Self-healing multiblock copolypeptide hydrogels via polyion complexation

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Abstract Diblock, triblock and pentablock copolypeptides were designed and prepared for formation of polyion complex hydrogels in aqueous media. Increasing the number of block segments was found to allow formation of hydrogels with substantially enhanced stiffness at equivalent concentrations. Use of similar length ionic segments also allowed mixing of different block architectures to fine tune hydrogel properties. The pentablock hydrogels possess a promising combination of high stiffness, rapid self-healing properties, and cell compatible surface chemistry that makes them promising candidates for applications requiring injectable or printable hydrogel scaffolds.

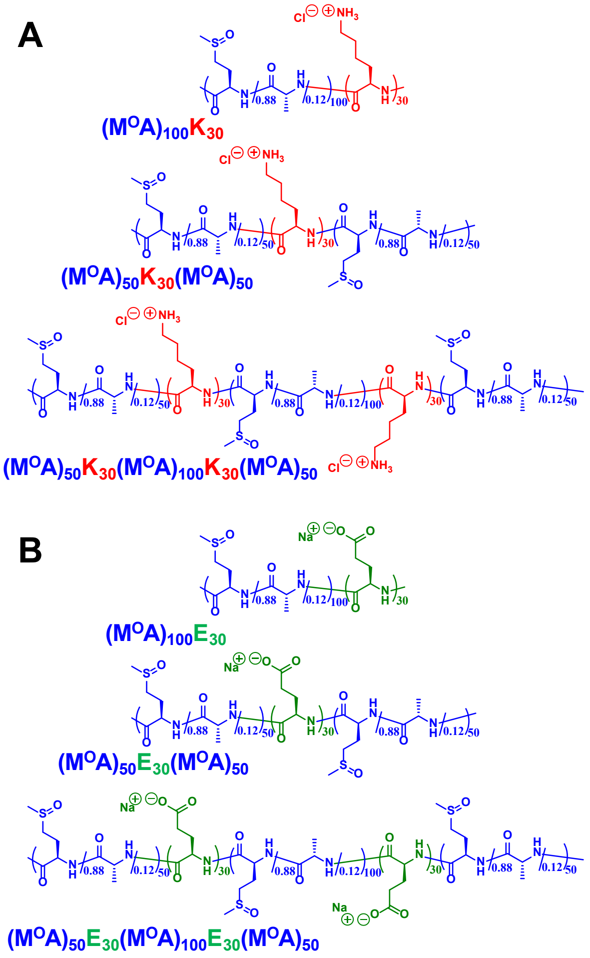
Polyion complex (PIC) assembly of dual hydrophilic block copolymers containing non-ionic and oppositely charged ionic segments has been developed as a facile method to prepare a diverse array of micelles, vesicles, and hydrogels in aqueous media.[1-3] Due to the high water solubility of precursors, PIC formation allows the preparation of supramolecular assemblies at high concentrations via simple mixing, and does not require the use of either heating or cosolvents. These assemblies are experiencing extensive development in applications, including as carriers for therapeutic molecules and as scaffolds for cell culture and tissue repair.[1-5] We recently reported the design of PIC diblock copolypeptide hydrogels

(DCH_{PIC}) that utilize formation of β-sheet structured solid complexes and were shown to be cell-compatible, self-healing, and resistant to dilution.[6] In effort to improve the efficiency of hydrogel formation and to enhance mechanical properties, we report here studies on the aqueous assembly of triblock and pentablock copolypeptides designed to form PIC hydrogels. These copolymers possess block architectures that have not previously been explored for PIC assembly, and were found to significantly enhance network formation and stiffness compared to diblock architectures at equivalent concentrations.

Most block copolymer PIC hydrogels have been prepared using one or two triblock copolymer components containing ionic end-blocks flanking a non-ionic, hydrophilic center block.[1-3,5,7-10] These hydrogels rely on the formation of phase-separated, PIC liquid coacervate spherical domains that act as physical crosslinks in the networks, where the charged segments are placed as end-blocks to facilitate bridging of spherical domains by the non-ionic segments leading to network formation.[5,7-11] Alternatively, hydrogels have also been prepared via the assembly of amphiphilic, hydrophobically assembled triblock copolymers in water, and these systems have recently incorporated more complex multiblock architectures to enhance and tune mechanical properties.[12-15] We and others have also found that use of multiblock and star copolymer architectures can enhance hydrogel properties in hydrophobically assembled copolypeptide systems.[16-19] However, these amphiphilic materials can be difficult to formulate, especially at higher concentrations, and the reported systems are not cell compatible.[18,20] Here, we sought to develop PIC multiblock copolypeptide hydrogels that could overcome these issues and allow the preparation of hydrogels with a broad range of tunable properties. Since the assembly of DCH_{PIC} in water promotes formation of solid PIC β-sheet fibrillar assemblies as opposed to the liquid coacervate spherical domains seen in other PIC hydrogels,[6] the design of PIC multiblock copolypeptide architectures is better modeled on related fibril forming amphiphilic block copolypeptide hydrogels[16-18] as opposed to the more conventional triblock architectures used to form PIC hydrogels.[1-3,5,7-10]

The design of PIC multiblock copolypeptide hydrogels was based on triblock and pentablock amphiphilic block copopolypeptides we had previously developed,[16-18] where cationic and hydrophobic segments were now replaced with non-ionic and ionic segments, respectively. The compositions of the non-ionic and ionic segments were based on those utilized previously in diblock $\mathrm{DCH_{PIC}}$,[6] namely poly(L-methionine sulfoxide-*stat*-L-alanine)$_{100}$-*block*-poly(L-lysine-HCl)$_{30}$, $(\mathrm{M^OA})_{100}\mathrm{K}_{30}$, and poly(L-methionine sulfoxide-*stat*-L-alanine)$_{100}$-*block*-poly(L-glutamate-Na)$_{30}$, $(\mathrm{M^OA})_{100}\mathrm{E}_{30}$ (Scheme 1). Notably, the triblock and pentablock copolypeptides were designed with non-associating (i.e. non-ionic) segments as end-blocks (Scheme 1, see Supporting Information (SI) Figure S1),[16-18] different from conventional PIC hydrogels that have associating ionic segments as end-blocks.[5,7-10] This change is due to the different mechanism of assembly in $\mathrm{DCH_{PIC}}$ compared to conventional PIC hydrogels, where the β-sheet structured solid PICs require substantial solubilizing segments to prevent further aggregation and precipitation.[6]

The triblock and pentablock compositions were designed so that (i) their mole fractions of non-ionic and ionic residues would be identical to the diblock samples, and (ii) ionic segments would be the same average length in all samples (Scheme 1). Matching of ionic and non-ionic copolypeptide content among all samples allows for meaningful and quantitative comparison of hydrogel properties since equivalent sample concentrations (wt %) will possess the same molar concentrations of amino acid components. Maintaining similar ionic segment lengths is also important since their variation has been shown to strongly affect hydrogel mechanical properties.[6,21] The copolypeptides in Scheme 1 were prepared by the stepwise addition of appropriate NCA monomers to growing chains initiated using $\mathrm{Co(PMe_3)_4}$,[22] and gave samples with segment lengths and compositions that agreed well with the predicted values (see Table S1). Subsequent oxidation of methionine residues resulted in their conversion to methionine sulfoxides,[23,24] and removal of protecting groups gave the final water soluble copolypeptides in high overall yields after purification (see Table S1).



Scheme 1. Chemical structures and compositions of diblock, triblock, and pentablock copolypeptides used in this study. (A) cationic copolypeptides. (B) anionic copolypeptides. Characterization data are given in Table S1.

PIC assemblies were prepared by mixing aqueous solutions (1x PBS) of matched diblock, triblock, or pentablock copolypeptides at different concentrations. Stoichiometric mixtures of oppositely charged copolypeptides resulted in the formation of transparent hydrogels within seconds to minutes depending on the concentration and block architecture, with pentablock samples forming hydrogels *ca*. 6 times faster than triblock and diblock samples. These samples were evaluated using oscillatory rheology to quantify their mechanical properties, and all were found to display elastic behavior (G′ » G″) over a range of frequency (Figure 1, see Figure S2). The hydrogels were also found to break down under high strain, as expected for physical hydrogels.[6] While hydrogel stiffness (G′) was found to increase with sample concentration for all samples, hydrogel stiffness of pentablock samples was always greater than triblock samples, and both were greater than diblock samples at equivalent concentrations (Figure 1, see Table S2). Hence, multiblock architectures were found to allow the preparation of significantly stiffer hydrogels at equivalent amino acid contents and concentrations.

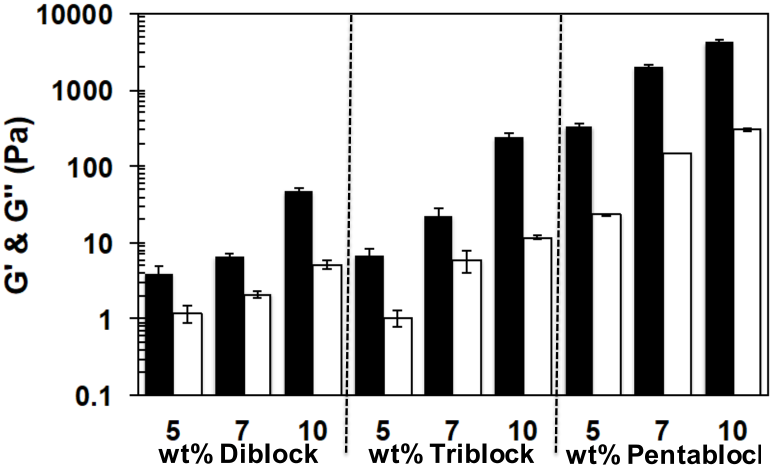


Figure 1. Mechanical properties of PIC block copolypeptide hydrogels. Storage modulus (G′, Pa, black) and loss modulus (G″, Pa, white) of diblock, triblock, and pentablock hydrogels were measured at varying sample concentrations in 1×PBS buffer at 25 °C. All G′ and G″ values were measured at an angular frequency of 5 rad/s and a strain amplitude of 0.01.

When comparing the diblock and triblock samples, both are composed of equal length chains. Here, the replacement of single long non-ionic segments in diblock samples with two shorter non-ionic segments in triblock samples is responsible for the observed modest increases in hydrogel stiffness. Although it has been found that longer non-associating (i.e. solubilizing) segment lengths in block copolypeptides enhance hydrogel formation, their effect on stiffness is relatively modest.[21] Consequently, it is thought that the higher density of solubilizing segments per associating segment found in the triblock samples increases solubilizing chain steric repulsion at the block junctions upon PIC formation, resulting in formation of more extended, stiffer fibril assemblies.[16-18,20]

The pentablock samples incorporate the features of the triblock samples, but also double the chain length. The presence of a central tether between associating domains also allows for bridging of fibrillar assemblies, and for and re-entry of chains into growing fibrils via chain folding during assembly.[18] Based on results from assembly of hydrophobically assembled pentablock copolypeptide hydrogels, we expect that the long central non-ionic tether segments present here will favor fibril re-entry over bridging, which was found previously to result in longer fibrils.[18] The architectural features of the pentablock hydrogels resulted in their stiffness being up to *ca*. 100 times greater than equivalent concentration diblock samples. We had previously found that hydrogel formation in diblock samples was driven by the assembly of solid β-sheet structures in the PIC domains,[6] which can be monitored by examination of polypeptide Amide I bands using FTIR spectroscopy. Analysis of lyophilized diblock, triblock, and pentablock samples revealed strong Amide I bands at 1653 cm^{-1} due to the disordered chain conformations of the ($M^{O}A$) segments, and Amide I shoulders at 1630 cm^{-1} that are characteristic of β-sheet chain conformations (See Figure S3).[6] The intensity of the band at 1630 cm^{-1} was found to increase in samples from diblock to triblock to pentablock, consistent with improved β-sheet formation in the pentablock samples. Thus, it appears that pentablock samples allow for better chain ordering within the structured PIC domains, potentially due to kinetically favored chain re-entry during assembly,[18] which results in substantial enhancement of hydrogel stiffness.

To evaluate interactions between different block architectures, we prepared charge balanced mixtures of cationic pentablock chains (i.e. PK) with anionic diblock (i.e. DE) or triblock (i.e. TE) chains. At 7.0 wt% in 1 x PBS media, these 'mismatched' samples were found to form hydrogels with stiffness (G′) intermediate between the matched diblock, triblock, and pentablock samples (Figure 2A). In both cases, enhancement of stiffness was up to *ca*. 10 times greater compared to matched diblock and triblock samples. These results indicate that the different block copolypeptide architectures containing associating ionic segments of similar length can efficiently co-assemble into hydrogel networks. This behavior is similar to results obtained with amphiphilic copolypeptide hydrogels where samples with similar hydrophobic segment lengths could be mixed to tune mechanical properties.[16] Such behavior is beneficial for the fine tuning of mechanical properties without having to prepare many different compositions, and potentially allows for significant enhancement of hydrogel stiffness using small amounts of multiblock copolypeptides. To test this concept, 7.0 wt% triblock hydrogels were prepared using increasing amounts (wt%) of cationic pentablock (i.e. PK) copolypeptides, where the amount of cationic triblock (i.e. TK) chains was simultaneously decreased in the formulations to maintain charge balance. The data in Figure 2B show that hydrogel stiffness can be enhanced as the fraction of cationic PK chains is increased, with a significant increase in stiffness when the fraction of PK is above 50 wt%.

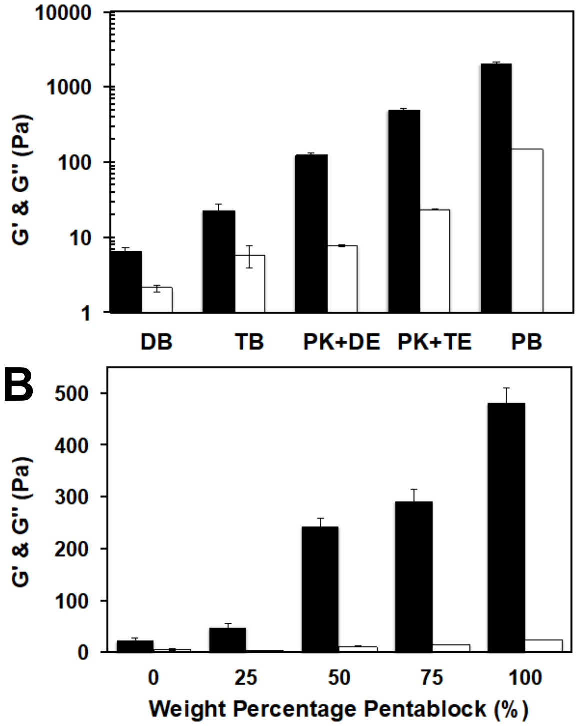


Figure 2. Mechanical properties of PIC block copolypeptide hydrogels prepared using matched and mismatched copolypeptides. (A) G′ (Pa, black) and G″ (Pa, white) of 7 wt% PIC hydrogels measured in 1×PBS buffer at 25 °C. In matched samples, diblock (DB), triblock (TB), and pentablock (PB) hydrogels were prepared by mixing cationic and anionic copolypeptides of the same block architecture. In mismatched samples, cationic pentablock (PK) was mixed with either anionic diblock (DE) or anionic

triblock (TE), where DE = $(M^OA)_{100}E_{30}$; TE = $(M^OA)_{50}E_{30}(M^OA)_{100}$; and PK = $(M^OA)_{50}K_{30}(M^OA)_{100}K_{30}(M^OA)_{50}$. (B) G′ (Pa, black) and G″ (Pa, white) of 7 wt% PIC hydrogels (PK+TB), with varying weight % incorporation of cationic PK component into triblock mixtures, were measured in 1×PBS buffer at 25 °C. All samples were prepared with stoichiometric E to K ratios. All G′ and G″ values were measured at an angular frequency of 5 rad/s and a strain amplitude of 0.01.

We previously observed that DCH_{PIC} were able to rapidly self-heal after mechanical breakdown when subjected to high amplitude oscillatory strain.[6] Rapid self-healing behavior is a desirable property in hydrogels as it allows deposition of hydrogel via injection through small bore needles, which has utility in biological applications as well as in additive manufacturing. Diblock, triblock, and pentablock hydrogel samples (at 10 wt%, chosen to obtain a reasonably stiff diblock hydrogel) were evaluated to determine if their self-healing properties were affected by block architecture. Each sample was subjected to high amplitude oscillatory strain, followed immediately by monitoring the recovery of elasticity over time by measuring G' at a much smaller strain amplitude (Figure 3). During the initial 100 s of high strain amplitude, G' for all samples dropped substantially to below the level of G'', indicating that they all became viscous liquids. Upon switching to low strain amplitude, all samples began recovering their elastic properties over time. Remarkably, the pentablock sample, which possessed the greatest stiffness, was the fastest (less than *ca*. 10 s) to fully recover its mechanical properties. For the diblock and triblock samples, recovery of elasticity continued to occur over a time scale of minutes. The rapid and complete self-healing of the pentablock hydrogels was unexpected due to their capacity to form network connections via bridging of PIC fibrils.[25] A possible explanation for rapid self-healing may that the pentablock hydrogels contain few chains that form bridges between fibrils, and instead most chains form loops on individual fibrils via kinetically favored chain re-entry during fibril growth, similar to fibrillar assemblies in hydrophobically associated pentablock copolypeptides.[18] The rapid self-healing properties of the pentablock hydrogels combined with the ability to prepare samples of high stiffness by varying concentration provide a promising combination of attributes for development of injectable or printable hydrogel scaffolds. Furthermore, for the compositions studied, the pentablock hydrogels were also found to be more resistant to dissolution in media compared to diblock and triblock samples (see Figures S4, S5).[6]

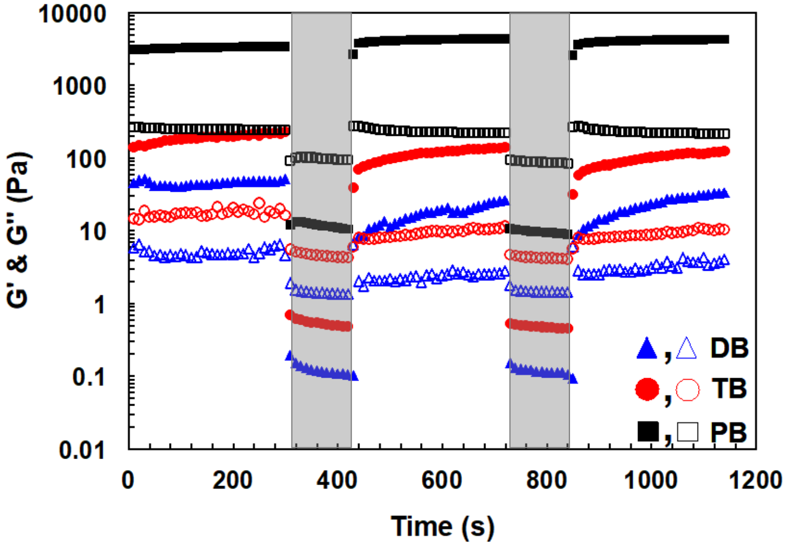


Figure 3. Mechanical recovery of diblock (DB), triblock (TB), and pentablock (PB) hydrogels. Samples prepared at 10 wt% in 1×PBS buffer at 25 °C (G′ = solid symbols; G″ = open symbols) after application of stepwise large-amplitude oscillatory breakdown (gray regions = strain amplitude of 10 at 10 rad/s for 120s) followed by low-amplitude linear recovery (white regions = strain amplitude of 0.01 at 5 rad/s for 300s).

In summary, new triblock and pentablock copolypeptides capable of forming PIC hydrogels in aqueous media were reported. With a design based on previously reported DCH$_{\mathrm{PIC}}$ and hydrophobically associated multiblock copolypeptides,[6,18] the pentablock hydrogels were found to possess substantially enhanced stiffness compared to diblock and triblock samples at equivalent concentrations. In addition to adjusting concentration, hydrogel properties could also be tuned by mixing 'mismatched' block architectures in different ratios. All hydrogels were capable of rapid self-healing after deformation, with pentablock samples showing the fastest complete recovery. The use of multiblock architectures in PIC copolypeptide hydrogels was found to impart these physical assemblies with significantly enhanced mechanical properties, while retaining self-healing ability and stability against dilution in aqueous media. It is expected that the multiblock hydrogels will also possess good cell compatibility, similar to that shown for the diblock DCH_{PIC}.[6]

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.#######.

> Experimental procedures, spectral data, rheology data, hydrogel swelling data, and methods for all hydrogel characterization (PDF).

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Notes

The authors declare no competing financial interest.

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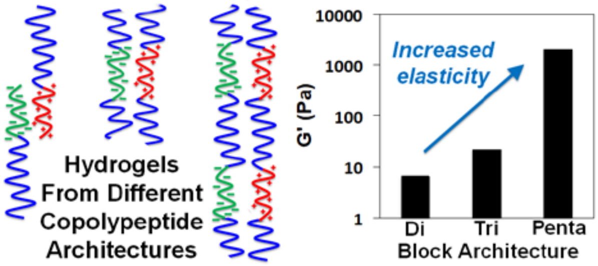
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