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Chitosan nanoparticles as a dual growth factor delivery system for tissue engineering applications

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ABSTRACT

Growth factors are essential in cellular signaling for migration, proliferation, differentiation and maturation. Sustainable delivery of therapeutic as well as functional proteins is largely required in the pharmacological and regenerative medicine. Here we have prepared chitosan nanoparticles (CNP) and incorporated growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), either individually or in combination, which could ultimately be impregnated into engineered tissue construct. CNP was characterized by Fourier transform infrared (FTIR) spectroscopy, Zeta sizer and high resolution transmission electron microscope (HRTEM). The particles were in the size range of 50–100 nm with round and flat shape. The release kinetics of both EGF and FGF incorporated CNP showed the release of growth factors in a sustained manner. Growth factors incorporated nanoparticles did not show any toxicity against fibroblasts up to 4 mg/ml culture medium. Increased proliferation of fibroblasts *in vitro* evidenced the delivery of growth factors from CNP for cellular signaling. Western blotting results also revealed the poor inflammatory response showing less expression of proinflammatory cytokines such as IL-6 and TNFα in the macrophage cell line J774 A-1.

1. Introduction

Tissue engineering promises the regeneration of injured organs to avoid organ transplantation and its complications and that merges the fields of cell biology, engineering and materials science and surgery to fabricate new functional tissue (Chaignaud et al., 1997). Cell-based tissue engineering has been grown to be the most promising alternative therapy for the treatment of many diseases (Pittenger et al., 1999). Cells communicate each other through molecular interactions or the secretion of hormones or mediators that act locally or systemically to adjust the environment. Growth factors refer to an expanding class of proteins that modulate cell differentiation, migration, adhesion, and gene expression (Rudland and Deasua, 1979; Babensee et al., 2000; Nimni, 1997). Growth factors are natural proteins and, in the purified form, these are heat and pH sensitive and highly prone to dissolution (Logeart-Avramoglou and Jozefonvicz, 1999). Therefore, techniques using delivery systems have been employed in order to protect and control the release of growth factors to efficiently exert their biological effects (Tabata, 2000).

EGF and FGF, the combination of these growth factors, can induce multiple cells and tissues because of its pletrophic effect. Vascularisation of bioengineered tissue is generally a prerequisite for achieving fully functional tissue regeneration. Angiogenic factors for example basic fibroblast growth factor (bFGF) have great potential to induce angiogenesis for biomedical applications (Folkman and Klagsbrun, 1987; Thompson et al., 1988). In addition to promoting neovascularization, bFGF is also involved in physiological processes such as wound healing and morphogenesis by controlling the proliferation, differentiation, and migration of various cell types (Wilkie et al., 1995; Rusnati and Presta, 1996; Broadley et al., 1989; Fernig and Gallagher, 1994). Recombinant epidermal growth factor (rhEGF) is a single-chain polypeptide, which stimulate the proliferation and differentiation of epithelial cells from the skin, cornea, lung, tracheal tissue and gastrointestinal tract. The clinical use of EGF has been extensively investigated in wound healing processes, especially in the treatment of gastric and diabetic ulcers (Na et al., 2006). Moreover, cellular interaction is highly limited by their ability to cross biological barriers and reach the target site. As such, the utility of these molecules as therapeutic agents clearly depends on the design of an appropriate carrier for their delivery to the body.

The strategy of utilizing nanoparticles as a carrier system for cell specific targeting and sustainable delivery of drugs has gained an increased interest (Moghimi et al., 2001; Ramge et al., 2000). The biocompatibility of nanoparticles very closely relates to cell behavior and particularly to cell adhesion to their surface (Hallab et al., 1995). Several studies have been reported in the development of

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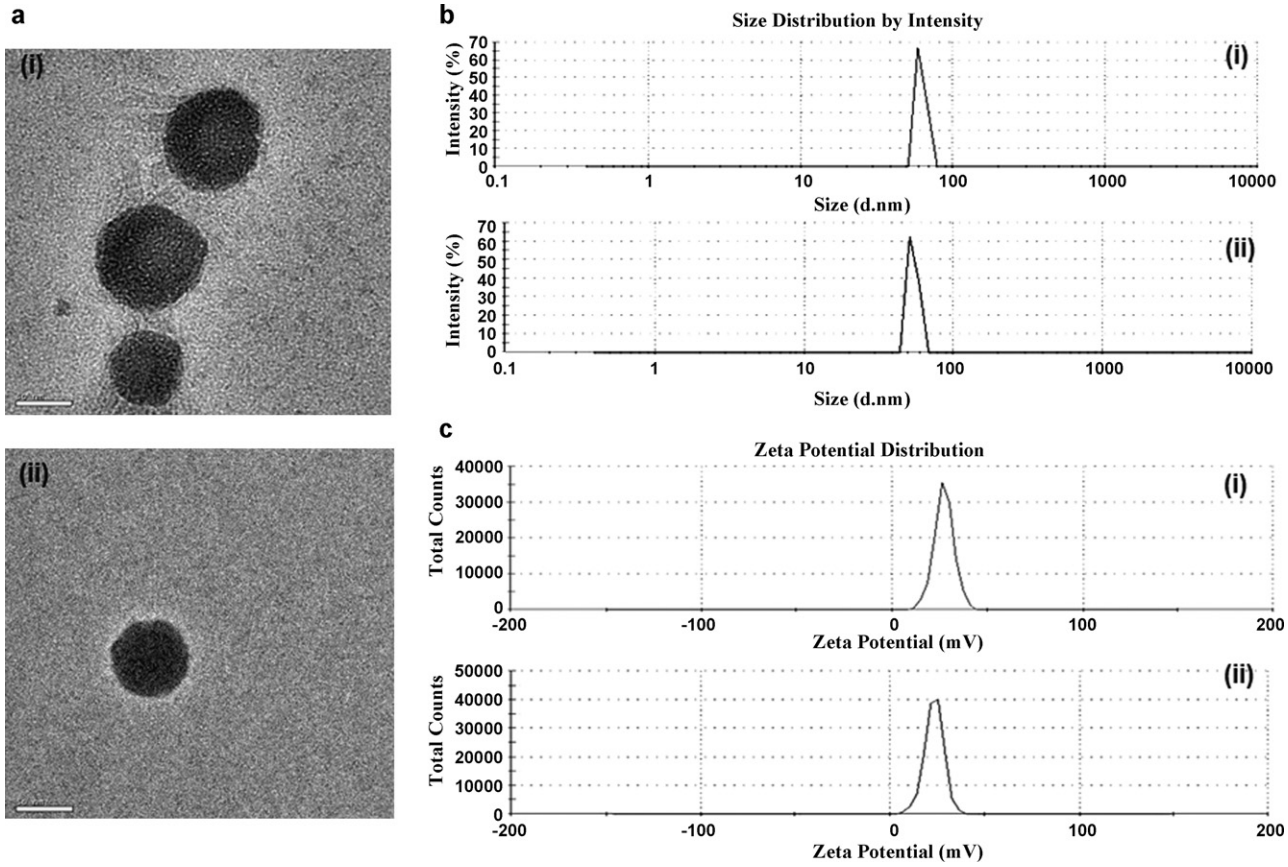

Fig. 1. (a) HRTEM of (i) EGF and (ii) FGF loaded chitosan nanoparticles (scale bar indicates 50 nm). (b) Particle size distribution of (i) EGF-CNP with a mean size of about 61 nm and (ii) FGF-CNP with a mean size of about 53 nm. (c) Zeta potential distribution of (i) EGF-CNP with a mean charge of 27 mV and (ii) FGF-CNP with a mean charge of 23 mV.

the carriers, among which the design of biodegradable nanoparticles has drawn considerable interest. Surface characteristics of materials, such as topography, chemistry or surface energy play an important role in cell adhesion on biomaterials (Rochev et al., 2004). The attachment, adhesion and spreading belong to the first phase of nanoparticle–cell interaction and the quality of this first phase will influence the cell's capacity to proliferate and differentiate itself in presence of the particles (Hallab et al., 1995).

Nanoparticles from biodegradable and biocompatible polymers are ideal candidate to deliver and carry drugs because they are expected to be adsorbed in an intact form in the gastrointestinal tract after oral administration (Florence et al., 1995). Chitosan, deacetylated form of chitin, is natural, nontoxic, highly biocompatible and has been widely used in the development of controlled drug delivery systems including films, sponges, beads, microbeads (microspheres), nanoparticles (Lai et al., 2003; Illum et al., 2001; Ravikumar, 2003) etc. Calvo et al. (1997) first developed the nanoparticles using a tripolyphosphate (TPP) cross-linking method. With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites. The hydrophilic nanoparticles generally have longer circulation in blood (Allemann et al., 1993). Such systems could not only control the rate of drug administration that prolongs the duration of the therapeutic effect but also delivers the drug to specific sites.

The objective of the present study was to develop biodegradable CNP for the incorporation of EGF and FGF and evaluate their potential as delivery system *in vitro*. High resolution transmission electron microscopy (HRTEM) and Fourier transform infrared (FTIR) spectroscopy were used to determine the physical and chemical nature of the particle, while Zeta sizer was used to determine the size distribution. Cytotoxicity and proliferation studies were performed against fibroblast cells and inflammatory reactions were also carried out against macrophage cells.

2. Materials and methods

2.1. Preparation and characterization of EGF and FGF incorporated nanoparticles

CNP were prepared according to the procedure followed by Pan et al. (2002). Single growth factor loaded CNP was formed by incorporation of either FGF (R&D systems, USA) loaded 0.1% TPP (Sigma–Aldrich, USA) to 0.2% chitosan (Sigma–Aldrich, USA) solution in 1% acetic acid (Sigma–Aldrich, USA) or EGF (R&D systems, USA) loaded chitosan to TPP. Dual growth factor loaded CNP were formed spontaneously upon incorporation of TPP (0.1%) containing FGF to 0.2% chitosan solution containing EGF under stirring. Appearance of turbidity was taken as an indicator for the formation of nanoparticles. The solution was then subjected to centrifugation at 20,000 rpm for 20 min to separate the supernatant and nanoparticles. The supernatant was saved to quantify the unbound free growth factor by enzyme-linked immunosorbent assay (ELISA) method using Human EGF and FGF Quantikine ELISA kit (R&D systems, USA) as per manufacturer's instructions. All measurements were carried out in triplicate.

2.2. Physicochemical characterization of CNP

Average particle size and the morphological characteristics of the nanoparticles were examined using HRTEM (JEM 3010, JEOL, USA). The aqueous dispersion of the particles was drop cast onto a carbon copper grid and the grid was air-dried. The sample was

stained with 1% muranyl acetate solution for 5 s at 7 °C before viewing on the HRTEM.

Particle size distribution and zeta potential of EGF and FGF-incorporated CNPs were determined using Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). For particle size analysis, measurements were carried out at 25 °C at the light scattering angle of 90 °C. The surface charge of the nanoparticles was analyzed by Zetasizer (Malvern, UK). All the measurements were performed in triplicate.

FT-IR spectra of raw chitosan, CNP, EGF-CNP and FGF-CNP were obtained using a Perkin-Elmer FT-IR spectrometer (SPECTRUM 1000). Each sample was made into pellet with potassium bromide (Sigma–Aldrich, USA) and scanned between 500 and 4000 cm^{-1} wavelength and the spectrum was recorded.

Differential scanning calorimetry (DSC) was employed to investigate the endothermic peak temperature shift of chitosan and CNP samples. Chitosan and CNP samples weighing about 3 mg each were placed in an aluminum pan. The aluminum pan was pressed to seal it and thus the samples were crushed to ensure complete contact with the aluminum pan. Analysis was performed in differential scanning calorimeter (DSC Q200 V23.10 Build 79 instruments) from 0 °C to 200 °C, at controlled heating rate of 10 °C/min.

Thermogravimetric analysis (TGA) of chitosan and CNP was performed using TGA (Q50 V20.6 Build 31) instruments. About 3 mg of each sample was heated at the rate of 10 °C/min under the atmosphere of nitrogen at temperature range of 30–400 °C.

2.3. Encapsulation and release studies

Release of EGF and FGF from growth factor incorporated CNP samples was determined by resuspending 1 mg of either EGF-CNP or FGF-CNP in 1 ml of phosphate buffered saline (PBS) solution and incubated at 37 °C under slight agitation. At appropriate time intervals, the samples were subjected to centrifugation and the supernatant was replayed by fresh PBS. The amount of EGF or FGF released into the medium was determined by using an ELISA kit (R&D systems, USA). The growth factor-loading efficiency of nanoparticles and their entrapment efficiency were calculated from the following equations (Cetin et al., 2007).

$$\text{Loading efficiency } (\%) = \frac{\text{total growth factor} - \text{free growth factor}}{\text{weight of nanoparticles}} \times 100$$

$$\text{Entrapment efficiency } (\%) = \frac{\text{total growth factor} - \text{free growth factor}}{\text{total growth factor}} \times 100$$

2.4. Cell culture

Murine NIH 3T3 fibroblast cells and murine J774 A-1 macrophage cells were obtained from NCCS (Pune, India) and cultured in Dulbecco's modified Eagles's medium (DMEM) (Sigma–Aldrich, USA) supplemented with 10% foetal bovine serum (FBS) (Invitrogen Corp., USA) penicillin (100 IU/ml), streptomycin (100 μg/ml) (Sigma–Aldrich, USA). Cells were maintained at 37 °C in a 5% CO_2 incubator. The whole adherent cells were detached by trypsinization 0.25% trypsin- ethylene diamine tetra acetic acid (EDTA) solution (Sigma–Aldrich, USA) after reaching 90% confluence.

2.4.1. In vitro cytotoxicity and cell compatibility studies

The biocompatibility of CNP with mouse 3T3 fibroblast cell line was tested using the methylthiazol tetrazolium (MTT), (Sigma–Aldrich, USA) assay. 3T3 fibroblast cells were cultured in 24-well plates (Thermanox™, Nunc, USA) at an initial seeding density of 5×10^4 cells/well. The growth medium was removed after 24 h and replaced with fresh medium. Then, CNP at different concentrations such as 2, 4 and 6 mg/ml was added and incubated at 37 ° C for 3 days. After 3 days, the growth medium was removed and replaced with 900 μl DMEM, 100 μl MTT solution (1 mg/ml). After incubation for 4 h at 37 °C, the upper medium was removed carefully and the intracellular formazan was dissolved by adding acidified isopropanol (Sigma–Aldrich, USA). The absorbance of the resultant solution was measured at 570 nm using the spectrophotometer. Experiments were run in triplicate per sample. The cells inoculated without CNP was taken regarded as control. All data were expressed as the mean ± standard deviation (SD) for $n = 3$.

After optimization of CNP concentration, the cells were treated with growth factors- incorporated CNP and allowed to grow in culture medium and analyzed by MTT on days 2, 4, 6 and 8 to monitor the proliferation. The percentage of cell viability was illustrated as the ratio of absorbance of nanoparticle-treated cells relative to those untreated.

2.4.2. Fluorescent microscopy by acridine orange/ethidium bromide (AO/EtBr) staining

Cell viability was studied morphologically using a fluorescent dye that intercalates DNA. AO stains DNA bright green, allowing visualization of the nuclear chromatin pattern. EtBr stains DNA orange, but is excluded by viable cells (Vento et al., 1998). Cells (5×10^4) were stained with 1.5 ml of AO/EtBr solution [AO in PBS (100 mg/ml)/EtBr in PBS (100 mg/ml) = 1:1, v/v], mixed gently and then examined through fluorescence microscope (AHPT-514, Olympus). (For interpretation of the references to colour in this text, the reader is referred to the web version of the article.)

2.5. Cell viability and proinflammatory cytokines analysis

Cell viability was evaluated on macrophage cell line J774 A-1 by fluorescent microscopy using AO/EtBr staining as described above. The cell culture supernatant was collected at 48 h and protein content in the samples was estimated by Lowry's method. Samples with matched protein concentration (20 μg) were mixed with Laemmeli's reducing buffer and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel by electorophoresis (SDS–PAGE). Protein were then transferred to nitrocellulose membrane (Hybond ECL, Pharmacia Biotech, Amersham) for 1 h and the membrane was incubated with primary antibody for IL6 and TNFα (1:200 dilution) (Santa Cruz Biotechnology, USA) individually at 37 °C for 4 h. After incubation, blots were washed with tris buffered saline (TBS) containing 0.5% Tween 20 and then incubated with alkaline phosphatase-conjugated secondary antibodies (1:1000 dilution) for 1 h and then detected by 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma–Aldrich, USA) substrate system.

2.6. Data analysis

The experimental results were expressed as mean ± SD. Analysis of variance (ANOVA) was performed by one-way ANOVA procedures (SSPS 11.5 for Windows). Significant differences between means were determined by Duncan post hoc test. $p < 0.05$ implies statistically significance.

3. Results

3.1. Physicochemical characteristics of growth factor (EGF and FGF) incorporated CNP

CNP was prepared by ionic gelation method using TPP. The formation of CNP is based on an ionic interaction between positively charged chitosan and negatively charged TPP. CNP prepared in this experiment exhibited a white powdered form and were

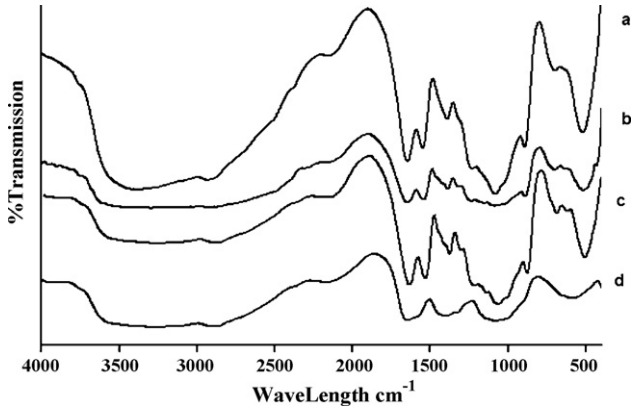

Fig. 2. FTIR spectra of (a) chitosan, (b) CNP, (c) EGF-CNP and (d) FGF-CNP.

insoluble in water. The morphological characteristics of growth factor-incorporated CNP were examined using HRTEM (Fig. 1a and b) and it revealed that both EGF-CNP and FGF-CNP have a similar morphology of spherical shape and a size range between 50 and 100 nm. The particle size distribution profile for EGF-CNP showed narrow size distribution with mean diameter of 61 nm (Fig. 1c) and for FGF-CNP, it was 53 nm as shown in Fig. 1d. The particle size analyzer showed slightly higher value. These could be due to the swelling of the particle. The zeta potential of the particles is also showed in Fig. 1e and f. EGF-CNP and FGF-CNPs have positive charges of about 27 mV and 23 mV respectively. The incorporation of growth factors did not alter the surface charge.

3.2. FT-IR spectrum of CNP

FTIR spectroscopic analysis of chitosan, CNP, EGF-CNP and FGF-CNP was performed to study the chemical structure of the particles. Fig. 2a shows characteristic peaks of chitosan at 3439 cm^{-1}, which correspond to the peak of the $-NH_2$ and –OH groups stretching vibration, and a peak at 1657 cm^{-1} is attributed to the $-CONH_2$ group, and a sharp peak of $-NH_2$ bending vibration at 1568 cm^{-1}. In Fig. 2b, a shift from 3439 to 3467 cm^{-1} in the CNP indicated that the hydrogen bonding is enhanced. And two absorption bands at 1642 and 1547 cm^{-1} appeared to show that the ammonium groups are cross-linked with TPP molecule. Ammonium groups of chitosan interact with polyphosphoric group of sodium polyphosphate to enhance both inter- and intramolecular interactions in CNP. The peak at 3439 cm^{-1} became slightly wider in both EGF-CNPs and FGF-CNPs (Fig. 2c and d) and it is due to the increase in the number of hydrogen bonding contributed by the growth factors. This indicated that the EGF and FGF are also linked with CNP through the electrostatic interaction.

3.3. Thermodynamic properties of CNP

The glass transition temperature (*Tg*) of the nanoparticles was measured by differential scanning calorimetry (DSC). The values were taken from heating the sample at the rate of 10 °C/min from room temperature to 200 °C. Fig. 3a shows the endothermic peaks for chitosan and CNP at 92 °C, and 95 °C respectively. CNP showed an increase in the melting temperature, compared to its parent polymer compound. TGA of chitosan and CNP were investigated to determine the pyrolytic changes in weight in relation to change in temperature and the data are given in Fig. 3b. It showed that the chitosan dehydrates its absorbed as well as bound water up to a temperature of about 265 °C and losing 9% of its total weight. Followed by this, a sudden weight loss was observed in the temperature range of 265–425 °C which was computed to be 57%, whereas CNP showed a weight loss of about 10% until 230 °C which was followed by a sudden drop in weight at the temperature range between 230 °C and 350 °C and the loss of which was accounted to about 40%.

3.4. In vitro EGF/FGF release kinetics

The loading amount and loading efficiency of EGF or FGF in CNP were found to be about 30 ng ± 2.5/mg of CNP and 91 ± 1.5% respectively. Release of EGF and FGF from CNP was determined *in vitro* by incubating CNP in PBS (pH 7.4) at 37 °C for 35 days. The release rate was monitored on alternative days. Fig. 4a and b depicts the release of EGF and FGF in a sustained manner without any burst release in the initial stage. The cumulative release of EGF and FGF were 13% and 11% respectively on the 2nd day and 56% and 54% respectively on 12th day. A cumulative release of EGF and FGF was 83.1% and 84% respectively on day 35.

3.5. In vitro fibroblast viability and proliferation studies

The viability of fibroblasts was measured by MTT assay after culturing for 3 days and the data are presented in Fig. 5. As it is evident from Fig. 5a, cells incubated with CNP were nearly 100% viable up to 4 mg/ml when compared to the control group. However, the concentration increased to 6 mg/ml, the toxicity was observed. Fig. 5b shows the cytocompatibility of CNP, EGF-CNP, FGF-CNP and EGF + FGF-CNP on 2, 4, 6, 8 days post incubation. The cell proliferation on the CNP increased with the increase in time interval which was observed through an increase in the optical density at 570 nm. However, single growth factor incorporated nanoparticles exhibited better cell growth compared to it control and CNP alone. Similarly, CNP incorporated dual growth factors was observed to be more compatible and promoted cell proliferation than the nanoparticle with single growth factor. Cell viability against CNP and its growth factor combination was analyzed using AO/EtBr staining (Fig. 5c). The fibroblast cells in the absence or presence of CNP analyzed using AO staining showed a least number of cell death compared to the total cell population (Fig. 5c1 and 2). It is observed from the result that the proliferation of fibroblasts in the presence of CNP was better than its control. Likewise, the CNP containing EGF or FGF and their combination were also evaluated for their effect on cell viability and proliferation. Among these three groups, the dual growth factor incorporated CNP exhibited high proliferation compared to its single growth factor containing CNP as was also observed comparatively in MTT assay.

3.6. In vitro evaluation of proinflammatory effects of CNP in macrophage

Macrophage cells play key roles in mediating host tissue response to implants in the foreign body reaction, hence the viability of macrophage cells exposed to growth factor-incorporated CNP was confirmed by using AO/EtBr staining, and the results are shown in Fig. 6a. The cells proliferated very well on the nanoparticles of all types, and only few dead cells were observed. Cellular activation on each sample was analyzed based on the amount of cytokines and chemokines produced by the adherent cells. Expression of TNFα and IL-6 by the macrophage cells under different experimental conditions is shown in Fig. 6b and c. Cytokines like TNFα and IL-6 are released when the foreign material comes into contact with the macrophages which were detected by western blotting. The expressions of these markers were low in the CNP. There was no visible difference in the expression of inflammatory markers between the plain CNP and growth factors-incorporated CNP indicating the negligible effect of growth factors in the activation of inflammation.

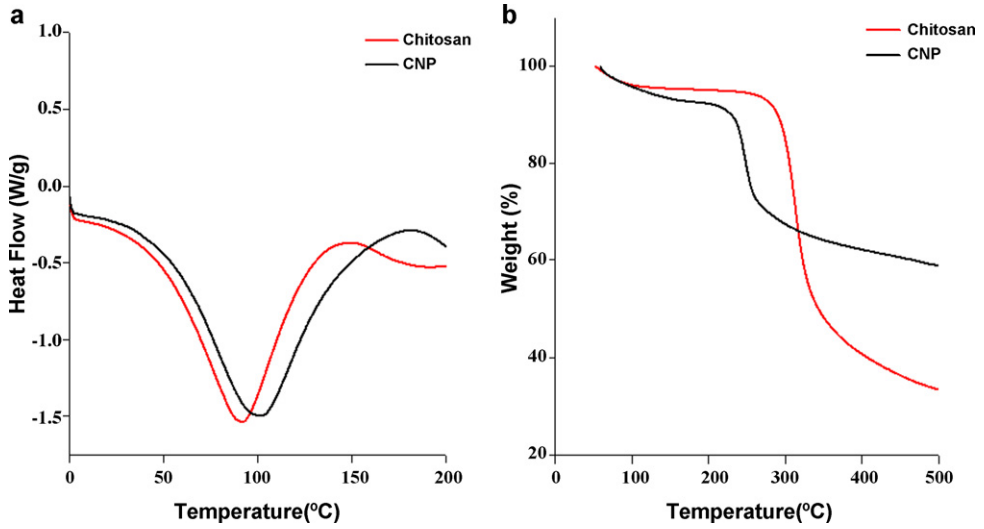

Fig. 3. (a) Differential scanning calorimetric profiles of chitosan and CNP. (b) Thermogravimetric profiles of chitosan and CNP.

4. Discussion

Our hypothesis was that an appropriate combination of different selective growth factors could increase the versatility of the resulting system in terms of the ability to interact with biological surfaces and cell membranes. In this study, CNP was synthesized with and without incorporation of growth factors and studied for morphology, surface charge, growth factor entrapment and release property, *in vitro* proliferation and inflammation response. In the recent past, there has been considerable interest in developing biodegradable nanoparticles as effective drug delivery devices (Panyam and Labhasetwar, 2003; Lamprecht et al., 2001). The advantages of using nanoparticles for drug delivery result from their two main basic properties. First, because of their small size, they can penetrate through smaller capillaries and are taken up by cells, which allow efficient drug accumulation at the target sites (Panyam et al., 2003; Thomas and Klibanov, 2003). Due to its cationic nature and controlled degradation rate, chitosan based materials with growth factors have occupied a reasonable place in drug delivery as well as regenerative medicine (Lee et al., 2000, 2002).

In our study, we explored the use of biocompatible CNP, as a vehicle to carry growth factors and subsequently deliver them to fibroblast *in vitro*. The size (50–100 nm) of the growth factor-incorporated CNP as examined by using HRTEM was comparable to that of the size measured by photon correlation spectroscopy using the Zetasizer. It suggested that particles should be 100 nm or less in diameter for longer circulation time and for the possibility of clearance by macrophages (Brannon-Peppas and Blanchette, 2004). The average size of the nanoparticles was around 61 nm for EGF-CNP and 53 nm for FGF-CNP respectively. Hence the CNP prepared in this investigation is considered suitable for longer circulation to accomplish the sustainable delivery of growth factors. The EGF-CNP and FGF-CNP with respective zeta potential of 27 mV and 23 mV can greatly influence their stability in suspension through electrostatic repulsion between the particles. A higher positive surface charge of CNP obtained in

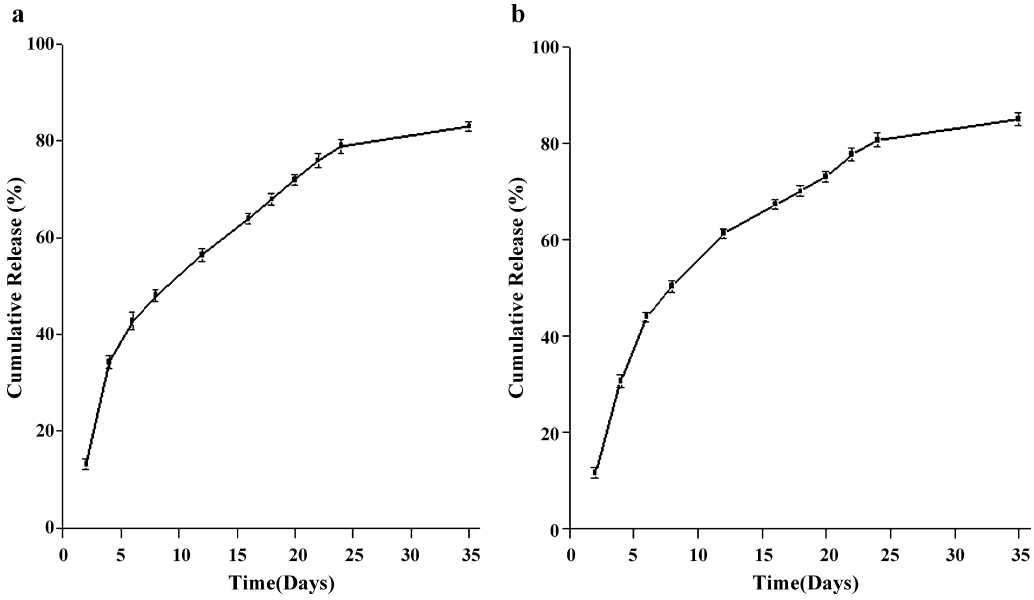

Fig. 4. Cumulative release profile of (a) EGF and (b) FGF from CNP in PBS, pH 7.4 at 37 °C. Each data point represents a mean ± standard deviation ($n = 3$).

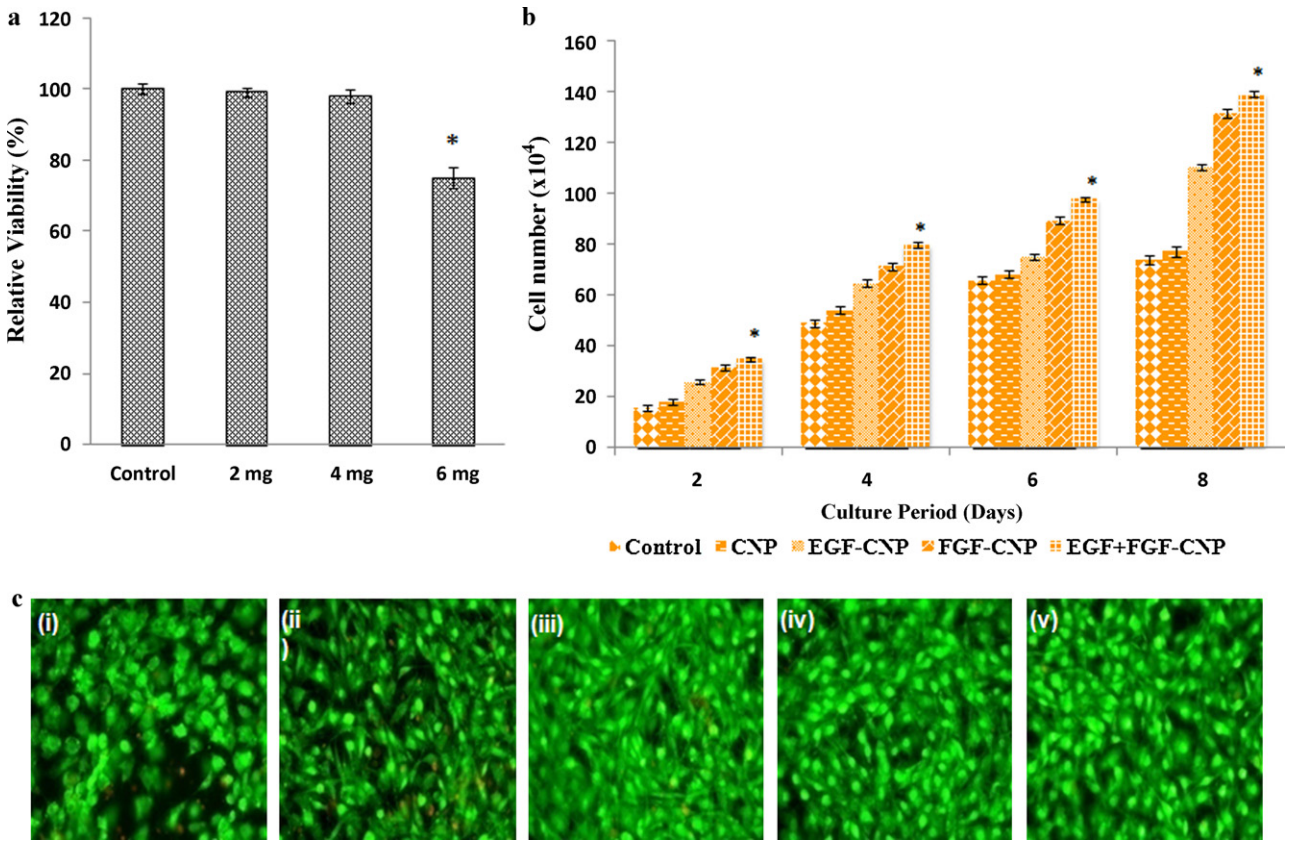

Fig. 5. Cytotoxicity of CNP in fibroblast cells: (a) the absence (control) or presence of different concentrations of CNP: 2 mg/ml, 4 mg/ml, 6 mg/ml for 3 days; error bar represent the mean ± SD for $n = 3$. $^*P < 0.01$ compared with control. (b) Proliferation of fibroblast cells on CNP with or without growth factors at different time intervals (control; CNP; EGF-CNP; FGF-CNP; and EGF + FGF-CNP). Each data point represents a mean ± standard deviation ($n = 3$). $^*P < 0.01$ compared with control. (c) Morphology of fibroblasts cultured on CNP with or without growth factors: (i) control, (ii) CNP, (iii) EGF-CNP, (iv) FGF-CNP and (v) EGF + FGF-CNP on day 11, visualized by fluorescent microscope. Original magnification: 200 ×.

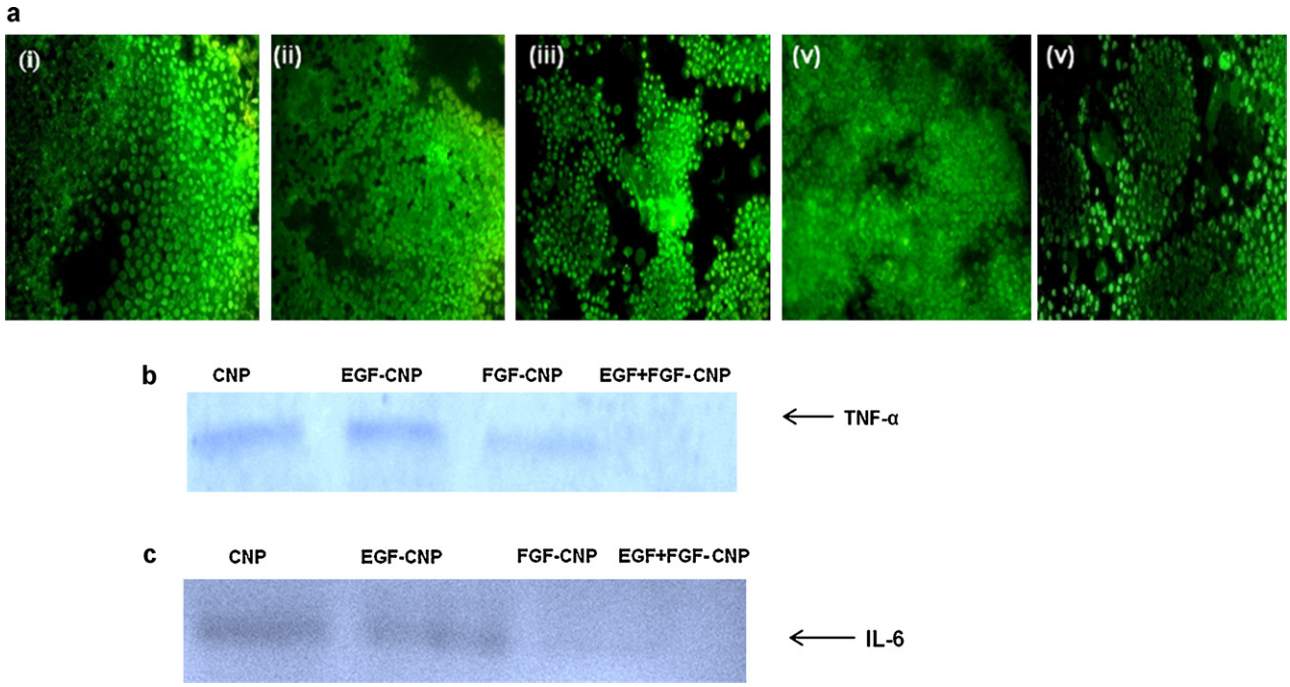

Fig. 6. (a) Morphology of macrophages cell line J774 A-1 cultured on CNP with or without growth factors: (i) control, (ii) CNP, (iii) EGF-CNP, (iv) FGF-CNP and (v) EGF + FGF-CNP on day 11 visualized by fluorescent microscope. Original magnification: 100 ×. Western blotting analysis of (b) TNFα and (c) IL-6 expression in macrophage cell line.

our study can increase interactions with the negatively charged cell membrane and a smaller particle diameter may increase probable entry into the cell resulting in enhanced drug delivery.

FTIR spectrum was performed to characterize the chemical structure of chitosan and nanoparticles. The free amino group seen at 1568 cm^{-1} in chitosan becomes ammonium ion, and hence new bands appeared at 1642 and 1547 cm^{-1} in the CNP. In the case of growth factors-incorporated CNP, EGF and FGF contributed to extra hydrogen bonding which would have made the nanocomposites more rigid and thus was stable for at least 35 days in PBS medium (pH 7.4) and was able to deliver the growth factors in a sustainable manner. Therefore the CNP, compared to chitosan as such, is more stable at higher temperature and thus shows slightly increased melting temperature (+3 °C) in DSC. The single endothermic peak obtained for chitosan or the CNP denote that the samples are pure and homogeneous but varies in their *Tg* for the reasons that they differ in the type- and degree- of interactions between the active group of molecules. The pyrolytic pattern of the biopolymer and its CNP, though showed similarity, varied in the moisture content and degradation temperature and the level of organic matter. However, they seem to be stable up to 100 °C after which they release water. The single step pyrolysis also confirms the purity of the organic matter present in the biopolymer. Thus the chemistry and its thermal property of the CNP support its use in drug delivery and tissue regenerative applications.

Nanoparticles provide a novel system to increase the persistence of growth factor in the circulation and to deliver it at the target site for prolonged period. The loading efficiency of >90% of EGF/FGF in CNP was obtained by trapping of growth factors by the chitosan along with interaction with TPP. This characteristic may be beneficial to the retention or accumulation of biologically active molecules for their sustained release from chitosan matrix. The growth factor release kinetics from CNP depends on the rate of water uptake, drug dissolution/diffusion rate and the nanoparticle degradation rate. The release profiles of both EGF-CNP and FGF-CNP illustrated in Fig. 4 clearly showed the sustained release of growth factors where approximately 50% of EGF and FGF was released from CNP on day 12. Followed by this, a constant slow release observed up to 35 days confirmed the typical sustained and prolonged drug release which depends on drug diffusion and matrix erosion mechanisms (Musumeci et al., 2006).

CNP prepared in this study was tested for its toxicity against fibroblast cells. Based on the results the toxicity of the nanoparticles was low and hence no decrease in cell viability was observed up to 4 mg/ml. Instead, the cells were healthy and maintained their morphology and adhesion capacity. It is known that cell adhesion is mediated by the interaction of surface proteins such as integrins with proteins in the extracellular matrix or on the surface of other cells or particles such as CNP (Absolom et al., 1987; Haas and Plow, 1994). Growth factor-incorporated CNP viz. EGF-CNP, FGF-CNP and EGF + FGF-CNP influenced the cell's capacity to proliferate and differentiate itself followed by better attachment and adhesion. The dual growth factor (EGF, FGF) incorporated CNP had a maximum cell proliferation compared to other groups. This was due to continuous release of both growth factors from CNP that synergistically stimulate the genes responsible for cellular functions. A large number of studies indicated that cells tend to attach onto hydrophilic surfaces than hydrophobic surfaces (Altankov et al., 1996; Chen et al., 2003; Wei et al., 2007).

Since CNP or the growth factor-incorporated CNP is hydrophilic/charged in their surfaces, these could have activated cellular adhesion and, thereby promoted the proliferation of macrophages. It is known that material surface chemistries are capable of modulating surface-adherent macrophage activation as measured by cytokine, chemokine, and matrix protein production. The cytokines such as TNFα and IL-6 are known to be two of the most important signaling molecules involved in conducting responses to foreign materials and their upregulation is considered to be an accurate measure of inflammation (Goodman, 1996; John et al., 2002). The absence of expression of TNFα and IL-6 cytokines in the presence of dual growth factor incorporated CNP confirm the high degree of compatibility of the system as a biomaterial for tissue regeneration. Among the EGF-CNP and FGF-CNP system, the latter was considered more compatible than the former as it exhibited suppressed expression of proinflammatory cytokines from the macrophages. These findings suggested that biomaterial surface chemistry plays stronger role in macrophage activation.

5. Conclusion

CNP incorporated with and without EGF/FGF or the combination of these growth factors was prepared for sustained release of growth factors. The *in vitro* study demonstrated the efficiency of CNP to deliver dual growth factors which could be successfully used for tissue engineering and drug delivery applications. Cell culture studies prove that the CNP has no toxicity and exhibit good biocompatibility. The attachment and growth of fibroblast in EGF + FGF-CNP system were greatly improved, while the inflammatory induction was not found compared with other groups. This study suggested that the feasibility of incorporation of combinative dual growth factors and their controlled release are considered significant for tissue engineering application. *In vivo* evaluation is to be carried out to prove this *in vitro* result for further development or fool proof concept.

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