pH-Responsive Poly(2-ethylacrylic acid-*co*-alkyl methacrylate) Copolymers as Biomembrane Switches

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ABSTRACT: The intracellular delivery of active biomacromolecules from endosomes into the cytoplasm generally requires a membrane-disrupting agent. Since endosomes have a slightly acidic pH, anionic carboxylated polymers could be potentially useful for this purpose because they can destabilize membrane bilayers by pH-triggered conformational change. In this study, different pH-sensitive 2-ethylacrylic acid and alkyl methacrylate [butyl methacrylate and hexyl methacrylate] copolymers were synthesized by reversible addition-fragmentation chain transfer polymerization with high yields. pH-dependent membrane disruptive activity was investigated with respect to their physicochemical and membrane lytic properties as a function of pH, concentration, and molecular weight. Hemolysis assays demonstrated that the presence of the hydrophobic monomer and sufficient protonation of the carboxylic acid groups were important parameters for efficient membrane destabilization. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 2522–2527, 2009

Key words: pH responsive; reversible addition-fragmentation chain transfer; hemolysis; copolymer

INTRODUCTION

Biological membranes, responsible for the compartmentalization of organs, cells, and cellular organelles, are natural barriers for most molecules that are not actively imported by living cells.[1] Many new therapeutic strategies, such as gene and interfering RNA-based therapies, and vaccine development require the delivery of polar macromolecules such as DNA, RNA, and proteins to intracellular sites.[2–4] These biological agents are typically taken up by targeted cell via endocytosis and trafficked through the endosomal-lysosomal pathway, which leads to the degradation by lysosomal enzymes and loss of therapeutic activity.[5] Endosomal escape remains a significant challenge to intracellular delivery of biomolecular therapeutics.

Synthetic polymers have significant potential for the delivery of molecules to cells both *in vitro* and *in vivo*. A variety of synthetic polymers and peptides have been used to deliver biological agents to cells.[6] Protonable amine-containing polycations including poly(ethylene imine), poly(amidoamine), and polyhistidine have proton-buffering capacities and become protonated at endosomal pH. It is thought that these polymers buffer the endosome against the pH drop, leading to an increased flux of protons and their counterious into the endosome.[7–9] Another approach of the use of carboxylic acid-containing polyanions such as poly(2-alkylacrylic acid) and methacrylic acid copolymers demonstrate pH-induced coil-to-globule transitions.[10,11] These polymers contain a critical balance of acidic carboxyl groups and hydrophobic alkyl or aromatic groups. The carboxylate ion of these polymers become protonated at endosomal pH values, and the polymer undergo a change from a hydrophilic, biologically inert state to a hydrophobic and endosomal membrane-destabilizing one.[12] The polymerization reactivity of α-alkylacrylic acid monomers is relatively low because of the steric hindrance of the growing chain end for chain propagation. In addition, polymers made from such monomers have low ceiling temperatures, where the polymerization is reversed,

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i.e., from growing chain polymer back to monomer. Homopolymerization of 2-ethylacrylic acid (EAA) worked only under bulk polymerization conditions with low polymer yields and uncontrolled molecular weight.[13] Because of the significant potential use of these hydrophobic poly(carboxylic acid), copolymerizing α-alkylacrylic acid with other monomers may offer a facile approach to copolymers with versatile structures and applications.[13–18] As these polymers are beginning to be used in fine biological and medicinal applications, it has been increasingly important to have control over the lengths, weights, and functionality of polymer chains. The most important technique developed to obtain that control has been the living radical polymerization (LRP). With LRP, this control is achieved simply by controlling the concentrations of the monomer and initiator of the polymerization in the reaction mixture. In this work, we prepare a series of EAA and hexyl/butyl methacrylate copolymers with high yield and controlled molecular weight by reversible addition-fragmentation chain transfer (RAFT) polymerization method. The pH-dependent membrane disruptive activity was investigated with respect to their physicochemical and membrane lytic properties as a function of pH, concentration, and molecular weight.

EXPERIMENTAL

Materials

The trithiocarbonate RAFT chain transfer agent (CTA), 2-dodecyl-sulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP), was prepared by the reported method.[15] Hexyl methyacrylate (HMA), butyl methyacrylate (BMA), 2,2′-azo-bis(isobutyronitrile) (AIBN), and all other solvents were purchased from Sigma-Aldrich Chemical (St. Louis, MO). All reagents were used as delivered without further purification except AIBN, which was crystallized from methanol before use.

Synthesis of 2-ethylacrylic acid monomer

EAA was prepared from diethyl ethylmalonate by a procedure published earlier.[14] The monomer was vacuum distilled and placed in the ampules. ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.10 (3H, —C*H*$_3$), 2.30 (2H, —C*H*$_2$—), 5.65 and 6.28 (2H, —C*H*$_2$=C<), and 11.65 (1H, —COO*H*).

Synthesis of poly(ethylacrylic acid-*co*-alkyl methyl acrylate) copolymers

Poly(2-ethylacrylic acid-*co*-alkyl methacrylate) copolymers were synthesized by RAFT polymerization using 2,2′-azobisisobutytonitrile as the initiator and 2-dodecylsulfanylthiocarbonyl-sulfanyl-2-methyl propionic acid (DMP) as RAFT chain transfer agent (CTA).[15] These reactions were performed in all glass Schlenck flasks, using a series of three freeze-pump-thaws to remove any oxygen present before heating in an oil bath at 80°C. The resulting crude polymer was dissolved in dimethyl formamide (DMF), precipitated in diethyl ether, and then dried under vacuum. Poly(EAA-*co*-BMA): δ (DMSO-d_6, ppm) 0.5–1.2 (a + f + g, 9H, —C*H*$_3$), 1.3 (e, 2H, —COOCH$_2$CH$_2$C*H*$_2$CH$_3$), 1.5–2.2 (b + d + h + i, 8H, in EAA: C*H*$_2$—C(CH$_3$)— and CH$_3$—C*H*$_2$, and —COOCH$_2$—C*H*$_2$— in BMA), 3.8 (c, 2H, —COOC*H*$_2$), and 12.2 (j, 1H, —COO*H*). Poly(EAA-*co*-HMA): δ (DMSO-d_6, ppm) 0.5–1.2 (a + f + g, 9H, —C*H*$_3$), 1.3 (e, 6H, —COOC*H*$_2$C*H*$_2$C*H*$_2$CH$_3$), 1.5–2.2 (b + d + h + i, 8H, in EAA: C*H*$_2$—C(CH$_3$)— and CH$_3$—C*H*$_2$, and —COOCH$_2$—C*H*$_2$— in HMA), 3.8 (c, 2H, —COOC*H*$_2$), and 12.2 (j, 1H, —COO*H*).

Characterization

Purity and composition of all the synthesized polymers were examined based on their ^{1}H-NMR spectra in $CDCl_3$ or DMSO-d_6. The weight-average molecular weight and molecular weight distribution of each polymer were examined following size exclusion chromatography principles using Waters 1525 binary HPLC pump connected to a Waters 717 plus auto sampler, a Waters 2487 dual λ absorbance detector, a Waters 2414 refractive index detector, and a Waters fraction collector III under the control of Breeze software run by an external PC. We used Ultrahydrogel 500 columns (Waters Corporation, Milford, MA) to determine the molecular weight of each polymer against a series of poly(ethylene glycol) standards (Polymer Laboratories, UK) using DMF as a mobile phase at a flow rate of 0.5 mL/min.

Hemolysis assay

Membrane-destabilizing polymers cause hemolysis of red blood cells at pH values similar to those found in the endosome, and endosomal release has been correlated to red blood cell (RBC) hemolysis. The capacity of the copolymers and comb-like polymers to induce pH-dependent membrane destabilization was determined using an RBC hemolysis assay described elsewhere.[5] Briefly, whole human blood was collected from volunteer donors using protocols in accordance with Zhejiang Sci-Tech University guidelines. RBCs were isolated by blood centrifugation and washed three times with 0.15*M* saline solution. After the final wash, the RBCs were diluted (1 : 10) in 0.1*M* phosphate buffer solution (PBS) at the desired pH values (5.8, 6.6, or 7.4) to yield a final RBC concentration of 10^8 RBCs per 200 μL. In eppendorf tubes, 800 μL of PBS at a particular pH value was mixed with 200 μL of RBC

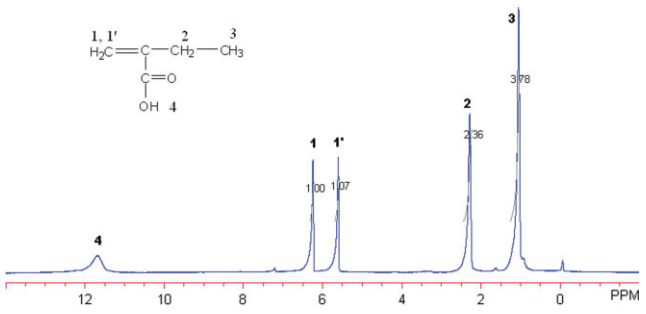


Figure 1 ^{1}H-NMR spectrum of 2-ethylacrylic acid monomer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

solution followed by the addition of polymer solution. The polymer and RBC mixtures were incubated in 37°C water bath for 1 h. During this incubation time, membrane-destabilizing polymers interact with the RBC membranes, releasing the hemoglobin (Hb) into solution. The tubes were centrifuged for 5 min at 13,500 × *g* to separate intact RBCs and disrupted membranes from the solution. The supernatant containing the released Hb was collected and transferred to 96-well plates, and the absorbance was measured on a Multiskan plate reader (ThermoFisher, New York) at 541 nm, which is the characteristic wavelength for Hb. The observed hemolysis of RBCs in PBS solutions and in DI water was used as negative and positive controls, respectively. The observed hemolytic activity of a given polymer composition at a given concentration and pH value was normalized to that of the positive control, DI water. All hemolysis assays were done in triplicate, and the data are reported as the average (the standard error of the mean). Statistical analysis of the hemolytic activity of different polymer compositions was done using Student's *t*-tests with a 95% confidence interval as the threshold for significance.

RESULTS AND DISCUSSIONS

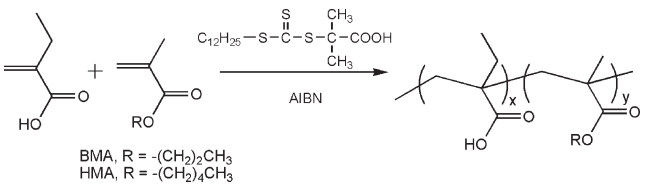
First, EAA was prepared from diethyl ethylmalonate by a procedure published earlier.[14] The monomer was purified by vacuum distillation. The ^{1}H-NMR spectrum of EAA can be observed in Figure 1, which indicates that the monomer has been successfully synthesized. What makes the synthesis of hemopolymer of α-alkylacrylic acid difficult is that attempting to polymerize the monomer directly often results in by-products and difficult purification and characterization procedures. The hemopolymer of 2-ethyacrylic acid that prepared by RAFT has broad polydispersity (PDI = 2.75) and low product yield (14.5%) as shown in Table I. The polymerization reactivity of EAA monomer is relatively low due to the steric hindrance of the growing chain end for chain propagation. In addition, PEAA have low ceiling temperatures, where the polymerization is reversed, i.e., from growing chain polymer back to monomer.[13]

However, after adding other monomer, BMA or HMA, the product yield increased greatly. A series of EAA and alkyl methacrylate copolymers with high yield and controlled molecular weight are prepared by RAFT polymerization method (Scheme 1), and the corresponding products were obtained and the results are summarized in Table I. The ratios of monomer reactivity were determined from the average composition of the copolymers listed in Table I with the nonlinear least-squares analysis to be $r_{EAA} = 0.23$ and $r_{BMA} = 0.46$ and $r_{EAA} = 0.30$ and $r_{HMA} = 0.48$, respectively. The possible reason for this is the decreasing of the steric hindrance in the random polymers after adding the BMA or HMA monomer. Furthermore, the resultant copolymers have higher ceiling temperatures which make the resultant copolymers more stable than the hemopolymers. The copolymers were investigated by ^{1}H-NMR as shown in Figures 2 and 3. A broad peak at around 12.0 ppm originating from the carboxyl groups of EAA and a narrow peak around at 4.0 ppm are assigned to the methylene protons linked to oxygen of ester group in BMA or HMA. Because of the signal overlap of main-chain protons, we choose the methylene protons linked to oxygen of ester groups and protons from carboxyl groups of EAA to confirm the composition of copolymer. The comparison of the integration values of peaks j and c, which

TABLE I
Chemical Compositions and Respective MWs of the Polymers Used in This Study

Polymer	M_n[a]	PDI	Ratio x : y		Yield (%)
Poly(ethylacrylic acid) (PEAA)	40,000	2.64	100 : 0[b]	100 : 0[c]	14.50
Poly(EAA$_x$-*co*-BMA$_y$)-1	35,520	1.14	50 : 50	45.65 : 54.35	95.65
Poly(EAA$_x$-*co*-BMA$_y$)-2	39,720	1.19	65 : 35	53.27 : 46.73	92.40
Poly(EAA$_x$-*co*-HMA$_y$)-1	33,850	1.13	50 : 50	46.82 : 53.18	90.28
Poly(EAA$_x$-*co*-HMA$_y$)-2	38,540	1.16	65 : 35	55.34 : 44.66	93.70

EAA, 2-ethylacrylic acid; BMA, butyl methyacrylate; HMA, hexyl methyacrylate.
[a] M_n Evaluated in DMF.
[b] Feed ratio of two monomers.
[c] Composition evaluated from the ^{1}H-NMR data.



Scheme 1 Chemical structure of the EAA copolymers tested.

provide building units ratios of PEAA and BMA, or PEAA and HMA, are listed in Table I.

Destabilization of membrane bilayers by the copolymers was first studied at the desired pH values (5.8, 6.6, or 7.4), using RBC as the endosomal membrane model. A useful endosomolytic agent should have membrane destabilizing properties at the mildly acidic pH (5.0–6.5) found in endosomes.[17] Figure 4 shows the copolymers' hemolytic activity data at three different pH values (5.8, 6.6, or 7.4) and three different concentrations (10, 20, 50 μg/mL). The copolymer [poly(EAA-*co*-BMA)-1] is most hemolytic at pH 5.8, whereas the copolymer presented limited hemolytic activity at neutral pH and the lowest hemolytic at pH 7.4 at all concentrations tested.

To determine the effect of hydrophobic monomer on the hemolytic activity properties of the copolymers, poly(EAA-*co*-BMA)-1 and poly(EAA-*co*-HMA)-1 with similar molecular weight were chosen to evaluate at concentrations ranging from 5 to 200 μg/mL at acidic pH (5.8). As shown in Figure 5, poly(EAA-*co*-HMA)-1 was more hemolytic at the concentrations from 10.0 to 100.0 μg/mL, where similar hemolytic activities were obtained at the lowest (5.0 μg/mL) and highest concentrations (200.0 μg/mL). Because the two copolymers have the similar molecular weight and composition, the higher hemolytic activity of poly(EAA-*co*-HMA)-1 should come from the hydrophobic units in the polymer. HMA has six-carbon chain in ether group, which has greater hydrophobicity than the four-carbon chain one. As the environmental pH is lowered to and below the pK_a

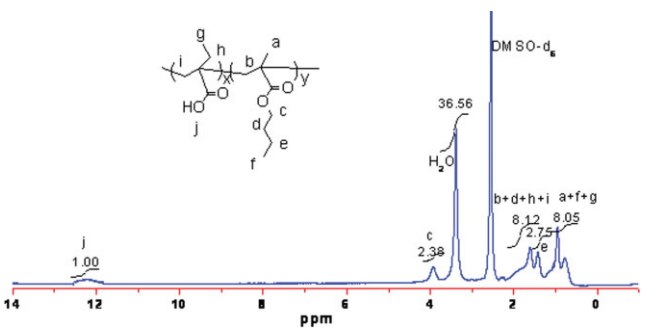


Figure 2 The chemical structure of poly(EAA-*co*-BMA)-1 copolymer was confirmed by ^{1}H-NMR spectrum in DMSO-d_6. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

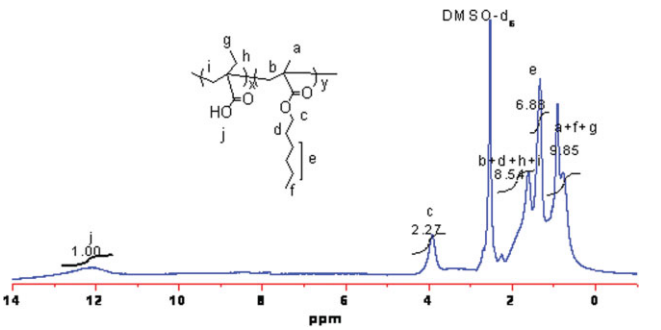


Figure 3 The chemical structure of poly(EAA-*co*-HMA)-1 copolymer was confirmed by ^{1}H-NMR spectrum in DMSO-d_6. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the carboxylic acid groups on the polymer backbone, poly(EAA-*co*-HMA)-1 become more protonated, and the copolymer results in greater hydrophobicity. In this hydrophobic state, poly(EAA-HMA/BMA)-1 are capable of interacting with biological membranes such as the RBC membrane, causing disruption.[19] Why did the two copolymers exhibit the similar hemolytic activity with the concentration at 5 and 200 μg/mL? The answer to this question probably lies in the conformation adopted by the copolymer in solution. When the copolymer concentration is very low, the copolymer is probably in a globule configuration and exhibits a relatively hydrophobic surface owing to the low concentration of EAA units. Such a conformation would make copolymers more susceptible to phagocytosis by the macrophages. Thus, the limited RBC hemolysis observed may be explained by strong intramolecular interactions and poor insertion into the phospholipids membrane. The ability of such copolymer to interact with the cell membrane at low concentrations is limited. However, at the concentration of

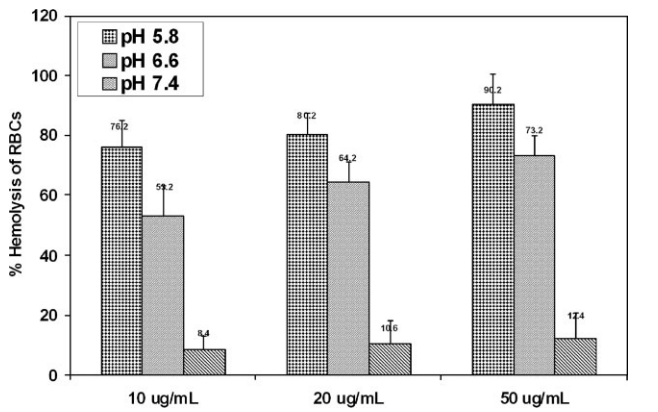


Figure 4 Hemolysis of red blood cells as a function of poly(EAA-*co*-BMA)-1 with the concentration at 10, 20, and 50 μg/mL. Each data point represents the mean of three determinations.

200 μg/mL, the copolymer concentration is too high, and the copolymers achieved complete RBC lysis.

The hemolysis assay was used to compare the membrane disrupting activity of the different copolymers with the hemopolymer, which were of different composition. Figure 6(A) shows the membrane disrupting activity of poly(EAA-*co*-BMA)-1 and poly(EAA-*co*-BMA)-2 when compared with the hemopolymer at the desired pH values (5.8, 6.6, or 7.4) with the concentration at 10 μg/mL. At pH 6.6 and 5.8, which is representative of the lower pH values encountered in early and late endosomes,[6] the copolymers had the highest hemolytic activity of all the polymers tested, whereas only low to negligible levels of hemolysis were achieved at pH 7.4. As the pH decreased, the hemolytic activity of PEAA increased, reaching a peak at pH 5.8. PEAA showed little hemolytic activity at pH 6.6 and 7.4, reaching a maximum of about 10% at pH 6.6, whereas hemolytic activity of the copolymers increased as the pH decreased. The copolymers showed significant levels of hemolysis at both pH 6.6 and 5.8. The membrane-destabilizing activity of copolymers on both RBCs could be explained by the presence of hydrophobic BMA units in the backbone. It has been suggested that alkyl chains facilitate interaction with hydrophobic components of the membrane. Interestingly, the copolymer with higher hydrophobic units in the polymer chain has higher level of hemolysis at all pHs tested. The similar results can also be observed in poly(EAA-*co*-HMA)-1 and poly(EAA-*co*-HMA)-2, as shown in Figure 6(B). It further demonstrated that membrane lytic activity could be enhanced by increasing the composition of hydrophobic units in the polymer chain.

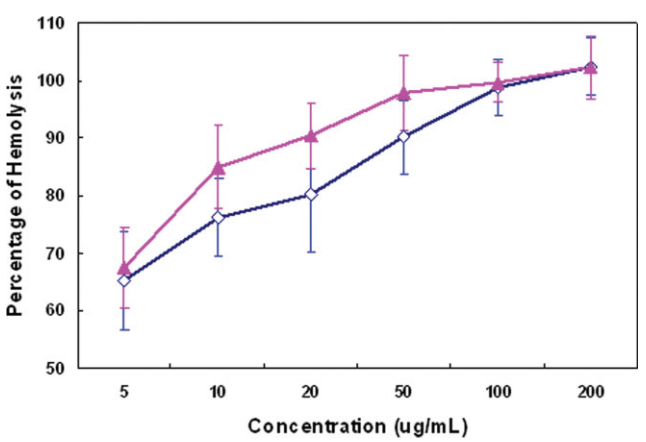


Figure 5 Hemolysis of red blood cells as a function of poly(EAA-*co*-BMA)-1 (squares) and poly(EAA-*co*-HMA)-1 (triangles) with the concentration at 5, 10, 20, 50, 100, and 200 μg/mL. Each data point represents the mean of three determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

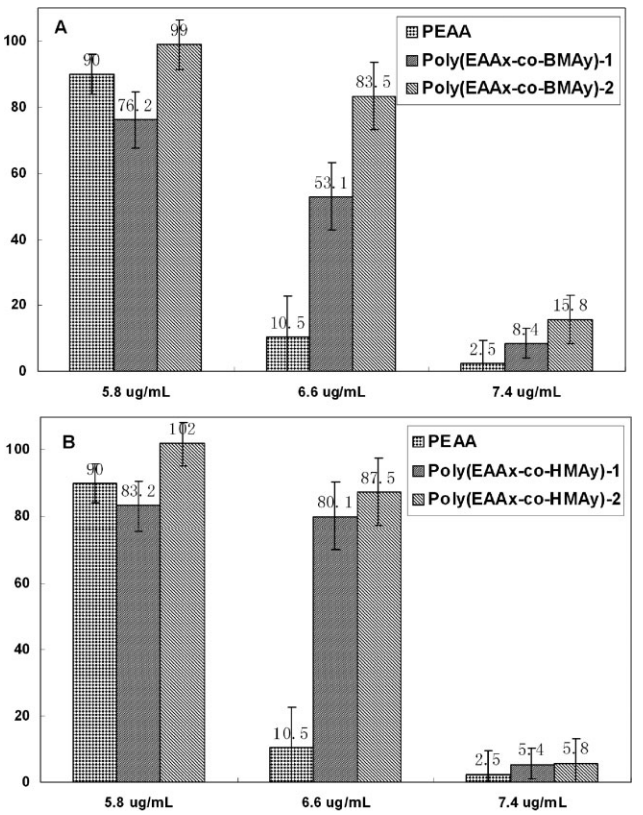


Figure 6 Hemolysis of red blood cells as a function of poly(2-ethyacrylic acid), poly(EAA-*co*-BMA)-1, and poly-(EAA-*co*-BMA)-2 with the concentration at 10 μg/mL (A). Lysis of red blood cells as a function of poly(2-ethyacrylic acid), poly(EAA-*co*-HMA)-1, and poly(EAA-*co*-HMA)-2 with the concentration at 10 μg/mL (B). Each data point represents the mean of three determinations.

CONCLUSIONS

In conclusion, different pH-sensitive EAA and alkyl methacrylate (BMA and HMA) copolymers were synthesized by RAFT polymerization with high yields and narrow polydispersity. The pH-dependent membrane disruptive activity was investigated with respect to their physicochemical and membrane lytic properties as a function of pH, concentration, and molecular weight. This study revealed that multiple parameters influence EAA copolymer–lipid interaction at neutral and acidic pH. The presence of hydrophobic units in the polymer chain was found to greatly increase their abilities to destabilize membrane bilayers.

References

1. Bulmus, V. Aust J Chem 2005, 58, 411.
2. Luten, J.; van Nostrum, C. F.; De Smedt, S. C.; Hennink, W. E. J Controlled Release 2008, 126, 97.
3. Lomas, H.; Canton, I.; MacNeil, S.; Du, J.; Armes, S. P.; Ryan, A. J.; Lewis, A. L.; Battaglia, G. Adv Mater 2007, 19, 4238.

4. Lackey, C. A.; Murthy, N.; Press, O. W.; Tirrell, D. A.; Hoffman, A. S.; Stayton, P. S. Bioconjugate Chem 1999, 10, 401.
5. Henry, S. M.; El-Sayed, M. E. H.; Pirie, C. M.; Hoffman, A. S.; Stayton, P. S. Biomacromolecules 2006, 79, 2407.
6. Jones, R. A.; Cheung, C. Y.; Black, F. E.; Zia, J. K.; Stayton, P. S.; Hoffman, A. S.; Wilson, M. R. Biochem J 2003, 372, 65.
7. Li, J.; Chuan Y.; Li, H.; Wang, X.; Goh, S. H.; Ding, J. L.; Wang, D. Y.; Leong, K. W. Adv Mater 2006, 18, 2969.
8. Lin, C.; Blaauboer, C.-J.; Timoneda, M. M.; Lok, M. C.; van Steenbergen, M.; Hennink, W. E.; Zhong, Z.; Feijen, J.; Engbersen, J. F.J. J Controlled Release 2008, 126, 166.
9. Tian, H.; Xiong, W.; Wei, J.; Wang, Y.; Chen, X.; Jing, X.; Zhu, Q. Biomaterials 2007, 28, 2899.
10. Kang, H. C.; Bae, Y. H. Adv Funct Mater 2007, 17, 1263.
11. Lee, L. K.; Williams, C. L.; Devore, D.; Roth, C. M. Biomacromolecules 2006, 7, 1502.
12. Kusonwiriyawong, C.; van de Wetering, P.; Hubbell, J. A.; Merkle, H. P.; Walter, E. Eur J Pharm Biopharm 2003, 56, 237.
13. Yin, X.; Hoffman, A. S.; Stayton, P. S. Biomacromolecules 2006, 7, 1381.
14. Murthy, N.; Robichaud, J. R.; Tirrell, D. A.; Stayton, P. S.; Hoffman, A. S. J Controlled Release 1996, 61, 137.
15. Lai, J. T.; Filla, D.; Shea, R. Macromolecules 2002, 35, 6754.
16. Rijcken, C.; Soga, O.; Hennink, W. E; van Nostrum, C. F. J Controlled Release 2007, 130, 131.
17. Yessine, M.-A.; Lafleur, M.; Meier, C.; Petereit, H.-U.; Leroux, J.-C. Biochim Biophys Acta 2003, 1613, 28.
18. Chantal, N. M.; Lackey, A.; Press, O. W.; Tirrell, D. A.; Hoffman, A. S.; Stayton, P. S. Bioconjugate Chem 1999, 10, 401.
19. Murthy, N.; Robichaud, J. R.; Tirell, D. A.; Stayton, P. S.; Hoffman, A. S. J Controlled Release 1999, 61, 137.