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α-Helical Polypeptide Microcapsules Formed by Emulsion-Templated Self-Assembly

Masa-aki Morikawa,[a] Masakuni Yoshihara,[b, c] Takeshi Endo,[b, d] and Nobuo Kimizuka*[a]

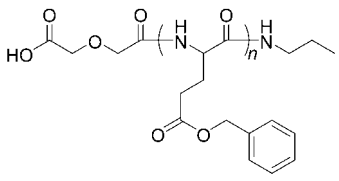
Abstract: α-Helical peptide microcapsules were prepared by the emulsion-templated self-assembly of amphiphilic poly(γ-benzyl L-glutamate)s (PBLG) **1**. By mixing solutions of **1** in dichloromethane (in the form of a sodium salt) with water, oil-in-water emulsions were obtained. Spontaneous stripping of the dichloromethane phase caused a decrease in the diameter of the microdroplets and finally stable microcapsules formed. The microcapsules contain an inner aqueous phase as observed by confocal laser scanning microscopy (CLSM). Binding of hydrophobic pyrene molecules to the polypeptide shell was also demonstrated. The present polypeptide microcapsules are stable even after drying in air and they would serve as supramolecular vehicles for both hydrophobic and water-soluble molecules.

Keywords: amphiphiles · emulsion · microcapsules · polypeptides · self-assembly

Introduction

Polymer microspheres are an important class of functional materials that are widely used in encapsulation, separation, and biological applications.[1] Hollow microcapsules of peptides are of particular interest, because of their intrinsic compatibility with biomolecules, high biodegradability, and thermal responsiveness that can be endowed by designing suitable primary (and secondary) structures. In spite of these promising features, studies on the self-assembly of polypeptides are rather limited to short peptides[2,3] or amphiphilic diblock peptides that contain large non-peptide segments.[4] In addition, formation of polypeptide self-assemblies are so far limited to aqueous bilayer vesicles,[2] crystalline tubes,[5] β-sheet aggregates,[6] and hydrogel networks.[7] Although the formation of vesicles from high-molecular-weight diblock polypeptides has been recently reported by Bellomo et. al,[2e] it still remains a challenge to develop stable microcapsules that are self-assembled from simple polypeptides with well-defined secondary structures.

In this study, we have synthesized anionic α-helical poly(γ-benzyl L-glutamate)s (**1**, number-averaged degree of polymerization: $n_{1a}=12$, $n_{1b}=32$) and investigated their emulsion-templated self-assembly into hollow microspheres. Poly(γ-benzyl L-glutamate)s (PBLG) are typical α-helical peptides that provides hydrophobic rigid-rod structures.[8] A diglycolic acid unit was introduced to enhance the hydrophilicity of the N-terminus. These polypeptides give oil-in-water microemulsions, and hollow microspheres are spontaneously formed upon rapid evaporation of the organic phase.



1: n = 12, 32

[a] Dr. M.-a. Morikawa, Prof. N. Kimizuka
Department of Chemistry and Biochemistry
Graduate School of Engineering, Kyushu University
Fukuoka 812-8581 (Japan)
Fax: (+81) 92-642-2011
E-mail: kimitcm@mbox.nc.kyushu-u.ac.jp

[b] Prof. M. Yoshihara, Prof. T. Endo
Henkel Research Center of Advanced Technology
Molecular Engineering Institutes, Kinki University
Fukuoka 820-8555 (Japan)

[c] Prof. M. Yoshihara
Department of Applied Chemistry
School of Science and Engineering, Kinki University
3-4-1 Kowakae, Higashi-Osaka, 577-8502 (Japan)

[d] Prof. T. Endo
Department of Polymer Science and Engineering
Faculty of Engineering, Yamagata University
4-3-16 Jonan, Yonezawa, 992-8510 (Japan)

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Results and Discussion

Formation of oil-in-water emulsions stabilized by amphiphilic α-helical polypeptides: The amphiphilic peptides **1a** and **b** are poorly soluble in pure water even in the form of sodium salts. This is due to the overwhelming hydrophobicity of the PBLG unit relative to the small hydrophilic group. On the other hand, these peptides are readily soluble in dichloromethane. Figure 1 shows circular dichroism (CD) spectra of

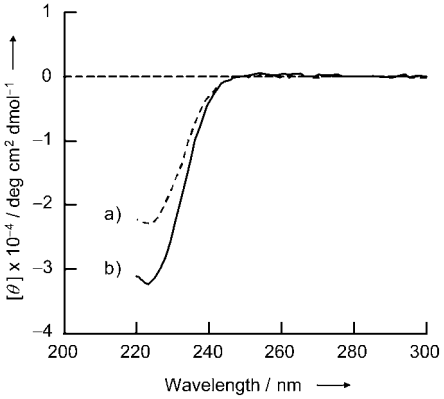

Figure 1. Circular dichroism spectra of the peptides in dichloromethane: a) **1a**; b) **1b**. Concentration, 0.5 unit mM. Cell length, 1 mm.

1 (concentration: 0.5 unit mM in dichloromethane). CD spectra below 220 nm were not obtained because of the interference of solvent. These spectra possess minima at 222 nm, which is characteristic of α-helical structures. The helicities determined by the molar ellipticity at 222 nm were approximately 68% for **1a** ($n=12$; $[\theta]_{222}=2.3\times10^4$ deg cm^2 dmol^{-1}) and 97% for **1b** ($n=32$; $[\theta]_{222}=3.3\times10^4$ deg cm^2 dmol^{-1}). It is reasonable that the longer peptide gives a higher content of α-helical structures.

Upon mixing solutions of **1** in dichloromethane (5 unit mM, 1 mL) and an aqueous phase containing equimolar sodium hydroxide (CH_2Cl_2/water = 1:2 or CH_2Cl_2/methanol/water = 1:1:1, v/v), microemulsions were obtained. After keeping the emulsions at room temperature for about 5 min (CH_2Cl_2/water = 1:2, v/v) or for a day (CH_2Cl_2/methanol/water = 1:1:1, v/v), aqueous layers separate from the microemulsion layers. These emulsion layers were stable for 2–3 days. In microscopic observations, these emulsion layers were placed on a glass slide without using a cover glass. This is to allow rapid evaporation of the dichloromethane phase.

Figure 2 shows dark-field optical microscopy images of emulsions in the course of dichloromethane evaporation. The emulsions initially contained dichloromethane droplets with diameters of 5–60 μm (Figure 2a, **1b**, CH_2Cl_2/water = 1:2, v/v). The average diameter of the droplets became smaller when methanol was added to the mixture (less than 30 μm, CH_2Cl_2/methanol/water = 1:1:1, v/v). The diameter of the droplets also decreased with time (5–30 μm after 2 min, Figure 2b) and reached a final size (less than 10 μm) after 3 minutes. These particles are hollow as can be seen from a

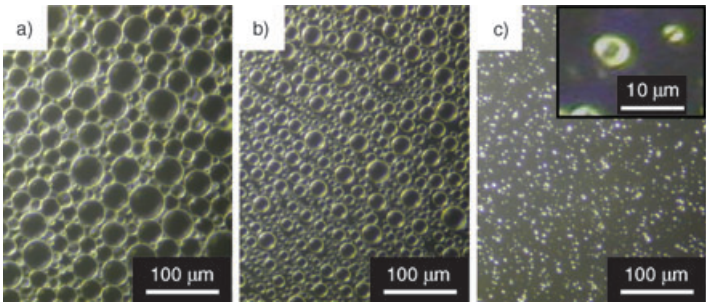

Figure 2. Dark-field optical micrograph images of the oil–water emulsions: a) as prepared; b) after 2 minutes; c) after 3 minutes. [**1b**] = 5 unit mM, CH_2Cl_2/water = 1:2, v/v.

magnified image (Figure 2c, inset). The reduction in size of dichloromethane droplets and the formation of hollow peptide capsules were also observed for **1a**.

The formation process of microcapsules was investigated in more detail by using confocal laser scanning microscopy (CLSM, λ_{ex} at 488 nm). The aqueous phase in the microemulsions was stained with sodium fluorescein (0.1 mM). Figure 3a, b shows CLSM images of the emulsions which have

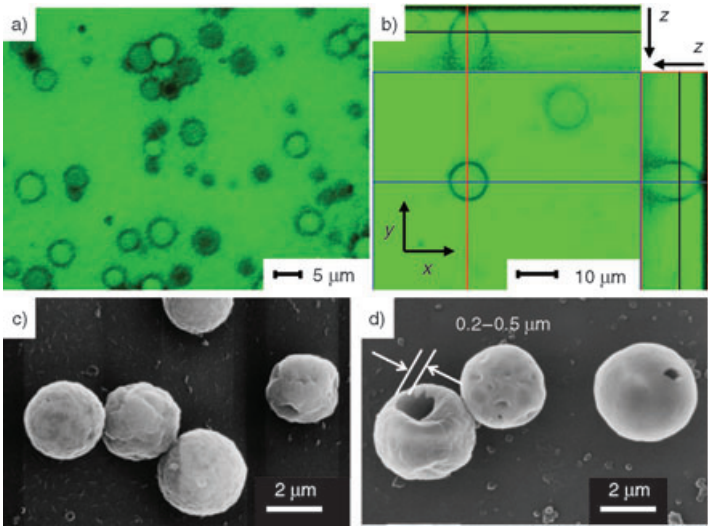

Figure 3. CLSM fluorescence images of aqueous peptide **1b** microcapsules after removal of the dichloromethane phase (a, b), and SEM images (c: **1a**; d: **1b**) of the peptide microcapsules. Sodium fluorescein (0.1 mM) was dissolved in the aqueous phase and emulsified at a CH_2Cl_2/water ratio of 1:2, v/v. The fluorescence images were obtained with the excitation wavelength of 488 nm and fluorescence intensity was visualized at wavelengths below 505 nm.

been kept for one hour prior to observation. Microparticles with diameters of approximately 1–5 μm are abundantly observed. The presence of an aqueous phase inside the microparticles is confirmed by the fluorescence image and height profiles (scanned in Figure 3b).

Interestingly, polypeptide microcapsules are stable in air, even after drying. A droplet of the emulsions (CH_2Cl_2/

water = 1:2, v/v) was placed on a carbon-coated copper grid and the specimens were then dried and observed by scanning electron microscopy (SEM, acceleration voltage, 25 kV, Figure 3c, d). Interestingly, microspheres with diameters of 2–7 μm (**1a**) and 1–4.5 μm (**1b**) are abundantly observed, with a small number of fractured particles (Figure 3d). The thickness of the shells is estimated to be approximately 200–500 nm, which is considerably larger than the molecular lengths of amphiphilic polypeptides (ca. 2.5 nm for **1a**; ca. 6 nm for **1b**). Therefore, the shells of the microcapsules are composed of an assembly of polypeptides.

FTIR spectra of the dried microcapsules prepared on CaF_2 plates are shown in Figure 4. The peaks observed around 1700–1750 cm^{-1} are assigned to the C=O stretching

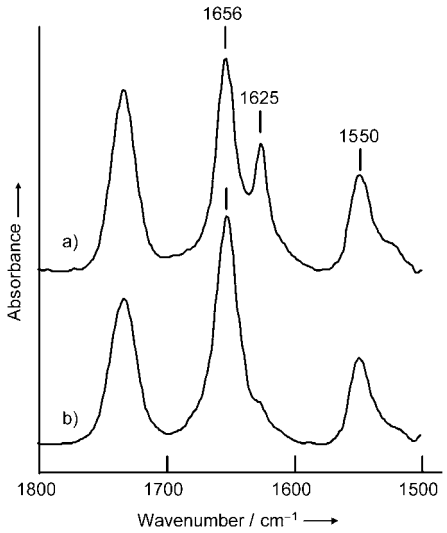

Figure 4. FTIR spectra of the peptide microcapsules immobilized on CaF_2 plates: a) **1a**; b) **1b**.

band of the side-chain ester groups. In the case of **1b**, the amide I band is observed at 1656 cm^{-1} (Figure 4b) and is assigned to α-helical structures maintained in the capsules. On the other hand, a new amide I band at 1625 cm^{-1} is additionally observed for the shorter oligopeptide **1a**, which is typical of β-sheet structures (Figure 4a). As described already, the α-helical content of **1a** in dichloromethane is lower than that of **1b**. Casting of the solutions of **1a** in dichloromethane on CaF_2 plates leads to the observation of the amide I band, which is absent in solutions, and it is likely that the intermolecular hydrogen bonds are formed from non-helical fractions of **1a** during the evaporation of solvent. A similar mechanism would be responsible for the formation of β-sheet structures in the cast microcapsules.

Figure 5 schematically illustrates the emulsion-templated self-assembly of amphiphilic polypeptides. At first, amphiphilic polypeptides are adsorbed onto the oil/water interface of the microdroplets. As the spontaneous stripping of dichloromethane proceeds, the rest of the monomerically dissolved polypeptides are accumulated at the interface and

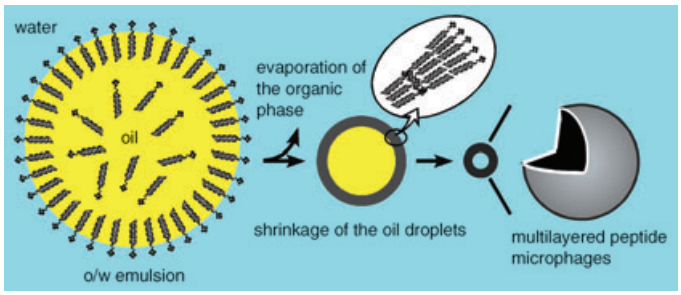

Figure 5. Schematic illustration of the emulsion-templated self-assembly process for **1b**. Oil–water (o/w) emulsions are converted to smaller polypeptide microcapsules upon spontaneous stripping of dichloromethane. In the case of the shorter oligopeptide **1a**, β-sheet structures are partly formed during solvent evaporation.

multilayered shells are formed. The self-assembled polypeptide shells are stable enough to maintain the microcapsule structures (diameters smaller than 10 μm) and the inner aqueous phase is formed during the assembling process. The use of emulsion templating is indispensable for the formation of microcapsules, as it is difficult to dissolve powdery samples of **1a** and **b** directly in pure water. It is also worth noting that the hollow micro-architectures remain intact on the surface even after solvent evaporation. These observations clearly demonstrate the pronounced stability of the present polypeptide microcapsules, in contrast to the conventional aqueous lipid vesicles.

Doping of hydrophobic pyrene molecules in the layered polypeptide shell: The peptide microcapsule can trap hydrophobic molecules. As a hydrophobic fluorescent probe, pyrene was dissolved in dichloromethane (concentration of pyrene and amphiphilic peptides, 10 μM and 5 unit mM, respectively) and was emulsified as described earlier. Figure 6 shows the CLSM image of the obtained microcapsules. The

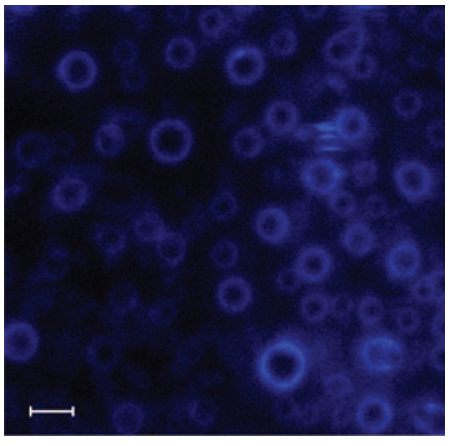

Figure 6. CLSM image of aqueous peptide microcapsules after the removal of the dichloromethane phase. Peptide **1b** (5 unit mM) and pyrene (10 μM) were dissolved in dichloromethane and emulsified at a CH_2Cl_2/water ratio of 1:2, v/v. The fluorescence image was obtained with an excitation wavelength of 364 nm and the fluorescence intensity was visualized at wavelengths below 385 nm. The scale bar represents 2 μm.

ultraviolet excitation wavelength available for the CLSM apparatus employed was 364 nm, and the fluorescence intensity was monitored at wavelengths longer than 385 nm. Though the excitation wavelength of 364 nm is redshifted compared to those commonly used for pyrene chromophores (ca. 330–340 nm),[9] blue fluorescence images of the microcapsule structures were successfully observed. The fluorescence spectrum showed a broad maximum around 470 nm, which is characteristic of the pyrene excimer emission.[9,10] It is reported that ground-state pyrene dimers display a redshifted absorption and are excitable even at longer wavelengths of 340–380 nm.[10] Therefore, the observed blue fluorescence in Figure 6 indicates that pyrene molecules are concentrated and form aggregates in the polypeptide shells.

Microscale hollow spheres of organic and inorganic polymers have been prepared by using the layer-by-layer (LBL) adsorption technique,[11] emulsion-templated interfacial polymerization, or sol–gel reactions.[12,13] In the LBL procedures, sacrificial colloidal templates need to be subsequently removed by thermal or chemical etching. This etching process is not compatible with the amphiphilic self-assembly of peptides. As far as we are aware, application of oil–water emulsions for interfacial self-assembly has been limited to latex particles.[14] The present emulsion-templated self-assembly technique allows facile doping of hydrophobic molecules in the stable polypeptide shells, and we envision these properties will possess attributes useful for microcapsule-related technologies.

Conclusion

The main conclusion of the present study is that stable microcapsules are easily obtained by emulsion-templated self-assembly of α-helical polypeptides. The emulsion-directed approach provides an opportunity to prepare stable microcapsules, even if amphiphilic components are not readily soluble in water. We expect numerous applications of the polypeptide microcages, such as robust supramolecular reservoirs and micro-scaffolds for biomolecules, which will be reported in due course.

Experimental Section

Synthesis of the peptide amphiphiles (1): γ-Benzyl L-glutamate *N*-carboxylic anhydride (BLG–NCA) was prepared by the reaction of triphosgene (2.1 g, 7 mmol, Wako Pure Chemical Ind., Ltd.) with γ-benzyl L-glutamate (5.0 g, 21 mmol, nacalai tesque) in dry tetrahydrofuran at 55 °C. BLG–NCA was then polymerized with freshly distilled *n*-propylamine (Wako Pure Chemical Ind., Ltd.) in chloroform at room temperature. The degree of polymerization of the peptide segments was controlled by adjusting the feed molar ratio of the monomer (BLG–NCA; [M]) to the initiator (*n*-propylamine; [I]): [M]/[I] = 10 (**1a**) or 30 (**1b**). After stirring for 3 h, the reaction mixture was poured into a large amount of diethyl ether and purified by repeating the precipitation. The obtained poly(γ-benzyl L-glutamate) was then treated with diglycolic anhydride (10 equiv relative to the peptides, Tokyo Kasei Kogyo Co., Ltd.) in dry chloroform at room temperature for 7 days. The reaction mixture was purified by washing with water at pH 3.0 and then dried in vacuo. Chemical structures and purity of the samples were confirmed by ^{1}H NMR and FTIR spectra for **1a** and **b**. ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C, TMS): δ = 0.8 (t, 3H; C*H*$_3$-), 1.4 (m, 2H; CH$_3$C*H*$_2$-), 1.8–2.6 (b, 4H × *n*; -C*H*$_2$C*H*$_2$-), 3.0 (b, 2H; -NHC*H*$_2$-), 3.8–4.4 (b, H × *n*; -(O=C)C*H*RNH-), 4.0 (s, 2H; -NH(C=O)C*H*$_2$O-), 4.1 (s, 2H; -OC*H*$_2$-(COOH)), 5.0 (b, 2H × *n*; benzyl-C*H*$_2$-), 7.3 (b, 5H × *n*; benzyl-*H*), 7.8–8.6 ppm (b, 1H × *n*; NH); IR (film): $\tilde{\nu}_{max}$ = 3300, 1730, 1650, 1550 cm^{-1}. The number-average degree of polymerization for polypeptide **1** was determined by using an ^{1}H NMR method, by comparing proton peaks of the terminal methyl and benzyl groups.

Preparation of the microemulsions: The polypeptides are soluble in dichloromethane or chloroform at a concentration of 5 unit mM. To obtain microemulsions, equimolar sodium hydroxide was dissolved in an aqueous phase. Upon mixing dichloromethane solutions of **1** with the aqueous phase, by shaking by hand or ultrasonication, oil-in-water (o/w) microemulsions were easily obtained. The addition of methanol was effective in enhancing the stability of the microemulsions. Water was purified with a Direct-Q system (Millipore, Co.) and was used throughout this study.

Measurements: Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter equipped with a Peltier-type thermostatic cell holder. A quartz cell of 1 mm path length was used. ^{1}H NMR spectra were obtained on a Varian INOVA 400 spectrometer. Infrared spectra were recorded on a JASCO FTIR 460 Plus spectrometer. Dark-field optical microscopy and confocal laser scanning microscopy (CLSM) were conducted with Olympus BHF and Carl Zeiss LSM510 instruments with a 63x oil-immersion objective, , respectively. Scanning electron microscopy (SEM) was conducted on a Hitachi S-5000 instrument operated at 25 kV.

Acknowledgements

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