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Studies on clarification of gelation mechanism of temperature-responsive biodegradable smart biomaterials and their utility as drug releasing devices

温度応答型生分解性スマートバイオマテリアルのゲル化機序の解明と薬物放出デバイスとしての利用に関する研究

研究科・専攻　：理工学研究科・総合理工学専攻

研究領域　：生体材料化学

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博士論文要旨

理工学研究科 総合理工学専攻
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≪論題≫

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≪概要≫

ある種のポリマー水溶液は, 温度等の外部刺激に応答してゾル状態からゲル状態へと転移することが知られている。これらのうち，室温 (生体外) ではゾル，体温付近 (生体内) ではゲルとなる生分解性のポリマーは，注射による投与が可能であり，Injectable Polymer (IP)としての利用が検討されている。生分解性 IP は，投与後は注入部位に留まり，役割を果たした後には生体内で分解して代謝されるため，低侵襲な組織再生用足場材料や薬物徐放デバイスとしての応用が期待されている。その代表例として，疎水性セグメント (A) に乳酸-グリコール酸共重合体 (PLGA) ，親水性セグメント (B) にポリエチレングリコール (PEG) を有する ABA 型トリブロック共重合体 (PLGA-*b*-PEG-*b*-PLGA) などの脂肪族ポリエステルと PEG とのブロック共重合体が知られている。このような IP については，既に数多くの研究成果が報告されており，臨床応用への期待も高まってきているが，解決すべき幾つかの課題が残っている。

論文提出者の所属する研究室では，疎水性セグメントにカプロラクトン-グリコール酸共重合体 (PCGA) を用いることで, 凍結乾燥による粉末化を可能とし, さらに PEG を添加することにより，凍結乾燥後の粉末試料から注射用分散製剤を調製する時間を著しく短縮することを見出し，臨床現場において即時調製可能な温度応答型ポリマー溶液の開発に成功した。また，温度に応答してゲル化を起こす際に，共有結合を形成し，体液等で高度に湿潤した環境下においても長時間ゲル状態を維持することができる IP 製剤の開発にも成功している。

このような温度に応答して共有結合ゲルを形成するブロック共重合体システムでは, 低温における架橋反応の生起を抑えつつ，温度上昇に伴ってゲル化が起こる時にのみに架橋反応を進行させる必要があり，ミセル状に溶解したポリマーの交換反応の抑制が鍵となる。しかしながら，ミセル様の溶解状態におけるポリマー鎖交換反応やその温度依存性に関する知見は未だ十分には得られておらず，さらに機能を高めた IP 製剤を合理的に設計・開発するためには，そのメカニズム解析が重要である。また，開発した共有結合形成 IP システムが，非共有結合型 IP システムと比較して，低分子および高分子薬物の徐放化にもたらす効果についてはまだ検討されていない。

そこで本論文では，PCGA-*b*-PEG-*b*-PCGA ポリマーを基軸としたポリマー群を用いて，ミセル状態でのポリマーの交換過程を含むゲル化メカニズムの解明と，実際の薬物徐放デバイスとしての応用を意図したペプチド性薬物の徐放化に関して検討を加えた。

本論文は，4 つの Chapter から構成される。以下，本論文の内容を Chapter ごとに要約する。

≪Chapter 1≫

Chapter 1 では，徐放性注射製剤の概要を紹介するとともに，温度応答型生分解性 IP の課題や望まれる諸特性を示し，本研究の目的について述べる。

≪Chapter 2≫

Chapter 2 では，温度応答型生分解性 ABA 型トリブロック共重合体がゾル-ゲル転移するメカニズムの解析を検討した結果について述べる。ABA 型トリブロック共重合体は水溶液中でフラワーミセルを形成することが知られているが，フラワーミセルを形成するミセル間のポリマー鎖交換については議論されていない。そこで本研究では，蛍光共鳴エネルギー移動 (Fluorescence Resonance Energy Transfer, FRET) 現象を利用し，ミセルを形成するポリマー鎖の交換状態から PCGA-*b*-PEG-*b*-PCGA のゲル化過程を考察した。FRET が起こる蛍光物質の組み合わせとして，ナフタレンとダンシルを選択し，各蛍光物質を PCGA-*b*-PEG-*b*-PCGA の末端ヒドロキシル基 (tri-PCG) に修飾した。ナフタレンを修飾した tri-PCG-nap のミセル溶液およびダンシルを修飾した tri-PCG-dan のミセル溶液を各々調製し，混合後，温度を上昇させることにより FRET が起こることを確認した。さらにゲル化するポリマー濃度にて，各温度で 1 分後の FRET の挙動を確認したところ, ゲル化温度の前後で立ち上がりが認められるシグモイド様曲線を描くことが判明した。しかし，ゲル化する前からミセルを形成するポリマー鎖の交換が起こることも明らかとなった。ゲル化する前からミセル間においてポリマー鎖の交換が起こることは，共有結合ゲルを形成するブロック共重合体システムでは不利である。事実，共有結合ゲルを形成するブロック共重合体システムでは，室温下 3 時間程度でゲル化する現象が認められた。一方，凍結乾燥から得られた温度応答型生分解性ポリマーの粉末を PBS で分散した溶液では，室温下 24 時間経過した場合もゲル化は認められなかった。そこで，FRET によるポリマー鎖の交換を調査したところ，溶液に比べ, ポリマー鎖の交換が抑えられていることを明らかにした。以上より, 温度応答型生分解性 ABA 型ブロック共重合体が形成するミセルは，ポリマー鎖の交換が起こっていること，凍結乾燥粉末の分散液ではポリマー鎖の交換を押えることが出来ることを明らかとし，今後の温度応答型生分解性ポリマーの設計に役立つと考える [1)]。

≪Chapter 3≫

Chapter 3 では，薬物徐放デバイスとしての応用にむけた検討結果について述べる。論文提出者の所属する研究室において，tri-PCG の末端のヒドロキシル基にアクリロイル基を修飾した tri-PCG-Acryl 水溶液と，水に不溶なポリチオールである Dipentaerythritolhexakis (3-mercaptopropionate) (DPMP) を内包した tri-PCG ミセル水溶液を混合したシステムは，温度上昇に伴ってゲル化が起こる際に共有結合を形成することにより，tri-PCG 単独に比べ，ゲル状態における力学強度の向上および生体内でのゲル状態の維持期間を長くすることに成功している。さらに tri-PCG-Acryl と DPMP の含有率を変化させることで, ゲル状態の維持期間を自在に調整できることを明らかにしている。この系を，高分子および低分子の薬物を徐放する製剤として利用した

場合には，共有結合の形成によるゲル状態の長期間維持効果により，これまでの IP 製剤と比較して，長期間の薬物徐放が達成されることが期待できる。そこで本研究では，tri-PCG-Acryl 及び DPMP 内包 tri-PCG システムにおいて，GLP-1 を薬物モデルペプチドとして選択し，その製剤作成と薬物放出挙動について *in vitro* 及び *in vivo* において検討した。結果，従来の温度応答性ポリマーに比べ，tri-PCG-Acryl と DPMP の含有率が高い温度応答性ポリマー，すなわち，ゲルの維持期間が長い製剤の方が，GLP-1 の血中維持期間が長いことが分かった。このことから，後期における薬物放出は，ゲルの崩壊と共に薬物が放出されていると考えられた [2)]。

≪Chapter 4≫

Chapter 4 では，本研究で得られた知見や成果についてその内容を総括し，温度応答型生分解性スマートバイオマテリアル分野に対する本研究の貢献とその意義を述べる。

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以　上

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Chapter 1

General Introduction

1.1 Parenteral depot formulation

There are various parenteral dosage forms, such as solutions, suspensions, emulsions, liposomes, micelles, implants and microspheres. However, long-term (over 1 month) sustained release formulations are limited to suspensions[1-3], implatns[4,5], and microspheres[6,7]. These formulations are deposited at the subcutaneous or intramuscular and gradually release their drug. Parenteral depot formulations have the potential to avoid the need for constant infusion of the drug or a very high frequency of injections, thereby enhancing patient compliance. Furthermore, depot formulations can minimize undesirable side effects caused by fluctuating drug blood levels typical of immediate release products, and formulations with various drug release duration times have been developed.

Parenteral depot formulations for hydrophilic compounds are limited to implants and microspheres. Implants allow the longest duration drug release of all parenteral depot formulations (e.g., one year)[4]. However, subcutaneous implants require use of a large needle gauge or surgery (e.g., Zoladex®), causing patient anxiety and discomfort. In contrast, microspheres are administrated using a smaller needle gauge, causing less stress to the patient. However, the disadvantages of microspheres are high manufacturing cost, a difficult administration procedure, and a danger of incomplete dispersion and syringe clogging[8,9]. Given the drawbacks of implants and microspheres, there has been significant effort to develop alternative depot systems with the following characteristics:

1) Painless and easy administration through small needle gauges

2) Easy to manufacture at low cost

1.2 In situ forming implant

Some formulations are a low-viscosity liquid prior to injection and solidify into a semi-solid or solid depot at the injection site after administration. These drug-loaded formulations can be injected using a small-gauge needle and the drug is released gradually from the depot at the injection site. Formulations with such properties are typically known as in situ forming implant (ISFI) systems. ISFI systems are expected to be less invasive and less painful than implant or microsphere depots, and many injectable and biodegradable ISFI systems have been developed. ISFIs are classified according to their mechanism of implant formation[8-11].

(1) In situ polymer precipitation

(2) Thermoplastic pastes

(3) In situ cross-linked systems

(3-1) Chemically cross-linked injectable polymer systems

(3-2) Physically cross-linked injectable polymer systems

1.2.1 In situ polymer precipitation

An in situ polymer precipitation system was proposed by Dunn et al.[12] and formed the basis of ISFI systems. A water-insoluble and biodegradable polymer is dissolved in an organic solvent, and the a drug is mixed in. When this formulation is injected into the body, the organic solvent diffuses and water penetrates into the organic phase. This leads to phase separation and precipitation of the polymer, forming a depot at the injection site. ATRIX Laboratories developed a technology called Atrigel® based on this method[13,14]. The most advanced product using Atrigel® technology, Eligard®, contains the luteinizing hormone-releasing hormone (LHRH) agonist leuprolide acetate and poly(DL-lactide-*co*-glycolide: 50/50, 75/25 and 85/15) (PLGA) dissolved in N-methyl-2-pyrrolidone (NMP). Eligard® is achieved to maintain the serum level of testosterone for 6 months with safety and efficacy. However, the NMP solvent has raised concerns with respect to biocompatobolity[15].

1.2.2 Thermoplastic pastes

Thermoplastic pastes are based on polymers with a melting point or glass transition temperature in the range of 25 °C to 65 °C. These polymers are heated above their melting point or glass transition to a liquid state before injection. After administration, they cool to body temperature and form a semi-solid depot in the body. Drugs are mixed with the molten polymer without the use of organic solvents. A semi-solid poly(ortho esters) (POE) was developed by Heller et al. as a biodegradable material[16]. Four generations of POEs have been developed, starting in the 1970s. The third- and fourth-generation POEs are highly biocompatible, and the fourth-generation POE can be manufactured reproducibility. These POEs are in a semi-solid state at room temperature (r.t.), allowing therapeutic agents to be mixed in without heating or the use of solvents. However, POEs cannot be injected through a small-gauge needle:

for example, third-generation POEs cannnot be easily injected through a 20-gauge needle[17].

1.2.3 In situ cross-linked systems

There are two types of in situ cross-linked systems: covalently (chemically) crosslinked systems and non-covalently (physically) cross-linked systems.

1.2.3.1 Chemically cross-linked systems

Chemically cross-linked systems can be categorized into two groups: radical polymerization systems (including photopolymerization) and chemical reaction systems.

In general, radical polymerization systems require two materials: polymers containing double-bonds and a free radical initiator. However, cross-linking agents used for free radical initiation are typically toxic[18,19]. In contrast, ultraviolet (UV) or visible light are required for photopolymerization and photosensitive initiators (e.g., eosin dyes) are less toxic than free radical initiators. Consequently, photopolymerization systems hold promise for various biomedical applications[20]. Although the use of in situ gels formed by UV irradiation is mainly limited to dental applications, photopolymerization systems can be also used for minimally invasive surgery attendant therapies such as peritumoral injection after surgery by using catheters or laproscopic devices, allowing direct exposure of the formulations to UV light[9].

Chemical reactions such as amine-succinimide reaction[21], Michael-type addition, disulfide formation, Diels-Alder reaction, azide-alkyne cycloaddition[22], Schiff base formation between an amine and aldehyde[23] were reported as in situ gelation systems. In such systems, two water-soluble polymers with different functional groups (OH, SH, COOH, NH_2) are separately dissolved in aqueous solution, and then mixed to allow the functional groups to react before injection. A solid (gel) state is formed from the liquid (sol) state by the formation of a covalent cross-linked network in the body. For example, Michael-type 1,4-addition occurs upon nucleophile attack on the β position of an α,β-unsaturated carbonyl compound. Thiols, alcohols and amines are often used as the nucleophile. Thiol groups are suitable for use in cross-linking biomaterial hydrogels because the thiol groups have a lower pKa (about 8.0) and stronger nucleophilicity than amine and hydroxyl groups, respectively. Therefore, this reaction

is highly selective even under physiological conditions (37 °C, pH = 7.4). Michael-type addition between thiol and maleimde[24], acryloyl[25] and vinyl sulfone groups[26] has been reported for biomaterial applications, but it is generally difficult to control the gelation time.

1.2.3.2 Physically cross-linked systems

Physical cross-linking between polymers is due to hydrophobic interactions[27], ionic interactions[28], or stereocomplex formation[29] triggered by external stimuli under physiological temperature, pH and ionic length. Temperature-responsive polymers can be especially attractive for use in ISFI system as they do not require organic solvents, copolymerization agents, or externally applied triggers[9]. Such polymer solutions with a sol-to-gel transition point between r.t. and body temperature have been extensively investigated as injectable polymer (IP) systems. An aqueous solution of a polymer is in the sol state at r.t. and thus water-soluble bioactive reagents such as proteins, peptides or living cells can be mixed and easily injected into the body to form a hydrogel at the desired site. Furthermore, biodegradable IP systems are more likely to be applied clinically as implantable materials compared to non-biodegradable IPs such as poly(N-isopropyl acrylamide)[30] and triblock poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)[31] because they can be excreted from the body after completing their function.

Such biodegradable thermo-gelling polymer systems have the advantage of rapid gelation in response to a temperature increase, although several issues remain regarding previously reported biodegradable IP systems intended for clinical application[32,33]. For example, once the hydrogel is formed in the body, it is likely to quickly revert to the sol state (typically in less than 24 h) in the presence of a large amount of water or body fluid, such as in the intraperitoneal space or the interior of a blood vessel. This reversion is due to gelation being dependent on non-covalent (hydrophobic) interactions, and gel formation is an equilibrium process controlled by the conditions, such as temperature and concentration of the gel. This may cause the hydrogel to disintegrate before it can perform its function, or the rapid release of bioactive agents.

1.3 Next-generation temperature-responsive biodegradable IP systems

Ohya et al. recently reported biodegradable temperature-triggered covalent gelation systems exhibiting longer and controllable duration times of the gel state by a "mixing strategy"[32,34]. They synthesized a tri-block copolymer of poly(ε-caprolactone-co-glycolide) (PCGA) and poly(ethylene glycol) (PEG), PCGA-*b*-PEG-*b*-PCGA (tri-PCG), by attaching acryloyl groups on both termini (tri-PCG-Acryl). A mixture of tri-PCG-Acryl micelle solution and tri-PCG micelle solution containing hydrophobic hexa-functional polythiols exhibited an irreversible sol-to-gel transition by covalent cross-linking in response to a temperature increase, and a longer and controllable duration of the gel state[34]. The acryloyl groups of tri-PCG-Acryl in the micelle did not react with the polythiol molecules immediately after mixing because the polythiols are present in separate micelle cores. However, upon inter-micellar aggregation during the sol-to-gel transition, covalent cross-linking occurs by thiol-ene reaction of acryloyl groups of tri-PCG-Acryl with the polythiol molecules.

1.4 Overview of this work

In this work, I attempt to understand the sol-to-gel transition behavior of temperature-responsive injectable polymer systems and apply these biodegradable temperature-triggered covalent gelation systems to drug release device. This thesis contains the following two chapters.

In Chapter 2, I investigated polymer chain transfer (exchange) between micelles composed of temperature-responsive biodegradable polymer during the gelling process. Amphiphilic triblock copolymers of PEG and aliphatic polyesters such as PCGA are typical examples of thermo-gelling polymers. The gelation mechanism of these polymers comprises two important steps: polymer chain transfer between the micelles, and subsequent aggregation of the polymer micelles. We previously reported an IP system exhibiting a temperature-responsive irreversible sol-gel transition that formed a covalently cross-linked hydrogel[34]. An IP formulation prepared by the "freeze-dry with PEG/dispersion" method (**D**-sample) retained its sol state at r.t. longer than a formulation prepared by the usual dissolution method (**S**-sample)[35]. I hypothesized that polymer chain transfer between micelles was suppressed in the **D**-samples because the micelle cores were in a solid-like semi-crystalline state. In this study, I

investigated polymer chain transfer using a fluorescence resonance energy transfer (FRET) method to reveal the role of polymer chain transfer in the sol-to-gel transition. I synthesized a triblock copolymer of PCGA and PEG (tri-PCG) with covalently attached naphthalene or dansyl groups at both termini, generating tri-PCG-nap and tri-PCG-dan for use as a FRET donor and acceptor, respectively. Investigation of the FRET behavior of a mixture of tri-PCG-nap/tri-PCG micelles and tri-PCG-dan/tri-PCG micelles revealed that polymer chain transfer between micelles was strongly accelerated at the gelation temperature, and polymer chain transfer in **D**-samples was suppressed compared to **S**-samples.

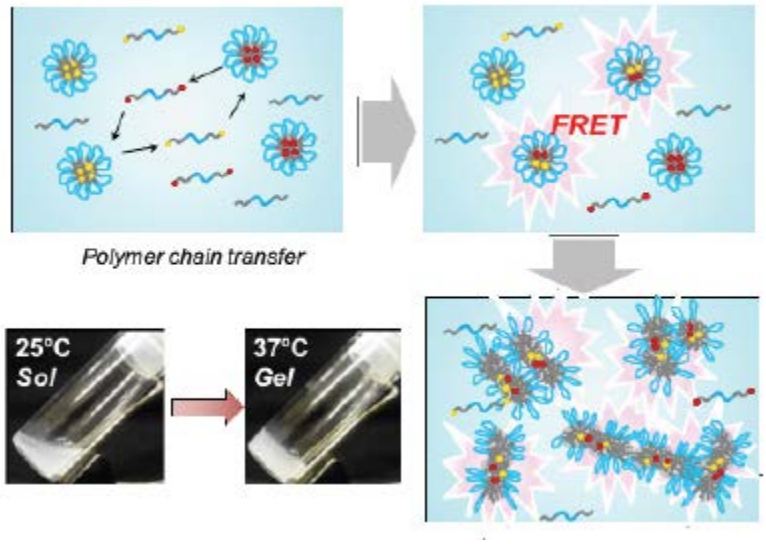

Figure 1. Illustration showing the experiments described in Chapter 2.

In chapter 3, I investigated the release behavior of glucagon-like peptide-1 (GLP-1) from a biodegradable injectable polymer (IP) hydrogel. This hydrogel shows temperature-responsive irreversible gelation due to covalent bonds formation through a thiol-ene reaction. *In vitro* sustained release of GLP-1 from an irreversible IP formulation was observed compared with a reversible (physical gelation) IP formulation. Moreover, pharmaceutically active levels of GLP-1 were maintained in blood after subcutaneous injection of the irreversible IP formulation into rats. This system holds promise the minimally invasive sustained release of peptide drugs and other water-soluble bioactive reagents.

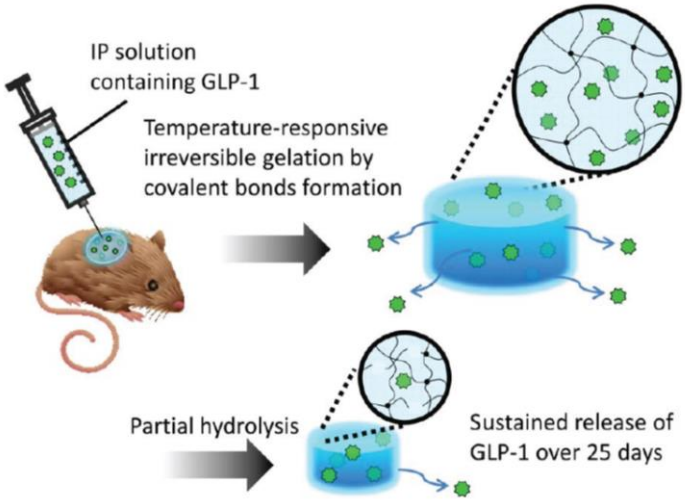

Figure 2. Illustration showing the experiments described in Chapter 3.

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Chapter 2

Analysis of the sol-to-gel transition behavior of temperature-responsive injectable polymer systems by fluorescence resonance energy transfer

2.1 Introduction

Some types of polymer solutions exhibit sol-to-gel transitions in response to a temperature increase. Thermo-gelling polymer systems having sol-to-gel transition points between room temperature (r.t.) and body temperature have been extensively investigated as injectable polymer (IP) systems for biomedical applications. An aqueous solution of such a polymer is in the sol state at r.t. and can easily be injected into the body to form a hydrogel at the desired site. Biodegradable IP systems are expected to be applied clinically as implantable materials, because they can be excreted from the body after their expected roles are completed. Water-soluble bioactive reagents, such as proteins and peptides or living cells, can be mixed with the polymer solutions and injected at a desired site to form a hydrogel. Therefore, such biodegradable IP systems have been investigated for adapting to minimally invasive drug delivery systems (DDS) exhibiting sustained release of drugs[1,2], biodegradable scaffolds in regenerative medicine[3,4], materials for adhesion prevention after surgical operations[5,6], embolization treatments of blood vessels[7], and endoscopic submucosal dissection (ESD) materials[8]. Amphiphilic block copolymers of poly(ethylene glycol) (PEG) as hydrophilic segments and biodegradable aliphatic polyesters as hydrophobic segments are known as typical examples of biodegradable IPs. For example, ABA triblock copolymers using poly(D,L-lactide) (PDLLA)[9], poly(D,L-lactide-co-glycolide) (PLGA)[10,11], poly(ε-caprolactone) (PCL)[12,13], and poly(ε-caprolactone-co-glycolide) (PCGA)[14-16] as hydrophobic segments have been reported.

Such biodegradable thermo-gelling polymer systems have an advantage of rapid gelation in response to a temperature increase, however there have been some issues in previously reported biodegradable IP systems for clinical applications. The hydrogel once formed in the body is likely to revert to the sol state in a short period (typically less than 24 h) in the presence of a large amount of water or body fluid, such as in the intraperitoneal space or the interior of a blood vessel, because the gelation is caused by non-covalent (hydrophobic) interactions, and gel formation is an equilibrium process controlled by the surrounding conditions such as temperature and concentration. This may cause the disappearance of the hydrogel before it can perform its role or the rapid release of bioactive agents. Another problem is with the preparative processes for IP formulations. Most biodegradable IPs are sticky pastes in the dry state at r.t., and it takes a long time to dissolve them in aqueous solution. These

characteristics are inconvenient in clinical settings and are obstacles for clinical applications of IP systems.

To solve these two problems, we recently reported biodegradable temperature-triggered covalent gelation systems exhibiting longer and controllable duration times of the gel state by a "mixing strategy"[17,18]. We synthesized a tri-block copolymer of PCGA and PEG, PCGA-*b*-PEG-*b*-PCGA (tri-PCG), attaching acryloyl groups on both termini (tri-PCG-Acryl). A mixture of tri-PCG-Acryl micelle solution and tri-PCG micelle solution containing hydrophobic hexa-functional polythiols exhibited an irreversible sol-to-gel transition by covalent cross-linking in response to a temperature increase, and exhibited a longer and controllable duration time for the gel state[18]. Just after mixing, the acryl groups of tri-PCG-Acryl in the micelle did not react with polythiol molecules because they existed in other micelle cores. Upon inter-micellar aggregation during the sol-to-gel transition, covalent cross-linking by the thiol-ene reaction of acryl groups of tri-PCG-Acryl with polythiol molecules occurred. Moreover, we applied the "freeze-dry with PEG/dispersion" method[19] as a quick extemporaneous preparation method developed in our previous studies on this system. We found that the IP formulations prepared by the "freeze-dry with PEG/dispersion" method (**D**-samples) retained their sol state at r.t. after preparation longer (more than 24 h) than formulations prepared by the usual heating dissolution method (**S**-samples), which undergo gelation typically within 3 h after preparation[20]. One of the differences between **D**-samples and **S**-samples is the state of the cores (solid-like or not). We confirmed that the micelle core of **D**-samples was in a solid-like semi-crystalline state. We hypothesized that gradual gelation in **S**-samples at r.t. was caused by transfer of tri-PCG-Acryl molecules from tri-PCG-Acryl micelles to tri-PCG micelles containing polythiols, and such polymer chain transfer was suppressed in **D**-samples. The polymer chain transfer rate for **D**-samples must be much slower than that for **S**-samples, because the micelle cores in **D**-samples were in a solid-like semi-crystalline state.

In this study, to confirm the hypothesis described above, we investigated the rate of polymer chain transfer (exchange) between micelles in the thermo-gelling polymer (tri-PCG) system. The previously proposed mechanism for sol-to-gel transition of biodegradable thermo-gelling polymer systems was a temperature-triggered micellar aggregation by dehydration of the PEG chain surrounding the micelles and subsequent growth of fibrous networks of the aggregation[17,21-24]. However, the polymer transfer

(exchange) behavior during the sol-to-gel transition has not been well investigated. Thus, we explore the effects of polymer transfer among micelles on the sol-to-gel transition, and reveal the primary factor for the stability of the **D**-sample.

To investigate polymer chain transfer in polymeric micelle systems, several methods have previously been reported, such as size exclusion chromatography (SEC)[25], fluorescence measurements[26-28], sedimentation[29], and small-angle neutron scattering[30]. In this study, we selected the fluorescence resonance energy transfer (FRET) method to investigate polymer chain exchange and transfer, because we can obtain real-time information using FRET for the polymer molecule transfer among the micelles in solution at various temperatures.

We employed naphthalene (nap) and dansyl (dan) groups as donors and acceptors, respectively, considering their overlapping fluorescence and absorption spectra[31] and relatively small molecular sizes. These fluorescence groups were covalently attached to both termini of tri-PCG to give tri-PCG-nap and tri-PCG-dan. The obtained tri-PCG-nap and tri-PCG-dan were mixed separately with tri-PCG to prepare tri-PCG/tri-PCG-nap micelle solution and tri-PCG/tri-PCG-dan micelle solution. After mixing the two micelle solutions, the fluorescence spectra of the mixed solution under various conditions were measured to evaluate polymer chain transfer (Figure 1).

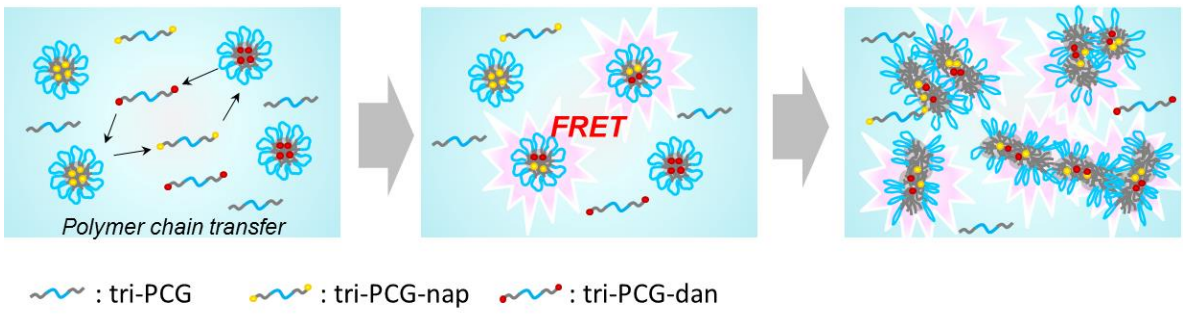


Figure 1. Schematic illustration for the polymer chain transfers in the tri-PCG micelle systems and the analysis by FRET.

2.2 Experimental procedure

2.2.1 Materials

PEG (molecular weight (MW) = 1,500 Da; PEG_{1500}), ε-caprolactone (CL), tin 2-ethylhexanoate ($Sn(Oct)_2$), N,N'-dicyclohexylcarbodiimide (DCC), and dimethylaminopyridine (DMAP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PEG (MW = 2,000 Da; PEG_{2000}) and glycolide (GL) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dansyl glycine and 1-naphthoic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Water was purified by a Milli-Q (Merck-Millipore, Darmstadt, Germany) system. All other reagents and organic solvents were commercial grade and used without further purification.

2.2.2 Measurements

^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a nuclear magnetic resonance spectrometer (400 MHz, JNM-GSX-400, JEOL) using deuterated solvent ($CDCl_3$). The chemical shifts were calibrated against tetramethylsilane (TMS) and/or solvent signal. The number-average molecular weights (Mn) were calculated from ^{1}H-NMR spectra. The weight-average molecular weights (Mw) and the polydispersity indexes (Mw/Mn) of the polymers were determined by size exclusion chromatography (SEC) (column: TSKgel Multipore HXL-M × 2; detector: RI). The measurements were performed using dimethylformamide (DMF) as an eluent at a flow rate of 1.0 mL min^{-1} at 40 °C using a series of PEG as standards. The fluorescence spectra were recorded by a FP-8300 (JASCO, Tokyo, Japan) spectrofluorometer at 25 °C. The excitation wavelength was 290 nm with excitation and emission bandwidths of 5 nm. The spectra were recorded from 300 to 600 nm with a scan rate of 1000 nm min^{-1}. Thermal analysis of the sample solutions was conducted using a differential scanning calorimeter (DSC-60, Shimadzu, Kyoto, Japan) with sealed aluminum pans.

2.2.3 Synthesis of tri-PCG-dan and tri-PCG-nap

PCGA-*b*-PEG-*b*-PCGA triblock copolymers (tri-PCG) were synthesized by ring-opening copolymerization of CL and GL in the presence of PEG_{1500} as a macroinitiator and $Sn(Oct)_2$ as a catalyst according to the method reported previously[19] (Scheme 1). Briefly, PEG_{1500} (16.7 g, 11.16 mmol) in a

100-mL flask with a stopcock was dried under vacuum at 120 ºC for 5 h. After cooling to r.t., CL (37.0 g, 324.5 mmol), GL (6.49 g, 55.9 mmol) (molar ratio of CL to GL (CL/GL) was 5.8) and $Sn(Oct)_2$ (159.9 mg, 394.7 μmol) were added to the flask which was then dried under vacuum at r.t. for 12 h. Polymerization was carried out at 160 ºC for 12 h by soaking the flask in an oil bath. The product was purified by reprecipitation using chloroform (100 mL) as a good solvent and diethyl ether (1000 mL) as a poor solvent to give a white solid of tri-PCG.

Tri-PCG-dan and tri-PCG-nap were synthesized through coupling reactions of terminal hydroxyl groups of tri-PCG with the carboxyl groups of dansyl glycine or 1-naphthoic acid (Scheme 2). Dansyl glycine (2.3 g, 7.47 mmol) was dissolved in DMF (7 mL). DCC (1.9 g, 8.98 mmol) was also dissolved in DMF (5 mL). These solutions were mixed and stirred at 4 ºC for 1 h. The obtained solution was added to the solution of tri-PCG (5.0 g, 0.94 mmol) and DMAP (63.1 mg, 0.75 μmol) in DMF (15 mL). The mixture was then stirred for 48 h at r.t. Dicyclohexylurea produced as a byproduct was removed by filtration, and the filtrate was evaporated under reduced pressure to remove DMF. The obtained products were purified by reprecipitation using chloroform as a good solvent and a mixture of diethyl ether and methanol (4/1, v/v) as a poor solvent. The reprecipitation procedure was repeated several times until no low-molecular-weight compound was detected. The resulting precipitate was washed with a cooled mixture of diethyl ether and methanol (4/1, v/v), and dried under vacuum overnight to give tri-PCG-dan. Similar procedures were carried out using 1-naphthoic acid instead of dansyl glycine to give tri-PCG-nap.

2.2.4 Sol-to-gel transition of tri-PCG and mixture

The sol-to-gel transition behavior of tri-PCG and the mixtures was investigated by a test-tube inverting method[17] as follows. A vial containing a micelle solution was immersed in a water bath at the desired temperature for 15 min, removed from the water bath, then inverted repeatedly to determine sol state or gel state based on the criteria of ‘‘flow’’ (= sol) and ‘‘no flow’’ (= gel) in 30 sec, with a temperature increment of 1 °C per step. Measurements were repeated three times at each temperature.

2.2.5 Preparation of samples for fluorescence measurements

S-samples were prepared by the heating dissolution method as follows. Tri-PCG-dan (64.2 mg) and tri-PCG (1542 mg) were dissolved in acetone (64 mL; the weight ratio of tri-PCG-dan/tri-PCG = 1/24). The obtained mixture (tri-PCG-dan/tri-PCG) solution was evaporated to remove acetone. Next, a predetermined amount of phosphate buffered saline (PBS) (pH = 7.4, 10 mM) was added to the flask (total polymer concentration = 0.025 - 15 wt%). After shaking with a vortex mixer for 1 min at r.t., the obtained suspension was heated to 65 °C and kept at 65 °C for 1 min, and further stirred by a vortex mixer for 1 min at r.t. The flask was immersed in ice-cold water for 2 min and further stirred by a vortex mixer for 1 min at r.t. These procedures were repeated until no insoluble particles were observed to give tri-PCG-dan/tri-PCG micelle solutions. Tri-PCG-nap/tri-PCG micelle solutions, where the weight ratio of tri-PCG-nap/tri-PCG was 0.7/24.3, were also prepared by the same procedure as for tri-PCG-dan/tri-PCG. Equal amounts of the obtained tri-PCG-dan/tri-PCG micelle solution and tri-PCG-nap/tri-PCG micelle solution were mixed together using a vortex mixer for 10 sec. The resulting **S**-sample solution was transferred to a glass cuvette and incubated in a water bath at a designated temperature. After incubation for predetermined periods, fluorescence spectra were recorded at r.t. after 60 min incubation at r.t. unless otherwise noted.

D-samples were prepared by the freeze-dry with PEG/dispersion method as follows. Tri-PCG-dan (28.6 mg), tri-PCG (686.4 mg) and PEG_{2000} (71.5 mg) were mixed (weight ratio = 1/24/2.5) and dissolved in acetone (7 mL). The solution was dissolved in pure water (70 mL) with stirring, then evaporated to remove acetone. The obtained aqueous solution was freeze-dried to give the mixture sample in powder form. A predetermined amount of PBS (pH = 7.4, 10 mM) was added to the powder (total polymer concentration = 15 wt%) in a test tube. The test tube was shaken by a vortex mixer for 1 min to give a macroscopically uniform milky dispersion. A dispersion of the mixture of tri-PCG-nap, tri-PCG and PEG_{2000} (weight ratio = 0.7/24.3/2.5) was prepared by the same procedure. Equal amounts of the obtained tri-PCG-dan/tri-PCG/PEG_{2000} dispersion and tri-PCG-nap/tri-PCG/PEG_{2000} dispersion were mixed with stirring using a vortex mixer for 10 sec to give **D**-samples. The resulting dispersion was transferred to a glass cuvette and incubated in a water bath at a designated temperature. After incubation for predetermined periods, fluorescence spectra were recorded at r.t. after 60 min incubation at r.t. unless

otherwise noted.

2.2.6 TEM observation

The micelle morphology was observed by transmission electron microscopy (TEM) (JEM-1400, JEOL, Tokyo, Japan) with acceleration voltage at 80 kV. The **S**-sample and **D**-sample for TEM were prepared by the same methods described above using pure water instead of PBS, and diluted with pure water to 2 wt%. An aliquot (4 μL) of the sample solution (2 wt%) was put on a STEM 150 Cu grid with hydrophilic polyvinylbutyral (PVB-C15) membrane (Okenshoji Co. Ltd., Tokyo, Japan). After 5 min incubation at r.t., the sample was dried by N_2 gas, and negatively stained with 1 wt% hexaammonium heptamolybdate tetrahydrate aqueous solution followed by 1 min incubation and drying with N_2 gas before observation.

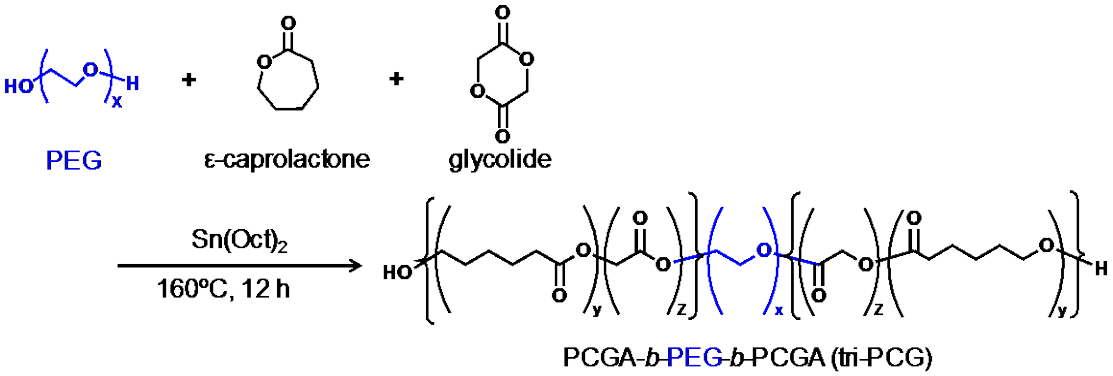
2.3 Results and Discussion

2.3.1 Synthesis of tri-PCG, tri-PCG-dan and tri-PCG-nap and sol-to-gel transition

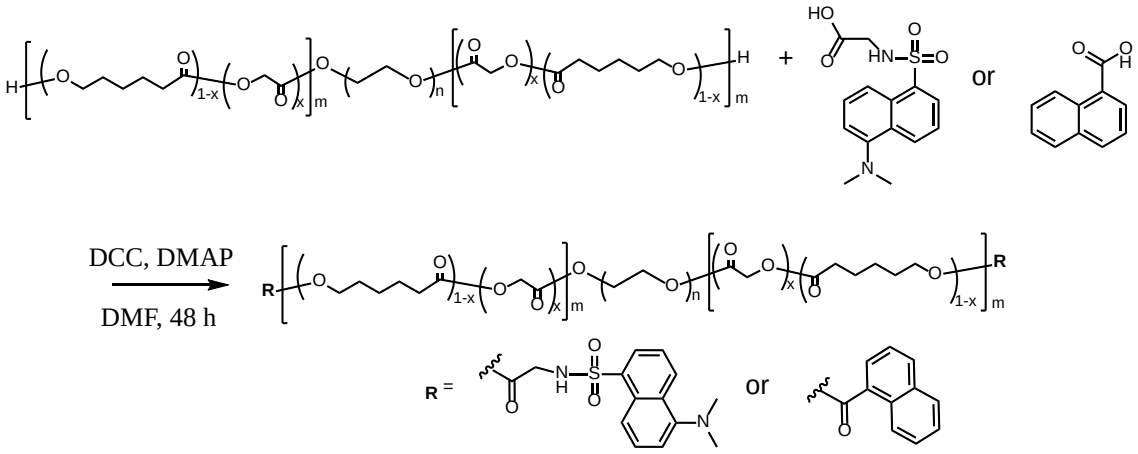
The synthesis of tri-PCG-dan and tri-PCG-nap were successfully carried out from tri-PCG according to Scheme 2. The characterization results of tri-PCG, tri-PCG-dan and tri-PCG-nap are shown in Table 1. ^{1}H-NMR spectra, SEC elution profiles and emission/excitation spectra of tri-PCG-dan and tri-PCG-nap are shown in Figures 2-5. The purities of tri-PCG-dan and tri-PCG-nap were confirmed by SEC analysis (Figure 4): no low-molecular-weight compounds were detected. The Mw of tri-PCG-dan was higher than those of the other copolymers, because the low-molecular-weight fraction was removed during the reprecipitation process. The degree of substitution of dan and nap groups per terminal OH groups was calculated to be 71.6% and 97.0%, respectively, by ^{1}H-NMR integration. The introduction of the nap group was quantitative, but the dan group incorporation was not. The tri-PCG-dan sample contained about 50% of tri-PCG molecules having only one dan group, but this will not affect the investigation of polymer chain transfer by FRET. The mixing ratios of tri-PCG-dan and tri-PCG-nap to tri-PCG were 1/24 and 0.7/24.3 to adjust the molar ratio of dan/nap = 1/1.

The tri-PCG solution (15 wt%) showed a sol-to-gel transition point at 35 °C (Figure 6), and the mixtures of tri-PCG-dan/tri-PCG micelle solution and tri-PCG-nap/tri-PCG micelle solution (**S**-sample)

showed the same (35 °C) sol-to-gel transition point as the tri-PCG solution. **D**-samples (mixture of tri-PCG-dan/tri-PCG micelles and tri-PCG-nap/tri-PCG micelles prepared by freeze-dry with PEG/dispersion method) showed sol-to-gel transition points at 36 °C.



Scheme 1. Synthesis of PCGA-*b*-PEG-*b*-PCGA triblock copolymer (tri-PCG)



Scheme 2. Synthesis of tri-PCG-dan and tri-PCG-nap.

Table 1. Characteristics of tri-PCG, tri-PCG-dan and tri-PCG-nap

Code	DP of CL[a)]	DP of GA[b)]	CL/GA[c)]	M_n (Da)[d)]	M_w (Da)[e)]	M_w/M_n[e)]	DS (%)[f)]
tri-PCG	14.2	4.2	3.4	5,200	6,100	1.44	-
tri-PCG-dan	19.9	6.3	3.2	7,400	8,000	1.28	71.6
tri-PCG-nap	13.0	4.0	3.3	5,300	6,300	1.47	97.0

a) Degree of polymerization of CL unit in a PCGA segment estimated by ^{1}H-NMR.

b) Degree of polymerization of glycolic acid (GA) unit in a PCGA segment estimated by ^{1}H-NMR.

c) Molar ratio of CL/GA in a PCGA segment calculated by ^{1}H-NMR.

d) Number-average molecular weight estimated by ^{1}H-NMR.

e) Weight-average molecular weight and polydispersity index estimated by SEC.

f) Degree of substitution of dan or nap group/OH estimated by ^{1}H-NMR.

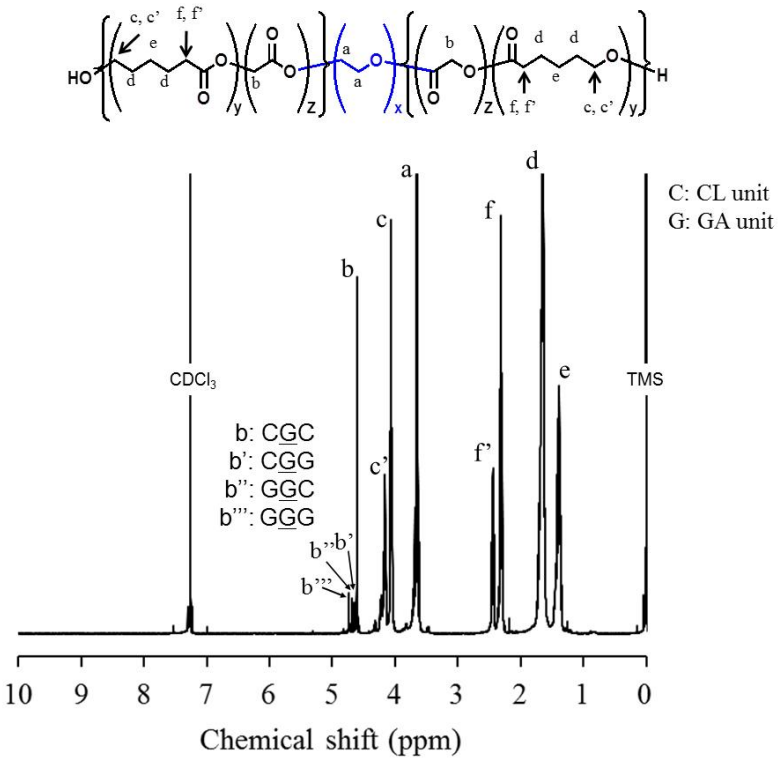
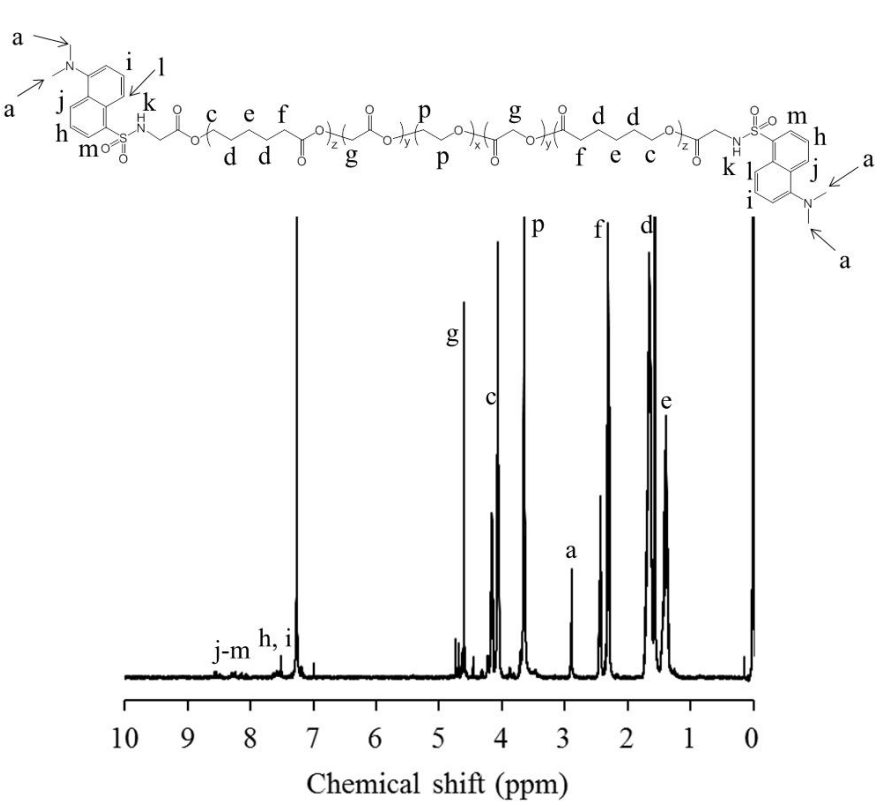


Figure 2. ^{1}H-NMR spectra of tri-PCG in $CDCl_3$.

(a)



(b)

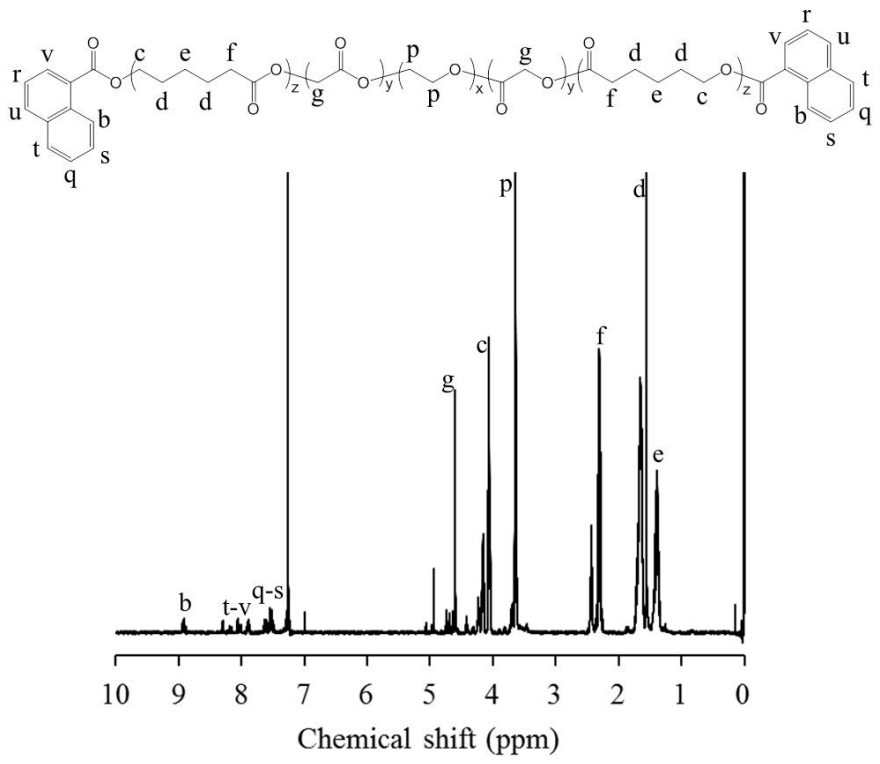


Figure 3. ^{1}H-NMR spectra of (a) tri-PCG-dan and (b) tri-PCG-nap in $CDCl_3$

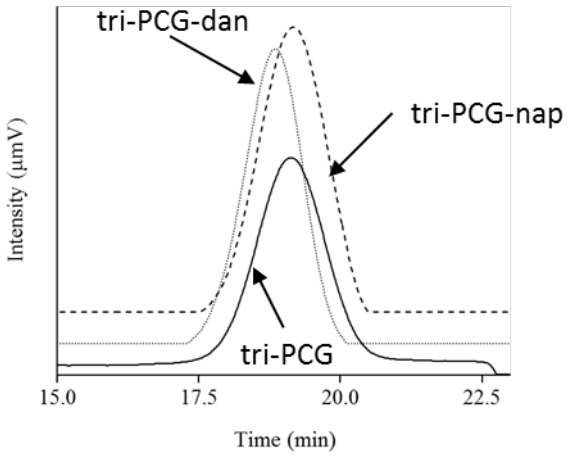


Figure 4. Elution profiles of size exclusion chromatography for tri-PCG, tri-PCG-dan and tri-PCG-nap.

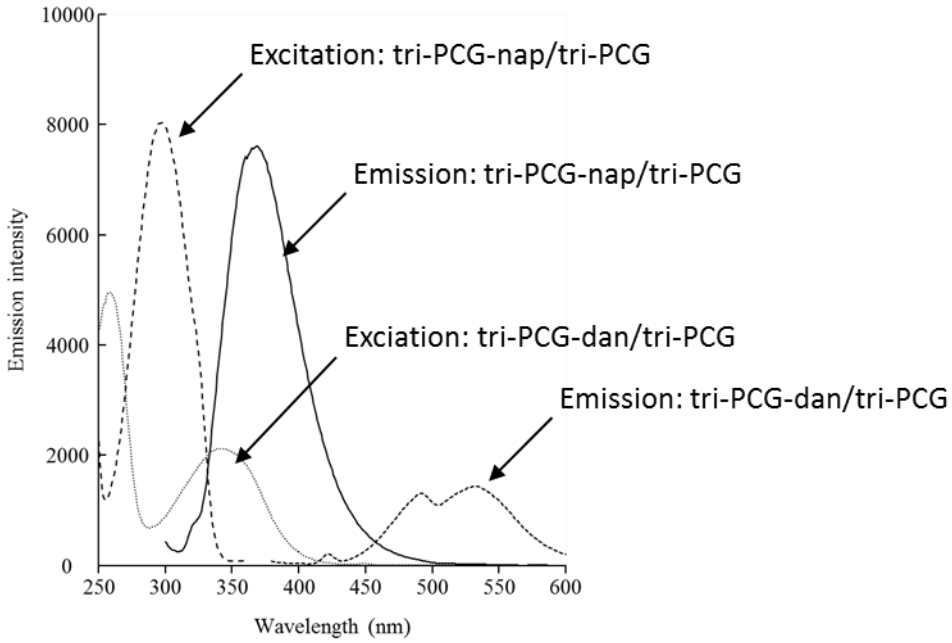


Figure 5. Emission and excitation spectra of the sample solutions.

Solid line: emission spectrum of tri-PCG-nap/tri-PCG micelle solution (excitation at 290 nm), long dashed line: excitation spectrum of tri-PCG-nap/tri-PCG micelle solution (emission at 370 nm), short dashed line: emission spectrum of tri-PCG-dan/tri-PCG micelle solution (excitation at 370 nm), dotted line: excitation spectrum of tri-PCG-dan/tri-PCG micelle solution (emission at 530 nm). Total polymer concentration = 0.025 wt%.

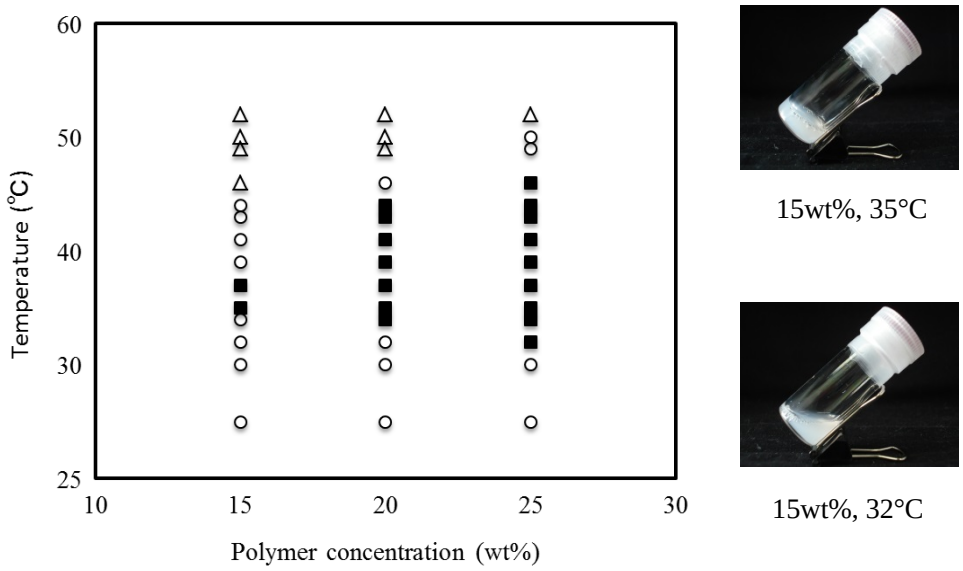


Figure 6. Phase diagram of tri-PCG in PBS. ○: sol, ■: gel, △: precipitate. Inserts are photographs of the polymer solutions (15wt%) at 32°C and 35°C.

2.3.2 Morphology of the micelles

To evaluate the morphology of the micelles in **S**-sample and **D**-sample, TEM observation of the sample solutions (or dispersions) was carried out. The results are shown in Figure 7. In Figure 7a, spherical particles with 40-60 nm diameters were observed for **S**-sample. The diameters were within reasonable level for flower-like polymeric micelles of tri-PCG. On the other hand, in Figures 7b and c, larger particles were observed for **D**-sample in addition to the similar size particles showed as seen in **S**-sample (Figure 2a). Some of the larger particles showed somewhat rugged shapes (Figure 2c). These results mean that some of **D**-sample micelles existed as aggregates and the other did not, showing good agreement with its turbid appearance.

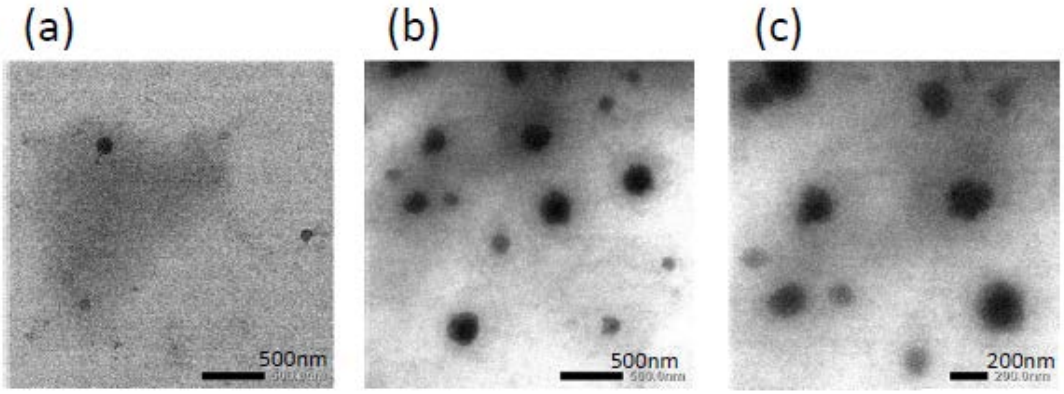


Figure 7. TEM images for **S**-sample (a) and **D**-sample (b, c).

2.3.3 FRET measurements on S-sample

The emission spectra of the **S**-samples (the mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution) after mixing at r.t. and further incubation at a predetermined temperature for 10 min were recorded. Figure 8a shows emission spectra of each sample before mixing, and Figure 8b shows typical examples of the emission spectra of diluted **S**-samples (copolymer concentration = 0.025 wt%) with excitation wavelength at 290 nm after incubation at various temperatures. As the incubation temperature increased, the emission intensities at 370 nm assigned to the nap group gradually decreased, and the emission at 530 nm assigned to the dan group gradually increased. These results indicate the occurrence of FRET from the nap group to the dan group. FRET occurs when nap and dan groups exist in close proximity (Förster distance). These results suggest that the polymer chain (tri-PCG-dan or tri-PCG-nap) transfers between the micelles, and that the rate of polymer chain transfer is dependent on temperature. Therefore, the degree of polymer chain transfer in the mixed micelle solution can be evaluated by monitoring FRET.

To evaluate the degree of polymer chain transfer (exchange) quantitatively, the emission intensity ratio for the peaks at 530 nm (dan) and 370 nm (nap) (I_{530}/I_{370}) was calculated as the polymer chain exchange index. Plots of I_{530}/I_{370} as a function of incubation temperature are shown in Figure 9. The data for the homogeneous mixtures of tri-PCG-nap, tri-PCG-dan and tri-PCG (weight ratio = 0.7/1.0/48.3) are shown to estimate the FRET efficiency at the equilibrium state after complete polymer chain exchange in Figure 9. The emission intensity ratio I_{530}/I_{370} for homogeneous samples was about 0.22 and remained

constant with temperature variation, confirming that FRET efficiency was not dependent on temperature. We regarded I_{530}/I_{370} = 0.217 (the average of observed values) as the theoretical maximum emission intensity ratio value where the degree of polymer chain exchange (Dex) = 100%, and I_{530}/I_{370} = 0.102 (theoretical value calculated from the additive spectra in Figure 8) as Dex = 0%, as shown on the Y-axis on the right side in Figure 9. Therefore, Dex can be defined by the following equation:

$$\text{Dex}\ (\%) = (\text{Rt} - \text{Rmin}) / (\text{Rmax} - \text{Rmin}) \times 100$$

where Rt is the observed I_{530}/I_{370} value, Rmax is the I_{530}/I_{370} value for a homogeneous mixture (= 0.217), and Rmin is the theoretical value of I_{530}/I_{370} with no polymer exchange (= 0.102).

I_{530}/I_{370} (and Dex) for the mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution (**S**-sample) increased with increasing temperature. This means that the rate of polymer chain transfer was greater at higher temperature. Over 48 °C, the Dex value reached a plateau at about 87%. Furthermore, the graph was a sigmoidal curve with a threshold at 40-45 °C. In a diluted condition (0.025 wt%, sufficiently below gelation concentration), the tri-PCG solution showed a higher transition point over 40 °C, which was detected by a diameter change in dynamic light scattering (DLS) (data not shown). These results suggest that the increase in Dex along with temperature was not only due to kinetic acceleration by the temperature increase, and the polymer transfer rate was significantly increased around the sol-to-gel transition temperature.

We also investigated the effects of polymer concentration on polymer chain transfer. Figure 10 shows the time course of the degree of polymer chain exchange (Dex) for various polymer concentrations after incubation at 35 °C. The emission spectra of the samples were recorded after dilution to 0.025 wt%, because the fluorescence intensity was too large when the concentration was high. Upon increasing polymer concentration, the Dex value reached a plateau in a shorter period of time. It is quite reasonable that the polymer exchange rate is higher at a higher polymer concentration. Mattice et al. reported that the polymer chain exchange rate was very slow at low polymer concentration[27] and the mechanism of polymer chain exchange was suggested to be micellar merger/splitting at higher polymer concentration by dynamic Monte Carlo (MC) simulations[32]. Our results showed good agreement with their hypothesis and provided evidence from an actual system that polymer chain exchange was promoted by merger and splitting between micelles at high polymer concentration. Therefore, we

confirmed that polymer chain transfer behavior can be examined under higher (15 wt%) polymer concentration.

Figure 11 shows the time course of Dex for the mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution (**S**-sample) with 15 wt% polymer concentration at various incubation temperatures. The increase in the Dex value was much faster at higher incubation temperature. The rate of polymer chain transfer between the micelles strongly depended on temperature. Polymer chain transfer was very slow at 25 °C. On the Basis of the results of DLS measurements, the diameter of the tri-PCG micelle was constant at 25 °C (Figure 12). These results suggest that the micelles neither merged nor aggregated at 25 °C. At a temperature sufficiently lower than the transition point, PEG chains existing at the shell layer of the micelles were adequately hydrated and the hydrophobic cores of the micelles were not exposed to the aqueous phase by exclusion volume effects of the PEG chains. This is the reason why merger/aggregation of micelles hardly occurred at low temperature even at high polymer concentration. After 1 min incubation at 35 °C, the micelle solution underwent gelation, but the Dex value still increased for an additional 9 min after gelation. These results indicate that polymer chain transfer occurred even after gelation, and the polymer chains could move in the hydrogel. On the other hand, at 30 °C, below the sol-to-gel transition temperature, the Dex value also increased gradually. These results suggest that some polymer chain transfer occurred below the gelation temperature.

We found that the rate of polymer chain transfer was significantly accelerated around the gelation temperature. In previous reports, the sol-to-gel transition occurred by association of micelles[21-24], and polymer chain transfer (exchange) occurred by merger between micelles[32]. However, it was suggested that polymer chain transfer began before the gelation temperature was reached and continued after gelation.

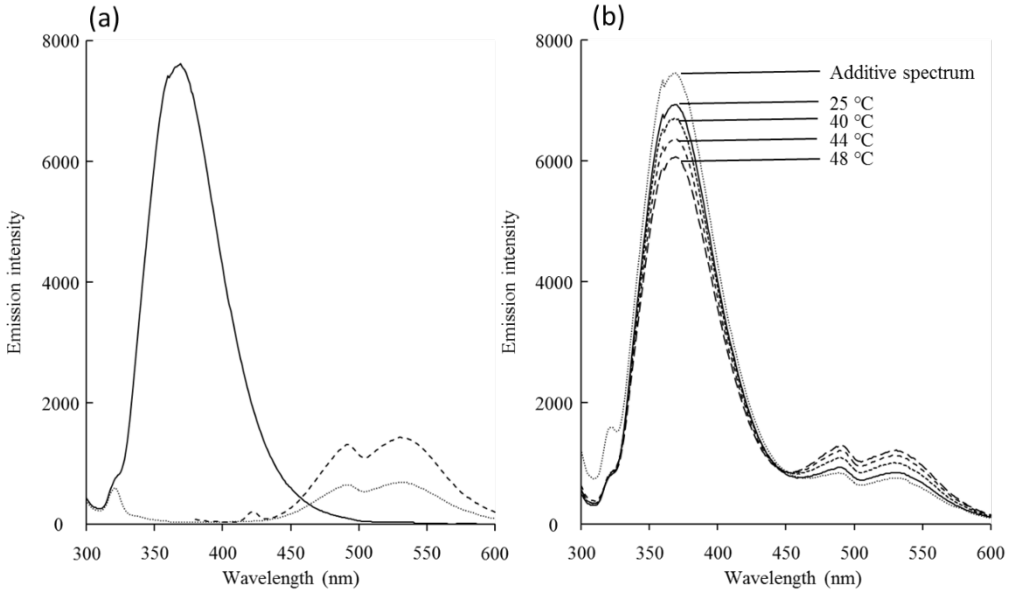


Figure 8. (a) Emission spectra for each samples before mixing. Solid line: tri-PCG-nap/tri-PCG micelle (excitation at 290 nm), dotted line: tri-PCG-dan/tri-PCG micelle (excitation at 290 nm), and dashed line: tri-PCG-dan/tri-PCG micelle (excitation at 370 nm). (b) Emission spectra for mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution (**S**-sample) after incubation at various temperature for 10 min, and the additive spectrum of tri-PCG-nap/tri-PCG micelle (excitation at 290nm) (solid line in (a)) and tri-PCG-dan/tri-PCG micelle (excitation at 290 nm) (dotted line in (a)).

Total copolymer concentration = 0.025 wt%.

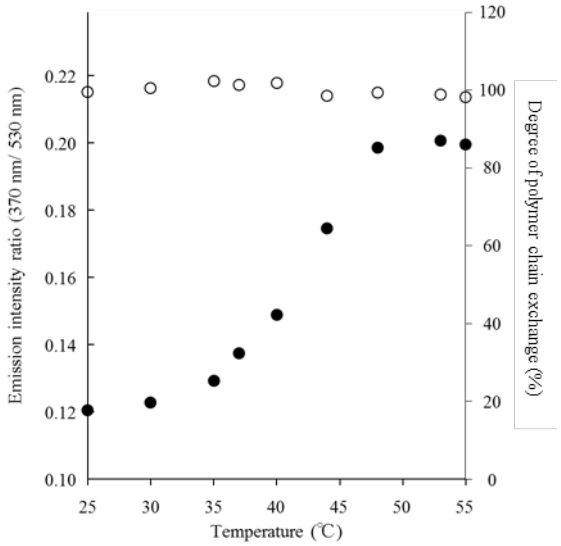


Figure 9. Emission intensity ratio (I_{530}/I_{370}) and degree of polymer chain exchange for homogeneous mixture of tri-PCG-nap/tri-PCG-dan/tri-PCG micelle (○), and mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution (**S**-sample) (●) after incubation at various temperature for 10 min.

Total polymer concentration = 0.025wt%.

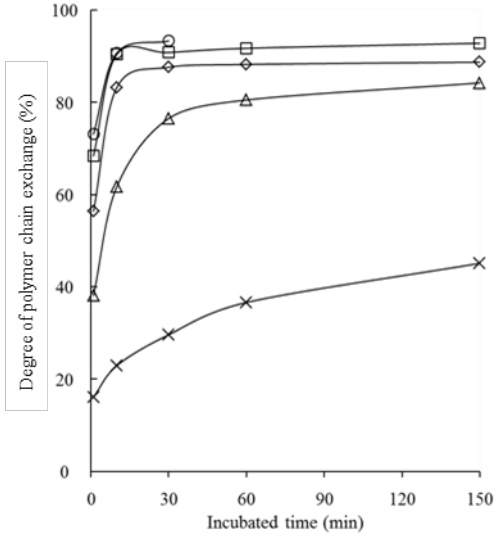


Figure 10. Degree of polymer chain exchange for S-sample with different polymer concentrations incubated at 35°C. ○: 15 wt%, □: 7.5 wt%, ◇: 2.5 wt%, △: 0.25 wt%, × : 0.025 wt%.

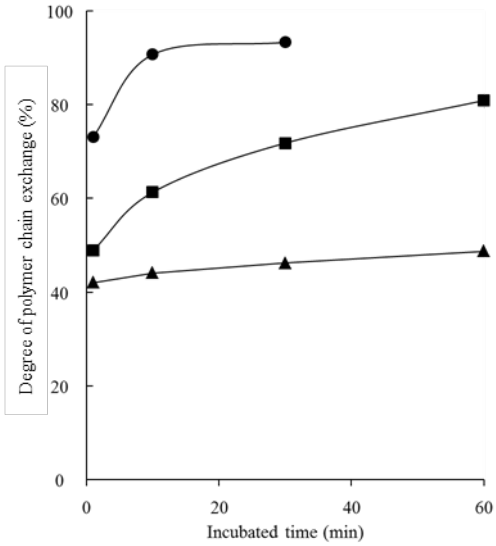


Figure 11. Time course of the degree of polymer chain exchange for the mixture of tri-PCG-nap/tri-PCG micelle solution and tri-PCG-dan/tri-PCG micelle solution (**S**-sample) incubated at 35°C (●), 30°C (■), or 25°C (▲). Total polymer concentration = 15 wt%.

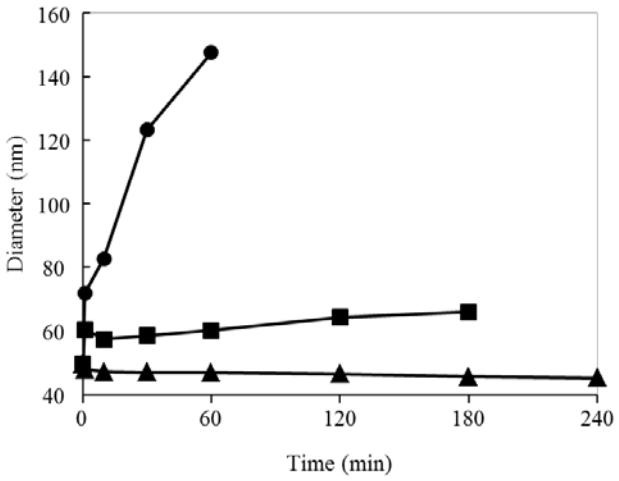


Figure 12. Time course of particle size change in tri-PCG micelle solution incubated at various temperatures. 35°C (●), 30°C (■), 25°C (▲). Polymer concentration = 0.5 wt%.

2.3.4 Comparison of D-samples and S-samples

As described in the introduction, we previously discovered that the temperature-responsive covalent cross-linking IP formulations prepared by the "freeze-dry with PEG/dispersion" method (**D**-samples) retained the sol state longer (more than 24 h) than the formulations prepared by the usual heating dissolution method (**S**-samples), which undergo gelation typically within 3 h after preparation[20]. We hypothesized that differences in stability (no gelation during incubation at r.t.) between **D**-samples and **S**-samples are due to differences in the rate of polymer chain transfer at r.t. To confirm this hypothesis, we investigated polymer chain transfer for both **D**-samples (15 wt%) and S-samples for an extended period of time at 25 °C. In this experiment, the emission intensity for the samples was measured immediately after dilution to 0.025 wt%. The results are shown in Figure 13. Clearly, the increase in the Dex value for the **S**-sample at the initial stage was significantly faster than that of the **D**-sample. After the initial (ca. 10 min) fast increase, the Dex value for the **S**-sample increased more gradually. For the **D**-sample, no rapid increase at the initial stage was observed and the Dex value increased gradually. After 180 min, the Dex values were about 45% and 20% for the **S**-sample and **D**-sample, respectively. Considering our previous results showing that spontaneous covalent gelation occurred in 3 h for **S**-samples[20], the critical degree of polymer exchange for gelation should exist between 20% and 45%. It was confirmed that the rate of polymer chain transfer in the **S**-sample was much faster than that in the **D**-sample at r.t especially at the initial stage. We confirmed that the **D**-sample showed an endothermic peak at 30 °C and the micelle core was in a solid-like semi-crystalline state by DSC measurements (Figure 14). Therefore, polymer chain transfer was suppressed in the **D**-sample because it was not in the usual equilibrium state between unimers and micelles. The initial stage polymer chain transfer was quite low for the **D**-sample because the amount of unimers in the **D**-sample micelle solution was extremely low. In addition, the unimers, if they existed, were difficult to incorporate into other micelles because the micelle cores were in the solid-like semi-crystalline state.

The main reason of the difference between **S**-samples and **D**-samples is the difference of the core states, which must be derived from the preparation process (freeze-dry or heating dissolution) as follows. **D**-sample was prepared by the freeze-dry with PEG/dispersion method. After freeze-dry, the core of the sample was partially crystalized. Although certain part of micelle was aggregated, the PEG molecules, as

additives, existed between the micelles in the aggregates, which helped rapid dispersion of the **D**-samples. During this dispersion process, the temperature was kept below the melting temperature. So, the crystal formed in the core was not melted, and no water molecule could penetrate into the micelle core. As results, the cores of **D**-sample micelles kept their semi-crystalline state. On the other hand, during the process for **S**-sample preparation (heating dissolution), temperature was raised above the melting temperature, and the crystal in the core was melted. But, during the cooling process, recrystallization in the micelle core was not proceeded in the presence of solvent (water). So, the core of the **S**-sample micelle became amorphous state.

To estimate the degree of polymer chain exchange in **S**-samples and **D**-samples during the sol-to-gel transition process, the Dex value under continuous temperature increase (from 25 °C to 42 °C, 1 °C min^{-1}) was monitored (Figure 15). The **S**-sample showed gelation at 35 °C, and its Dex value increased rapidly in the range of 30-37 °C. Thus, polymer chain transfer occurred in advance of gelation, and this result shows good agreement with the results shown in Figure 11. The percolation transition of associated micelles is considered to be the prevailing mechanism for the sol-to-gel transition. However, the association of micelles and polymer chain transfer began before reaching the transition temperature of percolation. In contrast, the Dex value of the **D**-sample gradually increased from 25 °C to 42 °C: no rapid increase at a certain temperature was observed. The **D**-sample showed gelation at 36 °C, where the Dex value was about 50%, which was significantly lower than that of the **S**-sample at the gelation temperature (78% at 35 °C). This result indicated that exchange of polymer chains between micelles is suppressed in **D**-samples compared to **S**-samples.

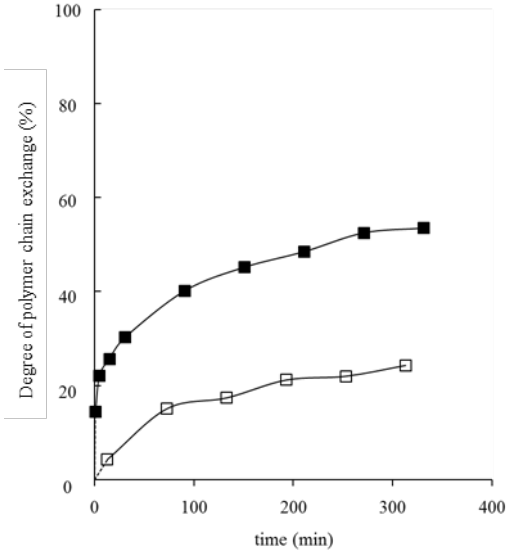


Figure 13. Time course of the degree of polymer chain exchange for **S**-sample (■) and **D**-sample (□) incubated at 25°C. Total polymer concentration = 15 wt%.

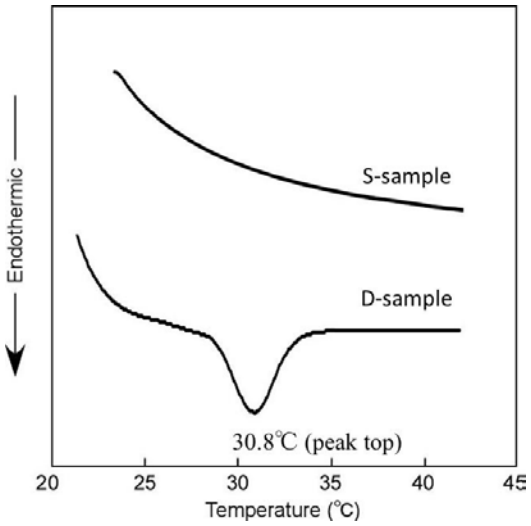


Figure 14. DSC curve for **S**-sample (mixture of tri-PCG-nap/tri-PCG and tri-PCG-dan/tri-PCG micelle solution) and **D**-sample (mixture of tri-PCG-nap/tri-PCG and tri-PCG-dan/tri-PCG micelle dispersion). Total polymer concentration = 15 wt%.

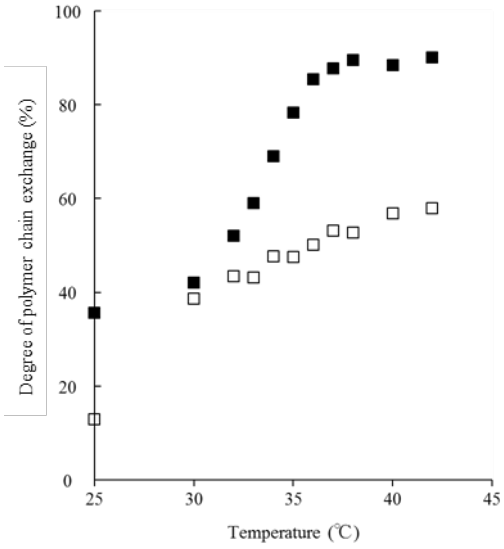


Figure 15. Degree of polymer chain exchange in **S**-sample (■)and **D**-sample (□) under continuous temperature increase (1 ℃ min^{-1}). The polymer concentration is 15 wt%.

2.4 Conclusion

We successfully synthesized PCGA-*b*-PEG-*b*-PCGA with naphthalene or dansyl groups covalently attached at both termini, tri-PCG-nap and tri-PCG-dan, as FRET donors and acceptors, respectively. The FRET observations provided an estimation of polymer chain transfer between the micelles in the mixture of tri-PCG-nap/tri-PCG micelles and tri-PCG-dan/tri-PCG micelles (**S**-sample or **D**-sample). Polymer chain transfer between micelles for the **S**-sample was strongly dependent on the polymer concentration and temperature, and was accelerated at the gelation temperature. It was revealed that polymer chain transfer began in advance of gelation, and continued even after gelation. We previously reported that covalent cross-linking IP formulations prepared by the "freeze-dry with PEG/dispersion" method (**D**-samples) retained the sol state at r.t. longer compared to **S**-samples prepared by the heating dissolution method[20], and hypothesized that polymer chain transfer between micelles was suppressed in **D**-samples, because the micelle cores were in a solid-like semi-crystalline state. In this study, we confirmed that polymer chain transfer in **D**-samples was in fact suppressed compared with **S**-samples. Therefore, the results obtained in this study strongly support our hypothesis.

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Chapter 3

Peptide drug release behavior from the biodegradable temperature-responsive injectable hydrogels exhibiting irreversible gelation

3.1 Introduction

Various temperature-responsive water-soluble polymers have been investigated for application in drug delivery systems (DDSs)[1-9]. Of these, several polymers in aqueous solution exhibit a temperature-responsive sol-to-gel transition between room temperature (r.t.) and body temperature and can thus be used as injectable polymer (IP) systems. Such polymer solutions can be mixed with water-soluble bioactive reagents such as proteins, peptides, or living cells before injection, and form a hydrogel entrapping these reagents at the injection site in the body. If the polymer can be hydrolyzed to low-molecular-weight compounds that can be metabolized or excreted from the body, such biodegradable IP systems could potentially act as implantable minimally invasive sustained drug release systems[10-12].

There have been many reports on drug release from hydrogels. The release rates of drugs from hydrogels are influenced by several factors, such as the hydrophilicity/hydrophobicity (solubility) and molecular weight of the drug, the degradation rates of the hydrogel, the mesh size of the network, and the diffusion constant of the drug in the hydrogel[13]. In general, temperature-responsive biodegradable IP hydrogels, once formed in the body, are likely to quickly revert to the sol state (typically in less than 24 h) at the injection site, where there is a large amount of body fluid[14]. This is because the gelation of such an IP system is caused by non-covalent (hydrophobic) interactions, and gel formation is an equilibrium process affected by local conditions such as concentration, pH, and temperature. This phenomenon may cause rapid disappearance of the hydrogel and more rapid release of bioactive agents than intended.

To address this problem, we recently reported the generation of biodegradable temperature-triggered covalent gelation systems exhibiting longer and controllable durations of the gel state by using a "mixing strategy"[14-16]. We synthesized a tri-block copolymer of poly(ε-caprolactone-*co*-glycolic acid) (PCGA), poly(ethylene glycol) (PEG), PCGA-*b*-PEG-*b*-PCGA (tri-PCG), and tri-PCG with acryloyl groups attached at both termini (tri-PCG-Acryl) (Figure 1). A mixture of tri-PCG-Acryl micelle solution and tri-PCG micelle solution containing dipentaerythritolhexakis(3-mercaptopropionate) (DPMP) (Figure 1) as a hydrophobic hexa-functonal polythiol exhibited an irreversible sol-to-gel transition by covalent cross-linking using a bio-orthogonal Michael-addition-type thiol-ene reaction in response to a temperature increase[15,16]. The mixed micelle

solution remained in the sol state just after mixing at r.t., but underwent gelation in response to a temperature increase. Once formed, the hydrogel stayed in the gel state even after cooling. In this system, the acryloyl groups at the copolymer termini and the thiol groups of DPMP existed separately in different micelles just after mxing. Covalent bond formation between the acryloyl and thiol groups occurred only upon sol-to-gel transition induced by a temperature increase, since this temperature rise induced inter-micellar aggregation due to hydrophobic interactions, resulting in a physically cross-linked hydrogel. During aggregation process, the micelle cores fused and subsequently the thiol groups of DPMP in the tri-PCG micelle core covalently cross-linked with the acryloyl groups via the Michael-addition-type thiol-ene reaction. This system existed for a longer and controllable duration time in the gel state. The duration time of the gel state after subcutaneous injection *in vivo* could be altered easily from 1 day to more than 60 days simply by changing the mixing ratio of DPMP/tri-PCG and tri-PCG-Acryl[15].

Peptides are becoming increasingly important as drugs due to their activity, target specificity, tolerability, and availability. However, peptides must be administered via the parenteral route because their half-life in the body is extremely short and their oral absorption is poor. Consequently, when continuous exposure to the peptide drug is needed, continuous infusion or multiple injections are required to achieve therapeutic efficacy, which is inconvenient and distressing for patients. Thus, there is a need to develop sustained release parenteral formulations, where a single injection allows the drug to be released over a period of weeks, months, or even years[17,18].

As described above, we previously developed a biodegradable temperature-triggered IP system exhibiting covalent gelation using a bio-orthogonal reaction and controllable duration times of the gel state. This system should have potential utility as a drug deposition method allowing the sustained release of peptide drugs. As far as we were aware, there has been no report on the drug release behavior using temperature-triggered covalent gelation system. In this study, we evaluated this IP system as a sustained peptide drug release device by studying the release behavior of a peptide from hydrogels prepared using our IP system. We chose glucagon-like peptide-1 (7-36 amide) (GLP-1) as the peptide model drug. GLP-1 holds promise for the treatment of type 2 diabetes but must be continuously infused or administered by multiple injections because of its extremely short half-life[19-21]. This is the first report

on the drug release behavior using biodegradable temperature-responsive covalent gelation system exhibiting long-term release of peptide drugs.

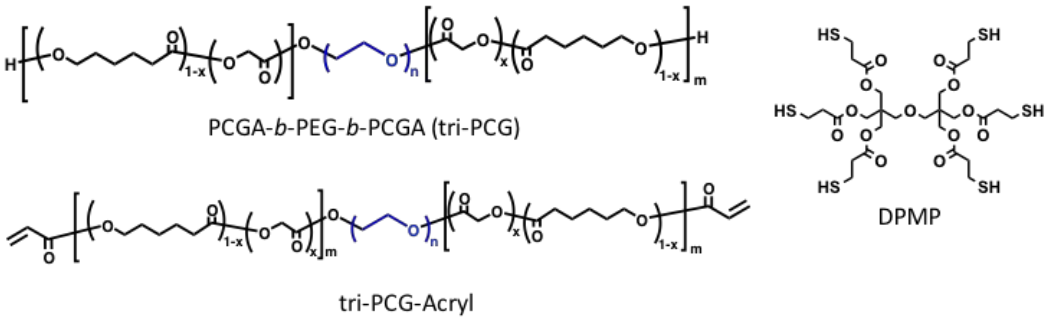


Figure 1. Structures of the polymers and polythiol used in this study.

3.2 Experimental procedure

3.2.1 Materials

Tri-PCG-1 and tri-PCG-2 were synthesized by the ring-opening polymerization of ε-caprolactone (CL) glycolide (GL) in the presence of PEG (molecular weight (Mw) = 1500 Da) according to the methods reported previously[14]. The Mn of PCGA segments, the total Mn, and the molar ratio of glycolic acid (GA) units to CL units in the tri-PCG-1 copolymer (CL/GA) were 1950, 5400 Da, and 3.4, respectively, and for tri-PCG-2 the values were 1250, 4000 Da, and 3.9, respectively (Table 1). Tri-PCG-Acryl was synthesized from tri-PCG-2 by the method described previously[15]. The total Mn and the degree of substitution by acryloyl groups were 4200 Da, and 91%, respectively (Table 2). GLP-1 (7-36 amide) was purchased from Aviva Systems Biology, Corp. (San Diego, CA, USA). DPMP was a gift from SC Organic Chemical Co., Ltd. (Osaka, Japan). Fetal calf serum (FCS) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Eagle's minimum essential medium (E-MEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Mouse fibroblast NCTC clone 929 (L929) cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Spague-Dawley (SD) rats (7 weeks old, female, 180 g average body weight) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Water was purified by a Milli-Q (Merck Millipore, Billerica, MA, USA) system. All other reagents and organic solvents were of commercial grade and were used without further

purification.

Table 1. Characterization of PCGA-*b*-PEG-*b*-PCGA triblock copolymers (tri-PCGs).

Code	DP of CL[a)]	DP of GA[b)]	CL/GA[c)]	M_n (Da)[d)]	M_w (Da)[e)]	M_w/M_n[e)]
tri-PCG-1	14.6	4.3	3.4	5,400	6,900	1.38
tri-PCG-2	9.9	2.6	3.9	4,000	4,800	1.38

a) The degree of polymerization of ε-caprolactone unit in a PCGA segment was calculated using ^{1}H-NMR.

b) The degree of polymerization of glycolic acid unit in a PCGA segment was calculated using ^{1}H-NMR.

c) Molar ratio of CL/GA in a PCGA segment estimated using ^{1}H-NMR.

d) Number-average of the molecular weight estimated using ^{1}H-NMR.

e) Weight-average of the molecular weight and the polydispersity index estimated using SEC.

Table 2. Charcterization of tri-PCG-Acryl

Code	M_n (Da)[a)]	M_w (Da)[b)]	M_w/M_n[b)]	DS (%)[c)]
tri-PCG-Acryl	4,200	5,100	1.40	91

a) Number-average of the molecular weight estimated using ^{1}H-NMR.

b) Weight-average of the molecular weight and the polydispersity index estimated using SEC.

c) Degree of substitution of the acryloyl group calculated using ^{1}H-NMR.

3.2.2 Preparation of the IP Formulations

The IP formulations were prepared as reported previously[15]. DPMP-loaded tri-PCG micelle solution (Solution A) was prepared as follows. Tri-PCG and DPMP were placed in a glass vial and dissolved with a small amount of acetone at r.t. The solution was dropped into pure water in a flask stirred at r.t. for 10 min, and then evaporated to remove the acetone. The aqueous solution was lyophilized to obtain powdery DPMP-loaded tri-PCG micelles. The powder was placed in a glass vial and a predetermined amount of PBS was added. After mixing with a vortex mixer for 1 min at r.t., the obtained suspension was heated to 65 °C and kept at 65 °C for 1 min, then mixed using a vortex mixer for 1 min at r.t. The glass vial was immersed in iced-cold water for 2 min and mixed with a vortex mixer for 1 min at r.t. These procedures were repeated until no insoluble particles were observed to give

DPMP-loaded tri-PCG micelle solution (Solution A).

Tri-PCG-Acryl micelle solution (Solution B) was prepared as follows. Tri-PCG-Acryl was placed in a glass vial and PBS was added. After mixing with a vortex mixer for 1 min at r.t., the obtained suspension was heated to 65 °C and kept at 65 °C for 10 s, then further mixed using a vortex mixer for 1 min at r.t. These procedures were repeated until no insoluble particles were observed to give tri-PCG-Acryl micelle solution (Solution B).

Finally, Solution A and Solution B were mixed at desired ratios to give IP formulations. The IP formulations are expressed as F(P1/D+PAx), where P1, /D, and +PAx denote the presence of tri-PCG-1 added, the presence of DPMP added, and the amount of tri-PCG-Acryl added was x wt% in total polymers.

3.2.3 *In vitro* Release Test of GLP-1

IP formulations **F(P1/D+PA$_{40}$)** containing GLP-1 were prepared as follows. A predetermined amount of GLP-1 was dissolved in Solution A (total polymer concentration = 26 wt%) by mixing and sonication, and the solution was then mixed with Solution B (total polymer concentration = 26 wt%). The pH was adjusted to 7.4 with 1N NaOH aqueous solution or 1N HCl aqueous solution, and the total polymer concentration was adjusted to 25 wt% by the addition of PBS. The mixing ratio of solution A/solution B was 3/2, with the content of tri-PCG-Acryl in the total polymer being 40 wt%, **F(P1/D+PA$_{40}$)**. As a control, IP formulation containing only tri-PCG-1 (without DPMP or tri-PCG-Acryl) and GLP-1 **F(P1)** was prepared by a similar method using only Solution A. The GLP-1 concentration in each IP formulation was 7.5 mg/mL.

Each formulation (200 μL) was placed in a glass vial and incubated at 37 °C for 30 min to obtain a hydrogel, then 1 mL of PBS as a release medium was gently added. At each sampling time, 0.6 mL of supernatant was removed and measured, and 0.6 mL fresh PBS was added to the vial, and the sample was then further incubated at 37 °C. The amount of GLP-1 in the sample solution was determined using a reversed-phase high-performance liquid chromatography (RP-HPLC) system (Waters, Milford, MA, USA) (column: Vydac 218TP54 (4.6 mm × 250 mm), eluent: acetonitrile containing 0.1% trifluoroacetic acid (TFA)/water containing 0.1% TFA, 1/4 to 4/1 linear gradient for 25 min, flow rate: 0.8 mL/min,

detector: UV at 210 nm).

3.2.4 Cytotoxicity

The viability of L929 mouse fibroblast cells after incubation with each sample for 24 h was investigated using a WST-8 assay (Dojindo, Tokyo, Japan). L929 mouse fibroblast cells (100 μL, 2.5 × 10^3 cells/well) in E-MEM containing 10% fetal bovine serum (FBS) were seeded in a 96-well microplate and cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. After preincubation for 24 h, all the medium supernatant was removed and added to 90 μL of fresh medium. Then, 10 μL of medium containing an IP formulation was added to each well and further incubated for 21 h. The medium supernatant was removed again from the wells, and the cells in the wells were washed with PBS twice. Thereafter, 90 μL of fresh medium and WST-8 reagent (10 μL) were added to the wells, and incubation was continued for a further 3 h. The microplates were read at 450 nm using a microplate reader. The average background absorbance from the control wells was subtracted from the sample data. The values for each sample were in the linear region of the standard curve for the WST-8 assay. Data are expressed as the means and SD (n = 6). Cell viability was calculated using the following equation:

$$\text{Cell viability}\ (\%)\ = Nt/Nc\ \times 100$$

where *Nt* and *Nc* are the number of cells with or without IP formulation after 21 h of incubation, respectively.

3.2.5 *In vivo* Experiments

The **F(P1/D+PA$_{40}$)** formulation (500 μL) containing GLP-1 was administrated by syringe with a 25 G needle subcutaneously in the back neck of a rat after anesthetizing with isoflurane. **F(P1)** containing GLP-1, **F(P1/D+PA$_{40}$)** without GLP-1, and GLP-1 in PBS (pH 7.4) were used as controls. The volume of all samples was 500 μL, and the concentration of GLP-1 was 7.5 mg/mL.

At each sampling time (2 h-25 days), 250 μL blood samples were obtained from the tail vein using a blood collection tube (BD Microtainer with K_2EDTA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The blood samples were treated with 5 μL of dipeptidyl peptidase IV inhibitor (Merck Millipore, Billerica, MA, USA) and centrifuged (9100 g, 10 min, 4 °C) to obtain the plasma. The amount

of active GLP-1 in the plasma was determined using an ELISA kit (GLP-1 active form assay kit, Immuno-Biological Laboratories Co., Ltd., Shizuoka, Japan). The results were expressed as mean ±SE (n = 4-6). Statistical comparisons were made using a Student's *t*-test. A value of $p < 0.05$ was considered significant. These experiments followed the guidelines for animal experiments at Kansai University. The experiment was approved by the Ethical Committee for Animal Experiments of Faculty of Chemistry, Materials and Bioengineering, Kansai University (17 April, 2017, Identification number 1709).

3.2.6 Rheological Measurements

The physical properties of the formulations after soaking in PBS (release test) or after subcutaneous injection into rats were investigated at 37 °C by rheological measurements using a dynamic rheometer (Thermo HAAKE RS600, Thermo Fisher Scientific, Waltham, MA, USA). A solvent trap was used to prevent solvent vaporization. The diameter of the parallel plate was 35 mm, and the gap was 0.2 mm. The controlled stress and frequency were 0.4 Pa and 1.0 rad/s, respectively.

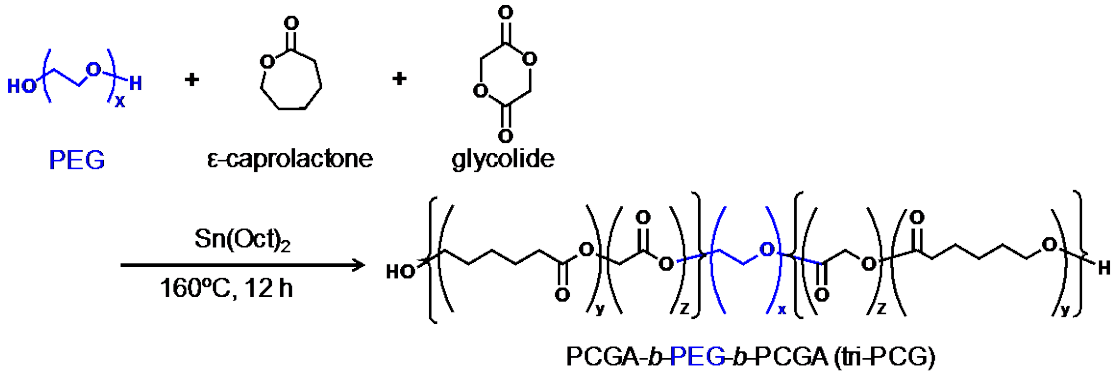
3.3 Results and Discussion

3.3.1 Synthesis of tri-PCGs and tri-PCG-Acryl and sol-to-gel transition containing GLP-1

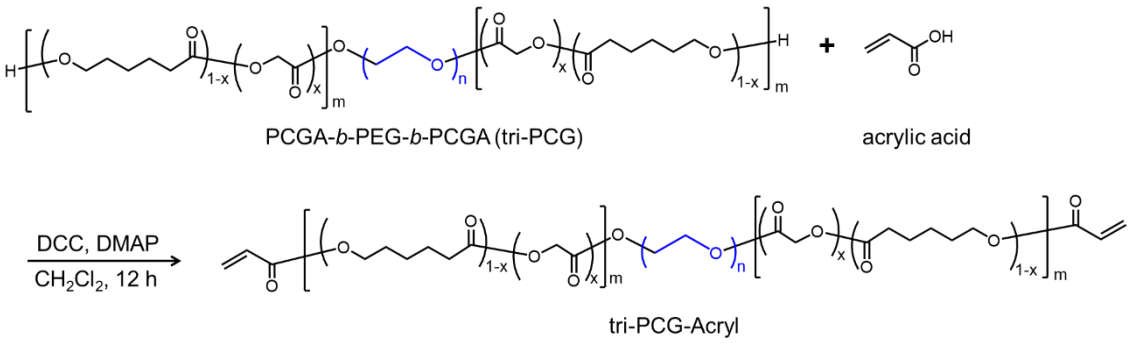
Tri-PCGs (tri-PCG-1 and tri-PCG-2) and tri-PCG-Acryl were successfully synthesized according to the methods reported previouly[15] (Schemes 1 and 2). Several characteristics of the polymers are shown in Table 1 and 2. The IP formulations were typically prepared by mixing DPMP-loaded tri-PCG micelles in Phosphate buffered saline (PBS) (Solution A) and tri-PCG-Acryl micelles in PBS (Solution B) at a mixing ratio A/B = 3/2, with the weight content of tri-PCG-Acryl in the total polymer being 40%. This IP formulation is denoted as **F(P1/D+PA$_{40}$)**, where P1, /D, and +PA$_{40}$ denote the presence of tri-PCG-1 added, the presence of DPMP added, and the amount tri-PCG-Acryl added was 40 wt% in total polymers, respectively. The control formation, prepared using only tri-PCG-1 or tri-PCG-Acryl, is denoted as **F(P1)** or **F(PA)**. The phase diagrams for tri-PCG-1 and tri-PCG-Acryl are shown in Figure 2. Both these polymer solutions (25 wt%) adopted a gel state at 37 °C.

The sol-to-gel transition behavior of the **F(P1/D+PA$_{40}$)** and **F(P1)** formulations containing GLP-1 are shown in Figure 3. Both formulations showed a sol-to-gel transition in response to a temperature

increase from 25 to 37 °C. The presence of GLP-1 had almost no effects on the sol-to-gel transitions of these formulations (Figure 4), and the transition temperatures were between 25 and 37 °C. After cooling to 4 °C, **F(P1)** containing GLP-1 adopted the sol state, showing that the sol-to-gel transition was reversible. In contrast, **F(P1/D+PA$_{40}$)** containing GLP-1 remained in the gel state after cooling, showing that the sol-to-gel transition was irreversible. These behaviors are in accordance with our earlier observations in the absence of GLP-1[15]. Consequently, the presence of GLP-1 had no effect on the phase transition irreversibility of the formulation.



Scheme 1. Synthesis of PCGA-*b*-PEG-*b*-PCGA triblock copolymer (tri-PCG).



Scheme 2. Synthesis of tri-PCG-Acryl

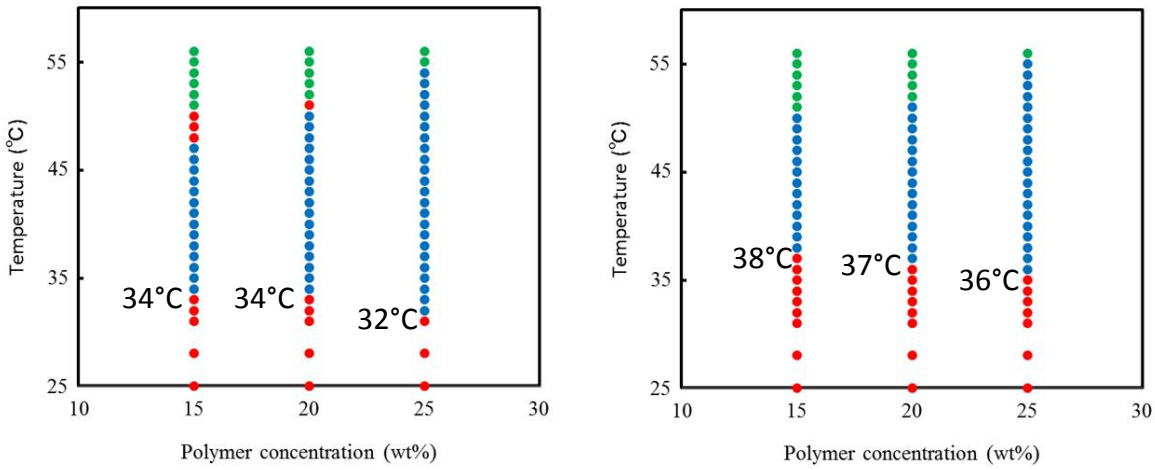


Figure 2. Phase diagram of (a) tri-PCG and (b) tri-PCG-Acryl. ●: sol, ●: gel, ●: sol (syneresis). Gelation temperature (T_{gel}) of each concentration was indicated.

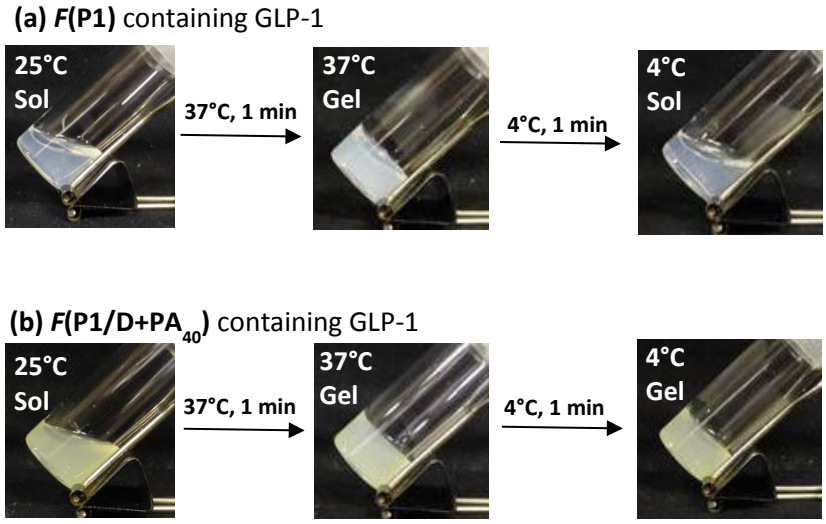


Figure 3. Photographs of (a) **F(P1)** hydrogel containing GLP-1 and (b) **F(P1/D+PA$_{40}$)** hydrogel containing GLP-1 after heating at 37°C for 1 min and subsequent cooling at 4°C for 1 min.

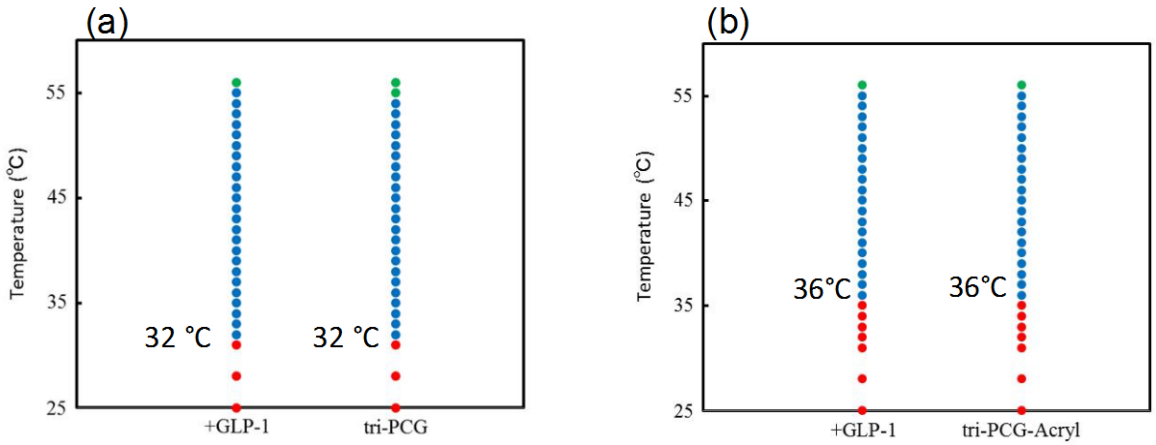


Figure 4. Comparison of gelation temperature in the presence or absence of GLP-1 for (a) tri-PCG and (b) tri-PCG-Acryl. ●: sol, ●: gel, ●: sol (syneresis). The polymer concentration = 25 wt%. Gelation temperature (T_{gel}) of each sample was indicated.

3.3.2 *In vitro* release test of GLP-1 from hydrogels

We next investigated the *in vitro* release behavior of GLP-1 from the formulations. Figure 5 shows the cumulative amount of GLP-1 released from **F(P1/D+PA$_{40}$)** and **F(P1)** hydrogels at 37 °C *in vitro*. Both hydrogels showed rapid release of GLP-1 during the first two days, and the release rate from **F(P1)** was higher than that from **F(P1/D+PA$_{40}$)**. On Day 2, the cumulative amount of GLP-1 released from **F(P1)** and **F(P1/D+PA$_{40}$)** was about 59% and 38%, respectively. We previously reported that irreversible **F(P1/D+PAx)** IP hydrogels with higher DPMP and tri-PCG-Acryl content showed a longer swelling ratio compared with hydrogel without cross-linking (**F(P1)**) and compared with hydrogels with lower DPMP and tri-PCG-Acryl content. This lower swelling was due to the higher cross-linking densities of these gels[15]. The mesh size of a hydrogel increases due to swelling and cross-linking density correlates with the swelling ratio[22]. The main reason for the more rapid release of GLP-1 from **F(P1)** compared to **F(P1/D+PA$_{40}$)** hydrogel is likely due to differences in the swelling ratios and diffusion coefficients of the two gels. Figure 6 shows photographs of the hydrogels taken during the release tests. The **F(P1)** hydrogel adopts a highly swollen state within 2 h and the formulation is essentially a sol after Day 1. By Day 2, the release of GLP-1 from **F(P1)** becomes gradual. As shown in Figure 6, although **F(P1)** reverts to the sol state completely by Day 30, 100% cumulative release is not attained. The GLP-1 molecule can

adsorb onto the polymer chains by hydrophobic interactions[23], and GLP-1 may be unstable due to spontaneous hydrolysis; consequently, 100% of the GLP-1 molecules could not be detected by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. On the other hand, the release of GLP-1 from the **F(P1/D+PA$_{40}$)** hydrogel became very slow after Day 2. As shown in Figure 6, **F(P1/D+PA$_{40}$)** remained in the gel state even after 30 days. The storage modulus (G') (4202 Pa) of **F(P1/D+PA$_{40}$)** was larger than the loss modulus (G") (326 Pa) and was similar to that of the sample on Day 0 (4696 Pa) (Table 3). As described above for **F(P1)**, not all the GLP-1 molecules could be detected by RP-HPLC analysis. Regardless, some GLP-1 remained inside the hydrogel and was released very slowly.

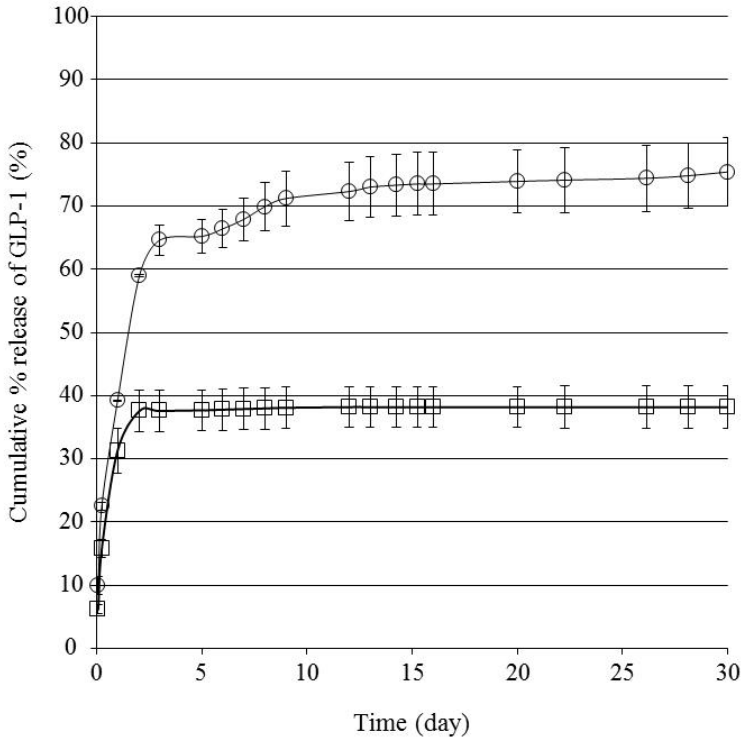


Figure 5. Cumulative release of GLP-1 (%) *in vitro* from **F(P1)** hydrogel containing GLP-1 (○) and **F(P1/D+PA$_{40}$)** hydrogel containing GLP-1 (□). The data are shown as the mean ±SD (n = 3).

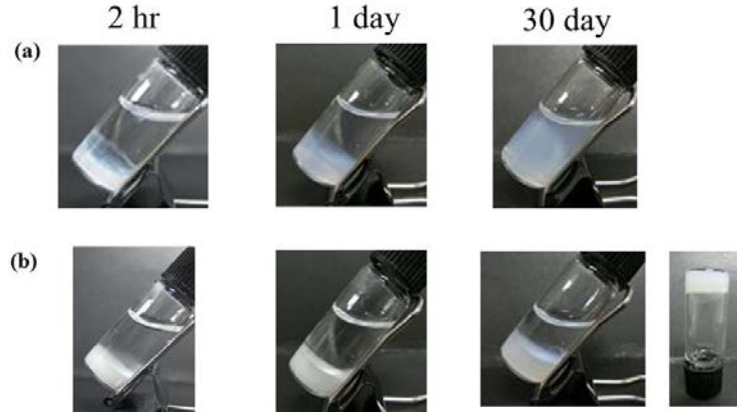


Figure 6. Photographs of (a) **F(P1)** hydrogel containing GLP-1 and (b) **F(P1/D+PA$_{40}$)** hydrogel containing GLP-1 during the *in vitro* release test.

Table 3. Physical properties of the hydrogels during release tests and *in vivo* experiments.

Formulation	*In vitro* or *vivo*	Day	G' (Pa)	G" (Pa)
F(P1)	*in vitro*	0	353	99
		30	N.D. [1]	N.D. [1]
	in vivo	25	N.D. [2]	N.D. [2]
F(P1/D+PA$_{40}$)	*in vitro*	0	4694	569
		30	4202	326
	in vivo	25	1147	364

[1] Not detected because of being dissolved, [2] not detected because of disappearance.

3.3.3 Cytotoxicity

To evaluate the cytocompatibilities of the IP formulations, we investigated the cytotoxicity of each formulation and their components towards L929 mouse fibroblast cells. The experiments were under dilute conditions (< 1 wt%), below the critical gelation concentration of each polymer. The results are shown in Figure 7. **F(P1)** (tri-PCG solution) exhibited no cytotoxicity over the concentration range tested. In contrast, **F(P1/D)** (tri-PCG micelle entrapping DPMP solution: (Solution A)) and **F(PA)** (tri-PCG-Acryl micelle solution: (Solution B)) showed weak cytotoxicity at concentrations above 0.01 wt% and 0.1 wt%, respectively. Liu et al. reported that oligo-thiols such as dithiothreitol (DTT) are

cytotoxic towards NIH/3T3 fibroblasts and rat bone marrow mesenchymal stem cells (BMSCs)[24]. Klouda et al. reported that polymers with multivalent acryloyl groups showed cytotoxicity[25]. Our results are therefore in agreement with these earlier reports. The thiol groups of DPMP in **F(P1/D)** and the acryloyl groups in **F(PA)** exhibited cytotoxicity, probably because of their interaction with the cell membrane. In contrast, the cytotoxicity of **F(P1/D+PA$_{50}$)** was negligible, similar to that of **F(P1)**. These results suggest that the thiol groups of DPMP and the acryloyl groups of tri-PCG-Acryl reacted with each other, decreasing the number of both functional groups, and that the reaction products exhibited no cytotoxicity. Consequently, **F(P1/D+PA$_{50}$)** showed no significant cytotoxicity.

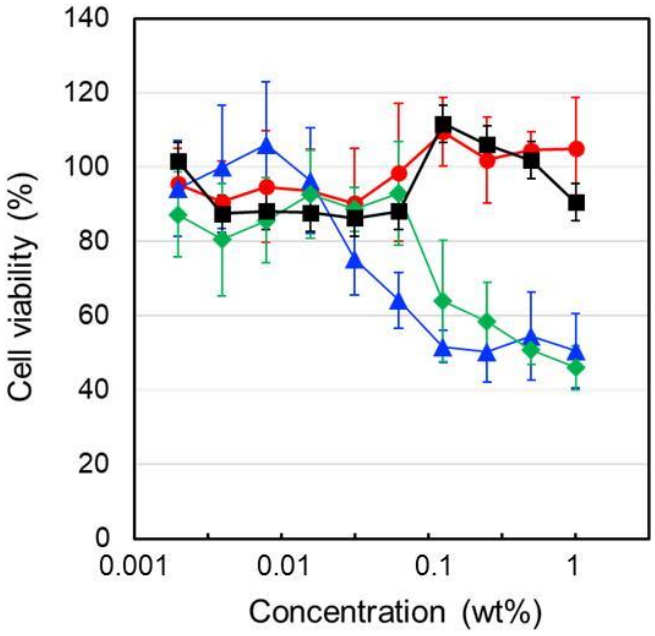


Figure 7. Cell viability of L929 fibroblast cells incubated in the presence of **F(P1)** (●), **F(P1/D)** (◆), **F(PA)** (▲) and **F(P1/D+PA50)** (■) in E-MEM containing 10% Fetal calf serum (FCS) at 37 °C for 21 h.

3.3.4 Plasma GLP-1 concentration in SD rats

We investigated the *in vivo* plasma concentration of GLP-1 after subcutaneous injection of the formulations into rats (Figure 8). We used an ELISA system to detect only active GLP-1; inactivated GLP-1 is not detectable by this system. GLP-1 disappeared from the plasma very quickly following the

injection of the GLP-1 solution: some GLP-1 was detected in the plasma after 2 h, but essentially none was detected after 1 day. This is as expected because the half-life of GLP-1 after subcutaneous injection is very short ($t_{1/2}$ = 9 min)[21]. The injection of **F(P1)** formulations resulted in much higher plasma GLP-1 levels compared to the GLP-1 solution at 2 h, suggesting that the **F(P1)** hydrogel provided a delayed effect compared to the solution, allowing GLP-1 to be absorbed from the injection site and transferred to the blood circulation. The plasma GLP-1 level subsequently decreased rapidly to 100 ng/L after 19 days, which is slightly higher than the level found in the control rats. The plasma GLP-1 level decreased to the control level 25 days after the injection of **F(P1)** hydrogel. These results are in relatively good agreement with the *in vitro* release tests (Figure 5): rapid, early release of GLP-1 from **F(P1)** hydrogel, then continuous sustained release from the sol state polymer chains acting as adsorbents in the subcutaneous space. **F(P1/D+PA$_{40}$)** hydrogel also provided a relatively high plasma GLP-1 level 2 h after injection, similar to that of the GLP-1 solution and much lower than that of the **F(P1)** hydrogel. This results suggests that the initial burst release of GLP-1 was suppressed by the lower swelling of the gel resulting from covalent cross-linking, in good agreement with the *in vitro* results. Interestingly, although **F(P1/D+PA$_{40}$)** hydrogel exhibited no detectable release of GLP-1 after 3 days *in vitro*, rats in the **F(P1/D+PA$_{40}$)** group showed higher plasma GLP-1 levels (about 300 ng/L) between Day 7 and Day 25 compared with the **F(P1)** and control rats. These results suggest that the hydrogel network in **F(P1/D+PA$_{40}$)** might be partially hydrolyzed by body fluids or by autocatalytic effects[24], resulting in the slow release of the GLP-1 entrapped in the hydrogel matrix *in vivo*. This speculation is supported by the results of a physical strength study (Table 3). The G' value of subcutaneously implanted **F(P1/D+PA$_{40}$)** hydrogel was 1147 Pa after 25 days, which is much lower than the G' value obtained *in vitro* after 30 days' incubation (4202 Pa), suggesting partial degradation. Kim et al. reported that the blood glucose level decreased significantly at a plasma GLP-1 level of around 200 ng/L[25]. Therefore, the plasma GLP-1 level (about 300 ng/L) obtained following the injection of **F(P1/D+PA$_{40}$)** hydrogel containing GLP-1 should be sufficient to be pharmacologically active over a period of 25 days. Figure 9 shows photographs of the sites where the formulations were injected subcutaneously in rats 25 days. All **F(P1)** hydrogel injected disappeared within 25 days, whereas all **F(P1/D+PA$_{40}$)** hydrogel remained at the injection site after 25 days and was in the gel state (G' (1147 Pa) was larger than G'' (364 Pa); Table 3). We can,

therefore, expect the continuous release of GLP-1 from **F(P1/D+PA$_{40}$)** hydrogel even after 25 days.

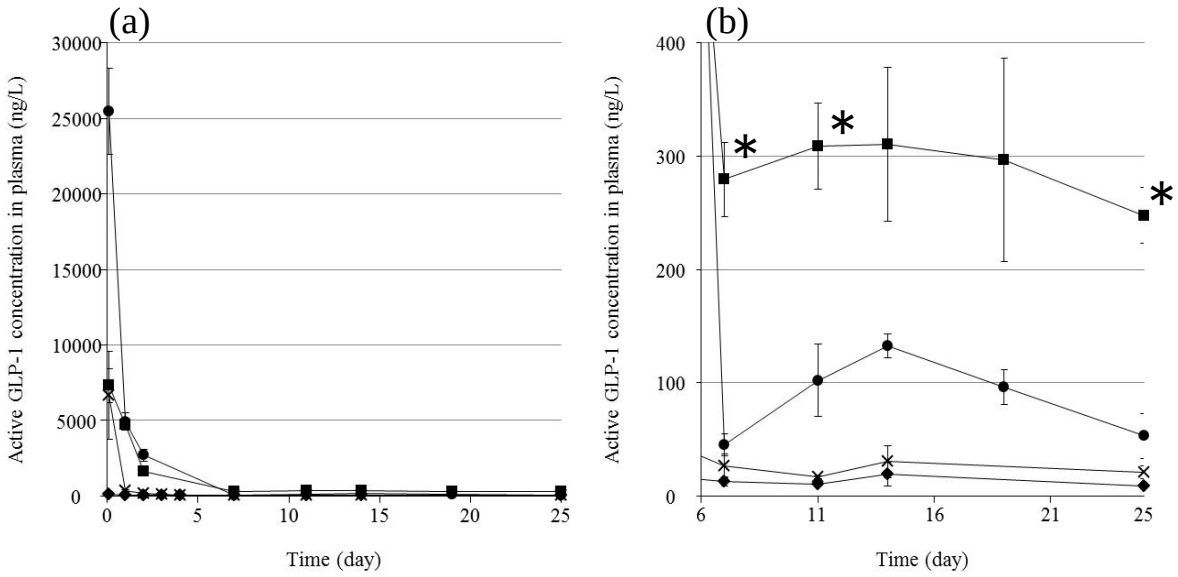


Figure 8. (a) Active GLP-1 concentration in plasma after subcutaneous injection for 25 days. (b) The Magnification of the area between Day 6 and 25. GLP-1 solution (×), **F(P1)** containing GLP-1 (●), **F(P1/D+PA$_{40}$)** containing GLP-1 (■), and **F(P1/D+PA$_{40}$)** without GLP-1 (◆). The data are shown as the mean ± SE (n = 4-6). ＊: $P < 0.05$ vs **F(P1)** containing GLP-1.

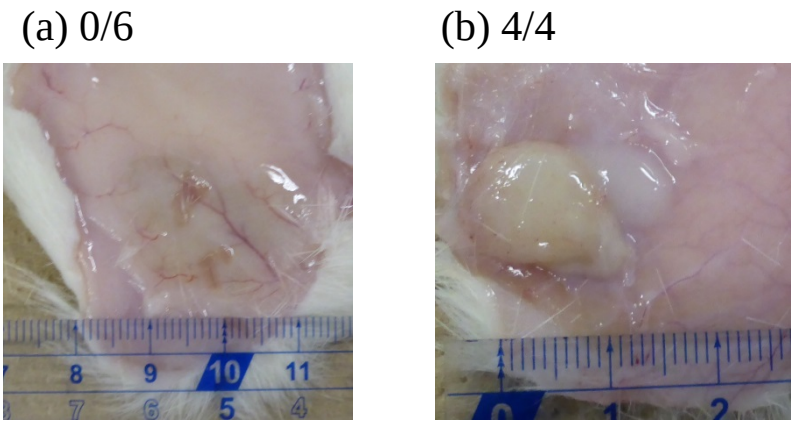


Figure 9. Photographs of hydrogels (a) **F(P1)**, (b) **F(P1/D+PA$_{40}$)** 25 days after subcutaneous injection in rats. The values indicate the number of rats harboring hydrogel/all rats.

3.4 Conclusions

We achieved long-term maintenance levels of plasma GLP-1 *in vivo* by using biodegradable IP system that shows irreversible gelation due to covalent bond formation. Compared with a conventional physical gelation system, this irreversible hydrogel system exhibits a lower swelling ratio early in the drug release process, a suppressed initial burst, and sustained release of the encapsulated drug. Moreover, the system remained in the gel state for a longer time and gradually degraded after subcutaneous injection, which could help maintain a therapeutic blood drug level for over 1 month. This IP formulation did not exhibit severe cytotoxicity. Therefore, this irreversible IP hydrogel system holds promise for use in a minimally invasive sustained drug release system for hydrophilic compounds such as peptides and proteins. Especially, the IP system with GLP-1 can be a new effective therapeutic system for type 2 diabetes providing good quality of life of patients without frequent injections.

3.5 References

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Chapter 4
Concluding Remarks

In Chapter 2, I investigated polymer chain transfer (exchange) between micelles composed of temperature-responsive biodegradable polymer through gelling process. Amphiphilic triblock copolymers of poly(ethylene glycol) (PEG) and aliphatic polyesters such as poly(ε-caprolactone-co-glycolide) (PCGA) are typical examples of thermo-gelling polymers. In the gelation mechanism of these polymers, polymer chain transfer between the micelles, and subsequent aggregation of the polymer micelles are important steps. We previously reported an IP system exhibiting a temperature-responsive irreversible sol-gel transition that formed a covalently cross-linked hydrogel. The IP formulation prepared by the "freeze-dry with PEG/dispersion" method (**D**-sample) retained its sol state at r.t. longer than a formulation prepared by the usual dissolution method (**S**-sample). I hypothesized that polymer chain transfer between micelles was suppressed in the **D**-samples, because the micelle cores were in a solid-like semi-crystalline state. In this study, I investigated polymer chain transfer by the fluorescence resonance energy transfer (FRET) method to reveal its role in the sol-to-gel transition. We synthesized a triblock copolymer of PCGA and PEG (tri-PCG) with covalently attached naphthalene or dansyl groups at both termini, tri-PCG-nap and tri-PCG-dan, as FRET donors and acceptors, respectively. The FRET behavior of the mixture of tri-PCG-nap/tri-PCG micelles and tri-PCG-dan/tri-PCG micelles was investigated. It was revealed that polymer chain transfer between micelles was strongly accelerated at the gelation temperature, and polymer chain transfer in **D**-samples was suppressed compared to **S**-samples.

In chapter 3, I investigated the release behavior of glucagon-like peptide-1 (GLP-1) from a biodegradable injectable polymer (IP) hydrogel. This hydrogel shows temperature-responsive irreversible gelation due to the covalent bond formation through a thiol-ene reaction. *In vitro* sustained release of GLP-1 from an irreversible IP formulation **F(P1/D+PA$_{40}$)** was observed compared with a reversible (physical gelation) IP formulation (**F(P1)**). Moreover, pharmaceutically active levels of GLP-1 were maintained in blood after subcutaneous injection of the irreversible IP formulation into rats. This system should be useful for the minimally invasive sustained drug release of peptide drugs and other water-soluble bioactive reagents.

List of publications

Chapter 2

Kazuyuki Takata, Keisuke Kawahara, Yasuyuki Yoshida, Akinori Kuzuya, Yuichi Ohya

Analysis of the sol-to-gel transition behavior of temperature-response injectable polymer systems by fluorescence resonance energy transfer.

Polym. J. **2017**, 49, 677-684

Chapter 3

Kazuyuki Takata, Hiroki Takai, Yuta Yoshizaki, Takuya Nagata, Keisuke Kawahara, Yasuyuki Yoshida, Akinori Kuzuya, Yuichi Ohya

Peptide drug release behavior from biodegradable temperature-responsive injectable hydrogels exhibiting irreversible gelation.

Gels **2017**, 3, 11pages doi:10.3390/gels3040038

Other publications

Yasuyuki Yoshida, Hiroki Takai, Keisuke Kawahara, Shintaro Mitsumune, Kazuyuki Takata, Akinori Kuzuya, Yuichi Ohya

Biodegradable Injectable Polymer Systems Exhibiting Longer and Controllable Duration Time of the Gel State.

Biomat. Sci. 2017, 5, 1304-1314.

Yasuyuki Yoshida, Kazuyuki Takata, Hiroki Takai, Keisuke Kawahara, Akinori Kuzuya, Yuichi Ohya

Extemporaneously Preparative Biodegradable Injectable Polymer Systems Exhibiting Temperature-Responsive Irreversible Gelation.

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