Platform Establishment of Analytical Development, Characterization, Quality and Cardiotoxicity Evaluation for Amphiphilic Poly(γ-glutamic acid) as Raw Material and Nanoparticles

Mayumi Ikeda

Osaka University

March 2019

Contents

Page

General Introduction

Nanomedicines are often considered to be complicated systems compared to traditional medicines [1]. The ability of nanotherapeutics to provide targeted drug delivery, improve drug solubility, extend drug half-life, improve a drug's therapeutic index, and reduce a drug's immunogenicity has resulted in the potential to revolutionize the treatment of many diseases [2]. The main goal is to improve drug bioavailability, pharmacokinetics, efficacy, and safety to promote the treatment of diseases which currently cannot be achieved with conventional dosage forms [3]. A colloidal delivery system containing nanoparticles (NPs), liposomes, microemulsions, polymeric self-assemblies, and so on [4] is one of the most promising because it may reduce unwanted toxic side effects and improve the therapeutic effect. These goals may be achieved by targeting them to the specific site of action, reducing their dose through improved bioavailability and improving shelf life by enhancing their stability. Nanotechnology by manipulation of characteristics of materials such as polymers and fabrication of nanostructures is able to provide superior drug delivery systems for better management and treatment of diseases [5].

Amphiphilic block or graft copolymers have been found to form self-assembled, nano-sized micelle-like aggregates of various morphologies in aqueous solution [6,7] and can be varied by changing the composition of the hydrophobic and hydrophilic blocks on the polymer chains. Various applications for NPs prepared by synthetic polymers such as poly(d,l-lactide-co-glycolide) (PLG), poly(lactic acid) (PLA), poly(d,l-lactic-coglycolic acid)(PLGA) have frequently been reported [8-11] in various technological and biomedical fields. Amphiphilic block copolymers such as poly(ethylene -glycol)-block-poly (lactic acid) (PEG-b-PLA), or PEG-b-polycaprolactone) (PEG-b-PCL) are very attractive for use in drug delivery applications [12,13] and have been shown to have great potential as vaccine delivery systems and immunostimulatory adjuvants [14]. These polymeric NPs entrap

antigens for delivery to certain cells or sustain antigen release by virtue of their slow biodegradation rate [15].

Our research group has already reported that biodegradable NPs consisting of molecules of hydrophilic poly(γ-glutamic acid) (γ-PGA) with a hydrophobic L-phenylalanine ethyl ester (Phe) side chain, i.e., γ-PGA-Phe NPs, which self-assemble into nanomicelles with a hydrophilic outer shell and a hydrophobic inner core, can be used for the sustained or targeted release of drugs [16,17]. The γ-PGA-Phe NPs have shown great potential as vaccine adjuvants. These NPs did not induce any cytotoxicity *in vitro* or *in vivo* [18,19] and the drug release rate can be controlled by varying the composition ratio and molecular weight (MW) of the graft or block copolymers [20-22].

On the other hand, for clinical application of the NP-based vaccine adjuvants, it is necessary to guarantee the quality and safety of the polymer as a raw material and the polymeric NPs. It is also important to be able to manufacture high-quality NP formulations with well-defined properties and functions for clinical development. Even though the use of biodegradable polymers for medicine is increasing and sufficient quality assurance is required for them, there are few examples of reliable analysis of polymers or NPs for guaranteeing their quality as pharmaceutical products. The properties of amphiphilic polymers as materials have not received much attention. While analytical developments in polymer research have been critical, advancements in analytical methods for biological research would lead to a better understanding of how biomaterials interact with biological systems. However, there are also enormous challenges in the development of new quantitative analytical methods, because it can be very difficult to create robust methods for assaying trace amounts of polymer and impurities [23]. Without an understanding of the distributions of polymer configurations, only the average structural features of copolymers can be determined. Though nanomedicine is a quickly evolving field where more and more possible applications are becoming evident and entering clinical trials or even the market; the analytical methods for NPs are not always

able to keep pace with the new formulations' demands. Therefore, it was necessary to identify the critical steps in the scale-up and manufacturing process for nanotechnology products. Understanding the physicochemical properties allowed the selection of the most appropriate technique for the synthesis of γ-PGA-Phe and manufacture of NPs. It is then particularly important to establish the shelf life of the product with an appropriate manufacturing process and formulation selection that ensures sufficient stability to maintain a desirable quality and physicochemical characteristics of the formulation. Keeping NPs stable during storage is a challenging issue in pharmaceutical product development. Low NP stability during storage seriously limits their practical application since this lack of stability can directly affect the efficacy and safety of the drug delivery system.

In addition, the actual and potential impurities are most likely to arise during the synthesis, purification, and storage of NPs. Despite the potential benefits that nanomedicine has to offer, the use of novel NPs and chemicals requires reliable data on their potential toxic effects on humans [24]. Innovative three-dimensional (3D) cell culture technology is giving scientists the ability to grow realistic human tissues for more effective drug testing while reducing the need for animal research. Our research group has already reported a cell-accumulation technique and the construction of 3D human induced pluripotent stem cells (hiPSCs)-cardiomyocyte (CM) tissues by coating extracellular matrices (ECM) nanofilms with fibronectin and gelatin (FN-G) onto a cell membrane using a layer-by-layer (LbL) technique for cells [25-27]. Therefore, the author considers that the recent availability of *in vitro* models based on hiPSCs opens up new opportunities for the development of *in vitro* models of screening for new DDS carriers and drugs, and patient-specific cardiac therapy.

In this study, the author focused on the establishment of a system for evaluating the quality of polymers and NPs, to assure the quality of γ-PGA-Phe as the raw material and the polymeric NPs composed of γ-PGA-Phe. Moreover, the author established the shelf life of the product with an appropriate manufacturing process and formulation selection that ensures sufficient stability and

assessed the safety of γ-PGA-Phe NPs by setting criteria for assessing the quality of NPs.

This thesis includes 3 chapters as follows:

Chapter 1 mainly focuses on the characterization of γ-PGA as a raw material for generating NP-based adjuvants as well as the development of quantitative analytical methods for γ-PGA-Phe, and the stability study. For clinical study, the quality of raw materials is a crucial factor and the characterization can support the selection of an appropriate formulation design. The novel analytical methods could quantitatively determine content of γ-PGA-Phe and impurity by reverse-phase chromatography (RP-HPLC) and absolute MW by SEC-RI/MALS system using size exclusion chromatography (SEC) coupled with a multi-angle light scattering (MALS) detector and refractive index (RI) detector. Using the reliable and practical methods established, the author clarified the MW of γ-PGA, the grafting degree of Phe for γ-PGA, and differences of the characterization in γ-PGA-Phe for different vendors of γ-PGA to develop robust manufacturing processes based on the physicochemical properties. Moreover, the author discussed the degradation mechanism of amphiphilic polymer (γ-PGA-Phe) observed in the stability studies as it has a significant impact on their safety and efficacy. The stability study could provide reliable evidence on how the quality of γ-PGA-Phe differs with time under environmental effects such as varying humidity, temperature or the pH of the buffer solution.

Optimization of the manufacturing process would reduce batch failures and improve yields but these goals are hampered by the lack of techniques for evaluating the characteristics and quality of NPs. **Chapter 2** demonstrates the establishment of new quantitative analytical methods for evaluating the quality of γ-PGA-Phe NPs, and the effects of manufacturing parameters and the quality of different formulations on ensuring high yields and consistently high quality in the production process.

The analytical methods were able to determine the component polymer (γ-PGA-Phe) content and

the impurities in the NPs as well as the absolute MW by fully dissociating the NPs to intact γ-PGA-Phe by the adsorption of SDS on γ-PGA-Phe. They were applied to optimize and evaluate the NP manufacturing process and were able to distinguish differences between NPs generated by different processes. As such, they allow the most suitable manufacturing process to be identified. In addition, the author suggested which critical parameters of the NPs need to be monitored throughout formulation development in order to achieve high reproducibility.

The establishment of adequate stability of formulation is a crucial component of drug development to help in selecting proper formulation. **Chapter 3** describes the assessment of the stability of NP formulations stored under various conditions, and how best to store NPs to retain efficacy while maximizing convenience, and find the optimal formulation and storage condition. The author designed appropriate stability protocols including test parameters and attributes of the formulation that are susceptible to change during storage and likely to influence quality, safety and efficacy for clinical application. It was shown that lyophilized NP formulation with trehalose provided a stable and high quality product for clinical studies and showed promise as an effective drug delivery system carrier.

Furthermore the use of novel NPs and chemicals requires reliable data on their potential toxic effects on humans. The cardiotoxicity of prospective impurities contained in NPs and reagents used in the manufacturing process was studied with vascularized iPSC derived 3D-CM by LbL. Toxicity was not observed and it was determined that the potential risk to humans from NPs is low. The author provided reasonable assurance that the products would remain at an acceptable level of quality for supply to patients throughout the period of a clinical study.

The analytical methods established in this study should facilitate the reliable and practical quality and safety testing of NP products, thus aiding the clinical development of γ-PGA-Phe-based drug-delivery systems. This approach, which establishes a platform for the practical application and

commercialization of vaccines using biodegradable NPs as vaccine adjuvants, should prove useful for product development.

References

[1] C. L. Ventola, *Pharmacy and therapeutics* **37**, 517 (2012).

[2] A. Hafner, J. Lovrić, G. P. Lakoš, I. Pepić, *Int. J. Nanomedicine* **19**, 1005 (2014).

[3] A. Hafner, J. Lovrić, G. P. Lakoš, I. Pepić, T. Lammers, F. Iessling, W. E. Hennink, G. Storm, *J. Controlled Release* **161**,175 (2012).

[4] S. T. Ram, R. Agrawal, *Current Drug Therapy* **10**, 20 (2015).

[5] N. A. Ochekpe, P. O. Olorunfemi, N. C. Ngwuluka, *Tropical Journal of Pharmaceutical Research* **8**, 275 (2009).

[6] L. F. Zhang, A. Eisenberg, *J. Am. Chem. Soc.* **118**, 3168 (1996).

[7] X. M. Liu, K. P. Pramoda, Y. Y. Yang, S.Y. Chow, C. He, *Biomaterials* **25**, 2619 (2004).

[8] C. Thomas, A. Rawat, L. H. Weeks, F. Ahsan, *Mol. Pharm*. **405**, 8(2011).

[9] S. Y. Kim, H. J. Doh, M. H. Jang , Y. J. Ha, S. I. Chung, H. J. Park, *Helicobacter* **4**, 33 (1999).

[10] J. M. Lü, X. Wang, C. M. Muller, H. Wang, P. H. Lin, Q. Yao, *Expert Rev. Mol. Diagn.* **4**, 325 (2009).

[11] R. L. McCall, R. W. Sirianni, *J. Vis. Exp*. **82**, 51015 (2013).

[12] M. Iijima, Y. Nagasaki, T. Okada, M. Kato, K. Kataoka, *Macromolecules* **32**, 1140 (1999).

[13] C. Allen, J. Han, Y. Yu, D. Maysinger, *J. Controlled Release* **63**, 275 (2000).

[14] V. Sokolova , T. Knuschke, J. Buer, A. M. Westendorf, M. Epple, *Acta Biomater*. **7**, 4029 (2011).

[15] S. L. Demento, W. Cui, J. M. Criscione, E. Stern, J. Tulipan, S. M. Kaech, *Biomaterials* **33**, 4957 (2012).

[16] T. Akagi, M. Baba, M. Akashi, S. Kunugi, *Polymers*, In: T. Yamaoka, editor, Nanomedicine, Berlin, Germany, Springer-Verlag Berlin, 3164 (2012).

[17] T. Akagi, T. Kaneko, T. Kida, M. Akashi, *J. Controlled Release* **108**, 226 (2005).

[18] T. Shima, M. Akagi, M. Akashi, *Biocon. Chem.* **26**, 890 (2015).

[19] T. Uto, T. Akagi, M. Akashi, M. Baba, *Clin. Vaccine Immunol*. **22**, 578 (2015).

[20] T. Akagi, X. Wang, R. I. Khalil, U. V. Irorere, I. Radecka, T. A. Burns, P. M. Kowalczuk, L. J. Mason, P. M. Khechara, *Int. J. Mol. Sci*. **18,** 313 (2017).

[21] L. Shih, Y. T. Van, *Bioresour. Technol.* **79**, 207 (2001).

[22] D. Ljubie, M. Pahovnik, E. Zigon, *The Scientific World Journal,* Article ID **932609**, 9 (2012).

[23] T. J. Oberlerchner, T. Rosenau, A. Potthast, *Molecules* **20**, 10313 (2015).

[24] P. Galvin , D. Thompson , K. B. Ryan, *Cell Mol. Life Sci.* **69**, 389 (2012).

[25] Y. Tsukamoto, T. Akagi, F. Shima, M. Akashi, *Tissue Eng. Part C Methods* **6**, 357 (2017).

[26] Y. Amano, A. Nishiguchi, M. Matsusaki, H. Iseoka, S. Miyagawa, Y. Sawa, M. Seo, T. Yamaguchi, M. Akashi, *Acta Biomaterial* **33**, 110 (2016).

[27] H. Narita, F. Shima, J. Yokoyama, S. Miyagawa, Y. Tsukamoto, Y. Takamura, A. Hiura, K. Fukumoto, T. Chiba, S. Watanabe, Y. Sawa, M. Akashi, H. Shimoda, *Scientific Reports* **7**, 13708, DOI:10.1038/s41598-017-14053-0.

Chapter 1.

Characterization and analytical development for amphiphilic poly(γ-glutamic acid) as raw material of nanoparticle adjuvants

1. 1. Summary

Amphiphilic graft copolymer consisting of poly(γ-glutamic acid) (γ-PGA) as the hydrophilic backbone and L-phenylalanine ethyl ester (Phe) as the hydrophobic side chain is an important biodegradable polymer with great potential in medical applications. In **Chapter 1**, the author established analytical methods for the characterization and quality control of γ-PGA-*graft*-Phe (γ-PGA-Phe), which forms nanoparticles in aqueous solution, as a deployment platform in practical applications for vaccine adjuvants. The SEC-RI/MALS system, which uses size exclusion chromatography (SEC) coupled with a multi-angle light scattering (MALS) detector and refractive index (RI) detector, was developed to evaluate the characteristics of various types of polymers. By this method, it was indicated that absolute molecular weight (MW) should be used to measure the branch polymer. A gradient reverse-phase chromatography (RP-HPLC) method was developed for the content of γ-PGA-Phe and the impurity levels to control product quality and safety. This quantitative approach could become key elements for identifying and characterizing γ-PGA-Phe. In addition, the degradation mechanism of γ-PGA-Phe was also identified as cleavage of main-chain of γ-PGA-Phe based on the stability study of γ-PGA-Phe in buffer solution with various pH values. The analytical developments described above will be important for use in both characterization and formulation design of biopolymers. Nanoparticles (NPs) composed of well-characterized

biodegradable γ-PGA-Phe are expected to have a variety of potential clinical applications such as their use as drug and vaccine carriers.

1. 2. Introduction

Recently, self-assembling block copolymers or hydrophobically modified polymers have been extensively investigated in the fields of biotechnology and pharmaceuticals. Amphiphilic block or graft copolymers have been found to form self-assembled, nano-sized micelle-like aggregates of various morphologies in aqueous solution [1,2]. Amphiphilic block copolymers such as poly(ethylene glycol)-*block*-poly (lactic acid) (PEG-*b*-PLA), or PEG-*b*-poly(ε-caprolactone) (PCL) are very attractive for use in drug delivery applications [3,4].

Poly(γ-glutamic acid) (γ-PGA) is an unusual anionic polypeptide produced by *Bacillus subtilis* comprising D/L-glutamate monomers polymerized through γ-glutamyl bonds [5] and is biodegradable, edible, water-soluble, and nontoxic for humans and the environment [6]. γ-PGA has been used widely in drug delivery platforms, because it has carboxyl groups on the side chains; this group offers attachment points for the conjugation of chemotherapeutic agents. The drug release rate can be controlled by varying the composition ratio and molecular weight (MW) of the graft or block copolymers [7-9].

In our previous study, nanoparticles (NPs) composed of γ-PGA conjugated with L-phenylalanine ethyl ester (Phe) as the hydrophobic segment (γ-PGA-Phe) were prepared for the development of vaccine and drug carriers [10-14]. Our group demonstrated that the γ-PGA-Phe NPs showed great potential as vaccine adjuvants, and these NPs did not induce any cytotoxicity *in vitro* or *in vivo* [15,16]. It has been demonstrated that the γ-PGA-Phe NPs are also effective for vaccines against human immunodeficiency virus (HIV) [17], influenza virus [18] or cancer [19] vaccines. The

preparation of the NPs and their potential application as vaccine adjuvants have recently been reviewed [20].

For clinical application of the NP-based vaccine adjuvants, it is needed to establish analytical methods of the γ-PGA-Phe copolymers in order to guarantee the quality and safety. Modifications added to the biodegradable polymers can be used to manipulate selected physical properties, such as toughness, flexibility, or barrier properties, thereby improving the functional capability. The advancement of analytical techniques for characterization has played an important role in the discovery, development and manufacture of pharmaceutical products. Characterization techniques should ideally be linked to the desirable properties of the polymers such as strength, impermeability, thermal stability, and optical properties. Using conventional methods of polymer characterization, only the average structural features of copolymers can be determined, without understanding the distributions of polymer configurations [21]. The average MW may be estimated by a number of techniques, such as light scattering, NMR, viscometric assay of the polymer's intrinsic viscosity, or chromatographic techniques [22]. Even though better characterization can improve the performance of polymers, the proprieties of amphiphilic polymers have not received much study. Especially, the characterization of branch polymers cannot be easily carried out because of the highly branched structures with a large number of functional end groups. As all of these measurements have experimental difficulties, they should be properly validated. There are also enormous challenges in analytical development, because it can be very difficult to create robust methods for assaying trace amounts of polymer and impurities [23]. Depending on the chemical nature of the polymer, good solvents for dissolution of samples may be difficult to find. Analysts must make extra effort to improve the method sensitivity and selectively of equipment used.

Chapter 1 mainly focuses on the development and evaluation of methods for characterization and quality analysis, which is important attributes that are built into the product. The development of

analytical techniques for determining content of γ-PGA-Phe and impurity by RP-HPLC and absolute MW by SEC-RI/MALS system was studied, and then optimization techniques was investigated by adjusting experimental parameters. Size exclusion chromatography (SEC) in combination with different detectors (e.g., ultraviolet (UV), refractive index (RI), multi-angle light scattering (MALS) can also provide information on the chemical composition as a function of the MW. SEC coupled with MALS detector is used as a tandem technique and is recognized as one of the most powerful techniques to obtain more extensive structure information for MWs of branched polymers with highly variable accuracy. The use of a MALS detector allowed for accurate MW determination independent of the column migration behavior [24].

Understanding the physicochemical properties allowed us to select the most appropriate technique for the synthesis of γ-PGA-Phe and manufacture of NPs. Information about the polymer chain structure can be determined based on the relationship between the MW and size. This information will allow the development of robust manufacturing processes based on the physicochemical properties and the quality of various polymers [25]. Therefore, it is necessary to clarify the MW of γ-PGA, the grafting degree of Phe for γ-PGA, and differences of the characterization in γ-PGA-Phe for different vendors of γ-PGA. The characterization will also support the selection of an appropriate formulation design.

Understanding the degradation mechanism is also important for biodegradable polymers but the degradation proprieties of amphiphilic polymers have rarely been studied. A method that seems to develop stable products can then be subjected to stability study for heat, humidity and pH in order to evaluate the potential degradation of the polymer. The purpose of the stability studies in **Chapter 1** is to provide reliable evidence on how the quality of γ-PGA-Phe differs with time under the effects of environmental factors such as humidity, temperature or the pH of the buffer solution.

1. 3. Experimental section

Materials

γ-PGAs (sodium salt form, D-Glu/L-Glu: 70/30) were commercial products, γ-PGA from vendor A (labeled MW: 480 kDa and 2,000 kDa) and γ-PGA from vendor B (labeled MW: 380 kDa). L-phenylalanine ethyl ester (Phe), phenylalanine (PA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), disodium hydrogen phosphate, hydrogen phosphate monosodium, trisodium citrate, citric acid and sodium dodecyl sulfate (SDS) for special grade respectively, and tetrahydrofuran (THF), ethanol (EtOH), acetonitrile (ACN) and distilled water for HPLC-grade were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). All other chemicals and solvents for analytical purity grade and polyethylene glycol/polyethylene oxide Standard ReadyCal Set Mp 200-1'200'000 were purchased from Sigma-Aldrich (St. Louis, MO). .

Synthesis of γ-PGA-Phe

Amphiphilic γ-PGA was synthesized as described previously [26,27] by the conjugation of Phe as the hydrophobic segment, which has biodegradable components containing carboxyl functional groups at the side chains, and γ-PGA as the hydrophilic backbone. Graft copolymers with different degrees of Phe grafting were prepared by changing the molar ratio of the glutamic acid units of γ-PGA to EDC. EDC reacts with the carboxyl groups of γ-PGA to form an active ester intermediate, which reacts with a primary amine group from the Phe to form an amide bond [10,27]. The chemical structures of purified γ-PGA-Phe was confirmed by ^{1}H NMR [27] and FT-IR spectroscopy [28], and characterized by ^{1}H NMR spectroscopy. The grafting degree of Phe was determined from the integral intensity ratio of the methylene peaks of γ-PGA to the phenyl group peaks of PA. The chemical structures of γ-PGA and γ-PGA-Phe are shown in Fig. 1-1 [26].

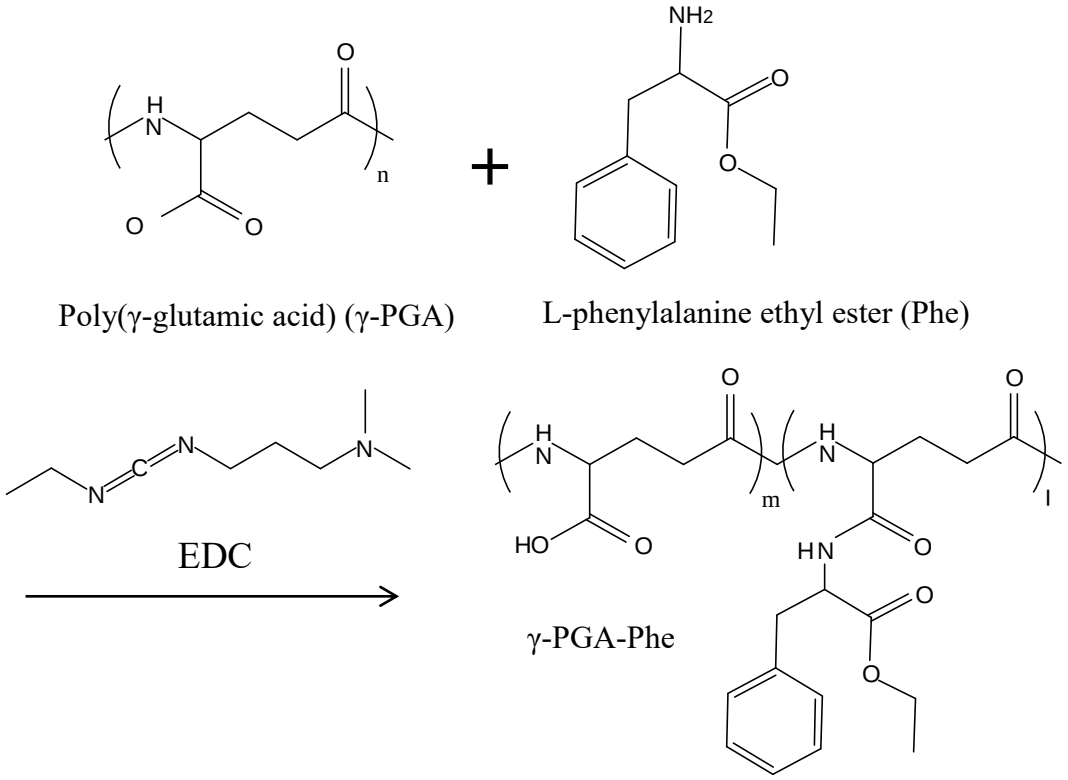


Fig. 1-1. Chemical structure of γ-PGA and synthesis scheme for γ-PGA-Phe. γ-PGA (4.7 unit mmol) dissolved in 50 mmol/L sodium carbonate solution was hydrophobically modified by Phe (4.7 mmol) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 24 hours at room temperature. Different amounts of EDC (4.7-9.4 mmol) per glutamic acid residue of γ-PGA were added. The purified γ-PGA-Phe was characterized by ^{1}H NMR and FT-IR spectroscopy. The grafting degrees of γ-PGA-Phe were controlled by altering the amount of EDC.

Determination of MW and MW distribution by SEC coupled to a MALS

SEC-RI/MALS measurements were performed using the Waters Alliance® system, which contains a 2695 pump, an auto sampler module and a degasser (Waters, Milford, MA), coupled to a Dawn Heleos II multi-angle light scattering (MALS) detector (Wyatt Technology Corp., Santa Barbara, CA), an Optilab T-rEX Refractive Index detector (RI) (Wyatt Technology Corp.) and a 2489 UV detector (Waters) operating at 225 nm and at a temperature of 25˚C.

The separations were carried out at flow rate of 0.4 mL/min using TSK gel columns (G2500 PWxL or G4000 PWxL, both 7.8 mm i.d. × 30 cm, 10 μm) in 20 mmol/L sodium phosphate buffer (pH8.0) and EtOH at a volume ratio of 1:1. γ-PGA powder and γ-PGA-Phe lyophilized powder were dissolved with the mobile phase for SEC-RI/MALS to prepare 2 mg/mL and filtrated using a GHP Acrodisc 13 mm syringe filter with a 0.2 μm GHP membrane (PALL Corp., Port Washington, NY). The mass of the samples injected onto the column was typically 0.6×10^{-4} g with a solution concentration of 3 or 1×10^{-3} g/mL.

The calculation of the average MW from MALS requires a sample-specific refractive-index increment (dn/dc), which was determined using the RI detector. The value was used for all subsequent MWs determinations. The SEC column was calibrated using standard containing 10 kinds of polyethylene glycol with a concentration of 1.0 mg/mL and polyethylene oxide with a concentration of 3.0 mg/mL. Absolute MWs and relative MWs were analyzed by ASTRA6 (Wyatt Technology Corp.) and Empower 2 software for GPC (Waters), respectively. The MW of γ-PGA-Phe was determined by a Debye plot obtained from the intensity of the scattered light. Both absolute and relative MWs for the estimation were weight average MW. PDI was calculated by dividing weight average MW by number average MW. The degradation ratio was calculated based on the shift of the main polymeric peak with time.

Evaluation of γ-PGA-Phe content and impurities by reverse phase chromatography

To quantify the γ-PGA-Phe content, a specific analytical method was developed. Briefly, γ-PGA-Phe lyophilized powder was dispersed in 20 mmol/L sodium phosphate buffer (pH 7.5). Then, 20 mmol/L sodium phosphate buffer (pH 7.5) containing 0.25% SDS (w/v) and 2% ACN (v/v) was added to these samples (0.1 mg/mL) and filtered. Content of γ-PGA-Phe and impurities were measured by reverse-phase chromatography (RP-HPLC) using a Waters Alliance® system. The separations were carried out at flow rate of 1 mL/min using Inertsil WP300 C4 (4.6 mm X 150 mm, 5 μm, GL Sciences, Japan) at a temperature of 40°C. Peaks were detected at 214 nm using a UV detector. The composition of eluent A was 0.25% SDS (w/v) and 2% ACN (v/v) in 20 mmol/L sodium phosphate buffer (pH 7.5) and the composition of eluent B was 0.25% SDS (w/v) and 60 % ACN (v/v) in 20 mmol/L sodium phosphate buffer (pH 7.5). A linear gradient was used: from 2 to 4% buffer B up to 4 min, from 4 to 100% buffer B up to 20 min and 100% buffer B up to 35 min. The samples (2×10^{-5} g) were injected into the column with a solution concentration of 1 or 0.2×10^{-3} g/mL. The degradation ratio was calculated based on the shift of the main polymeric peak was expressed as a percentage of degradation for γ-PGA-Phe. Empower 3 (Waters) was utilized for the data acquisition and analysis. The content and impurities of γ-PGA-Phe were calculated from area percentage peaks of in a chromatogram obtained by UV detector. Then, the content at each point (% of initial) for stability studies was calculated as [(content of γ-PGA-Phe at each point) / (initial content of γ-PGA- Phe)] × 100.

Sample preparation of MWs comparison of γ-PGA and γ-PGA-Phe for characterization and quality assessment

Vendor A's γ-PGA (labeled MW: 480 kDa and 2000 kDa), vendor B's γ-PGA (labeled MW: 380 kDa), and γ-PGA-Phe synthesized from each vendor and five kinds of γ-PGA-Phe with 23, 36, 40,

53 and 70% various grafting degrees of Phe were dissolved with mobile phase for SEC-RI/MALS and filtrated. These samples were analyzed by SEC-RI/MALS for characterization. γ-PGA-Phe synthesized by the fixed process was also dissolved with the mobile phase, filtrated and analyzed by SEC-RI/MALS and RP-HPLC respectively for quality assessment.

Stability of γ-PGA-Phe lyophilized powder

γ-PGA-Phe lyophilized powder with the grafting degree of 58% synthesized by vendor A's γ-PGA (labeled MW: 480 kDa). γ-PGA-Phe lyophilized powder was stored in the amber glass vials with a closed cap and a opened cap at 5°C and 25°C/60% relative humidity (RH) for 0 (Initial), 1 and 2 months, respectively. The cold chamber setting 5°C was used and the condition of 75% RH was controlled in a desiccator containing saturated sodium bromide solution to hold the humidity at 60% RH in a chamber setting at 25°C. "Close" in conditions means that the substance was not exposed to RH, even if the vial was placed in that climate. "Open" in conditions means that substance was exposed to RH.

γ-PGA-Phe was dissolved in a mixture of 20 mmol/L sodium phosphate buffer (pH 8.0) and EtOH solution (1:1, v/v) with the same volume of distilled water and mixed for 10 minutes. The mixed solution was filtrated. MW, content and impurity of samples were analyzed by SEC-RI/MALS and RP-HPLC.

Stability of γ-PGA-Phe in buffer solution

With regard to the preparation of γ-PGA-Phe buffer suspension, γ-PGA-Phe was dissolved in DMSO and 10 mg/mL was added to the same volume of distilled water and mixed for 10 minutes. The mixed solution was then dialyzed overnight with a 20 kDa cut-off dialysis cassette. The solution was freeze-dried overnight after dialysis. After freeze-drying, the powder was re-suspended with

distilled water. The resulting suspension was dispensed with 1 mL in each 3.5 mL glass vial (Daiwa Special Glass Co., Ltd, Japan). The dispensed vials were then freeze-dried again for five days.

Stability studies of γ-PGA-Phe, with the grafting degree of 58% synthesized by vendor A's γ-PGA (labeled MW: 480 kDa), in buffer solutions with pH values ranging from 4 to 9 were conducted to identify the degradation mechanism of γ-PGA-Phe. The resulting freeze-dried powder of γ-PGA-Phe was then suspended at concentration of 10 mg/mL with the following buffers at the specified pH values: 20 mmol/L citrate buffer (pH 5.0), 20 mmol/L citrate buffer (pH 6.0), 20 mmol/L sodium phosphate buffer (pH 7.0), 20 mmol/L sodium phosphate buffer (pH 8.0), and 20 mmol/L sodium phosphate buffer (pH 9.0). These γ-PGA-Phe samples in buffer solutions were stored at 5℃ for 1 month and at 25℃ for 1 month and at 40℃ for 2 weeks under accelerated conditions, to estimate the effects of temperature. Each sample was stored in the chambers setting each temperature. Then MW, content and impurity of samples were analyzed by SEC-RI/MALS and RP-HPLC.

1. 4. Results and Discussion

Development of analytical method for the characterization of γ-PGA and γ-PGA-Phe

It is important to develop analytical methods for γ-PGA and γ-PGA-Phe to understand their characterization and to support the effective development of self-assembled NPs for clinical application. To establish analytical methods of γ-PGA and γ-PGA-Phe, the SEC-RI/MALS system using 3 types of detectors, MALS (multi-angle light scattering), UV (ultraviolet) and RI (refractive index), was developed to evaluate the physicochemical properties of γ-PGA and γ-PGA-Phe (Fig. 1-2). The solubility of polymers can be significantly altered by the use of mixed solvents and the composition of solvent also effects the self-assembly properties of amphiphilic copolymers [29]. The

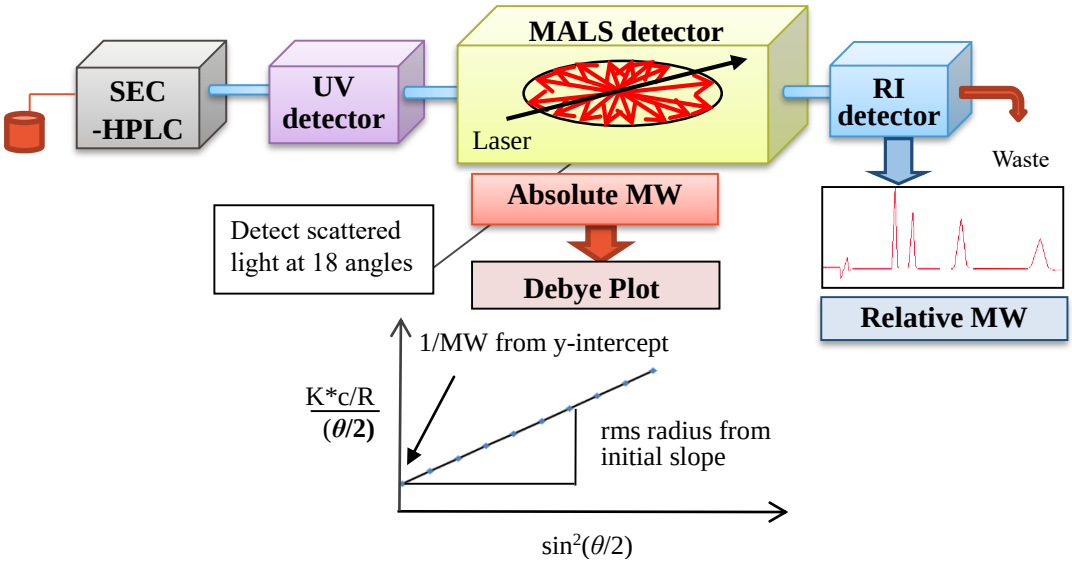


Fig. 1-2. SEC-RI/MALS System. The SEC column was linked to the Waters Alliance® system, which contains a 2695 pump, an auto sampler module and a degasser coupled to a Dawn Heleos II Multi-Angle Light Scattering (MALS) detector, an Optilab T-rEX Refractive Index (RI) detector and a 2489 UV detector operating at 225 nm and at a temperature of 25°C.

MW: Molecular weight. MALS describes a technique for measuring the light scattered by a sample into a plurality of angles. The optical collection fiber replaces a MALS detector at any of the 18 detection angles.

solubility of γ-PGA and γ-PGA-Phe were investigated to select mobile phase for SEC-RI/MALS with view to application of NPs. It has already reported that γ-PGA-Phe is dissoluble at the concentration of more than 10 mg/mL in DMSO, DMF, DMAc and NMP [27]. γ-PGA-Phe could also be well dissolved in the mixture of 20 mmol/L sodium phosphate buffer (pH8.0) and ethanol (Table 1-1) and the mixed solvent was selected as the mobile phase for SEC-RI/MALS.

This system can evaluate relative MW, absolute MW, degradation and aggregation of γ-PGA and γ-PGA-Phe. MALS measurement data can be used to determine the absolute MW without the need to make assumptions of the polymer's physicochemical properties, such as the effect of branch-polymers and cohesion. A MALS detector determines an accurate MW and the conformational difference for the target polymer. Light scattering measures the *z* average of the root mean square (RMS) radius and average MW. The RI detector determines the relative MW, which is not sufficient to accurately determine the MW of the polymer since the value is calibrated to the MW of a standard polymer (e.g. PEG or polystyrene) whose physicochemical structure might be different from that of the tested polymer. In general, amphiphilic polymers show different behavior in solvents of different polarity. The difference in each vender's γ-PGA used as raw materials, the effect of the MW of γ-PGA-Phe on the MW of γ-PGA and the degree of grafting of the hydrophobic base, Phe were evaluated using the SEC-MALS system. This method for γ-PGA-Phe and γ-PGA was validated based on specificity, linearity, precision, and accuracy. Correlation coefficient (R) at 5 concentrations for γ-PGA-Phe was 0.999 in RI and MALS. No aggregation of polymers was also confirmed by the use of mixed solvent.

MW comparison of γ-PGA and γ-PGA-Phe between material vendors

For γ-PGA and γ-PGA-Phe, the absolute MW, relative MW, PDI, aggregation and degradation were evaluated using the SEC-RI/MALS system. The use of the MALS detector allowed accurate

Table 1-1. Maximum solubility for γ-PGA-Phe MW at room temperature for SEC-RI/MALS

Solvent		Maximum Solubility (mg/mL)
Composition	Ratio (v/v)	
20 mmol/L disodium phosphate	-	1.3
20 mmol/L disodium phosphate : EtOH	8 : 2	3.5
	5 : 5	11.0
	3 : 7	4.8
20 mmol/L disodium phosphate : MeOH	8 : 2	2.1
	5 : 5	2.4
	3 : 7	3.2
20 mmol/L disodium phosphate : IPA	8 : 2	4.1
	5 : 5	5.2
	3 : 7	5.4
20 mmol/L sodium phosphate buffer (7.0) : EtOH	5 : 5	2.2
20 mmol/L sodium phosphate buffer (7.5) : EtOH		3.2
20 mmol/L sodium phosphate buffer (8.0) : EtOH		6.7
	8 : 2	2.2

EtOH: Ethanol, MeOH: Methanol, IPA: Isopropyl alcohol

20 mmol/L sodium phosphate buffer and each solvent were mixed in the described Ratio (v/v).

MW determination, independent of the column migration behavior. γ-PGA from each material vendor, vendor A and vendor B, and γ-PGA-Phe synthesized from each γ-PGA were evaluated using the SEC-RI/MALS system. As shown in Fig. 1-3a and Table 1-2, the measured absolute MW of γ-PGA purchased was 340 kDa from vendor A (labeled MW: 480 kDa) and 240 kDa from vendor B (labeled MW: 380 kDa). γ-PGA from vendor B had a different elution curve and a high PDI compared to those of vendor A. It was found that the characterization of γ-PGA differed between vendors.

Relative MW, absolute MW and PDI of γ-PGA-Phe with grafting degree 50% synthesized from γ-PGA differed between vendors (Fig. 1-3b and Table 1-2). Absolute MW of γ-PGA-Phe from vendor A's γ-PGA was 41 kDa and that of γ-PGA-Phe from vendor B's γ-PGA was 87 kDa. The MWs of both γ-PGA-Phe's were smaller than those of γ-PGA's even though γ-PGA-Phe was grafted with Phe. Absolute MW and relative MW of γ-PGA-Phe from vendor A were almost same. In contrast, the values of absolute MW and PDI of γ-PGA-Phe from vendor B became larger compared to relative MW and the PDI. γ-PGA-Phe from vendor B showed high heterogeneity. Retardation of large highly branched molecules was found during SEC separation. Because of the retardation, the large branched molecules co-eluted together with the smaller molecules that normally eluted at high elution volumes, resulting in a high PDI of the eluting fraction [30,31].

Cleavage of γ-PGA during γ-PGA-Phe synthesis

Absolute and relative MWs of γ-PGA-Phe (grafting degree: 50%) synthesized from vendor A's γ-PGA (labeled MW: 480 kDa) and γ-PGA-Phe (grafting degree: 57%) synthesized from vendor A's high MW γ-PGA (labeled MW: 2,000 kDa) were evaluated in a similar way. The absolute MW of γ-PGA-Phe synthesized from high MW γ-PGA was 172 kDa and the PDI was 3.0, and the absolute MW was also smaller than that of γ-PGA. Absolute MWs of γ-PGA-Phe from both γ-PGA's were

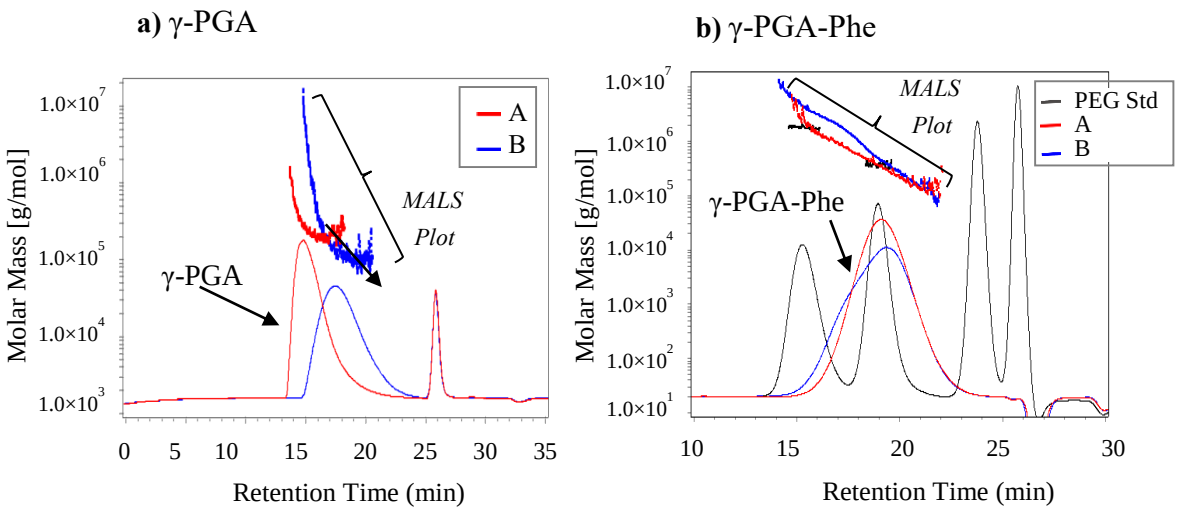


Fig. 1-3. SEC-RI/MALS chromatograms a) γ-PGA and b) γ-PGA-Phe. The solid lines shown in lower side indicate the RI response and the dot lines shown in upper side indicate the MALS response. The red line indicates vendor A's product and blue line indicates vendor B's product. Polyethylene glycol/polyethylene oxide samples with standard MWs of 200 Da, 2 kDa, 40.1 kDa and 220 kDa were used.

Table 1-2. Characterization results for γ-PGA and γ-PGA-Phe and Effect of Phe grafting degree on MW of γ-PGA-Phe

	Vendor	Labeled MW (kDa)	Phe Grafting Degree (%)	Absolute MW		Relative MW	
				MW (kDa)	PDI	MW (kDa)	PDI
γ-PGA	A	380	-	340	1.3	370	1.7
	B	480	-	240	1.7	130	1.7
γ-PGA-Phe	A	-	50	41	1.4	44	1.6
	B	-	50	87	2.4	64	1.7
			23	72	1.6	64	1.7
			36	59	1.4	54	1.6
			40	42	1.6	44	1.6
			53	72	2.3	43	1.8

Absolute MW, relative MW and PDI were determined by SEC-RI/MALLS analysis, and weight-average MWs were used for the estimation. γ-PGA-Phe's were synthesized by vendor A's γ-PGA and vendor B's γ-PGA, respectively. The degree of grafting of Phe was measured by ^{1}H NMR.

reduced to about 10% of labeled γ-PGA MWs (Table 1-3). The relative MW of γ-PGA-Phe from 2,000 kDa γ-PGA was also reduced to 83 kDa. High MW γ-PGA-Phe is more likely to be affected than low MW γ-PGA-Phe.

A study by J. Joyce *et al*. [32] shown definitively that aqueous carbodiimide treatment significant reduces both the MW and antigenicity of γ-PGA-Phe. In addition, a study by Y. Paterson *et al*. [33] previously demonstrated carbodiimide-induced cleavage of poly-L-glutamic acid at pH 5.0 in the presence or absence of ethanolamine. In discussing putative scission mechanisms, they suggested that a likely route was attack by free carboxyl groups on the *O*-acylisourea intermediate, basing this on the observation that cleavage was decreased in the presence of ethanolamine, which would suggest that as amide formation proceeded, fewer reactive free carboxyls were available for backbone cleavage. It was suggested that the main chain of γ-PGA-Phe was cleaved at a constant degree in the synthetic process.

In addition, it can be assumed that water molecules cannot as easily attack γ-PGA-Phe with a grafting degree of 53% in the synthetic process comparing to those with other grafting degrees, because hydrophobicity of γ-PGA-Phe is higher than that of γ-PGA.

Effect of Phe grafting degree on γ-PGA-Phe MW

Five kinds of γ-PGA-Phe's with grafting degrees of 23, 36, 40, 53 and 70% synthesized form vendor A's γ-PGA (labeled MW: 480 kDa) were used to evaluate the effect of the Phe grafting degree on γ-PGA-Phe MW. With an increase in the grafting degree of Phe, both the absolute MW and relative MW of γ-PGA-Phe decreased (Table 1-2). γ-PGA-Phe with 70% Phe grafting did not completely dissolve in the same way.

Absolute MW of γ-PGA-Phe with 53% Phe grafting showed the difference having a larger PDI and the threshold of the change compared to the other samples. On the other hand, the relative MW

Table 1-3. Effect of γ-PGA MW on γ-PGA-Phe MW

Labeled γ-PGA MW (kDa)	Phe Grafting Degree (%)	Absolute MW		Relative MW	
		MW (kDa)	PDI	MW (kDa)	PDI
480	50	41	1.5	39	1.7
2000	57	172	3.0	83	2.1

Absolute MW, relative MW and PDI were determined by SEC-RI/MALS analysis. Absolute and relative MWs for the estimation were weight-average MW. PDI was calculated by dividing weight average MWs by number average MW. Each γ-PGA-Phe was synthesized by vendor A's γ-PGA.

was decreased with increasing the grafting degree. The decrease of the γ-PGA-Phe MWs by the difference of grafting degree showed the different variability in the characterization of polymers. It was suggested that a conformational change of γ-PGA-Phe with grafting degree of 53% was occurred. As γ-PGA-Phe is a graft polymer, a conformational change by intermolecular interaction derived from the graft structure was expected, and the necessity of the absolute MW measurement was indicated. Therefore, both absolute MW and relative MW should be measured to characterize γ-PGA-Phe. When γ-PGA as the hydrophilic backbone and Phe as the hydrophobic side chain were synthesized by grafting Phe to γ-PGA-Phe, the grafting degree of the hydrophobic side chain at the carboxyl group depends on the concentration of the condensation agent. Moreover, as γ-PGA-Phe is a mixture of polymers with different grafting degrees and MWs, these differences are expected to affect the biodegradable characteristics of the polymer. The introduction of Phe into γ-PGA delayed polymer hydrolysis. It can be assumed that water molecules cannot as easily attack γ-PGA-Phe with a grafting degree of 53% in the synthetic process comparing to those with other grafting degrees.

The increased grafting degree of Phe may have enhanced the hydrophobic interactions between the Phe groups attached to the γ-PGA backbone [6], therefore, the author can suggest that with an increase in the degree of Phe grafting, absolute MW decreases. However, γ-PGA-Phe with a grafting degree of 53% becomes more stable due to the hydrophobic core.

Quality assessment of γ-PGA-Phe

MW and PDI for 2 lots of γ-PGA-Phe (degree of Phe grafting: 57% and 56%) synthesized by the large scaled process using vendor A's γ-PGA (labeled MW: 480 kDa) were evaluated by SEC-RI/MALS. Absolute MW of γ-PGA-Phe was 121 kDa and 112 kDa for the two lots, respectively (Table 1-4). The analytical results for the 2 lots were almost equivalent, and test reproducibility and consistency for the synthetic process were observed. The analytical method for

Table 1-4. Quality assessment of γ-PGA-Phe

γ-PGA-Phe.		Lot 1	Lot 2
Phe Gafting Degree (%)		57	56
Water Content (%)		2.5	4.2
Absolute MW (kDa)		121	112
Content (%)		94.6	95.5
Impurities (%)	PA	< 0.02	< 0.02
	U-1	5.10	4.50
	Phe	0.05	0.10
	U-2	0.09	0.10
	Others	0.25	0.34

-: Not determined.

Absolute MW and PDI were determined by SEC-RI/MALS analysis. γ-PGA-Phe's were synthesized by vendor A's γ-PGA, respectively. The degree of grafting of Phe was measured by ^{1}H NMR. Moisture content was determined by the Karl Fischer Method. Content of γ-PGA-Phe and impurity levels were determined by RP-HPLC. PA: Phenylalanine; Phe: Phenylalanine ethyl ester; U-1: Unknown-1; and U-2: Unknown-2.

measuring the content and impurities of γ-PGA-Phe was developed using RP-HPLC with acetonitrile gradient and a C4 column. Firstly, to select the proper HPLC column for separation of the content and impurities of γ-PGA-Phe, 4 columns, Proteonavi (4.6 mm id × 150 mm, 5 μm, Shiseido, Japan), Zorbax 3000SB-C3 (4.6 mm id × 150 mm, 5 μm, Agilent Technologies, Santa Clara, CA), YMC-Pack protein-RP (7.8 mm id × 150 mm, 10 μm, YMC Co. Ltd., Japn), and Inertsil WP300 C4, were tested. As the result, the columns except for YMC-Pack protein-RP were able to separate the impurities. In this study, Inertsil WP300 C4 column was selected from view of selectivity and retention of the content (data not shown). Each absorption spectrum of γ-PGA-Phe and impurities, PA, Phe, unknown peak (U)-1, U-2 and U-3 at 214 nm was qualitatively enough and wavelength selection is adequate. This method was validated by measuring specificity, linearity, recovery, limit of detection (LOD), limit of quantitation (LOQ), accuracy and recovery for PA or Phe. γ-PGA-Phe and the impurities were adequately resolved using the validated system. No degradant peaks were observed to coelute with γ-PGA-Phe, or known impurities after exposure to acidic, basic and heating conditions. The correlation coefficient (R) at 5 concentrations from 5 μg to 30 μg for γ-PGA-Phe was 0.997 and the percent y-intercept at the 1 mg/mL was 1.3%. LOD and LOQ were measured based on signal to noise ratio at about 3 and 10, respectively and LOQ was set at 0.02% for γ-PGA-Phe. Recovery of spiked 1% PA or 1% Phe for γ-PGA-Phe solution was quantitative respectively (data not shown). The quality of the 2 lots of synthesized γ-PGA-Phe was assessed by this analytical method. The peak of γ-PGA-Phe was detected at about 20.4 min. The content of γ-PGA-Phe in both lots was around 95% and the difference between the lots was fairly small (Fig. 1-4 and Table 1-4). γ-PGA-Phe synthesized by the process which can control Phe grafting degree, showed the same chromatographic patterns and high purity. Highly reproducible results were obtained. For the impurities, peaks for phenylalanine (PA), an unknown peak (U)-1, Phe and U-2 were detected at about 2.2 min, 8.2 min, 11.4 min and 16.7 min, respectively. Phe and PA, purchased

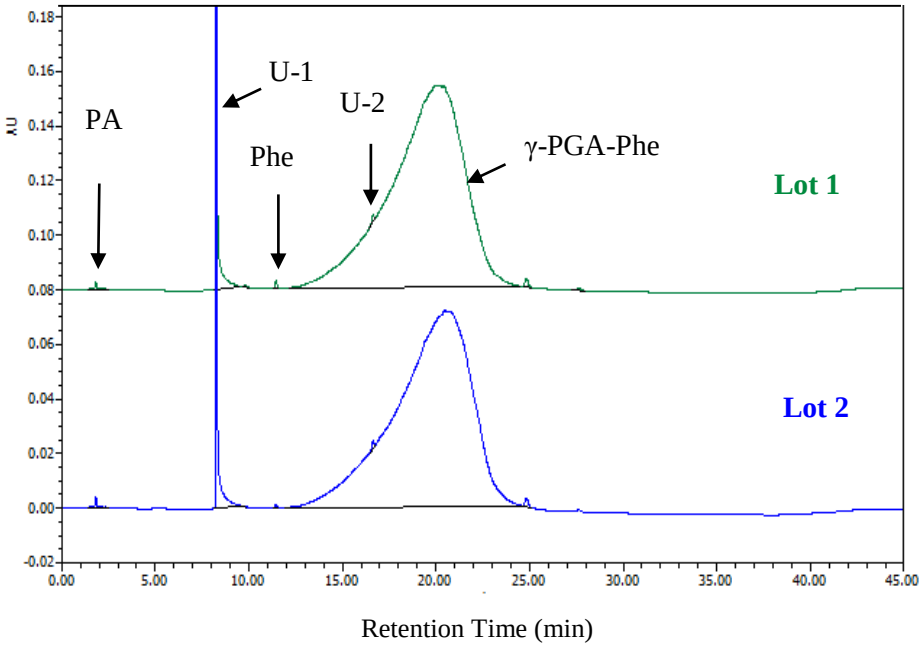


Fig. 1-4. Chromatograms of two lots of γ-PGA-Phe synthesized from vendor A's γ-PGA by RP-HPLC. γ-PGA-Phe was dissolved with 0.25% SDS (w/v) and 2% ACN (w/v) in 20 mmol/L sodium phosphate buffer (pH 7.5) at a final concentration of 0.2 mg/mL. The peaks for PA, U-1, Phe, U-2 and γ-PGA-Phe were detected at about 2.2 min, 8.2 min, 11.4 min, 16.7 min and 20.4 min, respectively. PA: phenylalanine; Phe: phenylalanine ethyl ester; U-1: Unknown-1; and U-2: Unknown-2.

were used as references for the identification of impurities. U-1 and U-2 were identified. U-1 was found to be the most abundant impurity, followed by U-2, Phe, and PA. U-1 at about 5% was detected in these lots and is considered to be derivative of EDC. U-2, Phe, and PA were less than 0.1%.

In general, many degradation tests already exist, but it was necessary to develop test methods to evaluate quality when dealing with biodegradable polymers. These methods can be used for targeted problems such as interaction between the material and the body, but may also cause due to an interference of rest-monomers, degradation-products or additives with biochemical pathways. These methods, which can be used to evaluate γ-PGA production improvement, will be helpful in developing methods to synthesize γ-PGA-Phe and develop NPs. In addition, purifying polymers to the highest possible level with respect to achieving reproducible physical and biophysical properties is important [34]. Even though it is difficult to find the dissolution solvent of samples, if repurifying this batch to remove the impurities by the isolation via this analytical method, it will be obtained in further high purity γ-PGA-Phe.

As it requires that the implementation of quality oversight and controls over the manufacture of drugs, including the safety of raw materials, materials used in drug manufacturing, and finished drug products for clinical application, these methods will be helpful.

Stability study of γ-PGA-Phe lyophilized powder

It is extremely important to select the most suitable storage conditions that will ensure sufficient stability of γ-PGA-Phe. The purpose of this part of our study was to assess the stability of γ-PGA-Phe lyophilized powder in different temperatures and relative humidity (RH). Appearance, absolute MW, content and impurities of stored γ-PGA-Phe were evaluated at each time point. The color of the γ-PGA-Phe turned from white to yellowish white after storage at 25°C/60% RH for 1

month and 2 months (Table 1-5). The absolute MW was reduced only at 25°C/60% RH with the opened cap. The content of γ-PGA-Phe did not change in any condition. As humidity and temperature increased, impurities slightly increased at 25℃ up to 2 months, while no degradation was observed at 5°C. Storage of γ-PGA-Phe at 25˚C resulted in degradation of γ-PGA-Phe and the combined thermal and relative humidity stress, 25°C/60% RH with the opened cap, accelerated the change of γ-PGA-Phe. It was suggested that temperature and humidity accelerate the hydrolytic degradation of γ-PGA-Phe. γ-PGA-Phe was sensitive to environmental conditions; hence it is recommended that it be stored in closed containers and treated under low humidity at low temperatures (5°C). In the preparation process of γ-PGA-Phe-based applications, it is particularly important to establish the shelf life of the product by conducting stability studies, because it is known that higher humidity accelerates the degradation of the peptides [35] and mechanical tablet strength.

Polymer degradation describes a series of events impacting the chemical, physical and mechanical properties of a polymer. Polymer degradation denotes changes in physical properties caused by chemical bond scission in the backbone of the macromolecule or by reaction with other chemical species. Scission and chemical reaction of pendent groups of linear polymers affect the physical properties only to a minor degree relative to backbone scission. Therefore, the author needs to clarify the degradation mechanism for γ-PGA-Phe and consider how to manufacture NPs to support further drug development by reducing the effect of temperature and humidity in promoting degradation during the manufacturing process of NPs. From the results described above, it should be noted that γ-PGA-Phe can be used as an effective DDS carrier by ensuring high quality of γ-PGA-Phe in the condition of low temperature and humidity.

Table 1-5. Stability study for γ-PGA-Phe lyophilized powder

Condition		Initial	5 ˚C		25 ˚C/60% RH, Close		25 ˚C/60% RH, Open	
			1 month	2 months	1 month	2 months	1 month	2 months
Appearance		White crystalline powder	White crystalline powder	White crystalline powder	White and yellowish white crystalline powder	White and yellowish white crystalline powder	White and yellowish white crystalline powder	White and yellowish white crystalline powder
Absolute MW (kDa)		264	-	249	258	241	203	158
Content (% of Initial)		100.0	97.6	100.2	101.3	101.9	97.5	105.6
Impurities (%)	PA	0.23	0.27	0.27	0.28	0.30	0.29	0.38
	U-1	0.37	0.39	0.39	0.43	0.46	0.79	1.02
	Phe	2.34	2.37	2.47	2.35	2.24	2.08	2.03
	U-2	0.84	0.85	0.90	0.87	0.90	0.95	1.09
	Others	0.08	0.13	0.09	0.09	0.08	0.14	0.13

-: Not determined.

Absolute MW, relative MW and PDI were determined by SEC-RI/MALLS analysis. Content of γ-PGA-Phe and impurity level were determined by RP-HPLC. γ-PGA-Phe was synthesized by vendor A's γ-PGA and the grafting degree of γ-PGA-Phe was 58%. "Close" in conditions means that the substance was not exposed to RH, even if the vial was placed in that climate. "Open" in conditions means that substance was exposed to RH.

Initial: Not treatment, PA: Phenylalanine; Phe: Phenylalanine ethyl ester; U-1: Unknown-1; and U-2: Unknown-2.

Stability study of γ-PGA-Phe in buffer solution with different pH values

A SEC chromatogram showed a γ-PGA-Phe peak at about 20 min for each sample. However, the peak shifted to higher elution volume which shows lower MW (Fig. 1-5) as compared to before the storage. Consequently, the absolute MW of γ-PGA-Phe (initial MW: 85.0 kDa) reduced gradually as the temperature increased for all pH values (Table 1-6). Higher temperatures made MW lower in these chromatograms. Even though the content of γ-PGA-Phe remained quite stable at 5°C for all pH values, degradation of γ-PGA-Phe was notably accelerated under acidic conditions (pH 4-5) and weak-alkaline conditions (pH 8) with an increase in temperature. For the sample in pH 4 buffer solution stored at 40°C for 2 weeks, a suspension appeared, making the solution cloudy. Some of the suspension settled out. The peak response of γ-PGA-Phe in the chromatogram became low and it was thought to be an effect of low recovery by aggregation. The formation of aggregate was considered to be due to the ionization of the carboxyl groups of γ-PGA located near the surfaces in the solution. As impurities, PA, U-1, Phe and U-2 were detected. The amount of U-1 detected was markedly increased with an increase in temperature and the increase was accelerated to more than 10% under acidic conditions and weak-alkaline conditions (Fig. 1-6 and Table 1-6). The pH had an effect on the hydrolytic degradation of γ-PGA-Phe. In addition, γ-PGA was not degraded in neutral buffer [12,36]. In my experiment, γ-PGA-Phe was stable under neutral condition (pH 6-7) at 5°C for 1 month. Exposure to elevated temperatures (40°C) was found to cause a significant loss of γ-PGA-Phe content and reduction in MW. The effect of temperature on the hydrolysis of γ-PGA has already been reported [37]. This result suggested that pH and temperature were important factors in the degradation behavior of γ-PGA-Phe. The rate of hydrolysis was accelerated with an increase in temperature. Therefore, it should be noted that hydrolytic degradation of γ-PGA-Phe can be controlled by changing the pH and temperature. To clarify the mechanism responsible for degradation, the author speculated on the chemical structures.

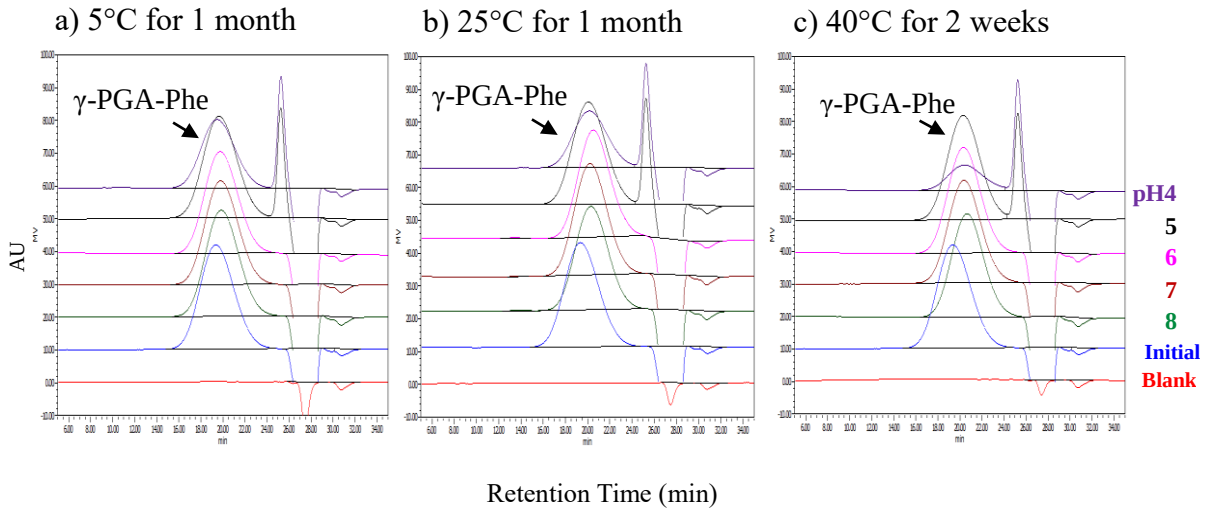


Fig. 1-5. SEC-RI chromatogram of γ-PGA-Phe for the accelerated stability study. The stability study for γ-PGA-Phe was conducted under (a) 5°C for 1 month, (b) 25°C for 1 month, and (c) 40°C for 2 weeks. Each chromatogram shows the results for γ-PGA-Phe stored at pH 4, 5, 6, 7 and 8 and for Initial (non-treatment) and blank (the mobile phase for SEC-RI/MALS) samples from top to bottom.

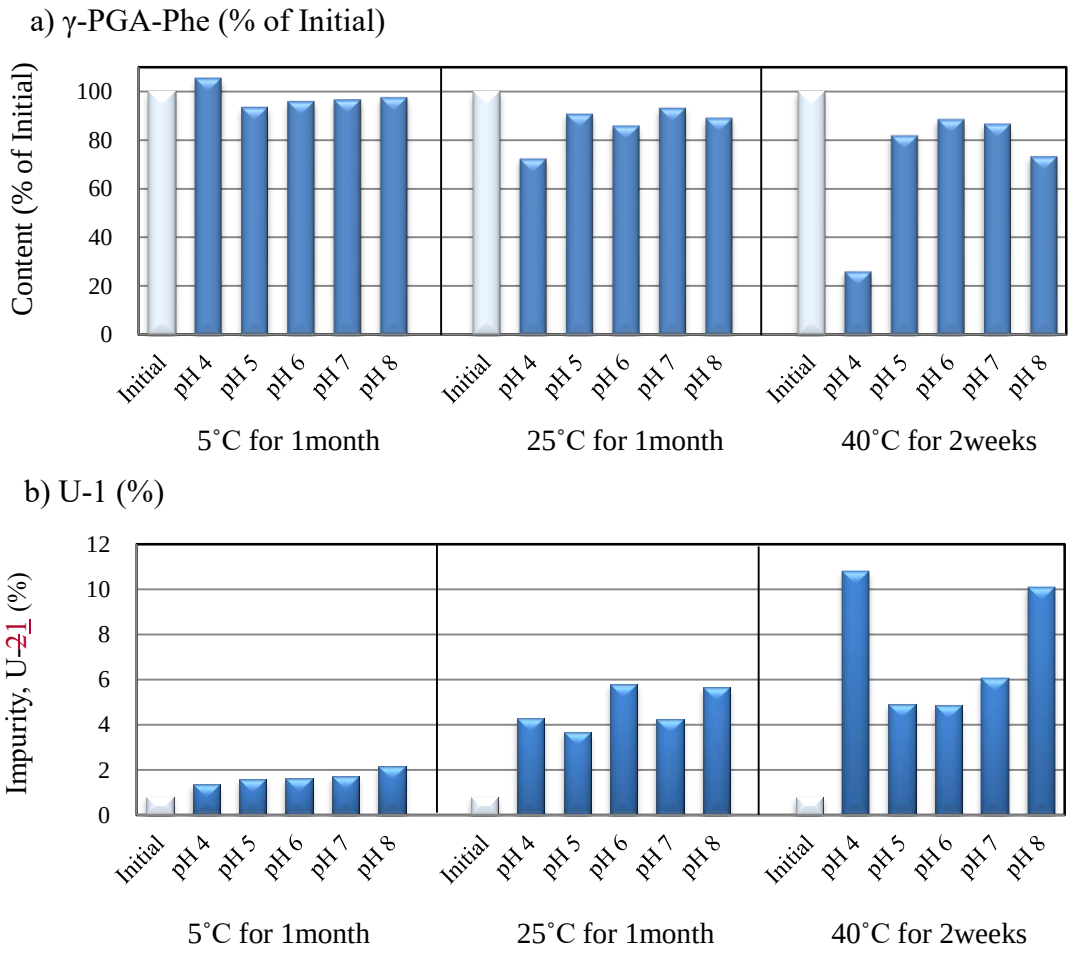


Fig. 1-6. γ-PGA-Phe content and impurity U-1 on the accelerated stability study of γ-PGA-Phe.

Table 1-6. Stability study of γ-PGA-Phe in buffer solution with different pH values

Condition			Initial	pH 4	pH 5	pH 6	pH 7	pH 8
5 ˚C 1 month	Absolute MW (kDa)		85.0	70.6	61.2	49.4	57.3	61.2
	Content (% of Initial)		100.0	105.3	93.4	95.8	96.4	97.4
	Impurities (%)	PA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		U-1	0.80	1.37	1.57	1.63	1.72	2.16
		Phe	0.44	0.50	0.44	0.34	0.09	0.05
		U-2	0.20	0.12	N.D.	N.D.	N.D.	N.D.
		Others	0.99	1.21	0.98	0.99	1.05	1.13
25 ˚C 1 month	Absolute MW (kDa)		85.0	51.4	49.4	58.6	48.1	-
	Content (% of Initial)		100.0	72.3	90.6	85.7	93.0	88.9
	Impurities (%)	PA	N.D.	0.05	0.04	N.D.	N.D.	N.D.
		U-1	0.80	4.29	3.67	5.78	4.25	5.65
		Phe	0.44	0.59	0.36	N.D.	N.D.	N.D.
		U-2	0.20	N.D.	N.D.	N.D.	N.D.	N.D.
		Others	0.99	1.71	1.23	1.62	1.33	1.62
40 ˚C 2 weeks	Absolute MWs (kDa)		85.0	46.8	44.7	46.2	45.6	42.0
	Content (% of Initial)		100.0	26.2	81.8	88.4	86.6	73.2
	Impurities (%)	PA	N.D.	0.05	0.04	N.D.	N.D.	0.02
		U-1	0.80	10.79	4.90	4.87	6.06	10.11
		Phe	0.44	1.11	0.26	N.D.	N.D.	N.D.
		U-2	0.20	N.D.	N.D.	N.D.	N.D.	N.D.
		Others	0.99	5.76	1.46	1.31	1.59	2.47

N.D.: Not detected. -: Not determined.

γ-PGA-Phe was synthesized by vendor A's γ-PGA and the grafting degree of γ-PGA-Phe was 58%.

In our several studies, it have shown that the γ-PGA backbone is composed of γ-linked glutamic acid and that the amide bond between the α-carboxylate side chains of the γ-PGA and Phe was not degraded [7]. The introduction of Phe groups into γ-PGA delayed polymer hydrolysis since water molecules cannot as easily attack the NPs manufactured by γ-PGA-Phe with a grafting degree of 53%, in comparison to γ-PGA [10,12]. Innate properties of the polymer backbone also regulate water accessibility to scission of individual polymer chains within DDS carrier [38]. In addition, it was clarified that the amounts of the impurities Phe and PA did not increase during the stability test (Table 1-6). Therefore, cleavage of the main-chain of γ-PGA-Phe was the major cause of degradation and the Phe side-chain had a minor contribution (Fig. 1-7).

For applications important characteristics of the polymer are stability, hydrophobicity, morphology, molecular weight, and degree of swelling in water of the polymer backbone. Susceptibility of the polymeric backbone toward hydrolytic cleavage is probably the most fundamental parameter [39]. In our research group enzymatic degradation of γ-PGA-Phe NPs has been extensively studied. As described above, γ-PGA-Phe should promise to become effective as a material of DDS carrier.

1. 5. Conclusion

There currently exists a wide range of degradable polymers that have potential as biomaterials. In **Chapter 1**, analytical methods for characterizing γ-PGA-Phe and measuring its quality, which evaluates the content of γ-PGA-Phe and level of impurities, were established using the SEC-RI/MALS system and RP-HPLC, respectively. These methods were robust and practical.

In addition, through a pH stability study, it was clarified that the major mechanism of biodegradation was cleavage of the main chain of γ-PGA-Phe. These characteristics make it a particularly attractive biodegradable polymer for a wide range of potential applications in the

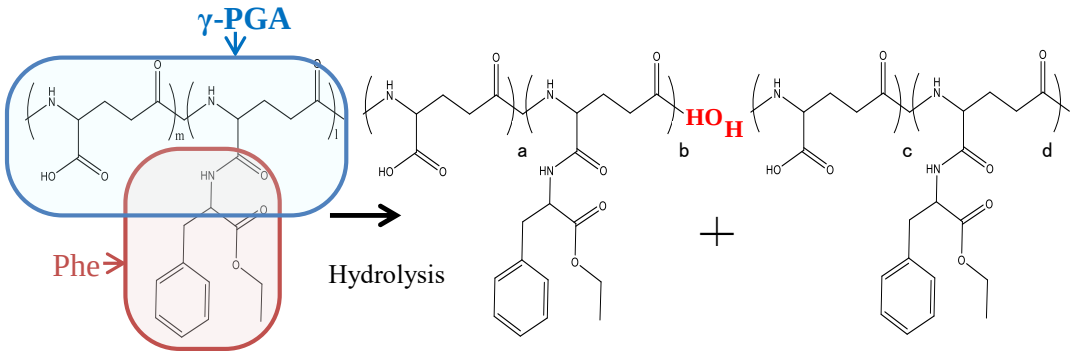


Fig. 1-7. Degradation route of γ-PGA-Phe. γ-PGA-Phe was mainly degraded by the cleavage of the main-chain of γ-PGA-Phe.

biomedical industry. While analytical developments in polymer research have been critical, advancements in analytical methods for biological research would lead to a better understanding of how biomaterials interact with biological systems. It is thought that biodegradable NPs have great potential as carriers for drug delivery systems.

The author's developed analytical methods and systems have been shown to be reliable. As numerous types of polymers are currently in use in virtually all fields of medicine, the author's developed analytical methods are expected to contribute to improving the safety of drug administration in clinical applications in the future. The author is also convinced that these techniques will be useful for evaluating the quality of other polymers, followed by various other functional and responsive polymers.

1. 6. References

[1] L. F. Zhang, A. Eisenberg, *J. Am. Chem. Soc.* **118**, 3168 (1996).

[2] X. M. Liu, K. P. Pramoda, Y. Y. Yang, S. Y. Chow, C. He, *Biomaterials* **25**, 2619 (2004).

[3] M. Iijima, Y. Nagasaki, T. Okada, M. Kato, K. Kataoka, *Macromolecules* **32**, 1140 (1999).

[4] C. Allen, J. Han, Y. Yu, D. Maysinger, A. Eisenberg, *J. Controlled Release* **63**, 275 (2000).

[5] M. H. Sung, C. Park, C. J. Kim, H. Poo, K. Soda, M. Ashiuchi, *Chem. Rec.* **5**, 352 (2005).

[6] X. Wang, T. Uto, T. Akagi, M. Akashi, M. Baba, *J. Med. Virology* **80**, 11 (2008).

[7] R. I. Khalil, U. V. Irorere, I. Radecka, T. A. Burns, P. M. Kowalczuk, L J. Mason, P. M. Khechara, *Int. J. Mol. Sci.* **18**, 313 (2017).

[8] L. Shih, Y. T. Van, *Bioresour Technol.* **79**, 207 (2001).

[9] T. S. Ljubie, D. Pahovnik, M. Zigon, E. Zagar, *The Scientific World Journal Article*, ID **932609**, 9 (2012).

[10] T. Akagi, M. Higashi, T. Kaneko, T. Kida, M. Akashi, *Macromol. Biosci.* **5**, 598 (2005).

[11] M. Uto, M. Toyama, Y. Nishi, T. Akagi, F. Shima, M. Akashi, M. Baba, *Results Immunol.* **3,** 1 (2013).

[12] T. Akagi, T. Kaneko, T. Kida, M. Akashi, *J. Controlled Release* **108**, 226 (2005).

[13] M. Ashiuchi, H. Misono, *Appl. Microbiol. Biotechnol.* **59**, 9 (2002).

[14] F. Shima, T. Akagi, M. Akashi, *Biomaterials Science* **2**, 1419 (2014).

[15] F. Shima, T. Akagi, M. Akashi, *Bioconjugate Chem.* **26**, 890 (2015).

[16] T. Uto, T. Akagi, M. Akashi, M. Baba, *Clin. Vaccine Immunol*. **22**, 578 (2015).

[17] T. Akagi, X. Wang, T. Uto, M. Baba, M. Akashi, *Biomaterials* **28**, 3427 (2007).

[18] S. Okamoto, M. Matsuura, T. Akagi, M. Akashi, T. Tanimoto, T. Ishikawa, M. Takahashi, K. Yamanishi, Y. Mori, *Vaccine* **27**, 5896 (2009).

[19] S. Yamaguchi, T. Tatsumi, T. Takehara, A. Sasakawa, M. Yamamoto, K. Kohga, T. Miyagi, T. Kanto, N, Hiramatsu, T. Akagi, M. Akashi, N. Hayashi, *Cancer Immunol. Immunother.* **59**, 759 (2010).

[20] T. Akagi, M. Akashi, *Engineered Cell Manipulation for Biomedical Applications*, In M. Akashi, T. Akagi, M. Matsusaki (Eds.), 205578 (2014).

[21] E. Szymańska, K. Winnicka, *Mar. Drugs* **13**, 1819 (2015) .

[22] Y. J. Zheng, *Formulation analytical development for low does oral drug products*, New Jersey, USA, John Wiley and Sons (2009).

[23] T. J. Oberlerchner, T. Rosenau, A. Potthast, *Molecules* **20**, 10313 (2015).

[24] S. Podzimek, T. Vleck, C. Johann, *Appl. Polym. Sci.* **81**, 1588 (2001).

[25] T. Yoshikawa, N. Okada, A. Oda, K. Matsuo, K. Matsuo, Y. Mukai, Y. Yoshioka, T. Akagi, M. Akashi, S. Nakagawa, *Biochem. Biophys. Res. Commun.* **366**, 408 (2008).

[26] M. Matsusaki, K. Hiwatari, M. Higashi, T. Kaneko, M. Akashi, *Chem. Lett.* **33**, 398 (2004).

[27] T. Akagi, M. Baba, M. Akashi, *Polymer* **48**, 6729 (2007).

[28] T. Kaneko, M. Higashi, M. Matsusaki, T. Akagi, M. Akashi, *Chem. Mater*. **17**, 2484 (2005).

[29] R. Hoogenboom, M. L. Hanneke, D. Wouters, S. Hoeppener, S. S. Ulrich, S. Schubert, *Soft Matter* **4,** 103 (2008).

[30] C. A. Makan, T. Otte, H. Pasch, *Macromolecules* **45**, 5247 (2012) .

[31] T. Otte, H. Pasch, T. Macko, R. Brüll, F. J. Stadler, J. Kaschta, F. Becker, M. Buback, *J. Chromatogr. A* **8**, 4257 (2011).

[32] J. Joyce, J. Cook, D. Chabot, R. Hepler, W. Shoop, Q. Xu, T. Stambaugh, M. Amezaga, S. Wang, L. Indrawati, M. Bruner, A. Friedlander, P. Keller, *J. Biol. Chem.* **20**, 4831 (2005).

[33] Y. Paterson, S. J. Leach, *J. Biochem. Biophys. Res. Commun.* **95**, 1722 (1980).

[34] P. R. Nagarkar, P. J. Schneider, *Methods Mol. Biol*. **47**, 461 (2008).

[35] G. Papadopoulos, G. Floudas, H. A. Klok, I. Schenell, T. P. Appel, G. Ponchel, *Biomacromolecules* **5**, 137 (2004).

[36] C. C. Chu, *J. Biomed. Mater. Res.* **15**, 795 (1981).

[37] K. Fan, D. Gonzales, M. Sevoian, *J. Environ. Polym*. **4**, 253 (1996).

[38] A. Goto, M. Kunioka, *Biosci. Biotechnol. Biochem*. **56**, 1031(1992).

[39] E. A. Simone, T. D. Dziubla, V. R. Muzykantov, *Drug Deliv.* **5,** 1283 (2008).

Chapter 2.

Development of analytical methods for evaluating the quality of dissociated and associated amphiphilic poly(γ-glutamic acid) nanoparticles

2. 1. Summary

A quantitative method of analyzing nanoparticles (NPs) for drug delivery is urgently required by researchers and industry. Therefore, in **chapter 2**, the author developed new quantitative analytical methods for biodegradable and amphiphilic NPs consisting of polymeric γ-PGA-Phe (phenylalanine attached to poly(γ-glutamic acid)) molecules. These γ-PGA-Phe NPs were completely dissociated into separate γ-PGA-Phe molecules by adding sodium dodecyl sulfate (SDS). The dissociated NPs were chromatographically separated to analyze parameters such as the γ-PGA-Phe content in the NPs, the impurities present using reverse-phase chromatography (RP-HPLC) with an ultraviolet (UV) detector, and the absolute MW using size-exclusion chromatography (SEC) with refractive index (RI) detector and multi-angle light scattering (MALS) detection, i.e., SEC-RI/MALS.

The chromatographic patterns of the NPs were equivalent to those of the component polymer (γ-PGA-Phe), and excellent chromatographic separation for the quantitative evaluation of NPs was achieved. To the best of our knowledge, this is the first report of the quantitative evaluation of NPs in the field of NP-based delivery systems. Furthermore, these methods were applied to optimize and evaluate the NP manufacturing process. The results showed that impurities were effectively removed from the γ-PGA-Phe during the manufacturing process, so the purity of the final γ- PGA-Phe NPs was enhanced. In addition, the appearance, clarity of solution, particle size, zeta potential, particle matter, osmolarity, and pH of the product were evaluated to ensure that the NPs were of the required

quality. The author's approach should prove useful for product and process characterization and quality control in the manufacture of NPs. γ-PGA-Phe NPs are known to be a powerful vaccine adjuvant, so they are expected to undergo clinical development into a practical drug-delivery system. The analytical methods established in **Chapter 2** should facilitate the reliable and practical quality testing of NP products, thus aiding the clinical development of γ-PGA-Phe-based drug-delivery systems. Moreover, since these analytical methods employ commonly used reagents and chromatographic systems, the methods are expected to be applicable to other NP-based drug-delivery products too.

2. 2. Introduction

Nanoparticulate drug carriers can now be created in various forms based on organic and inorganic materials, and unprecedented levels of control over carrier size, shape, surface properties, drug loading, and drug release are possible. Various nanostructured materials have been applied to drug delivery, such as nanoparticles (NPs), nanocapsules, nanotubes, micelles, microemulsions, and liposomes [1]. NPs have been the most extensively investigated of these materials due to their high biocompatibility and biodegradability. Biodegradable and biocompatible polymer-based NPs are one of the options available for controlled drug delivery and drug targeting [2-5]. While a wide variety of synthetic polymers such as poly(D,L-lactide-co-glycolide) (PLG) [6,7] and poly(D,L-lactic-co -glycolic acid)(PLGA) [8-10] have been used as NP components, clinical translation of the resulting NPs has been relatively slow, with only a handful of commercial products released thus far [11]. It is clear that the field of nanomedicine is still in its early stages, as success stories are few and far between.

Biodegradable NPs and polymeric NPs have great potential as vaccine delivery systems and immunostimulatory adjuvants [12]. Polymeric NPs have many advantages when utilized to develop

improved antigen delivery carriers, including the ability to flexibly control their physicochemical properties (e.g., particle size, shape, surface charge, hydrophobicity, and polymer composition). In our research group, biodegradable NPs consisting of molecules of hydrophilic poly(γ-glutamic acid) (γ-PGA) with a hydrophobic L-phenylalanine ethyl ester (Phe) side chain, i.e., γ-PGA-Phe NPs, which self-assemble into nanomicelles with a hydrophilic outer shell and a hydrophobic inner core, are used for the sustained or targeted release of drugs [13,14]. Our research group has already reported that γ-PGA NPs have the capacity to immobilize various proteins, peptides, and chemicals onto their surfaces and/or to encapsulate these substances [14]. They can also induce markedly higher levels of antigen-specific adaptive immune responses in mice and monkeys than achieved with antigens alone or when using antigens with other common adjuvants [15-17]. However, it is also important to be able to manufacture high-quality NP formulations with well-defined properties and functions for clinical development. Therefore, it is necessary to establish analytical methods for NPs in order to guarantee their quality and safety.

Biodegradable polymers are increasingly being used in medicine, so it is crucial to ensure that these polymers are of sufficient quality, but there are actually only a few examples of reliable analyses of polymers or NPs that can be used to guarantee the quality of NPs as pharmaceutical products. In most cases, microscopy techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) have been utilized as the only analytical tools to examine a few individual NPs [18-21]. However, modified NPs in particular need to be characterized and quantified by simple, accurate, and precise analytical methods [22]. The lack of available characterization techniques for NPs also hampers the optimization of the NP manufacturing process in order to, for example, reduce batch failures and improve yields. One reason why the analysis of NPs is difficult is their colloidal nature; they need to be dissociated sufficiently before analysis can be performed. One possible way to solve this issue would be to

dissociate the NPs into separate intact polymeric molecules using a surfactant, which would then allow further characterization and quality testing of the NPs. The presence of unwanted chemicals, even in small amounts, could influence the efficacy and safety of the pharmaceutical product.

Pharmaceutical analysis plays a prominent role in the quality control and assurance of pharmaceutical formulations and bulk drugs [23-25]. Indeed, in **Chapter 1**, the author has reported the characterization of γ-PGA-Phe as a raw material for generating NP-based adjuvants as well as the development of quantitative analytical methods for γ-PGA-Phe [26].

In addition, storage stability is crucial to ensuring the safety and efficacy of drug products [27-30], but there are no reliable analytical methods for NPs. The main stability characteristics of NPs include the sedimentation, agglomeration [31], and degradation of the NPs themselves as well as their components. Poor stability of biodegradable NPs in an aqueous medium presents a serious challenge to the clinical application of the NPs; hydrolysis during storage in an aqueous medium is thought to be a major degradation mechanism. A reliable method of analyzing NPs that can be used for quality testing should also be capable of assessing the stability of NPs stored under various conditions. Therefore, in **Chapter 2**, the author developed new quantitative analytical methods for biodegradable and amphiphilic NPs consisting of molecules of poly(γ-glutamic acid) with a phenylalanine side chain.

The main purpose of analytical methods applied during drug development and the drug manufacturing process is to provide information about the content, impurity, and stability of the drug as well as the effects of manufacturing parameters on the drug, to ensure that the production process yields drugs of a consistently high quality [32]. Therefore, the author also checked that the analytical methods established in **chapter 2** were able to determine the quality of the product in different formulations (in suspension and lyophilization). In addition, it was important to clarify which of the parameters of the NPs need to monitored throughout formulation development in order to achieve

high reproducibility. Furthermore, it was necessary to identify the critical steps in the scale-up and manufacturing process for nanotechnology products. The parameters that most strongly influence the efficacy, identity, purity, safety, and performance of the drug product were optimized and that it is important to identify the parameters that most strongly influence the quality of the drug product, as this enables analytical methods that focus on those parameters to be designed; such methods could be used to monitor the quality of the NPs production during the manufacturing process. Product quality is controlled by applying tight quality-control release specifications.

2. 3. Experimental Section

Materials

Special-grade γ-PGA (sodium salt form, labeled MW: 480 kDa, D-Glu/L-Glu: 70/30), L-phenylalanine ethyl ester (Phe), phenylalanine (PA), dimethyl sulfoxide (DMSO), 1-thyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dibutyrylamine, sodium dodecyl sulfate (SDS), sodium citrate, monosodium hydrogen phosphate, disodium hydrogen phosphate, sodium chloride (NaCl), and ethanol (EtOH), as well as HPLC-grade acetonitrile (ACN), EtOH, and distilled water, were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan), as was trehalose. Phosphate-buffered saline (PBS) pH 7.4 was purchased from Gibco (Brooklyn, NY). All other chemicals and solvents were of analytical grade. Polyethylene glycol/polyethylene oxide (ReadyCal standard set; Mp 200-1,200,000) was purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of γ-PGA-Phe

Amphiphilic γ-PGA was synthesized as described previously [33,34] by conjugating Phe (the hydrophobic segment with biodegradable components containing carboxyl functional groups on their side chains) with γ-PGA (the hydrophilic backbone). The chemical structure of the purified

γ-PGA-Phe [33] was characterized using ^{1}H NMR [34] and FT-IR spectroscopy [35]. The grafting degree of Phe was determined from the integral intensity ratio of the methylene peaks of γ-PGA to the phenyl group peaks of PA. In **Chapter 2**, γ-PGA-Phe with Phe grafting degrees of 40~60% was used.

Preparation of the γ-PGA-Phe NP suspension

Nine milliliters of γ-PGA-Phe solution (10 mg/mL in DMSO or a mixture of EtOH and 50 mmol/L sodium citrate 1:1 (v/v)) were added to 9 mL of 0.4 mol/L NaCl solution under stirring (400 rpm) at ambient temperature. The resulting NP suspension was stirred at room temperature for 10 min at 400 rpm and then centrifuged for 50 min at 10,000 rpm. After centrifugation, the supernatant was removed and the precipitate was re-suspended with distilled water. This centrifugation process was performed twice. The precipitate was finally suspended with 9 mL of PBS as a NP suspension (10 mg/mL or 2 mg/mL γ-PGA-Phe in feed). In some cases, the NP suspension was washed by centrifugal filtration and finally prepared as NPs in 9 mL of PBS (10 mg/mL or 2 mg/mL γ-PGA-Phe in feed). The size regulated γ-PGA-Phe NPs were prepared according to a previous report [14].

NPs were manufactured by three different processes: a centrifugation method (process-A) and a filtration method (process-B) with DMSO as an organic solvent for γ-PGA-Phe, and a filtration method with EtOH/buffer as a solvent for γ-PGAPhe (process-C).

Evaluation of NP dissociation and reconstitution

The NP suspension was dissociated with an equal volume of SDS solution at final concentrations of 2.0, 1.0, 0.5, and 0.2% (v/v) at room temperature. In the same manner, the NP suspension was dissociated with DMSO solution at final concentrations of 50, 60, 70, 80, and 90%. In addition, the

NP suspension was dissociated with an equal volume of 20 mmol/L sodium phosphate buffer solution (pH 7.0) and 50% ACN (v/v) containing 0.1% dibutyrylamine (3:7 v/v).

Each NP suspension was then diluted 25 times with distilled water and the NP diameter was determined with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). This analysis afforded the mean (Z-average) particle size, and an estimate for the width of the size distribution (polydispersity index, PDI) was obtained by dynamic light scattering (DLS). To assess the reconstitution of the dissociated NPs, 0.05 mol/L or 0.1 mol/L NaCl in 20 mmol/L sodium phosphate buffer (pH 7.4) were added to the dissociated NPs to encourage them to form NPs.

Determination of the MW and the MW distribution by SEC coupled to MALS detection

NPs (γ-PGA-Phe, polymer concentration of 10 mg/mL or 2 mg/mL) were dissociated with 2% SDS (final concentration) and diluted with a mixture of 20 mmol/L sodium phosphate buffer (pH 8.0) and EtOH 1:1 (v/v) to give a γ-PGA-Phe concentration of 3 mg/mL or 1 mg/mL. Lyophilized γ-PGA Phe powder was also dissolved in a mobile phase for HPLC in order to analyze the chromatographic patterns of the NPs. The diluted NP suspension and the γ-PGA-Phe solution were then filtered using a GHP Acrodisc 13 mm syringe filter with a 0.2 μm GHP membrane (PALL Corp., Port Washington, NY, USA). The mass of the sample injected onto the column was typically 0.6×10^{-4} g. Size-exclusion chromatography (SEC) was performed with a refractive index detector (RI) and a multi-angle light scattering (MALS) detector (SEC-RI/MALS) using a Waters Alliance® system (including a 2414 RI detector, a 2695 pump, an autosampler module, and a degasser; Waters, Milford, MA, USA) coupled to a Dawn Heleos II MALS detector and an Optilab® T-rEX RI detector (both instruments from Wyatt Technology Corp., Goleta, CA, USA). Separations were carried out with a mixture of 20 mmol/L sodium phosphate buffer (pH 8.0) and EtOH (1:1 v/v) at a flow rate of 0.4 mL/min using TSKgel columns (G4000 PWxL, 7.8 mm i.d. × 30 cm, 10 μm; Tosoh

Corp., Tokyo, Japan) at 30 °C. The calculation of the average MW and the Z average of the root mean square (RMS) radius via MALS required the sample-specific refractive-index increment (dn/dc), which was determined using the RI detector. The determined value was used for all subsequent MW determinations. The SEC column was calibrated using a standard kit containing ten kinds of polyethylene glycol at a concentration of 1.0 mg/mL and polyethylene oxide at a concentration of 3.0 mg/mL, which are the reference standards most commonly used in SEC for aqueous MW determination. The absolute MW was analyzed with the ASTRA6 software (Wyatt Technology Corp.) and Empower 2 software for GPC (Waters). The absolute MW of the γ-PGA-Phe in the NPs was determined using a Debye plot obtained from the intensity of the scattered light, and was estimated as the weight-average MW. The PDI was calculated by dividing the weight-average MW by the number-average MW.

Evaluation of the γ-PGA-Phe content of and impurities in the NPs by reverse-phase chromatography

After adding 2% SDS (v/v, final concentration) to the NP suspension, it was diluted with 20 mmol/L phosphate buffer (pH 7.5) containing 0.25% SDS (w/v) and 2% ACN (v/v) to 1 mg/mL or 0.2 mg/mL with shaking and then filtered using a GHP Acrodisc 13 mm syringe filter with a 0.2 μm GHP membrane (PALL, PN: 4554T). The mass of the sample injected onto the column was typically 2×10^{-5} g. The γ-PGA-Phe content of and the impurities in the NPs were measured by reverse-phase chromatography (RP-HPLC) using a Waters Alliance® system. The separations were carried out using Inertsil WP300 C4 (4.6 mm× 150 mm, 5 μm, GL Sciences,Tokyo, Japan) with the column at a temperature of 25 °C and with a flow rate of 1 mL/min. Peaks were detected at 214 nm using a UV detector. The compositions of eluents A and B were 0.25% SDS and 2% ACN in phosphate buffer (pH 7.5) (A) and 0.25% SDS and 60% ACN in 20 mmol/L sodium phosphate buffer (pH 7.5) (B). A

linear gradient was used: eluent B was increased from 2 to 4% until 2 min and from 4 to 100% until 20 min; 100% eluent B was maintained until 35 min. Empower 3 (Waters) was used for data acquisition and analysis. The content of γ-PGA-Phe and the impurity levels in the NPs were calculated from the area percentages of the peaks in a chromatogram obtained with a UV detector.

Variation in NP quality depending on the manufacturing process and preliminary stability study

The quality of the NPs manufactured using each of three processes (A-C) was evaluated. Each NP suspension was stored in an amber glass vial in a chamber maintained at 5°C for 0 (initial), 1, 2, and 3 months. Analysis of the Z-average size and the PDI of the NPs was carried out using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). After dissociating the NPs, the γ-PGA-Phe content and the impurities in the NPs were measured by RP-HPLC. The content (% of initial content) at a particular point in the stability study was calculated as [(γ-PGA-Phe content in NPs at that point in the study) / (initial γ-PGA-Phe content in NPs)] × 100%.

Comparison of lyophilized NPs and NPs in suspension

Lyophilized NP powder and NPs in suspension were stored at 5°C (long-term condition), 25°C (accelerated condition), and 40°C (stressed condition) for 6 months. After the storage period, the lyophilized NP powder was reconstituted at a concentration of 10 mg/ml in distilled water. The particle size distribution and relative scattering intensity (%) were also evaluated using a Zetasizer Nano ZS instrument (Malvern Instruments).

Evaluation of the optimal manufacturing process and formulation

NPs that were around 200 nm in diameter were manufactured by applying the optimized manufacturing parameters to the DMSO-based formulation (process-D) and the EtOH-based

formulation (process-E). 5% Trehalose (w/v), which is a formulation stabilizer, was added to some NP suspensions in PBS solution before the NPs were lyophilized overnight. Each lyophilized NP powder was reconstituted in distilled water before analysis. The characteristics of the NP suspension and the lyophilized NP powder were evaluated. The solution of NPs was inspected visually, and the author carried out DLS measurements using a Zetasizer Nano ZS instrument to examine the particle size, PDI, and zeta potential (via conductometry). The water content of the NPs was determined using a Karl Fischer titrator (AQV-2100C, Hiranuma Sangyo Co. Ltd., Japan), the γ-PGA-Phe content and impurities were analyzed by RP-HPLC, and the absolute MW was evaluated using SEC-RI/MALS. Particulate matter was measured via Light Obscuration Particle Count Test 1 using a liquid particle counter (HIAC System 9703, Hach Ultra, Tokyo, Japan), pH was monitored with a pH meter (F52, HORIBA, Kyoto, Japan), and the osmolality was tested with a 5007 OSMETTE XL osmometer (Precision Systems Inc., Natick, MA) based on the Pharmacopeia methods.

2. 4. Results and discussion

Dissociation of NPs

Further research into and development of diagnostic and therapeutic nanomedicines and their translation into clinical practice require appropriate characterization methods to ensure reproducible nanomedicine quality and performance. However, many of the methods that are currently available cannot provide a detailed analysis of the characteristics and quality of nanomedicines. The primary aim of **Chapter 2** was therefore to develop reliable and practical analytical methods for the quantitation of NP platforms. The ability to accurately evaluate the quality and characteristics of NPs could facilitate the creation of manufacturing processes that scale up and provide better yields with increased purity without adversely affecting the safety or efficacy of the product. Therefore, the sample preparation is important and more challenging. The dissociation condition of NPs as an

essential pretreatment method is necessary. Monodisperse NPs (157 nm in size) manufactured from γ-PGA-Phe with a grafting degree of 40% were used to study the optimal conditions for NP dissociation. The optimal dissociation conditions were identified by dissociating the NPs with SDS, DMSO, or ACN containing 0.1% dibutyrylamine (v/v) and determining the degree of NP dissociation in each case by evaluating the particle size using DLS.

SDS solution was added to the NP suspension at concentrations of 2.0, 1.0, 0.5, and 0.2% SDS (w/v) and the resulting solution was then diluted 25 times with distilled water. Given that the particle size changed from about 100 nm to <10 nm, it was evident that the NPs were completely dissociated at a concentration of ≥ 0.5% SDS (w/v) (Fig. 2-1). The variation in NP dissociation over time was evaluated from 0 to 15 min. The NPs quickly and completely dissociated to give molecules 10 nm in size as soon as the NPs suspended in PBS were exposed to the SDS solution. SDS is an anionic detergent with a polar head group and a nonpolar hydrocarbon tail. The negatively charged sulfate group and its positive sodium counterion both enhance the conductance of a SDS solution with respect to that of pure water. Also, the NPs that were completely dissociated by 2% SDS (w/v, final concentration) were not reconstituted by adding 0.1 mol/L NaCl (final concentration), which can aid the formation of NPs from γ-PGA-Phe. In short, the NPs were fully dissociated into individual γ-PGA-Phe polymeric molecules (the component material) as the adsorption of SDS on γ-PGA-Phe was irreversible, which prevented the NPs from reforming (Fig. 2-1). In the same manner, NPs were dissociated by adding 50, 60, 70, 80, and 90% DMSO (final concentrations). The NPs were dissociated into component molecules <10 nm in size when >70% DMSO (w/v) was added (data not shown here). DMSO is adsorbed by carboxylic groups of γ-PGA-Phe, which changes the surface properties of this compound and destroys the NP structure. The NPs dissociated by 70% DMSO (w/v) were not reconstituted upon the addition of 0.05 mol/L NaCl, but they were upon the addition of 0.1 mol/L NaCl. Furthermore, an equal volume of ACN containing 0.1% dibutyrylamine (v/v),

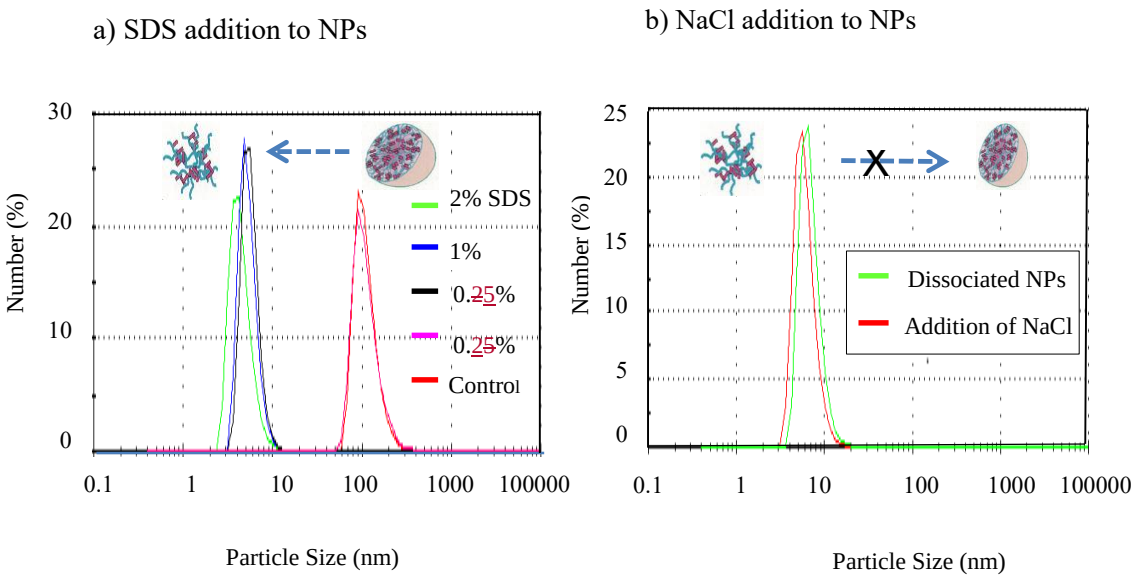


Fig. 2-1. Dissociation and reconstitution of NPs by size distribution analysis.

a) SDS solution was added to NPs in suspension at final concentrations of 2.0～0.2% SDS and they were diluted with distilled water. Then, the diameter was determined by Zetasizer Nano ZS (Malvern, UK).

b) 0.1 mol/L NaCl in 20 mmol/L sodium phosphate (pH7.4), which form NPs, were added to the dissociated NPs by SDS solution and they were diluted with distilled water. Then, the diameter was determined by Zetasizer Nano ZS (Malvern, UK).

which is highly soluble, was added to the NP suspension, leading to complete dissociation of the NPs. The dissociated NPs were not reconstituted upon the addition of 0.1 mol/L NaCl, but it was thought that they were degraded by alkaline hydrolysis of ester bonds of γ-PGA-Phe with an organic base such as ACN in this solution, so these dissociation conditions were deemed to be unacceptable. Consequently, 2% SDS (w/v, final concentration) was chosen as the optimal dissociation conditions for NPs.

By dissociating the NPs, the author was then able to develop analytical methods for determining the γ-PGA-Phe content and the impurities in the NPs (via RP-HPLC) as well as the absolute molecular weight (MW, via SEC-RI/MALS) based on methods the author developed in **Chapter 1** to analyze γ-PGA-Phe [26].

Development of methods for analyzing the NPs

In practice, the following properties of a liquid should be considered when choosing an appropriate sample solvent and mobile phase for HPLC: viscosity, reactivity, operating temperature, compatibility with the detector and the separation system, cost, smell, and safety. Multi-angle light scattering (MALS) technology is a powerful research tool, and the MALS-based methods that we have developed possess certain advantages over other techniques for evaluating some of the characteristics and the quality of NPs. Data on the absolute MW, the MW distribution, molecular structure and conformation, as well as the degradation of γ-PGA-Phe and its aggregation into NPs can be provided by a SEC-RI/MALS system coupled to concentration-sensitive and MW-sensitive detectors. While several examples of the use of HPLC for NP separation have been reported [36,37], and SEC is probably the most popular chromatographic technique that is used to fractionate NPs, a limited number of column materials are available for the SEC separation of larger particles. Surfactant-assisted SEC is a feasible method for separating NPs, and an appropriate SDS

concentration can be used to prevent aggregation in the column during SEC. As flow rate of HPLC and penetration time of NPs, which is the dissociated to γ-PGA-Phe by the addition of SDS for sample preparation, were influenced to the cleavage of polymer, they were optimized. When applied under the optimal conditions, these methods provided accurate values for the properties and quality of the dissociated NPs and the amphiphilic and biodegradable γ-PGA-Phe powder that was used to generate the NPs. The absolute MW, the number-average molecular weight (MN), and the PDI of the dissociated NPs were 148 kDa, 86 kDa, and 1.7; the corresponding values for the γ-PGA-Phe powder were similar to those of the NPs: 160 kDa, 86 kDa, and 1.9, respectively. The elution profiles of the RI and MALS plots for the NP suspension and γ-PGA-Phe powder were also found to be comparable, suggesting that the NPs did not degrade or aggregate during the analysis (Fig. 2-2).

Additionally, the author developed a method for analyzing the γ- PGA-Phe content and the impurities in the NPs, based on RP-HPLC with an ACN gradient and a C4 column. This method was validated by checking that its specificity, linearity, recovery, limit of detection (LOD), limit of quantitation (LOQ), and accuracy were all acceptable (data not shown here) when applying a mobile phase consisting of ACN and 20 mmol/L potassium dihydrogen phosphate (45:55, v/v) at a flow rate of 1 ml/min on an Inertsil WP300 C4 analytical column and a detection wavelength of 230 nm. This method exhibited linearity over an analytical range of 0.5–4 × 10^{-5} g based on linear regression (R^2 = 0.9996). The LOD and LOQ were measured based on signal-to-noise ratios of 3 and 10, respectively, and the LOQ was 0.02% for γ-PGA-Phe. Varying the injection volume while keeping the sample mass constant did not result in any significant variations in the chromatogram pattern. Upon analyzing the dissociated NPs, the peak from γ-PGA-Phe was detected at about 17.3 min. In terms of the main impurities in the dissociated NPs, peaks from PA, an unknown species (U-1), Phe, and another unknown species (U-2) were detected at about 1.9 min, 5.6 min, 9.8 min, and 15.2 min, respectively. Phe and PA obtained commercially were used as references when attempting to identity

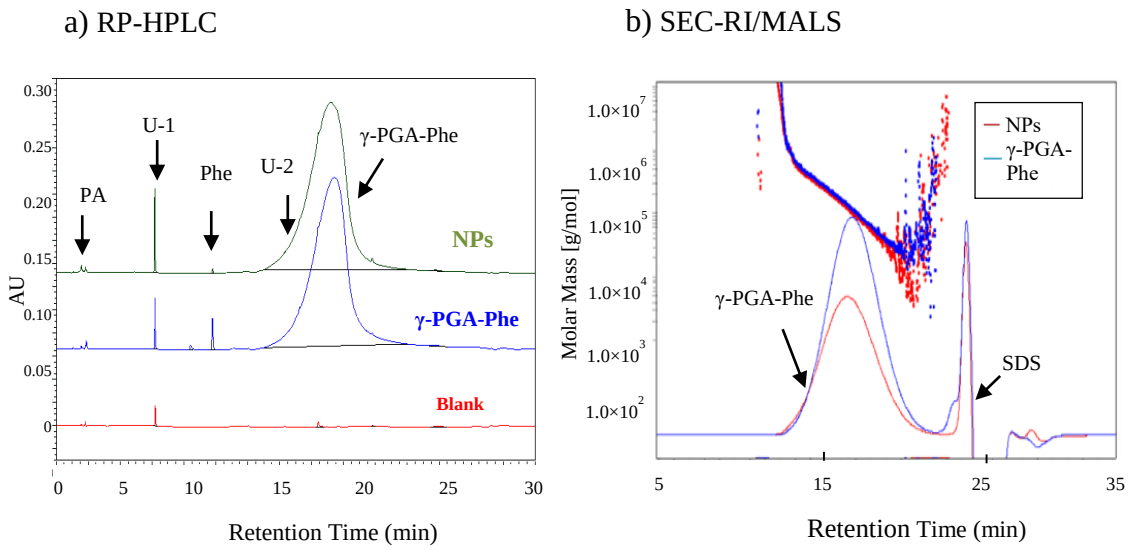


Fig. 2-2 a–b. Chromatograms for the dissociated NPs and lyophilized γ-PGA-Phe powder, as obtained using a) RP-HPLC and b) SEC-RI/MALS. In a, peaks for PA, U-1, Phe, U-2, and γ-PGA-Phe were detected at about 1.9 min, 5.6 min, 9.8 min, 15.2 min, and 17.3 min, respectively. PA: phenylalanine, Phe: phenylalanine ethyl ester, U-1: unknown species 1, U-2: unknown species 2. In b, the solid lines indicate the RI response and the dotted lines indicate the MALS response. The red line is for γ-PGA-Phe powder and the blue line is for NPs dissociated by SDS. HPLC mobile phase A was used as a blank

the impurities. EDC was used in the synthesis of γ-PGA-Phe, and derivatives of it did not overlap with any of the impurity peaks. These results show that elution profiles of the dissociated NP suspension and the γ-PGA-Phe powder are comparable. The HPLC method proved to be reliable for quantifying the γ-PGA-Phe content of and the impurities in the dissociated NP suspension, and provided useful insight into the quality of the NPs. The selection of an appropriate stationary phase, mobile phase, and gradient (either temperature or solvent gradient) is not always straightforward and often requires extensive experimental work. Phenomena such as the precipitation of polymers in the injector, breakthrough peaks, and irreversible adsorption of large polymers can result in incomplete recovery and therefore inaccurate quantitation.

After the NPs had been dissociated by SDS into intact γ-PGA-Phe molecules, chromatographic separation of the dissociated NPs by RP-HPLC and SEC-RI/MALS was found to be viable and permitted reliable quantification. These methods are expected to have considerable advantages over other commonly used nonqualitative methods.

Validations in NP quality depending on the manufacturing process

In previous research, we found that γ-PGA-Phe (in DMSO solvent) formed monodisperse NPs, and that the size of the NPs could easily be tailored (between 30 and 200 nm) by adjusting the NaCl concentration [38]. Three representative NP manufacturing processes (A-C, as mentioned in the "2-3. Experimental section") were evaluated based on the quality of the NPs they produced. The average particle size and the PDI (via DLS), the absolute MW (via SEC-RI/ MALS), as well as the content of γ-PGA-Phe and the impurities (via RP-HPLC) in the NP suspension were measured [39]. The Z-average size and the PDI of NPs manufactured by processes A, B, and C were 174 nm and 0.138, 173 nm and 0.192, and 109 nm and 0.209, respectively. Process-C yielded slightly smaller NPs, but they were still within the acceptable size range for confirmed immunological effects [40].

The yield of NPs from each process (A, B, and C) was calculated by comparing the γ-PGA-Phe content of the NP suspension with the initial mass of γ-PGA-Phe employed to prepare the NPs. The NP yields for processes B and C indicated that these processes permitted the reproducible and quantitative recovery of γ-PGA-Phe from NPs, whereas process-A afforded about 67% and a low recovery rate (Table 2-1) as the NPs either stuck to the membrane or flowed through it and were lost to the filtrate. The purity of the γ-PGA-Phe in each NP suspension was 80.4% for the NPs produced using process-A, 99.4% for the NPs produced using process-B, and 99.7% for the NPs produced using process-C. For process-A, the Phe and U-2 peak areas were 0.11% and 0.12% versus the area of the γ-PGA-Phe peak. A peak from an unknown species detected at 1.8 min was the most abundant impurity (15.4% of the area of the γ-PGA-Phe peak), but this peak was not detected when the other processes were implemented (Fig. 2-3). By contrast, in process-B, the peak areas (with respect to the γ-PGA-Phe peak) for U-1 (which eluted at 6.0 min), Phe, and U-2 (15.0 min) were 0.09, 0.04, and 0.02%, respectively. In process-C, the peak at 1.5 min and the U-1 peak had areas of 0.07% and 0.23%. The performance of each method (centrifugation and filtration) was observed. Purification of more concentrated samples by centrifugation required a large amount of buffer. Processes B and C, which both used filtration, produced NPs of a similar quality; when process-B or C was used, impurities were clearly decreased and the purity of the NPs was higher than when process-A was used. These results indicate that a DMSO-based centrifugation method (process-A) is not appropriate for purifying the NPs, whereas a DMSO-based filtration method (process-B) or an EtOH based filtration method (process-C) is appropriate for purifying the NPs. Therefore, the choice of purification method greatly affects the properties of the resulting NPs, and analytical methods which the author developed are able to distinguish differences in impurity levels and γ-PGA-Phe contents between NPs, thus allowing the most suitable manufacturing process to be identified.

Stress testing plays an important role in the drug development process, as it leads to a deeper

Table 2-1. The quality differences of NPs in three manufacturing processes

Process Method	γ-PGA-Phe Content		Impurities (%)					
	mg/mL	% of Total	Peak at 1.5min	Peak at 1.8 min	PA	U-1	Phe	U-2
▌	10.0	96.2	N.D.	N.D.	0.52	0.05	2.04	1.13
Process-A	6.7	80.4	N.D.	15.4	N.D.	0.01	0.11	0.12
Process-B	12.5	99.4	N.D.	N.D.	N.D.	0.09	0.04	0.02
Process-C	11.5	99.7	0.23	N.D.	N.D.	0.07	N.D.	N.D.

Quality of NPs manufactured by the following 3 different processes was evaluated: centrifugation method (process-A) and filtration method (process-B) with DMSO as an organic solvent for γ-PGA-Phe and filtration method with EtOH/buffer as a solvent for γ-PGA-Phe (process-C) by RP-HPLC. The peaks for PA, U-1, Phe, U-2 and γ-PGA-Phe were detected at about 1.9 min, 5.6 min, 9.8 min, 15.2 min and 17.3 min, respectively. PA: phenylalanine; Phe: phenylalanine ethyl ester; U-1: Unknown-1; and U-2: Unknown-2.

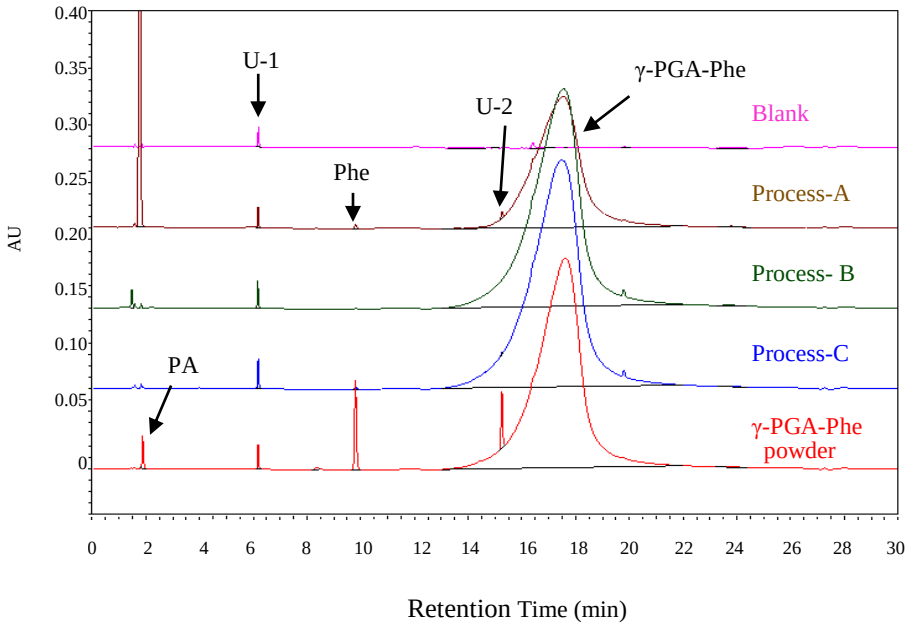


Fig. 2-3. Evaluation of the differences in quality of the NPs produced using various manufacturing processes. A centrifugation method (process-A), a filtration method with DMSO used as an organic solvent for γ-PGA-Phe (process-B), and a filtration method with EtOH/buffer used as a solvent for γ-PGA-Phe (process-C). Quality was assessed via RP-HPLC. PA: phenylalanine, Phe: phenylalanine ethyl ester, U-1: unknown species 1, U-2: unknown species 2.

understanding of the chemistry of the drug product and facilitates the development of methods which can analyze the stability of the product. Preliminary stability studies of NPs manufactured by processes B and C were therefore conducted at 0, 1, 2, and 3 months of storage (Fig. 2-4). The NPs were stored suspended in PBS at 5 °C. U-1, Phe, and U-2 impurity peaks were observed for the NPs generated by process-B, and the largest of these -the U-1 peak increased in area over time, from 0.09% to 0.5% (with respect to the γ-PGA-Phe peak) after 3 months (data not shown here). The other impurity peaks did not increase in area over time. For the NPs produced by process-C, the U-1 peak area increased from 0.07% to 0.47% over time, whereas the other impurities were not detected after 3 months of storage. The author previously reported to believe that U-1, the most abundant impurity in the γ-PGA-Phe, is a derivative of EDC. The amount of U-1 was observed to increase markedly with increasing temperature in a stability study of γ-PGA-Phe in an acidic or weakly alkaline buffer solution. The rate of hydrolysis accelerated with increasing temperature, i.e., the hydrolytic degradation of γ-PGA-Phe can be controlled by adjusting the pH and temperature [25]. Even though the degradation pathway of U-1 that leads to increasing levels of U-1 in NPs produced by process-B or C is unclear, this observation should help to improve the manufacturing process, formulation, and the storage conditions for the NPs.

In addition, for the NPs produced using processes B and C, the γ-PGA-Phe peak became broader and was eluted more quickly but the γ-PGA-Phe content did not change (data not shown here) as the storage time increased. The introduction of Phe groups onto γ-PGA delays polymer hydrolysis, as it makes it more difficult for water molecules to attack the NPs [14,41]. Also, even though the cleavage of the main chain of the γ-PGA-Phe was considered to barely impact the stability of the NPs produced using processes B and C, the particle size of the NPs stored at 5°C did not change and NPs particles containing low MW polymers partially are composed stablely. Key parameters for the stabilization of NPs are size and quality.

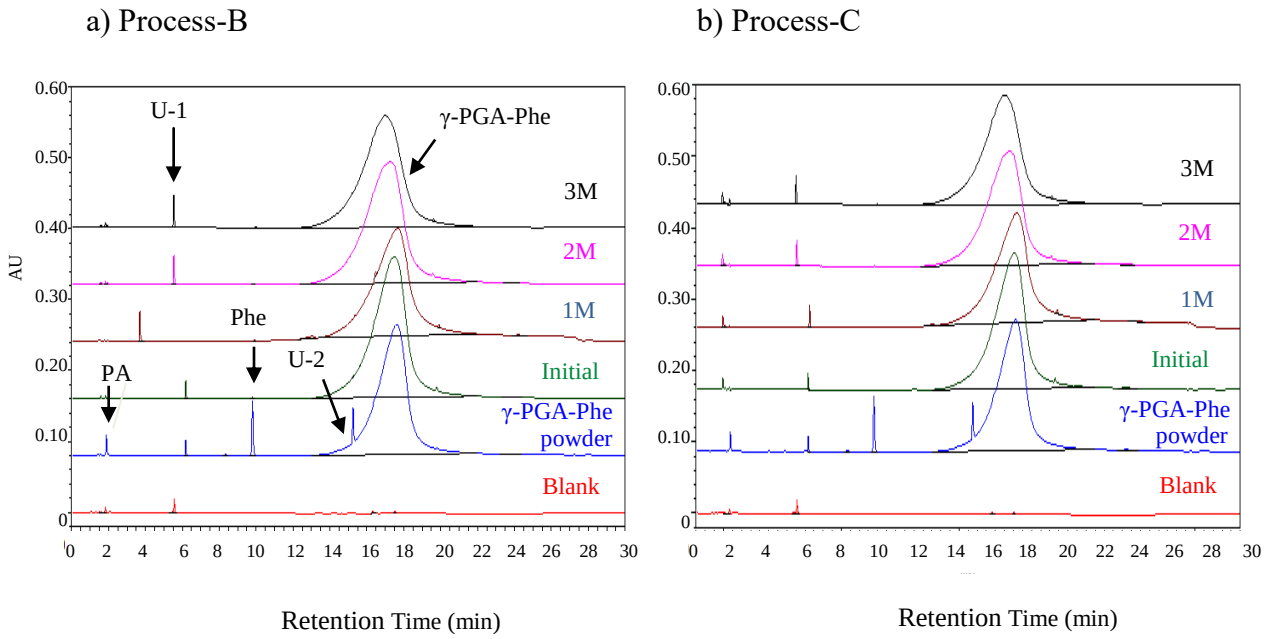


Fig. 2-4. Results of a stability study of γ-PGA-Phe NPs manufactured by either process-B or process-C. The study was conducted at 5 °C with the NPs suspended in PBS, and the stability was evaluated via RP-HPLC. Each chromatogram shows the results for NPs stored for 3 months (3 M), 2 months (2 M), 1 month (1 M), and 0 months (Initial), as well as for γ-PGA-Phe powder and a blank sample (HPLC mobile phase A).

NPs prepared by processes B and C remained stable for >3 months at 5°C and the quality of the NPs over this storage period did not change significantly. The NPs size is stable during the storage and it is considered that secondary intermolecular interaction is stable. In addition, the impurities in NPs did not increase and the quality is constant. The author found that the developed analytical methods were able to clearly distinguish differences in quality between NPs generated by different processes.

Storage stability of NPs in suspension and lyophilized NPs

Ensuring that NPs remain stable during storage is a challenging issue in pharmaceutical product development. When used in drug products, NPs are employed as either a dry powder or in suspension. Lyophilization is a commonly used stabilization technique for thermolabile pharmaceuticals. Low NP stability during storage seriously limits the practical application of the NPs, since this lack of NP stability can directly affect the efficacy and safety of the drug delivery system. In general, since DLS can be used to monitor changes in NP size, DLS can also be used to assess the stability of most NPs. Assuming that the sizes of the assembled NPs lie within a particular particle size range, deviations from the average NP size range can be interpreted as an indication of NP degradation in that particular environment. Another factor to consider is the PDI, which represents the dispersion homogeneity.

Therefore, we studied NPs suspended in different solvents and lyophilized NP powders, where the NPs were purified via processes A-C, in order to evaluate the effects of temperature and humidity on NP stability. Stability was assessed by monitoring changes in particle size and relative scattering intensity when the NPs were stored under various conditions at 5 °C, 25 °C, and 40 °C for 6 months. For the NPs produced by processes B and C, the mean size of the NPs in suspension remained stable at 5 °C for 6 months, and the NPs stored at 25 °C also remained virtually unchanged, although slight

degradation of the γ-PGA-Phe was observed (Fig. 2-5 and Table 2-2). When stored at 40 ℃ for 6 months, the mean size of the NPs in suspension reduced from 177 nm to 100 nm (for process-B NPs) or from 112 nm to 68 nm (for process-C NPs), and aggregation and degradation were observed. After 6 months, the relative scattering intensities of NPs that were stored in suspension at 5 ℃, 25 ℃, and 40 ℃ dropped to 93%, 52%, and 13% (for process-B NPs) or 79%, 29%, and 17% (for process-C NPs) of the original intensity.

On the other hand, regardless of whether process-B or C was applied to produce the NPs, the lyophilized NPs exhibited a consistent mean size even when stored at 40 ℃ for 6 months. For both process-B and process-C lyophilized NPs, the PDI was 0.16–0.26, indicating a relatively homogeneous dispersion in comparison to the NPs in suspension. Even though the relative scattering intensity decreased slightly to 91% (for process-B NPs) and 90% (for process-C NPs) of the original intensity following storage at 40℃ for 6 months, the scattering intensity was stable after storage at 5℃ and 25℃ for 6 months, and no large aggregates were detected. Consequently, the highest stability was observed for the lyophilized NPs stored at 5℃ and 25℃ for 6 months, as no change was noted in the particle size, relative scattering intensity, or any other characteristics. A comparison of the lyophilized NPs produced via processes B and C showed no significant difference in long-term stability. It is therefore clear that lyophilizing NPs obtained from suspension is a useful technique for enhancing NP stability and thus shelf life. The lyophilization process is assumed to enhance stability by preventing water catalyzed ester hydrolysis. The lyophilized formulation is thus the preferred approach for enhancing the stability of nanosuspensions during the manufacturing process and storage.

Evaluation of the optimal manufacturing process and formulation

High-quality NPs are needed for clinical applications of NP-based vaccine adjuvants.

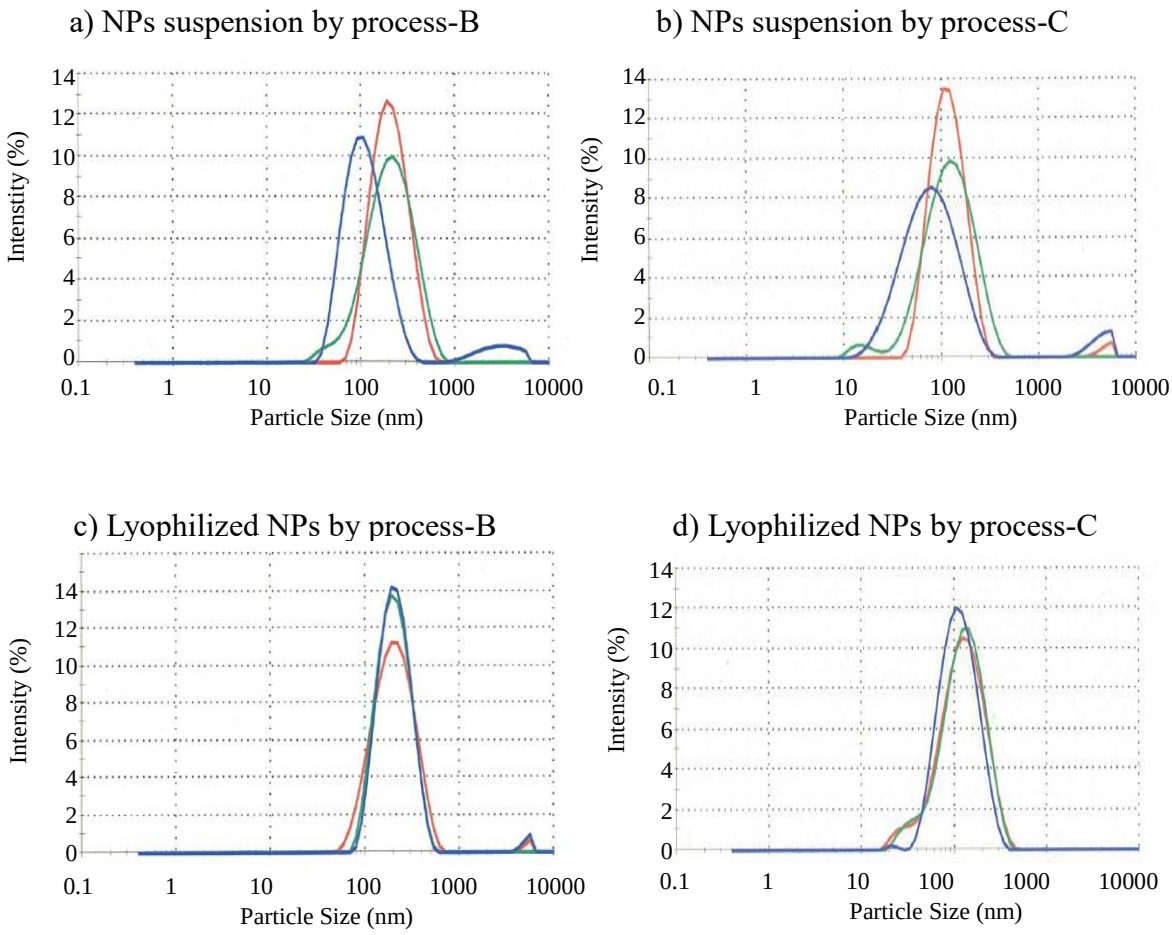


Fig. 2-5. Particle size distributions of NPs produced via different manufacturing processes (B or C) and stored as different formulations (lyophilized or in suspension) at various temperatures (red line 5°C, green line 25°C, or blue line 40°C) for 6 months. NP stability was probed by monitoring particle size changes during storage via DLS.

Table 2-2. Storage stability of NPs in suspension and lyophilized NP powder by EtOH based and DMSO-based processes

Process Method	Storage Condition		Suspension			Lyophilized Powder		
			Particle Size (nm)	PDI	Relative Scatter Intensity	Particle Size (nm)	PDI	Relative Scatter Intensity
Process-B (DMSO -based)	Initial		176.7	0.18	100	176.7	0.18	100
	5˚C	6M	177.9	0.21	93	179.6	0.23	106
	25˚C		166.3	0.25	52	177.5	0.16	107
	40˚C		99.6	0.28	13	178.6	0.26	91
Process-C (EtOH -based)	Initial		112.1	0.16	100	112.1	0.16	100
	5˚C	6M	105.6	0.22	79	100.2	0.24	102
	25˚C		95.6	0.27	27	104.0	0.25	102
	40˚C		68.1	0.34	19	99.5	0.21	92

M: Month and PDI: polydispersity index.

After the storage, lyophilized NP powder was reconstituted at 10 mg/ml as a polymer concentration in distilled water. The particle size distribution and relative scattering intensity (%) were also evaluated by Zetasizer Nano ZS.

A reproducible manufacturing process that yields a product of consistently high quality that displays the intended physicochemical characteristics, biological behaviors, and pharmacological profile is required for commercial use. The qualitative and quantitative differences between different NP formulations (NPs in suspension and lyophilized NP powder without trehalose) manufactured using the optimized DMSO-based process (process-D) and the optimized EtOH-based process (process-E) were assessed using the analytical methods developed in this work. It is well known that sugars can be introduced to prevent the aggregation of NPs during lyophilization and storage [42], so we employed this approach to enhance the stability of the nanosuspension. Also, to examine the effect of trehalose, it was added to NPs in suspension before lyophilization. The main aim was to control the size of the prepared NPs, but the product should also be as pure as possible. Determining the contents and the purity of the product, as well as the impurities in it, is considered crucial to ensuring product quality. Consequently, the content of γ-PGA-Phe and the impurities in the NPs in each formulation were examined by RP-HPLC, the absolute MW of the NPs was determined via SEC-RI/MALS, and the mean particle size was evaluated using DLS. Appearance and clarity of solution, particulate matter, particle size, zeta potential, pH, osmolality, and water content in the NP formulation were also measured to determine if the sample met the quality target. The NP yields achieved using the EtOH-based process and the DMSO-based process were around 92–93% and 81–82%, respectively, implying that the EtOH-based process gave higher yields than the DMSO-based process (Table 2-3). Levels of the impurities U-1 and PA in the NPs in suspension were 1.51% and 0.63% (for NPs produced via the DMSO-based process), and 2.42% and 0.02% (for NPs produced via the EtOH-based process). Compared to the impurity levels observed in the raw γ- PGA-Phe material, the impurity levels were somewhat lower in the manufactured NPs. The γ-PGA-Phe content and impurity levels were almost the same for NPs in suspension and for lyophilized NP powder with or without trehalose, regardless of the NP manufacturing process used (process-D or E).

Table 2-3. Characterization by NP Formulation

Process Method		γ-PGA-Phe Lyophilized Powder	NPs					
			DMSO (Process-D)			EtOH (Process-E)		
Formulation (Trehalose: -/+)			Suspension	Lyophilized Powder		Suspension	Lyophilized Powder	
				−	+		−	+
Appearance		White and off white crystalized powder	Free of visible particles	White to off-white cake or powder	White to off-white cake or powder	Free of visible particles	White to off-white cake or powder	White to off-white cake or powder
Clarity of Solution		N.A.	N.A.	Free of visible particles	Free of visible particles	N.A.	Free of visible particles	Free of visible particles
Water Content (%)		N.A.	N.A.	0.88	1.64	N.A.	0.52	1.16
pH		N.A.	7.24	7.23	7.14	7.09	7.08	6.86
Particle Size (nm)		N.A.	131.5	132.0	157.9	144.6	143.6	172.1
Osmolality (mOsm/L)		N.A.	278	273	293	276	256	300
Zeta Potential (mV)		N.A.	-19.8	-24.0	-28.0	-23.3	-25.3	-24.6
Particle Matter	≧10 μm	N.A.	23	6	14	1	5	8
	≧25 μm	N.A.	1	0	0	0	1	1
γ-PGA-Phe Content	mg/mL	1.00	0.818	0.805	0.816	0.929	0.921	0.934
	Recovery (%)	100	81.8	80.5	81.6	92.9	92.1	93.4
Impurities (%)	PA	N.D.	0.63	0.26	0.59	0.02	0.14	N.D.
	U-1	5.24	1.51	1.21	1.08	2.42	2.15	2.10
	Phe	0.03	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	U-2	0.11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Others	6.63	N.D.	0.51	0.28	0.89	1.17	1.03
Absolute MWs (kDa)		112	87	81	-.	81	128	92

-: Not determined.

Absolute MW was determined by SEC-RI/MALS analysis. Content of γ-PGA-Phe and impurity levels were determined by RP-HPLC. PA: Phenylalanine; Phe: Phenylalanine ethyl ester; U-1: Unknown-1, U-2: Unknown-2. N.A.: Not applicable.

The absolute MWs of the NPs were 81–128 kDa and the PDIs of the NPs were smaller than those of the γ-PGA-Phe. The absolute MW also reduced during the process. Polymer degradation is thought to occur, mainly through a hydrolytic mechanism due to the presence of hydroxyl groups on the NPs. NP size was found to be in the range 132–172 nm, and the PDI of the NPs was 0.14–0.16, suggesting that the particles were of the desired size. The particle size was slightly larger when trehalose was present. Each NP showed a negative zeta potential (20–28 mV), which can influence particle stability due to the ionization of the carboxyl groups close to the surface of γ-PGA-Phe (data not shown). The suspension and the solution used to generate the lyophilized powder were observed to be free of visible particles, and the appearance of the lyophilized powder was a white to off-white cake or powder. The same results were seen for the NPs produced via the EtOH-based process and those given by the DMSO-based process. Particulate matter with Method 1 solutions prepared based on insoluble particulate shielding particle counting method, which is described in US pharmacopeia 788 Particulate Matter for Injections, was almost clear and practically free from particles when examined under suitable conditions of visibility. pH values ranged from 6.86 to 7.24 and osmolality values from 256 to 300 mOsm/L; again, these parameters were almost the same for NPs produced by both processes (process-D and E). The water contents of NPs produced using the DMSO-based process were found to be slightly higher than those of the NPs produced by the EtOH-based process, indicating that NPs generated by the EtOH-based process are more stable during storage than NPs generated by the DMSO-based process. The lyophilized formulation was preferred for enhancing the stability of the nanosuspension during the manufacturing process and the storage period. Most reported stability concerns arise from nanosuspensions that are dispersed in a medium with or without stabilizers. In these cases, the stabilizers are usually added to the formulation to protect the NPs from lyophilizing stresses. In my formulations, the quality and characteristics of the NPs were unchanged by the lyophilization process, and no visible change occurred upon dispersing the

lyophilized powder in PBS solution.

Despite the lyophilization, there was no dependence of the quality and characteristics of the NPs on the manufacturing process or the formulation used (Table 2-3). Thus, my developed methods were clearly able to distinguish between manufacturing processes based on the quality and characteristics of the NPs produced. In addition, we previously reported that for protein encapsulated γ-PGA-Phe NPs prepared by a DMSO-based process with a centrifugation method, the encapsulation is between 30 and 60% efficient, and is stable across a range of acidic pH values, even after 10 days [15]. Even though the major obstacles to the clinical use of NPs are their physical and chemical instability with respect to the hydrolysis of the polymer materials that form the NPs, the author succeeded in producing stable γ-PGA-Phe NPs of high quality and with consistent characteristics using the development process and analytical methods.

It is known that high humidity and temperature accelerate peptide degradation [38]. It is therefore important to ensure a reasonable shelf life for products containing γ-PGA-Phe-based NPs (i.e., that the desired quality and physicochemical characteristics of the NPs are maintained under commonly encountered storage conditions) by applying an appropriate manufacturing process and selecting a suitable formulation. The author plans to assess the stability of NP formulations during long-term and accelerated storage at various temperatures and relative humidities in order to determine the optimal formulation and storage conditions for the NPs, and thus the shelf life of the NPs.

The author also intends to develop valid test methods for measuring relevant quality parameters and to establish generally accepted criteria for assessing the quality of NPs manufactured for clinical applications.

2. 5. Conclusion

The author has developed methods of analyzing the quality and characteristics of NPs that are

transferable to future clinical NP development. Dissociating the NPs to γ-PGA-Phe using SDS and then performing chromatographic separation via RP-HPLC or SEC-RI/MALS permits the precise, clear, and accurate quantitative characterization of NPs. These methods can be used to optimize the NP manufacturing process. To the best of our knowledge, these methods of quantitatively evaluating NPs are the first to be developed in the field of drug delivery. The availability of these methods is expected to accelerate the clinical development of NPs, which requires reliable analytical technologies to assure quality and safety.

2. 6 References

[1] L. Wu, J. Zhang, W. Watanabe, *Adv. Drug Deliv. Rev.* **63**, 456 (2011).

[2] C. Du, J. Zhao, J. Fei, Y. Cui, J. Li, *Adv. Healthcare Mater.* **2**, 1246 (2013).

[3] L. Gao, Y. Cui, Q. He, Y. Yang, J. Fei, J. Li, *Chem. A. Eur. J.* **17**, 13170 (2011).

[4] X. Yan, P. Zhu, J. Fei, J. Li, *Adv. Mater.* **22**,1283 (2010).

[5] D. Li, C. Li, A. Wang, Q. He, J. Li, *J. Mater. Chem.* **20**, 7782 (2010).

[6] C. Thomas, A. Rawat, L. H. Weeks, F. Ahsan, *Molecular Pharmaceutics* **8**, 405 (2011).

[7] S. Y. Kim, H. J. Doh, M. H. Jang, Y. J. Ha, S. I. Chung, H. J. Park, *Helicobacter* **4**, 33 (1999).

[8] S. L. Demento, W. Cui, J. M. Criscione, E. Stern, J. Tulipan, S. M. Kaech, *Biomaterials* **33**, 4957 (2012).

[9] J. M. Lü, X. Wang, C. M. Muller, H. Wang, P. H. Lin, Q. Yao, *Expert Review of Molecular Diagnostics* **4**, 325 (2009).

[10] R. L. McCall, R. W. Sirianni, *J. Vis. Exp.* doi:10.3791/51015 (2013).

[11] E. J. Cho, H. Holback, K. CLiu, S. A. Abouelmagd, J. Park, Y. Yeo, *Mol. Pharm*. **10**, 2093 (2013).

[12] V. Sokolova, T. Knuschke, J. Buer, A. M. Westendorf, M. Epple, *Acta Biomater.* **7**, 4029 (2011).

[13] T. Akagi, M. Baba, M. Akashi, S. Kunugi. *Polymers,* In: T. Yamaoka, editor, *Nanomedicine,* Berlin, Germany, Springer-Verlag Berlin **31** (2012).

[14] T. Akagi, T. Kaneko, T. Kida, M. Akashi, *J. Controlled Release* **108**, 226 (2005).

[15] T. Uto, X. Wang, K. Sato, M. Haraguchi, T. Akagi, M. Akashi, M. Baba, *J. Immun*. **178**, 2979 (2007).

[16] T. Uto, T. Akagi, T. Hamasaki, M. Akash, M. Baba, *Immunol. Lett.* **125**, 46 (2009).

[17] A. Himeno, T. Akagi, T. Uto, X. Wang, M. Baba, K. Ibuki, *Vaccine* **28**, 5377 (2010).

[18] S. Sagadevan, J. Podder, *J. Nano-Electron Phys*. **7**, 04008-1 (2017).

[19] W. C. Lin, M. C. Yang. *Macromol. Rapid Commun.* **26**, 1942 (2005).

[20] W. D. Pyrz, D. J. Buttrey, *Langmuir* **24**,11350 (2008).

[21] Y. E. Benstein, E. Nahum, U. Banin, *Nano Letters* **2**, 945 (2002).

[22] B. Zhang, B. Yan, *Anal. Bioanal. Chem.* **396**, 973 (2010).

[23] G. D. Watson, *Pharmaceutical Analysis, 3rd ed*. London, Churchill Livingstone (2012).

[24] A. H. Beckett, J. B. Stenlake, *Practical Pharmaceutical Chemistry, 4th ed*. New Delhi, CBS Publishers and Distributors pvt. Ltd. (1997).

[25] T. Higuchi, B. Hansen, *Pharmaceutical Analysis*, *3rd ed*. New Delhi: CBS Publishers and Distributors (1997).

[26] M. Ikeda, T. Akagi, T. Yasuoka, M. Nagao, M. Akashi, *J. Pharma. Biomed. Anal.* **150**, 460 (2018).

[27] M. Schwartz, I. S. Krull, *Analytical Regulatory and Validation Compliance,* New York, USA, Marcel Dekker (1997).

[28] C. M. Riley, T. W. Rosanske, editors, *Development and Validation of Analytical Methods,* Oxford, UK, Pergamon (1996).

[29] J. M. Miller, J. B. Crowther, editors, *Analytical Chemistry in a GMP Environment*, New York,

USA, Wiley (2000).

[30] J. Ermer, J. H. Miller, editors, *Method Validation in Pharmaceutical Analysis,* Weinheim, Germany, Wiley-VCH Verlag (2005).

[31] E. J. Cho, H. Holback, K. C. Liu, S. A. Abouelmagd, J. Park, Y. Yeo, *Mol. Pharmaceutics* **10**, 2093 (2013).

[32] Y. Kazakevich, B. R. Loburutto, *Stationary Phases* in Y. Kazakevich, B. R. Loburutto, editors, New Jersey, USA, John Wiley and Sons (2007).

[33] M. Matsusaki, K. Hiwatari, M. Higashi, T. Kaneko, M. Akashi, *Chem. Lett.* **33**, 398 (2004).

[34] T. Akagi, M. Baba, M. Akashi, *Polymer* **48**, 6729 (2007).

[35] T. Kaneko, M. Higashi, M. Matsusaki, T. Akagi, M. Akashi, *Chem. Mater.* **17**, 2484 (2005).

[36] V. L. Jimenez , M. C. Leopold, C. Mazzitelli, *Anal. Chem.***75**, 199 (2003).

[37] J. P. Wilcoxon, J. E. Martin, P. Provencio, *Langmuir* **16**, 9912 (2000).

[38] H. Kim, T. Akagi, M. Akashi, *Macromol. Biosci.* **9**, 842 (2009).

[39] H. Yan, Y. F. Hou, P. F. Niu, K. Zhang, T. Shoji, Y. Tsuboi, F. Y. Yao, L. M. Zhaoc, J. B. Chang, *J. Mater. Chem.* B **3,** 3677 (2015).

[40] H. Kim, T. Uto, T. Akagi, M. Baba, M. Akashi, *Adv. Funct. Mater.* **20**, 3925 (2010).

[41] T. Akagi, M. Higashi, T. Kaneko, T. Kida, M. Akashi, *Macromol. Biosci.* **5**, 598 (2005).

[42] S. Ghanbarzadeh, H. Valizadeh, P. Z. Milani, *Adv. Pharm. Bull*. **3**, 25 (2013).

Chapter 3.

Formulation stability of amphiphilic poly(γ-glutamic acid) nanoparticle and cardiotoxicity evaluation of NPs with human iPSC Derived 3D-cardiomyocyte tissues

3. 1. Summary

The author conducted a stability study of biodegradable and amphiphilic nanoparticles (NPs) consisting of phenylalanine attached poly(γ-glutamic acid) (γ-PGA-Phe) for drug delivery to find the optimal formulation, and define the optimal storage conditions and corresponding shelf-life using novel quantitative analytical and compendia methods. The stability of NPs suspension and lyophilized NP powder manufactured by a dimethyl sulfoxide (DMSO)-based process and an ethanol (EtOH)-based process using a filtration method was assessed under long-term conditions (5°C), accelerated conditions at 25°C/60% relative humidity (RH) and stressed conditions at 40°C/75% RH. The content of γ-PGA-Phe in NPs, impurities, absolute molecular weight (MW), appearance, clarity of solution, particle size, zeta potential, particle matter, osmolality, water content and pH were selected as evaluation parameters of NP stability.

The results indicated that suspension products were not stable at 5°C for 6 months based on the increase in impurities and the reduction of content. In contrast, lyophilized products were stable even at 40°C/75% RH for 6 months. Lyophilized NPs with trehalose showed better stability. The lyophilized NP formulation could therefore provide a stable and high quality product for clinical studies and shows promise as an effective drug delivery system carrier.

The cardiotoxicity of prospective impurities contained in NPs and reagents used in the

manufacturing process with human induced pluripotent stem cells (hiPSCs) derived three-dimensional (3D)-cardiomyocyte (CM) tissues by centrifugation Layer-by-Layer technique (LbL) was also evaluated. As a result, cardiotoxicity for NPs and reagents was not observed and it was clarified that the potential risk to human safety from NPs is low. The applicability of the approaches with hiPSCs derived 3D-CM tissues by centrifugation LbL is will be proposed for cardiotoxicity evaluation.

3. 2. Introduction

Stability studies are conducted to provide reasonable assurance that formulated products will remain at an acceptable level of quality for administration to patients throughout the period of a clinical study [1]**.** The most important steps during the developmental stages are pharmaceutical analysis and stability studies. These steps are required to determine and ensure the identity, potency and purity of ingredients, as well as those of the formulated products [2,3]. Therefore, establishing adequate stability of formulation is a crucial component of drug development to help in selecting proper formulation. The appropriate storage conditions and study duration should therefore be selected. The stability is affected by various factors such as dosage form (suspension vs. lyophilized powder), dispersion medium (aqueous vs. non-aqueous) and manufacturing technique (top-down vs. bottom-up) [4], and lyophilization is a commonly used stabilization technique for thermolabile pharmaceuticals [5]. Low stability of NPs during storage is a serious limiting factor for their practical application since it can directly affect the efficacy and safety of drug delivery systems. Even though it is known that higher humidity and temperature accelerate the degradation of peptides [6], the ideal conditions for NP storage including humidity, temperature, and physical state (e.g. in solution, lyophilized powder) are poorly understood.

Our group have already reported that biodegradable NPs (γ-PGA-Phe NPs) consisting of

hydrophilic poly(γ-glutamic acid)(γ-PGA), which self-assemble into nanomicelles with a hydrophilic outer shell and a hydrophobic inner core, show great potential as vaccine adjuvants [7]. In addition, in author's study, the developed novel quantitative analytical methods for γ-PGA-Phe as material and γ-PGA-Phe NPs were able to determine the quality of the product during both the drug development and manufacturing process [8,9]. The author succeeded in producing stable γ-PGA-Phe NPs of high quality and with consistent characteristics using our development process and analytical methods as shown in **Chapter 1 and Chapter 2.**

In γ-PGA-Phe NP applications, it is particularly important to establish the shelf life of the product with an appropriate manufacturing process and formulation selection that ensure sufficient stability to maintain a desirable quality and physicochemical characteristics. The purpose of **Chapter 3** was to assess the stability of NP formulations in various temperatures and relative humidity (RH) to determine how best to store NPs to retain efficacy while maximizing convenience, and find the optimal formulation and storage condition. Stability studies should include test parameters that evaluate attributes of the formulation that are susceptible to change during storage and likely to influence quality, safety and efficacy. Considerations for designing adequate stability protocols must include assessment of formulation. Therefore the author proposed test items in the stability study for relevant quality parameters for clinical application in **Chapter 3**.

The actual and potential impurities are most likely to arise during the synthesis, purification, and storage of NPs. Predicting NP toxicity remains one of the greatest limitations for efficient development of safe pharmaceuticals and is the main contributory factor in the high cost of drug development In particular, the cardiotoxicity of drugs is evaluated by an *in vitro* human ether-a-go-go related gene (hERG) assay, *in vivo* animal study, and then clinical studies [10,11]. hERG assays and animal studies have been known to fail to appropriately detect the cardiotoxicity of drugs under conditions of actual patient use, because there are boundaries to the use of single

non-myocyte cells in the hERG assay partially as well as species differences [12-14].

Innovative 3D cell culture technology is giving scientists the ability to grow realistic human tissues for more effective drug testing while reducing the need for animal research. The use of novel NPs and chemicals requires reliable data on their potential toxic effects on humans. Current test protocols are mainly based on *in vitro*–cultured animal-derived cells that either do not or insufficiently mirror the situation in humans. Therefore, the recent availability of *in vitro* models based on human induced pluripotent stem cells (hiPSCs) opens up new opportunities for the development of *in vitro* models of screening for new drugs, and patient-specific cardiac therapy.

Our research group has recently developed a cell-accumulation technique and already reported on the construction of 3D-hiPSC–cardiomyocyte (CM) tissues by coating extracellular matrices (ECM) nanofilms with fibronectin and gelatin (FN-G) onto a cell membrane using a layer-by layer (LbL) technique for cells [15-17]. This cell-accumulation technique was able to fabricate a 3D hiPSC-CM tissue with synchronous and periodic beating and it was found that the introduction of normal human cardiac fibroblasts (NHCFs) into hiPSC-CM tissues plays an important role in modulating organization and synchronous beating depending on the proportion of NHCFs [16]. The fabricated 3D hiPSC-CM tissues were prepared without any damage and with high yield. In addition, the 3D-hiPSC-CM tissues indicated significantly different toxicity responses compared to 2D-hiPSC-CM cells by addition of doxorubicin as a model of a toxic drug [17]. In order to evaluate the cardiotoxicity of impurities in NPs, the *in vitro* toxicity testing was conducted with our hiPSC-CM 3D-tissues.

3. 3. Experimental section

Materials

γ-PGA (sodium salt form, labeled MW: 480 kDa, D-Glu/L-Glu: 70/30), phenylalanine (PA), L-phenylalanine ethyl ester (Phe), dimethyl sulfoxide (DMSO), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), sodium dodecyl sulfate (SDS), sodium citrate, hydrogen phosphate monosodium, disodium hydrogen phosphate, and ethanol (EtOH) for special grade respectively, acetonitrile (ACN), EtOH, and distilled water for HPLC-grade and trehalose were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Phosphate buffer saline (PBS) pH7.4 was purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals and solvents were analytical purity grade and polyethylene glycol/polyethylene oxide Standard ReadyCal Set Mp 200-1'200'000 was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Fibronectin (FN) from human plasma (Mw = 4.6 x 10^5 Da) was purchased from Sigma-Aldrich Co. Gelatin (G) (Mw = 1.0 x 10^5 Da), Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). The 24-well cell culture insert with 0.4 μm, and cell culture plate were purchased from Corning (NY, USA). Normal human cardiac fibroblasts (NHCFs) were purchased from Lonza (Basel, Switzerland). The hiPSCs [3-factor (Oct3/4, Sox2, and Klf4), line: 253G1, established by Shinya Yamanaka] were purchased from RIKEN BioResource Center (Ibaraki, Japan).

Synthesis of γ-PGA-Phe

Amphiphilic γ-PGA was synthesized as described previously [7,18] by the conjugation of Phe as the hydrophobic segment, which has biodegradable components containing carboxyl functional groups at the side chains, and γ-PGA as the hydrophilic backbone. In this experiment, γ-PGA-Phe with 40%‑60% Phe grafting degree was used.

Preparation of γ-PGA-Phe NP suspension

γ-PGA-Phe solution (2 mg/mL in DMSO, or the mixture of EtOH and 50 mmol/L sodium citrate (1:1 v/v)) was added into an equal volume of 0.4 mol/L NaCl solution under stirring (400 rpm) at ambient temperature. The obtained NP suspension was stirred at room temperature for 10 minutes at 400 rpm, and then the obtained NP suspension was washed by centrifugal filtration and was finally added to PBS as a NP suspension (2 mg/mL γ-PGA-Phe in feed). These manufacturing methods with DMSO or with EtOH/buffer as a solvent for γ-PGA-Phe were conducted using optimized parameters to adjust the diameter to around 200 nm. The size-regulated γ-PGA-Phe NPs were prepared according to a previous report [7]. Some of the NPs in PBS suspension by both processes were lyophilized overnight with or without 5% trehalose (w/v), which is a stabilizer for formulation, based on NP solution.

Stability study of NP formulation

NP suspension and lyophilized NP powder by both processes were stored in amber glass vials with rubber stoppers and aluminum overseals to set the storage condition. NP suspension were stored at 5°C (long-term condition) for 0 (Initial), 1, 3 and 6 months, and at 25°C/60% relative humidity (RH) for 1 month (accelerated condition) respectively. Lyophilized NP powder (w/o trehalose) were stored at 5°C for 3 months and 6 months, 25°C/60% RH and 40°C/75% RH (stressed condition) for 1, 2, 3 and months (Table 3-1) according to the recommendations of the International Conference on Harmonization (ICH) [19].

Sample preparation for quantitative analysis

Each lyophilized NP powder was reconstituted with distilled water. Then, the stability of NP suspension and lyophilized NP powder was evaluated by the following parameters. The appearance

Table 3-1. Protocol of stability study

Sample		Storage Condition	Sampling Point (M)	Method
NPs Suspension		5˚C	1, 3, 6	Long term condition
		25˚C/60% RH	1	Accelerated condition
Lyophilized NPs	Trehalose (-)	5˚C	3, 6	Long term condition
		25˚C/60% RH	1, 2, 3, 6	Accelerated condition
		40˚C/75%RH	1, 2, 3, 6	Stressed condition
	Trehalose (+)	5˚C	3, 6	Long term condition
		25˚C/60% RH	1, 2, 3, 6	Accelerated condition
		40˚C/75%RH	1, 2, 3, 6	Stressed condition

M: Month. RH: Relative Humidity.

of NPs powder and the clarity of solution of NPs were observed by visual inspection, and the mean (Z-average) particle size, the width of the size distribution (polydispersity index, PDI) and zeta potential as a measure of conductivity were determined by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Particulate matter by Light Obscuration Particle Count Test with a Liquid Particle Counter (HIAC System 9703, Hach Ultra, Tokyo, Japan), water content by Karl Fischer titration method (AQV-2100C, Hiranuma Sangyo Co., Ltd., Japan), osmolality with a 5007 OSMETTE XL (Precision Systems Inc., MA, USA) and pH with a pH meter (F52 HORIBA, Kyoto, Japan) were measured according to the Pharmacopoeia method.

NP suspension and NP lyophilized powder were dissociated at 2% SDS as a final concentration and diluted with a mobile phase-based buffer. Determinations of MW and MW distribution by Size Exclusion Chromatography (SEC) with a refractive index detector (RI) and Multi-Angle Light Scattering (MALS) detector (SEC-RI/MALS) and content and impurities by reverse-phase chromatograph (RP-HPLC) with UV detector were subsequently undertaken as described previously [9,10]. γ-PGA-Phe content and impurities of NPs were calculated from area percentage peaks of a chromatogram obtained by UV detector. The content at each point (% of initial) for stability studies was calculated as [(γ-PGA-Phe content in NPs at each point)/ (initial γ-PGA-Phe content in NPs)] × 100.

Fabrication of 3D-hiPSC-CM tissues using a centrifugation-LbL method

The construction of hiPSC-CM 3D-tissue was conducted according to our previous reports [6,12, 15,17]. The FN-G-coated hiPSC-CMs and NHCFs were mixed and seeded onto a 24-well culture insert. In this study, tissue with a 50:50 ratio of hiPSC-CMs to NHCFs was fabricated. Thus, a total of 0.5×10^6 cells were seeded onto the 24-well culture insert, which corresponds to about 5 layers. After culturing for 5 days, the fabricated samples were analyzed.

Cardiotoxicity evaluation of NPs and reagents used in the production of NPs

To confirm whether the tissue models can be used as pharmaceutical tissue models, the cardiotoxicity of γ-PGA-Phe NPs and reagents used in the process of NPs was assessed. NPs in PBS suspension were prepared at concentrations of 10, 1, 0.1, 0.01, 0.001 and 0 (non-treatment) mg/mL. Each NP suspension was added to the hiPSC-CM tissue in a 24-well culture insert at final concentrations of 100, 10, 1, 0.1, 0.01 and 0 μg/mL. These tissues were incubated at 37°C for 15 mins. The movies were recorded by a high speed camera (HAS-L1, Digital Image Technology, Tokyo, Japan) and maximum contraction beating velocity, maximum relaxation beating velocity and beats per minute (BPM) were analyzed by particle image velocimetry (free and open-source software) using motion vector prediction (MVP) analysis to perform the quantitative image analysis of beating. Similarly, the cardiotoxicity for DMSO and EtOH was also evaluated at final concentrations of 0.1%.

3. 4. Results and Discussion

Storage stability of NP suspension

The high quality of NPs should be ensured for their use in clinical applications such as in NP-based vaccine adjuvants. For clinical applications, the important characteristics of the polymer in NPs are stability, hydrophobicity, morphology, molecular weight, and degree of swelling in water of the polymer backbone.

Our group has previously reported that protein-encapsulated γ-PGA-Phe NPs prepared by a dimethyl sulfoxide (DMSO)-based process with centrifugation method, and the resulting encapsulation has between 30 and 60% efficiency, and is stable over an acidic pH range even after 10 days [20].

A pharmaceutical product may undergo changes in appearance, consistency, content uniformity,

clarity of solution, water content, particle size and shape, pH and package integrity thereby affecting its stability. Such physical changes may be caused by shearing due to impact, vibration or abrasion and freezing or thawing as a result of temperature fluctuations. The author developed analytical methods were clearly able to distinguish the quality and characterization of NPs [10].

To identify the optimal formulation and most convenient storage conditions for NPs, the stability of NP suspension and lyophilized powder manufactured by both the DMSO-based and Ethanol (EtOH)-based processes was evaluated. Not all of the parameters for exact analysis are currently known. Therefore, to guarantee the consistent quality of NPs, test items of appearance, clarity of solution, particulate matter, mean particle size, zeta potential, pH, osmolality and water content in NP formulation were selected as evaluation parameters. Mean particle size and the PDI influence important properties of biphasic liquid. Nano systems include saturation solubility and dissolution velocity [21,22], and they play important roles in governing the physical stability and biological performance of nanosuspensions. Considerations for designing adequate stability protocols must include assessment of formulation.

The determinations of content, impurity and absolute MW have always been considered to be essential factors in ensuring product quality. As shown in **Chapters 1 and 2**, the author has already reported on a novel analytical technique for determining content and impurity by RP-HPLC and absolute MWs by SEC-RI/MALS after dissociating NPs by sodium dodecyl sulfate (SDS) was accomplished [19,20]. As a result of the stability study of NP suspension stored at 5°C for 0 (Initial), 1, 3 and 6 months, and at 25°C/60% relative humidity (RH) for 1 month respectively (Table 3-2), neither change in appearance nor visible foreign matter were observed at any point in the storage periods. Particulate matter was almost clear and practically free from particles, and pH values, osmolality values and zeta potentials were stable. The results were the same for the EtOH-based process and DMSO-based process at all points. However, particle size was stable even at 5˚C for 6

Table 3-2. Stability study for NP suspension at 5˚C and 25˚C/60% RH by EtOH based and DMSO-based processes

Process		DMSO			EtOH		
Storage Condition		Initial	5˚C	25˚C/ 60% RH	Initial	5˚C	25˚C/ 60% RH
			6M	1M		6M	1M
Clarity of Solution		Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles
pH		7.24	7.24	7.15	7.09	7.05	6.93
Particle Size (nm)		131.5	128.2	68.9	144.6	133.2	62.4
PDI of Particle Size		0.064	0.146	0.250	0.138	0.155	0.282
Osmolality (mOsm/L)		278	282	278	278	283	280
Zeta Potential (mV)		-19.8	-18.5	-28.1	-23.3	-22.6	-23.7
Particle Matter	≧10 μm	23	10	5	1	3	0
	≧25 μm	1	0	0	0	0	0
γ-PGA-Phe Content	mg/mL	0.818	0.722	0.614	0.929	0.867	0.768
	Recovery (%)	100.0	88.3	75.1	100.0	93.3	82.7
Impurities (%)	PA	0.63	0.73	0.91	0.02	N.D.	0.54
	U-1	1.51	6.88	12.3	2.42	6.34	11.5
	Phe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	U-2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Others	N.D.	1.32	1.02	0.89	1.71	1.94
Absolute MWs (kDa)		87	61	63	81	61	63

-: Not detected. M: Month. RH: Relative Humidity

Appearance and appearance of solution of NPs were observed by visual inspection, and particle size, the polydispersity index (PDI) and zeta potential as a conductometry were determined by DLS with Zetasizer Nano ZS. Particulate matter by Light Obscuration Particle Count Test with Liquid particle counter, water content by Karl Fischer titration method, osmolality with 5007 OSMETTE XL and pH with a pH meter were measured according to Pharmacopoeia method. Absolute MW was determined by SEC-RI/MALS analysis and Content of γ-PGA-Phe and impurity levels were determined by RP-HPLC.

PA: Phenylalanine; Phe: Phenylalanine ethyl ester; U-1: Unknown-1, U-2: Unknown-2.

months and it was reduced to half of the initial at 25˚C/60% RH for 1 month in both processes.

For γ-PGA-Phe, the contents at 5˚C for 6 months were reduced from the initial point to 88.3% in the DMSO-based process and 93.3% in the EtOH-based process. For main impurities, peaks for phenylalanine (PA), an unknown peak (U)-1, L-phenylalanine ethyl ester (Phe) and U-2 were detected at about 1.9 min, 5.6 min, 9.8 min, 15.2 min in NPs, respectively (Fig. 3-1). U-1 increased from 1.51% (Initial point) to 6.88 % in the DMSO-based process and from 2.42% (Initial point) to 6.34% in the EtOH-based process at 5˚C for 6 months. The EtOH-based process was slightly more stable than the DMSO-based process. At 25˚C/60% RH, the contents were 75.1% in the DMSO -based process and 82.7% in the EtOH-based process from the initial point, and U-1 as the impurity was increased to 12.3% and 11.5%, respectively. Absolute MWs in both processes showed about 20% reduction at 5˚C even for 1 month from the initial point and this was considered to be the result of hydrolytic degradation of γ-PGA-Phe main chain ester groups. From the above results, the EtOH-based formulation was slightly more stable than the DMSO-based formulation.

Storage stability of NP lyophilized powder

NP stability for lyophilized powder stored at 5°C for 3 and 6 months, 25°C/60% RH and 40°C/75% RH for 1, 2, 3 and 6 months with and without (w/o) trehalose on DMSO and EtOH based-NPs was evaluated to compare different dosage forms.

As a result (Table 3-3), no change in appearance nor visible foreign matter was observed at any point in the storage periods of either process. Particulate matter was almost clear and slightly increased but they were within the specification. pH values and osmolality values were stable at all points. The results were the same for both processes at all points regardless of whether trehalose was used. Even though particle size was slightly larger when trehalose was added at the initial point, they were constant during storage for both processes even at 40˚C/75% RH for 6 months.

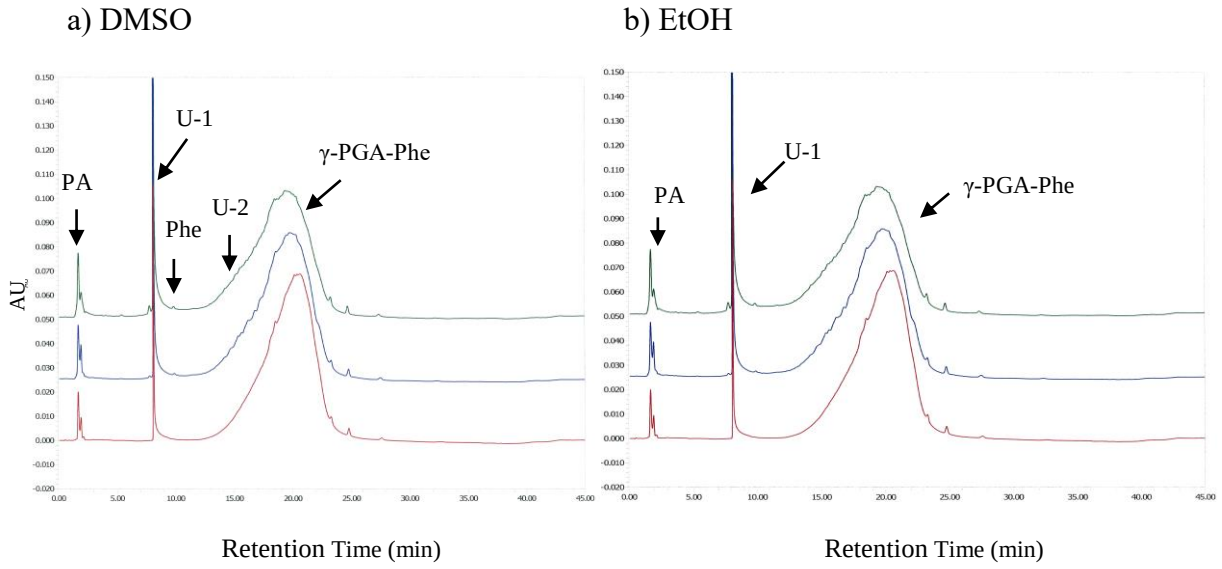


Fig. 3-1. A stability study of NPs in suspension manufactured by filtration method with DMSO and with EtOH as a solvent for γ-PGA-Phe was conducted, and evaluated by RP-HPLC. Each chromatogram shows the results for NPs in suspension stored for Initial (non-treatment), at 5°C for 6 months and 25°C/60% RH 6 months from bottom to top. Chromatogram of dissociated NPs and γ-PGA-Phe lyophilized powder by a) DMSO and b) EtOH. In the chromatogram of the peaks for PA, U-1, Phe, U-2 and γ-PGA-Phe were detected at about 1.9 min, 5.6 min, 9.8 min, 15.2 min and 17.3 min, respectively. PA: phenylalanine; Phe: phenylalanine ethyl ester; U-1: Unknown-1; and U-2: Unknown-2.

Table 3-3. Stability study for lyophilized NP powder w/o trehalose at 40˚C/75% RH by EtOH-based and DMSO-based processes

Process		DMSO				EtOH			
Formulation		PBS				PBS			
(Trehalose：-/+)		−		+		−		+	
Storage Condition		Initial	6M	Initial	6M	Initial	6M	Initial	6M
Appearance		White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder	White to off-white cake or powder
Clarity of Solution		Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles	Free of visible particles
Water Content (%)		0.92	0.48	1.64	2.21	0.52	0.61	1.16	2.09
pH		7.23	7.22	7.14	7.11	7.08	7.05	6.86	6.81
Particle Size (nm)		132.0	130.3	157.9	152.2	143.6	141.6	172.1	168.9
PDI of Particle Size		0.098	0.127	0.095	0.098	0.136	0.135	0.174	0.145
Osmolality (mOsm/L)		273	278	293	294	256	280	300	287
Zeta Potential (mV)		-24.0	-20.0	-28.0	-25.1	-25.3	-26.7	-24.6	-24.9
Particle Matter	≧10 μm	6	9	14	23	5	4	8	14
	≧25 μm	0	0	0	3	1	0	1	3
γ-PGA-Phe Content	mg/mL	0.805	0.826	0.816	0.839	0.921	0.877	0.934	0.969
	Recovery (%)	100.0	102.6	100.0	102.8	100.0	95.2	100.0	103.7
Impurities (%)	PA	0.26	0.10	0.59	0.57	0.14	0.13	N.D.	N.D.
	U-1	1.21	2.71	1.08	1.62	2.15	3.02	2.10	2.34
	Phe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	U-2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Others	0.51	1.48	0.28	0.61	1.17	2.02	1.03	1.30
Absolute MWs (kDa)		128	76	92	80	81	73	—	86

-: Not detected. M: Month. PDI: polydispersity index. The determination methods are shown in footnotes of Table 2. PA: Phenylalanine; Phe: Phenylalanine ethyl ester; U-1: Unknown-1, U-2: Unknown-2.

The γ-PGA-Phe content in EtOH-based NPs was slightly higher than that of DMSO-based NPs due to the difference of production yields, which was 92–93% in EtOH-based NPs and 81–82% in DMSO-based NPs, and they remained constant during storage at 25˚C/60% RH for 6 months and 40˚C/75% RH for 6 months. The γ-PGA-Phe content for the formulations w/o trehalose was almost the same in both manufacturing processes. Impurities were almost the same in both manufacturing processes and formulation w/o trehalose, and they showed little or no increase at 25˚C/60% RH for 6 months (Fig. 3-2). Except for U-1 in lyophilized NPs powder, the impurity slightly increased at 40˚C/75% RH for 6 months and this tendency of stability was similar to the results of 25˚C/60% RH for 6 months. Absolute MWs in DMSO-based NPs at 25˚C/60% RH and 40˚C/75% RH were reduced but those of EtOH-based NPs were nearly unchanged. Based on the above results, the chemical stability of lyophilized NPs was superior to that of NP suspensions, and the stability of EtOH-based NPs was better than that of DMSO-based NPs. In addition, EtOH-based NPs were stable even at 40˚C/75% RH for 6 months. These parameters for quality and characterization also remained unchanged during the storage periods.

The addition of trehalose, which is widely used as a cryoprotectant in the lyophilized formulation, was the preferred approach for enhancing the stability of nanosuspensions during both the lyophilization process and storage to improve the stability of NPs [23,24]. It was evident that lyophilized NPs based on suspension formulation are a successful technique for enhancing stability and thus in increasing the shelf life of NPs. With respect to impurities, NP formulation with trehalose showed better stability and there was no difference in the results between the two processes. It was indicated that lyophilized powder NPs with trehalose which was manufactured by the EtOH-based process is appropriate and it is stable even at 40˚C/75% RH for 6 months. Long-term storage at 5˚C or above may be achieved by the appropriate selection of molar ratio and sugar mixture sufficient to ensure the storage stability of NPs.

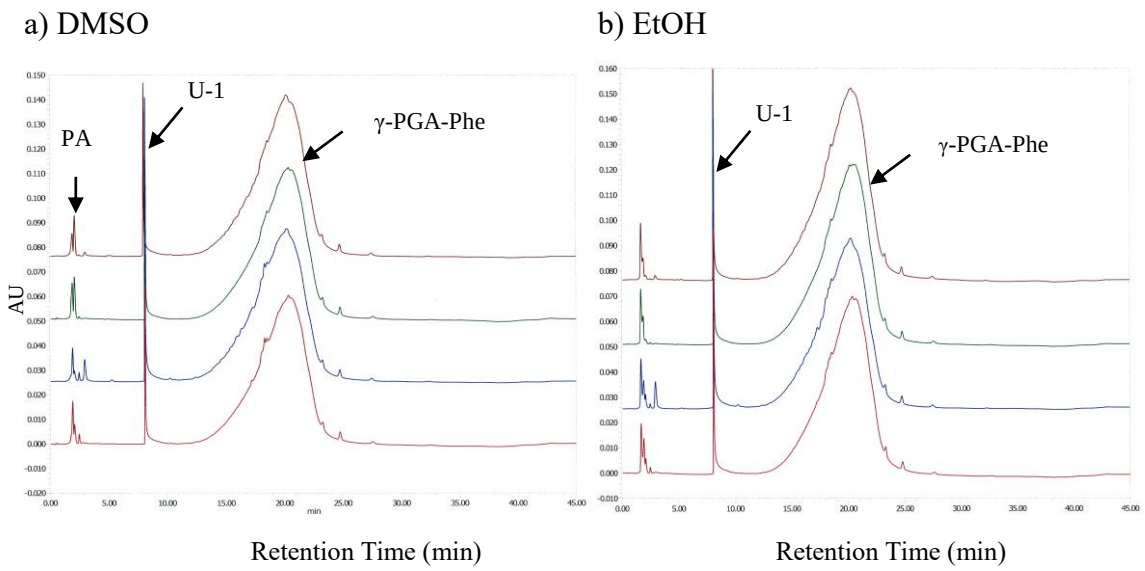


Fig. 3-2. A stability study of NPs in lyophilization powder manufactured by filtration method with DMSO and with EtOH as a solvent for γ-PGA-Phe was conducted, and evaluated by RP-HPLC. Each chromatogram shows the results for NPs lyophilization powder with trehalose stored for Initial (non-treatment), at 40°C/75% RH for 6 months (M), NPs lyophilization powder without trehalose stored for Initial (non-treatment), at 40°C/75% RH for 6 months from top to bottom. Chromatogram of dissociated NPs and γ-PGA-Phe lyophilized powder by a) DMSO and b) EtOH.

It is known that higher humidity and temperature accelerate the degradation of peptides [25]. In γ-PGA-Phe-based NP applications, it is particularly important to establish a stable shelf life for the Product with an appropriate manufacturing process and formulation selection to maintain the desired quality and physicochemical characteristics. Despite the major obstacles limiting the use of NPs being their physical instability and the chemical instability of the polymers that form them, the author succeeded in supplying stable, high quality γ-PGA-Phe NPs with constant characteristics using the developed process and novel analytical methods. Stability testing thus evaluates the effect of environmental factors on the quality of the formulated product which is then used for the prediction of its shelf life and to determine proper storage conditions. Moreover, the data generated during the stability testing is an important requirement for regulatory approval of any drug or formulation.

The construction of 3D-hiPSC-CM tissues using centrifugation-LbL

Fabrication of ECM nanofilms using a centrifugation-LbL method maintained high viability after the coating. After 5 days of incubation, the fabricated 3D-hiPSC-CM tissues with a 50:50 ratio of hiPSC-CMs to NHCFs exhibited synchronous and sustained beating (Fig. 3-3). The formation of an approximately 5 layered-structure was confirmed from the histological image.

Cardiotoxicity evaluation of NPs and reagents used in the manufacturing process of NPs

The actual and potential impurities are most likely to arise during the synthesis, purification, and storage of NPs. EDC, which is an Ames positive compound, was the best reagent for condensation in water, and the residual amount of EDC and its urea as genotoxic impurities in γ-PGA-Phe has already been determined by HPLC. Residual EDC in γ-PGA-Phe was not observed within the detection limit. The DMSO content in NP suspension after the filtration was also less than 0.00001

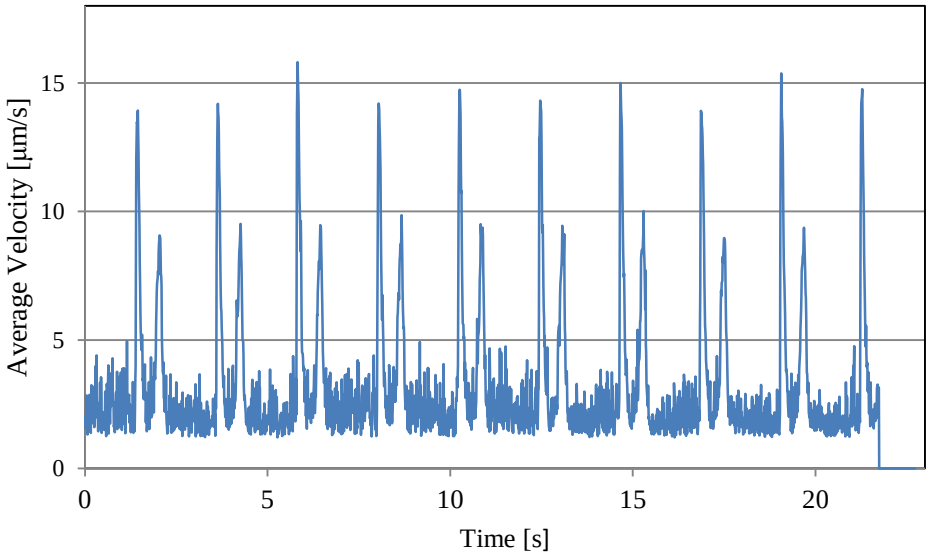


Fig. 3-3. Average velocity of the surface movement on 3D-hiPSC-CM tissues.

vol%. To confirm whether the tissue models could be used as pharmaceutical tissue models, a cardiotoxicity evaluation on hiPSC derived 3D-CM tissues for γ-PGA-Phe NPs and EtOH and DMSO used in the manufacturing process was performed. As the result, as shown in Fig. 3-4, maximum contraction beating velocity, maximum relaxation beating velocity and beats per minute (BPM) at the final concentrations of 100, 10, 1, 0.1, 0.01 μg/mL γ-PGA-Phe NPs were unchanged compared to those of non-treatment. In addition, maximum contraction beating velocity, maximum relaxation beating velocity and BPM at a final concentration of 0.1% of EtOH and DMSO were all unchanged.

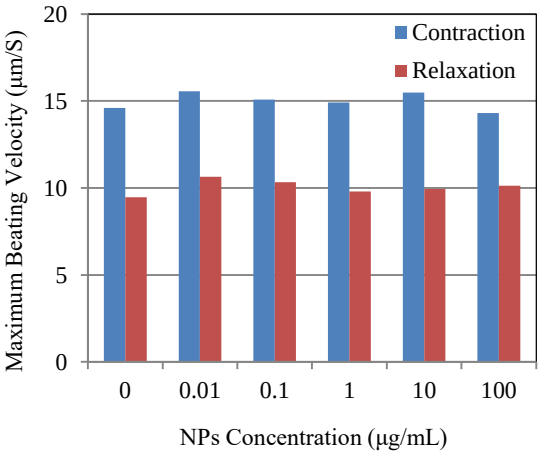
Cardiotoxicity of impurities possibly included in the NP manufacturing process was not observed and it was clarified that the potential risk to human safety of NPs is low.

3. 5. Conclusion

Test parameters and attributes of the formulation or the process that are susceptible to change during storage were selected. Stability studies based on these parameters, which are likely to influence quality, safety and efficacy, were then conducted to assure the quality of formulation. Appearance, clarity of solution, particle size, zeta potential, particle matter, osmolality and pH should be selected as evaluation parameters to guarantee the consistent quality of NPs. Using these parameters, the author was able to clarify that NP formulation with trehalose showed more better stability. The author's approach provides robust techniques for the quality control of NPs product formulations.

In addition, cardiotoxicity of potential impurities contained in γ-PGA-Phe NPs and reagents used in the manufacturing process was not observed. Maximum contraction beating velocity, maximum relaxation beating velocity and beats per minute (BPM) of the hiPSC derived 3D-CM tissues treated NPs and reagents were unchanged compared to those of non-treatment. Toxicity was not observed

a) Maximum Beating Velocity



b) Beat per Minutes

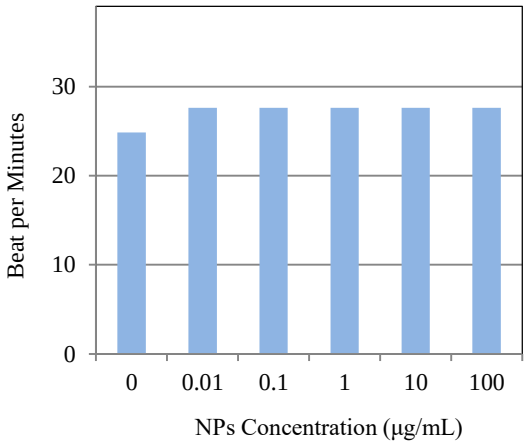


Fig. 3-4. Maximum contraction and relaxation velocity, and beats per minutes of the surface movement on 3D-hiPSC-CM tissues exposed NPs at final concentration 100, 10, 1, 0.1, 0.01 μg/mL and non-treatment. a) Maximum beating velocity and b) Beat per minutes.

and it was clarified that the potential risk to human safety of NPs is low. The availability of this model based on hiPSCs opens up new opportunities for the development of *in vitro* models of screening for new drugs.

3. 6. References

[1] B. Kommanaboyina, C. T. Rhodes, *Drug Development and Industrial Pharmacy* **25**, 857 (1999).

[2] B. Sanjay, S. Dinesh, S. Neha, *Journal of Applied Pharmaceutical Science* **02**, 1329 (2012).

[3] S. Singh, M. Bakshi, *Pharm. Technol. Asia* **24** (2000).

[4] P. R. Vishal, Y. K. Agrawal, *J. Adv. Pharm. Technol. Res.* **2**, 81 (2011).

[5] G. Saeed, V. Hadi, *Adv. Pharm. Bull.* **3**, 25 (2013).

[6] T. Kaneko, M. Higashi, M. Matsusaki, T. Akagi, M. Akashi, *Chem. Mater.* **17**, 2484 (2005).

[7] T. Akagi, M. Baba, M. Akashi, *Polymer* **48,** 6729 (2007).

[8] M. Ikeda, T. Akagi, T. Yasuoka, M. Nagao, M. Akashi. *J. Pharma. Biomed. Anal*. **150**, 460 (2018).

[9] M. Ikeda, T. Akagi, M. Nagao, M. Akashi, *Analytical and Bioanalytical Chemistry* **410**, 4445 (2018).

[10] Z. P. Qureshi, E. S. Vazquez, R. M. Rodriguez, K. B. Stevenson, S. L. Szeinbach, *Pharmacoepidemiol Drug Saf.* **20**, 772 (2011).

[11] J. K. Gwathmey, K. Tsaioun, R. J. Hajjar, *Expert. Opin. Drug Metab. Toxicol.* **5**, 647 (2009).

[12] M. Takeda, S. Miyagawa, S. Fukushima, A. Saito, E. Ito, A. Harada, R. Matsuura, H. Iseoka, N. Sougawa, N. Oda, M. Matsusaki, M. Akashi, Y. Sawa, *Tissue Eng. Part C Methods* **24**, 56 (2018).

[13] W. S. Redfern, L. Carlsson, A. S. Davis, *Cardiovasc. Res.* **58**, 32 (2003).

[14] B. M. Dumotier, M. Deurinck, Y. Yang, M. Traebert, W. Suter, *Pharmacol. Ther.* **119**, 152 (2008).

[15] Y. Tsukamoto, T. Akagi, F. Shima, M. Akashi, *Tissue Eng. Part C Methods* **6**, 357 (2017).

[16] Y. Amano, A. Nishiguchi, M. Matsusaki, H. Iseoka, S. Miyagawa, Y. Sawa, M. Seo, T. Yamaguchi, M. Akashi, *Acta Biomaterial* **33**, 110 (2016).

[17] H. Narita, F. Shima, J. Yokoyama, S. Miyagawa, Y. Tsukamoto, Y. Takamura, A. Hiura, K. Fukumoto, T. Chiba1, S. Watanabe, Y. Sawa, M. Akashi, H. Shimoda, *Scientific Reports* **7**, 13708 DOI:10.1038/s41598-017-14053-0.

[18] M. Matsusaki, K. Hiwatari, M. Higashi, T. Kaneko, M. Akashi, *Chem. Lett.* **33**, 398 (2004).

[19] ICH Topic Q 1 A (R2), *Stability Testing of new Drug Substances and Products, Current Step 4 ICH guidline* **68,** 65717 (2003).

[20] T. Uto, X. Wang, K. Sato, M. Haraguchi, T. Akagi, M. Akashi, M. Baba, *J. Immun.* **178**, 2979 (2007).

[21] R. H. Müller, 4th World Meeting ADRITELF/APV/APGI, Florence, **769** (2002).

[22] D. Leung, T. D. Nelson, T. A. Rhodes, E. Kwong, Nanosuspension process, Patent WO 2013066735 A1, 10 (2013).

[23] J. L. Cleland, X. Lam, B. Kendrick, J. Yang, T. H. Yang, D. Overcashier, D. Brooks, C. Hsu, J. F. Carpenter, *J. Pharm. Sci.* **90,** 310 (2001).

[24] A. Hedoux, L. Paccou, S. Achir, Y. Guinet, *J. Pharmaceutical Sciences* **102**, 2484 (2013).

[25] M. Kaneko, M. Higashi, M. Matsusaki, T. Akagi, M. Akashi, *Chem. Mater.* **17**, 2484 (2005).

Conclusion Remarks

The objective of this dissertation was to establish the NP protocols for evaluating the quality of polymers and NPs, to assure the quality of γ-PGA-Phe as the raw material and the polymeric NPs composed of γ-PGA-Phe. The author attempted to establish an appropriate manufacturing process and formulation selection that ensure sufficient stability, and assessed the cardiotoxicity of potential impurities contained in γ-PGA-Phe NPs and reagents used in the manufacturing process with human induced pluripotent stem cells (hiPSC) derived 3D-cardiomyocyte (CM) tissues by centrifugation Layer-by-Layer technique (LbL) technique.

In Chapter 1, the author described analytical methods for the characterization and quality control of γ-PGA-Phe, which forms NPs in aqueous solution and a deployment platform in practical applications for vaccine adjuvants was established. The established analytical methods using the SEC-RI/MALS system for absolute MW and RP-HPLC for the content of γ-PGA-Phe and the impurity levels were shown to be robust and practical. The SEC-RI/MALS system could evaluate the characteristics of various types of polymers and it was indicated that absolute MW should be used to measure the branch polymer. In addition, the degradation mechanism of γ-PGA-Phe was also identified as cleavage of the main-chain of γ-PGA-Phe based on the stability study of γ-PGA-Phe in buffer solution with various pH values. These characteristics made it a particularly attractive biodegradable polymer for a wide range of potential applications in the biomedical industry. This quantitative approach could become a key element in the identification and characterization of γ-PGA-Phe.

In **Chapter 2**, the author developed analytical methods for evaluating the quality of dissociated and associated amphiphilic γ-PGA-Phe NPs for quality control and characterization. Dissociating the NPs to γ-PGA-Phe using sodium dodecyl sulfate (SDS) and then performing chromatographic separation via RP-HPLC or SEC-RI/MALS is equivalent to those of the component polymer (i.e. γ-PGA-Phe), and

excellent chromatographic separation for the quantitative evaluation of NPs was achieved. These methods proved to be reliable for quantification with a precise, clear and accurate characterization of NPs. During the manufacturing process, impurities were effectively removed from the γ-PGA-Phe, so the purity of the final γ-PGA-Phe NPs was enhanced. These methods are very effective for the optimization of the NP manufacturing process and the selection of a suitable formulation. The author carefully selected the best parameters (appearance, clarity of solution, particle size, zeta potential, particle matter, osmolality, pH, water content, content as γ-PGA-Phe and impurities in NPs) to assure the consistent quality of NPs manufactured for clinical applications. To the best of my knowledge, these methods of quantitatively evaluating NPs and the approach are new developments in the field of drug delivery. This approach provides robust techniques for assuring the quality control of NP product formulations and evaluating the manufacturing process. In addition, these analytical methods employ commonly used reagents and chromatographic systems and are expected to be applicable to other NP-based drug-delivery products.

In **Chapter 3**, the author conducted a stability study of NPs to find the optimal formulation, and define the optimal storage conditions and corresponding shelf-life with novel quantitative analytical and compendia methods. NP stability was evaluated by selecting parameters and attributes of the formulation that are susceptible to change during storage. The results indicated that lyophilized NPs powder manufactured by EtOH-based process were stable even at 40°C/75% RH for 6 months and lyophilized NPs with trehalose showed even better stability.

In addition, cardiotoxicity of potential impurities contained in NPs and reagents used in the NP manufacturing process with hiPSC derived 3D-cardiomyocyte tissues by LbL technique was evaluated. Toxicity of impurities possibly included in the NP process was not observed and it was clarified that the potential risk to humans from NPs is low. The availability of this model based on hiPSCs opens up new opportunities for the development of *in vitro* models for the screening of new drugs.

As numerous types of polymers and NPs are currently in use in virtually all fields of medicine, the

author’s analytical methods are expected to contribute to the improvement of the safety of drug administration in future clinical applications. The availability of these methods is expected to accelerate the clinical development of NPs, which requires reliable analytical technologies to ensure quality and safety. The author is also convinced that these techniques will be useful for evaluating the quality of polymers and NPs, followed by various other functional and responsive polymers and NPs. Product quality is controlled by applying tight quality-control release specifications. It is thought that biodegradable NPs have great potential as carriers for drug delivery systems. In this respect, the lyophilized NP formulation could therefore provide a stable and high quality product for clinical studies and shows promise as an effective drug delivery system carrier.

Acknowledgements

I would like to express my gratitude to many people, for whom without their support and help, this dissertation would not have achieved.

First and foremost, I would like to express sincere gratitude to my supervisor Prof. Mitsuru Akashi of Osaka University, for providing me this precious research opportunity as a Ph.D. student in his laboratory, and the continuous guidance and invaluable encouragement.

I especially would like to express my deepest appreciation to my supervisor, Associate Prof. Takami Akagi of Osaka University for his elaborated guidance, considerable encouragement and invaluable discussion that make my research of great achievement.

I would like to express the sincere gratitude guidance to Dr. Tetsuo Miwa of Takeda Pharmaceutical Company and Prof. Masanori Baba of Kagoshima University for the kind help and advices of inestimable value on this research.

I would like to express appreciation and thank to Dr. Masami Kusaka of Takeda Pharmaceutical Company, Mr. Masao Nagao of Takeda Pharmaceutical Company, and Dr. Tomofumi Uto of University of Miyazaki for continuous guidance and valuable discussions.

I express gratefully acknowledgement to Dr. Fumiaki Shima of Osaka University, Mr. Jun Hirose, Mr. Hikaru Taira, Mr. Tatsuya Yasuoka and Mrs. Yoko Hara of Takeda Pharmaceutical Company for the insightful comments and suggestions.

I also express gratefully acknowledgement to Mr. Yoshinari Tsukamoto, Mrs. Kana Oji and Mrs. Kazuko Fukuda of Osaka University for the helpful advices and assistances.

During this research, I also thanks to Mr. Steven Glenn of Takeda Pharmaceutical Company, and all the members of the Akashi laboratory for the kind help in supporting research.

Finally, I would like to thank my family and friends who always support and encourage me and my colleague for their greatest love, patience, and getting along routine together.

List of Publications

Chapter 1:

M. Ikeda, T. Akagi, T. Yasuoka, M. Nagao, M. Akashi. Characterization and analytical development for amphiphilic poly(γ-glutamic acid) as raw material of nanoparticle adjuvants. *J. Pharma. Biomed. Anal.* **150**, 460 (2018).

Chapter 2:

M. Ikeda, T. Akagi, M. Nagao, M. Akashi, Development of analytical methods for evaluating the quality of dissociated and associated amphiphilic poly(γ-glutamic acid) nanoparticles, *Analytical and Bioanalytical Chemistry* **410**, 4445 (2018).

Chapter 3:

M. Ikeda, T. Akagi, M. Nagao, M. Akashi, Formulation stability of amphiphilic poly(γ-glutamic acid) nanoparticle and evaluation of cardiotoxicity of NPs with human iPSC derived 3D-cardiomyocyte tissues. *Under submission to J. Pharma. Biomed. Anal.*

Presentation at Conferences and Press Release

International Conference:

Mayumi Ikeda, Tatsuya Yasuoka, Masao Nagao, Takami Akagi, Mitsuru Akashi, "Analytical Development for γ-PGA, γ-PGA-PAE and Nanoparticles", 28th International Symposium on Polymer Analysis and Characterization, Houston, Texas, June 8-10, 2015.

Domestic Conference:

Mayumi Ikeda, Tatsuya Yasuoka, Yoko Hara, Masao Nagao, Takami Akagi, Mitsuru Akashi, "Analytical development of γ-PGA, γ-PGA-PAE and NPs", 24th Polymer Material Forum, Tokyo, Nov. 26-27, 2015.

(Poster Presentation Award was given from Society of Polymer Science, Japan.)

Press Release:

Mayumi Ikeda, Tatsuya Yasuoka, Yoko Hara, Masao Nagao, Takami Akagi, Mitsuru Akashi, "Analytical development of γ-PGA, γ-PGA-PAE and NPs", Press Release for 24th Polymer Material Forum, Tokyo, Nov. 16, 2015.

(This was chosen as the subject of press release for the public relations by Society of Polymer Science, Japan.)

End of file