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Research paper

Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB)

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ABSTRACT

Human serum albumin (HSA) nanoparticles were manufactured by desolvation. Transferrin or transferrin receptor monoclonal antibodies (OX26 or R17217) were covalently coupled to the HSA nanoparticles using the NHS-PEG-MAL-5000 crosslinker. Loperamide was used as a model drug since it normally does not cross the blood–brain barrier (BBB) and was bound to the nanoparticles by adsorption. Loperamide-loaded HSA nanoparticles with covalently bound transferrin or the OX26 or R17217antibodies induced significant anti-nociceptive effects in the tail-flick test in ICR (CD-1) mice after intravenous injection, demonstrating that transferrin or these antibodies covalently coupled to HSA nanoparticles are able to transport loperamide and possibly other drugs across the BBB. Control loperamide-loaded HSA nanoparticles with IgG2a antibodies yielded only marginal effects.

1. Introduction

The blood–brain barrier (BBB), formed by brain vessel endothelial cells linked together by tight junctions, restricts the transfer of most drug substances from the bloodstream into the brain [1,2]. These drugs include anticancer drugs, antibiotics, as well as peptides such as dalargin and other neuropeptides. Therefore, drug delivery across the BBB is a major challenge in the pharmaceutical research and development [3].

Colloidal drug carriers such as liposomes or nanoparticles (NPs) have been used to overcome this barrier [4–7]. The major advantage of these colloidal drug carrier systems is their possibility of the drug targeting by modifying the distribution of drugs in the body [8]. NPs overcoated with polysorbate 80 (Tween® 80) or poloxamer 188 (Pluronic® F 68) [9,10] are able to deliver many drugs across the BBB including loperamide, dalargin, kyotorphin, tubocurarine, and doxorubicin [6,11–13]. The mechanism of delivery across the BBB, with the surfactant-coated NPs appears to be adsorption of apolipoprotein E or A-1 after injection into the blood stream, followed by receptor-mediated uptake of the particles by the brain capillary endothelial cells [12,14]. This hypothesis was supported by the finding that covalent coupling of apolipoprotein E or A-1 to human serum albumin nanoparticles (HSA NPs) leads to similar effects [4,10,15,16]. Similarly to apolipoproteins, antibodies can be coupled to HSA NPs by covalent linkage with crosslinkers such as NHS-PEG3400-MAL [17].

Membrane transferrin receptor-mediated endocytosis is an efficient cellular uptake pathway for drug delivery of anticancer drugs [18]. The transferrin receptor (TfR) is over-expressed in many tumors [19] and has been widely studied. Transferrin or the antibodies against the transferrin receptor (for instance, R17217 and OX26 monoclonal antibody) have been investigated in a number of studies [20–22]. Since the transferrin receptor is also present in the blood–brain barrier, transferrin-conjugated solid lipid nanoparticles enabled an enhanced delivery of quinine dihydrochloride to the brain [23]. The mechanism of the enhanced efficacy of nanoparticles with conjugated transferrin appears to be due a higher cellular drug uptake [24]. The R17217 is a rat IgG2a antibody against the mouse TfR and binds to this receptor on mouse cells [25]. The first use of OX26, a murine mAb to the rat TfR, for the delivery of peptides across the BBB was reported in the early nineties [26,27]. Pardridge's group coupled the peptides to the OX26 via the avidin–biotin-system [26]. In later studies, they attached this antibody to liposomes [28].

The objective of the present study was the development of HSA nanoparticles to which transferrin was coupled, and to evaluate the potential of these nanoparticles to deliver drugs across the BBB. In addition, the possibility of achieving similar results by the coupling of the above-mentioned monoclonal antibodies (mAbs) against the transferrin receptor to the nanoparticles was

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investigated. Loperamide was chosen as the model drug since it does not cross the BBB [29] and its anti-nociceptive (analgesic) effects on the CNS can be easily quantified in the tail-flick test.

2. Materials and methods

2.1. Chemicals and reagents

Batch 016K7546 of HSA (fraction V, purity 96–99%), glutaraldehyde 8% solution, and transferrin (human: minimum 98%) were obtained from Sigma (Steinheim, Germany); apo-transferrin (human, >95.0% purity by SDS–PAGE) was from Calbiochem (Darmstadt, Germany), OX26 was from AbD Serotec (Oxford, UK), and the monoclonal antibody R17217 and the mouse isotype control IgG2a were from BioLegend (San Diego, USA). The crosslinker NHS-PEG-MAL-5000 was received from Nektar (Alabama, USA) and Traut's reagent (2-Iminothiolane) and Ellman's reagent from Pierce (Rockford, USA). The PD-10 Columns Sephadex™ G-25 M were from GE Healthcare (Buckinghamshire, UK), and all other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical grade and used as received.

2.2. Animals

Female ICR (CD-1) mice, 20–25 g (Harlan-Winkelmann, Borchen, Germany) were used for the in vivo study. Water and standard laboratory chow were freely available to the animals.

2.3. Nanoparticle preparation

Transferrin- and transferrin-mAb-modified HSA NPs were manufactured by a three step method: In the first step, unmodified HSA NPs were produced by a previously described desolvation technique [30]: For this purpose HSA was dissolved at a concentration of 100 mg/ml in 10 mM sodium chloride solution, and the pH of the solution was adjusted to 8.4. Two milliliters of the resulting solutions was filtered through a 0.22 μm filtration unit (Schleicher und Schüll, Dassel, Germany). For the formation of nanoparticles, 8.0 ml ethanol were added at room temperature with a peristaltic pump (Ismatec IPN, Glattbrugg, Switzerland) at a pump rate of 1 ml/min under constant stirring (550 rpm).

After the desolvation, 235 μl of 8% glutaraldehyde solution in water corresponding to 200% of the amount that is theoretically required for the quantitative cross-linking of the 60 amino groups in the HSA molecules of the particle matrix was added. The cross-linking was performed for at least 18 h under constant stirring at room temperature. Particles were purified by 3-fold centrifugation (16,100*g*, 8 min) and by redispersion of the pellets to original volume in water. The redispersion was performed using a vortex mixer and ultrasonication. The sediment and the supernatants of the desolvation process were analyzed. In the second step, sulfhydryl groups were introduced to the transferrin or to the antibodies by the reaction of the amino groups with 2-iminothiolane, and the amount of the introduced sulfhydryl groups was determined with Ellman's reagent.

In the third step, the nanoparticles were activated with the heterobifunctional crosslinker NHS-PEG-MAL-5000 using a 10-fold molar excess as described by Michaelis et al. [4], and the resulting sulfhydryl-reactive protein was covalently coupled to the nanoparticles [31].

2.4. Preparation of transferrin- and transferrin-mAb-coupled HSA NPs (TfR-mAb-coupled HSA NPs)

Transferrin was dissolved at a concentration of 1 mg/ml in phosphate buffer (pH 8.0) and incubated with 12.8 μl (50.85-fold molar excess) or 19.1 μl (76.2-fold molar excess) of 2-iminothiolane solution (6.9 mg in 1.0 ml phosphate buffer, pH 8.0) in the dark for 1.5 h at 20 °C under constant shaking (550 rpm) [32,33]. A 40-fold molar excess [34] of Traut's reagent (0.01 mg in 1.0 ml phosphate buffer) was added to 500 μl of 500 μg/ml concentrated antibodies [35]. Thereafter, the thiolated transferrin or thiolated antibody was purified by PD-10 Columns Sephadex™ G-25 M, using phosphate buffer (pH 8.0) as eluent. For the conjugation to the NPs, 500 μl of thiolated and purified solution of transferrin or of the antibodies was added to 500 μl of reactive HSA NPs. The mixture was incubated under shaking for 18 h at room temperature. Non-reacted thiolated transferrin or antibodies were removed by 2-fold NPs centrifugation and redispersion in water.

2.5. Size exclusion chromatography (SEC)

After centrifugation, the supernatants of the nanoparticles with the coupled transferrin or antibody were analyzed by SEC [31] at 280 nm (absorbance detector: Waters 468). The HPLC pump (Waters 600) was operated at 1 ml/min using phosphate buffer, pH 6.6, as the mobile phase. The supernatants were added to the mobile phase 1:1, and samples of 20 μl were injected with an autosampler (Thermo Seperation Products AS100).

2.6. Loperamide loading

Ten milligrams of the purified transferrin-modified or antibody-modified HSA NPs were incubated in 1000 μl of loperamide solution, which was dissolved in ethanol/water (42% ethanol v/v) at a concentration of 7.6 mg/ml. After incubation for 2 h the unbound loperamide was removed by centrifugation and redispersion, and determined by HPLC (see below) [4,36].

2.7. Determination of particle size and zeta potential

Particle size, polydispersity and the zeta potential of the loperamide-loaded nanoparticles were measured by a Malvern Zetasizer 3000 HSA (Malvern, Instruments Ltd., Malvern, UK) at a temperature of 25 °C and a scattering angle of 90° after an at least 200-fold dilution with purified water. For the zeta potential measurement, the Zetasizer was equipped with a dip-cell (Malvern Instruments Ltd., Malvern, UK).

2.8. Determination of loperamide loading

The unbound loperamide in the supernatant of the NPs was determined by HPLC [4,36]. Aliquots (20 μl) of the supernatants were injected into a Luna 250 × 4.6 mm 5 μm particle C18 (2) column (Phenomenex, Aschaffenburg, Germany). The flow rate was set to 1.0 ml/min using a mobile phase consisting of acetonitrile-sodium phosphate buffer (pH 2.3; 20 mM)-diethylamine (40:60:0.08, v/v/v). The resulting elution time was 22 min.

2.9. Animal testing

The animal experiments were performed in accordance with the German Tierschutzgesetz and the Allgemeine Verwaltungsvorschrift zur Durchführung des Tierschutzgesetzes, and were authorised by the Regierungspräsidium Darmstadt (V54 – 19c 20/15 – F 116/14). The mice were divided into 5 groups of 10 animals per group (Fig. 1 and 2): group A: loperamide-loaded HSA NPs linked to transferrin thiolated with a 50.85-fold molar excess, group B: loperamide-loaded HSA NPs linked to transferrin thiolated with a 76.2-fold molar excess,

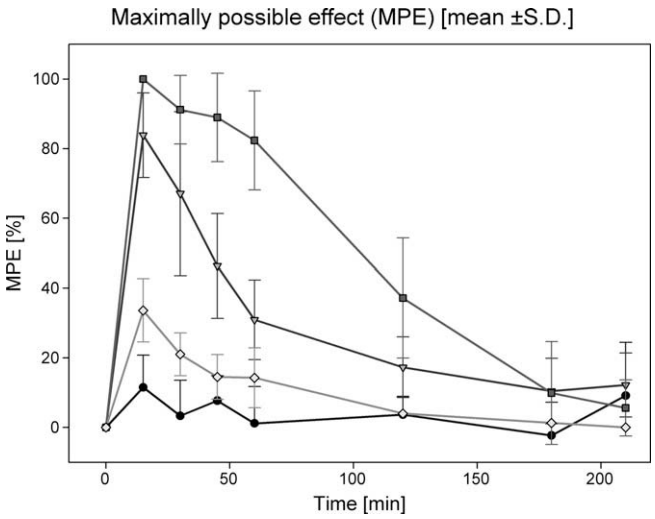

Fig. 1. Maximally possible anti-nociceptive effect (MPE) [mean ± SD] of loperamide in female ICR (CD-1) mice after injection of nanoparticles without attached transferrin or antibodies (negative control) ●, nanoparticles with covalently attached transferrin (▼ 50.85-fold molar excess, or ■ 76.2-fold molar excess of 2-iminothiolane) or with a covalently attached IgG2a antibodies (40-fold molar excess of 2-iminothiolane) ◇.

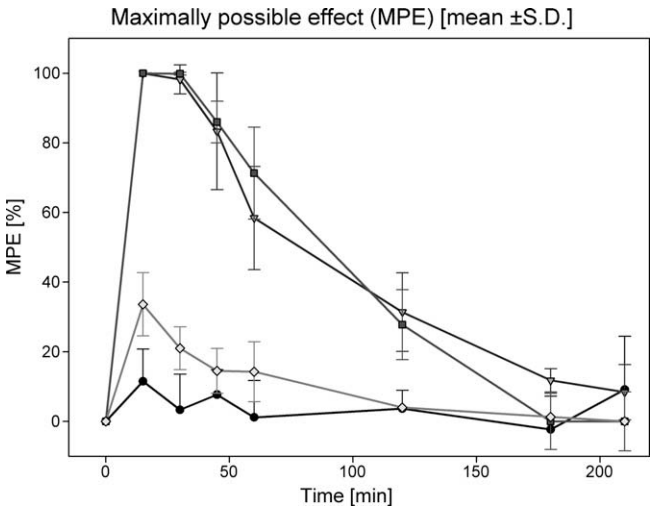

Fig. 2. Maximally possible anti-nociceptive effect (MPE) [mean ± SD] of loperamide in female ICR (CD-1) mice after injection of nanoparticles without attached transferrin or antibodies (negative control) ●, nanoparticles with covalently attached antibodies: ■ OX26 (40-fold molar excess of 2-iminothiolane), ▼ R17217 (40-fold molar excess of 2-iminothiolane) or ◇ IgG2a (40-fold molar excess of 2-iminothiolane).

group C: loperamide-loaded HSA NPs linked to OX26, group D: loperamide-loaded HSA NPs linked to R17217, and group E: loperamide-loaded HSA NPs linked to IgG2a. Each mouse received a dose of 7.0 mg/kg loperamide attached to 120 mg/kg NPs into the tail vein.

After the injection of the different preparations, the nociceptive threshold of each mouse was tested using the tail-flick test (Tail-Flick Analgesia meter, Ugo Basile). This instrument measures the pain sensitivity in mice as they respond to the application of heat to a small area of their tails. To prevent tail tissue damage, the experiments were terminated after 10 s if no response was evoked (cut off time). This time point was considered to indicate complete analgesia. For each animal, the tail-flick latency was determined before administration of the preparations (= pre drug latency). Tail-flick latencies were measured at 15, 30, 45, 60, 120, 180 and 210 min after injection of the loperamide formulations. The response latencies were converted to percent maximally possible effect (MPE) using the following term:

$$\%\mathrm{MPE} = \frac{\text{postdrug latency} - \text{predrug latency}}{\text{cut off time} - \text{predrug latency}} \times 100\%$$

3. Results

3.1. Nanoparticle preparation

Transferrin as well as the antibodies OX26 and R17217 or the negative control antibody IgG2a were covalently attached in a thiolated form to HSA NPs by sulfhydryl-reactive groups reacting with a PEG-Maleimide crosslinker (NHS-PEG-MAL-5000; Fig. 3). As shown in Table 1, the transferrin coupled HSA NPs are significantly larger than the empty nanoparticles, the TfR-antibody-coupled HSA NPs had a similar size, polydispersity, zeta potential and particle content as well as loperamide concentration. The number of mAb molecules bound on the surface of a single nanoparticle was calculated using the equation published by Cirstoiu-Hapca et al. [37].

3.2. Measurement of unbound free transferrin and free antibody

The SEC analysis of the supernatants shows that the transferrin and the antibody were quantitatively bound to the nanoparticles surface (Fig. 4). The number of sulfhydryl groups attached to the transferrin is an important factor of the bonding between transfer-

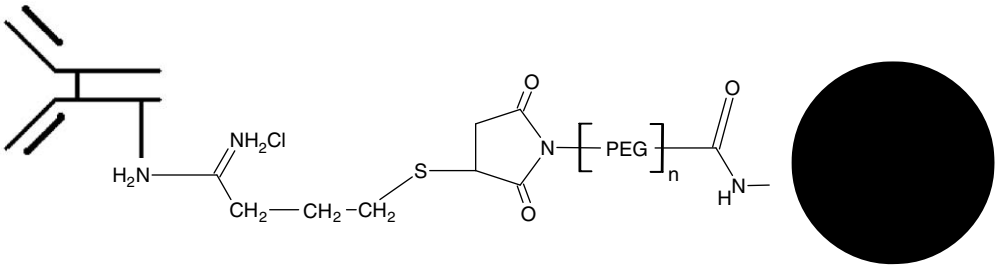

Fig. 3. Schematic representation of the antibody surface modification of the nanoparticles.

Table 1
Particle size before and after coupling ($n \geqslant 4$)

		Particle size [nm]	Polydispersity	Zetapotential [mV]	Particle content [mg/ml]	Ligand binding efficiency [%]	[μg/mg]	[Ab/NP]
Transferrin	Prior to thiolation	158 ± 5	0.022 ± 0.016	−38 ± 4				
	After thiolation	183 ± 10	0.087 ± 0.049	−28 ± 8	11.8 ± 2.6	84 ± 9	71.2 ± 16.4	
mAb OX26	Prior to thiolation	156 ± 7	0.052 ± 0.029	−34 ± 8				
	After thiolation	168 ± 5	0.072 ± 0.044	−28 ± 6	11.3 ± 1.9	92 ± 8	20.4 ± 1.6	267 ± 21
mAb R17217	Prior to thiolation	154 ± 3	0.019 ± 0.014	−41 ± 1				
	After thiolation	166 ± 7	0.054 ± 0.035	−32 ± 6	12.3 ± 0.3	75 ± 25	15.2 ± 3.8	192 ± 48
mAb IgG2a	Prior to thiolation	155 ± 4	0.026 ± 0.007	−40 ± 6				
	After thiolation	168 ± 3	0.044 ± 0.015	−33 ± 7	11.8 ± 0.5	84 ± 16	17.8 ± 2.8	232 ± 37

rin and the crosslinker. Figs. 5 and 6 show the dependence of the sulfhydryl coupling to transferrin on time and on the excess of Traut's reagent in comparison to the amount of transferrin. We discovered 50.85 and 76.2-fold excesses of 2-iminothiolane per mol of transferrin and a coupling time of 1.5 h to be optimal for the introduction of the sulfhydryl groups into the transferrin in order to avoid dimerisation or oligomerisation of the transferrin. The dimerisation and oligomerisation of the nanoparticles were also checked by the SEC method (Fig. 7).

3.3. Loperamide adsorption

Loading of loperamide to the NPs was found for all preparations to amount to 76 ± 6.92 μg loperamide/mg HSA.

3.4. Animal testing

Transferrin or TfR-mAb linked to loperamide-loaded HSA NPs via the NHS-PEG-MAL induced significant dose-dependent antinociceptive (analgesic) effects (Figs. 1 and 2). Treatment of animals with loperamide-loaded-NPs (7 mg loperamide/kg) using a 50.85-fold excess of transferrin (group A, Fig. 1) resulted in a maximally in possible effect (MPE) of 92% and the use of a 76.2-fold excess (group B) resulted in an MPE of 100% (Fig. 1) within 15 min after injection and achieved a marked prolonged effect compared to group A. The treatment of the animals with loperamide-loaded OX26-NPs (7 mg loperamide/kg, group C, Fig. 2) resulted in an MPE of 100% after 15 min. This effect decreased at longer time periods and disappeared after 180 min. Treatment with R17217 HSA

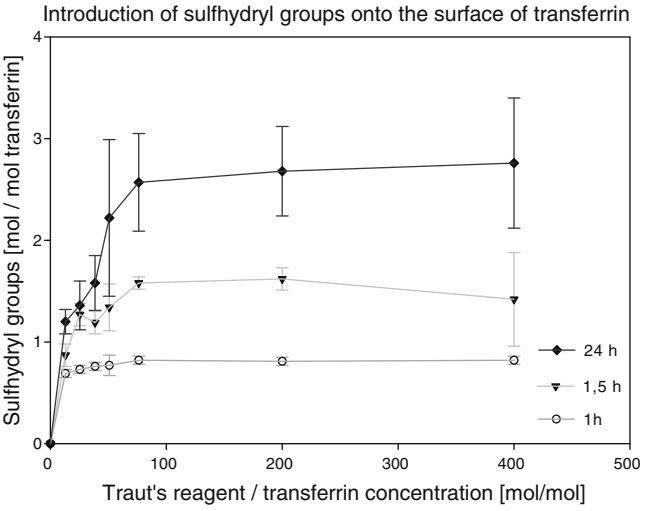

Fig. 5. Influence of the amounts of 2-iminothiolane and the reaction time on the thiolation of transferrin ($n = 3$). The following times were used: 1, 1.5 and 24 h. The time points 0.5, 2, 3 and 12 are not included in the figure for clarity.

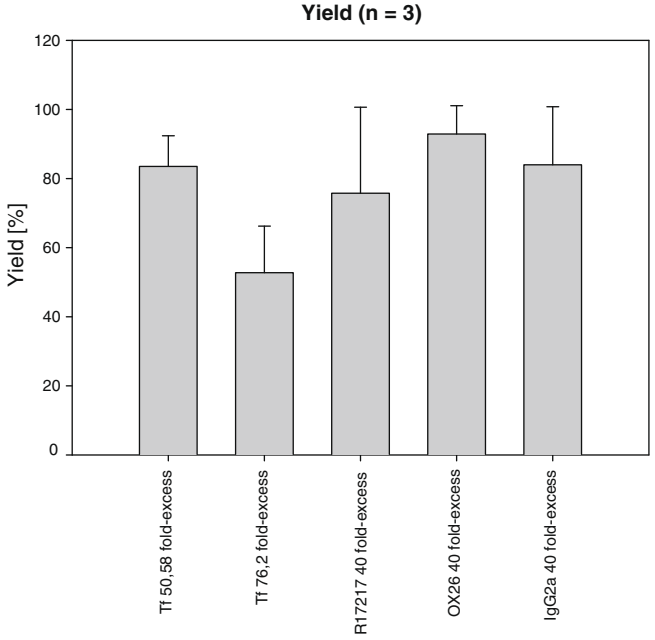

Fig. 4. Percentage of the bound transferrin or antibodies to the nanoparticles compared to the total ligand amount.

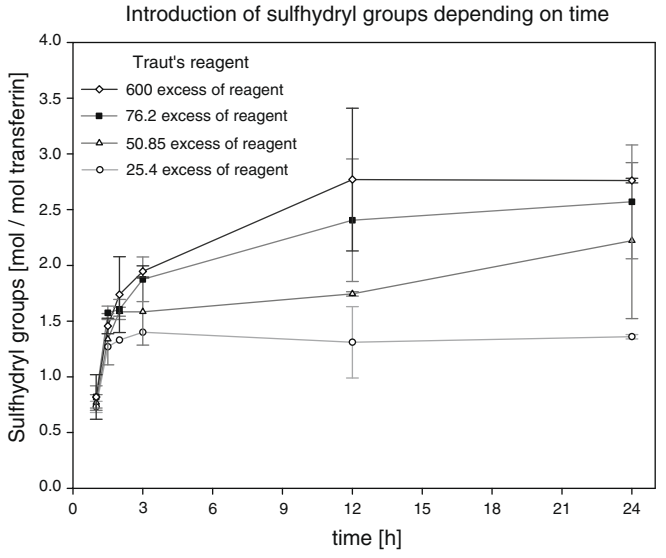

Fig. 6. Influence of the reaction time and amounts of 2-iminothiolane on the transferrin thiolation ($n = 3$). The following amounts were used: 25.4-, 50.85-, 76.2- and 600-fold excesses of 2-iminothiolane. The following amounts: 13-, 38.5-, 200- and 400-fold excesses of 2-iminothiolane are not included in the figure for clarity.

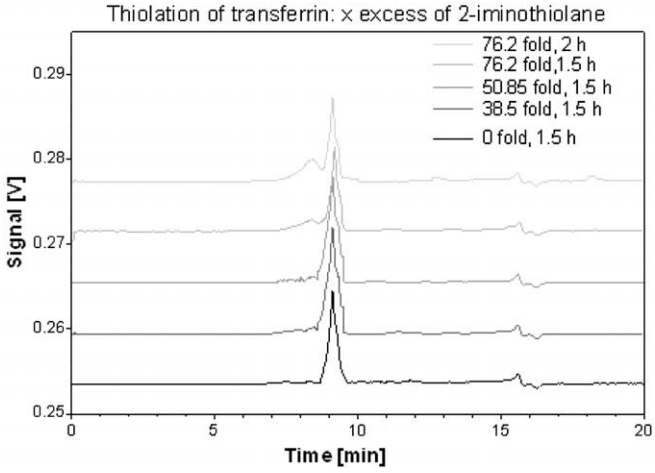

Fig. 7. Dimerisation of transferrin after 1.5 and 2 h reaction times and different amounts (molar excess) of 2-iminothiolane. Transferrin was analyzed by size exclusion chromatography at a retention time about 9 min. Dimers and higher conjugates were detected at shorter times.

NPs (group D) shows a similar effect as in group C (Fig. 2). In contrast, IgG2a coupled to the loperamide (7 mg/kg)-loaded HSA NPs (group E) did not achieve any statistically significant anti-nociceptive effects (Figs. 1 and 2).

4. Discussion

In this study, the possibility of using nanoparticles with covalently bound transferrin-modified or anti-transferrin-monoclonal antibodies (TfR-mAb) was investigated to transport drugs across the blood–brain barrier that normally cannot cross this barrier. Transferrin or TfR-mAbs (OX26 or R17217) were coupled using the NHS-PEG-MAL crosslinker to human serum albumin nanoparticles (HSA NPs) prepared by a desolvation procedure as described previously [31]. All the three preparations enabled the transport of loperamide across the BBB and achieved significant anti-nociceptive effects. In contrast, HSA NPs with attached IgG2a yielded no effects.

Covalent attachment of the ligands as in the present study enables the manufacture of stable systems. This method is advantageous over simple adsorption of antibody on to the nanoparticles surface as employed in the earlier studies [38]. The coupling of transferrin slightly increased the size of the nanoparticles from about 160 to about 185 nm, whereas the increase in size by the attachment of the antibodies was only around 10 nm. Other authors [24,39] also reported comparable small nanoparticle size increases after attachment of transferrin.

The efficiency of transferrin thiolation depended on time and excess of 2-iminothiolane (Figs. 5 and 6). With the exception of transferrin thiolated with a 76.2-fold excess of 2-iminothiolane, a high binding of the ligands was achieved (Fig. 4). The lower transferrin binding with higher amounts of Traut's reagent (2-iminothiolane) was caused most likely by the excessive formation of transferrin dimers due to the introduced sulfhydryl groups. These dimers then did not react with the crosslinker. Dimerisation and oligomerisation were caused by an increase in thiolation time and the amounts of 2-iminothiolane (Fig. 7). In order to avoid excessive dimerisation and oligomerisation a reaction time of 1.5 h was chosen.

The present study clearly demonstrates that different approaches may be employed to transport drugs across the BBB using nanoparticles. Both approaches, the binding of transferrin or the attachment of anti-transferrin-antibodies, were similarly efficient in delivering loperamide across the BBB. Moreover, in previous studies with nanoparticles the main strategy was to take advantage of lipoprotein receptors after binding of apolipoproteins or E [4,15] or of scavenger receptors after binding of apolipoprotein A-1 [10,16] to enable the drug transport. The interaction with these receptors was followed by the endocytotic uptake of the nanoparticles by the brain capillary endothelial cells [40,41] and even by transcytosis of the particles (unpublished results). Apart from covalent binding of the apolipoproteins, a drug delivery across the BBB also was possible by coating the nanoparticles with surfactants such as polysorbate 80 [5,9–13]. This coating leads to the adsorption of these apolipoproteins from the blood to the nanoparticle surface after intravenous injection. The attachment of transferrin or antibodies against the transferrin receptor represents an alternative approach that was already successful with liposomes [7]. To have these different possibilities represents a major advantage since it may facilitate simultaneous administration of different pharmaceutical or biological entities or constructs.

5. Conclusion

Human serum albumin nanoparticles (HSA NPs) coupled to transferrin or TfR-mAb are enabling a significant loperamide transport across the BBB into the brain. The loperamide-loaded, transferrin- or TfR-mAb-coupled HSA NPs achieved strong anti-nociceptive effects, whereas IgG2a-modified HSA NPs were not able to transport this drug across the BBB (Figs. 1 and 2). Therefore, these novel nanoparticles with attached transferrin or TfR-mAb represent very useful carriers for the transport of drugs into the brain.

Acknowledgement

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