Extending the self-assembly of coiled-coil hybrids

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UNITING POLYPEPTIDES WITH SEQUENCE-DESIGNED PEPTIDES: SYNTHESIS AND SELF-ASSEMBLY OF POLY(γ-BENZYL L-GLUTAMATE)-*b*-COILED-COIL PEPTIDE COPOLYMERS

A novel class of amphiphilic block copolymers has been synthesized, in which for the first time an N-carboxyanhydride (NCA) polymerization is initiated from a designed peptide. In this series of amphiphiles the hydrophobic block is poly(γ-benzyl L-glutamate) (PBLG), and the hydrophilic block is a coiled-coil forming peptide (denoted E). The synthetic approach was to synthesize the coiled-coil forming peptide on the solid phase, followed by NCA polymerization of γ-benzyl L-glutamate initiated from the N-terminal amine of the peptide on the solid support. The polypeptide-*b*-peptide was then cleaved from the resin, requiring no further purification. Peptide E contains 22 amino acids, while the average length of the PBLG block ranged from 36-250 residues. This synthetic approach was applied to create a modular system, in which the different PBLG block lengths are able to be connected noncovalently with various hydrophilic blocks via the specific coiled-coil folding of E with K or K-poly(ethylene glycol), where K is a peptide of complementary amino acid sequence to E. In this way nanostructures could be formed in water at neutral pH over the entire compositional range, which has not been demonstrated previously with such large PBLG blocks. It was found that the size, morphology (polymersomes or bicelles), and surface functionality could be specified by combining the appropriate modular building blocks. The self-assembled structures were characterized by means of dynamic light scattering, circular dichroism, scanning electron microscopy, cryogenic-transmission electron microscopy, fluorescence spectroscopy, and zeta-potential measurements. Finally, as the polymersomes are able to encapsulate water soluble compounds, and the surface of the polymersomes is easily functionalized via the coiled-coil binding, it is expected that these peptide-based polymersomes will be able to act as delivery vehicles to specific targets in the body.

INTRODUCTION

For materials scientists polypeptides are a fertile area for investigation as they can be programmed with the ability to adopt specific intra- and intermolecular conformations, which may allow heightened levels of control over the morphologies and properties of the self-assembled structures. The structure and functional properties of proteins and peptides are determined by their primary sequence of amino acids. Materials scientists are still unable to design the complex structures found in nature. Yet there has been some progress, particularly in understanding the folding of silks, elastins, collagens, and coiled-coil motifs.[1]

Two methods for the synthesis of peptides are the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCAs), and solid-phase synthesis. The ROP of NCAs is the most common method of synthesizing polypeptides containing a single amino acid residue.[2] The polymers can be readily prepared, and have no detectable racemization at the chiral centers.[3] Blocks based on glutamic acid (γ-benzyl L-glutamate) have been commonly synthesized as their polymerization is known to be the best controlled, and because they form well-defined rod-like α-helical secondary structures both in the solid-state and solution.[4] They have been initiated from traditional linear coil polymers,[5] polymer dendrimers,[6,7] modified lipids,[8] and polypeptides themselves synthesized by the ROP of NCAs.[9] Most commonly initiators with primary amine end-groups are used,[3] although the polymerization can also be initiated with transition metal-amine functionalized polymers.[10] Block copolymers have also been synthesized in the reverse manner, i.e. the ROP of NCA, followed by polymerization of another monomer from the polypeptide.[11,12] The ROP of NCAs has the disadvantage of multiple side-reactions and termination reactions, resulting in polypeptides with a wide range of polymer lengths. To reduce the range of lengths, which are likely to have different self-assembly properties, fractionation is often applied.[3] Additionally the abundance of side-reactions leads to contamination with homopolymers, which has to be separated from the desired block copolymers.[3]

There have been many studies of PBLG containing block copolymers in organic solution, but due to the hydrophobicity of PBLG blocks, there have only been limited reports of PBLG block copolymers in aqueous solution. Spherical,[13-15] cylindrical,[14] and whisker-like[16] micelles, solid spheres,[17] and vesicles[18,19] have been observed in aqueous solution from block copolymers which incorporate a PBLG block.

Using solid-phase synthesis protocols, peptides are designed with an exact molecular weight and specific amino acid sequence, which can result in a well defined shape and functionality. Importantly, the functionality can be more complex compared to NCA-based peptides, hence they are very interesting for incorporation into block-copolymers. The class of coiled-coil forming peptides, for which the design rules for the sequence-to-structure relationships are relatively well understood,[20] are accessible by solid-phase synthesis, and are of specific interest.

Coiled-coil forming peptides are strongly complementary in spatial packing and inter-peptide electrostatic interactions and form highly specific noncovalent complexes in aqueous solution. By designing the amino acid sequence appropriately many aspects of coiled-coil binding can be specified, such as the oligomerization state, complex size, orientation of binding, homo- or heterobinding, and stability. In Chapters 2 and 3 the coiled-coil peptide pair E and K were shown to form stable parallel heterodimers.[21,22] E and K fold to form amphipathic α-helices, and when E and K are mixed the driving force for the dimer structure is the shielding of the hydrophobic faces of the α-helices from the aqueous solution. The specificity of the complex is determined by the electrostatic interactions between the charged side chains of lysine and glutamic acid residues bordering the hydrophobic core. This renders the heterodimers energetically favorable and homodimers energetically unfavorable. The E/K heterodimer has been shown to retain the specific coiled-coil binding upon conjugation of macromolecules to E and/or K.[21-23]

In this Chapter, the combination of solid-phase synthesis with ROP to build up polypeptide-*b*-peptides is demonstrated for the first time. This new synthetic approach gives access to peptides that are not possible to synthesize using only solid-phase synthesis or only ROP. The large size range of polypeptides can be united with the complex functionality of sequence-specific peptides, leading to peptides with an increased range of potential properties.

The potential of this novel combination of peptide classes is demonstrated by the creation of a modular system with which one can mix-and-match hydrophobic and hydrophilic peptide blocks, with resultant control over the size, morphology, and surface functionality of the structures that self-assemble in aqueous solution.

In the polypeptide-*b*-peptides presented here one block is composed of hydrophobic PBLG, and the other block is composed of the hydrophilic coiled-coil forming designed peptide E (Table 1). The block copolymers are made by a two step process. Initially peptide E was synthesized using solid-phase synthesis protocols. While still anchored to the resin the amine terminus was used to initiate the ROP of γ-benzyl L-glutamate N-carboxyanhydride. This gives access to polypeptide-*b*-peptides with well-defined block sizes and functionalities. Additionally, it overcomes one of the main disadvantages of NCA polymerization, as any PBLG homopolymer that is formed as a side product can be readily washed away from the resin. PBLG-E block copolymers were synthesized with a range of hydrophobic block lengths, and cleaved from the resin, requiring no further purification.

Peptide E was chosen as the sequence-specific block because it folds into a stable coiled-coil dimer with peptide, K, due to their amino acid sequence designs, and the E/K pair was shown in the previous chapter to retain its coiled-coil binding upon conjugation of macromolecules to E and/or K. In Chapter 2 a short polystyrene chain with an average length of 9 monomers is coupled to the N-terminus of peptide E (denoted PS-E). PS-E forms spherical micelles in aqueous solution, and via coiled-coil folding with peptide K or K-poly(ethylene glycol)$_{77}$ the corona can be altered, resulting in larger spherical micelles,

or rod-like micelles. Furthermore, the E/K complex was shown to reversibly dissociate upon raising the temperature, again leading to a change in the self-assembled structures. Thus, by utilizing a coiled-coil forming peptide as the hydrophilic block of a block copolymer one can obtain a high degree of control over the hydrophilic block/s, and hence the nanostructures that form. However, the self-assembly of amphiphilic block copolymers depends not only on the corona chains, but also to a large extent on the hydrophobic blocks. As of yet, the conjugation of a range of hydrophobic block lengths to designed peptides has not been demonstrated. By combining polypeptides with sequence-designed peptides as presented here, we are able to create a modular system in which the hydrophobic PBLG length is readily controlled by ROP, and the hydrophilic block/s are readily controlled by coiled-coil folding (Figure 1), allowing one to piece together different components to access a range of self-assembled structures.

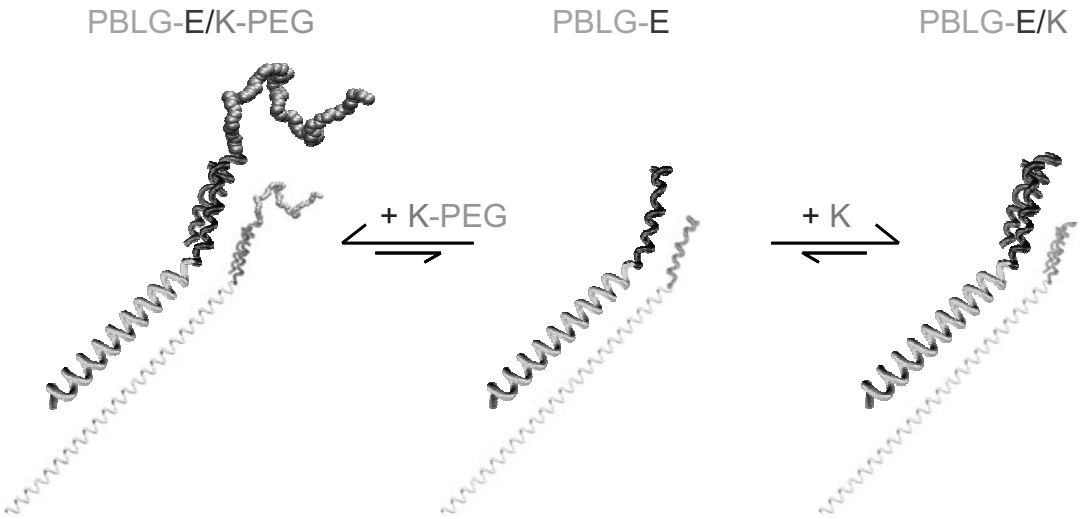


Figure 1. Schematic representation of a selection of the noncovalent amphiphiles obtained via the coiled-coil interaction between the building blocks.

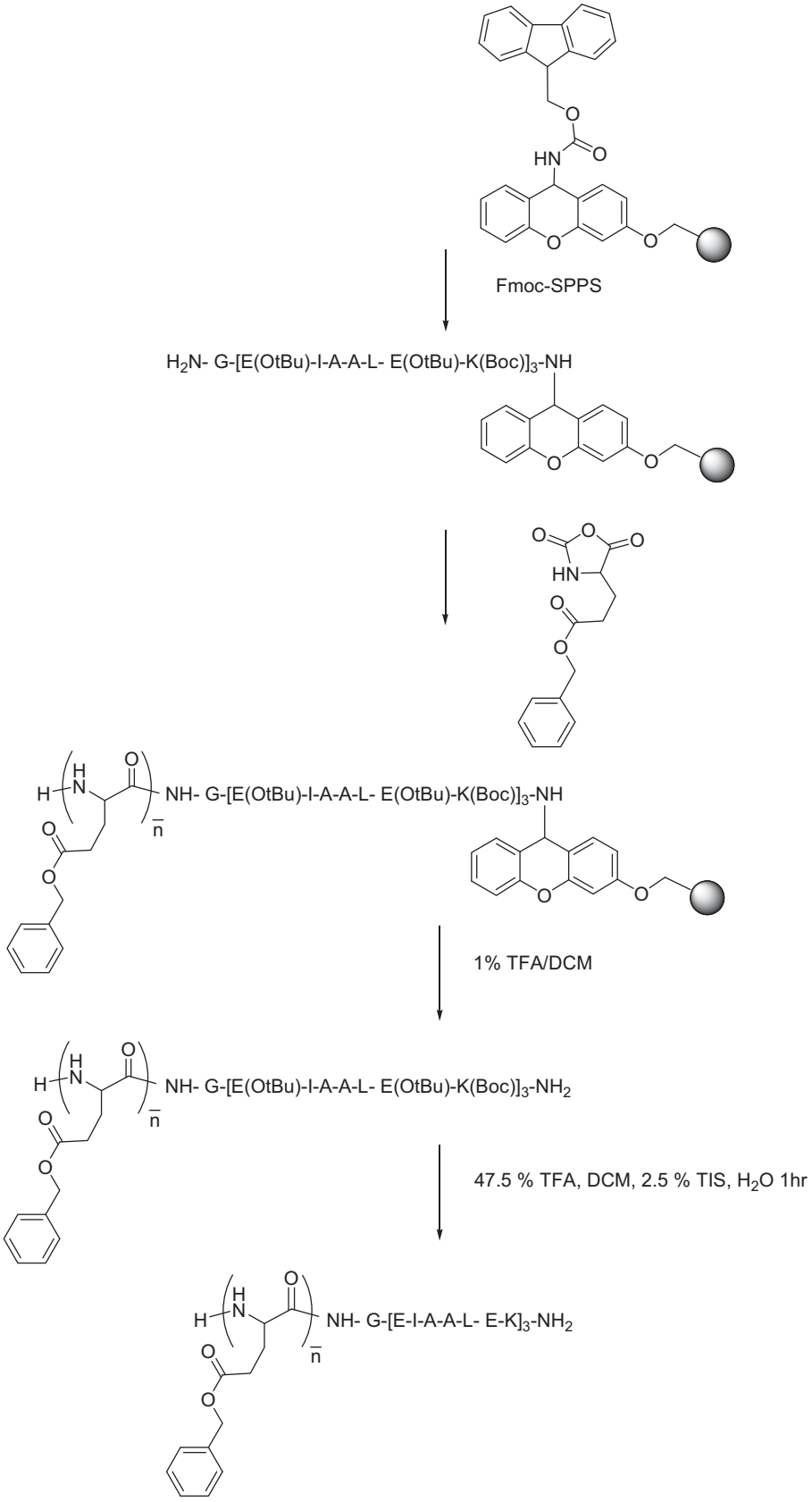
RESULTS AND DISCUSSION

Synthesis and Characterization of the protected PBLG-E block copolymer series

Poly(α-amino acid)s can be prepared by the ROP of NCAs starting from nucleophilic attack of the C_5 carbonyl group of the NCA by an initiator such as amines, alkoxide anions, alcohols, transitions metals, and water.[24] In the current approach to synthesize polypeptide-*b*-peptides, the coiled-coil peptide block E is first synthesized on a Sieber amide resin using standard Fmoc solid-phase peptide protocols, and the N-terminal Fmoc group is removed. ROP of the BLG-NCA is then initiated by the deprotected N-terminal amine of peptide E while still anchored to the resin (Scheme 1). The polymerization was conducted by shaking the resin-bound peptide with the NCA in DCM at room temperature under an argon atmosphere for one to three days. When all the BLG-NCA monomer was consumed, the resin was drained and washed thoroughly with DCM, NMP, and DMF. Typically 8% of the NCA monomer formed short oligomers during the polymerization reaction, due to trace amounts of water. These impurities were easily removed from the desired product by extensive washing of the resin.

The protected polypeptide-*b*-peptides were released from the solid support by shaking 10 times (2 minutes each) in 99:1 (v/v) DCM:TFA, with subsequent precipitation in cold methanol. The purity of each fraction was ascertained with GPC, from which it was found that within each synthesis the longer PBLG-E polypeptide-*b*-peptides were cleaved first from the resin, with a progressive shortening of the PBLG chain with each fraction collected, until finally peptide fragments from the solid-phase peptide synthesis of E were cleaved. In this way polypeptide-*b*-peptides with a lower polydispersity index (PDI) can be obtained by selecting which fractions to combine. Due to the washing away of homo-PBLG while the block copolymer is still attached to the resin, and the cleavage of peptide fragments from the resin only after the bulk of PBLG-E molecules have been cleaved, no further purification was necessary. The GPC chromatographs of the PBLG-E series are shown in Figure 2. Peaks are monomodal and the PDIs range from 1.1 for the hybrid with the shortest PBLG block to 1.7 for the hybrid with the longest PBLG block.

Scheme 1. Synthesis of PBLG-E by solid-phase peptide synthesis followed by N-carboxyanhydride ring-opening polymerization initiated from the N-terminal amine of the resin-bound peptide.



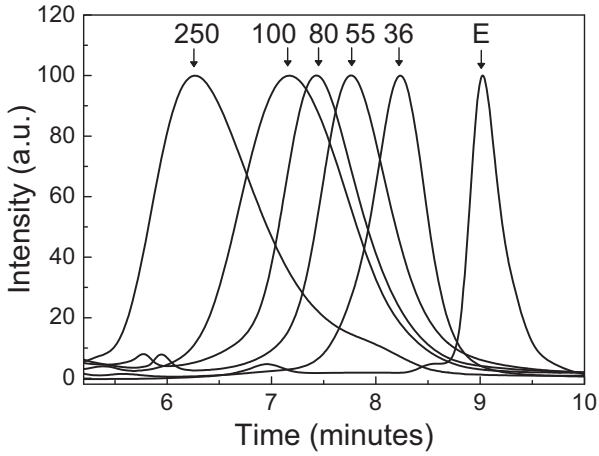


Figure 2. GPC chromatographs of the coiled-coil forming peptide E (not purified), and PBLG-E polypeptide-*b*-peptides in the protected form. The numbers above each trace refer to the degree of polymerization of the PBLG block.

Synthesis and Characterization of the PBLG-E block copolymer series

The protecting groups from the glutamic acid and lysine residues of peptide E were selectively removed using a mixture of TFA, DCM, water, and TIS, while retaining the benzyl protecting groups of the PBLG block, and the amphiphilic polypeptide-*b*-peptide was precipitated in cold methanol. The complete removal of the protecting groups was confirmed by the disappearance of the peak from the methyl groups of the OtBu and Boc moieties at 1.5 ppm from ^{1}H NMR spectra. To determine the degree of polymerization and M_n of the PBLG blocks, spectra were obtained for each compound in deuterated dichloromethane with increasing amounts of trifluoroacetic acid, ensuring the absence of aggregation of the amphiphilic block copolymer and hence that accurate peak comparisons between E and PBLG blocks could be made.[25-27] The peak arising from the leucine and isoleucine methyl protons of the E block was compared to the peak arising from the benzyl protons of the PBLG block (Figure 3). The degree of polymerization of the PBLG blocks determined using ^{1}H NMR spectroscopy matched the data obtained by GPC, indicating that the polystyrene standards used for GPC molecular weight comparison are reliable for these hybrids. The molecular characteristics of the compounds used in this study are shown in Table 1. The hydrophilic peptide E has 22 amino acid residues, while the hydrophobic PBLG block ranges from 36 to 250 benzyl glutamate residues.[28]

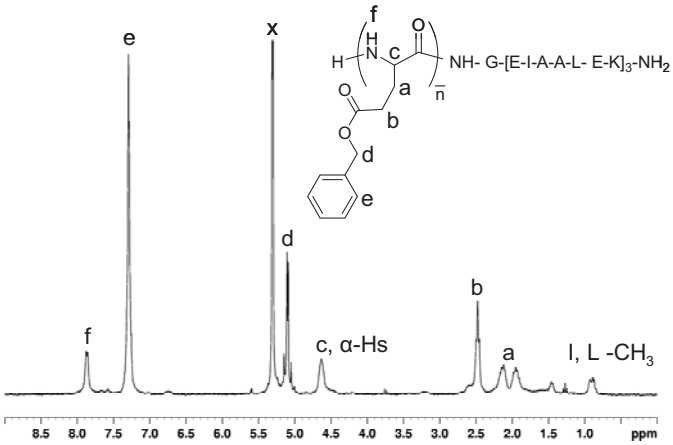


Figure 3. ^{1}H-NMR spectrum of $PBLG_{36}$-E in 7:3 (v/v) DCM-d_3:TFA with the proton assignments. X is from DCM.

Table 1. Molecular Characteristics of the Compounds used in this Study

Name	Structure	Yield (%)	M_n (g/mol)	PDI[3]
K	Ac-(K I A A L K E)$_3$G-NH_2	~ 40	2378.0[1]	
E	Ac-G(E I A A L E K)$_3$-NH_2	~ 40	2380.6[1]	
PEG-K	Ac-(K I A A L K E)$_3$G-PEG_{77}	~ 10	5832[1,2]	1.05[1]
$PBLG_{36}$-E	$PBLG_{36}$-G(E I A A L E K)$_3$-NH_2	28	10230[2,3]	1.1
$PBLG_{55}$-E	$PBLG_{55}$-G(E I A A L E K)$_3$-NH_2	30	14396[2,3]	1.3
$PBLG_{80}$-E	$PBLG_{80}$-G(E I A A L E K)$_3$-NH_2	56	19877[2,3]	1.4
$PBLG_{100}$-E	$PBLG_{100}$-G(E I A A L E K)$_3$-NH_2	69	24262[2,3]	1.4
$PBLG_{250}$-E	$PBLG_{250}$-G(E I A A L E K)$_3$-NH_2	74	57148[2,3]	1.7

[1] Obtained from MALDI-TOF MS. [2] Based on a comparison of ^{1}H-NMR peaks. [3] GPC calibrated with polystyrene standards. PEG: poly(ethylene glycol), PBLG: poly(γ-benzyl L-glutamate), amino acids in the designed peptides are represented by their one letter codes, Ac: acetyl.

Self-Assembling Properties of the Polypeptide-b-Peptides and Polymer Mixtures in Aqueous Solution

The synthesis and self-assembly of rod-rod block copolymers is an emerging topic in polymer chemistry, and the well-defined shapes of rod polymers leads to novel self-assembly properties. PBLG is hydrophobic and with a degree of polymerization (n) larger than 10 has an α-helical secondary structure,[29] resulting in a rod-like molecular shape. The length of PBLG α-helices is n x 1.5 nm[30] while the diameter is ~ 2 nm,[31] hence in the α-helical configuration the PBLG rod-like blocks in this study range in average length from 5.4 to 37.5 nm long. The peptide E was chosen as the hydrophilic block because it forms an α-helical coiled-coil dimer with K, a peptide with a complementary amino acid sequence (see Chapters 2 and 3). E/K is one of the shortest pairs of coiled-coil forming peptides that specifically forms heterodimers. E and K form complexes with a well defined rod-like geometry of cylinders 3.5 nm long with approximately the same diameter

as PBLG rods.[33] Poly(ethylene glycol) is a hydrophilic coil polymer, and the PEG used in this study, with an average of 77 monomers, has an average diameter of approximately 5 nm,[34] although it is more compact when attached to peptide K.[21] The peptides K and the hybrid K-PEG are predominantly hydrophilic and do not aggregate in aqueous solutions.[35] Due to the amphiphilic nature of the rod-rod PBLG-E polypeptide-*b*-peptides and the noncovalent complexes PBLG-E/K and PBLG-E/K-PEG, it was expected that the PBLG and hydrophilic blocks would phase separate in aqueous solution. The self-assembling characteristics of the PBLG-E series, both in isolation and with equimolar amounts of K or K-PEG were studied in phosphate buffered saline solution (PBS) at pH 7.0. The PBLG-E polypeptide-*b*-peptides, having large hydrophobic PBLG blocks, were not soluble in aqueous solutions. The standard methods for inducing self-assembly,[36] namely film hydration, or solvent injection followed by dialysis were ineffective as the PBLG-E block copolymers precipitated. Better results were achieved by injecting PBS into a tetrahydrofuran (THF) solution of the polymer with sonication. PBS is selective for the hydrophilic E, E/K, and E/K-PEG blocks and induces aggregation of the PBLG blocks. The initial ratio of THF to PBS was such that the E/K coiled coil is stable (Figure A1), and the THF was subsequently removed by continuous sonication for two hours in an open vessel. An example of circular dichroism spectra of the polymers and polymer mixtures is given in the Appendices (Figure A2).

Particle Sizes

The aggregation of the individual polypeptide-*b*-peptides (PBLG-E) was investigated with dynamic light scattering (DLS), as well as with the equimolar addition of the complementary peptide K or K-PEG. It was found that when the PBLG block length was 80 monomers or shorter the pure PBLG-E polypeptide-*b*-peptides had a suitable balance of hydrophobicity and hydrophilicity to form ordered supramolecular structures in aqueous solution. For $PLBG_{100}$-E, complexation with K resulted in a noncovalent block copolymer with a corona sufficient to form ordered structures. The amphiphilic block copolymer with the longest hydrophobic block, $PBLG_{250}$-E, required association with K-PEG in order to have a large enough corona to aggregate in an ordered manner. The average hydrodynamic diameter of the structures that self-assembled from the polymers and polymer mixtures ranged from 100 nm to 400 nm, and were significantly larger than the calculated sizes of spherical micelles. All size distributions were monomodal and the polydispersity index (PDI) of the samples was 0.35 or less.

As shown in Figure 4, increasing the PBLG block length resulted in larger aggregates. Additionally, for a particular PBLG block length, increasing the size of the head-group (going from E to E/K and E/K-PEG) leads to a smaller hydrodynamic diameter of the particles. These trends can both be explained by classical packing parameter considerations: the larger the head-group is in comparison to the hydrophobic PBLG, the greater is the curvature of the aggregate surface, and hence the particle size decreases.[37] The packing parameter was originally designed to predict the morphology and size of

nanostructures formed from lipids, and this approach is not always suited to block copolymers because it does not take into account the complexity of the interfacial energy and interaction free-energies of the blocks.[21] That being said, it is sufficient to explain the trends observed in the assembly of this system. This may be because in the case of both lipid structures and structures formed from the PLBG-E series the influence of stretching of the hydrophobic chains is minimal (lipid tails are stretched,[38] and the PBLG rods have a very well defined structure and size with no change in configuration expected upon aggregation[39]), hence the role of deformation of the core block on the free-energy of the structure is reduced.

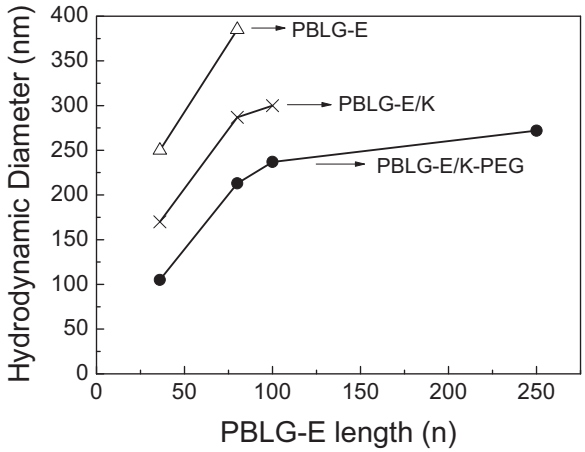


Figure 4. Typical hydrodynamic diameters of the self-assembled structures formed by the polypeptide-*b*-peptides and noncovalent complexes. ([Total Peptide] = 50 μM, PBS, 25 °C).

Particle Morphology

Information about the morphology of the supramolecular structures that formed was obtained by electron microscopy. The effects of PBLG chain length and the relative size of the corona on the ability of the molecules to controllably self-assemble as observed by scanning electron microscopy (SEM) were consistent with the DLS observations (Figure 5). Combining the shortest PBLG length with an E or E/K hydrophilic block results in spherical objects assembling in PBS, which collapse when dried (Figure 5A, B). $PBLG_{36}$-E/K-PEG has the same hydrophobic block length, but forms smaller structures, as explained in the DLS section, which are stable upon drying. This sample also contained disk-like aggregates, *vide infra*. To obtain further insight into the internal structure of the assemblies cryo-TEM studies were performed for a selection of the self-assembled structures (Figure 6). The shortest hybrid, $PBLG_{36}$-E, has a low PDI of 1.1, and self-assembles into vesicles with rather uniform membrane thicknesses, that seem to be independent of the vesicle diameter. The thicknesses observed increase slightly with increasing size of the hydrophilic block/s: 17.2 $\pm$ 2.6 nm for $PBLG_{36}$-E, 18.5 $\pm$ 2.4 nm for $PBLG_{36}$-E/K, and 21.5 $\pm$ 2.2 nm for $PBLG_{36}$-E/K-PEG (Figure 6A, B, C). The observed membrane thicknesses are in remarkably close accordance with the calculated bilayer

thicknesses, as seen in Table 2. These results show that the rigid hydrophobic PBLG rods are able to assemble into very well-defined bilayers through coupling to the water soluble peptide rods. In contrast to other block copolymer vesicles,[40] there does not appear to be any interdigitation of the two layers of the hydrophobic block, presumably due to the rod-like structure of the PBLG and the comparable diameter of the tail and corona rods.

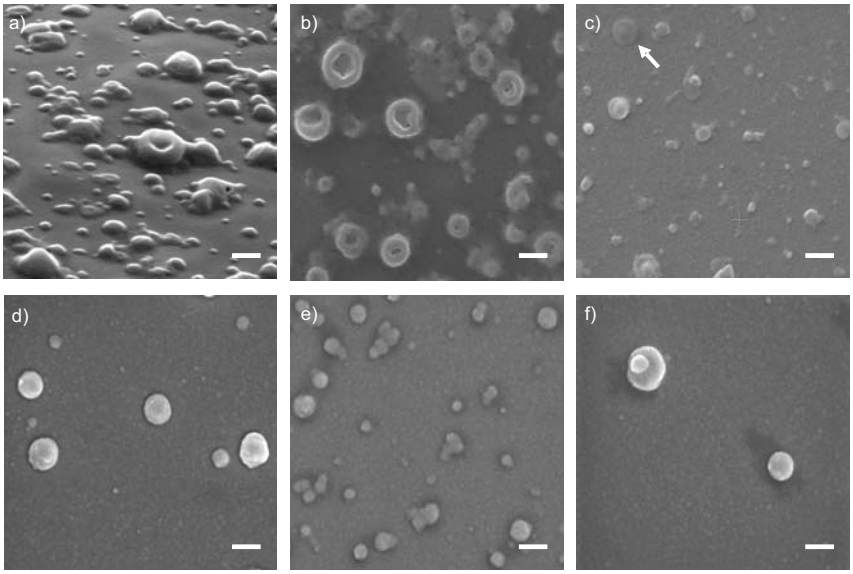


Figure 5. SEM images of the particles formed from (a) $PBLG_{36}$-E, (b) $PBLG_{36}$-E/K, (c) $PBLG_{36}$-E/K-PEG, (d) $PBLG_{100}$-E/K, (e) $PBLG_{100}$-E/K-PEG, (f) $PBLG_{250}$-E/K-PEG. Scale bars = 200 nm. ([Total Peptide] = 50 μM, PBS, 25 °C).

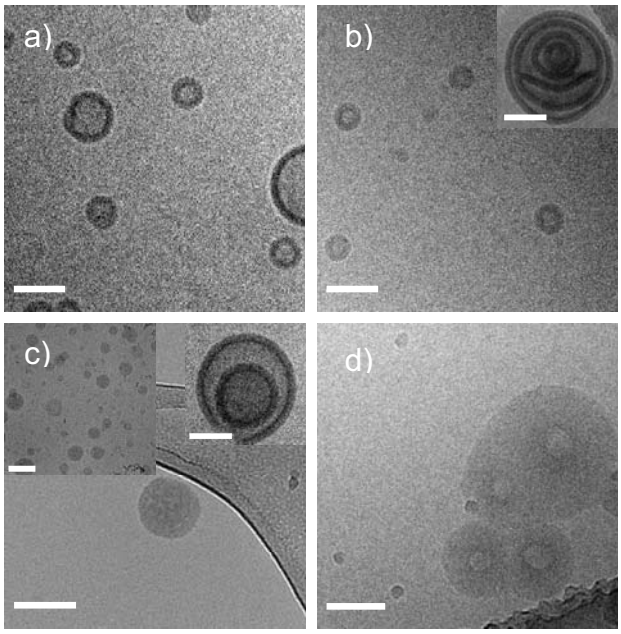


Figure 6. Cryo-TEM images of $PBLG_{36}$E (a), $PBLG_{36}$E/K (b), $PBLG_{36}$E/K-PEG (c), and $PBLG_{100}$E/K-PEG (d). Scale bars = 100 nm. ([Total Peptide] = ~ 1500 μM, PBS).

Table 2. Experimental and theoretical membrane thicknesses of the vesicles.

Sample	Membrane thickness	
	experimental (nm)[a]	theoretical (nm)[b]
$PBLG_{36}$-E	17.2 ± 2.6 nm	18
$PBLG_{36}$-E/K	18.5 ± 2.4 nm	18
$PBLG_{36}$-E/K-PEG	21.5 ± 2.2 nm	24
$PBLG_{100}$-E/K-PEG	68 ± 22 nm	42

[a] Determined by cryo-TEM. [b] PBLG length: n x 1.5 nm,[90] E and E/K length 3.5 nm,[27] PEG length: ~ 3 nm.[30]

In addition to polymersomes, disks of uniform height and density were observed in the SEM and cryo-TEM images of $PBLG_{36}$E/K-PEG (arrows, Figure 5C and Figure 6C). This is the sample with the shortest PBLG block and the longest hydrophilic component in comparison to the PBLG block. Presumably polymeric bicelles are observed only for this noncovalent block-copolymer because the length of the PBLG block is short enough that the hydrophilic blocks are able to fold over any exposed PBLG on the sides of the discs, shielding it from the aqueous buffer.[41] This eliminates the energetic need for the bilayers to close the hydrophobic sides by curving to form vesicles. This possibility was tested with computer modeling, which confirmed that PEG is able to cover the average length of the extended α-helical PBLG block without any chain stretching, i.e. while still in the random coil configuration (Figure 7). A theoretical study has found that for rod-coil block copolymers the only stable micellar form has disk-like cores and relatively large corona thicknesses. The disk-like core reduces the core-corona interfacial free energy of the rod blocks, as in this geometry the rods pack well together, and only large coil blocks can deform enough to balance the interfacial free energy.[39]

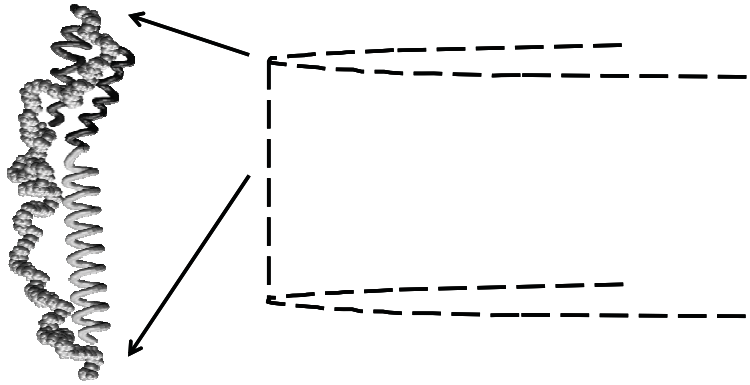


Figure 7. The space filling model of the $PBLG_{36}$-E/K-PEG complex illustrates that without chain stretching PEG readily extends the length of the coiled-coil peptides and the PBLG block. The E/K dimer structure is based on the work of Litowski and Hodges,[33] the PBLG adopts and α-helix, and the PEG has a random coil conformation. The $PBLG_{36}$-E/K-PEG model was built with Molden version 4.6,[42] and the image generated with VMD version 1.8.6.[43]

For the polypeptide-*b*-peptides and noncovalent complexes with longer PBLG lengths the SEM images exclusively show spherical objects that withstand the drying process (Figure 5D, E, F). Cryo-TEM was also conducted on the structures assembled from the complex $PBLG_{100}$-E/K-PEG. As seen in Figure 6D the spherical particles were observed to contain aqueous interiors, and have very bulky membranes of variable thickness (≈ 68 $\pm$ 22 nm). The range of membrane thicknesses likely arises from the polydispersity of molecular lengths in this sample. GPC of the protected polypeptide-*b*-peptide yielded a PDI of 1.4, and it was estimated that 95% of the $PBLG_{100}$-E lengths are between 9 nm and 80 nm when in the α-helical conformation.

An advantage of polymersomes over liposomes is that their membrane thickness varies depending on the composition, molecular weight, and degree of stretching of the blocks. The hydrophobic core of lipid bilayers is always approximately 3-4 nm thick, regardless of the lipid composition,[44] while the membrane thickness of polymersomes ranges from 3-5 nm[45] to 200 nm (in 1:1 v/v TFA/DCM).[46] In the present series the nature of the membrane can be dictated by the choice of hydrophobic and hydrophilic components, and the thickness of the membrane of $PBLG_{100}$-E/K-PEG vesicles is the largest reported for polymersomes in aqueous solutions.

Encapsulation

To demonstrate the ability of the supramolecular structures to encapsulate water soluble compounds, samples were prepared in the presence of the water soluble fluorescent dye rhodamine B. Following sonication the unencapsulated rhodamine B was removed by FPLC. The samples for which DLS indicated ordered structures (monomodal intensity distributions and PDI < 0.35) exhibited rhodamine B fluorescence (Figure A3), confirming that the polypeptide-*b*-peptides and noncovalent complexes had a suitable balance of the hydrophilic block size to the hydrophobic PBLG block to lead to controlled assembly, and that these self-assembled structures had aqueous interiors, i.e. were vesicles. As expected, the samples that did not show well defined assembly by DLS ($PBLG_{100}$-E, $PBLG_{250}$-E, and $PBLG_{250}$-E/K) contained insignificant amounts of rhodamine B, as verified by fluorescence spectroscopy (Figure A3). The hydrodynamic diameter of the nanocapsules did not change for at least 10 – 20 months at 4 °C as determined by DLS, showing the extensive stability of the samples.[47] As well as the particle size and morphology, the surface functionality was also retained over the same measurement period, with PBLG-E samples (having a net charge per molecule of -3) displaying a negative zeta potential, and PBLG-E/K and PBLG-E/K-PEG samples (with no net charge per complex) having zeta potentials closer to zero (Table A1).

CONCLUSIONS

A new class of peptides, polypeptide-*b*-peptides, has been synthesized by a straightforward synthetic approach. The ROP polymerization of an amino acid NCA is initiated from the N-terminal amine of a sequence specific peptide while still on the solid support. The concept is demonstrated by the solid-supported polymerization of γ-benzyl L-glutamate NCA from the coiled-coil forming peptide E, which was synthesized using standard solid-phase peptide synthesis protocols. Cleavage of the diblock from the resin and precipitation yielded block copolymers requiring no further purification. The polypeptide-*b*-peptides had PBLG block lengths ranging from 36 to 250 monomers. The chemical structure of the polypeptide-*b*-peptides was confirmed by GPC, NMR, FTIR, and MALDI-TOF MS. This new class of peptides will broaden the scope of peptide-based nanostructures, as the combination of polypeptides with designed peptides enables control over both the peptide size and functionality, which are both determining factors in molecular self-assembly. This potential is investigated by selecting 4 molecular building blocks and showing that a variety of structures could be accessed, relying on the size of the PBLG block and the noncovalent coiled-coil interaction of peptide E with the complementary peptide K or the hybrid K-PEG. Polymersomes with a range of sizes, membrane thicknesses, and surface functionality were formed, as well as disk-like micelles. The structures were characterized by means of CD, DLS, SEM, cryo-TEM, fluorescence spectroscopy, and zeta potential measurements. It was found that the diameters of the structures increased with decreasing hydrophilic to hydrophobic block size ratios, as is typical for traditional surfactants such as lipids. The form of the polymersome membrane depended on the length of the PBLG block, with the shortest PBLG length forming very well-defined membranes that were twice the calculated molecular length. The structures were demonstrated to encapsulate water soluble compounds, hence there is potential for use of these materials as drug delivery devices, and through E/K complexation the outside of the polymersomes can be decorated with targeting/stealth molecules.

EXPERIMENTAL SECTION

Materials and Methods

Materials

Fmoc-protected amino acids were purchased from Novabiochem. Tentagel PAP resin was purchased from Rapp Polymere. All other reagents and solvents were obtained at the highest purity available from Sigma-Aldrich or BioSolve Ltd. and used without further purification. Phosphate buffered saline, PBS: 30 mM $K_2HPO_4.3H_2O$, 19 mM KH_2PO_4, 100 mM KCl, pH 7.0.

General Methods

Gel permeation chromatography (GPC) was performed with a Shimadzu system equipped with a refractive index detector. A Polymer Laboratories column was used (3M-RESI-001-74, 7.5 mm diameter, 300 mm length) with DMF as the eluent, at 60 ºC, and a flow rate of 1 mL min^{-1}. Both the coiled-coil peptide and PBLG are soluble in DMF, and the runs were conducted at 60 ºC to prevent aggregation. The molecular weights were calibrated using polystyrene standards.

^{1}H-NMR spectra recorded on a Bruker AV-500 spectrometer and a Bruker DPX300 spectrometer at room temperature. The residual proton resonance of deuterated dichloromethane was used for calibration. In order to ensure that there were no aggregation artifacts in the spectra that were analyzed for molecular weight determination a range of ^{1}H-NMR spectra of the deprotected hybrids were recorded, from deuterated dichloromethane to 1:1 (v/v) deuterated dichloromethane:trifluoroacetic acid.

MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF spectrometer. Samples were dissolved in 1:1 (v/v) 0.1% TFA in water:acetonitrile (TA), at concentrations of ~10 mg mL^{-1}. Solutions for spots consisted of (v/v) 1:10 sample solution: 10 mg mL^{-1} α-Cyano-4-hydroxycinnamic acid (ACH) in TA.

FT-IR spectra were recorded on a BIORAD FTS-60A instrument equipped with a deuterated-triglycine-sulphate (DTGS) detector at a resolution of 20 cm^{-1}. The compounds were dried from dichloromethane onto an attenuated total reflectance (ATR) ZnSe crystal. A clean ATR ZnSe crystal was used as the background.

Synthesis

Solid-Phase Peptide Synthesis of the coiled-coil forming peptides E, K, and K-PEG

The peptides E and K, and the hybrid K-PEG were prepared and characterized as described in Chapter 3. After the peptide E was prepared, the resin was removed from the reaction vessel, swollen in 1:1 (v/v) DMF:NMP, and Fmoc deprotected. The amount of successfully synthesized E on a given weight of peptide-resin was estimated using the mass added to the resin during the synthesis of E, and by integration of HPLC peaks from an LCMS run of a test cleavage of 10 mg of resin-bound peptide.

Synthesis of γ-benzyl L-glutamate N-carboxyanhydride (BLG-NCA)

A suspension of γ-benzyl L-glutamate (ca. 5.0 g, 21.1 mmol) in anhydrous ethyl acetate was heated to reflux (120 °C) under an argon atmosphere with vigorous stirring. Triphosgene (ca. 2.1 g, 7.0 mmol) was added quickly and stirring was continued for 3 hours, until the suspension became clear. If the suspension remained turbid a small quantity of triphosgene was added every 15 minutes. The solution was filtered and concentrated to one third of the initial volume (oily yellow liquid). The product was transferred to a glovebox under an argon atmosphere and precipitated in hexane, filtered, recrystallized, and dried. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 7.3 (aromatic H, m); 5.1 (benzylic CH_2, s), 2.6 (γ-CH_2, t), 2.2 (β-CH_2, m), 4.4 (α-CH, t), 6.8 (N-H, br).

Solid-Phase Synthesis of Poly (γ-benzyl L-glutamate)-block-E (PBLG-E)

Poly(γ-benzyl L-glutamate) was synthesized via a one-pot NCA polymerization of γ-benzyl L-glutamate *N*-carboxyanhydride, initiated from the amine at the N-terminus of the peptide E while still on the resin. The resin-bound peptide was dried with reduced pressure at 40 °C overnight, and then in argon with reduced pressure for 5 hours. Under an argon atmosphere the peptide-resin was swollen in DCM (2.5 wt% NCA to DCM), and subsequently the appropriate weight of NCA (determined from the mass loading and HPLC peak integration) was added. The flask was shaken for 24 – 65hrs. A small volume of DCM was drained from the reaction vessel and the contents analyzed with FT-IR spectroscopy, showing that no NCA monomer remained (as determined by the absence of the carbonyl stretching absorption band of the C_2 carbonyl at 2000 – 1800 cm^{-1}, which is released as CO_2 during the reaction). The resin was drained and washed profusely with DCM, NMP, DMF, and finally with DCM. The initial DCM washes were dried to collect any homopolymer that formed in solution. The yields of the resin-bound block copolypeptides were 85% - 92%.

The hybrid material was cleaved in the protected form from the resin using 1:99 (v/v) TFA:DCM for 2 minutes, 10 times. Each cleavage mixture was precipitated drop-wise in cold methanol. The white precipitate was compacted with centrifugation and the supernatant removed. This was repeated three times with the addition of fresh methanol. The pellets were dried under reduced pressure. Molecular weights and their distributions of the protected PBLG-E polypeptide-*b*-peptides were determined using gel permeation chromatography.

The OtBu and BOC protecting groups of the glutamic acid and lysine residues of the E block were removed by stirring the block copolymer in 47.5:47.5:2.5:2.5 (v/v) TFA:DCM:water:TIS for 1 hour, and the product was precipitated drop-wise in cold methanol. The white precipitate was compacted with centrifugation and the supernatant removed. This was repeated three times with the addition of fresh methanol. The pellets were dried under reduced pressure, with yields ranging from 28% for the block copolymer with the shortest average PBLG length, which does not sediment well in cold methanol, to 74% for the block copolymer with longest PBLG block, which is the most hydrophobic of

the series. The purity and molecular weights of the deprotected polypeptide-*b*-peptides were checked using ^{1}H-NMR spectra. The absolute masses of the polypeptide-*b*-peptides with shorter PBLG blocks were determined using MALDI-TOF mass spectrometry. The yield and molecular weight of the compounds used in this study as determined by GPC, NMR, and MALDI-TOF spectroscopy are given in Table 1. The secondary structure of the polypeptide-*b*-peptides was determined using FT-IR spectroscopy.

Preparation of PBLG-E suspensions

0.1 μmol of each compound (PBLG-E, or PBLG-E and K, or PBLG-E and K-PEG) was dissolved in 200 μL tetrahydrofuran (THF). 2 mL of phosphate buffered saline (PBS, 50 mM PO_4, 100 mM KCl, pH 7.0) was added and the sample was immediately sonicated with an open lid for 2 hours in a Branson 1510 bath sonicator with an output of 70 W and 42 kHz. The final concentration of each polymer was 50 μM.

For the encapsulation of rhodamine B in the vesicles the samples were prepared as described above, with the addition of rhodamine B (0.2 mg mL^{-1}, 0.418 mM) to the buffer. The unencapsulated rhodamine B was removed over a fast protein liquid chromatography (FPLC) column. FPLC was performed with an Äkta prime, Amarsham Pharmacia Biotech apparatus with a Pharmacia XK 26 column (135 mm x 25 mm) packed with Sephadex G50-fine. PBS was used as the eluent. The flow rate was 5 mL min^{-1}, UV sensitivity was set on 0.1 AU, 1%, the conductivity was set on 15-20 mS cm^{-1} and the wavelength for UV recording was 254 nm. The amount of encapsulated rhodamine B in each sample was determined by fluorescence spectroscopy, with excitation at 555 nm, and emission monitored from 563 – 650 nm with 5 nm slits using a Cary-50 Spectrophotometer.

Characterization of PBLG-E suspensions

Dynamic Light Scattering

Dynamic light scattering was conducted as detailed in the experimental section of Chapter 3.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was conducted on a Nova NanoSEM FEI instrument with an accelerating voltage of 10 kV and spot size of 3.5. Samples for SEM were prepared by placing 5 μL of the solution on SEM stubs with a TEM grid on the carbon tape. After 30 minutes the excess buffer was removed. Samples were coated with gold for one minute, resulting in a layer ~ 15 nm thick.

Cryogenic Transmission Electron Microscopy

Cryogenic TEM was conducted as detailed in the experimental section of Chapter 3. Copper grids bearing lacey carbon films resulted in a greater number of self-assembled structures in the vitrified ice.

Zeta Potentials

Zeta potentials were measured at 25 °C using the same instrument. The laser wavelength was 633 nm and the scattering angle was 173˚. A Malvern universal dip cell (ZEN1002) was used, and the samples were diluted ten-fold with 1 mM NaCl to reduce the ionic strength of the buffer.

Circular Dichroism Spectroscopy

CD spectra were obtained as detailed in the experimental section of Chapter 2. For analysis each spectrum had the appropriate background spectrum (buffer or buffer/THF) subtracted.

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APPENDICES

Characterization of the PBLG-E block copolymer series

It was possible to characterize the PBLG-E polypeptide-*b*-peptides with the shortest PBLG blocks by MALDI-TOF MS. The masses observed do not correspond to an integer multiple of benzyl glutamate monomers in the PBLG chain. Additionally, the Kaiser test was negative (Kaiser, E.; Colescot.Rl; Bossinge.Cd; Cook, P. I. *Anal. Biochem.* **1970**, 34, (2), 595-598). These results indicate that the polymer chains do not end in a primary amine, as would be expected by the "amine" mechanism of ring-opening polymerization, but that another reaction, such as the "activated monomer" mechanism, has capped the growing chains. This is also consistent with the fact that there is not 100% monomer conversion, but some degree of oligomer formation. A given polymerization can alternate between these two mechanisms, and ROPs of NCAs using amines as initiators are notorious for their variable chain-end functionality and formation of homopolymer (Klok, H. A. *Angew. Chem., Int. Ed.* **2002**, 41, (9), 1509-1513. Deming, T. J., *Polypeptide and polypeptide hybrid copolymer synthesis via NCA polymerization*. **2006**; Vol. 202).

PBLG-E adopts a typical α-helical structure in the solid state as is evident from the amide I and amide II positions in FT-IR spectra (1651.1 cm^{-1} and 1546.9 cm^{-1} respectively). There is no shoulder on the amide I vibration, indicating that there is no random coil secondary structure in the hybrid, and thus that the secondary structure of E is helical in the solid state. The half width at half maximum of the amide II absorption depends on the stability of the α-helix, and at ~14cm^{-1} for the amide II band, this is on a par with the most stable helices (Nevskaya, N. A.; Chirgadze, Y. N. *Biopolymers 1976*, 15, (4), 637-648).

Self-assembling properties of the block copolymers and polymer mixtures in solution

Effect of THF on E/K and PBLG secondary and quaternary structures

The E/K heterodimer is a noncovalent complex driven by the packing of leucine and isoleucine residues forming a hydrophobic core in order to reduce contact with the aqueous environment. In PBS E/K exhibits a typical α-helical circular dichroism (CD) spectrum, with minima at 208 nm and 222 nm. The ellipticity ratio was 1.00 (Figure A2, thick line), consistent with interacting α-helices (Zhou, N. E.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1992**, 267, (4), 2664-2670). Upon the addition of THF, the secondary structure of the peptides remains α-helical, but the intermolecular interaction is disrupted, as evidenced by the decreasing ellipticity ratio (Figure A2). This is because adding THF to PBS reduces the polarity of the solvent so there is a decreased energetic penalty associated with the hydrophobic residues being exposed to the solvent.

PBLG is α-helical in THF, and aggregates in aqueous solutions. Samples were prepared using 5, 10, 20, 30, and 40 (v/v)% THF in PBS. Between 10 and 30% THF the particles had similar appearances, whereas with more THF the particles were larger (DLS) and had a different appearance (negative stained TEM, not shown). Based on these observations the initial ratio of THF:PBS was fixed at 10:90 (v/v). This strikes a balance between the necessity to perform experiments in an environment allowing coiled-coil pairing between

E and K, and the need for mobility of the hydrophobic PBLG blocks in order to reduce the formation of macro-aggregates.

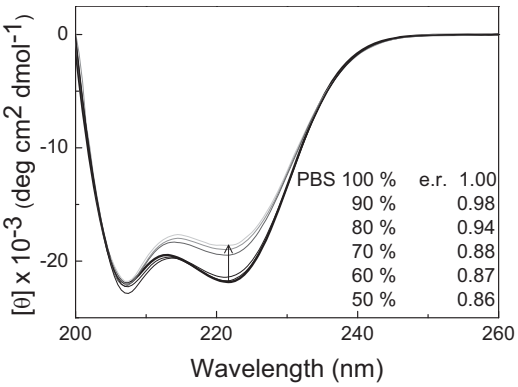

Figure A1. CD spectra of E/K in mixtures of PBS and THF. The intensity at 222 nm decreases with increasing THF concentrations (arrow). [Total Peptide] = ~ 0.5 mg mL^{-1}, PBS, 25 °C.

Peptide structure in the supramolecular assemblies

Circular dichroism (CD) spectra of the polypeptide-*b*-peptides and polymer mixtures in aqueous buffer after sonication are typical for aggregated α-helices: there is dampening of the spectrum and a red-shift of the 222 nm minimum compared to the typical solution spectra of α-helices. (Long, M. M.; Urry, D. W.; Stoeckenius, W. *Biochemical and Biophysical Research Communications* **1977**, 75, (3), 725-731. Frost, D. W. H.; Yip, C. M.; Chakrabartty, A. *Biopolymers* **2005**, 80, (1), 26-33. Potekhin, S. A.; Melnik, T. N.; Popov, V.; Lanina, N. F.; Vazina, A. A.; Rigler, P.; Verdini, A. S.; Corradin, G.; Kajava, A. V. *Chemistry & Biology* **2001**, 8, (11), 1025-1032. Pandya, M. J.; Spooner, G. M.; Sunde, M.; Thorpe, J. R.; Rodger, A.; Woolfson, D. N. *Biochemistry* **2000**, 39, (30), 8728-8734. Higashi, N.; Yamamoto, T.; Yokoyama, K.; Niwa, M. *Macromolecules* 1995, 28, (7), 2585-2587. Mattice, W. L.; Mccord, R. W.; Shippey, P. M. *Biopolymers* **1979**, 18, (3), 723-730. Cho, C. S.; Kobayashi, A.; Goto, M.; Akaike, T. *Thin Solid Films* 1995, 264, (1), 82-88. Urry, D. W. *Biochimica Et Biophysica Acta* **1972**, 265, (1), 115-&). The CD spectra of $PBLG_{36}$-E, $PBLG_{36}$-E/K, and $PBLG_{36}$-E/K-PEG are given in Figure S3. For $PBLG_{36}$-E the 222 nm peak is red-shifted, and both peaks are dampened. This is typical for membrane proteins, and the spectral artifacts are attributed to the particulate nature of the suspension (Long, M. M.; Urry, D. W.; Stoeckenius, W. *Biochemical and Biophysical Research Communications* **1977**, 75, (3), 725-731). For soluble proteins and peptides the intensity at 222 nm is directly proportional to the amount of helical structure (Chen, Y. H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, 13, (16), 3350-3359), but in this case the spectra are distorted due to the tight packing and therefore the amount of helical structure cannot be determined. Upon combining K with $PBLG_{36}$-E ($PBLG_{36}$-E/K) the distortions in the spectrum are reduced. With the addition of K-PEG ($PBLG_{36}$-E/K-PEG), the position of the minima is only slightly red-shifted (223 nm), and there is less dampening of the CD signal. These results show that as the size of the hydrophilic block/s increases in comparison to the hydrophobic PBLG block the helices become less constricted, yielding more standard CD spectra. Although the E/K complex can not be directly observed due to juxtaposition of the spectra of E/K with that of PBLG, it is clear that the molecules interact as the spectra are markedly different from the average of the individual components.

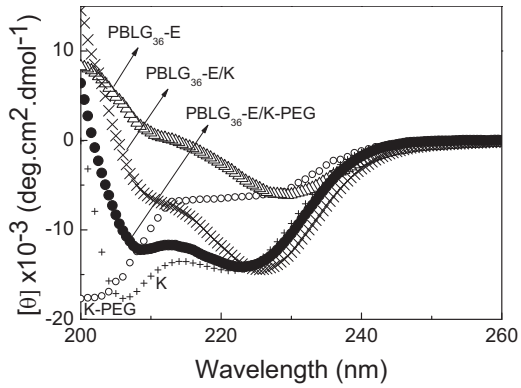

Figure A2. CD spectra of K (+), K-PEG (○), $PBLG_{36}$-E (Δ), $PBLG_{36}$-E/K (x), and $PBLG_{36}$-E/K-PEG (●) in PBS. [Total Peptide] = 50 μM, PBS, 25 °C.

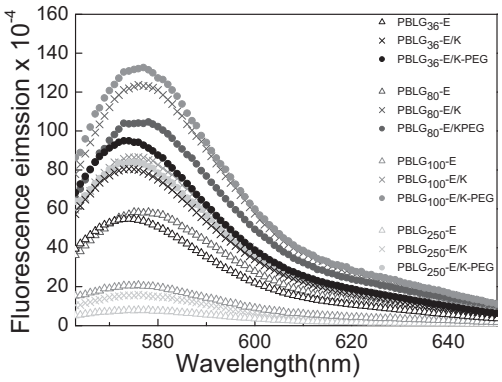

Figure A3. Fluorescence emission spectra of the polypeptide-*b*-peptides and polymer mixture suspensions. [Total Peptide] = 0.467 – 1.812 μM, PBS, 0.2 mg/mL rhodamine B, 25 °C.

Table A1. Zeta Potential of the Supramolecular Assemblies

Sample[a]	Zeta potential (mV)
$PBLG_{36}$-E	-26.1
$PBLG_{36}$-E/K	-9.5
$PBLG_{36}$-E/K-PEG	-7.2
$PBLG_{80}$-E	-28.5
$PBLG_{80}$-E/K	-10.2
$PBLG_{80}$-E K-PEG	-12.1
$PBLG_{100}$-E/K	-5.0
$PBLG_{100}$-E K-PEG	-5.7
$PBLG_{250}$-E K-PEG	-5.7

[a] [Total Peptide] = 0.0467 – 0.1812 μM, PBS: 5 mM phosphate, 10 mM KCl, pH 7.0, 0.02 mg/mL rhodamine B, 25 °C.