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This document is the Accepted Manuscript version of a Published Work that appeared in final form as Maria A. Bonifacio, Stefania Cometa, Andrea Cochis, Annachiara Scalzone, Piergiorgio Gentile, Alessandro C. Scalia, Lia Rimondini, Piero Mastroianni, Elvira De Giglio (2022). A bioprintable Gellan Gum/Lignin hydrogel: a smart and sustainable route for cartilage regeneration. International Journal of Biological Macromolecules 216, 336-346, doi: <https://doi.org/10.1016/j.ijbiomac.2022.07.002>

A bioprintable Gellan Gum/Lignin hydrogel: a smart and sustainable route for cartilage regeneration

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This work is dedicated to the memory of Prof. Emo Chiellini

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Abstract

Recent developments in tissue engineering are directed towards advanced biomaterials with highly-performing properties allowing the sustainable exploitation of renewable sources. In this work a composite hydrogel, based on a blend of two gellan gums with different acyl content embedding lignin, was developed for the regeneration of cartilage tissue. Different amounts of lignin (up to 0.4% w/v) were loaded into the polymeric matrix and the resulting hydrogels were physically crosslinked with magnesium ions. The physico-chemical characterizations established that, even if no chemical interaction between lignin and the polysaccharides was detected by XPS and SS-NMR analyses, lignin release in PBS resulted negligible and that lignin achieved up to 80% of ascorbic acid's radical scavenging activity *in vitro* on DPPH and ABTS radicals. Moreover, the formulated hydrogels were able to recover their original shape after compression. Lignin exploitation resulted cytocompatible as the viability of human mesenchymal stem cells (hMSC) 3D-seeded onto scaffolds resulted comparable to the lignin-free control hydrogel (>70% viable cells, $p>0.05$); moreover, the 0.4% lignin composites significantly improved the hMSC 3D-constructs chondrogenesis bringing to a significant ($p<0.05$) up-regulation of the collagen type II, aggrecan and SOX 9 chondrogenic genes. The presence of lignin conferred bacteriostatic properties to the hydrogel too, thus reducing the proliferation of the joint pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis* of >2 logs in comparison to the lignin-free control hydrogel. Finally, cellularized 3D constructs were manufactured via 3D bioprinting confirming the processability of the hydrogel formulation as a bioink and its unique biological features for creating a physiological milieu for cell growth.

Keywords: gellan gum; lignin; bioink; cartilage; bioprinting.

1. Introduction

Hydrogels are effective tools to address cartilage damages due to their biocompatibility and huge water content, which are very close to that of native tissues [1]. Moreover, hydrogels allow the design and fabrication of complex structures, ensuring high processability and versatility with 3D bioprinting technologies [2]. However, hydrogels' mechanical properties do not always fit with those required by cartilage tissue engineering, especially when articular defects should be repaired. In addition, bioactive hydrogels are often required to control inflammatory processes, hindering oxidative stress and radical species production [3]. Therefore, innovative hydrogels are being formulated to ensure the most suitable mechanical and bioactive performances, also delivering drugs and/or alive cells [4]. In the last decade, different studies have been addressed to the development of cartilage-replacing hydrogels based on gellan gum, a bacterial exopolysaccharide, in combination with different polymers and/or fibers to enhance its mechanical and biological performances [5-8]. On the other hand, our research group focused the attention on gellan gum-based composites reinforced with different inorganic clays to obtain promising systems for cartilage repair [9-12].

In this work, we have developed and characterized a gellan gum-based composite hydrogel combined with lignin since the need of highly-performing biomaterials should also be combined with environmental impact reduction, promoting the sustainable exploitation of renewable sources [13-15]. Thanks to its composition, lignin has been successfully blended to several polymers as a biocompatibilizer, enhancing the thermal resistance as well as the interfacial and mechanical properties of the resulting composites [16-17]. All these advantages have also prompted the additive manufacturing industry to develop lignin-based feedstocks as alternative 3D printing materials, able to replace plastics and nonrenewable polymers [18]. Some lignin-based, printable composites have been developed with nylon or polyethylene oxide for industrial purposes [19, 20].

Recently, Kouhi et al. proposed an injectable hydrogel based on gellan gum modified with lignocellulose nanofibrils enriched with melatonin loaded forsterite nanoparticle for cartilage tissue engineering [21]. However, to the best of our knowledge, no gellan gum/lignin printable composites

have been proposed for biomedical applications. We hypothesize that the addition of lignin to gellan gum provides a suitable chondrogenic activity and the proper mechanical features for printable hydrogels aimed at cartilage defects repair.

In our study, the printable hydrogel consists in a gellan gum/lignin blend, physically crosslinked with magnesium ions. Herein, since gellan gum is difficult to be printed as is, because of its high viscosity and sharp gelling temperature, two types of gellan gum, i.e. with high and low acyl content, have been mixed to lignin to obtain, for the first time, an easily bioprintable, eco-friendly hydrogel to manufacture constructs to be used in tissue engineering applications.

The hydrogel chemical composition has been studied through X-ray Photoelectron and Fourier Transform Infrared Spectroscopy, and Solid State Nuclear Magnetic Resonance, while its thermal behavior has been investigated by thermogravimetric analyses. In addition, the mechanical properties have been assessed by static compression tests and the *in vitro* radical scavenging activity of the lignin-containing hydrogels have been tested by DPPH and ABTS assays. Then, the cytocompatibility and chondrogenic activity of the developed hydrogel have been *in vitro* assessed on immortalised human mesenchymal stem cells (hMSCs) cultivated into 3D composites by means of metabolic assays, live/dead fluorescent imaging and extracellular matrix deposition by histological staining. Moreover, the bacteriostatic potential of the hydrogels conferred by lignin has been studied *in vitro* towards the joint pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis*. Finally, the optimized hydrogel encapsulating hMSC, as bioink, has been bioprinted in order to manufacture 3D porous constructs for cartilage regeneration.

2. Materials and Methods

2.1 Materials

Lignin (alkali, low sulphonate content) and low acyl content gellan gum (Phytigel™) has been purchased from Sigma Aldrich (Merck, Milan, Italy), while high acyl content gellan gum (KELCOGEL® LT100) has been supplied by CP Kelco (Atlanta, USA). The magnesium chloride

salt, exploited as cross-linker, is of Redi-dri™ grade. Hydrogels have been prepared with ultrapure water, obtained through a Milli-Q® distillation system (Millipore-Merck, Darmstadt, Germany). All solvents and reagents have been purchased from Sigma Aldrich (Merck, Milan, Italy), unless otherwise specified.

2.2 Hydrogel formulation

The formulation of the hydrogel composites has been optimized in terms of GG_H (high acyl content gellan gum) and GG_L (low acyl content gellan gum) ratios. Then, different amounts of lignin have been tested, until a proper hydrogel syringeability has been obtained. GG_H and GG_L have been dissolved in hot water (60°C) at 1.2% and 0.8% w/v (GG_H:GG_L equal to 3:2), respectively. Meanwhile, lignin aqueous solution 0.1, 0.2 or 0.4%w/v has been prepared and placed in an ultrasonic bath (Ultrasonic Cleaner AU-32, ARGO LAB, Carpi, Italy) to remove air bubbles. Then, the lignin solution has been added to the dissolved gellan gums and gently stirred on a magnetic plate (VELP Scientifica Srl, Usmate, Italy). Hydrogels have been poured into 24-well plates (Flat bottom, Corning Inc., NY, USA) and crosslinked with magnesium ions, as described in [10]. Based on the lignin content, the obtained gellan gum hydrogels were coded as GG_{HL}Lign0.1, GG_{HL}Lign0.2 and GG_{HL}Lign0.4. Gellan gums hydrogels without lignin (GG_{HL}) have been prepared as controls. For chemical characterizations, when dried samples are needed, the hydrogels have been frozen for 24h (-20°C), then freeze-dried at -55°C for 24h using an ALPHA 1-2 LD Plus freeze-drier (Martin-Christ, Osterode, Germany).

2.3 Physico-chemical and morphological characterization of the hydrogel

2.3.1 X-ray Photoelectron Spectroscopy (XPS)

The prepared hydrogels, once freeze-dried, have been sectioned and examined by means of XPS (PHI 5000 VersaProbe II, MN, USA). Analyses have been performed in high power mode with an AlK α X-ray radiation source and an instrument base pressure of 10⁻⁹ mbar. Wide scans, as well as high resolution scans have been recorded in fixed analyzer transmission (pass energy of 117.4eV and 29.35eV, respectively). The MultiPak software (v. 9.9.0) has been used for data mining, setting the

reference charge to 284.8eV (hydrocarbon peak). The software library has been exploited to normalize peak areas by elemental sensitivity factors and to calculate relative atomic percentages. Signals have been modelled as Gaussian-Lorentzian peaks having the same full width at half maximum (FWHM) and conducting non-linear least square fit iterations.

2.3.2 Solid-State Nuclear Magnetic Resonance (SS-NMR)

Solid-state NMR experiments were performed on a Bruker Avance I 400 spectrometer (operating at a frequency of 100.6 MHz for ^{13}C) using a 4.0 mm HX MAS probe at 298 K. For MAS experiments, samples were packed in zirconia rotors. ^1H - ^{13}C CP/MAS NMR experiments were performed using 3.25 μs proton $\pi/2$ pulse length, ν_{CP} of 55.0 kHz, contact time of 1.0 s, ν_{dec} of 76.9 kHz and recycle delay of 2.0 s. A two-pulse phase-modulation (TPPM) decoupling scheme was used for the ^1H decoupling. Chemical shifts for ^{13}C were referenced to the methylene signal of adamantane (δ 38.48).

2.3.3 Fourier Transform Infrared Spectroscopy (FT-IR/ATR)

Pure lignin and gellan gums powders, as well as the prepared hydrogels, have been tested through a Spectrum Two instrument (Perkin Elmer, Milan, Italy) equipped with the universal attenuated total reflectance accessory. Relevant spectra have been recorded at 4cm^{-1} resolution in the range 400 to 4000cm^{-1} .

2.3.4 Thermo-Gravimetric Analysis (TGA)

The thermal behavior of the dehydrated hydrogels has been assessed by a PerkinElmer TGA-400 instrument (Perkin Elmer, Milan, Italy), heating the samples within 30 and 600°C . The analyses have been performed in nitrogen, with a gas flow set at $20^\circ\text{C}/\text{min}$. Data have been recorded by means of the TGA Pyris software.

2.3.5 In vitro radical scavenging activity of the hydrogels and lignin in vitro release

The antioxidant performances of gellan gum and lignin/gellan gum hydrogels have been explored by *in vitro* ABTS and DPPH assays, as described by Zhong et al. [22]. Then, according to Wei and co-workers [23], the percentage radical scavenging activity (%RSA) of the prepared hydrogels has been assessed by the following equation (1):

$$\% \text{ DPPH} = \left[\frac{(A_{\text{rad}} - A_s)}{A_{\text{rad}}} \right] \times 100 \quad (1)$$

A

in which A_{rad} represents the absorbance of bare DPPH or ABTS reagent, while A_s is the absorbance of the sample. All the spectrophotometric analyses have been performed in triplicate, also on ascorbic acid, exploited as reference substance.

In addition, hydrogels' lignin release in PBS has been *in vitro* assessed up to 7 days at 37°C. At regular time intervals (2, 4, 8, 24, 48, 72, 120 and 168 h), aliquots of PBS were withdrawn and replaced by fresh PBS. Lignin aqueous solutions from 50 µg/mL to 2 µg/mL have been prepared and analyzed at 292 nm by a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, CA, USA) to obtain a five-points calibration curve ($r^2=0.999$). Release experiments were performed in triplicate and results are reported as mean $\pm$ standard deviation.

2.4 Mechanical characterization of the hydrogel

Mechanical properties of lignin-containing hydrogels (cylinder-shaped with diameter ~16mm and height ~20mm) have been analyzed by using the UniVert Test Device UV-200-01 (CellScale, Waterloo, Canada). Compression resistance of five samples for the selected composition was evaluated at room temperature. The crosshead speed has been set at 2.5mm/min, and the load was applied until the sample has been compressed up to reaching 35% of the strain. The resulting stress–strain curves have been plotted and the compressive modulus has been calculated as the slope of the initial linear portion of the stress–strain curve (0–10%), as reported previously by the authors [9]. Moreover, as previously described by Gentile et al. [24] the collapse strength (σ^*) and strain (ϵ^*) values have been calculated.

2.5 *In vitro* biological tests

2.5.1 Cell culture conditions

Human mesenchymal stem cells (hMSC) immortalized by hTERT lentiviral vectors as previously described [25] were used for the *in vitro* cytocompatibility and bioprinting assays. Cells were cultured using low glucose Dulbecco's Modified Minimal Essential Medium (DMEM) supplemented with

15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After reaching 80–90% of confluence, cells were enzymatically detached by trypsin-EDTA and used for biological evaluations.

2.5.2 Cytocompatibility of the hydrogel

Cylindric hydrogels of 0.6 mm height - 0.4 mm radius, were obtained and ethanol sterilized (70%) overnight. After air drying under sterile hood, a 3D model was realized as previously described by the authors [11, 12] seeding 3.5×10^6 cells/hydrogel using 200 μ L of PureCol EZ Gel solution (Merck, Darmstadt, Germany) as temporary substrate. The composites were maintained 24 hours in the incubator (37°C, 5% CO₂) to allow for complete collagen solidification and cells spreading; then, they were submerged with 2 mL of fresh medium and cultured for 7 days. The metabolic activity of the cells growing in the 3D matrix of the hydrogels was verified by the colorimetric metabolic assay alamar blue (alamarBlue™, ready-to-use solution from Thermo Fisher Scientific, Monza, Italy); briefly, the alamar solution was added to each composite (1 mL/each) and incubated in the dark for 3 hours. Then, the fluorescence was measured by a spectrophotometer (Spark, from Tecan, Basel, CH) at 570 nm wavelength. Results were expressed as relative fluorescent units (RFU) and GG-lignin-free hydrogels were considered as 100% control. The viability of the cells was visually checked by fluorescence staining using the Live/Dead assay (L/D from Thermo Fischer Scientific, Monza, Italy); briefly, living cells cultured within specimens' pores were stained with the fluorescent dyes for 4 hours and then observed with a confocal microscope (TCS SP8 LIGHTNING, from Leica Microsystems, Wetzlar, Germany) to detect the viable ones marked in green. To determine the % of viable cells, hydrogels were homogenized by trypsin (0.75%) – collagenase (1 mg/mL) solution and cells collected and counted by the Bürker chamber; after mixing with trypan blue to distinguish live and dead cells, the number of viable ones were normalized towards the GG-lignin free control and expressed as % of it. Finally, after fixation in 4% paraformaldehyde (20 min, room temperature) and embedding in the cryopreservation solution Kilik (from Biosigma, Milan, Italy), specimens were 5 μ m sliced with a cryostat machine and stained with Hematoxylin & Eosin to confirm the presence of

high-density cells and extracellular matrix within the hydrogels with lignin. Images were collected with a digital light microscope (EvoSFluid, from Thermo Fisher Scientific).

2.5.3 Chondrogenesis

3D-composites were prepared as previously described in section 2.5.2 and equilibrated 24 hours in maintenance medium (DMEM 15% FBS). Afterwards, basal medium was removed and replaced by the chondrogenic one composed of DMEM High Glucose (4.5 g/L) supplemented with 10% ITS+1 Premix Tissue Culture Supplement, 10^{-7} M dexamethasone, 1 μ M ascorbate-2-phosphate, 1% sodium pyruvate and 10 ng/mL transforming growth factor-beta 1 (Tgf- β 1) [26]. The hydrogels were cultured for 21 days in chondrogenic medium prior to evaluate hMSC chondrogenic differentiation by real-time PCR and histology. For the gene expression analysis, specimens were homogenized by TRIzol reagent (Merck), then RNA was isolated with isopropanol and reverse transcribed using a TaqMan kit (Applied Biosystems, Monza, Italy). For real-time PCR, TaqMan Gene Expression Assays (Applied Biosystems, Monza, Italy) were used on a GeneAmp 7500 Real Time PCR System (Applied Biosystems, Monza, Italy) using the 18S rRNA (Applied Biosystems cod. 4310893E) as housekeeping gene. The expression of the chondrogenic genes collagen II (COL 2), aggrecan (ACAN), and SRY-Box Transcription Factor 9 (SOX 9) was normalized towards the GG-lignin free control by means of the $\Delta\Delta$ Ct method. For histological analysis, specimens were cryo-embedded as previously described and 5 μ m cryo-slices were stained with Alcian blue, Safranin-O and Picrosirius red. Images were collected with digital light microscopy (EvoSFluid, from Thermo Fisher Scientific).

2.5.4 Antibacterial evaluation

Bacterial strains were obtained by the certified American Type Culture Collection (ATCC, Manassas, USA) and cultured following Manufacturer's instructions. Accordingly, the joint pathogens *Staphylococcus aureus* (*S. aureus*, ATCC 43300) and *Staphylococcus epidermidis* (*S. epidermidis* ATCC 14990) [27] were cultured 24 hours at 37°C, 95% humidity onto selective Mannitol Salt Agar plates (from Sintek, Milan, Italy) until the formation of round colonies. Afterwards, few colonies were collected, transferred into Luria-Bertani broth (LB) and cultured 2 hours at 37°C in agitation

(120 rpm) to reach the exponential growth phase; then, the optical density was adjusted diluting with fresh LB broth until 0.001 optical density at 600 nm corresponding to 1×10^5 bacteria/mL [26]. Accordingly, each specimen was submerged with 1 mL of the bacterial suspension and incubated for 48 hours at 37°C in agitation (120 rpm) to avoid bacteria aggregation. Afterwards, specimens were carefully washed 3 times with PBD to remove non-adherent bacteria whereas adherent biofilms were detached by vortex and sonicator (3 times each, 30 seconds). The number of viable bacteria was counted by the colony forming unit (CFU) method by the equation 2 [28]:

$$CFU = \frac{\text{OD} \times \text{Volume of sample} \times 10^6}{\text{OD of standard} \times \text{Volume of standard}} \times 10^{-\text{Dilution factor}} \quad (2)$$

2.6 Bioprinting of the hydrogel

Hydrogel's bioprinting was assessed with a Rokit Invivo 3D Bioprinter (RokitHealthcare, Seoul, South Korea), equipped with a temperature-controlled bio-dispenser to extrude bioinks formulation in a sterile environment. First, to evaluate the printability of the inks, the bare formulations of GG_{HL} with the addition of lignin at 0% (GG_{HL}), 0.1% (GG_{HL}Lign0.1), 0.2% w/v (GG_{HL}Lign0.2) and 0.4% w/v (GG_{HL}Lign0.4) were extruded at 42 °C on a printing bed, whose temperature was set at 30°C since the formulation's viscosity increases with the decrease of temperature. Two multi-layered structures were realized using the NewCreatorK software provided by Rokit Healthcare and printed with both formulations: a squared grid structure (12mm x 12mm x 2mm) and a concentric cylindrical structure (12mm diameter x 2mm height). A 27G tip was selected for the extrusion from the syringe with a printing speed of 7mm/s. Samples were printed on a plastic Petri dish and post-processing crosslinked with the addition of magnesium ions as described before. Images of the printed constructs, whose formulations were able of being extruded (GG_{HL}Lign0.2 and GG_{HL}Lign0.4) were obtained with a stereomicroscope equipped with a digital colour camera (Leica Microsystems, Wetzlar, Germania) and the grid-like shape with the GG_{HL}Lign0.4 was selected for the further bioprinting.

The bioprinting process was exploited loading the GG_{HL}Lign0.4 hydrogel formulation with human TERT immortalised bone marrow stromal cells (hMSC) to obtain a bioink. hMSC were

cultured as reported before in section 2.5.1. Briefly, cells were grown at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS, 2mM L-glutamine and a 1% P/S. When 80% confluent, cells (passage 84-85) were detached using trypsin EDTA, centrifuged at 1200 rpm for 5 min and suspended in the GG_{HL}Lign0.4 solution at a concentration of 7×10⁶ cells/mL for simulating the cells density of the middle layer of articular cartilage [29]. The resulted bioink formulation was transferred to the printing cartridge of the Rokit Invivo 3D bioprinter and extruded with the same parameters optimized for the acellular formulation. Once bioprinted, the constructs were cultured in a 24-well plate, changing the cell media every two days, at 37°C and 5% CO₂.

Cell viability assays were performed to evaluate the impact of the bioprinting process on cell viability at day 1, day 3 and day 7 of samples culture. It was used a qualitative fluorescence-based kit (ReadyProbes® Cell Viability Imaging Kit; Thermo Fisher, UK) to assess cells viability, according to manufacturer's instructions. This kit combines NucBlue® Live reagent (Hoechst 33342) which stains in blue the nuclei of all the cells and NucGreen™ Dead reagent which stains in green the nuclei of cells with only nuclei of cells with compromised plasma membrane integrity, in order to obtain viability information by comparing total vs dead cells. Images were collected using an EVOS M5000 imaging system (Thermo Fisher, UK). To quantify cells metabolic activity, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was used at day 1, day 3 and day 7 of constructs culture. MTS solution was prepared diluting CellTiter 96® AQueous One Solution Reagent in phenol red-free DMEM supplemented with 10% FBS and 1% P/S (1:5). 500µL of MTS solution were added to the samples, incubated at 37°C in a humidified 5% CO₂ atmosphere for 3 h and then 100µL of the solution was transferred into a 96-well plate in triplicate for each sample. Absorbance was recorded at 490nm using a FLUOstar® Omega multi-mode reader (BMG Labtech, Pero, Italy).

2.7 Statistical analyses

Biological evaluations were performed by using 6 replicates for each assay; first, data normal distribution and homogeneity of variance were confirmed by the Shapiro-Wilk's and the Levene's

test, respectively. Then, groups were compared by the one-way ANOVA using the Tukey's test as post-hoc analysis. For the cells viability of the bioprinted constructs, as well as for the in vitro antioxidant assays, the statistical significance of the results obtained was evaluated by GraphPad Prism Software (v. 8.4.1) using One-way ANOVA with repeated measurements. Tukey's post hoc test was carried out to highlight the main factors determining data variability. Statistical significance was set at $*p < 0.05$.

3. Results and Discussion

3.1 Physico-chemical and morphological characterization of the hydrogel

3.1.1 X-ray Photoelectron Spectroscopy (XPS)

XPS has been employed to investigate the surface chemical composition of the hydrogel. In Figure 1, C1s regions, peak fittings and attributions relevant to lignin (a), GG_{HL} (b) and GG_{HL}Lign0.4 (c) were reported. The deconvolution of the lignin C1s spectrum (Fig. 1a) was performed using a model consisting of five components.

The C1 component, having a binding energy of 284.5 eV, corresponds essentially to non-functionalized carbon atoms located in the lignin aromatic rings, representing the 33.4% of the total C1s area. The other components are reported in the relevant table and indicated the presence of alcoholic, cheton and carboxylic acid functionalities. The carboxylic acid groups were likely linked to lignin-derived phenolic acids (such as vanillic, p-hydroxybenzoic, syringic acid etc).

The fitting of the C1s of GG_L, with five peak components, was already reported [9] and showed a ratio between O-C-O and COOR groups equal to 5:1. In the case of GG_H (data not shown), the curve fitting, also performed with five components, highlighted a lower O-C-O/COOR ratio (i.e., 2:1), as expected by the high presence of acetate and glycerate groups.

Considering the C1s signal of GG_{HL} (Figure 1b), the peak deconvolution was still performed with five components and a O-C-O/COOR ratio of about 4:1 was indicative of the presence of both high

and low acyl gellan contributions and in good agreement with the feed GG_H/GG_L ratio (indeed, considering that $GG_H:GG_L$ was 3:2, a O-C-O/COOR ratio equal to 3.8 was expected).

In the case of the C1s signal of $GG_{HL}Lign0.4$ sample (Figure 1c), lignin presence was evidenced in the curve fitting, since an additional peak at 284.4 eV, referring to the carbon atoms located in aromatic rings, was clearly detected.

The $GG_{HL}Lign$ composites, containing lignin at 0.1 or 0.2% were also examined and showed the aromatic peak at 284.4eV, characteristic of the lignin, with an amount directly related to the lignin content.

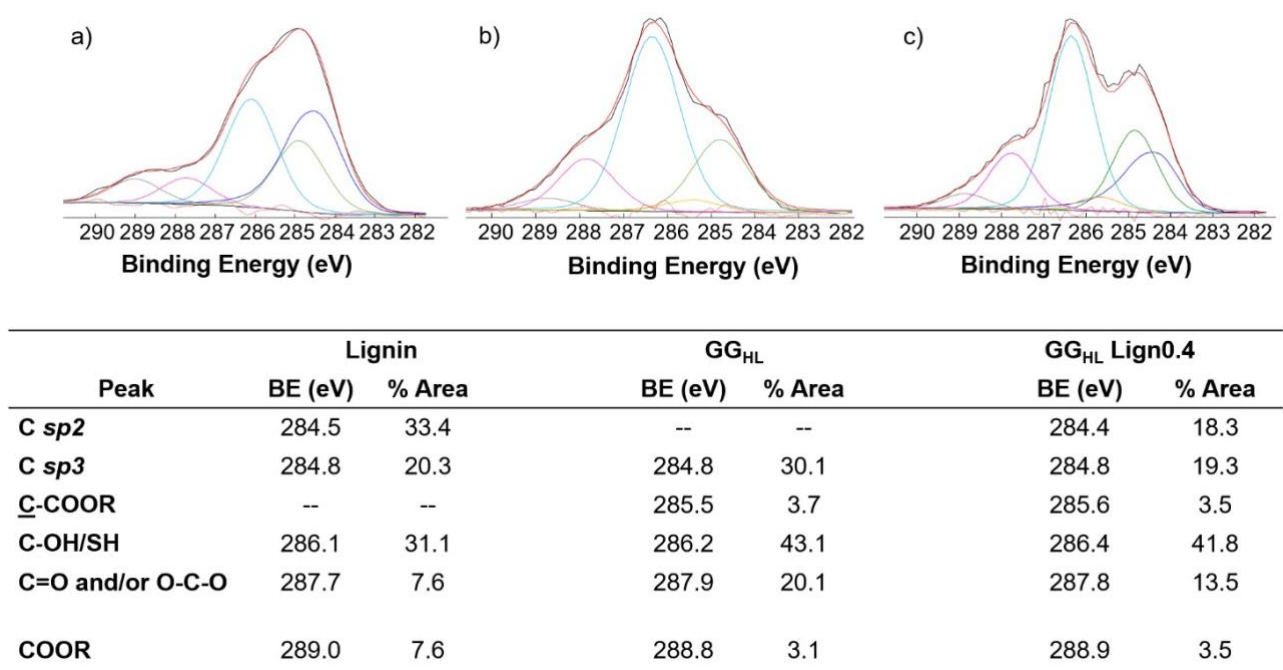


Figure 1: C1s curve fitting and relevant attributions of lignin (a), GG_{HL} (b) and $GG_{HL}Lign0.4$ (c). The maximum error on the binding energy values was equal to +0.2 eV.

3.1.2 Solid-State Nuclear Magnetic Resonance (SS-NMR)

Solid-state ^{13}C NMR spectra were recorded on lignin, GG_H and GG_L , as well as on the composite $GG_{HL}Lign0.4$, i.e., the system containing the highest lignin amount, in order to gain insight on the possible interactions occurring among the GG moieties and lignin. Figure 2a displays the 1H - ^{13}C CP MAS spectrum of lignin. Signals between 8 and 100 ppm, between 100 and 160 ppm, and between 160 and 220 ppm are ascribable to aliphatic carbons, aromatic carbons and carbonylic carbons (due

to cheton, aldehyde and carboxylic acid functionalities), respectively. The most intense peak of the entire spectrum is that at δ 56 attributable to OCH_3 moiety [30]. The ^1H - ^{13}C CP MAS spectra of GG_H and GG_L are displayed in Figure 2b-c. In these spectra are present signals ascribable to the methyl group of rhamnose (at 18 ppm), the CH_n carbons of glucose, rhamnose and glucuronic acid (between 53 and 93 ppm) and the anomeric carbons of the saccharides (at 103 ppm). The ^1H - ^{13}C CP MAS spectrum of GG_H showed an intense peak at δ 174 attributable to the carboxylic carbon of the acyl and glycerate moieties present in the “high acyl” gellan gum, whereas weak peaks at δ 175 and δ 180, ascribable to the carboxylate carbons of glucuronic acid were present in the ^1H - ^{13}C CP MAS spectrum of GG_L [31]. Sharp signals at δ 21, δ 42, δ 68 and δ 170, ascribable to 3-hydroxybutanoic acid (3-HBA) [32] are also present in the ^1H - ^{13}C CP MAS spectrum of GG_H .

Figure 2d shows the ^1H - ^{13}C CP MAS spectrum of the composite $\text{GG}_\text{HL}\text{Lign}0.4$, which appears as the superimposition of the spectra of lignin, GG_L and GG_H (including 3-HBA). The presence of lignin in the composite is demonstrated by the appearance, in the spectrum of the composite $\text{GG}_\text{HL}\text{Lign}0.4$, of the peak at 56 ppm, which belongs to the methoxy groups of lignin (*vide supra*) and of the peaks between 117 and 150 ppm, which belong to the aromatic carbons of lignin. No specific interaction between lignin and gellan network are highlighted by SS-NMR analysis.

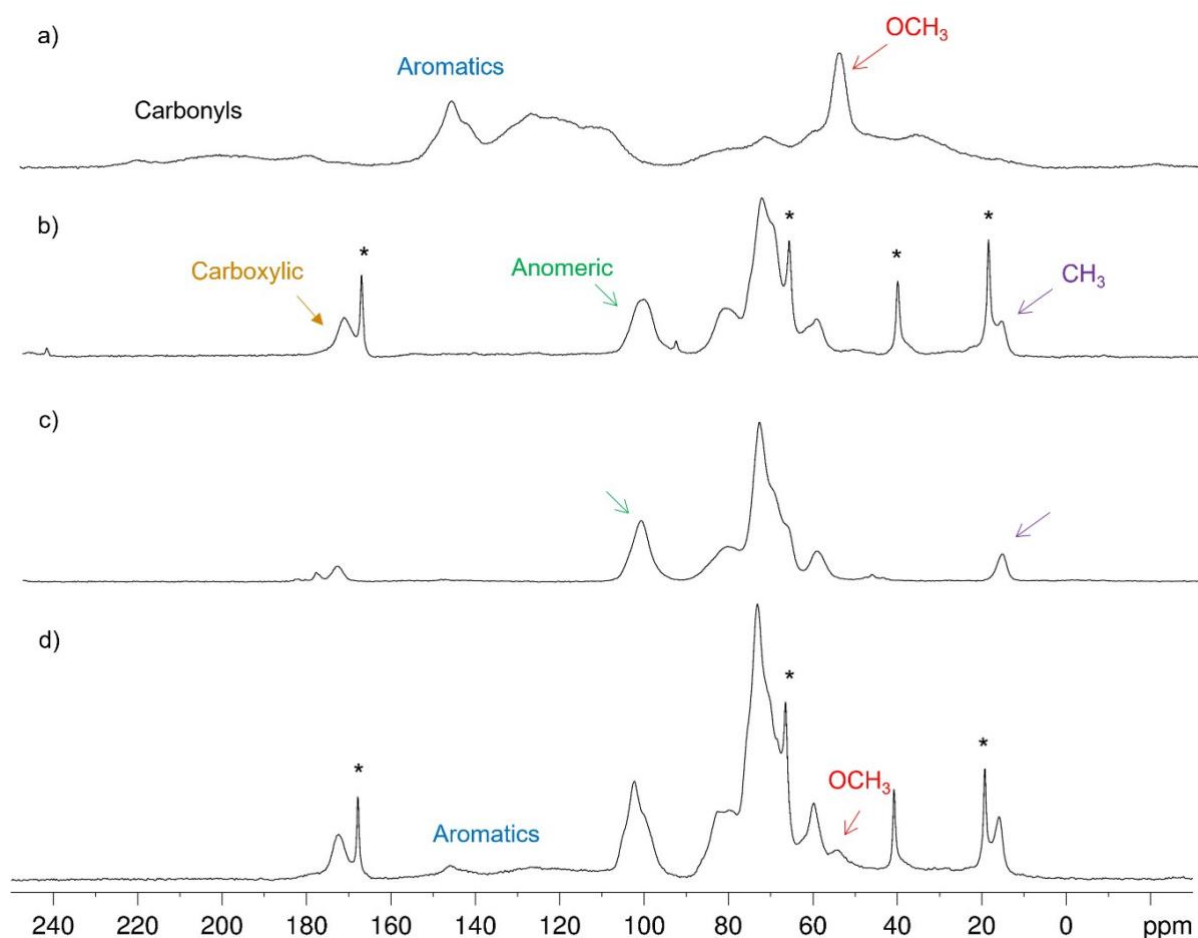


Figure 2. ^1H - ^{13}C CP MAS spectrum of lignin (a, 7.5 kHz), GG_H (b, 7.5 kHz), GG_L (c, 7.5 kHz) and GG_HL Lign0.4 (d, 10 kHz). Asterisked peaks belong to 3-HBA.

3.1.3 Fourier Transform Infrared Spectroscopy (FTIR/ATR)

FTIR spectra were recorded on both the feed materials and on the hydrogels, in order to identify the various functional groups and the specific moieties present in the composite hydrogels.

The typical bands of GG were present in both the low and high acyl forms (spectra reported in the Supplementary data). GG_L (or GG_H) spectra showed the band at 3345 (or 3348) cm^{-1} due to the presence of OH groups of glucopyranose ring. The band at 2924 (or 2925) cm^{-1} was due to the stretching vibrations of CH groups, while those appearing at 1598 (or 1609) and 1409 cm^{-1} were due to asymmetric and symmetric stretching of COO groups. The absorption at around 1032 (or 1024) cm^{-1} was attributable to C-OH stretching. Moreover, the intense peak at 1724 cm^{-1} was evident only in GG_H spectrum and it was assigned to the C=O stretching of acyl group of this GG form.

In Figure 3a, FTIR spectra of pure lignin, GG_{HL} and GG_{HL}Lign0.4 were reported. As far as the alkali lignin sample is concerned, the band at 3370 cm⁻¹ was assigned to the stretching vibration of OH groups, with high intensity due to the introduction of OH groups towards the lignin during alkali treatment. At 2937 cm⁻¹ the stretching vibrations of CH groups were shown, even if a shoulder at wavenumbers > 3000 cm⁻¹ was evident, due to the presence of aromatic CH groups. The occurrence of aromatic groups was also confirmed by the bands at 1590, 1512 and 1417 cm⁻¹, linked to aromatic skeletal vibrations. At 1634 cm⁻¹ the C=O stretching of conjugated carbonyl groups was present. The absorption band at 1264 cm⁻¹ can be attributed to the guaiacyl (G) unit and to the C–O stretch (guaiacyl ring breathing), while the band at 1136 cm⁻¹ could be related to the C–H in-plane deformation of the guaiacyl (G) unit [33].

GG_{HL} and GG_{HL}Lign0.4 samples showed typical absorption bands of the GG_L and GG_H forms, with the evident C=O stretching vibration of the GG_H moieties at 1724 cm⁻¹. Asymmetric and symmetric stretching of COO groups fell at about 1606 and 1408 cm⁻¹. The presence of lignin moieties in the composite hydrogel was observed by the presence of the C=O stretching of conjugated carbonyl groups at 1636 cm⁻¹.

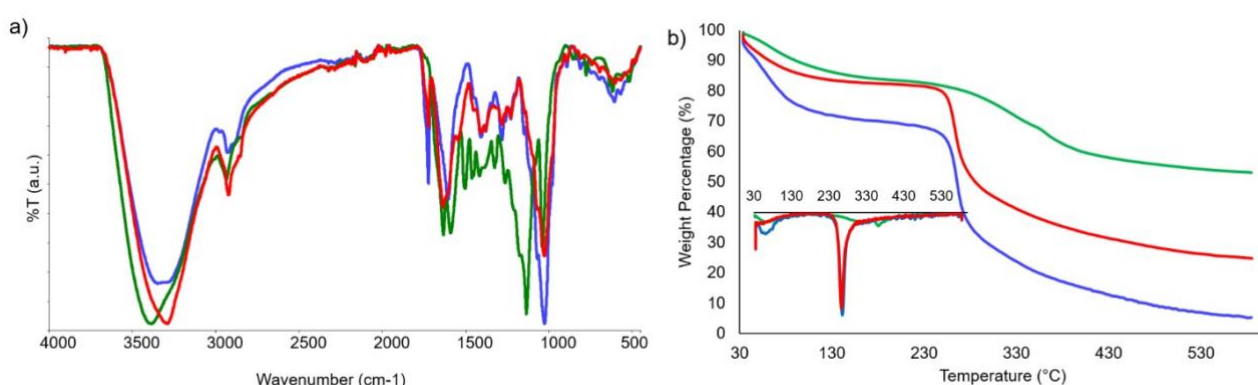


Figure 3: FTIR spectra acquired in ATR mode (a) and TGA (b) of lignin (green line) GG_{HL} (blue line) and GG_{HL}Lign0.4 (red line). In the panel (b), the inset reported the DTGA traces of the same samples.

3.1.4 Thermo-Gravimetric Analysis (TGA)

In Figure 3b, the TGA and DTGA traces of pure lignin, GG_{HL} and GG_{HL}Lign0.4 samples are reported. The weight loss of the lignin sample was due to the moisture removal, as observed by a small peak centred at 66°C on the DTG curve. This weight loss is considered as the moisture removal or drying stage. Then, a large peak in the temperature range of 200-400 °C was observed. This wide degradation step can be related to the fact that lignin is a complex structure composed of carbonyl, benzylic and phenolic groups [34]. The residue at 600°C was 54%. This high residue content could be related to the ability to cross-link exerted by the aromatic rings at high temperatures, giving rise to a re-polymerization, as reported by Amit and coworkers [33].

As regard as GG_L and GG_H, a water moisture of about 9 % for both the polymers and a main pyrolytic peak was observed at 269 and 266°C, with a residue at 600°C of 23 and 17% for low and high acyl GG, respectively (data not reported).

In GG_{HL} sample, a high moisture content was recorded (about 30%). The main decomposition peak fell at 265°C and a residue at 600°C of 8% was observed. Finally, GG_{HL}Lign0.4 showed a lower moisture content (about 16%), probably due to the incorporation of a more hydrophobic backbone, such as lignin [35]. The decomposition peak fell at 264°C and the residue a 600°C was 26%. The higher residue in the latter material can be related to the presence of lignin.

3.1.5 *In vitro* radical scavenging activity of the hydrogel and lignin *in vitro* release

Lignin consists in a complex network of polyaromatic molecules with powerful radical scavenging activity, due to its reactive functional moieties (i.e. phenolic, carbonyl, hydroxyl and aliphatic groups). Taking this into account, the antioxidant features of the bioprintable hydrogels embedding lignin have been investigated *in vitro* through DPPH and ABTS assays. Beyond the comparison with ascorbic acid, exploited as reference compound, the performances of the hydrogels embedding lignin have been compared to lignin-free hydrogels, to highlight the impact of lignin on the radical scavenging activity.

Lignin-free hydrogels, as expected, did not show antioxidant features, while GG_{HL}Lign0.1, GG_{HL}Lign0.2 and GG_{HL}Lign0.4 effectively scavenged DPPH and ABTS free radicals (Fig. 4a),

achieving up to 83% and 76% of ascorbic acid RSA% on DPPH and ABTS radicals, respectively. The obtained results were in good agreement with those reported by Dominguez-Robles et al. [36]. For both assays, it is worth to note that the antioxidant features were not explained by lignin content. Indeed, GG_HLign0.2 and GG_HLign0.4 equally scavenged both DPPH and ABTS radicals ($p>0.05$), while GG_HLign0.1 showed slightly reduced scavenging performances in both assays (64 and 65% for DPPH and ABTS, respectively; $*p<0.05$).

Furthermore, to shed light on the potential loss of antioxidant features due to lignin diffusion, the *in vitro* release of lignin from the bioprintable hydrogels was studied in PBS at 37°C up to 7 days (Fig. 4b). For the three prepared formulations, lignin release was scarce (<200 µg/mL). The plateau was reached after 72 h of incubation. Considering the low amount of lignin released *in vitro* after 7 days, it could be argued that the hydrogels' scavenging activity was mainly driven by contact mechanisms, in agreement with literature findings [37]. In addition, this hypothesis supported the obtained RSA% results, that did not directly correlate with lignin amounts added in the hydrogels.

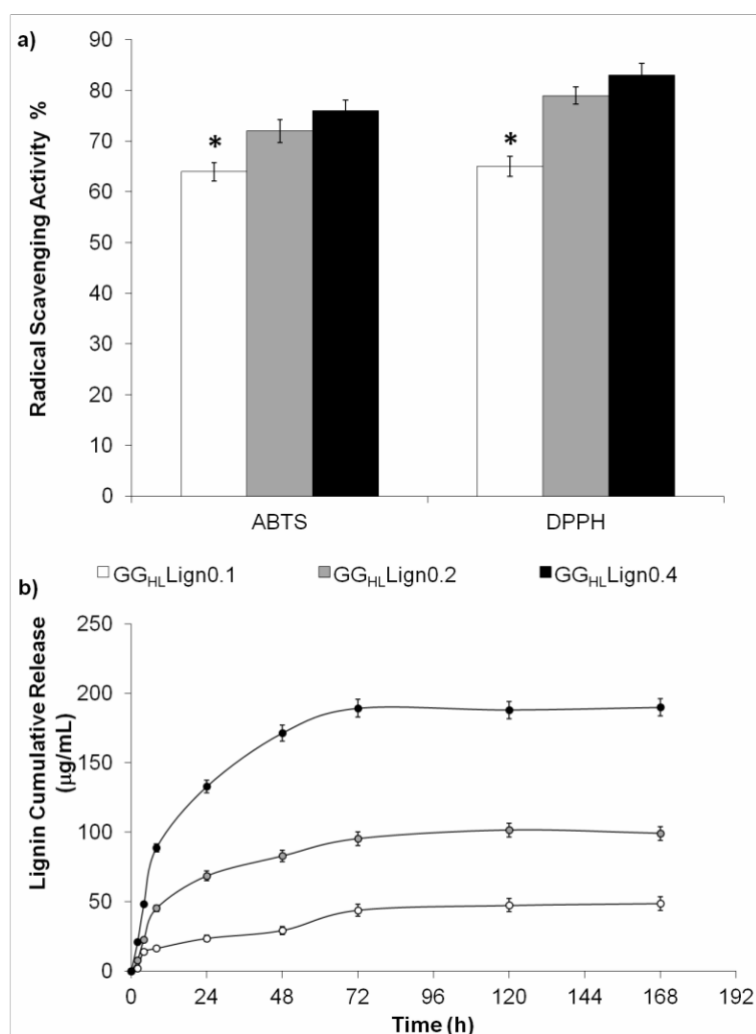


Figure 4: *In vitro* antioxidant activity on ABTS and DPPH radicals elicited by GG_{HL}Lign0.1, GG_{HL}Lign0.2 and GG_{HL}Lign0.4 (a). Data are expressed as % of ascorbic acid radical scavenging activity on the same radicals. (b) Lignin *in vitro* cumulative release from the bioprintable hydrogels, monitored up to 7 days. The test was performed in PBS at 37°C.

3.2 Mechanical characterization of the hydrogel

The mechanical properties of the obtained hydrogels were tested under static compression. Figure 5a shows the obtained stress-strain curves for both GG_{HL} and GG_{HL}Lign0.4 samples. During the test, the lignin-containing hydrogel underwent up to densification regime, which can be due to enhanced proximity between lignin components and polymer chains under complete compression with a strain increase, leading to a quick increase of the stress [38]. It is noteworthy that this hydrogel formulation completely recovered the original shape after removing the applied load. This behaviour was due to the addition of lignin, as also recently reported by Chen et al. [39]. In the mentioned work,

polyacrylamide/lignin nanoparticles (PAM/LNP) hydrogels demonstrated their high robustness and elasticity. In contrast, pure lignin-free GG_{HL} hydrogels were easily broken when a modest compression load was applied (break at ~23% strain). Furthermore, as shown previously by the authors [9], different mechanical properties can be calculated from the curves, including the elastic modulus, collapse strength and strain. These values are reported in Figure 5b.

A 3-fold increase of the compressive Young's modulus was noted after the addition of lignin into the gellan-gum based hydrogel formulation (37 ± 3 kPa for GG_{HL} and 119 ± 7 kPa for GG_{HL}Lign0.4). Moreover, the addition of lignin into the GG hydrogel formulation caused a significant shift in the transition point from linear to the collapse region to higher values (from 2.1 ± 0.4 kPa to 4.8 ± 0.9 kPa for σ^* while from $7.2 \pm 1.3\%$ to $11.6 \pm 1.8\%$ for ϵ^*). The resulting values are higher or within the range of the compressive properties reported for gel formulations proposed for cartilage regeneration [40, 41].

The observed mechanical strengthening can be due to the additional energy-dissipating mechanism introduced by the lignin fillers in the polymeric hydrogel formulation. Based on molecular dynamics principles, as described by Shah et al. [42], the fillers can orient and align within the deformation process during the compression tests. This phenomenon can create additional temporary crosslinking between the chains of the polymeric formulation, developing local areas of improved strength.

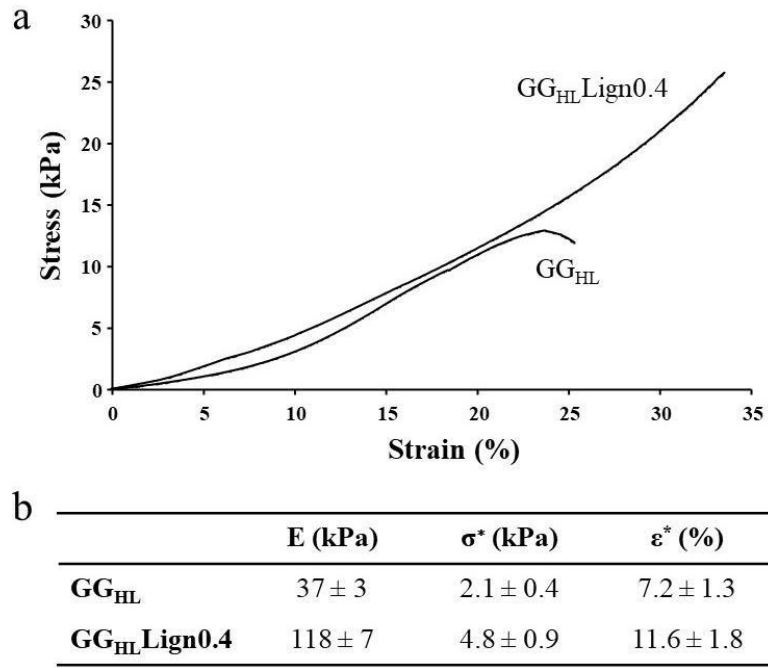


Figure 5: Stress-strain curves obtained by static compression test (a) and calculated values of the elastic compressive modulus (E), collapse strength (σ^*) and collapse strain (ϵ^*) for both GG_{HL} and GG_{HL}Lign0.4 samples (b).

3.3 In vitro biological evaluations

3.3.1 Cytocompatibility of the hydrogel

Despite the natural origin making lignin an interesting and abundant source for biomaterials applications, only a few literature deals with its exploitation for regenerative medicine purposes. To give some examples, Zhang et al. [43] proposed the use of lignin to produce spherical colloidal particles in combination with cellulose nanofibril-alginate hydrogel as a platform for 3D cultures; similarly, Quraishi et al. [44] combined lignin with alginate to produce cytocompatible aerogels for tissue engineering applications. Danti et al. [45] described the use of lignin-derived nanofibers for skin regeneration whereas Witzler et al. [46] recently reviewed lignin exploitation as drug delivery system. From these studies, the lignin was in general described as cytocompatible and suitable for the development of biomaterials; moreover, Inoue et al. [47] gave a first demonstration of the direct involvement of lignin within the Wnt pathway driving embryonic stem cells neuroectodermal differentiation. However, to the best of our knowledge, no previous literature reported about the formulation of a printable hydrogel, based on the combination of lignin with gellan gum. In addition,

the amount of lignin exploited radically changes in literature according to the aim. Therefore, a cytocompatibility evaluation of hydrogels embedding lignin at different concentrations was here assessed by directly culturing hMSC within the scaffolds' pores, in order to simulate the tissue ingrowth during the healing process. Results are reported in Figure 6.

In general, a lignin-dose dependent decrease in cells metabolic activity (a) and % of viability (b) was observed after 1-week culture in 3D condition. However, the values were not significant ($p>0.05$) and $>70\%$ in comparison with GG_{HL} lignin free hydrogels that were considered as positive control due to the well-established cytocompatibility of the gellan gum [48]. As a confirmation, when the 3D composites were stained by the fluorescent live/dead assay (c) and visually observed by confocal microscopy, a high-density of viable cells (stained in green) in tight contact to each other was appreciated within all the test (GG_{HL}Lign0.1, 0.2 and 0.4) and control (GG_{HL}) specimens' pores. Moreover, when the 3D composites were evaluated by Hematoxylin and Eosin staining (d), the entire structure of the hydrogels was observed, and the pores were confirmed as successfully filled by cells (nuclei stained in blue/dark violet) surrounded by extracellular matrix (ECM, stained in pink/light violet). Again, no macroscopic differences were observed in terms of cells' density and ECM deposition by comparing test and control specimens, thus giving a further confirmation of the cytocompatibility of the developed GG_{HL}Lign composites. Taking into account all of these results, the possibility to formulate a bioprintable hydrogel combining lignin 0.1-0.2-0.4% with gellan gum seems to be in line with previous literature suggesting the cytocompatibility of lignin aimed at biomedical applications.

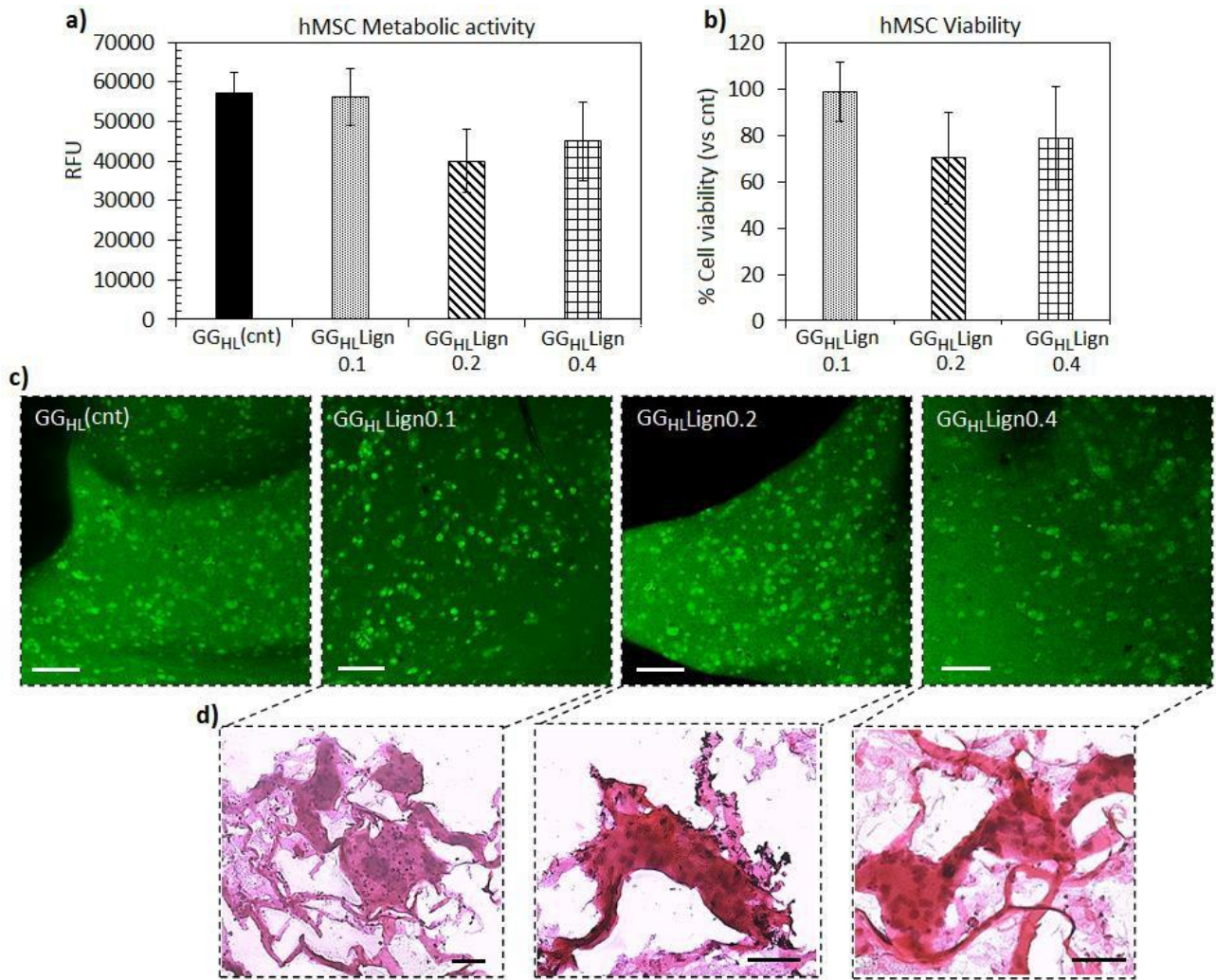


Figure 6. Cytocompatibility evaluation. The metabolic activity (a) and the % of viable cells (b) were not significantly decreased by the introduction of lignin ($p > 0.05$) in comparison with the lignin-free control. As a confirmation, the fluorescent live/dead assay (c) showed a high density of viable cells (stained in green) within all the test and control specimens, as well as the Haematoxylin and Eosin staining (d) revealed the presence of a dense ECM (stained in pink) surrounding the cells (nuclei stained in blue). Images bar scale=100 μ m.

3.3.2 Chondrogenesis evaluation

After confirming the cytocompatibility of the bioprintable GG_{HL}Lign 3D-composites, the opportunity to induce the chondrogenic differentiation of the seeded hMSC was investigated in view of their potential application for cartilage defects repair. Again, current literature provides very few examples of biomaterials carrying lignin aimed at cartilage repair; Liang et al. [49] reported about the possibility to promote osteochondral defects repair by the use of implantable membranes composed of poly (ϵ -caprolactone) and lignin nanofibers thanks to their antioxidant efficacy. Kouhi et al. [21] preliminary

evaluated the cytocompatibility of gellan-gum hydrogels doped with lignocellulose nanofibrils enriched with melatonin-loaded forsterite nanoparticles that were suggested for cartilage repair as potential future application. Due to the narrowness of these few studies, the ability to support chondrogenesis of the bioprintable GG_{HL}Lign composites was evaluated after 21 days of differentiation with chondrogenic medium and compared to the GG_{HL} lignin free control. The expression of the chondrogenic genes aggrecan (ACAN), collagen type II (COL 2) and SRY-Box Transcription Factor 9 (SOX 9) of cells seeded into GG_{HL}Lign (0.1-0.2-0.4) hydrogels were normalized towards the lignin-free control to highlight the contribute of the lignin to promote hMSC chondrogenic differentiation. Results are reported in Figure 7.

In general, the presence of lignin seems to improve the differentiation route, but in a dose-dependent manner. Indeed, only the 0.2 and 0.4% of lignin determined an over-expression of the above-mentioned genes, whereas the 0.1% was comparable to the GG_{HL} lignin free control. As a confirmation, the 0.4% determined the highest over-expression of chondrogenic genes ACAN (a), COL 2 (b) and SOX 9 (c) that resulted as significant towards both the 0.1 and 0.2% lignin ($p < 0.05$, indicated by # and §, respectively). To further confirm the strong pro-chondrogenic induction promoted by the 0.4% lignin, the hydrogels were stained by the cartilage ECM specific markers Alcian blue, Picrosirius red and Safranin-O (d). The positive Alcian blue staining confirmed the presence of sulfated and carboxylated acid mucopolysaccharides as well of sulfated and carboxylated sialomucins (glycoproteins) representative of the cartilage-like ECM. Similarly, the Picrosirius red confirmed the presence of high amount of collagen fibers (stained in red) and cytoplasm (stained in yellow). To confirm that such ECM combined with collagen was comparable to the cartilaginous one, the mucin-specific Safranin-O staining was further applied to hydrogels sections; as shown in Figure 7d, the stained ECM was highly positive to Safranin O (ECM in dark red, nuclei in black) thus confirming the previous findings suggesting the positive differentiation of the seeded hMSC towards the cartilage-like phenotype.

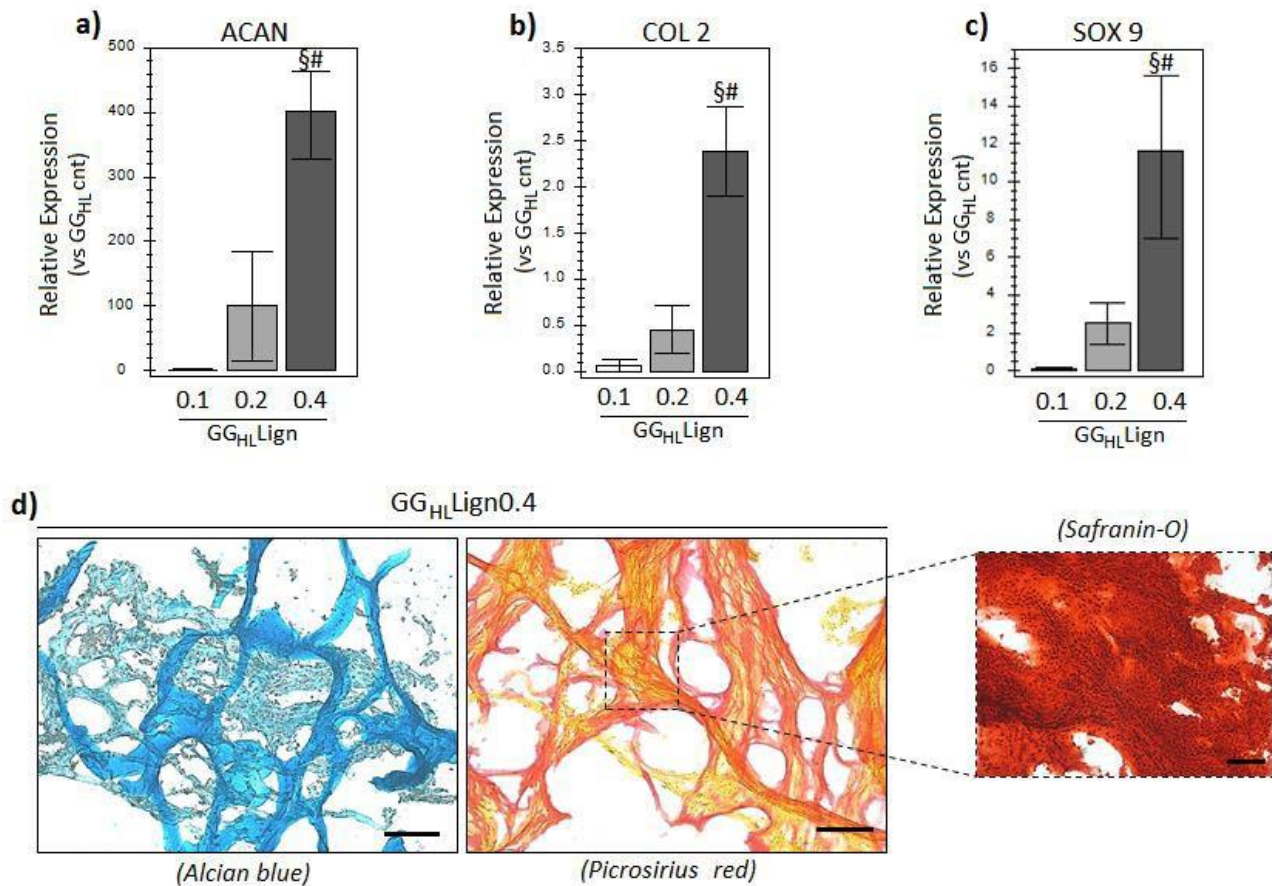


Figure 7. Chondrogenic differentiation. The presence of lignin improved the up-regulation of chondrogenic genes in a dose-dependent manner (a-c) in comparison to the GG_{HL}-lignin free control; accordingly, GG_{HL}Lign0.4 was significant towards both 0.1 and 0.2 ($p < 0.05$, indicated by § and #, respectively). The formation of a cartilage-like ECM was confirmed by histology (d): Alcian blue revealed the presence of mucopolysaccharides (light blue, nuclei in black), Picrosirius red the deposition of collagen fibers (red) within cytoplasm (yellow), while Safranin-O confirmed the incidence of mucins (in red, nuclei in black). Images bar scale=100 μ m.

3.3.3 Antibacterial evaluation

The exploitation of lignin intrinsic antibacterial efficacy for the doping of implantable biomaterials is supported by few evidences in the current literature. Abudula et al. [50] demonstrated that an injectable lignin-co-Gelatin cryogel containing 0.1 or 0.2% lignin was effective in preventing contamination from surgical site infection-associated pathogenic bacteria, showing an 8-10 mm inhibition halo. Furthermore, Eivazzadeh-Keihan et al. [51] demonstrated that a hydrogel based on lignin-agarose hydrogel and silk fibroin embedded zinc chromite nanoparticles was effective in reducing the growth of *P. aeruginosa* by the optical density analysis, but the effect can be mostly

ascribed to the presence of zinc. Other examples can be found dealing with packaging studies [52], whereas lignin is mostly used as carrier to deliver antibacterial ions such as silver and copper [53, 54].

Here the antibacterial properties of the bioprintable hydrogels were evaluated in light of the different lignin concentrations (0.1-0.2-0.4%) towards the main pathogens related to osteochondral infections (*S. aureus*, *S. epidermidis*) [27]. Results are reported in Figure 8.

Overall, a certain antibacterial effect due to lignin was noticed for both *S. aureus* (a) and *S. epidermidis* (b); indeed, a significant reduction was observed when the 0.2 or 0.4% were applied in comparison to the GG_{HL}-lignin free control ($p < 0.05$, indicated by §) or to the 0.1% lignin ($p < 0.05$, indicated by #). The detached colonies after growing into LB agar plates are visible in (c) and (d) for *S. aureus* and *S. epidermidis*, respectively.

Despite these encouraging results, if the starting inoculum ratio was considered (1×10^5 bacteria/hydrogel, indicated by the dashed lines in (a) and (b)), it is evident that a reduction of the bacterial number did not occur. The presence of lignin was effective in preventing bacteria proliferation as they were in the same log phase ($10E5$) of the starting inoculum, while in the GG_{HL}-lignin free controls bacteria number increased of about 2 logs ($10E7$). Therefore, it can be hypothesized that lignin applied in such concentration (in particular 0.2 and 0.4%) in the GG_{HL} hydrogels can confer a bacteriostatic efficacy to the hydrogel, but not an evident antibacterial effect.

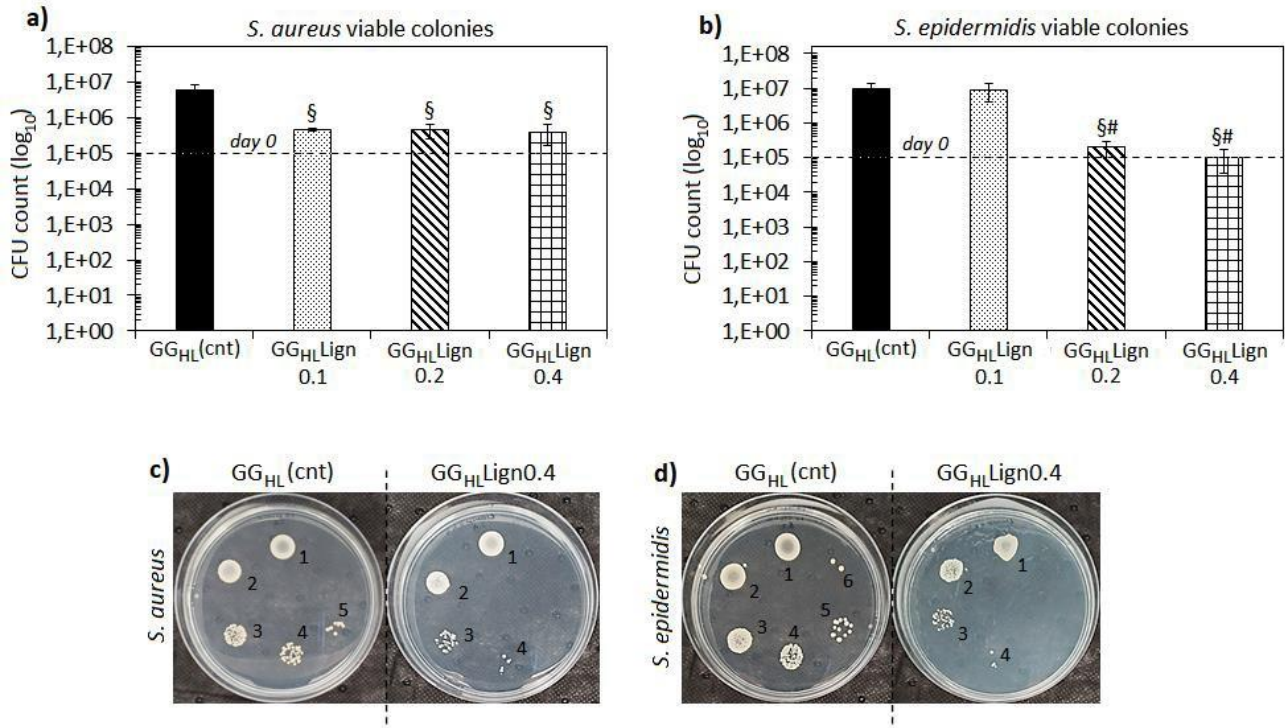


Figure 8. Antibacterial evaluation. The 0.2 and 0.4% lignin doping were effective in preventing both *S. aureus* (a) and *S. epidermidis* (b) proliferation in comparison to the GG_{HL}-lignin free controls ($p < 0.05$, indicated by §) and 0.1% lignin ($p < 0.05$, indicated by #) as visually confirmed by the viable colonies after detachment (c-d). However, the effect was mainly bacteriostatic as the number of starting bacteria (a-b, indicated by the dashed line) was not reduced but only maintained in the previous log ratio (10E5).

3.4 Bioprinting of the hydrogel

3D printing of the GG_{HL}-based formulations was exploited with the Rokit Invivo extrusion-based bioprinter, prior to embedding the formulations with cells for assessing the bioinks bioprinting capability. There were tested a variety of bioink blends at increasing concentrations of lignin (0-0.4% w/v) to determine the optimal composition for printability and promoting shape fidelity of the final construct. The printing process was unsuccessful for the formulations made of bare GG_{HL} and GG_{HL}Lign0.1. Indeed, these formulations were not extrudable continuously at 42 °C, showing the deposition of a discontinuous filament and an inhomogeneous highly viscous formulation. Although high viscosities are needed to extrude a continuous filament, to hold the deposited structure shape preventing its collapse and to obtain homogeneous cell distribution avoiding their sedimentation,

excessively viscous formulations obstruct the syringe nozzle resulting in unsuccessful bioprinting [55, 56].

Conversely, increasing the concentration of lignin to 0.2%, showed good results in terms of formulation printability, as it is shown in Figure 9a-d for both grid and concentric cylindrical shape structures. However, when the amount of lignin was doubled to 0.4% w/v, both the continuity of the filament extrusion and the shape stability of the final construct greatly improved (Figure 9e-h). As already reported in literature, the addition of lignin with the consequent increase in its concentration helps to obtain a shear-thinning behaviour of the polymer. This is one of the key properties for a newly formulated bioink [57, 58], because the shear-thinning behaviour allows (1) the free deposition of bioink filaments due to a temporal decrease of its viscosity inside the nozzle tip when a stress is applied, and (2) its post-printing structural integrity and stability [59]. Recent studies have shown that incorporation of lignin, as well as the incorporation of inorganic fillers, e.g., laponite, within the hydrogel network, can significantly promote the rheological and mechanical properties of the resulting biomaterials, resulting in a fundamental protection of the cells from shear stress caused by bio-extrusion [60].

After the optimization of the printing process parameters (Figure 9i), the obtained constructs were crosslinked with the addition of magnesium ions (via immersion in MgCl_2 solution), obtaining multilayered constructs of high accuracy in dimensions, suitable for biological application and *in vitro* studies.

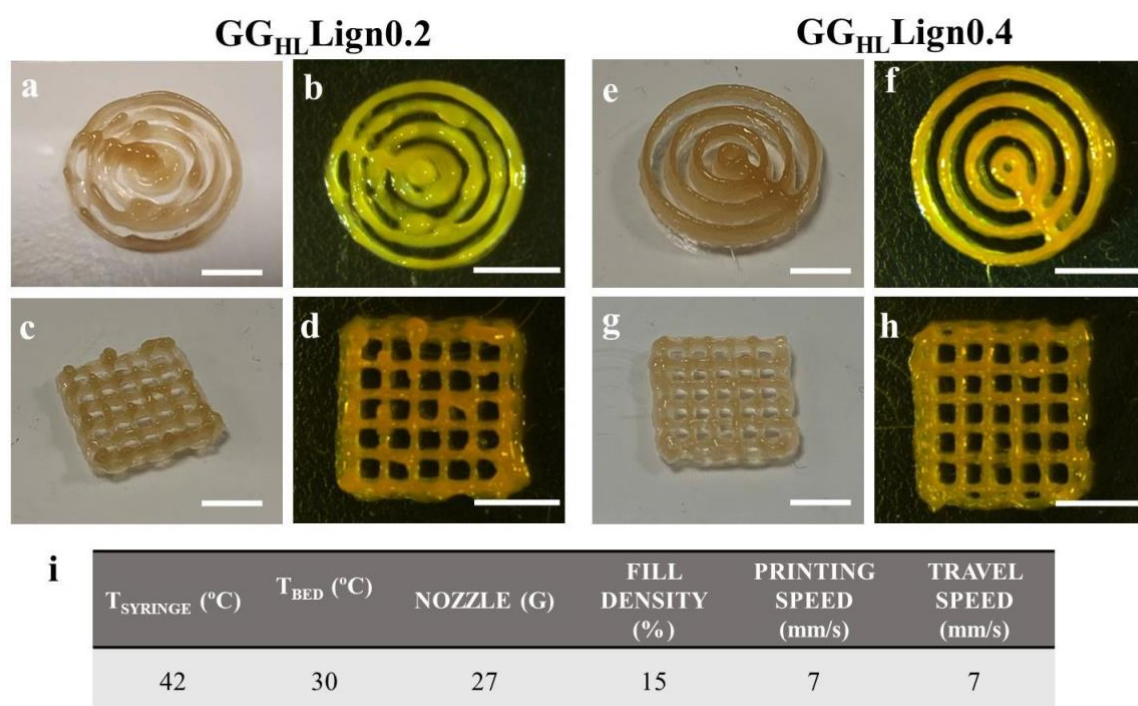


Figure 9. Images of the printing process for the formulation GG_{HL}Lign0.2: Concentric Cylindrical (a,b) and Grid (c,d) structures; and for the formulation GG_{HL}Lign0.4: Concentric Cylindrical (e,f) and Grid (g,h). Bars = 5 mm. Table (i) reports the printing process parameters.

The GG_{HL}Lign0.4 formulation was selected for the bioprinting due to its promising shear-thinning behaviour and chondrogenic features (see section 3.3.2). In this regard, an immortalised human stem cell line (Y201s) was used to demonstrate the viability of cells encapsulated in 3D bioprinted constructs comprising of 6 layers (Figure 10). A ReadyProbes® Cell Viability Imaging Kit was used instead of the Calcein/ethidium homodimer-1 (live/dead staining) for cell characterisation, because of a high background in the red field due to lignin that provided misleading results. Figure 10a demonstrates that cells (with nuclei stained in blue) were distributed homogeneously within the whole structure retaining a round, circular morphology over 7 days of culture. Few dead cells (green) were observed confirming the ability of the bioprintable formulation to promote cells survival and the printing process to not affect the cells viability. Furthermore, MTS results demonstrated the presence of metabolically active cells, which maintained a stable metabolic activity over the 7 days of culture without significant changes.

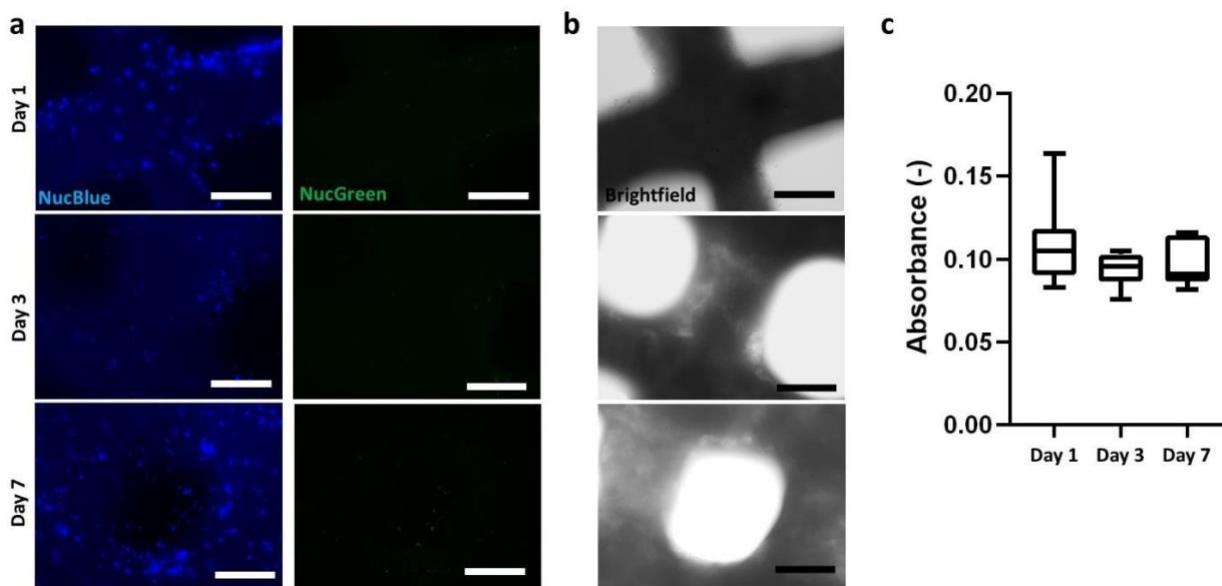


Figure 10 Assessment of the bioprinted constructs viability at day 1, day 3 and day 7. Cells viability assessed with ReadyProbes® Cell Viability Imaging Kit, staining in blue (NucBlue) all the cells nuclei and in green (NucGreen) the dead cells (a). Brightfield images of the structure of the stained constructs (b). Bars = 350 μ m. MTS assay to assess the metabolic activity of Y201s within the bioprinted construct (c).

4. Conclusions

A new gellan gum-based hydrogel formulation was developed in order to prepare 3D-printed scaffolds for the regeneration of cartilage tissue. This challenging matter has been achieved by the incorporation of lignin to a blend of low and high acyl gellan gums. FTIR, XPS and SS-NMR demonstrated no specific interaction between lignin and gellan network, while TGA analysis highlighted the high thermal stability of the composite. The bioprintable hydrogel showed remarkable antioxidant features, as demonstrated by the *in vitro* tests with DPPH and ABTS radicals. These results were due to lignin ability to quench radical species, more likely with contact mechanisms rather than by lignin diffusion. In addition, lignin provided the formulated hydrogels with mechanical properties suitable for cartilage regeneration. Biological evaluations confirmed that lignin is cytocompatible as well as its contribution seems to be effective in supporting and improving chondrogenesis. Moreover, a dose-dependent bacteriostatic effect was observed, suggesting that lignin can be helpful in preventing bacterial infections. Furthermore, the developed composite formulation fulfilled all the specifications for being considered as suitable bioink. Particularly, it

allowed bioprinting of 3D constructs with different geometry and resolution, thanks to the favorable viscosity due to the incorporation of lignin within the gellan gum blend. The bioprinted hydrogels did not adversely affect the encapsulated hMSC, allowing cell proliferation. Considering its processability, biocompatibility and antioxidant activity, this first printable gellan gum/lignin hydrogel will have potential in different applications in tissue engineering, beyond the regeneration of cartilage.

Acknowledgements

A. Scalzone acknowledges support from EPSRC for her Ph.D. studentship (EPSRC Grant EP/R51309X/1).

Data availability

The data required to reproduce these findings are available from the corresponding author upon request.

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All authors have read and agreed to the published version of the manuscript.