

Design of Nano-Sized Carrier for Delivery of Natural Compounds with Pharmacological Activity

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Design of Nano-Sized Carrier for Delivery of Natural Compounds with Pharmacological Activity

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CHAPTER 1

GENERAL INTRODUCTION

Since time immemorial, mankind has been using flora and fauna as a medicine for the treatment of various diseases. The first record, dating from 2600 BCE, shows that as many as 1000 types of substances derived from plants in Mesopotamia have been used for medicine, including the oils of *Cedrus species* (cedar) and *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora species* (myrrh), and *Papaver somniferum* (poppy juice). All of which are still in use today for the treatment of starting diseases from coughs and colds to parasitic infections and inflammation [1]. In 1500 BC, the record of Egyptian medicine contained in the document "Ebers Papyrus", which recorded more than 700 types of drugs generally came from plants. Moreover, more than 1300 kinds of Chinese traditional medicine [2] and more than 800 kinds of medicine from Indian Ayurvedic system have been documented [3].

Since the fundamental pharmacology has emerged in the last 18th century, the natural compound becomes a candidate for drug development. More than 60% of drugs in the market are derived from a natural source. The number of publications on natural compounds has continued to increase during past two decades (Fig. 1), reflecting the global increase of natural compounds research to produce new bioactive compounds in large quantities from year to year. This is driven by the urgent need for new drugs, in particular, to fight infections caused by multi-resistant pathogens. The emerging and re-emerging of infection diseases was occurred globally, by which they remain among the leading causes of death and disability worldwide. Therefore, natural

compounds, such as biological compounds (i.e. proteins) and natural products, serve great potential as a source in the development of new medicines.

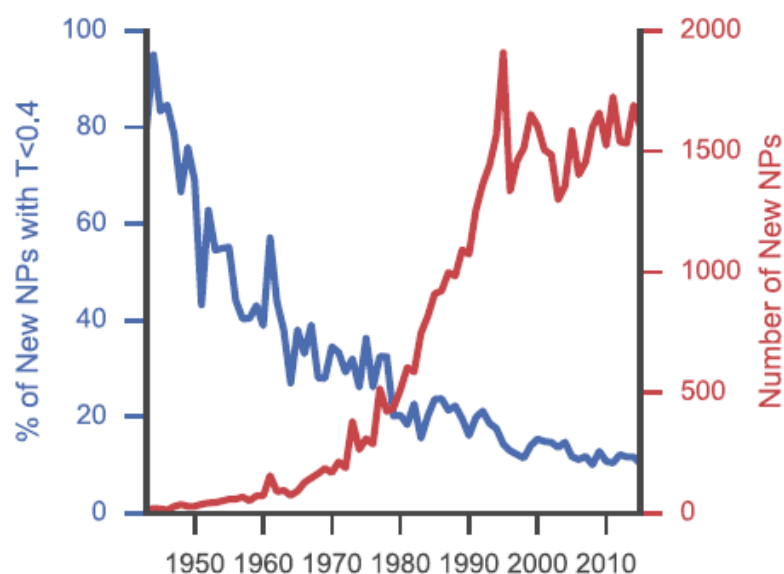


Fig. 1. Number of compounds published per year and rate of novel compound isolation as a percentage of total natural product isolation. Reuse from Proc. Natl. Acad. Sci. USA. 2017. Volume 114, No. 22, 5601-5606.

Natural compounds have many advantages over pure synthetic drugs. For example, proteins are known to have complex functions with high specific actions in the body, have a low potential to disrupt normal biological processes, and have good tolerance and low immunogenicity. Meanwhile, natural products, can be obtained from plants, animals, and microorganisms, have a huge diversity of chemical structures, of which the structural features of many natural products have been tailored through evolution for binding to biological macromolecules. When compared with synthetic drugs, natural products exhibit better structural and physicochemical features, where natural products have larger molecular size, greater three-dimensional complexity, lower hydrophobicity and increased polarity, fewer aromatic rings, more oxygen-rich, and more hydrogen bond donors. However, there are still many challenges in sending these natural components to the location of the disease. To overcome the obstacle in

the delivery of natural compounds, the use of nano-carrier delivery systems can be a solution in delivering natural compounds so that the efficacy of therapeutic effects of these components is achieved.

1.1 Challenges in the delivery of natural compounds

1.1.1 Biopharmaceutics

Protein drugs have attracted significant attention as a therapeutic agent because it has a series of very specific function and complexity, low potential to disrupt normal biological processes, and often well tolerated and lacking immunogenicity [4]. However, a number of challenges are encountered in the delivery of protein drugs. The main challenge in the delivery of protein therapy are related to their characteristics, such as size, polarity, and stability [4][5][6]. In addition, the protein drug administration route is crucial to the delivery of protein drugs to the target site. The challenge of protein delivery based on the administration routes summarized in Fig. 2 [7].

Generally, protein drugs are water-soluble biomolecules with high molecular weight. The large molecular weight of protein drugs limits its absorption in the intestinal and diffusion through the cell membrane. To reach the cellular membrane, proteins have to penetrate the mucus and glycocalyx, which are found at the surface of epithelial cells. Mucus is a complex hydrogel which contains proteins, carbohydrates, lipids, salts, antibodies, bacteria, and cellular debris, and glycocalyx is an acidic fibrous coating consisting of sulfated mucopolysaccharides. A difficult task for moieties with molecular weight larger than 5 kDa to penetration across to this surface barrier of the cells [8]. Protein also cannot easily pass between cells (paracellular pathway) due to the small paracellular space ranges (between 10 and 30-50 Å) [9] and tight junctions between adjacent cells [8]. The paracellular pathway restricted for small molecules, less than 100–200 Da [9]. In addition, the polarity of protein drug, which hydrophilic

nature with very low log P (<0) value [10], also limits its penetration across to the hydrophobic barrier function of the body, such as the stratum corneum layer of the skin [11] and the epithelium cell membrane in the intestinal [9].

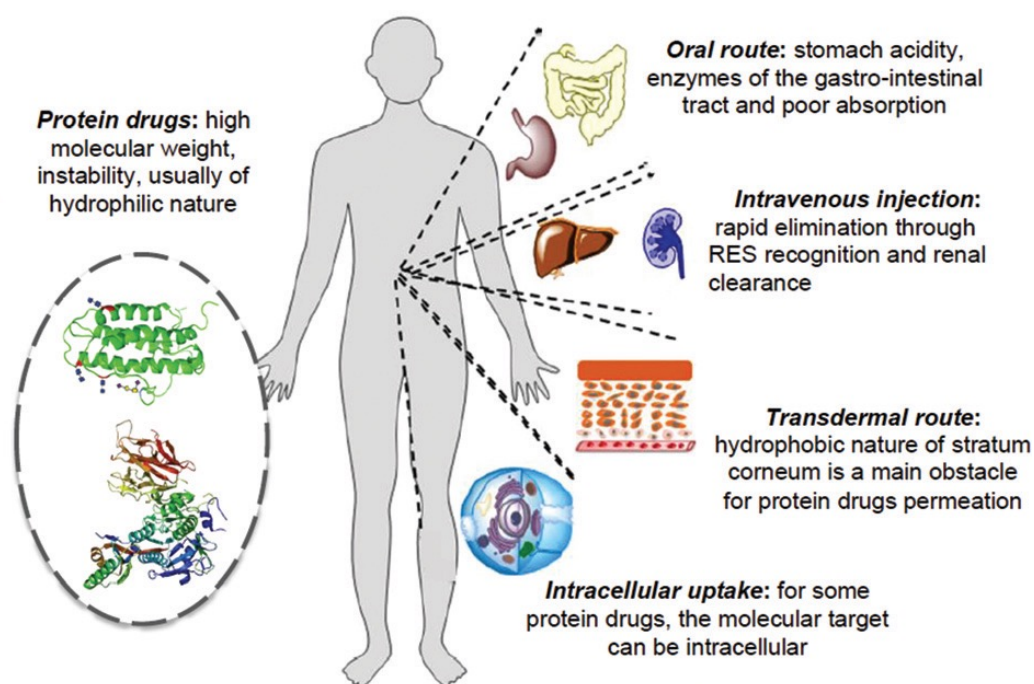


Fig. 2 Main human body challenges for protein drug delivery. RES: Reticulo Endothelial System. [7] Reproduced from *Biomaterial Science*.2016. 4, 2, 205-218 with permission of the Royal Society of Chemistry.

Protein drugs exhibit complex secondary and tertiary structures that must be maintained [10][12]. Conformation change structure protein causing loss of biological activity [13][6]. Denaturation of proteins can occur due to chemical or physical instability. When environmental conditions change, such as pH, ionic composition, or temperature [6], the native structure of the protein can be altered significantly. The protein becomes denatured under very acidic or alkaline conditions [14] or in the presence of some surfactant types [15]. When traversing the gastrointestinal tract, proteins experience a wide pH range (1–7), stomach pH 1–3, duodenal pH 6.0–6.5, and colonic pH 5.5–7.0, which affects their overall stability. At low pH, electrostatic

interactions result in an increased positive charge on the protein, whereas higher pH in a basic environment leads to the repulsion of negative charges. These repulsive forces create structural incongruity which damages the functional portion of the therapeutic entity [8]. Therefore, designing appropriate delivery strategies need to be counterbalanced these unfavourable intrinsic characteristics of proteins. Improper design or formulation of protein drugs can cause degradation, denaturation, and/or aggregation of the protein molecules, potentially causing both immunogenic side effects after administration and loss of pharmacological activity [10].

1.1.2 Natural products

Natural products are small molecules that can be isolated from the plants and other organisms, such as fungi, bacteria and marine organisms [16][17]. Historically, natural products have been used in treating human diseases for many years, due to their pharmacological or biological activities, such as antibacterial, anti-cancer, and antioxidant activity [16]. However, there still exist many challenges for application of natural products in the clinical practices, including solubility, bioavailability *in vivo*, hydrophilicity, physical and chemical instability. In addition, poor pharmacokinetics, first-pass metabolism, accumulation in tissues or low targeting efficacy can hamper therapeutic benefits of natural products.

Many of the studied natural compounds, such as curcumin, resveratrol, and genistein, are highly lipophilic. Highly lipophilic compounds are not ideal for drug delivery because they do not dissolve well in the bloodstream. These compounds have low bioavailability, for example, the administered of curcumin orally with doses of 3.6 g/day was obtained to the serum levels of 11.1 nmol/L. Patients who received lower doses of curcumin did not have detectable plasma levels [18][19], this result is similar for other common natural products, such as polyphenols and flavonoids [20][21].

Therefore, large quantities of the compounds must be administered in order to achieve the desired therapeutic effects. The large dose size of these compounds can lead to acute toxicity or low patient compliance [16]. In contrast, other classes of natural compounds, such as tannins and terpenoids, are highly hydrophilic. These compounds have low bioavailability because they cannot cross biological membranes [22].

On the other hand, many of the natural products have a poor chemical stability in the physiological condition. For example, Camptothecin (CPT) is an alkaloid compound isolated from the tree *Camptotheca acuminata*, Nyssaceae that is potential for anticancer drugs. CPT exists in two forms depending on pH value, namely an active lactone form at pH below 5 and an inactive carboxylate format basic pH (Fig. 3). The closed lactone E-ring of CPT is essential for antitumor activity while the carboxylate form is inactive to the tumour. The lactone ring of CPT is labile, which it hydrolyzed easily to form an inactive open carboxylate form at the physiological condition [23][24]. Another example of a natural product with chemical instability is curcumin. Curcumin is a polyphenol compound isolated from the rhizome of *Curcuma longa*, has low solubility in the physiological solution of 11 ng/mL [25] and unstable in the neutral and alkali condition [25][26]. When curcumin was incubated in 0.1 M phosphate buffer and serum-free medium, pH 7.2 at 37°C, about 90% decomposed within 30 min. The degradation products from curcumin are trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, vanillin, ferulic acid, feruloyl methane [26], as shown in Fig 4.

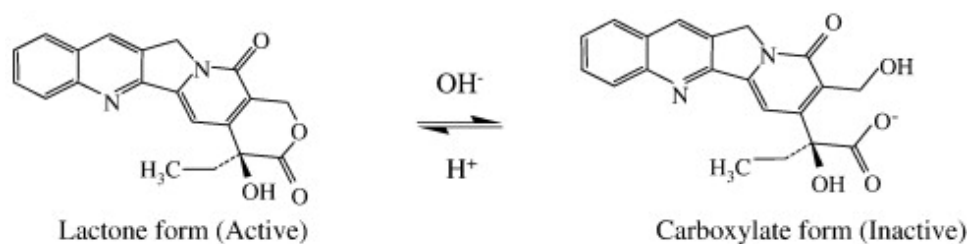


Fig. 3 Chemical structure of camptothecin (CPT) forms depending on the pH value.

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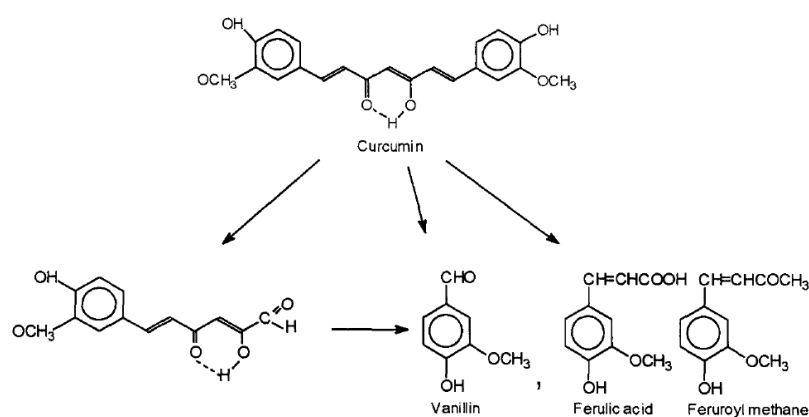


Fig. 4 Chemical products obtained from the degradation of curcumin in 0.1 M phosphate buffer, pH 7.2 at 37 °C. [26]-Reprint from *Journal of Pharmaceutical and Biopharmaceutical Analysis*, Vol 15, Issue 12, 1867-1876. Copyright 1997 with permission from Elsevier.

1.2 Drugs delivery system/ Nanocarriers

Nanocarriers as drug delivery systems are the key to overcoming the challenges associated with therapeutic agents, including therapeutic proteins and natural products [7]. Nanocarriers are delivery equipment or vehicles to facilitate the delivery of small compounds as well as large molecules such as proteins, which they carry therapeutic agents and release them at a specific rate at a particular location [12]. In addition, the nanocarrier should serve as a shield to protect the therapeutic agent from premature degradation and denaturing interactions with the biological

environment [5][27]. Various nanocarriers have been used in drug delivery research to improve targeted drug delivery, bioavailability, solubility, drug retention time, in addition to minimizing side effects, including toxicity risks. Moreover, nanocarriers also reduce the total concentration required to obtain therapeutic benefits [5].

Nanocarriers present at least one dimension in the nanoscale range (below 1 μm). As an ideal delivery system, they must be non-toxic, protecting their therapeutic payload and demonstrating biocompatibility, biodegradability, physicochemical stability, kinetics controlled release and pharmacokinetic enhancement [10][16][27]. Different types of nanocarriers have been effectively used (Fig 6) [27]. These include lipid-based nanocarriers such as liposome and solid lipid nanoparticle; surfactant-based nanocarrier, such as nanoemulsion; polymeric nanocarrier such as nanogel, polymer micelles, and layer-by-layer; inorganic nanocarriers such as carbon nanotube, gold nanoparticles; Protein-mediated nanocarriers such as engineering protein G, virus-like protein [27]. The loading of target therapeutic agents such as protein into various nanocarriers can use different methods, including direct conjugation via either chemical or genetic modifications, physical adsorption and covalent/noncovalent encapsulation [5][27] (Fig. 5).

Therapeutic agents such as protein and natural products are administered into the human body through different routes including parenteral, oral, pulmonary, topical or transdermal delivery [5][10]. The selection of nanocarriers for delivery of therapeutic agents should be tailored to the administrative route, where they must overcome obstacles on the route to allow the nanocarrier to accumulate and be eliminated at the target site. In addition, an appropriate administrative route should also be selected according to the location of the disease within the different target tissues in order for the efficacy of drug therapy to be achieved [5].

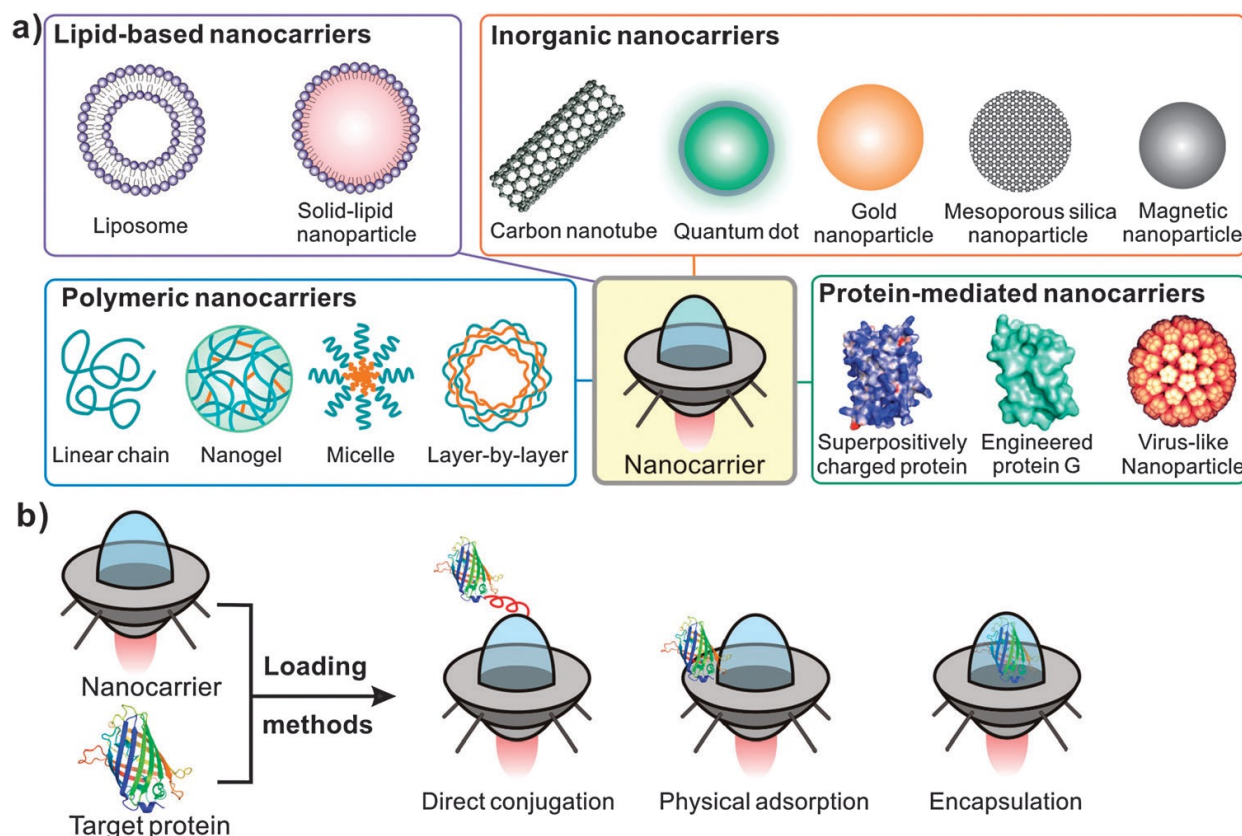


Fig. 5 (a) Schematic of various types of nanocarriers for therapeutic agent (b) Schematic of three main loading methods for preparing protein/nanocarrier composites. Different nanocarriers are represented as a “space shuttle”. [27]- Reproduced from Chem. Soc. Rev., 2011, 40, 3638–3655 with permission of the Royal Society of Chemistry.

1.3 Nano-carriers for delivery of biopharmaceutics

The introduction biopharmaceutics, such as therapeutic proteins, to the surface of the nanocarriers, can be used to target them to specific tissues. Given the characteristics of proteins previously described, the strategy in designing nanocarriers for proteins is determined by the route of administration.

a) Parenteral route

Protein therapeutic generally is delivered into the body through parenteral route, including the intravenous, intramuscular and subcutaneous, since they have large size molecule and instability in nature. Administred the protein using direct injection,

bypass the skin and mucus barrier, is faster to the delivery of therapeutic agent into the targeted site. However, the systemic bioavailability of protein therapeutic is often low and variable when administered subcutaneously or intramuscularly [28][27]. Therefore, it is essential to use nanocarriers for delivery of the protein therapeutic to the target site. For parenteral administration purpose, the protein can be loaded to nanocarrier using three different loading method as shown in Fig 5 including direct conjugation, physical absorption and interaction, and encapsulation.

The direct conjugated method is the attachment of protein therapeutics into the surface of nanocarriers, such as polymer-based and inorganic-based using covalent interaction [29]. The covalent attachment provides a stable linkage between carrier and protein, an important issue *for in vivo* applications. Covalent attachment of PEG polymers, known as PEGylation, is a classic example to improve protein stability and other pharmacokinetic properties. To date, various antibodies, cytokines and growth factors have been PEGylated, used for shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes [29]. Attachment of polymers to protein surfaces can be accomplished using well-established bioconjugation methods in the literature [27], by manipulating the reaction between side chains on proteins and polymers [5]. Reactive side chains on protein surfaces, such as amines, thiols, carboxylic acids and hydroxyl groups, can all serve as the tethering site, depending on the coupling chemistry.

Futami et al. [30] had widely explored intracellular protein transduction by chemically conjugating polyethyleneimine (PEI) to the surface of various proteins to create cationized macromolecules. The authors were able to deliver a diverse array of proteins including ribonuclease (RNase), enhanced green fluorescent protein (EGFP)

and IgG to the cytosol of murine and human fibroblast cells without observing significant differences in protein function. Another example of the direct attachment of protein drugs in nanocarrier is the covalent conjugation of a small mitochondrial heme protein, cytochrome C (Cyt C) to mesoporous silica nanoparticles (MSN), for induction of cancer cell death (Fig. 6).

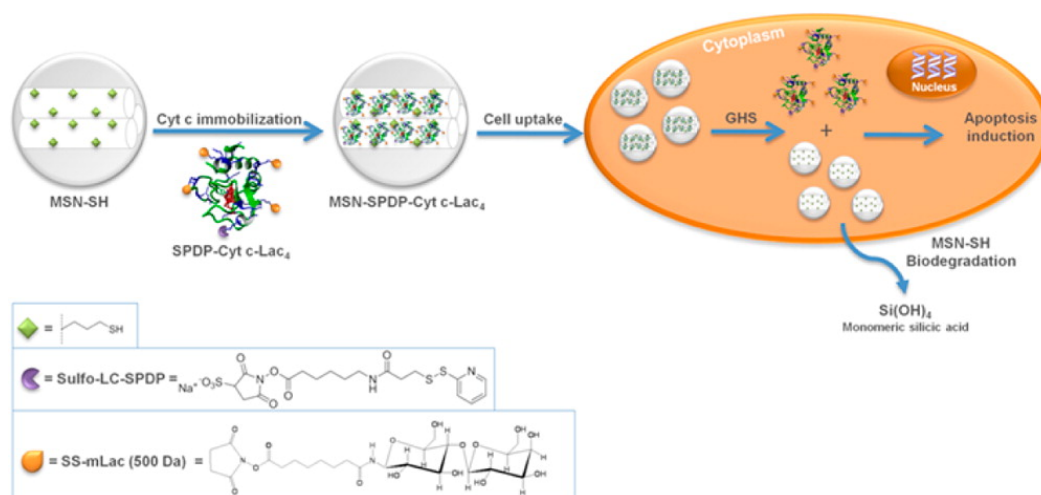


Fig. 6 Scheme of the immobilization of Cytc-Lac into MSN-SH via redox-sensitive smart bonds followed by its intracellular delivery into cancer cells. Reprinted with permission from *Molecular Pharmaceutics*, 11 (1), 102-111. Copyright 2014 American Chemical Society.

Physical absorption and interaction method is an alternative loading method without covalent modification, which is the self-assembly between proteins and the nanocarriers. The spontaneous assembly is typically facilitated by physical adsorption, which is driven by electrostatic forces or van der Waals interactions [27]. The main advantage of this method lies in the simplicity of preparation and applicability to any type of macromolecule without damage to the protein [5]. In addition, organic solutions and surfactants can be avoided, and absorbance is spontaneous in the process of physical adsorption[31]. Akiyoshi and coworkers [32] have utilized self-assembled cationic nanogels for encapsulating protein molecules using the functionalized cholesteryl group-bearing pullulan polymer (CHP) with an ethylenediamine group to

form CHPNH_2 for enhancing the cationic properties of the nanogel (Fig. 7). This, in turn, resulted in strong interaction with anionic FITC-BSA and led to the formation of narrow nanoparticles by self-assembly in water. The CHPNH_2 nanogels have more efficient intracellular uptake into HeLa cells compared to cationic liposomes and protein transduction domain-based carriers.

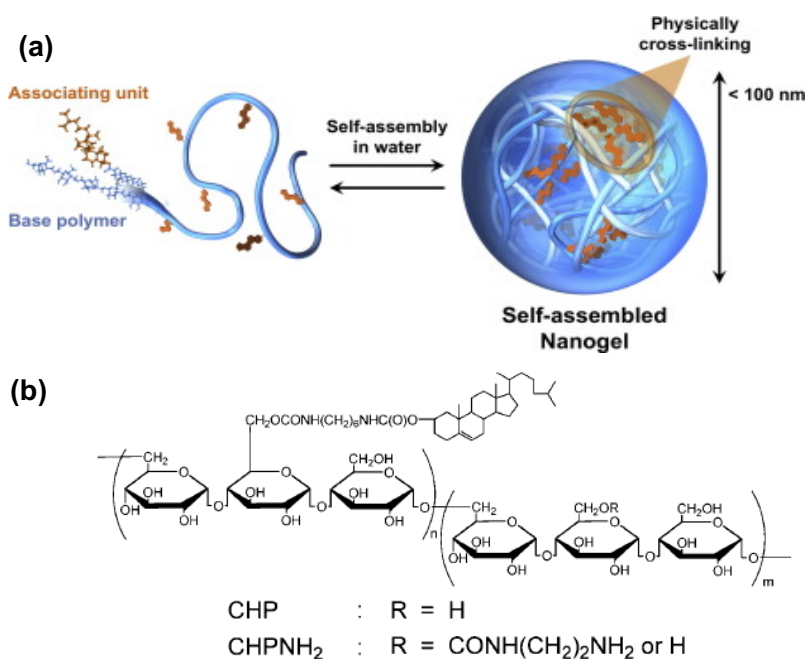


Fig. 7 (a) CHP-based nanogels: the representative self-nanogels.; and (b) chemical structure of cholesterol-bearing pullulan (CHP) and amino group-modified CHP (CHPNH_2) nanogel. (a) Reprint from *Advanced Drug Delivery Reviews*, Vol 95, 65-76. Copyright 2015 with permission from Elsevier. (b) Reprinted with permission from *Bioconjugated Chemistry*, 19, 882-890. Copyright 2008 American Chemical Society.

Encapsulation is another common method for loading the protein into nanocarriers, such as polymer-based nanocarrier, lipid-based nanocarriers, surfactant-based nanocarrier and protein-mediated nanocarriers. The strategies for the encapsulation of protein therapeutic commonly employed emulsification, polymer micelles, layer-by-layer, or the melting and injection technique. The emulsion technology is a common technique for encapsulating protein, in which ultra-sonicator

or homogenizer were used to prepare the nano-sized vehicles. Tahara et al utilized the double coating carrier for intracellular protein delivery. The preparation of this carrier was based on the emulsion technology. The protein or gene was coated with two different surfactants, produced by soli-in-oil (S/O) and solid-in-water (S/W) nanodispersion technology, as shown in Fig. 8. The effective gene expression and cellular uptakes of both anionic and cationic proteins were achieved by modification of carriers with functional molecules.

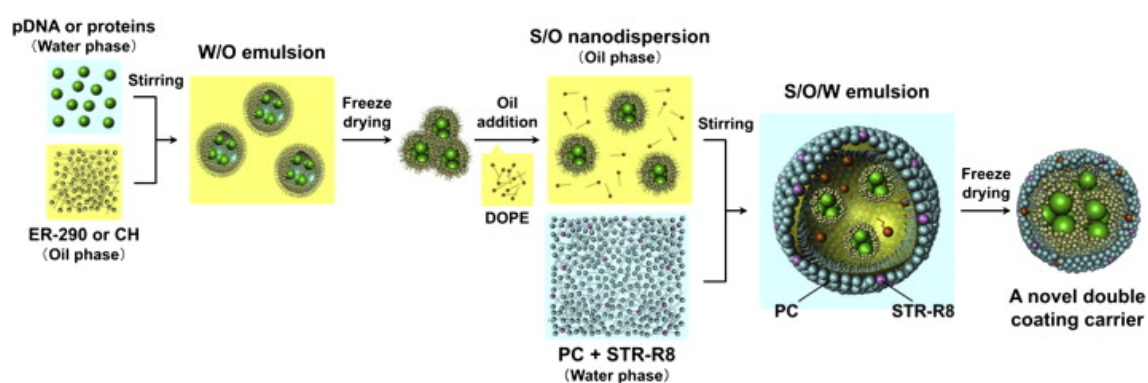


Fig. 8 Schematic showing the preparation method of a novel double coating carrier.
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b) Oral route

The oral route is the most conventional and safest route for drug delivery. The nanocarriers must overcome the obstacle in the stomach and gastrointestinal route, which they should protect the protein from premature denaturation and have a prolonged interaction with epithelial layers in the gastrointestinal track (GIT). For effective oral delivery, protein therapy is generally packed into nanocarriers for protecting against denaturation due to biological environments. Wang et al. [33] reported the oral insulin delivery using chitosan-based nanogels with both positive and negative charges. The charged chitosan-based nanogels were obtained by changing

the ratio of negatively charged carboxymethyl chitosan (CMCS) to positively charged chitosan in the nanogels using an ionic gelation method. Insulin was loaded through premixing with CMCS aqueous solution, then the formation of nanogel was occurred by the spontaneous interaction between CS, CMCS and insulin generated a cross-linked structure. In the oral efficacy test, charged CMCS/chitosan nanogel-loaded insulin exhibited a better effect on the reduction of blood glucose levels, especially for the negatively charged CMCS/chitosan nanogels. The loading of insulin into negatively charged CMCS/chitosan nanogels exhibited enhanced adhesion and permeation into the jejunum and ileum, which are important segments in the intestine and whose large surface areas enable efficient nutrient absorption. These results demonstrated that the surface charge property of nanogels determined the absorption sites of CMCS/CS-NGs in the small intestine.

c) Transdermal route

For the effective transdermal delivery of protein therapy, it is crucial to breaking through the main barrier stratum corneum layer in the skin, which is formed by corneocytes (bricks) and lipid (mortar). The lipid layer is packed with intracellular space and multiple intercellular lamellae. Nanocarriers must enhance the penetration of protein into the deep skin layer. Lipid-based nanocarrier and surfactant-nanocarrier have been used for transdermal protein delivery such as liposome, ethosome, santosome, solid lipid nanoparticles and solid-in-oil (S/O) nanodispersion. Castangia et al [34] reported the skin protein delivery using santosome-based nanocarrier. Santosomes were formulated using hydrogenated phosphatidylcholine, cholesterol and *Santolina insularis* essential oil. The essential oil was expected to modify the bilayer structure and improve the delivery and efficacy of the protein due to a synergistic effect of the phospholipid and *S. insularis* terpenes. Phycocyanin was

loaded into this lipid-based nanocarrier using sonication method, which it strongly interacted with the polar heads of the vesicle bilayer. The results demonstrated that the phycocyanin-loaded santosomes were more active than the free phycocyanin reducing skin oedema, probably because the santosomes might be promoted the internalization of phycocyanin in endothelial cells and ensured cell protection against oxidative stress, avoiding vascular congestion and oedema formation.

Goto and coworkers have developed the oil-based formulation using surfactant-based nanocarrier for transdermal protein delivery, namely solid-in-oil (S/O) nanodispersions. S/O nanodispersion is an oil-based formulation used for encapsulating the protein therapy with hydrophobic surfactant using emulsion technology and lyophilization to form the solid protein-surfactant complexes (Fig. 9). These nanocarriers have effectively enhanced the permeation of proteins into the skin, such as FITC-labeled insulin (MW ca. 6 kDa), enhanced green fluorescent protein (EGFP, MW ca. 27 kDa) and horseradish peroxidase (HRP, MW ca. 40 kDa).

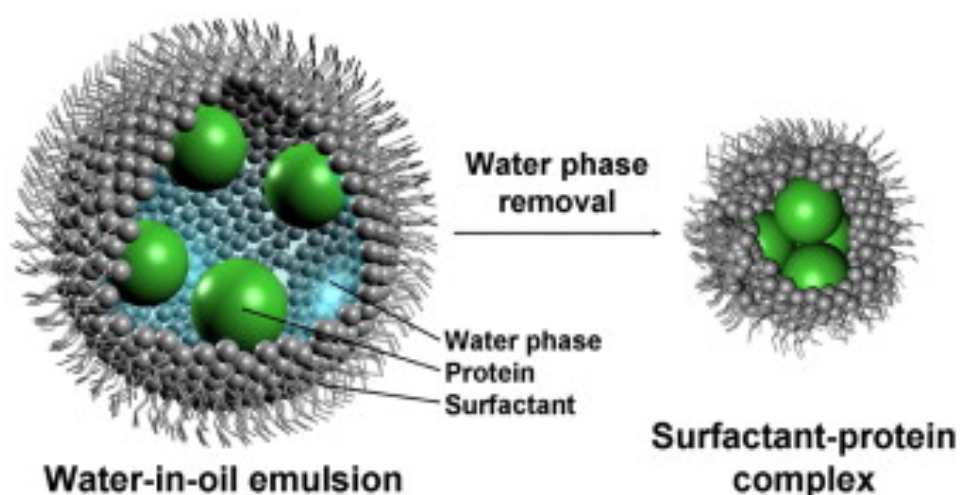


Fig. 9 Schematic illustration of the preparation of the surfactant–protein complex from a water-in-oil emulsion as a solid particle in an S/O nanodispersion. Reprint from *Journal of Controlled Release*, Vol 131, Issue 1, 14-18. Copyright 2008 with permission from Elsevier.

1.4 Nanocarriers for delivery natural products

Natural products are small molecular compounds derived from the plant and or others organism, which has several pharmacological activities. Generally, natural products have a hydrophobic nature, it causes their poor soluble in water and low bioavailability. For overcoming this problem, the natural products were loaded to the nanocarrier for improve its therapeutic effects and deliver it into the location of the diseases through different administration route.

a) Parenteral route

The administration of natural product into the body though parenteral route is an innovation strategy to enhance benefit of its therapeutics, since traditional natural products were administered though oral route. The natural product can load into the nanocarrier using different methods, such as physical absorption or interaction and encapsulation. Yin et al [35] developed an easily self-assembled and gelated octa-peptide FHFDFHFD for improve the anticancer effect of tanshinone IIA and total tanshinone extract (Fig 10). They are natural products isolated from *Salvia miltiorrhiza* Bunge, also known as Danshen in Chinese which is one of the most famous traditional Chinese herbs with more than 1000 years of clinical application. An octa-peptide hydrogel is formed simply by dissolving the gelator in water at room temperature (25 °C), in which tanshinone IIA was encapsulated into the triggered peptide-based hydrogel via non-covalent interaction. This nanocarrier showed increased loading capacity, sustained drug release and better anticancer capability. Zhang et al [36] reported the encapsulation of tanshinone IIA (TAN) using polymer-based nanocarriers. The authors was developed a mixed micelle system constituted with D- α -tocopheryl polyethylene glycol succinate-graft-poly(D,L-lactide-co-glycolide) (TPGS-g-PLGA) copolymer and Pluronic F68. TAN was encapsulated in the TPGS-g-PLGA/Pluronic

F68 mixed micelles by using the thin film hydration technology. The significant enhancement on pro-apoptosis by TAN micelles was evidenced by increased chromosome condensation, mitochondria membrane potential loss, cell apoptosis, and cleavages of caspase-3 and PARP. Furthermore, pharmacokinetic studies revealed that TAN mixed micelles significantly prolonged the circulation time and improved bioavailability of TAN in rats.

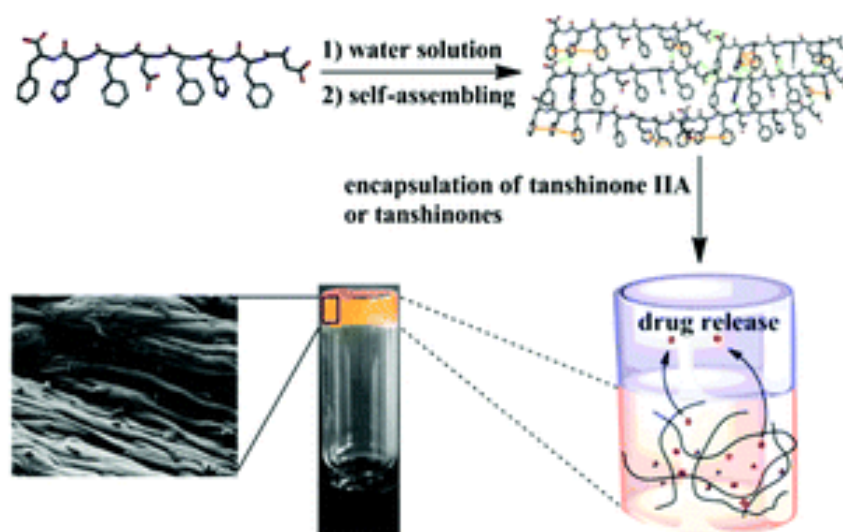


Fig. 10 Schematic illustration of the natural product-loaded peptide hydrogel. [35]- Reproduced from Chem. Commun., 2017, 53, 529-532 with permission of the Royal Society of Chemistry.

b) Oral route

Natural products, which are derived from plants, have been used as traditional medicine for many years. Oral delivery is the common route for administrated the natural products. However, the oral bioavailability of natural product is very low due to their hydrophobic nature formed an insoluble complex, which it is the subject to degradation during degradation process [37].

Nanocarriers with different material matrix can be used for oral delivery of natural products into the target tissue. The material of nanocarriers for encapsulation of natural products including protein-based, lipid-based, and polysaccharide-based

[37]. Dependent upon the chemical nature of matrix materials, the digestion site and rate of nanocarriers are predetermined in the first place (Fig. 11), for example, the integrity of protein-based nanocarriers readily destroyed by pepsin in the stomach. While the majority of polysaccharide-based nanocarriers experience degradation in small intestinal digestion. Moreover, non-starch polysaccharides, such as chondroitin sulfate, pectin, chitosan, alginate, etc., are retain structural integrity until reaching the colon as a result of the lack of digestive enzymes in the stomach and small intestine; thus, they serve as popular matrix candidates for colon-targeted nanocarriers [37][38].

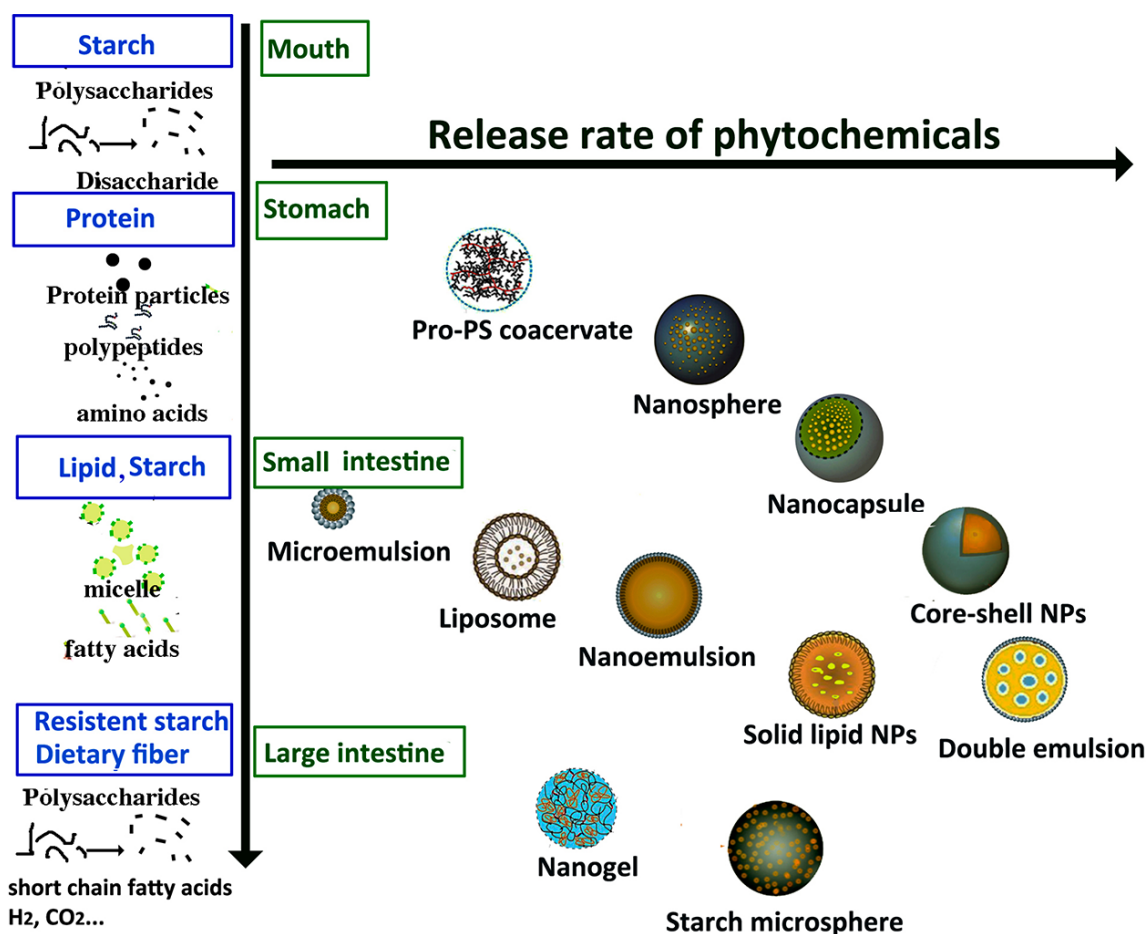


Fig 11. The influences of nanocarriers on the release profile of phytochemicals through programmed digestion of matrix materials and rational design of formula architecture. [37]-Reprinted with permission from J. Agric. Food Chem., 65, 6727–6735. Copyright 2017 American Chemical Society.

1.5 Objective and outline of the thesis

The main objective of the presenting thesis is to deliver natural compounds, such as protein and small molecular natural products using surfactant-based nano-carrier. For this purpose, phycocyanin (PC) and curcumin (Cur) were selected as model therapeutic agents, which are a naturally occurring protein and natural products, respectively. PC, a water-soluble protein-chromophore complex composed of hexameric $\alpha_6\beta_6$ subunits, has important biological functions in blue-green algae as well as pharmacological activities in biomedicine, such as anticancer, anti-inflammantory, and antidiabetic. In addition, Cur, a hydrophobic polyphenol compound, holds promising potential as an anticancer agent. The outline content of present work was summarized in Table 1.

Table 1. The outline content of present thesis

Chapter	Formulation	Target compounds	Type of molecule	Phase
2	Solid-in-Oil (S/O) nanodispersions	Phycocyanin	Protein	Solid/Oil
3	Gel-in-Oil (G/O) nanodispersions	Phycocyanin	Protein	Gel/Oil
4	Solid-in-Water (S/W) nanodispersions	Curcumin	Natural product (Polyphenol)	Solid/Water

The main content of this thesis consists of the following chapters:

In Chapter 2, the mechanism of transcutaneous protein delivery using a solid-in-oil (S/O) nanodispersion method was investigated. PC, a protein-chromophore complex, as a model protein was chosen to evaluate how the proteinaceous macromolecules formulated in an oil phase might permeate the skin. The solid-in-oil (S/O) nanodispersion was prepared by coating PC with hydrophobic surfactants. By

monitoring the absorption and fluorescence of PC, a mechanism for the transcutaneous delivery of the active form of PC using S/O nanodispersion into the skin was proposed.

In Chapter 3, the novel oil-based system for transcutaneous protein delivery, called as gel-in-oil (G/O) nanodispersions, was investigated. Since the conformational structure of protein is important on protein delivery, we proposed to use the gelatin matrix for the protection of the native structure of phycocyanin inside of the oil-based formulation. PC was loaded in G/O nanodispersion as model protein, and was evaluated by their functional integrity and skin permeability.

In Chapter 4, the formulation of curcumin using a solid-in-water (S/W) nanodispersion technique to enhance water solubility and therapeutic activity of curcumin was studied. This new aqueous formulation comprises simple preparation protocols: emulsification and freeze-drying for encapsulating hydrophobic biomolecules with a hydrophilic surfactant, followed by redispersion of the resultant solid complexes in an aqueous solution. The physicochemical properties and anticancer activity of the Curcumin-S/W nanodispersion were evaluated.

In Chapter 5, the main contents of this thesis were summarized and future perspectives regarding the design of nano-sized carrier for natural compounds was also discussed.

1.6 References

- [1] G.M. Cragg, D.J. Newman, Natural products: A continuing source of novel drug leads, *Biochim. Biophys. Acta - Gen. Subj.* 1830 (2013) 3670–3695. doi:10.1016/j.bbagen.2013.02.008.
- [2] C. Huang, *The Pharmacology of Chinese Herbs Second Edition*, CRC Press,

Boca Raton, FL, 1990.

- [3] S. Dev, Ancient-modern concordance in ayurvedic plants: Some examples, *Environ. Health Perspect.* 107 (1999) 783–789. doi:10.1289/ehp.99107783.
- [4] B. Leader, Q.J. Baca, D.E. Golan, Protein therapeutics: A summary and pharmacological classification, *Nat. Rev. Drug Discov.* 7 (2008) 21–39. doi:10.1038/nrd2399.
- [5] H. Zhao, Z.Y. Lin, L. Yildirimer, A. Dhinakar, X. Zhao, J. Wu, Polymer-based nanoparticles for protein delivery: Design, strategies and applications, *J. Mater. Chem. B.* 4 (2016) 4060–4071. doi:10.1039/c6tb00308g.
- [6] D.J. McClements, Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: A review, *Adv. Colloid Interface Sci.* 253 (2018) 1–22. doi:10.1016/j.cis.2018.02.002.
- [7] J.D.A. Pachioni-Vasconcelos, A.M. Lopes, A.C. Apolinário, J.K. Valenzuela-Oses, J.S.R. Costa, L.D.O. Nascimento, A. Pessoa, L.R.S. Barbosa, C.D.O. Rangel-Yagui, Nanostructures for protein drug delivery, *Biomater. Sci.* 4 (2016) 205–218. doi:10.1039/c5bm00360a.
- [8] V.K. Pawar, J.G. Meher, Y. Singh, M. Chaurasia, B. Surendar Reddy, M.K. Chourasia, Targeting of gastrointestinal tract for amended delivery of protein/peptide therapeutics: Strategies and industrial perspectives, *J. Control. Release.* 196 (2014) 168–183. doi:10.1016/j.jconrel.2014.09.031.
- [9] G. Camenisch, J. Alsenz, H. Van De Waterbeemd, G. Folkers, Estimation of permeability by passive diffusion through Caco-2 cell monolayers using the drugs' lipophilicity and molecular weight, *Eur. J. Pharm. Sci.* 6 (1998) 313–319. doi:10.1016/S0928-0987(97)10019-7.
- [10] M. Yu, J. Wu, J. Shi, O.C. Farokhzad, Nanotechnology for protein delivery:

- Overview and perspectives, *J. Control. Release.* 240 (2016) 24–37. doi:10.1016/j.jconrel.2015.10.012.
- [11] M.R. Prausnitz, R. Langer, Transdermal drug delivery, *Nat. Biotechnol.* 26 (2008) 1261–1268. doi:10.1038/nbt.1504.
- [12] M.A. Obeid, M.M. Al Qaraghuli, M. Alsaadi, A.R. Alzahrani, K. Niwasabutra, V.A. Ferro, Delivering natural products and biotherapeutics to improve drug efficacy, *Ther. Deliv.* 8 (2017) 947–956. doi:10.4155/tde-2017-0060.
- [13] H.A.D. Lagassé, A. Alexaki, V.L. Simhadri, N.H. Katagiri, W. Jankowski, Z.E. Sauna, C. Kimchi-Sarfaty, Recent advances in (therapeutic protein) drug development, *F1000Research.* 6 (2017) 113. doi:10.12688/f1000research.9970.1.
- [14] H. Neurath, J.P. Greenstein, F.W. Putnam, J.O. Erickson, The chemistry of protein denaturation, *Chem. Rev.* 34 (1944) 157–265. doi:10.1021/cr60108a003.
- [15] K. Takeda, Y. Moriyama, Kinetic Aspects of Surfactant-Induced Structural Changes of Proteins - Unsolved Problems of Two-State Model for Protein Denaturation -, *J. Oleo Sci.* 64 (2015) 1143–58. doi:10.5650/jos.ess15157.
- [16] C. Zhang, R.M. Davis, B. Xu, Natural product-based nanomedicine: recent advances and issues, (2015) 6055–6074.
- [17] Z. Guo, The modification of natural products for medical use, *Acta Pharm. Sin. B.* 7 (2017) 119–136. doi:10.1016/j.apsb.2016.06.003.
- [18] R.A. Sharma, S.A. Euden, S.L. Platton, R.A. Sharma, S.A. Euden, S.L. Platton, D.N. Cooke, A. Shafayat, H.R. Hewitt, T.H. Marczylo, B. Morgan, D. Hemingway, S.M. Plummer, M. Pirmohamed, A.J. Gescher, W.P. Steward, Phase I Clinical Trial of Oral Curcumin: Biomarkers of Systemic Activity and Compliance Phase I Clinical Trial of Oral Curcumin: Biomarkers of Systemic Activity and

- Compliance, 10 (2004) 6847–6854. doi:10.1158/1078-0432.CCR-04-0744.
- [19] P. Anand, A.B. Kunnumakkara, R.A. Newman, B.B. Aggarwal, P. Anand, A.B. Kunnumakkara, R.A. Newman, Bioavailability of Curcumin: Problems and Promises reviews Bioavailability of Curcumin: Problems and Promises, *Mol. Pharmacol.* 4 (2007) 807–818. doi:10.1021/mp700113r.
- [20] C. Manach, G. Williamson, C. Morand, A. Scalbert, C. Rémésy, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 intervention studies, *Am J Clin Nutr.* 81 (2005) 243S–255S. doi:10.1093/ajcn/81.1.230S [pii].
- [21] S.H. Thilakarathna, H.P. Vasantha Rupasinghe, Flavonoid bioavailability and attempts for bioavailability enhancement, *Nutrients.* 5 (2013) 3367–3387. doi:10.3390/nu5093367.
- [22] B.V. Bonifácio, P.B. da Silva, M. Aparecido dos Santos Ramos, K. Maria Silveira Negri, T. Maria Bauab, M. Chorilli, Nanotechnology-based drug delivery systems and herbal medicines: A review, *Int. J. Nanomedicine.* 9 (2013) 1–15. doi:10.2147/IJN.S52634.
- [23] D.J. Adams, M.L. Wahl, J.L. Flowers, B. Sen, M. Colvin, M.W. Dewhirst, G. Manikumar, M.C. Wani, Camptothecin analogs with enhanced activity against human breast cancer cells. II. Impact of the tumor pH gradient, *Cancer Chemother. Pharmacol.* 57 (2006) 145–154. doi:10.1007/s00280-005-0008-5.
- [24] Y. Çirpanli, E. Bilensoy, A. Lale Doğan, S. Çaliş, Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery, *Eur. J. Pharm. Biopharm.* 73 (2009) 82–89. doi:10.1016/j.ejpb.2009.04.013.
- [25] H.H. Tønnesen, M. Másson, T. Loftsson, Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: Solubility, chemical and photochemical

- stability, *Int. J. Pharm.* 244 (2002) 127–135. doi:10.1016/S0378-5173(02)00323-X.
- [26] Y.J. Wang, M.H. Pan, A.L. Cheng, L.I. Lin, Y.S. Ho, C.Y. Hsieh, J.K. Lin, Stability of curcumin in buffer solutions and characterization of its degradation products, *J. Pharm. Biomed. Anal.* 15 (1997) 1867–1876. doi:10.1016/S0731-7085(96)02024-9.
- [27] Z. Gu, A. Biswas, M. Zhao, Y. Tang, Tailoring nanocarriers for intracellular protein delivery, *Chem. Soc. Rev.* 40 (2011) 3638–3655. doi:10.1039/c0cs00227e.
- [28] J.T. Ryman, B. Meibohm, Pharmacokinetics of monoclonal antibodies, *CPT Pharmacometrics Syst. Pharmacol.* 6 (2017) 576–588. doi:10.1002/psp4.12224.
- [29] M.J. Roberts, M.D. Bentley, J.M. Harris, Chemistry for peptide and protein PEGylation.pdf, *Adv. Drug Deliv. Rev.* 54 (2002) 459–476. doi:10.1016/S0169-409X(02)00022-4.
- [30] J. Futami, M. Kitazoe, T. Maeda, E. Nukui, M. Sakaguchi, J. Kosaka, M. Miyazaki, M. Kosaka, H. Tada, M. Seno, J. Sasaki, N.H. Huh, M. Namba, H. Yamada, Intracellular delivery of proteins into mammalian living cells by polyethylenimine-cationization, *J. Biosci. Bioeng.* 99 (2005) 95–103. doi:10.1263/jbb.99.095.
- [31] A. Wang, Y. Cui, J. Li, J.C.M. Van Hest, Fabrication of gelatin microgels by a “cast” strategy for controlled drug release, *Adv. Funct. Mater.* 22 (2012) 2673–2681. doi:10.1002/adfm.201102907.
- [32] H. Ayame, N. Morimoto, K. Akiyoshi, H. Ayame, N. Morimoto, K. Akiyoshi, Self-Assembled Cationic Nanogels for Intracellular Protein Delivery Self-Assembled Cationic Nanogels for Intracellular Protein Delivery, 19 (2008) 882–890.

doi:10.1021/bc700422s.

- [33] J. Wang, M. Xu, X. Cheng, M. Kong, Y. Liu, C. Feng, X. Chen, Positive/negative surface charge of chitosan based nanogels and its potential influence on oral insulin delivery, *Carbohydr. Polym.* 136 (2016) 867–874. doi:10.1016/j.carbpol.2015.09.103.
- [34] I. Castangia, M.L. Manca, C. Caddeo, G. Bacchetta, R. Pons, D. Demurtas, O. Diez-Sales, A.M. Fadda, M. Manconi, Santosomes as natural and efficient carriers for the improvement of phycocyanin reepithelising ability in vitro and in vivo, *Eur. J. Pharm. Biopharm.* 103 (2016) 149–158. doi:10.1016/j.ejpb.2016.03.033.
- [35] Y. Yin, C. Wu, J. Wang, F. Song, W. Yue, W. Zhong, A simply triggered peptide-based hydrogel as an injectable nanocarrier of tanshinone IIA and tanshinones, *Chem. Commun.* 53 (2017) 529–532. doi:10.1039/C6CC08502D.
- [36] J. Zhang, Y. Li, X. Fang, D. Zhou, Y. Wang, M. Chen, TPGS-g-PLGA/Pluronic F68 mixed micelles for tanshinone IIA delivery in cancer therapy, *Int. J. Pharm.* 476 (2014) 185–198. doi:10.1016/j.ijpharm.2014.09.017.
- [37] J. Xiao, Y. Cao, Q. Huang, Edible Nanoencapsulation Vehicles for Oral Delivery of Phytochemicals: A Perspective Paper, *J. Agric. Food Chem.* 65 (2017) 6727–6735. doi:10.1021/acs.jafc.7b02128.
- [38] L. Zou, B. Zheng, R. Zhang, Z. Zhang, W. Liu, C. Liu, H. Xiao, D.J. McClements, Food-grade nanoparticles for encapsulation, protection and delivery of curcumin: Comparison of lipid, protein, and phospholipid nanoparticles under simulated gastrointestinal conditions, *RSC Adv.* 6 (2016) 3126–3136. doi:10.1039/c5ra22834d.

CHAPTER 2

MECHANISTIC INVESTIGATION OF TRANSCUTANEOUS PROTEIN DELIVERY USING SOLID-IN-OIL NANODISPERSION: A CASE STUDY WITH PHYCOCYANIN

2.1 Introduction

Phycocyanin (PC) is a water-soluble protein-chromophore complex derived from blue-green algae. PC from *Spirulina* is composed of α and β polypeptide subunits, of which hexamer ($\alpha_6\beta_6$) is the active form, with an approximate molecular weight of 262 kDa [1]. PC possesses a linear tetrapyrrole chromophore covalently bound to the apoprotein via a thioether bond, comprising one molecule on the α subunit and two molecules on the β subunit. These chromophores are an intrinsic and sensitive indicator for monitoring the tertiary structure of PC [2]. Furthermore, PC has several biological activities including antioxidant, anti-inflammatory, anticancer, neuroprotective [3], immunomodulating [4], and anti-diabetic activities. In particular, a potential anticancer effect of phycocyanin, both in vitro and in vivo, has been shown in a variety of cancer cell types, including skin [5], breast [6], lung [7], and pancreatic [8] cancer cells. Since the PC does not lead to toxicity when administered to normal human cells [8][9], it presents a promising protein for use in biomedicine.

In recent years, transdermal delivery systems have attracted much attention as a route for protein administration given their many advantages over injection or oral administration including the avoidance of gastrointestinal absorption, the provision of a painless, routine method of self-administration, and the possibility to ensure a steady drug concentration in the body by slow rates of drug release [10][11][12]. However,

proteinaceous macromolecule delivery remains challenging because of the intrinsic barrier function of the stratum corneum (SC) layer of the skin. The SC, the outermost layer of the epidermis, has a hydrophobic and layered structure with 15–20 μm thickness, and consists of 10–20 layers of corneocytes embedded with lipids such as ceramides, cholesterol esters, and fatty acids. It acts as a strong barrier against chemical compounds and pathogens, thus hindering the penetration of hydrophilic biomolecules ($M_w > 500 \text{ Da}$) [13][12]. Crossing the SC is key to efficient transdermal drug delivery [12]. Because PC is a hydrophilic biomacromolecule with a high molecular weight, it therefore may not easily penetrate the skin.

Nanosize carriers are among the most promising transcutaneous delivery systems for penetrating the skin barrier. A number of nanocarriers systems based on hydrophilic vesicles for the transdermal delivery of PC have been reported to date, including liposomes [14][15], ethosomes [15], hyalurosomes [16], santosomes [17], and penetration enhancer-containing vesicles (PEVs), the latter containing the penetration enhancer propylene glycol or Transcutol® P [15]. Given the hydrophobicity of SC, our approach to overcome the skin barrier is to develop an oil-based formulation of PC using a solid-in-oil (S/O) nanodispersion technique to enhance the skin permeability.

S/O nanodispersion is an oil-based nanodispersion of a solid powder of a hydrophilic molecule coated with hydrophobic surfactant molecules. S/O nanodispersion is achieved by the lyophilization of a water-in-oil (W/O) emulsion containing the protein and hydrophobic surfactant molecules followed by redispersion of the resultant solid complex in an oil phase. Through this process, hydrophilic proteins can be coated with surfactants without a loss in yield and homogeneously dispersed in a non-aqueous solvent [18]. Our previous studies demonstrated that the

use of an S/O nanodispersion system enhanced the skin permeation of peptides and proteins with molecular weights ranging from 1 to 45 kDa [19] [20] [21]. Another study has successfully demonstrated skin perfusion of a large protein, bovine serum albumin (BSA; 66 kDa), using an S/O nanodispersion system [22]. However, the permeability of a large macromolecular assembly composed of various proteinaceous subunits has not been validated to date.

Here, we demonstrate for the first time that PC, possessing a subunit structure, can be delivered through the skin by formulating it in as oil vesicles using an S/O nanodispersion technique. The resulting PC-in-oil (PC/O) nanodispersion was characterized by size, morphology, and structure, and skin permeability was evaluated in vitro using Yucatan Micropig (YMP) skin. As the PC chromophore is a sensitive probe for the retention of the native protein structure, it can indicate the formulation of PC when dissolved in an aqueous solution and dispersed in the oil phase by S/O nanodispersion. By monitoring the absorption and fluorescence of PC in different formulations, a mechanism for the transcutaneous delivery of the active form of PC to the skin was proposed.

2.2 Materials and Methods

2.2.1 Materials

PC from *Spirulina* was purchased from Tokyo Chemical Industries, Ltd. (Tokyo, Japan). Nonionic surfactant of sucrose laurate (commercial name, L-195) and sucrose erucate (commercial name, ER-290) were kindly supplied by Mitsubishi-Kagaku Foods (Tokyo, Japan). Cyclohexane was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and dibasic hydrate sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were purchased from Kishida Chemical Co., Ltd

(Osaka, Japan). Isopropyl myristate was purchased from Tokyo Chemical Industries, (Tokyo, Japan). A frozen Yucatan micropig (YMP) skin set (female; 5 months old) was purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan).

2.2.2 Methods

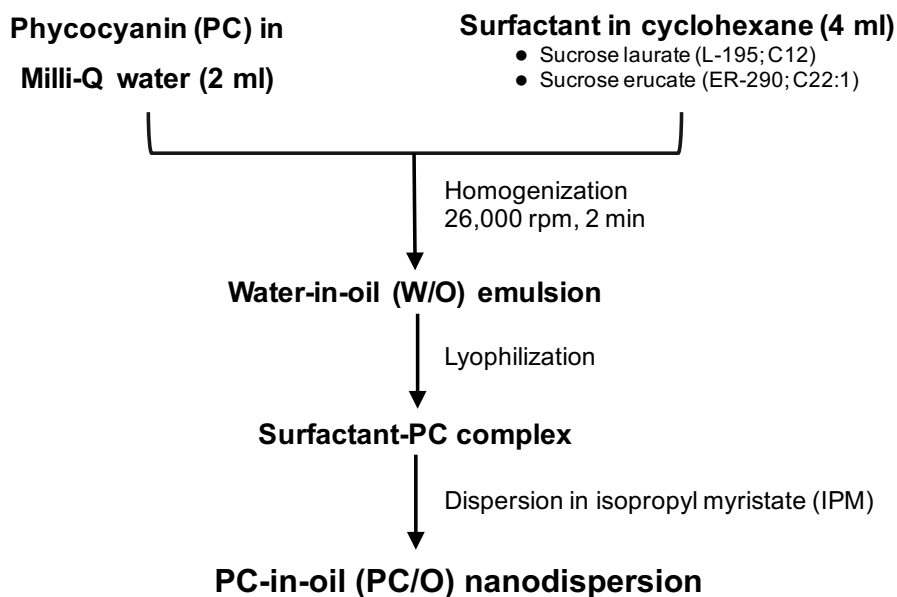
2.2.2.1 Preparation of PC/O nanodispersions

Sucrose laurate (L-195) or sucrose erucate (ER-290) were dissolved in cyclohexane to obtain a surfactant-cyclohexane solution (12.5 or 25 mg mL⁻¹). An aqueous solution of PC (2 mL) extracted from *Spirulina* with a concentration of 0.5 mg mL⁻¹ and 4 mL of the surfactant-cyclohexane solution were mixed using a homogenizer (Polytron, Switzerland) at 26,000 rpm for 2 min to form an W/O emulsion. The resultant emulsion was quickly frozen in liquid nitrogen for 20 min and lyophilized overnight using a freeze dryer (EYELA, Tokyo, Japan). The resulting viscous material was employed as a surfactant-PC complex. Finally, isopropyl myristate (IPM) was added to this complex to adjust the PC concentration to 1.0 mg mL⁻¹. A schematic illustration of the preparation of PC/O nanodispersion with different types of surfactant is shown in Scheme 1. The concentration of PC was quantified using an extinction coefficient ($\epsilon^{1\%}$) of 70 at 620 nm [23].

2.2.2.2 Characterization of PC/O nanodispersions

a) Size distribution

Particle size distribution and polydispersity index of PC/O nanodispersions were determined at 25±1 °C using a dynamic light scattering spectrophotometer (DLS) with an argon laser (DLS-8000, Otsuka Electronic Co., Osaka, Japan). For each nanodispersion, the average size was measured in triplicate.



Scheme 1. The preparation of PC/O nanodispersion with different types of surfactant.

b) Absorption and fluorescence spectroscopy

The spectroscopic properties of PC were recorded in the native state (PBS solution), in the S/O nanodispersion, and in extracted solution from the S/O nanodispersion on a UV-visible (UV-Vis) spectrophotometer (V-670, Jasco, Tokyo, Japan) (concentration 0.1 mg mL^{-1}) and a fluorescence spectrophotometer (LS55C, Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 590 nm (concentration $5 \mu\text{g mL}^{-1}$) at 25°C .

c) Encapsulation efficiency of surfactant-PC complexes

The encapsulation efficiency of surfactant-PC complexes was determined as previously reported [24]. The surfactant-PC complex containing 1 mg PC was added to 0.5 mL of ice-cold PBS, pH 7.4. The mixture was shaken for 10 min and cooled on ice for 5 min. After repeating these processes for 3 times, the mixture was centrifuged (4°C , 18000 g, 10 min). The free PC concentration in the supernatant solution was quantified by the fluorescence of PC (excitation wavelength of 590 nm at 25°C),

assuming that the liberated PC in the supernatant has the same fluorescent property of native PC. The encapsulation efficiency (EE) was calculated using the following equation:

$$EE [\%] = ([\text{Phycocyanin}]_{\text{feed}} - [\text{Phycocyanin}]_{\text{supernatant}}) / [\text{Phycocyanin}]_{\text{feed}} \times 100$$

d) Extraction of PC from PC/O nanodispersion

Extraction of PC from PC/O nanodispersion was evaluated with a biphasic system. Briefly, PC/O nanodispersion (1 mg mL⁻¹, 300 µL) was placed onto 3 mL phosphate-buffered saline (PBS). The biphasic system was gently stirred at 100 rpm for 30 min and then centrifuged at 15,000 g for 20 min at 25 °C. PC was released from the PC/O nanodispersion (oil phase) to the aqueous phase (PBS). The aqueous phase was collected and filtered using a filter unit (Avantec®, Toyo Roshi Kaisha, Ltd, Japan) with a pore size of 0.45 µm.

2.2.2.3 *In vitro* skin permeation study

Custom vertical diffusion Franz cells with a diffusion area of 0.785 cm² were used to perform *in vitro* skin permeation studies in YMP skin which was stored at -80 °C until use. Briefly, frozen YMP skin was allowed to thaw at room temperature and subcutaneous fat was carefully removed. Skin segments (2.5 × 2.5 cm) were mounted between the donor and receptor compartments of Franz cells, with the SC facing the donor side of the cell and the dermis facing the receptor. The receptor compartment was filled with 5 mL PBS (pH 7.4) and maintained at 32.5 °C with continuous stirring, referred to as the normal skin temperature [25][26]. An aliquot (300 µL) of aqueous solution or PC/O nanodispersion (1 mg mL⁻¹) was placed in the donor compartment (i.e., 0.3 mg/0.785 cm²), which was sealed with Parafilm. The skin

permeation study was performed for 24 h, with the Franz cells covered by aluminum foil to provide protection from light.

2.2.2.4 Histology of the skin

After the application of PC to skin as described above, skin segments were removed from the Franz cells, wiped with tissue paper, and embedded in Tissue-Tek® embedding compound before being placed in liquid nitrogen for 15 min. The embedded skin samples were sectioned using a cryostat microtome (CM1860UV, Leica, Wetzlar, Germany) with a longitudinal (20 μ m) and transverse (70 μ m) cutting angle. Sections of skin were mounted on glass slides, and the penetration of PC into the skin was examined by confocal laser scanning microscopy (CLSM) (LSM 700, ZEISS, Oberkochen, Germany). PC was detected at an excitation wavelength of 639 nm without a filter. Longitudinal skin section images were collected to determine the penetration of PC from the skin surface (SC) to the deep skin layer. Transverse skin section images were collected using the z-stack method and used to evaluate the accumulation of PC on the skin. Quantitative fluorescence analysis was performed using ImageJ software (National Institutes of Health: NIH, Maryland).

2.3 Results and Discussion

2.3.1 PC/O nanodispersion solubility and particle size

Given that PC is a hydrophilic biomacromolecule that cannot be directly dissolved in oil, an S/O nanodispersion technique was used to solubilize PC in an oil phase. We previously investigated the effect of alkyl chain length of surfactants used in PC/O nanodispersion on particle size distribution. Here, we used two types of a lipophilic sucrose fatty acid ester, sucrose laurate (L195, C12) and sucrose erucate (ER290, C22:1), to obtain a PC/O nanodispersion. These compounds are nonionic

surfactants with a hydrophilic/lipophilic balance (HLB) value of 1-2. The PC/O nanodispersion, prepared by the formation of a W/O emulsion and subsequent lyophilization, was dispersed in IPM as an oil vehicle. IPM-based S/O nanodispersion has shown the ability to interact with lipid in the SC to enhance the permeation of protein through the skin. Moreover, IPM is considered safe and is widely used in cosmetic and pharmaceutical products [12].

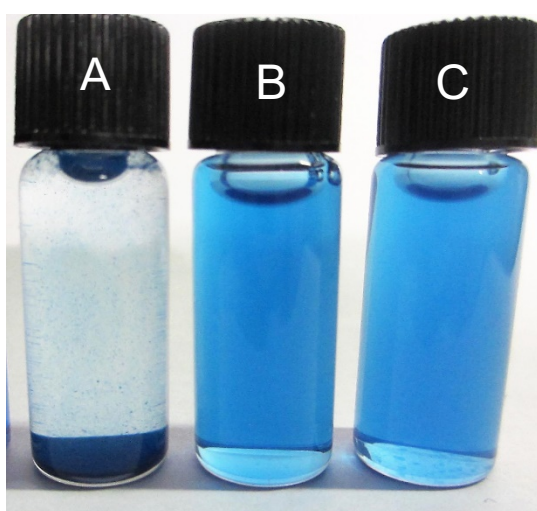


Fig. 1 Differences in physical appearance of phycocyanin in isopropyl myristate (IPM) (A), and phycocyanin-in-oil (PC/O) nanodispersions with sucrose laurate, L-195 (B) or sucrose erucate, ER-290 (C) in IPM. The weight ratio of protein to surfactant was 1:50. All samples contained 0.5 mg mL^{-1} of phycocyanin.

Fig. 1 represents the differences in physical appearance between native PC and PC/O nanodispersions using two types of surfactants in IPM. A translucent dispersion was obtained by dispersing surfactant-PC complexes (Fig. 1B and 1C), while a physical mixture of PC in IPM formed a blue precipitate (Fig. 1A). DLS analysis gave the mean particle size and polydispersity indexes (PDIs) of PC/O nanodispersions prepared with different PC-surfactant ratios, as summarized in Table 1. The mean particle size of the PC/O nanodispersion with the PC-ER290 complex

was smaller than that of the PC-L195 complex, indicating that the surfactant alkyl chain influenced the particle size of the PC/O nanodispersion. The PC/O formulation with ER290 (abbreviated as the PC-ER/O nanodispersion) resulted in a complex with small nanosized particles (220–270 nm on average) and narrower PDIs (0.23-0.39). S/O nanodispersion particles were formed from one droplet of W/O emulsion by freeze-drying, implying that the particle size of S/O nanodispersions was dependent on the size of the water droplets in the W/O emulsion. These findings showed that the surfactant alkyl chain length significantly influenced the size of the aqueous droplets in the W/O emulsion [27], with longer alkyl surfactant chains forming smaller-sized aqueous droplets.

Table 1. Size distribution of phycocyanin-containing S/O nanodispersions prepared with two surfactants

Sample code	Type surfactant	Weight ratio PC: surfactants	Average size (nm)	Pdl	EE (%)
PC-ER50/O	ER-290	1:50	274 ± 73	0.269-0.392	99.92±0.07
PC-ER100/O	ER-290	1:100	218 ± 14	0.232-0.305	99.97±0.01
PC-L50/O	L-195	1:50	857 ± 70	0.689-0.935	96.19±1.29
PC-L100/O	L-195	1:100	1030 ± 190	0.785-1.20	99.23±0.42

We also evaluated the encapsulation efficiency of PC in the surfactant-PC complex formation by solid-liquid extraction of PC from the surfactant-PC complexes to PBS. A fraction of non-encapsulated PC was extracted in PBS, and the PC was quantified by fluorescence spectroscopy to determine the encapsulation efficiency (EE). The results showed the quantitative encapsulation of PC for all the formulation prepared with ER290 (> 99% EE, Table 1). The EE slightly lowered when the L-195-

PC ratio was reduced (approx. 96% EE for PC-L50/O), implying more stable ER290-PC complex formulation compare to that with shorter alkyl chain surfactant, L-195.

2.3.2 Spectral characteristics of PC

Since the absorption and fluorescence spectra of PC are sensitive to the conformational change of the chromophore, we first characterized and compared the spectroscopic properties of native PC in aqueous solution and PC/O nanodispersion in isopropyl myristate (IPM). The ratio of visible to near-UV bands (A_{620}/A_{350}) of PC in the UV-vis spectra is used to evaluate the conformational change of chromophore. As shown in Fig. 2A, the absorption spectra of native PC dissolved in an aqueous solution exhibited an intense visible band and low near-UV band intensity. The ratio of these bands was around 5, which correlated to the linear conformation of the chromophore in PC (Fig. 2A). However, the intensity of the visible band was decreased and the near-UV band significantly increased when PC was solubilized in the oil phase by the S/O nanodispersion technique. The ratio of visible to near-UV bands of PC/O nanodispersion using L-195 and ER-290 were lower than 1 (Fig. 2A), suggesting a structural change in the chromophore from a linear to cyclic, porphyrin-type conformation [2].

We next characterized the PC extracted from PC/O nanodispersions using a biphasic extraction system. The extraction of PC from an oil-based nanodispersion to an aqueous phase showed that the PC-ER50/O nanodispersion released a smaller amount of PC ($5.1 \pm 0.6\%$) than the PC-L50/O nanodispersion ($44.5 \pm 2.7\%$). The results suggest that the stable entrapment of PC in the oil phase was achieved by the complex formation with longer C22:1 alkyl chain surfactant, ER290. By contrast, more PC was released to the aqueous phase from L195-based nanodispersion, implying the weaker interaction of PC with shorter C12 alkyl chain in the formulation [20][25]. Interestingly,

the absorption spectra of the extracted PC was similar to that of native PC, with the visible band stronger than the near-UV band (Fig. 3). Detailed characterization of the oligomerization of PC based on the UV-vis spectra [28] suggested that the PC extracted from PC/O nanodispersions is likely to be dissociated mainly into the $\alpha\beta$ monomeric unit, and partially associated to the oligomers (Table 2).

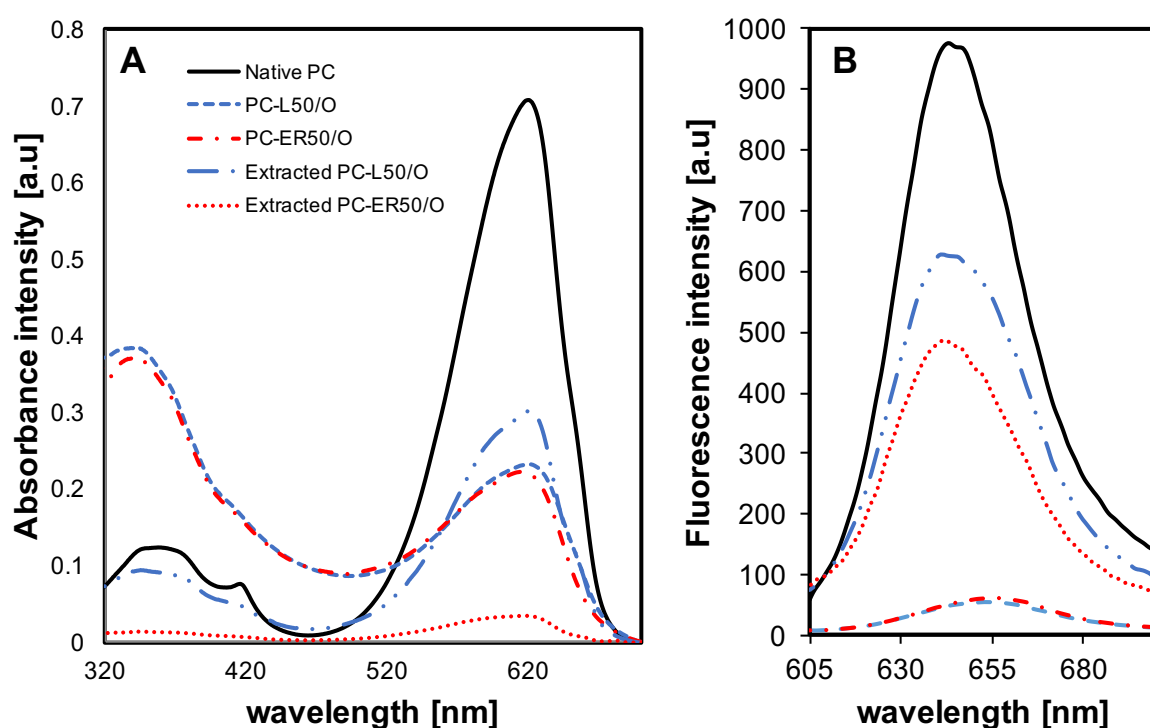


Fig. 2 UV-vis absorption (A) and fluorescence (B) spectra of phycocyanin (PC) in aqueous solution and solid-in-oil (S/O) nanodispersion in isopropyl myristate (IPM) at 25 °C. Solid line: native PC in PBS; dashed line: PC-L50/O nanodispersion in IPM; dashed-dotted line: PC-ER50/O nanodispersion in IPM; dotted line: PC extracted from PC-L50/O nanodispersion in PBS; and double dotted-dashed line: PC extracted from PC-ER50/O nanodispersion in PBS.

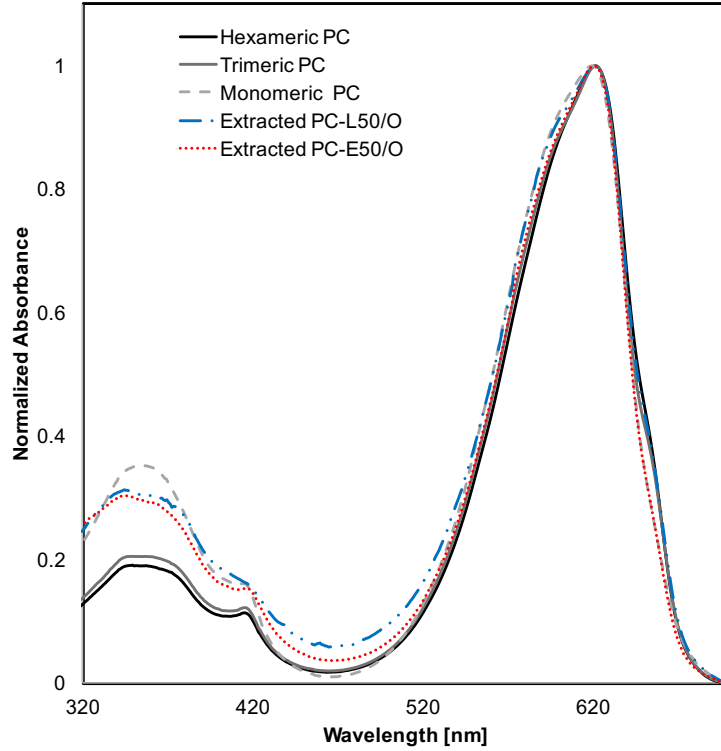


Fig. 3 Normalized absorption spectra of phycocyanin with different ($\alpha\beta$) oligomeric forms and the samples extracted from different PC/O formulations at 25 °C. The solvent conditions are as follows: hexamer, native form in PBS (pH 7.4); trimer: 1 M urea in PBS (pH 7.4); monomer: 5 M urea in PBS (pH 7.4); extracted PC in PBS (pH 7.4). The absorption spectrum of hexameric PC in PBS was used as a control of native form, with the absorbance at 620 nm was set to 1. Black solid line: hexameric PC; grey solid line: trimeric PC; grey dashed line: monomeric PC; red dot line: PC extracted from PC-ER50/O nanodispersion; and blue dotted-dashed line: PC extracted from PC-L50/O nanodispersion.

Fluorescence spectra of PC showed decreased fluorescence intensity after incorporation into the S/O nanodispersion (Fig. 2B). By contrast, after the release from PC/O nanodispersion the fluorescence intensity of PC sufficiently regained ($64 \pm 1\%$ from PC-L50/O and $46 \pm 5\%$ from PC-ER50/O, respectively). These results suggest a reversible conformational change of PC between the constrained form in the S/O formulation and the native form released from the S/O nanodispersion into the aqueous milieu. We reasoned that these unique fluorescent properties of PC should

be useful to track the active protein in the skin, leading to gain insight into the mechanism of protein delivery through the skin using S/O nanodispersion.

Table 2 The A_{620}/A_{350} ratio of different phycocyanin samples.

Sample	Ratio of A_{620}/A_{350}
Extracted PC-L50/O	3.3
Extracted PC-E50/O	3.3
Hexameric $(\alpha\beta)_6$ of PC	5.2
Trimeric $(\alpha\beta)_3$ of PC	4.8
Monomeric $(\alpha\beta)$ of PC	2.8

2.3.3 *In vitro* penetration studies of phycocyanin through pig skin

The permeation profile of PC containing S/O nanodispersions through the YMP skin layers was investigated by CLSM. YMP skin was selected as a skin model because of its similarities with human skin in structure and responsiveness in skin permeation studies [29][30]. The SC of YMP skin is about 20 μm thick and its epidermis ranges in thickness from 40 to 100 μm [29]. The YMP skin was treated with the PC in PBS or PC/O nanodispersion at concentration of 1 mg mL⁻¹ (300 μL), given the detection limit of PC on skin is 0.1 mg mL⁻¹ (Fig. 4). Longitudinal skin sections were used to examine the penetration of PC from the SC to the deep skin layer, shown as a 2D skin image. Fig. 3 shows that PC encapsulated in the ER290-S/O nanodispersion had a stronger fluorescent intensity than the L195-S/O nanodispersion. The highest PC fluorescence was observed when ER290 was used at a PC:surfactant ratio of 1:50 (abbreviated as PC-ER50/O in Table 1). In contrast, fluorescence intensity with a weak signal was observed on the skin after treatment with PC in PBS (Fig. 5).

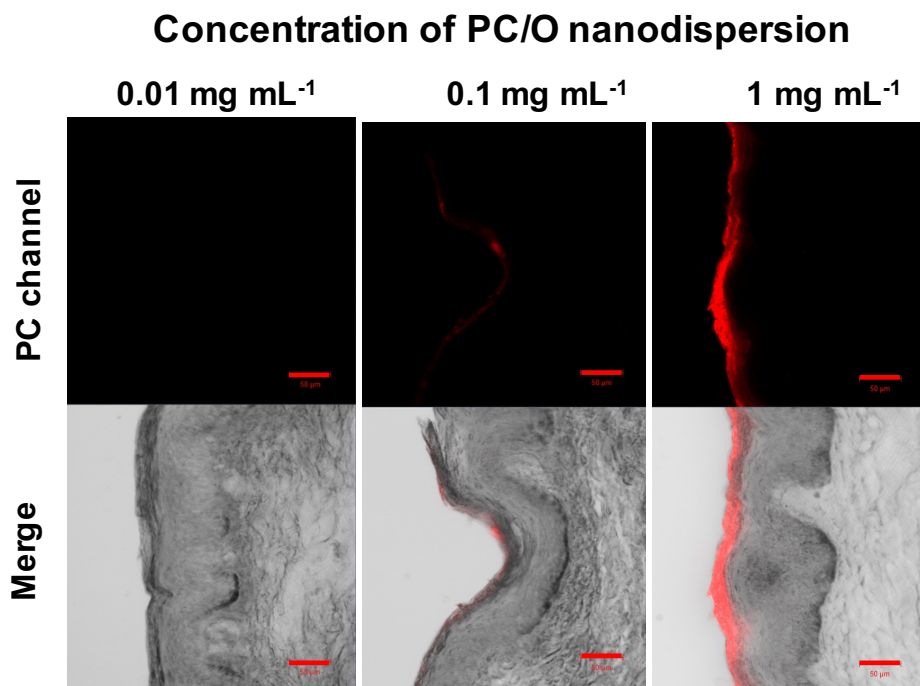


Fig. 4 The detection limit of PC/O nanodispersion on skin permeation study. The YMP skin was treated with PC-ER50/O for 24 h. Scale bar: 50 μ m.

We next performed quantitative fluorescence analyses of skin images to evaluate the penetration of PC through YMP skin. The average value of pixel fluorescence intensity was collected through the skin layers from surface of the skin (Fig. 6). Detailed analysis as a function of the distance from the skin surface to the deep skin layer clearly showed that the S/O nanodispersion enhanced the permeation of PC into the skin. ER290, with its longer erucate alkyl chains, showed a greater ability to deliver PC through the skin compared with L-195, with shorter lauryl chains, implying that the hydrophobicity of surfactant-PC complexes could be a key factor in the delivery of large proteins through the SC. This observation also suggested that stable encapsulation of PC in nano-size particles promoted the permeation into the deep skin. Moreover, a low PC-surfactant ratio facilitated deeper penetration into the skin, whereas a high PC-surfactant ratio promoted the accumulation of PC, mainly in the SC layer. These results indicate that excess surfactant in the S/O formulation could

reduce the penetration of PC into and across the skin, possibly due to the partition of drugs into the formulation phase rather than the SC layer [31].

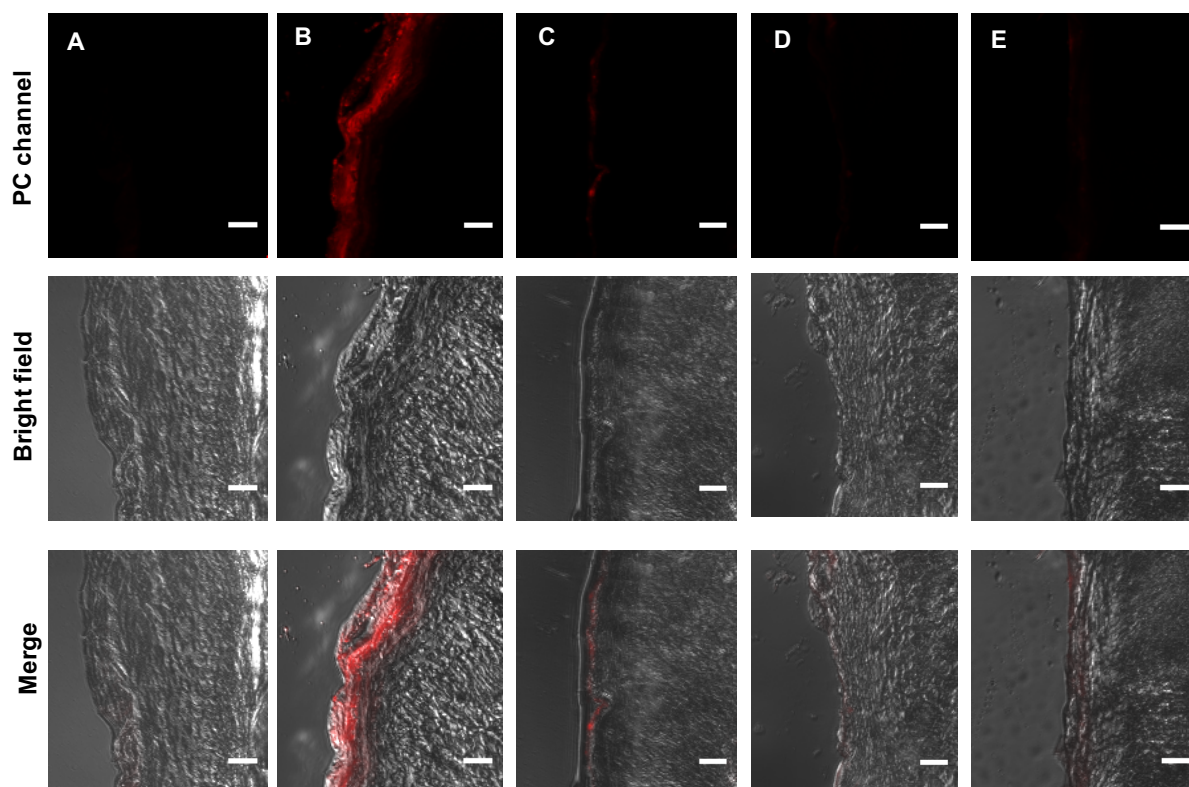


Fig. 5 Fluorescent images of Yucatan Micropig (YMP) skin cross-sections to which phycocyanin was applied in the following formulation: A) PBS solution; B) PC-ER50/O; C) PC-ER100/O; D) PC-L50/O; and E) PC-L100/O. Samples were applied for 24 h. Skin surface is displayed on the left side of the section. Scale bar: 25 μ m.

From our previous studies, we speculated that proteins would be released from S/O nanodispersions after crossing the SC layer, with hydrophobic surfactant molecules liberated at the interface between the SC and the epidermis [19]. For the PC-ER50/O nanodispersion, we observed a marked enhancement of fluorescence intensity after passing the SC layer, illustrating for the first time the release of biologically active forms of protein complexes from a S/O nanodispersion (Fig. 6). This result correlated with the fluorescent recovery of PC, in which fluorescence intensity

increased after the release of PC from S/O nanodispersion (Fig. 2B). The intense fluorescence of PC from the PC-ER50/O nanodispersion compared with the PC-ER100/O nanodispersion suggested that the lower PC-surfactant ratio promoted greater release and permeation of PC into the deep skin layer. The lower surfactant's concentration, the weaker are the resultant of the PC-surfactant complex [27].

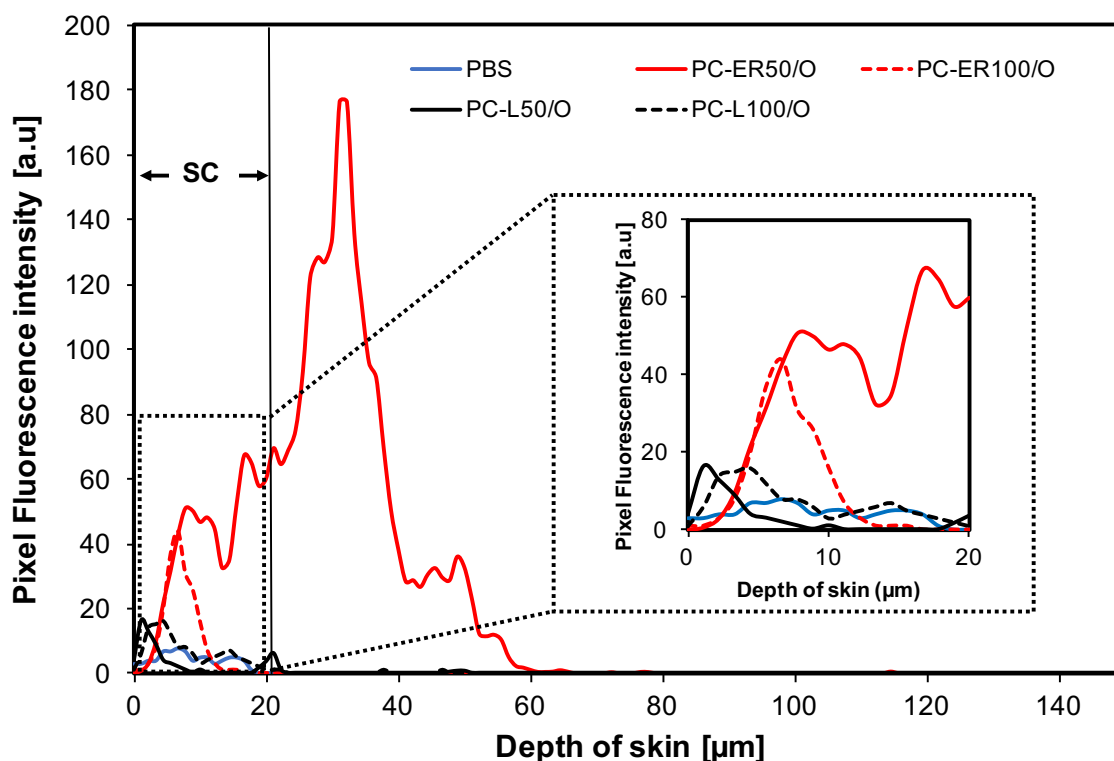


Fig. 6 Pixel fluorescent intensity of Yucatan Micropig (YMP) skin images. The fluorescent yield in the skin after each treatment was quantified using ImageJ software. The integration of pixel brightness values in arbitrary units (a.u.) provided the relative phycocyanin content in different depths of skin. SC: stratum corneum. Solid blue line: native PC in PBS; solid red line: PC-ER50/O; dotted red line: PC-ER100/O; solid line: PC-L50/O; dotted red line: PC-L100/O.

We further studied the accumulation of PC treated with the PC-ER50/O nanodispersion in YMP skin using the z-stack CLSM method. The calculated volume fluorescence intensity of PC is shown in Fig. 7. 3D fluorescence imaging clearly

showed the wide distribution of PC fluorescence in the skin layers, whereas weak fluorescence was observed when an aqueous PC solution was used. A comparison of the pixel fluorescence intensities of transverse skin sections treated with the PC/O nanodispersion showed a 43-fold increase compared with skin sections treated with aqueous PC solution (Fig. 7C).

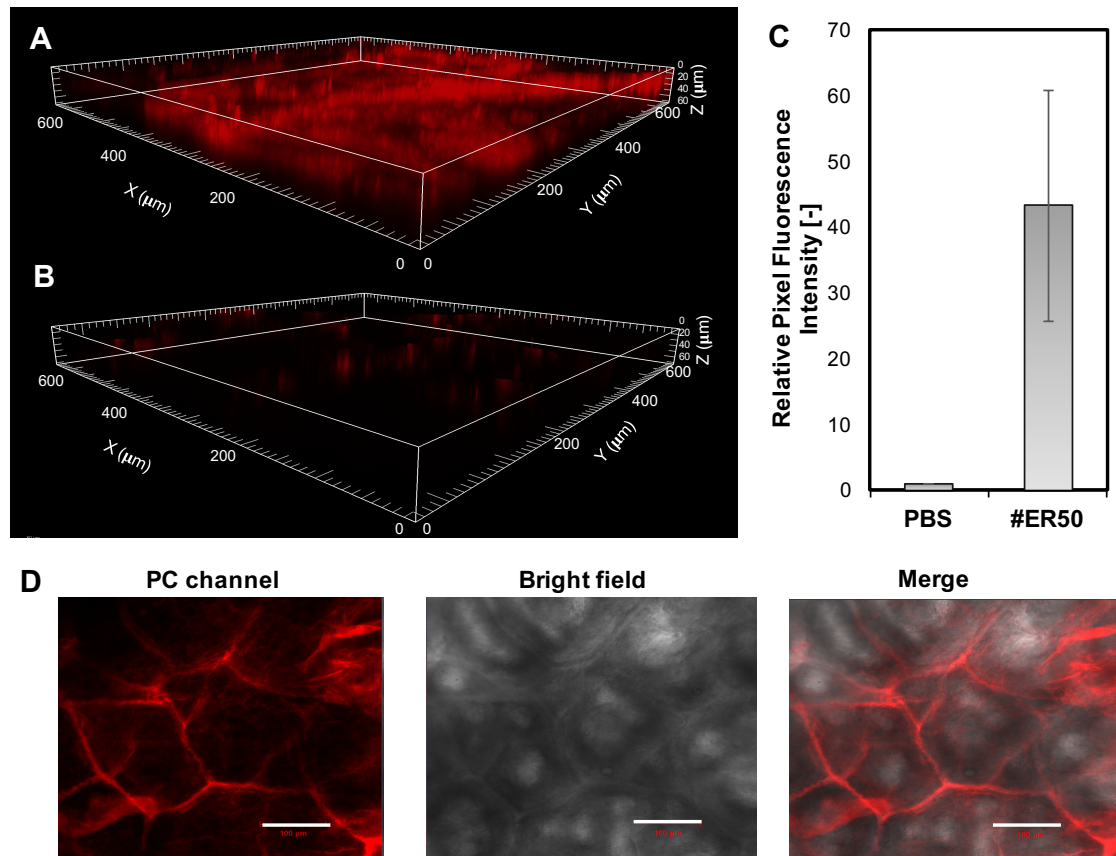


Fig. 7 Confocal 3D images of Yucatan Micropig (YMP) skin treated with (A) PC-ER50/O nanodispersion or (B) aqueous PBS solution of PC, and (C) comparison of the relative pixel fluorescence intensities obtained from confocal 3D images ($n = 5$). The PC in aqueous solution (PBS group) was used as a control, with pixel fluorescence intensity was set to 1. (D) CLSM images of YMP skin treated with PC-ER50/O on YMP skin. Scale bar: 100 μm.

The skin permeation route of PC-ER50/O nanodispersion on YMP skins was further investigated using CLSM. The red fluorescence from PC was observed between two corneocyte cells, implying the delivery of PC through intercellular

pathway (Fig. 7D). The use of IPM as an oil vehicle, known as a penetration enhancer by disrupting the lipid order and increasing the fluidity of SC layer [32][33], could promote the permeation of PC/O nanodispersion via intercellular routes. In contrast, the permeation of a hydrophilic formulation, PC in PBS, was not observed even after the pretreatment of the skin with IPM for 5 h (Fig. 8).

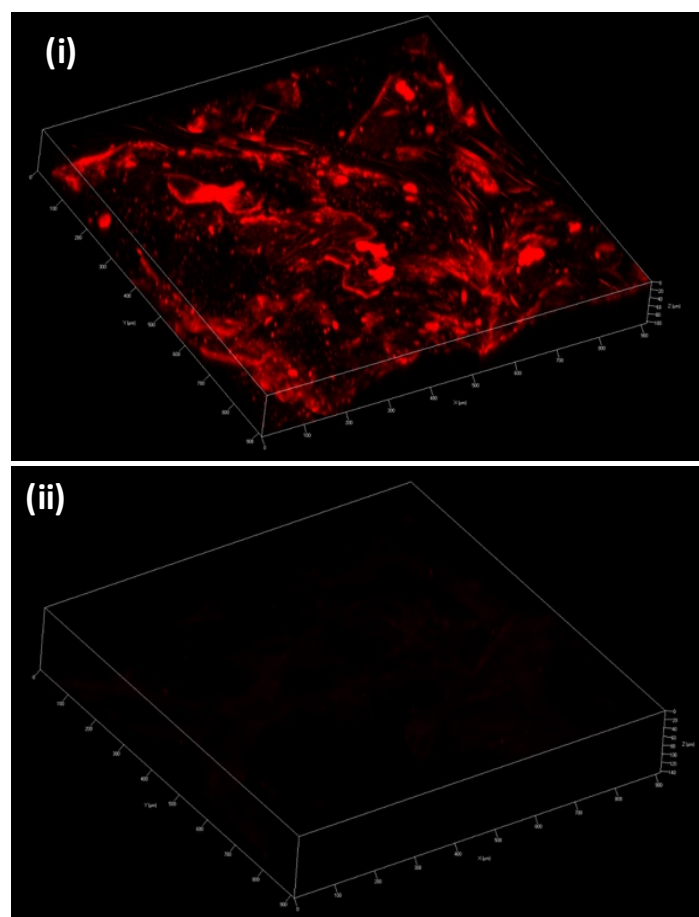


Fig. 8 Confocal 3D images of epidermis layer of YMP skin pretreated with IPM (5h), then treated with (i) PC-ER50/O and (ii) PC in PBS for 18 h.

As we discussed in Fig. 2 and Fig. 3, majority of PC released from the complex would be present in the fluorescently active, monomeric $\alpha\beta$ unit. Although the partial association to native hexameric $(\alpha\beta)_6$ form of PC would occur after the delivery into the skin, we couldn't confirm it at this stage. Taken together, the present findings

demonstrate that the active form of a protein with a subunit structure can be delivered through YMP skin if the protein of interest was formulated using a suitable surfactant.

2.4 Conclusions

Phycocyanin (PC), a high molecular weight protein with a subunit structure, was successfully delivered into the skin using an S/O nanodispersion technique. Nanosized particles in an oil vehicle, IPM, were formed by encapsulation of PC with a lipophilic surfactant, sucrose erucate (ER290). Spectroscopic studies indicated that PC folded in an inactive form in the S/O formulation but recovered its active structure when released into aqueous solution. Detailed in vitro skin permeation analyses clearly indicate that an S/O formulation with a low surfactant concentration facilitates the permeation of the active form of PC into the deep skin layers.

2.5 References

- [1] S. Boussiba, A.E. Richmond, Isolation and Characterization of Phycocyanins from Blue-Green Alga *Spirulina plantesis*, *Arch. Microbiol.* 120 (1979) 155–159.
- [2] M. Kupka, H. Scheer, Unfolding of C-phycocyanin followed by loss of non-covalent chromophore-protein interactions. 1. Equilibrium experiments, *Biochim. Biophys. Acta - Bioenerg.* 1777 (2008) 94–103. doi:10.1016/j.bbabi.2007.10.009.
- [3] S.K. Min, J.S. Park, L. Luo, Y.S. Kwon, H.C. Lee, S.H. Jung, I.D. Kim, J.K. Lee, H.S. Shin, Assessment of C-phycocyanin effect on astrocytes-mediated neuroprotection against oxidative brain injury using 2D and 3D astrocyte tissue model, *Sci.Rep.* 5 (2015) 14418. doi:10.1038/srep14418.

- [4] H.W. Chen, T.S. Yang, M.J. Chen, Y.C. Chang, E.I.C. Wang, C.L. Ho, Y.J. Lai, C.C. Yu, J.C. Chou, L.K.P. Chao, P.C. Liao, Purification and immunomodulating activity of C-phycoerythrin from *Spirulina platensis* cultured using power plant flue gas, *Process Biochem.* 49 (2014) 1337–1344. doi:10.1016/j.procbio.2014.05.006.
- [5] L.-C. Wu, Y.-Y. Lin, S.-Y. Yang, Y.-T. Weng, Y.-T. Tsai, Antimelanogenic effect of c-phycoerythrin through modulation of tyrosinase expression by upregulation of ERK and downregulation of p38 MAPK signaling pathways, *J. Biomed. Sci.* 18 (2011) 74. doi:10.1186/1423-0127-18-74.
- [6] B. Li, M.-H. Gao, X.-C. Zhang, X.-M. Chu, Molecular immune mechanism of C-phycoerythrin from *Spirulina platensis* induces apoptosis in HeLa cells in vitro., *Biotechnol. Appl. Biochem.* 43 (2006) 155–164. doi:10.1042/BA20050142.
- [7] R. Bingula, C. Dupuis, C. Pichon, J.Y. Berthon, M. Filaire, L. Pigeon, E. Filaire, Study of the Effects of Betaine and/or C-Phycocyanin on the Growth of Lung Cancer A549 Cells in Vitro and in Vivo, *J. Oncol.* 2016 (2016). doi:10.1155/2016/8162952.
- [8] G. Liao, B. Gao, Y. Gao, X. Yang, X. Cheng, Y. Ou, Phycocyanin Inhibits Tumorigenic Potential of Pancreatic Cancer Cells: Role of Apoptosis and Autophagy, (2016) 1–12. doi:10.1038/srep34564.
- [9] M. Ravi, S. Tentu, G. Baskar, S.R. Prasad, S. Raghavan, P. Jayaprakash, J. Jeyakanthan, S.K. Rayala, G. Venkatraman, Molecular mechanism of anti-cancer activity of phycocyanin in triple-negative breast cancer cells, (2015) 1–13. doi:10.1186/s12885-015-1784-x.
- [10] C. Liu, P. Quan, L. Fang, Effect of drug physicochemical properties on drug release and their relationship with drug skin permeation behaviors in hydroxyl

- pressure sensitive adhesive, *Eur. J. Pharm. Sci.* 93 (2016) 437–446. doi:10.1016/j.ejps.2016.08.048.
- [11] P. Karande, S. Mitragotri, Enhancement of transdermal drug delivery via synergistic action of chemicals, *Biochim. Biophys. Acta - Biomembr.* 1788 (2009) 2362–2373. doi:10.1016/j.bbamem.2009.08.015.
- [12] M. Kitaoka, R. Wakabayashi, N. Kamiya, M. Goto, Solid-in-oil nanodispersions for transdermal drug delivery systems, *Biotechnol. J.* 11 (2016) 1375–1385. doi:10.1002/biot.201600081.
- [13] G. Cevc, U. Vierl, Nanotechnology and the transdermal route. A state of the art review and critical appraisal, *J. Control. Release.* 141 (2010) 277–299. doi:10.1016/j.jconrel.2009.10.016.
- [14] M. Manconia, J. Pendás, N. Ledón, T. Moreira, C. Sinico, L. Saso, A.M. Fadda, Phycocyanin liposomes for topical anti-inflammatory activity: in-vitro in-vivo studies., *J. Pharm. Pharmacol.* 61 (2009) 423–430. doi:10.1211/jpp/61.04.0002.
- [15] C. Caddeo, M. Chessa, A. Vassallo, R. Pons, O. Diez-Sales, A.M. Fadda, M. Manconi, Extraction, purification and nanoformulation of natural phycocyanin (from Klamath algae) for dermal and deeper soft tissue delivery, *J. Biomed. Nanotechnol.* 9 (2013) 1929–1938. doi:10.1166/jbn.2013.1741.
- [16] I. Castangia, M.L. Manca, A. Catalán-Latorre, A.M. Maccioni, A.M. Fadda, M. Manconi, Phycocyanin-encapsulating hyalurosomes as carrier for skin delivery and protection from oxidative stress damage, *J. Mater. Sci. Mater. Med.* 27 (2016) 1–10. doi:10.1007/s10856-016-5687-4.
- [17] I. Castangia, M.L. Manca, C. Caddeo, G. Bacchetta, R. Pons, D. Demurtas, O. Diez-Sales, A.M. Fadda, M. Manconi, Santosomes as natural and efficient carriers for the improvement of phycocyanin reepithelising ability in vitro and in

- vivo, *Eur. J. Pharm. Biopharm.* 103 (2016) 149–158. doi:10.1016/j.ejpb.2016.03.033.
- [18] Y. Tahara, N. Kamiya, M. Goto, Solid-in-oil dispersion: A novel core technology for drug delivery systems, *Int. J. Pharm.* 438 (2012) 249–257. doi:10.1016/j.ijpharm.2012.09.007.
- [19] Y. Tahara, S. Honda, N. Kamiya, H. Piao, A. Hirata, E. Hayakawa, T. Fujii, M. Goto, A solid-in-oil nanodispersion for transcutaneous protein delivery, *J. Control. Release*. 131 (2008) 14–18. doi:10.1016/j.jconrel.2008.07.015.
- [20] M. Kitaoka, K. Imamura, Y. Hirakawa, Y. Tahara, N. Kamiya, M. Goto, Sucrose laurate-enhanced transcutaneous immunization with a solid-in-oil nanodispersion, *Medchemcomm.* 5 (2014) 20–24. doi:10.1039/c3md00164d.
- [21] Y. Hirakawa, R. Wakabayashi, A. Naritomi, M. Sakuragi, N. Kamiya, M. Goto, Transcutaneous immunization against cancer using solid-in-oil nanodispersions, *Med. Chem. Commun.* 6 (2015) 1387–1392. doi:10.1039/C5MD00168D.
- [22] M. Martins, N.G. Azoia, A. Ribeiro, U. Shimanovich, C. Silva, A. Cavaco-Paulo, In vitro and computational studies of transdermal perfusion of nanoformulations containing a large molecular weight protein, *Colloids Surfaces B Biointerfaces*. 108 (2013) 271–278. doi:10.1016/j.colsurfb.2013.02.032.
- [23] G. Faccio, M.M. Kämpf, C. Piatti, L. Thöny-Meyer, M. Richter, Tyrosinase-catalyzed site-specific immobilization of engineered C-phycoerythrin to surface, *Sci. Rep.* 4 (2014) 1–8. doi:10.1038/srep05370.
- [24] F. Yang, N. Kamiya, M. Goto, Transdermal delivery of the anti-rheumatic agent methotrexate using a solid-in-oil nanocarrier, *Eur. J. Pharm. Biopharm.* 82 (2012) 158–163. doi:10.1016/j.ejpb.2012.05.016.
- [25] N. Fujii, E. Aoki-Murakami, B. Tsuji, G.P. Kenny, K. Nagashima, N. Kondo, T.

- Nishiyasu, Body temperature and cold sensation during and following exercise under temperate room conditions in cold-sensitive young trained females, *Physiol. Rep.* 5 (2017) 1–13. doi:10.14814/phy2.13465.
- [26] Q. Zhang, M. Murawsky, T. LaCount, J. Hao, G.B. Kasting, B. Newman, P. Ghosh, S.G. Raney, S.K. Li, Characterization of Temperature Profiles in Skin and Transdermal Delivery System When Exposed to Temperature Gradients In Vivo and In Vitro, *Pharm. Res.* 34 (2017) 1491–1504. doi:10.1007/s11095-017-2171-x.
- [27] H. Yoshiura, M. Hashida, N. Kamiya, M. Goto, Factors affecting protein release behavior from surfactant-protein complexes under physiological conditions, *Int. J. Pharm.* 338 (2007) 174–179. doi:10.1016/j.ijpharm.2007.01.041.
- [28] K.L. Thoren, K.B. Connell, T.E. Robinson, D.D. Shellhamer, M.S. Tammara, Y.M. Gindt, The free energy of dissociation of oligomeric structure in phycocyanin is not linear with denaturant, *Biochemistry.* 45 (2006) 12050–12059. doi:10.1021/bi061140+.
- [29] M. Fujii, S. Yamanouchi, N. Hori, N. Iwanaga, K. Naruko, M. Matsumoto, Evaluation of Yucatan micropig skin for Use as an in vitro model for skin permeation study, *Biol. Pharm. Bull.* 20 (1997) 249–254. doi:10.1248/cpb.37.3229.
- [30] H. Takeuchi, M. Ishida, A. Furuya, H. Todo, H. Urano, K. Sugibayashi, Influence of Skin Thickness on the in Vitro Permeabilities of Drugs through Sprague-Dawley Rat or Yucatan Micropig Skin, *Biol. Pharm. Bull.* 35 (2012) 192–202. doi:10.1248/bpb.35.192.
- [31] H. Piao, N. Kamiya, A. Hirata, T. Fujii, M. Goto, A novel solid-in-oil nanosuspension for transdermal delivery of diclofenac sodium, *Pharm. Res.* 25

- (2008) 896–901. doi:10.1007/s11095-007-9445-7.
- [32] K. Sato, K. Sugibayashi, Y. Morimoto, Effect and mode of action of aliphatic esters on the in vitro skin permeation of nicorandil, *Int. J. Pharm.* 43 (1988) 31–40. doi:10.1016/0378-5173(88)90055-5.
- [33] I. Brinkmann, C.C. Müller-Goymann, An attempt to clarify the influence of glycerol, propylene glycol, isopropyl myristate and a combination of propylene glycol and isopropyl myristate on human stratum corneum, *Pharmazie*. 60 (2005) 215–220.

CHAPTER 3

A NOVEL GEL-IN-OIL NANODISPERSION FOR TRANSDERMAL PROTEIN DELIVERY

3.1 Introduction

Proteins have a high potential for treatment of various diseases, due to they have a particular set of functions and complexes; low potential to disrupt normal biological processes, and often well tolerated and lacking immunogenicity [1]. However, the delivery of the proteins into the target site remains a challenging, especially on transdermal delivery. Transdermal delivery is a non-invasive way for administration of proteins that can be utilized to bypass the variables of oral absorption and the pain and side effects associated with injections. The major challenge in transdermal delivery is the main barrier of skin, the stratum corneum layer (SC), which consists of corneocytes embedded in a highly organized lipid matrix, such as cholesterol, ceramide and free fatty acid[2][3]. Generally recognized that only relatively small molecular weight (typically <500 Da) could permeate through the skin by passive delivery[3][4]. Therefore, because of the protein has large molecular weight and hydrophilic nature, they might not easily to be penetrated into the skin.

Several approaches and delivery systems have been investigated and utilized to overcome the barrier of SC layer to attain higher skin permeability. An oil-based delivery system has been used for enhancing the permeability of water-soluble molecules into the skin, such as water-in-oil microemulsion[5][6], reverse micelles[7][8] and solid-in-oil nanodispersion[9][3][10]. In this formulation, the water-soluble molecules such as protein were solubilized into the oil phase by coating with

the hydrophobic surfactant. However, in this system the protein is surrounded by a surfactant in an oil vehicle with low water content. In addition, since the preparation process requires high-energy (i.e. high-speed homogenizers and the freeze drying[11][12]), the conformation of protein might be impaired, which affects the biological activity. For example, the tertiary structure of the proteins, such as phycocyanin[7] and phycoerythrin[5] might change when incorporated into reverse micelles with a molar ratio of water-to-surfactant (W_0) of <30 . Another sample showed that the enzymatic activity of horseradish peroxidase (HRP) was partially lost after released from a solid-in-oil suspension[13]. To overcome these issues, a novel oil-based drug system approach, a gel-in-oil (G/O) nanodispersion technique, is selected in the present study.

G/O nanodispersion is a gel-core oil-based system for protein delivery, in which protein drug is entrapped in the gelatin-based hydrogel coated with a hydrophobic surfactant using emulsion technology. A gelatin-based hydrogel is a semi-solid form of gelatin contains substantial water[14], which can protect the drug from hostile environments and also control drug release by changing the gel structure in response to environmental stimuli[15]. Gelatin is a natural hydrophilic polymer derived from the hydrolysis of collagen, which is a thermos-sensitive polymer with the reversible sol-gel transition. This natural polymer is highly biocompatible, biodegradable, non-immunogenicity and inexpensive biomaterial[14]; therefore, it has been recognized as a GRAS (Generally Regarded as Safe) material by the United States Food and Drug Administration. Gelatin has already been widely used in the pharmaceutical and medical application, such as it used as a stabilizer in vaccines against measles, mumps, rubella, Japanese encephalitis, rabies, diphtheria and tetanus toxin[16]. Uchida et al. also reported that the inactivation of Hepatitis B core antigen (HBcAg) by

methylene chloride was significantly reduced by addition of gelatin into the internal aqueous phase during a process of microencapsulation[17].

The aim of this study is fabrication of the G/O nanodispersion loaded with phycocyanin (PC) as a model protein drug, and evaluate their functional integrity and skin permeability. PC is a protein-chromophores complex derived from blue-green algae, such as *Spirulina*, contains α and β polypeptide subunit with three molecules of the chromophore on the $\alpha\beta$ units. PC has several pharmaceutical activities, including anticancer[18], anti-inflammatory[19], antioxidant[20] and wound healing effect[21]. The fluorescence intensity from these chromophores can be used as an indicator for monitoring tertiary structure of PC[22].

3.2 Materials and Methods

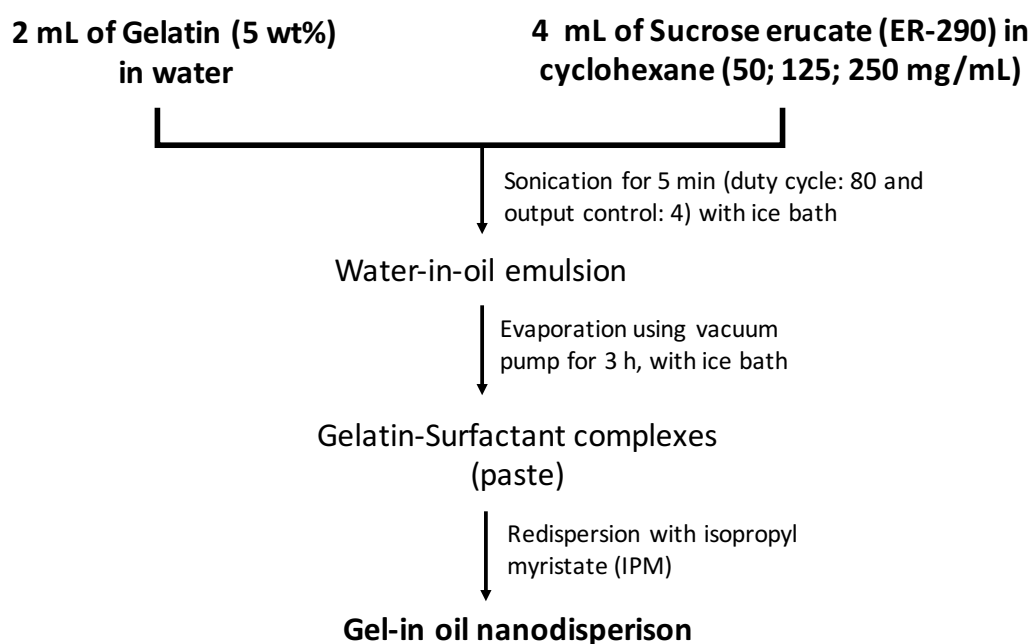
3.2.1 Materials

Gelatin from porcine skin (type A) was purchased from Sigma Aldrich (St Louis, USA). Phycocyanin (PC) from *Spirulina* was purchased from Tokyo Chemical Industries, Ltd. (Tokyo, Japan). Nonionic surfactant of sucrose erucate (commercial name, ER-290) was supplied by Mitsubishi-Kagaku Foods (Tokyo, Japan). Cyclohexane was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Isopropyl myristate was purchased from Tokyo Chemical Industries, (Tokyo, Japan). Yucatan Micropig (YMP) skin set (female; 5 months old) was obtained from Charles River Laboratories Japan, Inc (Kanagawa, Japan).

3.2.2 Methods

3.2.2.1 Preparation of gel-in-oil (G/O) nanodispersions

Briefly, gelatin (5 wt%) was dissolved in water solution at a temperature of 37 °C. Sucrose erucate (ER-290) in cyclohexane (50, 125 or 250 mg/mL) (4 mL) and 2 mL of gelatin solution were mixed using an Ultra-sonicator (TOMY Ultrasonic Disruptor UD-201, Tokyo, Japan) at duty cycle 80 and output control 4 with ice bath for 5 min to form a water-in-oil (W/O) emulsion. The cyclohexane in the W/O emulsion was removed using a vacuum pump (ULVAC DTC-21, Tokyo, Japan) with an ice-bath for 3-4 hours. The resulting viscous material was employed as a gelatin-surfactant complex. Finally, the paste of the complexes was dispersed with the isopropyl myristate (IPM) as oil phase (100 mL). A schematic illustration of the preparation of G/O nanodispersion is shown in Scheme 1.



Scheme 1. Preparation of gel-in-oil (G/O) nanodispersion

The size distribution and polydispersity index (PDI) of G/O nanodispersions were determined using dynamic light scattering (DLS) at 25 °C at detection angle 173°

with the run for ten measurement cycles (Zetasizer Nano-ZS, Malvern, Worcestershire, UK). The water content in G/O nanodispersion was evaluated by Karl Fischer titration method using a Karl Fischer moisture meter CA-200/VA-200 (Mitsubishi Chemical, Kanagawa, Japan). All samples were measured in triplicate.

3.2.2.2 Preparation of PC-loaded G/O nanodispersion

PC extracted from *Spirulina* was added to the gelatin solution with different ratio gelatin to PC to obtain the gelatin-PC solution. The total protein of gelatin-PC solution was fixed at 5 wt%. First, gelatin was dissolved in water at a temperature of 37 °C, then the aqueous PC was added into the gelatin solution. An aqueous gelatin-PC solution (2 mL) and four mL of sucrose erucate in cyclohexane (50 or 250 mg/mL) were mixed using an Ultra-sonicator (TOMY Ultrasonic Disruptor UD-201, Tokyo, Japan) to form a water-in-oil (W/O) emulsion containing phycocyanin. The cyclohexane in the emulsion was removed using a vacuum pump (ULVAC DTC-21, Tokyo, Japan) for 3-4 hours, then dispersed with 100 mL of isopropyl myristate as the oil phase to obtain phycocyanin loaded in gel-in-oil nanodispersion (PC-G/O nanodispersion).

3.2.2.3 Encapsulation efficiency of PC-G/O nanodispersion

Encapsulation efficiency of PC- G/O nanodispersion was determined following a previously described[10]. The gelatin-surfactant complex containing phycocyanin was added with five mL of ice-cold PBS, pH 7.4. The mixture was shaken and centrifuged (4°C, 18000 xg, 10 min). The free phycocyanin concentration in the supernatant solution was analysed by the UV-visible (UV-Vis) spectrophotometer (V-670, Jasco, Tokyo, Japan) at 25 °C, then quantified using an extinction coefficient ($\epsilon_{1\%}$) of 70 at 620 nm. The encapsulation efficiency (EE) was calculated using the following equation:

$$EE [\%] = ([\text{Phycocyanin}]_{\text{feed}} - [\text{Phycocyanin}]_{\text{supernatant}}) / [\text{Phycocyanin}]_{\text{feed}} \times 100$$

3.2.2.4 Extraction of PC from PC-G/O nanodispersion and S/O nanodispersion

Extraction of PC from PC-G/O nanodispersions was done by using a biphasic system described in our previously study[10]. Briefly, 1 mL of the oil-based formulation was placed onto five mL of warm PBS, pH 7.4. The biphasic system was vortexed for 5 min and incubated at 37 °C for 30 min, then centrifuged at 15,000x g for 20 min at 25 °C. The PC from the oil phase was released to the aqueous phase (PBS), which it was collected and filtered using a filter unit (Avantec®, Toyo Roshi Kaisha, Ltd, Japan) with a pore size of 0.2 µm. The concentration phycocyanin of in PBS was determined by the UV-visible (UV-Vis) spectrophotometer (V-670, Jasco, Tokyo, Japan) at 25 °C, then quantified using an extinction coefficient ($\epsilon^{1\%}$) of 70 at 620 nm.

3.2.2.5 Fluorescence spectroscopy

The spectroscopic properties of PC were recorded in the native state (PBS solution), in the PC-G/O nanodispersion and in the aqueous extraction solution (PBS) on a fluorescence spectrophotometer (LS55C, Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 590 nm (concentration of PC 10 µg/mL) at 25 °C.

3.2.2.6 *In vitro* skin permeation study

The *in vitro* skin permeation for G/O nanodispersion was evaluated using custom vertical diffusion Franz cells with the diffusion area of 0.785 cm². Yucatan Micropig (YMP) skin was used as a model skin due to their morphology of similar with human skin[23]. Briefly, the subcutaneous fat on YMP skin was carefully removed, then the skin was cut to 2 x 2 cm. Skin segments were mounted onto the receptor compartment of Franz cell with the stratum corneum (SC) layer facing the donor side of the cell, and then the donor compartment was clamped with the receptor

compartment. The receptor compartment was filled with five mL PBS (pH 7.4) and maintained at 32.5 °C with continuous stirring. An aliquot (300 µL) of an aqueous solution or G/O nanodispersion containing 0.2 mg/mL of PC was placed in the donor compartment, which was sealed with Parafilm and covered by aluminium foil to protect from light. For comparison, we also evaluated the skin permeation of phycocyanin dissolved in PBS.

After of the treatment for 24 h, the skin was washed and wiped with tissue paper to remove the excess of the sample on the top of the skin. The frozen of embedded skin samples were sectioned using a cryostat microtome at 15 µm (CM1860UV, Leica, Wetzlar, Germany), then examined by confocal laser scanning microscopy (CLSM) (LSM 700, ZEISS, Oberkochen, Germany). PC was detected at an excitation wavelength of 639 nm without a filter.

3.3 Results and Discussion

3.3.1 Preparation of G/O nanodispersion

We first optimised the preparation of G/O nanodispersion before incorporating a protein drug model. Sucrose fatty acid ester erucate (ER-290) was used as a hydrophobic surfactant for coated the gel core. ER-290 is a nonionic surfactant with the long alkyl chain, C22:1 and the hydrophilic/lipophilic balance (HLB) value of 2. ER-290 was chosen as a surfactant-based nanocarrier because it might be able to form the small droplet size of W/O emulsion[24] and enhanced delivery of large proteins through the SC layer of the skin[10].

G/O nanodispersion is prepared by forming water-in-cyclohexane emulsion using the ultrasonicator, then the removing of cyclohexane with the vacuum pump and

dispersion the paste of protein-surfactant complex with the oil phase as IPM (Scheme 1). Because of the ultra-sonication can be produced heat, we used the ice bath during emulsification to decrease the temperature of the emulsion. The temperature of the solution during emulsification is $\sim 34^{\circ}\text{C}$. The gelation of core part might be occurred during the cyclohexane removal using a vacuum pump with an ice bath, which the temperature of the emulsion was decreased. The gelation of porcine gelatin has occurred at the temperature below 31°C [25].

We evaluated the effect of three different weight ratio of the protein to ER-290 on G/O nanodispersions profiles. Dynamic light scattering (DLS, Zetasize Malvern) was used for analyzed the particle size of G/O nanodispersion. The mean size of G/O nanodispersions were in the nano-size range (140-190 nm) with narrow dispersity (Fig 1, Table 1). The particle size of G/O nanodispersion is depended on the surfactant concentration, and the increasing concentration of surfactant reduced the size of G/O nanodispersions.

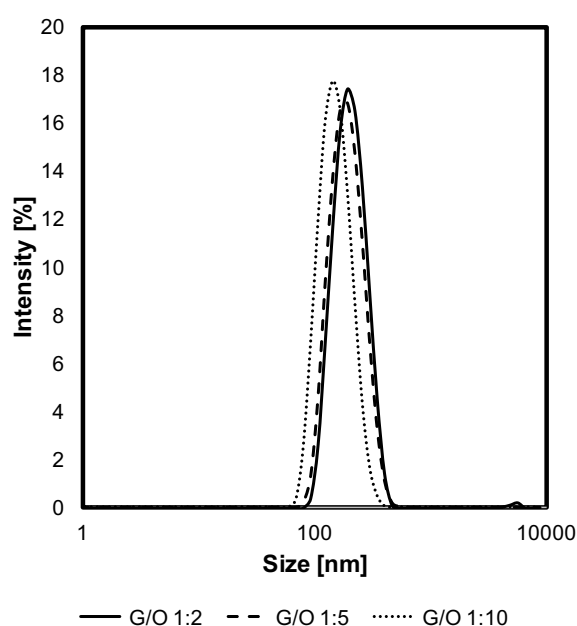


Fig. 1 The average size of G/O nanodispersion with three different weight ratio of the protein to surfactant

We also determined the water content of G/O nanodispersion using Karl Fischer titration method, to confirm the presence of gel-core of G/O nanodispersion. The water content of G/O nanodispersions is approximately 1.8 wt% for all formulations (Table 1), in other words, all water in gelatin solution might be entrapped in the core of G/O nanodispersion. These results indicated that the gel core-oil based system was successfully developed.

Table 1. Properties of G/O nanodispersion with different concentration of surfactant.

Sample code	Weight ratio gelatin to ER290	Z-size (nm)	PDI	Water content (%)
G/O 1:2	1:2	187.8 ± 3.4	0.08-0.188	1.84 ± 0.3
G/O 1:5	1:5	176.2 ± 94	0.09-0.150	1.83 ± 0.2
G/O 1:10	1:10	140.8 ± 9.9	0.043-0.147	1.79 ± 0.4

3.3.2 Characteristics of G/O nanodispersions loaded with PC (PC-G/O nanodispersion)

In this section, we investigated the loading of PC in G/O nanodispersion. The total protein concentration (gelatin and PC) was fixed at 5 wt%. Firstly, we observed the effect of weight percentage ratio of gelatin to PC on the gelation state of core composition before coated with G/O dispersions. The weight ratio of gelatin to PC affected to the gelation state of solution. The gel state of the solution obtained with the minimum concentration of gelatin at 3.5 wt% and loading concentration of PC of 1.5 wt%, as shown in Fig. 2. Therefore, we used three different weight percent ratio of gelatin to PC (with abbreviation G:P) on the core, i.e. G4.5: P0.5 (PC-G#1), G4: P1(PC-G#2); and G3.5: P1.5 (PC-G#3), to evaluate the functional integrity of PC in oil-based formulations .

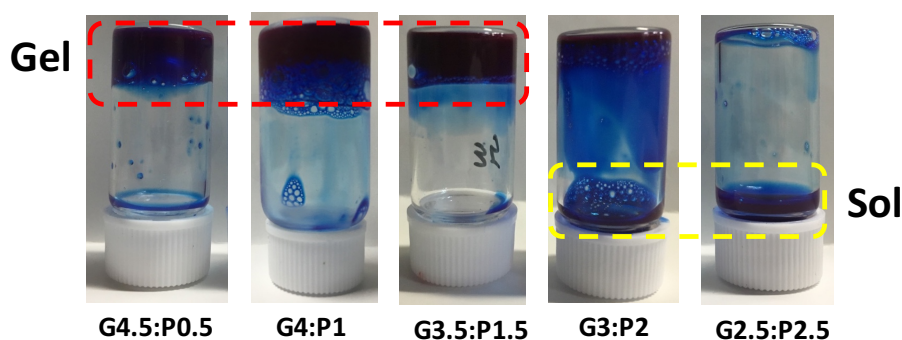


Fig. 2 Photograph of the gelatin and phycocyanin solution with different weight ratio at the temperature of 25 °C.

We prepared the PC in G/O nanodispersions with two weight ratio of total protein to surfactant for evaluating its effect on the encapsulation efficiency of PC in both oil-based formulations, i.e. 1:2 and 1:100. For the composition of the core part, we used the ratio of G:P of 3.5:1.5. The encapsulation efficiency of PC in G/O nanodispersions was determined by solid-liquid extraction method with PBS. The unencapsulation of PC in PBS was evaluated using a UV-Vis spectroscopy. As shown in Fig. 3, the encapsulation efficiency of PC in G/O nanodispersions was depended on the weight ratio of total protein to the surfactant, which the increase of weight ratio protein to surfactant might enhance the encapsulation efficiency of PC in both oil-based formulations. The high entrapment efficiency of PC was achieved by using G/O nanodispersion technique, even with a low concentration of surfactant (> 92%). Moreover, the different ratio of gelatin to phycocyanin did not affect the encapsulation efficiency of PC-G/O nanodispersion (Fig. 4). It suggested that the gel core of oil-based formulation might improve the entrapment of biomacromolecules.

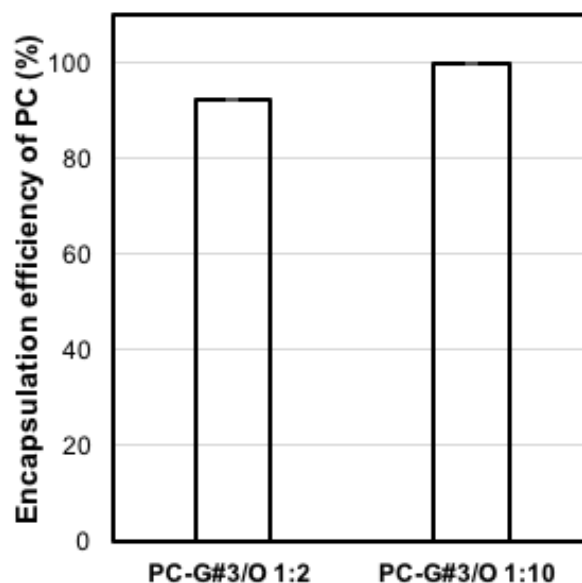


Fig. 3 The encapsulation efficiency of PC in the oil-based system with two different weight ratios of protein to surfactant. PC-G# 3/O: the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 3.5:1.5.

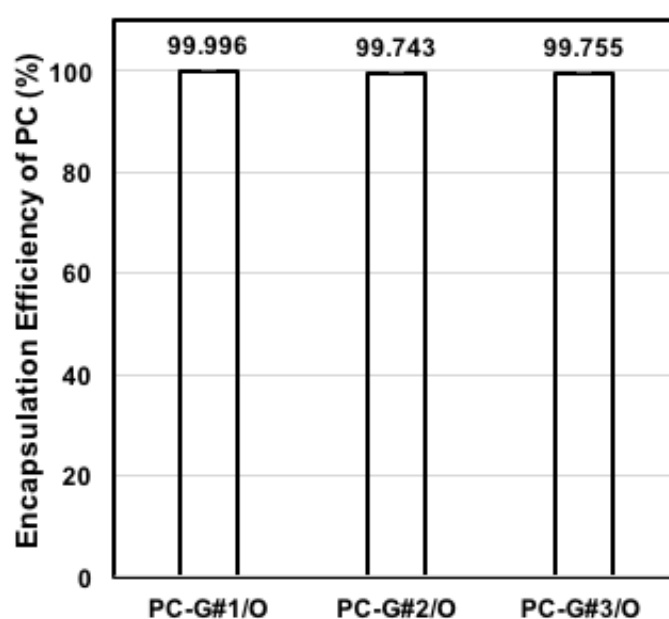


Fig. 4 The encapsulation efficiency of PC in the oil-based system with three different weight ratios of gelatin to phycocyanin (G:P). PC-G#1/O: G/O nanodispersion with the weight ratio of G:P of 4.5:0.5; PC-G#2/O: G/O nanodispersion with the weight ratio of G:P of 4:1; and PC-G#3/O: G/O nanodispersion with the weight ratio of G:P of 3.5:1.5.

We also evaluated the effect of the ratio of gelatin to PC in G/O nanodispersions on the water content. Fig. 5 showed that the concentration of gelatin affected to the water content on G/O nanodispersion. The decreasing the concentration of gelatin decreased the water content of G/O nanodispersions. This result indicated that the gelatin content affected to the absorption water ability of hydrogel. The gelatin polymer network absorbs water through hydrogen bonds formed between water molecules and carboxylic acid and amino groups [26].

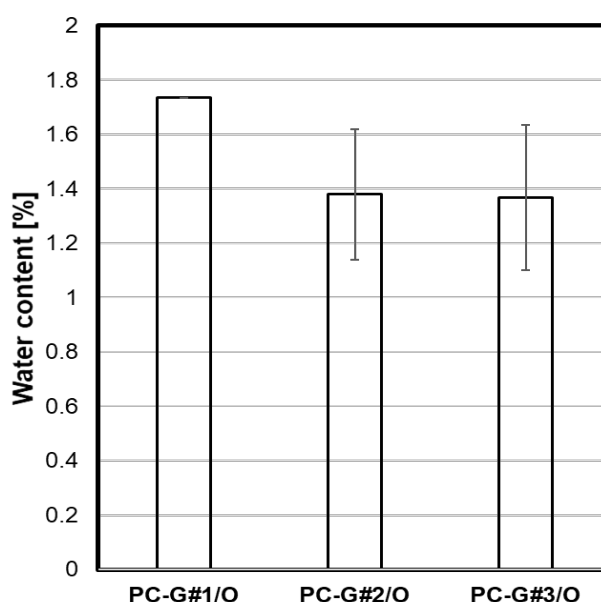


Fig 5. The water content of PC-G/O nanodispersions with three different weight ratio of gelatin to phycocyanin (G:P). PC-G/O was prepared by using weight ratio total protein to surfactant of 1:10. PC-G#1/O: G/O nanodispersion with the weight ratio of G:P of 4.5:0.5; PC-G#2/O: G/O nanodispersion with the weight ratio of G:P of 4:1; and PC-G#3/O: G/O nanodispersion with the weight ratio of G:P of 3.5:1.5.

3.3.3 Fluorescence spectra of phycocyanin (PC)

Given fluorescence intensity of PC related to the biological activity of PC, we used it to evaluate the functional integrity of the PC. Fluorescence spectroscopy might

be used to monitor the conformation state of PC on the oil-based formulation and PC extracted from that of formulations, which the conformation changed the structure of PC is indicated by decreasing the fluorescence intensity. The disappearance fluorescence intensity of PC shows the full unfolding of PC[22].

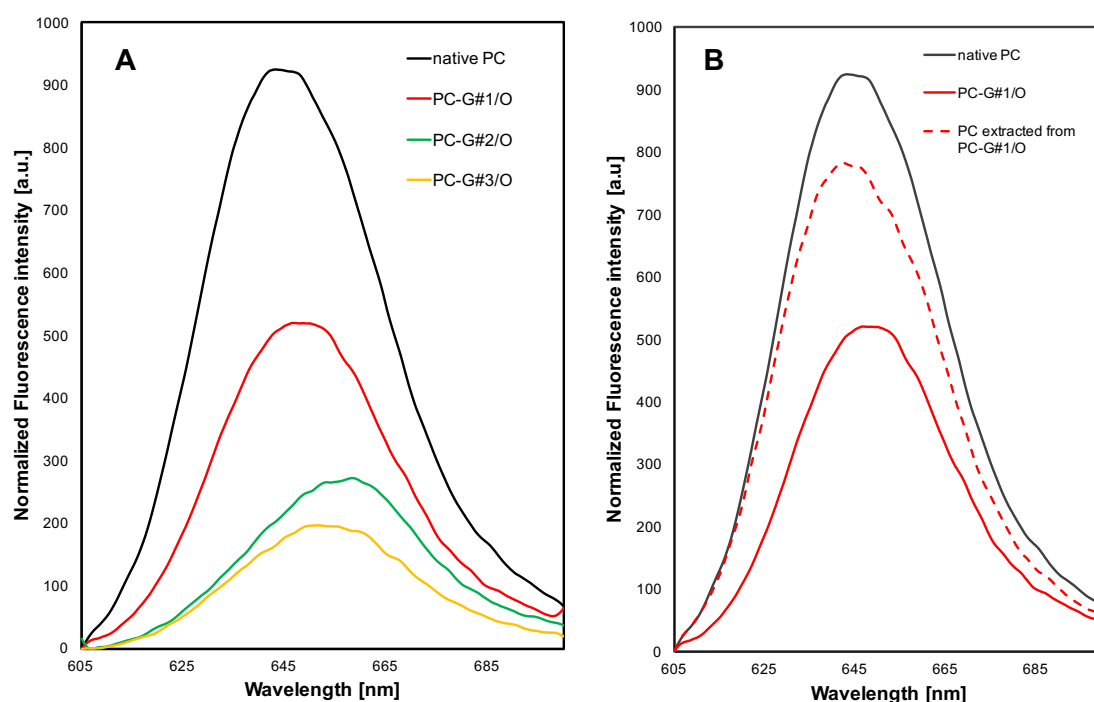


Fig. 6 Fluorescence spectra of PC in G/O nanodispersion with three different ratios of gelatin to PC (A) and fluorescence spectra of PC extracted from G/O nanodispersion at 25 °C. Concentration of PC of 10 μ g/mL. PC-G/O was prepared by using weight ratio total protein to surfactant of 1:2. PC-G#1/O: the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 4.5:0.5; PC-G#2/O: the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 4:1; PC-G#3/O: the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 3.5:1.5.

We prepared the G/O nanodispersion using three different weight per cent ratio of gelatin to PC in the core-part with weight ratio total protein and surfactant of 1:2. As shown in Fig. 6, the fluorescence of PC retained (up to 56%) when coated with G/O nanodispersion (with abbreviation PC-G#1/O). However, the fluorescence intensity of

PC-loaded G/O nanodispersions was decreased when the concentration of gelatin in the core decreased. Similar results were also obtained on PC-G/O naodispesions with weight ratio total protein to surfactant of 1:10 (data not shown). The fluorescent intensity of PC in PC-G/O nanodispersion may be correlated with the water content of the G/O nanodispersions (Fig 5), therefore we assumed that the retaining the PC structure in G/O nanodispersion depends on the water absorption ability of gelatin. The water content of the core-shell system is crucial for maintained the native structure of proteins[7][5].

To gain an insight of functional integrity of PC after released from G/O nanodispersions, we evaluated fluorescence intensity of the PC extracted from G/O nanodispersion using a biphasic extraction system. The PC extracted in aqueous phase showed the fluorescence intensity, and the extracted PC from G/O nanodispersion was well recovered to that of the native structure of PC, as illustrated in Fig 6B. This result suggested that the gelatin matrix might be able to protect the conformation of biomacromolecules such as PC in oil-based formulations. Gelatin is known as an amphiphilic molecule that can stabilize the protein during the emulsification by competing for container surfaces at the oil-water interfaces[17][27][28][29].

3.3.4 *In vitro* skin permeation studies

We evaluated the skin permeability of PC loaded G/O nanodispersion (PC-G#1/O) with two different of weight ratio total protein to surfactant, i.e. 1:2 and 1:10. For the comparison, we performed the aqueous solution of PC on the YMP skin for 24 h. Isopropyl myristate used as an oil phase of G/O nanodispersions, known as a penetration enhancer by disrupting the lipid order and increasing the fluidity of SC layer [30][31].

The permeation of PC into the skin was observed using CSLM, which the PC indicated by the red fluorescence. Fig. 7 showed PC-G/O nanodispersion had a stronger fluorescent intensity on the SC layer, compared than the aqueous solution of PC, implying that the encapsulating of protein into the oil-based delivery system could promote the penetration of protein through the main barrier of skin, i.e., stratum corneum layer. The highest penetration of PC was observed when G/O nanodispersion was formed with weight ratio total protein to ER-290 of 1:2, where indicated by the strong fluorescence intensity from the skin surface until the deep skin layer (Fig. 7). This suggested that the concentration of surfactant on G/O nanodispersion is a critical factor for increasing the permeability of PC through the skin. The excess surfactant in the G/O nanodispersion could reduce the diffusion of protein into the epidermis layer, which might be due to the slower release of the protein from the protein-surfactant complexes[24]:[32].

Since the melting point of porcine gelatin is 32 °C[25], we expected that the gelatin matrix of G/O nanodispersion was melted into the sol phase when it applied into the skin. The gelatin matrix-coated hydrophobic surfactant can penetrate into the lipid layer of the SC facilitated by the IPM as an oil phase. After crossing the SC layer, the protein would be released from G/O nanodispersion and diffused into the epidermis or dermis layer, while the hydrophobic surfactant on the G/O nanodispersion might be liberated at the interface between SC and epidermis. To confirm this point, we prepared the G/O nanodispersion using gelatin-FITC as a core part and adding rhodamine-labelled DOPE (0.05 wt.%) on the surfactant solution. The green and red fluorescences were detected at the same time. The results are shown in Fig. 8, and the red fluorescence associated with rhodamine was observed on the skin surface (0 μm) to a skin depth of 20 μm , which it is the SC layer of the skin. On the other hand,

the green fluorescence of FITC was observed more deeply into the epidermis layer. These results suggested that the hydrophobic surfactant could be separated from the protein at a point between SC and epidermis layer.

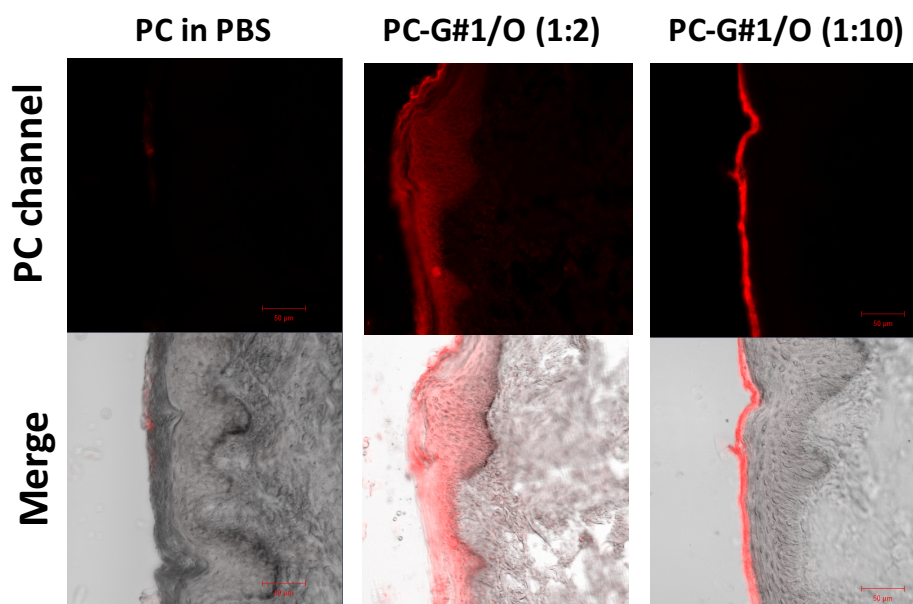


Fig. 7 Confocal image of the Yucatan Micropig (YMP) skin section after treated with phycocyanin with different formulation: PC in PBS: PC dissolved in PBS, pH 7.4; PC-G#1/O (1:2): the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 4.5:0.5 and weight ratio of protein to surfactant of 1:2; PC-G#1/O (1:10): the gel-in-oil nanodispersion containing phycocyanin with the weight ratio of G:P of 4.5:0.5 and weight ratio of protein to surfactant of 1:10.

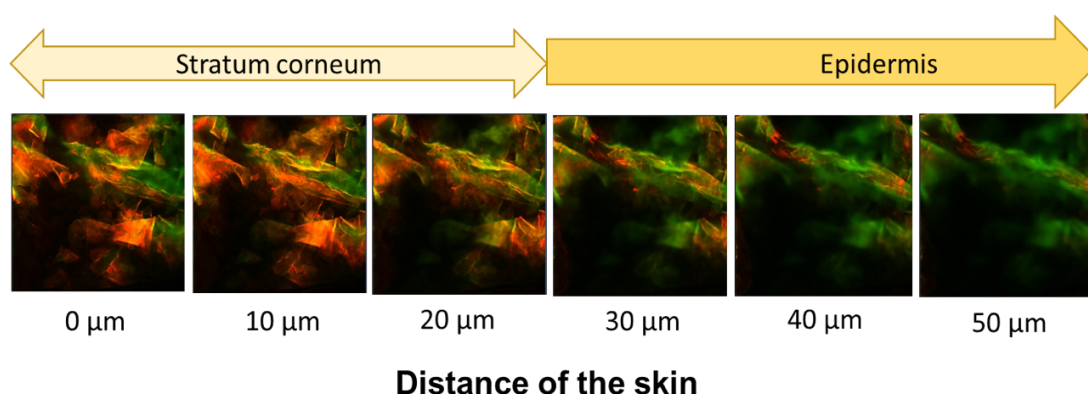


Fig. 8 CLSM image of YMP skin with different depth of skin layer. The red fluorescence showed Rhodamin-DOPE; and the green fluorescence showed the gelatin-FITC.

We also investigated the skin permeation route of PC-G#1/O nanodispersion on YMP skin using CLSM. Fig. 9 shows the red fluorescence of PC between corneocyte cell, which indicated that the PC-G/O nanodispersion was delivered PC through the skin via intercellular pathway. The use of IPM as an oil vesicle of G/O nanodispersion might promote the penetration of PC through intercellular route, because it is able to disrupt the lipid order and increase the fluidity of SC layer. Taken together the present finding demonstrated that the G/O nanodispersion has a high potential for transdermal protein delivery.

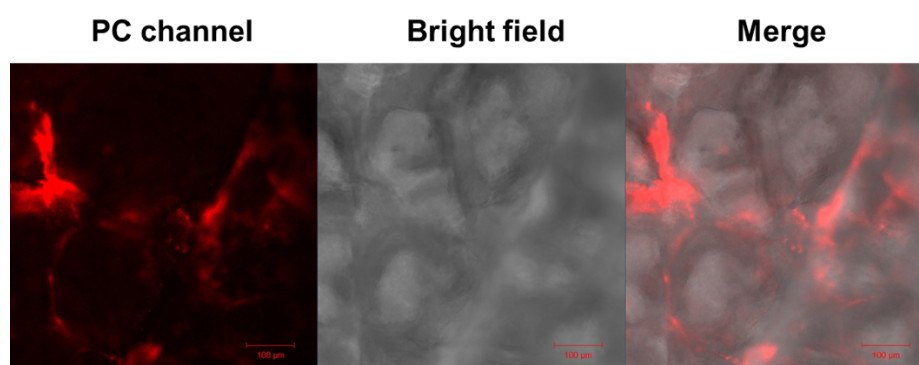


Fig. 9 CLSM images of transverse skin section after treated with PC-G#1/O nanodispersion with weight ratio total protein to surfactant of 1:2 on YMP skin for 24 h. Scale bar: 100 µm.

3.4 Conclusion

We demonstrated a novel oil-based delivery system for the delivery of large protein assembly through the skin. G/O nanodispersion achieved a nano-sized range of particle formation with high entrapment efficiency of protein. Spectroscopic studies indicated that G/O nanodispersion might be able to retain and protect an active form of the protein. *In vitro* skin permeation studies showed the enhanced permeability of protein delivery through the deep skin layer using G/O nanodispersion with a low

concentration of surfactant. These results suggested that the G/O nanodispersion has a high potential for transdermal protein delivery in pharmaceutical fields.

3.5 References

- [1] B. Leader, Q.J. Baca, D.E. Golan, Protein therapeutics: A summary and pharmacological classification, *Nat. Rev. Drug Discov.* 7 (2008) 21–39. doi:10.1038/nrd2399.
- [2] M. Janssens, J. van Smeden, G.S. Gooris, W. Bras, G. Portale, P.J. Caspers, R.J. Vreeken, T. Hankemeier, S. Kezic, R. Wolterbeek, A.P. Lavrijsen, J.A. Bouwstra, Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients, *J. Lipid Res.* 53 (2012) 2755–2766. doi:10.1194/jlr.P030338.
- [3] M. Kitaoka, R. Wakabayashi, N. Kamiya, M. Goto, Solid-in-oil nanodispersions for transdermal drug delivery systems, *Biotechnol. J.* 11 (2016) 1375–1385. doi:10.1002/biot.201600081.
- [4] J.D. Bos, M.M.H.M. Meinardi, The 500 Dalton rule for the skin penetration of chemical compounds and drugs, *Exp. Dermatol.* 9 (2000) 165–169. doi:10.1034/j.1600-0625.2000.009003165.x.
- [5] R. Bermejo, D.J. Tobaruela, E.M. Talavera, A. Orte, J.M. Alvarez-Pez, Fluorescent behavior of B-phycoerythrin in microemulsions of aerosol OT/water/isooctane, *J. Colloid Interface Sci.* 263 (2003) 616–624. doi:10.1016/S0021-9797(03)00391-6.
- [6] G. Russell-Jones, R. Himes, Water-in-oil microemulsions for effective

- transdermal delivery of proteins., *Expert Opin. Drug Deliv.* 8 (2011) 537–546.
doi:10.1517/17425247.2011.559458.
- [7] R. Bermejo, E.M. Talavera, C. DelValle, J.M. Alvarez-Pez, C-phycocyanin incorporated into reverse micelles: A fluorescence study, *Colloids Surfaces B Biointerfaces*. 18 (2000) 51–59. doi:10.1016/S0927-7765(99)00129-0.
- [8] M.C. Jones, H. Gao, J.C. Leroux, Reverse polymeric micelles for pharmaceutical applications, *J. Control. Release*. 132 (2008) 208–215. doi:10.1016/j.jconrel.2008.05.006.
- [9] Y. Tahara, S. Honda, N. Kamiya, H. Piao, A. Hirata, E. Hayakawa, T. Fujii, M. Goto, A solid-in-oil nanodispersion for transcutaneous protein delivery, *J. Control. Release*. 131 (2008) 14–18. doi:10.1016/j.jconrel.2008.07.015.
- [10] S.D. Hardiningtyas, R. Wakabayashi, M. Kitaoka, Y. Tahara, K. Minamihata, M. Goto, N. Kamiya, Mechanistic investigation of transcutaneous protein delivery using solid-in-oil nanodispersion: A case study with phycocyanin, *Eur. J. Pharm. Biopharm.* 127 (2018). doi:10.1016/j.ejpb.2018.01.020.
- [11] T. Wang, N. Wang, A. Hao, X. He, T. Li, Y. Deng, Lyophilization of water-in-oil emulsions to prepare phospholipid-based anhydrous reverse micelles for oral peptide delivery, *Eur. J. Pharm. Sci.* 39 (2010) 373–379. doi:10.1016/j.ejps.2010.01.006.
- [12] Y. Tahara, N. Kamiya, M. Goto, Solid-in-oil dispersion: A novel core technology for drug delivery systems, *Int. J. Pharm.* 438 (2012) 249–257. doi:10.1016/j.ijpharm.2012.09.007.
- [13] H. Yoshiura, Y. Tahara, M. Hashida, N. Kamiya, A. Hirata, T. Fujii, M. Goto, Design and in vivo evaluation of solid-in-oil suspension for oral delivery of

- human growth hormone, *Biochem. Eng. J.* 41 (2008) 106–110. doi:10.1016/j.bej.2008.04.001.
- [14] M. Foox, M. Zilberman, Drug delivery from gelatin-based systems, *Expert Opin. Drug Deliv.* 12 (2015) 1547–1563. doi:10.1517/17425247.2015.1037272.
- [15] Y. Qiu, K. Park, Environment-sensitive hydrogels for drug delivery, *Adv. Drug Deliv. Rev.* 64 (2012) 49–60. doi:10.1016/j.addr.2012.09.024.
- [16] J.L. Tlaxca, S. Ellis, R.L. Remmele, Live attenuated and inactivated viral vaccine formulation and nasal delivery: Potential and challenges, *Adv. Drug Deliv. Rev.* 93 (2015) 56–78. doi:10.1016/j.addr.2014.10.002.
- [17] T. Ushida, K. Shiosaki, Y. Nakada, K. Fukuda, Y. Eda, S. Tokiyoshi, N. Nageraya, K. Matsuyama, Microencapsulation of Hepatitis B COre Antigen for Vaccine Preparation, *Pharm. Res.* 15 (1998) 1708–1713.
- [18] L.C. Wu, Y.Y. Lin, S.Y. Yang, Y.T. Weng, Y.T. Tsai, Antimelanogenic effect of c-phycocyanin through modulation of tyrosinase expression by upregulation of ERK and downregulation of p38 MAPK signaling pathways, *J. Biomed. Sci.* 18 (2011) 1–11. doi:10.1186/1423-0127-18-74.
- [19] S. Hao, Y. Yan, W. Huang, F. Gai, J. Wang, L. Liu, C. Wang, C-phycocyanin reduces inflammation by inhibiting NF- κ B activity through downregulating PDCD5 in lipopolysaccharide-induced RAW 264.7 macrophages, *J. Funct. Foods.* 42 (2018) 21–29. doi:10.1016/j.jff.2018.01.008.
- [20] R. Thangam, V. Suresh, W. Asenath Princy, M. Rajkumar, N. Senthilkumar, P. Gunasekaran, R. Rengasamy, C. Anbazhagan, K. Kaveri, S. Kannan, C-Phycocyanin from *Oscillatoria tenuis* exhibited an antioxidant and in vitro antiproliferative activity through induction of apoptosis and G0/G1 cell cycle

- arrest, *Food Chem.* 140 (2013) 262–272. doi:10.1016/j.foodchem.2013.02.060.
- [21] I. Castangia, M.L. Manca, C. Caddeo, G. Bacchetta, R. Pons, D. Demurtas, O. Diez-Sales, A.M. Fadda, M. Manconi, Santosomes as natural and efficient carriers for the improvement of phycocyanin reepithelising ability in vitro and in vivo, *Eur. J. Pharm. Biopharm.* 103 (2016) 149–158. doi:10.1016/j.ejpb.2016.03.033.
- [22] M. Kupka, H. Scheer, Unfolding of C-phycocyanin followed by loss of non-covalent chromophore-protein interactions. 1. Equilibrium experiments, *Biochim. Biophys. Acta - Bioenerg.* 1777 (2008) 94–103. doi:10.1016/j.bbabi.2007.10.009.
- [23] M. Fujii, S. Yamanouchi, N. Hori, N. Iwanaga, K. Naruko, M. Matsumoto, Evaluation of Yucatan micropig skin for Use as an in vitro model for skin permeation study, *Biol. Pharm. Bull.* 20 (1997) 249–254. doi:10.1248/cpb.37.3229.
- [24] H. Yoshiura, M. Hashida, N. Kamiya, M. Goto, Factors affecting protein release behavior from surfactant-protein complexes under physiological conditions, *Int. J. Pharm.* 338 (2007) 174–179. doi:10.1016/j.ijpharm.2007.01.041.
- [25] G. Ninan, J. Joseph, Z.A. Aliyamveetil, A comparative study on the physical, chemical and functional properties of carp skin and mammalian gelatins, *J. Food Sci. Technol.* 51 (2014) 2085–2091. doi:10.1007/s13197-012-0681-4.
- [26] Q. Xing, K. Yates, C. Vogt, Z. Qian, M.C. Frost, F. Zhao, Increasing mechanical strength of gelatin hydrogels by divalent metal ion removal, *Sci. Rep.* 4 (2014) 1–10. doi:10.1038/srep04706.
- [27] J.D. Duncan, P.X. Wang, C.M. Harrington, D.P. Schafer, Y. Matsuoka, J.F.

- Mestecky, R.W. Compans, M.J. Novak, Comparative analysis of oral delivery systems for live rotavirus vaccines, *J. Control. Release.* 41 (1996) 237–247. doi:10.1016/0168-3659(96)01331-4.
- [28] L. Meinel, O.E. Illi, J. Zapf, M. Malfanti, H. Peter Merkle, B. Gander, Stabilizing insulin-like growth factor-I in poly(D,L-lactide-co-glycolide) microspheres, *J. Control. Release.* 70 (2001) 193–202. doi:10.1016/S0168-3659(00)00352-7.
- [29] S.Y. Tzeng, R. Guarecuco, K.J. McHugh, S. Rose, E.M. Rosenberg, Y. Zeng, R. Langer, A. Jaklenec, Thermostabilization of inactivated polio vaccine in PLGA-based microspheres for pulsatile release, *J. Control. Release.* 233 (2016) 101–113. doi:10.1016/j.jconrel.2016.05.012.
- [30] K. Sato, K. Sugibayashi, Y. Morimoto, Effect and mode of action of aliphatic esters on the in vitro skin permeation of nicorandil, *Int. J. Pharm.* 43 (1988) 31–40. doi:10.1016/0378-5173(88)90055-5.
- [31] I. Brinkmann, C.C. Müller-Goymann, An attempt to clarify the influence of glycerol, propylene glycol, isopropyl myristate and a combination of propylene glycol and isopropyl myristate on human stratum corneum, *Pharmazie.* 60 (2005) 215–220.
- [32] M. Kitaoka, K. Imamura, Y. Hirakawa, Y. Tahara, N. Kamiya, M. Goto, Sucrose laurate-enhanced transcutaneous immunization with a solid-in-oil nanodispersion, *Medchemcomm.* 5 (2014) 20–24. doi:10.1039/c3md00164d.

CHAPTER 4

ENHANCED POTENTIAL OF THERAPEUTIC APPLICATIONS OF CURCUMIN USING SOLID-IN-WATER NANODISPERSION TECHNIQUE

4.1 Introduction

Cancer is a severe disease with high mortality, accounting for 8.8 million deaths worldwide (about 17% of all deaths) in 2015 [1]. Polyphenol compounds are natural products that have shown promising anticancer activities. Diferuloylmethane or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, also known as curcumin, is a hydrophobic polyphenolic compound derived from the rhizome of the plant *Curcuma longa* L. the chemical structure of curcumin was shown in Fig. 1.

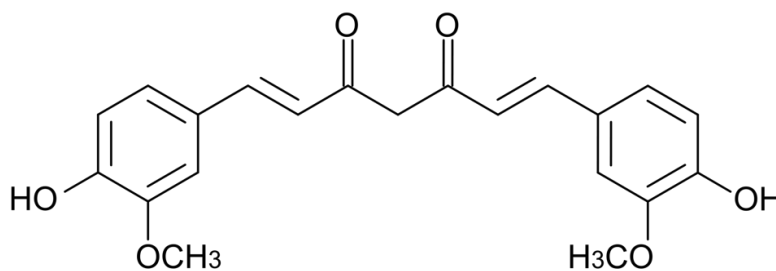


Fig. 1 The chemical structure of curcumin

Curcumin has a low molecular weight and is potentially a promising compound for cancer prevention and treatment [2,3]. *In vivo* studies have shown that curcumin suppresses the proliferation of various types of cancer cells, such as breast cancer [4], colon cancer [5] and pancreatic cancer [6]. Curcumin also has multiple pharmacological activities, including antibacterial, antioxidant, anti-inflammatory [2],

and antiviral activities [7]. Clinical trials have shown limited toxicity of curcumin even at very high doses of 12 g/day [8]. Despite the favorable pharmacodynamics and toxicity profiles, its therapeutic applications are restricted by its extremely low bioavailability and poor absorption. In two studies, little or no curcumin was detected in serum after oral or intravenous administration [8,9]. The low bioavailability of curcumin is attributed to its very low aqueous solubility at neutral or acidic pH, where the solubility of curcumin in a buffer of pH 5 is reportedly 3.8×10^{-8} M [10].

Over the past decade, there has been increasing development of formulation technology that aims to improve the solubility and pharmacokinetics of curcumin. Nanocarriers are among the most promising drug delivery systems for realizing curcumin's potential as a preventive or therapeutic agent. A number of nanocarrier systems loaded with curcumin have been reported to date, such as liposomes [6], polymer micelles [5,11], chitosan nanoparticles [12] and nanoemulsions [13].

In this study, we aimed to improve the solubility and bioavailability of curcumin by developing a new aqueous formulation using the solid-in-water (S/W) nanodispersion technique [14]. This novel technique comprises simple preparation protocols: emulsification and freeze-drying for encapsulating hydrophobic biomolecules with a hydrophilic surfactant, followed by redispersion of the resultant solid complexes in an aqueous solution. Our previous studies have demonstrated that this technique enables the encapsulation of the poorly soluble anticancer drug camptothecin (CPT) with high efficiency and stability [14]. Here, we applied this formulation strategy to study the nanodispersion of curcumin in the aqueous phase and characterize the S/W nanodispersion in detail.

4.2 Material and Method

4.2.1 Materials

Curcumin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Pluronic F68 and F127 were purchased from MP Biomedical, Inc (Solon, Ohio) and Sigma-Aldrich Co. (St. Louise, USA), respectively. Rhodamine-labelled dioleoyl phosphatidylcholine (Rho-DOPE) was purchased from Polar Lipid, Inc. (Alabama). HeLa cell lines were purchased from RIKEN Cell Bank and were cultured in minimum essential medium (MEM) supplement with 10% fetal bovine serum (FBS) and 1% of antibiotic-antimycotic mixed stock (Nacalai Tesque, Inc). All cells were incubated at 37 °C with humidified 5% CO₂ atmosphere.

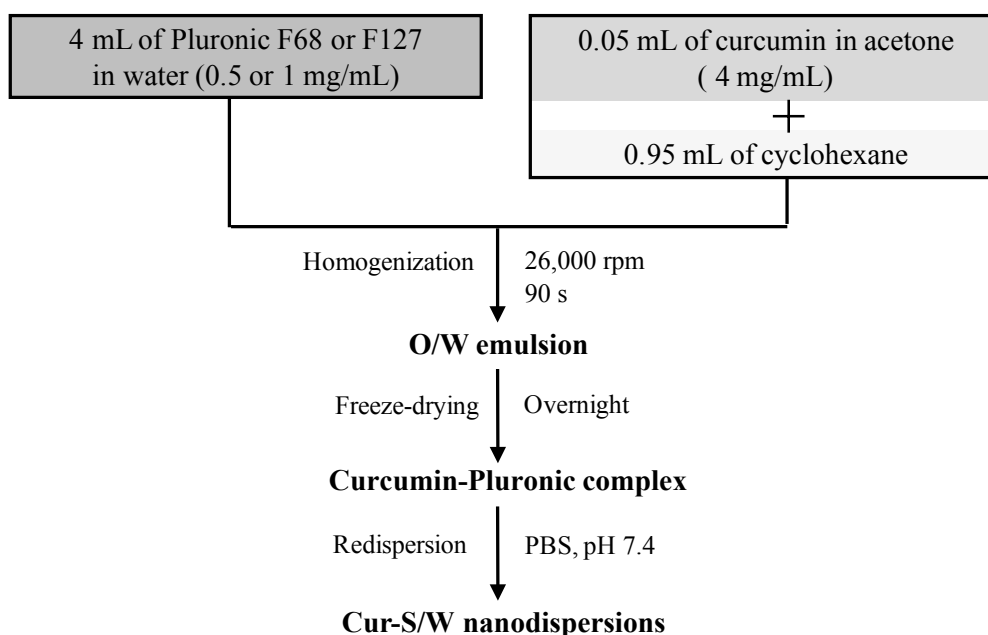
OPTI-MEM was purchased from Thermo Fisher Scientific, Inc. Cell counting kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Dulbecco's phosphate-buffered saline (D-PBS) was purchased from Nacalai Tesque, Inc (Kyoto, Japan). All other chemicals were of analytical grade.

4.2.2 Method

4.2.2.1 Preparation of curcumin loading solid-in-water nanodispersion

Briefly, curcumin solution was prepared by first dissolving curcumin in acetone to a concentration of 4 mg/mL. Curcumin in acetone (0.05 mL) was added to cyclohexane (0.95 mL). Then, 1 mL of the curcumin solution and 4 mL of an aqueous solution of Pluronic F68 or F127 (0.5 or 1 mg/mL) were mixed using a homogenizer (Polytron, Switzerland) at 26,000 rpm for 90 s to form an oil-in-water emulsion. The emulsion was immediately frozen in liquid nitrogen for 20 min and lyophilized overnight using a freeze dryer (EYELA, Tokyo, Japan) to obtain curcumin-pluronic complexes. The resultant complexes were dispersed in PBS (pH 7.4) to the final concentration of 0.1 mg/mL and this solution was employed as a curcumin-solid-in-water nanodispersion

(Cur-S/W nanodispersion). The preparation procedure of Cur-S/W nanodispersions is illustrated in Scheme 1.



Scheme 1. Preparation procedure of Cur-S/W nanodispersions.

4.2.2.2 Characterization of Cur-S/W nanodispersions

The size distribution and polydispersity index of Cur-S/W nanodispersions were determined using dynamic light scattering (DLS) at 25 °C at a detection angle of 173° with 10 measurement cycles per run (Zetasizer Nano-ZS, Malvern, Worcestershire, UK). The zeta potential was obtained using the same instrument. The measurement was performed in triplicate.

The encapsulation efficiency of Cur-S/W nanodispersions was determined by the syringe filtration method. The Cur-S/W nanodispersion was filtrated using a filter unit with pore size of 0.45 µm (Avatec®, Toyo Roshi Kaisha, Ltd.) to remove free curcumin. The concentration of curcumin was estimated by a reverse-phase high performance liquid chromatography (HPLC) unit equipped with an auto sampler (Jasco, LC-4000, Jasco Inc., Easton, MD, USA). The samples were diluted 2-fold using acetonitrile

before injection. 20 μ L of the sample was analyzed by a C₁₈ column (4.6 $\times$ 250 mm, Intersustain® C₁₈) with a mobile phase of acetonitrile containing 0.1% trifluoroacetic acid (55% v/v) and milli-Q-water at a flow rate of 0.8 mL/min. The encapsulation efficiency (EE) and loading capacity (LC) of curcumin in S/W nanodispersions were calculated using the equations below:

$$EE (\%) = W_s/W_o \times 100 \dots\dots\dots (1)$$

$$LC (\%) = W_s/(W_p + W_o) \times 100 \dots\dots(2)$$

where W_s is the curcumin weight in Cur-Pluronic complex, W_o is the weight of the initial feeding drug, and W_p is the weight of the initial feeding pluronic.

The morphology of particles was observed by transmission electron microscopy (TEM-2010, JEOL, Tokyo Japan). The Cur-S/W nanodispersions were directly deposited in a copper TEM grid with a carbon support and negatively stained with a 2% uranyl acetate solution. The excess solution was absorbed by filter paper and the grid was allowed to air-dry and then kept in a desiccator for subsequent microscopy.

4.2.2.3 *In vitro* release profile of curcumin from S/W nanodispersions

The release of free Cur and Cur-S/W nanodispersions was evaluated using the dialysis bag method under physiological conditions [15]. The Cur-S/W (0.1 mg/mL was placed in a dialysis bag with 12–14 kDa molecular weight cut-off (Spectra/Por®, Spectrum Laboratories, Inc.). It was incubated at 37 °C with equal volumes of PBS (pH 7.4) and ethanol as the release medium with sink condition, and shaken at 100 rpm. The control experiment was performed on curcumin solution with 10% dimethyl sulfoxide (DMSO) in PBS, which was placed in a dialysis bag and incubated under the same conditions. At each designated time point, 0.5 mL of the release medium was

collected, and then replaced with the fresh release medium. The amount of curcumin in the release medium was quantified by HPLC as described previously.

4.2.2.4 *In vitro* cytotoxicity studies

The *in vitro* cytotoxicity of free Cur, Cur-S/W nanodispersion and the carrier (pluronic) was evaluated in HeLa cell lines. Free Cur was dissolved in DMSO to yield a final concentration of less than 0.5%. HeLa cells were seeded at a density of 5000 cells in a 96-well plate (Greiner Bio-One GmbH, Germany) and incubated overnight. The medium was exposed to a series of free Cur, Cur-S/W nanodispersion and blank S/W nanodispersion in reduced serum medium (OPTI-MEM, GIBCO) at different concentrations. Cells were incubated with each treatment for 24 h. The samples were removed, and the cells washed with D-PBS (Nacalai Tesque, Inc), then 100 μ L of WST-8 cell counting solution (Dojindo Laboratories, Kumamoto, Japan) in OPTI-MEM was added to each well, followed by incubation for 3 h. The absorbance derived from WST-8 formazan was recorded on a microplate reader (Bio-Tek Instrument, Inc.) at 450 nm. As the control, non-treated cells were incubated under the same conditions. Cell viability was expressed as (sample/control) $\times$ 100%.

4.2.2.5 Cellular internalization studies

The Cur-S/W nanodispersions with Rho-DOPE were prepared as previously described [14], except that 10 μ L of 1 mg/mL Rho-DOPE in methanol was added to the pluronic aqueous solution. Cell internalization of Cur-S/W nanodispersion in HeLa cell was evaluated by confocal laser scanning microscopy (CLSM). The HeLa cells were seeded in a multi-well glass bottom dish at a density of 10,000 cells (Matsunami Glass Ind., Ltd, Osaka, Japan) and incubated overnight at 37 °C under a CO₂ atmosphere. The medium was removed, and 200 μ L of the samples dispersed in OPTI-MEM containing 10 μ g/mL of curcumin were added to the dish. After incubation

for 3 h, the samples were removed and fixation with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) was carried out for 10 min at 25 °C. A confocal image of HeLa cells was obtained using an LSM 700 confocal microscope (Carl Zeiss, Germany). Curcumin exhibited green fluorescence, which was observed using the Fluorocein isothiocyanate (FITC) filter. The rhodamine filter was used to observe the red fluorescence of curcumin-surfactant complexes labelled with Rh-DOPE.

4.3 Results and Discussion

Curcumin is well known for its poor solubility in water and its instability. The S/W nanodispersion technique is the encapsulation of hydrophobic drugs with hydrophilic surfactant by formation of an oil-water emulsion using a high-speed homogenizer, and subsequent freeze drying overnight to form the solid hydrophobic drug-surfactant complex. The complex was then redispersed in an aqueous solution to obtain a homogeneous nanodispersion. To validate the S/W technology as a delivery vehicle for curcumin, we used two different types of pluronics as surfactant to form curcumin-pluronic complexes. Pluronic is an A-B-A triblock copolymer with polymeric material A being a hydrophilic block poly(ethylene oxide) (PEO) and B being a hydrophobic block poly(propylene oxide) (PPO). It has been approved by the Food and Drug Administration (FDA) as an injectable material and is commercially available. Pluronics F68 and F127 were employed here for the encapsulation of curcumin.

4.3.1 Characteristics of Cur-S/W nanodispersions

Here, we used different types of pluronic block copolymers and investigated their effects on the properties of the Cur-S/W nanodispersions. These hydrophilic

surfactants are nonionic surfactants with different lengths of hydrophilic EO and hydrophobic PO chains, where the composition of EO and PO for Pluronic F68 and F127 are EO₇₆-PO₂₉-EO₇₉ and EO₁₀₀-PO₆₅-EO₁₀₀ respectively [16]. These varieties in the composition of EO and PO chains result in differences in the hydrophobicity of pluronics, and Pluronic F127 shows a higher hydrophobicity (Hydrophilic Lipophilic Balance value, HLB of 22) than F68 (HLB of 29). Curcumin was incorporated into the hydrophobic core of surfactant through the hydrophobic interaction of curcumin with the hydrophobic block PPO of pluronic. When the curcumin-pluronic complexes were dispersed in PBS, the difference in physical appearance of Cur-S/W nanodispersions prepared with the two types of surfactants is shown in Fig. 2. The translucent dispersion was obtained by dispersing curcumin-pluronic complexes in PBS. In contrast, a physical mixture of curcumin in PBS formed a yellow precipitate.

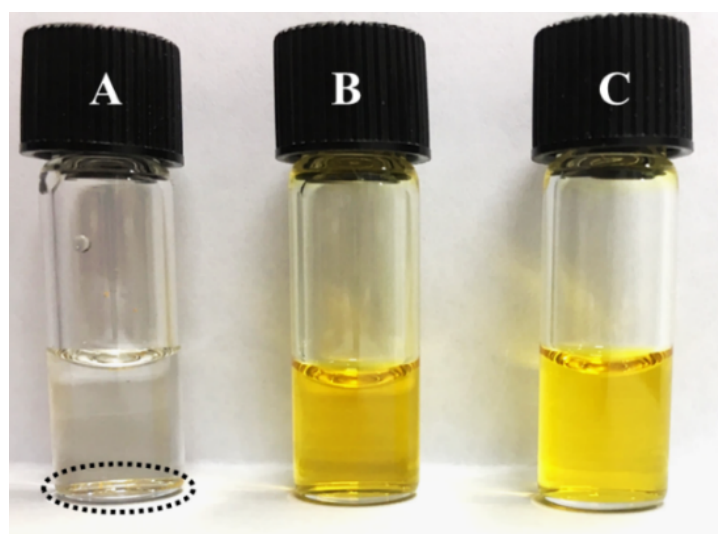


Fig. 2. Physical appearance of curcumin in PBS (A) and curcumin loaded S/W nanodispersions with Pluronic F68 (B) or Pluronic F127 (C) in PBS. The weight ratio of curcumin to pluronic was 1:10. All samples contained 0.1 mg/mL of curcumin.

The mean particle size of Cur-S/W nanodispersions prepared with different weight ratio of curcumin to pluronic was analyzed by DLS. The nanosize range of the

Cur-pluronic complexes with narrow dispersities were obtained using the nanodispersion technique, as summarized in Table 1. The mean particle size of Cur-S/W nanodispersion with Cur-F127 complex (av. 27–28 nm) was smaller than that of the Cur-F68 complex (av. 100–130 nm). In the present work, the Cur-S/W nanodispersions were prepared by lyophilization of cyclohexane-in-water emulsion with pluronic as a stabilizer. The hydrophobic surface of oil droplets was stabilized with the hydrophobic block PPO, while the two hydrophilic block PEO tails directed to the aqueous solution[17]. Therefore, the length of hydrophobic block PPO chain in polymer composition may have influenced the particle size of Cur-S/W nanodispersions.

The LC of curcumin in the formulations was also studied. The results in Table 1 suggest that a higher LC was obtained with a weight ratio of curcumin to pluronic of 1:10 for both surfactants (Cur-F68_#2 and Cur-F127_#2), and the conditions were selected for further characterization. When the loading concentration of curcumin was increased until the weight ratio of curcumin to pluronic reached 1:5, the complexes became extremely unstable, and the particle could not be characterized (data not shown). The increase in loading concentration of curcumin caused a notable increase in the particle size, as indicated in Table 1. In addition, the EE of Cur-S/W nanodispersion was evaluated by a filtration method using HPLC analysis. The results showed the quantitative EE of curcumin for all formulations prepared with the two types of surfactants and the different weight ratios of curcumin to pluronic (Table 1). High EE of curcumin was achieved by the S/W nanodispersion technique, with approximately 81% EE for cur-F68_#2 and 86% for cur-F127_#2, respectively. However, the EE slightly decreased when the loading concentration of curcumin was increased. The emulsification and freeze drying process can promote high entrapment

of the drugs in the presence of the surfactant molecules [14,18]. The zeta potential of Cur-S/W nanodispersions for cur-F68_#2 and cur-F127_#2 were -0.75 and 0.71 , respectively.

Table 1. Characteristics of Cur-S/W nanodispersions with two different surfactants

Sample	Type of surfactant	Weight ratio of curcumin:surfactant	Z-size (nm)	PDI	EE (%)	LC (%)
Cur-F68_#1	Pluronic F68	1:20	105 ± 2	0.09-0.12	95 ± 3	4.4 ± 0.4
Cur-F68_#2	Pluronic F68	1:10	113 ± 3	0.07-0.11	81 ± 4	7.3 ± 0.1
Cur-F127_#1	Pluronic F127	1:20	27 ± 0.7	0.2-0.26	88 ± 4	4.2 ± 0.4
Cur-F127_#2	Pluronic F127	1:10	28 ± 0.5	0.2-0.23	86 ± 5	7.9 ± 0.2

The morphology of Cur-S/W nanodispersions was analyzed using TEM. The sizes of Cur-S/W nanodispersions measured by TEM were in good agreement with the results of DLS analysis (Fig. 3). We observed spherical morphologies of Cur-S/W nanodispersions prepared with the two different types of pluronics. This finding is in contrast to that observed in our previous study, where the encapsulated CPT exhibited cubic lamellar morphologies, probably stemmed from recrystallization of CPT during the formulation process because of the poor solubility of CPT in cyclohexane [14]. However, here the curcumin is moderately soluble in cyclohexane, suggesting that the formation of the curcumin crystals at the early stage of preparation does not occur. The curcumin in cyclohexane is surrounded by the pluronic in water, resulting in the spherical morphologies of Cur-pluronic complexes.

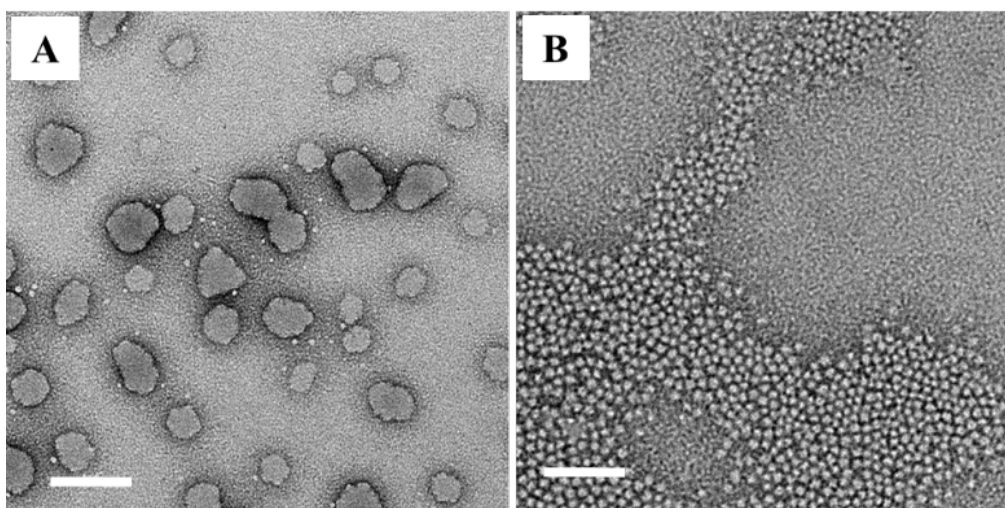


Fig. 3. TEM images of Cur-S/W nanodispersions prepared with Pluronic F68, Cur-F68_#2 (A) and Pluronic F127, Cur-F127_#2 (B). Scale bar: 200 nm.

4.3.2 *In vitro* release profiles

We investigated the *in vitro* release behavior of Cur-S/W nanodispersions using the dialysis method under sink condition. Figure 4 presents the percentage of curcumin release into the medium solution. We found that the free curcumin rapidly diffused into the medium within the first hour, because of the small molecular weight of curcumin (368.38 Da). In contrast, a slower and sustained release of curcumin was found for Cur-S/W nanodispersions (Fig. 4). We observed partial degradation of curcumin (approximately 20% after 350 min.) during incubation in the release medium at pH 7.4, possibly due to the unstability of curcumin under physiological conditions (Zebib et al. 2010). Besides, in the case of Cur-S/W formulations, some part of curcumin could be retained in the dialysis bag due to the high molecular weight of Pluronic. Therefore, we assumed that the equilibrium of curcumin released from Cur-S/W formulations reached at 50-60% in the current experimental conditions.

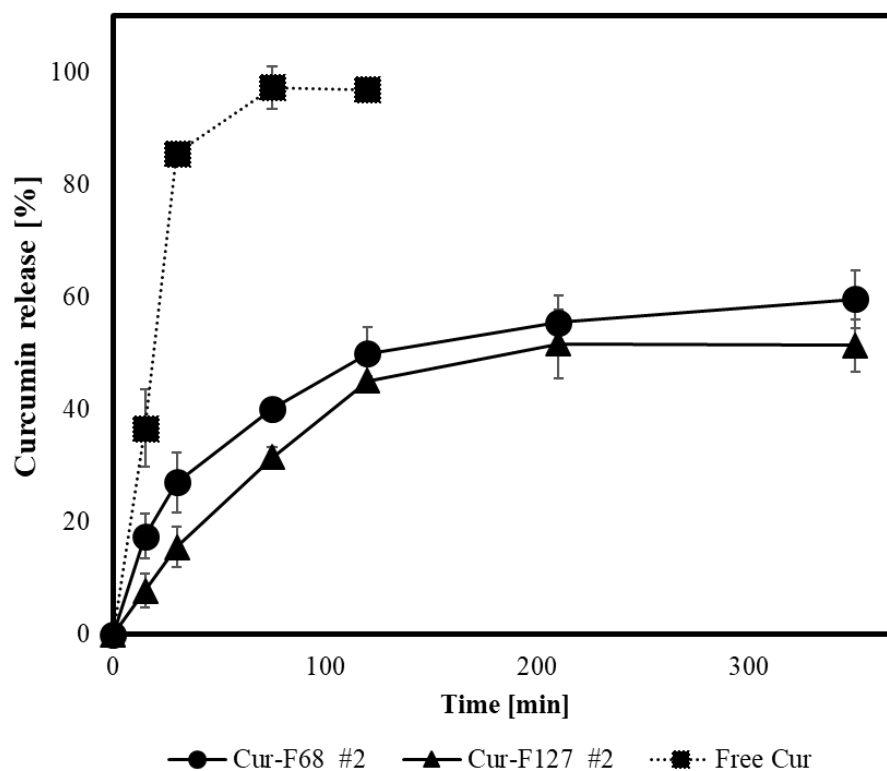


Fig. 4 *In vitro* release profile of free Cur and Cur-S/W nanodispersions.

The release rate of curcumin from Cur-F68 S/W nanodispersions was slightly faster than that of Cur-F127, approximately 60% and 51% of curcumin had been released into the medium from Cur-F68 and Cur-F127 S/W nanodispersions at 350 min, respectively. These results suggested that the hydrophobicity and molecular weight of pluronic could affect the release rate of curcumin from the S/W formulations. Pluronic F68 has a higher HLB value and smaller molecular weight compared with Pluronic F127 (Table 1), leading to slightly faster release kinetics.

4.3.3 Anticancer activity of Cur-S/W nanodispersions

The *in vitro* anticancer activity of Cur-S/W nanodispersions was evaluated by the WST-8 method using HeLa cells. As shown in Fig. 5, the cell viability decreased with increased curcumin concentration. In contrast, HeLa cells treated with the two types of pluronic as a carrier exhibited approximately 100% cell viability at concentration up

to 1000 $\mu\text{g/mL}$, suggesting that the carrier has no significant effects on the cell viability of HeLa cells (Fig. 6A). The non-toxic effect of the carrier was also noticed with the cell viability of normal cells (NIH3T3 cell line) at concentration up to 2000 $\mu\text{g/mL}$ (Fig. 6B).

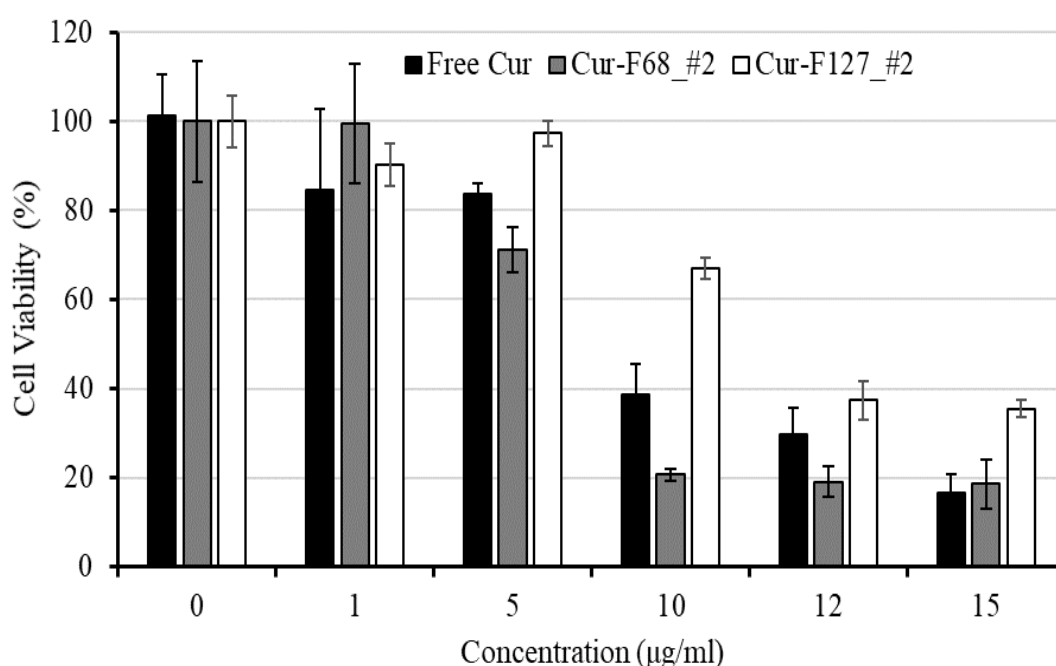


Fig. 5. Anticancer activity of free cur and Cur-S/W nanodispersions on HeLa cells with 24 h of incubation.

Free curcumin showed a half maximal inhibitory concentration (IC_{50}) value of ~ 10 $\mu\text{g/ml}$, which is consistent with the value reported previously (Razi et al, 2018). We found that clear difference between free Cur and Cur-S/W formulation (Fig. 5). For examples at the curcumin concentration of 10 $\mu\text{g/ml}$, the anticancer activity of Cur-F68 S/W nanodispersion was higher than that of Cur-F127 formulation and free curcumin, and free Cur was higher than that of the Cur-F127 S/W nanodispersions. The difference in anticancer activity of the Cur-S/W nanodispersions with two different types

of Pluronic might correlate to the release profile (Fig. 4) and the cellular uptake profile of the Cur-S/W formulations (Fig.7).

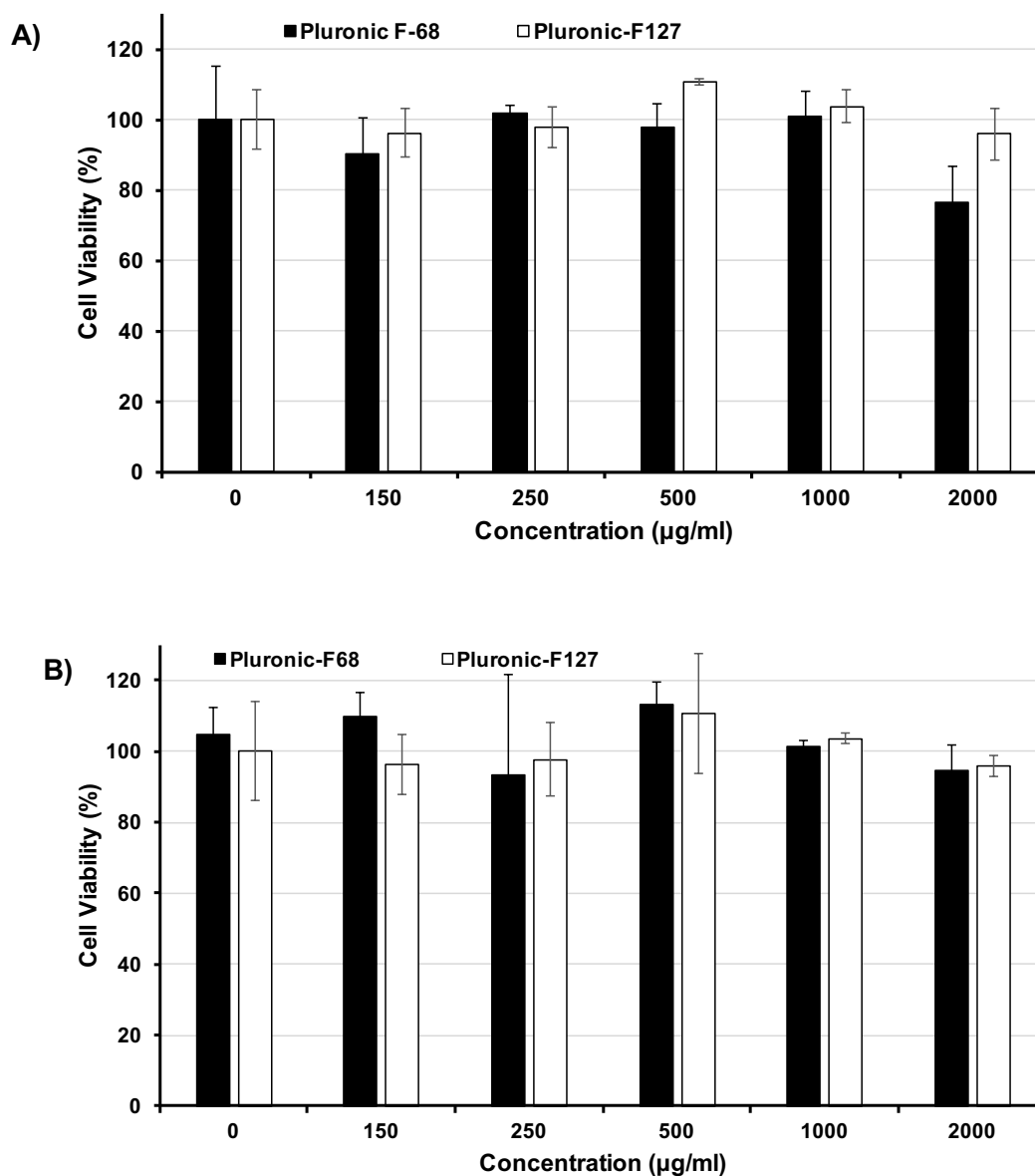


Fig. 6 (A) The anticancer activity of two type Pluronic as the carrier on HeLa cells for 24 incubation, (B) The toxicity of of two type Pluronic as the carrier on normal cells (NIH#T# cells line) for 24 h incubation.

4.4.4 Cellular internalization of Cur S/W nanodispersion

We evaluated the cellular internalization of Cur-S/W nanodispersions with two different surfactants using CLSM (Fig. 7). Since the curcumin is auto-fluorescent, it could be directly observed using a FITC filter, as green fluorescence. Moreover, the Rho-DOPE was incorporated with Cur-S/W nanodispersions for tracking the particle complex on the cells. The cellular uptake of Cur-S/W nanodispersion was observed after 3 h of incubation.

Fig. 7 shows that the green fluorescence derived from curcumin was located in the cytoplasm of HeLa cells, where the intensity of green fluorescence observed in the cells after treatment with Cur-F68 S/W nanodispersion was slightly higher than those treated with Cur-F127 S/W nanodispersion. In addition, stronger red fluorescence from Rho-DOPE was detected in the cells treated with Cur-F68 S/W nanodispersion compared with Cur-F127 S/W nanodispersion, indicating that the cellular internalization of Cur-F68 S/W was higher than Cur-F127 S/W nanodispersion. Cur-S/W nanodispersions was prepared as previously reported [14], by which the hydrophobic molecule was incorporated into the hydrophobic block PPO of Pluronic and the hydrophilic block PEO tails directed to the aqueous solution. We could assume that the cellular internalization of Cur-S/W nanodispersions was facilitated by endocytic pathway as observed in our previous study with the same Pluronic-based S/W nanodispersions, given that the basic structure of S/W nanodispersions for the solubilization of hydrophobic molecules was same.

The highest cellular uptake of the complex may be attributed to the higher cytotoxicity on the HeLa cells (Fig. 5). It has been previously reported that the more hydrophobic nanocarriers prepared with Pluronic F127 than those with F68 showed slightly higher cellular internalization because of the higher affinity for the cellular

membrane [19]. However, apart from the hydrophobicity of pluronic surfactants, the length of the hydrophilic block PEO chain of the pluronic nanocarrier also has an influence on the cellular uptake [20]. The longer PEO block in nanocarriers may result in more extensive hydration and less exposure of the hydrophobic core. Here, Pluronic F68 had a shorter hydrophilic block PEO compared with F127 (The average number of EO units for Pluronic F68 and F127 are 76 and 100 respectively). Moreover, the particle size of Cur-F127 S/W nanodispersions is much smaller than that of Cur-F68 S/W nanodispersions, implying a packed arrangement of the pluronic surfactant on the surface. These may result in the lower accessibility of the Cur-F127 nanocarrier to the cellular membrane. The slightly slower release kinetics of Cur-F127 suggests the superior stability of the nanocarrier and further supports this hypothesis. Taken together, the higher cellular internalization and the slightly higher release profile of Cur-F68 S/W nanocarriers resulted in the higher anticancer activity observed.

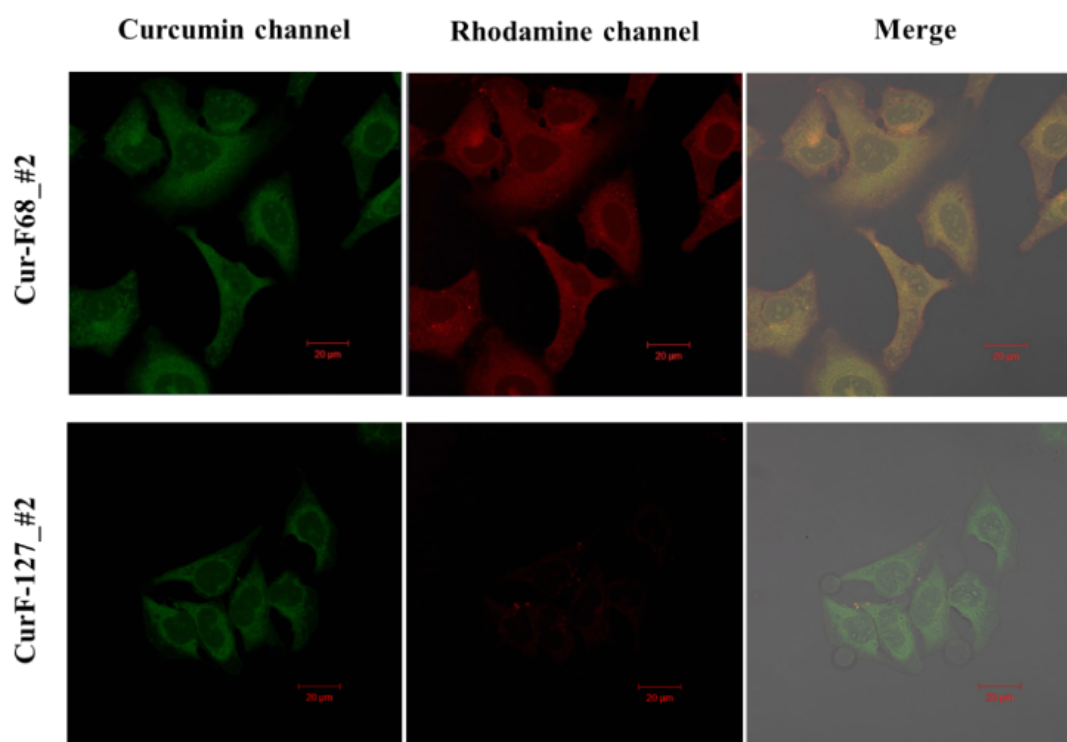


Fig. 7. Confocal images of HeLa cells treated with Cur-S/W nanodispersions for 3 h.

4.4 Conclusion

Curcumin was successfully encapsulated with high entrapment efficiency using the S/W nanodispersion technique. The enhanced water solubility of curcumin was achieved by the encapsulation of nonionic surfactant pluronic with the nanosized particles of curcumin in aqueous solution. *In vitro* release studies showed that the release rate of curcumin from the resultant complexes could be controlled by choosing a proper surfactant. A correlation between the release profile of the Cur-S/W nanodispersions and anticancer activity was observed. The improved water solubility of curcumin and observed anticancer activity of the Cur-S/W nanodispersions in this study have thus increased the potential of curcumin in pharmaceutical applications.

4.5 References

- [1] WHO, Global health estimates 2000 - 2015. Deaths by cause, age and sex., (2017) 8–10.
- [2] P. Anand, A.B. Kunnumakkara, R.A. Newman, B.B. Aggarwal, P. Anand, A.B. Kunnumakkara, R.A. Newman, Bioavailability of Curcumin: Problems and Promises reviews Bioavailability of Curcumin: Problems and Promises, Mol. Pharmacol. 4 (2007) 807–818. doi:10.1021/mp700113r.
- [3] S.C. Gupta, S. Patchva, B.B. Aggarwal, Therapeutic Roles of Curcumin: Lessons Learned from Clinical Trials, AAPS J. 15 (2013) 195–218. doi:10.1208/s12248-012-9432-8.
- [4] M. Bayet-Robert, F. Kwiatkowski, M. Leheurtur, F. Gachon, E. Planchat, C. Abrial, M.A. Mouret-Reynier, X. Durando, C. Barthomeuf, P. Chollet, Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and

- metastatic breast cancer, *Cancer Biol. Ther.* 9 (2010). doi:10.4161/cbt.9.1.10392.
- [5] X. Yang, Z. Li, N. Wang, L. Li, L. Song, T. He, L. Sun, Z. Wang, Q. Wu, N. Luo, C. Yi, C. Gong, Curcumin-encapsulated polymeric micelles suppress the development of colon cancer in vitro and in vivo, *Sci. Rep.* 5 (2015) 1–15. doi:10.1038/srep10322.
- [6] L. Li, F.S. Braiteh, R. Kurzrock, Liposome-encapsulated curcumin: In vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis, *Cancer*. 104 (2005) 1322–1331. doi:10.1002/cncr.21300.
- [7] D. Mathew, W.L. Hsu, Antiviral potential of curcumin, *J. Funct. Foods*. 40 (2018) 692–699. doi:10.1016/j.jff.2017.12.017.
- [8] C.D. Lao, M.T. Ruffin, D. Normolle, D.D. Heath, S.I. Murray, J.M. Bailey, M.E. Boggs, J. Crowell, C.L. Rock, D.E. Brenner, Dose escalation of a curcuminoid formulation, *BMC Complement. Altern. Med.* 6 (2006) 10. doi:10.1186/1472-6882-6-10.
- [9] L.C. Chen, Y.C. Chen, C.Y. Su, W.P. Wong, M.T. Sheu, H.O. Ho, Development and Characterization of Lecithin-based Self-assembling Mixed Polymeric Micellar (saMPMs) Drug Delivery Systems for Curcumin, *Sci. Rep.* 6 (2016) 1–12. doi:10.1038/srep37122.
- [10] H.H. Tønnesen, M. Másson, T. Loftsson, Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: Solubility, chemical and photochemical stability, *Int. J. Pharm.* 244 (2002) 127–135. doi:10.1016/S0378-5173(02)00323-X.
- [11] C. Gong, S. Deng, Q. Wu, M. Xiang, X. Wei, L. Li, X. Gao, B. Wang, L. Sun, Y.

- Chen, Y. Li, L. Liu, Z. Qian, Y. Wei, Improving antiangiogenesis and anti-tumor activity of curcumin by biodegradable polymeric micelles, *Biomaterials*. 34 (2013) 1413–1432. doi:10.1016/j.biomaterials.2012.10.068.
- [12] M.A. Razi, R.I.E. Wakabayashi, M. Goto, N. Kamiya, Formation and Characterization of Caseinate – Chitosan Nanocomplexes for Encapsulation of Curcumin, *J. Chem. Eng. Japan*. in press (n.d.).
- [13] T.P. Sari, B. Mann, R. Kumar, R.R.B. Singh, R. Sharma, M. Bhardwaj, S. Athira, Preparation and characterization of nanoemulsion encapsulating curcumin, *Food Hydrocoll.* 43 (2015) 540–546. doi:10.1016/j.foodhyd.2014.07.011.
- [14] R. Wakabayashi, R. Ishiyama, N. Kamiya, M. Goto, A novel surface-coated nanocarrier for efficient encapsulation and delivery of camptothecin to cells., *Medchemcomm.* 5 (2014) 1515–1519. doi:10.1039/C4MD00179F.
- [15] C. Chang, T. Wang, Q. Hu, M. Zhou, J. Xue, Y. Luo, Pectin coating improves physicochemical properties of caseinate/zein nanoparticles as oral delivery vehicles for curcumin, *Food Hydrocoll.* 70 (2017) 143–151. doi:10.1016/j.foodhyd.2017.03.033.
- [16] Z. Sezgin, N. Yüksel, T. Baykara, Preparation and characterization of polymeric micelles for solubilization of poorly soluble anticancer drugs, *Eur. J. Pharm. Biopharm.* 64 (2006) 261–268. doi:10.1016/j.ejpb.2006.06.003.
- [17] T. Tadros, Polymeric surfactants in disperse systems, *Adv. Colloid Interface Sci.* 147–148 (2009) 281–299. doi:10.1016/j.cis.2008.10.005.
- [18] Y. Tahara, N. Kamiya, M. Goto, Solid-in-oil dispersion: A novel core technology for drug delivery systems, *Int. J. Pharm.* 438 (2012) 249–257. doi:10.1016/j.ijpharm.2012.09.007.

- [19] J.Y. Kim, W. Il Choi, Y.H. Kim, G. Tae, S.Y. Lee, K. Kim, I.C. Kwon, In-vivo tumor targeting of pluronic-based nano-carriers, *J. Control. Release.* 147 (2010) 109–117. doi:10.1016/j.jconrel.2010.06.010.
- [20] A. Arranja, A.G. Denkova, K. Morawska, G. Waton, S. Van Vlierberghe, P. Dubruel, F. Schosseler, E. Mendes, Interactions of Pluronic nanocarriers with 2D and 3D cell cultures: Effects of PEO block length and aggregation state, *J. Control. Release.* 224 (2016) 126–135. doi:10.1016/j.jconrel.2016.01.014.

CHAPTER 5

CONCLUCIONS AND FUTURE PROSPECTIVE

5.1 Summary

Natural compounds have a great potential to cure or prevent a number of diseases because they have biological function as well as pharmacological activities in biomedicine. However, delivery of natural compounds into the target disease remains challenging. The aim of this work is to deliver natural compounds, such as protein and small molecular natural products using surfactant-based nano-carriers. For this purpose, phycocyanin (PC) and curcumin (Cur) were selected as model therapeutic agents, which are a naturally occurring protein and a natural compound, respectively. PC, a water-soluble protein-chromophore complex composed of hexameric $\alpha_6\beta_6$ subunits, has important biological functions in blue-green algae as well as pharmacological activities in biomedicine, such as anticancer, anti-inflammantory, and antidiabetic. Cur, a hydrophobic polyphenol compound, holds promising potential as an anticancer agent.

In Chapter 2, the mechanism of transcutaneous protein delivery using a solid-in-oil (S/O) nanodispersion method was investigated. PC, a protein-chromophore complex, was chosen to evaluate how the proteinaceous macromolecules formulated in an oil phase might permeate the skin. The solid-in-oil (S/O) nanodispersion was prepared by coating PC with hydrophobic surfactants. The extent of S/O nanodispersion of PC was dependent on the type of surfactant, suggesting that the selection of a suitable surfactant is crucial for encapsulating a large protein having a subunit structure. By measuring the intrinsic fluorescence of PC, we found that S/O nanodispersion facilitated the accumulation of PC in the stratum corneum (SC) of

Yucatan micropig skin. Furthermore, after crossing the SC layer, the fluorescent recovery of PC was evident, indicating the release of the biologically active form of PC from the SC into the deeper skin layer.

Based on the findings in Chapter 2, the conformational structure of proteins formulated in oil phase was influenced to their functional integrity after released from the formulation, thus in Chapter 3, a novel oil-based drug delivery system, namely gel-in-oil (G/O) nanodispersion, was investigated to stabilize proteins. G/O nanodispersion has a gel-core oil-based system for protein delivery, in which the trapping protein drugs was formulated in the gelatin-based hydrogel coated with a hydrophobic surfactant using emulsion technology. PC was loaded in G/O nanodispersion as model protein, and was evaluated their functional integrity and skin permeability. A nano-sized range of particle with high entrapment efficiency of protein was achieved by G/O nanodispersion. Spectroscopic studies indicated that G/O nanodispersion might be able to retain and protect an active form of PC. The gelatin concentration is a key factor for stabilizing the native structure of PC in G/O nanodispersion. *In vitro* skin permeation studies showed the enhanced permeability of protein delivery through the deep skin layer using G/O nanodispersion with a low concentration of surfactant.

In Chapter 4, the formulation of Cur using a solid-in-water (S/W) nanodispersion technique to enhance water solubility and therapeutic activity of Cur was studied. This new aqueous formulation comprises simple preparation protocols: emulsification and freeze-drying for encapsulating hydrophobic biomolecules with a hydrophilic surfactant, followed by redispersion of the resultant solid complexes in an aqueous solution. Pluronic F68 and F127 were used here for the encapsulation of curcumin. Enhanced aqueous solubility of Cur was achieved by the encapsulation with a hydrophilic surfactant using the S/W nanodispersion technique. The resultant nano-

sized formulation had a narrow size distribution and high entrapment efficiency of Cur. The highest loading capacity of Cur in S/W nanodispersion was obtained with a weight ratio of Cur to Pluronic of 1:10 for both surfactants. The release profile of the complexes was found to depend on the type of surfactant, suggesting that the selection of a proper surfactant is crucial for controlling the delivery. The anticancer activity of the S/W formulation with Cur was correlated with the drug release profiles and cellular uptake, which in turn influenced by the hydrophobicity and chemical structure of the surfactant.

5.2 Future prospective

The present study only focused on the evaluating mechanism of biomacromolecular protein formulated in oil phase using solid-in-oil (S/O) nanodispersion and characterized of the particles of the protein-surfactant complex after dispersion with oil vesicles, isopropyl myristate (IPM). Because of the characterization of intermediate products, the solid protein-surfactant complex is still missing, in the future, we can further conduct structural characterization of the solid protein-surfactant complexes to better understand the molecular characteristics of S/O nanodispersion.

Besides, in the present study, a new oil-based nano carrier, G/O nanodispersion, has shown a good ability to retain the native structure of protein by monitoring the fluorescence intensity of phycocyanin, however analysis was done only after the preparation of G/O nanodispersion. Since the stability of protein during the storage is crucial in the pharmacological fields, it is necessary to further investigate the stability of protein loaded into G/O nanodispersion during the long-term storage. In addition, the present work clearly demonstrated the possibility of delivering a large

protein assembly with a subunit structure through the skin using the oil-based delivery system. In future, this system could be expanded to the delivery of large protein drugs, such as antibody, virus-like protein or nanoparticles through the skin for pharmacological applications.

Finally, a new aqueous formulation for the encapsulation of hydrophobic molecules, such as curcumin, can further be evaluate to investigate the mechanism of S/W nanodispersion on the intercellular delivery of a small hydrophobic molecules. Exploring the other biocompatible surfactants and other natural products should gain further information on the possibility of this technique for delivering natural product-based medicines.

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