The Nanobiotechnology of Obtaining of Collagen Gels from Marin Fish Skin and Yours Reological Properties for using Like New Materials in Dental Medicine

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Abstract—This paper aims at presenting the biotechnology used to obtain collagen-based gels from shark (Squalus acanthias) and brill skin, marine fish growing in the Black Sea. Due to the structure of its micro-fibres, collagen can be considered a nanomaterial; in order to use collagen-based matrixes as biomaterial, rheological studies must be performed first, to state whether they are stable or not. For the triple-helix structure to remain stable within these gels at room or human body temperature, they must be stabilized by reticulation.

Keywords-Collagen, biotechnology, reticulation.

I. INTRODUCTION

TYPE I fibrillar collagen is a triple-helix structure protein present in the skin and in cartilaginous tissues. The acknowledged disadvantages related to obtaining collagen by extraction from fish skin consist mainly in the persistence of pigments and fish odour, and the fact that the denaturation temperature values are lower – usually below 30° C – which is a disadvantage concerning their use as bio-nanomaterials. Nonetheless, fish-derived collagen can be widely used due to its indisputable advantages:

- it is easier to extract and has shown better performance compared to animal-derived collagen;
- it entails a relatively low risk of containing unknown germs;
- the denatured collagen-based matrixes permit a better tissue regeneration compared to the native collagen-based ones [1], and the reticulum matrixes derived from fish collagen are likely to contain partially denatured structures due to the lower denaturation temperature.

Although fish-derived collagen does not form high-viscosity gels, it is extremely convenient for some applications, such as micro-packaging or obtaining photosensitive coatings. Films and porous matrixes can be obtained from collagen-based gels,

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just as from mammal-derived ones. They can be successfully used in dental medicine for treating oral diseases – they form bio-absorbable membranes and matrixes and they can incorporate different active ingredients which can be subsequently released in order to obtain the intended therapeutic effects.

Depending on the treatments applied, we can distinguish between four categories of bio-nanotechnologies used for obtaining collagen from marine fish:

- alkaline and acid treatments [2];
- acid treatment [3, 4-6];
- enzyme treatment [5, 6];
- combined treatments.

This paper aims at presenting the biotechnology used to obtain collagen-based gels from shark (*Squalus acanthias*) and brill skin, marine fish growing in the Black Sea. Due to the structure of its micro-fibres, collagen can be considered a nanomaterial – in order to use collagen-based matrixes as biomaterial, rheological studies must be performed first, to state whether they are stable or not. For the triple-helix structure to remain stable within these gels at room or human body temperature, they must be stabilized by reticulation.

II. MATERIALS AND METHODS

Reticulation has been performed by using glutaric aldehyde at 4-6°C for different concentrations of collagen derived from the two species of fish.

The rheological behaviour has been determined by using a Haake VT 550 rheo-viscometer with the following layout: S₃ (MV1) sensors system, d₁ and d₂ measuring ranges, shearing speed range, $\dot{\gamma}$, between 1.17 and 1872 s⁻¹. The measurements were initially performed at a $25 \pm 0.1^{\circ}$ C temperature, with a thermostatic time of 20 minutes, and then – using ice – at $20 \pm 0.1^{\circ}$ C. In order to establish whether the dispersed systems show a time-dependant rheological behaviour, measurements were performed when increasing and decreasing the shearing speed values, which allowed the tracing of the hysteresis loops for the film-forming systems with time-dependant rheological behaviour. The position of the two rheograms in Figs. 1 and 2 – meant to represent the hysteresis loop – proves that the gels obtained from shark skin (Fig. 1) and brill skin (Fig. 2) have a

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time-dependant rheological behaviour, namely a strongly tyxotropic one, without structure recuperation at low shearing speed values.

A. The Biotechnology used to Obtain Collagen from Black Sea Shark Skin

What we identified as the main problem regarding the shark (*Squalus acanthias*) skins was not just that of discoloration and removal of the odour, as in the case of other fishes, but especially the total removal of the epidermis, which contains placoid scales shaped like basal plates provided with a spike directed backwards and solid particles which do not dissolve during the collagen extraction process and impurify it; another

problem identified is that of finding a solution for extracting collagen that should not hinder the integrity of the epidermis, if such a solution is possible. Added to the above is the necessity of working at low temperature, due to the low denaturation temperature of collagen extracted from fish skin generally, as compared to that extracted from terrestrial mammals.

The first operation consisted of skinning the shark, followed by removal of muscle remains, then intensively washing it using tap and distilled water, and immediate storage in the freezer.

Below are the treatments to which the skin was submitted:

COLLAGEN EXTRACTION FROM SHARK SK	IN BY MEANS OF ALKALINE-ACID TREATMENTS
The alkaline-acid extraction of collagen from	Treating with 0.5M acetic acid
shark skin	
The first treatment applied was the one	As room temperature enabled – in the case of bovine
frequently employed as pre-treatment for bovine	skin – removal of the epidermis, this pre-treatment was
skins, as applied by Yoshimura, Terashima,	also applied to shark skin.
Hozan and Shirai [9] for the alkaline-acid	Considering the fact that the denaturation
extraction of collagen from shark skin: 20ml 0.5M	temperature of collagen extracted from fish skin is
concentrated solution of sodium hydroxide and	lower than that obtained from bovine skin, the 0.5M
15% sodium sulphate, with a pH of 11-12, was	acetic acid pre-treatment was applied both at room
inserted over the moist skin after dabbing it with	temperature (23-26°C), and in the frdige (5-7°C),
filter paper. A high moist skin/solution ratio was	without stirring, using variable skin/acid solution ratios,
used, of 1/21.4, in order to submerge the skin	between $1/7.5$ and $1/20$, aiming – each time – at
completely. I reatment was performed at room $(22^{\circ}C)$	submerging the skin entirely. Shark skin quickly
temperature (25 C). A $\theta = 2$ decreased a helf the shift have a situated with the second sec	becomes soaked in the 0.5M acetic acid solution,
After 3 days and a nall the skin became a little	significantly increasing its volume and weight. Thus, at 27° C it going in shout 1 hour aires 740 / in weight it
skin was kept in contact with the solution for a	27 C It gains – In about 1 nour – circa 74% in weight, it thickens and gets a jolly like aspect. Horny epidermia
skill was kept ill contact with the solution for a	can be cleaned (it was cleaned) and the solution will be
aspect did not undergo any significant changes. It	clear and of low viscosity so under these circumstances
was only the solution that got slightly coloured	collagen was not extracted completely (losses are
becoming nale beige. The skin was taken out of	negligible).
the solution and was washed with distilled water.	After cleaning the skin was washed twice in tan
which leads to its stiffening.	water and twice in distilled water to remove any
A new volume of solution was inserted over the	remains of horny layer, then it was finely chopped and
washed, squeezed and filter paper-dabbed skin.	divided into two batches in view of extracting collagen:
Another week later, the skin virtually disappears	one batch was still treated with acetic acid of the same
completely, the solution is coloured in an intense	concentration, while the other was treated with pH 3.5
hue of beige and its viscosity is reduced, while in	hydrochloric acid, in both cases the ratio between
the lower layer small deposits are formed of	soaked and filter paper-dabbed collagen and solution
particles previously existing in the epidermis.	being 1/7. The samples were stirred energetically for 1
Low viscosity is an indication of the fact that	hour and 40 minutes, and then they were placed into the
there is no collagen in the solution, but rather its	fridge. In the meanwhile viscosity increased, appearing
hydrolytic compounds, so the method cannot be	to be higher than in the case of the hydrochloric acid
applied for obtaining fibrillar or non-denatured	solution. On the second day they were removed, left to
collagen; it can only be used to obtain its	reach room temperature and forced through fine muslin
invarious compounds. In addition, the extract is	to remove the jeny-like component, whose suspension
The conclusion which can be drown is that this	were centrifuged for 30 minutes, then placed into the
treatment is too strong for obtaining fibrillar or	fridge while a new amount of acid was inserted over
non-denatured collagen from shark skin and that	the jelly-like component to continue extraction
new methods should be identified that could	Filtrates had low viscosity, which indicates the fact that
enable either the preliminary removal of the	collagen was denatured.

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epidermis, or the extraction of collagen without	
dissolving the epidermis.	

TABLE II Collagen ever action edom share sein dy means of all valine-activity atments			
Pre-treatment using 0.25M sodium acetate and 0.25M acetic acid solution			
Instead, the 24°C, the ease of cleaning being comparable to that in the case of soaking in acetic acid under the same circumstances. The supernatant is colourless, meaning no pigments are extracted. The extent of cleanliness and the aspect of cleaned skin are shown in the picture in Fig. 2, compared to the one soaked in 0.5M acetic acid.	Removal of non-collagen materials, which are soluble in a hydrous environment and are attached to skin fibrils by means of non-specific interactions, can be performed by extraction employing salt solutions, sodium chloride in particular. At the same time, they extract newly synthesized collagen molecules which are not yet incorporated into the fibrils by covalent bonds. Pre-treatment using 10% sodium chloride solution for 24h at room temperature is also specified for the process of extracting collagen from squid skin, but for the purpose of partially separating chromophores (dark brown cells containing pigments), followed by decolouring by means of hydrogen peroxide. The skin, unfrozen and dabbed with filter paper, was replaced into a 10% sodium chloride solution with a pH of 7, at room temperature (cca. 27°C), the skin/collagen ratio being 1/5.After 24h, the solution becomes coloured in beige and gives off an intense fish odour, while the skin is soft and does not appear soaked. Washing was first performed using tap water jet, then the skin was squeezed, washed with distilled water, squeezed again, left in distilled water for 10 minutes, checked for chloride ions, well squeezed, dabbed with filter paper and then weighed. The gain in weight is, indeed, very low, of only cca. 5.2%. The skin thus treated was then divided into two batches to undergo further treatment in view of extracting collagen		

Cold-treatment with 0.5M acetic acid	Treating with pH 3.5 hydrochloric acid
0.5M acetic acid solution at 6°C was added	The other batch of skin was inserted into a pH 3.5
over the filter paper-dabbed skin, at a	hydrochloric acid solution, using a wet skin/solution ratio
skin/acid ratio of 1/5.After 24 hours the skin	of 1/5. After 24 hours it seems unchanged and, indeed, its
becomes significantly soaked, its weight more	weight increased during this while by only 9%. The skin
than doubling (it increases about 2.1 times).	was removed from the hydrochloric acid, it was washed
The epidermis starts coming loose already,	entirely using tap and distilled water until all chlorine ions
although with some difficulty. After 48 hours	had disappeared, then the other treatment was applied,
it will clean well, but the skin seems thinned,	involving cold 0.5M acetic acid solution (skin/acid ratio =
and the portions from the abdominal area will	1/7) and storage in the fridge.
tear upon cleaning, which indicates that the	After 24 hours the skin increased its volume
soaking time was too long for them. The	considerably, it started peeling off, although with difficulty
supernatant, which is colourless and perfectly	and incompletely, which is why it was left for another 24 h
clear, has a rather high viscosity, which	in acetic acid, just as in the case of direct treatment with
means that a part of the collagen has already	acetic acid.
been extracted. The collagen was	After 48 hours the epidermis was cleaned, the clean skin
immediately separated by decanting, and then	was chopped into small pieces and then inserted into 0.5M
stored in the fridge.	acetic acid solution.
A new amount of cold acetic acid was	Both the vessel containing skin and acetic acid and the

inserted over the cleaned and chopped skin,

using the same ratio, and it was left in the

fridge for extraction (Fig. 1).

 TABLE III

 Collagen Extraction from Shark Skin by Means of Acid Treatments

Both the vessel containing skin and acetic acid and the colourless and relatively viscous supernatant were placed into the fridge. After 5 days the viscous supernatant was separated from the undissolved skin by decanting, and the two samples were mixed.



Fig. 1 Partly cleaned skin soaked in 0.5M acetic acid solution

III. BRILL SKIN

Given the experience gained in extracting collagen from shark skin, for brill only the sodium chloride pre-treatment was applied, followed by the extraction of collagen using 0.5M acetic acid solution.

The skin was peeled off the brill, as can be noticed in Fig. 3, then the muscle remains were cleaned off the skin chunks, the



Fig. 2 Partly cleaned skin soaked in 0.25M sodium acetate and 0.25M acetic acid solution

skin was washed using tap and distilled water, squeezed as thoroughly as possible, and placed in the freezer for conservation. In the case of brill, removing the epidermis prior to extracting collagen is out of the question, due to the bony scales which are very well rooted in the skin. Therefore, the skin was cut into very small pieces, to enable the removal of the scales.

 TABLE IV

 Collagen Extraction from Brill Skin by Means of Acid Treatments

Pre-treatment		Collagen extraction using 0.5M acetic acid	
		solution	
	As far as the pre-treatment is concerned, it was	After being squeezed and dabbed with filter	
	applied for 24 hours at room temperature, stirring	paper, the chunks of skin were inserted into cold	
	for 2 hours prior to removal, in two variants:	0.5M acetic acid solution, the first bony scaled	
	- using 0.4M (2.34%) sodium chloride, as	skin/solution ratio being of 1/2. For about 3 hours they	
	identified in specialised literature [13];	became well soaked, virtually absorbing all the	
	- using 10% sodium chloride, just as for shark	acetic acid solution. After adding acetic acid to	
	skin.	reach a ratio of $\frac{1}{4}-\frac{1}{4.3}$, they were left for another	
	During the stirring stage, the colour of the salt	31 hours in contact with the acid (for a total of 34	
	solution became more intense, particularly so for the	hours).	
	more diluted solution. At the same time, the skin in	After 24 more hours a very viscous mixture was	
	the 0.4M solution seemed slightly degraded (ragged)	obtained, from which the scales could be removed	
	on the muscle side and the smell of the solution was	manually, but incurring serious gel losses. To reduce	
	stronger, while the one in the 10% solution was	the viscosity of the mixture and thus enable an easier	
	firm, and the solution was somewhat less smelling;	removal of the scales, more acetic acid was added,	
	both chunks of skin were soft.	reaching a ratio of 1/5.8.	
	The chunks of skin were removed from the salt	The denaturation temperature of collagen	
	solutions, were washed with tap water, well	extracted from brill skin could not be found in the	
	squeezed, then washed with distilled water, left in it	disappearance of cal during continuous manual	
	for 24 hours, washed again with distilled water and	disappearance of get during continuous manual stirring this temperature can be deemed to be	
	an inspection was performed to check the absence of	between 22 °C and 23°C	
	chlorine ions.	Detween 22 C and 25 C.	

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The collagen thus obtained also includes, of course, remains of epidermis (coloured in a light grey), which is impossible to separate without a centrifugal device that can enable operation



Fig. 3 Brill: a – unskinned; b – skinned

IV. ANGUILLA SKIN

Anguilla can be skinned much more easily than the other two species of fish; two full strips can be obtained from each anguilla, virtually containing no muscles, as can be noticed in Fig. 4. The skin was washed well with tap water, then with distilled water, then it was squeezed and placed into the freezer. During the washing and squeezing procedures water got coloured, which means that a part of the pigments in the epidermis were removed.

at low temperature, in order to avoid collagen denaturation

(the centrifugal device in the laboratory only allows work at

room temperature) - see Fig. 3.

10% sodium chloride solution at		0.5M acetic acid solution
room temperature, for about		
A first, preliminary	The treatment performed in	The skin thus treated was inserted into
attempt consisted of	view of collagen extraction	0.5M acetic acid solution (1/5 ratio). For the
introducing a small	consisted of placing the skin in	next two hours it increased its volume very
amount of skin into 0.5M	10% sodium chloride solution at	much and remained unsubmerged, and
acetic acid (1/10 ratio)	room temperature for about 65	therefore more acetic acid solution was added,
and storing it in the	hours in order to extract non-	reaching a 1/6 skin/solution ratio.After 2 hours
fridge, in order to	collagenic proteins and fat, while	the epidermis would not peel off, only some of
establish whether this	the salt solution became slightly	the scales.
treatment allowed the	coloured – in a yellowish beige –	In 20 hours the skin soaked considerably,
removal of the epidermis,	and also somewhat opalescent.	increasing its weight 2.52 times, while the
which was intensely	After washing intensively	epidermis peeled off quite easily.
coloured. After about 65	using a tap water jet and after	After cleaning the epidermis, which causes
hours the skin was	squeezing well, the skin was	the loss of quite a large amount of gel from its
considerably soaked, the	washed with distilled water until	opposite side, the skin was washed in the
epidermis peeled off, but	achieving full removal of	acetic acid solution in which the soaking was
the one on the abdomen	chlorine ions (examination	performed, in order to remove any remains of
would partly tear upon	performed using a silver nitrate	epidermis, then it was dabbed with filter
cleaning, so the contact	solution), then it was squeezed	paper, chopped and inserted into 100 ml of
time with the acetic acid	again and dabbed with filter	0.5M acetic acid solution (skin/solution ratio
would have to be	paper. Following all these	1/3.25). After 18 hours the mixture is very
reduced.	operations, the weight of the skin	viscous and another 100 ml of solution was
	only increased by 3.4%, which	inserted - while stirring (skin/solution ratio
	indicates a very low level of	1/6.5).
	soaking.	

TABLE V Collagen Extraction from Anguilla Skin by Means of Alkaline and Acid Treatments

The denaturation temperature for collagen extracted from anguilla skin was not identified in specialised literature, either,

which is why the same manner was used as for brill skin to estimate that temperature, namely by visually observing the disappearance of the gel, and hence that temperature is between 25 and 26° C.

V. THE RHEOLOGICAL BEHAVIOUR OF GELS

As shown in the introduction, the collagen extracted from fish skin has a 10-25°C lower denaturation temperature than that of the collagen extracted from terrestrial mammals, which





creates difficulties for the extraction process and implies

special equipment – a thermostat-controlled room or chamber – in order to perform the extraction and the rheological measurements in the case of most species, for temperatures

about 20°C below room temperature. Besides, gels obtained

from fish skin have a considerably more reduced viscosity.

Fig. 4 Anguillas (a) and anguilla skin from which the epidermis was partially removed (b)

In order that the triple-helix structure remains stable within these gels at room or human body temperature, they must be stabilized by reticulation.

Reticulation has been performed by using glutaric aldehyde at 4-6°C for different concentrations of collagen derived from all three species of fish.

The rheological behaviour was determined by using a Haake VT 550 rheo-viscometer with the following layout:

- S₃ (MV1) sensors system, d₁ and d₂ measuring ranges, shearing speed range, $\dot{\gamma}$, between 1.17 and
- 1872 s^{-1} . The measurements were initially performed at a $25 \pm 0.1^{\circ}$ C temperature, with a thermostatic time of 20 minutes, and then using ice at $20 \pm 0.1^{\circ}$ C.

In order to establish whether the dispersed systems show a time-dependant rheological behaviour or not, measurements were performed when increasing and decreasing the shearing speed values, which allowed the tracing of the hysteresis loops for the film-forming systems with time-dependant rheological behaviour. For systems which are more resistant to shearing, a second and even third cycle of shearing speed increasedecrease was performed. At the same time, measurements were performed in the strict order of shearing speed variation (increase or decrease), moving from one measuring field to the other, given that the two fields have interposing values (for d₁ the shearing speeds vary between 11.69 and 1170 s⁻¹, while for d_2 they vary between 1.17 and 1872 s⁻¹). If only the d_2 field – which has the largest shearing speed interval - had been used, then the differences between these speeds would have been too large at high shearing speeds.

The viscosity values taken into account are those resulted from the values obtained when increasing shearing speed, as the systems are damaged to a less extent under shearing stress. In some cases, calculations were also made based on values obtained when decreasing shearing speed, or even when it was increased for a second time, but that was only to emphasize the time-dependent rheological behaviour, where applicable.

Figs. 5, 6 and 7 show the hysteresis loops for gels obtained from shark, brill and anguilla skin, respectively.



Fig. 5 Rheograms obtained for shark when: D – increasing; E – decreasing shearing speeds



Fig. 6 Hysteresis loop obtained for gel extracted from brill skin when: C – increasing; D – decreasing shearing speeds



Fig. 7 The hysteresis loop for anguilla during the second cycle of shearing speed increase-decrease: E – increase; F – decrease

VI. CONCLUSION

To obtain good performance and type I non-denatured fibrillar collagen, the extraction of collagen from the skin of marine fish species employed, namely shark, brill and anguilla, must imply the following operations:

- removal of non-collagen substances by pre-treatment with sodium chloride solution, preferably 10%, at room temperature for 24 hours; simultaneously, it is degreased and deodorized;
- removal of salt by exhaustively washing it with tap water and distilled water until all chlorine ions have disappeared, at the same time as fat and most of the smell;
- treatment of pre-treated skin with 0.5M acetic acid solution for 24-48 hours to remove the epidermis, in the case of shark and anguilla skin, and the placoid scales, in the case of brill skin;
- extracting collagen from the cleaned and chopped skin, at 4-6°C, by using 0.5M acetic acid solution, to prevent degradation;
- filtering the extracted collagen in order to separate it from skin remains either at the same temperature or by centrifugation at the temperature of the extraction process, if possible;

- storing the collagen in appropriate conditions, possibly preserving it by adding sodium nitride.

The conclusions we have reached confirm the fact that the gels based on collagen extracted from shark and brill skin have an ideal plastic behaviour, which allows their use for creating different pharmaceutical formulations.

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