pH-sensitive liposomes for receptor-mediated delivery to chicken hepatoma (LMH) cells

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Abstract pH-sensitive liposomes composed of dioleoylphosphatidylethanolamine and cholesterol hemisuccinate (3:2 mol/mol) bearing the *N*-acetylglucosamine derivative of bovine serum albumin (N-Ac-BSA) were applied for receptor-mediated delivery in chicken hepatoma (LMH) cells expressing the N-Ac-BSA-binding asialoglycoprotein receptor. Fluorescently labeled dextran was entrapped in liposomes by a modified freeze-thawing method (encapsulation efficiency of 23%). A novel method of coupling proteins onto the surface of preformed liposomes yielded a coupling efficiency of 60–70%. The association of pH-sensitive and lecithin liposomes with LMH cells was monitored by fluorescence-activated cell sorting and confocal microscopy. Prerequisites for receptor-mediated delivery to LMH cells were both the pH sensitivity of liposomes and the presence of N-Ac-BSA on the liposomal surface.

Key words: pH-sensitive liposome; Surface-available protein; Receptor-mediated targeting

1. Introduction

Liposomes have been studied for the past three decades in the field of controlled and site-specific delivery of drugs, peptides and proteins [1]. Liposomes function both as a controlled release system and as a delivery system transporting encapsulated substances into cells. However, the majority of liposomes internalized by cells enter through an endocytotic pathway and are then processed in the lysosomes, where enzymatic degradation of the lipids and the encapsulated compounds occurs [2]. Substances that are degraded or that cannot escape from the lysosomal compartment are therefore inactivated when delivered by most of the liposome compositions described to date. Specially designed liposomes, which are able to mediate cytoplasmic delivery, could achieve site-specific delivery. Two approaches are being proposed in order to achieve this goal: the creation of virosomes and the development of pH-sensitive compositions [3]. Virosome delivery is associated with the possibility of inherent toxicity and/or immunogenicity. Therefore, pH-sensitive liposomes were developed to mimic the behavior of viral particles. These pH-sensitive liposomes are endocytosed in coated vesicles and should fuse with lipid membranes in the acidic environment of the endosomes, thus facilitating the release of encapsulated compounds into the cytoplasm [4]. Another possibility is direct fusion of these liposomes with the plasma membrane if pH at the cell surface is decreasing. The liposomal content is then released directly into the cytoplasm. The commonly used lipid composition in pH-sensitive liposomes is dioleoylphosphatidylethanolamine (DOPE) and cholesterol hemisuccinate (CHEMS) [5].

It was demonstrated [6] that fusogenic lipids, such as DOPE, can improve the efficiency of the transfection. Oligodeoxynucleotides (ODN) with base sequences complementary (antisense) to specific cellular mRNA can modulate the expression of any individual gene. ODN are relatively large negatively charged hydrophilic molecules (MW 5000–10 000) which do not passively diffuse across cell membranes [7]. The association with drug carriers such as liposomes can protect them against degradation and advantageously utilize the cellular uptake mechanism. One more step forward is receptor-mediated targeting through specific ligands coupled onto the liposomal surface.

We chose to use the *N*-acetylglucosamine derivative of bovine serum albumin (N-Ac-BSA) which is responsible for binding to the avian membrane asialoglycoprotein receptor (ASGP-R) and internalization [8]. Coupling of N-Ac-BSA to preformed pH-sensitive liposomes enabled us to target the avian hepatoma cell line LMH expressing the ASGP-R. We compared the association of LMH cells with different liposomes, i.e. pH-sensitive liposomes (composed of DOPE and CHEMS) and conventional liposomes (composed of soy phosphatidylcholine, SPC), both bearing the specific ligand (N-Ac-BSA), pH-sensitive liposomes bearing BSA (non-specific ligand) or ligand-free. Liposomal bilayers were labeled with rhodamine-DHPE (Rh-PE) and the entrapped material was fluorescently labeled (FITC) as well. In the present work, we used FITC-dextran (MW 4400) to mimic antisense ODN of similar molecular weight and developed a liposomal system for receptor-mediated delivery. The fate of both the lipophilic and hydrophilic markers was followed by FACS analysis and confocal microscopy. pH-sensitive liposomes bearing N-Ac-BSA associated with the cells, whereas no association of conventional liposomes bearing the same ligand was found.

2. Materials and methods

2.1. Materials

N-Ac-BSA (bovine albumin-*p*-aminophenyl-*N*-acetyl-β-D-glucosamide) and LMH (chicken hepatoma) cells were generous gifts from the Department of Medicine II, University of Freiburg, Germany. BSA (bovine serum albumin), FITC-dextran (fluorescein isothiocyanate dextran; MW 4400), HEPES (*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)), sodium cholate, DOPE (dioleoylphosphatidylethanolamine) and CHEMS (cholesterol hemisuccinate) were products obtained from Sigma (Deisenhofen, Germany). SPC (soy phosphatidylcholine) came from Lipoid (Ludwigshafen, Germany). Lissamine rhodamine B (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; Rh-PE) was purchased from

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Molecular Probes (Leiden, The Netherlands). All other chemicals were of analytical grade.

2.2. Preparation of liposomes

Liposomes were prepared by a modified freeze-thawing method [9]. pH-sensitive liposomes (50 mM total lipid) were prepared as follows: solutions of DOPE (in chloroform), CHEMS (in tetrahydrofuran/methanol; 2:1 v/v) and Rh-PE (in methanol) in a molar ratio of 3:2:0.05 were mixed and evaporated to dryness. After vacuum desiccation, 0.5 ml HEPES (10 mM with 150 mM NaCl; pH 7.4) containing FITC-dextran (10 mM) was added and the lipid was suspended by vigorous shaking (room temperature). Liposome suspension was probe-sonicated 6×30 s (resting period 30 s) and left at room temperature (30 min) prior to freeze-thawing. The suspension was rapidly frozen in liquid nitrogen then brought to room temperature to thaw completely (approximately 20 min). The procedure was performed three times. SPC liposomes (50 mM lipid containing 1 mol% Rh-PE) were prepared under the same conditions. The non-entrapped marker was separated from the liposomes on a Sepharose CL-4B gel (Pharmacia, Uppsala, Sweden) and the entrapment efficiency was measured fluorimetrically. Size and polydispersity index of all preparations were determined by photon correlation spectroscopy (Zetamaster, Malvern, Germany; multimodal analysis).

2.3. Coupling of protein to liposomes

Protein (N-Ac-BSA or BSA) was coupled onto liposomes containing FITC-dextran by a novel method (Lung and Schubert, submitted) which was modified for pH-sensitive liposomes. In short, protein (20 mg/ml) was incubated with soy sterol-PEG 1100 tresylate (activated anchor molecule; 8 mg/ml), with the pH adjusted to 8.25 (borate buffer) and the mixture left overnight at 4°C. Free protein and anchor molecule were separated in HEPES buffer from the protein-anchor conjugate on a Sepharose CL-6B column (Pharmacia). Preformed liposomes were incubated with the protein-anchor conjugate for 6 h at 4°C and free protein was separated from liposomes bearing protein on a Sepharose CL-4B column.

2.4. Lipid and protein determination

The exact lipid concentration of liposomes was determined [10] prior to cell culture experiments. Liposomal and free protein were determined fluorimetrically or by BCA protein assay (Pierce, Rockford, IL, USA).

2.5. Cells and media

The chicken hepatoma cell line LMH [11] was grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. Approximately 5×10^5 cells per well were seeded into 6-well Falcon culture plates (Becton-Dickinson, Boole, Switzerland) 24 h prior to the experiments. Cells were maintained at 37°C in a humidified incubator (5% CO_2 atmosphere).

2.6. Cell experiments

Cells were washed with PBS and incubated with either liposomes or free FITC-dextran (all preparations in MEM, 1 ml per well) for 1 h at 37°C. After removing the liposomes and media, the cells were washed with PBS and harvested by trypsinization for 5 min at 37°C (0.02% EDTA containing 0.05% trypsin; w/v). The cells were centrifuged (5 min at 1200×*g*), washed and resuspended in PBS (0.5 ml), stored on ice and used for FACS analysis.

2.7. FACS

To assess the percentage of cell-associated fluorescence (both FITC-dextran entrapped in liposomes and Rh-PE in liposome bilayers), FACS analysis was performed using a FACS sorter (Becton-Dickinson, Heidelberg, Germany). 10 000 individual cells were detected by stray light and the percentage of cells which were additionally fluorescent was calculated by Lysis II software (Becton-Dickinson, Heidelberg, Germany).

2.8. Confocal microscopy

Liposomes bearing ligands and ligand-free liposomes were incubated for 1 h at 37°C (under the conditions described above) with LMH cells seeded on coverslips. After washing, cells were fixed with paraformaldehyde (2%, w/v) for 45 min at room temperature. Samples were examined by an Axiovert 100 (Zeiss, Jena, Germany) laser scanning confocal microscope.

3. Results

3.1. Entrapment of FITC-dextran in pH-sensitive liposomes

To deliver a sufficient amount of drug into the desired cells, high trapping efficiency of drug into carrier is required. We examined several liposome preparation methods (namely extrusion, detergent dialysis and freeze-thawing) to entrap FITC-dextran (MW 4400) as a model marker (data not shown). The highest efficiency was obtained with a modified freeze-thawing method. For pH-sensitive liposomes the encapsulation efficiency was 23%. Conventional liposomes (SPC) prepared by the same method entrapped only 15.8% of the marker, probably due to their smaller size when compared to pH-sensitive liposomes (Table 1).

3.2. Coupling of ligands to liposomes

The recently developed coupling method (Lung and Schubert, submitted) enables a highly efficient coupling of various proteins onto the surface of preformed vesicles. Coupling efficiency was approximately 60% and did not depend on liposome composition or protein used (Table 1). During the remote incorporation of the protein-linked lipid into the liposomal membrane, the release of the originally entrapped marker was less than 19% for pH-sensitive liposomes and 21% for SPC liposomes.

3.3. FACS

The cellular association of liposomes labeled with Rh-PE in bilayers and FITC-dextran in aqueous space (10 mM) was followed by FACS analysis. Fig. 1A–E shows the results of

Table 1
Characterization of pH-sensitive and control liposomes with coupled protein

Lipid composition	Size (nm)	PI[a]	Protein	Protein/lipid ratio (w/w)[b]	Coupling efficiency (%)	Release during coupling (%)[c]
pH-sensitive	304 ± 40	0.293	N-Ac-BSA	0.093	62.7 ± 3.8	16.8 ± 2.2
pH-sensitive	292 ± 54	0.290	BSA	0.090	60.9 ± 4.5	18.9 ± 0.6
SPC	256 ± 68	0.265	N-Ac-BSA	0.077	60.9 ± 3.3	20.7 ± 0.6
SPC	292 ± 54	0.248	BSA	0.078	61.3 ± 3.7	21.4 ± 1.6

[a]Polydispersity index.
[b]Coupled protein per liposomal lipid (total) ratio.
[c]Release of entrapped FITC-dextran during the coupling procedure.
pH-sensitive liposomes composed of DOPE/CHEMS/Rh-PE in a molar ratio of 3:2:0.05 (50 mM total) and SPC/Rh-PE liposomes in a molar ratio of 1:0.01 (50 mM) were prepared by a modified freeze-thawing method. Original liposomes with entrapped FITC-dextran (trapping efficiencies for pH-sensitive and SPC liposomes were 23.0 ± 2.7% and 15.8 ± 0.6%, respectively) were used in the coupling procedures. Size of liposomes before coupling was 228 ± 32 nm for pH-sensitive and 164 ± 22 nm for SPC liposomes. The values denote the mean of three preparations ± S.D.

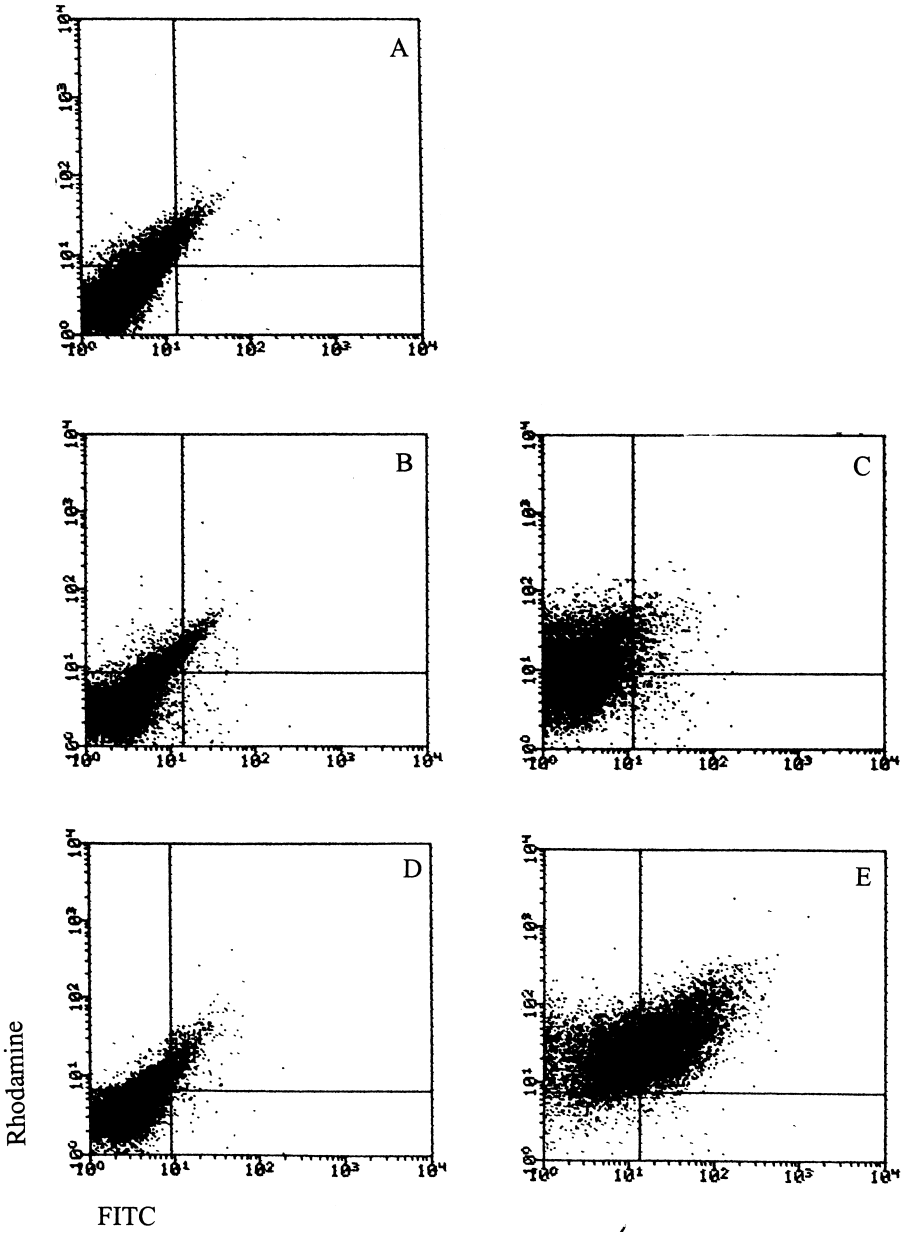

Fig. 1. FACS analysis of association of different liposome preparations with LMH cells. A: LMH cells (control). B: SPC liposomes bearing N-Ac-BSA. C: pH-sensitive liposomes. D: pH-sensitive liposomes bearing BSA. E: pH-sensitive liposomes bearing N-Ac-BSA. Liposomes (100 nmol lipid) labeled with Rh-PE (bilayer) and FITC-dextran (entrapped) were incubated with LMH cells for 1 h at 37°C.

Table 2
Association of liposomes with LMH cells

Preparation	Cells (%) in LL	Cells (%) in LR (FITC)	Cells (%) in UL (rhodamine)	Cells (%) in UR (FITC and rhodamine)
(1) Control (cells only)	91.0	1.1	3.9	4.0
(2) FITC-dextran	90.2	1.4	3.7	4.7
(3) pH-sensitive (empty)[a]	9.4	0.1	89.7	0.9
(4) pH-sensitive (empty)[a]+free FITC-dextran	13.4	0.0	86.0	0.5
(5) pH-sensitive[b]	27.7	0.2	71.2	0.9
(6) pH-sensitive-N-AC-BSA[c]	28.1	10.0	20.2	41.8
(7) pH-sensitive-BSA[d]	84.2	11.7	0.5	3.6
(8) SPC-N-Ac-BSA[e]	75.2	0.3	19.4	5.1

[a]pH-sensitive liposomes labeled with Rh-PE but without entrapped FITC-dextran (empty).
[b]Ligand-free pH-sensitive liposomes labeled with Rh-PE and entrapping FITC-dextran.
[c]pH-sensitive liposomes labeled with Rh-PE, entrapping FITC-dextran and bearing N-Ac-BSA.
[d]pH-sensitive liposomes labeled with Rh-PE, entrapping FITC-dextran and bearing BSA.
[e]SPC liposomes labeled with Rh-PE, entrapping FITC-dextran and bearing N-Ac-BSA.
Preparations (100 nmol lipid) were incubated with LMH cells for 1 h at 37°C.

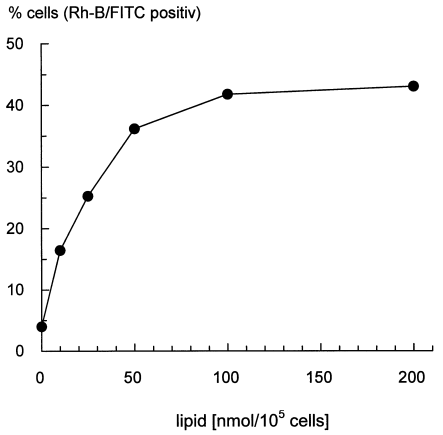

Fig. 2. Association of both lipid marker and entrapped marker of pH-sensitive liposomes bearing N-Ac-BSA with LMH cells, measured by FACS analysis.

FACS analysis. The cells in which both fluorescence of FITC-dextran (x-axis) and Rh-PE (y-axis) were detected signaled in a defined region (called upper right; UR) of the square. LMH cells (not incubated with liposomes but treated under the same exact conditions), which served as a negative control, showed 4% of the cells in the UR (Fig. 1A). SPC/N-Ac-BSA liposomes incubated under the same conditions showed a cell fluorescence signal in the UR region of only 5% (Fig. 1B; Table 2). pH-sensitive liposomes (ligand-free) incubated with the cells caused a shift of 71% of the cells toward the upper left region (Fig. 1C). Only 1% of the cells signaled in the UR, indicating that liposomal lipid associated with the cells but the entrapped marker was released, or lost, during the association (Table 2). pH/BSA liposomes did not associate with the cells (Fig. 1D). When LMH cells were treated with pH/N-Ac-BSA, 42% of the total cells were present in the UR region (Fig. 1E; Table 2). In order to follow the saturation of (receptor-mediated) uptake of pH-sensitive liposomes bearing N-Ac-BSA, liposomes of different lipid concentrations were incubated with LMH (5×10^5) cells. Fig. 2 shows that an increase in the amount of liposomes (lipid and/or protein) increased the number of cells emitting fluorescence in the UR region (presence of both markers). Liposomes (lipid concentration 10, 25, 50, 100 and 200 nmol) incubated with the cells caused signaling from 16% (10 nmol) to approximately 42% (100 and 200

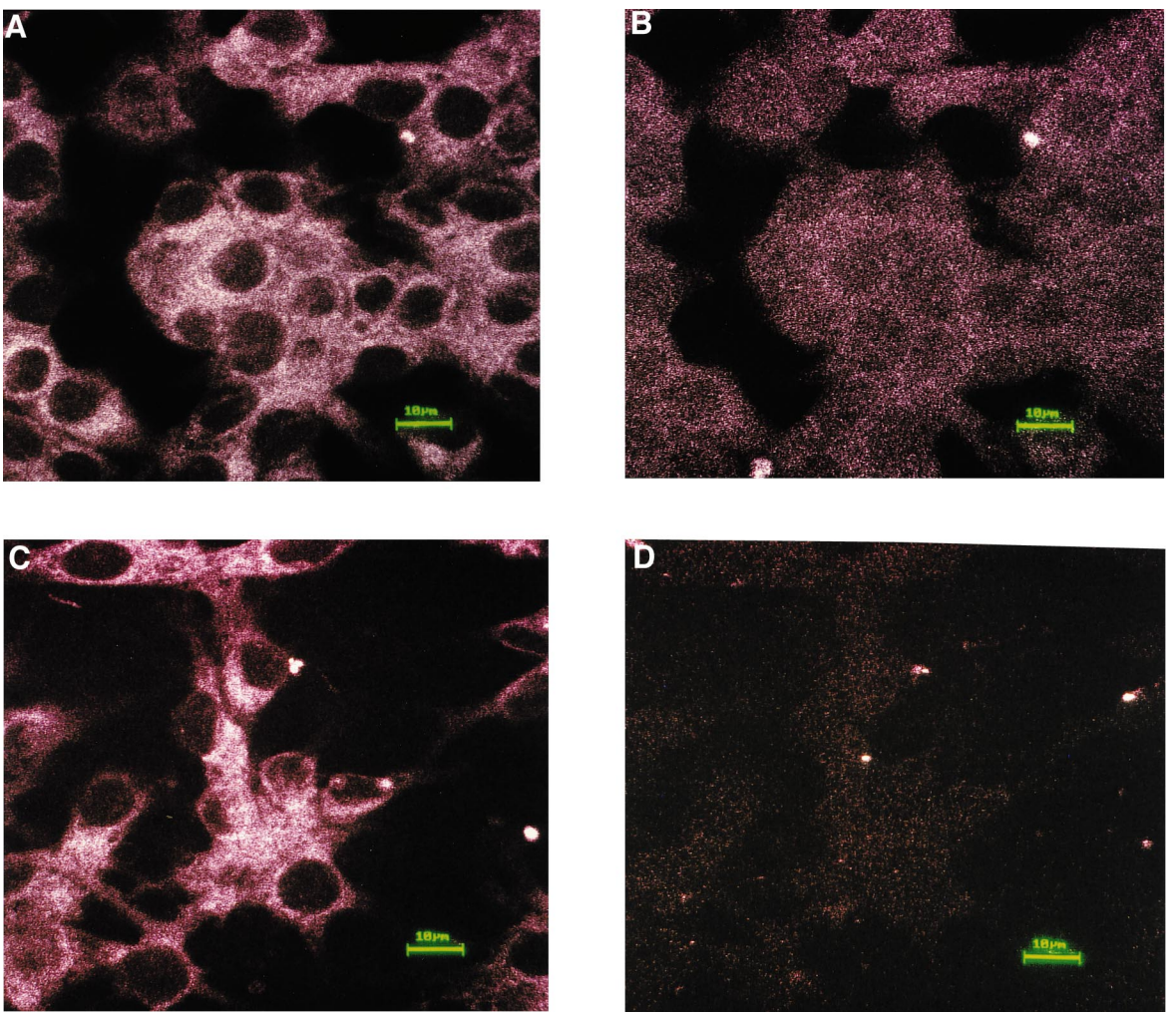

Fig. 3. Confocal micrographs of liposome-LMH cells association. A: Rhodamine fluorescence of cells incubated with pH-sensitive liposomes bearing N-Ac-BSA. B: FITC fluorescence of cells incubated with pH-sensitive liposomes bearing N-Ac-BSA. C: Rhodamine fluorescence of cells incubated with pH-sensitive liposomes. D: FITC fluorescence of cells incubated with pH-sensitive liposomes.

nmol) of the cells in the UR. When lipid concentration was increased to 500 nmol, no significant further increase in cellular uptake was observed (data not shown).

3.4. Confocal microscopy

Fig. 3A,B represents the confocal micrographs of LMH cells after incubation with fluorescently labeled pH/N-Ac-BSA liposomes. The lipophilic marker (Rh-PE) could be found near the cell membranes (Fig. 3A), whereas the green marker (FITC-dextran, which was entrapped in liposomes) was diffusely dispersed in the cytoplasm. Both Rh-PE and FITC-dextran were not concentrated in organelles (Fig. 3B). These findings indicate that the liposomal lipid was associated with the cells and delivered the entrapped material into the cytoplasm. SPC/N-Ac-BSA and pH/BSA liposomes did not associate with the cells (data not shown). Interestingly, in the case of ligand-free pH-sensitive liposomes, only red marker (Rh-PE) was detected (Fig. 3C,D). These data confirmed the quantitative data obtained by FACS analysis. Ligand-free pH-sensitive liposomes associate with the cells, but the entrapped material was lost.

4. Discussion

Liposomes coupled with ligands are widely applied in immunology or specific delivery (receptor-mediated targeting). The coupling of proteins onto the liposomal surface can be performed by different procedures and the presence of proteins on the liposomal surface can be confirmed by various methods [12,13]. The procedure used in this work represents a very fast and efficient strategy for coupling proteins onto the liposomal surface. The presence of protein on the liposomal surface was confirmed by protease assay [12] and corresponded with the data obtained fluorimetrically (data not shown). Most of the known methods require time-consuming coupling procedures and separation steps and are not suitable for pH-sensitive liposomes (inappropriate pH). When protein is coupled onto the preformed vesicles, the important parameter characterizing the method is the actual loss of originally entrapped material. In our case, it was always less than 20% (Table 1). To our knowledge, it is the first coupling method dealing with the coupling of protein to the surface of preformed pH-sensitive liposomes. The combination of the liposomal preparation method and coupling procedure enabled us to develop a liposomal delivery system carrying enough entrapped material and bearing a sufficient amount of protein to react with a corresponding cell-surface receptor.

Empty pH-sensitive liposomes (ligand-free) labeled with Rh-PE associated with the cells (Table 2) in the same manner as pH-sensitive liposomes entrapping FITC-dextran, namely the red fluorescence (Rh-PE) could be detected (Fig. 3C). We therefore concluded that the entrapped marker (FITC-dextran) was released, or lost, during the association. Incubation of empty pH-sensitive liposomes mixed with free FITC-dextran resulted in the same association (Table 2). Recently, the association of pH-sensitive and sterically stabilized pH-sensitive liposomes with monocytic human THP-1 cells was reported [14]. Diffuse cytoplasmic fluorescence was observed in the case of pH-sensitive liposomes, whereas only some punctuate fluorescence was visualized in the cells treated with sterically stabilized pH-sensitive liposomes.

In our study, pH-sensitive composition (DOPE-CHEMS) of the liposomes was a prerequisite for successful association (Fig. 1). Ligand-free pH-sensitive liposomes adsorbed to the cell surface but released the entrapped material outside the cells (Fig. 3D). When a specific ligand was present on the pH-sensitive liposomal surface, then, after specific ligand-receptor reaction, presumably stimulation of endocytosis or fusion of liposomal and plasma membrane occurred followed by cell internalization of liposomally entrapped material (Fig. 3B). We found that pH-sensitive liposomes adsorb to the cell surface to a greater extent than SPC liposomes. SPC/N-Ac-BSA incubated with the cells did not significantly associate with the cells (Table 2). The non-specific ligand (BSA) might cause steric hindrance between the receptor and ligand, preventing pH-sensitive liposomes to associate with the cells (Table 2). The pH sensitivity of liposomes bearing N-Ac-BSA was crucial for the delivery of entrapped material. The exact mechanism through which the pH-sensitive liposomes enter the cells is currently under examination. The fusion between two lipid membranes can occur in different ways, namely mixing of aqueous content *and* mixing of phospholipid components (complete fusion); exchange of bilayer components (without leakage of the aqueous content) *or* mixing of aqueous content (incomplete fusion) and exchange of bilayer components without mixing of the aqueous content but with leakage of the aqueous content (lysis). The first mechanism would be the most desirable for the delivery of antisense oligonucleotides. Furthermore, fusion of liposomes and cells can be influenced by the composition of pH-sensitive liposomes [4]. Based on our findings, we suggest the mechanism of delivery by ligand bearing pH-sensitive liposomes to LMH cells to be as follows: (i) attachment (adsorption) of pH-sensitive liposomes to the membrane, (ii) specific binding of the ligand to the cell surface receptor and stimulation of endocytosis or decrease in pH on the cell surface, (iii) fusion of the pH-sensitive liposomes with the endosomal membrane and/or plasma membrane, (iv) release of the pH-sensitive liposomal content into the cytoplasm.

For liver targeting (as in our case), fast uptake by the liver is desired and our findings indicate that the described delivery system has a potential in receptor-mediated targeting to LMH cells. Our data indicate that our carrier system is suitable for the delivery of entrapped material in vitro, therefore we are presently applying the system in in vivo delivery (liposomes containing FITC-dextran or antisense oligonucleotides).

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