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Novel hyaluronic acid–chitosan nanoparticles as non-viral gene delivery vectors targeting osteoarthritis

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ABSTRACT

Gene therapy is a promising new treatment strategy for common joint-disorders such as osteoarthritis. The development of safe, effective, targeted non-viral gene carriers is important for the clinical success of gene therapy. The present work describes the use of hybrid hyaluronic acid (HA)/chitosan (CS) nanoparticles as novel non-viral gene delivery vectors capable of transferring exogenous genes into primary chondrocytes for the treatment of joint diseases. HA/CS plasmid–DNA nanoparticles were synthesized through the complex coacervation of the cationic polymers with pEGFP. Particle size and zeta potential were related to the weight ratio of CS to HA, where increases in nanoparticle size and decreases in surface charge were observed as HA content increased. The particle size and the zeta potential varied according to pH. Transfection of primary chondrocytes was performed under different conditions to examine variations in the pH of the transfection medium, different N/P ratios, different plasmid concentrations, and different molecular weights of chitosan. Transfection efficiency was maximized for a medium pH of approximately 6.8, an N/P ratio of 5, plasmid concentration of 4 μg/ml, and a chitosan molecular weight of 50 kDa. The transfection efficiency of HA/CS-plasmid nanoparticles was significantly higher than that of CS-plasmid nanoparticles under the same conditions. The average viability of cells transfected with HA/CS-plasmid nanoparticles was over 90%. These results suggest that HA/CS-plasmid nanoparticles could be an effective non-viral vector suitable for gene delivery to chondrocytes.

1. Introduction

Osteoarthritis is one of the most common chronic, progressive, degenerative joint disease and is a leading cause of chronic disability in the elderly (Sarzi-Puttini et al., 2005). Due to its multifactorial etiology and complex pathogenesis, there are currently no satisfactory treatments. Gene therapy, which treats the causes of osteoarthritis by targeting specific pathological mechanisms, is attracting much attention as a promising new treatment approach (Zhang et al., 2006). A key issue of gene therapy is the selection of an appropriate and effective gene delivery vector. Viral vectors are the most common transfection agents currently in use, but safety risks are a major disadvantage (El-Aneed, 2004). Non-viral gene delivery systems have been proposed as safer alternatives to viral vectors as they can be administered repeatedly with minimal host immune response, are stable in storage, and can be easily produced in large quantities (Gao et al., 2008). As a non-viral vector for gene delivery, chitosan (CS) offers certain advantages such as non-toxicity (Hejazi and Amiji, 2003), good biocompatibility (Li and Zhang, 2005), biodegradability (Li and Zhang, 2005), high stability, and reasonable cost. However, low transfection efficiency currently limits the application of CS as a non-viral gene delivery vector (Gao et al., 2008; Lavertu et al., 2006; Zhao et al., 2009).

The transfection efficiency of CS vectors can be improved by combining CS with cationic or anionic biopolymers, such as polyethylenimine (Zhao et al., 2009) or arginine (Gao et al., 2008), prior to the addition of DNA. The choice of biopolymer greatly influences the specificity, stability, and size of the assembled nanoparticles (Duceppe and Tabrizian, 2009). Hyaluronic acid (HA) is another biocompatible anionic biopolymer used in a wide array of clinical applications (Almond, 2007; Menzel and Farr, 1998). HA of high molecular weight contained within healthy endogenous synovial fluids contributes to the elasticity and viscosity of synovial fluid. HA is also a natural mucopolysaccharide of the extracellular matrix of articular cartilage and plays an important role in its function. It is involved in cell adhesion, morphogenesis, and inflammation regulation (Menzel and Farr, 1998). In osteoarthritis, intra-articular injection of HA improves the viscoelasticity of synovial fluid, augments the flow of joint fluid, normalizes endogenous hyaluronate synthesis, inhibits hyaluronate degradation, reduces joint pain, and improves joint function (Abate et al., 2010). However, these biological properties of HA have been shown to be dependent on its molecular weight (Ghosh and Guidolin, 2002).

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Fragments of HA, or HA of low molecular weight, are able to bind various cellular receptors such as cluster determinant 44 (CD44) or toll-like receptor 4 (TLR-4) (Campo et al., 2010a, 2010b), which are highly expressed in osteoarthritic chondrocytes (Aruffo et al., 1990; Chow et al., 1995; Knudson and Knudson, 2004; Li and Huang, 2006). Therefore, the presence of specific ligands like HA on the nanoparticle surface may increase nanoparticle uptake in the osteoarthritic disease state. Such targeted gene carriers are of particular importance for gene therapy as targeting increases the efficiency and reduces side effects compared to non-targeted treatments (Li and Huang, 2006; Merdan et al., 2002). For example Kompella et al. (2006) have demonstrated that the cellular uptake of transferrin- or desorelin-modified polystyrene nanoparticles, which are able to specifically interact with transferrin and LHRH receptors, was significantly improved in comparison to plain nanoparticles.

CS–DNA complexes have been shown to be capable of transfecting chondrocytes under *in vitro* and *in vivo* conditions. Zhao et al. (2006) investigated the effects of several factors such as the pH of the transfection medium, the molecular weight of CS, and different plasmid concentrations on the transfection efficiency of CS–pEGFP nanoparticles in primary chondrocytes. When CS–DNA nanoparticles containing IL-1Ra or IL-10 genes were directly injected into the knee joint cavities of osteoarthritic rabbits, clear expression of IL-1Ra was detected in the knee joint synovial fluid of the CS-IL-1Ra injected group (Zhang et al., 2006). Overall, CS–DNA nanoparticles were found to have favorable characteristics for non-viral gene delivery to primary chondrocytes; however, the transfection efficiency of CS–DNA nanoparticles in chondrocytes was found to be unsatisfactory and restricted their clinical application (Zhang et al., 2006).

The present study has therefore designed a new non-viral gene carrier consisting of HA and CS for the osteoarthritis-targeted intracellular delivery of therapeutic genes into chondrocytes. This work investigates the characteristics of HA/CS–pEGFP nanoparticles and their transfection efficiency in primary chondrocytes under different conditions (pH of transfection medium, N/P ratio, and plasmid dose) to assess the feasibility of HA/CS-plasmid nanoparticles as non-viral agents capable of delivering therapeutic genes to chondrocytes for the treatment of osteoarthritis or other joint diseases.

2. Materials and methods

2.1. Materials

Chitosan, with a molecular weight (MW) of 50 kDa, and a deacetylation degree of 90%, was purchased from Sigma–Aldrich (USA). Notably, a variety of different CS molecular weights were used to assess the effect of CS molecular weight on transfection efficiency of primary chondrocytes. These were CS with molecular weights of 5 kDa, 100 kDa, 500 kDa, and 900 kDa, and deacetylation degrees of 85%, 85%, 82%, and 85%, respectively, which were all purchased from Sigma–Aldrich. Hyaluronic acid, with a molecular weight of 160 kDa, was purchased from C.P. Freda Pharmaceutical Co. Ltd. (Shangdong, China). Dulbecco's modified Eagle's medium (DMEM), D-Hanks, and antibiotics were from Gibco (USA). Fetal bovine serum (FBS) was from Hyclone (USA). Hyaluronidase and chitosanase were from Sigma–Aldrich (USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan). Lipofectamine™ 2000 was from Invitrogen (USA). The pReceiver-M29 vector carrying an enhanced green fluorescent protein (EGFP) was from Invitrogene (USA). The plasmid was propagated in *Escherichia coli* cells, isolated, and purified.

2.2. Nanoparticle preparation

Nanoparticles were prepared and DNA was incorporated according to the method described by Duceppe and Tabrizian (2009). In brief, 10 mg HA was dissolved in 4 ml of buffer (0.1 M sodium acetate, 0.1 M sodium chloride, pH7.2) under magnetic stirring. After HA was completely dissolved, 0.5 mg hyaluronidase (from bovine testes, activity 300–500 units/mg, Sigma–Aldrich, USA) was added to the solution, which was then kept for 24 h in a shaking incubator at 37 °C. After the enzymatic splitting reaction, the mixture was filtered (Amicon Ultra, MWCO 10 kDa, Millipore Corp., USA), and then the filtrated HA of low molecular weight (MW < 10 kDa) was centrifuged and collected by lyophilization (de la Fuente et al., 2008). The resulting substance was dissolved in distilled water (pH 5.5). 10 mg of CS was dissolved in 2% acetic acid (pH 5.5). Both the HA and CS solutions were filtered through a 0.22 μm membrane. The CS solution was stirred at a rate of 3000 rpm for 30 min, mixed into the HA solution and stirred for 10 min. Eight different mixtures were prepared with CS:HA weight ratios at: 1:2, 1:1, 2:1, 3:1,4:1, 5:1, 6:1, and 7:1. A constant concentration of 11.25 μg/ml of HA was used in all mixtures, and the CS concentration was varied as: 5.625, 11.25, 22.5, 33.75, 45, 56.25, 67.5, and 78.25 μg/ml. The required volume of 25 μg/ml plasmid DNA was gently added to CS/HA solution by gentle pipetting to form complexes of a selected N/P ratio. N/P ratio was defined as the molar ratio of the positive CS amino group and the negative DNA phosphate group. The mixture was vortexed rapidly for 3–5 s and left for 1 h at room temperature for the complexes to completely form.

2.3. Scanning electron microscopy (SEM)

Nanoparticles in solution were dropped onto a silica surface precoated with a thin layer of gold and palladium. The nanoparticles were lyophilized using a JFD-310 (JEOL, Japan) prior to analysis, and micrographs were obtained using a SEM (JSM-6360, Japan).

2.4. Particle size and zeta potential measurements

A Mastersizer 2000 laser diffractometer (Malvern Instruments, Worcestershire, UK) was used to measure the size and charge of the nanoparticles. The nanoparticles were prepared and analyzed in distilled water at 25 °C (pH 5.5). In order to find out the effect of pH on the particle size and zeta potential, HA/CS-plasmid nanoparticles prepared with CS:HA weight ratios of 4:1, for example, were prepared by changing the pH value of the nanoparticle solution from 5.5 to 8.0 shortly after preparation. The particle size and zeta potential were measured as described above.

2.5. Gel retarding analysis

HA/CS-plasmid nanoparticles were evaluated by agarose gel electrophoresis. The HA/CS nanoparticles were prepared at CS:HA weight ratios of: 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, and 7:1, as described above. The nanoparticles and the naked plasmid were loaded onto a 1% agarose gel containing ethidium bromide in Tris–borate EDTA buffer at pH 8.0. The samples were run on the gel at 120 V for 30 min. The gel was then photographed using a GDS-8000 (UVP, USA).

HA/CS-plasmid nanoparticles and naked pDNA solution (each containing 1 μg of pDNA) were added to 5 U of DNase I (Sigma–Aldrich, USA) and kept in a water bath for 1 h at 37 °C. Agarose gel (1%) electrophoresis was repeated, as described above.

In addition, for HA/CS-plasmid nanoparticles (N/P ratio 4) prepared with CS:HA weight ratios of 4:1, for example, different concentrations (56–336 ng/μl) of chitosanase (from streptomyces species, activity 18 units/mg, Sigma–Aldrich, USA) were added and the nanoparticle–chitosanase solution was maintained in a

water bath for 4 h at 37 °C. Agarose gel (1%) electrophoresis was repeated, as described above. In order to determine the combination between CS and pDNA, HA/CS-plasmid nanoparticles of different N/P ratios (from 1/2 to 8), prepared with CS:HA weight ratios of 4:1, were loaded and agarose gel (1%) electrophoresis was repeated as described above.

2.6. Cell culture

Cartilage from rabbit knee joints (from 2 New Zealand rabbits, 3 weeks old, purchased from the Experimental animal center of Guangdong province) was surgically isolated and placed into PBS. Cartilage slices were treated with 0.25% trypsin at 37 °C for 30 min, thoroughly washed, and treated with 0.1% collagenase for 12–16 h in an incubator at 37 °C and 5% CO_2. After washing twice with DMEM, the isolated chondrocytes were cultured in monolayer in medium consisting of DMEM supplemented with 10% FBS, and placed in an incubator at 37 °C and 5% CO_2 for 5–7 days. All procedures involving animals in this study were performed in accordance with the animal ethics regulations of Sun Yat-sen University, Guangzhou, China.

2.7. Cytotoxicity assays

Chondrocytes were seeded at a density of 5×10^4 cells per well in 100 μl of culture medium in 96-well plates and grown overnight. Immediately after culture medium was removed, either CS-plasmid or HA/CS-plasmid nanoparticles (N/P ratio 4, CS:HA weight ratios 4:1), at polymer concentrations ranging from 5 μg/ml to 40 μg/ml, suspended in fresh culture medium, were applied to the cells. In another group of cells, culture media was replaced by fresh serum-free media containing Lipofectamine™ 2000 (DNA 5 μg/ml). A group of cells treated with only fresh culture medium were used as controls. Cells were incubated with various complexes for 4 h, after which the various media were removed and replaced with fresh culture media. After the nanoparticles were removed, medium containing CCK-8 (10 μg per well) was added to the culture plates, and cells were maintained in an incubator at 37 °C and 5% CO_2 for 3 h. Optical density was measured using a microplate reader (Bio-RAD, model 680) at a wavelength of 570 nm, using a blank consisting of CCK-8 solution. Three replicates were counted for each sample. The mean value was reported in the final results.

2.8. In vitro transfection experiment

Chondrocytes were seeded into 24-well plates at a density of 1×10^5 cells per well in 500 μl of culture medium and incubated for 24 h prior to transfection. The medium was discarded, and cells were washed once with PBS of the same pH of the transfection medium. Nanoparticles containing DNA (HA/CS-plasmid nanoparticles with a CS:HA weight ratio of 4:1 were used as example) were added to the cells in DMEM containing 10% FBS without antibiotics and incubated for a period of 4 h. After this process, the nanoparticles solution was discarded, and fresh culture media, supplemented with serum and antibiotics, was added to the cells. The culture medium was changed every two days during the experiment. At different time points post transfection, EGFP-positive transfected cells were detected using fluorescence microscope (Nikon-TE2000-U, Japan).

Cells were collected and re-suspended in PBS (pH 7.4), and the transfection results were measured using a fluorescence activated cell sorting (FACS) apparatus (Calibur, BD, USA) through fluorescence channel 1 (FL1) 48 h later. Cells, which were exposed only to naked DNA or CS-plasmid nanoparticles, were analyzed as controls (at a concentration of 4 μg/ml of pEGFP). Lipofectamine™ 2000 was used as a positive control for transfection, and was added to DMEM without serum and antibiotics, following manufacturer procedures. Solutions were incubated for a period of 4 h, with a concentration of 0.8 μg/well of pEGFP. The medium was then discarded and replaced with a complete medium containing serum and antibiotics, according to instructions. All transfection experiments were performed in triplicate.

An additional transfection study was performed to investigate the potential utilization of CD44 in aiding transportation of pDNA into the cell by blocking the receptor with an excess of HA. Free HA (at concentrations 10, 20, and 50 times greater than the HA content of the nanoparticles) was added to the cells and incubated for 20 min at 37 °C. After incubation, polysaccharide solutions were aspirated, and nanoparticles containing DNA in solution were introduced to the cells and incubated for 4 h at 37 °C and 5% CO_2, as described above. The transfection efficiency was determined by fluorescent microscopy and FACS, as mentioned above.

2.9. Statistical analysis

The statistical significance was studied by one-way ANOVA and the *t*-test. All of the measurement data were displayed as means ± standard error of the mean ($\bar{x} \pm s$) and were derived using the SPSS software package (v.13). Differences were considered to be significant at a level of $p < 0.05$.

3. Results and discussion

3.1. Physicochemical properties of HA/CS-plasmid nanoparticles

The relationship between the weight ratio of CS to HA and the resulting nanoparticle size is shown in Fig. 1. There is a significant decrease in nanoparticle size with increasing amounts of CS, and the smallest particle size (115.6 ± 4.0 nm) formed with a 6:1 ratio of CS:HA (Fig. 1a). As demonstrated by the statistically significant differences in Fig. 1a, the zeta potential becomes more positive as the amount of CS within the polyelectrolyte complex increases. The highest zeta potential was obtained for CS:HA weight ratios higher than 6:1, yielding zeta values around 26.3 ± 0.5 mV for the 6:1 ratio. Hence, the size and zeta potential of the nanoparticles were dependent on the composition, with an increase in size and a decrease in the surface charge with increasing HA content. Fig. 1b demonstrates that when the pH value increased from 5.5 to 8.0, the particle size and zeta potential of the nanoparticles changed significantly, from 152.5 to 364.8 nm and 24.0 to −34.5 mV, respectively. These results illustrate that the physicochemical properties of the HA/CS-plasmid nanoparticles depend on their composition and the character of CS, and vary under different conditions. As the physicochemical properties of the nanoparticles are directly related to their bioactivities, investigating the transfection in different conditions in order to obtain the best transfection efficiency is necessary. SEM images revealed spherical particles with diameters of approximately 100–300 nm (Fig. 2).

The formation of nanoparticles as combinations of pEGFP and HA/CS was observed by agarose gel electrophoresis. Fig. 3a shows that the pEGFP was totally retained within the gel-loading well, which illustrates the complete combination of pEGFP with CS. After adding DNase I, the fluorescent light originating from the lane containing naked pDNA disappeared completely, whereas the fluorescence intensity of lanes containing HA/CS-plasmid nanoparticles decreased only slightly (Fig. 3b). This indicates that the pDNA within HA/CS-plasmid nanoparticles was subject only to slight nuclease degradation or to no degradation at all. These results indicate that HA/CS-plasmid nanoparticles entrap DNA. It is likely that pDNA was efficiently entrapped because of the strong interaction between pDNA phosphate groups and the amino groups of CS, in

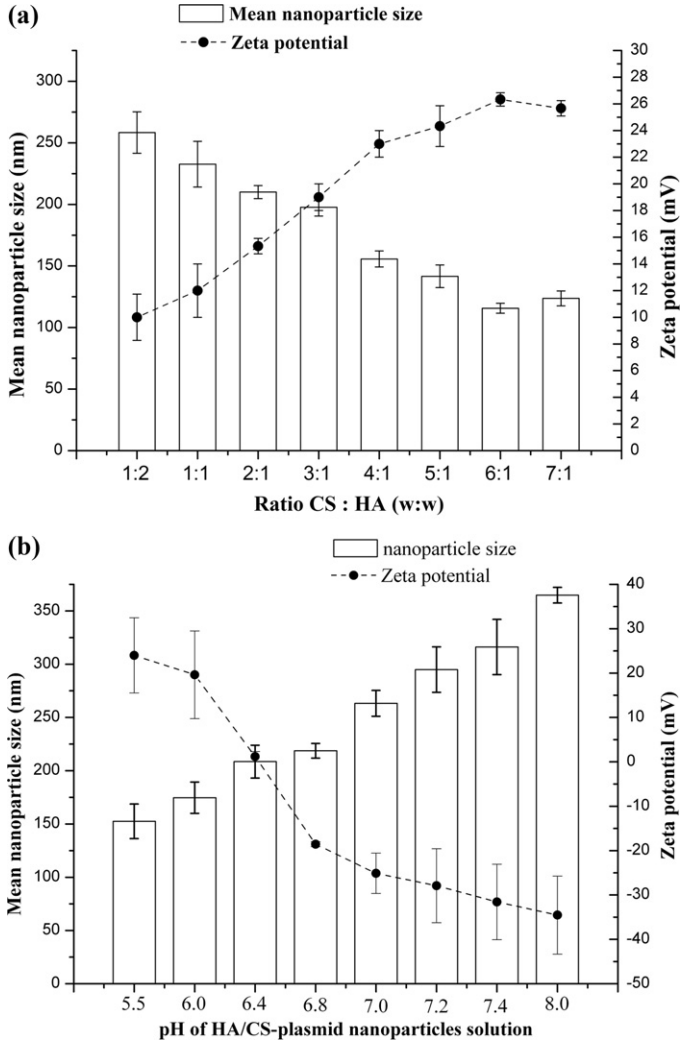

Fig. 1. Effect of the weight ratio of CS to HA (a) and pH value (b) on the particle size and zeta potential of HA/CS-plasmid nanoparticles ($n = 3$).

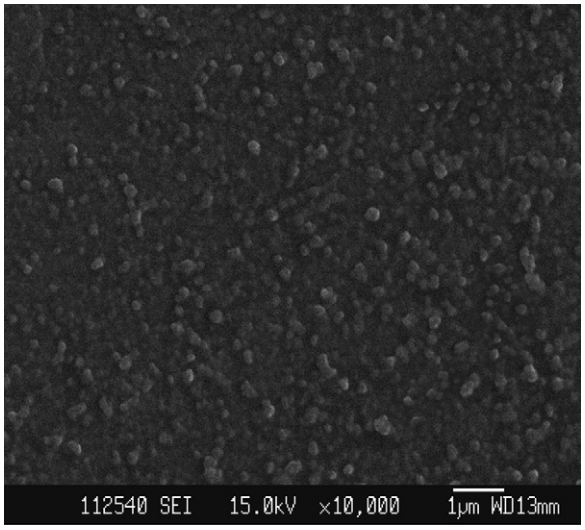

Fig. 2. Scanning electron microscopy (SEM) imaging of HA/CS-plasmid nanoparticles obtained with SEM imaging on a gold/palladium pre-coated silica surface at 5.0 kV. The image shown here is representative of a lyophilized nanoparticle sample with a weight ratio of CS:HA of 4:1 with 2.5 μg/ml of pEGFP.

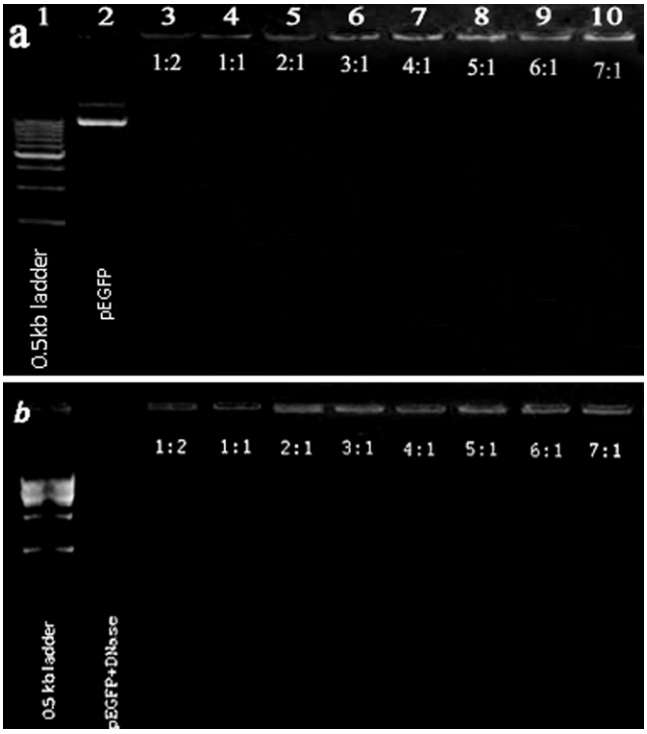

Fig. 3. Gel retarding analysis of HA/CS-plasmid nanoparticles. Lane 1: DNA marker; lane 2: naked DNA control; lane 3–10: HA/CS-plasmid nanoparticles prepared at CS:HA weight ratios of 1:2; 1:1; 2:1; 3:1; 4:1; 5:1; 6:1 and 7:1.

addition to hydrophobic and hydrogen bonds between CS and DNA nucleotides. Fig. 4a shows that the combination of pEGFP with CS was dependent on the N/P ratio. Only nanoparticles with N/P ratios higher than two could completely entrap DNA. Moreover, DNA could be released from the nanoparticles after HA/CS-plasmid nanoparticles were degraded by chitosanase (Fig. 4b).

3.2. Cell viability

In order to develop HA/CS-plasmid nanoparticles as a novel gene delivery carrier for joint disease, an assessment of any potential toxic interaction with chondrocytes is very important. In this study, the cytotoxicity of CS-plasmid and HA/CS-plasmid nanoparticles were estimated using the MTT assay. HA/CS-plasmid nanoparticles showed similar low cellular toxicity with CS-plasmid nanoparticles (Fig. 5). As the polyplex dose increased to 40 μg/ml, both CS-plasmid and HA/CS-plasmid nanoparticles showed a slight increase in cellular toxicity. However, the average cell viability of HA/CS-plasmid nanoparticles was more than 90%, which was much higher than that of Lipofectamine™ 2000, and showed less than 60% viability (Fig. 5). These cytotoxicity results indicate that HA/CS-plasmid nanoparticles should be a safer carrier than Lipofectamine™ 2000 and specify a safe range for the application of HA/CS-plasmid nanoparticles (5–40 μg/ml) to joint tissue/chondrocytes.

3.3. Transfection studies

The gene delivery potential of CS is known to depend on several factors, such as cell type, serum, N/P ratio, pH of transfection medium, the molecular weight of CS, and plasmid concentration (Gao et al., 2008; Mao et al., 2010; Zhao et al., 2006). In order to find optimal conditions for *in vitro* gene transfection efficiency of HA/CS-plasmid nanoparticles against primary chondrocytes, transfections were carried out under a variety of conditions (pH of

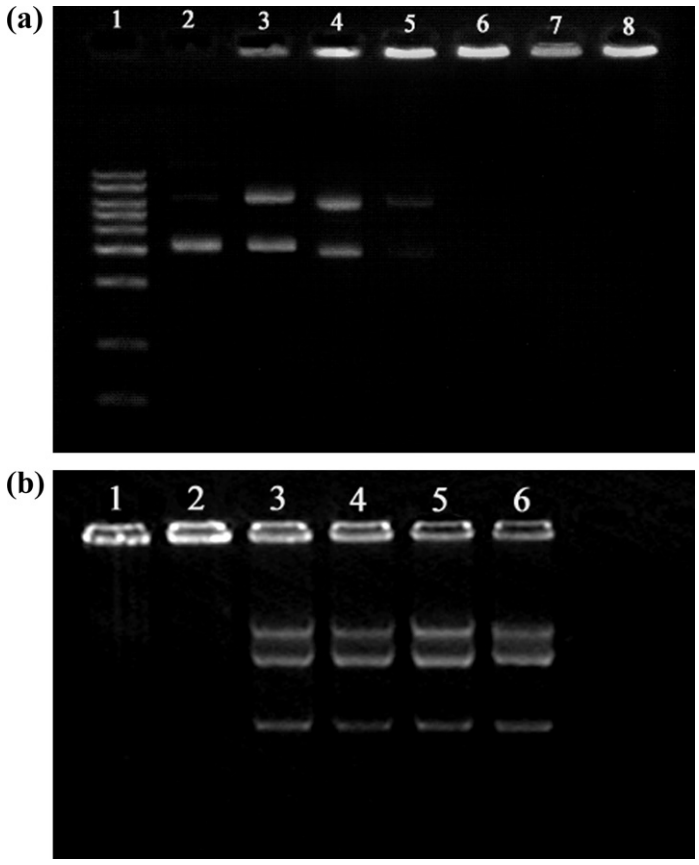

Fig. 4. Electrophoresis photo of HA/CS-plasmid nanoparticles prepared with different N/P ratios (a, pH = 5.5, lane 1: DNA marker; lane 2: naked pDNA; lane 3–8: HA/CS-plasmid nanoparticles prepared with N/P ratios of 1:2, 1:1, 2:1, 4:1, 6:1, and 8:1, respectively) and treated with different concentrations of chitosanase (b, lane 1–6: 56 ng/μl, 112 ng/μl, 168 ng/μl, 224 ng/μl, 280 ng/μl, and 336 ng/μl of chitosanase, respectively).

transfection medium, N/P ratio, plasmid concentration, nanoparticles prepared with chitosan of different molecular weights, and HA content). Because the effect of serum on the transfection efficiency of CS-plasmid nanoparticles in primary chondrocytes was already reported by Zhao et al. (2006) to be optimal for concentrations of 10% FBS, our experiments were carried out in medium with 10% FBS.

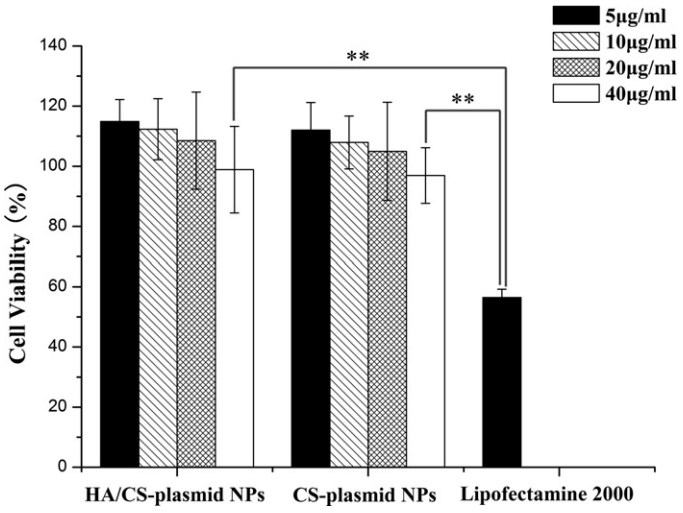

Fig. 5. Cell viability of HA/CS-plasmid nanoparticles, CS-plasmid nanoparticles, and Lipofectamine™ 2000 in primary chondrocytes. $^{**}p < 0.01$ compared with Lipofectamine™ 2000.

As shown in Fig. 6a, a pH below 7 (around 6.8) resulted in the highest transfection efficiencies, which was consistent with the results of Duceppe and Tabrizian (2009). However, this optimal pH is slightly more acidic than that reported by Zhao et al. (2006) in working with CS-plasmid nanoparticles in chondrocytes. The pK_a of the primary amines in CS is approximately 6.4 (Zhao et al., 2009), which suggests that the free amino groups in CS could not be fully protonized and therefore, when pH exceeded 7.0, the CS–DNA complexes were not positive enough to bind with the negatively charged cells through electrostatic interaction. For HA/CS-plasmid nanoparticles, the substantially lower pK_a value of HA ($pK_a = 3.0$) (Brown and Jones, 2005) could further decrease the pK_a value of HA/CS-plasmid nanoparticles. Therefore, the free amino groups in the CS backbone could only play an important role in condensing DNA into nanoparticles and interacting with cells at pHs even lower than those required for CS-plasmid nanoparticles.

Fig. 6b shows that transfection efficiency increased when N/P ratios increased from 1 to 5, with the highest level reached at an N/P ratio of 5. This is consistent with previous reports, which determined CS–DNA complexes with N/P ratios between 3 and 5 showed the highest transfection activity in 10% serum (Ishii et al., 2001). Changes in the N/P ratio would affect the surface charge of the polyplexes. This could affect transfection efficiency by influencing the ability of the nanoparticles to efficiently condense plasmid DNA and to interact with cell membranes (Huang et al., 2005). Consequently, lower N/P ratios may yield physically unstable complexes and poor transfection. On the contrary, N/P ratios higher than 5 should produce overly-stable complexes with reduced transfection (Koping-Hoggard et al., 2003).

In addition to the pH and N/P ratio, the amount of DNA incorporated within the nanoparticles plays an important role in the efficiency of the transfection process. As shown in Fig. 6c, the highest transfection efficiency was obtained for plasmid concentrations of 4 μg/ml. When the plasmid dose increased to 8 μg/ml or greater, the transfection efficiency decreased. At higher concentrations, nanoparticles may aggregate in the cell membrane, leading to inhibited cell uptake (Kompella et al., 2006).

To determine the effect of CS structure on the transfection efficiency of primary chondrocytes, transfections were performed in DMEM containing 10% FBS with HA/CS-plasmid nanoparticles prepared with CS of different molecular weights (Fig. 6d). At N/P ratio 5, the highest transfection efficiency was obtained with CS of molecular weight 50 kDa, which was consistent with previous reports on other cell lines (Sato et al., 2001). The molecular weight of CS may influence the stability of the nanoparticles, the efficiency of cell uptake, and the dissociation of DNA from the complex after endocytosis, thereby influencing the transfection efficiency of the final complex (MacLaughlin et al., 1998; Sato et al., 2001). High molecular weight CS has an enhanced ability to stabilize complexes, which beneficially protects DNA in the cellular endosomal/lysosomal compartments. However, this higher molecular weight CS also restricts or slows the release of DNA once inside the cell, resulting in low or delayed expression (MacLaughlin et al., 1998). In contrast, complexes formed with lower molecular weight CS are not sufficiently stable, and cannot provide effective protection for DNA due to early dissociation. Therefore complexes prepared with low molecular weight CS show little transgene expression. Consequently, an intermediate degree of stability needs to be achieved with CS of an appropriate molecular weight in order to obtain high levels of transfection.

To determine whether HA conjugated to the CS backbone could improve its transfection efficiency, we compared the transfection efficiency of HA/CS-plasmid nanoparticles with that of CS-plasmid nanoparticles, naked pDNA, and Lipofectamine™ 2000.

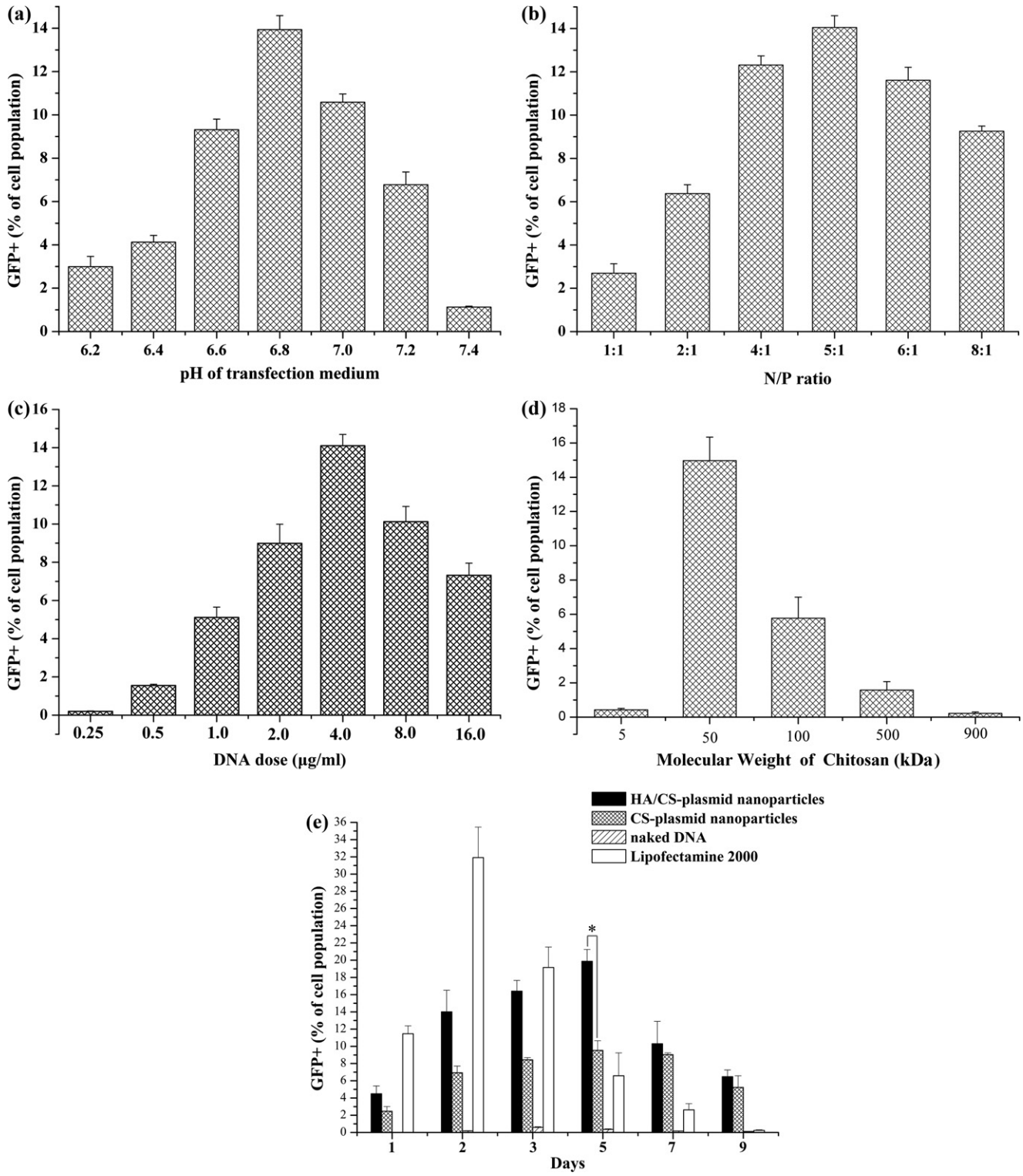

Fig. 6. Optimization of transfection parameters *in vitro*. Percentage of chondrocytes transfected *in vitro* using HA/CS-plasmid nanoparticles as measured by flow cytometry 48 h after transfection. The influence of different parameters on transfection was assessed. (a) Effect of pH of transfection medium (plasmid dosage was 4 μg/ml and N/P ratio 5), (b) varying N/P ratio of the nanoparticles (plasmid dosage was 4 μg/ml and transfection medium at pH 7.2), (c) influence of DNA concentration (N/P ratio 5 and transfection medium at pH 7.2), (d) effect of CS molecular weight (plasmid dosage was 4 μg/ml, N/P ratio 5, and transfection medium at pH 7.2), and (e) compared with that of CS-plasmid nanoparticles and naked plasmid DNA from 24 h to the ninth day. Lipofectamine™ 2000 as positive control. $^{*}p < 0.05$ when HA/CS-plasmid nanoparticles compared with CS-plasmid nanoparticles (at the second or the fifth day) ($n = 3$).

Transfection studies were performed against chondrocytes with an N/P ratio of 4 and pDNA dose of 4 μg/ml in DMEM (pH 7.2) containing 10% FBS. Twenty four to 48 h post transfection, the transfection efficiency of HA/CS-plasmid nanoparticles did not reach the same level as Lipofectamine™ 2000, but was significantly higher than that of CS-plasmid nanoparticles (Fig. 6e).

As shown in Fig. 6e, the ability of the nanoparticles to transfect chondrocytes was strongly influenced by their composition.

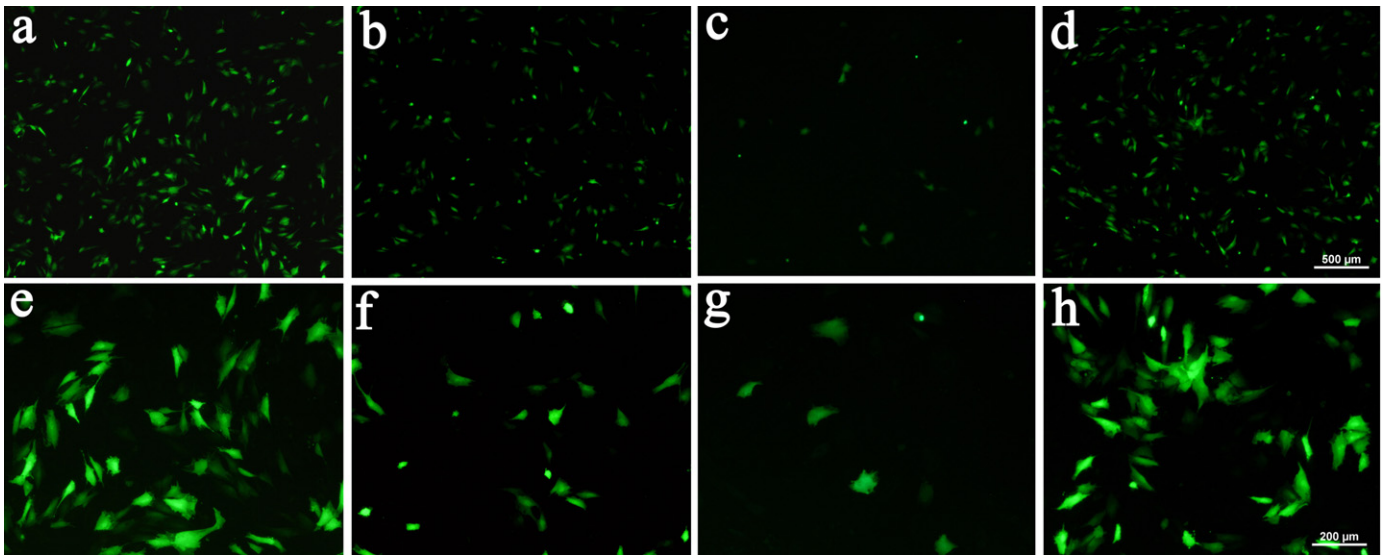

Fig. 7. Images of chondrocytes transfected with HA/CS-plasmid nanoparticles, CS-plasmid nanoparticles, naked plasmid DNA and Lipofectamine™ 2000 as observed under fluorescence microscope (100× magnification for (a)–(d); 200× magnification for (e)–(h)). HA/CS-plasmid nanoparticles (a and e); CS-plasmid nanoparticles (b and f); naked DNA (c and g) and Lipofectamine™ 2000 (d and h).

The level of EGFP expression increased significantly when HA was included in the nanostructures. This enhancement of the transfection efficiency by HA may be due to CD44 acting as the primary cell surface receptor for HA internalization and turnover (Culty et al., 1992; Knudson et al., 2002). In osteoarthritis, due to stimulation by inflammatory factors (such as IL-1), CD44 receptors are highly expressed and easily detected (Chow et al., 1995). The presence of HA in the nanoparticles could allow HA containing nanoparticles to gain facilitated access to the target cells via receptor-mediated endocytosis pathway (de la Fuente et al., 2008). In addition to this positive feature, it has been reported that the HA-CD44 interaction leads to a cellular signaling process (Aragona, 2004; Toole, 2001), which could promote the success of the gene transfection. Moreover, it has been shown that HA can be deposited onto the cationic surface of DNA–polyethyleneimine (PEI) complexes via electrostatic means, where it can minimize nonspecific interactions with serum proteins and behave like a transcription activator to enhance the transfection efficiency of HA-coated PEI complexes (Ito et al., 2006). The same type of mechanism could apply to the HA/CS-plasmid nanoparticles. Finally, HA could improve the internalization of the nanoparticles due to specific and non-specific interactions with the cells (de la Fuente et al., 2008). Polyanion charge density has a great impact on the stability and association between DNA and CS. As reported by Danielsen et al. (2005), the high charge density of anionic polymers like heparin, for example, result in a large decrease in the DNA release of CS-DNA complexes. Therefore, lower charge density polymers such as HA, which associate with CS by electrostatic interactions, should improve the transfection efficiency compared to nanoparticles involving higher charge density polyanions.

Nanoparticle transfections showed delayed EGFP expression. At 24 h post transfection, both CS-plasmid and HA/CS-plasmid nanoparticles expressed weak EGFP; and in cells transfected with naked pDNA, almost no expression of EGFP was observed. 48 h later, EGFP-expression increased significantly in the HA/CS-plasmid nanoparticles group; and its fluorescence intensity also increased significantly in comparison to that of CS-plasmid nanoparticles cells (Figs. 7 and 8). To function as viable gene-vectors, the nanoparticles must first escape endosome–lysosome enzymatic dissociation, and then be degraded by a polysaccharide enzyme before the DNA can be gradually released and enter into the nucleus. This time-dependent intercellular process might explain why the expression of foreign transfected genes remained lower at first in the HA/CS-plasmid and CS-plasmid nanoparticles groups with the transfection efficiency increasing after prolonged times (five days), unlike the rapid expression observed with Lipofectamine. This delayed-release characteristic of HA/CS-plasmid nanoparticles may greatly enhance its application potential in joint disease *in vivo*.

In order to ascertain if CD44 receptor-mediated endocytosis is involved in the internalization mechanism of HA/CS-plasmid nanoparticles by chondrocytes, a supplementary transfection study was performed. As shown in Fig. 9, after the CD44 receptor was blocked with an excess of HA (50-fold with respect to the amount of HA forming in the nanoparticle), the subsequent transfection efficiency of HA/CS-plasmid nanoparticles decreased significantly. This

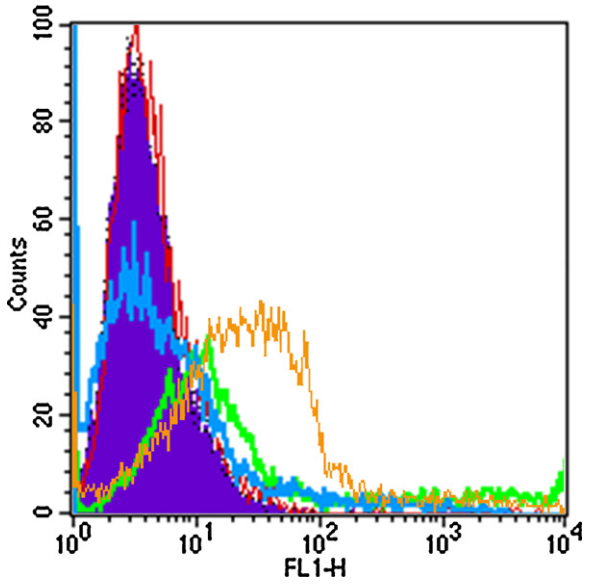

Fig. 8. EGFP fluorescent intensity of the cells for each group: (purple) untreated cells; (red) naked plasmid DNA; (blue) CS-plasmid nanoparticles; (green) HA/CS-plasmid nanoparticles; (yellow) Lipofectamine™ 2000 ($n = 3$, 48 h post transfection). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

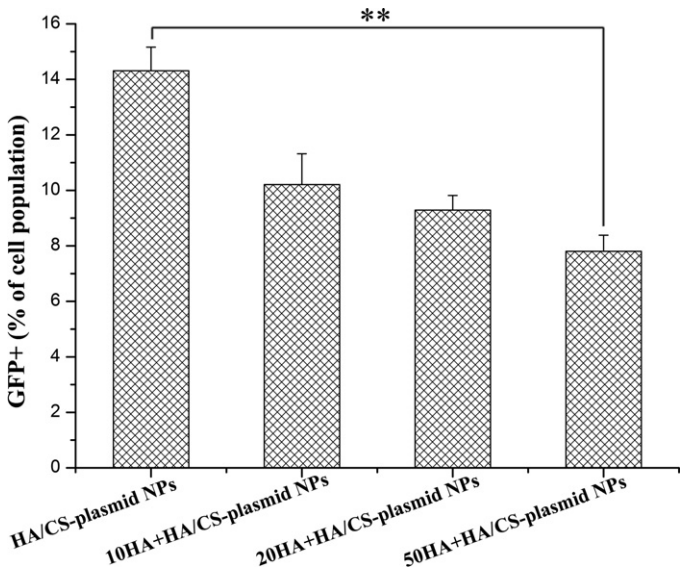

Fig. 9. Evaluation of the transfection efficiency of HA/CS–pEGFP nanoparticles upon incubation with chondrocytes. The CD44 receptor was blocked with an excess of HA (from 10-fold, 20-fold, to 50-fold with respect to the amount of HA forming the nanoparticle, respectively). The transfection efficiency was determined by FACS (mean ± SD, $n = 3$). $^{**}p < 0.05$ when the transfection efficiency of HA/CS–pEGFP nanoparticles compared with that of 50-fold HA + HA/CS–pEGFP nanoparticles 48 h post-transfection.

observation is reliable evidence to confirm that HA-CD44 mediated endocytosis was at least partially involved in HA/CS-plasmid nanoparticles transfection against chondrocytes.

In this study, HA/CS-plasmid nanoparticles were created as novel, non-viral gene delivery vectors targeted to osteoarthritis and other joint diseases. The size and zeta potential of HA/CS-plasmid nanoparticles were related to composition, and generally there was an increase in size and a decrease in the surface charge with increasing HA. The *in vitro* transfection efficiency of HA/CS-plasmid nanoparticles was found to be dependent on the pH of the transfection medium, N/P ratio, plasmid concentration, and molecular weight of CS. Transfection was maximized for a medium pH of approximately 6.8, an N/P ratio of 5, plasmid concentration of 4 μg/ml, and a CS molecular weight of 50 kDa. The transfection efficiency of HA/CS-plasmid nanoparticles was significantly higher than that of CS-plasmid nanoparticles under the same conditions. The average cell viability of HA/CS-plasmid nanoparticles was over 90%. These results suggest that HA/CS-plasmid nanoparticles could be a safe and effective non-viral vector for gene delivery to chondrocytes. Further studies would focus on evaluating the *in vivo* application of these novel HA/CS-plasmid nanoparticles in the treatment of joint diseases such as osteoarthritis.

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