In vivo evaluation of hydroxyapatite foams

P. Sepulveda,[1] A. H. Bressiani,[1] J. C. Bressiani[1] L. Meseguer,[2,*] B. König, Jr.[3]
[1]*Instituto de Pesquisas Energéticas e Nucleares, IPEN/CNEN, Cidade Universitária, C. P. 11049-Pinheiros, 05542-970 São Paulo, SP, Brazil*
[2]*Universidad de Murcia, Laboratorio de Cirurgia Ortopedica Experimental (Prof. M. Clavel-Sainz), Spain*
[3]*Department of Functional Anatomy: Structure and Ultra-structure, Institute of Biomedical Sciences, University of São Paulo, P. O. Box 66208, 05389-970, São Paulo, SP, Brazil*

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Abstract: Hydroxyapatite (HA) is widely applied as bone graft material due to its osteoconductive potential and well-established biocompatibility. In this work, macroporous hydroxyapatite structures made through foaming of aqueous suspensions and gelcasting were tested for *in vivo* osteointegration. These foams are composed of a three-dimensional array of spherical pores with diameters of approximately 100–500 μm, interconnected by windows of smaller size in the range of 30–120 μm. The HA foams were implanted in the tibia of albino New Zealand rabbits and removed after a period of 8 weeks. Histological analysis revealed that the pores in the foams were partially or completely filled progressively with mature new bone tissue and osteoid after the implanted period. No immune or inflammatory reactions were detected. The high osteoconductive potential of the HA foams provides a potential structure for use as bone substitute in orthopedic, oral, and cranio-maxillofacial reconstructive surgery, and as dento-alveolar implants. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 62: 587–592, 2002

Key words: porous hydroxyapatite; gelcasting of foams; osteointegration; rabbit tibia; bone grafts

INTRODUCTION

Development of suitable materials for reconstruction of bone defects has stimulated a wide field for research in the recent decades. Filling of bone defects is a routine procedure in arthroplasty revision surgery, healing of excised bone cysts or tumors, craniofacial reconstructions, traumatic bone loss, exodontia, osteotomy, periodontal intra-osseous resorption, among many other cases.[1]

Bone grafts can be autogenous, allogenic/xenogenous, or alloplastic. Alloplastic materials comprise the class of synthetic materials whose main advantage is the supply of high purity that avoids antigenicity problems and donor-site morbidity. Titanium, platinum, calcium phosphates, glass–ceramics, bioactive glasses, and a variety of polymers are examples of alloplastic implants. In general, ceramics such as alumina, zirconia, and calcium phosphates have an advantage over metals for repair and reconstitution of damaged parts of the skeleton principally because of their lack of toxicity to the physiological medium.[2,3] Hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$ and other calcium-phosphate based materials have attracted considerable interest because of the similarities with the mineral fraction of bone and their high osteoconductive potential.[4–6] Their use has been limited, however, as the materials are presented as granules or small dense or porous forms due to low inherent strength.

Recent advances in materials processing have been quickly incorporated into the biomedical field, to obtain more reliable grafts and implants. Several technologies are currently available to manufacture strong and reliable macroporous ceramics that have great potential to replace bone tissue. Special attention has been drawn to the novel route of gelcasting foams. This method yields compounds in various porosity fractions that are noncytotoxic, and have optimized strength and open spherical pores, as shown in previous works.[7–9] The macropores and the highly interconnected network provide the means of access for ingrowth of surrounding host tissues, facilitating further deposition of newly formed bone in the spherical cavities. Additionally, the intricate shape of the walls provides a framework that supports the organisation

*Academic Visitor at the Imperial College of Science, Technology and Medicine, London
Correspondence to: P. Sepulveda; e-mail: pilarsi@net.ipen.br
Contract grant sponsor: FAPESP (Brazil)

of growing tissue, improving biological fixation and avoiding drawbacks that may result from implant mobility.[2,4]

The current work addresses the *in vivo* evaluation of bone-like structures fabricated with hydroxyapatite, which is the result of a recent development in processing of macroporous materials. These porous ceramics meet many of the requirements to enable their use as aids in reconstitution and substitution of damaged bone tissue and various other biomedical fields such as membranes for enzyme cultivation, carriers for controlled delivery of drugs, and matrices for tissue engineering.

MATERIAL AND METHODS

Hydroxyapatite foam preparation and characterization

Macroporous bodies of biomedical-grade hydroxyapatite (Plasma Biotal Ltd., UK) were produced by a novel route that comprises foaming and setting through gelcasting.[9] The procedure involves dispersion of an aqueous suspension of the HA powder using polyacrylate derivatives as dispersing agent (Dispex A40, Allied Colloids, UK). Acrylic monomers (ammonium acrylate, Allied Colloids, UK) were also incorporated into the suspensions to promote gelation by *in situ* polymerisation. Prior to this, the mixture was foamed by agitation with a double-blade mixer, aided by the addition of a nonionic surfactant (Tergitol TMN10, Aldrich Chem. Co.) that reduces the surface tension of liquid–gas interfaces and stabilizes the foam. Gelation of the foamed suspensions was promoted by addition of initiator and catalyst for *in situ* polymerization of the monomers, using the redox system of ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylene diamine (TEMED) (both by Aldrich Chem. Co.). The bodies were cast into molds and dried at 100°C for 24 h. Sintering of the specimens was applied at 1350°C for 2 h for matrix consolidation. Scanning electron microscopy (SEM) (Leica—Stereoscan 440) of gold-coated specimens was carried out for observation of the morphology of the porous structure.

Animals and surgical procedure

For the animal experiments, cylindrical specimens of hydroxyapatite foams with dimensions of 3 mm in diameter were cut from sintered bodies with a diamond-coated core-drill. Bodies with porosity in the range of 80–85% vol were tested.

For care and use of laboratory animals, the guidelines and regulations of Institute of Biomedical Sciences, USP, have been observed. Six adult albino New Zealand male rabbits weighing between 2.5 and 3.0 kg were anaesthetized by a intravenous femoral injection of 1 mg/kg Rompun™ (Xylazyn Chloride) and 20 mg/kg Hypnol™ (3% sodium pentobarbital). The area of implantation was shaved and prepared with iodine wash (Betadine™). Through an anteromedial approach into the third proximal of the tibia, a cortical bone defect was drilled with diameter of 3 mm. The defect was filled with the HA foam (Fig. 1). The periosteum and the skin were carefully closed with simple uninterrupted sutures. The periosteum was preserved in order to achieve better postoperative healing with bone remodeling. Antibiotic therapy (0.5 mL of benzetacil (benzilpenicylin of 1,200,00 U.i.n.) was carried out during the immediate postoperative period (48 h). Rabbits recovering from the surgical procedure were kept warm with a heating pad and observed until ambulatory. The implants were left for a period of 8 weeks, following a standard procedure.[10] The animals were sacrificed by an anaesthetic overdose.

Histological analysis

The area of implantation was sectioned and the tibial segments were immediately fixed with Xylazyn, dehydrated, and embedded in methyl methacrylate resin. Block sections 0.8 mm thick were prepared perpendicular to the tibial axis with diamond-coated disks. The sections were ground and polished with diamond paper, then stained with Masson Trichrome method for observation under light and scanning electron microscopy. The chemical profile at the bone–material interface was studied by energy dispersive X-ray analysis (EDXA).

RESULTS

HA foam characterization

A representative specimen of the hydroxyapatite foams tested in this work is shown in Figure 2. The

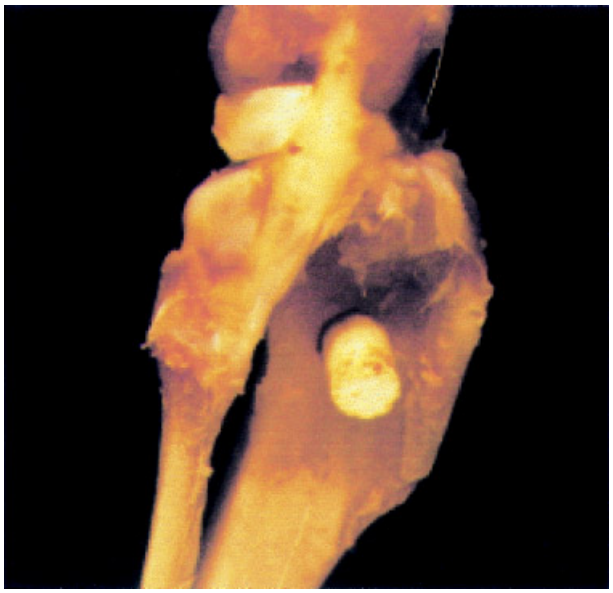

Figure 1. Illustration of the implant area in the anterior medial aspect of rabbit tibia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

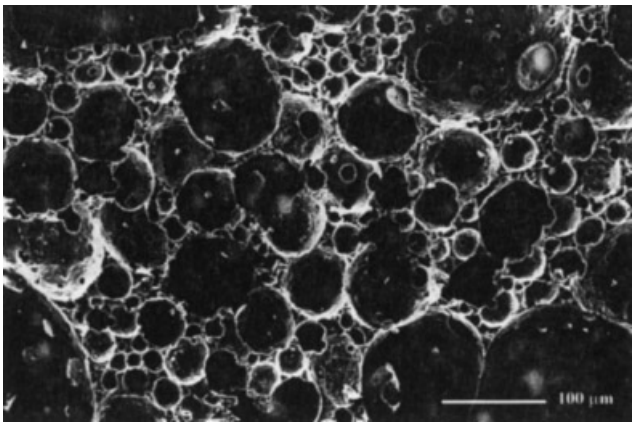

Figure 2. SEM micrograph of hydroxyapatite foams, showing a three-dimensional framework and highly interconnected spherical pores.

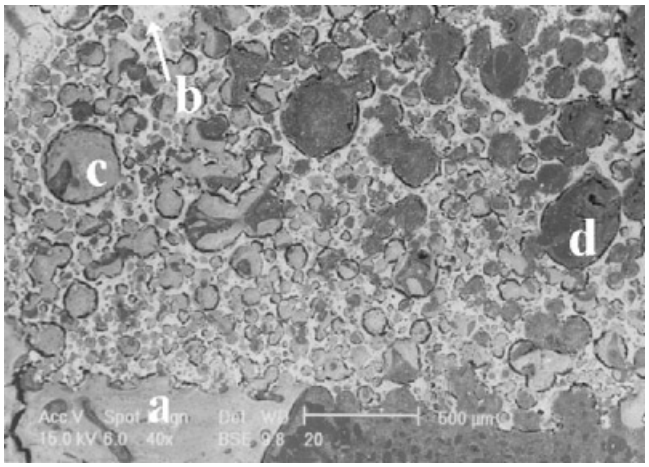

Figure 3. Section of HA foam implant after 8 weeks, showing osteo-integration and extensive bone ingrowth in within the pore and connections of the structure. The original bone is shown in (a), the porous implant appear in light-colored areas (b), whereas new bone tissue appears in light grey (c) and collagen in dark grey (d).

structure appears to be typically composed of large spherical pores that result from the bubbles in the foam, thoroughly interconnected through open channels, all enclosed by a compact framework of polycrystalline hydroxyapatite. Previous work reporting the characterisation of HA foams showed that pore size can be widely varied to suit different requirements and uses.[8] Large spherical pores vary within the size range of 100–500 μm and the smaller interconnecting windows between 30 and 120 μm, depending on the foam density. These structures show high levels of permeability ($k_1 = 1.22 \times 10^{-11}$ to 4.31×10^{-10} m^2, $k_2 = 1.75 \times 10^{-6}$ to 8.06×10^{-5} m), variable compressive strength (1.6–5.8 MPa), and elastic modulus on compression (3.6–21.0 GPa) depending on the density.[8] In general, other processing routes to manufacture highly porous ceramics lead to much lower mechanical properties than the gelcasting of foams.[7,8]

In vivo osteointegration

All animals survived the 8-week study period without evidence of inflammation or infection at the implantation site. No other immunological or adverse reactions were noted.

Observations of the implant sections reveal an extensive ingrowth of new-formed bone tissue into the pores of the HA implants. New bone formation can be detected around and within the implant, thoroughly connected by bridges across the implant open pores. Figure 3 illustrates bone–implant integration, new bone filling the foam structure progressively, from areas of neighboring old bone toward the inner part of the implant. In general, smaller pores are filled with bone more extensively than larger pores, since the latter require correspondingly more cellular activity to be filled. In spite of this, larger pores have been reported to provide higher integration strength.[11] The differences in density values tested in this work produced negligible effects on bone ingrowth fractions, as the pore structure was very similar in all materials.

In all implants, bone tissue deposition occurred mainly in the form of layers with trabecular architecture and a significant number of osteocyte lacunae were noticed. Newly formed bone tissue at various stages of maturation were also detected. Figure 4 gives an example of mature bone with regular osteon structure surrounded by areas where the new bony tissue was still in early stages of maturation.

The various calcification densities areas present in the implant pores can be demonstrated using Masson trichrome staining through different grades of staining that result from the differences in mineral concentrations (Fig. 5). In this figure, scattered areas of older bone tissue (dark blue) and newly formed bone (light blue) are clearly shown. The region also depicts the tendency of osteon-structured bone to form mainly in areas neighboring older bone. Implant pores filled with nonmineralized bone (osteoids) are demonstrated with dark red (Fig. 5).

A whole implant section is shown in Figure 6. This micrograph demonstrates healing from the stage of unfilled pores at the outermost zones of the implant (dark pores on the right of the illustration as indicated in (c) toward pores completely filled with trabecular bone (in b) neighboring the original bone (in a), within the 8 weeks of implantation. Bone formation within small pores was verified all throughout the implant, regardless of the distance of these pores from the older bone region.

Under scanning electron microscopy, EDXA analysis revealed no significant changes in the CA/P ratios of the HA implant surface, compared to the original

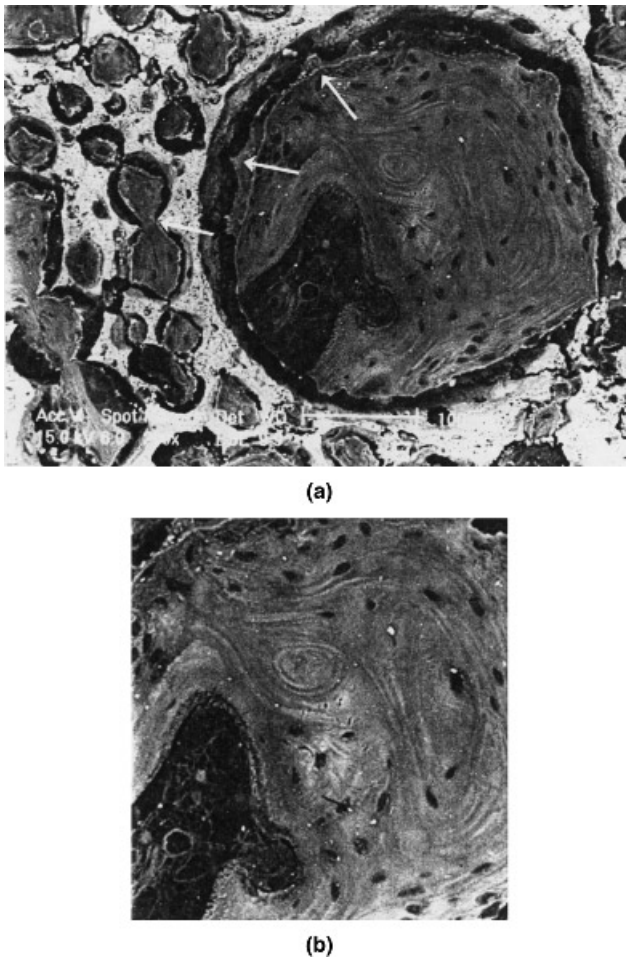

Figure 4. (a) Section of HA foam implant after 8 weeks, giving evidence of areas characterized by osteon regular structure surrounded by pores filled with bone that did not yet achieve full maturity. The arrows show how the bone tissue in the large spherical pores of the foam advances through the open channels. (b) Detail of (a) showing the remodeling in mature bone.

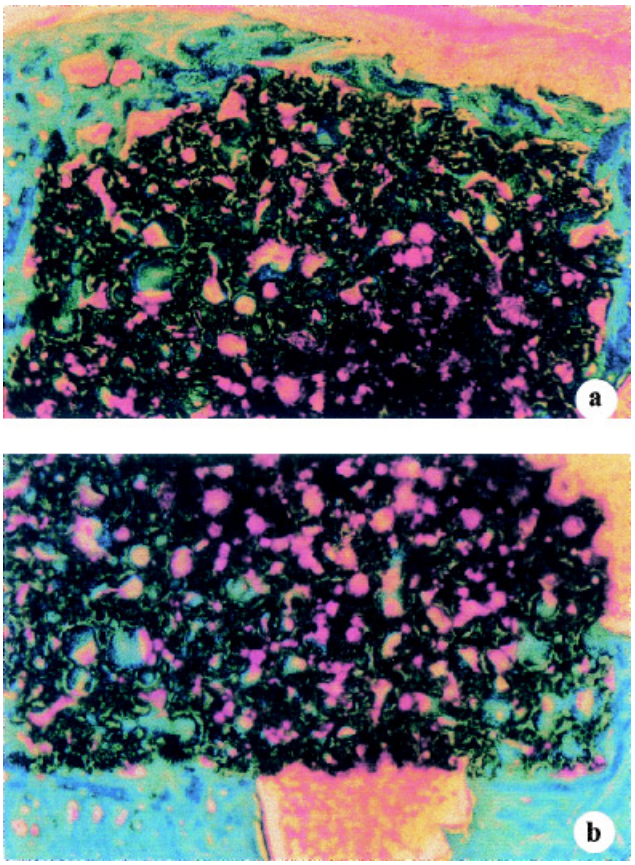

Figure 5. View of implant section stained with Masson Trichrome. Bone at various levels of calcification can be seen, with different intensification of mineral deposition. Zones of new bone tissue (light blue) appear within the pores. Dark blue areas identify islands of older bone tissue. The bone tissue appears to be deposited in lamellae, either in flat or in rounded configurations. Scattered calcification zones progressing from medullar area toward the implant region. (original magnification ×30).

material. This result implies that there was no measurable dissolution of chemical species from the HA implant that was in contact with physiological fluids and bone tissue, within the 8-week implantation period. Examples of the EDXA spectra for the implant area and bone are shown in Figure 7. The analysis reveals calcium and phosphate as the main elements in the implant area, whereas in bone significant quantities of silicon are observed. It has been suggested that silicon in immature bone is a bone mineralizing agent.[12]

DISCUSSION

Bone regeneration through the use of foams

An ideal candidate for bone graft implants must combine biocompatible compositions with specific macroporous structures, satisfying criteria of mechanical strength. Preferably, compositions should have bone-bonding and osteoconductive properties, although this is not an essential requirement.

A suitable porous network is required to promote extensive vascularization, for bone ingrowth, rapid bone regeneration, and good implant integration. Bone ingrowth rates depend greatly upon the pore morphology, the degree of pore connectivity, and pore volume. Pores larger than 100 μm are seen as necessary to allow blood and nutrient supply access for bone mineralization within the graft.[4] Nevertheless, processing of brittle materials with large pores always occurs with deleterious effects on the mechanical properties.

The manufacturing method presented in this work has been successfully applied to numerous compositions and has been shown to provide considerably higher mechanical strength than other routes.[7] Foaming as a technique to incorporate porosity into ceram-

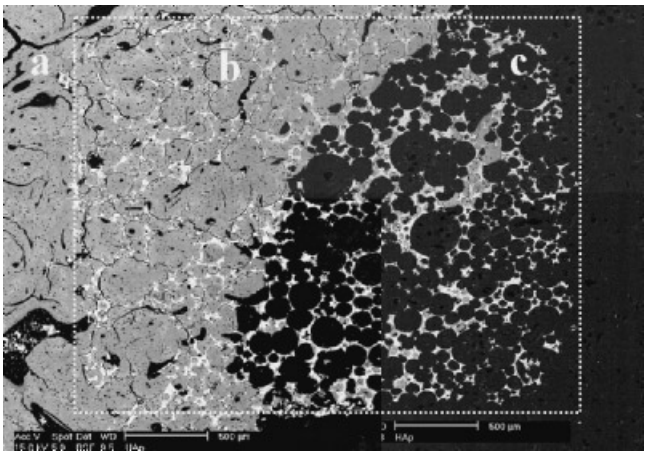

Figure 6. A panoramic view of HA foam implant (within dotted lines) being invaded by newly formed bone. The region of original bone appears in (a), pores completely filled with trabecular bone in (b), and unfilled pores in (c).

ics has many advantages, including its simplicity, the regular pore shape, and the ability to tailor pore size range. Although a precise replica of cancellous bone structure has not been synthesized to date, as synthetic materials differ from bone in terms of pore size distribution, interconnectivity, and porosity levels, foamed materials provide a good alternative for bone grafts.

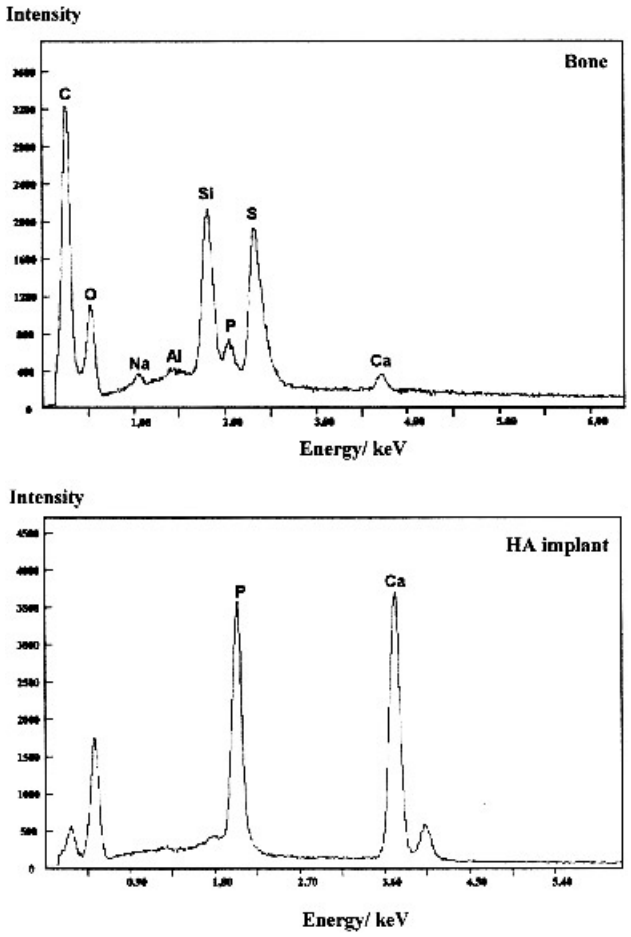

Figure 7. Spectra of energy dispersive EDXA of new deposited bone in the porous hydroxyapatite and in implant area that interfaces newly bone tissue.

The pore network of open-cell foams was previously shown to support bone integration using polyurethane.[13] However, porous forms of calcium phosphates, in particular hydroxyapatite and β-calcium phosphate, have been preferred as bone graft materials because of their high biocompatibility and osteoconductive properties.[14–16] Other compounds, such as bioactive glasses and glass–ceramics have even greater potential to regenerate bone tissue than hydroxyapatite, as demonstrated by *in vivo* experiments using particles.[17] Recent work reported the manufacture foams from sol–gel glasses to generate bioactive scaffolds for tissue engineering.[18]

The HA foam structure presented in this work was filled almost entirely with trabecular bone within the 8 weeks of implantation, confirming the high osteonconductive behavior of HA and the ability of the porous network to promote tissue ingrowth. Considering these results and the set of physical properties that characterizes these materials, the HA foams demonstrate great potential as a structure for bone grafting. Furthermore, given that the process is applicable to a variety of compounds, the compositions may be chosen as desired, to achieve various degrees of resorbability and osteoconductive properties.

CONCLUSIONS

In vivo evaluation of macroporous hydroxyapatite manufactured by the gelcasting of foams technique was carried out in the tibia of New Zealand rabbits. There was no evidence of immune or inflammatory responses to the materials used in this study. The HA foams allowed extensive osteointegration within 8 weeks of implantation, indicating their potential for use as a scaffold for *in vivo* bone growth. The use of different raw materials with a similar porous structure to the one shown here can provide a variety of chemical, mechanical, and bioactive properties in order to suit a wide variety of applications, such as tissue engineering scaffolds and drug-delivery systems.

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