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Unusual Salt Stability in Highly Charged Diblock Co-polypeptide Hydrogels

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Abstract: The stability and properties of dilute solution hydrogels, synthesized by transition metal mediated polymerization of amino acid *N*-carboxyanhydrides (NCAs), have been studied in deionized (DI) water as well as various ionic media. These hydrogels are diblock amphiphilic copolymers of hydrophilic, charged segments of poly(L-lysine HBr) or poly(L-glutamic acid sodium salt), and helical, hydrophobic segments of poly(L-leucine). While many of these samples are able to form strong gels in deionized water at polymer concentrations as low as 0.25 wt %, stability in salt or buffer solutions was found to be only achieved at moderately higher polymer concentrations (~3.0 wt %). We have adjusted relative copolymer compositions and molecular weights to optimize hydrogel strength and polymer solubility in salt concentrations up to 0.5 M NaCl, as well as in cell growth media and aqueous buffers of varying pH. These materials are unique since they do not collapse in high ionic strength media, even though gel formation is contingent upon the presence of highly charged polyelectrolyte segments. The remarkable properties of these hydrogels make them excellent candidates for use as scaffolds in biomedical applications, such as tissue regeneration.

Hydrogels are found in a broad array of applications, including in cosmetics, as food additives, and as biomedical materials.[1] Typically, these gels are composed of physically or chemically cross-linked hydrophilic polymers[2] or networks of fibers composed of self-assembled small molecules.[3] Often, an abundance of like-charged groups on the polymers or fibers is crucial to the formation of gel networks, which are swollen due to electrostatic forces.[4] The polyelectrolyte nature of many hydrogels also plays an important role in their use in applications by promoting or preventing interactions with charged polymers, biomolecules, or surfaces.[5] The high charge density can also limit the utility of these materials since the electrostatic interactions that stabilize these gels are in many cases highly sensitive to dissolved solutes. That is, the addition of salts, buffers, media, or serum proteins to polyelectrolyte hydrogels typically results in their collapse or precipitation.[2] Here, we report on polyelectrolyte hydrogels, made from block co-polypeptides, that are remarkably stable to high ionic strength media as well as charged biomolecules (i.e., serum proteins). This unprecedented stability results directly from self-assembly of hydrophobic α-helical domains, a unique gelation mechanism that does not rely on polyelectrolyte stretching to support the hydrogel network.

Recently, we discovered a class of amphiphilic diblock co-polypeptides composed of highly charged, hydrophilic (poly(L-lysine-HBr) or poly(L-glutamate-Na)), and α-helical hydrophobic (poly(L-leucine) domains that are able to form rigid hydrogels at low concentrations (>0.25 wt %) in deionized (DI) water.[6] The rodlike chain conformations of the hydrophobic segments were found to be crucial to hydrogel formation since they direct assembly of these polymeric surfactants into highly anisotropic network structures rather than spherical micelles.[7] Unlike many peptide systems, which form hydrogels via formation of long fibrils and tapes derived from assembly of β-sheets,[8] the packing of helices in our system allows fine adjustment of hydrogel strength, independent of polypeptide concentration, via manipulation of the size of the helical poly(L-leucine) segments.[6] Compared to protein-based hydrogels,[1b] which can also be composed of helical domains, our materials are unique in their stability to heat, displaying no loss in strength at temperatures (80−90 °C) that thin most protein gels (e.g.,

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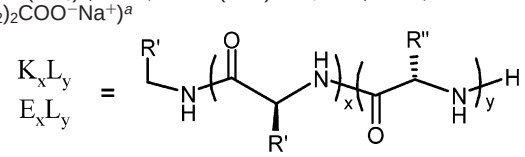
gelatin). In fact, they can be autoclaved without any loss of strength. The physical nature of these block copolymer assemblies imparts other attractive properties to the gels, such as rapid recovery of gel strength after shear thinning that permits application of the hydrogel via injection through a needle. This feature, in combination with the potential degradability of the peptide backbone and the inherent functionality of the gel surface (i.e., lysine residues that might provide cell adhesion points[5] or sites for drug or growth factor attachment[9]), makes these materials intriguing candidates for biomedical applications such as drug delivery and tissue engineering.[10]

A potential important issue in the use of block co-polypeptide hydrogels for these applications, however, is their compatability with buffers, salts, and serum. All previous studies of the hydrogels were performed in deionized water.[6] Based on the properties of other polyelectrolyte hydrogels,[2] it was possible that the gels would collapse in the presence of added ions (e.g., 100 mM NaCl). Added salt generally acts to screen the many like charges along the chains, weakening the forces responsible for chain stretching and interchain repulsion that typically support these gel networks. Such behavior is seen in chemically cross-linked poly(acrylic acid) gels, for example.[2] Since our hydrogels are supported by a mechanism entirely different from other polyelectrolyte hydrogels, we hoped that they might be able to tolerate high ionic strength solutions. To achieve this goal, an understanding of the key parameters affecting hydrogel properties was required. The helical conformation of the hydrophobic segments was known to drive formation of the membraneous network, and the size of these domains was found to correlate well with overall gel strength.[6] However, the role of the polyelectrolyte domains in hydrogel formation, strength, and stability was vague. Since added salt will primarily affect the properties of these charged domains, we focused our attention on this portion of the block copolymers.

Results

Polyelectrolyte Segment Length. In our previous studies, we found that the hydrophobic oligo(L-leucine) domain of a block copolypeptide must be at least 20 residues long to form a stable α-helical conformation.[6] The presence of this ordered structure was found to be a strict requirement for hydrogel formation in dilute solutions (<5 wt %) with gel strength proportional to the size of the hydrophobe domain. To demonstrate this quantitatively, we prepared three samples with the same overall average length (ca. 200 residues) but with hydrophobic compositions ranging from an average of 10−20 mol % (Table 1). As expected, the minimum concentrations for gelation decreased and the gel strengths increased dramatically with increasing hydrophobe content. Furthermore, as the hydrophobic segments were increased, the gels became more brittle (Figure 1). In the $K_{180}L_{20}$ sample, the storage modulus G' increased with strain amplitude γ_0 before the gel eventually broke down (strain-hardening). In contrast, gels with larger hydrophobic domains were stronger but broke down at much lower strain amplitude without exhibiting strain-hardening.

Table 1. Gelation Concentration and Gel Strength for a Variety of K_xL_y and E_xL_y Diblock Co-polypeptide Samples (R″ = $-CH_2CH(CH_3)_2$; for K, R′ = $-(CH_2)_4NH_3^+Br^-$; for E, R′ = $-(CH_2)_2COO^-Na^+$)[a]



sample	gelation concn (wt %)	gel strength (Pa) DI water	gel strength (Pa) 100 mM NaCl
$K_{80}L_{20}$	no gel at 6%	NA	NA
$K_{180}L_{20}$	2	12	37
$K_{170}L_{30}$	0.75	590	517
$K_{160}L_{40}$	0.25	4273	278
$K_{380}L_{20}$	0.25	146	153
$K_{370}L_{30}$	0.031	940	468
$K_{360}L_{40}$	0.125	480	242
$E_{180}L_{20}$	0.5	124	469
$E_{160}L_{40}$	0.25	265	47

[a] The gelation concentration was determined in DI water and is defined as the lowest concentration for which the storage modulus G' exceeds the loss modulus G'' at an angular frequency $\omega = 1$ rad/s. The gel strength refers to the value of G' at 1 rad/s and was measured for 3.0 wt % samples in both DI water and 100 mM NaCl. NA = experiments not applicable or not performed.

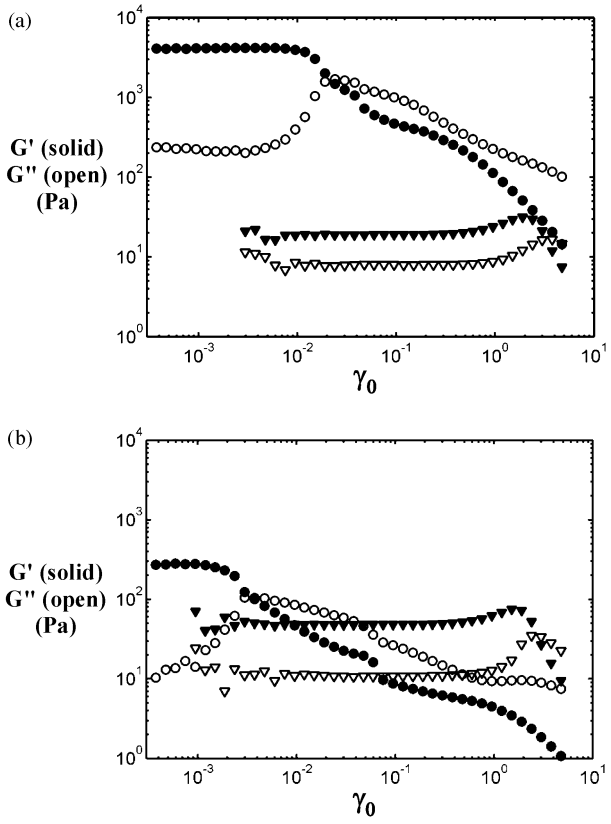


Figure 1. Strain behavior of diblock copolypeptides with and without added salt. (a) Strain amplitude (γ_0) sweep of 3.0 wt % samples in DI water at angular frequency $\omega = 6$ rad/s, storage modulus G' (solid symbols) and loss modulus G'' (open symbols): (▼) $K_{180}L_{20}$ and (●) $K_{160}L_{40}$. (b) Strain amplitude sweep of the same materials at 3.0 wt % in 100 mM NaCl solution.

To probe the role of the polyelectrolyte segments in these materials, we prepared three additional samples with the minimal hydrophobe length of 20 residues, where the size of the polyelectrolyte domain, poly(L-lysine), was varied from 80 to 380 average residues in length (Table 1). The most dramatic

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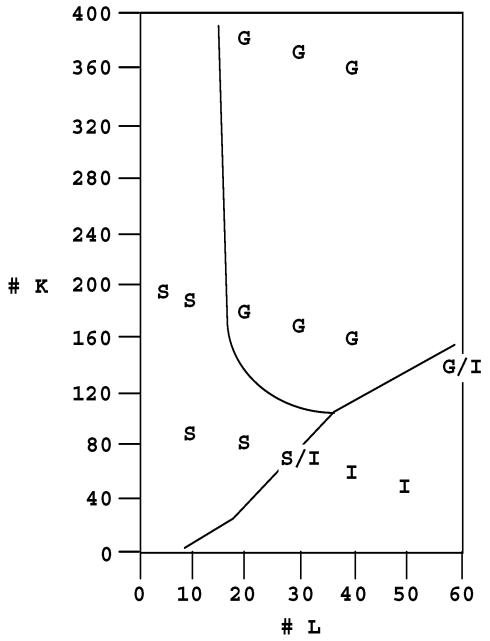

Figure 2. Properties of K_xL_y diblock co-polypeptides at 3.0 wt % in DI water. #K = average number of L-lysine residues in diblock copolymer. #L = average number of L-leucine residues in diblock copolymer. G = hydrogel. S = fluid clear solution. I = insoluble aggregates present. Solid lines delineate approximate boundaries between phases.

feature observed with these samples was the effect of polyelectrolyte length on minimum gelation concentration. The smallest sample, $K_{80}L_{20}$, although possessing the greatest percentage of hydrophobe, did not form a hydrogel even at concentations up to 6.0 wt %. This result was consistent with earlier observations that block co-polypeptides of average length of 100 residues or less are not good hydrogel formers.[6] The middle sample, $K_{180}L_{20}$, was able to form hydrogels at concentrations above 2.0 wt %. A more striking result, however, was that the largest sample, $K_{380}L_{20}$, which contains the smallest fraction of hydrophobe, formed stronger hydrogels at significantly lower concentrations (0.25 wt %). It should be noted that the increase in gel strength seen here was much smaller than had been seen for changes in the hydrophobe segment length (e.g., $K_{160}L_{40}$ vs $K_{180}L_{20}$ in Table 1). Regardless, it was apparent that lengths of the poly(L-lysine) segments correlated strongly with the ability of these block co-polypeptides to form hydrogel network structures. The effect of polyelectrolyte length on network strength, however, was small relative to changes in hydrophobe length.

The data presented above and those for other samples allowed construction of a crude "phase diagram" for these block copolymers as functions of lysine and leucine content, as well as polymer size (Figure 2). It can be seen that, at very high lysine fractions, the samples do not gel and will only form viscous fluids. At high leucine fractions, the samples form inhomogeneous precipitates that also do not gel water. The gel region exists for intermediate compositions containing at least 75 mol % lysine, a minimum leucine domain of 20 residues, and overall size greater than 100 residues. From a practical standpoint, gel-forming block co-polypeptides with chain lengths greater than 400 residues may not be as desirable due to their slow dissolution in water, which can take days compared to the few hours required for shorter samples (i.e., 200-mers). Also, the $K_{360}L_{40}$ sample was observed to have a lower gel strength

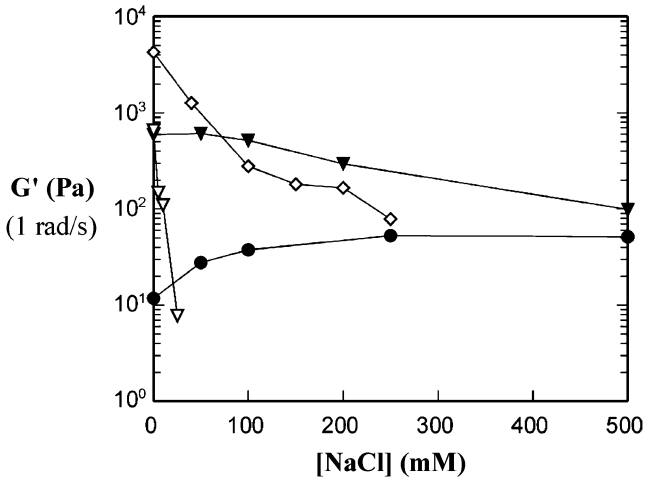

Figure 3. Hydrogel strength (G') as a function of salt concentration for different K_xL_y diblock copolypeptides. Gel strengths were measured at 1 rad/s: (●) 3.0 wt % $K_{180}L_{20}$, (▼) 3.0 wt % $K_{170}L_{30}$, (◇) 3.0 wt % $K_{160}L_{40}$, and (▽) 1.0 wt % $K_{160}L_{40}$.

than the $K_{160}L_{40}$ sample, possibly related to incomplete dissolution or hindered hydrophobe packing due to the longer lysine segment. Analogous samples prepared with negatively charged polyelectrolyte domains, i.e., poly(L-glutamate), were found to behave similarly (Table 1).

Hydrogels in the Presence of Added Salts. To evaluate the stability of the block co-polypeptide hydrogels to added salts, we first examined the rheological properties of the 200-residue copolymers ($K_{160}L_{40}$, $K_{170}L_{30}$, and $K_{180}L_{20}$) in the presence of NaCl (Table 1, Figure 3). The strongest gel-former in DI water, $K_{160}L_{40}$, was surprisingly the least stable to added salt. At a concentration of 1.0 wt %, the copolymer was seen to precipitate upon the addition of even small amounts of NaCl (ca. 50 mM) and the sample became fluid. At 3.0 wt %, the gel was more stable but became turbid and also weakened considerably with salt. The inherently much weaker gel formed from $K_{180}L_{20}$ showed the most surprising behavior with added salt. At 3.0 wt %, these weak gels actually became stronger when salt was added and were able to remain homogeneous to NaCl concentrations above 0.50 M. Thus, it appeared that salt stability and inherent gel strength were somewhat mutally exclusive. Advantageously, the $K_{170}L_{30}$ copolymer showed intermediate behavior at 3.0 wt %, forming a relatively strong gel that was able to maintain most of its strength and show very little turbidity upon addition of salt. This copolymer seemed to be the optimal choice for further study, since it possessed both good gel strength and salt tolerance.

Hydrogels formed from 3.0 wt % samples of $K_{170}L_{30}$ in water were subjected to a variety of buffers and ionic media to asses the range of their stability toward ions (Table 2). The divalent sulfate anion was found to decrease the strength of gels more significantly than chloride, yet the gels retained their mechanical integrity and did not become cloudy. The presence of divalent cations, such as calcium, qualitatively caused no appreciable effects other than those as seen with NaCl. The polymers were also dissolved and stable in a series of simple buffers of different pH, ranging from pH 4 to pH 9. Hydrogel samples were also prepared in DMEM (Dulbecco's modified eagles medium) and DMEM with 5% fetal calf serum and penicillin as representative cell culturing media. The $K_{170}L_{30}$ hydrogels were stable and remained transparent even in the presence of the varied ions,

Table 2. Gel Strength of $K_{170}L_{30}$ and $E_{180}L_{20}$ Diblock Co-polypeptide Samples in Various Media[a]

buffer	gel strength (Pa)	
	$K_{170}L_{30}$	$E_{180}L_{20}$
DI water	590	101
100 mM NaCl	517	438
50 mM Na_2SO_4	209	NA
50 mM $CaCl_2$	NA	253
pH 4.0 citrate	367	NA
pH 7.4 PBS	360	NA
pH 9.0 TRIS/HCl	729	NA
pH 9.0 glycine/NaOH	683	NA
DMEM	518	195
DMEM + 5% FCS	485	194

[a] All samples were prepared as 3.0 wt % solutions,and gel strength was measured at 1 rad/s. NA = experiments not applicable or not performed. All pH buffers were 50 mM. DMEM = Dulbecco's modified Eagles medium. FCS = fetal calf serum.

proteins, and other organics found in these complex solutions. As can be seen in Table 2, a similar but oppositely charged copolymer, $E_{180}L_{20}$, also showed excellent stability toward added salt and other ionic media.

Discussion

Relation of Hydrogel Strength to Molecular Architecture. Our measurements on lysine−leucine diblock co-polypeptides of different compositions and chain lengths revealed many trends pertinent to hydrogel formation and strength. Within the gel region, the size of the hydrophobic oligoleucine domain was found to dramatically affect hydrogel strength. This is reasonable since we have shown that the copolymers assemble into foamlike networks where the helical oligoleucine segments make up the core of the scaffold.[7] The side-by-side packing of the helical rods drives formation of these extended assemblies and disfavors formation of isotropic structures such as spherical micelles. The length of the oligoleucine domain controls the thickness and stability of the scaffold core, which in turn supports the hydrogel network. Thus, as the hydrophobic domain size is increased, the scaffold is strengthened and the modulus of the gel increases dramatically. When the size of the oligoleucine domain is smaller than ca. 20 residues, the chain is no longer in the α-helical conformation.[6] The consequence of this is that the block copolymers no longer pack into extended membranes and a gel network does not form. Even with helical 20 residue hydrophobic segments, packing was not efficient, as evidenced by the observed strain-hardening in the $K_{180}L_{20}$ sample. The deformable network of this sample, compared to the brittle networks of those with larger hydrophobic segments, suggests the helices are not densely packed at rest. At the other extreme of composition, when the hydrophobic domains are very large (i.e., ca. >50 residues), or make up >30% of the total composition, extended structures likely form but hydrophobic interactions dominate and the assemblies precipitate from solution. Hence, there is a limit to obtaining increased gel strength via hydrophobe tuning alone.

The charged poly(L-lysine) segments were also found to have some influence on hydrogel strength. This effect, however, was small when compared to the hydrophobe component (i.e., compare strengths of $K_{380}L_{20}/K_{180}L_{20}$ vs $K_{160}L_{40}/K_{180}L_{20}$, Table 1) and showed that polyelectrolyte repulsions are only mildly involved in strengthening the gel network. However, the dramatic effect of polyelectrolyte length on gelation concentration suggests that poly(L-lysine) length does play a significant role in dictating gel network morphology. In addition to providing solubility to the hydrophobic domains, the longer polyelectrolyte segments seem to be affecting the structure of the scaffolds themselves, either by changing curvature or defect density in membranes or by increasing twist in fibrillar assemblies.[3] Such processes are reasonable since one can view the polyelectrolyte segments as a densely grafted polymer "brush" on the self-assembled, hydrophobic surface.[11] Increasing the curvature or twist at this interface will give more volume to the polyelectrolyte segments, which is favored by the interchain electrostatic repulsions. The larger the polyelectrolyte segments, the greater the driving force to curve or distort the assemblies. A balance is achieved, however, since the rodlike hydrophobic segments are most stable as flat 2D membranes.[6,12] Thus, any distortion to stabilize the polyelectrolyte packing comes at the cost of destabilization of the hydrophobic helices. This frustration in self-assembly is the key to formation of these unique gel networks.

Hydrogel Salt Stability versus Copolymer Composition. We have found that the stability of block co-polypeptide hydrogels to added salt was highly dependent on hydrophobe composition. The $K_{160}L_{40}$ sample showed poor salt stability, both thinning and forming precipitate upon addition of NaCl. In many ways this behavior of $K_{160}L_{40}$ in salt was similar to the properties of the more hydrophobic material, $K_{140}L_{60}$, in salt-free water. As shown in Figure 2, $K_{140}L_{60}$ is partially insoluble in DI water and only forms viscous solutions, despite the large hydrophobic domain. With this sample, the polyelectrolyte segments are not large enough to effectively solubilize and stabilize the hydrophobic gel network. Qualitatively, it appears that added salt had a similar effect on the $K_{160}L_{40}$ sample by screening electrostatic interactions, resulting in reduced solubilizing ability and collapse of the network, essentially shifting the gel-insoluble transition in the "phase diagram" of Figure 2 to lower leucine content. Since both the $K_{170}L_{30}$ and $K_{180}L_{20}$ samples were able to maintain stable gel networks in added salt, we reasoned that salt was only detrimental to samples with very high hydrophobe compositions. In fact, at the other extreme of the gel region, it was found that salt enhanced gel strength in samples with high polyelectrolyte compositions (e.g., $K_{180}L_{20}$). In this case, we believe that the interchain repulsions of large polyelectrolyte segments in DI water prevent efficient packing of the small hydrophobic domains, resulting in very weak gels. Observance of strain hardening in $K_{180}L_{20}$ (Figure 1), but not in $K_{170}L_{30}$ and $K_{160}L_{40}$, also hinted that the hydrophobic helices were not inherently well-packed but could be packed into a stronger network under strain. Accordingly, $K_{180}L_{20}$ hydrogels displayed increasing brittleness as salt concentration was increased, indicating that the hydrophobes became better packed into stronger networks in ionic solutions. It seems plausible that the addition of salt relaxes the polyelectrolyte repulsions in these samples to allow better assembly of the hydrophobic network. The $K_{170}L_{30}$ sample, with intermediate composition, was consequently unaffected by these perturbations

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since they only affected samples with lysine/leucine ratios at the extremes of the gel-forming region.

The most remarkable feature of the $K_{170}L_{30}$ and $K_{180}L_{20}$ samples was the absence of any gel shrinkage upon addition of salt. Although a considerable fraction of charged polypeptide was found to be essential for gel formation in these materials, they differ significantly from all other polyelectrolyte hydrogels by being stable in both DI water and in high ionic strength media. Most polyelectrolyte-based gels rely on swelling of cross-linked charged chains in DI water to support the gel network.[2] When salt is added to such systems, the Coulombic interactions are screened and the gels collapse. Alternatively, in carrageenans, highly charged anionic polysaccharides, the polymers form viscous solutions in DI water and only gel upon addition of multivalent cations that act as cross-linking agents.[13] Similarly, some charged peptides will assemble into fibrillar gel networks upon addition of salt, which screens the like charges, but are soluble in DI water.[14] We have not found any other example where highly charged polymers are able to form hydrogels that are robust in both low and high ionic strength media.

The stability of the block co-polypeptide gels rests in their unique self-assembly mechanism. As we have found, the strength of the hydrogel scaffold arises from the packed α-helical hydrophobic domains. This scaffold is stabilized by the solubility and sterics of the polyelectrolyte chains but is not primarily supported by electrostatic interactions: the polyelectrolyte segments were found to only contribute a small amount to the strength of the gel scaffold. Therefore, it appears that as long as the polyelectrolyte segments are large enough to solubilize the hydrophobic scaffold, these hydrogels will not be substantially affected by ionic species in the solution. Similarly, gel volume is also controlled by the size of the hydrophobic membranous network and not dependent on polyelectrolyte stretching. The assembly mechanism seen in these block co-polypeptide hydrogels, namely, the association of α-helical segments into 3D networks, thus gives rise to a robust scaffold that is not critically dependent on environmental conditions. Although some protein-based hydrogels also assemble via helical associations, they differ greatly from our materials since they are all very temperature-sensitive, thinning at temperatures above 50 °C.[1b] The block co-polypeptide hydrogels do not thin up to the boiling point of water. These comparisons illustrate the novelty of the gelation mechanism in these amphiphiles and how it can give rise to properties that have not been realized in other materials.

Hydrogel Stability in Different Ionic Media. The optimized $K_{170}L_{30}$ copolymer was found to be reasonably stable in a variety of ionic media (Table 2). The most destabilizing media were those containing multivalent anions such as phosphate or sulfate. These ions are more efficient at condensing and possibly cross-linking the polyelectrolyte segments, effectively decreasing their solubility in water.[15] Once the solubility limit of the polyelectrolyte is reached, the gels will collapse, as we have noted. Even so, there is enough compositional flexibility in these materials to allow preparation of hydrogels stable to almost any ionic media. For example, the $K_{180}L_{20}$ hydrogel strengthens dramatically in the presence of divalent sulfate,[12] and $E_{180}L_{20}$ strengthens in divalent calcium (Table 2). The hydrogels were also stable over a broad pH range, essentially remaining intact as long as the polyelectrolyte segments were not neutralized. Gel strengths in these media were found to vary moderately but seemed to depend more on the composition of the buffer than the pH of the solution. This result is not too surprising since each buffer contains ions of different valency and since the charge density of the polyelectrolyte segments was unaffected in this pH range (pK_a(av) of poly(L-lysine) ≈ 10.5). The more unusual result was that the $K_{170}L_{30}$ gel was stronger in buffer at pH 9 than in deionized water, even with different salt compositions.

The stability of the $K_{170}L_{30}$ copolymer in cell culturing media and serum was also somewhat surprising, since these media contain numerous multivalent ions and anionically charged proteins. It is likely that the proteins coat the poly(L-lysine) segments in the gel since it is known that poly(L-lysine) homopolymer will coagulate many serum proteins in solution.[5] Apparently, the resulting polyelectrolyte complexes retain enough overall charge to solubilize the hydrophobic gel scaffold and prevent precipitation and collapse of the network. In the event that binding of serum proteins is disadvantageous, the glutamic acid based gels provide a good alternative. Their anionic character should act to repel the anionic serum proteins, as evidenced by the enhanced stability of $E_{180}L_{20}$ to DMEM + serum. Overall, the results detailed above illustrate the versatility of these unusual hydrogels for applications in a variety of ionic media. We have performed some initial cell culturing studies with these materials, which will be reported separately.[16]

Conclusions

The unique mechanism of gelation displayed in amphiphilic block co-polypeptide hydrogels was found to impart these materials with a number of desirable properties. They are injectable, can be sterilized by autoclave, are enzymatically degradable, require only small concentrations of material,[6] possess both nano- and microporosity,[7] and are stable both in the presence and absence of ionic species. Although some of these attributes can be found in other hydrogels, we know of no other single material that possesses all of these characteristics. While a balance of polyelectrolyte-to-hydrophobe ratio and overall chain length were found to be important in obtaining strong hydrogels, this system does allow considerable adjustment of these parameters to optimize the hydrogel properties for a specific application. Most notable is the ability to prepare either positively or negatively charged hydrogels, which may prove especially useful in biomedical applications.

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Supporting Information Available: Details of all materials and measurements (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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