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Self-assembly of random co-polymers for selective binding and detection of peptides

Bo Zhao¹, Mahalia A. C. Serrano¹, Jingjing Gao¹, Jiaming Zhuang¹, Richard W. Vachet^{1,2,3}, and S. Thayumanavan^{1,2,3}

¹Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003, USA

²Molecular and Cellular Biology Program, University of Massachusetts, Amherst, Massachusetts 01003, USA

³Center for Bioactive Delivery- Institute for Applied Life Sciences, University of Massachusetts, Amherst, Massachusetts 01003, USA

Abstract

Amphiphilic random co-polymers, which form stable reverse micelle-type assemblies, have been designed and synthesized. We demonstrate that the reverse micelles, formed by these co-polymers are capable of selectively binding peptides through electrostatic interactions, indicating that these random polymers can self-organize into functionally selective materials. Moreover, these random co-polymers also enable the ordered co-crystallization of matrix and extracted guest molecules, giving rise to substantial signal enhancements during MALDI-MS detection. Together, these observations represent an excellent example of how random polymers can self-assemble into ordered, functional materials.

TOC Graphic

INTRODUCTION

Detecting trace amounts of a specific molecule in a complex mixture is often challenging. Nowhere is this challenge more apparent than in biological samples. For example, peptide or protein biomarkers can be early indicators of disease, but are usually present at ng/mL or lower levels in complex matrices such as serum or other biological fluids,^{1–6} and are

therefore very difficult to detect.^{7–10} Because of the implications associated with detecting such molecules in complex mixtures, a variety of approaches have been developed over the years.^{11–14}

Self-assembling nanomaterials, in general, and polymer-based self-assembling materials, in particular, have attracted significant recent attention for detection and sensing,^{15–22} with block co-polymers being used most often.^{23–25} Block co-polymers rely on synthetic methods that allow for the continuous incorporation of hydrophilic monomers in a polymer, followed by continuous incorporation of hydrophobic monomers (or vice versa) to generate micelle or reverse micelle like structures. By comparison, amphiphilic homopolymers require the pre-incorporation of hydrophilic and hydrophobic moieties in the same monomer, enabling a greater density of the desired functional groups.^{26–28} Our group has successfully used self-assembling amphiphilic homopolymers to enrich peptides from a biphasic mixture for sensitive detection by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS);^{29–31} however, the synthetic and structural requirements of homopolymers limit the molecular design space, thereby constraining the ability to systematically tune the functional groups of a homopolymer. Similar limitations are present for block co-polymers as well. Consequently, we and others have become interested in studying the self-assembly of random co-polymers and assessing their utility in sensing and biomarker detection applications.^{32–35} Random co-polymers provide the synthetic simplicity in that the hydrophobic and hydrophilic monomers can be independently chosen and their relative ratios can be tuned by simply varying the feed ratios of the monomers in the polymerization reaction. Because of this versatility, we became interested in probing random co-polymer scaffolds for their ability to enrich peptides from a biphasic mixture. A question emerges, however, about the ability of random co-polymers to assemble appropriately to bind peptides of interest. Here, we describe our findings on random co-polymers forming ordered, functional assemblies that bind to and enhance the detection of peptides. Specifically, we are interested in using complementary electrostatic interactions in random co-polymers assemblies to selectively extract peptides and sensitively detect them.

RESULTS AND DISCUSSION

Random co-polymers, based on an electron-rich alkoxystyrenyl backbone, used in this study are shown in Figure 1. The hydrophobic monomer of the random co-polymers contains a p-alkoxy moiety, while hydrophilic monomer contains various charged and charge-neutral polar moieties. While polymers **P1–P4** contain carboxylates as the hydrophilic moieties, **P5** and **P6** were synthesized as control polymers with charge-neutral hydrophilic moieties. All monomers were synthesized through Wittig reactions of the corresponding aldehydes, and the polymerizations were carried out using nitroxide-mediated polymerization³⁶ (Scheme 1). The molecular weight of **P1** was found to be about 10 kDa with M_w/M_n of 1.1 (see Electronic Supplementary Information for details). For consistency, the ratio of hydrophilic and hydrophobic monomers was kept close to 1:1. The control polymers required a slightly different ratio of the monomer to achieve the targeted reverse micelle assembly.

The polymer-peptide binding occurs in these systems, when the polymer based reverse micelle assemblies selectively extract complementarily charged peptides from an aqueous

phase into the organic phase that has the polymeric assemblies. For this binding to occur with high fidelity, it is necessary that the reverse micelle assemblies are stable in the organic phase, especially during the biphasic extraction process. Because these amphiphilic random co-polymers can form normal micelles in an aqueous phase and reverse micelles in an organic phase, they could potentially distribute between the two phases during an extraction. However, for the selective peptide extraction, the polymer-peptide interactions should occur only in the organic phase and therefore the polymer should remain as a robust reverse micelle assembly in the organic phase. Consequently, we first tested whether the random co-polymers form reverse micelle-type assemblies in toluene. Solutions of polymers **P1–P6** were prepared by dissolving them in toluene for a final hydrophilic moiety concentration of 4.0 mM. One equivalent aqueous sodium hydroxide solution per hydrophilic moiety were also added to the solution to make the “water pool” of the reverse micelle assembly and to ensure that the carboxylic acid moieties are fully deprotonated. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) measurements of **P1** revealed assemblies of about 200 and 150 nm, respectively (Figure 2). Similar behavior was also observed with the polymers **P2–P6** (Figure S1).

Next, we tested the stability of the reverse micelle assemblies in a biphasic mixture. Accordingly, the solutions of reverse micelles in toluene were subjected to a biphasic extraction procedure with water. The possible transfer of the polymer **P1** into the aqueous phase was evaluated using UV-visible absorption spectroscopy. There is no discernible peak at ~280 nm, corresponding to the polymer, in the aqueous phase before and after extraction. This confirms that the polymer does not thermodynamically redistribute into the aqueous phase and that the polymer has an inherent preference for the organic phase, when it is initially assembled there. Similar behavior is observed for other amphiphilic random co-polymers as well (Figure S2). Note that toluene has an absorbance cut-off wavelength of 285 nm, which might interfere the assignment of the polymer peak. However, by blanking the baseline against the toluene solvent for the measurement of organic phases before and after equilibration, there is still a distinct peak at 280 nm, which confirms that the polymers are stably trapped in organic phase. To further confirm this assertion, dichloromethane (DCM) was used as the solvent with lower cut-off wavelength. Similar behavior of polymer assembly was indeed observed in DCM/water mixtures (Figure S3).

Given that the random co-polymers form reverse micelle like assemblies and remain in toluene, we next tested if they could behave as hosts that can selectively sequester guest molecules from an aqueous phase. To do this, we extracted an aqueous mixture of two hydrophilic dyes, rhodamine 6G and calcein (Figure 3). Because the reverse micelles of polymers **P1–P4** are negatively charged, we expected that the reverse micelles would selectively extract the positively charged dye, rhodamine 6G, while leaving the negatively charged calcein in the aqueous phase. After mixing the reverse micelles in toluene with an aqueous solution containing the two dyes, we indeed found that rhodamine 6G is selectively extracted to the organic phase and calcein remains in the aqueous phase (Figure 3c). In a control experiment, the extraction experiment without the reverse micelle in toluene did not result in the extraction of either dye (Figure 3d).

We next tested if the reverse micelles could selectively extract peptides with complementary charges. Five peptides (Table 1), with different isoelectric points (pI) values, were extracted from a 50 mM Tris buffer solution at pH 7.0. At this pH, peptides with pI values higher than 7.0 are expected to carry a net positive charge. On the other hand, peptides with pI values lower than 7.0 are expected to carry a net negative charge. Upon extraction and subsequent phase separation and MALDI-MS detection, we found that only peptides with pIs above 7.0 are effectively sequestered by the reverse micelles in the organic phase, whereas peptides with pIs below 7.0 remain in the aqueous phase (Figure 4). Similar behaviors were found for polymer **P2–P4**. (Table S1 and Figure S4)

Having shown that the random co-polymers can self-assemble into ordered structures and selectively extract guest molecules, we sought to test the effect of variations in the organization of charged moieties in the hydrophilic monomer. We first explored how these variations influence the extraction capacities of the reverse micelles. Capacity is important, as this is an indicator of how efficiently a particular type of supramolecular assembly can host a set of guest molecules. Moreover, capacity also indicates the extent to which enrichment of the oppositely charged peptide can occur in the organic phase, which is significant for detection purposes. Given the importance of charge complementarity in the extraction process, we examined how the positioning of the charged group could influence capacity using bradykinin (RPPGFSPFR, pI= 12.5) as the model peptide. Note that small peptides, such as bradykinin, commonly exhibit random coil structure in free aqueous solution. Therefore, secondary structure of the peptide does not affect binding capacity in these studies (Figure S5). When extractions are done with a homopolymer **P7**, which has the same amount of functionality as **P1** and a similar molecular weight, we find that the capacity of the homopolymer is two times less than the random co-polymer (Figure 5). This result suggests that random co-polymer design might be advantageous by allowing the polymer to organize more effectively to host a greater amount of peptide molecules. In other words, the random arrangement of carboxylate groups present in **P1** improves its ability to order itself into a functional host material. The importance of charge complementarity in producing a functional host material is further proven by considering that polymers **P5** and **P6** have almost no capacity to host the peptide (Figure 5).

Interestingly, polymer **P1** has a 30-fold higher capacity for the peptide than polymers **P2–P4**. The relatively low capacities of the polymers with multiple carboxylate groups is somewhat surprising, as one might expect that a greater number of the carboxylate functionality locally would increase the number of guest peptides that can be hosted within the assembly. Instead, our results indicate that either an optimum number of carboxylates packed within the assemblies or the capability that the polymer could position these carboxylate groups to an appropriate extent is necessary to achieve high guest capacity.

Finally, we tested if the random co-polymer design could organize into ordered structures that enhance the MALDI-MS detection of guest peptides. Previously, we demonstrated that amphiphilic homopolymers with electron donating alkoxy substituents on the aromatic ring (*e.g.* **P7**) cause a significant signal enhancement effect during the MALDI-MS analysis of peptides that are extracted into their interiors.^{37–38} This signal enhancement effect arises as a result of favorable ternary interactions between the polymer, peptide, and MALDI matrix

that cause peptides to concentrate into ‘hot spots’ that increase detection efficiency. We found that the polymer matrix interactions are mediated through aromatic donor-acceptor interactions, while the polymer/peptide interactions are electrostatic in nature. The co-polymer design described in this work allows us to explore whether such ordered interactions can arise from random polymers.

To test the ability of the random co-polymers to form ordered materials that enhance MALDI-MS detection, solutions of polymers **P1–P4** at constant carboxylate concentration were used to extract a 50 nM solution of fluorophore-labeled bradykinin (TMR-BK) (Chart S1). After the extractions, the organic phases were analyzed by MALDI-MS to determine the degree of signal enhancement and by fluorescence microscopy to determine the extent of hotspot formation. MALDI-MS measurements (Figure 6a) indicate that the extent of signal enhancement for extracted TMR-BK is the same for all the polymers **P1–P4**. Consistent with this observation is the fact that an equal extent of hotspot formation is observed for all the polymers in the corresponding fluorescence images (Figure 6b and Figure S7). The degree of signal enhancement and hotspot formation seen with the random co-polymers is similar to that observed for a homopolymer **P7**. Compared to fluorescence microscopy images of unextracted TMR-BK, polymers **P1–P4** can readily form “hot spots” that are enriched in peptides, indicating that the random co-polymers can organize in such a way to mediate the ternary interactions necessary for signal enhancement. Moreover, it appears that the organization of the hydrophobic and hydrophilic groups has little effect on signal enhancement as the random co-polymer and homopolymer behave similarly with regard to this phenomenon. Given the merit of signal enhancement effect, we evaluated the detection limit to be as low as 10 pM for **P1** (Figure S8).

CONCLUSIONS

In summary, we have designed and developed a new amphiphilic random co-polymer scaffold that can form supramolecular assemblies for sequestering guest molecules via a simple biphasic extraction method. The random co-polymers were synthesized by one-pot polymerization of both styrene-based hydrophobic moieties and hydrophilic functional groups, which are synthetically more accessible for both the preparations of monomers and functional group variations. With this scaffold, we have shown that: (i) the random co-polymers can self-assemble into reverse micelle-type assemblies; (ii) these reverse micellar structures are “kinetically trapped” in the organic phase, providing a necessary feature for biphasic extraction of guest molecules; (iii) these reverse micelles can selectively sequester guest molecules with complementary charge based on electrostatic interactions, demonstrating that random polymers can self-organize into functional materials; (iv) organization of the functional groups within the hydrophilic unit of the polymer influences the extraction capacity of reverse micelles; (v) random co-polymers can order themselves in a way that mediates ternary interactions with the MALDI matrix, giving rise to signal enhancements in MS. Given the simplicity with which these random co-polymers can be synthesized, these materials will make it easy to investigate how structural variations affect guest binding and detection. These future investigations will improve our fundamental understanding of these materials and will allow us to design better materials for the selective extraction of trace level molecules in complex mixtures.

MATERIALS AND METHODS

Polymer Synthesis and Characterization

General methods—All reagents were commercially available and used as received unless stated otherwise. ¹H-NMR spectra were recorded on a 400 MHz NMR spectrometer using residual proton resonance of the solvents as internal standard. Chemical shifts are re-ported in parts per million (ppm). Mass spectra were obtained by a Bruker AmaZon quadrupole ion trap mass spectrometer coupled with electrospray ionization source. Gel permeation chromatography (GPC) was used to estimate the molecular weight of polymers using THF as eluent and 1 μ L of toluene was added as the internal reference. Polystyrene standards were used for calibration and data analysis. Dynamic light scattering (DLS) was measured by a Malvern Zetasizer. Transmission electron microscopy (TEM) images were taken from JEOL JEM-2000FX.

Extraction method

Reagents—The peptide bradykinin (RPPGFSPFR) was purchased from the Sigma-Aldrich. TMR-bradykinin (TAMRA dye conjugated at the N-terminal of the peptide) was obtained from Biopeptek Inc. The following peptides were purchased from the Bachem: kinetensin (IARRHPYFL), malantide (RTKRSGSVYEPLKI), amyloid fragment 1–11 (DAEFRHDSGYE), preproenkephalin (SSEVAGEGDGDSMGHEDLY). Trifluoroacetic acid (TFA), and cyano-4-hydroxy-cinnamic acid (CHCA) were purchased from Sigma-Aldrich.

Preparation of Polymeric Reverse Micelles—An appropriate amount of amphiphilic random co-polymers was dissolved in toluene. One equivalent of aqueous NaOH solution or H₂O per functional group was added to the toluene solution. Sonication was conducted until the solution becomes homogeneously dispersed.

Matrix Solutions—CHCA in ACN solution was prepared at a concentration of 10 mg/mL in 50 μ L ACN: 47.5 μ L H₂O: 2.5 μ L TFA. CHCA in THF solution was prepared at a concentration of 30 mg/mL in 350 μ L THF:150 μ L H₂O:6 μ L TFA.

Peptide Extraction and Sample Preparation—The general extraction procedure has been described elsewhere,³⁸ but in short, the following procedure was used. Peptide solutions were prepared in 50 mM Tris buffer where the pH of the buffer is adjusted beforehand. 1 mL of the buffered peptide solution was mixed vigorously with 200 μ L of the polymeric reverse micelle solution for 2 hours. After mixing, the solution was centrifuged at 13,000 rpm for 60 minutes to break the emulsion. The aqueous phase was withdrawn and kept in a separate microtube. 10 μ L of the aqueous phase sample was then mixed with 10 μ L of ACN CHCA matrix solution. From this sample, 2 μ L was spotted on the MALDI target plate for MALDI-MS analysis. The remaining organic phase was dried by blowing N₂ gas. The dried residue of the mixture of polymer and the extracted peptides was re-dissolved in 10 μ L of THF and then 20 μ L of the THF CHCA matrix solution. From this sample, 1 μ L was spotted on the MALDI target plate for MALDI-MS analysis or on indium tin oxide (ITO)-coated glass slides for fluorescence microscopy.

Fluorescence Microscopy—Fluorescence images were obtained using an Olympus FluoView FV1000 confocal fluorescence microscope. 1 μ L of sample spotted on ITO-coated glass slides was allowed to dry and was visualized under 10 $\times$ magnification. The Cy3 channel (ex= 559 nm, em= 567 nm) and the ECFP channel (ex= 405 nm and em= 476 nm) were used to image TMR-bradykinin and CHCA matrix, respectively. Images obtained were 1024 $\times$ 1024 pixels in size with a scale of 0.478 m per pixel.

MALDI-MS Analysis—A Bruker Autoflex III time-of-flight mass spectrometer was used for the MALDI-MS analysis of all samples. Acquisition of all mass spectra was done in reflectron mode with an accelerating voltage of 19 kV. Each spectrum is the average of 500 laser shots at 40% laser power.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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P1 (x:y= 51:49)
(M_n = 10K Da, PDI= 1.1)

P2 (x:y= 46:54)
(M_n = 12K Da, PDI= 1.2)

P3 (x:y= 48:52)
(M_n = 14K Da, PDI= 1.1)

P4 (x:y= 52:48)
(M_n = 15K Da, PDI= 1.2)

P5 (x:y= 61:39)
(M_n = 10K Da, PDI=1.3)

P6 (x:y=65:35)
(M_n = 20K Da, PDI= 1.4)

Figure 1.
Chemical structures of amphiphilic random co-polymers **P1–P6**.

Figure 2.

(a) Dynamic light scattering (DLS) of reverse micelles of polymer **P1** (1×10^{-4} M) in toluene. (b) Transmission electron micrograph (TEM) of reverse micelles of polymer **P1** (1×10^{-5} M), after drying on the TEM grid. (c) UV-Vis measurements with reverse micelles of polymer **P1** (1×10^{-4} M) starting in toluene (ORG), before and after equilibration with aqueous phase (AQ).

Figure 3.

(a) Example of dye extraction experiments using toluene (left) and reverse micelles of **P1** (right). (b) Chemical structures of Rhodamine 6G and Calcein. (c) UV-Vis measurements of the dye mixture starting in water (AQ), before and after equilibration (Eq) with reverse micelles of **P1** (ORG). (d) UV-Vis measurements of the dye mixture starting in water (AQ), before and after equilibration (Eq) with toluene (ORG).

Figure 4.

(a) MALDI mass spectrum of the peptide mixture in the aqueous solution before extraction.

(b) MALDI mass spectrum of the organic phase after extraction using reverse micelles of polymer **P1** at pH 7.0. (c) MALDI mass spectrum of the aqueous phase after extraction using reverse micelles of polymer **P1** at pH 7.0.

Figure 5.

(a) Peptide binding capacities at pH 7.0 after extracting with 100 μL of 1.75×10^{-4} M of carboxylate or charge-neutral functional group concentrations of solution of different polymers. The peptide capacities for each polymer were determined in the same manner as that for Figure S6. (b) Chemical structure of homopolymer **P7**.

Figure 6.

(a) MALDI ion abundance of 50 nM of TMR-BK peptide extracted using different polymers. Unextracted TMR-BK: 100-fold higher concentration in the unextracted sample (5 μ M) relative to extracted sample (50 nM) to account for the 100-fold enrichment during extraction (See Figure S7). (b) Fluorescence images showing the degree of clustering and co-crystallization of TMR-bradykinin peptide (red) and α -cyano-4-hydroxy cinnamic acid (CHCA) matrix (green). Scale bar=100 μ m.

Scheme 1.
Synthesis of amphiphilic random co-polymer **P1**.

Table 1

Information of peptides used in extraction experiments in Figure 4.

Peak in Figures	Peptide	Sequence	Concentration (μ M)	m/z	pI ^a	Net charge at pH 7.0
1	Bradykinin	RPPGFSPFR	0.1	1060.57	12.5	+
2	Angiotensin I	DRVYIHPFHL	0.1	1269.68	7.7	+
3	Malantide	RTKRSQSVYEPLKI	0.1	1633.94	10.7	+
4	β -Amyloid 1–11	DAEFRHDSGYE	0.15	1325.54	4.1	-
5	Preproenkephalin	SSEVAGEGDGDSMGHEDLY	0.15	1954.76	3.6	-

(^a calculated using the program available at <http://pepcalc.com>.)