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(54) Title: INTERACTION BETWEEN BIOADHESIVE LIPOSOMES AND TARGET SITES

(57) Abstract

Recognizing substances, epidermal growth factor, gelatin, collagen and hyaluronic acid, have been covalently bound to liposomal surfaces and utilized to attach liposomes onto a cellular target site. These "bioadhesive" liposomes offer several advantages in the area of topically and locally administered free drug. These advantages include the mutual protection of both the drug and biological environment; an increase in drug bioavailability and retention at the target site; and improved adherence or adhesion to the designated target site.

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INTERACTION BETWEEN BIOADHESIVE LIPOSOMES AND TARGET SITES

BACKGROUND OF THE INVENTION

The present invention relates to a novel drug delivery system, particularly to microscopic drug delivery systems (MDDS) utilizing drug-encapsulating "bioadhesive" liposomes for topical and local drug administration.

Currently, the topical and local administration of a drug can be in its free form, dissolved or dispersed in a suitable diluent, or in a vehicle such as a cream, gel or ointment. Examples of therapeutic or designated targets for topical or local drug administration include burns; wounds; bone injuries; ocular, skin, intranasal and buccal infections; ocular chronic situations such as glaucoma; and topically and locally accessed tumors. Several difficulties exist with either the topical or local administration of a drug in its free form. For example, short retention of the drug at the designated site of administration reduces the efficacy of the treatment and requires frequent dosing. Exposure of the free form drug to the biological environment in the topical or local region can result in drug degradation, transformation into inactive entities and nondiscriminating and uncontrollable distribution of the drug. Such degradation and uncontrollable distribution of the drug can result in toxicity issues, undesirable side effects and loss of efficacy.

Microscopic drug delivery systems (MDDS) have been developed to overcome some of the difficulties associated with free drug administration. MDDS is divided into two basic classes: particulate systems, such as cells, microspheres, viral envelopes and liposomes; or nonparticulate systems which are macromolecules such as proteins or synthetic polymers. Using these specific systems, drug-loaded MDDS can perform as

sustained or controlled release drug depots. By providing a mutual protection of the drug and the biological environment, MDDS reduces drug degradation or inactivation. As a system for controlled release of the drug, MDDS improves drug efficacy and allows reduction in the frequency of dosing. Since the pharmacokinetics of free drug release from depots of MDDS are different than from directly-administered drug, MDDS provides an additional measure to reduce toxicity and undesirable side effects.

Liposomes offer a range of advantages relative to other MDDS systems. Liposomes are lipid vesicles composed of membrane-like lipid layers surrounding aqueous compartments. Composed of naturally-occurring materials which are biocompatible and biodegradable, liposomes are used to encapsulate biologically active materials for a variety of purposes. Having a variety of layers, sizes, surface charges and compositions, numerous procedures for liposomal preparation and for drug encapsulation within them have been developed, some of which have been scaled up to industrial levels. Through appropriate selection of liposome type and size, the encapsulated drug can also range in size. Liposomes can accommodate lipid-soluble drugs, aqueous soluble drugs and drugs with both hydrophilic and hydrophobic residues. Liposomes can be designed to act as sustained release drug depots and, in certain applications, aid drug access across cell membranes. Their ability to protect encapsulated drugs and other characteristics make liposomes a popular choice in developing MDDS, with respect to the previous practices of free drug administration.

Despite the advantages offered, utilization of drug-encapsulating liposomes does pose some difficulties. For example, liposomes as MDDS have limited targeting abilities, limited retention and stability in circulation, potential

toxicity upon chronic administration and inability to extravasate. In recent years, attempts have been made to couple different recognizing substances with liposomes to confer target specificity to the liposomes, namely antibodies, glycoproteins and lectins. Although the bonding of these recognizing substances to liposomes occurred, the resulting modified liposomes did not perform as hoped, particularly during in vivo studies. Other difficulties are presented when utilizing these recognizing substances. For example, antibodies can be patient specific and therefore, add cost to the drug therapy.

Several cell-associated entities can participate in the binding between cells and recognizing substances. These are generally divided into three major types: receptors and non-receptor components of the cellular system and extracellular matrix. Receptors can be present in several species or states, differing in populations per cell and in binding affinity. Binding to such receptor entities is usually referred to as "specific binding". Non-receptor cell membrane components also differ in populations and in affinity. Binding to such non-receptor entities is usually referred to as "non-specific binding".

To perform effectively, the topical or local administration of drug-encapsulating liposomes should have specificity for and the ability to adhere to the designated target area and should facilitate drug access to intracellular sites. Currently available liposomes and other MDDS systems do not meet these performance requirements of topical and local drug administration.

SUMMARY OF INVENTION

It has been learned that modifying regular liposomes by covalently anchoring certain recognizing substances to the

liposomal surface creates a "bioadhesive" liposome with target specificity and retention. The recognizing substances are molecules which can be utilized as an adhesive or glue, attaching a drug-encapsulating liposome onto a therapeutic target site. These "bioadhesive" recognizing substances can perform either through receptor mechanisms or through associations with components within the extracellular matrix. Regardless of the specific mechanism of adhesion, these substances are referred to as "bioadhesive recognizing substances" based on their common end result.

Through covalent anchoring, the bioadhesive recognizing substances become an integral part of the liposome, yet remain accessible to the interaction counterpart at the target site. They endow the liposome and encapsulated drug with the ability to adhere to the target site. Hence, "bioadhesive" liposomes have been developed which are target adherent, sustained release drug depots. The identification of recognizing substances and the methodologies of modifying liposomes has been disclosed in concurrently filed applications. These bioadhesive liposomes offer several advantages over previous practices of topically or locally administered free drug and other MDDS, whether with regular liposomes or other MDDS systems. These advantages include the mutual protection of both the drug and biological environment; an increase in drug bioavailability and retention at the target site; and improved adherence or adhesion to the designated target site. These advantages result in the potential reduction of undesirable biological side-effects of the drug being administered.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the binding of bioadhesive liposomes (EGF-modified; open double triangle) and regular liposomes (asterisk) of the LUVET type to A431 cells in culture (in

monolayers), as dependent upon liposome concentration. Bound liposomes, denoted as B, are in units of ng EGF per 10^6 cells. Free ligand concentration, denoted as L, are in units of ng EGF per 10^6 cells for bioadhesive liposome (first row of L values) and in units of umoles lipid per 10^6 cells for the regular liposomes (second row of L values).

FIG. 2 shows a time course of the binding of bioadhesive liposomes (collagen-modified) of the MLV type to A431 cells in culture (in monolayers). Collagen is tritium-labeled. The fraction of liposomes relative to the amount present in the initial reaction mixture at zero-time which is cell-associated is determined over time.

FIG. 3 shows the binding of bioadhesive liposomes (collagen-modified) and regular liposomes of the MLV type to A431 cells in culture (in monolayers). Collagen is tritium-labeled (^{3}-H) and liposomes are ^{14}-C labeled. Bound liposomes, denoted as B, are in units of ^{3}-H DPM per 10^5cells (left scale) and in units of ^{14}-C DPM per 10^5 cells (right scale). Free ligand concentration, denoted as L, are in units of ^{3}-H or ^{14}-C DPM per 10^5 cells. Bioadhesive liposome with collagen labeled is depicted with open double triangles; bioadhesive liposome with the liposome labeled is depicted with crosses; and, regular liposome is depicted with asterisks.

DETAILED DESCRIPTION

According to the present invention, bioadhesive liposomes have bound to cell cultures having receptors or extracellular matrix which accommodate the recognizing substance bonded to the liposome. Liposomes, in particular, multilamellar vesicles (MLV), microemulsified liposomes (MEL) or large unilamellar vesicles (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. Recognizing substances, each of which have been accepted for

human use, include epidermal growth factor (EGF), hyaluronic acid (HA), gelatin and collagen. Each of these recognizing substances have a biological origin and are biodegradable and biocompatible. Further, these recognizing substances have functional residues which can be utilized in covalent anchoring to the regular liposomal surfaces.

The methodologies of preparing the specific bioadhesive liposomes have been disclosed in separate applications concurrently filed with this disclosure and will not be repeated here.

A complete accounting of binding entities has been determined by the previously known multi-term Langmuir Isotherm equation, as applied for the quantitative description of the relationship between the free and dependent variables:

$$B = \sum_{i=1}^{n} \frac{Bmax_i\ [L]}{Kd_i + [L]} \qquad (1)$$

where n is the number of different cell-associated binding entities that a cellular system has for a specific recognizing substance; [L] is the concentration of free ligand, which can be recognizing substance, free liposomes or bioadhesive liposomes; B is the total quantity of bound recognizing substance per given number of cells, at a given [L]; and, $Bmax_i$ and Kd_i are the total number of sites of a given entity and the corresponding equilibrium dissociation constant. B and B_{max} are normalized for the same number of cells.

For cases in which receptors and non-receptor cell membrane components participate in the recognizing substance binding and in which the dissociation constant of the non-specific binding is sufficiently large with respect to the free ligand concentration, equation 1 can take the form:

$$B = \sum_{i=1}^{n-1} \frac{Bmax_i\,[L]}{Kd_i + [L]} + K_{ns}\,[L] \quad (2)$$

where the last term, K_{ns} [L], is the contribution of the non-specific binding to B and K_{ns} is the ratio of Bmax to Kd corresponding to the non-specific binding.

"Best-fit" values for parameters n, $Bmax_i$ and Kd_i are obtained by computer-aided data analysis, according to equations (1) and/or (2) above, applying nonlinear regression procedures.

The interaction of the bioadhesive EGF-modified liposomes has been established with cultures of A431 cells, in monolayers, as a biological model. This well-established cell line, originating from human epidermoid carcinoma, is enriched with EGF receptors. A431 cells have been repeatedly used for study of the interaction of free EGF and its receptor.

A431 cells have been shown to have three classes of EGF receptors, differing in their affinities and populations. The first of these classes is the ultra-high affinity sites with an equilibrium dissociation constant of 0.07 nM and a population of 150-4000 sites per cell. The next class is the high affinity sites with an equilibrium dissociation constant of 0.7 nM and a population of 1.5×10^5 sites per cell. The final class is the low affinity sites with an equilibrium dissociation constant of 5.9 nM and a population of 2×10^6 sites per cell.

Example One

To compare the binding ability of regular liposomes and bioadhesive liposomes, A431 cell cultures were grown in monolayers, in flasks, applying usual procedures for this cell

line. Two to three days prior to an experiment, the cells were seeded into multiwell culture plates and the experiments were done when the systems were confluent.

For purposes of assaying the modified liposomes, the EGF-recognizing substance was labeled with a generally known radioactive marker. Preparation of EGF-modified LUVET was completed as disclosed in the concurrently filed applications.

Prior to the addition of a reaction mixture of EGF-modified liposomes, free liposomes or free EGF, media was removed from the A431 cells and the cells were washed with a binding buffer. The reaction mixture and cells were incubated for 1-2 hours, at room temperature. Upon dilution and withdrawal of the reaction mixture at the end of incubation, 2-3 successive washings with a binding buffer,of the wells were completed. Lysis of cells or detachment of cells from the wells was then followed by withdrawal and collection of the well content, denoted as the cell fraction. Assays of the cell fraction were completed by label counting of the fraction as compared with the counting of the immediate products created through the preparation process.

A comparison between the binding of free liposomes and EGF-modified liposomes to the A431 cells is illustrated in Figure 1. The EGF-modified liposomes adhere to the A431 cells considerably better than free liposomes as no free liposomes were found at cell fraction. It is speculated that if free liposomes do associate with the cells, the dilution brought by the washings is sufficient to cause quantitative dissociation.

Example Two

Binding studies of EGF-modified liposomes to A431 cells were carried out as described in example 1 and the data were processed according to equation (1) above. The experimental conditions were such that the contribution of non-specific

binding was negligible. Indeed, the data were found to fit unambiguously with a single type of binding site for each liposome system studied. Results for several systems are listed in Table 1.

TABLE 1

BINDING PARAMETERS OF BIOADHESIVE LIPOSOMES TO A431 CELLS IN CULTURE

BIOADHESIVE LIPOSOME SYSTEM (a)	K_d (nM)	SITES PER CELL ($\times 10^{-5}$)
EGF-MLV	0.60 ± 0.017	0.17 ± 0.03
EGF-MLV	5.03 ± 1.9	1.07 ± 0.03
EGF-LUVET	2.91 ± 0.003	0.18 ± 0.001
EGF-MEL	0.04 ± 0.007	0.042 ± 0.0042
EGF-MEL	0.40 ± 0.13	3.7 ± 0.90
EGF-MEL	0.48 ± 0.05	0.28 ± 0.01

(a) Each bioadhesive liposome system is a different preparation; recognizing substance in each system is EGF.

An EGF-modified liposome is considerably larger than and different from free EGF, which is expected to affect the binding parameters. For a given class of receptors, the magnitudes of the dissociation constants for EGF-modified liposome systems are expected to be similar to or higher than those of free EGF. For a given class of receptors, the number of receptors per cell that are available for the EGF-modified liposomes is expected to be equal to or lower than the number of available for free EGF. Based on these considerations, the

binding data of the present example fit with the receptor classes of ultra-high and high affinities.

Regardless of the specific cell-associated binding entity involved, the binding data listed in Table 1 show that EGF-modified liposomes bind to this cellular system with high affinity and with a sufficient number of sites for these modified liposomes to perform as the desired bioadhesive liposomes.

Example Three

Binding collagen-modified liposomes to A431 cells was carried out essentially according to the procedures detailed above. The A431 cell line is not known to contain receptors for collagen. The interaction of either free collagen or liposomally bound collagen with the A431 cell line is expected to result from association of collagen with components within the extracellular matrix. Referring to Figure 2, incubation periods up to 4 hours were completed with 3 hours being the optimal period for binding and collagen-liposome concentrations.

Quantitative evaluations of binding of collagen-modified liposomes to A431 cells in culture are compared to regular liposome and exemplified in Figure 3. The data were processed according to equation (1) above. Through double labeling, 3-H-collagen and 14-C-cholesterol, it was possible to monitor the collagen and liposome simultaneously. The binding of the collagen-modified liposomes to the cells is greater than the binding of the corresponding regular liposomes.

For free and collagen-modified liposomes, the binding entities are of the extracellular matrix type of cell-associated entity. As in the case of EGF-modified liposomes discussed in example 2, the dissociation constant for collagen-modified liposomes is expected to be similar to or higher than those of free collagen. Likewise, the number of

available sites in the extracellular matrix available for collagen-modified liposomes is expected to be similar to or lower than free collagen. The example given in Table 2 fits with these considerations. The data for free collagen demonstrate that binding of this bioadhesive recognizing substance to this cellular system does occur and is a measurable phenomena, which can be processed to yield quantitative and meaningful parameters. Moreover, the data in Table 2 show quite clearly that the binding of collagen-modified liposomes to this cellular system is of sufficiently high affinity and with a large enough number of sites, for the collagen-modified liposomes to perform as the desired bioadhesive liposomes.

TABLE 2

BINDING PARAMETERS OF FREE RECOGNIZING SUBSTANCES AND BIOADHESIVE LIPOSOME TO A431 CELLS IN CULTURE

BIOADHESIVE LIPOSOME SYSTEM	K_d (uM)	NUMBER OF SITES ($\times 10^{-5}$)
FREE COLLAGEN	8.5 ± 2.3	179 ± 11
COLLAGEN-MLV	67.6 ± 31.35	548 ± 160

While the preferred embodiments have been described, various modifications and substitutions may be made without departing from the scope of the invention. For example, the mouse EGF and human urogasterone used in the disclosed examples could be substituted with EGF from other natural or synthetic sources. Similarly, the collagen, gelatin and HA could come from other natural or synthetic sources. Accordingly, it is to be understood that the invention has been described by way of illustration and not limitation.

CLAIMS

What we claim is:

1. A method for topical substance administration for a designated cellular target site comprises the topical application of a encapsulating liposome component and a recognizing substance component bonded to the liposomal surface where the recognizing substance component confers to the liposome component target specificity for and retention at the target site.

2. The method of administration of claim 1 wherein the liposome component is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

3. The method of administration of claim 1 wherein the liposome component includes phosphatidylethanolamine.

4. The method of administration of claim 1 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through receptor mechanisms at the cellular target site.

5. The method of administration of claim 1 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through associations with components within an extracellular matrix at the cellular target site.

6. The method of administration of claim 1 wherein the recognizing substance component is selected from the group consisting of gelatin, collagen, hyaluronic acid and epidermal growth factor.

7. The method of administration of claim 1 where the liposome component and the recognizing substance component are covalently linked.

8. The method of administration of claim 1 where the liposome component and the recognizing substance component are covalently linked by a crosslinking reagent.

9. A method of local substance administration for a designated cellular target site comprises the local application of a liposome component and a recognizing substance component bonded to the liposomal surface where the recognizing substance component confers to the liposome component target specificity for and retention at the target site.

10. The method of administration of claim 9 wherein the liposome component is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

11. The method of administration of claim 9 wherein the liposome component includes phosphatidylethanolamine.

12. The method of administration of claim 9 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through receptor mechanisms at the cellular target site.

13. The method of administration of claim 9 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through associations with components within an extracellular matrix at the cellular target site.

14. The method of administration of claim 9 wherein the recognizing substance component is selected from the group consisting of gelatin, collagen, hyaluronic acid and epidermal growth factor.

15. The method of administration of claim 9 where the liposome component and the recognizing substance component are covalently linked by a crosslinking reagent.

16. The method of administration of claim 9 where the liposome component and the recognizing substance component are covalently linked by a crosslinking reagent.

17. A liposome for substance administration for a designated cellular target site comprising a liposome component and a recognizing substance component bonded to the liposomal surface where the recognizing substance component confers to the liposome component target specificity for and retention at the target site.

18. A liposome of claim 17 wherein the method of administration of claim 1 wherein the liposome component is selected from the group consisting of multilamellar vesicles, microemulsified liposomes and large unilamellar vesicles.

19. A liposome of claim 17 wherein the method of administration of claim 1 wherein the liposome component includes phosphatidylethanolamine.

20. A liposome of claim 17 wherein the method of administration of claim 1 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through receptor mechanisms at the cellular target site.

21. A liposome of claim 17 wherein the method of administration of claim 1 wherein the recognizing substance component confers target specificity and retention by a mechanism of adhesion through associations with components within an extracellular matrix at the cellular target site.

22. A liposome of claim 17 wherein the method of administration of claim 1 wherein the recognizing substance component is selected from the group consisting of gelatin, collagen, hyaluronic acid and epidermal growth factor.

23. A liposome of claim 17 wherein the method of administration of claim 1 where the liposome component and the recognizing substance component are covalently linked.

24. A liposome of claim 17 wherein the method of administration of claim 1 where the liposome component and the recognizing substance component are covalently linked by a crosslinking reagent.

FIG. 1

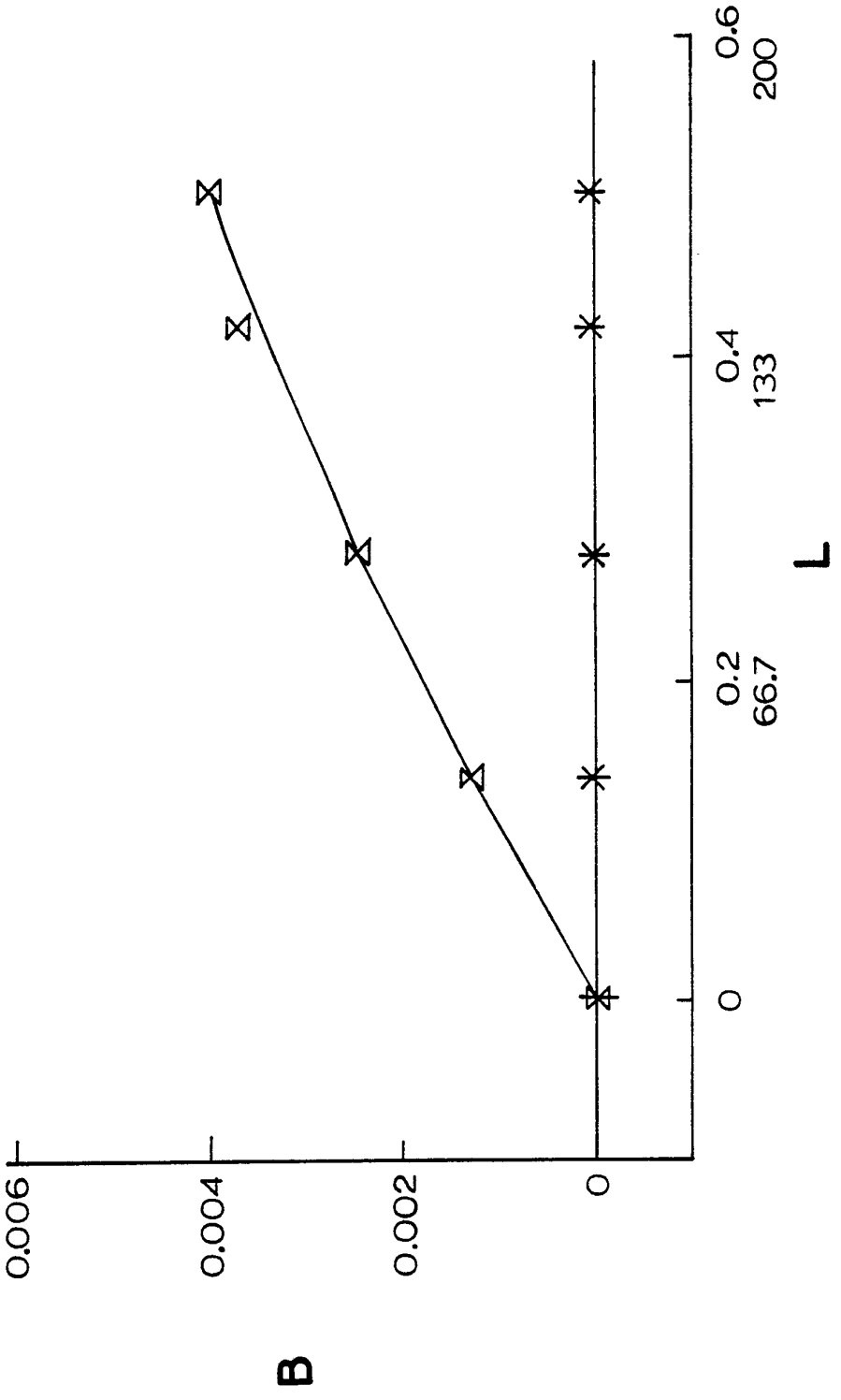


FIG. 2

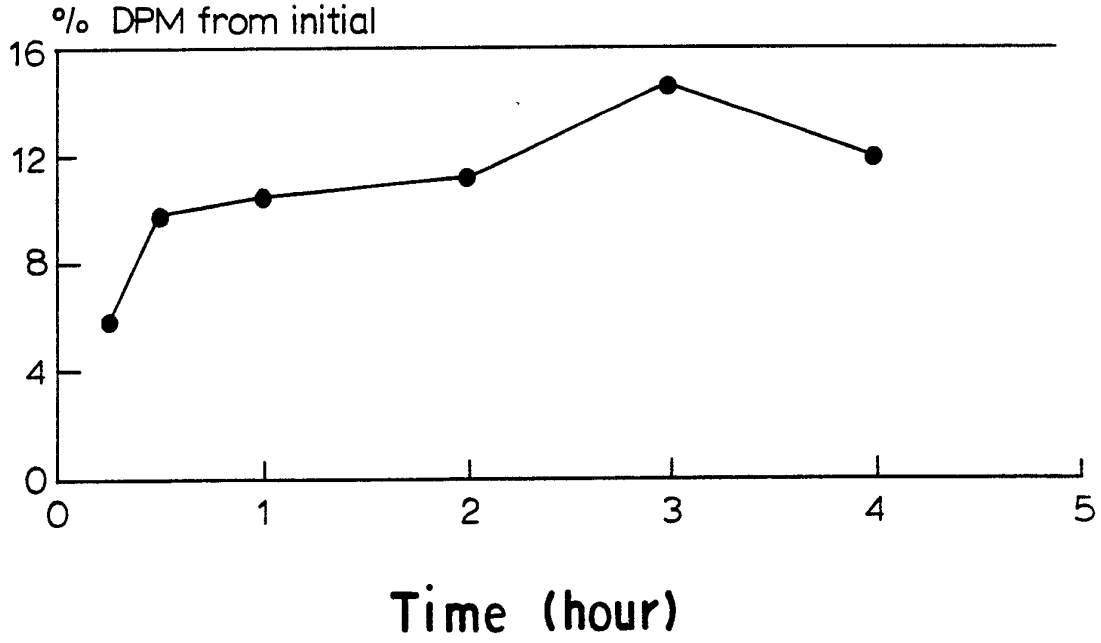
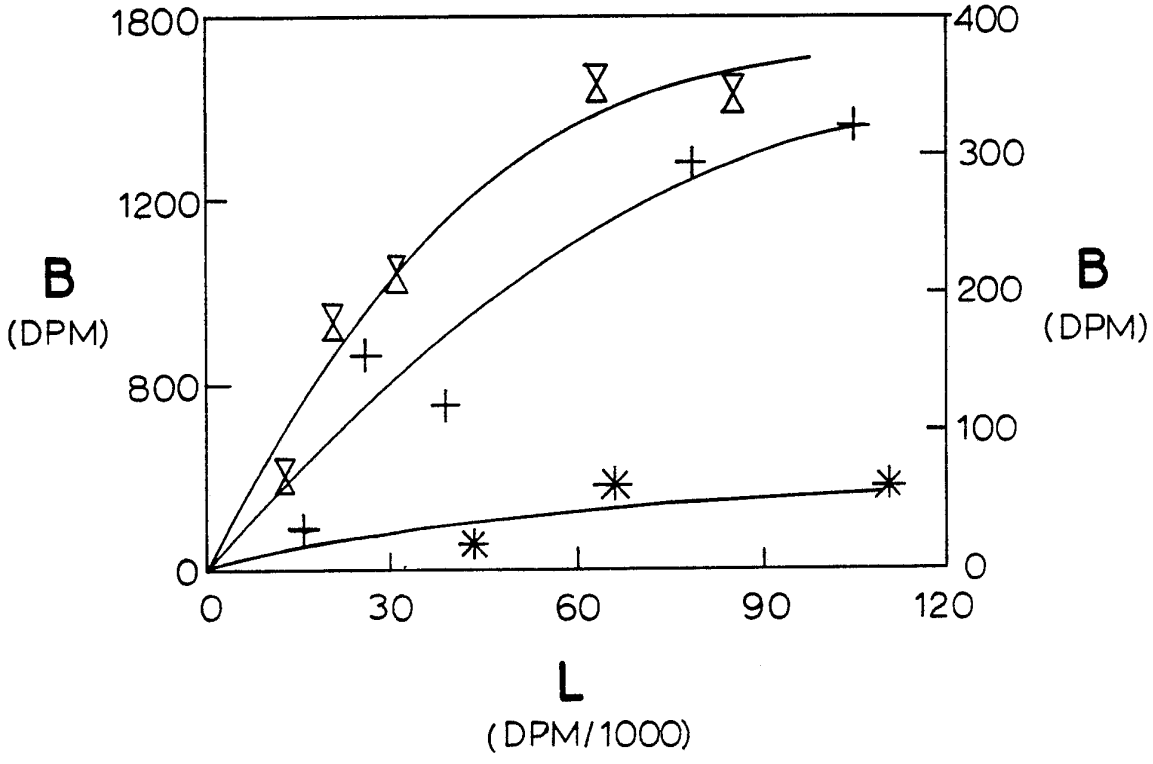

% DPM from initial
16
12
8
4
0
0
1
2
3
4
5
Time (hour)

FIG. 3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1200
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B
(DPM)
0
30
60
90
120
L
(DPM/1000)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/08112

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 9/127

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Biochemical and Biophysical Research Communications, vol. 160, no. 2, 28 April 1989, (Duluth, Minn. US), Y. ISHII et al.: "Preparation of EGF labeled liposomes and their uptake by hepatocytes", pages 732-736, see the whole article	17-24
Y	---	7,15,16
X	Patent Abstracts of Japan, vol. 9, no. 74 (C-273)[1797], 3 April 1985, & JP,A,59210013 (AJINOMOTO K.K.) 28 November 1984, see the abstract	17-24
X	--- G. GREGORIADIS: "Liposome Technology", vol. III, 1984, CRC Press, Inc., Boca Raton, US, see pages 75-94, in particular pages 88-89	17-24
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05-06-1992	30. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	---	1-16
X	WO,A,9009782 (LIPOSOME TECHNOLOGY, INC.) 7 September 1990, see the whole document, in particular page 4, line 14 - page 5, line 27	1-6,9-14
Y	---	7,15,16
A	EP,A,0295092 (UNILEVER PLC) 14 December 1988, see page 15, example 1	1-16

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-16 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9108112 SA 56485

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/06/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9009782	07-09-90	US-A- 4944948	31-07-90
		US-A- 5064655	12-11-91
		AU-A- 5182590	26-09-90
		EP-A- 0460100	11-12-91
EP-A- 0295092	14-12-88	AU-B- 613920	15-08-91
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