Chitosan Micro- and Nanospheres: Fabrication and Applications for Drug and DNA Delivery

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Abstract: Polysaccharides and other cationic polymers have been recently used in pharmaceutical research and industry for their properties to control the release of antibiotics, DNA, proteins, peptide drugs or vaccines. They have been also extensively studied as non viral DNA carriers for gene delivery and therapy. Chitosan is one of the most used since it can promote long-term release of incorporated drugs. Here, we reviewed the recent literature on the preparation of chitosan micro- and nanospheres using different manufacturing processes (nanofabrication). Moreover, the preparation of chitosan and chitosan/DNA nanospheres using a novel and simple osmosis-based method has been recently reported. This novel nanofabrication method may be a useful alternative to obtain small DNA-containing nanospheres (38 ± 4 nm) for biomedical applications. The reported method has general applicability to various synthetic or natural biopolymers. Solvent, temperature and membrane cut-off are the physicochemical parameters able to control the overall osmotic process leading to obtain several nanostructured systems with different size and shape that may be used in several biotechnological applications.

Key Words: Chitosan, cationic polymers, nanospheres, microspheres, gene delivery, DNA.

INTRODUCTION

Important efforts and advances in biotechnology have facilitated the production of macromolecules like polypeptides, proteins and polysaccharides and their effective use as carriers for biotechnological applications [1, 2]. Also in the field of gene therapy the development of efficient and safe gene carrier systems able to transfer DNA into cell is a major goal [3-6]. Different systems were developed in the last years: cationic liposomes [7-10], polylysine and its conjugates [11-13], diethylaminoethyl-dextran (DEAE-dextran) [14], dextran-spermine polycations [15], polyethyleneimine (PEI) [16, 17], polyamidoamine dendrimers [18], lipopolyamines [19-21] and chitosan [22]. Chitosan is a biodegradable polysaccharide obtained from deacetylated chitin (66% to 95% deacetylation) and the commercial product has an average molecular weight ranging between 4 and 20 kDa. It contains several amino groups that in acidic pH may undergo protonation leading to its solubilization in water. Chitosan may also establish electrostatic interactions with the negatively charged DNA to form complexes (polyplexes). Being non toxic, chitosan is also widely used in pharmaceutical research and in industry for the controlled release of antibiotics, DNA, proteins, peptide drugs or vaccines [23,24].

Size and shape of these systems are important factors for several medical applications: to improve bioavailability (i.e., overcoming enzymatic or adsorption barriers and in the case of nasal administration the mucociliary clearance) and to prolong the residence time of drug delivery systems at the site of drug absorption [25]. Different approaches have been used to obtain micro- and nanostructured chitosan-based nanoparticles: emulsions [26, 27], reverse micelles [28], solvent evaporation [29, 33, 34], spray-drying [30] and thermal processes [31]. On industrial scale, it is generally difficult to obtain nanoparticles with uniform dimensions and without surfactant traces in the final product.

Recently, we reported the use of a novel patented osmosis-based method to obtain well-defined chitosan and chitosan/DNA nanospheres without the use of surfactants [32]. The novelty of the reported method resides in the general applicability to various synthetic or natural biopolymers. Solvent, temperature and membrane cut-off are the physicochemical parameters able to control the overall osmotic process leading to obtain several nanostructured systems with different size and shape that may be used in several biotechnological applications.

Chitosan Nanospheres Fabrication Using Emulsions/ Surfactants

One of the methods used for the preparation of chitosan microsphere is the emulsion/surfactants solutions. The latter are mixtures of solvents and detergents where spheres begin to form in small vesicular compartments. Jameela and Jayakrishnan reported the preparation of chitosan microspheres from 74% deacetylated chitin by the glutaraldehyde cross-linking of an aqueous acetic acid dispersion of chitosan in a mixture of liquid paraffin and petroleum ether stabilized using sorbitan sesquioleate as the surfactant [26]. Cross-linking and hardening of the spherical particles were achieved by the addition of glutaraldehyde-saturated toluene through the organic phase. A relatively novel antineoplastic agent, mitoxantrone, was incorporated into the microspheres and the drug release was studied *in vitro* into phosphate buffer for over 4 weeks at 27°C. Drug release was found to

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be effectively controlled by the extent of cross-linking. Only about 25% of the incorporated drug was released over 36 days from microspheres of high cross-linking density. Implantation of placebo chitosan microspheres in the skeletal muscle of rats was carried out in order to assess the biocompatibility and biodegradability of the microspheres. Histological analysis showed that the microspheres were well tolerated by the living tissue. However, no significant biodegradation of the material was noticed over a period of 3 months in the skeletal muscle of rats. Their data indicate the possibility of using cross-linked chitosan microspheres as a drug carrier for sustained drug release for very long periods.

The development of nonviral vectors for the efficient and safe delivery to cells has long been pursued to facilitate gene therapy. Recently, many non-viral vectors modified with cationic lipids (i.e., dextran-spermine [15] and lipopolyamines [19-21]) and cationic polymers (i.e., polylysine and its conjugates [11-13], chitosan [22], polyethyleneimines [16,17] and polyamidoamines [18]) have been reported. However, stability, longer duration of gene expression, and reduced cytotoxicity need to be improved in non-viral cationic vectors. Chitosan has been successfully employed by some authors to prepare mucoadhesive poly-(lactide-co-glycolide) nanospheres (PLGA-NS). Chitosan was coupled to the nanoparticle's surface resulting into improved mucosal peptide absorption after oral and pulmonary administration [27].

Furthermore, nucleic acids are able to form complexes with cationic lipids. Nucleic acids for gene therapy (plasmid DNA, antisense oligonucleotide, small interfering RNA, etc.) can be encapsulated into the matrix of the polymer particles with the emulsion solvent diffusion method depicted in Fig. (**1**). The main advantage of this method resides in its easiness of preparation and also avoids the submicronization of particles by ultrasonication. The resultant nanospheres show better cellular uptake and different gene therapeutic effects compared with conventional vectors due to their improved adherence to cells and sustained release of polynucleic acid in the cells. Therefore, these chitosan-coated PLGA-NS can potentially be applied in nonviral vectors for gene therapy.

Chitosan Nanospheres Fabrication Using Reverse Micelles

Another method for the preparation of chitosan nanosphere has been reported by Mitra *et al*. [28] who employed a reverse-micelles technology. This technology is similar to the emulsion/surfactant method discussed above. The only exception is that the surfactant head groups are located toward the centre of the particle with the tails extending out (water-in-oil micelles) as depicted in Fig. (**2**).

This technology is very useful to reduce undesirable side effects like those induced by doxorubicin (DXR) commonly used in cancer therapy. In fact, this drug, a member of the anthracycline ring antibiotics, produces dose-dependent adverse effects such as cardio-toxicity and myelosuppression [39,40]. Attempts have been made to minimize them by coupling the drug with dextran (DEX) and then encapsulating the conjugate in hydrogel nanoparticles. By encapsulation of the drug conjugate in biodegradable, biocompatible long circulating hydrogel nanoparticles, the authors improved the therapeutic efficacy of the conjugate. The size of these nanoparticles as determined by quasi-elastic light scattering, was found to be 100±10 nm diameter, which favours the enhanced permeability and retention effect (EPR) as observed in most solid tumours. The antineoplastic effect of these DEX–DXR nanoparticles was evaluated in J774A.1 macrophage tumour cells implanted in Balb/c mice. The *in vivo* efficacy of these nanoparticles as antineoplastic drug carriers was determined by tumour regression and increased survival time as compared to free drug. The encapsulation efficiency of some conjugates in nanoparticles not only reduces the side effects, but also improves the therapeutic efficacy in the treatment of solid tumours.

So far, there are no examples on the incorporation of DNA into chitosan nano/microspheres prepared with the reverse micelle method. However, we suggest that the nanovesicles prepared according to Mertins *et al*., might be amenable to DNA delivery studies, and might be easily applied to this purpose [33].

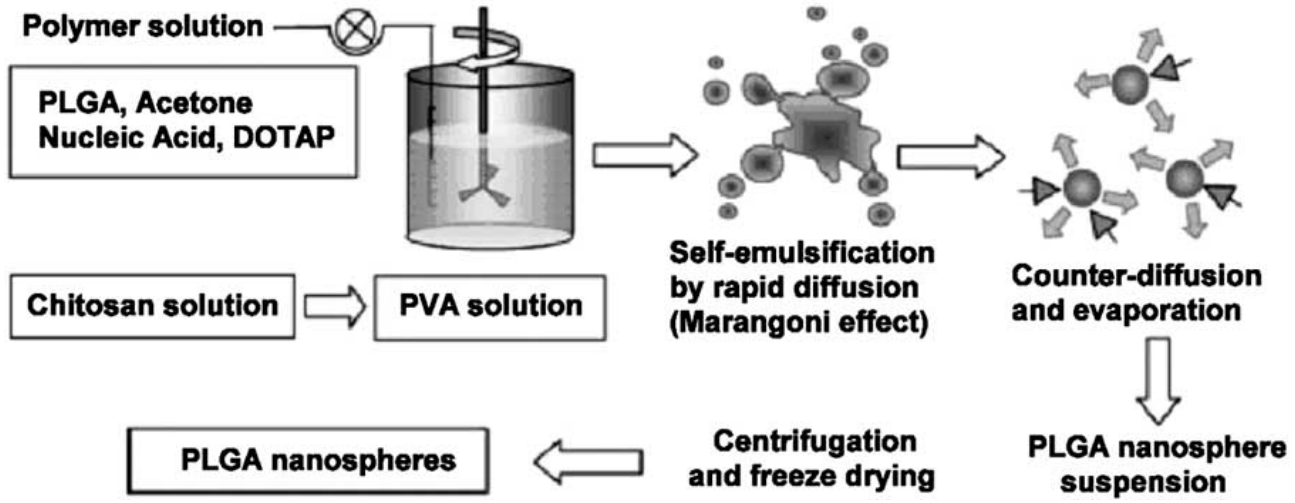

Fig. (1). Schematic Representation of PLGA Nanospheres Preparation Process. The polymer solution is added under vigorous stirring into a proper mixture of PLGA, Nucleic acid and DOTAP in acetone up to complete emulsification. After solvent evaporation the PLGA nanospheres are recovered by centrifugation and freeze drying.

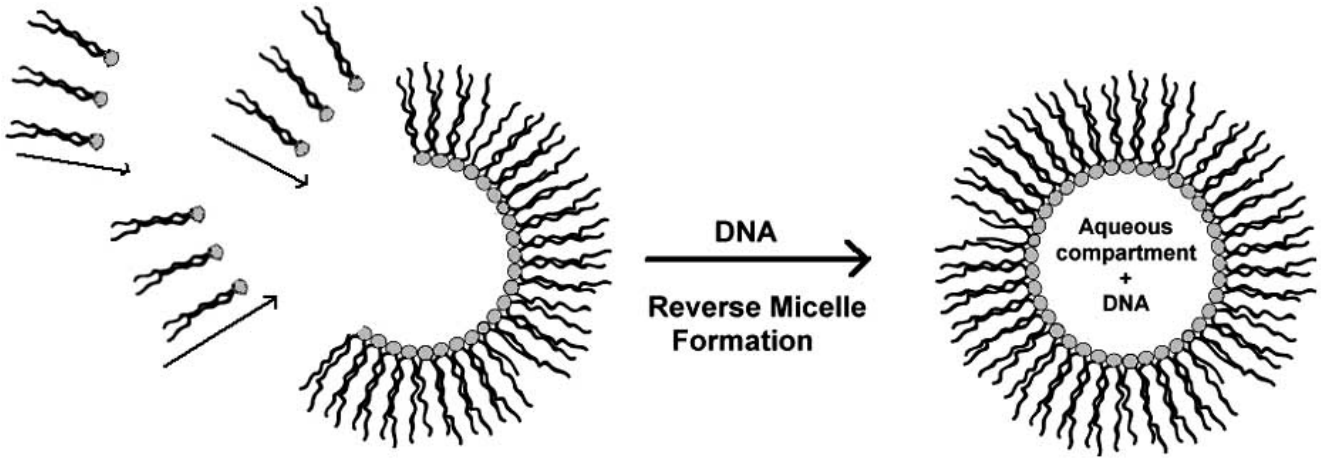

Fig. (2). Schematic Representation of reverse micelle formation process. The incorporation of DNA into the aqueous compartment is achieved during the spontaneous reverse micelle formation obtained adding the mixture of lipids in buffered solutions.

In fact, the authors described the preparation of composite nanovesicles containing soybean phosphatidylcholine and polysaccharide chitosan by the reverse phase evaporation method followed by the formation of an organogel, which is dispersed in water to yield the final liposome structures. The hydrodynamic radius of the composite nanovesicles is in the range of 174-286 nm, depending on the chitosan contents. A comparison with nanovesicles free from chitosan indicates the existence of higher contents of multi-lamellar structures in the composites, as well as improved stability in water.

Chitosan Nanospheres Fabrication Using Solvent Evaporation

Chitosan nanospheres have been also obtained using the "solvent evaporation" technique for the study of bioactive molecules delivery [34]. The principle of this technique is based on the formation of the polymeric particle by evaporation of the most volatile component of the reaction mixture. Solvent evaporation generates a localized oversaturation that induces the formation of a polymeric precipitate in the form of small spheres.

The time-regulated delivery of bioactive macromolecules from the porous scaffolds is very important in tissue engineering. Tetrandrine (Ted) is a plant-derived bisbenzylisoquinoline alkaloid that is obtained from the roots of *Stephania tetrandria*. Ted can be used as a modifier of poly-(l-lactic acid) scaffolds to promote chondrocyte differentiation and the secretion of type II collagen. The effect of Ted on chondrocyte's behaviour strongly depends on the concentration of Ted in the culture media. Amphiphilic di-block copolymer (PLAE) composed of l-lactide and methoxy poly-(ethylene glycol) (MePEG) has been prepared and the Ted-loaded copolymeric nanospheres have been obtained by self-emulsification and solvent evaporation. The mean sizes of core/shell PLAE nanospheres and Ted-loaded nanospheres are about 60 and 100 nm, respectively. Chitosan–gelatine (Cs–Gel) porous scaffolds loaded with PLAE–Ted nanospheres were fabricated through freeze drying. Ted-loaded nanospheres could be embedded within Cs–Gel scaffolds and no initial burst release (an initial high and rapid release) could be observed in the release patterns. Results obtained by these authors indicate a potential application for these scaffolds to sustained release of bioactive component in tissues.

Another recent work reports the preparation and characterization of cationic chitosan-modified poly-(D,L-lactide-co-glycolide) copolymer (PLGA/CS) nanospheres as DNA carriers [35]. It was shown that the diameter of the PLGA/CS nanoparticles can be optimized in the range of 150-200 nm, as determined by dynamic light scattering. The zeta potential of PLGA/CS nanoparticles increased with increasing CS concentration ([CS]) or decreasing pH: 55 mV at [CS]=3 mg/mL at pH 4 and changed polarity at about pH 8. The optimum conditions for fabricating the relatively small diameter and high zeta potential cationic nanoparticles were [CS]=3 mg/mL, [PLGA]=10 mg/mL, and the volume ratio of organic solution to aqueous medium 1/4. X-ray photo electron spectroscopy and fluorescence inverted microscope observations confirmed that CS molecules were adsorbed on the surface of PLGA nanoparticles. The DNA-condensing ability of the PLGA/CS nanoparticles and cell transfection efficiency of the nanoparticle-DNA complexes were estimated by gel electrophoresis and transfection experiment to 293FT cell, respectively. An efficient binding was obtained at a nanoparticle's concentration of 50 mg/mL (particles: DNA ratio = 5:1), with DNA fully complexed at a concentration of 100 mg/mL (10:1). However, even if the PLGA/CS nanoparticle–DNA–complexes are suitable for transfection, are less effective compared to commercial Lipofectamine 2000. The approach described in this study can be exploited for the preparation of CS-modified PLGA nanoparticles with controlled size and zeta potential, and it is beneficial in improving the DNA-binding ability and, to a minor extent, transfection efficiency.

Chitosan Microspheres Fabrication Using Spray-Drying and Thermal Processes

Spray drying is a common method used for drying solutions, colloids or suspensions through a hot gas (i.e., air or nitrogen) after fine droplets have been produced by an atomizer device. Non-crosslinked and crosslinked chitosan microspheres prepared by a spray drying method have been reported in the literature [30]. In this study, the microspheres

are positively charged, and have a good sphericity even if they display a smooth but distorted surface morphology. This method afforded particles with a size ranging from 2 to 10 μm. It was reported that the size and zeta potential of the particles were influenced by the crosslinking level: by decreasing the amount of crosslinking agent (either glutaraldehyde or formaldehyde), both particle size and zeta potential were increased. Preparation conditions also had influence on the particle size. DSC studies revealed that the H2-antagonist drug cimetidine, as well as famotidine was molecularly dispersed inside the microspheres, in the form of a solid solution. The release of model drugs (cimetidine, famotidine and nizatidine) from these microspheres was high and rapid (the so called "burst" effect).

Another paper reported the encapsulation of moxifloxacin to obtain sustained release of this drug after intrapulmonary administration [36]. The microspheres were produced by the spray-drying method using glutaraldehyde as the crosslinking agent. The particles were spherical with a smooth but distorted surface morphology and were of small size, ranging from 2.5 to 6.0 μm, thus suitable for inhalation. *In vitro* release studies showed a significant burst effect for all crosslinked systems, followed by a prolonged moxifloxacin release, particularly in the presence of the highest glutaraldehyde concentration. Lipid vesicles prepared with dipalmitoylphosphatidylcholine (DPPC) were used as an *in vitro* biomembrane model to evaluate the influence of chitosan microspheres on the interaction of moxifloxacin with biological membranes. Differential scanning calorimetry was used as a simple and non-invasive technique of analysis. Moxifloxacin freely permeates through DPPC liposomes, interacting with the hydrophobic zone of the bilayers (lowering of the ΔH value and loss of the cooperativity of the main transition peak). Uncrosslinked microspheres rapidly swelled and dissolved releasing free chitosan that was able to interact with liposomes (increase of ΔH value), probably altering the biomembrane permeability to the drug. Crosslinked microspheres did not show this property. Pulmonary absorption of moxifloxacin-loaded chitosan microspheres was evaluated compared to the free drug. A monolayer of Calu-3 human bronchial epithelial cells mounted on Franz diffusion cells was used as an *in vitro* bronchial epithelium model. Microspheres retard the absorption of moxifloxacin and within 6h the cumulative amount of permeated drug was about 18%, 11% and 7% (w/w) for free moxifloxacin, moxifloxacin-loaded crosslinked and moxifloxacin-loaded uncrosslinked microspheres, respectively.

Also dry powder formulations may be used for pulmonary gene delivery. The use of the cationic polymer chitosan as a readily available and biocompatible dispersing enhancer was investigated [37]. Lactose-lipid:polycation:pDNA (LPD) powders were prepared by spray-drying and post-mixed with chitosan or spray-dried chitosan. Moreover, the water-soluble trimethyl chitosan derivative was added to the lactose-LPD formulation before spray-drying. Spray-dried chitosan particles, displaying an irregular surface morphology and diameter of less than 2 μm, readily adsorbed to lactose-LPD particles following mixing. In contrast with the smooth spherical surface of lactose-LPD particles, spray-dried trimethyl chitosan-lactose-LPD particles demonstrated increased surface roughness and a unimodal particle size distribution (mean diameter 3.4 μm), compared with the multimodal distribution for unmodified lactose-LPD powders (mean diameter 23.7 μm). The encapsulated dose and *in vitro* deposition of chitosan-modified powders was significantly greater than that of unmodified powders. Moreover, the inclusion of chitosan mediated an enhanced level of reporter gene expression. This study emphasized that chitosan enhances the dispersion and *in vitro* pulmonary deposition performance of spray-dried powders.

Uniform-sized amino-quaternized chitosan microspheres were prepared by combining Shirasu porous glass (SPG) membrane emulsification technique and a novel thermal-gelation method [31]. In this preparation process, the mixture of quaternized chitosan solution and alpha-beta-glycerophosphate (alpha-beta-GP) was used as water phase and dispersed in oil phase to form uniform W/O emulsion by SPG membrane emulsification technique. The droplets solidified into microspheres at 37°C by thermal-gelation method with a simple and mild process. The influence of process conditions on the property of prepared microspheres was investigated and the optimized preparation conditions were obtained. As a result, the coefficient of variation (CV) of obtained microsphere diameters was below 15%. The obtained microspheres have porous structure and show an apparent pH-sensitivity. They dissolved rapidly in acid solution (pH=5) while are stable in neutral solution (pH=7.4). The pH-sensitivity of microspheres also affected its drug release behaviour. Bovine serum albumin (BSA), as a model drug, was encapsulated in microspheres, and it was released rapidly in acid solution and slowly in neutral medium. These novel quaternized chitosan microspheres with pH-sensitivity may be employed as drug delivery system in the biomedical field, such as tumour-targeted drug carrier.

Osmosis-Based Method for Chitosan and Chitosan/DNA Nanospheres Fabrication

On industrial scale, it is generally difficult to obtain nanoparticles with uniform dimensions and without surfactant traces in the final product. Recently, a novel osmosis-based method designed to obtain well-defined chitosan and chitosan/DNA nanospheres without the use of surfactants was reported [32]. The process conditions may be easily modulated by varying the solvent/non–solvent couple, temperature and membrane cut-off affording nanoparticles with different morphology for several biotechnological applications.

In particular, different solvent couples (i.e., H_2O/CH_3CN, H_2O/EtOH, H_2O/THF) may allow obtaining different morphologies (rods, disks or large spheres) as shown in Fig. (**3**).

To obtain the precipitation of nanospheres, the chitosan solution is transferred in a cellulose acetate dialysis bag and immersed into methanol. The osmotic equilibrium was reached after approximately 72 h, after which the precipitated polymer was recovered, washed several times with methanol and the pellet freeze-dried. For chitosan/DNA nanospheres preparation, deoxyribonucleic acid was added under vigorous stirring to the solution of chitosan.

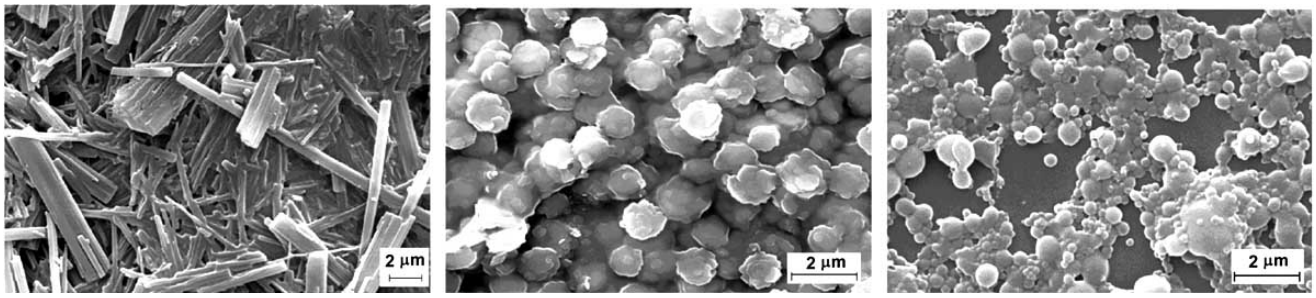

Fig. (3). SEM images of chitosan "rod-like" structures (H_2O-CH_3CN) (left), "disk-like" structures (H_2O-EtOH) (center) and large spheres (H_2O-THF) (right) obtained using a cellulose acetate membrane (MWCO=12 kDa). See ref. 32 for details.

Size and size distribution of chitosan and chitosan/DNA nanospheres were characterized by dynamic light scattering (DLS) resulting 45 ± 9 nm, and 38 ± 4 nm, respectively. Nanoparticles morphology was also investigated by scanning electron microscopy (SEM) in both the secondary and the backscattered electron modes to obtain the elemental analysis of chitosan/DNA nanoparticles. Size distribution and average particle diameter were determined analyzing 5-10 images, representing a population of more than 2000 particles. Fig. (**4**) reports the SEM images of chitosan/DNA nanospheres at two different magnitudes.

Macroscopically, chitosan/DNA particles appear as a long chain of interacting spheres but at a higher magnification these chains appear to be composed of small nanospheres with an assessed diameter of 45 (±10) nm (Fig. (**4**)). The amount of phosphorous obtained from SEM microanalysis and elemental analysis was almost 4% with respect to other investigated elements (C,N,O), indicating a calculated ratio DNA/chitosan of almost one phosphate group every four D-glucosamine monomers.

In order to assess the DNA payload, freeze-dried chitosan/DNA nanospheres (1 mg) were incubated in PBS under magnetic stirring up to their complete dissolution and DNA concentration was determined by spectrophotometry. Generally, the DNA payload amounted to 30% (±0.5%).

The kinetic of DNA release from chitosan/DNA nanospheres was also obtained. The kinetic follows a pseudo first order law according to the diffusion through the porous polymeric matrix. The maximum release of DNA was 86% of the initial feeding amount. The release of DNA from chitosan/DNA nanospheres showed an initial high and rapid release within the first 3 hours of incubation. Then, DNA was constantly released up to 72 hours with more than 70% of the encapsulated material released within 24 hours. These data are in good agreement with DNA agarose gel retardation assays (data not shown).

To investigate if the release process was pH-dependent, chitosan/DNA nanospheres were also incubated in PBS at pH=6.5 and pH=5.6 and the DNA release profiles were monitored and results are shown in Fig. (**5**).

The release of DNA from chitosan/DNA nanospheres at pH=6.4 showed an initial rapid release within the first 9 hours then DNA was constantly released up to 72 hours with more than 40% of the encapsulated material released within 24 hours. Interestingly, the profile at pH=5.6 accounts for the existence of only one release mechanism with a rapid DNA release up to 54% within 3 hours without displaying any initial burst. This peculiar release mechanism is most likely due to the rapid dissolution of chitosan/DNA nanospheres at pH=5.6 leading to a different release mechanism that does not involve the diffusion of DNA fragments through the polymer matrix.

Therefore, these data indicate that the DNA release process is pH-dependent influencing the protonation degree of the biopolymer. As a consequence, at the lowest pH values

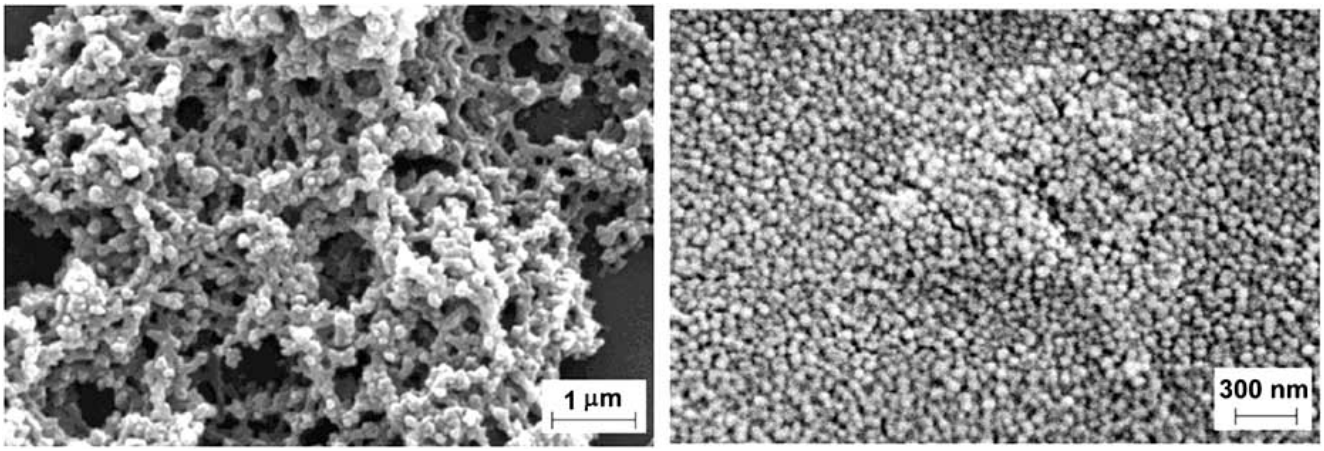

Fig. (4). SEM images of chitosan/DNA nanospheres (H_2O/EtOH) obtained using a cellulose acetate membrane (MWCO=12 kDa) at different magnification. See ref. 32 for details.

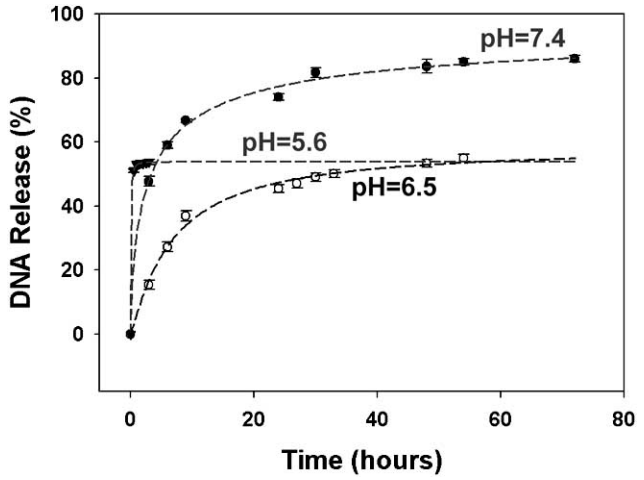

Fig. (5). DNA release (%) from chitosan/DNA nanospheres at a polymer/DNA ratio of 4:1 (w/w) after incubation in PBS at different pH values: (•) pH=7.4, (▼) pH=6.5, (o) pH=5.6. Values and standard deviations are reported together with the fitting curves (green R^2=0.9866, blue R2=0.9942, red R^2=0.9999). See ref. 32 for details.

considered, the soluble DNA fragments may strongly interact with the polymer matrix leading to a minor release.

Finally, chitosan/DNA nanospheres prepared following the proposed methodology are able to encapsulate DNA with improved efficiency in comparison with polyethylene vinyl co-acetate (EVAc) polymeric nanoparticles employed by Y.S.Jong *et al*. [38] These data are also in good agreement with similar works recently reported [31].

CONCLUSIONS AND PERSPECTIVES

Several methods may be employed to fabricate chitosan nano- and microspheres and this paper is aimed to give a short review of the recent literature.

Independently of specific biotechnological and biomedical applications, chitosan is generally more efficient in a solid nano- or microstructured morphology respect to different soluble forms. For DNA delivery, nanospheres demonstrated to be promising vectors but technological difficulties should be overcome to obtain homogeneous particles. Here, we commented also a recent paper published by our group concerning a versatile method for chitosan nanosphere fabrication [32]. Due to the intrinsic delivery properties of the reported DNA/chitosan nanospheres, it is easy to envisage their use as long-term delivery vehicles. The properties of reported compounds are not limited to delivery. In fact, chitosan being a naturally occurring mucopolysaccharide, has also muco-adhesive properties [41]. Such molecules may be therefore efficiently employed i.e. for oral or nasal gene (or other drugs) delivery.

In the near future, we expect that the scalable and industrially applicable method we recently reported will enable to produce other bio-polymeric nanostructures with improved properties for biomedical applications. Therefore, these novel micro- and nano-spheres undoubtedly will pave the way to novel therapeutic applications in various fields of medicine.

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