

Research paper

Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum

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Abstract

A previously described isolation procedure for collagen of the marine sponge *Chondrosia reniformis* Nardo was modified for scaling-up reasons yielding 30% of collagen (freeze-dried collagen in relation to freeze-dried sponge). Light microscope observations showed fibrous structures. Transmission electron microscopy studies proved the collagenous nature of this material: high magnifications showed the typical periodic banding-pattern of collagen fibres. However, the results of the amino acid analysis differed from most publications, presumably due to impurities that still were present. In agreement with earlier studies, sponge collagen was insoluble in dilute acid mediums and all solvents investigated. Dispersion of collagen was facilitated when dilute basic mediums were employed. The acid–base properties of the material were investigated by titration. Furthermore, a sponge extract was incorporated in two different formulations and compared with their extract-free analogues and a commercially available collagen containing product with respect to their effects on biophysical skin parameters. None of the preparations had a noticeable influence on the physiological skin surface pH. Skin hydration increased only slightly. However, all tested formulations showed a significant increase of lipids measured by sebumetry. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Collagen is a fibrous protein found ubiquitously in all multicellular animals. It is a particularly rigid and inextensible extracellular matrix protein that serves as a major constituent of many connective tissues. The characteristic feature of a typical collagen molecule, tropo collagen, is its long, stiff, triple-stranded helix, in which three collagen polypeptide chains are wound around one another in form of a ropelike superhelix. These peptides are extremely rich in proline and glycine, both of which are important for the formation of the collagen-specific helical structure [2–5].

Collagen is a natural material of low immunogenicity [6]. Many pharmaceutical applications are known for collagen, e.g. shields, injectable dispersions, sponges and microparticles [7]. Furthermore, collagen is used as a moisturiser in

cosmetic creams [8]. Moisturisers are employed for the therapy of so-called ‘dry skin’. Lipids work by the principle of occlusion, whereas humectants, such as collagen, attract water in the stratum corneum. In contrast to low molecular weight humectants, such as urea or glycerine, humectants of higher molecular weight, such as collagen, are not absorbed by the stratum corneum but rather stay on the skin surface, where they bind water by hydration and so increase the degree of skin-humidity [8]. Collagen thus represents an excellent film-building polymer in cosmetics.

The use of cattle as the main source for collagen has to be reconsidered because of the risks of BSE (bovine spongiform encephalopathy) and TSE (transmissible spongiform encephalopathy). One alternative is the use of porcine collagen or, much safer, collagen from sea animals, such as marine sponges.

These sessile animals here existed for 600 million years and are believed to be the earliest metazoans [9]. Several forms of molecular organisation and/or types have been attributed to sponge collagen [10,11]. Later studies prove the existence of at least two gene families in sponges by

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characterizing cDNA and genomic clones for sponge collagen [12]. Even type IV collagen was demonstrated by cDNA and genomic DNA studies in the homoscleromorph sponge *Pseudocortidium jarrei* [13].

Until now, published isolation-procedures for sponge collagen were very laborious involving a lot of steps, and therefore, were difficult to scale up [1,14–17]. The objective of the present work was firstly to simplify the collagen isolation procedure and secondly to evaluate if the marine sponge *Chondrosia reniformis* Nardo can be employed as an alternative collagen source. After physico-chemical characterization of the isolated material, a possible application as a moisturiser in cosmetic preparations was investigated using non-invasive in vivo measurement techniques.

2. Materials and methods

2.1. Materials

The sponge *Chondrosia reniformis* Nardo (Demospongiae: Hadromerida: Chondrosiidae) was collected in the Aegean Sea and stored in 50% ethanol until used. Acetone, methanol, *n*-hexane, and tris(hydroxymethyl)aminomethane were purchased from Roth (Karlsruhe, Germany) and ethanol 96% from Bundesmonopolverwaltung für Branntwein (Frankfurt, Germany). Ethyl acetate, sodium hydroxide pellets, 0.1 N NaOH, 0.1 N HCl, potassium carbonate, tartaric acid, and hydrochloric acid 37% were obtained from Merck AG (Darmstadt, Germany). All amino acid-analysator chemicals (e.g. buffers, ninhydrin reagent, amino acid reference) as well as the ion exchange column were purchased from Beckman (Munich, Germany). Avocado oil, dexpantenol, cocoa butter, and WAFU Variogrundlage® were obtained from Caesar and Lorentz GmbH (Hilden, Germany) and Eucerinum® O/W from Beiersdorf AG (Hamburg, Germany). Tegomuls 90S was purchased from Th. Goldschmidt AG (Essen, Germany) and Shoynear® Collective Intensive Day Care Cream from Shoynear Cosmetic GmbH (Augsburg, Germany). All other chemicals used were of analytical reagent grade.

2.2. Isolation of marine sponge collagen

Collagen was isolated by a previously described method [1]. Modifications were introduced in order to simplify the procedure. About 50 g of ethanol-conserved sponge material were washed three times under running tap water, cut into small pieces and homogenized using a blender (Kenwood BL 901, New Lane, Havant Hants, England) while 250 ml of a 100 mM Tris–HCl buffer (pH 9.5; 10 mM EDTA; 8 M urea; 100 mM 2-mercaptoethanol) were added. The pH of the resulting dark-coloured dispersion was raised from pH 7 to 9 with NaOH. After 24 h of continuously stirring at room temperature the viscous extract was centrifuged (5000 × *g*; 5'; 2°C; Sorvall RC-5B, Du Pont Instruments, Wilmington, USA). The pellet was discarded

and collagen was precipitated from the supernatant by adjusting the pH to 4 with acetic acid and collected by centrifugation (20,000 × *g*; 30'; 2°C). The pellet was resuspended in distilled water, centrifuged (20,000 × *g*; 30'; 2°C) and lyophilized for preservation.

2.3. Characterization of sponge collagen

2.3.1. Light microscopy

Aqueous dispersions of isolated material were investigated by bright field microscopy (Standardmikroskop CH, Olympus Optical Co. GmbH, Hamburg, Germany) and by phase contrast microscopy (AH3-RFCA, Olympus Optical Co. GmbH, Hamburg, Germany).

2.3.2. Electron microscopy

Twenty microliter aliquots of aqueous collagen dispersions were sampled for negative staining, using the single-droplet procedure [18]. The sample was attached to 20 s glow-discharge treated carbon support films, washed rapidly with three consecutive 20-μl droplets of distilled water, and stained with a 20-μl droplet of a 2% phosphotungstic acid solution (pH 7). Studies were performed using the Zeiss EM900 at room temperature, under minimal conventional irradiation conditions [19].

2.3.3. Solubility of sponge collagen

The isolated material was dispersed in the following solvents at room temperature to investigate their solvent action: acetone, methanol, ethanol 96%, ethyl acetate, *n*-hexane, double-distilled water, a 100 mM Tris–HCl buffer (pH 9.5), aqueous solutions of sodium hydroxide (pH 12), potassium carbonate (pH 9.5), tartaric acid, citric acid and hydrochloric acid 37%.

2.3.4. Amino acid composition

The isolated material was hydrolyzed by the method of Moore and Stein [20] with 6N HCl/2% phenol in the absence of oxygen (130°C; 20 h). The amino acid composition was determined on a Beckman amino acid analyser 6300 (Beckman, Munich, Germany) using a cation exchange column. After post-column-derivatisation with ninhydrin, proline and hydroxyproline were determined at 440 nm, whereas primary amino acids were determined at 570 nm wavelength. The amino acid quantification was performed using a standard amino acid mixture as reference and the software 'System Gold 8.0' (Beckman, Munich, Germany).

2.3.5. Titration

Each of two samples of 150 mg freeze-dried material was dispersed in 15 ml MilliQ^R-water by ultrasonication (Transonic digital, Elma, Singen, Germany). One sample was titrated with 0.1 N NaOH, the other with 0.1 N HCl. The pH was registered (pH meter 765 Calimatic, Knick Elektronische Meßgeräte GmbH & Co., Berlin, Germany) and the

resulting pH versus amount of NaOH, respectively HCl, plot evaluated [6,21,22]. Additionally, a blank sample without collagen was titrated under the same conditions. The titration range was from pH 2 to 12. All experiments were performed at room temperature.

2.4. Effects of sponge collagen on biophysical skin parameters

2.4.1. Sponge extract

The sponge extract was prepared according to the above described isolation procedure excluding 2-mercaptoethanol from the extraction buffer (pH 9.5; 100 mM Tris-HCl; 10 mM EDTA; 8 M urea). The supernatant of the first centrifugation step was for the formulations described below. The collagen content of the sponge extract was estimated gravimetrically: the protein was precipitated using 25.0 ml extract, collected, purified, lyophilized as described above, and weighed. The trial was performed in triplicate ($n = 3$).

2.4.2. Preparation of cosmetic formulations

Five different cosmetics (F1–F5) were employed. F1 and its extract containing analogue F2 were based on a previously published brochure [23], whereas F3 and F4 based on the ready to use Eucerinum® O/W-basis. The exact compositions of the employed formulations are given in Table 1.

In addition to the above mentioned four preparations, Shoynear® Collective Intensive Day Care Cream – a commercially available collagen-containing product – was included in the study as formulation F5.

2.4.3. In-vivo measurements

Eleven female and six male volunteers from 24 to 52 years of age participated in the trials. Seventy-five mg of each preparation were applied to a 25 cm² area of the volar forearm. One area of the same size – the reference side – was kept blank for control-measurements to detect environmental influences on the investigated results [24]. The subjects were asked not to wash the forearm or to apply cosmetics to the volar forearm for a period of at least 12 h prior to the study.

The skin parameters sebum, hydration state and pH were measured before and after 1, 3 and 6 h of application of the test preparations using the sebumeter-corneometer-pH-

meter-combination unit SM810® CM820® PH900® (Courage + Khazaka, Köln, Germany).

The integrated sebumeter SM810® measures the amount of sebum on the skin by absorbing it onto a thin plastic strip and measuring its transparency photometrically [25]. The result is directly displayed in µg sebum/cm².

For skin-hydration measurements many different methods are described in the literature [26]. The well-known corneometer-method (corneometer CM820®), which is based on the principle of constant dielectric measurements, was used for the present study. The changes in capacitance, which are recorded by this type of instrument, are converted in hydration units varying from 0 to 120 [27], where 0 units corresponds to very dry, and 120 units to very humid skin areas. Unlike the sebumeter-method, the results are only given in arbitrary units. As the in vitro-calibration of the corneometer CM820® is known to be difficult to accomplish [27], control measurements are paramount and were included in the present study.

The skin-pH was measured using the integrated PH900®-pH meter, equipped with a planar surface electrode, which enables a fast and reliable potentiometric determination of the actual skin pH [28,29].

For each instrument, the measurements were made according to manufacturer's instructions. The blank areas were investigated analogously. While sebum-measurements were determined once, skin hydration and pH were determined in triplicate ($n = 3$).

All measurements were performed in the same air conditioned room at room temperature and at comparable external relative humidity. The volunteers were allowed to adjust to the room climate for about 20 min prior to measurements.

3. Results and discussion

The marine sponge *Chondrosia reniformis* Nardo was chosen for this study due to some advantages: This sponge can be found ubiquitously in the Mediterranean sea and contains a lot of collagen. Studies on the cortex of the sponge *Chondrosia reniformis* showed that the hydroxyproline content approximately corresponds to a 40% of collagen content [17]. This estimation is in good agreement with our experience (data not shown). Furthermore, it is the only edible sponge [30] as confirmed by native inhabitants. This fact reduces the risk of undesired toxic compounds.

3.1. Isolation and characterization of sponge collagen

The isolation procedures for sponge collagen as described in the literature start from freshly frozen sponge material. By examining different ways of conservation of the collected sponge material, e.g., freezing, drying, application of salt, the use of ethanol turned out to be the most useful technique. In the present study collagen was isolated by a method previously described [1] not involving intensive purification steps. The output of isolated material was

Table 1
Composition of formulation F1–F4

Ingredients	F1	F2	F3	F4
Tegomuls 90 S	8.0 g	8.0 g	–	–
Avocado oil	19.0 g	19.0 g	–	–
Cocoa butter	6.0 g	6.0 g	–	–
D-Panthenol	2.7 g	2.7 g	–	–
Sponge extract	–	5.0 g	–	5.0 g
Aqua dest.	ad 100.0 g	ad 100.0 g	–	–
Eucerinum® O/W	–	–	100.0 g	ad 100.0 g

about 30% (freeze-dried material in relation to freeze-dried sponge material).

Collagen fibrils can assemble into large fibers that can be visualized by light microscopy [3]. This investigation represents a first approach to the isolated material. Fig. 1 shows fibrous structures that can already be seen at low magnifications ($300\times$).

To obtain evidence about the collagenous nature of the isolated material the sample was investigated by transmission electron microscopy (TEM). The observed structures were similar to those already published for collagen isolated from *Chondrosia reniformis* [17]. High magnifications showed the typical striation of collagen fibres (Fig. 2), which conclusively proved the collagenous nature of the material. Low magnifications showed large amounts of collagen. Only few impurities were visible. Huge alterations of the collagen structure as described to be typical for the urea treatment of insoluble collagen from human skin [31] were not observed.

This sponge collagen could not be dissolved using the conditions described above. Even excess amounts of different solutions and solvents and long dissolution periods of up to two weeks had no effect. However, there were differences between the investigated solutions: while dilute acidic solutions and different solvents had absolutely no effect on sponge collagen, mild basic mediums – between pH 8 and 10 – facilitated the dispersion of collagen in water, without changing the electromicroscopic appearance. Concentrated basic and acidic solutions hydrolyzed sponge collagen to fragments. These fragments were not observable by electromicroscopy. These results agree with the literature, which report a striking insolubility for sponge collagen in any medium [1,10,32–34]. Therefore, typical biochemical

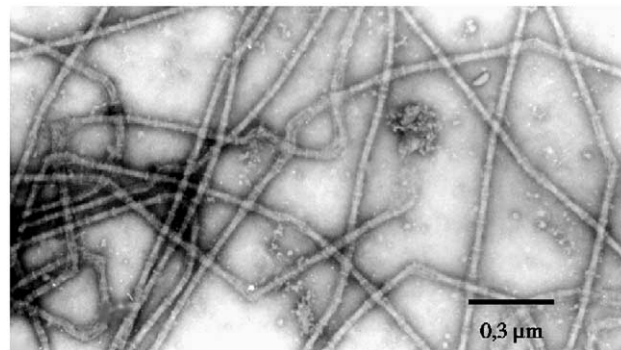


Fig. 2. Transmission electron micrograph of negatively stained sponge collagen. The bar indicates $0.3\ \mu\text{m}$ (courtesy of Dr R. Harris).

analyses at the protein level, e.g. SDS-polyacrylamide gel electrophoresis, isoelectric focussing and standard protein assays (Bradford, Lowry, UV) could not be employed with sponge collagen in the present or in previous studies [12].

In order to study the acid-base properties of the isolated material, a titration of an aqueous collagen dispersion was performed. Titration of insoluble collagen can even be performed to determine the isoelectric point of insoluble collagen [6]. To exclude any influence of primarily added basic solutions, the acid and alkaline segments of the titration curve were determined separately [21]. The titration curves are shown in Fig. 3. The pH of the freshly prepared collagen dispersion was $4.44 (\pm 0.05)$. It was significantly lower than that of MilliQ^R-water, probably resulting from the high content of acidic amino acids (aspartic acid or glutamic acid). The pH-chart of the collagen dispersion was less steep compared to the control titration and shows a flattening area between pH 5 and 3, which was confirmed

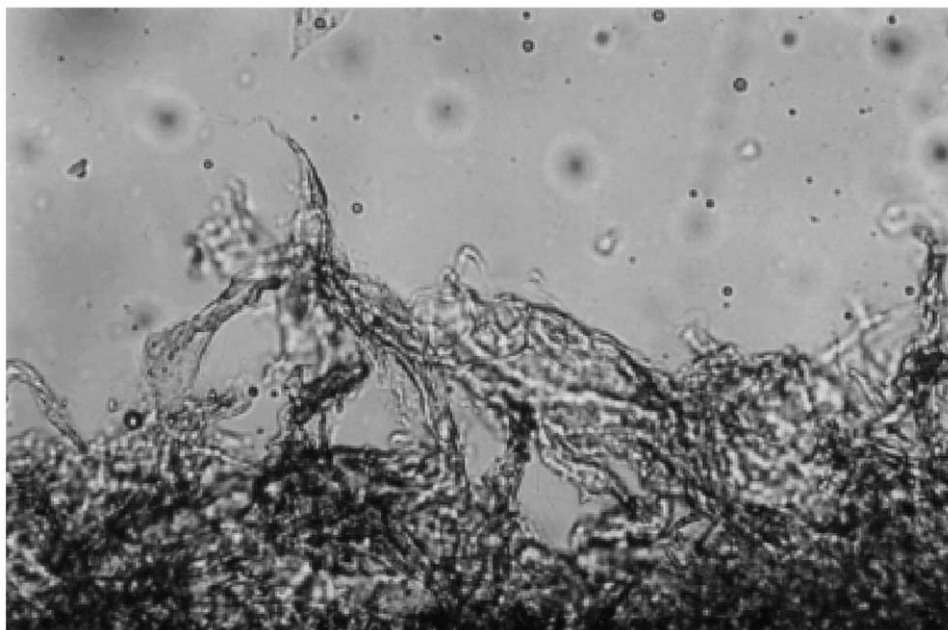


Fig. 1. Light microscopy photograph of sponge collagen.

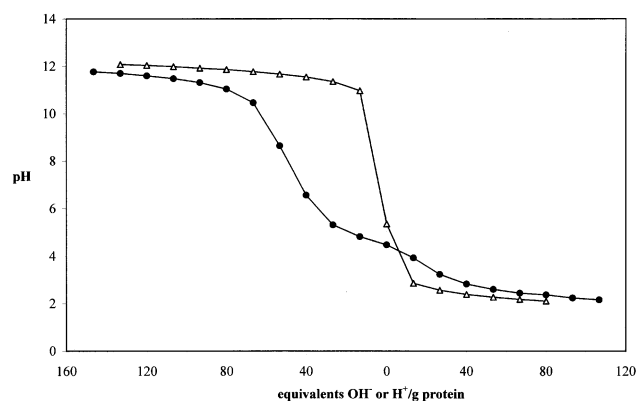


Fig. 3. Titration curve of an aqueous sponge collagen dispersion. Blank (Δ) and sponge collagen dispersion ($\bullet$).

to be typical for the collagen dispersions by two reiterations. Both curves levelled off at about pH 12 and bottomed out approximately at pH 2. From the titration curve it is possible to formulate an isoelectric range. The behaviour of the sample has isoelectric properties in the pH range between 6.5 and 8.5. This result is in agreement with findings of Friess [6] who determined the isoelectric point of insoluble bovine collagen to be 7.00 ± 0.09 .

The results of the amino acid analysis are shown in Table 2. The amounts of glycine and hydroxyproline are lower than those already described for collagen from the sponge *Chondrosia reniformis* [11]. The amount for glycine is closer to the results of Gross et al. [10]: investigation of collagen from the grass sponge *Spongia graminea* resulted in only 16.3% glycine. However, the reduced values for glycine and hydroxyproline can also be explained by impurities of glycoproteins, which are known to be strongly associated with collagen [34]. On the other hand, the amount of

L-hydroxylysine is higher than described before [11]. The rest of the results are in good agreement to the above mentioned studies, especially the amounts of aspartic acid, glutamic acid, proline, threonine, serine and arginine.

3.2. Effects of sponge collagen on biophysical skin parameters

3.2.1. Preparation of the sponge extract and the cosmetic formulations

Due to the physico-chemical properties, especially its striking insolubility, any attempt to incorporate isolated sponge collagen homogeneously into cosmetics failed. On the other hand, the centrifuged sponge extract was easy to incorporate in any tested formulation.

Excluding β -mercaptoethanol from the extraction buffer, the yield decreased from approximately 30–9.7%. The estimation of the collagen content in extracts was 1.94 (± 0.13)% (w/v). This underlines the importance of β -mercaptoethanol in the extraction buffer, partially solubilizing insoluble collagen by reduction of some disulfide bridges. This technique of cleaving covalent bounds is also well known for the isolation of some vertebrate collagens [31].

3.2.2. In-vivo study

The inner forearm was selected as the test site because it is easily accessible and relatively hair free. The physiological skin pH of about 5.5 protects the skin from microorganisms, allergens and toxic substances. Consequently, the determination of the skin pH represents an important factor of safety for the application of cosmetic products, facial soaps or clarifying lotions [28]. Therefore, modern products should not change the physiological pH [29]. All tested formulations showed almost no influence on the physiological pH-value (Fig. 4). No statistically significant differences were found between the skin areas with formulations and the blank area. The mean values ranged from pH 4.86

Table 2
Amino acid composition of the isolated material

Amino acid	%
Asx	9.5
Thr	4.7
Ser	4.2
Glx	10.3
Pro	5.4
H-LPro	2.4
Gly	18.9
Ala	7.7
Cys	0.3
Val	5.5
Met	1.4
Ile	5
Leu	6.2
Tyr	2.1
Phe	3.2
His	1.1
L-HLys	4.3
Lys	2.8
Arg	4.9

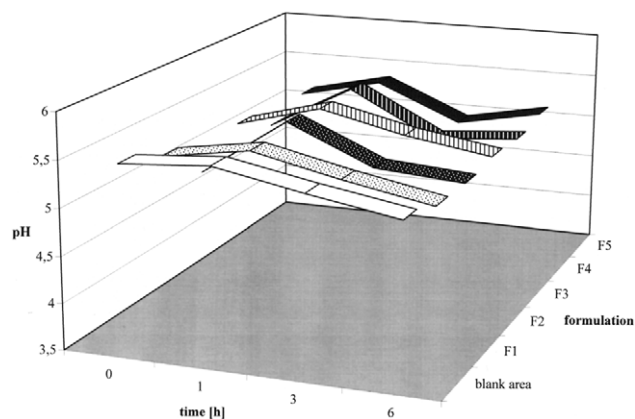


Fig. 4. Short-term in-vivo investigation of skin surface pH after application of the following formulations: Blank area ($\square$), formulation F1 ($\square$), formulation F2 ($\square$), formulation F3 ($\square$), formulation F4 ($\square$) and formulation F5 ($\square$).

(minimum of all mean values) to pH 5.52 (maximum of all mean values) and the standard deviation from 0.21 to 0.65 (not presented in the figure for clearness). Thus, no formulation led to a significant change of the physiological skin-pH.

Measurement of the stratum corneum hydration can be used for evaluation of the efficiency of skin moisturising [35]. The results are presented in Fig. 5: The starting corneometer value of 60 units pointed out that the average skin hydration state of the volunteers was, as expected for this skin area, between low and normal. The influence of all applied formulations on the stratum corneum hydration was low. In comparison to the untreated control site, the test sites showed only a slight increase of hydration. Only non-significant differences were observed between sponge collagen, the sponge collagen free bases F1 and F3, and the commercial skin care product Shoynear Collective®.

Skin surface lipids mainly originate from sebum. Sebum is an oily mixture of lipids, keratin, and cellular membrane structures excreted by the sebaceous glands [36]. The results are presented in Fig. 6: due to the fact that the number of sebaceous glands in the forearm is very small compared to other body-sizes, e.g., the forehead, the sebum values of the untreated areas were very low. All test-areas showed a significant increase in sebumeter transparency indicating a sebum increase between 140 and 180 $\mu\text{g}/\text{cm}^2$ within 1 h after application whereas no such effect could be observed on the control skin. However, since the sebumeter just measures the lipid content on the skin, no discrimination between sebum production and lipid increase due to the lipids contained in the formulations is possible. Hence, it is more likely that the above increase in sebumeter transparency was caused by the latter lipids. After 3 h the sebumeter reading decreased to a value of about 75 $\mu\text{g}/\text{cm}^2$, which kept on decreasing to a value of about 27 $\mu\text{g}/\text{cm}^2$ after 6 h. Only

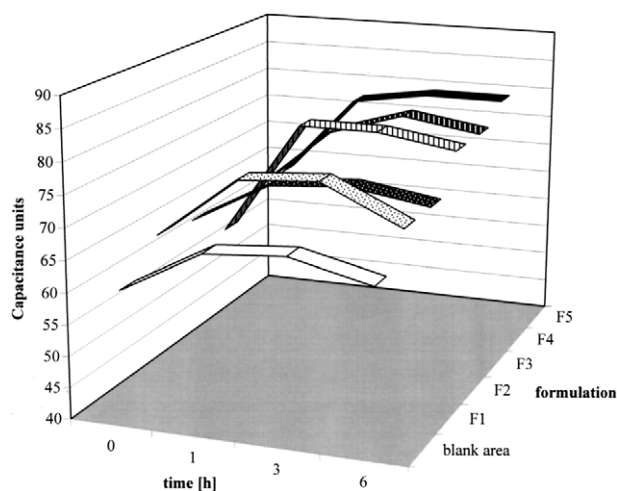


Fig. 5. Short-term in-vivo investigation of skin hydration after application of the following formulations: Blank area ($\square$), formulation F1 ($\square$), formulation F2 ($\square$), formulation F3 ($\square$), formulation F4 ($\square$) and formulation F5 ($\square$).

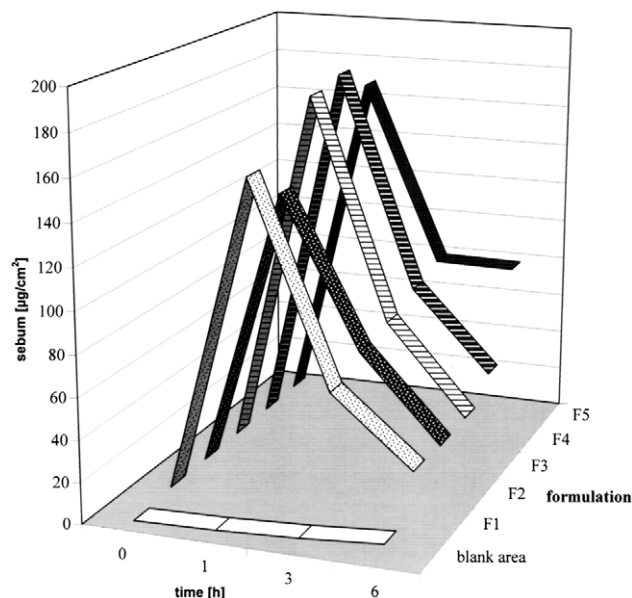


Fig. 6. Short-term in-vivo investigation of skin lipid content after application of the following formulations: Blank area ($\square$), formulation F1 ($\square$), formulation F2 ($\square$), formulation F3 ($\square$), formulation F4 ($\square$) and formulation F5 ($\square$).

Shoynear Collective® showed the tendency of higher values for the amounts of sebum even after 6 h.

The results from the pH-measurements show that no significant or adverse changes were caused by any of these formulations. Consequently, all formulations are suitable for repeated applications, as usually recommended for the therapy of 'dry skin'. Concerning skin-hydration both sponge collagen containing preparations as well as the commercial collagen containing preparation Shoynear Collective® showed a slight increase. The enhancement in lipids indicated by the sebumeter represents an advantage, since the 'dry skin' not only lacks skin hydration but also sufficient sebum [37,38].

This study clearly demonstrates that conventional collagen can be substituted by marine collagen. In addition, the currently available complicated isolation procedure can be simplified according to the method described in this publication.

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References

- [1] B. Diehl-Seifert, B. Kurelec, R.K. Zahn, A. Dorn, B. Jericevic, G. Uhlenbruck, W.E.G. Müller, Attachment of sponge cells to collagen substrata: effect of a collagen assembly factor, *J. Cell Sci.* 79 (1985) 271–285.
- [2] B. Rössler, J. Kreuter, D. Scherer, Collagen microparticles: preparation and properties, *J. Microencapsul.* 12 (1995) 49–57.
- [3] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson, *The molecular biology of the cell*, Garland Publishers, New York, 1994, pp. 971–990.
- [4] G.L. Zubay, *Biochemistry*, WCB Wm. C. Brown Publishers, USA, 1998, pp. 90–92.
- [5] A.L. Lehninger, *Biochemie*, VCH Verlagsgesellschaft mbH, Weinheim, Germany, 1987, pp. 107–108.
- [6] W. Frieß, *Drug delivery systems based on collagen*, Habil.-Schr., Friedrich-Alexander-Universität Erlangen-Nürnberg, Nürnberg, 2000.
- [7] W. Friess, Collagen – biomaterial for drug delivery, *Eur. J. Pharm. Biopharm.* 45 (1998) 113–136.
- [8] P. Elsner, E. Berardesca, H. Maibach, *Bioengineering of the Skin: Water and the Stratum Corneum*, CRC Press LLC, Boca Raton, 1994.
- [9] W.E.G. Müller, H.C. Schröder, Bioaktive Substanzen aus Schwämmen: Gene weisen den Weg bei der Suche nach neuen Arzneimitteln, *Biologie in unserer Zeit* 27 (1997) 389–398.
- [10] J. Gross, Z. Sokal, M. Rougvié, Structural and chemical studies of the connective tissue of marine sponges, *J. Histochem. Cytochem.* 4 (1956) 227–246.
- [11] R. Garrone, *Phylogenesis of Connective Tissue, Morphological Aspects and Biosynthesis of Sponge Intercellular Matrix*, Vol. 5, S. Karger, New York, 1978.
- [12] J.-Y. Exposito, R. Garrone, Characterization of a fibrillar collagen gene in sponges reveals the early evolutionary appearance of two collagen gene families, *Proc. Natl. Acad. Sci. USA* 87 (1990) 6669–6673.
- [13] N. Boute, J.-Y. Exposito, N. Boury-Esnault, J. Vacelet, N. Noro, K. Miyazaki, K. Yoshizato, R. Garrone, Type IV collagen in sponges, the missing link in basement membrane ubiquity, *Biol. Cell* 88 (1996) 37–44.
- [14] S. Junqua, L. Robert, R. Garrone, M. Pavans de Ceccatty, J. Vacelet, Biochemical and morphological studies on the collagens of horny sponges. *Ircinia* filaments compared to spongines, *Connect. Tissue Res.* 2 (1974) 193–203.
- [15] S. Junqua, J. Fayolle, L. Robert, Structural glycoproteins from sponge intercellular matrix, *Comp. Biochem. Physiol.* 50B (1975) 305–309.
- [16] S. Junqua, J. Fayolle, L. Robert, Isolation and characterization of structural glycoproteins from sponges, in: H. Peeters (Ed.), *Protides of the Biological Fluids*, Pergamon Press, New York, 1975, pp. 337–341.
- [17] R. Garrone, A. Huc, S. Junqua, Fine structure and physicochemical studies on the collagen of the marine sponge *Chondrosia reniformis* Nardo, *J. Ultrastr. Res.* 52 (1975) 261–275.
- [18] J.R. Harris, Negative staining and cryoelectron microscopy: the thin film techniques, Vol. 35, BIOS Scientific Publisher Ltd, Oxford, 1997.
- [19] J.R. Harris, M. Adrian, S. Bhakdi, M. Palmer, Cholesterol–Streptolysin O interaction: an EM study of wild-type and mutant streptolysin O, *J. Struct. Biology* 121 (1998) 343–355.
- [20] S. Moore, W.H. Stein, Chromatographic determination of amino acids by the use of automatic recording equipment, *Meth. Enzymology* (1963) 819–831.
- [21] C.M. Ofner, H. Schott, Shifts in the apparent ionization constant of the carboxylic acid groups of gelatin, *J. Pharm. Sci.* 74 (1985) 1317–1321.
- [22] T. Schmidt-Mende, C.S. Leopold, B.C. Lippold, Dissociation constants of enteric coating polymers, *Proceedings of the 2nd World Meeting, APGI/APV*, Paris, 1998, pp. 391–392.
- [23] J. Pütz, C. Niklas, *Cremes und sanfte Seifen*, vgs verlagsgesellschaft, Köln, Germany, 1988.
- [24] K.-H. Schrader, Untersuchungen wasser-retinierender Kosmetika auf der Haut, *Parfümerie Kosmetik* 62 (1981) 265–272.
- [25] S. Dikstein, W. Courage, Verteilung von Talgspiegelmessungen bei gesunden erwachsenen Frauen, *Ärztliche Kosmetologie* 15 (1985) 41–44.
- [26] E. Berardesca, G. Borroni, Instrumental evaluation of cutaneous hydration, *Clinics Dermat.* 13 (1995) 323–327.
- [27] A.O. Barel, P. Clarys, In vitro calibration of the capacitance method (Corneometer CM 825) and conductance method (Skicon-200) for the evaluation of the hydration state of the skin, *Skin Res. Technol.* 3 (1997) 107–113.
- [28] T. Fischer, C. Greif, W. Wigger-Alberti, P. Elsner, Instrumentelle Methoden zur Bewertung der Sicherheit und Wirksamkeit von Kosmetika, *Aktuelle Dermatologie* 8/9 (1998) 1–11.
- [29] H.P. Nissen, *Physikalische Meßmethoden in der Kosmetik*, *Kosmetik Int.* 11 (1988) 18–22.
- [30] K.G. Grell, H.-E. Gruner, E.F. Kilian, Band I: Wirbellose Tiere, 1. Teil: Einführung Protozoa, Placozoa, Porifera, in: H.-E. Gruner (Ed.), *Lehrbuch der speziellen Zoologie*, Gustav Fischer Verlag, Stuttgart, 1993.
- [31] J.P. Borel, A. Randoux, Methodological approach to the primary structure of collagen, in: L. Robert, M. Moczar, E. Moczar (Eds.), *Methods of Connective Tissue Research*, Vol. 10, S. Karger, New York, 1985, pp. 1–58.
- [32] R. Garrone, Formation and Involvement of Extracellular Matrix in the Development of Sponges, a Primitive Multicellular System, Alan R. Liss, New York, 1984, pp. 461–477.
- [33] R. Garrone, The Collagen of the Porifera, *Nato ASI Ser., Ser. A* 93, 1985, pp. 157–175.
- [34] S. Junqua, M. Lemonnier, L. Robert, Glycoconjugates from *spongia officinalis* (phylum porifera). Isolation, fractionation by affinity chromatography on lectins and partial characterization, *Comp. Biochem. Physiol.* 69B (1981) 445–453.
- [35] A.O. Barel, P. Clarys, Ann de Romsée, Wessels, Non-Invasive Electrical Measurements for Evaluating the Water Content of the Horny Layer: Comparison Between Capacitance and Conductance Measurements. Abstracts of the Conference on Prediction of Percutaneous Penetration, Brüssel, 1991.
- [36] P. Elsner, *Sebum, Bioengineering of the skin: Methods and Instrumentation*, CRC Press, Boca Raton, 1995.
- [37] R. Voigt, *Pharmazeutische Technologie für Studium und Beruf*, Deutscher Apotheker Verlag, Stuttgart, Germany, 2000, pp. 326–327.
- [38] E.G. Jung, *Dermatologie*, Hippokrates Verlag, Stuttgart, Germany, 1995, pp. 27–29.