The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

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Chapter 5

Coupling of metal containing homing devices to liposomes via a maleimide linker: use of TCEP to stabilise thiol-groups without scavenging metals

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Abstract

Liposomes for drug delivery are often prepared with maleimide groups on the distal end of PEG to enable coupling of homing devices, such as antibodies, or other proteins. EDTA is used to stabilise the thiol group in the homing device for attachment to the maleimide. However, when using a homing device that contains a metal, EDTA inactivates this by scavenging of the metal. Holo-transferrin (Tf) containing 2 iron atoms (Fe^{3+}), has a much higher affinity for the transferrin receptor than apo-Tf (which does not contain any Fe^{3+}). To couple Tf to a liposome, the introduction of a thiol group is necessary. During this process, by using N-succinimidyl S-acetylthioacetate (SATA), followed by 2 - 3 hr coupling to the liposomes, Fe^{3+} is scavenged by EDTA. This causes a decreased affinity of Tf for its receptor, resulting in a decreased targeting efficiency of the liposomes.

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) is a sulfhydryl reductant that is often used in protein biochemistry. We found that TCEP (0.01 mM) does not scavenge Fe^{3+} from Tf and is able to protect thiol groups for the coupling to maleimide. Furthermore, TCEP does not interfere with the maleimide coupling itself.

In this communication we describe the preparation of liposomes, focussing on the coupling of Tf to the maleimide linker at the distal end of PEG, without loosing Fe^{3+} from Tf. This method can be applied to other metal-containing homing devices as well.

Introduction

Drug delivery to the brain is very difficult due to the presence of the blood-brain barrier (BBB). Zhang *et al* (2003) have shown that liposomes targeted to the transferrin receptor (TfR) at the BBB can enhance the delivery of plasmid DNA (1). However, they used an antibody against the rat TfR as a homing device. We are interested in the mechanism of endocytosis via the TfR on bovine brain capillary endothelial cells *in vitro*. For the bovine TfR only polyclonal antibodies exist, which are not specific. Therefore, our goal was to develop a drug delivery system using the endogenous ligand for this receptor, transferrin (Tf), as a homing device. Tf is a 80 kDa protein folded into 2 identical subunits, which each can carry one iron (Fe^{3+}) atom. Holo-Tf contains 2 iron atoms and has a high affinity for the TfR at physiological pH, while apo-Tf does not contain any iron and has at least a 1000-fold lower affinity for the TfR (2).

For the preparation of liposomes we have adapted the method used by Heeremans *et al* (1992) and Mastrobattista *et al* (1999) who have used maleimide to couple a homing device to liposomes (3, 4). Certain homing devices such as Fab-fragments contain a thiol group for coupling to maleimide. Tf does not expose a free thiol group. However, by using N-succinimidyl S-acetylthioacetate (SATA) it can easily be introduced to a primary amine in an one-step reaction. It is important that the thiol group is protected from oxidation. This can be achieved by avoiding contact with oxygen (working under constant N_2) and/or by addition of EDTA (1 mM) to all aqueous solvents that are used. However, EDTA has the capacity to scavenge iron from Tf, thereby, decreasing its affinity for the TfR. Other SH-protectants often have a thiol group themselves (e.g. β-mercaptoethanol, dithiothreitol (DTT)), that competes for maleimide coupling. In contrast, tris(2-carboxyethyl)phosphine (TCEP, figure 1), that is often used in protein biochemistry, does not contain a thiol group. Furthermore, TCEP has proven to be a good reductant (5). Therefore, we have applied TCEP for the preparation of Tf-tagged liposomes.

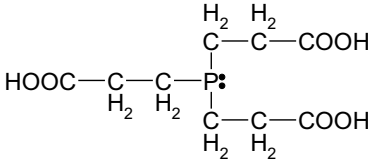

Figure 1: Structure of Tris(2-carboxyethyl)phosphine (TCEP)

For this communication, we have determined the amount of thiol groups introduced in Tf, as well as their stability. Furthermore, we determined whether Tf retained its iron during the preparation of Tf-tagged liposomes. Finally, the coupling efficiency of Tf to liposomes containing maleimide was determined in the presence of EDTA and TCEP.

Experimental

Transferrin

Tf was modified with a thiol group using SATA (6). Tf and SATA (1:8 molar ratio) were incubated in HBS pH 7.0 for 1 h at room temperature under constant shaking (step 1, figure 2). Free SATA was removed by centrifugation over 30 kDa cut-off filters. Directly before coupling to maleimide the thiol group was activated by incubation with 0.1 M hydroxylamine for 45 min at room temperature (step 2, figure 2). The amount and stability of the thiol groups was determined with Ellman's reagent (7). The presence of EDTA or TCEP did not affect the Ellman's determination of thiol groups. The iron determination was based on the inspection of UV-VIS absorption spectra at 280 and 460 nm (8). Holo-Tf (10 mg/ml) was incubated for 24 h at room temperature with EDTA (1 mM) or TCEP (0.1 or 0.01 mM). As a control apo-Tf (10 mg/ml) was loaded with $FeCl_3$ and sodium citrate (9). $FeCl_3$ and sodium citrate were mixed in a 1:10 ratio, before addition of 2 mol Fe^{3+} per mol Tf.

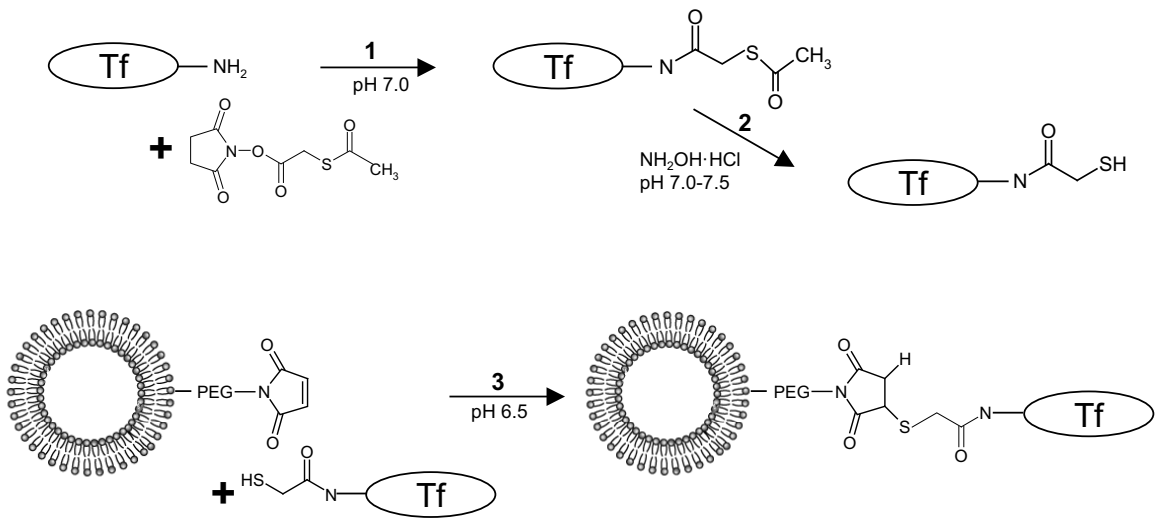

Figure 2: Reaction scheme of the thiolation of Tf with N-succinimidyl-S-acetylthioacetate (SATA, step 1), the activation of thiolated Tf with hydroxylamine HCl (step 2) and the conjugation of Tf to liposomes, bearing maleimide-PEG-DSPE (step 3).

Liposome preparation

Liposomes were prepared according to Heeremans *et al* (1992) and Mastrobattista *et al* (1999) with some modifications (3, 4). Briefly, liposomes were prepared of EPC-35 and cholesterol in a 2:1 molar ratio, with 2.5 % PEG_{2000}-DSPE and 2.5 % PEG_{2000}-maleimide-DSPE. Briefly, after evaporation of organic solvents the lipid film was resuspended in HBS pH 6.5 and liposomes were extruded 8 times through 200 & 100 nm polycarbonate filters using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). Directly after extrusion liposomes (8 – 12 mM phospholipid (PL)) were incubated with SATA modified Tf at a final amount of 1 – 4 mg Tf per 9 μmol PL in the presence of EDTA (1 mM) or TCEP (0.01 mM) for 2 h at room temperature under constant shaking (step 3, figure 2). The incubation volume was 1 – 2 ml, depending on the concentrations PL and Tf. After incubation with Tf, the liposomes were incubated with L-cystein for 30 min, to block all excess maleimide groups. N-ethylmaleimide (NEM, 8 mM) was added to the liposomes to stop the coupling reaction before separation of free Tf and liposomes by ultracentrifugation (60.000 g, 30 min, 10 °C). After ultracentrifugation liposomes were resuspended in HBS pH 7.0 without any additives.

Characterisation of liposomes

After preparation, the phospholipid content was determined according to Rouser (10), and the protein content was determined with the Biorad protein assay. Furthermore, size and polydispersity were determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK).

Materials

Bovine holo- and apo-transferrin, $FeCl_3$ and hydroxylamine-HCl were purchased from ICN Pharmaceuticals (Zoetermeer, the Netherlands). SATA, EDTA, TCEP, HEPES, L-cystein and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands), sodium citrate from J.T. Baker (Deventer, the Netherlands) sodium chloride from Merck (Amsterdam, the Netherlands). The Biorad protein assay was obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands) and DiD from Molecular Probes (Leiden, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany), PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and PEG_{2000}-maleimide-DSPE from Shear Water Corporation (Huntsville, AL, USA). Vivaspin columns are obtained from Vivascience AG (Hannover, Germany) and polycarbonate filters for extrusion from Nuclepore (Pleasanton, USA).

Results

Transferrin

Tf:SATA ratios of 1:4, 1:8, 1:12 and 12:1 were used (data not shown), but introduction of thiol groups was most effective at a Tf:SATA ratio of 1:8. After de-protection with hydroxylamine in the presence of EDTA 2.2 mol SH per mol Tf was obtained after 1 h incubation (table I). Using TCEP in a similar concentration (1 mM) 5.6 mol SH per mol Tf was detected after 1 h. This number dropped slightly over 24 h. With 0.1 and 0.01 mM TCEP 2.5 and 2.3 mol SH per mol Tf was detected after 1 h incubation, respectively. After 24 h at room temperature the amount of SH groups in the presence of EDTA was decreased with 50%, while with all concentrations of TCEP this decrease was less. Moreover, when heating Tf for 2 h at 37 °C TCEP proved to be a better stabiliser for thiol groups than EDTA (table I). Without hydroxylamine present, TCEP (0.1 and 0.01 mM) were able to de-protect the thiol group in SATA-modified Tf: after 1 h incubation 1.3 mol SH per mol Tf was detected.

Table I: Tf was modified with SATA (1:8 molar ratio) and subsequently incubated for 1 h with hydroxylamine to de-protect the thiol group in the presence or absence of EDTA or TCEP. The amount of SH groups per Tf molecule were determined directly after de-protection (1 h), 24 hours after de-protection (24h), or after 24 hours at room temperature, followed by 2 hours at 37 °C (24 h + 2 h 37 °C).

SATA-modified Tf in the presence of	SH/Tf (mol/mol)		
	1 h	24 h	24 h + 2 h 37 °C
no stabiliser	2.3	1.3	0.7
EDTA (1 mM)	2.2	1.1	0.6
TCEP (1 mM)	5.6	4.9	3.9
TCEP (0.1 mM)	2.5	1.9	1.6
TCEP (0.01 mM)	2.3	1.6	1.4
Tf (not-modified)	0.2	0.3	0.3

From UV-VIS analysis it was shown that both holo- and apo-Tf showed an absorption maximum at 280 nm (typical for proteins) but only holo-Tf showed a second absorption maximum at 460 nm (figure 3). This latter maximum is an indication for the presence of Fe^{3+}, although this was only visible at concentrations higher than 5 mg/ml. Incubation of holo-Tf with EDTA showed a small decrease in the iron peak already after 2 hour (data not shown), after 24 h this decrease was approximately 50 % (figure 3). The absorption spectra of Tf in the absence or presence of TCEP (0.1 or 0.01 mM) showed no difference (figure 3). Apo-Tf, incubated for 24 h with $FeCl_3$, had a similar absorption spectrum compared to holo-Tf. $FeCl_3$ and sodium citrate without Tf present did not show an absorption peak at 460 –470 nm (data not shown).

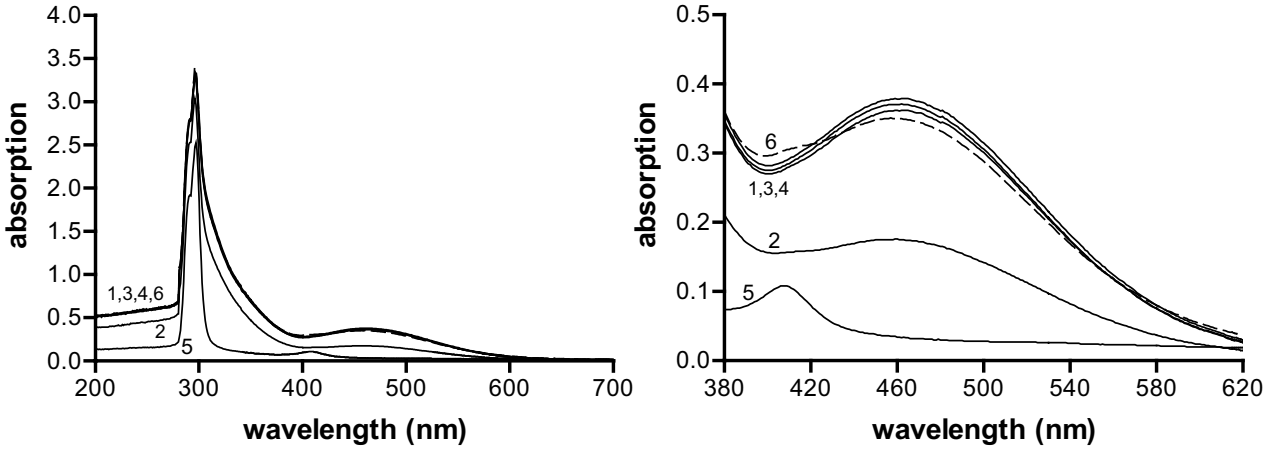

Figure 3: UV-VIS spectra of holo-Tf (10 mg/ml) in the absence (1) or presence of EDTA (1 mM, 2) or TCEP (0.1 or 0.01 mM, lines 3 and 4, respectively) and apo-Tf (10 mg/ml) in the absence (5) or presence (6) of $FeCl_3$ and sodium citrate. A typical protein peak is visible around 280 nm (left panel), while only for holo-Tf and apo-Tf incubated for 24 h with Fe^{3+} a peak around 460 nm is visible (right panel). EDTA is able to scavenge Fe^{3+}, as is shown by the decrease in absorption at 460 nm, TCEP does not scavenge Fe^{3+} after 24 h incubation (right panel).

Liposomes

Coupling of SATA modified-Tf to liposomes containing maleimide groups reached a maximum at 0.33 mg/μmol PL (figure 4). As expected, no difference in the extent of

coupling was obtained when this was performed either in the presence of EDTA or TCEP.

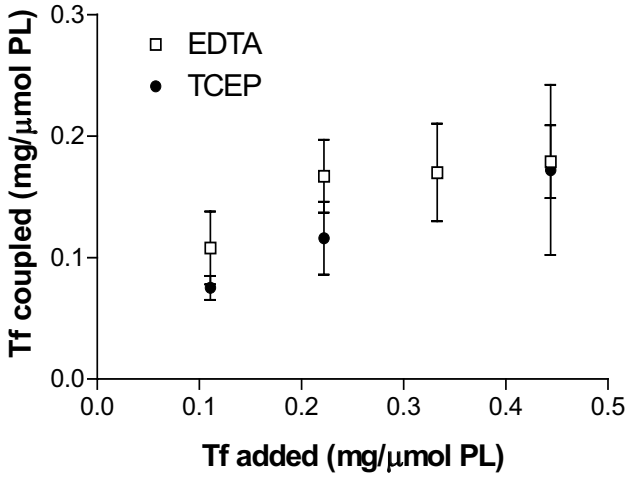

Figure 4: The amount of coupled Tf per μmol phospholipid (PL) is plotted against the added amount of Tf per μmol PL. This shows that the coupling of Tf is saturable at amounts higher than 0.25 mg Tf per μmol PL. The amount of coupling is not changed in the presence of TCEP (0.01 mM) compared to EDTA (1 mM).

Discussion

Targeting drugs to the brain is often aimed at the TfR on the BBB (1, 11). Recently, we have characterised the TfR on BBB endothelial cells (12). Furthermore, we have validated the use of the TfR for targeting drugs to the BBB by using Tf conjugated to horseradish peroxidase (13). By applying liposomes, it is possible to increase the ratio of target site delivered drug molecules per Tf homing device molecule. This is particularly true for small drug molecules. Therefore, we are interested in the preparation of Tf-tagged liposomes. During the preparation of Tf-tagged liposomes, it is important that Tf retains its iron, since holo-Tf (diferric Tf) has at least a 1000-fold higher affinity for the TfR than mono-ferric or apo-Tf (*2*). Each molecule Tf can bind two iron atoms with simultaneous binding of CO_3^{2-} (14). At physiological pH the binding of iron and the anion is strong, therefore, we do not expect that during the preparation of liposomes the binding of iron and the anion is changed. However, metal scavengers that can reach the iron binding site of Tf might affect the binding of iron to Tf.

Initially, we used EDTA during the preparation of the liposomes as a stabilising agent for the introduced thiol groups (6), but EDTA also has metal-scavenging properties. Alternatively, TCEP (figure 1) is a new thiol stabilising agent which does not

have the capacity to scavenge iron from Tf, as is shown in the UV-VIS spectra in figure 3. Holo-Tf has 2 peaks, one around 280 nm, which is typical for proteins, and a second peak between 460 and 470 nm. The latter is specific for iron, and is decreased to approximately 50 % after 24 h in the presence of EDTA (1 mM). This process is time dependent (data not shown), indicating that Tf is loosing its iron. In the presence of TCEP (0.1 and 0.01 mM) Tf retains its iron, since the peak at 460-470 nm does not change during incubation for 24 h.

The amount of thiol groups introduced after the SATA reaction and de-protection with hydroxylamine, is similar in the presence of EDTA (1 mM) or TCEP (0.1 and 0.01 mM) or "no stabiliser" (table I, 1h). After 24 h at room temperature and after 24 h at room temperature in combination with 2 h at 37 °C, more thiol groups per mol Tf were found in the presence of TCEP (0.1 and 0.01 mM) than in the presence of EDTA (table I). This indicates that TCEP is a better stabiliser of thiol groups. Kratz *et al* (1998) have prepared Tf conjugates of doxorubicin in the presence of EDTA (1 mM) (15). However, the EDTA was only present during the insertion of a thiol group with iminothiolane (1 hour incubation) and not during the coupling to maleimide. Our method required a longer incubation in the presence of EDTA, which caused a dissociation of iron from Tf. Therefore, we have used TCEP (0.1 mM) for the preparation of Tf-tagged liposomes.

A higher concentration TCEP (1 mM) caused a strong increase in the amount of thiol groups (5.6 mol SH per mol Tf after 1 h), indicating that Tf might be modified, and was probably not suitable for targeting anymore. Therefore, we have decided to use concentrations of 0.1 or 0.01 mM TCEP. When no hydroxylamine was present TCEP (0.1 and 0.01 mM) alone could also activate thiol groups in Tf (1.3 mol SH per mol Tf), although this occurred less efficiently. These experiments suggest that, although TCEP is a known reductant for disulphides (5, 16), it can also cleave a thioester bond.

Coupling of Tf to maleimide on the distal end of PEG-liposomes in the presence of EDTA (1 mM) of TCEP (0.1 mM) was concentration dependent, as is shown in figure 4. The coupling in the presence of TCEP tended to be slightly lower, although this difference was not significant. Highest coupling efficiencies were obtained at the lower concentrations, where more than 75 % of the added Tf was coupled, while at Tf concentrations higher than 3 mg/ 9 μmol PL coupling efficiency was 40 – 50 %. Burmeister Getz et al (5) have shown that TCEP (0.1 mM) inhibited the labelling of

myosin (a protein with a free thiol group) with a fluorescent label, containing a maleimide group, substantially. They also found that increasing the TCEP concentration led to a decrease in fluorescent label. However, they have used a ratio of maleimide to thiol of 3:1, while we have used a 4.4 – 17.5 fold excess of maleimide during the coupling of Tf to liposomes. Other reductants such as β-mercaptoethanol or DTT are very effective in their protection of free thiol groups, but they possess a thiol group themselves. Therefore, it is not possible to use β-mercaptoethanol or DTT for maleimide coupling. All liposomes were similar in size (170 – 200 nm) and had a polydispersity index lower than 0.25, indicating that after coupling of Tf in the presence of EDTA or TCEP we obtained similarly sized liposomes.

The objective of our research is to explore the use of the TfR for drug delivery to the brain. For this we initially used horseradish peroxidase (HRP) conjugated to a primary amine of Tf, which was specifically endocytosed by the TfR (13). To extent our research we want to apply liposomes loaded with HRP and tagged with Tf. For the preparation of these liposomes Tf is modified at a primary amine, similar as for the preparation of Tf-HRP conjugates. Therefore, we expect that the binding to and endocytosis by the TfR of Tf-tagged liposomes is not altered due to the SATA reaction and subsequent binding to maleimide-PEG-PE on the liposomal surface.

In conclusion, we have successfully applied the use of TCEP for the coupling of homing devices containing metal ions to maleimide groups on liposomes. In addition, for an efficient coupling it is important to protect the thiol groups in the homing device from oxidation. We have shown for our ligand Tf that TCEP has the advantage of stabilising thiol groups without scavenging iron from Tf.

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