Chapter 24

TAT-Peptide Modified Liposomes: Preparation, Characterization, and Cellular Interaction

Marjan M. Fretz and Gert Storm

Abstract

In general, cellular internalization of macromolecular drugs encapsulated in liposomes proceeds via endocytosis. This potentially leads to degradation of the liposome-encapsulated macromolecular content within the endosomal/lysosomal compartment. Therefore, bypassing the endocytic route by conferring a direct plasma membrane translocation property to the liposomes would be very beneficial. Cell penetrating peptides, e.g. TAT-peptide, are exploited in the drug delivery field for their capacity of plasma membrane translocation. Here, we describe the preparation of TAT-peptide modified liposomes and their cellular interaction using live cell flow cytometry and imaging techniques.

Key words: Endocytosis, Cell-penetrating peptides, TAT-peptide, Membrane translocation

1. Introduction

Cellular uptake of (targeted) liposomes is generally mediated by (receptor-mediated) endocytosis. Upon internalization, the liposomes and encapsulated drug will be routed from endosomes to lysosomes. Macromolecules, like proteins, peptides or nucleic acids, encapsulated in liposomes ending up in the endocytic pathway will be degraded, which causes inefficient intracellular delivery. In the recent years, attempts are being made to apply targeted liposomes for cytosolic delivery of macromolecules (1, 2). One approach reported to avoid endocytosis and to achieve direct cytosolic delivery via direct plasma membrane translocation is the use of so-called cell-penetrating peptides (CPP) of which the HIV-1 derived TAT-peptide is an example (3). These CPP have

V. Weissig (ed.), *Liposomes,* Methods in Molecular Biology, vol. 605,
DOI 10.1007/978-1-60327-360-2_24, © Humana Press, a part of Springer Science+Business Media, LLC 2010

been reported as cytosolic delivery vector for a variety of cargos, like fluorophores, proteins, oligonucleotides and particulate systems (3–9). Torchilin et al. were the first to report on cytosolic delivery of liposomes modified with a CPP on the surface of the liposomes (8). However, the plasma membrane translocation mediated via those CPP was questioned when it was shown that cell fixation could induce rigorous artefacts in the cellular distribution of fluorescently labelled CPP (10, 11). Several studies now pointed out the importance of using live cells to study the route of uptake of CPP and their cargoes (10–12).

TAT-peptide modified liposomes were prepared and cellular association and intracellular distribution of (double) fluorescently labelled particles were assessed by flow cytometry and confocal laser scanning microscopy.

2. Materials

2.1. Preparation and Characterization of Liposomes

1. Egg phosphatidylcholine (EPC), 1,2-distearoyl-glycero-3-phosphoethanolamine-*N*-(poly(ethylene glycol)2000) (PEG_{2000}–DSPE) (Lipoid GmbH, Ludwigshafen). Lipids can be stored as powder or as stock solution in ethanol at −20°C.
2. Maleimide–PEG_{2000}–DSPE (Shearwater Polymers, Huntsville, AL, USA, currently Nektar Pharmaceuticals). This lipid can be stored as powder or as stock solution in ethanol at −20°C.
3. Stock solutions containing fluorescent labels DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine 4-chlorobezenesulfonate salt) (Molecular Probes, Eugene, OR, USA) or Lissamine rhodamine B labelled glycerophosphoethanolamine (Avanti Polar Lipids, Alabaster, USA) are made in ethanol and kept at −20°C until use. These lipids are used to label the bilayer of the liposomes.
4. Hepes buffered saline (HBS): 135 mM NaCl and 10 mM Hepes. pH is set to 6.5, 7.0 or 7.4 as stated in the text.
5. FITC–dextran (Mw 70,000 Da, Sigma, St. Louis, MO, USA) is dissolved in HBS pH 7.0 to a concentration of 10 mg/mL. This solution is used for encapsulating a fluorescent marker in the aqueous interior of the liposomes.
6. To remove uncapsulated FITC–dextran or non-coupled TAT-peptide, a column of Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) is packed in 20% ethanol. Before use the column is equilibrated with HBS pH 6.5 or 7.4.

7. Hydroxylamine solution: 0.5 M Hepes, 0.5 M Hydroxylamine–HCl and 0.25 mM EDTA at pH 7.0. Always prepare a fresh solution before use.
8. Thiol-acetylated TAT-peptide (sequence YGRKKRRQRRRK-*S*-acetylthiolacetyl) (Ansynth BV, Roosendaal The Netherlands); referred to as TAT-sata is dissolved in HBS, pH 7.4 to a concentration of 5 mg/mL and 1 mg aliquots were kept at –20°C until use.

2.2. Cell Culture

1. Serum free culture medium: Dulbecco's Modified Eagle's Medium (DMEM) containing 3.7 g/L sodium bicarbonate, 4.5 g/L l-glucose supplemented with 2 mM l-glutamine, penicillin (100 IU/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) (Gibco, Grand Island, MY, USA).
2. Complete culture medium: The serum free medium is supplemented with 10% heat-inactivated foetal calf serum (Gibco, Grand Island, MY, USA).
3. Solution of trypsin (0.25% (w/v)) and ethylenediamine tetraacetic acid (EDTA; 0.02% (w/v)) in PBS (Gibco, Grand Island, MY, USA).

2.3. Flow Cytometry

1. Rhodamine–PE labelled liposomes; control (pegylated) and TAT-peptide modified liposomes.
2. Phosphate buffered saline (PBS): 140 mM NaCl, 8.7 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4 pH 7.4 (Braun, Melsungen, Germany).

2.4. Microscopy: Intracellular Distribution

1. Nunc Lab-Tek 16-well chamber slides from Fisher Scientific (Landsmeer, The Netherlands).
2. Phosphate buffered saline (PBS): 140 mM NaCl, 8.7 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4 (Braun, Melsungen, Germany).
3. Lysostracker Red (Invitrogen, Eugene, OR, USA) solution in PBS. Dilute the stock solution (1 mM) provided by Invitrogen to a final concentration of 75 nM in PBS. This solution can be stored at –20°C.
4. Double labelled TAT-peptide modified liposomes.

2.5. Microscopy: Metabolic and Endocytosis Inhibitors

1. Co-Star 6-well low adherence plates (Corning Life Science BV, Schiphol-Rijk, The Netherlands).
2. Cytochalasin D (Sigma Aldrich, St. Louis, MO, USA) is dissolved in DMSO to a concentration of 5 mg/mL and stored in the fridge until use.
3. For iodoacetamide (Sigma, St. Louis, MO, USA), a fresh stock solution with a concentration of 0.1 M is made in PBS.

4. TAT-peptide modified liposomes labelled with Rhodamine–PE.
5. Phosphate buffered saline (PBS): 140 mM NaCl, 8.7 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4 (Braun, Melsungen, Germany).

3. Methods

TAT-peptide modified liposomes are prepared by coupling the TAT-peptide to the distal end of PEG-chains on the liposomal surface. For this, maleimide-functionalized PEG-chains are incorporated in the lipid bilayer and the TAT-peptide has been functionalized with a thio-acetyl group at the C-terminus. Those thio-acetyl groups can be converted to sulfhydryl groups. This method has been described in the literature to couple targeting ligands like e.g. antibodies and peptides to liposomes (1, 13). Cellular association, which can include both binding and uptake, can be assessed by flow cytometry. Furthermore, to distinguish between binding and uptake and additionally to evaluate the intracellular localization of the liposomes, live cell confocal laser scanning microscopy can be applied. The use of double fluorescently labelled liposomes, with both the liposomal bilayer and the aqueous compartment labelled, can be used to study the integrity of the liposomes upon incubation with cells and additionally will give information about the possible cytosolic delivery of the encapsulated hydrophilic fluorescent label. Co-localization with markers of the endocytic pathway and the use of metabolic or endocytosis inhibitors will give information about the cellular internalization mechanism.

3.1. Preparation and Characterization of Liposomes

1. EPC, cholesterol, PEG2000–DSPE, and maleimide–PEG2000–DSPE are weighed and dissolved in absolute ethanol in a round bottom flask. The molar ratio of the lipids is 1.85: 1.00: 0.09: 0.06, respectively.
2. The bilayer of the liposomes is fluorescently labelled by adding either DiD or Rho–PE to a final ratio of 0.1 mol percentage.
3. Form a lipid film by evaporation of the ethanol using a rotavapor.
4. Before hydration, flush the lipid film with nitrogen for at least 30 min to obtain a complete dry lipid film.
5. Form liposomes by hydration of the film with either 1 mL of HBS pH 6.5 or 1 mL of HBS (pH 7.0) containing 10 mg/mL FITC dextran in case of rhodamine–PE labelled or DiD labelled liposomes, respectively.
6. Size the liposomes by extrusion to an average size of 150 nm and a polydispersity index below 0.2 (see Note 1). Size distribution should be checked with dynamic light scattering.

7. In case of FITC–dextran containing liposomes: remove the non-encapsulated FITC–dextran using a Sepharose CL-4B column using HBS pH 6.5 as eluent.
8. Split the batch of liposomes into two parts. One part will serve as control liposomes without any TAT-peptide coupled. For the control liposomes, continue with step 11. The second part is used to prepare TAT-peptide modified liposomes by following step 9–11.
9. For the TAT-peptide modified liposomes, deacetylate 1 mg of TAT-sata peptide by adding 20 μL of hydroxylamine solution to 200 μL of peptide solution and leaving it on a rollerbench for 1 h at room temperature to obtain free sulfhydryl groups.
10. Add the deacetylated peptide to the liposomes (1 mg peptide to 21 μmol total lipid) and leave the dispersion overnight at 4°C (see Note 2). The sulfhydryl groups will react with the maleimide-groups present at the distal ends of the PEG-chains resulting in a stable covalent linkage between the peptide and the PEG-chains (see Note 3).
11. Remove non-coupled peptide by size exclusion chromatography using Sepharose CL-4B column but with HBS pH 7.4 as eluent.
12. Characterize the liposomes with respect to size (e.g. dynamic light scattering) and lipid concentration (e.g. determination of phospholipid content using a method described by Rouser et al.) (14).

3.2. Cell Culture

1. The human ovarian carcinoma cell line NIH:OVCAR-3 originates from ATCC (Manassas, USA). OVCAR-3 cells are passaged when confluency is reached to provide new maintenance cultures in 75 cm^2 culture flasks. For maintenance, in general, 1:10 part of the last passage is transferred into a new flask. This procedure is done twice a week.
2. For passaging and seeding, the cells are first washed with PBS and detached from the culture flask by incubating with 3 mL trypsin/EDTA solution for approximately 10 min in the incubator. The trypsin/EDTA solution is inactivated by adding 13 mL of complete culture medium. For maintenance, 1:10 part is transferred into a new culture flask. For seeding or for use in a flow cytometry experiment, the cells are centrifuged (5 min, 300 × *g*), resuspended in the required medium, counted and diluted to the appropriate number of cells/ml.

3.3. Flow Cytometry

1. Prepare a cell suspension of 1×10^6 cells/mL in complete culture medium.
2. Add 100 μL cell suspension (1×10^5 cells) to a FACS tube.

3. For both TAT–liposome and control liposomes, the liposome stock is diluted in complete culture medium: concentrations of liposomes (total lipid) used are 1 mM, 500, 250, 125 and, 62.5 μM.
4. 100 μL of each liposome dispersion made in step 3 is added to the cells in the FACS tubes to obtain final liposome concentrations ranging from 31 to 500 μM total lipid.
5. Incubate the samples for 1 h at 4°C.
6. Centrifuge the samples for 5 min at 300 × *g*.
7. Remove the supernatant and add 400 μL ice-cold PBS.
8. Centrifuge (5 min, 300 × *g*) again and repeat step 7.
9. Resuspend the cells in 400 μL PBS and leave the samples on ice.
10. Analyze the cells by FACS, e.g. a FACScalibur (Becton&Dickinson) by counting at least 5000 viable cells and leave the samples on ice during analysis. For Rhodamine–PE labelled liposomes, the signal is detected in the FL-2 detector of the flow cytometer.
11. Data can be analyzed by flow cytometry programmes like Cell Quest or WinMDI and data is expressed as the mean fluorescence intensity (see Fig. 1).

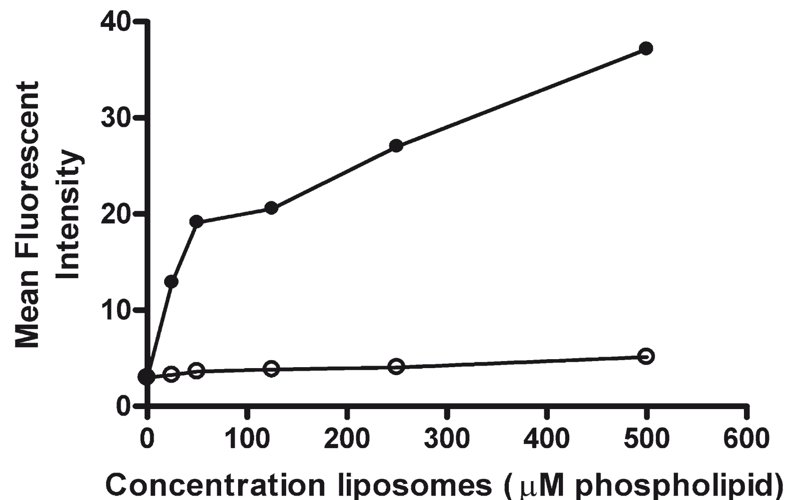

Fig. 1. Coupling of the TAT-peptide to the distal end of PEG-chains on liposomes increases the cellular association with OVCAR-3 cells. OVCAR-3 cells are incubated with various concentrations rhodamine–PE labelled control (*open circle*) or TAT–liposomes (*closed circle*) for 1 h at 4°C, washed and analyzed by flow cytometry. Each data point represents the mean fluorescence intensity of 5,000 cells (mean ± SD; $n = 3$). Error bars are within plot symbols when not visible. (Reproduced from (12) with permission from Elsevier Science)

3.4. Microscopy: Intracellular Distribution

1. Cells (10,000 cells/well) are seeded into a 16-well chamber slide and cultured overnight in complete culture medium.
2. Before applying the liposomes in serum-free medium, the cells are washed with 200 μL PBS.
3. 150 nmol of double labelled TAT–liposomes is added to the cells in serum free culture medium. The liposomes are labelled with the bilayer label DiD and FITC–dextran in the aqueous interior of the liposomes. Incubate for 1 h with the labelled TAT–liposomes.
4. Subsequently, incubate the cells for either 1 or 23 h in complete culture medium.
5. Thirty minutes before visualization, incubate with 100 μL Lysotracker Red solution (75 nM) at 37°C.
6. Wash the cells with PBS and mount them in PBS.
7. Cover the sample with a coverslip and seal using transparent nailpolish.
8. Analyze the live cells directly after step 7 with a confocal laser scanning microscope equipped with 488 nm, 568 nm and 633 nm lasers (see Fig. 2) (see Note 4).

3.5. Microscopy: Metabolic or Endocytsosis Inhibitors

1. Cells (300,000 cells/well) are added in a total volume of 5 mL serum free medium in 6-well Co-Star Low adherence plates (see Note 5).
2. In case of low temperature incubation, pre-incubate the cells for 30 min at 4°C prior to the liposome incubation (described in step 4).
3. In case of iodoacetamide or cytochalasin D, incubate the cells with either 1 mM iodoacetamide or 25 μg/mL cytochalasin D for 30 min before continuing with step 4.
4. RhodaminePE labelled liposomes are added (450 nmol total lipid).
5. Cells are incubated for 5 h at either 4°C or 37°C.
6. To remove the non-associated liposomes, the cells are washed twice by centrifugation (5 min, $300 \times g$) with PBS.
7. After the last centrifugation step, the cells are resuspended in approximately 100 μL PBS and an aliquot of the cell suspension is mounted on a glass slide and covered with a coverslip.
8. Seal with transparent nailpolish.
9. Directly visualize the samples using a confocal microscope equipped with a 568 nm laser, suitable for monitoring Rhodamine–PE labelled liposomes (see Fig. 3).

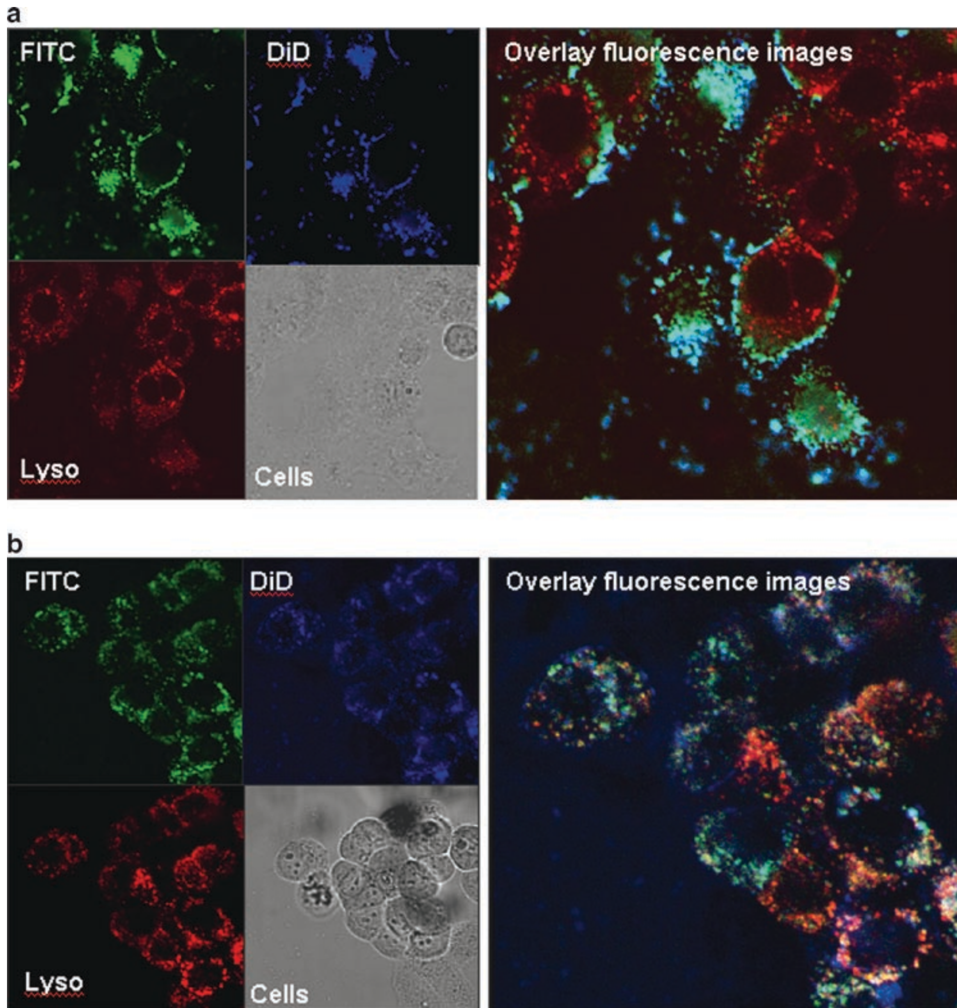

Fig. 2. Intracellular localization of TAT–liposomes. OVCAR-3 cells are incubated with 150 nmol of double fluorescently labelled TAT–liposomes for 1 h and subsequently incubated for 1 h (**a**) or 23 h (**b**) in liposomes-free medium. Thirty minutes before visualization the endocytic pathway is labelled with Lysotracker Red. Live cell imaging is performed with confocal laser scanning microscopy. Double labelled liposomes are used to study the integrity of the liposome during the uptake process: co-localization of both liposomal labels would indicate that the liposomes are intact. 1 h both liposomal labels are localized at the plasma membrane, which represent intact cell-bound TAT–liposomes. The electronically merged image clearly shows lack of co-localization with the endocytic pathway marker, Lysotracker Red. This opposite to the 24 h incubation, both liposomal labels can be seen intracellularly in a punctuate pattern. In the electronically merged image, co-localization with Lysotracker Red is clearly visible. This indicates that the TAT-peptide modified liposomes bind to the plasma membrane and after internalization are present in endocytic vesicles. Therefore, we conclude that the liposomes are internalized by endocytosis. (Reproduced from (12) with permission from Elsevier Science)

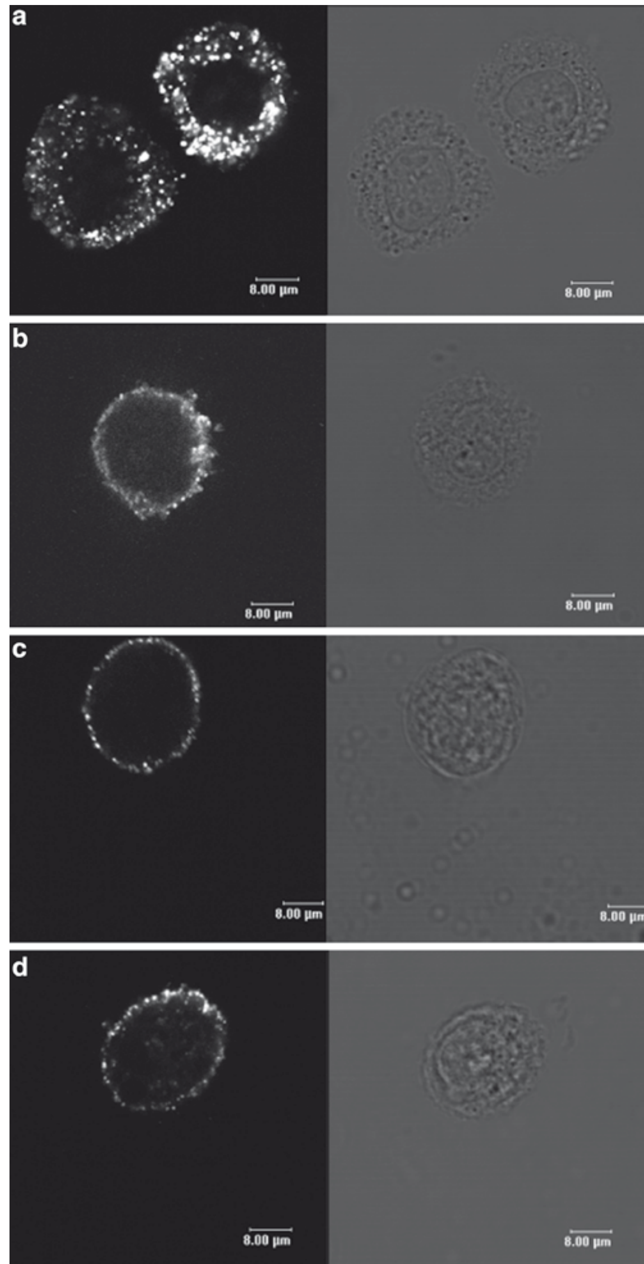

Fig. 3. Low temperature and the presence metabolic or endocytosis inhibitors prevent cellular uptake of TAT–liposomes. OVCAR-3 cells are incubated with Rhodamine–PE labelled TAT–liposomes for 5 h at 37° (**a**, **c**, **d**) or at 4°C (**b**). *Left panels* are confocal images, right panels are phase contrast images. Incubation at 37°C without any inhibitor results in intracellular vesicular localization of the TAT–liposomes (**a**). Only plasma membrane binding is observed in case of incubation at 4°C (**b**) and incubation with the metabolic inhibitor iodoactemide (**c**) or endocytosis inhibitor cytochalasin D (**d**). These results indicate that the cellular uptake of TAT–liposomes occurs via endocytosis. (Reproduced from (12) with permission from Elsevier Science)

4. Notes

1. Polydispersity index is an indication of the size distribution of the liposomes. The polydispersity can range from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse suspension.
2. Incubation time, temperature, and lipid concentration can be varied depending on the experimental set-up.
3. Maleimide groups are rather unstable in aqueous solution and, therefore, the peptide should be coupled to the liposomes on the day of lipid hydration.
4. When a triple labelling is used, the use of sequential scanning is preferred. In the normal modus, all the signal are simultaneously acquired in different channels, which in the case of multiple labelling can result in crosstalk, which can ultimately lead to misleading co-localization results.
5. The treatment of the OVCAR-3 cells with cytochalasin D or iodoacetamide resulted in detachment of the cells from normal microscope slides. Therefore, the incubation should be performed in the Low-adherence plates as described.

References

1. Mastrobattista E, Koning GA, Van Bloois L, Filipe AC, Jiskoot W, Storm G (2002) Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. J Biol Chem 277:27135–27143
2. Simoes S, Moreira JN, Fonseca C, Duzgunes N, de Lima MC (2004) On the formulation of pH-sensitive liposomes with long circulation times. Adv Drug Deliv Rev 56:947–965
3. Vives E, Brodin P, Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272:16010–16017
4. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B et al (1994) Tat-mediated delivery of heterologous proteins into cells. Proc Natl Acad Sci U S A 91:664–668
5. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285:1569–1572
6. Astriab-Fisher A, Sergueev D, Fisher M, Shaw BR, Juliano RL (2002) Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. Pharm Res 19:744–754
7. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT et al (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat Biotechnol 18:410–414
8. Torchilin VP, Rammohan R, Weissig V, Levchenko TS (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc Natl Acad Sci U S A 98:8786–8791
9. Tseng YL, Liu JJ, Hong RL (2002) Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and tat: a kinetic and efficacy study. Mol Pharmacol 62:864–872

10. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ et al (2003) Cell-penetrating peptides: a re-evaluation of the mechanism of cellular uptake. J Biol Chem 278(1):585–590
11. Lundberg M, Johansson M (2002) Positively charged DNA-binding proteins cause apparent cell membrane translocation. Biochem Biophys Res Commun 291:367–371
12. Fretz MM, Koning GA, Mastrobattista E, Jiskoot W, Storm G (2004) OVCAR-3 cells internalize TAT-peptide modified liposomes by endocytosis. Biochim Biophys Acta 1665:48–56
13. Koning GA, Morselt HW, Gorter A, Allen TM, Zalipsky S, Scherphof GL et al (2003) Interaction of differently designed immunoliposomes with colon cancer cells and Kupffer cells: an in vitro comparison. Pharm Res 20:1249–1257
14. Rouser G, Fkeischer S, Yamamoto A (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494–496