From the Sea to the Bee: Gellan gum-Honey-Diatom composite to deliver resveratrol for cartilage regeneration under oxidative stress conditions

Maria A. Bonifacio\textsuperscript{a,b}, Andrea Cochis\textsuperscript{c,\#}, Stefania Cometa\textsuperscript{a,\#}, Piergiorgio Gentile\textsuperscript{d}, Annachiara Scalzone\textsuperscript{a}, Alessandro C. Scalia\textsuperscript{c}, Lia Rimondini, Elvira De Giglio\textsuperscript{a,*}

\textsuperscript{a}Department of Chemistry, University of Bari “Aldo Moro”, Italy
\textsuperscript{b}Jaber Innovation s.r.l., Italy
\textsuperscript{c}Department of Health Sciences, Center for Translational Research on Autoimmune & Allergic Diseases CAAD, Università del Piemonte Orientale UPO, Novara, Italy
\textsuperscript{d}School of Engineering, Newcastle University, UK

These authors equally contributed to the work.

Contacts:

*Elvira De Giglio
Department of Chemistry, University of Bari “Aldo Moro”, via E. Orabona 4, Bari, 70126, Italy
elvira.degiglio@uniba.it
Tel./Fax: +39 0805442021
Abstract

Carbohydrate-based porous scaffolds are promising biomaterials to support cartilage regeneration. In this respect, their composition could be designed to face clinical challenges, i.e., articular load bearing, infections and oxidative stress. Herein, an innovative scaffold has been developed, combining raw materials belonging to different kingdoms of life. Indeed, gellan gum, a bacterial-derived carbohydrate, was blended with a beehive product (Manuka honey) with prominent antibacterial features. Moreover, resveratrol, a phytoalexin with powerful antioxidant activity, was loaded into the silica shells of diatoms, unicellular microalgae with cytocompatible features. The developed composite porous scaffolds demonstrated mechanical properties suitable for cartilage regeneration. Furthermore, they allowed the controlled release of resveratrol, hindering bacterial proliferation and oxidative stress damage, while supporting stem cell colonization and chondrogenic differentiation.

Keywords

Gellan gum; diatoms; composite; resveratrol; antioxidant; cartilage regeneration.

1. Introduction

Cartilage diseases represent an unmet clinical need, affecting both young and ageing individuals, with subsequent heavy costs for healthcare. Current treatment strategies fail to achieve full tissue regeneration, leading to function loss while paving the way for chronic pathologies (Martín et al., 2019). In this respect, inflammation is one of the main actors involved in cartilage degeneration (van der Kraan, 2019). Indeed, the ultimate strategy to reduce joint injuries consists in controlling inflammation in situ, e.g. by intra-articular injection of glucocorticoids or cytokine inhibitors (Rudnik-Jansen et al., 2019). Therein, non-steroidal anti-inflammatory drugs and other anti-inflammatory molecules are already being used to enhance joint functions while soothing pain (Prince et al., 2019). Resveratrol (RESV), a polyphenolic compound, is a powerful anti-inflammatory, immunomodulatory and antioxidant molecule commonly found in plants, where it works as a phytoalexin against
pathogens. Csaki and coworkers (2008) demonstrated that RESV regulates inflammation signaling in human chondrocytes in vitro. However, the limited water solubility of RESV, combined with its rapid removal from human blood, hinders the exploitation of RESV in regenerative medicine. In recent years, few studies used electrospinning to load RESV during the preparation of scaffolds for cartilage repair (Poornima & Korrapati, 2017). Nevertheless, a composite scaffold made of an organic network and an inorganic vehicle, loaded with RESV, has never been described. In this work, we report for the first time the preparation of a resveratrol-loaded composite scaffold, focusing the attention on the interaction between the carbohydrate-based three-dimensional network and inorganic clays. To this regard, diatomaceous earth microparticles were embedded within the gel, aiming to load, protect and deliver RESV in situ. The scaffold composition was designed with a cross-kingdom approach, exploiting only Nature-derived materials, from bacteria, sea microalgae, plant extracts and beehive products. Indeed, the organic network of the composite scaffold consists in the combination of gellan gum, a bacterial-derived polysaccharide, and Manuka Honey, a powerful antibacterial mixture of carbohydrates. Previous works demonstrated the biocompatibility and the ability to support chondrogenesis of gellan gum-based systems (Bonifacio et al., 2020a;2018). Herein, diatomaceous earth was added to achieve an optimized loading and release of RESV, while reinforcing the mechanical properties of the composite scaffold. To study the antioxidant features of the scaffold, hydrogen peroxide (H₂O₂) was used to induce oxidative stress in vitro, thus simulating an inflammatory condition.

This work focuses on the preparation and characterization of the first resveratrol-loaded composite scaffold reported in literature, with a particular attention to the interactions between the carbohydrate-based three-dimensional network and the inorganic clay, loaded with the bioactive molecule. A schematic representation of the experimental design of this study is reported in Fig. 1. We hypothesized that a gellan gum-based composite scaffold holds suitable mechanical properties to stimulate cartilage formation, providing antioxidant properties through resveratrol release.
Figure 1. Experimental design of the study. The DE/RESV system was optimized, characterized and embedded in the GG-MH-DE porous scaffold. The latter was characterized from the physico-chemical, mechanical and biological point of view.

2. Materials and methods

2.1 Materials

Gellan gum is hereafter named GG (Phytageel™, formula weight 1,000Kg/mol, low acylation degree).

Medical grade Manuka honey (MH) was purchased from ManukaGuard® ( Ndal Manufacturing Industries). Diatomaceous Earth (DE), median particle size ≤45.3μm, 82% acc. to Cilas, was purchased from Sigma-Aldrich. MgCl₂ salt, used as cross-linker, was of Redi-dri™ grade. Trans-resveratrol (RESV), was supplied by Farmalabor (98%, natural dry extract of Polygonum cuspidatum).

2.2 DE/RESV preparation

Encapsulation of RESV into DE was performed to improve its water-dispersibility, chemical and thermal stability, and to guarantee the biomolecule antioxidant activity. A RESV saturated solution in ethanol was prepared, in which DE was then dispersed in a DE:RESV weight ratio of 1.5:1 w/w (i.e., 6.25:1 mol/mol), by magnetic stirring for 30min. This procedure was optimized to maximize the drug loading, comparing the latter with those obtained by other loading strategies (i.e., using ultrasonic bath
for 15min or magnetic stirring for 30min followed by ultra-sonication for 15min). Finally, the exceeding ethanol was allowed to evaporate in an oven at 60°C until constant weight.

2.3 GG-MH-DE/RESV scaffold preparation

Porous scaffolds were prepared as previously reported for GG-based systems (Bonifacio et al., 2020a;2018;2017) with some modifications. Briefly, GG powder (2%w/v) was dissolved under stirring in water at 90°C. After cooling the solution at 60°C, MH (2%w/v) was added and vigorously stirred. Clay-reinforced samples were obtained mixing the dissolved components with an aqueous suspension of DE or DE/RESV (0.5% w/v). The gels were then poured in plastic molds and crosslinked through the external gelation method (Kaklamani et al., 2014) using MgCl₂ solutions as cationic source (0.5%w/v). After 24h, the porous scaffolds were obtained through freezing at -20°C for 24h and freeze-drying for 10h (Christ ALPHA1-2/LD+, Martin Christ). In all the experiments, GG-MH-DE was considered as control.

2.4 Spectroscopic and thermal characterizations

Pure DE and RESV powders, the DE/RESV system as well as the dehydrated GG-MH-DE and GG-MH-DE/RESV porous scaffolds were characterized by Fourier-Transform Infrared Spectroscopy (FT-IR) in Attenuated Total Reflectance mode (ATR), X-ray Photoelectron Spectroscopy (XPS) and Thermo-Gravimetric Analysis (TGA). As far as the experimental details for morphological characterization of the unloaded and RESV-loaded specimens is concerned, experimental details are reported in Supplementary Material (section 1.1).

FT-IR (ATR) was performed on dry samples, without pretreatment, with a Spectrum Two PE instrument (PerkinElmer) equipped with the universal ATR accessory (UATR, Single Reflection Diamond/ZnSe). FT-IR/ATR spectra were acquired from 400 to 4000cm⁻¹, with a resolution of 4cm⁻¹.

The surface characterization was carried out by XPS on powders or sections of dried scaffolds, in high power mode by a scanning microprobe PHI 5000VersaProbe II (Physical Electronic) with monochromatized AlKα X-ray radiation source. Survey scans and high-resolution spectra were
acquired in Fixed Analyzer Transmission (FAT) mode, with pass energy of 117.4eV and 29.35eV respectively. The base pressure during analysis was 10^{-9} mbar. The MultiPak software (v.9.9.0) was exploited for data analysis. Normalized peak area was used to calculate atomic percentages, considering empirically derived sensitivity factors of the MultiPak library.

Finally, TGA analysis was obtained heating 5-10mg of samples in air-saturated atmosphere using PerkinElmer TGA-400 instrument (PerkinElmer Inc.). The heat range was 30-800°C at a flow rate of 20°C/min. The gas flow was set at 20mL/min. Thermograms (TG) with respective derivative (DTG) curves were recorded and data were analysed using the software TGA Pyris series.

2.5 Resveratrol loading, release and antioxidant activity

The efficiency of RESV encapsulation into DE was assessed by HPLC, establishing RESV loading, as well as its release kinetics. A protocol described by Mark et al. (2014) was carried out (Supplementary material, section 1.3). Moreover, to assess RESV release kinetics, scaffolds were incubated in three release media, i.e., simulated synovial fluid (SSF), PBS and mobile phase (Supplementary material, section 1.4).

As far as RESV in vitro antioxidant activity is concerned, the DPPH assay was performed as previously reported (Shrikanta et al., 2015) (Supplementary material, section 1.5).

2.6 Mechanical characterisation

All mechanical tests were executed using the mechanical tester EZ-SX (Shimadzu) equipped with a 20N load cell. For the uniaxial compression tests, three cylinder-shaped porous scaffolds (1.6 x 1.2cm) with and without RESV were tested at room temperature. The crosshead speed was set at 1mm·min^{-1} and the load was applied while the sample was compressed until break (~30% of its original height). The compressive Young’s moduli (E) were calculated as the slope of the initial linear region of the σ–ε curve (0-8%). Single-step stress-relaxation tests were performed as proposed by Bonifacio et al. (2020a), with a single compression ramp at a speed of 0.4mm·min^{-1} (cylinder-shaped samples 1cm x 0.8cm) until reaching 5% of strain. Then, the strain was held constant for 900s, while the load was
recorded as a function of time. Within 10% compression, the stresses versus strain relations of the gels were almost linear. The peak stress (Ep) was obtained at 5% strain. Recorded data were processed by MATLAB R2017 software, as shown in Bonifacio et al. (2020a) to calculate the relaxation times, indicating the poroviscoelastic behaviour of the analysed samples. Multiple-step stress-relaxation tests were performed following the protocol reported by Li et al. (2020) where the strain for each compression process was set to 5% and repeated for 5 cycles. Typically, the samples were quickly preloaded to the selected strain at a rate of 3.9mm/min and kept for 900s. A spherical and impermeable stainless-steel indenter (radius 2.5mm) was used during the experiments. Three consecutive measurements spaced 2mm were performed on each sample (random measurements between indentations). The indentation tests were conducted in load-unload configuration with a depth of less than 300µm and a rate of about 5µm/s. The generated force-indentation depth (P-h) curves were analysed as suggested by Drirai & Yadavalli (2013), for the measurement of the relative stiffness (N/m).

2.7. Biological characterization

2.7.1 Specimens

For biological assays, specimens were prepared as 1cm height–0.5cm diameter disks, sterilized by 100% ethanol for 1 hour (1mL/specimens), air dried under biological hood flow overnight and stored at room temperature until use. HPLC analyses were performed to check the eventual loss of resveratrol and honey components due to the sterilization procedure. No significant amounts of honey components, nor of resveratrol, were released from the scaffolds after 1h of dipping in 100% ethanol.

2.7.2 Cells culture and cytocompatibility assessment

Human mesenchymal stem cells (hMSCs) immortalized through hTERT lentiviral vectors were used for in vitro experiments. hMSCs were cultured in low glucose DMEM supplemented with 15% fetal bovine serum (FBS, Merck) and 1% antibiotics (penicillin/streptomycin). When cells reached 80–90% confluence, they were detached with trypsin-EDTA and used for in vitro evaluations. To assess in vitro
specimens’ cytocompatibility, sterile dehydrated disks were dropwise filled with 200μL of cold (4°C) PureCol™ EZ Gel solution (Merck) containing 1×10^6 cells. Further details are reported in Supplementary material, section 1.6.

2.7.3 Oxidative stress conditions

To evaluate specimens’ oxygen and nitrogen active species (ROS/RNS) scavenge properties, oxidative stress was induced through of H₂O₂, which was applied directly into the medium at a final concentration of 300mM (Zhu et al., 2016) while cells stimulation was assessed at 3h/day. Briefly, specimens were filled with cells (as reported in 2.7.2 and Supplementary material, section 1.6) and medium was doped with H₂O₂. Further details are described in Supplementary material, section 1.7.

2.7.4 Bacterial culture

Specimens’ antibacterial activityThe antibacterial activity of the specimens was evaluated towards a pathogenic, biofilm-former strain of Staphylococcus aureus (S. aureus), selected as the main responsible for cartilage infections. The strain was purchased from the American Type Culture Collection (ATCC, reference strain ATCC 43300) and cultured on selective Mannitol Salt Agar plate (Sintek) until the formation of single colonies. The detailed protocol is reported in section 1.8 of Supplementary material. The same section describes cells-bacteria co-culture assay, also performed under oxidative stress conditions.

2.7.5 Chondrogenesis

Lastly, composites’ ability to support chondrogenesis under oxidative stress was assayed. Afterwards, chondrogenesis was verified through gene expression and histology (section 1.9 Supplementary material).

2.8 Statistical analysis of data

Physico-chemical and mechanical characterizations were performed on at least three samples, reporting mean±standard deviation. Biological experiments were carried out using six sample replicates. Statistical
analysis of data was performed using the SPSS software (v.20.0, IBM). Data were compared by ANOVA, followed by Tukey’s test. The significance level was set at $p<0.05$.

3. Results and Discussion

3.1 Resveratrol loading optimization

DE particles were used as carriers for the preparation of RESV-loaded GG-based porous scaffolds. In literature, different methods to improve the availability of poorly soluble drugs have been described (e.g. surfactant solubilisation, complexation, micronisation, etc.) (Kharb et al., 2006). Recently, drug amorphization has been proposed as a more efficient method to increase drug availability. This could be obtained solubilizing the drug in an appropriate solvent and then allowing its recrystallization in presence of inorganic supports.

Here, RESV loading procedure has been optimized considering three different preparation methods, i.e., dispersion of DE in ethanol solution of RESV by (i) magnetic stirring, (ii) ultra-sonication and (iii) a sequence of (i) and (ii) procedures. Magnetic stirring resulted the optimal loading procedure in terms of loading efficiency (LE%) values (LE% formula was reported in Supplementary material, section 1.3): magnetic stirring: 99.8±0.4%; ultrasonication: 64.2±0.2%; magnetic stirring + ultrasonication: 53.0±0.4%. Moreover, the influence of chemical and morphological structure of the carrier on the loading capacity and release kinetic of RESV was investigated comparing DE with mesoporous silica (MS), already employed as inorganic filler for the GG-MH based system (Bonifacio et al., 2020a).

Although the chemical nature of these two clays was fairly the same, RESV loading capacity of DE resulted significantly higher than that exerted by MS (i.e., LE% of 99.8±0.4% and 5.92±0.03% for DE and MS, respectively). These different loading capacities could be explained considering that the two clays displayed different surface area, morphology, and pore size distribution.

3.2 Physico-chemical characterizations of the porous scaffolds

Spectroscopic characterizations (FT-IR/ATR and XPS) and thermogravimetric analyses have been performed to shed light on the chemical composition, as well as on the thermal stability of the
developed scaffolds. Fig.2a shows the comparison of RESV, DE and DE/RESV FT-IR (ATR) spectra. DE/RESV spectrum indicated the presence of most of the characteristic bands of the stilbene derivative (i.e. NH$_2$ and OH stretching vibration bands at 3400 and 3290cm$^{-1}$, respectively, and those of -CH$_2$ ($\nu$CH$_2$) at 2951 and 2880cm$^{-1}$). The $\nu$ C-O-C, $\nu$ C-O-H, and $\nu$ C-N resulted in an intense wide band at 1250cm$^{-1}$, as well as two less intense and finer bands around 1030 and 1070cm$^{-1}$. The $\nu$C$-$O$-$C, $\nu$C$-$O$-$H, and $\nu$C$-$N resulted in an intense wide band at 1250cm$^{-1}$, as well as two less intense and finer bands around 1030 and 1070cm$^{-1}$. The C=C aromatic and inter-aromatic rings vibrations were observed at 1560cm$^{-1}$ and 1600cm$^{-1}$, respectively. The trans-olefin band of RESV was found at 964.4cm$^{-1}$. Moreover, the C-H aromatic rings bending ($\delta$ C-H) was observed at 830cm$^{-1}$. All these bands were also detected for pure RESV and were consistent with literature data (Zhou et al., 2016). Therefore, comparing FTIR spectra of RESV and DE/RESV, no changes in the RESV spectrum, especially in the fingerprint region (900-1400cm$^{-1}$), were observed. As a result, DE acted as an inert vehicle and no chemical interaction with RESV occurred. Concerning the GG-MH-based systems, the presence of RESV in the composite was not detectable from IR analyses, due to the predominance in the spectrum of the polysaccharide absorption bands (data not shown). XPS analysis was employed to gain information on the surface chemical composition of DE/RESV and GG-MH-DE/RESV systems. In this respect, unloaded GG-MH-DE scaffold, pure RESV and DE powders were analysed as well. The surface atomic composition of the systems was summarized in Fig.2b. The C/O ratio detected on RESV was 4.6, according to the stoichiometry of the pure compound (i.e., 4.7). Moreover, the Si/O ratio on DE was equal to 0.48, suggesting that the clay was essentially silica. The presence of carbon species could be due to DE purification procedures and to hydrocarbon contamination, ubiquitous in XPS analysis. In the DE/RESV system, RESV presence is clearly evidenced by the increase in carbon content and by the decrease of Si/O ratio (0.27), due to the organic oxygen species present in the system. Finally, in GG-MH-DE and GG-MH-DE/RESV, Si/O ratios further decreased to 0.023 and 0.019, respectively, due to the predominance of organic oxygen functionalities of the polysaccharide matrix. Considering RESV C1s curve fitting (Fig.2c), two main components were observed, aliphatic and/or aromatic C-H (284.8eV) and C-OH (286.4eV) groups, as expected. The DE/RESV system (Fig.2d)
showed two contributions as well. No BE shifts were detected for both components, thus suggesting both the integrity of the loaded RESV and the absence of chemical interaction with the clay, according to FT-IR results. In GG-MH-DE, no significant differences with the previously reported GG-MH samples loaded with different clays were found (Bonifacio et al., 2020; 2018). Moreover, the addition of RESV into the scaffold did not add any additional contribution to the C1s curve fitting (data not shown).

TGA analysis was employed to examine possible changes in thermal properties of the investigated systems and to estimate RESV loading into the clay. Considering DE/RESV, Fig. 2e and 2f reported the comparison of TGA and DTGA curves of RESV, DE and DE/RESV. RESV resulted stable up to 252°C, with a 1% weight loss. The first step, in the temperature range of 250–380°C with mass loss of about 20% is attributed to melting and to the beginning of drug thermal decomposition. The second step, falling at 573°C and with a mass loss of 78%, was related to the oxidation of the carbonized compound (da Silva et al., 2017). A similar TG curve was observed when RESV was loaded in DE. On the other hand, analysis indicated a thermal destabilization of RESV in the DE/RESV system. Indeed, the inset temperature (i.e., the temperature at 1% weight loss) shifted to 231°C and the decomposition peak temperatures shifted to 296°C and 521°C. These lower decomposition temperatures detected in DE/RESV could be related to a partial loss of RESV crystallinity in the diatomaceous system and this is an indirect evidence of a good dispersion of the drug inside the DE (Popova et al., 2014). Anyway, the DE/RESV system yet revealed a high thermal stability. Finally, the residues (Rx) at 800°C of DE, RESV and DE/RESV were 99.7, 1.8 and 60.4%, respectively. These data allowed an estimation of the loading capacity (LC%), using the following formula (Gutiérrez et al., 2017).

\[ LC\% = \left[ \frac{(R_{DE/RESV} - R_{DE})}{(R_{RESV} - R_{DE})} \right] \times 100 \]

The LC% was equal to 39.4±0.7%, according to HPLC results and the theoretical one, indicating a LE% of 98.4±1.8%. Considering the TGA results of the unloaded and RESV-loaded GG-MH-DE
porous scaffolds (see Supplementary Material, section 2.1 and Fig. S1), the main decomposition steps were similar to the other previously studied clay-reinforced GG-MH systems (Bonifacio et al., 2020b).

Figure 2. FT-IR, XPS and TGA analyses. (a) FT-IR (ATR) spectra of DE (black line), RESV (red line) and DE/RESV (blue line); (b) XPS atomic percentages (At%) of RESV, DE, DE/RESV, GG-MH-DE and GG-MH-DE/RESV samples. (c) XPS C1s curve fitting of RESV and (d) DE/RESV system. (e) TGA and (f) DTGA of DE (black line), RESV (red line) and DE/RESV (blue line).

Additional information related to the scaffolds’ morphology were gained through SEM analyses (sections 1.1 and 2.2 of the Supplementary material). The scaffolds displayed a highly porous architecture, suitable for cell colonization (Fig. S2). Moreover, swelling studies were performed (sections 1.2 and 2.3 of the Supplementary material), highlighting an effective water uptake for both GG-MH-DE and GG-MH-DE/RESV scaffolds (Fig. S3).
3.3 Resveratrol loading, release and antioxidant activity

The amount of RESV loaded in the DE/RESV system was assessed by HPLC, resulting equal to 39.7±0.5%w/w, as expected from the theoretical DE:RESV ratio (1.5:1). Furthermore, when the DE/RESV system was embedded into the porous scaffold, RESV loading resulted 4.3±0.7%w/w, according to the preparation protocol (see section 2.3).

As far as RESV release is concerned, simulated synovial fluid (SSF) was selected as release medium, in order to mimic the physiological conditions in which the porous scaffolds would be implanted. In this respect, Fig.3a reports the kinetics obtained from GG-MH-DE/RESV and DE/RESV. Regarding the GG-MH-DE/RESV scaffold, the presence of the organic network delayed RESV release, reaching a plateau within the first week. It is worth to note that, RESV amount released in SSF from the scaffold was only the 3% of the loaded molecule, because of its poor aqueous solubility. Nevertheless, this amount provided the sought antioxidant activity, as demonstrated in the biological experiments reported below. It can be hypothesized that the developed scaffold, once implanted, will behave as a resveratrol reservoir. This feature could be particularly useful, since RESV is cleared with a zero-order kinetic, resulting in a short half-life in human blood (Amiot et al., 2013). Moreover, performing RESV release in more favorable media, the plateau increased up to ~60% in PBS:ethanol 10% and to ~80% in mobile phase (water:methanol 40:60), in good agreement with Kamath et al. (2014) (see Supplementary material, section 2.4 and Fig. S4).

Figure 3. RESV release and antioxidant activity. In a), in vitro cumulative release of RESV from bare RESV, DE/RESV and GG-MH-DE/RESV, performed up to 30 days at 37°C in simulated synovial fluid.
fluid (SSF). From day 7 up to day 30 the differences between the three samples are significant (p<0.05); b) In vitro scavenging activity of the scaffolds on DPPH radical. Data are expressed as Radical Scavenging activity percentage (%RSA) (p<0.001).

As far as the in vitro antioxidant activity is concerned, DPPH assay showed that, after 10 min of incubation, a Radical Scavenging Activity (RSA) equal to 96.4±1.8% was reached (Fig.3b). The porous scaffolds without RESV showed an antioxidant activity of 3.5±0.6%, likely due to the scavenger molecules belonging to MH. The ability of RESV to quench DPPH, already described by Chung et al., 2020, was preserved by the scaffold itself; hence the preparation process did not alter RESV antioxidant features.

3.4 Mechanical characterisation

Stress-strain curves recorded during static compression test are shown in Fig.4a. The samples were compressed until break (~30% of the strain) indicating good load bearing ability (Li et al., 2020). Both scaffolds compositions showed similar Young’s modulus (138±9kPa for GG-MH-DE and 132±5kPa for GG-MH-DE/RESV), in accordance with previous results (Bonifacio et al., 2020a), without significant differences due to the addition of RESV. These values were in agreement with Kelly et al. (2013) and Scalzone et al., (2019), which reported a suitable compressive Young’s modulus ranging from 100–1000 kPa for native articular cartilage.
Figure 4. Mechanical tests on GG-MH-DE and GG-MH-DE/RESV scaffolds. σ/ε curve obtained after static compression test (a); σ/t curves after single-step (b) and multiple-step (c) stress-relaxation analysis at 5% strain; and indentation curve showing the load applied to the scaffold’s surface vs penetration depth (d). Report of the viscoelastic relaxation time (τ₁) and poroelastic relaxation time (τ₃) calculated during the multiple stress-relaxation analysis on the GG-MH-DE and GG-MH-DE/RESV scaffolds at each cycle for 5 cycles at 5% strain (e).

Viscoelasticity and poroelasticity effects have been found in articular cartilage: the first property is fluid-flow independent and related to macromolecular conformational changes, while the second one is fluid-flow dependent and related to small molecules migration (Richard et al., 2013). To assess the effect of the presence of the RESV on the poroviscoelastic behaviour of the porous scaffolds, we performed single- and multiple-step stress-relaxation analysis and indentation test. Fig.4b shows the typical stress-relaxation curve obtained from the single stress-relaxation test and the relative mechanical parameters were calculated: peak elastic modulus (Ep), viscoelastic relaxation time (τ₁) and poroelastic relaxation time (τ₃). The Ep values, calculated following the protocol of Bian et al. (2011),
were 127±8kPa for GG-MH-DE and 131±9kPa for GG-MH-DE/RESV, without showing significant differences as in the static compression tests. Furthermore, from the stress-relaxation curves computational analysis done using Matlab software, the relaxation times $\tau_1$ and $\tau_3$ were calculated fitting the experimental values with a third order exponent polynomial equation. The obtained relaxation times ($\tau_1=12±2s$, and $\tau_3=860±60s$ for GG-MH-DE and $\tau_1=24±5s$ and $\tau_3=1900±200s$ for GG-MH-DE/RESV), calculated following the generalised Maxwell model, consisting in three relaxation times ($\tau_1=1–10s$, $\tau_2=10–100s$ and $\tau_3>100-1000s$) were in the range of those reported in literature for polymeric gels (Bonifacio et al., 2020a;Richard et al., 2013) and were comparable with the values obtained by the same authors in previous works (Bonifacio et al., 2020a).

To compare the time-dependent mechanical properties of the proposed scaffolds, step-wise stress-relaxation experiments were performed, consisting of 5 cycles of 5% compressive strain ramps, each followed by an equilibrium period in an unconfined compression. Fig.4c showed the stress versus time ($\sigma/t$) profiles. Both scaffolds showed a sharper stress decay after each compression ramp reaching the equilibrium with rapid relaxation over time, demonstrating recovery capability and fast recovery time ($<10s$) after the relaxation, and a good stability during the whole compression process (Li et al., 2020). The relaxation times were calculated for each cycle for each sample as explained before for the singles stress-relaxation process and reported in Fig.4e, confirming that the obtained viscoelastic relaxation time $\tau_1$ (from 9.3±1.2s at step 1 to 7.4±0.6s at step 5 for the GG-MH-DE and from 10±2s at step 1 and 7.8±1.0s at step 5 for the GG-MH-DE/RESV) and the poroelastic relaxation time $\tau_3$ (from 850±70s at step 1 to 352±11s at step 5 for the GG-MH-DE and from 800±100s at step 1 and 342±5s at step 5 for the GG-MH-DE/RESV) were in the range of the articular cartilage tissue (Hu & Suo, 2012). The fast stress-relaxation of the scaffolds would benefit the stress unloading during the compression, suitable characteristic for cartilage-biomimicking materials. Indeed, the literature reports that high degree of stress relaxation upon loading may enhance cells spreading and proliferation as well as the differentiation of stem cells (De Giglio et al., 2018).
Although the majority of the studies reported in literature have been focused on properties derived by compression tests on the top of the tissue or sample (Sim et al., 2014), in this work depth-sensing indentation tests were performed, where a small indenter tip was brought down onto a flat surface loading a very small volume of the sample. This indentation test revealed local changes in the analysed samples (Boi et al., 2019). The areas of the hysteresis loop almost remained constant with the addition of the RESV and both scaffolds demonstrated to be able to dissipate energy (Fig.4d). This load and unload cycle analysis can be used to further analyse the gels viscoelastic properties, as reported by Qasmi et al. The selected load rate (5µm/s) fully activates viscoelastic phenomena (Richard et al., 2013) without showing differences in the stiffness values of both systems (2.4±0.6N/m and 2.6±0.3N/m with and without RESV respectively).

3.5 Specimens’ cytocompatibility

Due to the novelty of the GG-MH-DE/RESV formulation, cytocompatibility was first in vitro assayed to exclude any toxic compound affecting cells behavior. Stem cells were selected as cells representative for the healing process undergoing tissue repair. Results are reported in Fig.5.

In absence of oxidative stress (Fig.5a-b), specimens showed a similar behavior. Indeed, a significant difference was noticed at day 3 (Fig.5a, *p<0.05) but in the following days 7 and 10 cells showed a comparable metabolic activity when seeded in both GG-MH-DE or GG-MH-DE/RESV (p>0.05). The difference in cells metabolism detected at the earlier time-point can be probably due to the fact that RESV can affect stem cells metabolism by the Wnt/β-catenin senescence pathway (Yoon et al., 2015); to counteract this negative effect, cells express SIRT1 gene that is activated by RESV to enhance proliferation thus switching off the senescence cascade.

Hematoxylin and eosin staining representative for day 10 (Fig.7b) confirmed the presence of a high density of cells forming aggregates within the scaffold’s pores. Conversely, when oxidative stress was induced, a different behavior was observed comparing the specimens (Fig.5c). Indeed, within the first 4 days of culture, no significant differences were noticed
normalizing cells metabolism results to the seeding day (day 0), suggesting a similar response or
tolerance to the induced stress. However, after day 4, a clear inversion between GG-MH-DE and GG-
MH-DE/RESV was observed by looking at the alamar blue assay results (Fig. 5c). Indeed, while the
metabolism of cells seeded onto GG-MH-DE started to drop, it increased on GG-MH-DE/RESV
(Fig.5b, red arrow) thus suggesting that the RESV scavenge activity was effective in reducing the
amount of toxic active species affecting cells metabolism thus making hMSC able to proliferate.

Consequently, results were significant at day 9 and 10 comparing values obtained for GG-MH-DE and
GG-MH-DE/RESV (Fig.4b, *p<0.05). These results confirmed that the role of RESV was appreciable
when the oxidative stress was applied due to its protective scavenge role.

Figure 5. Cytocompatibility. When cells were cultured within porous scaffolds in absence of H₂O₂
(a), similar results were obtained with the only exception of 24h (a, p<0.05, indicated by *) as
confirmed by haematoxylin and eosin staining representative for day 10 (b). Conversely, the protective
activity of RESV was effective in protecting cells from H$_2$O$_2$ oxidative stress after 4 days culturing in presence of hydrogen peroxide (c); differences were significant at day 9 and 10 (p<0.05, indicated by *).

To confirm the direct link between cells metabolism results and RESV active species scavenge, the amount of released ROS/RNS was evaluated. Results are reported in Table 1.

In general, significant results were obtained at all the tested time-points comparing the active species released from GG-MH-DE and GG-MH-DE/RESV (*p<0.05). These results agreed to the DPPH assay (Fig.3b), also confirming GG-MH-DE/RESV performances in biological environment. Thus, RESV-loaded porous scaffolds were immediately effective in counteracting the oxidative stress. This protective activity allowed cells to maintain their metabolism over time, thus explaining the differences observed with Alamar blue assay after some days culturing.

### Table 1. ROS/RNS evaluation. Fluorescence (RFU) results are expressed as means ± standard deviations.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>GG-MH-DE (RFU)</th>
<th>GG-MH-DE/RESV (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9500±500</td>
<td>4300±900*</td>
</tr>
<tr>
<td>2</td>
<td>6500±200</td>
<td>2690±120*</td>
</tr>
<tr>
<td>3</td>
<td>6200±500</td>
<td>2530±150*</td>
</tr>
<tr>
<td>4</td>
<td>6730±90</td>
<td>2486±16*</td>
</tr>
<tr>
<td>7</td>
<td>4700±500</td>
<td>1850±180*</td>
</tr>
<tr>
<td>9</td>
<td>4700±400</td>
<td>1500±200*</td>
</tr>
<tr>
<td>10</td>
<td>4600±400</td>
<td>1500±200*</td>
</tr>
</tbody>
</table>

3.6 Antibacterial activity

Porous scaffolds synthesis steps and the surgical procedures aimed at the implantation open to the possibility of bacterial infection. This eventuality is not very common (Stutz et al., 2000) but can be of particular severity when septic arthritis occurs (Wyatt et al., 2017). Commonly, infections have been found to be caused by *Staphylococcus aureus* (Balato et al., 2017). The here presented scaffolds hold as common antibacterial agent MH, that owes this effect to methylglyoxal (MGO) (Bonifacio et al.
Moreover, stem cells hold intrinsic antibacterial properties that act in synergy with MGO. Therefore, the preservation of cells metabolism in presence of stress conditions represents itself an antibacterial tool. The use of RESV as an antibacterial compound was recently validated by literature. Indeed, Tang et al. (2019) showed that *S. aureus* virulence can be attenuated by RESV through the inhibition of alpha-hemolysin expression. Similarly, subinhibitory concentrations of RESV were effective in downregulating saeRS that is directly involved in alpha-hemolysin production (Duan et al., 2018). Accordingly, here we tested specimens’ antibacterial properties towards the joint pathogen *S. aureus* strain (Fig.6) by applying a co-culture method resembling clinical one and to monitor the metabolic activity of cells and bacteria that are challenging in the same environment for the same system colonization (Cochis et al., 2020). First, antibacterial activity was assessed without H2O2 after 48h samples equilibration after cells seeding (Fig.6a). GG-MH-DE and GG-MH-DE/RESV offered a similar protection to the seeded cells, as the initial number was not lowered after 48h of direct infection (Fig.6b, dashed lines indicates day 0 cells number). Accordingly, bacterial number was lowered of about 1 log by both specimens, likely as a combined activity of MGO, cells and RESV (Fig.6c).
Figure 6. Antibacterial activity. When composites were infected without H\textsubscript{2}O\textsubscript{2} pre-treatment (a), similar results were obtained comparing viable cells (b) and CFU number (c). Conversely, when infection was induced after 7 days H\textsubscript{2}O\textsubscript{2} pre-treatment (d), a significant difference was noticed for both cells (e) and CFU count (f) comparing GG-MH-DE/RESV porous scaffolds with control GG-MH-DE ones (p<0.05, indicated by *).

To repeat the experiment in a stress condition, we decided to pre-treat cells with H\textsubscript{2}O\textsubscript{2} for 7 days (after the 48 hours of cells equilibration) referring to the previous cytocompatibility evaluation reported in Figure 5c where the first evidence of the RESV protective activity in comparison to untreated specimens was observed. In this oxidative stress environment, GG-MH-DE/RESV composites showed
a higher activity with respect to GG-MH-DE ones. Indeed, viable cells number was still preserved by GG-MH-DE/RESV, while a significant decrease was observed for GG-MH-DE (Fig. 6e, *p<0.05).

Therefore, it can be speculated that the superior antibacterial activity of GG-MH-DE/RESV porous scaffolds is due to the synergistic activity of MGO and RESV (Bonifacio et al. 2018; Duan et al., 2018; Tang et al. 2019) which preserved metabolically-active cells (Fig. 6f).

3.7 Chondrogenesis

The presence of stress conditions has been demonstrated to interfere with chondrogenesis (Mateos et al., 2013; Zákány et al., 2007). Accordingly, we assayed stem cells differentiation towards cartilage lineage in absence or after 7 days of H₂O₂ pre-treatment to compare the healing process in physiological or stress conditions. Results are summarized in Fig. 6. In the absence of H₂O₂, chondrogenic genes COL 2 (Fig. 7a) and ACAN (Fig. 7b) were up-regulated in a similar manner for both GG-MH-DE and GG-MH-DE/RESV, thus suggesting that stem cells were correctly undergoing differentiation towards cartilage-like phenotype. Histology (Fig. 7e) showed that cells were positive for Alcian blue staining, confirming that cells expressed cartilage-like matrix sulfated mucins. This outcome confirms that materials developed for chondral regeneration, i.e. composites with porous architecture, supported matrix deposition (e.g. glycosaminoglycans). However, it is difficult to quantify GAGs on porous scaffolds, as previously debated by Cochis et al. (2017), when a polyurethane-methylcellulose hydrogel device was considered for cartilage repair under biomechanical stimulation.

Conversely, when oxidative stress was induced, an interference in chondrogenesis was observed for GG-MH-DE. Indeed, the expression of chondrogenic (COL 2) was reduced in comparison to the non-stressed specimens (Fig. 7a, *p<0.05) while the expression of (COL 1) was increased (Fig. 7c), thus suggesting an interference towards cells differentiation. On the opposite, looking at the GG-MH-DE/RESV results, the presence of oxidative stress did not modify the gene expression profile: COL 2 was not significantly down regulated in comparison with untreated porous scaffolds as well as the expression of COL 1 was comparable. This evidence was supported by the COL2:COL1 ratio that confirmed how Resveratrol-doped scaffold were able to preserve the cells maturation also in the stress.
conditions (Fig. 7d). Therefore, it can be hypothesized that the presence of RESV was effective in preserving chondrogenesis by reducing oxidative stress. From this point of view, it must be mentioned that some chemicals contained into the chondrogenic medium such as TGF-β belongs to the family of anti-inflammatory molecules; accordingly, the effect of RESV can be underestimated by the activity of such molecules, as previously debated (van der Kraan et al., 2019). However, in our experimental plan, H$_2$O$_2$ pre-treatment was applied using basal medium for 7 days while chondrogenic medium was added afterwards. Therefore, we suppose that this two-step strategy minimized interferences. Finally, histological analysis confirmed PCR results (Fig. 7f). Indeed, looking at both Alcian blue (upper panel) and Safranin-O (lower panel) staining, a denser marking for GG-MH-DE/RESV could be observed in comparison with GG-MH-DE (Fig. 7f, identified by the stars) where less positive areas were identified (Fig. 7f, indicated by the arrows), thus suggesting better cell maturation towards cartilage-like matrix. However, despite the promising results here obtained here, it must be highlighted that experiments were conducted by simulating an in vitro simple model of inflammation due to hydrogen peroxide that of course does not completely resemble the complexity of the inflammatory cascade that can strongly impair cartilage repair, as discussed by van der Kraan (2019). Accordingly, more proofs both in vitro and in vivo are still required to verify whether this resveratrol-loaded system is able to maintain the same protective effect in a more physiologically stressed environment.
**Figure 7. Chondrogenesis.** The expression of chondrogenic collagen 2 (COL 2, a) and aggrecan (ACAN, b) genes demonstrated that the presence of RESV allowed for their up regulation despite stress conditions. On the opposite, cells hosted in the GG-MH-DE specimens suffered from oxidative stress by lowering COL 2 expression and increasing COL 1 (c) thus showing an interference in the differentiation as confirmed by the COL2:COL1 ratio (d). Histology (e, f, low magnification=10x, high magnification=40x) confirmed a mode dense presence of GAGs for GG-MH-DE/RESV (stained by Alcian blue and Safranin-O, indicated by the arrows and stars).

### 4. Conclusions

Porous scaffold composites, based on GG, MH and RESV-loaded DE were developed with the aim to replace injured cartilage. High and replicable loading efficiency, as well as ameliorated drug release, were obtained. The DE/RESV system was embedded into the organic network, leading to porous scaffolds with noteworthy and reliable mechanical properties (i.e. stiffness, stress bearing, recovery
capability and energy dissipation). Moreover, the hypothesis that these scaffolds could contrast oxidative processes was confirmed, since resveratrol preserved scaffolds pro-chondrogenic and antibacterial properties, even if exposed to oxidative stress, which simulated the injured tissue.

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