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# Dual crosslinked iminoboronate-chitosan hydrogels with strong antifungal activity against *Candida* planktonic yeasts and biofilms

Daniela Ailincai[a], Luminita Marin[a,*], Simona Morariu[a], Mihai Mares[b], Andra-Cristina Bostanaru[b], Mariana Pinteala[a], Bogdan C. Simionescu[a,c], Mihai Barboiu[a,d]

[a] "Petru Poni" Institute of Macromolecular Chemistry of Romanian Academy, 41A, Aleea Gr. Ghica Voda, Iasi, Romania
[b] "Ion Ionescu de la Brad" University, Laboratory of Antimicrobial Chemotherapy, 8, Aleea Sadoveanu, Iasi, Romania
[c] "Gheorghe Asachi" Technical University of Iasi, 73, Bd. Dimitrie Mangeron, Iasi, Romania
[d] Institut Européen des Membranes, F-34095, Place Eugène Bataillon, Montpellier, France

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ABSTRACT

Chitosan based hydrogels are a class of cross-linked materials intensely studied for their biomedical, industrial and environmental application, but their biomedical use is limited because of the toxicity of different organic crosslinkers. To overcome this disadvantage, a new strategy to produce supramolecular chitosan hydrogels using low molecular weight compounds able to form covalent linkages and H-bonds to give a dual crosslinking is proposed. For this purpose we used 2-formylphenylboronic acid, which brings the advantage of imine stabilization *via* iminoboronate formation and potential antifungal activity due to the presence of boric acid residue. FTIR and NMR spectroscopy indicated that the gelling process took place by chemo-physical crosslinking forming a dual iminoboronate-chitosan network. Further, X-ray diffraction demonstrated a three-dimensional nanostructuring of the iminoboronate network with consequences on the micrometer-scale morphology and on the improvement of mechanical properties, as demonstrated by SEM and rheological investigation. The hydrogels proved strong antifungal activity against *Candida* planktonic yeasts and biofilms, promising to be a friendly treatment of the recurrent vulvovaginitis infections.



## 1. Introduction

Hydrogels are three-dimensional polymeric networks which are able to hold a large amount of water or biological fluids, with applicability in a high number of biomedical, industrial and environmental purposes starting with drug delivery, wound dressing, soft contact lenses or diapers, as well as in restorative dentistry, tissue engineering, water waste treatment and soil conditioning (Buenger, Topuz, & Groll, 2012; Chawla, Ranjan Srivastava, Pandey, & Chawla, 2014; Ullah et al., 2015). Many polymers proved the ability to form hydrogels, in the presence or absence of a crosslinking agent (Palumbo et al., 2015). Among them, chitosan, the second most abundant natural polymer, is recognised as an excellent option due to its rich therapeutic properties: biocompatibility and biodegradability, hemostatic, hypolipidemic, hypoglycemic, antitumoral, antimicrobial and fungicidal activity – to mention only some (Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli et al., 1994 Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994). Chitosan based hydrogels can be obtained by either physical or chemical crosslinking. The physically crosslinked chitosan hydrogels present the advantage of being temperature responsive, but their application is limited due to their weak mechanical properties and uncontrolled dissolution (Bhattarai, Gunn, & Zhang, 2010). The chemically crosslinked chitosan hydrogels show slower degradability and possibility to control their pore size being recommended for *in vivo* long-term applications (Beauchamp, St Clair, Fennell, Clarke, & Morgan, 1992). Some attempts to combine the two crosslinking ways resulted in dual-network hydrogels with improved mechanical properties, promising to be a reliable route to high performance materials (Bai et al., 2016; Fajardo, Favaro, Rubira, & Muniz, 2013).

Since chitosan is a polysaccharide which contains functional amine groups, the primary pathway of its crosslinking is the acid condensation with dialdehydes, especially glutaraldehyde, forming imine bonds. Due to the reversibility of the imine bond formation, the obtained hydrogels have the advantage of being pH-responsive

* Corresponding author.
*E-mail addresses:* lmarin@icmpp.ro, lmarin2011973@yahoo.com (L. Marin).

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and biodegradable (Mi, Kuan, Shyu, Lee, & Chang, 2000). Nevertheless, the toxicity of dialdehydes, and especially of glutaraldehyde, related to the human body restricts their use for biomedical applications (Beauchamp et al., 1992; Berger et al., 2004) and imposes the necessity to find new friendly crosslinking agents. Thus, the preparation of hydrogels with impact in the biomedical field remains a challenge of current interest (Azevedo & Kumar, 2012; Berger et al., 2004; Mahkam, 2010; Mikhailov et al., 2016).

In finding a pathway toward hydrogels for biomedical applications, we propose the use of 2-formylphenylboronic acid as chitosan crosslinker, based on the assumption that its structure should facilitate a dual crosslinking – a covalent one *via* imine forming and a physical one *via* H-bonding, giving rise to a chemo-physical chitosan network. Additionally, due to the *ortho* position of the boric acid residue, the further stabilization of the imine linkage through intra-molecular H-bonds or dative linkages *via* an iminoboronate motif is possible. Recently considered as a powerful tool for bio-orthogonal dynamic covalent chemistry, the iminoboronates proved the ability to specifically target lipids, peptides and proteins as well as cancer-cells (Bandyopadhyay, McCarthy, Kelly, & Gao, 2015; Cal et al., 2014). The dynamic iminoboronate unit may allow the reorganization and the adaptation in response to various external stimuli like pH or temperature, creating materials for new applications in biotechnology and medicine. On the other hand, considering the anticancer activity of boronic-imine compounds, and their low toxicity (Pasa et al., 2016) and the antifungal activity of the boric acid in the treatment of recurrent and resistant yeast vaginitis (De Seta, Schmidt, Vu, Essmann, & Larsen, 2009), it is expected that the combination of 2-formylphenylboronic acid with chitosan to create novel products with improved biological activity.

In this paper, we present a novel synthetic strategy to develop chitosan based hydrogels using 2-formylphenylboronic acid as a dual crosslinking agent. The chemical and supramolecular structure of the iminoboronate-chitosan hydrogels, their morphology, rheological behaviour, swelling ratio, as well as antifungal activity against both planktonic and biofilm *Candida* yeasts were evaluated and discussed. Three novel aspects brought by the paper must be highlighted here: (i) the obtaining of iminoboronate derivatives of chitosan, (ii) the chitosan double crosslinking to give chemo-physical hydrogels, and (iii) the strong antifungal activity of the obtained hydrogels.

## 2. Experimental part

### 2.1. Materials

2-Formylphenylboronic acid (2-FPBA) (95%), low molecular weight chitosan (263 kDa, DA: 83%), D-glucosamine hydrochloride and phosphate buffer solution have been purchased from Aldrich and used without further purification. All the reagents used in antifungal measurements – Yeast Peptone Dextrose Agar (YPD), RPMI-1640, 3-(*N*-morpholino)propanesulfonic acid (MOPS), 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT), menadione, calcofluor (Fluorescent Brightener 28) – were purchased from Sigma-Aldrich and used as received.

### 2.2. General procedure for hydrogel and xerogel obtaining

To a 2% solution (g/mL) of chitosan (0.06 g, 0.29 mmol of glucosamine repeating units) in acidic water (0.7% acetic acid solution: 21 μL of acetic acid in 3 mL of water) was added drop wise a 1% solution (g/mL) of 2-formylphenylboronic acid in ethanol (see Table 1), under vigorous magnetic stirring (500 rpm) at 55 °C. The molar ratio between $NH_2$ and CHO functional groups has been varied (keeping constant the amount of chitosan and changing the amount of aldehyde to achieve hydrogels with different crosslinking densities (see Table 1)). The reaction mixture reached the gelation point in less than 5 min for a $NH_2$/CHO ratio of 1/1, and after 3 h for the 2/1; 2.5/1; 3/1; 3.5/1; 3.75/1 ratios. No hydrogel has been obtained for the 4/1 molar ratio, the reaction mixture remaining a viscous liquid even after 24 h. The visual examination revealed transparent semisolid materials with smooth texture, without air bubbles or other macroscopic particles. The hydrogels were kept uncovered for one day up to the initial volume of chitosan solution was reached.

The corresponding xerogels of the obtained hydrogels were prepared by lyophilisation. As the NMR indicated the increase of the imine linkage density during a week, xerogels of the hydrogels kept covered for one week, were also obtained. A 2% chitosan in 0.7% acetic acid solution has been also lyophilized, to be used as a control reference. The codes of the hydrogels obtained for different molar ratios of the $NH_2$/CHO functional groups are given in Table 1. Symbol * was used to designate the hydrogels kept one week before lyophilisation.

### 2.3. Methods

The hydrogels were frozen in liquid nitrogen and further submitted to lyophilization using a Martin Christ, ALPHA 1-2LD equipment for 24 h at −57 °C and 0.050 mbar (Dinu, Pradny, Dragan, & Michalek, 2013).

FTIR spectra of the xerogels have been registered using a FT-IR Bruker Vertex 70 Spectrofotometer, by ATR technique and processed using OPUS 6.5 software.

The NMR spectra were obtained on a Bruker Avance DRX 400 MHz Spectrometer equipped with a 5 mm QNP direct detection probe and z-gradients. The chemical shifts are reported as δ values (ppm) relative to the residual peak of the deuterium oxide used as solvent.

Wide angle X-ray diffraction (WXRD) of the xerogel pellets was performed on a Bruker D8 Avance diffractometer with the Ni-filtered Cu-Kα radiation (λ = 0.1541 nm), in the range of 2–40° (2 theta degrees). The working conditions were 36 kV and 30 mA and data were handled by the FullProf 2000 program. The xerogel pellets were obtained in a manual Hydraulic Press, by applying a pressure of 10 N/m$^2$.

The xerogel morphology was studied with a field emission Scanning Electron Microscope SEM EDAX – Quanta 200 at accelerated electron energy of 12.5 or 20 KeV.

Rheological tests were carried out at 37 °C by using a Bohlin CVO rheometer with a parallel plate geometry (60 mm diameter and 500 μm gap) and thermal control by the Peltier effect in closed system. An exhaustive description of the measurements is given in Supporting information file.

Time-kill studies were performed using a previously described method (Canton, Peman, Gobernado, Viudes, & Espinel-Ingroff, 2004), slightly modified as follows. A synthetic vagina-simulative medium (SVSM) was prepared (Moosa, Sobel, Elhalis, Du, & Akins, 2004; Marques, Loebenberg, & Almukainzi, 2011) to assure biomimetic conditions. Two clinical isolates *Candida albicans* RTCC 1112 and *Candida glabrata* RTCC 1532 were used as testing microorganisms (RTCC: Romanian Type Culture Collection). From each strain, a 5 McFarland suspension in SVSM was prepared and adjusted to $2.5 \times 10^7$ CFU/mL using the TC20 automated cell counter (Bio-rad, USA). Subsequently, equal volumes of yeast suspension and hydrogel solution in SVSM were mixed and incubated at 36 ± 1 °C, to obtain final mixtures with 2-FPBA concentrations of 0.142% and 0.071%, respectively. A drug free control was also prepared by mixing equal volumes of yeast suspension and SVSM. At predetermined time intervals (0, 6, 12 and 24 h), a 1 mL aliquot from each test and control tube was serially diluted in sterile water,