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# An *in vivo* study of a bone grafting material consisting of hydroxyapatite and reconstituted collagen

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This study aims to evaluate the performance of our recently developed microspheres of hydroxyapatite/reconstituted collagen as a bone grafting material. The microspheres were fabricated into a circular disc and implanted in a pre-drilled hole in a rat's calvaria. The bone tissue had regenerated and grown into the disc bone graft 4 weeks following implantation. After 16 weeks of implantation, the regenerated bone had integrated with the remaining material and made close contact with it. The disc had been completely absorbed with almost no visible bone graft left after 24 weeks of implantation. In contrast, a hydroxyapatite disc still remained intact on the 24th week after implantation. These results suggested that the hydroxyapatite/reconstituted collagen microsphere can be used as an excellent bone grafting material.


## 1. Introduction

Reconstruction of bone defects caused by trauma or surgical resectioning of a bone tumor is a major issue in maxillofacial orthopedic surgery. Several grafting materials are currently available to improve bony topography. The bone grafts used today include autografts [1], allografts [2, 3], xenografts and synthetic composites [4]. While autogeneous bone grafts are well acceptable for bone regeneration, it has to be dissected from another bone tissue of the patient. This not only causes donor site morbidity, but it is also difficult to adjust the dissected tissue to form the desired shape. On the other hand, both allografts and xenografts have disadvantages and may cause an immune reaction and/or the transfer of pathogens.

Collagen and hydroxyapatite are commonly used as bone-filling materials in orthopedic surgery [5–7]. Hydroxyaptite, almost identical to the bioapatite of bone, has been widely used as an artificial bone substitute. Although small particulate hydroxyapatites can be absorbed and remodeled in the host [8], these ceramic particles dislocate within the tissue if implanted directly. To eliminate such undesired mobility, hydroxyapatite powders are often mixed with collagen [9], gelatin [10] and fibrin glue [11] when used as bone grafts.

We have recently developed a novel protocol to prepare composite microspheres (200–300 $\mu$m) containing hydroxyapatites and atelocollagen. These microspheres contain hydroxyapatite that is micron in size, and dispersed in a matrix of reconstituted fibrous collagen. *In vitro* test results indicate that these microspheres are excellent carriers for culturing osteoblast cells [12]. In this study, we fabricated composite discs made of the hydroxyapatite/collagen microspheres, and evaluated their *in vivo* performance by implanting the composite discs into pre-drilled cavities in a rat's calvaria. We have demonstrated that these microspheres exhibited excellent biocompatibility, suggesting that they have a potential application as bone grafting material.

## 2. Materials and methods

### 2.1. Materials

Collagen was prepared from rat's tail tendon according to the procedure described previously [13]. Hydroxyapatite was purchased from Merck (Darmstadt, Germany) and its structure was confirmed by X-ray diffraction analysis as having a Ca/P ratio of 1.62. The average particle size and surface area were determined to be 5.3 $\mu$m and 68 $m^2/g$, respectively. All other chemicals used were of reagent grade.

### 2.2. Preparation of microsphere

The particulate hydroxyapatite was mixed and suspended in a collagen solution with a weight ratio of

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35:65 (collagen to hydroxyapatite) at 4 °C. This suspension was added drop by drop to a bath of 100 ml olive oil (37 °C) while stirring at 350 rpm. Reconstitution of collagen in the suspended aqueous droplets was achieved by incubating at 37 °C for one hour. The hydroxyapatite/collagen gel beads thus formed were collected and transferred to a solution of 2.5% glutaraldehyde, and then incubated at 37 °C for three more hours. The cross-linked gel beads (approximately 500 $\mu$m in diameter) were washed repeatedly with 0.02 M PBS; they were then lyophilized and stored at −20 °C. These microspheres were fabricated into circular disc by placing 20 mg of hydroxyapatite/collagen particles into a tungsten steel model and pressed them into a cylindrical form (4 mm in diameter, 1 mm in length). The resulting discs were sterilized by UV irradiation before implantation.

*Figure 1* Photograph of examples of the hydroxyapatite disks (HAP) and hydroxyapatite/collagen disks (COHAP) used in this study. The size is 4 mm in diameter, 1 mm in length.

### 2.3. Animal study

Twelve Sprague-Dawley rats of approximately 200 g in weight were used in this experiment. The animals were anesthetized by an intramuscular injection of ketamine (25 mg/kg) and Combelen® [*N*-(3′-dimethylaminopropyl)-3-propionylphenothiazine] (5 mg/kg). The head of the rat was then fixed in a stereotaxic instrument (KOPF, model 900), and the calvarial skin and periosteum were incised after shaving. The temporal bone was exposed and a bone defect of about 4 mm (in diameter) was created using a 0.5 mm drill on both side of the median suture. The composite disc was then placed into this hole. To trace the newly formed bone structure, the rats were injected intramuscularly with tetracycline HCl (20 mg/kg) and xylenol orange (90 mg/kg) every two weeks until they were sacrificed.

### 2.4. Histological methods

The animals were sacrificed at 2, 4, 16, 24 weeks after implantation. The cranium specimen containing the implanted material and its surrounding bone tissue was dissected from the rat and first examined radiographically. For histological observation, this dissected tissue was fixed in a 10% buffered (pH 7) formalin solution. The fixed specimens were then dehydrated in alcohol over a successive concentration change from 50 to 100% alcohol, and washed with acetone before embedded in polymethylmethacrylate. After hardening, the specimen was cut into slices of approximately 1 mm thick using a microtome. The tissue sections were glued to a slide and polished manually to a thickness of about 50 $\mu$m under cooling water. Some animals, after implantation, were injected with a fluorescence dye mixture and the specimens were then assessed for intra-vital staining with tetracycline using a fluorescent microscope [14, 15].

## 3. Results

### 3.1. Radiographic results

Fig. 1 shows a pair of discs prepared separately from hydroxyapatite particles and hydroxyapatite/collagen microspheres. These two different discs were placed independently into two pre-drilled holes in the calvaria of the same rat. The X-ray scans of the skulls of the rats sacrificed at 2, 4, 16 and 24 weeks after implantation are shown in Fig. 2. As indicated by the roentgenographs, the dark areas found within the composite disc are the implants being penetrated by bone tissue and fluid during the early stages after implantation. The hydroxyapatite disc, on the other hand, remained almost intact even after 24 week of implantation.

### 3.2. Histological results

The ingrowth of bone tissue into the composite disc was further investigated by the histological staining. As shown in Fig. 3, two weeks after the implantation, blood clots and connective tissues were found surrounding the composite discs within the defect area. Although the bone callus around the fracture site of calvaria could be clearly seen in the vicinity of the trauma cavity, the formation of new bone can not be detected at this early stage of bone repair. When the specimen was examined under a fluorescence microscope, the collagen (displaying green color under the fluorescence microscope) appeared to be distributed throughout the implant material. At the 4th week after implantation, bone tissue was beginning to grow into the disc. The bony callus has become more prominent and a bony sleeve has formed outside the implant. In addition, the hydroxyapatite/collagen microspheres have been absorbed significantly and the implanted material is surrounded by new bone over its peripheral portion (Fig. 4). After 16 weeks of implantation, the regenerated bone has already integrated into the remaining material. As shown in Fig. 5, new bone tissue has grown into the matrix and there is obvious close contact between the two. Overall, the bony callus and the composite disc has been absorbed to an even greater extent and only a few osteocytes can be found embedded within the remaining implant materials (Fig. 5). After 24 weeks of implantation, the composite discs has been completely absorbed, there is almost no visible bone graft left.

The regeneration of bone in the implanted rat calvaria was studied in more detail using the fluorescence

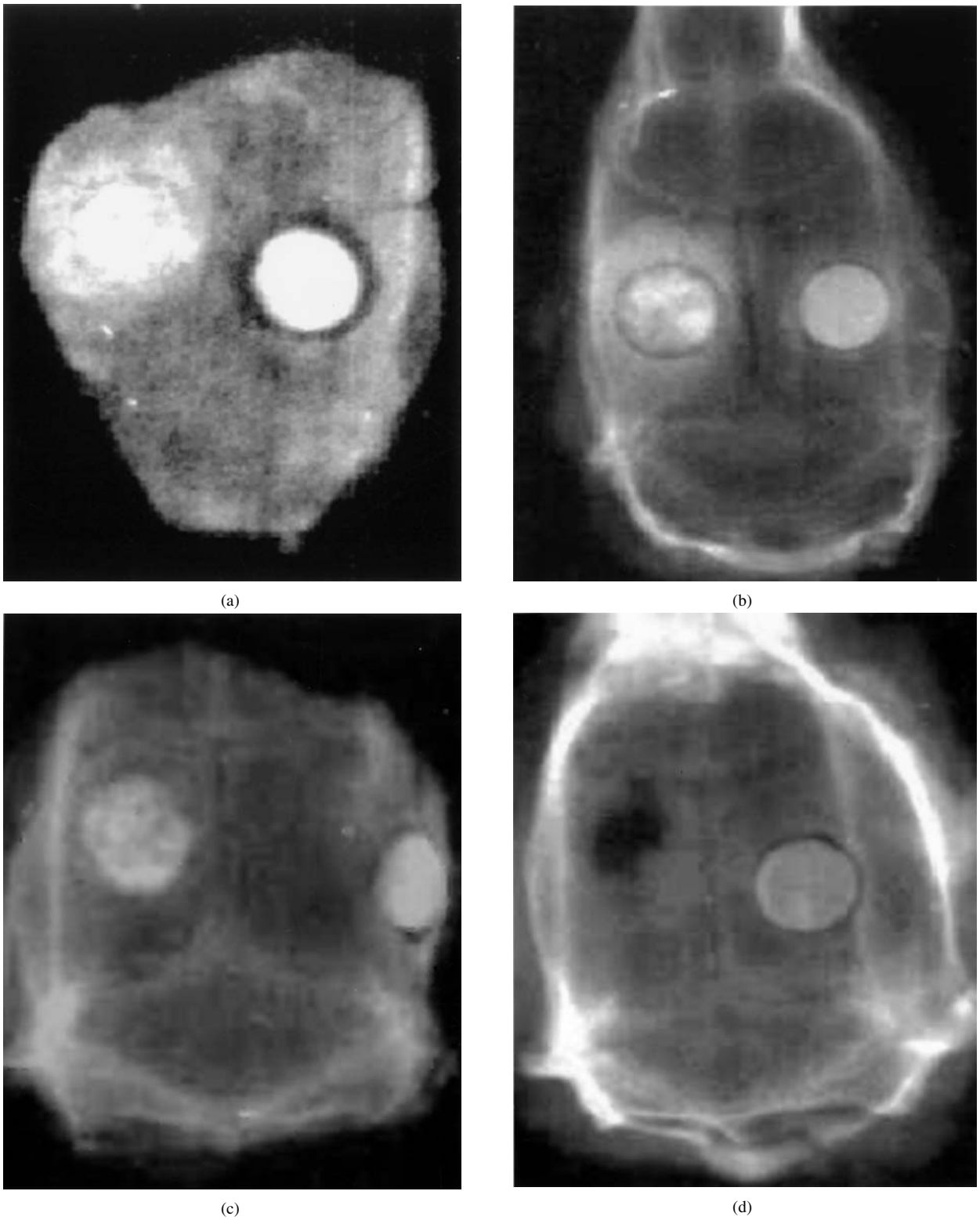
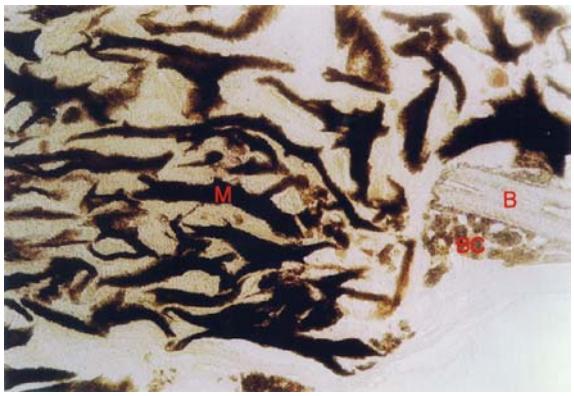
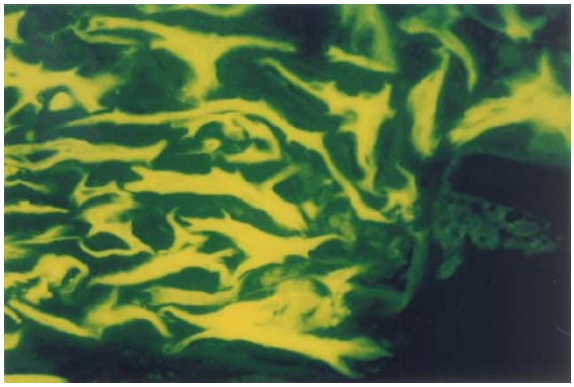


*Figure 2* Roentgenography of SD-rats' skull showing trephine defect filled with the collagen/hydroxyapatite microsphere disk (left side) and the hydroxyapatite disk (right side). (a) 2 weeks, (b) 4 weeks, (c) 16 weeks and (d) 24 weeks.

labeling method described by Milch [14] based on the ability of new bone tissue to take up tetracycline. This works because tetracycline can chelate biological active ions such as calcium and after tetracycline has been administered into the blood stream, the tetracycline bound to the calcium is deposited in the area where new bone is mineralizing [15]. Since tetracycline emits fluorescence, lines of concentrated tetracycline (bright green line in Fig. 6) are deposited in the zone of newly formed bone and can be seen clearly under a fluorescence light microscope. In addition, the Harvesian system with trapped osteocytes and an outer circumferential system could be found in this specimen using optical microscope (Fig. 6). A prominent cement line, which is specific to bone remodeling [16], can also be located in the specimen after toluidine blue staining. As shown in Fig. 7, a cement line was clearly seen at the 24th week after implantation. In contrast, no bone repair was found around the hydroxyapatite disc even after 24 weeks of implantation (Fig. 8).

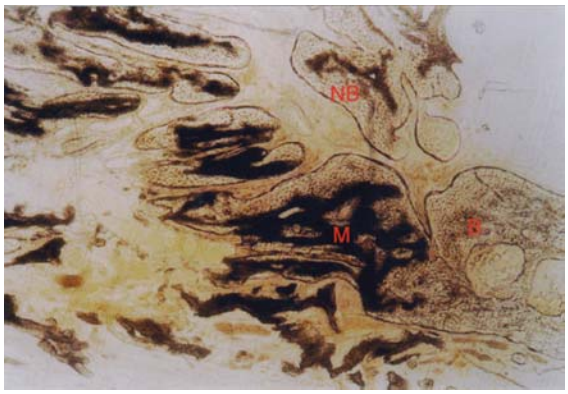



(a)

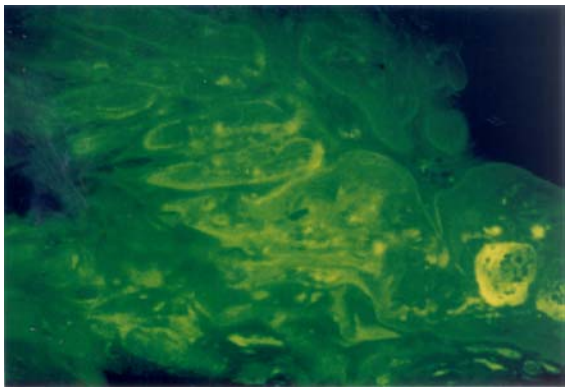


(b)

*Figure 3* Photomicrograph of collagen/hydroxyapatite microsphere disk after 2 weeks of implantation (a) optic microscopy, (b) same field as in (a) by fluorescence microscopy. M: implant materials, B: bone, BC: bone callus.

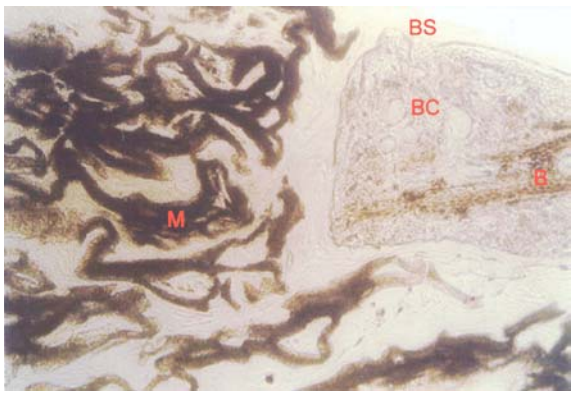



(a)

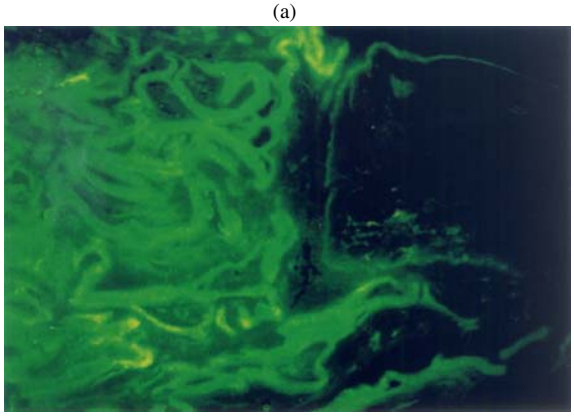


(b)

*Figure 5* Photomicrograph of collagen/hydroxyapatite microsphere disk after 16 weeks of implantation (a) optic microscopy, (b) same field as in (a) by fluorescence microscopy. M: implant materials, B: bone, NB: new bone.

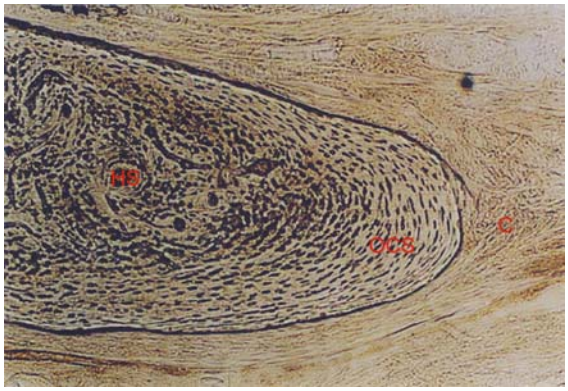



(a)

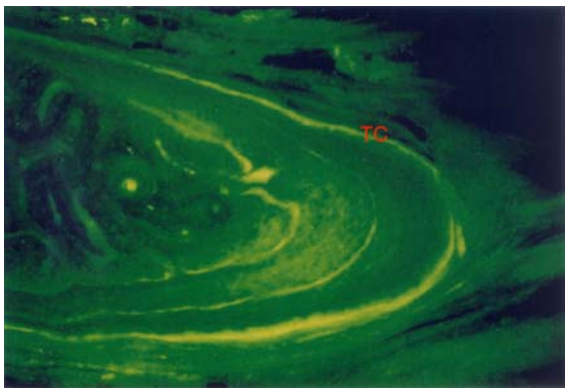


(b)

*Figure 4* Photomicrograph of collagen/hydroxyapatite microsphere disk after 4 weeks of implantation (a) optic microscopy, (b) same field as in (a) by fluorescence microscopy. M: implant materials, B: bone, BC: bone callus, BS: bony sleeve.




(a)




(b)

*Figure 6* Photomicrograph of collagen/hydroxyapatite microsphere disk after 16 weeks of implantation (a) optic microscopy, (b) same field as in (a) by fluorescence microscopy. TC: tetracycline, C: cavity, HS: haversian system, OCS: outer circumferential system.

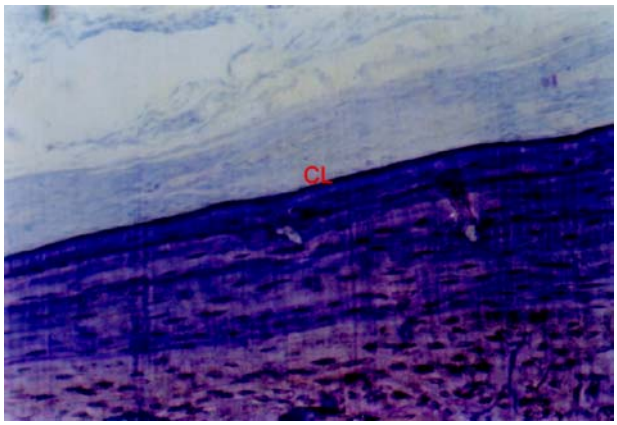



*Figure 7* Histological view of bone regeneration in the bone defect (Tolidine blue stain) after 24 weeks of implantation. CL: cement line.

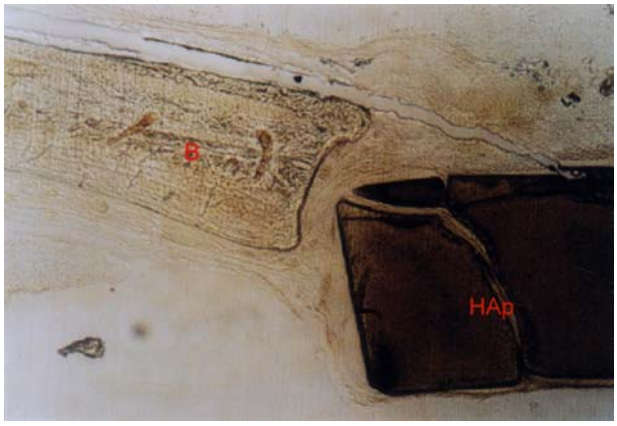



*Figure 8* Photomicrograph of a hydroxyapatite disk after 24 weeks of implantation. HAp: hydroxyapatite disk, B: bone.

## 4. Discussion

An ideal bone substitute must satisfy the following requirements: (1) the surface should be capable of supporting attachment and proliferation of osteogenic cells (2) the substitute should resorb as soon as it has served its purpose of providing a template for regeneration; and (3) neither substitute nor its degradation products should provoke an inflammatory and/or toxic effect.

Because of its similarity to natural bone composition, a synthetic composite of collagen and hydroxyapatite is conceivably an excellent bone substitute. Flautre *et al.* [17] recently prepared hydroxyapatite powders coated with collagen in ethyl-2-hexyl cocoate. In their study, a significant amount of hydroxyapatite was not absorbed probably because that the hydroxyapatite they used had a size of about 45 $\mu$m. Pohunkov [8] reported that hydroxyapatite of a size smaller than 25 $\mu$m could be phagocytosed by macrophage. This explains why our composite disc of microspheres containing hydroxyapatite particulates of approximately 5 $\mu$m could be absorbed *in vivo*. It is however noteworthy to mention that in our study the control hydroxyapatite is not absorbable, probably because the compressed disc forms a dense structure and has the particles within it so tightly aggregated that macrophage are unable to engulf them.

We have found that hydroxyapatite/collagen microspheres serve as a good temporal defect scaffold during early bone regeneration immediately after trauma. Collagen is a component of the extracellular matrix that acts as a cell adhesive and has been shown to be able to induce osteoblasts proliferation, which should accelerate bone regeneration. *In vivo*, collagen will be degraded by collagenase and the smaller hydroxyapatite particles can be resorbed by macrophages or osteoclasts. These together contribute to the excellent biocompatibility and potential bone graft application of the collagen/hydroxyapatite microspheres.

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