



Enhanced initial bone regeneration with inorganic polyphosphate-adsorbed hydroxyapatite

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ABSTRACT

Inorganic polyphosphate (poly(P)) can promote binding between fibroblast growth factors and their receptors and enhance osteoblastic cell differentiation and calcification. This study evaluated the possibilities for poly(P) adsorbed onto interconnected porous calcium hydroxyapatite (IP-CHA) as a new bone regeneration material. Prepared 1%, 5%, 25% and 50% poly(P)/IP-CHA composites showed the elution peak of poly(P) between 15 and 20 min, respectively, with the highest value from 50% poly(P)/IP-CHA *in vitro*. Histologically, at 1 week of placement into the femur of rabbits, granulation tissue had penetrated into the pores in all composites and IP-CHA as a control. In contrast, at 2 weeks of placement, newly formed lamellar bone was found in all groups, although a higher amount of bone regeneration was obviously formed in the 25% and 50% poly(P)/IP-CHA with a significantly higher value of bone regeneration ratio of 50% poly(P)/IP-CHA. These results indicate that 25% and 50% poly(P)/IP-CHA composites may enhance initial bone regeneration.

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1. Introduction

Bone regeneration is of key interest in implant dentistry and orthopedics. Autogenous bone grafting is the most predictable procedure for bone regeneration, but has disadvantages, such as limited supply of bone from harvesting surgery, persistence of pain at the donor site, and possible nerve damage or fracture or cosmetic disability at the donor site [1,2]. Bone graft substitutes, including allogeneic bone, xenogeneic bone and some types of ceramics such as hydroxyapatite (HA) and tricalcium phosphate, are currently available to avoid the above problems. However, none has been proved fully satisfactory for clinical demands [3]. For these reasons, the development of new artificial bone which is biocompatible and predictable is anticipated.

In recent years, a number of growth factors or therapeutic agents, such as bone morphogenetic protein-2 (BMP-2) [4–6], basic fibroblast growth factor (bFGF) [7–9], platelet-derived growth factor (PDGF) [10], polyphosphate (poly(P)) [11,12], have been introduced with tissue engineering techniques to enhance bone regeneration. Among these, inorganic poly(P), an orthophosphate polymer found in mammalian nuclei, mitochondria, lysosomes and plasma membranes [13], can stabilize bFGF and promote binding between bFGF and their receptors [11]. Moreover, poly(P) has been shown to enhance osteoblastic cell differentiation and cell calcification in osteoblast-like cells [14] and promote bone regeneration in the alveolar bone of rats [12]. These findings suggest that poly(P) can be expected

to promote biological activity, accelerating bone regeneration. However, little reporting on bone regeneration through use of poly(P) *in vivo* can be found in the literature. In terms of bone regeneration via utilization of tissue engineering, a key element in the process is scaffolding to transport growth factors or therapeutic agents. Recently, a form of interconnected porous calcium HA, IP-CHA, has been introduced to serve as graft material and scaffold for bone formation [15]. Since IP-CHA has a systematic arrangement of spherical uniform pores with interconnections between them, it may provide favorable scaffold, allowing cells or agents into internal structures. Based on recent findings relating to poly(P) and IP-CHA, the combined application of these two materials is expected to become a new bone substitute for bone regeneration.

This study was designed to clarify the possibilities for poly(P) adsorbed onto IP-CHA as a new bone regeneration material. For preparation of this new material, the IP-CHA was soaked in 1–50% poly(P) solutions and dried to fix the poly(P) to the IP-CHA. To examine the basic characteristics, the adsorbed ratio of poly(P) to IP-CHA and the dynamic elution ratio of poly(P) from IP-CHA were evaluated *in vitro*. In addition, bone regeneration was evaluated *in vivo* by implanting the material into the femurs of New Zealand rabbits.

2. Materials and methods

2.1. Materials

Poly(P) with sodium salt with an average chain length of 60 phosphate residues and the distribution of 40–80 polymer size

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phosphate residues (Regenetiss Inc., Nagano, Japan) was selected, with its concentration reported in terms of phosphate residues. Cylinder-type (diameter, 3 mm; height, 5 mm) IP-CHA (NEO-BONE®, Covalent Materials Corporation, Tokyo, Japan) with 75% porosity, a mean pore diameter of 150 μm and interconnected pores 40 μm in diameter was used as a scaffold.

2.2. Manufacture of poly(P)/IP-CHA composites

Each IP-CHA was soaked in a poly(P) solution with adjusted concentrations of 1%, 5%, 25% and 50% (w/w), respectively. The IP-CHA soaked solutions were de-aerated for 2 h with an aspirator. Afterwards, the solution that remained internally was removed by centrifuging (2500 rpm, 2 min), and the poly(P) adsorbed onto IP-CHA was dried for at least 3 days at 37 °C. As a result, 1% poly(P)/IP-CHA, 5% poly(P)/IP-CHA, 25% poly(P)/IP-CHA and 50% poly(P)/IP-CHA composites were obtained, in which poly(P) was adsorbed onto the surface of IP-CHA pores. The material was then sterilized using gamma-ray irradiation.

2.3. Evaluation of outer structure by scanning electron microscopy

The outer structures of 1%, 5%, 25% and 50% poly(P)/IP-CHA composites and IP-CHA as control were observed using scanning electron microscopy (SEM) (JMS-7300, Nihon Denshi Oyo Co. Ltd., Tokyo, Japan) at 100 \times and 1000 \times magnification before and after poly(P) absorption.

2.4. Evaluation of adsorbed poly(P) by toluidine blue staining

Each poly(P)/IP-CHA composite and IP-CHA was cut in the center, and the central portion was exposed. These composites were then soaked in a 0.05% toluidine blue solution (w/v). The solution was de-aerated for 30 min. Afterwards, the material was centrifuged (2000 rpm \times 5 min) three times and dried at 37 °C for 24 h or more. The adsorbed condition of poly(P) was confirmed by toluidine blue staining.

2.5. Measurement of adsorbed amount of poly(P) in vitro

To measure the amount of poly(P) that was adsorbed onto the IP-CHA, 100 mg of poly(P)/IP-CHA was broken down into smaller pieces and dissolved in 0.2 ml of water. To elute poly(P) completely from the IP-CHA, brief sonication was performed. After centrifugation (5000g \times 5 min), 10 μl of supernatant was collected, and 10 μl of 2 N HCl was added. To hydrolyze poly(P) completely into orthophosphate, the acid solution was heated at 100 °C for 30 min. The phosphate concentrations of acid hydrolyzed solution were measured using malachite green (BIOMOL GREEN™, BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) according to the manufacturer's protocol. To estimate the background phosphate level eluted from IP-CHA itself, the phosphate concentration eluted from 100 mg of IP-CHA was also measured according to the procedure described above. The amount of poly(P) adsorbed into the IP-CHA was calculated by subtracting this background level from the total phosphate level eluted from poly(P)/IP-CHA.

2.6. Assay for dynamic elution of adsorbed poly(P)

Each poly(P)/IP-CHA composite was soaked in 1 ml of ultrapure water and de-aerated for 10 min. Then each sample was set in a silicon tube (inner diameter 3 mm, length 35 mm). A constant flow (0.1 ml min⁻¹) of 0.9% NaCl in the tube was achieved with a liquid chromatography system (BioLogic Duo Flow, Bio-Rad Laboratories Japan, Tokyo, Japan). Outflow solution was collected by fraction collector (0.5 ml fraction⁻¹) at 61 time points in every 5 min for a

total for 305 min. Then, each sample solution (70 μl) was hydrolyzed at 100 °C for 30 min with HCl with a final concentration of 1 N. Finally, the phosphate concentration of the hydrolyzed solution was measured using malachite green (BIOMOL GREEN™, BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) according to the manufacturer's protocol. The poly(P) elution profile was determined by calculating the phosphate concentration in each fraction.

2.7. Animal experiment to evaluate bone regeneration in vivo

The protocol for this animal study was approved by the Research Facilities Committee for Laboratory Animal Science, Hiroshima University School of Medicine. Fifteen New Zealand white rabbits (sex, male; weight, 3–3.5 kg) were used. The surgical procedures were performed with the rabbits under general anesthesia with sodium pentobarbital (10 mg kg⁻¹) and local infiltration anesthesia with 2% lidocaine and 1:80,000 noradrenaline. Muscle and periosteal flaps were made on the left and right femurs, and two bone sockets at each side (diameter, 3 mm; length, 5 mm) were prepared. Then 1%, 5%, 25% and 50% poly(P)/IP-CHA composites and IP-CHA as a control were placed into the bone sockets (Fig. 1).

After 1, 2 and 3 weeks of placement, rabbits were anesthetized and perfused with 10% neutral formalin through the aorta. Femurs were harvested and further fixed in 10% neutral formalin for 1 week. Tissue blocks from each bone socket were cut and decalcified by K-CX solution (FALMA, Tokyo, Japan) for 1 week. The blocks were then dehydrated through a graded ethanol series, cleared in xylene and embedded in paraffin. Sections 5- μm thick were obtained and stained with hematoxylin and eosin. Images of bone regeneration were digitized and histomorphometrically analyzed using NIH ImageJ (National Institutes of Health, Bethesda, MD). New bone regeneration in the pores of scaffold at the cortical area was quantified as the ratio of regenerated bone to total cortical bone area (Fig. 2). These bone regeneration ratios were statistically analyzed at the 5% significance level using one-way analysis of variance and the Tukey HSD multiple comparison assay ($n = 4$).

3. Results

3.1. IP-CHA structures

SEM pictures show large spherical pores of IP-CHA divided by thin walls and interconnected with one another. After adsorption



Fig. 1. Poly(P)/IP-CHA composite and IP-CHA placed into bone sockets in the femur.

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