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Research paper

Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy ☆

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Abstract

Site-specific delivery of drugs and therapeutics can significantly reduce drug toxicity and increase the therapeutic effect. Transferrin (Tf) is one suitable ligand to be conjugated to drug delivery systems to achieve site-specific targeting, due to its specific binding to transferrin receptors (TfR), expressed on several cell types of therapeutic interest. TfRs have been reported to be highly expressed on the surfaces of tumour cells and the well-characterised and efficient mechanism of internalisation of Tf has been exploited for the delivery of anticancer drugs, proteins, and therapeutic genes into primarily proliferating malignant cells. Liposomes are effective vehicles for drugs, genes and vaccines and can be easily modified with proteins, antibodies, and other appropriate ligands, resulting in attractive formulations for targeted drug delivery. In this study, we used atomic force microscopy (AFM) and transmission electron microscopy (TEM) to confirm the conjugation of Tf to liposomes by three different coupling methods. In addition, the conventional assays for quantification of protein amount (BCA) and phospholipid content (according to Steward) were performed. AFM and TEM were able to display Tf-molecules on the liposomal surfaces and can be routinely used to obtain additional visual information on the protein–drug carrier conjugation in a fast and reliable manner.
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Keywords: Drug targeting; Transferrin; Atomic force microscopy; Transmission electron microscopy; Anticancer therapy; Liposomes

1. Introduction

The high specificity of endocytic uptake of transferrin (Tf) by the transferrin receptor (TfR) has made it a subject of interest for targeted drug delivery. TfR-targeted drug conjugates can be delivered across epithelial barriers or accumulated in proliferating tissues [1]. Serum transferrin is a globular glycoprotein (80 kDa) which has often been used as a model protein because of its high aqueous solubility, low affinity for lipids and the ability to bind to specific receptors on cell membranes [2]. The TfR is expressed in many tissue types in the body, particularly, in areas that feature a high turnover rate of cells. Elevated expression levels of TfR in neoplastic carcinomas [3] show a good correlation between the number of TfR expressed and the proliferative ability of the tumour [4]. The knowledge of TfR-over-expression in tumour tissues had led to a focused targeting of TfR in anticancer therapy [5], and subsequently to a number of small drugs (e.g. adriamycin) and colloidal carrier systems (e.g. liposomes and nanoparticles) linked to Tf [6,7].

Liposomes have been used as vehicles for drug delivery because of their biocompatibility and the possibility to incorporate both water-soluble and hydrophobic materials [8]. They received much attention as carrier systems for advanced drug delivery in the context of anticancer therapy [9]. Several methods have been developed to link water-soluble proteins to the surface of liposomes in an attempt to prepare stable liposomes with high binding efficiency to cellular membranes. Such proteoliposomes may serve as efficient carriers for the transfer of drugs, enzymes, and

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nucleic acids into cells. In general, formation of ligand–liposome-complexes should be simple, fast, efficient, reproducible and yield stable, non-toxic bonds. The biological properties of the ligands (i.e. target recognition and binding efficiency) should not be substantially altered and the drug loading efficiency and drug release rates of the vesicle should not be negatively affected. Lastly, the ligand-targeted liposomes should be stabile and their circulation half-lives should be long enough to allow them to reach and interact with the target cells. A particularly useful protein–liposome coupling procedure involves the covalent modification of functional groups of proteins using polyethylene glycol (PEG)-linked lipid residues [10]. Although many techniques of conjugating proteins to liposomes have been published, there is no rapid and reliable way to display the actual conjugation. In the majority of cases, a protein assay is performed to estimate the amount of bound ligand. Such a procedure, however, bears the risk of over-assessment due to unspecific binding.

Here, three different methods of linking Tf to DSPC/cholesterol liposomes were used in a comparative manner [10–15]. The goal of this study has been to visualise the actual protein binding, using two independent microscopic methods, i.e. atomic force microscopy (AFM) and transmission electron microscopy (TEM). AFM is a surface analytical method that can generate nano-scale topographic images by scanning a fine silicon tip across a surface. For its ability of high-resolution imaging under physiologic, non-destructive conditions without the necessity of previous fixation of the sample, AFM has developed into a powerful tool for studying structural details. TEM is widely used to image structures near to the atomic level. While the maximum resolution is lower in TEM than in AFM, the contrast and image formation is much better understood in TEM than in AFM. Thus, both imaging methods may provide additional/overlapping information and support each other.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-glutaryl (*N*-glutaryl-PE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol) 2000] (DSPE-PEG_{2000}-COOH) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), human holo-transferrin, 2-iminothiolane (Traut's reagent), Sepharose CL-4B, goat anti-human transferrin antibody and the bicinchoninic acid (BCA) kit for protein determination were from Sigma (Seelze, Germany). Cholesterol (chol) was obtained from Riedel-de Haën (Seelze, Germany). Sulpho-*N*-hydroxysuccinimide (S-NHS) was obtained from Perbio Science (Bonn, Germany). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine-(polyethylene glycol) 2000-maleimide (DSPE-PEG_{2000}-MAL) was from Nektar (Huntsville, AL). Centrisart-10 and -20 (molecular weight cut off: 10 and 20 kDa, respectively) concentrators were from Vivascience (Hannover, Germany).

2.2. Liposome preparation

The liposomes were prepared from cholesterol, DSPC and the according linker lipid at the following molecular ratios: DSPC:chol:linker (6:3:0.6 mol%). Briefly, a mixture of phospholipid (PL) and cholesterol (chol) in chloroform:methanol (2:1) was dried to a thin lipid film in a rotary evaporator (Büchi, Essen, Germany). The solution to be encapsulated was then added to result in a final lipid concentration of 10 mg lipid/ml corresponding buffer. After vortexing, the sample was incubated for 10 min at a temperature above the transition temperature of the used lipids (53°C for DSPC:chol) in a cabinet drier [16]. Unilamellar liposomes were prepared by extruding the resulting multilamellar vesicles 11 times through a 200 nm polycarbonate membrane, followed by 11 times extrusion through a 100 nm membrane using a Liposofast Basic device (Avestin, Mannheim, Germany).

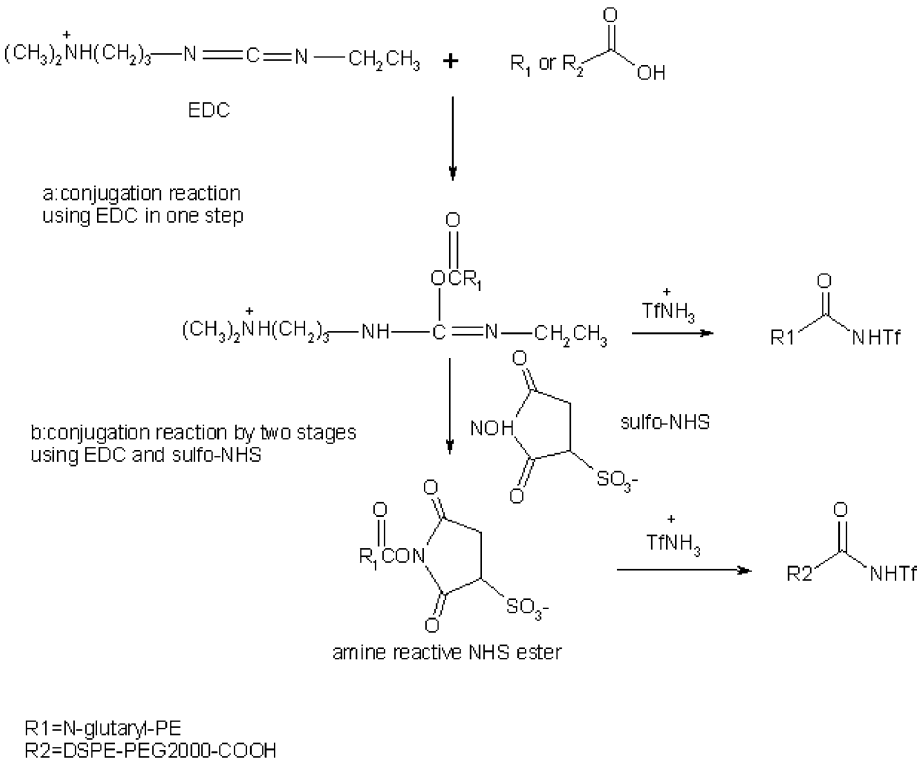
2.3. Coupling of transferrin to the liposomes

In the first two methods, an amide bound between *N*-glutaryl-PE or DSPE-PEG_{2000}-COOH as linker lipid is formed to link Tf to the liposome surface. In both methods, a bond between free amino groups of the protein and carboxylic groups of the linker is formed in the presence of a water-soluble carbodiimide (Scheme 1). The difference, however, is method 2 uses sulpho-*N*-hydroxysuccinimide (S-NHS) in addition to *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC). S-NHS is a water-soluble analogue to NHS which is used to modify carboxyl groups to amine-reactive NHS-esters. This is accomplished by mixing the NHS with a carboxyl containing molecule and a dehydrating agent, EDC, which reacts with the carboxyl group first and forms an amine-reactive intermediate, an *O*-acylisourea.

Method 3 relies on the formation of thioether-bonds between the protein and the liposome. Here, the Tf molecules are thiolated using 2-iminothiolane (Traut's reagent) and react with maleimide groups on the PEG-terminus (Scheme 2).

2.3.1. Method 1: N-glutaryl-PE

Two milligrams EDC were added per 1 μmol of lipid in phosphate buffered saline (PBS; 129 mM NaCl, 2.5 mM KCl, 7.4 mM $Na_2HPO_4 \times 7\ H_2O$, 1.3 mM KH_2PO_4; pH 7.4) and incubated for 6 h at room temperature. Excess EDC was then removed by ultrafiltration using a Centrisart-10

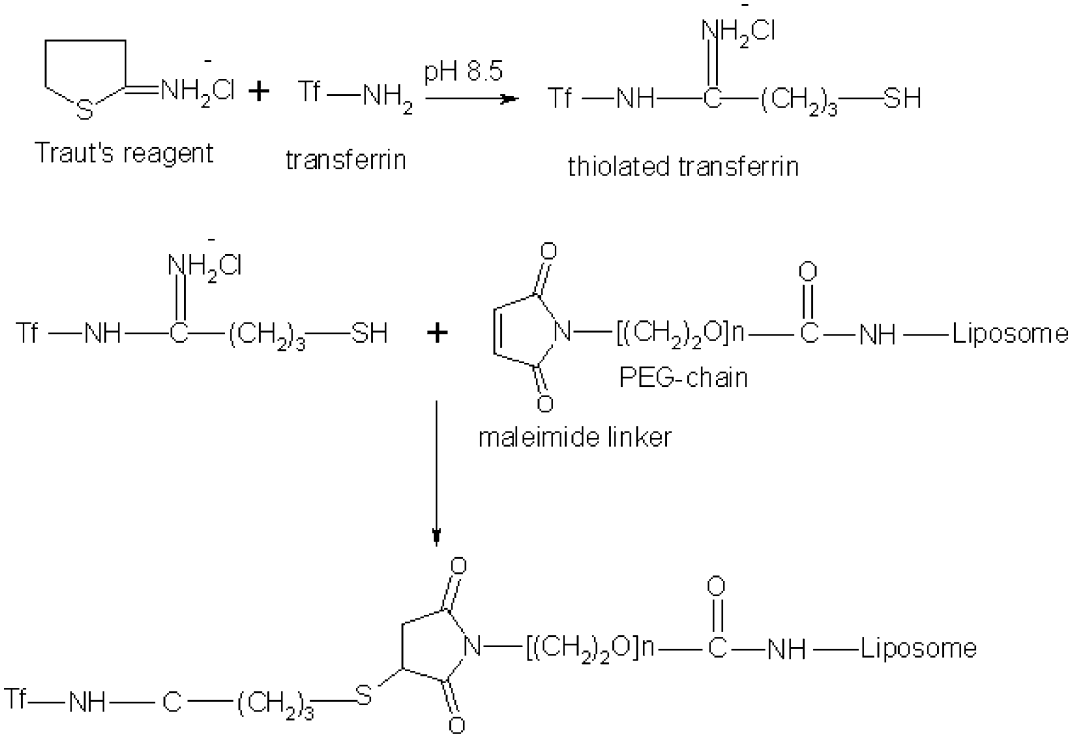


Scheme 1. The expected coupling reaction of transferrin to the surface of liposomes using *N*-glutaryl-PE and DSPE-PEG_{2000}-COOH as linker lipids.

concentrator. In the next step, 125 μg Tf/μmol PL was added and incubated overnight at room temperature. Free Tf was separated from the liposomes by passing the liposome suspension through a Sepharose CL-4B gel [11,12].

2.3.2. Method 2: DSPE-PEG_{2000}-COOH

The lipids were re-suspended in an appropriate amount of 400 mM citrate/5 mM phosphate buffer (pH 4.0). Then, 1 ml PBS (pH 7.5) and 360 μl of both EDC (0.25 M in H_2O)



Scheme 2. The expected coupling reaction of transferrin to the surface of liposomes using DSPE-PEG_{2000}-MAL as linker lipid.

and S-NHS (0.25 M in H_2O) were added per 10 μmol of lipid. The mixture was allowed to incubate for 10 min at room temperature, before adjusting to pH 7.5 with 1 M NaOH. PL of about 125 μg Tf/μmol was added and gently stirred for 8 h at 4 °C. Unbound protein was removed by passing the liposome suspension through a Sepharose CL-4B gel column [13].

2.3.3. Method 3: DSPE-PEG_{2000}-MAL

The lipid mixture was hydrated with PBS (pH 7.4). Then, 125 μg Tf/μmol PL (in PBS) was added to 2 ml borate–EDTA buffer (0.15 M Na borate, 0.1 mM EDTA, pH 8.5) containing 400 nmol of fresh Traut's reagent. This mixture was incubated in the dark for 60 min on a rotational shaker (at 110 rpm). The thiolated Tf was concentrated by ultrafiltration (Centrisart-20) to a volume of 0.2 ml, diluted with 2 ml PBS (pH 8.0) and concentrated again to 0.2 ml. The Tf was immediately added to the liposomes and left to react overnight at room temperature without further agitation. Lastly, the transferrin-conjugated liposomes were separated from free Tf by Sepharose CL-4B gel filtration [14,15].

2.4. Photon correlation spectroscopy

Liposomal size determination of Tf-conjugated and plain liposomes was carried out using a Zetasizer 3000 HS (Malvern Instruments, Herrenberg, Germany) equipped with a photon correlation spectroscopy unit. The scattered light was detected at a scattering angle of 90°. Measurements were performed at 25 °C. For all measurements, samples were diluted 50-fold in distilled water to obtain comparable viscosities. PCS gives information about the mean diameter of the bulk population and the width of distribution via the polydispersity index (PI). Mean values and standard deviation were calculated from at least three determinations.

2.5. ζ-Potential measurements

The ζ-potential measurements of the Tf-conjugated and plain liposomes were carried out in the standard capillary electrophoresis cell of a Zetasizer 3000 HS at pH 7.4 in the presence of NaCl to adjust the conductivity to 50 μS/cm. Measurements were performed at 25 °C with automatic duration. The instrument was routinely calibrated with a −50 mV latex standard (Malvern). The electrostatic mobility was converted into the ζ-potential using the Helmholtz–Smoluchowski equation (not shown). The mean values and standard deviation were calculated from three independent measurements (three runs each).

2.6. Phospholipid concentration

Phospholipid (PL) concentration was assayed according to Stewart's protocol [17]. Briefly, the standard curve was obtained by adding 2 ml of ferrothiocyanate reagent to different concentrations of DSPC, ranging from 0 to 0.5 mg/ml. The test tube's content was vortexed vigorously for 15 s, centrifuged at 1000 rpm for 5 min and the lower layers were removed with a Pasteur pipette. Absorbance of the formed complex was assessed using a plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at a wavelength of 485 nm. Liposome samples were treated similarly after dehydrating the aliquots of liposome suspension (100 μl) under a nitrogen stream and re-dissolving them in 2 ml of chloroform.

2.7. Protein assay and Tf-binding efficacy

The average amount of transferrin conjugated to the liposome (i.e. amount of PL) was quantified as described by Derycke et al. [15]. One hundred microlitres of liposome suspension were added to 400 μl of methanol. The mixture was vortexed and centrifuged (10 s at 9000 × *g*). Then, 200 μl of chloroform were added and the sample was vortexed and centrifuged again (10 s at 9000 × *g*). For phase separation, 300 μl of water were added and the sample was vortexed again and centrifuged for 1 min at 9000 × *g*. The upper phase was carefully removed and discarded. Three hundred microlitres of methanol were added to the chloroform phase and the interphase with the precipitated protein. The sample was mixed and centrifuged to pellet the protein (2 min at 9000 × *g*). The supernatant was removed and the protein pellet was dried under a stream of air. The pellet was then dissolved in 20 μl of PBS (pH 7.4) and the concentration was determined with a bicinchonic acid (BCA) protein assay using pure holo-transferrin as standard. The coupling efficiency was calculated as μg Tf/μmol PL.

2.8. Transmission electron microscopy

The coupling of transferrin to liposomes was assessed by transmission electron microscopy using two approaches; negative staining and immunoelectron microscopy.

For negative staining, a formvar coated copper grid (300 mesh, hexagonal fields) was placed on 15–30 μl droplets of liposome suspensions for 2–3 min at room temperature. To improve adhesion of the liposomes on the formvar film, grids were pre-treated with glow discharge in the argon plasma of a sputter coater for 2 min. After adhesion of liposomes, grids were washed on four droplets of distilled water before they were placed on a drop of uranyl acetate (2%) for 2 min. Finally, grids were dried at room temperature after removing the excess liquid.

Immunoelectron microscopy was done with liposome suspensions after filtration through a centrifugal filter (molecular weight cut off 100 kDa; Microcon, Millipore, Schwalbach, Germany) in order to reduce free unbound transferrin. Droplets (10–15 μl) of liposomal suspension were put on parafilm and brought in contact with a copper grid for 2–3 min (see above). Grids were then washed twice

Table 1
Size and polydispersity index (PI) of liposomes before and after coupling of transferrin (values represent the mean ± SD of three batches)

Liposome composition	Before addition of transferrin		After addition of transferrin	
	Size (nm)	PI	Size (nm)	PI
DSPC:chol:*N*-glutaryl-PE	121.40 ± 5.57	0.250 ± 0.03	129.00 ± 1.66	0.230 ± 0.01
DSPC:chol:DSPE-PEG_{2000}-COOH	148.43 ± 2.97	0.197 ± 0.02	154.80 ± 0.85	0.157 ± 0.01
DSPC:chol:DSPE-PEG_{2000}-MAL	166.00 ± 10.41	0.133 ± 0.02	169.33 ± 8.76	0.147 ± 0.01

with PBS and pre-treated for 2 min with PBS/glycine (50 mM) followed by TBS/BSA-C (0.1% acetylated bovine serum albumin in Tris–buffered saline; Aurion, Wageningen, The Netherlands) for 2 min to block unspecific binding. The anti-transferrin antibody was diluted at 1:20 in TBS/BSA-C and applied for 15 min at room temperature, followed by washing twice with TBS/BSA-C. Liposomes were finally negatively stained with uranyl acetate (see above).

Transmission electron microscopy was performed with a Tecnai 12 Biotwin (FEI Co., Eindhoven, The Netherlands) using 120 kV acceleration voltage. Images were recorded with a CCD camera (Megaview III; Soft Imaging Systems, Münster, Germany) at a resolution of at least 1376 × 1032 pixels.

2.9. Atomic force microscopy

The liposomal formulations with and without Tf modification were prepared as described above and diluted in ultrapure water (MilliQ, 18.4 MΩ, pH 5.5). Not later than 1 h after preparation, the liposomes were directly transferred onto a silicon chip by dipping it into the liposome suspension. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA). The microscope was vibration-damped. Commercial pyramidal Si_3N_4 tips (NCH-W, Veeco Instruments) on a cantilever with a length of 125 μm, a resonance frequency of about 220 kHz and a nominal force constant of 36 N/m were used. All measurements were performed in tapping mode to avoid damage of the sample surface. The scan speed was proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded as described previously [18]. The results were visualised either in height or in amplitude mode.

2.10. Statistical analysis

Results are expressed as mean ± SD Significance ($P < 0.05$) of differences in the size, polydispersity (PI) and zetapotential values from several ($n \geq 3$) data groups were determined by one-way analysis of variances (ANOVA), followed by Neumann–Keuls–Student post hoc tests.

3. Results

3.1. Liposome size and ζ-potential

The physical properties of the liposomes before and after coupling of Tf are given in Tables 1 and 2. The size of the liposomes before conjugation of Tf was in the range of 120 nm for method 1 (*N*-glutaryl-PE) and 150 nm for method 2 (DSPE-PEG_{2000}-COOH), while liposomes prepared according to method 3 (DSPE-PEG_{2000}-MAL) were of a size around 165 nm. After the addition of Tf, the average sizes increased between 5 and 10 nm for all samples under investigation, with the lowest increase (i.e. ∼3 nm) for method 3 and increases of ∼7 nm for methods 1 and 2, respectively. The PI did not show any significant alteration, indicating that the stability of the liposomes has not been negatively affected by any of the methods.

Values for the ζ-potential were between −20 and −40 mV for the Tf-free liposomes. In case of *N*-glutaryl-PE (method 1) and DSPE-PEG_{2000}-COOH (method 2), the conjugation of negatively-charged Tf (IP 5.9) resulted in a significant decrease of the ζ-potential, while no significant change could be observed for liposomes prepared according to method 3 (DSPE-PEG_{2000}-MAL).

3.2. Tf-binding efficacy

The amounts of Tf (assessed by BCS assay) in correlation to the amount of total phospholipid (assessed according to Ref. [17]) in the different formulations are given in Table 3. While the amount of PL was comparable between all three methods, there were significant differences

Table 2
ζ-Potential of liposomes before and after coupling of transferrin at pH 7.4 (values represent the mean ± SD of three batches)

Liposome composition	Before coupling (mV)	After coupling (mV)
DSPC:chol:*N*-glutaryl-PE	−31.67 ± 1.53	−42.33 ± 2.08
DSPC:chol:DSPE-PEG_{2000}-COOH	−20.67 ± 1.15	−32.33 ± 2.08
DSPC:chol:DSPE-PEG_{2000}-MAL	−21.67 ± 1.15	−21.33 ± 1.53

Table 3
Transferrin amount, phospholipid amount and coupling efficiency using three different linker lipids (values represent the mean ± SD of three batches)

Liposome composition	Tf amount (μg/ml)	PL amount (mg/ml)	Coupling efficiency (μg Tf/μmol PL)
DSPC:chol:*N*-glutaryl-PE	840.57 ± 43.43	8.26 ± 0.20	84.93
DSPC:chol:DSPE-PEG_{2000}-COOH	1075.69 ± 35.59	7.44 ± 0.22	108.76
DSPC:chol:DSPE-PEG_{2000}-MAL	124.82 ± 12.45	8.35 ± 0.10	11.87

in the protein content of the samples. Method 2 resulted in 108.76 μg Tf/μmol PL, method 1 in 84.93 μg Tf/μmol PL, and method 3 exhibited the lowest protein content with 11.87 μg Tf/μmol PL.

3.3. Transmission electron microscopy

The different preparations of liposomes were visualised by transmission electron microscopy after negative staining with uranyl acetate. Transferrin-conjugated liposomes revealed a particulate surface coating, which was absent in the corresponding unconjugated liposome preparations (Fig. 1A and B). Surface particles on transferrin-conjugated liposomes showed a maximal length of about 10 ± 3.6 nm ($n = 50$). The space between the transferrin-conjugated liposomes was also filled with particulate material, while in preparations of unconjugated liposomes, the background between the liposomes appeared to be unstructured. The degree of the particulate decoration varied among the three coupling methods. Liposomes produced according to method 2 showed a higher density of particles on their surfaces than liposomes produced according to methods 1 and 3 (data not shown).

In order to investigate whether the particulate decoration found on the conjugated liposomes was due to a binding of transferrin, specific antibodies raised against human transferrin were added before the negative staining procedure. Liposome suspensions were filtered with a centrifugal filter to remove unbound protein or reagents prior to the measurement. As a result, the transferrin-conjugated liposomes were clearly decorated by fluffy dark structures. In some cases, these structures were arranged on the liposome surface like spokes on a hub (Fig. 1C). Unconjugated liposomes only occasionally showed a slight decoration with similar structures at their surface (Fig. 1D). The different decoration of conjugated and unconjugated liposomes is indicative for antibody complexes in conjugated liposomes. Taken together, this data suggests that transferrin molecules were exposed on the surface of transferrin-conjugated liposomes.

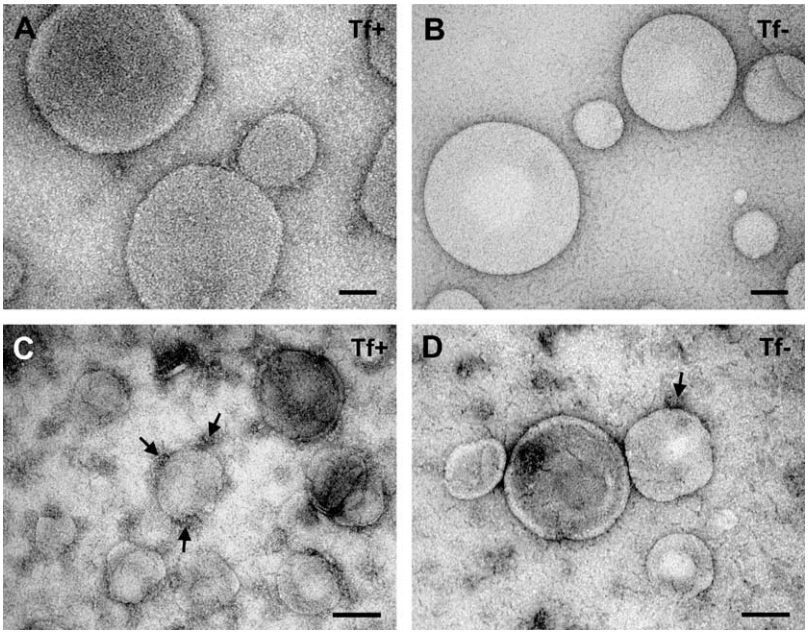


Fig. 1. TEM of liposomes prepared with DSPE-PEG_{2000}-COOH (method 2). (A, B) Liposomes after negative staining with uranyl acetate. Liposomes that have been conjugated with transferrin (A, Tf+) display small particles on their surface, while control preparations (B, Tf-) appear to be smooth and undecorated. Bar = 50 nm. (C, D) Liposomes immunolabelled with anti-transferrin antibody. The conjugated liposomes (C, Tf+) reveal a fluffy darkly stained decoration (arrows). In the control (D, Tf-) only a few liposomes are slightly decorated. Note the background between the liposomes is decorated equally in both preparations.

3.4. Atomic force microscopy

Atomic force microscopy was used to visualise all liposomal formulations under wet conditions and to confirm the particle size and morphology measurements performed by PCS and TEM. For size determination, all visible particles within a representative scan area were individually evaluated. The obtained data can generally deviate from the results of PCS measurements, because of an interaction of the soft and flexible liposomes with the surface of the silicon wafer. However, the individual particle evaluation allowed excluding such artefacts from further analysis.

The plain liposome formulations using *N*-glutaryl-PE, DSPE-PEG_{2000}-COOH, and DSPE-PEG_{2000}-MAL showed average vesicle sizes of 165 ± 16 nm ($n = 45$), 157 ± 12 nm ($n = 57$), and 172 ± 9 nm ($n = 29$), respectively. PCS produced respective values of 121, 148 and 166 nm (Table 1). The covalent coupling of Tf to the liposome surface led to an increase in diameter between 5 and 15% (192 ± 22 nm for *N*-glutaryl-PE, 168 ± 17 nm for DSPE-PEG_{2000}-COOH, and

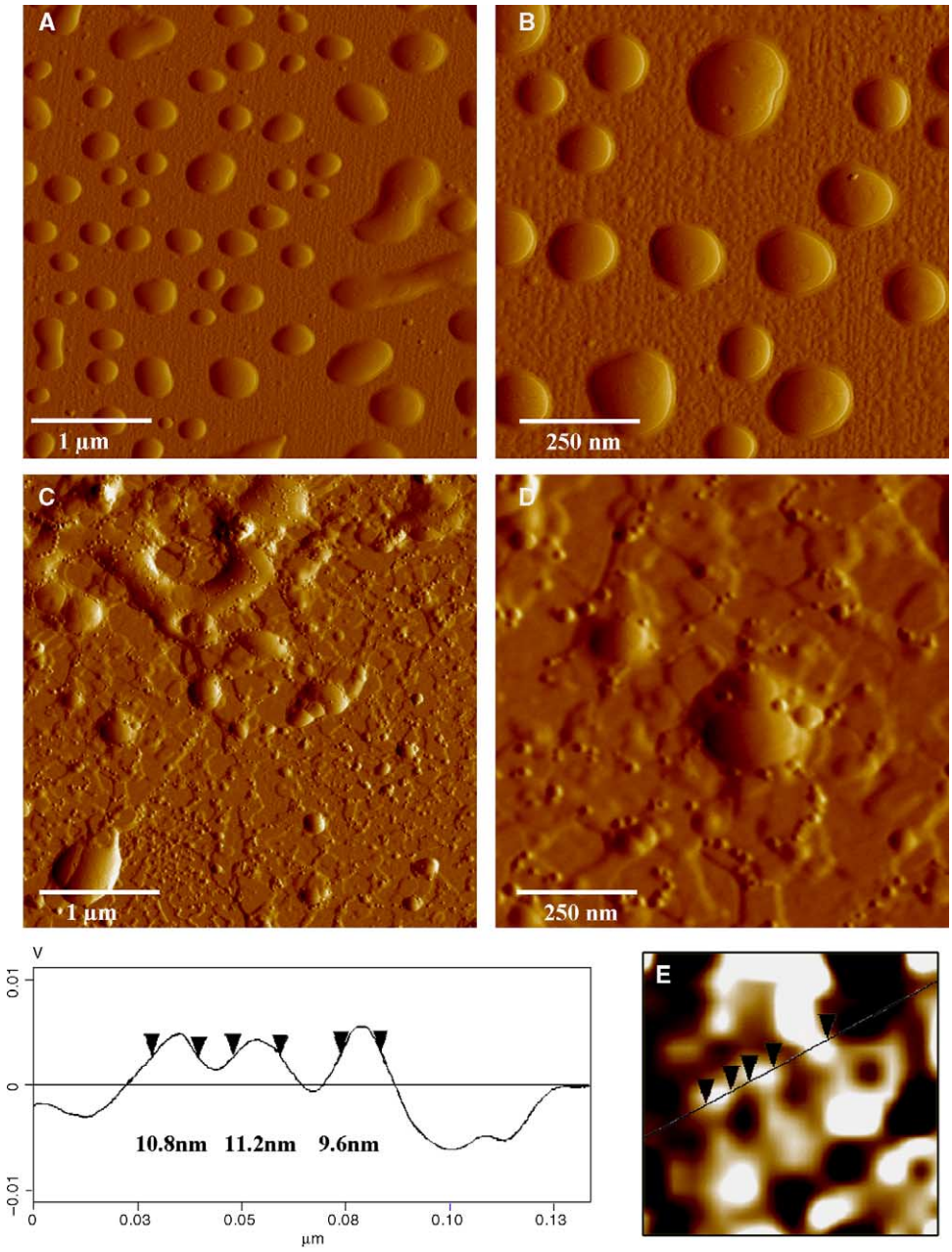


Fig. 2. AFM images of liposomes prepared with DSPE-PEG_{2000}-COOH. (A, B) Plain liposomal formulations. The liposomes show a smooth surface morphology. (C, D) Liposomes covalently modified with Tf. Small globular structures are visible at the surface. (E) Line scan of liposome surface. The globular structures were measured to be 10.53 ± 1.45 nm in diameter.

180 ± 12 nm for DSPE-PEG$_{2000}$-MAL). In the case of both plain and modified liposomes produced according to method 1, the vesicle morphology was relatively unstable on the silicon support, resulting in a liposome spreading into flat rafts with sizes between 300 and 900 nm (data not shown). The incorporation of PEGylated lipids into the liposomes induced a sterical stabilisation in the other two formulations with liposomes found to be round and of spherical shape (Fig. 2).

The surface of plain liposomes was always smooth and no structures could be observed (Fig. 2A and B). When Tf was linked to the liposomes, small globular structures, localised on the liposomal surface became visible, exhibiting the highest rate of appearance in liposomes produced according method 2 (Fig. 2C and D). These particles had a size of 10.53 ± 1.45 nm ($n = 27$), comparable to data assessed by TEM. From the molecular weight, the size of a single Tf molecule can be calculated to be 4–5 nm [19]. The determined size was slightly larger, indicating the formation of associates comprising of two or three Tf molecules.

4. Discussion

It has been the goal of this study to visualise the binding of a protein, transferrin, to a colloidal drug carrier system using two independent microscopic methods, atomic force microscopy (AFM) and transmission electron microscopy (TEM). To achieve this, ligand-modified DSPC/cholesterol liposomes were prepared according to three previously published methods and investigated with regard to their physico-chemical properties and efficacy of the Tf-modification.

AFM and TEM were able to detect Tf at the liposomal surface on the molecular level in a fast and reproducible manner. Both microscopic techniques can deliver semi-quantitative visual information on the actual functionalisation of nano-scale drug carriers with protein- or antibody molecules. While the use of AFM does not require any fixation or preparation of the sample prior the measurement, the advantage of TEM is the possibility to reach a higher level of specificity when using antibodies raised against the protein under investigation.

Intriguingly, the three chosen conjugation reactions resulted in significant differences with regard to their coupling efficacy. This became apparent using both the conventional characterisation methods such as BSA assay and assessment of ζ-potential and also by employing the newly introduced microscopic methods. Method 2, using DSPE-PEG$_{2000}$-COOH as linker lipid, exhibited the highest amount of bound Tf, while method 3, using DSPE-PEG$_{2000}$-MAL, still showed significant amounts of bound Tf on the liposomal surfaces, but in comparison on a three-times lower scale. A possible explanation of this phenomenon is that in method 3, free thiol groups may react amongst themselves to produce disulphide bonds leading to cross linking of protein molecules before the actual conjugation to the linker. In addition, the random introduction of thiol groups in the Tf molecule may interfere with the biological properties of the molecule resulting in a lower affinity to its receptor [20]. COOH-groups at the terminal ending of PEG-chains are sterically less accessible, but the higher chemical reactivity of the NHS-intermediate in method 2 was able to compensate this disadvantage [21,22].

Summarised, it can be concluded that the microscopic methods introduced herein, AFM and TEM, are able to present fast and reliable complementary visual information on the protein-modification of colloidal drug carriers. When used in addition to conventional techniques, they can significantly enhance the process of characterisation of new systems for advanced drug delivery.

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