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siRNA delivery using polyelectrolyte-gold nanoassemblies in neuronal cells for BACE1 gene silencing

Aparna Chaudhary, Sanjeev Garg *

Department of Chemical Engineering, IIT Kanpur, Kanpur, UP 208016, India

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ABSTRACT

Small interfering RNA (siRNA) mediated RNA interference is a versatile therapeutic tool for many intractable genetic disorders. Various nanoassemblies specifically designed to deliver the siRNAs could be utilized for efficient siRNA delivery which is one of the major concern for the success of this therapeutic. Thus, in the present study, polyelectrolyte-gold nanoassemblies (PE-Gold NAs) were selected for siRNA delivery of an *in vitro* verified siRNA. Three different polyelectrolytes (polyethyleneimine, citraconic anhydride modified poly (allylamine) hydrochloride and poly L-arginine) were used to formulate the PE-Gold NAs using the layer-by-layer technique. Successful physico-chemical characterizations of these PE-Gold NAs were performed using UV-Visible, FTIR, ^{1}H-NMR spectroscopies, XRD, TEM, DLS and Zeta potential measurements. *In vitro* studies for the cytotoxicity, the uptake of these nanoassemblies and the gene silencing were carried out using these PE-Gold NAs in N2a and NB4 1A3 (murine neuronal) cell lines. The three selected PE-Gold NAs showed significant BACE1 (β-site APP cleaving enzyme 1) gene silencing (50–60%). This work demonstrates the potential of PE-Gold NAs to deliver siRNA targeting BACE1 in neuronal cells. Finally, it was concluded that different polyelectrolytes used in the PE-Gold NAs achieve different gene silencing due to the variation in their delivery efficiencies.

1. Introduction

RNA-targeting therapeutics are currently being explored for intractable genetic disorders where conventional therapy is ineffective [1–5]. RNA interference, using small interfering RNA (siRNA), is one such RNA-targeting therapy that interferes at the post-transcriptional level by degrading the target mRNA. The siRNA therapy could be a choice for Alzheimer's disease (AD), a neurodegenerative disorder. It is an accepted hypothesis that BACE1 (β-secretase) initiates the enzymatic cleavage of amyloid precursor protein (APP) and leads to the generation of amyloid beta peptides (Aβ). These peptides subsequently oligomerize and are responsible for the formation of neurotoxic plaque in the brain of an AD patient. The BACE1 gene has been identified and reported as an effective target for AD [3,6]. Thus, BACE1 gene was selected as the target in the present study using siRNA therapeutics.

The active sites in BACE1 protein (β-secretase) are less hydrophobic compared to the other proteases. Moreover, the active sites in β-secretase are open in comparison to the other protein targets. Thus, β-secretase is not a suitable target for the conventional therapeutics [6,7]. Therefore, a suitable gene therapy such as siRNA mediated RNAi could be utilized to target the BACE1 gene. The success of any siRNA based therapy primarily depends on the efficient siRNA design and delivery to the target. The efficient design aspects were discussed previously elsewhere [8].

There are various known barriers to a successful siRNA delivery. These are its nuclease degradation, high molecular weight, and presence of negative charge due to the phosphate backbone [4,9,10]. Therefore, an ideal delivery vehicle should overcome these known barriers. Moreover, the delivery vehicle should also breach the different biological barriers, *e.g.* serum instability, tissue permeability, cell membrane permeability, lysosomal degradation, RISC (RNA-induced silencing complex) loading [4,9–11]. In the open literature, various such delivery vehicles (viral and non-viral) for siRNA delivery targeting BACE1were reported in the last few years. Singer et al. [3] reported siRNA delivery targeting BACE1 using Lentivirus as a viral delivery vehicle. The viral delivery provides a sustained silencing effect and has few side-effects, namely, insertional mutagenesis, uncontrolled expression and immunogenicity [12,13]. Non-viral delivery vehicles were, thus, developed to mitigate the side-effects associated with the viral delivery vehicles. Several non-viral delivery vehicles were developed for siRNA delivery targeting BACE1. The non-viral delivery vehicles used for siRNA delivery were exosome [14], siRNA conjugated with high-density lipoprotein-α-tocopherol [15], PEGylated-CdSe/ZnS quantum dots [16], lipophilic

Abbreviations: AD, Alzheimer's disease; BACE1, β-site APP cleaving enzyme 1; LbL, Layer-by-layer; PAH-Cit, Citraconic anhydride modified poly (allylamine) hydrochloride; PE-Gold NAs, Polyelectrolyte-gold nanoassemblies; PEI, Polyethyleneimine; PLA, Poly L-arginine; siRNA, Small interfering RNA.

* Corresponding author.
E-mail address: sgarg@iitk.ac.in (S. Garg).

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boron cluster [17], PEGylated-anionic peptide-siRNA nanocomplex [18], linear polyethyleneimine (LPEI)-g-polyethylene glycol (PEG) copolymer-based micellar nanoparticle system [19] and trans activated response element-RNA binding protein-conjugation [20]. The non-viral delivery vehicles, in general, circumvent the side effects associated with the viral delivery vehicles [5,21–25]. Still most of the non-viral delivery vehicles suffer with a few limitations, *e.g.*, their efficacy is low leading to high and repeated doses leading to toxicity [26,27], and non-specificity towards other target sites [13].

In the present study, inorganic nanoparticles modified with layer-by-layer (LbL) technique as a non-viral delivery vehicles (inorganic nanoassemblies) were selected for siRNA delivery targeting BACE1. Similar LbL inorganic nanoassemblies have been widely reported in the recent past for siRNA delivery. Gold, silica, calcium carbonate and iron oxide inorganic nanoparticles were used as the core for LbL formation with alternate polyelectrolyte layers [28–32]. The gold nanoparticle provides many advantages over other inorganic nanoparticles, due to its high biocompatibility, optical properties, easy surface functionalization capacity and simple synthesis to achieve tuneable size [32–35]. Cationic polyelectrolytes were selected for layering the inorganic nanoparticles because of their efficiency to cross the cellular barriers and protect the siRNA from nuclease degradation [10,32,36–41]. Moreover, the LbL modified PE-Gold NAs are more compact and use controlled polyelectrolyte loading and are less toxic as compared to the self-assembled polyelectrolyte nanoassemblies as used by Shyam et al. [19]. The PE-Gold NAs does not change the functionality of siRNA as compared to lipophilic boron cluster conjugation system [17]; better loading capacity as compared to siRNA conjugated with high-density lipoprotein-α-tocopherol [15] and trans activated response element-RNA binding protein-conjugation [20]; easy synthesis as compared to exosome [14]. Poor siRNA neuronal cell transfection efficiency is observed with the naked siRNA delivery. Thus, the aim of this study was to establish if the LbL functionalized PE-Gold NAs could be used to deliver siRNA in the neuronal cells for BACE1 gene silencing. Moreover, the study was designed to establish the differences in BACE1 silencing due to different polyelectrolytes used for layering the PE-Gold NAs. Earlier, a siRNA design tool was developed [8] and *in silico* predictions were made for the BACE1 gene as an AD target. These predictions were subsequently verified in an *in vitro* transfection study using Lipofectamine®2000 (unpublished data). In the present work, one of the *in vitro* verified siRNA was chosen to establish the viability of the siRNA delivery using LbL synthesized PE Gold NAs in neuronal cells (N2a and NB4 1A3) expressing BACE1 gene. Three different polyelectrolytes (branched-polyethyleneimine, PEI; citraconic anhydride modified poly (allylamine) hydrochloride, PAH-Cit; and poly L-arginine, PLA) were selected to formulate the PE Gold NAs. The first two nanoassemblies (PEI-Gold NA and PAH-Cit-Gold NA) were formulated using the reported methods in Elbakry et al. [31] and Guo et al. [32], respectively, while the third (PLA-Gold NA) was similarly formulated using PLA. PEI is reported to possess high charge density which provides high siRNA loading capacity and has previously been used successfully for silencing [31,42–46]. PAH-Cit is a charge reversal polymer which is reported to provide endosomal escape resulting in high silencing [32]. PLA is a biodegradable poly-amino acid with reported high siRNA loading, stability and silencing with low cytotoxicity [47].

2. Materials and method

2.1. Materials

Chloroauric acid ($HAuCl_4 \cdot xH_2O$) was obtained from Loba Chemie (Au content 49%). Sodium borohydrate was obtained from Merck. Tri-sodium citrate was obtained from Fisher Scientific, 11-Mercaptoundecanoic acid (11-MUA), PEI (MW = 25 kDa), PAH (MW = 55 kDa), PLA hydrochloride (PLA, MW = 15–70 kDa) were obtained from Sigma-Aldrich. All the chemicals were used as received. The optimum molecular weight of PEI recommended for efficient gene transfection with minimal toxicity is 5–25 kDa [48]. Hence, PEI with a molecular weight of 25 kDa was selected and utilized in the present work. This selection was in agreement with the reported value of the molecular weight of PEI in the open literature for the synthesis of LbL NAs [31,32]. Autoclaved deionized (MilliQ) RNase free water was used for all the preparation. DMEM and HAM's F-10, antibiotic/antimitotic solution and MTT were obtained from Sigma-Aldrich. FBS was obtained from Gibco® cell culture media. DMSO was obtained from Merck. The cell lines used were N2a and NB 41A3 obtained from NCCS, Pune. Citraconic anhydride (Spectrochem Pvt. Ltd., India) modified PAH (Appendix A, Section A.1.1) and Rhodamine isothiocyanate (RITC) (Sigma-Aldrich) tagged PEI were formulated in the lab (Appendix A, Section A.1.2). Dialysis membrane (12 k MWCO) was obtained from Sigma-Aldrich. Single stranded DNA (sense strand) for cell viability assay and 6-FAM dsDNA (Sigma-Aldrich) for uptake experiments were used in place of siRNA. The sequences were, sense strand: 5′-TGGAGATGGTGGACAACCTTT-3′, antisense strand: 5′-AGGTTGTCCACCATCTCCATT-3′. The strands sequence of siRNA (Sigma-Aldrich) used in the study for sense was 5′-UGGAGAUGGUGGACAACCU(dTdT)-3′ and for antisense was 5′-AGGUUGUCCACCAUCUCCA(dTdT)-3′. The non-target siRNA (Eurofin MWG Operon) was selected against EGFP gene, a negative control for gene silencing. The sequences were as, sense strand: 5′-CAAGCUGACCCUGAAGUUC(dTdT)-3′ and antisense strand: 5′-GAACUUCAGGGUCAGCUUG(dTdT)-3′.

2.2. Synthesis of the PE-Gold nanoassemblies

The gold nanoparticles were synthesized by Yang et al.'s method [49]. Briefly, 100 ml of 1 mM aqueous solution of $HAuCl_4$ was mixed with 8 ml of 38.8 mM aqueous sodium citrate solution that was used as a stabilizer and capping agent. 3 ml of 100 mM aqueous solution of ice-cold $NaBH_4$ was added dropwise under vigorous stirring and incubated for 24 h under constant stirring to decompose residual sodium borohydrate. A red colour colloidal solution of gold nanoparticles was formed. The solution was centrifuged at 2500*g* for 5 min and the supernatant was used for further modifications in the ligand exchange process with 11-MUA. The supernatant pH was brought to 11 with 1 M NaOH and 11-MUA was added to this alkaline solution at a final concentration of 0.1 mg/ml. Thiol capped- AuNP colloidal solution was purified at 7000 g for 20 min and pellets were re-suspended in 1 mM NaCl. First nanoassembly, PEI-Gold NA (AuNP/PEI/siRNA/PEI), was prepared by a method reported in the literature [31] with a few modifications. PEI (1 mg/ml) and siRNA (2 μM) were added to the thiol capped- AuNP solution in equal ratio and was incubated for 20 min under thorough mixing for each layer formation. After each step, the colloidal solutions were washed with 10 mM NaCl at 5000 g for 20 min and resuspended in 10 mM NaCl. Second nanoassembly, PAH-Cit-Gold (AuNP/PEI/PAH-Cit/PEI/siRNA), was prepared by a method reported by Guo et al. [32] with a few modifications. PAH-Cit, anionic polyelectrolyte, was synthesized as described earlier [50] and used at a concentration of 1 mg/ml for layering. The layers were deposited using PEI, PAH-Cit and siRNA similarly as the first nanoassembly. Finally, the nanoassembly was dispersed in 10 mM NaCl. The third nanoassembly, PLA-Gold NA (AuNP/PLA/siRNA/PLA), was also synthesized similarly using LbL deposition of PLA (1 mg/ml) and siRNA (2 μM) over thiol capped-AuNP.

2.3. Characterization of the PE-Gold nanoassemblies

PE-Gold NAs were characterized by UV-Visible spectroscopy, TEM (Transmission electron microscopy), X-ray diffraction (XRD), Zeta potential and dynamic light scattering (DLS) measurements. The UV-Visible spectra were produced on Multiskan (Thermo Scientific). The electron micrographs were obtained with Tecnai G2 20 S-TWIN TEM (FEI Company, Hillsboro, OR). Sufficiently diluted samples were prepared by depositing them onto carbon-coated copper grid (200 mesh)