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ORIGINAL PAPER

Cationic β-Cyclodextrin–Chitosan Conjugates as Potential Carrier for pmCherry-C1 Gene Delivery

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Abstract In this study cationic β-cyclodextrin-chitosan-mediated nanoparticles were used to transfer pmCherry-C1 into glioblastoma cells and their transfection efficiency were compared to lipofectamine and electroporation. Physico-chemical characteristics of nanoparticles were evaluated by photon correlation spectroscopy and scanning electron microscopy. Electrophoretic nuclease resistance and stability assays were used to check the protection of DNA from nucleases digestion. mCherry reporter construct was used for visualization, followed by quantitation of cell survival and gene expression by fluorescence-activated cell sorting analysis and fluorescence microscopy. Particle size was approximately 200 nm and did not change at 4 °C even after 12 weeks. Importantly, the positively charged complexes interacted with DNA could serve as an efficient DNA delivery systems. Most of the gene was associated with the nanoparticles and was efficiently protected from DNAse I digestion. More than 80 % of transfected cells expressed mCherry efficiently.

Keywords Chitosan · Cyclodextrin · Gene delivery · Glioblastoma multiforme · Experimental design

Abbreviations

CS	Chitosan
STPP	Sodium tripolyphosphate
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
DMSO	Dimethyl sulphoxide
pDNA	Plasmid DNA
CD	β-Cyclodextrin
PCS	Photon correlation spectroscopy
LDA	Laser doppler anemometry
SEM	Scanning electron microscopy
mCh	pmCherry-C1 plasmid
CCD	Central composite design
FACS	Florescence-activated cell sorting

Introduction

Gene delivery systems can be divided into viral, non-viral, and combined hybrid systems [21]. Non-viral gene delivery systems were introduced as an alternative to viral-based systems; where the most important advantage of these systems is improvement in transfection efficiency [1]. Physical methods such as electroporation, microinjection, gene gun, and impalefection, and chemical methods like lipoplexes and

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polyplexes are known as non-viral methods [34]. Physical methods refer to the delivery of the gene via application of physical forces to increase permeability of the cell membrane. Chemical methods utilize natural or synthetic carriers to deliver genes into cells [32]. Electroporation is temporary destabilization of the targeted cell membrane and genes could be able to penetrate into the cell [21]. Optimum transfection and subsequent cell viability should be estimated by a number of experimental variables e.g., cell density, reagent and DNA concentrations, reagent–DNA complexing time, voltage, and the pulse in electroporation methods [7]. Felgner and Wu introduced the use of lipoplexes and polyplexes (complexes of lipid or polymer with DNA) for the efficient delivery of DNA through plasma membrane in mammalian cells in 1987 [14]. These vectors rely on the self-assembling; at physiological pH they are cationic [8] and after removal of small counter ions, they spontaneously form complexes with anionic nucleic acids [3]. Chemical systems are more common and they are generally stable enough to protect nucleic acids from degradation [6]. Liposomes as the most important delivery systems have unique characteristics e.g., capability to incorporate hydrophilic and hydrophobic drugs and their low toxicity [20, 21], but the rapid degradation of liposomes is a drawback of these delivery systems. Poly (L-lysine), polyethyleneimine, amidoamine dendrimers, poly (L-histidine), polyvinyl pyridine, and cationic polysaccharides are cationic polymers which are commonly used in gene delivery [13]. Cationic polysaccharides are natural, non-toxic, biodegradable, and biocompatible materials [4]. Most of these conjugates form stable complexes with various plasmids, and are found active in efficient transfecting cells in vitro and in vivo. High in vitro transfection efficiency was achieved with dextran–spermine conjugates using different cell lines [23, 27]. Pullulan–spermine conjugates act as very promising carriers for delivering DNA to brain endothelial cells [31]. Chitosan as a natural linear copolysaccharide β-(1 → 4)-2-amine- 2-deoxy-D-glucose (GlcNac) and β-(1 → 4)-2-acetamino-2-deoxy-D-glucose (GlcN) exhibits several favorable biological properties and used in the gene delivery effectively [28]. In the present study the transfection efficiency of CS/CD/TPP/pmCherry-C1 complex as gene delivery system was compared with electroporation and lipoplexes delivery systems as common delivery systems reported so far, and the conditions for reproducible stable delivery of exogenous genes into U87 cells, as a mammalian cell model of transfection were also studied.

Materials and Methods

Chemicals

Materials were purchased from Sigma-Aldrich Company (Sigma-Aldrich Mo, USA) unless stated otherwise. Chitosan (low molecular weight, viscosity 20–300 cP, 1 wt% in 1 % acetic acid), sodium tripolyphosphate (STPP), dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (100 μg/ml), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), G418 disulfate salt solution and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1 reagent) were all purchased from Sigma-Aldrich. β-cyclodextrin (CD) with molecular weight $M_w = 1135$ g/mol were obtained from Merck Company (Darmstadt, Germany). Plasmid DNA (pDNA) encoding Cherry fluorescent protein (pmCherry-C1) driven by a CMV promoter was prepared as instructed by Invitrogen Corporation (Carlsbad, CA, USA). Lipofectamine™ 2000 reagent was purchased from Invitrogen Corporation (Carlsbad, CA, USA). U87 glioblastoma cells were obtained from Pasteur institute of Iran.

Preparation of Chitosan Complex

Nanoparticles were obtained by ionotropic gelification [5]. Two aqueous phases of CS and CD solution with the cross-linker TPP were mixed under magnetic stirring and maintained under agitation to allow complete formation of system. CS acidic solution (1 % glacial acetic acid) was prepared at a concentration of 2 mg/ml and the volume employed was fixed at 3 ml. The corresponding volumes of the CD aqueous solution (6–12 mg/ml) and TPP solution (1.5 mg/ml) were mixed at a final volume of 1 ml. For nanoparticles encapsulating a pDNA model, the required amount of the plasmid was incorporated directly into the CD/TPP phase. The theoretical loadings were fixed at 5 % (w/w, based on the weight of all nanoparticle components: CS, CD, and TPP).

Chitosan Complex Characterization

Particle size and the size distribution of the nanoparticles were determined by photon correlation spectroscopy (PCS). Zeta potential values of the nanoparticles were obtained by laser doppler anemometry (LDA), measuring the mean electrophoretic mobility. Samples of the nanoparticle suspensions were diluted at the appropriate concentration with filtered water for PCS and with 1 mM KCl for LDA. PCS and LDA analyses were performed with a Zetasizer® 3000 HS (Malvern Instruments, UK). The surface and cross-sectional morphology of the chitosan nanoparticles (sonicated) were analyzed using field emission scanning electron microscopy (FE-SEM, MIRA 3 XM, Tescan USA Inc.) after sputter coating with gold for 5 min. All samples were examined at an acceleration voltage of 15 kV. The images were analyzed using the Image J software to assess the diameter of the chitosan

nanoparticles. Rheological parameters (shear stress, shear rate, viscosity) of chitosan were measured by viscometric measurements using Brookfield Engineering labs DV-III Ultra Rheometer. The viscometer was operated between 2 and 26 rpm and shear stress, shear rate, and viscosity data were obtained directly from the instrument; the SC4-18 spindle was selected for the measurement. Viscosity-average molecular weight was calculated by Mark–Houwink equation (1) [33]:

$$[\eta] = KM_v^a, \quad (1)$$

where $K = 4.74 \times 10^{-5}$ (dm^3/g) and $a = 0.72$ determined in 0.2 M acetic acid solution at 25 °C.

The association of pDNA to the nanoparticles was studied by a conventional agarose gel electrophoresis that ran for 30 min at 100 V (Sub-Cell GT 96/192, Bio-Rad Laboratories Ltd., England).

Cytotoxicity Studies

Cells were seeded on each well of 96-well culture dish at a density of 1×10^4 and incubated for 24 h. One hundred microliter of the CS/CD/TPP and CS/CD/TPP/pmCherry-C1 composites at the various concentrations of 0.01, 0.05, 0.125, 0.625, 1.25, 2.5, and 5 mg/ml was applied to each well, followed by 24 h incubation. The medium was refreshed (100 µl) and 10 µl of WST-1 solution was added and the cells were allowed to incubate for 3 h. The absorbance of samples was measured by ELISA reader (BioTek® Elx 800) at 450 nm wavelength and the reference of 630 nm. Absorbance of non-treated cells (as control) was estimated as 100 % viability and the treated cells were calculated according to it. Experiments were replicated four times with at least three wells for each sample. All samples were run in three replicates and the experiments were repeated twice. The survival rate (%) was calculated following Eq. (2) [12]:

$$\%\ \text{Survival rate} = (\text{OD in treatment group} / \text{OD in control group}) \times 100 \quad (2)$$

The inhibitory concentration required for 50 % cytotoxicity (IC50) value was determined using the Prism dose–response curve (Prism Graphpad, Prism version 6 for windows, GraphPad Software, Sa Diego, CA, USA), and by plotting the percentage of inhibition versus the concentration. Lactate dehydrogenase (LDH) release of U87 cells after 24 h incubation with nanoparticles was measured using the supernatant of the cell culture media by LDH Cytotoxicity Detection Kit (Pars Azmoon, Tehran, Iran). The absorbance was read at 492 nm using an automatic analyzer RA-1000 (Technicon, Ireland). All samples were run in three replicates and the experiments were repeated twice. Triton X (1 %) was used as positive control (high control) and untreated cells were used as negative control (low control). The mortality rate (%) was calculated following Eq. (3):

$$\text{Motrality rate } (\%) = ((A - C)/(B - C)) \times 100, \quad (3)$$

where A = Test sample − Medium; B = High control − Volume control; C = Low control − Medium

Transfection Studies

Transfection studies were performed with nanoparticles loaded with 5 % (w/w) pmCherry-C1 and naked pmCherry-C1 (positive control). The cells were maintained at 70 % confluence in 12-well tissue culture plates 24 h before the experiment. To transfect the cells, they were washed and freshly prepared CS/CD/TPP and CS/CD/TPP/pmCherry-C1 nanoparticles (0.625 mg/ml) supplemented by DMSO (0.5, 0.75, 1, and 1.25 %) were added to 400 µl cell suspension in 12-well microplate. The plates were shook gently by shaker (160 rpm/10 min) and incubated for 24 h. After 24 h the complex was removed and 1 ml of fresh culture medium was added. The medium was changed every other time if the experiments exceeded 2 days. At the indicated time point (48, 72 h), cells were investigated under a fluorescence microscope (Olympus TH 1400) for the number of Cherry-positive cells. To obtain stably transfect cells, the samples were incubated first in complete medium for 72 h and then kept in the complete medium supplemented with 300 µg/ml G418 solution for 21 days.

Electrophoretic Nuclease Resistance and Stability Assays

To investigate the ability of the copolymer to protect DNA from enzymatic degradation, 3 µl of naked pmCherry-C1 (control) and CS/CD/TPP/pmCherry-C1 complexes were combined with 2 U DNase I in 4 µl 50 mM Tris–HCl at pH 7.6, then incubated for 1 h at 37 °C and run on an agarose gel. For stability assays, CS/CD/TPP/pmCherry-C1 complexes (4 mg/ml) in serum-free medium were prepared and incubated for up to 7 days. The fluorescent intensity of bands corresponding to DNA and their electrophoretic mobility was observed and compared with those obtained with naked and CS/CD/TPP/pmCherry-C1 complexes in the absence of serum-free medium.

In Vitro Release Studies

To quantify the DNA release from the obtained nanostructured systems, 4 mg of CS/CD/TPP/pmCherry-C1 nanoparticles were incubated in PBS (pH 7.4) under gentle magnetic stirring for 72 h. Samples were centrifuged for 10 min at 13,000 rpm, the supernatant was then removed

and replaced with fresh PBS. At fixed time points 5, 9, 18, 23, 30 days supernatant was collected, analyzed by spectrophotometer, and the kinetic of DNA release from CS/CD/TPP/pmCherry-C1 nanoparticles was determined.

Stable Transfection by Lipoplexes

Cytotoxicity evaluations were done as described above. The medium was changed to the fresh medium and the different volume (1, 1.5, 2, 2.5, and 5 μl) of lipofectamine solution was applied to each well, follow by 24 h incubation. The absorbance of non-treated cells was estimated as 100 % viability and for the treated cells was calculated according to it.

As both the total amount of DNA and the DNA–lipofectamine ratios were important factors in the calculation of transfection efficiency, various DNA–lipofectamine (μg:μl) ratios using 2, 4, 8, 16, and 20 μg of mCh plasmid with different volume of lipofectamine (1, 1.5, 2, 2.5) were examined. Also, the effects of increasing amounts of mCh plasmid (0.01–0.1 μg) were measured while maintaining a constant volume of lipofectamine (2.5 μl according to the cytotoxicity study). One day before transfection, 0.2–2 × 10^5 viable cells were cultured in 700 μl DMEM without antibiotics to attain 90–95 % confluency at the time of transfection. DNA–lipofectamine complexes were prepared and diluted in serum-free medium supplemented with DMSO (0.5, 0.75, 1, and 1.25 %), mixed gently and incubated for 5–20 min at room temperature. Transfection complex (200 μl/well) was added to each well and mixed gently by rocking the plate back and forth. The cells were incubated for 24–48 h at 37 °C; 5 % CO_2. Medium was replaced with complete medium the next day. To obtain stably transfected cells, the samples were incubated first in complete medium for 72 h and then in complete medium supplemented with 300 μg/ml G418 for 21 days.

Stable Transfection by Electroporation

Electroporation was performed using the Eppendorf Multiporator®, which provides a constant square pulse wave and ease of optimization. Initially, the pulse length and strength was optimized to limit cell death, while being intense enough to allow the DNA to cross the cell membrane. For electroporation, the cells were counted and re-suspended in iso-hypo-osmolar buffer. The appropriate concentration of DNA, rate of voltage, and the length of the pulse were chosen according to the experimental design analysis. After pulsed, cells suspension was spread out in complete medium with 0.5, 0.75, 1, and 1.25 % DMSO and then allowed to stand for 5 min at room temperature. After 24 h, the cells were washed twice with PBS and re-fed with complete medium. To obtain stably transfected cells, the samples were incubated first in complete medium for 72 h and then in complete medium supplemented with 300 μg/ml G418 for 21 days.

Experimental Design and Data Analysis

The central composite design (CCD) was used to investigate the significance of the effects of parameters including DNA concentration (1, 30.5, 60 μg), voltage (400, 800, 1200 V), and pulses of the exponential wave (30, 165, 300 μs) into three levels (low, basal, and high) as coded values of −1, 0, +1 and star points of $\alpha = \pm 1$ and the responses of all 11 experiments were recovered (Table 1).

The main and interaction effects were evaluated in this design. To find the most prominent effects and interactions, ANOVA was calculated using STATISTICA 7.0 software. A *p*-value less than 0.05 indicates the statistical significance of an effect at 95 % confidence level. *F* test was used to estimate the statistical significance of all terms in the polynomial equation within 95 % confidence interval. The mathematical relationship between the three independent variables was approximated by the second-order polynomial model Eq. (4):

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} x_i^2, \quad (4)$$

where y is the predicted response (removal percentage) and X_i's are the independent variables (DNA concentration, voltage, and pulse) that were known for each experimental run. β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the cross-product coefficient.

Cell Culture

The non-transformed U87 cells were maintained in DMEM supplemented with 10 % FBS and 100 μg/ml penicillin/

Table 1 Data statistics of model variables

Runs	Voltage (v)	Pulse (μs)	DNA (μg)	Removal (%)
1	1200	30	60	0.5
2	800	165	30.5	4.8
3	400	300	1	0.3
4	1200	300	60	0.1
5	400	30	1	2.75
6	1200	30	1	2.75
7	800	165	30.5	4.8
8	800	165	30.5	4.8
9	400	30	60	0.1
10	1200	300	1	0.1
11	400	300	60	0.1

streptomycin at 37 °C in a humidified atmosphere with 5 % CO_2.

Plasmid Preparation

The pmCherry-C1 plasmid (4.722 kb) encoding Cherry fluorescent protein driven by a CMV promoter was used. The pmCherry-C1 was propagated in an *Escherichia coli* strain Top 10 (ATCC® PTA-10989™) and purified by phenol–chloroform method according to the protocol. Both the yield and purity of the pmCherry-C1 plasmid were evaluated by UV spectroscopy. The absorbance ratio of 260–280 nm wavelengths for pmCherry-C1 plasmid solution was measured to be between 1.8 and 2.0.

Isolation of mch+ Single Cells Using Florescence-Activated Cell Sorting (FACS)

mCh+ cells were isolated on a cell sorter (BD FACS Calibur, USA). Data were acquired on an automated cell analyzer (LSRII; BD Biosciencs) and analyzed with WinMDI software (Treestar). Cells were harvested from the plates using trypsin–EDTA treatment, and were suspended in 3 ml PBS in centrifuge tubes, centrifuged at 1200 rpm for 10 min, and the cell pellet was washed twice in 3 ml PBS to remove background fluorescence from the media. Cells were suspended in 0.5 ml PBS and transferred to flow cytometry cuvettes for analysis. mCh was excited at 587 nm, and emission was detected using a 610/20 band-pass filter.

Statistical Analysis

Statistical differences were examined using one-way ANOVA, followed by Tukey test for multiple comparisons. All analyses were run using the IBM SPSS Statistics Version 21, and differences between the groups were judged significant at $p < 0.05$.

Results and Discussion

The overall goal of this study was to evaluate the potential of β-cyclodextrin-grafted chitosan conjugated as a vehicle for the delivery of the genes of interest to the glioblastoma cells, and comparing its transfection ability with other transfection methods such as electroporation and lipoplexes. In this regards the ability to associate genes to the proposed nano-carrier, the interaction of the nano-carrier with the targeted cells, and the capacity of the gene-loaded nano-carrier to be expressed in the target cells was taken into consideration [30]. Results showed that CS/CD/TPP/pmCherry-C1 complexes are an efficient method for introducing gene into cells of interest comparing to the other methods [16]. Near 80 % transfection efficiency was obtained with optimized conditions, was significantly higher than that of lipofectamine™ 2000, electroporation, and naked DNA towards U87 cell line. The experiments show that, not only transferring gene into target cells using chitosan-based nanoparticles was considerable, but also measuring the level of gene transfer was much easier in comparison with lipoplexes and electroporation because of their low toxicity and high flexibility.

Stable Transfection by Chitosan Complex

Figure 1 reports the SEM micrographs of CS/CD/TPP and CS/CD/TPP/pmCherry-C1 complexes. The average diameter of CS/CD/TPP nanoparticles was 200 ± 14 nm ($\delta = 7$ %) (Fig. 1a). Macroscopically CS/CD/TPP/pmCherry-C1 particles appear as a long chain of interacting particles which are composed of small nanoparticles (Fig. 1b).

CS/CD nanoparticles were prepared by ionic gelation in the presence of TPP and the ability of chitosan to gel rapidly upon contact with TPP relied on the formation of inter- and intramolecular cross-linkages mediated by the anionic molecules [25]. The mechanism of formation of the nano-systems combines the electrostatic interaction between CS and CDs groups of opposite charge, with the ability of CS to undergo a liquid–gel transition due to its ionic interaction with TPP [9]. The results of these preparation and characterizations indicate that nanoparticles with an average size (200 ± 14 nm) were consistent with previous findings that chitosan with low molecular weight produced smaller nanoparticles [17].

Hydrodynamic size readings of the synthesized nanoparticles as determined by DLS are even more significant than values obtained by SEM. Results showed that the particle size of CS/CD/TPP complexes was approximately 207 ± 39 nm and did not increase after 12-week storage at 4 °C. The average diameters determined by DLS were larger than the sizes determined from the SEM images for the corresponding samples. This was presumably because DLS gave the mean hydrodynamic diameter of the nanoparticle core surrounded by the organic and solvation layers, whereas SEM gave the diameter of nanoparticles alone in the dry state. The zeta potential of CS/CD/TPP was approximately −3.40 mV overall. All complexes retained a particle size near to 200 nm and positive charge suitable for efficient cellular uptake [29]. The polydispersity index (PDI) was low, indicating that a homogeneous dispersion was obtained [26]. PDI was 0.2 in correspondence with the chitosan. Regarding the surface charge, chitosan samples gave positively charged nanoparticles. When chitosan, cyclodextrin, and TPP were mixed with

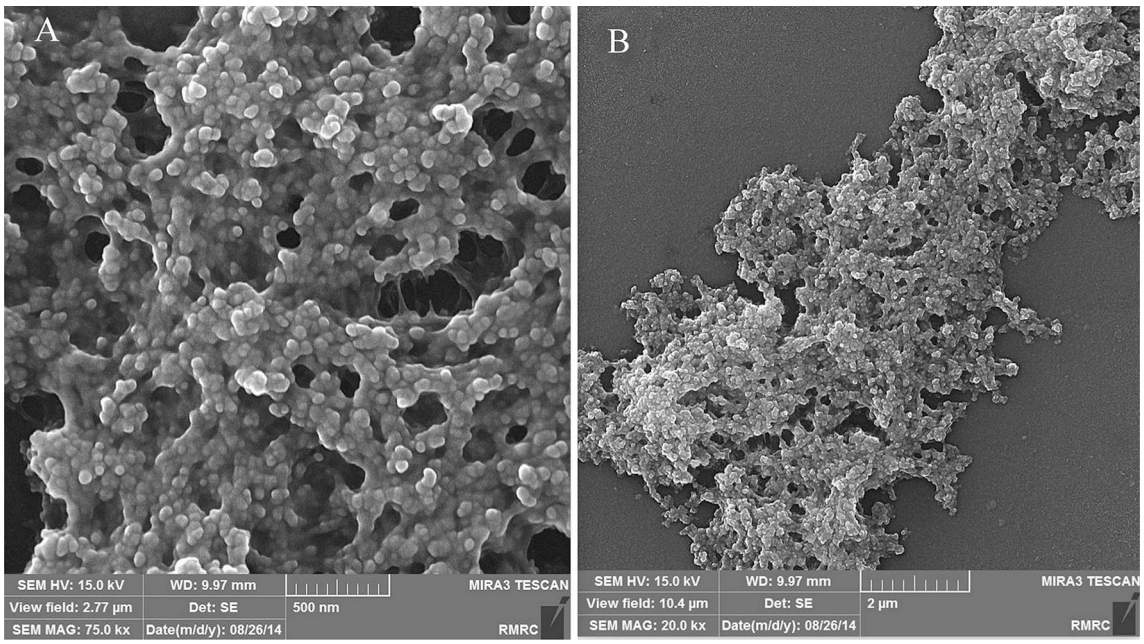

Fig. 1 SEM micrographs of CS/CD/TPP nanoparticles (**a**) and CS/CD/TPP/DNA complexes (**b**) (*scales* 500 nm, 2 µm)

each other, they formed compact complexes with an overall positive surface charge, as confirmed by measurements of zeta potential values. Size stability as a function of time has been studied and the samples presented an increase in size without formation of large aggregates. The size evolution during storage is depended on many factors e.g., particle aggregation which provides a more efficient rearrangement, the interaction of free polymer chains with the particle network that leads to a reorganization of intermolecular entanglements, syneresis, and swelling due to the presence of TPP that generates an inflow of water by osmosis [26]. Therefore, the size change is usually due to balance between the above-mentioned forces. The time span in which the growing stage finished, is depended on the size of the initial nanoparticle that is in turn depended on the molecular weight of chitosan [26].

The relation between shear rate and apparent viscosity is shown in Fig. 2. The results indicate that the solution exhibited pseudoplastic fluids behavior, since the viscosity decreased as shear rate increased. It is related to the breakage of intermolecular interactions by shearing a typical behavior of pseudoplastic fluids. These results are in accordance with the findings of [11].

It was found that chitosan has an average molecular weight of 4.17×10^5 g/mol determined by viscometry (Fig. 3) which was increased by increasing its intrinsic viscosity.

The toxicity of various concentrations of nanoparticles prepared in this study was evaluated after 24 h incubation of the trypsinized U87 cells with the transfection

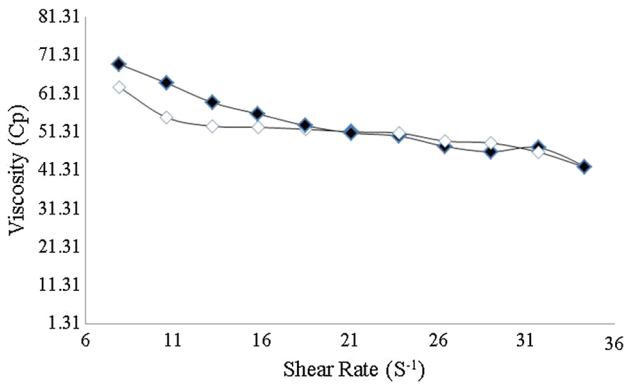

Fig. 2 Influence of the shear rate on the rheological curves of chitosan solution

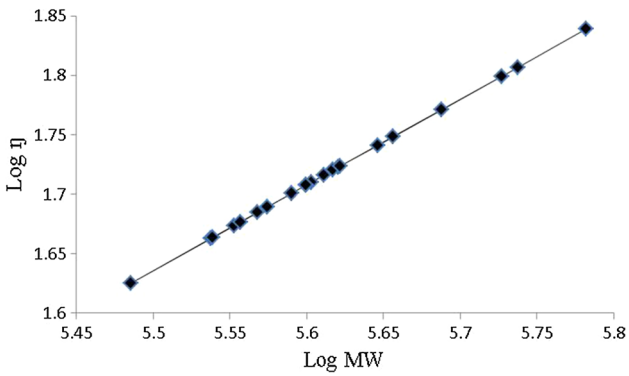

Fig. 3 Intrinsic viscosity of chitosan in 0.2 M acetic acid solution at 25 °C as a function of M_v determined by viscometry

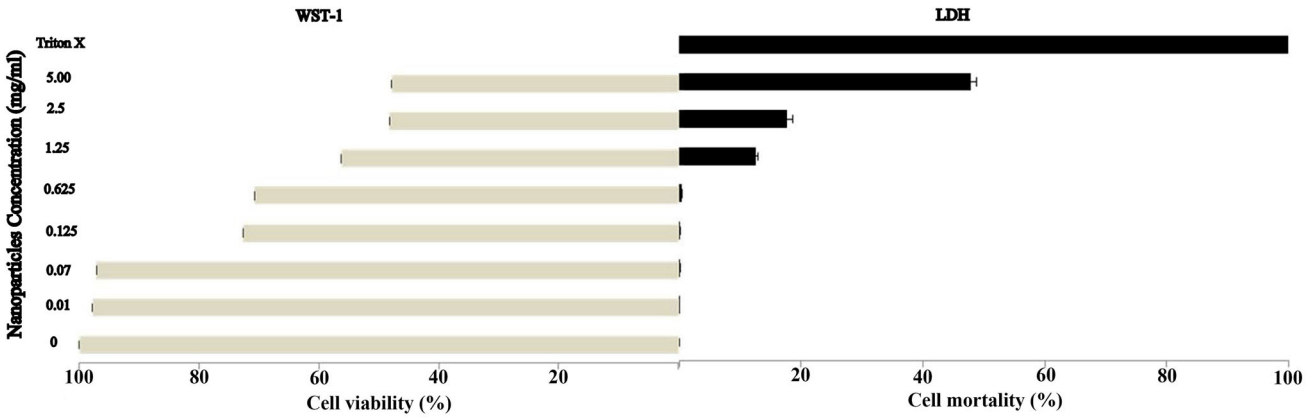

Fig. 4 U87 cell viability (WST-1) and mortality (LDH) after treatment with the different concentrations of chitosan complexes in the same conditions. Data represented as mean ± SD

complexes, using WST-1 and LDH tests to quantify cell viability and cell mortality, respectively (Fig. 4). The results showed that as the concentration of chitosan complexes rose from zero to 5 mg/ml on the basis of the initial concentration, the survival rate of the U87 cells decreased. Both the primary effect of the nanoparticles or their side effects could be responsible for the observed cytotoxicity [22]. A balance between cell membrane integrity and cytotoxicity of the therapeutic agent is a crucial aspect in gene delivery. The survival rate with regard to cytotoxicity was 70 % at 0.625 mg/ml, with an IC_{50} value of 4.6 mg/ml. Chitosan complex showed toxic effect more than 40 % with concentration 5 mg/ml. It confirmed that nanoparticles did not exhibit any sever toxicity at employed concentration (0.652 mg/ml) with LDH test and are in combination with previous studies [24].

The transfection efficiency of the CS/CD/TPP/pmCherry-C1 complexes were investigated at varying concentrations of chitosan complex up to 0.625 mg/ml. Transfection efficiency increased in the presence of DMSO and the relative transfections were measured by means of fluorescence which increased approximately at 1 % DMSO. The viability of transfected cells with CS/CD/TPP/pmCherry-C1 complexes with and without DMSO was approximately 90.0 and 80 % of cells were typically mCh+ (Fig. 5). In a separate experiment, the cytotoxicity of DMSO in non-transfected cells was tested and it was found that DMSO had no significant effect on viability up to 1.25 %, which was the maximum concentration tested.

In vitro transfection efficiency of CS/CD nanoparticles was found to be dependent on the CS/CD:DNA (w/w) ratio. The average post-treatment cell viability was ≥90 % as nanoparticle dose increased to 0.625 mg/ml [9]. From the results it was demonstrated that CS nanoparticles were efficiently internalized into U87 cell line and the uptake efficiency as measured by flow cytometry was about 80 %.

The ability of the copolymer to entrap pmCherry-C1 plasmid was studied using the agarose electrophoresis technique. From the photograph of the agarose gel, depicted in Fig. 6b–d, it could be stated that most of the DNA plasmid was associated with the nanoparticles, since no migration of free DNA was observed. Nanoparticles present a strong fluorescence localized in the well, indicating the formation of adducts, and in correspondence to the free DNA band, suggesting that DNA might be partially bound also on the surface of the nanoparticle as expected [19]. The stability of the nanoparticle complex was evaluated 7 days later in the serum-free medium (Fig. 6e).

DNA release from the constructed nano-structured systems was analyzed by spectrophotometer. At fixed time points of 5, 9, 18, 23, 30 days the supernatant was collected, and the kinetic of DNA release from CS/CD/TPP/pmCherry-C1 complexes was measured. The release of DNA from nanoparticles showed an initial release within the first 5 days and continued during 9 days of incubation. DNA was then constantly released up to 30 days with more than 25 μg of the encapsulated DNA released.

Nanoparticle stability and nucleic acid protection are important parameters for efficient nucleic acid delivery [2]. Results indicate that our CS formulation was able to protect DNA plasmid at supra-physiological concentration of nuclease. Nuclease protection is of great importance for nucleic acid delivery systems through maintenance of cargo bioavailability and improved pharmacokinetic profile, thereby increasing the therapeutic potential of these nanoparticles [2]. According to the agarose gel experiments, most of the DNA was associated with the nanoparticles, since no migration of free DNA was observed. This fact is in agreement with the previous results obtained for other CS-based nanometric systems, and it can be easily explained by the high affinity of CS for DNA [2]. Indeed, it is known that the strong electrostatic

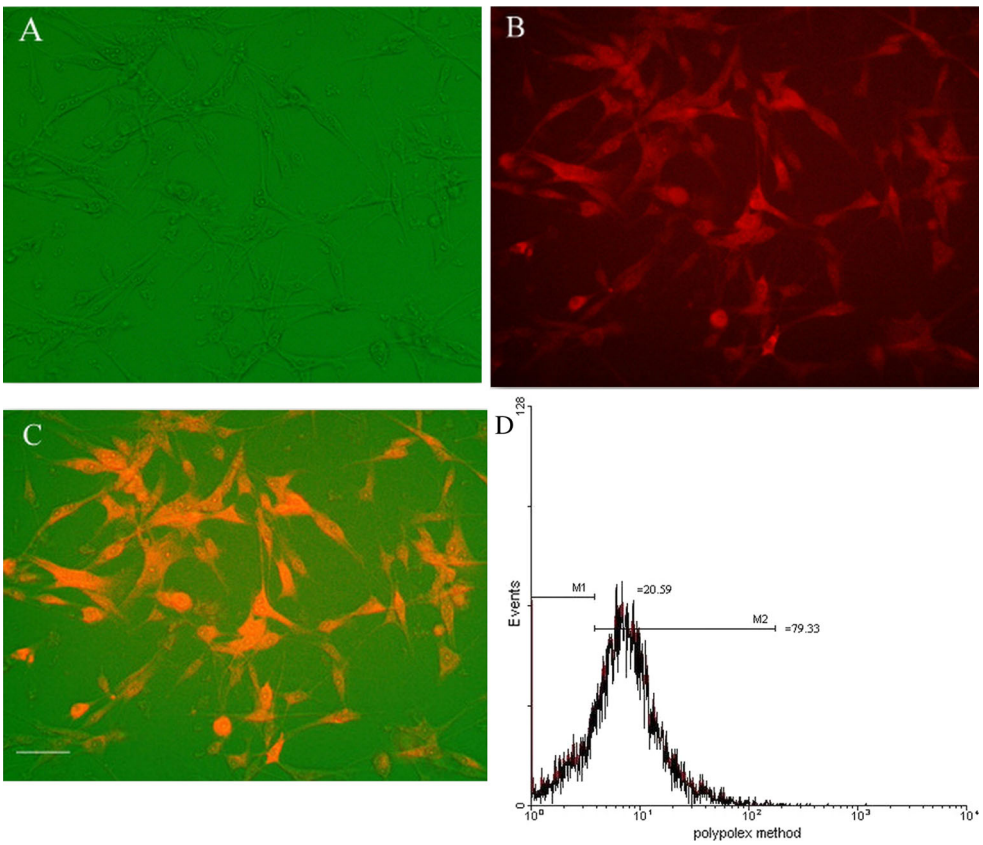

Fig. 5 Fluorescent and light micrograph images of transfected U87 cells. Light microscopy (**a**), fluorescence microscopy (**b**), merge (**c**), cells analyzed by flow cytometry (**d**) (*scale* 200 μm)

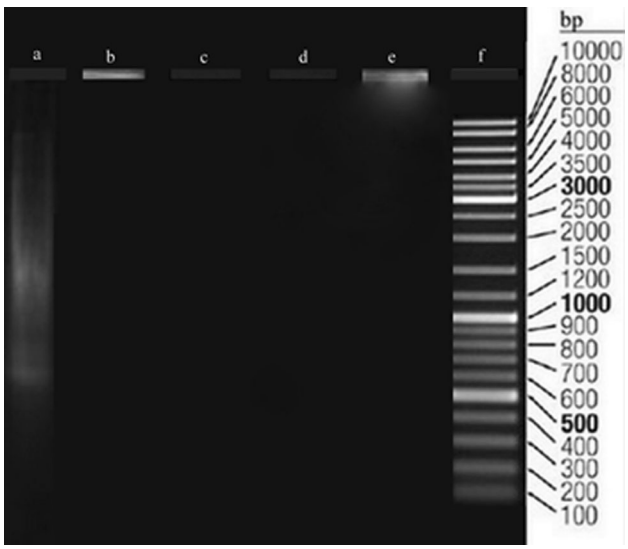

Fig. 6 Agarose gel electrophoresis of the nanoparticles encapsulating plasmid DNA. DNase I digested naked pmCherry-C1 (negative control) (*a*) DNase I digested freshly prepared CS/CD/TPP/pDNA (*b*) DNase I digested CS/CD/TPP/pDNA in PBS was stable at 4 °C for 3 months, (*c*) DNase I digested CS/CD/TPP (positive control) (*d*), CS/CD/TPP/pDNA in the absence of serum (*e*), DNA marker (*f*)

interaction exists between the phosphate groups of DNA and the amino groups of chitosan, as well as hydrophobic and hydrogen bonds. DNA release study suggests that nanoparticles are able to encapsulate DNA with improved efficiency. The maximum release of DNA was 86 % of the initial feeding amount. The first one, occurring within 9 days is likely due to DNA release from the nanoparticle surface, while, at a later stage DNA was constantly released from the core of nanoparticles as a consequence of chitosan hydration and swelling.

Stable Transfection by Lipoplexes

Up to 2.5 μl of lipofectamine could be used without affecting cell viability or morphology. Maximum efficiency in constant volume of lipofectamine (2.5 μl) was found to be occurring on 0.1 μg of mCh plasmid per 12-well microplate. The efficiency of transfection increased with increasing DNA/lipofectamine ratios. At 20 μg DNA concentrations, the proportion of mCh+ cell was slightly reduced at all DNA/lipofectamine ratio test. Therefore a combination of 2–4 μg DNA and the highest possible DNA/lipofectamine ratio of 2.5 μl were used in subsequent experiments. Transfection efficiency was monitored for mCh plasmid by fluorescent microscopy (Fig. 7) and FACS. FACS analysis (38 %) of mCh revealed an excellent correlation with microscopic examination.

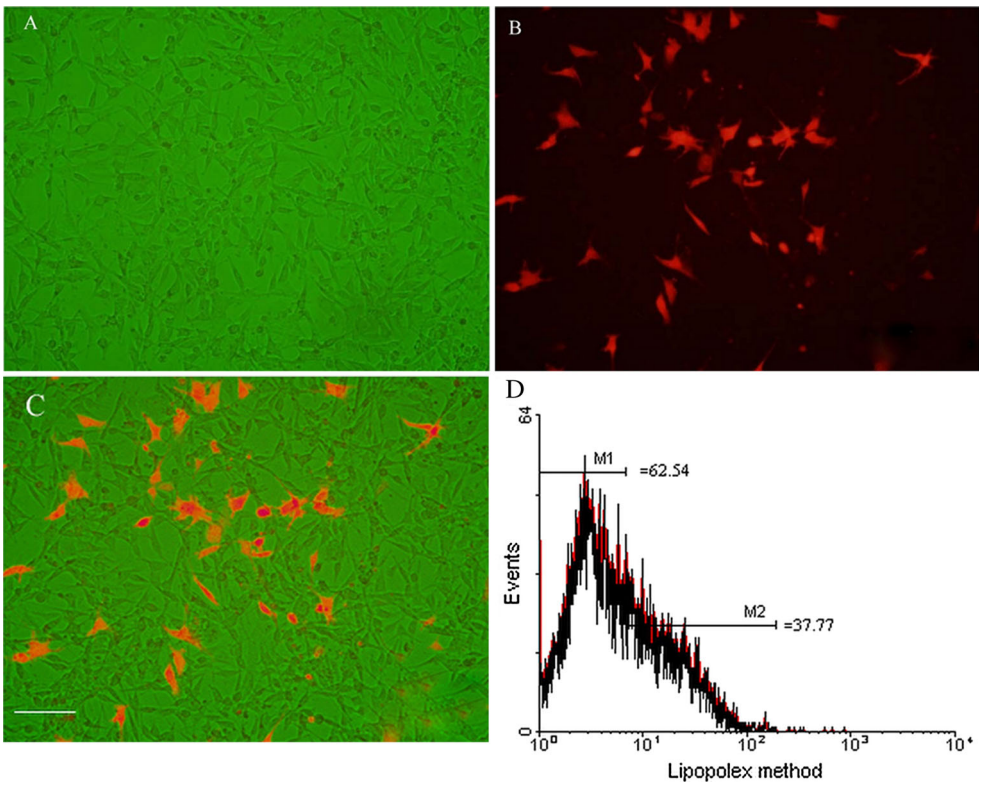

Fig. 7 Fluorescent and light micrograph images of transfected cells with lipofectamine/pmCherry-C1 plasmid complexes and viewed 24 h later. Light microscopy (**a**), fluorescence microscopy (**b**), merge (**c**), cells analyzed by flow cytometry (**d**) (Scale: 200 μm)

Table 2 Analysis of variance (ANOVA) for CCD

Source of variance	Sum of squares	df	Mean square	*F*-value	*p*-value
A-voltage	0.005	1	0.005	3.00	0.1817
B-pulse	3.78	1	3.78	2268.75	<0.0001
C-DNA	3.25	1	3.25	1950.75	<0.0001
AB	0.045	1	0.045	27.00	0.0138
AC	0.045	1	0.045	27.00	0.0138
BC	2.76	1	2.76	1656.75	<0.0001
A^2	34.26	1	34.26	20,554.57	<0.0001
B^2	0.000	0	0.000		
C^2	0.000	0	0.000		
Lack of fit	0.005	1	0.005		
Pure error	0.000	2	0.000		
Total SS	44.15	10			

Lipid-based delivery vectors are developed as most efficient non-viral methods. Most of them are synthetic such as lipofectamine, Cellfectin II, HiPerFect, and FuGENE [18]. They represent a promising approach to practical gene therapy because of their low toxicity, biodegradability, high cellular uptake, and easy surface functionalization [10]. As both the total amount of DNA and the DNA/lipofectamine ratios were important factors in transfection efficiency, therefore to achieve the acceptable lipid-based transfection, the total amount of DNA and the DNA/lipofectamine ratios were optimized [15]. The capability of lipofectamine™ 2000 for gene delivery into U87 cells was analyzed and compared with other methods as well. U87 cells were transfected at a suitable rate (37.8 %) in comparison with electroporation method.

The experiments were planned in a random manner to minimize the effect of uncontrolled variables. ANOVA was calculated and a *p*-value less than 0.05 indicate the statistical significance of an effect at 95 % confidence level (Table 2).

Data analysis gave a semi-empirical expression of extraction recovery (ER%) with following Eq. (5):

Table 3 Experimental design and results of the 2^3 full factorial central composite design

Run	Variables			Transfection	
	Voltage (v)	Pulse (μs)	DNA concentration (μg)	Experimental	Predicted
1	1200	30	60	0.5	0.47
2	800	165	30.5	4.8	4.8
3	400	300	1	0.3	0.33
4	1200	300	60	0.1	0.13
5	400	30	1	2.75	2.73
6	1200	30	1	2.75	2.78
7	800	165	30.5	4.8	4.8
8	800	165	30.5	4.8	4.8
9	400	30	60	0.1	0.13
10	1200	300	1	0.1	0.075
11	400	300	60	0.1	0.075

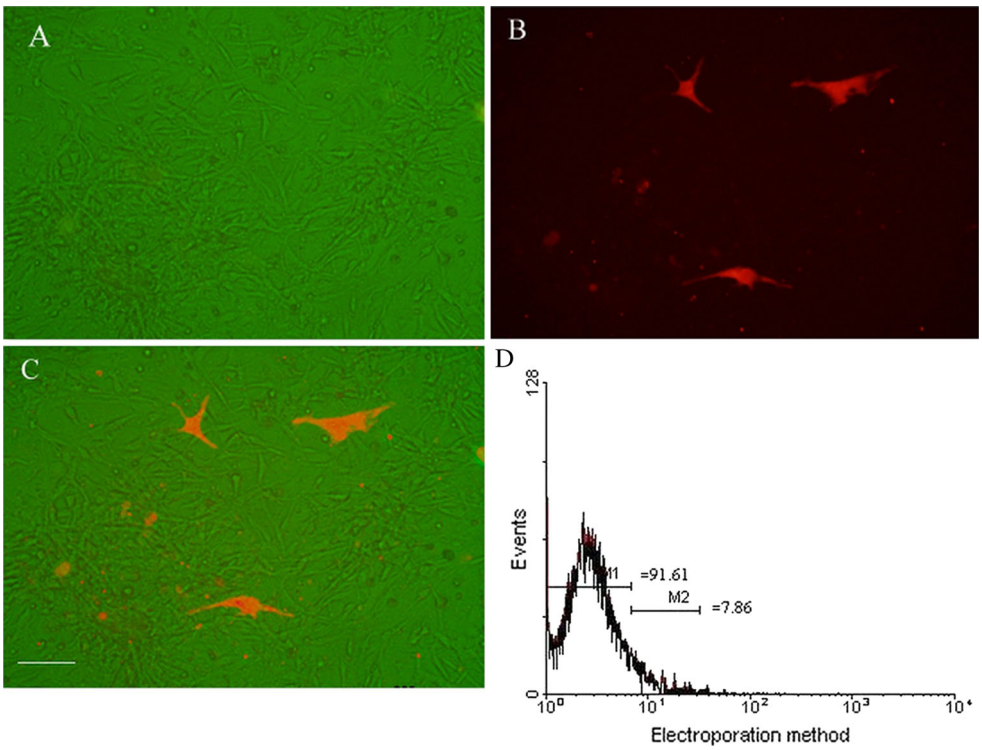

Fig. 8 Fluorescent and light micrograph images of transfected U87 cells. Cells were electroporated with mCh plasmid DNA. Bright field image of U87 cells (**a**) fluorescence image, (**b**) merge, (**c**) cells analyzed by flow cytometry, (**d**) (*scale* 1.0 mm)

$$Y = 4.8 + 0.025A - 0.69B - 0.64C - 0.075AB + 0.075AC + 0.59BC - 3.96A^2 \quad (5)$$

The transfections (%), as predicted by the final quadratic model along with the corresponding observed values, are given in Table 3

Therefore the best electroporation conditions for transfection of mCh were 800 V with time constant of 165 μs and DNA concentration of 30.5 μg (4×10^5 cells/800 μl for U87 cell line). Transfection efficiency was monitored for mCh plasmid, by fluorescent microscopy (5 %) and FACS (Fig. 8). The FACS analysis (8 %) of mCh revealed a close correlation with microscopic examination. The expression of mCh was reduced by time. Longer time points were tested in this experiment, and the fluorescence of mCh could persist in cells up to 10 day.

High transfection efficiency in U87 cell lines was achieved by identifying the most favorable electroporation voltage and pulse duration. The results confirm that our refined conditions maximized both transfection efficiency and cell viability [16]. The best electroporation conditions for U87 cell line was obtained using an open plate-based system which allows adjustment of parameters including voltage and pulse duration. The electroporation conditions

reported in this study used voltage setting between 400 and 1200 V and 30 and 300 μs with DNA concentration of 1 and 60 μg. Only 10 % of electroporation efficiency was obtained with optimized conditions for U87 cells which was different from previous studies on the other cell lines [16]. Statistical analysis of uptake efficiency intercell showed meaningful differences when comparing the CS nanoparticles with lipoplexes and electroporation methods.

Conclusions

Chitosan-based nanoparticles as non-viral gene delivery systems was examined on the U87 cells, and compared with lipoplex and electroporation as common transfection methods. Besides their great DNA association capacity, these nanoparticles exhibit low toxicity, deliver the associated DNA, and cause high levels of protein expression. Therefore, results suggest that chitosan-based nanoparticles might be a safe and efficient non-viral vector for gene delivery to U87 cell line.

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