European Journal of Medicinal Chemistry 121 (2016) 132–142

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Fragment-based solid-phase assembly of oligonucleotide conjugates with peptide and polyethylene glycol ligands

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form
25 April 2016
Accepted 1 May 2016
Available online 3 May 2016

Keywords:
Antisense oligonucleotides
PEGylation
Disulfide bioconjugates
Solid phase synthesis
Gene silencing

ABSTRACT

Ligand conjugation to oligonucleotides is an attractive strategy for enhancing the therapeutic potential of antisense and siRNA agents by inferring properties such as improved cellular uptake or better pharmacokinetic properties. Disulfide linkages enable dissociation of ligands and oligonucleotides in reducing environments found in endosomal compartments after cellular uptake. Solution-phase fragment coupling procedures for producing oligonucleotide conjugates are often tedious, produce moderate yields and reaction byproducts are frequently difficult to remove. We have developed an improved method for solid-phase coupling of ligands to oligonucleotides via disulfides directly after solid-phase synthesis. A 2′-thiol introduced using a modified nucleotide building block was orthogonally deprotected on the controlled pore glass solid support with N-butylphosphine. Oligolysine peptides and a short monodisperse ethylene glycol chain were successfully coupled to the deprotected thiol. Cleavage from the resin and full removal of oligonucleotide protection groups were achieved using methanolic ammonia. After standard desalting, and without further purification, homogenous conjugates were obtained as demonstrated by HPLC, gel electrophoresis, and mass spectrometry. The attachment of both amphiphilic and cationic ligands proves the versatility of the conjugation procedure. An antisense oligonucleotide conjugate with hexalysine showed pronounced gene silencing in a cell culture tumor model in the absence of a transfection reagent and the corresponding ethylene glycol conjugate resulted in down regulation of the target gene to nearly 50% after naked application.

1. Introduction

Oligonucleotide gene silencing and splice-switching technologies have grown into indispensable tools in biomedical research as well as promising therapeutic modalities. However, their widespread therapeutic application is still significantly challenged by their poor pharmacokinetics, particularly insufficient cellular uptake [1–3]. Considering the overall moderate clinical success of oligonucleotides so far, it seems imperative to develop novel oligonucleotide derivatizations that properly shield the active agent up to its site of action without perturbing its pairing avidity.

One of the most promising approaches to improve the cellular uptake of the ONs is the development of bioconjugates with uptake enhancing ligands [4,5]. By attaching specific ligands, oligonucleotide distribution and cellular uptake can be rationally modulated to a certain extent. Depending on the ligand, efficient shielding from degrading enzymes, increased circulation times, receptor-specific cell binding and uptake, or enhanced membrane permeation can be conferred to oligonucleotides. Revusiran is the prime example of GalNAc-siRNA conjugates which make use of highly efficient receptor-mediated uptake in hepatocytes through the asialoglycoprotein receptor [6–8].

The generation of ligand-oligonucleotide conjugates requires efficient fragment-(or stepwise-) based synthesis schemes, which are ideally versatile to enable coupling of a variety of different ligands [9,10]. Precise and site-specific conjugation of small molecule ligands, or mono-disperse peptides and polymers with a defined chemical structure allows the generation of homogeneous ON conjugates for structure-activity relationship investigations and further preclinical development [11]. Arginine or lysine rich cell-penetrating peptides, glycoclusters, lipids, (linear or branched)

Abbreviations: ON, oligonucleotide; PEG, polyethylene glycol; CPG, controlled pore glass; LCAA, long chain amino alkyl; TCEP, tris(2-carboxyethyl)phosphin; TBP, tri-*n*-butylphosphine; HOBt, 1-hydroxybenzotriazole; DIPEA, N,N-diisopropylethylamine; BOP, benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate.

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http://dx.doi.org/10.1016/j.ejmech.2016.05.001
0223-5234/

polyamines, (linear or branched) poly- or oligo-ethylene-glycol chains and numerous small molecules have all received interest as possible oligonucleotide ligands [12].

Although conventional postsynthetic coupling reactions on the 3′- and 5′- positions of oligonucleotides in solution seem to be straightforward, there are some serious limitations in many cases, particularly for ligands with poor solubility in aqueous solutions. Often, multistep deprotection and activation procedures with subsequent purification steps are necessary, and electrostatic interactions of some ligands (particularly basic peptides) with oligonucleotides often hamper the efficiency of the coupling reactions [13]. These standard methods, typically making use of amino- or, less frequently, thiol-linkers introduced during oligonucleotide synthesis, are limited to attaching a single ligand at a terminal nucleotide position. Small molecules such as cholesterol or carbohydrates can also be tethered to the 5′-position of oligonucleotides by using respective phosphoroamidite building blocks [14,15]. Attachment on the 2′-position via linkers attached to the hydroxyl group of modified ribose nucleotides offer more versatile conjugation at any nucleotide within the sequence as well as multiple attachments within a single sequence [16]. Ligands are usually attached to the cleaved and purified oligonucleotide by solution-phase fragment coupling. In addition, stepwise solid-phase synthesis of peptide-oligonucleotide conjugates is possible, starting either with assembly of the ON followed by on-line ligand synthesis or vice versa [10,17]. The use of appropriately derivatized phosphoramidite building blocks carrying the desired ligand is another possibility to generate conjugates with small molecule ligands. In this strategy, ligands are tethered to the oligonucleotides via phosphate linkages, and it has been recently widely employed particularly for preparing single- and double stranded GalNAc conjugates for hepatocyte targeting [8,18–21].

There are a variety of chemical linkages used to join ligands with oligonucleotides, including amides, hydrazines, oximes, and many more [10,11,22]. Thiols afford an orthogonal reaction site to form ON-ligand conjugates through thioether, thioester or disulfide linkages [17,23]. Due to its ease of formation and their favorable stability profile for therapeutic applications, native ligation via a disulfide bond is perhaps the most extensively explored linkage in oligonucleotide peptide bioconjugations [22,24,25]. Disulfide linkages between ligands and oligonucleotides have been predominantly formed by employing post-synthetic approaches [25]. Although the exact extent and the kinetics of cleavage within endosomes are not fully elucidated, disulfide linkages are designed to at least partially release the delivered oligonucleotide from the ligand within endosomal compartments after successful cellular internalization [26]. Basic molecules are believed to contribute to subsequent endosomal escape through the proton sponge effect [27]. Linker cleavability is crucial for at least conjugates with large ligands such as PEGylated oligonucleotides and peptide- or protein-oliognucleotide conjugates, because bulky substituents impede interaction with the respective effector nucleases [28].

Solid-phase fragment coupling offers an alternative method for the stepwise synthesis on a single support. In this approach, the entire conjugate is assembled on a single support, either by conjugation of a prefabricated conjugate group or by assembling the conjugate moiety by a stepwise process prior to or after the ON synthesis [9]. The advantage of this method compared to solution fragment coupling is the application to a broad spectrum of ligands including lipophilic molecules and the ease of separating excess reagents and byproducts. We have recently communicated a facile post-synthetic disulfide formation method for solution based fragment coupling [29]. An S-sulfonate protected cysteine is utilized as the complementary ligand part to attach a peptide ligand onto the 2′-thioethyl arm of the ON in aqueous buffered solutions. In order to circumvent the problems associated with solution-phase approaches, i.e. the cumbersome purification and its limited applicability to lipophilic ligands, we evolved the method to allow on-bead coupling. Besides enabling facile purification, this method is also applicable to a wider range of ligands including those not easily soluble in aqueous buffers [10,30].

2. Results and discussion

To enable efficient formation of disulfide linked oligonucleotide conjugates and facilitate removal of ligand educts and reaction side product, we developed a feasible method for fragment coupling on the controlled pore glass (CPG) solid support. A resin-bound, still protected oligonucleotide with a free thiol group facilitates the coupling of lipophillic ligands in organic solvents. This is of particular importance considering the high hydrophilicity and anionic character of oligonucleotides which renders them unfavorable for conjugation reactions with other charged ligands due to aggregation [31]. In contrast, the resin-bound ON is practically neutrally charged since the internucleosidic oxygen atom is still protected as cyanoethyl ether. For the sake of achieving high coupling efficiencies, the ligand is usually used in considerable excess, which complicates the purification when the conjugation reaction is carried out in solution. In contrast, on a solid support, the unreacted conjugate group and the side products are easily removed by simple filtration, which markedly facilitates purification procedures.

A crucial point is the necessity of orthogonal cleavage of the thiol protection group and the linkage compatibility with the final global deprotection and cleavage step which is usually achieved in concentrated aqueous ammonia. The stability of peptide amide bonds during standard deprotection/cleavage is well documented [32]. However, when preparing disulfide linked conjugates, the oligonucleotide deprotection/cleavage step has to be adapted due to cleavage of the disulfide bond under those basic conditions.

In order to enable on-resin coupling, an adequate orthogonal oligonucleotide protection/deprotection strategy had to be developed. Among the conventional sulfur protecting groups adapted to ON synthesis and post-synthetic in-solution coupling, the S-trityl group has found widespread use due to its stability towards phosphoramidite ON synthesis strategy and subsequent deprotection/cleavage and conjugation procedures, particularly with respect to RNA ligation reactions [33]. However, with respect to solid-phase conjugation approach, this strategy bears important shortcomings; the standard argentometric deprotection of the trityl group followed by regeneration produces insoluble side products which cannot be thoroughly washed off the resin. As a result, poor reagent diffusion results in drastic reduction of yields during the subsequent coupling steps [33,34]. We chose the *tert*-butyl sulfanyl group because it possesses favorable characteristics for post-synthetic on-bead deprotection as well as adequate stability for withstanding the conditions of phosphoramidite based oligonucleotide synthesis. In contrast to solid-phase coupling, the risk of the generation of homodimeric oligonucleotides is minimized.

By using S-sulfonate derivatized ligands [29], the required deprotection and activation steps of other disulfide-building procedures are avoided, as is the risk of homodimerization. We selected a highly cationic peptide and an amphiphilic ethylene glycol ligand for coupling to prove the use of the method for compounds with different solubility profile. In particular, basic peptides are difficult to attach to oligonucleotide because of their high charge density, which makes them prone to aggregation and hampers the use of aqueous solutions. The S-sulfonate group withstands the deprotection conditions of the Fmoc peptide synthesis strategy thus enabling peptide synthesis products to directly