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Poly(vinylphosphonic acid) immobilized on chitosan: A glycosaminoglycan-inspired matrix for bone regeneration

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ABSTRACT

Glycosaminoglycans modulate the attraction of bone precursor cells including the actions of proteins essential for bone regeneration. In this study, poly(vinylphosphonic acid) was entrapped and immobilized in chitosan prior to formation of a porous three-dimensional matrix. Mimicking glycosaminoglycan interactions occurring *in vivo*, we evaluate its bone regeneration potential. Immobilized phosphonate was characterized by the absence of covalent linkage in Fourier transform infrared spectroscopy and polyanion effect in X-ray diffraction analysis. Higher surface and bulk protein adsorption was observed for the matrices containing phosphonate groups, consequently improving the proliferation and attachment of MC3T3-E1 cells. The porous poly(vinylphosphonic acid)-chitosan matrix was able to promote significant bone formation after *in vivo* implantation in rat calvarial defect as observed from the reconstructed images and histological analysis of tissue sections taken after 4 and 8 weeks. The unique porous matrix showed potential for bone tissue engineering applications.

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1. Introduction

Glycosaminoglycans (GAGs) are a group of extracellular matrix (ECM) polysaccharides that are involved in numerous biological activities and are important as molecular co-receptors in cell–cell interactions through their ability to interact with ECM proteins and peptide growth factors. GAGs play vital roles in the binding and activation of growth factors in cell signal transduction required for biological development such as cell adhesion, migration, growth and differentiation [1,2]. GAG bioactivity is associated with the interaction of its negatively charged groups to the positively charged amino groups of proteins. These charged functional groups are believed to have a crucial role for the formation of proteoglycans and hence the key for biochemical processing/signaling related to cell functionality and survival [3]. Chitosan, a linear polysaccharide composed of *N*-glucosamine and *N*-acetylglucosamine units, is similar in structure to GAGs, containing repeating disaccharide units and hexosamine [3–5]. Chitosan, the deacetylated derivative of chitin, has been extensively used in pharmaceutical formulations, drug delivery systems, protein recognition and separation, tissue engineering, transplant and cell regeneration, due to its excellent properties and biocompatibility and biodegradability [6,7]. But for mineralized tissue regeneration such as bone, osseointegration and osteoconduction are also important other than protein–polysaccharide interactions [3]. Both processes are triggered by other negatively charged groups, specifically the phosphate groups. Phosphates have been recognized by the biomaterials scientific community for purposes of bone regenerative medicine [8,9] and studies have been conducted to modify chitosan with phosphate or phosphonic groups to improve its properties [10]. Thus, incorporation of carboxyl or sulfate groups to chitosan has been investigated in several studies mimicking GAG structure and functionality [11,12].

Amongst the various functional synthetic polymers known today, poly(vinylphosphonic acid) and its derivatives are growing in interest for various applications. Poly(vinylphosphonic acid) (PVPA) is one of the simplest polymeric diprotic acid synthesized by either free radical polymerization of its monomer, VPA or saponification of VPA methyl ester monomer followed by hydrolysis [13]. The abundance of phosphate groups of PVPA has been shown to be beneficial due to its similarity to the phosphate groups of natural bone hydroxyapatite [14]. Due to the cationic nature of chitosan, it readily forms a polyelectrolyte complex with negatively charged polyanions such as PVPA and finally intercomplex aggregation, thus PVPA was immobilized prior to scaffold fabrication.

This study was conducted with the goal of developing a porous matrix resembling glycosaminoglycan structure and functionality

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with chitosan and PVPA. The smaller molecules of PVPA were posited to be possibly trapped in chitosan by increasing the sieving property and immobilized by electrostatic attraction. This system is designed to provide a means of retaining and concentrating desirable factors, such as proteins and other bioactive molecules in biological fluids, either by protein entanglement in the surface or electrostatic interactions to enhance cellular activity involved in bone formation. Moreover, the physically entrapped molecules might adopt a conformation similar to their natural form wherein bioactivity could be retained because the molecular interactions are similar to ECM–protein interactions and the system may even be capable of recruiting desirable growth factors from surrounding tissue fluids *in vivo*.

A range of analyses, including functional and histological, to evaluate the *in vivo* effect in a rat cranial defect model was conducted. In this study, it was hypothesized that this unique system will significantly enhance and accelerate bone repair within a critical-size defect by simulating interactions *in vivo*, providing a highly relevant approach to tissue repair.

2. Experimental

2.1. Chitosan purification

Purification was done to ensure that chitosan was of high purity and free of protein contamination prior to use. Chitosan (DD > 75%; M_w 10^5 Da; Sigma-Aldrich, USA) was dissolved in alkali solution (1 g chitosan/10 ml 1 M NaOH solution). This solid–liquid mixture was heated and continuously stirred for 2 h at 70 °C and then filtered with suction using a Buchner funnel. Recovered solids were washed thoroughly then dried. The recovered chitosan powder was dissolved in 0.1 M acetic acid solution, filtered and precipitated with 1 M NaOH solution. The precipitate was washed thoroughly with deionized water and vacuum dried at room temperature for 24 h. The resulting solid, purified chitosan (CS, DD 85%) is used for the experiment.

2.2. PVPA immobilization

Immobilization of PVPA was done by simultaneous adsorption and cross-linking. PVPA (M_w 24 KDa, Polysciences, Inc., USA) was adsorbed by mixing CS (1:10) with PVPA (800 μg/ml as optimized) in 0.3 M EDC (N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide; Sigma-Aldrich, USA) and MES (2-morpholinoethanesulfonic acid; Sigma-Aldrich, USA) solution for 5 min at 37 °C with gentle tumbling. The mixture was centrifuged to retrieve the supernatant and the resulting PVPA/chitosan (PVPACS) was dried at room temperature for 24 h. The concentration of free PVPA was deduced by colourimetric analysis of phosphate using KH_2PO_4 phosphorus standard. The supernatant was incubated with ammonium molybdate (Sigma-Aldrich, USA) at 37 °C for 2 h to produce a phosphomolybdate colored complex that was analyzed at 820 nm wavelength via spectrophotometer (NanoPhotometer Pearl, IMPLAN, Germany). Using linear regression analysis from known PVPA concentrations, the amount of free PVPA in the supernatant was obtained. Thus, the amount of immobilized PVPA was calculated as the difference of free PVPA and initial amount of PVPA in solution.

2.3. Characterization

Spectra were collected from 675 to 4000 cm^{-1} using a Fourier transform infrared spectrophotometer (FTIR; Nicolet iS10–Smart iTR, Thermo Fisher Scientific, USA). FTIR data of CS before and after exposure to PVPA solution (PVPACS) was collected. PVPACS was rinsed three times with deionized water to remove excess PVPA and EDC and dried prior to analysis. X-ray diffraction (XRD) patterns of CS and PVPACS were also obtained using a desktop x-ray diffractometer (MiniFlexII, Rigaku, Japan).

Electrostatically bound PVPA was further verified by desorption test using three salt concentrations: deionized water (0 mM NaCl), 140 mM NaCl and 500 mM NaCl [15]. PVPACS powder was briefly washed with deionized water and immersed in desorbing solutions at 37 °C with agitation for 24 h. The amount of desorbed PVPA was determined by analysis of the phosphorus content in the respective supernatants, as previously described using colourimetric assay with ammonium molybdate compound.

2.4. Porous matrix preparation

Solution of PVPACS (2% w/v) and CS (2% w/v) were prepared by dissolution in 1% acetic acid. Aliquots of each solutions were placed in poly(vinyl chloride) (PVC) molds and subjected to thermally induced phase separation (−80 °C) and subsequent sublimation of ice crystals for 24 h to form interconnected pores. Lyophilized samples were sequentially immersed in ethanol series (pure, 70%, 50%) and finally Milli-Q ultrapure water (Millipore, USA) to remove acid and once again lyophilized.

2.5. Matrix properties

2.5.1. Morphology and porosity

CS and PVPACS were mounted on sample holders and coated with thin layer of platinum in a sputter coater (Cressington Scientific Instruments, UK). The pore morphology of the matrices was observed under a scanning electron microscope (SEM, JSM-6701F, JEOL, Japan). Pore size distribution range of the matrices was determined by mercury intrusion porosity meter (Quantachrome Porosity Meter, USA) with accompanying Poremaster software.

2.5.2. Swelling behavior in culture medium

Matrices with known diameter and height (1.30 cm in diameter, 0.30 cm in thickness) were washed with phosphate buffered saline (PBS, pH 7.25; Carlsbad, CA) in 24-well plates then incubated in 2 ml MEM culture media (Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (Equitech-Bio, USA). Samples were placed in humidified incubator at 37 °C with 5% CO_2 for 24 h to allow complete swelling of the samples. The changes in diameter and thickness were recorded.

2.5.3. Surface and bulk protein uptake

Protein uptake into CS and PVPACS matrices were examined by measuring the total amount of proteins associated with the matrices. The matrices were extensively washed with PBS and serum-free MEM medium containing 1% of penicillin/streptomycin (Multicell, Wisent, USA) for 2 h. The samples were allowed to swell in serum-free MEM medium for 24 h in a humidified incubator at 37 °C with 5% CO_2 to take expansion into account when calculating the surface and bulk protein concentration. The matrices were then washed three times with serum-free medium before being placed in a 12-well plate with 2 ml MEM medium supplemented with 10% fetal bovine serum and placed in a humidified incubator. After 24 h of incubation, the samples were transferred to a clean 12-well plate and gently rinsed with PBS to remove loosely attached proteins. Proteins in the matrices were measured by Bio-Rad protein microassay kit (Bio-Rad Laboratories, USA). Serum protein absorption on culture plates were also measured for comparison.

2.5.4. Degradation by lysozyme

In vitro degradation rates of CS and PVPACS matrices were determined by measuring the change in sample weight over time under treatment with lysozyme. Initial lyophilized weight of neutralized

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