ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2017) xxx–xxx

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Chitosan-based nanocomposites for the repair of bone defects

L. Keller[a,1], A. Regiel-Futyra[b,1], M. Gimeno[c], S. Eap[a], G. Mendoza[d,e,f,*], V. Andreu[d,e,f,*], Q. Wagner[a], A. Kyzioł[b], V. Sebastian[d,e,f], G. Stochel[b], M. Arruebo[d,e,f], N. Benkirane-Jessel[a]

[a]*INSERM, Unité Mixte de Recherche UMR 1109, Team "Osteoarticular and Dental Regenerative NanoMedicine," Fédération de Médecine Translationnelle de Strasbourg, Faculté de Chirurgie Dentaire, UDS, Strasbourg, France*
[b]*Faculty of Chemistry, Jagiellonian University, Kraków, Poland*
[c]*Faculty of Veterinary, Department of Animal Pathology, University of Zaragoza, Spain*
[d]*Department of Chemical Engineering, Aragon Institute of Nanoscience (INA), University of Zaragoza, Zaragoza, Spain*
[e]*Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, 28029, Madrid, Spain*
[f]*Aragon Health Research Institute (IIS Aragón), 50009, Zaragoza, Spain*

Received 13 December 2016; accepted 8 June 2017

Abstract

Chitosan scaffolds of different deacetylation degrees, average molecular weights and concentrations reinforced with silica nanoparticles were prepared for bone tissue regeneration. The resulting nanocomposites showed similar pore sizes (<300 μm) regardless the deacetylation degree and concentration used in their formulation. Their mechanical compression resistance was increased by a 30% with the addition of silica nanoparticles as nanofillers. The biocompatibility of the three-dimensional chitosan scaffolds was confirmed by the Alamar Blue assay in human primary osteoblasts as well as the formation of cell spheroids indicative of their great potential for bone regeneration. *In vivo* implantation of the scaffolds in a mice calvaria defect model provided substantial evidences of the suitability of these nanocomposites for bone tissue engineering showing a mature and dense collagenous tissue with small foci of mineralization, vascularized areas and the infiltration of osteoblasts and osteoclasts. Nevertheless, mature bone tissue formation was not observed after eight weeks of implantation.

Key words: Chitosan; Silica nanoparticles; Scaffold; Bone regeneration; Human primary osteoblasts

The prevalence of bone diseases is markedly growing due to the increase in life expectancy and the population aging, becoming a global public health problem and representing a burden for health care systems. Current clinical treatments for bone healing, replacement or regeneration are based on the use of bone grafts (autografts or allografts) and bone graft substitutes, which differ in their strength and osteoconductive, osteoinductive and osteogenic potential.[1] However, due to the limitations associated with their clinical use, the ongoing search of other therapeutic strategies is focused on the development of novel graft substitutes for their implantation in the damaged bone region. One of the main approaches for tissue regeneration includes the development of 3-dimensional (3D) scaffolds that closely mimic the composition and the fibrillary structure of bone extracellular matrix.[2,3] Besides, a highly porous structure and suitable surface chemistry and topography are required for facilitating cell adhesion, cell growth and proliferation, diffusion of oxygen and nutrients, and for the removal of metabolic waste created during the regenerative process. Regardless of the porous structure, porosity greater than 75% and pore sizes of above 150 μm appear to be necessary to achieve osteoconductive properties, although these morphological characteristics are

Conflict of interest: There is no conflict of interest to be declared.

Financial support: This work was supported by ERC Consolidator Grant program (ERC-2013-CoG-614715, NANOHEDONISM). CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008-2011 financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

*Corresponding authors at: Universidad de Zaragoza, Centro de Investigación Biomédica de Aragón-CIBA, Planta A-Sala Postdoctorales, 50009 Zaragoza.
E-mail addresses: gmmenc@unizar.es (G. Mendoza), vandreu@unizar.es (V. Andreu).

[1] Both authors contributed equally to the work.

http://dx.doi.org/10.1016/j.nano.2017.06.007
1549-9634/

going to depend on the repair, rate of remodeling and rate of degradation of the scaffolding material.[4,5] From a mechanical point of view, it is also necessary that the mechanical properties of the scaffold are comparable to the ones of the defective site to act as a temporary skeleton and to provide sufficient mechanical integrity to withstand the loadings during the bone remodeling process, restoring the normal biomechanical function of the bone.

Several biomaterials have been used for the fabrication of the aforementioned scaffolds, including natural materials derived from animals or plants (collagen, fibrin, hyaluronan and chitosan) and synthetic materials, such as bioactive ceramics and a wide range of synthetic polymers.[6] Scaffolds composed of natural origin polymers are attractive owing to their biocompatibility, biodegradability, low toxicity and chronic inflammatory response, and due to their biological characteristics and structural similarities with human tissues.

In that sense, chitosan (CS), a derivative of chitin, is a polycationic aminopolysaccharide produced *via* alkaline N-deacetylation process of the naturally occurring biopolymer.[7] Its linear chains consist of β-1,4-linked N-acetyl-D-glucosamine and D-glucosamine units which are susceptible of biodegradation. A wide range of CS applications results from its unique set of versatile physicochemical and biological properties. In contrast to chitin, CS is soluble in most dilute acidic solutions at pH below 6.5 (pKa value ~6.3).[8] The physicochemical and biological properties of CS significantly depend on its average molecular weight and deacetylation degree (DD) being both characteristics dependent on the biological origin of the polymer and on the deacetylation process conditions used.[9] CS is known to be biocompatible, biodegradable and antimicrobial against several bacterial and fungal strains.[10–12] Its biological inertness and low toxic effects have been supported by numerous *in vitro* and *in vivo* studies. No allergic and inflammatory reactions after CS-based materials implantation, injection or topical application on the human body were proven.[13,14] However, its use as scaffold is currently limited by having reduced mechanical properties. In that sense, due to its ease of processing and the above-mentioned properties, there has been a growing interest in the combination of CS with other materials in an attempt to increase its mechanical resistance and widen its application in the biomedical field as scaffolding. This disadvantage has been overcome by modifying CS scaffolds with bioactive inorganic materials (hydroxyapatite, tricalcium phosphate) or synthetic polymers (such as poly(vinyl alcohol) and poly(ethylene glycol)) and natural polymers (collagen).[15,16] In that way, it has been proven that the addition of nanofillers such as carbon nanotubes, clay and silica significantly enhances the mechanical, electrical, and thermal stability of the polymers.[17–19] It is important to mention that several evidences suggest that silicon plays an important role in bone formation and health. To date, its potential biological action on the bone turnover is unclear but it has been suggested that it is involved in collagen synthesis and matrix mineralization.[20] So, Beck *et al* reported that bioactive silica based nanoparticles stimulate osteoblast differentiation and mineralization and suppress osteoclast differentiation *in vitro* as well as enhance bone mineral density *in vivo*.[21] Furthermore, composite mesoporous silica nanoparticle/CS nanofibers have been proposed as effective scaffolds in bone tissue applications due to their promotion of osteoblast attachment, proliferation osteogenic differentiation and bone formation.[22] Also, it has been described that the incorporation of nano-silica in CS/chondroitin sulfate nanocomposites improves the mechanical property of scaffolds and enhances the rate of mineralization and serum protein adsorption.[23]

On the other hand, recently, a next generation of biomaterials is emerging in the medicine regenerative field, focusing on the design of bioactive and resorbable scaffolds. These hybrid materials incorporate inert nanostructured scaffolds, bioactive factors and living cells capable of constituting the bone tissue matrix, guiding and accelerating the bone healing process.[24] In that way, specific cell types such as mesenchymal stem cells, osteoblasts or osteogenic progenitor cells are seeded directly on 3D scaffolds prior to *in vivo* implantation in bone defects. Several studies have observed proliferation, migration, and differentiation of osteoblast-like cells in collagen type I scaffolds likewise the synthesis of osteocalcin, a specific protein of osteoblastic function.[24] In addition, it has been demonstrated that MG-63 osteoblasts-like cells are able to attach and proliferate in CS scaffolds regardless of its degree of acetylation (DA), retaining their osteoblastic phenotype.[25]

In this work, we report the synthesis and characterization of tridimensional scaffolds composed of CS reinforced with SiO_2 nanoparticles to favor bone tissue regeneration. *In vitro* biocompatibility studies of these nanocomposites pre-seeded with human primary osteoblasts were carried out before *in vivo* implantation. Subsequently, their osteogenic, osteoinductive and osteoconductive potential for the reconstruction of full-thickness calvarial defects was evaluated in a mice model after histopathologic examination.

Methods

Materials

CSs (from *Sigma-Aldrich*) with low (L, ~369 kDa, from chitin of crab shells), medium (M, ~1278 kDa, from chitin of crab shells) and high (H, ~2520 kDa, from chitin of crab shells) average molecular weights were used for the scaffolds preparation. The deacetylation degrees of CS L, M and H grades were 86 ± 3%, 89 ± 2% and 85 ± 3%, respectively.[26] An aqueous solution of acetic acid (99.8% *Sigma-Aldrich*) was used as a solvent for the polymer. Sucrose (>99.0% *Sigma-Aldrich*) was used as a cryoprotectant during the lyophilization process. Tetraethoxysilane (TEOS, Sigma-Aldrich), ammonium hydroxide (Ammonia, ACS reagent 28%) and absolute ethanol (EtOH, Sigma-Aldrich) were used to synthetize SiO_2 nanoparticles (SiO_2 NPs).

Synthesis of silica nanoparticles

~100 nm SiO_2 NPs were produced following the well-known Stober method,[27] which is based on the hydrolysis and condensation reaction of TEOS in the presence of NH_4OH in EtOH (molar ratio TEOS: EtOH: NH_3:H_2O = 1:280:18:56). Briefly, 2.6 mL of ammonia was added to 15 mL of ethanol and