

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/41172261>

# Uniting Polypeptides with Sequence-Designed Peptides: Synthesis and Assembly of Poly( $\gamma$ -benzyl L-glutamate)-b-Coiled-Coil Peptide Copolymers

ARTICLE in JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · FEBRUARY 2010

Impact Factor: 12.11 · DOI: 10.1021/ja909540a · Source: PubMed

---

CITATIONS

44

---

READS

52

5 AUTHORS, INCLUDING:



**Jan-Willem Handgraaf**

Culgi B.V., Leiden, Netherlands

37 PUBLICATIONS 982 CITATIONS

SEE PROFILE



**Nico Sommerdijk**

Technische Universiteit Eindhoven

248 PUBLICATIONS 7,832 CITATIONS

SEE PROFILE



**Alexander Kros**

Leiden University

109 PUBLICATIONS 2,363 CITATIONS

SEE PROFILE

### Uniting Polypeptides with Sequence-Designed Peptides: Synthesis and Assembly of Poly( $\gamma$ -benzyl L-glutamate)-*b*-Coiled-Coil Peptide Copolymers

Hana Robson Marsden,<sup>†</sup> Jan-Willem Handgraaf,<sup>‡</sup> Fabio Nudelman,<sup>§</sup>  
Nico A. J. M. Sommerdijk,<sup>§</sup> and Alexander Kros\*,<sup>†</sup>

*Department of Soft Matter Chemistry, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands, Culgi B.V., P.O. Box 252, 2300 AG Leiden, The Netherlands, and Soft Matter cryoTEM Research Unit, Department of Biomedical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands*

Received November 10, 2009; E-mail: a.kros@chem.leidenuniv.nl

**Abstract:** A new class of peptide has been created, polypeptide-*b*-designed peptides, which unites the useful qualities of the two constituent peptide types. We demonstrate the synthesis and self-assembly possibilities of this class of peptide chimera with a series of amphiphilic polypeptide-*b*-designed peptides in which the hydrophobic block is poly( $\gamma$ -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 the ring-opening polymerization of  $\gamma$ -benzyl L-glutamate *N*-carboxyanhydride, initiated from the N-terminal amine of the peptide E 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 to 250 residues. This new class of peptide was applied to create a modular system, which relied on juxtaposing the properties of the component peptide types, namely the broad size range and structure-inducing characteristics of the polypeptide PBLG blocks, and the complex functionality of the sequence-designed peptide. Specifically, the different PBLG block lengths could 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 dynamic light scattering, circular dichroism, scanning electron microscopy, cryogenic-transmission electron microscopy, fluorescence spectroscopy, and zeta-potential measurements. Finally, as the structures are able to encapsulate water-soluble compounds, and the surfaces are easily functionalized via the coiled-coil binding, it is expected that these peptide-based nanocapsules will be able to act as delivery vehicles to specific targets in the body.

#### Introduction

For materials scientists, peptides 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.<sup>1</sup> 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.<sup>2</sup>

Two methods for the synthesis of peptides are the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhy-

drides (NCAs) and solid-phase synthesis. The ROP of NCAs is the most common method of synthesizing polypeptides containing a single amino acid residue.<sup>3,4</sup> The polymers can be readily prepared and have no detectable racemization at the chiral centers.<sup>5</sup> Blocks based on glutamic acid ( $\gamma$ -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  $\alpha$ -helical secondary structures both in the solid-state and in solution.<sup>6</sup> They have been initiated from traditional linear coil polymers,<sup>7</sup> polymer dendrimers,<sup>8,9</sup> modi-

<sup>†</sup> Leiden University.

<sup>‡</sup> Culgi B.V.

<sup>§</sup> Eindhoven University of Technology.

(1) Cavalli, S.; Kros, A. *Adv. Mater.* **2008**, *20*, 627–631.

(2) van Hest, J. C. M.; Tirrell, D. A. *Chem. Commun.* **2001**, 1897–1904.

(3) Smeenk, J. M.; Ayres, L.; Stunnenberg, H. G.; van Hest, J. C. M. *Macromol. Symp.* **2005**, *225*, 1–8.

(4) Marsden, H. R.; Kros, A. *Macromol. Biosci.* **2009**, *9*, 939–951.

(5) Deming, T. J. *Adv. Polym. Sci.* **2006**, *202*, 1–18.

(6) Gallot, B. *Prog. Polym. Sci.* **1996**, *21*, 1035–1088.

(7) Caillol, S.; Lecommandoux, S.; Mingotaud, A. F.; Schappacher, M.; Soum, A.; Bryson, N.; Meyrueix, R. *Macromolecules* **2003**, *36*, 1118–1124.

**Table 1.** Molecular Characteristics of the Compounds Used in This Study

name	structure	yield (%)	$M_n$ (g/mol)	PDI <sup>c</sup>
K	Ac-(K I A A L K E) <sub>3</sub> -G-NH <sub>2</sub>	~40	2378.0 <sup>a</sup>	
E	Ac-G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	~40	2380.6 <sup>a</sup>	
PEG-K	Ac-(K I A A L K E) <sub>3</sub> -G-PEG <sub>77</sub>	~10	5832 <sup>a,b</sup>	1.05 <sup>a</sup>
PBLG <sub>36</sub> -E	PBLG <sub>36</sub> -G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	28	10 230 <sup>b,c</sup>	1.1
PBLG <sub>55</sub> -E	PBLG <sub>55</sub> -G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	30	14 396 <sup>b,c</sup>	1.3
PBLG <sub>80</sub> -E	PBLG <sub>80</sub> -G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	56	19 877 <sup>b,c</sup>	1.4
PBLG <sub>100</sub> -E	PBLG <sub>100</sub> -G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	69	24 262 <sup>b,c</sup>	1.4
PBLG <sub>250</sub> -E	PBLG <sub>250</sub> -G(E I A A L E K) <sub>3</sub> -NH <sub>2</sub>	74	57 148 <sup>b,c</sup>	1.7

<sup>a</sup> Obtained from MALDI-TOF MS. <sup>b</sup> Based on a comparison of <sup>1</sup>H NMR peaks. <sup>c</sup> GPC calibrated with polystyrene standards. PEG, poly(ethylene glycol); PBLG, poly( $\gamma$ -benzyl L-glutamate); amino acids in the designed peptides are represented by their one letter codes, Ac: acetyl.

fied lipids,<sup>10</sup> and polypeptides themselves synthesized by the ROP of NCAs.<sup>11</sup> Most commonly initiators with primary amine end-groups are used,<sup>5</sup> although the polymerization can also be initiated with transition metal-amine-functionalized polymers.<sup>12</sup> Block copolymers have also been synthesized in the reverse manner, that is, the ROP of NCA, followed by polymerization of another monomer from the polypeptide.<sup>13,14</sup> The ROP of BLG-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.<sup>5</sup> Additionally, the abundance of side-reactions leads to contamination with homopolymers, which have to be separated from the desired block copolymers.<sup>5</sup>

Using solid-phase peptide synthesis (SPPS) 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 as 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,<sup>15</sup> are accessible by solid-phase synthesis and are of particular interest. Coiled-coil forming peptides are strongly complementary in spatial packing and interpeptide 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 this Article, we combine for the first time solid-phase synthesis with ROP to build up polypeptide-*b*-peptides. This new synthetic approach gives access to peptides that are not possible to synthesize using only solid-phase synthesis or only ROP. The well-defined shape and large size range of polypeptides can be united with the complex functionality of sequence-specific peptides, leading to peptides with an increased range of 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, the hydrophobic block is composed of PBLG, and the second 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  $\gamma$ -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. A series of PBLG-E block copolymers were synthesized with hydrophobic block lengths ranging from tens to hundreds of monomers, 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 has been shown to retain its coiled-coil binding upon conjugation of macromolecules to E and/or K.<sup>16–18</sup> Of particular relevance is our previous coupling of a short polystyrene chain with an average length of 9 monomers to the N-terminus of peptide E (denoted PS<sub>9</sub>-E).<sup>17</sup> PS<sub>9</sub>-E forms spherical micelles in aqueous solution, and via coiled-coil folding with peptide K or K-poly(ethylene glycol)<sub>77</sub> 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

- (8) Huang, H.; Dong, C. M.; Wei, Y. *Comb. Chem. High Throughput Screen.* **2007**, *10*, 368–376.
- (9) Higashi, N.; Koga, T.; Niwa, M. *Langmuir* **2000**, *16*, 3482–3486.
- (10) Dimitrov, I. V.; Berlinova, I. V.; Iliev, P. V.; Vladimirov, N. G. *Macromolecules* **2008**, *41*, 1045–1049.
- (11) Sun, J.; Chen, X. S.; Lu, T. C.; Liu, S.; Tian, H. Y.; Guo, Z. P.; Jing, X. B. *Langmuir* **2008**, *24*, 10099–10106.
- (12) Brzezinska, K. R.; Deming, T. J. *Macromolecules* **2001**, *34*, 4348–4354.
- (13) Kros, A.; Jesse, W.; Metselaar, G. A.; Cornelissen, J. J. L. M. *Angew. Chem., Int. Ed.* **2005**, *44*, 4349–4352.
- (14) Imanishi, Y. J. *Macromol. Sci., Chem.* **1984**, *A21*, 1137–1163.
- (15) Woolfson, D. N. *Fibrous Proteins: Coiled-Coils, Collagen And Elastomers*; Elsevier Academic Press Inc.: San Diego, CA, 2005; Vol. 70, p 79.

- (16) Yano, Y.; Yano, A.; Oishi, S.; Sugimoto, Y.; Tsujimoto, G.; Fujii, N.; Matsuzaki, K. *ACS Chem. Biol.* **2008**, *3*, 341–345.
- (17) Robson Marsden, H.; Korobko, A. V.; van Leeuwen, E. N. M.; Pouget, E. M.; Veen, S. J.; Sommerdijk, N. A. J. M.; Kros, A. J. *Am. Chem. Soc.* **2008**, *130*, 9386–9393.
- (18) Robson Marsden, H.; Elbers, N. A.; Bomans, P. H. H.; Sommerdijk, N. A. J. M.; Kros, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 2330–2333.

the hydrophobic PBLG length is readily controlled by ROP, and the hydrophilic block(s) are readily controlled by coiled-coil folding, allowing one to piece together different components to access a range of self-assembled structures.

## Experimental Section

**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. PBS: 50 mM phosphate, 100 mM KCl, pH 7.0.

**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 previously.<sup>17</sup> 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  $\gamma$ -Benzyl L-Glutamate N-Carboxyanhydride (BLG-NCA).** A suspension of  $\gamma$ -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 h, until the suspension became clear. If the suspension remained turbid, a small quantity of triphosgene was added every 15 min. 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. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>,  $\delta$ ): 7.3 (aromatic H, m), 5.1 (benzylic CH<sub>2</sub>, s), 2.6 ( $\gamma$ -CH<sub>2</sub>, t), 2.2 ( $\beta$ -CH<sub>2</sub>, m), 4.4 ( $\alpha$ -CH, t), 6.8 (N-H, br).

**Solid-Phase Synthesis of Poly( $\gamma$ -benzyl L-glutamate)-block-E (PBLG-E).** Poly( $\gamma$ -benzyl L-glutamate) was synthesized via a one-pot NCA polymerization of  $\gamma$ -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 under reduced pressure at 40 °C overnight, and then in argon with reduced pressure for 5 h. 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 24–65 h. A small volume of DCM was drained from the reaction vessel, and the contents were 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<sub>2</sub> carbonyl at 2000–1800 cm<sup>-1</sup>, which is released as CO<sub>2</sub> 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 min, 10 times. Each cleavage mixture was precipitated dropwise in cold methanol. The white precipitate was compacted with centrifugation, and the supernatant was removed. This was repeated three times with the addition of fresh methanol. The pellets were vacuum-dried.

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 h, and the product was precipitated dropwise in cold methanol. The white precipitate was compacted with centrifugation, and the supernatant was removed. This was repeated three times with the addition of fresh methanol. The pellets were vacuum-dried, 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 (Table 1).

## Characterization of the PBLG-E Polypeptide-*b*-Peptides.

Molecular weights and their distributions of the protected PBLG-E polypeptide-*b*-peptides were determined using gel phase chromatography (GPC). 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 °C, and a flow rate of 1 mL min<sup>-1</sup>. Both the coiled-coil peptide and the PBLG are soluble in DMF, and the runs were conducted at 60 °C to prevent aggregation. The molecular weights were calibrated using polystyrene standards.

The purity and molecular weights of the deprotected polypeptide-*b*-peptides were checked using <sup>1</sup>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. To ensure that there were no aggregation artifacts in the spectra that were analyzed for molecular weight determination, a range of <sup>1</sup>H NMR spectra of the deprotected hybrids were recorded, from deuterated dichloromethane to 1:1 (v/v) deuterated dichloromethane:trifluoroacetic acid.

The absolute masses of the polypeptide-*b*-peptides with shorter PBLG blocks were determined using MALDI-TOF mass spectrometry. 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 ~3 mg mL<sup>-1</sup>. Solutions for spots consisted of (v/v) 1:10 sample solution: 10 mg mL<sup>-1</sup>  $\alpha$ -cyano-4-hydroxycinnamic acid (ACH) in TA.

The secondary structure of the polypeptide-*b*-peptides was determined using FT-IR spectroscopy. FT-IR spectra were recorded on a BIORAD FTS-60A instrument equipped with a deuterated-triglycine-sulfate (DTGS) detector at a resolution of 20 cm<sup>-1</sup>. The compounds were dried from dichloromethane onto an ATR ZnSe crystal. A clean ATR ZnSe crystal was used as the background.

**Preparation of PBLG-E Suspensions.** 0.1  $\mu$ mol of each compound (PBLG-E, or PBLG-E and K, or PBLG-E and K-PEG) was dissolved in 200  $\mu$ L of tetrahydrofuran (THF). Two milliliters of phosphate buffered saline (PBS, 50 mM PO<sub>4</sub>, 100 mM KCl, pH 7.0) was added, and the sample was immediately sonicated with an open lid for 2 h in a Branson 1510 bath sonicator with an output of 70 W and 42 kHz. The final concentration of each polymer was 50  $\mu$ M.

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<sup>-1</sup>, 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, Amersham Pharmacia Biotech apparatus with a Pharmacia XK 26 column (135 mm  $\times$  25 mm) packed with Sephadex G50-fine. PBS was used as the eluent. The flow rate was 5 mL min<sup>-1</sup>, UV sensitivity was set on 0.1 AU, 1%, the conductivity was set on 15–20 mS cm<sup>-1</sup>, and the wavelength for UV recording was 254 nm.

**Characterization of PBLG-E Suspensions.** Experimental diffusion coefficients, *D*, were measured at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS equipped with a peltier-controlled thermostatic cell holder. The laser wavelength was 633 nm, and the scattering angle was 173°. The Stokes–Einstein relationship  $D = k_b T / 3\pi\eta D_h$  was used to estimate the hydrodynamic radius, *D<sub>h</sub>*. Here, *k<sub>b</sub>* is the Boltzmann constant, and  $\eta$  is the solvent viscosity.

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 10-fold with 1 mM NaCl to reduce the ionic strength of the buffer.

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  $\mu$ L of the solution on SEM stubs with a TEM grid on the carbon tape. After 30 min, the excess buffer was removed. Samples were coated with gold for 1 min, resulting in a layer  $\sim$ 15 nm thick.

Samples for cryogenic TEM were concentrated by centrifuging in Centricon centrifugal filter devices MWCO 3000 g mL<sup>-1</sup> at 4 °C. Sample stability was verified by DLS and TEM. The cryogenic transmission microscopy measurements were performed on a FEI Technai 20 (type Sphera) TEM or on a Titan Krios (FEI). A Gatan cryo-holder operating at  $\sim$ –170 °C was used for the cryo-TEM measurements. The Technai 20 is equipped with a LaB<sub>6</sub> filament operating at 200 kV, and the images were recorded using a 1k  $\times$  1k Gatan CCD camera. The Titan Krios is equipped with a field emission gun (FEG) operating at 300 kV. Images were recorded using a 2k  $\times$  2k Gatan CCD camera equipped with a post column Gatan energy filter (GIF). The sample vitrification procedure was carried out using an automated vitrification robot: a FEI Vitrobot Mark III. TEM grids, both 200 mesh carbon coated copper grids and R2/2 Quantifoil Jena grids, were purchased from Aurion. Copper grids bearing lacey carbon films were homemade using 200 mesh copper grids from Aurion. Grids were treated with a surface plasma treatment using a Cressington 208 carbon coater operating at 25 A for 40 s prior to the vitrification procedure.

Circular dichroism (CD) spectra were obtained using a Jasco J-815 spectropolarimeter equipped with a peltier-controlled thermostatic cell holder. Spectra were recorded from 260 to 200 nm in a 1 mm quartz cuvette at 25 °C. Data were collected at 0.5 nm intervals with a 1 nm bandwidth and 1 s readings. Each spectrum was the average of five scans. For analysis, each spectrum had the appropriate background spectrum (buffer or buffer/THF) subtracted.

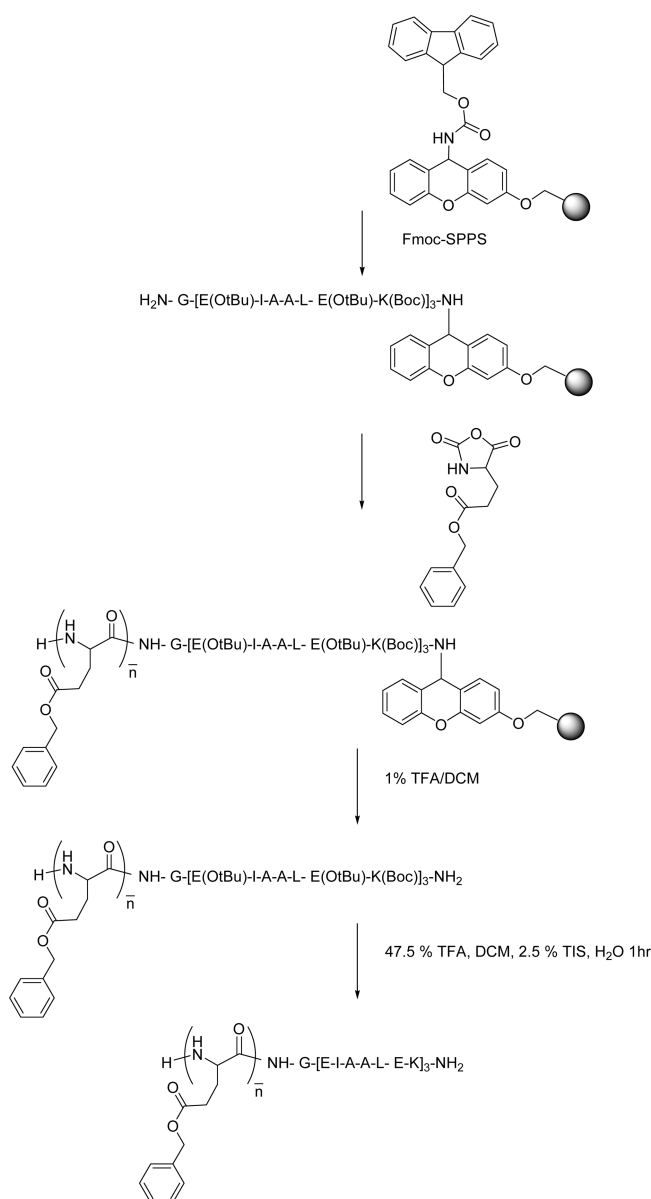
The amount of encapsulated Rhodamine B in each sample was determined by fluorescence spectroscopy, with excitation at 555 nm, and emission monitored from 563–650 nm with 5 nm slits using a Cary-50 spectrophotometer.

## Results and Discussion

**Synthesis and Characterization of the Protected PBLG-E Block Copolymer Series.** Poly( $\alpha$ -amino acid)s can be prepared by the ROP of NCAs starting from nucleophilic attack of the C<sub>5</sub> carbonyl group of the NCA by an initiator such as amines, alkoxide anions, alcohols, transition metals, and water.<sup>19</sup> In our approach to synthesize polypeptide-*b*-peptides, we first synthesized the coiled-coil peptide block E on a Sieber amide resin using standard Fmoc solid-phase peptide protocols and removed the N-terminal Fmoc group. ROP of the BLG-NCA was 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 1–3 days. When all of 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 min 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

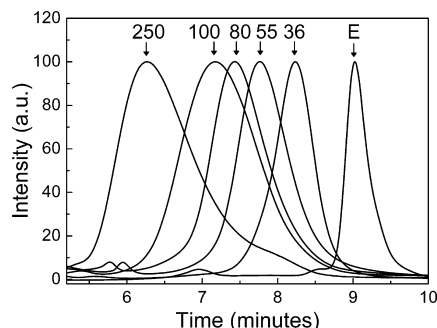
**Scheme 1.** Synthesis of PBLG-E by Solid-Phase Peptide Synthesis Followed by *N*-Carboxyanhydride Ring-Opening Polymerization Initiated from the N-Terminal Amine of the Resin-Bound Peptide



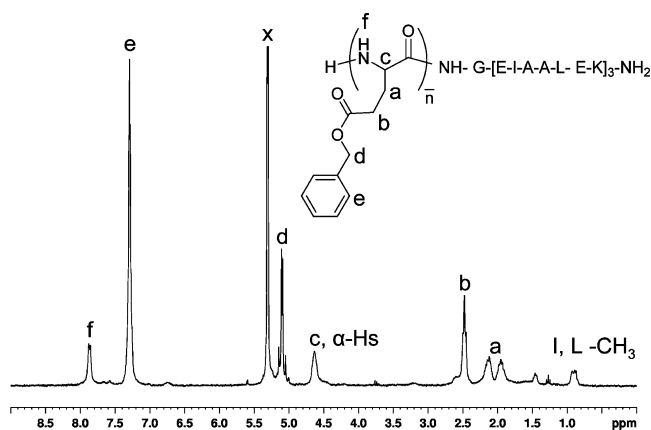
it was found that within each synthesis the longer PBLG-E polypeptide-*b*-peptides were cleaved first from the resin, with a progressive shortening of the PBLG chain with each fraction collected, until finally peptide fragments from the solid-phase peptide synthesis of E were cleaved. In this way, polypeptide-*b*-peptides with a lower polydispersity index (PDI) can be obtained by selecting which fractions to combine. Because of 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 1. 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.

**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

(19) Blout, E. R.; Karlson, R. H. *J. Am. Chem. Soc.* **1956**, 78, 941–946.



**Figure 1.** 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.



**Figure 2.**  $^1\text{H}$  NMR spectrum of PBLG<sub>36</sub>-E in 7:3 (v/v) DCM- $d_3$ :TFA with the proton assignments. X is from DCM.

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  $^1\text{H}$  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.<sup>20–22</sup> 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 2). The degree of polymerization of the PBLG blocks determined using  $^1\text{H}$  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.<sup>23</sup>

**Modular Assembly 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 lead to novel self-assembly properties.<sup>24</sup> PBLG is hydrophobic and with a degree of polymerization ( $n$ ) larger than 10 has an  $\alpha$ -helical secondary structure,<sup>25</sup> resulting in a rod-like molecular shape. The length of PBLG  $\alpha$ -helices is  $n \times 1.5$  nm  $\text{\AA}$ <sup>26</sup> while the diameter is  $\sim 2$  nm,<sup>27</sup> hence in the  $\alpha$ -helical configuration the PBLG rod-like blocks in this study range in average length from 5.4 to 37.5 nm long. The hydrophilic block, peptide E, forms an  $\alpha$ -helical coiled-coil dimer with K, a peptide with a complementary amino acid sequence (Figure S1).<sup>17,28</sup> 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.<sup>29</sup> 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,<sup>30</sup> although it is more compact when attached to peptide K.<sup>17</sup> The peptides K and the hybrid K-PEG are predominantly hydrophilic and do not aggregate in aqueous solutions.<sup>31</sup>

Because of the amphiphilic nature of the rod-rod PBLG-E polypeptide-*b*-peptides and the noncovalent complexes PBLG-E/K and PBLG-E/K-PEG, we 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,<sup>32</sup> 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

(23) See the Supporting Information.

(24) Scherf, U.; Adamczyk, S.; Gutacker, A.; Koenen, N. *Macromol. Rapid Commun.* **2009**, *30*, 1059–1065.

(25) Rinaudo, M.; Domard, A. *Biopolymers* **1976**, *15*, 2185–2199.

(26) Murthy, N. S.; Knox, J. R.; Samulski, E. T. *J. Chem. Phys.* **1976**, *65*, 4835–4839.

(27) Chang, Y. C.; Frank, C. W. *Langmuir* **1996**, *12*, 5824–5829.

(28) The secondary and quaternary structures of the peptides E and K in PBS were evaluated by circular dichroism spectroscopy. Peptide E adopts a predominantly random coil conformation, while K exhibits a predominantly  $\alpha$ -helical spectrum. Both peptides are in the monomeric state as indicated by the observed ellipticity ratios ( $[\theta]_{222}/[\theta]_{208}$ ) of 0.59 and 0.74, respectively. When peptides E and K were combined in an equimolar ratio, denoted E/K, a typical  $\alpha$ -helical spectrum is exhibited, with minima at 208 and 222 nm. The ellipticity ratio was determined to be 1.00, consistent with interacting  $\alpha$ -helices. This clearly shows that E and K specifically interact to form a hetero- $\alpha$ -helical coiled coil. The formation of the dimeric species was confirmed by determining the molecular weights using sedimentation equilibrium, revealing that separate solutions of E and K are purely monomeric while the mixture of E/K exists as dimers.

(29) Lindhout, D. A.; Litowski, J. R.; Mercier, P.; Hodges, R. S.; Sykes, B. D. *Biopolymers* **2004**, *75*, 367–375.

(30) The hydrodynamic diameter of the PEG block was determined by DLS.

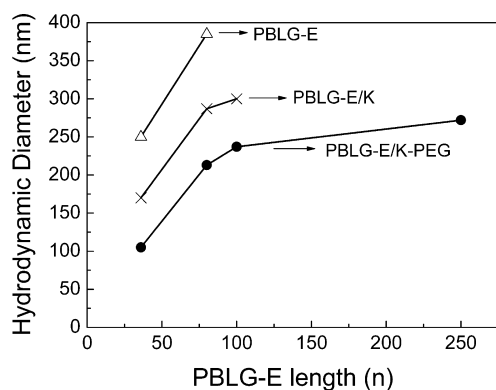
(31) Kaiser, E.; Colescot, R.; Bossinge, C.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.

(32) Kita-Tokarczyk, K.; Grumelard, J.; Haefele, T.; Meier, W. *Polymer* **2005**, *46*, 3540–3563.

(20) Note that when PBLG-E is in the  $\alpha$ -helical conformation, for example, in DCM or DMSO, the  $\alpha$ -H resonance peak is at 4.0 ppm, and by adding TFA the peak position is shifted low-field to 4.7 ppm, indicating that the hybrids have random coil conformation in this solvent mixture and are not aggregated.

(21) Higashi, N.; Kawahara, J.; Niwa, M. *J. Colloid Interface Sci.* **2005**, *288*, 83–87.

(22) Bradbury, E. M.; Cranerob, C.; Goldman, H.; Rattle, H. W. E. *Nature* **1968**, *217*, 812–816.

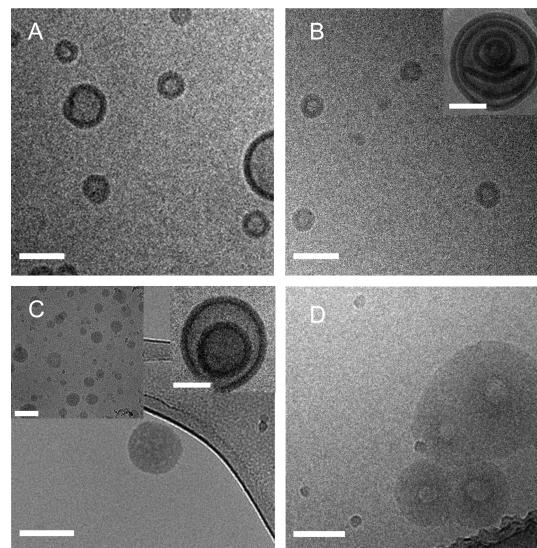


**Figure 3.** Typical hydrodynamic diameters of the self-assembled structures formed by the polypeptide-*b*-peptides and noncovalent complexes ([total peptide] = 50  $\mu$ M, PBS, 25  $^{\circ}$ C).

such that the E/K coiled coil is stable (Figure S2), and the THF was subsequently removed by continuous sonication for 2 h in an open vessel. An example of circular dichroism spectra of the polymers and polymer mixtures is given in the Supporting Information (Figure S3).

**Particle Sizes.** The aggregation of the individual polypeptide-*b*-peptides (PBLG-E) was investigated with dynamic light scattering (DLS), as well as with the addition of equimolar amounts of the K or K-PEG components. It was found that when the PBLG block length was 80 monomers or shorter, each of the three permutations had a suitable balance of hydrophobicity and hydrophilicity to form ordered supramolecular structures in aqueous solution. For PLBG<sub>100</sub>-E, complexation with K or K-PEG resulted in a noncovalent block copolymer with a corona sufficient to form ordered structures. The amphiphilic block copolymer with the longest hydrophobic block, PBLG<sub>250</sub>-E, required association with K-PEG 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 to 400 nm and were significantly larger than the calculated sizes of spherical micelles (10.8–85 nm<sup>33</sup>). All size distributions were monomodal, and the polydispersity index (PDI) of the samples was 0.35 or less.

As shown in Figure 3, increasing the PBLG block length resulted in larger aggregates. Additionally, for a particular PBLG block length, increasing the size of the headgroup (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 headgroup is in comparison to the hydrophobic PBLG, the greater is the curvature of the aggregate surface, and hence the particle size decreases.<sup>34</sup> 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.<sup>17</sup> 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



**Figure 4.** Cryo-TEM images of (A) PBLG<sub>36</sub>E, (B) PBLG<sub>36</sub>E/K (with multilamellar vesicle inset), (C) PBLG<sub>36</sub>E/K-PEG (with bilamellar vesicle and bicelles inset), and (D) PBLG<sub>100</sub>E/K-PEG. Scale bars = 100 nm ([total peptide] =  $\sim$ 1500  $\mu$ M, PBS).

hydrophobic chains is minimal (lipid tails are stretched,<sup>35</sup> and the PBLG rods have a well-defined structure and size with no change in configuration expected upon aggregation<sup>36</sup>), hence the role of deformation of the core block on the free-energy of the structure is reduced.

**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 S4).

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 S4). PBLG<sub>36</sub>-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 4). The shortest polypeptide-*b*-peptide, PBLG<sub>36</sub>-E (with a low PDI of 1.1), self-assembles into vesicles with rather uniform membrane thicknesses, which 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<sub>36</sub>-E,  $18.5 \pm 2.4$  nm for PBLG<sub>36</sub>-E/K, and  $21.5 \pm 2.2$  nm for PBLG<sub>36</sub>-E/K-PEG (Figure 4A–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,<sup>37</sup> there does not appear to be any interdigitation of the two layers of the hydrophobic block,

(33) Based on PBLG length,  $n \times 0.15$  nm; E and E/K length, 3.5 nm; PEG length, 3 nm.

(34) Israelachvili, J. N.; Mitchell, D. J.; Ninham, B. W. *J. Chem. Soc., Faraday Trans. 2* **1976**, 72, 1525–1568.

(35) Opsteen, J. A.; Cornelissen, J. J. L. M.; van Hest, J. C. M. *Pure Appl. Chem.* **2004**, 76, 1309–1319.

(36) Halperin, A. *Macromolecules* **1990**, 23, 2724–2731.

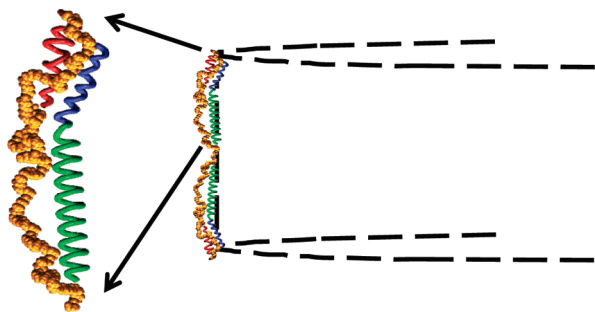
(37) Srinivas, G.; Discher, D. E.; Klein, M. L. *Nat. Mater.* **2004**, 3, 638–644.



**Table 2.** Experimental and Theoretical Membrane Thicknesses of the Vesicles

sample	membrane thickness	
	experimental (nm) <sup>a</sup>	theoretical (nm) <sup>b</sup>
PBLG <sub>36</sub> -E	17.2 ± 2.6 nm	18
PBLG <sub>36</sub> -E/K	18.5 ± 2.4 nm	18
PBLG <sub>36</sub> -E/K-PEG	21.5 ± 2.2 nm	24

<sup>a</sup> Determined by cryo-TEM. <sup>b</sup> PBLG length,  $n \times 1.5 \text{ \AA}$ ; <sup>26</sup> E and E/K length, 3.5 nm; <sup>29</sup> PEG length,  $\sim 3 \text{ nm}$ .<sup>17</sup>



**Figure 5.** The space-filling model of the PBLG<sub>36</sub>-E/K-PEG complex illustrates that without chain stretching PEG readily extends the length of the coiled-coil peptides and the PBLG block, as is proposed to occur at the edges of the polymeric bicelles. The E/K dimer structure (red and blue helices) is based on the work of Litowski and Hodges,<sup>29</sup> the PBLG adopts an  $\alpha$ -helix (green), and the PEG has a random coil conformation (orange). The PBLG<sub>36</sub>-E/K-PEG model was built with Molden version 4.6,<sup>39</sup> and the image was generated with VMD version 1.8.6.<sup>40</sup>

presumably due to the rod-like structure of the PBLG and the comparable diameter of the hydrophobic and corona rods.

In addition to polymersomes, bicelles, disks of uniform height and density, were observed in the SEM and cryo-TEM images of PBLG<sub>36</sub>-E/K-PEG (arrow in Figure S4C, and Figure 4C; note that the bicelles were predominantly situated on the carbon film of the TEM grids, as shown in the inset of Figure 4C). 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 lengths of the PBLG blocks are short enough that the hydrophilic PEG block is able to fold over any exposed PBLG on the sides of the discs, shielding it from the aqueous buffer.<sup>38</sup> This eliminates the energetic impetus 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  $\alpha$ -helical PBLG block without any chain stretching, that is, while still in the random coil configuration (Figure 5). 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,

as in this geometry the rods pack well together, and large coil blocks are less affected by confinement free energy.<sup>36</sup>

For the polypeptide-*b*-peptides and noncovalent complexes with longer PBLG lengths, the SEM images exclusively show spherical objects that withstand the drying process. Cryo-TEM was also conducted on the structures assembled from the complex PBLG<sub>100</sub>-E/K-PEG. As seen in Figure 4D, the spherical particles were observed to contain aqueous interiors and have very bulky membranes of variable thickness ( $\sim 68 \pm 22 \text{ nm}$ ). 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<sub>100</sub>-E lengths are between 9 and 80 nm when in the  $\alpha$ -helical conformation.

An advantage of block copolymer capsules 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,<sup>41</sup> while the membrane thickness of polymersomes ranges from 3–5 nm<sup>42</sup> to 200 nm (in 1:1 v/v TFA/DCM).<sup>43</sup> In the present series, the nature of the membrane can be dictated by the modular choice of hydrophobic and hydrophilic component(s), and as far as we are aware the breadth of the membrane of PBLG<sub>100</sub>-E/K-PEG aggregates is the largest reported for polymersomes in aqueous solutions.

**Encapsulation, Stability, and Surface Functionality.** 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 S5), 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. As expected, the samples that did not show well-defined assembly by DLS (PBLG<sub>100</sub>-E, PBLG<sub>250</sub>-E, and PBLG<sub>250</sub>-E/K) contained insignificant amounts of rhodamine B, as verified by fluorescence spectroscopy (Figure S5). 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.<sup>44</sup> As well as the particle size and morphology, the surface functionality is also determined by which molecules are chosen to complex with the E block and was likewise 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-

(38) There is likely to be sorting within the bicelles such that the polymer-*b*-peptides with shorter hydrophobic blocks are located at the rim. The shortest PBLG length in the PBLG<sub>36</sub>-E polymer-*b*-peptide is 23 monomers (as determined by MALDI-TOF MS), which corresponds to an  $\alpha$ -helical length of  $\sim 3.5 \text{ nm}$ . The persistence length of bulk PBLG is  $\sim 2 \text{ nm}$  and increases when under thermodynamic confinement, as is the case in this nanophase separated system. Floudas, G.; Speiss, H. W. *Macromol. Rapid Commun.* **2009**, *30*, 278–298. It is possible that the PBLG rods are kinked, further reducing/eliminating the area of solvent-exposed PBLG.

(39) Schaftenaar, G.; Noordik, J. H. *J. Comput.-Aided Mol. Des.* **2000**, *14*, 123–134.

(40) Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graph.* **1996**, *14*, 33–38.

(41) Discher, B. M.; Hammer, D. A.; Bates, F. S.; Discher, D. E. *Curr. Opin. Colloid Interface Sci.* **2000**, *5*, 125–131.

(42) Schillen, K.; Bryskhe, K.; Mel'nikova, Y. S. *Macromolecules* **1999**, *32*, 6885–6888.

(43) Jenekhe, S. A.; Chen, X. L. *Science* **1998**, *279*, 1903–1907.

(44) The hydrodynamic diameter of PBLG<sub>36</sub>-E, PBLG<sub>36</sub>-E/K, and PBLG<sub>36</sub>-E/K-PEG was monitored over 10 months, while the remainder of the samples were monitored (and were stable) over 20 months.



E/K-PEG samples (with no net charge per complex) having zeta potentials closer to zero (Table S1).

## Conclusions

A new class of peptides, polypeptide-*b*-peptides, has been synthesized by a straightforward synthetic approach. The new peptide class is achieved by initiating ring-opening polymerization of NCAs from a designed peptide. The concept is demonstrated by the solid-supported polymerization of  $\gamma$ -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 the functionality, which are both determining factors in molecular self-assembly. This potential is investigated by selecting four 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 the 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.

**Supporting Information Available:** Characterization of the compounds. Circular dichroism spectra of the coiled-coil forming peptides in PBS and THF. Circular dichroism spectra, SEM images, fluorescence spectra, and zeta potential measurements of the self-assembled nanostructures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA909540A