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Enhancement of efficiency of chitosan-based complexes for gene transfection with poly(γ-glutamic acid) by augmenting their cellular uptake and intracellular unpackage

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ABSTRACT

As a cationic polysaccharide, chitosan (CS) has been identified for its potential use as a non-viral vector for exogenous gene transfection. However, owing to their electrostatic interactions, CS complexes may cause difficulties in gene release upon their arrival at the site of action, thus limiting their transfection efficiency. In this work, an attempt is made to facilitate the release of a gene by incorporating a negatively-charged poly(γ-glutamic acid) (γPGA) into CS complexes in order to diminish their attractive interactions. The mechanisms of exploiting γPGA to enhance the transfection efficiency of CS complexes are elucidated. The feasibility of using this CS/γPGA-based system for DNA or siRNA transfer is explored as well. Additionally, potential of the CS/γPGA formulation to deliver disulfide bond-conjugated dual PEGylated siRNAs for multiple gene silencing is also examined. Moreover, the genetic use of pKillerRed-mem, delivered using complexes of CS and γPGA, to express a membrane-targeted KillerRed as an intrinsically generated photosensitizer for photodynamic therapy is described.

1. Introduction

Gene therapy using exogenous genes such as DNA or small interfering RNA (siRNA) is a highly promising means of treating various inheritable or acquired diseases [1]. However, in addition to their easy degradation by nucleases present in the biological environment, naked genes cannot be internalized into cells effectively, subsequently lowering their transfection efficiency [2]. Therefore, a reliable gene delivery system is required to protect and deliver therapeutic genes. Most gene delivery systems use either viral or non-viral vectors. Although viral vectors allow for an efficient gene transfection [3], their clinical applications are limited by concerns of immunogenicity [4]. While considered as non-viral vectors for carrying genes, cationic polymers are less immunogenic than viral vectors, yet still have a low efficiency [5,6].

As a cationic polysaccharide, chitosan (CS, Fig. 1a) is highly promising for use in condensing anionic genes into a compact structure *via* electrostatic interactions, subsequently providing effective protection against nucleases [7,8]. However, the transfection efficiency of such binary CS/gene complexes is still relatively low [9,10]. We speculate that the strength of attractive interactions between CS and DNA (siRNA) prevents their dissociation within the cells, thus precluding transcription of DNA (interference of RNA) and leading to low transfection (gene silencing). An ideal gene delivery system should provide an adequate means of protecting loaded genes during delivery and releasing them when appropriate.

To facilitate an effective release of genes intracellularly, our previous work has developed an approach by incorporating a negatively charged poly(γ-glutamic acid) (γPGA) into CS complexes, capable of weakening their attractive interactions [7]. γPGA is a naturally occurring peptide with a γ-glutamyl unit at its *N*-terminal end (Fig. 1a). It has been used as a carrier for the oral delivery of insulin [11–14] and intravenous transport of an anti-cancer drug [15–17]. This work demonstrates feasibility of using this ternary CS/γPGA-based system for DNA or siRNA transfer.

2. Characteristics of ternary CS/DNA/γPGA complexes and their transfection efficiency

2.1. Binding capacity, morphology, size, and zeta potential

Based on an ionic-gelation method, CS/DNA/γPGA complexes were prepared at various known charge ratios (N:P:C), which were expressed

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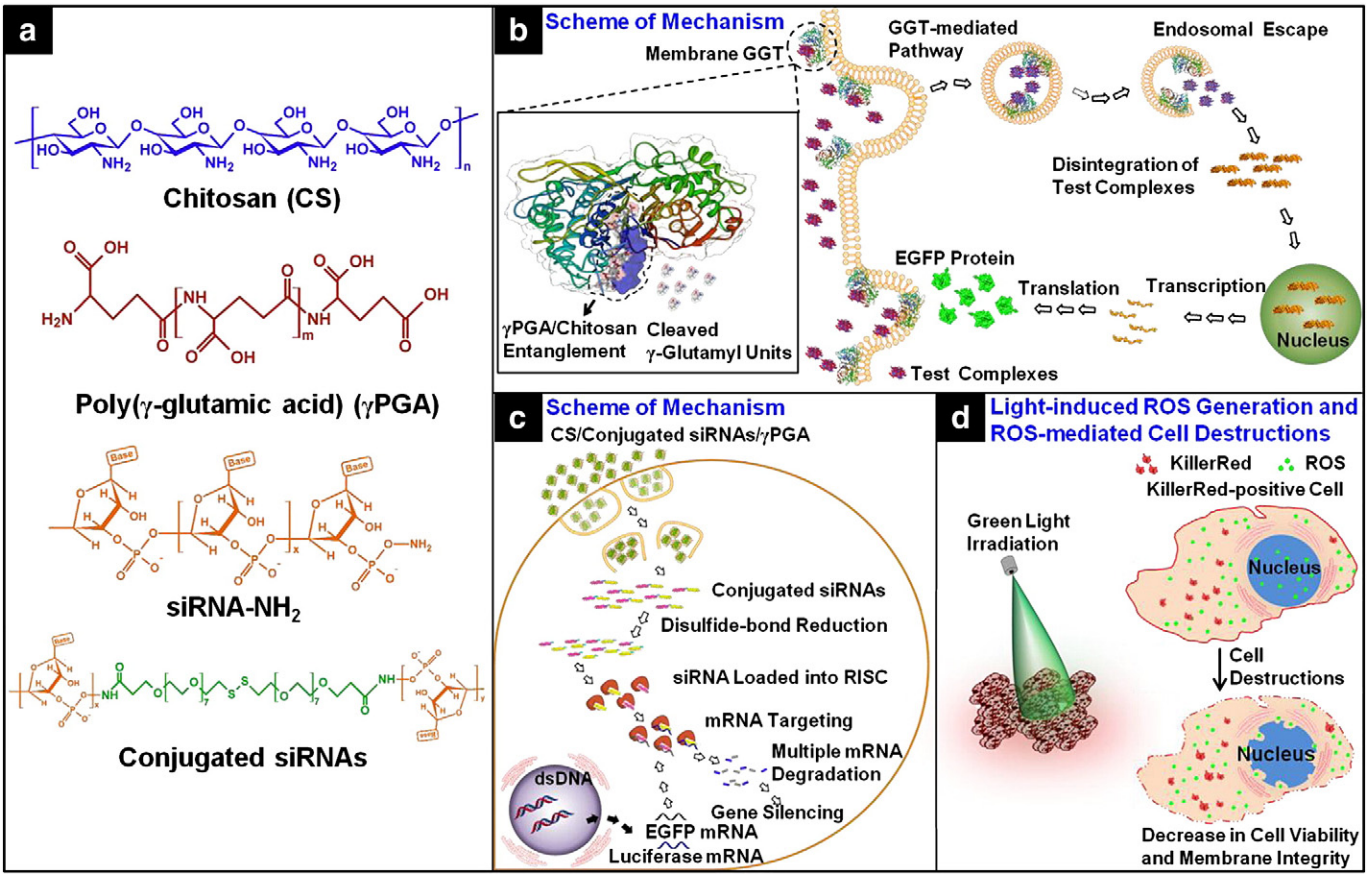

Fig. 1. Schematic illustrations showing (a) the chemical structures of chitosan (CS), poly(γ-glutamic acid) (γPGA), siRNA-NH2, and disulfide bond-conjugated dual PEGylated siRNAs (conjugated siRNAs) used in the study; (b) the mechanism of cellular uptake via the membrane γ-glutamyl transpeptidase (GGT) as delivered by the CS/γPGA complexes and their gene expression; (c) the mechanism on multiple gene silencing by conjugated siRNAs as delivered via the CS/γPGA complexes. RISC: RNA-induced silencing complex; (d) the mechanism of a genetically encoded KillerRed protein as an intrinsically generated photosensitizer for photodynamic therapy. (a,c) Reproduced with permission from Ref [31], Copyright 2013 Elsevier; (b) Reproduced with permission from Ref [10], Copyright 2012 Elsevier; (d) Reproduced with permission from Ref [32], Copyright 2014 Elsevier.

as the molar ratios of the amino groups (N) on CS (15 kDa, with a degree of deacetylation of 85%) to the phosphate groups (P) on plasmid DNA (pEGFP-N2 or pGL4.13 luciferase) and the carboxyl groups (C) on γPGA (20 kDa). The binding capacity of CS with DNA, prepared at various N:P ratios, was evaluated using gel retardation assay. Increasing the N:P ratio to 10:1 retarded the migration of DNA entirely (Fig. 2a). Therefore, test complexes were prepared using an N:P ratio of 10:1. Fig. 2b revealed no significant DNA release when incorporating γPGA in test complexes (N:P:C ratios of 10:1:0.5 to 10:1:6). Encapsulation efficiency of the plasmid DNA in test complexes was nearly 100% (Table 1).

Morphology of the as-prepared complexes was examined using transmission electron microscopy (TEM). According to Fig. 2c, the binary CS/DNA complexes containing no γPGA had a heterogeneous size distribution with a donut, rod, or pretzel shape. Conversely, when γPGA was incorporated, the ternary complexes became condensed and were spherical in shape with a homogeneous size distribution. The size distribution and zeta potential of test complexes in an aqueous environment were examined by dynamic light scattering (DLS). Generally, with an increasing amount of γPGA incorporated, the size of the ternary complexes increased relatively, while their zeta potential value decreased markedly (Table 1). Notably, the diameter of test complexes detected by DLS was relatively larger than that observed by TEM. This is mostly likely owing to that the size of complexes measured by DLS reflected their hydrodynamic diameter, while that observed under TEM was the diameter of dried complexes.

2.2. Intracellular release of DNA

Following their treatment by restriction enzymes (*Bam*HI and *Hind*III) at pH 7.0, simulating the intracellular cytosol environment, test complexes were analyzed by gel electrophoresis. Following digestion by restriction enzymes, three bands of DNA (4.6, 2.7, and 1.9 kb) were clearly observed on the electrophoresis gel images (Fig. 2d). With an increasing amount of γPGA in test complexes, the DNA band intensity increased, implying that incorporating polyanionic γPGA may enhance the release of DNA from test complexes within the cells.

2.3. Transfection efficiency and cytotoxicity

Two numerical indicators were introduced to present the transfection efficiency of test complexes, *i.e.* percentage of cells transfected and gene expression level [18,19]. The percentage of cells (*i.e.* human HT1080 fibrosarcoma cells) that expressed the transgene was determined by counting the number of EGFP-positive cells by using flow cytometry at 48 h post transfection. Only up to 15% of the cells produced EGFP when transfected with the binary CS/DNA complexes (N:P:C ratio of 10:1:0, Fig. 2e). Also, incorporating γPGA in test complexes significantly increased the percentage of cells that expressed EGFP. Among all studied groups, the cells receiving the ternary CS/DNA/γPGA complexes with an N:P:C ratio of 10:1:4 had the highest percentage of cells transfected (55%); their gene expression level