# Elastolytic-sensitive 3D-printed chitosan scaffold for wound healing applications

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(Accepted 28 October 2021)

## Abstract

The combination of a chitosan 3D-printed scaffold with a hydrogel matrix containing an elastin-like polypeptide functionalized with the epidermal growth factor (HEGF) was evaluated as a possible strategy to obtain a bioactive platform with stimuli-responsive properties. We designed a chitosan/HEGF hybrid scaffold and examined the physico-chemical properties and the in vitro behavior when in contact with simulated biological fluids. Primary human dermal fibroblasts (hDFs) were used to test the in vitro cytocompatibility. Overall, these data provide first insights into the integration of HEGF-based hydrogel with 3D-printed scaffolds, contributing towards the rational design of a new smart functional wound dressing.

## Introduction

Recently, bio-inspired polypeptides such as the elastin-like polypeptides (ELPs) have proved to be excellent as components for drug delivery and tissue engineering applications due to their good cytocompatibility and biocompatibility, their ease of handling and design, production, and modification.[1–3] The interest towards these recombinant biopolymers is based on the fundamental role of the native elastin protein in the extracellular matrix that confers rubber-like elasticity to the tissues, allowing them to be subjected to indefinite cycles of deformation/relaxation without rupture. Most of the elastin-like polypeptides currently used for tissue engineering applications are derived from the recombinant expression of the repeated bovine aminoacidic motifs, and the human recombinant version of these elastin-like sequences has been developed as an alternative to be used in tissue engineering. Human Elastin-Like Polypeptides (HELPs) are artificial, recombinant biopolymers based on the hexapeptidic VAPGVG repeated motif of human elastin.[1] Interestingly, thanks to the presence of glutamine and lysine residues in their primary structure, HELPs can be cross-linked under the action of transglutaminase (TG) to form stable hydrogels without the use of harsh chemicals like glutaraldehyde or analogous cross-linking agents.[4] HELP-based biomaterials have already shown high potential to be employed for many applications in tissue engineering, regenerative medicine, and cell encapsulation,[5] as well as to prepare biomimetic surfaces for cell culture,[6] and for the delivery of biological therapeutic agents.[7,8] Moreover, the use of recombinant HELP opens the possibility to incorporate bioactive sequences that contribute to the development of new bioactive materials that can be applied in tissue regeneration.[9]

The features of ELPs make them also useful for the design of swellable, adaptable, and elastic wound dressings, finely tailoring their structural and mechanical properties. However, despite all these promising characteristics, very little work has been performed on ELPs in the field of wound healing.[2] One of the main drawbacks lies in the poor rheological characteristics of the derived hydrogel matrix that results inadequate for applications on difficult-to-heal wounds. The management of chronic wounds is currently based on the use of robust wound dressings as they provide better exudate management and prolonged residence at the wound site.[10] To overcome this problem, the integration of the HELP-based hydrogel with 3D-printed polymeric scaffolds may represent a successful strategy to preserve the bioactive properties of the HELP hydrogel as well as to increase the mechanical and handling performance of the scaffold. The application of 3D printing in the wound healing field is particularly interesting, especially when combined with 3D scanning, to create personalized dressings, adapted in shape and size to individual patients.[11] Recently, an innovative extrusion-based 3D printing technique combined with freeze-gelation has been proposed to prepare chitosan scaffolds to be applied in the regenerative skin tissue field.[12] Chitosan is a very versatile semi-synthetic polymer derived from the alkaline N-deacetylation of chitin, the main structural component of the crustacean exoskeleton, and finds most of its application in wound dressings, scaffold, and as antimicrobial agent.[13] It is a biodegradable and biocompatible

polymer that possesses antibacterial, hemostatic, and bioadhesive characteristics,[14] all desirable features for ideal wound dressings. In addition, the easy chemical modification and favorable rheological characteristics have prompted the use of chitosan and its composites as bio-inks for 3D-printed biomaterials.[15,16] For this reason, chitosan-based wound dressings are extensively studied to favor wound closure, prevent wound infections, and control the release of drugs and growth factors at wound sites to stimulate and improve wound healing.

The use of recombinant techniques to produce HELPs offers several advantages as well. Modified versions of HELP functionalized with bioactive molecules can be easily prepared, as in the case of the fusion of HELP with the epidermal growth factor (EGF) that has been recently synthesized in our laboratory.[17] EGF, together with its receptor (EGFR) plays an essential role in wound healing by stimulating epidermal and dermal regeneration[18,19] but its use in wound care has been limited so far by its short half-life, resulting from the rapid in vivo degradation, and by the limited efficacy of the delivery methods. By introducing EGF sequence within the backbone of HELP, we obtained a fusion protein (HEGF) maintaining both the EGF bioactivity and the responsivity to a proteolytic environment, such as a wound site.[20]

The study reported here focuses on the development of a new composite scaffold that combines the flexibility of the 3D printing technique with the stimuli-induced release ability of a HEGF hydrogel. High-porosity chitosan 3D-printed scaffolds (CHIT) were embedded by enzymatic cross-linking in HEGF-enriched HELP matrix and the interaction between these components as well as the release of EGF from the scaffold was evaluated. Finally, primary human Dermal Fibroblasts (hDFs) were used to test the in vitro cytocompatibility of the scaffolds.

## Materials and methods

### Material development and scaffold fabrication via extrusion-based 3D printing

HELP recombinant biopolymer and its modified fusion with the EGF (HEGF) were prepared as previously reported.[7] The recombinant products were expressed in a C3037 *E. coli* strain (New England Biolabs, Ipswich, MA) and then subjected to an extraction and purification procedure. The separation of the recombinant biopolymers of interest from the supernatant was obtained exploiting the inverse phase transition properties using a series of temperature-dependent transition cycles. Three of these cycles were sufficient to obtain the pure recombinant protein. The polypeptide was frozen overnight at −80°C, and then lyophilized at 0.01 atm and −60°C in a Modulyo apparatus (Edwards, Crawley, UK) for long-term storage. The yield and the purity of the recombinant polypeptides obtained were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Chitosan Chitoclear™ (CAS 9012-76-4, degree of deacetylation 95%; molecular weight 150–200 kDa) from PRIMEX Ehf (Siglufjordur, Iceland) was used for 3D printing. The preparation and characterization of the 3D-printed scaffolds employed in this study were already described by Elviri et al.[21] The 3D printing system was conceived as an automation of a freeze-gelation method for the preparation of chitosan scaffolds with controlled porosity, to have a precise and accurate control of the scaffold geometry. Briefly, a chitosan solution (4% w/v) in 1% v/v aqueous acetic acid solution was loaded in a 5 mL syringe mounting a 26 G needle (inner diameter 192 µm). The syringe was fixed in an in-house built 3D printer and the solution was extruded on a cooled surface and instantly frozen to fix the grid structure composed by overlapping orthogonal filaments with an inter-filament distance of 200 µm. For the performed experiments, 5-layer scaffolds were produced. At the end of each printing process, the frozen hydrogel underwent ionotropic gelation that occurred in potassium hydroxide 1.5 M (pH 14). After 1 h of immersion, scaffolds were washed in ultrapure water till neutrality.

### Composite chitosan/HEGF wound dressings preparation

To prepare the CHIT/HEGF composites, HELP and HEGF were enzymatically cross-linked resulting in a matrix embedding the CHIT scaffolds. Before the cross-linking, the scaffolds were cut in a 5-mm-diameter disk using a stainless-steel punch, frozen overnight at −80°C, and lyophilized at 0.01 atm and −60°C in a Modulyo apparatus (Edwards, UK).

To prepare the matrix, a 4% (w/v) solution of HEGF and HELP was prepared dissolving the lyophilized proteins in cold 10 mM Tris/HCl (Sigma-Aldrich, USA), pH 8. HEGF-loaded composites were fabricated employing 20 µL of a precooled 4wt% HEGF/HELP (1:19) solution that was mixed with 2 µL of microbial transglutaminase (60 mg/mL) and quickly dropped onto the surface of the CHIT 3D-printed scaffold in a vertically placed cylindrical mold. The mold was then centrifuged at 1500 rpm for 3 min to achieve a homogenous gel distribution in the porous scaffold. The cross-linking was completed after 2-h incubation at room temperature. After the reaction, the composite dressings were gently removed from the mold, washed extensively with ultrapure water at 4°C overnight, frozen overnight at −80°C, and finally lyophilized at 0.01 atm and −60°C in a Modulyo apparatus (Edwards, UK). The CHIT/HEGF composites scaffolds were stored in desiccators over silica gel at room temperature until use.

### Physico-chemical characterization of the printed CHIT/HEGF composites

The surface morphology of CHIT/HEGF composites was analyzed through a stereoscopic microscope (Olympus SZ61TR) and scanning electron microscopy (SEM) (Philips Model 501). To collect the SEM images, the samples were mounted on a metal stub by means of carbon adhesive tape and coated with a 20-nm-thick gold/palladium.

The average porosity and the density of CHIT/HEGF composite wound dressings were determined by a fluid

displacement method, using ethanol as the displacement liquid.[22] The pore average diameters were calculated measuring at least 100 pores from three different SEM images using the public domain *ImageJ software* 1.52v (*NIH*, Bethesda, MD, USA).

Water uptake was determined by placing the CHIT and CHIT/HEGF composite wound dressing in water. The initial weight of each sample was accurately recorded using an analytical balance, and then they were placed in 20 mL of water in a thermostatic bath at 37°C. Samples were taken out, excess water was carefully removed using tissue paper, and after being weighed were re-immersed in water. The sample weight was recorded after 15 and 30 min, 1, 2, 4, 6 h and from there onwards until equilibrium was established after 24 h. The percentage swelling ratio (SR%) at each time point was calculated using Eq. (1):

$$SR\% = \frac{W - W_0}{W_0} \times 100, \quad (1)$$

where $W$ is the mass of the swollen sample and $W_0$ is the mass of the initial dry sample. The equilibrium water content (EWC) percent was calculated by Eq. (2):

$$EWC(\%) = \frac{W_e - W_d}{W_e} \times 100, \quad (2)$$

where $W_e$ is the mass of the swollen sample at equilibrium and $W_d$ is the mass of the dry sample at equilibrium.

The interaction of CHIT and CHIT/HEGF composite with the proteins was evaluated using the solution depletion technique. Both the 3D-printed CHIT scaffold and the CHIT/HEGF composite were immersed in 5 mL of Bovine Serum Albumin (BSA) solution 1 mg/mL (Sigma-Aldrich, USA) in PBS pH 7.4. After 24 h of incubation at 37°C, the amount of adsorbed protein was calculated from the differences in the BSA concentration before and after immersion of the composites. The Bradford reagent was used for the quantification of the protein absorbed by hydrogels. Briefly, 5 µL of the samples were mixed with 250 µL of the Bradford reagent and incubated in the dark at room temperature for 1 h before analysis. The absorbance (ABS) of the samples was measured at 595 nm using a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments, Inc., USA). Results were expressed as the difference between the ABS of the control 1 mg/mL BSA solution and the ABS of the same solution that came into contact with the samples.

The in vitro stability of the CHIT/HEGF composite was tested immersing the constructs in simulated wound fluid (SWF) composed as follows: 0.4 M NaCl, 2 mM $CaCl_2$, 8 mM TRIS, all obtained from Sigma-Aldrich (USA). To simulate a proteolytic environment, elastase (0.5 µg/mL) was added to the SWF. The weight loss during immersion in SWF was measured by recording the weight changes of the dry specimen after the specified incubation time. Briefly, different sets of samples ($n=4$) were immersed in 5 mL of SWF containing 2.5 µg of elastase at 37°C for 48 h. Material dissolution was evaluated in terms of weight loss in relation to the immersion time. After 6, 24, and 48 h, the composites were removed from the fluid, rinsed with ultrapure water, and finally, lyophilized at 0.01 atm and −60°C in a Modulyo apparatus (Edwards, UK) after overnight freezing at −80°C. The percentage of weight loss (WL) was calculated according to Eq. (3):

$$WL\% = \frac{W - W_0}{W_0} \times 100, \quad (3)$$

where $W$ is the mass of the sample at time $t$ and $W_0$ is the mass of the initial dry sample. Degradation studies were conducted with CHIT/HEGF composite and with the unloaded CHIT scaffolds.

## Evaluation of EGF release from the CHIT/HEGF composite wound dressing

The CHIT/HEGF composite wound dressings were washed with excess water to ensure the removal of any unbound or unreacted component. The lyophilized dressings described above were first soaked in 500 µL of digestion buffer (50 mM Tris/HCl pH 7.5, 1 mM $CaCl_2$) at 37°C for 16 h, the supernatant (named To/n) was sampled and stored at −20°C before analysis. Then, the CHIT/HEGF dressings were immersed in 700 µL of the 50 mM Tris/HCl pH 7.5, 1 mM $CaCl_2$ buffer added of elastase from porcine pancreas (Sigma-Aldrich, USA, ≥4 units/mg) to a final concentration of 0.5 µg/mL. 200 µL of this supernatant was immediately removed (T0) and stored at −20°C for subsequent analysis. The composite was further incubated at 37°C in the remaining 500 µL with the elastase enzyme. After 2 h, 50 µL of supernatant was collected and stored at −20°C for subsequent analysis. For volume replacement, 50 µL of the fresh buffer with elastase were added to the sample to continue the incubation at 37°C. The same procedure was repeated after 4 and 8 h.

The supernatants deriving from the EGF release assays were analyzed for EGF content by a dot blot procedure as described in the supplementary material.

## Cell culture and cytotoxicity assay

Biological investigations on HEGF-loaded composite wound dressing were performed using human dermal fibroblasts (hDFs). Primary human fibroblasts were isolated, with informed consent, from a healthy, normolipemic 45-year-old female. hDFs, coded as C84, were grown in Minimum Essential Eagle Medium (MEM) (Thermo Fisher Scientific, USA) with 10% Fetal Bovine Serum (FBS), antibiotic solution (streptomycin 100 IU/mL and penicillin 100 IU/mL), and 2 mM L-glutamine (all obtained from Aurogene, Italy), at 37°C in a wet atmosphere with 5% $CO_2$. The cytotoxicity assay was performed by first culturing hDFs on CHIT/HEGF dressings and then evaluating the viability of the cells using the resazurin

assay. The detailed procedure is reported in the supplementary material.

## Results

An interesting strategy for enhancing the structural integrity and fracture toughness of hydrogels is forming a composite by incorporating a 3D-printed scaffold as a structural element.[23,24] Following the deposition scheme provided by the 3D printer (Fig. 1(a)), CHIT scaffolds show a regular grid structure (Fig. 1(b)) and can be handled without the risk of breaking. On the other hand, cross-linked HELP-based matrices were proved to be structurally too weak, and for this reason, we decided to strengthen this hydrogel preparing a composite material by using a mixture of HELP and HEGF and performing the enzymatic cross-linking directly on the 3D CHIT scaffolds.[4] The cross-linking takes place through the formation of isopeptide bonds among lysine and glutamine residues available on the HELP domain, present in HEGF as well, mimicking a process that occurs in nature avoiding the use of chemical or physical harsh conditions. After washing and freeze-drying, CHIT and CHIT/HEGF dressings have a regular and elegant shape, with adequate handling properties to withstand the requirement for a wound application (Fig. 1(c) and (d), respectively). The preparation method developed here is simple and leads to the formation of a complex porous architecture characterized by two well-separated microstructures.

In Fig. 2, representative SEM images of the surface of the CHIT/HEGF composites are shown. In these images, the complex microstructure is well highlighted, with the porous interconnecting network of polymeric strands having irregularly shaped pores with thin walls. It has already been reported that the activity of transglutaminase on HELP solutions resulted in hydrogel matrices with a porous structure.[4] In the magnification of Fig. 2, the pore size difference of the two materials is clearly visible, with an open cell structure with an average diameter of 86.5 ± 23.3 μm formed by the HEGF-loaded HELP matrix and a smaller chitosan interconnected network characterized by pores with an elongated shape and an average diameter of 30.3 ± 10.9 μm. This peculiar morphology of CHIT/HEGF composite has a significant impact on the ability of the dressing to absorb water, increasing the swelling ratio after 24 h from 494.4% ± 72.2% to 1125.0% ± 52.9% (Table I).

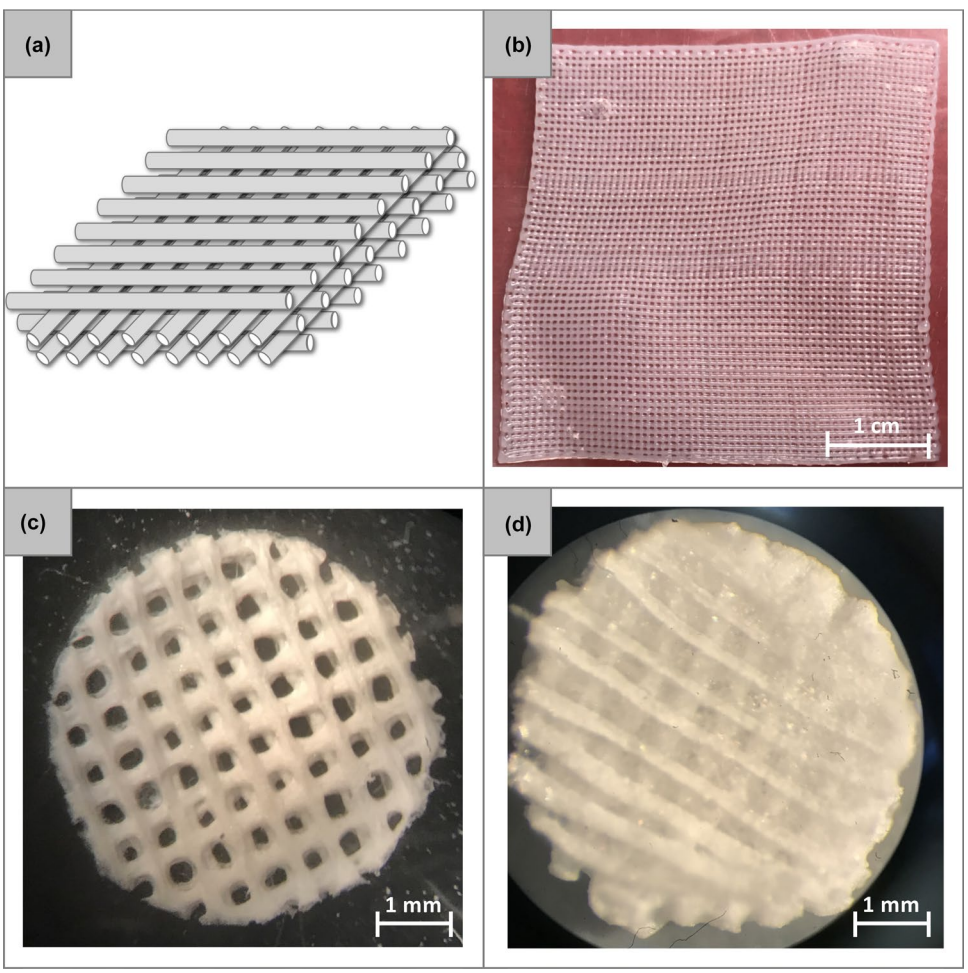


**Figure 1.** (a) Schematic representation (not in scale) of the 3D-printed structure, (b) macroscopic appearance of a 3D-printed CHIT scaffold after ionotropic gelation in KOH 1.5 M, (c) lyophilized CHIT 3D-printed scaffold, and d) CHIT/HEGF composite after freeze-drying visualized by stereoscopic microscope.

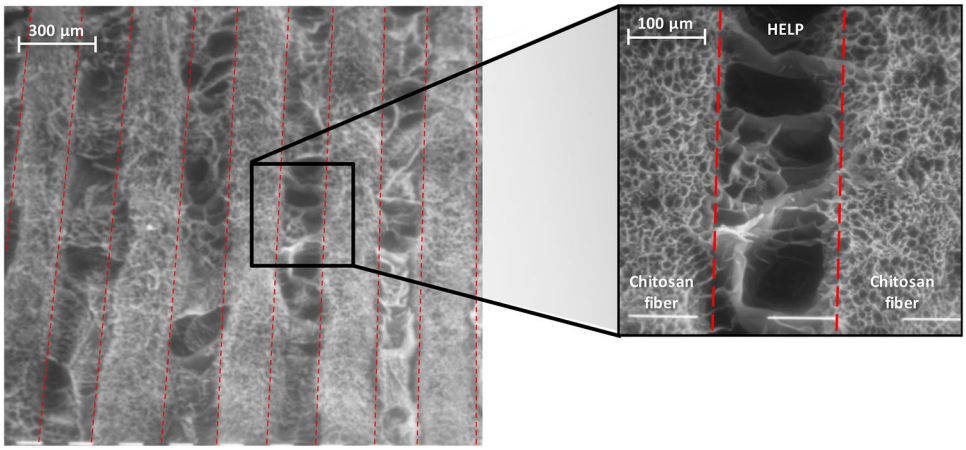


**Figure 2.** Representative SEM images of the CHIT/HEGF composite. The morphological differences are particularly evidenced in the magnification, where the HEGF-loaded HELP matrix form an open cell structure with an average diameter of 86.5 ± 23.3 µm, while the CHIT scaffold has a smaller interconnected network characterized by pores with elongated shape and an average diameter of 30.3 ± 10.9 µm.

**Table I.** Comparison of physical properties between CHIT/HEGF and CHIT.

| | Swelling ratio (%) | Equilibrium water content (%) | Porosity (%) | Apparent density (mg/cm$^3$) |
|---|---|---|---|---|
| CHIT | 494.4 ± 72.2 | 82.6 ± 1.3 | – | – |
| CHIT/HEGF | 1125.0 ± 52.9 | 90.7 ± 1.5 | 36.77 ± 5.97 | 41.83 ± 3.34 |

For a dressing, the ability to absorb fluids and retain moisture without leaking is essential for the final application, as the accumulation of excess exudate on the wound site slows down the healing process and causes skin maceration.[10] At the same time, the rate and duration of swelling determine the ability of the dressing to control drug release over a prolonged period, a process generally driven by fluid uptake and diffusion. When looking at swelling profiles of scaffolds with respect to time (Fig. 3(a)), besides the higher swelling capacity by CHIT/HEGF composite compared to CHIT scaffold, it is interesting to notice that the swelling rate is significantly different. CHIT dressing reaches its maximum within 30 min, while CHIT/HEGF reaches it in an hour at least. The comparison of CHIT and CHIT/HEGF with the HELP plain matrix was not possible due to the very poor handling properties of the HELP after hydration, confirming the need for chitosan 3D-printed scaffolds as an essential structural component of the proposed system. Among the parameters that can affect the kinetics of water uptake, the different pore size of the material is one of the most prominent. The EWC represents the amounts of fluids that a material can absorb in relation to its weight. As for the swelling profiles, the results reveal that the EWC is significantly higher for the CHIT/HEGF dressing (Table I). An EWC value similar to the fluid contents of living tissues (about 60%) is considered a good indicator of the compatibility of these materials with the wound area. Finally, the low porosity and density of CHIT/HEGF, calculated with the fluid replacement method, suggest a relatively compact material, but which still maintains the ability to absorb fluids, due to the significant differences in water absorption and EWC.

When a dressing comes into contact with wound exudate, the exudate proteins almost immediately start to adsorb to the surface, eliciting foreign body reactions, triggering the inflammatory response, and delaying the wound healing process. This phenomenon is strongly related to the adsorption of water molecules but is also dependent on the surface composition of the (bio)material that comes in contact with the wound environment.[25] Because of its high concentration in wound exudate and moderate size, albumin dominates initial interactions with the surface and for this reason, we simulated the adsorption of exudate proteins onto CHIT and CHIT/HEGF composites using a Bovine Serum Albumin (BSA) solution at pH 7.4 and 37°C. However, in this case, both the samples tested resulted to have a negligible interaction with the BSA, independently from the swelling properties of the materials (Fig. S1), which is usually the main driving force for protein adsorption at most interfaces.

A feature of HELP-based hydrogels is their susceptibility to the action of neutrophil elastase, which causes the release of bioactive moieties.[26] The activity levels of neutrophil elastase were found significantly elevated in chronic wounds such as pressure ulcers and leg ulcers,[27,28] with no association with the condition of the wound.[29] CHIT/HEGF composites were investigated for their stability in SWF containing elastase to simulate the proteolytic environment generally present in a chronic wound (Fig. 3(b)). Throughout the experiment, the activity of elastase on the degradation rates of 3D-printed CHIT structure is negligible and the slight chitosan weight loss could be explained by solubilization of non-cross-linked chitosan chains. In contrast, at each time point, there is a significant difference in weight loss on CHIT/HEGF dressings due to a selective HEGF-loaded HELP matrix degradation by elastase that does not affect the chitosan scaffold (Fig. 3(b), Fig. S2). Our results showed a progressive HELP-based hydrogel matrix loss, which achieved complete degradation after 24 h, despite

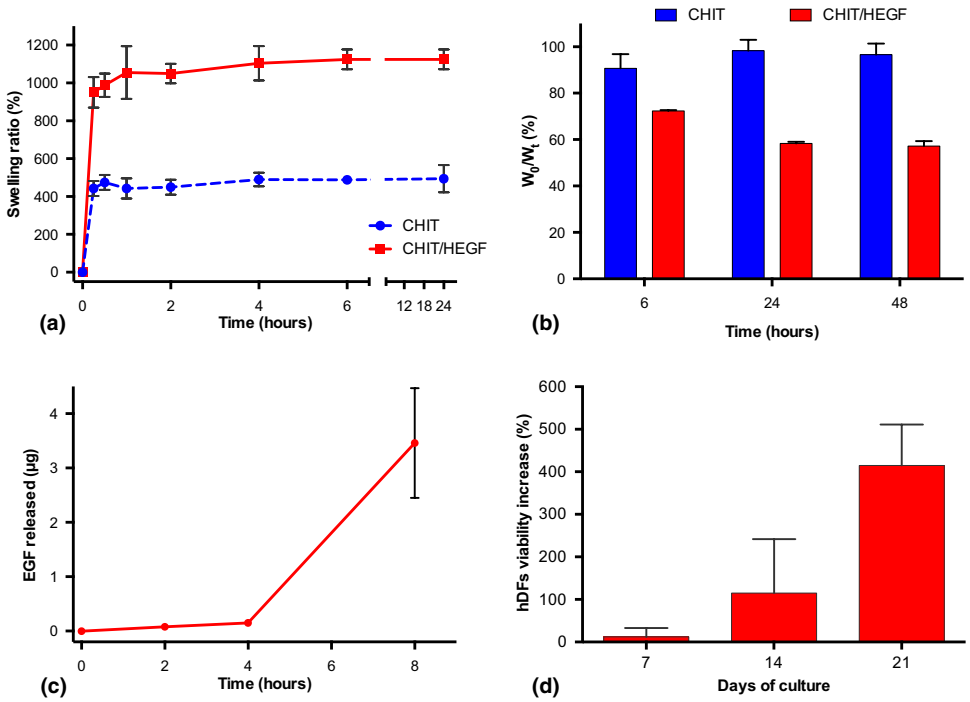


**Figure 3.** Physico-chemical and biological characterization of the 3D-printed chitosan scaffold (CHIT) and of CHIT/HEGF composite wound dressings. (a) Water uptake, (b) stability of the CHIT and CHIT/HEGF in SWF containing elastase, (c) cumulative release of EGF from CHIT/HEGF composites, (d) effect of the CHIT/HEGF on fibroblast viability. Bars represent the mean ± SD of triplicate determination in three independent experiments.

an inhibitory effect of chitosan on elastase activity has been reported.[30] Interestingly, this susceptibility to proteolysis of the HEGF-loaded HELP matrix can be exploited to trigger the release of active compounds loaded in the matrix itself.[7] In our case, the CHIT/HEGF dressings were tested to verify the ability of an elastolytic stimuli-induced release of the EGF. The samples were first soaked in the release buffer alone, without any enzyme that can trigger the release. The dot blot analysis of the supernatants derived from the 16-h incubation of the composites in the absence of the elastase (To/n) did not show any chemiluminescent signal (data not shown) confirming that the enzymatic action of elastase is essential to trigger the EGF release from the dressings. On the contrary, after enzyme addition, chemiluminescent signals became detectable after about 8 h of incubation, which is also the time in which a large part of the HEGF matrix is degraded by the action of elastase (Fig. 3(c)). Taken together, the in vitro stability and the EGF release experiments confirmed that the proteolytic degradation of the HEGF-loaded matrix leads to the EGF local release from the composite. Results suggest that if applied in vivo, this composite may be activated by the elastolytic activity of the wound exudate, representing an attractive smart dressing with stimuli-responsive properties. Further studies are underway on the activity of the CHIT/HEGF composites applied to in vivo wound models for the evaluation of EGF release and its effects on wound progression.[20]

Preliminary biological investigations were performed to evaluate the occurrence of any cytotoxic effects and the ability of CHIT/HEGF dressing to support fibroblast viability, with respect to CHIT scaffold, whose cytocompatibility had been previously demonstrated.[12] The in vitro cytotoxicity evaluation is a fast method to provide predictive evidence of material biocompatibility. For wound healing applications, good cytocompatibility is desirable, as well as adequate physical properties and biodegradability. As shown in Fig. 3(d), CHIT/HEGF not only did not show any cytotoxic effect on hDFs, but also significantly improved their proliferation with respect to CHIT dressing. This suggests that in the hDF cultures the release of EGF in the growth medium may be induced, promoting cell proliferation.

## Conclusions

Overall, here we describe a method to prepare a composite material by reinforcing the HEGF hydrogel with a 3D-printed chitosan scaffold, to ensure adequate mechanical strength to withstand the requirement for its application as a wound dressing. The peculiar morphology of CHIT/HEGF composite observed using SEM microscopy has a significant impact on the ability of the dressing to absorb body fluids. The susceptibility to enzymatic degradation of the HEGF-loaded matrix makes this composite sensitive to the proteolytic environments, an attractive feature to realize smart dressings with stimuli-responsive properties. Further investigation will follow to better clarify the effect of the proteolytic environment on the EGF release mechanism and the in vivo activity of dressings based on these composites in a rabbit splinted-wound model.

## Acknowledgments

This work was funded by "Commissariato del Governo della Regione Friuli Venezia Giulia - Fondo Trieste" and managed by AREA Science park in the frame of "Made in Trieste" program. The authors wish to thank Dr. M. Stebel for technical assistance in HELP and HEGF polypeptide production and Prof. S. Passamonti for scientific assistance during the project.

## Data availability

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

## Declarations

### Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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