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Patente de Invención ☒ Patente de Modelo de Utilidad ☐
Vía Nacional ☐
Vía PCT (Fase Nacional) ☒ Cap. I ☒ Cap. II ☐

No. Solicitud Internacional PCT/JP2008/056156
Fecha de Presentación Internacional 28 Marzo 2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 41 a 62	30/Octubre/2009
Reivindicaciones:	Folios 63 a 65	30/Octubre/2009
Dibujos:	Folio 67	30/Octubre/2009
Prioridad:	JP 2007-090874	30/Marzo/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Liposoma transpulmonar para controlar la llegada de fármaco"

El problema planteado en esta solicitud consiste en la necesidad de suministrar liposomas que mejoren las características de suministro de fármacos o genes en el sitio de acción objetivo a nivel pulmonar.

Para resolver este problema técnico la solicitud en estudio proporciona un liposoma de administración pulmonar modificado superficialmente con por lo menos un polímero seleccionado del grupo que consiste de alcoholes polivinílicos hidrofobizados terminales y quitosán.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 61 K 47/32, 45/00, 47/24, 47/28, 47/36, 48/00, 9/127.

4. Excepción a la Patentabilidad (Art 20 D 486 literal d)

El objeto de la reivindicación 16, que hace referencia a un método de tratamiento, no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 13 a 15 no es patentable, de acuerdo con el Art 14.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 a 12 no son claras porque el término "para administración pulmonar" indica el uso que puede darse al producto reivindicado, el cual no es una característica técnica que defina a la invención. Se sugiere hacer la aclaración respectiva.

Las reivindicaciones 1, 4 a 8 y 10 no son claras porque la expresión "la superficie del liposoma se modifica" hace referencia a un resultado a alcanzar. De igual forma, las expresiones "la superficie de tejido pulmonar o células de superficie son sitios objetivos del liposoma" de las reivindicaciones 4 y 10, "el liposoma es liposoma de liberación sostenida para administración pulmonar" de la reivindicación 5, "la superficie y el interior del tejido pulmonar son sitios objetivos del liposoma" de las reivindicaciones 6 y 11 y "el liposoma es de acción rápida para administración pulmonar" de la reivindicación 7, son resultados a alcanzar. Se sugiere eliminar dichas expresiones y reemplazarla por las características esenciales estructurales que contribuyen a la obtención de esos efectos.

La reivindicación 1 no es clara porque reclama un liposoma, el cual es una composición, pero no se definen sus componentes y proporciones. Adicionalmente, se reclaman "alcoholes polivinílicos hidrofobizados terminales" como parte del liposoma, pero no se incluyen las opciones preferidas de dichos compuestos, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere incluir los componentes y proporciones que hacen parte de la invención, identificando cada compuesto por medio de su nombre IUPAC o estructura química.

La reivindicación 2 no es clara porque reclama un fosfolípido sin incluir las opciones preferidas que hacen parte de la invención. Se sugiere incluir el nombre IUPAC o la estructura de los fosfolípidos que hacen parte del liposoma. Adicionalmente, la expresión "como un componente constituyente de una membrana liposómica" hace referencia a la función del fosfolípido y no a sus características esenciales estructurales. Se sugiere eliminar dicha expresión.

La reivindicación 8 no es concisa porque reclama diferentes grupos hidrófobos que tienen entre 1 y 30 átomos de carbono, obteniéndose gran cantidad de posibilidades y dificultando determinar la extensión del objeto que se busca proteger. Se sugiere incluir las opciones preferidas de los grupos hidrófobos.

La reivindicación 9 no está soportada en la descripción porque las realizaciones preferidas no divulgan un fármaco o un gen encapsulado en el liposoma. Se sugiere modificar el capítulo descriptivo para dar soporte a esta reivindicación y definir los fármacos y/o genes que hacen parte de la invención por medio de su denominación común internacional y en el caso de los genes por medio de su secuencia de nucleótidos.

La reivindicación 12 no es clara porque reclama un procedimiento para la preparación de un liposoma pero se define únicamente por la etapa de “mezclar”. Se sugiere incluir cada una de las etapas y condiciones de elaboración necesarias para la preparación del liposoma. Adicionalmente, se reclama “un componente o componentes constituyentes de una membrana liposómica” pero no se especifican cuales son dichos componentes”. Se sugiere incluir el nombre IUPAC o la estructura química de dichos componentes.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (Marzo 30 de 2007) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Takeuchi H. et al. European Journal of Pharmaceutics and Biopharmaceutics. 1999;48(2):123-9.	Prolonged circulation time of doxorubicin-loaded liposomes coated with a modified polyvinyl alcohol after intravenous injection in rats	Septiembre/1999
D2	Takeuchi H. et al. Advanced Drug Delivery Reviews. 2001;47(1):39-54.	Mucoadhesive nanoparticulate systems for peptide drug delivery	23/Marzo/2001
D3	WO0172283	Cationic liposomes	04/Octubre/2001

D1 <http://www.ncbi.nlm.nih.gov/pubmed/10469930> divulga (Pág. 123, abstract) un liposoma para ser cargado con el fármaco anticancerígeno doxorubicina; el liposoma adicionalmente se modifica con un material de recubrimiento como un alcohol polivinílico modificado que contiene una especie hidrofóbica.

D2[http://www.ncbi.nlm.nih.gov/pubmed?term=Adv%20Drug%20Deliv%20Rev.%202001%20Mar%2023%3B47\(1\)%3A39-54](http://www.ncbi.nlm.nih.gov/pubmed?term=Adv%20Drug%20Deliv%20Rev.%202001%20Mar%2023%3B47(1)%3A39-54) divulga (Pág. 42, columna 2, párrafo 2) liposomas, los cuales se cubren con polímeros mucoadhesivos como quitosán o alcohol polivinílico con una cadena alquílica larga.

D3<http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=0172283A1&KC=A1&FT=D&ND=3&date=20011004&DB=EPODOC&locale=en> EP divulga (Pág. 2, líneas 15 y 16; Pág. 2, líneas 19 – 22) liposomas para la administración intrapulmonar de ácidos nucleicos o productos genéticos, donde dichos liposomas han sido modificados por la adición de un glicosaminoglicano, el cual se une covalentemente a la superficie del liposoma.

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un liposoma para administración pulmonar en donde la superficie del liposoma se modifica con por lo menos un polímero seleccionado del grupo que consiste de alcoholes polivinílicos hidrofobizados terminales y quitosán

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (Frente a D1):

Características esenciales	D1
Un liposoma en donde la superficie del liposoma se modifica con por lo menos un polímero seleccionado del grupo que consiste de alcoholes polivinílicos hidrofobizados terminales	Un liposoma en donde la superficie del liposoma se modifica con un alcohol polivinílico hidrofobizado

Las características **esenciales** de la composición de la reivindicación 1 habían sido divulgadas previamente en D1, el cual enseña un liposoma para ser cargado con el fármaco anticancerígeno doxorrubicina; el liposoma adicionalmente se modifica con un material de recubrimiento como un alcohol polivinílico modificado que contiene una especie hidrofóbica (Pág. 123, abstract). D1 divulga que un liposoma puede contener fosfatidilcolina, colesterol, colesteril-antraceno-9-carboxilato (Pág. 124, columna 1, párrafo 2), diacetil fosfato (Pág. 125, columna 2, párrafo 1) y que el alcohol polivinílico modificado contiene un grupo hidrófobo como C₁₆-H₃₃-S- (Pág. 124, columna 1, párrafo 2). También, D1 da a conocer un método para preparar dichos liposomas, que comprende la formación del liposoma, la adición del fármaco doxorrubicina y simultáneamente, la adición del polímero de recubrimiento (Pág. 124, columna 1, párrafos 3-5; columna 2, párrafo 1). D1 divulga que el polímero de recubrimiento que modifica la superficie del liposoma, prolonga el tiempo de circulación de los liposomas cargados con doxorrubicina (Pág. 128, columna 2, párrafo 4).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 5, 8 a 10 y 12 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (Frente a D2):

Características esenciales	D2
Un liposoma en donde la superficie del liposoma se modifica con por lo menos un polímero seleccionado del grupo que consiste de alcoholes polivinílicos hidrofobizados terminales y quitosán	Un liposoma en donde la superficie del liposoma se modifica con un alcohol polivinílico hidrofobizado o quitosán

Las características **esenciales** de la composición de la reivindicación 1 habían sido divulgadas previamente en D2, el cual enseña que nanoesferas cubiertas con quitosán son útiles para la liberación pulmonar de fármacos (Pág. 39, abstract). D2 divulga liposomas que comprenden por ejemplo, L- α -dipalmitoilfosfatidilcolina y diacetil fosfato, los cuales se cubren con polímeros mucoadhesivos como quitosán o alcohol polivinílico con una cadena alquílica larga (Pág. 42, columna 2, párrafo 2). Adicionalmente, D2 da a conocer un procedimiento para preparar liposomas recubiertos (Pág. 40 – 43).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2 a 12 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La reivindicación 9 consiste en una preparación de liposoma para administración pulmonar, en donde un fármaco o un gen se encapsula en el liposoma para administración pulmonar de la reivindicación 1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2	D3
Preparación de liposoma, en donde	Un liposoma en donde	Un liposoma en donde
un gen se encapsula en el liposoma	<i>un fármaco se encapsula en el liposoma</i>	un gen se encapsula en el liposoma
la superficie del liposoma se modifica con por lo menos un polímero seleccionado del grupo que consiste de alcoholes polivinílicos hidrofobizados terminales y quitosán	la superficie del liposoma se modifica con un alcohol polivinílico hidrofobizado o quitosán	la superficie del liposoma se modifica con un <i>glicosaminoglicano</i>

Los documentos D2 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 9 porque:

D2 divulga que nanoesferas cubiertas con quitosán son útiles para la liberación pulmonar de fármacos (Pág. 39, abstract). D2 divulga liposomas que comprenden por ejemplo, L- α -dipalmitoilfosfatidilcolina y diacetil fosfato, los cuales se cubren con polímeros mucoadhesivos como chitosán o alcohol polivinílico con una cadena alquílica larga (Pág. 42, columna 2, párrafo 2). D2 da a conocer liposomas que contienen fármacos peptídicos como insulina (Pág. 48, columna 2, párrafo 2) y calcitonina (Pág. 49, columna 1).

La diferencia entre la invención, definida en la reivindicación 9 y la composición de D2 consiste en que se encapsula un gen dentro del liposoma.

D3 divulga liposomas para administración intrapulmonar de ácidos nucleicos o productos genéticos (Pág. 2, líneas 15 y 16). D3 da a conocer que dichos liposomas han sido modificados por la adición de un glicosaminoglicano, el cual se une covalentemente a la superficie del liposoma. D3 enseña que el glicosaminoglicano puede ser cualquiera de su grupo, como ácido hialurónico, condroitina, quitina, etc. (Pág. 2, líneas 19 – 22). D3 también divulga métodos para preparar formlaiones liposomicas con ácido nucleicos encapsulados (Pág. 2, líneas 23 – 24).

La diferencia entre la invención, definida en la reivindicación 9 y la composición de D3 consiste en que la superficie del liposoma se modifica específicamente con quitosán en vez de usar cualquier glicosaminoglicano.

El efecto que logra el incluir un gen dentro del liposoma es que se obtienen formulaciones liposómicas alternativas con diferentes principios activos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar la composición conocida en D2 para obtener formulaciones liposómicas alternativas que permitan la encapsulación de genes".

Sin embargo, la encapsulación de genes dentro de un liposoma, ya está divulgada en el documento D3 que se refiere a liposomas para administración intrapulmonar de ácidos nucleicos o productos genéticos (Pág. 2, líneas 15 y 16), donde dichos liposomas han sido modificados por la adición de un glicosaminoglicano que se une covalentemente a la superficie del liposoma (Pág. 2, líneas 19 – 22).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría los genes de D3 en la composición de D2 para llegar así al objeto de la reivindicación 9 de la solicitud; por lo cual la reivindicación 9 se considera obvia.

Puesto que las reivindicaciones dependientes 10 a 12 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 9 a 12 cumplen el requisito de novedad (Art. 16) cuando se refieren a la encapsulación de un gen en el liposoma, pero no cumplen con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Takeuchi H. et al. European Journal of Pharmaceutics and Biopharmaceutics. 1999;48(2):123-9.	1 – 5, 8 – 10, 12	Novedad
D2	Takeuchi H. et al. Advanced Drug Delivery Reviews. 2001;47(1):39-54.	1 – 12	Novedad
		9 – 12	Nivel Inventivo
D3	WO0172283	9 – 12	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

~~09 OCT. 2012~~

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Research paper

Prolonged circulation time of doxorubicin-loaded liposomes coated with a modified polyvinyl alcohol after intravenous injection in rats

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Abstract

The purpose of this study was to evaluate the functions of a modified polyvinyl alcohol (PVA-R), which has a hydrophobic moiety, as a coating material for liposomes to be loaded with the anticancer drug, doxorubicin. The size controlled liposomes (egg phosphatidylcholine: cholesterol = 1:1 molar ratio) were prepared by the hydration method followed by extrusion. Drug encapsulation and surface modification with polymers (PVA and PVA-R) were carried out simultaneously using a modified pH gradient method. The existence of a thick polymer layer on the surface of the liposomes was confirmed by an increase in particle size and the amount of polymer on the liposomal surface, especially for the PVA-R-coated liposomes. The effects of polymer coating on the behavior of the liposomes *in vivo* were evaluated by measuring the circulation time and biodistribution of the drug after *i.v.* administration of the liposomal drug in rats. The PVA-R-coated liposomes showed a more prolonged circulating time for the drug with less uptake by the reticuloendothelial system after *i.v.* administration in rats, compared with non-coated liposomes. These results confirm that polymer possessing a hydrophobic anchor at its end, like PVA-R, is a suitable material for modifying the surface of doxorubicin-loaded liposomes to improve their stability in the circulating blood. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Liposomes; Polymer coating; Doxorubicin; Reticuloendothelial system avoidance; Modified polyvinyl alcohol

1. Introduction

Liposomes have been extensively investigated as drug carriers for a variety of drugs including the anticancer drug, doxorubicin. The drug-loaded liposomes are usually injected intravenously for systemic application. However, there is usually rapid clearance from the circulating blood by the reticuloendothelial system (RES) located mainly in the liver and spleen [1,2], although this depends on the particle size and lipid composition of the liposomes [3,4]. This appears to be effective for the delivery of drugs to the RES, but is of no use for targeting drugs to specific parts of the body such as solid tumors other than in the RES. To avoid this disadvantage, 'stealth technology' has been developed. It has been reported that the formulation of ganglioside G_{M1} , polyethylene glycol attaching phospholipids (PEG-lip) and gluconic acid derivatives lead to a reduc-

tion of RES uptake and prolongation of the plasma half-life of liposomes administered intravenously [5–9]. A highly water-soluble polymer grafted onto the lipid bilayer of the liposomes exerts a long range mutual repulsion between adjacent bilayers and, presumably, prevents the interaction of liposomal surfaces with macromolecules, such as proteins, in the blood [10].

Polymer coating is an alternative method of modifying the surface of liposomes. It is a characteristic of the polymer coating method that the manner of coating is very simple, just mixing a liposome suspension and a polymer solution, without chemically linking the polymers to the lipid molecules. We [11] have reported the feasibility of coating the surface of liposomes with modified hydrophilic polymers such as polyvinyl alcohol or polyacrylic acid having hydrophobic anchor(s) (PVA-R or PAA-R). Previously [12], we confirmed the formation of a thick coating layer on the surface of liposomal particles and the improved physical stability of PVA-R coated liposomes.

In this study, we examined the effectiveness of PVA-R-coated liposomes as a carrier for the anticancer drug, doxorubicin, when administered intravenously in rats. After

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confirming the formation of doxorubicin-loaded, PVA-R coated and size-controlled (100 nm in diameter) liposomes composed of egg phosphatidylcholine and cholesterol in a molar ratio of 5:5, the fate of the PVA-R-coated liposomes in vivo was evaluated by measuring the circulation time and biodistribution of the drug after i.v. administration of the liposomal drug. The data suggested that coating of liposomes with PVA-R effectively improved the drug circulation time with reduced uptake of liposomes by the RES.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (COATSOME NC-10S) was purchased from the Nippon Oil and Fats Co., Japan. Cholesterol was obtained from the Sigma Chemical Co. Doxorubicin hydrochloride was kindly donated by the Kyowa Hakko Kogyo Co., Tokyo, Japan. Cholesteryl-anthracene-9-carboxylate (CA), used as a lipid marker, was purchased from LAMBDA, Austria. Polyvinyl alcohol (PVA205) and polyvinyl alcohol bearing a hydrophobic anchor ($C_{16}H_{33}$ -S-) at the terminal of the polymer (PVA-R) were supplied by Kuraray Co., Japan. The degree of polymerization of PVA and PVA-R was 450, 480, respectively. Male Wistar rats (body weight 230–260 g, 11 weeks old) were obtained from Japan SLC Ltd., Japan and were maintained in standard housing. All other chemicals were commercial products of reagent grade.

2.2. Preparation of liposomes

The size-controlled liposomes with egg phosphatidylcholine and cholesterol in a 1:1 molar ratio were prepared by the hydration method followed by extrusion. In brief, the lipid mixture was dissolved in a small amount of chloroform in a 200 ml round-bottom flask and dried in a rotary evaporator under reduced pressure at 40°C to form a thin lipid film. The thin lipid film was dried in a vacuum overnight to ensure complete removal of the solvent. Multilamellar vesicles (MLV) were formed by adding a saline solution containing 50 mM citric acid to the flask followed by vortexing at 60°C and incubation for 1 h at 10°C. The MLVs were then extruded 12 times using an Extruder (Lipex Biomembranes, USA) equipped with a polycarbonate membrane filter (pore size: 1 μ m or 0.4 μ m, Nuclepore®) to produce small size liposomes (SUV).

For the preparation of CA-containing liposomes, a small amount of CA as a lipid marker was added to the lipid mixture before evaporation. CA-containing liposomes of diameter 100 nm were prepared by the hydration method followed by sonication (UR-200P, Tomy Seiko Co.).

Polymer-coated liposomes were prepared by mixing the resultant liposomal suspension (1 ml) with an equal amount (1 ml) of polymer solution, followed by incubation at 10°C for 1 h.

Doxorubicin was loaded into the liposomes by the pH gradient (interior acidic, pH 2.1) method [13] with some modifications. In the preparation, the liposomal suspension (1 ml) was mixed with an equal amount of doxorubicin aqueous solution, followed by adding a saline solution containing 0.2 mmol sodium carbonate (2 ml) diluted with distilled water (2 ml). This mixture was incubated with periodic mixing for 10 min at 60°C, followed by incubation for 1 h at 10°C. For the preparation of polymer-coated and doxorubicin-loaded liposomes, polymer coating and drug loading were carried out simultaneously. To coat the liposomes with polymer, a saline solution containing both 0.2–0.4 mmol sodium carbonate and an appropriate amount of PVA or PVA-R (4 ml) was used instead of the sodium carbonate solution in the drug-loading process described above.

In order to investigate the effect of temperature on the encapsulation efficiency of doxorubicin into liposomes, incubation after mixing the solutions was carried out at 10°C for 1 h instead of heating at 60°C.

2.3. Properties of liposomes

The particle size and distribution of the liposomes were measured with a dynamic light scattering method using LPA-300 equipment (Otsuka Electron Co.). An aliquot of each liposomal suspension was diluted with a large amount of distilled water.

The amount of doxorubicin loaded in the liposomes was determined by measuring the concentration of non-entrapped drug. In measuring the drug concentration, 3 ml of the liposomal suspension diluted 50 times with phosphate buffer (pH 5.0) was mixed with 0.7 ml 1 N sodium hydroxide, and the drug concentration in the samples was measured spectrophotometrically at 600 nm (UV-160A, Shimadzu), taking into consideration the turbidity of the system, which was also spectrophotometrically measured at 750 nm. The total amount of doxorubicin in the system was determined in the same manner after collapsing the liposomal structures by adding 0.06 ml 10% Triton solution to the samples. The percentage of drug trapped was calculated by dividing the amount of the substance in the interior of the liposomes by the total amount in the system.

2.4. Animal experiments

All animal experimental protocols were approved by the animal welfare commission of Gifu Pharmaceutical University.

In monitoring the plasma levels of doxorubicin in rats, free or liposomal (non-, PVA- or PVA-R-coated) doxorubicin at a concentration of 2 mg/ml was injected via the tail vein at a dose of 5 mg doxorubicin/kg body weight. At appropriate intervals, blood was collected from the retro-orbital sinus. Subsequently, plasma was separated by centri-

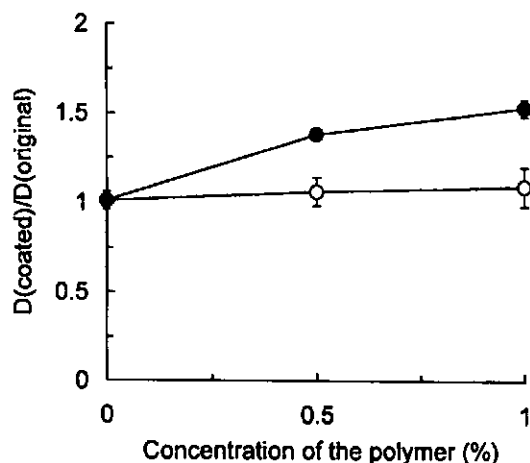


Fig. 1. Particle size change in liposomes following coating with PVA-R or PVA ($n = 3$). Lipid composition of liposomes: EPC/cholesterol = 5:5. ●, PVA-R, ○, PVA.

fugation ($3000 \times g$, 10 min) and the drug concentration analyzed.

In monitoring the plasma pharmacokinetics of the carriers, CA-containing liposomes with or without polymer coating were injected into the rats at the same dose of total lipid as in the doxorubicin evaluation, followed by collection of plasma as described above.

In the drug distribution study, rats were injected in the same manner at a dose of 1 mg doxorubicin/kg body weight. At 1, 8 and 24 h after injection, the rats were sacrificed and the liver, spleen and heart were immediately removed, weighed and homogenized with citric acid buffered saline (CBS) (pH 8.0) (0.5 ml/g tissue).

The quantitative analysis of doxorubicin in plasma or each tissue was carried out by the HPLC method previously reported by Masuike et al. [14]. In short, the drug-in-plasma and tissue homogenates, both of which were diluted with CBS, pH 8.0, were extracted with an appropriate amount of toluene/ butanol (1:1, v/v) by vigorously mixing for 40 min in a reciprocal shaker (SR-2, TAITEC, Japan). The organic phase was then dried in a rotary evaporator (VEC-310, IWAKI, Japan) under reduced pressure at 60°C . The residues were dried in a vacuum overnight to ensure complete removal of the solvent, and dissolved in a carrier solution ($0.03 \text{ mM NH}_4\text{H}_2\text{PO}_4\text{:MeOH} = 3:7$) for HPLC analysis. The samples were subjected to HPLC analysis with a fluorospectrophotometer (FP-920, JASCO, Japan) using daunorubicin as an internal standard.

To determine the quantity of the CA in plasma, 50 μl aliquots of the plasma samples were extracted with an appropriate amount of chloroform/methanol (1:1, v/v), followed by centrifugation (3,000 rev./min, 10 min). The concentration of CA was measured by the fluorescence in the resultant supernatant with a fluorospectrophotometer (Hitachi F3010).

The significance of the in vivo data was evaluated using Student's t -test and $P < 0.05$ was considered significant.

3. Results

3.1. Polymer coating of liposomes

The feasibility of polymer coating of the chosen liposomes with polyvinyl alcohol (PVA) and a modified polyvinyl alcohol (PVA-R) was evaluated by comparing the particle size of the liposomes before and after polymer coating. The liposomes used in this study were composed of EPC and cholesterol in a molar ratio of 5:5 and the particle size was $107.3 \pm 8.2 \text{ nm}$. Mixing the liposomal suspension with the polymer solution and following incubation, i.e. the polymer coating process, led to an increase in the particle size of the liposomes. The change in particle size depended on the type and concentration of polymer used. The particle diameter of liposomes coated with PVA-R at a final concentration of 1% was $163.9 \pm 12.9 \text{ nm}$, i.e. 1.53-fold that of the original liposomes (Fig. 1). The increase in particle size of PVA-coated liposomes was relatively small. These results suggest the existence of a coating layer on the surface of the liposomes, especially for the PVA-R-coated liposomes. These results were relatively consistent with our previous observation for liposomes of dimilistoylphosphatidylcholine (DMPC)/dicetyl phosphate (DCP)/cholesterol in a molar ratio of 7:3:1 [12].

The amount of polymer coating the surface of the liposomes was estimated by measuring the polymer concentration of the supernatant in the centrifuged liposomal suspension after coating. The amount of PVA-R coating increased with the increasing concentration of PVA-R used in coating (Fig. 2). When the amount of coating was calculated on a molar basis by assuming the molecular weight of the polymer to be about 20000, the molar ratio of PVA-R to phospholipids is 1/270 at a final polymer concentration of 1%. The value is almost the same for the DMPC/DCP/cholesterol (7:3:1) liposomes. Although only a thin coating layer was detected for PVA in measuring the particle size change, about one-third the amount of PVA relative to PVA-R was observed as the coating layer at the same polymer concentration. These differences in coating

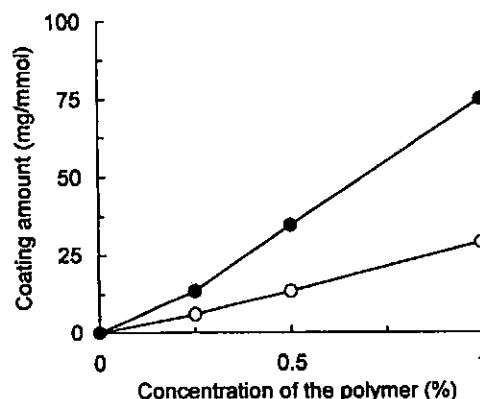


Fig. 2. Amount of PVA-R- or PVA coated onto liposomes ($n = 3$). Lipid composition of liposomes: EPC/cholesterol = 5:5. ●, PVA-R, ○, PVA.

Table 1

Doxorubicin concentration in plasma of rats after intravenous administration of doxorubicin solution (sol), non-coated liposomal doxorubicin (100 nm, non-100), PVA-coated liposomal doxorubicin (100 nm, PVA100), PVA-R-coated liposomal doxorubicin (100 nm, PVA-R100), non-coated liposomal doxorubicin (300 nm, non-300) and PVA-R-coated liposomal doxorubicin (300 nm, PVA-R300)*

Time (h)	Conc. of doxorubicin in plasma ($\mu\text{g/ml}$)					
	sol	non-100	PVA100	PVA-R100	non-300	PVA-R300
0.5	0.15 $\pm$ 0.01	73.1 $\pm$ 2.7	76.2 $\pm$ 3.1	88.8 $\pm$ 5.2**	21.9 $\pm$ 2.0	83.0 $\pm$ 6.8
1	0.15 $\pm$ 0.00	63.5 $\pm$ 2.1	60.8 $\pm$ 5.5	79.1 $\pm$ 4.0***	22.3 $\pm$ 2.9	71.2 $\pm$ 2.0
2	0.14 $\pm$ 0.00	42.7 $\pm$ 2.8	45.3 $\pm$ 3.4	57.0 $\pm$ 3.6**	17.0 $\pm$ 4.5	46.8 $\pm$ 2.3
4	0.13 $\pm$ 0.01	28.8 $\pm$ 2.2	34.0 $\pm$ 2.0	38.2 $\pm$ 2.2**	13.3 $\pm$ 5.9	25.2 $\pm$ 3.6
8	0.13 $\pm$ 0.01	13.5 $\pm$ 0.8	16.5 $\pm$ 0.7	19.4 $\pm$ 2.3*	3.3 $\pm$ 1.7	13.8 $\pm$ 1.1
12	0.11 $\pm$ 0.00	4.1 $\pm$ 0.7	5.2 $\pm$ 0.4	7.7 $\pm$ 1.2**	1.1 $\pm$ 0.3	5.7 $\pm$ 1.3
24	0.00 $\pm$ 0.00	0.7 $\pm$ 0.0	1.1 $\pm$ 0.5	1.4 $\pm$ 0.2**	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
AUC ($\mu\text{g/ml h}$) ^{a,24}	2.22	353.2	386.7	473.3	121.9	363.2

* The number of animals is given in the caption to Fig. 4. Lipid composition of liposomes: EPC:cholesterol = 5:5. Dose of doxorubicin: 5 mg/kg weight.

amount could be attributed to the different coating procedures for these polymers [12].

When doxorubicin was encapsulated into the liposomes (EPC/cholesterol = 5:5) using the pH gradient method, the encapsulation efficiency of drug was 50.3 % for the non-coated liposomes. The encapsulation efficiency was markedly improved to 93.5% by heating the system during the encapsulation of drug as described in Section 2.2. The increased temperature made the lipid membrane more fluid, which could facilitate drug transport through the lipid membrane of the liposomes. Similar encapsulation efficiency was observed for PVA-coated (93.2%) and PVA-R-coated (93.3%) liposomes using the modified pH gradient method.

Although the data presented in Figs. 1 and 2 were observed for the corresponding empty liposomes, almost

the same values for the particle size change and polymer coating amount were found for the doxorubicin-loaded ones under typical conditions. Thus, we confirmed the preparation of PVA- and PVA-R-coated, and doxorubicin-loaded liposomes using the modified pH gradient method.

3.2. Plasma pharmacokinetics

The doxorubicin solution, or that encapsulated into the liposomes with or without polymer coating, was injected into male Wistar rats via the tail vein at the same dose. The plasma pharmacokinetics of doxorubicin is presented in Table 1. In the case of i.v. administration of doxorubicin solution, the drug disappeared from the circulating blood very rapidly owing to its short half-life. In contrast, liposomal doxorubicin showed a long circulating time, although it depended on the particle size of the liposomes. When the liposomes were coated with PVA-R, the drug levels in plasma were higher than those for the non-coated liposomes at each sampling time. The effect of polymer coating on the circulation time of doxorubicin was evident from the relatively larger liposomes (approx. 300 nm in diameter), which were prepared by extruding with a membrane of 400 nm pore size (Table 1). In the case of PVA coating, the effect was not as large as that for PVA-R coating. The area under the doxorubicin concentration-time curve (AUC) from 0 to 24 h post-injection in Table 1 clearly shows the effects of polymer coating on the systemic drug circulation described above.

One possible explanation for the enhanced drug circulation with PVA-R-coated liposomes is the prolonged circulation time of the liposomes following PVA-R coating. In order to evaluate the actual circulation profiles of the carriers, CA-containing liposomes with or without polymer coating were prepared and injected into the rats in the same manner and at the same dose of total lipid as used to study the pharmacokinetics of doxorubicin. The plasma pharmacokinetics of the liposomes is shown in Fig. 3. When the

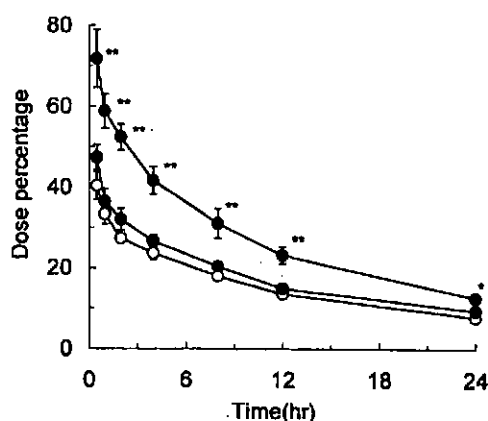


Fig. 3. Effect of polymer coating on the circulation profiles of liposomes in plasma after i.v. injection ($n = 3$). Lipid composition of liposomes: EPC/cholesterol = 5:5. ●, PVA-R-coated liposomes, shaded circle, PVA-coated liposomes and ○: non-coated liposomes. * $P < 0.05$, ** $P < 0.01$; significantly different from the level for PVA-coated liposome. The level for PVA-R-coated liposomes is significantly different from that for non-coated liposomes, $P < 0.01$.

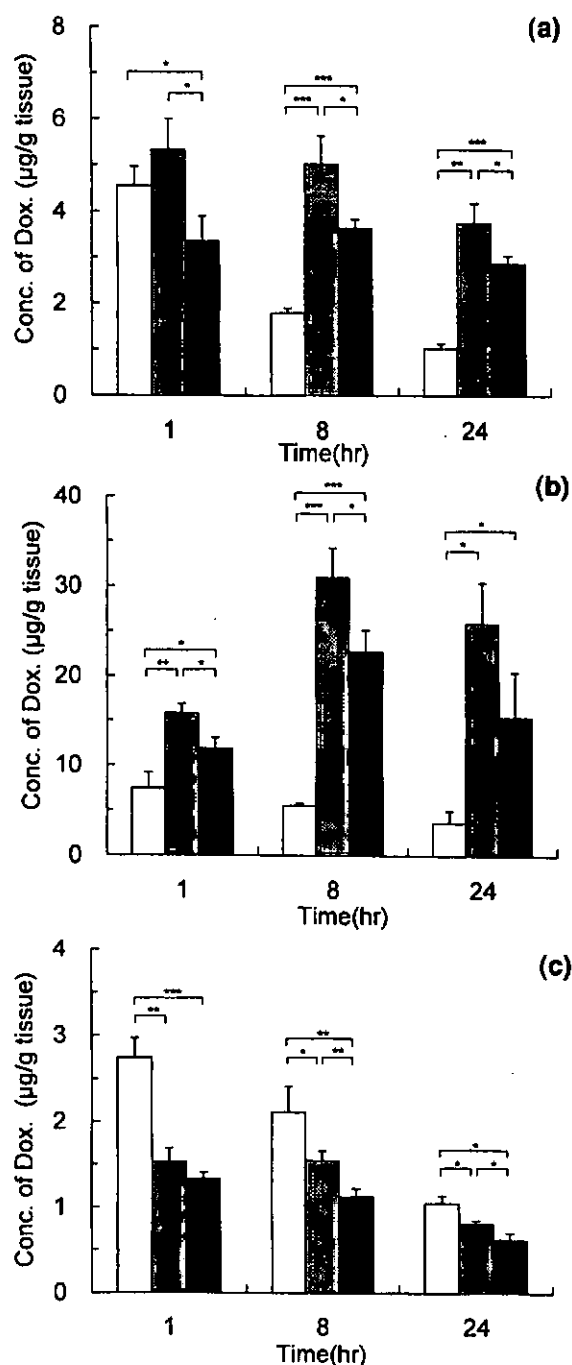


Fig. 4. Doxorubicin concentration in the tissues (a, liver, b, spleen and c, heart) of rats after intravenous administration of doxorubicin solution or liposomal doxorubicin ($n=3$). Lipid composition of liposomes: EPC:cholesterol = 5:5. ■, PVA-R-coated liposomal doxorubicin; shaded square, non-coated liposomal doxorubicin and □, doxorubicin solution. Particle size of the liposomes: approx. 100 nm. Dose of doxorubicin: 1mg/kg weight.

CA-containing liposomes were coated with PVA-R, the liposomal circulation time became much longer than that for the non- and PVA-coated liposomes. These profiles confirm the effectiveness of PVA-R coating in improving the liposomal stability in the bloodstream.

3.3. Distribution study

The prolonged systemic circulating time of polymer-coated liposomes may be attributed to the reduction in uptake by the RES in the liver and spleen. To clarify the reduction in RES uptake following polymer coating, the drug concentrations in liver and spleen were measured at appropriate intervals after injection of liposomal doxorubicin, with or without PVA-R coating or doxorubicin solution (Fig. 4a,b). Comparing the drug concentration for PVA-R-coated liposomal doxorubicin with that for non-coated, the former was significantly lower than the latter at each sampling point. These observations confirm the reduction in RES uptake of liposomes following polymer coating with PVA-R. On administering doxorubicin solution to rats, the highest concentration of drug in liver was observed at 1 h and the concentration decreased comparatively rapidly with time. On the other hand, when doxorubicin was encapsulated into liposomes, the drug concentration in liver decreased more slowly compared with the injection of free doxorubicin. This may be due to the continuous influx of drug from the blood via RES uptake of liposomal doxorubicin.

The drug concentration in spleen after injection of liposomal doxorubicin was considerably higher than that for the doxorubicin solution. This result clearly shows the RES uptake of the particulate systems in spleen. A significant difference in drug concentration was observed between non-coated and PVA-R-coated liposomes as well as in the liver, which suggests again the inhibitory effect of the polymer coating on RES uptake. As far as the time-course profiles of drug concentration are concerned, the liposomal doxorubicin produced a maximum drug concentration at 8 h after injection, while the free doxorubicin steadily decreased with time. This can be also attributed to the continuous uptake of liposomal doxorubicin by the RES.

Drug levels in the heart as a parameter of cardiac toxicity are shown in Fig. 4c. Following administration of free doxorubicin, the drug concentrations in heart were very high at any sampling time. Formulating doxorubicin as a liposomal system significantly reduced drug distribution to the heart. At 1 h, in particular, the drug concentrations of non- and PVA-R-coated liposomes were 0.55- and 0.49-fold that of free doxorubicin. It was interesting that the drug concentration in heart following injection of PVA-R-coated liposomes was lower than that for non-coated liposomes although the corresponding drug concentration in blood was higher at any of the sampling points.

4. Discussion

In a previous paper, we confirmed that a steric coating layer was formed on the surface of liposomes composed of DMPC, DCP and cholesterol (7:3:1 molar ratio) with PVA-R. The mechanism for the formation of the thick coating

layer with PVA-R involved anchoring the hydrophobic portion of the polymer to the lipid membrane at the surface of the liposomes. When PVA was used as the coating polymer, the resulting coating layer was not thick owing to the coating mechanism, simple physical adsorption. Similar coating properties of these polymers were observed for the EPC:cholesterol (5:5) liposomes (Figs. 1 and 2).

Much attention has been paid to the reduction in RES uptake of liposomes, since Allen et al. [5] reported that the formulation of the monosialo-ganglioside (G_M1) in liposomal systems was effective in prolonging their blood circulation. The synthetic lipids linking with polyethylene glycol (PEG-lip) have been found to form sterically stabilized liposomes with both natural and synthetic phospholipids, which can markedly prolong the circulating time in blood after i.v. injection. The clearance and tissue distribution of the sterically stabilized liposomes following i.v. administration has been examined by a number of research groups [15]. It is well accepted that the polymer layer formed on the liposomal surface may effectively protect the liposomes from interacting with plasma proteins in the blood, leading to a reduction in RES uptake and prolonged circulating time in blood [10].

When doxorubicin in various formulations was injected into rats via the tail vein, higher retention of the drug in blood was observed for the PVA-R-coated liposomes than for the non-coated liposomes (Table 1). This result strongly suggests that the circulation time of liposomes in the bloodstream is prolonged by coating with PVA-R as well as by using PEG-lip-containing liposomes. This was confirmed by measuring the circulation time of the liposomes themselves using a lipid marker CA (Fig. 3).

The coating effect on the circulation time of doxorubicin was more marked for the relatively larger liposomes (Table 1). This is because these larger liposomes (usually more than 200 nm in diameter) disappeared rapidly from the blood circulation compared with the smaller vesicles after i.v. administration because of the enhanced RES recognition [3,4]. Measuring the doxorubicin concentration in liver and spleen after i.v. administration, a statistically significant difference in concentration was observed between PVA-R-coated and non-coated liposomes at all sampling intervals (Fig. 4). The lower concentration of PVA-R-coated liposomes suggests a reduction in the RES uptake of liposomes following coating with PVA-R.

The reduction in RES uptake of sterically stabilized liposomes, such as PEG-lip-containing liposomes, is generally attributed to the reduced binding of plasma proteins to the liposomes [10]. The coating layer of PVA-R-coated liposomes would be steric enough to prevent this unfavorable adsorption of protein under biological conditions. In considering the steric stabilizing effect of polymer coating, both the hydrophilicity and flexibility of the coating polymer layer may be responsible for reducing the RES uptake of particles. However, when the liposomes were coated with PVA, no significant improvement in the circulation time of the particles was observed (Fig. 3). Since PVA forms a coating layer

on the surface of the liposomal particles (Fig. 2) and PVA is a typical hydrophilic polymer, this suggests that the flexibility of the coating layer is more important for the longer circulation of the particles in bloodstream.

An alternative explanation for the ineffectiveness of PVA-coating is the dissociation of the polymer from the liposomes when the liposomes enter the bloodstream. It is presumed that the tendency to detach depends on the coating mechanism, i.e. PVA-R is not easily dissociated from the liposomal surface because of anchoring of the hydrophobic moiety to the phospholipid bilayers, while it is relatively easy for the PVA in the coating layer to detach since simple physical adsorption is involved. This tendency may be reflected in the retention of the coated liposomes in the bloodstream.

Generally, doxorubicin tends to be taken up by heart muscle, resulting in severe toxicity [16,17]. The use of drug carriers including liposomes is expected to reduce this side-effect by controlling the distribution of the drug. In comparing the drug concentrations in heart post i.v. administration of the drug, encapsulation of the drug into liposomes was found to significantly reduce distribution to the heart (Fig. 4c). As we confirmed that the liposome particles could not be distributed to the cardiac cells, by using CA-containing liposomes (data not shown), it is suggested that the free doxorubicin leaked from the liposomes is responsible for the small amount of doxorubicin detected in the heart following the use of liposomal doxorubicin.

In conclusion, polymer coating is effective in prolonging the circulation time of doxorubicin-loaded liposomes. It was demonstrated that a polymer possessing a terminal hydrophobic anchor like PVA-R is a suitable material for modifying the surface of liposomes. Although the effectiveness of the polymer-coated liposomes is not yet as high as PEG-derivative-containing liposomes in terms of retention in the bloodstream after i.v. injection, polymer coating is expected to be a promising method for preparing drug carriers to avoid RES uptake because of its simplicity. It may be possible to enhance the effectiveness of polymer-coated liposomes by improving the liposomal formulation by optimizing factors including lipid composition, the particle size and molecular weight of the polymer.

References

- [1] G. Poste, C. Bucana, A. Raz, P. Bugelski, R. Kirsh, I.J. Fidler, Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery, *Cancer Res.* 42 (1982) 1412–1422.
- [2] J.H. Senior, Fate and behavior of liposomes in vivo: a review of controlling factors, *Crit. Rev. Ther. Drug Carrier Syst.* 3 (1987) 123–193.
- [3] L.D. Mayer, L.C.L. Tai, D.S.C. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, M.B. Bally, Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice, *Cancer Res.* 49 (1989) 5922–5930.
- [4] G. Storm, F.H. Roerdink, P.A. Steerenberg, W.H. de Jong, D.J.A. Crommelin, Influence of lipid composition on antitumor activity

- exerted by doxorubicin-containing liposomes in a rat solid tumor model, *Cancer Res.* 47 (1987) 3366–3372.
- [5] T.M. Allen, A. Chonn, Large unilamellar liposomes with low uptake into the reticuloendothelial system, *FEBS Lett.* 223 (1987) 42–46.
- [6] T.M. Allen, C. Hansen, J. Rutledge, Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues, *Biochim. Biophys. Acta* 981 (1989) 27–35.
- [7] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, *Biochim. Biophys. Acta* 1066 (1991) 29–36.
- [8] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthey, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J. Martin, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, *Proc. Natl. Acad. Sci. USA* 88 (1991) 11460–11464.
- [9] Y. Namba, T. Sakakibara, M. Masada, F. Ito, N. Oku, Glucuronate-modified liposomes with prolonged circulation time, *Chem. Pharm. Bull.* 38 (1990) 1663–1666.
- [10] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles, *Biochim. Biophys. Acta* 1062 (1991) 77–82.
- [11] H. Takeuchi, H. Yamamoto, T. Hino, Y. Kawashima, Mucoadhesion of polymer-coated liposomes to rat intestine in vitro, *Chem. Pharm. Bull.* 42 (1994) 1954–1956.
- [12] H. Takeuchi, H. Yamamoto, T. Toyoda, H. Toyobuku, T. Hino, Y. Kawashima, Physical stability of size controlled small unilamellar liposomes coated with a modified polyvinyl alcohol, *Int. J. Pharm.* 164 (1998) 103–111.
- [13] L.D. Mayer, M.B. Bally, P.R. Cullis, Uptake of Adriamycin into large unilamellar vesicles in response to a pH gradient, *Biochim. Biophys. Acta* 857 (1986) 123–126.
- [14] T. Masuike, J. Odake, M. Kohagura, T. Noda, Y. Takemoto, Determination of Adriamycin and its metabolites in biological samples using high performance liquid chromatography II. Analysis of tissues by extraction method (in Japanese), *Yakugaku Zasshi* 104 (1984) 620–623.
- [15] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, *Biochim. Biophys. Acta* 1113 (1992) 171–199.
- [16] A. Rahman, A. Kessler, N. More, B. Sikić, G. Rowden, P. Woolley, P.S. Schein, Liposomal protection of Adriamycin-induced cardiotoxicity in mice, *Cancer Res.* 40 (1980) 1532–1537.
- [17] A. Gabizon, A. Dagan, D. Goren, Y. Barenholz, Z. Fuks, Liposomes as in vivo carriers of Adriamycin: reduced cardiac uptake and preserved antitumor activity in mice, *Cancer Res.* 42 (1982) 4734–4739.



Mucoadhesive nanoparticulate systems for peptide drug delivery

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Abstract

This chapter describes the preparation of and methods for evaluating mucoadhesive nanoparticulate systems, including liposomes and polymeric nanoparticles. Mucoadhesive ability is conferred on the particulate systems by coating their surface with mucoadhesive polymers such as chitosan and Carbopol. The feasibility of this surface modification was confirmed by measuring the zeta potential. Several methods of evaluating the mucoadhesive properties of particulate systems have been reported in the literature. We have also developed some novel evaluation procedures including a particle counting method using a Coulter counter for polymer-coated liposomes. The mucoadhesive properties of the polymer-coated liposomes and polymeric nanoparticles were confirmed by means of these mucoadhesion tests. In applying these mucoadhesive nanoparticles to the oral and pulmonary administration of peptide drugs, more effective and prolonged action was observed in comparison with non-coated systems, thereby confirming the usefulness of mucoadhesive nanoparticulate systems for the delivery of peptide drugs. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Polymeric nanoparticle; Liposomes; Mucoadhesion; Chitosan; Peptide; Insulin; Calcitonin; Oral delivery; Pulmonary delivery

Contents

1. Introduction	40
2. Preparation of nanoparticulate peptide delivery systems	40
2.1. Polymeric nanoparticles	40
2.1.1. Emulsion solvent diffusion method	40
2.1.2. Phase separation method	41
2.2. Effect of preparation method of polymeric nanoparticles on the encapsulation efficiency of peptides	41
2.3. Surface modification of nanoparticulate systems with mucoadhesive polymers	42
3. Evaluation of mucoadhesive particulate systems	44
3.1. Evaluation of mucoadhesive properties of particulate systems	44
3.2. Mucoadhesive properties of polymer coated liposomes	45
3.3. Mucoadhesive properties of polymeric nanoparticles coated with mucoadhesive polymers	48
4. Peptide drug delivery with mucoadhesive nanoparticulate systems	48
4.1. Effectiveness of polymer-coated liposomes for the absorption of peptide drugs	48
4.2. Mucoadhesive polymeric nanoparticles for oral administration of peptide drugs	50
4.3. Pulmonary delivery of peptide drugs with mucoadhesive polymeric nanoparticles	50
5. Conclusion	52
References	52

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1. Introduction

The bioadhesion of the oral dosage forms of poorly absorbable drugs has received much attention as have transdermal and buccal systems. Bioadhesion to mucous membranes in the gastrointestinal tract can be described in term of mucoadhesion. Since mucoadhesion can prolong the residence time of drug carriers at the absorption sites, improved drug absorption is expected from a combination of mucoadhesiveness and controlled drug release from the devices used. Longer et al. [1] were the first to show that a delayed gastrointestinal transit induced by bioadhesive polymers could lead to the increased oral bioavailability of a drug.

A tablet for oral or buccal use is one of the most extensively studied mucoadhesive dosage forms [2–4]. These devices are prepared by formulating mucoadhesive polymers such as hydroxypropyl-methylcellulose and Carbopol, which is a high molecular weight poly (acrylic acid) copolymer, loosely cross-linked with allyl sucrose. A multi-unit bioadhesive system has also been prepared by coating microspheres of poly (hydroxyethyl-methacrylate) with mucoadhesive polymers using laboratory-scale equipment [5,6]. Akiyama et al. [7] prepared a polyglycerol ester of fatty acid-based microspheres coated with Carbopol®934P (CP) and CP-dispersing microspheres to evaluate their mucoadhesive properties.

In developing colloidal drug delivery systems, Lenaerts et al. [8] demonstrated the mucoadhesive properties of poly (alkylcyanoacrylate) nanoparticles in a series of autoradiographic studies and confirmed that the bioavailability of vincamine was improved by the nanoparticulate systems. Pimienta et al. [9] investigated the bioadhesion of hydroxypropylmethacrylate nanoparticles or isohexylcyanoacrylate nanocapsules coated with poloxamers and poloxamine to rat ileal segments in vitro using a labeled compound. We have developed mucoadhesive liposomes by coating the anionic liposomal surface with a cationic mucoadhesive polymer, chitosan, to evaluate the mucoadhesive properties both in vitro and in vivo [10,11]. The feasibility of chitosan coating for nanospheres of poly (lactic-co-glycolic acid) (PLGA) and the mucoadhesiveness of the resultant particles have been confirmed [12].

One of the most attractive applications of mucoadhesive nanoparticulate systems is for the oral administration of peptide drugs. Peptide drugs are intrinsically poorly absorbable owing to their high molecular weight and hydrophilicity; they are also susceptible to enzymatic degradation in the gastrointestinal tract. The drug carriers are expected to remain in the gastrointestinal tract while protecting the entrapped peptide drugs from enzymatic degradation until they are absorbed in released or intact particulate form. The concept of this system could be also applied to other peptide delivery systems, such as nasal and pulmonary administration systems via the mucous membranes.

In this chapter, we will review a variety of attempts to prepare and evaluate the function of mucoadhesive nanoparticulate systems mainly aimed at the delivery of peptide drugs via oral and pulmonary administration.

2. Preparation of nanoparticulate peptide delivery systems

2.1. Polymeric nanoparticles

Polymeric nanoparticles with biodegradable and biocompatible polymers are good candidates for particulate carriers to deliver peptide drugs. Such particles are expected to be adsorbed in an intact form in the gastrointestinal tract after oral administration [13]. Various preparation methods for polymeric nanoparticles, such as polymerization and solvent diffusion methods, have been reported [14]. We have successfully prepared nanoparticles with poly (lactic-co-glycolic acid) (PLGA) containing peptide drugs by using the emulsion solvent diffusion method, which is based on polymeric spherical crystallization, and the phase separation method [15–18].

2.1.1. Emulsion solvent diffusion method

Emulsion solvent diffusion (ESD) methods can be classified into two categories: the “in water” method and the “in oil” method. To prepare PLGA nanoparticles by the ESD in water method, PLGA and drug are dissolved in a mixed solvent of acetone and ethanol (or methanol), both of which dissolve in aqueous solution. When this polymer solution is

poured into aqueous poly (vinyl alcohol) (PVA) solution, a submicronized o/w emulsion is spontaneously formed due to immediate reduction of the interfacial tension with rapid diffusion of organic solvent into the aqueous phase (the Marangoni effect). Following solvent diffusion, the nanospheres are obtained by coprecipitation of polymer and drug following the reduction in solubility. During preparation of the nanoparticles, PVA prevents the aggregation and fusion of emulsion droplets by adsorbing on the surface of the droplets. Then, the resultant nanosphere suspension is centrifuged and resuspended in distilled water to remove the solvent and free drug in the upper aqueous phase. Finally, the nanospheres are powdered by freeze-drying.

The advantages of this method are its simplicity, narrow distribution and ready dispersibility of the resultant particles. The particles obtained are monodispersed spheres having a mean diameter of 250 nm with a polydispersity index of between 0.02 and 0.06 (Fig. 1). The powdered nanospheres can be readily dispersed to form a colloidal suspension just by rehydrating. However, a disadvantage of this method is that water-soluble drugs tend to leak from the emulsion droplets during the solvent diffusion process, leading to a low degree of entrapment of drug in the resultant particles.

To improve the encapsulation efficiency of water-soluble drugs into nanospheres, the dispersing medium can be changed from an aqueous solution to a medium chain triglyceride (MCT, caprylate and caprate triglyceride). PLGA-drug nanoparticles can be prepared by pouring the organic solution con-

taining PLGA and drug into an oily medium. The nanospheres collected from the oily suspension by centrifugation are washed with *n*-hexane. Finally, the nanospheres are suspended in PVA solution and then powdered by freeze-drying.

The difference between the two methods, ESD in water and in oil, involves the submicronized emulsion formation process. In the “water” method, the emulsion is spontaneously submicronized due to the rapid solvent diffusion at the surface of droplets. In the “oil” method, however, such rapid diffusion does not occur. Therefore, there is a need to add a surfactant (Span®80) to the polymer phase in the in oil method. Nanospheres with an average diameter of ca. 700 nm were obtained. The polydispersity index of this suspension ranges from 0.2 to 0.3.

2.1.2. Phase separation method

The phase separation method was devised to modify the drug encapsulation properties. In this method, aqueous drug solution is poured into a dichloromethane/acetone solution containing PLGA which is being agitated using a high-shear homogenizer. After preparation of the w/o emulsion, MCT is added to the system while stirring to induce phase separation. The coacervated droplets of PLGA surround the drug aqueous droplets due to the high activation energy of the surface of the aqueous droplets. Following the phase separation process, PLGA is precipitated by removing the organic solvent under vacuum.

The nanospheres prepared by the phase separation method have a bimodal particle size distribution with a weight mean diameter and polydispersity index of 800 nm and 0.3–0.5, respectively, due to a degree of coalescence of coacervated droplets.

2.2. Effect of preparation method of polymeric nanoparticles on the encapsulation efficiency of peptides

The encapsulation efficiency of peptide drugs into PLGA nanospheres was evaluated using TRH (molecular weight, 363.4) and elcatonin (molecular weight, 3363.82) as model peptide drugs. The results obtained suggest that the encapsulation efficiency is highly dependent on the method used to prepare the nanospheres (Fig. 2).

The ESD in oil method showed the highest

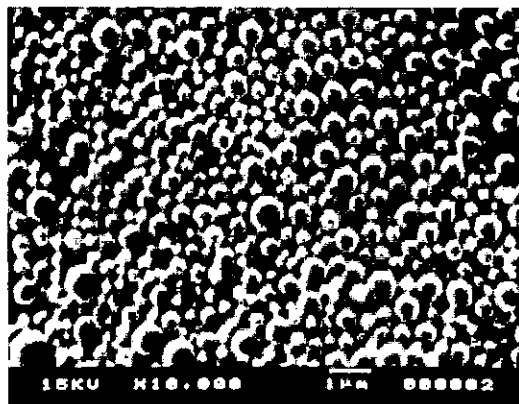


Fig. 1. Scanning electron micrograph of PLGA nanospheres.

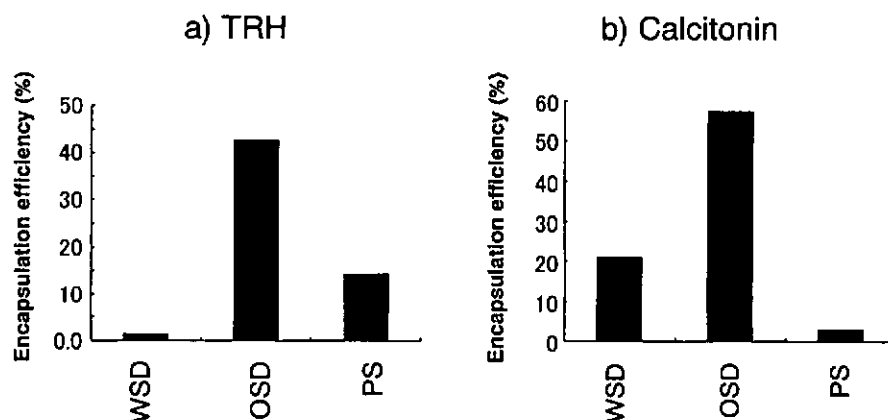


Fig. 2. Effect of preparation method on the encapsulation efficiency of nanospheres.; WSD: Emulsion solvent diffusion method in water; OSD: Emulsion solvent diffusion method in oil; PS: Phase separation method in oil.

encapsulation efficiency for each drug compared with the other methods. Although the ESD in the water method is suitable for the encapsulation of hydrophobic drugs, such as indomethacin, hydrophilic drugs may leak into the aqueous dispersing medium during the particle formation process. In the phase separation method, a relatively low encapsulation efficiency was observed for calcitonin, although MCT was used as a dispersing medium. These results suggest that the encapsulation efficiency in the phase separation method depends on the interaction of the drug with the polymer. When the polymer-drug interaction is low, the emulsion droplets of drug are not adequately covered with the coacervates of PLGA, resulting in the formation of thinner polymer walls on the surface of the nanoparticles. Indeed, in measuring the interaction between PLGA and the model drugs with DSC, an interaction could only be detected for TRH.

2.3. Surface modification of nanoparticulate systems with mucoadhesive polymers

One of the most promising strategies for designing mucoadhesive particulate systems is surface modification, or coating, of the drug carrier particles with mucoadhesive polymers. For coarse particles, a conventional coating method, such as fluidized bed coating, can be used. In reviewing the surface modification of colloidal particles, a number of investigations of the polymer coating of liposomes

have been published. Sunamoto et al. [19,20] have demonstrated an improvement in the chemical and physical stability of polymer coated liposomes prepared with polysaccharide derivatives such as mannan or amylopectin. So far several compounds, such as poloxamer [21], polysorbate 80 [22], carboxymethyl chitin [23], carboxymethyl chitosan [24] and dextran derivatives [25], have been reported to be suitable for the liposomal system.

With the aim of designing a mucoadhesive liposomal system, we examined the feasibility of polymer coating of liposomes with a variety of mucoadhesive polymers [7,8]. Negatively charged liposomes were prepared with L- α -dipalmitoylphosphatidylcholine (DPPC) and diacetyl phosphate (DCP) by the thin-film hydration method. A phosphate buffer (1 ml, pH 7.4) was used in the hydration. The polymers used for liposome coating are chitosan (CS), poly (vinyl alcohol) with a long alkyl chain (PVA-R) and poly (acrylic acid) bearing cholesterol (PAA-Chol), which are prepared from poly (acrylic acid) [26]. The properties of these polymers are summarized in Table 1. PVA-R and PAA-Chol dissolved in a phosphate buffer (pH 7.4) and CS dissolved in an acetate buffer (pH 4.4) were used to coat the liposomes. In coating with Carbopol (CP), positively charged liposomes with stearyl amine (SA) were prepared. Polymer coating was carried out by mixing the resultant liposomal suspensions with the same amount of polymer solution, followed by incubation.

The formation of a polymer layer on the surface of the liposomes was confirmed by comparing the zeta potential of liposomes before and after polymer coating [10]. The values of the zeta potential of the polymer-coated liposomes were found to change on increasing the concentration of polymer solution used in coating (Fig. 3). The change in zeta potential value of PAA-Chol-coated liposomes was mainly attributed to the electrical charge of the polymers fixed on the surface of the liposomes. Formation of an ion complex of CS with DCP on the surface of the liposomes during the coating process could explain the reduced zeta potential of the CS-coated liposomes. In the case of the uncharged polymer, PVA-R, the formation of a polymer layer on the surface of the liposomes could move the sheared plane to the solution side, which could be responsible for the change in the zeta potential. These results confirm the existence of a fixed polymer layer on the surface of liposome particles. In changing the pH of the dispersing medium of the liposomes from acidic to neutral, the zeta potential of CP-coated liposomes shifted from zero to more negative values, supporting the formation of the polymer layer on the

surface of positively charged liposomes. Coating of positively charged liposomes with an anionic polymer, Carbopol, was also confirmed in a similar manner [27]. It was also possible to coat submicronized liposomes with these mucoadhesive polymers.

We have also demonstrated the feasibility of coating poly (lactic-co-glycolic acid) (PLGA) nanoparticles with mucoadhesive polymers (chitosan, poly (vinyl alcohol) and poly (acrylic acid)) [12]. The polymer-coated PLGA nanoparticles were prepared by emulsion solvent diffusion methods (ESD) in water and in oil by adding the polymer to the systems during preparation of the nanoparticles (ESD in water) or by mixing the nanoparticles with the polymer solution (ESD in oil). In both cases, the polymer coating may be completed by physical adsorption of polymers on the surface of the nanoparticles. Using a higher concentration of the mucoadhesive polymer during the process caused aggregation of the nanoparticles. Thus, the polymer concentration in the coating was less than 0.5% in all cases. As formulation of PVA is necessary for the ESD process to prevent adhesion of particles to the apparatus, it was co-formulated with other polymers

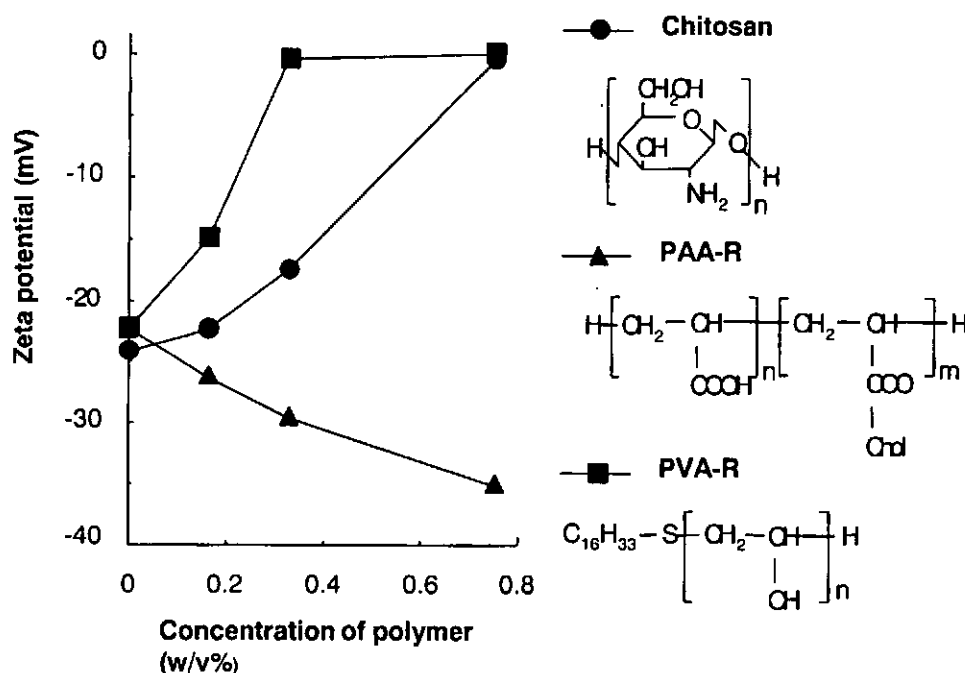


Fig. 3. Zeta potential of polymer-coated liposomes.

during the process. Surface coating with the polymers was confirmed by measuring the zeta-potential in the similar manner to that used for the polymer-coated liposomes.

3. Evaluation of mucoadhesive particulate systems

Various methods have been proposed to evaluate the mucoadhesive properties of tablets or mucoadhesive polymers themselves [28]. However, the number of studies dealing with the mucoadhesion of particulate systems is limited. In particular, special methods may be required to evaluate the mucoadhesive properties of nanoparticulate systems.

3.1. Evaluation of mucoadhesive properties of particulate systems

Rao and Buri [29] have evaluated the mucoadhesive properties of particulate systems. The particles were prepared by coating glass beads (0.45–0.5 mm) or crystals of acetyl salicylic acid ($> 630 \mu\text{m}$) with various hydrophilic polymers such as Polycarbophil (PC), NaCMC, HPMC, MC. In the adhesion test, the particles were placed on rat jejunum or stomach in a humid environment *in vitro*. The percentage of particles retained on the tissue was considered to be an index of bioadhesion. They observed stronger adhesion for polycarbophil and sodium carboxymethylcellulose-coated particles than other polymer coated particles.

Lehr et al. [30] used an *in situ* perfused ileal loop in the rat to study the mucoadhesion of multiple-unit bioadhesive systems. Microspheres of poly (2-hydroxyethyl methacrylate) (315–400 μm) were synthesized by suspension polymerization and coated with several mucoadhesive polymers in an air-suspension process. The polymer-coated microspheres were perfused with isotonic saline, and the number of collected particles was counted. Based on the mean residence time (MRT) calculated from the residence curves of the particles, the polycarbophil-coated particles exhibited strong mucoadhesive properties. They also proposed an *in-vitro* model for

intestinal absorption testing with rat intestine that was applicable to adhesion studies.

Durrer et al. [31,32] investigated the adsorption isotherms of poly (styrene) latex to rat intestinal mucosa under static conditions using turbidimetry and FTIR-ATR analysis. In the turbidimetric analysis, the amount of latex particles adsorbed was measured after completely dissolving the mucosa. FTIR analysis was carried out with the dried mucosa samples. A good correlation was observed for the absorbed amounts of particles measured by the two methods. The adsorption studies indicated that the shape of the absorption isotherms depend on the size and surface properties of the particles. It was also confirmed that the adsorption of particles was irreversible.

Based on these adsorption studies, Ponchel et al. [33] proposed a particle adsorption model with a porous adsorbent for fine particles, such as 200 nm poly (isobutyl cyanoacrylate) nanoparticles or poly (styrene) latexes with different particle sizes (Fig. 4). Fine particles less than 1 μm penetrate into the mucous layer, a porous adsorbent. On the other hand, larger particles, such as 2 μm polystyrene, showed a Langmuir-type adsorption. This adsorption pattern suggests that adsorption involves a monolayer of the particles on the smooth surface.

Investigations of the behavior of fine particles in the gastro-intestinal tract *in vivo* are usually carried out using radiolabelled particles. The particle movement profiles in the intestinal tract can be determined after oral administration of poly (hexyl-[3- ^{14}C]-cyanoacrylate) nanoparticles in mice [34]. The recovery of radioactivity from the orally administered nanoparticles in the intestine is measured at appropriate intervals. The total radioactivity recovered in the intestine after 90 min amounted to about 60–80% of the administered dose. Then, it was rapidly eliminated up to 240 min. Although the radioactivity had fallen to a trace level at 24 h after administration, 0.04% of the 90 min radioactivity was still detectable after 6 days.

Ponchel et al. [32] also studied the intestinal mucoadhesion profiles of ^{14}C -labeled poly (lactic acid) (PLA) particles (mean diameter: 1.5 μm) as a function of time after administration in the rat. After intragastric administration of 2 ml of a 10 mg/ml microsphere suspension, the rats were sacrificed at

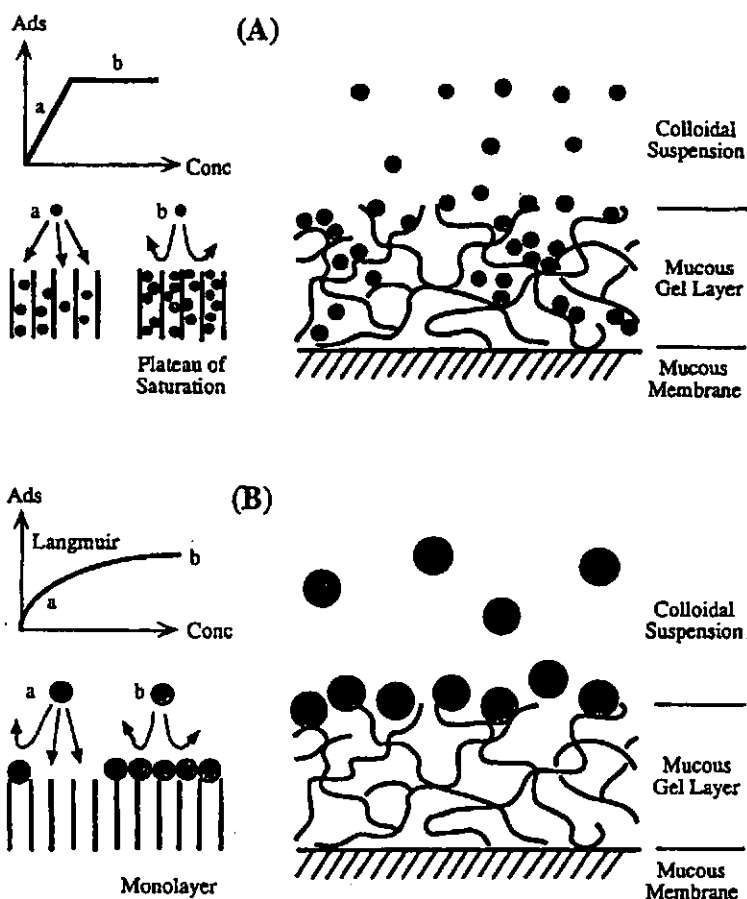


Fig. 4. Adsorption isotherms and corresponding adsorption models. A: particles $< 1 \mu\text{m}$. B: particles $> 1 \mu\text{m}$.

different times. Their intestines were removed and divided into five segments from duodenum to colon. The five segments were rinsed with saline and then the radioactivity of the rinsed fractions and the rinsed segments were counted to determine the amount of non-adhering particles. At 1 h after administration, 97% of the PLA particles administered remained in the GI-tract, while the percentage of recovered radioactivity in lumen and mucosa was 88.2 and 8.8%, respectively. At 0.5 h, particles adhered to the entire mucosal surface. However, the peak of the particle distribution moved to the lower sections with time and most of the particles passed through the small intestine within 6 h. The poor adsorption of PLA particles compared with model poly (styrene) latexes or poly (isobutyl cyanoacrylate) nanospheres

was attributed to its hydrophilic surface due to the presence of PVA on the surface of the particles.

3.2. Mucoadhesive properties of polymer coated liposomes

We tried to develop a simple in vitro method to evaluate the mucoadhesiveness of particulate systems such as polymer-coated liposomes [10,11]. In this test, an intestine tube (15 cm) isolated from Wistar rats was filled with the liposomal suspensions diluted 100-fold with an appropriate buffer solution after washing the inside of the tube with saline solution. The tube was sealed with closers and then incubated in saline solution at 37°C for more than 15 min. By measuring the number of liposomal particles before

and after incubation using a Coulter counter, the mucoadhesive % could be calculated by the following equation.

$$\text{Adhesive \%} = (N_0 - N_s) / N_0 \times 100$$

where N_0 and N_s are the number of liposomes before and after incubation, respectively.

The CS-coated liposomes showed the highest adhesive % (ca. 45%) of the three different polymer-coated liposomes tested under the same conditions. The mucoadhesive function is thought to be provided by the polymer layer fixed on the surface of the liposomes, because non-coated liposomes have an adhesive % of zero. When the coating amount of CS was increased by increasing the concentration of CS, the adhesive % of CS-coated liposomes increased. This result strongly suggests that CS on the surface of liposomes has mucoadhesive properties. Mucoadhesive properties of CS-coated liposomes were exhibited in phosphate buffer at pH 7.4, in which CS is not dissociated. Hence, it was concluded that interpenetration between the polymeric and mucous networks is responsible for the adhesion.

The mucoadhesive properties of positively and negatively charged liposomes have been compared by the particle counting method (Fig. 5) [27]. The

positively charged liposomes showed a higher adhesive % to the mucin layer than the negatively charged ones, and this was attributed to the negative charge of the surface of the mucin layer of the intestine. However, CP-coated, and thus negatively charged liposomes, as well as positively charged CS-coated liposomes, showed a higher adhesive % than positively charged non-coated liposomes. This result suggests again that a physical entanglement between the polymer and mucous layer is an important factor in promoting mucoadhesion of the polymer coated liposomes.

Confocal laser scanning microscopy is a useful technique for detecting the mucoadhesion of particulate systems. Recently, we observed the penetration of the CS-coated liposomes into the mucous layer in the rat after oral administration of the liposomes [35]. In comparing the non-coated and chitosan-coated liposomes, more liposomal particles penetrated the mucus layer in the case of chitosan-coated liposomes. As far as the amount remaining in the intestinal tube was concerned, the chitosan-coated liposomes tended to reside in the upper part compared with the non-coated liposomes (Fig. 6) [36]. These results confirm the mucoadhesive function of the chitosan-coated liposomes. It was also found that the amount of liposomal particles penet-

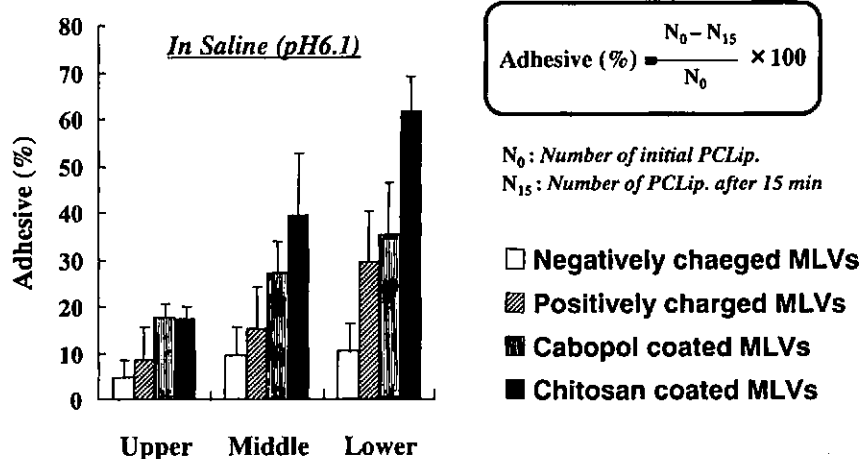
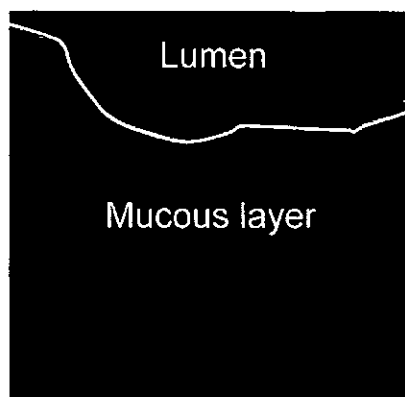
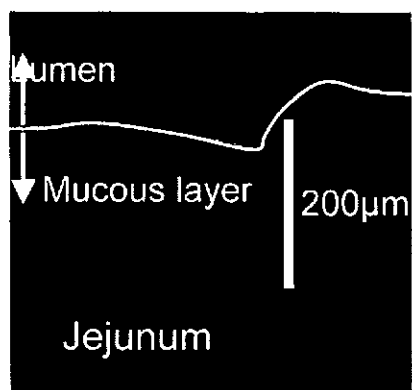


Fig. 5. Mucoadhesion investigation of polymer coated liposomes in rat intestine. Lipid composition; DPPC:DCP=8:2, DPPC:SA=40:1, Carbopol; Carbopol®971P, Chitosan; M.w.: 150 000, deacetylation degree: 85%.

A) Non-coated MLVs (4 μ m)

B) Chitosan-coated MLVs



C) Chitosan-coated SUV

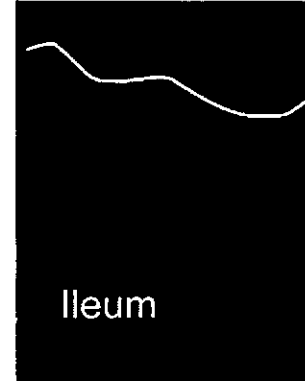
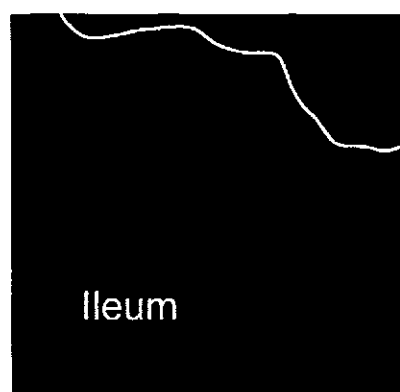
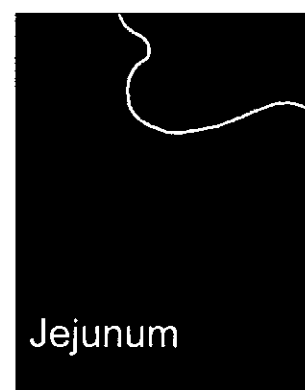


Fig. 6. Confocal laser scanning micrographs of uncoated and chitosan-coated MLV (A,B) or SUV (C) liposomes at 2 h after intragastric administration. TL (A) and 339.2 nm (B), respectively. [DSPC:DCP:Chol. = 8:2:1 Chitosan; M.w.: 150,000, deacetylation degree: 85% (0.3%)].

rating to the mucous layer increased when the size of the liposomes was reduced to ca. 100 nm in both non- and chitosan-coated liposomes [35].

3.3. Mucoadhesive properties of polymeric nanoparticles coated with mucoadhesive polymers

The mucoadhesiveness of surface-modified polymeric nanoparticles was also evaluated using the isolated rat intestinal sac [9]. As it is difficult to count the number of the submicron sized particles in suspension, the amount of adhering nanoparticles was measured fluorophotometrically by using fluorescence-labeled nanospheres. In this test, the everted rat intestinal sac was immersed in a suspension of fluorescence-labeled nanospheres and gently agitated for 15 min. Then, the amount of nanoparticles adhering to the sac was determined fluorophotometrically after acetone extraction. The chitosan-coated nanospheres showed higher mucoadhesion to the everted intestinal sac in saline than the uncoated nanospheres or nanospheres with other polymer coatings (Fig. 7). These findings are consistent with the results for polymer-coated liposomes. There was no distinct site-specificity for the mucoadhesion of chitosan-coated nanospheres to the gut sacs, although the mucoadhesion of the particles decreased slightly in the following order: duodenum > jejunum > ileum.

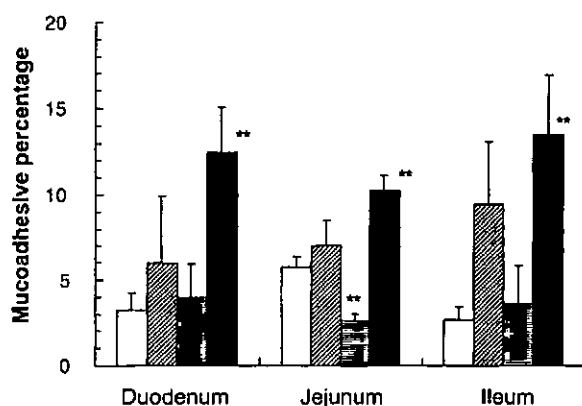


Fig. 7. Effect of the type of modifying polymer on the mucoadhesion of PLGA nanospheres (expressed as a %). (□) Control (PVA), (▨) PAA, (▩) NaAlg, (■) Chitosan, (Mean $\pm$ S.D., $n=3$, **: $P<0.01$).

The mucoadhesive properties of polymer-coated nanoparticles were also evaluated using mucin particles in vitro [37]. A nanoparticle suspension (0.01 w/v%) was mixed with a mucin suspension (0.05 w/v%) and then the mixed suspension was centrifuged at 1000 rpm for 10 min to separate the nanospheres adhering to the mucin particles (ca. 6 μ m). The amount of nanospheres adsorbed to the mucin particles was determined by fluorometry. The trend in the adhesive properties of nanospheres to the mucin particles was the same as their mucoadhesive properties measured using the intestine immersing method described above. These results indicate that this method is useful for screening the bioadhesiveness of nanoparticles without the need to use animals.

4. Peptide drug delivery with mucoadhesive nanoparticulate systems

4.1. Effectiveness of polymer-coated liposomes for the absorption of peptide drugs

We evaluated the effectiveness of polymer-coated liposomes as carriers of peptide drugs following their oral administration in rats [11]. Insulin-encapsulating liposomes were prepared by the thin-film method with 1 mg/ml of insulin solution, obtained by mixing a 0.01 N HCl insulin solution with the same volume of acetate buffer (pH 4.4). Polymer coating was carried out in the same manner as described above. The insulin liposomal suspension (24 IU/rat) was administered intragastrically to male Wistar rats fasted for the previous 24 h and the plasma glucose levels were measured using a commercial kit (glucose CII-test WAKO) at appropriate intervals. A marked reduction in blood glucose was observed 30 min after administration of the CS-coated liposomal insulin, while little reduction was detected in the case of the uncoated insulin liposomes (Fig. 8). The most striking result was that the reduced basal blood glucose level was maintained up to at least 12 h after administration of the CS-coated insulin-loaded liposomes. This prolonged effect of the CS-coated liposomal system could be attributed to its mucoadhesive properties. It is presumed that an insulin molecule released from the liposomes in the

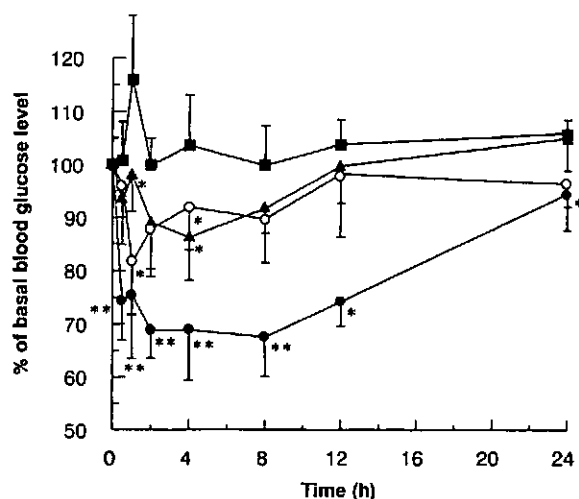


Fig. 8. Change in serum glucose (%) after oral administration of insulin (24IU/rat). (■) Control, (●) CS-coated liposomes, (▲) Uncoated liposomes, (○) Insulin soln. Each value represents the mean S.E. of four or five rats; *, $P < 0.05$, **, $P < 0.01$. Lipid composition: DPPC:DCP = 8:2. Polymer concentration: 0.75%.

mucous layer is absorbed without being enzymatically degraded.

In an additional absorption experiment, calcitonin (Elcatonin)-loaded liposomes, with or without polymer coating, were administered intragastrically to rats to confirm the effects of the mucoadhesive properties of the devices on the enteral absorption of poorly absorbable drugs [27]. At appropriate time intervals, the calcium concentration in blood was monitored with a commercial kit (Wako Pure Chemicals). In this test, negatively charged and positively charged uncoated liposomes and CP-coated liposomes, as well as CS-coated liposomes, were compared under the same experimental conditions. (Fig. 9a, b). The positively charged liposomes reduced the calcium concentration by a greater amount than the negatively charged liposomes, which reflects the difference in the mucoadhesive properties of these liposomes. The CP-coated liposomes, as well as the CS-coated liposomes, had a more prolonged effect than the positively charged liposomes, confirming the effectiveness of the polymer coating.

In conclusion, both chitosan- and Carbopol-coated liposomes have mucoadhesive properties which improve the enteral absorption of model peptide drugs, insulin and calcitonin, formulated in liposomes, in rats.

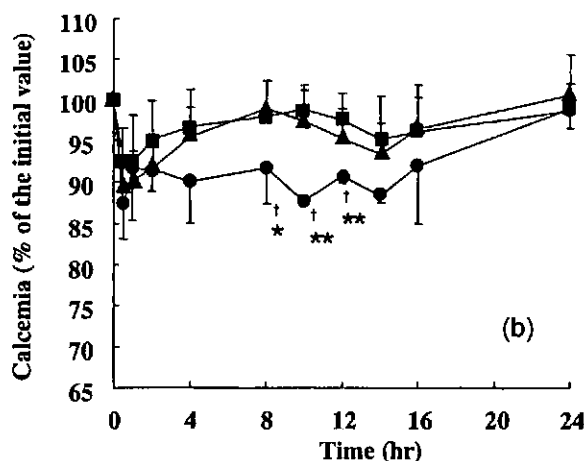
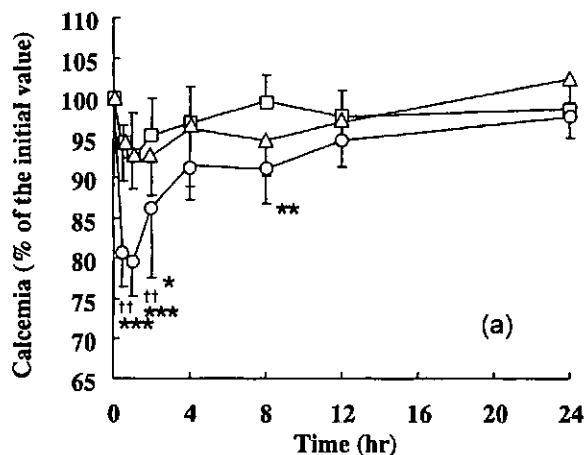


Fig. 9. (a) Chitosan-coated MLV liposomes containing calcitonin: lipid compositions. (□) calcitonin solution, (○) negatively charged MLVs, (●) positively charged MLVs, (▲) chitosan-coated MLVs; DPPC:DCP = 8:2, Chitosan; M.w.: 150,000, deacetylation degree: 85%, Dose; 500IU/kg, (b) Carbopol-coated MLV liposomes containing calcitonin. (■) calcitonin solution, (○) positively charged MLVs, (●) Carbopol-coated MLVs; Lipid composition: DPPC:SA = 40:1, Carbopol; Carbopol®971P, Dose; 500IU/kg. *, $P < 0.05$, **, $P < 0.01$: Significantly different from calcitonin solution. †† $P < 0.01$: Significantly different from negatively or positively charged MLVs.

4.2. Mucoadhesive polymeric nanoparticles for oral administration of peptide drugs

We evaluated the effectiveness of mucoadhesive polymeric nanospheres in the absorption of a peptide drug by using Elcatonin-loaded PLGA nanospheres coated with chitosan [12]. The physiological action, i.e., reduction in blood calcium, was monitored after

oral administration of the uncoated and chitosan-coated PLGA nanospheres in rat. The blood calcium fell temporarily by about 80–85% for 1 h after intragastric administration of uncoated nanospheres containing elcatonin, as shown in Fig. 10. The blood calcium-time profile was almost the same as that following the administration of elcatonin solutions of 250 IU/Kg or less. On increasing the dose of the uncoated nanospheres, the reduction in Ca concentration was prolonged over 10 h. On the other hand, a significantly prolonged reduction in blood Ca was obtained over 12 h after administration of the chitosan-coated nanospheres at doses of 125 IU/Kg and 250 IU/Kg. On increasing the dose, e.g. to 500 IU/Kg, the reduction in calcium was prolonged. This finding indicates that the chitosan-coated PLGA nanospheres are able to overcome their mucoadhesion ability due to the turnover of the mucosal surface in the small intestine. This might be due to deep penetration of chitosan-coated nanospheres into the mucus layer and their adhesion to mucus tissue, where the drug is slowly released from the system. The increase in residence time and intimate contact of the chitosan-coated nanospheres with the wall of

the small intestine (adsorption site) could significantly improve the degree of drug absorption, although the literature reports that there is an enhancement of nanospheres taken up by gut tissue.

These findings suggest that mucoadhesive nanoparticulates are useful peptide carriers for mucosal peptide delivery due to their prolonged retention in the gastro-intestinal tract and penetration into the mucus layer.

4.3. Pulmonary delivery of peptide drugs with mucoadhesive polymeric nanoparticles

Aerosol inhalation therapy via the respiratory tract has been used frequently to treat lung disease, and systemic drug delivery has also been used. This route is desirable for delivering peptide drugs since it has the following advantages over other routes. (1) The surface area of the lung is extremely large and mucosal permeation of drug is comparatively easy, because the vascular system is well developed and the walls of alveoli are extremely thin. (2) The activity of drug-metabolizing enzymes intracellularly or extracellularly is relatively low. To deliver drugs into the deep lung, which is the main absorption site, requires the generation of aerosols less than 7 μm .

We have tried to use mucoadhesive nanoparticles for pulmonary delivery [38]. There are three different methods for delivering drugs to the lung, pressurized metered dose inhalers, nebulizers and dry powder inhalers. Pressurized metered-dose inhalers (MDI) using chlorofluorocarbons (CFC) as a propellant have been widely used as pulmonary drug delivery devices. However, because of the environmental concerns about CFC, the development of nonpressurized delivery systems without CFC propellants has been welcomed as an alternative to MDI. Therefore, for aerosolization of nanosphere, the nebulizer method was chosen because nebulizers are routinely used without the need for special training. Furthermore, the nebulizer is suitable for very fine suspensions as well as aqueous solutions.

The administration system was constructed as shown in Fig. 11. In this system, aerosolized mists from nebulizers accumulate in the chamber, where a constant mist concentration is maintained. Then, the mist is drawn up and administered to the animal via

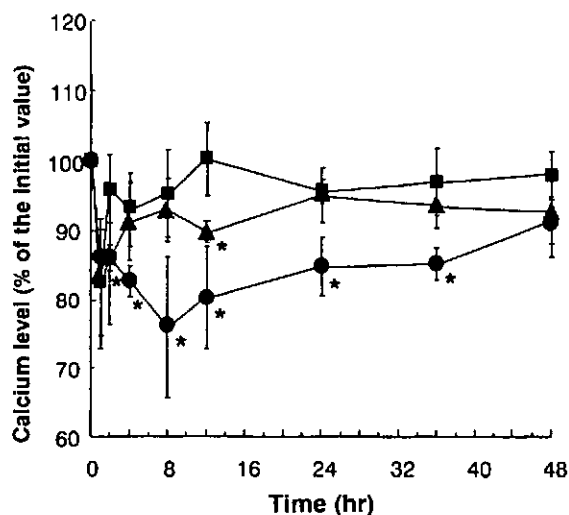


Fig. 10. Blood calcium profiles after intragastric administration of calcitonin loaded PLGA nanospheres modified with or without chitosan and calcitonin solution. (■) Calcitonin solution, (▲) Unmodified nanospheres, (●) Chitosan modified nanospheres ($n = 3$, Mean $\pm$ S.D. (*: $P < 0.05$), administered dose: 500 IU/Kg.

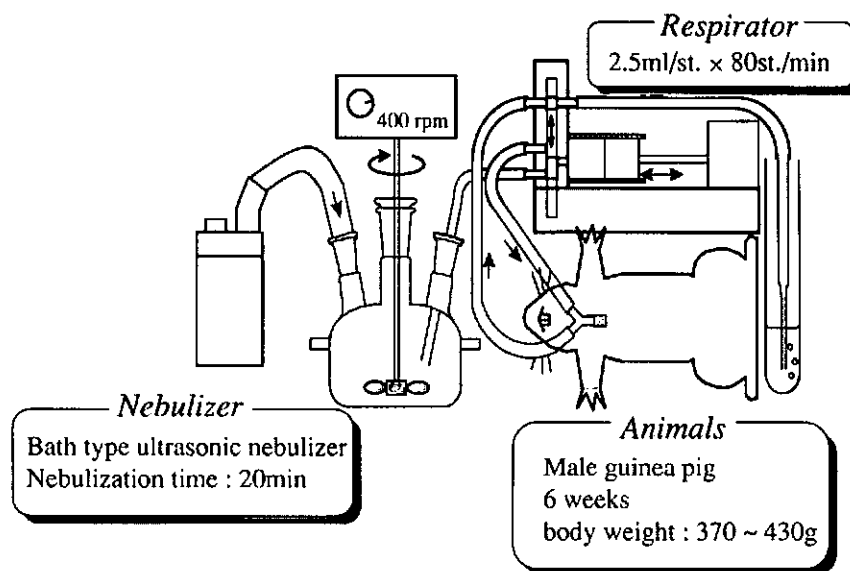


Fig. 11. Device for the delivery of PLGA NS to the lungs of guinea pigs.

the trachea using a respirator. This system was confirmed to be able to readily administer a constant amount of nanoparticle systems.

CS-modified nanospheres labeled with a fluorescent dye were administered to guinea pig lungs using this administration system. The clearance rate of the CS-modified nanospheres was lower than that of unmodified nanospheres. Most of the unmodified nanosphere were cleared soon after administration (<1 h). However, the CS-modified nanospheres exhibited slower elimination from the lung than the unmodified nanospheres. The time-course of the nanospheres remaining in the lung followed first-order kinetics. Then, the elimination rate of CS modified nanospheres, which was calculated from the data on their retention behavior, was about half that of unmodified nanospheres. These results indicate that the CS-nanospheres adhere to the mucus in the trachea and lung tissues due to the mucoadhesive properties of CS and release the drug in the lung over a prolonged period.

The elcatonin loading nanospheres and elcatonin solution were administered to the lungs (100 IU/Kg) in the same manner and the blood calcium concentration was monitored (Fig. 12). The particle size of elcatonin-loaded nanospheres was ca. 650 nm.

The drug solution produced a temporary fall in blood calcium after administration. Unmodified nanospheres also produced a temporary reduction in blood which was longer than that obtained by the drug solution. These reduced blood calcium levels had returned to normal at 8 h after administration. However, a significantly prolonged reduction in blood calcium after administration of the CS-nanospheres enclosing elcatonin was produced for over 24 h compared with the drug solution and the unmodified nanospheres. It is believed that the unmodified nanospheres are rapidly removed from lung before they are able to release the drug. The prolonged pharmacological effect of CS-nanospheres could be attributed to their adherence to lung tissue resulting in them remaining there for a considerable period.

These results show that mucoadhesive nanospheres are useful for the pulmonary delivery of peptide drugs as well as for oral administration.

5. Conclusion

We have developed mucoadhesive nanoparticulate systems, including liposomes and PLGA nanoparti-

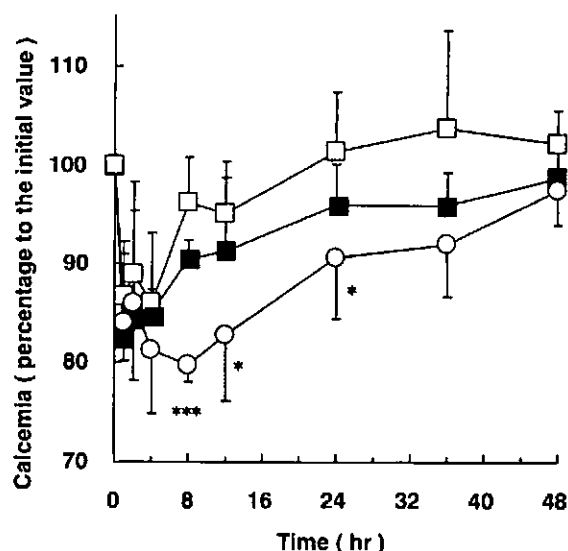


Fig. 12. Blood calcium profiles (% of initial value) after pulmonary administration (Dose: 100 IU/Kg). (□): Elcatonin solution, (■) unmodified nanospheres, (○) Chitosan modified nanospheres ($n=4$, Mean $\pm$ S.D., *: $P<0.05$, ***: $P<0.001$).

cles, to improve peptide delivery via mucus membranes, e.g. oral administration. PLGA nanoparticles were prepared by a novel emulsion solvent diffusion (ESD) method in water or in oil as well as the phase separation method. In the ESD methods, PLGA nanoparticles were prepared by coprecipitation of drug and polymer in emulsion droplets of an organic phase miscible with the dispersing medium either produced spontaneously in water or following the addition of surfactant in the oil phase; then the organic solvent diffused into the dispersing medium. The ESD in oil method has the advantage of improving the encapsulation efficiency of water soluble peptides compared with the ESD in water method.

A mucoadhesive liposomal system was prepared by treating (coating) a negatively charged liposome suspension with DPPC and DCP with a mucoadhesive polymer solution, such as CS, PVA-R, or PAA-Chol. Positively charged liposomes with SA were coated with CP. Mucoadhesive surface modification of PLGA nanoparticles was carried out by adding the polymer (CS, Na Alg, PAA) to the systems during preparation of the nanoparticles (ESD in water) or by

mixing the nanoparticles with the polymer solution (ESD in oil). The surface coating of nanoparticulate systems with polymers was confirmed by monitoring the change in zeta potential before and after surface modification. This change was due to neutralization of the surface charge or a sharing plane shift by the action of the polymer layer adsorbed on the surface of the particles.

We have developed novel methods to evaluate the mucoadhesive properties of polymer-coated nanoparticulate systems, including a particle counting method using a Coulter counter, measuring free particles after an adhesive test in an intestine tube, adsorption methods involving measuring the particles adsorbed to the everted rat intestinal sac immersed in a suspension of particles, and by measuring the adsorbed particles on mucin fragments dispersed in medium. Using the mucoadhesion test, it was found that CS-coated particulate systems exhibited superior adhesion ability, compared with other systems, followed by CP-coated particles. The penetration of CS-coated liposome into a layer of mucus in the rat after oral administration was confirmed directly by confocal laser scanning microscopy.

Mucoadhesive nanoparticulate systems, such as chitosan- and Carbopol-coated liposomes and PLGA nanoparticles, were found to be useful peptide (insulin, calcitonin) carriers for improving oral mucosal delivery due to their prolonged retention in the gastro-intestinal tract and excellent penetration into the mucus layer. Furthermore, mucoadhesive PLGA nanoparticles were shown to be useful for the pulmonary delivery of peptide drugs as well as oral administration due to sharing the same mechanism as in the enteral absorption of peptides.

References

- [1] M.A. Longer, H.S. Ch'ng, J.R. Robinson, Bioadhesive polymers as platforms for oral controlled drug delivery III: Oral delivery of chlorothiazide using a bioadhesive polymer, *J. Pharm. Sci.* 74 (1985) 406–411.
- [2] M. Ishida, N. Nambu, T. Nagai, Mucosal dosage form of lidocaine for toothache using hydroxypropyl cellulose and carbopol, *Chem. Pharm. Bull.* 30 (1982) 980–984.
- [3] K. Satoh, K. Takayama, Y. Machida, Y. Suzuki, M. Nakagaki, T. Nagai, Factors affecting the bioadhesive property of tablets consisting of hydroxypropyl cellulose and

- carboxyvinyl polymer, *Chem. Pharm. Bull.* 37 (1989) 1366–1368.
- [4] W.G. Chen, G.C.C. Hwang, Adhesive and in vitro release characteristics of propranolol bioadhesive disc system, *Int. J. Pharm.* 82 (1992) 61–66.
 - [5] C.M. Lehr, J.A. Bouwstra, J.J. Tukker, H.E. Junginger, Intestinal transit of bioadhesive microspheres in an in situ loop in the rat—A comparative study with copolymers and blends based on poly acrylic (acid), *J. Controlled Release* 13 (1990) 51–62.
 - [6] C.M. Lehr, J.A. Bouwstra, W. Kok, A.G. De Boer, J.J. Tukker, J.C. Verhoef, D.D. Breimer, H.E. Junginger, Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat, *J. Pharm. Pharmacol.* 44 (1992) 402–407.
 - [7] Y. Akiyama, N. Nagahara, T. Kashiwara, S. Hirai, H. Toguchi, In vitro and in vivo evaluation of mucoadhesive microspheres prepared for the gastrointestinal tract using polyglycerol esters of fatty acids and a poly(acrylic acid) derivatives, *Pharm. Res.* 12 (1995) 397–405.
 - [8] V. Lenaerts, P. Couvreur, L. Grislain, P. Maincent, Nanoparticles as a gastroadhesive drug delivery system, in: V. Lenaerts, R. Gurny (Eds.), *Bioadhesive Drug Delivery Systems*, CRC Press, Florida, 1990, pp. 93–104.
 - [9] C. Pimienta, F. Chouinard, A. Labib, V. Lenaerts, Effect of various poloxamer coatings on in vitro adhesion of isohexylcyanoacrylate nanospheres to rat ileal segments under liquid flow, *Int. J. Pharm.* 80 (1992) 1–8.
 - [10] H. Takeuchi, H. Yamamoto, T. Niwa, T. Hino, Y. Kawashima, Mucoadhesion of polymer-coated liposomes to rat intestine in vitro, *Chem. Pharm. Bull.* 42 (1994) 1954–1956.
 - [11] H. Takeuchi, H. Yamamoto, T. Niwa, T. Hino, Y. Kawashima, Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes, *Pharm. Res.* 13 (1996) 896–901.
 - [12] Y. Kawashima, H. Yamamoto, H. Takeuchi, Y. Kuno, Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin, *Pharm. Dev. Technol.* 5 (2000) 77–85.
 - [13] A.T. Florence, A.M. Hillery, N. Hussain, P.U. Jani, Nanoparticles as carriers for oral peptide absorption: studies on particle uptake and fate, *J. Controlled Release* 36 (1995) 39–46.
 - [14] J. Kreuter, Nanoparticles, in: J. Kreuter (Ed.), *Colloidal Drug Delivery Systems*, Marcel Dekker Inc, New York, 1994, pp. 219–342.
 - [15] Y. Kawashima, New processes – application of spherical crystallization to particulate design of pharmaceuticals for direct tableting and coating, and new drug delivery system, in: D. Chulia, M. Deleuil, Y. Pourcelot (Eds.), *Handbook of Powder Technology*, Vol. 9, Elsevier, Amsterdam, 1994, pp. 493–512.
 - [16] T. Niwa, H. Takeuchi, T. Hino, N. Kunou, Y. Kawashima, Preparations of biodegradable nanospheres of watersoluble and insoluble drugs with DL-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior, *J. Controlled Release* 25 (1993) 89–98.
 - [17] T. Niwa, H. Takeuchi, T. Hino, M. Nohara, Y. Kawashima, Biodegradable submicron carriers for peptide drugs: Preparation of DL-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate by a novel emulsion-phase separation method in an oil system, *Int. J. Pharm.* 121 (1995) 45–54.
 - [18] Y. Kawashima, H. Yamamoto, H. Takeuchi, T. Hino, H. Niwa, Properties of a peptide containing DL-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods, *Eur. J. Pharm. Biopharm.* 45 (1998) 41–48.
 - [19] J. Sunamoto, K. Iwamoto, M. Takada, T. Yuzuriha, K. Katayama, Improved drug delivery to target specific organs using liposomes as coated with polysaccharides, *Polymer Sci. Technol.* 23 (1983) 157–168.
 - [20] J. Sunamoto, K. Iwamoto, M. Takada, T. Yuzuriha, K. Katayama, Polymer coated liposomes for drug delivery to target specific organs, in: J.M. Anderson, S.W. Kim (Eds.), *Recent Advances in Drug Delivery Systems*, 1984, pp. 153–162.
 - [21] M. Jamshaid, S.J. Farr, P. Kearney, I.W. Kellaway, Poloxamer sorption on liposomes: comparison with polystyrene latex and influence on solute efflux, *Int. J. Pharm.* 48 (1988) 125–131.
 - [22] B. Kronberg, A. Dahlman, J. Carlfors, J. Karlsson, P. Artursson, Preparation and evaluation of sterically stabilized liposomes: colloidal stability, serum stability, macrophage uptake, and toxicity, *J. Pharm. Sci.* 79 (1990) 667–671.
 - [23] C. Dong, J.A. Rogers, Polymer-coated liposomes: stability and release of ASA from carboxymethyl chitin-coated liposomes, *J. Controlled Release* 17 (1991) 217–224.
 - [24] S. Alamelu, K.P. Rao, Studies on the carboxymethyl chitosan-containing liposomes for their stability and controlled release of dapson, *J. Microencapsul.* 8 (1991) 505–515.
 - [25] W.G.L. Elferink, J.G. de Wit, G. In't Veld, A. Reichert, A.J.M. Driessen, H. Ringsdorf, W.N. Konings, The stability and functional properties of proteoliposomes mixed with dextran derivatives bearing hydrophobic anchor groups, *Biochim. Biophys. Acta* 1106 (1992) 23–30.
 - [26] S.C. Arnold, M.S. Ferritto, R.W. Lenz, D.A. Tirrell, pH dependent modification of phospholipid vesicle membranes by poly(carboxylic acid)s bearing pendant cholesteryl esters, *Polymer Reprint* 27 (1986) 42–43.
 - [27] H. Takeuchi, Y. Matsui, H. Yamamoto, Y. Kawashima, Mucoadhesive liposomes coated with chitosan or Carbopol for oral administration of peptide drugs, in: *Proceedings of 26th International Symposium on Controlled Release of Bioactive Materials*, 1999, pp. 988–989.
 - [28] N.A. Peppas, A.G. Mikos, Experimental methods for determination of bioadhesive bond strength of polymers with mucus, *Stp Pharma* 5 (1989) 187–191.
 - [29] K.V. Ranga Rao, P. Buri, A novel in situ method to test polymers and coated microparticles for bioadhesion, *Int. J. Pharm.* 52 (1989) 265–270.
 - [30] C.M. Lehr, J.A. Bouwstra, J.J. Tukker, H.E. Junginger,

- Intestinal transit of bioadhesive microspheres in an in situ loop in the rat — a comparative study with copolymers and blends based on poly(acrylic acid), *J. Controlled Release* 13 (1990) 51–62.
- [31] C. Durrer, J.M. Irache, F. Puisieux, D. Duchene, G. Ponchel, Mucoadhesion of latexes: I. Analytical methods and kinetic studies, *Pharm. Res.* 11 (1994) 674–679.
- [32] C. Durrer, J.M. Irache, F. Puisieux, D. Duchene, G. Ponchel, Mucoadhesion of latexes: II. Adsorption isotherms and desorption studies, *Pharm. Res.* 11 (1994) 680–683.
- [33] G. Ponchel, M.J. Montisci, A. Dembri, C. Durrer, D. Duchene, Mucoadhesion of colloidal particulate systems in the gastro-intestinal tract, *Eur. J. Pharm. Biopharm.* 44 (1997) 25–31.
- [34] J. Kreuter, U. Muller, K. Munz, Quantitative and microautoradiographic study on mouse intestinal distribution of polycyanoacrylate nanoparticles, *Int. J. Pharm.* 55 (1989) 39–45.
- [35] H. Takeuchi, Y. Matsui, H. Yamamoto, Y. Kawashima, unpublished data.
- [36] Y. Matsui, H. Yamamoto, H. Takeuchi, Y. Kawashima, Particulate design of chitosan coated liposomes and their mucoadhesive property, *J. Pharm. Sci. Technol.* 59 (Supplement) (1999) 66.
- [37] Y. Kawashima, H. Yamamoto, H. Takeuchi, Y. Kuno, Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin, *Pharm. Dev. Technol.* 5 (2000) 77–85.
- [38] H. Yamamoto, Y. Kawashima, H. Takeuchi, Y. Kuno, S. Sugimoto, Improved pulmonary delivery of peptide with surface-modified lactide/glycolide copolymer nanospheres with chitosan, *Proceedings of the 27th International Symposium on Controlled Release of Bioactive materials*, Paris, 2000, #8029.