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**Full Paper**

# Enzymatically crosslinked Ulvan hydrogels as injectable systems for cell delivery.

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**Abstract:**The present study represents a challenging effort aiming at converting the waste algal biomass Ulva sp. into a source of high added value materials for biomedical applications by means of a clean and sustainable process. Ulvan, a sulphated polysaccharide extracted from Ulva sp., is investigated as in situ gelling material by using the enzyme Horseradish Peroxidase (HRP) as catalyst and $H_2O_2$ as reagent. The polysaccharide is successfully modified with tyramine units in order to be susceptible to enzymatic recognition and crosslinking through oxidative coupling. The times of gelation of Ulvan-tyramine (UT) solutions are optimized by adjusting the amount of $H_2O_2$ used for ensuring their practical administration as injectable systems. The rheological properties of the polymeric solutions and the relevant hydrogels evaluated under operative conditions prove to be suitable as injectable in situ forming 3D-hydrogels. The preliminary biological assays evidence the full cytocompatibility of the developed hydrogels and their feasibility for being used as 3D-hydrogels for cell delivery.

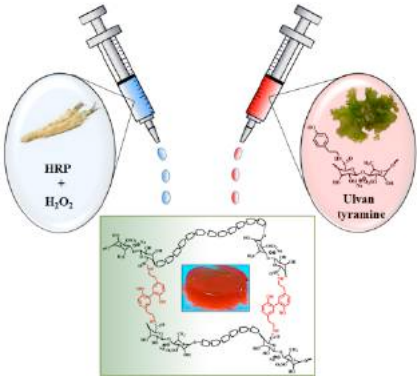
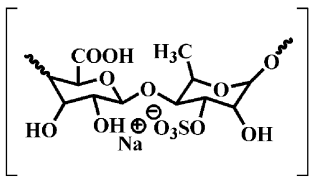


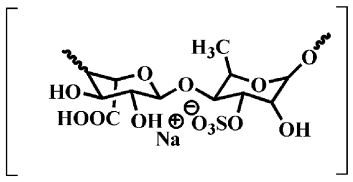

FIGURE FOR ToC_ABSTRACT

# 1. Introduction

The exploitation of biomasses as resources of energy and materials represents an unavoidable need due to the irreversible depletion of petroleum-based feedstock and the increasing demand of naturally derived products. Algae do represent promising candidates due to their abundance and content of chemical precursors of energy and bioactive materials although a large part of them still remains unused as being mainly involved in processes harmful for the environment such as uncontrolled eutrophication of coastal and lagoon waters and wild degradation over seashores.[1,2] A promising class of polymeric materials of algal origins is represented by sulphated polysaccharides since they are not easily found in other organisms and they are naturally displaying a wide range of biological activities.[3] The present study reports the investigation of ulvan, a sulphated polysaccharide extracted from green algae belonging to *Ulva sp.*, as polymeric matrix for the development of in situ gelling 3D-hydrogels for cells delivery. The exploitation of *Ulva* has been so far limited in Eastern countries for food consumption and dietary supplements although it represents the most abundant green macroalgae being ubiquitous in coastal benthic communities around the world.[4-5]. The sugar composition of ulvan is extremely variable but rhamnose, xylose, glucuronic and iduronic acid and the presence of sulphate groups have been identified as the main constituents of the polymer.[6] A structural motif characterizes the heteropolymer chain provided by the presence of disaccharide repeating units constituted by aldobiuronic acid sequences designated as type A (glucurorhamnose 3-sulphate, A3s) and type B (iduronorhamnose 3-sulphate, B3s) (**Figure 1**).




Glucurorhamnose 3-sulphate, A3s




Iduronorhamnose 3-sulphate, B3s

**Figure 1.** Main disaccharide units constituting Ulvan

Ulvan represents a unique material due to the content of uncommon sugar such as iduronic and sulphated rhamnose displaying a wide range of biological activities and excellent physicochemical properties[7]. The presence of carboxyl, hydroxyl and sulphate groups makes ulvan a versatile material whose chemical structure, close to that of mammalian glycosaminoglycans such as hyaluronan and chondroitin sulphate, could be easily modified to fulfil the requirements of the envisaged applications. Although the degradation of ulvan in vivo has not been yet investigated, the polysaccharide is considered as a biodegradable material by action of naturally occurring mammalian enzymes responsible for the degradation of glycosaminoglycans[8]. Accordingly ulvan has been recently investigated as polymeric matrix for the development of gelling materials for biomedical applications. Both physical and chemical crosslinking were involved in the preparation of ulvan hydrogels by developing polyelectrolyte complexes with chitosan, hybrid nanofibers by physical mixture with biocompatible polymers and covalent networks by using reactive crosslinkers and UV light [9-16] Our aim was the development of novel injectable hydrogels by means of environmentally safe procedures in order to convert the algal biomass from waste material into a particularly advantageous biomedical device by means of sustainable processes. Indeed the present study reports the unexploited preparation of in situ gelling system based on ulvan crosslinked by enzymatic action aimed at the development of injectable hydrogels.[17] The use of injectable hydrogels is particularly recommended in biomedical applications since they can be administered in vivo in a minimally invasive manner, are able to properly fill irregular shaped defects, can easily encapsulate drugs and cells in the liquid state and are effective delivery depots after solidification. In situ gelling could be triggered by chemical (crosslinking agents) and physical stimuli (UV light, temperature, pH).[18] Chemical cross-linking is a more effective process to synthesize hydrogels with better mechanical stability and improved control over biodegradation. However, this approach often requires the use of toxic chemical

agents that can alter the activity of encapsulated bioactive molecules and cells. The use of enzymes could represent a straightforward strategy to obtain covalent crosslinking in a rapid and clean manner. Accordingly, ulvan was modified with phenol moieties in order to be susceptible to recognition by Horseradish Peroxidase (HRP) enzyme whose combination with hydrogen peroxide catalyzed gel formation through the covalent coupling between the grafted phenols.[17,19]. The developed polymeric systems were investigated as suitable in situ gelling materials by evaluating their time of gelification, injectability and rheological properties. The rat pheochromocytomaPC12 cell line, chosen as a model of cells able to proliferation in suspension, was used to preliminary assess the cytocompatibility of the developed hydrogels and their ability to maintain viable cells upon the occurring of the enzymatic cross-linking.

## 2. Experimental Section

### 2.1 Materials

Ulvan batch in powder as extracted from *Ulva armoricana* was kindly supplied by CEVA within the framework of the EU-founded project BIOPAL. The number average molecular weight (Mn) of ulvan was determined as 60 kDa by using size exclusion chromatography. Pullulan standards (Polymer Laboratories, UK) were used to obtain the calibration curve (range 6000-400000 g/mol). HRP was purchased from Sigma Aldrich (Type II; 181 U/mg) and used as received. Hydrogen peroxide (30 wt%) was purchased from Sigma Aldrich and used as received.N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98%) and N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS, ≥ 98%) were purchased from Sigma Aldrich and used as received. Spectra/Por® dialysis membranes made of cellulose esters (MWCO: 10 kDa) were purchased from Spectrum Labs. Phosphate Buffer Saline 0.1 M, pH 7.4 (PBS 0.1M) was prepared by dissolving 2.0 g of KCl, 2.0 g of $KH_2PO_4 \cdot H_2O$, 80.0

g of NaCl and 15.6 g of $NaH_2PO_4 \cdot 12H_2O$ in 1 liter of deionized water and used diluted 1:10 with deionized water . The final pH was adjusted to 7.4 with NaOH 5 N and the resulting solution was steam sterilized (121°C for 20 min) before use. Dulbecco's Modified Eagles Medium (DMEM) was purchased from Sigma-Aldrich. Alamar Blue® and Live-Dead Assays (InVitrogen) commercial products were stored at -20°C in the dark and used according to the procedure indicated by the producers.

Rat pheochromocytoma cell line PC12 (CRL 1721, American Type Culture Collection) was cultured in DMEM, modified with 2mM L-glutamine, and added with fetal bovine serum (10%), penicillin/streptomycin solution (100 U/100 $\mu g ml^{-1}$) and anti-mycotic. PC12 cell line was grown in suspension in 25 $cm^2$ tissue culture flasks at 37°C, 5% CO2 until confluence. Cells were centrifuged at 1000 rpm for 5 min and re-dispersed in DMEM to obtain the appropriate cell number for the seeding.

## 2.2 Synthesis

### 2.2.1 Preparation of UT

In a 250 ml round bottomed, three-necked flask provided with a magnetic stirrer 2.0 g of ulvan (5.0 mmol of major disaccharide repeating units corresponding to 5.0 mmol of carboxyl groups) were dissolved in 100 ml of MES buffer (0.1 M, pH 6.0). After dissolution, EDC (1.93 g, 10.0 mmol) and sulfo-NHS sodium salt (1.09 g, 5.0 mmol) were added to the reaction mixture. The obtained solution was left under stirring for 1 h at room temperature. A large excess of tyramine hydrochloride (18.0 g, 100 mmol) was then added to the mixture and the resulting solution was kept stirring for 22 h at room temperature. The raw material was recovered after precipitation into absolute ethanol (10:1 v/v), filtration and washing with absolute ethanol.

The product was further purified by exhaustive dialysis against deionized water. After freeze-drying (-50 °C, 0.100 mbar) a white powder was recovered. Yield: 75%. $^{1}$HNMR (200 MHz, $D_2O$, δ): 1.0-1.3 (3H,$CH_3$), 2.8 (2H, $CH_2$), 2.9-4.0 (14H, overlapped), 7.2-7.3 (H, ArH), 7.4-7.5 (2H, ArH) ppm. FT-IR (KBr): ν 3440 (br, $\nu_s$O-H), 1670 (s, ν C=O, amide I band), 1560 (s, bend N-H, amide II band), 1506 (s, C=C, ring vibration), 1250 (s, $\nu_s$S=O), 1030-1060 (br s, $\nu_{as}$C-O), 847 (br s, bend C-O-S) cm$^{-1}$.

### 2.2.2 Characterization of UT

NMR spectra were recorded on a Varian Gemini 200 spectrometer operating at 200 MHz at 300 K. NMR samples were prepared in $D_2O$ at concentrations of 15–20 mg/ml.

FT-IR spectra were recorded on solids dispersed in KBr pellets (1/50 mg/mg) by using a Jasco FT-IR 410 spectrophotometer.

## 2.3 Hydrogels preparation

UT-based hydrogels were prepared both in PBS 0.01 M, pH = 7.4 and in the cell growth medium DMEM.

### 2.3.1 Preparation of UT-based hydrogels in PBS 0.01 M

Hydrogels were prepared in a 1 ml vial by sequential addition of a solution of ulvan-tyramine in PBS 0.01 M (0.160 ml, 125 mg ml$^{-1}$stock solution), a solution of HRP in PBS 0.01 M (0.019 ml) at different concentrations (**Table 1**) and a solution of hydrogen peroxide in PBS 0.01 M (0.021 ml, 97.9 mM stock solution).The obtained mixtures were gently stirred and kept at room temperature or at 37 °C until the gel was formed as evidenced by the tilting method (2.4.1).

**Table 1.**Times of gelation of UT in PBS 0.01 M solutions determined by using different HRP concentrations

| Entry | HRP [mg/ml][a] | HRP [U/ml][b] | Gelation time(s) 25°C | 37°C |
|---|---|---|---|---|
| UT_1 | 0.60 | 5.0 | 55 | 21 |
| Blank 1 | 0.60 | 5.0 | -[c] | -[c] |
| UT 2 | 0.42 | 3.5 | 69 | 42 |
| Blank 2 | 0.42 | 3.5 | -[c] | -[c] |
| UT_3 | 0.30 | 2.5 | 89 | 63 |
| Blank 3 | 0.30 | 2.5 | -[c] | -[c] |
| UT 4 | 0.27 | 2.2 | 95 | 67 |
| Blank 4 | 0.27 | 2.2 | -[c] | -[c] |
| UT 5 | 0.25 | 2.0 | 100 | 75 |
| Blank 5 | 0.25 | 2.0 | -[c] | -[c] |
| UT_6 | 0.15 | 1.2 | 264 | 177 |
| Blank 6 | 0.15 | 1.2 | -[c] | -[c] |
| UT_7 | 0.075 | 0.6 | 1796 | 877 |
| Blank 7 | 0.075 | 0.6 | -[c] | -[c] |

a: Enzyme concentration of stock solution
b: Concentration of the enzyme in the tested solution expressed as enzymatic units per volume
c: Gelification did not occur

2.3.2. Preparation of UT based hydrogels in DMEM

Hydrogels were prepared in a 10 ml vial by sequential addition of a solution of UT conjugate in DMEM (1 ml, 50 mg $ml^{-1}$stock solution) a solution of HRP in PBS 0.01 M (0.048 ml, 0.3 mg $ml^{-1}$stock solution) and a solution of hydrogen peroxide in PBS 0.01 M (0.052 ml, 97.9 mM stock solution). The obtained mixture was gently stirred and kept at room temperature until the gel was formed as evidenced by the tilting method.

2.3.3. Preparation of cell loaded enzymatically crosslinked UT hydrogels

Prior to cells seeding, UT was sterilized under UV light for 15 minutes. Experiments were performed in a 24 wells tissue culture polystyrene plate. For each well containing the polymeric solution (0.500 ml, 5% w/v), $1x10^5$ of PC12 cells were added. A solution of HRP in PBS 0.01 M (0.024 ml, 0.3 mg $ml^{-1}$ stock solution) and hydrogen peroxide in PBS 0.01 M (0.026 ml, 97.9 mM stock solution) were sequentially added to each well. Hydrogels were allowed to form for 1 minute and incubated for 1 hour at 37°C and 5% $CO_2$. Medium was refreshed and the samples were maintained in the incubator.

## 2.4 Characterization of enzimatically crosslinked UT based hydrogels

### 2.4.1. Determination of gelation time

The gelation times of the prepared UT conjugates were determined in PBS 0.01 M at different concentrations of HRP (**Table 1**) both at room temperature and at 37 °C by using the tilting method. Accordingly the criterion for a positive result was the absence of flowing of the solution containing the polymer within 1 minute of inverting the vial.[20]. An experimental control was done for each HRP concentration by using pristine ulvan (Table 1).

### 2.4.2. Determination of swelling degrees (SD%)

Hydrogels were prepared in a 24 wells tissue culture polystyrene by sequentially adding a solution containing UT in PBS 0.01 M (1 ml, 50 mg $ml^{-1}$stock solution), a solution of HRP in PBS 0.01 M (0.048 ml, 0.3 mg $ml^{-1}$stock solution) and a solution of hydrogen peroxide in PBS 0.01 M (0.052 ml, 97.9 mM stock solution) into each well. The obtained mixtures were allowed to gelify at room temperature for 10 minutes and then freeze dried (-50 °C, 0.100 mbar) resulting in cylindrically shaped dried scaffolds. The swelling degree experiments were carried out on the obtained samples at 37 °C by using DMEM as immersing medium. The weight of each sample was determined prior to immersion (Wd) and at predetermined time

intervals (Ws) after first blotting with tissue paper to remove excess water. The swelling degrees (SD%) were calculated according to Equation (2):

$$SD\% = \frac{(Ws - Wd)}{Wd} \times 100 \qquad (2)$$

The experiments were performed in triplicate and the SD % was reported as the mean ± standard deviation.

2.4.3. Rheological analysis

Rheological characterizations were carried out on UT hydrogels and UT solutions by using a Rheometric Scientific Instruments RM500 rheometer with a plate to plate geometry (steel, 20 mm diameter).UT hydrogels were prepared in DMEM according to the procedure previously described (2.3.2). Prior to analysis the samples were allowed to swell to equilibrium in DMEM at 37 °C for 24 hours.

To prevent sample slippage, self-adhesive sandpaper was attached to both the plates according to a procedure reported in literature [21]. Prior to analysis, each tested hydrogel was loaded between the plates, and the height of the top plate was adjusted to ensure a good contact of the sample with both plates and avoid gel squeezing (normal force of 0.25 N). All the tests were performed in a temperature-controlled chamber.Strain sweep measurements were carried out in the range 0.01 – 50 % strain at an angular frequency of 3.14 rad/s in order to determine the linear viscoelasticity region.

Frequency sweep measurements were performed over an angular frequency of 0.5 – 100 rad $s^{-1}$ at a fixed shear strain of 1 %, whose value was found to lie within the linear viscoelastic region. The complex modulus G*, the storage modulus G‘, the loss modulus G‘‘ and the phase degree (δ) were determined.Data were processed by using Rheoexplorer 3.0 software and the results were reported as the mean ± standard deviation calculated for 3 replicates.

The viscosity of UT conjugate in DMEM solution (50 mg $ml^{-1}$) was measured in the temperature range 15 to 40 °C, by employing a plate to plate gap of 0.052 µm and applying different shear stresses to the tested samples (1, 2, 5 and 10 Pa).[22]

Data were reported as the mean ± standard deviation calculated for 3 replicates.

#### 2.4.4 Biological evaluation

PC12 cell viability and proliferation assays were carried out using the alamarBlue® assay. The reagent resazurin diluted in the culture medium (1:10) was added to cell seeded hydrogels (paragraph 2.3.3). After 4 hours of incubation at 37°C and 5% $CO_2$, measurements of resorufin dye absorbance were detected at 565 nm, with 595 nm as reference wavelength, using a Biorad Microplate Reader. Cells grown on tissue culture polystyrene were used as control.

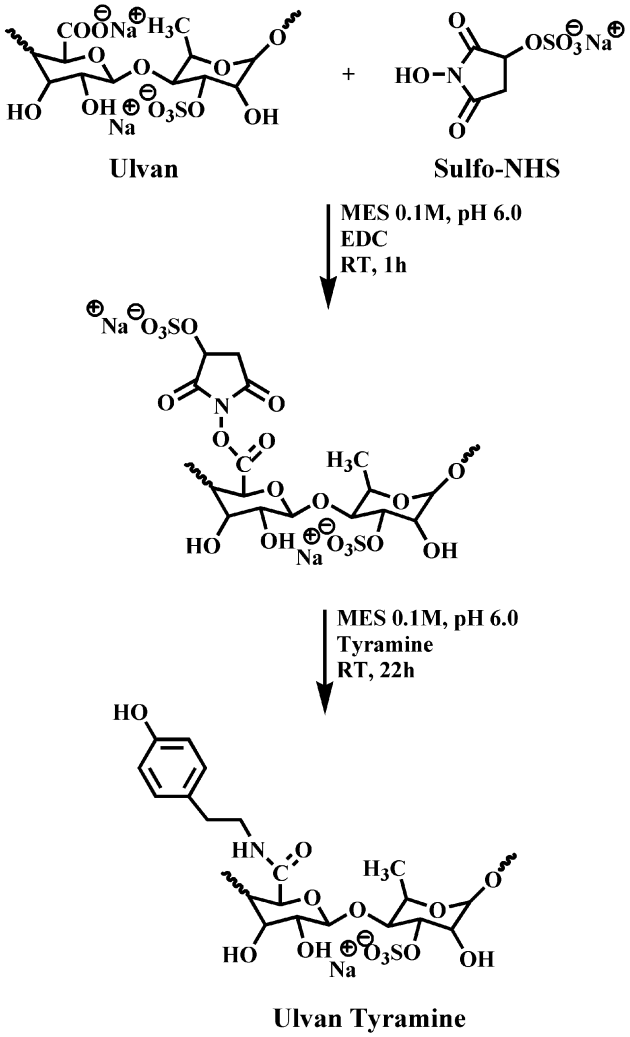
Visualization of viable cells in UT hydrogels was evaluated at 24 and 48 hours after cells seeding, by means of the LIVE/DEAD®Assay. Briefly, LIVE/DEADviability assay was performed by incubating cell seeded hydrogels with a solution of calcein AM (2µM) and ethidium homodimer-1 (2µM) in PBS (pH 7.4, 0.01 M) for 30 minutes at 37°C. The presence of viable (green) and dead (red) cells were immediately assessed using a Nikon Eclipse TE2000 inverted microscope equipped with an EZ-C1 confocal laser (Nikon, Japan).

Biological tests were performed on quadruplicate for each concentration, and the data were represented as mean ± standard deviation. Statistical difference was analyzed using one way analysis of variance (ANOVA) and a p value $< 0.05$ (*) or $p < 0.001$ (**) were considered significant.[23]

## 3. Results and Discussion

### 3.1 Synthesis and characterization of UT

Hydroxyphenyl compounds represent optimal substrates for HRP, a plant enzyme conventionally used in green chemistry processes. The conjugation of hydroxyphenyl units to polymeric materials has been reviewed as a straightforward strategy for the preparation of in situ gelling systems susceptible to crosslinking by enzymatic action.[19] Tyramine has so far represented the hydroxyphenyl compound most used in the development of in situ gelling materials crosslinked by HRP, due to its safe profile and high reactivity in the step of conjugation provided by the terminal amine group.[24] Polysaccharide-Tyramine conjugates were reported as ideal materials for the development of in situ gelling systems due to their inherent biocompatibility and susceptibility to enzymatic catalysis.[19] Dextran, alginate and hyaluronic acid represent so far the most investigated polysaccharides involved in the preparation of in situ gelling precursors containing tyramine.[25-30] To the best of our knowledge this strategy has never been applied on sulphated polysaccharides of algal origins. In the present study ulvan was selected as a model material due to the abundance of the *Ulva sp* biomass used as source of the polysaccharide and its reported bioactivity.[7] UT conjugates were successfully synthesized through formation of amide bonds between the carboxyl group of ulvan and the amine group of tyramine by using conventional carbodiimide chemistry. To this aim the carboxyl groups of ulvan were properly activated by converting them into reactive sulfo-NHS esters after treatment with EDC and sulfo-NHS in slight acidic conditions according to a procedure optimized for alginate (**Scheme 1**).[31]




**Scheme 1.** Synthetic route for the preparation of UT conjugates.

The successful grafting of Tyramine units onto the pendant carboxyl group of ulvan was confirmed spectroscopically by $^{1}$HNMR and FT-IR analysis.

The overlay of the $^{1}$H-NMR spectra of the pristine ulvan and UT conjugate is shown in (**Figure 2**).

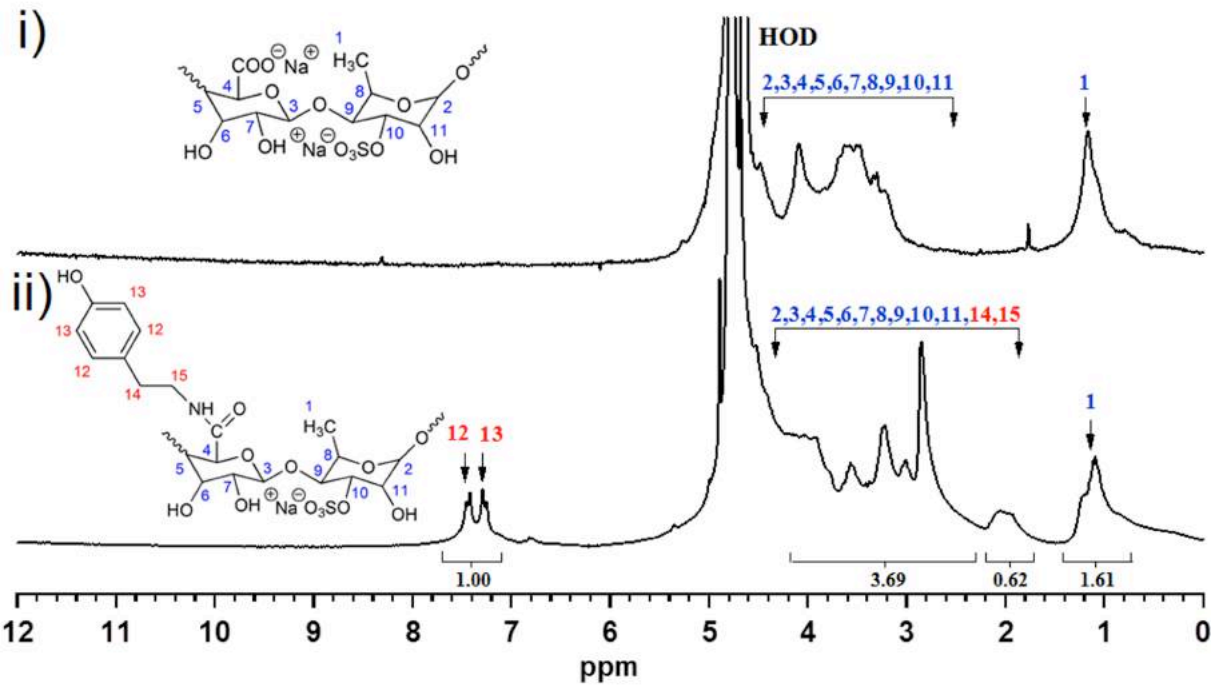



**Figure 2.** [1]HNMR spectra of (i) ulvan and (ii) UT conjugate recorded in $D_2O$.

The presence of tyramine units was revealed by the signals recorded in the spectral region typical of aromatic protons comprised between 7–8 ppm. The signals relevant to ulvan protons were found unresolved in the spectral region comprised between 3-4 ppm.

The degree of substitution (DS) of tyramine units grafted onto Ulvan backbone was calculated by [1]HNMR analysis. DS was defined as the mean number of tyramine units linked to each disaccharide repeating units and it was calculated according to Equation (1):

$$DS = \frac{(H12 + H13)}{4}\frac{3}{H1} \qquad (1)$$

where H12 and H13 were the integral values of the signals relevant to the aromatic protons of tyramine and H1 was the integral value of the signal of the methyl protons of the repeating unit of ulvan (Figure 2). The signal of the methyl group of ulvan was taken as reference since it lied on a spectral region free from overlapping signals and it was considered as characteristic of the repeating unit of the polysaccharide (Figure 1). The maximum obtainable

DS value was estimated to be 1, since each disaccharide repeating unit of ulvan contains one reactive carboxyl group.

Typically obtained DS values ranged from 0.3 to 0.4 indicating the occurring of one linkage of tyramine unit every three repeating units of ulvan.

The FT-IR analysis provided further evidences regarding the occurred conjugation between ulvan and tyramine. The overlay of the FT-IR spectra of pristine ulvan and UT conjugate is shown in (**Figure 3**).

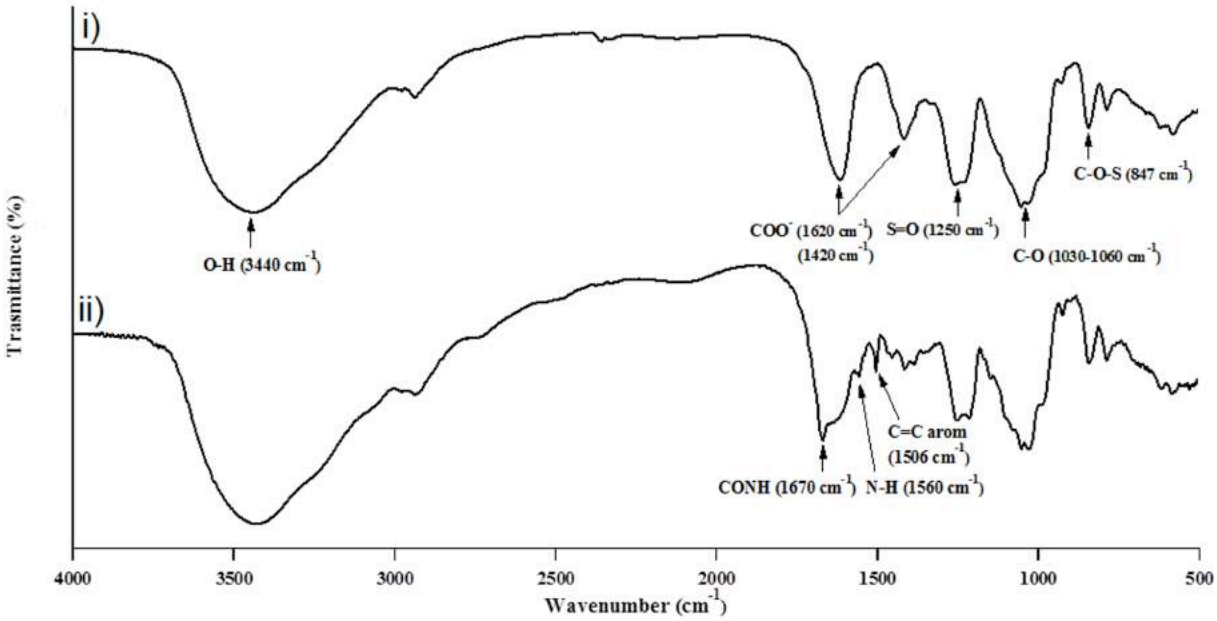



**Figure 3.** FT-IR spectra of i) ulvan and ii) UT.

The spectrum of ulvan is characterized by the absorption bands recorded at 1620 $cm^{-1}$ and 1420 $cm^{-1}$ respectively attributed to the asymmetrical and symmetrical stretching vibrations of the carboxylate group and at 1250 $cm^{-1}$ and 847 $cm^{-1}$ respectively attributed to the stretching vibration of S=O and the bending vibration of C–O–S of sulfate groups.[32]

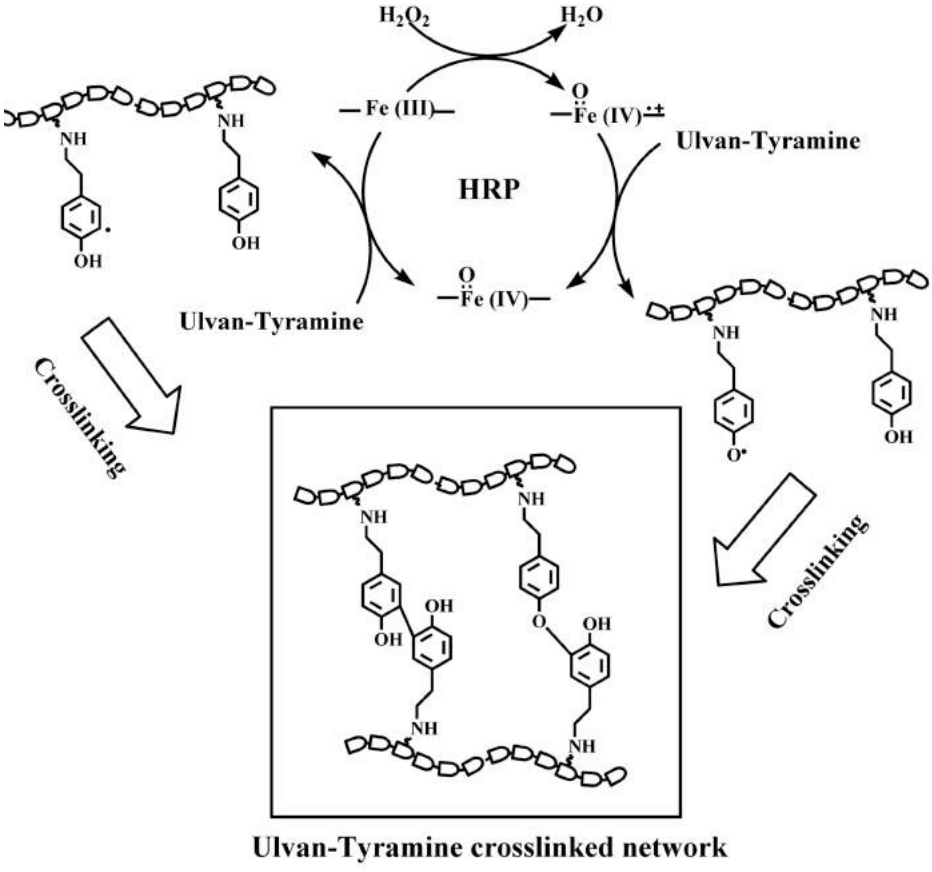
The formation of amide bonds between Ulvan and tyramine was revealed by the presence of two bands recorded at 1670 $cm^{-1}$ and 1560 $cm^{-1}$ respectively attributed to the C=O stretching vibrations (Amide I Band) and NH bending vibrations (Amide II Band). The sharp decrease

of the intensity of the absorption band recorded at 1420 $cm^{-1}$ evidenced the consumption of carboxylate group of ulvan during the reaction. Significant absorptions relevant to the presence of tyramine in UT were found at 1506 $cm^{-1}$and 1350 $cm^{-1}$ attributed respectively to the skeletal vibrations of the carbon atoms within the aromatic ring and to C-N stretching vibrations of tyramine.

### 3.2 UT based hydrogels

#### 3.2.1 Hydrogels preparation

In presence of $H_2O_2$, HRP is reported to catalyze the oxidative coupling of hydroxyphenyl compounds by following a radical pathway.[17] The covalent bonds can occur between the carbon atoms in ortho position to the hydroxyl group or the carbon atom in ortho position to the oxygen atom of the phenol group (**Scheme 2**).




**Scheme 2.** Mechanism of crosslinking of UT mediated by HRP.

The amount of crosslinks formed whose value unavoidably affects the physical properties of the polymeric network could be modulated by simply tuning the concentration of the enzyme and/or $H_2O_2$ .[19]

3.2.2 Determination of gelation times

Within the purpose of developing injectable hydrogels for biomedical applications, the gelation times are required to be adequate for ensuring their practical administration to the patient. To this aim the injectable system must crosslink rapidly to avoid gelation away from the injection sites. The gelation rates could be adjusted by simply varying the amount of enzyme and/or $H_2O_2$ concentrations. In our experiments the concentration of $H_2O_2$ was maintained low (1-10 mM) in order to increase the gelation times and reduce possible negative effects on cell viability.[33-35] The optimal concentration of HRP was established by measuring the times of gelation of the UT solutions upon variation of the concentration of the enzyme. The tests were carried out both at 25°C and 37°C by using PBS 0.01 M as medium in order to mimic the physiological conditions (Table 1). The time of gelation was determined as the moment in which the solution containing the polymer did not flow within 1 minute upon inversion of the vial. An experimental control for each HRP concentration was carried out by using not functionalized ulvan. As expected the blank solutions did not display gelification during the experiments thus indicating the decisive role of tyramine in the crosslinking reaction.

The kinetics of gelation of UT solutions was markedly affected by HRP concentration (**Figure 4**).

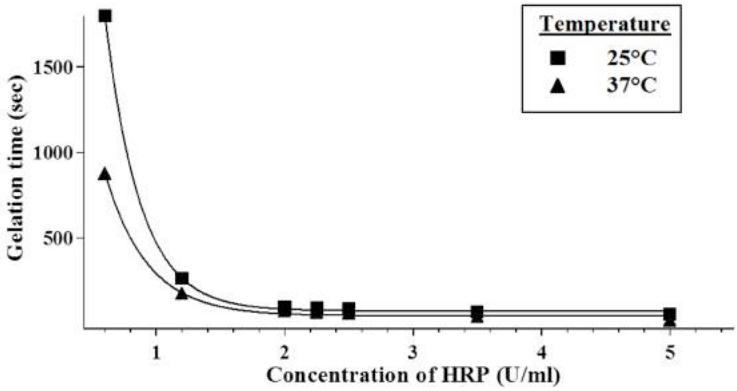



**Figure 4.** Dependence of gelation times of UT in PBS 0.01 M solution on HRP concentration at constant $H_2O_2$ concentration (9.8 mM).

The behavior recorded both at 25 °C and at 37 °C revealed an exponential dependence between the gelation times and HRP concentration similarly to what observed for in situ gelling systems based on hyaluronic acid-tyramine conjugates.[33] The gelation time was found to be nearly constant at HRP concentrations above 2.5 U/ml notwithstanding the temperature of the medium. The rate of crosslinking recorded at 37 °C was higher compared to that evidenced at 25 °C thus enabling the required time for practical administration at room temperature followed by a fast gelation in situ at physiological conditions. As evidenced in Figure 4, the enzymatic concentration of 2.5 U/ml represented the minimum amount of HRP displaying the higher activity. The combination of HRP and $H_2O_2$ at concentrations fixed respectively at 2.5 U/ml and 9.8 mM was found to effectively produce stable UT gels within 90 sec from their mixing, thus representing a valuable system for the development of in situ gelling materials suitable for biomedical applications.

The optimized composition for a rapid crosslinking of UT in PBS 0.01 M was tested also in DMEM since it represents a widely used basal medium for supporting the growth of different mammalian cell lines. The times of gelation proved to be not affected by changing the medium since stable gels were formed in situ after 1 minute of mixing the components (**Figure 5**).

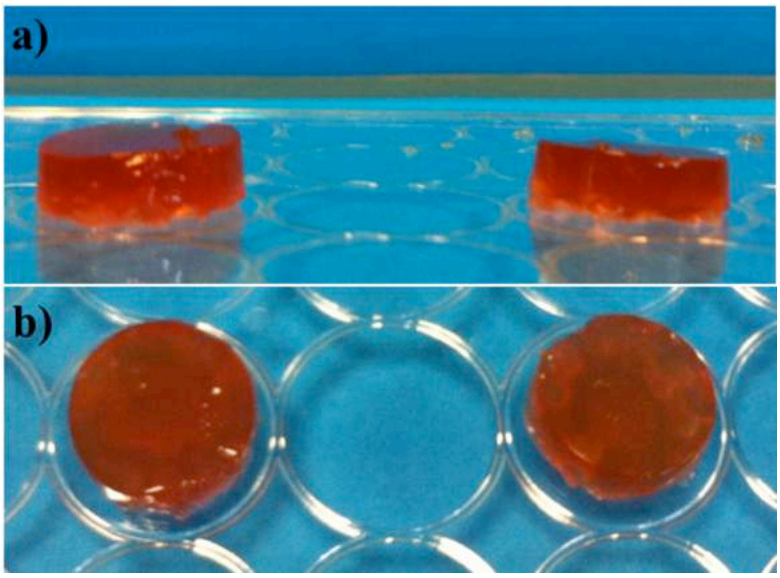



**Figure 5.** Pictures of in situ formed UT based hydrogels swollen in DMEM taken from a) lateral, b) top view

3.2.3 Swelling experiments

The amount of water absorbed by hydrogels, objectively measured through swelling degree experiments, definitely represents a decisive parameter for their efficacy in drug/cells delivery since its presence allows for a more efficient diffusion of nutrients, signals and/or drugs within the polymeric matrix and regulates both the mechanical properties and rates of degradability of the hydrogels. The swelling degrees (SD%) of UT conjugates were measured in PBS 0.01 M by weighing the hydrogels at regular intervals of time and their values were calculated according to Equation (2).

The amount of water absorbed by hydrogels increased exponentially with time and reached an equilibrium value after about 30 hours of immersion (**Figure 6**).

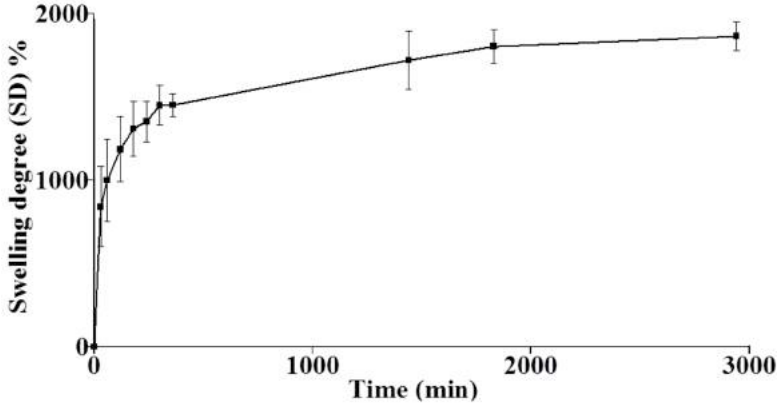


**Figure 6.** Swelling behavior of UT hydrogels immersed in PBS (0.01M, pH 7.4).

The SD% value calculated at the equilibrium was about 2000 in agreement with those reported for Ulvan based hydrogels crosslinked by UV allowing for the classification of Ulvan-based hydrogels as super absorbent materials.[7,16,37]

3.2.4 Rheological analysis

The viscoelastic behavior of UT hydrogels was characterized under dynamic shear conditions by means of a parallel plates rheometer. Prior to analysis, the hydrogels were swollen in DMEM in order to investigate the rheological properties of the materials under operative conditions. The complex modulus G*, elastic storage modulus G‘, viscous loss modulus G‘‘ and phase degree δ of the hydrogels were experimentally determined at 37 °C. A strain sweep test was carried out at constant angular frequency (3.14 rad $s^{-1}$) to determine the region of linear viscoelasticity. Both G‘ and G‘‘ were found to be constant in the range 0.1-5% strain. A strain value of 1% was adopted for the frequency sweep experiments. The frequency sweep experiments were investigated within the range 0-100 $rads^{-1}$. G‘ and G‘‘ values of UT sample were found to be nearly constant in the angular frequency range of 0.5-10 rad $s^{-1}$ with an average value of 602.1 Pa and 19.7 Pa, respectively (**Table 2**).

**Table 2 .**Rheological properties of UT hydrogels measured within the linear viscoelastic region (shear strain 1%) under frequency sweep conditions

| **Sample** | **\|G*\| [Pa]** | **G'[Pa]** | **G'' [Pa]** | **δ[°]** |
|---|---|---|---|---|
| UT | 602.6 ±141.1 | 602.1 ± 141.3 | 19.7±14.9 | 2.0± 1.7 |

Mean values calculated within the angular frequency range 0.5-10 $rads^{-1}$

The hydrogel was found to display elastic behavior when subjected to a periodical stress having an angular frequency value lower than 10 rad/s. At higher angular frequencies, i.e. low

relaxation time, the sample flexibility was diminished and the swollen sample becomes increasingly rigid, as evidenced by the sharp increase of both G' and G'' [37]. Moreover. the viscous loss modulus G'' was rapidly approaching the elastic storage modulus G' of the hydrogels indicating the increase of the viscous component on the rheological properties of the materials (**Figure 7**).

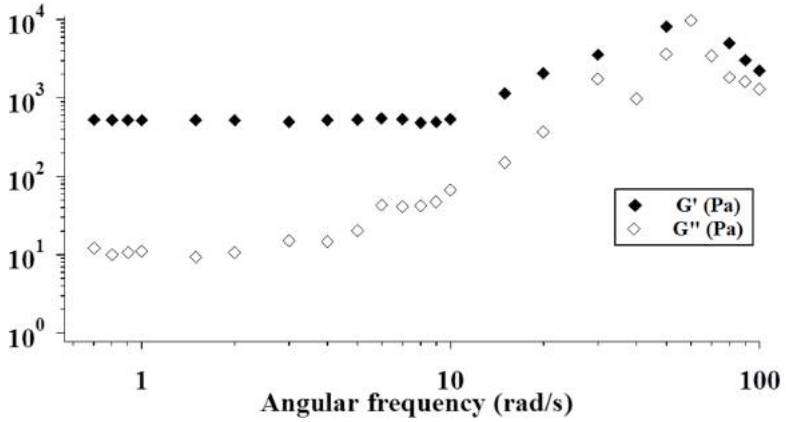



**Figure 7.** Representative curves of elastic storage modulus G' and viscous loss modulus G'' as a function of the applied frequency (rad/s) of UT hydrogel swollen in DMEM at 37 °C

The injectability of the pre-gelling mixtures was investigated by means of a parallel plate rheometer as well by measuring the viscosity of the solutions subjected to different shear stress and temperature.[22] The viscosity of water was measured at 1 Pa and 10 Pa in the same temperature range and used as reference. The maximum limit value of viscosity to ensure hydrogels injectability was assumed 0.05 Pas as the threshold allowed for subcutaneous injection via a 25 to 27-gauge syringe.[38-39] The data evidenced that the viscosities of the tested solutions lied far below the maximum limit of injectability suggesting the suitability of UT as injectable system for the preparation of in situ forming hydrogels (**Figure 8**).

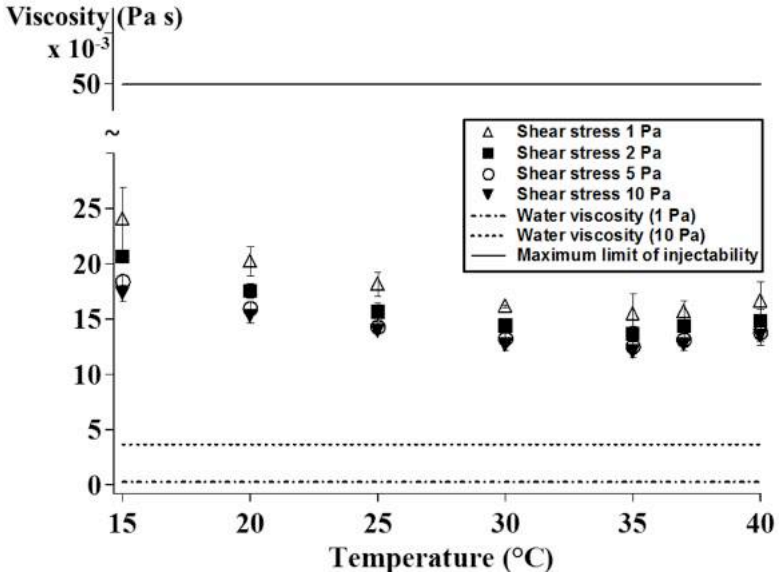



**Figure 8.** Viscosity of UT solutions in DMEM measured at different shear stresses and temperatures.

3.2.5 Biological analysis

In view of employing the developed UT hydrogels as systems for cell delivery, samples were preliminary submitted to biological investigations by using the rat pheochromocytoma PC12 cell line, selected as model for cell growing in suspension.

The ability of UT hydrogels to sustaining the viability and proliferation of PC-12 cell line was investigated by Alamar Blue assay.

PC12 cells cultured into UT hydrogels highlighted appreciable values of cell proliferation in comparison to the cells cultured on tissue culture polystyrene (TCPS) (**Figure 9**).

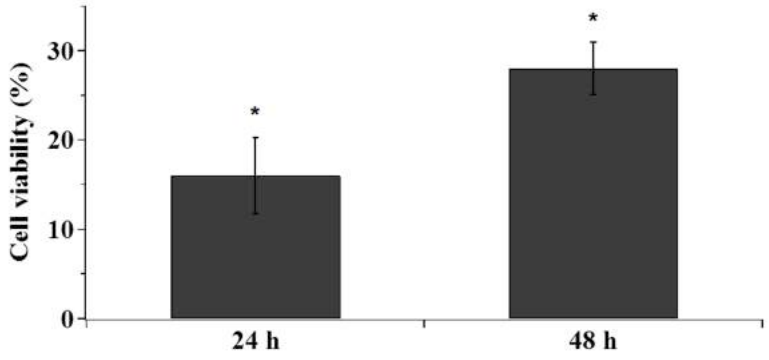



**Figure 9.** Cell viability of PC12 cultured on UT based hydrogels by Alamar Blue® assay.

After 24 and 48 hours of culture, cells reached a proliferation rate of about 16% and 28 % respectively, showing a significant increase with time ($p < 0.05$). These results confirmed the absence of toxic compounds released by the gelling systems and the ability of the developed hydrogels to sustain the viability and proliferation of PC12 cells upon the hydrogel formation in situ.

A live/dead assay was carried out in order to visualize the seeding efficiency and the proliferation of PC12 cells into the prepared hydrogels. The results were consistent with those obtained by the metabolic assay. Hydrogels revealed an appreciable number of viable PC12 cells (**Figure 10**) embedded in the formed hydrogel network. Red stains were not detected indicating the absence of dead cells.

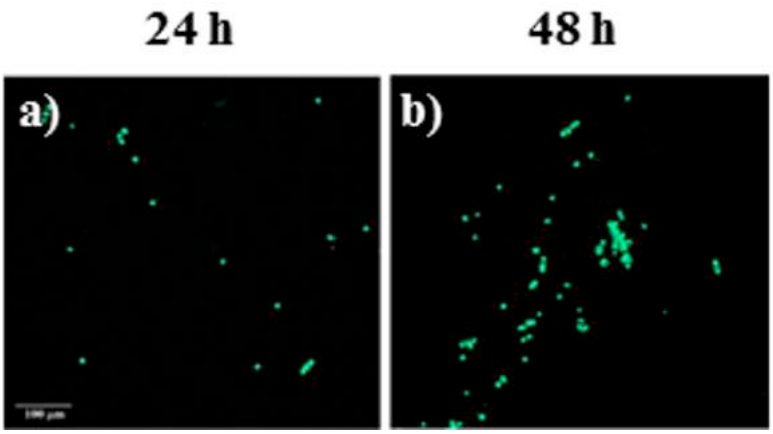



**Figure 10.** Confocal laser scanning microscopy micrographs of viable cells (green) in UT hydrogels revealed by live/dead assay at a) 24 hours and b) 48 hours (20X magnification).

## 4. Conclusions

The present study reports the conversion of ulvan, a sulphated polysaccharide of algal origin, into an in situ gelling material suitable for biomedical applications. Ulvan was successfully modified with tyramine units in order to be susceptible to crosslinking reactions catalyzed by enzymatic action in presence of $H_2O_2$. The amount of enzyme and $H_2O_2$ used were optimized

in order to induce times of gelation appropriate for the development of injectable hydrogels. The preliminary biological investigations indicate also the suitability of the enzymatically crosslinked ulvan hydrogels to act as vehicle for viable cells supporting the possibility of their application as injectable cell delivery systems. The reported results and findings may contribute to open new scenarios for the biomedical applications of polysaccharides derived from renewable and sustainable waste algal biomasses.

Keywords: algae biomass, ulvan, horseradish peroxidase, in situ forming hydrogels, cell delivery

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## Table of Contents

**The paper describes for the first time the preparation of an injectable system based on a sulphated polysaccharide of algal origin, ulvan, whose in situ gelling is catalyzed by enzymatic action.** The whole process is designed to limiting environmental issues through the exploitation of renewable materials and the development of sustainable procedures.