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Mechanical behavior of bioactive poly(ethylene glycol) diacrylate matrices for biomedical application

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ABSTRACT

The biomedical applications of physically entangled polymeric hydrogels are generally limited due to their weak mechanical properties, rapid swelling and dissolution in physiologically relevant environment. Chemical crosslinking helps stabilizing hydrogel structure and enhancing mechanical properties, thereby allowing a higher stability in phisiological environment. In this context, it is known that the mechanical properties of the hydrogel are affected by both the molecular weight (MW) of the starting polymer and the concentration of the crosslinker. Here, our aim was to assess the influence of polymer MW and concentration in the precursor solution on the mechanical features of the final hydrogel and their influence on cells-material interaction. In detail, 3D synthetic matrices based on poly(ethylene glycol) diacrylate (PEGDA) at two molecular weights (PEG 700 and PEG 3400) and at three different concentrations (10, 20, 40 w/v %), which were photopolymerized using darocour as an initiator, were studied. Then, infrared and swelling analyses, along with a comprehensive mechanical characterization of the obtained hydrogels (i.e. oscillatory shear and confined compression tests), were performed. Finally, to evaluate the influence of the mechanical features on the biological behaviour, the hydrogels were characterized in terms of cell adhesion percentage and cell viability after functionalizing the substrates with RGD peptide at three different concentrations. Results have demonstrated that both the Young's modulus (E) in compression and the elastic modulus (G') in shear of the hydrogels increase with increasing polymer precursor concentration. E decreased as MW increased, and the differences are more relevant for more concentrated hydrogels. On the contrary, G' appears to increase with increasing PEGDA MW and in particular for the lowest polymer precursor concentration. The biological results have demonstrated that cells cultured for longer times seem to prefer PEG 3400 hydrogels with a larger mesh size structure that posses higher viscoelastic properties in shear.

1. Introduction

Hydrogels are attractive platforms for tissue engineering due to their highly porous and swollen three-dimensional matrix, which emulates the structure of soft tissues, and also provides effective transport of water, nutrients and metabolic waste products (Hou et al., 2010; Vashist and Ahmad, 2015; Zhu and Marchant, 2011). As swollen polymer networks, physically entangled hydrogels generally possess very weak mechanical properties (De France et al., 2018; Hoffman, 2002; Peppas et al., 2000) and undergo rapid swelling and dissolution in physiologically relevant environment. Therefore, chemical crosslinking of hydrogels is often preferable aiming to stabilize their structure/architecture, and to endow the obtained structures with higher mechanical properties (Hennink and van Nostrum, 2002; Machado et al., 2004; Oryan et al., 2018; Paradossi et al., 2009). In this context, it is well known that both the molecular weights of the used polymer and the concentration of the

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crosslinking agent do influence the mechanical properties of the obtained hydrogel (Drira and Yadavalli, 2013; Jang et al., 2017). Here, we aim to shed light on polymer concentration in the precursor solution as a further convenient design parameter to tailor the macroscopic characteristics of the final hydrogel. More in detail, we have speculated that the viscosity of the starting polymeric solution in which the crosslinking moiety is present does affect the crosslink density and, consequently, the mesh size of the obtained hydrogel which, in turn, determines the mechanical properties of the final hydrogel.

Among biopolymers used in biomedical field, FDA-approved synthetic hydrophilic hydrogelpoly(ethylene glycol) (PEG) is very attractive for its well-known biocompatibility (Drury and Mooney, 2003; Xu et al., 2018). Thus, PEG has been largely employed as a 3D support for tissue engineering, also because of its adjustable mechanical properties that allow an easy regulation of scaffold architecture (Gombotz et al., 1991; Lamprecht et al., 2016; Raic et al., 2014; Zhu, 2010). Due to their strongly hydrophilic nature, PEG hydrogels are normally bioinert and cannot support protein adsorption and cell adhesion (Beamish et al., 2010; Zhu, 2010; Zhu and Marchant, 2011), but can be properly designed to mimic the properties of the extracellular matrix (ECM) if their surface is decorated with short bioactive peptides acting as biological cues and enhancing cellular recognition *in vitro* and *in vivo* (Bott et al., 2010; Gobin and West, 2002; Zhu, 2010). For example, PEG-based scaffolds on which RGD peptide sequence (R: arginine; G: glycine; D: aspartic acid) has been attached can promote cell migration according to a dose-dependent scheme, provided that the chemical bond between PEG and RGD is stable enough to withstand contraction forces of cells during attachment and migration (Burdick and Anseth, 2002; Guarnieri et al., 2010; Mann et al., 2001). PEG-based hydrogels have been widely employed as materials for drug delivery and regenerative medicine (Hoffman, 2002; Zant and Grijpma, 2016) so that, in plenty of reports, a strong effort has been devoted to investigate on their mechanical properties through, in particular, rheological shear tests (Coutinho et al., 2010; Lin-Gibson et al., 2004; Shin et al., 2011; Zant and Grijpma, 2016). However, to the best of our knowledge, confined compression tests have not been taken into account enough to characterize the mechanical features of PEG-based hydrogels. Actually, confined compression tests are extremely useful for hydrogels designed for biomedical applications, as they can simulate body physiological stimuli thereby enhancing the prediction of their performance *in vivo* once in contact with the biological fluids. Consequently, the aim of this work was to assess the influence of the concentration and molecular weight of the starting polymer on the mechanical features of the final hydrogel and their influence on cell behavior. To this aim, 3D hydrogels based on poly (ethylene glycol) diacrylate (PEGDA) were produced and characterized for the effectiveness of cross-linking reaction, as well as their swelling and mechanical properties in confined compression conditions. Furthermore, oscillatory shear experiments were used to provide useful information about network characteristics of the hydrogels. Moreover, a biological characterization of the hydrogels, in terms of cell adhesion and viability, has been performed after the functionalization of the substrates with RGD peptide, in order to correlate the mechanical features of the hydrogels with their biological behavior.

2. Material and methods

2.1. Materials

DAROCUR1173 (2-hydroxy-2-metil-propiofenone) photoinitiator has been chosen due to its low toxicity and was kindly provided by CIBA (Switzerland). Oligo(ethylene glycol diacrylate) (PEGDA) (Mw = 700 Da, Sigma Aldrich, USA; Mw = 3400 Da, Nektar Therapeutics, San Carlos, CA) and conjugated peptide-PEG-acrylate molecules (PEG RGD), obtained as previously reported (Guarnieri et al., 2010), were used. Phenol red-free DMEM (Low glucose Dulbecco's Modified Eagles Medium) from Hyclone (Germany). 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer solution and NIH 3T3 murine cell line from Swiss mouse embryo from Sigma were used. Isopropanol was obtained from Sigma-Aldrich (USA).

2.2. PEGDA hydrogel preparation

For hydrogel preparation, PEGDA has been dissolved in HEPES buffer (10mM) at pH 7.4, to obtain 10, 20 and 40% w/v solutions. Then, DAROCUR1173 photoinitiator was added (3% w/w with respect to the polymer). RGD-activated PEG hydrogels were obtained by adding acryloyl-PEG-RGD (0.5, 1 and 5 mM for each concentration of polymer) to the precursor solution. The hydrogel was formed by placing this solution in cylindrical molds of desired size (d = 10 mm; h = 1 mm) and by UV light exposure (20 s, 365 nm, 3 mW/cm^2) for photopolymerization initation and hydrogel formation.

2.3. Hydrogel characterization

2.3.1. Hydrogel swelling

The swelling of the hydrogels has been determined by immersing PEG specimens, previously dried under a hood at room temperature (RT), in bidistilled water at RT. At predetermined time intervals, the amount of absorbed water has been quantified by the swelling ratio Q, defined as:where W_s is the mass of the swollen sample and W_d is the mass of the samples in the dry state.

2.3.2. Infrared (IR) analyses

Infrared (IR) spectroscopy of PEG hydrogels has been performed in solid phase. Prior to analyses, hydrogel specimens were dried for 48 h at 30 °C under vacuum and, subsequently, the dehydrated samples were crushed with potassium bromide (1:100 weight ratio). IR spectra have been recorded using a spectrometer in the 4000–400 cm^{-1} wavenumber range.

2.3.3. Uniaxial confined compression measurements

Stress-relaxation tests under confined compression have been carried out by a dynamometer (Instron 4042) at controlled deformation rate and temperature. Cylindrical hydrogel samples (h_0 = 3 mm) were placed in a cylindrical steel cell, covered with a porous and permeable septum and immersed in distilled water. To avoid non-linearity, each deformation was set to 75 μm (deformation rate: 19 μm/min) and kept constant for 1800 s. This cycle was repeated 4 times to a final 10% deformation.

2.3.4. Rheology

The rheological properties of PEG hydrogels have been evaluated with a GEMINI rotational rheometer (Bohlin Instruments, Malvern, USA), using a humidity chamber. Dynamic rheological tests were carried, so as to observe the sample behavior in the regime of linear viscoelasticity. A sinusoidal deformation law, $\gamma = A\sin(\omega t)$, was superimposed, where A is the amplitude (which was set constant), ω the angular frequency and t the time. Initially, an amplitude sweep test has been taken at 1 Hz frequency, to identify the range of the linear viscoelasticity regime. Subsequently, the rheological tests have been performed at 25 °C with a fixed amplitude, in the 0.1-10 Hz frequency range, using a plate-plate geometry (15 mm diameter). Oscillatory shear deformation in this frequency range has been chosen since they can emulate the actual mechanical stresses occurring during normal physiological processes. To prevent water evaporation during the tests, a humidity chamber was used, and the relative humidity was set at 90%.

2.4. Biological characterization of the hydrogel

2.4.1. Cell culture

Mouse embryo fibroblasts NIH3T3 (NIH Swiss) were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, BioWhittaker,

Walkersville, MD), 2 mM L-glutamine (Sigma, St. Louis, MO), 1000 U l^{-1} penicillin (Sigma, St. Louis, MO) and 100 mg l^{-1} streptomycin (Sigma, St. Louis, MO). For the experiments, 70–80% confluent cells were used.

2.4.2. Cell adhesion and viability

For cell adhesion experiments, 5x10^4 cells/cm^2 of mouse fibroblast NIH-3T3 were seeded on RGD-PEGDA at different concentration of RGD (0,5, 1, and 5 mM) and of PEGDA (10, 20 and 40 %w/v) and on control hydrogels without RGD, pre-incubated in serum-free medium for 6 h, to avoid unspecific cell adhesion depending on serum protein adsorption, and then incubated in DMEM-10% FBS for the analyses. The samples have been incubated at 37 °C with controlled atmosphere at 5% CO_2. After 6 and 24 h of incubation, adhered cells were manually counted using a Burkër chamber. Cell morphology was evaluated by optical microscope observations by using a 10X objective. The cell viability was measured by MTT assay. NIH-3T3 cells (5x10^4 cells/cm^2) were seeded on the different substrates and incubated for 24 and 72 h. Then the cells were treated with 0.5 mg/mL MTT reagent (KeyGEN BioTECH, Nanjing, China) for 4 h. The formazan product was dissolved with 150 μl of DMSO per well, and the absorbance was detected with a spectrophotometer plate reader (Multilabel Counter, 1420 Victor, PerkinElmer) at 570 nm.

3. Results

3.1. Hydrogel swelling

The results of swelling tests, depicted in Fig. 1, show that in all cases the retention ability of the hydrogels decreases with increasing polymer concentration. As shown in the histogram, PEG 700 samples at 10, 20 and 40 (% w/v) possess Q values decreasing from 5.88 to 3.21 and 2.05, respectively. The same trend was observed for the samples with PEG 3400 at 10, 20 and 40 (%w/v) which displayed decreasing Q values of 9.26, 5.48 and 3.60, correspondingly.

Results showed that PEG 3400 possessed a higher water retention capacity compared to PEG 700, and this can be reasonably ascribed to the fact that PEGDA with higher molecular weight forms hydrogels with a larger mesh size.

Water absorption experiments have been carried out to assess the influence of the production conditions, and the obtained results allowed to define the main microstructural parameters of the final hydrogel, i.e. the calculation of the average molecular weight between crosslinks, M_c and, hence, the molecular mesh size, ξ. The following equation has been used to this aim (Canal and Peppas, 1989):

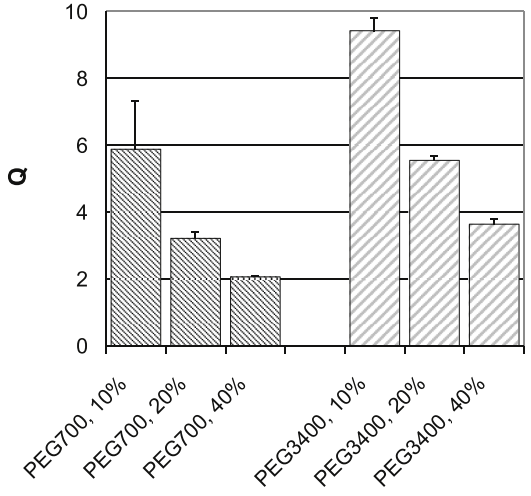

Fig. 1. Swelling ratios.

$$\frac{1}{M_c} = \frac{2}{\overline{M}_n} - \frac{\frac{\bar{v}}{v}\left[\ln(1-\phi_s) + \phi_s + \chi\phi_s^2\right]}{\phi_r\left[\left(\frac{\phi_s}{\phi_r}\right)^{1/5} - \frac{1}{2}\left(\frac{\phi_s}{\phi_r}\right)\right]} \quad (1)$$

where $\bar{v}$ is the specific volume of PEG in the amorphous state (0.893 cm^3 g^{-1}), v is the molar volume of water (18 cm^3 mol^{-1}), while ϕ_r and ϕ_s are the volume fractions of the polymer in the hydrogel in the relaxed and swollen state, respectively; ϕ_r was estimated using the percentage of PEG diacrylate in the precursor solution, and then immediately after gelation, while ϕ_s was calculated using the weight of the dry hydrogel and the water content in the swollen state. The value of χ, i.e. the Flory-Huggins polymer-solvent interaction parameter was set to be 0.426, as determined in a previous publication (Cima and Lopina, 1995).

Analogously, hydrogel mesh size [Å] was calculated with the following equation (Canal and Peppas, 1989):

$$\xi = \left(\bar{r}_0^2\right)^{1/2}\phi_s^{-1/3} \quad (2)$$

where:

$$\left(\bar{r}_0^2\right)^{1/2} = l\left(2\frac{M_c}{M_r}\right)^{1/2} C_n^{1/2} \quad (3)$$

In equation (3), $\left(\bar{r}_0^2\right)^{1/2}$ is the average end-to-end distance of PEG chains in the unperturbed (solvent-free) state, M_r the molecular weight of the repeating unit and C_n the characteristic ratio. Table 1 summarizes the calculated values of M_c and ξ, which were found, respectively, to be about 3 times and 2 times higher for PEG 3400, at all polymer concentrations.

3.2. Infrared (IR) analyses

As a representative example of all the samples, Fig. 2 reports IR spectra of PEG 700 hydrogel.

Acrylate groups, which are involved in the crosslinking, are characterized by the C = C bonds, while C–O–C bonds are not involved in the process. Therefore, the comparison between peak areas of C = C vs. C–O–C bonds provides indication on the degree of conversion of acrylate groups, i.e. on hydrogel crosslinking. Table 2 summarizes qualitative information on the degree of conversion of the double bonds, which are related to the relative importance of C═C vs C–O–C peaks.

The evidenced trend indicates that the degree of conversion of the double bonds C = C and, consequently the efficiency of the crosslinking reaction, is increasing with increasing concentration of polymer.

3.3. Uniaxial compression measurements

Stress – relaxation curves, obtained from uniaxial compression test are reported in Fig. 3.

Stress – strain (Fig. 4) graphs have been obtained by reporting the stress values at equilibrium as a function of sample strain defined as $\varepsilon = (h_0\text{-}h)/h_0$, where h is the height of the sample at equilibrium and h_0 the initial height of the sample. As shown in Table 3 Young's modulus (E), evaluated by the Stress-Strain curve, increases with increasing concentration for both PEG 700 and PEG 3400. More in detail, E values of the

Table 1
Mc and ξ values for PEG hydrogels as calculated with equations (2) and (3).

	Polymer concentration (w/v %)	M_c [g/mol]	ξ [Å]
PEG700	10	230	19
	20	190	14
	40	120	10
PEG3400	10	840	39
	20	600	28
	40	440	22

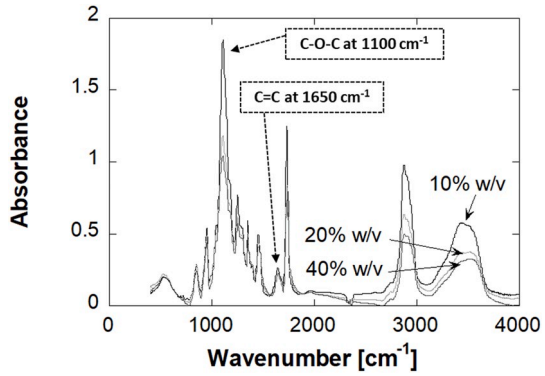

Fig. 2. IR spectra of PEG hydrogels at 10, 20 and 40% w/v.

Table 2
Peak areas for C═C and C–O–C bonds as calculated from IR spectra.

	Polymer concentration (w/v %)	C═C peak areas	C–O–C peak areas	Area ratio C═C/ C–O–C
PEG700	10	7.77	64.4	0.121
	20	7.57	34.6	0.219
	40	7.86	30.1	0.261

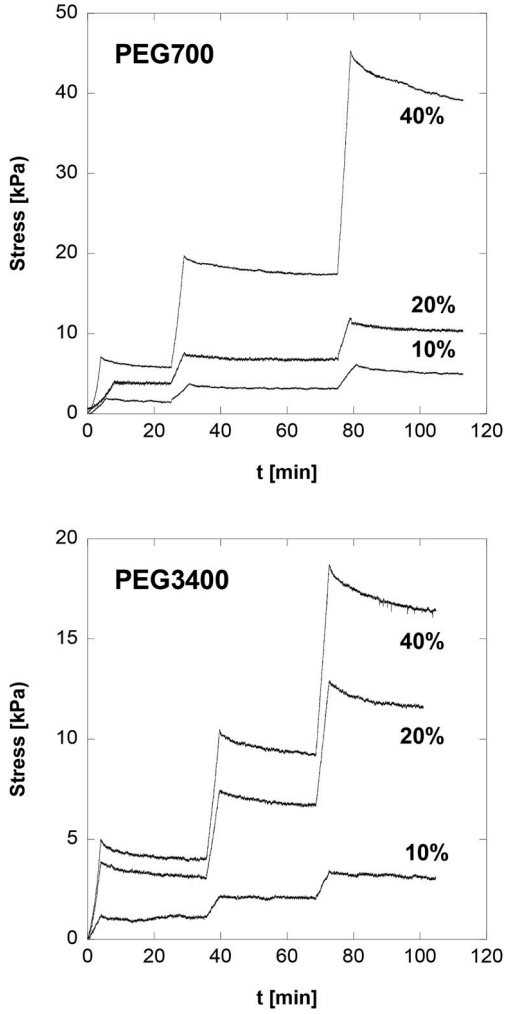

Fig. 3. Stress-relaxation curve.

hydrogels at 10, 20 and 40 (w/v%) increase respectively from 28, 76, 118 kPa for PEG 700, and 22, 62, 80 kPa for PEG 3400. Clearly, for a fixed MW (700 or 3400) and for an increasing polymer concentration in the precursor solution, the obtained material is increasingly stiff. On the contrary, for constant polymer concentration, and comparing the two different molecular weights (PEG 700 and PEG 3400), a stiffer hydrogel is obtained with the lowest MW. In particular, under compression test, E values are higher in PEG 700 compared to PEG 3400, being for PEG 700 at 10, 20 and 40 (%w/v) 1.3, 1.2 and 1.5 times respectively higher than PEG 3400. These can be ascribed to tha fact that PEG 700 absorbs less water due to its narrower molecular mesh.

3.4. Rheological properties

A representative mechanical spectrum, that is the dependence of G' and G'' as a function of frequency of PEG 700 sample (20% w/v), is reported in Fig. 5.

The rheological spectra displayed in Fig. 5, qualitatively similar for all the prepared hydrogels, show that, over the entire frequency range, the elastic modulus is always higher than the viscous modulus, i.e. no crossover occurs under the used experimental conditions. Moreover, there is a weak dependence of both viscoelastic moduli upon oscillation frequency, while G' and G'' values differ by roughly one order of magnitude, thereby pointing at the rheological behavior characteristic of strong gels. In Fig. 6, the comparison of G' for all samples is shown.

Results displayed in Figs. 5 and 6 show that elastic and viscous moduli can be increased by properly choosing the MW of PEG prepolymer and the concentration of the precursor solution. In Table 3, G' and Tan δ values estimated at the frequency equal to 1 Hz are reported. As can be noted, G' increases with increasing concentration for both PEG 700 and PEG 3400 samples. Indeed, 10, 20 and 40 (%w/v) G' values increase, respectively, from 5.97, 44.1, 95.9 kPa for PEG 700, and 9.28, 59.7, 99.8 kPa for PEG 3400. The G' values under shear test, unexpectedly, at 10 and 20 (w/v%) are higher by 1.5, 1.3 times for the PEG 3400 compared to the PEG 700. Moreover, Tan δ values for hydrogels at 10 and 20 and 40 (%w/v) are respectively 0.06, 0.07, 0.16 for PEG 700 and 0.02, 0.08, 0.11 for PEG 3400. It is possible to notice that, for both molecular weights of the precursor solution at 10 and 20 %w/v, the value of Tan δ is about 10^{-2} while for the gels at 40% w/v Tan δ increases of one order of magnitude. At the lowest concentration of the precursor solution, it is possible to obtain stronger gels having lower dissipation capability.

3.5. Biological characterization of the hydrogel

It has to be noted that the addition of RGD peptide to hydrogel provides a remarkable cell adhesion capability, which indeed occurred for boh PEG 700 and PEG 3400 and for all RGD peptite concentrations. As it can be seen in the histograms shown in Fig. 7, for all control samples (without RGD) the percentage of cell adhesion never exceeds 10%, while already the lowest RGD concentration led to about 70% of cell adhesion, after 24 h. All histograms show that for all samples there is a maximum of adhesion for the intermediate concentration of PEG-RGD (1mM).

In Fig. 8 bright-field image shows NIH3T3 cell morphology seeded on representative PEGDA + RGD after 24 h. As it can be seen, the cells attached to the PEGDA + RGD appear elongated. Indeed, their morphology seems to be in line with the morphological characteristics of NIH3T3 cells *in vitro*, which is spindle-shaped, often characterized by numerous extension processes, which consists of a cellular protrusion that adheres to the flat surface, typical of fibroblast cells. This qualitative assessment of the morphology is in accordance with the viability data, shown below.

As shown in Fig. 9, a good viability has been observed for PEG 3400 and PEG 700 for all polymer concentrations. For both PEG 3400 and 700 the results, normalized for the control samples without RGD

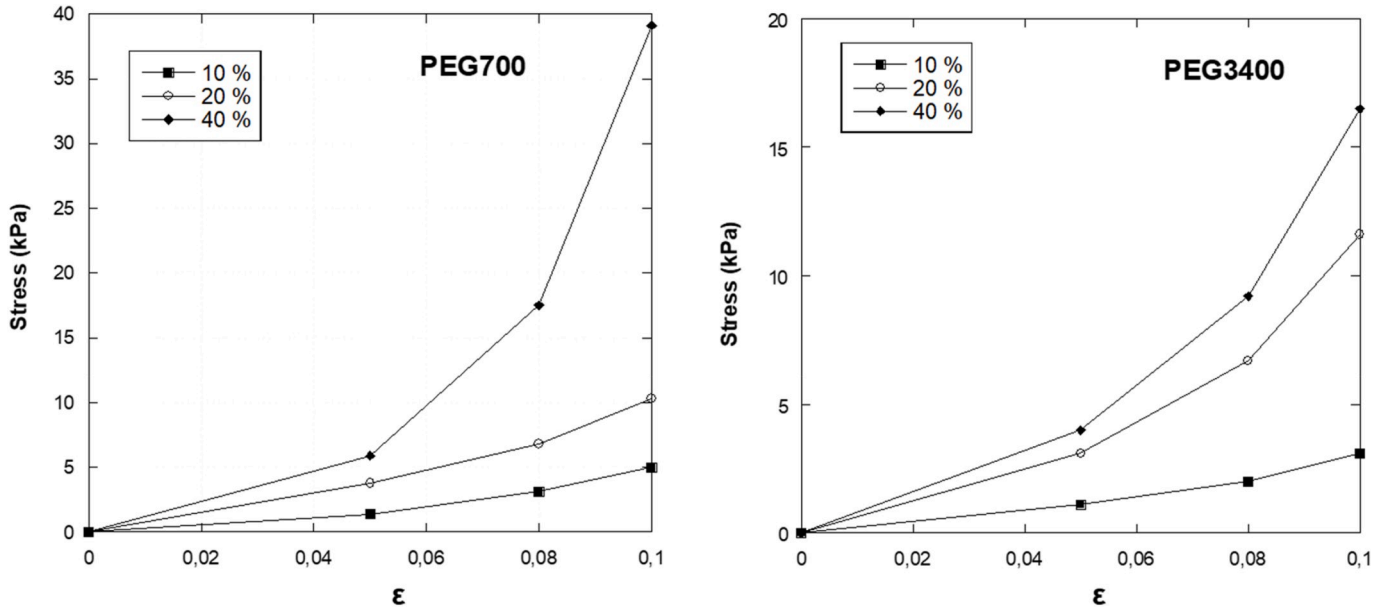

Fig. 4. Stress-strain curves.

Table 3
Mecchanical properties values. G' and Tan δ were estimated at frequency of 1 Hz at 25 °C.

	PEG 700			PEG 3400		
	E (kPa)	G' (kPa)	Tan δ	E (kPa)	G' (kPa)	Tan δ
10%	28 ± 8	5.97 ± 0.06	0.06	22 ± 5	9.28 ± 0.07	0.02
20%	76 ± 7	44.1 ± 0.07	0.07	62 ± 6	59.7 ± 0.06	0.08
40%	118 ± 12	95.9 ± 0.05	0.16	80 ± 3	99.8 ± 0.08	0.11

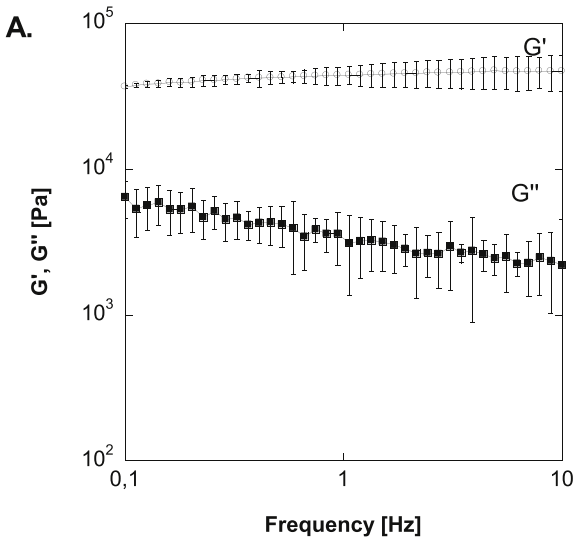

Fig. 5. G' and G'' as a function of oscillation frequency for PEG700 sample, 20% w/v.

functionalization, show that when the cells are seeded on the RGD funcionalized PEG, the viability increses with the increasing of RGD peptide concentrations, from 0,5 until 5 mM. In particular, for PEG 3400 the trend appears to further increase after 72 h of cell culture. With regards to the influence of the different MW of the hydrogels on cell viability, it has been observed that on PEG 3400, the viability appears to be slighty better compared the PEG 700 after 24 h and the differences between PEG 3400 hydrogels become more evident after the 72 h of adhesion, compared to PEG 700 hydrogels.

4. Discussion

In this work, PEGDA hydrogels at two different molecular weights and three concentrations have been produced by photo-polymerization with the main aim to correlate their mechanical behavior, under oscillatory shear and confined compression experiments, to their biological performance.

The comparison of the results displayed in Figs. 4–6 indicates that the mechanical properties of PEG hydrogels can be modulated by properly choosing the molecular weight of PEG prepolymer and its concentration in the precursor solution. As to the effect of PEG prepolymer molecular weight, it seems that the results of oscillatory shear tests contradict the findings of uniaxial compression tests. Indeed, the results of compression tests, at fixed concentration, indicate that the elastic modulus is decreasing with increasing MW of PEG prepolymer, while the viscolestic properties in shear increase with increasing of the MW, especially at 10 and 20 (w/v %) (Table 3). It must be underlined that compression and shear tests provide different information; indeed, shear experiments account for the internal friction among the macromolecular coils that are also influenced by topological interaction. Indeed, it is well known that in polymeric systems, at a fixed concentration as the MW increases, topological interactions (entanglement) occur thus increasing friction and consequently the viscoelastic parameters of the systems (Xin et al., 2004). Results indicate that, under the experimental shear conditions used in this work, a higher MW of the prepolymer leads to a less effective chemical molecular interaction and also that the effect of chain coil and friction prevails over the effect of molecular mesh size.

More in detail, for hydrogels with higher PEG MW, the E values are lower than those of PEG 700, presumably due to a decrease in the cross-link density as the MW increased. However, this correlation between MW and E is less pronounced for hydrogels with lower precursor polymer concentration PEG contents (i.e., 10 and 20%w/v). Differently, G' values increase with increasing PEG MW for the lower polymer concentrations (10 and 20 %w/v) with no significant differences for the highest polymer concentration (40 %w/v). As to the effect of the prepolymer concentration of the precursor, it has been shown that hydrogels at higher prepolymer concentrations have higher E in compression test and G' in shear test. This trend could be related to the

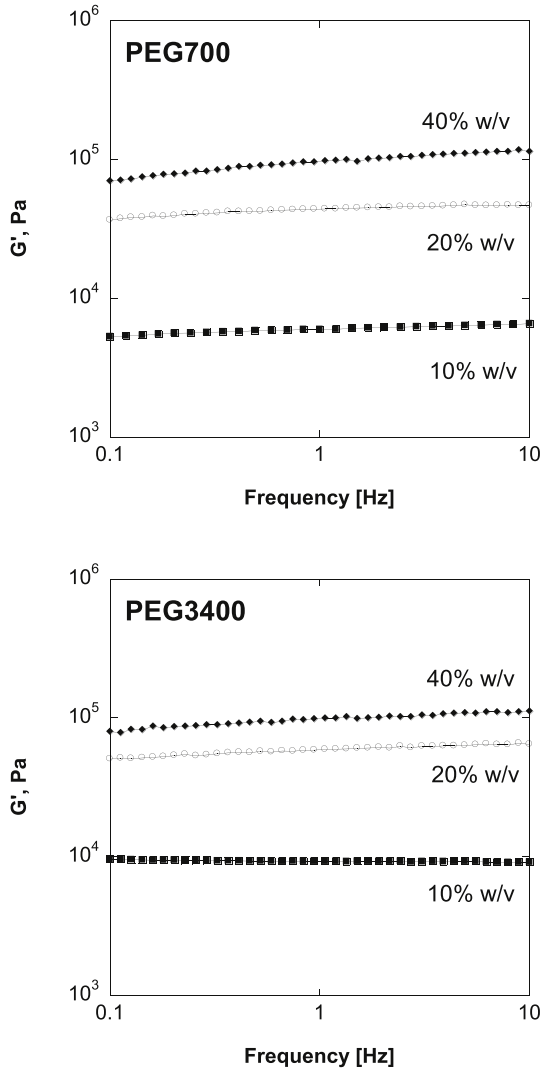

Fig. 6. G' as a function of oscillation frequency for PEG700 and PEG3400 samples (10, 20, 40% w/v).

superimposition of two competitive effects: (i) from a static viewpoint, with increasing polymer concentration the overlapping of the chains increases, thus favoring the cross-linking process; (ii) from a dynamic viewpoint, the increased viscosity of the mixture reduces the mobility of the chains, thus reducing the probability that, during the UV irradiation, a macroradical can react with other chains resulting in a cross-linking reaction. Thus, the formation of cross-links between chains does not occur homogeneously within the irradiated polymer solution; it is likely, indeed, that the proximity between two cross-linked chains enhances the probability that other cross-links could form (D'Errico et al., 2008; Lin-Gibson et al., 2004; L). Consequently, at higher polymer concentration, it is reasonable to assume that hydrogels contain cross-linked clusters which reinforced the network. As the concentration decreases, the amount of oligomer present becomes insufficient to form uniformly dispersed clusters, thereby creating large "defects" in the network (Lin-Gibson et al., 2005).

Mechanical test results correlate with the results of the swelling test. The cross-link density in fully swollen networks is directly proportional to the gel modulus and inversely proportional to the swelling. PEG 3400 possesses an higher water retention capacity, associated with a worse mechanical behaviour of the samples during confined compression tests, and this can be reasonably ascribed to the fact that PEGDA with higher molecular weight forms hydrogels with a larger mesh size, while PEG 700 absorbs less water, due to its narrower molecular mesh size, resulting in a higher modulus E in compression. Indeed, at fixed concentration, ξ of the hydrogel obtained using PEG 3400 is twice as high as that using the PEG 700. Futhermore, as evidenced by the swelling data, as the prepolymer concentration increases, the mesh sizes decrease. In particular by increasing the concetration from 10 to 40 (%w/v), ξ is halved for both PEG 700 and 3400. Swelling data results show that with increasing polymer concentration Q values of the hydrogels decrease. As the polymer concentration increases, the probability of crosslink formation in the hydrogel increases, and also the correlated mechanical properties of the hydrogels; this involves the formation of a more rigid structure able to retain less amount of water. On the contrary, when the polymer concentration is lower, the cyclization of the molecular structure is favored, which relates to a less rigid and more porous structure with a higher capability of swelling. This means that a change in the initial polymer concentration leads to the obtainment of a material with a different chemical - physical features molecular structure (Canal and Peppas, 1989). As it is known, the degree of swelling of the hydrogel depends primarily on its porosity, and results from several conflicting factors. The water, penetrating within the material, tends to stretch the links among polymer chains, that spontaneously react by opposing to this phenomenon, which leads then in a configuration at a lower entropy. Swelling equilibrium is obtained when this resistant force is balanced by the force exerted by the osmotic pressure of the water. The degree of swelling is also very important as for cellular adhesion and invasion, since it affects the depth and the amount of cell density within the hydrogel following a complex balance between mesh size, mechanical properties and durotaxis propensity of the cells (Giarra et al., 2018). In particular, cell invasion within a hydrogel-like bulk material is governed by cell deformability and cavity-to-cavity migration, along with the ability of polymer chains to disentangle with a subsequent pore enlargement (da Silva et al., 2010). A material with a high degree of swelling is also very porous, and therefore allows a more homogeneous cell invasion, along with a facilitated exchange of nutrients and metabolic wastes with the living tissue surrounding the hydrogel. Basically, material properties can be modulated by properly choosing the MW of the starting polymer or its concentration in the precursor solution.

Moreover, IR results showed a slight increase of the conversion of acrylate groups with increasing concentration of the prepolymer in solution, and therefore an increase in the crosslinking degree. However, this increase was not high enough to explain the strong differences in gel physical-mechanical properties. This seemingly puzzling phenomenon can be explained considering that during the polymerization of multivinyl monomers there is a high possibility of primary cyclizations, which occur when a pendant double bond reacts with the radical on its own polymer chain during propagation reaction. The occurrence of primary cyclizations likely generates a material with a lower crosslinking density and, therefore, these results into a less rigid material, which is capable of absorbing higher amounts of water. In more detail, this behavior can be explained through the kinetic model developed by Elliott & Bowman (Elliott and Bowman, 1999), which accounts for the different reactivity between the double bonds freely pendant and during polymerization. In particular, at higher solvent concentrations (i.e. lower monomer concentrations), freely pendant double bonds are more prone to primary cyclization because the effective local concentration of radicals decreases slowly, while the concentration of monomer double bonds is drastically reduced by the presence of the solvent. This model is consistent with experimental results and can explain the changes in physical-chemical properties of the hydrogels at different prepolymer concentration in the precursor solution. It is known that the incorporation of RGD adhesive peptides on polymer substrates, such as polyacrylamide surfaces and PEG hydrogels (Brandley and Schnaar, 1988; Hern and Hubbell, 1998), assists the cell attachment which is essential for the maintenance of the cell viability, proliferation and migration. Furthermore, it has also been demonstrated that also the mechanical characteristics of the materials influence cell behavior (Guarnieri et al.,

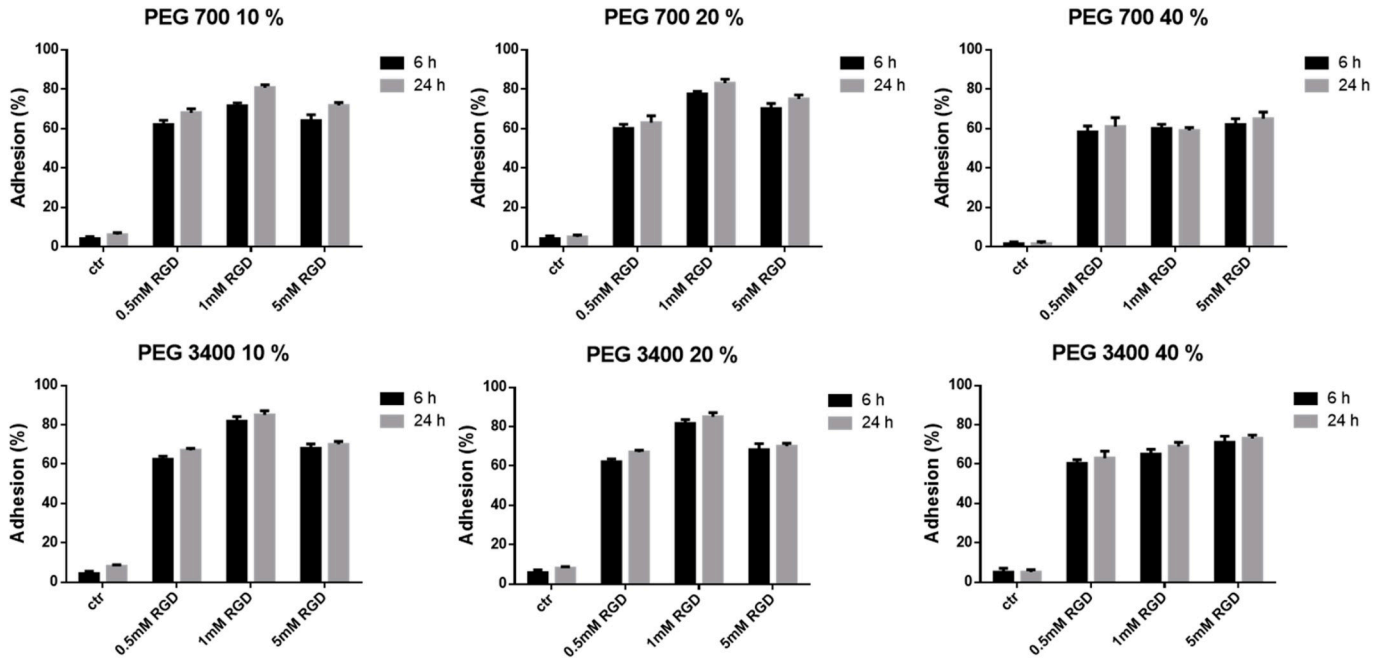

Fig. 7. Histograms of the adhesion percentage of NIH3T3 cells, after 6 and 24 h of culture on the PEG 700 and PEG 3400 hydrogels functionalized with different concentrations of RGD (0.5, 1 and 5 mM).

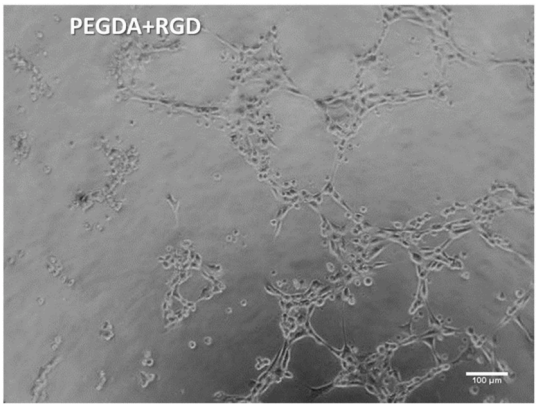

Fig. 8. Bright-field image of NIH3T3 cells seeded on representative PEGDA + RGD.

2010; Panzetta et al., 2017). Here, adhesion results showed a similar trend in terms of percentage of adhesion on hydrogels after 6 and 24 h of culture, indicating a preference for the intermediate concentration of 1 mM of RGD peptide, probably due to a balance between the RGD exposure and the integrin transmembrane receptors of the cells. The viability results have demonstrated, for both hydrogels, an increase of the cell viability with the increasing of the concentration of RGD peptide functionalization. Moreover, a predilection of the cells for the PEG 3400 hydrogel compared to PEG 700 after long time of culture (72h) has been noted. Whereas, there are no significant differences for cell viability after 24 h of culture on both hydrogels. Thus, cells would seem to prefer, after longer culture times, hydrogels with larger molecular meshes and lower E values, such as those of PEG 3400, probably because the nutrient recirculation is more efficient in these substrates. From these results it appears clear that cell behavior is influenced not only by the presence and the different concentration of RGD peptides, but also by the mechanical and structural features of the materials. Indeed, on matrix with a higher stiffness, the cell actomyosin apparatus, dedicated to the contractility of cells, converges on cell spreading, which is tightly coupled to proliferation (Mih et al., 2012). The restriction of the spread area of adherent cells can lead to growth arrest, while if the spread area increases, the rate of proliferation is positively affected. On the other hand, these results have demonstrated that although PEG 700 appears to have higher mechanical properties in compression than PEG 3400, cells cultured for longer times seem to prefer hydrogels with a larger mesh size structure that posses higher viscoelastic properties in shear. Therefore, we can conclude that parameters like the MW and the concentration of a polymer influence the mechanical properties that are reflected in different materials structure able to affect cells mechanosensing. Moreover the interesting aspect to underline is that, although many works are devoted to the study the stiffness of the biomaterials as a parameter to direct and control cell growth and migration, the stiffness of material depends intrinsically on the technique with which this is measured.

5. Conclusion

In this work, we have studied the influence of polymer molecular weight and concentration of the precursor solution on the mechanical features of the final hydrogel and their effect on cells-material interaction. 3D synthetic matrices based on PEGDA at two molecular weights (PEG 700 and PEG 3400) and at three different pre-polymer concentrations (10, 20, 40 w/v %) were employed. In particular, we focused our attention on the mechanical performance in oscillatory shear and confined compression tests. Indeed, oscillatory shear experiments can provide useful information about network characteristics of the hydrogel. Differently, confined compression tests are extremely useful for hydrogels designed for biomedical applications, as they can simulate body physiological stimuli thereby enhancing the prediction of their performance *in vivo,* once in contact with the biological fluids. We found that Young's modulus (E) in compression and elastic modulus (G') in oscillatory shear increased with increasing polymer precursor concentration for both PEG 700 and PEG 3400. As to the influence of the polymer precursor MW, E decrase with increasing of the MW, especially at higher prepolymer concentration (40 %w/v). Inversely, G'in shear increased with increasing PEG MW for the lower polymer concentrations (10 and 20 %w/v) while there are no significant differences for the

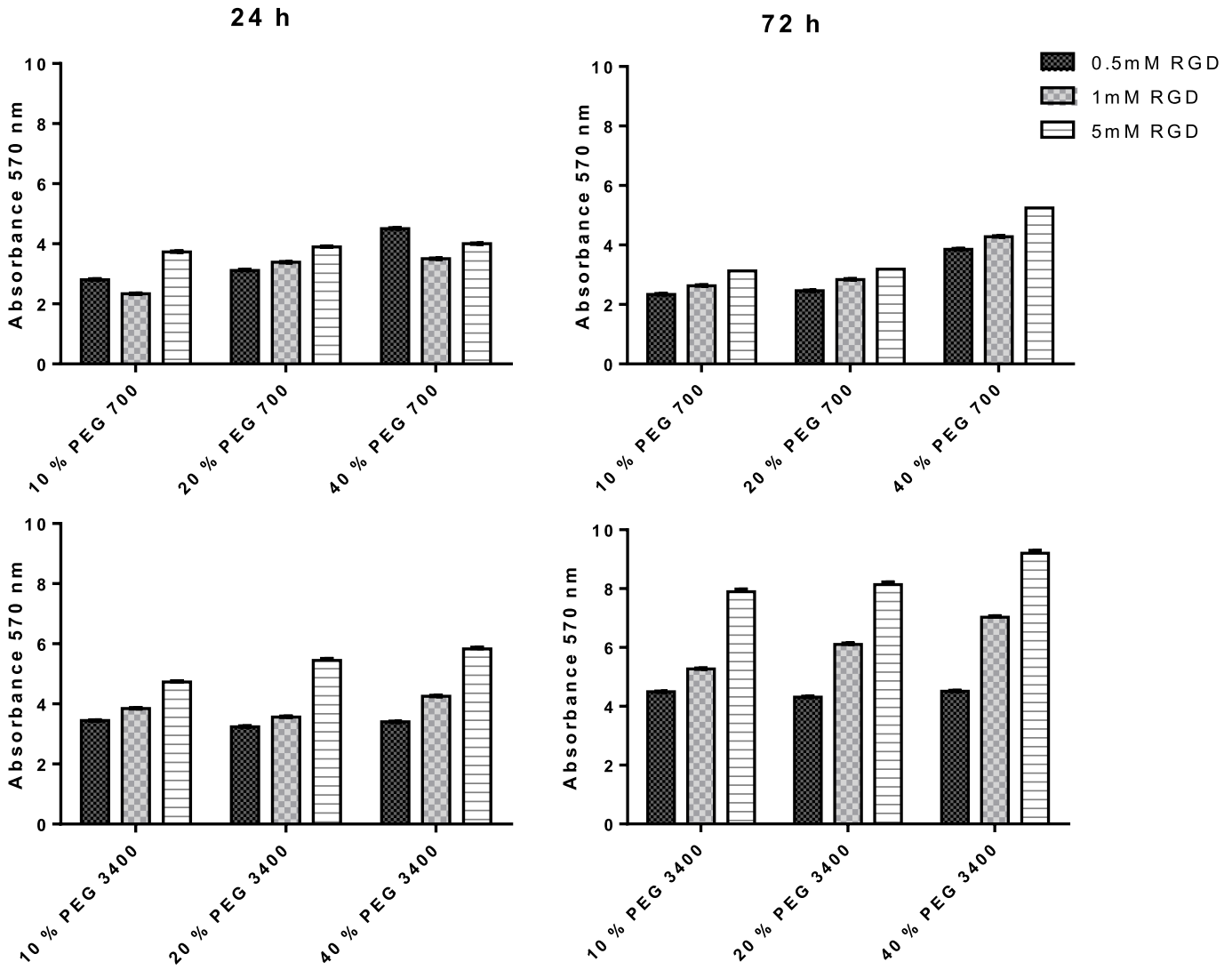

Fig. 9. The histograms show the viability of NIH 3T3 cells after 24 and 72 h of culture on the PEG 700 and PEG 3400 hydrogels at different concentrations of the functionalized RGD peptide (0.5, 1 and 5 mM). Absorbance at 570 nm is directly proportional to cell viability.

highest polymer concentration (40 %w/v). The biological results have demonstrated that cells, cultured on substrates functionalized with RGD peptide, seem to prefer PEG 3400 hydrogel at longer times of culture as a substrate suitable for their development and proliferation, although PEG 700 appears to have higher mechanical properties in compression. This behavior could be ascribed to the wider molecular mesh size of PEG 3400 which allows a more efficient recirculation of nutrients. The overall results here presented indicate that the molecular weight and the concentration of a polymer do influence the final architecture of the hydrogels, which, in turns, affect the mechanical properties of the substrate and, thus, cell mechanosensing. The interesting aspect evidenced in this work is that, although many works are devoted to the study of the biomaterial stiffness as a parameter to direct and control cell growth and migration, the stiffness study alone is not enough. Indeed, oscillation shear and confined compression tests of a substrate can account for different and complementary information, both necessary for better design the macroscopic characteristics of the final hydrogel for biomedical application.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Francesca Della Sala: Writing - original draft, Investigation, Writing - review & editing. **Marco Biondi:** Writing - original draft, Investigation. **Daniela Guarnieri:** Formal analysis. **Assunta Borzacchiello:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Luigi Ambrosio:** Resources, Visualization. **Laura Mayol:** Project administration, Writing - review & editing.

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