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Research paper

Novel peptide-dendrimer/lipid/oligonucleotide ternary complexes for efficient cellular uptake and improved splice-switching activity

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ABSTRACT

Despite the advances in gene therapy and in oligonucleotide (ON) chemistry, efficient cellular delivery remains an obstacle. Most current transfection reagents suffer from low efficacy or high cytotoxicity. In this report, we describe the synergism between lipid and dendrimer delivery vectors to enhance the transfection efficiency, while avoiding high toxicity. We screened a library of 20 peptide dendrimers representing three different generations and evaluated their capability to deliver a single-stranded splice-switching ON after formulating with lipids (DOTMA/DOPE). The transfection efficiency was analyzed in 5 reporter cell lines, in serum-free and serum conditions, and with 5 different formulation protocols. All formulations displayed low cytotoxicity to the majority of the tested cell lines. The complex sizes were $<$ 200 nm; particle size distributions of effective mixtures were $<$ 80 nm; and, the zeta potential was dependent on the formulation buffer used. The best dendrimer enhanced transfection in a HeLa reporter cell line by 30-fold compared to untreated cells under serum-free conditions. Interestingly, addition of sucrose to the formulation enabled – for the first time – peptide dendrimers/lipid complexes to efficiently deliver splice-switching ON in the presence of serum, reaching 40-fold increase in splice switching. Finally, *in vivo* studies highlighted the potential of these formulae to change the biodistribution pattern to be more towards the liver (90% of injected dose) compared to the kidneys (5% of injected dose) or to unformulated ON. This success encourages further development of peptide dendrimer complexes active in serum and future investigation of mechanisms behind the influence of additives on transfection efficacy.

1. Introduction

By 2005, at least one hundred different dendrimer structures and more than 1000 surface modifications were reported [1]. The rapidly growing field of dendrimers started when polypropylenimine (PPI) was successfully synthesized by Fritz Vögtle and his team in the late 1970s [2]. Several structures and modifications followed afterwards [3]. However, it was not until the early 1990s when dendrimers were introduced to the field of gene delivery [4]. Unlike many widely used transfection reagents, dendrimers are synthesized with a well-defined molecular structure and are thus monodisperse, so that control over their size and structure [5] makes structure-function relationships possible [6,7]

Secondly, the buffering capacity of dendrimers has been postulated to enhance drug/gene delivery: The 'proton sponge hypothesis' introduced by Hansler and Szoka [4] and confirmed by Sonawane et al. [8] highlighted the functional role of the tertiary amines of dendrimers under acidic conditions in the endosome. Tertiary amines get

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protonated and act as buffers, allowing chloride counter ions accumulate and cause endosomal osmotic swelling, then lysis [9,10]. Finally, the outer surface of dendrimers has the capacity to be modulated. Dendrimers are composed of three main units: inner core, interior branching units, and outer shell [10]. During synthesis, desired surface groups can be added in the outer dendrimer shell. Consequently, the dendrimer surface may be equipped with an overall positive, negative or neutral charge. Multivalency provides the dendrimers with additional avidity. For example, decoration of the dendrimer surface with positively charged groups enhances interaction with negatively charged ONs and cell membranes, leading to desirable complexation and improved intracellular delivery [5].

A major drawback of the conventionally used, polymeric, positively charged dendrimers is their cytotoxicity [11]. Several cationic dendrimers such as the well-known polyamidoamine (PAMAM, StarburstE®), poly(propyleneimine) (PPI) and poly(ethylene oxide) (PEO) are associated with cytotoxicity, which is dependent on the generation and surface modifications [12,13]. This toxicity has strongly urged researchers to either modify the existing structures or to introduce new ones aiming to obtain functional, yet less toxic dendrimers. Modifications of the dendrimer structure involved engineering of the outer surface with safer substitutes, replacement of cationic surface groups with anionic ones, or conjugation of the negatively charged ON to be delivered to the dendrimer surface [11]. Furthermore, a variety of safer, biocompatible/biodegradable dendrimer structures has been reported including *e.g.* polyether, polyester, phosphate, citric acid, melamine, triazine, and peptide dendrimers [11].

Peptide dendrimers have been greatly developed since their first synthesis by Denkewalter et al. [14] and Aharoni et al. [15]. These dendrimers can be divided into three main classes. (1) grafted or surface functionalized peptide dendrimers, (2) pseudo-peptide dendrimers, and (3) full peptide dendrimers. The first two classes have amino acids on the surface only, or on the surface and in the core, respectively. Only the third type (full peptide dendrimers) is entirely composed of amino acids, from the core through branching units to the outer shell [16]. These full peptide dendrimers display a variety of interesting functions such as cell-penetration and drug delivery [17,18]. Structures based on the amino acids as Lysine, Arginine, Histidine, or alternating sequences between them have been commonly used to protect and deliver genetic material [19–21]. Although the use of peptide dendrimers reduces cytotoxicity compared to normal dendrimers, these structures are still facing the same biological barriers as other delivery vectors. These barriers include limited resistance to serum, inadequate cellular uptake, insufficient endosomal escape ability and degradation by cytosolic nucleases [22]. In fact, a single delivery vector faces many challenges to mediate a successful gene transfection. Surface functionalization of dendrimers has been one proposed way to combine different structures and many compounds have been tested as surface ligands [23]. However, synthetic methods, product yield and purity have been sub-optimal.

Combining different delivery vector structures is another way to surmount the above-mentioned biological barriers. By mixing different vector structures, we can utilize their unique properties, to achieve synergistic effects on gene delivery, and minimize synthetic complications. The vector combination strategy has been successfully applied in a number of studies. A synergistic enhancement in the transfection efficiency of plasmid DNA (pDNA) was achieved upon mixing low-molecular weight polyethylenimines (PEIs) with different cationic lipids [24,25]. Equally, higher transfection efficiencies were shown for a 1:1 M ratio mixture of the cationic lipid (DOTMA) and the neutral lipid (DOPE) with linear and branched-peptides [26]. More recently, Kwok et al. assessed the transfection efficiency of both pDNA [6] and siRNA [7] using a library of peptide dendrimers mixed with cationic and neutral lipids. For efficient transfections, it was critical to have both hydrophobic and charged amino acids in the peptide dendrimer structures. Furthermore, a helper lipid mixture, DOTMA/DOPE (Lipofectin), was crucial for pDNA and siRNA transfections [27]. While much work has focused on plasmid DNA and siRNA transfection, to our knowledge, the delivery of splice-switching ON has, however, never been addressed by such a synergistic strategy between peptide dendrimers and lipids.

Moreover, previous studies have not investigated the potential effect of the order of addition of the different components in the formulation, cell line selectivity, or the transfection efficacy in the presence of serum that often negatively affects the transfection efficacy by various mechanisms [28]. In this report, we explored the transfection efficacy of formulated splice-switching ON using a library of 20 different peptide dendrimers mixed with Lipofectin as the lipid component. The screening involved five different cells lines, and two transfection conditions (serum-free and serum-associated conditions). We also tested five different orders of complexation and how they affected the transfection efficiency. In addition, we examined the effect of different buffers and additives on the enhancement of transfections under serum conditions. Finally, the knowledge gained from exploration of this matrix was probed by *in vivo* biodistribution of the optimal formula after intravenous injection in mice.

2. Materials and methods

2.1. Materials

The 18-mer splice-switching ON with a sequence (CCUCUUACCUC AGUUACA) was synthetized at GE Healthcare, Sweden. The ON has a fully modified phosphorothioate backbone and 2′-*O*-methyl modified ribose sugar units.

Lipofectin (a liposomal formulation of a 1:1 (w/w) ratio of DOTMA (N-[1-(2,3-dioleyloxy)- propyl]-N,N,N-trimethylammonium chloride) and DOPE (dioleoyl phosphotidylethanolamine)), and Lipofectamine 2000 (L2000) were both purchased from Invitrogen, Sweden. Sucrose, glucose and sodium chloride were obtained from Sigma-Aldrich, Sweden.

2.2. Cell lines and culture conditions

Five luciferase reporter cell lines representing different tissue origins (HeLa Luc/705, U-2 OS_705, C2C12_705, HuH7_705, and Neuro 2a_705 cell lines) [29] were cultured and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C in a humidified incubator with 5% CO_2. The preference of enzymatic reporter gene system over fluorescent proteins (as GFP) is due to their higher sensitivity, better representation of transcription rates by minimizing influence of extreme signals, and applicability in the presence of interfering fluorophores [30,31].

2.3. Synthesis and characterization of peptide dendrimers

Solid phase synthesis was used for preparation of peptide dendrimers while applying the Fmoc/Boc protection strategy as reported [6]. Out of the prepared 20 dendrimers, two were synthesized to represent the 1st generation, 8 as the 2nd generation, and 10 as the 3rd generation dendrimers (Fig. 1). Details about synthesis, characterization, chemical structures and yields are provided in supplementary information (SI-peptide dendrimers synthesis).

2.4. ON transfection using HeLa Luc/705 reporter cell line under serum-free conditions

One day before transfection, cells were seeded in sterile, clear bottom, white TC-Treated 96-well plates (Corning®, Sweden). Seeding was done at a density of 1.5×10^4 cells/well, and a final volume of 100 µL full growth medium/well to obtain 70–80% confluency/well the next day.

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