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Influence of viscoelastic properties of an hyaluronic acid-based hydrogel on viability of mesenchymal stem cells

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Abstract.
BACKGROUND: The present research is involved in the framework of the biotherapy using mesenchymal stem cells (MSCs). Here, MSC encapsulation in a hydrogel based on hyaluronic acid (HA) is investigated to optimize the composition of the biomaterial.
METHODS: Several formulations candidates of the hydrogel (9 in total) are postulated as a scaffold for the 3D MSC culture in order to investigate their potential to mimic the *in vivo* cellular environment. Rheological measurements in oscillation mode of complex modulus and complex viscosity are performed on the different hydrogels. Biological tests are carried out for the measurement of the cell viability of MSC encapsulated in the hydrogels.
RESULTS: Rheological and biological findings are correlated together in order to establish relationships between the viscoelastic properties of the hydrogel and the cellular viability of MSC.
CONCLUSIONS: In the light of the viaility results, the composition of the hydrogel was related to the MSC proliferation. Thus, such relations are useful tools for scientists offering them more flexibility in the design of their hydrogels while ensuring an acceptable level of MSC viability.

Keywords: Mesenchymal stem cells (MSCs), hydrogel based on hyaluronic acid, rheology, cellular viability

1. Introduction

One of the greatest challenges of the regenerative medicine is to enable the repair of injured native tissue using cellular therapies particularly by combining stem cells with biomaterials scaffolds [1,2].

Thus, researches over the past 10 years focused their attention on cells cultured *in vitro* in 3D scaffolds which resemble the physiology of their counterparts *in vivo* much better than cells cultured conventionally on 2D surfaces [3]. For that, various hydrogels have been proposed to mimic aspects of the *in vivo*

cellular environment with more and less success [4]. Note that essential criteria exist in the design of hydrogels as their ability to allow cell proliferation, a sufficient porosity to permit diffusion of nutrients and gases, an absence of toxicity of the resulting material and/or degradation, and no reaction inflammatory reaction in contact with the biological tissue.

Nowadays, no biomaterial is really representative of the *in vivo* physiological environment in which cells will be injected. Consequently, in order to improve the reconstituted tissue architecture and to better control the interactions between cells and their environment in such *in vitro* structures, several technologies based on the micro and nano-elaboration of hydrogels have been developed such as emulsification, photolithography, microfluidics synthesis and microinjection. For further information, the reader could consult the review paper of Deligkaris et al. [5] where hydrogel-based devices for biomedical applications are described with a presentation of comprehensive, qualitative, theoretical overview of hydrogels' synthesis and operation. On the other hand, to help the design of hydrogels for cell encapsulation and highlight their applications in tissue engineering, different animal models were used. As an illustrative example, the xenograft rat model [6] has been investigated to study aneurysm evolution and expansion in the abdominal aorta. Thus, it has been proved that the injection of mesenchymal stem cells (MSCs) cultured in hydrogel based on hyaluronic acid (HA) can regenerate injured vascular tissue and particularly stabilize the AAA improving thereby the mechanical resistance of the weakened arterial wall [7–9]. In this context, authors [10] focused especially on the optimization of the hydrogel based on hyaluronic acid (HA) from a biological point of view through the investigation of the MSC differentiation potential, their phenotype and viability when encapsulated in a hydrogel. Note that the design of hyaluronic acid (HA)-based hydrogel scaffolds to investigate cell response is a major field of interest in developing tissue engineering and regenerative medicine applications. Thus, Lam et al. [11] presented an overview of the biological context of HA, which is needed to better understand how to engineer cell-matrix interactions in the scaffolds. It is worth to be emphasized that the optimization of the hydrogel composition is also dependent on its viscoelastic properties [12] which represent a determinant criterion for the MSC culture and their potential to repair the AAA *in vivo* while exhibiting the suitable phenotype adapted to the *in vitro* mechanicalstress. In fact, the elaboration and optimization of the hydrogel composition require the knowledge of its rheological properties over a wide range of temperatures and time solicitation. For instance, the dynamic measurements of viscosity are needed for the monitoring of the gelation process of a hydrogel. More generally, the knowledge of the rigidity modulus is an important indicator for the cohesion degree in the material structure [13]. In the case of viscoelastic materials, the evolution of this cohesion is governed by the stress-strain relationship under different thermal and frequency scenarios.

The aim of this present work is threefold.

(i) To study different hydrogel formulations based on hyaluronic acid (HA), gelin and an agent of crosslinking polyethylene glycol diacrylate (PEGDA), as candidates to the MSC culture, through an experimental investigation based on both rheological and biological tests. For that, rheological measurements of viscoelastic properties of different hydrogel formulations and cellular viability tests with Alamar Blue protocol were performed.

(ii) To identify the best formulation of hydrogel which can be used as a 3D structure for the MSC preservation for the treatment of AAA created by the xenograft rat model.

(iii) To draw conclusions from experimental investigation especially by establishing correlations and relationships between the rheological properties of the hydrogel and/or its composition and its potential, expressed in terms of cellular viability, to encapsulate MSCs.

The results showed that the formulation of 40% of HA and 1/8 of PEGDA exhibits the best cellular viability rate recorded after 28 days of MSC culture. Furthermore, the selected formulation is distinguished by the lowest structure rigidity and viscosity in comparison with the others hydrogels, that may constitute rheological criteria in the choice of the hydrogel.

2. Materials and methods

2.1. Recovery and culture of MSCs

MSCs were obtained from the femurs and tibias of 344 male Fischer rats (Charles River, France) aged between 7 and 10 weeks. After epiphyses section, the marrow was recovered by centrifugation at $500g$ for 5 min according to the method described by Peister et al. [14]. The cell suspension taken up in 10 ml of culture medium was filtered through a sieve (70 µm) and then homogenized by successive passages in a 5 ml pipette. Rat MSCs were incubated with α-MEM supplemented with 10% FBS, antibiotics and 50 ng/ml TGFß1 (Sigma-Aldrich). The medium was renewed regularly in order to keep the cells in culture. When the cellular confluence reached 80%, the cells were seeded. After washing using the PBS1 X, the cells were dissociated by the action of 0.25% trypsin – EDTA 1 mM (Invitrogen, France) at 37°C, then, the action of the trypsin was inhibited by the medium culture. After centrifugation for 5 min at $200g$, the cells were replaced in culture medium and incubated at 37°C and 5% CO_2.

2.2. Hydrogel preparation

Hydrogel compositions for different HA and gelin

	Designation	HA	PEGDA
F_{11}	HA:gelin (40:60), r 1:4	40%	1/4
F_{12}	HA:gelin (40:60), r 1:6	40%	1/6
F_{13}	HA:gelin (40:60), r 1:8	40%	1/8
F_{21}	HA:gelin (50:50), r 1:4	50%	1/4
F_{22}	HA:gelin (50:50), r 1:6	50%	1/6
F_{23}	HA:gelin (50:50), r 1:8	50%	1/8
F_{31}	HA:gelin (60:40), r 1:4	60%	1/4
F_{32}	HA:gelin (60:40), r 1:6	60%	1/6
F_{33}	HA:gelin (60:40), r 1:8	60%	1/8

2.3. Rheological measurements

Measurements of viscoelastic properties were performed on the nine hydrogel samples using a Dynamic Shear Rheometer (Bohling CVOR from Malvern) in shear oscillatory mode using a plate and plate geometry (diameter = 8 mm, gap = 1 mm). Experiments were carried within a linear viscoelastic regime in a strain controlled oscillation mode with a frequency sweep (f ranging between 0.1 Hz and 5 Hz). The imposed strain of 1% was chosen such as a linear viscoelastic (LVE) response is expected and also to prevent the destruction of the hydrogel structure during the test. It is worth noticing that the rheological tests were repeated at least three times by using a new hydrogel sample at each new test so that the repeatability test can be checked and average result are obtained. The shear rheometer is connected to computer for the real-time monitoring of the rheological properties evolutions of the different hydrogels.

Accordingly, the shear stress (τ) and strain (γ) are given as:

$$\begin{cases} \tau = \tau_0 \exp i(\omega t + \delta), \\ \gamma = \gamma_0 \exp i\omega t, \end{cases} \tag{1}$$

where δ denotes the phase angle between the solicitation and its response ranging between 0° and 90°, ω the load pulsation ($\omega = 2\pi f$) and i the imaginary unit.

The shear complex modulus is defined as:

$$G^*(\omega, T) = \frac{\tau}{\gamma} = \frac{\tau_0}{\gamma_0} \exp i\delta = G' + iG''. \tag{2}$$

The modulus of the shear complex modulus corresponds to the ratio τ_0/γ_0 in Eq. (2) while G' and G'' denote respectively the elastic (or storage) modulus and the viscous (or loss) modulus. The knowledge of G' and G'' allows the definition of the loss modulus expressed as:

$$\text{Tan}(\delta) = \frac{G''}{G'}. \tag{3}$$

The complex viscosity of the material is expressed as:

$$\eta^*(\omega, T) = \frac{\tau(t)}{\dot{\gamma}} = \frac{\tau_0 \exp i(\omega t + \delta)}{\gamma_0 i\omega \exp i\omega t} = \frac{1}{i\omega} G^*(\omega, T), \tag{4}$$

where $\gamma^{\cdot}$ is the rate of the shear strain. The combination of Eqs (2) a n d (4)

yields to:

$$\eta^*(\omega, T) = \frac{G''}{\omega} - i\frac{G'}{\omega} = \eta' - i\eta''. \tag{5}$$

2.4. Alamar Blue tests

The Alamar Blue test used in the present study is from Invitrogen (France). Alamar Blue is a non-toxic reagent indicating metabolic activity and therefore cell viability. The reduction of the active non-fluorescent of Alamar Blue, called resazurin in the mitochondria of living cells, leads to the release of the resorufin which is a highly fluorescent substance in the red. MSCs were incubated for 3 h in the culture medium with the presence of 10% Alamar Blue. In each experimental condition, the fluorescence of 100 μl of the product was measured using the fluorescence reader Perkin Elmer Victor. To determine the optimal composition of the hydrogel for cell culture, we evaluated the viability of MSCs of rat, encapsulated in different hydrogel formulations by the Alamar Blue test 1, 7, 14, 21 and 28 days after 3D cell culture. The objective was here to quantify the resorufin amount after the reduction of the active component of Alamar Blue (resazurin) by mitochondria. During this reaction, the color of the culture medium turned from blue to fluorescent pink.

2.5. Statistical analysis

It shall be emphasized that the aforementioned tests were performed by considering two-tailed and unpaired Student's t-tests having a significance level of 5%. In addition, the quantitative results are given as mean – standard error of the mean.

3. Results and discussion

3.1. Rheological properties

The evolutions of the complex modulus versus the frequency of solicitation for the nine hydrogels are shown in Fig. 1. The statistical data have shown a small dispersion less than 1% for all the rheological measurements. It can be seen from the different cases that the complex modulus increases with frequency. This finding is an expected result for viscoelastic materials in general which are strongly dependent on the time solicitation. In fact, for a shorter time loading (i.e. a higher frequency), the structure rigidity of the hydrogel, here expressed by the complex modulus, is more important. Besides, one can see that the higher is the hyaluronic acid concentration in the solution, the less pronounced is the effect of crosslinking agent PEGDA. This result can be noticed from the comparison of the curves deviations for a given hyaluronic acid concentration. A particular attention is worth to be paid to the hydrogel F_{13} (40% HA and 1/8 PEGDA) for which the complex modulus results are much deviated from F_{11} and F_{12}. Furthermore, one can see that hydrogel F_{13} exhibits the lowest complex modulus and therefore a softer structure comparing to the eight other formulations.

Plots of the elastic and viscous modules, respectively G' and G'' of the hydrogel F_{13} are shown in Fig. 2. The experimental results highlight commonly the predominance of the elastic behavior of the different hydrogels ($G' > G''$) ($Tan(\delta) < 1$). This finding is in accordance with the typical solid-like behavior of a gel already formed. Let recall that prior to gelation, the material exhibits a typical liquid-like
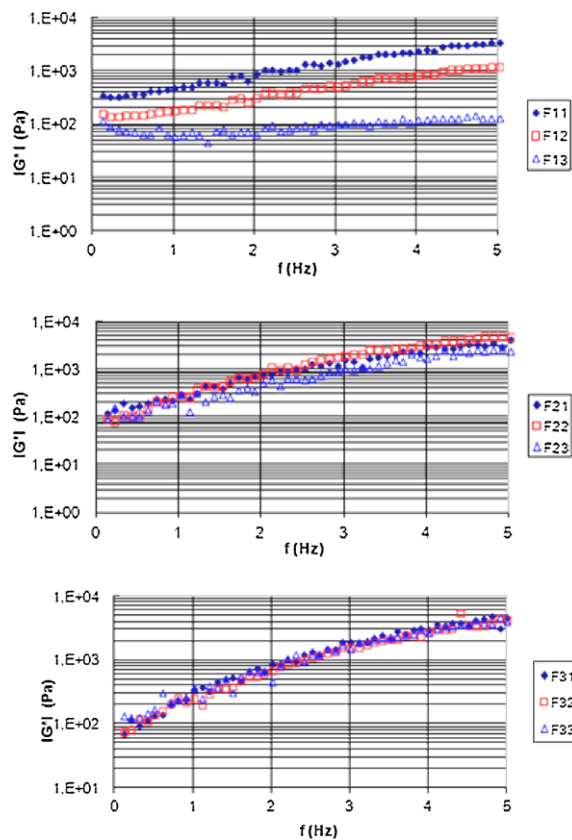
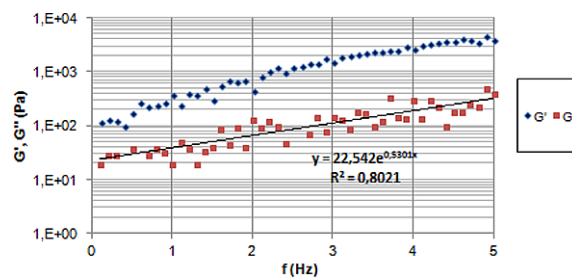Fig. 1. Complex modulus of hyaluronic acid hydrogels at 37°C. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BME-151557.)

Fig. 2. Elastic and viscous modulus of hyaluronic acid hydrogel F_{13} at 37°C. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BME-151557.)

behavior characterized by a storage modulus less important than the viscous one. At the gelation point, a cross-over point can be observed ($G' = G''$), [15–17]. Then, when the gelation progresses, the curves G' ad G'' are deviated with a predominance of G'. Besides, it can be noticed the experimental dispersion in the viscous modulus results fitted here by a mathematical power function (in continuous line on the same graph). This dispersion may be associated to the parametric uncertainties of the rheological test or also to the error measurements.
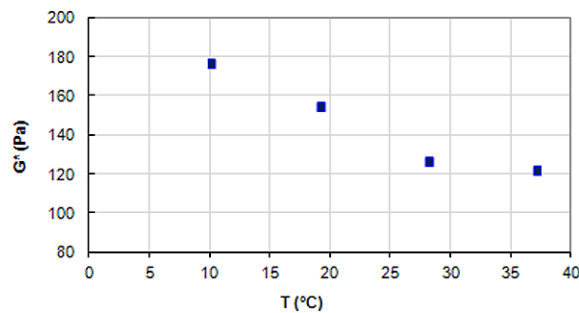
Fig. 3. Complex modulus of hyaluronic acid hydrogel F_{13} at $f = 0.1$ Hz. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BME-151557.)

In Fig. 3, are presented the complex modulus evolutions versus temperature ranging from 10°C and 37°C for the case of the hydrogel F_{13} at a frequency of 0.1 Hz which is associated to a time loading of 10 s. It can be noticed that G^* decreases when temperature is higher reflecting thus the softening of the hydrogel structure versus the thermal conditions. Furthermore the loss of the hydrogel rigidity recorded between 10°C and 37°C is about 31%.

From the plots of Fig. 4, it can be noticed that the different formulations exhibit nearly the same tendencies. First, one can distinguish a first phase for which the viscosity decreases in the range 0.1–1 Hz followed by a second longer phase ranging between 1 and 5 Hz for which the viscosity increases with the frequency. In our sense, the first phase can be associated to an accommodation stage for which the material starts to take place in the rheometer and memory the oscillatory solicitation. By analogy with the complex modulus, it can be observed the increase of the complex viscosity when the solicitation time decreases. Besides, the hydrogel F_{13} can be distinguished by the lowest viscosity comparing to the other hydrogels reflecting by this way a lower internal resistance to flow.

3.2. Cellular viability

The results obtained by Alamar Blue test are presented in Fig. 5. The values are given here in normalized form with respect to the results obtained at the 4th day which corresponds to the first day of the MSC culture. One can distinguish by the observation of the different curves a first step corresponding to the first week for which the cellular viability rate decreases for the nine hydrogels. This first step can be probably attributed to an accommodation phase of the MSC with the hydrogel environment. Note that the loss of viability is the lowest in the case of the hydrogel F_{13} where 80% of living cells are recorded after 7 days.

Then, a second phase can be observed for which the number of living MSC increases excepting for the cases of hydrogels F_{11}, F_{21} and F_{31} (bottom grey curves) characterized by a reticulation rate of 1/4 where a cell depletion can be observed. This finding indicates that the PEGDA reticulation of 1/4 does not provide the suitable environment for the 3D MSC culture and may be not sufficient to develop the required matrix rigidity of the MSC. On the contrary, the hydrogel F_{13} represents the best case with a great cellular vitality and seems to be the more suitable environment for the MSC encapsulation. Therefore, from a particular medical point of view, some conclusions can be drawn in the light of these findings since it can be possible to use the selected optimized hydrogel as an appropriate matrix for the preservation of MSC in order to treat the abdominal aortic aneurysm (AAA) therapy created by the rat xenograft model [18].
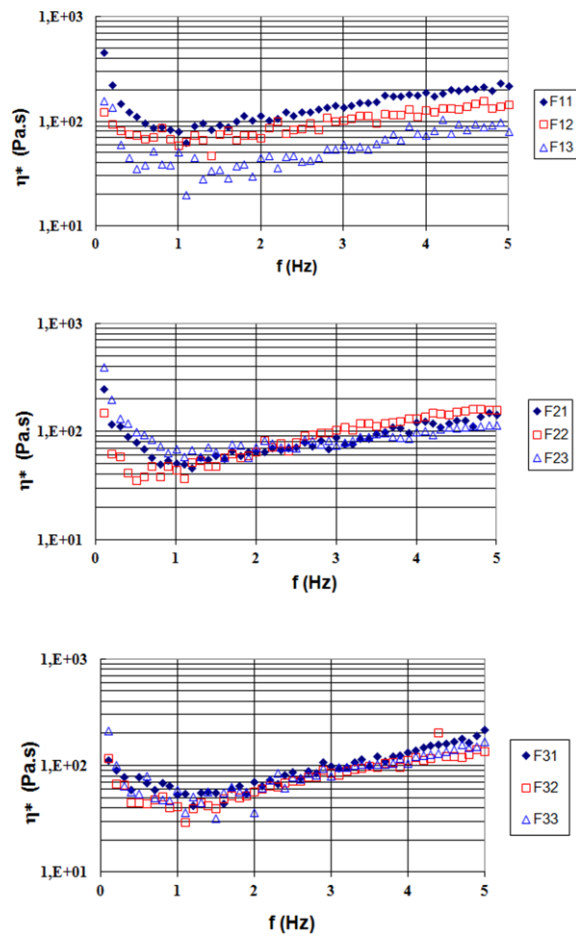
Fig. 4. Complex Viscosity of hyaluronic acid hydrogels at $T = 37°C$. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BME-151557.)
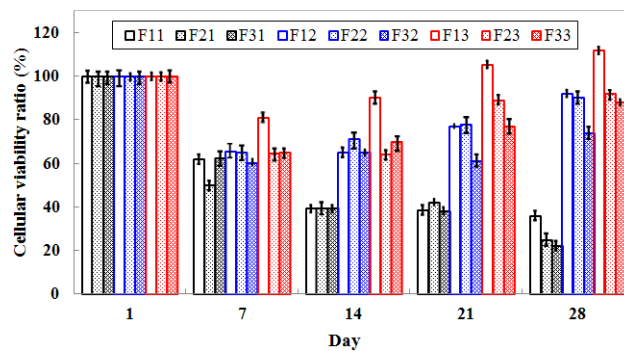

Fig. 5. Comparison of the cellular viability for the nine hydrogels. (Results presented in form of the average $\pm$ standard deviation of the average.) (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BME-151557.)
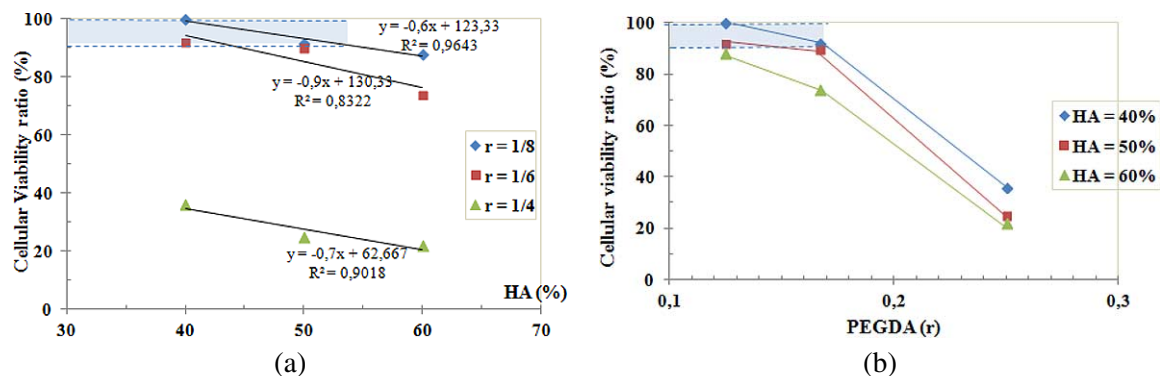
Fig. 6. Dependence of the cellular viability at 28 days on hydrogel composition: (a) with HA concentrations, (b) with PEGDA reticulation rates. (The continuous lines are referred to the tendency curves resulted from the data fitting.)

3.3. Relation hydrogel composition/cellular viability

The results of cellular viability test obtained at 28 days can be used to establish a relation between the MSC survey in a given hydrogel on the one hand, and its composition on the other hand. Such relations can be a useful tool for scientists, for instance, in order to identify a set of potential and good hydrogels for the MSC encapsulation in the light of their constituents. Figure 6(a) presents the evolution of the cellular viability of the cultured MSC versus the HA concentrations for the nine hydrogels. A similar tendency can be noticed for a given rate of PEGDA, highlighting a reduction of the cellular viability with increasing the HA concentration in the hydrogel. This reduction is highlighted when the PEGDA rate is equal to 1/4 which let deduce that this rate is not suitable for the MSC preservation in the associated hydrogel. However, the rates of PEGDA ranging between 1/8 a n d 1 /6 seem to be potential solutions for the composition of the hydrogel as highlighted by the blue colored region in this figue. The lower and upper bounds of this region correspond to target cellular viabilities respectively of 90% and 100%. The identification of the tendency curves by a least squares method allows the determination of the maximal HA concentrations in the hydrogel to ensure a cellular viability of at least 90%. For instance, maximal values for HA concentrations shall not exceed 55.5% and 44.8%, respectively when PEGDA rates are equal to 1/8 a n d 1 /6 in order to reach a cellular viability of 90% as a minimum.

3.4. Relation rheological properties/cellular viability

Figure 7 presents the relationship between the MSC viability and the rheological properties of the hydrogel in which they were encapsulated. Here, the loss modulus ($Tan(\delta)$) and the average complex viscosity (η^*_Moy) computed over the frequency range 0.1–5 Hz were considered. It can be pointed out from the upper Fig. 7(a) that the cellular viability follows a decreasing function of a quadratic form with regards to the loss modulus. This finding let suppose that predominately elastic hydrogels represent more suitable environments for the culture of MSC than the fluid-like ones. The dashed red lines are referred to the inverse method used for the computation of the potential range of the loss modulus in
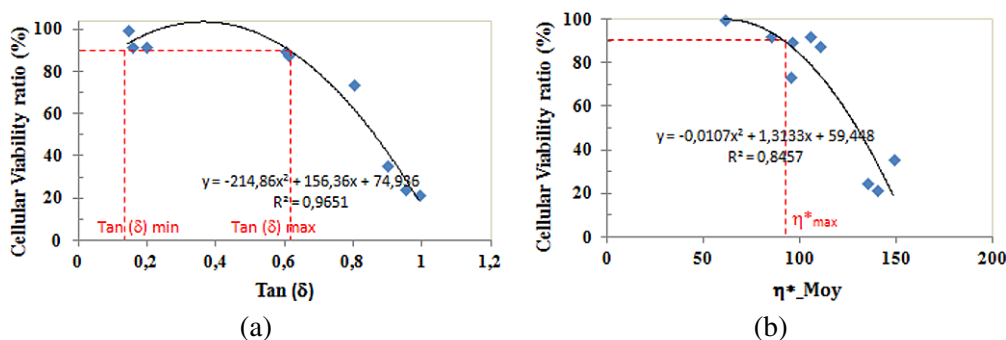
Fig. 7. Evolution of the cellular viability at 28 days versus the rheological properties of the hydrogels: (a) with loss modulus, at $T = 37°C$ and $f = 0.1$ Hz, (b) with the complex viscosity at $T = 37°C$. (The continuous lines are referred to the ten-dency curves resulted from the data fitting.)

which the cellular viability is higher than 90%. Therefore, the "best" Tan(δ) is shown to be comprised between 0.11 and 0.61.

The results plotted in the bottom curve of Fig. 7(b) provide a supplementary link between rheological and biological findings since a better viability is noticed for hydrogels with low viscosities. Accordingly, the less viscous hydrogels seem to be more favorable to allow the cell proliferation and therefore to preserve the MSC phenotype and viability. The threshold value of the complex viscosity for which a 90% of cellular viability is reached is equal to 91.5 Pa · s.

4. Limitations

Some highlights based on rheological and biological tests are presented in this study but several limitations can be noticed. In fact, the investigation of the hydrogel was limited to nine formulations with specific concentrations of HA, gelin and crosslinking agent PEGDA. Therefore, the drawn conclusions, relating rheological properties of hydrogel and composition to the biological properties, were based on this set of experiences. However, attention shall be also paid to other compositions and particularly to the cases for which the hyaluronic acid and PEGDA concentrations are respectively less than 40% and 1/8 in order to allow a broader view and understanding of the potential of hydrogel to preserve the MSC. Accordingly, the readers are cautioned that it may be not appropriate to use the aforementioned forward-looking relations for the prediction of the relationship between composition and cellular viability. Another limitation of this study could be the lack of full microstructure information about the different tested hydrogels which could have been useful to find out the potential relation between the hydrogel porosity and the cellular viability for instance. In addition, other parameters were not taken into account in the present study as the diffusion of serum and oxygen in the hydrogel to faithfully reproduce the *in vivo* environment of MSC. Thus, a comparative study with the MSC recovered directly from the extraction of the bone marrow without culturing must be carried out.

Despite these limitations, this work opens up interesting research tracks to improve our knowledge of the hydrogel biomaterial properties and offers challenging prospects for the development of stem-cell based therapies for the treatment of diseased or damaged tissues as for AAA in the xenograft rat model.

5. Conclusion

A combined rheological and biological study for the optimization of hyaluronic acid hydrogels used as a 3D structure for the culture of MSC was presented. The hydrogel formulations candidates were suggested using nine different concentrations of gelin and PEGDA reticulation rates. The viscoelastic properties of hydrogels gathered from complex modulus and complex viscosity measurements highlighted that the hydrogel F_{13} including 40% of HA and 1/8 of PEGDA is distinguished by the lowest structure rigidity and viscosity in comparison with the other hydrogels. On the other hand, the biological results of the cellular viability measurements proved that the hydrogel F_{13} exhibits the best viability rate recorded after 28 days of MSC culture. The correlation between rheological and biological properties allows the definition of a potential range of viscoelastic properties for hydrogels which can be considered as potential solutions for the MSC culture. Accordingly, for a given hydrogel, one can predict its potential to preserve the MSC by the only knowledge of its rheological properties, thus saving a great laboratory biological work. Alternatively, when the viscoelastic properties are not available but the hydrogel composition is known, one can make use of the aforementioned relationship linking the cellular viability of MSC on the one hand and their artificial niche composition one the other.

These different relations can provide a fast and efficient tool for engineers in order to optimize the design of the hydrogel biomaterial with regards to the wished viability of MSCs.

Thus, at this study stage, the optimal hydrogel formulation based on hyaluronic acid (HA) could be a good candidate in the vascular tissue repair protocol as in the xenograft rat model.

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