Comparative Evaluation of siRNA-loaded Chitosan Tripolyphosphate/Dextran Sulfate Nanoparticles Prepared by Adsorption Method: *In Vitro* Characterizations

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ABSTRACT

Human malignancies are one of the potent targeted diseases for therapeutics based on RNA. There are many barriers for effective delivery of siRNA to cell, since it is rapidly degraded by RNAse and macrophages. Polymeric nanoparticles are used extensively for the transport of genes across cell membranes. Among all polycationic polymers chitosan (CS) has been widely used. Cross-linkers are very important in the production of stable nanoparticles. The most commonly used cross-linkers in the preparation of CS nanoparticles are sodium tripolyphosphate (TPP) and dextran sulfate (DS). In current study, the researchers aim to formulate siRNA-loaded CS-TPP and CS-DS nanoparticles by adsorption method and to compare these nanoparticles on the basis of physicochemical characterizations. By changing the CS concentration from 0.1 to 0.4% w/v, the siRNA loaded CS-TPP and CS-DS nanoparticles were successfully prepared with average diameter of +24.8 ± 1.5 to +43.2 ± 0.5 and +44.7 ± 4.0 to +63.0 ± 1.2, respectively. siRNA-loaded CS-TPP nanoparticles showed spherical morphology while CS-DS nanoparticles showed irregular morphology. All nanoparticles showed high encapsulation efficiency. The release profile for nanoparticles prepared by both cross-linkers were also observed. Based on the results, siRNA-loaded CS-TPP nanoparticles show a great potential for clinical use in siRNA-based cancer therapies.

Key Words: siRNA, Tripolyphosphate, Dextran Sulfate, Chitosan Nanoparticles.

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INTRODUCTION

RNA interference (RNAi) is the process of specific post transcriptional gene silencing triggered by double stranded RNAs (dsRNAs). Non-specific effects triggered by small interfering RNAs (siRNAs) complicate the use of RNAi to specifically down regulate gene expression [1]. Recently, it has been shown that chemically synthesized Dicer substrate siRNA (DsiRNA) (27-bp duplexes) is more effective than traditional siRNA (21 bp duplexes) [2].

Human malignancies are one of the targeted diseases for RNA-based therapeutics [3]. Vascular endothelial growth factor (VEGF) is an important angiogenic factor associated with tumor growth and metastasis in a wide variety of solid tumors [4]. Although, angiogenesis is a fairly complex process, however the VEGF biochemical pathway has been found to play an early key role in tumor growth [5]. It has been shown that the anti-tumor efficacy is achieved for a variety of malignant diseases, by targeting angiogenesis [6, 7].

Polycationic polymers are widely used materials for the transportation of material across cell membranes. Commonly utilized synthetic polymers include linear macromolecules such as polyethyleneimine (PEI), poly-L-lysine (PLL), and diethylaminoethyl-dextran (DEAE-

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dextran), as well as branched polymers such as poly(amidoamine) (PAMAM) dendrimers. Surprisingly, it seems that different classes of natural polycationic polymers play a similar role by different mechanisms, including the cell-penetrating peptides (CPPs) and chitosan (CS). These polycationic polymers are known as carriers in drug delivery [8].

CS, a cationic polysaccharide, is biocompatible, less toxic, non-immunogenic, and degradable by enzymes. It has been used widely in many drug delivery applications, especially in gene delivery systems, because its positively charged amines allow electrostatic interactions with negatively charged nucleic acids to form stable complexes. For more than a decade, CS has been known as a gene vector for oligonucleotides (ON) and siRNA [9].

Cross-linkers play an important role in the preparation of stable nanoparticles. The most widely used cross-linker in the preparation of CS nanoparticles is sodium tripolyphosphate (TPP), which is a small, anionic, non-toxic cross-linking agent [10]. The main constraint of TPP is limited sites for ionotropic gelation [11]. Another polyanion commonly used to cross-link CS is dextran sulfate (DS), which is a biodegradable and biocompatible compound [12].

In the present study CS nanoparticles with two different cross-linkers were formulated. Nanoparticles were loaded with siRNA by using adsorption method. The prepared nanoparticles were characterized on the basis of their particle size, surface charge, morphology, and in vitro release study.

MATERIALS

Low molecular weight CS of Mw 190 kDa with a 75–85% DD was purchased from Sigma-Aldrich (USA). TPP and DS (Mw = 500 kDa) were obtained from Merck (Germany). Double stranded siRNA (sense strand 5´-GAUUAUGUCCGGUUAUGUAUU-3´, antisense strand 3´-UACAUAACCGGACAUAAUCUU-5´) of 21 bp in length targeting against VEGF gene, was purchased from Dharmacon (USA). phosphate-buffered saline (PBS; pH 7.4) and acetic acid and other chemicals used were of analytical grade and used as received.

METHODS

Ionic Gelation Method

CS nanoparticles were prepared via ionic gelation method with some modifications [9]. A series of CS solutions (0.1%, 0.2%, 0.3%, 0.4%, w/v) were prepared by dissolving the required amount of CS in 2% v/v glacial acetic acid. Three cross-linking agents (TPP/DS) were used to prepare CS nanoparticles (CS-TPP, CS-DS). TPP and DS solutions (0.1% w/v) were prepared by separately dissolving them in deionized distilled water. Each type of CS nanoparticles was prepared by adding 1.2 mL of their respective cross-linker aqueous solution dropwise using a pipette into 3 mL of CS solution (0.1%, 0.2%, 0.3%, and 0.4% w/v) at 25˚C under a constant magnetic stirring (MS MP8 Wise Stir Wertheim, Germany) at 700 rpm for 30 min. The resulting nanoparticles were left for another 30 min at 25˚C before further analysis.

siRNA Adsorption

The CS-TPP/DS nanoparticles were prepared using ionic gelation method prior to siRNA adsorption. A volume of 500 μL siRNA solution (10 µg/mL) was pipetted dropwise to 500 μL of CS nanoparticles suspension and the interaction tube was quickly inverted up and down for 10 sec. The particles were then left for two hours at 25˚C before further analysis.

In Vitro Characterisation

Determination of Particle Size and Polydispersity Index

The mean particle diameter (z-average) and polydispersity index (PDI) of freshly prepared unloaded CS-TPP/DS nanoparticles, siRNA-loaded CS-TPP/DS nanoparticles were determined by photon correlation spectroscopy (PCS) using ZS-90 Zetasizer (Malvern Instruments, Worcestershire, UK). Each sample was measured in triplicate at 25°C and data are reported as mean ± standard deviation (SD).

Determination of Zeta Potential

The mean zeta potential (surface charge) of freshly prepared unloaded CS-TPP/DS nanoparticles, siRNA loaded CS-TPP/DS nanoparticles were determined by photon correlation spectroscopy (PCS) using ZS-90 Zetasizer (Malvern Instruments, Worcestershire, UK). Each sample was measured in triplicate at 25°C, and data are reported as mean ± SD.

Determination of Particle Morphology

Morphological characterization of siRNA-loaded CS-TPP/DS nanoparticles, was carried out using a TEM (Tecnai Spirit, FEI, Eindhoven, The Netherlands).

Entrapment Efficiency

The entrapment efficiency of siRNA (% entrapped) was measured using a UV-vis spectrophotometer (Shimadzu UV-1800, Shimadzu Scientific Instruments, Japan) at 260 nm:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Csample} - \text{Csupernatant}}{\text{Csample}} \times 100 \quad (1)$$

where C_{sample} is the concentration of siRNA added and $C_{supernatant}$ is the concentration of siRNA in the supernatant. All measurements were performed in triplicate, and data were reported as mean ± SD.

In Vitro Release Studies

The release profile of siRNA- loaded CS-TPP nanoparticles (CS concentration 0.3% w/v) was studied in PBS at pH 7.4. Samples (4 mL) were centrifuged at 35,000 rpm for 30 min at 25˚C, and the pellets were re-suspended in PBS (3 mL), pH 7.4. The mixture was placed on a magnetic stirrer with a stirring speed of 100 rpm at 37˚C for 15 days. At pre-determined time intervals, samples were centrifuged at 35,000 rpm for 30 min at 25˚C. Then, a whole volume of supernatant was taken for analysis and replaced with an equivalent volume of fresh buffer solution. The amount of released siRNA in the supernatant was analyzed by a UV-vis spectrophotometer (Shimadzu 1800) at a wavelength of 260 nm.

RESULTS

1. Particle size and PDI

The mean particle size of siRNA-loaded CS-TPP/DS nanoparticles was increased significantly by increasing the CS concentration from 0.1% to 0.4% w/v, as shown in Table1. PDI values of these nanoparticles were ranged from 0.3 to 1, and the values also increased as CS concentration increased from 0.1% to 0.4% w/v (Table 1).

Table 1. Particle size, PDI, and zeta potential of siRNA-loaded CS-TPP/DS nanoparticles prepared at different CS concentrations. The siRNA was loaded onto the CS nanoparticles by adsorption method, n=3.

CS concentration (% w/v)	Particle size (nm) ± SD	PDI ± SD	Zeta potential (mV) ± SD
CS-TPP-siRNA			
0.1	114.9 ± 6.1	0.3 ± 0.17	+24.8 ± 1.5
0.2	258.6 ± 2.7	0.4 ± 0.20	+31.7 ± 1.1
0.3	310.6 ± 4.3	0.5 ± 0.13	+37.5 ± 1.2
0.4	410.2 ± 8.6	0.6 ± 0.18	+43.2 ± 0.5
CS-DS-siRNA			
0.1	832.9 ± 26.2	0.3 ± 0.14	+44.7 ± 4.0
0.2	876.5 ± 16.0	0.4 ± 0.08	+48.9 ± 2.0
0.3	1011.6± 83.9	0.7 ± 0.19	+61.9 ± 1.1
0.4	1361.0± 35.3	0.8 ± 0.26	+63.0 ± 1.2

2. Zeta potential

The zeta potential of siRNA adsorbed onto CS-TPP/DS increased as the concentration of CS increased (Table 1), because of the increase of unneutralized NH_3^+ groups of CS that did not counteract with the fixed amount of negatively charged siRNA [13].

3. Morphology

Unloaded CS-TPP nanoparticles were spherical in shape. Similar morphology was also observed when nanoparticles were loaded with siRNA by adsorption method, as shown in Figure 1. However, CS-DS nanoparticles had irregular morphology and were aggregated as shown in Figure 2.

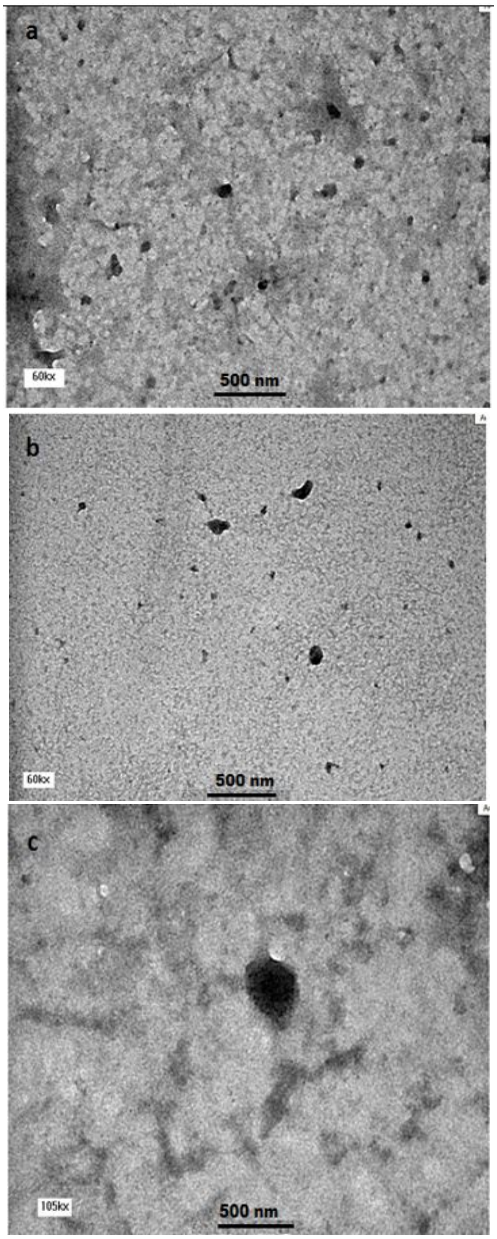
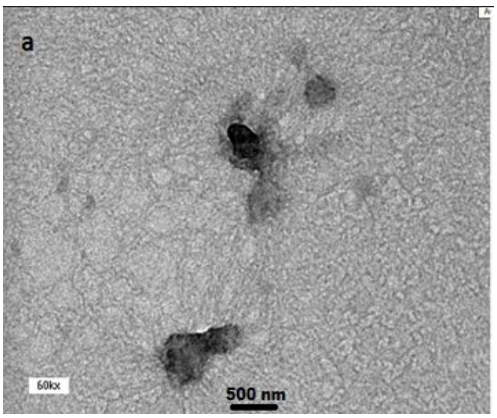


Figure 1. TEM images of CS-TPP-siRNA nanoparticles prepared from 0.1% w/v CS (a), 0.2% w/v CS (b), and 0.3% w/v CS (f) at different magnifications (60 kx, 105 kx).



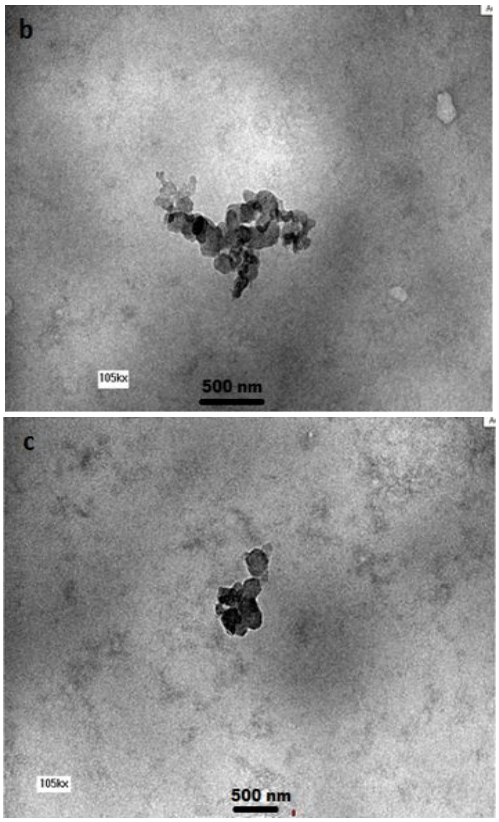


Figure 2: TEM images of CS-DS-siRNA nanoparticles prepared from 0.1% w/v CS (a), 0.2% w/v CS (b), and 0.3% w/v CS (f) at different magnifications (60 kx, 105 kx).

siRNA encapsulation efficiency

siRNA encapsulation efficiency achieved for CS-TPP/DS nanoparticles by adsorption method was in the range of 82% to 96%. as shown in Figure 3.

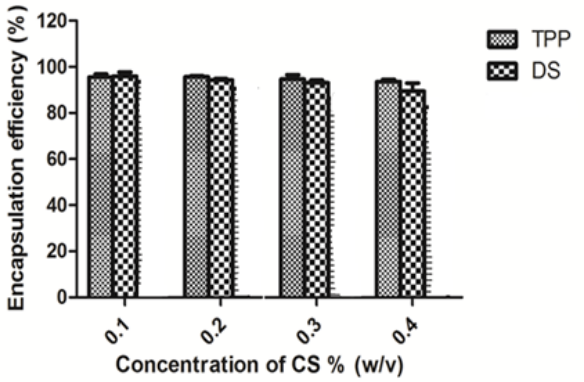


Figure 3. Encapsulation efficiency of siRNA adsorbed onto CS-TPP/DS nanoparticles prepared using different CS concentrations (0.1% to 0.4% w/v CS), n=3.

In vitro release

The *in vitro* release profiles of siRNA-loaded CS-TPP/DS nanoparticles in PBS at pH 7.4 are shown in Figure 4. The release of siRNA was divided into two stages based on the release rate. In the first stage, rapid siRNA release was observed from CS-TPP and -DS nanoparticles in the first 12 h, resulting in 18% and 24% of cumulative release, respectively, due to the diffusion of siRNA bound at the particle surface.

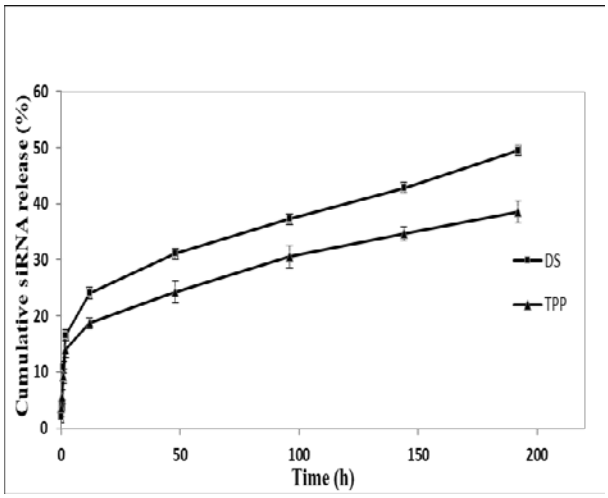


Figure 4: The release profile of siRNA-loaded CS-TPP/DS nanoparticles at pH 7.4, *n*=3.

DISCUSSION

The significant increase of mean particle size of siRNA-loaded CS-TPP/DS nanoparticles by increasing the CS concentration from 0.1% to 0.4% w/v was expected due to the lower viscosity at lower concentrations of CS. CS-TPP-siRNA nanoparticles had the smallest particle size at all concentrations studied (114.9 ± 6.1 to 410.2 ± 8.6nm). In contrast, CS-DS-siRNA nanoparticles had significantly larger particles which were in the range of 832.9 ± 26.2 to 1361.0 ± 35.3 nm (Table 1), respectively, due to larger size of DS molecules or tendency of these particles to aggregate. PDI values of formulations using TPP as a cross-linking agent were within the acceptable range, indicating narrow distribution of particle size. The high PDI values and large particle sizes might be attributed by the presence of aggregations. These results showed the effect/influence of different cross-linkers on the physical features of CS nanoparticles.

The zeta potential of siRNA adsorbed onto CS-TPP/DS increased as the concentration of CS increased (Table 1), because of the increase of unneutralized NH_3^+ groups of CS that did not counteract with the fixed amount of negatively charged siRNA [13].

The decrease in zeta potential after siRNA adsorption was due to neutralization of siRNA phosphate groups with CS NH_3^+ groups, lowering the positive charges of cationic CS [13, 14]. Moreover, the nanoparticles containing TPP exhibited low zeta potential in comparison with

nanoparticles containing DS (Table 1). This might be due to the strong cross-linking between TPP and the NH_3^+ groups of CS, which would result in higher extent of neutralization of NH_3^+ groups and thereby, lowering the zeta potential. Higher zeta potential was also observed for other cross-linkers, due to the lower degree of cross-linking between DS and CS [14]. Moreover, siRNA adsorbed CS-TPP nanoparticles showed spherical morphology. However siRNA adsorbed CS-DS nanoparticles showed irregular morphology and aggregation due to inefficient cross-linking as shown in Figure 2**.**

In general, the encapsulation efficiencies of all formulations decreased when CS concentration was increased, as shown in Figure 3. When CS concentration was increased from 0.1% to 0.4% w/v, the encapsulation efficiency of siRNA-loaded CS-TPP and CS-DS, formulations was decreased from 95% ± 1.2 to 93% ± 1.0, and 95% ± 1.2 to 89.4% ± 3.1, respectively.

The initial release of siRNA from CS-DS-siRNA was greater than that from CS-TPP-siRNA nanoparticles, which could be due to the lower binding efficiency and poor control of diffusion-based release of DS [15]. In the second stage, siRNA was released at a sustained constant rate from CS-TPP/DS nanoparticles for up to 8 days. TPP contributed to the least cumulative siRNA release (38%) in the second stage, followed by DS (49%) as shown in Figure 4

CONCLUSION

CS nanoparticles loaded with siRNA were successfully prepared by adsorption methods using two different cross-linkers (TPP, and DS). CS-TPP nanoparticles produced the smallest particle size, with high entrapment and binding efficiencies. Of the cross-linkers studied, nanoparticles prepared by TPP created the most stable system, leading to slow burst release of siRNA. Based on the results, siRNA-loaded CS-TPP nanoparticles showed a great potential for clinical applications in siRNA-based cancer therapies.

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Conflict of Interest

Authors don't have any conflict of interest

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