Development of Injectable Thermogelling Chitosan–Inorganic Phosphate Solutions for Biomedical Applications

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Thermosetting polymers are attractive candidates for biomedical applications as noninvasive therapeutic delivery vehicles. In the present study, the feasibility of developing a neutral physiological temperature setting injectable formulation based on chitosan and an inorganic phosphate salt have been demonstrated. The in situ gelling system was developed by adding different concentrations of ammonium hydrogen phosphate (AHP) to chitosan solution. The resulting solutions have pH in the range of ~7–7.2. The gelling time of the chitosan–AHP solution was determined by incubating the solutions at 37 °C. Depending on the concentrations of AHP added, the gelling time varied from 5 min to 30 h at 37 °C. Addition of various diluents to chitosan–AHP solution did not significantly change the gelling time of the solutions. The gels were found to be cytocompatible as evidenced from in vitro cytocompatibility evaluation using MC3T3-E1 mouse osteoblast like cells. The feasibility of using the gels as a stem cell carrier vehicle as well as a macromolecular delivery vehicle has been demonstrated.

Introduction

Injectable in situ setting hydrogels have tremendous potential as carrier matrices for a wide range of biomedical and pharmaceutical applications such as drug delivery and tissue engineering.[1–3] The advantages of injectable gelling systems over preformed matrices include introduction into the body in a minimally invasive manner, ability to provide a good fit, and delivery of bioactive molecules or cells to the defect site under mild conditions.[1,2] For effective clinical application, injectable systems should be able to gel at mild physiological conditions in a clinically relevant time period. The gel should maintain its integrity for a desired period of time, show good biocompatibility, and biodegrade into nontoxic degradation products.[1,2] Several natural and synthetic polymers have been investigated for developing in situ gelling systems induced by stimuli such as temperature, light, or pH.[4–12] Among these, the thermogelling injectable system is one of the most extensively investigated and highly preferred system for in vivo applications.[12–15]

Chitosan is a biodegradable and biocompatible natural polymer composed of D-glucosamine and *N*-acetyl-D-glucosamine, is attracting a great deal of interest for biomedical applications.[16] Chitosan is derived from chitin, which is the main component of crustacean exoskeleton and is the second most abundant natural polymer.[17] The intrinsic properties of chitosan such as solubility in weak acids, biocompatibility, bioadhesivity, enzymatic degradability, and antibacterial activity makes it one of the most preferred biomaterial for developing drug and cell delivery matrices.[18] The presence of amino groups in chitosan renders it chemically reactive, and several different types of cross-linking agents have been identified to develop chitosan-based hydrogels. These include chemical cross-linking of chitosan using the amino groups of chitosan and various dialdehydes, ionic cross-linking of chitosan using various ions such as citrates and polyphosphates, and formation of polyelectrolyte complexes using anionic polymers.[19–22] Among these, ionic cross-linking is highly preferred for biomedical applications, being a simple and mild process and as it avoids the use of toxic chemical cross-linking agents.

Because of the excellent biocompatibility of chitosan, injectable stimuli sensitive systems based on chitosan has raised significant interest as therapeutic delivery vehicles. Poly(ethylene glycol) (PEG) grafted chitosan having improved aqueous solubility and ability to undergo thermoreversible sol–gel transition near physiological temperature due to the hydrophobic interactions between the polymer chains has been reported as a drug delivery vehicle.[23] Chenite et al. reported the feasibility of developing neutral, thermogelling chitosan solution by the addition of polyol counterionic monohead salts such as β glycerophosphate (βGP).[12] Chitosan–βGP solutions are found to be stable at low temperatures and can transform into a gel at physiological temperature. Because of its unique properties, the thermogelling chitosan–βGP system has raised significant biomedical interest, however, the high concentrations of the bioactive molecule (βGP) required for the gelation process may need to be avoided.[24,25]

The purpose of this study was to evaluate the feasibility of developing a thermal and pH responsive chitosan in situ gelling system using low concentrations of inorganic phosphate salts as the gelling agent and to evaluate the efficacy of the thermogelling system as a cell and or macromolecular delivery matrix.

Materials and Methods

Chitosan from crab shells (minimum 85% deacetylation), ammonium hydrogen phosphate (AHP), tripolyphosphate, β-glycerophosphate (βGP), FITC-dextran, and FITC-albumin were procured from Sigma (St. Louis, MO). Human, bone marrow-derived mesenchymal stem cells (hMSCs) and hMSC basal growth medium (BGM) were obtained from

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Cambrex (East Rutherford, NJ). MC3T3-E1 cells were procured from ATCC. Dulbecco's phosphate buffered saline (PBS) without calcium or magnesium and minimum essential media (MEM) were purchased from Gibco (Carlsbad, CA). All other chemicals used were of reagent or pharmaceutical grade obtained from Sigma.

Preparation of Chitosan Solution. Chitosan was dissolved in 0.5% acetic acid solution (~2.8%) under magnetic stirring for 48 h at room temperature. The resulting solution (pH ~ 5.6) was filtered and stored at 4 °C. The viscosity of the solution was found to be 5300 cps when measured at a shear rate of 4 s^{-1} (Brookfield DV-II + Pro Viscometer).

Preparation of Thermogelling Solution. Chitosan (5 mL) was aliquoted into a glass vial and magnetically stirred in an ice bath. An appropriate amount of ammonium hydrogen phosphate (AHP), either as a powder (0.075 g/5 mL chitosan) or as a solution (60% in water, pH ~ 8.6) was slowly added to the chilled chitosan solution. The pH of the resulting mixture was found to be in the range of 7–7.2.

Gelling Time Determination. Chitosan solution (5 mL, pH ~ 5.6) was reacted with different amounts of AHP (0.06, 0.075, 0.09, 0.12 g or equivalent volume) in an ice bath (~0 °C) with magnetic stirring for 2 min The pHs of the corresponding solutions were found to be ~7.09 ± 0.03, 7.13 ± 0.05, 7.25 ± 0.02, and 7.3 ± 0.04. Test tube inverting method was used to determine the time of sol–gel transition.[23] The chitosan–AHP mixture was incubated in a water bath at 37 °C, and time measurements were started. The flowability of the solution was checked every 30 s for 30 min by tilting the vial. The time at which the solution stopped flowing was taken as the gelling time and the values reported are the average of four determinations ±SD. The gelling time of chitosan–βGP solution was also determined as above.

Rheological Analysis. A controlled-stress rheometer (AR 2000, TA Instruments, Inc.) was used to demonstrate the sol–gel transition property of chitosan–AHP solution at 37 °C by following the viscosity of the solution with time. Briefly, the chilled chitosan–AHP solution containing 0.075 g of AHP was introduced into the rheometer equipped with a parallel plate geometry immediately after preparation. The variation in viscosity was measured as a function of time at 37 °C with a constant oscillation frequency of 1 Hz.

Addition of Diluents. To 5 mL of chitosan in an ice bath was added 0.075 g of AHP with magnetic stirring. The solution was stirred magnetically and different volumes of the diluents were added. The diluents used were water (pH 7.0), phosphate buffered saline (PBS), and minimum essential media (MEM). The gelling time of the resulting solutions were determined using the test tube inversion method.

Cytotoxicity Studies. Cytotoxicity evaluation of the chitosan–AHP gel was performed using MC3T3-E1 cells (mouse osteoblastic cell line). The extent of toxicity was evaluated qualitatively using a direct contact assay and semiquantitatively by following the viability of cells cultured in the extraction media of the gels prepared using different concentrations of AHP.

Briefly, chitosan solution in acetic acid was sterilized by autoclaving and AHP solution by filter sterilization using a 0.22 μm sterile filter. Different concentrations of AHP (0.075 and 0.09 g) were then added to 5 mL each of chitosan solutions with stirring in an ice bath. The solutions were poured into sterile petri dishes and allowed to gel at 37 °C overnight. Circular discs were bored from the resulting gels.

MC3T3-E1 cells were subcultured from a stock culture (ATCC, Manassas, VA), by trypsinization (passage 14) and seeded (50000 cells/well) on to multiwell tissue culture plates. The cells were fed with minimum essential media (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin, and cultured in a humidified atmosphere and 5% CO_2 at 37 °C.

The direct contact assay was performed as follows. When the subcultured MC3T3-E1 cells attained a monolayer, the bored circular gels were placed into the well in contact with the cells and incubated at 37 °C for 24 h. After 24 h, the media was removed and the viability of the cells adjacent to the gel matrix was evaluated qualitatively using the live/dead viability/cytotoxicity assay (Molecular Probes) following the manufacturer's instructions. Briefly, the media was removed and cells were washed with PBS and incubated in calcein-AM ethidium homodimer mixture (2 μM calcein-AM solution and 4 μM ethidium homodimer-1) for 30 min at room temperature. The intracellular esterase activity of live cells converts the nonfluorescent cell-permeant calcein-AM into cell impermeant and highly fluorescent calcein. The polyanionic calcein dye thereby accumulates inside live cells that have intact membranes and results in an uniform green fluorescent signal. Ethidium homodimer-1 enters dead cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acid leading to a red fluorescent signal. After incubation, the viability of the cells directly in contact with the gels was determined using a BioRad microRadiance confocal scanning system (model MRA1).

For the test on extracts, the extract was prepared by incubating the gel in MEM supplemented with 10% FBS and 1% antibiotics at an extraction ratio of ~1.57 cm^2/mL for 24 h. The extraction media was then used to feed a subconfluent monolayer of MC3T3 E1 cells seeded on tissue culture polystyrene (TCPS) and cultured for 24 h. The cell viability was then compared to cells cultured in media alone by determining the metabolic activity of the cells using the MTS assay (3-4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium) according to the manufacturer's instructions (Promega). After 24 h of incubation, the media was removed and the cells were washed with sterile phosphate buffered saline (PBS). The PBS was then replaced by 1 mL of fresh media and 200 μL of MTS reagent and incubated at 37 °C for 2 h. At the end of incubation time, the reaction was stopped by adding 250 μL of sodium dodecyl sulfate. The resulting solution was diluted in a 4:1 ratio using distilled water and the absorbance was read at 490 nm.[26] Results are reported as absorbance ±SD.

Viability of Encapsulated Cells in the Chitosan–AHP Gel. Briefly, the gelling solution was prepared by adding sterile AHP solution to sterile chitosan solution with magnetic stirring. The gelling mixture was then heated to 37 °C in a water bath and MC3T3-E1 cells (1×10^7 cells/mL of the gelling solution) were uniformly suspended in the solution. The resulting solution was poured into sterile Petri dishes and allowed to gel at 37 °C. Circular discs were bored from the gels and the discs were cultured in MEM at 37 °C in a humidified atmosphere and 5% CO_2. Gels without cells were also prepared in a similar manner. At days 1 and 14, the encapsulated cells were stained using calcein-AM ethidium homodimer mixture as described earlier, and the cell viability and distribution were visualized using a laser scanning confocal microscope (model MRA1).

hMSC Culture in Gel Matrices. Passage 4 hMSCs were used for this study. The thermogelling solution was equilibrated at 37 °C and hMSCs (9.2×10^6/mL of the gelling solution) were added. The cells were uniformly suspended in the chitosan–AHP mixture and poured into sterile 35 mm diameter plates. The solution was allowed to gel at 37 °C in a humidified incubator with 5% CO2. Circular disks were bored from the gel and placed into 24 well plates and cultured in either basal mesenchymal stem cell media or osteogenic differentiation media for various periods of time at 37 °C in a humidified atmosphere and 5% CO_2. Control gels without cells were prepared in a similar manner.

Histology. The osteogenic potential of the encapsulated mesenchymal stem cells in chitosan–AHP gel cultured in osteogenic media was determined as follows. At predetermined time points, the culture media was removed and circular disks were washed with D-PBS and fixed in formalin. The gels were paraffin embedded, sectioned, and then stained with Alizarin red to detect the presence of calcium as an indication of osteogenic differentiation per Armed Forces Institute of Pathology protocols. Briefly, the slides were deparaffinized and hydrated in distilled water. Alizarin red S solution was then added and allowed to incubate for 30 s to 5 min. The slides were examined under a microscope, and when red-orange color appeared, the excess stain was removed and dehydrated using acetone, acetone–xylene solution and xylene.[28] Slides were visualized using the Nikon Eclipse E600 epifluorescence microscope (Nikon Corporation) and the MetaVue

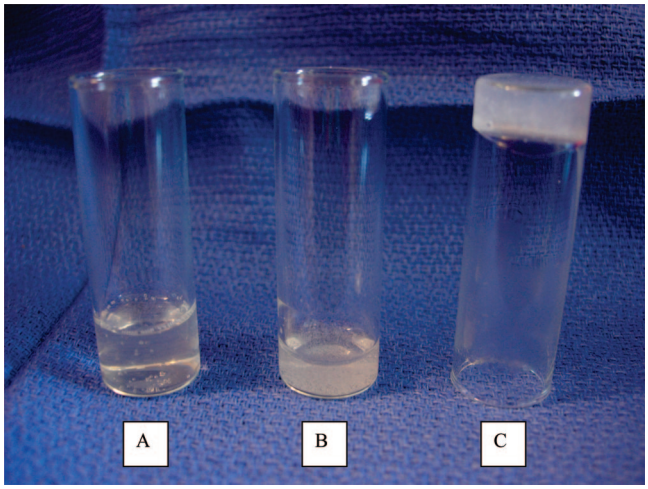


Figure 1. Photograph showing the thermogelation of chitosan–AHP solution. (A) Chitosan solution in 0.5% acetic acid. (B) Chitosan–AHP solution (0.075 g of AHP added to 5 mL of chitosan solution in an ice bath and stirred for 2 min followed by incubating at 37 °C for 4 min. (C) Thermogelled chitosan–AHP solution after incubating the chitosan–AHP solution at 37 °C for 11 min.

Imaging System (Molecular Devices Corporation, Sunnyvale, CA). Slides were viewed at 40× magnification.

In Vitro Release Studies. Chitosan–AHP solution was prepared as described earlier (0.075 g of AHP/5 mL chitosan) and appropriate amounts of FITC-dextran/FITC-albumin was added to the solution and gelled at 37 °C. Preweighed FITC-dextran/FITC-albumin loaded circular discs (10, 20, and 30 mg macromolecule/mL of the gelling solution) were placed separately in 10 mL of phosphate buffer saline (PBS) at 37 °C in a shaking water bath. At predetermined times, the media was removed and replaced with fresh PBS. The FITC-dextran/FITC-albumin concentration in the release media was determined spectrophotometrically at 491 nm and the percentage cumulative release was calculated based on the total FITC albumin content.

Statistical Analysis. Statistical analysis was performed using a one way ANOVA with a minimum confidence level of ($p < 0.05$) for statistical significance.

Results

Thermogelation of Chitosan Solution in the Presence of AHP. The feasibility of developing a thermogelling chitosan solution in the presence of AHP was demonstrated (Figure 1).[27] The effect of AHP concentration on the gelling time of the chitosan solution was determined by varying the amount of AHP solution added to chitosan while forming the themogelling solution. Figure 2 shows the gelling time of chitosan–AHP solution as a function of the amount of AHP added. The pH of the corresponding solutions varied from ~7.00 to 7.2. As shown in Figure 2, the concentration of AHP has a significant effect on the gelling time of the chitosan–AHP solution. Thus, increasing the AHP concentration significantly decreased the gelling time within the concentration range studied. It has been found that at a lower concentration of 0.045 g of AHP, the solution took ~30 h to gel when incubated at 37 °C (data not shown). However, no significant difference in gelling time was observed between AHP concentrations of 0.075 and 0.09 g. Because our aim was to develop systems with a gelling time of approximately 10 min, 0.075 g of AHP/5 mL of chitosan was used in all the subsequent studies. However, it has been found that increasing the concentration to 0.12 g/5 mL of chitosan could further decrease the gelation time. Figure 3 shows the thermogelation of chitosan–AHP solution determined as a function of time using oscillatory rheometer. The concentration of the AHP used was 0.075 g. The viscosity of the chitosan–AHP solution was found to increase after ~8 min and showed a significant increase within 15 min of incubation at 37 °C, demonstrating the sol–gel transition.

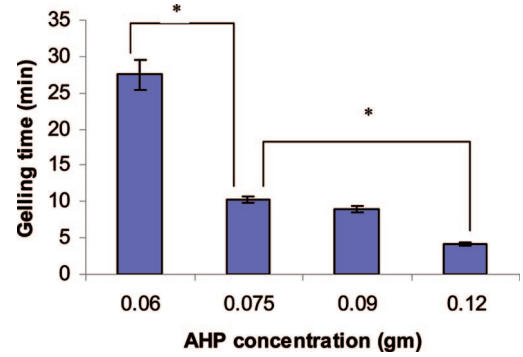


Figure 2. Gelling time of chitosan–AHP solution as a function of the amount of AHP added to the chitosan solution (0.06, 0.075, 0.09, 0.12 g of AHP were added to 5 mL each of chitosan solution). Gelling time was determined by incubating the solutions at 37 °C. Statistical significance at $p < 0.05$.

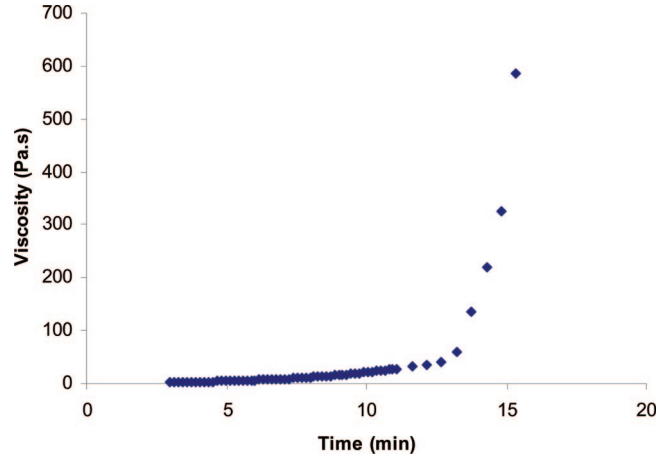


Figure 3. Variation of viscosity of chitosan–AHP solution with time as measured using an oscillatory rheometer at a fixed frequency of 1 Hz (0.075 g of AHP was added to 5 mL of chitosan solution).

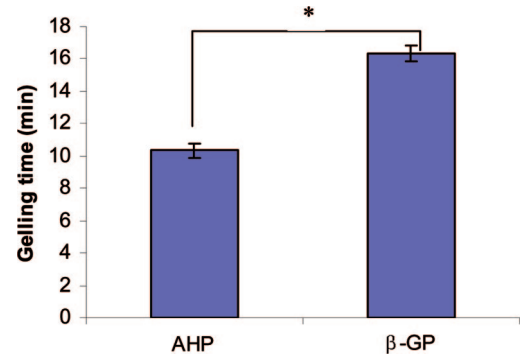


Figure 4. Comparison of the gelling time of AHP- and βGP-treated chitosan solutions (5 mL each of chitosan solution was treated with 0.075 g of AHP and 0.367 g of βGP in an ice bath. Gelling time was determined by incubating the solutions at 37 °C). Statistical significance at $p < 0.05$.

Figure 4 shows the gelling time of chitosan solutions treated with AHP compared to the gelling time of chitosan treated with βGP at 37 °C. The amount of βGP used was 0.367 g where as the amount of AHP was 0.075 g. The concentration of βGP used was 3 mol equiv to the concentration of AHP used. ACDV

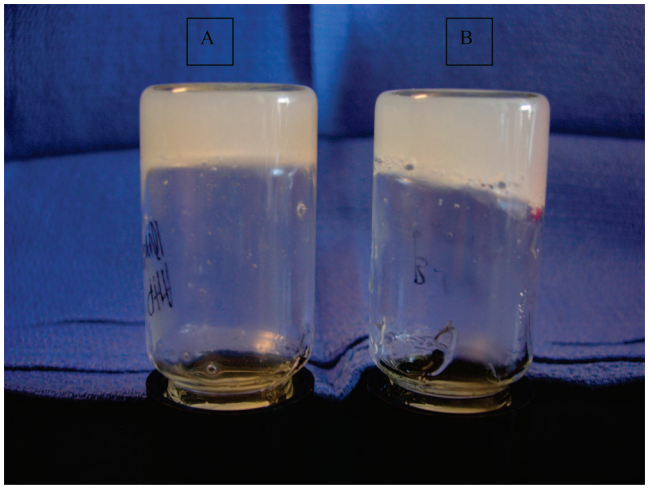


Figure 5. Photograph showing the gross morphology of gels formed from the thermogelation of chitosan–AHP and chitosan–βGP systems.

Table 1. Effect of Diluents on the Gelling Time of Chitosan–Phosphate Solution[a]

diluent	gelling time (min)
2 mL PBS	10.6 ± 0.5
5 mL PBS	11.8 ± 0.5
2 mL MEM	11.5 ± 0.4
2 mL water	10.8 ± 0.6

[a] Five mL of chitosan solution was treated with AHP solution (AHP 0.075 g), and appropriate volumes of diluents were added and incubated at 37 °C for gelling time determination.

lower concentration of βGP, (2 mol equiv to AHP) gelation did not happen within 10 h at 37 °C (data not shown). The data shows that AHP induced gelation takes place at a significantly lower concentration of the gelling agent.

Figure 5 shows a photograph of chitosan gels formed using AHP and βGP as the gelating agents, indicating the similarity in the gross morphology of chitosan–AHP and chitosan–βGP gels.

Effect of Diluents on the Gelation Time of Chitosan–AHP Solution. In addition to the concentration of AHP and the pH of the solutions, the effect of addition of various diluents such as distilled water (pH 7.0), cell culture media (MEM), and PBS were studied. Table 1 shows the effect of diluents on the gelling time of chitosan–AHP solution determined by the test tube inversion method. The ratio of the chitosan to AHP was kept constant in all these determinations. The table shows that dilution of chitosan–AHP using various diluent solutions did not significantly affect the gelling time of the solution under the conditions studied.

Cytotoxicity of Chitosan–AHP Gels. Figure 6 shows the photomicrographs of calceinAM–ethidium bromide homodimer stained MC3T3-E1 cells in direct contact with the chitosan–AHP gels. As can be seen, viable cells are present in close proximity to the gels as confirmed by the live/dead staining. The nontoxicity of the gels was further confirmed using the MTS assay after growing MC3T3-E1 cells in 1 mL of the media containing the gel extracts for 24 h. Figure 7 shows the metabolic activity of cells cultured for 24 h in media containing extracts of gels formed by adding various concentrations of AHP to 5 mL of chitosan (extraction ratio ~1.57 cm^2 of the gel/mL of the media). As shown in the figure, cells grew well in the gel extracts, even at the high concentration range of 0.2 g, showing that the gel extracts are nontoxic.

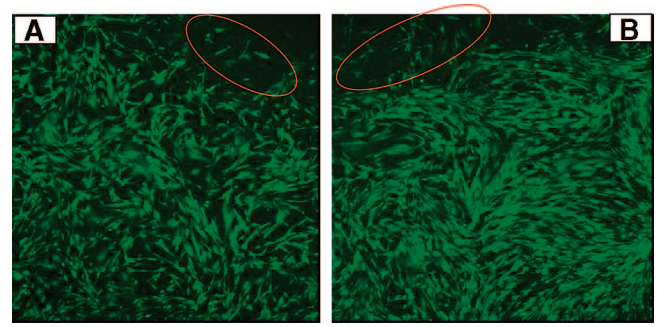


Figure 6. Photomicrographs showing the viability of MC3T3-E1 cells cultured in direct contact with chitosan–AHP gels having different concentrations of AHP added as determined by live/dead staining: (A) 0.075 g of AHP for 5 mL of chitosan and (B) 0.09 g of AHP for 5 mL of chitosan. Circles indicate the position of gels (20×).

The viability of MC3T3-E1 cells encapsulated inside the gels after 3 and 14 days in culture was evaluated by fluorescence staining using live/dead assay. Figure 8 shows the confocal fluorescence image of cells encapsulated in chitosan–AHP. It can be seen that the cells were homogeneously distributed within the gels and more than 90% of the cells remained viable inside the gels. A patch of dead cells were found along the border, presumably due to the boring of cell encapsulated discs from the gels.

The feasibility of chitosan–AHP gel as a stem cell delivery vehicle was evaluated, and the ability of stem cells to differentiate into appropriate lineage within the gel has been demonstrated. Figure 9 shows the Alizarin red staining of hMSC encapsulated chitosan–AHP gel after 28 days in culture using basal control medium and osteogenic medium. The cells encapsulated in chitosan–AHP gels cultured in the osteogenic media showed red staining, qualitatively indicating the mineralization of the encapsulated cells. The mineral deposits were found to be localized in the area adjacent to the encapsulated cells.

Figure 10 shows the feasibility of the chitosan–AHP system as a macromolecular drug delivery vehicle. FITC-albumin and FITC-albumin were used as model macromolecules. Figure 10A shows the percent cumulative release profiles of different concentration of FITC-dextran from thermogelled chitosan–AHP matrices as a function of time at 37 °C. FITC-dextran showed a faster release profile, with more than 80% of the macromolecule released from the gel within 72 h. Figure 10B shows the percent cumulative release profiles of different concentration of FITC-albumin from thermogelled chitosan–AHP matrices as a function of time at 37 °C. Unlike FITC-dextran, FITC albumin showed a very slow release profile from chitosan–AHP gel. Only ~50% of the protein released from the gel during the first 10 days, indicating the potential of the gel as a long-term protein delivery vehicle.

Discussion

Chitosan is an aminated polysaccharide that is soluble in weak acids due to the protonation of the amino groups. Poly- or multivalent anions are therefore extensively used to develop ionically cross-linked chitosan matrices utilizing the cationic nature of acidic chitosan solutions. Acidic solutions of chitosans are stable only up to a pH of about 6.2.[22] Increasing the pH of chitosan solution above its pK_a results in spontaneous gelation of the solution. However, a recent study has shown that neutralization of acidic chitosan solution at low temperature

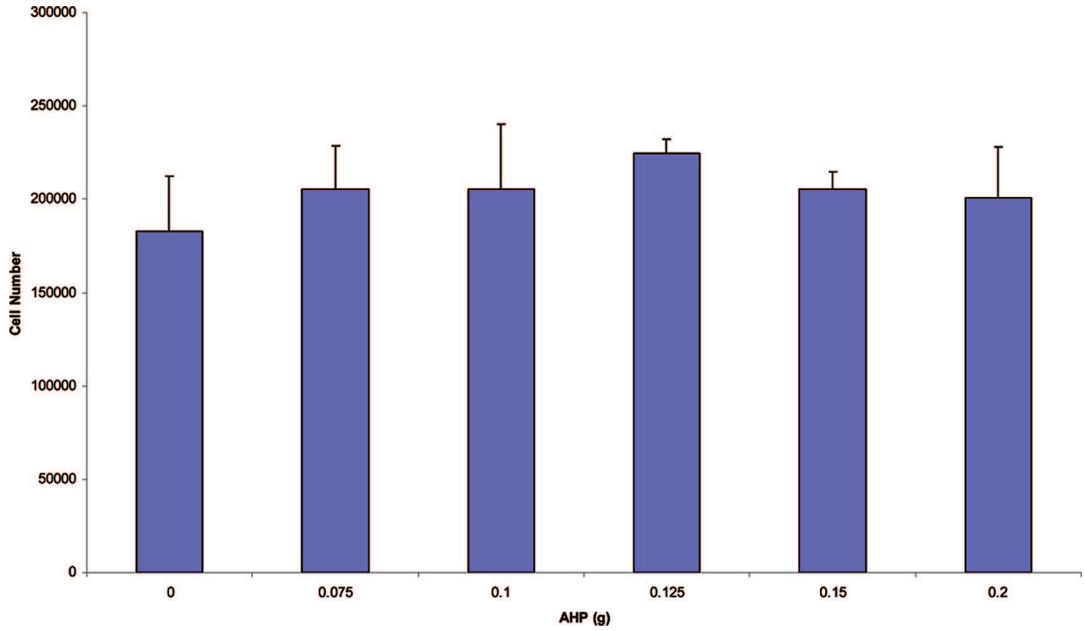


Figure 7. Viability of MC3T3-E1 cells cultured in the presence of the extraction media of chitosan–AHP gels prepared by adding different concentrations of AHP (0.075, 0.09, and 0.12 g) to 5 mL of chitosan solutions as determined by MTS assay.

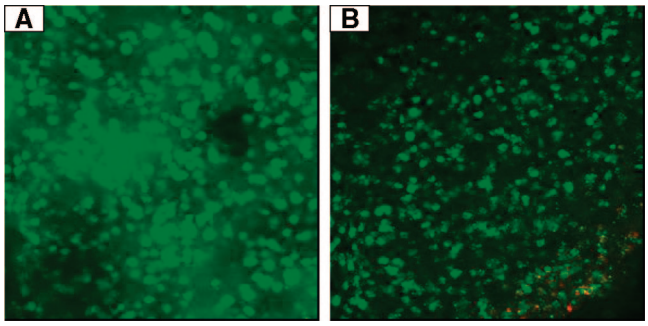


Figure 8. Viability of MC3T3-E1 cells encapsulated within the chitosan–AHP gels cultured for different time periods as determined by live/dead staining: (A) 3 days in culture (×20), (B) 14 days in culture (×20).

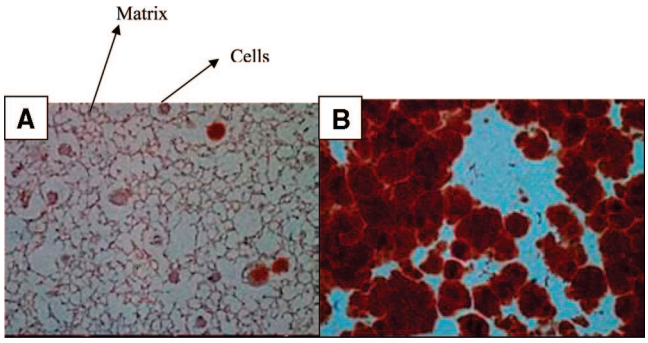
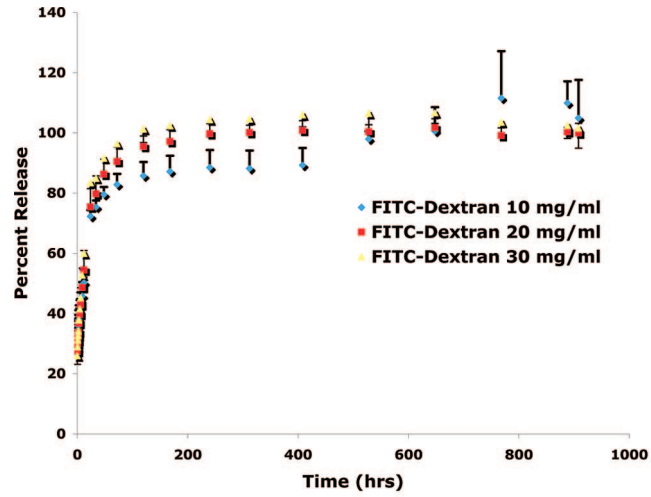


Figure 9. Alizarin red staining of hMSC encapsulated in chitosan–AHP gels for 28 days: (A) cultured in basel control media (40×) and B. cultured in osteogenic media (40×).



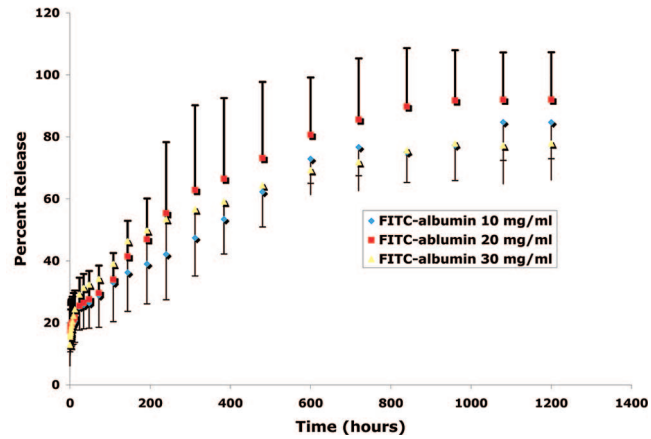


Figure 10. Cumulative macromolecular release profiles from chitosan–AHP gels at 37 °C as a function of time: (A) release of different concentrations of FITC dextran, (B) release of different concentrations of FITC-albumin.

using certain organic phosphates such as βGP can prevent the precipitation of chitosan at a pH above 6.2. These solutions have been shown to transform into a gel as the temperature of the solution was increased.[12,13,24,25,28] In this study, we investigated for the first time the effect of an inorganic phosphate salt on developing a thermogelling neutral injectable chitosan solution.

It has been found that addition of basic AHP solution (pH ~ 8.6) to chitosan solution at low temperature could increase the pH of the chitosan solution above pH 6.2 without spontaneous gelation. At the concentration of AHP studied, chitosan solutions having pH in the range of 7–7.2 has been prepared at low

temperature. Unlike multivalent phosphates such as tripolyphosphate, which is known to form pure ionic cross-linking with chitosan, the divalent phosphate ions from AHP did not induce a pure ionic cross-linking. The addition of tripolyphosphate to chitosan solution under the same experimental conditions resulted in the rapid gelation of the polymer governed by ionic cross-linking. Thus the addition of AHP salt to chitosan solution is presumably neutralizing the solution without significant electrostatic interactions between the chitosan chain and the divalent phosphate ion. This is evident from the feasibility of preventing the precipitation of chitosan at higher solution pH due to the addition of AHP and the temperature controlled gelation process of the chitosan–AHP system when incubated at 37 °C due to chitosan chain interactions (Figures 1 and 3). The gelation time and pH of the chitosan–AHP solution was found to depend significantly on the concentration of the AHP added (Figure 2). It has been found that low concentrations of AHP (<0.03 g, pH 6.8) did not induce the thermogelation of chitosan and increasing the concentration of AHP correspondingly decreases the gelation time and increases the pH of the chitosan–AHP system. Also, no gelation was observed at 37 °C when the pH of the chitosan–AHP solution was below 7.00 and the gels formed above pH 7.0 did not show any thermoreversibility. This suggests that the combined effect of electrostatic attraction and increased hydrophobic interactions is involved in promoting the thermogelation of the chitosan–AHP solution as in the case of βGP–chitosan solution.

Because the thermogelling injectable system has the potential to be used as carrier agents for cells or molecules, the effect of addition of various diluents on the gelling time of chitosan–AHP solution was evaluated. The addition of diluents to the chitosan–AHP system without changing the pH of the system did not significantly change the gelation time (Table 1). This also suggests that the pH is one of the important parameter that determine the temperature setting process in the chitosan–AHP gelling system.

The properties of chitosan solutions are known to depend on many different factors, including molecular weight of the polymer, degree of deacetylation, crystallinity of the polymer, viscosity and concentration of the solution, concentration of acetic acid, and pH. All these parameters can affect the gelling time of the thermosetting system. Therefore, a study was performed to compare the gelation time of the chitosan–AHP system to the previously reported chitosan–βGP system with the same chitosan solution. It has been found that the concentration of AHP required for inducing thermogelation of chitosan solution was significantly lower when compared to the βGP system (Figure 4). Thus the addition of 0.075 g of AHP induced thermogelation of 5 mL of chitosan when incubated at 37 °C in approximately 11 min, whereas 0.367 g of βGP (3 mol equiv) was needed to induce gelation in approximately 16 min. This may be presumably due to the increased electrostatic interactions and less stabilization effect of AHP compared to the βGP system. The chitosan–βGP system has been reported to be highly stable at 4 °C, which has been attributed to the stabilizing effect of the polyol moiety in the molecule.[12,13,24,25,28] Thus the study demonstrated that in chitosan–AHP system, thermogelation can be achieved in a clinically relevant time period of 5−15 min by incubation at 37 °C maintaining osmolarity in the physiologically acceptable range (270–340 mOsmol/kg).

The direct contact assay (Figure 6) and cytotoxicity of the gel extract study (Figure 7) demonstrated that chitosan–AHP gel or gel extract is not toxic to osteoblast-like cells. The fast gelling chitosan–AHP solution is therefore a potential candidate for a wide range of biomedical applications as a cell carrier vehicle. The cell encapsulation study using MC3T3E1 cells clearly demonstrated the efficacy of chitosan–AHP gels to encapsulate cells and maintain cellular viability for a prolonged period of time (Figure 8). The encapsulated cells showed more or less a rounded morphology even after 14 days in culture when encaspulated in the gel, presumably due to lower interactions with the matrix for anchorage dependent cells like ostoeblasts. The chitosan–AHP gel also supported human mesenchymal stem cell (hMSC) differentiation over a 28 day period into osteogenic lineage when exposed to the proper growth factors demonstrating the efficacy of the gel system as a stem cell delivery vehicle (Figure 9). The biodegradation of chitosan raises significant interest while developing an injectable delivery vehicle. The in vivo degradation of chitosan has been reported to be primarily due to lysozyme and takes place through the hydrolysis of the acetylated residues. The rate of degradation of chitosan inversely depends on the degree of acetylation and crystallinity of the polymer.[29] The effect of AHP on the degradation of chitosan in vitro and in vivo is currently under evaluation.[30]

The potential of chitosan–AHP as a drug delivery vehicle was demonstrated using FITC-dextran and FITC-albumin. No statistically significant differences in release kinetics were observed between gels loaded with different concentrations of FITC-dextrans, presumably due to the large pore size of the thermogels. However, the neutral hydrophilic macromolecule FITC-dextran showed a faster release from the gel compared to FITC-albumin. FITC-albumin carries a net negative charge at pH 7.4 and hence could interact with the cationic chitosan backbone. This is evident from the slow release of FITC-albumin from the gels compared to the neutral molecule FITC-dextran having the same molecular weight. Because of the ionic interactions with the matrix polymer, the complete release of albumin might be possible only after the enzymatic degradation of the chitosan–AHP matrix.

Conclusions

An injectable thermogelling solution was developed by the addition of AHP to chitosan solution in acetic acid. The addition of AHP to chitosan resulted in the formation of near-neutral solution that gelled in a clinically relevant time period when incubated at 37 °C. The in vitro cytotoxicity studies indicate that chitosan–AHP systems are nontoxic toward MC3T3-E1 cells. The encapsulation study performed using MC3T3-E1 and hMSCs demonstrated the potential of chitosan–AHP system as a cell carrier matrix. The chitosan–AHP could also function as a prolonged protein delivery vehicle. These studies collectively indicate the good biocompatibility of the chitosan–AHP system and with the potential to function as a carrier matrix for multiple biomedical and tissue engineering applications.

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