Chitosan-Coated Gold Nanoparticles

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Delivery of small interfering RNA (siRNA) provides one of the most powerful strategies for downregulation of therapeutic targets. Despite the widely explored capabilities of this strategy, intracellular delivery is hindered by a lack of carriers that have high stability, low toxicity and high transfection efficiency. Here we propose a layer by layer (LBL) self-assembly method to fabricate chitosan-coated gold nanoparticles (CS-AuNPs) as a more stable and efficient siRNA delivery system.

Keywords: gold nanoparticles ; Chitosan ; layer by layer assembly ; siRNA ; gene delivery ; endosomal escape

1. Introduction

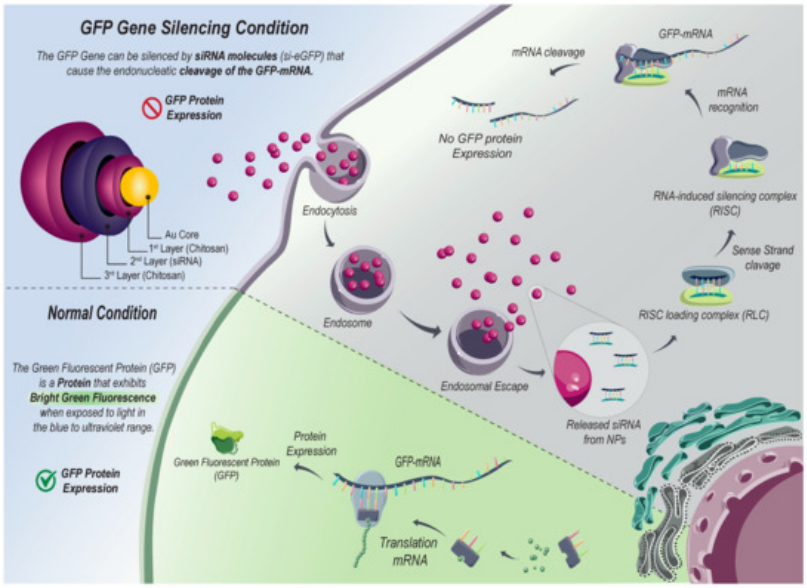
Small interfering RNA (siRNA) is a powerful therapeutic technology which induces post-transcriptional gene silencing via translation inhibition or by cleavage of the target mRNA by incorporating into the RNA-induced silencing complex (RISC) in the cytoplasm [1][2]. Fast enzymatic digestion, limited cellular uptake, inefficient release from endosomes, and lack of cell-specific targeting are the major obstacles of using naked (free) siRNA [3][4][5]. Thus, the therapeutic application of siRNA molecules requires suitable carriers to allow them to be delivered inside target cells in a safe and effective manner. Many different delivery systems have been explored to date. These include viral [5] and non-viral vectors [6][7], each of which come with their own set of advantages and disadvantages. For example, although viral vectors are highly efficient, they are associated with safety concerns such as inherent immunogenicity [8], mutagenesis [9], oncogenic potential, and inflammation [10]. These concerns have triggered the development of non-viral vectors for siRNA delivery including cationic lipids, polymers, peptides and inorganic nanoparticles [7][11][12]. While non-viral vectors are considered to be safer with a reduced immunogenic response, they come with their own drawbacks. For instance, cationic liposomes can suffer from low colloidal stability, difficulties in controlled release, fast elimination in the body, and poor targeting [13]. Polypeptides for gene delivery have limited efficiency and are associated with toxic side effects [14]. In addition, cationic polymer-based carriers can suffer from low stability [15] as well as poor cell recognition and internalization [16]. In spite of those challenges, non-viral vectors remain of great current interest given their better safety profiles and ease of production at an affordable costs [17].

One of the most widely used polymers for the design of nanocarriers for siRNA delivery is chitosan [18][19][20]. Chitosan is a linear biopolymer consisting of randomly repeating D-glucosamine and N-acetyl-d-glucosamine units [21]. Chitosan is a weak polybase with a pKa around 6.5 which offers the advantage of being biodegradable and biocompatible while at the same time being highly positively charged at a pH below the pKa so that it can easily form electrostatic complexes with nucleic acids [22]. On the downside, it suffers from poor stability [23][24] and an undefined structural composition [25]. While improvements have been suggested by making blends with other polymers or modifying its chemical structure [23][26], there is still a need for increasing the stability of chitosan as a gene delivery vehicle. Inorganic nanoparticles like gold nanoparticles (AuNPs) have attracted great interest because of several advantages including their simple synthesis, tunable size and surface properties, good biocompatibility, and multifunctional capabilities [27][28][29]. These unique properties make AuNPs attractive stabilizing scaffolds for gene delivery vehicles. In particular, AuNPs can be used as scaffolds for layer-by-layer (LBL) self-assembly, which is a widely used technique to deposit multiple layers of positively and negatively charged polymers onto surfaces of films or nanoparticles [30][31][32]. Such hybrids of cationic polymers and inorganic nanoparticles combine the advantages of both systems to achieve increased gene delivery efficacy [33]. For instance, LBL assembled AuNP-siRNA have been prepared with polymers that can induce endosomal escape, such as polyethylenimine (PEI) [34][35][36][37][38]. However, release of siRNA in the cellular cytoplasm remains limited due to the high binding affinity between AuNPs-PEI and siRNA [39]. In addition, PEI is often associated with high cytotoxicity due to inducing membrane perturbations and chromosome aberrations [40].

Therefore, in this study we propose LBL assembled AuNP-siRNA in combination with chitosan as a biocompatible and biodegradable cationic polymer to obtain highly stable gene delivery carriers for efficient intracellular siRNA delivery. AuNPs capped with chitosan (CS-AuNPs) are synthesized by using chitosan as both the reducing and stabilizing agent

[41][42]. Next, negatively charged siRNA is incorporated as the next layer on top of the positively charged CS-AuNPs. Finally, a third chitosan layer is applied to protect the siRNA and endow the particles with a net positive charge which allows them to easily adsorb to the negatively charged cell membrane, hence facilitating endocytic uptake.

Long term stability in storage buffer, stability in different media, and siRNA release are investigated. siRNA gene silencing is subsequently evaluated in H1299 cells stably expressing green fluorescent protein (H1299-eGFP) (as shown in Scheme 1) as a model that allows for an easy readout of siRNA silencing. Cellular uptake, endosomal escape transfection efficiency and cell viability are all studied. We find that the here presented AuNP-based carriers show better stability and efficacy than commercial transfection agents (Lipofectamine (cationic lipid mediated transfection) and jetPEI® (cationic polymer)) or nanocarriers prepared from chitosan alone (CSNPs).



Scheme 1. Schematic representation of LBL-CS-AuNPs for siRNA delivery. Under normal conditions, H1299-eGFP cells continuously express enhanced Green Fluorescent Protein (eGFP). When LBL-CS-AuNPs are taken up by cells via endocytosis, chitosan can induce endosomal escape. Then, the released siRNA (si-eGFP) is processed by the RNA-induced silencing complex (RISC), which targets and cleaves GFP-mRNA. The cleavage of GFP-mRNA leads to decreased GFP expression and a diminished green fluorescence intensity of H1299-eGFP cells.

2. Nanoparticle Formation, Characterization and Stability

CS-AuNPs have been synthetized in a one-step synthetic method which used chitosan (CS) both as the reducing agent and stabilizer to generate CS-capped AuNPs. The appearance of a LSPR peak at 524 nm in the UV-Visible spectrum and the absence of plasmonic bands associated with agglomeration of nanoparticles confirmed that the CS-AuNPs are stable and do not show aggregation. For the second layer of the LBL coating, different ratios of siRNA were evaluated for siRNA attachment on the surface of the CS-AuNPs. Then, a final CS layer was applied to protect the loaded siRNA from preventing fast release and ensuring efficient uptake by cells and efficient endosomal escape.

Evaluation of siRNA release profiles and colloidal stability indicated that LBL-CS-AuNPs were more stable than CSNPs which are composed of chitosan alone. We hypothesize that the macromolecular organization of the polymer layer on the surface of the gold nanoparticles confers to their high colloidal stability due to the high cationic charge and the steric effect of the chitosan.

3. Uptake and Transfection Efficiency of Nanoparticles

The biocompatibility of a vector for siRNA delivery is an important consideration. Investigation of metabolic activity and induction of apoptosis showed that CSNPs induced very little toxicity, even at the highest concentration, while for LBL-CS-AuNPs, the toxicity gradually increased with increasing concentration. Chitosan is known to be a biocompatible polymer so the low toxicity by CSNPs is not surprising. The fact that LBL-CS-AuNP induced more toxicity is very likely due to enhanced cellular uptake, as we indeed could observe by using fluorescently labeled siRNA. For CSNPs, the rMFI did not increase substantially with increasing NP concentration, indicating that under the studied concentrations, the uptake machinery was already saturated. Indeed, it has been previously suggested that endocytic uptake pathways may be different for particles of different sizes [43][44]. Therefore, it cannot be excluded that the larger CSNPs are taken up via a

different endocytic pathway, which perhaps may saturate more quickly, than the smaller LBL-CS-AuNPs. Regardless of the underlying mechanism, enhanced uptake of LBL-CS-AuNPs resulted in a maximum gene silencing of 76% for LBL-CS-AuNP with 24 nM siRNA, while this remained limited to 49% for CSNPs with 50 nM siRNA.

When comparing transfection efficiencies with two commercial transfection reagents, jet-PEI and Lipofectamine, it was found that CSNPs showed similar effects as jetPEI®, while being much less effective than Lipofectamine. LBL-CS-AuNPs at 8 nM, on the other hand, performed even better than Lipofectamine, with similar knockdown efficiencies at higher siRNA concentrations. Importantly, unlike the other carriers (commercial transfection reagents or CSNPs), LBL-CS-AuNPs were still as functional 7 days after synthesis, which is yet another demonstration of superior stability of LBL-CS-AuNPs.

4. Endosomal Escape Efficiency

In order to explain the experimental observations of the silencing effect, we proceeded to determine and quantify the endosomal escape capacity of all the NPs evaluated. After image analysis (~500 cells for each sample), a direct correlation was found between the extent of endosomal escape and the transfection efficiencies, indicating that the escape from endosomes is one of the main factors in the effectiveness of the siRNA delivery carriers evaluated here.

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