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# Is there a cause-and-effect relationship between physicochemical properties and cell behavior of alginate-based hydrogel obtained after sterilization?

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**Abstract:** Alginate-based hydrogel scaffolds are widely used in the field of cartilage regeneration and repair. If the effect of autoclaving on the alginate powder is well known, it is not the same for the possible effects of the sterilization UV treatment on the properties of the hydrogel after polymerization. To select an effective sterilization treatment of alginate-based materials, one must find what are inter-relationship between the characteristics (chemical, physical and mechanical) of alginate-based hydrogel during sterilization, and what consequences have affected on cell behavior. In this study, we investigated the influence of UV sterilization treatments (UV-1 and UV-2: 25 and 50 min, respectively) and autoclaving to obtain alginate (Alg) / hyaluronic acid (HA) hydrogel, as well as further evaluated the relationship between physicochemical properties and cell behavior of Alg/HA hydrogel after UVs and autoclaving. The physicochemical properties of this mixture at the powder or polymerized states were analyzed using ATR-FTIR, HPLC-SEC, rheological, indentation testing and sterility testing. The cell behaviors of hydrogels were evaluated by cell viability and proliferation, and chondrogenic differentiation. The effects of treatment parameters and their correlation with the others characteristics were determined statistically by Principal Component Analysis (PCA). In this study, we have shown that the cell behavior in alginate-based hydrogels was not only regulated by physicochemical properties (as molar mass or/and viscosity), but also associated with the controlling of sterilization time. It can provide a basis for choosing an effective method of sterilization, which can keep the mechanical or physical-chemical properties of Alg-based hydrogel scaffold and maintain its cytocompatibility and its ability to induce chondrogenesis from mesenchymal stem cells.

**Keywords:** Sterilization, alginate, hydrogel, autoclave, UV

## 1. Introduction

Articular cartilage has poor ability to self-repair, and damage due to tissue lesion, trauma, or natural degeneration. In order to repair the damaged cartilage, many researchers have promoted alginate-based scaffold to use in field of tissue-engineered cartilage due to it is easily to construct the hydrogels which support cell encapsulation and culture. Several studies have shown that alginate-based scaffold can promote mesenchymal stem cells (MSC) differentiation to chondrocyte phenotype, and synthesis of cartilage-specific matrix (Zhao et al., 2013; Van Vlierberghe et al., 2011; Sun and Tan et al., 2013; Xu et al., 2008; Reppel et al., 2005). Meanwhile, sterilized alginate-based composite powder is imposed. So the effective sterilization methods play an important role in the application of biomedical that not only eliminate the risk of infection, but also maintain structure, function and use of materials (Ahmed et al., 2013). However, it is reported that alginate is difficult to sterilize and to handle (Puppi et al., 2010). Even if Draget K L *et al*. (Draget et al., 1990) produced sterilized alginate by filtration *via* an appropriate submicron filter, it is not very convenient for highly viscous of alginate hydrogel. Generally, moist heat (Hu et al., 2014; Ofori-Kwakye and Martin, 2005), autoclaving (Leo et al., 1990; Vandenbossche et al., 1993; Ofori-Kwakye and Martin, 2005), ethylene oxide (Leo et al., 1990), gamma-irradiation (Lee et al., 2003) and UV (Mao et al., 2012) are used to sterilize the alginate-powder, but for both of them lead to the chain scission and hemolysis of alginate due to the high pressure and temperature which the process of it is called de-polymerization. However, among all these sterilization process, there is no statistic analysis that studies the inter-relationship between the physicochemical characteristics of alginate hydrogel after sterilization and cell behavior.

Alginate (Alg) could be obtained from brown algae and some soil bacteria. This is a linear polysaccharide containing blocks of (1,4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) residues which are believed to take part in intermolecular cross-linking with $Ca^{2+}$ to form hydrogels (Lee and Mooney et al., 2012). The structure of alginate hydrogel is similar to the glycosaminoglycan (GAG), which is long unbranched polysaccharides makes up the cartilage (Wang et al., 2009). Several studies reported that alginate-based hydrogel have a good biocompatibility, non-immunogenic and chondrogenic differentiation, when MSC were seeded into hydrogel (Xu et al., 2008; Follin et al., 2015; Guo et al., 2014; Du et al., 2016). Similarly, hyaluronic acid (HA) is a glycosaminoglycan consisting of D-glucuronic acid and D-N-acetylglucosamine units. It is an important component of proteoglycan organization which composed cartilage tissue. Meanwhile, it can contribute to the function of the tissue itself (Collins et al., 2013). It is considered as a chondrogenesis inductor of MSC (Chung and Burdick, 2009). Borzacchiello et al. (Borzacchiello et al., 2015) made the HA hydrogel through the cross-linking HA molecules with divinyl sulfone (DVS) and have shown it has a good biocompatibility, mechanical and injectability properties suited to biomedical application.

So, both alginate and HA natural polymers exhibit properties of biocompatibility, biodegradability, non-immunogenicity and low cost. They can be an attractive hydrogel candidate for cartilage tissue engineering. In our work, we planned to combine the alginate with hyaluronic acid as raw materials to form hydrogel scaffold. Even if the effects of autoclaving on alginate powder properties are known, very few is know about the effect of UV as sterilization treatments on alginate-based composite powder properties and on its capacity to maintain chondrogenic differentiation *in vitro*. Meanwhile, our ultimate goal was to estimate the correlation between physicochemical properties and cell behavior of alginate-based hydrogel during sterilization.

## 2. Materials and methods

### 2.1. Sterilization treatments of powder

The powder of 1.5% (m/v) alginate (medium viscosity, Sigma-Aldrich, France) and hyaluronic acid (Acros organics, USA) (ratio 4:1) was sterilized by UV-1 treatment (ultraviolet during 25 min), UV-2 treatment (ultraviolet during 25 min and was turned over during 25 min) (Ultraviolet lamp = 2×15 w, wavelength= 254 nm, Distance = 30 cm, Vilber lourmat, France), autoclaving at 121°C for 30 min (Advantage-Lab, AL02-07-100, France), non-sterilized (without any treatments for the powder, as to be control). Then, they were homogeneously dissolved in 0.9% sodium chloride (Merck, Germany) to form gel solution by constant stirring in a sterile glass tube.

### 2.2. Hydrogel scaffold construct, cell culture and chondrogenic differentiation

To prepare gel solution as we described above. A syringe was used to absorb the gel solution before being dropped in a 102 mmol/L $CaCl_2$ (Sigma-Aldrich, France) solution for 10 min, forming spherical beads (Alg/HA) completely. Then the hydrogel beads were washed three times by 0.9% NaCl solution, which was further studied. In addition, we used the spraying method to make the hydrogel scaffold which was previously described with cells (Tritz et al., 2010; Tritz-Schiavi et al., 2010). The spraying system is composed of an airbrush working with a compressor (Mjahed et al., 2008). The spraying bottle containing the gel solution was connected to the airbrush, and then solution was sprayed on a sterile glass plate with the spraying pressure is equal to 0.9 bar, which was quickly and horizontally putted into a bath of 102 mM $CaCl_2$ for 10 minutes, forming the 3D-Alg/HA hydrogel scaffold. And then scaffolds were washed two times with a 0.9% NaCl solution. These scaffold without cells were kept in 1 mM of $CaCl_2$ at room temperature before Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) analysis and mechanical testing.

Wharton's jelly mesenchymal stem cells (WJ-MSC) were isolated by the explant methods from the umbilical cord as previously described (Reppel et al., 2014). Briefly, human umbilical cords were collected after patents' informed consent which was regarded as surgical waste, as well as on the basis

of the guidelines for the care and use of an ethic committee of Nancy Hospital. Umbilical cords were washed with 70% ethanol and Hanks' balanced salt solution (HBSS). The umbilical cord vessels were removed and Wharton's jelly aseptically cut into very small pieces which were cultured in a six-well plate with complete medium (Alpha Modified Eagle Medium, α-MEM, Lonza, belgium) with 10% Fetal bovine serum (Sigma-Aldrich, France), 100 IU/mL penicillin (Gibco, France), 100 µg/mL streptomycin (Gibco, France), 2 mM L-glucose (Sigma-Aldrich, France), and 2.5 µg/mL amphotericin B (Gibco, France). They were incubated at 37℃ in 5% $CO_2$, 5% $O_2$ incubator. After 7 days, cells have migrated from Wharton's jelly and adhered on plate. Then, Wharton's jelly pieces have been removed and culture medium was changed twice a week until cell subconfluence (80%). After 2 weeks, WJ-MSC were harvested with 0.25% Trypsin-EDTA (Sigma-Aldrich, France) and seeded in a new culture flask with a density 1000 cells/cm². Media were changed three times weekly. This process was repeated until culture up to the third passage. For viability, proliferation and chondrogenic differentiation analysis, WJ-MSC were encapsulated into Alg/HA hydrogel beads, manufactured as previously described (Wang et al., 2008). Scaffolds were cultivated in 6-well plates with induced chondrogenic culture medium which contained DMEM-high glucose (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 1 mM $CaCl_2$, 0.1 µM dexamethasone (Sigma-Aldrich, France), 50 µg/mL ascorbate-2-phosphate (Sigma-Aldrich, France), 100 µg/mL sodium pyruvate (Sigma-Aldrich, France), and 40 µg/mL L-proline (Sigma-Aldrich, France). Scaffolds were incubated at 37℃ in a humidified incubator with 5% $CO_2$, 5% $O_2$ for 30 days. The differentiation medium was changed three times weekly.

### 2.3. Chemical properties of hydrogel scaffold

#### *2.3.1. Fourier Transform infrared (FTIR) spectroscopy*

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectra were recorded on a Fourier transform infrared spectrometer (170SX, Nicolet Co., Madison, WI, USA) to analyse the chemistry of prepared 3D-Alg/HA hydrogel scaffolds during these sterilization methods. Each sample was dried on vacuum desiccator at 60℃ for 24 h, then cut into small pieces and moved onto ATR crystal, which were measured with a FTIR spectrometer. The infrared spectra of the raw materials (dry alginate and hyaluronic acid powders) were also respectively measured with a FTIR spectrometer; the spectra were collected over range of 4000-500 $cm^{-1}$.

#### *2.3.2. Molecular weights evolution*

Size Exclusion Chromatography (SEC) was performed at room temperature to quantify some possible degradation of Alg/HA during treatments. SEC was performed using a Waters HPLC pump equipped

with a serial set of SP 806, 805 and 804 OH pack columns (Shodex). Elution was carried out with 0.1 M $NaNO_3$ containing $NaN_3$ as a bactericide (0.4 g/L) at 0.7 mL/min, and was monitored by Multi Angle Laser Light Scattering detector (MALLS Mini Damn, Wyatt) and differential refractometry (Waters 410) dual detection.

Solution (2 mg/mL) were prepared by dissolution in the same eluent and left under vigorous stirring for 24 h. Filtration of solutions was carried out right before injection, in order to get rid of possible aggregates. Refractive index increments (dn / dc) equal to 0.154 mL/g and 0.145 mL/g were measured in the same eluent for alginate and hyaluronic acid, respectively. dn / dc used for Alg/HA (ratio 4/1) blend was estimated by the following equation:

$(dn / dc)_{Alg/HA} = 0.8\ (dn / dc)_{Alg} + 0.2\ (dn / dc)_{HA} = 0.1522$

**2.4. Physical properties of hydrogel scaffold**

***2.4.1. Viscosity characterization***

The shear viscosity of Alg/HA hydrogel has been measured after treatments of autoclave, UV-1, UV-2 and control (hydrogel obtained from a no sterilized Alg/HA powder). Sample was sheared in between a cone and a plate geometry (diameter = 40 mm, angle = 2°) mounted on a controlled stress rheometer DHR-3 (TA Instrument). The preparation of Alg/HA gel was manufactured as previously described and all the measurements are done at a temperature of 20°C. In order to measure the steady-state shear viscosity we performed the following protocol: shear rate $\dot{\varepsilon}$ ($s^{-1}$) was increased step by step from 0.1 to 100 $s^{-1}$ with a maximum time of 180 s at each shearing point and the shear stress $\tau$ (Pa) has been recorded. This protocol ensures that transients have disappeared. The shear viscosity $\eta$ (Pa. $s^{-1}$) is defined by the ratio between the shear stress and shear rate: $\eta = \tau / \dot{\varepsilon}$ (De Kee et al., 1994).

***2.4.2. Mechanical testing***

The mechanical behavior of 3D-Alg/HA hydrogel scaffolds were characterized for various sterilization treatments using the spherical indentation technique as the following figure 1.A-B. The tests were performed on a Zwick/Roll machine, with 5.5 mm diameter steel ball. The indenter was introduced onto the surface of the hydrogel scaffold at different velocities varying from 0.25 to 5 mm/min. Three force-displacement curves were obtained for all velocities and treatments. The effects of the sterilization treatments had been evidenced using the force response corresponding to an indenter displacement of 0.35 mm for all specimens whose thicknesses were between 2 and 3 mm. At the same time, it has been clearly shown that the gel exhibits a well-known viscoelastic mechanical behavior.

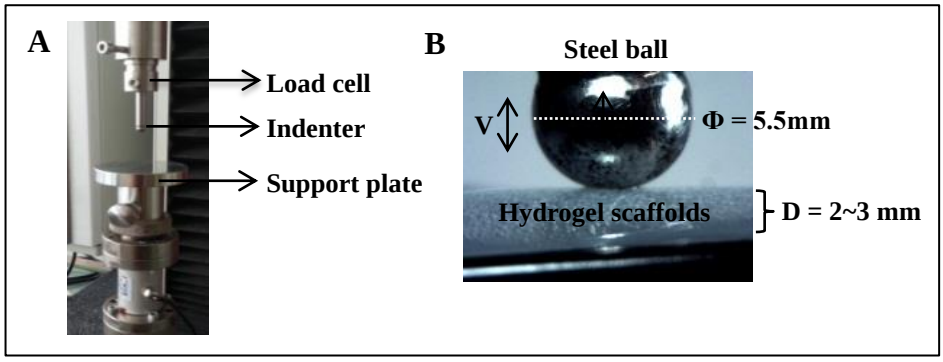



**Fig.1** (A) Apparatus for indentation testing and (B) the illustration of using 5.5 mm diameter spherical indenter attached on the surface of 3D-Alg/HA hydrogel scaffolds, which an indenter with a displacement of 0.35 mm at different velocities.

### 2.5. Sterility testing

Hydrogel beads ($n \geq 8$) without cells were immersed in Fluid Thioglycollate medium (FTM) (Sigma-Aldrich, France) which was designed to test the growth of bacteria and in Sabouraud 2% Glucose Agar (SGA) (Sigma-Aldrich, France) for fungal cultivation for a period of 7 days at 37 °C. Culture mediums alone served as negative control whereas unsterilized samples served as positive control. Every 2 days, we examined the clouding of the FTM and the appearance of the colonies in the Sabouraud agar indicated contamination and inefficient sterilization.

### 2.6. Biocompatibility and histological

#### *2.6.1. Cell viability assessment*

To assess the viability of encapsulated cells the PI/Annexin-V kit (Life technologies, France) was used. Cells were extracted from Alg/HA hydrogel beads using 55 mM sodium citrate (Sigma-Aldrich, France) and 50 mM EDTA solution (Merck, Darmstadt, Germany) after 3, 5, 10, 20 and 30 days of culture. After centrifugation (320 g, 5 min), cells were suspended in 100 µL of 1X Annexin-liant buffer with 2.5 µL of Annexin V-Alexa 488 and 1 µL of propidium iodide (PI) for 15 min at the room temperature. Cells were detected with fluorescence emission at 530 nm and 575 nm by Gallios flow cytometer (Beckman Coulter, Brea, CA, USA).

#### *2.6.2. Cell proliferation analysis*

The proliferation of encapsulated cells in the Alg/HA hydrogel beads was evaluated by using the Alamar Blue™ assay Kit (Thermo scientific, France) for 3, 5, 10, 20 and 30 days. The samples were washed twice with PBS in culture plate, and then Alamar Blue solution was added (10% v/v of DMEM without phenol red) for 4 hours at 37 ℃ in a humidified incubator with 5% $CO_2$, 5% $O_2$. The reaction of

Alamar Blue was read in a microplate reader at 570 nm and 600 nm. To calculate the percentage reduction of Alamar Blue™ as following equation:

$$\% \text{ reduction} = \frac{(OD570_{(control)} \times 155677) - (OD600_{(control)} \times 14652)}{(OD570_{(sample)} \times 117216) - (OD600_{(sample)} \times 80586)} \times 100$$

The absorption value of negative control is culture medium and Alamr Blue™. The experiment was performed in quadruplicate.

#### *2.6.3. Histochemical staining*

It was performed after 30 days of differentiation of WJ-MSC seeded into each kind of hydrogel bead. Samples were washed with PBS, then kept in the 4% paraformaldehyde (Sigma-Aldrich, France) for 4 hours, and lastly washed overnight in 100 mM sodium cacodylate (Sigma) 10 mM $CaCl_2$ and 50 mM $BaCl_2$ (Sigma-Aldrich, France) buffer. The scaffolds were dehydrated, after embedded in paraffin blocks. Samples were cut into serials sections (5µm) which was stained with Hematoxylin Eosin Saffron (HES), Sirius red and Alcian blue for cell morphology, total collagen and sulfated proteoglycans (GAG), respectively. Microphotographs were taken using a light microscope (LEICA, DMD108, USA). Image analysis was performed using free software Image J 1.50i, and the percentage stain area was estimated using the following relation: sem percentage= (sem / mean) × 100 (Goldring et al., 2006). Where sem is the standard error mean for the measured parameter.

### 2.7. PCA component analysis

The effects of treatment parameters and their correlation with the others characteristics were determined statistically by means of the Principal Component Analysis method (PCA), which through the XLSTAT software (Addinsoft, New York, USA).

### 2.8. Statistical analysis

Data are reported and presented as the mean ± standard deviation (SD) ($N \geq 3$) and were statistically analyzed by one- or two-way ANOVA (analysis of variance) using GraphPad prism 6 software (GraphPad, San Diego, CA, USA). A value of $p$ less than 0.05 were considered to be significant. If significance existed, a post-hoc analysis was performed using the Bonferonni post-tests to evaluate significance for all experiments.

## 3. Results and discussion

### 3.1. FTIR spectra

The FTIR spectra of the Alg powder, HA powder and Alg/HA hydrogel scaffolds are shown in Fig. 2A. For the pure alginate, peaks at 3444, 1030, 1618 and 1416 $cm^{-1}$ were attributed to the stretching of -OH groups, the stretching of C-O-C bonds, the asymmetric and symmetric stretching vibrations of $CO_2^-$,

respectively (Daemi and Barikani, 2012). In case of HA spectrum, peaks at 3444, 1041, 1627 and 1411 $cm^{-1}$ were assigned respectively to the stretching of -OH groups, the stretching of C-O-C bonds, the asymmetric and symmetric stretching vibrations of C=O of amide I functions (Wu, 2012). From the ATR-FTIR spectrum of Alg/HA hydrogel scaffolds, we can observe the asymmetric stretching of C=O bond shifted to 1630 $cm^{-1}$ and symmetric stretching of C=O bond shifted to 1424 $cm^{-1}$. In addition, the bands of –OH groups shifted from 3444 $cm^{-1}$ to 3435 $cm^{-1}$. The representative spectra of Alg/HA hydrogel under different sterilization treatments were shown in Fig. 2B. In case of Alg/HA hydrogel, peaks characteristic of carboxylate and amide I groups appeared in the same range. Most important is that no significant shift of characteristic FTIR peaks of Alg/HA hydrogel was observed under different sterilization treatments, which indicated that these sterilization methods have no effect on the chemical functional groups of them. As Mao S *et al* (Mao et al., 2012) demonstrated using UV spectroscopy there was no change in chemical structure of the alginate during de-polymerization process. In addition, we analysis the FTIR peaks of originally pure Alg powders and pure HA powders under treatments of autoclave, UV-1, UV-2 and control, which also further prove without chemical groups changing. These results are shown in supplemental figure (Fig. S1A&1B).

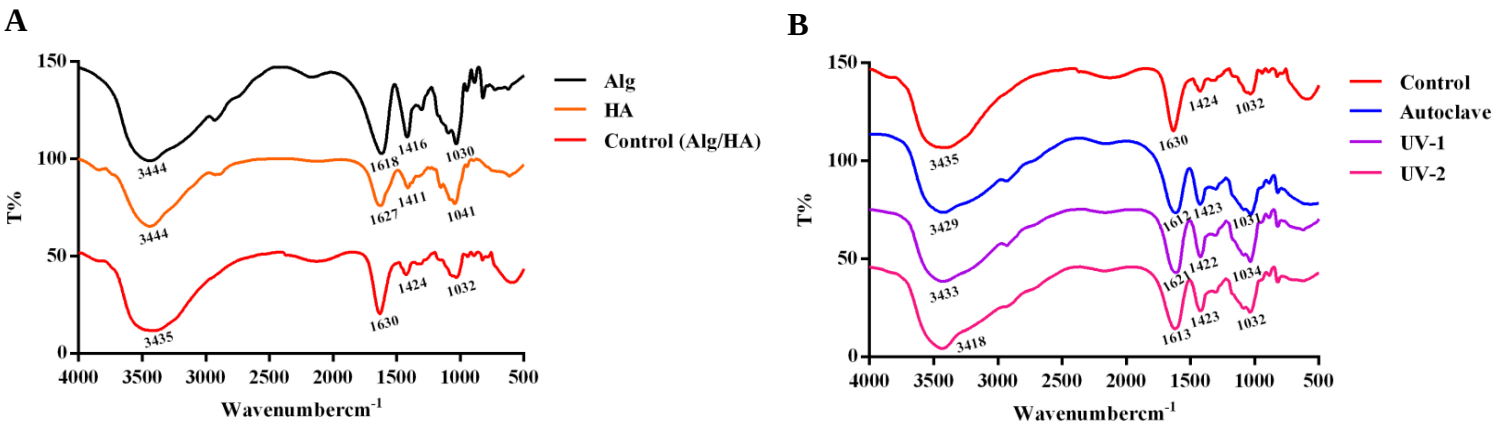



**Fig.2** (A): FTIR spectra of Alg powder, HA powder and Alg/HA hydrogel scaffolds; (B): FTIR spectra of Alg/HA hydrogel scaffolds under exposure to the sterilization methods.

### 3.2. Analysis of molar masses

Table 1 shows the result of weight average molecular weight ($\overline{M_w}$) and number average molecular weight ($\overline{M_n}$) of alginate (as supplemental table. S1) and Alg/HA chains under various sterilization treatments. Here, we found that each sterilization treatment (UV-1, UV-2, and autoclave) led to the degradation of alginate chains even in the Alg/HA blend. In comparison to control, $\overline{M_n}$ and $\overline{M_w}$ for Alg/HA under the treatment of autoclave diminished by about 63% and 52%, respectively, that is much

more pronounced that under other treatments (UV-1 and UV-2). Otherwise, the value of $\overline{M_n}$ and $\overline{M_w}$ for Alg/HA under the treatments of UV-1 and UV-2 are very nearly. UV-1 and UV-2 sterilization treatments led to a much less degradation of Alg/HA chains. This phenomenon can be attributed to the absorption of UV energy due to polysaccharide. Since, the formation of free radical through UV light may cleave covalent bonds, which can initiate de-polymerization reaction (Wasikiewicz et al., 2005). While autoclave has more deleterious effect, this is due to the de-polymerization which leads to the breakdown of the main chain backbone of alginate. In conclusion, the autoclave treatment caused an important reduction in $\overline{M_n}$ and $\overline{M_w}$ as compared with control. However, the molecular weight is the most important property of the polymers in the sense that it also governs their mechanical properties, which will be discussed later.

**Table 1** Number average molecular weight ($\overline{\mathbf{M_n}}$), weight average molecular weight ($\overline{\mathbf{M_w}}$) and dispersity (D = ($\overline{\mathbf{M_w}}/\overline{\mathbf{M_n}}$) for Alg/HA under various sterilization methods.

| Samples + treatments | $\overline{M_n}$×105 (g/mol) | $\overline{M_w}$×105 (g/mol) | D |
|---|---|---|---|
| Alg/HA + Control | 4,15 | 4.96 | 1.19 |
| Alg/HA + Autoclave | 1,54 | 2,39 | 1.55 |
| Alg/HA + UV-1 | 2,76 | 3,78 | 1.37 |
| Alg/HA + UV-2 | 2,31 | 3,44 | 1.49 |

### 3.3. Rheological characterization

The shear viscosity of Alg/HA in gelling state is plotted on Fig. 3 for the three sterilization protocols as for the control sample. Compared to the control samples, the viscosity after UV treatments is roughly decreased, but the fluid keeps its shear thinning behavior, i.e. the shear viscosity decreases with the increasing shear rate. The viscosity after autoclave treatment is strongly decreased by one order of magnitude and the shear thinning behaviour is lost. These results are confirmed by the previous study (Lee et al., 2012), which showed that the alginate polymer exhibits a reduction of its viscosity. These methods lead to the alginate chain scission (linked by β (1→4) glycosidic bonds) and hemolysis (de-polymerization).

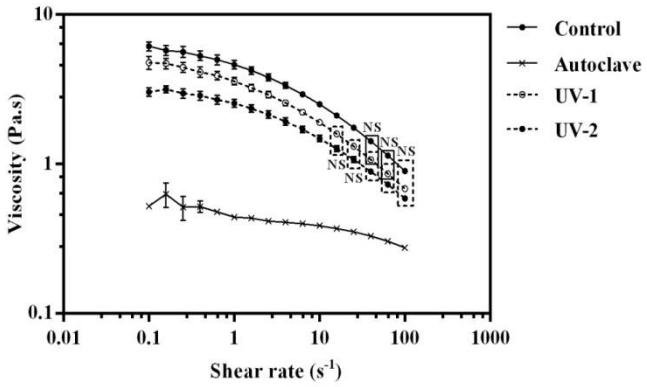


**Fig.3** Shear viscosity of Alg/HA in gelling state under the sterilization treatments (autoclave, UV-1, UV-2 and control) as a function of the shear rate. Control correspond to a hydrogel obtained from a no sterilized Alg/HA powder. The result is expressed as mean ± SD (n =3). No Significant (NS, $p > 0.05$ for UV-1 *vs* UV-2 from 10 $s^{-1}$ to 100 $s^{-1}$; as $\dot{\varepsilon}$=100 $s^{-1}$, control *vs* UV-2, control *vs* UV-1 and UV-1 *vs* UV-2)

### 3.4. Indentation character of hydrogel scaffolds

The hydrogel scaffolds are fairly soft viscoelastic media that require appropriate mechanical characteristics techniques to be investigated. That's why we selected the indentation technique, which is often used for living and synthetic soft materials. This mechanical test can be done at different scales (nano by AFM, micro or macro) and presents the advantage of being fast and non-destructive (Merino et al., 1991; Oyen, 2014; Ahearne et al., 2005), although the strain and stress fields are not uniform. In the present study, we used it to compared the effects of treatment without explicity characterize the mechanical behaviour. Note that the determination of the mechanical behaviour of the hydrogel could only be done by a numerical method such as the finite element method. The main results are given as a function of sterilization treatments in Fig.4. It illustrates the force-velocity curves of the compression force of 3D-Alg/HA hydrogel scaffolds under these sterilization methods (control, UV-1, UV-2, and autoclave). As mentioned above, we found the mechanical strength (force measured at 0.35 mm indenter displacement). This strain rate sensitivity is significantly lower for autoclave comparing to the other treatments ($p < 0.05$). Moreover, a significant difference in the strength may be observed after UV-2 and autoclave when compared to control. On the other hand, there is not statistically significant difference between UV treatments and control, as well as the value of force for hydrogel under the treatment of UV-2 is almost same to the control at the each velocity.

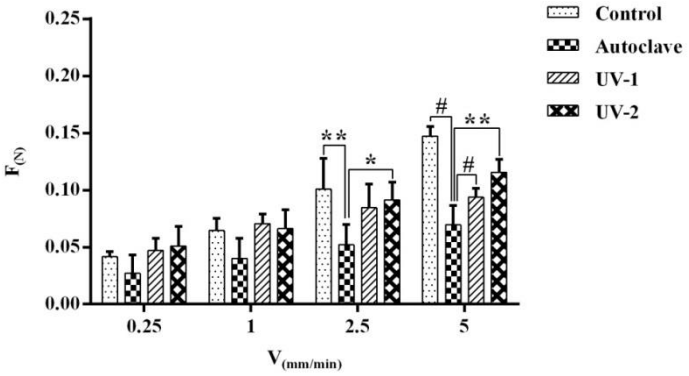



**Fig.4** Typical force-velocity profile of Alg/HA hydrogel scaffolds through the testing of indentation, Date are presented as mean ± SD with n ≥3, analysis of variance (ANOVA). (V= 2.5mm/min, *$p < 0.05$, UV-2 *vs* autoclave; **$p < 0.01$ control *vs* autoclave; and V= 5mm/min, $^{\#}p < 0.001$, control *vs* autoclave, UV-1 *vs* autoclave ; **$p <$ 0.01, UV-2 *vs* autoclave).

### 3.5. Sterilization testing

The efficiency of sterilization approaches on the Alg/HA hydrogel beads without cells were investigated by FTM and SGA from 1 to 7 days to detect the growth of bacteria or funga, respectively. The results are shown in Table 2 and Fig. 5A-B. After 3 days of culture, there is presence of clouding of the thioglycollate broth mediums with hydrogel scaffolds by UV-1 treatment, which indicated that obligate anaerobes grew in the lower areas of the tube. Otherwise, until 7 days, we found that there is no clouding of the thioglycollate broth in the medium for hydrogel scaffolds sterilized by UV-2 and autoclave treatments. Meanwhile, for all of treatment methods, there is no appearance of colonies in the Sabouraud 2% Glucose agar until 7 days of culture while for positive control, we observed colonies at 7 days. In conclusion, autoclaving and UV-2 treatment keep hydrogel beads from contamination, which can protect them infection from bacterial and fungal. These results are in agreement with De Moraes MA et al. study on silk fiber membranes (Moraes et al., 2014). Moreover, our results showed that UV-radiation for 25 min was not enough to eliminate microorganisms for hydrogel scaffold. It also has an effect on cell behavior of Alg/HA hydrogel scaffolds, which it will be shown later.

**Table 2** Efficiency of sterilization approaches on Alg/HA hydrogels. "+" present contamination of FTM medium or in SGA, "-" present non-pollution when the samples were immersed into the FTM medium or in SGA. (Negative control: culture medium; Positive control: unsterilized sample)

| | Incubation time (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Thioglycollate broth | | | | Sabouraud 2% Glucose Agar | | | |
| Treatments | 1 | 3 | 5 | 7 | 1 | 3 | 5 | 7 |
| Autoclave | - | - | - | - | - | - | - | - |
| UV-1 | - | + | + | + | - | - | - | - |
| UV-2 | - | - | - | - | - | - | - | - |
| Positive control | + | + | + | + | + | + | + | + |
| Negative control | - | - | - | - | - | - | - | - |

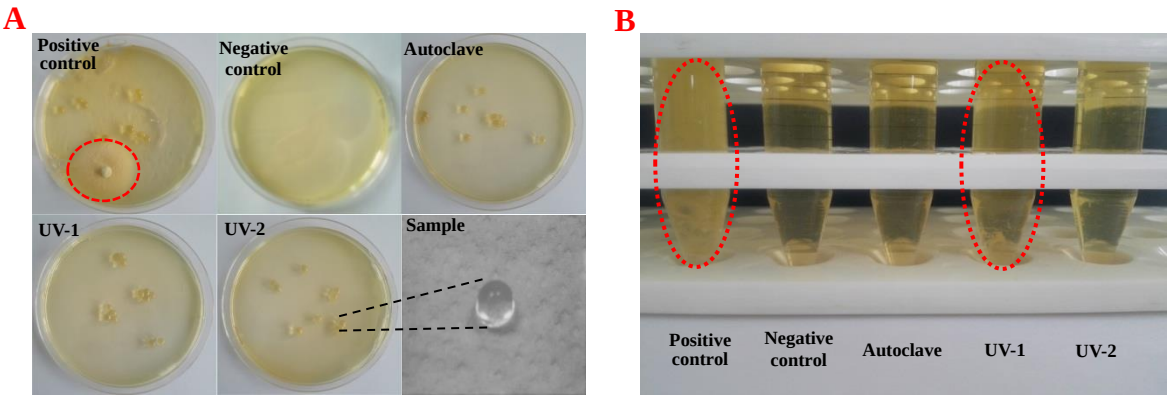


**Fig.5** Efficiency of sterilization treatments on the Alg/HA hydrogel scaffold at 7 days. (fig.5A: SGA, there is appearance of white colonies in positive control; fig.5B: FTM, there are appearance of phenomena of white haze or flocculent precipitate in positive control and UV-1 treatment)

### 3.6. Viability and proliferation of WJ-MSC encapsulated into hydrogel beads

Fig. 6A shows viability of cells encapsulated into hydrogels under three kinds of sterilization treatments (autoclave, UV-1 and UV-2). At the beginning of culture, we found that cell viability in monolayer was significantly higher than other treatments ($^{++++}P < 0.0001$). Meanwhile, it was observed that cell viability increased with the days of culture except for UV-1. After 3 days of culture, the proportion of viable cells after autoclave, UV-1 and UV-2 treatments, were 64.3 ± 1.4%, 50.2 ± 0.2% and 55.3 ± 1.2%, respectively. On D30, cell viability reached 81.3 ± 3.9% for autoclaved samples and 89.3 ± 5.5% for UV-2 treatment. These viability rates were close to those observed during monolayer expansion ($P > 0.05$). In contrast, the cell viability of UV-1 samples always kept lower proportion as compared with others ($^{\#}P< 0.0001$) or monolayer ($^{+++}P< 0.001$). Fig. 6B shows the viable/necrotic/apoptotic of cells seeded in Alg/HA hydrogel scaffolds after 30 days of culture. It exhibited that the proportion of viable cells were 73.4% (autoclave), 47.7% (UV-1), 94.8% (UV-2) and 99.9% (monolayer cells). As well as, the percentages of necrotic and apoptotic cell seeded in hydrogels sterilized by UV-1 treatment are much more than the others maintained in hydrogels sterilized by UV-2 and autoclave treatment. Fig. 6C presents Alamar Blue reduction of encapsulated cells culture in Alg/HA hydrogels. It was found that the Alamar Blue reduction of cells in the hydrogel scaffolds increased for the treatment method of UV-2 and autoclave from 5 days to 30 days, with an increase about 2 fold between UV-2 and autoclave on D 3 *vs*. D 30 of culture. Moreover, the results obtained in UV-2 and autoclave are always higher than UV-1 ($p < 0.001$ from D 5). All these results are correlated with the sterilization efficiency. In fact, Stoppel WL *et al* (Stoppel et al., 2014) showed that UV method during 20 min is not enough to eliminate bacterial persistence of hydrogel, but the long duration of UV treatment (25+25 min) seems to be beneficial for living cells-laden Alg/HA hydrogel scaffolds. The authors agree on the fact that the sterilization time is a prime parameter in clinical medicine (Braghirolli et al., 2014).

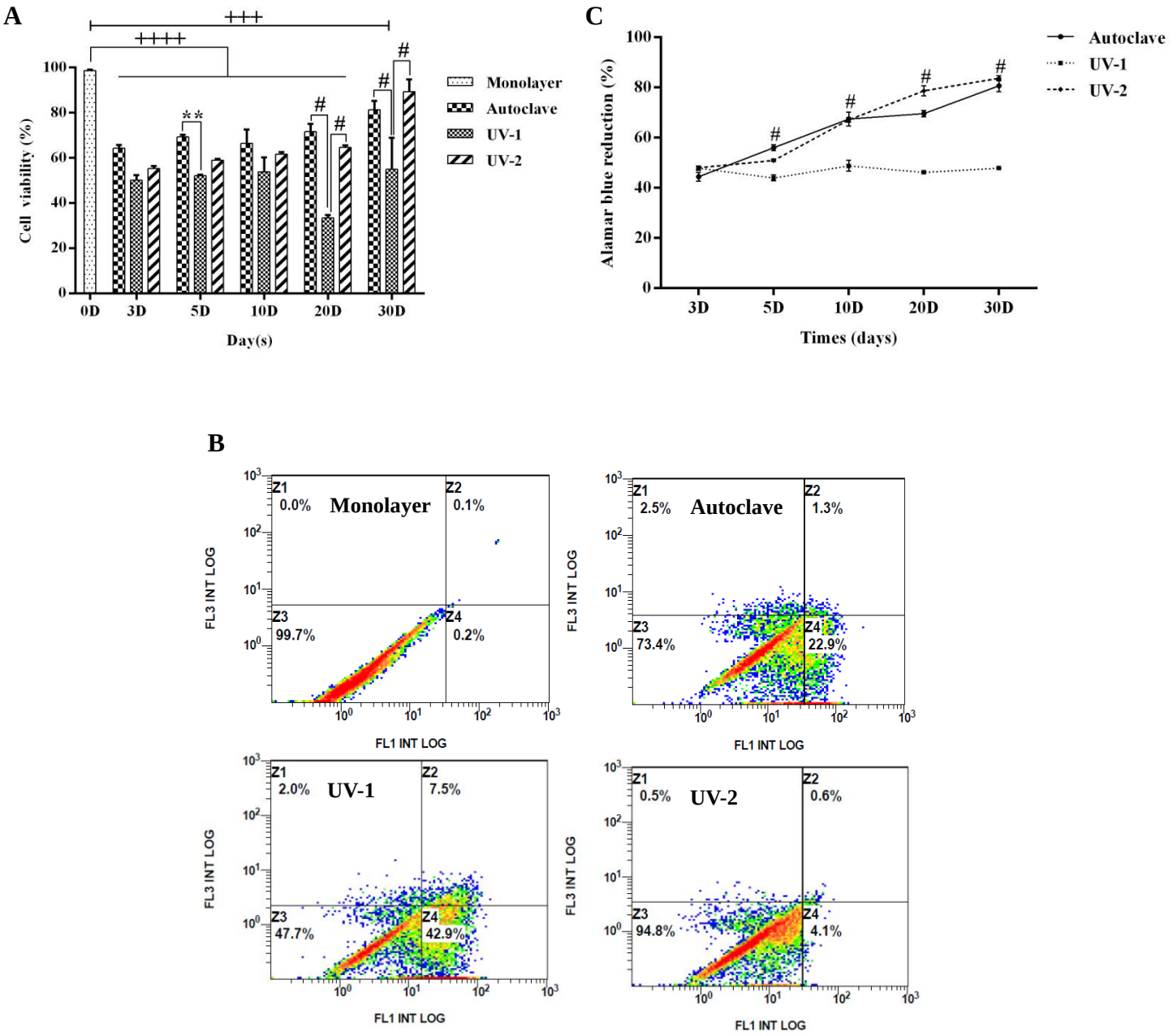
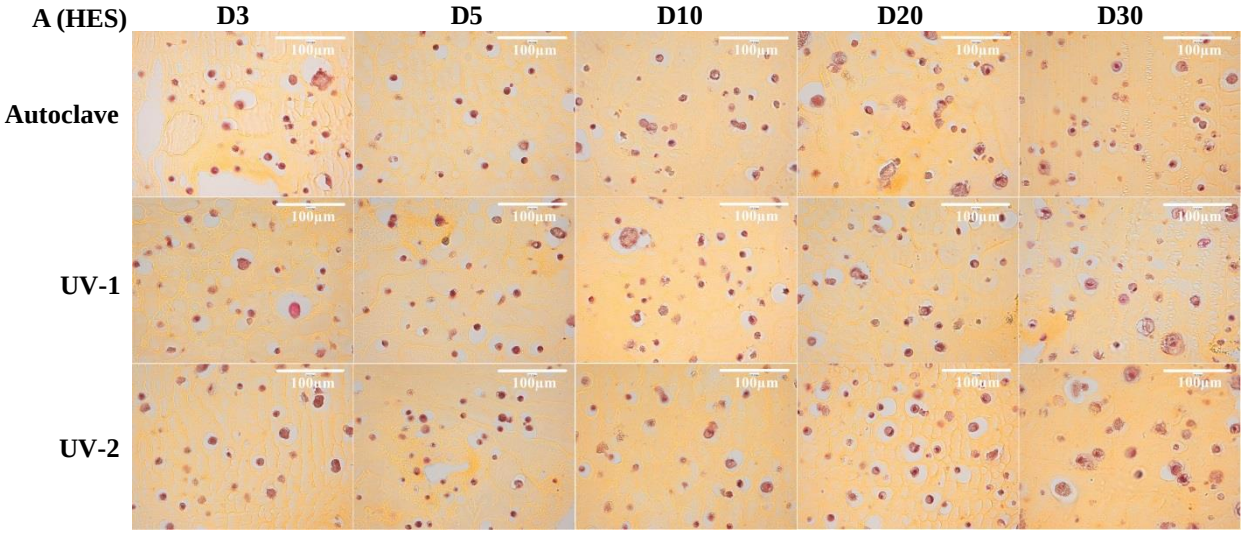



**Fig.6** The viability (A) of encapsulated cells in Alg/HA hydrogel scaffolds was measured by flow cytometry after 3, 5, 10, 20, and 30 days of culture; (B) Flow cytometry analysis of viable/necrotic/apoptotic cells seeded in hydrogel scaffolds at 30 days and (C) the Alamar Blue reduction of encapsulated WJ-MSC culture in Alg/HA hydrogel scaffold by Alamar Blue assay after 3, 5, 10, 20 and 30 days. * or # represent a significant difference in each group (Two-way ANOVA, $^{++++}p < 0.0001$, day x *vs* day 0 (monolayer) for the any treatment; $^{+++}p < 0.001$, day 30 *vs* day 0 for UV-1 treatment; $^{**}p < 0.01$ and $^{\#}p < 0.0001$, autoclave *vs* UV-1, UV-2 *vs* UV-1, autoclave *vs* UV-1 for the same culture time, bars represent the standard deviation, N=3 (viability), N= 4 (Alamar Blue).

### 3.7. Chondrocytes differentiation of WJ-MSC seeded into hydrogel scaffolds

After 3, 5, 10, 20 and 30 days of culture of WJ-MSC seeded into Alg/HA hydrogel scaffolds, we evaluated the cells organization, cells morphology, GAG and collagen synthesis by histology. Fig. 7A has shown the results obtained from HES coloration. Whatever the sterilization treatments, we found that the repartition of WJ-MSC is uniform into the scaffold and that the cell morphology keeps a round shape. Detected GAG (Fig. 7B) and collagen (Fig. 7C), respectively by Alcian blue and Sirius red, gradually increased with culture time. Moreover, GAG and collagen synthesis seemed to be greater in hydrogel scaffolds whose powder was sterilized by UV-2 and autoclave compared to hydrogel scaffolds whose powder was sterilized by UV-1. In particular, hydrogel scaffolds whose powder was sterilized by UV-2 stained much more for GAG and collagen than the others treatments after 30 days of culture. Furthermore, after 30 days of chondrogenic differentiation, we measured the percentage of positive stain using ImageJ analysis. The results showed that UV-2 treatment has a significant higher percentage of positive area for collagen (Fig.8A) as compared with the others treatments ($P < 0.001$). As well as, for the synthesis of GAG (Fig.8B), highly significant differences were also observed between UV-2 and UV-1 treatment ($^{**}P < 0.01$). From the results above, we can conclude that hydrogel scaffolds with UV-2 treatments may be a good alternative to ensure the cell viability and chondrocyte differentiation of the WJ-MSCs-laden hydrogel.



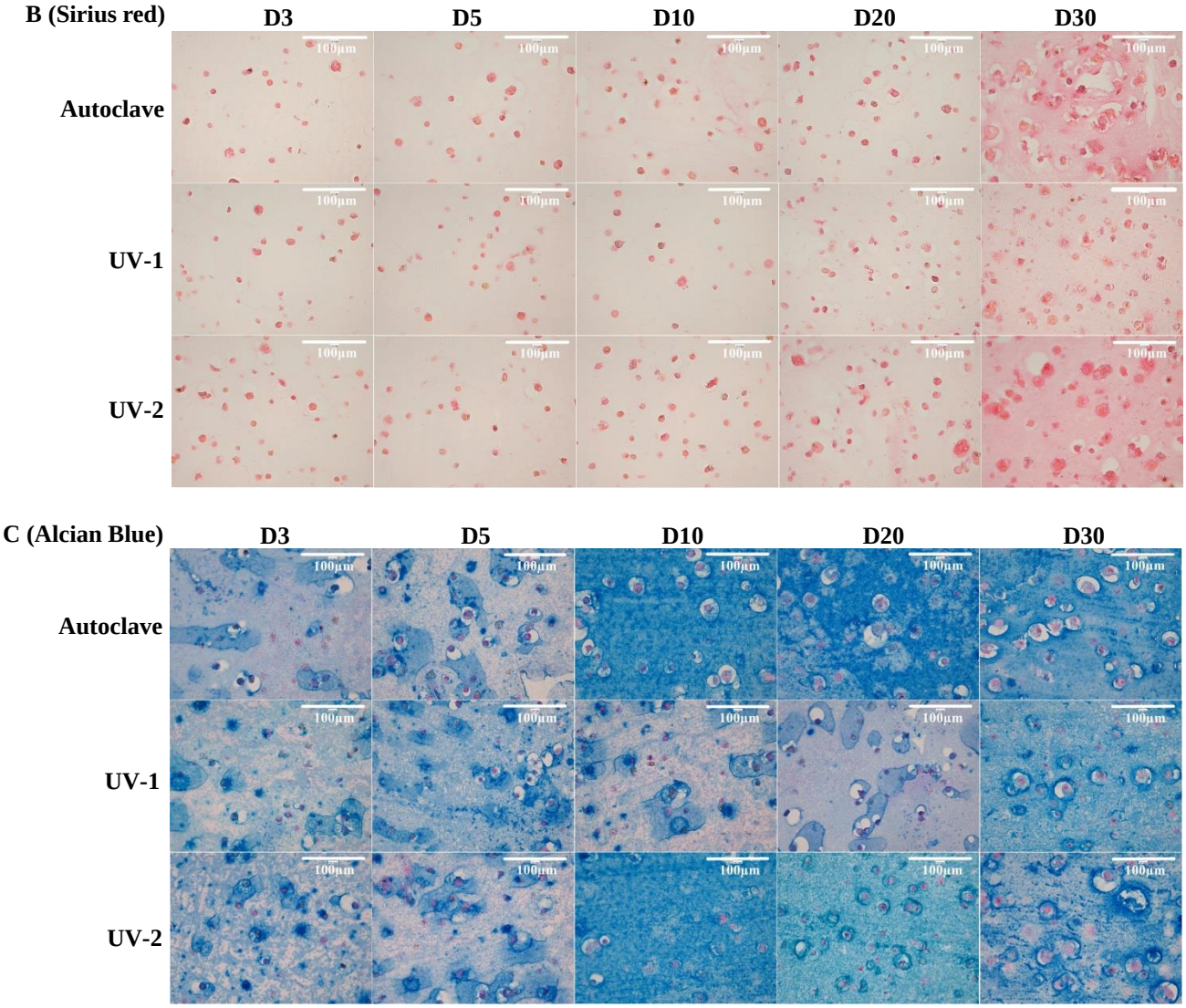
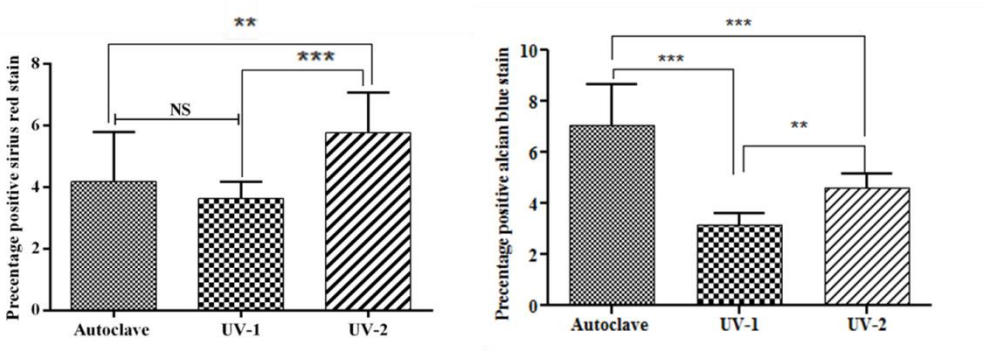



**Fig.7** Histological images of matrix molecules expression by WJ-MSCs encapsulated in Alg/HA hydrogel scaffolds and cultured under the differentiation liquid of chondrocyte after 3, 5, 10, 20 and 30 days. (A) The morphologies of cells in hydrogel scaffold by staining with HES. (B) Collagen synthesis in hydrogel scaffolds by Sirius red staining. (C) Glycosaminoglycan synthesis in hydrogel scaffolds by Alcian Blue staining. Scale bar is 100 μm




**A** **B**

**Fig. 8** Sirius red (collagen) positive stain area percentage (A) and Alcian blue (GAG) positive stain area percentage (B) in histological samples. All results are expressed as mean ± SD of the mean ($n \geq 20$). $^{**}p < 0.01$, $^{***}p < 0.001$ and NS ($p > 0.05$).

### 3.8. PCA component analysis

The effects of treatment parameters and their correlation with the others characteristics were determined statistically by means of the Principal Component Analysis method (PCA), as shown in Fig.9. In fact, the well-known PCA is used to reveal the internal structure of the present multivariate dataset as a set of coordinates in the corresponding high-dimensional data space. This was done by using an orthogonal transformation to convert the set of observations into a set of uncorrelated variables called principal components (F1 *vs* F2 which represents 84.09% of total variability). In the present case, this procedure was applied to the results of experiments with 16 variables: Control, Autoclave, UV-1, UV-2, $M_n$, $M_W$, Viscosity, Viability, Proliferation, Mechanical strength, GAG, Collagen, FTIR $_{(-OH)}$, FTIR $_{(C=O)}$, FTIR $_{(-COO)}$ and FTIR $_{(C-O-C)}$. The PCA bio-plot clearly showed variable parameters are clustered into two groups as A and B (Fig.9). From view of observation points (1, 2, 3 and 4), physical-chemical parameters (Group A) are negative variables (axis F1). But, the autoclave treatment is relatively further from physical-chemical parameters (Group A), when compared with other treatments. As we described above this is due to de-polymerization of alginate at high temperature. Meanwhile, UV-2 treatment is closed to Group B, which performed the best physical-chemical properties as same as control. For the second principal component (axis F2), biology parameters (Group B) reflected positive variables. It appears that the treatment of UV-2 is located near the viability, proliferation, GAG and collagen parameters as compared with the UV-1 treatment. It seems that UV-2 treatment is beneficial for viable cell as same as autoclave treatment. Moreover, the collagen synthesis seemed to be greater in UV-2 treatment than in the others. It is agreement with results given in Fig.6 and Fig.8A, respectively. There is correlation matrix for the studied parameters as shown in Table. 3. Without any treatments (control), the Alg/HA hydrogel kept nature properties as $\overline{M_n}$

(0.910), $\overline{M_w}$ (0.867), viscosity (0.717) and strength (0.650). For the autoclave treatment, from the view of the biomechanical, it can be seen that autoclave has strong negative affected on molar masses ($\overline{M_n}$: -0.668, $\overline{M_w}$: -0.745), viscosity (-0.884) and strength (-0.915), which is a logical correlation between them. For example, the mechanical properties of alginate hydrogel depending on the viscosity which were determined by weight average molecular weight ($\overline{M_w}$) (Lee and Mooney et al., 2012). More interestingly, this table showed autoclaving produces a concomitant effect on FTIR(C=O) (-0.558), which is also correlated with $\overline{M_n}$ (0.965) and $\overline{M_w}$ (0.942). Actually, when the main chains of Alg/HA or pure alginate are broken during the depolymerization reaction of Alg/HA by autoclaving, each chains will give 2 shorter chains keeping the same chemical composition that the initial ones. It means that shorter chains will have the same chemical groups (FTIR are not changing) with lower $M_n$. So, decreasing $M_n$ will modify the viscosity or other physical and mechanical properties of Alg/HA hydrogels. From the view of cell behavior, autoclaving has positive effect on the cell viability (0.412) and proliferation (0.451). For UV-1 treatment, one can observe a positive influence on FTIR (C-O-C) (0.927) and negative effect on FTIR (-COO) (-0.816). However, it is not beneficial for cell viability (-0.024) and proliferation (-0.104). For UV-2 treatment, it is can be seen that not only it has positive affected on strength (0,270), but also slightly affected on viscosity (-0.023), $\overline{M_n}$ (-0.243) and $\overline{M_w}$ (-0.157). More importantly, it is beneficial for cell viability (0.544) and proliferation (0.554), but also it has positive affected on the synthesis of GAG (0.647) and collagen (0.429), which depends on a suitable sterilization time (25min+25min) and viscosity of hydrogel. Thus, a high viscosity produces a negative effect on cell viability (-0.787) and proliferation (-0.808). In other words, a high of viscosity of gel solution to form hydrogel is not beneficial for cell viability due to the high shear forces required to mix cells with these solutions (Kong et al., 2003; Schneider et al., 2003). Similarly, high molecular masses ($M_n$ and $M_w$) have negative effect on the cell viability (-0.941, -0.901), proliferation (-0.942, -0.906). In addition, negative correlations between FTIR (-OH), FTIR(C=O) and cell viability (-0.769, -0.982), and proliferation (-0.786, -0.992), and GAG (-0.819, -0.925), and collagen (-0.697, -0.998) may be observed. According to our PCA analysis, it is acquired that a worse viscosity of alginate may be obtained after autoclave treatment. This has consequences on the gel stability and mechanical properties, thereby compromises the use of the gel as a scaffold after this treatment. Furthermore, note that the UV treatment time is an important parameter with regard to the cell behavior (viability, proliferation, GAG and collagen). The duration of 25 + 25 min (UV2) seems to give better results than simply 25 min (UV1). So, we chose an appropriate shear viscosity of gel solution which is easy to handle and to immobilize cells, but also keep a good mechanical property.

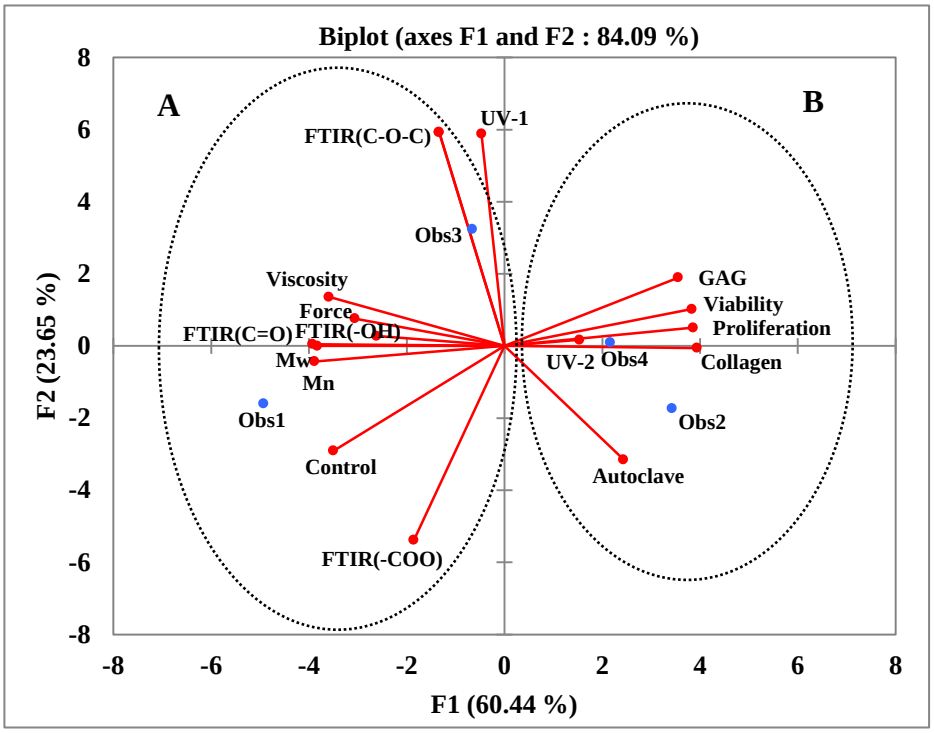



**Fig.9** Illustration of the data set under the new coordinate system defined by the principal components analysis. (axes F1 and F2 representing 84.09%).

**Table 3** The correlation matrix for the studied parameters. (Values in bold are different from 0 with a significance level alpha= 0.05; Strength: the force of hydrogel scaffolds with a 0.35 mm displacement at 5mm/min)

| Variables | Control | Autoclave | UV-1 | UV-2 | $M_n$ | $M_w$ | Viscosity | Viability | Proliferation | Strength | GAG | Collagen | FTIR(-OH) | FTIR(C=O) | FTIR(-COO) | FTIR(C-O-C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 1 | -0,333 | -0,333 | -0,333 | **0,910** | **0,867** | **0,717** | **-0,931** | **-0,901** | 0,650 | **-0,929** | **-0,881** | 0,549 | **0,877** | **0,816** | -0,132 |
| Autoclave | -0,333 | 1 | -0,333 | -0,333 | **-0,668** | **-0,745** | **-0,884** | 0,412 | 0,451 | **-0,915** | 0,215 | 0,594 | 0,022 | -0,558 | 0,000 | -0,662 |
| UV-1 | -0,333 | -0,333 | 1 | -0,333 | 0,001 | 0,035 | 0,190 | -0,024 | -0,104 | -0,005 | 0,067 | -0,142 | 0,373 | 0,159 | **-0,816** | **0,927** |
| UV-2 | -0,333 | -0,333 | -0,333 | 1 | -0,243 | -0,157 | -0,023 | 0,544 | 0,554 | 0,270 | 0,647 | 0,429 | **-0,944** | -0,478 | 0,000 | -0,132 |
| $M_n$ | **0,910** | -0,668 | 0,001 | -0,243 | 1 | **0,994** | **0,937** | **-0,941** | **-0,942** | **0,854** | **-0,859** | **-0,977** | 0,536 | **0,965** | 0,556 | 0,267 |
| $M_w$ | **0,867** | -0,745 | 0,035 | -0,157 | **0,994** | 1 | **0,969** | **-0,901** | **-0,906** | **0,903** | **-0,799** | **-0,959** | 0,466 | **0,942** | 0,510 | 0,324 |
| Viscosity | 0,717 | **-0,884** | 0,190 | -0,023 | **0,937** | **0,969** | 1 | **-0,787** | **-0,808** | **0,952** | -0,644 | **-0,896** | 0,351 | **0,873** | 0,323 | 0,503 |
| Viability | **-0,931** | 0,412 | -0,024 | 0,544 | **-0,941** | **-0,901** | **-0,787** | 1 | **0,997** | -0,627 | **0,978** | **0,976** | -0,769 | **-0,982** | -0,556 | -0,183 |
| Proliferation | **-0,901** | 0,451 | -0,104 | 0,554 | **-0,942** | **-0,906** | **-0,808** | **0,997** | 1 | -0,636 | **0,965** | **0,986** | -0,786 | **-0,992** | -0,488 | -0,262 |
| Strength | 0,650 | **-0,915** | -0,005 | 0,270 | **0,854** | **0,903** | **0,952** | -0,627 | -0,636 | 1 | -0,470 | -0,750 | 0,060 | 0,713 | 0,401 | 0,360 |
| GAG | **-0,929** | 0,215 | 0,067 | 0,647 | **-0,859** | **-0,799** | -0,644 | **0,978** | **0,965** | -0,470 | 1 | **0,911** | **-0,819** | **-0,925** | -0,610 | -0,032 |
| Collagen | **-0,881** | 0,594 | -0,142 | 0,429 | **-0,977** | **-0,959** | **-0,896** | **0,976** | **0,986** | -0,750 | **0,911** | 1 | -0,697 | **-0,998** | -0,453 | -0,349 |
| FTIR(-OH) | 0,549 | 0,022 | 0,373 | **-0,944** | 0,536 | 0,466 | 0,351 | -0,769 | -0,786 | 0,060 | **-0,819** | -0,697 | 1 | 0,735 | 0,108 | 0,288 |
| FTIR(C=O) | **0,877** | -0,558 | 0,159 | -0,478 | **0,965** | **0,942** | **0,873** | **-0,982** | **-0,992** | 0,713 | **-0,925** | **-0,998** | 0,735 | 1 | 0,439 | 0,348 |
| FTIR(-COO) | **0,816** | 0,000 | **-0,816** | 0,000 | 0,556 | 0,510 | 0,323 | -0,556 | -0,488 | 0,401 | -0,610 | -0,453 | 0,108 | 0,439 | 1 | -0,649 |
| FTIR(C-O-C) | -0,132 | -0,662 | **0,927** | -0,132 | 0,267 | 0,324 | 0,503 | -0,183 | **-0,992** | 0,360 | -0,032 | -0,349 | 0,288 | 0,348 | -0,649 | 1 |

## 4. Conclusions

The sterilization treatment is crucial process for tissue engineering. We have shown that the mechanical properties of alginate hydrogel depend on the viscosity which was determined by weight average molecular weight ($\overline{M_w}$). Otherwise, the cell behavior in hydrogels depends not only on a suitable sterilization time (25min+25min) but also on viscosity of hydrogel. Furthermore, we demonstrated that UV-2 treatment could to be an effective method to sterilize alginate powder, with (i) minimal impact on the mechanical integrity or physical-chemical properties and (ii) ability to maintain alginate cytocompatibility and its ability to induce chondrogenesis from mesenchymal stem cells. In conclusion, the cell behavior of Alg-based hydrogels were not only regulated by physicochemical properties (as molar mass or/and viscosity), but also associated with the controlling of sterilization time.

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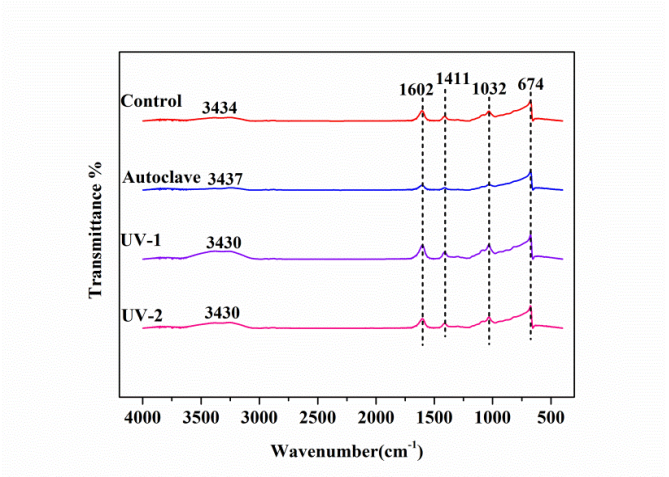
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## Appendix A. supplementary data

**Fig. S1.** (S1A) FTIR spectra of pure hyaluronic acid powder under exposure to the sterilization methods; (S1B) FTIR spectra of pure alginate powder under exposure to the sterilization methods.



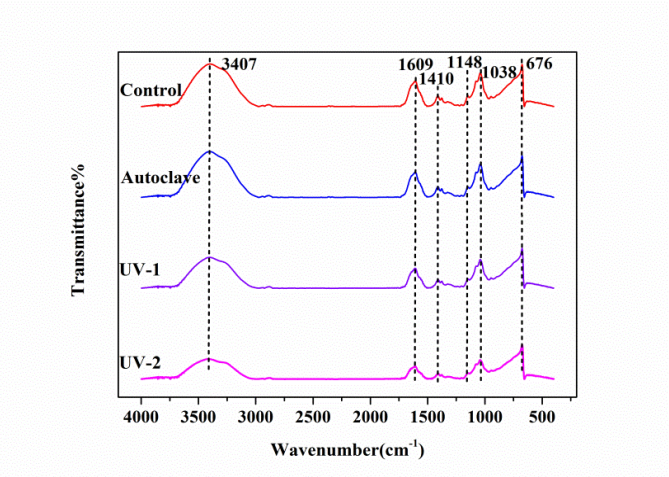




**Table S1** Number average molecular weight ($\overline{M_n}$), weight average molecular weight ($\overline{M_w}$) and dispersity (D = ($\overline{M_w}/\overline{M_n}$) for pure alginate under various sterilization methods.

| Samples + treatments | $\overline{M_n}$×105 (g/mol) | $\overline{M_w}$×105 (g/mol) | D |
|---|---|---|---|
| Alg + Control | 2.22 | 3.08 | 1.39 |
| Alg + Autoclave | 0.95 | 1.96 | 2.07 |
| Alg + UV-1 | 1.92 | 2.56 | 1.33 |
| Alg + UV-2 | 1.94 | 2.81 | 1.45 |