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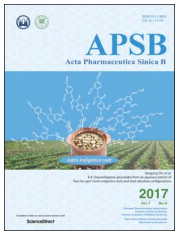

ORIGINAL ARTICLE

Improved method for synthesis of low molecular weight protamine–siRNA conjugate

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Abstract RNAi technology has aroused wide public interest due to its high efficiency and specificity to treat multiple types of diseases. However, the effective delivery of siRNA remains a challenge due to its large molecular weight and strong anionic charge. Considering their remarkable functions *in vivo* and features that are often desired in drug delivery carriers, biomimetic systems for siRNA delivery become an effective and promising strategy. Based on this, covalent attachment of synthetic cell penetrating peptides (CPP) to siRNA has become of great interest. We developed a monomeric covalent conjugate of low molecular weight protamine (LMWP, a well-established CPP) and siRNA *via* a cytosol-cleavable disulfide linkage using PEG as a crosslinker. Results showed that the conjugates didn't generate coagulation, and exhibited much better RNAi potency and intracellular delivery compared with the conventional charge-complexed CPP/siRNA aggregates. Three different synthetic and purification methods were compared in order to optimize synthesis efficiency and product yield. The methodology using hetero-bifunctional NHS–PEG–OPSS as a crosslinker to

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synthesize LMWP–siRNA simplified the synthesis and purification process and produced the highest yield. These results pave the way towards siRNA biomimetic delivery and future clinical translation.

1. Introduction

Post-transcriptional gene silencing occurs naturally in a process called RNA interference (RNAi), first reported by Fire and Mello in 1998[1]. RNAi holds promise as a powerful tool for gene therapy for novel treatments of various diseases[2–5]. RNAi is triggered by double-stranded RNA (dsRNA), after which dsRNA is cleaved by dicer, a member of the RNase III family of ribonucleases, to generate 21–23 double-stranded short interfering RNA duplexes (siRNA)[6]. Due to the high efficiency, specificity and the ability to perform numerous rounds of mRNA cleavage, siRNA has been recognized as the most attractive candidates for modulating disease-related mRNAs[7–9]. The site of action of siRNA is the cytosol. However, since polyanionic siRNAs are large molecules (MW: 1.3×10^4 to 1.5×10^4 Da) with negative charges from the phosphate backbone (about –40 charges)[10], they cannot readily enter cells by passive diffusion. In addition, nuclease susceptibility and poor penetration into many tissues are also biological barriers to siRNA delivery[11]. To realize its therapeutic potential, it is important to establish an efficient siRNA biomimetic delivery system.

Biomimetic drug delivery systems based on natural particulate range from pathogens to mammalian cells, as they possess specific functions *in vivo* that are worth examining in more depth. In conjunction with the availability of advanced biotechnology tools, investigators have exploited natural particulates for multiple applications in the delivery of proteins, siRNA and other therapeutic agents. For siRNA delivery, the biomimetic systems are generally divided into two major types based on viral and non-viral vectors. The viral systems use transfection of shorthairpin RNA (shRNA)-expressing vectors to produce siRNA in a cell[12]. However, one of the major problems of the viral vector system is the unwanted side effects that are caused by off-target reactions due to the natural tropism[13], even if it has a high efficiency for siRNA delivery. A wide range of non-viral vectors systems including liposome and lipids[14,15], cholesterol-conjugated[16], cationic polymers[17], RNA aptamers[18] and peptides[19] have been developed for siRNA delivery. These non-viral systems with improved safety, reduced immunogenicity, enhanced efficacy on target sites have shown potential for applications in biomimetic siRNA delivery. Among these, cell penetrating peptide (CPP) -mediated siRNA delivery is noteworthy. CPPs are capable of carrying a wide range of macromolecules into a variety of cells, with less cytotoxicity and high efficiency.

In general, biomimetic siRNA delivery mediated by CPP mainly occurs through two strategies, by noncovalently complexed *via* charge interactions[20] or by covalent conjugation[21]. Most of the studies involving CPPs have utilized the non-covalent conjugation method. The formation of noncovalent electrostatic complexes is a technically simple approach, and it may induce effective intracellular uptake. However, the formulation process of covalent conjugates can be well controlled in terms of homogeneity and reproducibility[22]. Besides, CPP-mediated transport efficiency of the covalent compound is higher than that of physical mixtures[23–25].

To achieve the efficient siRNA biomimetic delivery that vectorized with CPPs, it is necessary to formulate a soluble, 1:1 monomeric CPP–siRNA conjugate through a cytosol-cleavable disulfide linkage. After the siRNA is deliveried by the CPP into the cell, the siRNA can be retained in the cytosol with the disulfide linkage cleaved in the reductive enviroment, performing gene silencing treatment function through the RNA-induced silencing complex (RISC) system. PEGylation is known to have a shielding effect on charged molecules and reduce host immune response, so that PEG was introduced as a crosslinker. It has been shown that the conjugates did not generate coagulation, yet exhibited much better RNAi potency and intracellular delivery compared with the conventional charge-complexed CPP/siRNA aggregates[26].

In the present study, the abovementioned conjugation method was further improved in order to simplify the process and increase the yield. As depicted in Scheme 1, three methods were applied with either different purification steps or different linkers. We aimed to find a method combining efficient synthesis and purification steps with the highest production yields.

2. Materials and methods

2.1. Materials

LMWP (VSRRRRGGRRRRRR) was produced according to our developed protocol[27,28]. Heterobifunctional PEG derivatives maleimide PEG succinimidyl carboxymethyl ester (MW = 3500 Da) and ortho-pyridyl disulfide PEG succinimidyl carboxymethyl ester (MW = 3500 Da) were purchased from Jenkem technology Co., Ltd. (Beijing, China). Anti-enhanced green fluorescent protein (EGFP) siRNA–cysteine was synthesized by Guangzhou Ribobio Co., Ltd. (Guangzhou, China). The sense and anti-sense strands of siRNA was: 5′-GGCUACGUCCAGGAGCGCACC-3′ (sense), 3′-UUCCGAUGCAGGUCCUCGCGU-5′(anti-sense). For coupling, the sense strand of the siRNA was modified with an extra cysteine residue at its 5′-end. *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and dithiothreitol (DTT) were purchased from Thermo–Fisher Scientific Inc. Dimethyl sulfoxide (anhydrous solvent) was bought from Aladdin Industrial Corporation (Shanghai, China). Hi-Trap heparin™ HP columns, Hi-Trap™ DEAE FF columns, DEAE Sepharose Fast Flow were obtained from GE Healthcare Bio-Sciences Corp (Stockholm, Sweden). Affinity columns were obtained from Sangon Biotech (Shanghai, China). Agrose B, low EEO (Biotech Grade), was supplied by BBI Life Sciences Corporation (Shanghai, China). 20 bp DNA ladder, nucleic acid dyestuffs SYGR GreenII, 6 × DNA loading buffer and poly-lysine were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 5-(and-6)-Carboxytetramethylrhodamine, succinimdyl ester (TAMRA) was from AnaSpec Inc. (CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and

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