



Title	Development of hydrogels and cell aggregates driven by triple helix formation of collagen model peptides
Author(s)	吉田, 和暉
Citation	大阪大学, 2026, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/105417
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

<https://ir.library.osaka-u.ac.jp/>

The University of Osaka

Doctoral Dissertation

**Development of hydrogels and cell aggregates driven
by triple helix formation of collagen model peptides**

**(コラーゲンモデルペプチドの三重らせん形成を
駆動力としたハイドロゲルおよび
細胞凝集体の創製)**

KAZUKI YOSHIDA

January 2026

**Department of Applied Chemistry
Graduate School of Engineering
The University of Osaka**

Content

General Introduction	1
Chapter 1	7
1.1 Introduction	7
1.2 Experiments.....	9
1.2.1 Materials.....	9
1.2.2 Synthesis of F(PPG) ₁₀ and F(POG) ₁₀	10
1.2.3 Functional characterizations of FCMP	10
1.2.4 Evaluation of hetero-triple helix formation.....	10
1.2.5 Evaluation of conditions for hetero-triple helix formation	11
1.3 Results and discussion.....	13
1.3.1 Synthesis and functional characterizations of FCMPs.....	13
1.3.2 Functional characterizations of FCMPs	17
1.3.3 Characterization of hetero-triple helices with (PPG) ₁₀ and (POG) ₁₀	18
1.3.4 The concentration and temperature dependence of the triple helix formation.....	21
1.3.5 Evaluation of time dependence of triple helix formation at 37 °C.....	23
1.4 Conclusion.....	25
1.5 References	25
Chapter 2	27
2.1 Introduction	27
2.2 Experiments.....	29
2.2.1 Materials.....	29
2.2.2 Synthesis and functional characterizations of AzidoEG4(PPG) ₁₀ and DBCOEG4(PPG) ₁₀	29
2.2.3 Synthesis of 4armPEG10k-triazole-EG4(PPG) ₁₀	30
2.2.4 Sol-gel transition analysis of 4armPEG10k-triazole-EG4(PPG) ₁₀	30
2.2.5 Synthesis of 4armPEG10k-triazole-EG4(POG) ₁₀	31
2.2.6 Evaluation of sol-gel transition property in response to CMP's amino acid sequences	31
2.2.7 Sol-gel transition analysis of 4armPEG10k-triazole-EG4CMPs with CMPs	32
2.2.8 Effect of the additional single strand CMPs on 4armPEG10k-triazole-EG4CMPs gel	32
2.3 Results and discussion.....	34
2.3.1 Synthesis and functional characterizations of AzidoEG4(PPG) ₁₀ and DBCOEG4(PPG) ₁₀	34
2.3.2 Synthesis of 4armPEG10k-triazole-EG4(PPG) ₁₀	38

2.3.3 Sol-gel transition analysis of 4armPEG10k-triazole-EG4(PPG) ₁₀	40
2.3.3 Synthesis of 4armPEG10k-triazole-EG4(POG) ₁₀	41
2.3.4 Evaluation of sol-gel transition property in response to CMP's amino acid sequences	42
2.3.5 Sol-gel transition analysis of 4armPEG10k-triazole-EG4CMPs with CMPs	44
2.3.6 Effect of the additional single strand CMPs on 4armPEG10k-triazole-EG4CMPs gel	46
2.4 Conclusion.....	48
2.5 References	48
Chapter 3	50
3.1 Introduction	50
3.2 Experiments.....	52
3.2.1 Materials	52
3.2.2 Synthesis of 4arm-PEG-DCA	52
3.2.3 Fmoc protection of (PPG) ₁₀	53
3.2.4 Synthesis of 4arm-PEG-DCA/-(PPG) ₁₀	53
3.2.5 Functional characterizations of 4arm-PEG-DCA/-(PPG) ₁₀	54
3.2.6 Evaluation of cytotoxicity response	54
3.2.7 Polymer adsorption to cell.....	54
3.2.6 Cell-cell assembly formation	55
3.3 Results and discussion.....	55
3.3.1 Synthesis of 4arm-PEG-DCA	55
3.3.2 Fmoc protection of (PPG) ₁₀	57
3.3.3 Synthesis of 4arm-PEG-DCA/-(PPG) ₁₀	58
3.3.4 Functional characterizations of 4arm-PEG-DCA/-(PPG) ₁₀	60
3.3.5 Evaluation of cytotoxicity response	60
3.3.6 Polymer adsorption to cell.....	61
3.3.7 Cell-cell assembly formation	63
3.4 Conclusion.....	63
3.5 References	64
Concluding Remarks	65
List of Publications.....	67
Acknowledgements	68

General Introduction

Biofabrication is the field of creating tissues and organs from biological materials such as cells and proteins, and artificial tissues and organs that reproduce complex structures and functions are expected to be applied in transplantation therapy and drug efficacy evaluation. Recently, the fabrication of tissues that mimic the complex three-dimensional structures of biological tissues using hydrogels as scaffold materials have been reported.^[1, 2] However, it is still challenging to completely reproduce the functions of real tissues. One of the reasons is that the abundant presence of scaffold materials probably hinders cell–cell interactions (Figure 1). For a tissue to function as a tissue, it is important for individual cells to interact closely with each other to induce differentiation. Therefore, the tailor-made approaches for the fabrication of cell aggregates to facilitate intercellular interactions are needed.

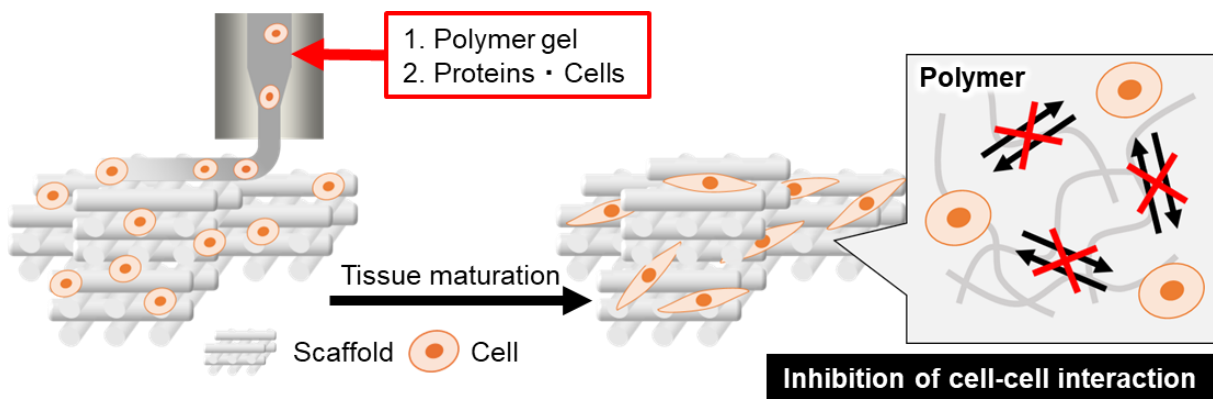


Figure 1. Schematic illustration of biofabrication method using scaffold polymer.

In these days, the methods for constructing higher-order tissue structures from cell aggregates are being studied (Figure 2).^[3] Especially, the cell surface modification approaches have been attracted much attention because they can manipulate and control cell interactions and phenotypes for various biomedical applications.^[4] As Biologically-Driven cell surface modifications, genetic engineering methods and metabolic engineering methods^[5] which introduce functional groups through sugar metabolism are reported, but they have some challenges such as cell-type dependency, risk of trigger immunogenic responses and tumorigenesis, and chemical insertion efficiency that can vary depending on cell metabolism.^[4] Accordingly, chemically-driven cell modifications have also been studied.^[4] Among them, covalent-bond formation is considered as a simple and straightforward method, but it has the lack of targeting specificity and cytotoxicity. On the other hand, hydrophobic insertion in the

cell surface using phospholipids, alkyl chains, and cholesterol have low cytotoxicity.^[6,7] Previously, it was also reported that the hydrophobic insertion using a cholesterol.^[8]

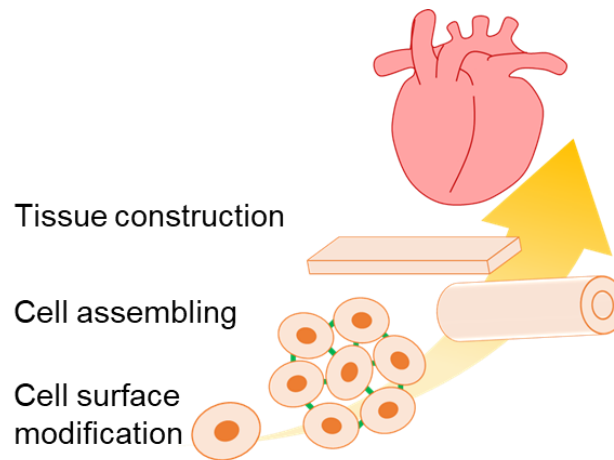


Figure 2. Schematic illustration of constructing higher-order tissue structures from cell aggregates

Some researchers are using DNA chain as a crosslinking site to make cell-cell assemblies.^[6,9] However, there are some concerns about the usage of DNA such as DNA degradation and potential risk of immune response.^[10] On the other hand, it is known that a collagen-like triple-helix is resistant to most proteases except for collagen-specific enzymes,^[11] the application of collagen-like triple helices is considered promising.

Collagen is a protein found in all multicellular organisms and accounts for about 20-30% of all proteins in animals.^[12] The triple helix structure characteristic of collagen consists of three polyproline II-like left-handed helices forming a right-handed triple helix. The triple helix region of collagen has a glycine (Gly, G) repeating every three residues in the amino acid sequence, which can be expressed as Xaa-Yaa-Gly. In vertebrates, proline (Pro, P) appears selectively at Xaa and hydroxyproline (Hyp, O) at Yaa, enhancing the thermal stability of the triple helix. To study the structure of collagen, Collagen model peptides (CMPs) with repeating triple-helical amino acid sequences have been synthesized. The first synthesis and use of these peptides date back to the 1960s.^[13] In the amino acid sequence of CMPs, the side chains of Xaa and Yaa residues are outside the triple helix, while Gly residues are buried inside.^[14] The carbonyl of the Xaa residue forms a hydrogen bond with glycine NH in the parallel chain, and the three chains are staggered by one amino acid to form a triple helix.^[15] For example, (PPG)₁₀'s crystal structure exhibits a rod-like shape with a length of approximately 90 Å and a diameter of approximately 10 Å, and the three chains are oriented in the same direction from the C-terminus to the N-terminus (Figure 3).^[16] The stability of the CMPs assembly can be

manipulated by amino acid composition, peptide length, and external conditions. CMPs are known to exhibit repeatable triple helix formation and dissociation upon heating and cooling. It is known that an increase in the number of repeating units enhances the stability of the triple helix,^[17] and it has been reported that mixed triple-helix formation between chains with different numbers of repeating units occurs only with difficulty.^[18] The folding of each chain into a polyproline-II helix based on the $n \rightarrow \pi^*$ interaction and the interchain hydrogen bonding contribute to the stability in triple-helix formation.^[14] In particular, (PPG)₁₀ and (POG)₁₀ are considered standard collagen models. Since collagen is one of the most widespread proteins in nature, CMPs are considered suitable for biological applications. In addition, the use of fully synthesized CMPs circumvents batch-to-batch heterogeneity, potential allergic reactions, and possible zoonotic disease transmission seen with extracted materials.^[19]

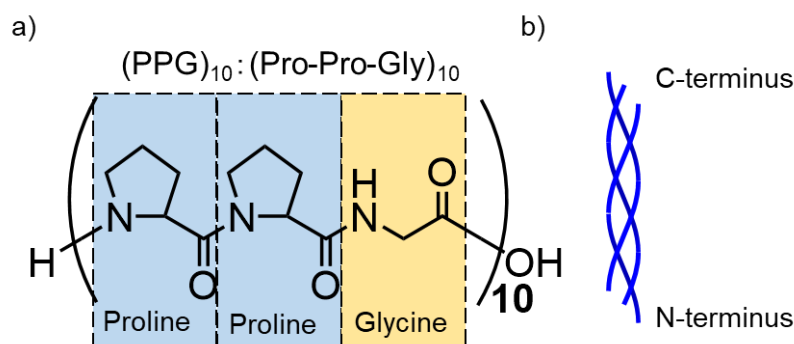


Figure 3. Schematic illustrations of (a) the structure of (PPG)₁₀ and (b) the organization of (PPG)₁₀'s triple helices.

However, a cell assembly method using CMP as a cell assembling motif has not yet been reported. In addition, there are few reports about the analysis of CMP hydrogels by homo- and hetero triple helix formation, and few studies have clarified the effect of additional CMP strands using homo and hetero triple helix formation.

Outline of this thesis

In this thesis, the author analyzed the assembly properties via homo- and hetero-triple helix formation of CMP and investigated the hydrogels and cell assembling method with crosslinking characteristics driven by triple helix formation toward the control of material properties through homo- and hetero-triple helices formation of CMP and their application.

Chapter 1

Analysis of homo- and hetero-triple helix formation of collagen model peptides and

evaluation of their stability in a biological environment

In Chapter 1, the author evaluated the triple helix formation of two collagen model peptides, (PPG)₁₀ and (POG)₁₀, by fluorescence and circular dichroism (CD) spectroscopic measurements for the single and mixed conditions. Fluorescein isothiocyanate labeled (PPG)₁₀ and (POG)₁₀ exhibit an increase or decrease in fluorescence intensity as the triple helix dissociates and forms. By combining fluorescence and CD spectroscopic measurements, the ratio of (PPG)₁₀ to (POG)₁₀ chains in the hetero-triple helix, the concentration and temperature dependence of the homo- and hetero-triple helix formation were able to analyze. In addition, even when the temperature during triple helix formation was set to 37 °C, which is above the melting temperature (T_m) of (PPG)₁₀, the formation of the hetero-triple helix was suggested, indicating that (POG)₁₀ might play a major role in the formation of the hetero-triple helix at 37 °C. From these results, it was suggested that heterotrimers have different association properties from homotrimers and are expected to be applied in nanotechnology and biomaterials as new self-assembled materials.

Chapter 2

Analysis of the Assembly Properties and Control of Sol–Gel Transition via Homo- and Hetero-Triple Helix Formation of Collagen Model Peptide-Modified 4arm Poly(ethylene glycol)

In Chapter 2, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands toward the further application for cell assembling method with crosslinking characteristics driven by triple helix formation. First of all, the 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ were successfully synthesized. Subsequently, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands. The 4arm-PEG10k-triazole-EG4CMPs showed gel-sol transition by the internal triple helix formation of CMPs. The gel-sol transition behavior was controlled by the amino acid sequences of CMP and the presence of additional non-modified CMPs. Furthermore, it's possible that the gel properties of 4arm-PEG10k-triazole-EG4CMP can be controlled by additional single-strand CMP. This phenomenon is expected to be applied in biomaterials for the development of new self-assembled materials and to further understanding of triple helix formation-driven sol-gel transition.

Chapter 3

Fabrication of a Thermoresponsive Cell Assembly Method Driven by Triple Helix Formation of a Collagen Model Peptide

In Chapter 3, the author fabricated the thermoresponsive cell assembly method driven by triple helix formation of CMP. As results of the evaluation, more cell aggregates were formed after cooling under conditions with 4arm-PEG-DCA/-(PPG)₁₀ than without 4arm-PEG-DCA/-(PPG)₁₀. Accordingly, to the best of our knowledge, this is the first report of the thermoresponsive cell assembly method driven by triple helix formation of CMP. The use of CMP is expected to be applied in a new cell assembling approach for tissue engineering.

References

- [1] S. Choi, K. Y. Lee, S. L. Kim, L. A. MacQueen, H. Chang, J. F. Zimmerman, Q. Jin, M. M. Peters, H. A. M. Ardoña, X. Liu, A.-C. Heiler, R. Gabardi, C. Richardson, W. T. Pu, A. R. Bausch, K. K. Parker, *Nat. Mater.* **2023**, 22, 1039.
- [2] N. Noor, A. Shapira, R. Edri, I. Gal, L. Wertheim, T. Dvir, *Adv. Sci.* **2019**, 6, 1900344.
- [3] V. M. Gaspar, P. Lavrador, J. Borges, M. B. Oliveira, J. F. Mano, *Adv. Mater.* **2020**, 32, 1903975.
- [4] J. Almeida-Pinto, M. R. Lagarto, P. Lavrador, J. F. Mano, V. M. Gaspar, *Adv. Sci.* **2023**, 10, 2304040.
- [5] J. A. Prescher, D. H. Dube, C. R. Bertozzi, *Nature* **2004**, 430, 873.
- [6] Y. Teramura, *Biomaterials* **2015**, 48, 119.
- [7] Y. Teramura, K. N. Ekdahl, K. Fromell, B. Nilsson, K. Ishihara, *Langmuir* **2020**, 36, 12088.
- [8] H. Nakatsuji, Y. Shioji, N. Hiraoka, Y. Okada, N. Kato, S. Shibata, I. Aoki, M. Matsusaki, *Mater. Horiz.* **2021**, 8, 1216.
- [9] Z. J. Gartner, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.* **2009**, 106, 4606.
- [10] P. Shi, Y. Wang, *Angew. Chem. Int. Ed.* **2021**, 60, 11580.
- [11] L. L. Bennink, D. J. Smith, C. A. Foss, M. G. Pomper, Y. Li, S. M. Yu, *Mol. Pharm.* **2017**, 14, 1906.
- [12] K. Okuyama, T. Kawaguchi, *Kobunshi Ronbunshu* **2010**, 67, 229.
- [13] S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai, K. Kakiuchi, *BCSJ* **1968**, 41, 1273.
- [14] V. Kubyshkin, *Org. Biomol. Chem.* **2019**, 17, 8031.
- [15] S. A. H. Hulgán, J. D. Hartgerink, *Biomacromolecules* **2022**, 23, 1475.
- [16] R. Berisio, L. Vitagliano, L. Mazzarella, A. Zagari, *Protein Sci.* **2002**, 11, 262.
- [17] T. Koga, S. Kingetsu, N. Higashi, *Int. J. Mol. Sci.* **2021**, 22, 4533.
- [18] Y. Kobayashi, Y. Suezaki, K. Inouye, K. Tanaka, K. Kakiuchi, Y. Kyogoku, *Biopolymers* **1977**, 16, 247.
- [19] D. Kalita, B. K. Sarma, *Langmuir* **2025**, 41, 9162.

Chapter 1

Analysis of homo- and hetero-triple helix formation of collagen model peptides and evaluation of their stability in a biological environment

1.1 Introduction

Self-assembled materials have attracted much attention and have been extensively studied in recent years, because the reversibility of non-covalent interactions can impart a variety of functions, including stimulus responsiveness and self-healing properties. Functionalized self-assembled materials can be used for many applications, especially in drug delivery, biotechnology, catalysis, and energy.^[1] For example, in the field of photonic materials, luminescent shape-memory polymers based on composites of liquid-crystalline cellulose nanocrystal film and polyacrylate matrices have been reported.^[2,3] In biomedical applications, the fabrication of doxorubicin prodrug micelles for tumor cascade photothermal chemotherapy has also been reported.^[4] In the case of natural and naturally occurring building blocks, natural and synthetic DNA, proteins or peptides, phospholipids, and polysaccharides have been utilized for self-assembly.^[5] Peptides have been used as biomimetic building blocks for supramolecular self-assembly^[5] because they are short sequences of amino acids that are domains of naturally occurring protein structure and function. In particular, collagen model peptides (CMPs), which form a unique triple helix structure, have attracted attention as supramolecular self-assemblies.

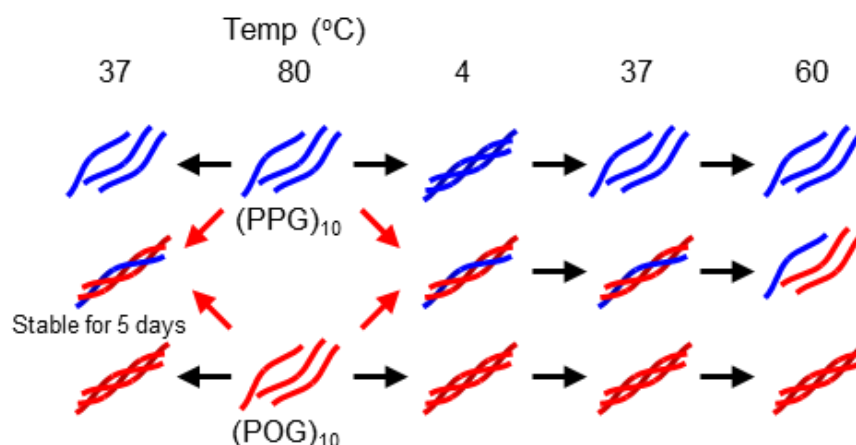
CMPs are known to exhibit repeatable triple helix formation and dissociation upon heating and cooling. In particular, (PPG)₁₀ and (POG)₁₀ are considered standard collagen models. Since collagen is one of the most widespread proteins in nature, CMPs are considered suitable for biological applications. Various CMPs have been investigated as drug delivery carriers, thermosensitive gelators, collagen modification tools, and supramolecular assemblies due to their unique thermo-responsive triple helix formation and collagen binding properties via hydrogen bonding.^[6-13] For example, a thermo-responsive gelator, a CMP-modified 8-arm PEG multi-arm polymer, has been reported in a study using CMP as a cross-linking site.^[8] Previously, it was also reported that multi-arm poly(ethylene glycol) (PEG) (4arm-PEG-

(POG)₁₀ end-modified with (POG)₁₀ induces gelation by binding between triple helices when mixed with fibrous collagen and has potential for tissue engineering applications.^[11] The formation of ultra porous laminated tubes or bilayer nanosheets by self-assembly of heteromeric pairs of charge-complementary CMPs^[12] and the hierarchical self-assembly of CMPs with various aromatic groups at the N-terminal into nanofibers and the encapsulation of hydrophobic molecules^[13] have also been reported. Furthermore, the properties of CMPs to form triple helices with denatured collagen have been studied for the detection of denatured collagen,^[9] denatured collagen recognition by CMPs modified on quartz crystal microbalance sensor substrates,^[14] and tumor localization polymer carriers that selectively bind to denatured collagen in the tumor extracellular matrix.^[15] However, many studies were based on homo-triple helices.

Studies on hetero-triple helices have also been reported, and it has been reported that (PPG)₁₀ and (POG)₁₀ form a hetero helix by annealing under mixed conditions.^[16, 17] Type I collagen is a heterotrimer consisting of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain and Sharma *et al.* identified key amino acid residues in the $\alpha 1$ and $\alpha 2$ C-propeptides that determine homo- and heterotrimerization of collagen type I.^[18] For more detailed studies of collagen, a hetero-triple helix of CMP has been produced using electrostatic interactions^[19] and templates.^[20] Unlike the homo-triple helix, the hetero-triple helix has the advantage that different molecules attached to the ends of each chain can be selectively brought into proximity. It could be possible to selectively react or accelerate reactions between reactive molecules by concentrating them through triple helix formation. However, the detailed properties of the hetero-triple helix have not been fully characterized. If the characteristics of the heterotrimers can be revealed, they are expected to be applied as new self-assembled materials.

Herein, the author synthesized fluorescent-labeled CMPs to characterize the hetero-triple helix formation in detail by fluorescence quenching associated with triple helix formation. Previously, Xiao *et al.* reported triple helix formation using terminal fluorescent-labeled CMPs,^[21, 22] but there was no information about the nature of hetero- and homo-triple helices by (PPG)₁₀ and (POG)₁₀. In this study, fluorescence labeling was performed on the simple repeating peptides (PPG)₁₀ and (POG)₁₀ to reveal in detail the characteristics of hetero- and homo-triple helices by CMPs and their association properties (Scheme 1). When CMPs with equal or higher triple helix stability were added to FCMPs, the fluorescence intensity reached a plateau at more than 2 equivalent CMPs, while higher concentrations were required at lower

CMPs. It was indicated that the triple helix formation concentration of the heterotrimer prepared under 1:2 mixing conditions of (PPG)₁₀ and (POG)₁₀ is almost half that of [(POG)₁₀]₃ but 45 times higher than [(PPG)₁₀]₃. Furthermore, the heterotrimer formed at 37 °C was stable after 5 days which was the same as [(POG)₁₀]₃. These results suggest that heterotrimers have different association properties from homotrimers and are expected to be applied in nanotechnology and



biomaterials as new self-assembled materials.

Scheme 1. Schematic illustration of temperature-dependent triple helix formation and dissociation of (PPG)₁₀, (POG)₁₀, and (PPG)₁₀ and (POG)₁₀ mixture. Even when the temperature during triple helix formation was set to 37 °C, which is above the melting temperature (T_m) of (PPG)₁₀, the hetero-triple helix was formed, and was stable after 5 days.

1.2 Experiments

1.2.1 Materials

The (PPG)₁₀ (Ref. 4006) and (POG)₁₀ (Ref. 4033) were purchased from Peptide Institute, Inc. (Osaka, Japan). Boric acid (Ref. 021-02195), sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄, and 5M sodium hydroxide (Ref. 196-05375) were purchased from Wako Chemicals (Osaka, Japan). Fluorescein 5-isothiocyanate (FITC) (Ref. 16151-66), dimethyl sulfoxide (DMSO) (Ref. 13445-45), and D-PBS without Ca and Mg, powder (Ref. 07269-84) were purchased from Nacalai Tesque (Kyoto, Japan). Acetone (Ref. 010-00306) was purchased from Kishida Chemical (Osaka, Japan). Deuterium oxide (Ref. 151882) was purchased from Sigma-Aldrich (St. Louis, USA). Fluorescein (Ref. F0095) was purchased from Tokyo Chemical Industry (Tokyo, Japan). All chemicals were used without further purification.

1.2.2 Synthesis of F(PPG)₁₀ and F(POG)₁₀

The (PPG)₁₀ (10 mg, 3.95 μ mol) was dissolved in 5 mL of 50 mM boric acid (pH 9) and preincubated at 60 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 15.4 mg of FITC in 0.5 mL DMSO was added to the solution immediately, and then the reaction mixture was wrapped in foil and stirred for 24 h at room temperature. After the reaction, the whole reaction mixture was poured into acetone and centrifuged at 10,000 rpm for 15 min to precipitate the resulting FITC-(PPG)₁₀ (F(PPG)₁₀). This F(PPG)₁₀ was washed twice with acetone, and then dried under vacuum. The structure of the F(PPG)₁₀ was analyzed by FT-IR (HORIBA, FT-702, Kyoto, Japan), MALDI-TOF Mass (Bruker, Autoflex III, Massachusetts, USA), fluorescence, CD, and ¹H-NMR (Bruker, AVANCE III 600, Massachusetts, USA). F(POG)₁₀ was also synthesized in a similar manner to F(PPG)₁₀.

1.2.3 Functional characterizations of FCMP

F(PPG)₁₀ and F(POG)₁₀ were dissolved in PBS at 1 mM and stored at 4 °C. F(PPG)₁₀ and F(POG)₁₀ were both diluted to obtain a final concentration of 500 nM. The solutions were incubated at 25 °C, heated to 80 °C for 10 min, and re-equilibrated at 4 °C for 1 day. Fluorescence and CD spectra were measured by a spectrofluorometer (JASCO, FP-8500, Tokyo, Japan) and a circular dichroism spectrometer (JASCO, J-725, Tokyo, Japan) at 25 °C, 80 °C, and 4 °C, respectively. Samples were incubated at 4 °C and then were measured at 25 °C. The emission spectra were obtained for each peptide from 450 to 600 nm at an excitation wavelength of 492 nm.

1.2.4 Evaluation of hetero-triple helix formation

The solution was prepared by mixing 1 mM (PPG)₁₀ in PBS and 1 mM (POG)₁₀ in PBS at a 1:2 molar ratio. Thermal transition curves were obtained by recording the ellipticity at 225 nm using a J-820 AC (JASCO, Tokyo, Japan), while the temperature was continuously increased in the range 25-90 °C at a heating rate of 1.0 °C min⁻¹. Measurements were performed under two different conditions: immediately after mixing and after heating at 80 °C for 10 min and cooling at 4 °C for 24 h. The melting temperature (T_m) was determined by the following equation: $F(T) = (\theta(T) - \theta_U(T)) / (\theta_F(T) - \theta_U(T))$. $F(T)$ is the fraction folded. $\theta(T)$, $\theta_U(T)$, and $\theta_F(T)$ are the observed ellipticity, the ellipticities calculated from the linear approximation of the region at triple helix dissociation and formation, respectively. T_m was defined as the temperature

at which $F(T_m)=1/2$.

Three types of peptide solution were also prepared: F(PPG)₁₀, F(POG)₁₀, and a mixture of F(PPG)₁₀ and F(POG)₁₀. The mixing ratio of F(PPG)₁₀ to F(POG)₁₀ was 1:2. The samples were prepared in the concentration range of 1,000 μ M to 0.1 μ M in PBS for each peptide solution. The solutions were incubated at 25 °C, heated to 80 °C for 15 min, and re-equilibrated at 4 °C for 1 day. Fluorescence spectra were measured by the spectrofluorometer at 25 °C, 80 °C, and 4 °C. F.I.*(%) is calculated by the following equation: $F.I.*=100*\{(F.I._{25\text{anneal}})-(F.I._{25\text{mix}})\}/\{(F.I._{80})-(F.I._{25\text{mix}})\}$. F.I._{25anneal} is fluorescence intensity at 25 °C after annealing. F.I._{25mix} is fluorescence intensity at 25 °C before annealing. F.I.₈₀ is fluorescence intensity at 15 min at 80 °C. The critical triple helix formation concentration (CTC) was determined as the concentration at the intersection of two approximate straight lines.

XRD (AERIS, Malvern Panalytical, Malvern, U.K.) measurements were performed under three conditions: (PPG)₁₀, (POG)₁₀, and a mixture of (PPG)₁₀ and (POG)₁₀. The mixing ratio of (PPG)₁₀ to (POG)₁₀ was 1:2. The mixture was obtained by pre-heating at 80 °C for 15 min, then cooling at 4 °C for 1 day and lyophilizing the 1 mM aqueous solution.

Furthermore, three types of peptide solution were prepared: (PPG)₁₀, (POG)₁₀, and a mixture of (PPG)₁₀ and (POG)₁₀. The mixing ratio of F(PPG)₁₀ to F(POG)₁₀ was 1:2. The total concentrations of solutions were 1 mM in PBS. The solutions were incubated at 25 °C, heated to 80 °C for 15 min, and re-equilibrated at 4 °C for 1 day. The spectra were measured using a Zetasizer nano ZS (Malvern Panalytical, Worcs, UK) at 25 °C, 80 °C, and 4 °C. Samples were incubated at 4 °C and then were measured at 25 °C.

1.2.5 Evaluation of conditions for hetero-triple helix formation

To evaluate the effects of mixing ratio changes, four types of peptide solution were prepared: a mixture of F(PPG)₁₀ and (PPG)₁₀, a mixture of F(PPG)₁₀ and (POG)₁₀, a mixture of F(POG)₁₀ and (PPG)₁₀, and a mixture of F(POG)₁₀ and (POG)₁₀. The mixing molar ratio of FCMPs and CMPs were 1:0, 1:0.5, 1:1, 1:2, 1:3, and 1:4. The concentration of FCMPs was fixed at 300 μ M. The solutions were incubated at 25 °C, heated to 80 °C for 15 min, and re-equilibrated at 4 °C for 1 day. Fluorescence spectra were measured by spectrofluorometer at 25 °C, 80 °C, and 4 °C. Samples were incubated at 4 °C and then were measured at 25 °C. F.I.** (%) is calculated by the following equation. $F.I.**=100*\{(F.I._{25\text{anneal}})-(F.I._{25\text{mix}})\}/\{(F.I._{80})-(F.I._{25\text{mix}})\}$. F.I._{25anneal} is fluorescence intensity at 25 °C after annealing.

F.I._{25mix} is fluorescence intensity at 25 °C before annealing. F.I.₈₀ is fluorescence intensity at 15 min at 80 °C.

To evaluate the effect of temperature, three types of peptide solution were prepared: F(POG)₁₀, a mixture of F(POG)₁₀ and (PPG)₁₀, and a mixture of F(POG)₁₀ and (POG)₁₀. The mixing molar ratio of FCMP and CMP was 1:2. The concentration of FCMP was fixed at 300 μM. The solutions were incubated at 25 °C, heated to 80 °C for 15 min, and stored at 4 °C or 37 °C for 1 day. Samples were incubated at 4 °C and then were measured at 25 °C.

Furthermore, three types of peptide solution were prepared: F(PPG)₁₀, F(POG)₁₀, and a mixture of F(PPG)₁₀ and F(POG)₁₀. The mixing ratio of F(PPG)₁₀ to F(POG)₁₀ was 1:2. The total concentration of each solution was fixed at 1 mM in PBS. The solutions were incubated at 25 °C, heated to 80 °C for 15 min, and stored at 4 °C or 37 °C. Fluorescence spectra were measured by spectrofluorometer at 25 °C, 80 °C at 5, 10, and 15 min, and 4 °C or 37 °C at 5, 10, 20, 30, and 1,440 min, respectively.

In addition, Temperature-dependent change of fluorescent intensity of fluorescein and FCMPs at the condition of gradual temperature decrease at 1.0 °C min⁻¹ from 80 to 25 °C were measured. Four types of samples were prepared: fluorescein, F(PPG)₁₀, F(POG)₁₀, and a mixture of F(PPG)₁₀ and F(POG)₁₀ in PBS at 100 μM.

1.3 Results and discussion

1.3.1 Synthesis and functional characterizations of FCMPs

The F(PPG)₁₀ and F(POG)₁₀ were successfully synthesized, as characterized by FT-IR, MALDI-TOF-MASS, fluorescence, and CD measurements (Figures 1-1, 1-2, 1-3, and 1-4). Figure 1-1b shows the FT-IR spectra of (PPG)₁₀, FITC, and F(PPG)₁₀. In F(PPG)₁₀, the isothiocyanate-derived peak around 2,100 cm⁻¹ seen in FITC disappears, and the C=S stretching vibration seen around 1,110 cm⁻¹ is observed, suggesting modification by FITC. MALDI-TOF Mass spectra also indicated the presence of F(PPG)₁₀ (Figures 1-1c and 1-1d). The F(POG)₁₀ also showed the C=S stretching vibration, and the Mass spectrum also indicated the presence of F(POG)₁₀ (Figures 1-3a, 1-3b, and 1-3c). The grafting ratio of fluorescein on the labeled (PPG)₁₀ and (POG)₁₀ were calculated to be 70% and 78% by ¹H NMR, respectively (Figures 1-2 and 1-4). The F(PPG)₁₀ and F(POG)₁₀ showed lower fluorescence intensity than FITC at the same concentration (Figures 1-1e and 1-3d). This suggests that concentration quenching occurred due to the triple helix formation of F(PPG)₁₀ or F(POG)₁₀. The CD spectrum of collagen-like triple helices in solution has a unique positive peak at about 220 nm.^[13, 23] As shown in Figures 1-1f and 1-4e, a peak characteristic of the collagen triple helix was observed, but it was less intense than in CMP at the same concentration, suggesting that triple helix formation was partially prevented by fluorescent groups at the ends.

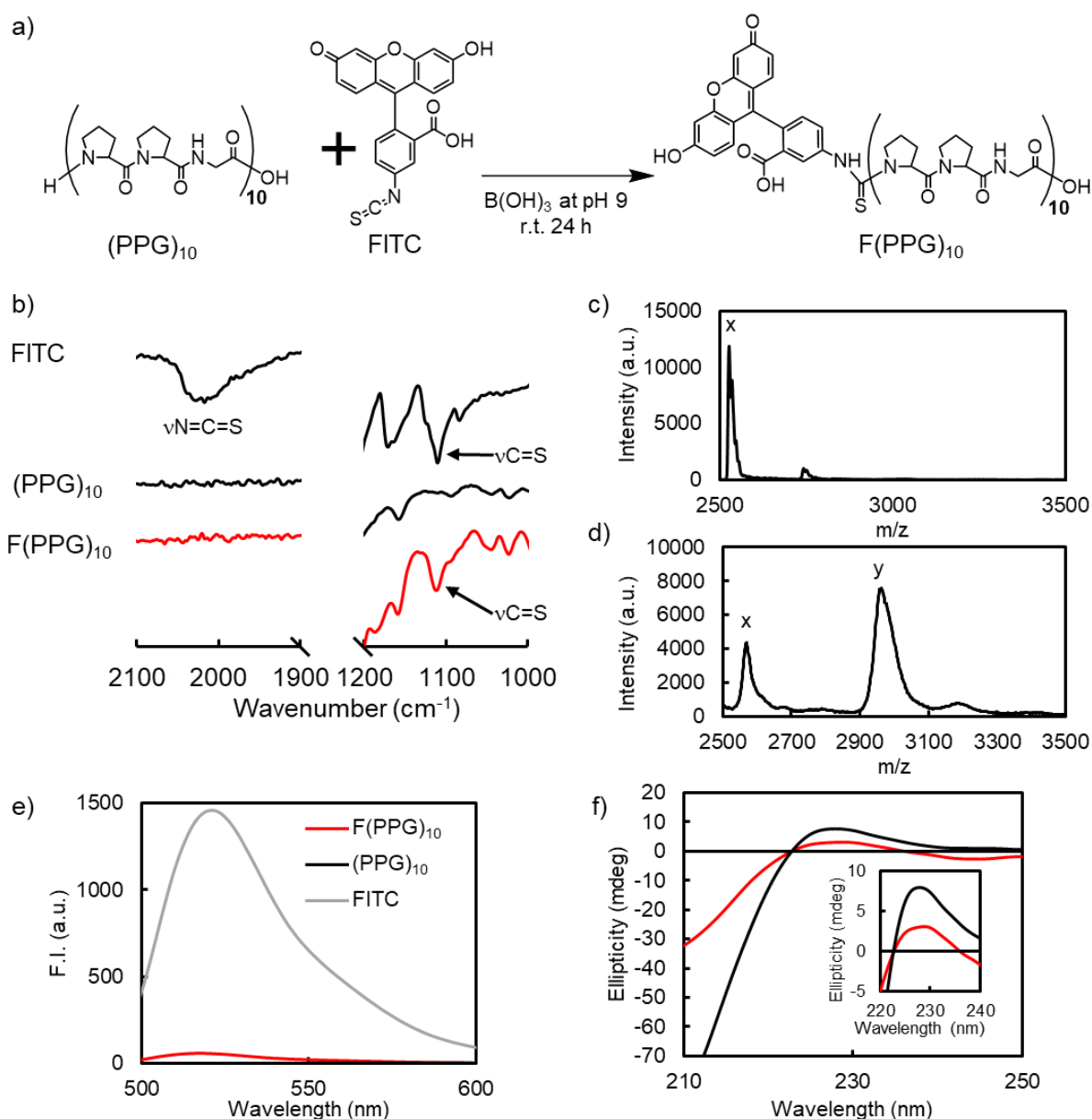


Figure 1-1. Synthesis and functional characterizations of FCMP. a) Synthesis of F(PPG)₁₀. b) FT-IR spectra of FITC, (PPG)₁₀, and F(PPG)₁₀ over the range of 1,000-1,200 cm⁻¹ and 1,900-2,100 cm⁻¹. MALDI-TOF-MASS spectrum of c) (PPG)₁₀ and d) F(PPG)₁₀. Peak x and y were assigned to be (PPG)₁₀ and F(PPG)₁₀, respectively. e) Fluorescence spectra of (PPG)₁₀, FITC, and F(PPG)₁₀. Concentration of each sample is 500 nM in PBS. The excitation wavelength was 492 nm. f) CD spectrum of (PPG)₁₀ and F(PPG)₁₀. Concentration of each sample is 10 μM in PBS.

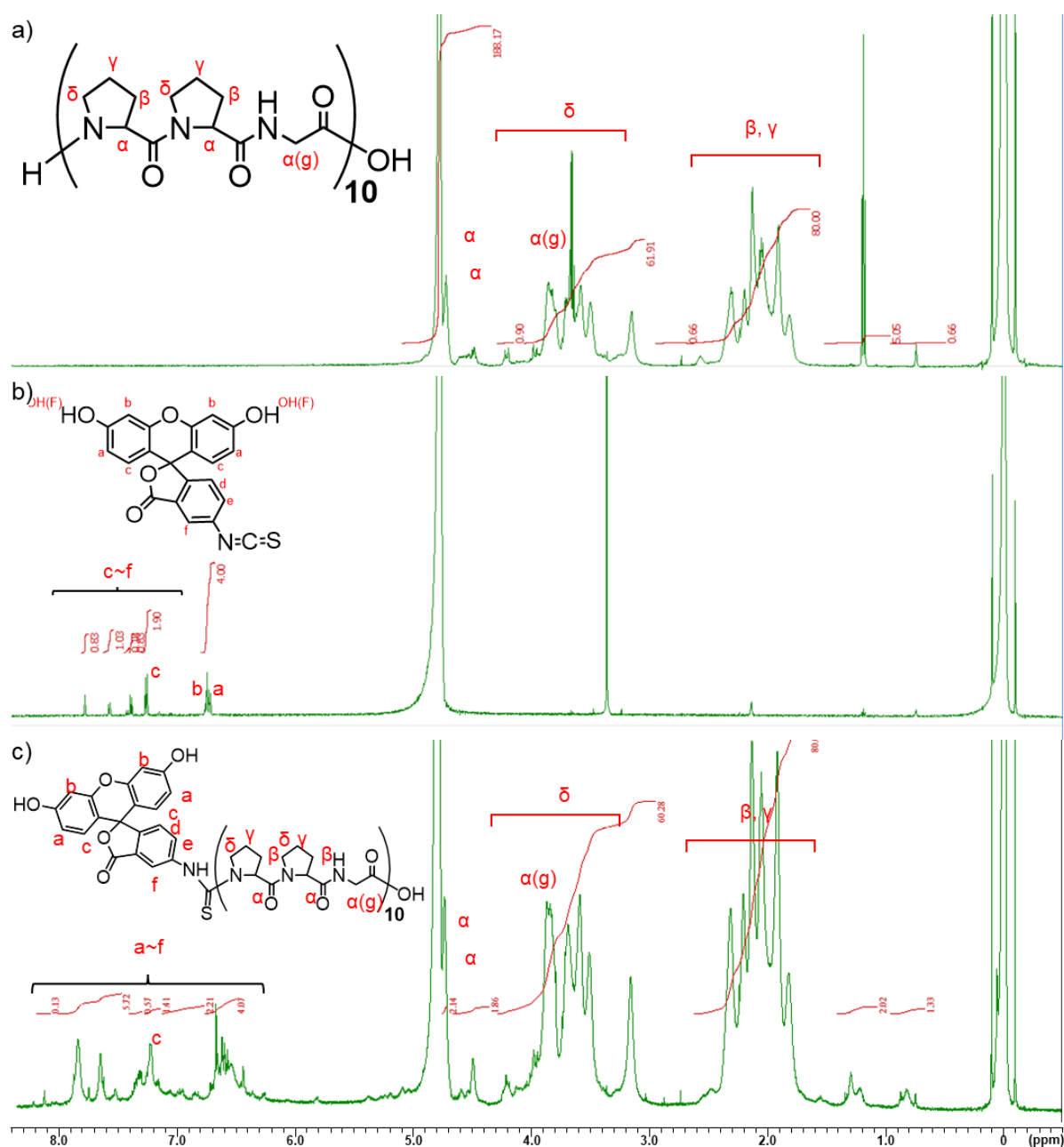


Figure 1-2. ^1H -NMR spectra of (a) (PPG) $_{10}$, (b) FITC and (c) F(PPG) $_{10}$ in D_2O at 25 $^\circ\text{C}$.

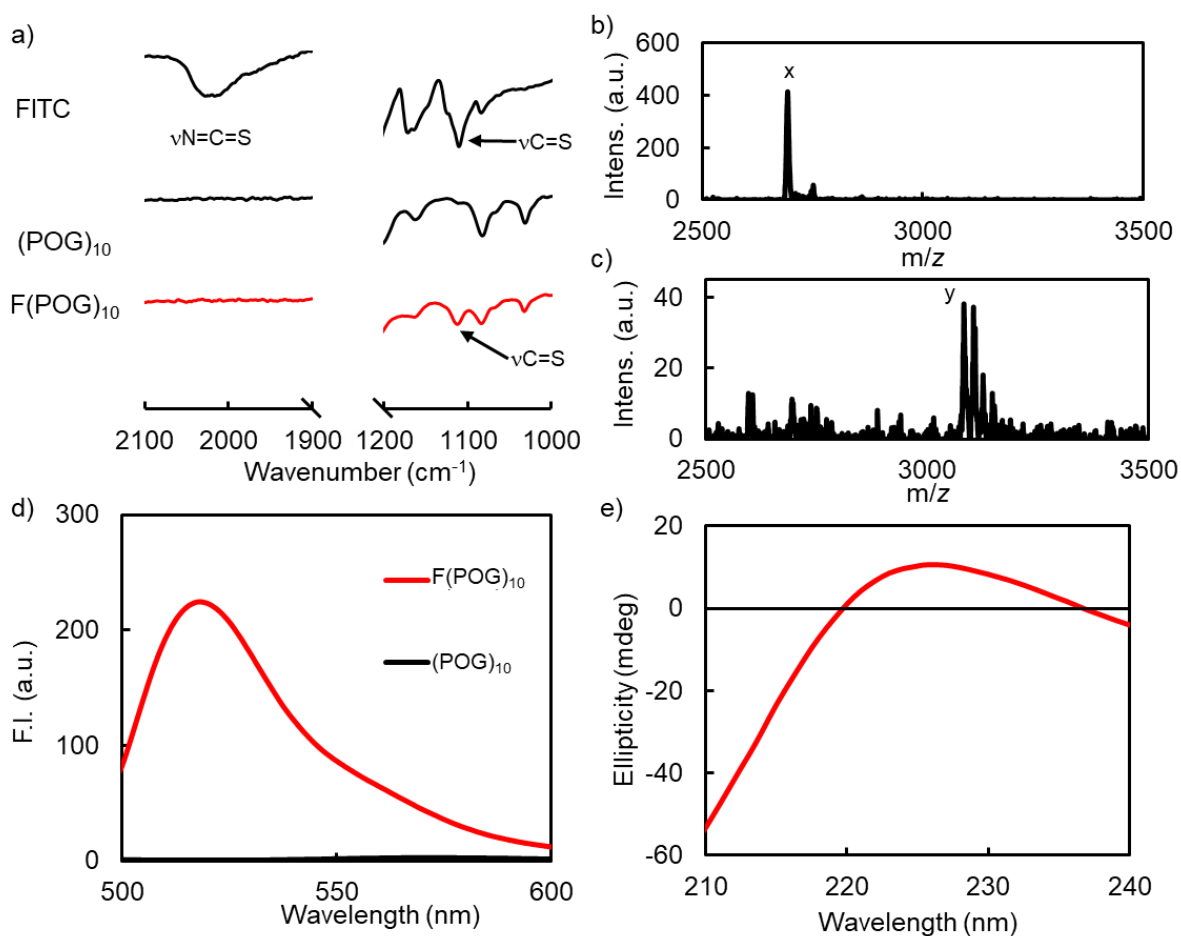


Figure 1-3. Synthesis and functional characterizations of F(POG)₁₀. a) FT-IR spectra of FITC, (POG)₁₀, and F(POG)₁₀ over the range of 1,000-1,200 cm⁻¹ and 1,900-2,100 cm⁻¹. b) MALDI-TOF-MASS spectrum of b) (POG)₁₀ and c) F(POG)₁₀. Peak x and y were assigned to be (POG)₁₀ and F(POG)₁₀, respectively. d) Fluorescence spectra of (POG)₁₀ and F(POG)₁₀. The concentration of each sample is 500 nM in PBS. The excitation wavelength was 492 nm. e) CD spectrum of F(POG)₁₀. The concentration is 10 μM in PBS.

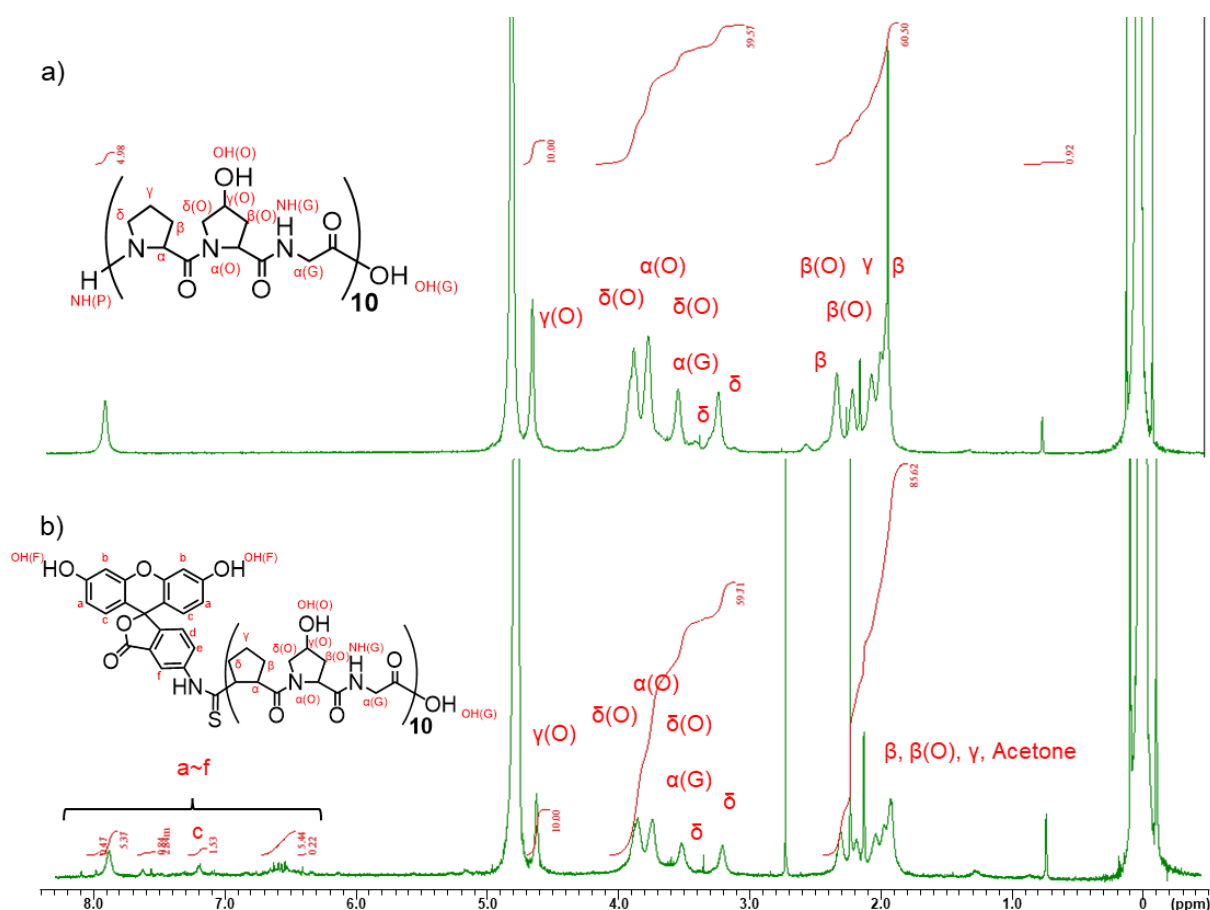


Figure 1-4. ^1H -NMR spectra of (a) $(\text{POG})_{10}$ and (b) $\text{F}(\text{PPG})_{10}$ in D_2O at 25°C .

1.3.2 Functional characterizations of FCMPs

Next, the properties of $\text{F}(\text{PPG})_{10}$ and $\text{F}(\text{POG})_{10}$ as a homo-triple helix were checked (Figure 1-5). $\text{F}(\text{PPG})_{10}$ exhibited a fluorescence with a maximum of 518 nm at 25°C , and after incubation for 10 min at 80°C , the maximum value increased about 5-fold. On the other hand, in fluorescein, the fluorescence did not decrease with increasing temperature from 25°C to 80°C , suggesting that by incubation at 80°C the triple helix and the aggregation of the fluorescent groups dissociated. In addition, when the $\text{F}(\text{PPG})_{10}$ solution was heated at 80°C and then cooled at 4°C for 1 day, the fluorescence intensity decreased markedly, suggesting the reformation of the triple helix.

To confirm the dissociation and formation of the triple helix in this FCMP at different temperatures, CD spectra at 25°C , 80°C and 4°C were measured (Figure 1-5). Positive ellipticity associated with a triple helix was observed at 225 nm at 25°C , but disappeared after

incubation at 80 °C, and recovery of positive ellipticity was observed after cooling at 4 °C. These results indicated that F(PPG)₁₀ and F(POG)₁₀ exhibit an increase or decrease in fluorescence intensity as the triple helix dissociates and forms.

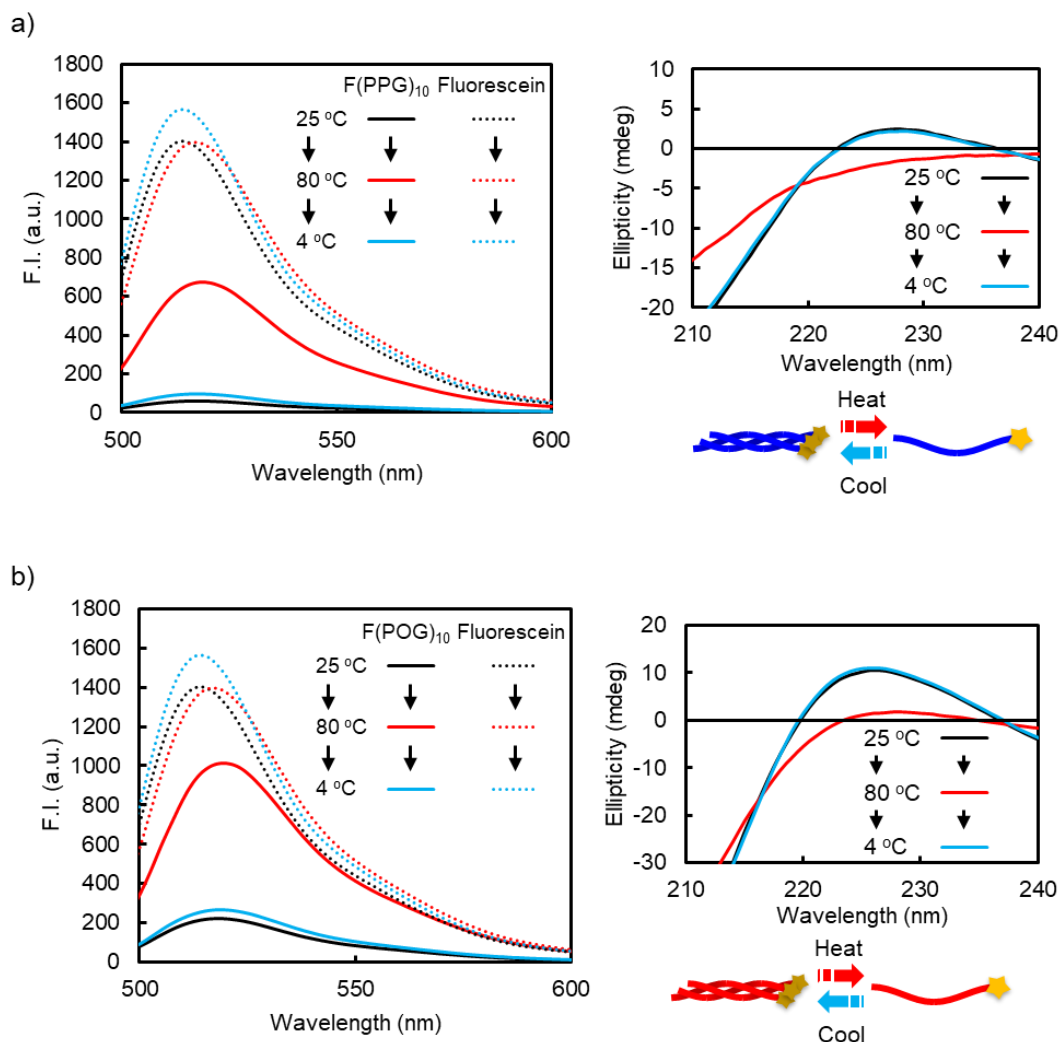


Figure 1-5. Fluorescence characterization of fluorescence-labeled CMPs during homo-triple helix formation. a) Fluorescence and CD profiles at 25 °C, 80 °C, and 4 °C of F(PPG)₁₀ and a schematic illustration of triple helix formation and dissociation. The concentration of each sample is 500 nM in PBS at fluorescence measurement. b) Fluorescence and CD profiles at each temperature of F(POG)₁₀ and a schematic illustration of triple helix formation and dissociation.

1.3.3 Characterization of hetero-triple helices with (PPG)₁₀ and (POG)₁₀

To evaluate the T_m of the hetero-triple helices with (PPG)₁₀ and (POG)₁₀, the author

performed CD spectral measurements at varying temperatures (Figure 1-6a). In the spectra of (PPG)₁₀ and (POG)₁₀ in the mixed state, the T_m of (PPG)₁₀ was observed at 36 °C and that of (POG)₁₀ at 64 °C. On the other hand, the same measurements were performed after heating and subsequently cooling the mixed aqueous solution of (PPG)₁₀ and (POG)₁₀. From this result, the T_m of the hetero-triple helix was calculated to be 59 °C, which is between the T_m of the homo-triple helix of (PPG)₁₀ (36 °C) and that of (POG)₁₀ (64 °C), confirming the change in T_m due to hetero-triple helix formation. It is considered that hetero-triple helix formation adds the stabilizing effect contributed by the (POG)₁₀ chain compared with the (PPG)₁₀ homo-triple helix. Several factors contribute to triple-helix stability, such as stabilization of polyproline-II helices, hydrogen bonding between the glycyl NH and an Xaa carbonyl from a parallel chain, enhanced packing between the Xaa and Yaa side chains, and formation of an energetically favorable side-chain–side-chain association.^[24] Hyp side chain stabilizes the triple helix through the stereoelectronic effect (gauche effect) of its 4-hydroxy group and by restricting the backbone conformation. This stabilization here occurs due to the packing effect. Although a total of four types of triple helices, two homo-triple helices and two hetero-triple helices, were observed by HPLC after heating and cooling processes,^[16, 17] similar homo-triple helices were not observed in this experiment. Thus, the main component should be the hetero-triple helices even there is a possibility of the presence of each homo-triple helix.

The concentration dependence in the triple helix formation was also evaluated by fluorescence measurements (Figures 1-6b-d). The values of CTC for F(PPG)₁₀ and F(POG)₁₀ were calculated to be 320 μM and 4 μM, respectively. On the other hand, the CTC was calculated to be 7 μM for the mixture, indicating that mixing F(PPG)₁₀ and F(POG)₁₀ changed the CTC. It was also indicated that the triple helix formation concentration of the heterotrimer prepared under 1:2 mixing conditions of (PPG)₁₀ and (POG)₁₀ is almost half that of [(POG)₁₀]₃ but 45 times higher than [(PPG)₁₀]₃. This result suggested that concentrations above 7 μM are required for hetero-triple helix formation under these mixed conditions.

The distance and size of the helices were evaluated by XRD and dynamic light scattering (DLS). For (PPG)₁₀ and (POG)₁₀, a strong peak was observed at approximately 8 degrees (Figure 1-6e). This is reported to be very similar to the peak derived from the distance between triple helices in the hexagonal arrangement of collagen triple helices.^[25] On the other hand, a similar peak of about 8 degrees was observed for the (PPG)₁₀ and (POG)₁₀ hetero-triple helix. These results confirmed a triple helix arrangement similar to (PPG)₁₀ and (POG)₁₀ in the hetero-

triple helix condition. As shown in Figure 1-6f, the particle size of the hetero-triple helix decreased at 80 °C and subsequently returned to the same size as before heating after cooling at 4 °C. The particle size was similar to that of (PPG)₁₀ and (POG)₁₀ (Figure 1-7), suggesting that the hetero-triple helix undergoes a size change similar to that of the homo-triple helix.

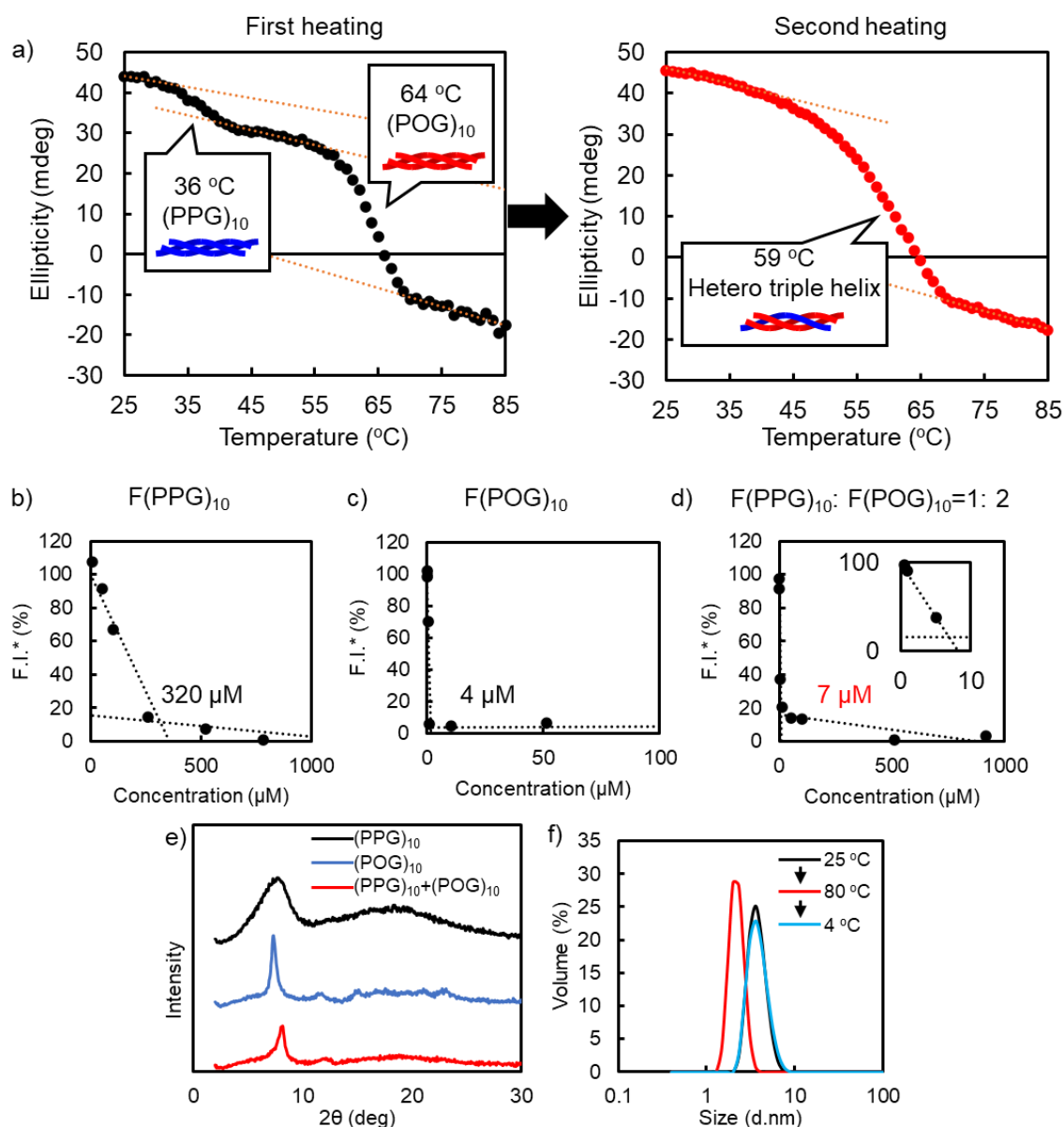


Figure 1-6. Characterization of hetero-triple helices with (PPG)₁₀ and (POG)₁₀. a) Melting profiles of (PPG)₁₀ and (POG)₁₀ mixture ((PPG)₁₀:(POG)₁₀=1:2) and (PPG)₁₀ and (POG)₁₀ mixture after annealing obtained by CD measurement at 225 nm. CTC values of b) (PPG)₁₀, c) (POG)₁₀, and d) (PPG)₁₀ and (POG)₁₀ mixture after annealing calculated by fluorescence measurement. e) XRD profiles of (PPG)₁₀, (POG)₁₀, and (PPG)₁₀ and (POG)₁₀ mixture. f) DLS profiles at 25 °C, 80 °C and 4 °C of (PPG)₁₀ and (POG)₁₀ mixture.

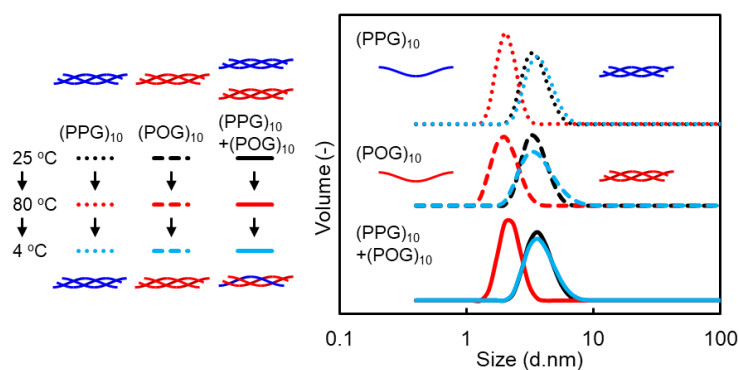


Figure 1-7. DLS profiles at 25 °C, 80 °C, and 4 °C of (PPG)₁₀, (POG)₁₀, and (PPG)₁₀:(POG)₁₀=1:2 mixture.

1.3.4 The concentration and temperature dependence of the triple helix formation

To evaluate the concentration dependence of CMPs in the hetero-triple helix, fluorescence spectra were measured by varying the mixing ratio of fluorescent unmodified CMPs to fluorescent modified CMPs at a fixed concentration (Figure 1-8a). In the case of the combination of F(PPG)₁₀ and (POG)₁₀, a plateau was reached in regions where the ratio of (POG)₁₀ to F(PPG)₁₀ was greater than 2. This result suggested that aggregation of the fluorescent groups at the ends of the CMP had not happened, possibly because F(PPG)₁₀ and (POG)₁₀ formed a 1:2 hetero-triple helix. Even in the case of homo-triple helix combinations such as F(PPG)₁₀-(PPG)₁₀ and F(POG)₁₀-(POG)₁₀, the fluorescence intensity reached nearly 100% at mixing ratios of 3 or more (Figure 1-8b). On the other hand, when (PPG)₁₀ was added to F(POG)₁₀, the ratio increased to 4 and did not reach a plateau. This suggested aggregation of terminal fluorescent groups in the combination of F(POG)₁₀ and (PPG)₁₀, which was due to the formation of a hetero-triple helix with F(POG)₁₀:(PPG)₁₀=2:1, although there is a possibility of the presence of homo-triple helix F(POG)₁₀. It was reported that folding of homo-(POG)₁₀ occurs with a kinetics faster than that of homo-(PPG)₁₀.^[17] As shown in Figure 1-8a, although an increase in the amount of additional (PPG)₁₀ would be expected to raise the population of the (PPG)₁₀:F(POG)₁₀=2:1 triple helix, the 1:2 triple helix is considered to preferentially form at the amount of (PPG)₁₀ used in this study. These results suggest that the hetero-triple helix formation changed due to the difference in the mixing ratio of (PPG)₁₀ and (POG)₁₀.

Next, temperature-dependent hetero-triple helix formation during triple helix formation was evaluated (Figures 1-8c and 1-8d). As shown in Figure 1-8c, the triple helix formation at 4 °C was about 5%, 38%, and 77% for F(POG)₁₀ alone, F(POG)₁₀:(PPG)₁₀=1:2, and F(POG)₁₀:(POG)₁₀=1:2, respectively. These results indicated that F(POG)₁₀ alone caused aggregation of terminal fluorescent groups by homo-triple helix formation, while the presence of (PPG)₁₀ or (POG)₁₀ inhibited the aggregation of terminal fluorescent groups by triple helix formation between F(POG)₁₀ and the respective CMPs. On the other hand, when triple helices were formed at 37 °C, F(POG)₁₀ alone, F(POG)₁₀:(PPG)₁₀=1:2 and F(POG)₁₀:(POG)₁₀=1:2 resulted in about 6%, 27%, and 66%, respectively. Even though the *T_m* of (PPG)₁₀ was lower than 37 °C, the ratio of fluorescence intensity increases compared to F(POG)₁₀ alone, suggesting hetero-triple helix formation between (PPG)₁₀ and (POG)₁₀ at 37 °C. When the samples were kept at 37 °C for 5 days, there was no significant change in the percentage of fluorescence intensity, confirming that the formed triple helices were stable for 5 days.

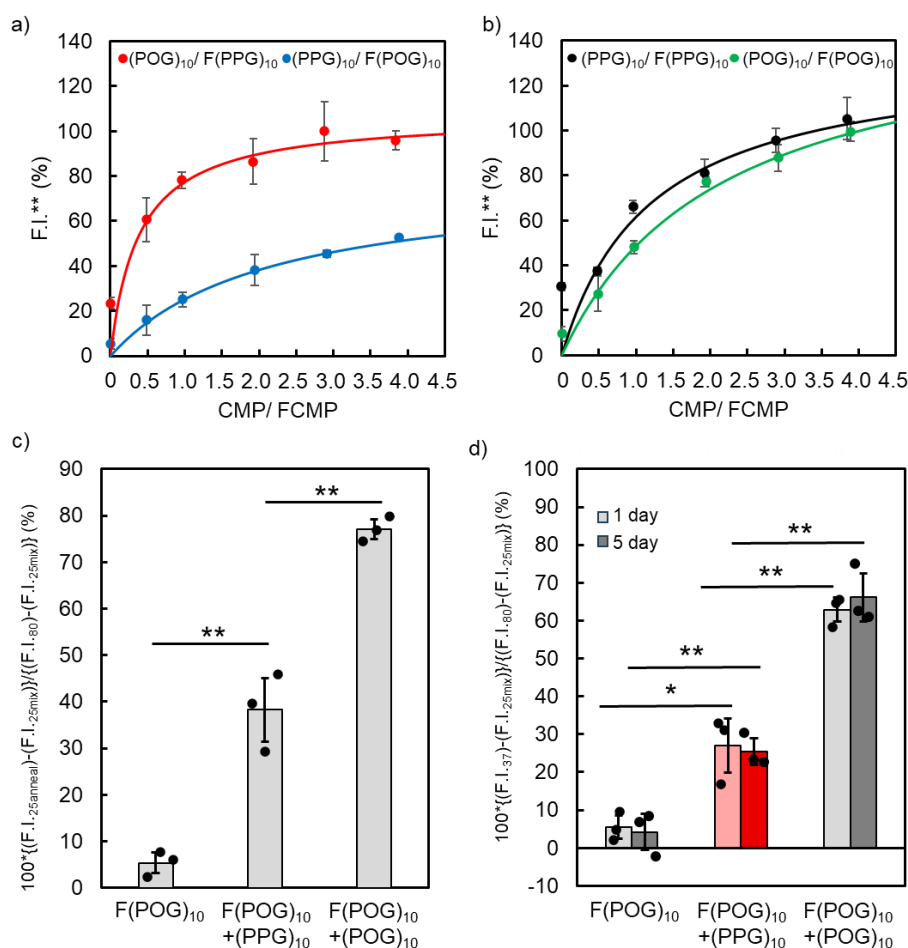


Figure 1-8. Composition of hetero-triple helix structures and their stabilities. a) Hetero-triple helix forming properties of (PPG)₁₀ and (POG)₁₀ mixture and b) homo-triple helix forming properties of (PPG)₁₀ and (POG)₁₀ after annealing at varying mixing ratios calculated by fluorescence measurement (n=3). F.I.** (%) is calculated by the following equation. F.I.**=

$100 * \{(F.I._{25\text{anneal}}) - (F.I._{25\text{mix}})\} / \{(F.I._{80}) - (F.I._{25\text{mix}})\}$. $F.I._{25\text{anneal}}$ is fluorescence intensity at 25 °C after annealing. $F.I._{25\text{mix}}$ is fluorescence intensity at 25 °C before annealing. $F.I._{80}$ is fluorescence intensity at 15 min at 80 °C. Hetero-triple helix forming properties of (PPG)₁₀ and (POG)₁₀ mixture at different cooling temperatures of c) 4 °C (n=3, ** p <0.01) and d) 37 °C (n=3, * p <0.05, ** p <0.01) calculated by fluorescence measurement. $F.I._{37}$ is fluorescence intensity after cooling at 37 °C.

1.3.5 Evaluation of time dependence of triple helix formation at 37 °C

As shown in Figure 1-9, the time dependence of triple helix formation at 4 °C and 37 °C were evaluated by measuring fluorescence spectra. At 37 °C, F(PPG)₁₀ did not show a significant decrease in the fluorescence intensity ratio within 30 min, while F(POG)₁₀ showed a sharp decrease within 10 min. On the other hand, the mixture of F(PPG)₁₀:F(POG)₁₀=1:2 showed a rapid decrease by 10 min, like the behavior of F(POG)₁₀. This result confirmed that there is no significant difference in the formation rate of the (POG)₁₀ homo-triple helix and the (PPG)₁₀ and (POG)₁₀ hetero-triple helix at 37 °C. It is thought that this is because the main driving force for triple helix formation at 37 °C is due to (POG)₁₀. In addition, the temperature and time dependence of triple helix formation was measured by fluorescence during the process of descending temperature, suggesting that F(POG)₁₀ forms triple helices more quickly than the mixture of F(PPG)₁₀ and F(POG)₁₀ (Figure 1-10).

Finally, the properties of the triple helix at (PPG)₁₀, (POG)₁₀, and (PPG)₁₀:(POG)₁₀=1:2 are listed in Table 1. For all entries in the table, the (PPG)₁₀:(POG)₁₀=1:2 condition showed values between (PPG)₁₀ and (POG)₁₀ and close to (POG)₁₀.

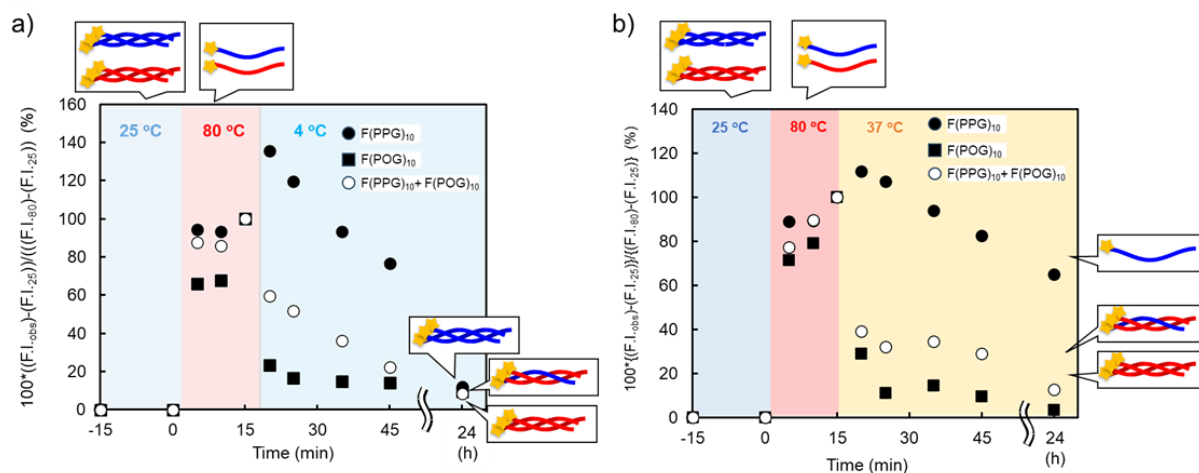


Figure 1-9. Temperature and time-dependent increase and decrease of fluorescence intensity of

F(PPG)₁₀, F(POG)₁₀, and a mixture of F(PPG)₁₀ and F(POG)₁₀. The samples were kept at (a) 4 °C or (b) 37 °C after they were incubated at 80 °C. The mixing ratio of F(PPG)₁₀ to F(POG)₁₀ was 1:2. F.I._{obs} is fluorescence intensity at the time. F.I.₂₅ is fluorescence intensity. at 25 °C. F.I.₈₀ is fluorescence intensity at 15 min at 80 °C.

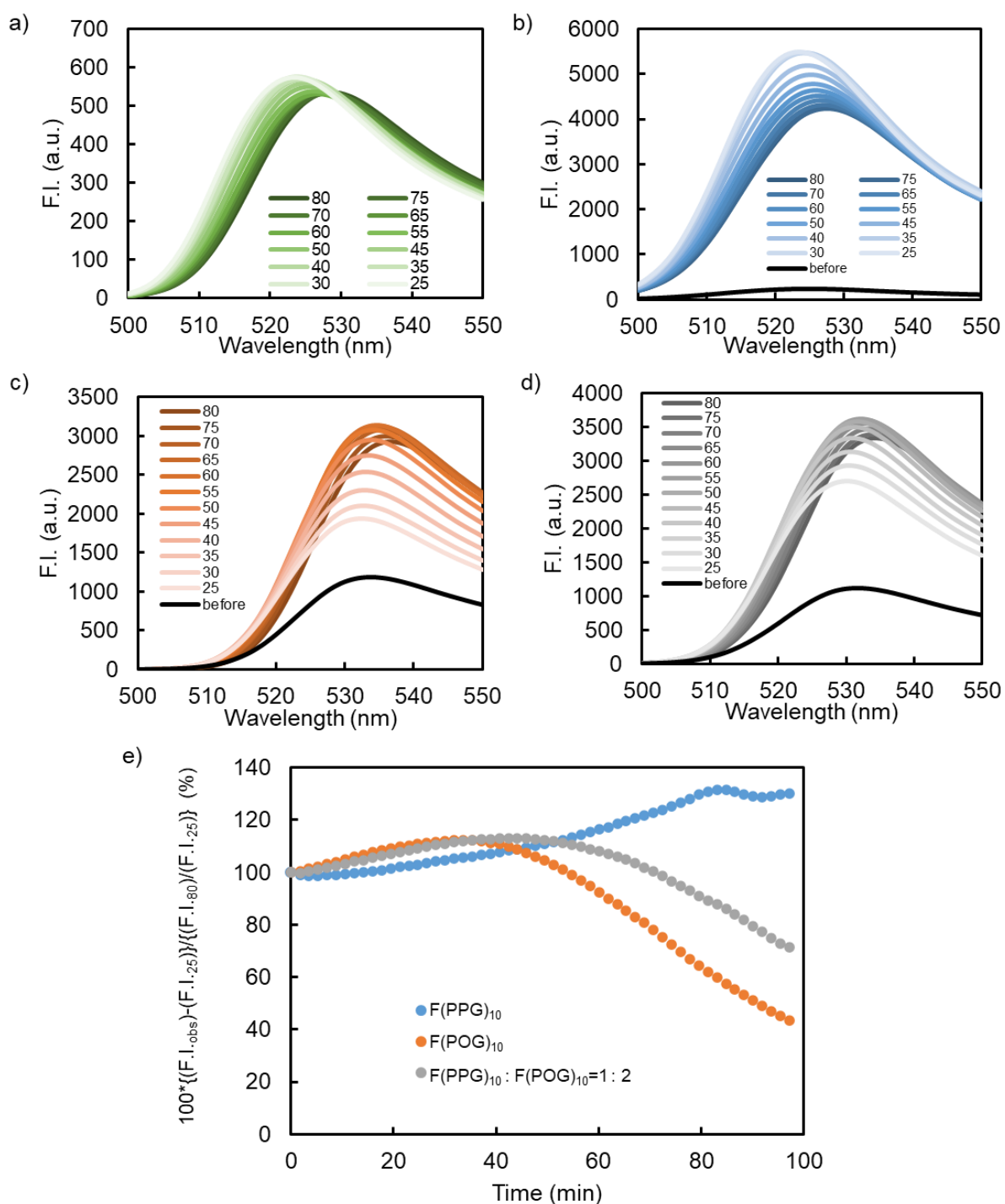


Figure 1-10. Temperature-dependent change of fluorescent intensity of fluorescein and FCMPs at the condition of gradual temperature decrease at 1.0 °C min⁻¹ from 80 to 25 °C. Fluorescent spectra of a) fluorescein, b) F(PPG)₁₀, c) F(POG)₁₀, and d) a mixture of F(PPG)₁₀ and F(POG)₁₀

in PBS at 100 μ M. e) Time-dependent fluorescent intensity change of the peak top of each sample at each temperature. F.I._{obs} is fluorescence intensity at the time. F.I.₂₅ is fluorescence intensity at 25 °C before annealing. F.I.₈₀ is fluorescence intensity at 80 °C.

Table 1. CTC values, melting points (T_m), and 50%F.I. times ($t_{50\%}$) of (PPG)₁₀, (POG)₁₀, and (PPG)₁₀ and (POG)₁₀ mixture.

	(PPG) ₁₀	(POG) ₁₀	(PPG) ₁₀ +(POG) ₁₀
CTC (μM)	320	4	7
T_m (°C)	36	64	59
$t_{50\%}$ at 37 °C (min)	> 1440	2.6	3.5
$t_{50\%}$ at 4 °C (min)	77	2.0	10

1.4 Conclusion

In chapter 1, the author evaluated the triple helix formation of two collagen model peptides, (PPG)₁₀ and (POG)₁₀, by fluorescence and CD spectroscopic measurements for the single and mixed conditions. This result was consistent with the trend inferred from the ratio of (PPG)₁₀ to (POG)₁₀ chains in the hetero-triple helix suggested by the fluorescence spectroscopy measurements. In addition, even when the temperature during triple helix formation was set to 37 °C, which is above the T_m of (PPG)₁₀, the formation of the hetero-triple helix was suggested, indicating that (POG)₁₀ might play a major role in the formation of the hetero-triple helix at 37 °C. These results confirm that heterotrimers have different association properties from homotrimers and are expected to be applied in nanotechnology and biomaterials as new self-assembled materials.

1.5 References

- [1] Y. Mai, Z. An, S. Liu, *Macromol. Rapid Commun.* **2022**, *43*, 2200481.
- [2] Q. Lyu, M. Li, L. Zhang, J. Zhu, *Macromol. Rapid Commun.* **2022**, *43*, 2100867.
- [3] M. Xu, Z. Xu, M. A. Soto, Y.-T. Xu, W. Y. Hamad, M. J. MacLachlan, *Adv. Mater.* **2023**, *35*, 2301060.
- [4] W. Zhang, X. Lyu, L. Zhang, W. Wang, Q. Shen, S. Lu, L. Lu, M. Zhan, X. Hu, *Macromol. Rapid Commun.* **2022**, *43*, 2100918.

- [5] N. J. Sinha, M. G. Langenstein, D. J. Pochan, C. J. Kloxin, J. G. Saven, *Chem. Rev.* **2021**, *121*, 13915.
- [6] C. Kojima, S. Tsumura, A. Harada, K. Kono, *J. Am. Chem. Soc.* **2009**, *131*, 6052.
- [7] J. Hwang, H. Huang, M. O. Sullivan, K. L. Kiick, *Mol. Pharmaceutics* **2023**, *20*, 1696.
- [8] C. M. R. Pérez, L. A. Rank, J. Chmielewski, *Chem. Commun.* **2014**, *50*, 8174.
- [9] A. Y. Wang, X. Mo, C. S. Chen, S. M. Yu, *J. Am. Chem. Soc.* **2005**, *127*, 4130.
- [10] A. Y. Wang, C. A. Foss, S. Leong, X. Mo, M. G. Pomper, S. M. Yu, *Biomacromolecules* **2008**, *9*, 1755.
- [11] M. Matsusaki, R. Amekawa, M. Matsumoto, Y. Tanaka, A. Kubota, K. Nishida, M. Akashi, *Adv. Mater.* **2011**, *23*, 2957.
- [12] A. D. Merg, G. Touponse, E. van Genderen, T. B. Blum, X. Zuo, A. Bazrafshan, H. M. H. Siaw, A. McCanna, R. Brian Dyer, K. Salaita, J. P. Abrahams, V. P. Conticello, *J. Am. Chem. Soc.* **2020**, *142*, 19956.
- [13] T. Koga, S. Kingetsu, N. Higashi, *Int. J. Mol. Sci.* **2021**, *22*, 4533.
- [14] S. Fuji, K. Tanaka, S. Kishikawa, S. Morita, M. Doi, *J. Biosci. Bioeng.* **2022**, *133*, 168.
- [15] N. Subrahmanyam, B. Yathavan, J. Kessler, S. M. Yu, H. Ghandehari, *J. Control. Release* **2023**, *353*, 278.
- [16] D. A. Slatyer, C. A. Miles, A. J. Bailey, *J. Mol. Biol.* **2003**, *329*, 175.
- [17] R. Berisio, V. Granata, L. Vitagliano, A. Zagari, *Biopolymers* **2004**, *73*, 682.
- [18] U. Sharma, L. Carrique, S. Vadon-Le Goff, N. Mariano, R.-N. Georges, F. Delolme, P. Koivunen, J. Myllyharju, C. Moali, N. Aghajari, D. J. S. Hulmes, *Nat. Commun.* **2017**, *8*, 14671.
- [19] V. Gauba, J. D. Hartgerink, *J. Am. Chem. Soc.* **2007**, *129*, 2683.
- [20] Y. Li, X. Mo, D. Kim, S. M. Yu, *Biopolymers* **2011**, *95*, 94.
- [21] X. Sun, J. Fan, X. Li, S. Zhang, X. Liu, J. Xiao, *Chem. Commun.* **2016**, *52*, 3107.
- [22] X.-X. Sun, J. Fan, Y.-N. Hou, S. Liang, Y.-P. Zhang, J.-X. Xiao, *Chin. Chem. Lett.* **2017**, *28*, 963.
- [23] S. Sakakibara, Y. Kishida, K. Okuyama, N. Tanaka, T. Ashida, M. Kakudo, *J. Mol. Biol.* **1972**, *65*, 371.
- [24] V. Kubyshkin, *Org. Biomol. Chem.* **2019**, *17*, 8031.
- [25] L. Ruiz-Rodriguez, P. Loche, L. T. Hansen, R. R. Netz, P. Fratzl, E. Schneck, K. G. Blank, L. Bertinetti, *MRS Bull.* **2021**, *46*, 889.

Chapter 2

Analysis of the Assembly Properties and Control of Sol–Gel Transition via Homo- and Hetero-Triple Helix Formation of Collagen Model Peptide-Modified 4arm Poly(ethylene glycol)

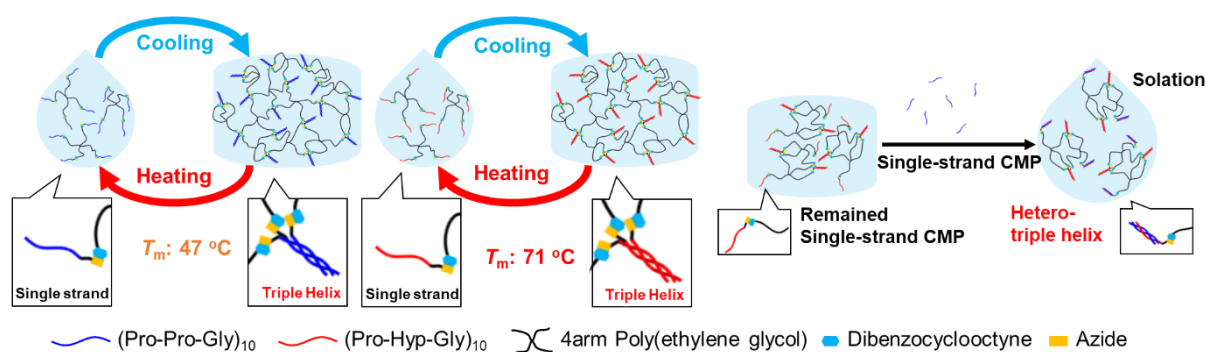
2.1 Introduction

In Chapter 1, the author evaluated the homo- and hetero- triple helix formation of two collagen model peptides, (PPG)₁₀ and (POG)₁₀. In this chapter, toward the further application for cell assembling method with crosslinking characteristics driven by triple helix formation, the assembly and control of material properties through homo- and hetero-triple helices formation of CMP were analyzed using CMP-Modified 4arm Poly(ethylene glycol) via sol-gel transition.

Hydrogels are widely used materials for medical applications in the field of tissue engineering, and many variants have been used, from synthetic polymers to biopolymers such as hyaluronic acid, fibrin, and collagen. Various interactions, such as covalent, ionic, and non-covalent bonds, have been studied as driving forces for the formation of hydrogels, and non-covalent physical approaches are expected to be more useful for tissue engineering compared to covalent chemical approaches because they can maintain the activity of encapsulated biomolecules.^[1] Among the non-covalent bonding approaches, CMPs have attracted attention as a supramolecular self-assembly moiety for a non-covalent physical approach. There are some reports about thermo-responsive gelations using CMP as a cross-linking site.^[2, 3] Although CMPs have such unique properties for gel construction, there are few reports about the analysis of CMP hydrogels by homo- and hetero triple helix formation, and few studies have clarified the effect of additional CMP strands using homo and hetero triple helix formation.

Here, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm PEG-(PPG)₁₀ and 4arm PEG-(POG)₁₀, and the effect of additional CMP strands. First, the author synthesized CMPs grafted onto 4arm-PEG; 4arm-PEG10k-triazole-(Ethylene glycol)4(PPG)₁₀ (4arm-PEG10k-triazole-EG4(PPG)₁₀) and 4arm-PEG10k-triazole-EG4(POG)₁₀, end-terminal CMP conjugated polymers, and analyzed their sol-gel

transition properties by the self-assembling of homo and hetero triple helices. The 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ showed gel-sol transition by the triple helix formation and dissociation, but these gels changed to a liquid state following incubation at 50 °C and 80 °C, respectively, due to their triple helix stability. Furthermore, the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with the single-strand (PPG)₁₀ solution gradually changed to a liquid state at 37 °C for 1 day. These findings suggest the hydrogel with crosslinking properties driven by triple-helix formation of CMP is controlled by CMP hetero triple helix formation using single-strand CMPs and amino acid sequence variation. This phenomenon is expected to be applied in biomaterials for the development of new self-assembled materials and to further understanding of triple helix formation-driven sol-gel transition.



Scheme 2-1. A schematic illustration of triple-helix crosslinked hydrogels with sol-gel transition in response to CMPs.

2.2 Experiments

2.2.1 Materials

The (PPG)₁₀ (Ref. 4006) and (POG)₁₀ (Ref. 4033) were purchased from Peptide Institute, Inc. (Osaka, Japan). AzidoPEG4NHS (Ref. A2388) was purchased from Tokyo Chemical Industry (Tokyo, Japan). DBCONHCOPEG4NHS (Ref. BP-23726) was purchased from BroadPharm (San Diego, USA). Dimethyl sulfoxide (DMSO) (Ref. 043-07216), 5 M hydrochloric acid (Ref. 081-05435), sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄, and 5 M sodium hydroxide (Ref. 196-05375) were purchased from Wako Chemicals (Osaka, Japan). D-PBS without Ca and Mg, powder (Ref. 07269-84) and sodium hydrogen carbonate (NaHCO₃) (Ref. 31213-15) were purchased from Nacalai Tesque (Kyoto, Japan). Acetone (Ref. 010-00306) was purchased from Kishida Chemical (Osaka, Japan). Deuterium oxide (Ref. 151882) was purchased from Sigma-Aldrich (St. Louis, USA). 4Arm PEG-DBCO, MW 10k (Ref. PSB-4073) was purchased from Creative PEGWorks (Chapel Hill, USA). All chemicals were used without further purification.

2.2.2 Synthesis and functional characterizations of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀

The (PPG)₁₀ (20 mg, 7.9 μmol) was dissolved in 5 mL of 50 mM sodium bicarbonate (pH 8.5) and preincubated at 60 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 15.84 μmol of AzidoPEG4NHS or DBCOEG4NHS in 0.5 mL DMSO was added and kept at room temperature for 1 day. After the reaction, the whole reaction mixture was dialyzed (MWCO: 1000 Da) for 3 days with water, methanol, and water. The resulting solution was freeze-dried. The AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ were characterized by CD, UV-vis, MALDI-TOF Mass, and ¹H-NMR measurements. The CD, UV-vis, MALDI-TOF Mass, and ¹H-NMR spectra were measured by a CD spectrometer (JASCO, J-1100, Tokyo, Japan), a UV-Vis Spectrophotometer (JASCO, V-670, Tokyo, Japan), a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer (JEOL, JMS-S3000, Tokyo, Japan), and a Nuclear Magnetic Resonance Spectrometer (Bruker, AVANCE III 600, Massachusetts, USA), respectively.

2.2.3 Synthesis of 4armPEG10k-triazole-EG4(PPG)₁₀

The AzidoEG4(PPG)₁₀ (5 mg, 1.78 μ mol) was dissolved in 2.5 mL of PBS (pH 7.4) and preincubated at 60 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 4.76 mg (0.446 μ mol) of 4armPEG10k-DBCO in 2.5 mL PBS was added to the solution immediately, and then the reaction mixture was wrapped in foil and stirred for 24 h at 37 °C in an incubator. After the reaction, the whole reaction mixture was dialyzed (MWCO: 3500 Da) for 3 days with water at 37 °C. The resulting solution was freeze-dried. The structure of the 4armPEG10k-triazole-EG4(PPG)₁₀ was analyzed by ¹H-NMR. As a control of 4armPEG10k-triazole-EG4(PPG)₁₀ for further experiments, (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ was also synthesized and characterized. Solutions of 667 μ M AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ in PBS buffer were prepared. After they were mixed at a ratio of 1:1, the reaction progress was traced by UV-vis and CD measurements for 24 h at room temperature. The reaction solution was kept at 4 °C during the click reaction. The reaction product was characterized by MALDI-TOF Mass and CD measurement. The melting temperature (T_m) was calculated using the thermal denaturation analysis program of the machine. The solutions were incubated at 25 °C, heated to 80 °C for 10 min, and re-equilibrated at 4 °C for 1 day. This annealing was repeated twice. CD spectra were also measured at each point.

2.2.4 Sol-gel transition analysis of 4armPEG10k-triazole-EG4(PPG)₁₀

The solution of 4armPEG10k-triazole-EG4(PPG)₁₀ was prepared in PBS buffer at a concentration of 50 mg/mL. After the polymer was dissolved, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1 day. After that, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1 further day. This annealing was repeated twice. A tipping test confirmed the shape at each step. The CD profiles at each point were also measured by the solution of 4armPEG10k-triazole-EG4(PPG)₁₀ in PBS. The stock solution's concentration was 3.2 mg/mL. CD measurements were carried out with diluted samples.

The solution of 4armPEG10k-triazole-EG4(PPG)₁₀ was prepared in PBS buffer at a concentration of 50 mg/mL. After the polymer was dissolved, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1 day. After that, the CD denaturation curve measurement was taken. The denaturation condition was 1 °C/min from 20 °C to 90 °C. Moreover, the 50 mg/mL and 100 mg/mL 4armPEG10k-triazole-EG4(PPG)₁₀ in PBS were prepared. After the polymer was dissolved, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1

day. After that, the rheology test was carried out with 2% strain and 10 Hz frequency using a Modular Compact Rheometer (MCR 302, Anton Paar). Temperature was kept at 20 °C during the measurement.

2.2.5 Synthesis of 4armPEG10k-triazole-EG4(POG)₁₀

The (POG)₁₀ (21.3 mg, 7.9 μmol) was dissolved in 5 mL of 50 mM sodium bicarbonate (pH 8.5) and preincubated at 60 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 15.84 μmol of AzidoPEG4NHS in 1 mL DMSO was added and kept at room temperature for 1 day. After the reaction, the whole reaction mixture was dialyzed (MWCO: 1000 Da) for 3 days with water, methanol, and water. The resulting solution was freeze-dried. The AzidoEG4(POG)₁₀ (5 mg, 1.69 μmol) was dissolved in 2.5 mL of PBS (pH 7.4) and preincubated at 80 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 4.5 mg (0.422 μmol) of 4armPEG10k-DBCO in 2.5 mL PBS was added to the solution immediately, and then the reaction mixture was wrapped in foil and stirred for 24 h at 37 °C in an incubator. After the reaction, the whole reaction mixture was dialyzed (MWCO: 3500 Da) for 3 days with water at 37 °C. The resulting solution was freeze-dried. The structure of the 4armPEG10k-triazole-EG4(POG)₁₀ was analyzed by ¹H-NMR.

2.2.6 Evaluation of sol-gel transition property in response to CMP's amino acid sequences

The solutions of 4armPEG10k-triazole-EG4(PPG)₁₀ and 4armPEG10k-triazole-EG4(POG)₁₀ were prepared in PBS buffer at concentrations of 50 mg/mL. Then, the 4armPEG10k-triazole-EG4(POG)₁₀, and the mixture of 4armPEG10k-triazole-EG4(PPG)₁₀ and 4armPEG10k-triazole-EG4(POG)₁₀ (1:1) were prepared. After the polymer was dissolved, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1 day. After that, the solution was heated at 80 °C for 10 min and cooled at 4 °C for 1 further day. This annealing was repeated twice. A tipping test confirmed the shape at each step.

The solutions of 4armPEG10k-triazole-EG4(PPG)₁₀ and 4armPEG10k-triazole-EG4(POG)₁₀ were prepared in PBS buffer. Then, the 4armPEG10k-triazole-EG4(POG)₁₀, and the mixture of 4armPEG10k-triazole-EG4(PPG)₁₀ and 4armPEG10k-triazole-EG4(POG)₁₀ (1:1) were prepared. After the polymer was dissolved, the solution was heated at 80 °C for 10

min and cooled at 4 °C for 1 day. After that, the CD denaturation curve measurement was taken. The denaturation condition was 1 °C/min from 20 °C to 90 °C. The measurements were carried out with the diluted samples.

2.2.7 Sol-gel transition analysis of 4armPEG10k-triazole-EG4CMPs with CMPs

The solutions of 4armPEG10k-triazole-EG4(PPG)₁₀ and 4armPEG10k-triazole-EG4(POG)₁₀ were prepared in PBS buffer at concentrations of 100 mg/mL. Then, the solution of (PPG)₁₀ or (POG)₁₀ was also prepared by PBS buffer. The concentrations of CMPs were 9.27 mM (0.5 eq.) and 18.54 mM (1 eq.). After the 4armPEG10k-triazole-EG4(PPG)₁₀ or 4armPEG10k-triazole-EG4(POG)₁₀ were dissolved, the solutions were heated at 90 °C for 15 min and cooled at 4 °C overnight. After the samples formed a gel, the same volume (50 µL) of the 0.5 eq. (PPG)₁₀, 0.5 eq. (POG)₁₀, or PBS was added. The 1 eq. (PPG)₁₀ and (POG)₁₀ were also added only the 4armPEG10k-triazole-EG4(PPG)₁₀ gels. The sample entries are as follows: (4armPEG10k-triazole-EG4(PPG)₁₀+0.5 eq. (PPG)₁₀, 4armPEG10k-triazole-EG4(PPG)₁₀+0.5 eq. (POG)₁₀, 4armPEG10k-triazole-EG4(PPG)₁₀+PBS, 4armPEG10k-triazole-EG4(PPG)₁₀+1 eq. (PPG)₁₀, 4armPEG10k-triazole-EG4(PPG)₁₀+1 eq. (POG)₁₀, 4armPEG10k-triazole-EG4(POG)₁₀+0.5 eq. (PPG)₁₀, 4armPEG10k-triazole-EG4(POG)₁₀+0.5 eq. (POG)₁₀, and 4armPEG10k-triazole-EG4(POG)₁₀+PBS). After that, the samples were heated at 90 °C for 15 min and cooled at 4 °C overnight. Then, the samples were heated at 37 °C for 15 min, and at 50 °C for 15 min, and at 80 °C for 15 min. A tipping test confirmed the shape at each step.

2.2.8 Effect of the additional single strand CMPs on 4armPEG10k-triazole-EG4CMPs gel

The gel of the 100 mg/mL 4armPEG10k-triazole-EG4(POG)₁₀ was prepared in the same way. After the samples formed a gel, the same volume of the 0.5 eq. (PPG)₁₀, 0.5 eq. (POG)₁₀, or PBS was added, respectively. Before the addition, the CMP solutions were heated at 80 °C for 15 min and cooled for 5 seconds by liquid nitrogen. The sample entries are as follows: (4armPEG10k-triazole-EG4(POG)₁₀ gel +PBS, 4armPEG10k-triazole-EG4(POG)₁₀ gel +0.5 eq. (PPG)₁₀, 4armPEG10k-triazole-EG4(POG)₁₀ gel +0.5 eq. (POG)₁₀). After that, the samples were kept at 37 °C for 1 day. The gel of the 100 mg/mL 4armPEG10k-triazole-EG4(PPG)₁₀ was prepared in the same way. After the samples formed a gel, the same volume of the 0.5 eq. (POG)₁₀ was added. Before the addition, the (POG)₁₀ solution was heated at 80 °C for 15 min

and cooled for 5 seconds by liquid nitrogen. After that, the sample was kept at 25 °C for 1 day. About the 4armPEG10k-triazole-EG4(POG)₁₀ gel +0.5 eq. (PPG)₁₀, the CD denaturation curve measurement was taken after the incubation at 37 °C for 10 days. Before the measurement, the sample solution was diluted and kept at 20 °C for 10 minutes. The denaturation condition was 1 °C/min from 20 °C to 90 °C. Synthesis and functional characterizations of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀

2.3 Results and discussion

2.3.1 Synthesis and functional characterizations of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀

First, AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ were synthesized by amide coupling reaction (Figure 2-1a). From the peak of x and z in Figures 2-1b and 2-1c, the presence of both AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ were confirmed. The (PPG)₁₀ and AzidoEG4(PPG)₁₀ showed no peaks around 300 nm in the UV-vis spectra, but DBCOEG4(PPG)₁₀ showed the DBCO peak around 300 nm (Figure 2-1d). The grafting ratios of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ were calculated to be ~100% by ¹H-NMR and UV-vis spectra using DBCO's calibration curve, respectively (Figures 2-2, 2-3, and 2-4). The CD spectra shown in Figure 2-1e suggested that their triple helix formation abilities were maintained after the reaction. The AzidoEG4(POG)₁₀ was also successfully synthesized (Figures 2-5 and 2-6).

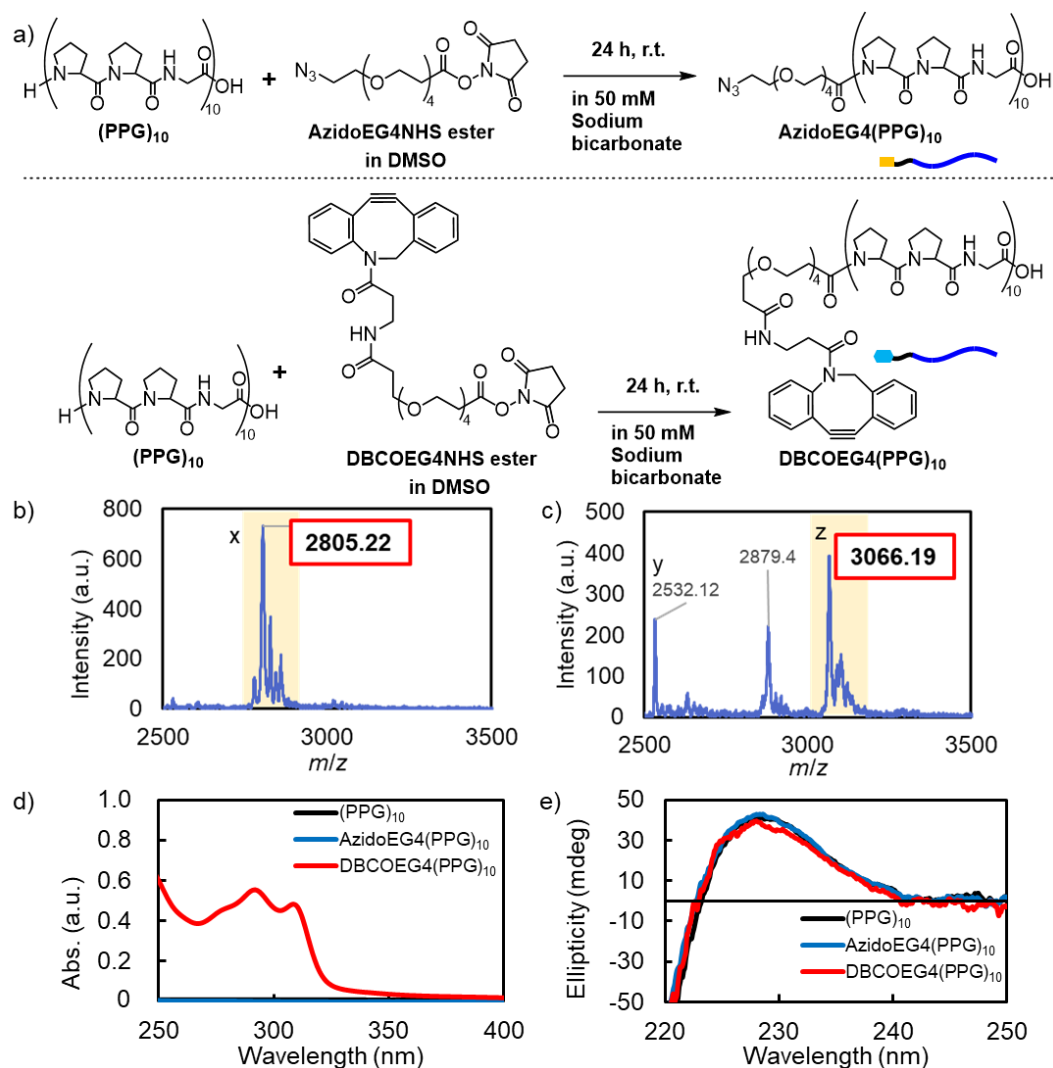


Figure 2-1. Functional characterizations of Azido(PPG)₁₀ and DBCO(PPG)₁₀. a) Synthesis of

AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀. MALDI-TOF-MASS spectrum of b) AzidoEG4(PPG)₁₀ and c) DBCOEG4(PPG)₁₀. Peaks x, y, and z were assigned to be AzidoEG4(PPG)₁₀, (PPG)₁₀, and DBCOEG4(PPG)₁₀, respectively. d) UV-vis spectra of (PPG)₁₀ (black line), AzidoEG4(PPG)₁₀ (blue line), and DBCOEG4(PPG)₁₀ (red line). e) CD profiles of (PPG)₁₀ (black line), AzidoEG4(PPG)₁₀ (blue line), and DBCOEG4(PPG)₁₀ (red line).

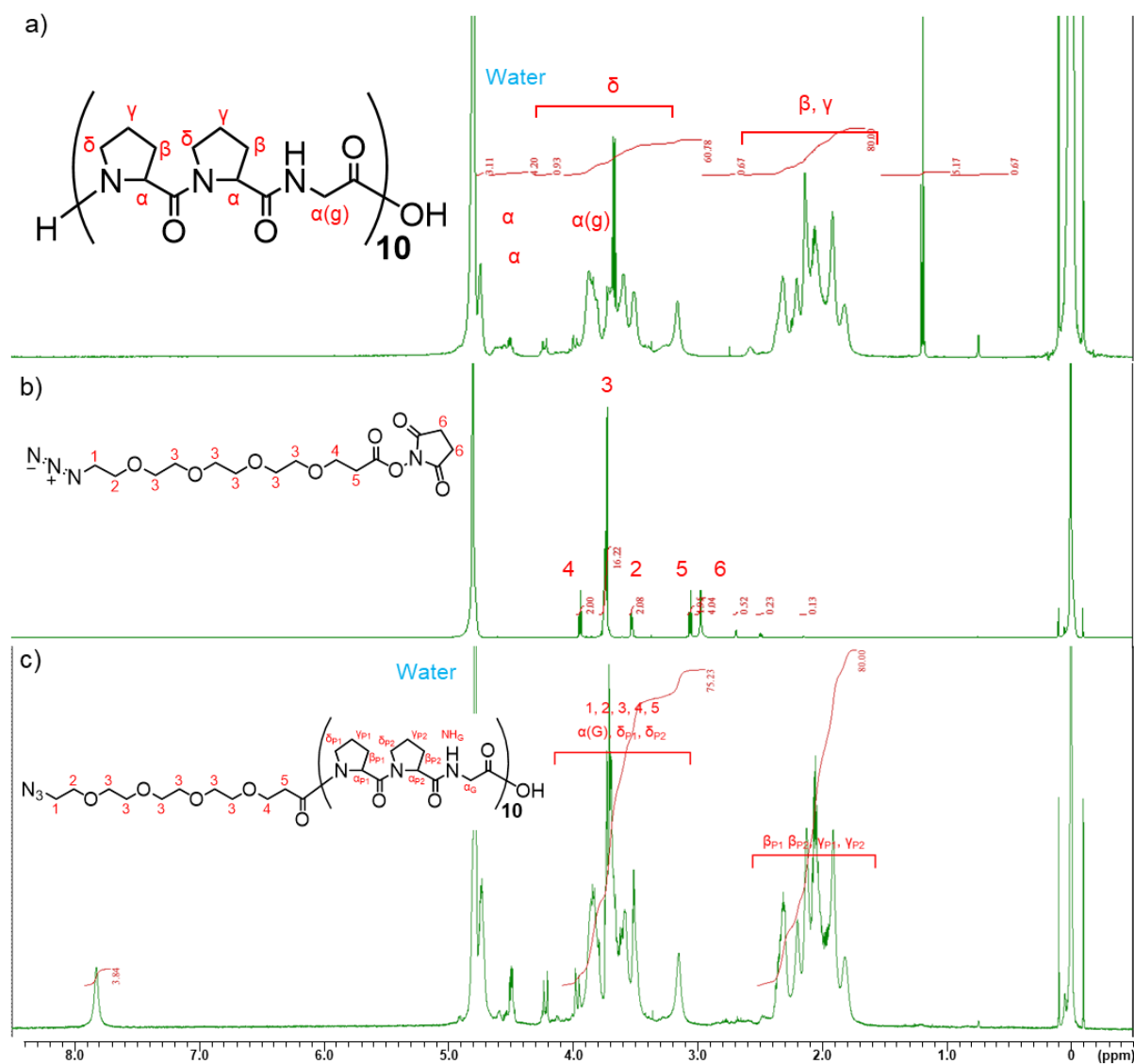


Figure 2-2. ¹H-NMR spectra of (a) (PPG)₁₀, (b) AzidoEG4NHS and (c) AzidoEG4(PPG)₁₀ in D₂O at 25 °C in a magnetic field at 600 MHz. The grafting ratio of the AzidoEG4(PPG)₁₀ was calculated to be 94% by comparison between the integral of H(1~5) of AzidoEG4 moiety and H(α(G), δ_{P1}, and δ_{P2}) of AzidoEG4(PPG)₁₀.

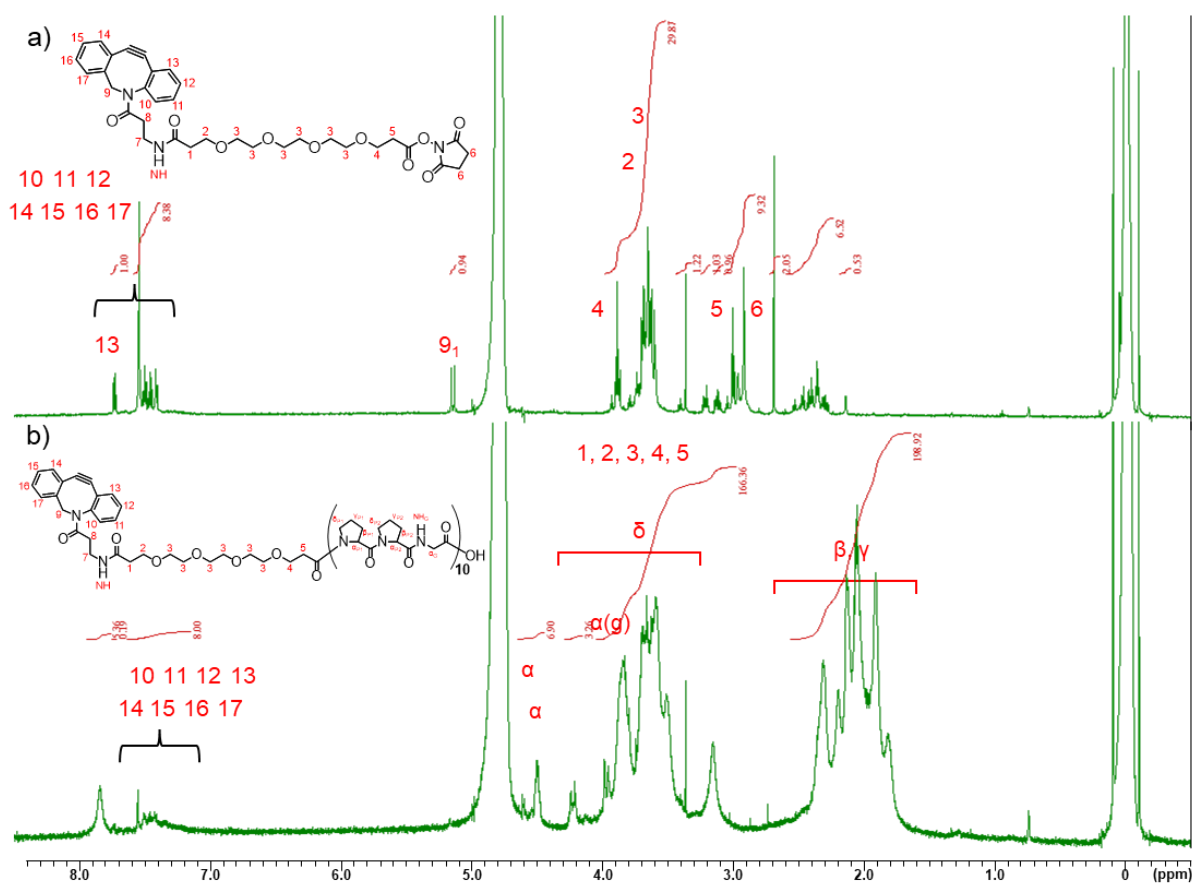


Figure 2-3. ^1H -NMR spectra of (a) DBCOEG4NHS and (b) DBCOEG4(PPG)₁₀ in D_2O .

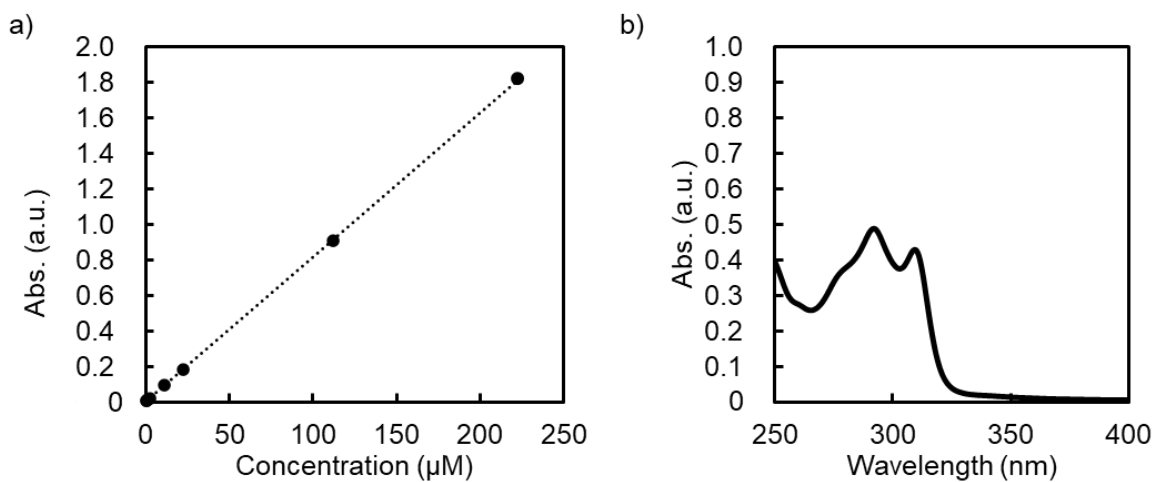


Figure 2-4. (a) UV-vis calibration curve of DBCOEG4NHS in PBS at 308 nm and (b) UV-vis spectrum of DBCOEG4(PPG)₁₀ in PBS. The grafting degree of DBCOEG4(PPG)₁₀ was calculated to be around 106%.

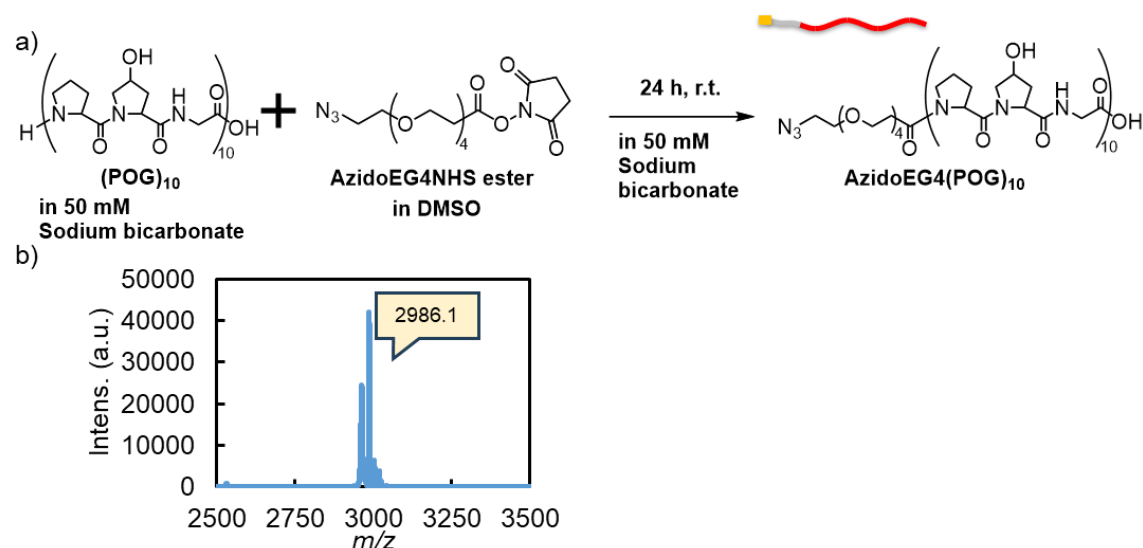


Figure 2-5. (a) Synthesis of AzidoEG4(POG)₁₀. b) MALDI-TOF-MASS spectrum of AzidoEG4(POG)₁₀.

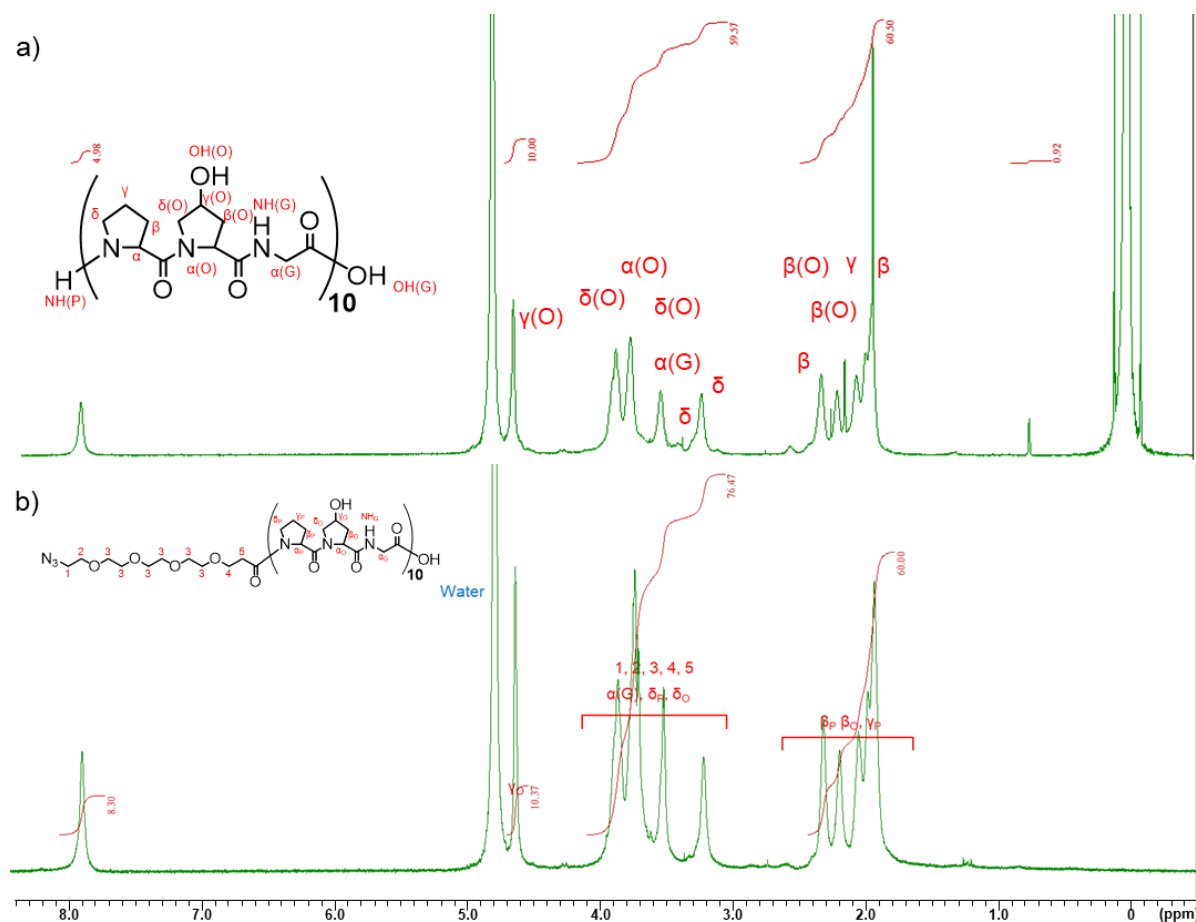


Figure 2-6. ¹H-NMR spectra of (a) (POG)₁₀, (b) AzidoEG4NHS and (c) AzidoEG4(POG)₁₀ in D₂O at 25 °C by the magnetic field at 600 MHz. The grafting ratio of the AzidoEG4(POG)₁₀ was calculated to be 96% by comparison between the integral of H(1~5) of AzidoEG4 moiety and H(α(G), β_p, and δ_o) of AzidoEG4(POG)₁₀.

2.3.2 Synthesis of 4armPEG10k-triazole-EG4(PPG)₁₀

The 4arm-PEG10k-triazole-EG4(PPG)₁₀ was successfully synthesized by alkyne-azide click reaction using 4arm-PEG10k-Dibenzocyclooctyne and Azido-EG4(PPG)₁₀ (Figure 2-7). These grafting degrees were calculated to be around 100% from ¹H-NMR.

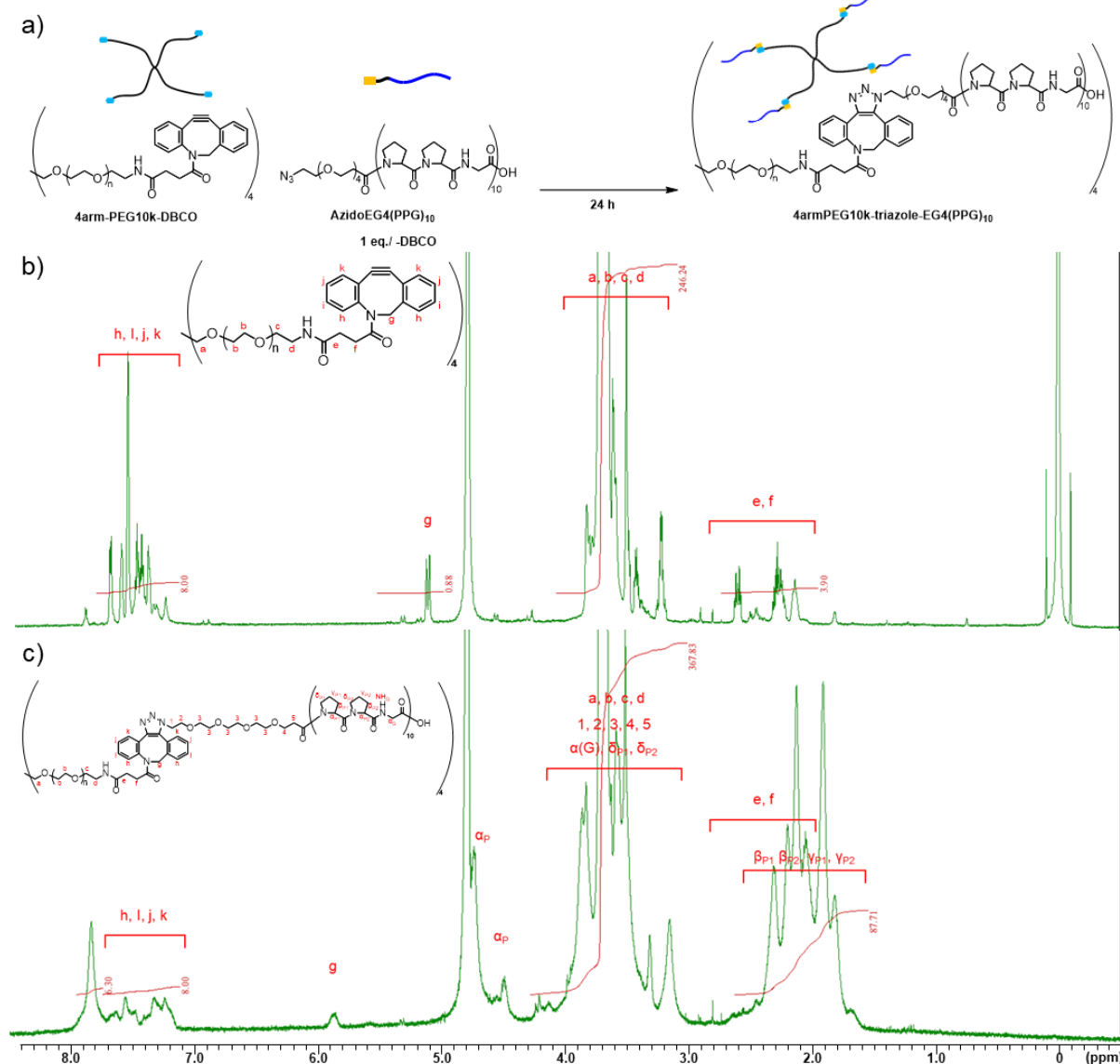


Figure 2-7. (a) Reaction scheme of AzidoEG4(PPG)₁₀ and 4arm-PEG10k-DBCO. ¹H-NMR spectra of (b) 4armPEG10kDBCO and (c) 4armPEG10ktriazole-EG4(PPG)₁₀ in D₂O. The grafting ratio of AzidoEG4(PPG)₁₀ was calculated by comparison between the integral of H(h, i, j, and k) of DBCO moiety and the sum of H(e and f) of ethylene moiety and H(β and γ) of AzidoEG4(PPG)₁₀.

As a control of 4armPEG10k-triazole-EG4(PPG)₁₀ for further experiment, (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ was also synthesized by alkyne-azide click reaction using DBCO-

EG4(PPG)₁₀ and Azido-EG4(PPG)₁₀ and characterized (Figure 2-8). The presence of (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ was checked by MALDI-TOF Mass (Figure 2-8b). During the click reaction, the triple helix structure was retained (Figures 2-8c and 2-8d). The T_m didn't decrease during heating and cooling cycles, and the triple helix structure was completely recovered (Figures 2-8e and 2-8f). Previous studies have reported that introducing aromatic rings at the CMP terminus increases T_m .^[4, 5] It is also reported that the triple helices are stabilized by the introduction of a fluorescent group via a spacer at the N terminus.^[6] AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ also contain EG4 as a spacer, and DBCO contains an aromatic ring. Therefore, it is considered that the T_m of DBCOEG4(PPG)₁₀ becomes higher than that of AzidoEG4(PPG)₁₀. From these results, (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ was characterized, and it was indicated that the modification and reaction at the N-terminus could contribute to the stabilization.

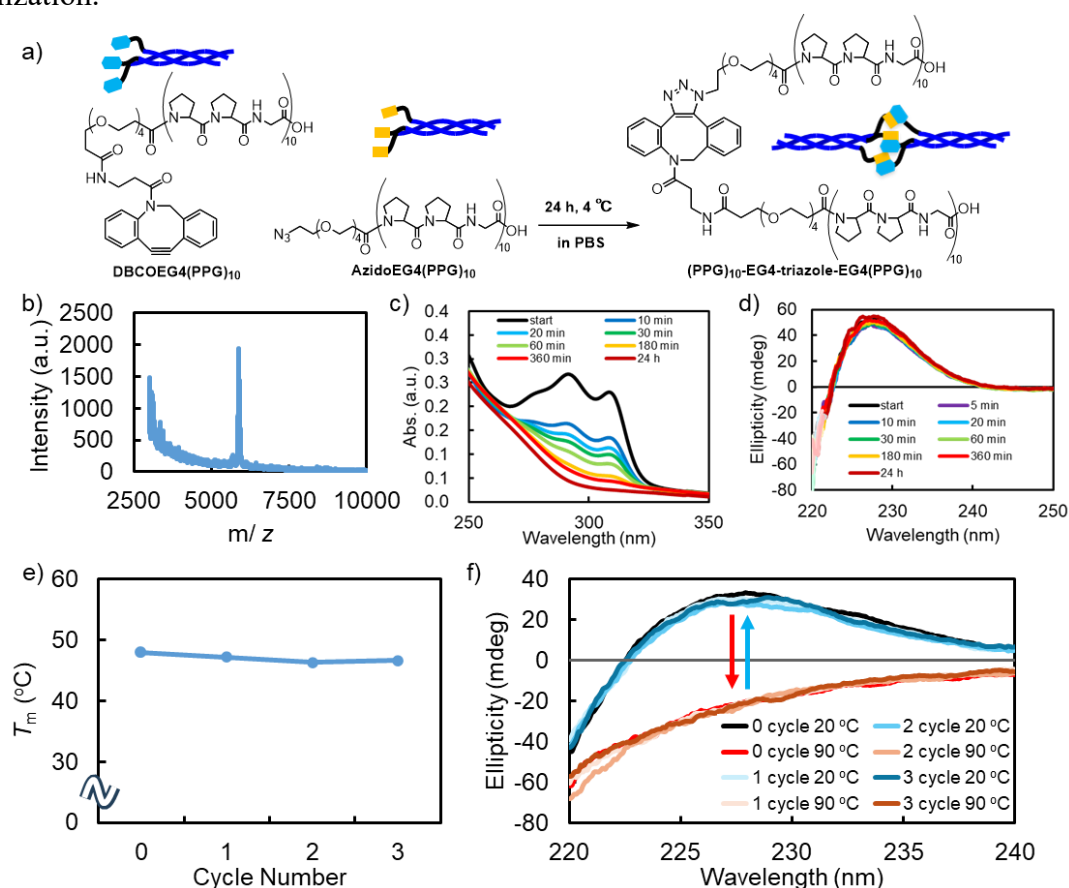


Figure 2-8. (a) Reaction scheme of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀. b) MALDI-TOF Mass spectrum of (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀. c) UV-vis and d) CD profiles at each time point of AzidoEG4(PPG)₁₀ and DBCOEG4(PPG)₁₀ mixed solution. e) The T_m values at each annealing cycle of (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ and f) CD profiles at 20 °C and 90 °C in the cycles.

2.3.3 Sol-gel transition analysis of 4armPEG10k-triazole-EG4(PPG)₁₀

The gel-sol transition properties were evaluated at 50 mg/mL in PBS. After heating and cooling the 4arm-PEG10k-triazole-EG4(PPG)₁₀ solution, the sample showed gelation, and the gel became a liquid state after heating (Figure 2-9a). This gel-sol transition was repeatable. In contrast, the (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀ didn't form a gel (Figure 2-9b). From the CD spectra, the triple-helix formation and dissociation were also repeatable (Figure 2-9c). The CD denaturation curve suggested that the triple helices were dissociated at around 40 °C (Figure 2-9d). These results suggested that 4arm-PEG10k-triazole-EG4(PPG)₁₀ gel was formed by the internal triple helices (Figure 2-9e). Moreover, the storage modulus increased with increasing concentration (Figure 2-10). The 4arm-PEG10k-triazole-EG4(PPG)₁₀ showed gelation at 50 and 100 mg/mL, but not at 10 mg/mL (data not shown).

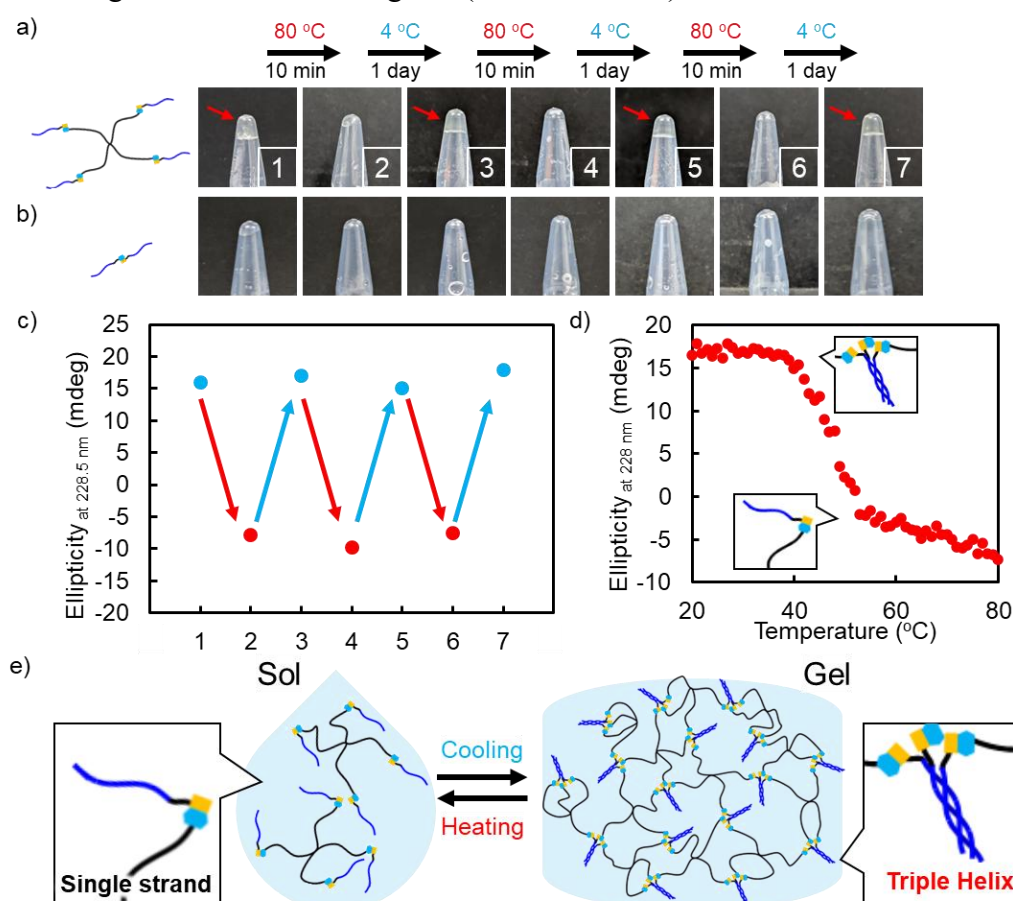


Figure 2-9. Composition of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and gelation analysis. Gel-sol profiles and schematic illustrations of a) 4arm-PEG10k-triazole-EG4(PPG)₁₀ and b) (PPG)₁₀-EG4-triazole-EG4(PPG)₁₀. The polymer concentration was 50 mg/mL, and the CMP concentrations were fixed between the samples. The tube was kept inverted for more than 1 min. c) CD profiles of 4arm-PEG10k-triazole-EG4(PPG)₁₀ during heating and cooling cycles. d) CD denaturation curve of 4arm-PEG10k-triazole-EG4(PPG)₁₀. e) Gel-sol transition scheme

of 4arm-PEG10k-triazole-EG4(PPG)₁₀.

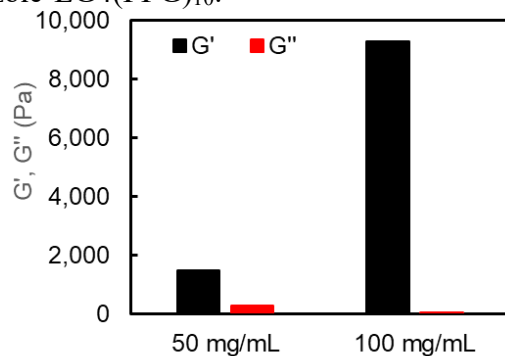


Figure 2-10. Rheology analysis of 4armPEG10k-triazole-EG4(PPG)₁₀ gel. The concentrations were 50 mg/mL and 100 mg/mL. Temperature was kept at 20 °C during the measurement.

2.3.3 Synthesis of 4armPEG10k-triazole-EG4(POG)₁₀

The 4arm-PEG10k-triazole-EG4(POG)₁₀ was successfully synthesized by alkyne-azide click reaction (Figure 2-11). AzidoEG4(POG)₁₀ was synthesized by amide coupling reaction. These grafting degrees were calculated to be around 100% from ¹H-NMR.

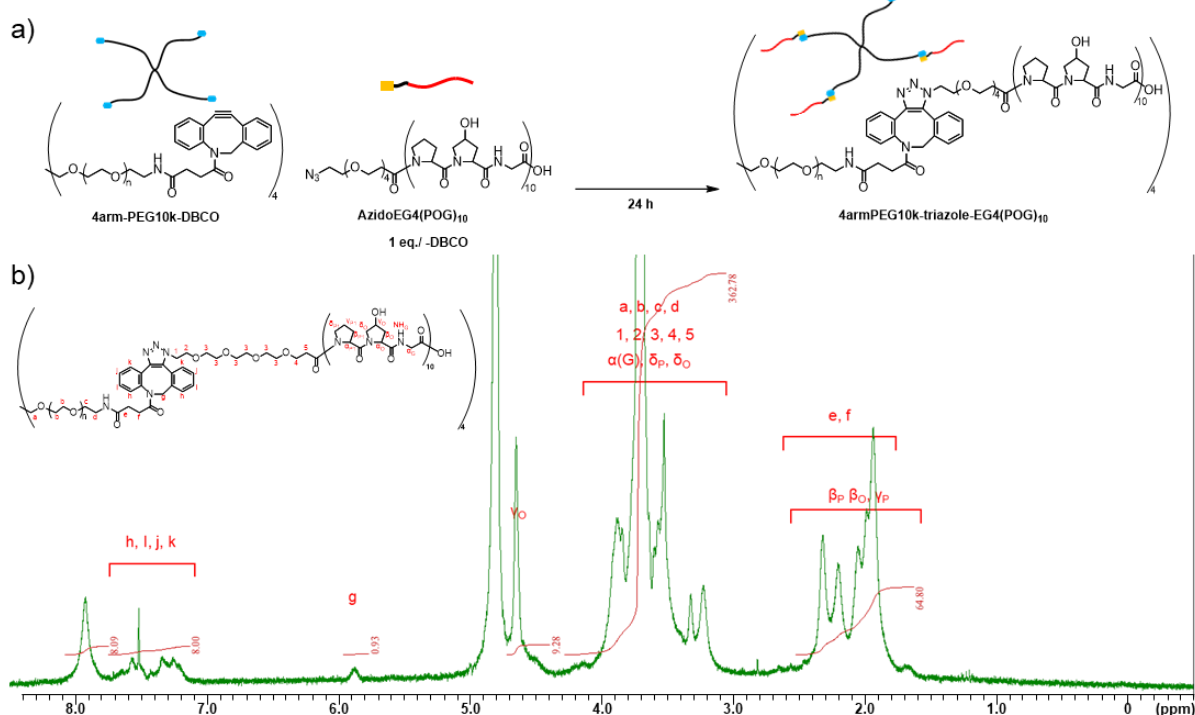


Figure 2-11. (a) Reaction scheme of AzidoEG4(PPG)₁₀ and 4arm-PEG10k-DBCO. (b) ¹H-NMR spectrum of 4armPEG10ktriazole-EG4(POG)₁₀ in D₂O. The grafting ratio was calculated by comparison between the integral of H(h, i, j, and k) of DBCO moiety and the sum of H(e and f) of ethylene moiety and H(β_p, β_o, and γ_p) of AzidoEG4(POG)₁₀.

2.3.4 Evaluation of sol-gel transition property in response to CMP's amino acid sequences

The 4arm-PEG10k-triazole-EG4(POG)₁₀ also showed thermoresponsive gel-sol transition (Figure 2-12a). It has been reported that dendrimers modified with (POG)₁₀ form gels, and in that study, the gelation was suggested to be driven by entropy-driven association of the triple-helical structures upon heating.^[7] In contrast, the 4arm-PEG10k-triazole-EG4CMPs used in this study formed gels upon cooling under both the (PPG)₁₀ and (POG)₁₀ conditions, suggesting that the gelation is driven by cross-linking through CMP triple helices. The gels are expected to possess a network structure in which CMP triple helices serve as cross-linking points.

The effect of CMP's amino acid sequence in gel-sol transition was also evaluated. The 4arm-PEG10k-triazole-EG4(PPG)₁₀ gel turned into a liquid state after incubation at 50 °C. On the other hand, the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel didn't turn into a liquid state after incubation at 50 °C but 80 °C (Figures 2-12b and 2-12c). From the CD denaturation curves, these solation temperatures correspond to these T_m (Figure 2-12d). The stabilities of (PPG)₁₀ and (POG)₁₀ are 36 °C and 64 °C, respectively, which correspond to the denaturation temperatures of the respective 4armPEGEG4-CMPs. The increased stability results from terminal modifications of the CMPs as described in section 2.3.2. These results confirmed that the gel-sol transition is driven by the triple helix formation, and the amino acid sequences of CMP affect the gel-sol transition behavior. Moreover, the 1:1 mixture of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ also showed gel-sol transition and the T_m was calculated at 60 °C (Figures 2-12d and 2-12e). The rounded denaturation curve suggested that several combinations of homo- and hetero-triple helix were formed. From these results, it was suggested that the gel-sol transition property is also controlled by the hetero-triple helix formation (Figure 2-12f).

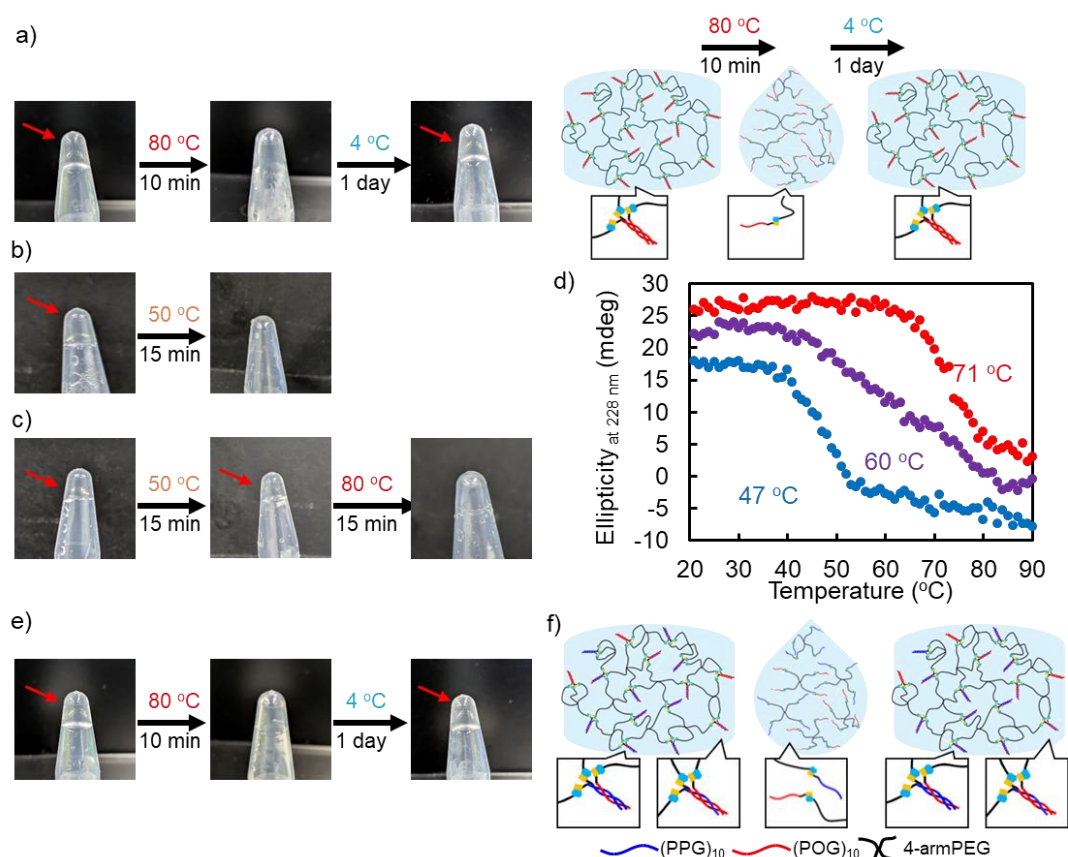


Figure 2-12. Composition of 4arm-PEG10k-triazole-EG4(POG)₁₀ and gelation analysis of 4arm-PEG10k-triazole-EG4CMPs. a) Reaction scheme of AzidoEG4(POG)₁₀ and 4arm-PEG10k-DBCO. Gel-sol profiles and schematic illustrations of b) 4arm-PEG10k-triazole-EG4(POG)₁₀ and c) 1:1 mixture of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀. The polymer concentration was 50 mg/mL, and the CMP concentrations were fixed between the samples. The tube was kept inverted for more than 1 min. CD profiles of d) 4arm-PEG10k-triazole-EG4(PPG)₁₀, e) 4arm-PEG10k-triazole-EG4(POG)₁₀, and f) the mixture of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ in PBS at 25 °C, 80 °C, and 4 °C. g) CD denaturation curve of 4arm-PEG10k-triazole-EG4(PPG)₁₀ (blue dot), 4arm-PEG10k-triazole-EG4(POG)₁₀ (red dot), and 1:1 mixture of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ (violet dot). h) CD melting profiles of (PPG)₁₀ (blue dot), (POG)₁₀ (red dot), and (PPG)₁₀ and (POG)₁₀ mixture ((PPG)₁₀:(POG)₁₀=1:1) after annealing (violet dot).

2.3.5 Sol-gel transition analysis of 4armPEG10k-triazole-EG4CMPs with CMPs

The effect of additional CMPs was evaluated (Figures 2-12 and 2-13). Among the 4arm-PEG10k-triazole-EG4(PPG)₁₀ containing samples, gelation was not observed in 4arm-PEG10k-triazole-EG4(PPG)₁₀+1 eq. (PPG)₁₀ after annealing. However, the samples 0.5 eq. (PPG)₁₀ or PBS added showed gelation. These results suggested that the hetero-like triple helices among Polymer's (PPG)₁₀ and additional (PPG)₁₀ were formed and they inhibited the inter-polymer triple helix formation as a non-covalent crosslinking point (Figure 2-12d). The additional (POG)₁₀ also inhibited the gelation by inter-polymer triple helix formation (Figures 2-13a and 2-13b). The 4arm-PEG10k-triazole-EG4(PPG)₁₀+1 eq. (POG)₁₀ showed gelation after 4 °C incubation, but the gel was cloudy. The 1 eq. (POG)₁₀ in PBS was like a suspended solution, suggesting that (POG)₁₀ homo-triple helices were formed so fast at this concentration. It was hypothesized that the limited inhibition of interpolymer triple helix formation by hetero triple helix formation between 4arm-PEG10k-triazole-EG4(PPG)₁₀ and (POG)₁₀ occurred because the ratio of homo-triple helices of (POG)₁₀ increased.

The 4arm-PEG10k-triazole-EG4(POG)₁₀+0.5 eq. (PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀+0.5 eq. (POG)₁₀ samples also showed gelation inhibition after 4 °C incubation (Figures 2-13c and 2-13d). Among the 4arm-PEG10k-triazole-EG4(PPG)₁₀ + 0.5 eq. CMPs samples, gelation happened after 4 °C incubation, but the 4arm-PEG10k-triazole-EG4(POG)₁₀+0.5 eq. CMPs samples didn't form a gel. It is suggested that the difference in the formation kinetics of triple helices between (PPG)₁₀ and (POG)₁₀ could have changed the hetero triple helix formation behavior.^[8] These results suggested that the sol-gel transition was controlled by additional CMPs due to the hetero-like triple helix formation among Polymer's CMPs and additional CMPs.

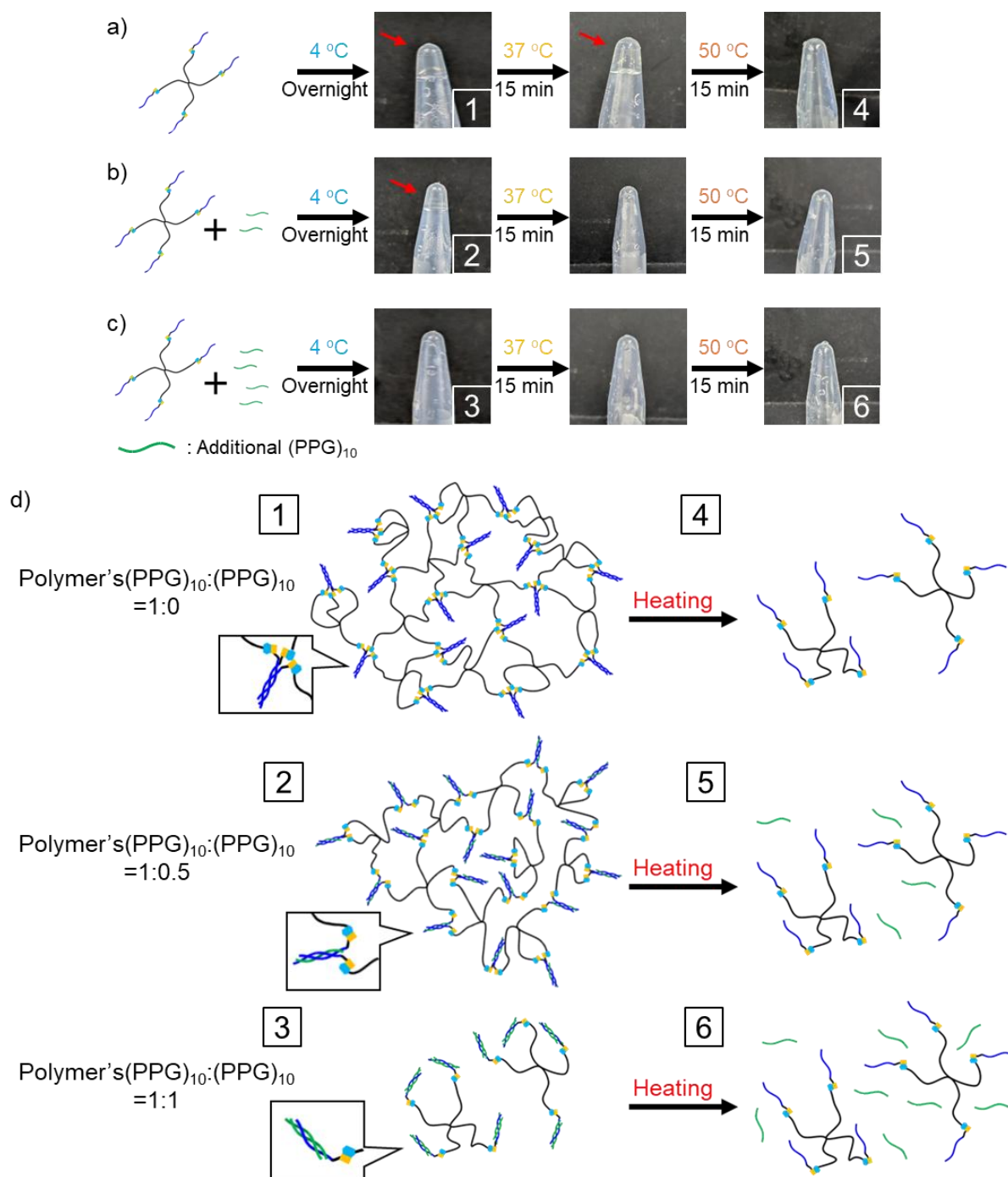


Figure 2-12. Gel-sol profiles of a) 4arm-PEG10k-triazole-EG4(PPG)₁₀+0.5 eq. (PPG)₁₀, b) 4arm-PEG10k-triazole-EG4(PPG)₁₀+1 eq. (PPG)₁₀, c) 4arm-PEG10k-triazole-EG4(PPG)₁₀+PBS. The final polymer concentration of each sample was 50 mg/mL. The tube was kept inverted for more than 1 min. d) Schematic illustrations of the additional CMP-dependent control of sol-gel transition by inhibition of interpolymer triple helix formation. The numbers correspond to the numbers shown in the above figures.

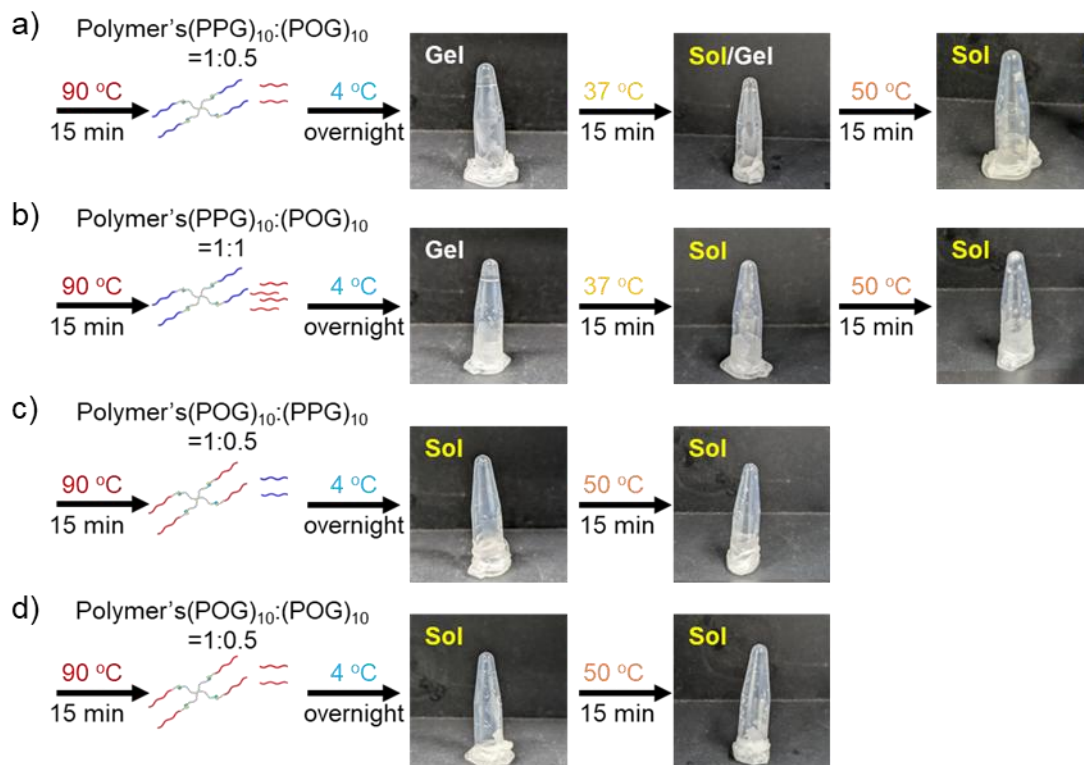


Figure 2-13. Gel-sol analysis of 4armPEG10k-triazole-EG4CMPs with CMPs. Gel-sol profiles of a) 4armPEG10k-triazole-EG4(PPG)₁₀+0.5 eq. (POG)₁₀, b) 4armPEG10k-triazole-EG4(PPG)₁₀+1 eq. (POG)₁₀, c) 4armPEG10k-triazole-EG4(POG)₁₀+0.5 eq. (PPG)₁₀, d) 4armPEG10k-triazole-EG4(POG)₁₀+0.5 eq. (POG)₁₀. The final polymer concentration of each sample was 50 mg/mL. The tube was kept inverted for more than 1 min.

2.3.6 Effect of the additional single strand CMPs on 4armPEG10k-triazole-EG4CMPs gel

Furthermore, the effect of the additional single strand CMPs on 4armPEG10k-triazole-EG4CMPs gel was evaluated (Figure 2-14). When the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with PBS was kept at 37 °C for 1 day, the gel maintained a gel state, and the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with (POG)₁₀ solution sample showed similar behavior (Figure 2-14a and 2-14b). On the other hand, the gels turned into a liquid state about the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with the single-strand (PPG)₁₀ solution (Figure 2-14c). As shown in Figure 2-14d, the CD denaturation curves of the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with the single-strand (PPG)₁₀ solution showed the loose denaturation curve from 40 °C to 60 °C, the flat area from 65 °C to 70 °C, and the shape denaturation curve from 75 °C. The denaturation

profile from 75 °C was similar with that of 4arm-PEG10k-triazole-EG4(POG)₁₀. This result suggested that the presence of several combinations of homo- and hetero-triple helices of the 4arm-PEG10k-triazole-EG4(POG)₁₀ and (PPG)₁₀. From these results, it is expected that the single-strand state (POG)₁₀ was present in the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel, and the gel became weaker after the hetero-triple helices formation (Figure 2-14e). On the other hand, the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel with (POG)₁₀ maintained a gel state because the (POG)₁₀ was kept triple-helix structure (Figure 2-14f). From these results, it's possible that the gel properties of 4arm-PEG10k-triazole-EG4CMP can be controlled by additional single-strand CMP.

From these results, it was suggested that gelation inhibition by CMP hetero triple helix formation and tuning of gelation properties by amino acid sequence variation are possible.

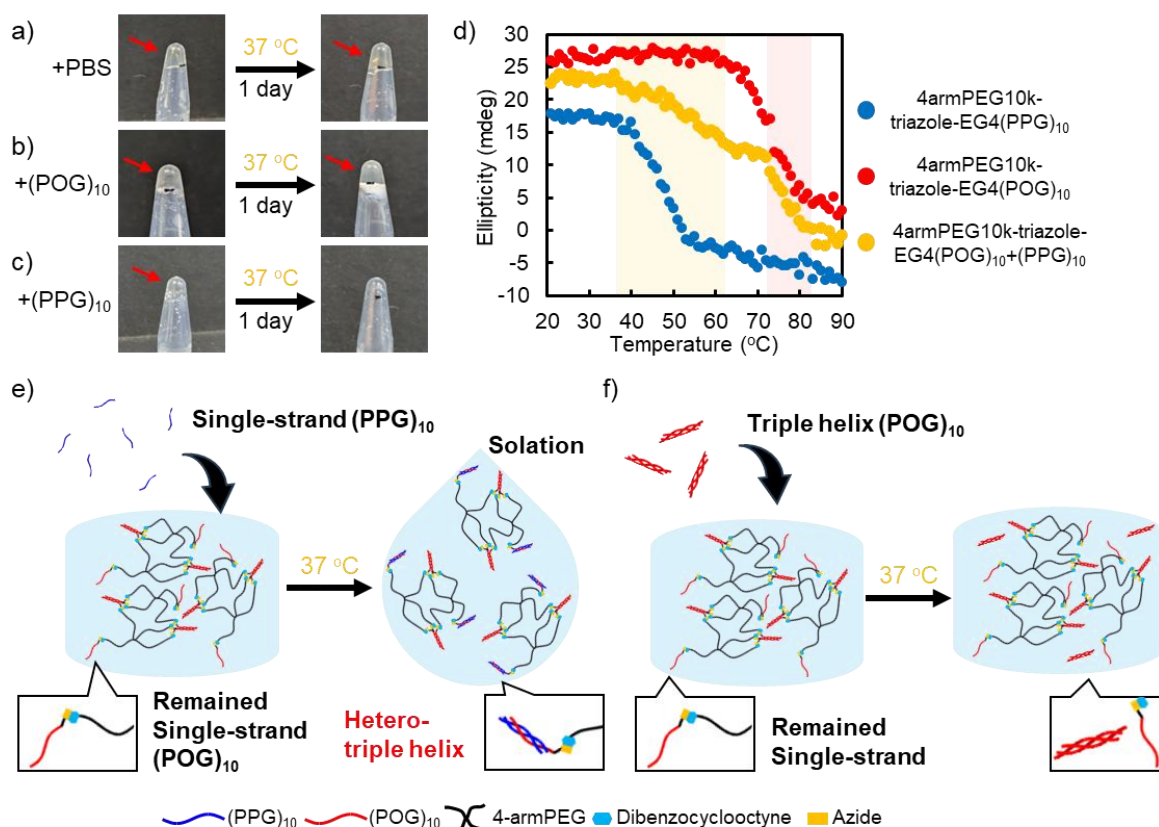
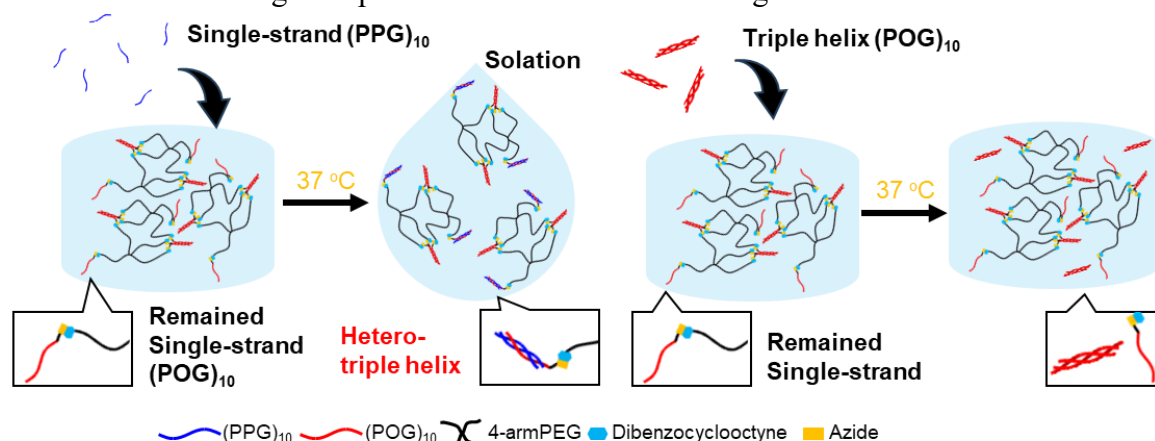


Figure 2-14. Effect of the additional single strand CMPs on 4arm-PEG10k-triazole-EG4CMPs gel. Gel-sol profiles of a) 4arm-PEG10k-triazole-EG4(POG)₁₀+PBS, b) 4arm-PEG10k-triazole-EG4(POG)₁₀ gel+0.5 eq. (POG)₁₀, and c) 4arm-PEG10k-triazole-EG4(POG)₁₀ gel+0.5 eq. (PPG)₁₀. d) CD denaturation curves of 4arm-PEG10k-triazole-EG4(PPG)₁₀ (blue dot), 4arm-PEG10k-triazole-EG4(POG)₁₀ (red dot), and 4arm-PEG10k-triazole-EG4(POG)₁₀ gel+0.5 eq. (PPG)₁₀ (yellow dot). e) A schematic illustration of a possible mechanism about the

4arm-PEG10k-triazole-EG4(POG)₁₀ gel degradation by single strand (PPG)₁₀. f) A schematic illustration of the 4arm-PEG10k-triazole-EG4(POG)₁₀ gel and triple-helix (POG)₁₀.

2.4 Conclusion

In summary of chapter 2, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands. First of all, the 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ were successfully synthesized. Subsequently, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands. The 4arm-PEG10k-triazole-EG4CMPs showed gel-sol transition by the internal triple helix formation of CMPs. The gel-sol transition behavior was controlled by the amino acid sequences of CMP and the presence of additional non-modified CMPs. Furthermore, it's possible that the gel properties of 4arm-PEG10k-triazole-EG4CMP can be controlled by additional single-strand CMP. This phenomenon is expected to be applied in biomaterials for the development of new self-assembled materials and to further understanding of triple helix formation-driven sol-gel transition.



Scheme 2-2. Triple-helix crosslinked hydrogels with sol-gel transition in response to single-stranded CMPs via hetero-like triple helix formation.

2.5 References

- [1] L. Haines-Butterick, K. Rajagopal, M. Branco, D. Salick, R. Rughani, M. Pilarz, M. S. Lamm, D. J. Pochan, J. P. Schneider, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7791.
- [2] C. M. R. Pérez, L. A. Rank, J. Chmielewski, *Chem. Commun.* **2014**, *50*, 8174.

- [3] M. Matsusaki, R. Amekawa, M. Matsumoto, Y. Tanaka, A. Kubota, K. Nishida, M. Akashi, *Adv. Mater.* **2011**, *23*, 2957.
- [4] T. Koga, S. Kingetsu, N. Higashi, *Int. J. Mol. Sci.* **2021**, *22*, 4533.
- [5] J. M. Keever, P. D. Banzon, M. K. Hales, A. L. Sargent, W. E. Allen, *J. Org. Chem.* **2023**, *88*, 11885.
- [6] L. B. Boll, L. W. Kreft, H. Wennemers, *Angew. Chem. Int. Ed.* **2025**, *64*, e202500856.
- [7] C. Kojima, T. Suehiro, T. Tada, Y. Sakamoto, T. Waku, N. Tanaka, *Soft Matter* **2011**, *7*, 8991.
- [8] S. Boudko, S. Frank, R. A. Kammerer, J. Stetefeld, T. Schulthess, R. Landwehr, A. Lustig, H. P. Bächinger, J. Engel, *J. Mol. Biol.* **2002**, *317*, 459.

Chapter 3

Fabrication of a Thermoresponsive Cell Assembly Method Driven by Triple Helix Formation of a Collagen Model Peptide

3.1 Introduction

In chapter 2, the author found that the material properties was able to be controlled by CMP's amino acid sequences and additional CMPs. In chapter 3, the author investigated the application of CMP triple helix formation as a thermoresponsive cell assembly method.

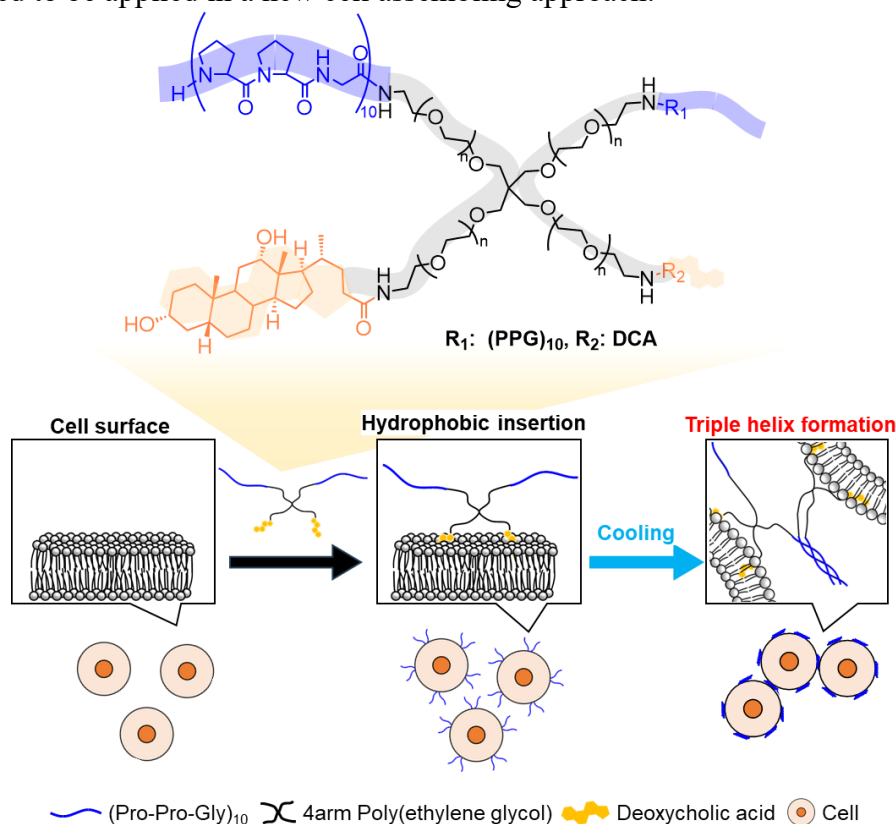
As described in general introduction, “Biofabrication” is the field of creating tissues and organs from biological materials such as cells and proteins, and artificial tissues and organs that reproduce complex structures and functions are expected to be applied in transplantation therapy and drug efficacy evaluation. Recently, the fabrication of tissue constructs that mimic the complex three-dimensional structures of biological tissues using hydrogels as scaffold materials have been reported.^[1, 2] However, it is still challenging to completely reproduce the functions of real tissues. One of the reasons is that the abundant presence of scaffold materials probably hinders cell–cell interactions. For a tissue to function as a tissue, it is important for individual cells to interact closely with each other to induce differentiation. Therefore, the tailor-made approaches for the fabrication of cell aggregates to facilitate intercellular interactions are needed.

In these days, the methods for constructing higher-order tissue structures from cell aggregates are being studied.^[3] Especially, the cell surface modification approaches have been attracted much attention because they can manipulate and control cell interactions and phenotypes for various biomedical applications.^[4] As Biologically-Driven cell surface modifications, genetic engineering methods and metabolic engineering methods^[5] which introduces functional groups through sugar metabolism are reported, but they have some challenges such as cell-type dependency, risk of trigger immunogenic responses and tumorigenesis, and chemical insertion efficiency that can vary depending on cell metabolism.^[4] Accordingly, chemically-driven cell modifications have also been studied. Among them, covalent-bond formation is considered a simple and straightforward method, but it has the lack

of targeting specificity and cytotoxicity. On the other hand, hydrophobic insertion in the cell surface using phospholipids, alkyl chains, and cholesterol have low cytotoxicity.^[6,7] Previously, it was also reported that the hydrophobic insertion using cholesterol.^[8]

Some researchers are using DNA chain as a crosslinking site to make cell-cell assemblies.^[6,9] However, there are some concerns about the usage of DNA that DNA degradation and potential risk of immune response.^[10] On the other hand, it is known that a collagen-like triple-helix is resistant to most proteases except for collagen-specific enzymes,^[11] the application of collagen-like triple helices is considered promising. However, a cell assembly method using CMP as a cell assembling motif has not yet been reported.

Herein, the author synthesized a 4armed poly(ethylene glycol) (PEG) terminally co-modified with (PPG)₁₀ and deoxycholic acid (DCA) (4arm-PEG-DCA/-(PPG)₁₀) and evaluated the characteristics and the cell-assembling property (Scheme 1). As results of the evaluation, more cell aggregates were formed after cooling under conditions with 4arm-PEG-DCA/-(PPG)₁₀ than without 4arm-PEG-DCA/-(PPG)₁₀. Accordingly, the author fabricated the thermoresponsive cell assembly method driven by triple helix formation of CMP. The use of CMP is expected to be applied in a new cell assembling approach.



Scheme 3-1. A schematic illustration of thermo-responsive cell assembling method driven by triple-helix formation of CMP.

3.2 Experiments

3.2.1 Materials

The (PPG)₁₀ (4006) was purchased from Peptide Institute, Inc. (Osaka, Japan). Deuterium oxide (536-7474), *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyloxy]succinimide (Fmoc-NHS) (064-02461), 5 mol/L hydrochloric acid (081-05435), 4% paraformaldehyde phosphate buffer solution (163-20145), piperidine (160-02776), sodium carbonate (199-01585), sodium deoxycholate (DCA-Na) (192-08312), tetrahydrofuran (206-05106), and trypsin (9002-07-7) were purchased from Wako Chemicals (Osaka, Japan). Cell Count Reagent SF (07553-44), Dulbecco's Modified Eagle Medium (High Glucose) (DMEM) (08458-16), sodium bicarbonate (31213-15), fluorescein 5-isothiocyanate (FITC) (16151-66), D-PBS without Ca and Mg, powder (07269-84), and Antibiotic-Antimycotic Mixed Stock Solution (100x) (02892-54) were purchased from Nacalai Tesque (Kyoto, Japan). Ethylenediaminetetraacetic acid tetrasodium salt, 2-hydrate (000-29295) and hexane (010-36766) were purchased from Kishida Chemical (Osaka, Japan). Dimethyl sulfoxide-d₆ (151874-25G), and 4arm-PEG20k-NH₂ HCl salt (JKA7026-1G) were purchased from Sigma-Aldrich (St. Louis, USA). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (D2919) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 4',6-Diamidino-2-phenylindole (DAPI), fetal bovine serum (FBS) (10270-106) and FluoroBrite™ DMEM (FluoroBriteDMEM) (A18967-01), and trypan blue stain 0.4% (T10282) were purchased from Thermo Fisher Scientific Inc. (Waltham, U.S.A). Agarose (50090) was purchased from Cambrex Bio Science Rockland, Inc. (ME, U.S.A). Epithelial fibrosarcoma (HT1080) and normal human dermal fibroblasts (NHDF) (CC-2509) were obtained from Lonza (Basel, Switzerland).

3.2.2 Synthesis of 4arm-PEG-DCA

After dissolving DCA-Na (8.3 mg, 20 μmol) in 5 mL of 50 mM Na₂CO₃/NaHCO₃ (aq.) (pH 8.5), DMT-MM (27.7 mg, 100 μmol) in 0.5 mL of Milli-Q water was added and stirred at ice bath for 10 min. After the activation, the 4arm-PEG20k-NH₂ (100 mg, 5 μmol) in 4.5 mL of 50 mM Na₂CO₃/NaHCO₃ was added, and then the reaction mixture was stirred for 24 h at room temperature. After the reaction, the whole reaction mixture was dialyzed (MWCO: 3.5 kDa) for 3 days against methanol and water. The resulting solution was freeze-dried. The 4arm-PEG20k-DCA was characterized by ¹H-NMR measurement. The ¹H-NMR spectra were measured by a Nuclear Magnetic Resonance Spectrometer (Bruker, AVANCE III 600,

Massachusetts, USA). The synthesis 4arm-PEG-DCA using 0.5 eq or 2 eq of DCA relative to the -NH₂ group of 4arm-PEG20k-NH₂ was also carried out.

3.2.3 Fmoc protection of (PPG)₁₀

The (PPG)₁₀ (50 mg, 19.8 μ mol) was dissolved in 12.5 mL of 100 mM NaHCO₃ (pH 8.5) and preincubated at 80 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, 66.6 mg of Fmoc-NHS in 12.5 mL THF was added to the solution immediately, and then the reaction mixture was stirred for 24 h at room temperature. After the reaction, the whole reaction mixture was dialyzed (MWCO: 1000 Da) for 3 days against methanol and water. The resulting solution was freeze-dried. The Fmoc(PPG)₁₀ was characterized by ¹H-NMR and MALDI-TOF Mass measurements. The MALDI-TOF Mass spectrum was measured by a Time-of-Flight Mass Spectrometer (JEOL, JMS-S3000, Tokyo, Japan).

3.2.4 Synthesis of 4arm-PEG-DCA/-(PPG)₁₀

After dissolving the Fmoc(PPG)₁₀ (8.4 mg, 3.0 μ mol) in 1 mL of 50 mM Na₂CO₃/NaHCO₃, the solution was preincubated at 80 °C for 5 min to dissociate the triple helices. After a cool-down of the solution in a refrigerator for 5 min, DMT-MM (4.2 mg, 15 μ mol) in 0.1 mL of Milli-Q water was added and stirred at ice bath for 5 min. After the activation, the 4arm-PEG-DCA (21 mg, 1 μ mol) in 0.9 mL of 50 mM Na₂CO₃/NaHCO₃ was added, and then the reaction mixture was stirred for 24 h at room temperature. After the reaction, the whole reaction mixture was dialyzed (MWCO: 3.5 kDa) for 3 days against water at 37 °C. The resulting solution was freeze-dried. The obtained product was characterized by ¹H-NMR and UV-vis measurements. The UV-vis spectrum was measured by a UV-Vis Spectrophotometer (JASCO, V-670, Tokyo, Japan). The UV-vis spectra of Fmoc-NHS in DMSO at 250, 100, 75, 50, 25, 10, 1 μ M at 25 °C were also measured for calibration curve.

Subsequently, after the 4arm-PEG20k-DCA/-Fmoc(PPG)₁₀ (15 mg, 0.65 μ mol) was dissolved in 4 mL of DMSO, excess piperidine (1 mL) was added dropwise and stirred for 2 h. After the deprotection, the whole reaction mixture was washed three times with hexane using a separating funnel. 5 mL of Milli-Q water was added to the lower phase solution, and then dialysis (MWCO: 3.5 kDa) for 3 days against methanol at room temperature and water at 37 °C was carried out. The resulting solution was freeze-dried. The obtained product was

characterized by ^1H -NMR measurement.

3.2.5 Functional characterizations of 4arm-PEG-DCA/-(PPG)₁₀

The 4arm-PEG-DCA/-(PPG)₁₀ was dissolved in PBS at 20 mg/mL. The solution was incubated at 25 °C, heated to 60 °C for 15 min, and re-equilibrated at 4 °C for 1 day. The CD spectra were measured by a CD spectrometer (JASCO, J-1100, Tokyo, Japan) at 25 °C, 80 °C, and 4 °C. Thermal transition curves were obtained by recording the ellipticity at 225 nm using a J-1100 (JASCO, Tokyo, Japan), while the temperature was continuously increased in the range 25-90 °C at a heating rate of 1.0 °C min⁻¹.

3.2.6 Evaluation of cytotoxicity response

NHDF was maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Cells were cultured in 5% CO₂ and 95% humidified air at 37 °C. NHDF was seeded on 96-well plate at 5×10^3 cells/well. After 24 h, 100 μL of 4arm-PEG20k-DCA/-(PPG)₁₀ at 500, 100, 50, 0 μM was added in DMEM medium supplemented with 10% FBS and 1% antibiotics at pH 7.4 in each well and incubated for 24 h at 37 °C. After the incubation, the plates were washed with PBS, and cell counting kit-8 solution diluted 10 times by FluoroBriteDMEM with 1% antibiotics was added. After 2 h at 37 °C, 80 μL of the supernatant was collected to 96-well plates, and the absorbance at 450 nm was measured using a Synergy HTX microplate reader (Agilent Technologies, Santa Clara, U.S.A).

3.2.7 Polymer adsorption to cell

The stock solution of 4arm-PEG20k-DCA/F(PPG)₁₀ (500 μM) in PBS was prepared. The stock solution was preincubated at 50 °C for 15 min and diluted 10 times by FluoroBriteDMEM with 1% antibiotics before the addition. NHDF was seeded on 35 mm glass bottom dish at 2.5×10^5 cells. After 24 h incubation, the dishes were washed with PBS, and 1 mL of the 50 μM 4arm-PEG20k-DCA/FITC (PPG)₁₀ was added. After incubation at 37 °C for 6 h, the plates were washed with PBS, and fixation using 4% Paraformaldehyde phosphate buffer solution was carried out. For the fluorescence imaging, the nuclei were stained with DAPI. After the staining, the dishes were observed using a FV-10i confocal laser microscope (OLYMPUS, Tokyo, Japan). Images were acquired using the same equipment.

The stock solution of 4arm-PEG20k-DCA/Fmoc(PPG)₁₀ (500 μM) in PBS was also

prepared. The stock solution was diluted 10 times by FluoroBriteDMEM with 1% antibiotics before the addition. The 1.0×10^6 HT1080 cells were transferred to 1.5 mL tubes. 1 mL of the 50 μ M 4arm-PEG20k-DCA/Fmoc(PPG)₁₀ was added to the tubes, and keep them at 37 °C for 0, 2, 6 h. After the corresponding points, 200 μ L of the samples was transferred to the new tubes and centrifuged (3500 rpm, 1 min). The supernatant was removed and the precipitate was washed with PBS twice gently. After that, 100 μ L of PBS was added into the tube, and the cells were re-suspended and transfer the suspension to the 96-well plate. And then, the fluorescence intensity was measured using microplate reader (excitation wavelength: 275 nm, emission wavelength: 315 nm).

3.2.6 Cell-cell assembly formation

The 96-well plates were coated with 50 μ L of 0.5wt% Agarose in PBS before the experiments. The stock solution of 4arm-PEG20k-DCA/(PPG)₁₀ (5000 μ M) in PBS was prepared. The stock solution was preincubated at 50 °C for 15 min and diluted 10 times by DMEM with 10% FBS, 1% antibiotics, and 500 μ M EDTA before the addition. NHDF was seeded on the 96-well plate at 5×10^3 cells/well. 100 μ L of 4arm-PEG20k-DCA/-(PPG)₁₀ at 500 μ M was added in each well and incubated for 2 h at 37 °C. After the incubation, the plates were kept at 4 °C for 15 min, and then at room temperature for 2 h with gentle agitation. After incubation, the dishes were observed using a phase-contrast microscope (Thermo Fisher Scientific Inc., Waltham, U.S.A). Images were acquired using the same equipment.

3.3 Results and discussion

3.3.1 Synthesis of 4arm-PEG-DCA

First, 4arm-PEG20k-DCA was synthesized for the introduction of a hydrophobic insertion site and characterized by ¹H NMR measurement (Figures 3-1, 3-2, and Table 3-1). The incorporation amount of DCA was calculated to be 62% from the ¹H NMR spectra (Figure 3-2). The grafting degree of DCA was controlled by the ratio of DCA (Table 3-1). From these results, the synthesis of 4arm-PEG20k-DCA was confirmed.

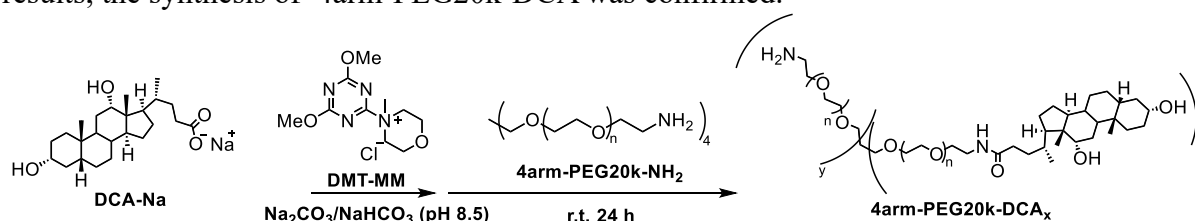


Figure 3-1. Synthesis of 4arm-PEG20k-DCA.

Table 3-1. Synthesis of 4arm-PEG20k-DCA.

	Feed of DCA (eq.) (/NH ₂)	Feed of DMT-MM (eq.) (/DCA)	Grafting Degree (%)
4arm-PEG20k-DCA ₂₂	0.5	5	22
4arm-PEG20k-DCA ₆₂	1	5	62
4arm-PEG20k-DCA ₁₀₀	2	5	~100

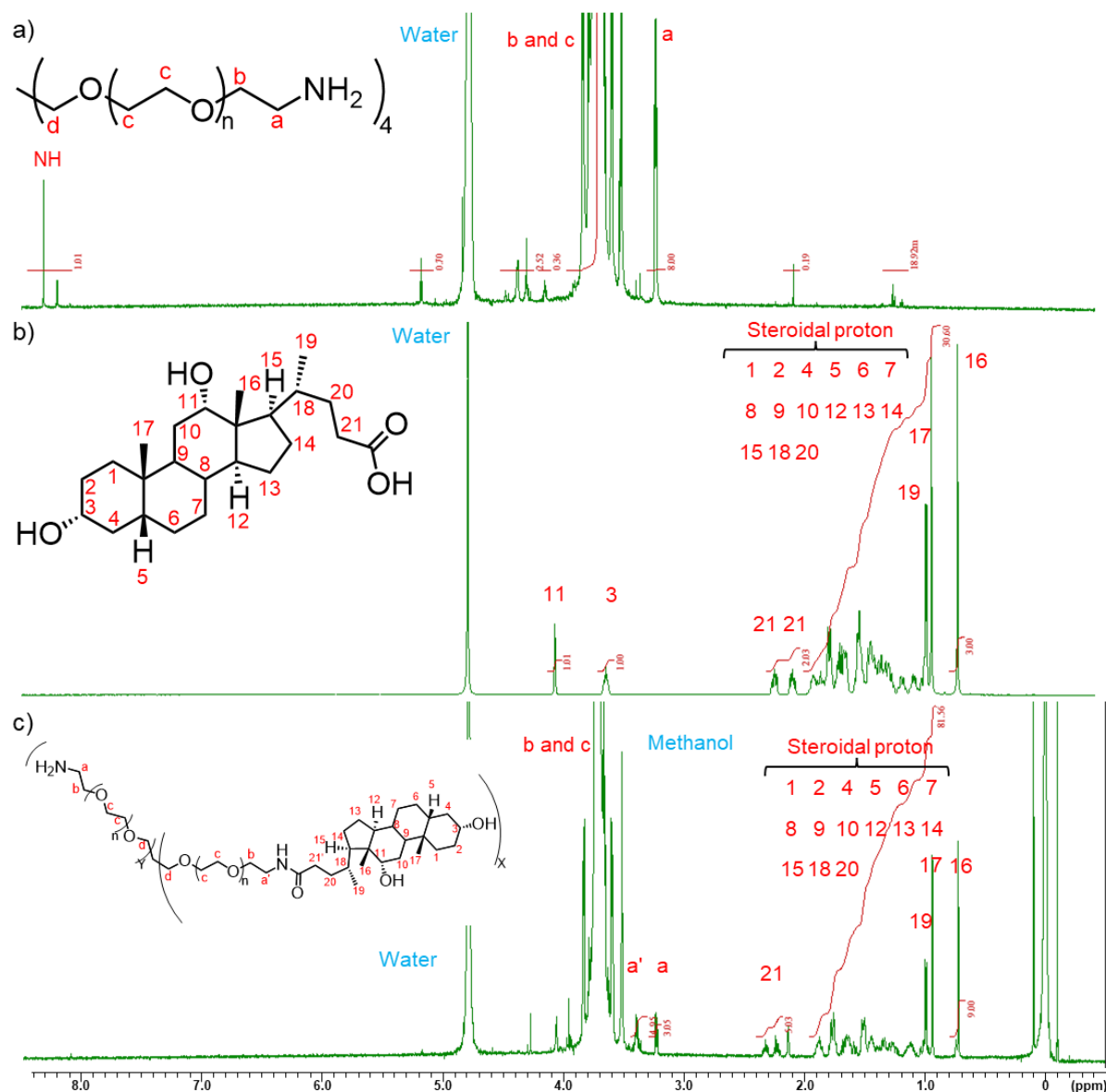


Figure 3-2. ¹H NMR spectra of a) 4arm-PEG20k-NH₂, b) DCA, and c) 4arm-PEG20k-DCA_x in D₂O at 25 °C by the magnetic field at 600 MHz. The grafting ratio of DCA on the modified 4arm-PEG was calculated to be 62% by comparison between the integral of H(a)+ H(a') of 4arm-PEG molecule and H(16) of DCA.

3.3.2 Fmoc protection of (PPG)₁₀

Before introducing (PPG)₁₀ in 4arm-PEG20k-DCA, for the purpose of preventing side reaction, the Fmoc-protection of (PPG)₁₀ was carried out by Fmoc-NHS (Figure 3-3a). The obtained Fmoc(PPG)₁₀ was characterized by ¹H NMR and MALDI-TOF Mass spectra (Figures 3-3 and 3-4). The presence of Fmoc(PPG)₁₀ was confirmed by the Mass spectrum and the grafting degree was determined to be around 100% from ¹H NMR spectra.

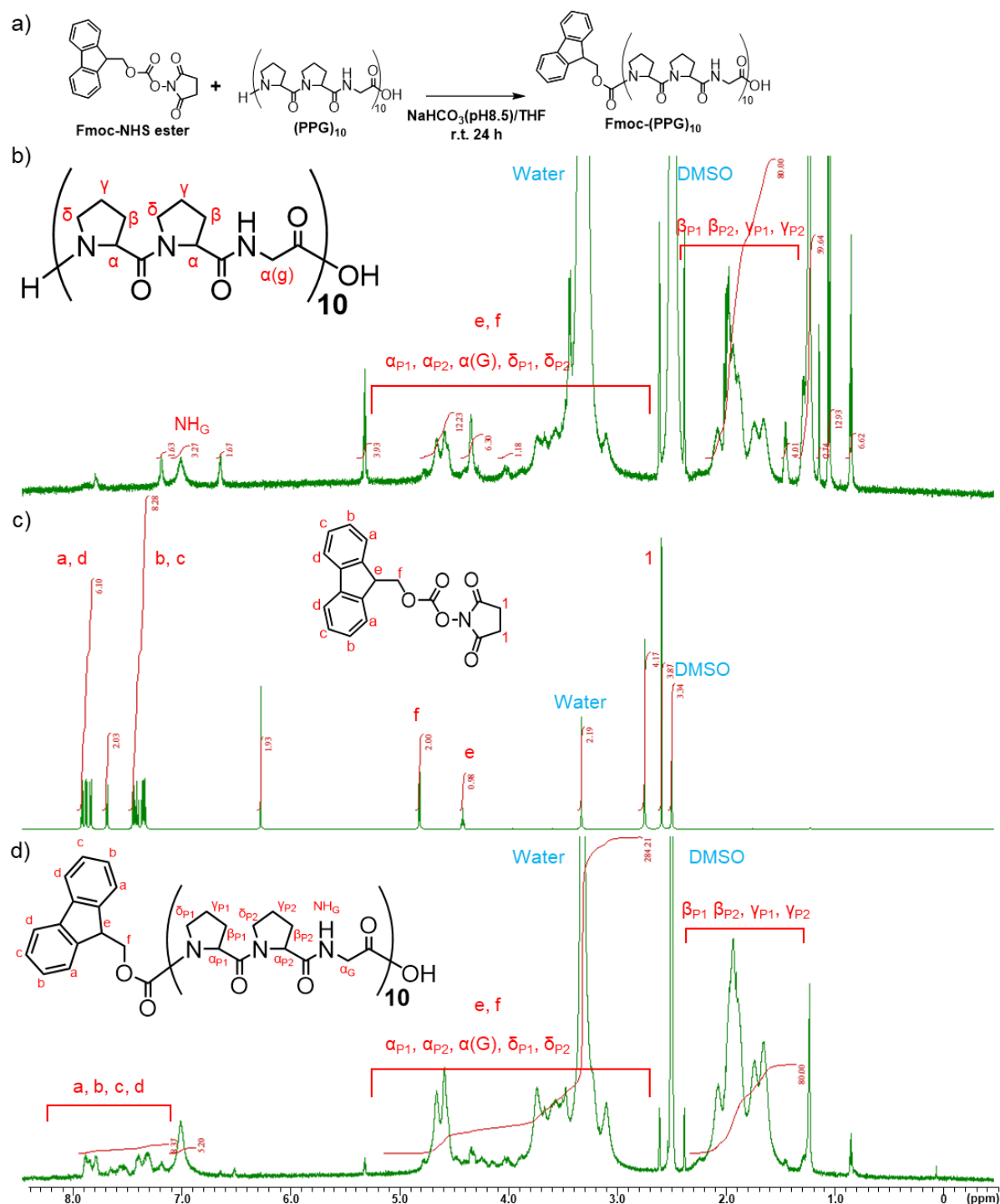


Figure 3-3. (a) Synthesis of Fmoc(PPG)₁₀. ¹H-NMR spectra of (b) (PPG)₁₀, (c) Fmoc-NHS

ester, and (d) Fmoc(PPG)₁₀ in DMSO-*d*₆. The grafting ratio of Fmoc on the labeled (PPG)₁₀ was calculated to be 105% by comparison between the integral of H(a)+H(b)+H(c)+H(d) of Fmoc molecule and H(β and γ) of (PPG)₁₀.

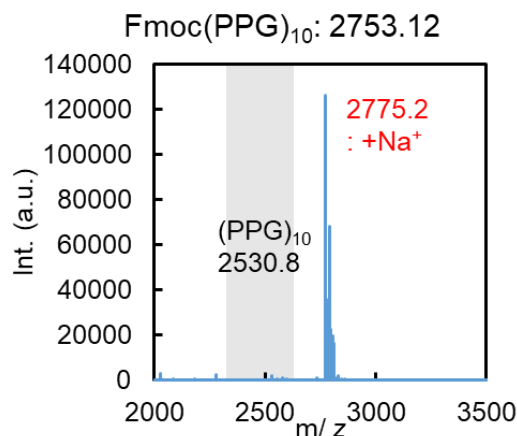


Figure 3-4. MALDI-TOF Mass spectrum of Fmoc(PPG)₁₀.

3.3.3 Synthesis of 4arm-PEG-DCA/-(PPG)₁₀

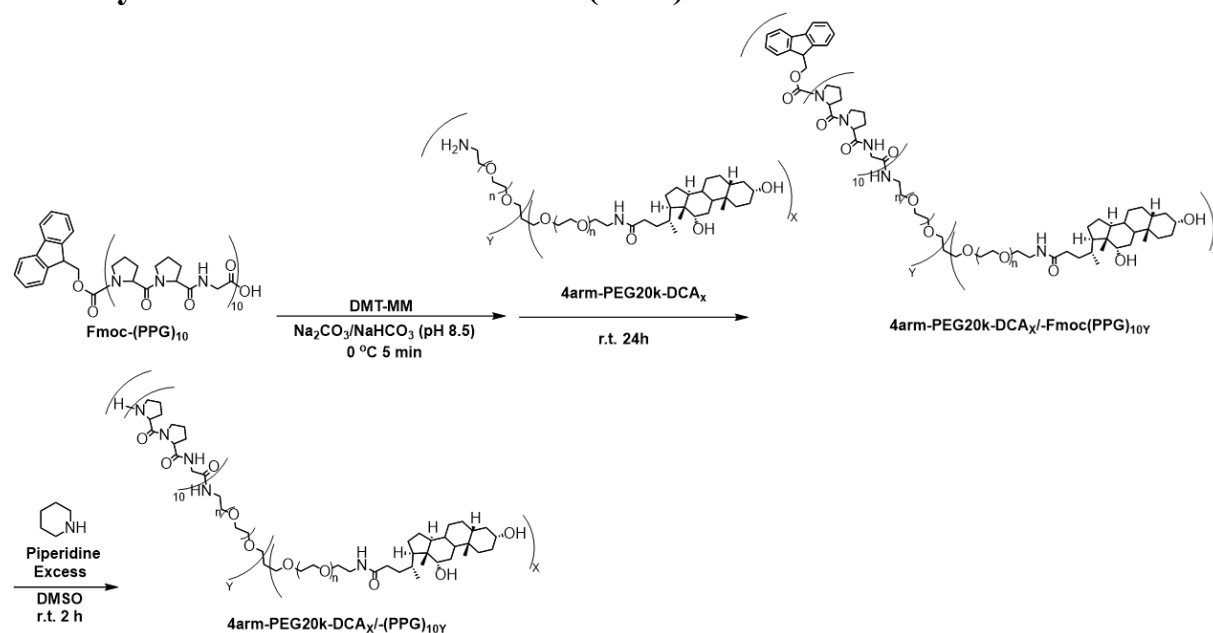


Figure 3-5. Synthesis of 4arm-PEG20k-DCA/-(PPG)₁₀.

The Fmoc(PPG)₁₀ was grafted to 4arm-PEG20k-DCA by amide coupling reaction, and then Fmoc group was deprotected by piperidine (Figure 3-5). The introduction of Fmoc(PPG)₁₀ was suggested by the presence of the Fmoc-derived peak at 7-8ppm (Figure 3-6a) and at around 300 nm (Figure 3-7). The grafting degree was calculated to be around 53.8% from UV-vis measurement (Figure 3-7), and it was expected that the presence of remained non-grafted

Fmoc(PPG)₁₀. The Fmoc-derived peak disappeared in Figure 3-6b after the Fmoc-deprotection reaction. From these results, the synthesis of 4arm-PEG20k-DCA/(PPG)₁₀ was confirmed.

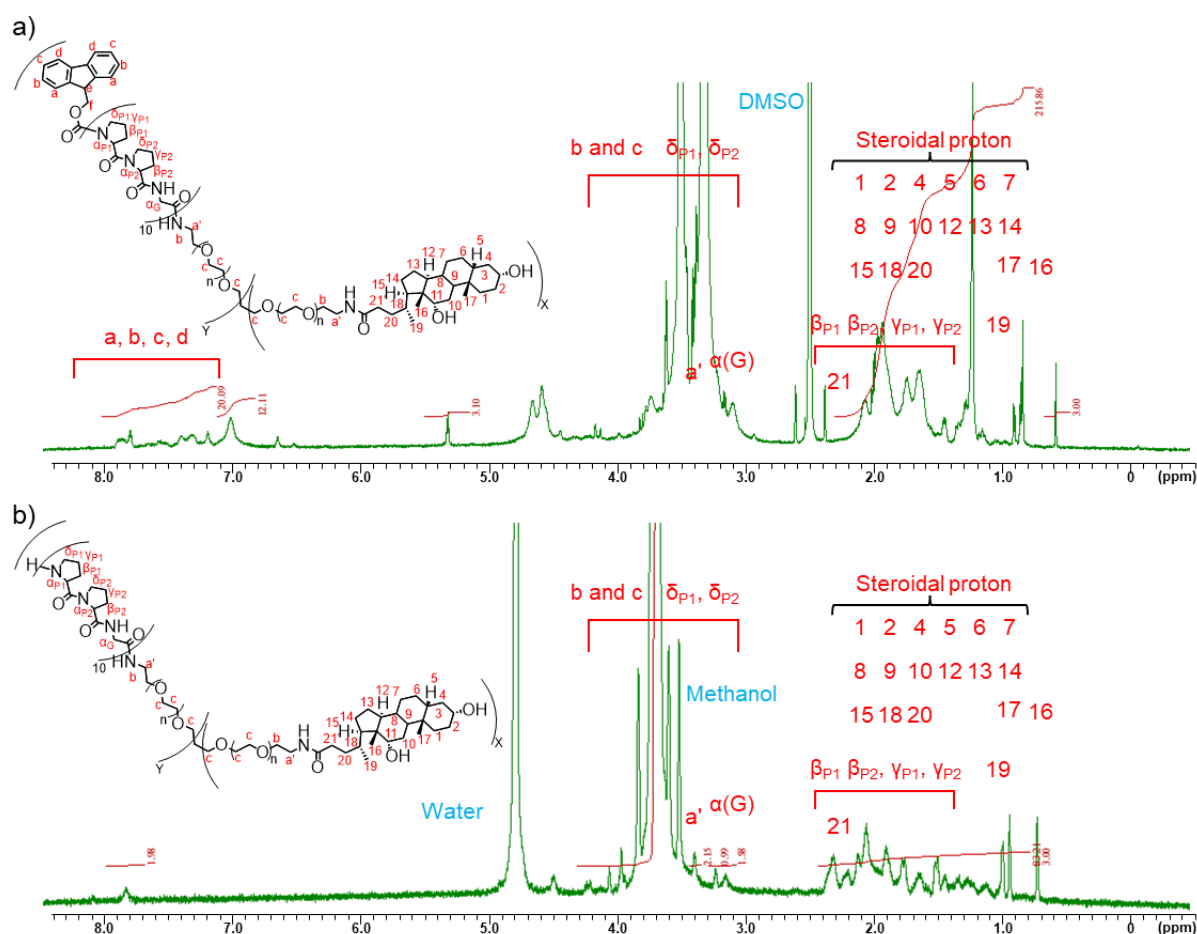


Figure 3-6. ¹H NMR spectra of 4arm-PEG20k-DCA/Fmoc(PPG)₁₀ in DMSO-d₆ and 4arm-PEG20k-DCA/(PPG)₁₀ in D₂O. The grafting ratio of 4arm-PEG20k-DCA/(PPG)₁₀ was calculated to be 38% by comparison between the integral of H(16) of DCA molecule and H(β and γ) of (PPG)₁₀.

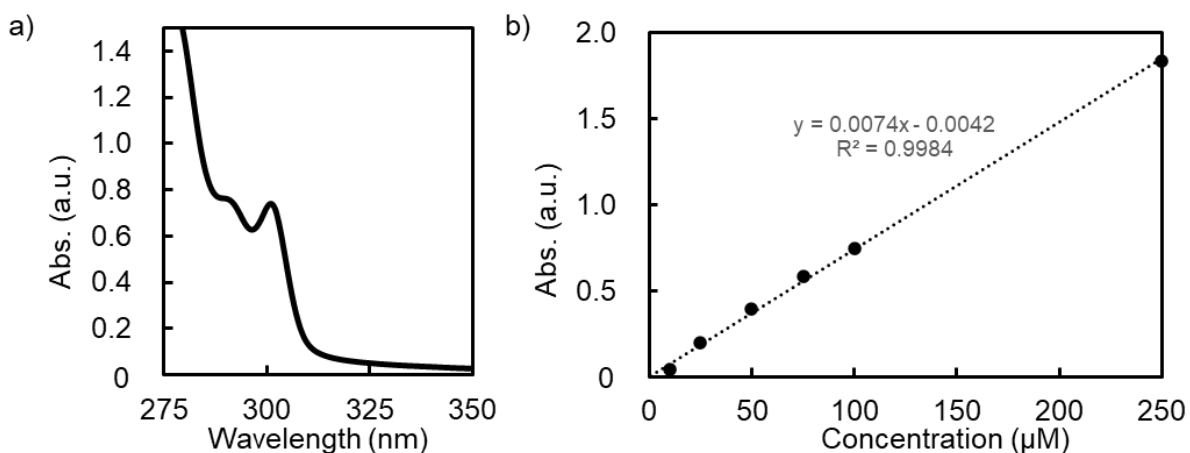


Figure 3-7. (a) UV-vis spectrum of 4arm-PEG20k-DCA_x/Fmoc(PPG)₁₀_Y in DMSO at 25 °C and (b) a calibration curve of Fmoc-NHS in DMSO at 301 nm.

3.3.4 Functional characterizations of 4arm-PEG-DCA/-(PPG)₁₀

Next, the author characterized the triple-helix formation property of 4arm-PEG-DCA/-(PPG)₁₀ by CD measurement (Figure 3-8). To confirm the dissociation and formation of the triple helix at different temperatures, CD spectra at 25 °C, 60 °C and 4 °C were measured. As shown in Figure 3-8a, positive ellipticity associated with a triple helix was observed at 228 nm at 25 °C, but disappeared after incubation at 60 °C, and recovery of positive ellipticity was observed after cooling at 4 °C. These results indicated that 4arm-PEG-DCA/-(PPG)₁₀ exhibits triple-helix formation and dissociation property. The triple-helix stability was also evaluated by CD denaturation measurement (Figure 3-8b). In the spectrum, the denaturation behavior of (PPG)₁₀ moiety was observed, and the T_m was calculated to be 42 °C. From these results, the triple-helix formation property of 4arm-PEG-DCA/-(PPG)₁₀ was confirmed.

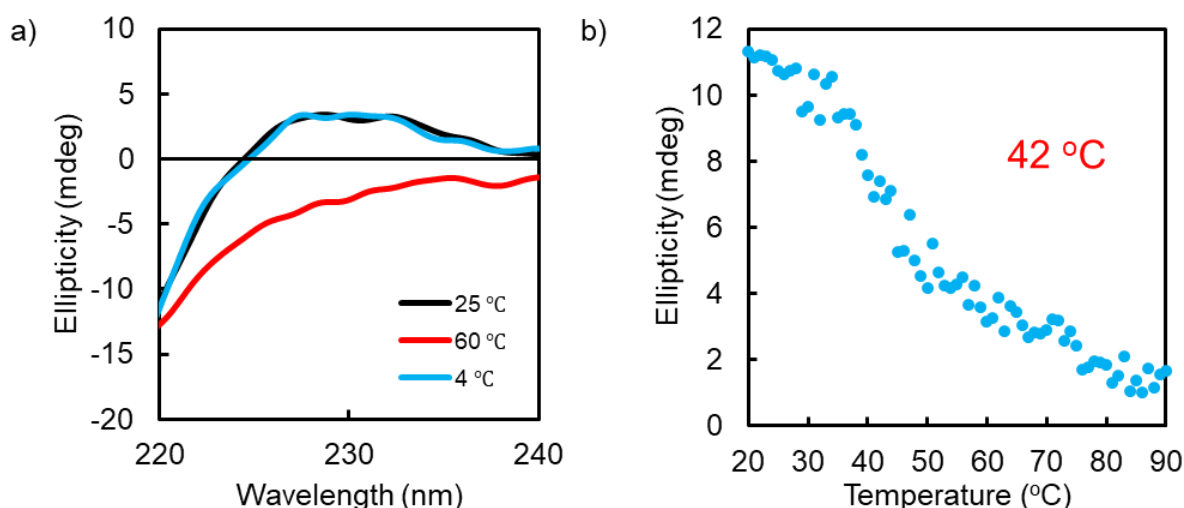


Figure 3-8. Characterization of 4arm-PEG20k-DCA/-(PPG)₁₀. (a) CD profiles of 4arm-PEG20k-DCA/-(PPG)₁₀ at 25 °C, 60 °C, and 4 °C. (b) The melting profile of 4arm-PEG20k-DCA/-(PPG)₁₀ obtained by CD measurement at 228 nm.

3.3.5 Evaluation of cytotoxicity response

Toward applications in cell modification, the cytotoxicity effect of 4arm-PEG20k-DCA/-(PPG)₁₀ was assessed on *in vitro* monolayer cultures of normal human dermal fibroblasts (NHDF) by incubating 4arm-PEG20k-DCA/-(PPG)₁₀ at 500, 100, 50, 0 μM for 24 h (Figure 3-9). Importantly, 4arm-PEG20k-DCA/-(PPG)₁₀ showed a low cytotoxicity at 500, 100, 50, 0 μM

(Figure 3-9). The results suggested that the 4arm-PEG20k-DCA/-(PPG)₁₀ had a low cytotoxicity at concentrations no more than 500 μ M.

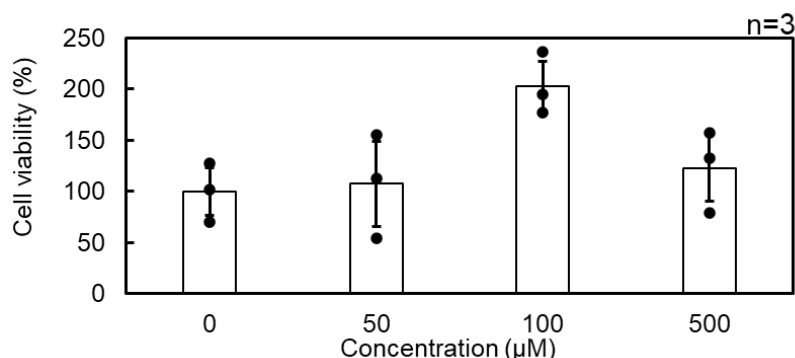


Figure 3-9. The cell viability test after incubation with the 500, 100, 50, 0 μ M 4arm-PEG20k-DCA/-(PPG)₁₀ for 24 h.

3.3.6 Polymer adsorption to cell

To check the polymer adsorption property to cell, first, fluorescein isothiocyanate labeled 4arm-PEG20k-DCA/-(PPG)₁₀ (4arm-PEG20k-DCA/-F(PPG)₁₀) was synthesized and characterized by ¹H NMR (Figure 3-10 and 3-11). After the incubation for 6 h, the confocal laser microscopy image of the NHDF cells showed the fluorescence intensity derived from the 4arm-PEG20k-DCA/-F(PPG)₁₀ on the cells (Figure 3-12a). Furthermore, the polymer adsorption property was also evaluated using 4arm-PEG20k-DCA/-Fmoc(PPG)₁₀ with HT1080 cell (Figure 3-12b). Compared with the control samples, The fluorescence intensity of the cell suspension in PBS was increased after 2 and 6 h incubation with 4arm-PEG20k-DCA/-Fmoc(PPG)₁₀ (Figure 3-12b). These results indicated that the 4arm-PEG20k-DCA/-(PPG)₁₀ was adsorbed to cells.

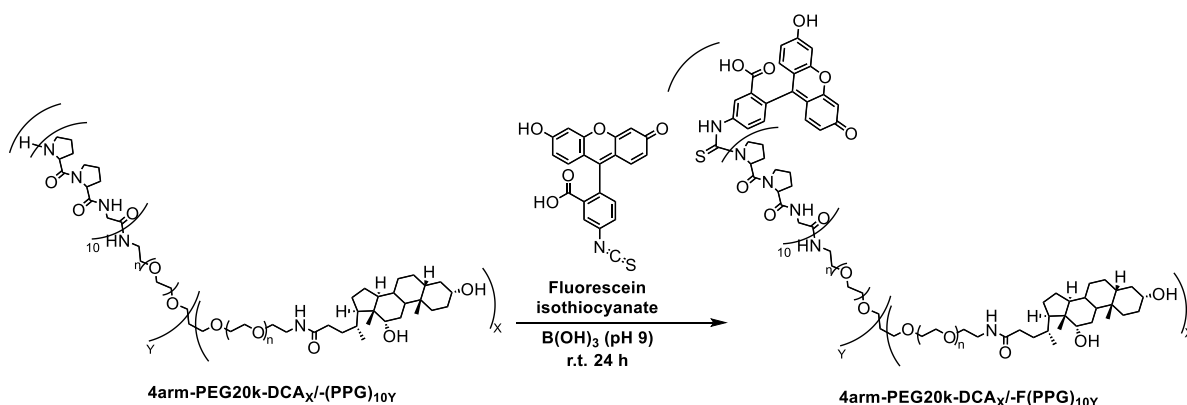


Figure 3-10. Synthesis of fluorescein isothiocyanate labeled 4arm-PEG20k-DCA/-(PPG)₁₀.

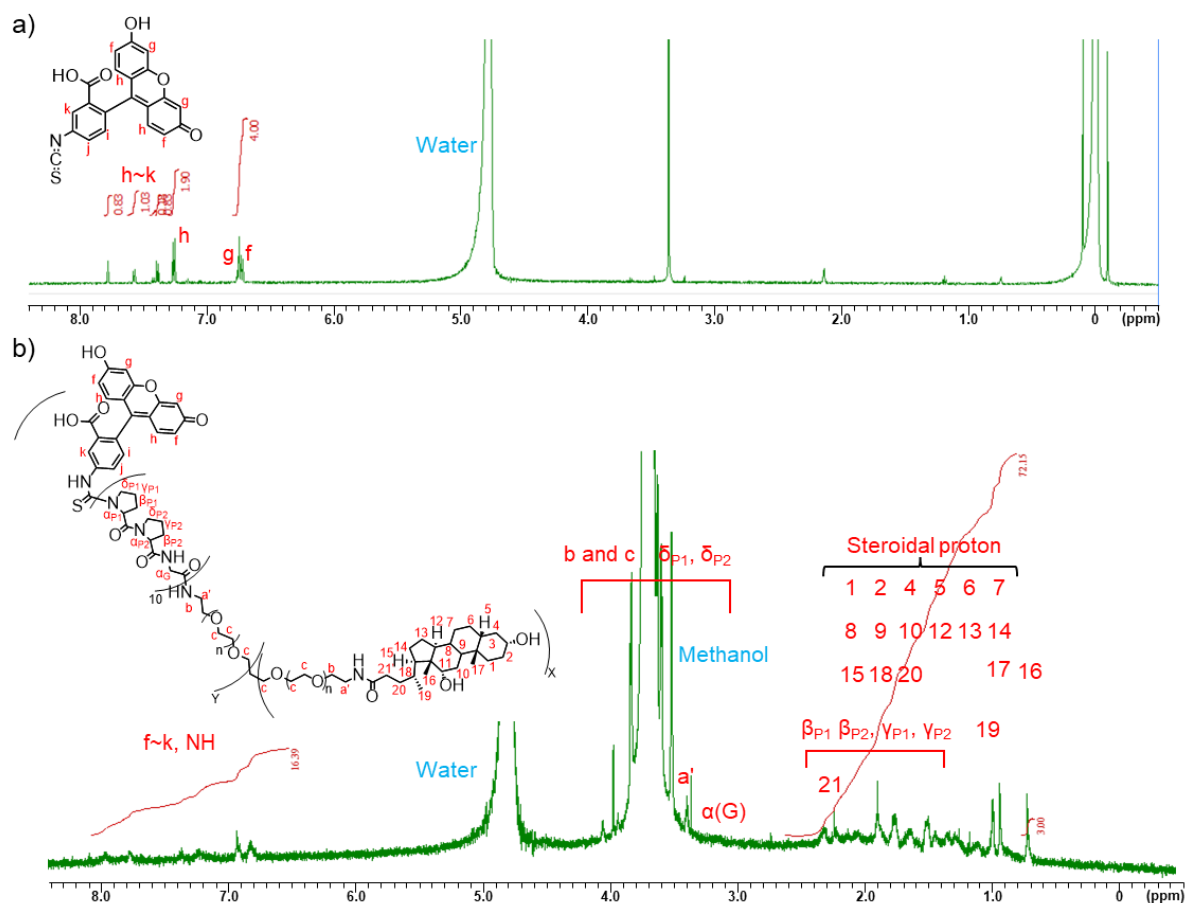


Figure 3-11. ^1H -NMR spectra of (a) Fluorescein isothiocyanate and (b) 4arm-PEG20k-DCA/-F(PPG)₁₀.

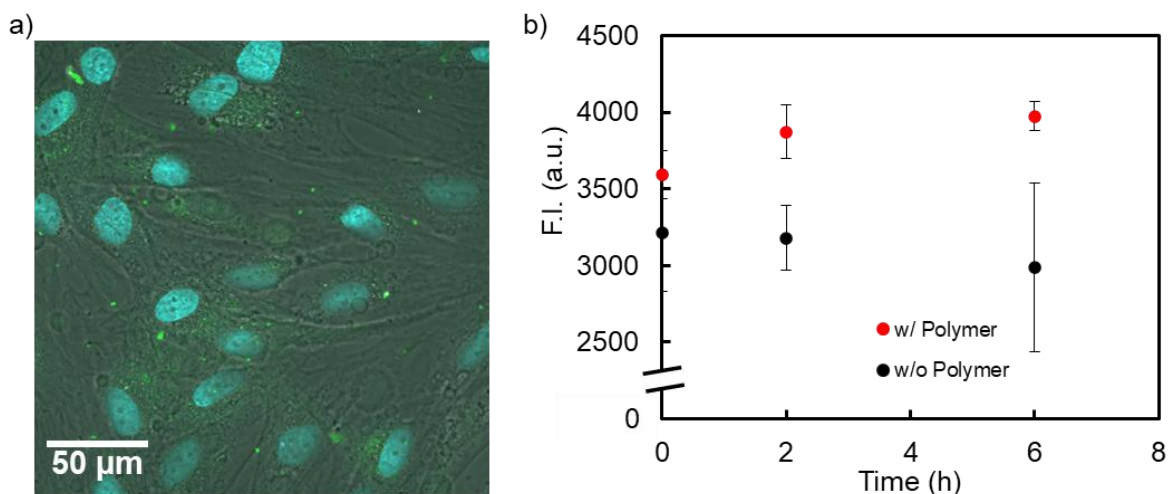


Figure 3-12. (a) The fluorescence image of cells with 4arm-PEG20k-DCA/-F(PPG)₁₀ after incubation for 6 hours. (b) Fluorescence profiles of cell suspension with 4arm-PEG20k-DCA_x/(PPG)_{10Y} or without 4arm-PEG20k-DCA/-Fmoc(PPG)₁₀ at 0, 2, and 6 h after incubation.

3.3.7 Cell-cell assembly formation

The cell-cell assembly formation was assessed by the evaluation of the amount of cell aggregates by the phase contrast microscopy observation. As shown in Figure 3-13a, the phase contrast image of the 4arm-PEG20k-DCA/-(PPG)₁₀-modified NHDF showed the presence of cell aggregates after cooling at room temperature for 2 h. The amounts of cell aggregates with 4arm-PEG20k-DCA/-(PPG)₁₀-modification were 4.3 times higher than the samples without 4arm-PEG20k-DCA/-(PPG)₁₀-modification (Figure 3-13b). From these results, it was suggested that the thermoresponsive cell assembly driven by triple helix formation of CMP.

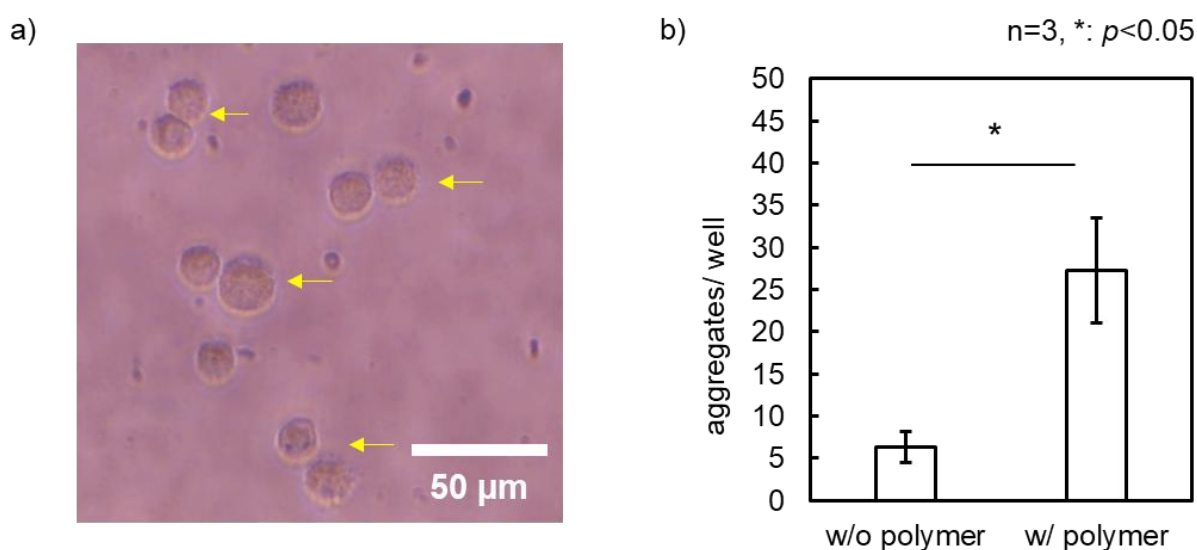
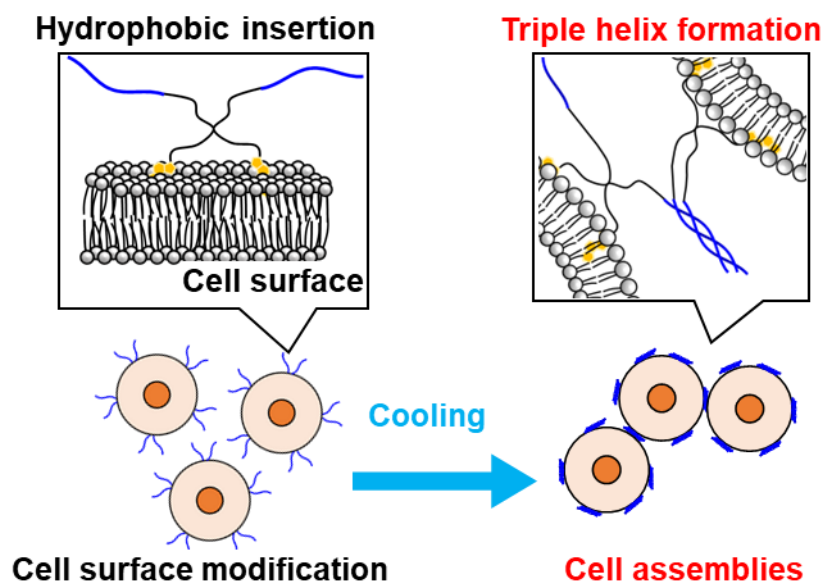


Figure 3-13. (a) A phase contrast image of cell aggregates with 4arm-PEG20k-DCA/-(PPG)₁₀. (b) Number of cell aggregates with 4arm-PEG20k-DCA/-(PPG)₁₀ or without 4arm-PEG20k-DCA/-(PPG)₁₀.

3.4 Conclusion

In chapter 3, the author fabricated the thermoresponsive cell assembly method driven by triple helix formation of CMP. As results of the evaluation, more cell aggregates were formed after cooling under conditions with 4arm-PEG-DCA/-(PPG)₁₀ than without 4arm-PEG-DCA/-(PPG)₁₀. Accordingly, to the best of our knowledge, this is the first report of the thermoresponsive cell assembly method driven by triple helix formation of CMP. The use of CMP is expected to be applied in a new cell assembling approach for tissue engineering.



Scheme 3-2. Thermo-responsive cell assembling method driven by triple-helix formation of CMP.

3.5 References

- [1] S. Choi, K. Y. Lee, S. L. Kim, L. A. MacQueen, H. Chang, J. F. Zimmerman, Q. Jin, M. M. Peters, H. A. M. Ardoña, X. Liu, A.-C. Heiler, R. Gabardi, C. Richardson, W. T. Pu, A. R. Bausch, K. K. Parker, *Nat. Mater.* **2023**, 22, 1039.
- [2] N. Noor, A. Shapira, R. Edri, I. Gal, L. Wertheim, T. Dvir, *Adv. Sci.* **2019**, 6, 1900344.
- [3] V. M. Gaspar, P. Lavrador, J. Borges, M. B. Oliveira, J. F. Mano, *Adv. Mater.* **2020**, 32, 1903975.
- [4] J. Almeida-Pinto, M. R. Lagarto, P. Lavrador, J. F. Mano, V. M. Gaspar, *Adv. Sci.* **2023**, 10, 2304040.
- [5] J. A. Prescher, D. H. Dube, C. R. Bertozzi, *Nature* **2004**, 430, 873.
- [6] Y. Teramura, *Biomaterials* **2015**, 48, 119.
- [7] Y. Teramura, K. N. Ekdahl, K. Fromell, B. Nilsson, K. Ishihara, *Langmuir* **2020**, 36, 12088.
- [8] H. Nakatsuji, Y. Shioji, N. Hiraoka, Y. Okada, N. Kato, S. Shibata, I. Aoki, M. Matsusaki, *Mater. Horiz.* **2021**, 8, 1216.
- [9] Z. J. Gartner, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.* **2009**, 106, 4606.
- [10] P. Shi, Y. Wang, *Angew. Chem. Int. Ed.* **2021**, 60, 11580.
- [11] L. L. Bennink, D. J. Smith, C. A. Foss, M. G. Pomper, Y. Li, S. M. Yu, *Mol. Pharm.* **2017**, 14, 1906.
- [12] S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai, K. Kakiuchi, *BCSJ* **1968**, 41, 1273.

Concluding Remarks

In this thesis, toward the control of material properties through homo- and hetero-triple helices formation of CMP and their application, the author analyzed the assembly properties via homo- and hetero-triple helix formation of CMP, and investigated the hydrogels and cell assembling method with crosslinking characteristics driven by triple helix formation.

In chapter 1, the author evaluated the triple helix formation of two collagen model peptides, (PPG)₁₀ and (POG)₁₀, by fluorescence and CD spectroscopic measurements for the single and mixed conditions. This result was consistent with the trend inferred from the ratio of (PPG)₁₀ to (POG)₁₀ chains in the hetero-triple helix suggested by the fluorescence spectroscopy measurements. In addition, even when the temperature during triple helix formation was set to 37 °C, which is above the T_m of (PPG)₁₀, the formation of the hetero-triple helix was suggested, indicating that (POG)₁₀ might play a major role in the formation of the hetero-triple helix at 37 °C.

In chapter 2, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands. First of all, the 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀ were successfully synthesized. Subsequently, the author analyzed the detailed Sol-Gel transition properties driven by triple helices formation of 4arm-PEG10k-triazole-EG4(PPG)₁₀ and 4arm-PEG10k-triazole-EG4(POG)₁₀, and the effect of additional CMP strands. The 4arm-PEG10k-triazole-EG4CMPs showed gel-sol transition by the internal triple helix formation of CMPs. The gel-sol transition behavior was controlled by the amino acid sequences of CMP and the presence of additional non-modified CMPs. Furthermore, it's possible that the gel properties of 4arm-PEG10k-triazole-EG4CMP can be controlled by additional single-strand CMP. This phenomenon is expected to be applied in biomaterials for the development of new self-assembled materials and to further understanding of triple helix formation-driven sol-gel transition.

In chapter 3, the author fabricated the thermoresponsive cell assembly method driven by triple helix formation of CMP. As results of the evaluation, more cell aggregates were formed after cooling under conditions with 4arm-PEG-DCA/-(PPG)₁₀ than without 4arm-PEG-DCA/-(PPG)₁₀. Accordingly, to the best of our knowledge, this is the first report of the

thermoreponsive cell assembly method driven by triple helix formation of CMP. The use of CMP is expected to be applied in a new cell assembling approach for tissue engineering.

List of Publications

Chapter 1

Analysis of homo- and hetero-triple helix formation of collagen model peptides and evaluation of their stability in a biological environment

Kazuki Yoshida, Satoshi Fujita, and Michiya Matsusaki

Langmuir **2024**, *40*, 3823.

Chapter 2

Triple-helix crosslinked Hydrogels with Sol-gel Transition in Response to Collagen Model Peptides

Kazuki Yoshida and Michiya Matsusaki

Chem. Lett. Accepted.

Chapter 3

Triple Helix Formation of a Collagen Model Peptide Induces Cell Aggregation Formation

Kazuki Yoshida, Edward Vermeersch, Sandra Van Vlierberghe, and Michiya Matsusaki

Biochem. Biophys. Res. Commun. Accepted.

Acknowledgements

This study was carried out from 2020 to 2026 at Department of Applied Chemistry, Graduate School of Engineering, the University of Osaka. In the past six years, I have received a lot of assistance and support during my research. Upon the completion of this thesis, I would like to express my gratitude to all those who have helped me.

Foremost, I am extremely grateful to my supervisor, Prof. Michiya Matsusaki for his invaluable advice, continuous support, and encouragement during this work. I also appreciate that Prof. Matsusaki gave me an opportunity to study abroad at Ghent University. These absolutely broaden my horizon and enrich my life experience.

I am deeply grateful to the thesis committee: Prof. Mamoru Fujitsuka and Prof. Tadashi Mori, for their insightful comments and their donation of time.

I would like to extend my sincere thanks to Prof. Sandra Van Vlierberghe in Ghent University for her kind support for my research in Ghent and article writing. I also thank all the members in Prof. Sandra's research group for their kind support in research in Ghent.

I am deeply grateful to Dr. Satoshi Fujita in National Institute of Advanced Industrial Science and Technology for his insightful comments and suggestions in the experiment, data discussion and article writing. Thanks to Prof. Toshiyuki Kida and Associate Prof. Hajime Shigemitsu for their assistance in XRD measurement.

Besides my supervisor, I am deeply grateful to Associate Prof. Masahiko Nakamoto and Assistant Prof. Kenta Homma for the comments and suggestions. Thanks to Specially Appointed Assistant Prof. Fiona Louis, Dr. Naoko Sasaki, Dr. Hirotaka Nakatsuji, Dr. Dong Hee Kang, Dr. Zhengtian Xie, Dr. Zeng Jinfeng, Dr. Marie Piantino, Dr. Abdul Sisak Muhammad Asri, Dr. Quentin Muller, Dr. Miali Marco Elvino, Dr. Yucheng Shang, Dr. Zhang Zhuying, Dr. Asli Sena Karanfil, Dr. Daisuke Tomioka and Dr. Zhang Huiting for their comments and guidance to my experiments.

I would like to offer my special thanks to Ms. Eri Enomoto, Ms. Chika Sugiki and Ms. Eri Hosokawa for their kind support and help in the laboratory affairs, scholarships application and daily life.

My appreciation also goes to the previous and current fellows in the laboratory: Thanks to Guest Associate Prof. Shiro Kitano, Guest Associate Prof. Shinji Irie, Guest Associate Prof. Akiko Takasuga, Research fellow Takuya Sugiura, Visiting researcher Yoshihiro Kodama, Asuka Kato, Koichi Hattori, Mizuki Mizobuchi, Miki Mizukami, Yasuyuki Naito, Kazumasa Nodake, Ryoko Oodera, Yuka Yoshinouchi and Mizuho Suzuki, Naoya Takakusaki, Hisami Ueno.

Thanks to Ms. Yukiko Sorayama, Ms. Lisa Thibes, Ms. Mizue Onishi, Ms. Saori Nanbu, Ms. Haruka Fujii, Ms. Yukie Shimahara, Ms. Mami Kikkawa, Mr. Kazuma Ishiguro, Mr. Noboru Hiraoka, Mr. Tomoyuki Suezawa, Mr. Shogo Shimada, Mr. Ryo Mitsuyasu, Mr. Rei Miyata, Ms. Yuning Zhang, Ms. Lingyu Pei, Mr. Yuki Koba, Mr. Tomoya Matsuo, Mr. Kazuki Moroishi, Ms. Nozomi Kasahara, Mr. Tamaki Kumauchi, Ms. Misa Miyamoto, Mr. Ryosuke Isobe, Mr. Yusuke Kajiura, Ms. Kaori Hayazaki, Mr. Kazuki Otake, Ms. Chika Nakadozono, Ms. Sukulya Bunuasunthon, Ms. Panitporn Laowpanitchakorn, Ms. Li He, Mr. Rentaro Sakamoto, Ms. Sugandha Singh, Ms. Wu Chun-Yi, Ms. Riko Akehi, Mr. Ryoto Itani, Mr. Kanta Iwamoto, Ms. Aya Nagura, Ms. Itsuki Miyaguni, Ms. Hong Young Kyoung, Ms. Tomoka Iida, Mr. Kanta Ogura, Ms. Rino Kamada, Ms. Ryo Sekiya, Mr. Takuro Kawamoto, Mr. Tomoya Ushiroda, Mr. Kohei Uno, Ms. Sayaka Oshitani, Ms. Reina Funatomi, Mr. Kakeru Yasumoto, Mr. Yoshihide Arai, Mr. Masahiro Yagi, Ms. Mako Kawakami, Mr. Masachika Miyai, Ms. Rinka Motoyama, Ms. Yukino Yoneda, Ms. Ayumi Watanabe and so on. Thanks to their accompany and kind support for both research and daily life.

I would like to thank the research and financial support from Japan Science and Technology Agency (JST, JST SPRING, JPMJSP2138).

I would like to express my deep appreciation to my friends, who endured this long process with me and always offered their support.

I would like to express my heartfelt gratitude to my grandparents, Yosuke Mori and Tomoko Mori, who have offered great support.

Finally, I would like to express my deepest gratitude to my parents, Tadanori Yoshida and Mariko Yoshida, who have been my greatest supporters throughout the completion of my thesis, standing by me with their emotional encouragement and helping me overcome every challenge.

January 2026

Kazuki Yoshida