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Controlled synthesis and properties of poly(L-homoserine)

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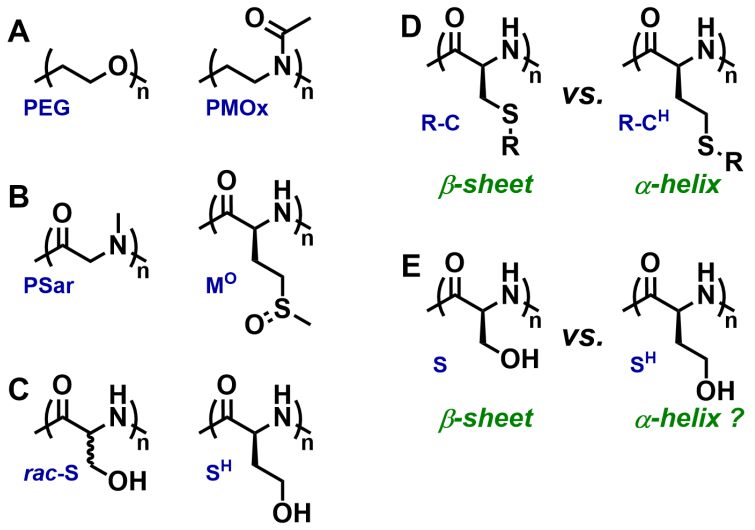
Abstract We report preparation of a new water soluble, non-ionic homopolypeptide poly(L-homoserine) as well as poly(L-homoserine) block copolymers with controllable segment lengths. The conformational preferences of poly(L-homoserine) were also determined in both the solid-state and in solution. Poly(L-homoserine) is soluble in water and adopts a disordered conformation that makes it a promising addition to the small class of non-ionic, water soluble homopolypeptides with potential for development for applications in biology. Toward this goal, a poly(L-homoserine) containing block copolypeptide was prepared and found to assemble into micro- and nanoscale vesicles in water.

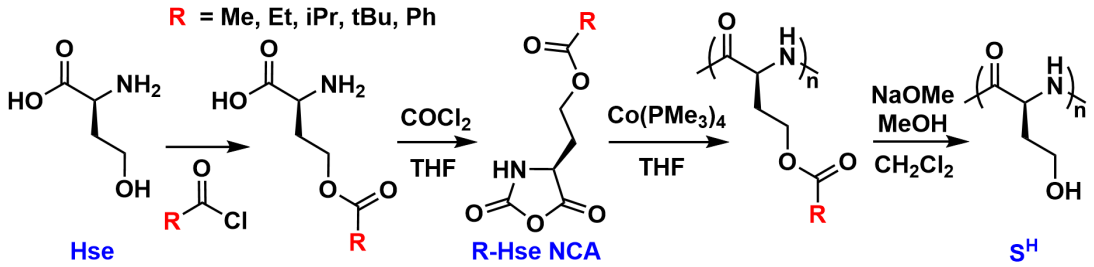
Water soluble, non-ionic polymers that possess non-fouling properties and compatibility with biological systems are useful for a range of biomaterial and therapeutic delivery applications.[1,2] Such polymers can be used for steric stabilization and solubilization of nanostructures as well as shielding of therapeutics from host immune systems. Polyethylene glycol (**PEG**) has been widely used in such applications (Scheme 1a), but its widespread use, lack of degradability and consequent accumulation in organs has led to the development of allergic reactions to **PEG** in an increasing percentage of the human population.[3-5] Due to concerns about widespread use of **PEG** in humans, a variety of hydrophilic polymers

are under investigation as alternatives to **PEG**, including polyoxazolines (e.g. **PMOx**),[6] polysarcosine (**PSar**),[7,8] poly(L-methionine sulfoxide) (**M^{O}**),[9-11] as well as oligoethylene glycol modified polypeptides[12-16] (Scheme 1a,b). In addition to their non-fouling properties, amino acid based **PSar** and **M^{O}** are especially promising since they can be readily degraded to natural metabolites in biological environments,[7-11] thus avoiding accumulation *in vivo*. Recently, water soluble poly(D/L-serine), ***rac*-S**, has also been proposed as a biocompatible polymer (Scheme 1c),[17-19] where incorporation of heterochiral residues is essential to prevent formation of water insoluble β-sheet structures as observed for poly(L-serine), **S**.[20-22] Here, we have investigated the synthesis and properties of poly(L-homoserine), **S^{H}**, (Scheme 1c), a water soluble, non-ionic homopolypeptide that shows potential for applications in biology.



Scheme 1. Non-ionic polymers under investigation for biological applications. a) Non-degradable polyethylene glycol (**PEG**) and polymethyloxazoline (**PMOx**). b) Degradable polysarcosine (**PSar**) and poly(L-methionine sulfoxide) **M^{O}**. c) More recently investigated poly(D/L-serine) (***rac*-S**) and poly(L-homoserine) (**S^{H}**). d) Poly(S-alkyl-L-cysteines) (**R-C**) typically form β-sheets while poly(S-alkyl-L-homocysteines) (**R-C^{H}**) form α-helices. e) Poly(L-serine) (**S**) forms β-sheets while poly(L-homoserine) (**S^{H}**) is hypothesized to be α-helical.

Homopolymers of the non-ionic, polar amino acid L-serine have attracted attention for some time.[20-22] Early efforts to prepare **S** gave water soluble polymers that were of interest as protein mimics.[21] However, it was later discovered that these soluble polymers had undergone racemization to ***rac*-S**, and that the homochiral **S** is insoluble in water due to formation of β-sheet structures.[22] While ***rac*-S** has been found to be promising for biomaterials applications,[17-19] its widespread usage may be limited by its lack of stereoregularity due to heterogeneity of D- and L-residue sequences within and among chains as well as its susceptibility to degradation under mild aqueous conditions.[20] Inspired by prior research that showed homologs of β-sheet forming poly(S-alkyl-L-cysteines), **R-C**, i.e. poly(S-alkyl-L-homocysteines), **R-C^{H}**, can adopt soluble α-helical conformations in water,[23,24] we were curious if a similar conformational change would occur upon homologation of **S** to **S^{H}** (Scheme 1d,e). This hypothesis is supported by anecdotal information from Fasman and Blout, who reported that a sample of **S^{H}** adopted an α-helical conformation in HFIP and remarked it possessed "a small helical content" in water.[25,26] However, no synthetic details or molecular characterization data for this **S^{H}** sample were provided. In related work, Goodman and Felix reported the synthesis and properties of the further homolog poly(δ-hydroxy-L-α-aminovaleric acid), i.e. "poly(L-homohomoserine)", which was found to adopt an α-helical conformation in aqueous 2 M LiBr.[27] Due to the increased hydrophobic content of its side-chains, this polypeptide was not soluble in water at LiBr concentrations less than 0.75 M. Due to the anticipated water solubility and limited available data for **S^{H}**,[25,26] we decided to pursue the controlled synthesis of this polypeptide and investigate its properties, particularly in aqueous media.



Scheme 2. Synthesis of L-homoserine (Hse) derivatives, their corresponding R-Hse NCA monomers, and polymerization of NCAs to give poly(L-homoserine) (**S^{H}**) after side-chain deprotection.

With the ultimate goal of preparation of **S^{H}** containing block copolypepides with controlled compositions and end groups, we recognized the importance of obtaining a soluble **S^{H}** precursor to facilitate chain length control and efficient chain extensions. Since the ester protected monomer, O-acetyl-L-homoserine N-carboxyanhydride (Me-Hse NCA, Scheme 2) had been reported but not polymerized,[28] we began our studies with this derivative. Although Me-Hse NCA is prepared in good yields and is readily purified by recrystallization (see supporting information (SI) Table S1), we found that the resulting poly(O-acetyl-L-homoserine) formed gels during polymerizations in THF, DCM or DMF, which in some cases also prevented complete monomer conversion (see SI Table S2). Consequently, we synthesized a series of new ester protected R-Hse NCAs (Scheme 2) to identify the best monomer(s) for high polypeptide solubility and yield (see SI Tables S1, S2). In terms of both monomer yield and ease of purification, the Me- and tBu-Hse NCAs were most promising. However, homopolymers of both these NCAs gelled during polymerization, while homopolymers of Et- and iPr-Hse NCAs were found to give soluble polymers in THF. Rather than use the more difficult to purify Et- and iPr-Hse NCA oils, we chose to instead focus on mixtures of the crystalline Me- and tBu-Hse NCAs as a means to increase polypeptide solubility, as has been shown with other NCA copolymerizations.[10,29]

It was found a 1:9 mixture of Me- and tBu-Hse NCAs was found to readily copolymerize using $Co(PMe_3)_4$ in THF at 20 °C,[30] giving soluble poly(pivaloyl-L-homoserine$_{0.90}$-*stat*-acetyl-L-homoserine$_{0.10}$), **tBu/Me-S^{H}**, copolypeptides with chain lengths that were controlled by variation of monomer to initiator ratio (Figure 1a), and molecular weight distributions that remained narrow and monomodal (Figure 1a,b, see SI Figure S1). This comonomer composition was used for all subsequent polymerization studies. Conformational data for **tBu/Me-S^{H}** were obtained from analysis of FTIR and

circular dichroism (CD) spectra (Figure 1c,d), which were consistent with this polypeptide adopting an α-helical chain conformation in both solution and solid state.[26]

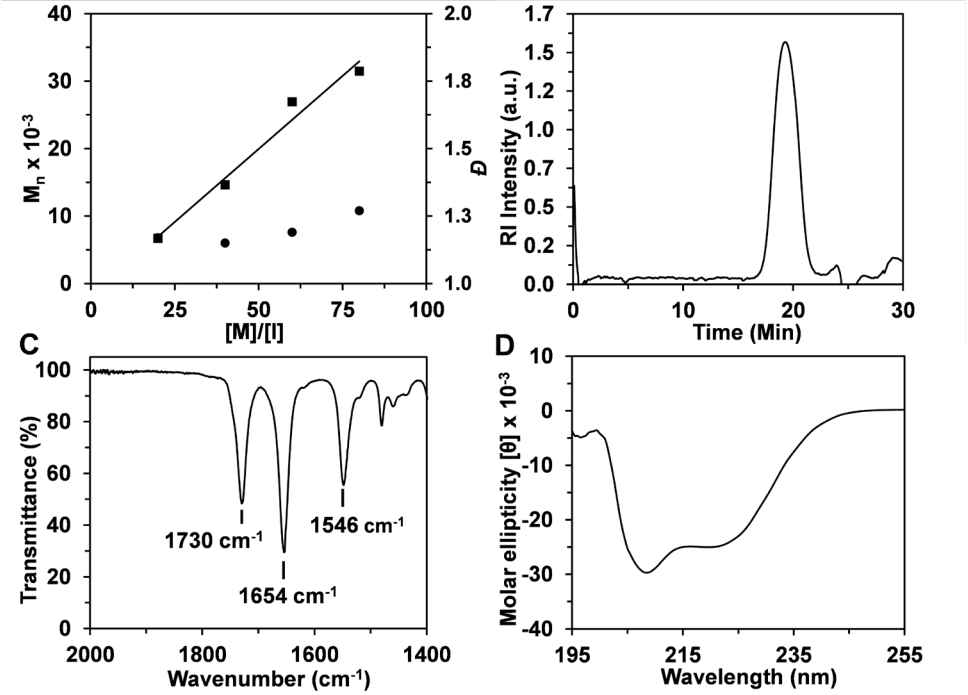


Figure 1. Synthesis and properties of poly(pivaloyl-L-homoserine$_{0.90}$-*stat*-acetyl-L-homoserine$_{0.10}$), **tBu/Me-S^{H}**. a) Variation in molecular weight, determined by ^{1}H NMR, and dispersity, determined by GPC, of **tBu/Me-S^{H}** as a function of monomer to initiator ratio (M:I) using $Co(PMe_3)_4$ in THF at 20 °C. b) GPC chromatogram of **(tBu/Me-S^{H})$_{174}$** in HFIP containing 0.5% (w/w) KTFA. c) Solid State FTIR spectrum showing the amide band region of **(tBu/Me-S^{H})$_{120}$**. The amide bands at 1654 and 1546 cm^{-1} are consistent with an α-helical conformation.[31] d) CD spectrum of **(tBu/Me-S^{H})$_{47}$** (0.1 mg/mL) in HFIP at 22 °C. Minima at 208 and 224 nm are consistent with the polymer adopting an α-helical conformation.[31] Molar ellipticity [θ] units: (deg·cm^2 ·dmol^{-1}).

The **tBu/Me-S^{H}** precursors were then deprotected under basic conditions, which gave the desired **S^{H}** homopolymers in excellent yields (*ca*. 90%, Scheme 2). At degrees of polymerization less than *ca*. 50, **S^{H}** was found to freely dissolve in DI water, yet **S^{H}** at higher degrees of polymerization only formed cloudy suspensions in water. We found that higher molecular weight **S^{H}** could be fully dissolved in water

by first dissolving the solid with a small amount of trifluoroacetic acid (TFA) to disrupt any preformed structures, and then diluting with DI water to give a solution of $\mathbf{S^H}$ in *ca.* < 10 % v/v aqueous TFA. Dialysis of this solution against DI water resulted in a solution of $\mathbf{S^H}$ in DI water. To better understand this phenomenon, we examined solid-state FTIR spectra of $\mathbf{S^H}$ as a function of chain length. The FTIR spectrum of $\mathbf{S^H_{47}}$ showed Amide I and Amide II bands characteristic for a disordered chain conformation, as well as a weak Amide I band indicating a small amount of β-sheet content (See SI Figure S2).[31] However, the FTIR spectrum for longer chain $\mathbf{S^H_{100}}$ showed weaker Amide I and Amide II bands for the disordered chain conformation, and a much stronger Amide I band indicating a significant amount of β-sheet content (Figure 2a, see SI Figure S2). Thus, it appears that $\mathbf{S^H}$ increasingly favors adoption of the β-sheet conformation in the solid state as chain length increases, which can explain the poor direct dissolution of longer $\mathbf{S^H}$ chains in water.

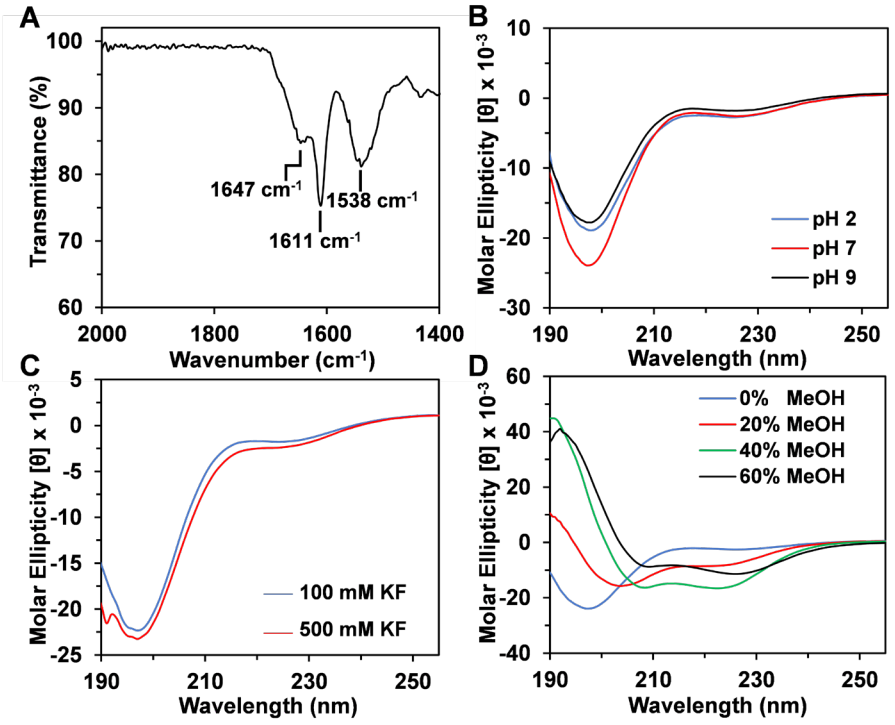


Figure 2. Properties of poly(L-homoserine), $\mathbf{S^H}$. (a) Solid State FTIR spectrum of the amide band region for $\mathbf{S^H_{100}}$. The bands at 1647 and 1538 cm^{-1} are indicative of a disordered conformation.[31] The Amide I

band at 1611 cm^{-1} is indicative of a β-sheet conformation.[31] (b) CD spectra of $\mathbf{S^H_{105}}$ at pH 2, 7, and 9 in DI water. (c) CD spectra of $\mathbf{S^H_{105}}$ in DI water containing either 100 mM or 500 mM KF. (d) CD spectra of $\mathbf{S^H_{105}}$ in DI water containing 0, 20, 40 or 60% (v/v) methanol. All CD spectra of $\mathbf{S^H}$ solutions (0.1 mg/mL) were recorded at 22 °C. Molar ellipticity [θ] units: ($deg \cdot cm^2 \cdot dmol^{-1}$).

In spite of β-sheet formation in the solid state, all lengths of $\mathbf{S^H}$ could be fully dissolved in DI water by initially using TFA to dissolve the solids as described above (see SI). Once dissolved in water, $\mathbf{S^H}$ was found by CD spectroscopy to primarily adopt disordered chain conformations that were minimally affected by variation of either solution pH or ionic strength (Figure 2b,c).[31] This result is somewhat consistent with Fasman and Blout's comment of $\mathbf{S^H}$ possessing "a small helical content" in water.[25,26] To understand how solvation affects the conformation of $\mathbf{S^H}$, we also recorded CD spectra of aqueous solutions of $\mathbf{S^H}$ with increasing concentrations of methanol (Figure 2d). Methanol is known to promote formation of α-helical conformations due to its low relative permittivity compared to water, resulting in weaker H-bonding with polypeptide functionality.[32] With increasing methanol content, the CD spectra of $\mathbf{S^H}$ showed increasing α-helical content, with $\mathbf{S^H}$ being predominantly helical in 40% v/v methanol.[31] The decreased intensity of CD bands in 60% v/v methanol is likely due to aggregation of $\mathbf{S^H}$, since the polypeptide was found to precipitate at methanol volume fractions greater than 60%. These results show that $\mathbf{S^H}$ can adopt α-helical conformations when side-chain solvation is less than in water, which also agrees with the finding of Fasman and Blout that their $\mathbf{S^H}$ sample was α-helical in HFIP.[26] However, the finding that non-ionic $\mathbf{S^H}$ possesses a disorded conformation in water is advantageous for its use as a solubilizing component in aqueous block copolymer assemblies.[33]

To confirm the ability to extend active polypeptide chains with $\mathbf{S^H}$ segments, block copolypeptides were prepared using L-methionine (Met) and O-*tert*-butyl-L-serine (tBu-Ser) NCA comonomers (Table 1). Block copolymerizations with Met NCA showed that $\mathbf{S^H}$ segments could be introduced either before or after Met NCA polymerization (see SI Schemes S1, S2). Chain lengths were controlled by variation of

monomer to initiator ratios, and monomodal distributions were observed by GPC confirming efficient chain extensions (see SI Figure S3). Note that protecting groups were removed and methionine residues were methylated to obtain samples with adequate solubility for GPC analysis (see SI Scheme S3). Since Poly(L-serine), **S**, is not water soluble,[22] we used this property to prepare 'amphiphilic' block copolypeptides composed solely of $\mathbf{S^H}$ and **S** segments (Table 1, see SI Scheme S4, Figure S3). The diblock copolypeptide $\mathbf{S^H_{110}S_{20}}$ was designed to possess a larger solubilizing $\mathbf{S^H}$ block relative to the water insoluble **S** block since we have previously determined that such compositions are amenable to formation of regular assemblies in water.[33]

Table 1. Synthesis of $\mathbf{S^H}$ containing block copolypeptides using $Co(PMe_3)_4$ initiator in THF at 20 °C.

First Segment			**Second Segment**		**Diblock Copolypeptide**[c]			
Monomer	**eq.**[a]	**DP**[b]	**Monomer**	**eq.**[a]	M_n[d] **(kDa)**	**DP**[d]	M_w/M_n[e]	**Yield (%)**[f]
tBu/Me-Hse NCA[g]	20	34	Met NCA	60	20.4	143	1.23	90
Met NCA	20	42	tBu/Me-Hse NCA	60	30.6	181	1.28	92
tBu/Me-Hse NCA	50	110	tBu-Ser NCA	8	23.3	130	1.27	85

Met NCA = L-methionine NCA. tBu-Ser NCA = O-*tert*-butyl-L-serine NCA. [a] Indicates total equivalents of monomer per $Co(PMe_3)_4$ initiator. [b] Degree of polymerization (DP) determined by end-group analysis after complete polymerization of first segments. [c] Data for diblock copolypeptides after complete polymerization of second segments. [d] Number average molecular weight (M_n) and DP determined by end-group analysis. [e] Determined by GPC-MALS. [f] Total isolated yield of diblock copolypeptides. [g]Mixture of tBu-Hse NCA and Me-Hse NCA (9 to 1 molar ratio).

To study assembly of $\mathbf{S^H_{110}S_{20}}$, the sample was labeled at the N-terminus with fluorescein-5-isothiocyanate (FITC), and then dispersed in water by first dissolving in TFA as described above followed by mild sonication of the resulting aqueous suspension. This procedure gave a slightly turbid suspension

of 1.0 mg/mL of FITC labeled $\mathbf{S^{H}_{110}S_{20}}$ in DI water (see SI). Imaging of this suspension using differential interference contrast (DIC) microscopy showed the presence of many small circular objects *ca*. 2.9 μm in diameter (Figure 3a). Using laser scanning confocal microscopy (LSCM), images of a z-slice through the objects revealed they are hollow, water filled assemblies, consistent with vesicle structures (Figure 3b). These assemblies were found to be stable upon storage at 20 °C for at least a week. In effort to reduce the size of these assemblies to nanoscale range, which would be more amenable for biological applications, a suspension of as-formed vesicles at 2.0 mg/mL in DI water was extruded through a series of polycarbonate track etch membranes with pore sizes ranging from 1.0 to 0.2 μm. Analysis of the resulting sample using dynamic light scattering (DLS) showed a monomodal distribution of assemblies with average diameter of 290 nm and PDI = 0.35 (Figure 3c). Based on these results we propose that $\mathbf{S^{H}_{110}S_{20}}$ can assemble into vesicles in DI water with diameters that can be adjusted from microns from hundreds of nanometers (Figure 3d).

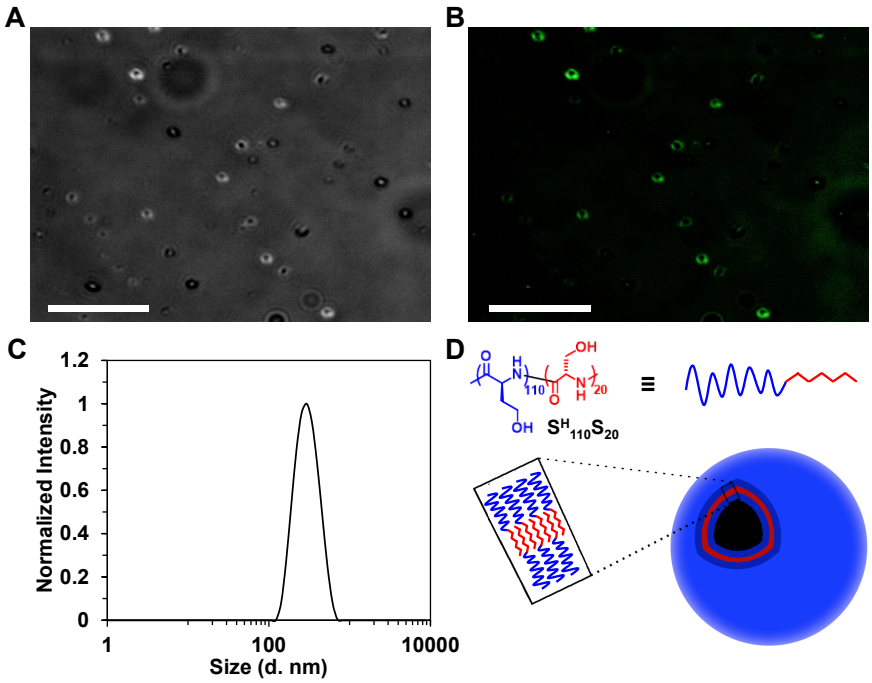


Figure 3. Assembly of diblock copolypeptide $\mathbf{S^{H}_{110}S_{20}}$ in DI water. (a) DIC microscopy image and (b) LSCM image, z-thickness = 0.78 μm, of fluorescein-5-isothiocyanate (FITC) labeled $\mathbf{S^{H}_{110}S_{20}}$ at 1.0 mg/mL in DI water at 20 °C (bars = 20 μm). (c) DLS data for assemblies of $\mathbf{S^{H}_{110}S_{20}}$ after extrusion

through polycarbonate track etch membranes of 1.0, 0.4 and 0.2 μm pore size at 2.0 mg/mL in DI water at 20 °C. (d) Schematic showing proposed vesicular assembly of $\mathbf{S^H_{110}S_{20}}$ in water.

In summary, we successfully prepared new $\mathbf{S^H}$ homopolymers and $\mathbf{S^H}$ containing block copolymers with controllable segment lengths. The conformational preferences of $\mathbf{S^H}$ were also determined in both the solid-state and in solution. The ability of $\mathbf{S^H}$ to adopt β-sheet conformations in the solid-state, disordered conformations in water and α-helical conformations in aqueous MeOH mixtures highlights how the different conformations of $\mathbf{S^H}$ are finely balanced and are strongly influenced by environment. The conformational preferences of $\mathbf{S^H}$ were also found to lie between its homologs **S** and poly(δ-hydroxy-L-α-aminovaleric acid). The disordered conformation and solubility of $\mathbf{S^H}$ in water make it a useful addition to the small class of non-ionic, water soluble homopolypeptides being investigated for biological applications. Toward this goal, we prepared a proof of concept $\mathbf{S^H_{110}S_{20}}$ block copolypeptide that was found to assemble into micro- and nanoscale vesicles in water.

Supporting Information

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

Experimental procedures, spectral data, and methods for polypeptide characterization (PDF).

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Notes

The authors declare no competing financial interest.

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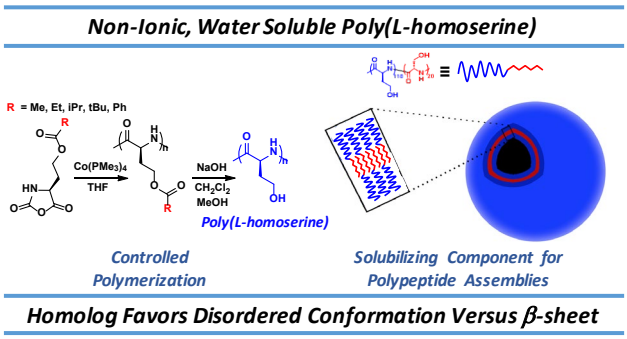
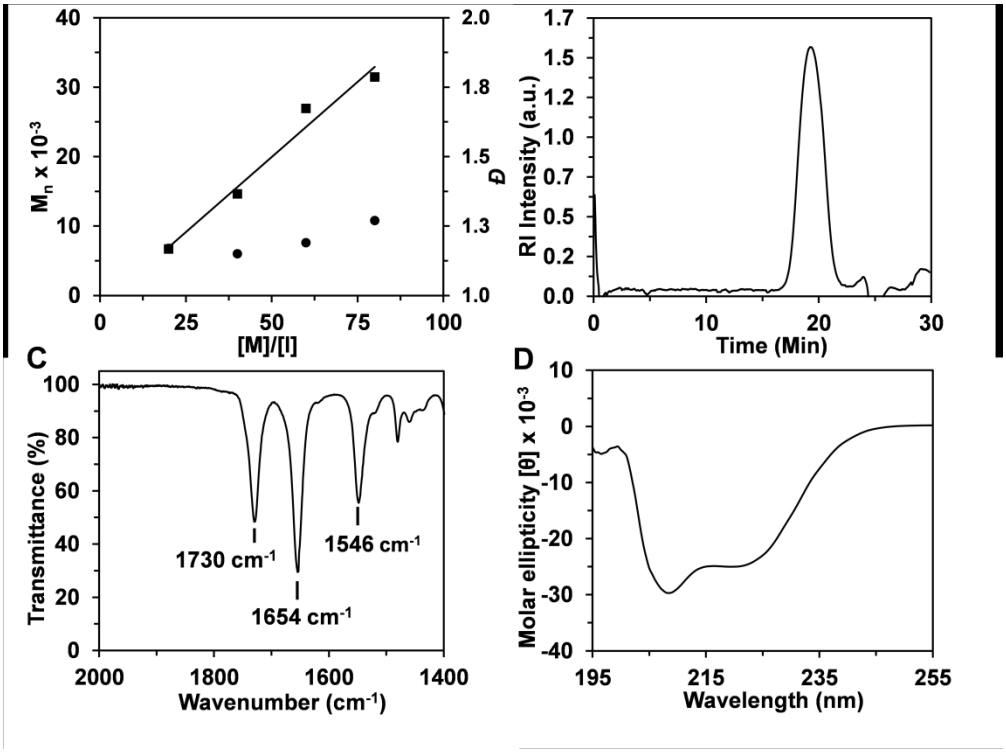
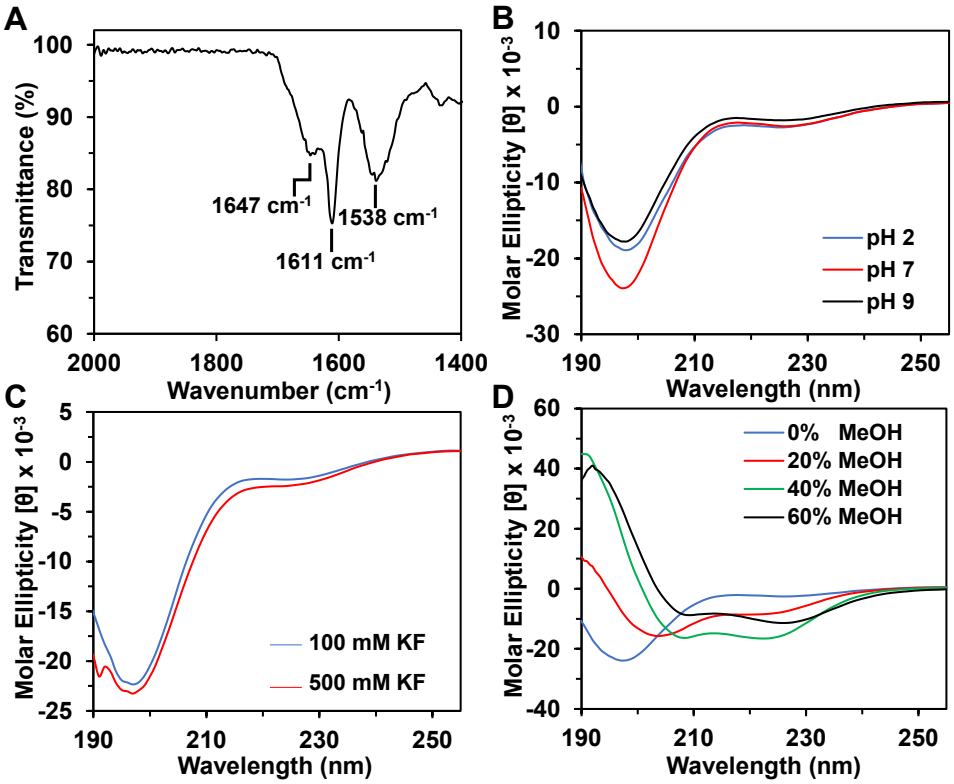
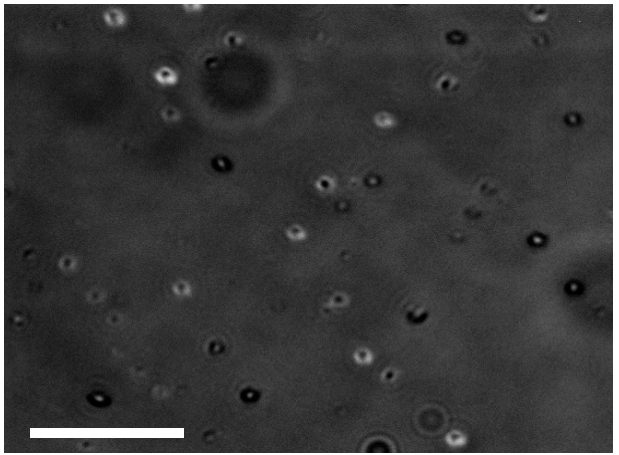
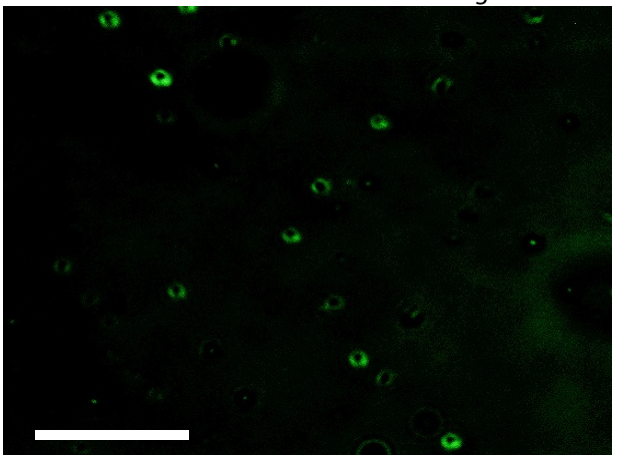

Non-Ionic, Water Soluble Poly(L-homoserine)
R = Me, Et, iPr, tBu, Ph
Co(PMe3)4
THF
NaOH
CH2Cl2
MeOH
Poly(L-homoserine)
Controlled Polymerization
Solubilizing Component for Polypeptide Assemblies
Homolog Favors Disordered Conformation Versus β-sheet

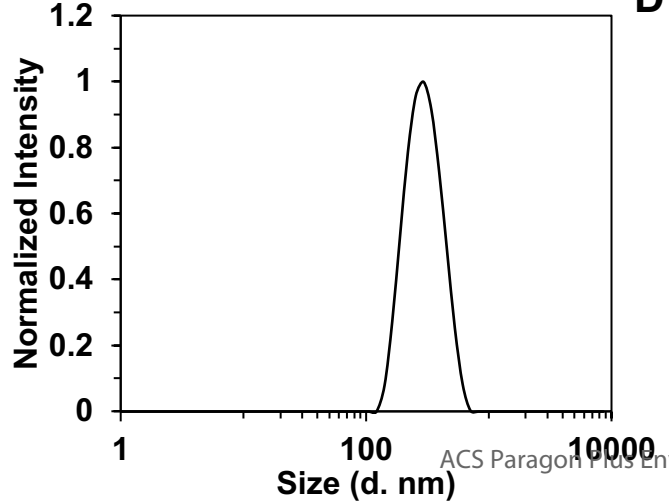


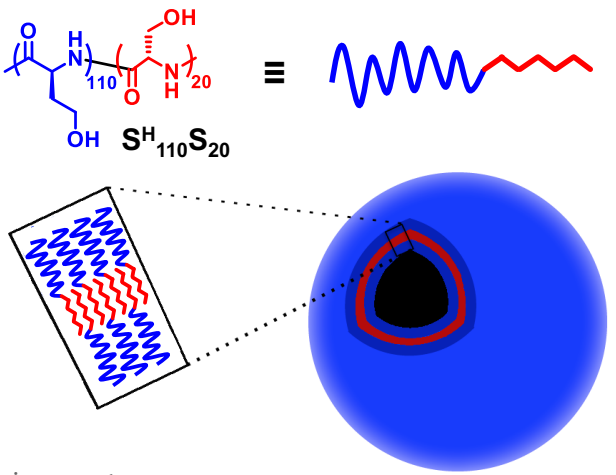
781x581mm (144 x 144 DPI)

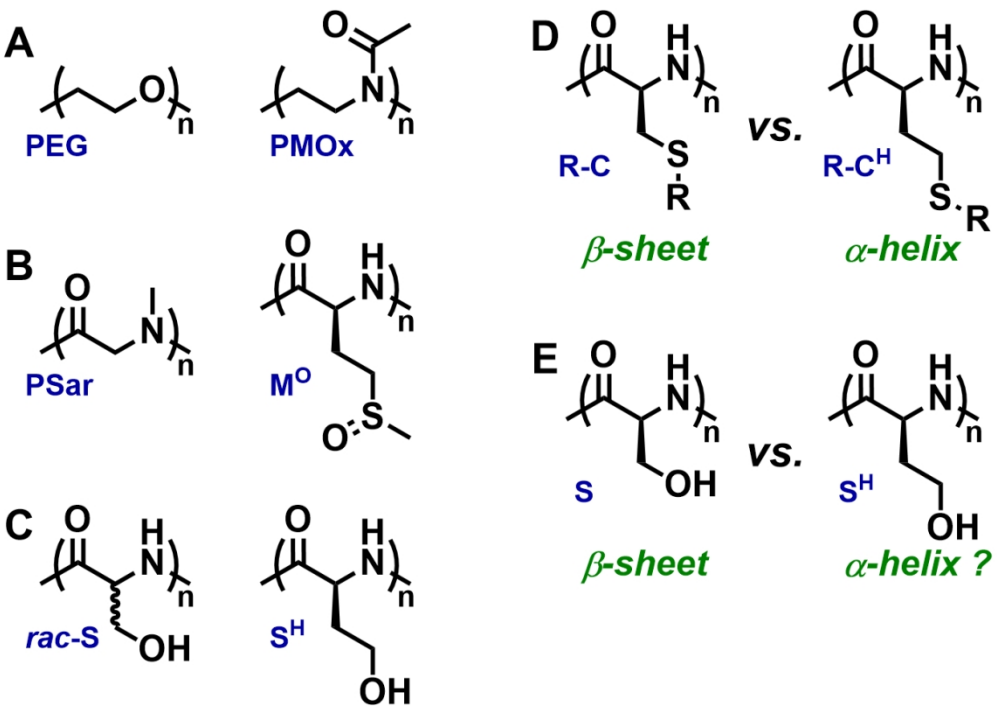

A
Transmittance (%)
100
90
80
70
60
2000
1800
1600
1400
Wavenumber (cm-1)
1647 cm-1
1538 cm-1
1611 cm-1
B
Molar Ellipticity [θ] x 10-3
10
0
-10
-20
-30
190
210
230
250
Wavelength (nm)
pH 2
pH 7
pH 9
C
Molar Ellipticity [θ] x 10-3
5
0
-5
-10
-15
-20
-25
190
210
230
250
Wavelength (nm)
100 mM KF
500 mM KF
D
Molar Ellipticity [θ] x 10-3
60
40
20
0
-20
-40
190
210
230
250
Wavelength (nm)
0% MeOH
20% MeOH
40% MeOH
60% MeOH





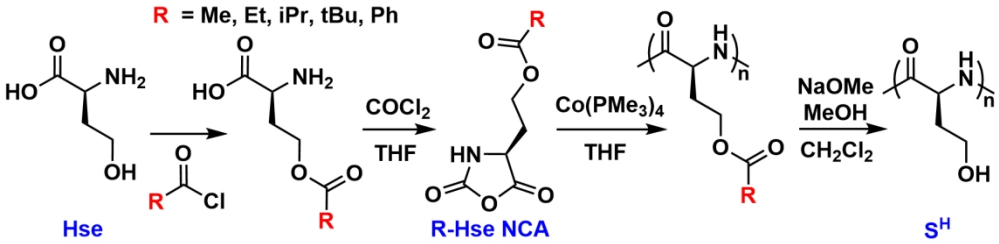






Scheme 1

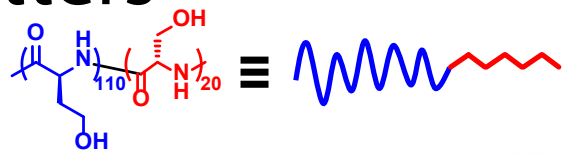
117x83mm (300 x 300 DPI)

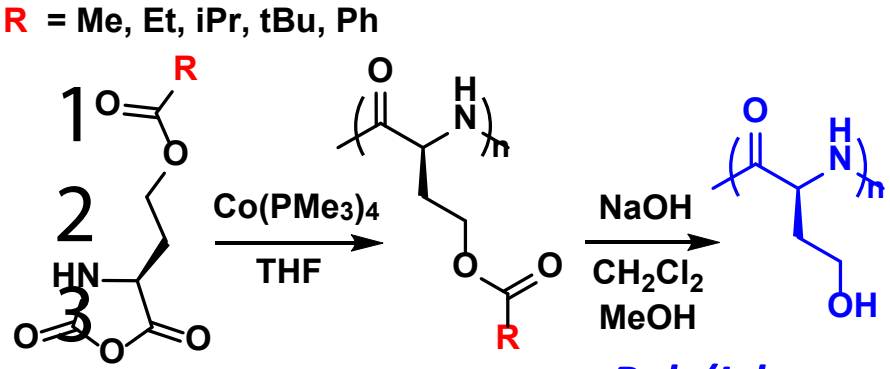


Scheme 2

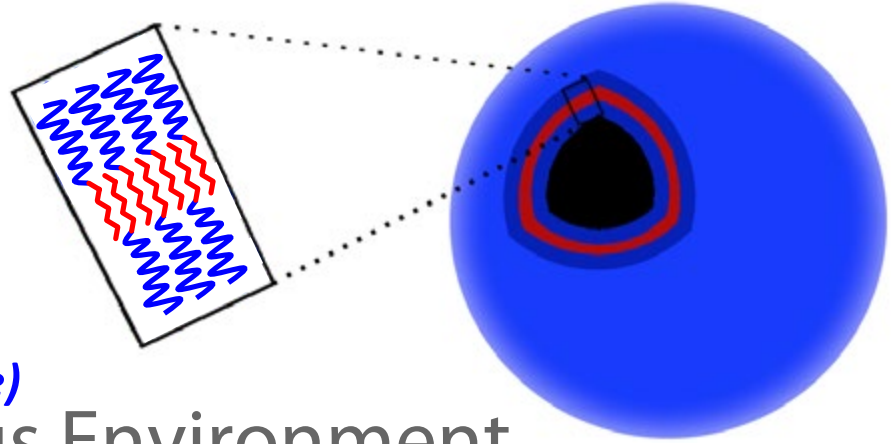
158x39mm (300 x 300 DPI)

Non-Ionic, Water Soluble Poly(L-homoserine)





Poly(L-homoserine)



ACS Paragon Plus Environment

Controlled Polymerization

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