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ORMOPLEXEs for gene therapy: *In vitro* and *in vivo* assays

J.C. Matos [a,b], A.R. Soares [c], I. Domingues [c], G.A. Monteiro [d,e], M.C. Gonçalves [a,b,*]

[a] Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[b] CQE, Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, Portugal
[c] Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[d] Departamento de Bioengenharia, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisboa, Portugal
[e] IBB — Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Portugal

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ABSTRACT

Gene therapy stays on the cutting edge of biomedical research, being the design of the optimal gene delivery vector one of the key requests. Silica-based nanoparticles (NPs) have emerged as promising non-viral gene delivery vector, due to their high biocompatibility, nontoxicity, non-immunogenicity, biodegradability and enormous bioconjugation versatility. In this work a sol-gel methodology for the synthesis of amino-functionalized silica NPs (NH_2-ORMOSIL NPs) was optimized, and NPs were characterized by TEM and FTIR. In a first step NH_2-ORMOSIL NPs were bioconjugated with a plasmid DNA, pVAX1-GFP, assembling an ORMOPLEXE, confirmed by agarose gel electrophoresis. In a second step, *in vitro* studies have been performed with cultured CHO cells, where ORMOPLEXEs transfection was proved by CLSM. *In vivo* transfection efficiency and bio-distribution were performed in Zebrafish (*Danio rerio*) embryos, assessed by FM. Finally, NPs ecotoxicity was studied in zebrafish embryos by following the mortality and developmental endpoints.

1. Introduction

Almost three decades have passed since the approval of the first gene therapy clinical trial, in 1990 [1]. Since then, the number of clinical protocols initiated and approved worldwide has increased exponentially, focusing on different etiology diseases [2].

So far, the vast majority (75%) of gene therapy clinical trials have been performed by viral vectors [2]. Notwithstanding, viral delivery and therapy systems usually carry disadvantages as the potential risk of excessive immune response (adenovirus), insertional mutagenesis (retroviruses), as well as gene size limit and high cost which have restricted some of their applications. Non-viral vectors are far less efficient than viral ones, but they have the advantages of low immunogenicity, high capacity for gene insertion, along with the possibility of large series production with reduced costs [3,4].

In the last thirty years numerous non-viral gene delivery systems have been designed, targeted to reduce toxicity and to improve gene therapeutic effects. Polydispersity, reproducibility, and chemical stability, along with high physical performance, are some of the characteristics required to non-viral gene carriers. Among non-viral gene delivery systems, cationic polymers (able to complex with pDNA forming POLYPLEXES) [4], lipids (self-assembled to pDNA forming LIPOPLEXES) [5], solid lipid nanoparticles (NPs) [6–8], dendrimers [9], polyethylenimine [10] and cationically modified silica NPs [11–14] play a special role. Lately, hybrid systems have been developed; VIBROSOMES, combining liposome with an inactivated HIV or influenza virus, or just the mixture of viral vectors with cationic lipids, are some of the examples. However, non-viral systems may risk molecular degradation, as its molecules may be degraded by extra- or intracellular nucleases. Cationic systems, in particular, are associated with inflammatory processes [15]. Therefore, the lack of an efficient and safe non-viral gene delivery vector is still a crucial concern for gene therapy.

Among the diversity of nanoparticles (NPs) currently in the market, silica-based NPs present several attributes that promote their use as platform for DNA gene therapy [15,16]. They offer high biocompatibility, hydrophilicity, and enormous flexibility for surface modification with a high payload capacity, a prolonged blood circulation time, giving rise to a wide range of biomedical and pharmaceutical applications [16–20]. *In situ* silica functionalization, by introducing organic functional groups during the sol-gel process (forming an ORganically MOdified SILica matrix, known as ORMOSIL [21]), allows easy chemical conjugation/decoration of biomolecules at the ORMOSIL surface [22–24] and/or the load with either hydrophilic or hydrophobic drugs/dyes.

Among the commonly used functionalizing groups, amine (—NH_2) is the one promoting more efficient pDNA interactions [14,25–27]. The —NH_2 group electrostatically interact with proteins, enhancing

* Corresponding author at: Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal.
E-mail address: clara.goncalves@ist.utl.pt (M.C. Gonçalves).

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their adsorption [28–30], binding and protecting pDNA from enzymatic digestion allowing cell transfection *in vitro* [31–33].

The present work aimed at developing NH_2-ORMOSIL NPs to be used as gene delivery vectors. To achieve this objective the following steps were performed:

i) synthesis and characterization of NH_2-ORMOSIL NPs, observed by Transmission Electron Microscopy (TEM) and, Fourier transform infrared spectroscopy (FTIR);
ii) conjugation of NH_2-ORMOSIL NPs with pDNA (pVAX1-GFP) forming an ORMOPLEXE, controlled by electrophoresis mobility;
iii) *in vitro* transfection efficiency assessed by Chinese hamster ovary (CHO) cells, followed up by confocal laser scanning microscopy (CLSM);
iv) *in vivo* transfection efficiency and bio-distribution assessed by zebrafish (*Danio rerio*) embryos, followed up by CLSM;
v) ecotoxicity assessed by zebrafish embryos, by following the mortality and developmental endpoints by Stereoscopic Zoom Microscope (SZM).

The Zebrafish model presents many features that make it well suited on studies for nanomedical applications. They have a short generation time (about 3 months) and a large hatch size (more than 200 eggs), which allows low-cost assays. Zebrafish also presents a rapid embryogenesis, with most of the organs developed in 48 h post fertilization (hpf) [34,35].This rapid development and the optical transparency of the embryos allow the monitoring of organ development including delays and malformations induced by several types of factors.

2. Materials and methods

All chemicals, tetraethoxysilane ($Si(OC_2H_5)_4$, TEOS, reagent grade 98%, Aldrich Chemistry), 3-aminopropyltriethoxysilane ($NH_2(CH_2)_3Si(OC_2H_5)_3$, APTES, ≥98%, Sigma-Aldrich), ammonia hydroxide solution (NH_4OH, 28–30%, Sigma-Aldrich), and absolute ethanol (CH_3CH_2OH, EtOH, 99.5%, Merck) were used without further purification.

2.1. Transmission Electron Microscopy (TEM)

Size (static diameter) and size distribution were determined by TEM. To analyze the samples, a drop of the NPs suspension was placed on a copper grid and left to dry at room temperature. TEM Hitachi H-8100 model was used and the micrographs were obtained using an applied tension of 200 kV.

2.2. Fourier transform infrared spectroscopy (FTIR)

NPs *fingerprint* were recorded by FTIR, with Nicolet 5700 in transmission mode in the medium infrared (IR) range. The NH_2-ORMOSIL NPs samples were finely ground and mixed with potassium bromide (KBr, optical grade, ≥99.9%, Sigma-Aldrich), and then pressed into a disc (5 mg NH_2-ORMOSIL NPs: 200 mg KBr).

2.3. Confocal Laser Scanning Microscopy (CLSM)

In vitro studies were followed with CLSM. The images were obtained with a Leica TCS SP5 laser scanning microscope.

2.4. Microinjector

IM 300 Microinjector from Narishige was used to microinject Zebrafish embryos. This microinjector enables the injection of very small volumes of liquid with great precision, being the time and the pressure set in advance. Borosilicate glass capillaries were pulled using a needle puller and the resulting flexible, thin, closed tip was snapped off to open the capillary for injecting. Each tip was calibrated for the release of fluid in oil.

2.5. Stereoscopic Zoom Microscope (SZM)

Zebrafish eggs were observed under the SMZ 1500, Nikon Corporation, Japan.

2.6. Fluorescence Microscope (FM)

Zeiss Imager Z1 FM was used to analyze biodistribution in zebrafish embryos.

2.7. Synthesis methodology

NH_2-ORMOSIL NPs were synthesized, characterized and further conjugated with pVAX.1-GFP (pDNA) forming ORMOPLEXEs (for short). ORMOPLEXEs were screened sequentially through gel agarose electrophoresis and *in vitro* tests. Then the ORMOPLEXEs compositions with better performances were studied in *in vivo* assays in Zebrafish model. Ecotoxicity was assessed by zebrafish embryos. Fig. 1 and Table 1 illustrate and summarize the work methodology.

2.8. NH_2-ORMOSIL NPs synthesis and characterization

The synthesis of NH_2-ORMOSIL NPs was performed by modifying Huang et al. [32] protocol. TEOS and APTES sol-gel precursors were used with two different molar ratios (TEOS:APTES 9:1, 7:3). The silica NPs synthesis was performed by mixing 44.7 mL of ethanol, 1.15 mL of ammonia solution and 2.25 mL of bi-distilled water. The solution was then placed under heated-magnetic stirring until a temperature of 50 °C was reached. At that moment, 1.9 mL of precursor's mixture was quickly added. For the TEOS:APTES 9:1 M ratio, 1.71 mL of TEOS and 0.19 mL of APTES, were used; for the TEOS:APTES 7:3 M ratio, 1.31 mL and 0.59 mL (values based on the TEOS:APTES 9:1 and 7:3 M ratios). The solution was left in magnetic stirring at 50 °C (during 30 min for the TEOS:APTES 7:3 M ratio, and 60 min for 9:1 M ratio). NPs were then centrifuged and finally washed twice with ethanol 70% (2 × 10 mL) and with bi-distilled water (0–2 μS/cm conductivity at pH at 5.8–6.5) (2 × 10 mL). NH_2-ORMOSIL NPs were dried in the incubator at 40 °C for a period of 72 h.

The hybrid nature of the ORMOSIL NPs was confirmed by FTIR, while size and size distribution were observed by Transmission Electron Microscopy (TEM). Size measurements were performed with *Adobe Photoshop CS5®*.

2.9. Plasmid purification

The plasmid (pDNA) used in the present study was pVAX1-GFP (3697 bp), which contains the human cytomegalovirus (CMV) immediate-early promoter, a ColE1 type origin of replication and the kanamycin resistance gene for bacterial selection [36]. The plasmid was obtained by growing *Escherichia coli* DH5α cells in 5 mL of Luria Bertani (LB) broth medium (from Sigma-Aldrich) and 30 μg/mL of kanamycin (from Invitrogen), at 37 °C, overnight in an orbital shaker (250 rpm). The plasmid purification was performed using the procedure described in the High Pure Plasmid Isolation Kit (Roche, Germany).

2.10. ORMOPLEXES assembling

The bio-conjugation of the NH_2-ORMOSIL NPs with pDNA gives rise to pDNA:NH_2-ORMOSIL NPs assemble (ORMOPLEXEs for short). ORMOPLEXEs were made by direct incubation of pDNA and NH_2-ORMOSIL NPs, in a wide range of pDNA:NPs mass ratios (from 1:20 up to 1:350). 1 μg of pDNA was placed in each well (of the