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Bioadhesive, collagen-modified liposomes: molecular and cellular level studies on the kinetics of drug release and on binding to cell monolayers

Noga Yerushalmi and Rimona Margalit *

Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel

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Liposomes, modified by covalently-anchoring collagen to their surface, were investigated for their abilities to be bioadhesive and to act as sustained-release drug carriers. These bioadhesive liposomes have the potential to induce significant improvements in topical and regional therapies. The major findings for uni- (ULV) and multilamellar (MLV) bioadhesive liposomes are: (a) Both ULV and MLV release small molecular weight drugs over prolonged periods. For example, rate constants of $(6 \pm 0.5) \cdot 10^{-3}$ and $(2.6 \pm 0.8) \cdot 10^{-3}$ h^{-1}, were obtained for the release of vinblastine and fluconazole, respectively, from collagen-ULV. (b) For a given drug, that rate constant can be shifted (up or down) by the choice of liposome type and collagen-surface density and the latter, if high enough, lead to the formation of an additional liposome-associated drug reservoir. (c) Using monolayers of the A431 cell line to model the in vivo targets, the bioadhesive (but not the regular) liposomes were found to bind with high affinity to the monolayers. For example, equilibrium dissociation constants of $6.3(\pm 3)$ μM and $2.7(\pm 0.5)$ μM were determined for bioadhesive MLV and ULV, respectively, with corresponding saturation occupancies of $3.7(\pm 1)$ and $4.0(\pm 0.2)$ pmoles liposomal collagen/monolayer of 10^5 cells. (d) Following the retention of bioadhesive MLV at A431 monolayers for 24 h, it was found that: at 4°C, 24 h did not suffice to reach equilibrium, but at 37°C equilibrium binding was obtained within 3–5 h and there was quantitative liposome retention (per viable monolayer) thereafter. It is concluded that these liposomes are bioadhesive sustained-release carriers, as desired, meriting further cellular and in vivo studies.

Introduction

The topical application of therapeutic agents to wounds and burns, be it an established antibiotic to treat infections ** [1–6], or the yet-experimental growth-factors for the acceleration of the self-healing processes [7–13], requires the agent to get across a short, but critical, obstacle course. That course stretches from the site of administration, through the wound fluid, to the sites of drug action, which are the receptors of the viable cells engaged in the healing process, or the adherent bacterial colonies. Along that path the drugs are subject to dilution, degradation or inactivation, diversion to other locations and scavenging by components other than their targets. The cumulative effect of these events is a reduction in drug availability at the target, which in turn reduces the efficacy of the therapy (and at the extreme abolishes it). Attempts to counter such an outcome by frequent dosings and re-dressings can result in further trauma to the injured tissues. This situation might be improved were the agent administered, still topically, but in a depot form that would: (a) be capable of protecting its load from the hostile environment (b) be localised and retained as close as possible to the site(s) of drug action and (c) be capable of releasing its load over a time span that can extend to several days. Needless to say, the depot cannot be toxic and should not interfere with the self-healing processes. Nor should its application and re-application require steps that could further traumatize the injured tissue.

* Corresponding author. Fax: +972 3 6415053.

Abbreviations: BSA, bovine serum albumin; CH, cholesterol; ECM, extracellular matrix; GDA, glutaraldehyde; LUVET, large unilamellar vesicles, extruded; MLV, multilamellar vesicles; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

** The literature on wounds, with respect to healing, infections and growth factors, and on liposomes as drug delivery systems, is very extensive. In the interest of controlling the number of citations, an attempt has been made to cite recent reviews and articles that, together, give a broad view of each field.

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As a depot, liposomes meet most, but not all, of the requirements set above. Liposomes are nontoxic, biodegradable, biocompatible, able to protect encapsulated matter from hostile external environments and capable of acting as sustained-release systems [14–17]. The critical factor that regular liposomes lack is an ability to localise and be retained at the injured tissues in close proximity to the target sites of growth factors and of antibiotics. The approach taken in the present study, aimed at endowing liposomes with this ability, was to modify their surface by the covalent attachment of a bioadhesive ligand. Collagen has bee n selected for this task on the basis of its ability to bind to components of the extracellular matrix (ECM) [18–22]. An ability which, if retains in the liposome-anchored protein, could localize the liposomes at the ECM within the newly formed tissue, in close proximity to the adherent bacterial colonies and to the cells in need of growth factors. Furthermore, being of biological origin, the addition of collagen to the liposomes should not compromise their nontoxic, biocompatible and biodegradable nature.

In order to perform as designated, the collagen-modified (bioadhesive) liposomes should be capable of adhering to their designated sites with high affinity (and significantly better than regular, non-modified liposomes) and be retained there in face of cellular activities such as cell motion, cell death and cell proliferation. Also, the modified liposomes should be capable of acting as sustained-release systems and it is noted that even if the 'parent' non-modified liposome has the desirable release capability, its modification could impair this property.

The study reported here was focused on investigating at the molecular and cellular levels, the binding, retention and drug-release properties of collagen-modified liposomes. Two liposome types, multilamellar (MLV) and extruded unilamellar (LUVET) were studied and a monolayer of the A431 cell line (originating from human epidermoid carcinoma) was used as a model system for the liposome binding and retention studies. The choice of an adherent cell line was to provide an environment that would have living cells and extracellular matrix, yet undergo changes with time, such as cell migration, cell death and cell proliferation.

Experimental procedures

Materials

High-purity soybean phosphatidylcholine (PC) was purchased from American Lecithin (Atlanta, GA, USA). All other high-purity lipids, collagen type VIII (soluble type I, from rat tail), a 25% solution of glutaraldehyde (GDA) and vinblastine were purchased from Sigma (St. Louis, MO, USA). Fluconazole was a gift from Baxter Healthcare. The following radiolabels were purchased from Amersham (Arlington Heights, IL, USA) and found to be stable: [^{3}H]cholesterol, [^{14}C]cholesterol, [^{3}H]vinblastine and [^{3}H]acetic anhydride. Spectra/Por4 dialysis tubings (molecular weight cutoff of 12000 to 14000) were purchased from Spectrum Medical Industries (Los Angeles, CA, USA). Polycarbonate membranes were purchased from Nucleopore (Pleasanton, CA, USA). All other reagents were of analytical grade.

A431 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Fetal Calf Serum, Dulbecco's Modified Eagle's Medium (high glucose), L-glutamine, penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Biological Industries (Beth Haemek, Israel). Culture flasks and multiwell culture plates were from Sterilin (Hounslow, UK) and Costar (Cambridge, MA, USA).

Absorbance spectra were measured using a Bausch and Lomb 601 spectrophotometer. High speed centrifugation was performed using a Sorvall RC-58 centrifuge. Beckman L-8 and L-5 centrifuge models were used for ultracentrifugation. Liquid scintillation counting was performed with a Kontron Analytical Betamatic I. The Extruder used for the preparation of extruded liposomes was from Lipex. Samples were extruded through polycarbonate membranes. Liposomes were sized by quasi-elastic light scattering, using a Malvern Automeasure 4700.

Methods

Liposome preparation and drug encapsulation. Multilamellar (MLV) and extruded unilamellar (LUVET) liposomes were prepared essentially as previously described [23,24], from lipid compositions of PC/PE/CH at mole ratios of 3:1:1. For drug-free liposomes the swelling solution was phosphate-buffered saline at a pH of 7.2. The same buffer was used for the drug-encapsulating liposomes and the drugs were introduced through the swelling solution. When required, the liposomes were separated (and washed, usually two or three washes) from excess unencapsulated drug by high speed (for the MLV) and ultrahigh speed (for the LUVET) centrifugations, using the following conditions: 4°C and 1 h at 27000 $\times g$ and at 250000 $\times g$, for the high and ultrahigh speed centrifugations, respectively.

Liposome modification. Collagen-modified liposomes were prepared and characterized as previously described [24]. Briefly, liposomes, GDA and collagen were incubated in phosphate-buffered saline (pH 7.2), with constant stirring at 4°C, for 24 h. At the end of the incubation, the liposomes were separated from excess reagents and by-products by centrifugations (as described above) and repeated washings. When modifying drug-encapsulating liposomes, all steps were car-

ried in the presence of drug in the external medium, in order to minimize drug loss during the process. A specific activity, defined as the quantity (in μg) of collagen bound per μmol lipid, was determined for each preparation, using the lipid and collagen assays defined in a following section.

Drug diffusion. The kinetics of drug diffusion were studied according to previously reported procedures [23,24]. Briefly, a suspension of liposomes (0.5–1.0 ml) was placed in a dialysis sac and the sac was immersed in a constantly-stirred receiver vessel which contained a 10–16-fold volume of drug-free buffer (phosphate-buffered saline at pH 7.2). At designated periods, the dialysis sac was moved from one receiver vessel to another containing fresh (i.e., drug-free) buffer. Drug concentration was assayed in each dialysate and in the sac (at the beginning and end of each experiment).

Liposome binding to cell monolayers. The A431 cells were grown in monolayers as previously described [24]. Several days prior to an experiment the cells were seeded into 24-well multiwell culture plates at densities in the range of $5 \cdot 10^4$ cells/ml and the experiments were performed upon reaching confluency. All wells in the plate were washed with 'binding buffer' which was phosphate-buffered saline (pH 7.2) with 0.2% BSA. The cells were incubated with 200 μl of reaction mixtures per well, the mixtures being either collagen-modified or regular lipsomes, suspended in the binding buffer or in serum-supplemented cell growth media.

For concentration-dependance studies the wells were incubated with increasing concentrations of liposomes (in triplicates) and incubation was for 3 h at 25°C. Immediately prior to the experiment the cells from two wells (per plate) were detached by trypsinization. The detached cells were collected and counted for viable cells, using the Trypan blue method. Upon termination of the reaction the diluted reaction was aspirated and the wells were subjected to three washes with cold binding buffer, each wash volume being 5–8-fold that of the reaction mixture. Following the last wash the cells from each well were detached as described above and the radioactivity (which would result from cell associated liposomes) was counted. Several wells, serving as controls, received buffer alone.

Time-dependent studies were conducted at 4°C, 25°C and 37°C. All the wells in a plate received the same liposome concentration and the experiment was terminated for several wells at a time, at designated periods. The terminated wells were washed and the cells detached as described above. Six wells were used for each time point, three of which were used for radioactive counting (each well separately) and the remainder three for counts of viable cells (also each well separately).

Quantitative determinations. Two procedures were used for collagen assay: (a) By radiolabel, the labeling done through acetylation with [^{3}H]acetic anhydride essentially according to the method of Gisslow and McBride [25], resulting in specific activities within the range of $(5–8) \cdot 10^7$ dpm/mg protein. (b) By a modified Lowry procedure [26]. Both procedures could be applied to free as well as to liposome-associated collagen. Lipids, the tritium-labeled collagen and vinblastine were assayed by the respective radiolabel (see Materials above). Fluconazole was assayed by its absorbance at 262 nm. Molar absorption coefficients for the drug in buffer and in 5% DOC were determined to be 610 and 596, respectively.

Results and Discussion

I. Drug-release properties of collagen-modified liposomes

The kinetics of drug release were studied for collagen-modified liposomes, encapsulating vinblastine or fluconazole as models of small molecular weight drugs. Similar experiments were conducted with non-modified liposomes, from the same original batches. Based on our previous experience [23], care was taken to keep the lipid concentration below 30 mM, in order to minimize the effects of liposome–liposome interactions. Typical results are exemplified in Fig. 1, for

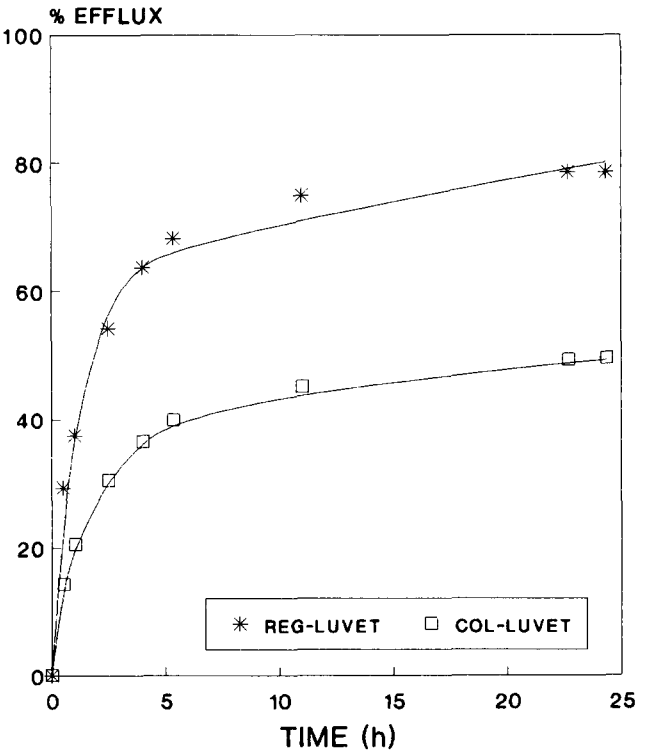

Fig. 1. Kinetics of vinblastine release from bioadhesive (collagen-modified) and regular unilamellar liposomes of the LUVET type. Ordinate: Cumulative release normalized to the total drug in the system, at time = 0. Abscissa: Time. The points are the experimental data and the solid curves are the theoretical expectations, according to Eqns. 1 and 2 in the text, for the regular and the bioadhesive liposomes, respectively, using the parameters listed in Table I.

TABLE I

Drug release from collagen-modified and from regular liposomes: kinetic parameters

Liposome type	Lipid (mM)	Drug	f_a (%)	f_l (%)	k_a (h^{-1}) ($\times 1000$)	k_l (h^{-1}) ($\times 1000$)
MLV	6	vinblastine		51 ± 6		50 ± 10
Coll.-MLV	6	vinblastine		50 ± 2		34 ± 3
MLV	26	fluconazole		61 ± 0.6		4.4 ± 0.7
Coll.-MLV	26	fluconazole		59 ± 0.3		6.6 ± 0.4
LUVET	9	vinblastine		38 ± 4		27 ± 7
Coll.-LUVET	9	vinblastine	32 ± 0.8	59 ± 0.6	415 ± 24	6.0 ± 0.5
LUVET	17	fluconazole		13 ± 0.8		21 ± 4
Coll.-LUVET	17	fluconazole	39 ± 0.6	27 ± 7	610 ± 126	2.6 ± 0.8

vinblastine-encapsulating collagen-modified, as well as non-modified, LUVET. The data, which show the accumulation of released drug (normalized to the total drug in the system at time = 0) with time, clearly indicate continuous (although at a varying pace) drug release over a time-frame of 24 h. Furthermore, upon comparing the regular vs. the bioadhesive liposomes, it becomes clear that the modification process did not cause any severe damage with respect to drug release. It did not bring immediate depletion, nor did the modification result in blockage of drug release. Rather, the patterns of drug release from the control and from the bioadhesive liposomes are quite similar, with some decrease in the latter, which is beneficial for performance as a sustained-release depot.

In order to obtain a quantitative evaluation of drug release, experimental data of the type illustrated in Fig. 1 were analysed according to previously-derived multi-pool kinetic models [23,24].

Briefly, in the first case which is termed the 'two-pool model', the drug within the dialysis sac is distributed at time zero between two pools, a liposome-associated pool and a pool of free (i.e., unencapsulated) drug *. The overall drug release should correspond to the following equation:

$$f = f_f(1 - \exp[-k_f t]) + f_l(1 - \exp[-k_l t]) \quad (1)$$

where f is the accumulated product (i.e., the drug in the dialysate), normalized to the total drug in the system at time = 0. The distribution of the drug, at time = 0, between the free and the liposome-associated drug pools is given by f_f and f_l, respectively (obviously, f_f and f_l sum up to unity); k_f and k_l are the corresponding rate constants.

In the second case, termed a 'three-pool model' the drug within the dialysis sac is distributed at time zero between three pools. A pool of free drug (as in the former case) and two independent pools of liposome-associated drug. For the case in which only the releases from the liposome-associated pools are rate-limiting, the overall drug release should correspond to the following equation:

$$f = f_f + f_a(1 - \exp[-k_a t]) + f_l(1 - \exp[-k_l t]) \quad (2)$$

where f_a is the fraction of the total drug in the system at time = 0, which occupies the additional (compared to the two-pool model) liposome-associated drug pool, and k_a is the corresponding rate constant. All other parameters are as defined for Eqn. 1 above.

For the MLV, both the bioadhesive and the control systems were found to fit the two-pool model. As can be seen from Table I, for each drug species, the magnitudes of k_l for corresponding bioadhesive and regular liposomes are quite similar. These magnitudes support the observation made upon the raw data, that the modification does not damage drug release. For both drugs the level of encapsulated drug (i.e., f_l) is > 50%. This is noted to be an appreciable encapsulation for the relatively low lipid concentration range used. As detailed under methods, care was taken to have drug in the external medium during the modification and purification steps in order to minimize drug loss. The insignificant change in the magnitude of f_l, from the regular to the bioadhesive liposomes, is clear support that depletion or significant loss have been prevented.

The situation with the LUVET is different. The control systems still fit the two-pool model while the bioadhesive systems, irrespective of the encapsulated drug, fit the three-pool model. The data, listed in Table I, show that both f_l and k_l change, the former increasing and the latter decreasing, from the control to the bioadhesive LUVET.

The following hypothesis is proposed to account for the phenomena described above, in particular for the

* As previously discussed [23], the liposome-associated drug is the pool of interest. The unencapsulated drug present in the liposome preparation at time = 0 is considered an unavoidable, but manageable, contamination.

differences between the collagen-modified MLV and the collagen-modified LUVET:

The bioadhesive properties of collagen can lead to self-interactions among collagen molecules. Whether such interactions would come into expression among the collagen molecules anchored to the liposomal surface is expected to depend on the collagen density at that surface. Where that density is sufficient, the collagen self-interactions could result in a bioadhesive layer surrounding the liposomal surface. It is therefore suggested that for the LUVET, but not for the significantly larger MLV, the current levels of collagen surface-density are sufficient to form such a layer. This bioadhesive layer at the surface of the LUVET is, furthermore, proposed to act as a drug-entrapment entity becoming that additional liposome-associated drug pool which was detected for the LUVET (but not for the MLV). The existence of this bioadhesive layer makes the bioadhesive LUVET a different liposome species than the corresponding control LUVET. The effects of these liposome-species differences on properties of the systems, are discussed below.

In a previous communication [23] it was shown on theoretical grounds, and supported by experimental data, that liposome concentration and drug partition coefficient (K_p) are the two major factors affecting the efficiency of drug encapsulation. The effect of the partition coefficient was observed through comparisons among different drug species. The present case offers an opportunity to observe the effects of K_p for the same liposome concentration and drug species, through the changes in liposome surface-properties due to the bioadhesive layer. Comparing the magnitudes of f_l in the control systems, to the sum of $f_l + f_a$ in the bioadhesive ones (the bioadhesive layer taken to be an integral part of the liposome), it is clear from the data (Table I) that the fraction of liposome-associated drug has increased from the control to the bioadhesive systems. As (presently) both the liposome concentrations and the drug species are the same, this is interpreted to result from an increase in the coefficient for the partition of the drug between the external aqueous phase and the bioadhesive LUVET, from the corresponding coefficient for the regular liposomes. The observation that the partition coefficients have increased for a neutral (fluconazole) as well as a charged (vinblastine) drug (at the pH range studied) implies that among the factors contributing to the new magnitudes of K_p, the change in the standard chemical potential component is the dominant over the change in the electrical potential component.

The parameters f_l for the regular and $(f_l + f_a)$ for the bioadhesive liposomes are thermodynamic properties, as they are the equilibrium distribution of drug between the liposomes and the external aqueous phase. In the present study it was not only possible to obtain

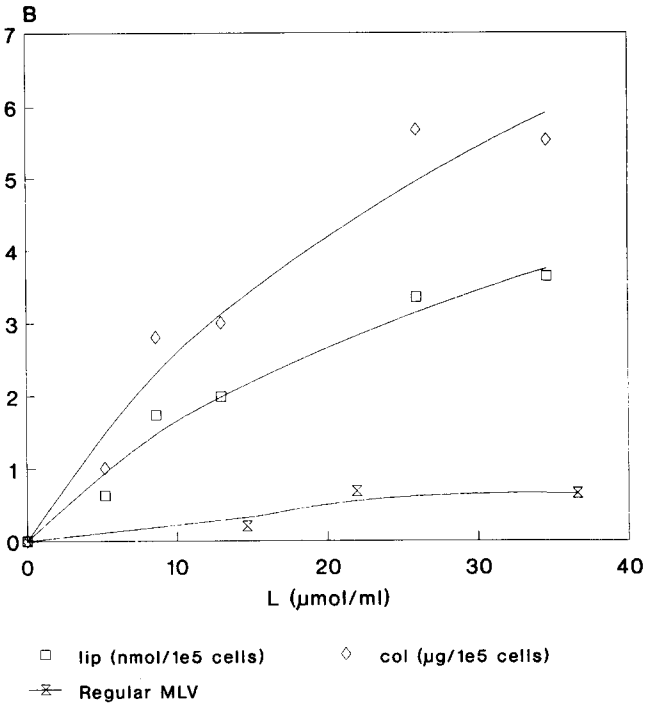

Fig. 2. Equilibrium binding of bioadhesive (collagen-modified) and of regular MLV to monolayers of the A431 cells. Points are the experimental data and the solid curves for the bioadhesive liposomes are the theoretical expectations drawn according to Eqn. 3 in the text, for the parameters listed in Table II.

thermodynamic (i.e., macroscopic) properties, from kinetic (i.e., microscopic) experiments, but to also determine the intraliposomal equilibrium distribution for those cases with more than one liposomal pool (f_a and f_l). The magnitudes obtained for these distributions (Table I) indicate that not only the bioadhesive LUVET as a whole has become more favorable for the drugs, but that the intraliposomal drug pool (i.e., f_l) has also become more favorable for drug entrappment compared to the control LUVET.

The decrease in k_l is also linked to the existence of the bioadhesive layer and to the changes in the liposome surface-species it reflects. In previous studies [23], it was shown that the rate constant of the release of encapsulated drug decreases with the increase in the interactions among liposomes. In those studies, the increase in liposome–liposome interactions resulted from an increase in liposome concentration and it required liposome concentrations higher than those studied here, for the phenomena to be noticeable. It is suggested that the decrease in k_l, seen here, is also a result of an increase in liposome–liposome interactions, not because of concentration changes but due to the presence of the bioadhesive layer.

II. Interactions of collagen-modified liposomes with cell monolayers: liposome-concentration effects

In general, we found the bioadhesive liposomes to

TABLE II

Interactions of collagen-modified liposomes with monolayers of A431 cells: parameters of equilibrium binding

Liposome type	Collagen/lipid (nmol/μmol)	K_d (μM)	B_{max} (pmol collagen/10^5 cells)
Free collagen		0.85 ± 0.23	3.0 ± 0.15
MLV	0.19	6.8 ± 3.4	9.1 ± 2.8
MLV	7.46	6.3 ± 3.0	3.7 ± 1.0
LUVET	0.08	0.08 ± 0.03	1.1 ± 0.08
LUVET	1.09	2.7 ± 0.5	4.0 ± 0.2

bind to the cell monolayers whereas regular (i.e., non-modified) liposomes showed only negligible binding, if at all. This is illustrated by the typical data shown in Fig. 2. The saturating pattern observed there for the increase in monolayer-associated liposomes with the increase in liposome concentration is indicative of an equilibrium binding process. The data show quite clearly the parallel tracks of the lipid and collagen components of the bioadhesive systems and the difference in the binding of the lipid component between a non-bioadhesive and a bioadhesive liposomes. These data are taken as experimental support that the binding observed is of the collagen-liposome entity.

In order to evaluate the bioadhesivity of a variety of collagen-modified liposomes species, both uni- and multilamellar liposomes were explored and for each, a high and a low (relatively) level of collagen surface density (expressed in nmol collagen/μmol lipid). Each combination of liposome type and collagen density is expected to produce a different liposomal system.

Data of the type shown in Fig. 2 were processed according to the Langmuir isotherm, in the form given in Eqn. 3 below, where B denotes the quantity of liposomes (in mol of lipid or collagen) bound, per 10^5 cells and L denotes the concentration of liposomes remaining unbound at equilibria. B_{max} is the total number of binding sites per 10^5 cells, K_d is the dissociation equilibrium constant and n is the number of different types of binding sites.

$$B = \sum_{j=1}^{n} \frac{B_{max_j}[L]}{K_{d_j} + [L]} \tag{3}$$

The data were found to fit the case for a single type of binding site and the magnitudes of B_{max} and K_d obtained are listed in Table II together with data for the binding of free collagen. As seen from the Table (and Fig. 2) the collagen-modified liposomes are indeed bioadhesive. They bind to the monolayers with sufficiently high affinities, especially taking into consideration the probable nature of the interaction, namely binding to ECM components rather than to membrane-embedded receptors.

The data show that, qualitatively, the binding phenomena is observed for all systems, irrespective of liposome type and collagen density. Moreover, as expected for the binding of different liposomal species there are quantitative differences in the magnitude of B_{max} and K_d, that indicate that indicates that the bioadhesivity is not restricted to a narrow range of liposome types or collagen loading. Some of the system-to-system differences probably result from the differences in liposome species and in the collagen/lipid ratio. However, it is proposed that the major underlying cause of those differences, as well as the scatter within a system, result from the nature of the binding site(s) offered by the cellular system, namely the ECM. There could be some well-to-well and plate-to-plate variations in the quantity and composition of the ECM and such variations would be reflected in scatter within a system (as more than one well received the same liposome concentration) and for the differences among systems. In fact, similar well-to-well variation can also be seen in the data for free collagen (also shown in Table II). Taking into account that in an in vivo situation ECM variations will definitely occur from one wound to another, and for the same wound from one day to another, gives an upside view to the present in vitro results.

III. Interactions of collagen-modified liposomes with cell monolayers: retention

Initial studies to determine the time-course of the binding of collagen-MLV to the A431 monolayer have shown that equilibrium is obtained at room temperature, in a phosphate-buffered saline binding media at 3 h. In the present set of experiments, the objective was to determine the retention of the liposomes at the cell monolayer, going beyond the 3 h limit. To gain some insight into both the kinetics of binding and into retention, the experiments were conducted at 4°C and at 37°C at sterile conditions and at non-sterile conditions *. Also, the cells were maintained and the binding was conducted in serum-supplemented cell growth environment.

A temperature 4°C was found to be unfavorable both in terms of cell survival and in terms of liposome binding, and 24 h did not suffice for the binding to reach equilibrium at this temperature (data not shown).

* It is noted that the liposomes added to each cell were not a sterile product, nor were the experiments themselves conducted in sterile environment. On the other hand, the culture media itself contained, as usual, antibiotics. It is suggested that, despite the risk of bacterial contamination, these conditions mimic the real in vivo situations (such as in wounds) better than the addition of sterile produces alone together with strict adherence to sterile conditions throughout the experiments. Obviously, such conditions will be required for longer-term studies.

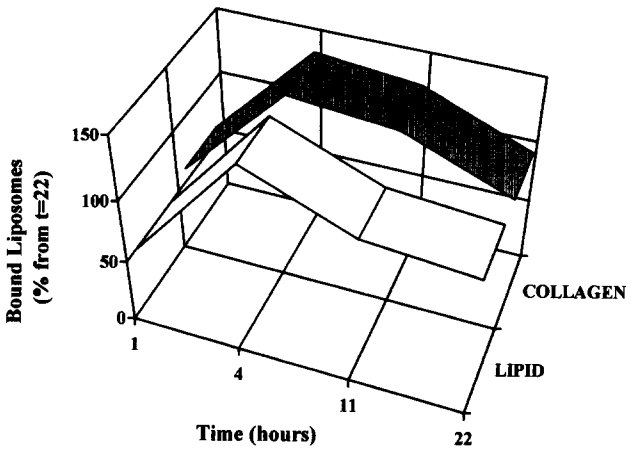

Fig. 3. Retention of bioadhesive (collagen-modified) MLV at monolayers of A431 cells, in the presence of serum-supplemented cell culture media and at 37°C. Bound liposomes are given in units of μmol lipid/1·10^5 viable cells (for the lipid component) and nmol collagen/1·10^5 viable cells (for the collagen component), each normalized to the data at the highest time point.

In contrast, as shown in Fig. 3, 37°C sufficed to reach equilibrium and the liposomes remained bound thereafter, at the same level and with parallel tracks for the lipid and collagen components, also supporting (as previously shown) that the collagen-liposome entity is bound and retained.

As indicated above, two parallel lines of experiment were designed for the pursuit of the liposome retention at 37°C: In the first (Fig. 4A), care was taken to prepare the liposomes and to carry out all steps in the experiment under aseptic conditions. These will be refereed to as the 'favorable' conditions. In the second line (Fig. 4B), liposome preparation and all steps in the experiment were conducted 'on the bench' without specific care to reduce bioburden. These will be referred to as the 'unfavorable' conditions.

Scanning, first, the cell dynamics, it is clear in both cases there was continuous cellular activity in the course of the experiment. For the cells maintained under the favorable conditions (Fig. 4A) the cells continue to thrive, proliferating at a vigorous pace for the first 19 h, leveling off towards 24 h with a modest decline over the last 6 h, which is attributed to contact inhibition and/or depletion of nutrients. In contrast, under the unfavorable conditions (Fig. 4B) there was a continuous decline in the level of viable cells. Since the only difference between these two experiments was in the aseptic conditions used for both the liposomes and the cells, it is proposed that the decline in the 'unfavorable' conditions could be a combination of poor conditions for cell growth together with some bacterial contamination. Yet, for both the favorable and the unfavorable conditions, once the liposome binding has reached equilibrium, the quantity of liposomes bound per given quantity of viable cells remained rather constant. Thus the liposomes continue to hold tenaciously (and in a quantitative manner) onto the viable cells, even towards the end of the test period at the unfavorable set, where significant cell death has occurred.

Besides affirming the ability of these liposomes to act as site-retained systems, the data above carry the following implications: The data of Fig. 4A make it clear that the binding of the bioadhesive liposomes does not prevent cell proliferation carrying the implication that such liposomes will not interfere with self-healing processes if applied to wounds and burns. The 'favorable' conditions go towards mimicking conditions that would prevail in surgical wounds while the 'unfavorable' ones go towards mimicking the conditions

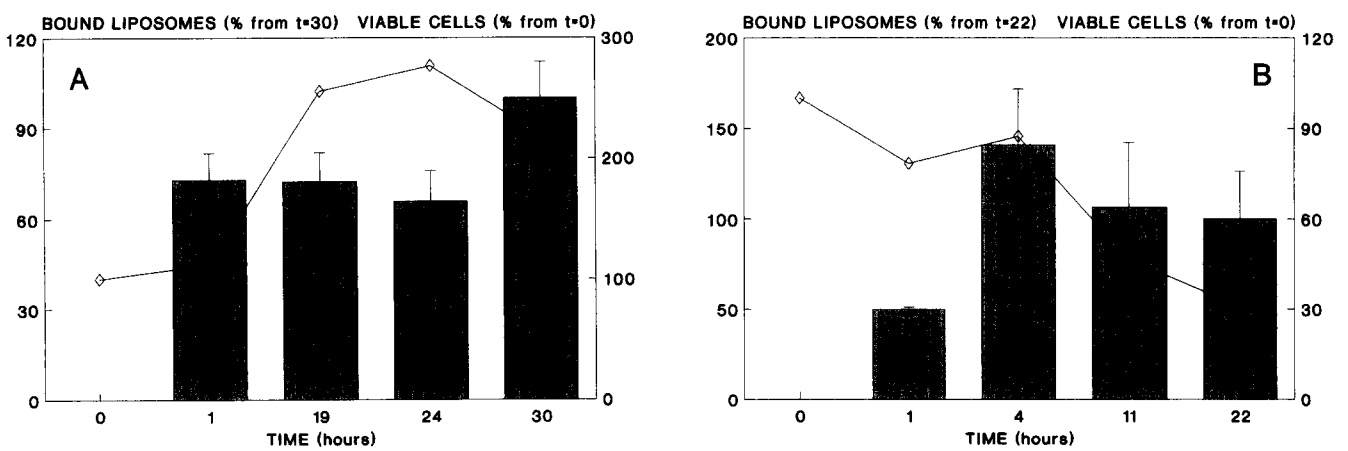

Fig. 4. Retention of bioadhesive (collagen-modified) MLV at monolayers of A431 cells, in the presence of serum-supplemented cell culture media and at 37°C. Bars/Left-hand ordinate: Bound liposomes/1·10^5 viable cells, normalized to the value determined at the last time point. Line(and points)/Right-hand ordinate: % of viable cells per well, normalized to the cells/well at time = 0. Abscissa: Time. The points are the experimental data and the lines are non-theoretical, drawn to emphasize the trends in the data. (A) Studies done under aseptic conditions. (B) Studies done under non-aseptic conditions (see text for further details).

that would prevail in trauma wounds and in burns. The observation that the liposomes are well-retained under both sets of conditions implies that the use of these liposomes could be wide-ranged and not limited to a small number of therapeutic targets.

Conclusions

Testing, at the molecular and cellular levels, collagen-modified liposomes for their potential as site-adherent sustained-release drug depots, it is concluded that these liposomes can meet several critical requirements:

(1) They can act as sustained-release depots, releasing encapsulated small molecular weight drugs at half-lives that range from 0.6 to 11 days. Furthermore, the data indicate that the collagen surface-density (manipulated through the choice of liposome type and the degree of surface modification) can be used as a tool to moderate liposome–liposome interaction and, through that, the rate of drug release. This finding fits with previous demonstrations of the effect of liposome–liposome interactions on the rate of drug release, achieved through the control of liposome concentration [23].

(2) The collagen-modified (but not the corresponding non-modified) liposomes act as site-adherent depots, capable of binding with high affinity (the ΔG° gain upon binding is on the order of 7 kcal/mol) to monolayers of cells in culture. The binding is not limited to a specific liposome type, nor to a degree of surface modification. The binding is seen to withstand ECM-attributed well-to-well in vitro variations. This is taken as a positive trait, carrying the implication that the present liposomes have the potential to be site-adherent for a broad range of the in vivo designated targets, as those are certain to express variations in their ECM.

(3) The collagen-modified liposomes have shown an ability to remain bound at the target, well after reaching equilibrium and despite continued cellular activities in the monolayer. Although this is a much-simplified, limited, model of the cell-related dynamics anticipated to take place at the in vivo targets these data, combined with the high-affinity binding, imply that the collagen-modified liposomes have the potential to act as site-adherent systems at dynamic in vivo targets.

Further studies, aimed at extending the present investigation to other cell cultures, to platforms of biologically-produced ECM and to in vivo models are underway.

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