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POLYACRYLAMIDE-GELATIN HYDROGELS AND MINERALIZED HYBRIDS. INFLUENCE OF GELATIN TYPE

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The paper present (1) the structural and rheological characterization of cold water fish and bovine gelatin and (2) the synthesis of both non-mineralized and mineralized hybrid compositions based on the two types of gelatin and polyacrylamide. Hydrogels mineralization was performed by the immobilization of Na_2HPO_4 in the polymerization mixture followed by incubation in $CaCl_2$ in the crosslinking bath of gelatin. Subsequently, the synthesized materials were characterized in order to establish the influence of the type of gelatin on the properties of the final material.

Keywords: bovine gelatin, fish gelatin, mineralization, rheology

1. Introduction

As a derivative of collagen, gelatin is one of the preferred polymers in the biomedical and pharmaceutical fields [1]. Its use can vary from drug delivery [2, 3] and wound dressing [4, 5] to soft [6, 7] and hard tissue engineering [8]. The largest amount of gelatin used in the medical field has either bovine or porcine provenience. However, due to religious or social issues on the one hand, and the transmission of bovine spongiform encephalopathy (BSE) on the other hand, the use of mammalian gelatin has been limited [1, 9]. Fish gelatin might be considered an alternative since it possesses similar characteristics as the mammalian gelatin and in the same time is accepted by the halal and kosher consumers [10].

In order to obtain materials with controlled, tailorable properties, a synthetic polymer was added to the system. Polyacrylamide (PAAm) is well

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known for its easily tunable properties, such as network density and water affinity [11].

In this context, in the present work we report the network-forming polymerization of the synthetic monomer – acrylamide (AAm) with methylenebisacrylamide (MBA) in the presence of gelatin, leading to the formation of a semi-interpenerating polymer network (semi-IPN). Subsequently, an interpenetrated polymer network (IPN) is formed through the crosslinking of gelatin with glutaraldehyde (GA). In addition, polymers - nano-apatite seems appealing nanocomposites for a wide range of tissue regeneration applications. Therefore, in the second part of this work, we investigated the potential of the hybrid polymers to be loaded with nano-apatite using an innovative method, the immobilization of a phosphate salt in the hybrid polymer precursors followed by the mineralization in a calcium containing bath. The synthesized scaffolds were characterized through FT-IR and SEM. In addition, their water affinity was estimated and their rheologic behavior was investigated.

2. Materials and methods

2.1. Materials

Bovine (BG) and fish gelatin (FG), AAm and MBA, all supplied by Sigma-Aldrich, were used as such. o-Phthalaldehyde 98.5% (OPA) (Acros Organic), n-butylamine (Sigma) and mercaptoethanol (Merk) were used in order to determine the primary amine content. The photo-polymerization initiator, 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959) was purchased from Ciba Specialty Chemicals and used as 0.8% (w/v) aqueous solution. Sodium phosphate dibasic and calcium chloride (Sigma-Aldrich) were used as mineralization precursors. Double distilled water (ddH$_2$O) obtained with a GFL 2102 apparatus was used as solvent.

2.2. Methods

2.2.1. Characterization of biopolymers

^{1}H Nuclear Magnetic Resonance (H-NMR)

^{1}H NMR spectra were recorded on a 300 MHz Varian Gemini 300 BB spectrometer, using deuterium oxide (D$_2$O) as solvent, at 25 °C.

Quantitative detection of primary amines

The quantitative determination of –NH$_2$ groups in BG and FG, respectively, was performed using OPA method, as already reported [12]. UV-VIS spectroscopy was performed on a CINTRA 101 spectrometer, at a fixed wavelength of 335 nm. n-butylamine solutions of known concentrations in the range 1-10 mM were used to draw the calibration curve. The experiment was performed in triplicate, at room temperature.

Sol-gel temperature for concentrated aqueous solutions of BG and FG

In order to estimate the sol-gel transition temperature aqueous solutions (50% w/v) of FG and BG respectively, were subjected to rheometric measurements. The determinations were performed using a Kinexus Pro rheometer equipped with parallel plate geometries. To ensure an accurate temperature control within the sample, a Peltier element was used. Evaporation of the sample was prevented using a water-lock. The temperature sweep tests were performed in the linear viscoelastic region (previously determined), at a constant frequency of 1 Hz, increasing the temperature in the studied interval at a rate of 1^oC/ min.

2.2.2. Synthesis of gelatin-PAAm membranes

Hybrid natural-synthetic hydrogels were prepared using various ratios of AAm and BG and FG, respectively (Table 1). MBA was used as synthetic crosslinking agent in a 2:100 molar ratio with respect to AAm. In a first step a semi-IPN was obtain through the polymerization of the monomer in the presence of a photoinitiator (Irgacure 2959), added in a 0.1:100 molar ratio with respect to C=C bonds. The total solid content (T) was kept constant at 30% in all compositions. The polymerization mixtures were degassed for 10 minutes in an ultrasound bath and subsequently poured between two glass plates. All polymerization reactions were performed for 60 minutes, using an ECX-F26 transilluminator, at a wavelength of 312 nm. After the polymerization was complete the obtained films were incubated in a 2% aqueous solution of GA for two hours, leading to an IPN of natural-synthetic polymer. Subsequently, the films were washed in large amounts of ddH_2O under gentle stirring and dried overnight at room temperature. For simplicity, Fi and Bi state for the hybrid IPNs obtained using FG and BG, respectively, where i indicates the compositions as described in Table 1.

Table 1

Compositions of the polymerization mixtures used to generate the hybrid membranes

Sample index (i)	AAm:FG (w/w)	AAm:BG (w/w)
1	30:70	30:70
2	50:50	50:50
3	70:30	70:30

2.2.3. Mineral loading of PAAm-gelatin membranes

In order to estimate the potential of hybrid PAAm-gelatin membranes to be loaded with apatite mineral, Na_2HPO_4 was immobilized in the polymerization mixture. The ratio Na_2HPO_4: polymer mixture was kept constant, at a value of 1:10 (w/w). The compositions were obtained in a similar manner as previously described and also, the same polymerization procedure was followed. Briefly, the mineral salt was added to the polymer precursors described in Table 1. Anhydrous

$CaCl_2$was added in the crosslinking bath to ensure a molar ratio Ca^{2+}:PO_4^{3-}= 1.67. After the complete dissolution of $CaCl_2$, the pH of the crosslinking bath was adjusted to 7.4. After the crosslinking cycle was completed, the samples were removed from the bath, washed in large volumes of ddH_2O under gentle stirring and the dried at room temperature overnight. For simplicity, the mineralized materials are further denoted FiM and BiM.

2.2.4. Characterization of PAAm-gelatin membranes

Fourier Transform Infrared Spectroscopy (FT-IR)

The presence of apatite mineral in the gelatin-PAAm hydrogels was investigated through FT-IR using a JASCO 4200 spectrometer equipped with a Specac Golden Gate attenuated total reflectance (ATR) device in the 4000 – 600 cm^{-1} wave number region. The recorded spectra were compared with the control samples (PAAm, FG and BG) and with the spectra recorded for commercial nano-HA.

Scanning electron microscopy (SEM)

SEM analysis was used as additional method to investigate the presence of mineral on the FiM and BiM materials. The micrographs were recorded using a QUANTA INSPECT F SEM device equipped with a field emission gun with 1.2 nm resolution and with an X-ray energy dispersive spectrometer (EDX). The specimens for SEM were coated with a thin layer of gold.

Water affinity of gelatin-PAAm and gelatin-PAAm-HA films

Both mineralized and non-mineralized PAAm-gelatin materials were investigated with respect to their water affinity. In this respect, samples of each composition were weighed immediately after the washing was complete and again after complete drying, and their equilibrium water content (EWC, %) was calculated using equation (1). In order to perform rehydration tests the dried samples were subsequently immersed in ddH_2O for 12 hours and weighed at equilibrium. Their rehydration degree (RD, %) was computed using equation (2).

$$\boldsymbol{EWC}, \% = \frac{w_{post-synthesis} - w_{dehydrated}}{w_{post-synthesis}} \cdot \mathbf{100} \qquad (1)$$

$$\boldsymbol{RD}, \% = \frac{w_{rehydrated} - w_{dehydrated}}{w_{rehydrated}} \cdot \mathbf{100} \qquad (2)$$

Rheology of gelatin-PAAm and gelatin-PAAm-HA films

Dynamic oscillatory measurements were performed on a Kinexus Pro rheometer equipped with Peltier element, at a pre-established temperature of 37°C. Samples with the diameter of 20 mm were placed on the bottom plate of the rheometer and parallel plate geometry was used. Hydrogel's dehydration was prevented using a water-lock. The storage modulus (G') was estimated. In a first step, amplitude sweep tests were performed in order to establish the linear viscous region (LVR) of the compositions. In this respect, the samples were subjected to an increasing oscillatory stress ($10^0 \div 10^3$ Pa) while temperature and frequency are kept constant (37°C, 1Hz). Subsequently, frequency sweep tests were performed keeping the oscillatory deformation constant, at a lower value then the LVR limit. The frequency was gradually increased from 0.10 to 10 Hz.

3. Results and discussions

3.1. Characterization of the biopolymers

^{1}H Nuclear Magnetic Resonance (H-NMR)

A typical ^{1}H-NMR spectrum of a 1 % gelatin solution at 25°C is shown in Fig. 1. Most of the proton signals are well resolved and can be assigned to specific amino acids as follows: the peak with the smallest chemical shift (0.88 ppm) to the methyl resonances of the amino acids leucine, valine, and isoleucine; the peaks at 1.18 and 1.35 ppm to the methyl resonance of threonine and alanine, respectively. The next two peaks at 1.62 and 1.79 ppm are assigned to the methyl resonances of lysine and arginine, while the peaks at 2.67, 2.95, 3.17 and 3.59 ppm correspond to methylene resonances of aspartic acid, lysine, arginine and proline, respectively [13]. Studies concerning the amino acids content in different types of gelatins have highlighted that phenyl alanine content is the same in both fish and bovine gelatin [14]. Therefore the content of the amino acids listed above can be assessed based on the ratio between the integral of the corresponding signals and the signal of phenyl alanine. Accordingly, one can notice that FG when compared to BG has a lower content of proline, valine, leucine and a higher content of threonine and aspartic acid residues. However, both fish and bovine gelatins have the same proportion of alanine, lysine and arginine residues.

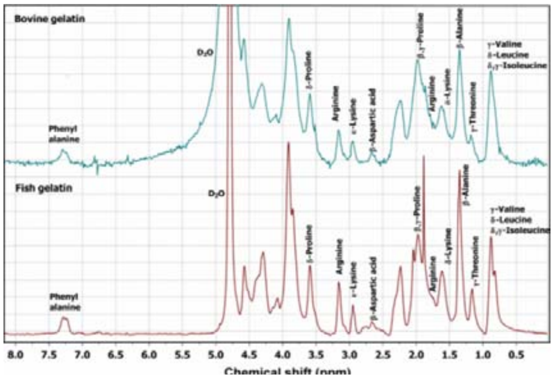


Fig.1. ^{1}H-NMR spectra of fish and bovine gelatin in D_2O.

The obtained data are consistent with the results of other research groups. For example, Avena-Bustillos et al. also reported that FG has lower proline, valine and leucine content then BG, but has a higher content of threonine and a similar content of alanine residues [15].

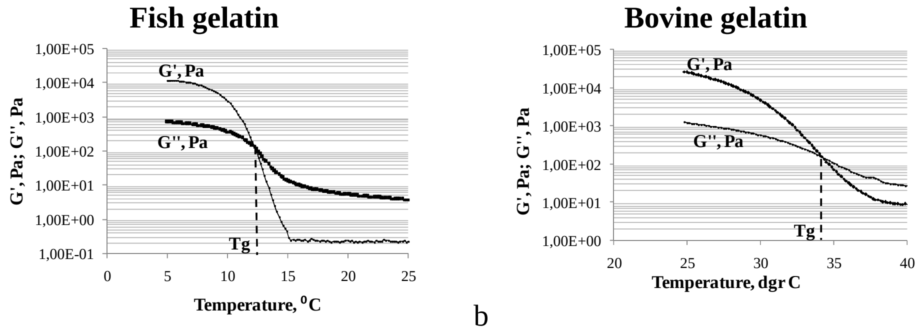
Detection of primary amine content using OPA method

The OPA method indicated, for the content of primary amines, that BG has 6.5 x 10^{-4}moles NH_2/ 1 g protein, while FG has only 4.1 x 10^{-4} moles NH_2/ 1 g protein. These results are consistent with the above-described results, the difference regarding the primary amines content residing, most probably, in the different content of amino acids. Moreover, the amount of primary amines becomes essential when hydrogels are formed through $–NH_2$ crosslinking. Accordingly, it appears that BG can generate denser networks with respect to FG.

Sol-gel temperature for concentrated aqueous solutions of BG and FG

For the synthesis of biomaterials based on gelatin and for the processing of such materials, it is of major importance to compare the sol-gel transitions of the two biopolymers.

The rheological measurements confirmed that the type of gelatin strongly influences the sol-gel transition (Tg). Temperature sweep data showed that Tg is reached at a higher temperature for the solution of BG then for the solution of FG of the same concentration ($Tg_{BG} \approx 34^oC$ and $Tg_{FG} \approx 12^oC$, respectively) (Fig.2, a and b). In addition, for both types of gelatin, the complex modulus (G*) decreases with the increase of temperature, stating for the temperature – viscousness dependency of the studied materials (Fig. 2, c and d). This significant difference in the thermal behavior of the two types of gelatin has an important effect both on the processing of such materials as well as on the elastic properties of the corresponding hydrogels.



a

b

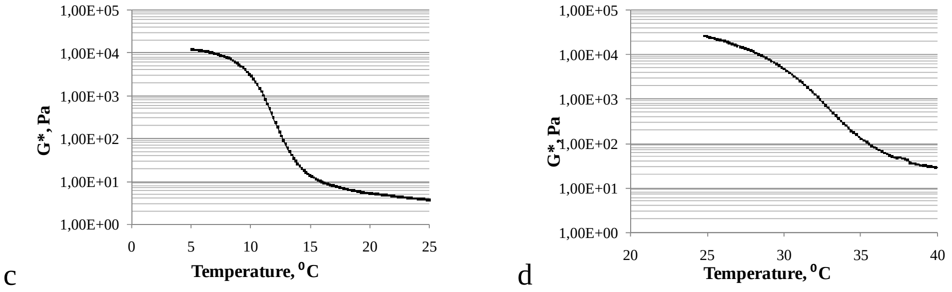


Fig. 2: Rheologic behaviour for fish gelatin (left column) and bovine gelatin (right column)

Similar results regarding the rheological behavior of FG and BG have been obtained by other research groups. For example, Haug et al. found that a lower content of proline and hydroxyproline in FG leads to lower sol-gel temperature [16].

3.2. Synthesis of gelatin-PAAm films and mineralization of hybrid hydrogel films

IPNs of gelatin-PAAm were successfully obtained through the described two-step procedure that involves (1) the formation of a semi-IPN consisting in gelatin embedded in the synthetic network PAAm-MBA obtained through free radical photo-polymerization, and (2) the crosslinking of gelatin in a bath of GA aqueous solution. The potential of such hydrogels for biomedical use was already emphasized [12, 17-19]. To broaden even more the field of applications, mineralized scaffolds were prepared. In this respect, the same synthesis procedure was followed, but the polymerization mixture was supplemented with Na_2HPO_4 as a mineralizing precursor, while the crosslinking bath was supplemented with $CaCl_2$, to allow simultaneous mineralization. PO_4^{2-} anions in the polymeric network act as mineralization nucleators, attracting the Ca^{2+} cations available in the crosslinking bath. It was expected that the migration of the cations into the hydrogel scaffold towards the anions, would lead to a progressive loading of the material with nano-apatite particles.

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR analysis was used both to identify the macromolecular components of the natural-synthetic IPNs, as well as the presence of mineral phase in the FiM and BiM composite materials.

Natural-synthetic IPNs. The spectra recorded for the two studied types of gelatin are almost identic, the spectrum of FG being slightly shifted. The spectra of BG and FG present as main characteristic signals broad with spike at 3283 cm^{-1} (common signal for O-H and N-H stretching), 3079 cm^{-1} and 3076 cm^{-1}, respectively (N-H), 2946 cm^{-1} and 2942 cm^{-1} respectively (saturated C-H stretch), 1630 cm^{-1} (amide I) and 1528 cm^{-1} and 1526 cm^{-1} (amide II). On the other hand, PAAm is characterized by one broad peak with two spikes at 3333 cm^{-1}and 3190 cm^{-1} (N-H stretch) and a much stonger C=O stretch at 1645-1604 cm^{-1} [18]. The spectra of all the hybrig materials show a combination of broad O-H and N-H vibrations of mixed origin (gelatin, PAAm), while in the wavenumber interval 1530 cm^{-1} to 1640 cm^{-1} the vibrations characteristic to amide I and II from gelatin and to C=O from PAAm are noticed (Fig. 3, a and b)

FiM and BiM composites. In addition to the natural and synthetic polymers as control samples, HA was also subjected to FT-IR measurements. The spectrum of HA present one major spike at 1022 cm^{-1} (Fig. 3, b), wich can also be found in all mineralized compositions, thus proving the formation of mineral.

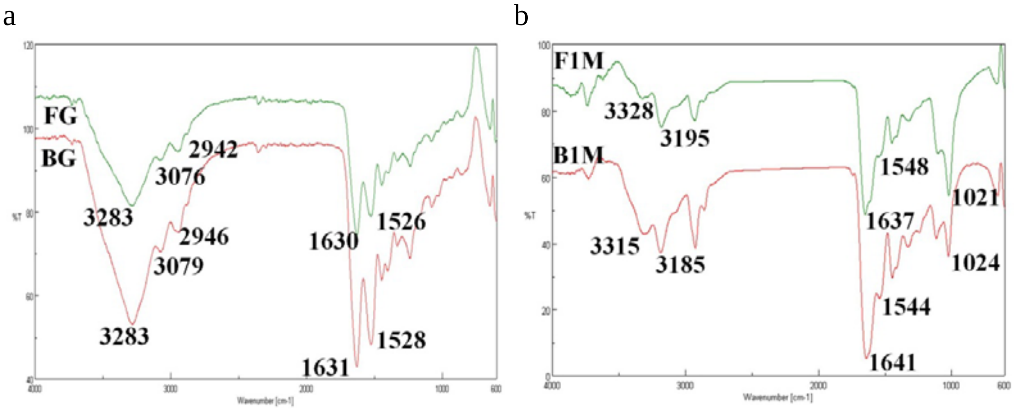


Fig. 3: ATR FT-IR spectra registered for: F1 and B1 (a) and F1M and B1M (b)

Scanning electron microscopy (SEM)

The SEM micrographs of the synthesized compositions confirmed the presence of HA mineral. Representative morphologies for nanometric biologic-like apatite are presented below for the compositions with the highest content of natural polymer. The surface of both fish and bovine-based materials is almost entirely covered with HA (Fig.4, A and D), while within the samples nanometric nucleation centers can be observed (Fig. 6, B and E). This findings prove that this synthesis method permits the obtaining of composite materials. In addition, EDX microanalysis performed on both FiM and BiM scaffolds indicated experimental Ca/P molar ratio of approximately 1.6 and 1.5, respectively (Fig. 4, C, F).

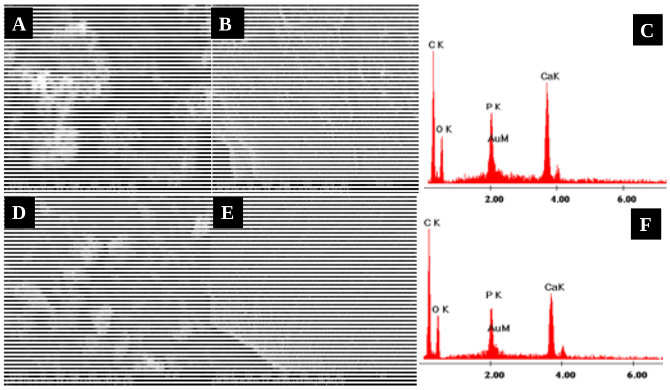


Fig. 4: SEM images and EDX spectra of F1M (first row) and B1M (second row): surface (A, D); section (B, E); EXD spectra (C, F)

Water affinity of gelatin-PAAm and gelatin -PAAm-HA films

The EWC and RD of the both mineralized and non-mineralized hybrid hydrogels were estimated using equations (1) and (2), respectively. The obtained data are graphically represented in Fig. 5. In the case of the non-mineralized compositions, the hydration/ rehydration behaviour of the synthesized materials is dependent of the type of gelatin used, namely the compositions in which the natural component is represented by FG have a higher EWC and RD when compared with the mammalian correspondent. The difference between the EWC and the RD decreases with the increase of PAAm for both types of gelatin, indicating that the increase of synthetic polymer leads to materials that are more stable when subjected to hydration-rehydration cycles (see Fig. 5 a). The presence of mineral has little influence on the water affinity of both fish, and bovine-based materials (Fig.5, b). The values computed for both EWC and RD are smaller in the case of mineralized samples when compared with the non-mineralized ones, but the differences are not significant. For example, EWC_{F1} = 90% while EWC_{F1M} = 84%, while RD_{F1}=84% and RD_{F1M} = 82%.

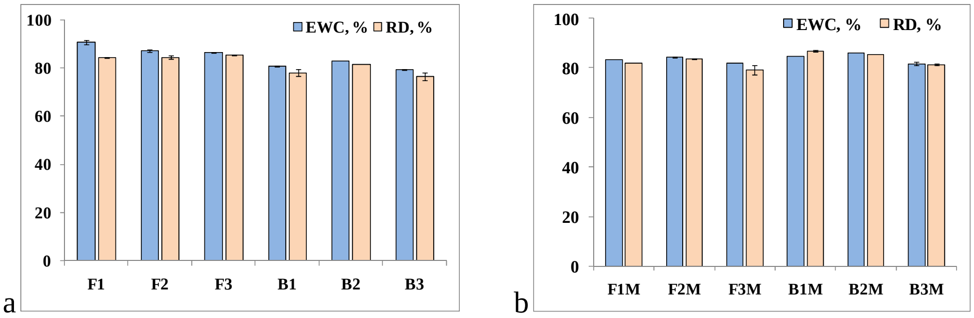


Fig. 5.: Water affinity as determined by EWC and RD for both Fi and Bi (a) and FiM and BiM (b) series

Rheology of gelatin -PAAm and gelatin -PAAm-HA films

The rheology tests performed on the rehydrated non-mineralized samples based on both FG and BG showed that increasing the amount of PAAm leads to higher G'(Fig. 6, a, b), being consistent with the results registered for water affinity. Furthermore, the tests performed on the mineralized samples showed that the presence of the mineral has little influence on the rheologic behavior of both fish and bovine gelatin based composite materials. All materials presented an elastic behavior.

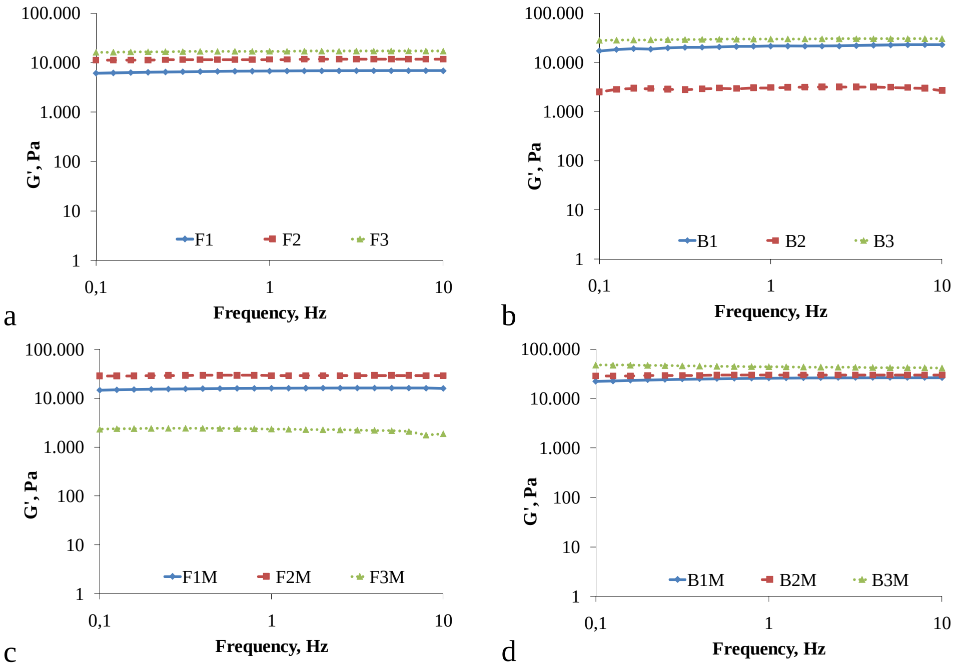


Fig. 6: Rheologic behaviour of the Fi (a), Bi (b), FiM (c) and BiM (d) series

6. Conclusions

The present paper present (1) the characterization of two types of gelatin (fish and bovine) and (2) the synthesis of hybrid hydrogels based on the characterized gelatin and PAAm. The scaffolds were loaded with mineral phase and the resulting materials were characterized. In this respect, PO_4^{3-} anions were added in the polymerization mixture and Ca^{2+} cations were added in the crosslinking baths. SEM micrographs and EXD spectra confirmed the presence of mineral in the synthesized materials. The water affinity tests showed that (1) increasing the ratio of synthetic polymer with respect to the natural counterpart leads to an increase of the swelling capacity and in the same time (2) the presence of mineral in the natural-synthetic materials does not have a significant influence on the water affinity. The rheology tests are in agreement with the previously reported result, showing that (1) the increase of PAAm ratio leads to softer, more elastic materials and in the same time (2) the presence of Ca/P mineral in both fish and bovine gelatin based materials does not significantly influences the rheologic behavior of the composite materials.

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