# Antibiofilm poly(carboxybetaine methacrylate) hydrogels for chronic wounds dressings

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***Abstract:***

The current study demonstrates the benefits of poly(carboxybetaine methacrylate) hydrogels in chronic wound healing. These hydrogels demonstrate high absorbing capacity upon swelling in salt solutions thus revealing great potential as dressings for highly exuding chronic wounds. Moreover, upon swelling they expand, increasing their volume by 25%, which makes them patient friendly ensuring also the proper wound healing. Poly(carboxybetaine methacrylate) hydrogels were also shown to absorb collagenase and myeloperoxidase, two enzymes that are specific for chronic wounds, reducing in this way their amount by 30-45 % in the wound bed without entirely inhibiting their activity, as the latter is necessary for the wound healing process. The hydrogels were also shown to be non-cytotoxic as well as to prevent the biofilm formation of S. Aureus. The *in vivo* implantation in rats showed no immune response to moderate immune reaction for both studied PCB hydrogels. Thus, the properties of the PCB networks revealed in the study demonstrate their potential as chronic wounds dressing materials.

***Keywords:*** polyzwitterions, chronic wound dressings, antipolyelectrolyte behavior, pH and salt responsiveness, antibiofilm activity, biocompatibility.

## *1. Introduction*

Wound healing is a dynamic process comprising of inflammation, proliferation and remodeling/scar formation stages, during which the injured skin and related tisues are repaired and the wound is closed. Factors of different origin could disrupt the normal wound healing process and result into hard-to-heal chronic wound. Chronic wounds remain a significant challenge as they hamper the patient's quality of life and present substantial financial burden to the healthcare systems. Chronic wounds have several distinctive characteristics as compared to the acute wounds, namely:

• elevated matrix metalloproteinases (MMPs) activity [1, 2] due to an imbalance between the MMPs synthesis and their tissue inhibitors levels. The increased MMPs' proteolytic activity results into extracellular matrix as well as growth factors degradation which impairs the proper healing process [3, 4].

• elevated myeloperoxidase (MPO) activity: MPO catalyzes the formation of reactive oxygen species, such as hypochloric acid (HClO), which plays a crucial role in the host defense against bacteria and fungi. However, their prolonged presence in the wound results into tissue necrosis and permanent tissue damage [5].

• increased pH of the wound: the non-healing wound environment has a pH in the range of 7.15 to 8.93 [6]. In contrast, the pH of intact skin ranges from 4 to 6, while the pH of underlying tissue is 7.4. The acidic environment is considered favorable for skin fibroblasts proliferation [7], promoting epithelialization and angiogenesis [8], controlling bacterial colonization [9] and facilitating the release of oxygen from oxyhaemoglobin [10]. In contrast, alkalinity has an adverse effect on the wound tissue by depriving the wound of oxygen [10] and providing a favorable environment for bacterial growth [8].

• often chronic wounds have an excessive exudate production: high levels of exudate trapped under the dressing cause wound overhydration, i.e. "softening" or "sogginess" (maceration), thus making the skin more prone to damage [11]. Moreover, as the exudate contains high levels of harmful substances, including enzymes that break down the cell supporting extracellular matrix as well as the growth factors, it may cause skin stripping (excoriation) leaking out onto the periwound skin.

• the moist environment of chronic wounds is highly susceptible to bacterial infections. Bacterial biofilms complicate further and delay the wound healing [12].

Advanced approaches for chronic wounds management are focused on the development and application of multifunctional wound dressings, based on bio- and synthetic polymers. The ideal dressing for chronic wound management should promote the healing process, reduce the pain and enhance the skin restoration. At the same time, wound dressings in general should prevent the wound site from dryness, ensure thermal insulation and protection from mechanical trauma and bacterial infiltration, provide gaseous permeability and should be easily exchangeable without sticking on the wound. They are also required to be biocompatible and eventually biodegradable in regard to their after use disposal.

Recently, we have revealed the potential of poly(sulfobetaine methacrylate) (PSB) as a dressing material for chronic wound management [13]. PSB belongs to the class of polyzwitterions (PZI) – polymers known for their excellent antifouling properties and biocompatibility. The current study aims to evaluate the performance of another type of PZI, namely polycarboxybetaines, as a chronic wound dressing. Polycarboxybetaines possess a carboxylic group in their zwitterionic moiety which defines several advantages over the PSB when applied as chronic wound dressing material, namely:

- higher hydrophilicity, due to the carboxylic group, higher swelling ability and thus capacity to absorb and retain the wound exudate.
- pH responsiveness, which affects their swelling behavior.

All advantageous characteristics, such as the antipolyelectrolyte behavior, non-adhesiveness, transparency and hydrogel nature that make PSB networks appropriate material for chronic wound dressings are also valid for polycarboxybetaines. However, polycarboxybetaine hydrogels have not been explored for chronic wound management so far and the attempts for their application in wound healing in general are scarce. For example, mats obtained via electrospinning of polycarboxybetaines copolymers with methyl methacrylate were applied for acute wound healing [14]. The study has shown that the non-cell-adherent nature of polycarboxybetaines makes the wound dressings easily removable. Moreover, the *in vivo* results obtained for these copolymers showed the formation of new tissues within two weeks, thus evidencing that the polycarboxybetaine based dressings could be successfully used as wound dressing materials that minimize the scar formation. Although the paper claims the possibility for application of these dressings for “large area of chronic wounds” no experimental tests to support this claim are performed within the study. Moreover, the

polycarboxybetaine-poly(methyl methacrylate) dressings performance is not discussed in the light of the specific characteristics of chronic wounds outlined above. Recently, two papers reported the development of polycarboxybetaines and PSB hydrogels modified with antimicrobial silver nanoparticles for management of burn wounds [15,16], demonstrating the advantages of polycarboxybetaines hydrogels. Nevertheless, polycarboxybetaines have not been explored as chronic wound dressings materials.

The aim of the current study was to demonstrate and preclinically validate the advantageous properties and functionality that polycarboxybetaines hydrogels possess in regard to their application as chronic wound dressings. In this respect, their intelligent (pH and salt-responsive) behavior, ability to physically absorb and partially inhibit *in vitro* and *ex vivo* collagenase and MPO, their antibiofilm activity, cytotoxicity and *in vivo* biocompatibility were studied.

## *2. Experimental part*

### *2.1. Materials*

Sodium metabisulfite ($Na_2S_2O_5$, 97 %), ammonium persulfate ($(NH_4)_2S_2O_8$, 98 %), poly(ethylene glycol) diacrylate (PEGDA, with average Mn = 575), 2-(dimethylamino) ethyl methacrylate (DMAEMA), calcium chloride dihydrate ($CaCl_2.2H_2O$), zinc chloride ($ZnCl_2$), phosphate buffered saline (PBS 0.1M, pH=7.4), guaiacol (98 %) and tryptic soy broth (TSB) were purchased from Sigma-Aldrich (Spain). Ethylmethylketon (≥99 %) was purchased from Fluka. Beta-propiolactone (97%) was obtained from Alfa Aesar (Germany). Ethylene glycol (anhydrous 99.8 %) and ethanol were purchased from Laborchemie Apolda (Germany).

Collagenase (Coll) from Clostridium hystolyticum type I (EC 3.4.24.3 U/mg solid: 1 U hydrolyzes 1.0 μmol of furylacryloyl-Leu-Gly-Pro-Ala per min at 25 ºC) was purchased from Sigma Aldrich. EnzChek Gelatinase/Collagenase Assay Kit was provided by Molecu-lar Probes (USA).

Staphylococcus aureus (ATCC 25923) and human foreskin fibroblast cells line BJ-5ta (ATCC CRL-4001) were obtained from American Type Culture Collection ATCC (LGC Stand-ards S.L.U, Spain). AlamarBlue Cell Viability Reagent was purchased from Invitrogen, Life Technologies Corporation (Spain). Dulbecco's Modified Eagle's Medium and L-glutamine were purchased from Sigma Aldrich (Spain).

### *2.2. Synthesis of carboxybetaine methacrylate (CBMA) monomer*

). The reaction mixture was stirred at 10 ˚C for 3 h then left at 0 ºC for 12 h. The a

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### *2.3. Synthesis of poly(carboxybetaine methacrylate) networks*

Poly(carboxybetaine methacrylate) (PCB) networks were synthesized via free radical crosslinking polymerization. To this purpose, 1M CBMA solution in a mixed solvent ethylene glycol/ethanol/H2O (6 : 2 : 2 volume ratio) was prepared. The initiator system $Na_2S_2O_5$ (0,665 mol. % with respect to monomer) + $(NH4)_2S_2O_8$ (1.445 mol. %) was dissolved into the monomer solution. Different amounts of the crosslinking agent PEGDA were added to the reaction mixture in order to obtain hydrogels with different crosslinking density (Table 1).

**Table 1.** PCB networks with varying crosslinking density*

| **Designation** | **4PCB** | **5PCB** | **6PCB** | **7PCB** |
|---|---|---|---|---|
| **PEGDA concentration (mol.%)** | **4** | **5** | **6** | **7** |

* 1M CBMA; $Na_2S_2O_5$ (0.665 mol. %) + $(NH_4)_2S_2O_8$ (1.445 mol. %) with respect to CBMA.

The obtained solution was homogenized for 30 min and injected between two glass plates, separated by a rubber spacer. The polymerization took place at 60 °C for 15 h. The obtained PCB hydrogels (with size 80 mm/ 50 mm/ 2 mm for length/width/ thickness) were immersed in a large volume (~300 ml) of distilled water for two weeks, exchanging water every day in order to remove the residuals (proved by UV of the waste waters).

### *2.4. Characterization of PCB networks*

#### *2.4.1. Swelling kinetics*

The swelling kinetic of PCB networks was followed gravimetrically. To this purpose, a dry sample (~25 mg) was swollen in distilled water at 37 °C for 24 h. Hydrogels were withdrawn from the aqueous medium at defined time intervals and weighted after removing the excess of water on their surface with a fiber free paper. The swelling ratio (SR) was calculated by using the following equation:

$$SR = (m_s - m_d)/m_d \qquad (1)$$

where $m_s$ is the weight of the swollen hydrogel and $m_d$ is the weight of the dry PCB network.

### *2.4.2. Swelling behavior of PCB networks in salt solutions*

To investigate the influence of the salt (NaCl) concentration on the PCB networks swelling ability, aqueous solutions of NaCl with different concentrations were prepared. PCB dry samples (~ 0.1 g) were soaked in the NaCl aqueous solutions (0.1 to 5.0 M) for 24 h at ambient temperature and their SR was determined using equation (1). For each NaCl concentration, at least 3 pieces from each PCB network (Table 1) were tested and the results were averaged.

### *2.4.3. pH influence on PCB swelling behavior*

The swelling behavior of PCB hydrogels in aqueous solutions with different pH value ranging from 2 to 10 was studied. The desired pH of the solutions was adjusted using either $H_3PO_4$ or NaOH. Dry PCB disks (~ 0.1 g) (Table 1) were allowed to swell in the solution with the lowest pH at ambient temperature for 24 h. After that, they were taken out from the solution, carefully wiped with fiber free paper in order to remove the water excess on their surface and weighted. The samples were then placed into the next solution with higher pH and the same procedure was repeated, gradually increasing pH of the used solution. All measurements were performed with three pieces from each PCB network (Table 1) and the results were averaged.

### *2.4.4. Thermal properties of PCB hydrogels*

Differential scanning calorimetry (DSC) was used to study the influence of the NaCl concentration on the water binding ability of PCB hydrogels. Dry PCB networks were left to swell until reaching their equilibrium swelling ratio (ESR) in aqueous NaCl solutions within the concentration range from 0.1 to 5 M for 72 h at room temperature. Then each sample (~7.5 mg) was transferred into a DSC standard aluminum pan and sealed tightly. An empty pan was used as a reference during the DSC analysis. Both pans were first cooled to - 90 °C and then heated to 160 °C at a heating rate of 10 °C/min. A constant flow of 50 mL/min $N_2$ gas was purged through the system during the DSC run. The amounts of freezable (WFS) and non-freezable (WNFS) water were calculated using the following equations [18]:

$$W_s\ [\%] = ((m_s - m_d)/m_w)*100 \quad (2)$$

$$W_{fs}\ [\%] = (\Delta H_{hydrogel}/\Delta H^\circ H_2O)*100 \quad (3)$$

$$W_{nfs}\ [\%] = W_s - W_{fs} \quad (4)$$

where $m_w$ is the weight of the hydrogel after 72 h of swelling into the salt solution; $\Delta H^o_{H_2O}$ = 333.5 J/g is the water melting enthalpy; $\Delta H_{hydrogel}$ is the sum of the melting enthalpies for all endothermic peaks which appear in the DSC heating run; $W_{fs}$ is the freezable/unbounded water and $W_{nfs}$ is the non-freezable/bounded water.

### *2.4.5. Collagenase absorption by PCB hydrogels*

Collagenase solution (1 mg/mL) was prepared by dissolving collagenase powder in PBS, pH 7.4. Dry PCB samples (~ 30 mg) were immersed into 1 mL collagenase solution at 37 °C for 72 h. The collagenase absorption by the PCB hydrogels was determined by using the Bradford's method [19]. To this purpose, 30 µl from the collagenase solution where PCB sample was incubated were mixed with 270 µl Comassie Brilliant Blue G. The resulting solution was left at room temperature for 5 min and then its absorbance at 595 nm was measured. The quantity of the collagenase in the solution before and after incubation was determined by using a calibration curve, obtained for bovine serum albumin (BSA) ($Y = 0.637x - 0.078$; $R^2 = 0.9987$). Each experiment was triplicated and the results were averaged. The collagenase absorption (CA) by each PCB network was calculated using the equation:

$$CA = ((m_o - m_t)/m_o)*100 \qquad (5)$$

where $m_o$ is the collagenase quantity in the initial collagenase solution (1mg/mL) and $m_t$ is the residual collagenase quantity left in the solution after 72 h incubation of the PCB hydrogels.

### *2.4.6. Volume change of the PCB hydrogels upon swelling*

The ability of PCB to change the volume (expand) upon swelling in 0.15 M NaCl was studied by video recording. To this purpose, a dry piece of PCB with size 2x2cm was placed into 10 mL 0.15 M NaCl aqueous solution with dissolved methylene blue to make the observation clearer. The volume expansion after 3 min was registered by measuring the size of the obtained hydrogel. The swelling ratio of the piece was estimated by measuring the change in its weight.

### *2.4.7. In vitro collagenase activity (CA) determination*

EnzCheck Gelatinase/Collagenase Kit was used to determine the inhibitory effect of PCB hydrogels towards collagenase. Briefly, PCB samples (~ 30 mg) were incubated in 600 µl 32 U/mL collagenase solution at 37 ºC for 24 h 100 µL of the collagenase

solution after incubation with the PCB hydrogel were transferred into a 96-well microplate and mixed with 80 µL PBS. 20 µL of 250 µg/mL gelatin substrate were subsequently added to start the enzymatic reaction. The absorbance of the resulting solution at 528 nm was taken immediately after the substrate addition (i.e. at 0 s) and then every 15 s for 75 s in total (the catalyzed reaction initially follows a linear relationship, from which its velocity is obtained). The absorbance values for each reaction time were measured in triplicate. For the sake of comparison, the activity of collagenase in solution without incubation with PCB hydrogel was used as a control. The % of the retained collagenase activity (EA) after incubation with PCB hydrogels was calculated using the following equation:

$$EA = (\Delta A_{PCB}/\Delta A_{control})*100 \qquad (6)$$

where

$$\Delta A_{PCB} = A_{PCB}^{75} - A_{PCB}^{0}$$

$$\Delta A_{control} = A_{control}^{75} - A_{contro}^{0}$$

In these equations, $A_{PCB}^{0}$ and $A_{PCB}^{75}$ are respectively the absorbance values obtained at 0 s and at 75 s of the enzyme reaction of the collagenase, incubated with PCB samples. Similarly, $A_{control}^{0}$ and $A_{conrol}^{75}$ are respectively the absorbance at 0 s and 75 s of the enzyme reaction performed with neat collagenase.

### *2.4.8. Ex vivo activity of the PCB hydrogels against collagenase in wound exudates*

UrgoClean (from Urgo Medical) dressings loaded with wound exudate from patients with leg ulcers were provided from Hospital of Terrassa, Spain. The wound exudate was extracted following the procedure described below. A patch from the wound dressing (~1 g) was soaked in 5 mL deionized water for 15 min. After the dressing removal, the supernatant was vortexed for 2 min and then centrifuged for 5 min at 10 000 rpm allowing to erythrocytes, bacteria and other macromolecular materials to precipitate. The sludge was removed and the supernatant (liquor) was centrifuged again using the same conditions. The extracted exudate was stored at 4 ºC until used. The wound exudate was diluted 8 times in order to fall within the linear part of the enzyme reaction progress. PCB hydrogels (30 mg) were incubated in 600 µL exudate at 37 ºC for 24 h. 100 µL from the exudate solution, after these 24 h of incubation, were mixed with 80 µL PBS with pH 6.5. To each solution, 20 µL of 250 µg/mL gelatin

solution were added as a substrate and the fluorescence was measured at 528 nm as already described above for *in vitro* collagenase activity assay. Similarly, an exudate solution not incubated with PCB hydrogel was used as a control.

### *2.4.9. Bacterial growth inhibition*

*S. aureus* single colony was inoculated in sterile TSB at 37 ºC and 240 rpm for 24 h. The bacterial suspension was diluted with fresh portion TSB to give a final concentration of 106 CFU/mL. PCB samples (30 mg) were mixed with 1 mL of *S. aureus* suspension at 37 ºC for 24 h. For the sake of comparison, a silicone piece with similar weight was also soaked in 1mL bacterial suspension at the same conditions to be used as a control. At the end of the incubation period, the bacterial medium was removed and all hydrogels samples were washed three times with 1 mL sterile 0.9 % NaCl, pH=6.5 in order to get rid of any loosely bound cells. PCB hydrogels were then transferred into sterile vials, containing 1 mL 0.9 % NaCl, pH 6.5 and put in an ultrasonic bath at 37 ºC for 20 min. 30 µl aqueous medium were subsequently withdrawn from each vial and spread on Braid Parker agar plates. After 18 h at 37 ºC, the number of colonies on the agar plates was counted and used to calculate the concentration of live bacterial cells using the following equation:

$$Inhibition\ (\%) = (CFU_{PCB} - CFU_S)\ x100\ /\ CFU_S \qquad (7)$$

where $CFU_{PCB}$ is the number of the bacteria colonies grown on the respective PCB hydrogel, while $CFU_s$ represents the number of grown colonies found for silicone. All presented results are obtained as the average value of three independent measurements.

### *2.4.10. Cytotoxicity evaluation*

Humans foreskin fibroblasts were used to assess the cytotoxicity of PCB hydrogels. Briefly, cell cultures were maintained at 37 ºC under 5 % $CO_2$ atmosphere in DMEM medium containing 4 mM L-glutamine, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 1 mM sodium pyruvate, 10 % (v/v) of FBS and 10 g/L hygromycin. After reaching a confluence, the cells were harvested using trypsin-EDTA solution (ATCC-30-2101, 0,25 % (w/v) trypsin/0.53 mM EDTA solution in Hank's BSS without calcium or magnesium) and then seeded at a density of 2 x $10^4$ cells/well in a sterile 24-well polystyrene plate. 24 h later the hydrogel samples (30 mg) and a silicone piece

(positive control) were incubated together with the cells and left for another 24 h at 37 ºC under 5 % $CO_2$ atmosphere. Cells were subsequently washed twice with PBS and mixed with 500 µL of 10 % (v/v) AlamarBlue reagent in DMEM to assess their viability being in contact with PCB hydrogels. The obtained solutions were left at 37 °C for 4 h. Finally, their absorbance at 570 nm was measured, using 600 nm as a reference wavelength. BJ5ta cells relative viability (CRV) (%) was determined for each PCB sample as compared to the cell viability (CV) when incubated with DMEM using the following formula:

$$CRV\ (\%) = (CV_{PCB}/\ CV_{DMEM})\ x\ 100 \qquad (8)$$

Where $CV_{PCB}$ is the absorbance obtained for the samples incubated with the PCB hydrogel and $CV_{DMEM}$ is the absorbance value obtained for the control - viable cells incubated only with DMEM. All results are reported as the mean values of three independent measurements.

#### *2.4.11. In vivo biocompatibility testing*

##### *2.4.11.1. Animal design and implantation of biomaterials*

All procedures in the *in vivo* experiments were consistent with the regulations of International Standard ISO 10993 -1:2009 for biological evaluation of medical devices and criteria for evaluation set out in ISO 7405:1997 concerning tissue and inflammatory reactions, and ISO 10003-6, 2007 Part 6 - Tests for local effects after implantation and the requirements of local institutional, national Regulation № 20/01.11.2012 regarding laboratory animals and animal welfare and European legislation. All procedures were confirmed by the Institutional Animal Care and Use Committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences (Permit number: 11 30 127). In the in vivo experiments, 18 Wistar male rats were used divided into 3 groups: the 1st group – control without implantation of materials, the 2nd group – with implantation of 6PCB, the 3rd group – with implanted 7PCB. The 6PCB and 7PSB hydrogels were chosen for intermuscular implantation and biosafety evaluation of the materials on the basis of their swelling behavior and the rate of the increase of their weight upon swelling and possible subsequent pressure atrophy in surgically fixed tissues.

Aseptically, after standard atropine-xylazine-ketamine anesthesia in appropriate doses, sterilized implants were inserted in the fossa poplitea between *m. biceps*

femoris and *m. semitendinosus*. After 8 weeks of evaluation period all animals were humanely euthanized.

#### *2.4.11.2. Histological procedures*

Tissue samples (1 $cm^3$ in size) with incorporated implants were routinely fixed in 10% buffered formalin, dehydrated in ethanol and were embedded in paraffin. Tissue sections (3-5 μm thick) were stained in hematoxylin and eosin and examined by light microscope (Leica DM 5000B, Wetzlar, Germany).

The sections were scored for the presence of fibrotic and inflammatory reactions and foreign body response, newly formed vessels and their caliber. To evaluate the findings after the microscopic observations, the following criteria regarding inflammatory reactions were used:

1. *No reaction or slight reaction* - fibrous capsule formation and absence of inflammatory cells or presence of a fibrous capsule formation with few lymphocytes and plasmocytes.
2. *Moderate reaction* - fibrous capsule formation with the presence of macrophages, polymorphonuclear leukocytes (PMNL), lymphocytes, and plasmocytes.
3. *Severe reaction* - presence of large accumulations of PMNL, lymphocytes, plasmocytes, macrophages, foreign-body giant cells, and congested capillaries.

## *3. Results and discussion*

### *3.1. Swelling kinetics*

The influence of the crosslinking degree on the swelling behavior of PCB networks is well seen from the swelling kinetics curves presented in Fig. 1. The higher the PEGDA concentration, the lower the water uptake of PCB networks. The sample with the loosest network, 4PCB, increased 6 times its initial weight after 24 h swelling in water. In contrast, the densest PCB network increased “only” 4 times its initial weight.

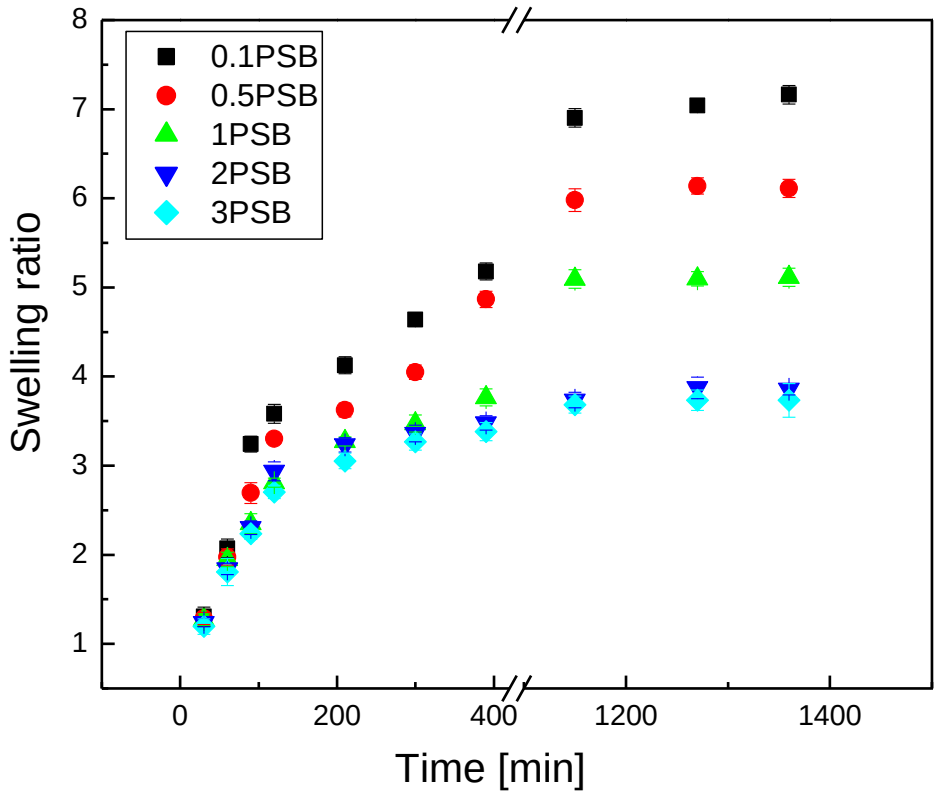



**Fig.1** Swelling kinetics of PCB in water at room temperature (25 $^{o}$C).

The crosslinking density also influenced the swelling rate of the PCB networks. The looser PCB networks (4PCB and 5PCB) needed ~8 h to reach their ESR, while the denser ones (6PCB and 7PCB) reached their ESR for ~2 h (Fig. S1). This effect could be explained by the more rigid structure of the denser PCB networks as well as by their lower ESR as compared to the looser PCB. Thus, it could be concluded that PCB's swelling behavior could be effectively controlled by the crosslinking agent PEGDA concentration.

The rheological study of the PCB hydrogels with different crosslinking density showed that they are stable and mechanical string to be applied as dressing materials (See Supplementary Information, Fig. S2).

### *3.2. Salt effect on the swelling behavior of PCB hydrogels*

The swelling degree of PCB networks increases as the salt concentration increases and this is a demonstration of the antipolyelectrolyte behavior which is typical for PZI

(Fig. 2). The highest ESR were obtained for the PCB swollen in the most concentrated NaCl solution (5M), while their lowest swelling was observed in pure water.

The highest swelling ability is demonstrated by the loosest (4PCB) network, which increased more than 11-fold its initial weight in 5M NaCl as compared to that in water. Respectively, the lowest swelling ability was shown by the densest (7PSB) network obtained with the highest PEGDA concentration, which increased “only” 8-fold its initial weight in salt solution in comparison with pure water.

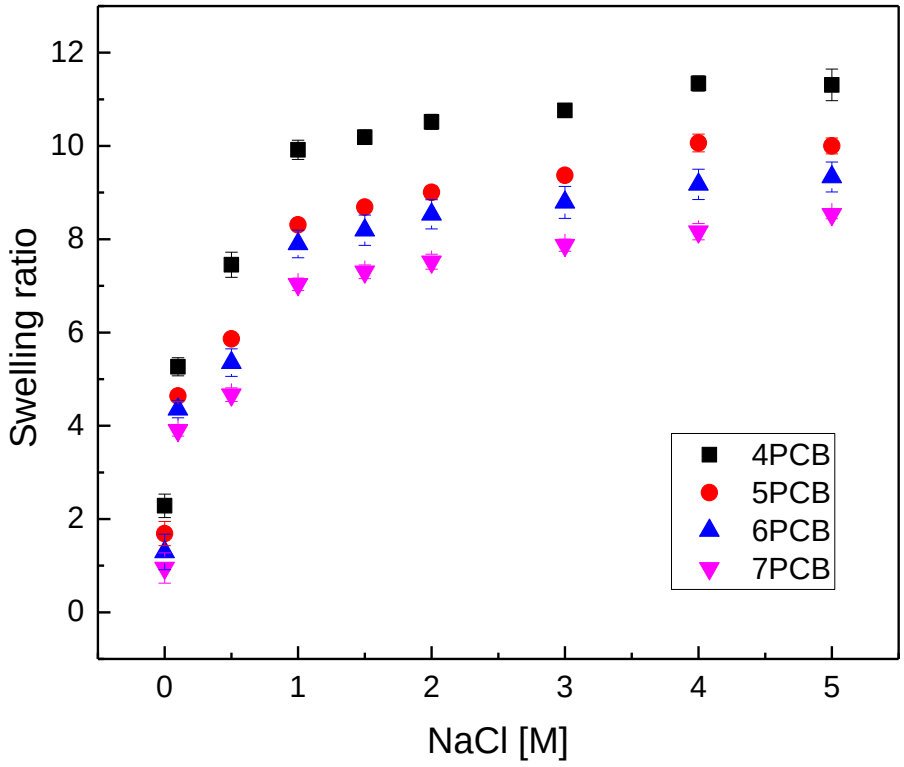



**Fig. 2** Equilibrium swelling ratio of PCB networks as a function of NaCl concentration.

All curves change their slope at 1M NaCl: below this salt concentration all curves are very steep and the SRs increase fast as the salt concentration increase. Above 1M NaCl, the slope of the swelling curves becomes less steep and the SR dependence on NaCl concentration is not very strong. The different slopes before and after 1M NaCl could be explained by the “saturation” of the zwitterionic moieties of PCB with the salt ions at this salt concentration, which reduced the salt effect on the SR upon firther salt concentration increase.

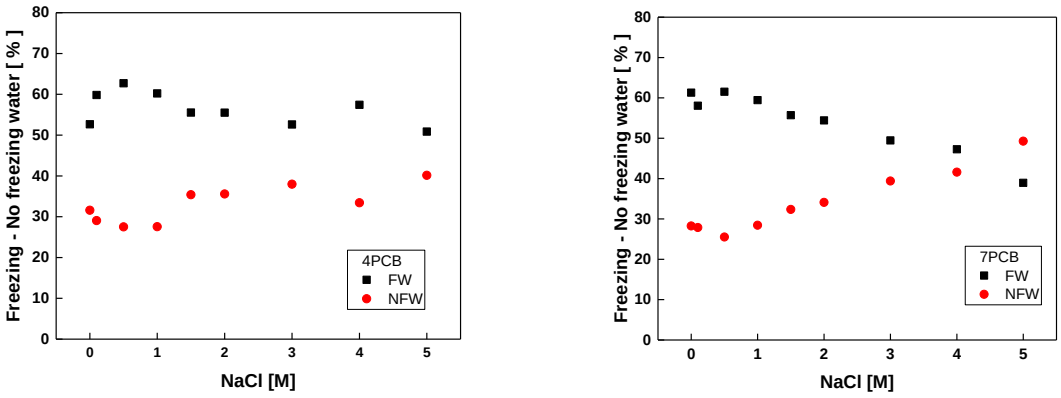
In summary, PCB hydrogels are suitable candidates as dressing material for highly exuding wounds since they were able to absorb large amounts of salty aqueous solutions similar to the wound exudate.

### *3.3. "Non-bound" and "bound" water in PCB hydrogels*

The water-polymer interactions may define many important hydrogel properties such as the absorption and diffusion of solutes (e.g. drugs and enzymes), the biocompatibility, and the mechanical performance. When strong interaction between water molecules and the polymer exists, the so called "bound" water is obtained which crystallizes at temperature much lower than 0 ºC.

On the other side, when water molecules do not interact with the polymer, it is called "non-bound" or "free" water that crystallizes at 0 ºC. The PZI's excellent biocompability is often explained by the large amount of bound water which they contain. The latter is considered to make the PZI surface very soft and gentle for proteins from the body fluids (e.g. blood, lymph and saliva). The highly hydrated PZI surface is believed to define their excellent non-adhesiveness and low biofouling. Therefore, the "bound"/"non-bound" water ratio for the PCB hydrogels and the effect of the network density and the salinity of the medium on this ratio, have been studied.

Two PCB hydrogels, 4PCB and 7PCB, were swollen in NaCl aqueous solutions with concentrations ranging from 0.1 to 5M, and the amounts of freezable (non-bound) water (FW) and non-freezable (bound) water (NFW) were determined by using DSC (Fig. 3). For both samples, the loosest (4PCB) and the densest (7PCB) PCB networks, the amount of bound water was between 30 % and 40 %. This amount slightly increased with NaCl concentration due to the interaction between the water molecules and the salt ions which immobilized additional amount of water, and became more significant as the salt concentration increased. The crosslinking density, however, did not significantly influence the amount of free and bound water in the samples, which means that the nature of the polymer plays more important role here.




**A** **B**

**Fig. 3** Dependence of FW and NFW on NaCl concentration for: (A) 4PCB and (B) 7PCB PCB hydrogels.

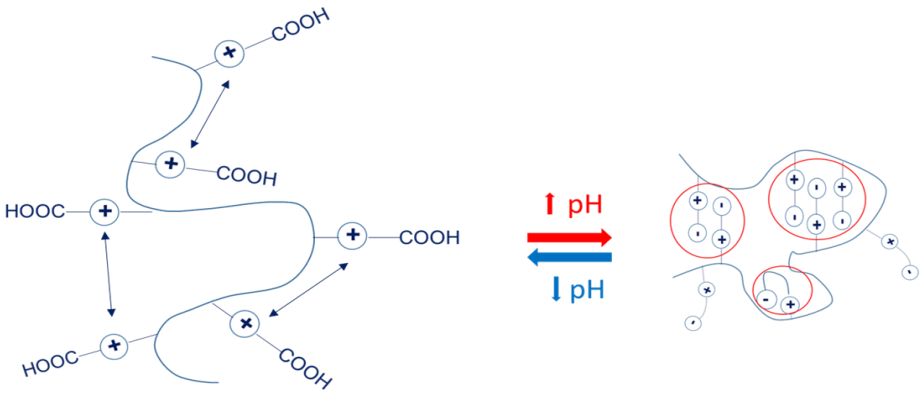
Under physiological conditions, i.e. at salt concentration 0.15M NaCl, both PCB hydrogels possess almost equal amounts of bound water (~ 30 %). Exactly the opposite trend was observed for PSB hydrogels, where the amount of non-freezable water was higher (~ 40 %) as compared to PCB but the amount of “free” water was much lower (~ 30-40 %) [13].

### *3.4. pH sensitivity of PCB hydrogels*

The variation of pH is expected to significantly influence the swelling behavior of PCB hydrogels due to their side chains -COOH groups. While carboxylic groups could be protonated or deprotonated following the acid-base equilibria with respect to the pH of the swelling medium, the -$R_3N$+ groups in PCB’s zwitterionic moiety are insensitive to such pH changes. Thus, the pH variation results into polycation to polyzwitterion transformation of PCB.

The swelling behavior of PCB networks with different crosslinking degree was studied in the pH range 2÷12 at ambient temperature. All PCB hydrogels decreased their swelling degree gradually as the pH increased from 2 to 9 (Fig 4). When the swelling medium became alkaline, i.e. pH>9, no further change of PCB’s swelling was observed. At the lowest pH value, i.e. 2 (pH < $pK_a^{COOH}$ which is ~ 4.5) all carboxyl groups are protonated, and thus PCB behaves as a polycation due to the permanent positive charge of their quaternary ammonium groups (Scheme 1). The electrostatic repulsions between the neighbor -$R_3N$+ groups are the reason for the high swelling ability and the expansion of the hydrogels. When pH gradually increased, the number

of deprotonated carboxylic groups also increased and PCB was gradually transformed from polycation to polyzwitterion (Scheme 1).




**Scheme 1.** Influence of pH on the –COOH groups protonation/deprotonation and the related change in the PCB conformation.

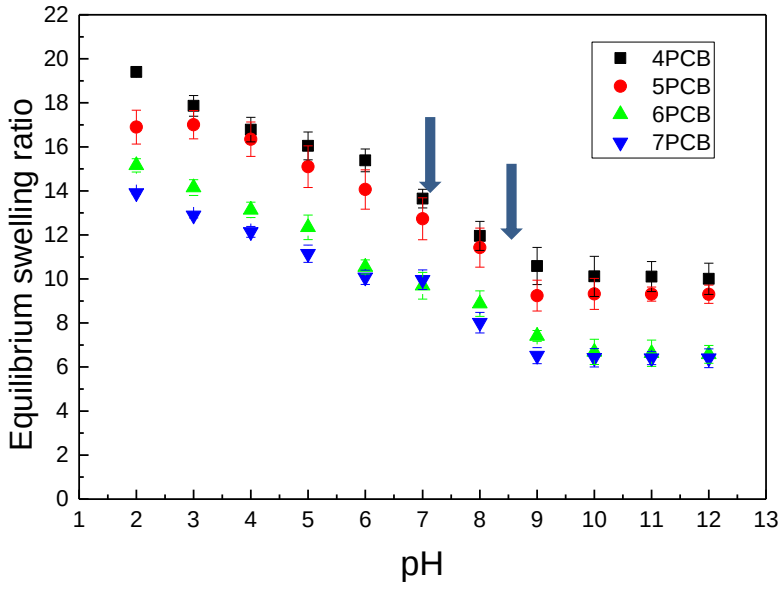



**Fig. 4** pH dependence of PCB swelling at 25 °C.

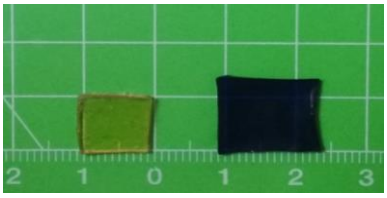
This resulted into hydrogel shrink because the number of electrostatic repulsions diminished. At pH>9, the lowest PCB swelling degree was reached and it remained constant even when pH increased up to 12. This constant value was attained when all –COOH groups were deprotonated, i.e. all side chains were transformed into zwitterions and they all participated in the formation of zwitterionic clusters.

The change of the overall charge of PCB (from positively charged to neutral) is expected, on the one hand, to *switch off* the antibacterial performance of these hydrogels and to *switch on*, on the other hand, their antibiofilm activity. The pH range of this polycation-polyzwitterion transformation coincides with the change in pH values from acute (~4 to 6) to chronic wound (~7.15 to 8.93 designated in Fig. 4) and the transition of bacteria from being motile cells to becoming sessile cells forming the biofilm typical for the chronic wounds.

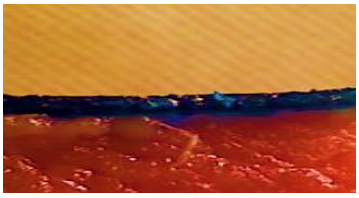
The impact of the crosslinking degree on the PCB hydrogels swelling behavior as a function of pH was consistent with the results obtained for the swelling kinetics (Fig 1). The highest crosslinking degree results in the lowest swelling ability of hydrogels due to the densest polymer network. As the pH environment of chronic wounds is slightly alkaline (above 7.15), it can be concluded that the most appropriate candidate for chronic wound dressing is 4PCB hydrogel as it demonstrated the highest swelling ability in the pH region of chronic wound (7.15 to 8.93) [6]. However, all PCB samples showed great swelling capacity in salt solution depending on their network density, and could swell 8 to 12-fold their own weight. This behavior differs from the behavior of all polyelectrolyte based wound dressings currently on the market, which shrink in salt solutions.

### *3.5. Volume change of the PCB hydrogels*

One of the most important advantages that PZI, and in particular PCB, could provide as wound dressings is their ability to expand, but not to shrink upon wound exudate absorption. In Fig. 5A, the volume change of 4PCB networks is presented. The 4PCB sample swollen for 3 min in Methylene blue 0.15M NaCl was shown to expand upon absorption of the physiological solution by 25 %. For comparison, the same experiment was performed with three commercially available wound dressings (see Supplementary Information, Fig. S3). It was observed that AQUACEL® Ag Extra™ and Durasoft shrunk upon swelling, while ALLEVYN Adhesive expanded slightly. The PCB expansion, however, is larger and thus it could provide better comfort to the patients. Wound dressing shrinkage is one of the bottlenecks for management of exuding wounds as the dressing shrinkage causes mechanical injury to the wound as well as pain and discomfort to the patient.




(A)



(B)

**Fig. 5** Expanding the 4PCB hydrogel for 3 min in Methylene blue 0.15M NaCl solution (A) enabling (B) full coverage of the wound.

Even if the wound already started to heal, the wound dressing shrinkage could compromise the proper healing process as well as it could cause disruption of the healing tissues. Thus, another important advantage of PCB hydrogels as wound dressing materials is their expandability upon swelling in physiological solution and it was demonstrated here.

The hydrogel nature of the PCB based material defines another their advantage as chronic wound dressing, namely the close cover of the wound surface. It is known that if the dressing does not fully cover the wound surface, the warmth and moisture promotes bacteria colonization of the wound. The empty spaces between the wound and the dressings form niches where bacteria could colonize easier, so an important practical aspect of a chronic wound dressing is its ability to closely follow the wound curvature. This is an easy task for the hydrogels (Fig. 5B) but not for other wound dressings (see Fig S3B in the Supplementary Information). On the pictures (Fig. 5B and Fig. S3B) the wound is mimicked via a piece of meat with non-even surface which was fully covered by the PCB hydrogels (Fig. 5B) and no niches between both surfaces were observed.

### *3.6. Enzyme absorption in PCB hydrogels*

Collagenase is one of the MMPs that plays an important role for the re-epithelialization in wound healing. However, the excessive collagenase activity delays the wound closure and leads to wound chronicity [20]. Wound dressings based on PCB hydrogels are expected to regulate the high collagenase activity in two ways: i) they can absorb and retain the wound exudate, containing the enzyme, and ii) directly inhibit the collagenase activity.

Taking into account that PCB hydrogels demonstrated good swelling ability, especially in presence of salts, we studied their absorption potential towards collagenase. It can be seen from Fig. 6 that the amount of collagenase, absorbed by PCB hydrogels,

depended on the polymer network density: as the crosslinking degree of the polymer network increased, the amount of loaded/absorbed enzyme decreased. The observed difference between the collagenase loading of the different PCB networks is statistically significant as determined by one-way ANOVA (see Tables S1 and S2, Supplementary Information (SI)). The loosest (4PCB) network absorbed ~38 % from the initially available in the solution collagenase, while the densest, 7PCB, network was able to load ~29 % of collagenase.

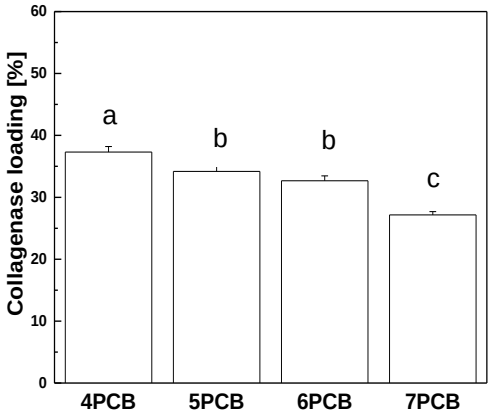



**Figure 6.** Collagenase loading (%) in PCB networks (a, b, c: the same letter designation means that the values are not statistically different (p = 0.05), Table S2, SI).

In both cases, PCB hydrogels demonstrate ability to physically remove part of the overexpressed collagenase from the wound. The enzyme loading potential of PCB hydrogels is expected to be influenced by their salt-dependent ability to swell as well as by their pH sensitivity. Both parameters are expected to enhance the PCB networks swelling ability. Thus, PCB demonstrates appropriate swelling ability in the pH region of chronic wounds, which combined with their salt enhanced swelling capacity contributed to the reduced collagenase concentration in the chronic wounds.

### *3.7. Collagenase inhibitory activity of PCB hydrogels*

The physical retention of collagenase from PCB networks was further complemented by examination of their ability to inhibit it. The *in vitro* tests show that collagenase incubated with PCB hydrogels with different crosslinking densities preserved between 60 to 75 % of its initial activity (Fig. 7A). There is no statistically significant difference between the inhibitory effect of PCB hydrogels as determined by one-way ANOVA

(Tables S3 and S4, Supplementary Information), i.e. the inhibitory effect of PCB hydrogels on the collagenase activity does not depend on the PCB networks crosslinking density.

Similar tests were performed *ex vivo* using a real wound exudate instead of collagenase solution. This experiment most closely mimics the real chronic wounds conditions as there are no reliable animal models for chronic wounds testing. The results obtained from the *ex vivo* experiments coincide well with the *in vitro* results as the former also show that PCB hydrogels inhibit around 30 % of the total proteolytic activity of the chronic wound exudate (Fig. 7B). Again, there is no statistically significant difference between the samples as determined by one-way ANOVA (Tables S5 and S6). This inhibition activity of PCB samples towards collagenase can be assigned to the simultaneous action of these two factors: enzyme physical absorption in the hydrogel and removal from the wound bed as well as the inhibition activity of PCB itself. However, as no clear dependence of the inhibitory effect on the crosslinking density is observed, it could be assumed that the enzyme physical absorption does not interfere with the observed enzyme inhibition by the PCB hydrogels.

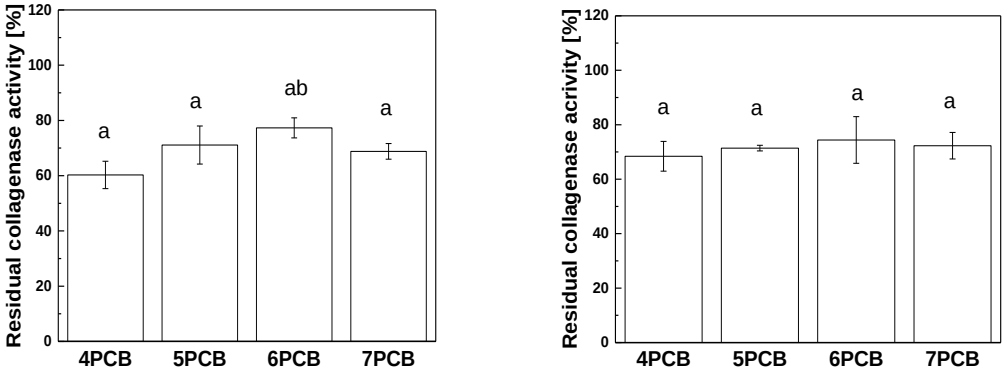



**Fig. 7** *In vitro* (A) and *ex vivo* (B) tested collagenase activity after incubation with PCB hydrogels (a, b: the same letter designation means that the values are not statistically different (p = 0.05), Tables S4 and S6, SI).

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Thus, the PCB networks could be effectively used as collagenase absorbents without significantly hampering the enzyme activity which ensures proper wound healing at reduced collagenase levels in the wound.

Similar experiments were performed with MPO as this is another enzyme which overexpression is known to be characteristic of chronic wounds. Again, the results from both in vitro and ex vivo tests showed that PCB hydrogels reduced MPO activity by 20 to 40 %, depending on their network density.

### *3.8. Antibiofilm activity of PCB hydrogels*

The non-fouling behavior of PZI, and in particulate of PCB, is often explained by the hydration layer formed on the polymer surface [21] due to the bound to the polymer water. This layer does not allow the adhesion of proteins and bacteria on the polymer surface which is considered as the reason for bacterial adhesion prevention and hence for the antibiofilm activity of PCB. All studied PCB networks demonstrated a significant inhibition of S. aureus biofilm growth on their surfaces in comparison with a pristine silicone used as a control (Fig. 8). The Tukey post hoc test shows that the difference between the control value and any mean value of PCB samples is significant at 0.05 significance level (p=0) (Table S12, Supplementary Information).

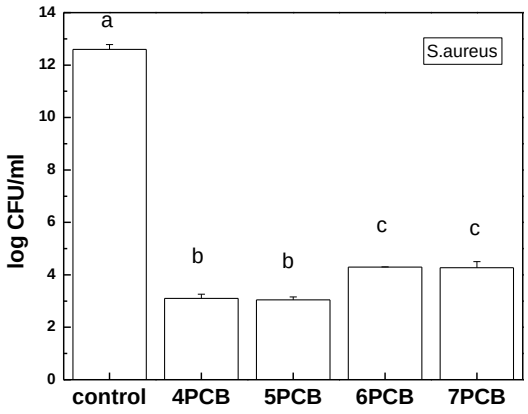


**Figure 8.** *S. aureus* growth on PCB hydrogels (a, b, c: the same letter designation means that the values are not statistically different (p = 0.05), Table S12, SI).

The one-way ANOVA analysis shows that there is a statistically significant difference between the samples (Table S11, Supplementary Information) and the crosslinking density has an effect on the antibiofilm performance of PCB hydrogels. It seems that the increase of the crosslinking density slightly decreases the antibiofilm performance of PCB hydrogels. However, all PCB hydrogels demonstrate potential to exert antibiofilm activity which make them appropriate as chronic wound dressings materials.

### *3.9. Cytotoxicity of PCB hydrogels*

As wound dressing candidates, PCB networks were tested for their cytotoxicity (Fig. 9). The BJ-5ta cells viability was above 75 % for all PCB samples, ranging from 75 to 97 %. These results suggest that PCB hydrogels are non-cytotoxic and could be used as wound dressing materials.

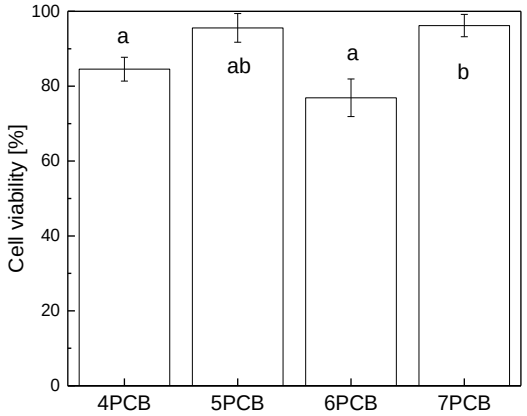


**Fig. 9.** Cell viability of BJ-5ta cells, incubated with PCB hydrogels for 24 h as determined by alamar blue reagent (a, b: the same letter designation means that the values are not statistically different (p = 0.05), Table S14, SI).

The performed ANOVA indicates that cytotoxicity of PCB hydrogels is significantly influenced by the degree of crosslinking (p=<0.0005) (Tables S13 and S14) but no clear dependence is seen in Fig. 9.

### *3.10. Biological evaluation of PCB hydrogels*

#### *3.10.1. In vivo experiments*

The regulatory approval process for different materials and their usage as medical devices needs to pass via biocompatibility testing. The implantation sites of all treated animals revealed normal tissue structures and implantable materials with preserved dimensions located among loosely arranged collagenous fibers of connective tissue (Fig. 10).

In all cases, thinner or thicker fibrous capsules, composed of collagenous fibers, fibroblasts and fibrocytes were formed around the implanted PCB hydrogels as a part of the chronic inflammation and foreign body reaction. Other signs of acute inflammatory reaction as polymorphonuclear neutrophils and lymphocyte infiltration,

necrosis, and calcification were not observed, as well as any congestion and evidential intolerance towards the implanted materials.

Both implanted PCB hydrogels, namely 6PCB and 7PCB (designated with 5-point stars in Fig. 11 (a) and (b)) provoked the formation of thin fibrous capsules populated with fibroblasts and fibrocytes (designated by +). Both capsules have intra- and extracapsular neoformed capillaries and small blood vessels (designated with arrows) which vary in diameters and level of development. The cell populations in the examined sections were mainly presented by fibrocytes, rarely lymphocytes, monocytes and plasma cells. Reabsorption and remodeling of the implants were not found neither any histopathological signs of local or systemic toxicity. The materials were assessed as successful as they have shown good biocompatibility according to the 1st and the 2nd criteria of ISO_7405.

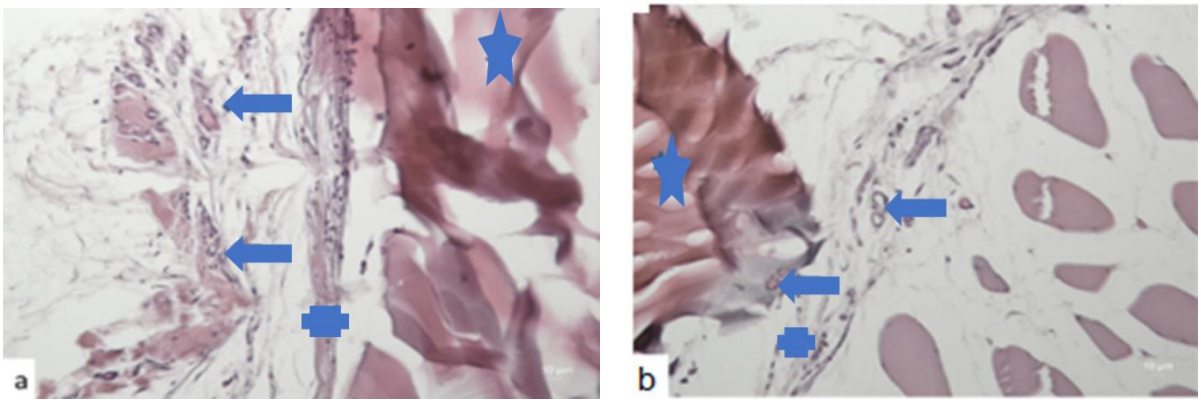


**Figure 11.** (a) Implanted 6PCB hydrogel, designated with 5-point star (a) provoked the formation of two layers of a thin fibrous capsule (designated by +) connected via loose connective tissue and populated with fibroblasts and fibrocytes. The presence of intra- and extracapsular neoformed capillaries and small blood vessels (designated with arrows). (b) - implanted 7PCB hydrogel (designated by 5-point star) provoked the formation of a thin fibrous capsule (designated by +) inhabited with fibroblasts and fibrocytes; plenty of neoformed capillaries and small blood vessels were seen (designated with arrows).

The formation of fibrous capsulation and the newly formed vascularity in the sites of implantation were result from the interactions between the PCB hydrogels and the local environment. These events usually are a part of specific cellular and molecular

dynamics in tissues under cell mediated profibrogenic factors, proteins and specific processes which remodel the extracellular matrix. Similar to these histological aspects after implantation observed here were reported in various *in vivo* experiments with other materials [22-24] evaluated as an indication for the good tolerance from the organisms to the implants and are biological tissue responses with a particular role in the wound healing process [25].

## *4. Conclusions*

The study has revealed the potential of PCB hydrogels as dressing materials for chronic wounds management. The PCB hydrogels were shown to possess high ability to absorb large amounts of physiological solution (mimicking the wound exudate), expanding their dimensions, but not shrinking as most of the available at the market wound dressings. Due to their high swelling ability in salt solutions, the PCB hydrogels were able to absorb the collagenase and MPO, which makes them appropriate for controlling the overexpression of these deleterious enzymes in chronic wounds. Moreover, PCB hydrogels were demonstrated not to allow the biofilm formation from S. aureus which is one of the most prevalent bacterial species identified in chronic wounds. The PCB hydrogels were shown to be non-cytotoxic and biocompatible as they could be classified as provoking no reaction or slight reaction to moderate reaction after their implantation *in vivo* in rats for 2 months. Additionally, the PCB hydrogels possess all characteristics of hydrogel wound dressings such as transparency, preserving wound moisture, and non-adherence to the wound. All these properties are advantageous for the envisaged application of PCB hydrogels as chronic wound dressings.

## 5. Associated content

**Supporting information**. Volume change of commercially available AQUACEL® Ag Extra™ ConvaTec, ALLEVYN Adhesive Smith & Nephew, DURAFIBER, Smith & Nephew wound dressings in 0,15 M NaCl. Rheological properties of 4PCB and 7PCB PCB hydrogels.

## 6. Acknowledgment

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