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IGF-1 Release Kinetics from Chitosan Microparticles Fabricated Using Environmentally Benign Conditions

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Abstract

The main objective of this study is to maximize growth factor encapsulation efficiency into microparticles. The novelty of this study is to maximize the encapsulated growth factors into microparticles by minimizing the use of organic solvents and using relatively low temperatures. The microparticles were fabricated using chitosan biopolymer as a base polymer and cross-linked with tripolyphosphate (TPP). Insulin like-growth factor-1 (IGF-1) was encapsulated into microparticles to study release kinetics and bioactivity. In order to authenticate the harms of using organic solvents like hexane and acetone during microparticle preparation, IGF-1 encapsulated microparticles prepared by the emulsification and coacervation methods were compared. The microparticles fabricated by emulsification method have shown a significant decrease ($p < 0.05$) in IGF-1 encapsulation efficiency, and cumulative release during the two-week period. The biocompatibility of chitosan microparticles and the bioactivity of the released IGF-1 were determined *in vitro* by live/dead viability assay. The mineralization data observed with Von Kossa assay, was supported by mRNA expression levels of osterix and runx2, which are transcription factors necessary for osteoblasts differentiation. Real time RT-PCR data showed an increased expression of runx 2 and a decreased expression of osterix over time, indicating differentiating osteoblasts. Chitosan microparticles prepared in optimum environmental conditions are a promising controlled delivery system for cells to attach, proliferate, differentiate and mineralize, thereby acting as a suitable bone repairing material.

Keywords

IGF-1; chitosan; microparticles; controlled release; *in vitro*; degradation

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1. Introduction

Chitosan is widely being investigated for use in bone tissue engineering applications. From the orthopedic point of view, chitosan can be fabricated in many forms including films, rods, porous foams, and gels, which makes its usage flexible with respect to the type and size of defect it is used to heal in bone repair [1-3]. Chitosan is an aminopolysaccharide obtained from the alkaline N-deacetylation of natural biopolymer chitin that is found in crab shells, lobsters, and shrimps. Several techniques have been employed to prepare particles, such as emulsification, ionic gelation, spray drying and so on [4]. These particles can be obtained by both physical and chemical cross-linking. Since chitosan is a cationic molecule, it can be physically cross-linked with negatively charged multivalent ions such as tripolyphosphate (TPP). This is advantageous as the cross-linking is reversible and may largely avoid use of toxic chemicals.

Chitosan microparticles are also being considered as a good platform for controlled release of growth factors by the virtue of their loading capacity, good adsorption potential and long shelf life. There are mainly two ways by which proteins can be encapsulated, first by interspersing them in the matrix and second by immobilizing them by electrostatic or chemical bonding [5]. But depending on the growth factor and their mechanism of interaction with cells, either sustained release or immobilization of the growth factor can be beneficiary [6,7].

Controlled release of growth factors is also essential for its gradient formation, which helps in providing a mechanism by which cells can obtain spatial and directional cues. *In vivo*, as the growth factors are released from the source, it diffuses, degrades or binds to extracellular matrix forming local gradients. Previous studies have found that insulin like-growth factor 1 (IGF-I) plays an important role in stimulating chemotactic movement of osteoblasts. *In vivo* mechanism indicates that IGF-1 entrapped in bone matrix is released during resorption process that induces migration of osteoblasts to the repair site [8]. Specifically, linear growth of the bone in the epiphyseal growth plate is regulated by IGF-1 [9,10]. Studies have also shown that the narrowing of the growth plate that occurs with age is affiliated to the decreased local production and circulating levels of IGF-1 [11,12]. Several studies support the hypothesis that certain threshold circulating levels of IGF-1 is necessary to attain peak bone mass density and strength [13]. Generally, *in vivo* IGFs exist in bound state forming a complex extending the half-life ($t_{1/2}$) of the circulating IGF-1 [14,15]. Therefore, when injecting IGFs into the body, it becomes necessary to encapsulate and inject them to maintain their endocrine activity [16]. Critical factors that need to be considered in the successful use of growth factors using microparticles are the optimum dosage, exposure period, and release kinetics.

From the literature search, we understood that even though there are many techniques by which chitosan microparticles have been prepared, care has not been taken to protect the growth factor being encapsulated to improve its encapsulation efficiency and bioactivity. Also, there are many factors which environmental preparation conditions that influence microparticle morphology and release kinetics. Thus, the main objective of this study is to prepare chitosan microparticles in environmentally favorable conditions for delivery of

biologically active growth factors and to study their influence on pre-osteoblast cells. First, in-order to prove the hypothesis that various organic solvents being used during microparticles fabrication on effect the stability of growth factor IGF-1, microparticles were fabricated by the emulsification and coacervation method and their release studies were compared. Later, the coacervation particles were studied particularly to observe the effect of various formulation and environmental parameters in order to optimize the controlled release conditions. This study was carried out for two weeks and the time period was based on a study which revealed that mRNA coding for IGF-1 increased 10-15 fold on day 8 after tibial fracture in rats [17]. And finally, to test the bioactivity of the IGF-I being released and the biocompatibility of the chitosan microparticles formulated, live/dead cell assay was performed using OB-6 cell line. Gene expression study was performed using real time reverse transcription-polymerase chain reaction (RT-PCR) and finally the mineralization study was done using von kossa assay.

2. Materials and Methods

2.1. Materials

Chitosan (85% deacetylated, medium molecular weight), sodium tripolyphosphate (TPP), acetic acid, phosphate buffered saline (PBS) and silver nitrate were purchased from Sigma-Aldrich (USA). IGF-1 was purchased from Invitrogen (USA), IGF-1 ELISA kit (DY291) was supplied by R&D systems (USA), and LIVE/DEAD cytotoxicity/viability assay (Invitrogen, USA).

2.2. Fabrication of chitosan microparticles by emulsification method and coacervation technique

Microparticles were prepared by emulsification and coacervation method. Emulsification procedure followed was similar to the method described by Jayasuriya et al. [18], where 2% chitosan solution was mixed with equal volume of acetone. This mixture was then added drop wise into 500 ml of cottonseed oil, which was mixed with 4 ml of span. The oil suspension was stirred at a speed of 900 rpm for 14 h at 37°C. 64% (w/w) TPP was added to the reaction mixture and four hours after the addition of TPP, chitosan microparticles were purified using hexane followed by vacuum filtration and air-drying.

In the coacervation method, 2% chitosan was prepared by dissolving in acetic acid (1% v/v) at room temperature. The mixture was passed through a nylon mesh to remove insoluble substances. This mixture was then added drop wise to the TPP solution. The microparticles were allowed to cross-link overnight and then were air-dried. For optimizing the microparticles preparation, various parameters were varied including the amount of TPP, temperature at which the microparticles were being cross-linked and needle size. IGF-1 was dissolved in water and was added to the chitosan solution to encapsulate the growth factor.

2.3 Characterization of chitosan microparticles

The surface morphology and particles size was analyzed using the scanning electron microscopy (SEM) Hitachi S-4800, Hitachi Ltd., Japan. The samples were prepared by fixing the microparticles using the double sided tape and sputter coating with gold before

analysis. The SEM operated at 15 kV voltage and 10 μ A current. Size distribution data was obtained from measuring diameter of particles obtained using SEM images (n=12), with each image having a minimum of 10 particles.

2.4 Degradation study

The optimized microparticles were also tested for degradation *in vitro*. In order to do so, the microparticles were suspended in PBS of pH 7.4 and 5.1 for a period of 30 weeks. Lysozyme is added to PBS at a concentration of 10mg/L and the samples were incubated at 37°C under continuous shaking at 50 rpm. Lysozyme is usually one of the enzymes involved in degradation of chitosan *in vivo*. The averaged pH changes for microparticles immersed in PBS solutions of pH 7.4 and 5.1 were plotted for 30 weeks.

2.5 Determination of IGF-1 encapsulated in the microparticles

Microparticles (20 mg) containing IGF-1 were dissolved in 2 ml of 1% acetic acid and kept in incubator at 37°C for 20-24 h. The microparticles dissolved completely releasing the encapsulated IGF-1, which was quantified using ELISA according to manufacturer's protocol. Triplicates of each sample were analyzed. Encapsulation efficiency (EE) was calculated as follows:

$$EE = \frac{\text{Actual amount of IGF-1 encapsulated into the microparticles}}{\text{Total amount of IGF-1 used for encapsulation of microparticles}} * 100\%$$

2.6 In vitro release study of IGF-1

IGF-1 encapsulated microparticles (20 mg) were suspended in PBS (pH 7.4) and incubated at 37°C with continuous rotation at 25-50 rpm for a period of two weeks. At predetermined time points, PBS in the vials was collected and replaced with 2 ml of fresh PBS to maintain the *in vitro* conditions. Samples were stored at -20°C until further analysis. The IGF-1 remaining in the microparticles after the study period was determined by dissolving the microparticles in 1% (v/v) acetic acid at 37°C for 24-48 h. To quantify IGF-I being released ELISA was used. Manufacturer's protocol was followed to do the ELISA (R & D Systems). Triplicates of each sample were used to do the assay and the results are expressed as mean + SD. IGF-1 release kinetics was studied by changing the parameters of the experiment: different amounts of IGF-1 were added to determine the optimum amount for efficient encapsulation given the amount of chitosan and TPP percent; microparticles were suspended in PBS solutions of different pH to study its effect on release; different TPP amounts were used to prepare microparticles as it cross-links the chitosan molecules; and the effect of temperature was studied by preparing the IGF-1 encapsulated microparticles at different temperatures.

2.7 Bioactivity of IGF-1 released in vitro from coacervation microparticles

To confirm the bioactivity of the IGF-1 released from chitosan microparticles formed by coacervation method, pre-osteoblasts (OB-6) were used for assays *in vitro*. IGF-1 has been found to play an important role in stimulating osteoblast proliferation and matrix mineralization. Live/dead assay and von kossa assay were performed in order to check the

effect of IGF-1 encapsulated microparticles on proliferation of viable cells and mineralization of osteoblasts respectively. Real time RT-PCR was carried out to specifically determine the expression of certain mineralization markers in osteoblasts.

2.7.1 Osteoblast cell culturing—*In vitro* studies were performed with coacervation microparticles prepared using 2% chitosan and 50% TPP with 0.05% IGF-1 encapsulated (as observed from release data, this amount was found to be optimum). IGF-1 was encapsulated during microparticle preparation. Dr. Lecka Czernik of the Department of Orthopedic Surgery at University of Toledo kindly provided pre-osteoblast OB-6 murine cell line vial, which was used for propagation and our further studies. α -MEM medium supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used for culturing cells at 37°C in humidified atmosphere of 5% CO₂. For all the experiments, microparticles were sterilized under UV light for 45 min. In a 24-well plate, 25 mg microparticles were added to each well and then seeded with a cell suspension containing 10⁵ cells/ml, unless otherwise mentioned. In all the experiments, the microparticles were soaked in the medium for 45 min prior to seeding them with cell suspension. This was done particularly because the particles absorbed some medium, swelled and later settled into the bottom of the well plate. Thus prior to addition of cell suspension, it was ensured that the particles settled to the bottom of the well plate.

2.7.2. Cell attachment and proliferation assay—Attachment of cells to the microparticles was determined using a phase contrast light microscope (FSX 100, Olympus). On day 3, 5, and 7, the culture medium was aspirated; cells were washed twice with 1X PBS and then fixed using 2.5% glutaraldehyde. After fixing, the microparticles were washed thoroughly in water and then cells were observed under light microscope. The viability and proliferation of attached cells on the chitosan microparticles was confirmed by intracellular esterase activity as indicated by the green fluorescence of calcein that had been enzymatically converted from calcein AM. Dead cells were simultaneously recognized by their red fluorescence caused by the binding of nucleic acids to ethidium homodimer 1 that has entered the cells through damaged membranes. Chitosan microparticles without growth factor, cells in medium without growth factor, and medium containing IGF-1 without microparticles were used as controls. Medium was changed every three days. Live/Dead viability/cytotoxicity (Invitrogen, USA) assay was performed according to the manufacturer's protocol on day 5 and day 10 by transferring the microparticles to a new well in order to distinctly see the cells attached to microparticles only.

2.7.3. Determination of mineralization by von kossa staining—Von Kossa staining was carried out to characterize mineralization of differentiated osteoblasts. On day 5 and 10, the cells were washed thrice with 1× PBS solution and then the cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min. Later, the microparticles were washed thoroughly in water and then 2% silver nitrate solution was added and the plate was placed on aluminum foil exposed to light for 10 min after which silver nitrate was removed and the plate was rinsed with water carefully before observation under the bright field microscopy.

2.7.4. Real time RT-PCR analysis—Cells were seeded at 2×10^5 cells/ml and cultured in the presence of microparticles (with and without IGF-1) for 5, 7, and 10 days. Total RNA was isolated using RNeasy Mini Kit (Qiagen, USA) following manufacturer's instructions. The purity and concentration of total cellular RNA was determined using Nanodrop-1000, version 3.6.0. The minimum RNA amount obtained was considered as a base, for calculations to prepare reverse transcribe complementary DNA (cDNA) using the Verso cDNA kit (Thermo Scientific, USA) according to manufacturer's protocols. The forward and reverse primers for the selected genes were designed from Integrated DNA Technologies (IDT, USA) and are listed in Table 1. Expression was quantified using real time RT-PCR analysis with SYBR green master mix kit (Applied Biosystems, USA). Data analysis was carried out using Applied Biosystems StepOne Plus thermal cycler and detection system. The real time RT-PCR analysis was carried out for two independent experiments with each sample run in duplicates. The gene expression levels were normalized to the expression of the housekeeping gene GAPDH and were expressed as fold changes relative to the expression of the genes in cells only on the respective days.

2.8 Statistical analysis

Data are reported as means $\pm$ standard deviation for $n = 3$ unless otherwise stated. One-way analysis of variance (ANOVA) was followed by Tukey's Honest Significant Test (HSD) to determine the statistical significance among means of different groups. Two-way ANOVA analysis was also conducted followed by Tukey's HSD. A probability value of $P < 0.05$ or $P < 0.001$ was used to determine significance, which was specified each time.

3. Results

3.1. Characterization of chitosan microparticles by SEM

3.1.1. Morphology of chitosan microparticles—SEM images of the microparticles fabricated by emulsification method at room temperature showed that they were in the size range of 50-80 μm . Their shape was a little distorted but overall shape was spherical (Fig. 1a). The microparticles prepared by coacervation method were spherical. The size range of these microparticles was between 500-700 μm (Fig. 1b). SEM data has shown (Fig. 1c) that the surface of the microparticles is groovy and rough. SEM images of microparticles prepared by varying parameters did not show much of a difference in shape and size but the microparticles appear to be smoother when prepared at cold temperature when compared to the microparticles prepared at room temperature. The size of the microparticles obtained from one needle size had consistency and thus less variation in size (Fig. 2). This is advantageous as reproducibility of microparticles is necessary, which will result in repeatability of the release behavior.

3.1.2. Degradation of microparticles—The degradation study was conducted to determine the stability of the microparticles *in vitro*. The data shows that there is not much variation observed in the pH of the solution during the 30 weeks period (Fig. 3). There was also not much change in the physical structure of the microparticles immersed in PBS solution of pH 7.4 after 30 weeks, but the particles suspended in PBS solution of pH 5.1

degraded in 15 weeks (Fig. 4). At the lower pH, there was a higher percent of swelling observed when compared to that at pH 7.4, indicating the uptake of solution.

3.2 In vitro release of IGF-1 from microparticles

3.2.1 Comparing release of IGF-1 from emulsification and coacervation microparticles

microparticles—The amount of IGF-1 encapsulated in both types of microparticles was determined by dissolving the IGF-1 encapsulated microparticles in acetic acid and it was found that the amount of IGF-1 encapsulated in emulsification microparticles (70.02 ng) was approximately 50% less than that encapsulated in coacervation microparticles (168.7 ng). This result indicates that though same amount of IGF-1 was added for encapsulation, IGF-1 was not encapsulated in emulsification microparticles, indicating the adverse effect of the organic solvents used during microparticle preparation, which denatures IGF-1. At the end of 14 days, it was found that only 3.31% of the encapsulated proteins were released, whereas for coacervation microparticles, 30.68% of growth factor was released (Fig. 5). This result indicates that there is a 10 times increase in the percent of IGF-1 release, which may either indicate growth factor degradation in emulsification microparticles or slower release of growth factors.

3.2.2. Release of IGF-1 by coacervation microparticles prepared by varying conditions

—In this experiment, only one parameter is varied at a time, keeping the other parameters same. In this experiment, microparticles are prepared at low temperatures by coacervation method, unless otherwise mentioned to maximize encapsulation efficiency.

3.2.2.1 Effect of the amount of IGF-1 used during encapsulation: Direct proportionality is observed between the amount encapsulated and the amount released till 0.05% w/v IGF-1 but then a decrease is observed as the amount encapsulated is increased further to 0.2% w/v (Fig. 6a). The release data showed that there is a significant difference ($P < 0.05$) between the cumulative amount released by day 14 between 0.05% and 0.2% w/v IGF-1 encapsulated microparticles. The encapsulation efficiency (Table 2) of the particles showed that 0.05% w/v IGF-1 particles have greater amount of IGF-1 encapsulated than 0.2% w/v IGF-1 (Table 2).

3.2.2.2 Effect of TPP concentration on IGF-1 release from microparticles: The release profile shows that as the TPP amount increased, the IGF-1 being released from the microparticles decreased (Fig. 6b). In 50% TPP-chitosan microparticles, in comparison with other groups, we could observe maximum release of 75.18% IGF-1 by day 14, but by day 2 itself, we observe that 42.22% of IGF-1 has been released. On the other hand, in case of 60% TPP-chitosan microparticles, we observe that by day 14, only 39.2% of IGF-1 is released, but by day 2, only 14.79% of IGF-1 is released and in case of 80% TPP, it was observed that the cumulative release percent decreased to 15.57%.

3.2.2.3 Effect of pH on release of IGF-1: The release behavior of microparticles studied in PBS solution of different pH showed significant difference in the release ($p < 0.05$) between pH 5.07 and 7.4 and 10.7 on day 14. The result showed that at a lower pH, the release was largest and the release decreased with increase in pH (Fig. 6c). This can be explained by the

indicative difference in the swelling behavior *in vitro*. For pH 5.07, the microparticles swelled significantly in the first 2 h indicating a burst release and later swelling degree did not change greatly, thus indicating a steady release over the time period.

3.2.2.4 Effect of temperature on IGF-1 encapsulation and release: A significant difference ($p < 0.05$) is observed in the release of IGF-1 when microparticles are prepared at room temperature and when prepared at lower temperatures (Fig. 6d). This can be explained by the significant difference observed in the encapsulation efficiency of the growth factor (Table 2).

3.3 Bioactivity of IGF-1 released *in vitro* from coacervation microparticles

3.3.1. Cell attachment and viability—Light microscopy images taken on day 3 (Fig. 7a) showed that the cells attached to the microparticles had round morphology and were just beginning to attach, while on day 5 and day 7, the cells appeared to flat and were spreading well with higher cell-substrate contact area (Fig. 7b, c).

The number of viable cells increased, as observed by the live dead cell assay during the ten-day culture period, except for the control with just the cells, which showed a number of dead cells (cells with red stained nuclei) by day 10, which is probably due to overpopulation of the cells (Fig. 8a). Proliferation of cells on three-dimensional microparticles (with and without IGF-1) increased through the ten-day period, with most number of live cells (cells with green cytoplasm) observed on day 10 (Fig. 8b). Microparticles are three-dimensional and in order to capture all the cells attached on its surface, a number of images beginning from the top of the microparticle need to be taken and thus Fig. 8b shows four such images. There was also a difference seen in the number of viable cells attached to the IGF-1 encapsulated particles than to particles with no growth factor on day 5 and 10 (Fig 8c, 8d), thereby proving that the IGF-1 being released from the microparticles is biologically active and is helping in the proliferation of osteoblasts.

Image J software was used to analyze the % area of microparticle occupied by cells to determine any significant difference in the cell attachment and as it can be seen from Fig 9, a significant increase in cell attachment was found on microparticles with IGF-1 on day 5 and day 10 ($p < 0.05$) in comparison with cells attached to microparticles without IGF-1. This indicates that the IGF-1 being released from the microparticles is biologically active and is beneficial for attachment and proliferation of pre-osteoblasts.

3.3.3 Von Kossa staining—The staining assay indicated that not much of mineralization is observed on day 5 in any of the samples (Fig 10A), but on day 10, all the samples showed signs of mineralization (Fig 10B). Pairwise analysis of the mineralized area in different samples was conducted using SPSS and the results indicated that there is a significant difference ($p = 0.019$) between the mineralization observed in microparticles with and without IGF-1 on day 10 (Fig 11).

3.3.4. Real time RT-PCR analysis—Fig. 12a shows that mRNA level of osterix expression decreases with time from day 5 to day 10. SPSS two-way ANOVA indicates a significant difference ($p < 0.001$) between all the samples on day 5, 7 and 10. Post hoc

Tukey's HSD indicates that the expression of *osx* in cells cultured on microparticles without any growth factor is significantly more ($p < 0.001$) in comparison with cells cultured on without any microparticles, indicating the influence of microparticles on cell differentiation. Also there is a significant increase ($p < 0.001$) in the expression of the gene in microparticles with IGF-1 in comparison with both the normal microparticles and cells only, indicating that IGF-1 being released from the microparticles is biologically active and plays an important role in cell differentiation. Tukey's HSD pairwise comparison between the expressions at different time points also shows that there is a significant decrease ($p < 0.001$) in the expression of the gene with time.

As opposed to osterix gene, *runx 2* gene expression was found to increase with time (Fig. 12b) from day 5 to day 10. Two way ANOVA analysis indicates that there is a significant difference ($p < 0.05$) in the expression of *runx 2* between all the samples at different time points. Tukey's posthoc pairwise comparison indicated that there is a significant increase ($p < 0.05$) in the gene expression for cells cultured on microparticles (with and without growth factor) in comparison with cells only control. There is also a significant increase ($p < 0.001$) in the expression of *runx 2* on cells cultured on microparticles with IGF-1. Tukey's HSD multiple comparison also indicated that there is a significant increase ($p < 0.05$) in the expression of the gene with time.

4. Discussion

Chitosan is being widely investigated as a promising implantable biomaterial with a number of applications in tissue engineering [19]. The most intriguing properties that attract chitosan to researchers are its tissue compatibility, biodegradability and functionality [20]. Diverse efforts have been made to obtain chitosan-TPP cross-linked microparticles to explore its potential as a delivery system. Optimizing the fabrication conditions and the comprehensive properties of these microparticles is still an ongoing important research topic to obtain better bone scaffolds, as these parameters influence the loading and encapsulation efficiency of the growth factors, release profiles as well as govern the interaction of the particles with the biological tissue. Therefore, in this study, we focused on optimizing various parameters that influence microparticle morphology as well as have an effect on release kinetics. The bioactivity of the IGF-1 released was also tested by *in vitro* experiments.

Microparticles prepared at room temperature as well as at low temperatures indicated that the microparticles had a rough surface, which is an important parameter known to influence cell attachment, including bone cells [21]. The particles were also found to possess internally less dense structure, which is advantageous for delivery of proteins as well as play an important role in its degradation [22-24]. The microparticles thus fabricated have very good physical characteristics for protein encapsulation and cell attachment and proliferation, which were proved experimentally.

Lysozyme is an amphoteric enzyme that includes ionizable groups at its active site. Therefore, variation in the pH of the PBS solution may result in changes in the ionic form of the active site, thus affecting its activity. Studies have also found that its activity also depends on the substrate on which it is acting (in our case, chitosan microparticles). The

maximum activity of native lysozyme was observed at pH 4.5, but depending on the substrate, its activity was found to be optimum between pH 4-7 [25]. Previous studies also indicate the fact that the substrate specificities of lysozyme in degradation of soluble chitosan are independent of pH between 4.5 and 7. Lower pH 5.1 was considered for this study to stimulate an environment induced by macrophages accumulating around the polymer *in vivo* after implantation. Degradation study indicates that in the presence of lysozyme and at lower pH 5.1, microparticles degrade faster in comparison with that at higher pH 7.4. Lysozyme contains a hexameric-binding site, which is a hexasaccharide sequence that presents 4 or more acetylated units, necessary for the initial degradation of partially N-acetylated chitosan. Apart from the activity of lysozyme on the microparticles, pH also has a direct influence on microparticle degradation. At low pH, the free amine groups in chitosan will be protonated, resulting in high positive charge and also charge number on TPP reduces. This hydrolysis of chitosan-TPP bonds lead to microparticle degradation at a faster rate at low pH [26].

In order to develop a better method to fabricate microparticles for growth factor encapsulation, we compared microparticles prepared using emulsification and coacervation methodology. Emulsification process can be harmful to the stability of the protein due to the presence of multiple organic-aqueous interfaces and also the presence of organic solvents itself can denature number of protein molecules [27,28]. In case of coacervation microparticles, initial burst release was observed till day 1 release 15% of IGF-1 but later, we observed a sustained released till day 14. The release of proteins from these microparticles suspended in PBS solution of pH 7.4 and the degradation study indicates that the release of IGF-1 is mainly due to desorption and diffusion and not by degradation or erosion of microparticles. The proteins adsorbed on the surface of the chitosan particles are first released in the process of desorption and contribute mainly to the burst release phase while the next phase, IGF-1 encapsulated in the microparticle is released mainly by diffusion and minor contribution by degradation process [29-31], together contributing to the controlled release.

Two mechanisms control the encapsulation of IGF-1 in chitosan microparticles. Physical entrapment of IGF-1 in the mesh like structure of the microparticle and the hollow structure inside the microparticle also helps in encapsulating some IGF-1, which flows in during the process of diffusion as the microparticle swells in TPP solution. This indicates that there is a saturation level for the amount of IGF-1 that can be encapsulated in the 700 μm size microparticles. Some of the IGF-1 can escape to the TPP solution where it attains negative charge (isoelectric point = 8.6) [32,33]. Thus partially, IGF-1 can be incorporated due to electrostatic interactions between the negatively charged IGF-1 and positively charged chitosan molecules. There is also ionic interaction between the chitosan molecules and the TPP, which chemically constitute the microparticle. This explains the reason for saturation of positive sites in the molecule and thus a saturation level for the amount of IGF-1 that can be incorporated for a given amount of chitosan-TPP in a microparticle, thereby limiting the amount of IGF-1 that can be encapsulated.

The action of growth factors is concentration dependent as it decides the distance around the tissue that gets influenced by that growth factor. Very little concentration of the drug might

not be sufficient to activate the osteoblast cells while too much of IGF-1 can saturate cell receptors and disrupt cell guidance [34]. Studies have shown that certain concentration of IGF-1 have shown significant increase in bone matrix formation and stimulated bone formation [35] and this concentration was found to coincide with the concentration of IGF-1 being released by day 14 from the microparticles encapsulated with 0.05% w/v IGF-1.

In the experiment to determine the effect of varying amounts of TPP used for cross-linking, we can understand that chitosan and TPP cross-link to form interconnected mesh like network and the density of this network depends on the concentration of TPP and chitosan. The growth factor encapsulated before cross-linking gets distributed in the mesh like network and its movement through this network occurs based on its hydrodynamic diameter. When the hydrodynamic diameter of the growth factor is smaller than the average mesh size, release occurs through diffusion and when the diameter is greater than or equal to the mesh size, release occurs via enzymatic degradation of the chitosan microparticle [5]. The cross-linking density in the microparticle is determined by the concentration of TPP and chitosan, thus higher the concentration of TPP, smaller is the mesh size thus restricting the motion of growth factors through it [36].

In the experiment to determine the effect of pH on growth factor release, we could clearly observe a difference in the release profile at pH 5.1, 7.4 and 10.1. This can be explained from the fact that chitosan-TPP complex has a pI value of 7.5 [37]. According to Henderson-Hasselbalch equation, it can be concluded that at acidic pH, this complex attains a positive charge. Thus chitosan molecules attain an extended conformation at lower pH due to charge repulsion of highly protonated amino groups, which subsequently yield an increase in the mesh size. IGF-1, which is physically entrapped in the microparticle and has a pI of 8.6 is also conveniently released due to its positive charge. At pH 7.4, the chitosan-TPP complex behaves like a zwitterion. Therefore swelling behavior is not observed and IGF-1 is released in sustained manner. At pH 10.7, the complex attains a negative charge and the chitosan molecules become increasingly globular decreasing the mesh size and the entrapped protein could not be released easily [37,38].

The experiment to determine the influence of microparticle preparation temperatures also indicated a difference in the release profile and amounts. This result is in agreement with the fact that the protein integrity is dependent on the temperature [39].

The *in vitro* study is also proves the bioactivity of the released growth factor. Studies have shown that 2D substrates differ from 3D scaffolds in the manner they support cell migration, localization and function [40,41]. Literature also supports that surface topography and dimensions have a regulatory effect on cell behavior. Groovy surface is known to influence osteoblast movement independent of the surface material [42]. Surface roughness modulates the production of growth factors and cytokines, thus influencing proliferation, differentiation and matrix production by osteoblasts [43]. Also the cell binding properties of chitosan may be explained by its polycationic nature, which is obtained by deacetylation because of which the cells that carry a negative charge at physiological pH binds to it [44]. Von kossa assay indicated a significant difference in mineralization observed in IGF-1 containing microparticles in comparison with controls. Previous study has shown that mice lacking

IGF-1R in osteoblasts have increased osteoid thickness but fails to mineralize it, showing that IGF-1 plays an important role in signaling matrix mineralization [45,46]. Previous studies have shown that osterix and runx 2 are transcription factors necessary for osteoblast differentiation [47,48]. When IGF-1 binds to the IGF-1R, it activates two distinct pathways, the phosphatidylinositol 3 kinase (PI3K)/Akt and ERK signaling pathway [49,50]. Both these pathways are responsible for osteogenic differentiation from uncommitted mesenchymal stem cells and for maturation of committed osteoblasts [51]. Activation of Akt signaling pathway induces osteoblast differentiation by expression and activity of several bone specific genes like Runx 2, osterix. Runx 2 and osterix are transcription factors required for osteoblast differentiation [52]. Studies have shown that signaling via Akt can potentiate osteoblast differentiation by cooperation with Runx 2 [53]. Thus increased expression of runx-2 and osterix in the presence of IGF-1 accelerate osteoblast differentiation by regulating factors required for the process. Our lab has been involved in improving mechanical and biological properties of chitosan [54-57], and these results indicate that IGF-1 encapsulated chitosan microparticles improve osteoblasts proliferation and differentiation.

5. Conclusions

In conclusion, although there have been studies with chitosan microparticles, majority of the studies used conditions that are not suitable for the growth factor encapsulation. We prepared particles in ideal conditions, by not using any organic solvents for particle preparations and also employing lower temperatures during microparticle encapsulation, thus ensuring the structural and functional stability of the growth factors. The particles obtained were in the size range of 500-700 μm , balancing the effect of maximum release of encapsulated growth factor and bone filling material requirement. Preparation of microparticles at lower temperatures also ensured increased encapsulation efficiency and release when compared to the release at higher temperatures. The method used is also a cost effective procedure as it avoided the use of chemicals like oil, hexane, acetone and use of other equipment necessary for particles preparation. The cell study proves that the microparticles help in enhancing the proliferation and differentiation ability of the cells. The difference in the IGF-1 incorporated particles and normal particles shows that the IGF-1 being releases in biologically active and helps in the cell multiplication. Thus the prepared microparticles may allow for a wider range of application for *in vivo* bone regeneration and other regeneration of tissue applications.

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Highlights

- Coacervation chitosan microparticles were biocompatible and biodegradable.
- IGF-1 encapsulation efficiency increased with coacervation chitosan microparticles.
- Coacervation chitosan microparticles support osteoblast attachment and differentiation.
- Coacervation chitosan microparticles support osteoblast mineralization.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

a

b

c

d

Figure 8.

Figure 9.

a

b

Figure 10.

Figure 11.

Figure 12.

Table 1

Primers used for real time rt-PCR

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	ACGACAGTCCATGCCATCAC	TCCACCCTGTTGCTGTA
Osterix	AGCGACCACTTGAGCAAACAT	GCGGCTGATTGGCTTCTTCT
RUNX 2	GGGCACAAGTTCTATCTGGAAAA	CGGTGTCACGCGCTGAA

Table 2

Encapsulation efficiency of IGF-1 in coacervation microparticles.

Parameters		Encapsulation efficiency
IGF-1 amount used for encapsulation (w/v %)	0.01%	58.10%
	0.02%	49.8%
	0.05%	19.57%
	0.2%	14.9%
Temperature	below room temperature	59.57%
	room temperature	8.3%
TPP percent	50%	56.97%
	60%	49.86%
	80%	40.09%
pH of PBS solution	5.07	44.5%
	7.4	49.8%
	10.7	47.09%