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New gene delivery system based on oligochitosan and solid lipid nanoparticles: 'In vitro' and 'in vivo' evaluation

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ABSTRACT

In the present work, we evaluated the potential utility for gene delivery of three oligochitosans (OligoCh) that differs in the M_n (OligoChA: 6.1 kDa, OligoChB: 11.5 kDa, and OligoChC: 13.7 kDa), with deacetylation degree of 85%. OligoCh were complexed directly with the pCMS-EGFP plasmid to form OligoCh–DNA carriers. Taking into account the features and benefits of both Ch and SLNs, we also combined the OligoCh with SLNs. The three OligoCh presented a great ability to condense and protect the DNA. The OligoCh of highest M_n (OligoChC) complexed with SLNs at a OligoChC:DNA:SLN ratio 2.5:1:5 induced the highest transfection level in HEK-293 cells at day 3; being transfection 2-fold higher at day 7. After the intravenous administration to mice, OligoChC–DNA and OligoChC–DNA–SLN vectors were able to induce the expression of EGFP in the spleen, lung and liver, which was maintained for at least 7 days. In spite of the difference in the "in vitro" transfection levels between both vectors, no difference was detected in transfection after "in vivo" administration. Moreover, the OligoChC improved the "in vivo" transfection efficacy of the DNA–SLN vector. This work shows the potential utility of the combination of SLNs and OligoCh for the development of new non-viral vectors for gene therapy.

1. Introduction

The development of new drug delivery systems usually involves the combination of components of different nature, with the aim of taking advantage of beneficial properties of each one. In the field of gene therapy, non-viral systems with different composition are under study; e.g. hyaluronic acid and chitosan (de la Fuente et al., 2010), peptides and solid lipid nanoparticles (del Pozo-Rodríguez et al., 2009a; Delgado et al., 2011) or lysine-based peptides and PLGA (Nie et al., 2009), among others.

SLN-based vectors developed by our group have shown capacity for transfection 'in vitro' in several cell lines, and 'in vivo' as well after intravenous and ocular administration (del Pozo-Rodríguez et al., 2010; Delgado et al., 2012a,b). Their ability to condense and protect DNA (del Pozo-Rodríguez et al., 2007), and their entry efficiency into several cell lines Delgado et al., 2012a), along with the possibility to be decorated with other compounds (del Pozo-Rodríguez et al., 2009a; Delgado et al., 2011, 2012b), make this nanoparticular system an interesting alternative to viral vectors. From the point of view of the technological application, SLNs have good stability and are subject to be lyophilized (del Pozo-Rodríguez et al., 2009b), which facilities their industrial production.

Chitosans (Ch) are polysaccharides comprising copolymers of glucosamine and N-acetylglucosamine. They are biodegradable, biocompatible, nontoxic, and cheap polycationic polymers with low immunogenicity (Jreyssaty et al., 2012). These properties are considered of great interest for many scientists working in biomedical fields, and specifically in drug delivery (Dutta et al., 2004; de la Fuente et al., 2008, 2010; Csaba et al., 2006, 2009a). Moreover, the capacity of Ch to cross cell membranes (Thanou et al., 2001) improves the entry of active substances into several types of cells. Due to the positive charge of Ch, it binds DNA efficiently and protects it from nuclease degradation; therefore, this polymer is considered very interesting for gene therapy (Ramesan and Sharma, 2012). It has been shown that low molar mass Ch are more efficient for transfection than high molar masses Ch (Csaba et al., 2009b; Turan and Nagata, 2006; Strand et al., 2010; Duceppe and Tabrizian, 2009). This effect can be attributed to the easier release of pDNA from the nanocarrier upon cell internalization (Strand et al., 2010; Thibault et al., 2010). Moreover, low molar mass Ch

Abbreviations: Ch, chitosan; EGFP, green fluorescent protein; MM, molar mass; OligoCh, oligochitosan; SLN, solid lipid nanoparticle; RT, room temperature.

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plays an important role for the endosomal escape of Ch nanocarriers (Thibault et al., 2011). Although highly deacetylated Ch have shown better transfection in 'in vitro' studies, the effect of Ch deacetylation degree is still unclear (Garcia-Fuentes and Alonso, 2012). After 'in vivo' administration, Ch nanocarriers have demonstrated good transfection capacity (Garcia-Fuentes and Alonso, 2012). Most of the Ch-based nanocarriers for gene therapy are based on direct complexation of Ch and the nucleic acid (Leong et al., 1998). As mentioned above, the efficacy for gene delivery depends on the capacity of the Ch to complex genetic material and to cross biological barriers. Additionally, its pH buffering capacity favours the endosomal escape (Chang et al., 2010). It is known that the positive charge of Ch nanocarriers is reduced in some physiological fluids due to their intrinsic pH (Germershaus et al., 2008), and this may reduce the colloidal stability of the Ch nanoparticles. Several strategies have been proposed in order to increase the stability of Ch nanocarriers, such as the conjugation with polyethylene glycol (Csaba et al., 2009c), or the incorporation of amine groups (i.e. quaternized chitosan) in the polysaccharide backbone, making the positive charge independent of pH (Garcia-Fuentes and Alonso, 2012). Finally, its cationic nature, that provides a strong electrostatic interaction with negatively charged mucosal surfaces, joint with its bioadhesive properties, could prolong the contact time with tissues (Mansouri et al., 2004).

In the present work, we evaluated the potential utility for gene delivery of three oligochitosans (OligoCh) that differs in the M_n (OligoChA: 6.1 kDa, OligoChB: 11.5 kDa, and OligoChC: 13.7 kDa), with deacetylation degree of 85%. OligoCh were complexed directly with the pCMS-EGFP plasmid to form OligoCh–DNA carriers. Taking into account the features and benefits of both Ch and SLN, we also combined the OligoCh with SLNs and we evaluated these two kinds of vectors, OligoCh–DNA and OligoCh–DNA–SLN, in terms of 'in vitro' and 'in vivo' transfection after intravenous administration to mice.

2. Materials and methods

2.1. Synthesis of OligoCh

Chitosan of M_n of 1.200 kDa and degree of deacetylation 85% (Yuhuan Ocean Biochemical CO. Ltd. CH-K05011512, China) was used as starting material for preparation of three samples of oligochtiosans. Chitosans with varying molar masses were prepared by controlled radical degradation via continuous addition of various concentration of hydrogen peroxide to 2.5% high MM Ch solution. The reaction was carried out for 2 h at 80 °C. All samples, after degradation and purification had various molar masses and similar polydispersities of MM in range 1.5–2.2. The detailed procedure of degradation and purification of Ch is described elsewhere (Bartkowiak and Hunkeler, 2000). The molar mass of final oligochitosan samples was determined by GPC method using Knauer SmartLine HPLC system (Knauer, Germany) equipped with refractometric detection at a flow-rate of 1 cm^3/min. Two SEPARON HEMA BIO columns 1000 and 40 (TESSEK Ltd., Praha, Czech Republic) with PTFE guard column (Supelco, USA) were employed as the stationary phase with aqueous solution of 0.5 M acetic acid/0.5 M sodium acetate as an eluent. The water-soluble GPC standards pf dextran (PSS, Mainz, Germany) were selected and used for column calibration and as a relative reference for MM calculation of low molar mass OligoCh. Table 1 represents the Gel Permeation Chromatography (GPC) results for the three synthesised OligoCh samples.

Table 1
The GPC results for the three OligoCh samples.

Sample	M_n[a] (g/mol)	M_w[b] (g/mol)	Polydispersity
OligoChA	6,100	40,000	6.6
OligoChB	11,500	100,000	8.7
OligoChC	13,700	125,000	9.1

[a] M_n: Number average molecular weight.
[b] M_w: Weight average molecular weight.

2.2. Production of solid lipid nanoparticles (SLNs)

The SLNs, composed by the solid lipid Precirol® ATO 5 (Gattefossé; Madrid, Spain) and the surfactants 1,2-Dioleoyl-3-Trimethylammonium-Propane Chloride Salt (DOTAP) from Avanti Polar Lipids (0.4% w/v) and Tween 80 (0.1% w/v), were produced by a solvent emulsification–evaporation technique previously described (del Pozo-Rodríguez et al., 2007).

2.3. Preparation of vectors

OligoCh–DNA vectors were obtained by mixing the pCMS-EGFP plasmid, which encodes the enhanced green fluorescent protein (EGFP), with an aqueous solution of OligoCh. Different OligoCh to DNA ratios (w/w) were applied 1:1, 2.5:1, 5:1, 7.5:1, 10:1, 12.5:1 and 15:1.

OligoCh–DNA–SLN vectors were prepared by first forming OligoCh–DNA complexes at different ratios, and then, incorporating the SLN under agitation for 30 min. The SLN to DNA ratio, expressed as the ratio of DOTAP to DNA (w/w), was fixed at 5:1. These vectors have OligoCh–DNA complexes adsorbed on the surface of nanoparticles.

2.4. Study of DNA binding to OligoCh

The resulting OligoCh–DNA complexes were subjected to electrophoresis on a 1% agarose gel (containing ethidium bromide for visualisation) for 30 min at 120 V. The gel electrophoresis materials were acquired from Bio-Rad (Madrid, Spain). The bands were observed with an Uvitec Uvidoc D-55-LCD-20 M Auto transilluminator.

2.5. Size and potential measurements

Sizes of OligoCh–DNA and OligoCh–DNA–SLN vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials of OligoCh–DNA and OligoCh–DNA–SLN were measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcesteshire, UK). All samples were diluted in 0.1 mM NaCl solution.

2.6. DNase protection study and SDS-induced release 'in vitro'

Deoxyribonuclease I (DNase I) from Sigma–Aldrich (Madrid, Spain) was added to OligoCh–DNA–SLN complexes to a final concentration of 1U DNase I/2.5 μg DNA, and the mixtures were incubated at 37 °C for 30 min. Thereafter, a lauryl sulphate sodium (SDS) solution was added to the samples to a final concentration of 1% to release DNA from the vectors. Samples were then analysed by electrophoresis on agarose gel (described above) and the integrity of DNA in each sample was compared with untreated DNA as control.

2.7. Cell culture and transfection protocol 'in vitro'

'In vitro' assays were performed with Human Embrionic Kidney (HEK-293) cells, obtained from the American Type Culture Collection (ATCC). Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain).

HEK-293 cells were maintained in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM)