Development of Novel Drug Delivery System Using Silicone

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List of Abbreviations

Alum: aluminum hydroxide gel

Avi: avidin

BHT: 2,6-Di-*t*-butyl-*p*-cresol

BSA: bovine serum albumin

CA: citric acid

CLSM: confocal laser scanning microscopy

Gly: glycine

HSA: human serum albumin

IFN: natural α-type interferon

Man: mannitol

PBS: phosphate buffered saline

PLGA: poly (lactic/glycolic acid)

PSTB: polystyrene beads

SA: sodium bicarbonate

SC: sodium citrate

TP: DL-α-tocopherol

VD_3: 1α, 25-$(OH)_2$ vitamin D_3

General Introduction

Aiming at improving drug efficacy, minimizing side effects and promoting patient compliance, extensive studies on drug delivery system (DDS) have been conducted. The studies are generally classified into three categories, that is, 1) controlled release system, the purpose of which is to keep appropriate drug concentration in a body, 2) pro-drug, the purpose of which is to improve pharmacokinetics by modification of chemical structure of the drug, and 3) targeting, the purpose of which is to concentrate drug at diseased site. Among these, the concept of controlled release systems occurred in early period, and various methods have been reported, for example applications of polymer membrane [1], osmotic pressure [2], and external stimulation such as magnetism or glucose [3, 4].

In order to develop a commercially practical DDS, to effectuate desirable drug release profile is not sufficient. A lot of other subjects, such as studies on biocompatibility, sterility method, and industrial manufacturing condition should be thoroughly investigated. Because of the burdens, kinds of materials applicable to DDS carrier are limited to only a few. Among those, silicone and poly-(lactic/glycolic acid) (PLGA) are extensively used. Features of these polymers are indicated as follows.

PLGA is hydrolyzed in a body and therefore has been utilized as a material for suture and so on. Bioerosion rate is controllable by changing copolymer composition and/or molecular weight [5, 6]. PLGA is easily fabricated to microspheres. However, because of its large surface areas, its administration accompanies significant initial burst. Lupron

Depot® [6] is an example of DDS which utilizes PLGA as a carrier material. Lupron Depot® releases leuprorelin acetate continuously for one or three months after significant initial burst. This release profile attributes to immediate downregulation of hormone receptors and to maintain the chemical castration state, which is effective for the treatment of prostate cancer. PLGA microspheres can be used as carriers of drugs with such specific pharmacological effects and those with a wide therapeutic index (e.g., growth hormone [7]). However, the number of such drugs is limited.

Silicone is biologically inert, not biodegraded and mechanically flexible. These features make silicone to be extensively utilized for many biomedical applications, including indwelling catheter and prosthesis, which are deposited in a body for long period. Norplant® [8] and Compudose® [9] are examples of DDS which has been developed using silicone as a drug carrier. So far, silicone has been used for controlled release system of lipophilic and low molecular weight drugs. On the other hand, since water-soluble, high molecular weight drugs such as proteins are neither soluble nor diffusible in silicone polymer networks, silicone was not considered to be suitable for the delivery of protein drugs [10, 11]. Namely, silicone could not be an effective drug carrier material, depending on physicochemical properties of the drug.

This study has been performed with the purpose to develop a novel and commercially

practical DDS using silicone as a carrier material, which can be applied for various kinds of drugs. Especially, the application of silicone as a carrier material for protein drugs was investigated in detail.

In part I of this thesis, techniques to control release of protein drugs from silicone formulation were investigated. Chapter 1 concerns a matrix formulation in which protein drug powder is uniformly distributed in silicone. Matrix formulation released interferon (IFN), which was an example of protein drugs, over long period of time *in vitro*. Chapter 2 deals with a covered-rod formulation, which was developed in order to improve drug release profiles of a matrix formulation. Covered-rod formulation released human serum albumin (HSA) or IFN at a constant rate for 30-100 days *in vitro* without significant initial burst.

In part II of this thesis, efficacy of silicone formulation was investigated. Chapter 3 deals with a silicone formulation as a novel vaccine delivery vehicle. Covered-rod formulation, which released avidin (Avi) as an antigen model over a long period, induced antibody production responses that were more strongly enhanced than the conventional alum-adjuvanted group.

In part III of this thesis, extension of the technique in controlled release of silicone

formulation was investigated. Chapter 4 concerns controlled release of lipophilic drug which is highly potent in low concentrations. 1α, 25-$(OH)_2$ vitamin D_3 (VD_3) was used as an example of such lipophilic drugs. Addition of HSA and devices on formulation type and material were effective to suppress initial burst and to modify drug release. Chapter 5 deals with controlled release of insoluble drugs or simultaneous release of two kinds of water-soluble drugs. Polystyrene beads (PSTB) which was a model of insoluble drugs was released from silicone formulation when citric acid (CA) and sodium bicarbonate (SB) were added as additives. Release pattern of two kinds of water-soluble drugs was independently controllable by using a double-layered formulation.

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Part I

Design of Silicone Formulation

and

Controlled Release of Protein Drugs

Chapter 1

Controlled Release of Protein Drugs Using Matrix Formulation

1. Introduction

Recent advances in genetic engineering technologies have led to the discovery and development of novel therapeutic protein and peptide drugs. Since protein and peptide drugs are chemically and biologically unstable and have extremely short half-lives *in vivo*, many studies to develop a drug delivery system (DDS) for protein drugs have been performed in order to improve their therapeutic effects. Especially, DDSs using biodegradable polylactic acid or poly(lactic/glycolic acid) (PLGA) as a carrier have recently been extensively researched [1-5]. For example, Lupron Depot® [1-3], which consists of injectable microspheres of leuprorelin acetate, was developed using PLGA and is currently used for the treatment of prostate cancer. However, the number of protein drugs to which this technology can be applied is limited, since PLGA microspheres usually accompany significant initial burst. The initial burst is, in general, not desirable from the standpoint of safety. In addition, the need to use organic solvents, which is indispensable in the manufacturing process of PLGA microspheres, is another drawback because of the poor stability of protein drugs.

To develop sustained-release formulations that can be applied to a wide range of protein drugs, silicone, a non-biodegradable polymer was focused on. Since silicone has a long history as a material used for implants, its safety has been carefully investigated. Silicone is already in use as a carrier in DDSs for lipophilic drugs [6-8]. However, since protein drugs do not diffuse through most polymer chains, it was at first believed to be unsuitable for the delivery of protein drugs [9, 10]. Hsieh et al. reported that proteins such as bovine serum albumin (BSA) and chymotrypsin were released from silicone *in vitro* [11], when incorporated in concentrations as high as 30-50% (w/w) in the formulations. Incorporation at such high contents may be practicable for proteins such as BSA or chymotrypsin, however, it is not practical from the point of view of safety and economy to apply this method to protein drugs such as cytokines and growth factors that exert potent physiological activities in trace quantities [12].

In this chapter, a new technique, by which protein drugs effective in small doses are released from silicone over a long period, was reported. Interferon (IFN) was used as an example of a cytokine, and IFN release profiles from silicone formulations *in vitro* and *in vivo* were investigated.

2. Experimental

2.1. Materials

Silicone elastomers (Silastic® MDX4-4210 and Silastic® Q7-4750) were supplied by Dow Corning Corporation (MI, USA). The MDX4-4210 is cross-linked by hydrosilylation when a base and a curing agent are mixed in a mass ratio of 10:1. The base consists mainly of dimethylvinylsiloxy-terminated polydimethylsiloxane (Fig. 1), and the curing agent consists of a platinum catalyst and a cross-linker. Product Information from Dow Corning Corporation indicates that MDX4-4210 is well cured by keeping at 25 °C for 24 hours. Q7-4750 is cross-linked by hydrosilylation when Part A and Part B are mixed in equal portions. Part A consists of dimethylvinylsiloxy-terminated poly(dimethylsiloxane-co-methylvinylsiloxane) (Fig. 2) and a platinum catalyst. Part B consists of the same siloxane as Part A and a cross-linker. The cross-linking density of Q7-4735, which is a product of the same series as Q7-4750, has been reported to reach a plateau value by treatment at 25 °C for 3 days [13], and Product Information from Dow Corning Corporation indicates Q7-4735 and Q7-4750 cure at almost the same rates. The tensile strength of MDX4-4210 and Q7-4750 is higher than 550 and 1250 psi, respectively. Human lymphoblastoid interferon, a natural alpha-type interferon (IFN) (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan) was used. Human serum albumin (HSA) (Buminate® 25%, Baxter Healthcare Corporation, IL, USA) was used to control IFN release

from silicone. Glycine (Gly, Nacalai Tesque Inc., Kyoto, Japan) was used as an additive.

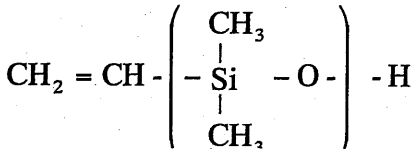

Fig. 1. Structure of siloxane (MDX4-4210)

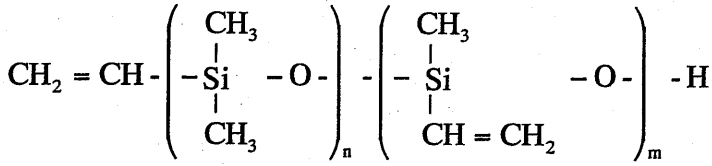

Fig. 2. Structure of siloxane (Q7-4750)

2.2. Preparation of IFN silicone formulations

The formulations used in this study are shown in Table 1. Typically an IFN silicone formulation (A-1) was prepared as follows. Aqueous solutions of IFN (56×10^6 IU/ml; 209.5 ml) and HSA (25%; 55.9 ml) were mixed and lyophilized. The lyophilized cake was sieved through a mesh to obtain 53-150 μm IFN/HSA powder (0.69×10^6 IU/mg). MDX4-4210 10.5 g was mixed with a curing agent (1.05 g) and IFN/HSA powder (4.98 g; IFN/HSA powder content was 30%(w/w)). After deforming in a vacuum chamber, the mixture was filled into a polytetrafluoroethylene tube (inner diameter: 5 mmϕ) and cured at room temperature for three days. After curing, the IFN silicone rod was removed from the tube and cut into 1cm-long pieces and stored at 5°C. All of the samples except D-1, D-2, D-3 and D-4 were prepared in the same manner. However, the mixing ratio of the IFN and

Table 1

Compositions of IFN silicone formulations

	IFN (IU)	Content (%)		Particle size	Type of
		IFN/HSA powder	Gly	of powder (μm)	Silicone
A-1	0.49×10^6	30	0	53-150	MDX4-4210
A-2	5.5×10^6	30	0	53-150	MDX4-4210
A-3	47×10^6	30	0	53-150	MDX4-4210
B-1	4.2×10^6	5	0	<300	MDX4-4210
B-2	4.2×10^6	15	0	<300	MDX4-4210
B-3	4.7×10^6	30	0	<300	MDX4-4210
C-1	44×10^6	30	0	<53	MDX4-4210
C-2	42×10^6	30	0	53-150	MDX4-4210
C-3	41×10^6	30	0	106-212	MDX4-4210
D-1	6.1×10^6	30	0	<2	Q7-4750
D-2	6.8×10^6	30	2	<2	Q7-4750
D-3	6.4×10^6	30	5	<2	Q7-4750
D-4	5.9×10^6	30	10	<2	Q7-4750
E-1	0.43×10^6	30	0	<300	MDX4-4210
E-2	4.7×10^6	30	0	<300	MDX4-4210

HSA solutions, or that of powder and silicone was changed according to the composition.

IFN silicone formulation D-2 was prepared as follows. Aqueous solutions of IFN (106×10^6 IU/ml; 126 ml) and HSA (10%; 14 ml) were mixed. Gly (0.23 g) was dissolved in the IFN/HSA solution (35 ml) and the mixture was spray-dried using a spray dryer (Mini-spray DL-21, Yamato Scientific Co., Ltd., Tokyo, Japan). Observation by scanning electron microscopy revealed the size of the spray-dried powder to be <2 μm. Q7-4750 Part A (2.1 g) and Part B (2.1 g) were mixed. The percentage of spray-dried powder (1.8 g) mixed with silicone (4.2 g) was 30%(w/w). The mixture was fed into an extruder, extruded through a 1.6 mm die, and cured at room temperature for three days. After curing, the IFN silicone rod was cut into 1cm-long pieces and stored at 5°C. Silicone formulations D-1, D-3 and D-4 were prepared in the same manner, except that mixing ratios of IFN, HSA and Gly were changed according to the composition.

Reproducibility of tensile-strength measurement of silicone formulations was sufficiently high.

2.3. Measurement of IFN content in silicone formulations

An IFN silicone formulation was placed in a centrifugal mill, ZM-1 (NIHON SEIKI SEISAKUSYO Co., Tokyo, Japan), with liquid nitrogen. After the formulation was completely frozen, it was centrifuged inside a 500-μm-mesh screen at 10,000rpm for 1 minute,

and the pulverized particles were collected. The particles were then immersed in 10 ml of phosphate-buffered saline (PBS; pH 7.4), 0.5% HSA and 0.01% sodium azide. The solution was assayed for IFN concentration by radioimmunoassay (RIA), using an IFN-α kit (Dinabot Co., Ltd., Tokyo, Japan). RIA was performed in doublet.

2.4. *In vitro* release study

An IFN silicone formulation was placed in a tube containing 10 ml of PBS (pH 7.4), 0.5% HSA and 0.01% sodium azide. The tube was placed in an incubator at 5°C. At designated times, an aliquot was collected and transferred to a new tube containing fresh buffer. The solution was assayed for IFN concentration by RIA, using an IFN-α kit. RIA was performed in doublet. IFN is stable in the buffer during the sampling intervals.

2.5. Release rate analysis

Release rate was analyzed from the *in vitro* release profile for the first five days. It was calculated from linear regression analysis [14].

2.6. Measurement of osmotic pressure

IFN/HSA or IFN/HSA/Gly powder (200 mg) was dissolved in 2 ml of PBS (pH 7.4), 0.5% HSA and 0.01% sodium azide. The osmotic pressure of the solution was measured

three times by an auto-osmometer (Osmostat OM-6020, Daiichi Kagaku Inc., Kyoto, Japan) for each sample.

2.7. Confocal laser scanning microscope (CLSM) analysis

Silicone formulations were prepared with Q7-4750 and HSA powder (powder size; <20 μm, HSA content; 30%(w/w)). The HSA silicone formulations were placed in tubes containing 3 ml of PBS (pH 7.4) and 0.01% sodium azide. The tubes were placed in an incubator at 5°C for 12 days. The initial and *in vitro* tested formulations were cut in the center and observed under a CSLM (LSM-GB200, Olympus Optical Co., Ltd., Tokyo, Japan). HSA possesses inherent fluorescence, while silicone does not. Therefore, the HSA powders distributed in silicone could be detected against the black background of silicone.

2.8. Experimental animals

Female athymic nude mice (nu/nu, BALB/c background) were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed in a negative-pressure safety rack under specific pathogen-free conditions.

2.9. Measurement of serum IFN levels

The IFN silicone formulations (A-1, A-2 and A-3) were administered subcutaneously

into 5 week-old-mice. Then, blood samples were collected at preset time points: 0.5, 1, 3, 10, 30, 60 and 90 days. Sera were collected and stored at -40°C until the IFN assay. The serum IFN levels were measured by RIA, using an IFN-α kit.

2.10. Pharmacokinetic analysis

The maximum concentration (C_{max}) and its time of occurrence (T_{max}) were read directly from the serum concentration-time curve. The apparent half-life ($T_{1/2}$) was calculated using a one-compartment model as follows. The elimination rate (K_e) was calculated by the least squares method from the linear terminal phase of the serum concentration-time curve and used to calculate $T_{1/2}$ from the following relationship: $T_{1/2}=\ln 2/ K_e$.

2.11. Tumor transplantation

A mass of RCC-1 tumor was excised and cut into about 2 mm×2 mm pieces in Hanks' solution. Then, a fragment was implanted subcutaneously with a trocar needle at a ventral site in a nude mouse.

2.12. Antitumor therapy

When the tumor nodules grew to 5-8 mm in diameter, nude mice were separated randomly into groups of six mice, and IFN therapy was started. IFN solutions were injected

subcutaneously into nude mice at a dose of 10^3 IU, 10^4 IU or 10^5 IU/mouse/day at sites distant from the tumors for 21 consecutive days. The IFN silicone formulation A-1, A-2 or A-3 was administered subcutaneously at sites distant from the tumor. Control groups were given placebo silicone formulations in the same way or left untreated.

2.13. Measurement of tumor growth

The length and width of the tumors were measured with a sliding caliper, and the estimated tumor volume (V) was expressed as $V = 1/2 \times \text{length(mm)} \times [\text{width(mm)}]^2$. Statistical analysis was carried out by Mann-Whitney's U-test.

3. Results

3.1. IFN silicone formulations

Aqueous solutions of IFN and HSA were mixed at predetermined volumes and lyophilized or spray-dried to obtain IFN/HSA powders with specific IFN concentrations. Particle size could be controlled by the sieve mesh size or the spray-drying conditions. Then, predetermined amounts of IFN/HSA powders and silicone were mixed homogeneously and cured at room temperature to obtain cylindrical silicone formulations. Silicone formulations could be easily fabricated, regardless of the kind of silicone used. Figure 3 shows an example of an IFN silicone formulation, and Table 1 lists the composition of various IFN

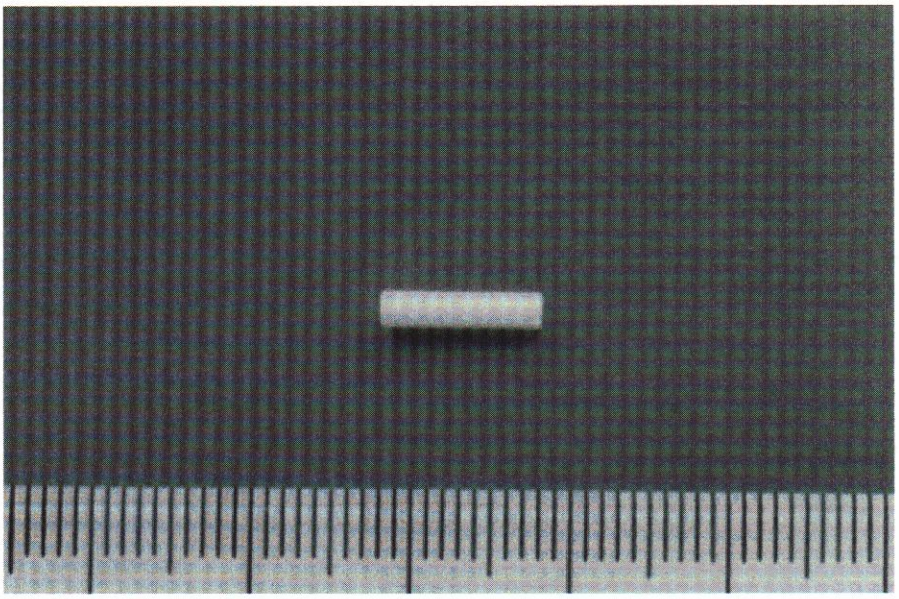

Fig. 3. Photograph of a silicone formulation.

silicone formulations.

In order to evaluate the stability of IFN during the preparation of the silicone formulation, the amounts of IFN recovered from the IFN silicone formulations were determined. The IFN silicone formulations (A-1, A-2 and A-3) were pulverized in a grinder. The pieces were immersed in a buffer, and the resultant solution was assayed for IFN concentration by RIA (Table 2). The recovery of IFN from the IFN silicone formulations was 94% or higher for A-1, A-2 and A-3, indicating that IFN remained chemically stable during the preparation of the silicone formulations.

Table 2

Recovery of IFN from IFN silicone formulations[a]

Sample	IFN content	
	Theoretical value (IU)	Recovery (%)
A-1	0.49×10^6	100±6
A-2	5.5×10^6	94±7
A-3	47×10^6	110±19

a Results are expressed as the mean ± S.D. if three independent samples.

3.2. Control of IFN release

3.2.1. Effect of the amount of IFN/HSA powder loaded

IFN silicone formulations with different amounts of IFN/HSA powder loaded, i.e., B-1 (5%), B-2 (15%) and B-3 (30%), were immersed in a buffer for assay of IFN release. Figure 4 shows the results.

IFN was continuously released from the IFN silicone formulations over a period of one month. The total amount of IFN released over one month in the case of B-1 or B-2 was as low as 5% or less; however, that in the case of B-3 (IFN/HSA content: 30%) was 42%.

3.2.2. Effect of IFN/HSA particle size

IFN silicone formulations that contained IFN/HSA powders of different particle sizes, i.e., C-1 (<53 mm), C-2 (53<<150 mm) and C-3 (106<<212 mm), were immersed in a buffer for assay of IFN release. Figure 5 shows the results.

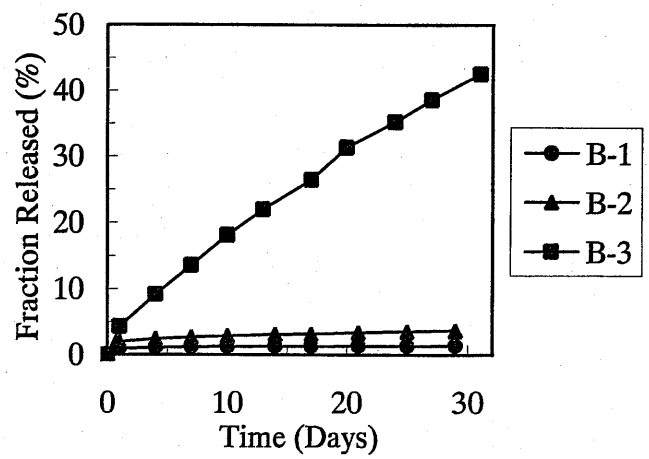

Fig. 4. IFN release profiles for IFN silicone formulations containing different amounts of IFN/HSA powder.

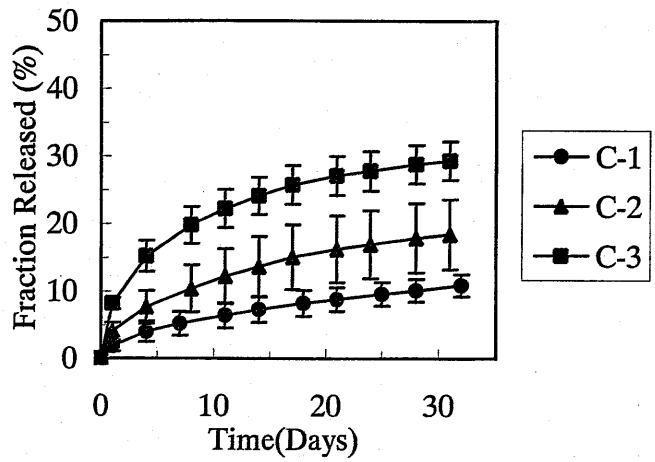

Fig. 5. IFN release profiles for IFN silicone formulations containing IFN/HSA powders of different particle sizes. Each point represents the mean ± S.D. of data from two independent samples.

The IFN release rate increased with increase in particle size of the IFN/HSA powder. The total amount of IFN released over one month in the case of C-1 (smallest particle size) was approximately 10%, whereas that in the case of C-3 (largest particle size) was approximately 27%. Within the range of particle sizes considered in this study, the IFN release rate could be controlled to approximately 3 times.

3.2.3. Effect of different concentrations of Gly

IFN silicone formulations that contained different amounts of Gly, i.e., D-1 (0%), D-2 (2%), D-3 (5%) and D-4 (10%), were prepared by using Q7-4750 silicone. Although chemical structure and tensile strength were different between Q7-4750 and MDX4-4210 that was used to prepare other silicone formulations, IFN release patterns were similar between them. Figure 6 shows the results. The IFN release rate increased with increase in the amount of Gly added. Table 3 summarizes the initial rates of IFN release, calculated from linear regression analysis of Fig. 6. Table 4 summarizes the osmotic pressures of the buffers in which IFN/HSA or IFN/HSA/Gly powders, used to prepare D-1-D-4, were dissolved.

The osmotic pressure of the buffer in which IFN/HSA powder was dissolved was the same as that of the native buffer; the osmotic pressure of the buffer increased as the amount of Gly contained increased.

Figure 7 shows the relationship between IFN release rates for the first five days of the *in vitro* study and the osmotic pressures (the difference in the osmotic pressures between the

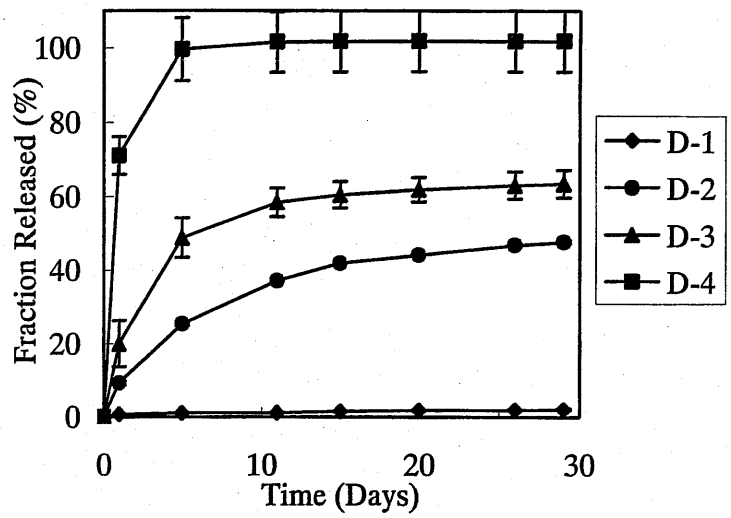

Fig. 6. IFN release profiles for IFN silicone formulations containing different amounts of Gly. Each point represents the mean ± S.D. of data from two independent samples.

Table 3

IFN release rates from IFN silicone formulations containing different amounts of Gly[a]

Sample	Release Rate (%/day)
D-1	0.2±0.0
D-2	4.8±0.0
D-3	9.0±0.7
D-4	16.3±1.5

a Results are expressed as the mean ± S.D. of two independent samples.

Table 4

Osmotic pressure of solution with dissolved IFN/HSA(/Gly) powders[a]

	Osmotic pressure (mOsm)	Difference between buffer (mOsm)
Buffer	282±1	-
D-1	283±0	1±1
D-2	352±0	70±1
D-3	458±1	176±1
D-4	591±0	309±1

a Results are expressed as the mean ± S.D. of three independent samples.

buffers in which different concentrations of Gly were dissolved and that of the native buffer). The IFN release rate increased with increase in the osmotic pressure.

The osmotic pressure of the buffer in which IFN/HSA powder was dissolved was the same as that of the native buffer; the osmotic pressure of the buffer increased as the amount of Gly contained increased.

3.2.4. Effect of IFN concentration

IFN silicone formulations with different IFN contents were examined, i.e., E-1 (0.43×10^6 IU/formulation) and E-2 (4.7×10^6 IU/formulation), were immersed in a buffer for assay of IFN release. Figure 8 shows the results. The release profiles for E-1 and E-2 were nearly the same when the content and particle size of the IFN/HSA powders were the same, regardless of the difference in IFN content between the two (the content of IFN in E-2 was 10 times that in E-1).

3.3. Mechanism of IFN release

The changes in distribution of the HSA powder within the silicone formulations with time were analyzed by CLSM. Figure 9 shows the crosssectional micrographs.

Before immersion of the formulations in the buffer, HSA powders were homogeneously distributed throughout the silicone (Figs. 9(a), 9(b)). After immersion for 12 days, the amount of HSA powder decreased and the particles became spherical, indicating that the

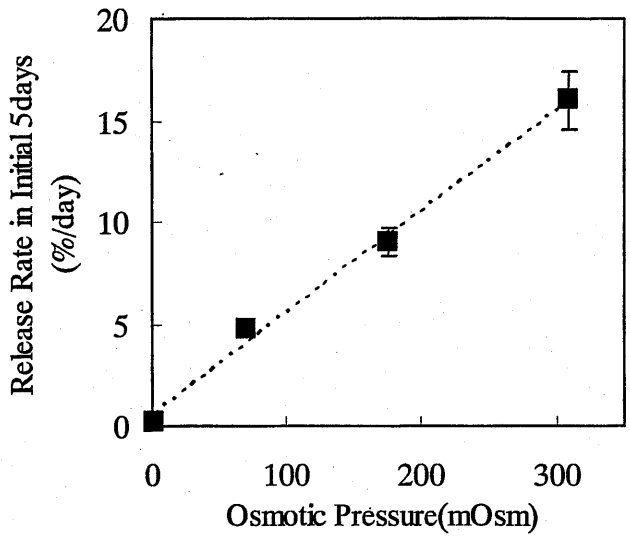

Fig. 7. Correlation between IFN release rates and osmotic pressure.

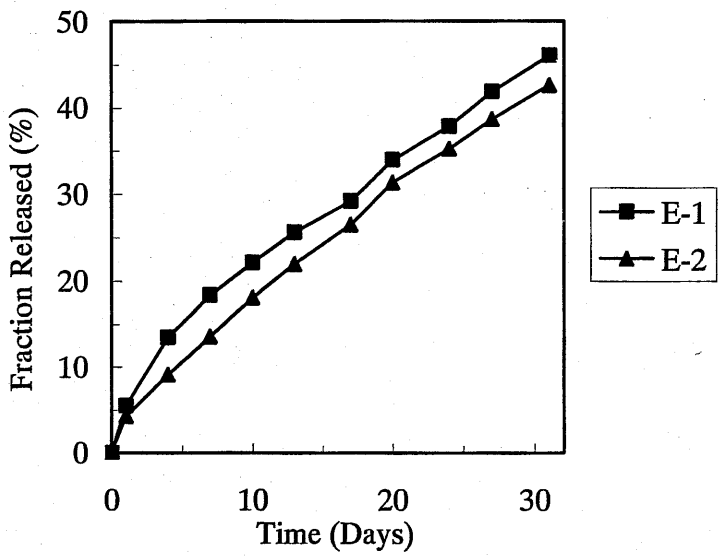

Fig. 8. IFN release profiles for IFN silicone formulations containing different amounts of IFN.

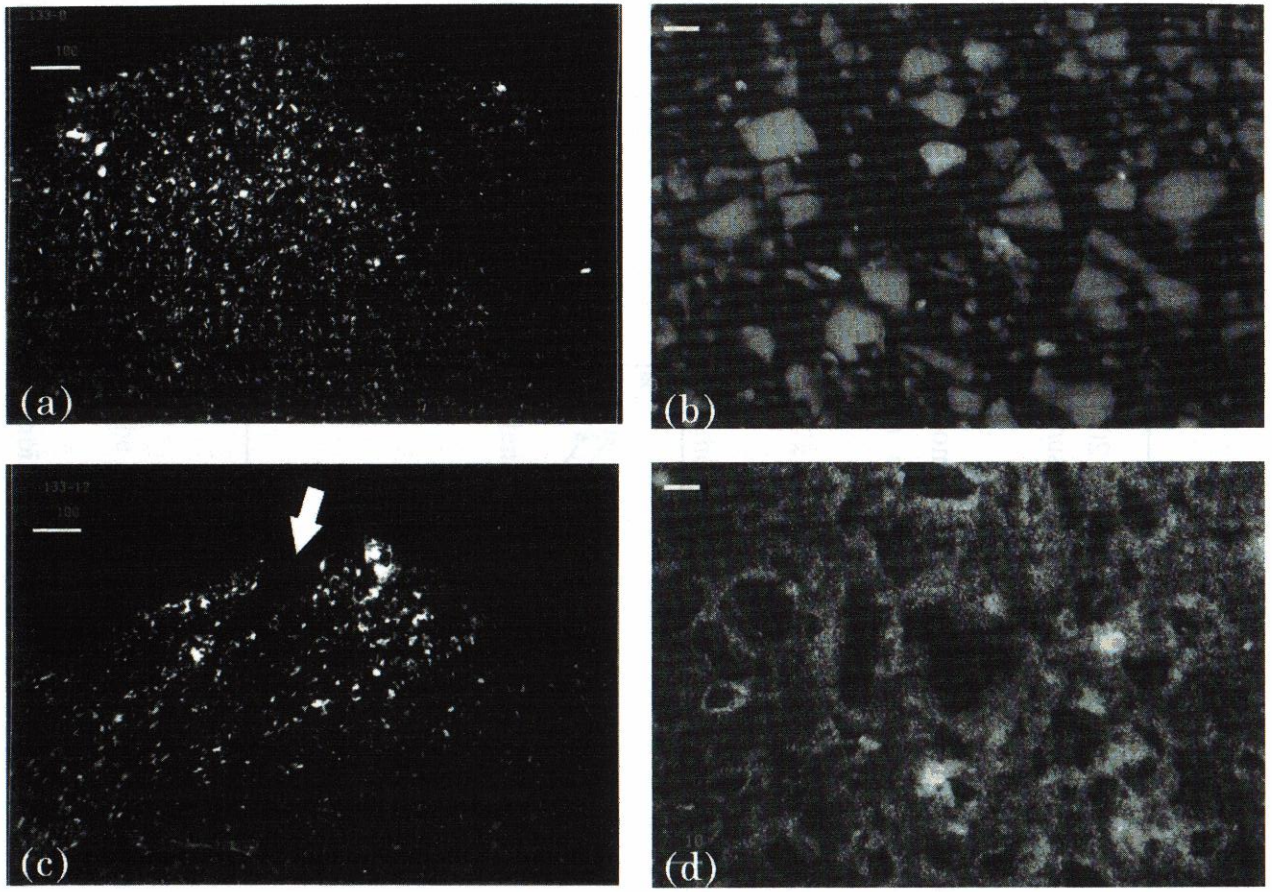

Fig. 9. Time course of CLSM images of the silicone formulations. (a) Before release test, scale bar = 100 μm. (b) Before release test, scale bar = 10 μm. (c) Incubated for 12 days in vitro, scale bar = 100 μm. (d) Incubated for 12 days in vitro, scale bar = 10 μm.

powders were dissolved in the buffer and released. As shown by the arrow in the figure, a channel, a continuous black path, was recognized in the formulation (Fig. 9(c)). Magnified images around the channel revealed several white spots showing HSA particles dissolved in the buffer, as well as many holes where the HSA particles had dissolved and been released from (Fig. 9(d)).

3.4. Change in serum IFN concentration

The IFN silicone formulations (A-1, A-2 and A-3) were administered subcutaneously into the back of nude mice. Blood samples were collected from the nude mice at predetermined time points after the administration, and the serum IFN concentrations were measured. Figure 10 and Table 5 show the time-courses of changes in the serum IFN concentrations and pharmacokinetic parameters, respectively.

In the group of mice in which A-1 (0.49×10^6 IU) was administered, the maximum concentration (C_{max}) of IFN was observed at the initial sampling point, i.e., 12 h after administration, and IFN remained detectable for 10 days, but could no longer be detected at the next sampling point (30 days). In the group in which A-2 (5.5×10^6 IU) was administered, the serum IFN concentrations at 12 h and 24 h were almost the same, however, C_{max} was observed at 24 h after administration. IFN was still detectable in

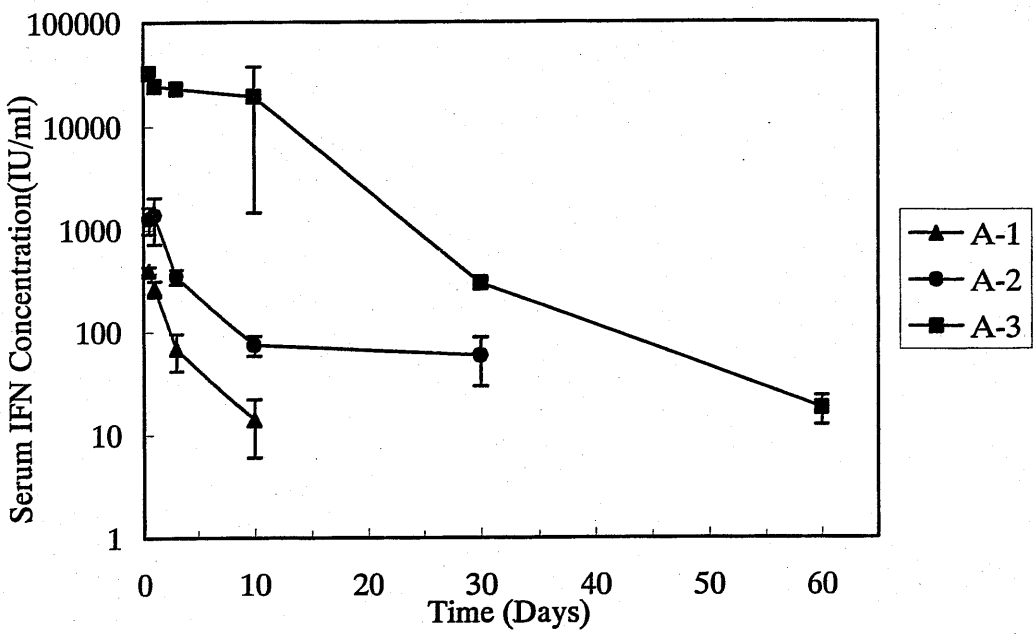

Fig. 10. The time course of serum IFN levels in nude mice after administration of IFN silicone formulations. Each point represents the mean ± S.D. if data from three nude mice.

Table 5

Pharmacokinetic parameters[a]

Parameter		Solution[21]	A-1	A-2	A-3
Dose	(IU)	10^5	0.49×10^6	5.5×10^6	47×10^6
T_{max}	(h)	1	12	24	12
C_{max}	(IU/ml)	4390	400 ± 33	1379 ± 657	32139 ± 4753
$T_{1/2}$	(h)	4	50	190	127

a C_{max} are expressed as the mean ± S.D. of three nude mice.

the serum at 30 days after the administration, but not at 60 days. In the group in which A-3 ($47x10^6$ IU) was administered, C_{max} was observed at the initial sampling point, i.e., 12 h after administration. IFN was still detectable in the serum at 60 days, but not at 90 days. The half-lives of A-1, A-2 and A-3 were 50, 190 and 127 h, respectively; the half-lives of the drug were 12-42 times longer than those in mice that had been administered an aqueous solution of IFN.

3.5. Antitumor activity

Proliferation of tumor cells was suppressed in mice treated with IFN silicone formulations, compared to those treated with placebo or left untreated as shown in Figure 11. The antitumor activity of A-1 ($0.49x10^6$ IU) was similar to that of an aqueous solution of IFN which was injected into mice at a dose of 10^4 IU/day for 21 consecutive days; the tumor volume in the mice was significantly decreased (<0.05), compared to that in the untreated group at 37 days after tumor transplantation (28 days after IFN administration). The antitumor activity of A-2 ($5.5x10^6$ IU) was similar to that of an aqueous solution of IFN which was injected into mice at a dose of 10^5 IU/day for 21 consecutive days; the tumor volume in the mice was significantly decreased (<0.05) compared to that in the untreated group at every sampling point except the first

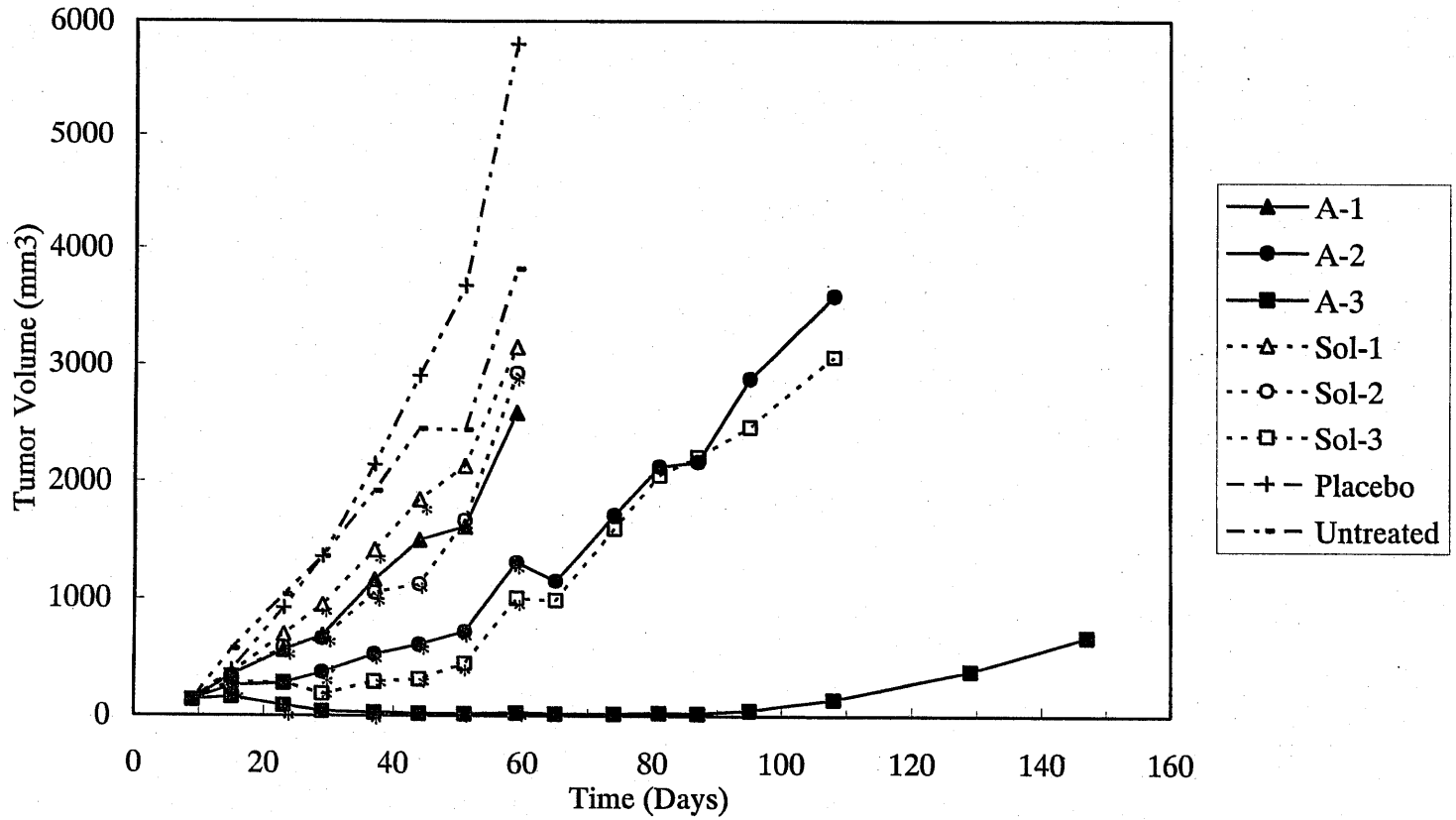

Fig. 11. Anti-tumor effect of the IFN silicone formulations in nude mice. IFN solutions were administered for 21 consecutive days at daily doses of 10^3 IU (Sol-1), 10^4 IU (Sol-2), and 10^5 IU (Sol-3). For control, placebo and untreated groups were plotted. Each point represents the mean ± S.D. of data from six nude mice. *P<0.05.

(on the day of IFN administration). In the mice treated with A-3 ($47x10^6$ IU), the tumor volume started to decrease immediately after IFN administration. The tumors in 5 out of 6 mice were not visible to the naked eye, and remained invisible to the naked eye until 95 days after tumor transplantation (89 days after IFN administration). Similar to the mice treated with A-2, the tumor volume was significantly decreased (<0.05) in mice treated with A-3 compared to that in the untreated group at every sampling point except the first (on the day of IFN administration).

4. Discussion

In this study, a new technique has been developed, by which protein drugs effective at very low concentrations are released from silicone. IFN, which exhibits strong physiological activity in the order of micrograms, was used as the model protein drug in this study. IFN (molecular weight: 17,000-23,000) has various biological effects, such as antiviral, antitumor and immunomodulatory activities; it is currently used widely to treat viral infections and malignant diseases [15-17]. When IFN is used for treatment, a dose of $3\text{-}6x10^6$ IU is generally administered every day or every two days, depending on the type and severity of the disease. If the total dose of IFN ($90\text{-}180x10^6$ IU: 1 mg or less in weight), to be administered over one month period to one patient, is mixed with silicone, the percentage of IFN in the

silicone formulation is still extremely low; IFN would be trapped in the silicone and can not be released easily. In order for IFN to be released from the silicone formulation, a water-soluble substance that promotes the penetration of water into silicone is considered to be necessary. IFN would then be expected to dissolve and be released along with the dissolution and release of the water-soluble substance. HSA was selected as the water-soluble substance in this study, since it has a stabilizing effect on various protein drugs including IFN. IFN silicone formulations were prepared (Fig. 3), in which IFN is homogeneously dispersed, by preparing IFN/HSA powders that contained a small amount of IFN.

In general, protein drugs including IFN are easily denatured and deactivated by heating and use of organic solvents. Since silicone formulations can be prepared under mild conditions without heating or using organic solvents, the silicone formulation is expected to be applicable to a large variety of protein drugs. In fact, recovery of IFN from the IFN silicone formulation was 94% or higher in the case of A-1 to A-3 (Table 2), indicating that IFN was not denatured to any significant extent during the preparation of the IFN silicone formulation.

Then, with the objective of evaluating the release profiles of protein drugs, fifteen kinds of IFN silicone formulations were prepared and investigated *in vitro* and *in vivo*. When IFN silicone formulations were immersed in a buffer, IFN was released continuously. IFN

dissolution and release are considered to occur along with HSA dissolution and release. This is suggested by the finding that IFN release rates were the same for IFN silicone formulations that contained IFN amounts varying up to 10-fold but the same amount of IFN/HSA powder (Fig. 8). In addition, it was found that the IFN release rate depends on the IFN/HSA powder content, the particle size and the amount of Gly added. These results are analyzed here, considering the mechanism by which the water-soluble substance is released from the silicone formulation.

In regard to the IFN/HSA powder content, the following results were obtained. IFN silicone formulations that contained either 5% (B-1) or 15% (B-2) IFN/HSA powders showed a 1-2% IFN release rate on the first day, and an extremely low release rate thereafter, while IFN silicone formulation that contained 30% IFN/HSA powder (B-3) showed continuous IFN release and the IFN release rate was accelerated compared to that of B-1 and B-2 (Fig. 4). The reason for the initial release observed in the cases of B-1 and B-2 may be that IFN/HSA powders existing on the surface of the formulations were dissolved and released into the buffer instantaneously. However, since the content of IFN/HSA powders was low and particles were isolated from each other in the formulations, interconnecting channels may not have been formed among the particles, leading to the extremely low release rate. On the other hand, in the case of B-3, since the content of IFN/HSA powder in the formulation was sufficiently high, interconnecting channels were formed among the particles, leading to

continuous release of IFN. The IFN release rate increased as the IFN/HSA particle size increased (Fig. 5). This could be because it takes more time for interconnecting channels to be formed as the particle size decreases. When Gly was added to the IFN/HSA powder, the IFN release rate was increased (Fig. 6). This effect was prominent even when the Gly content was as low as 2% (D-2); the total amount of IFN released over 30 days from D-1 (in which Gly was not added) was approximately 2%, whereas that from D-2 was approximately 48%. The effect of Gly could be explained not only by the difference in the IFN/HSA(/Gly) powder content in the formulations (D-1: 30%, D-2: 32%), but also by the increase in osmotic pressure that occurs as a result of dissolution of the powder in water. When the osmotic pressure increases with dissolution of the powder in water, it is thought to cause cracks on the surrounding silicone wall [18], leading to easy formation of interconnecting channels and acceleration of IFN release. This speculation is supported by the finding that the IFN release rate increased with increase in the osmotic pressure of the IFN/HSA/Gly solution (Fig. 7). In other experiments (data not shown), the protein drug release rate could be controlled by using additives other than Gly, such as sodium chloride, which also increases the osmotic pressure.

Based on the above results, the release mechanism of water-soluble substances from silicone formulations is considered to be as follows. Although several studies on the release mechanism of water-soluble substances from a hydrophobic polymer such as silicone have

been reported [10, 18-20] and the formation of water-filled channels was speculated to play an important role, this hypothesis has so far not been established in detail. First, the particles of drug powders existing on the surface of the silicone formulations are dissolved and released into the surrounding medium (i.e., water). Second, water fills the pores from which particles have been eluted, leading to dissolution and release of the particles that exist near the pores. Repetitive occurrence of this process results in the formation of interconnecting channels and particles of the drug powder which exists inside the silicone formulation are sequentially released. When the drug powders dissolve, it causes osmotic pressure and bears stress on the surrounding silicone. When the stress is strong enough, small cracks generate accompanied by break of cross-links. Since cracking contributes to the connection of the formation of interconnecting channels, it accelerates drug release. Thus, drug release from silicone is considered to be related to both the generation of cracks and the formation of interconnecting channels. A silicone formulation containing HSA powder was immersed in a buffer and the cross section of the formulation was observed by CLSM (Fig. 9). The micrographs indicate that channels extended from the surface of the silicone formulation and pores were formed at positions where particles of the HSA powder might have originally existed. Since channels are formed by dissolution of drug powder, cross-linking density of silicone matrix should not change significantly. On the other hand, cracking is formed by physical strength, that is, osmotic pressure induced by dissolved drug powder. Therefore, it

might accompany break of cross-links leading to slightly decreased cross-linking density. However, since silicone is highly cross-linked three dimensionally, cracking may not alter the shape of formulations. Fig. 9 shows that silicone formulation after *in vitro* release experiments is not swollen.

The serum IFN concentrations were measured in nude mice in which IFN silicone formulations were administered. The results indicate that the serum IFN concentrations were maintained in the detectable range for 10-60 days after the administration of the IFN silicone formulations (Fig. 10). This result shows that IFN can be released continuously from the IFN silicone formulations, not only *in vitro* but also *in vivo*. The half-lives ($T_{1/2}$) were 12-42 times longer than the half-life of an aqueous solution of IFN. The $T_{1/2}$ of the IFN silicone formulation A-1 was short, since its IFN content was low and the serum IFN concentration decreased to below the detectable range soon after s.c. administration of this formulation.

Furthermore, the antitumor activities of the IFN silicone formulations were examined. In the untreated or placebo groups, the volume of a human renal cell carcinoma (RCC-1) xenografted into the nude mice increased exponentially, while in the mice treated with IFN silicone formulations, significant inhibition of growth of the RCC-1 was observed; this effect was dependent on the IFN content of the IFN silicone formulations (Fig. 11). The IFN silicone formulation A-3 ($47x10^6$ IU) exhibited the most pronounced growth inhibitory effect;

tumors in 5 out of 6 mice disappeared completely and the tumor growth remained suppressed for 90 days after a single administration. The aforementioned results indicate that physiologically active IFN is released over a long period of time from IFN silicone formulations *in vivo*, proving the feasibility of using these silicone formulations.

Since the preparation conditions of silicone formulations are mild, this technique can be applied to a variety of protein drugs. The use of silicone formulations is expected to be especially effective for the treatment of chronic diseases and for the prophylactic treatment of high-risk patients. In the case of a DDS that delivers a drug over a long period of time, discontinuation of treatment might become necessary. Since silicone is inert, unlike carriers that decompose *in vivo*, it can be removed immediately and easily. From this standpoint, silicone formulations are superior to other biodegradable formulations.

5. Conclusion

A new method has been developed in which silicone is used for sustained release over a long period of time of protein drugs that exhibit potent physiological activity in trace quantities. In this study, silicone formulations were prepared using IFN as an example of such protein drugs. The drug release rate from the silicone formulation could be controlled by changing parameters such as the content of the IFN/HSA powder, particle size and the amount of additive. In addition, the IFN silicone formulation was found to suppress the

proliferation of human renal cell carcinoma xenografed into nude mice for a long period after a single administration. This indicates that physiologically active IFN is released over a prolonged period of time from the IFN silicone formulation *in vivo*. These results indicate that silicone formulations might be novel and practically feasible sustained-release formulations of therapeutic proteins and peptides.

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Chapter 2

Controlled Release of Protein Drugs Using Covered-rod Formulation

1. Introduction

The purpose of sustained-release formulations is to maintain an appropriate drug concentration in the body over a long period, therefore, in general, a formulation which releases drugs at a constant rate is considered ideal. However, unlike lipophilic drugs, which can be released at a constant rate from hydrophobic polymer, the number of reports on the successful zero-order release of protein drugs is limited [1-2].

To devise a practical sustained-release formulation for protein drugs, silicone has been studied as a drug carrier in Chapter 1 [3]. Interferon (IFN) was used as an example of a protein drug and produced a matrix formulation in which IFN/human serum albumin (HSA) powder is uniformly dispersed in a silicone matrix. The IFN release rate could be controlled by changing parameters such as the amount of additive and particle size of the IFN/HSA powder. However, the release of protein drugs from a matrix formulation is a first-order release, i.e., the release rate decreases with time. The release mechanism of protein drugs from the matrix formulation was investigated and a new drug delivery system that enables a constant release of protein drugs over a long period was developed, by improving the geometry of the formulation. In this chapter, a technology to control the release of protein

drugs from the new silicone formulation, which is named the covered-rod formulation, is reported.

2. Experimental

2.1. Materials

Silicone elastomer (Silastic® Q7-4750) was obtained from Dow Corning Corporation (MI, USA). Composition and structure of Q7-4750 are described in Chapter 1. Human lymphoblastoid interferon, a natural alpha-type interferon (IFN) (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan), was used. Human serum albumin (HSA) (Buminate® 25%, Baxter Healthcare Corporation, IL, USA) was used to control IFN release from silicone. Glycine (Gly), sodium chloride, and sodium glutamate (Nacalai Tesque Inc., Kyoto, Japan) were used as additives.

2.2. Preparation of silicone formulations

The formulations used in this study are listed in Table 1. HSA, IFN/HSA and IFN/HSA/additive powder were prepared using the procedure outlined in Chapter 1 [3]. The preparation method of a matrix formulation is also described in the chapter. Typically, a covered-rod formulation C-1 was prepared as follows. Q7-4750 Part A (4.02 g) was mixed with Part B (4.02 g) and IFN/HSA/additive powder (3.96 g). The IFN/HSA/additive

Table 1 Composition of silicone formulations

	IFN (IU)	Composition		Powder size (μm)	Formulation type
		HSA (%)	Additive (%)		
M-1	-	30%	-	53-150	Matrix
M-2	6×10^6	30%	-	53-150	Matrix
M-3	-	30%*	Gly 10%	<2	Matrix
C-1	9×10^6	30%	Gly 2%, NaCl 1%	<2	Covered-rod
C-2	-	30%	-	53-150	Covered-rod
C-3	1×10^6	20%	-	<20	Covered-rod
C-4	1×10^6	30%	-	<20	Covered-rod
C-5	1×10^6	40%	-	<20	Covered-rod
C-6	1×10^6	50%	-	<20	Covered-rod
C-7	1×10^6	30%	-	<20	Covered-rod
C-8	1×10^6	30%	-	20-53	Covered-rod
C-9	1×10^6	30%	-	53-150	Covered-rod
C-10	1×10^7	30%	Gly 10%	<2	Covered-rod
C-11	1×10^7	30%	Sodium glutamate10%	<2	Covered-rod
C-12	1×10^7	30%	NaCl 10%	<2	Covered-rod
C-13	1×10^7	30%	-	<2	Covered-rod
C-14	-	30%*	Gly 10%	<2	Covered-rod

*Texas-Red-labeled HSA

powder content was 33%(w/w). A kneaded mixture of the IFN/HSA/additive powder and silicone was filled into a syringe. Separately, a mixture of Part A (25 g) and Part B (25 g) was filled into an another syringe. The mixtures were extruded through concentrically arranged die (opening diameter was 1.9 mm) so that the IFN/HSA/additives containing silicone formed the inner part and the IFN/HSA/additive-free silicone the outer part. The extruded rod was cured at room temperature for 3 days. After curing, the rod was cut to 1cm in length and stored at 5°C. The mixing ratio of IFN, HSA and additive solutions, or that of powder and silicone was changed according to the composition of each sample.

Texas-Red-labeled HSA was used to prepare M-3 and C-14 so that HSA could be identified by confocal laser scanning microscopy (CLSM). The labeling method has been reported by Titus et al. [4].

2.3. Measurement of IFN content in the silicone formulations

The IFN covered-rod formulation C-1 was frozen with liquid nitrogen and then milled for 30 seconds using Cryo-Press CP-100 (Microtech Co., Ltd., Chiba, Japan). The milled pieces were immersed in 30 ml of phosphate-buffered saline (PBS, pH 7.4), 0.3% Tween20 and 0.01% sodium azide. The solution was diluted with PBS containing 0.5% bovine serum albumin (BSA) and assayed for IFN concentration by bioassay at Mitsubishi Kagaku BCL Inc. (Tokyo, Japan).

2.4. *In vitro* release study

An IFN silicone formulation (C-3~13) was placed in a tube containing 10 ml of PBS (pH 7.4), 0.5% HSA and 0.01% sodium azide. Since IFN is unstable at 37°C in a solution form, the tube was placed in an incubator at 5°C to avoid degradation of IFN and to measure the precise amount of IFN released. At designated times, an aliquot was collected and transferred to a new tube containing fresh buffer. The solution was assayed for IFN concentration by radioimmunoassay (RIA), using an Interferon alpha kit (Dinabot Co., Ltd., Tokyo, Japan). RIA was performed in duplicate. IFN is stable in the buffer during the sampling intervals.

In vitro release study of HSA silicone formulation (M-1, C-2) was performed in the same manner except that the release medium was 3 ml of PBS (pH 7.4) and 0.01% sodium azide, and that HSA concentration was assayed by the Bradford method.

2.5. Release rate analysis

The release rate was analyzed based on the *in vitro* release profile. It was calculated from linear regression analysis.

2.6. Measurement of osmotic pressure

IFN/HSA or IFN/HSA/additive powder (300 mg) was dissolved in 0.6 ml of PBS (pH

7.4), 0.5% BSA and 0.01% sodium azide. The osmotic pressure of the solution was measured three times, using an auto-osmometer (Osmostat OM-6020, DAIICHI Kagaku Inc., Kyoto, Japan), for each sample.

2.7. Experimental animals

Female athymic nude mice (nu/nu, BALB/c background) were purchased from CLEA Japan Inc., Tokyo, Japan, and were housed in a negative-pressure safety rack under specific pathogen-free conditions.

2.8. Measurement of serum IFN levels

The IFN covered-rod formulation C-1 was administered subcutaneously into 5-week-old mice. Then, blood samples were collected at preset time points of 1, 4, 7, 14 and 28 days. Sera were collected and stored at -40°C until the IFN assay. The serum IFN levels were measured by RIA, using an Interferon-α kit.

2.9. Pharmacokinetic analysis

The apparent half-life ($T_{1/2}$) was calculated using a one-compartment model as follows. The elimination rate (K_e) was calculated by the least-squares method from the linear terminal phase of the serum concentration-time curve and used to calculate $T_{1/2}$ from the following

relationship: $T_{1/2}$=ln2/ K_e.

2.10. Confocal laser scanning microscope (CLSM) analysis

Silicone formulations M-3 and C-14 were prepared with Q7-4750 and Texas-Red-labeled HSA/Gly powder (powder size; <2 μm, Texas-Red-labeled HSA content; 30%(w/w), Gly content; 10%(w/w)). The silicone formulation was placed in a tube containing 10 ml of PBS (pH 7.4), 0.5% BSA. The tube was placed in an incubator at 5°C for 5 days. The initial and *in vitro* tested formulations were cut in the center and observed using a CLSM (LSM-GB200, OLYMPUS OPTICAL Co., Ltd., Tokyo, Japan). HSA was rendered fluorescent by Texas Red, while silicone elastomer was not. Therefore, the HSA powders distributed in silicone elastomer could be detected against the black background of silicone elastomer.

3. Results

3.1. Preparation of covered-rod formulation

HSA was used as a model protein drug. IFN was used as an example of a protein drug which exerts physiological activities at very low concentrations. The preparation method of a covered-rod formulation is simple and the reproducibility of the formulation is

good. The thickness of the outer layer was uniform and there was no space between the outer and the inner layers. Figure 1 shows an example of a covered-rod formulation.

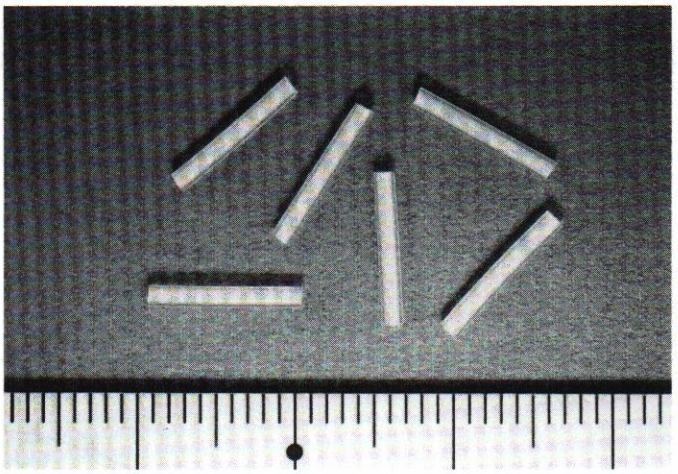
In order to evaluate the stability of IFN during the preparation of the covered-rod formulation, the content of IFN in the formulation was determined. The IFN covered-rod formulation C-1 was crushed using a grinder. The pieces were then immersed in buffer, and the resultant solution was bioassayed for IFN content (Table 2). The recovery of IFN from the IFN covered-rod formulation was approximately 100%, indicating that IFN was stable during the preparation of the covered-rod formulation.

3.2. *In vitro* release behavior

HSA was continuously released from the matrix formulation M-1 over a period of approximately one month (Fig. 2a). The release rate was high during the initial stage but decreased with time. On the contrary, the covered-rod formulation C-2 continued to release HSA at a constant rate without the first day; the total amount of HSA released in 99 days was 82% (Fig. 2b). Fig. 2c shows comparison of released fraction per day between M-1 and C-2. The rapid release of C-2 on the first day should be contributed by HSA powder existed on the surface of the formulation. Except the day, the rate of HSA release of C-2 was constant, comparing to that of M-2.

The profiles of HSA release of the matrix and covered-rod formulations are

(a)



(b)

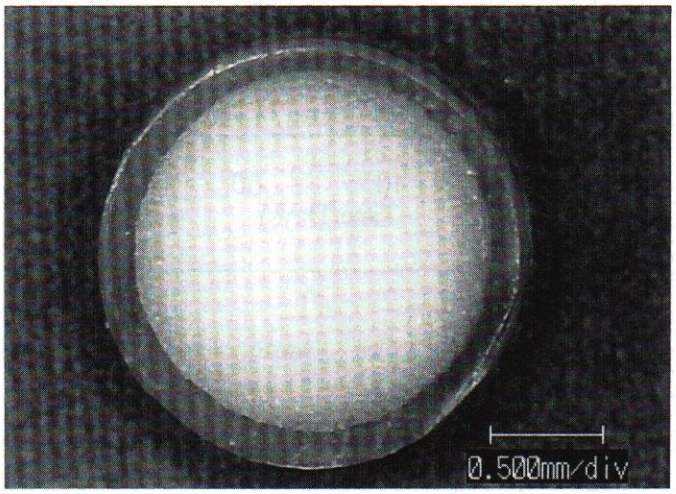


Fig. 1. Photograph of a covered-rod formulation: (a) the appearance, and (b) a cross-section.

Table 2

Recovery of IFN from IFN covered-rod formulation[a]

Sample	IFN content	
	Theoretical value (IU)	Recovery (%)
C-1	9.25×10^{6}	102±6

a Results expressed as mean ± deviation of two independent samples.

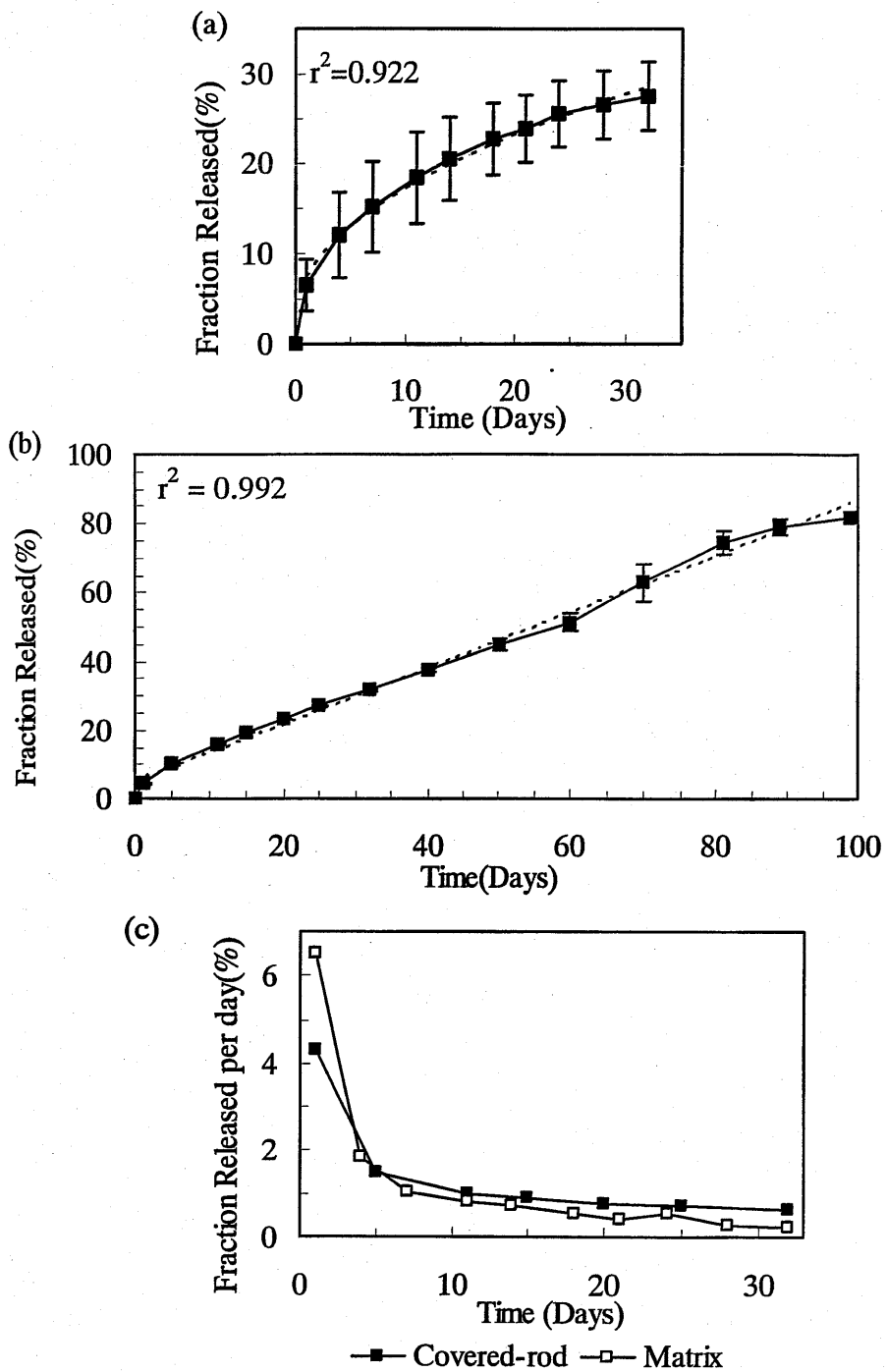


Fig. 2. HSA release profiles for HSA silicone formulations: (a) matrix (M-1), (b) covered-rod (C-2), (c) released fraction per day of each formulation. Each point represents mean ± deviation of data from two independent samples.

approximated by Eqs. (1) and (2), respectively. The squares of the correlation coefficients between the calculated and measured values are indicated in the figures. The HSA release profiles of the matrix and covered-rod formulations correspond very well to Eqs. (1) and (2), respectively.

$$y = a_m \cdot t^{1/2} + b_m \quad (1)$$

$$y = a_c \cdot t + b_c \quad (2)$$

3.3. Control of drug release

3.3.1. Effect of IFN/HSA powder content

Covered-rod formulations containing different amounts of IFN/HSA powder were prepared. These formulations were immersed in buffer to assay for IFN release. Figure 3a shows the results.

The total amount of IFN released over one month for C-3 (IFN/HSA powder content: 20%) was extremely small, 0.65%; however, IFN was released continuously at a constant rate from C-4, C-5 and C-6, whose IFN/HSA powder contents were 30% or more. The IFN release rate increased with increasing IFN/HSA powder content. The total amount of IFN released over one month for C-6 (the highest content of IFN/HSA powder) was 57%.

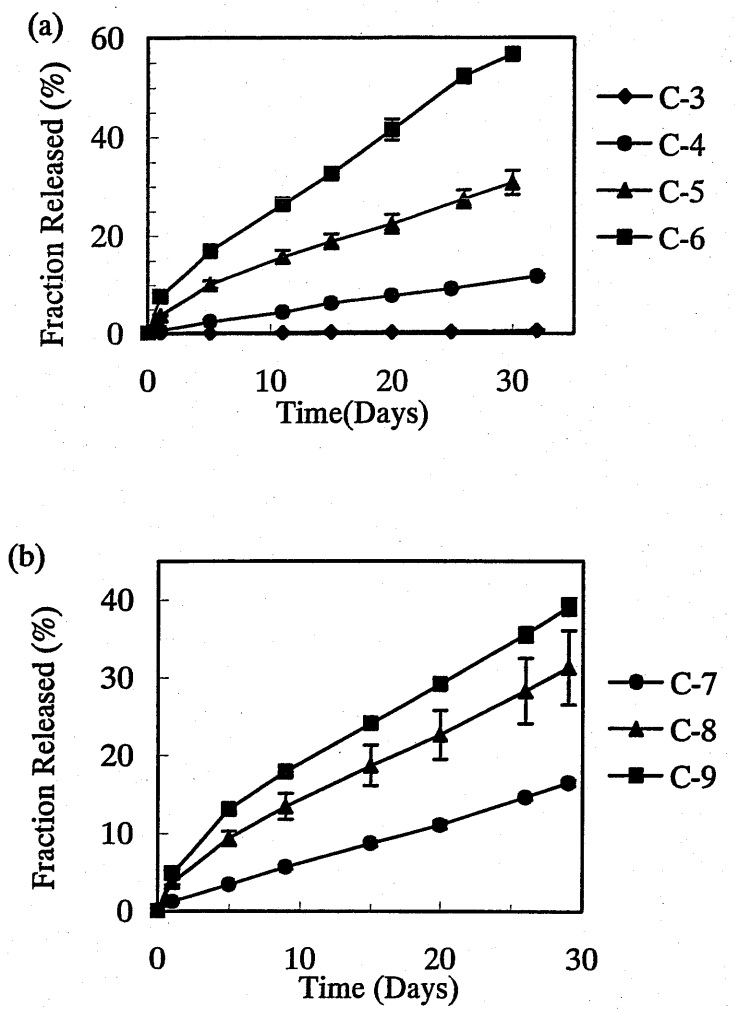


Fig. 3. IFN release profiles for IFN covered-rod formulations containing (a) different amounts of IFN/HSA powder, and (b) IFN/HSA powders of different particle sizes. Each point represents mean ± deviation of data from two independent samples.

3.3.2. Effect of IFN/HSA particle size

Covered-rod formulations containing IFN/HSA powders of different particle sizes, i.e., C-7 (<20 μm), C-8 (20-53 μm) and C-9 (53-150 μm), were immersed in buffer to assay for IFN release. Figure 3b shows the results.

The IFN release rate increased with increasing particle size of IFN/HSA powder. The total amount of IFN released over one month for C-7 (smallest particle size) was 17%, whereas that for C-9 (largest particle size) was 39%.

3.3.3. Effect of kinds of additives

Covered-rod formulations were prepared using IFN/HSA powder containing different types of additives: C-10 (additive: Gly), C-11 (additive: sodium glutamate), C-12 (additive: sodium chloride) and C-13 (no additive). The formulations were immersed in buffer to assay for IFN release. Figure 4 shows the results. The IFN release rate depends on the kinds and presence/absence of additives.

IFN/HSA/additive or IFN/HSA powders used to prepare C-10~C-13 were dissolved in a buffer, and the osmotic pressures of the buffers were measured. Table 3 summarizes the IFN release rates and differences in osmotic pressure (which is equal to the osmotic pressure of the buffer containing IFN/HSA/additive minus the osmotic pressure of the buffer itself). The osmotic pressure of the buffers in which IFN/HSA/additive powder (C-10~C-12) was

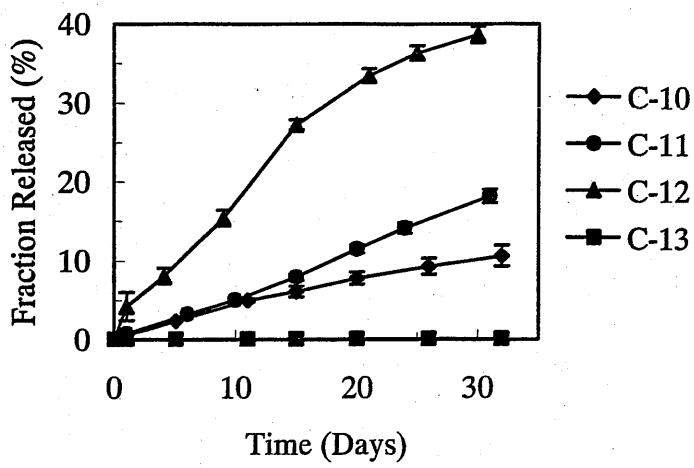


Fig. 4. IFN release profiles for IFN covered-rod formulations containing different additives. Each point represents mean ± deviation of data from two independent samples.

Table 3

IFN release rates of IFN covered-rod formulations and osmotic pressure of solutions with dissolved IFN/HSA/additive powder[a]

	Release rate, a_c (%/day)	Osmotic pressure (mOsm)
C-10	0.335±0.061	1468±2
C-11	0.586±0.040	1761±8
C-12	1.329±0.003	4841±5
C-13	0.001±0.000	148±3

[a] Results of release rates expressed as mean ± deviation of two independent samples. Results of osmotic pressures expressed as mean ± S.D. of three measurements.

dissolved was higher than that without additive (C-13).

The relationship between IFN release rate (corresponding to a_c in Eq. (2)) and the osmotic pressure is shown in Fig. 5. The IFN release rate increased with increase in the osmotic pressure.

3.4. Effect of length of the covered-rod formulation

Covered-rod formulations C-8 of different lengths (1 and 2 cm) were immersed in buffer to assay for IFN release. Figure 6 shows the change in IFN released per day with time.

Over the first month, both formulations released IFN at a constant rate, except on the first day; the amount of IFN released per day from 1-cm and 2-cm formulations were similar. However, after one month, the amount of IFN released from the 1-cm formulation declined gradually, whereas that released from the 2-cm formulation remained constant over two months.

3.5 Change in serum IFN concentrations

The IFN covered-rod formulation C-1 was administered subcutaneously into the back of nude mice. Blood samples were collected from the nude mice at predetermined time points after administration, and the serum IFN concentrations were measured. Figure 7 and

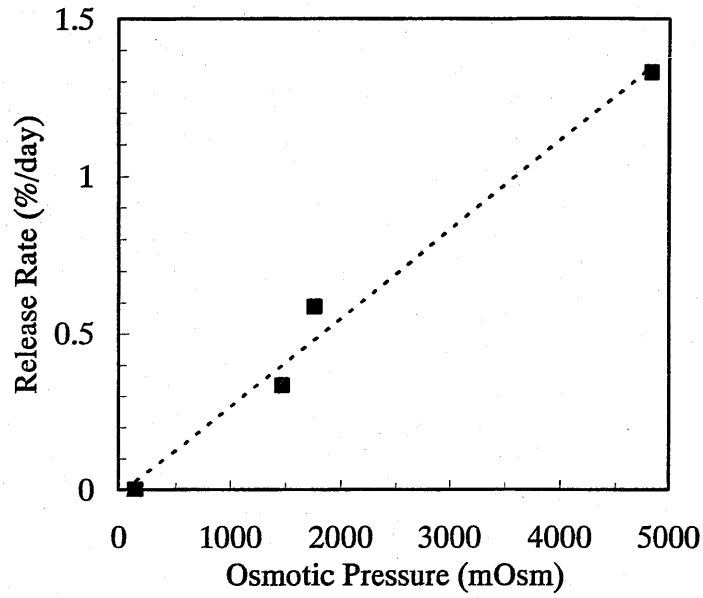


Fig. 5. Correlation between IFN release rates and osmotic pressure.

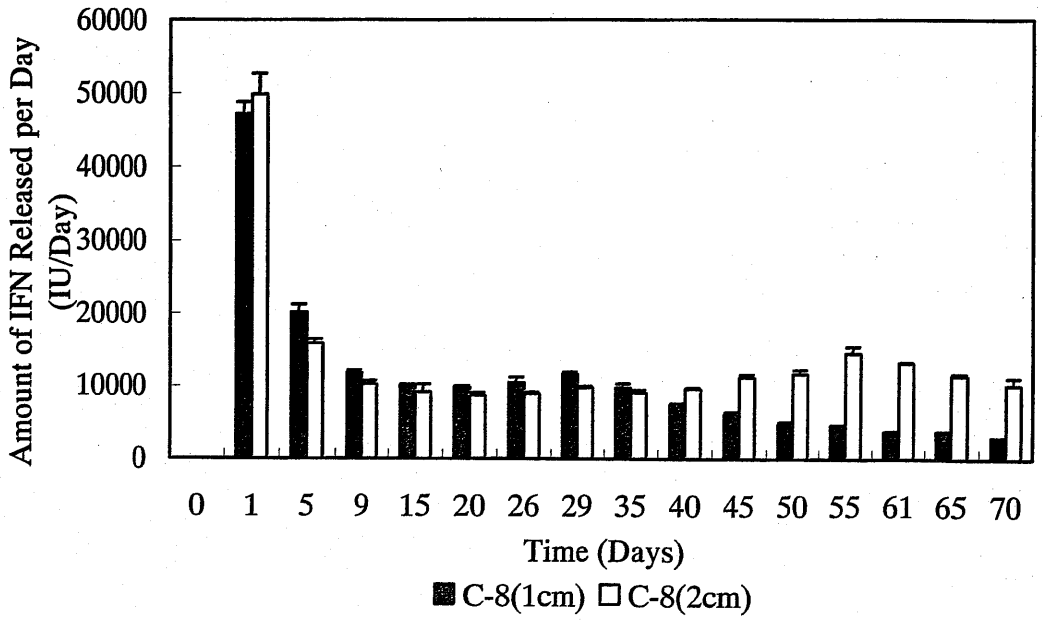


Fig. 6. The amount of IFN released per day from IFN covered-rod formulations with different rod lengths. Each point represents mean ± deviation of data from two independent samples.

Table 4 show the changes in serum IFN concentrations and the half-lives, respectively. For reference, the data for the IFN matrix formulation M-2 and IFN aqueous solution previously reported are also shown in Fig. 7 and Table 4.

In the group of mice in which IFN aqueous solution (1×10^5 IU) was administered, the serum IFN concentration decreased rapidly. In the group of mice in which the IFN matrix formulation M-2 (6×10^6 IU) was implanted, the maximum serum concentration of IFN (1379 ± 657 IU/ml) was observed at the initial sampling time, i.e., 24 h after implantation; the concentration decreased to approximately 1/19 of the maximum value at ten days after implantation; subsequently, serum IFN could be detected for 30 days. On the other hand, in the group of mice in which the IFN covered-rod formulation C-1 (9×10^6 IU) was implanted, the maximum serum concentration (1763 ± 137 IU/ml) was observed at 24 h, and an approximately constant level was maintained; even on the 28th day after implantation, the serum IFN concentration was approximately 1/18 of the maximum value.

3.6. Mechanism of drug release

The change in distribution of Texas-Red-labeled-HSA powder in silicone with time was analyzed by CLSM. Figure 8 shows micrographs of the formulations.

Before immersion of the formulations in buffer, Texas-Red-labeled-HSA powder was homogeneously distributed throughout silicone; there was no difference in the distribution of

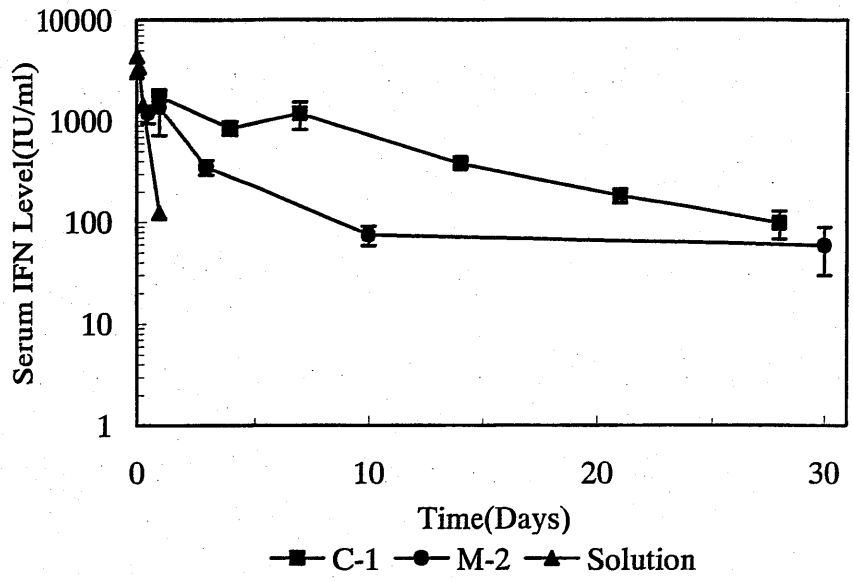


Fig. 7. The time course of serum IFN levels in nude mice after administration of IFN silicone formulations. Each point represents mean ± S.D. of data from three nude mice. ◆; C-1, ●; M-2, ▲; Solution.

Table 4

Pharmacokinetic parameter

Parameter		C-1	M-2	Solution [5]
Dose	(IU)	9×10^6	6×10^6	1×10^5
$T_{1/2}$	(h)	158	190	4

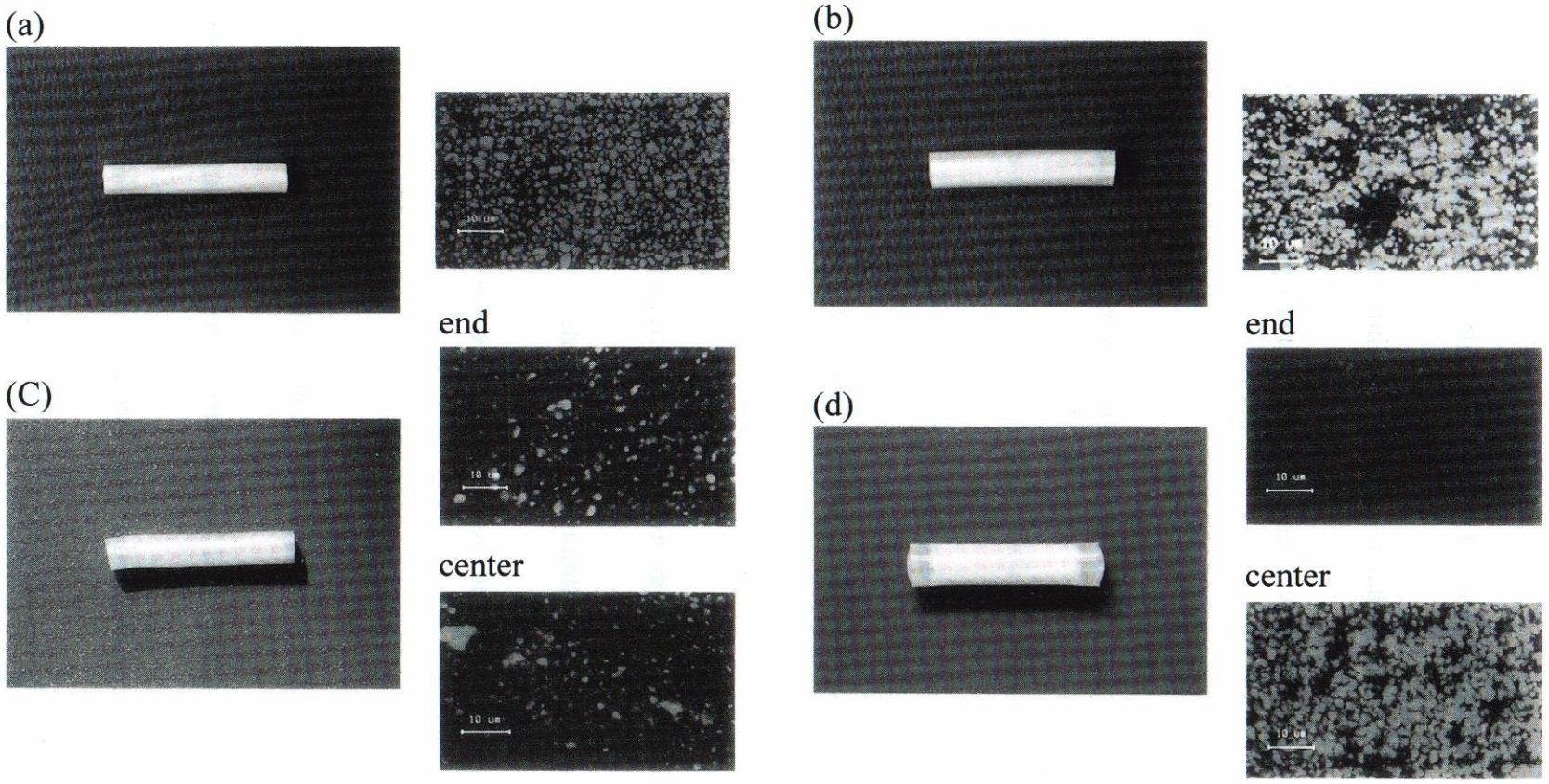


Fig. 8. Time-course images of the silicone formulations. (a) Before release test, M-3. (b) Before release test, C-14. (c) Incubated for 5 days *in vitro*, M-3. (d) Incubated for 5 days *in vitro*, C-14. Bars in CLSM images indicate 10 μm.

Texas-Red-labeled-HSA powder between the matrix formulation M-3 (Fig. 8a), and the inner layer of the covered-rod formulation C-14 (Fig. 8b). After immersion for 5 days, Texas-Red-labeled-HSA powder was released, leading to a decrease in the density of the powder in M-3 (Fig. 8c). On the other hand, in C-14, water infiltrated from the ends of the formulation, and the area where powder was released became transparent. At the ends of the formulation, little powder was present; in contrast, as much powder as under the initial condition remained in the central part of the formulation (Fig. 8d).

4. Discussion

In general, peptide and protein drugs are unstable and have an extremely short half-life in the body. To resolve these clinical problems, many researchers have studied sustained-release formulations of protein drugs in order to increase the duration of the drug effect and reduce the frequency of administration and side effects. Examples of formulations which have already been practically applied include microspheres of poly(lactic/glycolic acid) (PLGA) such as Lupron Depot® [6-9] and Nutropin Depot™ [10-14]. These microspheres of PLGA release drugs via an initial burst immediately after administration. It is reported that the initial burst of PLGA microspheres is attributed to their large surface area and/or porous structure [8, 11-12]. Due to the initial burst, microspheres of PLGA can be used as carriers of drugs with specific pharmacological effects (e.g., leuprorelin acetate) and those

with a wide therapeutic index (e.g., growth hormone); however, an initial burst is generally undesirable from the point of view of safety.

In order to develop a sustained-release formulation of protein drugs that is effective over a long time of period, a matrix formulation using silicone as a carrier has been developed. The matrix formulation was designed to suppress initial burst, by reducing surface area compared with microspheres, and making the structure not porous. Although the level of the initial burst is not as high as that of microsphere formulations, the release rate of the protein drugs decreases with time. Based on the knowledge accumulated through the development of matrix formulations, the geometry of formulations was improved and a new formulation, i.e., a covered-rod formulation has been developed. Since the covered-rod formulation was designed to further reduce surface area compared with the matrix formulation, initial rapid release of the covered-rod formulation is very little. In addition, the covered-rod formulation enables the release of protein drugs at a constant rate over a long period.

The protein drug release profiles from matrix and covered-rod formulations were examined using HSA (Fig. 2). The amount of HSA released from the matrix formulation M-1 decreased with time, while the covered-rod formulation C-2 continued to release HSA at a constant rate for approximately 100 days. It is reported that when a drug dispersed in polymer is released through channels, the percentage of drug released is proportional to the square root of time [15]; $y = a_m \cdot t^{1/2}$. On the other hand, when a drug is released at a

constant rate regardless of time (zero-order release), the percentage of drug released is in proportion to time; $y = a_c \cdot t$. Theoretically these equations intersect the origin. However, since protein drug particles which exist on the surface of formulations are released immediately after immersion into an aqueous environment, coefficients b_m and b_c were necessary in the equations to make corrections for an initial rapid release (Eqs. (1) and (2)). The HSA release profile of the matrix and covered-rod formulations, M-1 and C-2, corresponded very well to Eq. (1) and (2), respectively.

When covered-rod formulations containing IFN/HSA powder were immersed in buffer, IFN was released continuously in the zero-order manner. The IFN release rate depended on the IFN/HSA powder content and its particle size. In addition, when covered-rod formulations were prepared using IFN/HSA powder containing different types of additives, e.g., Gly, sodium glutamate and sodium chloride, the IFN release rate was influenced by the kind of additive (Fig. 4). The degree of influence was greater for formulations prepared using an additive that induces a higher osmotic pressure (Table 3 and Fig. 5). Similar experiments were performed using matrix formulations and reported the results were reported in Chapter 1 [3]. It was shown that powder content, particle size and additives had similar effects on the protein drug release rates of these two formulations; however, the release profiles of the matrix formulations were of the first order, while those of the covered-rod formulations were of zero order.

IFN covered-rod formulations were implanted in nude mice and the serum IFN concentrations were measured. In the group of mice in which IFN solution was administered, the serum IFN concentration decreased rapidly. In the group of mice in which the IFN matrix formulation M-2 or IFN covered-rod formulation C-1 was implanted, the serum IFN concentration was maintained above the detection limit during the period of observation, i.e., about 30 days after a single administration (Fig. 7). The half-lives ($T_{1/2}$) were 48 and 40 times longer than that of an IFN aqueous solution, respectively (Table 4). This indicates that the both formulations released IFN continuously *in vivo*. In the group of mice in which the IFN covered-rod formulation was implanted, the serum IFN concentration was maintained at an approximately constant level for 28 days, indicating that IFN was released from the covered-rod formulation at a constant rate not only *in vitro* but also *in vivo*.

Microspheres are difficult to apply for protein drugs such as cytokine and insulin that manifest strong physiological activity at low doses, because of a large initial burst. However, it is expected that the covered-rod formulation can be practically applied for these protein drugs.

The release mechanism of protein drug from a covered-rod formulation is discussed with respect to the formation process of channels, water infiltration patterns and diffusion characteristics of the protein drug, by comparing these aspects with those of a matrix formulation, as follows.

The release process of protein drugs from a matrix formulation is considered to be as follows. First, protein drug particles existing near the surface of the formulation are dissolved and released into the surrounding water. Second, adjacent particles are dissolved and released into the surrounding water. When high osmotic pressure is induced by the dissolution of protein drug powder into water, cracks occur on the surrounding silicone walls, which promotes the connection of the channels. By repeating this process, pores (where the protein drug powder had been) and cracks connect to form channels, and the protein drug inside the formulation is sequentially released. In case of a covered-rod formulation, protein drugs are considered to be released through channels which are formed in an inner layer by the same process as the matrix formulation. In fact, the release rates of protein drugs can be controlled by the same factors such as the content of protein drug powder, the particle size, and the kind of additives, with respect to both the matrix and covered-rod formulations as described above. The results indicate that the release processes of the two formulations are related to channel formation. Protein drugs are not released through an outer layer of the covered-rod formulation, because channels are not formed in the outer layer and protein drugs can not diffuse through polymer network.

In order to investigate the water infiltration patterns of the matrix and the covered-rod formulations, Texas-Red-labeled-HSA powders were used to prepare both formulations. The change in the distribution of Texas-Red-labeled-HSA powder within the formulations was

analyzed by CLSM, tracing the fluorescence of Texas Red (Fig. 8). Before the immersion of the formulations in buffer, the images of the matrix formulation and of the inner layer of the covered-rod formulation were similar; Texas-Red-labeled-HSA powders were homogeneously distributed throughout silicone. For the matrix formulation incubated for five days *in vitro*, the image of the central part is similar to that of the two ends; Texas-Red-labeled-HSA powder was released and its density decreased. On the other hand, for the covered-rod formulation incubated for five days *in vitro*, the image of the central part is different from that of the ends. In the central part, Texas-Red-labeled-HSA powder existed at a density similar to the initial density; it is considered that the release of Texas-Red-labeled HSA does not occur from the central part at that time. In contrast, at the two ends, only a slight amount of dissolved Texas-Red-labeled HSA remained. This may be explained as follows. In the matrix formulation, water infiltration proceeded on the entire surface of the formulation; water infiltrated into all parts of the formulation within a relatively short period, and therefore a similar image was observed for all parts of the formulation at day 5. In contrast, in the covered-rod formulation, water infiltration proceeded from the ends of the formulation, the rate of water infiltration was slow and at the central part the initial condition was maintained at day 5.

The water infiltration patterns of matrix and covered-rod formulations are summarized as follows. For a matrix formulation, the formation of channels occurs over the entire

surface of the formulation; since the water penetration front proceeds from the surface of the cylindrical formulation into its interior, the area of the water penetration front decreases with time (Fig. 9a). For a covered-rod formulation, since the formulation is surrounded by silicone through which water does not penetrate, the area from which water can infiltrate is limited to the two ends; the area of the water penetration front always remains constant (Fig. 9b). The above discussions are also supported by the observation obtained in the *in vitro* experiments, that water infiltrates throughout the matrix formulation, but water infiltrates only from the two ends of the covered-rod formulation (Fig. 8).

Protein drug inside a covered-rod formulation is dissolved upon water infiltration. However, the dissolution of the protein drug is not the rate-limiting step of drug release, because the release of protein drug from a covered-rod formulation continues even after water infiltrates into all parts of the formulation, as evident by visual observation. In addition, if the dissolution of protein drug powder is the rate-limiting step of the drug release, the release rate should decrease with time, since the dissolved protein drug must diffuse through a longer channel as the water penetration front of a covered-rod formulation moves inward with time. However, the results were inconsistent with this assumption; a covered-rod formulation continued to release protein drug for a long period at a constant rate. These findings indicate that the diffusion rate of the dissolved protein drug inside the formulation is extremely low compared to that outside the formulation.

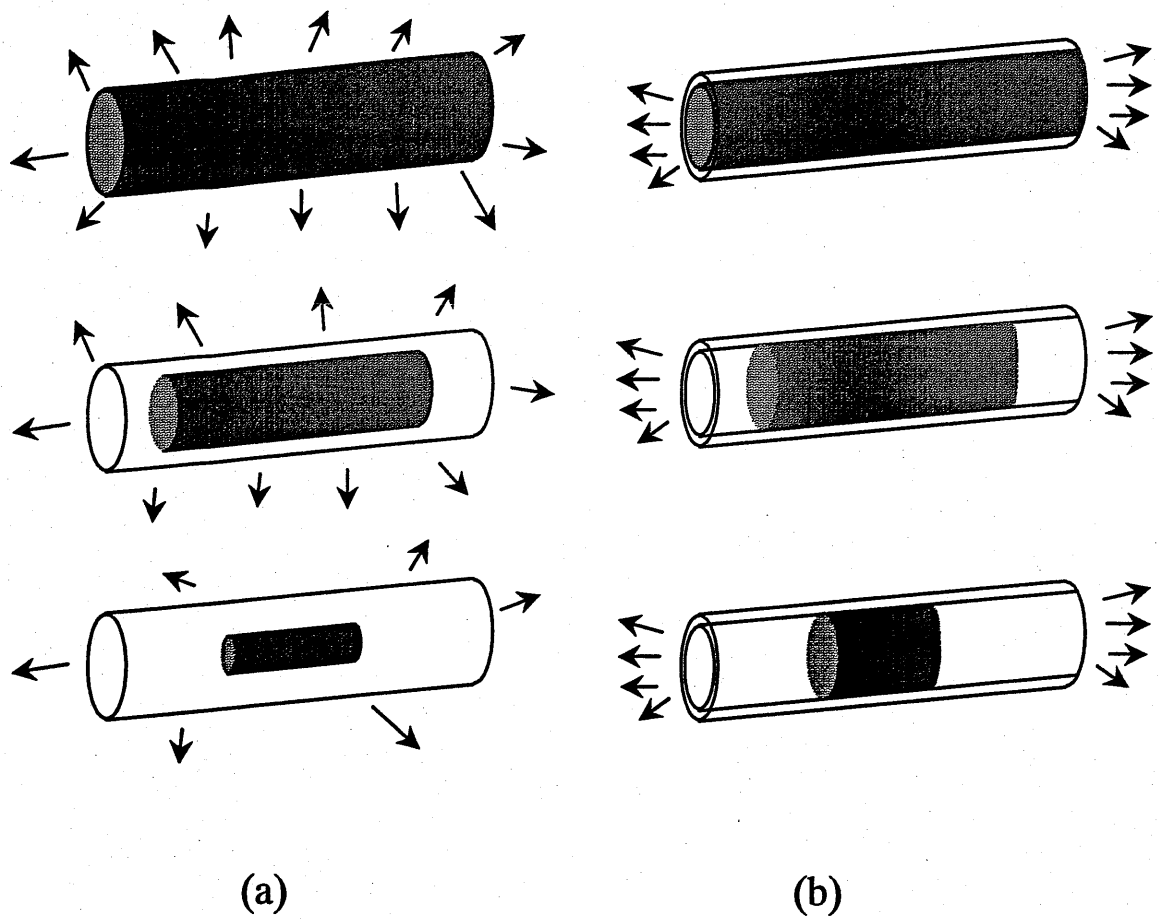


Fig. 9. Images of water penetration into and protein release from the silicone formulations: (a) matrix, (b) covered-rod. The patterned domain indicates the area where intact protein drug powders remain. Arrows indicate from where the protein drug is released.

In the case of a matrix formulation, protein drug is released from the entire surface of the formulation; the concentration of the protein drug inside the formulation decreases within a relatively short period.

In contrast, diffusion of protein drug is suppressed in a covered-rod formulation, since 1) the area from which the drug is released is extremely small compared to that of the matrix formulation and 2) the channels are rendered narrow by the pressure of the outer layer on the inner layer. Consequently, the concentration of the dissolved protein drug inside the channels might be saturated and remains constant, resulting in the constant release of the protein drug.

The mechanism explained above is also supported by the finding that the release duration can be controlled by the length of the formulation. When 1-cm and 2-cm covered-rod formulations C-8 were immersed in buffer to assay for the amount of IFN released per day, the amounts of IFN released from the two formulations were similar over the first month; however, subsequently, the IFN released from the 1-cm formulation gradually decreased, whereas that from the 2-cm formulation was maintained at the same level as during the first month (Fig. 6). Over the first month, there was no remaining IFN/HSA powder to be dissolved inside the 1-cm formulation and the IFN concentration inside the channels gradually decreased, as did the release rate. In contrast, the IFN concentration inside the channels was still maintained at a constant level inside the 2-cm formulation, which leads to a

continuous zero-order release.

The coefficient a_m in Eq. (1) is considered to be dependent on the drug release area, the solubility of the protein drug in water (body fluid), the channel volume inside the formulation, the tortuosity of the channels and the diffusion rate of the dissolved protein drug inside the channels. The coefficient a_c in Eq. (2) seems to be influenced by the same factors as those of a_m in Eq. (1); however, even if the matrix formulation and the inner layer of the covered-rod formulation have the same composition, the values of a_c and a_m would not be the same. It is considered that the difference between a_c and a_m depends not only on the difference between the drug release area but also on other factors, such as the diffusion rate of the dissolved protein drugs in the channels, as discussed above. For example, a_m of the matrix formulation D-4 was 16.3%/day, as reported in Chapter 1 [3], whereas a_c of the covered-rod formulation C-10, having an inner layer of the same structure as that of D-4, was 0.335%/day. Even if the coefficients a_m and a_c are corrected by the difference in the drug release area (the drug release area of D-4 is approximately 14 times that of C-10), the coefficient for the matrix formulation is higher than that for the covered-rod formulation. This may be because the IFN diffusion rate inside the channels of the covered-rod formulation is lower than that of the matrix formulation.

In general, protein drugs including IFN are denatured and deactivated by treatments such as heating or the use of organic solvents. Since the covered-rod formulation can be

prepared under mild conditions without heat treatment or the use of organic solvents, it is possible to prepare formulations of protein drugs that are sensitive to these treatments without reducing the physiological activity. In fact, the evaluation of the stability of IFN during the preparation of the formulation revealed that the recovery of IFN from the formulation was approximately 100% of the theoretical value (Table 2).

5. Conclusion

In order to develop a long-term delivery system for protein drugs that enables the zero-order release, a covered-rod formulation was developed.

Since a covered-rod formulation can be prepared under mild conditions without heat treatment or the use of organic solvents, it can be applied to protein drugs that are sensitive to these treatments which generally cause degradation of their physiological activities. Furthermore, the covered-rod formulation can release protein drug at a constant rate regardless of the molecular weight of the drug without significant initial burst. The drug release rate can be controlled by changing parameters such as the particle size of protein drug powder, its content and the kind of additives. When the IFN covered-rod formulation was implanted *in vivo*, the serum IFN concentration was maintained at a constant level.

The covered-rod formulation is expected to be practically applied for protein drugs with

narrow therapeutic indices, and can be used effectively and safely for treatments of chronic diseases.

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Part II

Efficacy of Silicone Formulation

Chapter 3

Silicone Formulation As A Novel Vaccine Delivery Vehicle

1. Introduction

Matrix and covered-rod silicone formulations release protein drugs in first-order and zero-order profiles for long time of periods, respectively. In addition, both formulations released IFN *in vivo* without loss of its physiological activity [1-2]. From these results, the silicone formulations are expected to utilize in therapy of chronic diseases or prophylactic treatments, with improved quality of life and less administration frequency. Other example of expecting application of the silicone formulations is vaccine.

The optimization of the immune response to any vaccine involves careful selection of the method of delivering the antigen. However, the optimum delivery profile has not been established and the relationship between antigen delivery and the phenotype of the ensuing immune response is yet to be determined. Controlled release systems such as silicone formulations will introduce prolonged antigen delivery profiles as an alternative delivery technique, in contrast to bolus administration by injection or short-term delayed release resulting from injection with adjuvants such as alum or oil emulsions. The limited literature on vaccination by continuous antigen release in fact includes a number of examples in which mature and protective immune responses have been elicited, using products such as pumps [3]

or implants [4-5]. These results suggest that controlled release systems have potential to allow complete vaccination with a single antigen delivery, an ultimate goal in vaccine research. The study presented in this chapter examines the potential for the silicone formulations as vaccine delivery systems.

2. Experimental

2.1. Antigens and adjuvants

Immunopure™ avidin (Avi) was obtained from Pierce Chemical Company (NY, USA) and used as a model antigen at levels of 10-500μg per sheep dose, as indicated. For clostridial vaccination, toxoided culture supernatants were obtained from a vaccine manufacturer and used at levels equivalent to that of the commercially available vaccines for sheep. Aluminum hydroxide gel (alum) was prepared as described in Harlow and Lane [6] and incorporated into vaccine formulations at a 1:2 dilution. Recombinant ovine interleukine-1β (rovIL-1β) was prepared at the Centre for Animal Biotechnology as described in elsewhere [7] and incorporated as adjuvant into silicone formulations at levels of either 20 or 50 μg as indicated.

2.2. Preparation of silicone formulations

Silicone formulations were prepared as follows. Solutions of antigens and adjuvants,

along with additives mannitol (Man) and/or sodium citrate (SC) were mixed and lyophilized. The resulting lyophilized cake was milled to form fine and uniform particles and blended with silicone (Silastic® Q7-4750, Dow Corning Corporation, MI, USA), composed of a 1:1 ratio of Part A (containing catalyst) and Part B (containing cross-linking agent). The mixture was then pressed through an extruder with a 1.6 mm die, and cured at 25°C for 3-4 days. The extruded rod was then cut to lengths (approximately 10 mm) to produce matrix formulations. For covered-rod formulations, the mixture was co-extruded with an outer covering of silicone that was impermeable to water. The extruded rod was then cut to 10 mm lengths so that the cut surfaces exposed the central core of permeable inner layer.

2.3. *In vitro* release study

A silicone formulation (Group 7 and 10 in Table 1) was placed in a tube containing 10 ml of PBS (pH 7.4), 0.3% Tween 20, 0.01% sodium azide. At designated times, an aliquot was collected and transferred to a new tube containing fresh buffer. The solution was assayed for Avi and IL-1β concentration by ELISA. ELISA was performed in duplicate. Avi and IL-1β are stable in the buffer during the sampling intervals.

2.4. Animals

The 12-24-month-old merino ewes and wethers were obtained from commercial farms

and housed at the Howard Florey Experimental Farm, Tooradin, Australia. Sheep were maintained on pasture and mustered for vaccination and blood sampling. Individuals were identified by eartag and randomly allocated to experimental groups of seven sheep.

2.5. Vaccination

Silicone formulations were delivered subcutaneously to the back of the neck using a prototype handheld implant delivery gun prepared by N.J. Phillips Pty. Ltd., Gosford, Australia. Conventional vaccinations were administered to the back of the neck in a total volume of 1 ml using syringes fitted with 18 g needles.

2.6. Sample collection

Blood was collected prior to vaccination and at intervals following vaccination via the jugular vein using 18 g needles and syringes. An amount of 10 ml blood samples were collected and allowed to clot, prior to centrifugation to remove remaining red cells. Serum samples were decanted and stored at -20°C prior to use in immunoassays.

2.7. Immunoassays

Anti-avidin antibody responses were assessed by ELISA as previously described [7]. Isotype-specific antibody responses were assessed by ELISA as for total antibody responses,

except that isotype-specific anti-sheep monoclonal antibodies were applied after the test serum, followed by an anti-mouse antibody conjugated to horseradish peroxidase. Anti-clostridial antibody responses were assessed as for anti-avidin responses except that the plate was coated with a standard solution of toxoided culture supernatant of *C. novyi* or *C. tetani*. Neutralising antibody titres were determined by a commercial vaccine manufacturer using passive transfer of immune sera to mice and subsequent challenge infection.

3. Results

An immunization study was established to determine the efficacy of silicone formulations as vaccine delivery vehicles in sheep, using the model antigen Avi. Groups of sheep were immunized as described in Table 1. As the silicone formulations are prepared using dry powder raw materials, alum, which is the most commonly used commercial vaccine adjuvant, was not applicable. Therefore, the rovIL-1β was used as adjuvant. Control animals were vaccinated using conventional liquid injectable vaccines, either antigen in saline alone, antigen in saline with rovIL-1β adjuvant or antigen in saline with alum. Two types of silicone formulation, the matrix and the covered-rod were compared. However, dose-response analysis was undertaken using matrix formulations only. Formulations as indicated in Table 1 were delivered subcutaneously at day 0. At day 28, all sheep received a secondary administration of Avi in saline alone. This was designed to simulate an antigenic

Table 1

Vaccine formulations used to immunize sheep for comparison of implants to conventional vaccination and for dose-response studies of antigen and adjuvant in the formulations.

Group number	Formulation		
	Type	rovIL-1β (μg)	Avi (μg)
1	Matrix	0	500
2	Matrix	0	100
3	Matrix	0	10
4	Matrix	20	500
5	Matrix	20	100
6	Matrix	20	10
7	Matrix	50	500
8	Matrix	50	100
9	Matrix	50	10
10	Covered-rod	50	500
11	Saline	0	500
12	Saline	20	500
13	Alum	0	500

challenge and adjuvant was deliberately omitted so that the effects of the primary vaccine treatments could be readily distinguished. Blood samples were collected at intervals throughout the vaccination study to determine total anti-avidin antibody responses. Due to the large number of serum samples, sera from each group was pooled and titrated so that 50% response titres could be determined for each group in the same assay. In addition, serum samples from individual sheep were tested at a single dilution (1:400) so that statistical analysis could be performed, using first an analysis of variance (ANOVA) followed by Fisher's technique of pairwise comparison. Minitab™ software was used to perform the analysis. While the 50% response titres are shown in the figures, significant differences are indicated in the text and use a level of significance of 5%.

3.1. Comparison of silicone formulations to conventional vaccination methods

Figure 1 shows Avi and IL-1β release profiles of matrix (Group 7) and covered-rod (Group 10) formulations. Matrix formulation rapidly released Avi and IL-1β, and the release was almost finished within 7 days. On the other hand, covered-rod formulation constantly released them for 15 days.

Figure 2 shows antibody titres for sheep vaccinated with formulations of group 7 and 10, that received 500 μg of Avi and 50 μg of rovIL-1β in matrix or covered-rod formulations respectively. Also shown are groups 11, 12 and 13, which received 500 μg of Avi only, Avi

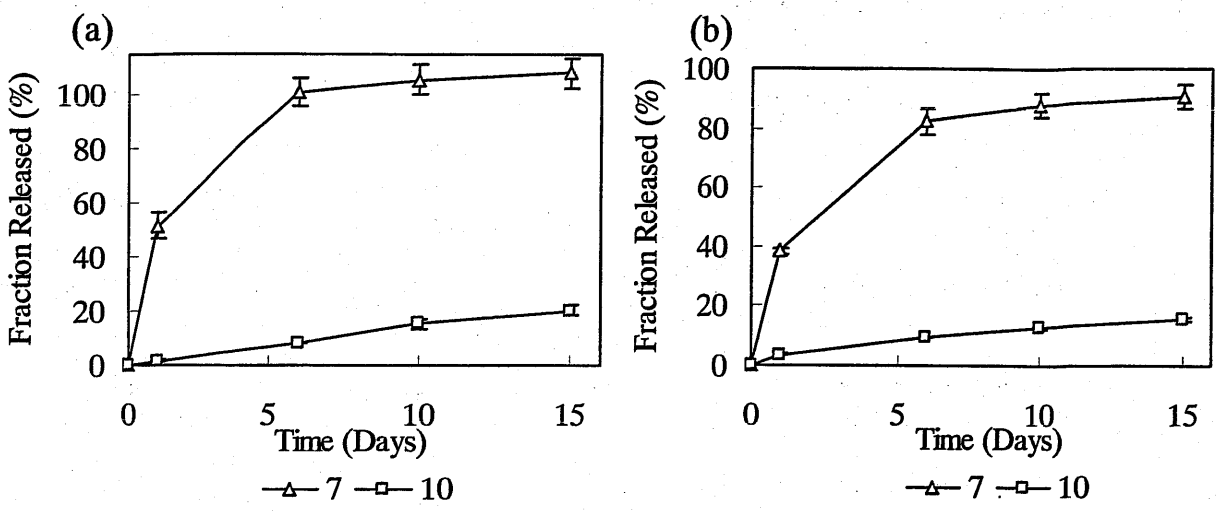

Fig. 1. (a) Avi and (b) IL-1β release profiles for matrix (Group 7) and covered-rod (Group 10) formulations. Each point represents mean ± deviation of data from two independent samples.

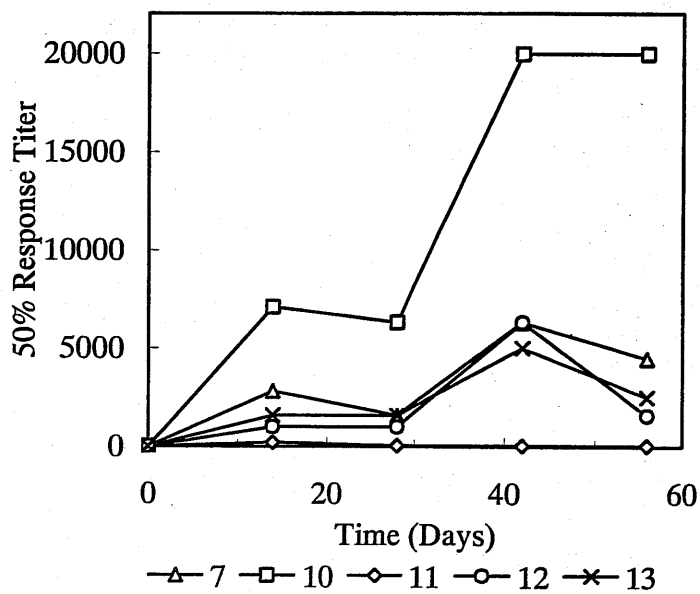

Fig. 2. Anti-avidin antibody responses induced following primary vaccination of sheep with either Avi in saline (◇, group 11), Avi in saline plus rovIL-1β (○, group 12), Avi in alum (×, group 13), or Avi and rovIL-1β in either the matrix (△, group 7) or covered-rod (□, group 10), followed by secondary vaccination at day 28 with Avi in saline. Results were obtained using serum pools from each experimental group.

in saline plus 20 μg of rovIL-1β, or Avi with alum adjuvant. As expected, priming of sheep with Avi in saline in the absence of adjuvant did not elicit any marked antibody response to the antigen (group 11). In contrast, the addition of rovIL-1β as adjuvant to this saline formulation resulted in a considerable enhancement of the antibody response, ranging from a 5-fold enhancement by day 14 post-primary vaccination to 30-fold by 14 days after the secondary administration of antigen (group 12). This confirms the adjuvant activity of rovIL-1β. The antibody response to antigen delivered in alum (group 13) was similar to that of the saline + rovIL-1V group (group 12), after both primary and secondary immunizations. When Avi and rovIL-1β were incorporated into the matrix formulation (group 7), the antibody response was slightly higher than those of the saline + rovIL-1V group (group 12) and alum adjuvanted group (group 13) after both primary and secondary immunizations. A marked enhancement was observed using covered-rod formulations comprising 50 μg of rovIL-1β and 500 μg of Avi, the only dose levels tested in the covered-rod in this experiment. Compared with the alum control group (group 13), the antibody titre elicited following a single delivery of the covered-rod formulation was equivalent to that observed after secondary immunization of the alum controls. After a second delivery of antigen to the covered-rod treatment group, the antibody response was rapidly elevated to a level 4-fold above that of the alum controls at day 14 post-secondary. Individual sample analysis showed that due to high variability typical of outbred sheep populations, the difference between the covered-rod group

response and the alum adjuvanted controls was not statistically significant. The covered-rod response was significantly higher than for the group that did not receive adjuvant in the immunizing formulation. While the antibody response then declines slowly in the matrix immunized group and the alum control group, there was no drop in titre in the covered-rod treatment group over days 14-28 post-secondary immunization. The use of the covered-rod implant, therefore, induced an antibody response markedly superior to that of conventional alum-based vaccination and demonstrated sustained antibody titres.

3.2. Antigen and adjuvant dose-response analysis with matrix formulations

In this initial trial, the dose-response relationship was assessed for only the matrix formulation. Results for sheep vaccinated with matrix formulations with either 10, 100 or 500 μg of Avi are shown in Figures 3a, 3b and 3c for groups adjuvanted with 0 μg, 20 μg or 50 μg of rovIL-1β, respectively.

The comparison of antibody responses induced by different doses of antigen in the formulation yielded a surprising result. Although there was no significant difference between groups, there was a trend towards the lowest dose of antigen (10 μg) inducing the highest antibody titres. This was observed at each dosage of rovIL-1β used and the difference was evident following primary vaccination and generally became more marked as the trial progressed. In contrast, elevating adjuvant dosages also elevated antibody titres.

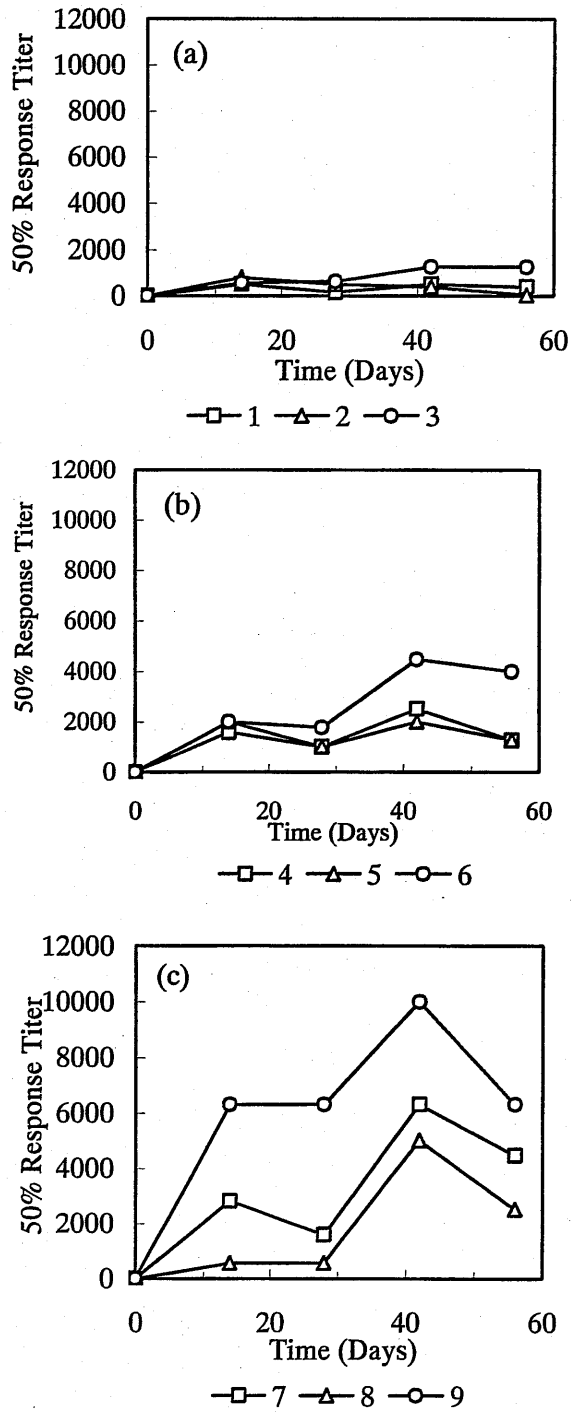

Fig. 3. Dose-response analysis for Avi and rovIL-1β administered in matrix formulations. Avi was administered at primary vaccination at either 10 μg (○), 100 μg (△) or 500 μg (□) per dose with either no adjuvant (a), or 20 μg of rovIL-1β (b) or 50 μg of rovIL-1β (c), followed by secondary vaccination with Avi in saline. Results were obtained using serum pools from each experimental group.

While there was minimal antibody induced in the absence of rovIL-1β (Fig 3a), titres observed at day 14 post-secondary vaccination ranged from 2000 - 4500 when 20 μg of rovIL-1β was used (Fig 3b), to 5000 - 10,000 when a dosage of 50 μg was used (Fig 3c).

3.3. Duration of immune response to silicone formulation vaccination

A vaccination study was undertaken that was similar to the first study except that a longer interval (69 days, rather than 28 days) was left between primary vaccination with the silicone formulations or conventional vaccine and secondary vaccination with antigen in saline alone. In this way, the duration of the response to the formulation could be better assessed. The Avi dose used was 500 μg for all groups, and formulations had either no adjuvant, or 50 μg of rovIL-1β as adjuvant, as indicated in Table 2. This experiment also included a control group that received alum plus rovIL-1β as an adjuvant combination (group 3). Group mean 50% response titres are shown in Figure 4. Similar to the initial experiment, there was minimal response to immunization with Avi in saline only (group 1). In addition, delivery of antigen without rovIL-1β adjuvant from either the matrix or covered-rod formulations did not elicit a higher response than the saline-vaccinated controls.

A moderate enhancement in antibody response was observed in the alum control group (group 2) and the matrix plus rovIL-1β group (group 5). For these groups, the antibody tires were 4 to 10-fold higher than for the saline control group at day 14 post-primary vaccination,

Table 2

Vaccine formulations used to immunize sheep for study of duration of response to silicone formulations.

Group number	Formulation		
	Type	rovIL-1β (μg)	Avi (μg)
1	Saline	0	500
2	Alum	0	500
3	Alum	50	500
4	Matrix	0	500
5	Matrix	50	500
6	Covered-rod	0	500
7	Covered-rod	50	500

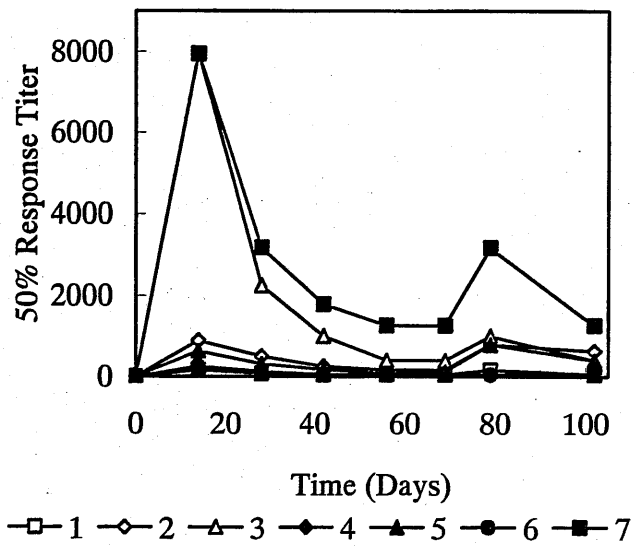

Fig. 4. Anti-avidin antibody responses induced following primary vaccination with either Avi in saline (□, group 1) or alum (◇, group 2) or alum and rovIL-1β (△, group 3) or Avi formulated without adjuvant into matrix (◆, group 4) or covered-rod (▲, group 5) formulations, or Avi formulated with rovIL-1β into matrix (●, group 6) or covered-rod (■, group 7) formulations, followed by secondary vaccination at day 69 with Avi in saline. Avi was administered at 500 μg per dose and rivIL-1β was administered at 50 μg per dose. Results were obtained using serum pools from each experimental group.

with these titres returning to pre-vaccination levels by day 56 post-primary. Following secondary vaccination with antigen and saline at day 69, antibody responses for groups 2 and 5 were 5-6 fold higher than for the saline control response.

A marked enhancement in antibody response was observed by day 14 post-primary for the treatment groups that received either an injection with Avi plus alum and rovIL-1β as adjuvant (group 3) and the covered-rod plus rovIL-1β group (group 7). At this time point, equivalent titres were recorded for both these groups, that were 9-fold higher than for the Avi plus alum vaccinated sheep (group 2). Beyond this time point, there was a rapid drop in titre for both groups, however, by 42, it was evident that the fall in titre for the covered-rod plus rovIL-1β group was occurring at a reduced rate compared to the alum/rovIL-1β controls. By day 69 post-primary vaccination, the covered-rod plus rovIL-1β group had a titre 4-fold higher than the alum/rovIL-1β group, and this titre was higher than that observed for any of the other treatment groups after secondary vaccination. Following secondary vaccination, a boost in titre was observed for the covered-rod plus rovIL-1β group. The titre did not reach the very high levels observed after the primary vaccination, but was 3-fold higher than for any other treatment group at 10 days post-secondary vaccination. In contrast to the first experiment where the post-secondary titre was maintained in the covered-rod + IL-1β group, where the secondary immunization was given at 69 days rather than 28 days the titre in the equivalent group began to decline immediately after vaccination. However, the titre was

remained consistently higher than for other treatment groups at both time points assessed after the secondary immunization.

3.4. Isotype-specific antibody response to silicone formulation vaccination

Serum samples collected 10 days following the second vaccination from the experiment shown in Table 2 were used to assess the isotype-specific antibody responses. These results are shown in Figure 5. As there is no reference serum available for sheep isotyping studies, the actual amount of each isotype present is unknown and the relationship between O.D. values and antibody quantities may not necessarily be the same for each isotype. The O.D. of each isotype and the relative proportions of each isotype however, may be compared between treatment groups. Low levels of IgM were observed for all treatment groups, with group 2, the alum-adjuvanted controls showing the lowest IgM titres. IgG1 was the dominant isotype for all treatment groups. While IgG2 was measured in all groups, the relative proportions differed, ranging from low levels (relative to IgG1 response) in the matrix/rovIL-1β group (group 5, G1/G2 ratio=7) to high levels in the covered-rod/rovIL-1β group (group 7, G1/G2 ratio=1.95). The covered-rod formulation, therefore, induces a response that favors production of both sheep IgG isotypes.

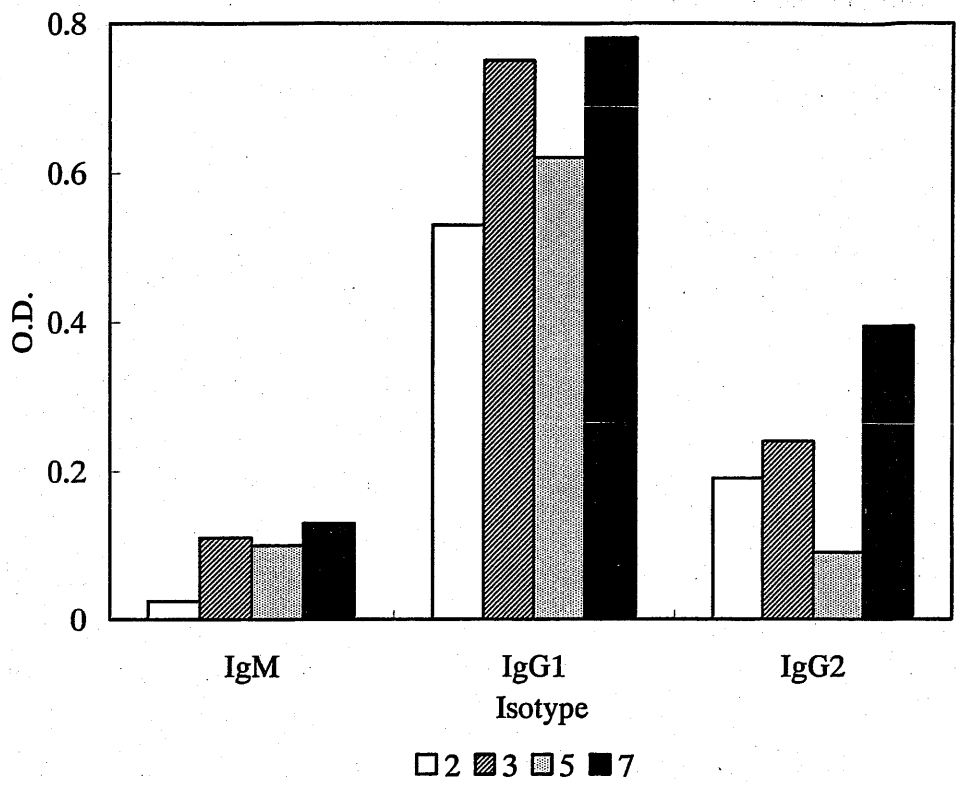

Fig. 5. Isotype-specific anti-avidin responses induced following primary vaccination with either Avi in either alum (group 2, white bars) or alum and rovIL-1β (group 3, diagonal striped bars) or Avi and rovIL-1β in either matrix (group 5, dotted bars) or covered-rod (group 7, black bars) formulations, followed by secondary vaccination at day 69 with Avi in saline. Avi was administered at 500 μg per dose and rovIL-1β was administered at 50 μg per dose. Results were obtained using serum pools from each experimental group.

3.5. Use of silicone formulations for delivering clostridial antigens

The antibody response to delivery of commercially relevant antigens was next assessed by administering either *C. tetani* toxoid, or *C. novyi* toxoid to sheep as described in Table 3. The optimum implant, covered-rod plus rovIL-1β was used and compared to delivery of the toxoids in either alum, similar to commercially available vaccines, or in alum plus rovIL-1β. Sheep were immunized at day 0 and again at day 28 with a formulation identical to the primary vaccination. Fig. 6a and 6b show the total antibody responses elicited against *C. tetani* and *C. novyi* toxoids, respectively. While after primary vaccination, similar responses were observed for each treatment group, following secondary vaccination a marked boosting in titres occurred in animal vaccinated with the covered-rod formulation. By day 42 (14 days post-secondary vaccination), the titre for the covered-rod groups (groups 3 and 6) exceeded those of the alum-adjuvanted controls (groups 1 and 4) by 5-fold (*C. tetani*) to 10-fold (*C. novyi*). While the titres rapidly dropped over the next 14 days, the rate of reduction decreased and by day 78 of the trial (50 days post-secondary), the titres for the covered-rod groups remained 5 to 10-fold higher than the alum controls. The addition of rovIL-1β to the alum formulation (groups 2 and 5) did not provide a significant elevation of antibody response over the alum controls as was observed for immunization with Avi. The protective ability of clostridial vaccination may be assessed by measuring the neutralizing antibody titre induced, and these results, obtained from samples collected 14 days after the

Table 3

Vaccine formulations used to immunize sheep for study of response to clostridial vaccination

Group number	Formulation		
	Type	rovIL-1β (μg)	Antigen
1	Alum	0	Tetanus toxoid
2	Alum	50	Tetanus toxoid
3	Covered-rod	50	Tetanus toxoid
4	Alum	0	Novyi toxoid
5	Alum	50	Novyi toxoid
6	Covered-rod	50	Novyi toxoid

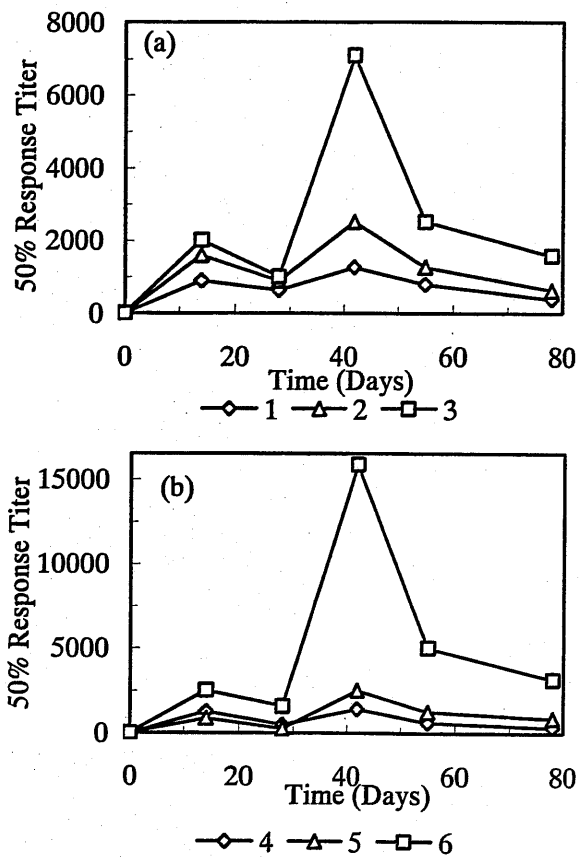

Fig. 6. Total antibody responses induced against two vaccinations at day 0 and 28 with either (a) *C. tetani* toxoid or (b) *C. novyi* toxoid administered in either alum (group 1 and 4, ◇) alum plus rovIL-1β (group 2 and 5, △) or covered-rod with rovIL-1β (group 3 and 6, □). Results were obtained using serum pools from each experimental group.

secondary vaccination are shown in Figure 7. For alum-adjuvanted groups, typical protective levels were observed, however, these levels could be enhanced by 2-fold to 4-fold by either combining rovIL-1β into the conventional alum formulation, or by administering the antigens with rovIL-1β using the covered-rod formulation.

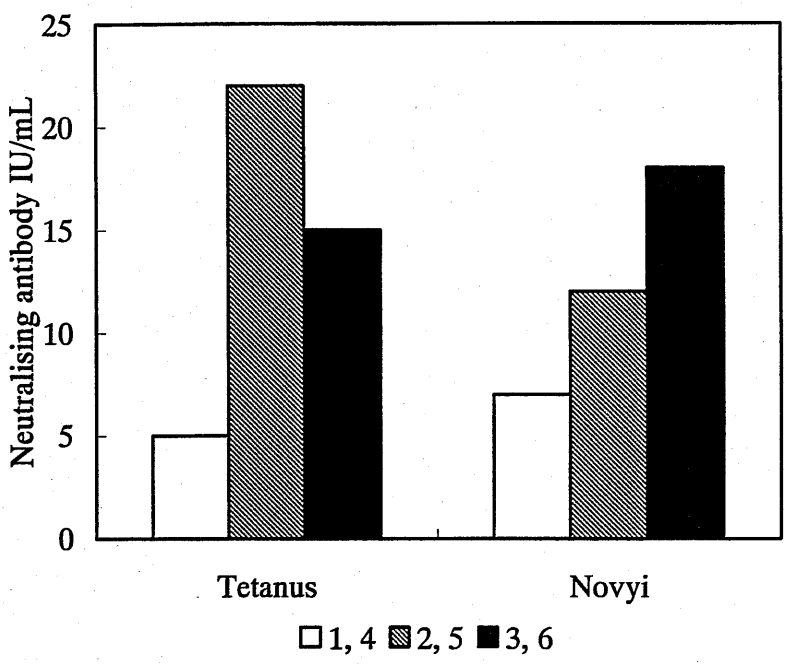

Fig. 7. Neutralizing antibody titres induced 14 days after secondary vaccination with clostridial toxoids formulated in either alum (group 1 and 4, white bars), alum plus rovIL-1β (group 2 and 5, diagonal striped bars) or covered-rod formulation with rovIL-1β (group 3 and 6, black bars).

4. Discussion

The delivery of vaccine antigen via controlled release formulations offers many potential benefits, including enhanced stability and shelf-life and reduction in dosage of antigen or adjuvant. Primarily, the use of formulations may result in delivery of vaccine in a single administration, eliminating problems of non-compliance and reducing administration costs substantially. While the current study examines the primary response elicited by silicone vaccine formulations followed by booster immunizations with either antigen in saline or in a second formulation, the covered-rod formulation has also shown potential as a single-dose vaccine delivery device [8].

Since the silicone formulation is restricted to use of dry powder materials, the use of alum adjuvant, whose structure does not retain integrity upon freeze-drying [9], is therefore excluded. However, there are a number of adjuvants that are suitable for use in the silicone formulations, including Quil A, a product that has been widely used in veterinary vaccines [10]. In the study described here, rovIL-1β, that has previously demonstrated adjuvant activity with both Avi and clostridial antigens were used [7, 11]. The silicone formulations are an ideal way to deliver cytokine adjuvants as they are able to maintain stability in the lyophilized state and are required in extremely small dose levels and may be easily incorporated into the silicone formulations. In addition, the silicone formulation confers increased safety for cytokine adjuvants that may have adverse effects if excessive amounts are

administered. The silicone formulation offers exquisite control over dose levels and there is also reduced risk to the operator as self-injection is less likely.

The immune response elicited by silicone formulations was compared to those elicited by antigen without adjuvant, antigen with soluble rovIL-1β and antigen with alum. The alum groups were used as controls as this adjuvant represents the most widespread adjuvant in use in human or animal vaccines. It is universally used as an adjuvant for clostridial toxoid vaccines. Alum typically induces moderate antibody responses, primarily of the IgG1 isotype, and little cellular immunity [12], and is reasonably safe for use with the exception of occasional sterile abscess or granuloma formation at the injection site [13].

The results indicate a typical variability in responses between different experiments which is not unusual when using outbred animals derived from a number of different breeding stocks. However, there was a consistent pattern of responses evident between similar treatment groups in different experiments. While the use of the matrix formulations elicited responses equivalent to those induced by alum, it was the covered-rod that induced a marked enhancement in response over alum control groups. This is surprising, because only low levels of antigen are released over a long period of time from the covered-rod formulation (Fig. 1). Similarly, it was the lowest dose of antigen released from the matrix formulations that induced the highest antibody response. From conventional immunological theory, increased antibody responses are generally elicited in response to increasing antigen doses,

although at the high and low dose extremes, antigenic tolerance is induced [14-16]. Low antigen doses have also resulted in a change to a cellular based response [17]. The difference in this case may have been the fact that release was continuous rather than pulsatile as occurs with needle immunization. In addition, the gradual release of antigen from the lyophilized state into the limited available body fluids would have resulted in high concentrations of antigen being delivered to the lymph node and may have been as effective at inducing a response as delivery of antigen highly diluted in saline. There was not, however, a similar inverse dose-response relationship for delivery of rovIL-1β. This may be attributed to the short in vivo half-life of rovIL-1β (2 minutes following intravenous delivery, [18]) so that the amount delivered was still a limiting factor in the response.

Although the silicone formulations are designed to released antigen continuously over a long period, the antibody response elicited declined soon after the primary immunization. While the titres induced by the covered-rod formulation were sustained at higher levels and for a longer time period that for other forms of immunization, it may be expected that continual antigen stimulation would maintain peak titres for some time. When very low levels of antigen are released, however, it is likely that this antigen would be adsorbed by the high titre circulating antibody and this may serve to limit the response. In addition, feedback mechanisms exist within the immune system to turn off the immune response and prevent the induction of pathology that may result from chronic stimulation of the antibody response

(reviewed in [19]).

Figures 2, 4 and 6 show significant antibody response in covered-rod groups after secondary immunization. Similar result was previously observed [8] and the mechanism is speculated as follows. Antigen delivered by soluble vaccination is transient. Therefore, the persistence of antigen was short and thus only limited number of B cells would be selected, expanded and become memory cells. On the other hand, when antigen was delivered by covered-rod formulations, whole repertoire of B cells was constantly stimulated, which should lead to production of superior number of memory cells having high affinity.

5. Conclusion

Silicone formulations offer a new method for vaccination that induces improved antibody responses and has the potential for single-dose immunization. They also will provide an essential tool in studying the immune response elicited by different antigen delivery profiles. Further studies will focus on the development of single-dose delivery systems and the use of alternative adjuvants to modulate the response to vaccination. In addition, the silicone formulations will also serve as a useful tool to assess the immune response induced by using a combination of continuous and conventional vaccination in either the primary and/or secondary vaccination.

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Part III

Development of Technique in Controlled Release of Silicone Formulation

Chapter 4

Novel method to control release of lipophilic drugs with high potency from silicone

1. Introduction

In the 1970s, much interest was focused on the use of silicone as a delivery system for lipophilic drugs. Two types of systems have been developed: reservoir type and matrix type. In the former, a core of drug is surrounded by a polymer membrane. Norplant® [1] is an example of commercialized reservoir system which is effective for five years with single administration. The principal advantage of reservoir-type systems is the ease with which constant release rates can be obtained. However, they are potentially dangerous because a tear in the reservoir could cause rapid release of all the incorporated drug. In the matrix-type systems, the drug is uniformly distributed in the polymer matrix. The principal advantage of matrix-type system is low cost. The principal disadvantage of matrix system is the first-order kinetics of drug release. Compudose® [2] is an example of commercialized matrix-type systems effective over longer than 100days with single administration. In this way silicone has been extensively investigated as a practical DDS carrier material. However, additional study is necessary for lipophilic drugs which are highly potent at low concentrations, because drug release should be strictly controlled. It has been reported that factors such as physicochemical properties of drugs and additives, membrane thickness and

drug-loading method influence the rate of drug release from silicone matrix [3-8]. Among these factors, the use of additives seems to be the most practical way to control drug release. Amphiphilic substances such as PEG or glycerin have been shown to enhance release of steroids from silicone matrix [5, 6, 8]. However, it was reported that these substances weaken physical strength of the silicone formulations [6]. In the present chapter, using vitamin D_3 (VD_3) as a lipophilic drug, which is highly potent at low concentrations, novel methods effective to suppress initial burst and to control rate of drug release were searched for.

HSA was used as an additive in order to establish unique method to control release of lipophilic drugs, avoiding problems caused by amphiphilic substances. Core-rod-type formulation was prepared and the drug release was investigated. Since the core is cured silicone, rapid leak would not occur in this type of formulation even if the membrane is broken. In addition, novel method to suppress initial burst and rate of drug release of reservoir-type system was investigated.

2. Experimental

2.1. Materials

Silicone samples (Silastic® MDX4-4210 Medical Grade Elastomer with Catalyst, Silastic® RX-50 Medical Grade Tubing, Silastic® Medical Adhesive Silicone Type A and

Silastic® Q7-4750 Biomedical Grade ETR Elastomer Kit) were provided by Dow Corning Corporation (MI, USA). Compositions and structures of MDX4-4210 and Q7-4750 are described in Chapter 1. RX-50 is a tube sample prepared using Q7-4750. Medical Adhesive Silicone Type A is a one-component silicone adhesive.

1α,25-$(OH)_2$ vitamin D_3 (VD_3) was purchased from Biomol Research Laboratories, Inc. (PA, USA). 2, 6-Di-*t*-butyl-*p*-cresol (BHT), DL-α-tocopherol (TP) and sodium azide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sudan II, which is a dye modeling for lipophilic drugs, was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) Buminate® were purchased from SIGMA CHEMICAL CO. (MO, USA) and Baxter Healthcare Corporation (IL, USA), respectively.

2.2. Preparation of VD_3 silicone formulations

Figure 1 shows the structure of the silicone formulations, and Table 1 lists their compositions.

Matrix formulation (VD-1 and VD-2)

The base and the curing agent of MDX4-4210 were mixed in a mass ratio of 10:1. TP and BHT (0.5%w/w each) were added to this mixture as anti-oxidants. Freeze-dried HSA

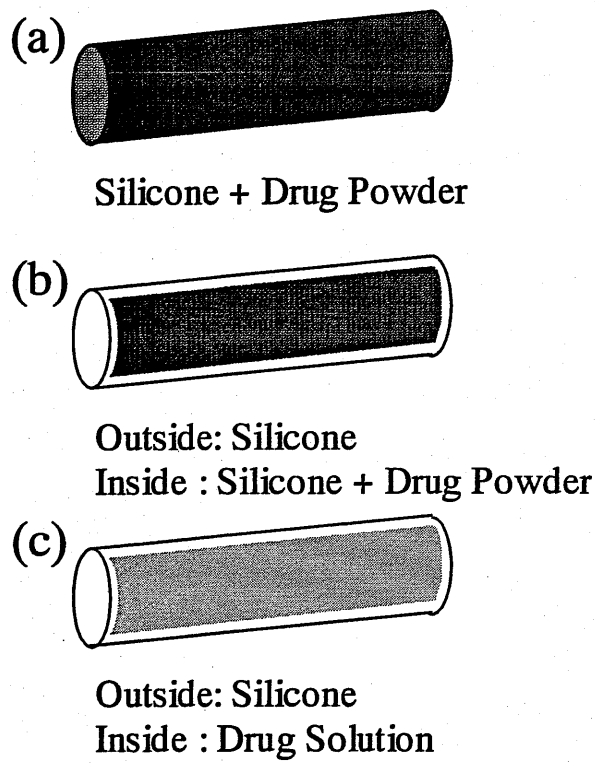

Fig. 1. Silicone formulation: (a) matrix formulation (VD-1 and VD-2), (b) core-rod formulation (VD-3), (c) reservoir formulation (VD-4).

Table 1 Composition of VD_3 Silicone Formulations

		Formulation			
	Content	Type	Size	Additive	Silicone
VD-1	43μg	Matrix	5ϕ × 10	-	MDX4-4210
VD-2	45μg	Matrix	5ϕ × 10	HSA 30%w/w	MDX4-4210
VD-3	32μg	Core-rod	3ϕ × 15	-	Inside: MDX4-4210 Outside: Q7-4750
VD-4	38μg	Reservoir	3ϕ × 15	TP	Q7-4750

powder (434.8 mg) was mixed with 1.00 g of MDX4-4210 to prepare VD-2. Each mixture was cast into a sheet, and 130 μl of VD_3 ethanol solution (concentration: 1.0 mg/ml) were placed on each film. Then, ethanol was removed under nitrogen flow and each mixture was kneaded to have VD_3 homogeneously distributed. Each mixture was then deformed by centrifugation, filled into a Teflon tube with an inner diameter of 5.0 mm, and allowed to stand for 24 h to cure. After curing, each mixture was taken out of the Teflon tube and cut into 10-mm-long pieces.

Core-rod formulation (VD-3)

MDX4-4210 (277 mg) containing 0.5%w/w TP and 0.5%w/w BHT was mixed with 186 μg VD_3 and deformed by the same method as for matrix formulation. The mixture (52 mg) was filled in the middle of an RX-50 tube (3.0 mm outer diameter, 2.0 mm inner diameter, 50 mm length) and allowed to stand for 24 h to cure the core section. MDX4-4210 was introduced into the tube at both ends to seal; the mixture was allowed to stand for 24 h and the ends of the tube were cut off to adjust the length of the sealed portion at 2 mm.

Reservoir formulations (VD-4)

A small amount of MDX4-4210 was filled into an RX-50 tube (3.0 mm outer diameter, 2.0 mm inner diameter, 50 mm length) from both ends of the tube and cured to prepare a

container with the length of the hollow cylinder of 15 mm. The container was degassed by sticking one end with a needle, and a TP solution (46 μl) of VD_3 (concentration: 0.94 mg/ml) was injected from the other end using an syringe. The two holes were sealed with Medical Adhesive Silicone Type A.

2.2.3. Diffusion of Sudan II in silicone

MDX4-4210, MDX4-4210 containing 30%w/w HSA, and Q7-4750 were cured in a 35-mm plastic petri dish. Sudan II (1 mg) was placed at the middle of the petri dish, and the diffusion of Sudan II was observed either visually or with a digital microscope VH-6200 (KEYENCE CORPORATION, Osaka, Japan).

2.2.4. VD_3 release study

Formulations were placed in a brown-colored glass test tube containing 3 ml of phosphate buffered saline (PBS, pH 7.4) containing 0.05% BSA and 0.01% sodium azide and allowed to stand at 37°C. The amount of VD_3 released into PBS during a specified period was determined by reversed-phase HPLC. HPLC was performed by an isocratic condition and the mobile phase was 55% acetonitrile. The flow rate was 1.0 mL/min. Waters μ-Bondasphere 5μ C18-100Å column (3.9 × 150 mm) was used and UV detection was performed at 265 nm.

3. Results

3.1. Diffusion of Sudan II in silicone

The effect of HSA added to MDX4-4210 on the diffusion of lipophilic drugs was investigated using Sudan II as a model. The effects of different silicone matrices on the diffusion rate of Sudan II were also investigated. Since silicone is transparent and Sudan II colors red, the trace of Sudan II diffusion can be detected by coloration. The area Sudan II diffusion in 3 days was largest with MDX4-4210. There was not large difference between MDX4-4210 containing 30%w/w HSA and Q7-4750 (Fig. 2-b1). Observation by a digital microscope (x300) showed that the MDX4-4210 sheet was dyed homogeneously (Fig. 2-b2). On the other hand, in the MDX4-4210 sheet containing 30%w/w HSA, only silicone was dyed, keeping HSA unchanged (Fig. 2-b3). These results showed that diffusion profile of Sudan II, modeling for a lipophilic drug, could be controlled by adding HSA to a silicone matrix.

3.2. Controlled release of VD_3 by HSA

Matrix formulations VD-1 without additives and VD-2 with 30%w/w HSA continuously released VD_3 for one month (Fig. 3a). While VD-1 released 11.8% of VD_3 in the first day, VD-2 released only 5.5%. The average rates of VD_3 release in the later stage,

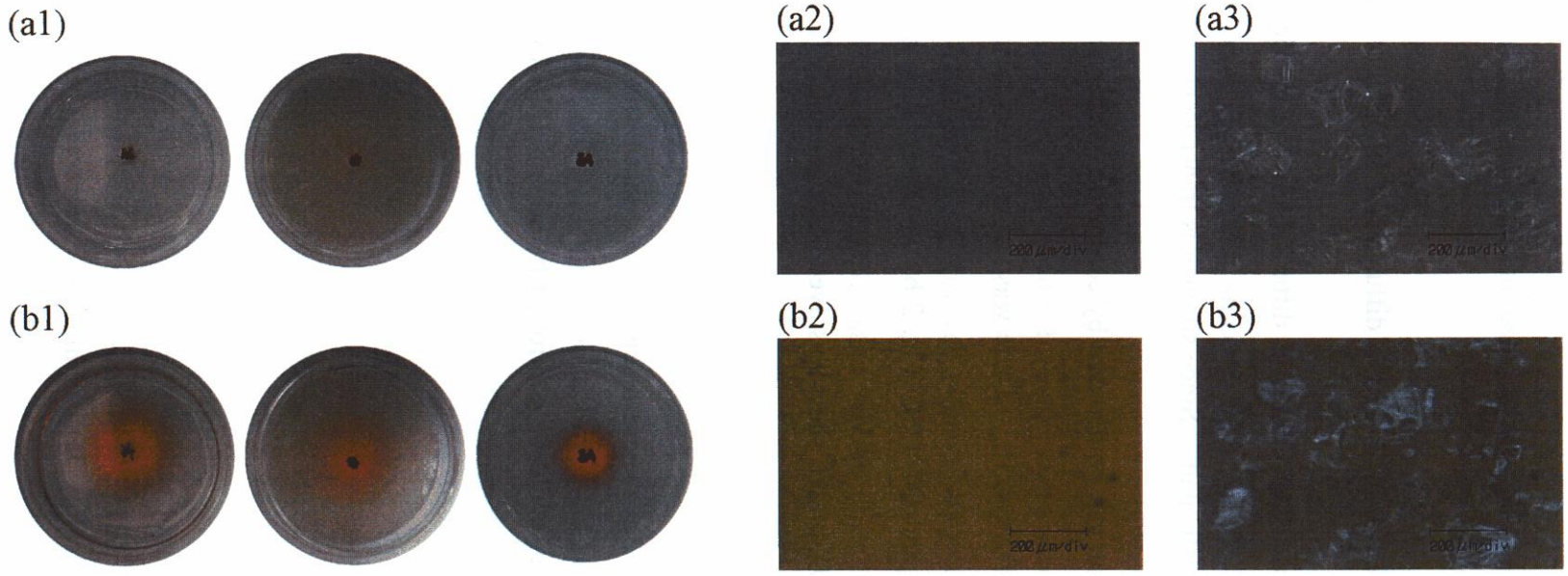

Fig. 2. Diffusion of Sudan II in silicone: (a1) initial; from the left, MDX4-4210, 30%w/w HSA MDX4-4210, Q7-4750, (a2) MDX4-4210 initial (x300), (a3) 30%w/w HSA MDX4-4210 initial (x300), (b1) after 72 h; from the left, MDX4-4210, 30%w/w HSA MDX4-4210, Q7-4750, (b2) MDX4-4210, after 72 h (x300), (b3) 30%w/w HSA MDX4-4210, after 72 h (x300).

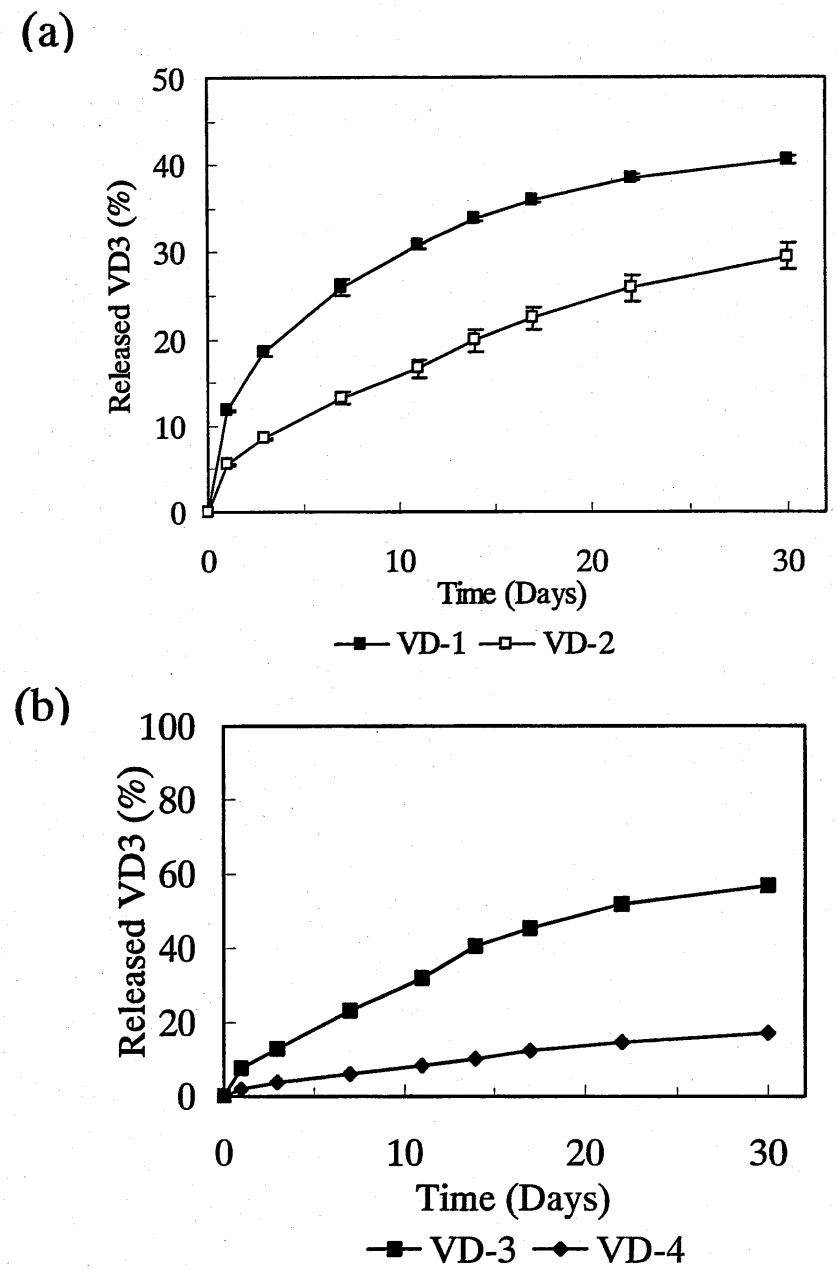

Fig. 3. Release of VD_3 from silicone formulations: (a) controlled release by addition of HSA, (b) controlled release from different types of formulations (average for n=2).

that is, 14-30 days, were calculated from Fig.3a. The results were 0.42%/day for VD-1 and 0.61%/day for VD-2. The monthly amount of release was 41% for VD-1 and 30% for VD-2. Thus, the release of VD_3 representing a lipophilic drug could be controlled by adding HSA to a silicone matrix.

3.3. Controlled release of VD_3 from different types of formulation

The release of VD_3 from VD-1 (matrix formulation) was initially rapid and gradually decreased (Fig. 3a). On the other hand, the release from VD-3 (core-rod formulation) and VD-4 (reservoir formulation) continued almost constantly after small initial burst (Fig. 3b). The release of VD_3 from VD-4 was slower than that from VD-3, and the monthly amount of release was 57% for VD-3 and 17% for VD-4. Thus, the release of VD_3 could also be controlled by VD_3 incorporating form in silicone matrix.

4. Discussion

A number of investigations have been carried out on the sustained release of lipophilic drugs from silicone matrices. It has been reported that substances such as glycerin and PEG promoted the release of lipophilic drugs by inducing swelling of the formulation [5, 6]. Pfister et al. [6] reported that the release rate of testosterone was decreased by adding titanium dioxide or barium sulfate to a

silicone carrier. However, they did not clarify what factors were affected by these additives. When a drug is dissolved in a cylindrical matrix formulation, the rate of drug release is given by eq. 1 [9],

$$\frac{M_t}{M_\infty} = 4\left(\frac{Dt}{pr^2}\right)^{\frac{1}{2}} - \frac{Dt}{r^2} \quad \left(\text{in case of } \frac{M_t}{M_\infty} \leq 0.4\right) \tag{1}$$

where M_t is the amount of drug released at time t, M_∞ is the total amount of drug contained in the formulation, D is the diffusion coefficient in a carrier material, and r is the diameter of the formulation. Eq. 1 shows that the rate of drug release can be controlled by varying D under a constant r.

In general, matrix formulations show an initial rapid release. According to eq. 1, decrease of D in the initial period should be effective to suppress initial rapid release. From the fact that the release rate of testosterone was decreased by insoluble substances such as titanium dioxide, D of testosterone in silicone should be suppressed by these substances. It is considered that titanium dioxide, which is immiscible with testosterone and silicone, blocked the diffusion of testosterone. HSA, which is water-soluble and therefore immiscible with lipophilic drugs and silicone, is expected to have a similar effect. Since HSA does not swell strongly, significant promotion of drug release would not occur unlike glycerin or PEG. In addition, when 30%w/w HSA is added to silicone, it is gradually released from silicone

matrix as shown in Chapter 2. Therefore, reduction of D by HSA addition should occur only in the early period of release experiments.

In this investigation, it was found that the addition of HSA to a silicone matrix delayed the diffusion of Sudan II (Fig. 2-b1). This might have occurred by the HSA particle blocking Sudan II diffusion in the silicone matrix. In a digital microscope observation, silicone matrix in the area of Sudan II diffusion colored red, but HSA particle was not dyed (Fig. 2-b3). From this observation, VD_3 is considered not to diffuse through HSA particle, but through silicone phase. This finding supports the above consideration, that is, HSA blocked the diffusion of VD_3 in a silicone matrix.

The change in D by the addition of HSA was reflected in the release of VD_3 from a silicone carrier. The initial release rate of VD-2 was slower than that of VD-1 which does not contain any additives (Fig. 3a). However, the rate of VD_3 release of VD-2 in the period of 14-30 days was 1.5 times as large as that of VD-1. This reversal could be explained as following. When HSA exists in silicone matrix, it disturbs VD_3 diffusion. However, once HSA is released, VD_3 diffusion is no longer suppressed. Since water-filled channels are formed inside the formulation, the contact of remaining VD_3 with water becomes easier than the initial stage to accelerate VD_3 release (Fig. 4). Because HSA has high molecular weight,

only low osmotic pressure is induced by its solution. Even so, cracking might slightly occur during release of HSA, which leads to decrease of cross-linking density of silicone matrix. This incidence might increase VD_3 diffusion rate in silicone matrix and enhance release of VD_3.

Aiming at a constant release, the core-rod (VD-3) and reservoir (VD-4) formulations were prepared. When a lipophilic drug is enclosed within a polymer container beyond the saturated concentration, the release rate of the drug is given by eq. 2 [9],

$$\frac{dMt}{dt} = \frac{2\pi hDK\Delta C}{\ln\left(r_o/r_i\right)} \qquad (2)$$

where dM_t/dt is the drug-release rate at time t, h is the length of core in the formulation, K is the distribution coefficient of the drug between the membrane and the core, ΔC is the difference in concentration between the outside and the inside of the membrane, and r_0 and r_i are the outer and inner diameters of the formulation, respectively. While drug exists over the saturated concentration, ΔC is constant and the drug is released at a constant rate during the steady state. In the case of core-rod formulations, diffusion rate of drugs in membrane material (D_2 in Fig. 4b) has to be smaller than that in the core material (D_1 in Fig. 4b) in order to have a constant release. When the diffusion rates of Sudan II in MDX4-4210 and Q7-4750 were compared, it was found that the distance of Sudan II diffusion during 3 days

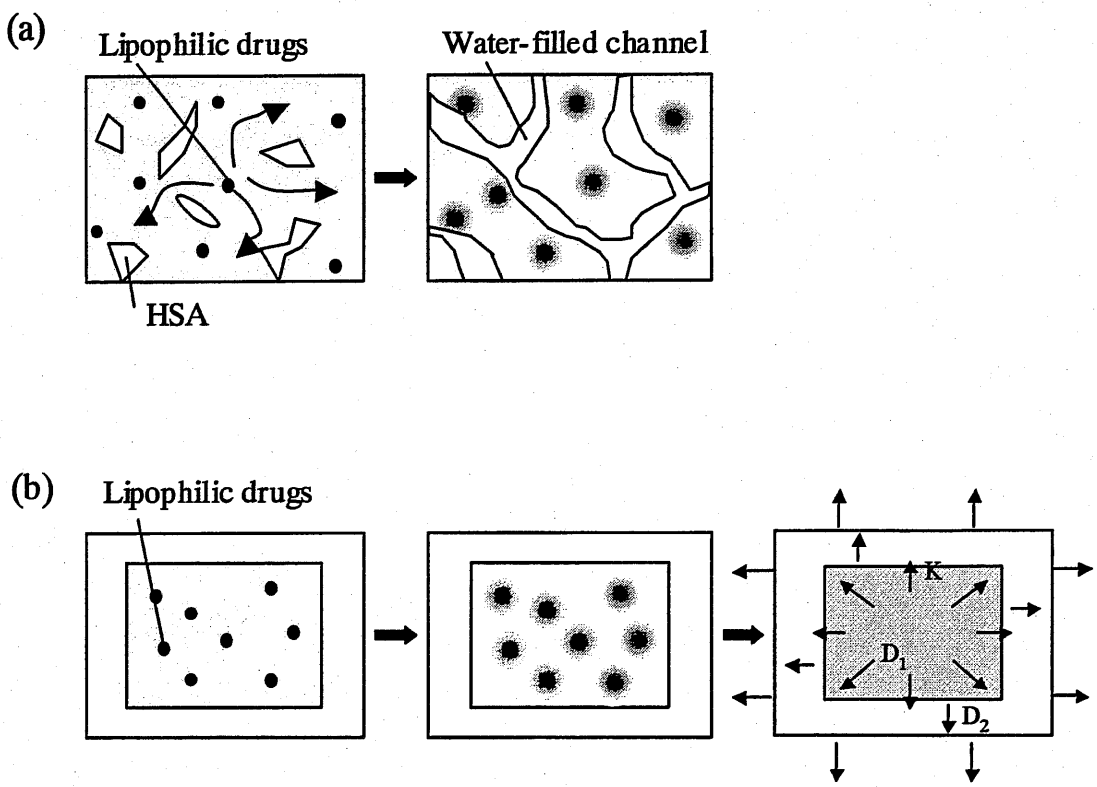

Fig. 4. Diagram showing the release process of lipophilic drug from silicone formulations: (a) addition of HSA suppresses initial burst and enhances release rate in the later stage, and (b) covering a matrix formulation with a membrane of low diffusivity (core-rod formulation) suppresses initial burst and releases drug in a constant rate. In addition, using materials for which the drug has high affinity (reservoir formulation), the drug-release rate is reduced.

was shorter than that in MDX4-4210 (Fig. 2-b1). Therefore, the diffusion of lipophilic drugs in Q7-4750 should be slower than that in MDX4-4210. While a base siloxane of MDX4-4210 has vinyl groups only at ends of molecules, that of Q7-4750 has vinyl groups not only at ends but also at side chains (Figs. 1 and 2 in Chapter 1). This fact should result in higher cross-linking density of Q7-4750 than MDX4-4210. Based on the result, an RX-50 tube made from Q7-4750 was used as a membrane. It was found that VD_3 was released from VD-3 *in vitro* at an almost constant rate (Fig. 3b). Based on this finding, the VD_3 concentration in the core of VD-3 was considered to exceed the saturation value, and the RX-50 tube acted as a diffusion barrier. This is also supported by the fact that, although VD_3 content per unit volume of the VD-3 core is higher than that of VD-1, the amount of VD_3 released per unit surface area on the first day was smaller for VD-3 (0.016 $\mu g/mm^2$) than for VD-1 (0.025 $\mu g/mm^2$). In contrast, with respect to VD-3, the release rate gradually decreased after 14 days (accumulated amount of release: approximately 40%). Based on this finding, approximately 60% of the initial amount of VD_3 in VD-3 (approximately 400 $\mu g/cm^3$) is considered to be the saturated concentration in MDX4-4210. VD_3 was also released at a constant rate from VD-4 as well as VD-3, but the release rate of VD-4 was approximately 1/3 as low as that of VD-3. Among the parameters shown in eq. 2, VD_3 content, formulation size and membrane material are the same in VD-3 and VD-4. The only difference exists in K. VD_3 exists in MDX4-4210 in the case of VD-3, and in TP in the case

of VD-4. Probably K is smaller in the RX-50/TP pair than in the RX-50/MDX4-4210 pair (VD_3 has a higher affinity to TP than to MDX4-4210).

5. Conclusion

Addition of HSA to a silicone matrix suppresses the initial burst of VD_3 by reducing the diffusion rate. Having HSA released, the rate of drug release is increased. Namely, addition of HSA enables to release VD_3 from matrix formulation in almost constant rate. Covering the surface of a matrix formulation with a membrane of low diffusivity suppresses initial burst and maintain constant rate of drug release. In addition, using materials for which the drug has high affinity as dissolution solvent, the drug-release rate can be reduced.

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Chapter 5

Novel drug delivery device using silicone: controlled release of insoluble drugs or two kinds of water-soluble drugs

1. Introduction

The mechanism of drug release from silicone carrier differs depending on the physiochemical properties of the drugs as illustrated in Fig. 1. In the 1980s, although silicone was considered to be unsuitable for the delivery of water-soluble macromolecules, Hsieh et al. [1] and Fujioka et al. [2-4] reported that proteins could be continuously released from silicone. In this way, many studies have been conducted on DDS using silicone. However, investigations have not been perfect to satisfy various medical requirements. First, sustained release of insoluble drugs has been desired. On purpose to develop new vaccine formulations which are effective for a long period of time, sustained release of antigens from silicone carrier has been attempted. It was reported that a superior immunity was obtained by sustained release of avidin (Avi) as an antigen model from silicone carrier compared to injection of an aqueous Avi solution [5, 6]. Similar effects are expected by sustained release of live vaccines and inactivated vaccines. Such vaccines contain viruses or bacteria as antigens, which are insoluble either in water or in organic solvents. However, sustained release of insoluble drugs from silicone has not been reported so far. Second, simultaneous release of two kinds of water-soluble drugs is often required. Depending on the nature of

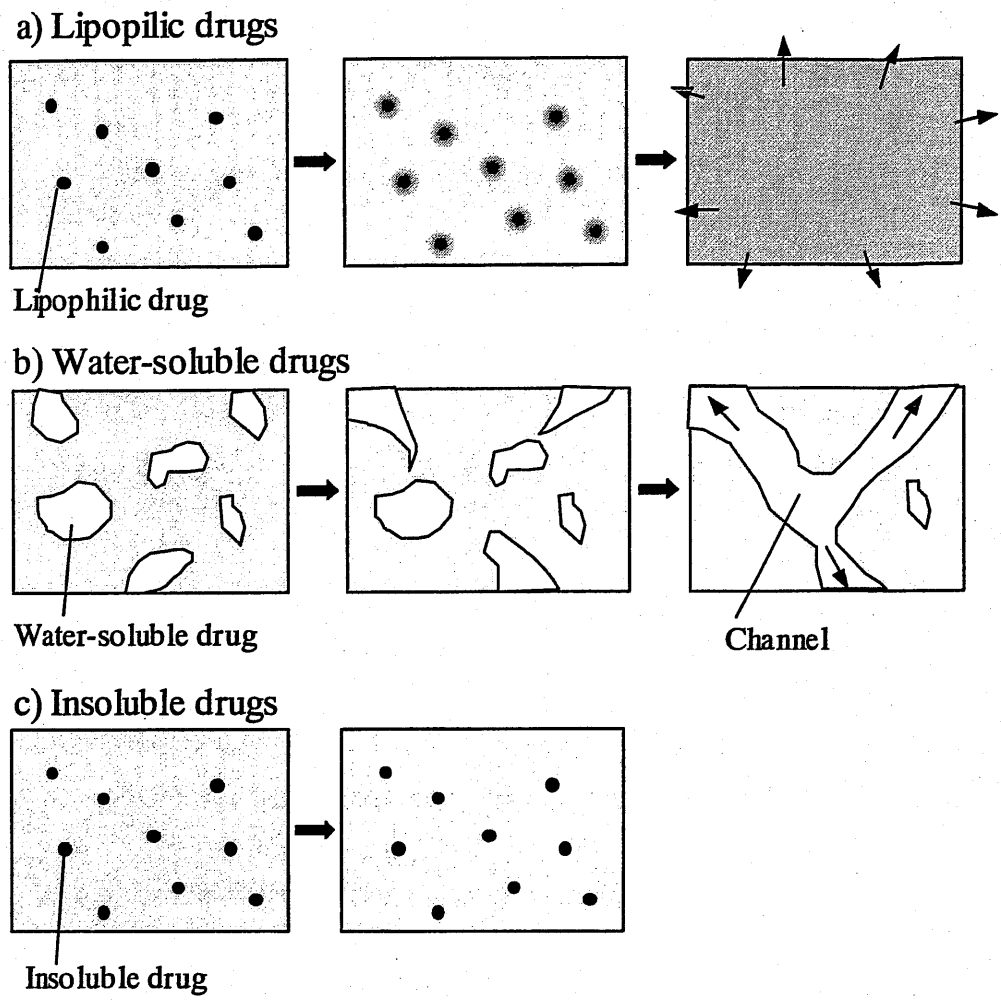

Fig. 1. Release mechanism of drugs with different physiochemical properties from a silicone carrier. (a) Lipophilic drugs are solubilized in silicone, diffuse, and are released. (b) Water-soluble drugs neither dissolve nor diffuse in silicone. At first, water-soluble drug existing on the surface area, and then, that existing in the near-surface area dissolve into water. Repetition of these processes leads to channel formation, allowing water-soluble drugs in the bulk to be released. (c) Insoluble drugs neither dissolve nor diffuse in silicone. They are not released because channels are not formed due to insolubility in water.

disease, combined use of multiform drugs could yield synergistic effect. For example, simultaneous release of antigens and ajuvants is expected to improve its efficacy. When multiform drugs are released, the release pattern of each drug should be optimized to obtain the maximum therapeutic effect. For example, since adjuvant sometimes induces strong inflammatory reactions at the administration site, a short release period is preferable. On the other hand, sustained release of antigens for a long period is desirable to maintain the effects. In this way, the development of the method to release two kinds of water-soluble drugs in a suitable release profile should be indispensable.

In this chapter, technologies to control the release of drugs with various physicochemical properties from silicone carriers were investigated.

2. Experiments

2.1. Materials

Silastic® Q7-4750 Biomedical Grade ETR Elastomer Kit was provided by Dow Corning Corporation (MI, USA). Composition and structure of Q7-4750 is described in Chapter 1.

Citric acid (CA), sodium bicarbonate (SB), glycine (Gly), sodium citrate (SC), mannitol (Man) and sodium azide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). As a model for insoluble drugs, polystyrene beads (PSTB) were used. For PSTB, Bacto

Latex® 0.81 (0.81 μmϕ) and Fluoresbrite® plain microspheres YG (1 and 20 μmϕ) were purchased from Difco Laboratories, Inc. (MI, USA) and PolyScience, Inc. (IL, USA), respectively. Human lymphocyte interferon (natural α-type interferon, IFN) obtained from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan) was used. IL-1β was provided by Dr. Lofthouse of The University of Melbourne. Bovine serum albumin (BSA) purchased from SIGMA CHEMICAL CO. (MO, USA), human serum albumin (HSA) Buminate® from Baxter Healthcare Corporation (IL, USA), high-grade gelatin from Nippi, Inc. (Tokyo, Japan), and avidin (Avi) from Boehringer Mannheim GmbH (Werk Penzberg, Germany) were used.

2.2. Preparation of formulations

Figure 2 shows the structure of the silicone formulations, and Table 1 lists their compositions.

2.2.1. Preparation of PSTB silicone formulations

Bacto Latex® 0.81 was used for the preparation of PSTB-1 and 2, and Fluoresbrite® was used for the preparation of other formulations. For example, PSTB-6 was prepared as follows. PSTB (1-μm diameter) was washed with water, filtered using a 0.22-μm membrane filter and dried under vacuum. This PSTB (60 mg) was then mixed with 3.63 g of CA solution (100 mg/ml) and freeze-dried. The freeze-dried PSTB/CA mixture was

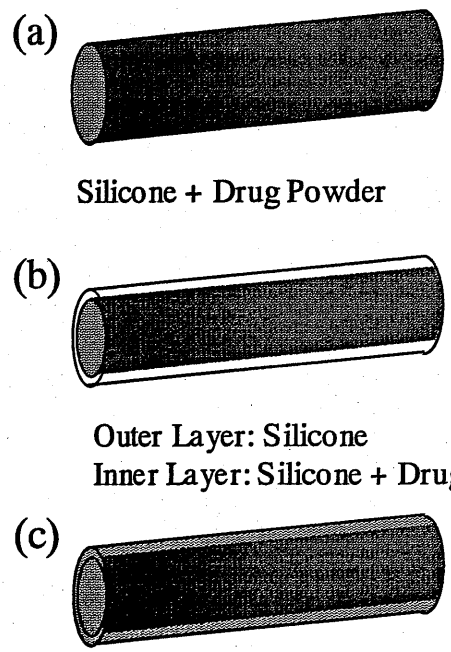

Fig. 2. Silicone formulation: (a) matrix formulation, (b) covered-rod formulation, (c) double-layered formulation.

Table 1 Composition of silicone formulations

	Model Drug			Formulation		
		Solubility	Content	Type	Size(mm)	Additive**
PSTB-1	PSTB (1μm)*		437μg	Matrix	2ϕ × 10	Gly 30%w/w
PSTB-2	PSTB (1μm)*		469μg	Matrix	2ϕ × 10	CA:SB=1:1.5, 30%w/w
PSTB-3	PSTB (1μm)		886μg	Matrix	2ϕ × 10	-
PSTB-4	PSTB (1μm)	Insoluble	650μg	Matrix	2ϕ × 10	Gly 30%w/w
PSTB-5	PSTB (1μm)		508μg	Matrix	2ϕ × 10	CA:SB=1:3, 15%w/w
PSTB-6	PSTB (1μm)		676μg	Matrix	2ϕ × 10	CA:SB=1:3, 30%w/w
PSTB-7	PSTB (20μm)		672μg	Matrix	2ϕ × 10	CA:SB=1:3, 30%w/w
IFN/HSA-1	IFN		6MU	Matrix	2ϕ × 10	Gly 10%w/w
	HSA		7.8mg			
IFN/HSA-2	Inside: IFN		6MU	Covered-rod	2ϕ × 10	Inside: Gly 10%w/w
	Inside: HSA	Water-soluble	7.2mg			
IFN/HSA-3	Inside: IFN		7MU	Double-layer	2ϕ × 10	Inside: Gelatin 30%w/w
	Outside: HSA		4.6mg			Outside: -
IL-1β/Avi-1	Inside: Avi		868μg	Double-layer	2ϕ × 10	Inside: SC:Man=1:2, 30%w/w
	Outside: IL-1β		17μg			Outside: SC:Man=1:2, 30%w/w

*PSTB; not fluorescent

**Additives; kind of additives and their content in the formulation

passed through a sieve with a 300-μm mesh to obtain PSTB/CA powder of the diameter less than 300 μm. In the same way, SB powder was prepared. After mixing with 0.70 g each of Q7-4750 Part A and Part B, PSTB/CA powder (282.25 mg) and SB powder (317.75 mg) were added and homogeneously mixed. The mixture was filled into a syringe, extruded through a 1.6-mm-diameter nozzle, and cured at 37°C for 1 day. The cured mixture was cut into 10-mm-long pieces.

2.2.2. Preparation of double-layered silicone formulations

The preparative methods for IFN/HSA-1 (matrix type) and IFN/HSA-2 (covered-rod type) have been described in Chapters 1 and 2 [3, 4]. For example, double-layered formulations IFN/HSA-3 was prepared as follows. An aqueous solution (28.2 g) of IFN (114 MU/ml) was mixed with 2.5 g gelatin and freeze-dried. The freeze-dried cake was ground under a nitrogen to obtain IFN/gelatin powder. Q7-4750 Part A and Part B (1.05 g each) were mixed with 0.90 g IFN/gelatin powder and filled into a syringe. Q7-4750 Part A and Part B (17.5 g each) were mixed with 15.0 g of HSA powder and filled into other syringe. The mixtures were extruded through a concentrically arranged nozzle and die (orifice diameter of 1.6 and 1.9 mm) to have IFN/gelatin-containing silicone inside and HSA-containing silicone outside. This was cured at 25°C for three days. The resultant sample was cut into 10-mm-long pieces.

2.3. Evaluation of drug release from formulations

2.3.1. PSTB silicone formulations

Formulations were placed in a test tube containing 2 ml of PBS (pH 7.4) containing 0.1% pluronic and 0.01% sodium azide. The test tube was gently shaken at 37°C. The amount of PSTB released into PBS during a specified period was determined using Fluoroscan II (LabSystems, Co., Finland) with the emission wavelength at 485 nm, and the detection wavelength at 538 nm.

2.3.2. Double-layered silicone formulations

Formulations were placed in a test tube containing 2 ml of PBS (pH 7.4) containing 0.3% Tween 20 and 0.01% sodium azide and allowed to stand at 5°C. At specified time periods, the amount of IFN released was determined by radioimmunoassay (RIA) and the amounts of HSA, IL-1β and Avi released were determined by enzyme immunoassay (EIA). Since IFN and IL-1β are rather unstable proteins, their stability in the buffer solution during the sampling intervals was confirmed in advance of the *in vitro* release study.

3. Results

3.1. PSTB silicone formulations

Figure 3a shows the appearance of PSTB-1, 2 and 3 after immersion in PBS for 15 days. PSTB-1 gently swelled and retained its original shape. PSTB-2 significantly swelled and the surface layer was broken. Change was not observed in PSTB-3. Figure 3b shows PSTB-releasing profile of PSTB silicone formulations. PSTB was not released from PSTB-3, which contained no additives. Similarly, from PSTB-4 containing 30%w/w Gly, PSTB was not released. On the other hand, PSTB was released from PSTB-5, 6 and 7 in which PSTB/CA powder and SB powder were distributed. The amount of released PSTB from PSTB-6 and 7, both containing 30%w/w PSTB/CA and SB powders, was larger than that from PSTB-5, containing 15%w/w PSTB/CA and SB powders. These results indicate that PSTB can be release from silicone by using CA and SB as additives, and its release rate can be controlled by amount of CA and SB incorporated in silicone matrix. The particle size of PSTB (1 μm or 20 μm) did not affect the amount of PSTB released.

3.2. Simultaneous controlled release of two kinds of water-soluble drugs

3.2.1. Release from matrix and covered-rod formulations

Figure 4a shows the release of IFN and HSA from IFN/HSA-1 (matrix) and IFN/HSA-2 (covered-rod). The drug release from the matrix formulation was much faster than that from the covered-rod formulations. In the matrix formulation, an initial burst

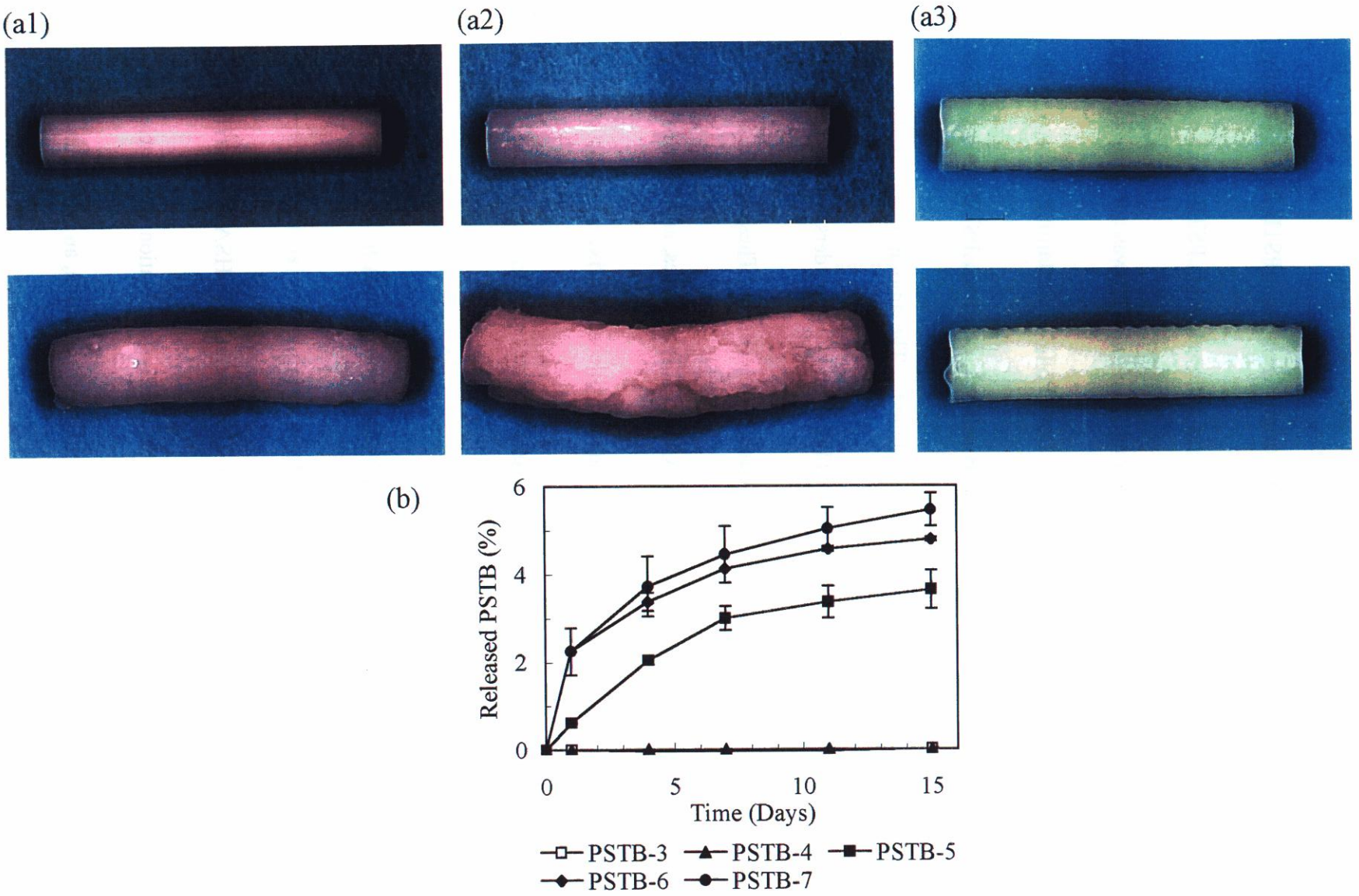

Fig. 3. (a) Appearance of PSTB silicone formulations; top, before, bottom, after 15-day immersion in PBS; (a1) PSTB-1, (a2) PSTB-2, (a3) PSTB-3, (b) release of PSTB from silicone formulations (average for n=2).

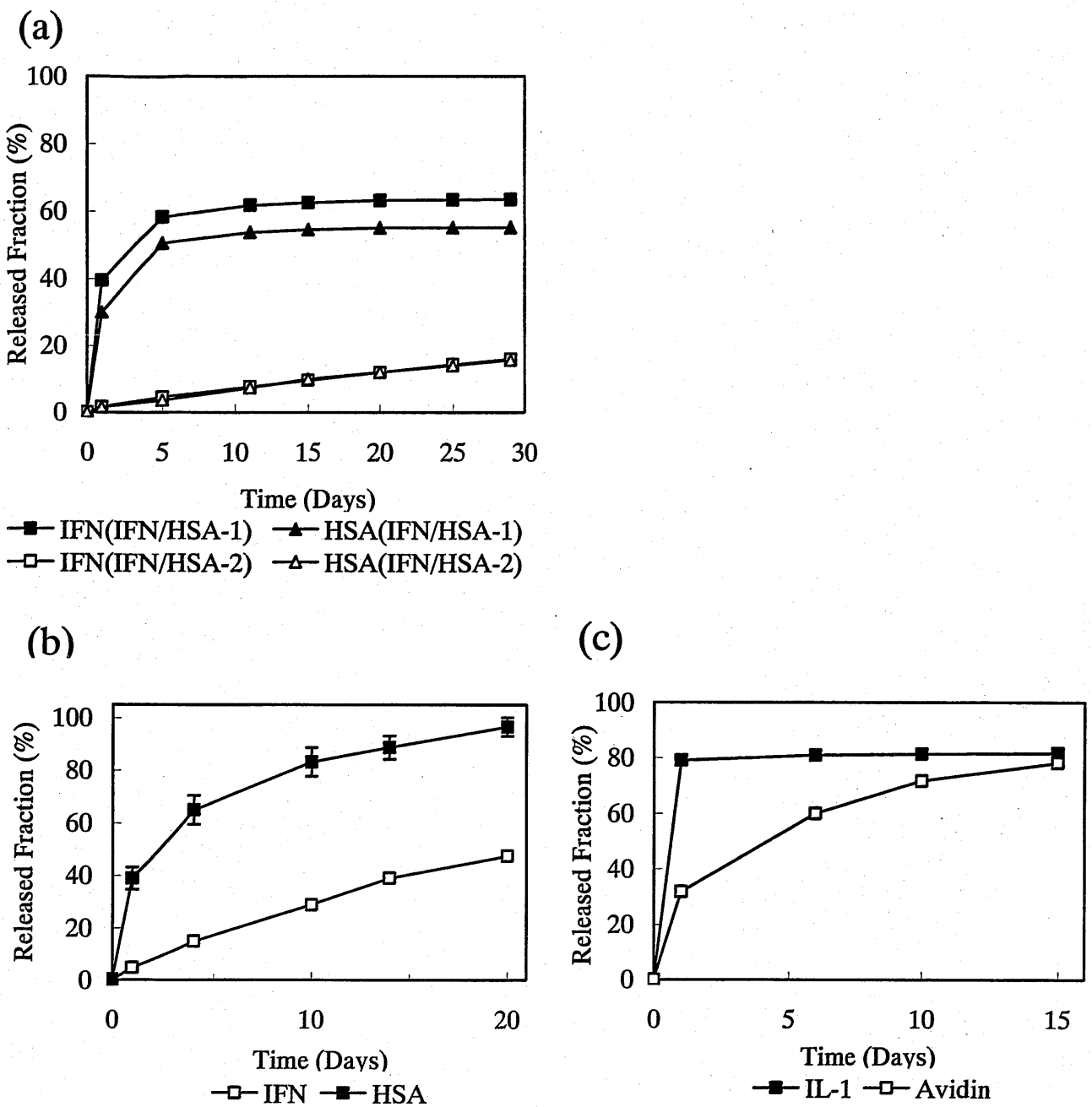

Fig. 4. Simultaneous release of two kinds of drugs from various silicone formulations: (a) matrix-type IFN/HSA-1 (¦ , ▲) and covered-rod-type IFN/HSA-2 (□, Δ) (average for n=2), (b) double-layered IFN/HSA-3 (average for n=2), (c) double-layered IL-1β/Avi-1 (n=1).

release followed by a slow release took place, while in the covered-rod formulations a slow and monotonous release was observed. For both types of formulations, IFN and HSA were released in the same pattern. Therefore, when two kinds of drugs should be released in the same pattern, matrix or covered-rod formulation would be useful depending on required release profile (first-order or zero-order).

3.2.2. Release from double-layered formulations

Figures 4b and 4c show the release of drugs from double-layered IFN/HSA-3 and IL-1β/Avi-1. For both formulations, the release of the outer-layer drug showed an initial burst profile. With IFN/HSA-3, HSA in the outer layer was released rapidly and 97% of HSA was released within 20 days, while IFN in the inner layer was released gradually. With IL-1β/Avi-1 too, IL-1β in the outer layer was rapidly released within one day, and Avi in the inner layer was released gradually over 15 days.

From this result, it was released that a double-layered formulation enabled control release of two kinds of drugs in different patterns.

4. Discussion

PSTB as a model of insoluble drug was not released from a silicone carrier when it was dispersed in the silicone without additives (PSTB-3, Fig. 3b). In the *in vitro* release

experiment, when PSTB-3 was immersed in a buffer solution, no swelling was observed (Fig. 3a), suggesting that channels and cracks were not formed in the formulation under these conditions. Even in the presence of 30%w/w Gly as an additive (PSTB-4), PSTB was not released. PSTB-1, which contained 30%w/w Gly as in the case of PSTB-4, swelled after immersion into the buffer solution, suggesting the formation of channels and cracks inside the formulation. Since the original particle size of Gly is 300 μm, PSTB particles having a diameter of 1 μm can pass through channels formed by Gly release. The absence of PSTB release indicates an extremely slow diffusion of PSTB in the channel. It is considered that some driving force could accelerate release of insoluble substances. According to this idea, SB and CA were used as additives. Water-soluble SB and CA might form channels and cracks inside the formulation and react upon mixing to generate carbon dioxide in the channels. The gaseous carbon dioxide would promote release of PSTB. This appears to explain the experimental findings that the silicone formulation containing CA and SB released PSTB in the rate depending on the contents of CA and SB (Fig. 3b). PSTB-2 swelled significantly and many cracks are observed on the surface (Fig. 3a), indicating a generation of high pressure caused by carbon dioxide gas. Although intense swelling and surface cracks were not observed in PSTB-5, 6 and 7, these formulations should have released PSTB by the same mechanism. A faster release could be attained by generating a larger amount of carbon dioxide, that is, by suitable choice of the kind and amount of

carbonate and acid.

In order to meet various medical requirements, matrix formulations, covered-rod formulations, and double-layered formulations containing two kinds of drugs were prepared and investigated their release behaviors. In contrast to covered-rod formulations, water penetrates through an outer layer of double-layered formulations. Therefore, it is expected that the drug in the inner layer would be released with a first-order profile. However, IFN released was more like zero-order kinetics (Fig. 4b). This could have arisen from a slow channel formation in the outer layer, making the inner layer of IFN/HSA-3 similar to that in a covered-rod formulation. In the case of double-layered formulation of IL-1β/Avi-1 containing SC and Man, which promote drug release, the drug release was faster than that of IFN/HSA-3. Almost all of IL-1β existing in the outer layer was released within one day. The release pattern of Avi in the inner layer obeyed the first-order kinetics (Fig. 4c), supporting the above consideration.

5. Conclusion

Novel methods by which insoluble drugs and two kinds of water-soluble drugs are released in a controlled way were found (Fig. 5). For insoluble drugs, by gas generation in

the carbonate/acid reaction in silicone carrier achieved the sustained released (Fig. 5a). To release two kinds of water-soluble drugs in the same pattern, powderic drugs should be homogeneously distributed in the formulation. For their release in different patterns, double-layered formulations should be adopted, in which the drug to be released faster is placed in the outer layer, and the drug of slower release in the inner layer (Fig. 5b).

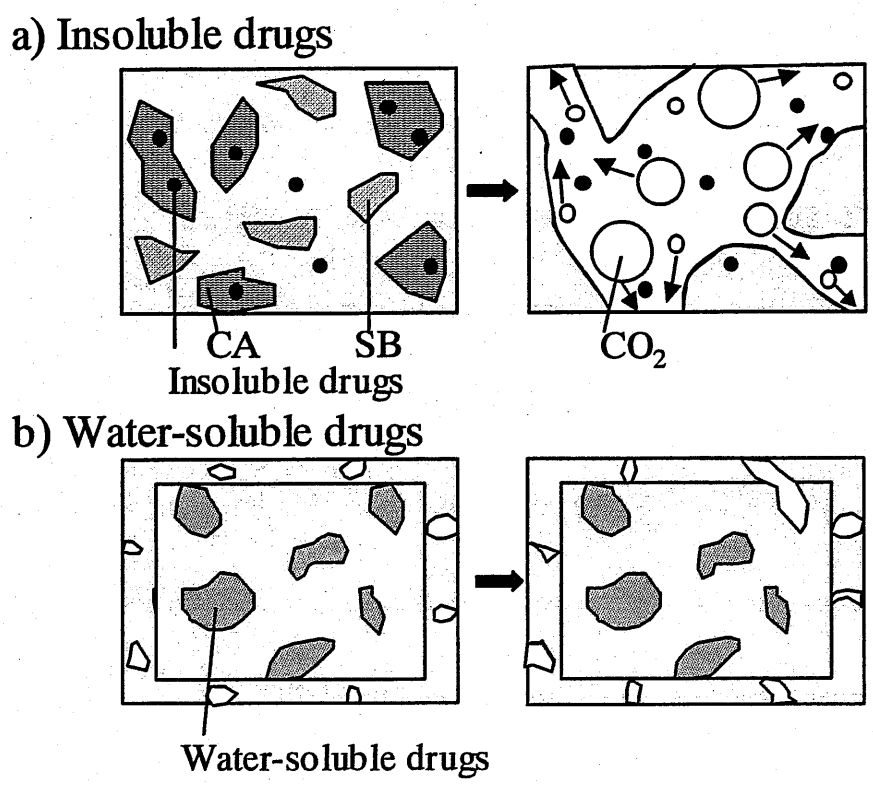

Fig. 5. Diagram showing the process of drug release from a silicone formulation: (a) insoluble drugs can be released using sodium bicarbonate and citric acid as additives; (b) two kinds of water-soluble drugs can be released with different patterns from double-layered formulations, drug placed in the outer layer is released rapidly, and that in the inner layer slowly.

6. References

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Concluding Remarks

This thesis summarizes research achievements on the application of silicone to DDS carrier.

In part I, aiming at developing DDS which releases protein drugs over a long period of time, two types of silicone formulations were designed and investigated. In Chapter 1, a novel technique, by which protein drugs effective in small doses can be released over a long period, was developed. Preparation of silicone formulation was conducted under mild conditions without heat treatment or the use of organic solvents. In this study, interferon (IFN) was used as a model of the protein drugs. The IFN silicone formulation released IFN continuously *in vitro* and suppressed the tumor growth in nude mice for about 100 days after a single administration. This suggests that physiologically active IFN is released over a prolonged period of time from the IFN silicone formulation *in vivo*.

In Chapter 2, in order to achieve zero-order release of protein drugs, covered-rod formulation was developed. The covered-rod formulation released human serum albumin (HSA) or IFN at a constant rate over 30-100 days *in vitro* without significant initial burst. When the IFN covered-rod formulation was implanted in nude mice, the serum concentration of IFN was maintained at a constant level during the period of observation, i.e., 28 days. The covered-rod formulation enabled precise control of release of protein drugs and a possible use in practical DDS is expected.

In part II, silicone formulations were assessed as vaccine delivery vehicles in sheep, using avidin (Avi) as a model antigen as well as *Clostridium tetani* and *Clostridium novyi* toxoids. The matrix and covered-rod formulations were prepared using lyophilized antigen and adjuvant (recombinant ovine interleukin-1β; rovIL-1). The matrix formulation was capable of inducing antibody responses equivalent to those induced by conventional vaccination with aluminum hydroxide (alum) adjuvant. The covered-rod formulations, that release very low levels of antigen over a long period, induced stronger responses than the alum control groups. Prolonged duration of the antibody response was also observed with covered-rod formulations. Silicone formulations offer a new method for vaccination that induces improved antibody responses and has the potential for single-dose immunization.

In part III, extension of technique in controlled release of silicone formulation was attempted. In Chapter 4, using vitamin D_3 (VD_3) as an example of lipophilic drugs which have high potency in low concentrations, novel method to suppress initial burst and to modify the rate of drug release from silicone matrix was investigated. As a result, it was found that (a) addition of HSA suppressed initial burst and enhanced release in the later stage, resulting in constant release of VD_3, (b) covering a matrix formulation with a membrane of low diffusivity (core-rod formulation) suppressed initial burst and released drug in a constant rate,

and (3) using materials for which the drug has high affinity as dissolution solvent (reservoir formulation), the rate of drug release was reduced.

In Chapter 5, methods for (1) continuous release of insoluble drug, and (2) simultaneous release of two kinds of water-soluble drugs from silicone carrier were established. Polystyrene beads (PSTB) and proteins such as IFN and HSA were used as model drugs. PSTB was released from silicone only when citric acid (CA) and sodium bicarbonate (SB) existed as additives. The release patterns of IFN and HSA were almost the same in the cases of matrix and covered-rod formulations. On the other hand, double-layered formulation released drugs in different pattern.

To conclude, drug release from silicone formulation is summarized as follows.

1) Control factors for drug release from silicone

   (i) water-soluble drugs (protein drugs): formation of channels and cracks in silicone matrices.

   (ii) lipophilic drugs: diffusion rate in silicone matrices.

   (iii) insoluble drugs: generation of carbon dioxide gas in silicone matrices.

2) Protein drug release profiles

   (i) matrix formulation: first-order.

   (ii) covered-rod formulation: zero-order.

(iii) double-layered formulation: drug in outer layer is quickly released and drug in inner layer is released slowly.

List of Publications

Chapter 1 Development of a new drug delivery system for protein drugs using silicone (I).

J. Controlled Release, **66**, 49-61 (2000).

Chapter 2 Development of a new drug delivery system for protein drugs using silicone (II).

J. Controlled Release, **73**, 279-291 (2001).

Chapter 3 Injectable silicone implants as vaccine delivery vehicles.

Vaccine, **20**, 1725-1732 (2002).

Chapter 4 Novel method to control release of lipophilic drugs with high potency from silicone

Chem. Pharm. Bull., submitted

Chapter 5 Novel drug delivery device using silicone: controlled release of insoluble or two kinds of water-soluble drugs

Chem. Pharm. Bull., submitted

List of Other Publications Related to This Thesis

Continuous antigen delivery from controlled release implants induces significant and anamnestic immune response

Vaccine, **20,** 1089-1098 (2002).

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