**RELAZIONE SCIENTIFICA**

Bando Short Term Mobility – CNR2016

# Development of Antimicrobial Ionic Liquids in Bioactive Sol-Gel Hydroxyapatite for Tissue Engineering

## Introduction

Bacteria and fungi can often adhere to biomaterials and have the capability of forming biofilms on foreign bodies [1]. Biofilms are structured microbial communities that are attached to a surface. Individual microorganisms in biofilms are embedded within a matrix of – often slimy – extracellular polymers, and characteristically display a phenotype that is markedly different from that of planktonic cells [2]. Mature biofilms contain numerous microcolonies with interspersed water channels to allow circulation of nutrients. On other surfaces (e.g. cellulose fibres) microcolonies consisting entirely of yeast cells are produced. The subsequent detachment of single cells or cell aggregates from these biofilms can result in the development of highly resistant local or systemic infections in patients. It has been suggested that the local application of antimicrobials can provide higher local antibiotic concentrations than those through intravenous application, and can also avoid the toxicity accompanied with high plasma levels. Furthermore, biofilms are resistant to a range of antifungal agents currently in clinical use [3], including amphotericin B and fluconazole, and there appear to be multiple resistance mechanisms. Biofilm cells are supposed to grow slowly because of the limited availability of nutrients, particularly at the base of the biofilm [4]. A slow growth rate is often accompanied by changes in cell surface composition that could, in turn, affect the susceptibility of the microorganisms to antimicrobial agents. Another recent suggestion is that a small number of 'persister' cells are responsible for resistance [5]. Multiple mechanisms appear to operate in bacteria, and these vary with the bacteria present in the biofilm and the nature of the antimicrobial agent being administered. It has been demonstrated that some imidazolium, pyridinium and quaternary ammonium ionic liquids (IL) have antimicrobial activity [6]. Some of these compounds possessed good antimicrobial potency, which was dependent on the 1-n-alkyl chain-length. In particular, antimicrobial activities of a series of 1-n-alkyl-3-methylimidazolium chlorides [CnMIm]Cl (where

[CnMIm] = 1-n-alkyl-3-methylimidazolium and Cn = CnH(2n+1); n = 4, 6, 8, 10, 12, 14, 16, 18) were evaluated against a panel of clinically significant bacterial and fungal pathogens (E. coli, S. typhimurium, S. Aureus and Candida Albicans).

Nowadays, the IL represent an important class of substances with a large variety of biological activities and applications, such as antimicrobial [7-9], antifungal [9,10], antitumor [7], antioxidant [7], antifibrous [7], and bioengineering (drug/gene delivery or biosensors) [7,9].

Thus, the aim of this study is creating injectable **ionic liquid-hydroxyapatite-based biocomposite** with antimicrobial activity and able to regenerate the tissue in bone defects. The sol-gel synthesis approach appears to be the most suitable route towards performing injectable and bioactive hydroxyapatite-based biocomposites [11-].

The in situ application of IL into sol-gel processes allows nanoparticles' structure control, driven by the IL' self-assembling property and selective IL-substrate interactions, while preserving their specific properties. During my stay at UFRGS, the simultaneous opposite responses toward osteoblasts and microbial proliferation of hybrid gel materials was studied.

## Material and Methods

The HA/IL gel materials were obtained by re-suspending IL [$C_n$MIm]Cl (where [$C_n$MIm] = 1-*n*-alkyl-3-methylimidazolium and $C_n = C_nH_{(2n+1)}$; n = 4, 6, 8, 10, 12, 14, 16, 18) at 1 and 2wt% with different chain-lengths in the gelling HA water solution. IL interact with the growing particles through the hydrogen bond "co-π-π stacking" mechanism, which creates an IL-layer on the HA surface. Thus, differences in the size, geometry, polarity and Coulomb coupling forces between the IL' anions and cations contribute directly to the final HA particle size and morphology.

The biological properties in terms of cytotoxicity, proliferation and osteogenic differentiation of human Mesenchymal Stem cells (hMSC), were investigated. Furthermore, antimicrobial investigations on bacteria (E. Coli and S. Aureus) and fungi (Candida Albicans and Tropicalis) were performed.

The Calcium release from materials was also investigate for evaluating the different effect of gel materials on osteogenic differentiation.

## Results and Discussion

In general, the insertion of IL into the setting gels induced a shorter gelification at room temperature and an increase in crystallinity in comparison with the neat HA system. IL

interact with the growing particles through the hydrogen bond "*co-π-π stacking*" mechanism, which creates an IL-layer on the HA surface. Thus, differences in the size, geometry and Coulomb coupling forces between ILs' anions and cations contribute directly to the final HA particle size and morphology. The biological analyses showed no cytotoxic effects and good biological response on hMSCs. In particular, we have found that CaP-ILs showed better cell attachment than CaP after 24hrs (fig.1A) of culture time; after that the cell proliferation over culture time as demonstrated in figure 1B. Moreover, osteogenic differentiation was evaluated in basal medium by expression of early (alkaline phosphatase, ALP) and later (osteocalcin, OCN) markers. The results showed that ALP and OCN levels are higher in gel materials treated with IL having longer alkyl chain. The sperimental *in vitro* model of bone inflammation performed after 2days of culture time with hMSC demonstrated that CaP_IL materials reduce nitrites production induced by LPS (1μg/ml) compared to CaP alone (Fig.2A). By contrast CaP alone and in presence of ionic liquids reduced ROS levels increased by LPS (1μg/ml) (Fig.2B). Meanwhile CaP significantly increased anti-inflammatory IL-10 cytokine levels reduced by LPS (1μg/ml) and presence of ionic liquid C16 2% determined a significant production in IL-10 levels compared CaP alone (Fig.2C).

The antimicrobial investigation performed on two fungi strains (C. Albicans and C. Tropicalis) and Gram positive (S. Aureus) and Gram negative (E. Coli) bacteria showed an antimicrobial effect for IL and CaP-IL with longer chain as CaP_$C_{16}$, CaP_$(C_{10})_2$ at 1 and 2wt%, after 48hrs of culture time. SEM images confirmed a presence of biofilm on CaP, CaP_$C_4$ and CaP_$C_{10}$, meanwhile any traces of biofilm production was detected for materials at longer chain CaP_$C_{16}$ and CaP_$(C_{10})_2$.

**Conclusions**

During my stay at UFRGS I had the possibility to complete the biological studies of injectable calcium phosphates complexed with Ionic Liquid. Altogether, this scientific mission strengthened the collaboration between IPCB-URFGS and will allow the preparation of a manuscript that will be submitted to high impact journal. In the same time an abstract was submitted to ESB 2017 congress (European Society of Biomaterials).

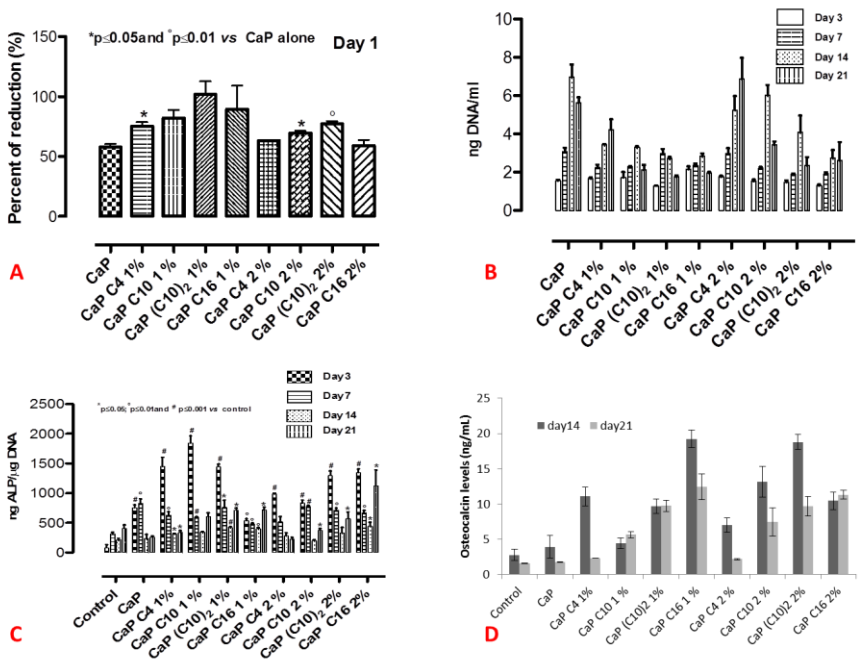


Fig.1: Biological studies performed on hMSC A) cell attachment after 24hrs; B) cell proliferation at long time; C-D) expression of ALP and OCN at 3, 7, 14 and 21 days of culture time, respectively.

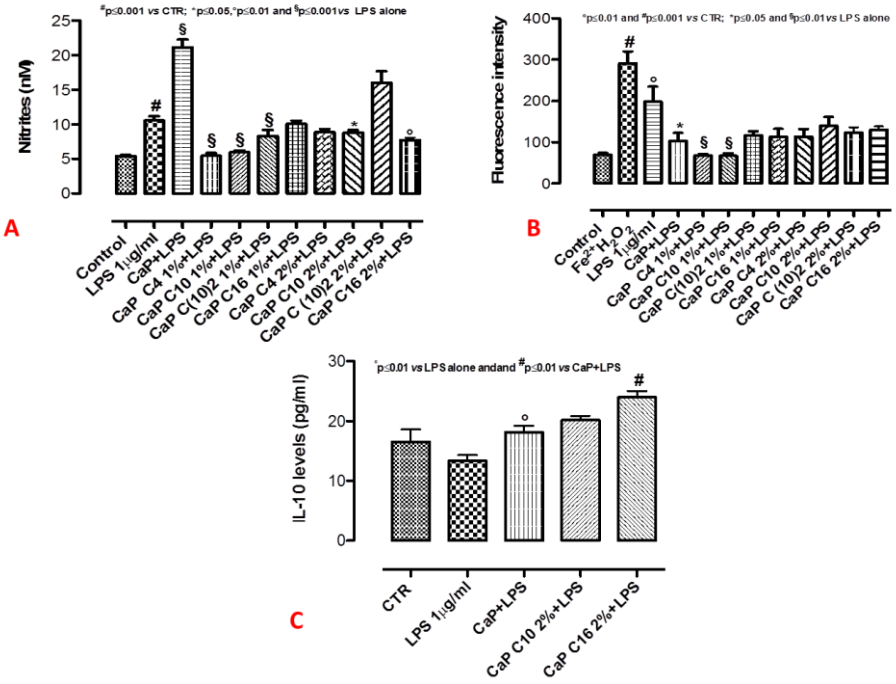


Fig.2: *In vitro* inflammation model by A) expression of nitrites and B) reactive oxygen species (ROS); C) expression of anti-inflammatory interleukin IL-10.

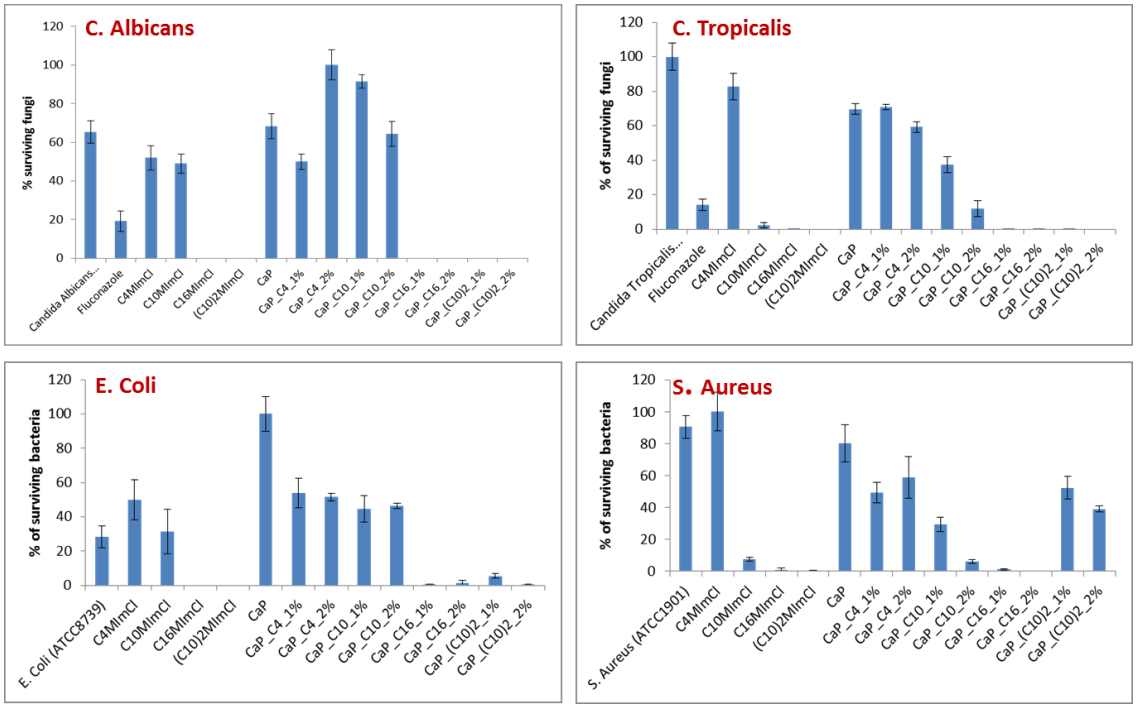


Fig.3: Antimicrobial results on fungi (C. Albicans and C. Tropicalis) and bacteria (E.Coli and A. Aureus) after 48hrs of culture time.

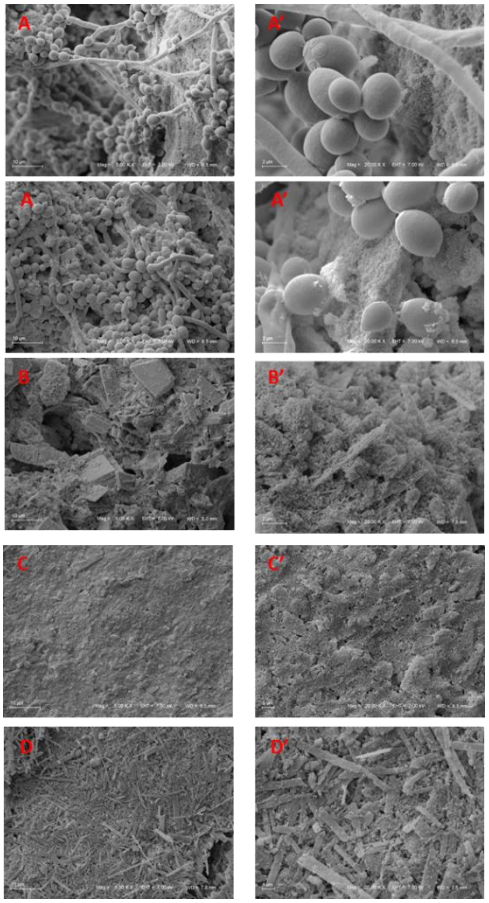


Fig.4: Biofilm production after 48hrs of culture time.

**References**

[1] M. Katsikogianni and Y.F. Missirlis. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria material interactions. M. European Cells and Materials. 2004;8:37-57.

[2] Hans-Curt Flemming, Jost Wingender. The biofilm matrix. Nature Reviews Microbiology 2010;8:623-633.

[3] Jyotsna Chandra, Duncan M. Kuhn, Pranab K. Mukherjee, Lois L. Hoyer, Thomas McCormick, and Mahmoud A. Ghannoum. Biofilm Formation by the Fungal Pathogen *Candida albicans*: Development, Architecture, and Drug Resistance. J. Bacteriol. 2001;183:5385-5394.

[4] L. Julia Douglas. Candida biofilms and their role in infection. TRENDS in Microbiology 2003;11:30-36.

[5] Kim Lewis. Persister cells, dormancy and infectious disease. Nature Reviews Microbiology 2007;5:48-56.

[6] Schrekker CML, et al., Multitask Imidazolium Salt Additives for Innovative Poly(l-lactide) Biomaterials: Morphology Control, Candida spp. Biofilm Inhibition, Human Mesenchymal Stem Cell Biocompatibility, and Skin Tolerance. ACS Appl. Mater. Interfaces. 2016;8:21163-76.

[7] Riduan, S. T.; Zhang, Y. Imidazolium Salts and Their Polymeric Materials for Biological Applications. Chemical Society Reviews. 2013;42:9055-70.

[8] Pendleton, J. N.; Gilmore, B. F. The Antimicrobial Potential of Ionic Liquids: A Source of Chemical Diversity for Infection and Biofilm Control. International Journal of Antimicrobial Agents. 2015;46:131-139.

[9] Smiglak, M.; Pringle, J. M.; Lu, X.; Han, L.; Zhang, S.; Gao, H.; MacFarlane, D. R.; Rogers, R. D. Ionic Liquids for Energy, Materials, and Medicine. Chemical Communications- Royal Society of Chemistry, 2014;50:9228-50.

[10] Liu, L.; Wu, H.; Riduan, S. N.; Ying, J. Y.; Zhang, Y. Short Imidazolium Chains Effectively Clear Fungal Biofilm in Keratitis Treatment. Biomaterials. 2013;34:1018-1023.

[11] M.G. Raucci , M.A. Alvarez-Perez , S. Meikle, L. Ambrosio, M. Santin . Poly(epsilon-Lysine) dendrons tethered with phosphoserine increase mesenchymal stem cell differentiation potential of calcium phosphate gels. Tissue Eng Part A. 2014 Feb;20(3-4):474-85. doi: 10.1089/ten.TEA.2012.0450.

[12] M.G. Raucci, D. Giugliano, M.A. Alvarez-Perez and L. Ambrosio. Effects on growth and osteogenic differentiation of mesenchymal stem cells by the strontium-added sol-gel hydroxyapatite gel materials. J Mater Sci: Mater in Med 2015;26(2):90.

[13] M. G. Raucci, D. Giugliano, A. Longo, S. Zeppetelli, G. Carotenuto and L. Ambrosio. Comparative facile methods for preparing graphene oxide–hydroxyapatite for bone tissue engineering. Tissue Eng Reg Med (2016). Published online in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/term.2119.