Cyclooxygenase inhibition by diclofenac formulated in bioadhesive carriers

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Abstract

Adverse effects and gastrointestinal toxicity limit the use of Diclofenac, a frequently-used NSAID for treatments of rheumatic disorders and other chronic inflammatory diseases. Diclofenac-carrier formulations may alleviate adverse effects, increase efficacy and allow local administration. We report here our first step, biophysical and biochemical investigations of Diclofenac formulated in our previously-developed bioadhesive liposomes carrying hyaluronan (HA-BAL) or collagen (COL-BAL) on their surface. Both liposome types encapsulated Diclofenac at high efficiency, encapsulated doses reaching 13mg drug/ml, and performed as sustained-release Diclofenac depots, half-lives of drug release (under fastest conditions) ranging from 1 to 3days. Therapeutic activity of liposomal Diclofenac was evaluated in CT-26 cells that possess the CD44 hyaluronan receptors and integrins, and are a bench-mark for intracellular COX enzymes. HA-BAL and COL-BAL showed high cellular-affinity that was 40 fold and 6 fold over that of regular liposomes. Free, and liposome-encapsulated, Diclofenac showed similar activities. For example: 2–3nM Diclofenac given to intact cells generated COX-inhibition levels in the range of 60–70% for free drug and for encapsulated drug in COL-BAL and in HA-BAL. We propose these novel Diclofenac formulations possess key physicochemical and biochemical attributes for task performance, meriting the next step into in vivo studies.
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1. Introduction

Age, major trauma, and repetitive joint use, are the main risk factors in osteoarthritis (OA), the most common form of joint disease. OA is an organ disease caused by the violation of balance between synthesis and degradation of cartilage, in which all tissues are affected — synovial joint, subchondral bone, synovium, meniscus and cartilage [1,2]. Current available treatment is of symptoms, directed to relieve the pain and regain function. For example: oral administration of NSAID tablets relieves inflammation and pain but causes adverse effects [3,4]; intra-articular injection of corticosteroids, also relieves inflammation, but cannot be used for chronic treatment due to adverse effects such as cartilage damage and joint breakdown [1,2]; intra-articular injection of hyaluronan (HA) to increase viscoelasiticy of the synovial fluid lessened mechanical damage [5,6].

The NSAID Diclofenac, a reversible inhibitor of both COX1 and COX2, is frequently used in the treatment of OA [2,3,7]. It's oral administration, however, is accompanied by NSAID-common adverse effects that include gastrointestinal toxicity, gastric ulcers and anaphylaxis [8,9]. Enteric-coated sustained-release tablets may alleviate some of these effects, but do not resolve the problem of gastrointestinal toxicity [10].

Local administration (via intra-articular injection) of Diclofenac formulated in a carrier, may be a viable alternative to oral administration. It could deliver therapeutic Diclofenac doses to the target tissue while simultaneously overcoming the drug's gastrointestinal toxicity and other adverse effects, provided the carrier possesses the following attributes: an ability to adhere with high affinity to recognition sites present in the target area such as extracellular matrix, cartilage, and membrane-embedded receptors; ability to perform as a site-retained sustained-release Diclofenac depot; composition and structure that would render the carrier itself biocompatible, biodegradable, non-toxic and free of adverse effects. Several Diclofenac-carrier formulations attest to the feasibility of the approach proposed

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here, albeit for different routes of administration: hydrogel CS-Ca^{2+} designated for oral administration and transfersomes designated for topical application to intact skin [11,12].

Hypothesizing that our previously-developed bioadhesive liposomes have the potential to meet the carrier requirements defined above, we set out to pursue them as Diclofenac carriers. To that end, we selected multilamellar liposomes (MLV) that have hyaluronan (denoted HA-BAL) or collagen (denoted COL-BAL) anchored covalently to their surface [13–15,18,19]. The rationale driving these selections was based on several considerations: (i) MLV can provide multiple barriers to drug diffusion compared to unilamellar liposomes, thus MLV have higher potential for sustained-release performance and (ii) The presence of recognition sites at the target zone – such as hyaluronan receptors (CD44), integrins, extracellular matrix and cartilage components – to which HA-BAL and COL-BAL can bind with high affinity [20–25].

We report here the first steps in developing and characterizing Diclofenac-loaded bioadhesive liposomes, focusing on the molecular and cellular levels of organization. For the latter we made use of monolayers of intact cells modeling tissues to which the liposomal Diclofenac will be administered in the future (by local injection). The molecular studies included studies on encapsulation efficiency, sustained-release performance and serum stability. The cellular studies included identification of CD44 in the cell membrane, cell–liposome binding and, most notably, therapeutic activity of the liposome-loaded diclofenac (i.e. COX inhibition).

2. Materials and methods

2.1. Materials

Phospholipon 90G (high purity Soybean phosphatidylcholine (SPC)) was a kind gift from Nattermann Phospholipid GmbH (Cologne Germany). Dipalmitoyl phosphatidylethanolamine (DPPE), collagen, Glutaraldehyde, Diclofenac, Arachidonic acid (AA) and Indomethacin were from Sigma Chemical Co. (St. Louis, USA.) Hyaluronan (HA) was a kind gift from Genzyme, Cambridge MA. USA). FITC-tagged CD44 antibody (HCAM clone IM7), rat IgG$_{2b}$ was from Santa Cruz Biotechnology (Santa Cruz, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) containing 10% Heat inactivated Fetal Bovine Serum (FBS), 1% Mem-Eagle Non essential amino acids (X100), 1% L-glutamine, 1% Penicillin (10,000U/ml) + Streptomycin (10mg/ml) + Nystatin (1250U/ml) solution, and 0.25% Trypsin – EDTA solution were from Biological Industries (Beit Haemek, Israel). [^{14}C]Diclofenac, [^{3}H]PGE$_2$ and [^{3}H] Cholesterol were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Costar tissue culture flasks and plates were from Corning (Corning, USA). Dialysis tubing (molecular weight cutoff of 12,000–14,000) was from Spectrum Medical Industries (Los Angeles, USA).

Ultracentrifugation was performed with a Sorval Discovery M120 SE micro ultracentrifuge (Tennessee, USA). Lyophilization was performed with a HETO Drywinner 3 (Alleraod, Denmark). Fluorescence emission was measured using a FL 500 microplate fluorescence reader BioTek instruments (Vermont, USA).

2.2. Methods

2.2.1. Regular and bioadhesive drug-free liposomes

Regular MLV (denoted RL) were composed of SPC and DPPE, at the mole ratio of SPC:DPPE 95:5 and the total lipid concentration of 100mg lipid/ml. The liposomes were prepared by the "novel" method [16,18], essentially as previously described except: ethanol replaced chloroform:methanol mixtures for lipid dissolution, the swelling solution was 0.1M borate buffer at pH 9, and

incubation of the dry lipid film was for 2h at 65°C, in a shaker bath. A portion of these RL was set aside as a control and the remainder was taken to surface modification, binding hyaluronan or collagen, according to the previously-reported processes [13–15,19,21,26]. Briefly: to obtain HA-BAL, HA was dissolved in acetate buffer (0.1M, pH 4.5) at the concentration of 2mg/ml, was preactivated by incubation with EDC (ethyl-dimethyl-aminopropyl-carbodiimide) [15–17] for 2h at 37°C. The preactivated HA was mixed with the RL suspension, at the ratio of 1:1 (v/v).

To obtain COL-BAL, collagen was dissolved in 0.01% acetic acid to the final concentration of 2mg/ml, allowing 24h at 4°C for complete dissolution. The collagen was mixed with the RL suspension at a ratio of 1:1 (v/v), followed by the addition of Glutaraldehyde to a final concentration of 1%.

Each reaction mixture was incubated for 24h under shaking or stirring, at 37°C and 4°C, for the HA-BAL and for COL-BAL, respectively. The bioadhesive liposomes were freed from excess materials and by-products by ultracentrifugation for 30min at 4°C and a g force of 160,850, followed by several successive washes and re-centrifugations in PBS pH = 7.6, suspending the final pellets in this buffer. The control RL, were subjected to the same centrifugation and washing processes. Aliquots of 1ml of the three liposome types – RL, HA-BAL and COL-BAL – were frozen for 2h at − 80°C, followed by lyophilization. The resultant liposome powders were stored at − 18°C until further use.

2.2.2. Drug encapsulation and release

Drug encapsulation was performed as follows: The lyophilized liposomes, brought to room temperature, were re-hydrated with an aqueous solution of Diclofenac, incubating the systems for 2h at 37°C.

Kinetics of drug release were studied according to our previously-developed experimental set up and data processing [27–29]. Briefly: a suspension of Diclofenac-encapsulating liposomes was placed in a dialysis sac that was immersed in a continuously-stirred receiver vessel containing drug-free buffer (PBS pH 7.6), receiver to liposomes volume ratios was 15. At designated time points, the dialysis sac was transferred from one receiver vessel to another containing fresh drug-free buffer. Drug concentration was determined in each dialysate and in the sac (at the beginning and end of the run). The data were analyzed according to a previously derived multi-pool kinetics model, expressed in Eq. 1 [14,19,13,19, 27–29]:

$$f_{(t)} = \sum_{j}^{1-n} f_j \left(1 - \exp^{-k_j t} \right) \tag{1}$$

where $f_{(t)}$ is the fraction of drug that diffused from the sac to the reservoir at time = t, normalized to the total drug in the system at time = 0; n is the number of independent drug pools in the system, f_j is the fraction of the total drug in the system occupying the jth pool at $t = 0$ and k_j is the rate constant for drug diffusion from the jth pool. These kinetic experiments can also yield the encapsulation efficiency, which is defined as the ratio of liposome-encapsulated drug to the total drug in the system: When the kinetic study is performed on the complete system (i.e. encapsulated and un-encapsulated drug), the value of f_j for the encapsulated pool is also the encapsulation efficiency. The dialysis approach was also used for the serum stability studies, except the liposome sample in the dialysis sac was suspended in 1:1 (v/v) PBS:Fetal bovine serum (FBS) and the dialysis was either against a similar mixture of PBSFBS or against PBS alone. In all studies diclofenac was assayed using a trace of radioisotope [^{14}C] Diclofenac.

2.2.3. Cell culture growth and maintenance

Monolayers of CT-26 cells (mouse colon carcinoma), PANC-1 cells (human pancreatic adenocarcinoma), HT-29 cells (human colon cancer) and COS-7 cells (African green monkey, kidney) were grown in T75 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% Heat inactivated Fetal Bovine Serum (FBS), 1% Mem-Eagle Non essential amino acids (X100), 1% L-glutamine, 1% Penicillin (10,000U/ml) + Streptomycin (10mg/ml) + Nystatin (1250U/ml) solution. Cultures were maintained at 37°C in 5% CO$_2$. For all experiments cells were harvested from subconfluent cultures using 0.25% Trypsin – EDTA solution and were resuspended in fresh full serum-supplemented growth medium before plating.

2.2.4. Liposomes binding to cell monolayers

2.2.4.1. CD44 expression.
To verify that the CT-26 cells express and harbour CD44 receptors in their cell membrane, CT-26 cells were plated in a 24-well plate and the experiment was initiated upon confluency. The cells were

incubated for 20min at 4°C with an anti CD44 antibody at the dose 5μg/ml Ab/10^6 cells. Similar monolayers of COS-7, HT-29 and of PANC-1 cells were used as negative and positive controls. At the end of the incubation the medium above the cells was aspirated, the cells were subjected to extensive washing by PBS, and the fluorescence was determined in a fluorescence plate reader, with excitation and emission at 485nm and 530nm, respectively.

2.2.4.2. liposome-cell binding. The experiments were carried out as previously described [14,15,20]. Briefly, 48–72h prior to the experiment CT-26 cells were seeded onto 24-multiwell plates and the experiment was initiated upon confluency. The cell-growth media was replaced with 500μl of serum-free medium containing the desired liposomes at increasing concentrations. Incubations were for 60min at 37°C. Upon termination, the medium from each well was aspirated, and each well was washed three times with PBS. The cells in each well were lysed with 1N NaOH over night at 37°C. The content of each well was collected and assayed for total cell protein and for cell-associated liposomes. Protein assay was by the Bradford method. Liposome assay was by inclusion of trace [^{3}H]Cholesterol in the formulation which is stable in these liposomes [14,15,20] applying radioactive counting. Wells receiving free medium alone served as controls. The results were analyzed according to the Langmuir isotherm [13,15,18,20].

2.2.5. COX inhibition by free and by liposome-encapsulated Diclofenac in intact cells

CT-26 cells were seeded as described above for binding, except the experiment was initiated at 70% confluency. The cell-growth media was replaced with 500μl serum-free media containing a desired Diclofenac formulation (free or liposomal), ranging from 0.1–10nM Diclofenac. Wells receiving drug-free medium or "empty" (i.e. drug-free) liposomes served as controls. Incubations were for 30min at 37°C, followed by addition of the substrate arachidonic acid (AA) at a final concentration of 30mM. The reaction was arrested after an additional incubation of 20min at 37°C, by the addition of Indomethacin at a final concentration of 5μM. The medium of each well was collected and subjected to a radioimmunoassay (RIA) [30] using trace [^{3}H]PGE$_2$ to determine the PGE$_2$ concentration. Determination of total cell protein was as described in section 4 above.

3. Results and discussion

3.1. Molecular properties of Diclofenac-liposome formulations

Diclofenac encapsulation efficiency was explored as function of liposome type, liposome concentration and drug concentration. At the liposome concentration corresponding to 20mg lipid/ml, Diclofenac encapsulation efficiencies were 40–50%, even at the low end of drug concentrations (0.1–5mg/ml) (Data not shown). Increasing the liposome concentration to 100mg lipid/ml generated an increase in encapsulation efficiencies as expected from prior studies [29], for all liposome types tested (Fig. 1). Clearly, it is possible to encapsulate Diclofenac in liposomes, at high efficiency and – irrespective of drug dose level – rendering the liposomes bioadhesive does not impair drug encapsulation (Fig. 1). At low drug doses, encapsulation efficiency is relatively high and close to complete. With the increase in drug concentration there is some drop in encapsulation efficiency, yet even close to the limit of drug solubility (20mg Diclofenac/ml) satisfactory encapsulation efficiencies of ≥ 50% are obtained. Moreover, the tendency of encapsulation efficiency to decrease at the high end of initial drug concentrations, had no adverse effect on drug loading. The latter increases continuously with the increase in initial concentration, as also shown in Fig. 1.

Unidirectional Diclofenac efflux from liposomal formulations showed a multi-phase pattern which was similar in all three liposome types (Fig. 2A). This pattern is quite similar to our previous findings for other small molecular weight drugs corresponding to a two-pool model, one assigned to the unencapsulated drug present in the system at time = 0 and the other to the liposome-encapsulated drug. Data from experiments of the type shown in Fig. 2, analyzed according to Eq. (1) above, fit the case of $n = 2$, as expressed in Eq. (2) below:

$$f_{(t)} = f_1\left(1 - \exp^{-k_1 t}\right) + f_2\left(1 - \exp^{-k_2 t}\right) \qquad (2)$$

The magnitudes obtained for k_2, the parameter indicative of the sustained-release nature of the systems, are shown in Fig. 3. As in the case of encapsulation, and as indicated from the raw data of Fig. 2, rendering the liposomes bioadhesive did not affect drug release — neither blocking it, nor accelerating it. As
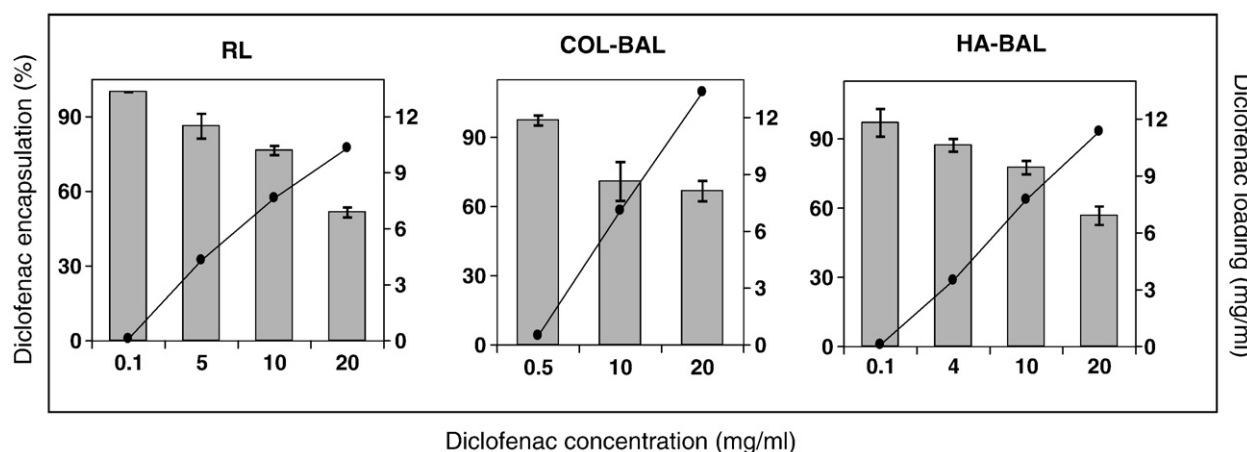

Fig. 1. Diclofenac (SD) encapsulation efficiency and Diclofenac drug loading, in regular and in bioadhesive liposomes, as function of initial drug concentration. Left-hand panel: regular liposomes (RL), middle panel: Bioadhesive liposomes with collagen as the bioadhesive ligand (COL-BAL), right-hand panel: Bioadhesive liposomes with hyaluronan as the bioadhesive ligand (HA-BAL). In each panel the bars (left-hand ordinate) represent the encapsulation efficiency, each an average of 3 determinations (using the kinetic method) and the error bars represent the standard deviations, and the lines (right-hand ordinate) represent the drug loading (i.e. concentration of encapsulated drug). Liposome concentrations were constant at 100 mg lipid/ml, and the lipid composition for all liposome types was SPC:DPPE at the mole ratio of 95:5.
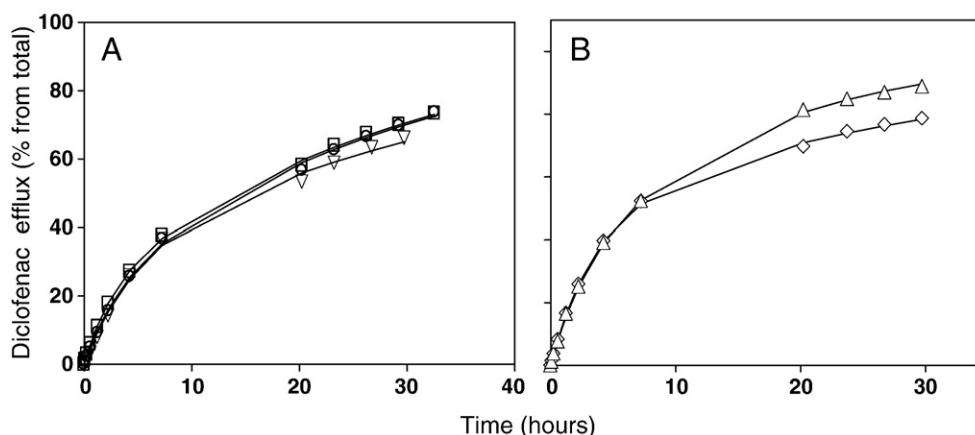
Fig. 2. Kinetics of Diclofenac (SD) efflux from regular and from bioadhesive liposomes, in buffer and in buffer:serum media. Liposome details as those listed in the legend to Fig. 1. Points are experimental, and the solid curves are the theoretical expectations, drawn according to Eq. (2) in the text. A. Efflux into buffer media. RL: open squares, HA-BAL: open circles, COL-BAL: open inverted triangles. B. Efflux into buffer or serum media. HA-BAL suspended in PBS and dialyzed against PBS — open diamonds, HA-BAL suspended in PBS:serum 1:1 and dialyzed against PBS:serum 1:1 — open triangles.

also seen in Fig. 3, the efflux rate constant of the encapsulated Diclofenac was found to increases with the increase in initial drug concentration. Given that such efflux is a multi-step process with a single rate-limiting step [14,19,27–29], this increase implies a reduction in energetic costs either at the same rate-limiting step or a shift in the location of that step. Yet we wish to stress that for the entire span of systems, k_2 remained within the range of $0.01–0.03 h^{-1}$ corresponding to half-life of drug release in the range of 1–3 days. This is clear support for the sustained-release nature of these liposomal Diclofenac formulations. The unidirectional flux conditions under which these experiments were conducted, represent the fastest drug release expected due to the significant dilution that generates a massive driving force for diffusion. Such conditions would be expected to reign when liposomes are administered systemically. The smaller the dilution of an administered liposome formulation, the smaller the driving force for drug diffusion, hence the slower the net efflux. A case of lesser dilution is local, rather than systemic, administration of drug carrier systems. Based on these physicochemical considerations, we expect that designated local administration of diclofenac–liposome formulations, where dilution is significantly less than in the case of systemic administration, will extend the time span for drug delivery from each dose well beyond that of 1–3 days.

Proteins that are part of the biological environment into which the liposomal formulations will be administered, may pose a risk of destabilizing the formulations, which is liposome-specific rather than drug-specific. To assess this risk at the molecular level, the kinetic experiments described above were repeated, suspending the liposomes within the dialysis sac in a media modeling the protein contents of biological fluids, made of serum:buffer 1:1 (v/v). Were the liposomes significantly destabilized, this would have appeared as a significant increase in the rate of drug release due to liposomal depletion. The results shown in Fig. 2B are clear indication that this was not the case, as rates of drug efflux in the presence and absence of serum are quite similar. Further support for stability of the bioadhesive liposomes in biological media is drawn from

previous in vivo studies of antibiotic-encapsulating COL-BAL [29].

3.2. Cellular properties of Diclofenac–liposome formulations

The hyaluronan-specific receptor family CD44 is known to be over-expressed in many types of tumors while it is usually poorly-expressed in normal cells [21,23,26]. In the test cell line used here, CT-26, CD44 expression is anticipated to correlate with favored binding of HA-BAL compared to the other types of liposomes used in this study (i.e., RL and COL-BAL). Such favored binding may, furthermore, affect COX inhibition by the liposomal Diclofenac.

The expression of CD44 in the tested CT-26 cells was verified, as shown in Fig. 4, by the binding of anti CD44 antibody to these cells as well as to positive controls (i.e., the PANC-1 and the HT-29) and by the lack of binding to cells devoid of this receptor family (i.e., the COS-7 cells).
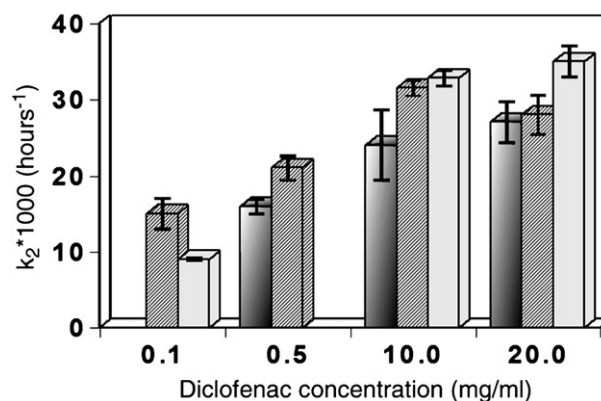

Fig. 3. The relationship between k_2 – the efflux rate constant of liposome-encapsulated Diclofenac (SD) – and the initial drug concentration, for regular and for bioadhesive liposomes. Liposome details are similar to those listed in the legend to Fig. 1. Light-shaded bars: regular liposomes, dark-shaded bars: hyaluronan bioadhesive liposomes (HA-BAL), diagonal-patterned bars: collagen bioadhesive liposomes (COL-BAL). Each bar is an average of 3 determinations and the error bars represent the standard deviations.
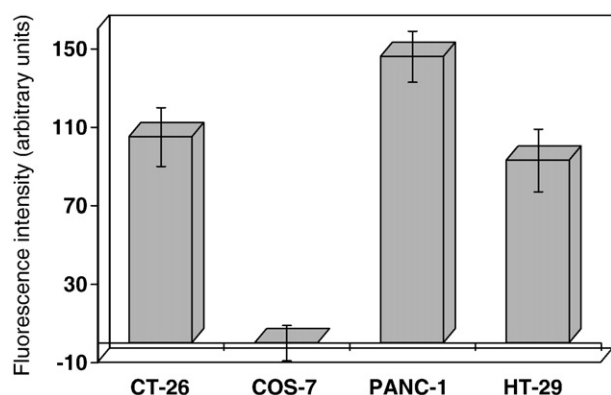
Fig. 4. Binding of FITC-tagged anti CD44 antibody (HCAM clone IM7, rat IgG$_{2b}$) to cell monolayers. Each bar is an average of four determinations and the error bars represent the standard deviations.

All liposome types studied (RL, HA-BAL and COL-BAL) were found to bind to monolayers of CT-26 cells, with a saturating pattern (albeit with quantitative differences among the liposome types). Data analysis fit a one-term Langmuir Isotherm [13,15,18,20], and the resultant binding parameters are listed in Table 1. The interaction of regular liposomes (RL) with the CT-26 cells is, as expected, non-specific and relatively weak. As anticipated from the presence of CD44 receptors, HA-BAL bound to the CT-26 cells with high affinity, that was 40 fold higher than that of the RL, with a concomitant drop in the capacity. The latter is expected, given that the number of specific sites a cell offers a ligand (i.e. receptors) is usually smaller than the number of sites for non-specific adsorption. Binding of these HA-BAL to COS-7 cells (the negative control cell line lacking CD44 receptors), was of considerable lower affinity, yielding a Kd of 1.4 mM lipid and a Bmax of 1.2 nmol lipid/10^5 cells. These correspond to a 64 fold lower affinity than to the test CT-26 cells, with a concomitant 11 fold higher Bmax. Both these parameters, lower affinity and higher capacity are indicative of non-specific binding. The binding of COL-BAL to these cells falls in-between those of the RL and the HA-BAL, showing an affinity which is 6 fold higher than that of RL with a drop in capacity less-pronounced than in the case of HA-BAL. These data indicate presence of components on the surface of the CT-26 cells that are capable of binding collagen (hence COL-BAL) such as integrins and ECM, but with a lower specificity than HA-BAL. Nevertheless, both types of bioadhesive liposomes show sufficient binding to perform as site-retained Diclofenac depots.

Table 1
Binding of regular and of bioadhesive multilamellar liposomes to monolayers of the CT-26 cell line

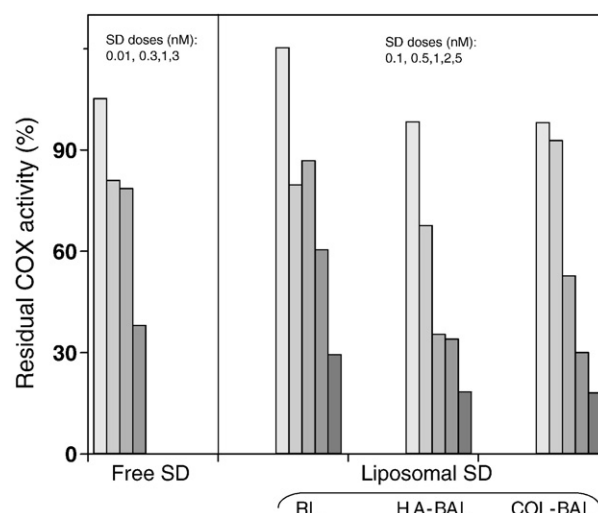| Liposome type	Bioadhesive ligand	Binding parameters	
		K_d (mM lipid)	B_{max} (nmoles lipid/10^5 cells)
RL	None	3.9 (±0.7)	0.23 (±0.02)
COL-BAL	Collagen	0.62 (±0.06)	0.15 (±0.01)
HA-BAL	Hyaluronan	0.11 (±0.02)	0.022 (±0.001)

Fig. 5. COX inhibition, expressed as residual COX activity, by free and by liposome-encapsulated Diclofenac (SD), in intact CT-26 cells. Each set of bars represents, for each formulation, the increase in enzyme inhibition as function of increasing drug concentrations. Drug concentrations increase from left to right, as represented by the gradual increase in the bar-shade density, and the specific values are listed on the figure. Liposome details are as listed under Fig. 1. Enzyme activity was determined using arachidonic acid as the substrate, following the increase in the PGE$_2$ product. Each bar is an average of 6 determinations and the standard deviations were <10%.

Even when, as in the present case, drug encapsulation within a carrier and drug efflux from the carrier are both highly satisfactory, questions still remain with respect to the desired biological activity. The processes of encapsulation within a carrier may damage a drug and cause loss of its activity. In addition, drug release from the carrier to the cell may be too slow and too low to provide intracellular activity within a relevant time frame. The *in vitro* studies we conducted (Fig. 5) to evaluate COX inhibition in intact cells, were designed to address these questions. Free SD, tested to verify proper response of all assay components, generated the expected clear dose-dependant inhibition of COX activity in the intact CT-26 cells (Fig. 5, left-hand bar set). All three liposomal SD formulations (Fig. 5, right-hand bar sets) were also clearly active in COX inhibition, on a par with that of the free drug. These results indicate that formulation within the liposomes did not damage drug activity and that the encapsulated drug gained access into the cells. It may be, moreover, that within the time span of the experiment only part of the encapsulated drug moved from the liposomes into the cells. The potency of liposomal SD may, therefore, be higher than that of free drug. Whether the higher affinity of HA-BAL to cells expressing the CD44 receptor translates into a therapeutic advantage for SD formulated in these liposomes awaits studies in animal models.

4. Conclusions

It follows, from the data presented and discussed, that HA-BAL and COL-BAL provide high efficiency encapsulation of SD at therapeutically-relevant doses, can act as sustained-release depots with half-lives of drug release in the range of 1–

3 days and are stable in biological media. The bioadhesive, but not the regular, liposomes bind with high affinity to the CT-26 cell line that models target cells in two respects: having the CD44 receptors for hyaluronan and integrins for collagen; containing intracellular COX which is the site of Diclofenac action. The liposome-encapsulated Diclofenac, moreover, remains active and functional as a COX inhibitor. There also seems to be a correlation, which needs further future corroboration, that the higher the liposomal affinity to the target cell, CT-26 in the present case, the better it can function as a site-adherent drug depot.

Taken together, we offer the conclusion that these systems merit further studies in animal models. Such investigations, applying local injections of these novel formulations in a rat model of osteoarthritis, are underway.

References

[1] K.D. Brandt. Osteoarthritis, in: E. Braunwald, A.S. Fauci, D.L. Kasper, S.L. Hauser, D.L. Longo, J.L. Jameson (Eds.), Harrison's : Principles of internal medicine, Vol. 2, McGraw-Hill Professional Publishing; 15th edition. pp. 1987-1992.

[2] Z. Ge, Y. Hu, B.C. Heng, Z. Yang, H. Ouyang, E.H. Lee, T. Cao, Osteoarthritis and therapy, Arthritis Rheum. 55 (2006) 493–500.

[3] M. Saravanan, K. Bhaskar, G. Maharajan, K.S. Pillai, Ultrasonically controlled release and targeted delivery of diclofenac sodium via gelatin magnetic microspheres, Int. J. Pharm. 283 (2004) 71–82.

[4] S.E. Bove, S.L. Calcaterra, R.M. Brooker, C.M. Huber, R.E. Guzman, P.L. Juneau, D.J. Schrier, K.S. Kilgore, Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis, Osteoarthr. Cartil. 11 (2003) 821–830.

[5] S.F. Sun, Y.J. Chou, C.W. Hsu, C.W. Hwang, P.T. Hsu, J.L. Wang, Y.W. Hsu, M.C. Chou, Efficacy of intra-articular hyaluronic acid in patients with osteoarthritis of the ankle: a prospective study, Osteoarthr. Cartil. 14 (2006) 867–874.

[6] L.W. Moreland, Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action, Arthritis. Res. Ther. 5 (2003) 54–67.

[7] T.E. Towheed, Pennsaid therapy for osteoarthritis of the knee: a systematic review and metaanalysis of randomized controlled trials, J. Rheumatol. 33 (2006) 567–573.

[8] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, Annu. Rev. Biochem. 69 (2000) 145–182.

[9] J.R. Vane, Y.S. Bakhle, R.M. Botting, Cyclooxygenases 1 and 2, Annu. Rev. Pharmacol. Toxicol. 38 (1998) 97–120.

[10] T. Khazaeinia, F. Jamali, Effect of drug release rate on therapeutic outcomes: formulation dependence of gastrointestinal toxicity of diclofenac in the rat, Inflammopharmacology 12 (2004) 69–80.

[11] M.F. Tsai, Y.L. Chiang, L.F. Wang, G.W. Huang, P.C. Wu, Oral sustained delivery of diclofenac sodium using calcium chondroitin sulfate matrix, J. Biomat. Sci. 16 (2006) 1319–1331.

[12] G. Cevc, G. Blume, New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes, Biochim. Biophys. Acta 1514 (2001) 191–205.

[13] N. Yerushalmi, A. Arad, R. Margalit, Molecular and cellular studies of hyaluronic acid-modified liposomes as bioadhesive carriers for topical drug delivery in wound healing, Arch Biochem Biophys 313 (1994) 267–273.

[14] N. Yerushalmi, R. Margalit, Bioadhesive, collagen-modified liposomes: molecular and cellular level studies on the kinetics of drug release and on binding to cell monolayers, Biochim. Biophys. Acta 1189 (1994) 13–20.

[15] N. Yerushalmi, R. Margalit, Hyaluronic acid-modified bioadhesive liposomes as local drug depots: effects of cellular and fluid dynamics on liposome retention at target sites, Arch. Biochem. Biophys. 349 (1998) 21–26.

[16] K.L. Carraway, D.E. Koshland, Carbodiimide modification of proteins, Methods Enzymol. 25 (1972) 616–623.

[17] V. Weissing, J. Lasch, G. Gregoriadis, Covalent coupling of sugars to liposomes, Biochem. Biophys. Acta. 1003 (1989) 54–57.

[18] R. Margalit, Liposome-mediated drug targeting in topical and regional therapies, Crit. Rev. Ther. Drug.Carrier Syst. 12 (1995) 233–261.

[19] D. Peer, R. Margalit, Physicochemical evaluation of a stability-driven approach to drug entrapment in regular and in surface-modified liposomes, Arch. Biochem. Biophys. 383 (2000) 185–190.

[20] S. Fischer, A. Arad, R. Margalit, Lipsome-formulated enzymes for organophosphate scavenging: butyrylcholinesterase and Demeton-S, Arch. Biochem. Biophys. 434 (2005) 108–115.

[21] D. Peer, R. Margalit, Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal doxorubicin in syngeneic and human xenograft mouse tumor models, Neoplasia 6 (2004) 343–353.

[22] S. Dedhar, E. Ruoslahti, M.D. Pierschbacher, A cell surface receptor complex for collagen type I recognizes the Arg-Gly-Asp sequence, J. Cell. Biol. 104 (1987) 585–593.

[23] C.M. Isacke, H. Yarwood, The hyaluronan receptor, CD44, Int. J. Biochem. Cell. Biol. 34 (2002) 718–721.

[24] D.J. White, S. Puranen, M.S. Johnson, J. Heino, The collagen receptor subfamily of the integrins, Int. J. Biochem. Cell. Biol. 36 (2004) 1405–1410.

[25] B.P. Toole, Hyaluronan: from extracellular glue to pericellular cure, Nat. Rev. Cancer 4 (2004) 528–539.

[26] D. Peer, R. Margalit, Loading mitomycin C inside long circulating hyaluronan targeted nano-liposomes increases its antitumor activity in three mice tumor models, Int. J. Cancer 108 (2004) 780–789.

[27] D. Peer, A. Florentin, R. Margalit, Hyaluronan is a key component in cryoprotection and formulation of targeted unilamellar liposomes, Biochim. Biophys. Acta 1612 (2003) 76–82.

[28] R. Margalit, Liposomal drug delivery: thermodynamic and chemical kinetic consideration, J. Cont. Rel. 17 (1991) 285–296.

[29] R. Margalit, Bioadhesive Liposomes for Topical Treatment of Wounds, in: S.B. Chohen, H. Bernstein (Eds.), Microparticulate Systems for the Delivery of Protein and Vaccines, vol. 77, CRC press, 1996, pp. 425–461.

[30] G. Levin, N. Kariv, E. Khomiak, A. Raz, Indomethacin inhibits the accumulation of tumor cells in mouse lungs and subsequent growth of lung metastases, Chemotherapy 46 (2000) 429–437.