

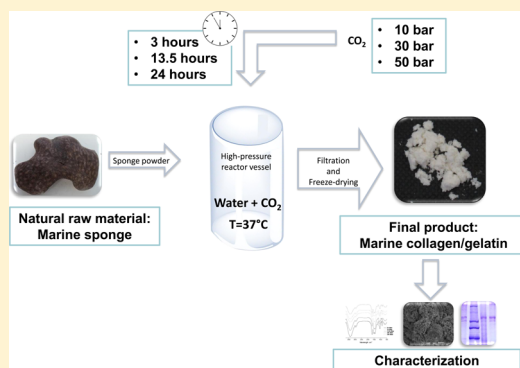
# Extraction of Collagen/Gelatin from the Marine Demosponge *Chondrosia reniformis* (Nardo, 1847) Using Water Acidified with Carbon Dioxide – Process Optimization

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**ABSTRACT:** Marine sponges are a rich source of natural bioactive compounds. One of the most abundant valuable products is collagen/gelatin, which presents an interesting alternative source for pharmaceutical and biomedical applications. We have previously proposed an innovative green technology for the extraction of collagen/gelatin from marine sponges based in water acidified with carbon dioxide. In this work, we have optimized the process operating conditions toward high yields and collagen quality as well as to reduce extraction procedure duration and energy consumption. The process extraction efficiency is higher than 50%, corresponding to a yield of approximately 10% of the sponge dry mass, obtained for mild operating conditions of 10 bar and 3 h. The extracted material was characterized by scanning electron microscopy (SEM), rheology, Fourier transformed infrared spectroscopy (FTIR), circular dichroism (CD), amino acid analysis, and SDS-PAGE. The extracts were found to be composed of highly pure mixtures of collagen and gelatin with similar properties to collagen isolated from other marine sources. The cytotoxicity evaluation, performed with L929 cells, has proven the biocompatibility of the material extracted. Overall, the results obtained demonstrate the efficiency of this approach and the high industrial potential of this technology to obtain marine collagen/gelatin with properties suitable for biomedical applications.



## 1. INTRODUCTION

In recent years, an increased concern about society's dependence on fossil fuel resources and a consequent forthcoming shortage motivated the search for alternative and sustainable sources, in particular, more attention has been addressed to the marine ecosystem.<sup>1,2</sup> Accordingly, marine resources appeared as novel raw materials in a wide range of areas, including food industry,<sup>3</sup> biomaterials development,<sup>4</sup> pharmaceuticals,<sup>5</sup> and biomedicine.<sup>6,7</sup> In fact, several blue biotechnology strategies have been focused on different marine organisms, including marine sponges, which are considerably rich in natural products, particularly collagen.<sup>8</sup>

Collagen is the main structural protein of the extracellular matrix (ECM) of animals, being responsible for the mechanical properties, organization, and shape of tissues and accounts for around 30% of the total protein content of the human body.<sup>9</sup> In mammals, it is the major component of bone, cartilage, and skin, with 28 different types of collagens described in the literature.<sup>9,10</sup> Collagen is a high hierarchical protein that has a complex structural organization based on fibril monomers constituted by a triple helix, which consists of three  $\alpha$ -chains with a characteristic glycine-proline-hydroxyproline repeating unit.<sup>11</sup> Gelatin is a soluble peptidic compound, which is

obtained after the partial hydrolysis of collagen.<sup>12</sup> The high availability of natural sources, coupled with its versatility, biodegradability, biocompatibility, and low immunogenicity make collagen a superior material for several biomedical applications,<sup>13,14</sup> including tissue engineering strategies.<sup>15–17</sup> Additionally, collagen is also used in other fields such as the food industry, cosmetics, and pharmaceutical applications, which leverages its yearly current industrial demand for more than 326,000 tons.<sup>18</sup> Currently, most of the collagen used (about 98%) in industrial exploitation is from mammal origin, derived from bovine and porcine hides and bones.<sup>10</sup> However, mammalian collagen has been associated with a high pathological risk of transmitting diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and avian/swine influenzas.<sup>19</sup> Moreover, mammalian origin collagen presents other disadvantages related with social and religious constraints as well as extraction and purification processes, which are complex, requiring

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chemical processing and thus are quite expensive.<sup>10,18,20</sup> In this sense, in order to overcome all these drawbacks, marine-derived collagen is being considered highly attractive as an important alternative source. Despite the limitations in terms of source-dependent composition variation as well as lower melting temperatures, marine origin collagen has the advantages of eliminating the risks of disease transmission and being obtained in simpler and cheaper procedures.<sup>4,20</sup>

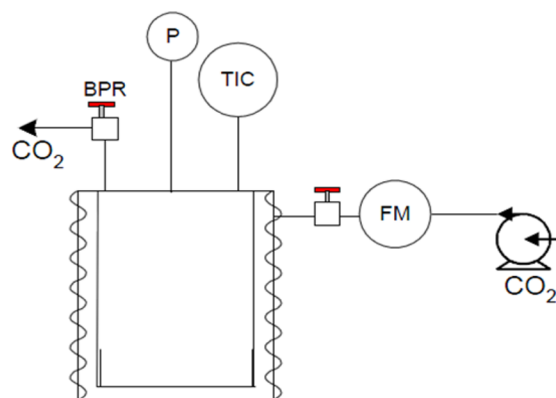
To date, collagen has been successfully isolated from several other marine species beside marine sponges, the richest marine source.<sup>8,18,19</sup> These include fish scales and skin,<sup>21–23</sup> starfish,<sup>24</sup> jellyfish,<sup>25,26</sup> eel fish,<sup>27</sup> cuttlefish skin,<sup>28</sup> shark skin,<sup>29,30</sup> and squid skin.<sup>31</sup> However, despite its unique physicochemical properties,<sup>8,32–35</sup> sponge-derived collagen is not available in large quantities and an industrial extraction process has yet to be established, mainly due to the lack of efficient and dedicated purification procedures that might trigger investments in the more sustainable sponge mariculture trials projects.<sup>36</sup> In fact, the current methods are mainly based on enzymatic and acidic or basic treatments, often resulting in low extraction yields.<sup>10</sup> These isolation strategies are very laborious, time consuming, and presents several drawbacks that hamper the process scale up, such as the requirement of multiple operation steps, energy, and large amounts of water and other solvents.<sup>19</sup> Moreover, these methods result in low selectivity and low extraction yields.<sup>8,19</sup> Therefore, there was a need for a more effective extraction method comprising fewer operational stages and being more environmentally sustainable and compliant with the green chemistry principles. In that sense, we have recently proposed a new technology<sup>37</sup> for the extraction of marine sponge collagen/gelatin based on water pressurized with carbon dioxide. This methodology comprises one single extraction step, which proceeds under mild operating conditions and avoids the use of any organic solvents.<sup>18,37</sup> In this view, our approach can also be considered as a first step for the establishment of a biorefinery approach, which aims to optimize the cultured sponge biomass exploiting all the compounds that might have valuable properties. However, to scale up the process and translate this technology to an industrial scale, a broad study of the effect of the operating conditions is required. Therefore, the major objective of this work is to perform an optimization of the process operating conditions (pressure and extraction time) in order to obtain high extraction yields and high amounts of collagen/gelatin with the appropriate properties. For that, we tested the method on the marine sponge *Chondrosia reniformis* since it has been already used as a potential source of collagen/gelatin for biomedical applications in tissue engineering and regenerative medicine, as previously described by the works of Heinemann et al.<sup>38,39</sup> and Swatschek et al.<sup>40,41</sup>

## 2. MATERIALS AND METHODS

**2.1. Materials.** *Chondrosia reniformis* (Nardo 1847) (*C. reniformis*) specimens were collected from the Mediterranean Sea (Alassio, Italy) and were kindly provided frozen or lyophilized by Dr. Antonio Sarà and Dr. Martina Millanese (Studio Associato GALA, Italy). All other reagents, obtained from commercial sources, were high quality reagent grade chemicals for laboratory use respecting the specifications of the American Chemical Society (ACS).

**2.2. Extraction of Collagen/Gelatin.** Collagen/gelatin extraction was performed following the previously established procedure as described elsewhere.<sup>18,37</sup> Briefly, freeze-dried *C.*

*reniformis* specimens were washed with distilled water overnight to remove salt and other contaminants and then were freeze-dried again. The salts removal was evaluated by measuring the washing water conductivity by means of a SCANSCI conductivity meter (DDS-11AW). Salts extraction was considered completed when conductivity values reached those of pure water. Prior to the extraction, the sponge material was processed using an Ultra Centrifugal Mill ZM200 (Retsch) to grind the material into small pieces of approximately 0.5 mm. Afterward, the sponge material (approximately 1 g) was loaded into the high pressure vessel (Figure 1) together with 20 mL of



**Figure 1.** Schematic representation of the high-pressure vessel system (total volume of 30 mL) used for the extraction of marine collagen/gelatin. BPR, back pressure regulator; P, pressure transducer; TIC, temperature controller; FM, flow meter.

distilled water (solid–liquid ratio of 1:20). The system was then heated to 37 °C and pressurized with carbon dioxide with constant agitation of 300 rpm. The water acidification, achieved by the addition of CO<sub>2</sub> to the mixture, promoted collagen/gelatin solubilization, as observed in our previous study.<sup>18</sup> The optimization was performed by selecting three pressures (10, 30, and 50 bar) and three extraction times (3, 13.5, and 24 h) and combined as indicated by the experimental design performed using the Design-Expert Software Version 9 (Stat-Ease). After the extraction period terminated, the vessel was rapidly depressurized, and the aqueous solution extract was filtered twice, using first a paper filter, followed by a second filtration with a 0.45 μm syringe filter. The solution was then frozen, and the final collagen/gelatin powder extracts were obtained after freeze-drying.

### 2.3. Characterization of Collagen/Gelatin Extracts.

**2.3.1. Extraction Yield, Total Protein, and Collagen/Gelatin Quantification.** The extraction yield was determined as the ratio between the final dry mass of extract obtained and the initial dry mass of sponge loaded in the high-pressure vessel. The total protein content of each batch relative to the dry mass of extract was assessed using the Micro BCA Protein Assay Kit (ThermoFisher Scientific), according to the instructions provided by the manufacturer. The amount of collagen/gelatin present in each sample in respect to the extracted dry mass was quantified using the Sircol Collagen Assay Kit following the protocol described by the manufacturer (Sircol, Soluble Collagen Assay, Biocolor, Life Science Assays, UK). This assay, developed for quantification of type I to type V mammalian collagens, consists of a dye-binding method that allows the analysis of acid and pepsin-soluble collagens.

**2.3.2. Scanning Electron Microscopy Analysis.** The morphology of powder extracts obtained from the protocols using different extraction conditions was assessed by a Nova NanoSEM 200 scanning electron microscope (SEM). The samples were fixed on aluminum stubs using a mutual conductive adhesive tape and covered with a gold nanolayer using a Cressington 108 A sputter coater.

**2.3.3. Fourier Transform Infrared Spectroscopy.** The infrared spectroscopy characterization was performed on a Shimadzu-IR Prestige 21 spectrometer. The extract samples were powdered, mixed with potassium bromide (KBr) in pellets, and analyzed in the spectral region of 4000–500  $\text{cm}^{-1}$ , with resolution of 2  $\text{cm}^{-1}$ , and the final spectrum was recorded as the average of 32 individual scans.

**2.3.4. Circular Dichroism Analysis.** The capability of our extraction process to preserve the triple helical conformation of collagen/gelatin extracts was evaluated by the acquisition of circular dichroism (CD) spectra using a Jasco Model J-865 spectropolarimeter (JASCO). CD measurements were performed using a quartz cylindrical cuvette with a path length of 0.1 mm. For each measurement, the cuvette was filled with 150  $\mu\text{L}$  of 3.3 mg/mL of extract in distilled water. CD spectra were obtained by continuous wavelength scans (with triplicates) from 190 to 260 nm at a scan rate of 50 nm/min. Before CD spectra acquisition, the extract samples were equilibrated at room temperature for 1 h.

**2.3.5. Amino Acid Analysis.** The amino acid content was determined by quantitative analysis using a Biochrome 30 (Biochrome Ltd., Cambridge, U.K.). Briefly, the samples were completely hydrolyzed and separated by an ion exchange column. After post-column derivatization by ninhydrin, the samples were analyzed at two wavelengths: 440 and 570 nm. An internal standard of norleucine was used to determine the concentration of amino acids in the sample.

**2.3.6. Gel Permeation Chromatography–Size Exclusion Chromatography.** The molecular weight distribution was determined by size exclusion chromatography (GPC-SEC) on a Viscotek TDA 305 gel permeation chromatograph (Malvern) with a refractometer, right angle light scattering, and viscometer detectors on a set of four columns: precolumn Suprema 5  $\mu\text{m}$  8  $\times$  50 S/N 3111265, Suprema 30  $\text{\AA}$  5  $\mu\text{m}$  8  $\times$  300 S/N 3112751, Suprema 1000  $\text{\AA}$  5  $\mu\text{m}$  8  $\times$  300 S/N 3112851 PL, and Aquagel–OH MIXED 8  $\mu\text{m}$  7.5  $\times$  300 S/N 8M-AOHMIX-46-51. The elution was performed with an aqueous solution of 0.01 M monopotassium phosphate, 0.3 M of disodium phosphate, and 0.2 M of sodium chloride (pH 5.3) at flow rate of 1.0 mL/min, and the chromatographic profile was recorded with a refractive index detector (RI-Detector 8110, Bischoff). The samples were prepared at a concentration of 1 mg/mL using the eluent, and the injection volume was 100  $\mu\text{L}$ . The elution times and the RI detector signal were calibrated with a commercial calibration polysaccharide set from Varian containing 10 Pullulan calibrants with narrow polydispersity and Mp (molecular mass at peak maximum) ranging from 180 Da and 708 kDa.

**2.3.7. SDS PAGE.** Two discontinuous mini-sodium (100 mm  $\times$  750 mm  $\times$  1.5 mm) dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared using reagents from Sigma SDS-PAGE kit and casted on a Biorad Mini Protean II system. Freeze-dried collagen was dissolved in deionized water or 1 mM hydrochloric acid (HCl) at 6 mg/mL. The solutions were then mixed 1:1 (v/v) with loading buffer and heated for 4/5 min at 100  $^{\circ}\text{C}$  to completely unfold the

proteins. After being cooled, samples were centrifuged at 10,000 rcf for 1 min to precipitate eventual undissolved material. Aliquots were applied in gels and run at 60 V (for 20 min) and at 140 V until the frontline reached the lower part of the gel. Additionally, the Mini-Protean cell was immersed in cold water to prevent overheating of gel while running at high voltage.

Gels were stained consecutively with different dyes to show protein, glycosaminoglycans (GAGs), and negatively charged glycoproteins profile. Proteins were stained with G-250 coomassie (Biorad) according to Candiano et al.<sup>42</sup> GAGs were detected by staining gels with 0.5% toluidine (Sigma) in 2% acetic acid (Flucka) for 30 min followed by 1 h destaining in 2% acetic acid. Negative glycoproteins were stained with Alcyan blue. For that, the gels were treated with 1% sodium metaperiodate (Sigma) in 3% acetic acid for 1 h, and after several washes in  $\text{dH}_2\text{O}$ , they were immersed in 1% sodium bisulfite (Sigma) for 30 min and washed again thoroughly with  $\text{dH}_2\text{O}$ . Finally, gels were treated with a solution containing 0.5% Alcyan blue (Sigma) in 3% acetic acid for 4 h. Excess staining was removed overnight placing the gels in 7% acetic acid. Between the different staining procedures, the gels were treated with 12.5% trichloroacetic acid for 30 min to remove as much as possible background noise. All the steps described above were performed at RT on an orbital shaker.

**2.3.8. Rheology.** The collagen/gelatin extracts rheological/viscoelastic properties were characterized on a Kinexus Prot Rheometer (Kinexus Prot, MAL1097376, Malvern) fitted with parallel plate geometry with 20 mm diameter (PU20 SR1740 SS) and 1 mm gap. The steady-state flow measurements were performed under controlled-stress conditions where the torque amplitude was imposed by using a logarithmic ramp of shear rate in a range from 0.1 to 100  $\text{s}^{-1}$ . All experiments were performed at a controlled temperature of 37  $^{\circ}\text{C}$ , and results represent the average of at least three measurements.

**2.3.9. Cytotoxicity.** The cytotoxicity of the extracts was assessed based on the bio-reduction of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (cell titer 96 aqueous solution cell proliferation assay, Promega, U.S.A.). MTS assay was performed in accordance with ISO/EN 10993 Part 5 guidelines<sup>43</sup> using an immortalized mouse lung fibroblasts cell line (L929 cell line) purchased from the European Collection of Cell Cultures. Briefly,  $1.5 \times 10^4$  cells/mL were cultured in a 48-well plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Alfagene, U.S.A.) and 1% antibiotic/antimycotic (AA) solution (Gibco, U.K.) for 24 h. At this time, the medium was replaced by the collagen/gelatin solutions, with a concentration of 1:15 m/v (collagen solution:medium culture). Latex was used as positive control for cell death, and cell culture medium was used as a negative control representing the standard condition for cell proliferation. Cell viability was evaluated by assessment of cell metabolic activity using the MTS assay after 72 h in culture. The bio-reduction of MTS yields a water-soluble brown formazan product. This was quantified by UV-spectroscopy, reading the formazan absorbance at 490 nm in a microplate reader (Synergy HT, Bio-Tek Instruments, U.S.A.).

**2.4. Statistical Analysis.** All data values are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using Graph Pad Prism 6.00 software (San Diego, U.S.A.). Statistical significances (\* $p$  < 0.05, \*\* $p$  < 0.01, and

\*\*\* $p < 0.001$ ) were determined using one-way analysis of variance (ANOVA) for an average of three to 12 replicates, followed by the post hoc Tukey's test for all pairwise mean comparisons.

### 3. RESULTS AND DISCUSSION

#### 3.1. Extraction Yield, Protein, and Collagen Content.

In a previous study, we have described for the first time the possibility to extract collagen/gelatin from marine sponges using water acidified with carbon dioxide.<sup>18</sup> Interestingly, carbon dioxide-mediated strategies have already been described for the isolation of bioactive compounds. However, this was achieved using harsher temperature and pressure conditions.<sup>44,45</sup> In this work, we extend our previous study and report the optimization of the extraction conditions. During the extraction process, the pressurization of the mixture (sponge material and distilled water) with carbon dioxide promotes the acidification and consequently the solubilization of the acid soluble collagen/gelatin. According to previous studies,<sup>46,47</sup> for the temperature of 37 °C and pressures of 10, 30, and 50 bar employed in this work, the predicted pH values of the mixture range between 3 and 3.4.

The yield, protein, and collagen content of the extracts obtained under different operating conditions are reported in Table 1.

**Table 1. Extraction Yield (%), Protein, and Collagen Content (%) at Different Operating Conditions<sup>a</sup>**

operating conditions		extraction yield (%)	protein content (%)	collagen content (%)
extraction time (h)	pressure (bar)			
3	10	11.0 (±1.6)	24.2 (±1.8)	22.8 (±5.4)
3	50	9.0 (±1.1)	31.2 (±6.6)	20.5 (±0.5)
13.5	30	12.4 (±0.3)	22.3 (±3.2)	20.5 (±2.5)
24	10	11.4 (±3.0)	31.1 (±9.5)	30.4 (±0.8)
24	50	10.8 (±0.8)	32.5 (±3.3)	28.6 (±9.5)

<sup>a</sup>Values are presented as average ± standard deviation ( $n = 3$ ).

We found no relevant differences in the extraction yield when comparing the different extraction conditions. Nonetheless, batches obtained with prolonged extraction time led to a relevant increase in the collagen/gelatin content. The results obtained are in agreement with our previous work, where

extractions performed at 37 °C and 50 bar for 16 h have produced an extraction yield of 11.6% with a collagen content of 36%.<sup>18</sup> Interestingly, even at very low carbon dioxide pressures, such as 10 bar, the collagen/gelatin recovered is nearly 50% of the collagen content present in this sponge species (about 40 wt %)<sup>19</sup> and represents a major development over other extraction methodologies reported in the literature.<sup>8,19,48</sup> This improvement is certainly related to the fact that our methodology<sup>18,37</sup> is composed by a single extraction step operating at mild conditions and using water as the only solvent, while conventional methods<sup>8,19,48</sup> are based in complex acidic/basic treatments and involving multiple stages for the isolation and precipitation of collagen, each one of them with associated product losses, which surely contributes to the low extraction yields obtained.

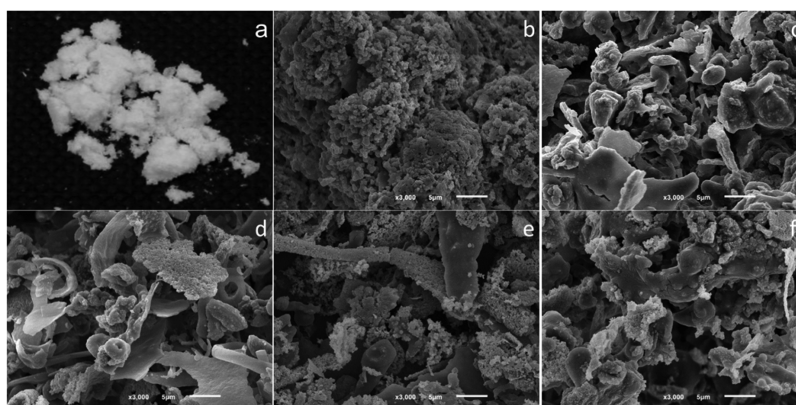
#### 3.2. Morphological Analysis of Collagen/Gelatin Extracts.

The collagen/gelatin extracts, after freeze-drying, are recovered as a free-flowing white powder. As an example, an image of the sample obtained after extraction for 3 h at a pressure of 50 bar is presented in Figure 2a. At a microscale level, the SEM micrographs reveal that the samples present a nodular aspect with two different patterns: smooth or rough. Collagen extracted from *C. reniformis* has been reported to present, mainly, a nonfibrillar form.<sup>8,35</sup> Accordingly, in this work, and similarly to what has been previously described in the literature,<sup>18,19</sup> the typical fibrillar structure of collagen was not observed for any of the extracts.

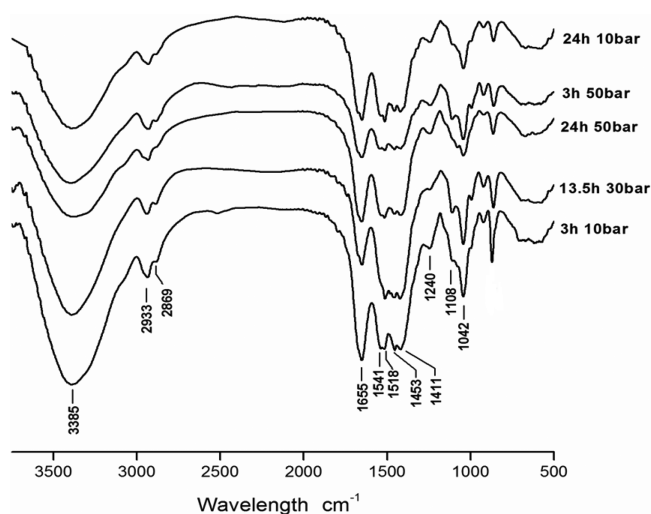
#### 3.3. Chemical Characterization of Extracts. 3.3.1. FTIR.

The extracts were characterized by FTIR, and the different spectra are presented in Figure 3. It is apparent that despite the different extraction conditions, the overall spectra profiles are very similar, suggesting that the extracts present similar structures and chemical compositions.

The spectra contain the representative peaks attributed to amide A, amide B, amide I, amide II, and amide III as previously described for marine collagen/gelatin obtained from other sources.<sup>49–51</sup> The broad band of amide A (3000–3500  $\text{cm}^{-1}$ ), from N–H stretching, is typical for the presence of intermolecular hydrogen bonds, while the amide B bands from the asymmetrical and symmetrical stretching of  $\text{CH}_2$  are, respectively, observed at 2933 and 2869  $\text{cm}^{-1}$ . The peak for amide I, from the stretching vibrations of the carbonyl groups ( $\text{C}=\text{O}$ ) in proteins is observed at 1655  $\text{cm}^{-1}$ . The presence of amide II is observed from the peak at 1541  $\text{cm}^{-1}$  attributed to NH bending vibration coupled with CN stretching, 1453  $\text{cm}^{-1}$



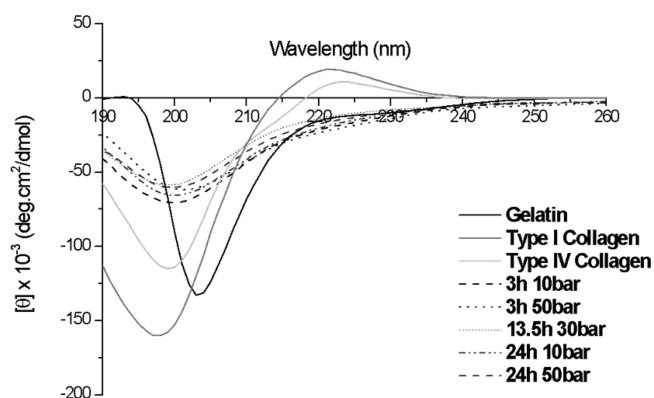
**Figure 2.** Morphological analysis of the collagen/gelatin extracts: (a) powder form. SEM micrographs (magnification of 3000 $\times$ ) of the samples obtained at (b) 3 h, 10 bar, (c) 3 h, 50 bar, (d) 13.5 h, 30 bar, (e) 24 h, 10 bar, and (f) 24 h, 50 bar (scale bar, 5  $\mu\text{m}$ ).



**Figure 3.** FTIR spectra for collagen/gelatin extracts obtained using different extraction conditions.

from  $\text{CH}_2$  bending, and  $1411\text{ cm}^{-1}$  from  $\text{COO}^-$  symmetrical stretching. Finally, the presence of the amide III group was identified from the  $\text{NH}$  bending at  $1240\text{ cm}^{-1}$  (associated with  $\text{CN}$  stretching) and  $\text{C}=\text{O}$  stretching at  $1108\text{ cm}^{-1}$ .

**3.3.2. Circular Dichroism.** Circular dichroism is a valuable spectroscopy tool that can be used to determine the optical isomerism and the secondary structure of macromolecules since it originates specific patterns and peaks according to the presence of  $\alpha$  helix,  $\beta$  sheet/turn, and/or random coils regions. CD spectra of extracts were compared with reference collagen/gelatin materials, namely, bovine type I collagen, human placenta type IV collagen, and bovine-derived gelatin. We can observe in Figure 4 that there are no significant differences in



**Figure 4.** CD spectra of different extracts, type I collagen, type IV collagen, and gelatin.

the CD spectra of the material extracted at different conditions. The extracted materials appear to have a rather complex secondary structure having an evident peak close both to the typical peak of native collagen and the one of gelatin. Indeed, all of our extracts showed the presence of negative peaks in the region of 197–203 nm, which falls close to the 192 nm peak of native collagen and the 203 nm peak of gelatin. On the other hand, none of the samples has a positive peak in the region around 222 nm that is the typical fingerprint of collagen in the  $\alpha$  helix conformation. Whether those results are given by a higher amount of triple helix in comparison with bovine gelatin

and originate from peaks superposition is still unclear. Other studies have reported similar profiles for collagen isolated from other sources using different extraction procedures.<sup>52–54</sup> Accordingly, the acid soluble marine collagen isolated from black drum skin by Ogawa et al.<sup>53</sup> presented a CD spectrum containing only a negative peak around 201 nm.

**3.3.3. Amino Acid Composition of Collagen/Gelatin Extracts.** The results of the amino acid analysis in terms of mol % are presented in Table 2. The results can be compared to the ones previously reported by Swatschek et al.<sup>19</sup> for *C. reniformis* sponge-derived collagen.

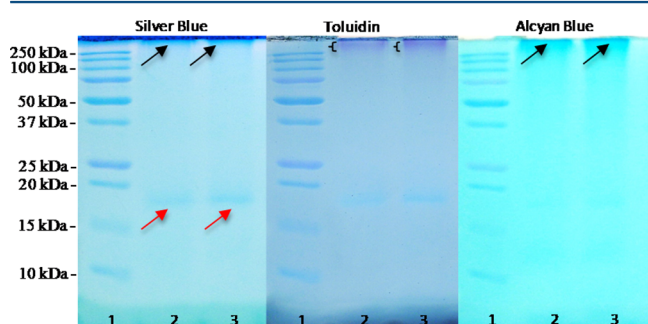
**Table 2.** Amino Acid Composition of Extracts Obtained at Different Times and Pressures and a Previously Reported Measurement on *C. reniformis* Sponge Collagen

time (h)	3	3	13.5	24	24	ref 19
pressure (bar)	10	50	30	10	50	
amino acid	mol %					
Asx	10.8	15.0	16.7	17.3	11.0	9.5
Thr	4.6	4.3	3.9	3.0	4.2	4.7
Ser	6.3	7.7	6.1	5.5	5.6	4.2
Glx	7.5	9.3	6.6	7.4	11.2	10.3
Gly	22.8	16.7	17.4	21.5	31.6	18.9
Ala	8.1	8.4	8.3	8.5	8.6	7.7
Cys	0.7	0.7	1.0	0.9	0.9	0.3
Val	4.7	3.9	4.6	3.6	3.9	5.5
Met	1.2	1.2	1.3	1.4	1.0	1.4
Ile	1.9	2.0	1.9	1.7	2.8	5
Leu	2.8	3.3	4.1	3.5	3.5	6.2
Nleu	12.7	13.4	14.6	14.6	5.5	*
Tyr	1.8	1.1	0.9	0.6	0.8	2.1
Phe	3.6	3.9	4.6	3.4	2.3	3.2
OHlys	6.6	2.4	3.1	2.4	1.6	4.3
His	0.4	0.8	0.7	0.6	0.4	1.1
Lys	1.2	3.0	1.9	1.5	1.4	2.8
Arg	2.4	2.8	2.2	2.7	3.7	4.9
	mol %					
OhPro	39.8	31.7	28.8	38.8	47.3	*
Pro	60.2	68.3	71.2	61.2	52.7	*

Overall the results suggest a good agreement between our samples and the data reported in the literature. Differences may be originated from the inherent variability of natural products and/or coextraction of some contaminants, such as proteins and collagen-like proteins. Furthermore, the method reported by Swatschek et al.<sup>19</sup> does not concern the extraction of the acid soluble fraction of collagen, which may hence be another source of variation for the results obtained. As expected, the amino acids which are present at a higher concentration are glycine followed by alanine.<sup>55</sup> Proline and hydroxyproline are two of the most important amino acids in the composition of collagen as they are responsible for the formation and stabilization of the triple helix.<sup>56</sup> The ratio of OhPro/Pro allows us to compare the results with others reported in the literature. Interestingly, in the present study, all the extracts presented OhPro/Pro ratios between 0.4 and 0.9, obtained at operating conditions of 13.5 h/30 bar and 24 h/50 bar, respectively. Veeruraj et al.<sup>27</sup> report a ratio of 0.98 OhPro/Pro for acid-soluble collagen obtained from eel fish, while Park and co-workers<sup>24</sup> in a comparative study of the physicochemical properties of collagen from different sources refer to OhPro/Pro ratios of 0.71, 0.66, 0.73, and 0.5 for bovine skin, porcine skin, antiotic membrane, and

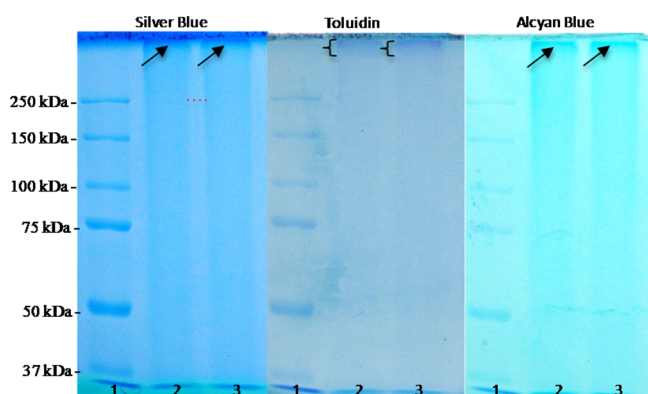
star fish, respectively. This ratio is however highly variable not only between collagen/gelatin isolated from different species but also when different extraction conditions are employed, as it can be confirmed in different works previously reported in the literature.<sup>57,58</sup>

**3.4. SDS-PAGE Analysis.** SDS-PAGE was used as a qualitative tool to check the extracts content. The extracts were easily suspended both in dH<sub>2</sub>O or 1 mM HCl, and the formation of protein precipitates after the denaturation and the following centrifugation step were never observed. Looking at the overall bands stained with the different dyes (Figure 5),



**Figure 5.** SDS-PAGE (15%) stained consecutively with different dyes; 120  $\mu$ g of material, previously resuspended in distilled water (lane 2) or in 1 mM HCl (lane 3) and loading buffer, were loaded in each well. Lane 1 = 5  $\mu$ L of Dual Precision Plus Marker (Biorad). Black arrow = putative collagen macromolecules; red arrow = possible contaminant protein; brace = GAGs.

despite the rather high amount loaded in each well (120  $\mu$ g), the presence of significant bands was not observed, thus suggesting the possible presence of other compounds that are not sensitive to the staining procedures employed. In terms of the protein profile, it is possible to observe the presence of a weak band around 18 kDa and the typical, although weak, pattern of high molecular weight gelatin in the upper part of the gels. Interestingly, the gelatin pattern is significantly more evident using Alcian blue staining suggesting that the sponge collagen/gelatin obtained with our method is highly glycosylated. The results presented in Figure 6, where the high molecular weight region was better resolved, indicate that the



**Figure 6.** SDS-PAGE (7.5%) stained consecutively with different dyes; 120  $\mu$ g of material, previously resuspended in distilled water (lane 2) or in 1 mM HCl (lane 3) and loading buffer, were loaded in each well. Lane 1 = 5  $\mu$ L of Dual Precision Plus Marker (Biorad). Black arrow = putative collagen macromolecules; brace = GAGs; red broken line = collagen MW as calculated from GP-SEC.

glycosylated gelatin, revealed by G-250 and Alcian blue staining, is only partially matching the pattern of the sulfated GAGs detected with Toluidin. Glycosylated gelatin peptides, obtained from hydrolyzed fish skin gelatin and functionalized via transglutaminase enzyme, have demonstrated interesting bioactivity properties, namely, antioxidant and antimicrobial activity.<sup>59</sup> To summarize the results of the SDS-PAGE analysis, we can conclude that our data suggest the presence of a natural-derived glycosylated gelatin. Moreover, we found a possible contaminant band around 18 kDa and the presence of a high molecular weight sulfated GAGs.

### 3.5. Molecular Weight Determination by GPC-SEC.

The number-average molecular weight ( $M_n$ ) and weight-average molecular weight ( $M_w$ ) of the extracted material were determined by GPC-SEC and compared with human collagen type IV. The results obtained are presented in Table 3 and

**Table 3.** Average Molecular Number ( $M_n$ ) and Average Molecular Weight ( $M_w$ ) of Different Extracts and Human Type IV Collagen<sup>a</sup>

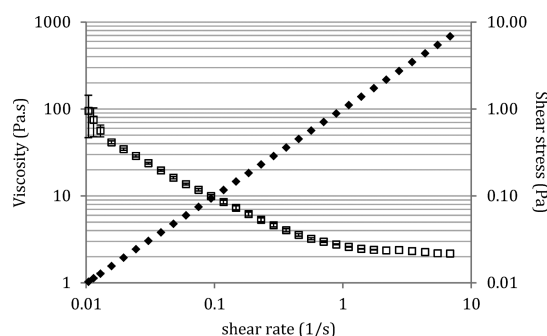
sample	$M_n$	$M_w$
3 h 10 bar	104 ( $\pm 15$ )	457 ( $\pm 34$ )
3 h 50 bar	88 ( $\pm 9$ )	359 ( $\pm 23$ )
24 h 10 bar	89 ( $\pm 13$ )	385 ( $\pm 16$ )
24 h 50 bar	97 ( $\pm 12$ )	366 ( $\pm 20$ )
human collagen type IV	158 ( $\pm 5$ )	325 ( $\pm 8$ )

<sup>a</sup>Values are expressed in kDa; standard deviation are represented in the brackets.

show that the extraction conditions did not impact the molecular weight of the collagen/gelatin, with the differences observed being within the experimental error. The GPC-SEC results are in agreement with the values obtained by SDS-PAGE, whose technique is limited to the analysis of  $M_w$  below 250 kDa. From our previous work,<sup>18</sup> we have reported values of  $M_n$  and  $M_w$  of 60.57 and 208.92 kDa, respectively, for collagen/gelatin extracted from *C. reniformis* for 16 h at 50 bar and 37 °C. The molecular weight of the extracts obtained in this work is slight higher than on our previous report and is attributed to the natural variability and use of sponges that were collected at different moments.

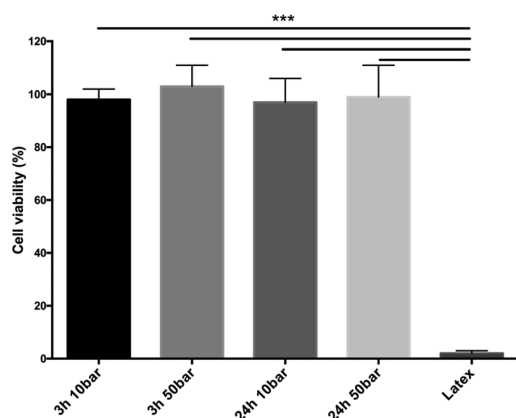
**3.6. Rheological Behavior of Collagen/Gelatin Extracts.** The viscosity of the collagen/gelatin extracts is an important parameter not only in terms of characterization of the materials but also toward application development. Figure 7 presents the behavior viscosity of a 0.5 wt % aqueous solution of the extract obtained after 24 h at 50 bar as a function of shear rate.

The sample exhibits a typical shear thinning behavior for low shear rates, characterized by a decrease in the viscosity with increasing shear rate and a linear relationship between the shear rate and shear stress. A comparison of the viscosity values obtained with the ones reported in the literature for other samples of marine origin collagen is not straightforward. The analysis of the rheological behavior reported was carried out using different geometries and different parameters, which make the comparison difficult. Nonetheless, the extracts obtained present interesting rheological properties to be processed further. Particularly, the extracts could be used for the synthesis of hydrogels for cosmetic and biomedical applications.



**Figure 7.** Flow curve of collagen/gelatin solution (0.5 wt %) extracted from *C. reniformis* (open symbols represent viscosity; closed symbols represent shear stress). Bars indicate standard deviation.

**3.7. Cytotoxicity.** The possible cytotoxicity of the obtained materials was evaluated in accordance with the protocol described in ISO/EN 10.993 guidelines.<sup>43</sup> The viability of the cells cultured in the presence of the extracts was compared to that of cells cultured in DMEM supplemented with 10% FBS and 1% AA. The results obtained (Figure 8) show that for all



**Figure 8.** MTS cytotoxicity assay of the collagen/gelatin extracts obtained: Cell viability (%) after 72 h culture of L929 cells in contact with collagen/gelatin extracts. Latex was used as positive control. Values have been normalized to the values obtained from the negative control (L929 cells cultured in standard DMEM media); \*\*\* $p < 0.001$ .

extracts, after a 72 h culture, the cells present comparable viability to those cultured under standard conditions. Indeed, we did not find any statically significant differences between the negative control and the cells exposed to the extracts. The results presented clearly show that the extracts obtained with our methodology are noncytotoxic.

#### 4. CONCLUSIONS

The extraction of marine origin collagen/gelatin was successfully achieved through a process based on carbon dioxide acidified water. In fact, the isolation procedure was effective even at the mildest operating conditions, which allowed reducing extraction time and therefore processing costs. Additionally, it was confirmed that the proposed technology resulted in a 30% improvement on extraction yield when compared to traditional acid/enzymatic procedures, resulting in the isolation of more than 50% of the collagen content of the marine sponge studied in this work. All the operating conditions produced a mixture of glycosylated collagen/gelatin

with similar properties in terms of morphology, chemical composition, molecular weight, and cytotoxicity. Therefore, the operating parameters have limited impact on the collagen/gelatin quality, hence, confirming the versatility and robustness of the proposed organic solvent-free methodology. For this reason, attending to the physicochemical properties of the extracts and the processing conditions, the optimal extraction conditions are 10 bar and 3 h of extraction.

Overall, the results obtained herein, not only provide important data on the obtained materials and extraction process parameters, opening the possibility to scale up this simple, cheap, and environmentally sustainable process to an industrial scale but also suggest that marine sponge-derived collagen/gelatin is a promising material for biomedical, cosmetic, and pharmaceutical applications.

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##### Notes

The authors declare no competing financial interest.

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