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PEGylated iminodiacetic acid zinc complex stabilizes cationic RNA-bearing nanoparticles

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ABSTRACT

Self-assembling nanoparticles comprising cationic polymers are of interest for the delivery of oligonucleotide-based therapeutics. Unfortunately, exposure of the nanoparticle cationic surface to plasma and plasma proteins compromises particle stability and circulating half-life. Herein, we report that improved nanoparticle stability can be achieved through temporary grafting of PEG to the nanoparticle surface. Grafting is induced through zinc complexation between PEG–IDA and the exposed polyhistidylated polylysine (H-K) cationic polymer of pre-formed nanoparticles.

Over the past 10 years, therapeutic siRNA has attracted much interest as a new class of pharmaceutical agents.[1–3] However, systemic delivery of siRNA to biologically relevant tissues and cell types is difficult due to many obstacles including rapid degradation and clearance.[4] In order to counter these problems, groups have explored strategies which include chemical modification of siRNA,[5,6] encapsulation within liposomes[7,8] and complexation of siRNA within nanoparticles comprising cationic polymers.[9,10] In addition, variations and combinations of these main strategies are under investigation.

Regarding both native and chemically modified siRNA, challenges relating to circulation time and tissue delivery must be addressed. While these challenges are known to be mitigated through encapsulation within liposomes, this strategy can be problematic due to difficulties associated with achieving high encapsulation efficiency. Nanoparticles comprising cationic polymers, due to the self assembly of stoichiometric mixtures of their components, have the potential to bypass historical problems associated with liposomes. Unfortunately, different challenges are present.

Among the challenges associated with self-assembling cationic, nanoparticles are particle size, particle stability, cell binding, cellular uptake, circulating half-life and endosomal escape.[11] Several of these issues can be addressed through PEGylation.[12] Specifically, coating of the nanoparticle surface with polyethylene glycol (PEG) has the effect of shielding the cationic nature of the particles from the electrolytes and anionic environment of the blood stream. This effect also reduces indiscriminant cell binding and potentially improves circulating half-life. However, while PEGylation potentially improves nanoparticle performance in these areas, it can significantly inhibit cellular uptake.[13] This problem can be addressed through reversible PEGylation.

Various cationic polymers have been explored for encapsulation of siRNA into nanoparticles. Of these polymers, polyethyleneimine (PEI) is presently the most studied.[14] However, use of PEI is limited by its toxicity, non-biodegradable nature and high polydispersity. Another intensively studied cationic polymer for delivering nucleotides is poly(L-Lysine) (PLL).[15] Although biodegradable, PLL has limited use due to its toxicity. Additionally, it is believed that endosomal escape is essential for an efficient siRNA delivery vehicle.[16] Unfortunately, PLL is too basic to significantly influence this process on its own.

Histidine–lysine (H-K) peptides have been studied due to their biodegradability and ability to complex siRNA.[17] In fact, it is the inclusion of histidine that lowers the polymer pK_a (compared to PLL) to a range capable of influencing siRNA endosomal release.

Zinc is known to complex histidine residues in biological settings. This is illustrated in the structures of the family of zinc-bound metalloendopeptidases which includes collagenase. In these structures, zinc complexes with three histidine residues within the active site. Additional zinc valences are available for complexation with zinc-chelating small molecules.[18] Based on this observation, we now report that zinc-bound iminodiacetic acid provides a viable strategy for the reversible PEGylation of cationic siRNA-bearing nanoparticles comprising H-K peptides. In addition to effective grafting of mPEG on nanoparticles, Zn–His chelation enhances nanoparticle stability in serum.

The mPEG–IDA–Zn complexes of the present study were prepared using the three-step process shown in Scheme 1. As illus-

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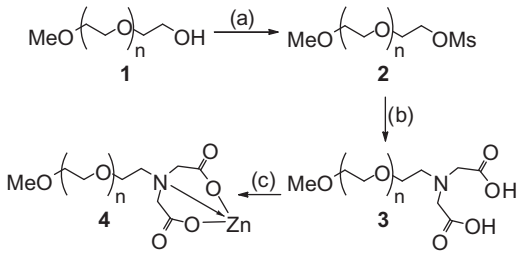

Scheme 1. Reagents and conditions: (a) Ms-Cl, DIEA, CH_2Cl_2, (98%); (b) (i) Iminodiacetic acid, NaOH, H_2O, 80 °C, (ii) DEAE-Sephadex purification (H_2O then 25% brine) (80%); (c) $ZnCl_2$ or $Zn(OAc)_2$, 10 mM HEPES, pH 7.

trated mPEG–OH, **1**, was treated with methanesulfonyl chloride yielding the corresponding mesylate, **2**. The mesylate was then treated with iminodiacetic acid. The crude mPEG–IDA complex was purified by ion exchange chromatography (DEAE-Sephadex, ammonium bicarbonate gradient) giving pure **3**. Finally, treatment of **3** with zinc chloride at pH 7, followed by dialysis and lyopholization yielded mPEG–IDA–Zn. In the present study, mPEG–IDA–Zn was prepared from both 2 kD and 5 kD mPEG–OH.

Nanoparticles were prepared by mixing equal volumes of siRNA solution (25mer, 0.4 mg/mL) and H-K polymer[17] solution (2.4 mg/mL) at pH 4.5. The pH was then increased to 6.5 to induce particle formation. These particles, prior to mPEG grafting, were imaged by transmission electron microscopy demonstrating spherical structures with diameters of approximately 100 nm (Fig. 1).

To obtain mPEG-grafted nanoparticles, compound **4** (2 k or 5 k) was added to the naked nanoparticle formulations at pH 6.5 and the pH was increased to 7.5 to induce chelation. The molar ratio of compound **4** to H-K polymer was varied to obtain different degrees of PEG grafting.

Once formed, nanoparticles were characterized by size, salt stability and serum stability. Particle sizes were measured by dynamic light scattering.[19] Salt stability was determined by suspending particles in aqueous sodium chloride solutions and measuring changes in particle size over time as determined by dynamic light scattering.[20] Serum stability was established by measuring amounts of free siRNA and siRNA degradation as determined by PAGE.[21]

As indicated in Figures 2 and 3, initial nanoparticle sizes fell within a range of approximately 90–120 nm. This was consistent with the TEM findings illustrated in Figure 1.

As shown in Figure 2, salt stability was dependent upon the size of the grafted PEG. Specifically, naked (non-PEGylated) particles rapidly aggregated from the initial time point while particles grafted with 2 kD mPEG had a delayed response. However, when nanoparticles grafted with 5 kD mPEG were challenged with

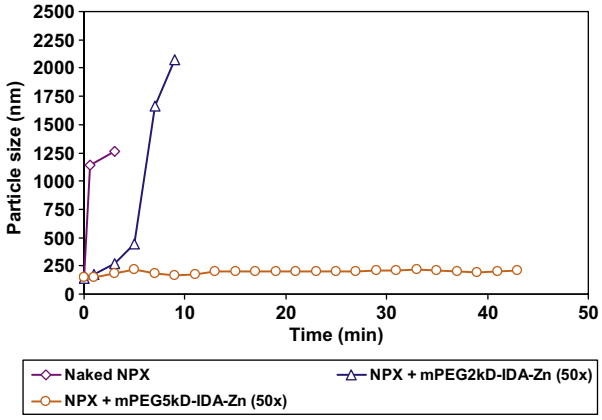

Figure 2. Stability of naked and mPEG-grafted nanoparticles in 214 mM sodium chloride solution.

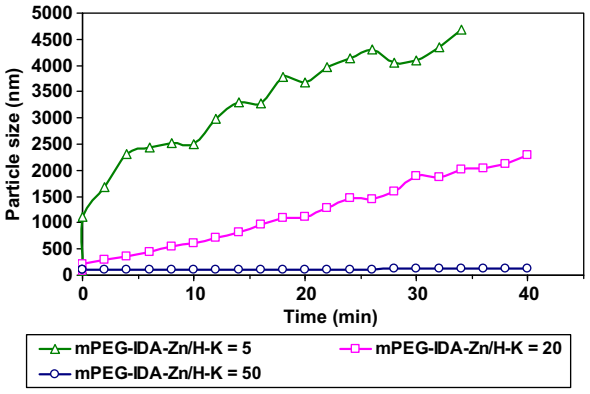

Figure 3. Stability of naked and 5 kD mPEG-grafted nanoparticles in 100 mM sodium chloride solution as a function of degree of mPEG grafting.

214 mM sodium chloride solution, no aggregation was observed over 45 min.

Expanding upon the stability of 5 kD mPEG-grafted nanoparticles, particle stability was measured as a function of the degree of grafting. As shown in Figure 3, while a 5:1 molar ratio of compound **4** to H-K polymer showed little stabilization, a 20:1 molar ratio slowed aggregation compared to 5:1. Finally, a 50:1 molar ratio was sufficient to completely inhibit nanoparticle aggregation when particles were exposed to 100 mM sodium chloride solution.

PAGE was used to assess the degree siRNA leakage from nanoparticle formulations when exposed to serum (Fig. 4). In this study,

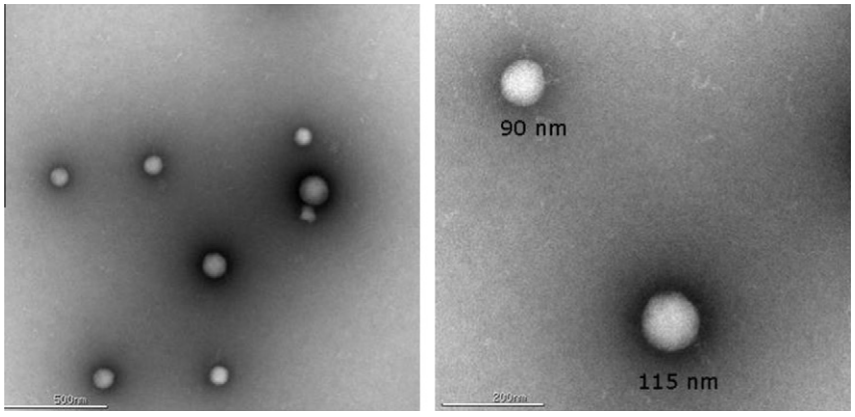

Figure 1. TEM images of naked siRNA/H-K nanoparticles.

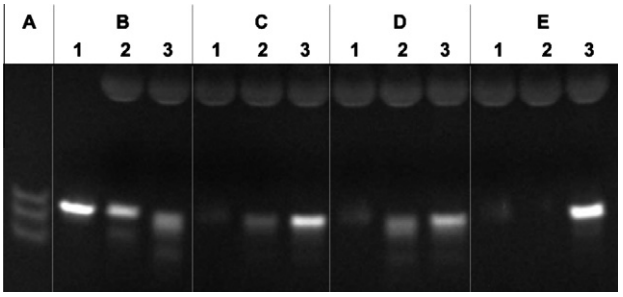

Figure 4. PAGE analysis of nanoparticles (NPXs) with varying degrees of grafted 5 kD mPEG–IDA–Zn complex. Column A: RNA ladder; column B: lane 1—siRNA standard in water, lane 2—siRNA standard in serum, lane 3—siRNA incubated at 37 °C in serum for 2 h; columns C (Naked NPX), D (NPX grafted with 2 × mPEG5 kD–IDA–Zn complex) and E (NPX grafted with 50 × mPEG5 kD–IDA–Zn complex): lane 1—NPX in serum, lane 2—NPX incubated at 37 °C in serum for 2 h, lane 3—NPX incubated at 37 °C in serum for 2 h followed by treatment with heparin.

nanoparticle formulations (columns C–E) were exposed to serum with no incubation (lane 1), incubated in serum (lane 2) and dissociated with heparin following serum incubation (lane 3). The results in column C indicate that naked nanoparticles tend to leak siRNA as indicated by the presence of a siRNA band. Furthermore, the amount of free siRNA increases during the incubation time as indicated by the increased intensity of the siRNA band in lane 2. However, while siRNA leakage does occur, naked nanoparticles are still able to retain some siRNA during the incubation period. This is illustrated by the stronger band in lane 3 (compared to lanes 1 and 2) where incubated nanoparticles are treated with heparin to induce total siRNA release. When naked nanoparticles were grafted with 2 × mPEG5 kD–IDA–Zn complex, no significant reduction in siRNA leakage was observed (column D). In contrast, nanoparticles grafted with 50 × mPEG5 kD–IDA–Zn complex (column E), showed reduced leakage as indicated by minimal siRNA bands in lanes 1 and 2 as compared to the corresponding bands in columns C and D. Finally, column E showed remarkable improvement of nanoparticle serum stability when grafted with 50 × mPEG5 kD–IDA–Zn. Lanes 1 and 2 showed minimal siRNA leakage while lane 3 demonstrated nearly full release of intact siRNA (intensity similar to lane 1 of column B) with minimal siRNA degradation. This clearly indicates that the grafted nanoparticles were able to retain and protect almost all siRNA used in the formulation.

In summary, mPEG–IDA–Zn complexes were prepared from both 2 kD mPEG–OH and 5 kD mPEG–OH. These zinc complexes were shown to graft onto siRNA/H-K polymer nanoparticle formulations. The resulting mPEG-grafted nanoparticles showed enhanced stability to salt and serum dependent upon the size of the grafted mPEG and the degree of mPEG grafting. The best results were obtained using 5 kD mPEG in a 50-fold molar excess over the H-K polymer.

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20. Nanoparticle suspensions (5 μL, 0.2 mg/mL siRNA) were added to sodium chloride (50 μL, 110 mM or 238 mM in water). Particle sizes were then monitored by dynamic light scattering at 2 min intervals over 45 min. Stability was assessed by the rate at which particle sizes increased.
21. Stability of nanoparticles in serum was established by measuring the amount of siRNA leakage. siRNA was detected using PAGE. For these studies, nanoparticle formulations (0.5 μL, 0.2 mg/mL siRNA) were added to mouse serum (9.5 μL). Mixtures were then incubated at 37 °C for 2 h. Following incubation, samples were treated with heparin to effect total release of siRNA from nanoparticles. PAGE analyses (20% TBE polyacrylamide gel at 160 V and 12 mA for 60 min) were performed on formulations prior to incubation, after incubation and after treatment with heparin.