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GalNAc bio-functionalization of nanoparticles assembled by electrostatic interactions improves siRNA targeting to the liver

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ABSTRACT

RNA interference (RNAi) has the potential to reversibly silence any gene with high efficiency and specificity. To fulfill the clinical potential of RNAi, delivery vehicles are required to transport the short interfering RNA (siRNA) to the site of action in the cells of target tissues. Here, we describe the features of novel liver-targeted siRNA nanoparticles (NPs), co-assembled due to the complexation of alginate sulfate (AlgS) with siRNA, mediated by calcium ions bridges (AlgS–Ca^{2+}-siRNA NPs) and then bioconjugation of a targeting ligand onto the AlgS upon the NP surface. To gain insight into the complexation process and confirm AlgS accessibility on NP surface, we investigated different schemes for fabrication. All resulting NPs, independently of the component addition order, were of average size of 130–150 nm, had surface charge of < -10 mV, exhibited a similar atomic composition on their surface, were efficiently uptaken by HepG2 cells and induced approx. ~90% silencing of STAT3 gene. Ca^{2+} and AlgS concentrations in NPs affected cell uptake and gene silencing. Bioconjugation of N-acetylgalactosamine (GalNAc), a ligand to the asialoglycoprotein receptor (ASGPR) overexpressed on hepatocytes, was validated by XPS analysis and cell uptake by receptor-mediated mechanism. After intravenous (*i.v.*) injection to BALB/c mice, GalNAc-NPs were targeted to liver by a factor of ~3 with lesser renal clearance compared to non-targeted NPs. We foresee that the combined advantages of site-specific targeting and reversibility of the tri-component NPs as well as the simplicity of their fabrication make them an attractive system for targeted delivery of siRNA.

1. Introduction

Over the last decade or so, small interfering RNA (siRNA) has attracted intense interest owing to its therapeutic potential in disease therapy [1,2]. Yet, siRNA delivery in vivo is still a major challenge due to its rapid degradation by nucleases, poor cellular uptake and rapid renal clearance following systemic administration [3–5]. In an effort to overcome these limitations and to improve the delivery of RNAi-based therapeutics, researchers have explored various carriers including polymers, liposomes, dendrimers and siRNA conjugates to name a few [6,7]. In particular, recent research has focused on biocompatible anionic carriers that offer longer circulation time, less interaction with serum components and lower cytotoxicity compared to cationic carriers [8–12].

We recently described the co-assembly in solution of stable, slightly anionic Ca^{2+}-siRNA nanocomplexes, which maintain particle size and surface charge in physiological relevant conditions [13]. We showed that calcium ion concentration in the final formulation was critical, affecting both the physical properties (size, surface charge) of nanocomplexes, their ability to protect siRNA from RNase degradation and their gene silencing efficiency [14].

In the present paper, we investigated the inclusion of the semi-synthetic alginate sulfate (AlgS) in order to provide functional groups available for the attachment of cell-targeting ligands to the nanoparticles (NPs). As the first stage, to ensure maximal accessibility of AlgS for ligand attachment on NPs, we investigated the co-assembly process of the three components, Ca^{2+}, siRNA and AlgS, by applying various component addition strategies and compared the resulting NPs in terms of size, surface charge, surface atomic composition by XPS, their intracellular uptake and gene silencing. Then, we bio-functionalized the NPs with *N*-acetylgalactosamine (GalNAc), a ligand to the asialoglycoprotein receptors (ASGPRs) overexpressed on hepatocytes [15–17]. GalNAc is known for its ability to bind to the ASGPR receptor with higher selectivity than unmodified galactose [15,18] and recent

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studies have used the conjugated GalNAc moiety in an effort to design a delivery system targeting hepatocellular carcinoma [17,19–23]. Thus, the GalNAc moiety was conjugated directly to the AlgS-Ca^{2+}-siRNA NPs to form a novel tumor-specific delivery vehicle (GalNAc-NPs). Here, we present the bioconjugation protocol and the physico-chemical properties of the resultant GalNAc-NPs, their uptake/internalization by ASGPRs overexpressing hepatoma cells (HepG2) via receptor-mediated endocytosis, and their capacity to induce gene silencing. A biodistribution study after intravenous (*i.v.*) injection in BALB/c mice confirmed targeting of GalNAc-NPs into the liver by a factor of ~3 compared to non-targeted formulations.

2. Materials and methods

2.1. Materials and cells

Alginate (sodium salt, VLVG < 75 kDa) was from NovaMatrix, FMC Biopolymers (Drammen, Norway). Alginate sulfate (AlgS) was prepared as described in [24]. Fluorescent labeling of AlgS with HiLyte Fluor 647 nm amine dye (Anaspec, Fremont, CA) by the carbodiimide chemistry was previously described [25]. For XPS measurements, undoped double-side polished silicon oxide wafer was obtained from Virginia Semiconductors, Inc. (Fredericksburg, VA). **Quant**-iT RiboGreen assay was purchased from Life Technologies. The siRNAs: targeting eGFP, STAT3, transfection control (Cy3/Cy5-labeled siRNA), non-targeting (siNC5), GAPDH were from Dharmacon (Lafayette, CO) (Supplementary Table S1). Cell culture reagents Minimum Essential Medium (MEM), L-glutamine, penicillin/streptomycin, heat inactivated fetal bovine serum (FBS) were from Biological Industries (Kibbutz Beit-Haemek, Israel). Other reagents, unless specified otherwise, were from Sigma. All reagents were of analytical grade.

Human hepatocellular carcinoma cells (HepG2) from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in MEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Cells were incubated, at 37 °C, in a humidified air atmosphere containing 5% CO_2.

2.2. Preparation of AlgS-Ca^{2+}-siRNA NPs

AlgS-Ca^{2+}-siRNA-Reg NPs (Reg-for regular procedure) were prepared by first mixing equal volumes of siRNA (10 μM) and $CaCl_2$ (1 M) using a vortex for 30 s, followed by 20 min-incubation at room temperature (RT) to allow complex formation. Then, an equal volume of AlgS (50 μg/ml (50 μM) in 10 mM HEPES) was added and mixed by vortexing for 30 s, followed by 30 min incubation at RT. AlgS-Ca^{2+}-siRNA NPs were freshly prepared before use.

AlgS-Ca^{2+}-siRNA-Rev NPs (Rev- for reversal procedure) were prepared by first mixing equal volumes of AlgS (50 μg/ml in 10 mM HEPES) and $CaCl_2$ (1 M) by vortexing for 30 s, followed by 20 min-incubation at RT to allow complex formation. Then, an equal volume of siRNA (10 μM) was added and mixed by vortexing for 30 s, followed by 30 min incubation at RT.

AlgS-Ca^{2+}-siRNA-Both NPs (Both-for mixing the two anionic species) were prepared by mixing equal volumes of AlgS (50 μg/ml in 10 mM HEPES) and siRNA (10 μM) by vortexing for 30 s followed by 20 min incubation at RT. Then, equal volumes of the $CaCl_2$ (1 M) were added and mixed by vortexing for 30 s, followed by 30 min incubation at RT prior to use. The final tri-component concentration in all NPs was 12.5 μM siRNA, 0.25 M $CaCl_2$, 12.5 μM AlgS.

We also prepared NPs with a greater molar ratio of AlgS to siRNA, 5:1 or 10:1, as well as NPs with varying Ca^{2+} concentrations in the NPs. Ca^{2+}-siRNA nanocomplexes (without AlgS) were prepared as the Reg procedure with addition of 10 mM HEPES as a final step.

2.3. Bioconjugation of GalNAc to AlgS-Ca^{2+}-siRNA NPs

A volume of 470 μl of NPs (AlgS-Ca^{2+}-siRNA-Reg NPs, AlgS:siRNA molar ratio 1:1, 5 mM Ca^{2+}) were prepared as described in section 2.2. The molar ratio of AlgS:EDC:sulfo NHS:ligand in the bioconjugation reaction was 1:2.9:1.46:2.9. First, sulfo NHS and EDC were added into the NP suspension and the mixture was stirred for 30 min. Then, 10 μl of 2-acetamido-1-amino-1,2-dideoxy-β-D-glucopyra (2.53 mg/ml) was added to EDC/NHS-functionalized NPs and stirred for 4 h. The galactosamine-functionalized NPs (denoted as GalNAc-NPs) were added to Ultra-0.5 filter device (Millipore, Billerica, Mass., US) with 3 kDa cut off and spun at 14,000 × g for 15 min and then recovered according to the manufacturer's instructions. The recovered GalNAc-NPs were diluted 1:1 (v:v) with $CaCl_2$ (250 mM) to maintain final Ca^{2+} of 5 mM in NPs or with HEPES (10 mM) to obtain NPs with 2.5 mM Ca^{2+}.

2.4. Particle size and zeta (ζ) potential measurements

Particle size distribution and mean diameter of AlgS-Ca^{2+}-siRNA NPs, after dilution 1:50 in 10 mM HEPES, were measured on a CGS-3 (ALV, Langen, Germany) instrument. Particles were analyzed by scattered laser light (He-Ne laser, 20 mW, 632.8 nm) and detected at an angle of 90°, during 10s for 20 times, at 25 °C. Correlograms were calculated by ALV/LSE 5003 correlator and fitted with a version of the program CONTIN. The surface charge (ζ potential, mV) was measured on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) using electrophoretic cells (DTS 1060, Malvern Instruments Ltd., UK). Zeta potentials were recorded three times, 10 to 100 measurements in each run (depending on standard deviation).

2.5. Transmission electron microscopy

AlgS-Ca^{2+}-siRNA NPs composed of gold-labeled AlgS or unlabeled AlgS were prepared at a final concentration of 2.5 μM AlgS/0.5 M Ca^{2+}/2.5 μM siRNA. A 5 μl-sample was placed on carbon-coated films on copper EM grids hydrophilized by glow discharge. The excess liquid was blotted and the grids were allowed to dry at RT. The samples were imaged at RT using a FEI Tecnai 12 G^2 TWIN TEM (Gatan model 794 CCD, bottom mounted) at acceleration voltage of 120 kV. Specimens were studied in a low-dose imaging mode to minimize beam exposure and electron beam radiation damage. Images were recorded digitally using the Digital Micrograph 3.6 software (Gatan, Munich, Germany).

2.6. X-ray photoelectron spectroscopy (XPS)

The experiment was conducted according to a protocol we recently reported [26]. In brief, a 5-μl droplet of each sample was placed on a silicon oxide wafer and dried overnight in an entry lock chamber before conducting the XPS measurements. XPS data were collected using an X-ray photoelectron spectrometer ESCALAB 250 ultrahigh vacuum (1×10^{-9} bar) apparatus with an AlKα X-ray source and a monochromator. The X-ray beam size was 500 μm and survey spectra were recorded with pass energy (PE) 150 eV. High energy resolution spectra were recorded with pass energy (PE) 20 eV. The spectral components of C1s, O1s, N1s, Ca2p, Cl2p, S2p and P2p signals were found by fitting a sum of single component lines to the experimental data by means of nonlinear least-squares curve-fitting. To correct for charging effects, all spectra were calibrated relative to a carbon 1s peak positioned at 284.6 eV.

2.7. Entrapment efficiency

siRNA entrapment efficiency in AlgS-Ca^{2+}-siRNA NPs was evaluated by Quant-IT Ribogreen assay (Invitrogen) using the manufacturer's protocol. The assay is based on the strong fluorescence of Ribogreen upon intercalation with siRNA. AlgS-Ca^{2+}-siRNA NPs were