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Dynamic constitutional chemistry towards efficient nonviral vectors

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A B S T R A C T

Dynamic constitutional chemistry has been used to design nonviral vectors for gene transfection. Their design has been thought in order to fulfill *ab initio* the main requirements for gene therapy. As building blocks were used hyperbranched PEI as hydrophilic part and benzentrialdehyde and a diamine linear siloxane as hydrophobic part, connected through reversible imine linkages. The obtaining of the envisaged structures has been confirmed by NMR and FTIR spectroscopy. The dynamic synthesized amphiphiles proved to be able to self-assemble in nano-sized spherical entities as was demonstrated by TEM and DLS, characterized by a narrow dimensional polydispersity. Agarose gel electrophoresis proved the ability of the synthesized compounds to bind DNA, while TEM revealed the spherical morphology of the formed polyplexes.

As a proof of the concept, the nonviral vectors promoted an efficient transfection on HeLa cells, demonstrating that dynamic constitutional chemistry can be an important tool in the development of this domain.

1. Introduction

Conceptualized in 1972 and being at this moment at the forefront of medicine, gene therapy is an innovative procedure used in the treatment or for the amelioration of the patients' state of health [1,2]. Gene therapy uses nucleic acids as drugs by their delivery into the patient's pathological cells, aiming to replace the defective gene or to correct a genetic defect or a chronic disease, including cancer [3–5]. The key step in gene therapy is by far the development of an appropriate carrier for DNA, which has to fulfill mainly two major and contradictory requirements: firstly it has to bind strong enough the semirigid chains of the anionic DNA and secondly it has to bind it by reversible interactions in order to allow the release of the DNA into the cell nucleus [6,7]. To skip the barrier from fundamental design to application, they must fulfill many other and equally important requirements: the carrier should be nontoxic, biodegradable, it should facilitate endocytosis and also protect the genetic material from enzymatic degradation and so on. Moreover, the surface properties of a carrier are also highly important, along with its size and shape [8]. To this aim, great effort has been dedicated toward a simple design with efficient transfection. As the viral vectors proved important drawbacks, being even dangerous for the human body, the researchers' attention turned to a nonviral design which presents important advantages such as an easy preparation, high versatility regarding their design and structure, limited immunogenicity and ability to carry larger amounts of DNA [9,10]. The major drawback of the nonviral vectors is their transfection efficiency which is considerable lower than in the case of the viral vectors. This is why, in the last years the obtaining of new nonviral vectors for gene therapy with high transfection efficiency is one of the main targets in medicine related research.

The main candidates used in gene therapy for the nonviral approach are the cationic polymers such as chitosan [11], cationic lipids [12], polyethyleneimines [13,14], polypropyleneimines [15] or polyamidoamines [16].

Among these, polyethyleneimine (PEI) is by far the most frequently used polymer in nonviral vectors preparation, presenting a high ability to complex the genetic material, intrinsic endosomal activity and also a unique buffering capacity known as “proton sponge” effect [17,18]. More than this, studies demonstrated that PEI interacts with DNA with forces lower than 25 pN, forming stable polyplexes in physiological conditions but which are susceptible to be degraded by replisomes [19].

Intensive studies on the transfection ability and on the cytotoxicity of PEI demonstrated a close correlation of these two parameters with its molecular weight, both the transfection efficiency and cytotoxicity increasing with the molecular weight [20–24]. More than this, the transfection efficiency of the polyplexes based on PEI and DNA depends also on the N/P ratio. Thus, the polyplexes formed at an N/P ratio higher than 3 contain an excess of amine groups from PEI, which seems to facilitate the endosomal escape [25], fact which explains the superior transfection efficiency at higher N/P ratios.

In order to increase the transfection efficiency, different structures trying to mimic somehow the viral carriers were designed and obtained, based mainly on the cationic mediated transferring strategies. Therefore, liposomes [26], polymersomes [27], comb-like structures, star shaped structures [28] or dendrimers [29] have been prepared and demonstrated superior transfection efficiencies compared to the simply

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cationic polymers. This superior behavior has been attributed to the obtaining of spherical three-dimensional morphologies, which have a high density charge on the surface promoting the DNA binding and transfecting. Moreover, in the case of the dendrimers, an improved transfection was obtained for higher dendrimer generation [30], especially when hydrophobic units are comprised in each generation [31]. This is closely related to the vectors' ability to form small and stable nanoentities which assure a large distribution of the functionalities and allows further the DNA binding.

In this context, the present paper proposes a new design for nonviral vectors, by using hydrophobic and hydrophilic building blocks linked through reversible imine linkages, by dynamic constitutional chemistry (DCC). DCC is based on the use of reversible bonds at both molecular and supramolecular levels and was involved up to now in the development of many and diverse classes of molecules such as: metallo-cycles, molecular hosts, liquid crystals, hydrogels and many others [32–36]. The use of this synthetic pathway allowed in this case the obtaining of dynamic amphiphiles able to self-assemble into almost monodisperse spherical nanoentities, overcoming the synthesis and purification difficulties encountered in the obtaining of dendrimers or nanoparticles [30,37]. In order to fulfill *ab initio* the main requirements for nonviral vectors among which the ability to bind DNA, to facilitate the endosomal escape and to penetrate cell membrane, PEI was chosen as a hydrophilic part for the amphiphiles, while a linear siloxane was used as the main moiety for the hydrophobic part. The dynamicity at molecular level is conferred by the fact that the building blocks are linked together through reversible imine linkages, while the one at supramolecular level by the establishment of hydrophobic/hydrophobic interactions between the parts of the molecule which contain siloxane.

1.1. Rational design

The vectors' design has been thought to be formed from a hydrophobic core and a hydrophilic shell. As literature data indicate that the nonviral vectors containing siloxane units induced high transfection efficiency [11,38], a siloxane based hydrophobic core has been synthesized. To this aim a siloxane bearing diamine has been reacted with a trialdehyde in 1/1 molar ratio, to create an oligomeric chain with aldehyde functionalities (**A**), capable to bind the hydrophilic shell in multiple sites (Scheme 1). Hyperbranched PEI with low molecular weights of 800 Da and 2000 Da were used as hydrophilic moieties, being known that this polycationic polymer, presents the ability to act as a binder for DNA by electrostatic interactions and also to generate transfection, due to its "proton sponge" effect [13]. To attach the hydrophilic part to the hydrophobic one, the hydrophobic oligomer **A** has been reacted with hydrophilic PEI, considering 1 mol of PEI per 1 mol of structural unit of A, in diluted solution (1%). The reaction conditions were chosen in order to facilitate the yielding of a core-shell hydrophobic/hydrophilic structure with a maximum of PEI units, by imination and transimination reactions [39] toward a spherical stable entity. The idea was to use low molecular weight PEI chains, which are known as nontoxic but which, because of being linked on the same molecular entity, should be able to generate a high transfection efficiency, similar to the one of high molecular weight PEI. On the other hand, in reaching the desired spherical design, an import role is played by the hydrophobic/hydrophilic segregation of the resulted amphiphile in aqueous medium. Therefore, by virtue of the dynamicity at both molecular (reversible imine bonds) and supramolecular level (hydrophobic-hydrophilic forces), along with the natural tendency of energy minimization, the resulted oligomeric species should be able to self-assemble in water, forming spherical nanoentities [40]. In this manner, should result nano-sized nonviral vectors with a hydrophobic core based on the linear siloxane and the aldehyde, and a hydrophilic shell formed by PEI800 or PEI2000. The synthetic strategy has been designed in the Scheme 1.

2. Experimental

2.1. Materials

Benzene-1,3,5-tricarboxaldehyde from Manchester Organics, hyperbranched PEI (MW = 800 Da, and 2000 Da 50 wt% in H_2O), cell culture plates supplied by Corning (New York, USA) were used as received. Siloxane from Sigma-Aldrich Chemie (Germany; alpha-MEM, penicillin-streptomycin-amphotericin B mixture and Trypsin-Versene from Lonza; fetal bovine serum (FBS) from Gibco; HeLa cells from CLS-Cell-Lines-Services-GmbH, Germany; CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit and Bright-Glo(TM) Luciferase Assay System kit from Promega.. Single stranded low molecular wight (almost 250 base pairs), salmon sperm DNA was purchased from Fluka (St. Louis, USA). Plasmids pCS2 + MT-Luc (pLuc) encoding for firefly luciferase and pCS2 + NLS-eGFP (pEGFP) which encodes for enhanced green fluorescent protein were kindly provided as a gift from Prof. Adrian Salic (Harvard University, Boston) and were multiplied in *E. Coli* DH5a (gift supplied by Dr. Anca Gafencu, "Nicolae Simionescu" Institute of Cellular Biology and Pathology, Bucharest), and further purified using E.Z.N.A. Endo-free Plasmid Mini II kit (Omega Bio-Tek, Inc.).

2.2. Synthesis

2.2.1. Synthesis of the hydrophobic core

In the first step, the hydrophobic core has been synthesized by the condensation reaction between benzene 1,3,5-tricarboxaldehyde (0.162 g, 1 mmol) and 1,3-bis-(3-aminopropil)-1,1,3,3-tetramethyl siloxane (0.261 g, 1 mmol) in chloroform (8.46 mL) at a final concentration of the reagents of 5% *w/v*, at 20 °C, overnight (24 h). The compound was obtained as a yellow viscous liquid. The resulted compound will be further noted **A**.

A: yellow viscous liquid, η = 89%

^{1}H NMR (400.13 MHz, $CDCl_3$, ppm): δ 10.2($Ph(C\underline{H}O)_3$); 10.15 (1H, $Ph(C\underline{H}O)_2CH=N$-; 10.05 (1H, $Ph(C\underline{H}O)(CH=N\text{-})_2$); 8.63, 8.48, 8.3, 8.22, 8.16, 8.08, 7.94 (13H, $C\underline{H}=N$ and Ph-$\underline{H}$); 3.6, 3.5 (11H, $H_2N\text{-}C\underline{H}_2$), 1.7 (11H, $H_2N\text{-}CH_2\text{-}C\underline{H}_2$), 0.5 (11H, $H_2N\text{-}CH_2\text{-}CH_2\text{-}C\underline{H}_2$).

FTIR (KBr, cm^{-1}) 3430 (ν_{NH2}), 2949, 2830 ($\nu_{Si\text{-}C}$, ν_{CH3}, ν_{CH2}), 1705 ($\nu_{C=O}$), 1647 ($\nu_{CH=N}$), 1055 ($\nu_{Si\text{-}O}$), 838, 739 ($\nu_{CH\ aromatic}$).

GPC: M_n = 2558 g/mol; M_w = 2877 g/mol; M_z = 3360 g/mol; M_p = 1757 g/mol; M_w/M_n = 1.125; M_z/M_w = 1.168.

2.2.2. Synthesis of the amphiphiles

In the second step, PEI of two different molecular weights (800 and 2000 Da) has been reacted with the hydrophobic compound A, by a condensation reaction in a 1:1 molar ratio of the structural unit of A to the PEI. For that, the hydrophobic part **A** (0.039 g, 0.1 mmol) has been dissolved in 3.92 mL THF in order to obtain a solution with the concentration of 1% *w/v*, while the hydrophilic PEI with a molecular weight of 800 Da (PEI800) (0.06 g, 0.1 mmol) or of 2000 Da (PEI2000) (0.18 g, 0.1 mmol) was solved in 6 mL and 18 mL, respectively of ethanol, in order to form also a solution of 1% w/v concentration. The reactions were conducted in diluted systems in order to facilitate the imination and transimination processes and to give rise to individual nanoentities by the self-assembling of the resulted amphiphiles [40]. The two diluted solutions have been mixed together and kept under magnetic stirring for 10 days, at room temperature. The reaction mixture was dried under vacuum. From now on, the code **B** stands for the compound obtained using PEI800, while the code **C** for the one using PEI2000. Both compounds were soluble in water.

B: yellowish solid