7], those with an UCST are more rare despite the recent increasing interest they received [8, 9]. UCST polymers find applications in biomedicine [10] such as drug delivery [11-13], catalysis [14] and 3D-printed scaffolds [15]. This UCST behavior is promoted by either hydrogen bonding or Coulomb interactions [8, 9]. Non-ionic polymers with hydrogen bonding-induced UCST behavior, *e.g.* poly(*N*-acryloyl glycinamide) [16, 17] and poly(acrylamide-*co*-acrylonitrile) [18], have attracted much attention due to their relative insensitivity to pH and ionic strength [9]. Copolymerization of hydrogen bonding and charged monomers yields to pH-sensitive UCST materials through a delicate balance between electrostatic interactions and hydrogen bonding. Copolymerization of ureido- and allyl-based acrylates allows the rational design of copolymers with a targeted UCST depending on the hydrophobic parameter [19]. Seuring and Agarwal provide guidelines for the design of such copolymers indicating that they should "possess strong hydrogen donors and acceptors, contain no or very few ionic groups, be hydrolytically stable and consist of chains with homogeneous copolymer composition" [9]. Fully charged polymers are not expected to exhibit UCST behavior in water between 0 and 100 °C. Yet, zwitterionic polymers possess this phase transition due to attractive Coulomb interactions between groups of opposite charges [9, 20, 21]. UCST behavior of fully charged polyelectrolytes [22] is obtained by adding specific counterions, *e.g.* hydrophobic [23-25] or multivalent [26, 27] ions or for poly(ionic liquids) [28] with chloride counterions.

Amino acid-containing polymers are of particular interest for biomedical applications, *e.g.* polymers with arginine residues are used for their antimicrobial activity [29] and their ability to promote drug vectorization [30]. Sequence heuristic study shows that resilin-like polypeptides enriched in arginine residues exhibit an UCST behavior under physiological conditions [31]. Fully charged cyanophycin, a biosynthetic zwitterionic polymer, is a poly(L-aspartic acid) bearing arginine and lysine showing an increase of UCST as the arginine/lysine ratio increases [32, 33]. Only a few UCST polymers containing α-amino acid residues have been developed so far [34], either by modification of polypeptides or (co)polymerization of monomers bearing only one amino acid on their side chains. These polymers can be classified according to the interactions triggering the UCST mentioned previously, *i.e.* hydrogen bonding and Coulomb interactions. Non-ionic vinyl polymers bearing one amino acid residue have been synthesized to study their thermoresponsiveness in water [35]. Among those, only alanine-based polymers exhibit an UCST at pH 2.0 due to the protonation of the terminal carboxylic acid group. To introduce hydrogen bonds, cationic poly(L-ornithine)s have been partially modified on their side chains by ureido groups revealing an UCST behavior in physiological media. Recently, the synthesis and reversible addition-fragmentation chain transfer (RAFT) polymerization of serine-based acrylate [36] and tryptophan-based styrene [37] monomers has been described affording zwitterionic polymers exhibiting an UCST only at low pH. Water-soluble polypeptides bearing imidazolium [38] and pyridinium [39] groups have been polymerized to obtain fully charged polymers with an UCST behavior in the presence of BF_4^- counterions.

We describe here the first fully charged polyelectrolyte with oligopeptide side chains exhibiting a reversible UCST behavior in pure water without addition of any specific counterions. This polymer is a cationic comb polymer with oligoarginine pendent chains synthesized by RAFT polymerization of methacrylate-*g*-pentaarginine (**MA-R5**). The preparation of methacrylate-based monomers bearing a peptide sequence is not trivial requiring the development of synthesis strategies to minimize Michael side reactions affecting the purity of the macromonomers synthesized. We propose an original strategy combining solid-phase peptide synthesis and copper-assisted alkyne-azide cycloaddition.

2. RESULTS AND DISCUSSION

Methacrylate-*g*-pentaarginine protected with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) groups was prepared by iterative addition of Fmoc-Arg(Pbf)-OH onto a 2-chlorotrityl chloride resin using HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyl uronium hexafluorophosphate) as coupling agent and HOBt (1-hydroxy-benzotriazole hydrate) as racemization inhibitor in DMF. The on-resin protected pentaarginine was amidated with 6-azido-hexanoic acid using the same reaction conditions followed by a copper-assisted alkyne-azide cycloaddition with propargyl methacrylate in the presence of copper(I) bromide and *N*,*N*,*N*′,*N*′′,*N*′′-pentamethyldiethylenetriamine. The protected macromonomer was cleaved from the resin using a solution of 2,2,2-trifluoroethanol in dichloromethane. RAFT polymerizations of this macromonomer were unsuccessful despite the different polymerization conditions attempted. Polymerizations were then conducted on the deprotected macromonomer **MA-R5** that was obtained by changing the cleavage conditions using a solution of trifluoroacetic acid (TFA) and isolated by precipitation in cold acetone in 36% yield. The structure of the macromonomer and its purity (>90%) were confirmed by NMR spectroscopies, mass spectrometry and liquid chromatography-mass spectrometry (Fig. S1, ESI). The optimal polymerization conditions of **MA-R5** were identified and performed in a solution of water and methanol (1/1 v/v) at pH 3 for 24 h at 60 °C using 4-(cyanopentanoic acid)-4-dithiobenzoate (CPABD) as chain transfer agent and 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) as initiator (Scheme 1) aiming a degree of polymerization (DP_n) of 10, 20, 50, and 100. Poly(methacrylate-*g*-pentaarginine) **P(MA-R5)$_n$** (where n is the degree of polymerization calculated from monomer conversion) was isolated by precipitation from the polymerization medium on cooling. These polymers were insoluble in organic solvents and in water at 25 °C, (except for **P(MA-R5)$_3$** corresponding to $DP_{n,target}$ = 10 showing visual solubility in water at room temperature). Their degree of polymerization (Table 1) was determined by ^{1}H NMR spectroscopy at 65 °C either via the conversion of the polymerization ($DP_{n,th}$) assuming the "living" character of RAFT polymerization or by analyzing the integrals of the terminal group (7.83 ppm, aromatic proton of RAFT agent) and the repeat units (1.09-1.20 ppm, methyl of the polymethacrylate backbone) of the purified polymer ($DP_{n,exp}$, Fig. S2, ESI). Due to the high molecular weight of **MA-R5** (1061.6 g mol^{-1}), the determination of $DP_{n,exp}$ was not possible when targeting high $DP_{n,target}$. **P(MA-R5)$_n$** with $DP_{n,target}$ of 10 and 20 were however soluble in a solution of acetonitrile/water (20/80) with 0.1 M NaCl and 0.1% TFA. Their number-average molecular weights were determined by size-exclusion chromatography (SEC) using this solution as eluent on hydrophilic silica-based SEC columns as 2,700 (Đ = 1.69, DP_n = 2.5) and 11,400 (Đ = 1.51, DP_n = 10) respectively (Fig. S3, ESI). However, even though **P(MA-R5)$_{29}$** with a $DP_{n,target}$ of 50 seemed visually soluble in the SEC eluent the molecular weight observed was above 300,000 g mol^{-1} suggesting its aggregation making the determination of the molecular weight of **P(MA-R5)$_n$** of high degree of polymerization difficult. Determination of the molecular weight of the polymers by matrix-assisted laser desorption/ionization mass spectrometry was unsuccessful as no peak was observed.



Scheme 1. Synthesis of poly(methacrylate-*g*-pentaarginine) **P(MA-R5)$_n$** based on solid phase peptide synthesis of methacrylate-*g*-pentaarginine (**MA-R5**) and its polymerization under RAFT conditions.

Table 1. Chemical characteristics of **P(MA-R5)$_n$**.

Sample	DP_{target}[a]	Conversion[b] [%]	$DP_{n,th}$[c]	$DP_{n,exp}$[d]	N_R[e]	M_n[f] [g mol^{-1}]	Đ[f]
P(MA-R5)$_3$	10	25	3	3	15	2700	1.69
P(MA-R5)$_{10}$	20	52	10	7	50	11400	1.51
P(MA-R5)$_{29}$	50	57	29	n.d.	145	n.d.	n.d.
P(MA-R5)$_{50}$	100	50	50	n.d.	250	n.d.	n.d.

[a] Targeted degree of polymerization if 100 % monomer conversion was reached. [b] Determined by ^{1}H NMR spectroscopy. [c] Degree of polymerization obtained from monomer conversion. [d] Degree of polymerization determined by analysis of the terminal group on the ^{1}H NMR spectrum. [e] Number of arginine residues per polymer chain calculated from $DP_{n,th}$. [f] Number-average molecular weight (M_n) and dispersity (Đ) determined by SEC measurement in 20/80 acetonitrile/water with 0.1 M NaCl and 0.1% TFA on hydrophilic silica-based SEC columns.

Surprisingly, the dissolution of **P(MA-R5)$_n$** in pure water led to a cloudy solution at 20 °C becoming clear at 85 °C (Fig. 1a inset photos). The UCST behavior of these polymers was investigated in water by turbidimetry at 600 nm. Polymer solutions, prepared in pure water at 1, 2 and 5 mg mL^{-1}, underwent a heating and cooling cycle between 10 and 85 °C at 1 °C min^{-1}. Although **MA-R5** had no UCST (Fig. S4), **P(MA-R5)$_{10}$** displayed sharp UCST-type transitions with a narrow hysteresis during heating and cooling cycles for each concentration (Fig. 1a). Cloud (T_{CP}) and clearing (T_{CL}) points were determined at the inflection point[40] on the cooling and heating curves, respectively. T_{CP} increased from 31 to 51 °C (32 to 52 °C for T_{CL}) with the polymer concentration (Table 2). Interestingly at 2 mg mL^{-1}, **P(MA-R5)$_{10}$** had a phase transition temperature at 36 °C on heating, near the body temperature. **P(MA-R5)$_3$**, **P(MA-R5)$_{29}$** and **P(MA-R5)$_{50}$** exhibited a UCST behavior with a less sharp transition (Fig. S4, ESI and Table 2). Even with a degree of polymerization of 3, a phase transition was observed between 23 and 17 °C due to the presence of fifteen arginine residues per polymer chains. The same dependency of T_{CP} on the polymer concentration was observed in the range of 10 to 85 °C with an absence of UCST behavior at 1 mg mL^{-1}. T_{CP} was also dependent on the molecular weight of the polymers (Fig. 1b). At 5 mg mL^{-1} in **P(MA-R5)$_n$**, T_{CP} increased from 22 °C for $DP_{n,target}$ = 10 up to a plateau around 87 °C for $DP_{n,target}$ = 50. This evolution of T_{CP} could be attributed to the influence of the end groups for the shortest polymer chains that became insignificant above a certain degree of polymerization. Although arginine residues were protonated, **P(MA-R5)$_n$** possessed an UCST behavior even for a high number of arginine residues (at least up to 250 cationic charges per chain).

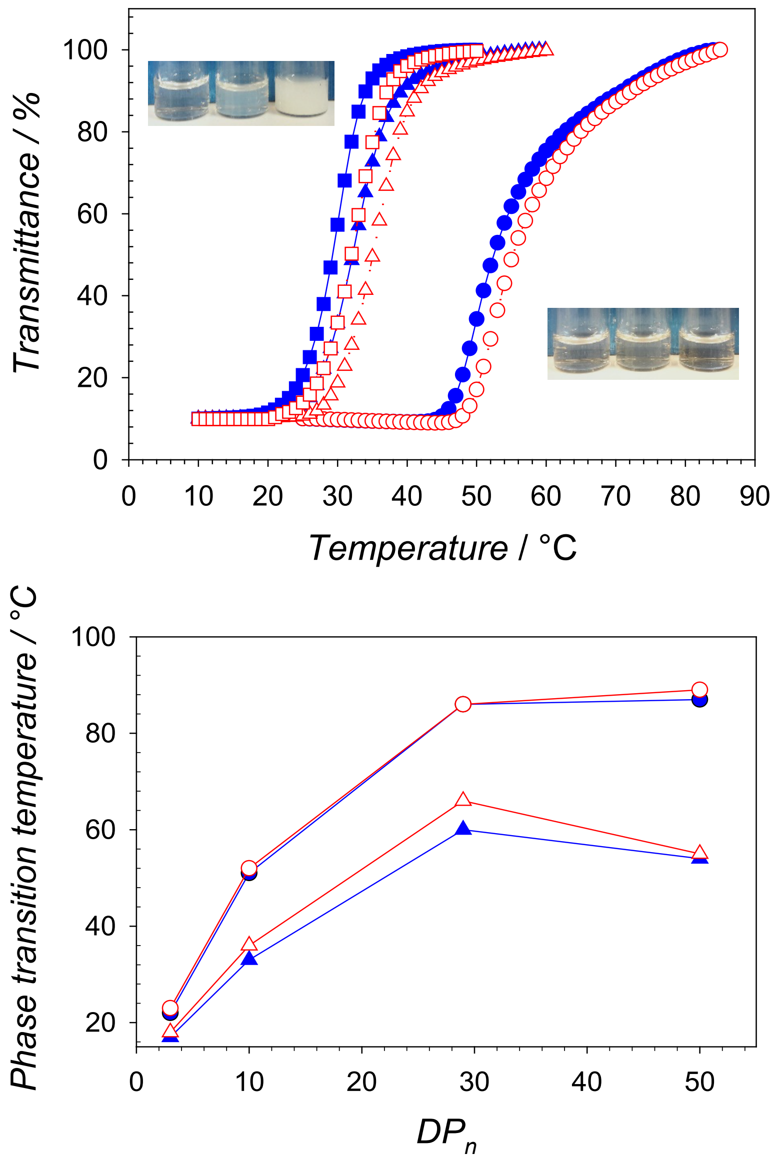


Fig. 1. UCST characterization of **P(MA-R5)$_n$** aqueous solutions at 1 (squares), 2 (triangles) and 5 (circles) mg mL^{-1} by (a) turbidimetry on heating (open red symbols) and cooling (filled blue symbols): transmittance versus temperature curves for **P(MA-R5)$_{10}$** (inset photos of solutions at 20 °C and 85 °C) and (b) evolution of the phase transition temperature as a function of the degree of polymerization (DP_n); as the phase

transition at 1 mg mL^{-1} was only observed for **P(MA-R5)$_{10}$**, the data points at this concentration were not depicted.

Table 2. Thermoresponsive properties of **MA-R5** and **P(MA-R5)$_n$**.

Sample	Concentration [mg·mL^{-1}]	T_{CP}[a] [°C]	T_{CL}[b] [°C]	D_h[c] [nm] (PDI)
MA-R5	2	none	none	-
P(MA-R5)$_3$	5	22	23	-
	2	17	18	168 ± 48 (0.06)
	1	none	none	-
P(MA-R5)$_{10}$	5	51	52	-
	2	33	36	118 ± 34 (0.07)
	1	31	32	-
P(MA-R5)$_{29}$	5	86	86	-
	2	60	66	154 ± 72 (0.30)
	1	none	none	
P(MA-R5)$_{50}$	5	87	89	-
	2	54	55	115 ± 37 (0.09)
	1	none	none	-

Cloud (T_{CP}) and clearing (T_{CL}) temperatures at the inflexion point upon [a] cooling and [b] heating, determined by turbidimetry in water (600 nm, 1°C min^{-1}). Hydrodynamic diameters (D_h) and polydispersities (PDI) determined by dynamic light scattering in water at 25 °C.

Dynamic light scattering of the polymer solution of **P(MA-R5)$_n$** at a concentration of 2 mg mL^{-1} revealed the formation of aggregates at 25 °C. **P(MA-R5)$_3$** and **P(MA-R5)$_{10}$** are mainly individual polymer chains at 85 °C (Fig.2a, Table 2 and Fig. S5, ESI) supporting the UCST behavior. **P(MA-R5)$_{10}$** has a hydrodynamic diameter of 118 nm with a positive zeta potential of 32 mV. Characterization of the polymer structures was performed by atomic force microscopy (AFM, Fig. 2 and Fig. S6, ESI). A drop of **P(MA-R5)$_n$** solution was deposited on silicon wafer and the structures were observed in liquid state at room temperature. Polymer aggregates were observed for all samples on the whole surface. **P(MA-R5)$_{10}$** aggregates were visualized having a lateral size of 124 ± 10 nm and around 20 nm in height.

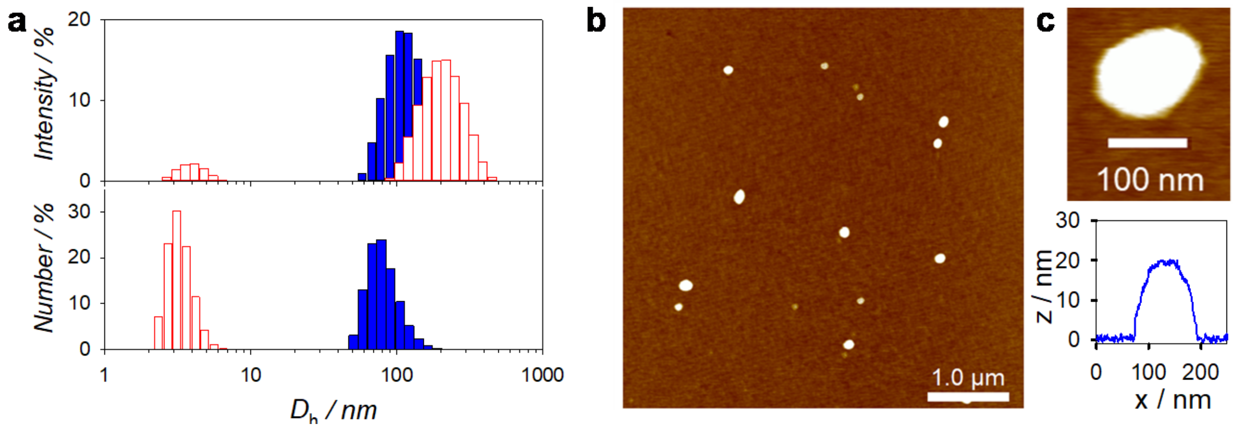


Fig. 2. (a) Dynamic light scattering results showing the intensity (top) and number (bottom) fraction of the hydrodynamic diameter of **P(MA-R5)$_{10}$** in water at 25 °C (filled blue bars) and 85 °C (open red bars) at 2 mg mL^{-1}. It should be noted that the intensity signal is more sensitive to larger objects, thus a strong scattering peak can be observed for a small number of aggregates. (b) AFM height image, obtained in tapping mode and liquid state, of **P(MA-R5)$_{10}$** aggregates (1 mg mL^{-1}) observed at room temperature (z scale of 20 nm) and (c) zoom and cross-section profile of an isolated aggregate.

Arginine is composed of guanidinium (Gdm^+) groups in the side chain. The existence of contact ion pairing of Gdm^+ ions in water was evidenced by several computational studies [41] and confirmed experimentally at high concentration in GdmCl [42]. Recently, Tesei *et al.* have demonstrated the self-association of decaarginine peptides (R10) at low ionic strength by small-angle X-ray scattering and molecular dynamic (MD) simulations. This self-association is explained by the formation of contact ion pairs of Gdm^+ groups [43]. This counterintuitive contact ion pairing is due to Gdm^+ planar structure and specific hydration. This cation interacts strongly with water as hydrogen donor, only in its molecular plane while its two faces remain hydrophobic [44]. The non-occupied hydrophobic faces favor the self-association of Gdm^+ into a stacked pair configuration [42]. To gain more insights on the interaction between **P(MA-R5)$_{10}$** chains at the molecular level, MD simulations were performed on a system composed of four polymer chains with ten repeat units, 200 Cl^- as counterions of arginine residues, and 28 681 H_2O molecules during 1.5 µs at 293.15 K and 358.15 K. The distance between arginine residues was determined via radial distribution functions of Gdm^+-Gdm^+ distance averaged over the last 1.25 µs (Fig. 3a).

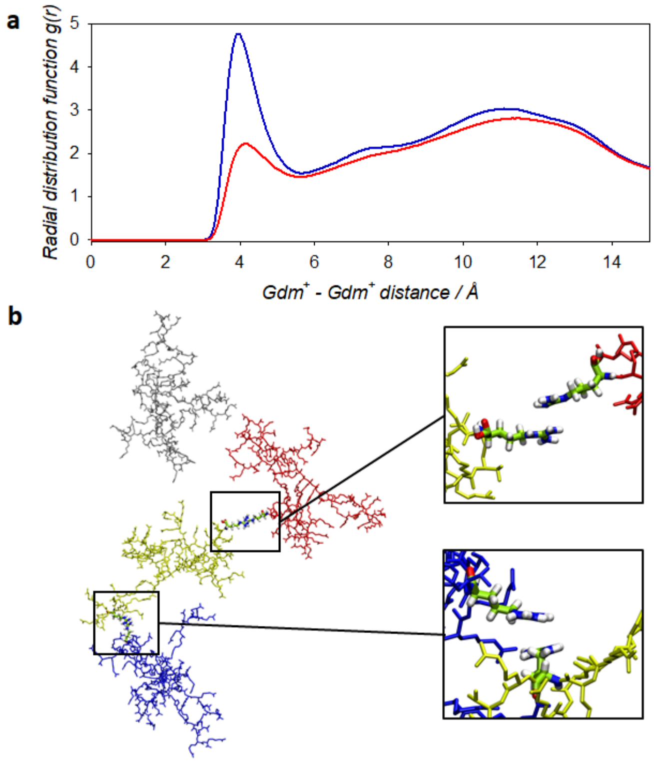


Fig. 3. (a) Radial distribution function of Gdm^+-Gdm^+ distance at 293.15 K (blue) and 358.15 K (red). (b) Snapshot of **P(MA-R5)$_{10}$** trimer formation observed during MD simulations highlighting the Gdm^+-Gdm^+ stacking between two polymer chains.

At both temperatures, a first peak was observed around 4 Å corresponding to the stacking of Gdm^+ groups of the arginine residues [42]. The integration of this peak up to its first minimum at about 5.5 Å indicated a larger number of Gdm^+-Gdm^+ interactions at 293.15 K than at 358.15 K with 0.32 and 0.18, respectively. The majority of the stacking was present on the same **P(MA-R5)$_{10}$** chains, but also observed between different polymer chains. This led to the formation of **P(MA-R5)$_{10}$-P(MA-R5)$_{10}$** dimers and in some cases even trimers (Fig. 3b). The average lifetime of inter-**P(MA-R5)$_{10}$** Gdm^+-Gdm^+ stacking was nearly twice longer at 293.15 K in comparison to 358.15 K with 36 ± 4 and 20 ± 3 ps, respectively. These results indicated a higher stability of **P(MA-R5)$_{10}$** aggregation at lower temperature. Thus, the unexpected UCST behavior in water of **P(MA-R5)$_{10}$** can be explained by the "Coulomb-defying" self-association of Gdm^+. The original structure of **P(MA-R5)$_{10}$** bearing R5 side chains favored the self-association of arginine moieties through hydrophobic interactions in water. By increasing the temperature, the hydrophobic interactions between R5 side chains were likely disrupted leading to the solubilization of the polymer chains.

When **P(MA-R5)$_{10}$** solution was prepared at physiological salt concentration (150 mM NaCl), no UCST behavior was observed (Fig. S8a, ESI). Furthermore, the addition of a chaotropic agent, *i.e.* one equivalent of TFA per Gdm^+ group, led also to completely soluble polymers with no phase transition (Fig. S8b, ESI).

The presence of salts prevented the Gdm^+ stacking. According to recent publications, the pKa of Gdm^+ is reported to be at around 13.5 [45, 46]. At 25 °C, 2 mg mL^{-1} **P(MA-R5)$_{10}$** solution prepared in MilliQ water had a pH of 2.3 with positively charged aggregates with a zeta potential of +32 mV. When the pH was increased to pH 7 (near the physiological pH), a decrease of the cloud (T_{CP}) and clearing (T_{CL}) points was observed from 33 to 31 °C and 36 to 29 °C, respectively (Fig. S9a, ESI). At this pH, the zeta potential of the aggregates decreased to +25 mV. The terminal carboxylic acid group of pentarginine side chains are deprotonated leading to negatively charged COO^- groups which induces the decrease of the overall charge of the aggregates. At pH 12 closed to the pKa of Gdm^+, **P(MA-R5)$_{10}$** solution had no UCST behavior. The zeta potential dropped to +15 mV at pH 12. Near its pKa, some Gdm^+ were deprotonated leading to a decrease of the number of positively charged moieties. The formation of neutral guanidine which is non-planar and has five possible tautomeric forms [47], disturbs the stacking leading to the loss of the phase transition.

3. CONCLUSION

In summary, cationic comb polymers with pentaarginine side chains were prepared by RAFT polymerization from peptide-bearing macromonomer based on methacrylate obtained by solid-phase peptide synthesis and copper-assisted alkyne-azide cycloaddition reaction. These polymers displayed an unexpected UCST behavior in water up to pH 12 promoted by hydrophobic interactions through Gdm^+-Gdm^+ stacking, confirmed by MD simulations. The addition of salt induced the complete disappearance of the UCST behavior of **P(MA-R5)$_{10}$**. This study opens the way for the design of cationic aqueous UCST polymers and their potential uses for biomedical applications, *e.g.* materials possessing antimicrobial properties and drug delivery systems.

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DATA AVAILABILITY

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

APPENDIX A. Supplementary material

Supplementary data to this article can be found online at .

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