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An aquaporin 4 antisense oligonucleotide loaded, brain targeted nanoparticulate system design

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Received June 25, 2013, accepted September 1, 2013

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Pharmazie 69: 340–345 (2014) *doi: 10.1691/ph.2014.3149*

Aquaporins (AQPs), members of the water-channel protein family, are highly expressed in brain tissue especially in astrocytic end-feet. They are important players for water hemostasis during development of cytotoxic as well as vasogenic edema. Increased expression of AQPs is important in pathophysiology of neurological diseases such as neuroinflammation and ischemia. Unfortunately, there are a few pharmacological inhibitors of AQP4 with several side effects limiting their translation as a drug for use in clinical conditions. Another therapeutic approach is using antisense oligonucleotides (ASOs) to block AQP4 activity. These are short, synthetic, modified nucleic acids that bind RNA to modulate its function. However, they cannot pass the blood brain barrier (BBB). To overcome this obstacle we designed a nanoparticulate system made up of chitosan nanoparticles surface modified with PEG and conjugated with monoclonal anti transferrin receptor-1 antibody *via* streptavidin-biotin binding. The nanocarrier system could be targeted to the transferrin receptor-1 at the brain endothelial capillaries through monoclonal antibodies. It is hypothesized that the nanoparticles could pass the BBB *via* receptor mediated transcytosis and reach brain parenchyma. Particle size, zeta potential, loading capacity and release profiles of nanoparticles were investigated. It was observed that all types of chitosau (CS) nanoparticles had positive zeta potential values and nanoparticle particle size distribution varied between 100 and 800 nm. The association efficiency of ASOs into the nanoparticles was between 80–97% and the release profiles of the nanoparticles exhibited an initial burst effect followed by a controlled release. The results showed that the designed chitosan based nanocarriers could be a promising carrier system to transport nucleic acid based drugs to brain parenchyma.

1. Introduction

Several strategies exist for drug delivery to the brain. Invasive techniques such as distruption of the blood brain barrier (BBB) temporarily by injecting mannitol solution or direct drug delivery into the brain were effective techniques but they are associated with an increased risk of infection and high cost (Garcia-Garcia et al. 2005). Chemical methods (lipidization) (Misra et al. 2003), or biological methods like direct conjugation of drug molecules with antibodies (Pardridge 2002), have shown interesting results but they also have some drawbacks. Linking of a lipophilic moiety or a ligand to the drug can provide a loss of therapeutic effect. Another promising strategy could be to bind drugs to colloidal carriers. They are able to deliver drug molecules to a specific site by coupling ligands to the surface of the colloids which can be administered intravenously. Also receptor mediated targeting of polymeric nanosystems provides selective targeting to tissues.

Aquaporins (AQP) are a family of homologous water channel proteins, expressed in many epithelial and endothelial cell types involved in fluid transport (Agre 1997; Agre and Kozono 2003; Verkman 2002). AQPs increase the plasma membrane osmotic permeability by controlling the water flow to intra-and extra-cellular areas (Agre et al. 2002). AQPs basic structure is a homo-tetramer forming a central pore through which water, cations, and gases such as CO_2 flow. The major type of AQPs in the brain is AQP4 which has the highest water permeability coefficient (Amiry-Moghaddam and Ottersen 2003; Jung et al. 1994; Yang and Verkman 1997). AQPs, the predominant water channel protein in normal brain tissue, is especially expressed in astro-glial foot processes (Amiry-Moghaddam and Ottersen 2003; Haj-Yasein et al.; Nielsen et al. 1997). They have an important role in water hemostasis as well as edema formation under pathological conditions (Manley et al. 2000; Ribeiro Mde et al. 2006; Verkman et al. 2006). AQP4 knockout mice were shown to have dramatically reduced brain edema (Manleyet al. 2000;

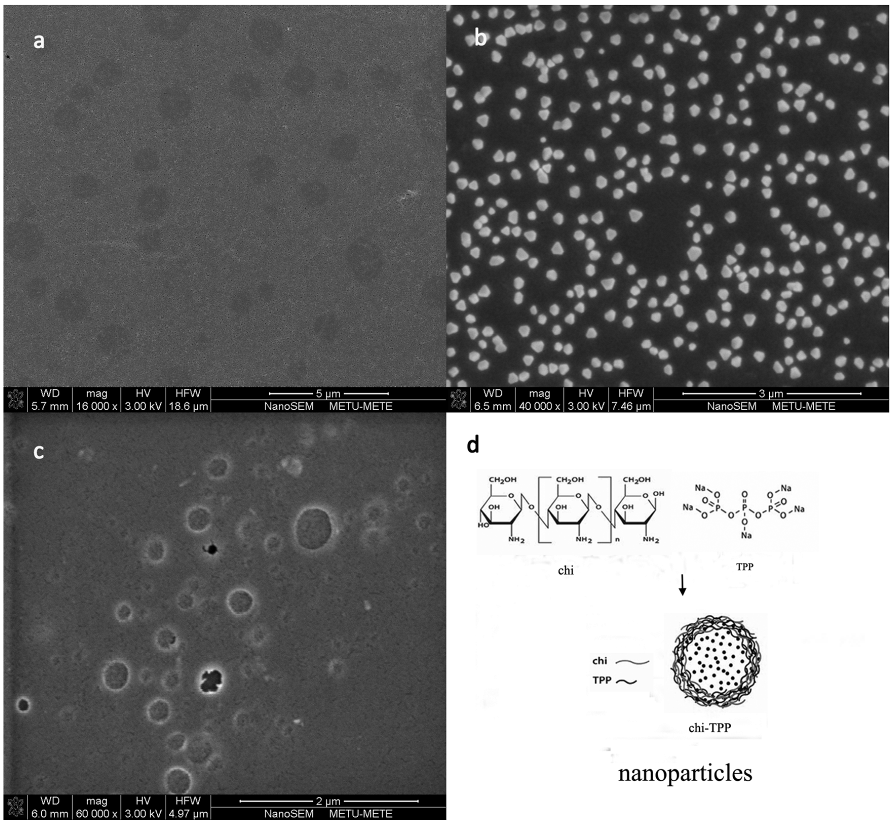

Fig. 1: Scanning electron micrographs of (a) CS nanoparticles, (b) CS-PEG nanoparticles, (c) CS-PEG-BIO nanoparticles, and (d) schematic illustration of CS nanoparticle preparation procedure.

Papadopoulos and Verkman 2007). It would be a great innovation to prevent edema formation in the brain by inhibiting AQP4 synthesis. As the brain swells inside the noncompliant skull, intracranial pressure rises, causing brain ischemia, herniation and eventually death (Saadoun et al. 2002; Tait et al. 2008). AQP4 antagonists cannot be used *in vivo* as they are toxic (Migliati et al. 2009; Yool et al.). Hence, a novel carrier nanoparticulate system was developed for AQP4 antisense oligonucleotides in this study.

Antisense oligonucleotides (ASOs) are short, single-stranded, about 15–25 nucleotide long synthetic molecules having complementary sequences of targeted mRNA or pre-mRNA by which the selective hybridization yields the inhibition of gene expression. These molecules are promising molecules in the treatment of diseases such as cancer, AIDS, etc. However, major problems in using such molecules in the treatment of diseases are lack of stability and poor penetration into the cell, thus a specific carrier is needed (Agrawal and Iyer 1997; Dias and Stein 2002a; Dias and Stein 2002b; Jason et al. 2004; Lee and Roth 2003; Lochmann et al. 2004). Enzymatic instability problems of oligonucleotides could be partially solved by chemical modifications like phoshorothioate binding (Campbell et al. 1990; Crooke 2000).

In this context, we used an oligonucleotide (5 /56-FAM/T*C*A*T*A*C*G*G*A*A*G*A*C*A*A*T*A*C*C*T *C-3) that was designed for the inhibition of AQP4's membrane linkage side subunit synthesis. Protection of ASOs from exo- and endo- nuclease activity is managed by means of phosphorothioate modification (Monia et al. 1996). As these kind of drugs are encapsulated into a polymer based nanoparticulate systems such as Chitosau (CS), their stability in blood would be enhanced. However, conventional polymeric systems are removed from the bloodstream by reticulo-endothelial system and this leads to an important reduction in pharmaceutical efficiency (Tosi et al. 2008). When nanoparticulate drug carrier systems are coated with a hydrophilic polymer such as polyethylene glycol (PEG), system stays longer in blood circulation and also pharmacokinetic properties are optimized (Torchilin et al. 2001). A conjugation of a nanoparticulate carrier system to a monoclonal antibody is needed to overcome the blood-brain barrier (BBB). This is accomplished *via* targeting them to the transferrin receptors located in the BBB, specifically on endothelial lining (Aktas et al. 2005; Olivier et al. 2002; Ulbrich et al. 2009).

The purpose of this study was to evaluate particle size, zeta potential, morphological properties, encapsulation efficiencies and release profiles of chitosan nanocarriers loaded with a phosphorothioate modified antisense oligonucleotide which could be a promising carrier system to transport nucleic acid based drugs to brain parenchyma in the case of edema and other brain diseases.

2. Investigations and results

2.1. Nanoparticle characterization

All types of CS nanoparticles were prepared according to the ionic gelation procedure. Nanoparticles were formed *via* the interactions between the positive amino groups of CS and negative anionic groups of tripolyphosphate (TPP). Nanoparticle surface was modified with PEG to provide enhanced blood residence time. To prepare "stealth" nanoparticles, the polymer (CS) was PEGylated before nanoparticle preparation.

SEM images of blank and loaded CS, CS-PEG, and CS-PEG-BIO nanoparticles are shown in Fig. 1a-c, respectively. The

Table 1: Characteristics (zeta potential and particle size) of blank and 5 μg ASO loaded nanoparticles

Polymer	Zeta potential (mV)		Particle size (nm)	
	Blank	Loaded	Blank	Loaded
CS	26 ± 2	23 ± 6	267 ± 14	338 ± 9
CS-PEG	18 ± 2	15 ± 3	116 ± 4	170 ± 9
CS-PEG-BIO	15 ± 3	14 ± 4	612 ± 6	671 ± 7
CS-PEG-BIO/MAb	18 ± 1	16 ± 1	686 ± 9	784 ± 5

Table 2: Association efficiency values (%) with the change of polymer, the amount of ASO loaded, and the type of the solution that ASO added

ASO amount	The solution that the ASO added into					
	Polymer solution			TPP solution		
	5 μg	10 μg	15 μg	5 μg	10 μg	15 μg
CS	98 ± 2	97 ± 1	92 ± 3	90 ± 2	92 ± 4	88 ± 2
CS-PEG	97 ± 2	95 ± 2	90 ± 3	89 ± 5	90 ± 4	82 ± 4
CS-PEG-BIO	93 ± 2	90 ± 3	87 ± 3	83 ± 4	85 ± 4	80 ± 4

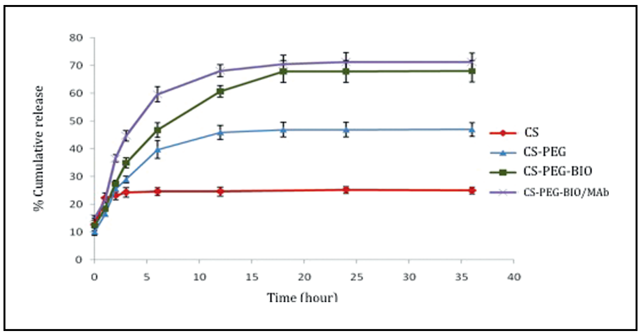

Fig. 2: *In vitro* release profiles of ASO from CS, CS-PEG, and CS-PEG-BIO nanoparticles (37 °C, pH 7.4).

particles showed a dense and generally spherical structure. Particle size and zeta potential of nanoparticles are given in Table 1.

2.2. ASO Encapsulation

There was a significant increase in the ASO encapsulation efficiency for the conjugated nanoparticles as compared with the non-conjugated nanoparticles (Table 2). Also, there was a significant increase of association efficiency when the oligonucleotide molecule was firstly added into the polymer solution during the manufacturing process.

2.3. In vitro release of ASO from nanoparticles

The release profiles of oligonucleotide from the different types of CS nanoparticles in phosphate buffered saline (PBS) at a pH of 7.4 are shown Fig. 2. A fast initial release for all types of polymers was determined. A slow release was observed after 6 h and the profiles reached plateau values after 24 h.

The percentage of ASO released from the system was increased by modification of the polymer. CS-PEG-BIO nanoparticles exhibited higher amounts of release than other derivatives. The percentage of the released drug was enhanced by the increasing hydrophobic characteristic of modified polymers.

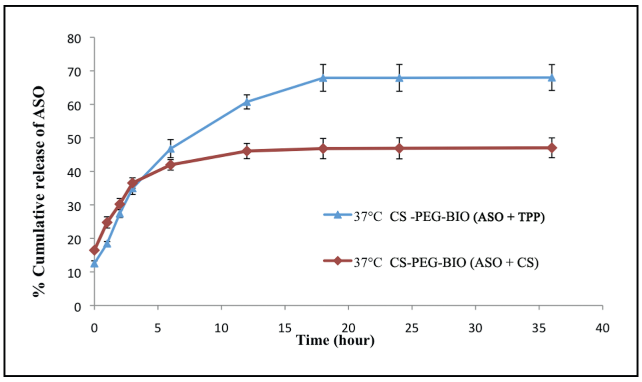

Fig. 3: *In vitro* release profiles of ASO from CS-PEG-BIO nanoparticles with different manufacturing processes (with the change of the solution ASO added) (37 °C, pH 7.4).

One more explored parameter that may affect the release profiles was the addition of ASO to TPP solution or to the polymer solution. Higher concentration of ASO was obtained in release medium when ASO is added into the TPP solution even they have lower association efficiencies and *vice versa* for the addition into the polymer solution.

3. Discussion

AQPs have important roles in brain water hemostasis under physiological conditions. During disease states like ischemia, trauma or brain tumor, they can lead to brain edema formation through AQP4. Up to date there is no specific anti edema treatment. Inhibition of AQP4 channels either with non-selective inhibitors or knocking down the gene and expression in transgenic animal models demonstrated promising results. Hence, new therapeutic methods that will specifically inhibit AQP4 activity could be used in clinical conditions.

ASOs are short, synthetic, modified nucleic acids that bind RNA and modulate its function by either gene silencing or regulation of RNA metabolism. They should have enough half-life within tissues, appropriate biodistribution, and efficient uptake in order to be effective as therapeutics. Modification of the phosphate backbone can increase the tissue half-life of ASOs. One of the mostly used modifications is phosphorothioate modification, which is the substitution of a non-bridging oxygen in the phosphate backbone with a sulfur atom. Phosphorothioate modification greatly improves the stability of ASOs and still allows cleavage by RNAse so that the antisense can reach the desired target without degradation.

One of the obstacles during treatment of neurological and psychiatric diseases is the penetration of therapeutics to the brain tissue. BBB is the main limitation and only allows penetration of a limited number of substances and drugs. However, there are several specialized transport systems located in the endothelia of the BBB such as receptor mediated transcytosis (Abbott et al. 2006). Transferrin receptor is used for this purpose.

In this study, we combined novel brain targeted nanoparticles with ASO designed against AQP4 binding site to syntrophin located on the dystrophino-glycan complex of astrocytic membranes. Through using this type of ASO, we have the advantage of loss of function rather than knockdown.

CS is a natural polysaccharide that allows the production of biocompatible and biodegradable drug delivery systems. Water solubility and positive charge of chitosan is extremely important in order to enable an interaction of negatively charged polymers and macromolecules with polyanions in an aqueous environment (Prabaharan 2008). Also the endothelial cells in the BBB have negative surface charge so that the positive surface charge of chitosan nanoparticles could increase the possibility of the interaction between the nanoparticles and brain vascular endothelium. On the other hand, mild conditions of chitosan nanoparticle preparation procedure through aqueous solubility of polymer provides enhanced stability of encapsulated drug.

Blank and ASO-loaded CS, CS-PEG, and CS-PEG-BIO nanoparticles formed spontaneously upon incorporation of the counter anion TPP into the corresponding CS, CS-PEG, and CS-PEG-BIO polymer solutions. Nanoparticles were formed as a result of the ionic interactions between the negative TPP and the positively charged amino groups of CS. The ratio of CS/TPP was established according to the preliminary studies.

ASO loading did not affect the general morphological properties of nanoparticles. Thus, it can be mentioned that both loaded and blank particles have spherical shape and show uniformity in particle size distribution.

It was observed that CS-PEG nanoparticles were smaller than CS nanoparticles. This may be explained by the colloid stabilization exerted by the PEG. On the other hand, when the surface of nanoparticles were modified with PEG-BIO or PEG-BIO/MAb, a significant increase in nanoparticle size was observed. This may be attributed to an increase in the hydrophobicity of the surface of the nanoparticles leading to aggregation due to presence of bulky groups on the surface. As shown in Table 1, blank nanoparticles displayed a zeta potential in the range of 26–18 mV. The addition of the negatively charged ASO resulted in a significant decrease in the zeta potential of the nanoparticles to the range 23–16 mV.

Under optimal conditions, higher values of ASO and chitosan were encapsulated inside the nanoparticles due to ionic interactions between positively charged chitosan and negatively charged oligonucleotide molecules. There were several factors which could affect the encapsulation of the oligonucleotide in the CS nanoparticles, such as CS/TPP ratio, auxiliary molecules, and interaction between the ASO and CS. Also blocking the ionic interaction between chitosan and ASO by modifying the groups would cause an increase in the interaction and the association as well. In this study, the AE% could be correlated with the surface modification of the CS. Also, the type of the solution (polymer or crosslinker) to which ASO were added was also important.

A fast initial release from all type of polymers suggests that some amount of the ASO could be located onto the surface of the nanoparticles to result in a burst release. After 6 h a slow release due to degradation of nanoparticles was determined.

The higher release amount when ASO was added into the TPP solution instead of into the polymer solution could be associated with the strong ionic interaction between chitosan and oligonucleotide molecules.

As a consequence of these results, this system could be regarded as a preferable pharmaceutical carrier system for nucleic acid based drug molecules. CS-PEG-BIO derivative of CS was the most suitable polymer for our purpose in the sense of higher EA and closer to intended release profile. The BIO modification was also provided to attach the Ab *via* streptavidin-biotin linkage and increased the preferability of the derivative.

This novel nanocarrier system demontrated a capacity for the association of the antisense oligonucleotide molecule. Due to the high interaction ability of the chitosan with the negatively charged oligonucleotide molecule and the release profiles suggested this oligonucleotide carrier system as promising and could be used as a drug carrier for nucleic acid based therapeutics like plasmid DNA, siRNA, etc. as well as antisense oligonucleotides. *In vivo* experiments are needed to prove the effectiveness and safety of this nanoparticulate carrier system and further studies are needed to exhibit the efficacy of the system for penetration through the BBB.

4. Experimental

4.1. Materials

Chitosan (Protasan Cl 113, Mw 150 kD, deacetylation degree: 75- 90%) was purchased from FMC Biopolymers (Norway). Pentasodium TPP, streptavidin, maleimidobenzoyl *N*-hydroxy-succinimide ester (MBS) were purchased from Sigma-Aldrich and Traut's Reagent (2-iminothiolane) was purchased from Thermo Fisher Scientific Inc, Pierce (USA). Functional-grade purified anti-mouse CD71 (transferrin receptor) (clone: R17217, catalog no: 16–0711) antibody was received from eBioscience (USA). Acetonitrile was HPLC grade, triethylamine (TEA) was synthesis grade and glacial acetic acid was analytical grade and they were all purchased from Merck KGaA (Darmstadt, Germany). Water was purified to 18.2 Ω.cm of electrical resistivity in-house using a Millipore Simplicity® UV system (USA). Oligonucleotide molecule was synthesized and purchased from IDT-DNA (USA). The antisense oligonucleotide was synthesized with phosphorothioate bond modification to increase molecule stability against nucleases and prolong plasma residence time (Campbellet al. 1990). Additionally, the molecule was tagged with a fluorescence emitting 6-FAM group.

4.2. Methods

4.2.1. Synthesis of CS-PEG

CS · HCl (100 mg, 0.50 mmol) was dissolved in H_2O (14.3 ml). MeO-PEG-OCH_2CO_2H has been synthesized from a commercially available MeO-PEG-OH according to known procedures (Royer and Anantharamaiah 1979). MeO-PEG-OCH_2CO_2H (17.8 mg, 3.47 µmol, M_n 5114) and *N*-hydroxysuccinimide (NHS) (2.03 mg, 0.018 mmol) were then added to the solution. Finally, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC · HCl) (27.1 mg, 0.141 mmol) was added in portions due to its instability. The resulting solution was stirred at room temperature for 22 h and then was ultrafiltered (Amicon, YM30) and lyophilized to yield CS-*g*-PEG (CS-PEG) as white foam (105 mg). The degree of PEGylation of this sample was 0.6% as determined by ^{1}H NMR (2% DCl in D_2O) (Fernandez-Megia et al. 2007; Novoa-Carballal et al. 2013).

4.2.2. Synthesis of CS-PEG-BIO

CS-PEG-BIO was synthesized following the same procedure as described above for CS-PEG from CS · HCl (100 mg, 0.50 mmol), HO_2C-PEG-biotin (14.6 mg, 3.65 µmol, M_n 4030) (Fernandez-Megiaet al. 2007), NHS (1.87 mg, 0.016 mmol), and EDC · HCl (27.0 mg, 0.141 mmol). After ultrafiltration and lyophilization CS-PEG-BIO was obtained as white foam (110 mg). The degree of PEGylation of this sample was 0.6% as determined by ^{1}H NMR (2% DCl in D_2O) (Fernandez-Megiaet al. 2007; Novoa-Carballalet al. 2013).

4.2.3. Preparation of chitosan and surface-modified chitosan nanoparticles

CS, CS-PEG, or CS-PEG-BIO nanoparticles were prepared by ionic gelation of CS, CS-PEG, or CS-PEG-BIO with TPP according to a procedure previously developed for the preparation of CS nanoparticles (Calvo et al. 1997). Preliminary experiments were performed in order to identify the optimal concentrations of CS and TPP for nanoparticle formation. Practically, CS nanoparticles were formed upon dropwise addition of 1 mL TPP aqueous solution (0.4 mg/mL) to 1 mL of CS aqueous solution (1.75 mg/mL). Likewise, CS-PEG and CS-PEG-BIO (1 mg/mL each) nanoparticles were prepared by dropwise addition of 0.4 mL TPP aqueous solution (0.84 mg/mL) to 1 mL of each of the corresponding aqueous polymer solutions. These solutions were then stirred under magnetic stirring at medium speed and room temperature. Nanoparticles were isolated by ultracentrifugation (10,000 rpm, 4 °C, 60 min) and resuspended in water by manual shaking.

4.2.4. Preparation of TfRMAb-conjugated nanoparticles

The SA/TfRMAb conjugates were prepared according to the procedure described by Karatas et al. (2009). Briefly, 7 mg of SA was dissolved in 250 µL 0.1 M EDTA (Solution I). Traut's reagent solution (100 µL, 4 mg/mL) and borate buffer (1.9 mL) were mixed and stirred for 5 min on magnetic stirrer at room temperature (Solution II). Equal volumes of Solution I and II were mixed and stirred for 90 min. To transform some of the amino groups into maleimide groups, antibody solution (100 µL, 1 mg/mL) was mixed with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide solution (5 µL, 5 mg/mL in dimethylformamide) 12.5 µL of streptavidin solution and 12.5 µL of antibody solution were added to CS-PEG-BIO nanoparticle suspension (500 µL); this suspension was vortexed for

30 s and incubated for 30 min at room temperature. Loading of ASO was performed in the same conditions as for TfRMAb-free chitosan nanoparticles.

4.2.5. ASO entrapment in chitosan–TPP nanoparticles

Different amounts of ASO (5, 10, 15 μg) were dissolved in ultrapure water for the association of ASO with the chitosan-TPP nanoparticles. ASO solution was added into crosslinker solution or polymer solution considering the final concentration of these solutions must be remained unchanged. Crosslinker (TPP) solution was added onto the polymer solution under constant magnetic stirring at room temperature. The particles were then incubated at room temperature for 10 min before use or further analysis.

4.2.6. Characterization of nanoparticles

Measurements of the size and zeta potential of the nanoparticles were performed in triplicate by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer Nanosystems 3000 HS (Malvern Instruments, UK). The size measurements were performed at 25 °C at a 90 ° scattering angle, and each measurement was recorded for 180 s.

4.2.7. Quantitative analysis of ASO

For the quantitative analysis of ASO, an HPLC method was developed and validated. An Agilent Technologies 1100 Series liquid chromatograph with a degasser (Tokyo, Japan), a quaternary pump, a thermostatted autosampler, a thermostatted column compartment, and a fluorescent detector FLD (Germany) was used. For data processing ChemStation (Agilent Technologies, USA) software was used. The column (Nucleodur C_{18}, 5 μm, 250 x 4.6 mm) was purchased from Higgins Analytical Inc. (California, USA). The flow rate of mobile phase was 1.0 mL/min at isocratic conditions. The injection volume was 20 μL, the column thermostat temperature was maintained at 55 °C and the detector wavelength was set at 495 nm for the excitation and 520 nm for emission wavelengths. The mobile phase comprised 0.1 M triethylammonium acetate (TEAA), pH 7.0, and acetonitrile (70:30, *V/V*).

4.2.8. Determination of ASO loading efficiency

The loading capacity of ASO (%) entrapped into the chitosan nanoparticles was obtained from the determination of free ASO concentration in the supernatant recovered after particle centrifugation (10,000 rpm at 4 °C for 1 h). Association efficiency was the percentage of entrapped ASO, (difference between the total amount of ASO added for the nanoparticles preparation and the amount of non-entrapped ASO remaining in supernatant after centrifugation) to the total amount of ASO added. The amount of free peptide was then measured in the clear supernatant by RP-HPLC method mentioned. The association efficiency (AE) was calculated according to the following equation:

$$\%AE = 100 \times (\text{total peptide amount} - \text{free peptide amount})/\text{total peptide amount}.$$

4.2.9. Evaluation of in vitro ASO release from CS-PEG-BIO/SA-TfRMAb nanoparticles

The nanoparticles were collected by centrifugation at 10,000 rpm for 1 h and every batch of nanoparticles were then resuspanded in 500 μL of PBS, pH 7.4, at 37 °C to quantify the released amount of ASO. Nanoparticle suspension was immobilized on a shaking water bath, the supernatant was isolated by centrifugation at 10,000 rpm for 20 min at 4 °C at varying time points, and the amount of ASO released was measured by RP-HPLC as described earlier.

Acknowledgements: This study is supported by The Scientific and Technological Research Council of Turkey (TUBITAK, Project Number: 110S460).

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