

ACRYLIC COMPOSITE MATERIALS MODIFIED WITH BEE POLLEN FOR BIOMEDICAL APPLICATION

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Abstract

The aim of this paper is to present the influence of bee pollen on the physicochemical and in vitro properties of poly(acrylic acid) (PAA) hydrogel composites enriched with hydroxyapatite and modified with bee pollen as a prospective material for biomedical application with beneficial features including good osseointegration and anti-inflammatory effect. The phase and chemical composition of hydroxyapatite synthesized by wet-precipitation method was confirmed by means of X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FT-IR). Proposed materials were investigated towards in vitro properties by immersion in the incubation fluids including artificial saliva, Ringer's solution and distilled water. The composites swelling ability was determined. Additionally, the chemical structure of the polymer matrix composites was confirmed by FT-IR method. Moreover, to characterize composite degradation process during 21-day incubation the FT-IR technique was used. In order to describe bee pollen feature, both scanning electron microscopy and X-ray fluorescence spectrometry were used. Presented research revealed that hydroxyapatite, as well as PAA undergo biodegradation during in vitro test. Moreover, matrices degradation results in incubation fluids pH decrease associated with anionic nature of PAA which is further enhanced by bee pollen release. The strongest pH drop effect was observed for Ringer's solution. Increase in conductivity of distilled water confirmed composites degradation process.

Keywords: hydrogel, hydroxyapatite, bee pollen, composite, biomaterial

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Introduction

In recent years hydrogels, due to their biocompatibility, cell-controlled degradability, and intrinsic cellular interaction have become one of the most important groups of materials used in medicine. Nowadays, hydrogels play an important role in dynamically developing field of tissue engineering where they are used as three-dimensional porous scaffolds to guide the growth of new tissues, as implantable materials in cartilage wound healing, drug delivery systems, wound dressings and in bone regeneration [1-6].

The structure and functions of those polymeric materials can be modified in a precisely controlled way. To foster new tissue formation hydrogels must meet a number of criteria that include physico-chemical (e.g. degradation and mechanics) and biological (e.g. cell adhesion) guidelines [7]. Hydrogels are made from natural polymers (e.g. collagen, chitosan, keratin) as well as synthetic polymers (e.g. poly(vinyl alcohol), PVA, poly(ethylene glycol), PEG, poly(acrylic acid), PAA). [8-15].

PAA has been intensively examined by many scientists in the context of biomedical applications, due to its biocompatibility, pH-sensitivity, solubility in water and aqueous solutions of inorganic salts and simultaneous lack of solubility in most organic liquids [16-17]. In neutral and alkaline solution acidic carboxyl groups of PAA are ionized and this leads to electrostatic repulsion of the polymer chains [18-19]. Over recent years, various PAA-based materials for the use in tissue engineering have been proposed [20].

Nowadays, polymeric hydrogels are mainly combined with different type of nanocompounds (e.g. graphene oxide, carbon nanotubes, gold-silica nanoshells, nano-hydroxyapatite, ultra-high-molecular-weight polyethylene nanofibres) [21-25], biologically active proteins or peptides [26-27], and reinforced with inherently biocompatible particles (e.g. hydroxyapatite - HAp) [28-30].

HAp is the inorganic component of bones and teeth affecting their hardness and strength. HAp due to its porosity, bioactivity, biocompatibility and ability to form a good connection with the living tissues is willingly used as a component of different types of biomaterials, especially in dental and orthopaedic surgery as a filling material for biocompatible matrix [30-33].

In this research paper, we describe in detail the preparation, characterization and biocompatibility studies of three-dimensional hydrogel composite material prepared from acrylic acid, particles of HAp and agar (as a stabilizing agent), cross-linked by poly(ethylene glycol) diacrylate (PEGDA) under microwave irradiation and modified by different amounts of bee pollen. Experimental pharmacological studies performed on bee pollen confirmed its prospective application in hyper-lipidemia and atherosclerosis treatment as well as detoxification activity against organic solvents such as carbon tetrachloride and trichlorethylene [34-35]. Moreover, it is proved that bee pollen can be applied in the early old age symptoms and neurasthenic inertia treatment [36]. Pollen also shows high anti-inflammatory effect comparable to naproxen and indomethacin. Its anti-inflammatory effect is about inhibiting the activity of enzymes responsible for turning arachidonic acid into toxic compounds that induce inflammatory conditions in tissues [37]. Bee pollen containing hydrogel materials were presented in [38]; however hydroxyapatite addition in such materials can enhance their functionality by promoting osseointegration.

Materials and Methods

Materials

In experiments, all the reagents were of analytical grade and used without further purification. The chemicals: calcium oxide (CaO, to prepare aqueous solution of calcium hydroxide), 85% phosphoric acid (H₃PO₄), 25% ammonia solution, acrylic acid (AA), potassium hydroxide (KOH) and ammonium persulfate (APS) were purchased from POCh Gliwice, Poland. PAA was derived from Merck and agar was acquired from Sigma Aldrich.

Synthesis of hydroxyapatite

Hydroxyapatite was obtained by widely used synthesis technique called chemical or wet precipitation, based on the chemical reaction between calcium hydroxide $\text{Ca}(\text{OH})_2$ (0.5M) and phosphoric acid H_3PO_4 (0.3M) in the presence of ammonia solution, according to the equation (1).



Preparation of PAA/HAp bee pollen modified hydrogel

Synthesis of PAA/HAp hydrogel was conducted in accordance with the procedure: 45 cm³ of acrylic acid was neutralized with 50 cm³ potassium hydroxide aqueous solution (40wt.%) and cooled slowly down to 30°C. After that 1 wt.% initiator (APS), 1wt.% crosslinking agent (PEGDA) and 20 g of stabilizing agent (agar) were added. Consequently, the obtained solution was finally mixed with previously prepared powder mixtures with various content of hydroxyapatite and ground bee pollen (TABLE 1). The resulting mixtures of powders and PAA, with other additions, were cross-linked by microwave irradiation at 600 W for 1 min, which resulted in formation of three-dimensional porous hydrogel structures.

Hydrogel swelling characteristics

1.0 g hydrogel samples were prepared in order to determine the swelling ratio of materials in simulated body fluid solution (TABLE 2), Ringer's solution (TABLE 3), artificial saliva (TABLE 4) and distilled water after twenty-three days of incubation at 37°C. The swelling ratio (X) was calculated according to the equation:

$$X = \frac{m - m_0}{m_0} \quad (2)$$

where m_0 is the weight of hydrogel sample before incubation and m is its weight after incubation time.

Measurements of the ionic conductivity and pH value

Measurements of ionic conductivity and pH values of SBF, Ringer's solution, artificial saliva and distilled water were carried out during the hydrogels samples incubation in the mentioned fluids. The pH values changes were measured using Elmetron CP-401 waterproof pH Meter equipped with ERH – 111 composite electrode, and the ionic conductivity of solutions was investigated using Elmetron EC- 60 conductivity meter.

X-ray diffraction

The X-ray diffraction method was used to identify the hydroxyapatite particles in powder mixtures used to prepare hydrogel materials and phase identification of bee pollen. Analysis was performed using Philips X'Pert X-ray diffractometer equipped with a graphite monochromator PW 1752/00 with Ni filter (40 kV and 30 mA). All XRD patterns were recorded in the 2θ range 10° – 60°.

IR Spectroscopy

The FT-IR studies, by KBr pellet technique, were utilized to determine the changes in the composition of the hydrogels after incubation time in comparison to their initial chemical composition using a Scimitar Series FTS 2000 Digilab spectrophotometer in the middle infrared range of 4000-400 cm⁻¹.

X-ray fluorescence spectrometry

In this paper XRF technique was used to analyze the chemical composition of bee pollen. The investigations were performed using Mini Pal PW 4025/00 X -ray PANalytical spectrometer with a sample changer, built-in helium system and analytical area of Na - U. Spectrometer was equipped with a semiconductor Si-PIN detector and X-ray lamp with a side window (9W) with Rh cathode.

TABLE 1. The composition of the powders used in synthesis.

Sample	HAp content [g]	Bee pollen content [g]
s1 1	4.0	2.0
s1 2	4.0	2.5
s1 3	4.0	3.0
s1 4	4.0	3.5
s1 5	4.0	4.0
s2 1	1.0	2.5
s2 2	1.0	3.5
s2 3	1.0	4.5
s2 4	1.0	5.5
s2 5	1.0	6.5

TABLE 2. The composition of the SBF solution.

Component	Concentration [g/L]
NaCl	8.035
NaHCO ₃	0.355
KCl	0.225
K ₂ HPO ₄ ·3H ₂ O	0.231
MgCl ₂ ·6H ₂ O	0.311
HCl (1M)	39 ml
CaCl ₂	0.292
Na ₂ SO ₄	0.072
Tris	6.118
HCl (1M)	0-5 ml

TABLE 3. The composition of the Ringer's solution prepared according to [39].

Component	Ions content [mM/dm ³]
Na ⁺	147.0
K ⁺	4.0
Ca ²⁺	3.0
Cl ⁻	157.0

TABLE 4. The chemical composition of the artificial saliva prepared according to [40].

Component	Concentration [g/dm ³]
NaCl	0.400
KCl	0.400
CaCl ₂ ·H ₂ O	0.765
NaHPO ₄ ·H ₂ O	0.780
Na ₂ S·H ₂ O	0.005
(NH ₂) ₂ CO ₃	1.000

Result and Discussion

SEM images of raw bee pollen at different magnifications are shown in FIG. 1. As it can be observed, bee pollen particles reveal elliptical shape and very uniform size distribution. Grains uniformity in size and shape is caused by gathering of bee pollen of the same plant species. In FIG. 1b it is shown that bee pollen particles reveal well-ordered porous distribution on their surface, which may result in significant improvement in adhesion to acrylic hydrogel matrix.

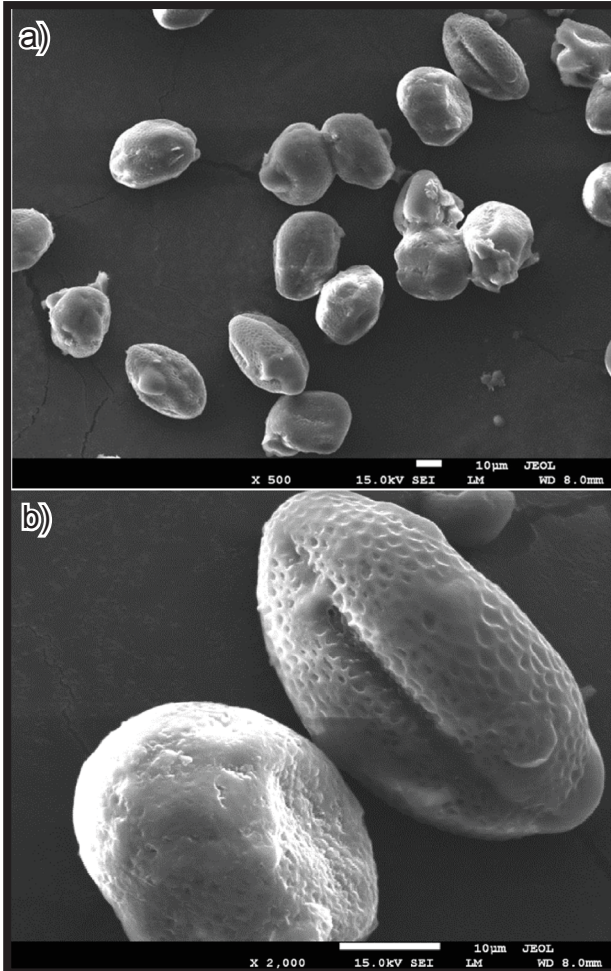


FIG. 1. SEM images of bee pollen at 500x magnification (a), and single particles of bee pollen at 2000x magnification (b).

The analysis of bee pollen chemical composition was carried out with X-ray fluorescence technique. XRF studies (FIG. 2) have shown that potassium and calcium are the most abundant elements in the pollen tested. There is a noticeable difference between content of K and Ca and the content of the remaining elements in tested sample. In addition, sulfur, phosphorus, iron, copper and zinc were also detected in smaller amounts as compared to the previously discussed elements. The content of other elements such as manganese and silicon is negligible. What is important and worth mentioning is that the bee pollen chemical composition differs from their counterparts in different parts of the world. Therefore, the currently occurring season, plant species and climatic zone have a significant impact on the chemical composition of bee pollen.

X-ray diffraction patterns of hydroxyapatite and HA/bee pollen powder mixtures used in composites syntheses are shown in FIG. 3. The XRD pattern found in FIG. 3a is typical for crystalline monophasic hydroxyapatite. FIG. 3e shows XRD pattern of a raw bee pollen. The presence of pollen in the analyzed samples caused increase in the intensity of the background of pattern 1 b to 1 d in angular range 10° - 30° .

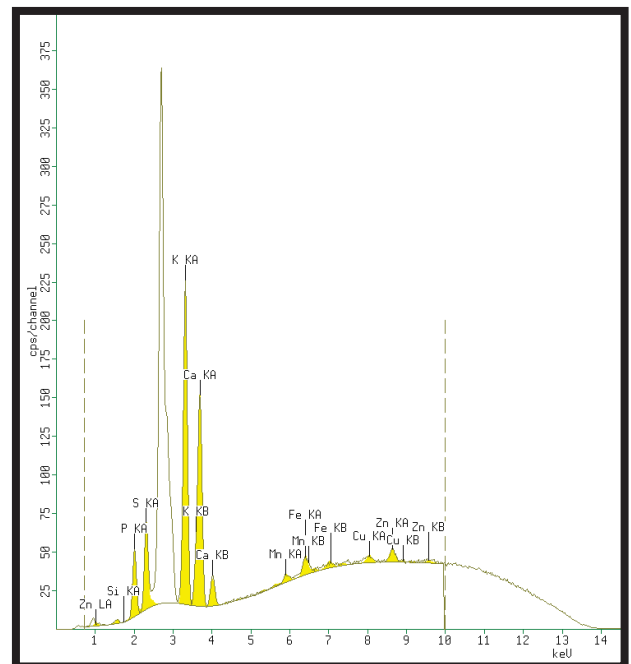


FIG. 2. X-ray fluorescence analysis of raw bee pollen.

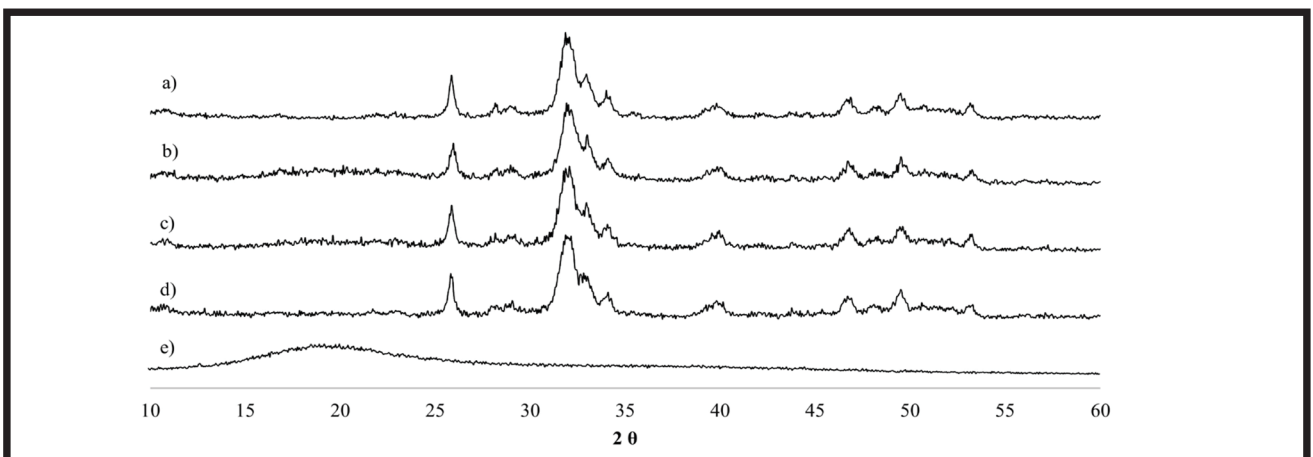


FIG. 3. XRD pattern of synthetic hydroxyapatite (a), S2 5 (b), S1 5 (c), S1 1 (d) and raw bee pollen (e).

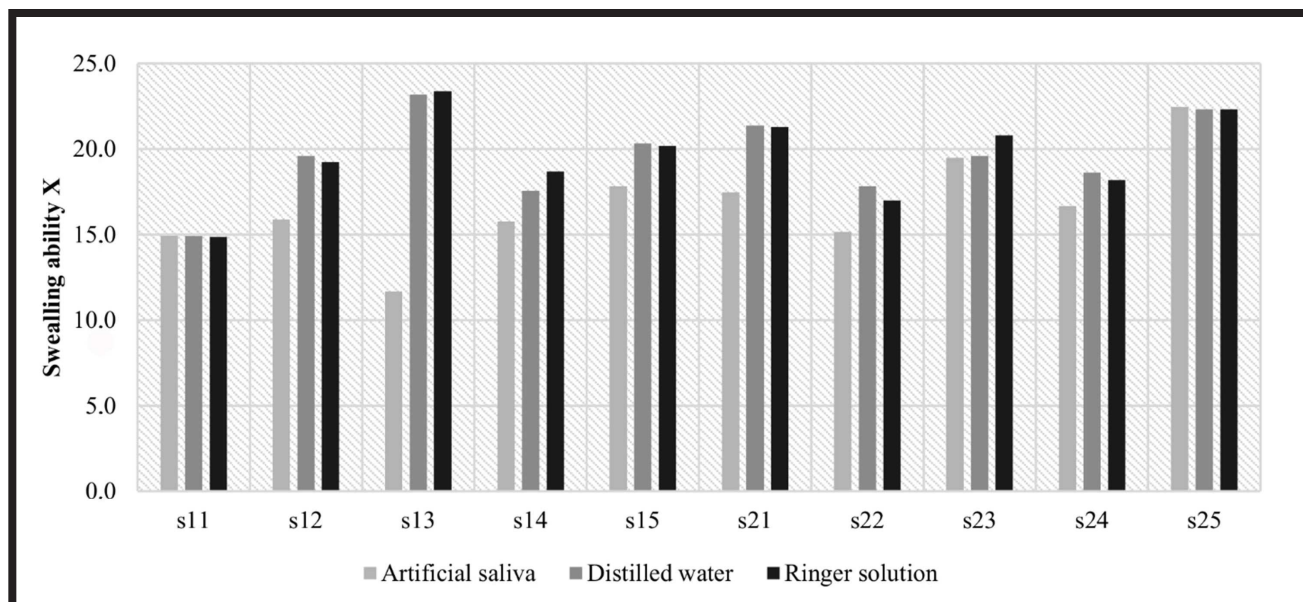


FIG. 4. Swelling ability of hydrogel composites.

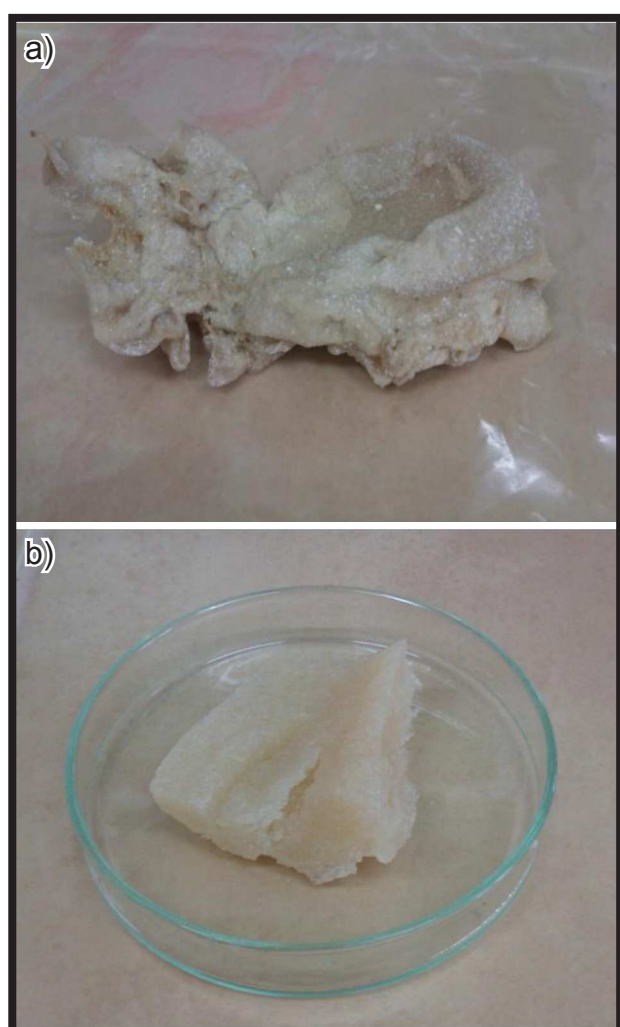


FIG. 5. Hydrogel composite before incubation (a) and after 21 days immersion in distilled water (b).

FIG. 4 shows swelling ability (X) of hydrogel composite materials incubated for 21 days in artificial saliva, distilled water and Ringer's solution. Measurement of specimens mass was carried out immediately after incubation without samples drying and revealed mass increase for all hydrogels.

High swelling ability of hydrogels is well-known feature of this type of materials. However, when measuring the mass of hydrogels, the possible degradation of the matrix should also be taken into account. The highest weight gain was observed for composites incubated in distilled water and Ringer's solution, which is due to pH of this solution. FIG. 5 shows hydrogel composites before incubation and after 21-day immersion in distilled water.

PAA is classified as a pH sensitive polymer which is caused by hydrophilic groups $-\text{COOH}$ in polymer structure. At low pH, the interaction between the non-ionized carboxyl groups causes attraction of polymer segments, resulting in the shrinking of the macromolecule to compact form. With the increase in environmental pH, the carboxyl groups are ionized and repelled, causing the polymer chain expanding and water binding. At a $\text{pH} < 4$, the PAA is in a non-dissociated form, but at a $\text{pH} > 8$ the chain is fully ionized.

The pH changes of incubation fluids during samples immersion are associated with the presence of bioactive hydroxyapatite particles and biodegradable matrix, as well as releasing of bee pollen from the polymer matrix (FIG. 6). The changes in pH were more pronounced, the greater bee pollen/hydroxyapatite mass ratio was used for the composites synthesis, which confirms acidic nature of bee pollen. PAA, due to its anionic nature, also affected the pH drop. Changes in pH in individual solutions were not significant. The largest decrease in pH from 11.01 to 8.46 was recorded for the Ringer's solution.

FIG. 7 presents changes in the ionic conductivity of distilled water around the tested samples as a function of time. As a result, continuous growth of ionic conductivity was observed, along with an increase in the incubation time of composites in distilled water, although the rate and range of growth were varied. The different growth rate of ionic conductivity was due to different composition of composites. Composites with a medium and low content of bee pollen, for which both pH and conductivity were less pronounced, were more chemically stable.

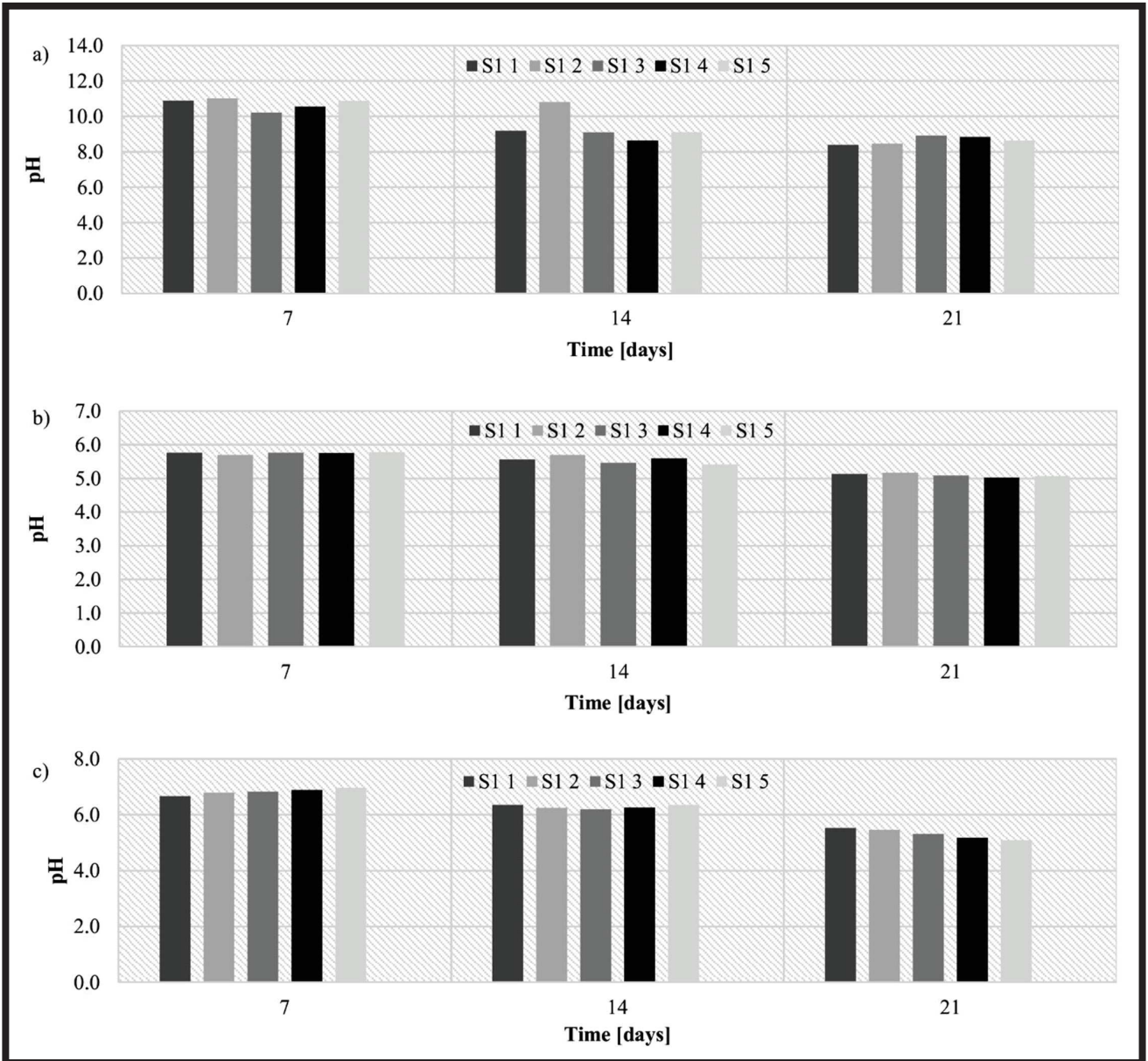


FIG. 6. The pH changes of Ringer's solution (a), artificial saliva (b) and distilled water (c).

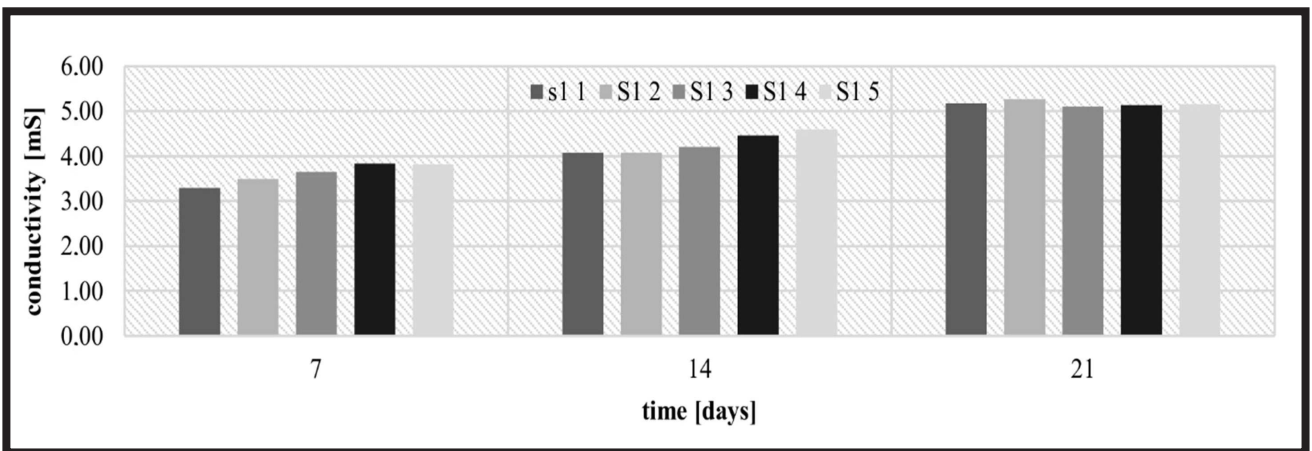


FIG. 7. Changes in conductivity of water during incubation s1 samples series.

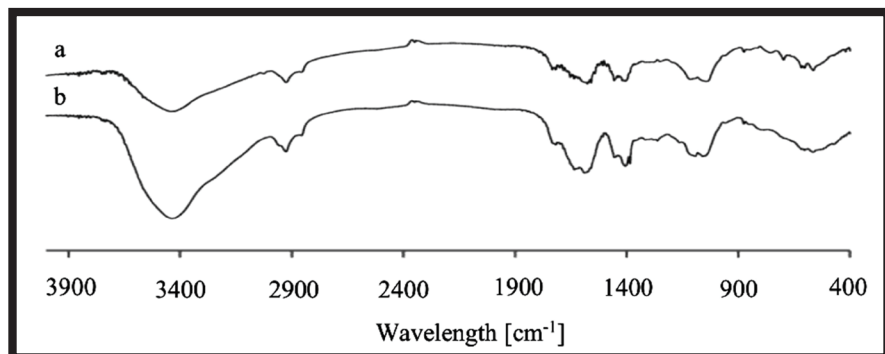


FIG. 8. FT-IR spectra of S2 5 (a) and S1 1 (b) samples before incubation.

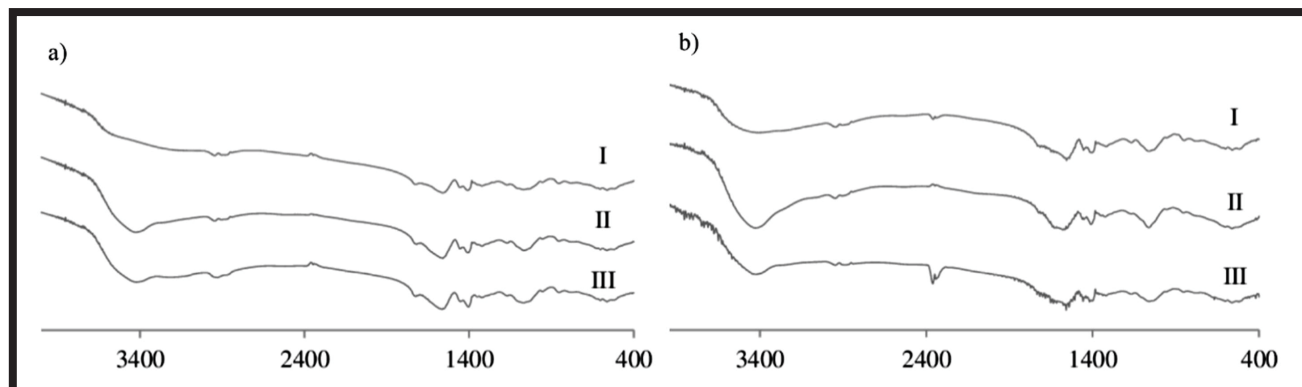


FIG. 9. FT-IR spectra of S2 5 (a) and S1 1 (b) samples after incubation in Ringer's solution (I), artificial saliva (II) and distilled water (III).

TABLE 5. IR band assignment of the samples.

Transmittance bands [cm ⁻¹]	Peak assignment
560-600	bending vibration of PO ₄ ³⁻ (O-P-O)
690, 740	in-plane deformation bending mode of CO ₃ ²⁻ (O-C-O)
870	bending mode of CO ₃ ²⁻ (A-type)
1030	triply degenerate asymmetric stretching mode of PO ₄ ³⁻ (P-O)
1100	bending mode of PO ₄ ³⁻ (P-O)
1375	symmetric bending deformation of C-H
1450	symmetrical scissor mode vibrations of the -CH ₂ -
1487	stretching mode of CO ₃ ²⁻
1550	vibrations of C=O
1715	ester groups stretching C=O
2785	symmetric stretching of -CH ₂ -
2962	asymmetric stretching of -CH ₂ -
3450	stretching mode of O-H

In order to fully characterize the HA-incorporated polymer matrix composites, FT-IR spectroscopy was employed. The FT-IR spectra of the chosen materials before immersion in incubation fluids are shown in FIG. 8.

FIG. 9 shows the FT-IR spectra after incubation in artificial saliva, distilled water and Ringer's solution. These spectra exhibit the presence of all bands that are found in HA specimen and pure PAA with intensity dependent on the content of the individual components of materials. Moreover, based on the FT-IR spectra, any interactions between materials ingredients that can lead to new chemical compounds formation are not observed thus PAA/HA/bee pollen composite materials may be considered as a mixture of these ingredients. FIG. 9a and 9b show the FT-IR spectra of specimens after 21-day incubation.

The degradation rate of materials is slightly dependent on the proportion of HA and bee pollen in the composite and a significant decrease in band intensity is observed after 21 days of immersion for all materials, indicating that both PAA and HA undergo degradation process in the incubation fluids. Transmittance bands revealed in the FT-IR spectra are assigned and summarized in TABLE 5.

Conclusions

The aims of this research were, firstly, to prepare hydrogel matrices made of PAA containing hydroxyapatite and modified with bee pollen and, secondly, to evaluate their prospective application in the field of biomedicine. Synthesis proposed in this paper allows to obtain 3D hydrogels enriched with bioactive phosphate and bee pollen that is recognized to be antiseptic and anti-inflammatory agent. Prepared composite materials present good sorption capacity against different fluids used in biomedical testing, such as Ringer's solution and artificial saliva. In vitro tests performed in this research proved that the investigated materials are stable in body-simulated conditions during 21 days of incubation. Given the fact that acrylic hydrogels are already applied as wound dressing materials, further investigations should be focused on adoption this structure in the field of biomaterials supporting bone regeneration, which can be achieved by bioactive phosphates incorporation. It can be presumed that such modified hydrogels would possess beneficial properties and would support osseointegration.

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