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Water and Carbon Dioxide: Green Solvents for the Extraction of Collagen/Gelatin from Marine Sponges

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ABSTRACT: Marine sponges are extremely rich in natural products and are considered a promising biological resource. The major objective of this work is to couple a green extraction process with a natural origin raw material to obtain sponge origin collagen/gelatin for biomedical applications. Marine sponge collagen has unique physicochemical properties, but its application is hindered by the lack of availability due to inefficient extraction methodologies. Traditional extraction methods are time consuming as they involve several operating steps and large amounts of solvents. In this work, we propose a new extraction methodology under mild operating conditions in which water is acidified with carbon dioxide (CO_2) to promote the extraction of collagen/gelatin from different marine sponge species. An extraction yield of approximately 50% of collagen/gelatin was achieved. The results of Fourier transformed infrared spectroscopy (FTIR), circular dichroism (CD), and differential scanning calorimetry (DSC) spectra suggest a mixture of collagen/gelatin with high purity, and the analysis of the amino acid composition has shown similarities with collagen from other marine sources. Additionally, *in vitro* cytotoxicity studies did not demonstrate any toxicity effects for three of the extracts.

KEYWORDS: *Green solvents, Extraction, Collagen, Gelatin, Marine sponges, Carbon dioxide, Water, Natural products*

INTRODUCTION

Sustainable development relies not only on the development of novel processes but also on the use of alternative sources of raw materials, which decrease dependence from fossil fuel resources. In this sense, the sea provides a plentiful resource of potential new products for society including biomaterials.[1,2]

In the past decade, comprehensive manuscripts[3] indicate that sponges (*Porifera*) are the most promising avenue for blue biotechnology, and their leading role within marine biotechnology stems from their long evolutionary history. These sponges are particularly rich in collagen, which is one of the most important and abundant proteins in the human body, with 20 genetically distinct forms known today. The current industrial demand for collagen is up to 326.000 tons per year[4] for different fields of application, including alimentary, cosmetics, pharmaceutical, and biomedical.[5−7,2,8]

The advantages of the use of collagen include the fact that it is highly abundant in nature, nonantigenic, biodegradable, nontoxic, and biocompatible.[9] However, there are some constrains hindering the development of new products. Nowadays, 98% of the collagen is of mammalian origin, for instance, from calf skin and bone. These carry a high risk of disease transmission such as bovine spongiform encephalopathy, as well as social and/or religious constraints.[10,11] Both Judaism and Islam forbid the consumption of porcine-derived products, while Hindus do not consume bovine-related products.

Aiming to overcome these drawbacks, alternative sources have been suggested, such as collagen from marine sources, including jellyfish,[7] fish scales[12] and skin,[13] cuttlefish skin,[14] and starfish.[15] Particularly, sponge collagen has unique physicochemical properties[16−18] and is a promising resource, but sponge collagen is not available in large quantities because of the lack of efficient extraction methodologies.

Traditionally, marine collagen/gelatin can be isolated from marine resources after acid, base, or enzymatic treatments. For sponges, a treatment with a complex Tris-HCl buffer solution (pH 9.5, 10 mM EDTA, 8 M urea, and 100 mM 2-mercaptoethanol) is commonly proposed,[1,19] but such methods are generally time consuming as they involve several operating steps and have low selectivity and low extraction yields.[20] Furthermore, they require the use of large amounts of solvents.[21] Environmental concerns and strict legislation on the use of volatile organic solvents are forcing chemical industries to move toward the application of alternative processing methodologies, which comply with the green chemistry philosophy. Chemical industries have been moving

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toward the development of innovative processes as the awareness that sustainable development is becoming mandatory and essential for their competitiveness.[22]

The major objective of this work is hereafter the exploitation of a new extraction process, using water and carbon dioxide as solvents with a natural origin raw material to obtain sponge origin collagen/gelatin. To the best of our knowledge, it is the first time that this approach is reported in the literature. We propose, hereafter, a new extraction methodology for the extraction of acid soluble collagen/gelatin, which normally denatures at high temperatures (human collagens)[23,24] under mild operating conditions, in which water is acidified with carbon dioxide to promote the extraction of collagen/gelatin from different marine sponge species.

EXPERIMENTAL SECTION

Materials. Sponge samples of the species *Thymosea* sp. were collected in the Atlantic Ocean in the Azores and were kindly provided by Joana Xavier (Azores University, Portugal). Samples of *Chondrila nuculla* were collected in the Mediterranean Sea. *Chondrila nuculla* from Alassio was grown in aquaculture, while *Chondrila nuculla* from Portofino is the wild type. Samples of *Chondrosia reniformis* were collect on the Israeli coast. The species were kindly provided frozen or freeze-dried by Ronald Osinga (Porifarma, The Netherlands), Micha Ilan (Tel Aviv University, Israel), and Antonio Sarà and Martina Millanese (Studio Associato GAIA, Italy).

All further reagents were high quality reagent grade chemicals for laboratory use (chemicals meet the specifications of the American Chemical Society, ACS) and were used as received.

Collagen/Gelatin Extraction. Marine sponges (*Thymosea* sp. (TIM), *Chondrosia reniformis* (CR), *Chondrila nuculla*, Alassio (ConAL), and *Chondrila nuculla*, Portofino (ConPF)) were ground in small pieces. Five grams of sponge material was weighed and washed with distilled water overnight to remove salt and any contaminants present and were then were lyophilized. Afterward, the samples were placed in a high pressure vessel (30 cm^3), and 10 mL of distilled water was added. The vessel was heated to 37 °C. The system was pressurized with carbon dioxide to 50 bar. The extraction was performed in batch mode for 16 h. After this time, the high pressure vessel was rapidly depressurized. The extract obtained was filtered with a 0.45 μm filter and frozen. Collagen/gelatin powder was obtained after freeze-drying of the extracts.

Characterization of Marine Sponge Collagen/Gelatin. *Extraction Yield and Collagen/Gelatin Quantification.* The yield of extraction was quantified as the ratio of dry extract obtained per weight of initial dry sample. The quantification of collagen/gelatin on the different extracts was performed using the Sircol assay kit. The Sircol assay is a dye-binding method specific for the analysis of collagen/gelatin. The determination of collagen/gelatin was carried out according to the protocol described by the manufacturer (Sircol, Soluble Collagen Asay, Biocolor, Life Science Assays, U.K.).

Scanning Electron Microscopy. The powder obtained from the different extractions was analyzed by a Nova NanoSEM 200 scanning electron microscope (SEM). The samples were fixed by mutual conductive adhesive tape on aluminum stubs and covered with gold using a sputter coater prior to microscopic analysis.

Fourier Transform Infrared Spectroscopy. Fourier transform infrared spectroscopy (FTIR) spectra of the extracts were obtained with a Shimadzu-IR Prestige 21 spectrometer in the spectral region of 4000−800 cm^{-1} with resolution of 2 cm^{-1} as the average of 32 individual scans. The samples were analyzed in KBr pellets.

Circular Dichroism. Circular dichroism (CD) measurements of the extracted material were performed using a Jasco Model J-865 spectropolarimeter (Jasco, U.K.) using a quartz cylindrical cuvette (Hellma, Germany) with a path length of 0.1 mm. The cuvette was filled with 150 mL of sample (0.01 g/mL) for each measurement. CD spectra were obtained by continuous wavelength scans (average of three scans) from 180 to 260 nm at a scan-rate of 50 nm/min. The samples were equilibrated for 1 h at room temperature before the CD spectra were acquired.

Differential Scanning Calorimetry. Differential scanning calorimetry experiments were carried out using a DSC Q100 equipment (TA Instruments, U.S.A.). The experiments were conducted under a nitrogen atmosphere on samples (5−10 mg) packed in aluminum pans. The samples were heated at a constant heating rate of 5 °C/min from 0 to 90 °C, followed by an isothermal period at 90 °C. The samples were then cooled at the same rate to the initial temperature.

Amino Acid Analysis. The amino acid content was determined by quantitative amino acid analysis using a Biochrom 30 (Biochrom Ltd., Cambridge, U.K.).[22] Briefly, the samples were hydrolyzed and separated by an ion exchange column. After postcolumn derivatization by ninhydrin, the samples were analyzed at two wavelengths: 440 and 570 nm. An internal standard of norleucine was used to determine the concentrations of amino acids in the sample.

Isoeletric Point Determination. The isoelectric point of the samples was determined following a titration protocol described in the literature.[24] For each extract, 10 mg of collagen/gelatin was dissolved in 6 mL of Milli-Q water. Each sample was titrated with a solution of 0.02N NaOH and with a solution of 0.02 N HCl. The pH was registered (pH meter, 3510 Jenway), and the resulting pH values were plotted versus the amount of NaOH and HCl. The titration range was from pH 2 to 12. All experiments were performed at room temperature.

Gel Permeation Chromatography−Size Exclusion Chromatography. The determination of the molecular weight of the samples was carried out by size exclusion chromatography (GPC-SEC). Briefly, 1 mg of extract was dissolved in 1 mL of an aqueous solution of sodium nitrate 0.2 M containing 0.02% of sodium azide. The solutions were filtered through a 0.22 μm membrane and analyzed on a size exclusion chromatograph (Viscotek TDA 305) equipped with three detectors: light scattering, refractive index, and viscometer. Elution was performed at 30 °C using a flow rate of 1 mL/min of the following eluents: aqueous solution of sodium nitrate 0.2 M containing 0.02% sodium azide. The column set was composed by a guard precolumn Aq. Guard col 50 mm × 6.0 mm (Viscotek) and a PLaquagel−OH mixed 8 μm (300 mm × 7.5 mm, Polymer Laboratories).

In Vitro Cytotoxicity. The cytotoxicity of the extracts was assessed on bioreduction of a novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H-tetrazolium (MTS) (cell titer 96 aqueous solution cell proliferation assay, Promega, U.S.A.). MTS assay was measured in accordance with ISO/EN 10993 Part 5 guidelines[27] using an immortalized mouse lung fibroblasts cell line (L929 cell line) purchased from the European Collection of Cell Cultures. Briefly, 1.5 × 10^4 cells/mL were cultured in a 48 well plate in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Alfagene, U.S.A.) and 1% antibiotic/antimycotic solution (Gibco, U.K.) for 24 h. At this time, 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 ideal situation for cell proliferation. Cell viability was evaluated by assessment of cell metabolic activity using the MTS assay after 72 h in culture. The bioreduction 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.).

Statistical Analysis. Statistical analysis of the data was performed using IBM SPSS Statistics Version 20. Normality was verified by the Shapiro−Wilk test. Normal distributed data were then analyzed using one-way ANOVA with a Bonferroni's post-test. When normality was not observed, a nonparametric test, namely, Kruskall−Wallis test was performed. Differences between the groups with $p < 0.05$ were considered to be statistically significant.

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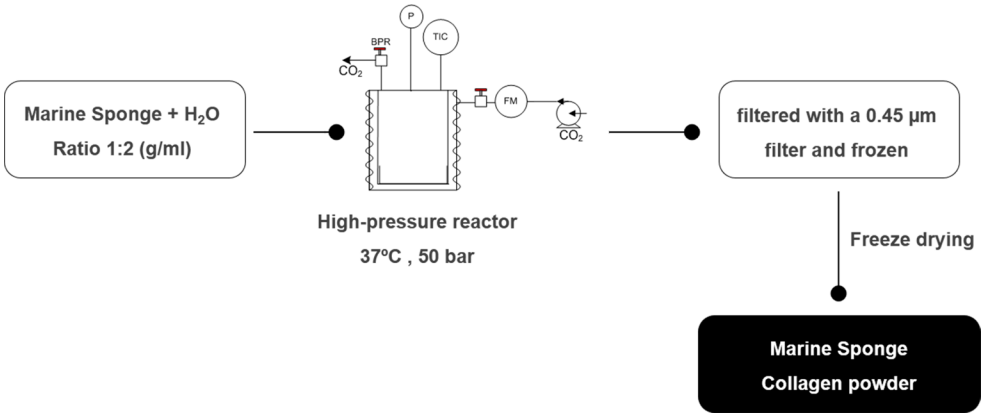

Figure 1. Schematic representation of the extraction procedure for marine sponge collagen/gelatin.

Table 1. Extraction Yield and Collagen/Gelatin Content on the Different Extracts

source	gram extract/100 g sample[a]	collagen/gelatin content (%)[b]
Thymosea sp.	16.6	82
Chondrosia reniformis	11.6	36
Chondrilla nuculla (Alassio)	9.0	54
Chondrilla nuculla (Portofino)	17.3	82

[a]Values are indicated as a percentage of gram of collagen/gelatin per gram of wet tissue. [b]Assessed by Sircol collagen quantification kit.

RESULTS AND DISCUSSION

Marine origin collagen/gelatin has been reported to be a great promise, but so far, there has been a gap between the scientific interest and the wide industrial application of this source of collagen/gelatin

Extraction. Different papers in the literature report the possibility to solubilize a fraction of collagen from different sources in acidic solutions, most of which are dilute acetic acid solutions, but these methods in marine sponges report low yield around 2%.[25] The procedures, however, do not lead to high yields of extraction, and the process requires several post-operation steps for the purification of the products. In this work, we propose the use of water acidified with carbon dioxide at 50 bar to extract collagen/gelatin from the three species of marine sponges mentioned. In Figure 1, a schematic representation of the extraction procedure is represented.

Extraction Yield and Collagen/Gelatin Content. The quantification of the extraction yield was calculated as the mass of extract obtained per gram of sponge extracted. The extraction yields are presented in Table 1.

In our study, results demonstrate that the lowest extraction yields were obtained for the *Chondrilla nuculla* (Alassio) sponge, and the highest yield was obtained from *Chondrilla nuculla* (Portofino). The results obtained are significantly higher than the ones presented for example by Addad and co-workers, who report the extraction of collagen from different organs of jellyfish.[7] A careful comparison with the data from the literature however has to be carried out. In most cases, it is not clear whether the authors refer to the concentration of collagen in the extracts or in fact the amount of extract per weight of raw material. Regarding the extraction of the acid-soluble fraction from marine sponges from dilute acetic acid solutions, the proposed technology represents an improvement of nearly 30% in extraction yield.[1,4,20] Besides the determination of the extraction yield, it is also important the quantification of the collagen/gelatin present in the extracts. The quantification of the amount of collagen/gelatin present in each extract was performed using a specific detection kit (Sircol assay kit), which revealed that the extracts recovered from *Thymosea* sp. and *Chondrilla nuculla* (Portofino) present a considerable high value of collagen/gelatin near 82%. Considering that the collagen/gelatin content of these species may vary from 30 to 40 wt %,[1] the extraction performed represents hereafter an extraction yield of approximately 50% of the collagen/gelatin present in the sponge. Hence, the proposed technology for the extraction of marine sponges could be a valuable source of collagen/gelatin for industrial exploitation. Such industrial exploitation is also dependent on the sustainability of the raw material, which in the case of marine sponges can be addressed by aquaculture. The obtained results indicated that the amount of collagen/gelatin extracted from the wild sponge (ConPF) is about three times the amount extracted from the cultured one (ConAL). However, the current study did not account for the time at which marine sponges were collected, and the sustainability can be only correctly assessed with a study over time (covering different seasons) and embracing multiple sponge generations.

Morphological Analysis of the Extracts. The morphology of the obtained powder was observed by scanning electron microscopy, and the respective images are presented in Figure 2. From the images presented, it is noticeable that the typical fibrillar structure of collagen/gelatin was not observed in any of the samples obtained. However, nodular collagen has been reported in the literature in a work by Heinemann et al[19] and Pozzolini et al,[18] in which collagen was extracted from *Chondrosia reniformis*.

Chemical Characterization of Extracts. *Infrared Spectroscopy.* The representative FTIR spectra of the four marine sponges extracted (TIM, CR, ConAL, and ConPF) in the 4000−400 cm^{-1} wavenumber regions are presented in Figure 3. The main bands are labeled in the figure and are described in Table 2. The FTIR spectra of marine sponge extracts are relatively complex and comprise several bands corresponding not only to collagen chemical groups but also probably to other

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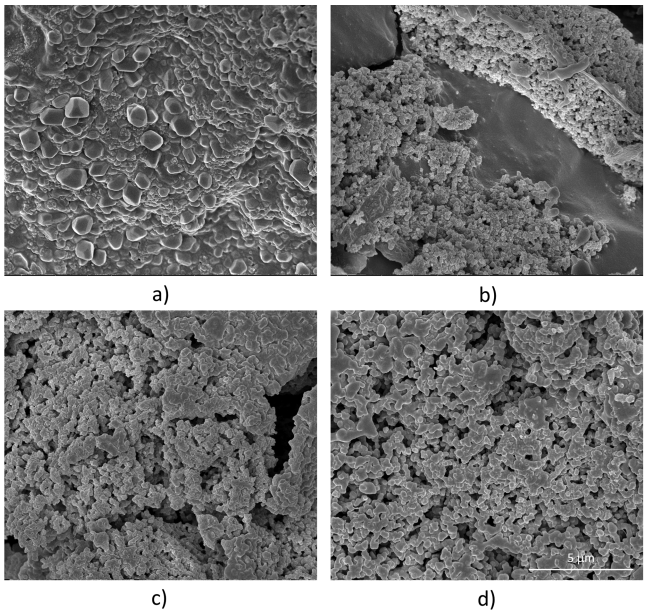

Figure 2. SEM images of the marine sponge collagen/gelatin extracts of (a) TIM, (b) CR, (c) ConAL, and (d) ConPF. Bar = 5 μm.

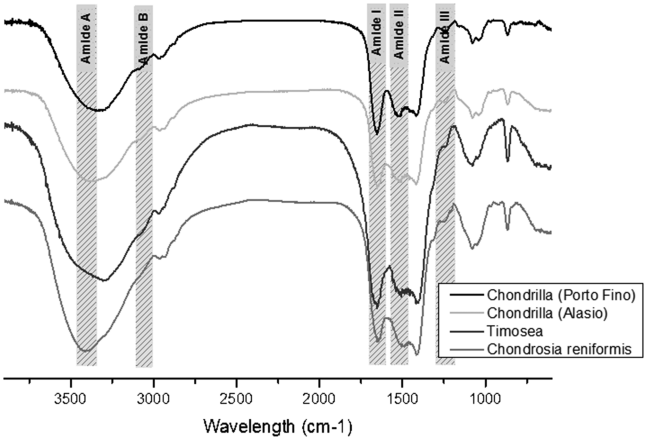

Figure 3. Fourier transform infrared spectra of of the marine sponge collagen/gelatin extracts.

proteins, lipids carbohydrates, and nucleic acids, as shown in Figure 3.

Fourier transform infrared spectra of collagen/gelatin extracted from *Thymosia* sp., *Chondrosia reniformis*, and *Chondrilla nuculla* (Alasio and Portofino) marine sponges had great similarity to each other, which suggested their chemical compositions were relatively similar. The FTIR spectra of different collagen/gelatin contained several bands representing amide A, amide B, amide I, amide II, and amide III, which were similar to those collagen/gelatin from other marine sources reported in the literature.[26,28] The amide A band is generally associated with the N−H stretching vibration and shows the existence of hydrogen bonds[28] and in our extracts was found at 3425 cm^{-1}. The amide B band was observed at 3283, 3277, 3294, and 3294 cm^{-1} for TIM, CR, ConAL, and ConPF, respectively, which represents the asymmetrical stretch of CH_3.[4]

The amide I band, associated with stretching vibrations of the carbonyl groups (C═O bond),[29] was observed at 1653, 1644, 1647, and 1653 cm^{-1} for TIM, CR, ConAL, and ConPF, respectively. The amide II band appeared at 1522 cm^{-1} for all extracts and is a result of the N−H bending vibration coupled with C−N stretching vibration.[28] Amide III was observed at 1240, 1249, 1240, and 1242 cm^{-1} for TIM, CR, ConAL, and ConPF, respectively. Furthermore, the presence of the amide III (C−H stretching) observed by IR absorption suggests the helical structure of the collagen/gelatin extracted.[4,28] The marine collagen/gelatin extracted with CO_2 acidic water showed a secondary structure.

Circular Dichroism. The circular dichroism spectra (CD) of the four marine sponges extracted (TIM, CR, ConAL, and ConPF) in the wavelength range of 180−260 nm are shown in Figure 4. CD spectra of the collagen/gelatin controls (collagen

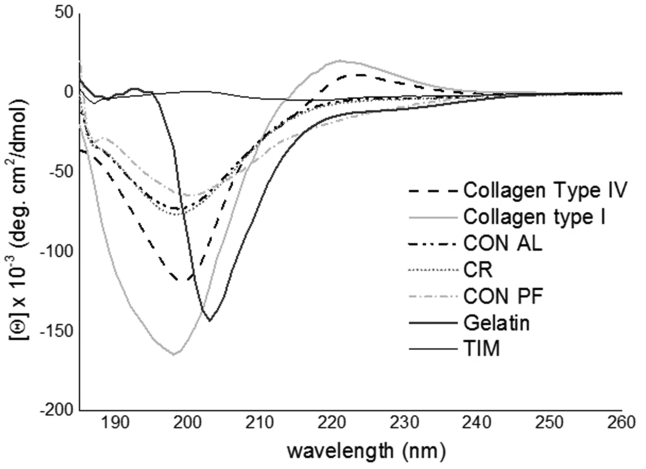

Figure 4. CD spectra of the marine sponge collagen/gelatin extracts measured at room temperature.

Table 2. General Peak Assignments of FTIR Spectra Consist of Marine Sponge Collagen/Gelatin Extracts

ConPF	ConAL	TIM	CR	region peak assignments
3425	3425	–	3425	amide A: N−H stretching (in proteins)
3294	3294	3283	3277	N−H stretching when involved in hydrogen bonding
3082	3085	3076	3080	amide B: CH_3 asymmetric stretching
2934	2931	2929	2925	amide B: CH_2 asymmetric stretching
2870	2873	2873	2873	CH_3 asymmetric bending
2835	–	–	2853	CH_3 symmetric stretching
1653	1647	1653	1644	amide I: C═O stretching (in proteins)
1522	1522	1522	1522	amide II: N−H bending
1242	1240	1240	1249	amide III: C−H stretching
1078	1075	4080	1080	PO_2 symmetric stretching
1034	1034	1046	1040	C−O−H from carbohydrates

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type I from bovine and collagen type IV from human placenta) present two peaks, a positive peak at 221 nm and a negative peak at 192 nm. This is a characteristic profile of the collagen triple helix.[26] On the other hand, gelatin, used as a control, and the extracted material from ConAL, ConPF, and CR have positive peaks (220 nm) that are absent, suggesting the existence of random coils.[25] The negative peak of the extracted materials is present close to 192 nm, around the value of the negative peak for collagen type IV. A similar profile was obtained by Zhang et al.[25] in a study with collagen from bovine-limed split wastes, called collagen hydrolysate. In the case of TIM, the CD spectra is not conclusive due the presence of other peaks that may suggest the presence of others compounds.

Thermal Transition Temperature. The denaturation temperature of collagen/gelatin was determined by differential scanning calorimetry. The marine sponge collagen/gelatin extracted had different denaturation temperatures (T_d) depending on the source (Figure 5 and Table 3). The T_d of TIM

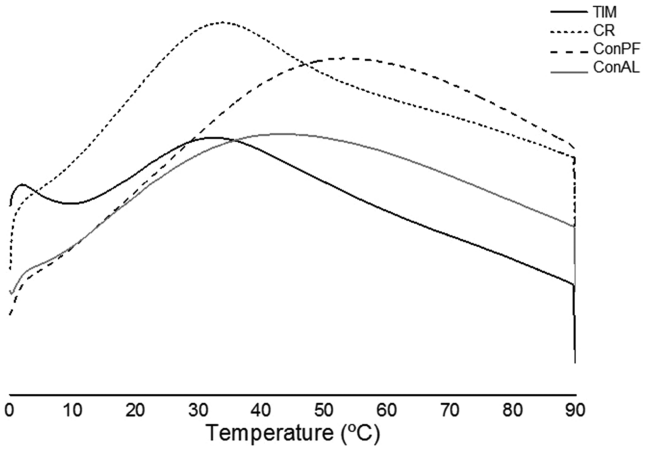

Figure 5. DSC thermogram of marine sponge collagen/gelatin extracts.

Table 3. Denaturation Temperature of the Different Sponge Extracts

source	dehydrated samples, T (°C)
Thymosea sp.[a]	31.02
Chondrosia reniformis[a]	30.48
Chondrilla nuculla (Alasio)[a]	38.93
Chondrilla nuculla (Portofino)[a]	50.05

[a]Onset point determination.

(31.02 °C) and CR (30.48 °C) are similar, but the T_d values of ConPF (50.05 °C) and ConAL (38.93 °C) show higher values. Interestingly, comparing the T_d of *Chondrilla nuculla*, the species cultivated in aquaculture (Alassio) to the one harvested in the wild (Portofino) present different values. The value observed for ConPF is the higher reported in the literature as far as we know. The TIM and CR denaturation temperatures are near collagen type IV from human placenta (28.5 °C). The T_d of the collagen/gelatin extracted from the marine sponges, depending on the source, was close to the other marine sources like muscle of carp (32.50 °C) and Japanese sea bass (30.00 °C) and also calf skin collagen (40.00 °C) as reported in the literature. Nevertheless, this comparison should be carefully made because the scan-rate used in the calorimetric analysis is susceptible of influencing the temperature peak.[29] The results suggest that intramolecular hydrogen bonds stabilizing the triple helix structure of collagen might be disrupted to some levels, mainly due to the repulsion of collagen molecule in acidic solution. This phenomenon was observed by different authors in extraction of collagen in various marine sources.[4,7,26,30]

Amino Acid Analysis. The imino acid content of proline and hydroxyproline has been correlated with thermal denaturation temperature of marine origin collagen.[26,31,35] The amino acid composition for the four marine sponges collagen/gelatin extracted was thus determined. The amino acid composition of collagen/gelatin from TIM, CR, ConAl, and ConPF had similar amino acid profiles between them and are presented in Table 4.

Table 4. Amino Acid Composition of Different Sponge Extracts (residues/1000 residues)

amino acid	TIM	CR	ConAL	ConPF
Ala	101	118	118	113
Thr	78	58	61	69
Ser	61	59	33	30
Glu	117	60	89	85
Gly	149	235	239	237
Asp	69	66	48	43
Cys	5	3	3	4
Val	29	40	25	26
Met	4	6	3	2
Ile	11	16	9	10
Leu	26	22	20	22
Tyr	3	4	7	7
Phe	17	15	11	9
Lys	30	19	18	16
His	2	6	4	5
Lys	22	27	24	21
Arg	36	33	39	33
Ohpro	81	60	83	93
Pro	160	151	166	175
imino acid	241	211	249	268

Comparing these results with other collagen/gelatin compositions from marine sources,[4,6,7,26,32,36] we observed an analogous behavior. Collagen is composed by a triple helical sequence of amino acid repeat, (Gly-Pro-Hyp)*n*,[26] the most abundant of which is glycine (Gly). This was observed in all extracts where the amounts of 149, 235, 239, and 237 total amino acid residues per 1000 residues were determined. Considering the imino acid content (Proline (Pro) + hydroxiproline (Hyp)), the results show values around ∼240 total amino acid residues per 1000 residues, which was slightly higher than in the case of others marine sources[1,4,8,26,28] and can also justify the higher values of denaturation temperatures. The imino acid contributes to the formation and stabilization of cross-links in the collagen.[35] The alterations in imino acid content are associated with the different living environments, particularly the temperature of the habitat.[28]

Isoeletric Point Determination. Isoelectric point is an important parameter of proteins because it is related with amino acid content.[25] All the samples present a similar behavior, showing an isoelectric point around pH 8, in the basic range due to the acidic conditions of the extraction, because this maintained intact the amide residues.[25] This observation has been reported by Swatschek for collagen

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extracted from *Chondrosia reniformis*, who indicates an isoelectric range between 6.5 and 8.5.[1] The titration curves show a flattening area at pH 6 that eventually levels off at pH 2. The behavior of the extracts present a second critical point noted at pH 5, common to the four samples of collagen/gelatin. Highberger et al.[3] in 1939 reported the existence of two different critical points in the pH mobility of collagen and gelatin, corresponding to a pH of 4.7 and 7.7, respectively. Our observations are in agreement with the finding herein reported and may suggest the presence of a mixture between collagen and gelatin in the extracts.

Molecular Weight Determination. The molecular weight of the extracts was determined by size exclusion chromatography (GPC-SEC). The molecular weight of a substance, particularly a polymer, is a key chemical characteristic that can dramatically influence the material mechanical performance, particularly the viscosity and rheological behavior.[7] In this sense, size exclusion chromatography was used to determine the number-average molecular weight (M_n) and weight-average molecular weight (M_w) for the four different collagens extracted (Table 5).

Table 5. M_n and M_w of Different Sponge Extracts[a]

sample	M_n (kDa)	M_w (kDa)
Thymosea sp.	48.98 (±10.63)	155.40 (±20.42)
Chondrosia reniformis	60.57 (±15.73)	208.92 (±14.96)
Chondrilla nuculla (Portofino)	34.29 (±6.52)	112.77 (±12.60)
Chondrilla nuculla (Alassio)	42.02 (±9.76)	110.59 (±8.75)

[a]Standard deviations are in parentheses.

Higher M_n and M_w were obtained in collagen/gelatin extracted from *Chondrosia reniformis* with 60.57 and 208.92 kDa, respectively. The molecular weight of the extracts is similar to the one of collagen/gelatin extracted from other marine sources, such as eel fish skin (~95 kDa/~210 kDa),[4] skin of strip catfish (~26 kDa/~95 kDa),[11] and calf skin collagen and skin of largefin longbarbel catsfish (~116 kDa/~200 kDa).[32] Zhang et al.[25] reported a molecular weight of ~50 kDa for collagen hydrolysate derived from bovine-limed split wastes. The values of the molecular weight reported in the literature are very wide. This is mostly due to the fact that the extraction processes have a high impact on the final extracts. The result of the molecular weight of collagen/gelatin extracted from marine sponges by CO_2 acidic water at room temperature demonstrated that they could be used as appropriate materials for biomaterial applications.

***In Vitro* Cytotoxicity Studies.** The cytotoxicities of the four different collagen/gelatin extracts obtained were evaluated in accordance with the protocol described in ISO/EN 10-993.[27] The viability of the cells cultured in a tissue culture plate in the presence of the collagen/gelatin was determined as a function of the cells cultured in the DMEM culture medium. Figure 6 presents the cell viability after 72 h in contact with the material. The obtained results were compared to cell growth on the tissue culture plate in the absence of sponge extract, as positive control, and latex, which was used as negative control.

The results show that collagen/gelatin extracted from *Chondrosia reniformis* and *Chondrilla nuculla* (Alassio and Portofino) do not compromise the metabolic activity of the cells. On the contrary, cell viability is higher than 100%, which indicates an increase in the metabolic activity of the cells in the presence of this material. The only exception was the extract from *Thymosia* sp. Even though 82% of the obtained extract

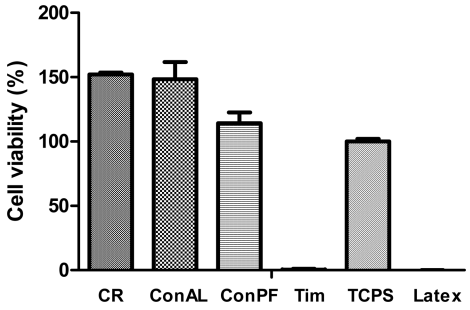

Figure 6. Cytotoxicity screening of the different extracts from marine sponges. Latex extract and standard culture medium (TCPS) were used as positive and negative controls, respectively.

was quantified to be collagen/gelatin, there may be some cytotoxic compounds in the extract responsible for the results obtained. In fact, marine sponges are widely recognized as a marine source of cytotoxic compounds with potential antitumoral interest.[33,34,37,38] Further purification steps in this case would be required to overcome the toxicity observed.

CONCLUSIONS

The extraction of sponge origin collagen/gelatin with high pressure carbon dioxide-acidified water was successfully achieved. The proposed methodology allows an extraction of nearly 50% of the collagen/gelatin content of the sponges tested, representing an increase of more than 30% over conventional acid extractions using dilute acetic acid solutions. The extracted material was confirmed to be a mixture of collagen and gelatin by different physical and chemical analysis techniques. Cytotoxicity behavior demonstrated that the collagen/gelatin obtained is in most cases noncytotoxic, with the exception of collagen/gelatin extracted from *Thymosea* sp. The results presented suggest that sponge collagen extracted with water acidified by carbon dioxide is a promising material for biomedical applications.

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Notes

The authors declare no competing financial interest.

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