A macroporous hydrogel for the coculture of neural progenitor and endothelial cells to form functional vascular networks in vivo

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A microvascular network is critical for the survival and function of most tissues. We have investigated the potential of neural progenitor cells to augment the formation and stabilization of microvascular networks in a previously uncharacterized three-dimensional macroporous hydrogel and the ability of this engineered system to develop a functional microcirculation in vivo. The hydrogel is synthesized by cross-linking polyethylene glycol with polylysine around a salt-leached polylactic-co-glycolic acid scaffold that is degraded in a sodium hydroxide solution. An open macroporous network is formed that supports the efficient formation of tubular structures by brain endothelial cells. After subcutaneous implantation of hydrogel cocultures in mice, blood flow in new microvessels was apparent at 2 weeks with perfused networks established on the surface of implants at 6 weeks. Compared to endothelial cells cultured alone, cocultures of endothelial cells and neural progenitor cells had a significantly greater density of endothelial cells cultured alone, cocultures of endothelial cells and NPCs would promote the stabilization of engineered tissues and to test the hypothesis that coculture of endothelial cells and NPCs promote the formation of endothelial cell tubes in coculture and the development of a functional microcirculation in vivo. We demonstrate a previously undescribed strategy for creating stable microvascular networks to support engineered tissues of desired parenchymal cell origin.

Vascularization is vital for tissue function. A microvascular network is critical for oxygen delivery, nutrient exchange, and, ultimately, for the long-term survival of tissue cells. Whereas the overall size and specific application of engineered tissues depend on the ability to create stable and functional microvascular networks (1, 2), vascularization has proven difficult to achieve. Approaches for engineering de novo microvascular networks have included the delivery of angiogenic growth factors from polymer constructs (3–5), implanting biodegradable matrices seeded with endothelial cells or their progenitors (6–9), and combining growth factors and cells (4). Although these approaches have shown promise, the long-term stability and function of microvascular networks remains a challenge.

Recent work has implicated a strong functional interaction between neural progenitor cells (NPCs) and endothelial cells (10) and has shown spatial proximity between established neural and vascular networks (11). We sought to capitalize on this potential interaction to tissue engineer a functional vascular network. Our goal was to design a scaffold that would support the development of microvessels by providing a spatial environment that enabled neural progenitor cells to interact with endothelial cells during tube formation.

Hydrogels, because of their highly water-saturated nature, are well suited for the transport of soluble factors, nutrients, and waste (12, 13). One of the challenges with hydrogels has been to obtain controlled pore architectures. The use of light to cross-link, inscribe, or remove material has been studied as a means to obtain distinct pore architectures in hydrogels (14, 15). These techniques lead to two-dimensional patterns of high-density and low-density regions. An alternative approach by using the dissolution of polycaprolactone fibers has also been used to create a similar pore architecture (16). However, to develop functional microvascular networks that are integral to tissue and organ function, a three-dimensional pore architecture is required.

Our goal in these experiments was to engineer a three-dimensional scaffold that is suitable for microvascular networks in engineered tissues and to test the hypothesis that coculture of endothelial cells and NPCs would promote the stabilization of microvascular networks in vivo. We have developed a two-component enzymatically degradable hydrogel that is cast around a hydrolytically degraded polymer scaffold to create the requisite architecture. Our findings show that the resulting macroporous hydrogel supports the coculture of endothelial cells and neural progenitor cells. Furthermore, these cocultures encourage endothelial cell tube formation and the development of stable functional microvascular networks as confirmed after 6 weeks of implantation in the mouse.

Results

Hydrogel Characterization. The mechanical properties, swelling, and degradation were determined for the isotropic hydrogel. The mechanical data by following Flory’s equations as described in ref. 17, we found the effective density of crosslinks to be 1.85 mol/m³. From this calculation, we found that the effective molecular weight between crosslinks was 540 g/mol. The hydrogel was found to degrade in trypsin at a concentration of 0.01 mg/ml over the course of 24 h (Fig. 6b). However, we saw no weight loss or degradation when the gels were stored in PBS for up to 1 week. The swelling equilibrium was 14.93 ± 0.09.

Macroporous Hydrogel Synthesis. Fig. 1 shows a schematic of the macroporous hydrogel formation process. The reaction of the activated polyethylene glycol (PEG) with polylysine produces an activated polyelectrolyte complex (complex). The complex is further activated with a water-soluble initiator (18) to form a hydrogel that is biodegradable and degradable in a sodium hydroxide solution. The hydrogel was synthesized by cross-linking polyethylene glycol with polylysine around a salt-leached polylactic-co-glycolic acid scaffold that is degraded in a sodium hydroxide solution. An open macroporous network is formed that supports the efficient formation of tubular structures by brain endothelial cells. After subcutaneous implantation of hydrogel cocultures in mice, blood flow in new microvessels was apparent at 2 weeks with perfused networks established on the surface of implants at 6 weeks. Compared to endothelial cells cultured alone, cocultures of endothelial cells and neural progenitor cells had a significantly greater density of endothelial cells cultured alone, cocultures of endothelial cells and NPCs would promote the stabilization of engineered tissues and to test the hypothesis that coculture of endothelial cells and NPCs promote the formation of endothelial cell tubes in coculture and the development of a functional microcirculation in vivo. We demonstrate a previously undescribed strategy for creating stable microvascular networks to support engineered tissues of desired parenchymal cell origin.

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Abbreviations: BEC, brain-derived immortalized microvascular endothelial cell; NPC, neural progenitor cell; PECAM, platelet endothelial cell adhesion molecule; PEG, polyethylene glycol; SEM, scanning electron microscopy.

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isotropic hydrogel that exhibits a classic, uniform morphology in scanning electron microscopy (SEM) micrographs. Because dehydration for SEM leads to artifact in the highly water-saturated gels, their morphology can be better viewed by cryosectioning the gels followed by staining with fluorescein isothiocyanate (FITC), which reacts with the free amines in the polylysine (Fig. 1 f and g). The as-reacted gel exhibits regions depleted of polylysine on the order of 100 μm corresponding to the absence of free amines for reaction with FITC. In contrast, reaction around the polyactic-co-glycolic acid salt-leached scaffold followed by degradation in NaOH leads to a continuous macroporous network with depleted polylysine regions ranging from 200 to 500 μm.

In Vitro Cell Seeding: Effects of Architecture. For the in vitro experiments, the macroporous hydrogels were seeded with brain-derived immortalized microvascular endothelial cells (BECs), NPCs, or the coculture, NPC:BEC. When the macroporous structure is seeded with BECs, the cells form continuous tube-like structures by 3 days (Fig. 2 a–c). This structural continuity is in contrast to the seeding of endothelial cells in the isotropic gel. In the isotropic gels, BECs and NPCs exhibited limited migration and tubular structures into the gel and no networks formation (data not shown). SEM images (Fig. 2 a and b) demonstrate that the BECs line the macropores forming tube-like networks and the dimensions of the hydrogel pore structure roughly determine the dimensions of the cell network.

Sectioning and immunostaining for platelet endothelial cell adhesion molecule-1 (PECAM-1) confirmed tubule formation within the gels (Fig. 2 c–g). PECAM-1 is a well-established endothelial marker that organizes at cell-cell junctions (18). NPCs were also found in the NPC and NPC:BEC groups near the vessels. The NPCs are negative for nestin and positive for glial fibrillary acidic protein (GFAP) and neurofilament 200 at 7 days in vitro, suggesting that they are differentiating (Fig. 7, which is published as supporting information on the PNAS web site). This differentiation is expected for NPCs cultured in serum-containing media. In preliminary studies, seeded hydrogels were cultured for a total of 6 weeks. Optimal tubule formation as defined by the greatest density of tubules was observed at 3 days, and, hence, this was the designated time point for implantation.

Tubule formation was quantified in the BEC and NPC:BEC groups. Tubule formation was not seen in the NPC group in vitro. There was no statistical significance between tube length, diameter, and the density of tubules at the 3-day time used for implantation (Fig. 2 and h). After 3 days in culture, hydrogels seeded with BECs and NPCs:BECs had average tubule lengths of 91.5 ± 10.7 μm and 75.9 ± 6.9 μm, average tube diameters of 23.8 ± 1.9 μm and 22.7 ± 1.9 μm, and average tubule densities of 1.2 tubes per area ± 0.1 μm for both where the area is 5.2 × 10^2 μm².

In Vivo Imaging. At time points of 1, 2, 4, and 6 weeks after implantation, microvascular function in the gels was assessed by intravital fluorescence microscopy. Blood flow was visualized by labeling the blood plasma (Fig. 3 a–c). In mature vessels, the FITC-dextran should be constrained to the lumen of the vessels because of size-limited permeability of the luminal wall. Although the hydrogel does autofluoresce slightly, it was readily distinguished from much higher fluorescence within the vasculature and did not impede the visualization of red blood cell flow, which appear dark against the fluorescent plasma. At 1 week, no blood vessels were observed within the hydrogel. Although there was vascularized connective tissue surrounding the implant, blunt dissection easily removed this connective tissue with little or no bleeding. However, at 2, 4, and 6 weeks, the interfacial layer between the gel and surrounding tissue was noticeably thicker and the vascularized tissue surrounding the implants was firmly connected to the hydrogels. Furthermore, functional vessels were found penetrating the gels as deep as the working distance of the optics permitted (100–200 μm) (Fig. 3 b and c).

No obvious differences were apparent between implants from the three treatment groups during the intravital microscopy portion of the experiment except with regard to the formation of clots/angiogenic sinks as seen in Fig. 3 d. The clot shown in Fig. 3 d is representative of those observed both adjoining and enveloping the hydrogel. Of the 40 implants in this study, clots formed around 3 of the 18 implants at the 2, 4, and 6 week time points. Clot formation was observed after 2 weeks in vivo and only in BEC or NPC:BEC implants, suggesting the presence of BECs may promote the formation of these structures.

Intravital microscopy indicated that hydrogels encouragedvascularization from all surrounding tissue, including skin, muscle, and fat. Fig. 3 e shows a multitude of vascular networks under fluorescent illumination. Several vessels observed during surgical preparation did not contain fluorescence (white arrows in Fig. 3 e), indicating that they had been disrupted during surgery and before injection of the FITC-dextran. Nevertheless, robust red blood cell flow was observed in many vessels even after the skin and connective tissue had been cleared. The diameter of vessels entering the
gel were estimated visually to be on the order of 30 μm, with the vascular networks closely associated with the hydrogel having smaller diameters estimated to be closer to 20 μm. All three groups, BECs, NPCs, and NPCs:BECs, demonstrated similar signs of integration and visible blood flow. A video of representative areas of blood flow into the hydrogels at 2, 4, and 6 weeks is available as Movie 1, which is published as supporting information on the PNAS web site.

Attempts to visualize flow near the center of the gel required cutting into the gel and caused an efflux of FITC-dextran into the observed incision site, indicating disruption of the vascular supply. This physical disturbance produced intense background fluorescence and obviated further intravital observations. Therefore, histology and immunocytochemistry were critical to confirm the function of vascular networks within the gels.

Postimplantation Characterization: Immunohistochemistry. Three samples were selected at random from each in vivo group for immunohistochemistry and histology from samples that did not exhibit clots to elucidate vessel formation within the hydrogels. Images in Fig. 4 are representative of each group at the stated time point. The hydrogels appeared to be intact with no observable signs of degradation at any of the time points studied.

Vessel formation in the hydrogels was quantified for density of vessels as well as average length and diameter of vessels in the in vivo experiments. No differences were found for the average diameter of length of vessels between the groups for any time point (Fig. 4b). In most cases, the tubules spanned the macro-pores of the hydrogel, suggesting that the architecture played a significant role in defining the diameter of the tubes.

Although there were no differences in the dimensions of the tubules, there were significant differences in the density of vessels. The NPC group had a low density of PECAM-1 expression and vessel structures after 2 weeks and then a gradual increase up to 6 weeks, which is most likely due to the migration of host endothelial cells into the gel (Fig. 4a and d). The BEC group showed a similar density of PECAM-1-positive vessels throughout, but at 6 weeks, the centers of BEC hydrogels had few cells (Fig. 4c). The average density of vessels was constant for the BEC group over time, but there was greater variation in density with the inner areas exhibiting lower density of vessels (Fig. 4d). It is possible that the vessels in the BEC group are regressing at 6 weeks.

In contrast, there was a statistically significant increase in the density of vessels for the NPC:BEC group as compared to the BEC group at 6 weeks (Fig. 4d). Furthermore, the density of vessels in the NPC:BEC group was higher at 6 weeks as compared to 4 weeks, and no empty regions of the gels were found (Fig. 4e). Fig. 4g is a larger magnification of one of these vessels. There is also a marked increase in junctions between vessels in the gels at 6 weeks in the coculture group.

Fig. 4f demonstrates the recruitment of cells into the implant up to 4 weeks for all groups. However, this graph confirms the decrease in the area of cells within the implant observed with the BEC group.
at 6 weeks. There are statistical differences between both NPC and NPC:BEC groups with the BEC group at the 6 week time point.

**Postimplantation Characterization: Hematoxylin/Eosin for Red Blood Cell Visualization.** Hematoxylin and eosin staining permitted visualization of functional blood vessels. As described above, hydrogels harvested 1 week after implantation did not exhibit blood flow, and sectioning revealed little to no cell integration from the tissue into the gel. After 2 weeks, endothelial cells permeated the gel in all three groups. In the BEC and NPC:BEC groups, cells oriented along the pores of the hydrogel. Implants harvested 4 weeks after implantation show functional blood vessels as evident by the presence of red blood cells in the vessel lumen (Fig. 5a and c). Blood vessels remained functional at the 6 week time point for NPCs and NPCs:BECs (Fig. 5d). However, as previously observed with PECAM staining, there were few cells found in the center of BEC hydrogels (Fig. 5b) at 6 weeks.

**Discussion**

By casting a macroporous network into the polylsine-PEG hydrogel, we were able to create a previously uncharacterized pore architecture in a hydrogel that was well suited to the formation of microvascular networks in vivo. This technique allows the creation of any three-dimensional pore network that can be defined by a degradable polyester scaffold. Because the degradable polyesters are readily processed to form a wide range of architectures (19), this technique is extremely versatile and permits a variety of three-dimensional tailored pore architectures not previously obtained with hydrogels. By using an enzymatically degradable hydrogel that does not degrade by hydrolysis, we demonstrate the application of a viable system for enabling the formation and maturation of microvessel networks suitable for tissue engineering applications.

By having a tailored porous network in the gel, the BECs were physically directed into vessel-like structures before implantation. Although we have seen cells, including the NPCs, migrate into the gel itself, the path of least resistance is clearly the open, porous architecture. The attraction of using a hydrogel as opposed to a hydrophobic polymer system such as polylactic acid is that the gel does not degrade by hydrolysis, which requires that the host vasculature be intimately associated with the implants and not degenerate with histological evaluation after implantation, the current model further supports their biocompatibility. This behavior illustrates the importance of a biocompatible scaffold in engineering microvascular networks. For the vessels to be functional, they must anastomose with the host vasculature, which requires that the host vasculature be intimately associated with the implants and not isolated by a dense collagen-rich capsule. As confirmed in vivo and with histological evaluation after implantation, the current model displays the requisite properties.

It has proven challenging to create microvascular networks that are stable. There is often regression of vessels. We saw what
are seen at 4 and 6 weeks in NPC:BEC hydrogels. Arrows denote red blood cells.

Fig. 5. Hematoxylin and eosin staining of in vivo implants. (a) Four-week BEC hydrogel. Red blood cells are evident in the vessel lumen. (b) Six-week BEC hydrogel. Few cells or vessels are found at the center of the implant. (c) Four-week NPC:BEC hydrogel. (d) Six-week BEC:NPC hydrogel. Red blood cells are seen at 4 and 6 weeks in NPC:BEC hydrogels. Arrows denote red blood cells in the blood vessel lumen.

Hydrogel Characterization. The morphology of the hydrogels was evaluated by SEM (FEI XL-30 environmental). Hydrogels seeded with cells were fixed in 10% buffered formalin and dehydrated by using graded ethanol steps followed by the addition of hexamethyldisilizane. The morphology of hydrated gels was evaluated by using fluorescence microscopy. Gels were incubated in an excess of FITC, which reacts with the free amines of the gel. The gels were sectioned, and images were taken by using a Zeiss Axiosvert 200 Inverted Microscope. Elastic and viscoelastic moduli were obtained by using a Dynamic Stress Rheometer (TA Instruments, New Castle, DE). Moduli were calculated at constant stress (1 Pa) for a frequency range of 0.01–10 Hz by using a 0.625-ml sample volume and a 20-mm-diameter parallel plate geometry. The resulting data were analyzed by using RSI ORCHESTRA (TA Instruments).

To determine the degradation profile, lyophilized hydrogels (5–15 mg) were submerged in an excess of PBS for 24 h at 37°C. Samples were blotted dry to remove any free water and were weighed to obtain a wet mass. A 0.01 mg/ml solution of trypsin in PBS was used to digest the peptide bonds of the hydrogel. Gels were incubated in 2 ml of the trypsin solution. Samples were blotted dry and weighed at periodic time intervals until complete digestion occurred.

To measure the swelling equilibrium ratio, hydrogels were lyophilized overnight and weighed to record their dry weight (Wd). The gels were then submerged in an excess of PBS. The wet weight (Ww) was recorded throughout a 24-h time period to obtain the rate of swelling and the final equilibrium swelling ratio. To determine the Ww, the gels were removed from the PBS, lightly blotted on a kimwipe to remove free water, and weighed. The equilibrium swelling ratio (ES) was calculated from the equation ES = Ww/Wd.

Cell Culture and Seeding of Hydrogels. BECs were a generous gift from Britta Engelhardt (Theodor Kocher Institute, Bern, Switzerland) (23, 24), and they were maintained in brain endothelial cell medium (DMEM/10% FBS/10 mM Hepes/10−5 M 2-mercaptoethanol/1% penicillin/streptomycin). Green fluorescent protein (GFP) NPCs were isolated and maintained according to Lu et al. (25) in serum-free epidermal growth factor (EGF) containing media.

Hydrogel components were sterilized by using a 0.22-μm sterile syringe filter before crosslinking. Gels were further sterilized by UV exposure just before seeding. Hydrogel discs 1 mm thick and 5 mm in diameter were seeded with BECs (1 million cells per gel at a concentration of 1 × 10⁶ cells/ml), NPCs (100,000 cells at a concentration of 1 × 10⁵ cells/ml), or a coculture of NPCs:BECS at a ratio of 1:10 (1 million BECs and 100,000 NPCs per implant). This coculture ratio was chosen based on unpublished data observed by Q.L., M.C.F., M.Y., E.B.L., and J.A.M. Seeded gels were maintained in 12-well plates coated with 1% BSA to reduce cell adhesion to the wells.

For in vitro experiments, seeded gels were maintained under static conditions in the BEC media with a media change every 3–4 days. Hydrogels were fixed in 10% buffered formalin at 3 days and at 1, 2, 4, and 6 weeks for histological analysis.

For in vivo experiments, nine hydrogels were prepared for each of the three treatment groups, BECs, NPCs, or NPCs:BECs;
respective homocellular cultured hydrogel constructs served as controls for the cocultured hydrogel constructs. The implants were cultured under static conditions in BEC media for 3 days at 37°C and 5% CO2. The six hydrogels from each group that exhibited the most uniform macroporous architecture were selected by the naked eye and implanted into mice. Preliminary experiments with Dil-labeled cells as well as SEM imaging indicated that regardless of the uniformity of the gel, at 3 days, all of the gels were uniformly covered in cells.

**Surgical Procedures.** Thirty-six 8- to 12-week-old female C57 black mice (C57BL/6) (Charles River Laboratories, Wilmington, MA) were used. Each mouse was anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). An incision of ~8 mm in length was made through the skin overlying the thoracic spine, and s.c. pockets were created bilaterally (caudal to each scapula) by clearing connective tissue under the skin. Two hydrogels from the same treatment group (see above) were implanted in each mouse, one in each pocket. Each implant was a 1-mm by 5-mm disk. The incision was closed with surgical clips, and the animals were maintained on a heating pad until they regained mobility. All mice were maintained with a heat lamp. The exposed tissue was irrigated with sterile saline, and overlying connective tissue was removed by dissection. To visualize blood flow, 100 μl of 0.5% FITC-dextran (70 kDa) was injected retro-orbitally to label the plasma compartment (red blood cells appear dark against this fluorescent background). The completed preparation was transferred to the stage of an intravital microscope (modified ACM; Zeiss, Thornwood, NY). Video images were acquired using a ×63 (Zeiss Neofluor, NA = 0.20) and ×20 (Nikon Plan SLWD, NA = 0.35) objectives coupled to a Water WAT-902H monochromatic CCD camera (Edmund Optics, Barrington, NJ) or a Hitachi KP-D 50U chromatic camera (Hitachi-Densi, Tokyo).

**Histology and Immunocytochemistry.** Excised implants were fixed in 10% buffered formalin overnight, soaked in a 30% sucrose solution, and embedded in OCT, and 20-μm thick cross sections were made with a Microm cryotome. Sections were either stained with hematoxylin and eosin for general pathology or immunostained with antibodies against mouse PECAM-1 (Pitter et al., ref. 18; 1:100), nestin (BD Pharmingen; 1:200), giall fibrillary acidic protein (Sigma; 1:80), and neurofilament 200 (Sigma; 1:200). Secondary antibodies included goat anti-rabbit and goat anti-mouse Alexa Fluor 647 (Molecular Probes; 1:200).

Briefly, 20-μm sections were blocked in 5% BSA and 3% normal goat serum (Vector Laboratories) for 1 h at room temperature. Samples then were incubated with the primary antibody at 4°C overnight followed by incubation with the appropriate secondary antibody for 1 h at room temperature.

Three implants were processed for histology and immunocytochemistry per group at random from the implants that did not exhibit clot formation. At least five sections, 200 μm apart from each other, were stained for hematoxylin/eosin and each immunocytochemical marker. The results from the histological and immunocytochemical analysis were quantified as described below.

**Quantification of Histology.** The number, diameter, and length of the microvessels in both the in vitro and in vivo hydrogels were quantified by using a ×10 objective (Zeiss, NA = 0.45) on the Zeiss Axiosvert 200 microscope with an MRC camera and AIVIATION 4.8 software for image capture and analysis. Vessels were identified by the immunostaining for PECAM-1 and their morphology. The vessel length was measured from the outer end of the cell body on either end of the tube, and the most direct path was chosen. The diameter was measured from outer cell body to outer cell body normal to the length of the tube. The density of tubes was calculated by determining the number of tubes per viewing area (812 μm by 643 μm). The number of cells in the implants was determined by assessing the number of nuclei present as marked by staining for DAPI. The total area of DAPI in each image was determined, and this area was divided by the mean area of an individual nucleus. For each determination, a total of 8–12 areas were counted in three to five sections per implant to obtain representative samples for statistical analysis.

**Statistics.** All experiments were done in triplicate. Data were analyzed by using a one-way ANOVA followed by the Tukey test for determining differences between groups. Differences were accepted as statistically significant with P < 0.05. Summary data are presented as means ± standard error of the mean.

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Supporting Text

Using the relationship between the frequency-independent elastic modulus and the effective crosslinking density (or network junction density) (as presented in Calveat et al., ref. 1) based on Flory’s theory of rubber elasticity, $n_e$, the effective crosslinking density is defined as:

$$n_e = \frac{G'}{\left(1 - \frac{2}{f} \left(\frac{f}{2}\right)RT\right)},$$

where $f$ is the number of strands linked to a crosslinker. For our calculations, the number is close to 3. (The core of the four-arm polyethylene glycol (PEG) has four strands attached, and the junction between an arm and polylysine has three arms. Therefore, there is one four-arm junction for every four three-armed junctions (between the polylysine and a PEG arm) assuming complete reactivity making $f$ equal to 3.2, however it is very unlikely that all of the arms reacted. Assuming a 75% coupling rate, then $f$ becomes 3.25.) Using $f = 3.25$ and $T = 298$ K, and $G' = 2,870$ Pa.

$$n_e = 1.85 \text{ mol/m}^3.$$

The effective molecular mass between crosslinks is defined by the polymer concentration in kg/m$^3$ divided by $n_e$ so that $M_e = 540$ g/mol.

Interestingly, the molecular mass of one PEG arm is supposed to be 2,500 g/mol. The difference in the calculations from the mechanical data and theoretical calculations is likely due to the limitations in the Flory equation, which assumes that the chains are freely jointed. Both the polylysine and PEG exist as $\alpha$-helices and not as freely jointed chains. This steric hindrance is likely to lead to the difference.