Aquaporins (AQP), 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 AQP 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 chitosan (CS) nanoparticles had positive zeta potential values and nanoparticle 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 nanocarrier 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. Imvasive techniques such as disruption 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) (Mitra et al. 2003), or biological methods like direct conjugation of drug molecules with antibodies (Partridge 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). AQP increase the plasma membrane osmotic permeability by controlling the water flow to intra- and extra-cellular areas (Agre et al. 2002). AQP basic structure is a homo-tetramer forming a central pore through which water, cations, and gases such as CO2 flow. The major type of AQP in the brain is AQP4 which has the highest water permeability coefficient (Amary-Moghaddam and Ottersen 2003; Jung et al. 1994; Yang and Verkman 1997). AQP, the predominant water channel protein in normal brain tissue, is especially expressed in astro-glial foot processes (Amary-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 (Manley et al. 2000; Yilmaz Capan, PhD, Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Sihhiye, Ankara, 06100 Turkey ycapan@hacettepe.edu.tr 

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 phosphorothioate binding (Campbell et al. 1990; Crooke 2000). In this context, we used an oligonucleotide (5′-5′-FAM/5′-C′A′C′G′G′A′A′A′C′A′T′C′T′-3′) that was designed for the inhibition of AQP4’s membrane linkage side subunit synthesis. Protection of ASOs from exonuclease 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 Chitosan (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 nanoparticleulate 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 triplyphosphate (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, respectively. The
Table 1: Characteristics (zeta potential and particle size) of polymers and resulting nanoparticles.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Zeta potential (mV)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>Loaded</td>
</tr>
<tr>
<td>CS</td>
<td>26 ± 2</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>CS-PEG</td>
<td>18 ± 2</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>CS-PEG-BIO</td>
<td>15 ± 3</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>CS-PEG-BIO/MAb</td>
<td>18 ± 1</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

Table 2: Association efficiency values (%) with the change of the solution that ASO added.

<table>
<thead>
<tr>
<th>ASO amount</th>
<th>The solution that the ASO added</th>
<th>5 μg</th>
<th>10 μg</th>
<th>15 μg</th>
<th>5 μg</th>
<th>10 μg</th>
<th>15 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>Blank</td>
<td>98 ± 2</td>
<td>97 ± 1</td>
<td>92 ± 1</td>
<td>90 ± 2</td>
<td>92 ± 4</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>CS-PEG</td>
<td>Blank</td>
<td>97 ± 2</td>
<td>95 ± 2</td>
<td>90 ± 3</td>
<td>89 ± 5</td>
<td>90 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>CS-PEG-BIO</td>
<td>Blank</td>
<td>93 ± 2</td>
<td>90 ± 3</td>
<td>87 ± 3</td>
<td>83 ± 4</td>
<td>85 ± 4</td>
<td>80 ± 4</td>
</tr>
</tbody>
</table>

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. We also developed novel brain targeted nanoparticles using transferrin co-assembly to improve drug delivery to brain tissue. The use of ASOs for the treatment of neurological and psychiatric diseases is promising. However, more research is needed to optimize the delivery of ASOs to the brain tissue.
charge of chitosan nanoparticles could increase the possibility of the interaction between the nanoparticles and brain vascula-
lar endothelium. On the other hand, mild conditions of chitosan nanoparticle preparation procedure through aqueous solubility of
delayed the 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.
AS0 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
taxted by the PEG. On the other hand, when the surface of nanoparticle 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 pres-
ence 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 inter-
actions 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 AS0 by modifying the groups would cause an increase in the interaction and the asso-
ciation 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 AS0 were added was also important.
A fast initial release from all type of polymers suggests that some amount of the AS0 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 associ-
ated 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 B/O modification was also provided to attach the Ab via streptavidin-biotin linkage and
increased the preference of the derivative.
This novel nanocarrier system demonstrated 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 sug-
gested this oligonucleotide carrier system as promising and effective delivery system for ASO. Due to the charge of chitosan
and the negatively charged AS0 molecule and the release profiles sug-
gested this oligonucleotide carrier system as promising and effective delivery system for AS0. Due to the charge of chitosan
and the negatively charged AS0 molecule and the release profiles sug-

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-hydroxysuccinimide ester (MBS) were purchased from Sigma-Aldrich and Thermo Fisher Scientific (2-iminothiolane) was purchased from Thermo Fisher Scientific Inc, Pierce (USA). Functionally
grade purified anti-mouse CD71 (transferrin receptor) (clone: R17217, catalog no: 16-0711) antibody was received from Biovendor (USA). Accu-
tinum was HPLC grade, methylcellulose (TEA) was synthesis grade and all the other materials were of analytical grade.
MeO-PEG-OH (17.8 mg, 3.47 µmol, 95% purity) and N-hydroxysuccinimide (NHS) (2.03 mg, 0.016 mmol) were then added to the solution. Finally, N-$\text{\text{`-dimethylamino}p}y\text{rovyl-vinc-
eric acid} (6-FAM) (17.8 mg, 0.14 mmol) was added in portions due to its instability. The resulting solution was stirred at room temperature for 22 h and then ultrafiltered (Amicon, YM30) and lyophilized to yield CS-$\text{\text{`-PEG (CS-PEG) as white foam (105 mg). The degree of PEGylation of this sample was 0.6% as determined by }^1\text{H NMR (2% DCI in D}_2\text{O (Fernandez-Megia et al. 2007, Novoa-Carballo 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. CS-PEG-BIO was further modified with PEG-BIO (4.35 µmol, Mw 4000) (Fernandez-Megia et al. 2007), NHS (1.87 mg, 0.016 mmol), and EDC (3-dimethylaminopropyl)-N-hydroxysuccinimide ester (MBS) were
den by 1H NMR (2% DCI in D$_2$O (Fernandez-Megia et al. 2007, Novoa-Carballo et al. 2013).

4.2.3. Preparation of chitosan and surface-modified chitosan nanopartic1es

CS, CS-PEG, or CS-PEG-BIO nanoparticles were prepared by ionic gela-
ation 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 iden-
tify the optimal concentrations of CS and TPP for nanoparticle formation. Practically, CS nanoparticles were formed upon dropwise addition of 1 mL, TPP aqueous solution (0.84 mg/mL) to 1 mL of CS aqueous solu-
tion (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 poly-
mer solutions. These solutions were then stirred under magnetic stirring at medium speed and room temperature. Nanoparticles were isolated by ultracentrifugation (100,000 rpm, 4 °C, 60 min) and resuspended in water by manual shaking.

4.2.4. Preparation of TIRMAs-conjugated nanoparticles

The S/ATIRMAs conjugates were prepared according to the proce-
dure described by Karatas et al. (2009). Briefly, 7 mg of SA was dissolved
in 250 mL of 0.1 M EDTA (Solution I). Traut’s reagent solution (100 µL, 4 mg/mL) and bovine buffer (1.9 mL) were mixed and stirred for 15 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 sodium/benzoyl-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

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

4.2.9. Evaluation of in vitro ASO release from nanoparticle suspension recovered after particle centrifugation (10,000 rpm at 4°C for 20 min). Association efficiency was the percentage of entrapped ASO, (difference between the amount of non-entrapped ASO remaining in supernatant after centrifugation and the total amount of ASO added) divided by the amount of ASO released into the supernatant at 55°C and the detector wavelength was set at 495nm for the excitation and 520nm for emission wavelengths. The flow rate of mobile phase was 1.0mL/min at isocratic conditions. The injection volume was 20µL 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 separated by centrifugation at 10,000 rpm for 20min at 4°C at varying time points, and the amount of ASO released was measured by RP-HPLC as described earlier.

4.2.10. Determination of ASO loading efficiency. The loading capacity of ASO (%) entrapped into the chitosan nanoparticles was calculated according to the following equation:

\[
\text{AE} = \frac{\text{total peptide amount} - \text{free peptide amount}}{\text{total peptide amount}} \times 100
\]

4.2.9. Evaluation of in vitro ASO release from CS-PEG-BIO3-TPP nanoparticles

The nanoparticles were collected by centrifugation at 10,000 rpm for 1h and each batch of nanoparticles was then resuspended in 500mL 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 separated by centrifugation at 10,000rpm for 20min at 4°C by varying time points, and the amount of ASO released was measured by RP-HPLC as described earlier.

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

References


