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Fabrication and Characterization of Injectable Biomaterials for Biomedical Applications

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Abstract

The aim of this study is to evaluate the injectable cross-linked chitosan (CS) microparticles (MPs) to apply for biomedical applications specifically for bone regeneration. The CS MPs were fabricated by emulsification method and formed the cross-links between the amide groups in the CS and phosphate groups in the sodium tripolyphosphate (TPP) ionic cross-linking agent. The MPS were analyzed for morphology by Scanning Electron Microscope (SEM). The fabricated CS MPs were in the spherical shape with the size range of 20–100 μm . These CS MPs were analyzed for biodegradation by immersing in phosphate buffered saline (PBS, pH = 7.4) at 37°C for 30 weeks. The biodegradation of CS MPs in PBS was initiated at week 25. Mesenchymal stem cells (MSCs) were harvested from the bone marrow of mice tibia and femurs. The MSC attachment on CS MPs was tested using LIVE/DEAD cell assay with a Fluorescence Microscope. The murine MSCs attachment onto CS MPs at day 2 was confirmed by visualizing fluorescence images. The CS MPs were also analyzed for the injectability and retainability at the site using a subcutaneous injection in a rat model. The fabricated CS MPs possess injectability, biodegradability and biocompatibility. Therefore, these CS MPs have a great potential to apply for various biomedical applications including bone regeneration by injection.

Keywords

Chitosan; emulsification; biodegradation; microparticles; mesenchymal stem cells; subcutaneous injection

Introduction

There are several different types of implant materials used for biomedical applications. For example, the two main types of bone grafts currently used are autografts and allografts [1,2]. In addition, synthetic materials including, metals, ceramics, plastics, and composites are used for repair the bone defects [3]. An autograft is a section of bone taken from the patient's own body, whereas an allograft is taken from a cadaver. These types of grafts are limited due to some uncontrollable factors. For autografts, the key limitation is donor site morbidity [4], in which the remaining tissue at the harvest site is damaged by the removal of

the graft, resulting in surgical scars, blood loss, pain, prolonged surgical time and rehabilitation, and infection risk. A limitation of allografts has been the immunologic response to the foreign tissue of the graft. The tissue is often rejected by the body and is subject to the inflammatory reactions. Synthetic materials also have major disadvantages including mechanical breakdown, corrosion, toxicity, and non-resorbability.

Emerging technology, tissue engineering and regenerative medicine has been introduced to use both life science techniques and engineering principles to produce the cell-scaffold constructs [5]. These cell-scaffold constructs can be used for several tissue regeneration applications including bone and cartilage regeneration. The three-dimensional (3D) bulk scaffolds can be implanted at the defect site.

These scaffolds should exhibit the following properties [6]: biocompatibility - they must not be rejected by the immune system; osteoconductivity - they should provide an appropriate environment for attachment, proliferation and function of living cells; they should have an ability to incorporate osteoinductive factors to direct and enhance new bone growth; they should allow for ingrowth of vascular tissue to ensure survival of transplanted cells; they should have sufficient mechanical integrity to support loads encountered at the implant site; they should be biodegradable, with a controlled predictable, and reproducible rate of degradation; they should degrade into non-toxic molecular species that are easily metabolized or excreted; and they should manufacture conveniently with low cost.

The main advantage of an injectable scaffold is that the implantation can be completed by minimally invasive surgery (MIS) at a defect site using a suitable vehicle [7]. MIS limits pain, prolonged hospitalization, recovery time, blood loss, and scar formation compared with conventional open surgeries, which require implanting 3D conventional scaffolds.

In this study we selected CS as a base material to fabricate the MPs. CS is used in several medical applications including both systemic and local drug delivery [8,9], and wound dressing [10]. CS scaffolds appear to favor the differentiation of osteoprogenitor cells and bone formation [11]. CS is a deacetylated derivative of chitin, a high molecular weight and the second most abundant natural biopolymer commonly found in the shells of marine crustaceans and cell walls of fungi. Another interesting property of CS is its intrinsic antibacterial activity. In general, these materials evoke a minimal foreign body reaction [12], with little or no fibrous encapsulation. Biodegradability and biocompatibility are very important properties that make CS a useful material for bone regeneration.

In this paper we describe the fabrication of cross-linked CS MPs by emulsification method and characterization of these MPs for the biocompatible and biodegradable properties.

Materials and Methods

Fabrication

The chemical structure of CS and TPP was given in Fig. 1. The CS MPs were fabricated using our scale-up procedures in emulsification method as described previously [13]. Briefly, the CS solution (1.5%, w/v) was prepared by dissolving CS in dilute acetic acid

(1%, v/v) at room temperature and filtering through nylon cloth to remove any insoluble components. The CS solution (25 ml) was mixed with an equal volume of acetone. The mixture, 36 ml out of 50 ml, was then added drop wise into 600 ml of cottonseed oil which mixed with 4 ml of span 85. The oil suspension was stirred for 14 h at 37°C and an agitation speed of 870 rpm. 64% (w/w) of TPP was mixed with 4 ml deionized water and added to the reaction mixture. Four hours after the addition of TPP, the MPs were purified using hexane followed by vacuum filtration and air drying. The CS MPs were analyzed for morphology with scanning electron microscope (SEM).

Biodegradation Study

The fabricated and neutralized hybrid MPs (30 mg) were added to 11 ml glass vial shells. The MP samples were incubated with 9 ml of Phosphate Buffered Saline (PBS) at 37°C in the incubator under continuous shaking at 50 rpm for predetermined time points up to 30 weeks. The vial shells were closed with a plastic plug designed for the vial shells, to prevent evaporation of the PBS over the long time period of the experiment. In order to mimic *in vivo* physiological environment, every week 4 ml of incubated PBS solution was removed from each sample and replaced with an equal volume of fresh PBS medium.

Mesenchymal Stem Cells (MSCs) – Isolation and Attachment

The C57/BL-6 strain, 6 weeks old, male mice were purchased from the Charles Rivers Laboratory, Wilmington, MA. The mice were housed in the animal care facilities at the University of Toledo Health Science Campus. The MSCs were isolated from mouse femurs and tibia as previously published [14]. The sterilized CS MPs were placed in 96 well plates with 6 mg of MPs. Prior to cell seeding 50 µl of cell culture medium was added into each well, to ensure the MPs settle down at the bottom of the plate. The cell density used was 20,000 cells/well. Attachment of cells on MPs was studied using the live/dead cell assay (Molecular probes). After washing cells with PBS, 100 µl of 2 µM calcein was added to each well and incubated at room temperature for 20 min. Cells seeded in wells without MPs served as controls. Fluorescence images of the cells were taken using Leica fluorescence microscope with x400 original magnification.

Subcutaneous Implantation of CS MPs

Dark Agouti male rats were purchased from Harlan Laboratories, Indianapolis, IN, USA. All injections were performed under anesthesia and normal sterile conditions. Anesthesia was administered via 4% isoflurane as an inhalant using a rodent vaporizer. The sterilized MPs and PBS slurry were injected into four sites in the back of the animal. After injection, the anesthesia was removed from the animals and they were returned to their cages and monitored until conscious. Monitoring of the animals continued daily until they were euthanized for further studies.

At week 2 and 4, tissue samples surrounding the injection site were taken and placed into enough 10% formalin to cover the sample. Each tissue sample was then embedded in paraffin and sectioned into 5 µm slices and mounted on slides. After deparaffinizing and washing, the tissue samples were stained with hematoxylin and eosin and imaged using an optical microscope.

Results and Discussion

Fabrication and Characterization

The size and shape of the CS MPs containing 64% TPP for scaled-up batches were examined using a SEM, and confirmed the spherical shape and the smooth surface of MPs. The CS MPs were in a diameter range of 20–100 μm (Fig. 2). We were able to produce around 300 mg of CS MPs using our scale up method. The ionic cross-links were formed between the amide groups in the CS and phosphate groups in the TPP. These cross-links provide the structural integrity for CS MPs. These MPs have an ability to inject as a therapeutic scaffold for various biomedical applications including bone and cartilage regeneration.

In Vitro Biodegradation

We examined the biodegradation of CS MPs during 25–30 week period. SEM results indicated the slight surface morphology changes in CS MPs at week 25 (Fig. 3) compared to the morphology of CS MPs without any treatment at week 0 (Fig. 2). Therefore, biodegradation of MPs appears to begin at week 25. This result suggests that MPs will be relatively stable *in vivo* for at least 25 weeks, thus providing sufficient time for bone regeneration. Generally, the degradation of CS is related to the molecular weight and deacetylation. These MPs were strong not only having the higher deacetylation (> 85%) but these MPs were ionically cross-linked with TPP. This interaction leads to the fabrication of physically strong particles according to our optimization and scale-up procedures [13]. Many investigations have published that the factor in controlling the rate of degradation has been shown to be inversely associated with CS's degree of deacetylation. Highly acetylated CS degrades rapidly, and highly deacetylated (> 80%) CS has low degradation rates and may remain several months *in vivo* [15].

MSC attachment on CS MPs

The murine MSCs attachment onto CS MPs at day 2 was confirmed by visualizing fluorescence images (Fig. 4). The fluorescence images of the MSCs were taken using Leica fluorescence microscope with x 400 original magnification. The cells seem to be started to spread on the MPs at day 2. It should be noted that MPs produced autofluorescence for the calcein and appeared green.

CS MP retaining at the site

We also studied the retention of MP-cell constructs at the injection site using the subcutaneous injections in a rat model at week 2 and 4. The histology sample confirmed that retention of MPs at the injection site without diffusing. In all of the H & E stained slides (Fig. 5), large MPs can be readily seen dyed red. From prior experimental data and their size and shape, it can be concluded that the red spherical shaped particles are in fact CS MPs. Taken together, these data demonstrated that the CS MPs did stay localized to the site of injection.

Summary

The CS MPs are in spherical shape with 20–100 μm size range. They are biodegradable and stable *in vitro* at least for 25–30 weeks in PBS. There are several advantages use of an injectable MP system compared to the traditional block scaffolds. Since the size of MPs is small they can be combined with a vehicle and be administered by injection. Therefore, these MPs can be used as filler for bone defects of different shapes and sizes through MIS. *In vitro* studies confirmed MSC attachment on CS MPs. We also confirmed that CS MP retention at the injected site. These results confirmed that the biocompatibility of these MPs. The CS MPs developed have a great potential to be used as an injectable scaffold for bone regeneration including orthopaedic, craniofacial and cartilage applications using minimally invasive conditions.

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References

- Greenwald SA, Boden SD, Goldberg VM, Khan Y, Laurencin CT, Rosier RN. Bone-graft substitutes: facts, fictions, and applications. *J Bone Joint Surg.* 2001; 83-A:98. [PubMed: 11712842]
- Rees DC, Haddad FS. Bone transplantation. *Hosp Med.* 2003; 64:205. [PubMed: 12731131]
- Kohn, DH.; Ducheyne, P. Materials for Bone, Joint and cartilage replacement. In: Williams, DF., editor. Medical and Dental materials. VCH Verlagsgesellschaft; FRG: 1992. p. 29
- Younger EM, Chapman MW. Morbidity at bone graft donor sites. *J Ortho Trauma.* 1989; 3:192.
- Langer R, Vacanti JP. Tissue Engineering. *Science.* 1993; 260:920. [PubMed: 8493529]
- Ishaug-Riley SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res.* 1997; 36:17. [PubMed: 9212385]
- Xu JW, Zaporozhan V, Peretti GM, Roses RE, Morse KB, Roy AK, Mesa JM, Randolph MA, Bonassar LJ, Yaremchuk MJ. Injectable tissue-engineered cartilage with different chondrocyte sources. *Plast Reconstr Surg.* 2004; 113:1361. [PubMed: 15060348]
- Orienti I, Cerchiara T, Luppi B, Bigucci F, Zuccari G, Zecchi V. Influence of different chitosan salts on the release of sodium diclofenac in colon-specific delivery. *Int J Pharm.* 2002; 238:51. [PubMed: 11996810]
- Portero A, Remuñán-López C, Criado MT, Alonso MJ. Reacetylated chitosan microspheres for controlled delivery of antimicrobial agents to the gastric mucosa. *J Microencap.* 2002; 19:797.
- Conti B, Giunchedi P, Genta I, Conte U. The preparation and in vivo evaluation of the wound-healing properties of chitosan microspheres. *STP Pharma Sci.* 2000; 10:101.
- Di Martino A, Sittertinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials.* 2005; 26:5983. [PubMed: 15894370]
- Li Z, Ramay HR, Hauch KD, Xiao D, Zhang M. Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials.* 2005; 26:3919. [PubMed: 15626439]
- Jayasuriya AC, Bhat A. Optimization of scaled-up Chitosan Microparticles for Bone Regeneration. *Biomed Mater.* 2009; 4:55006.
- Jayasuriya AC, Bhat A. Mesenchymal stem cell function on hybrid organic/inorganic microparticles in vitro. *J Tissue Eng Regen Med.* 2010; 4:340. [PubMed: 20033925]
- Ren D, Yi H, Wang W, Ma X. The enzymatic degradation and swelling properties of chitosan matrices with different degrees of N-acetylation. *Carbohydrate Research.* 2005; 340:2403. [PubMed: 16109386]

Fig. 1.
Chemical structure of CS (A); and TPP (B)

Fig. 2.
SEM Image of cross-linked CS MPs at week 0 (without any treatment).

Fig. 3.
SEM Image of cross-linked CS MPs immersed in PBS at week 25.

Fig. 4.
Fluorescence microscopy image for MSC attachment on CS MPs at day 2 when treated with LIVE/DEAD cell assay (original magnification x400).

Fig. 5. Histology image of remaining CS MPs at week 4. CS MPs were subcutaneously injected to the back of the rat.