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Enzymatically Fabrication of Encapsulating Injectable Nanocomposite Hydrogels in Osseous Cell Regeneration

Shiv Prakash Mishra
Assistant Professor, Dept. of Physics & Electronic, Dr. Ram Manohar Lohia Avadh University, Ayodhya, India

***Abstract*: A various biodegradable materials have been applied for regenerating of soft and hard tissues in biomedical applications. There are different biological advantage of macromolecules like polypeptide (gelatin) and polysaccharide (chitosan or cellulose), biphasic calcium phosphate nanoparticles (BCPNPs) and injectable nanocomposite hydrogel (IN hydrogel) are being enzymatically fabricated from a phenolic chitosan (PC) and phenolic gelatin (PG) derivative and encapsulation of BCPNPs. Since enzymatic peroxidase catalyzed reaction may proceed due to free functional groups of gelatin and chitosan binding to –OH group in BCP resulting crosslinking density of IN hydrogels, indicating supporting effect on cell growth or migration of bioactive components of IN hydrogels. The formation of BCPNPs-encapsulated PG-PC IN hydrogel enhanced bio mineralization in a phosphate buffer saline (PBS) at 37℃ about pH 7.4 on the composite surface in the simulated bio fluid which have significant in proliferation of bone marrow mesenchymal cells for typical bone cell regeneration in specific tissue engineering.**

***Keywords*: Gelatin, Chitosan, Injectable nanocomposite hydrogel, Biphasic calciumphosphates.**

1. Introduction

In unique chemistry of biological apatite ($Ca_5(PO_4)_3$ X; where X= F or OH) which is the main inorganic component of hard tissues such as bone and teeth [1]. The formation of hydroxyapatite ($Ca_{10}(PO_4)_6$ $(OH)_2$) crystals from octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4.5H_2O$) is well reported [2], [3]. Including several other functional polymer used in artificial organs, tissue engineering, medical devices and dentistry etc. [4]. Although, tyrosine based polyphosphates pertinent to potential biomaterial and polysaccharide (chitosan)-tripolyphosphate have attention [5], [6]. Here, we have reported a newly enzyme mediated hydrogels which play an important role in biomedical and engineering application, due to their practical performance such as delivery of bioactive components treatment or replacement of damaged tissue, organs or regenerative typical tissue cells[7] and encapsulation of nanoparticles in order to enhance cell attachment and osteoblast proliferation[8].The hydrogels consist of hydrophilic polymers that swell in aqueous solution thus facilitating the transportation of substance such as nutrients and by-products from cell metabolism.

Attentionally, the polysaccharides (chitosan, dextran, heparin and chondroitin sulphate) are playing an important role in preparation of injectable peroxidat enzyme mediated highly biocompatable and biodegradable hydrogels, that exhibited a great potential in tissue regeneration [7], [9]. For hard tissue regeneration, the combination of mineral nanoparticles and the hydrogels has recently been a new strend in fabricating nanocomposite hydrogel for hard tissue substitutes such as dentistry, orthopedics and reconstructive surgery, which improved strength & mechanical properties [10]. The β-tricalcium phosphate dispersed hydrogels significantly improved the compressive strength and biomeneralization in simulated body fluid. Although, the vancomycin–encapsulated nanocomposite hydrogel performed antimicrobial activity against *staphylococcus aureus* [11]. The nanocomposite hydrogels have also compatible with high adhesion density of mesenchymal stem/human cells [12]. It is well known that, the chitosan is tissue adhesive, hemostatic, anti-infective, biodegradable and supportive for cell attachment, but, however, not it all cell types [13], [14].

The proteins as collagen and its denatured gelatin are also widely used for pharmaceutical and medical application due to their high biocompatibility, fast biodegradability and enhancement of cell attachment and proliferation [15]. The gelatin posseses more integrin binding domains for cell attachment and it enhances cell attachment as well as they are quickly degraded by collagenase (enzyme) within 3-4 days [16]. The calcium phosphate (CP) and biphasic (BCP) nanoparticles both have been used in orthopedic application because of its repairing, biocompatibility, osteoconductivity and osteointegration, with producing osteoinduction as compared to hydroxyapatite or α-, β-tricalcium phosphate [17].

In this paper we have reported the protein (gelatin) – polysaccharide (chitosan) based nanocomposite hydrogels are prepared from 4-hydroxyl phenyl acetamide – conjugated – chitosan and tyramine or p-hydroxyl phenyl acetic acid (HPA) – functionalized gelatin in presence of BCPNPs, horscradish peroxidase (HRP) enzyme /H_2O_2. The chitosan /gelatin and BCPNPs - based IN hydrogels can be adjustable gelatin time, appropriate collagenase – mediated degradation rates and enhancement of biomeneralization and bone cell growth that

enable it to be a great platform for regenerative medicine to overcome some limitations of gelatin - chitosan based materials.

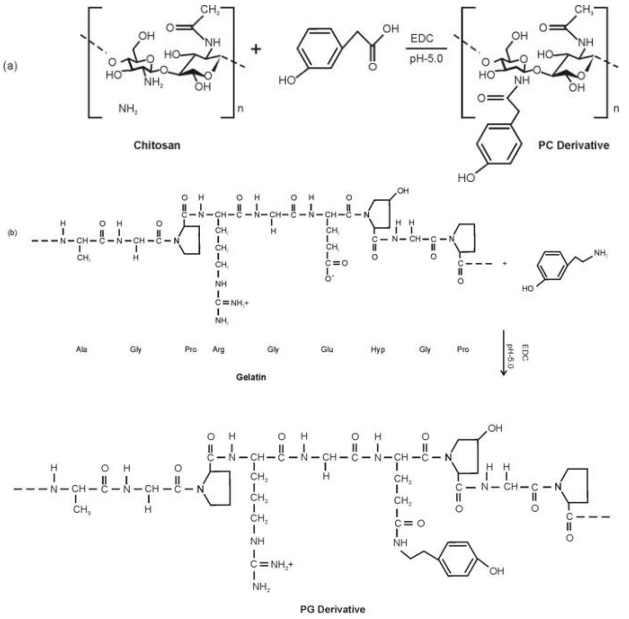

Fig. 1. (a) Chemical structure of chitosan and gelatin with synthetic scheme of PC, (b) PG derivative

2. Material and methods

In experimental procedure, which are adapted from the work described by Nguyen et al [8], where all the required chemicals /reagents are standarized laboratory based. In prilimanary, chitosan (1.0g) is dissolved in a solution of 40 ml distilled water and 0.5ml of 1M HCl. Then 4- hydroxyl- phenyl acetic acid (HPA) 0.45g was added into the mixture and pH of solution is adjusted to 5.0 and then 1-ethyle-3-(3- dimethylaminopropyl) carbodiimide (EDC) 0.90g is added to the chitosan solution under stirring for 24h (one day) at room temperature 25℃ . The solution is dialyzed against distilled water using membrane dialysis, for 3 days to obtained PC. Now, gelatin (2.0g) and tyramine (1.0g) are dissolved in 30ml distilled water, with adjusted pH to 6, following addition of ED Carbodiimide 0.50g under strring for 24h. Then, the solution is dialyzed against deionized water using membrane dialysis for 3 days. Subsequently, the dialyzed solution is freeze and dried to obtained PG. In preparation of BCP nanoparticles, the using of calcium chloride and tricalcium phosphate salts at molar ratio of Ca/P=1.57. The PH of the reaction mixture is maintained at pH 7. Calcination process is conducted at 750℃ to obtained BCP nanoparticles, where the BCPNPs is obtained below 80nm in diameter by ball-milling process.

In preparation of gelatin or chitosan based hydrogels, the PG (40mg) is dissolved in distilled water (300μL) and it separated into two vials equally. Then enzyme HRP (30μL . of 0.05mg/ml stock solution) and H_2O_2 (30 μL of 0.05- 0.15% w/v stock solution) are added into each tube separately. The gelatin based hydrogel was formed by mixing two HRP & H_2O_2 contained vials. Phenyl gelatin polymer concentration is 10% w/w in hydrogel. Chitosan based hydrogel is prepared by the same process as described for PG hydrogel, in which 8.0mg phenyl chitosan is prepared in (150μL) distilled water with HRP & H_2O_2 as above mention. The final concentration of the polymers solution was 8% w/w. The gelatin time may determined by using the vial tilting method.

The PG-PC hydrogels formation have occurred when solution A (contained PG, PC and HRP) mixed with solution B (contained PG, PC and H_2O_2) at same volume of the precursor polymer solution as demonstrated in above experiment. Practically, BCPNPs encapsulating PG- PC hydrogels are prepared from the same hydrogels process, in which contained 10 w/w % of the BCPNPs. The gelatin time behaviours of the hydrogels and IN hydrogels are characterized at the different concentration of HRP and H_2O_2 from 0.05- 0.15 to 0.20% wt/vol., with relativity that enzyme mediated hydrogels contained approximately 8 w/w % of the polymer concentration.

The biodegradation of injectable nanocomposite hydrogels have been studied in a collagenase-mediated, in which these materials were immersed in phosphate buffer saline (PBS) solution pH 7.4 containing collagenase (0.2μ/ml) at 37℃ and then monitoring their weight losses at different incubation times. Samples with different mass ratios having accurately weighted (wi) before immersing in 1ml of enzymatic solution. At the predetermined intervals, samples being removed from the incubation medium. Then, weight of remaining hydrogels and IN hydrogels (wt) is:

$$\text{Degradation rate (rate of weight loss \%)} = \frac{wi - wt}{wi} \times 100$$

Where, wi and wt are initial or remaining weights of hydrogels/ IN hydrogels, respectively.

3. Results and discussion

The characterization study of polymers have reported that in crystalline phase of β-TCP & HAP with small particle size, the nanoparticles may be highly potential in fabrication of nanocomposite biomaterials. The study of HRP/H_2O_2 mediated coupling reaction of phenolic moieties-modified polymer is an high efficiency to prepare injectable hydrogels [7] where the fast gelation time obtained to be 60s and 12s for preparation of (1:5wt/wt) PC and PG solution at around 0.125- 0.15 wt% of the used stocke H_2O_2 concentration, respectively, with molar ratio of H_2O_2 and phenolic moiety to crosslinked polymer chains at 0.5 is optimal condition [18]. Generally, PCD/PGD and IN hydrogels could be formed with in gelation time 40s. Figure-2 show that the representative data for gelation time(s) versus concentrate H_2O_2 (wt %). The role of HRP enzyme which catalizes to decompose H_2O_2 in coupling phenolic or aniline derivatives for formulation and fabrication of several types of phenolic derivation based biomaterials as well as due to free function groups of gelatin and chitosan binding to – OH groups in BCP resulting in increasing crosslinking density of

IN hydrogels, indicating supporting effect on cell growth and migration or sustainable release of bioactive components of these IN hydrogels [18, [19].

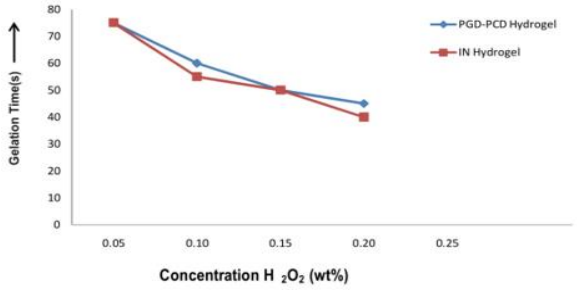

Fig. 2. Representative data for Gelation time vs. Concentration H_2O_2 (wt%) of PG-PC derivative and IN Hydrogel

The biodegradation and cellular compatibility behaviours of the gelatin or chitosan based biomaterials are partially different, where the collagenase induced degradation rate profiles that differently performed as changed mass ratios of PC-PG (1PC: 2.5PG and 1PC: 5PG) in the gels, approximately 60 wt/wt % of its weight at the end of this survey. The different behaviours could be derived from binding of calcium ions (released from BCPNPs) and collagenase leading to inhibition of its proteolytic activity [20]. Hence, such studies clarified that gelatin based materials have a fast biodegradable characteristic in a collagenase containing media while the biodegradation of chitosan-formulated hydrogel could be modulated to enhance mineralization and implant for regenerating specific tissue.

The biomineralization studies could indicate, the PG-PC hydrogels and its nanocomposities sample are immersed in a PBS solution (pH 7.4) at 37℃. After 4 weaks (28 days) of incubation, soaking in the buffer solution, these materials have collected and washed with distilled water to remove soluble inorganic salts and then it charactrized by SEM and other as X-ray diffraction method adapted. A highly crystallized phase of HAP encapsulated gelatin matrix could be observed which indicated a higher deposition of calcium and phosphate ion, which confirmed that the highly PG and PC formulated hydrogels samples performance in enhancing biomineralization ability [21]. The study of gelatin enrich biomaterials have induce outgrowth of cells because gelatin owns more integrin binding domains for cell attachment and enhancing outgrowth of cells [16], [22]. Resulting, it offers several different formulations of the PG-PC hydrogels /IN hydrogels with a various collagenase medicated degradation rate and the high cytocompatibility with mesenchymal stem cells. It because living cells are stained with green fluorescence by an intracellular esterase enzymatic reduction of a nonfluorescent calcein which enables implant with minimally invasive ways exhibiting its greatly potential for regenerating several kinds of typical tissues as combined with bioactive molecules, or/and nanoparticles such as growth factor, bioglass nanoparticles, genes and BCPNPs etc.

4. Conclusion

In conclusion, we have been reported the enzymatically fabricated phenolic protein (gelatin)-polysaccharide (chitosan) and encapsulation of biphasic calcium phosphate to injectable hydrogels which were prepared via enzymatic peroxidase-catalyzed reaction, depending on the amount of chitosan–formulated derivatives, where the IN hydrogels practically performed an appropriated biodegradation rate over a long period of time. But, here, an increase of the newly formulated gelatin based encapsulated BCPNPs nanoparticles biocomposite can enhance biomineralization and proliferation on the composite surface. Regarding to these finding results and there osteoinduction and osteointegration and ostioconductivity characteristics of BCPNPs with PG-PC derivative hydrogels to applying as in bone cell regeneration under specific typical tissue engineering.

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