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Silencing of the metastasis-linked gene, AEG-1, using siRNA-loaded cholamine surface-modified gelatin nanoparticles in the breast carcinoma cell line MCF-7

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ABSTRACT

Cholamine surface-modified gelatin nanoparticles prepared by the double desolvation method using acetone as a dehydrating agent were selected and potentially evaluated as non viral vectors of siRNA targeting a metastatic gene AEG-1 in MCF-7 breast carcinoma cells. The ability of modified gelatin nanoparticle to complex and deliver siRNA for gene silencing was investigated. Hence, Particle size, surface charge (zeta potential) and morphology of siRNA/Gelatin nanoparticles (siGNPs) were characterized via dynamic light scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscope (TEM). Moreover, the nanoparticles cytotoxicity, loading efficiency and interaction with MCF-7 human breast carcinoma cells were evaluated. Cationized GNPs of mean size range of 174 nm and PDI of 0.101 were produced. The loading efficiency of siGNPs at a Nitrogen/Phosphate (N/P) ratio (w/w) of 200:1 was approximately 96%. Cellular uptake was evaluated after FITC conjugation where the particles produced high transfection efficiency. Finally, ELISA analysis of AEG-1/MTDH expression demonstrated the gene silencing effect of siGNPs, as more than 75% MTDH protein were inhibited. Our data indicate that cholamine modified GNPs pose a promising non-viral siRNA carrier for altering gene expression in MCF-7 breast cancer cells with many advantages such as relatively high gene transfection efficiency and efficient silencing ability.

1. Introduction

Systemic administration of siRNA holds great challenges for both extracellular and intercellular delivery. After I.V. injection, siRNA is distributed to the body organs via the blood circulation where it is rapidly degraded by serum endonucleases having a plasma half-life of about 10 min [1–3]. At the same time it undergoes clearance via glomerular filtration and renal excretion. Moreover, naked siRNA has low intracellular uptake through passive diffusion. Solely, it cannot induce RNAi phenomena inside the cell due to its high molecular weight (~13 kDa) and the presence of negative charge on the sugar phosphate backbone under normal physiological conditions creating electrostatic repulsion, making it difficult to cross the lipid bilayer of cell membrane whose head groups are also negatively charged. Furthermore, after internalization by the cell through endocytosis, siRNA must escape from the endosomes and be released from its carrier to the cytosol (site of action) in order to be loaded onto RISC and initiate mRNA degradation [4]. Due to the aforementioned facts, the majority of siRNA is either degraded or entrapped before entering the cell and reaching the site of action. Therefore, the development of safe and effective carriers (viral or non-viral) or adopting modification strategies are necessities for efficient delivery of siRNA-mediated gene therapy [5].

Abbreviations: AEG-1, astrocyte elevated gene-1; bp, base pair; DEPC, diethyl pyrocarbonate water; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescent isothiocyanate; GFP, green fluorescent protein; GLA, gluteraldehyde; GNPs, gelatin nanoparticles; GRAS, generally recognized as safe; IL-8, interleukin 8; MBC, metastatic breast cancer; MTDH, metadherin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N/P, nitrogen/phosphate ratio; PEG, poly ethylene glycol; pGNPs, plain gelatin nanoparticle; RES, reticlo-endothelium system; RFU, relative fluorescent unit; siGNPs, siRNA loaded gelatin nanoparticle; siRNA, small interfering RNA.

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Despite their high transfection efficiency and effective targeting, viral vectors possess high toxicity risks and many immunity problems. These substantial reasons made the development of non viral alternatives highly desirable [6], specifically, cationic polymeric non-viral vectors. This is due to the fact that these carriers hold advantageous properties concerning safety, simplicity of preparation and high encapsulation capability [7]. Non-viral siRNA vectors typically involve complexing siRNA with a positively charged nanoparticulate carriers. Therefore, siRNA can be either encapsulated inside or conjugated to the nanoparticles surface through electrostatic binding between the positive charges of the nanoparticles and the negative charges of siRNA.

Protein based nanoparticles have attracted high interest over the past decades for therapeutics delivery especially for genetic products. This is due to their excellent characteristics, their highly precise structures, compatibility with biological systems, biodegradability, non-antigenicity, stability *in-vivo* and on storage and finally for their abundant renewable resources [8]. Furthermore, the presence of high number of functional groups on their surface available for modifications makes them attractive for targeting purposes. They can also be tailored to escape the reticuloendothelial system (RES) by creating an aqueous steric barrier [9].

In particular, gelatin is one of the interesting protein hydrocolloid materials that can be used as a potential carrier of siRNA based nanoparticle delivery, not only because it possesses no to low toxicity but also due to its simple production techniques with low costs and possible modification opportunities owing to its intrinsic nature. Therefore, gelatin based nanoparticles can offer exceptional contributions as a safe and simple carrier for siRNA delivery systems.

Accordingly, in the current study, cholamine surface-modified (cationic) gelatin nanoparticles prepared by the double desolvation method were chosen and potentially explored as a non viral vector of siRNA targeting a metastatic gene AEG-1 in MCF-7 breast carcinoma cells due to its different merits and excellent bio-safety profile. Astrocyte elevated gene-1 (AEG-1, also known as metadherin MTDH, and 3D3/Lyric), a newly discovered gene that was cloned over a decade ago, has emerged in recent years as a potentially crucial mediator of tumor malignancy and a key converging point of a complex network of oncogenic signaling pathways [10]. Furthermore, AEG-1 was proven to contribute in several hallmarks of metastatic cancers, including cell proliferation, and survival under stressful conditions such as serum starvation and resistance to chemotherapy [11].

To this end, selected siRNA-loaded surface-modified gelatin nanoparticles were tested *in-vitro* by monitoring their cytotoxicity on MCF-7 cell, effective internalization after fluorescence labeling and finally, studying the biological activity of AEG-1 siRNA entrapped in the prepared cationized gelatin nano-carriers using enzyme linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Materials

Gelatin type A from porcine skin (bloom 300), gelatin type B from bovine skin (bloom 175), ovalbumin (Albumin from chicken egg white, lyophilized), gluteraldehyde (25% solution in water), glycine, cholamine chloride hydrochloride (2-aminoethyl)-trimethylammoniumchloride hydrochloride, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), hydrofluoric acid ≥48%, DiEthyl Pyrocarbonate (DEPC) and collagenase from Clostridium histolyticum were purchased from Sigma-Aldrich, Zwijndrecht, Netherlands. AEG-1 siRNA solution (10 μM) and 10 μM solution of control siRNA consisting of a scrambled non-targeting 20–25 nucleotide sequence were obtained from Santa Cruz, Germany. Fluorescein isothiocyanate (FITC) was obtained from Thermo-Fischer Scientific Inc., Waltham, MA. Fetal Bovine Serum (FBS), Penicillin-streptomycin antibiotic (pen-strep), Phosphate buffer saline (PBS), Trypan Blue 0.4% and Dulbecco's Modified Eagle Medium (DMEM) having 4.5 g/L glucose with L-Glutamine were all purchased from Lonza, Germany. 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium-bromid (MTT) was obtained from Serva Electrophoresis, Germany. Spectra/Por® dialysis membrane, 12,000–14,000 molecular weight cut off was purchased from Spectrum Laboratories Inc., Rancho Dominguez, Canada. MCF-7 breast cancer cell lines together with the RPMI media were obtained from VACSERA, Cairo, Egypt.

2.2. Methods

2.2.1. Preparation of gelatin nanoparticles (GNPs)

Gelatin nanoparticles were prepared using the double desolvation methods as described by Coester and his colleagues with slight modification [12] as follows; where 1.25 g of Gelatin type A (bloom 300) was dissolved in 25 ml of purified deionized water at 40 °C under constant mild stirring. After, 25 ml of acetone was added to this solution in order to achieve the first desolvation and the rapid sedimentation of the high molecular weight of gelatin, where the supernatant that contained the low molecular weight gelatin was discarded. The remaining sediment was re-dissolved again in 25 ml of purified deionized water at 40 °C under mild heating. The pH of the gelatin solution was adjusted to 2.5 using 5 M HCL. Then, the dissolved gelatin solution was desolvated again using 60 ml acetone by drop wise addition under vigorous stirring at 40–45 °C. In order to harden the nanoparticles, different amounts of gluteraldhyde (GLA) 25% (v/v) a non zero cross-linker was added to crosslink the particles for different time intervals (1,4,8,12,and 16) hours at 500 rpm. After 12 h, Glycine solution (751 mg/100 ml) was added and stirred at 500 rpm for 1 h to block the residual aldehyde groups (CHO) of glutaraldehyde and thereby stopping its action. Finally; the dispersion was centrifuged at 15,000 rpm for 25 min. The particles were purified by threefold centrifugation and re-dispersion cycles in purified water.

2.2.2. Determination of the practical yield of the prepared gelatin nanoparticles

In order to determine the amount of gelatin nanoparticles produced by the adopted double desolvation method, lyophilization (freeze drying) was employed (Eyela FDU-2100, Japan). An amount of 20 ml of the gelatin nanoparticles prepared with a ratio $Gelatin_{[NH2]}$:$GLA_{[CHO]}$ equals 1:1 and a cross-linking time of 12 h was stored in −80 °C freezing temperature for 1 h and then transferred immediately to the freeze dryer to be left for 2 days until complete dryness and the subsequent formation of lyophilized GNPs. The mass of the prepared GNPs was determined gravimetrically by determining the difference in the weight of the flask before and after freeze drying. The percentage yield of the produced nanoparticles was determined with respect to both the weight of the original added amount of gelatin (1.25 gm) and also with respect to the weight of the hydro-gel like sediment produced in the first desolvation step that contains the high molecular weight fragment of gelatin responsible for producing the homogenous gelatin nanoparticles. Hence, the percentage yield was calculated according to the following Eqs. (1) and (2):

Yield percentage with respect to the original gelatin amount

$$= \frac{\text{weight of the produced GNPs}}{\text{weight of the original gelatins used}} \times 100 \quad (1)$$