



Double functionalization in chitosan scaffolds for bone tissue engineering improvement

Annj Zamuner · Matteo Zoboli · Elena Zeni · Teresa Russo · Ignazio Castagliuolo · Andrea Porzionato · Francesca Ravanetti · Antonio Cacchioli · Gabriella D'Auria · Lucia Falcigno · Chiara Battocchio · Giovanna Iucci · Martina Marsotto · Antonio Gloria · Paola Brun · Monica Dettin

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Abstract Chitosan is a natural polysaccharide produced by the deacetylation of chitin. It has been employed for several biomedical applications since it is biocompatible and biodegradable. In this work, two synthetic peptides were covalently grafted to chitosan to enhance cellular response. The first sequence, the nonapeptide (351–359) of human vitronectin (HVP), was demonstrated to enhance osteoblast adhesion and migration. The second peptide, GBMP1 α , a 22 mer

sequence reproducing the fragment (48–69) of BMP-2, increased the osteogenic differentiation of mesenchymal stem cells. The anchoring chemistry involves the formation of a Schiff base between the amino groups of chitosan and aldehyde groups specifically introduced into the peptide backbone, followed by a subsequent reduction step. One of the advantages of this method is the specific anchoring of a bioactive sequence in a single-step reaction carried out in an aqueous solution and mild conditions. Six different 3D-porous scaffolds were designed and obtained by various combinations of both functionalized chitosan

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A. Zamuner
Department of Civil, Environmental, and Architectural Engineering, University of Padova, Via Marzolo 9, 35131 Padua, Italy

A. Zamuner · E. Zeni · M. Dettin (✉)
Department of Industrial Engineering, University of Padova, Via Marzolo 9, 35131 Padua, Italy
e-mail: monica.dettin@unipd.it

M. Zoboli · F. Ravanetti · A. Cacchioli
Department of Veterinary Science, University of Parma, Strada del Taglio 10, 43126 Parma, Italy

T. Russo
Institute of Polymers, Composites and Biomaterials, National Research Council of Italy, Viale J. F. Kennedy 54, 80125 Naples, Italy

I. Castagliuolo · P. Brun
Department of Molecular Medicine, University of Padova, Via Gabelli 63, 35131 Padua, Italy

A. Porzionato
Department of Neuroscience, Section of Human Anatomy, University of Padova, Via Gabelli 65, 35121 Padua, Italy

G. D'Auria · L. Falcigno
Department of Pharmaceutical Sciences, University Federico II of Naples, Via Montesano 49, 80131 Naples, Italy

C. Battocchio · G. Iucci
Department of Science, Roma Tre University, Via Della Vasca Navale 79, 00146 Rome, Italy

M. Marsotto
Department of Chemical Science and Technologies, University of Tor Vergata, Via Della Ricerca Scientifica 1, 00133 Rome, Italy

A. Gloria
Department of Industrial Engineering, University of Naples Federico II, P.Le Tecchio 80, 80125 Naples, Italy

and chitosan simultaneously functionalized with both peptides. Scaffolds were characterized through X-ray photoelectron spectroscopy (XPS), nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FT-IR), and mechanical measurements. Biological assays revealed that functionalized scaffolds exhibited significantly improved adhesion, proliferation, and mineralization in human jaw-derived osteoblasts compared to pristine chitosan. Results from mechanical measurements highlighted the ability to modulate compressive modulus and maximum stress. Notably, scaffolds containing both peptides demonstrated significant synergistic effects *in vivo*, enhancing material cellular colonization and reducing fibrotic capsule thickness. This study highlights the potential of peptide-functionalized chitosan scaffolds in advancing bone tissue engineering and addressing current limitations in bone repair strategies.

Keywords Chitosan · Covalent functionalization · Bioactive peptides · Design methods · Mechanical measurements · Bone tissue engineering

Introduction

Bone diseases remain a significant clinical challenge, accounting for half of all chronic conditions in individuals over the age of 50 (Qu et al. 2019). Currently, autologous bone grafting is regarded as the gold standard for bone reconstruction, yet it presents several disadvantages, including the risk of secondary injuries and donor site morbidity (Keating et al. 2001; Oryan et al. 2014). To address the limitations of grafts, bone tissue engineering (BTE) has proposed various scaffolding materials strategically enriched with bioactive molecules and cells (Amini et al. 2012). Recent advances in tissue engineering have increasingly emphasized the importance of multifunctional scaffolds capable of providing complex and coordinated biochemical signals to direct cellular behavior. In this context, nanobiomaterials and bioink-based scaffolds used in 3D bioprinting have demonstrated promising potential due to their ability to incorporate bioactive components that modulate cell adhesion, proliferation, and differentiation. As highlighted in recent literature, the integration of nanostructured cues within biomaterial systems enables more precise control over the cellular

microenvironment, supporting the development of engineered tissues and artificial organs (Salahshour et al. 2024). In this study, chitosan, a natural polysaccharide derived from the deacetylation of chitin (de Alvarenga 2011), was selected as the biocompatible and biodegradable base biomaterial (Ravi Kumar 2000; Rinaudo 2006). Chitosan has osteoconductive properties because it supports the attachment, proliferation, and differentiation of osteoblasts (bone-forming cells) (Aguilar et al. 2019) and finally promotes the formation of new bone tissue, which is essential in bone regeneration and repair. Although chitosan alone has limited mechanical strength, it can be combined with other materials (*e.g.* hydroxyapatite, tricalcium phosphate or carbon nanotubes) or crosslinked (Krishna et al. 2024) to overcome this issue, making it more suitable for load-bearing applications in bone tissue engineering (Deepthi et al. 2016; Hu et al. 2020; Lee et al. 2000; Li et al. 2005; Oryan et al. 2017; Pighinelli et al. 2013). Chitosan has inherent antimicrobial properties, which can help to reduce the risk of infection at the implantation site (Ke et al. 2021). This is particularly advantageous in bone tissue engineering, where infections can lead to severe complications. Chitosan features two types of key functional groups in its monomeric residue: an amine group (NH_2) at the C2 position and two hydroxyl groups (OH), which facilitate its functionalization with peptides for engineering applications (Brun et al. 2021a), making it a versatile platform for delivering therapeutic agents directly to bone repair sites. Additionally, chitosan can be molded into various forms, such as 3D sponges, hydrogels, films, nanofiber matrices, or nanoparticles (Croisier and Jérôme 2013; Ahmed et al. 2018; Li et al. 2025). This adaptability allows it to be customized for specific bone tissue engineering applications, such as scaffolds that mimic the natural bone extracellular matrix. Notably, the porosity of lyophilized chitosan scaffolds supports cell migration and the exchange of nutrients and waste (Oryan et al. 2017).

Functionalizing chitosan with proteins or peptides for various applications, particularly in bone tissue engineering, gained a lot of interest in recent years (Kiryk et al. 2025). S Kim et al., developed chitosan hydrogels photo-crosslinked and functionalized with the RGD peptide and phosphoserine that showed bone healing capacity in a mouse calvaria defect model (Kim et al. 2016). In another study, researchers

covalently functionalized a chitosan/hydroxyapatite biomimetic composite with the BMP2-derived peptide (P24) for controlled delivery, achieving significantly higher ectopic osteogenesis in rat dorsal muscle pockets when compared to the unfunctionalized control (Chen et al. 2017).

The development of biomimetic scaffolds capable of effectively supporting tissue regeneration hinges on their ability to provide multiple, simultaneous biochemical cues to guide cellular behavior (Wang et al. 2025). In the context of bone tissue engineering, achieving a multifunctional scaffold that promotes both osteogenic differentiation and cell adhesion is particularly crucial. Despite the recognized importance of multi-stimuli systems, most current strategies rely on single-factor modifications, limiting their regenerative potential. This study presents one of the first studies into the double covalent functionalization of chitosan with two bioactive peptides (named HVP and GBMP1 α). HVP is an adhesive sequence derived from human vitronectin (fragment: 351–359), able to improve osteoblast adhesion, primarily through interactions with cell-surface glycosaminoglycans (GAGs) such as heparan sulfate, and migration (Brun et al. 2013; Zamuner et al. 2017, 2021). GBMP1 α reproduces the fragment (48–69) of BMP-2 protein and increases osteogenic differentiation of mesenchymal stem cells through activation of BMP signaling via SMAD 1/5/8 phosphorylation (Rampazzo et al. 2017).

Various methods have been employed to covalently bind a bioactive sequence to chitosan, including the use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxy succinimide (NHS) (Ho et al. 2005; Tigli et al. 2008). In this work, an alternative functionalization chemistry is proposed: the formation of Schiff bases, which are subsequently reduced. This reaction occurs between the amino groups of chitosan and the aldehyde group of peptides, the latter produced through oxidative cleavage of the serine residue appropriately added at the N-terminal of both sequences (Geoghegan et al. 1992; Spears et al. 2016). One key advantage of this method is the ability to specifically anchor a bioactive sequence in a single-step reaction, performed in an aqueous solution under mild conditions (Brun et al. 2021b).

In a previous study, we demonstrated that chitosan functionalized with HVP or GBMP1 α promoted

the adhesion, proliferation, and differentiation of osteoblasts *in vitro* (Brun et al. 2021b). In this study, hybrid scaffolds containing different percentages of each functionalized chitosan were prepared to identify the conditions that maximize the benefit for the cells. In addition, chitosan was functionalized simultaneously with both bioactive peptides. The scaffolds prepared with this simpler approach gave the best results in *in vitro* and *in vivo* assays.

Materials and methods

Materials

The Fmoc-protected amino acids, the Rink Amide MBHA resin, all coupling agents (*i.e.*, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate, HBTU; and oxima pure), N, N-dimethylformamide (DMF), and N-methyl pyrrolidone (NMP) were purchased from Merck (Merck KGaA, Darmstadt, Germany). All the remaining reagents and solvents necessary for the solid phase peptide synthesis (*i.e.*, piperidine; dichloromethane (DCM); N, N-diisopropylethylamine (DIPEA); trifluoroacetic acid (TFA); diethyl ether; triethyl silane (TES)) were from Biosolve (Leenderweg, Valkenswaard, The Netherlands). All other solvents, such as acetonitrile, methanol, ethanol, and acetic acid, were supplied from Merck (Merck KGaA, Darmstadt, Germany). Reagents such as sodium (meta)periodate (NaIO₄) and sodium cyanoborohydride (NaCNBH₃) were from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Chitosan 70/1000 was supplied by Heppe Medical Chitosan (HMC⁺, Halle, Germany).

Peptide synthesis and characterization

S-X-HVP peptide (sequence: H-Ser-X-Phe-Arg-His-Arg-Asn-Arg-Lys-Gly-Tyr-NH₂, where X=7-Ahe, 7-amino heptanoic acid) was synthesized and characterized as reported elsewhere (Brun et al. 2021b), following the scheme reported in Fig. S1 (Supplementary Material). Two syntheses of S-X-HVP were carried out. S-X-GBMP1 α peptide (sequence: H-Ser-X-Pro-Phe-Pro-Leu-Ala-Asp-His-Leu-Asn-Ser-Thr-Asn-His-Ala-Ile-Val-Gln-Thr-Leu-Val-Asn-Ser-NH₂, where X=7-Ahe, 7-amino heptanoic acid) was synthesized using a SYRO I automatic synthesizer. The

peptide was synthesized through solid-phase peptide synthesis (SPPS) with Fmoc chemistry as reported in Fig. S2 (Supplementary Material). The total amount of crude peptide obtained was 223.95 mg. The crude peptide was characterized using a reverse-phase high-performance liquid chromatography (One chromatographic analysis for each peptide. RP-HPLC, conditions: column, Vydac C₁₈, 5 μ m, 300 Å, 4.6 mm×250 mm, W. R. Grace & Co.-Conn; flow rate, 1 mL/min; eluent A: 0.05% TFA in H₂O milliQ; eluent B: 0.05% TFA in acetonitrile; linear gradient: from 22 to 44% of eluent B in 40 min; λ =214 nm; retention time: 24.9 min), and the identity of the peptide was ascertained through MALDI-TOF mass analysis (4800 MALDI-TOF/TOF TM analyzer provided with 4000 Series Explorer TM software, Applied Biosystem/MDS Sciex, CA, USA): experimental mass, 2601.69 Da; theoretical mass, 2601.94 Da. One mass analysis for each crude peptide was carried out. The synthesis of S-X-GBMP1 α was repeated 4 times.

Serine oxidative cleavage and peptide purification

Serine oxidative cleavage and purification of S-X-HVP were performed as previously reported in (Brun et al. 2021b). The procedure adopted to obtain the alpha-oxo-aldehyde group at the N-terminal of the S-X-GBMP1 α is reported briefly: the crude peptide (40 mg) was dissolved in H₂O milliQ up to a final concentration of 1 mg/mL, then NaIO₄ (21.4 mg, 2.5 mM) was added to the peptide solution. The mixture was stirred magnetically at room temperature for 4 min. Subsequently, the solution was filtered, and the peptide carrying the alpha-oxo-aldehyde group at the N-terminal was purified through RP-HPLC (conditions: column, Zorbax C₁₈, 5 μ m, 300 Å, 9.4 mm×250 mm, Agilent Technologies, Inc.; flow rate, 4 mL/min; eluent A: 0.05% TFA in H₂O milliQ; eluent B: 0.05% TFA in acetonitrile; linear gradient: from 22 to 37% of eluent B in 60 min; λ =214 nm. The serine oxidative cleavage and the following HPLC purification were repeated 5 times for each synthesis). The pure peptide was characterized by RP-HPLC (conditions: column, Vydac C₁₈, 5 μ m, 300 Å, 4.6 mm×250 mm, W. R. Grace & Co.-Conn; flow rate, 1 mL/min; eluent A: 0.05% TFA in H₂O milliQ; eluent B: 0.05% TFA in acetonitrile; linear gradient: from 27 to 37% of eluent B in 20 min; λ =214 nm; retention time: 11.0 min. One

chromatographic analysis for each peptide), and the identity of the peptide was ascertained by mass spectrometry (experimental mass: 2570.10 Da, theoretical mass: 2570.94 Da). One mass analysis for each purified peptide was carried out.

Chitosan functionalization

The chitosan functionalization with each peptide was carried out following the procedure described by (Secchi et al. 2019). Briefly, 21.89 mg of pristine chitosan (Ch) were reacted with 19.95 mg of alpha-oxo-aldehyde X-GBMP1 α peptide to obtain 22 mg of chitosan functionalized with the X-GBMP1 α peptide (ChG). This reaction was repeated 4 times. In the other case, a total amount of 14.41 mg of alpha-oxo-aldehyde X-HVP was allowed to react with 37.65 mg of pristine chitosan to produce X-HVP conjugated to chitosan (ChH, 30.11 mg). This reaction was repeated twice. In both cases, the amount of reducing agent (NaCNBH₃) added was in a 1:1 (w/w) ratio with the peptide.

For the simultaneous functionalization of chitosan with both peptides (ChHG), the same protocol was used: 24.72 mg of pristine chitosan reacted with 10.16 mg of alpha-oxo-aldehyde X-GBMP1 α (4×10^{-6} mol) and 5.58 mg of alpha-oxo-aldehyde X-HVP (4×10^{-6} mol) at the same time, and 15.58 mg of NaCNBH₃ was added to reduce the newly formed Schiff bases to amine bonds. The yield of ChHG was 25 mg. This reaction was repeated 4 times.

Scaffold preparation

Porous scaffolds were prepared with different blends of functionalized chitosan (*i.e.*, ChH, ChG, and ChHG) and pristine chitosan (Ch). For all scaffolds, except for ChH/ChG and ChHG, a percentage of 50% of pure chitosan was kept in the matrix mixture, as it was previously demonstrated to be the concentration that represents a compromise between enhancing the bioactivity of chitosan without affecting its antibacterial properties (Brun et al. 2021a). The quantity of each material used to prepare one matrix of every condition is reported in Table 1.

NMR analyses were performed on chitosan, Ch, and on chitosan doubly functionalized with HVP and GBMP1 α peptides, ChHG. The Ch sample was prepared by dissolving approx 0.8 mg in 700 μ L

Table 1 Amounts of differently functionalized chitosan (*i.e.*, ChH, ChG, and ChHG) and pristine chitosan (Ch) used to prepare one scaffold

Scaffold	Description	Ch [mg]	ChH [mg]	ChG [mg]	ChHG [mg]	0.2 M acetic acid [μ L]
Ch	100% Ch (control)	1.68				242
Ch/ChH	50% Ch 50% ChH	0.84	0.84			242
Ch/ChG	50% Ch 50% ChG	0.84		0.84		242
ChH/ChG	50% ChH, 50% ChG					242
Ch/ChH/ChG	50% Ch 25% ChH 25% ChG	0.84	0.42	0.42		242
ChHG	100% ChHG				1.68	242
Ch/ChHG	50% Ch 50% ChHG	0.84			0.84	242

D₂O containing 2.2% (v:v) of HCl 12 M. The ChHG sample was prepared by dissolving 2.7 mg of product in 700 μ L of deuterated acetic acid 2 M in D₂O. Acetic acid-d₄ (99.9% isotopic purity) and D₂O (99.9% isotopic purity) were purchased from Merck Life Science Srl, Milan, Italy. All spectra were acquired at 298 K with Bruker Avance NMR spectrometers operating at the Larmor proton frequency of 400 or 700 MHz. ¹H spectra were acquired with 16–32 scans, 10 s interscan delay, and water suppression with z gradients. The proton spectra of Ch have been internally referenced against trimethylsilyl propanoic acid (TSP) at 0.0 ppm. The proton spectra of ChHG were referenced considering the residual methyl resonance of deuterated acetic acid (CHD₂-COOD) at 2.03 ppm. The acetylation degree and peptide functionalization of Ch were obtained by signal integration performed using MESTREN-OVA 6.0 software (Mestrelab Research, S.L., Santiago de Compostela, Spain). One specimen was analyzed for each group.

Fourier-transform infrared (FT-IR) spectroscopy

Reflection–Absorption InfraRed Spectroscopy (RAIRS) measurements were performed by means of a VECTOR 22 (Bruker) FT-IR interferometer operating in the wavenumber range 400–4000 cm⁻¹, with a resolution of 1 cm⁻¹, equipped with a Specac P/N 19650 series monolayer/grazing angle accessory and with a DTGS detector. Spectra were recorded at incidence angles of 70° with respect to the normal to the sample surface. One specimen was tested for each group.

Scanning electron microscope (SEM) analysis

To evaluate the microstructure and the surface topography of the spongy chitosan and functionalized chitosan scaffolds, a scanning electron microscope (SEM, Mod JSM-6490 Jeol, Peabody, MA, USA) analysis was performed. Each sample was sputter-coated with gold (Quorum sputter coater model Q150R E, Quorum Technologies, Laughton, UK), and all analyses were performed using a tungsten thermionic emission SEM system suitable for high-vacuum operations, equipped with an energy-dispersive X-ray detector (EDX), model IXRF Systems 500 (Jeol, Peabody, MA, USA). One specimen was tested for each group: images of three different areas were collected at multiple magnifications.

Mechanical measurements

Compression tests were performed on the different kinds of chitosan scaffolds (radius $R_0 = 5.0$ mm, height $H_0 = 0.3$ mm). Samples were immersed in physiological solution at 37 °C and compressed using an INSTRON 5566 testing machine at a rate of 1 mm/min up to a strain of 50%. Considering the measured force F , the initial cross-sectional area of the specimen ($A_0 = \pi R_0^2$), the height variation (ΔH), and the initial height (H_0) of the specimen, the engineering stress (σ) and the engineering strain (ϵ) were calculated as follows: $\sigma = F/A_0$ and $\epsilon = \Delta H/H_0$. Five specimens were tested for each group.

Biological assays *in vitro*

Primary human osteoblast culture

Primary human osteoblast cells were isolated from cortical mandibular bone fragments obtained during routine oral surgical procedures. The study protocol was approved by the Ethics Committee Azienda Ospedale, Padova University (4899/AO/20 on May 26, 2022), and written informed consent was obtained from the donor prior to sample collection. Bone explants were placed in Dulbecco's Modified Eagle Medium/Ham's F12 (1:1) supplemented with 20% (v/v) fetal bovine serum, 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, 1% (v/v) antibiotic–antimycotic solution, and 1 U/mL insulin (ThermoFisher Scientific, Milan, Italy). Cultures were maintained under standard conditions until osteoblastic cells migrated from the explants and reached adequate expansion. For osteogenic induction, cells were cultured in complete medium supplemented with 50 µg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerophosphate (Merck, Milan, Italy). For experimental assays, cells were detached with trypsin–EDTA (Thermo Fisher Scientific, Milan, Italy), washed with culture medium, and seeded onto the different substrates at a density of 1×10^5 cells/mL. The culture medium was replaced every 2 days when required. Cells between passages 3 and 5 were used for all experiments.

Cell adhesion and proliferation assay

For the adhesion assay, primary human osteoblasts were plated onto the different substrates and allowed to attach for 2 h at 37 °C under standard culture conditions. After incubation, non-adherent cells were removed by washing with phosphate-buffered saline (PBS). Adherent cells were then incubated for 4 h with MTT solution (5 mg/mL; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Merck, Milan, Italy). The reaction was terminated by adding 0.01 N HCl in 10% (v/v) sodium dodecyl sulfate (SDS). After 16 h of solubilization, absorbance was measured at 620 nm using a microplate reader.

Cell proliferation on the different matrices was analyzed using carboxyfluorescein diacetate succinimidyl ester (CFSE, ThermoFisher Scientific), a membrane-permeable fluorescent dye that is

progressively diluted during cell division. Osteoblasts were incubated with 25 µM CFSE at 37 °C for 10 min in pre-warmed PBS containing 0.1% (v/v) bovine serum albumin (BSA). Staining was quenched by adding five volumes of ice-cold complete culture medium. Cells were subsequently washed, counted by Trypan blue exclusion, and seeded onto the matrices at a density of 1×10^5 cells/mL. After 4 days of culture, samples were collected, rinsed with PBS, and incubated with trypsin–EDTA for 5 min under gentle agitation to detach cells. Proliferation was quantified using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) by analyzing 10,000 events and determining the percentage of CFSE-positive cells.

Mineralization assay

Primary human osteoblasts were cultured on the different matrices for 7 days, corresponding to the time point previously identified as optimal for calcium deposition (Brun et al. 2021a). At the end of the incubation period, samples were rinsed with phosphate-buffered saline (PBS) and stained with 40 mM Alizarin Red solution (pH 4.2) for 40 min at room temperature in the dark to detect calcium deposits. Following staining, cells were incubated with 10% (v/v) acetic acid for 30 min at −20 °C to extract the bound dye. The resulting suspensions were heated at 85 °C for 10 min and subsequently centrifuged at $13,000 \times g$ for 10 min at 4 °C. Supernatants were collected and their pH adjusted to neutral. Alizarin Red quantification was performed by measuring absorbance at 405 nm using a microplate reader (Victor X2 MultiPlate Reader, PerkinElmer, Milan, Italy).

Gene expression analysis

The expression of Runt-related transcription factor 2 (*RUNX2*), vitronectin (*VTN*), and osteopontin (*SPP1*) transcripts was evaluated in primary human osteoblasts after 24 h of culture on the different matrices. Following incubation, samples were rinsed with PBS, and total RNA was isolated using the SV Total RNA Isolation System (Promega, Milan, Italy), according to the manufacturer's instructions. Residual genomic DNA contamination was eliminated by DNase I treatment. Reverse transcription and quantitative PCR amplification were carried out in a single step using

the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Milan, Italy). Each reaction included 200 nM of forward and reverse primers, iTaq Universal SYBR Green reaction mix, iScript reverse transcriptase, and 200 ng of total RNA as template. Quantitative real-time PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative gene expression levels were calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal control. Primer sequences are reported in Table 2.

Biological assays *in vivo*

Surgical procedures and animal care were conducted following the guidelines of the Animal Care and Use Committee of Padova University (244/2015-PR), under the oversight of the Italian Ministry of Health and in adherence to the European directive for animal experiments. All authors complied with the ARRIVE guidelines. A total of 21 eight-week-old C57BL/6 J male mice were anaesthetized using 5% isoflurane inhalation. The dorsal skin was shaved and cleaned with 70% ethanol. Then, the skin was cut, creating a 1 cm long incision on the left and right sides of the lumbar region. Indeed, each animal received two scaffolds (one for each dorsal site) to align with the 3R principles (replace, reduce, refine) for ethical animal research. Three replicates were performed for each scaffold; each replicate was implanted in 3 different animals. Scaffolds were immediately positioned, and the incisions were sutured. During the surgical procedure, the mice were placed on a heated pad and subsequently monitored closely for 12 h. Thereafter, animals were returned to their cages and observed for signs of dehydration or pain, such as hunched posture or reduced mobility. Body weight was recorded weekly. Animals were sacrificed, and scaffolds were harvested 40 days after the surgical procedure, a time

established in our and other previous studies (Scott et al. 2012; Zamuner et al. 2021).

Histological processing

The specimens were fixed in 10% formalin for 24 h before histological processing with a Leica HistoCore PEARLS system. Sections of 5 µm thickness were obtained with a rotating microtome (Slee Cut 6062; Slee Medical, Mainz, Germany) and stained with Haematoxylin and Eosin (H&E), according to the manufacturer's protocol (Histo-Line Laboratories). The sections were then acquired as whole slide images (WSI) using a digital slide scanner (Slideview VS200, Evident, Japan).

Digital image analysis

The scanned WSI images were used for histological quantification in QuPath version 0.5.0 (Bankhead et al. 2017). Identification of the scaffold, capsule, and connective tissue on WSI was performed by two independent morphologists. Each component (capsule, scaffold, and connective tissue) was detected in QuPath using threshold-based tools on digital RGB image channels. Cell segmentation was performed using the dedicated cell segmentation interface (Cell Detection), setting optimized parameters for the isolation of nuclei.

The capsule thickness was assessed by manually measuring the thickness at 5 different points for each capsule identified. The capsule cellularity, defined as the number of cells per square millimeter, was determined by calculating the ratio of the total number of cells identified through nuclear segmentation to the occupied area of the capsule. Similarly, scaffold cellularity was assessed by relating the number of segmented cells within the scaffold to the surface area occupied by the scaffold. The residual scaffold material was quantified by measuring the total implant area in relation to the scaffold area visualized in the

Table 2 Oligonucleotide sequences for quantitative PCR assay

Gene	Sequence
<i>GAPDH</i>	Fw: 5'-agtgccagcctcgctcccgta-3' Rv: 5'-caggcgcccaatacggccaa-3'
<i>RUNX2</i>	Fw: 5'-cagtgcacacatgtcagcaa-3' Rv: 5'-gctcacgtcgctcatttg-3'
<i>VTN</i>	Fw: 5'-ggaggacatcttcgagcttct-3' Rv: 5'-gctaatgaactggggctgtc-3'
<i>SPPI</i>	Fw: 5'-aagtttcgacagacctgacatc-3' Rv: 5'-ggctgtcccaatcagaagg-3'

samples. The inflammation score and tissue response were performed on 3 ROIs at 40× for each sample, following the guidelines from DIN ISO 10993–6 (2016).

Statistical analysis

Biological data are reported as the mean ± standard deviation (SD) of at least three independent experiments, each performed in triplicate (the number of replicates for each experiment is specified in the corresponding section). The statistical analysis of data from mechanical tests was performed using ANOVA and Tukey's HSD test. Statistical analysis of data from bioassays was performed using a one-way ANOVA test followed by Dunnett's multiple comparison test, using Graph-Pad Prism 10.2.3. Statistical significance was considered for p -values < 0.05.

Results and discussion

Chitosan functionalization assessment

XPS analysis

XPS spectra of plain chitosan (Ch) and functionalized chitosan (ChH, ChG, and ChHG) were collected at C1s, N1s, and O1s core levels and analyzed using Gaussian profiles for fitting. When different elements were identified in the spectrum, the same width at half-height was used for all individual photoemission bands.

A complete collection of Binding Energy (BE), full-width half maximum (FWHM) values, and proposed assignments are reported in Table S1 in the Supplementary Material for all samples. N1s and O1s spectra are considered the most interesting in order to probe the successful functionalization of Ch surfaces; as an example, N1s and O1s spectra collected on plain Ch and Ch/ChHG are shown in Fig. 1 and will be discussed in detail here, in comparison with ChG and ChHG data reported in Table S1. As can be observed in Table S1 and Fig. 1a, c, N1s spectra of all samples consist of a single signal assigned to nitrogen bound to a carbon with a single-bond (C–N) (BE = 398 eV). This signal is not distinguishable from the amide group diagnostic for peptide bonds (NHCO) (Brun et al. 2021b), so its persistence is

expected in the modified Ch surfaces. O1s spectra (Fig. 1b, d) for Ch and Ch/ChHG are composite, and two spectral components can be identified in all samples by applying a peak-fitting procedure.

The component at low BE values (530 eV, in green in Fig. 1b, d) refers to the oxygen of a carbonyl (C=O) functional group; Ch spectrum (Fig. 1b) has a very low C=O signal, while the intensity of this peak considerably increases in Ch/ChHG (Fig. 1d), suggesting the successful anchoring of the peptides on the Ch surface, as previously observed and reported in the literature for similar systems (Secchi et al. 2019).

The O1s spectral component observed in all samples at higher BE values (531 eV) is assigned to C–O functional groups of chitosan. These data confirmed the successful chitosan functionalization. In particular, the increase in the N/O ratio is particularly interesting as the peptides possess a higher nitrogen content with respect to pristine chitosan (Table 3).

NMR analysis

Ch and ChHG were both characterized by solution NMR: the first to estimate the degree of Ch acetylation and therefore the percentage of free amino groups available to bind peptides; the second to evaluate the percentage of HVP and GBMP1 α peptides bound to Ch. For these purposes, the same methodology applied for the characterization of ChH and ChG was used (Brun et al. 2021b). The 1D proton spectrum of Ch is shown in Fig. 2a. The 4.0–3.5 ppm region shows signals due to the 2H–6H protons of the GlcN and GlcNAc units except for the 2H proton of GlcNAc, which resonates at ca. 3.15 ppm. The protons of the CO–CH₃ acetyl group of GlcNAc are observed at 2.05 ppm, while the remaining 1H protons are not visible because the water suppression procedure canceled them.

The degree of acetylation of Ch [1] is easily obtained from the ratio between the integral of the acetyl methyl protons, I_{CH_3} , compared to the integral of the remaining 6 protons of each saccharide unit, $I_{\text{H}_2\text{--H}_6}$. From the calculation:

$$\frac{I_{\text{CH}_3}}{3} \times \frac{6}{I_{\text{H}_2\text{--H}_6}} \quad (1)$$

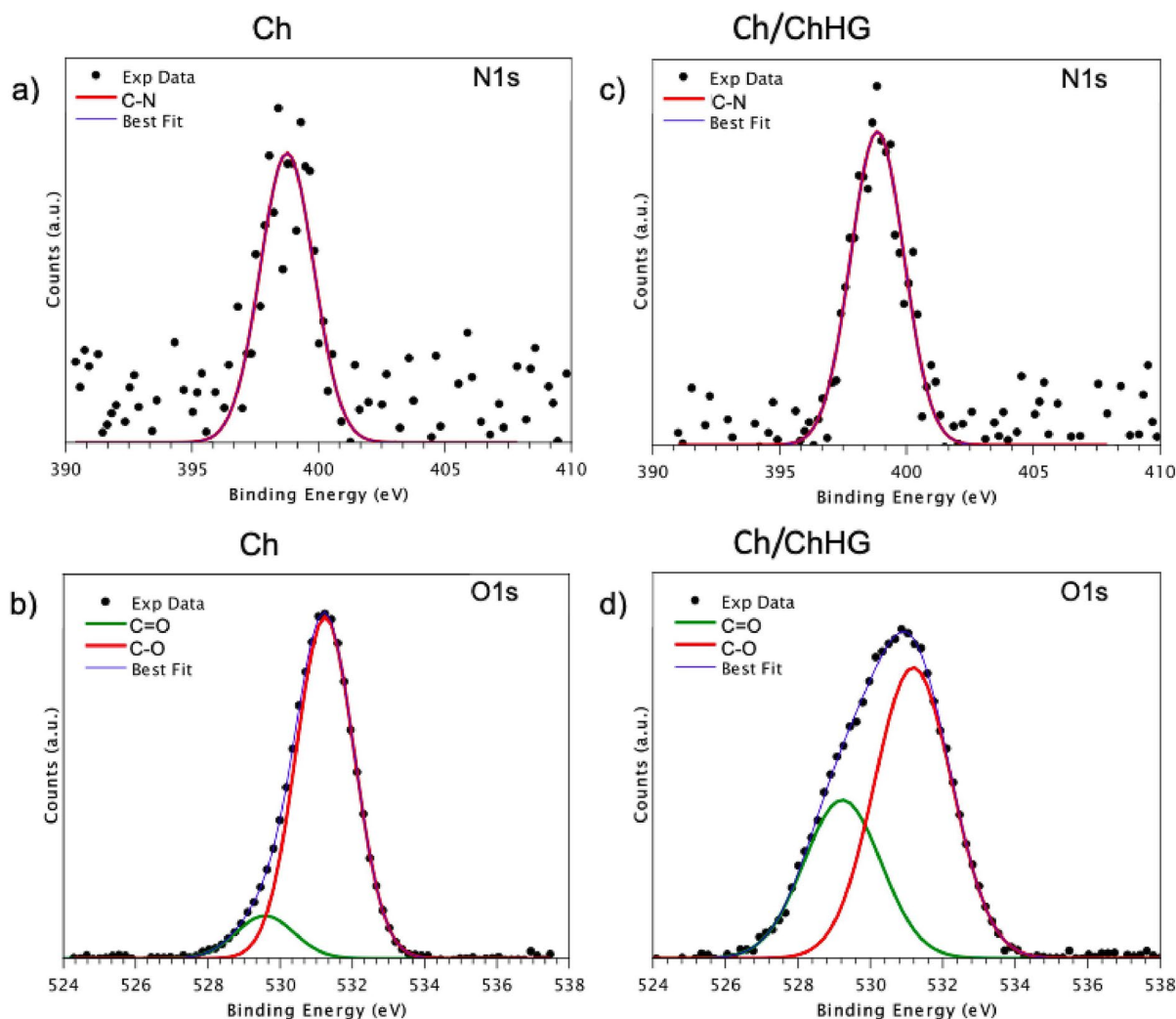


Fig. 1 XPS analysis. N1s **a, c** and O1s **b, d** experimental spectra collected on Ch and Ch/ChHG, respectively, with curve-fitting analysis. Markers represent experimental points; lines represent fitting components and best-fit

Table 3 N/O ratio of each sample determined by XPS analysis

Sample	N/O
Ch	0.05
Ch/ChH	0.1
Ch/ChG	0.09
ChH/ChG	0.2
Ch/ChH/ChG	0.1
ChHG	0.2
Ch/ChHG	0.1

an acetylation degree of $36 \pm 4\%$ is obtained, indicating that the percentage of amino groups useful for the functionalization of Ch represents $64 \pm 6\%$, in line with the indication of the commercial product (70%).

The average number of peptides covalently linked to the chitosan chains was obtained by considering the ratio between the integral corresponding to one proton of the HVP or GBMP1 α peptide, compared to the integral corresponding to one proton of the chitosan, $R = I_{\text{H-pept}}/I_{\text{H-Ch}}$. The proton spectrum of ChHG ($\text{D}_2\text{O}/\text{CD}_3\text{COOD}$) reported in Fig. 2b shows two isolated doublets at 7.1 and 6.8 ppm. These signals, due to the aromatic protons 2,6 H and 3,5 H of

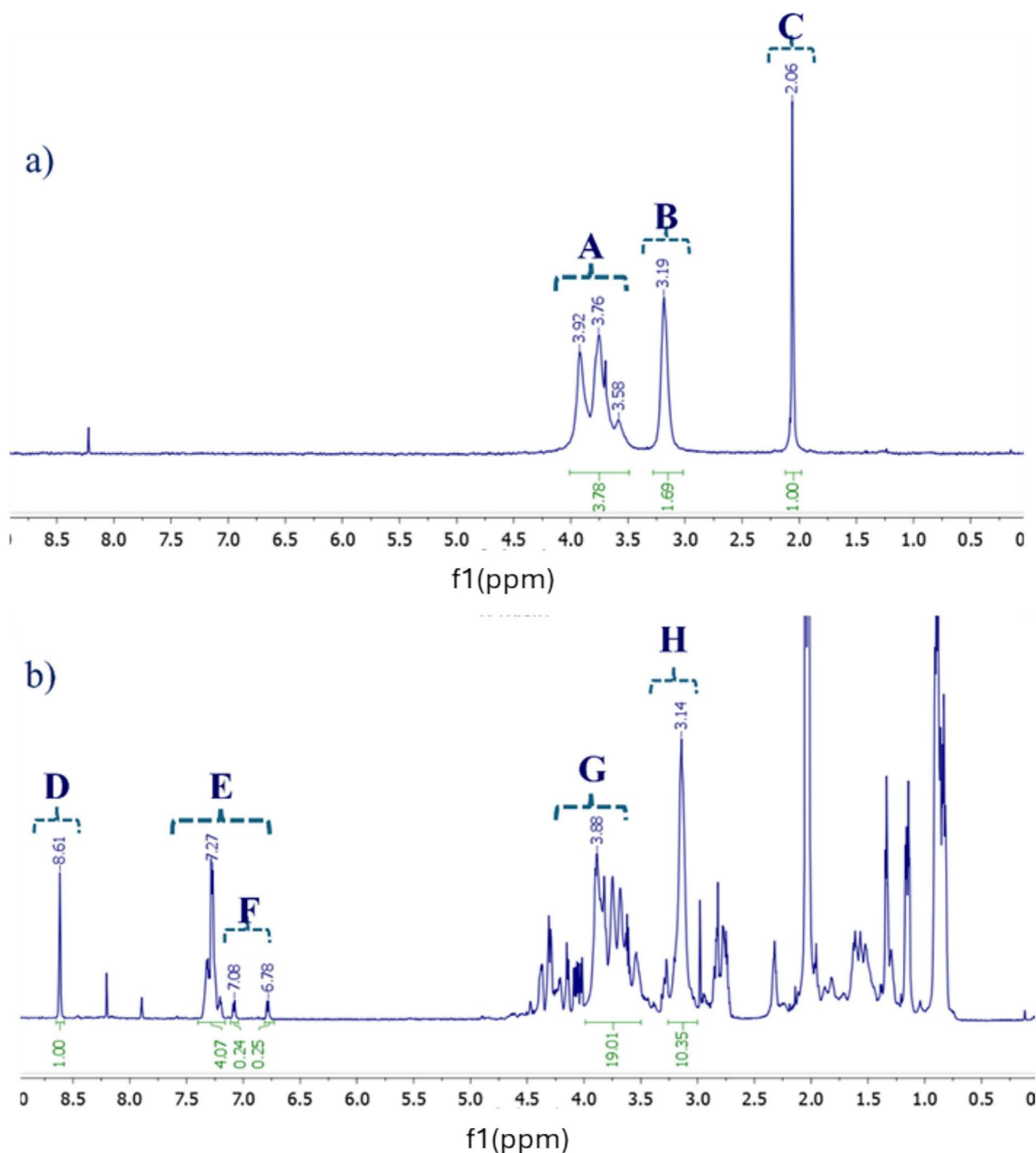


Fig. 2 NMR analysis. **a** ^1H -NMR spectrum of Ch in HCl- D_2O 2.2% (v:v) acquired at 298 K and 400 MHz; **b** ^1H -NMR spectrum of ChHG in CD_3COOD 2 M in D_2O acquired at 298 K

and 700 MHz. Green numbers below the signals refer to integral values. Blue numbers above the peaks refer to chemical shift in ppm. Prot=proton

Tyr¹¹ of HVP, allow us to estimate the integral corresponding to 1 proton of HVP, I_{HVP} . According to the spectrum, the I_{HVP} value is 0.12. The entire 8.7–6.8 ppm spectral region picks up the resonances

of 10 aromatic protons from HVP (Phe³, His⁵, Tyr¹¹) and 7 from GBMP1 α (Phe⁴, His⁹, His¹³). Therefore, the integral corresponding to a single GBMP1 α proton, $I_{\text{GBMP1}\alpha}$, was obtained by subtracting the

contribution of the HVP protons from the integral of the entire region and dividing the result by 7 ($I_{\text{GBMP1}\alpha}=0.44$). Finally, the integral equals to 1 proton of Ch, I_{Ch} , was evaluated considering that the resonances of Ch overlap with 12 protons of HVP and 16 of GBMP1 α . For that, the I_{Ch} was obtained by subtracting from the total integral of the chitosan regions, the contribution of both peptides and dividing the result by 6, *i.e.*, the number of protons for each monosaccharide unit, for an I_{Ch} value of 3.11. The calculation returns a substitution ratio of approximately 4% for HVP and 14% for GBMP1 α . Considering that the chitosan sites available for functionalization are approximately $64 \pm 6\%$, we can conclude that HVP binds ca. 6–7% and GBMP1 α ca. 20%. To note that these findings refer to the soluble components of the adducts.

Scaffold characterization and mechanical measurements

The infrared spectra of all chitosan blends are reported in Fig. 3. All spectra showed a broad band around $3600\text{--}3400\text{ cm}^{-1}$ due to the O–H stretching vibrations, and a peak at 1080 cm^{-1} related to C–O stretching vibrations.

Both pristine chitosan and blends containing functionalized chitosan presented the band at 1650 cm^{-1} related to C=O stretching, as chitosan is only partially deacetylated, but in the case of blends enriched with functionalized chitosan, a new

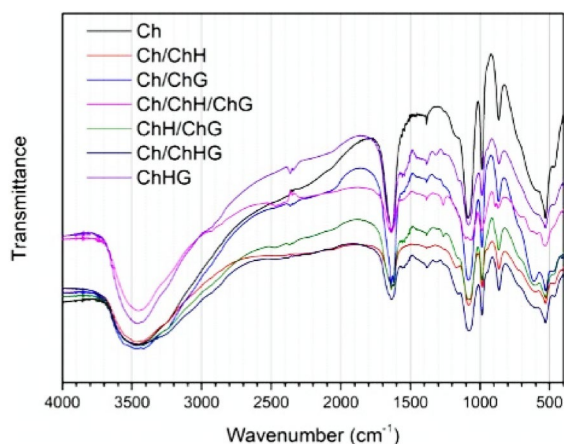


Fig. 3 FT-IR analysis. spectra of pristine chitosan (Ch, as control) and all the different chitosan blends

band related to the Amide II present in the peptide sequences appeared at $1530\text{--}1550\text{ cm}^{-1}$. FT-IR characterization of pure ChH and pure ChG was reported elsewhere (Brun et al. 2021b).

The morphological appearance of the scaffolds was evaluated using a scanning optical microscope (SEM), as reported in Fig. 4.

The pure chitosan matrix (Ch, Fig. 4) exhibits the typical open-cell porous structure. For the control matrix Ch, porosity was measured in terms of both average length ($22.176 \pm 3.62\text{ }\mu\text{m}$) and width ($13.79 \pm 3.58\text{ }\mu\text{m}$). The porosity is significantly lower compared to chitosan matrices frozen in dry ice ($-80\text{ }^{\circ}\text{C}$), as reported in the literature (Hsieh et al. 2007; Qian et al. 2011; Gong et al. 2014). Indeed, the lower freezing temperature of liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) leads to faster freezing with rapid ice nucleation and small crystal formation, whereas slower cooling at higher temperatures results in slower ice nucleation and larger crystals.

The Ch/ChH matrix is structurally similar to the pure chitosan control; the S-X-HVP peptide is highly basic and positively charged, similar to pristine chitosan. In this case, the pore size is slightly smaller, with an average length of $16.71 \pm 3.18\text{ }\mu\text{m}$ and an average width of $9.63 \pm 2.69\text{ }\mu\text{m}$. Ch/ChG matrix results in a less spongy structure with pores having an average length of $14.53 \pm 2.08\text{ }\mu\text{m}$, and an average width of $11.07 \pm 2.89\text{ }\mu\text{m}$. Conversely, the Ch/ChH/ChG scaffold presents a morphologically distinct and ‘filamentous’ structure. Pore measurements in this case yielded an average length of $20.46 \pm 2.17\text{ }\mu\text{m}$ and an average width of $15.94 \pm 2.96\text{ }\mu\text{m}$. The ChH/ChG matrix is similar to the control structure but denser, with smaller pores: the average pore length is $11.38 \pm 2.88\text{ }\mu\text{m}$, and the width is $11.21 \pm 2.37\text{ }\mu\text{m}$. The last two conditions, Ch/ChHG and ChHG, are visually very similar to each other and to the control matrix: they have average lengths of $20.46 \pm 2.17\text{ }\mu\text{m}$ and $19.52 \pm 2.55\text{ }\mu\text{m}$, respectively, and average widths of $12.76 \pm 2.99\text{ }\mu\text{m}$ and $15.07 \pm 3.17\text{ }\mu\text{m}$, respectively.

The mechanical behavior of all scaffolds is similar to that of a flexible foam, as reported in Fig. 5. The compressive modulus (E) was calculated as the slope of the initial linear region of the stress–strain curve. Table 4 reports the compressive modulus (E) and maximum stress (σ_{max}) as mean value $\pm$ standard deviation.

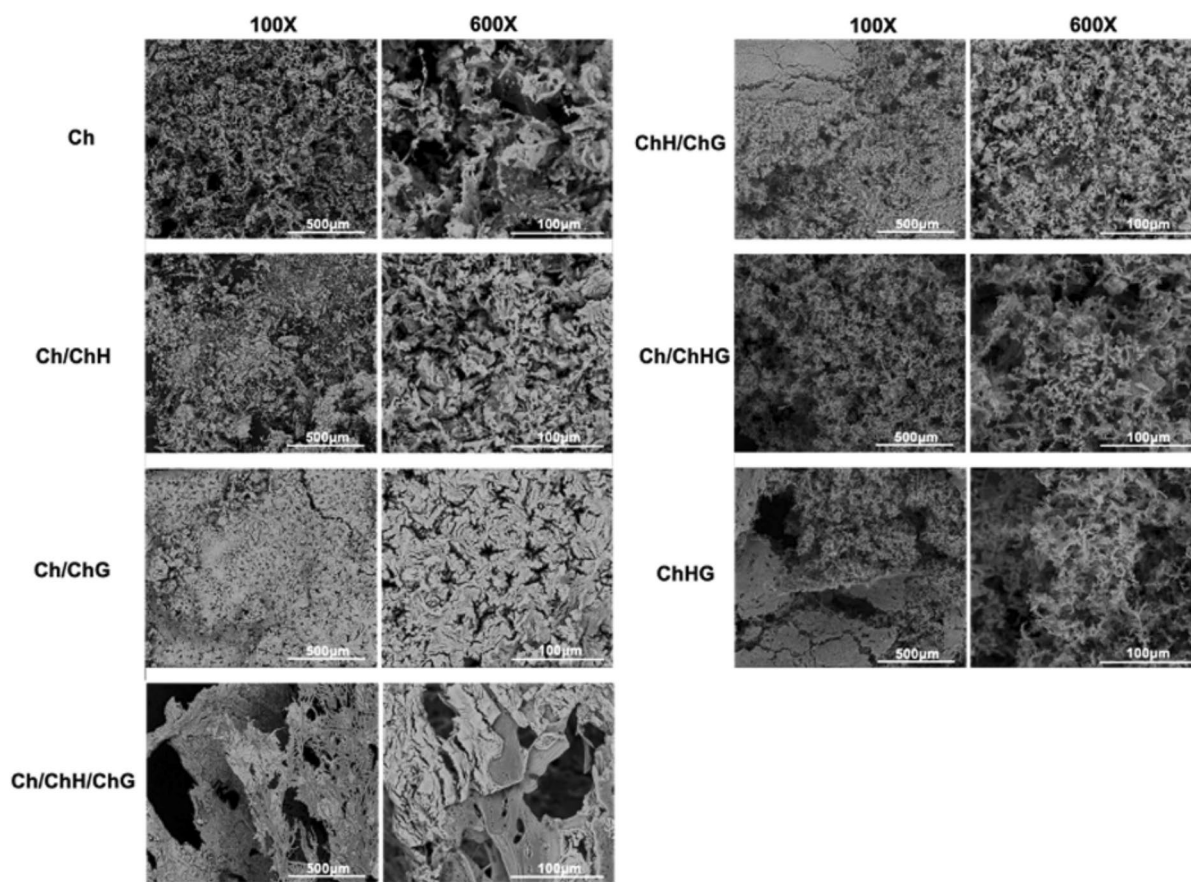


Fig. 4 SEM analysis. Surface morphology of all scaffolds. Images were acquired using a SEM at two enlargements (100× and 600×)

The highest value of maximum stress (8.9 ± 0.5 kPa) was observed for Ch (Table 4, Table S2). However, the highest values of compressive modulus were found for Ch (22.6 ± 2.0 kPa), Ch/ChH (21.3 ± 2.1 kPa), Ch/ChG (23.0 ± 2.3 kPa), and Ch/ChHG (22.7 ± 2.3 kPa) as no statistically significant differences were observed among these groups (Table 4, Table S2). Concerning maximum stress, there were no significant differences among Ch/ChH (7.3 ± 0.4 kPa), Ch/ChG (7.1 ± 0.4 kPa), and Ch/ChHG (6.9 ± 0.5 kPa) (Table 4, Table S2). Furthermore, the values of compressive modulus and maximum stress obtained for Ch/ChH/ChG (16.4 ± 1.4 kPa and 5.6 ± 0.3 kPa) were significantly lower than those measured for Ch, Ch/ChH, Ch/ChG, and Ch/ChHG (Table 4, Table S2).

However, the compressive modulus and maximum stress found in the case of Ch/ChH/ChG were

significantly higher than those obtained for ChH/ChG (10.1 ± 1.0 kPa and 4.0 ± 0.3 kPa, respectively) and ChHG (8.5 ± 0.8 kPa and 4.1 ± 0.3 kPa, respectively). In addition, no statistical differences were found between ChH/ChG and ChHG in terms of compressive modulus and maximum stress (Table 4, Table S2).

The obtained results underline the need to find a compromise that can optimize the scaffold design to balance the biological and mechanical properties.

Biological assays *in vitro*

All the scaffolds containing ChH significantly enhanced the initial human osteoblast adhesion (at 2 h of incubation) compared to the control (Ch), confirming the adhesion property of the HVP peptide anchored to the substrate (Fig. 6a). This result is in

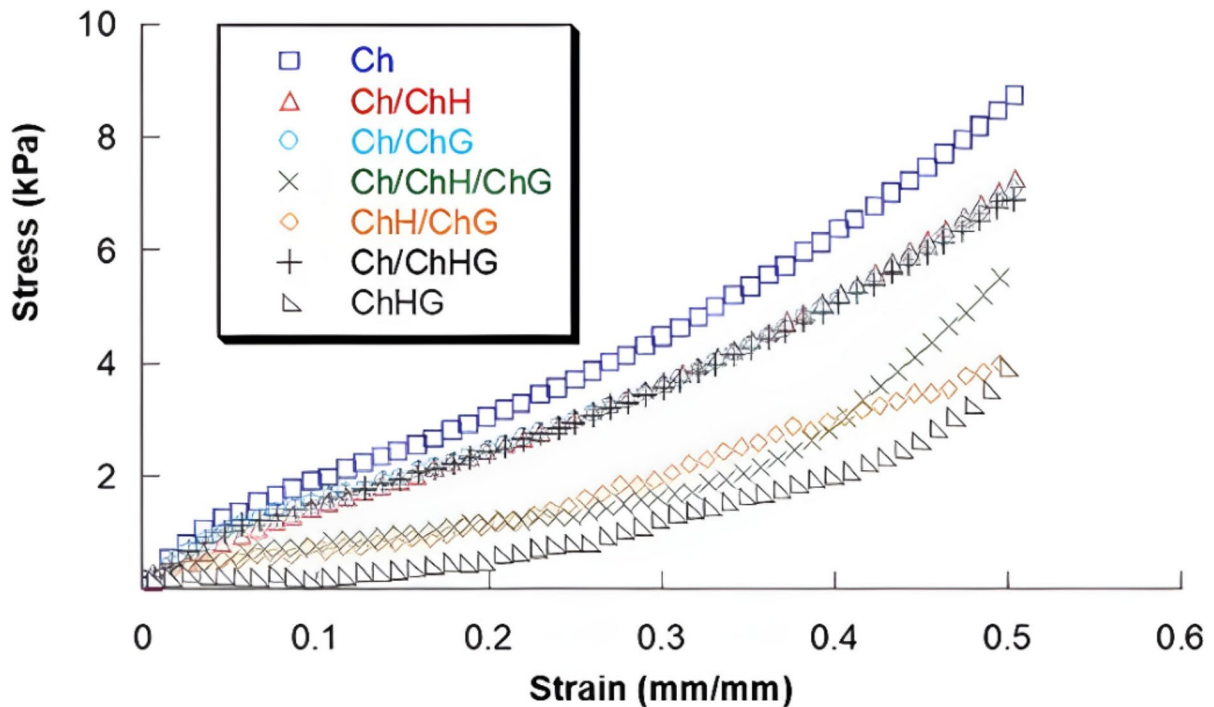


Fig. 5 Mechanical measurements. Typical stress–strain curves obtained from compression tests on the different kinds of chitosan scaffolds (rate of 1 mm/min, final strain of 50%)

Table 4 Results from mechanical measurements on the different kinds of chitosan scaffolds: compressive modulus (E) and maximum stress ($\sigma_{\max}$) reported as mean value $\pm$ standard deviation

Scaffold	E (kPa)	$\sigma_{\max}$ (kPa)
Ch	22.6 ± 2.0	8.9 ± 0.5
Ch/ChH	21.3 ± 2.1	7.3 ± 0.4
Ch/ChG	23.0 ± 2.3	7.1 ± 0.4
Ch/ChH/ChG	16.4 ± 1.4	5.6 ± 0.3
ChH/ChG	10.1 ± 1.0	4.0 ± 0.3
Ch/ChHG	22.7 ± 2.3	6.9 ± 0.5
ChHG	8.5 ± 0.8	4.1 ± 0.3

line with previously published data where chitosan functionalized with HVP peptide gave the best osteoblast adhesion result (Brun et al. 2021b). Ch/ChG is the only scaffold not containing ChH that showed a significant increase in osteoblast adhesion, whilst the contemporary presence of both peptide sequences (Ch/ChHG and ChHG) seems not to induce any dramatic positive cellular response in terms of adhesion.

These differences in results could be due to different surface peptide concentration or distribution.

All scaffolds showed a statistically significant increase in osteoblast proliferation when compared to the pure chitosan scaffold Ch (Fig. 6b and c). It's interesting to note that although the ChHG and Ch/ChHG did not improve cell adhesion within 2 h of incubation, they yielded the best results in terms of cell proliferation after 4 days of cell culture. In Fig. 7a, data obtained from the calcium deposition assay are reported. Calcium deposition is a key marker of osteoblast differentiation and the formation of bone-like mineralized matrix. Indeed, higher levels of calcium deposition suggest that the substrates not only support osteoblast adhesion and proliferation but also promote osteoblastic-like phenotype in primary human bone-derived cells. This is critical for the scaffold's effectiveness in bone tissue engineering. Our data indicated that all tested scaffolds support an osteoblastic-like phenotype, thereby inducing a mineralized extracellular matrix necessary for bone regeneration and loading.

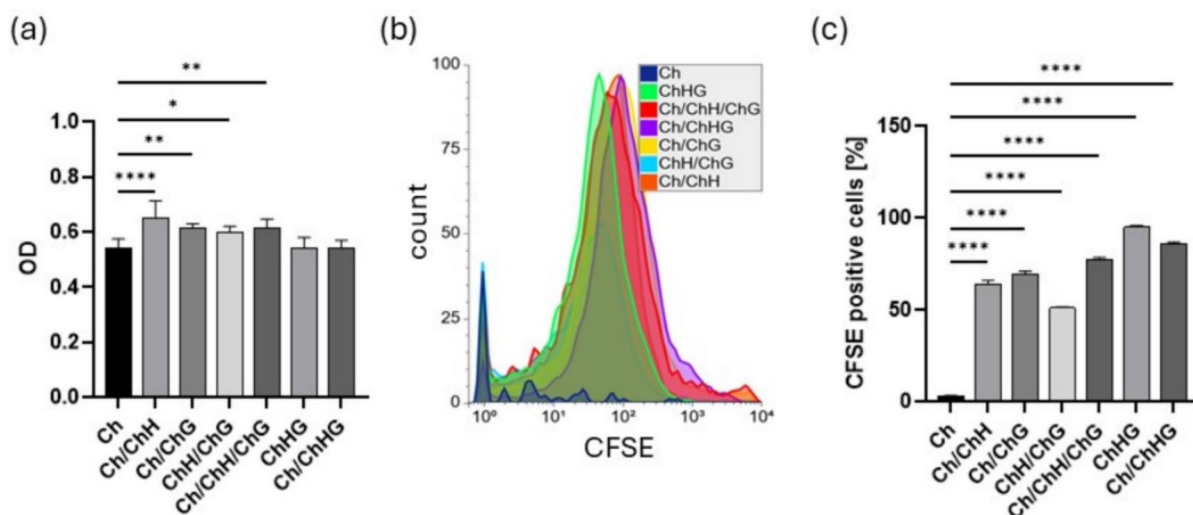


Fig. 6 Biological assays *in vitro* with human osteoblasts on porous scaffolds of different blends of functionalized chitosans. Human osteoblast adhesion at 2 h **a** and proliferation at 4 days **b** and **c** from cell seeding. The percentage of CFSE-positive cells was determined by flow cytometry, and data were

analyzed using Floreada.io **b**. Data were graphed **c**. Data are reported as mean $\pm$ standard deviation of 3 independent experiments each performed in triplicate ($n=9$). * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.0001 vs Ch/ChG and/or ChHG

Since bone regeneration and matrix mineralization are secondary to gene expression, we quantified mRNA levels of genes involved in osteoblast differentiation (*RUNX2*), cell adhesion and spreading (*VTN*), and bone homeostasis (*SPPI*). As shown in Fig. 7b–d, all matrices were able to significantly increase the expression of the tested genes as compared with Ch.

Overall, the *in vitro* studies reported that scaffolds containing the highest concentration of ChG sustained better bone regeneration and extracellular bone matrix mineralization than scaffolds containing the highest concentration of ChH ($p < 0.0001$). Therefore, whereas ChH scaffolds primarily promote cell adhesion (Fig. 6a), Ch/ChG and ChHG substrates likely set the cellular signaling to express proteins involved in cell proliferation (Fig. 6c) and differentiation (Fig. 7b–d), leading to calcium deposition (Fig. 7a). At the same time, Ch/ChH/ChG and Ch/ChHG scaffolds do not sum up the single contribution of the functionalization, providing insights into the functional combination.

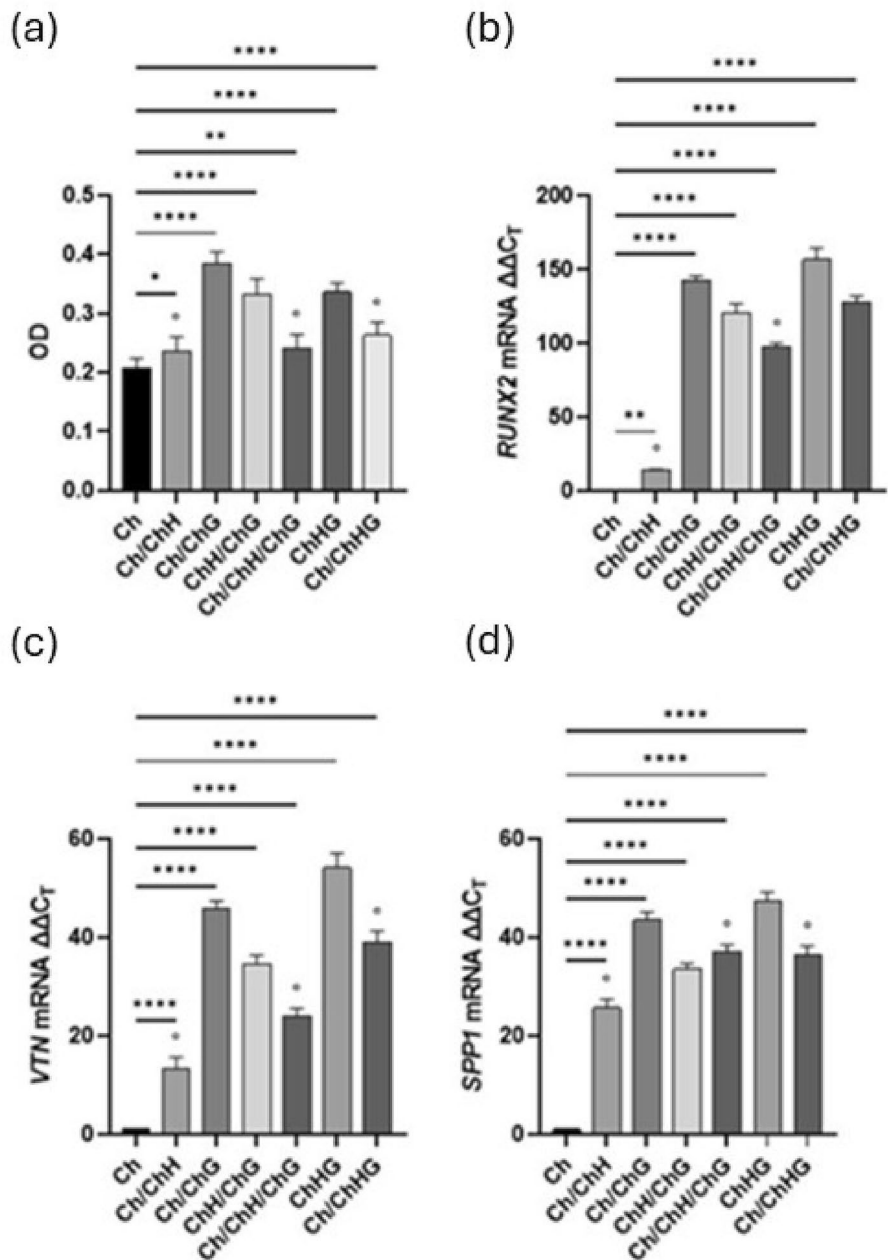
In the work of Mochizuki et al. (Mochizuki et al. 2003), adhesive peptides of laminin are conjugated alone or as a mixture to chitosan. The authors demonstrate not only a different response to the biochemical stimulus by different cells (fibroblasts versus neurites)

but, above all, that the mixtures of two anchored sequences provide results dependent on the concentrations of the individual peptides: if the 9:1 ratio behave by promoting the biological activity of the predominant peptide, the 1:1 ratio can create hybrid responses. In our case, a 1:1 ratio between the two peptides does not provide equal peptide densities: surprisingly, the NMR analysis indicates a substitution ratio of 6–7% for HVP and 20% for GBMP1 α . Therefore, it is logical that the ChHG matrix appears to possess characteristics more similar to ChG.

In vivo bioassays

Following the surgical procedures, the animals rapidly recovered with no abnormalities or signs of pain observed in the subsequent days. By monitoring the body weight, we observed slight, non-significant differences compared to non-treated mice, limited to the first week after the procedure. No alterations in posture and locomotion and no signs of dehydration, such as sunken eyes, were detected. At the site of implantations, we did not observe discharge, excessive redness, or swelling; the wounds closed and healed effectively. At the time of sacrifice, the tissue surrounding the implantation site appeared

Fig. 7 Biological assays *in vitro* with human osteoblasts on porous scaffolds of different blends of functionalized chitosans. Cell calcium deposition **a** at 7 days. Gene expression at 24 h of *RUNX2* **b**, *VTN* **c**, and *SPP1* **d**. Data are reported as mean $\pm$ standard deviation of 3 independent experiments each performed in triplicate (n=9). *p-value < 0.05, **p-value < 0.01, ***p-value < 0.0001 vs Ch/ChG and/or ChHG



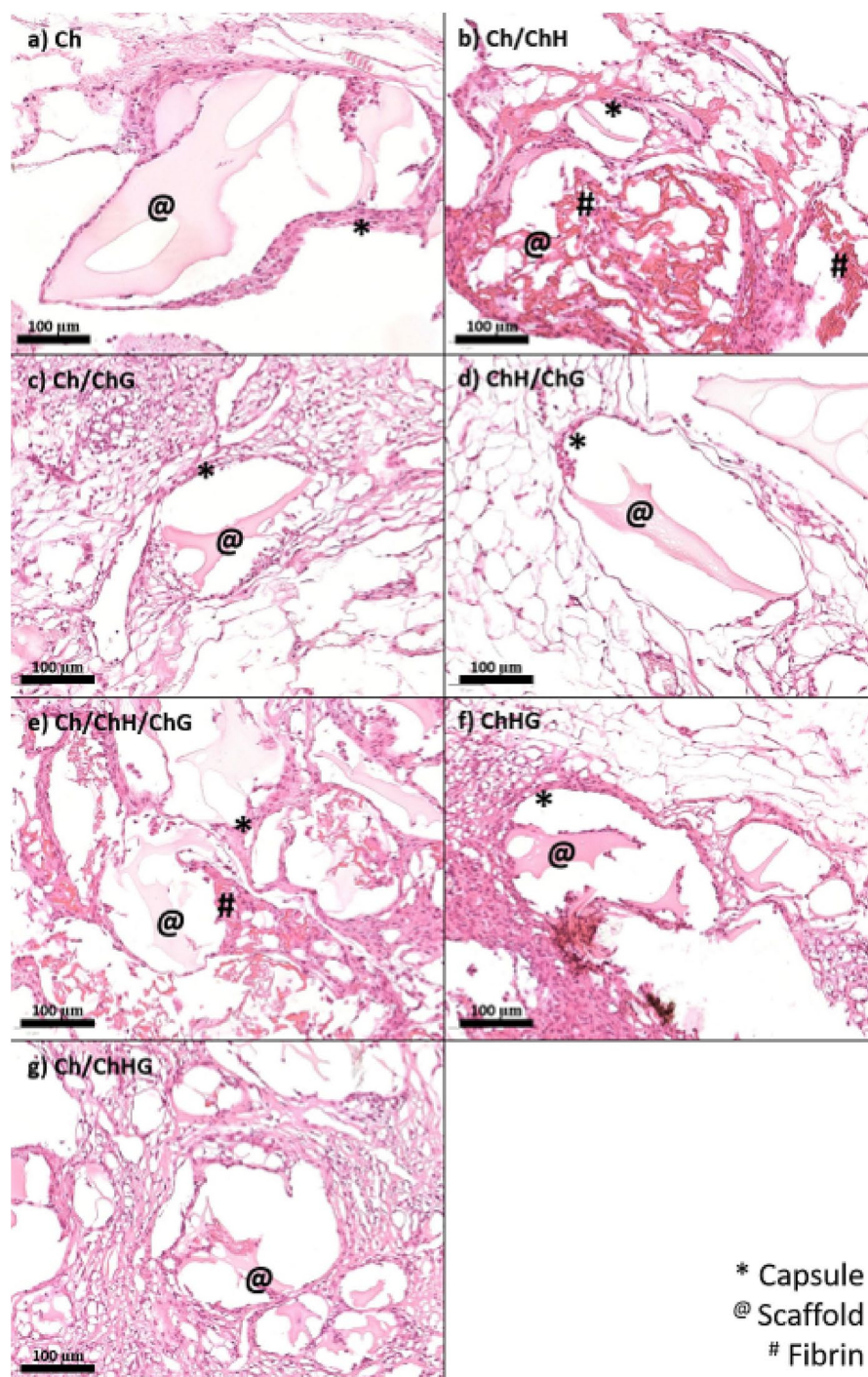
macroscopically normal, with no evidence of inflammation or necrosis.

Histological analysis demonstrated the subcutaneous encapsulation of all the grafted material (Fig. 8) in all experimental conditions; the newly forming tissue was characterized by fibrous connective tissue. Regardless of the chitosan functionalization, the capsule exhibited a reduction in thickness as compared to the control (Ch), as visible in

Fig. 8a-g. Quantitative assessment of the capsule thickness and cellularity corroborated this finding, revealing a statistically significant decrease in all functionalized scaffolds compared to the pure chitosan scaffold (Fig. 9a, b).

From a morphological point of view, the pure chitosan capsule was characterized by dense, irregular connective tissue, while the functionalized chitosan capsule was characterized by loose connective tissue,

Fig. 8 Histological analysis. Implant site and scaffold morphology visualized on H&E staining for each combination of chitosan, HVP, and GBMP1 α . Capsule, scaffold, and fibrin are marked respectively with *, @, and #. Scale bar = 50 μ m



promoting the reparation/regeneration of the implant site.

Regarding the cellular colonization of the scaffolds, the H&E staining revealed an enhanced cellular ingrowth in the Ch/ChH and ChHG combinations

compared to the control (Fig. 8b, e). The quantification of scaffold cellularity was statistically higher in these samples in comparison with pure chitosan (Fig. 9c), indicating these combinations could promote scaffold colonization. In all peptide

combinations, the amount of residual scaffold at the implant site appears slightly reduced compared to pure chitosan (Fig. 8a-g). However, the quantification of residual material showed no statistical reduction among samples (Fig. 9d), suggesting an increase in scaffold absorption and consequent enhancement in biocompatibility.

The overall evaluation of the inflammatory reaction to the implant revealed only a slight reaction that, for all the functionalized materials, resulted comparable to that of pure Chitosan. The ChH/ChG and ChHG combinations showed a lower inflammatory response, although not statistically significant compared to the control (Fig. 9e). Concerning the evaluation of tissue response, although the presence of loose connective tissue was mainly observed in the Ch/ChH, Ch/ChH/ChG, and ChHG combinations (Fig. 8a-g), all experimental conditions were non-irritant (Fig. 9f).

Conclusions

Recent works have been published (Lai et al. 2025) in which designed scaffolds for tissue engineering were able to provide cells with multiple stimuli through sequences that promote cell growth as well as anti-bacterial action and angiogenesis. This complex scenario offers a better interpretation of the extracellular matrix, which can stimulate multiple cellular activities through the subtle quantitative balance of soluble factors and insoluble molecules, influencing many cell types simultaneously present.

This work concerns the biological action of two peptides used separately to graft chitosan. The two functionalized chitosan were mixed in various proportions to prepare hybrid scaffolds, or alternatively, the two peptides were used to functionalize the polysaccharide in a simpler and faster procedure simultaneously. Biological assays further elucidated the benefits of peptide functionalization. Ch/ChH scaffolds notably enhanced initial osteoblast adhesion whereas Ch/ChG and ChHG demonstrated superior calcium

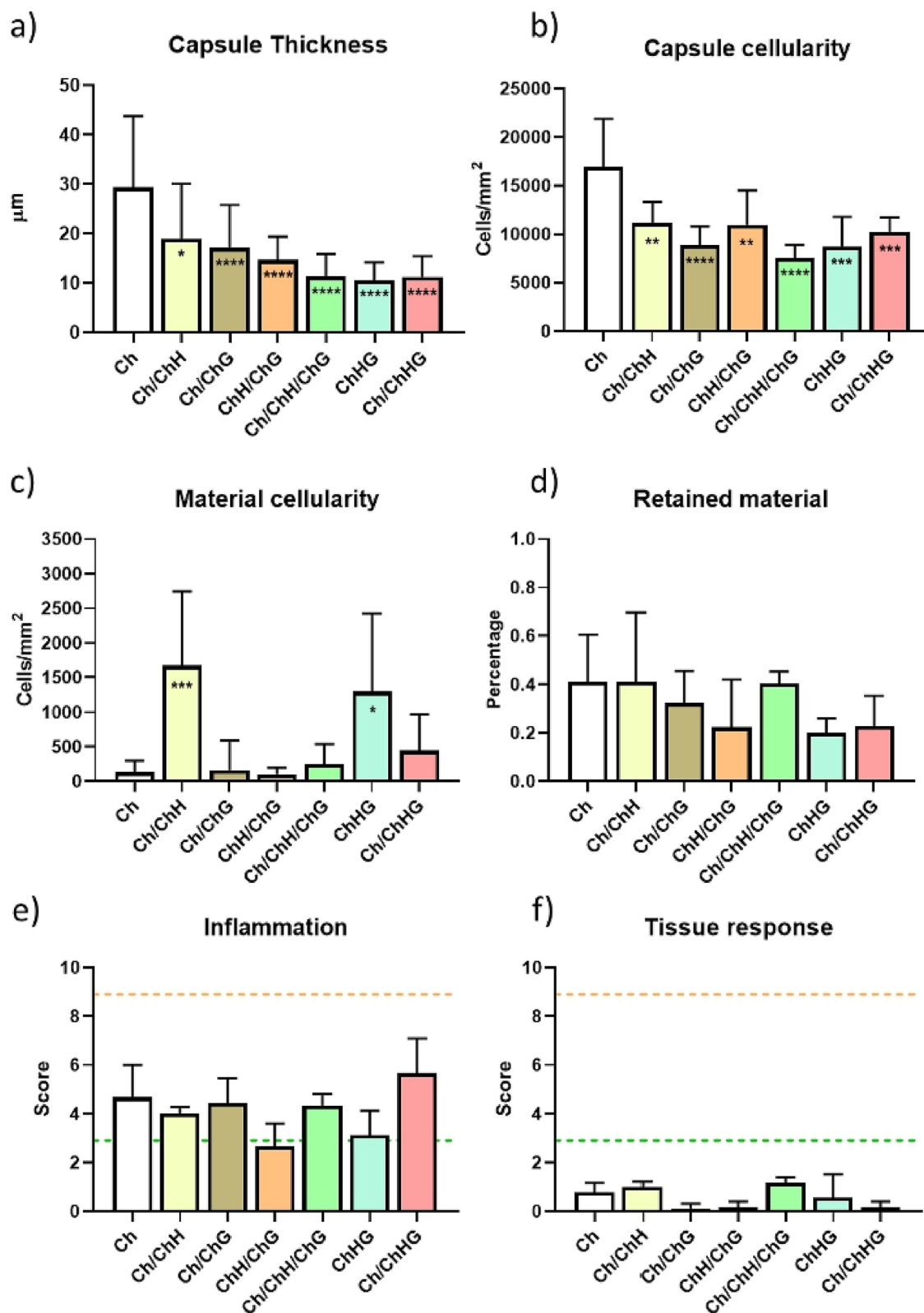
deposition and gene expression of *RUNX2*, *VTN*, and *SPPI*, essential indicators of effective new bone generation and matrix mineralization.

In the last cases, the incorporation of HVP and GBMP1 α peptides into chitosan scaffolds also suggests a synergistic effect. Thus, histological analysis of subcutaneously implanted scaffolds revealed how peptide functionalization of chitosan, although not completely suppressing the formation of a capsule surrounding the scaffold, reduced its thickness and cellularity, while not altering its inflammatory or tissue responsiveness. Furthermore, the Ch/ChH and ChHG conditions exhibited a significant increase in scaffold cellularity, demonstrating increased *in-vivo* colonization.

Even though the ChHG group provided interesting results in terms of biological performances, it has a compressive modulus and a maximum stress that are lower than those of the Ch group. However, it is also worth noting that the aim of the work was to explore several design alternatives according to several applications for bone regeneration (Schulze et al. 2023), and the biomechanical environment during endochondral ossification is characterized by a rather low modulus of approximately 8 kPa, differently from healthy mature bone tissue (Petersen et al. 2018; Schulze et al. 2023).

In addition, the presence of cells in the scaffold would generally lead to an increase of its overall stiffness, as cells may proliferate and populate the porous network, thus contributing to a higher, more rigid structure through the deposition of the extracellular matrix and the application of forces to the scaffold, which would clearly increase the resistance of the construct to deformation.

These findings provide a foundation for further development and optimization of peptide-functionalized chitosan scaffolds, potentially leading to more effective solutions for bone regeneration and repair. In the near future, the implant of functionalized chitosan in an *in vivo* bone defect model could evaluate the intricate interplay of various cellular and molecular signaling pathways able to promote bone healing.



◀**Fig. 9** Histological quantification based on WSI images and performed in QuPath. Capsule thickness, expressed in μm , and number of cells identified within the capsule surrounding the scaffold, expressed as the number of cells detected with nuclei segmentation on the scaffold area **a**, **b**. Number of cells identified in the colonization of the scaffold material, expressed as the number of cells on the scaffold area **c**. Percentage of scaffold material identified within the implant side **d**. Inflammation and tissue response to scaffold implant, divided into non-irritant (green line) and slight irritant (orange line), in accordance with DIN ISO 10993–6 (e, f). *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001

Author contributions Conceptualization: Monica Dettin; Data curation: Annj Zamuner, Matteo Zoboli, Teresa Russo, Giovanna Iucci, Paola Brun; Formal analysis: Annj Zamuner, Matteo Zoboli, Paola Brun; Funding acquisition: Andrea Porzionato, Paola Brun, Monica Dettin; Investigation: Annj Zamuner, Matteo Zoboli, Elena Zeni, Teresa Russo, Ignazio Castagliuolo, Francesca Ravanetti, Gabriella D'Auria, Lucia Falcigno, Giovanna Iucci, Chiara Battocchio, Martina Marsotto, Antonio Gloria, Paola Brun; Methodology: Francesca Ravanetti, Paola Brun, Monica Dettin, Antonio Gloria; Project administration: Monica Dettin; Resources: Andrea Porzionato, Francesca Ravanetti, Paola Brun, Monica Dettin; Supervision: Antonio Gloria, Antonio Cacchioli, Paola Brun, Monica Dettin; Validation: Paola Brun, Monica Dettin; Visualization: Annj Zamuner, Matteo Zoboli.; Writing—original draft: Annj Zamuner, Teresa Russo, Francesca Ravanetti, Gabriella D'Auria, Giovanna Iucci, Monica Dettin; Writing—review and editing: Annj Zamuner, Francesca Ravanetti, Antonio Gloria, Gabriella D'Auria, Giovanna Iucci, Paola Brun, Monica Dettin. All authors have given approval to the final version of the manuscript.

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Data availability The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interests There are no conflicts to declare.

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References

- Aguilar A, Zein N, Harmouch E, Hafdi B, Bornert F, Offner D, Clauss F, Fioretti F, Huck O, Benkirane-Jessel N, Hua G (2019) Application of chitosan in bone and dental engineering. *Molecules* 24:3009. <https://doi.org/10.3390/molecules24163009>
- Ahmed S, Annu AA, Sheikh J (2018) A review on chitosan centred scaffolds and their applications in tissue engineering. *Int J Biol Macromol* 116:849–862. <https://doi.org/10.1016/j.ijbiomac.2018.04.176>
- Amini AR, Laurencin CT, Nukavarapu SP (2012) Bone tissue engineering: recent advances and challenges. *Crit Rev Biomed Eng* 40:363–408
- Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, James JA, Salto-Tellez M, Hamilton PW (2017) QuPath: open source software for digital pathology image analysis. *Sci Rep* 7:16878. <https://doi.org/10.1038/s41598-017-17204-5>
- Brun P, Scorsetto M, Vassanelli S, Castagliuolo I, Palù G, Ghezzi F, Messina GML, Iucci G, Battaglia V, Sivoletta S, Bagno A, Polzonetti G, Marletta G, Dettin M (2013) Mechanisms underlying the attachment and spreading of human osteoblasts: from transient interactions to focal adhesions on vitronectin-grafted bioactive surfaces. *Acta Biomater* 9:6105–6115. <https://doi.org/10.1016/j.actbio.2012.12.018>
- Brun P, Zamuner A, Battocchio C, Cassari L, Todesco M, Graziani V, Iucci G, Marsotto M, Tortora L, Secchi V, Dettin M (2021a) Bio-functionalized chitosan for bone tissue engineering. *Int J Mol Sci* 22:5916. <https://doi.org/10.3390/ijms22115916>
- Brun P, Zamuner A, Cassari L, D'Auria G, Falcigno L, Franchi S, Contini G, Marsotto M, Battocchio C, Iucci G, Dettin M (2021b) Chitosan covalently functionalized with peptides mapped on vitronectin and BMP-2 for bone tissue engineering. *Nanomaterials* 11:2784. <https://doi.org/10.3390/nano11112784>
- Chen Y, Liu X, Liu R, Gong Y, Wang M, Huang Q, Feng Q, Yu B (2017) Zero-order controlled release of BMP2-derived peptide P24 from the chitosan scaffold by chemical grafting modification technique for promotion of osteogenesis *in vitro* and enhancement of bone repair *in vivo*. *Theranostics* 7:1072–1087. <https://doi.org/10.7150/thno.18193>
- Croisier F, Jérôme C (2013) Chitosan-based biomaterials for tissue engineering. *Eur Polym J* 49:780–792. <https://doi.org/10.1016/j.eurpolymj.2012.12.009>
- de Alvarenga ES (2011) Characterization and properties of chitosan. In: Elnashar M (ed) *Biotechnology of Biopolymers*. InTech
- Deepthi S, Venkatesan J, Kim S-K, Bumgardner JD, Jayakumar R (2016) An overview of chitin or chitosan/nano ceramic composite scaffolds for bone tissue engineering. *Int J Biol*

- Macromol 93:1338–1353. <https://doi.org/10.1016/j.ijbio.2016.03.041>
- Geoghegan KF, Stroh JG (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconj Chem* 3:138–146. <https://doi.org/10.1021/bc00014a008>
- Gong H, Agustin J, Wootton D, Zhou JG (2014) Biomimetic design and fabrication of porous chitosan–gelatin liver scaffolds with hierarchical channel network. *J Mater Sci Mater Med* 25:113–120. <https://doi.org/10.1007/s10856-013-5061-8>
- Ho M-H, Wang D-M, Hsieh H-J, Liu H-C, Hsien T-Y, Lai J-Y, Hou L-T (2005) Preparation and characterization of RGD-immobilized chitosan scaffolds. *Biomaterials* 26:3197–3206. <https://doi.org/10.1016/j.biomaterials.2004.08.032>
- Hsieh C-Y, Tsai S-P, Ho M-H, Wang D-M, Liu C-E, Hsieh C-H, Tseng H-C, Hsieh H-J (2007) Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds. *Carbohydr Polym* 67:124–132. <https://doi.org/10.1016/j.carbpol.2006.05.002>
- Hu D, Ren Q, Li Z, Zhang L (2020) Chitosan-based biomimetically mineralized composite materials in human hard tissue repair. *Molecules* 25:4785. <https://doi.org/10.3390/molecules25204785>
- (2016) ISO 10993–6:2016; Biological evaluation of medical devices—Part 6: tests for Local effects after implantation. international organization for standardization: Geneva, Switzerland
- Ke C-L, Deng F-S, Chuang C-Y, Lin C-H (2021) Antimicrobial actions and applications of chitosan. *Polymers* 13:904. <https://doi.org/10.3390/polym13060904>
- Keating JF, McQueen MM (2001) Substitutes for autologous bone graft in orthopaedic trauma. *J Bone Joint Surg Br* 83-B:3–8. <https://doi.org/10.1302/0301-620X.83B1.0830003>
- Kim S, Cui Z-K, Fan J, Fartash A, Aghaloo TL, Lee M (2016) Photocrosslinkable chitosan hydrogels functionalized with the RGD peptide and phosphoserine to enhance osteogenesis. *J Mater Chem B* 4:5289–5298. <https://doi.org/10.1039/C6TB01154C>
- Kiryk J, Michalak M, Majchrzak Z, Laszczyńska M, Kiryk S, Szotek S, Gerber H, Nawrot-Hadzik I, Matys J, Dobrzyński M (2025) Functionalization strategies of chitosan-based scaffolds with growth factors for bone regeneration: a systematic review. *Mar Drugs* 23:396. <https://doi.org/10.3390/md23100396>
- Krishna VS, Subashini V, Hariharan A, Chidambaram D, Raaju A, Gopichandran N, Nathanalaxmi MP, Lekhavadhani S, Shanmugavadivu A, Selvamurugan N (2024) Role of crosslinkers in advancing chitosan-based biocomposite scaffolds for bone tissue engineering: a comprehensive review. *Int J Biol Macromol* 283:137625. <https://doi.org/10.1016/j.ijbiomac.2024.137625>
- Lai S, Shi C, Yuan L, Li K, Wang X, Yu X, Liu P, Wang H, Wang L, Yu H (2025) Five-in-one hydrogel integrating bacteriostasis, self-healing capability, growth factor release, electrical stimulation, and photothermal stimulation tailored for complex wound repair. *ACS Biomater Sci Eng*. <https://doi.org/10.1021/acsbomaterials.5c00244>
- Lee YM, Park YJ, Lee SJ, Ku Y, Han S-B, Klokkevold PR, Chung C-P (2000) The bone regenerative effect of platelet-derived growth factor-BB delivered with a chitosan/tricalcium phosphate sponge carrier. *J Periodontol* 71:418–424. <https://doi.org/10.1902/jop.2000.71.3.418>
- Li Z, Ramay HR, Hauch KD, Xiao D, Zhang M (2005) Chitosan–alginate hybrid scaffolds for bone tissue engineering. *Biomaterials* 26:3919–3928. <https://doi.org/10.1016/j.biomaterials.2004.09.062>
- Li Y, Li X, Zhu L, Liu T, Huang L (2025) Chitosan-based biomaterials for bone tissue engineering. *Int J Biol Macromol* 304:140923. <https://doi.org/10.1016/j.ijbiomac.2025.140923>
- Mochizuki M, Kadoya Y, Wakabayashi Y, Kato K, Okazaki I, Yamada M, Sato T, Sakairi N, Nishi N, Nomizu M (2003) Laminin-1 peptide-conjugated chitosan membranes as a novel approach for cell engineering. *FASEB J* 17:1–20. <https://doi.org/10.1096/fj.02-0564fje>
- Oryan A, Sahviah S (2017) Effectiveness of chitosan scaffold in skin, bone and cartilage healing. *Int J Biol Macromol* 104:1003–1011. <https://doi.org/10.1016/j.ijbiomac.2017.06.124>
- Oryan A, Alidadi S, Moshiri A, Maffulli N (2014) Bone regenerative medicine: classic options, novel strategies, and future directions. *J Orthop Surg* 9:18. <https://doi.org/10.1186/1749-799X-9-18>
- Oryan A, Alidadi S, Bigham-Sadegh A, Moshiri A, Kamali A (2017) Effectiveness of tissue engineered chitosan–gelatin composite scaffold loaded with human platelet gel in regeneration of critical sized radial bone defect in rat. *J Control Release* 254:65–74. <https://doi.org/10.1016/j.jconrel.2017.03.040>
- Petersen A, Princ A, Korus G, Ellinghaus A, Leemhuis H, Herrera A, Klaumünzer A, Schreivogel S, Woloszyk A, Schmidt-Bleek K, Geissler S, Heschel I, Duda GN (2018) A biomaterial with a channel-like pore architecture induces endochondral healing of bone defects. *Nat Commun* 9:4430. <https://doi.org/10.1038/s41467-018-06504-7>
- Pighinelli L, Kucharska M (2013) Chitosan–hydroxyapatite composites. *Carbohydr Polym* 93:256–262. <https://doi.org/10.1016/j.carbpol.2012.06.004>
- Qian L, Zhang H (2011) Controlled freezing and freeze drying: a versatile route for porous and micro-/nano-structured materials. *J Chem Technol Biotechnol* 86:172–184. <https://doi.org/10.1002/jctb.2495>
- Qu H, Fu H, Han Z, Sun Y (2019) Biomaterials for bone tissue engineering scaffolds: a review. *RSC Adv* 9:26252–26262. <https://doi.org/10.1039/C9RA05214C>
- Rampazzo E, Dettin M, Maule F, Scabello A, Calvanese L, D'Auria G, Falcigno F, Porcù E, Zamuner A, Della Puppa A, Boso D, Basso G, Persano L (2017) A synthetic BMP-2 mimicking peptide induces glioblastoma stem cell differentiation. *Biochim Biophys Acta Gen Subj* 1861:2282–2292. <https://doi.org/10.1016/j.bbagen.2017.07.001>
- Ravi Kumar MNV (2000) A review of chitin and chitosan applications. *React Funct Polym* 46:1–27. [https://doi.org/10.1016/S1381-5148\(00\)00038-9](https://doi.org/10.1016/S1381-5148(00)00038-9)
- Rinaudo M (2006) Chitin and chitosan: properties and applications. *Prog Polym Sci* 31:603–632. <https://doi.org/10.1016/j.progpolymsci.2006.06.001>

- Salahshour P, Abdolmaleki S, Monemizadeh S, Gholizadeh S, Khaksar S (2024) Nanobiomaterials/bioinks based scaffolds in 3D bioprinting for tissue engineering and artificial human organs. *Adv Biol Earth Sci* 9:97–104. <https://doi.org/10.62476/abes9s97>
- Schulze F, Lang A, Schoon J, Wassilew GI, Reichert J (2023) Scaffold guided bone regeneration for the treatment of large segmental defects in long bones. *Biomedicines* 11:325. <https://doi.org/10.3390/biomedicines11020325>
- Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, Soo C, James AW (2012) Brief review of models of ectopic bone formation. *Stem Cells Dev* 21:655–667. <https://doi.org/10.1089/scd.2011.0517>
- Secchi V, Franchi S, Ciccarelli D, Dettin M, Zamuner A, Serio A, Iucci G, Battocchio C (2019) Biofunctionalization of TiO₂ surfaces with self-assembling layers of oligopeptides covalently grafted to chitosan. *ACS Biomater Sci Eng* 5:2190–2199. <https://doi.org/10.1021/acsbiomaterials.9b00430>
- Spears RJ, Fascione MA (2016) Site-selective incorporation and ligation of protein aldehydes. *Org Biomol Chem* 14:7622–7638. <https://doi.org/10.1039/C6OB00778C>
- Tiğli RS, Gümüşderelioğlu M (2008) Evaluation of RGD- or EGF-immobilized chitosan scaffolds for chondrogenic activity. *Int J Biol Macromol* 43:121–128. <https://doi.org/10.1016/j.ijbiomac.2008.04.003>
- Wang S, Liu Y, Zhou Y, Zheng W, Mao Y (2025) Biomimetic peptides in bone tissue engineering: from function to application. *Colloids Surf B Biointerfaces* 256:115017. <https://doi.org/10.1016/j.colsurfb.2025.115017>
- Zamuner A, Brun P, Scorzeto M, Sica G, Castagliuolo I, Dettin M (2017) Smart biomaterials: surfaces functionalized with proteolytically stable osteoblast-adhesive peptides. *Bioact Mater* 2:121–130. <https://doi.org/10.1016/j.bioactmat.2017.05.004>
- Zamuner A, Brun P, Ciccimarra R, Ravanetti F, Veschini L, Elsayed H, Sivoilella S, Iucci G, Porzionato A, Di Silvio S, Cacchioli A, Bernardo E, Dettin M (2021) Biofunctionalization of bioactive ceramic scaffolds to increase the cell response for bone regeneration. *Biomed Mater* 16:055007. <https://doi.org/10.1088/1748-605X/ac1555>

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