

## TITLE PAGE

### **Effect of functionalized PHEMA micro- and nano-particles on the viscoelastic properties of fibrin-agarose biomaterials**

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## **ABSTRACT**

Two types of PHEMA-based particles, exhibiting either carboxyl or tertiary ammine functional groups, were incorporated to fibrin-agarose hydrogels, and the effect of the addition of these synthetic particles on the viscoelastic and microstructural properties of the biomaterials was evaluated. Experimental results indicated that the incorporation of both types of polymeric particles to fibrin-agarose scaffolds was able to improve the biomechanical properties of the biomaterials under steady state and oscillatory shear stresses, resulting in scaffolds characterized by higher values of the storage, loss and shear moduli. In addition, the microstructural evaluation of the scaffolds showed that the nanoparticles exhibiting carboxyl functional groups were homogeneously distributed across the fibrous network of the hydrogels. The addition of both types of artificial polymeric particles was able to enhance the viscoelastic properties of the fibrin-agarose hydrogels, allowing the biomaterials to reach levels of mechanical consistency under shear stresses in the same range of some human native soft tissues, which could allow these biomaterials to be used as scaffolds for new tissue engineering applications.

## **KEYWORDS**

Fibrin, agarose, functionalized particles, hydrogel, scaffold.

## INTRODUCTION

Hydrogels are three-dimensional networks formed by hydrophilic homopolymers, copolymers, or macromers (pre-formed macromolecular chains) crosslinked to form polymer matrices. These polymers, generally used above their glass transition temperature ( $T_g$ ), are typically soft and elastic due to their thermodynamic compatibility with water and have found use in many biomedical applications. Biological hydrogels of biomedical interest have been formed from agarose, alginate, chitosan, hyaluronan, fibrin, and collagen, among others <sup>1,2</sup>.

Fibrin-based hydrogels have been successfully used as biomaterials for several tissue engineering applications, because of their inherent biocompatibility and biomimetic extracellular matrix structure <sup>3</sup>. Moreover, fibrin has recently gained more attention for being used as scaffold in the first stem-cell therapy recommended for approval by the European Medical Agency <sup>4-6</sup>. In previous studies, fibrin-agarose (FA) scaffolds with 0.1% of agarose have shown great potential for the generation of a variety of bioengineered soft human tissues such as cornea, skin, oral mucosa and peripheral nerve: in fact, the addition of agarose to fibrin scaffolds has demonstrated to enhance their consistency and biomechanical properties under tensile, shear and compression stresses <sup>7-13</sup>.

Due to the limitations of hydrogels in terms of mechanical properties, sometimes it is necessary to combine natural and synthetic polymers in order to acquire the desired properties to generate biomimetic scaffolds <sup>1,2</sup>. Synthetic polymers used in tissue engineering include, among others, poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(lactic-co-glycolic acid) (PLGA) and polyacrylates such as poly(2-hydroxyethyl methacrylate) (PHEMA). Moreover, the addition of nanoparticles made of synthetic polymers to fibrous scaffolds is an extremely effective method to design hybrid materials with drug delivery capabilities and develop controlled-

delivery systems of growth factors and therapeutic agents for a wide range of applications<sup>14-20</sup>. A recent study showed that fibrin-based scaffolds containing PLGA nanoparticles loaded with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were able to stimulate wound healing in diabetic mice<sup>21</sup>. In addition, the incorporation of particles in fibrin-based biomaterials is able to modify their structural and mechanical properties: in fact, in physiological conditions during hemostasis, microstructures such as platelets and red blood cells are not only necessary components in the fibrin clots formation, but they also cause a dramatic effect on their morphological and rheological properties<sup>22-24</sup>. However, recent studies suggested that blood elements show several problems when incorporated in scaffolds for tissue engineering applications, first because the cellular components from other source than the patient would cause host response to the implanted biomaterial, but also because autologous platelets transplant was demonstrated to amplify pro-inflammatory responses in vivo<sup>25,26</sup>. Moreover, the presence of platelets into fibrin-based matrices, as for platelet-rich plasma injections, resulted in a limited impact for the treatment of different types of injuries<sup>27-29</sup>. The addition of fully biocompatible polymeric nanoparticles is capable of improving the biomechanical resistance and providing drug delivery capabilities to hydrogel-based biomaterials, which makes it an interesting strategy that can be applied to FA scaffolds and enhance their potential for a variety of tissue engineering and drug delivery applications. In this regard, there is a significant scope in the application of nanoparticle surface modifications and the possibility of incorporating proteins, growth factors, enzymes and antibiotics through binding or adsorption, to provide more cues to tissue response. PHEMA micro- and nano-particles are potential candidates for this purpose, as PHEMA polymeric formulations have been extensively used as biomaterials since their introduction as biomedical materials by Wichterle and Lim<sup>30,31</sup>, and were recently studied to generate soft

contact lenses with controllable drug delivery capabilities<sup>32</sup>. The use of PHEMA zinc- and calcium-loaded nanoparticles with a diameter of 130 nm has been recently described for dental adhesive applications, to provide durable adhesion to dentin and facilitate the hybrid layer remineralization of demineralized dentin<sup>33,34</sup>.

In the case of FA biomaterials, a recent study demonstrated that the incorporation into the scaffolds of synthetic nanoparticles with a mean diameter of 115 nm, composed by a non-biodegradable magnetite core covered by a crosslinked vinylic polymeric shell (methyl methacrylate-co-hydroxyl ethyl methacrylate-co-ethylene glycol dimethacrylate), was able to increase the viscoelastic moduli of FA scaffolds under shear stresses, and this increase was proportional to the concentration of nanoparticles in the constructs<sup>35</sup>. In the present study, a novel biomaterial composed of fibrin, agarose and two different types of PHEMA-based particles is proposed and evaluated in terms of its biomechanical and microstructural properties, to study its potential as biomaterial for tissue engineering applications.

## **MATERIALS AND METHODS**

### **Scaffolds generation**

The scaffolds generated in this study were composed of fibrin-agarose based hydrogel (at 0.1% of agarose) and 2% of PHEMA-based particles. Two types of biocompatible nano- and micro-particles, called PolymP-*n*Active and PolymP-Amine3 respectively, were purchased from NanoMyP (Granada, Spain). The particles were fabricated through a polymerization precipitation process, and are composed by 2-hydroxyethyl methacrylate (HEMA) as backbone monomer, ethylene glycol dimethacrylate (EGDMA) as crosslinker and methacrylic acid (MAA) as functional monomer. The PolymP-*n*Active (NP01) are spherical and uniform nanoparticles, with

an average diameter of 130 nm, that exhibit a high concentration of functional groups (-COOH), thus are capable of complexing metal cations, principally  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ . PolymP-Amine3 microparticles (NP8) are spherical and uniform micro-beads with tertiary amine functionalized surfaces and an average diameter of 8  $\mu\text{m}$ . Both NP01 (with carboxyl groups) and NP8 (with tertiary amine groups) possess protonatable functional groups, which means that the absolute value of their charge depends on the pH. Thus, the negative charge of the NP01 increases when increasing pH, and decreases with decreasing pH, reaching almost neutral charge at very low pH values. On the contrary, the NP8 show higher positive charge at low values of pH, and their charge decreases as the pH increases, reaching virtually neutral charge at very high pH values. The particles, which were provided in a 70% ethanol solution, were subjected to a washing process before utilization: this process consisted of 3 cycles of centrifugation at 17000 g for 40 minutes, where each centrifugation cycle was followed by substitution of the supernatant with bi-distilled water, in a sterile environment, in order to eliminate ethanol from the solution before use. The re-dispersion of the particles in bi-distilled water was achieved by submerging the centrifugal tubes containing the particles in an ultrasonic bath during 5 minutes, followed by vortex shaking during 3 minutes. Finally, water was substituted with Dulbecco's Modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% of FBS, 1% of L-glutamine and 1% of antibiotic, through a final centrifugation cycle, and the particles were re-suspended in the medium as previously described. FA samples, containing 2% w/w of NP01 or NP8, were fabricated following the same procedure, to study the importance of the dimension and composition of the particles on the rheological behavior of FA based hydrogels and on the microstructure of the scaffold.

FA scaffolds with a final volume of 5 mL were generated adapting a method previously developed by Alaminos et al. <sup>7</sup>. To produce each scaffold, 3.8 mL of human plasma from donors (provided by the Human Tissue Bank of Granada, Spain) were mixed with 0.3 mL of DMEM, 75µL of tranexamic acid (Fides-Ecofarma) to avoid fibrinolysis, 0.25mL of CaCl<sub>2</sub> (Sigma-Aldrich) at 2% in bi-distilled water to activate the coagulation cascade, and 0.25mL of type VII agarose (Sigma-Aldrich) at 2% in PBS. Finally, a final volume of 5 mL was obtained by adding 2% w/w of particles, suspended in 0.325 mL of DMEM, to the solution. FA scaffolds without particles were generated as control samples. All the hydrogels produced were placed in 3.5 cm diameter petri dishes and incubated at 37°C for 24 hours, in order to obtain the scaffolds used in this study.

#### **Microstructural evaluation**

FA control samples and the scaffolds containing particles were fixed in a solution of 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 24 h, rinsed 3 times in PBS during 5 minutes each time, dehydrated at 4°C in increasing concentrations of acetone solutions (30, 50, 70, 90, 96 and 100%) during 15 minutes at each concentration, and subjected to critical point drying (CPD30, Balzers Union, Liechtenstein). The analysis of the scaffolds transversal section was achieved by fracturing the dried samples, to expose their cross-section. The specimens were then placed on aluminum stubs, sputter-coated with gold (Nanotech Polaron-SEMPREP2, Polaron Equipment Ltd., UK) and observed in a scanning electron microscope (SEM) (Quanta 200, FEI, USA) at 5 kV and 9-11 mm working distance. Additionally, other specimens were coated with carbon and observed with a field emission scanning electron microscope (FE-SEM) (GEMINI, Carl Zeiss SMT, Germany), at 2-3 kV and 3-4.5 mm working distance, in order to obtain higher magnifications for nanoparticles visualization.

### **Biomechanical evaluation**

The mechanical properties of the scaffolds were measured with a rheometer Haake Mars III (Thermo Scientific, USA) adapting the method described by Lopez-Lopez et al.<sup>35</sup> for the rheological evaluation of FA based scaffolds. The surface of the parallel plates of the measuring system (diameter of 3.5 cm) were serrated to avoid wall slip and to enhance gripping of the hydrogel samples. The specimens were placed between the parallel plates of the rheometer at 37°C in a water vapor-saturated atmosphere, and the top plate was lowered until the biomaterial responded to the compressive stress with a normal force of 5 N: following this procedure, the gap at measurement between the plates in all tests was approximately 300 μm. Two types of tests were conducted: (i) oscillatory test to measure the viscoelastic moduli ( $G'$  and  $G''$ ), and (ii) steady state tests to quantify the shear modulus ( $G$ ) of the samples. All the FA samples (i.e., without particles and containing NP01 and NP8) were tested by triplicate for each of the tests. For the oscillatory measurements, an oscillatory strain,  $\gamma = \gamma_0 \cos(2\pi ft)$ , of fixed frequency ( $f = 1$  Hz) and logarithmically increased amplitude (between 0.01 and 0.1),  $\gamma_0$ , was first applied. This amplitude sweep allowed delimitation of the viscoelastic linear region (i.e., the region for which the viscoelastic moduli are independent of the stress amplitude  $\gamma_0$ ). Frequency sweeps, in which  $\gamma_0$  was fixed at a value within the viscoelastic linear region and the frequency was varied in a logarithmic way, were also conducted. These tests allowed the evaluation of the behavior of the viscoelastic moduli when subjected to a changing frequency. For all the oscillatory tests the sinusoidal stress in each step of the measuring ramps was maintained for a time equivalent to 8 periods of oscillation. The viscoelastic moduli were recorded during the last 5 periods to discard transient values.

Steady state measurements of the shear modulus were undertaken by applying a constant shear rate,  $\dot{\gamma}$  of  $0.01 \text{ s}^{-1}$  for 30 s and the resulting shear stress,  $\sigma$ , was recorded within the linear regime to avoid disruption of the specimens.

#### **Statistical analysis**

To analyse the effect of the incorporation of the particles on the measured storage, loss and shear moduli of the different FA-based scaffolds fabricated, the Mann-Whitney test was operated and a value of  $p < 0.05$  was considered as statistically significant in all cases.

## **RESULTS**

#### **Structural evaluation**

The macroscopic evaluation of the FA based scaffolds containing NP01 showed that the hydrogels were physically stable and consistent, macroscopically similar to the control samples without nanoparticles. The SEM analysis allowed to observe that the NP01 nanoparticles were homogeneously distributed across the FA network, as shown in **figure 1**. Additionally, it was found that the NP01 adhered to both fibrin and agarose components of the biomaterial, preserving the homogeneity of the network. The morphological observation of the SEM images of FA scaffolds containing NP01 showed that they were homogeneously dispersed, and firmly attached to the fibers of the network. In addition, the microscopical distribution of the fibers of the network was not affected by the incorporation of the nanoparticles. On the contrary, the SEM analysis of the FA samples containing NP8 showed disruption of the fibrillar network, aggregation and precipitation of the microparticles (**figures 1c and 1f**).

The FE-SEM evaluation allowed analyzing the network in details at a higher magnification, and it was possible to observe how the NP01 integrated into the network of fibrin fibers and spots of agarose, as shown in **figure 2**. The increase of the microscopic magnification applied with the FE-SEM microscope allowed the observation of individual nanoparticles and how they attached to both the fibrin fibers and the agarose areas of the network, modifying the appearance of the scaffolds at the micro- and nano-structural level.

### **Biomechanical evaluation**

All the prepared hydrogels showed an elastic, solid-like, response when subjected to mechanical stresses of up to  $\sim 10$  Pa. For oscillatory stresses, the solid-like behavior of the hydrogel (i.e., connected to its elastic response) is usually quantified by the storage modulus,  $G'$ , which can be taken as a measure of its mechanical strength. The loss modulus,  $G''$  is related to the dissipation of energy upon the oscillatory mechanical stimulus and to the liquid-like behavior of the specimen. All the samples showed higher values of  $G'$  than those of  $G''$  for all the measured range of the stress amplitude (**figure 3a**). However, the values of  $G'$  for sample FA+NP01 were  $\sim 50\%$  higher than for samples FA and FA+NP8. No significant changes were observed between samples FA and FA+NP8, which indicated that the  $8 \mu\text{m}$  particles did not have any effect on the strength of the material. As for  $G''$ , sample FA+NP8 showed higher values than the FA control, and for FA+NP01 even higher values were measured. This would indicate that the dissipation of energy increased with the addition of the particles to the FA matrix, and such an increase was higher for the smaller particles. Both moduli remained independent of the amplitude of the stress, which proves that the applied oscillatory stresses were within the viscoelastic linear region. Only at the highest values of  $\sigma_0$ , a slight decrease of  $G'$  was observed, which could be taken as the

onset of the non-linear regime, indicating microscopically disruption of the hydrogel structure. This change took place at lower stress amplitudes for both the FA and the FA+NP8 samples, which would again prove weaker mechanical properties. The solid-like behavior was also maintained when the frequency of the oscillatory stimulus was varied within the viscoelastic linear region (**figure 3b**). Similarly to the amplitude sweeps, the highest values of  $G'$  and  $G''$  were observed for sample FA+NP01, while sample FA exhibited the lowest ones.  $G'$  appeared to be almost independent of the frequency, while  $G''$  displayed a maximum, typical of gel samples<sup>36</sup>. Such a maximum appeared at ~3 Hz for samples FA and FA+NP8 and at ~4 Hz for sample FA+NP01.

For steady-state stimuli, the shear stress showed a linear trend at low strain values where it was proportional to the strain (**figure 3c**). The proportionality constant, the shear modulus ( $G$ ) was similar for samples FA and FA+NP8. However, it appeared to be higher for sample FA+NP01. These results are in good agreement with those shown in **figure 3a** and b for oscillatory stresses, because  $G$  is also considered a measure of the hydrogel strength.

Therefore, all the rheological moduli were significantly higher for sample FA+NP01 than for the FA control, as shown in **figure 3d**. More specifically, the average values of  $G'$  and  $G''$  (within the viscoelastic linear region) and  $G$  measured for the scaffold containing NP01, equal to 145.68, 42.01 and 122.80 Pa respectively, were significantly higher ( $p < 0.001$  for  $G'$  and  $G''$ ,  $p < 0.05$  for  $G$ ) than those measured for the FA control samples, equal to 95.74, 24.90 and 76.40 Pa respectively. The addition of NP8 showed a lower effect in magnitude on the rheological parameters of the FA based scaffolds (as it is possible to observe in **figure 3d**), but the average values of  $G'$ ,  $G''$  and  $G$ , which are equal to 98.35, 29.84 and 87.60 Pa respectively, demonstrated that the NP8 did also significantly increase the rheological moduli of the scaffolds ( $p < 0.05$  for  $G'$

and  $G'$ ,  $p < 0.001$  for  $G''$ ), when compared to the FA control samples. However, the presence of NP01 nanoparticles resulted in significantly higher average values ( $p < 0.001$  in all cases) of the storage, loss and shear moduli, when compared to the average moduli values of the scaffold generated with NP8.

## **DISCUSSION**

### **Effect on the microstructural properties**

The microstructural analysis by SEM of the FA non-loaded samples showed a homogeneous and isotropic morphology of fibers distributed in random direction. This is in accordance with a previous study where the same biomaterial was used to generate a bioengineered oral mucosa consisting of oral mucosa fibroblasts cultured within a scaffold with magnetic and non-magnetic particles of 115 nm and 130 nm in diameter. In the absence of magnetic field during jellification of the hydrogels, both types of nanoparticles affected the FA network in a similar way as the NP01 used in this work, distributed themselves homogeneously without the formation of aggregates, and did not disrupt the random alignment of the fibers mesh<sup>35</sup>.

The analysis by FE-SEM also confirmed that the NP01 were homogeneously distributed across the network of the samples. The high affinity and binding of NP01 to fibrin gel observed in the FESEM images may also account for the attained improvement in mechanical properties. This could be due to the formation of peptide bonds between the carboxylic groups of the nanoparticles and the ammine groups of the fibrin molecules, or it could be explained in terms of ionic charge, as the negatively charged NP01 (zeta potential of -43.3 mV at pH=7.4, according to the provider) facilitate fibrin binding, through high affinity to the positively charged E domains of the fibrin monomers<sup>37</sup>. Osorio et al. recently reported that the high affinity of the same

nanoparticles (NP01) to demineralized dentin collagen could be a result of the electrostatic forces, due to the negative charge of the nanoparticles and the positive charge of demineralized collagen, or a consequence of the formation of covalent bonds between the COO<sup>-</sup> groups of the NP01 and the NH<sup>+</sup> sites of the dentin collagen<sup>33,34</sup>. This situation might not occur when considering the tertiary amine functionalized microparticles (NP8), whose affinity to the fibrin-agarose hydrogel could be affected by their positive charge and lack of carboxylic groups on their surface.

#### **Effect on the biomechanical properties**

The macroscopic mechanical properties are related to the microstructure of the samples and, thus, the differences observed in **figure 2** could explain such trends. Indeed, the nanoparticles of sample FA+NP01 appeared to be homogeneously distributed around the fibrin network and attached to the fibrin fibers. They were very probably acting as connectors between fibers, which could have had a failure retardation effect as described in previous works<sup>38,39</sup>.

In the case of sample FA+NP8, the particles settled down due to gravity during gelation. As a result, the particles were at the bottom of the hydrogel sample. Thus, the increase in the viscoelastic moduli measured by the upper plate of the rheometer, compared to that measured for a control sample without particles (sample FA), was lower than the increase registered for the sample FA+NP01. Therefore, the size of the particles included in the formulation must be carefully chosen to avoid sedimentation. In this regard, previous studies have highlighted the unique property of resilience and elasticity of fibrin networks, provided by the inherent capability of fibrin fibers to distribute the load across the network and activate a sequential stiffening process of the hydrogels under external tensile stresses<sup>40,41</sup>. On the other hand, it has

been recently demonstrated that some modifications to the fibrin-based hydrogels, such as the addition of agarose at concentrations higher than 0.3% w/w, caused an alteration of the fibrin-based network organization at the micro-level, which resulted in a decrease in the values of the Young's moduli under tensile stresses<sup>11,42</sup>. The formation of inhomogeneities in the fibrillar morphology of the network caused by the incorporation of NP8 (**figures 1c** and **1f**) could interfere on the distribution of tensile loads along the network, affecting the tensile resistance of the fibrin-based scaffolds.

Thus, the modification of the dimension and composition of the particles incorporated to the FA scaffolds is an effective strategy to enhance the rheological properties of the hydrogels, allowing the application of these scaffolds for different purposes. In fact, the addition of NP01 to the fibrin-agarose hydrogels has led to a significant increase of all the viscoelastic moduli measured in this study ( $G'$ ,  $G''$  and  $G$  increased by 51%, 68% and 62%, respectively). In previous studies, fibrin-agarose scaffolds (at 0.1% of agarose) have already demonstrated to possess biomechanical resistance to shear stresses in the same range of magnitude as native tissues such as human cornea and oral mucosa<sup>42,43</sup>, but the addition of PHEMA nanoparticles of 130 nm in diameter (NP01) proved to be a novel technique able to make the new formulation of the fibrin-agarose scaffolds more biomimetic of other native human soft tissues, as the enhanced values of the shear modulus ( $G=122.80$  Pa) fall in the same range of values corresponding to native human brain tissue and nerves ( $G =100-1000$  Pa), as shown in **figure 4**<sup>44</sup>.

The addition of NP01 showed not only to preserve the natural crosslinking and formation of the fibrin-based network, but also to increase the viscoelastic moduli of the biomaterial under shear stresses. In vivo tests will be needed in the future to assess the effect of the addition of NP01 when implanted into a foreign body, and to investigate the possibility of surface modifications

and incorporation of a wide range of biomolecules, to provide more cues to tissue response. The homogeneous and dispersed distribution of the NP01, and their efficient attachment to the FA polymeric structure, may add a great potential to the generated scaffolds. There is a significant scope in the application of nanoparticles with the possibility of incorporating proteins (i.e. growth factors, enzymes) through binding or adsorption, to provide cues to tissue response. Studying the cells-biomaterial interactions will also be the focus of future studies.

## **CONCLUSIONS**

Although fibrin-agarose hydrogels have previously shown a great potential as biocompatible scaffolds for the generation of a selection of bioengineered soft human tissues, the formulations of fibrin-agarose scaffolds produced to date are not able to adequately mimic the biomechanical properties and the extracellular matrix micro-fibrillar environment of the majority of native human tissues, characterized by higher values of the viscoelastic parameters. Here is reported a straightforward methodology to develop new fibrin-agarose based biomaterials which can address this problem thanks to their increased storage, loss and shear viscoelastic moduli under shear stresses. Two different types of PHEMA-based nanoparticles were successfully added to the fibrin-agarose scaffolds, and in the case of the nanoparticles of 130 nm in diameter with carboxylic functional groups, their addition resulted in a homogeneous distribution of the nanoparticles, which bound efficiently to the microstructural network of the biomaterial, as observed by SEM and FE-SEM. The biomechanical characterization demonstrated that the addition of both types of nanoparticles to fibrin-agarose scaffolds resulted in a significant increase of the rheological moduli measured under shear stresses. The addition of PHEMA-based nanoparticles to the fibrin-agarose based hydrogels allowed reaching viscoelastic properties in

the range of those of several human native soft tissues, which could also enhance their potential as biomaterials for future tissue engineering and drug delivery applications.

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## FIGURE LEGENDS

**Figure 1:** SEM images of fibrin-agarose hydrogels with 0.1% of agarose (transversal section). (a, d) Fibrin-agarose hydrogel without nanoparticles; (b, e) fibrin-agarose hydrogel containing NP01; (c, f) fibrin-agarose hydrogel containing NP8. Scale bars: 50  $\mu\text{m}$  (a, b, c) and 10  $\mu\text{m}$  (d, e, f).

**Figure 2:** FE-SEM images of fibrin-agarose hydrogels with 0.1% of agarose (transversal section). (a) Fibrin-agarose hydrogel without nanoparticles; (b) fibrin-agarose hydrogel containing NP01. Scale bar: 1  $\mu\text{m}$ .

**Figure 3:** (a) Amplitude sweeps for all the samples. The storage modulus,  $G'$ , was higher than the loss modulus,  $G''$ , for all the measured amplitudes of the oscillatory stress, which confirms their solid-like behavior. (b) Frequency sweeps for all the samples. The solid-like behavior was maintained along all the range of frequencies. (c) Shear stress vs shear strain for steady-state measurements for all the samples. The slope of the curves is the shear modulus,  $G$ . (d) Summary of the rheological moduli for all the samples (the values of  $G'$  and  $G''$  correspond to the average within the viscoelastic linear region). (\*) and (\*\*) indicate  $p < 0.05$  and  $p < 0.001$ , respectively.

**Figure 4:** Values of storage, loss and shear moduli of native human tissues, compared to the corresponding values of the different scaffolds formulations: i) FA 0.1% (striped area), ii) FA 0.1% with NP8 (dotted area) and iii) FA 0.1% with NP01 (entirely filled area).