



Bioderived Green Algae Metabolite as a Latent Cross-Linking Agent for Protein-Based Hydrogels with High Potential for Skin Repair Applications

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Giuseppe Melilli, Patricia Rousselle, Mohamed Mehiri, Nathanael Guigo, Didier Pin, et al.. Bioderived Green Algae Metabolite as a Latent Cross-Linking Agent for Protein-Based Hydrogels with High Potential for Skin Repair Applications. ACS Applied Bio Materials, 2025, 8 (3), pp.2558-2568. <10.1021/acsabm.5c00010>. <hal-05236555>

HAL Id: hal-05236555

<https://hal.science/hal-05236555v1>

Submitted on 2 Sep 2025

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Bio-derived green algae metabolite as a latent crosslinking agent for protein-based hydrogels with high potential for skin repair applications.

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Keywords: hydrogels; biomimicry; stiffness; cell adhesion; skin regeneration

ABSTRACT

Despite advances in wound treatment through tissue engineering, the rapid colonization of biomaterials by host cells remains a crucial step towards complete wound healing. Thanks to their excellent biocompatibility, biodegradability, low antigenicity and cost-effectiveness, crosslinked hydrogels have attracted much attention as a viable solution for wound treatment. In this work, we have developed an innovative crosslinking method for gelatin-based hydrogels inspired by the wound closure mechanism of the green algae *C. taxifolia*. Caulerpenyne (CYN), a metabolite extracted from the algae, was used as a latent crosslinking agent for gelatin. The covalent crosslinking process is triggered by an in situ and on-demand deacetylation of the enol acetate functionalities of CYN in oxytoxin 2 (OXY) containing 1,4-dialdehyde, which immediately reacts with the lysine residue in gelatin. The content of ϵ -amino groups in gelatin was monitored as a function of CYN concentration. Swelling and gel content were analyzed

as a function of CYN concentration. Morphology, rheological and biological properties were evaluated by in vitro and in vivo tests. Cell adhesion and viability tests performed with OXY-crosslinked hydrogels and compared with non-crosslinked and genipin-crosslinked gelatin showed excellent performance. Their use in whole skin wounds in pigs showed that CYN-crosslinked hydrogels promoted complete skin regeneration without any cytotoxicity, making them extremely promising matrices in the field of regenerative medicine.

1. INTRODUCTION

Perfected over millions of years of evolution, marine biological systems possess the extraordinary ability to fulfil their functional needs through a sophisticated interplay of chemical, physical, and mechanical strategies, seamlessly integrated into seawater or high-moisture environment. These systems exhibit adaptive multifunctionalities and mechanisms that surpass even our most advanced manufacturing technologies to design innovative synthetic materials ¹. Understanding the mechanisms that control biological systems in the sea offers fascinating approaches to mimicking the performance of natural underwater organisms. Many marine organisms have inspired the development of synthetic materials technologies, such as response to stimuli ^{2, 3}, antimicrobial shell ⁴, strength and toughness ⁵, microgrooves and microchannels ⁶, and wet adhesion ^{7, 8}. Underwater protein crosslinking is not exclusively limited to bioadhesive processes as similar mechanisms have been observed in certain species of macroalgae, such as siphonous green algae. Upon injury, these unicellular organisms initiate a series of biochemical cascade reactions aimed at triggering rapid chemical crosslinking of extracellular matrix (ECM) proteins, leading to the formation of a biopolymer known as external wound plug (EWP) ⁹⁻¹¹. Beyond the wound healing mechanism, the plug-forming process is an efficient strategy for the rapid proliferation of green algae ¹². *Caulerpa taxifolia*, is a tropical green invasive alga that has spread rapidly in the Mediterranean ¹³. The formation of EWP produced by *C. taxifolia* occurs thanks to its main second metabolite – Caulerpenyne (CYN) – which plays a key role in the underwater crosslinking process ¹⁴. This sesquiterpene is stabilized by enol acetate functionalities and stored in this latent chemical configuration until an external physical damage threaten the survival of the alga. Within few seconds after injury, cells damages trigger an esterase-mediated deacetylation of CYN producing a 1,4-

dialdehyde derivative, referred to oxytoxine 2 (OXY). The 1,4-dicarbonyl moiety promptly reacts with nucleophilic functional groups of ECM proteins promoting covalent bonding. This seals the damage tissue so that the cytoplasm does not come into contact with the sea water.

The use of synthetic and biological crosslinkers has been widely used to promote stability of the physical architecture of protein-based hydrogels used for the treatment of deep skin wounds ¹⁵. Biomaterials traditionally used for wound healing can act as a temporary barrier to stop bleeding, prevent infection and promote tissue regeneration. Despite advances in wound treatment, colonization of materials by host cells remains a critical challenge as the behavior of skin cells is significantly influenced by the physical, chemical and mechanical properties of hydrogels such as stiffness, porosity, shear stress, hydrophobicity and permeability ¹⁶. Although several biomaterials such as collagen and synthetic polymers have been developed for wound treatment, only a few have reached the market for clinical use ¹⁷. Hydrogels are good candidates for wound healing as they not only provide a physical barrier but also drain excess exudate and create a moist environment ^{18,19}. However, their low mechanical strength limits their use in clinical practice. Hydrogels with low covalent bonds promote high porosity, which enables cell viability and molecular diffusivity, but their low crosslinking density reduces their stiffness, which impairs the tissue repair process. In fact, hydrogels with optimal stiffness can promote wound closure through keratinocyte proliferation and migration, enhance ECM synthesis and remodeling, improve myofibroblast transformation, and reduce scar formation ²⁰⁻²³.

Gelatin hydrogels offer promising properties, including excellent biocompatibility, biodegradability, low antigenicity and cost-effectiveness, but when physically crosslinked, they exhibit poor stability and mechanical properties. The introduction of covalent bonds between polypeptide chains using crosslinkers solves this problem ²⁴, and fossil-based glutaraldehyde (GTA) is one of the most commonly used aldehyde compounds to produce stable protein-based hydrogels ²⁵⁻²⁷. However, this chemical reagent impairs the biocompatibility of these hydrogels and leads to cytotoxic effects ²⁸.

Biological alternatives with low cytotoxicity have been explored, such as genipin (GP) ²⁹⁻³¹, tannic acid ³², enzymes ^{33, 34}, dialdehyde of natural sources ³⁵, carrageenan ³⁶, and methacrylate anhydride ³⁷.

However, these approaches are designed for a rapid and uncontrolled crosslinking process once the crosslinker has been physically mixed into an aqueous gelatin solution or has come into contact with the gelatin scaffold. An important aspect in the development of future hydrogels is the introduction of latent compounds that are able to activate a crosslinking process in-situ and on demand, triggered by simple and mild external stimuli. A recent study reported the development of gelatin–poly(ethylene glycol) (PEG) composite hydrogels with different solid contents and crosslinking degrees by crosslinking a four-arm-PEG-succinimidyl glutarate (4-arm-PEG-SG) with gelatin for controlled release of drugs ³⁸.

In order to strengthen and diversify the options for meeting this challenge, we developed a novel crosslinking method for gelatin-based hydrogels based on the wound closure mechanism of *C. taxifolia*. We designed a biomimetic gelatin-based hydrogel by promoting its covalent crosslinking through *in situ* and on-demand deacetylation of the metabolite CYN, mimicking the CYN-crosslinked matrix scaffold of the alga. To achieve this, we further refined the extraction of CYN from the alga and tested its ability to trigger chemical crosslinking in a protein-based hydrogel by acting on a pH change through a non-toxic and completely environmentally friendly process. We characterized the absorbability and tunable mechanical properties of the obtained 3D hydrogel, investigated its biocompatible and skin cell growth-inducing properties and questioned its potential to promote wound healing *in vivo*.

2. MATERIALS AND METHODS

2.1. Raw materials

Gelatin (type B, 225 Bloom) was purchased from Sigma (Sigma Aldrich Chimie S.a.r.l., Saint-Quentin-Fallavier, France). GP was purchased from Apollo Scientific (Cheshire, UK) and CYN was isolated from *C. taxifolia* in Villefranche-Sur-Mer (France) according to our previously described protocol ³⁹ under current regulatory framework. In brief, freeze-dried *C. taxifolia* (~ 30 kg) was crushed and extracted six times with 1 L of a mixture of MeOH–CH₂Cl₂ (1:1, v/v). The combined fractions were evaporated under reduced pressure to obtain a crude organic extract, which was purified by CPC using Arizona U (heptane, EtOAc, MeOH, H₂O; 4/1/4/1; v/v/v/v) as a two-phase solvent system to obtain 7 g of pure CYN.

2.2 Preparation of crosslinked gelatin

2.2.1. CYN-crosslinked gelatin

Two mL of aqueous gelatin solution (20 %, w/v) was heated at $T = 60\text{ }^{\circ}\text{C}$ for 3 hours to ensure complete solubilization. A mixture of CYN/EtOH (10, 20, 30 $\text{mg}\cdot\text{mL}^{-1}$) was then added to the solution to achieve a final CYN concentration of 5, 10 and 15 wt% (based on gelatin), designated as gel/CYN5, gel/CYN10 and Gel/CYN15, respectively. The formulation was then poured into a silicone mold and cooled to $T = 4\text{ }^{\circ}\text{C}$ for 3 hours. The physical hydrogel was then removed from the silicone mold and immersed in NaOH solution (0.1 M) at $T = 4\text{ }^{\circ}\text{C}$ for 24 hours. To re-equilibrate the pH and remove all traces of unreacted CYN, the hydrogel was purified in water at $T = 37\text{ }^{\circ}\text{C}$ for 24 hours. As a control, pure gelatin was mixed with CYN without treatment with aqueous NaOH solution. The control samples were stored at $4\text{ }^{\circ}\text{C}$ for 1 week.

2.2.2. GP crosslinked gelatin

Two mL of aqueous gelatin solution (20 %, w/v) was heated at $T = 60\text{ }^{\circ}\text{C}$ for 3 hours to ensure complete solubilization. A mixture of GP/EtOH (10, 20, 30 $\text{mg}\cdot\text{mL}^{-1}$) was added to the solution to achieve a GP concentration of 5, 10, 15 % (relative to the gelatin), designated as gel/GP5, gel/GP10 and gel/GP15, respectively. The formulation was then poured into a silicone mold and incubated at $37\text{ }^{\circ}\text{C}$ for 24 hours. To remove all traces of unreacted GP, the hydrogel was purified in water at $T = 37\text{ }^{\circ}\text{C}$ for 24 hours.

2.3. Methods for biomaterial characterization

2.3.1. Trinitrobenzene sulfonic acid (TNBS) assay

The extent of reacted ϵ -amino groups in gelatin was determined using TNBS % (Sigma), which interacts with lysine and hydroxylysine residues to generate trinitrophenyl derivatives that absorb in the UV range. In brief, 11 mg of uncrosslinked and crosslinked gelatin samples were mixed with 1 mL of a 4 % sodium bicarbonate solution and 1 mL of 0.5 % TNBS and incubated at $40\text{ }^{\circ}\text{C}$ for 4 hours. Subsequently, 3 mL of 6-N hydrochloric acid was added to the mixture and autoclaved at $120\text{ }^{\circ}\text{C}$ for 1 hour to dissolve all insoluble parts of the hydrogel. The hydrolysate was then diluted with 5 mL of water and extracted

with diethyl ether. To remove the remaining ether, 5 mL of the aqueous phase was heated in a hot water bath for 15 minutes. After cooling to room temperature, the aliquot part of the aqueous phase was diluted with 15 mL of water. The absorbance was measured at 346 nm in a UV-Vis spectrometer using a quartz cuvette with 1 cm path length. The blanks were prepared using the same procedure, except that HCl was added before TNBS to prevent the amino groups of the gelatin from reacting with TNBS. Three replicates were used for each determination. The same procedure was used for the pure gelatin samples, which served as controls.

$$\text{reacted } \varepsilon - \text{amino groups (\%)} = \left(1 - \frac{\text{Abs. CYN-crosslinked gelatin}}{\text{Abs.unreacted gelatin}}\right) \quad (1)$$

2.3.2. Rheological properties of CYN-crosslinked gelatin

A HAAKE MARS rheometer (Thermo Fisher Scientific, Villebon-sur-yvette, France) with a plate geometry (25 mm plate diameter and 1 mm gap) was used to characterize the rheological properties of CYN-crosslinked gelatin. An integrated Peltier plate was used to set the temperature. The storage modulus (G') and the loss modulus (G'') were investigated as a function of frequency. A frequency sweep experiment was performed by varying the frequency from 0.1 to 10 Hz at constant strain (1 %) at $T = 37^\circ\text{C}$. The hydrogel was equilibrated at 37°C for 10 minutes. A solvent trap was used to avoid water evaporation during the measurement.

2.3.3. Compression Tests

The HAAKE MARS rheometer was used for the mechanical compression test at 37°C . The cylindrical test specimens (25 mm diameter, 3 mm thickness) were inserted into the lower plate of the rheometer. The hydrogels were compressed vertically by the upper plate at a constant speed of 0.1 mm.s^{-1} to 40 % of the initial gap to prevent the sample from being squeezed out.

2.3.4. Gel content and Swelling

Freeze-dried crosslinked samples with a cylindrical shape (20 mm diameter x 10 mm thickness) were prepared and weighed for the gel content and swelling tests. The samples were hydrated in deionized water at 37°C for 24 hours, removed from water, wiped with tissue paper and weighed. The gel content

was determined by immersing the CYN-crosslinked gelatin samples and the control sample in hot deionized water ($T = 50\text{ }^{\circ}\text{C}$) for 48 h. The remaining crosslinked hydrogels were then removed, frozen for 24 h and freeze-dried for 48 h. The gel content (%) and SD (%) were calculated using the following equations:

$$\text{gel content (\%)} = \frac{W_{final}}{W_{initial}} \times 100 \quad (2)$$

$$\text{SD (\%)} = \frac{W_{swollen} - W_{initial}}{W_{initial}} \times 100 \quad (3)$$

$W_{swollen}$ was the weight of the swollen sample after 24 hours at $37\text{ }^{\circ}\text{C}$, $W_{initial}$ was the initial weight of the dry samples, and W_{final} was the weight of the dry samples after 48 hours at $T = 37\text{ }^{\circ}\text{C}$. The results of the degree of swelling and gel content were averaged over the measurements carried out in triplicate. The results are given as mean $\pm$ standard error.

2.3.5. Scanning Electron Microscopy (SEM)

CYN-crosslinked samples (5, 10 and 15%) were swollen in water at $37\text{ }^{\circ}\text{C}$ for 48 hours, cut into cylindrical shapes (20 mm diameter), frozen overnight and freeze-dried for 48 hours. Cross-sections were performed at room temperature using a razor blade. The cross sections were gold plated (3 nm) with a Q150R S sputter coating (Quorum Technologies) prior to observation with a Phenom ProX Desktop SEM. Image-J software was used to estimate the pore size. The results are given as mean $\pm$ SD.

2.4. Methods for analyzing biocompatibility and functionality of gels

2.4.1. Cell culture

Normal human keratinocytes (NHK) were obtained from human foreskin according to previously published procedures⁴⁰ and grown in supplemented keratinocyte growth medium containing 0.15 mM CaCl_2 (KGM-GOLD BulletKit, Lonza Biosciences, Basel, Switzerland). Normal human fibroblasts (NHF) were obtained from human foreskin explants and cultured using standard techniques in DME supplemented with 2 mM glutamine, an antibiotic cocktail and 10 % fetal calf serum⁴¹. Skin biopsies

were obtained in compliance with ethical and safety guidelines according to the French donor regulation (declaration n° DC -2008-162, submitted to the Cell and Tissue Bank of the Hospices Civils de Lyon).

2.4.2. Cell adhesion experiment

The NHF and NHK were detached from culture plates and counted. Cells were seeded in 96-well plates with bottoms previously coated with gelatin, CYN-crosslinked gelatin (5, 10 and 15 %) and with GP-crosslinked gelatin (5, 10 and 15 %). Both NHFs and NHKs were seeded (4×10^4 cells per well) in their respective culture medium (5 wells per condition). After 1 hour at 37 °C in a humidified incubator with 5 % CO₂, the non-adherent cells in each well were counted. The number of adherent cells was determined by subtracting the non-adherent cells from the total number of seeded cells.

2.4.3. Cell viability test

NHFs and NHKs were separated from their culture plate and placed in a 96-well plate whose bottom had previously been coated with gelatin, CYN-crosslinked gelatin (5, 10 and 15 %) and with GP-crosslinked gelatin (5, 10 and 15 %) and sterilized with ethanol 70 %. NHFs were seeded with 10^5 cells per well and NHKs with 4×10^5 cells per well in the respective culture media (5 wells per condition). After 2 hours at 37 °C in a humidified incubator with 5 % CO₂, the cell culture medium was removed and replaced with culture medium containing 12.5 % Alamar Blue cell viability reagent (Bio-Rad France Laboratories, Marnes-La-Coquette, France). To monitor cell growth, the absorbance of the culture medium was measured at 590 nm at different time points. NHFs and NHKs were photographed using a Zeiss Axiovert 40 microscope coupled to a Coolsnap Fx camera (Roper Scientific, Evry, France).

2.4.4. In vivo assay

Two full-thickness wounds (punch biopsy of 11 mm diameter and 4 mm thickness) were made on the backs of 2 female pigs. In each pig, one wound was treated with the gel/CYN5 and a control wound was treated with the gel/GP5. The gel-treated wounds were covered with a secondary dressing consisting of a microporous polyurethane film (Tegaderm), sterile dressings and Elastoplast to protect and secure them. The quality of each wound was clinically assessed by photographs on day (D)7 and D10 after

wound treatment, and a secondary dressing change was performed on D7. A biopsy including all wounds treated with the two gels from the same pig was performed on D7 and D10. The samples were immediately processed for microscopic examination. The experiments were conducted at the Institut Claude Bourgelat VetAgro Sup Campus Vétérinaire de Lyon, where the pigs were kept under pathogen-free conditions in facilities approved by the accreditation for the care of laboratory animals and compliant with current regulations and standards of the French Ministry of Agriculture, which comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of VetAgro Sup, Campus Vétérinaire de Lyon, France. For histologic analysis, biopsies were processed for paraffin embedding, and paraffin sections were processed for hematoxylin-eosin-phloxin-saffron (HES) staining. The stained skin sections were scanned with a ZEISS Axioscan 7 and the images were processed with Fiji software as previously described ⁴².

2.4.5. Transmission electron microscopy

The tissue was cut into small pieces and fixed in 2 % glutaraldehyde at 4 °C for 2 hours. The samples were washed three times at 4 °C and fixed in 2 % OsO₄ for 1 hour at 4 °C. The fixed tissues were dehydrated in a series of graded ethanol washes, transferred to propylene oxide and impregnated with Epon A (50 %), Epon B (50 %), and DMP30 (1.7 %). Inclusions were obtained by polymerization at 60 °C for 72 hours and sections were made using a UC7 (Leica) ultramicrotome. Areas were first selected on semi-thin sections (1000 nm thick) stained with methylene blue Azur II. Ultrathin sections (~100 nm) were mounted on 150 copper grids coated with 1:1,000 polylysine, stabilized at RT and treated with uranyl acetate and lead citrate. The sections were examined with a Jeol 1400JEM (Tokyo, Japan) transmission electron microscope, 100 kV, equipped with an Orius 600 camera and Digital Micrograph.

2.5. Statistical treatment of the data

All data in the Results section are presented as mean $\pm$ st. dev., unless otherwise specified. The one-way method ANOVA and Student tests were used to analyse the cell biology data, and a p-value of less than

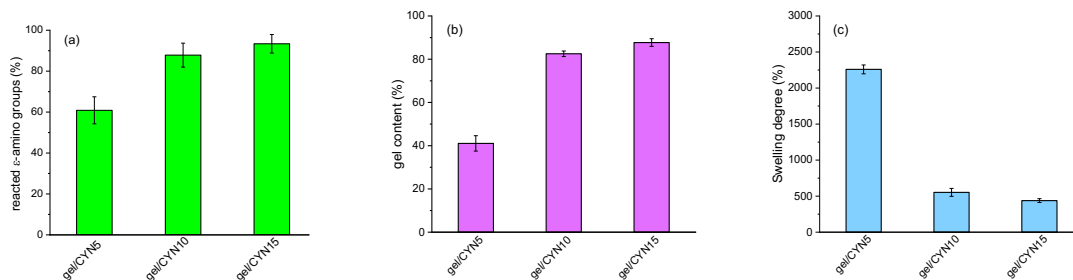


Figure 2. (a) TNBS assay results, (b) gel content, and (c) swelling degree function of the CYN concentration (5, 10, 15 %wt).

The degree of crosslinking of hydrogels can be efficiently estimated by measuring the gel content and swelling. In general, a low degree of crosslinking leads to a high-water absorption capacity and thus to a decrease in gel content, while highly crosslinked hydrogels allow an increase in gel content and reduce swelling capacity. As shown in Figure 2b, an increase in gel content was observed as a function of the concentration of the latent crosslinking agent. Gel/CYN5 shows a gel content of about 60 %, while with gel/CYN15 almost 90 % gel was achieved. As with the algae, no crosslinking reaction was observed between the CYN stabilized by enol acetate functionalities and the gelatin, even when incubated for 1 week at 4 °C. This result could be easily verified by evaluating the gel content of the control samples (without treatment in aqueous NaOH solution). As expected, the mixture was completely dissolved, indicating the absence of crosslinking network. The swelling capacity of the CYN-crosslinked gelatin hydrogels was calculated using Equation 3 and shown in Figure 2c. The degree of swelling of the three tested samples is $2260 \% \pm 62 \%$, $552 \% \pm 55 \%$ and $438 \% \pm 27 \%$ for gel/CYN5, gel/CYN10 and gel/CYN15, respectively. According to the gel content results (Figure 2b), the increase in crosslink density with the increase in CYN concentration contributes to the decrease in swelling capacity. The TNBS determination, the gel content and the swelling capacity (Figure 2) show a similar trend with a strong variation between 5 and 10 wt%. However, a less pronounced variation in gel content and crosslinking ability was observed at CYN content higher than 10 wt (Figures 2b, c), which is a direct consequence of the lower ability to find ϵ -amino groups for the crosslinking reaction (Figure 2a). This indicates that deacetylated CYN is unable to form a fully crosslinked hydrogel, likely due to the

shielding effect of the gelling tertiary structure^{43, 44}, which prevents some of the lysine residues from reacting with OXY.

The morphology of the CYN-crosslinked gelatin was examined by scanning electron microscopy (SEM). Figure 3 shows the cross-section SEM images of freeze-dried gelatin hydrogels prepared with different CYN concentrations (5, 10 and 15 %). The images show interconnected open pores, which are essential for the transport of nutrients and oxygen to the cells in the hydrogel⁴⁵. Similar morphologies have already been described for hydrogels crosslinked with gelatin⁴⁶⁻⁴⁸. From 50 to 80 pores were measured in SEM images of each condition using the Image J software (threshold technique). The pore sizes were estimated to be 400 ± 34 , 362 ± 23 and 348 ± 41 μm for gel/CYN5, gel/CYN10 and gel/CYN15, respectively. An increase in CYN concentration leads to a decrease in average pore size, indicating an increase in crosslink density. As previously reported, such average pore sizes enable cell proliferation and angiogenesis⁴⁹.

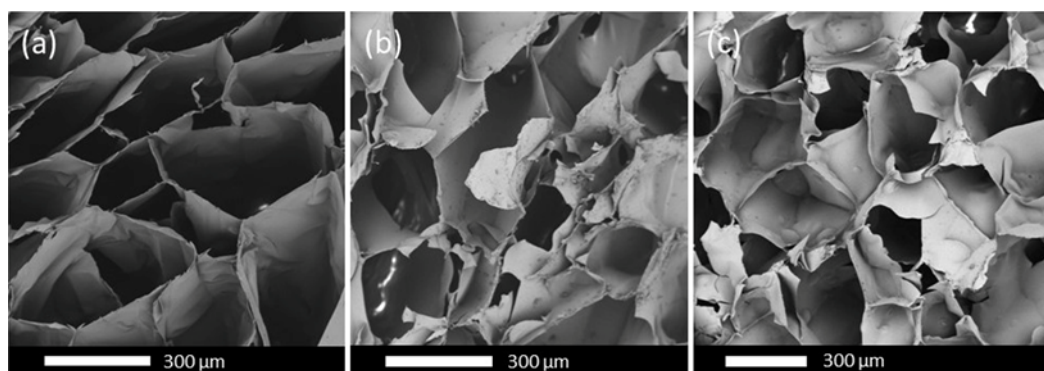


Figure 3. SEM images of cross-section morphology of the gel-cyn hydrogels: (a) gel/CYN5, (b) gel/CYN10, (c)gel/CYN15.

The rheological properties of the CYN-crosslinked gelatin hydrogels were investigated by frequency sweep experiments. The measurements were performed at 37 °C to evaluate the reaction of the hydrogel at physiological temperature. Above a certain temperature (denaturation temperature, T_d), the physically crosslinked gelatin hydrogel transitions from a triple helix to a random coil. The T_d of gelatin depends on several factors, such as gel straightness (Bloom), concentration, moisture content and pH^{50, 51}, and is generally below 37 °C. It is worth noting that the frequency sweep experiment for CYN-crosslinked

samples shows an almost constant value of G' in the frequency range studied (0.1-10 Hz), indicating the formation of a stable crosslinked network in gel/CYN samples (Figure 4a). The storage modulus of the crosslinked gelatin hydrogels increases as a function of CYN concentration. The G' ($f = 1$ Hz) was measured at 64, 463 and 1426 Pa for gel/CYN5, gel/CYN10, and gel/CYN15, respectively. This result reflects the degree of crosslinking of the samples, which increases with increasing CYN concentration.

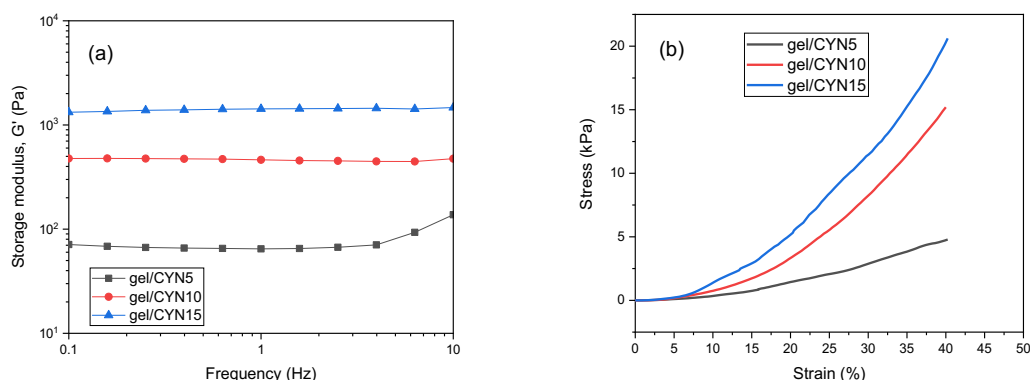


Figure 4.(a) Frequency sweep experiment of cyn-crosslinked gelatin at 37 °C (strain 1%); (b) compression test of cyn-crosslinked gelatin at 37 °C.

A compression test was carried out with a constant compression rate of up to 40 % of the strain (Figure 4b) at 37 °C. The stress-strain curves follow a similar trend as reported in the literature for protein-based hydrogels^{30, 52}. Due to the viscoelastic properties of the crosslinked hydrogels, a non-linear variation of the curves was observed and no breaking point was observed at 40 % of the strain. As can be seen in Figure 4b, different behaviors were detected depending on the CYN concentration. When the CYN concentration is increased from 5 to 15 %, the compressive stress rises from 4.8 to 20.6 kPa at 40 %, indicating a significant increase in mechanical strength.

3.2. *In vitro* characterization of the biological properties of the CYN-crosslinked gelatin

To investigate the compatibility of CYN-crosslinked hydrogels with human skin cells, we first examined the ability of dermal NHFs and epidermal NHKs to interact with the gels compared to control GP-crosslinked gels (Figure 5). As expected, NHF adhesion was significantly increased regardless of the concentration of CYN or GP used compared to non-crosslinked gelatin, which is known to be less

effective⁵³. (Figure 5a). While cell adhesion to CYN-crosslinked gels remained high, it was increased with the stiffness of the gel. This stiffness-dependent adhesion property was not found for GP gels (Figure 5a). As for NHKs, adhesion to all CYN- or GP-crosslinked gels was as good as to gelatin alone, which is known to be a good adhesion substrate for NHKs (Figure 5b)⁵². Similar to that seen with NHFs, the adhesion of NHKs to the CYN-crosslinked gels was progressive and correlated with the degree of crosslinking achieved in the 15 % crosslinked gel, which was higher than in the corresponding GP control.

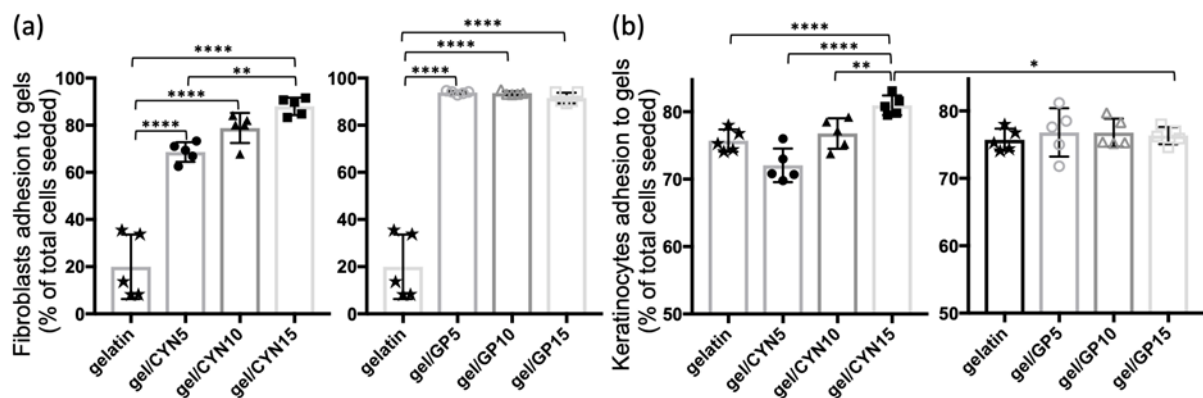


Figure 5. CYN-crosslinked gelatin promotes human skin cells adhesion in a gel stiffness-dependent manner. (a) Human primary dermal fibroblasts and (b) epidermal keratinocytes were seeded for 1 hour on non-crosslinked gelatin, gel/CYN5, gel/CYN10 and gel/CYN15 as well as gel/GP5, gel/GP10 and gel/GP15. Adherent cells were quantified and expressed as a percentage of total cells. Shown are mean $\pm$ SD with **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ determined by Student's t test.

To assess the cytocompatibility and the ability of the CYN-crosslinked gels to promote cell growth, we examined the viability of NHFs and NHKs seeded on the CYN-crosslinked gels compared to non-crosslinked and GP-crosslinked gelatin (Figure 6). All gels tested allowed the cells to live and grow over

a long period of time, albeit to varying degrees. Higher NHF growth was observed on the CYN gels in the first few hours of culture, but this quickly reached a plateau (Figure 6a).

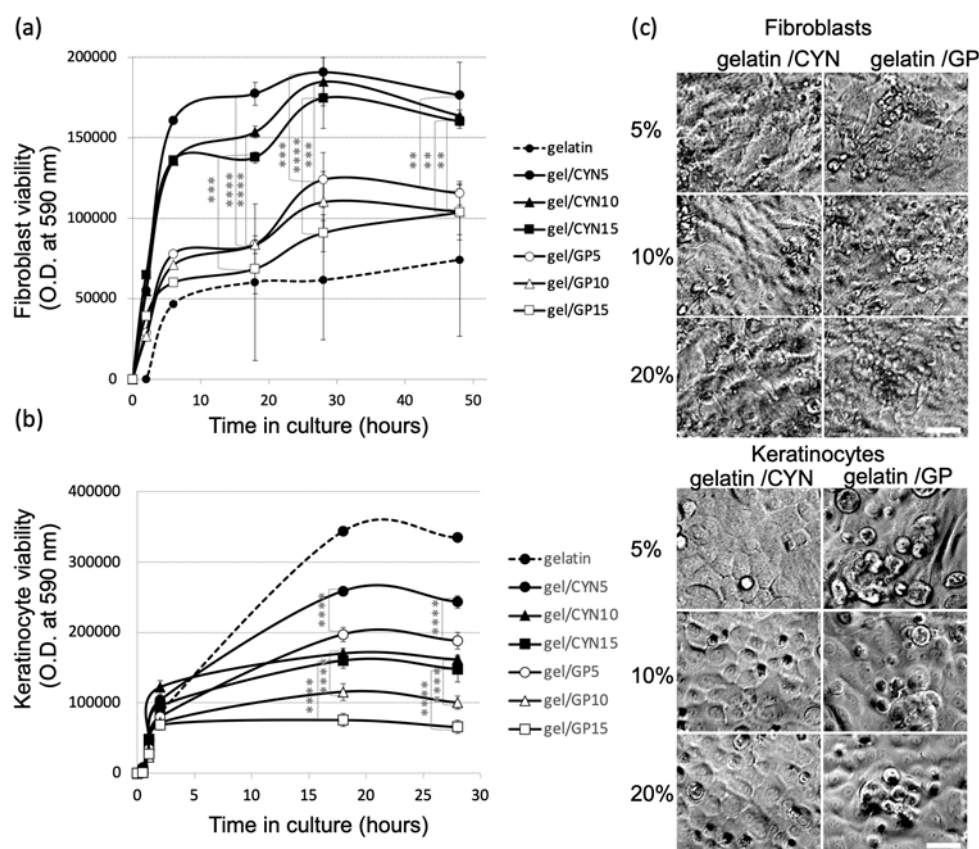


Figure 6. CYN-crosslinked gels biocompatibility over time. Human primary dermal fibroblasts (a) and epidermal keratinocytes (b) were seeded on gelatin, gel/CYN and gel/GP as indicated. Cell viability and growth were monitored using the Alamar-Blue assay during the indicated period. Shown are mean $\pm$ SD with **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ determined by Student's t test. Note the presence of cell aggregates on the gels crosslinked with GP. Bar, 50 μ m. (c) Phase contrast images of cells at confluency. Note the presence of cell aggregates on the gels crosslinked with GP. Bar, 50 μ m.

Growth on the GP gels was slower over time and remained significantly lower at each time point measured and for each concentration of crosslinking agent. Non-crosslinked gelatin was again the least effective growth substrate. The situation was different for the NHKs, which showed a profile of survival and maximum growth on non-crosslinked gelatin (Figure 6b). Although the cell viability profiles for all crosslinked gels were relatively close to each other, we found that the growth rate on the CYN-

crosslinked gels was systematically slightly and significantly higher than on their GP-crosslinked counterparts. Observation of the cells after 24 hours of culture shows that confluent cell layers are present under all crosslinking conditions, indicating a good growth rate of the cells (Figure 6c). We note that cell aggregates are present on the GP-crosslinked gels, which could be related to the high adhesion capacity of these gels and could be the cause of the slowdown in the proliferation process.

3.3. Analysis of the skin healing effect of CYN-crosslinked gelatin *in vivo*

The healing-promoting activity of the gel/CYN5 was evaluated in a porcine model of a deep wound in comparison to the gel/GP5 (Figure 7). The two gels were equally easy to apply to the wounds, remained in place throughout the experiment and showed no macroscopically significant differences in the healing process, which lasted until D10.

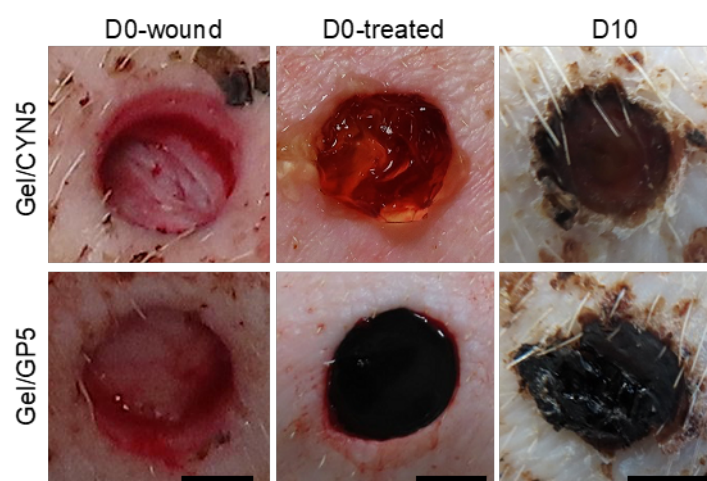


Figure 7. Full thickness punch wounds on the back of two pigs at D0 and immediate treatment with GEL/CYN5 or GEL/GP5. Appearance of the two healed skin wounds at D10 post-injury. Bar 5 mm.

Histological analysis of HES-stained sections of biopsies taken at D7 (a, b) and D10 (c, d) after injury showed clear differences in the healing process (Figure 8A). From the seventh day after treatment (a,b), the wounds treated with the two gels showed well-developed granulation tissue filling the entire wound space. The wounds treated with gel/CYN5 were already completely reepithelialized by a thin epidermal layer (a), whereas the epidermalization of the wounds treated with gel/GP5 only started at the two adjacent wound edges (b). The analysis of the biopsies on D10 shows very clear differences (c, d). The

epidermis of the gel/CYN5-treated wounds was completely regenerated and reconstructed (c), whereas the epidermis of the gel/GP5-treated wounds had not even covered the granulation tissue (d). The maturation of the granulation tissue of wounds treated with the gel/CYN5 hydrogel was remarkably advanced and showed an appearance close to the normal dermis when compared to the gel/GP5, which was still in the inflammatory phase of the repair process. These results show that CYN-crosslinked gelatin is a hydrogel that can promote skin repair. Wounds treated with this hydrogel were already covered with epidermis on the seventh day after injury, and complete healing was accomplished on the tenth day without any abnormalities. Wound re-epithelialization is an essential step that determines the overall healing process and influences the quality of the restored tissue²¹. The CYN-crosslinked gelatin showed a clear advantage over its GP-crosslinked counterpart, in which the re-epithelialization of the wound was not even completed on the tenth day under the same conditions and the inflammatory phase was still ongoing.

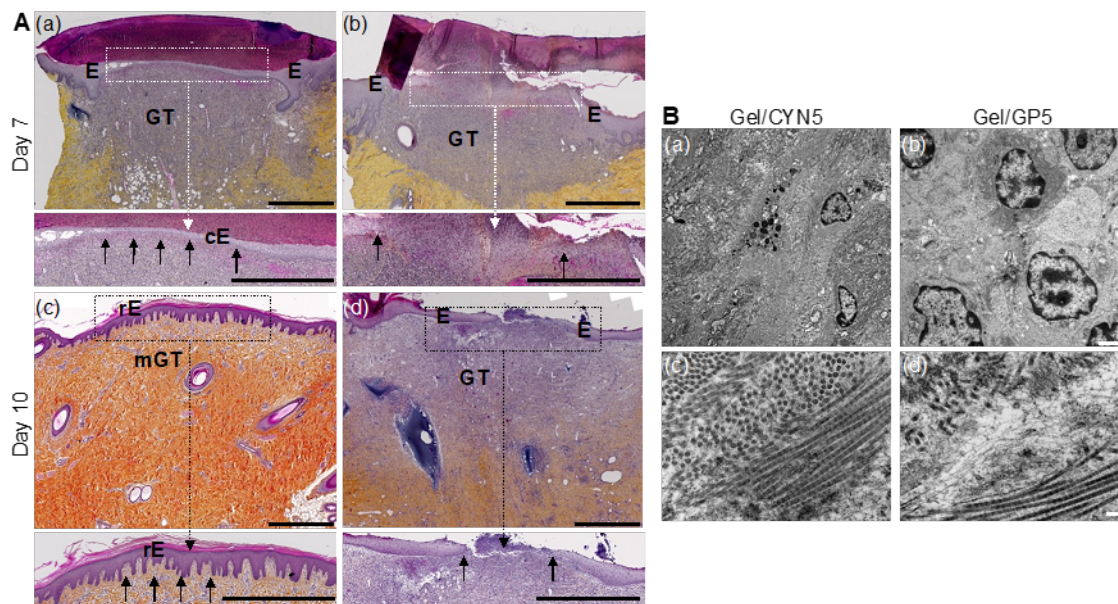


Figure 8. (A) HES-stained histological sections of the whole wound biopsies treated with gel/CYN5 (a, c) and gel/GP5 (b, d) at D7 and D10 post injury. GT granulation tissue; mGT, mature granulation tissue; E, epidermis; cE, complete epidermisation; rE, regenerated epidermis. Bar 1 mm. (B) Transmission electron microscopy images of the granulation tissue of wound treated by gel/CYN5 (a, c) and gel/GP5 (b, d) at day 10 post-injury. Bar 2 μ m (a, b), 0.2 μ m (c, d).

To assess the quality and maturation stage of the granulation tissue on the 10th day after injury, we performed transmission electron microscopy (Figure 8B). Low magnification images of the granulation tissue of wounds treated with gel/GP5 show a sparse matrix containing numerous inflammatory cells (b), whereas it is very dense and much less cellularized in wounds treated with gel/CYN5 (a). A closer look at the ECM shows a network of longitudinal and transverse collagen fibers that is already well organized after treatment with gel/CYN5, while it still appears rudimentary and early in the granulation tissue formed after treatment with gel/GP5.

4. DISCUSSION

Our study reports the first crosslinking method of protein-based hydrogels based on the wound closure mechanism of the Mediterranean algae *C. taxifolia*. The successful spread of *C. taxifolia* is indeed attributed to a combination of factors, including high temperature tolerance, asexual reproduction and an exceptionally fast response to wounds⁵⁴. This ability to heal by forming a protective matrix depends on CYN, an important metabolite that plays a key role in the repair of damaged tissue. The three acetate groups of this sesquiterpene are enzymatically cleaved within minutes after mechanical damage, and its deacetylated structure (OXY) immediately reacts with nucleophilic functional groups of ECM proteins, promoting the process of underwater covalent crosslinking thereby building the 3D network EWP. After extracting CYN, we successfully used it to crosslink a gelatin polymer and mimic the structural and mechanical properties of the algae's EWP matrix. By acting on external stimuli such as pH with an aqueous NaOH solution, we achieved *in situ* deacetylation of CYN, leading to the formation of OXY, which immediately crosslinked gelatin molecules (Figure 1). As previously reported⁵⁵, chemical bases were efficiently used to convert 1,4-bis-enol acetate to 1,4-dialdehyde. The development of several physical gelatin hydrogels with different concentrations of organic latent CYN crosslinker from 5 to 15 % showed that the crosslinking density increases as a function of CYN concentration, leading to a gradual decrease in the degree of swelling from 2260 % $\pm$ 62 % to 438 % $\pm$ 27 %. These data place the tested gels in the category of highly swelling hydrogels, whose properties are extremely interesting for wound care, as they have a high absorption capacity for wound exudates and a high activity to promote cell recruitment and migration⁵⁶. In addition, the excellent water retention capacity is the most

remarkable feature of hydrogels, which is the basis for mimicking the softness and water content of ECMs ⁵⁷. Also, the pore size, ranging from 400 ± 34 to 348 ± 41 μm in diameter, makes these macroporous gels excellent candidates for direct cell colonization, ECM production, and the development of a network of blood and lymphatic vessels ranging from 20 to 150 μm in diameter ⁵⁸. The rheological and mechanical properties showed a similar trend with an increase in storage modulus and mechanical strength as a function of CYN concentration. A storage modulus of 64, 463 and 1426 Pa for gel/CYN5, gel/CYN10 and gel/CYN15, respectively, offers a wide range of possibilities for clinical applications. Several cell types are involved in the wound healing process, including inflammatory cells, keratinocytes, fibroblasts, myofibroblasts, and endothelial cells, which respond to the mechanical forces of the microenvironment by initiating a cascade of cell cytoskeleton-dependent events and pathways leading to cell proliferation and migration, ECM synthesis and remodeling, and termination of inflammation ^{20, 21, 59}. Regardless of their CYN content, the gels were found to induce adhesion and growth of human dermal and epidermal cells, consistent with the process of tissue repair. These properties were steadily better than those of GP-crosslinked gels. Our cell adhesion assays showed that both NHFs and NHKs immediately adhered and spread on CYN-crosslinked gels. More than 70 % of the plated cells adhered within one hour after contact with the gel/CYN5 % and increased to 80 % and 100 % when gel stiffness increased with gel/CYN 10 and 15, respectively. This stiffness-dependent cell adhesion property confirms that CYN-crosslinked gels are powerful tools with tunable mechanical properties that are sensed and responded to by the cells. This represents a significant advantage, as substrate stiffness influences the organization of the cell cytoskeleton, the migration behavior and the fate of the cells ⁶⁰. The dependence on the percentage of crosslink was also evident in our cell viability and proliferation assays, where we found optimal cell growth with CYN5 gels, a result most likely related to a stiffness more suitable for cell proliferation. Our results showing that all CYN-crosslinked hydrogels exhibit excellent cytocompatibility and growth-promoting properties towards skin cells prompted us to consider their use for skin repair *in vivo*. Our preliminary preclinical tests with the gel/CYN5 in a deep porcine wound model have shown that the gel can excellently promote skin regeneration compared to its counterpart, the gel/GP5. Immunohistochemical analysis of the healing process showed that the CYN-crosslinked gel already promoted complete epidermal coverage of

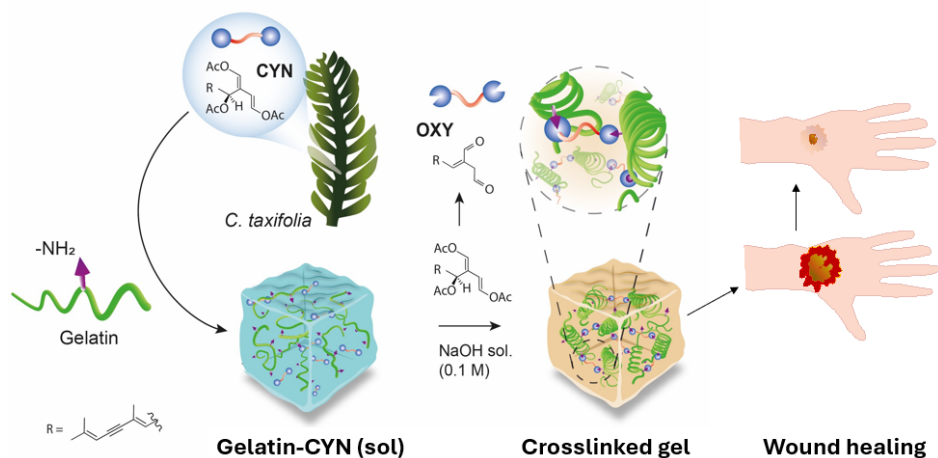
granulation tissue on the seventh day. Furthermore, the gel/CYN5 led to a complete regeneration of the epidermis on a fully reconstituted dermis of mature ECM, whose inflammatory phase seems to have subsided on the tenth day after the lesion, when the gel/GP5 had not yet completed its re-epithelialization phase. All our *in vitro* and *in vivo* results make CYN-crosslinked hydrogels extremely promising matrices for the field of regenerative medicine. With its specific properties, CYN joins the list of biological crosslinking agents obtained from natural resources that have no toxic effects and therefore guarantee safety for humans and the environment⁶¹. Our work, which shows a functional superiority of the CYN-crosslinked hydrogels compared to hydrogels crosslinked with GP, one of the most commonly used natural crosslinking agents, opens up interesting perspectives to be explored in the near future with regard to the range of possible applications. The mechanical and ultrastructural properties of CYN-crosslinked hydrogels, which can be modulated according to the degree of crosslinking, are similar to those recently described in a series of new gelatin-PEG hybrid hydrogels for the delivery of molecules³⁸. However, CYN-crosslinked hydrogels differ from gelatin-PEG hybrid hydrogels in their ease and speed of synthesis and their exclusive gelatin content. Besides, their already proven ability to promote skin regeneration *in vivo* makes them candidates for immediate large-scale preclinical testing.

5. CONCLUSION

Caulerpenyne plays a key role in the repair of damaged tissue in *C. taxifolia*. Its deacetylated structure (OXY) promptly reacts with nucleophilic functional groups of ECM proteins, promoting the process of underwater covalent crosslinking. Inspired by the wound closure mechanism of *C. taxifolia*, we developed a crosslinked gelatin hydrogel by *in situ* and on-demand deacetylation of CYN. In this study, we determined a simple method to convert CYN to OXY by changing the pH with an aqueous NaOH solution. The results show an increase in crosslinking density as a function of CYN concentration, leading to a decrease in the swelling degree and pore size. The rheological and mechanical properties showed a similar trend with an increase in storage modulus and mechanical strength as a function of CYN concentration. The gels crosslinked in this way exhibited such cytocompatibility properties that we could consider their use for skin repair. Regardless of their CYN content, the gels were found to induce adhesion and growth of dermal and epidermal cells, consistent with the process of tissue repair.

These properties were consistently better than those of GP-crosslinked gels. Preliminary preclinical tests conducted with the gel/CYN5 in a porcine deep wound model demonstrated the gel's ability to promote skin regeneration in an exceptional manner.

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Author Contributions

The conception, design, achievement of experiments, and analysis of data were performed by all authors.

GM and PR wrote the manuscript. PR and DP performed *in vitro* and *in vivo* experiments respectively.

All authors revised the manuscript and gave their approval of the submitted version.

Notes

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data required to reproduce these findings are available on request.

ACKNOWLEDGMENTS

The work was supported by Université Côte d'Azur and SATT Sud-Est funds to MM and NS and by Auvergne-Rhône Alpes and Cares HORIZON-TMA-MSCA-SE grants to PR. We acknowledge E. Errazuriz of SFR Santé Lyon-Est (UAR3453 CNRS, US7 Inserm, UCBL) facility for EM experiments.

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