Preparation and in *vitro* characterizations of VEGF siRNA-loaded Chitosan- Poly-D-glutamic acid sodium salt nanoparticles

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ABSTRACT

Human diseases have been one of the most commonly targeted for therapeutics based on RNA. Polymeric nanoparticles either natural or synthetic have been used widely for the genes' transport across cell membranes. CS has been widely used as a polycationic polymer for drug delivery. Cross-linkers play a vital part in the preparation of stable nanoparticles. The aim of the present study was to formulate siRNA-loaded CS- poly-D-glutamic acid sodium salt (PGA) nanoparticles using ionic gelation (adsorption method) and assess it considering the physicochemical properties. The siRNA loaded CS-PGA nanoparticles were successfully prepared, and the results showed that the average diameter of nanoparticles was in the range from 400.0 ± 55.6 to 3575.5±71.2 by changing the CS concentration from 0.1 to 0.4% w/v. The zeta potential observed was in the range of +60.8 ± 1.1 to +38.2 ± 1.4 by changing the CS concentration from 0.1 to 0.4% w/v. siRNA-loaded CS-PGA nanoparticles showed aggregation and irregular morphology. All nanoparticles showed the high encapsulation efficiency in the range of 82% to 96%. The release profile for CS-PGA nanoparticles showed sustained release of siRNA. Based on the results, siRNA-loaded CS-PGA nanoparticles could be used for further optimization studies such as stability and steric hindrance.

Key Words: siRNA, VEGF, polyD- glutamic acid, chitosan nanoparticles.

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INTRODUCTION

RNA interference (RNAi) is the process of sequencing 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]. It has been recently shown that chemically synthesized Dicer substrate siRNA (DsiRNA) (27-bp duplexes) is more potent than traditional siRNA (21 bp duplexes) [2]. Applying DsiRNA to trigger RNAi should lead to the increased effectiveness and longer stability of RNAi, at lower concentration compared with siRNA [3].

Human malignant tumors have been tried to be treated by RNA-based therapeutics [4]. Vascular endothelial growth factor (VEGF) has been a major angiogenic factor connected with tumor growth and metastasis in a wide variety of solid tumors [5]. There are however barriers to the effective delivery of siRNA to cell, since it is rapidly degraded by RNA*se* and macrophages [6, 7].

Polycationic polymers are used extensively for the transport of materials across cell membranes. Synthetic polymers widely employed have included linear macromolecules such as polyethyleneimine (PEI), poly-L-lysine (PLL), diethylaminoethyl-dextran (DEAE-dextran), and branched polymers such as poly (amidoamine) (PAMAM) dendrimers. Interestingly, several classes of natural polycationic polymers have appeared to play a similar role, although, by a different mechanism, including the cell-penetrating peptides (CPPs) and chitosan (CS). These polycationic polymers have gained

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popularity as carriers in drug delivery [8].

CS has been known to be biocompatible, less toxic, non-immunogenic, and degradable by enzymes. CS is a cationic polysaccharide, and because it includes positively charged amines that make the electrostatic interactions possible by negatively charged nucleic acids that make stable complexes, it has been widely applied, particularly in gene delivery systems in several delivery applications. CS has been studied for more than a decade as a gene vector for oligonucleotides (ON) and siRNA.

The stability of nanoparticles at all times remained a vital issue for the efficient drug delivery. Studies have verified that stability and efficiency of nanoparticles have been greatly dependent on types of cross linkers used. Cross-linkers have a very essential function in preparing strong nanoparticles. Lately, several research studies have been done on poly-D-glutamic acid sodium salt (PGA) as an anion which is used in preparing CS nanoparticles. PGA is an anionic polypeptide which is naturally solved in water, and it is biodegradable and edible, and there has been no report of toxicity. PGA has good tissue affinity, which has attracted researcher interests [9].

MATERIALS

Low molecular weight CS of Mw 190 kDa with a 75–85% DD was purchased from Sigma-Aldrich (USA). PGA was 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 Thermoscientific Dharmacon (USA) phosphate-buffered saline (PBS) pH 7.4 and acetic acid and other chemicals used were of the analytical grade and used as received.

METHODS

Ionic Gelation Method

CS nanoparticles were prepared via ionic gelation method with some modifications [10]. A series of CS solutions (0.1%, 0.2%, 0.3%, 0.4%, w/v) was prepared by dissolving the required amount of CS in 2% v/v glacial acetic acid. A cross-linking agent (PGA) was investigated to prepare CS nanoparticles (CS-PGA). PGA solution (0.1% w/v) was prepared by separately dissolving them in deionized distilled water. Each type of CS nanoparticles was prepared by adding 1.2 mL in their cross-linker aqueous solution dropwise using a pipette in 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-PGA nanoparticles were prepared using ionic gelation method prior to siRNA adsorption. A volume of 500 μL siRNA solution (10µg/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 h at 25˚C before further analysis.

IN VITRO CHARACTERISATIONS

Determination of Particle Size and Polydispersity Index

Photon correlation spectroscopy (PCS) using ZS-90 Zetasizer (Malvern Instruments, Worcestershire, UK) was used to identify the mean particle diameter (z-average) and polydispersity index (PDI) of freshly prepared unloaded CS-PGA nanoparticles, and siRNA-loaded CS-PGA nanoparticles. Each sample was measured in triplicate at 25°C, and the data was reported as mean ± standard deviation (SD).

Determination of Zeta Potential

The mean zeta potential (surface charge) of freshly prepared unloaded CS-PGA nanoparticles, and siRNA loaded PGA 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 the data was reported as mean ± SD.

Determination of Particle Morphology

Morphological characterization of siRNA-loaded CS-PGA 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 the added siRNA and $C_{supernatant}$ is the concentration of siRNA existing in the supernatant. All measurements were done in triplicate, and the obtained data was represented as mean ± SD.

In Vitro Release Studies

The release profile of siRNA- loaded CS-PGA nanoparticles (CS concentration 0.1% w/v) was studied in PBS at pH 7.4. The 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 obtained mixture was stirred on a magnetic stirrer with a stirring speed of 100 rpm at 37˚C for 15 days. At the time intervals which were determined before, the samples were centrifuged at 35,000 rpm for 30 min at 25˚C. After that, the complete volume of supernatant was analyzed and substituted with the equal amount of fresh buffer solution. A UV-vis spectrophotometer (Shimadzu 1800) was used to analyze the amount of released siRNA in the supernatant at a wavelength of 260 nm.

RESULTS

Unloaded CS- PGA Nanoparticles

i. Particle size and PDI

The mean particle size of CS-PGA nanoparticles (ranged from 980.9 ± 55.6 to 1940.2 ± 91.2,) was increased significantly by increasing the CS concentration from 0.1% to 0.4% w/v, as shown in Table 1. This phenomenon was expected as the lower viscosity of lower CS concentration resulted in a better solubility and subsequently, better interaction between CS and cross-linkers, and thus, produced smaller particle size [11]. The PDI values of the formulations using PGA as a cross-linking agent were high, implying a broad distribution of the particle size.

Table 1. Particle size, PDI, and zeta potential of unloaded CS-PGA nanoparticles prepared at different CS concentrations, *n*=3

CS concentration (% w/v)	Particle size (nm) ± SD	PDI ± SD	Zeta potential (mV) ± SD
CS-PGA nanoparticles			
0.1	980.9 ± 55.6	0.6 ± 0.09	+68.7 ± 1.0
0.2	1466.7±23.7	0.8 ± 0.10	+74.4 ± 0.9
0.3	1571.7±40.5	0.8 ± 0.05	+77.8 ± 1.2
0.4	1940.2 ±91.2	1.0 ± 0.09	+83.0 ± 2.0

ii. Zeta potential

Higher zeta potential was seen for the other cross-linkers probably because of their lower degree of cross-linking between PGA and CS which resulted in more unneutralized NH_3^+groups [12].

siRNA-Loaded CS- PGA Nanoparticles by Adsorption Method

i. Particle size, PDI, Zeta potential

The mean particle size of siRNA-loaded CS- PGA nanoparticles was remarkably enlarged by boosting the CS concentration from 0.1% to 0.4% w/v, as presented in Table 2.

By increasing the concentration of CS, the zeta potential of siRNA adsorbed onto CS- PGA was boosted (Table 2), this occurred because unneutralized NH_3^+ groups of CS did not act against the stable amount of negatively charged siRNA [13].

Table 2. Particle size, PDI, and zeta potential of siRNA-loaded CS-PGA nanoparticles prepared at different CS concentrations and 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
0.1	400.0 ± 55.6	0.5 ± 0.08	+60.8 ± 1.1
0.2	961.2 ± 16.0	0.7 ± 0.20	+63.8 ± 2.2
0.3	2418.2±70.5	0.9 ± 0.01	+44.2 ± 1.0
0.4	3575.5±71.2	1.0 ± 0.04	+38.2 ± 1.4

ii. Morphology

CS-PGA showed the presence of aggregations after the adsorption with siRNA (Figure1).

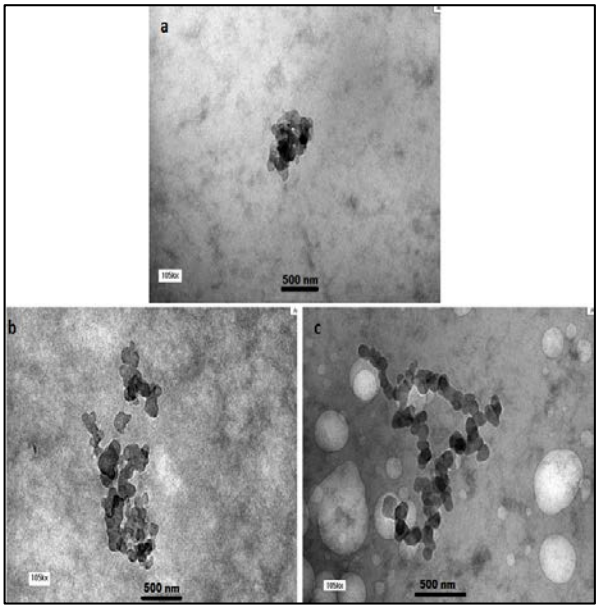

Fig. 1. TEM images of CS-PGA-siRNA nanoparticles prepared from 0.1% w/v CS (a), 0.2% w/v CS (b), and 0.3% w/v CS (c) at different magnifications (60 kx, 105 kx).

iii. siRNA encapsulation efficiency

siRNA encapsulation efficiency achieved for CS- PGA nanoparticles by adsorption method was in the range of 82% to 96%. In general, the encapsulation efficiencies of all the formulations decreased when CS concentration was increased, as shown in Figure 2. The encapsulation efficiency of siRNA-loaded CS-PGA formulations was decreased from 93.5% ± 1.1 to 82% ± 1.8, respectively,

when CS concentration was increased from 0.1% to 0.4% w/v.

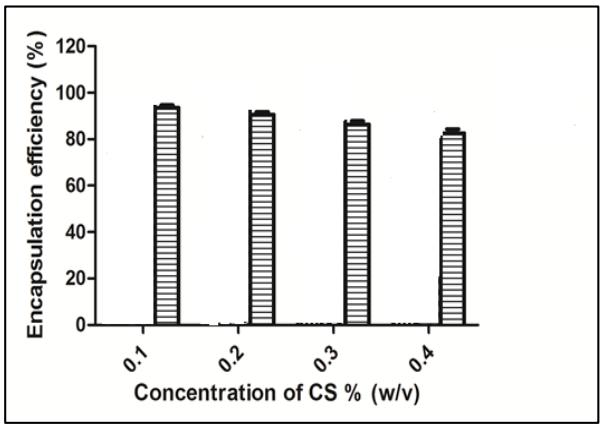

Fig. 2 Encapsulation efficiency of siRNA adsorbed onto CS-PGA nanoparticles prepared using different CS concentrations (0.1% to 0.4% w/v CS) and siRNA was adsorbed onto the CS nanoparticles, *n*=3.

iv. Stability of siRNA loaded CS-PGA nanoparticles

In this experiment, heparin was used to displace/dissociate the adsorbed siRNA from CS nanoparticles. In comparison to the control (untreated free siRNA), trailing bands were observed for adsorbed siRNA onto CS-/PGA nanoparticles (0.1% CS concentration) (Figure 3), indicating the release of siRNA from the nanoparticles but in a slower migration.

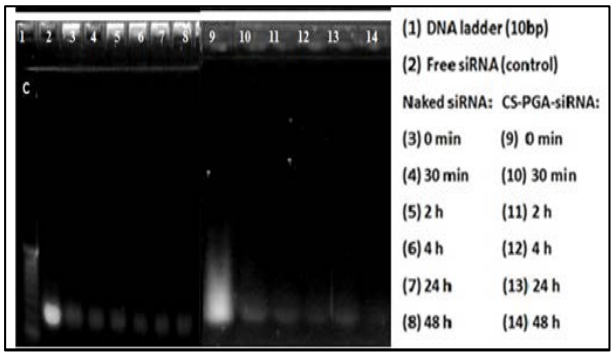

Fig. 3.Electrophoretic mobility of siRNA from CS-PGA nanoparticles, following incubation in RPMI medium containing 10% FBS for 48 h incubation.

v. *In vitro* release

The *in vitro* release profiles of siRNA-loaded CS-PGA nanoparticles in PBS at pH 7.4 have been shown in Figure 4. The release of siRNA was divided into 2 stages based on the release rate. In the first stage, rapid siRNA release in the first 12 h was observed from CS- PGA nanoparticles, resulting in 25% of the cumulative release, respectively due to the diffusion of siRNA bound at the particle surface. The initial release of siRNA from CS-PGA-siRNA nanoparticles was greater, which could be related to the lower binding efficiency and poor control of the diffusion-based release of PGA [14]. In the second stage, siRNA was released at a sustained constant rate from CS- PGA nanoparticles for up to 8 days.

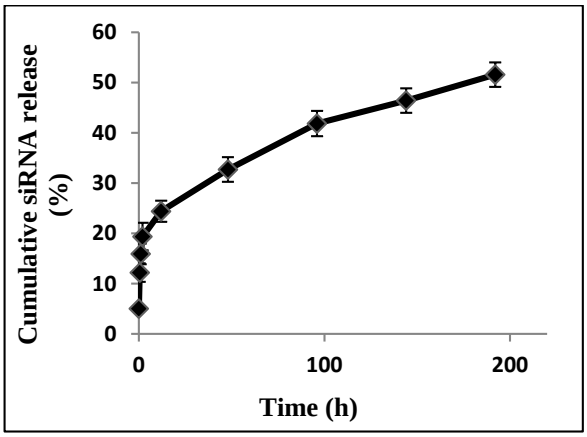

Fig. 4 :The release profile of siRNA-loaded CS-TPP/DS/PGA nanoparticles (by adsorption method) at pH 7.4, *n*=3.

DISCUSSION

The mean particle size of siRNA-loaded CS- PGA nanoparticles was increased significantly by increasing the CS concentration from 0.1% to 0.4% w/v, as shown in Table 2. This was expected due to the lower viscosity at lower concentrations of CS. CS-PGA-siRNA nanoparticles had significantly larger particles which were in the range of 400.0 ± 55.6 to 3575.5 ± 71.2 due to the larger size of PGA molecules or tendency of these particles to aggregate as shown in Table 2.

The decrease in zeta potential after siRNA adsorption was due to the neutralization of siRNA phosphate groups with CS NH_3^+ groups, lowering the positive charges of cationic CS [12, 13]. The only exception was observed for 0.3% and 0.4% w/v CS concentration of CS-PGA-siRNA which showed lower zeta potential when compared to 0.1% and 0.2% w/v CS concentration. This could be explained by the fact that CS concentration above the critical concentration would contribute to the spontaneous aggregation of the particles. At critical concentration, the particle size increased drastically, along with the sudden drop of zeta potential. This finding was in accordance with a previous study reported by Gan et al. (2005) as shown in Table 2 [15].

Additionally, because of the lower degree of cross linking between PGA and CS which was obtained from an excess positive charge from the unneutralised CS NH_3^+ groups, the aggregation was most probably to happen with PGA [15]. It has been represented that under acidic conditions, there would be electrostatic repulsion and interchain hydrogen bonding interactions in the equilibrium when

CS concentration was under a particular limit. At the points over this limit, NH3+ groups are likely to form surface shielding and the intermolecular hydrogen bonding is dominated during the cross linking process. Therefore, a lot of CS molecules get involved in the cross-linking of a single particle, and at the end, a big particle is formed. Because the electrostatic repulsion among particles is not enough for keeping the stability of these big particles, flocculent precipitates are formed [11]. siRNA encapsulation efficiency achieved for CS-PGA nanoparticles by the adsorption method was in the range of 82% to 96%. In general, the encapsulation efficiencies of all the formulations decreased when CS concentration was increased, as shown in Figure 2.

In heparin displacement assay, heparin was used to displace/dissociate the adsorbed siRNA from CS nanoparticles. In comparison to the control (untreated free siRNA), the trailing bands were observed for the adsorbed siRNA onto CS-PGA nanoparticles (Figure 3), indicating the release of siRNA from the nanoparticles but in a slower migration. Thus, it was assumed that siRNA had been partially unbound from the nanoparticles, and some of the unbound siRNA might have interacted with components in FBS. This also explains why fader bands were observed compared to the control as not all siRNA had been released and in its free form. Naked siRNA started to degrade as early as 0 min, right after the siRNA was mixed with serum and prior to the freezing steps [13]. Furthermore, most siRNA was degraded after 48 h of incubation. On the other hand, siRNA loaded CS-PGA nanoparticles were partially protected after 48 h incubation as less dense bands were observed, and the degradation started as early as 0 min as shown in Figure 3.

The release of siRNA (Figure 4) was divided into 2 stages based on the release rate. In the first stage, rapid siRNA release in the first 12 h was observed from CS- PGA nanoparticles, resulting in 25% of cumulative release, respectively due to the diffusion of siRNA bound at the particle surface. The initial release of siRNA from CS-PGA-siRNA nanoparticles was greater, which could be related to the lower binding efficiency and the poor control of diffusion-based release of PGA [14]. In the second level, CS- PGA nanoparticles released siRNA at a stable speed for up to 8 days.

CONCLUSION

CS nanoparticles loaded with siRNA were successfully prepared by the adsorption methods using PGA cross-linkers. CS-PGA nanoparticles loaded with siRNA showed small particle size of 0.1% CS concentration. The encapsulation efficiency observed was high, and sustained the release of siRNA from CS-PGA nanoparticles. Thus, CS-PGA nanoparticles of 0.1% w/v CS concentration would be used for the further evaluation; and this research presented the platform for the further optimization studies of PGA used as a cross-linker.

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Conflict of Interest

The authors did not have any conflicts of interest.

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