General paper

Structural Analyses on Aggregates of Graft Copolypeptides Containing Tryptophan in Aqueous Media and Efficiency of Drug Release

Shigeru OKAMOTO*, Takayoshi HANAI*, Fumio YAMAUCHI*,
Kenji NAGATA*, Shinichi SAKURAI**, Kazuo SAKURAI*** and Eiji NAKANISHI*

*Department of Material Science & Engineering, Nagoya Institute of Technology,
Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan

**Department of Polymer Science & Engineering, Kyoto Institute of Technology,
Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

***Chemotransfiguration Project, Japan Science and Technology Corporation,
Kurume Research Bldg., 2432 Aikawa, Kurume, Fukuoka 839-0861, Japan.

Abstract: The amphiphilic graft copolypeptide, consisting of poly(*N*-hydroxyethyl L-glutamine) (PHEG) and poly(L-tryptophan (L-Trp)) PTrp (denoted as PHEG-g-PTrp), was synthesized. At the L-Trp composition of 10 ~ 25 mol %, L-Trp residues aggregated to form the hydrophobic cores in the aqueous solution. The hydrophobic substance could be incorporated in this L-Trp core. This function had not been seen for the random copolypeptide consisting of the same constituent molecules. The aggregation of PHEG-g-PTrp was observed by dynamic light scattering, transmission electron microscopy and small-angle X-ray scattering. The film specimen was prepared from PHEG-g-PTrp by modifying L-Trp residues in trifluoroacetic acid. The hydrophobic substances were incorporated in the high pH region, and the control of the release was achieved by changing pH through the dissociation of the indole rings in the L-Trp residues.

Key words: Graft copolypeptide, Amphiphilic polymer, Aggregation, Transmission electron microscopy, Small-angle X-ray scattering, pH dependence, Drug release

1. INTRODUCTION

Block or graft copolymers are getting widely noticed as functional materials. They consist of different polymers connected by the covalent bonds. In the two phase state, the constituent polymers segregate from each other, and consequently the systems have internal structures formed by the interfaces between the different polymers [1,2]. These interfaces play a crucial role as the origin of the physical properties or the functions. Their morphology and the orientation should be taken into account, when they are put to practical use. The interfaces exhibit a variety of morphologies in accordance with the change of the composition of the constituent polymers [3] or temperature [4], etc. The formation or the disappearance of the interfaces [1,2,5-7], and the transition between the different morphologies [8] or the orientational change [7,9-12] under external fields have been of great interest to many theoreticians and experimentalists.

In recent years, the copolymers with such internal structures are intensively investigated for the purpose of the utilization as functional materials in the medical field. For example, Kataoka et al. [13,14] invented an anticancer compound, using the block copolymer consisting of polyethylene oxide and polyaspartic acid. Largest feature of the graft or the block copolymers is a tremendously huge area of interfaces. The effect or the efficiency of transportation or reaction through the interfaces are always multiplied by their interfacial area. Hence the block or graft copolymers can be expected as excellent functional materials. In this paper, we report the possibility of the application of graft copoly-

mers to a drug-release membrane [15].

L-tryptophan (L-Trp) residues, containing an indole ring, have the largest hydrophobicity among the natural amino acids. L-Trp displays various colors by the high electron density on the indole ring [16-20], when treated with strong acids, and shows reversible color change with the change of pH [21]. For example, L-Trp forms a tricyclic structure by the treatment of trifluoroacetic acid (TFA) and shows color change with the variation of pH by the dissociation of indole ring [22]. We reported that the application of this dissociation to the hydrogel membrane of random copolypeptides [23]. The membrane showed the drastic changes in color, degree of swelling, and mechanical properties. In this study, we prepared the amphiphilic graft copolypeptide with poly(L-Trp) (PTrp) and hydrophilic poly(*N*-hydroxyethyl L-glutamine) (PHEG) [24], and have examined the utility of the graft copolymer as a drug-release membrane.

2. EXPERIMENTAL

2.1. Materials

2.1.1. Synthesis of copolymers

The prepolymers, poly(γ -benzyl L-glutamate) (PBLG) and poly(L-tryptophan) (PTrp) were synthesized by the *N*-carboxyanhydride (NCA) method. The details of the method were described elsewhere [25-28]. Both γ -benzyl L-glutamate (γ -BLG) NCA and L-Trp NCA were prepared by reaction with bis(trichloromethyl) carbonate (triphosgene) at 40 °C and purified by recrystallization from ethyl acetate solution with petroleum ether. The polymerization of the γ -

BLG NCA was initiated by triethylamine (TEA) with the molar ratio of NCA/TEA = 50/1 in a 1/1 (vol/vol) solvent mixture of dioxane and dichloromethane at room temperature. The PBLG was precipitated in an excess amount of cold methanol and dried *in vacuo*. The molecular weight measured by a viscometry was 307,000 g/mol.

The polymerization of L-Trp NCA was initiated by *n*-butyl amine (NBA) with the molar ratio NCA/NBA = 5/1 in *N,N*-dimethyl formamide (DMF) [29,30]. The PTrp was precipitated in an excess amount of diethyl ether and also dried *in vacuo*. The degree of polymerization (D.P.) was estimated from the ¹H-Nuclear Magnetic Resonance (NMR) measurement by the comparison between the peak of the indole ring at 7 ppm and the peak of methylene of the initiator at 0.8 ppm. Thus evaluated D.P. was 12.

The graft copolymer, PBLG-*g*-PTrp, was prepared with the PBLG and PTrp synthesized above. First PBLG was dissolved in dioxane and underwent the 10 % of debenzilation by stirring for 1 h with addition of NaOH aq. (0.1 N). The solution was neutralized by HCl aq. In succession, hydroxybenzotriazole (HOBt) was added as a coupling accelerator and stirred for 1 h at 0 °C. Finally, PTrp in DMF was added at 0 °C and coupled on to debenzilated PBLG under the coupling agent of *N,N'*-dicyclohexylcarbodiimide (DCC). The graft copolymer was precipitated in an excess amount of methanol after the further stirring and aging for 72 h. Characteristics of the graft copolymers were summarized in Table 1.

The random copolymer was prepared by copolymerization of γ -BLG NCA and L-Trp NCA. The γ -BLG NCA and L-Trp NCA, at a molar ratio of 75/25, were dissolved in a 1/1 (vol/vol) mixture of 1,4-dioxane and dichloromethane. Polymerization was initiated by TEA with the molar ratio of NCA/TEA = 50/1 at the room temperature. The copolymer, poly(γ -BLG-*ran*-Trp), was precipitated in an excess amount of cold methanol after aging for 72 h and dried *in vacuo*. The molecular weight estimated by a viscometry was 200,000 g/mol.

2.1.2. Preparation of crosslinked amphiphilic copolymer membrane

The crosslinked amphiphilic copolymer membrane, PHEG-*g*-PTrp membrane, was prepared by aminolysis of PBLG-*g*-PTrp membrane cast from chloroform including the subtle amount of hexafluoroisopropanol. The PBLG-*g*-PTrp membrane was immersed in 2-amino-1-ethanol with 10 mol% of crosslinking agent, 1,8-octamethylenediamine,

at 50 °C for 168 h. Debenzylation of PBLG was confirmed by the disappearance of absorption due to ester groups at 1740 cm⁻¹ in an IR spectrum.

2.1.3. Preparation of water-soluble amphiphilic copolymer

The water-soluble amphiphilic copolymer, PHEG-*g*-PTrp, without crosslinking was also prepared by the same aminolysis procedure as in sub-section 2.1.2. The water-soluble product was purified by dialysis with the molecular cutoff of 8000. The composition of tryptophan residues was analyzed by ¹H-NMR. Debenzylation was also confirmed in the same way. Similarly, water-soluble Poly(HEG-*ran*-Trp) was prepared from Poly(γ -BLG-*ran*-Trp).

2.1.4. Preparation of the TFA-treated membrane

The PHEG-*g*-PTrp membrane was immersed in TFA with irradiation of ultra violet (UV) [22], where the wavelength was 365 nm and the intensity was 300 μ W cm⁻² for 72 h. The TFA-treated membrane (hereafter denoted as PHEG-*g*-PTrpT) was neutralized by sodium hydrogencarbonate and dried *in vacuo*.

2.2. Measurement

2.2.1. NMR

The confirmation of the generation of γ -BLG, γ -BLG NCA and L-Trp NCA, and the estimation of the composition of L-Trp residue in PHEG-*g*-PTrp were carried out by ¹H-NMR (JEOL Model JEOLJNM-GX400).

2.2.2. UV-VIS absorption spectrum

3 mg of azobenzene was dispersed in the 3 ml of PHEG-*g*-PTrp aqueous solution with a variety of concentration. The solution was kept at 50 °C for 72 h. The excessive azobenzene was removed by the filtration. UV-VIS absorption spectra of azobenzene incorporated by PHEG-*g*-PTrp was measured at the characteristic absorption wavelength, 433 nm by a JASCO Model V-520 spectrophotometer using a quartz cell with a path length of 1 cm.

2.2.3. Fluorometry

The fluorochrome, 1-Anilino-8-naphthalenesulfonic acid magnesium (ANS-Mg) dissolved in PHEG-*g*-PTrp aqueous solution at pH = 5.5 was detected by the spectrophotofluorometer (Shimadzu Model RF-5300FC). The excitation wavelength was 350 nm and the detected fluorescent wavelength was in a range from 400 to 600 nm.

The drug-release rate was measured 5 times for PHEG-*g*-PTrpT by fluorometry. The hydrophobic compound, testosterone, was incorporated by the PHEG-*g*-PTrpT membrane. The membrane was set in the cell for the fluorometer, where the water continuously supplied into the cell. The fluorescent from the drain was measured with the excitation wavelength of 292 nm and the fluorescent peak detected at 383 nm. For the calibration of fluorometry, we measured testosterone alone dissolved in water without the membrane. The peak intensity after the subtraction of the background intensity of the membrane without testosterone was proportional to the amount of testosterone dissolved in water. The pH of the water was varied between 2 and 5.

2.2.4. Dynamic light scattering

The hydrodynamic radius of PHEG-*g*-PTrp was measured in the aqueous solution at 25 °C by dynamic light scattering (DLS) (Otsuka Model DLS7000). The wavelength of the incident beam was 488 nm. The concentration

Table 1. L-Trp contents in graft copolymers PHEG-*g*-PTrp estimated by ¹H-NMR.

Copolymer	Feed (mol%)		L-Trp contents (mol%)
	PBLG	PTrp	
PHEG- <i>g</i> -PTrp30	70	30	26.2
PHEG- <i>g</i> -PTrp25	75	25	22.5
PHEG- <i>g</i> -PTrp20	80	20	17.0
PHEG- <i>g</i> -PTrp15	85	15	13.6
PHEG- <i>g</i> -PTrp10	90	10	10.9
PHEG- <i>g</i> -PTrp5	95	5	5.8

was 1×10^{-3} mol/l.

2.2.5. Transmission electron microscopy

The aqueous solution of PHEG-g-PTrp with the concentration of 1×10^{-3} mol/l was freeze-dried on the grids. The freeze-dried specimen was observed by TEM (JEOL Model JEM-1200EX).

2.2.6. Small-angle X-ray scattering

The SAXS from the PHEG-g-PTrp aqueous solution was measured at the beam line BL-15A of High Energy Accelerator Research Organization, Tsukuba, Japan. The concentration of the solution was 1×10^{-2} mol/l. The camera length was ca. 2300 mm and the wavelength of the incident beam was 0.1504 nm. Subtraction of the air scattering was conducted with taking account of the absorption of X-ray.

3. RESULTS

3.1. Aggregation of PHEG-g-PTrp

3.1.1. Critical aggregation concentration

The fluorochrome, ANS-Mg can be dissolved in various solvents, and the wavelength at the maximum fluorescent emission, $\lambda_{\max}$, of ANS-Mg depends on the solvents. For example, $\lambda_{\max}$ is observed at 530, 483, 474, and 460 nm in water, methanol, ethanol, and tetrahydrofuran, respectively. In other words, $\lambda_{\max}$ becomes smaller (blue shift) with lowering the solvent polarity. It can be expected that the blue shift is observed when ANS-Mg is incorporated in the hydrophobic core of L-Trp.

The ANS-Mg was dissolved in PHEG-g-PTrp aqueous solutions at pH = 5.5. The concentration of the PHEG-g-PTrp graft copolymers was varied from 1×10^{-7} to 1×10^{-3} mol/l. The measurement was conducted for all kinds of the graft copolymers. The PHEG-g-PTrp5 did not show any concentration dependence of $\lambda_{\max}$. This result suggests that the content of the L-Trp in PHEG-g-PTrp5 was so small that the L-Trp could not aggregate. Meanwhile, the PHEG-g-PTrp30 precipitated. This can be attributed to the large hydrophobicity due to the high content of L-Trp.

Figure 1 shows the concentration dependence of $\lambda_{\max}$

Fig. 1. Concentration dependence of the maximum fluorescence emission wavelength, $\lambda_{\max}$, for PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20, PHEG-g-PTrp25.

Table 2. Critical Aggregation Concentration.

L-Trp content (%)	10	15	20	25
CAC (mol/l)	3.1×10^{-4}	2.8×10^{-4}	2.2×10^{-4}	1.9×10^{-4}

Table 3. Hydrodynamic diameter and its distribution for aggregates of PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20, PHEG-g-PTrp25, as estimated by DLS.

L-Trp content (%)	<i>d</i> (nm)	polydispersity
10	192.4	0.41
15	74.3	0.20
20	67.2	0.12
25	90.3	0.11

for PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20, and PHEG-g-PTrp25. $\lambda_{\max}$ drastically decreased around 3×10^{-4} mol/l as the concentration increased. It seems reasonable to define the inflection point of each profile in Fig. 1 as the critical aggregation concentration (CAC) of the corresponding graft copolymer. The CAC's of the copolymers are presented in Table 2. There is a slight decrease of CAC with increasing the L-Trp content. It must be noted that these values are ten times larger than those of the corresponding block copolymers [31]. This is explained by the chain constraint of the PHEG main chains to form the hydrophobic L-Trp aggregates, and will be discussed later in sub-sections 3.1.3. and 3.1.5. in more detail.

3.1.2. Dynamic light scattering

The hydrodynamic size of the PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20 and PHEG-g-PTrp25 in water were measured by the dynamic light scattering method. The diameters and their distribution are displayed in Table 3. The diameter of PHEG-g-PTrp10 was twice as large as the others. A possible explanation for this is that PHEG-g-PTrp10 has only ca. 14 graft chains in each molecule and this number may be too close to the threshold to bring about aggregation, so that much more copolymers are needed to form PTrp aggregates. Actually, PHEG-g-PTrp5, which has fewer graft chains, could not aggregate in any concentration as discussed in sub-section 3.1.1.

Another distinctive feature is that the Trp-content dependence of the size had the minimum for PHEG-g-PTrp20. There are two competitive factors. One is that the cohesive force increases with the increase of the PTrp content. This leads to the decrease of the aggregates in size. The other is that the number of the graft copolymers joining the same core increases as the PTrp content increases, because the main chain gets more rigid as the graft chains becomes dense on the same main chain. Consequently, the graft copolymers participating in the same aggregates increases in number. By these two competitive factors, the size of PHEG-g-PTrp20 was the smallest.

3.1.3. Transmission electron microscopy

Figure 2 shows the typical TEM image of PHEG-g-PTrp15, freeze-dried from its aqueous solution at the concentration of 1×10^{-3} mol/l above CAC. The diameter of

Fig. 2. TEM image of PHEG-g-PTrp15, freeze-dried from the aqueous solution at the concentration of 1×10^{-3} . The scalebar in each image indicates 50 nm.

Fig. 3. *In situ* SAXS profile, I , as a function of the magnitude of the scattering vector, q , for PHEG-g-PTrp25 aqueous solution at the concentration of 1×10^{-2} mol/l (dotted line) and the fitted curve calculated based on the core-shell model (solid line).

the aggregates seems to be in good agreement with the result by DLS in Table 3. More noteworthy is that there were small bright particles, whose diameters were ca. 5 nm. The dark and the bright regions can be attributed to PHEG shells and PTrp cores of the aggregate, respectively (refer to Fig. 4 for the model). The TEM images of PHEG-g-PTrp20 and PHEG-g-PTrp25 looked similar to that shown in Fig. 2. Meanwhile, in the image of PHEG-g-PTrp10, there did not exist the bright small region. The diameters of PHEG-g-PTrp10 had wide distribution and varied from 100 to 300 nm. This may also be explained by the fact that the number of the graft chains is small, which may be close to the onset of aggregation as described in sub-section 3.1.2. Thus the aggregates of PTrp in PHEG-g-PTrp10 must have been unstable and could not keep its stable form.

3.1.4. Small-angle X-ray scattering

The structure of the aggregates have been examined *in situ* by SAXS. Figure 3 shows the SAXS intensity, I , from PHEG-g-PTrp25 aqueous solution (1×10^{-2} mol/l) as a function of q , where q is the magnitude of the scattering vector, as defined by

$$q = 4\pi \sin \theta / \lambda, \quad (1)$$

with θ and λ being half the scattering angle and the wavelength of the incident beam, respectively. In the low q region at $\log q < -1.0$, the intensity was almost constant, which is typical to spherical particles. The SAXS profile was fitted by the theoretical curve based on the core-shell model [32–34]. The diameter of the cores evaluated by the fitting

Fig. 4. Aggregation model for graft copolymers, PHEG-g-PTrp15, PHEG-g-PTrp20 and PHEG-g-PTrp25.

Fig. 5. Aggregation model for block copolymers.

was ca. 4.5 nm. This size was in good agreement with the diameters of the bright region in the TEM image, i.e., the PTrp cores.

3.1.5. Model for PHEG-g-PTrp aggregates in water

As shown in sub-sections 3.1.2. and 3.1.3., the average diameter of PHEG-g-PTrp aggregate was in a range of 70 to 90 nm, while that of the cores of PTrp graft chains was about 5 nm as described in sub-sections 3.1.3. and 3.1.4. A possible model for PHEG-g-PTrp15, PHEG-g-PTrp20 and PHEG-g-PTrp25 is shown in Fig. 4, while the conventional core-shell model for block copolymers is shown in Fig. 5 for the sake of a comparison. In the case of block copolymers, the constituent block chains segregate in the two-phase

Fig. 6. Concentration dependence of UV-VIS absorbance for PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20, PHEG-g-PTrp25, and Poly(HEG-ran-Trp).

state and form a concentric core-shell sphere. The water-insoluble component form the core with the corona chain of the soluble component forming the shell. Similarly, the graft chains are insoluble component and aggregate to form the cores in the case of the PHEG-g-PTrp graft copolymers. However, the entropy loss of the main chain (PHEG) is considered to be much bigger than that of the block copolymers. In other words, if the total content of L-Trp residues is the same in both systems and one PHEG-g-PTrp aggregate contains one PTrp core like conventional block copolymer systems, the number of PTrp chains participating in one core is much bigger than that of block copolymers. Hence, PHEG chains congest around the core and lose their conformational entropy. Thus, it is safely expected that one PHEG-g-PTrp aggregate contains a large number of PTrp small cores as displayed in Fig. 4. The interfacial area between the different constituent polymers is larger in the graft copolymer systems than in the conventional block copolymer systems. Since quick response of the drug release through the interface is required, the larger interfacial area seems to make the graft copolymers more effective for the quick drug-release behavior, as compared to block copolymers.

3.2. Incorporation of Hydrophobic Compound by PHEG-g-PTrp

PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20 and PHEG-g-PTrp25 were tested for the application as a drug reservoir. The hydrophobic substance, azobenzene, was dispersed in the PHEG-g-PTrp aqueous solutions. A portion of it was incorporated in the PTrp cores. The amount of the incorporated azobenzene was estimated based on the UV-VIS absorption spectrum. Figure 6 shows the relationship between the concentration of PHEG-g-PTrp and the UV-VIS absorbance. The absorbance linearly depended on the concentration and also increased with the increase of the L-Trp content. This fact strongly supports the existence of the PTrp cores in the graft copolymers. For comparison, the absorbance for the corresponding random copolymer,

Fig. 7. Released ratio (%) of testosterone upon a jump of pH from 5 to 2, monitored as a function of time.

Poly(HEG-ran-Trp), was shown together in Fig. 6 and exhibited almost steady at 0.04.

3.3. Drug Release of Hydrophobic Compound by PHEG-g-PTrpT

Finally, We prepared a membrane which can release drug molecules incorporated in the cores. We modified PHEG-g-PTrp, which have an ability to incorporate drug molecules as discussed in section 3.2. but does not have the function to release them. Note, however, that similar aggregates of the hydrophobic PTrp graft chains exist in the membranes. Here, we treated the PHEG-g-PTrp membrane with trifluoroacetic acid (TFA) to obtain a PHEG-g-PTrpT membrane. The membrane incorporates water molecules by the dissociation of the complex of indole rings, as described in chapter 1. The drug-release behavior of hydrophobic substance was examined upon a jump from pH = 2 (below 4) to pH = 5 (above 4), because the TFA-treated L-Trp dissociate below pH = 4 [22]. Note here that pH = 2 is close to the acidity of gastric acid. Testosterone was employed as the hydrophobic substance. Testosterone was incorporated by the PHEG-g-PTrpT in the methanol solution. This membrane was set in the flow cell made of quartz for the fluorometer, where water was continuously supplied into the cell. The pH of the water was sequentially altered between 2 and 5. The fluorescence from the drain was monitored. The results are shown in Fig. 7. No release of testosterone was observed at pH = 5 during first 4 h. Amazingly, the quick response and the continuous release was detected during the next 4 h after the change of pH from 5 to 2. Finally, pH was altered back to 5 at Time = 8 h again, then the quick cessation of the release was confirmed and no release was observed during the last 4 h.

4. DISCUSSION

As described in section 3.1, amphiphilic graft copolymers, PHEG-g-PTrp's, whose PTrp contents are in the range from 10 to 25 mol % aggregate above their CAC's. The SAXS and TEM results revealed that these aggregates con-

tain a large number of hydrophobic cores whose diameter is ca. 5 nm due to the conformational-entropy loss of the PHEG main chains, as discussed in sub-section 3.1.5. and illustrated in Fig. 4. These cores have an ability to incorporate hydrophobic substance and function as a hydrophobic-drug reservoir, as demonstrated in section 3.2. For the purpose of drug-release, however, the drug molecules incorporated into the cores should be released again when needed.

We are aiming to utilize the functional polymer as a capsule under the stimulus of gastric acid in a stomach. Accordingly, we prepared the membrane of PHEG-g-PTrp and modified the PTrp graft chains in the hydrophobic cores by the TFA treatment. Note that the structure similar to that observed in the aggregates is safely expected to remain in the membrane, though the structure was examined in the aggregates because the equilibrated structure was expected to be obtained in solution.

We briefly demonstrate the drug-release mechanism and its relationship with the structural change. At pH = 5, the PTrp graft chains cohere by the hydrophobic cohesive force in the cores, illustrated in Fig. 4, and incorporate the drug molecules inside. As the indole rings dissociate at pH = 2, PTrp side-chains become repulsive to each other by the charge on the indole rings and incorporate water molecules inside. Then, the hydrophobic drug molecules incorporated inside go out slowly by diffusion.

In this paper, we discussed the efficiency of drug release by the stimulus of pH change. The temperature was constant for all the systems. Temperature will be an important factor to control the drug release. This is still under investigation.

5. CONCLUSION

Amphiphilic graft copolymers, PHEG-g-PTrp's, whose L-Trp contents varies from 5 to 30 (mol %), were investigated by UV-VIS absorption spectrum, fluorometry, DLS, TEM, and SAXS. Fluorometry results exhibiting CAC suggested that PHEG-g-PTrp5 did not aggregate at all, and that PHEG-g-PTrp10, PHEG-g-PTrp15, PHEG-g-PTrp20, PHEG-g-PTrp25 had CAC at concentrations of 3.1×10^{-4} , 2.8×10^{-4} , 2.2×10^{-4} , 1.9×10^{-4} (mol/l), respectively. The DLS results showed the diameters of the aggregates for PHEG-g-PTrp15, PHEG-g-PTrp20, PHEG-g-PTrp25 were 70 to 90 nm, while that of PHEG-g-PTrp10 was about 190 nm. According to the TEM and SAXS results, it may safely be stated that the PHEG-g-PTrp aggregate includes a number of PTrp cores, whose diameter was ca. 5 nm. The UV-VIS absorption spectrum showed that PHEG-g-PTrp membranes could capture the hydrophobic compounds in the PTrp cores, while the corresponding random copolymer, Poly(HEG-*ran*-Trp) could not. The incorporated quantity depended on the concentration of PHEG-g-PTrp and the L-Trp contents. By using TFA-treated membranes, PHEG-g-PTrpT, we were able to control the drug release upon the stimulus by changing pH through the dissociation of the indole rings.

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