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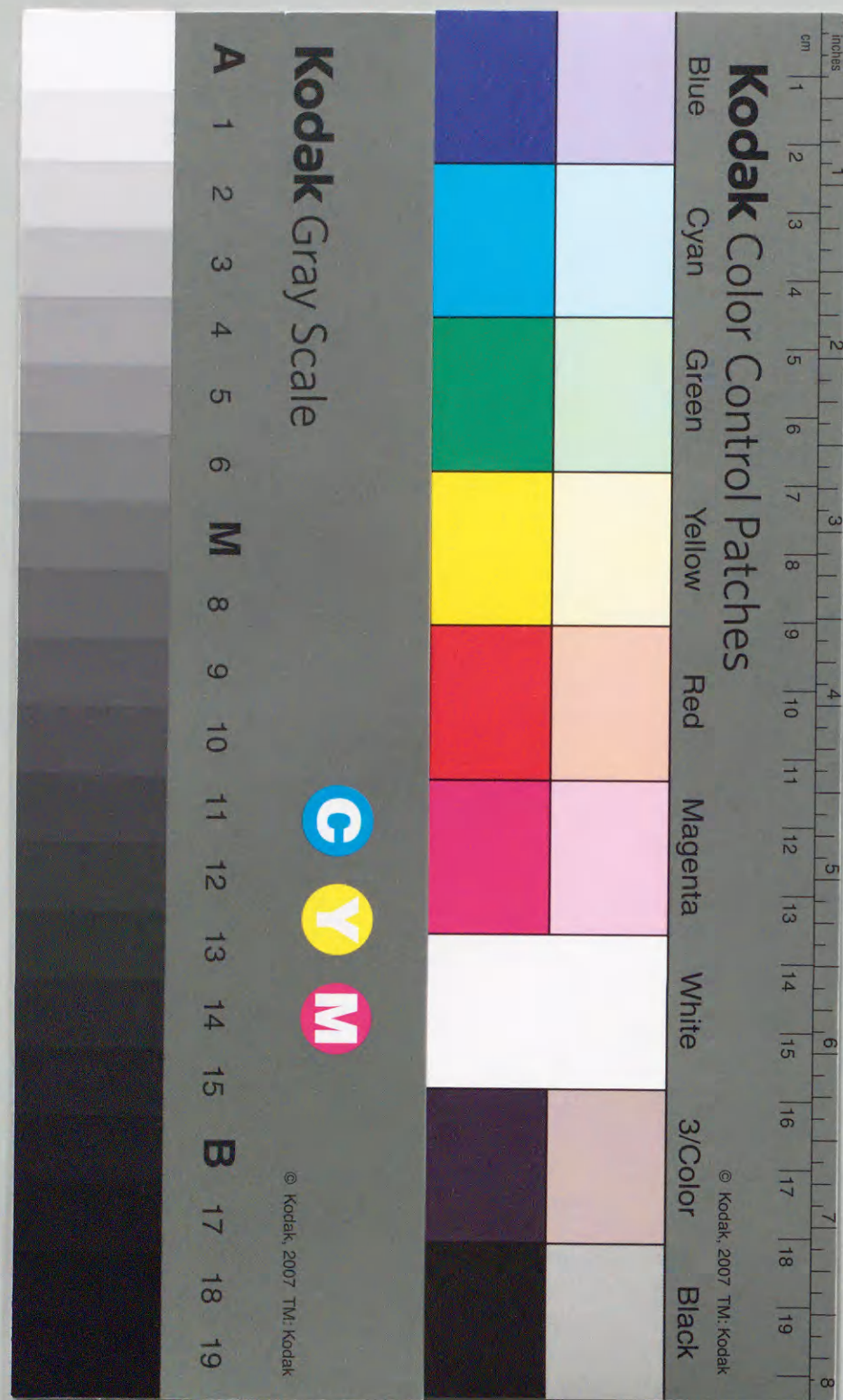
Study on Controlled Release from Biodegradable
Hydrogels with Microdomain Structures

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Japan Advanced Institute of Science and Technology

Study on Controlled Release from Biodegradable

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Hydrogels with Microdomain Structures

by

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PREFACE

The present dissertation is the result of the studies under the direction of Professor Dr. Nobuhiko Yui during 1993-1998. The purpose of this dissertation is to propose the novel controlled release formulations for clinical drug delivery, poly(ethylene glycol)-containing polysaccharide hydrogels and solutions. Fundamental matters for controlled drug delivery systems using biodegradable polymers and hydrogels, and aqueous polymer two-phase systems used as the method for construction of microdomain-structured vehicles are covered as a general introduction in the first chapter. Chapter 2 describes regulated insulin release from biodegradable dextran (Dex) hydrogels containing poly(ethylene glycol) (PEG) as a model of microdomain-structured devices. Chapter 3 describes the regulation of the phase-separated structure in PEG/Dex hydrogels with different water content and crosslink density. Demonstration of controlled release of bioactive peptide drugs using multi-layered hydrogel formulations is presented in chapter 4. Chapter 5 gives hyaluronic acid grafted with PEG as a novel peptide formulation, and suggests its use for an injectable therapeutic treatment. The last chapter summarizes the conclusive items of this dissertation.

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Chapter 1

General Introduction

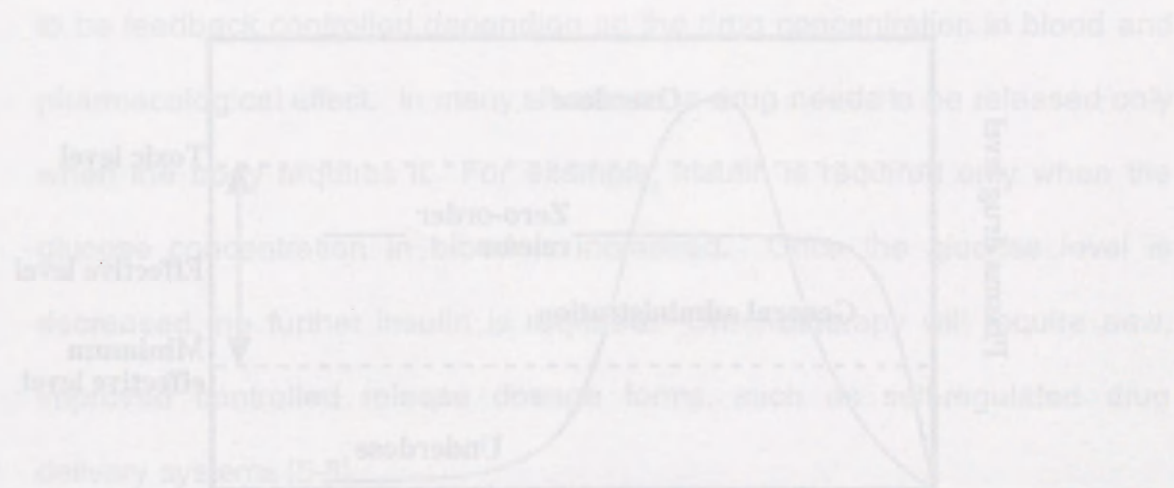


Figure 1-1. Typical time course of drug release from a polymer matrix.

The premise of the zero-order release is to maintain constant drug concentration in blood for an extended period of time. The zero-order release is achieved by using a polymer matrix that releases drug at a constant rate.

1-1: Controlled Release Drug Delivery Systems

1-1-1: Requirements of Controlled Drug Delivery

During the last two decades, significant advantages have been made in the controlled release of bioactive and therapeutic agents [1, 2]. In the early stages of research on controlled release drug delivery, major emphasis was focused on the development of zero-ordered release devices. Current technology has improved to such a level that delivery of some drugs at a constant rate for a certain period of time ranging from days to years is not a major issue anymore.

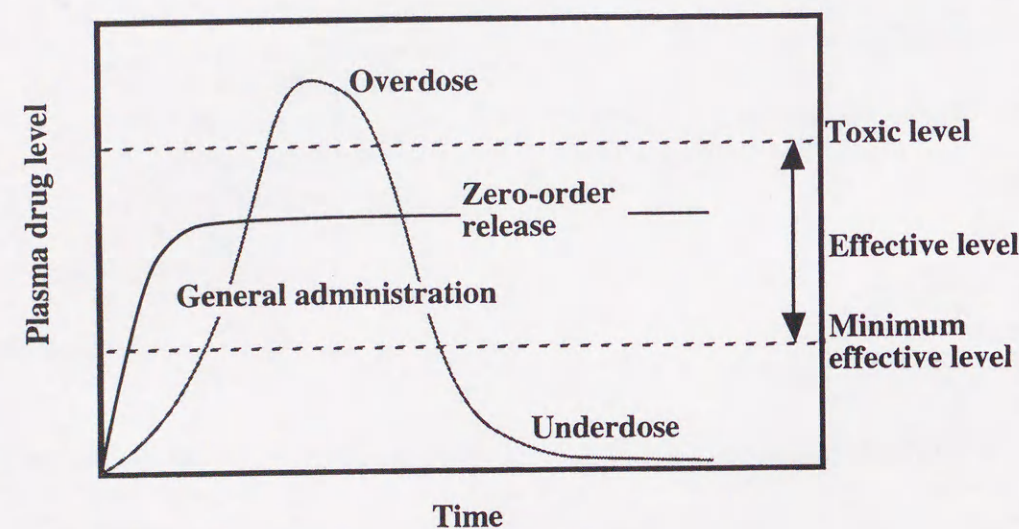


Figure 1-1. Typical time course of drug level with zero-order release formulation and general administration.

The premise of the zero-order release is to maintain constant drug concentration in blood for an extended period of time. The zero-order release

of a drug, however, does not necessarily result in a constant drug concentration in blood. The absorption of the drug by the body usually does not follow the zero-order kinetics, except when the drug is delivered directly into the blood stream by an infusion pump. The nonzero-order drug absorption is still effective in most cases as long as the drug concentration is maintained between the minimum effective and the maximum safe concentrations as shown in Figure 1-1. Cumulative evidence suggests that circadian rhythms need to be considered in drug delivery [3, 4] as described later. Many drugs show rhythmic changes in bioavailability, absorption, distribution, and excretion. Furthermore, the zero-order drug release is not always necessary and sometimes it is not desirable. The drug delivery needs to be feedback controlled depending on the drug concentration in blood and pharmacological effect. In many situations, a drug needs to be released only when the body requires it. For example, insulin is required only when the glucose concentration in blood is increased. Once the glucose level is decreased, no further insulin is required. Chronotherapy will require new, improved controlled release dosage forms, such as self-regulated drug delivery systems [5-8].

1-1-2: Chronopharmacology

Chronopharmacology is the study of how the effects of drugs vary with biological timing and endogenous periodicities. The goal is to improve

understanding of periodic and thus predictable (circadian, circa diem=about 24h) changes in both desired effects and tolerance of medications. Dosing time-dependent changes also include quantification of parameters characterizing endogenous circadian rhythms, in terms of pharmacological effects. Predictable temporal variations of effects and disposition of agents (medications, hormones and toxic substances) are governed by endogenous biological rhythms rather than by changes of external factors. On the 24h scale (as well as on the yearly scale) there are peaks and troughs of physiological variables that are not randomly distributed; their respective locations correspond to a temporal organization controlled by a set of pacemakers (biological clocks) that presumably are interconnected and hierarchized [9]. Periodic changes of environmental factors are used by organisms as signals and cues to reset the circadian clocks. For many plants and animals, prominent factors are the light-dark cycle, alteration of rest and activity related to constraints of work schedules, and/or habits of social life in humans [10]. It is now well established that nearly all functions of the body, including those influencing pharmacokinetic parameters, display significant daily variations. Thus, recently, drug delivery systems concerning with chronopharmacology is extensively studied in order to optimize drug delivery exhibiting pulsatile and/or self-regulated drug release.

1-1-3: Protein and Peptide Delivery

There are additional important issue that have to dealt with for further development of controlled release dosage forms. Controlled release devices should be able to deliver large molecular weight drugs, such as peptides and proteins, without altering the bioactivities. The continuing advances in genetic engineering make available an ever-increasing number of peptides, usually defined as compounds having fewer than 20 amino acid residues, and the much higher-molecular-weight proteins. The recombinant DNA technology has allowed production of many protein drug in large quantities which were not readily available previously. Because these compounds are not orally active, other routes of administration must be used and a number of alternative routes such as nasal [11], rectal [12], and transdermal [13] are under active development. A methods using the ocular route for the systemic delivery of peptides has also been described [14]. However, at this time, parenteral administration by subcutaneous or intramuscular administration is the only route that is generally applicable. Further, because proteins are very rapidly deactivated by proteolytic enzymes, prolonged release is highly desirable if daily administrations are required. A number of reviews covering the controlled parenteral delivery of peptides and proteins have been published [15-17].

Implants capable of releasing peptides or proteins in a controlled manner for the desired length of time is clearly a very-high-priority objective. However, controlled delivery is a major obstacle in the effective use of the protein drugs i. e., the development of such implants for high-molecular-weight

compounds is a difficult undertaking which becomes even more so with proteins that have complex tertiary structures that are essential for their physiological activity. Thus, controlled delivery of bioactive peptides and proteins is not as easy as the delivery of small molecular weight drugs. The loading of protein drugs into, and their release from, polymeric controlled release devices without altering the bioactivity still an unsolved problem. Moreover, zero-order release kinetics are not always the best delivery regimens and in some instances described above, due to down-regulation of biological receptors, pulsatile or self-regulated release is required.

1-2: Biodegradable Polymers for Controlled Drug Delivery

1-2-1: Biodegradation

Polymer degradation can be classified into photo- and photo-oxidative, thermo- and thermo-oxidative, mechanochemical, ozone-induced, radiolytic, ionic, and biodegradation [18]. Biodegradation of polymers is the classification of greatest importance for biomedical, pharmaceutical, and environmental applications. Biodegradation has been defined in various ways by different investigators. It has been defined as changes in surface properties or loss of mechanical strength, assimilation by microorganisms, degradation by enzymes, backbone chain breakage and subsequent reduction in the average molecular weight of the polymer, or extraction of low

molecular weight material leading to surface defects. Degradation can occur by each of the above mechanisms alone or in combination with one another.

While no consensus is found on the definition of biodegradation, it is clear that biodegradation can occur on many different structural levels, i. e., molecular, macromolecular, microscopic, and macroscopic, depending on the mechanism [19]. It was argued that the phenomenon of degradation *in vivo* may not be equated with the term biodegradation, since biodegradation implies the active participation of biological entities such as enzymes or organisms in the degradation process [20, 21]. However, it is difficult to identify the involvement and the role of biological species in the *in vivo* degradation. Both hydrolytic and enzymatic processes may contribute to the degradation to different extents during the different stages of the degradation process. Degradation may begin by hydrolysis, but as the polymer breaks up and surface area accessibility increase, enzymatic degradation may dominate [21]. Thus, at least for now, the definition of biodegradation should be broad enough to include all types of degradation occurring *in vivo* whether the degradation is due to hydrolysis or metabolic processes [22].

1-2-2: Enzyme-Catalyzed Hydrolysis

Enzymes in the body play vital roles in various reactions essential for daily activities. Enzymes are proteins which function as catalysts for a specific reaction or series of reactions, such as oxidation, reduction, hydrolysis, esterification, synthesis, and molecular interconversions [23]. Each enzyme is

different in specificity, location, and concentration in the body. They act in concert to process and metabolize proteins and polysaccharides [24]. In each enzyme there is an active site which binds substrates and either forms or cleaves particular bonds in the substrates. The binding between enzymes and substrates depends on the three-dimensional structures of the active sites and substrate segments. Thus, the enzymatic degradation of substrates is substrate-specific and specific enzymes exist in the body for the degradation of particular proteins and polysaccharides.

Since natural polymers, such as proteins and polysaccharides, are well known for their degradability in the body, they were the first to be exploited to prepare biodegradable biomaterials. The use of catgut as an absorbable suture led to the development of synthetic, biodegradable sutures [25]. Reconstituted collagen has been used widely as a bioabsorbable suture, a wound dressing, and a drug delivery systems [26]. From the enzymatic degradation of proteins, it is to be expected that enzymes will play a significant role in the degradation of synthetic poly(α -amino acids) such as poly-L-lysine, poly-L-arginine, poly-L-aspartic acid, or poly-L-glutamic acid [27]. The types of enzymes involved and the extent of degradation of poly(α -amino acids), however, are not easily predictable [28]. Of the many poly(α -amino acids), the enzymatic degradation of poly-L-lysine has been studied most extensively.

Hydrophobic polymers with no hydrolyzable bonds, such as polyethylene, polypropylene, poly(methyl methacrylate), or polystyrene, are expected to be the most stable.

Table 1-1. Examples of enzymatic degradation of water-insoluble, synthetic polymers

Nylon 6,6	chymotrypsin trypsin papain
Poly(ϵ -caprolactone)	esterase lipase
Poly(ester-urea)	chymotrypsin esterase subtilisin
Poly(ether urethane)	liver homogenate (acid phosphatase, esterase) cathepsin leucine aminopeptidase papain urease trypsin
Poly(ethylene terephthalate)	esterase papain leucine aminopeptidase
Poly(glycolic acid)	esterase ficin carboxypeptidase A clostridiopeptidase A bromelain esterase leucine aminopeptidase
Polyhydroxybutyrate	esterase
Poly(lactic acid)	pronase proteinase K bromelain lipase
Polylysine	trypsin chymotrypsin carboxypeptidase B elastase papain ficin

However, cumulative evidence suggests that even the most inert polymers suffer some degree of degradation after implantation into the body [29], or

under physiological conditions in the presence of the proper enzymes [30]. Furthermore, it is also suggested that enzymes play an important role in the biodegradation process. Although enzymatic degradation is known to be highly specific, there may be sufficient nonspecificity to make enzymes play a significant role in the degradation of many synthetic polymers [21]. Indeed, seemingly inert polymers, such as nylon, poly(ether urethane), poly(ethylene terephthalate), poly(hydroxybutyrate), poly(ester-urea), poly(ϵ -caprolactone) and poly(glycolic acid), are degradable by enzymes. Table 1-1 lists some synthetic polymers which are known to undergo significant enzymatic degradation *in vivo* as well as *in vitro*. Many of the synthetic polymers are degradable by esterases and this is not totally unexpected since they are known to be involved in the detoxification process in the body [28]. The ability of the enzymes to penetrate into synthetic polymers determines the extent of enzyme-catalyzed degradation and the mode of degradation, i. e., surface vs. bulk degradation. The degree of enzyme penetration into polymers depends on the size of the enzyme as well as the physical property of the polymer.

1-2-3: Surface Degradation for Controlled Drug Delivery

Biodegradable polymers have much potential for applications as implantable carriers for drug delivery systems as well as for surgical repair materials. Regulated drug release from biodegradable polymer matrices has been studied in order to release dispersed or dissolved drug via degradation of the polymer matrix [2]. This concept was first proposed as degradation-

controlled drug delivery. However, early work in this area indicated several problems with degradation-controlled drug delivery. In a series of newly designed biodegradable polymers intended for drug delivery, degradation in these polymers was found to occur uniformly throughout the polymer matrices, not from a surface front. This is known as bulk degradation described above. Further, drug release prior to polymer degradation has been quite significant, which makes degradation-controlled drug delivery quite difficult. Thus, the development of drug delivery formulations using surface degradable polymers must overcome several technical hurdles and remains an area of on-going research interest. Surface degradable polymers have been recently designed to effect drug release in proportion to the erosion rate at the polymer surface. These surface-degradable polymers are now recognized as materials which may readily degradation-controlled drug delivery. Differences between surface and bulk degradation, processes are schematically shown in Figure 1-2.

Drug delivery via surface erosion of biodegradable polymers may be advantageous with respect to delivery of drugs from matrices which degrade via the bulk mechanisms. Basically, the degradation of bulk degradable polymers occurs throughout the polymer matrix, which causes both burst effects and inactivation of incorporated drugs. Furthermore, drug diffusion through the polymer matrix is usually significant prior to degradation.

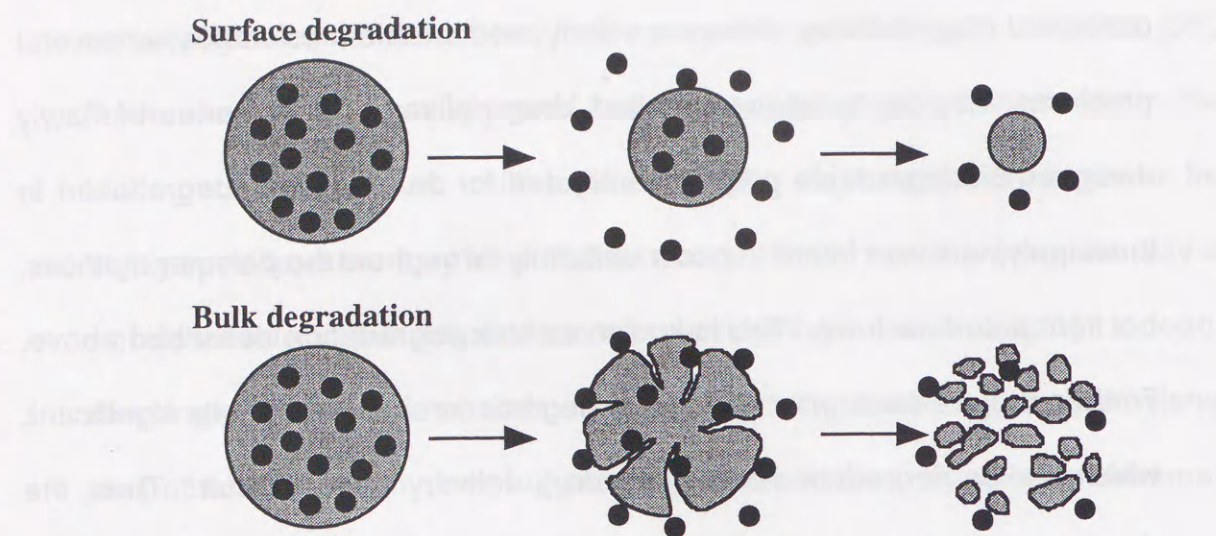


Figure 1-2. Differences between surface and bulk degradation.

Thus, drug release from such biodegradable matrices is difficult to predict, and therefore the successful development of surface degradable polymers for drug formulation is a highly priority. Polymers such as poly(ortho esters), polyacetals, and polyanhydrides have been designed so to effect drug release in proportion to the erosion rate of the polymer surface. Surface erosion (degradation) of these polymers take place through the hydrolysis of labile linkage in hydrophobic polymer backbone [31].

1-3: Drug Delivery Systems Using Biodegradable Hydrogels

There are two different modes of biodegradation, described previously: surface and bulk degradations. The surface and bulk degradations are also called heterogeneous and homogeneous degradations, respectively. These two degradation modes are based on the

location of polymer breakdown. The predominant mechanism of drug release from a bulk-degrading polymer is the simple diffusion of a drug occurring prior to or concurrent with the degradation of the polymer matrix. On the other hand, drug release from a surface-degrading polymer is determined by the relative contribution between the drug diffusion and the degradation of the matrix. In cases where drug molecules are covalently bound to the polymer chains, drug release is determined mainly by the degradation of labile bonds between the drug and the polymer backbone regardless of the degradation mode of the polymer matrix.

In biodegradable hydrogels, drugs are usually in contact with water and thus the drug solubility is an important factor in drug release. The release of drugs with appreciable water solubility will be rapid and independent of the matrix degradation rate. Thus, in general, hydrogels may not be suitable for the controlled release of most low molecular weight, water-soluble drugs. However, it is considered that the biodegradable hydrogel systems are useful for the delivery of macromolecular drugs, such as peptides and proteins, because of their high water content and feasibility to incorporate drugs.

1-3-1: Mechanisms of Drug Release from Biodegradable Systems

In the drug release from biodegradable polymeric systems, two main mechanisms have been proposed such as degradation-controlled and

diffusion-controlled systems. In degradation-controlled monolithic systems, the drug is dispersed uniformly throughout the polymer matrix, and diffusion of the drug is slow compared with polymer degradation. For surface-degrading systems, drug release is affected by the surface-to-volume ratio and the geometry of the device. The effect of the shape of the device on the dissolution of pharmaceutical tablets is well known [32]. It is briefly described an example of drug release from surface degrading devices following the analysis of Hopfenberg [33].

If S_t is the amount of drug released from a sphere of radius r in time t , then the kinetic expression describing release would be given by:

$$dS_t / dt = k_0 4\pi r^2 \quad (1.1)$$

where k_0 is a rate constant. Since the rate-determining erosion process occurring at position r is carried out at an ever decreasing radius, the drug release rate, dS_t / dt decrease continuously. From the mass balance, S_t is also given by:

$$S_t = (4\pi/3)C_0[a^3 - r^3] \quad (1.2)$$

where C_0 is the uniform initial concentration of drug dispersed throughout the device and a is the initial radius at time $t = 0$. Substitution of equation (1.2) into (1.1) and differentiation gives the following relationships:

$$r = a - (k_0 / C_0)t \quad (1.3)$$

Substituting equation (1.3) into (1.2) yields

$$S_t = (4\pi/3)C_0\{a^3 - [a - (k_0 / C_0)t]^3\} \quad (1.4)$$

and since $(4\pi/3)C_0a^3$ is equal to S_∞ , which is the cumulative amount of drug released as time approaches infinity, then following equation is obtained:

$$S_t / S_\infty = 1 - [1 - (k_0 t) / (C_0 a)]^3 \quad (1.5)$$

Similar derivation for a cylinder of radius a and length L provides the following equation:

$$S_t / S_\infty = 1 - [1 - (k_0 t) / (C_0 a)]^2 \quad (1.6)$$

and the equivalent expression for a slab with a thickness of $2a$ would be

$$\begin{aligned} S_t / S_\infty &= 1 - [1 - (k_0 t) / (C_0 a)] \\ &= k_0 t / (C_0 a) \end{aligned} \quad (1.7)$$

Plots of fraction of drug released from sphere, cylinder, and slab against time are given in Figure 1-3.

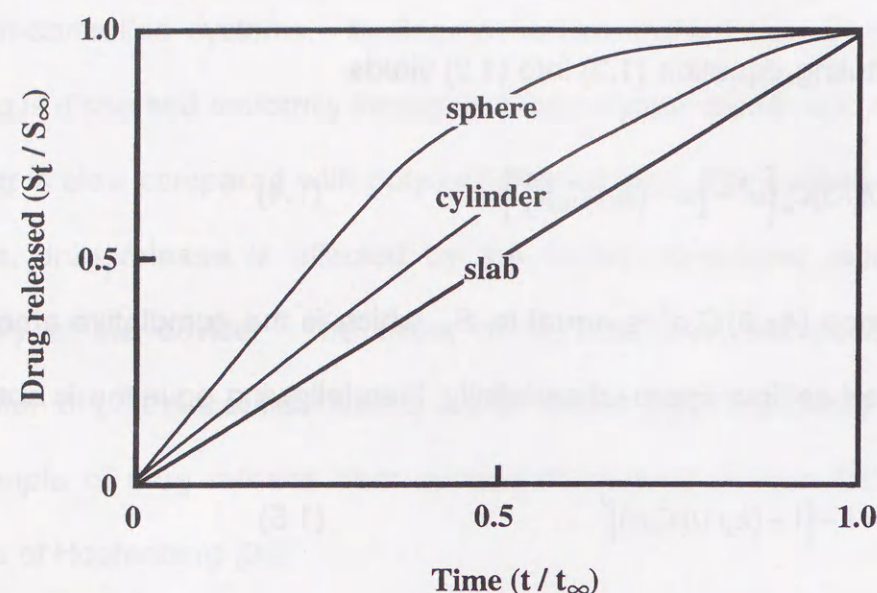


Figure 1-3. Drug release profiles for devices containing uniform dispersed drug and degrading via a surface-controlled degradation.

A detailed understanding of drug release from a matrix undergoing bulk degradation (without swelling) is difficult because drug release occurs by a combination of diffusion and degradation. If the drug diffuses rapidly from the device relative to the degradation of the polymer, the drug release is governed by the equations derived for simple diffusion-controlled systems. The situation becomes more complex if the drug is released during bulk degradation, since the matrix is gradually loosened by degradation. In this case, the drug permeability through the polymer will increase with time, offsetting the decrease in concentration gradient. The following is a model used by Baker for estimation of the permeability changing with time [2].

The release of dispersed drug from nondegradable devices is described by the following Higuchi equation:

$$M_t = A(2DC_sC_0t)^{1/2} \quad (1.8)$$

for $C_0 \gg C_s$

$$= A(2PC_0t)^{1/2} \quad (1.9)$$

where P is the drug permeability ($P = DC_s$). If the matrix is gradually loosened by degradation, the drug permeability will increase with time and such an increase will offset the decline of drug release. If it is assumed that the fractional increase in permeability is inversely proportional to the number of intact (undegraded) bonds in the polymer matrix, then

$$\begin{aligned} P/P_0 &= (\text{initial number of bonds})/(\text{number of bonds remaining}) \\ &= N/(N - Z) \end{aligned} \quad (1.10)$$

where P_0 is the original permeability and P is the permeability after Z cleavages of polymer chains. If it is further assumed that bond cleavage follows simple first-order kinetics, then

$$dZ/dt = k(N - Z) \quad (1.11)$$

where k is the first-order rate constant. This leads to

$$\ln[N/(N - Z)] = kt \quad (1.12)$$

Substitution of equations (1.10) and (1.12) into equation (1.9) yields

$$M_t = A(2P_0 e^{kt} C_0 t)^{1/2} \quad (1.13)$$

Equation (1.13) is plotted in Figure 1-4. The polymer degradation causes the drug permeability to increase. Figure 1-4 also shows the diffusional release of drug in the absence of erosion, which slowly declines and follows the $t^{1/2}$ kinetics. Figure 1-5 shows an example of hydrocortisone release from a degradable polyester device. It is noted that drug release was accelerated as a result of the increase in permeability above the initial value by matrix degradation. If the matrix degrades before drug depletion, a large burst in drug release will be observed [34].

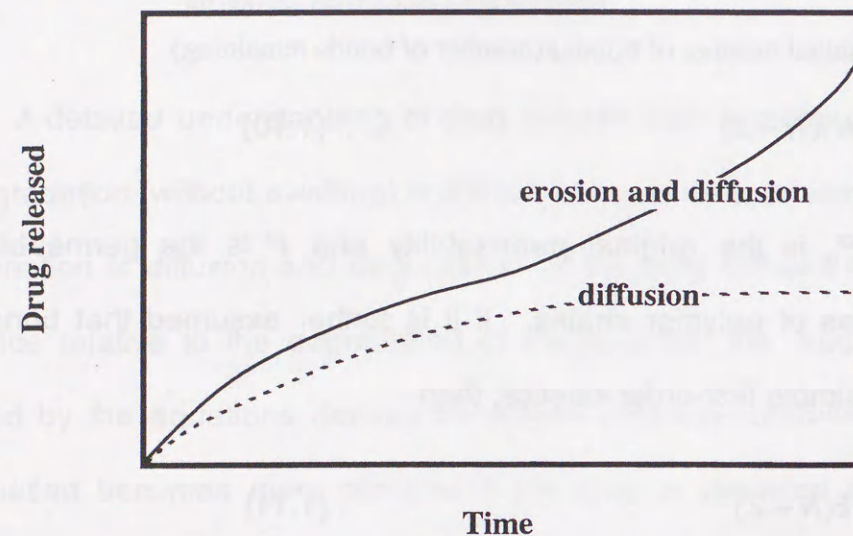


Figure 1-4. Theoretical drug release from a polymer slab by diffusion, and by combined erosion and diffusion.

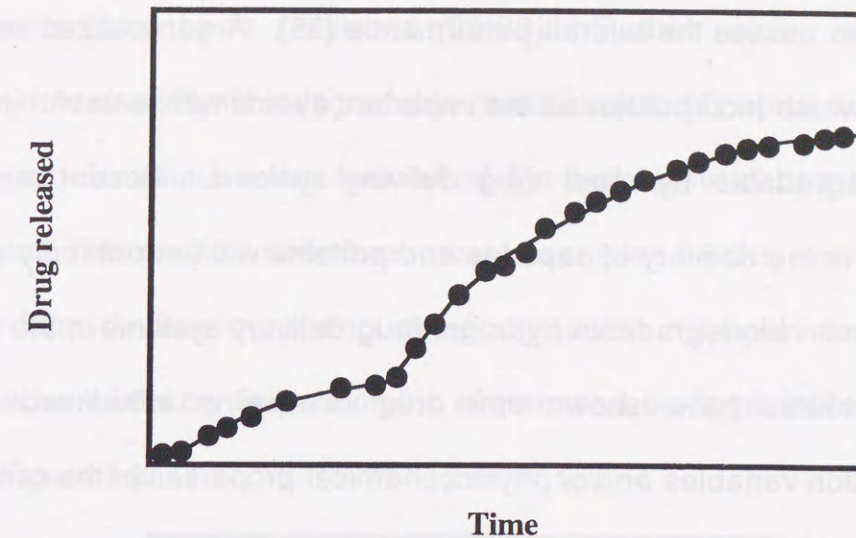


Figure 1-5. Theoretical drug release from a polymer slab by diffusion, and by combined erosion and diffusion.

1-3-2: Biodegradable Hydrogel Systems

Information on the degradation kinetics of biodegradable hydrogels and mathematical modeling of drug release from degrading hydrogels is rather limited at this time. A detailed understanding of drug release from a hydrogel undergoing a homogeneous degradation process is difficult. This is because the degradation and the release characteristics of most degradable hydrogel systems depend on a complex, interrelated set of events. They include, but are not limited to, chemical reaction, multicomponent diffusion, and physical changes in the system such as tortuosity and porosity. During the degradation process, the drug permeability continuously changes as a result of the combination of all these interrelated events and thus, it is often

difficult to assess the overall performance [35]. A generalized mathematical model which incorporates all the important events will be useful in the design of biodegradable hydrogel drug delivery systems. Recent rapid growing interest in the delivery of peptides and proteins will undoubtedly attract more attention on biodegradable hydrogel drug delivery systems in the near future. Many studies have shown that drug release is influenced by various formulation variables and/or physicochemical properties of the components in biodegradable drug delivery systems. In addition to the rate of polymer degradation, drug release is affected by the physical parameters of the polymer such as the water content, degree of crosslinking, crystallinity, and phase separation [2]. The physicochemical properties of the drug, particularly its solubility in the polymer and in the aqueous medium, and the amount of drug loading are also expected to have profound effects on the release characteristics of the drug-polymer composite. For these biodegradable systems the rate can be achieved by adjusting these parameters.

1-4: Optimized Modeling of Biodegradable Drug Release Formulations with Heterogeneous Structures

Much work has been done with biodegradable polymers since poly(glycolic acid) was first marketed as a biodegradable suture material. Many successful techniques for preparation of microparticles from biodegradable polymers such as poly(lactic acid), poly(glycolic acid), and their

copolymers have been published [36-39]. Further, recent research has brought new classes of biodegradable materials [40, 41] which demonstrate a surface erosion mechanism instead of the bulk erosion pattern of earlier materials. This section describes recent examples for the analysis of drug release from devices consisting of biodegradable microparticles which are contained within a continuous matrix of a second biodegradable polymer as shown Figure 1-6.

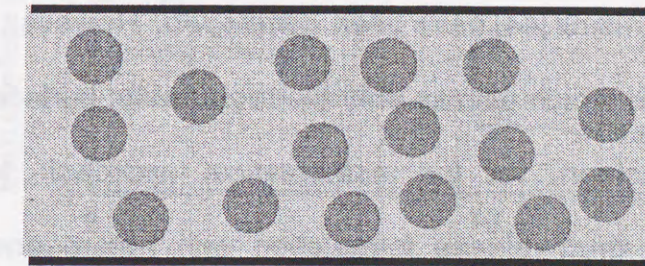


Figure 1-6. Biodegradable microspheres containing active agent formulated within a cylinder of a second biodegradable polymer.

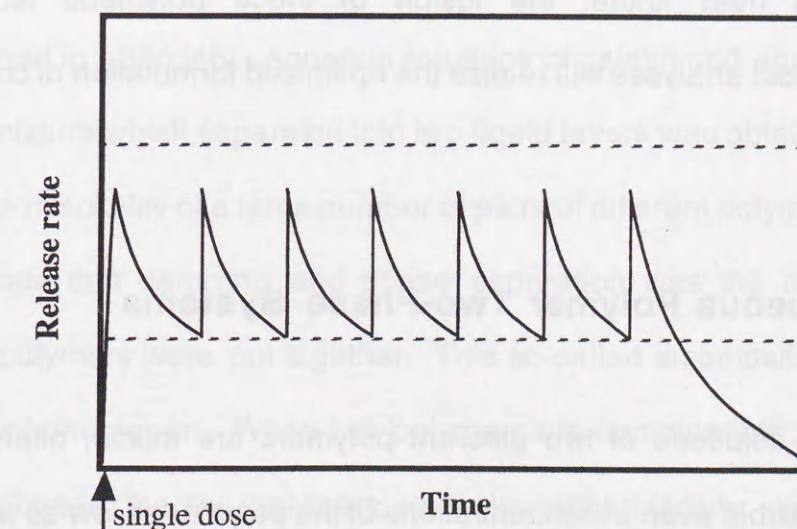


Figure 1-7. Rate of drug release from the formulation in Figure 1-6.

The purpose of the release devices presented here is to release drug within a desired range of release rates for an extended period of time, yet not be zero-order release. The geometry of this biodegradable polymer matrix described is that of a cylinder with one or both circular ends exposed for erosion. The general configuration has been mentioned in the patent literature, but only with non-degradable or bulk-eroding polymers [42]. Mathematical analyses of degradation of particular polymers [43] and modeling and simulation of various mechanisms [44] have been developed. However, no formulation has been described which utilizes only biodegradable, surface-eroding polymers in such a fashion. As for mathematical approach for construction of biodegradable drug release formulation with heterogeneous structure has been proposed [45] and Figure 1-7 shows the release pattern from the formulation as a result of the mathematical analysis.

In near future, the fusion of these polymeric technique and mathematical analyses will realize the optimized formulation of controlled drug release.

1-5: Aqueous Polymer Two-Phase Systems

If solutions of two different polymers are mixed, often the mixture becomes turbid even at concentrations of the polymer as low as a few percent. Such a mixture will eventually separate into two layers. Analysis of the phases at various concentrations of the polymers shows that the system

behaves as a three-component system (disregarding the polydispersity of the polymers) and that the two layers behave as two phases in equilibrium, conforming to the phase rule. An example of a phase diagram is given in Figure 1-8.

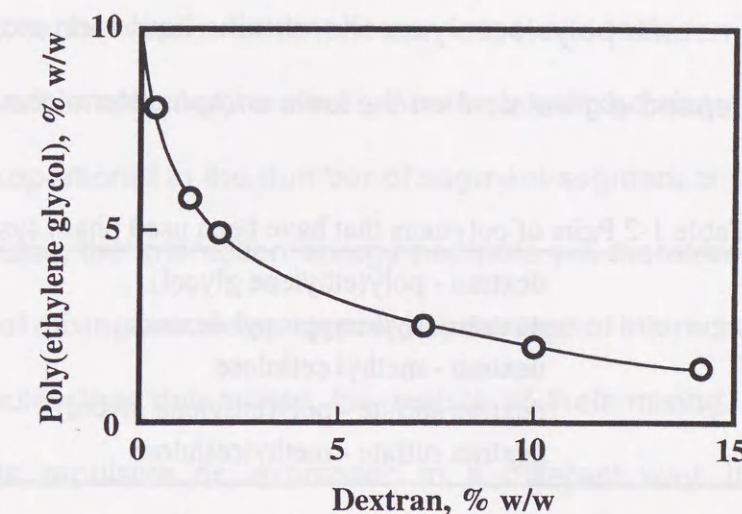


Figure 1-8. Phase diagram of the system PEG/Dex water.

Such liquid-liquid phase separation in macromolecular mixtures was first reported in 1896 [46]. Aqueous solutions of gelatin and agar were mixed, a turbid mixture which separated into two liquid layers was obtained. After that report, the miscibility of a large number of pairs of different polymers and could demonstrate that demixing and phase separation was the rule when two different polymers were put together. This so-called incompatibility is a very common phenomenon. When two polymers are incompatible they collect in different phases; the two polymers are only partly miscible. In some cases, however, the two polymers collect together in one of the phases, which therefore becomes polymer rich while the other phase consists mainly of

solvent. This phenomenon occurs frequently when the two polymers carry opposite charges. This kind of phase separation has been studied in detail.

Some phase systems that have been used in partition studies are listed in Table 1-2. Both nonionic polymers and polyelectrolytes may be used. In the systems with polyelectrolytes one obtains liquid ion exchangers. Their properties depend a great deal on the ionic composition of the system.

Table 1-2 Pairs of polymers that have been used phase systems

dextran - poly(ethylene glycol)
dextran - hydroxypropyl dextran
dextran - methyl cellulose
dextran sulfate - poly(ethylene glycol)
dextran sulfate - methylcellulose

Phase separation can also occur in polymer mixtures where one of the polymers, for example, is a protein. In some cases the protein will collect in a liquid, protein-rich phase, but more often the protein forms a thick gel or solid-like phase. This is frequently the result when proteins are mixed with high concentrations of poly(ethylene glycol) (PEG). It has been utilized with success for purification purposes. Since proteins are polyelectrolytes, their phase separation with PEG depends on the ionic composition [47], as do all other polyelectrolyte systems. The theory of phase separation in polymer mixtures has been treated by many authors [48-50]. Only a very qualitative account of them will be given here.

The result of mixing substances depends on the free energy of mixing. A mixture will split into two phases if this decreases the free energy of

the system. The free energy of mixing in turn depends on interaction energy term and entropy of mixing terms. As a first approximation the gain in entropy on mixing is related to the number of molecules involved in the mixing process. The entropy of mixing per mole is therefore, again as a first approximation, the same for small and large molecules. The interaction energy between molecules, however, increases with the size of the molecules since it is proportional to the number of segment-segment contacts. For very large molecules, the interaction energy per mole will therefore dominate over the entropy of mixing per mole. Hence, it is the type of interaction between the macromolecules that determines the results of their mixing. If the type of interaction is repulsive or, expressed in a different way, if the attraction between like molecules is larger than between unlike molecules, the system will have its energetically most favorable state when the two polymers are separated. The result of mixing of two such polymers is "incompatibility"; one phase contains predominantly the one polymer, and the other phase the other polymer. If, on the other hand, the attraction between unlike polymers is stronger than between like polymers, there will be a gain in free energy if the two polymers collect together and their separation into a common phase is favored.

1-5-1: The Effect of Molecular Weight of The Polymer as a Factor on The Phase Systems

Aqueous polymer two-phase systems are obtained by mixing two different water-soluble polymers in water, described above. Such phase systems can be used for partition of biomolecules such as proteins and nucleic acids and also cell particles such as cell organelles and membrane vesicles. Partition depends on many factors. In the case of proteins for a phase system containing the two polymers, poly(ethylene glycol) (PEG) and dextran (Dex), these have been studied in detail [47]. The partition coefficient (concentration of protein in upper phase divided by concentration in the lower phase) depends, among other factors, on the molecular weight and charges of the partitioned proteins, the ionic composition of the phase system and the type and molecular weight of the polymers [47]. The effect of the molecular weight of the polymers is such that when the molecular weight of one polymer is decreased the protein favors more the phase rich in this polymer. For example, in the PEG-Dex phase system, if the molecular weight of Dex is reduced a protein will favor more the lower Dex-rich phase. Conversely, if the molecular weight of the PEG is reduced the protein will favor more the upper, PEG-rich phase [47].

1-5-2: Physical Stability of Proteins and Peptides in Aqueous Polymer Two-Phase Systems

In recent years, the developments of protein or peptide drug formulations have become an important research area for pharmaceutical

sciences. The production of protein or peptide drugs as therapeutic agents has been accelerated because of recent advances in biotechnology and modern peptide synthetic chemistry. However, these proteins and peptides have such disadvantages as physical and chemical instability. Physical instability generally refers to changes in the secondary, tertiary and higher-ordered structure with denaturation, aggregation and precipitation. Chemical instability can be defined as any modification process including hydrolytic and oxidative degradation. To enhance the stability of proteins and peptides, conjugations or mixtures with water-soluble polymers have been proposed since 1970's. It is considered that the advantages of them involve prevention from their enzymatic degradation, rapid elimination from a body, and antigenicities of the peptides [51, 52]. In the aqueous polymer two-phase systems, physical stability of the proteins and peptides partitioned into PEG phase has been reported [47]. When proteins and peptides are partitioned into the PEG phase in an aqueous polymer two-phase system, it is considered that the surface of the proteins and peptides may be covered or the chain of them may be entangled with PEG. Therefore, the physical stability of them in PEG phase increases compare with them in PBS, saline or other aqueous solutions.

1-6: Objective of This Study

During the past two decades biodegradable polymeric systems for controlled drug delivery have been extensively studied because an invasive technique such as surgery is not required after their use. Specifically, biodegradable hydrogels have been represented as an attractive drug formulation because of their advantages, such as biocompatibility, high responsibility for specific degradation, and a feasible approach for incorporating drugs into matrices. However, unexpected drug leakage from the matrix caused by drug diffusion has been a significant clinical problem. In order to prevent drug diffusion and to achieve degradation-controlled drug release from a highly water-swollen matrix, this study proposes a new approach for preparing heterogeneous-structured hydrogels for bioactive peptide delivery. The concept of degradation-controlled drug release matrix, a goal of this study, is illustrated in Figure 1-9. This concept will lead to a stimuli-responsive drug delivery system with heterogeneous-structured formulations. As one promising way to achieve the degradation-controlled drug release, the design of biodegradable polymers with a heterogeneous structure which is based on an aqueous polymer two-phase system is suggested.

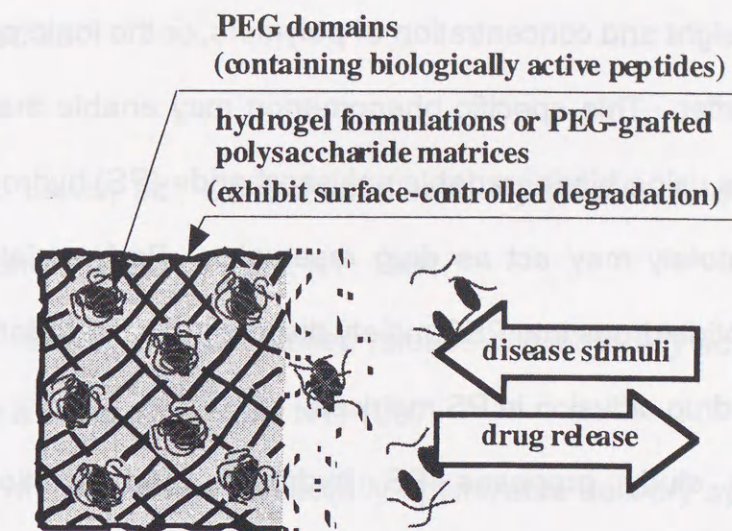


Figure 1-9. Stimuli responsive (degradation-controlled) drug delivery system with heterogeneous structures.

In the field of biochemical researches, several separation techniques have been of great importance to advance biochemistry and related fields. In particular, liquid-liquid distribution of macromolecules provides a procedure for the purification and study of proteins, cells, and cell particles, which is an aqueous polymer two-phase system. The two-phase system is obtained by mixing aqueous solutions of two different water-soluble polymers, such as PEG and dextran. The top phase is the PEG solution, and the bottom phase is the dextran solution in this two-phase system. The separation of proteins in such a system is based on differences in their distribution between two phases. For example, in the case of using PEG and dextran (or hyaluronic acid) two-phase systems in a phosphate buffer solution, it is expected that negatively charged proteins such as insulin will be preferentially partitioned into the PEG phase. Partition differences depend on such parameters as the

molecular weight and concentration of polymers, or the ionic composition and pH of the buffer. This specific phenomenon may enable the formulation of peptide drugs using biodegradable polysaccharide (PS) hydrogels containing PEG: PEG moiety may act as drug reservoirs. Preferential distribution of bioactive peptide drugs into PEG moiety dispersing in degradable PS matrices may prevent drug diffusion in PS matrices.

This study proposes PS hydrogels and solutions with PEG microdomains for regulated peptide delivery. A series of crosslinked PS hydrogels containing PEG, and PEG-grafted PS's were prepared as a model of heterogeneous-structured devices composed of drug reservoirs and degradable matrices, and as a model of the peptide drug, insulin release during their enzymatic degradation was examined in relation to their two-phase morphology. The preliminary results will enable a novel approach to design matrices which exhibit degradation-controlled peptide release.

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Chapter 2

Regulated Insulin Release from Biodegradable Dextran Hydrogels Containing Poly(ethylene glycol)

2-1: Introduction

Biodegradable polymeric systems for controlled release drug delivery have been extensively studied because an invasive technique such as surgery is not required after their use [1, 2]. Especially, biodegradable hydrogels have been represented as an attractive drug formulation because of their advantages such as biocompatibility, high responsibility for specific degradation, and feasible approach to incorporate drugs into matrices. However, it seems quite difficult to achieve degradation-controlled drug release from these hydrogels since their highly water swollen states cause drug diffusion through the matrices. In order to prevent drug diffusion in such a highly water swollen hydrogel, a heterogeneous-structured hydrogel of a degradable hyaluronic acid (HA) matrix containing drug microreservoirs (lipid microspheres) has been proposed as a newly designed implantable device for inflammation responsive drug delivery [3, 4]. The heterogeneous-structured HA hydrogel was found to enable degradation-controlled release of lipid microspheres. Furthermore, the heterogeneous-structured device was also feasible for regulating drug release from biodegradable hydrogels and for protecting the inactivation of drug by the hydrophobic nature of microreservoirs. When water-soluble drugs such as bioactive peptides are introduced in biodegradable hydrogels, another approach will be required because the application of lipid microspheres limits to water-insoluble hydrophobic drugs. This study will propose new approach of designing

biodegradable hydrogels for bioactive peptide delivery, which is based on an aqueous polymer two-phase system.

In the field of biochemical researches, several separation techniques have been of great importance to advance biochemistry and related fields. In particular, liquid-liquid distribution of macromolecules provides a procedure for purification and study of proteins, cells and cell particles, which is an aqueous polymer two-phase system [5-10]. The two-phase system is obtained by mixing aqueous solutions of two different water-soluble polymers such as poly(ethylene glycol) (PEG) and dextran. The top phase is PEG solution and the bottom phase is dextran solution in this two-phase system. The separation of proteins in such system is based on differences in their distribution between two phases. For example, in the case of using a PEG and dextran two-phase system in phosphate buffer solution, it is expected that negatively charged proteins such as insulin will be preferentially partitioned into PEG phase. Partition differences depend on some parameters such as the molecular weight and concentration of polymers, or the ionic composition and pH of buffer [6]. This specific phenomenon may enable the formulation of peptide drugs using biodegradable dextran hydrogels containing PEG: PEG moiety may act as drug reservoirs. Preferential distribution of bioactive peptide drugs into PEG moiety dispersing in degradable dextran matrices may prevent drug diffusion in dextran matrices.

In this chapter, a series of crosslinked dextran hydrogels containing PEG were prepared as a model of heterogeneous-structured devices

composed of drug reservoirs and degradable matrices, and insulin release during their enzymatic degradation were examined. The preliminary results will propose novel approach to design hydrogels which exhibit degradation-controlled peptide release.

2-2: Materials and Methods

2-2-1: Chemicals

All Chemicals were analytical-grade commercial materials and used without further purification. Poly(ethylene glycol) (PEG) (Mn=1000, 6000, 50000), glycidyl methacrylate, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TMEDA), fluorescein isothiocyanate (FITC) and insulin from bovine pancreas (26unit/mg), were purchased from Wako Pure Chemical Industries, Osaka, Japan. Dextran (Mn=40000) was purchased from Tokyo Chemical Industry Co., Tokyo, Japan. Dextranase from penicillium sp. (12.9unit/mg) was purchased from Sigma Chemical Co., St. Louis, U. S. A.

2-2-2: Partition of Insulin in PEG/dextran Two-Phase Systems

Partition of insulin between PEG and dextran solutions was determined by the fluorescence of FITC-labeled insulin, which was prepared as follows [11] : Insulin (100mg, 17.2mmol) was dissolved in 5 ml of 0.5M Na₂CO₃-NaHCO₃ buffer (pH9.2), and the final volume was adjusted to 7ml with 0.15M NaCl. FITC (7.0mg, 17.2mmol) was added to the insulin solution.

After 8h stirring at 22-24°C, the reaction was terminated by adjusting pH to 4.5 with HCl. Then, the solution was kept at 4°C for 16h to obtain FITC-labeled insulin as a precipitate. The reaction mixture was centrifuged at 2000rpm for 5min to remove the fluorescent impurities, and the precipitate was washed with a few milliliters of cold 0.01M ammonium acetate at pH4.5. After removal of the fluorescent impurities, the supernatant of the mixture was colorless, indicating that FITC-labeled insulin was already purified. Then drying *in vacuo*, FITC-labeled insulin was obtained as a fine powder.

40wt.% polymer solutions (1.0ml) of PEG and dextran in 0.1M phosphate buffer solution (PBS, pH7.4) and 1mg/ml FITC-labeled insulin solution (0.5ml) in PBS were mixed. The mixture was stirred for 1min and left to settle for at least 30min. Insulin concentration in both top and bottom phases was determined by FITC fluorescent intensity excited at 490nm and emitted at 520nm using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777).

2-2-3: Preparation of PEG/Dex Hydrogels

Methacrylic dextran (MA-dextran) was prepared as follows [12]: dextran (1.0g, 25mmol) was dissolved in 10ml of 0.01M carbonate buffer (pH11). Glycidyl methacrylate (0.5ml, 3.8mmol) was added to the dextran solution, and the mixture was stirred for 5days at room temperature. Dextran was precipitated with 400ml of ethanol and washed with 100ml of ethanol. After centrifugation at 3000rpm for 5min, the precipitated dextran was

dissolved in 5ml of distilled water, precipitated again, and washed with ethanol. The procedure was repeated at least four times to free the product from excess glycidyl methacrylate. The content of methacrylic groups in the dextran derivatives was measured by using UV spectrophotometer at 260nm. The calibration curve for methacrylic groups was determined using glycidyl methacrylate as a standard. It was confirmed that one methacrylic group was introduced per 46 glucose residues in dextran chains.

40wt.% PEG solution in 0.1M PBS (pH7.4), 40wt.% MA-dextran solution in PBS and 1mg/ml FITC-labeled insulin solution in PBS were mixed in the vol. ratio of 5:90:5, respectively, followed by the addition of APS (20mg) to the mixture. After vigorous stirring for 1min, the mixture was injected to a cell (inner diameter: 4cm, height: 1mm). In order to crosslink MA-dextran, 50 μ l of TMEDA was injected to the cell and left to settle at 4°C for 10min. It was confirmed that the turbidity of PEG/Dex solution mixture was constant for much longer than that the reaction time for crosslinking dextran, which suggests that the heterogeneity of PEG/Dex mixture by the vigorous stirring was maintained during the crosslinking reaction. The gel obtained was immediately placed into an excess of PBS. The weights of both equilibrated gel in PBS and its dried one were measured for calculating the water content. Crosslinked dextran hydrogel (Dex hydrogel) was transparent, but crosslinked dextran hydrogels containing PEG (PEG/Dex hydrogels) were opaque. The water contents of these hydrogels were approximately 93-94% independent of PEG

content. In spite of their high water contents, PEG/Dex hydrogels were mechanically tough and could be used as a slab shape.

2-2-4: Characterization of PEG/Dex Hydrogels

The morphology of PEG/Dex hydrogels in swollen state was observed with a phase-contrast microscope (PCM) (Olympus Co., Tokyo, Japan, U-SPT). After freeze drying, differential scanning calorimetric analysis of PEG/Dex hydrogels was also examined (Seiko Instruments Co., Tokyo, Japan, DSC120) in the temperature range from -25 to 160°C; sample weights were 6mg; and the scanning rate was 2°C/min. In order to estimate the heterogeneity of PEG/Dex hydrogels in swollen states, small-angle light scattering (SALS) measurements were carried out using SALS apparatus (IST Planning Co., Tokyo, Japan, SALS-100S) with He-Ne laser ($\lambda=632.8\text{nm}$, 2mW) as a light source.

2-2-5: Enzymatic Degradation of PEG/Dex Hydrogels and Insulin Release

The degradation of PEG/Dex hydrogels by dextranase was examined *in vitro*. Here, three different shapes were used with identical mass: slabs, rectangular solids, and cubes. After equilibration in 0.1M PBS (pH7.4), the hydrogels were mounted in a nylon mesh-bag attached to a thread for dipping into solutions and immersed into dextranase solution in PBS (2 or 0.4unit/ml)

with stirring to initiate the degradation. The degradation rate was estimated by measuring the residual weight of the hydrogel together with the nylon mesh-bag. Insulin release from the slab hydrogel was estimated by measuring FITC fluorescent intensity of the system solutions excited at 490nm and emitted at 520nm continuously using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777) equipped with a flow cell (Japan Spectroscopy Co., Tokyo, Japan, FSC-322). The total volume of this flow system was 15ml, and the flow rate was fixed at 30ml/min using a peristaltic pump (Iwaki Glass Co., Tokyo, Japan, PST-100).

2-3: Results and Discussion

2-3-1: Partition of Insulin in PEG/dextran Two-Phase Systems

In order to explain the partition of substances in aqueous polymer two-phase systems, it is necessary to discuss the basic mechanism of phase separation. Recent investigation has been made on the molecular mechanism of the phase separation in aqueous polymer systems [8]. If solutions of two different polymers are mixed, the mixture often becomes turbid even at concentration of polymer as low as a few percent. Such a mixture will eventually separate into two phases. The result of mixing solutions depends on the free energy of mixing. If the free energy of mixing is decreased, the mixture will separate into two phases. The free energy of mixing depends on interaction energy terms and entropy of mixing terms. This analysis is based

on a modified Flory-Huggins theory for polymer solutions [6]. The entropy of mixing per mole is the same for small and large molecules since the gain in entropy of mixing is related to the number of molecules in the mixture. However, the interaction energy between molecules increases with the size of the molecules since it is proportional to the number of segment-segment contacts. For large molecules, the interaction energy per mole will therefore dominate over the entropy of mixing per mole. Therefore, it is considered that the type of interaction between the macromolecules determines the results of their mixing. If the type of interaction is repulsive, the system will have its energetically most favorable state when the two polymers are separated. The result of mixing of such two polymers is immiscible: one phase contains predominantly the one polymer, and the other phase the other polymer. In PEG/dextran two-phase systems, although low molecular weight substances such as salts, amino acids, and nucleotides partition equally between the phases, larger molecules such as proteins usually have partition coefficients in the range between 0.1 and 10 [5]. Further, both the molecular weight of the polymers and the ionic strength of buffer have a strong influence on the partition [5]. If the preferential partition of drugs into PEG phase can be obtained in PEG/dextran systems, it is possible to design the heterogeneous-structured devices composed of drug reservoirs (PEG) and degradable dextran matrices. Then, as a model of bioactive peptide drugs, insulin was used in this study.

Figure 2-1 shows the partition of insulin in the PEG/dextran system with different PEG molecular weights ($M_n=1000, 6000, 50000$; PEG1, PEG6, PEG50, respectively) in 0.1M PBS. In this system, insulin as a model of bioactive peptide drugs was preferentially partitioned into the PEG phase. It is considered that negatively charged protein of insulin was mainly partitioned into the PEG phase because phosphate ions have been reported to be partitioned into the dextran phase [6]. The concentration of insulin in the PEG phase decreased with an increase in the PEG molecular weight, while little change occurred in the insulin concentration in the dextran phase. It seems likely that insulin in the PEG phase transferred to an interface between two phases with increasing the molecular weight of PEG. Generally, it is recognized that if the molecular weight of one of two polymers increases, the entropy of mixing protein and the polymer will be reduced [9]. Thus, it is obvious that the concentration of proteins in a certain phase decrease with the molecular weight of polymer.

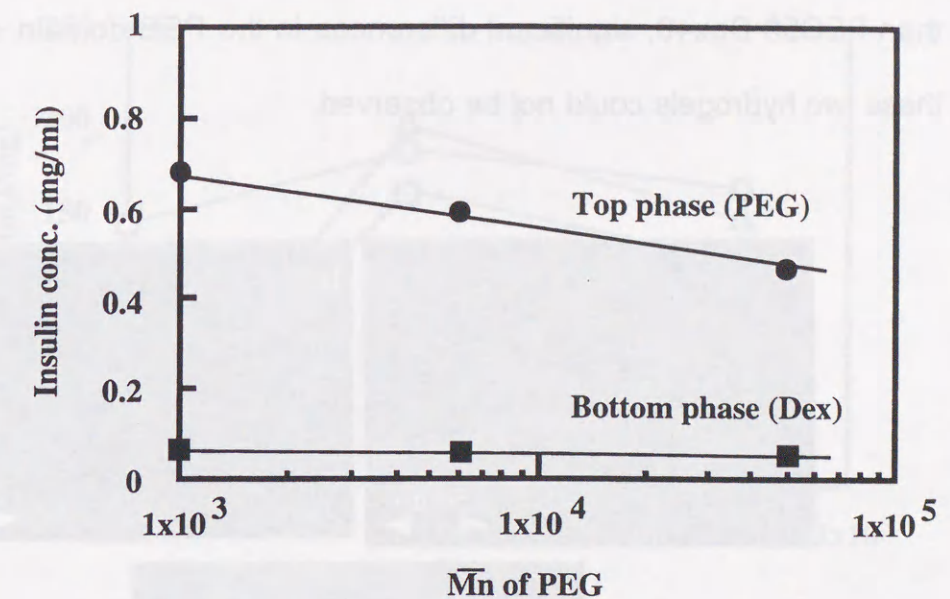


Figure 2-1. Partition of insulin in PEG/Dex two-phase systems in 0.1M PBS (pH 7.4).

2-3-2: Two-Phase Morphology of PEG/Dex Hydrogels

This study includes four different crosslinked hydrogels: a crosslinked dextran hydrogel (Dex40), and three types of PEG/Dex hydrogels with different molecular weights; 1000 (PEG1-Dex40), 6000 (PEG6-Dex40), and 50000 (PEG50-Dex40). Figure 2-2 shows phase-contrast micrographs of PEG/Dex hydrogels with different PEG molecular weights. Since the PEG content in PEG/Dex hydrogel was fixed at 5% in this study, spherical domains correspond to PEG moieties in these micrographs. PEG domains in PEG6-Dex40 and PEG50-Dex40 were observed with several dozens of micron-order whereas much smaller PEG domains were observed in PEG1-Dex40. Although larger PEG domains were somewhere observed in PEG6-Dex40

than PEG50-Dex40, significant differences in the PEG domain size between these two hydrogels could not be observed.

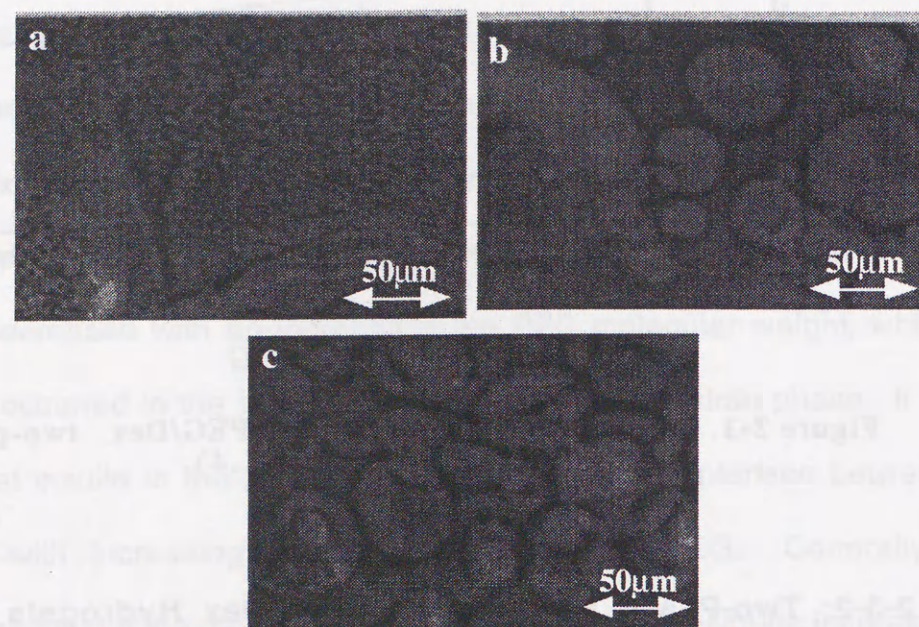


Figure 2-2. PCM pictures of PEG/Dex hydrogels with different PEG molecular weight. a, PEG1-Dex40; b, PEG6-Dex40; c, PEG50-Dex40.

Figure 2-3 shows the results of differential scanning calorimetric analysis: the heat of fusion (ΔH) of PEG moiety in PEG/Dex hydrogels. In the case of PEG homopolymer, there was not significant difference in ΔH of PEG (140 to 175 mJ/mg) among three different molecular weights. On the other hand, in the case of PEG/Dex hydrogels, significant decrease in ΔH of PEG was observed in PEG1-Dex40 compared with other two PEG/Dex hydrogels. In contrast, the magnitudes of ΔH of PEG in PEG6-Dex40 and PEG50-Dex40 were almost identical with those of homopolymers. This tendency was also

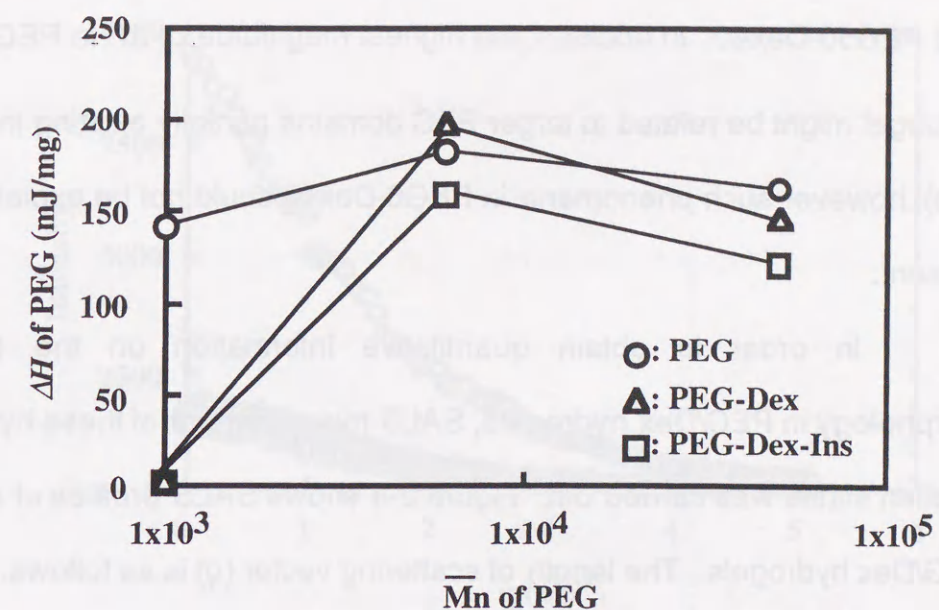


Figure 2-3. The heat of fusion (ΔH) of PEG moiety in PEG/Dex hydrogels with different molecular weight.

observed in the case of PEG/Dex hydrogels containing insulin. It is supposed that the magnitudes of ΔH reflect the degree of PEG crystallinity. The crystallinity of a certain polymeric chain in two-phase systems such as polymer-blends and/or block-copolymers in general is governed by miscibility with another component, and dominates some morphological characteristics such as the size and phase mixing of microdomain structures. Therefore, the results of Figure 2-3 may suggest that PEG6-Dex40 and PEG50-Dex40 exhibited PEG domains well phase-separated from dextran matrices whereas PEG crystallization was hindered by the occurrence of phase mixing with dextran to form much smaller domains in PEG1-Dex40. This consideration is supported by the PCM pictures in Figure 2-2, where the progress in well phase-separation between PEG and dextran were observed in PEG6-Dex40

and PEG50-Dex40. In addition, the highest magnitude of ΔH in PEG6-Dex40 hydrogel might be related to larger PEG domains partially existing in Figure 2-2 (b), however such phenomena in PEG6-Dex40 could not be explained at present.

In order to obtain quantitative information on the two-phase morphology in PEG/Dex hydrogels, SALS measurement of these hydrogels in swollen states was carried out. Figure 2-4 shows SALS profiles of a series of PEG/Dex hydrogels. The length of scattering vector (q) is as follows:

$$q = (4\pi/\lambda)\sin(\theta/2) \quad (2.1)$$

where λ is wave length of a light source and θ is a scattering angle. The scattering intensity of PEG1-Dex40 shows a maximum at a magnitude of $q_{max}=1.1 \times 10^{-3} \text{ nm}^{-1}$. The interdomain distance (r) is defined as the following equation [12-15]:

$$r = 2\pi / q_{max} \quad (2.2)$$

The result calculated from equation (2.2) was $5.7 \mu\text{m}$, which corresponds to average distance between PEG domains and exhibited agreement with Figure 2-2 (a). The scattering intensity of other two hydrogels has no significant peaks, which may indicate that the size of PEG domain as scattering particle had wide distribution. Thus, in order to estimate the heterogeneity of PEG/Dex hydrogels, the correlation distance (l_c) was obtained from Debye-Bueche plots [16, 17] of the results in Figure 2-4 as square root of the ratio of slope to intercept of the plots (Figure 2-5).

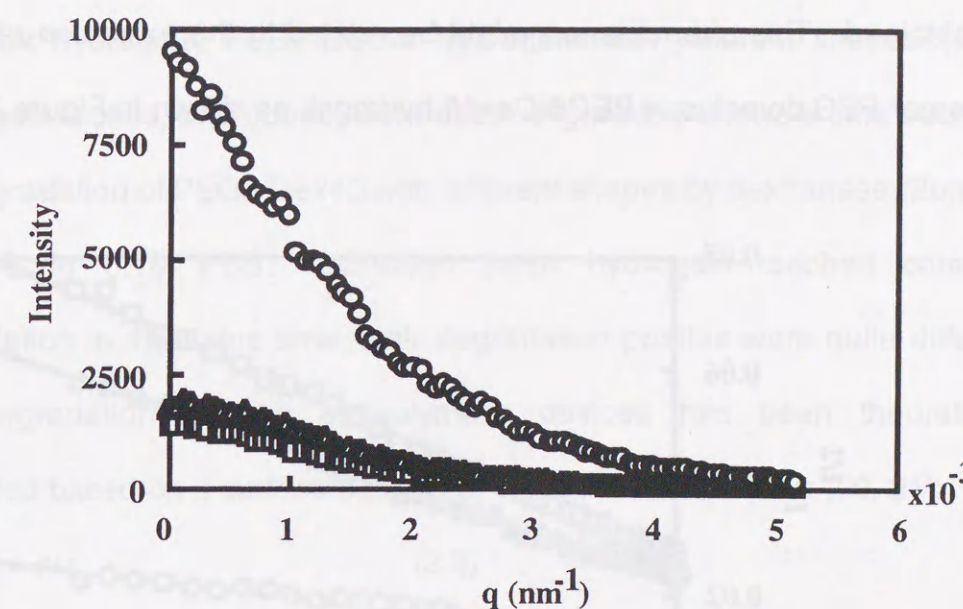


Figure 2-4. SALS profiles of PEG/Dex hydrogels with different molecular weight. $\circ$, PEG1-Dex40; Δ , PEG6-Dex40; $\square$, PEG50-Dex40.

In general, l_c is known as an index of heterogeneity, which can be defined as average length of randomly drawn vectors passing through the two phases [16, 17]. The values of l_c increased with PEG molecular weights but seemed to reach somewhat constant (427.8, 486.2, and 517.7nm for PEG1-Dex40, PEG6-Dex40, and PEG50-Dex40, respectively), which suggests the heterogeneity of PEG/Dex hydrogels also increase with PEG molecular weights. Since it has been previously reported that the interdomain distance and domain size in certain polymer-blend systems increase theoretically in proportion to $N^{2/3}$, where N is the degree of polymerization [18, 19], it may be expected that l_c also increases in proportion to $N^{2/3}$. However, in this experiment, the linearity between l_c and PEG molecular weights was not

obtained. This phenomenon might be related to the existence of somewhat larger PEG domains in PEG6-Dex40 hydrogel, as shown in Figure 2-2 (b).

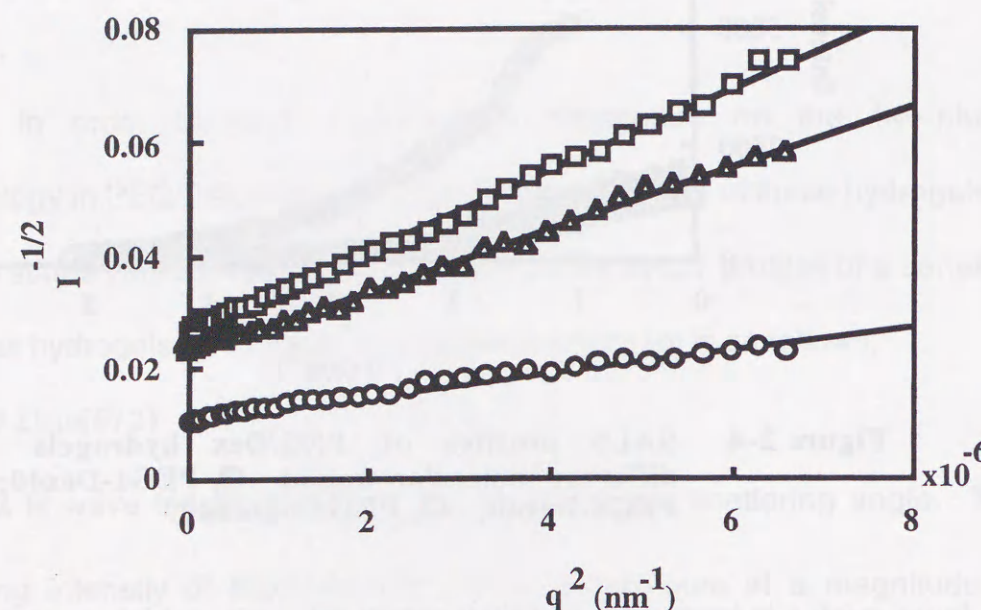


Figure 2-5. Debye-Bueche plots of the results of Figure 2-4.

2-3-3: Surface-Controlled Degradation of PEG/Dex Hydrogels by Dextranase

Previously, the degradation of crosslinked hydrogel was estimated by two independent procedures: one was the analysis of the degradation products by using high-performance liquid chromatography and the other was changes in residual weights of the hydrogel [3]. Since results from these two procedures were in good agreement in terms of the degradation profiles, thus, the degradation of PEG/Dex hydrogels was monitored by measuring the residual weights in this study. In order to examine degradation mechanism of

PEG/Dex hydrogels, PEG6-Dex40 hydrogels with different shapes (slabs, rectangular solids, and cubes) were used. Figure 2-6 shows a time course of the degradation of PEG6-Dex40 with different shapes by dextranase (2unit/ml) at 37°C in 0.1M PBS. Although these hydrogels reached complete degradation in the same time, their degradation profiles were quite different. The degradation kinetics of polymeric devices has been theoretically estimated based on a surface-controlled degradation as follows [20, 21]:

$$M_t / M_\infty = t / t_\infty \quad (2.3)$$

where M_t is the amount of polymer degraded from a slab at any time t , M_∞ is the total amount of polymer, and t_∞ is the time to complete degradation. In the similar manner, expressions for devices with cylindrical and spherical shapes, respectively, are introduced as follows [2, 22].

$$M_t / M_\infty = 2t / t_\infty - (t / t_\infty)^2 \quad (2.4)$$

$$M_t / M_\infty = 1 - [1 - (t / t_\infty)]^3 \quad (2.5)$$

Equations (2.4) and (2.5) are available for devices with rectangular and cubic shapes respectively, and yield another expression as follows:

$$1 - [1 - (M_t / M_\infty)]^{1/2} = t / t_\infty \quad (2.6)$$

$$1 - [1 - (M_t / M_\infty)]^{1/3} = t / t_\infty \quad (2.7)$$

Figure 2-7 shows plots of equations (2.3), (2.6) and (2.7) based on the data in Figure 2-6. The plots from equations (2.3), (2.6) and (2.7) using the data in Figure 2-6 fall on the theoretical line, as shown in Figure 2-7. These plots provide almost a linear relationship, indicating that the degradation of

PEG/Dex hydrogels by dextranase takes place via a surface-controlled mechanism. It is considered that intrusion of dextranase into the hydrogels was inhibited by steric restraints, resulting in the enzymatic hydrolysis from the surface front.

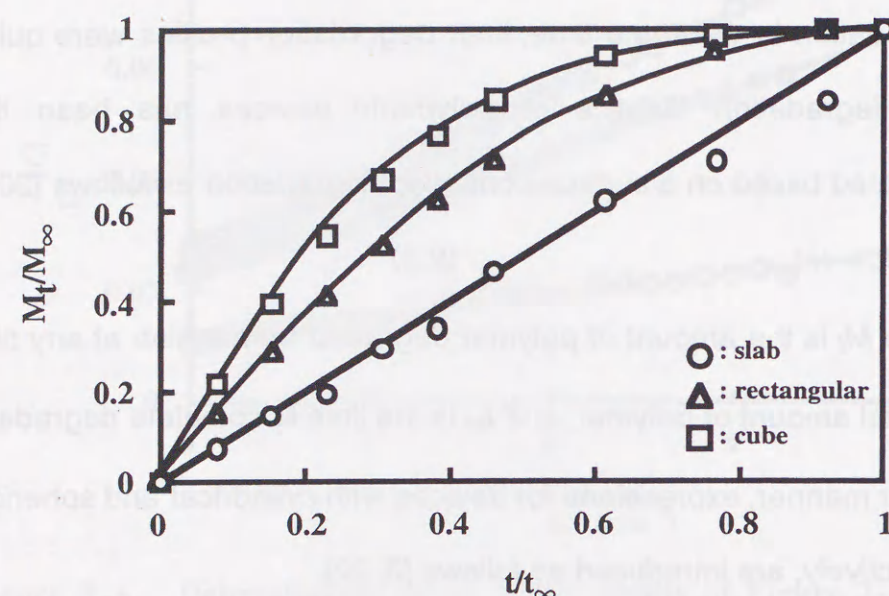


Figure 2-6. Enzymatic degradation of PEG/Dex hydrogels with three different shapes.

Figure 2-8 shows a time course of the degradation of PEG/Dex hydrogels with a slab shape but different PEG molecular weights. The degradation profiles of all the hydrogels were in proportion to the time, indicating that all the data followed the equation (2.3). This equation is based on the assumption that the degradation takes place via surface front and that the surface area is constant during the degradation process. Since every hydrogel had a slab shape of 25x20x2mm in this experiment, it is considered that the degradation was proceeded from the both sides of the slab, and the effect of degradation from the edges could be negligible in Figure 2-8. The

time to complete degradation in all PEG/Dex hydrogels was almost constant as for as this dextranase concentration.

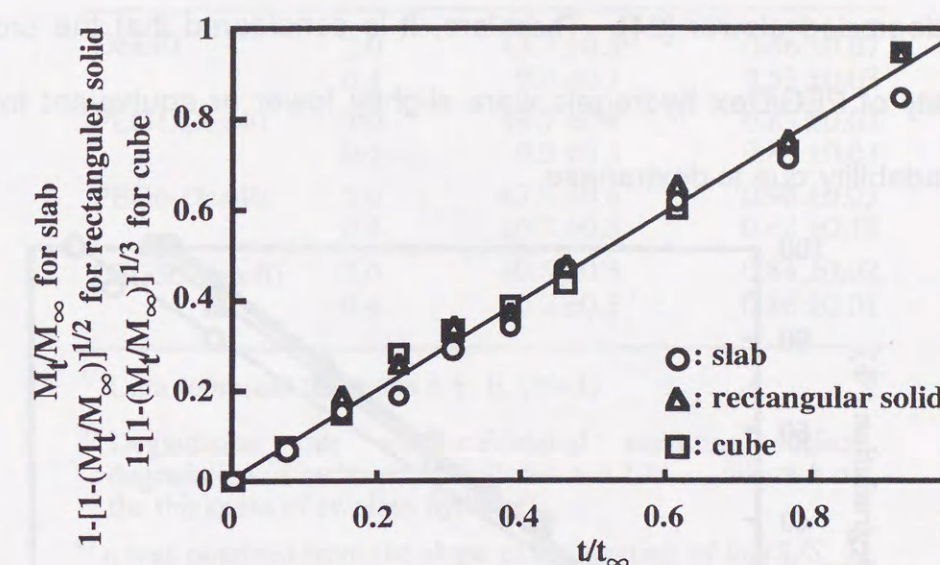


Figure 2-7. Surface-controlled degradation profiles of PEG/Dex Hydrogels.

The degradation rates (B) of these hydrogels can be calculated from the following equation:

$$B = h / 2t_{\infty} \quad (2.8)$$

where h is the film thickness. This equation is also based on the above assumption that degradation takes place from both sides of the slab, not from the edges. Table 2-1 summarizes the calculated rate of each hydrogel. PEG/Dex hydrogels had a little higher in degradation rate than Dex40, which is considered due to less dextran content in PEG-containing samples. The calculated rates of dextran degradation (the degradation rate B times dextran content) in PEG/Dex hydrogels were slightly higher but almost equal to Dex40 (ca. 46 to 47×10^{-7} cm/sec). For the degradation rate of dextran hydrogels using

dextranase, it has previously reported that as the degree of crosslinking increased, degradation time increased, *i. e.*, the more crosslinked hydrogels are degraded slower [24]. Therefore, it is considered that the crosslinking density of PEG/Dex hydrogels were slightly lower or equivalent in terms of degradability due to dextranase.

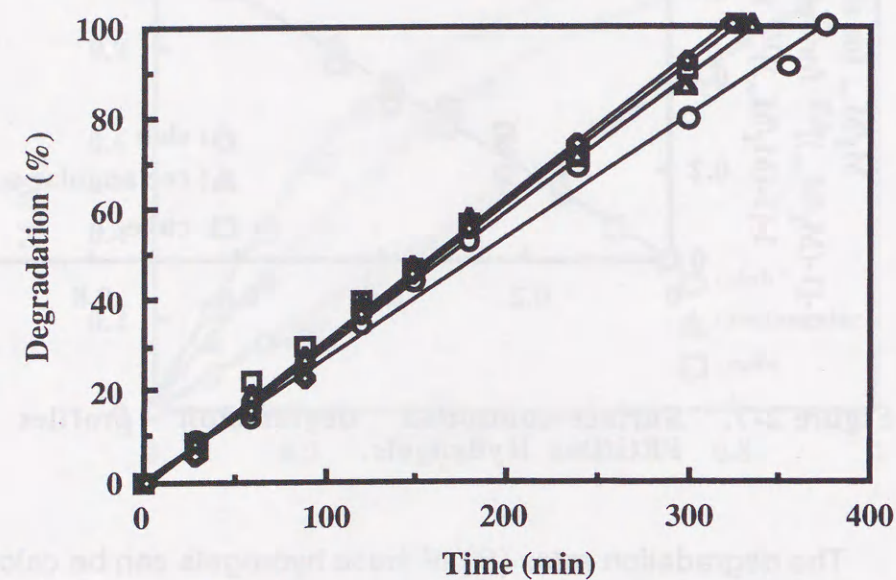


Figure 2-8. Degradation of PEG/Dex hydrogels by dextranase at 37°C in 0.1M PBS. Dextranase conc. was 20u/ml. ○, Dex40; ▲, PEG1-Dex40; □, PEG6-Dex40; ◆, PEG50-Dex40.

Table 2-1^{a)} Degradation rates and n values of PEG-Dex hydrogels.

code	dextranase conc. (u/ml)	B^b ($\times 10^{-7}$ cm/sec)	n^c
Dex40	2.0	43.7 ± 0.5	0.66 ± 0.07
	0.4	9.0 ± 0.1	0.53 ± 0.03
PEG1-Dex40	2.0	48.7 ± 0.4	0.83 ± 0.03
	0.4	9.9 ± 0.3	0.89 ± 0.03
PEG6-Dex40	2.0	47.9 ± 0.4	0.96 ± 0.05
	0.4	10.7 ± 0.3	0.68 ± 0.13
PEG50-Dex40	2.0	49.5 ± 0.3	0.84 ± 0.02
	0.4	10.2 ± 0.3	0.86 ± 0.01

^{a)} Data represent the mean $\pm$ S. E. (N=3).

^{b)} Degradation rate was calculated assuming surface degradation of hydrogel, as follows: $= h / 2t$, where h is the thickness of swollen hydrogel.

^{c)} n was obtained from the slope of the plotting of $\log(S/S_\infty)$ against $\log(t/t_\infty)$.

2-3-4: Insulin Release from PEG/Dex Hydrogels during Their Enzymatic Degradation

The release of insulin from PEG/Dex hydrogels during their enzymatic degradation (dextranase concentration of 2unit/ml) was examined. When the degradation was completed, the insulin release was also completed. Figure 2-9 summarizes the insulin release profiles of PEG/Dex hydrogels during their enzymatic degradation. The insulin release profile of Dex40 exhibited an upper convex curve, whereas that of other three PEG/Dex hydrogels were rather on the broken line in this figure. If the insulin release is caused by the surface degradation of dextran matrices, the plots should be on the broken line. However, these release profiles were deviated from this line, which indicates that the insulin release might be caused by not only the

surface degradation of dextran matrices but also the diffusion of insulin through dextran matrices.

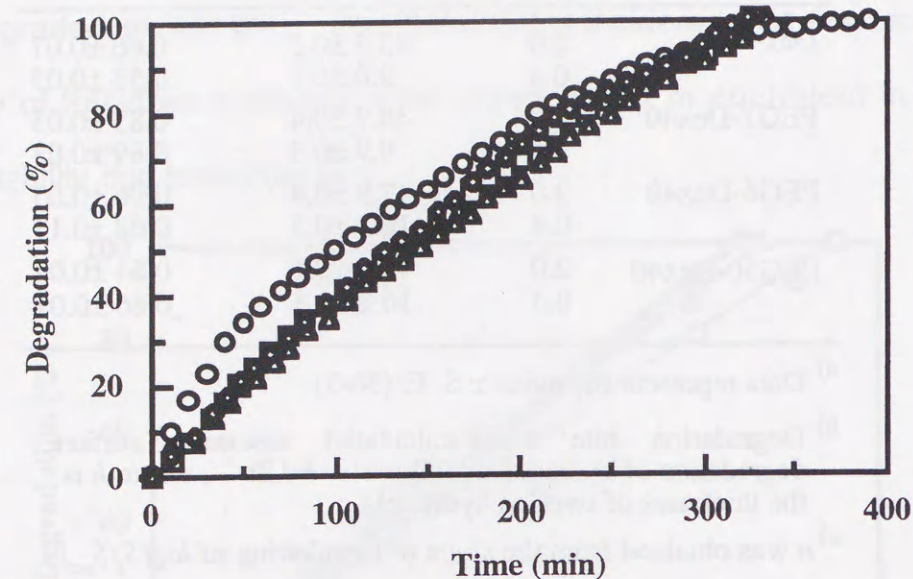


Figure 2-9. Insulin release from PEG/Dex hydrogels during enzymatic degradation at 37°C in PBS. Dextranase conc. was 2u/ml. ○, Dex40; △, PEG1-Dex40; □, PEG6-Dex40; ◇, PEG50-Dex40.

The analysis of insulin release based on kinetics was also investigated. Generally, drug release from a polymer matrix at any time t , during the first 60% of release, can be given by the equation (2.9).

$$S_t / S_\infty = k(t/t_\infty)^n \quad (2.9)$$

where S_t is the amount of insulin released from the hydrogels at any time t , S_∞ is the total amount of insulin released from the hydrogels, k is a constant characteristic of the drug-polymer system, t_∞ is the time to complete insulin release (the same as the time to complete degradation), and n an exponent characteristic of the release mechanism [20, 21]. For diffusion-controlled

release, fractional release can be characterized by a certain constant multiplied by the square root of time, thus, n value is 0.5. For degradation-controlled release, the fractional release is a linear function of time, thus, n value may be 1.0. The common logarithm of equation (2.9) is expressed by the equation (2.10).

$$\log(S_t / S_\infty) = n \log(t/t_\infty) + \log k \quad (2.10)$$

thus, we calculated the exponent n from the slope of the plots of $\log(S_t/S_\infty)$ against $\log(t/t_\infty)$, the results of which are summarized in Table 2-1. In the case of Dex40, n value was not completely 0.5 but 0.66. In separated experiments, during storage in 0.1M PBS at 37°C for 400min (almost the same time as t_∞ in this dextranase concentration), more than 60% of loaded insulin was actually leaked from Dex40, the result of which is shown in Figure 2-10. These two results indicate that insulin release from Dex40 was influenced by both matrix degradation and insulin diffusion through the matrix. The introduction of PEG into dextran hydrogel altered the exponent n to ca. 1.0, which can indicate the shift in the mode of transport from diffusion-controlled mechanism to degradation-controlled mechanism.

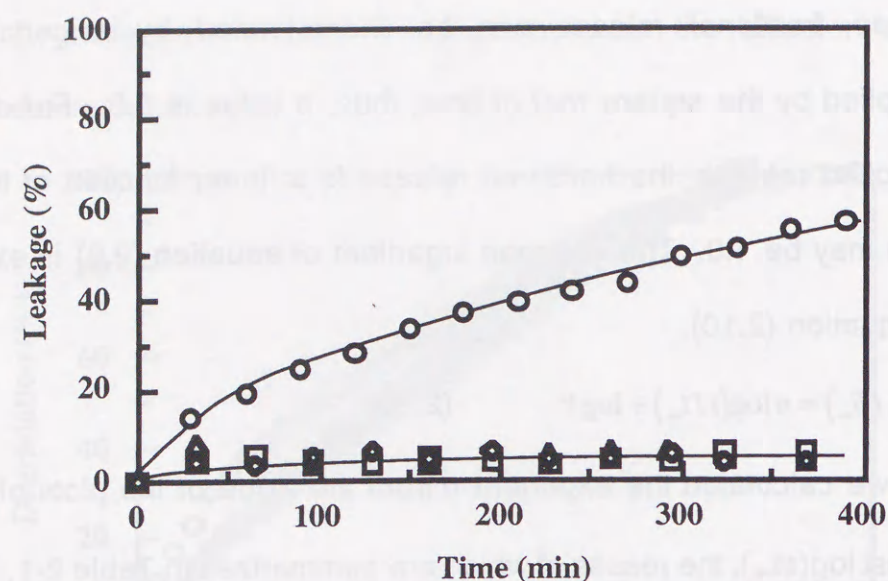


Figure 2-10. Insulin leakage from PEG/Dex hydrogels during storage at 37°C in PBS. ○, Dex40; ▲, PEG1-Dex40; □, PEG6-Dex40; ◆, PEG50-Dex40.

As shown in Figure 2-1, insulin was preferentially partitioned into PEG phase, thus, it is supposed that insulin diffusion through dextran matrices is not occurred when insulin was partitioned into PEG phase. It has been reported that certain water-soluble proteins as model drugs loaded into crosslinked polysaccharide hydrogels can diffuse through hydrogel matrices to be released [12]. On the other hand, it has been also reported that release of hydrophobic and hydrophilic drugs loaded into crosslinked dextran hydrogels could be regulated by swellability and degradability due to crosslinking density [23, 24]. In that case, release profiles of several drugs loaded into crosslinked dextran hydrogels could be varied with the crosslinking density. However, less than 10% of loaded insulin was leaked from PEG/Dex hydrogels during storage for 400min, as shown in Figure 2-10. This result also

supports the suggestion that PEG/Dex hydrogels can release insulin with different release mechanism from Dex hydrogel.

Theoretical drug release from biodegradable polymers has been already analyzed by Lee [25]. Figure 2-11 shows a simulation of drug release from planar biodegradable hydrogels by applying voluntary values to the equation (2.11), simplified from the reference [25].

$$S_t / S_\infty = [2(Dt/3)^{1/2} + Bt] / h \quad (2.11)$$

where D is the diffusion coefficient assuming $1 \times 10^{-6} \text{cm}^2/\text{sec}$ taking the molecular weight of insulin (ca. 6000) into account, B is the degradation rates assuming 1×10^{-3} to $1 \times 10^{-7} \text{cm/sec}$, and h is a slab thickness. This figure suggests that the release profile approaches degradation-controlled release mechanism when the magnitude of degradation rate times slab thickness (Bh) becomes larger than that of diffusion coefficient (D), while the release profile is followed by diffusional release mechanism when Bh is equal to or lower than D . Therefore, the above experimental condition in enzymatic degradation of PEG/Dex hydrogels might include the effect of the degradation rate on insulin release mechanism (n values).

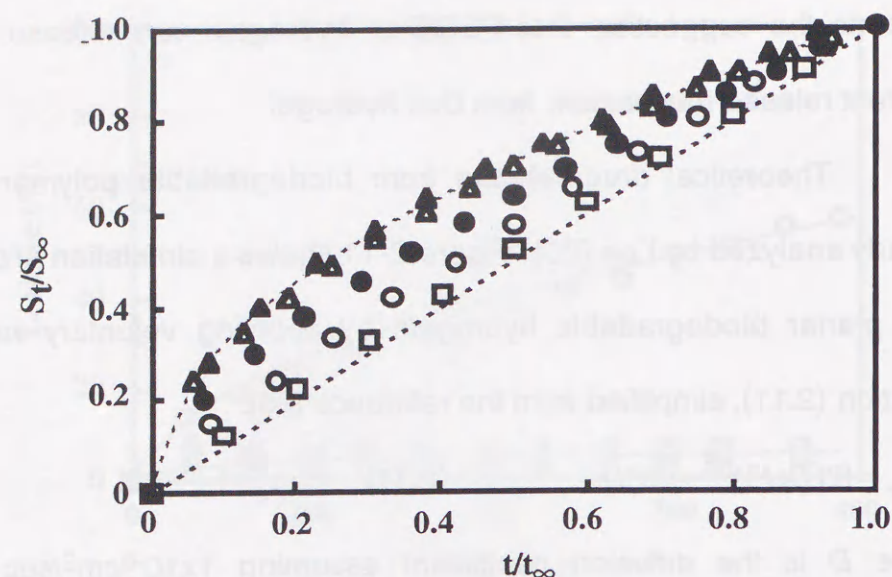


Figure 2-11. Simulation of drug release from planar biodegradable hydrogels. Diffusion coefficient: $D=1 \times 10^{-6}$ cm²/s, film thickness: $h=1$ mm. □, $B=1 \times 10^{-3}$; ○, $B=1 \times 10^{-4}$; ●, $B=1 \times 10^{-5}$; ▲, $B=1 \times 10^{-6}$; ▼, $B=1 \times 10^{-7}$.

Then, in order to eliminate the effect of degradation rate on insulin release, insulin release during much slower degradation of PEG/Dex hydrogels was examined by using much lower dextranase concentration (0.4 unit/ml). All hydrogels showed time-dependent degradation profiles but much longer degradation time than those in Figure 2-8. This indicates that the degradation of these hydrogels was dependent upon the enzyme concentration used. The degradation rate of each hydrogel in this condition was also summarized in Table 2-1. It is clear that the degradation rate of each hydrogel decreased in proportion to the dextranase concentration. Figure 2-12 summarizes the release profiles of insulin released from PEG/Dex hydrogels given by a similar manner to Figure 2-9. Release profiles of insulin shown in Figure 2-12 were almost the same as those in Figure 2-9, although

much longer time to complete degradation in Figure 2-12 than in Figure 2-9. The n values resulted from the plots of $\log(S_t/S_\infty)$ against $\log(t/t_\infty)$ in this dextranase concentration were also summarized in Table 2-1. The n value for Dex40 reached 0.5 with decreasing dextranase concentration while those for PEG/Dex hydrogels remained constant nearly 1.0, except for PEG6-Dex40. This indicates that the insulin release profiles of PEG/Dex hydrogels were hardly influenced by the degradation rate whereas that of Dex40 was influenced by the degradation rate. Exceptionally in the case of PEG6-Dex40, the n value decreased with decreasing dextranase concentration. There can be two possible reasons for the decrease in n value for PEG6-Dex40. The first possibility is that insulin diffusion through dextran matrices dominates the n value. However, the result in Figure 2-10 should discount this possibility. The second possibility comes from the consideration that the burst erosion of PEG moiety during the degradation process induces insulin release, which is not observed during a storage as in Figure 2-10.

From the measurement of solution turbidity using a spectrophotometer (500 nm), it was found that phase separation behavior using PEG6 was observed to be most rapidly proceeded in the series of aqueous PEG/dextran two-phase systems. As described previously, ΔH of PEG moiety was maximum at PEG6-Dex40 hydrogel, which supports better phase separability of PEG6 with dextran. It is easily imagined that such phase separability of PEG6 enhances the formation of larger PEG domains, as partly observed in Figure 2-2 (b), somewhat may form partially-continuous PEG

phases. Thus, at present, it must be suggested that the phase separability of PEG6-dextran two-phase system causes unexpected insulin release in PEG6-Dex40. Recently, it was found that similar phenomenon also occurred when hyaluronic acid (HA) was used as crosslinked polysaccharide matrices: crosslinked HA hydrogel containing PEG6 had the minimum n value for insulin release. This specific feature using PEG6 is still under investigation, and will be discussed in the next chapter. Taking all the results shown above into account, it is considered that insulin diffusion through dextran matrix was dominant in the case of Dex40, whereas insulin release from a series of PEG/Dex hydrogels were regulated by the surface degradation of PEG/Dex microdomain-structured matrices because of preferential partition of insulin into PEG phase.

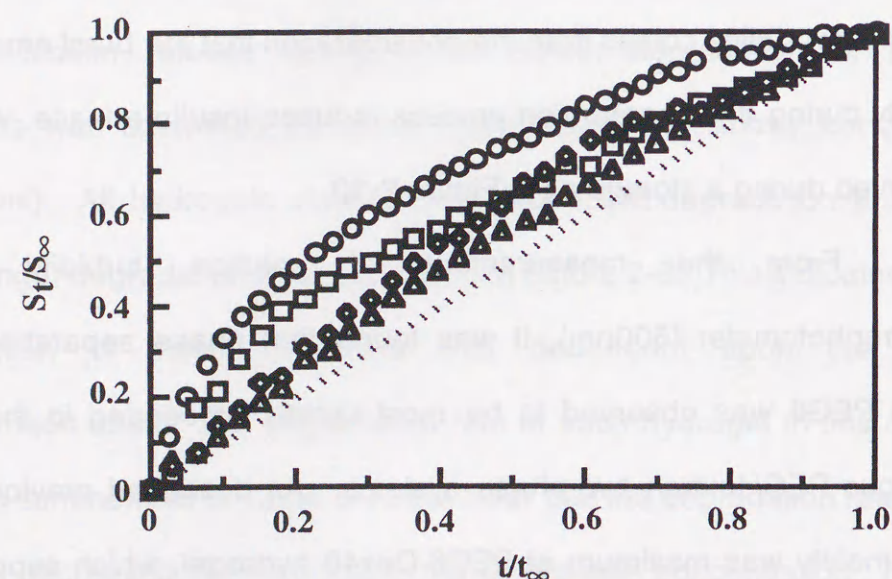


Figure 2-12. Insulin release from PEG/Dex hydrogels during enzymatic degradation at 37°C in PBS. $\circ$, Dex40; $\triangle$, PEG1-Dex40; $\square$, PEG6-Dex40; $\diamond$, PEG50-Dex40.

2-4: Conclusion

The formation of heterogeneous-structured hydrogels consisting of PEG as drug reservoirs and dextran as biodegradable matrices can achieve degradation-controlled insulin release, based on the knowledge of aqueous polymer two-phase systems. In addition, it is suggested that the insulin release from these hydrogels can be regulated by the extent of microdomain structures composed of PEG and Dex phases. In this study, the content of PEG moiety was fixed at 5% in every PEG/Dex hydrogel because PEG elution was occurred during the crosslinking reaction when PEG was introduced higher than 10%. It should be noted that the size and distribution of PEG domains as drug reservoirs in dextran matrices were not distinctly controlled in this study because PEG segment was not crosslinked nor covalently bound to dextran segment. PEG-grafted dextran is synthesized as well-defined PEG domain-dispersed biodegradable hydrogels, described latter chapter.

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Chapter 3

Degradation-Controlled Insulin Release from PEG/dextran Hydrogels with Different Phase Structures

3-1: Introduction

Biodegradable polymeric systems have been used frequently in the development of advanced drug delivery systems. The use of biodegradable polymeric systems in drug delivery is desirable, because an invasive technique such as surgery is not required after their use [1, 2]. Biodegradable polymeric systems also provide flexibility in the design of formulations for high molecular weight drugs such as peptides and proteins. Especially, biodegradable hydrogels have been represented as an attractive drug formulation because of their advantages such as biocompatibility, high responsibility for specific degradation, and feasible approach to incorporate drugs into matrices. However, it seems quite difficult to achieve degradation-controlled drug release from these hydrogels since their highly water swollen states cause drug diffusion through the matrices. In order to prevent drug diffusion in such a highly water swollen hydrogel, a heterogeneous-structured hydrogel of a degradable hyaluronic acid (HA) matrix containing drug microreservoirs (lipid microspheres) was proposed as a implantable device for inflammation responsive drug delivery [3, 4]. The heterogeneous-structured HA hydrogel was found to enable degradation-controlled release of lipid microspheres. Furthermore, the heterogeneous-structured device was also feasible for regulating drug release from biodegradable hydrogels and for protecting the inactivation of drug by the hydrophobic nature of microreservoirs. When water-soluble drugs such as bioactive peptides are introduced in

biodegradable hydrogels, another approach will be required because the application of lipid microspheres limits to water-insoluble hydrophobic drugs. The late paper has proposed a new approach of designing biodegradable hydrogels for bioactive peptide delivery, which is based on an aqueous polymer two-phase system [5].

Aqueous polymer two-phase systems are well known phase separation techniques in the field of biotechnology [6-9]. The two-phase system is obtained by mixing aqueous solutions of two different water-soluble polymers such as poly(ethylene glycol) (PEG) and dextran. The top phase is PEG solution and the bottom phase is dextran solution in this two-phase system. The separation of proteins in such system is based on differences in their distribution between two phases. For example, in the case of using a PEG and dextran two-phase system in phosphate buffer solution, it is expected that negatively charged proteins such as insulin will be preferentially partitioned into PEG phase. Partition differences depend on some parameters such as the molecular weight and concentration of polymers, or the ionic composition and pH of buffer [10, 11]. This specific phenomenon may enable the formulation of peptide drugs using biodegradable dextran hydrogels containing PEG: PEG moiety may act as drug reservoirs.

In the Chapter 2, biodegradable dextran hydrogels containing poly(ethylene glycol) were prepared as a model of heterogeneous-structured devices composed of drug reservoirs and degradable matrices. Insulin was preferentially loaded into poly(ethylene glycol) (PEG) phase, and the

degradation of both crosslinked dextran hydrogels containing PEG (PEG/Dex hydrogels) and crosslinked dextran hydrogel (Dex hydrogel) by dextranase were found to proceed from the surface front. However, release profiles of insulin from PEG/Dex hydrogels were different from that of Dex hydrogel: insulin release from PEG/Dex hydrogels was regulated by the surface degradation of PEG/Dex microdomain-structured matrix whereas diffusion was dominant factor in insulin release from Dex hydrogel. Furthermore, it was suggested that the phase structures in the PEG/Dex hydrogels might be changed with PEG molecular weight, water content and/or crosslink density.

In order to regulate the phase-separated structures and achieve degradation-controlled drug release from the water swollen hydrogels, a series of PEG/Dex hydrogels with different water content and crosslink density were prepared in this chapter, and insulin release during their enzymatic degradation were examined.

3-2: Materials and Methods

3-2-1: Chemicals

All Chemicals were analytical-grade commercial materials and used without further purification. Dimethylsulfoxide (DMSO), N,N-dimethylaminopyridine (DMAP), poly(ethylene glycol) (PEG) (Mn=1000, 6000, 50000), glycidyl methacrylate (GMA), ammonium persulfate (APS), fluorescein isothiocyanate (FITC) and insulin from bovine pancreas (26unit/mg), were

purchased from Wako Pure Chemical Industries, Osaka, Japan. Dextran (Mn=40000) was purchased from Tokyo Chemical Industry Co., Tokyo, Japan. Dextranase from penicillium sp. (12.9unit/mg) was purchased from Sigma Chemical Co., St. Louis, U. S. A.

3-2-2: Preparation of Glycidyl Methacrylated-Delivertized Dextrans

Glycidyl methacrylated dextrans (GMA-dex) were synthesized according to S. C. De Smedt *et al.* [12]. Seven batches of GMA-dex were prepared: for each batch, dextran (10g) and DMAP (2g) were dissolved in 100ml of distilled DMSO. Respectively 2.2, 1.1, 0.73, 0.55, 0.44, 0.37, and 0.29g of GMA were added to the reaction mixtures corresponding with a ratio of 1 mol of GMA per 2, 4, 6, 8, 10, 12.5, and 16.7mol of glucopyranose units of dextran. The reaction mixture was stirred at room temperature for 48h. After neutralizing of DMAP with an equimolar amount of 4N hydrochloric acid, the solution was poured into a large excess of ethanol. The precipitate was collected by filtration and dissolved in 20ml of distilled water. The solution was dialyzed against distilled water for 5days and finally lyophilized. The degree of GMA substitution in the dextran derivatives (i.e., the number of GMA groups per 100 dextran glucopyranose units) was determined by ¹H-NMR (Varian, Palo Alto, CA, U. S. A., 300MHz FT-NMR Gemini300).

3-2-3: Preparation of PEG/Dex Hydrogels

A certain weight percent of GMA-dex solution in 0.1M PBS (pH7.4), 40wt.% of PEG solution in PBS, and 1mg/ml FITC-labeled insulin solution in PBS were mixed in the vol. ratio of 90:5:5, respectively, followed by the addition of APS (20mg) to the mixture. After vigorous stirring for 1min, the mixture was injected to a cell (inner diameter: 10mm, height: 4mm). Crosslinking GMA-dex was initiated by exposing the mixture to a visible light from a high pressure mercury lamp (500W) for 10 min. It was confirmed that the turbidity of PEG/Dex solution mixture was constant for much longer than the reaction time for crosslinking GMA-dex, which suggests that the heterogeneity of PEG/Dex mixture by the vigorous stirring was maintained during the crosslinking reaction. The weights of both equilibrated gel in PBS and its dried one were measured for calculating the water content. Crosslinked dextran hydrogel (Dex hydrogel) was transparent, but crosslinked dextran hydrogels containing PEG (PEG/Dex hydrogels) were opaque. The water contents of these hydrogels were approximately 70-94%.

3-2-4: Characterization of PEG/Dex Hydrogels

The crosslink density of the hydrogels was calculated from the equilibrium compression modulus measured by using thermo mechanical analyzer (Seiko Instruments Co., Tokyo, Japan, TMA/SS150C). The morphology of PEG/Dex hydrogels in swollen state was observed with a phase-contrast microscope (PCM) (Olympus Co., Tokyo, Japan, U-SPT).

After freeze drying, differential scanning calorimetric analysis of PEG/Dex hydrogels was also examined (Seiko Instruments Co., Tokyo, Japan, DSC120) in the temperature range from -25 to 160°C; sample weights were 6mg; and the scanning rate was 2°C/min.

3-2-5: Enzymatic Degradation of PEG/Dex Hydrogels and Insulin Release

The degradation of PEG/Dex hydrogels by dextranase was examined *in vitro*. After equilibration in 0.1M PBS (pH7.4), the hydrogels were mounted in a nylon mesh-bag attached to a thread for dipping into solutions and immersed into dextranase solution in PBS (2unit/ml) with stirring to initiate degradation. The degradation rate was estimated by measuring the residual weight of the hydrogel together with the nylon mesh-bag. Insulin release from the slab hydrogel was estimated by measuring FITC fluorescent intensity of the system solutions excited at 490nm and emitted at 520nm continuously using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777) equipped with a flow cell (Japan Spectroscopy Co., Tokyo, Japan, FSC-322). The total volume of this flow system was 15ml, and the flow rate was fixed at 30ml/min using a peristaltic pump (Iwaki Glass Co., Tokyo, Japan, PST-100).

3-3: Results and Discussion

3-3-1: Preparation and Enzymatic Degradability of PEG/Dex Hydrogels

The hydrogels used in this study are divided two categories, one is crosslinked dextran hydrogel (Dex hydrogel), another is crosslinked dextran hydrogels containing poly(ethylene glycol) (PEG/Dex hydrogels). Both categories include dextran with different degrees of GMA introduction: 10, 7, 5, and 1 GMA groups per 100 glucopyranose units of dextran. Furthermore, the concentration of GMA-dex solutions in the preparation was varied from 15 to 40wt.% (15, 20, 25, 30, and 40wt.%). The shape of all the hydrogels was 4mm cube. Figure 3-1 shows a time course of the degradation of PEG/Dex hydrogels with different GMA-dex concentrations. In spite of constant GMA substitution, the degradation rate increased with increasing the concentration of GMA-dex solution. In the Chapter 2, surface-controlled degradation of Dex and PEG/Dex hydrogels by dextranase was confirmed with different shapes (slabs, rectangular solids, and cubes) [5]. In this chapter, used theoretical equations were 2.5 and 2.7, because the hydrogels were cubic shape.

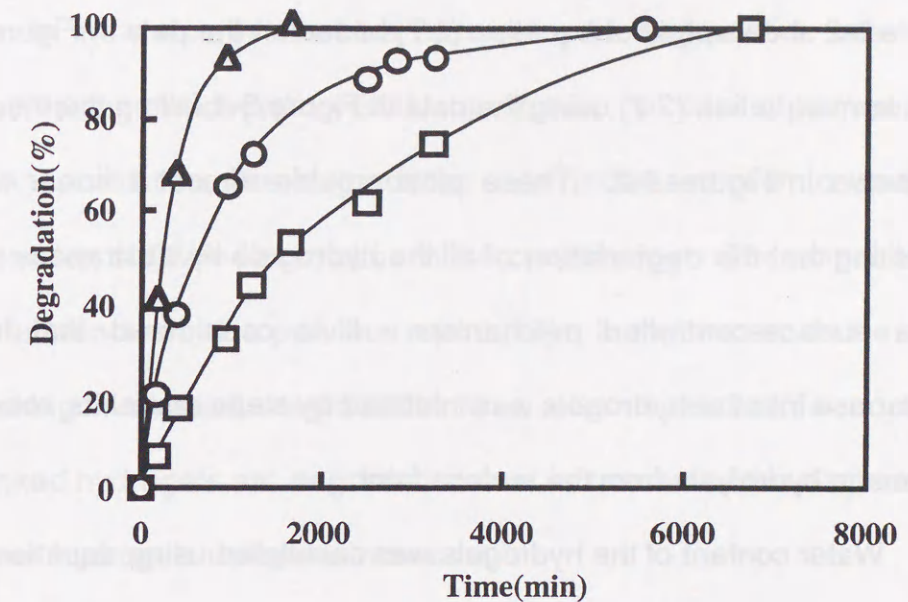


Figure 3-1. Degradation of PEG/Dex hydrogels by dextranase at 37°C in PBS. The concentration of GMA-dex solution were 15 (Δ), 20 ($\circ$), and 25 ($\square$) wt.%. The number of GMA groups per 100 glucopyranose units = 7.

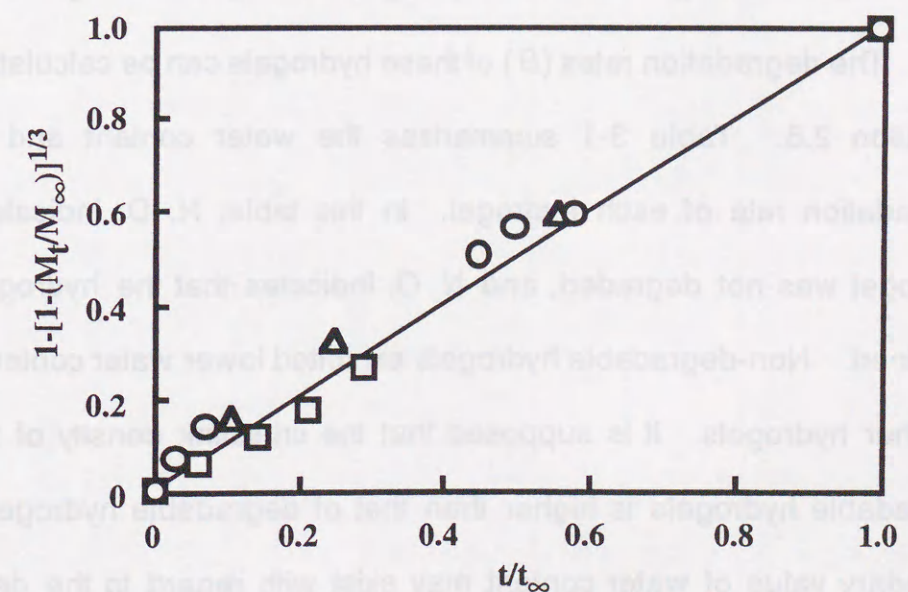


Figure 3-2. Surface-controlled degradation profile of PEG/Dex hydrogels. The conditions of GMA-dex are similar to Figure 3-1.

Figure 3-2 shows plots of equation (2.7) based on the data in Figure 3-1. The plots from equation (2.7) using the data in Figure 3-1 fall on the theoretical line, as shown in Figure 3-2. These plots provide almost a linear relationship, indicating that the degradation of all the hydrogels by dextranase takes place via a surface-controlled mechanism. It is considered that intrusion of dextranase into the hydrogels was inhibited by steric restraints, resulting in the enzymatic hydrolysis from the surface front.

Water content of the hydrogels was calculated using equation (3.1) [16]:

$$\text{water content} = 100 \times (W_s - W_d) / W_s \quad (3.1)$$

where W_s is the weight of swollen hydrogel, and W_d is the weight of dried one.

The degradation rates (B) of these hydrogels can be calculated from the equation 2.8. Table 3-1 summarizes the water content and calculated degradation rate of each hydrogel. In this table, N. D. indicates that the hydrogel was not degraded, and N. O. indicates that the hydrogel was not obtained. Non-degradable hydrogels exhibited lower water content than that of other hydrogels. It is supposed that the crosslink density of these non-degradable hydrogels is higher than that of degradable hydrogels and the boundary value of water content may exist with regard to the degradability against dextranase.. In the case of the degrees of GMA substitution are 5 and 1, the hydrogel was not obtained completely when the concentration of the GMA-dex solution below 25wt.%. With the similar concentration of GMA-dex

solution in the preparation of the hydrogels, the degradation rate increased with decreasing the degree of GMA substitution. In case of the same degree of GMA substitution, the degradation rate increased with decreasing the concentration of GMA-dex solution in the crosslinking. These results indicate similar tendencies in the previous report [17]. In that report, as the degree of crosslinking increased, degradation time also increased, *i. e.*, the more crosslinked hydrogels are degraded slower in respect of the degradation rate of dextran hydrogels using dextranase.

Table 1 Water content and degradation rate of Dex hydrogels

GMA-dex conc. (wt% of solution)	The number of GMA groups per 100 glucopyranose units					
	10		7		5	
	W.C. (%) a)	B ^{b)} (x10 ⁻⁶ cm/sec)	W.C. (%)	B (x10 ⁻⁶ cm/sec)	W.C. (%)	B (x10 ⁻⁶ cm/sec)
40	70	N.D. ^{c)}	76	N.D.	83	N.D.
30	73	N.D.	80	N.D.	87	0.77
25	77	N.D.	83	0.49	N.O. ^{d)}	N.O.
20	79	N.D.	87	0.77	N.O.	N.O.
15	85	0.51	90	1.84	N.O.	N.O.

a) Water content was calculated as follows: $= (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{wet}} \times 100 \%$.

b) Degradation rate was calculated assuming surface degradation of hydrogel, as follows: $= h / 2t$, where h is the thickness of swollen hydrogel.

c) N. D. indicates the hydrogel was not degraded.

d) N. O. indicates the hydrogel was not obtained.

3-3-2: Two-Phase Morphology of PEG/Dex Hydrogels

Crosslink densities (mol/cm³) of the hydrogels were estimated from following equation [16, 18].

$$E_c = Sh_s / A_s \quad (3.2)$$

$$v_e / V = Sh_0 / 3A_0 RT \quad (3.3)$$

Where E_c is the compression modulus, S is the initial slope of force-deformation curve, v_e/V is the effective network concentration (crosslink density), A_s is the swollen area, A_0 is the unswollen area, h_s is the undeformed swollen height, h_0 is the undeformed unswollen height, R is the gas constant, and T is the temperature. Figures 3-3, 3-4, and 3-5 summarize the relationship between the crosslink densities and water contents of PEG-Dex hydrogels with different PEG molecular weights and different degrees of GMA introduction to dextran chains. Crosslink densities of these hydrogels reduced with increase in the water contents. In the case that the water contents were nearly equal, crosslink densities of the hydrogels containing PEG6000 (PEG6/Dex) were the higher than those of PEG1/Dex and PEG50/Dex. Mechanical measurements of hydrogels and calculation of the crosslink densities in respect of chemical and physical crosslink can be found in a previous review [19]. It is considered that the compression modulus (crosslink densities) reflect chemical crosslink in the hydrogels while the water

contents reflect both chemical crosslink and physical chain entanglements between PEG and dextran. Thus, it is suggested that physical chain entanglements of PEG6/Dex is much less than that of PEG1/Dex and PEG50/Dex: well phase separated PEG domains may exist in the PEG6/Dex.

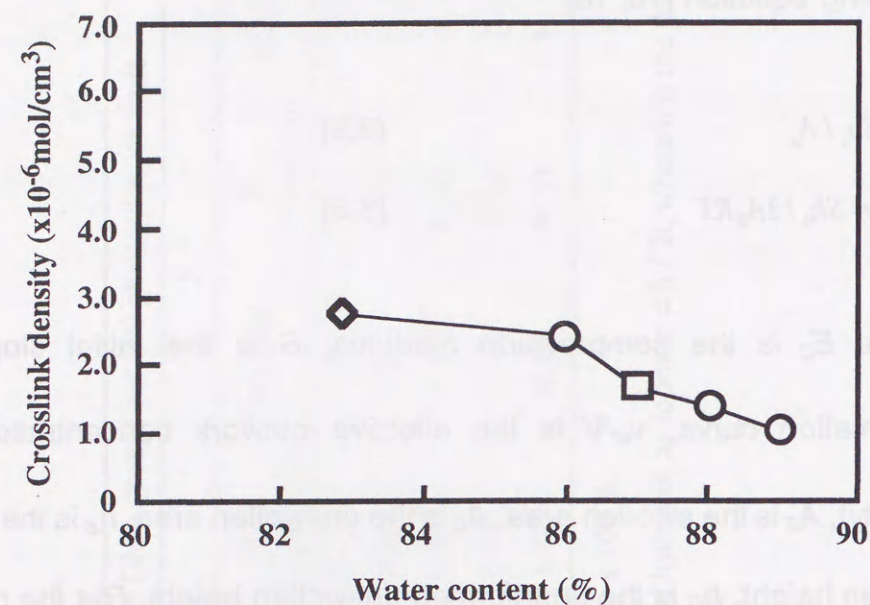


Figure 3-3. Relationship between the crosslink density and water contents of PEG1/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =10 (□), 7 (○), and 1 (◇).

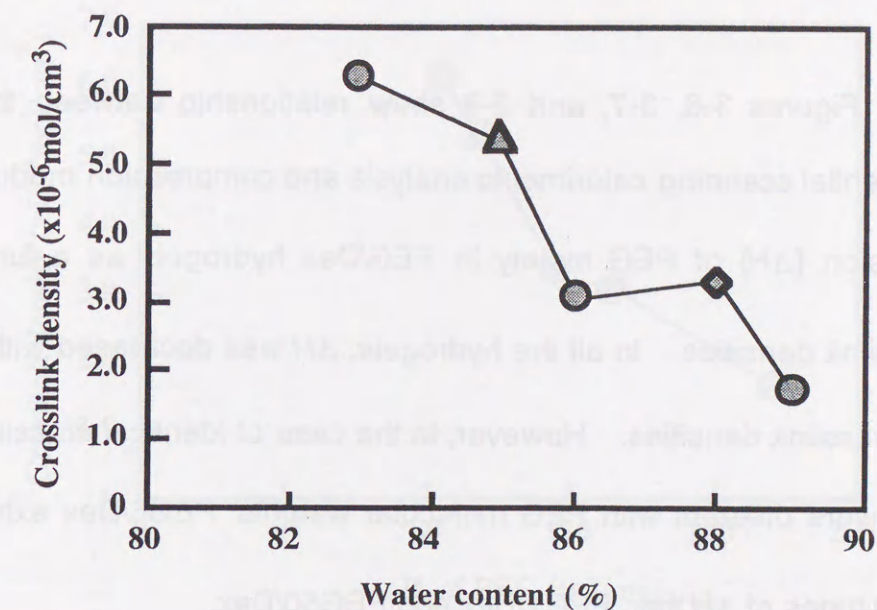


Figure 3-4. Relationship between the crosslink density and water contents of PEG6/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =7 (●), 5 (▲), and 1 (◇).

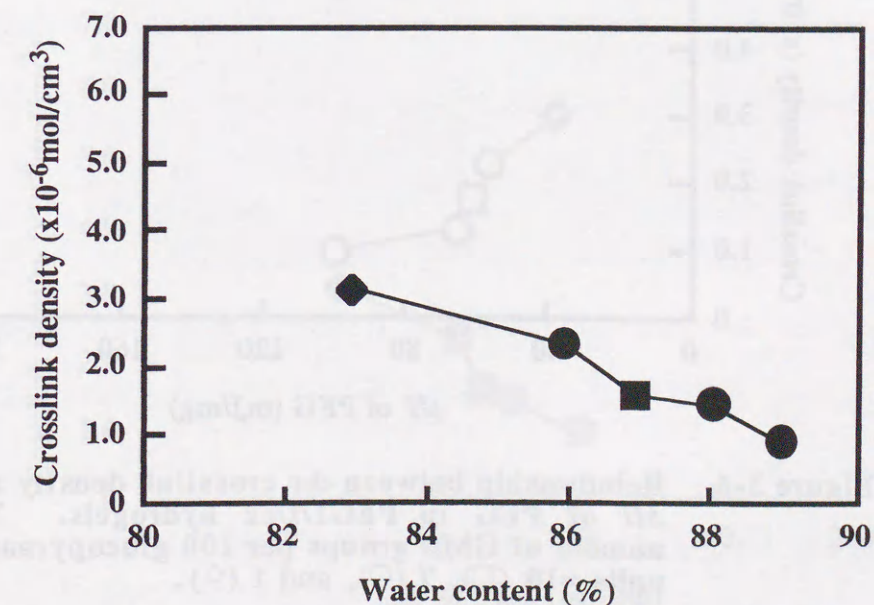


Figure 3-5. Relationship between the crosslink density and water contents of PEG50/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =10 (■), 7 (●), and 1 (◇).

Figures 3-6, 3-7, and 3-8 show relationship between the results of differential scanning calorimetric analysis and compression modulus: the heat of fusion (ΔH) of PEG moiety in PEG/Dex hydrogels as a function of the crosslink densities. In all the hydrogels, ΔH was decreased with increase in the crosslink densities. However, in the case of identical crosslink densities, ΔH s were different with PEG molecular weights: PEG6/Dex exhibited larger magnitudes of ΔH than PEG1/Dex and PEG50/Dex.

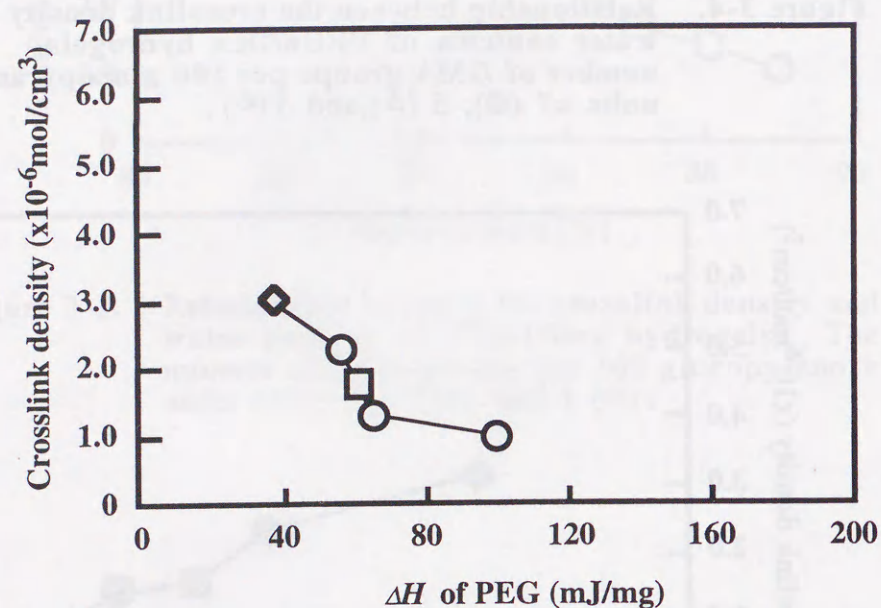


Figure 3-6. Relationship between the crosslink density and ΔH of PEG in PEG1/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =10 (□), 7 (○), and 1 (◇).

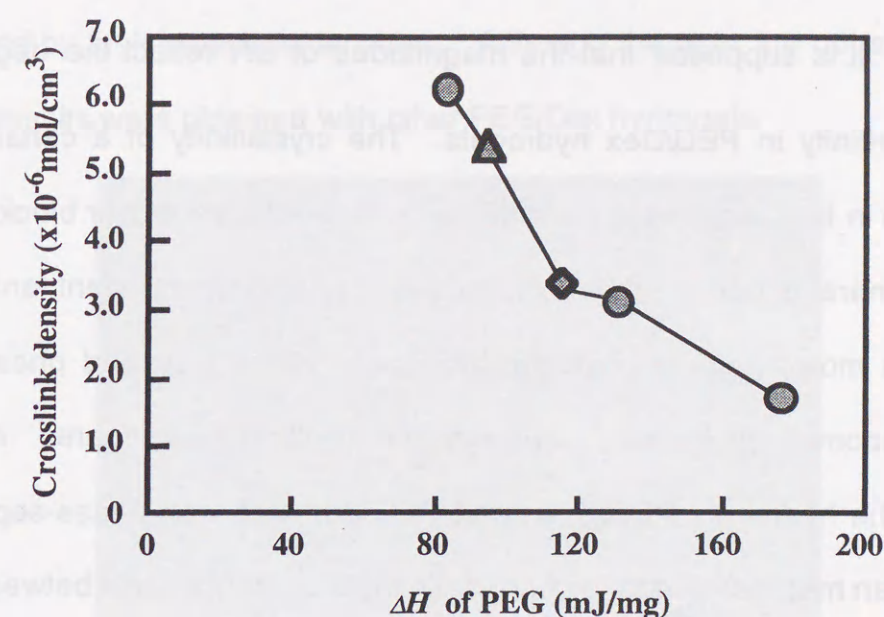


Figure 3-7. Relationship between the crosslink density and ΔH of PEG in PEG6/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =7 (●), 5 (△), and 1 (◇).

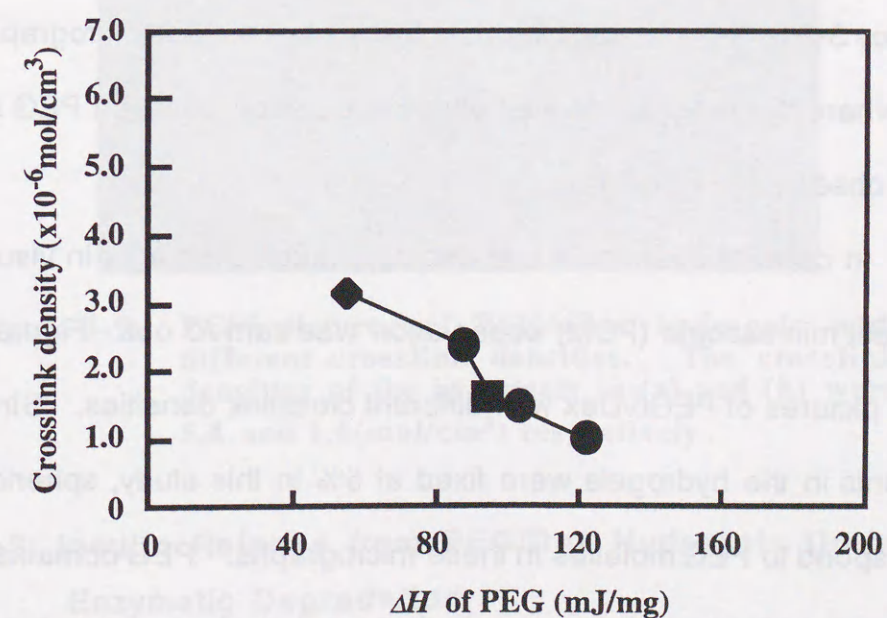


Figure 3-8. Relationship between the crosslink density and ΔH of PEG in PEG50/Dex hydrogels. The number of GMA groups per 100 glucopyranose units =10 (■), 7 (●), and 1 (◇).

It is supposed that the magnitudes of ΔH reflect the degree of PEG crystallinity in PEG/Dex hydrogels. The crystallinity of a certain polymeric chain in two-phase systems such as polymer-blends and/or block-copolymers in general is governed by miscibility with another component, and dominates some morphological characteristics such as the size and phase mixing of microdomain structures. Therefore, the results of these figures may suggest that the hydrogels PEG6/Dex had PEG domains well phase-separated from dextran matrices due to less physical chain entanglements between PEG and dextran whereas PEG crystallization was hindered by the occurrence of phase mixing with dextran to form much smaller domains in other hydrogels (PEG1/Dex and PEG50/Dex). These considerations from the results of Figures 3-3 to 3-8 are supported by the phase-contrast micrographs in Figure 3-9, where the progress in well phase-separation between PEG and dextran were observed in PEG6/Dex.

In order to examine the phase separation structures in visually, phase-contrast microscopic (PCM) observation was carried out. Figure 3-9 shows PCM pictures of PEG6/Dex with different crosslink densities. Since the PEG contents in the hydrogels were fixed at 5% in this study, spherical domains correspond to PEG moieties in these micrographs. PEG domains in (b) were observed with several dozens of micron-order whereas much smaller PEG domains were observed in (a). The crosslink densities of the hydrogels in (a) and (b) were 5.4 and 1.6(mol/cm³) respectively. These results indicate that the phase separation and size of PEG domains in PEG-Dex hydrogels were

regulated by their crosslink densities. Although the data are not shown, the similar results were obtained with other PEG/Dex hydrogels.

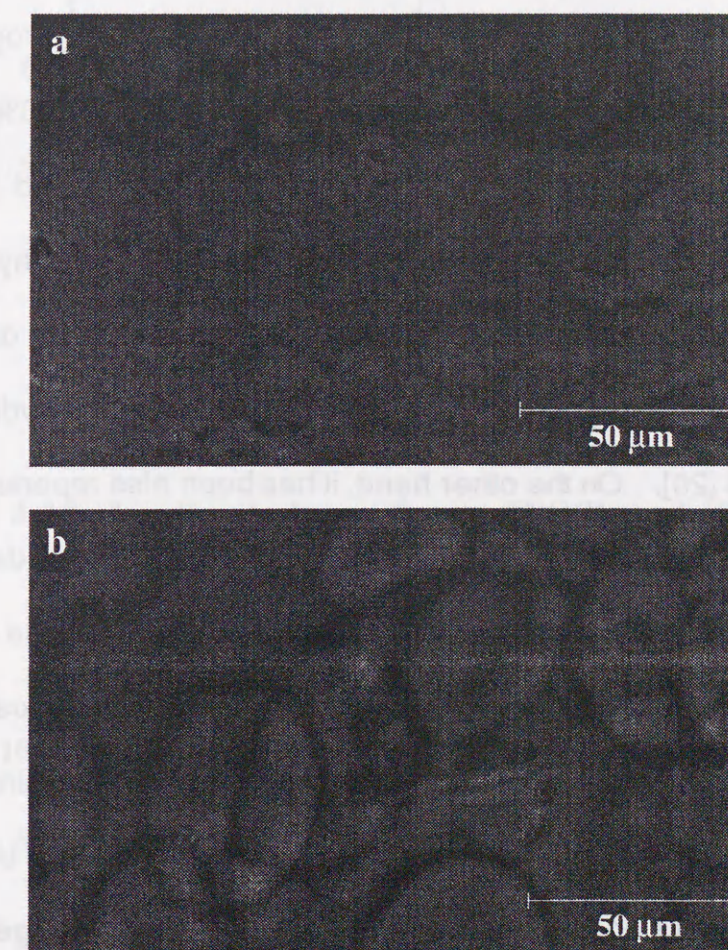


Figure 3-9. PCM pictures of PEG6/Dex hydrogels with different crosslink densities. The crosslink densities of the hydrogels in (a) and (b) were 5.4 and 1.6(mol/cm³) respectively.

3-3-3: Insulin Release from PEG/Dex Hydrogels During Their Enzymatic Degradation

The leakage of insulin from the hydrogels during storage in PBS was examined with different group of crosslink density. Figure 3-10 shows insulin leakage from PEG/Dex hydrogels during storage in 0.15M PBS at 37°C for

800min. The hydrogels are designated by the sequence codes such as PEG1/Dex-0.8, where -0.8 refers to the crosslink density 0.8 mol/cm^3 . Loaded insulin was completely leaked from Dex-1.2 hydrogel whereas the leakage from PEG/Dex hydrogels was almost less than 20%. This result is similar to the result of the Chapter 2, and it is suggested that insulin was preferentially partitioned into PEG domains in PEG/Dex hydrogels. It has been reported that certain water-soluble proteins as model drugs loaded into crosslinked polysaccharide hydrogels can diffuse through hydrogel matrices to be released [20]. On the other hand, it has been also reported that release of hydrophobic and hydrophilic drugs loaded into crosslinked dextran hydrogels could be regulated by swellability and degradability due to crosslinking density [17, 21]. Thus, in order to examine the effect of crosslink density on the insulin leakage, similar experiment was carried out using the hydrogels exhibited higher crosslink density than that of the hydrogels used in Figure 3-10. Figure 3-11 shows the insulin leakage from the hydrogels. The insulin leakage from Dex hydrogel (Dex-5.4) was reduced about 30%. In the case of PEG/Dex hydrogels, insulin leakage was completely prevented. These results suggest that the diffusion coefficient of insulin in the hydrogels reduced with increasing the crosslink density of these hydrogels. However, the hydrogels in Figure 3-11 were found to be non-degradable against dextranase and may not achieve degradation controlled release.

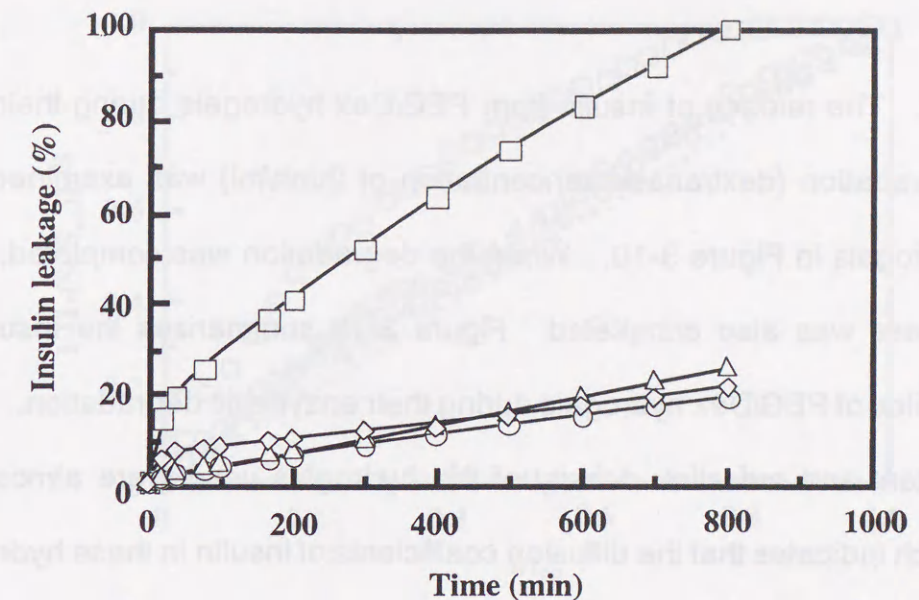


Figure 3-10. Insulin leakage from PEG/Dex hydrogels during storage at 37°C in 0.15M PBS. The hydrogels were Dex-1.2 (□), PEG1/Dex-0.8 (○), PEG6/Dex-1.6 (△), and PEG50/Dex-0.8 (◇).

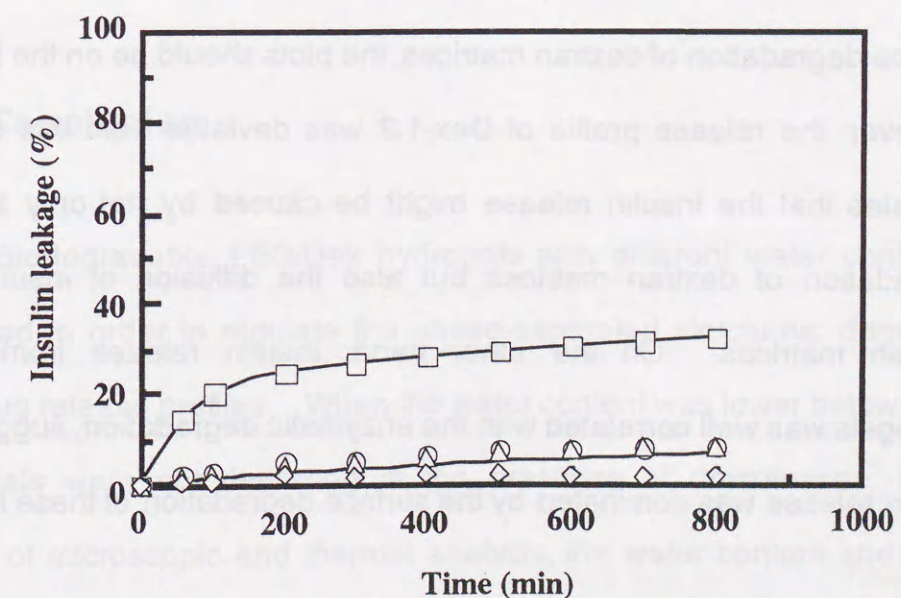


Figure 3-11. Insulin leakage from PEG/Dex hydrogels during storage at 37°C in 0.15M PBS. The hydrogels were Dex-5.4 (□), PEG1/Dex-3.6 (○), PEG6/Dex-5.4 (△), and PEG50/Dex-3.6 (◇).

The release of insulin from PEG/Dex hydrogels during their enzymatic degradation (dextranase concentration of 2unit/ml) was examined using the hydrogels in Figure 3-10. When the degradation was completed, the insulin release was also completed. Figure 3-12 summarizes the insulin release profiles of PEG/Dex hydrogels during their enzymatic degradation. The water content and crosslink density of the hydrogels used were almost constant, which indicates that the diffusion coefficients of insulin in these hydrogels were assumed to be equal. The insulin release profile of Dex-1.2 exhibited an upper convex curve, whereas that of other three PEG/Dex hydrogels were rather on the broken line in this figure. The broken line indicates surface degradation profile of these hydrogels. If the insulin release is caused by the surface degradation of dextran matrices, the plots should be on the broken line. However, the release profile of Dex-1.2 was deviated from this line, which indicates that the insulin release might be caused by not only the surface degradation of dextran matrices but also the diffusion of insulin through dextran matrices. On the other hand, insulin release from PEG/Dex hydrogels was well correlated with the enzymatic degradation, suggesting that insulin release was dominated by the surface degradation of these hydrogels.

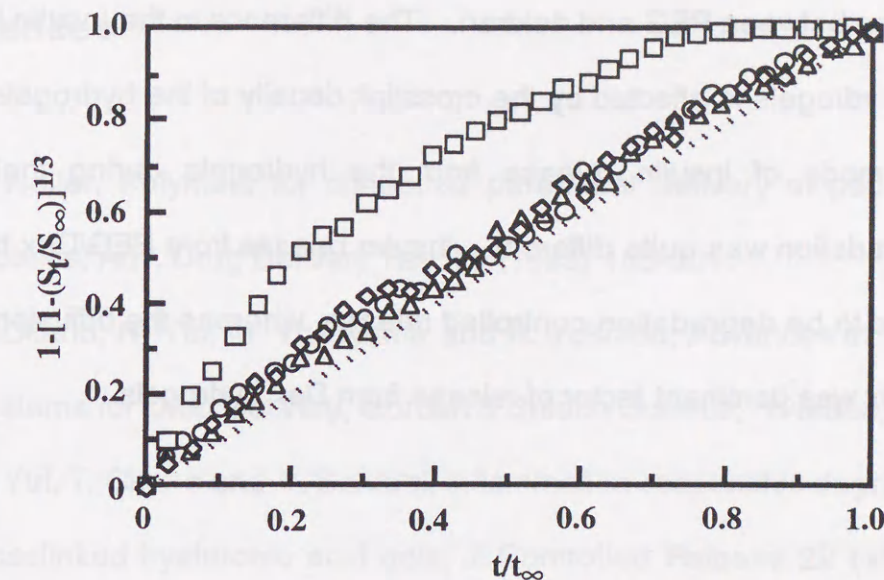


Figure 3-12. Insulin release profiles of PEG/Dex hydrogels during enzymatic degradation. The concentration of dextranase was 2.0U/ml. The hydrogels were Dex-1.2 ($\square$), PEG1/Dex-0.8 ($\circ$), PEG6/Dex-1.6 ($\triangle$), and PEG50/Dex-0.8 ($\diamond$).

3-4: Conclusion

Biodegradable PEG/Dex hydrogels with different water content were prepared in order to regulate the phase-separated structures, degradability, and drug release profiles. When the water content was lower below 83%, the hydrogels were not degraded in the presence of dextranase. From the results of microscopic and thermal analysis, the water content and crosslink density affected to the phase-separated structures of PEG/Dex hydrogels, i.e., higher crosslink density correlated with exhibiting lower phase separability and forming smaller PEG domains in PEG/Dex hydrogels because of phase

mixing between PEG and dextran. The difference in the insulin leakage from the hydrogels is affected by the crosslink density of the hydrogels. However, the mode of insulin release from the hydrogels during their enzymatic degradation was quite different. Insulin release from PEG/Dex hydrogel was found to be degradation-controlled release, whereas the diffusion through the matrix was dominant factor of release from Dex hydrogels.

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Chapter 4

Pulsatile Peptide Release from Multi-Layered Hydrogel Formulations Consisting of Poly(ethylene glycol)-Grafted and Ungrafted Dextrans

4-1: Introduction

The temporal control of drug delivery has been strongly desired as a novel approach to effective drug therapies [1]. Especially, many peptide and protein drugs must be chronically administrated based on the circadian rhythm in the human body. In fact, a number of hormones are known to be endogenously released in a pulsatile manner. Studies on such hormones as insulin [2], the growth hormone [3, 4], calcitonin [5] and the human corticotropin-releasing hormone [6] suggest that the therapeutic index remarkably increases if the hormones are administrated in a pulsatile manner, rather than at a constant rate. As for an approach to pulsatile drug release, N.A. Mazer has introduced the concept of the pulsatile input function and its link to pharmacokinetics and pharmacodynamics [7]. Furthermore, R.A. Siegel *et al.* have reported a general strategy for pulsatile drug release [8, 9]. They pointed out the demand of designing a special polymeric membrane, the permeation properties of which is oscillatively altered. Thus, pulsatile drug delivery is recognized as an important concept to achieve not only improved peptide delivery but also other drugs which are required to be released in a pulsatile manner.

Several kinds of pulsatile drug delivery systems have been reported [10-12]. In these systems, the rate of drug release is regulated by external stimuli. These include transdermal iontophoresis [13, 14], electrophoresis [15, 16], and sonophoresis [17]. The utilization of stimuli-responsive polymers

is one of the approaches to the periodic pulsatile drug release in response to glucose [18], pH [19], temperature [20, 21], ultrasound [22], magnetic or electrical fields, and so on [23-25]. Recently, other approach to pulsatile delivery without external stimulation have been considered by a few researchers. Y.H. Bae *et al.* reported biphasic drug release utilizing interpenetrating polymer networks [26]. Wuthrich *et al.* reported pulsatile drug release using bioerodible devices which can delay the release of peptide drugs [27]. As for mathematical approach for pulsatile drug release using biodegradable polymers, L. Brannon-Peppas has proposed a model of a heterogeneous-structured device containing surface-erodible microspheres [28].

Pulsatile drug delivery may be achieved by utilizing the biodegradation of polymeric substrates if drugs do not leak from the polymer matrix. From this perspective, this study has focused on biodegradable dextran (Dex) hydrogels containing poly(ethylene glycol) (PEG) as a model of heterogeneous-structured devices composed of drug reservoirs and degradable matrices. PEG and Dex are well known as water soluble, thermodynamically incompatible polymers which exhibit aqueous polymer two-phase systems. In these hydrogels, it was found that PEG showed phase separation from Dex matrix and acted as a drug reservoir. By using this specific system, the diffusion of insulin as a model peptide was completely hindered in these hydrogels, because insulin partitioned into the PEG domains can entangle or interlace with the PEG segments. It was found that

insulin release from the hydrogels was regulated by dextranase-catalyzed surface degradation of the hydrogels.

In this chapter, multi-layered hydrogel formulations consisting of PEG-grafted Dex (PEG-g-Dex) containing insulin and ungrafted Dex without insulin were prepared, and pulsatile insulin release due to their enzymatic degradation was demonstrated. The size of the PEG domains acting as a drug reservoir can be controlled by the coupling reaction of PEG with Dex. Furthermore, the multi-layered hydrogel formulation is considered to be suitable for pulsatile peptide release due to surface degradation. This approach proposes a novel design of biodegradable formulations for chronopharmacological peptide delivery systems.

4-2: Materials and Methods

4-2-1: Chemicals

All Chemicals were analytical-grade commercial materials and used without further purification. Methoxy-poly(ethylene glycol)-amine (M-PEG-NH₂) (Mn=5000) was purchased from Shear Water Polymers, Inc., Huntsville, AL, U. S. A. Dimethylsulfoxide (DMSO), *N,N*-dimethylaminopyridine (DMAP), ammonium persulfate (APS), *p*-nitrophenyl chloroformate, fluorescein isothiocyanate (FITC), and insulin from bovine pancreas (26unit/mg), were purchased from Wako Pure Chemical Industries, Osaka, Japan. Glycidyl methacrylate (GMA) and dextran (Dex, Mn=40000) were purchased from

Tokyo Chemical Industry Co., Tokyo, Japan. Dextranase from *penicillium* sp. (12.9unit/mg) was purchased from Sigma Chemical Co., St. Louis, MO, U. S. A. Dialysis tubes (molecular weight cutoff: 30000) were purchased from Spectrum Medical Industries, Inc., Houston, U. S. A.

4-2-2: Partition of Insulin in PEG/Dex Two-Phase Systems

The partition of insulin between PEG and Dex solution in phosphate buffer solution (PBS, pH 7.4) was determined by the fluorescence of FITC-labeled insulin, which was similar to the method described in the late chapter [29]. PEG and Dex solutions in 0.15 M PBS (1ml respectively) were mixed with 1 mg/ml FITC-labeled insulin solution (0.1 ml) in PBS. Preparatively, PEG content was adjusted to be 8, 13 and 40%, and total concentration of PEG and Dex were 30wt.%. The mixture was allowed to settle for at least 30 min. Insulin concentration in both the top (PEG) and bottom (Dex) phases was determined by FITC fluorescence excited at 490 nm and emitted at 520 nm using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777).

4-2-3: General Procedure for The Preparation of Multi-Layered Hydrogel Formulations

Preparation of GMA-introduced PEG-grafted dextran: PEG-grafted dextran (PEG-g-Dex) was synthesized according to the method previously reported by E. Schacht *et al.* [30]. Dex was activated by *p*-nitrophenyl

chloroformate, followed by the condensation reaction with M-PEG-NH₂. PEG contents were regulated by the initial amount of PEG and *p*-nitrophenyl carbonate groups in Dex. PEG-g-Dex (5.0g) and DMAP (1.0g) were dissolved in 50ml of distilled DMSO. GMA (0.29g) was added to the reaction mixtures at a ratio of 1mol of GMA per 16.7mol of glucopyranosyl units of Dex. The reaction mixture was stirred at room temperature for 48h. After neutralization of DMAP with an equimolar amount of 4N hydrochloric acid, the solution was poured into a large excess of ethanol. The precipitate was collected by filtration and dissolved in 20ml of distilled water. The solution was dialyzed against distilled water for 5days and finally lyophilized. The degree of GMA substitution in the Dex derivative was determined by solution state ¹H-NMR (Varian, Palo Alto, CA, U. S. A., 300MHz FT-NMR Gemini300), comparing the integration of the signals at δ 5.75 and 6.2ppm (CH=CH of GMA) with those at δ 5.0ppm (C(1)H of Dex) with D₂O as a solvent.

Preparation of the formulations: Thirty weight-percent of GMA-introduced PEG-g-Dex solution in 0.1M PBS (pH7.4) and 5mg/ml FITC-labeled insulin in saturated glutamic acid solution were mixed in a volume ratio of 9:1, followed by the addition of APS. After vigorous stirring for 1min, the mixture was injected to a silicone tube (inner diameter: 3mm, length: 10mm). The crosslinking reaction was initiated by exposing the mixture to visible light from a high pressure mercury lamp (500W) for 10 min. Insulin-free GMA-Dex solution (6 μ l) and GMA-introduced PEG-g-Dex solution containing FITC-insulin (15.6 μ l) were alternatively crosslinked within the tube

to prepare a multi-layered formulation. The total volume of the formulation was 70.6 μ l, and the total amount of incorporated insulin was 24 μ g.

4-2-4: Insulin Leakage from PEG-g-Dex Solutions

Insulin leakage from Dex and PEG-g-Dex solutions were estimated as follows: 30wt.% solution of Dex or PEG-g-Dex in PBS (4.5ml) was mixed with 3.0mg/ml of FITC-labeled insulin in saturated glutamic acid solution (0.5ml). The mixture was transferred to the dialysis tubes (MWCO 30000) subsequently immersed in vials containing PBS and stirred at 37°C. At appropriate time, an outer solution was sampled and diluted in order to determine the insulin leakage by FITC fluorescence excited at 490nm and emitted at 520nm using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777).

4-2-5: Enzymatic Degradation and Characterization of PEG-g-Dex Hydrogels

The degradation of PEG-g-Dex hydrogels by dextranase (0.1unit/ml) was examined *in vitro*. The degradation rate was estimated by measuring the residual weight of the hydrogel together with the silicone tube. In order to estimate the heterogeneity of PEG-g-Dex hydrogels in swollen states, small-angle light scattering (SALS) measurements were carried out using SALS

apparatus (IST Planning Co., Tokyo, Japan, SALS-100S) with He-Ne laser ($\lambda=632.8\text{nm}$, 2mW) as a light source.

4-2-6: Physical Stability of Insulin in PEG-g-Dex Solutions

Two wt.% solution of PEG-g-Dex in 0.15 M PBS containing the FITC-labeled insulin was prepared as mentioned above (insulin concentration: 0.6 mg/ml). As controls, 2 wt.% solution of Dex or PBS containing the FITC-labeled insulin was prepared as well as PEG-g-Dex solution. These solutions were settled for 60 h at 25°C and the conformational change of insulin was examined by circular dichroism (CD) spectroscopy. All CD spectra were obtained using a CD spectrophotometer (Japan Spectroscopy Co., Tokyo, Japan, J-720) at 25°C.

4-2-7: Insulin Release from Multi-Layered Hydrogel Formulations

Enzymatic degradation and insulin release from the multi-layered hydrogel formulation were estimated according to the previous method [29]: after equilibration in 0.1M PBS (pH7.4), the formulation was mounted in a nylon mesh-bag attached to a thread for dipping into solutions and immersed into dextranase solution in PBS (0.1unit/ml) with stirring to initiate the degradation. The degradation rate was estimated by measuring the residual weight of the formulation together with the nylon mesh-bag. Insulin release

from the formulation was estimated by measuring FITC fluorescence excited at 490nm and emitted at 520nm continuously using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777) equipped with a flow cell (Japan Spectroscopy Co., Tokyo, Japan, FSC-322). The total volume of this flow system was 15ml, and the flow rate was fixed at 30ml/min using a peristaltic pump (Iwaki Glass Co., Tokyo, Japan, PST-100).

4-3: Results and Discussion

4-3-1: Preparation and Characterization of PEG-g-Dex and Crosslinked PEG-g-Dex Hydrogels

In the chapter 2 and 3, it was found that one of the disadvantages of crosslinked dextran hydrogels which physically contain PEG (PEG/Dex-mixed hydrogel) was their limited PEG content with uncontrollable domain size: the PEG content in PEG/Dex-mixed hydrogel was fixed at 5wt.% because PEG leakage occurred during the crosslinking reaction when higher than 10wt.% of PEG was used. This was due to the fact that PEG was not crosslinked nor covalently bound to Dex. In this chapter, PEG was covalently bound to the Dex backbone as a grafted chain. The grafting of the PEG chain onto Dex could be qualitatively confirmed. An aqueous solution of GMA-introduced Dex coupled with PEG was observed to be opaque, although the mixture of GMA-introduced Dex and PEG solution was observed to be separated into two-phases. This observation indicates that PEG domains

were stably dispersed in the Dex matrix in the PEG-g-Dex solution, and the solution may be used as an injectable therapeutic formulation without further crosslinking procedures.

The PEG content in PEG-g-Dex was determined to be 8, 13, and 40wt.% by $^1\text{H-NMR}$. Based on the $^1\text{H-NMR}$ spectra, the peaks corresponding to methylene proton of PEG ($\delta 3.7\text{ppm}$) and anomeric proton of Dex ($\delta 5.0\text{ppm}$) were observed. These results indicate that the PEG content was regulated by feed condition of the reaction (initial amount of PEG and nitrophenyl groups in Dex). In order to obtain quantitative information on the two-phase morphology in crosslinked PEG-g-Dex hydrogels with different PEG content, SALS measurement of these hydrogels in swollen states was carried out. Figure 4-1 shows SALS profiles of a series of PEG-g-Dex hydrogels (PEG-g-Dex1, PEG-g-Dex2 and PEG-g-Dex3 indicate 8, 13 and 40wt.% of PEG content respectively). The length of scattering vector (q) is defined as the equation 2.1. The scattering intensity of PEG-g-Dex show a maximum at a magnitude of $q_{\text{max}}=1.61, 1.16$ and 0.73 ($\times 10^{-3}\text{nm}^{-1}$), related to PEG-g-Dex1, PEG-g-Dex2, and PEG-g-Dex3 respectively. The interdomain distance (r) is defined as the equation 2.2 [31-34]. The result calculated from equation 2.2 were $3.9, 5.4$ and $8.6\mu\text{m}$, respectively. It is confirmed that the size of PEG domain in PEG-g-Dex hydrogel increased with PEG content in PEG-g-Dex.

Using these PEG and Dex two-phase systems, the partition of insulin was determined. Figure 4-2 shows the partition of insulin in the PEG/Dex systems with different PEG content to dextran (PEG content were 8, 13 and

40wt.% respectively) in 0.15M PBS. In these systems, insulin as a model of bioactive peptide drugs was preferentially partitioned into the PEG phase. These phenomena were similar to the results of the chapter 2 and 3. The concentrations of insulin in both phases were approximately identical with the change in the PEG content.

In the PEG-g-Dex aqueous solution or its crosslinked hydrogels, the PEG domain may act as a drug reservoir. Figure 4-3 shows the insulin leakage from the PEG-g-Dex solutions in 0.15M PBS (pH 7.4) at 37°C . The insulin leakage from the PEG-g-Dex solutions was found to be less than 6%, whereas over 70% of the loaded insulin was actually leaked from the Dex solution. It is considered that PEG-g-Dex prevents insulin leakage due to the preferential partition of insulin into the PEG domains and successive entanglement of insulin with the PEG chain.

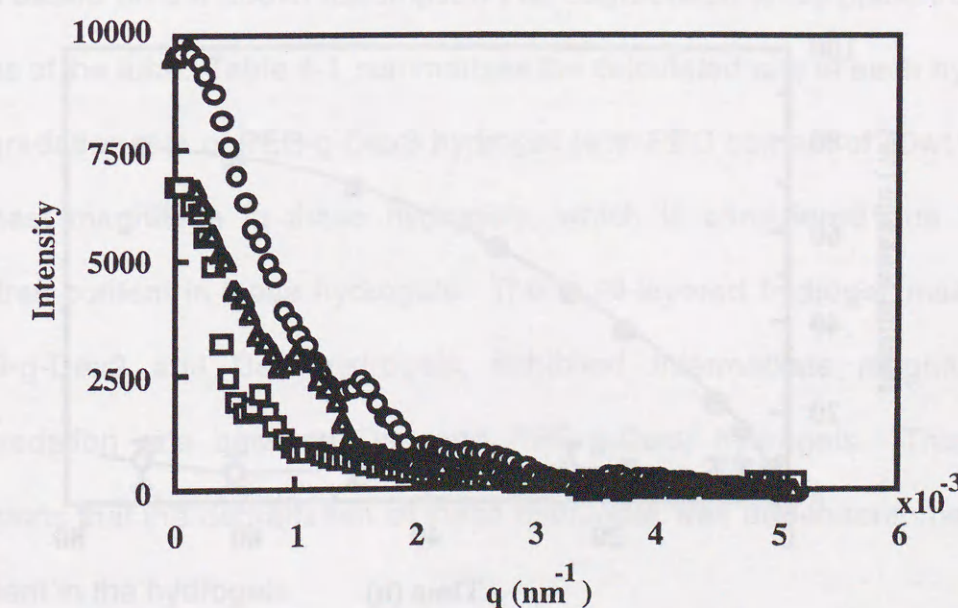


Figure 4-1. SALS profiles of PEG-g-Dex hydrogels with different PEG content. $\square$, PEG-g-Dex1; Δ , PEG-g-Dex2; $\circ$, PEG-g-Dex3.

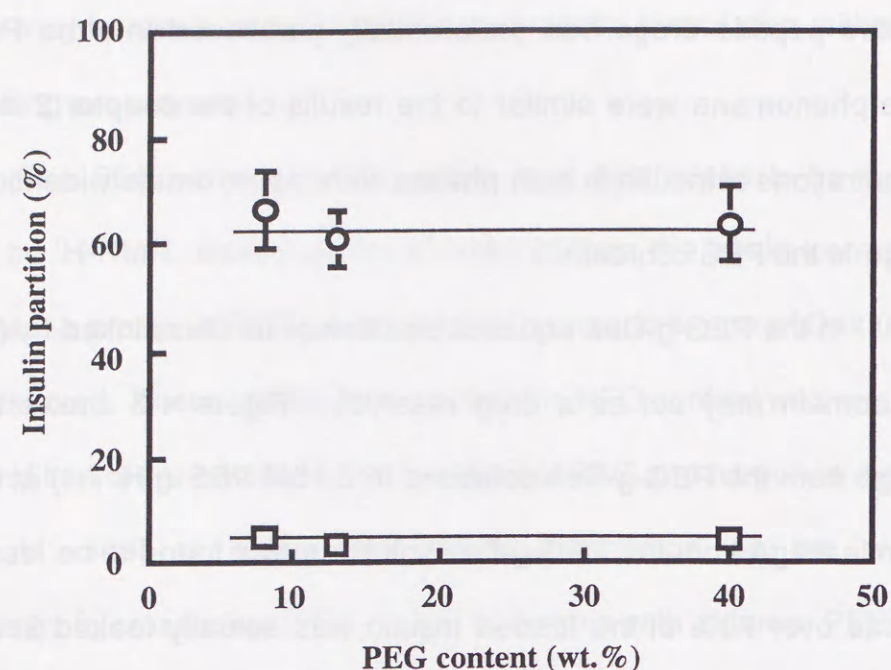


Figure 4-2. Insulin partition into PEG/Dex two-phase systems. $\circ$, top phase (PEG); $\square$, bottom phase (Dex). Total concentration of the polymer solution was 30wt.%. Total amount of insulin was 24 μ g.

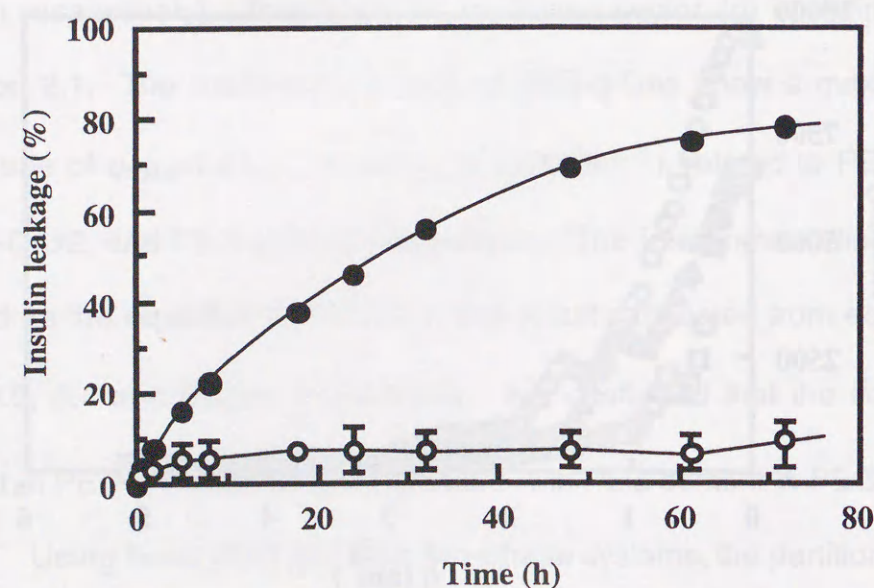


Figure 4-3. Insulin leakage from Dex and PEG-g-Dex solutions during storage at 37°C in PBS (0.15M, pH7.4). $\bullet$, Dex solution; $\circ$, mean of PEG-g-Dex1, PEG-g-Dex2 and PEG-g-Dex3.

4-3-2: Enzymatic degradation of PEG-g-Dex hydrogels

In order to examine degradation mechanism of hydrogels, enzymatic degradation *in vitro* was carried out with a series of PEG-g-Dex hydrogels. The samples were prepared with silicone tubes and shapes were cylindrical, but the degradation fronts were limited to both side of the tube. In this chapter, it is considered that the equation of the slab shape (equation 2.4 and 2.6) will be acceptable because the direction of the degradation front was limited to one axis. Figure 4-4 shows a time course of the degradation of the hydrogels (Dex hydrogel, PEG-g-Dex hydrogel with PEG content of 13wt.%, and Multi-layered hydrogel) at 37°C in 0.15M PBS. The degradation rates (B) of the hydrogels can be calculated from the equation 2.8. This equation is also based on the above assumption that degradation takes place from both sides of the tube. Table 4-1 summarizes the calculated rate of each hydrogel. Degradation rate of PEG-g-Dex3 hydrogel (with PEG content of 40wt.%) was highest magnitude in these hydrogels, which is considered due to less dextran content in these hydrogels. The multi-layered hydrogel, made from PEG-g-Dex2 and Dex hydrogels, exhibited intermediate magnitude in degradation rate between Dex and PEG-g-Dex2 hydrogels. This result supports that the degradation of these hydrogels was dependent upon PEG content in the hydrogels.

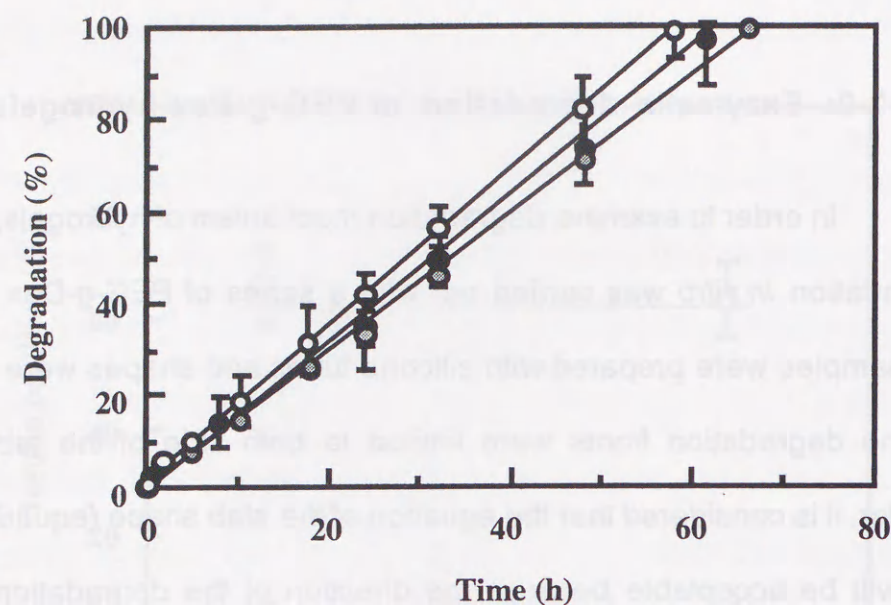


Figure 4-4. Enzymatic degradation of hydrogel formulations. ○, PEG-g-Dex2 hydrogel; ●, PEG-g-Dex and Dex multi-layered hydrogel; ●, Dex hydrogel.

Table 4-1 Degradation rate ($B \times 10^6$ cm/sec) of the formulations

Dex hydrogel	PEG-g-Dex hydrogels			Multi layered hydrogel
	PEG-g-Dex1	PEG-g-Dex2	PEG-g-Dex3	
	PEG content (wt.%)			PEG content (wt.%)
	8	13	40	8.6
2.13 ±0.14	2.30±0.14	2.42 ±0.17	2.59±0.11	2.33 ±0.15

4-3-3: Physical stability of insulin in PEG-g-Dex hydrogels

Conformational change and aggregation of peptides have been important subjects to be solved when considering peptide formulations. Physical stability of insulin during storage was examined in terms of aggregation and conformation change in the formulation. Figure 4-5 shows

the results of the CD spectroscopy of FITC-labeled insulin in PEG-g-Dex hydrogels. The CD spectra of initial insulin in PBS (a) and in the formulation (PEG-g-Dex) after 60 h (b) exhibited identical peak at 205-218 nm, whereas the CD spectrum of insulin in both Dex solution (c) and PBS (d) after 60 h was drastically changed with negative peak at 225-240 nm. It is known that the increase in the negative peak at 225 nm is attributed to an increase in β -structure of proteins. The result of Figure 4-5 indicates the conformational change of insulin occurred during 60h-storage in both Dex solution and PBS. In contrast, it is obvious that the conformational change of insulin was effectively prevented during 60h-storage in the PEG-g-Dex solutions. Chain entanglement between insulin and PEG may be related to this finding.

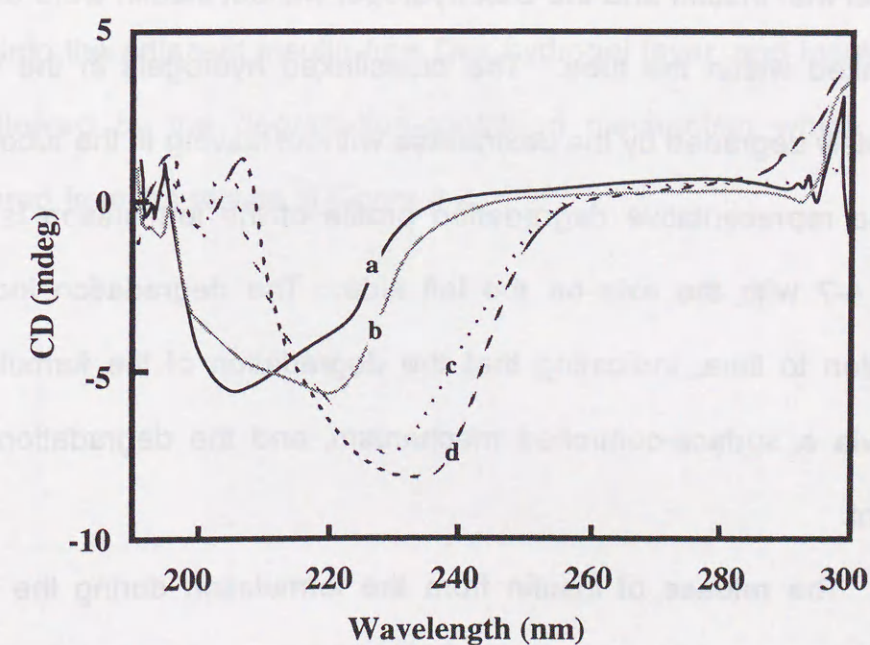


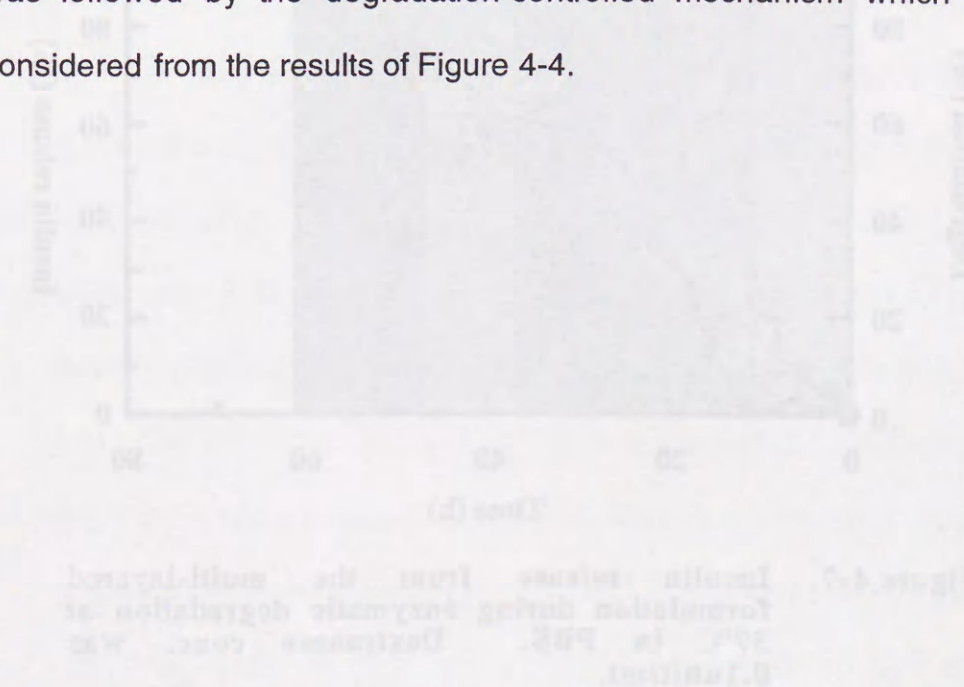
Figure 4-5. CD spectra of FITC-labeled insulin. (a), at initial in PBS; (b), after 60h in PEG-g-Dex2 solution; (c), after 60h in Dex solution; (d), after 60h in PBS.

4-3-4: Pulsatile insulin release from multi-layered hydrogel formulation

A multi-layered hydrogel formulation consisting of PEG-g-Dex hydrogel containing insulin and insulin-free Dex hydrogel was prepared inside a silicone tube as a cylindrical shape as shown in Figure 4-6. A commercially available silicone tube was cut and used as an outer shell in order to limit the direction of the degradation front as previously described. The crosslinked PEG-g-Dex hydrogel layer was observed to be yellow color due to the FITC-insulin, whereas the crosslinked Dex hydrogel layer was observed to be transparent. This observation indicates that the PEG-g-Dex hydrogel with insulin and the Dex hydrogel without insulin were alternatively crosslinked within the tube. The crosslinked hydrogels in the tube were completely degraded by the dextranase without leaving in the tube (Figure 4-4). The representative degradation profile of the formulation is shown in Figure 4-7 with the axis on the left side. The degradation increased in proportion to time, indicating that the degradation of the formulation took place via a surface-controlled mechanism, and the degradation rate was constant.

The release of insulin from the formulation during the enzymatic degradation is also shown in Figure 4-7 with the axis on the right side. In this figure, the dark-gray area corresponds to the degradation period of the PEG-g-Dex hydrogel layer, which was calculated by using the degradation rate

and the length of PEG-g-Dex hydrogel layer (2.2mm). Insulin release from the formulation was observed over the degradation period of the PEG-g-Dex hydrogel layer containing insulin. On the other hand, insulin release was not observed when the insulin-free Dex hydrogel layer was degraded. No difference in the insulin release profiles among a series of PEG-g-Dex hydrogels was observed. The formulation had two degradation fronts (the degradation proceeded via both terminals of the tube) as shown in Figure 4-6. The two pulses of insulin release shown in Figure 4-7 are considered to be due to the degradation of the outer two layers and inner layer of the PEG-g-Dex hydrogels. This is supported by the fact that the amount insulin release at the first dark-gray area in Figure 4-7 was twice as the next dark-gray area. These results indicate that insulin in the PEG-g-Dex hydrogel layer did not diffuse into the adjacent insulin-free Dex hydrogel layer, and insulin release was followed by the degradation-controlled mechanism which could be considered from the results of Figure 4-4.



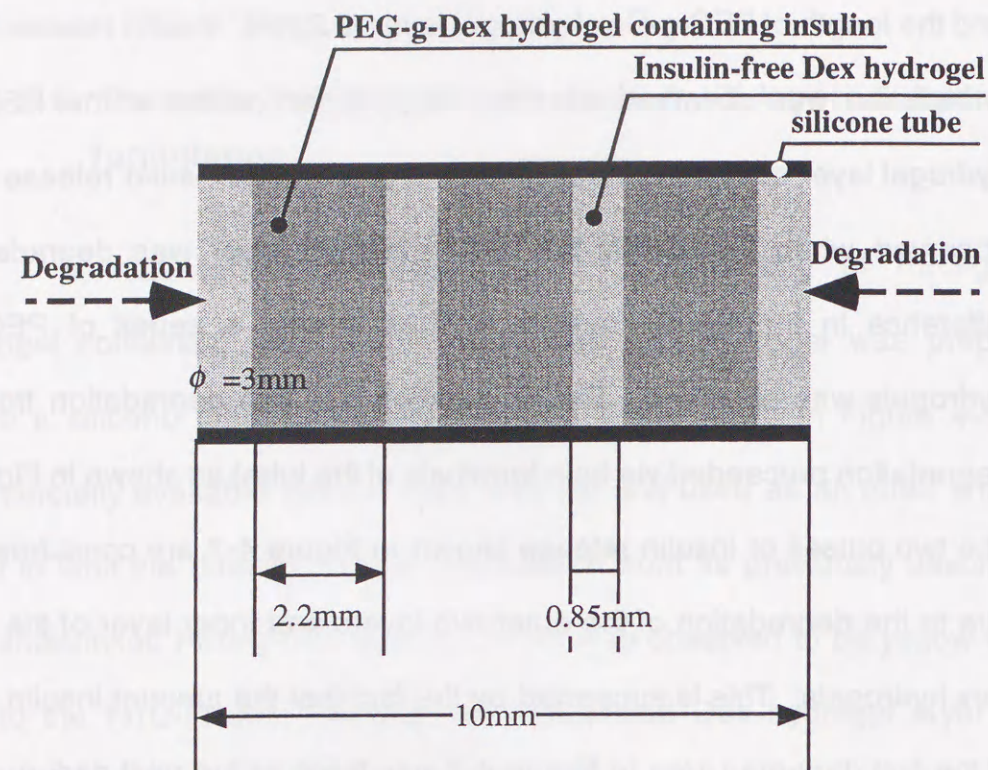


Figure 4-6. Illustration of the multi-layered formulation.

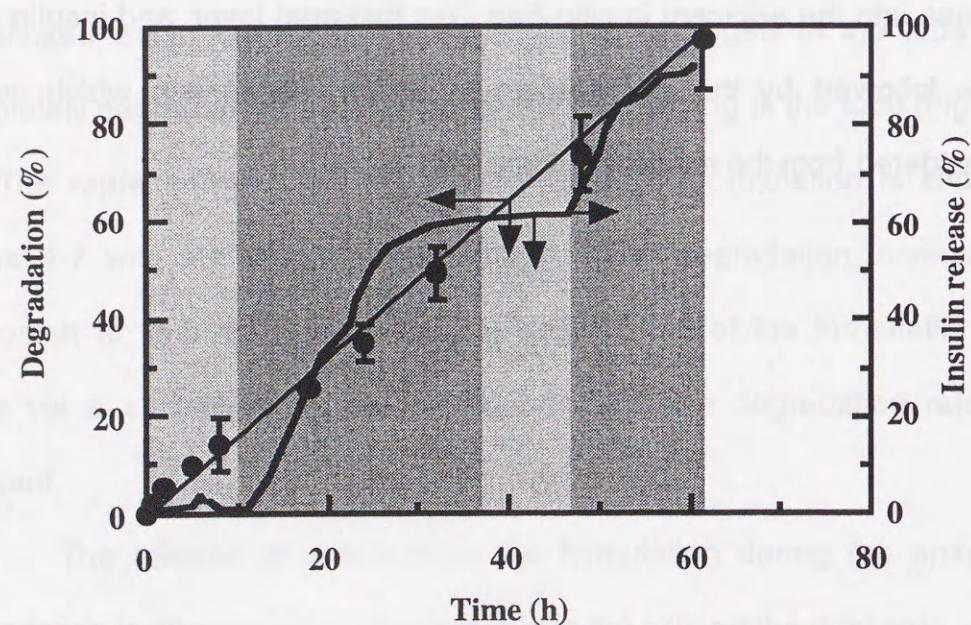


Figure 4-7. Insulin release from the multi-layered formulation during enzymatic degradation at 37°C in PBS. Dextranase conc. was 0.1unit/ml.

4-4: Conclusion

It is suggested that the multi-layered hydrogel formulations using PEG-g-Dex and Dex are suitable for regulating the peptide release in a pulsatile manner. Furthermore, it is obvious that the conformational change of insulin was effectively prevented during storage in the PEG-g-Dex solutions. Chain entanglement between insulin and PEG may be related to this finding. Further study on the PEG-Dex microdomain structure and mathematical analysis may enable us to realize a variation in the pulsatile peptide release at predetermined period which is useful for chronopharmacological drug delivery systems.

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Chapter 5

Hyaluronic Acid Grafted with Poly(ethylene glycol) as a Novel Peptide Formulation

5-1: Introduction

Biodegradable polymers have been represented as an attractive drug formulation because of their advantages such as biocompatibility, high responsibility for specific degradation, and feasible approach to incorporating drugs into their matrices. However, unexpected drug leakage from the matrix caused by drug diffusion can lead to serious side effects. In order to prevent the drug leakage and to achieve degradation-controlled drug release from a highly water-swollen matrix, a heterogeneously-structured hydrogel of a degradable dextran (dex) matrix containing poly (ethylene glycol) (PEG) as a drug reservoir has been proposed as a newly designed implantable device for water soluble peptide delivery [3].

Liquid-liquid distribution of macromolecules provides a procedure for purification of proteins and cell particles in PEG and polysaccharide (PS) system, which is an aqueous polymer two-phase system [4]. In the chapter 2 and 4, this specific phenomenon was used in biodegradable dex hydrogels containing PEG. PEG exhibited phase separation from dex matrix and acted as a drug reservoir. Leakage of insulin, as a model drug, caused by diffusion was completely prevented in these hydrogels; presumably, insulin partitioned into the PEG domains can entangle or interlace with PEG segments. In addition, it was suggested that the insulin release from PEG/dex heterogeneously-structured hydrogels can be regulated by the extent of microdomain structures composed of PEG and dex phases. The degradation-

controlled insulin release was observed when hyaluronic acid (HA) was used instead of dex matrices [3]. However, the size and distribution of the PEG domains in these hydrogels could not be controlled because the PEG segment was neither crosslinked nor covalently bound to the PS segment. In the chapter 4, multi-layered hydrogels consisting of dex grafted with PEG (PEG-g-dex) containing the peptide drug and ungrafted dex were prepared [5]. In these hydrogels, the PEG content in the PEG-g-dex layer was controllable by the reaction conditions. This result suggests that the size and distribution of the PEG domains may be controlled by the PEG content in PEG-g-dex. Furthermore, the concentration of the loaded insulin could be increased in proportion to the PEG content. From this point, it is expected that HA grafted with PEG can increase the amount of the drug with the PEG content. In this case, the HA matrix may act as an inflammation-responsive degradable formulation for drug delivery such as the previously reported HA hydrogels containing lipid microspheres [6].

HA is a viscoelastic biomaterial that is a major component of vitreous humor and of synovial fluid in joints. It has unique rheological properties that are unparalleled by those of any other natural or synthetic polymer. These unique properties indicate the potential use of HA as a surgical aid, especially for the replacement of the natural vitreous humor in ophthalmic procedures [7]. In synovial fluid, HA is generally believed to be essential for lubrication of joints to prevent friction and for shock absorption [8, 9]. The HA solutions are currently used for viscosupplementation in treating osteoarthritis; however,

they do not remain in the joint for prolonged periods because of their quick degradation in an inflammatory environment and thus fail to exert a long-lasting effect [10, 11]. In order to utilize the potential of HA in biomedical research, many approaches to the chemical modification of HA have been undertaken [12]. For example, HA has been crosslinked using a variety of reagents to prepare HA hydrogels [13]. However, none of the products were prepared as an injectable drug delivery material. On the other hand, solutions of native HA have been administered intra-articularly together with steroidal drugs [14]. In these cases, drug leakage from HA solution and fast disappearance of the drugs from the joint cavity are a clinical problems. From these perspectives, prevention of HA degradation and drug leakage are necessary to achieve advanced injectable drug delivery systems. HA grafted with PEG can be envisaged as a material for injectable formulations. It is expected that the grafted PEG acts as a drug reservoir as mentioned above, and degradability of HA vs. hyaluronidase may be reduced due to the grafted PEG chains.

In this chapter, HA grafted with PEG (PEG covalently bound as a graft chain to HA backbone) was prepared as a material for heterogeneously-structured formulations composed of drug reservoirs and degradable matrices. Enzymatic degradation and insulin leakage were examined in their aqueous solutions of these preparations *in vitro*. In this case, insulin is a model of antiinflammatory peptides such as lipocortin 1, and its role in antiinflammatory activity in joint inflammation has recently been investigated

[15-17]. Based on these preliminary results, a novel approach to designing a heterogeneously-structured HA solution which can be used as an injectable formulation will be proposed.

5-2: Materials and Methods

5-2-1: Chemicals

All chemicals were analytical-grade commercial materials and were used without further purification. Poly(ethylene glycol) (PEG) ($M_n=1000$, 6000, 50000), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), fluorescein isothiocyanate (FITC) and insulin from bovine pancreas (26unit/mg) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Sodium hyaluronate (HA) ($M_w=1.2 \times 10^6$) was purchased from QP Fine Chem. Co., Tokyo, Japan. Methoxy-PEG-hydrazide (M-PEG-HZ) ($M_w=5000$) was purchased from Shear Water Polymers, Inc., Huntsville, U. S. A. A pullulan standard sample was purchased from Fluka Chemie AG, Buchs, Switzerland. Bovine testicular hyaluronidase (300 unit/mg) was purchased from Sigma Chemical Co., St. Louis, U. S. A. Dialysis tubes (molecular weight cutoff: 10000 and 30000) were purchased from Spectrum Medical Industries, Inc., Houston, U. S. A.

5-2-2: Partition of Insulin in PEG/HA Two-Phase Systems

The partition of insulin between PEG and HA solution in phosphate buffer solution (PBS) was determined by the fluorescence of FITC-labeled insulin, which was similar to the method described in the previous report [3]. Thirty wt.% of PEG solution and 5 wt.% of HA solution in 0.15 M PBS (pH 7.4, 1 ml respectively) were mixed with 1 mg/ml FITC-labeled insulin solution (0.1 ml) in PBS. The mixture was allowed to settle for at least 30 min. Insulin concentration in both the top (PEG) and bottom (HA) phases was determined by FITC fluorescence excited at 490 nm and emitted at 520 nm using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777).

5-2-3: General Procedure for The Synthesis of HA Grafted with PEG (PEG-g-HA)

The synthesis of PEG-g-HA was carried out as follows: sodium hyaluronate (HA) (200 mg, 0.50 mmol of disaccharide repeat unit) was dissolved in pure water so that the concentration of the HA solution was approximately 4 mg/ml. To this mixture was added an equimolar amount of M-PEG-HZ (500 g, 0.10 mmol). The pH of the reaction solution was then adjusted to 4.75 using 0.1N HCl. EDC (161 mg, 1.0 mmol) was then added in solid form. The reaction was allowed to proceed for 2 h or until no further increase in pH was observed, at which time the pH of the reaction mixture was adjusted to 7.0 by the addition of 1N NaOH. A dialysis tube (molecular weight cutoff 10000) was prepared by soaking the membrane in distilled water at room temperature for 30 min and subsequent by rinsing with water.

The reaction mixture was then transferred to the pretreated dialysis tube and dialyzed exhaustively against pure water. The clear and viscous final mixture was lyophilized for 48 h. In the procedure, the pH of the reaction mixture was adjusted to 4.75, which had been previously determined to be optimal for the coupling reaction [19, 20]. The addition of EDC caused an increase in pH, and the pH was maintained at 4.75 by the addition of 0.1N HCl for ca. 2 h. The pH was then increased to 7.0, and the reaction mixture was purified by dialysis to remove reagents from products. The PEG-g-HA was isolated as a white fibrous mass in quantitative yield after lyophilization.

The average number of PEG chains bound to a HA chain was determined from $^1\text{H-NMR}$ (Varian 300MHz FT-NMR Gemini300, Palo Alto, U. S. A.) in D_2O , comparing the integration of the signals at 2.01 ppm (CH_3 , acetyl of HA) with that at 3.69 ppm (CH_2 of PEG).

5-2-4: Characterization of PEG-g-HA

In order to estimate the heat of fusion (ΔH) of PEG in the dry state of PEG-g-HA, differential scanning calorimetric analysis was carried out (Seiko Instruments Co., Tokyo, Japan, DSC120) in the temperature range from -25 to 160 °C. Sample weights were 6 mg, and the scanning rate was 2 °C/min. The heterogeneity of the PEG-g-HA solutions was determined by small-angle light scattering (SALS) measurements using an SALS apparatus (IST Planning

Co., Tokyo, Japan, SALS-100S) with a He-Ne laser ($\lambda=632.8$ nm, 2mW) as a light source.

5-2-5: Enzymatic Degradation of The PEG-g-HA Solutions

The degradation of the PEG-g-HA by hyaluronidase was examined *in vitro*. A 2 wt.% solution of HA or a series of PEG-g-HA in 0.15 M PBS (pH 7.4) was preincubated for 30 min at 37 °C. Enzymatic degradation was performed by incubation with 30 units of hyaluronidase per 1 mg of HA at 37 °C. Digestion was allowed to proceed for 48 h. In order to determine the degradation, the reaction mixture was sampled and digestion was terminated at the appropriate time by heating in a boiling water bath for 10 min. The sample was cooled to room temperature and centrifuged at 10,000 g for 10 min. The supernatant was separated into two portions, one was injected into an HPLC system, and the other was analyzed by light scattering measurement. Molecular weight measurements of degraded HA and PEG-g-HA were performed using a GPC system and light scattering apparatus. For the HPLC analysis, 20 μ l of the solution was applied after passage through a Millipore filter (pore size: 0.45 μ m). The HPLC systems (Japan Spectroscopy Co., Tokyo, Japan, a 2-line degasser DG-980-50, an intelligent HPLC pump PU-980, an intelligent sampler AS-950-10, a column oven CO-965, a multiwavelength detector MD-910, and an intelligent RI detector RI-930) were used with a GPC column (Tosoh, Tokyo, Japan, GMPW_{XL}) at a flow rate of 0.4

ml/min at 40 °C. A 0.1M NaNO₃ solution was used for the mobile phase. For the calibration of the HPLC system, pullulan standards were used for the determination of the relationship between the molecular weight of the polymer and the elution volume. The detection of samples was performed by an RI detector, and the obtained data were calculated using the analytical software BORWIN PDA version 1.0 (JMBS Developments, Le Fontanie, France). A multi angle light scattering photometer (Ohtsuka Electronics Co., Osaka, Japan, DLS-7000) was used at 632.8 nm (He-Ne laser as a light source) using $dn/dc=0.153$ cm³/g.

5-2-6: Insulin Leakage from PEG-g-HA Solutions

Insulin leakage from PEG-g-HA was estimated as follows: 2 wt.% solution of HA or PEG-g-HA in PBS (2 ml) was mixed with 3.0 mg/ml of FITC-labeled insulin in saturated glutamic acid solution (0.5 ml). The mixture was transferred to a dialysis tube (MWCO 30,000), subsequently immersed in a vial containing PBS and stirred at 37 °C. At the appropriate time, the outer solution was sampled and diluted in order to determine insulin leakage by FITC fluorescence excited at 490 nm and emitted at 520 nm using a spectrofluorometer (Japan Spectroscopy Co., Tokyo, Japan, FP-777).

5-2-7: Physical Stability of Insulin in PEG-g-HA Solutions

Two wt.% solution of PEG-g-HA in 0.15 M PBS containing the FITC-labeled insulin was prepared as mentioned above (insulin concentration: 0.6 mg/ml). As controls, 2 wt.% solution of HA or PBS containing the FITC-labeled insulin was prepared as well as PEG-g-HA solution. These solutions were settled for 60 h at 25°C and the conformational change of insulin was examined by circular dichroism (CD) spectroscopy. All CD spectra were obtained using a CD spectrophotometer (Japan Spectroscopy Co., Tokyo, Japan, J-720) at 25°C.

5-3: Results and Discussion

5-3-1: Partition of Insulin in PEG/HA Two-Phase Systems

Figure 5-1 shows the partition of insulin in the PEG/HA system with different PEG molecular weights ($M_n=1,000, 6,000, 50,000$; PEG1, PEG6, PEG50, respectively) in 0.15M PBS. In this system, insulin as a model of an antiinflammatory peptide drug was preferentially partitioned into the PEG (top) phase. It is considered that the negatively charged protein of insulin was mainly partitioned into the PEG phase because HA is a polyelectrolyte which is negatively charged. The concentration of insulin in the PEG phase was not influenced by the change in PEG molecular weights, while a significant change occurred in the insulin partition in the case of the PEG/dextran two-phase systems [3].

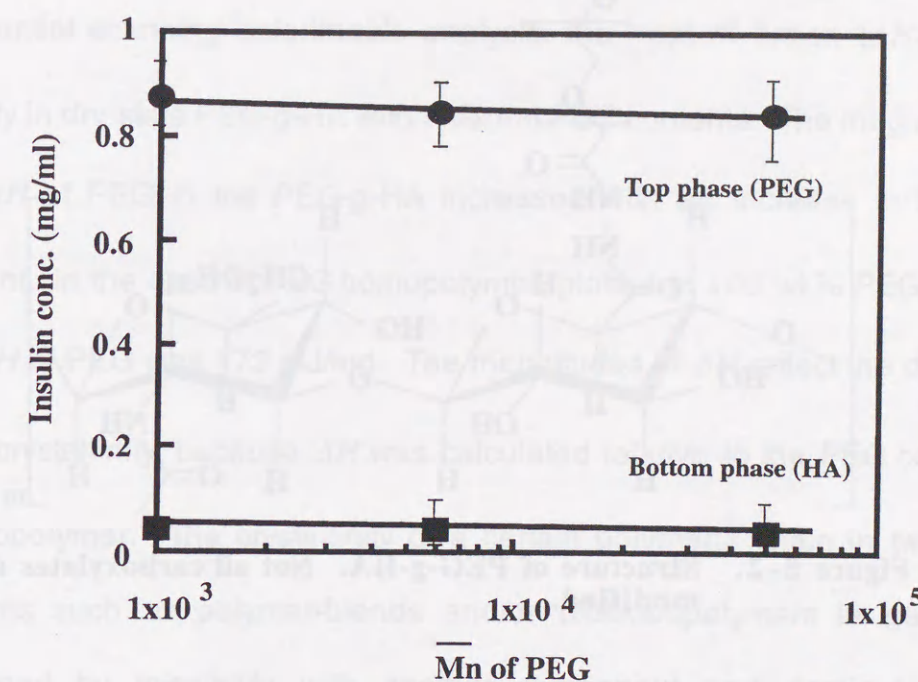


Figure 5-1. Partition of insulin in PEG/HA two-phase systems in 0.15M PBS (pH7.4). Top phase is PEG, bottom phase is HA.

5-3-2: Synthesis and Characterization of PEG-g-HA

In this chapter, a new methodology for introducing reactive hydrazido groups into HA oligosaccharide [18-20] was extended to the PEG introduction of high molecular weight HA (Figure. 5-2). The content of PEG in the copolymer was controlled by the initial amount of PEG reagent which is summarized in Table 5-1.

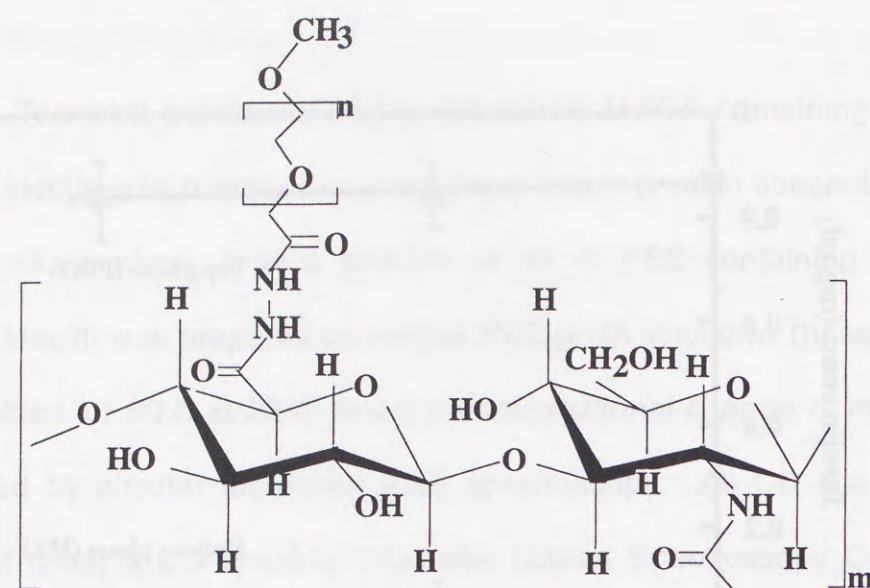


Figure 5-2. Structure of PEG-g-HA. Not all carboxylates are modified.

Table 5-1 Preparation of hyaluronic acid grafted with PEG

Code	PEG		
	$\overline{M}_n$	Ave number ^{a)}	Content (wt.% ^{b)}
PEG-g-HA1	5000	0.6	7
PEG-g-HA2	5000	2.1	22
PEG-g-HA3	5000	4.7	39
PEG-g-HA4	5000	10.1	58

a) Average number of PEG chains per 100 repeat units of HA calculated from the result of ¹H-NMR.

b) PEG contents were calculated from average number of PEG chains.

The PEG with a number average molecular weight of 5,000 used in this chapter is a semicrystalline polymer with a melting point in the range of 57-60°C. If phase separation occurs in PEG-g-HA in the solid state, the PEG

side chain may exhibit crystallization. Figure 5-3 shows the results of differential scanning calorimetric analysis: the heat of fusion (ΔH) of PEG moiety in dry state PEG-g-HA with different PEG contents. The magnitudes of the ΔH of PEG in the PEG-g-HA increased with an increase in the PEG content. In the case of PEG homopolymer, plotted at 100 wt.% PEG content, the ΔH of PEG was 172 mJ/mg. The magnitudes of ΔH reflect the degree of PEG crystallinity, because ΔH was calculated relative to the PEG content in the copolymer. The crystallinity of a certain polymeric chain in two-phase systems such as polymer-blends and/or block-copolymers in general is governed by miscibility with another component and dominates some morphological characteristics such as the size and phase mixing of microdomain structures. Therefore, the results in Figure 5-3 may suggest that PEG-g-HA with higher PEG content (PEG-g-HA4) exhibited PEG moieties well separated from the HA matrices, whereas PEG crystallization was hindered by phase mixing with HA to form much smaller domains in PEG-g-HA at lower PEG content (PEG-g-HA1).

In order to obtain quantitative information on the PEG-g-HA solution, SALS measurement of these solutions was carried out. Although these solutions were visibly turbid, the scattering intensity of these solutions was not obtained (data not shown). Thus, in order to estimate the heterogeneity of these solutions, the correlation distance (l_c) was obtained from Debye-Bueche plots [21, 22] in the results of SALS measurement as the square root of the

ratio of slope to the intercept of the plots (Figure 5-4). In general, l_c is known as an index of heterogeneity, which can be defined as the average length of randomly drawn vectors passing through the two phases [21, 22]. The values of l_c increased with PEG content (263.8, 305.3, 422.7, and 592.2 nm for PEG-g-HA1, PEG-g-HA2, PEG-g-HA3 and PEG-g-HA4, respectively), which suggests that the heterogeneity of PEG-g-HA solutions also increases with PEG content.

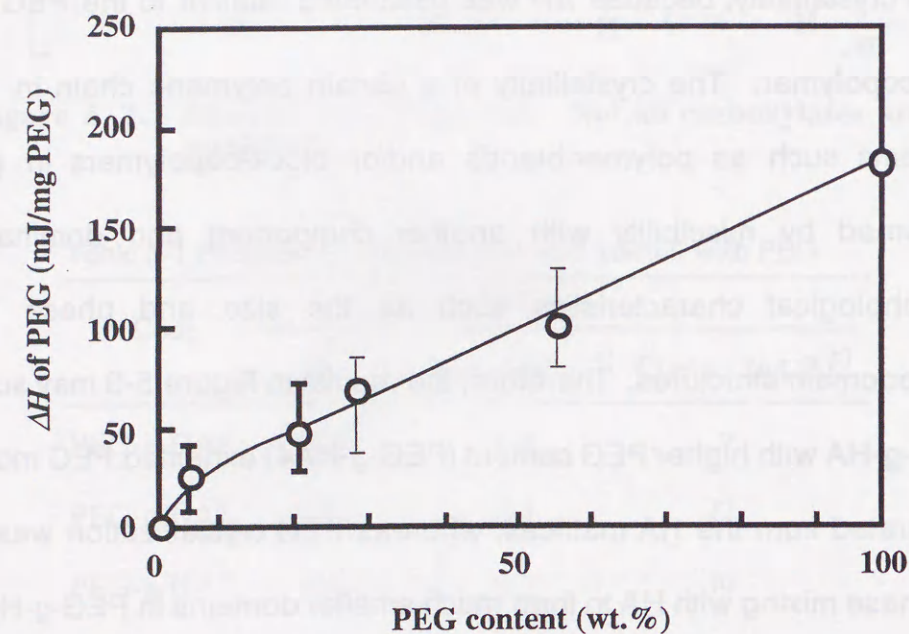


Figure 5-3. Relationship between ΔH of PEG and the PEG content in PEG-g-HA.

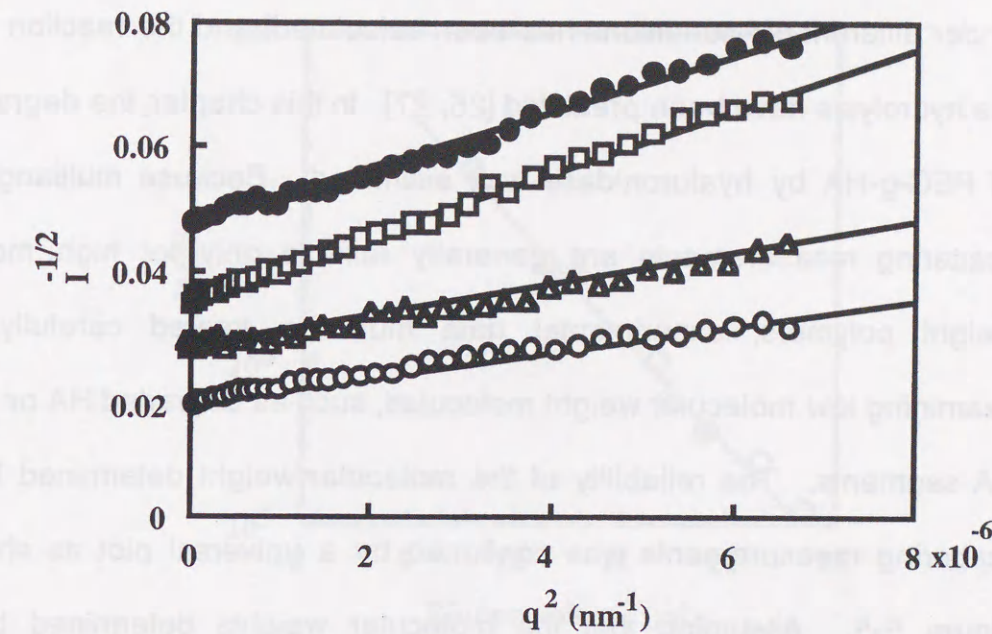


Figure 5-4. Debye-Bueche plots of SALS measurement. $\circ$, PEG-g-HA1; Δ , PEG-g-HA2; $\square$, PEG-g-HA3; $\bullet$, PEG-g-HA4.

5-3-3: Degradation of PEG-g-HA by Hyaluronidase

It is considered that enzymes play a major role in the biodegradation process of biomaterials *in vivo*. Hydrophobic polymers with no hydrolyzable bonds, such as polyethylene, polypropylene, or polystyrene, are expected to be stable in a living body. Cumulative evidence, however, suggests that even the most inert polymers suffer some degree of degradation after implantation into the body or under physiological conditions in the presence of the proper enzymes [23]. It has been reported that the degradation of HA is caused by inflammation-generated active oxygen in the form of hydroxyl radicals and hydrolysis with hyaluronidase, which is ubiquitous in cells and in serum [24, 25]. Recently, the activation energy for glycoside bond cleavage with HA

under different pH conditions has been calculated, and the reaction sites of the hydrolysis have been predicted [26, 27]. In this chapter, the degradability of PEG-g-HA by hyaluronidase was estimated. Because multiangle light scattering measurements are generally reliable only for high molecular weight polymers, experimental data must be treated carefully when examining low molecular weight molecules, such as degraded HA or PEG-g-HA segments. The reliability of the molecular weight determined by light scattering measurements was confirmed by a universal plot as shown in Figure 5-5. Assuming that the molecular weights determined by light scattering are correct, it can be obtained a universal plot using $[\eta]$ estimated by the Mark-Houwink-Sakurada equation;

$$[\eta] = K \times MW^a \quad (5.1)$$

where MW is the molecular weight, and for both PEG-g-HA and standard pullulan samples: $K=0.029$ and $a=0.80$ for PEG-g-HA applied to hypothetically native HA's values [28], and $K=0.022$ and $a=0.65$ for pullulan [29]. Because this consideration was not executed, the plot (Figure 5-5) of filled circles (PEG-g-HA) deviated from a straight line in the upper direction. As shown in Figure 5-5, the universal plot then gave a single straight line, which includes the plot of standard pullulan samples with calibrated MW values. This indicates that the MW determined in this chapter is reliable.

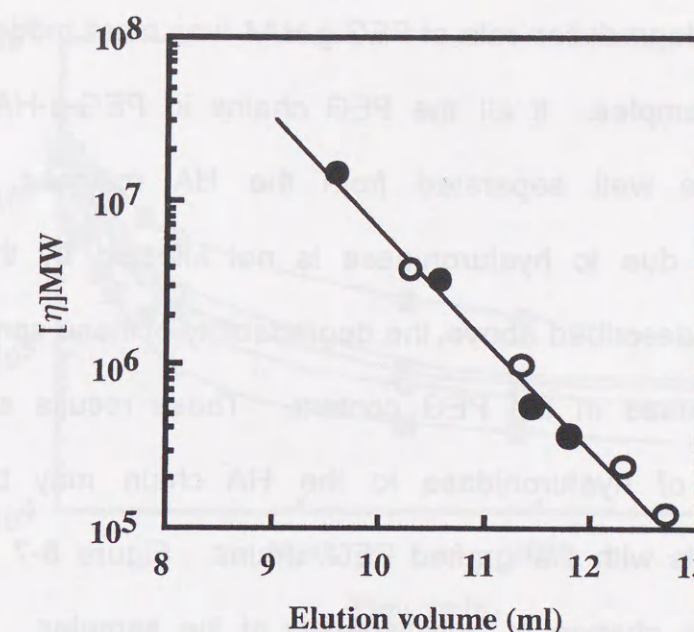


Figure 5-5. Plot of $[\eta]MW$ versus elution volume for HA and pullulan standard samples. ●, HA; ○, pullulan standards.

Figure 5-6 shows the time course of the molecular weight decrease HA and a series of PEG-g-HA by hyaluronidase (600 unit/ml) during a 48 h treatment at 37 °C in 0.15M PBS (pH 7.4). Initial molecular weights of HA and PEG-g-HAs were *ca.* 1×10^6 . In detail, the molecular weight of HA calculated from GPC measurement was 1.28×10^6 (3507 of repeat units), and in the same manner, the molecular weights of PEG-g-HA1, PEG-g-HA2, PEG-g-HA3 and PEG-g-HA4 were 1.30×10^6 , 1.70×10^6 , 1.96×10^6 and 2.1×10^6 , respectively. From this figure, the influence of PEG content on the enzymatic degradation was observed. The molecular weights of these polymers decreased to half of the initial values during 4 h of treatment. After 4 h, the samples exhibited a relatively slow decrease in the molecular weight. The degradation rate was decreased with an increase in the PEG content in PEG-g-HA. Especially, the

slope of the degradation rate of PEG-g-HA4 was more moderate than that of other four samples. If all the PEG chains in PEG-g-HA solutions form microdomains well separated from the HA matrices, it seems that degradability due to hyaluronidase is not affected by the PEG chains. However, as described above, the degradability of these samples decreased with an increase in the PEG content. These results suggest that the accessibility of hyaluronidase to the HA chain may be inhibited by entanglements with the grafted PEG chains. Figure 5-7 shows the time course of the change in polydispersity of the samples. These samples exhibited a quick increase in polydispersity during 3-4 h of treatment. During this time, the average molecular weight of the samples decreased quickly as shown in Figure 5-6. After 4 h, the polydispersity of the samples decreased, and after 24 h of treatment, it reached the value of ca. 1.4-1.5. Furthermore, the same value was obtained after 48 h. The change in the polydispersity of native HA during enzymatic degradation has already been reported by Chabreck *et al.* [30]. In their report, the dependence of the molecular weight on the time of exposure to a polymer degrading agent was described, and they concluded that HA was statistically degraded for the first time. However, after the molecular weight reached the order of 10^4 , the degradation process no longer statistically progressed, resulting in decreased polydispersity. Taking this report into account, these results indicate a similar phenomenon. Presumably, hyaluronidase can degrade HA to its degradation product by the order of 10^4 within 48 h.

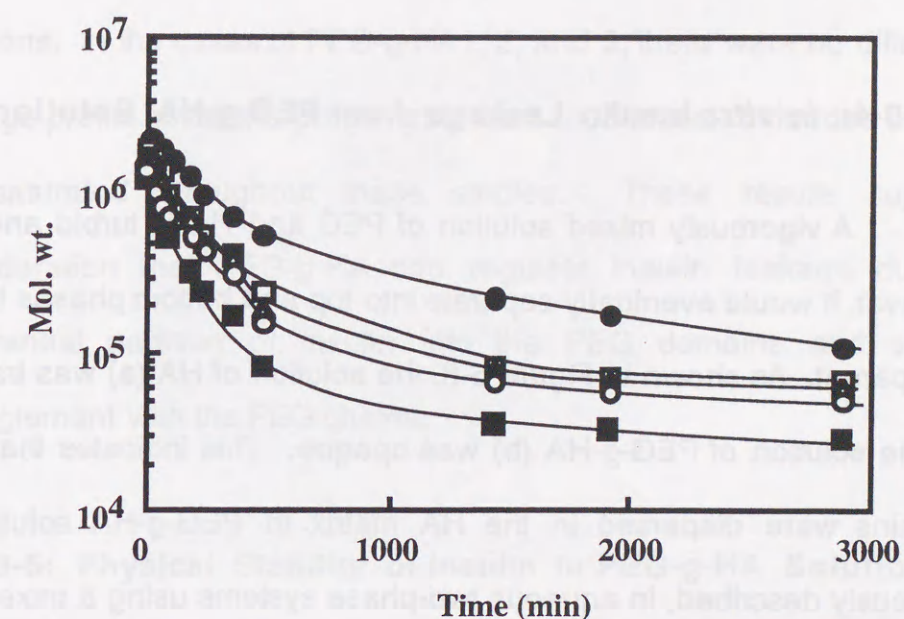


Figure 5-6. Molecular weight loss of HA and PEG-g-HA with hyaluronidase treatment in 0.15M PBS (pH7.4) at 37°C. ■, HA; ○, PEG-g-HA1; ▲, PEG-g-HA2; □, PEG-g-HA3; ●, PEG-g-HA4.

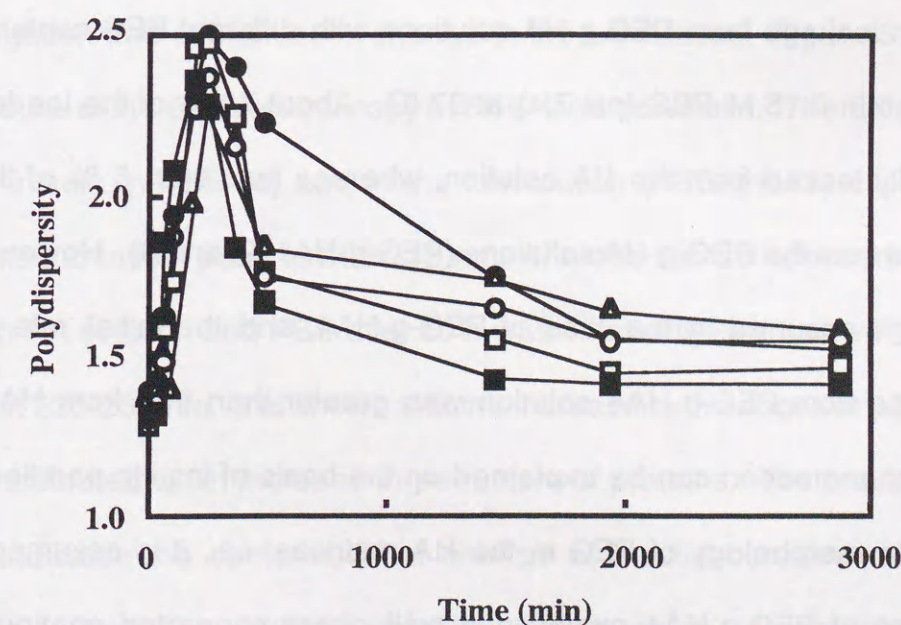


Figure 5-7. Time dependence of the polydispersity of HA and PEG-g-HA with hyaluronidase treatment in 0.15M PBS (pH7.4) at 37°C. ■, HA; ○, PEG-g-HA1; ▲, PEG-g-HA2; □, PEG-g-HA3; ●, PEG-g-HA4.

5-3-4: *in vitro* Insulin Leakage from PEG-g-HA Solutions

A vigorously mixed solution of PEG and HA is turbid and opaque; however, it would eventually separate into top and bottom phases to become transparent. As shown in Figure 5-8, the solution of HA (a) was transparent, but the solution of PEG-g-HA (b) was opaque. This indicates that the PEG domains were dispersed in the HA matrix in PEG-g-HA solutions. As previously described, in aqueous two-phase systems using a mixed solution of PEG and HA, insulin was preferentially partitioned into the PEG phase. From this point of view, the solution of PEG-g-HA may enable insulin to remain in PEG domains dispersed in the HA matrix. Figure 5-9 shows the insulin leakage from PEG-g-HA solutions with different PEG contents during storage in 0.15 M PBS (pH 7.4) at 37 °C. About 70 % of the loaded insulin actually leaked from the HA solution, whereas less than 5 % of the insulin leaked from the PEG-g-HA solutions (PEG-g-HA1, 2, and 3). However, insulin leakage occurred in the case of PEG-g-HA4, and the initial rate of insulin leakage from PEG-g-HA4 solution was greater than that from HA solution. This phenomenon can be explained on the basis of insulin partition and the domain morphology of PEG in the HA matrices, *i.e.*, it is assumed that the solution of PEG-g-HA4 exhibited a well phase-separated continuous PEG phase which contacted the outer aqueous environment and may have enabled the leakage of insulin, whereas relatively small PEG domains that entrapped insulin by chain entanglements existed in other PEG-g-HA

solutions. In the cases of PEG-g-HA1, 2, and 3, there were no differences in leakage profile since it is presumably the concentration of loaded insulin was not saturated throughout these studies. These results support the consideration that PEG-g-HA can regulate insulin leakage due to the preferential partition of insulin into the PEG domains and successive entanglement with the PEG chains.

5-3-5: Physical Stability of Insulin in PEG-g-HA Solutions

Conformational change and aggregation of peptides have been important subjects to be solved when considering peptide formulations. Physical stability of insulin during storage was examined in terms of aggregation and conformation change in the formulation. Figure 5-10 shows the results of the CD spectroscopy of FITC-labeled insulin. The CD spectra of initial insulin in PBS (a) and in the formulation (PEG-g-HA2) after 60 h (b) exhibited identical peak at 205-218 nm, whereas the CD spectrum of insulin in both HA solution and PBS after 60 h was drastically changed with negative peak at 225-230 nm. It is known that the increase in the negative peak at 225 nm is attributed to an increase in β -structure of proteins. The result of Figure 5-10 indicates the conformational change of insulin occurred during 60h-storage in both HA solution and PBS. In contrast, it is obvious that the conformational change of insulin was effectively prevented during 60h-storage in the PEG-g-HA solutions. Chain entanglement between insulin and PEG may be related to this finding.

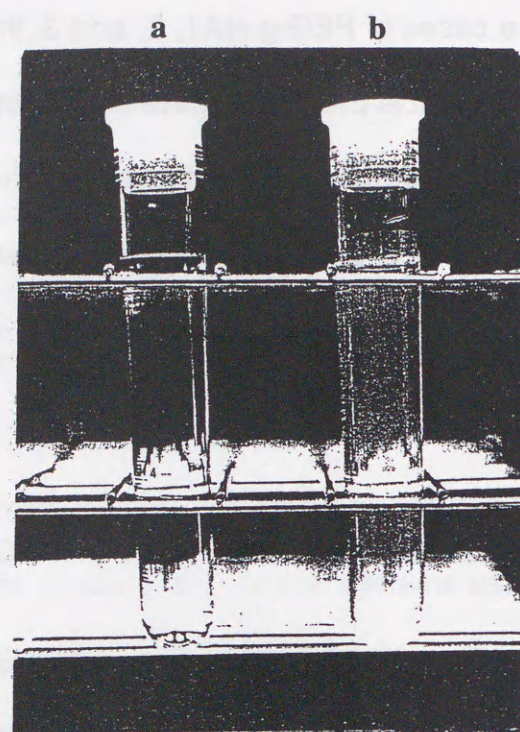


Figure 5-8. Photograph of HA (a) and PEG-g-HA (b) solutions. Both solutions contain 2.0 wt. % of HA.

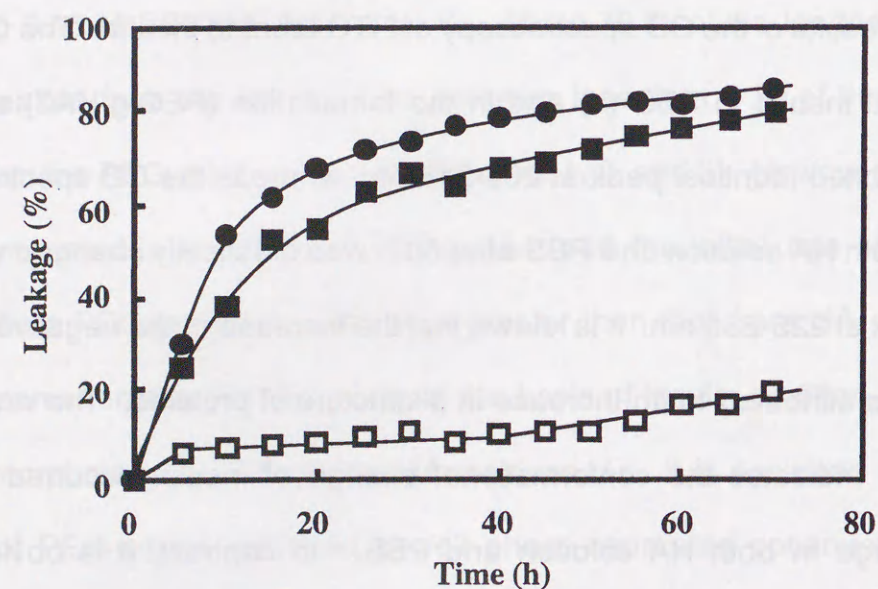


Figure 5-9. Insulin leakage from solutions of HA and PEG-g-HA with different PEG content during storage in 0.15M PBS (pH7.4) at 37°C. ■, HA; □, PEG-g-HA1, PEG-g-HA2 and PEG-g-HA3; ●, PEG-g-HA4.

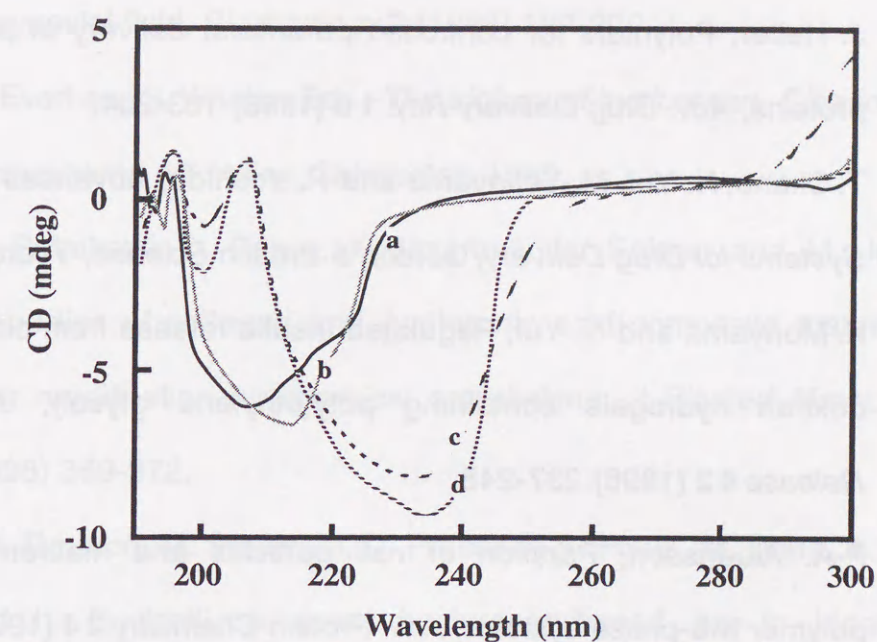


Figure 5-10. CD spectra of FITC-labeled insulin. (a), at initial in PBS; (b), after 60 h in PEG-g-HA2 solution; (c), after 60 h in HA solution; (d), after 60 h in PBS.

5-4: Conclusion

PEG-g-HA solutions consisting of PEG as a drug reservoir and HA as a biodegradable matrix are a feasible way to prevent drug leakage and to achieve degradation-controlled insulin release. Of special interest should be paid for the prevention of conformational change of insulin during 60h-storage in PEG-g-HA solutions. Such a system as a non-diffusive but degradation-controlled drug release in a solution state may be advantageous as injectable therapeutic formulations for intra-articular treatments with antiinflammatory peptide drugs.

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In this dissertation, biodegradable hydrogels with microdomain structures for controlled drug release were designed as advanced drug delivery materials. The chapters 2 and 3 concerned with degradation-controlled peptide release from heterogeneous-structured hydrogels. The chapter 4 concerned with demonstration of pulsatile insulin release from multi-layered hydrogel formulation. The chapters 5 and 6 concerned with drug-encapsulatable solution with microdomain structures.

The chapters from 2 to 6 provided the following findings:

In the chapter 2, biodegradable dextran hydrogels containing poly(ethylene glycol) (PEG) were prepared as a model of heterogeneous-structured devices composed of drug reservoirs and degradable matrices, and insulin release during their enzymatic degradation was investigated. Specific distribution of bioactive peptides has been well known in a combination of PEG and dextran as an aqueous polymer two-phase system. Insulin was preferentially loaded into PEG phase when methacrylic dextran was crosslinked in the presence of PEG and insulin. The degradation of both crosslinked dextran hydrogels containing PEG (PEG/Dex hydrogels) and crosslinked dextran hydrogel (Dex hydrogel) by dextranase were found to proceed from the surface front. However, release profiles of insulin from PEG-Dex hydrogels were different from that of Dex hydrogel: insulin release from PEG/Dex hydrogels was regulated by the surface degradation of PEG/Dex microdomain-structured matrix whereas diffusion was dominant factor in insulin release from Dex hydrogel.

In the chapter 3, biodegradable PEG/Dex hydrogels with different water content were prepared in order to regulate the phase-separated structures, degradability, and drug release profiles. When the water content was lower below 83%, the hydrogels were not degraded in the presence of dextranase. From the results of microscopic and thermal analysis, the water content and crosslink density affected to the phase-separated structures of PEG/Dex hydrogels, i.e., higher crosslink density correlated with exhibiting lower phase separability and forming smaller PEG domains in PEG/Dex hydrogels because of phase mixing between PEG and dextran. The difference in the insulin leakage from the hydrogels is affected by the crosslink density of the hydrogels. However, the mode of insulin release from the hydrogels during their enzymatic degradation was quite different. Insulin release from PEG/Dex hydrogel was found to be degradation-controlled release, whereas the diffusion through the matrix was dominant factor of release from Dex hydrogels.

In the chapter 4, multi-layered hydrogel formulations consisting of PEG-grafted Dex (PEG-g-Dex) and ungrafted Dex were investigated as a model of pulsatile drug release formulation. In these formulations, it is considered that the grafted PEG domains act as a drug reservoir dispersed in the Dex matrix based on aqueous polymer two phase systems. In the formulations, loaded insulin into PEG phase was more stable than the insulin dissolved in other aqueous solutions. It was found from the results of CD spectrum measurement that conformational change of insulin did not

occurred. The formulations exhibited surface-controlled degradation by dextranase, and insulin release was observed in a pulsatile manner because of the multi-layered structure; PEG-g-Dex hydrogel layers containing insulin and insulin-free Dex hydrogel layers. Thus, it is suggested that the multi-layered hydrogel formulations using PEG-g-Dex and Dex are feasible for chronopharmacological drug delivery systems.

In the chapter 5, hyaluronic acids (HA) grafted with PEG (PEG-g-HA) were synthesized. The materials characterization, enzymatic degradability and peptide (insulin) release from solutions of the copolymers were examined. Insulin was preferentially partitioned into the PEG phase in a PEG/HA solution system. Enzymatic degradation of the copolymers was strongly dependent on the PEG content. Thermal analysis revealed that PEG-g-HA exhibited a variation in phase-separated structures depending on the PEG content. The solution of PEG-g-HA enabled insulin to remain in the PEG moieties dispersed in the HA matrix. Leakage of insulin from the copolymers was dependent upon the PEG content. A dramatic increase in leakage rate occurred when the PEG content was increased to greater than 39% by weight. It is considered that the loaded insulin was partitioned into the PEG moieties and became entangled with the PEG chains. The conformational change of insulin was effectively prevented in PEG-g-HA solutions, although insulin was denatured in storage of both phosphate buffered solution and HA solution. Such a heterogeneous-structured

polymeric solution may be advantageous as an injectable therapeutic formulation for ophthalmic or arthritis treatment.

Conclusively, degradation-controlled drug release was achieved by designing biodegradable matrices with microdomain structures in this study. The novel method for making heterogeneous structures such as drug reservoirs and degradable matrices was established by using phase separation method; aqueous polymer two-phase systems. The prepared hydrogels and grafted polymer solutions exhibited the degradation-controlled drug release. Furthermore, it is a quite new finding that the conformational change and denaturation of loaded peptide drug were prevented by using these PEG-containing polysaccharide hydrogels and solutions. If these methods and mathematical analysis for pharmaceutical therapy are combined, extremely useful therapeutic formulations will be realized as advanced drug delivery materials in near future.

The novel method for making heterogeneous structures such as drug carrier and biodegradable matrix was established by using phase separator method. Aqueous copolymer two-phase systems were prepared by mixing PEG and PLGA in water. The prepared hydrogel and dried polymer sponges exhibited the degradation-controlled release. Furthermore, it is a quite new finding that the conformational change and denaturation of loaded protein drugs were governed by using phase separator method. These results indicate that the phase separator method is a useful method for preparing drug carrier and biodegradable matrix. The prepared hydrogel and dried polymer sponges exhibited the degradation-controlled release. Furthermore, it is a quite new finding that the conformational change and denaturation of loaded protein drugs were governed by using phase separator method. These results indicate that the phase separator method is a useful method for preparing drug carrier and biodegradable matrix.

List of Publications

1. K. Miyazawa, T. Ogasawara, and N. Yui, *Biodegradable matrix for drug delivery*, *Journal of Biomedical Materials Research*, **42**, 117-124 (1998).
2. K. Miyazawa, T. Ogasawara, and N. Yui, *Biodegradable matrix for drug delivery*, *Journal of Biomedical Materials Research*, **42**, 117-124 (1998).
3. K. Miyazawa, T. Ogasawara, and N. Yui, *Biodegradable matrix for drug delivery*, *Journal of Biomedical Materials Research*, **42**, 117-124 (1998).
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10. K. Miyazawa, T. Ogasawara, and N. Yui, *Biodegradable matrix for drug delivery*, *Journal of Biomedical Materials Research*, **42**, 117-124 (1998).

List of Publications

Original articles

- K. Moriyama and N. Yui, Regulated insulin release from biodegradable dextran hydrogels containing poly(ethylene glycol), *J. Controlled Release* **42**, 237-248 (1996).
- K. Moriyama and N. Yui, Regulated peptide delivery from biodegradable polysaccharides containing poly(ethylene glycol) microdomains in: T. Akaike, T. Okano, M. Akashi, M. Terano and N. Yui, Eds., *Advances in Polymeric Biomaterials Science*, pp. 523-536, CMC, Tokyo, 1997.
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- K. Moriyama, T. Ooya and N. Yui, Degradation-controlled peptide release from PEG/dextran hydrogels with different phase structures, to be submitted.

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動的粘弾性および光散乱測定によるミクロドメイン
構造化多糖ヒドロゲルの構造解析

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論文の内容の要旨

本研究では、ミクロドメイン構造を有する生体内分解性薬物放出ヒドロゲルの内部構造を、動的粘弾性測定および光散乱測定などのレオロジー的手法を用いて解析し、薬物送達システムに適用する材料の力学物性と内部構造の関係を明らかにすることを目的とした。測定したヒドロゲルは、架橋 HA マトリックス中に異種成分として PEG が存在し、これまでの検討によって一定濃度以上において両者は相分離することが確認されている。架橋ヒドロゲルの乾燥試料を用いて粘弾性測定を行なった結果、上記のようにミクロドメインを形成することを特徴を有する 2 相系ヒドロゲルの所定力場での粘弾性挙動から、PEG と HA 鎖の振る舞いが全体的物性と如何なる関わり合いを有しているか推察することを試みた。その結果、温度上昇によってゲル全体の結晶性が増大し、含有している PEG の融点以上ではその傾向が顕著となることが示唆された。この現象を熱分析や一定温度下での粘弾性の周波数依存性などから推測した結果、PEG の融点以下では架橋 HA マトリックス中に PEG 鎖が束縛されているが、融点以上では PEG 鎖の融解に伴い、HA 鎖との反発および PEG 同士の親和性によって架橋 HA マトリックス中で凝集し、両成分の結晶性が上昇するために、ヒドロゲル全体としての結晶性も上昇しているものと考えられた。



LEMONS, LEMONADE, AND LEMONADE

LEMONS