International Journal of Medical, Medicine and Health Sciences
ISSN: 2517-9969
Vol:2, No:7, 2008

Rheological Characterisation of Collagen Gels from Marine Resources of Black Sea and Chlohexidine Salt for using in Dental Medicine

Sirbu R., Negreanu-Pirjol T., Leca M., Bechir A., Maris M., and Maris D.

***Abstract*—**In the paper we presented the possibility of application collagen gels with active principle's from marine algae extract and chlorhexidine salt in dental medicine. The hydro-alcoholic extracts from marine algae have been used as they have been obtained. The extracts from marine algae and chlorhexidine salt (digluconate) are incorporated in type I non-denatured fibrillar collagen matrixes. In order to obtain therapeutic effects at nanostructure level, it is important to know the rheological characteristics of the relevant mixtures of collagen gels and extracts from marine algae selected for use. In this survey we have studied mixtures made of non-denatured fibrillar collagen hydro-gels where different concentrations of marine algae have been incorporated. Based on the data obtained for the shearing tensions, we have traced the rheograms – the diagrams for shearing tensions depending on the shearing speed values – from which we have calculated the apparent viscosities as ratios between shearing tension and speed values, which have been figured in relation to the shearing speed values, with a view to levelling dependency.

***Keywords*—**rheological properties, fibrillar collagen hydro-gel, marine algae, chlorhexidine salt, dental medicine.

I. INTRODUCTION

DISEASES of the oro-dental tissues are a great interest in dental medicine. The interest shown for the periodontal diseases has increased since it has been scientifically proved that there is a connection between cutaneous-mucous diseases and the periodontopaties, with different degrees of severity, within systemic morbidity: arteriosclerosis, myocardial infarction and cerebral haemorrhages.

Anti-inflammatory ingredients can be used in regenerative therapy for tissues affected by the periodonthal disease; these ingredients are obtained from natural resources such as marine algae, which have proved to have an anti-inflammatory action on some negative Gram and positive Gram germs [1].

Chlorhexidine (1,1-hexametilen-bis-[5-(p-clorfenil)-biguanide) is a bisguanidic derivative substitute which is used in antiseptic pharmaceutical products, for the treatment of oro-dental diseases (in stomatitis, gingivitis, periodontal syndromes and prevention of dental plaque). Chlorhexidine salt activity is due to the spectral action upon the vegetative forms of Gram positive bacteria *(Staphylococcus aureus*, *Streptococcus faecalis*) and fungi (*Candida albicans*) but with less action on the Gram negative bacteria (*Escherichia coli, Proteus, Bacillus, Pseudomonas aeruginosa*). The extracts from marine algae and chlorhexidine salt were incorporated in type I non-denatured fibrillar collagen matrixes.

R. Sirbu is with the "Ovidius" University of Constanta, Faculty of Pharmacy, 300, Mamaia Bvd., Constanta, Romania (corresponding author to provide phone: 0040-241-670344; e-mail: rodicasirbu3@yahoo.com).

T. Negreanu-Pirjol is with the "Ovidius" University of Constanta, Faculty of Pharmacy, 300, Mamaia Bvd., Constanta, Romania (e-mail: ticuta_np@yahoo.com).

M. Leca is with the University of Bucharest Faculty of Chemistry, Bucharest, Romania, (e-mail: minlec@yahoo.com).

II. MATERIALS AND METHODS

A. The Collagen Matrix Methods from Literature

One such work describes the preparation of collagen from the skin of a small-sized shark, usually known as spiny dogfish shark (*Squalus acanthias*), a cold water fish whose collagen is characterised by a very low denaturation temperature, namely 14.3oC, as found by viscosity measurements, or 16oC, as found by optical rotation measurements.

Extraction consists of the following operations: dissection from the muscle, cutting into small pieces, suspension in approximately 10 volumes of 0.5M acetic acid between a temperature of 0-5^{o}C. All operations were performed below 5^{o}C because of the collagen's low denaturation temperature, [2]. After soaking for a few days and stirring, the soaked pieces of skin were disintegrated using a glass homogenizer. After a few more days of stirring, the suspension was filtered through a thin-spun cloth and then centrifuged at 15000 rpm for one hour. The protein was precipitated by adding sodium chloride with a final concentration of 10%. The collagen was collected by centrifugation, then re-dissolved in 0.5M acetic acid, centrifuged and precipitated by dialysis against a large volume of disodic phosphate. Then it was dissolved in 0.5M acetic acid, dialysed for a long time against the same solvent, then centrifuged and lyophilised [3].

In a different paper, is a description of various methods deemed as optimum for the extraction of collagens solubilised by alkalis, prepared by means of both alkaline-acid extraction and direct alkaline extraction [4]:

(a) alkaline-acid extraction, which consists of pre-treatment using 0.5M concentrated solution of sodium hydroxide containing 15% sodium sulphate for 5 days at 20^{o}C, followed by acid extraction [5,6];

(b) directly alkaline extraction, involving treatment with concentrated solution of sodium hydroxide containing 10% sodium chloride for 20-30 days at 4°C [7,8].

B. Innovative Method

For preparing gels containing hydro-alcoholic marine algae extracts and chlorhexidine salt the concentrations were: 0.6% collagen, 5 and 10% by weight for algae extracts and 0,2 % chlorhexidine digluconat. We have not used higher concentrations for the algae extracts or for the ethylic alcohol because the extracting of the components in ethylic alcohol p.a. could involved a decrease of the collagen-based gel viscosity, big quantities of it being likely to actually lead to its destruction (dissociation into two phases, one rich in water, the other rich in collagen). The hydro-alcoholic extracts from marine algae have been used, without being previously diluted or concentrated in order to be brought to the same concentration, which means that the gels which contain prepared algae extracts do not have the same concentration of dry ingredients [9,10]. The characteristics of the hydro-alcoholic extracts obtained from the three marine algae are presented in Table 1. The gels with 0,6 % collagen, 5% respectively 10% hydro-alcoholic extract and 0,2 % chlorhexidine digluconat have been prepared at room temperature from the initial gel with 1,64 % collagen, by adding the relevant quantities of distilled water, marine algae hydro-alcoholic extracts and 0,2 % chlorhexidine digluconat solution, under shaking conditions.

The marine algae from Black Sea coast used in this work were: *ULVAE LACTUCA, CYSTOSEIRA BARBATA, CERAMIUM RUBRUM* (Table 1).

Ulvae lactuca has a pale green to dark green thalle, with the aspect of an irregular leafy blade. It can reach dimensions of 5 to 30 cm, and sometimes more. *Ulva lactuca* vegetates throughout the whole year, with its peak in winter-spring, at low depths, close to the surface of the thalle.

Cystoseira barbata is a large brown monoic alga, 1,5 – 2 m, which grows in the Black Sea on a rocky substratum, as multiannual associations.

Ceramium rubrum, Div. Rhodophyta, Subcl. Florideophycidae, Fam. Ceramiaceae (Huds.) is a red algae grow abundantly and spontaneous in the Black Sea. Rhodophyta are the source to produce agar-agar and carrageen used to improve the chemical and physical characteristics of several industrial products and in the production of tissue culture media.

Chlorhexidine (CHX) (Fig. 1) is a weak base, low soluble in water. To increase its water solubility, CHX forms salts with gluconic acid (CHx-digluconate 20g/100 mL), available on market as a 0,20% aqueous solution. One of the quality assays of European Pharmacopoeia regarding this solution is the content of *p*-chloroanyline that should be <500ppm (*p*-chloroaniline results as a decomposition under light action, during a long period of storage). Clorhexidine digluconate decomposition is related to the *p*H of the solution and of the temperature of storage. If the solution is stored la 400C, the amount of p-chloroanyline is over the value after one month. There are no solid composition of CHX (chemical stable), water soluble that can form clear solutions.

TABLE I
CHARACTERISTICS OF THE HYDRO-ALCOHOLIC EXTRACTS OBTAINED FROM THE THREE MARINE ALGAE

Algae species	Dry substance contents (g/100 g solution)	pH	Solution aspect
CYSTOSEIRA BARBATA	3,68	4,0	Brown in thick layer, yellowish-green in thin layer
ULVAE LACTUCA	2,51	4,5	Dark green with brownish hues in thick layer, green in thin layer
CERAMIUM RUBRUM	2,88	4,0	Dark green with slightly brownish hues in thick layer, green in thin layer

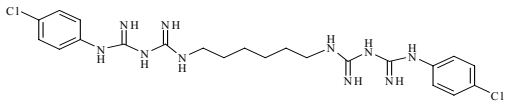

Fig. 1 Chlorhexidine base

C. Preparing gels of type i, non-denatured fibrillar collagen with algae extracts

The gels with 0.6% collagen, 5% respectively 10% by weight hydro-alcoholic algae extracts and 0,20% chlorhexidine digluconate were prepared at room temperature. Based on certain previous rheological measurements performed with non-denatured fibrillar collagen Type I gels, some of which do not and some of which do contain ethyl alcohol with a *p*H level of 3. Lyophilisation alone caused the solubility and emulsifying capacity decrease. Freeze drying is an important process in sample preparation and for the preservation and storage pharmaceuticals [11÷13]. We used Labconco Freeze-Dry systems with 12L capacity, -5 °C temperature. The gels studied are shown in (Table 2). Gels have been subjected to the rheological measurements at 25 ± 0.1^0C, after at least 15 minutes of thermostatic treatment at the above-mentioned temperature. To this effect, we have used a rotation viscosimeter Haake VT 550 with coaxial cylinders, which is capable of developing a shearing speed, $\dot{\gamma}$, with values between 0.6 and 3.0 x 10^4 s^{-1}, of measuring shearing tensions, τ, with values between 1 and 10^5 Pa and, depending on the sensors system used, of measuring apparent viscosities, η^*, between 1 and 10^9 mPa.s.

III. RESULTS AND DISCUSSIONS

First it is presented the rheological behaviour of gels containing ethyl alcohol in the mentioned quantities, but that do not contain algae extracts.

TABLE II
THE CHARACTERIZATION OF THE COLLAGENIC GELS

Algae extract	The gel's aspect	No. Samples
Gels without algae extract	Colourless, clear Colourless, clear Colourless, barely opalescent	SAMPLE 1 SAMPLE 2 SAMPLE 3
Gels with Brown algae *Cystoseira Barbata*	Opalescent, yellowish green More opalescent, brownish yellow	SAMPLE 1 SAMPLE 2
Gels with Green algae *Ulvae Lactuca*	Clear, barely green Barely opaque, barely green	SAMPLE 1 SAMPLE 2
Gels with Red algae *Ceramium Rubrum*	Barely opalescent, yellowish green A degree more opalescent, brownish yellow	SAMPLE 1 SAMPLE 2

A.1. Gel without ethyl alcohol

Collagen gels 0,6% from the first row of table 2 that do not contain ethyl alcohol and that have been maturated for at least 12 hours at the temperature of 4°C, have been subjected to the rheological measurements under the above mentioned conditions.

For the gel that does not contain ethyl alcohol solution, measurements could be carried out for shearing speed ranging between 48,6 and 1312 s^{-1} while the rheogram obtained is described in Fig. 2. As the curve inclination decreases according as the shearing speed increases, the rheogram in Fig. 2 confirms the pseudoplastic behaviour of the collagen gel. The collagen gel, being relatively diluted, presents an ideally plastic behaviour trend at high shearing speeds of over cca. 440 s^{-1}, which means that the gel does not continue to modify its structure at shearing speeds exceeding the mentioned value (very diluted gels present this behaviour)

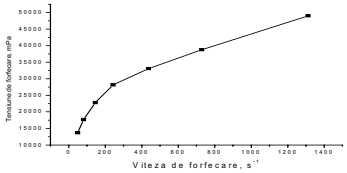

Fig. 2 Rheogram of gel with collagen concentration of 0,6%, without ethyl alcohol

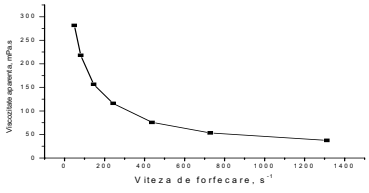

Fig. 3 Fluctuation of apparent viscosities with shearing speeds for the collagen gel without ethyl alcohol.

The apparent viscosities have been calculated for every point on the rheogram as ratio between the shearing stress and speed. Variation curve of apparent viscosities with shearing speeds for this gel is presented in Fig. 3. This is also representative for materials with pseudoplastic behaviour and it presents a more pronounced decrease for small shearing speeds and tends to a limit value at high shearing speeds.

A.2. Gel containing 5% ethyl alcohol

If 5% weight solution of 70% ethyl alcohol solution (3,5% alcohol) is introduced into gel, its viscosity decreases and the system crashes faster, as it can be seen in the rheogram in fig. 4 which shows both that this gel has lower values of the shearing stress for all shearing speeds and destructurizes considerably stronger at the highest shearing speed of 1312 s^{-1}, the value of the shearing stress at this speed turning just a little higher than 729 s^{-1} (Fig.4 and Fig. 5).

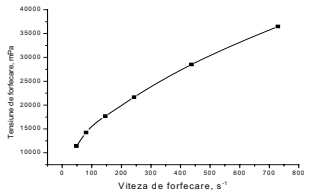

Fig. 4. Rheogram of 0,6% collagen gel containing 5% of 70% ethyl alcohol solution.

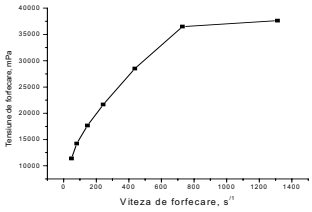

Fig. 5. Rheogram from figure 3, except for the last point.

The value of 2110,2 mPa s is obtained from the origin ordinate for the viscosity at zero shearing speed, a value 1,6 times smaller than the one for the gel with the same collagen concentration without ethyl alcohol. This proves that the presence of ethyl alcohol in collagen gel produces the decrease of viscosity, probably because of the higher interaction between water and ethyl alcohol than between water and collagen, so that water interacts prevailingly with alcohol.

A.3. Gel with 10% ethyl alcohol

If the concentration of ethyl alcohol within collagen gel is doubled, maintaining constant the concentration of the latter, the rheogram does not present any more a strong destructuration at the shearing speed of 1312 s^{-1}, but the behaviour is pseudoplastic along the entire field of shearing speeds on which measures have been performed, between 48,6 and 1312 s^{-1}, as it can be observed in Fig.6.

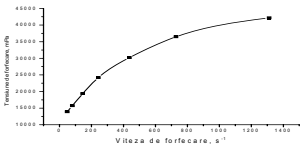

Fig. 6. Rheogram of collagen gel containing 10% of 70% ethyl alcohol solution.

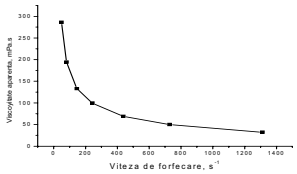

Fig. 7. Fluctuation of apparent viscosities with shearing speeds for the gel containing 10% of 70% ethyl alcohol solution.

The form of the rheogram in figure 5 is similar to that of 0,6% collagen without ethyl alcohol but the rheological behaviour is the same along the entire range of shearing speeds on which measures were performed. This means that the levelling of variation curve of apparent viscosities with shearing speeds will be better (the correlation coefficient will be higher). Fig. 7 shows that, indeed, the curve indicating the decrease of apparent viscosities with the increase of shearing speeds is very similar to that obtained for the collagen gel without ethyl alcohol, but the values are a little lower. In order to facilitate the comparison of rheological behaviour of the three collagen gels that do not contain algae extracts but contain or not 70% ethyl alcohol solution, viscosities at zero shearing speed and types of rheological behaviour of collagen gels with concentrations of 0,6% which do not contain/contain 70% ethyl alcohol solution are presented in Table 3.

TABLE III
CHARACTERISTICS OF 0,6 % COLLAGEN GELS WHICH DO NOT CONTAIN/CONTAIN THE MENTIONED RATES OF 70% ETHYL ALCOHOL SOLUTION

70% alcohol content in gel, % (g/100g gel)	Viscosity at zero shearing speed, mPa.s	Rheological behaviour
0	3377,29	Pseudoplastic, ideally plastic at high shearing speeds.
5	2110,20	Pseudoplastic, destructuration at high shearing speeds
10	3413,86	Pseudoplastic along the entire range of shearing speeds.

B. Rheological Behaviour Of Collagen Gels Containing Hydro-Alcoholic Extract Of Cystosteria Barbata

B.1. Gel with 5% alcoholic extract

By adding a 5% hydro-alcoholic extract of 3,68% of *Cystosteria Barbata* to the gel with final collagen concentration of 0,6% generates a certain opalescence of the system that can be explained as a proof of reduced compatibility or-if the opalescence is stronger – even incompatibility. Rheological measurements performed with this system, certainly after its maturation at 4°C, on the range of shearing stress between 81 and 1312 s^{-1}, have led to the rheogram in Fig. 8.

B.2. Gel with 10% alcoholic extract

By adding a 10% *Cystosteria Barbata* extract with concentration of 3,68% to dry matter results both in the intensification of gel colour and the increase of opalescence. Nonetheless, the values of shearing stress are slightly higher than those for the prior concentration for the majority of shearing speeds, which means that the viscosity is higher. Therefore, by using this quantity of extract, the components from the extract of this alga are not completely incompatible with the fibrillar collagen (does not reside collagen).

The rheogram obtained for the collagen gel containing 10% by weight hydro-alcoholic extract from the mentioned alga, with the rheological measurements performed on the same range of shearing speeds as well as for the prior system, is indicated in fig. 9.

Fig. 9 indicates that this gel has a pseudoplastic behaviour along the entire range of shearing speeds as the previous had.

The main characteristics of 0,6% collagen gels containing *Cytosteria Barbata* extract, prepared and studied from a rheological point of view, are indicated in Table 4.

TABLE IV
CHARACTERISTICS OF 0,6 % COLLAGEN GELS CONTAINING CYSTOSTERIA BARBATA EXTRACT

Content of extract in gel, % (g/100g gel)	Viscosity at zero shearing speed, mPa.s	Rheological behaviour
5	2084,97	Pseudoplastic
10	2813,94	Pseudoplastic

C. Rheological behaviour of collagen gels containing Ulvae lactuca extract

C.1. Gel with 5% alcoholic extract

By adding a 5% *Ulvae Lactuca* extract with concentration of 2,51% to the gel whose final concentration is 0,6% in collagen, the result is a clear, barely green gel, possibly indicating that there is a compatibility between collagen and the components extracted from the alga. The rheological measurements were performed for shearing speeds between 81 and 1312 s^{-1}, the same as for the corresponding gel with *Cystosteria Barbata* extract and they lead to rheogram in Fig. 10 which indicates that this gel also presents a pseudoplastic behaviour along the entire range of shearing speeds.

C.2 Gel with 10% alcoholic extract

If the amount of alga extract is doubled, the gel becomes slightly opalescent, which may indicate a slight incompatibility with this concentration of alga extract. At the same time, rheological measurements can be performed at lower shearing speeds, starting with 48,6 s^{-1} and to the maximum speed of 1312 s^{-1}, which indicates that this gel has a higher viscosity than the one containing 5% hydro-alcoholic extract. The resulting rheogram is shown in Fig. 11. The

results obtained for viscosities at zero shearing speeds of the two 0,6% collagen gels containing *Ulvae Lactuca* are summarized in Table 5.

RHEOLOGICAL BEHAVIOUR OF COLLAGEN GELS CONTAINING ALGAE EXTRACTS AND 0,2 % CHLORHEXIDINE DIGLUCONATE

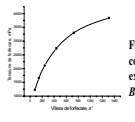

Fig. 8 Rheogram of collagen gel containing 5% extract from *Cystoseira Barbata.*

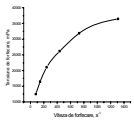

Fig. 9 Rheogram of Collagen gel containing 10% extract from *Cystoseira Barbata*

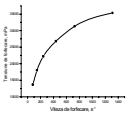

Fig. 10 Rheogram of collagen gel containing 5% extract from *Ulvae Lactuca*

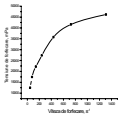

Fig. 11 Rheogram of collagen gel containing 10% extract from *Ulvae Lactuca*

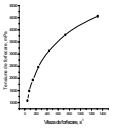

Fig. 12 Rheogram of collagen gel containing 5% extract from *Ceramium rubrum.*

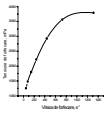

Fig. 13 Rheogram of collagen gel containing 10% extract from *Ceramium rubrum.*

TABLE V
CHARACTERISTICS OF 0,6 % COLLAGEN GELS CONTAINING

Content of extract in gel, % (g/100g gel)	Viscosity at zero shearing speed, mPa.s	Rheological behaviour
5	3248,96	Pseudoplastic
10	2978,05	Pseudoplastic

ULVAE LACTUCA EXTRACT

D. Rheological Behaviour Of Collagen Gels Containing Ceramium Rubrum Extract

D.1.Gel with 5% alcoholic extract

Ulvae Lactuca extract with concentration of 2,88% generates – when introducing an amount of 5% in the collagen gel – a certain opalescence but this is more reduced than the one generated by *Cystosteria Barbata* extract. Rheological measurements for this system were performed at shearing speeds between 48,6 and 1312 s^{-1}, which means that the gel is more viscous than the corresponding ones which contain extracts from the other two algae. The resulted rheogram with the measured data for the 5% collagen gel is shown in Fig.12.

D.2. Gel with 10% alcoholic extract

The gel containing 10% *Ceramium Rubrum* extract is a little more opalescent than the gel containing 5% but less opalescent than the one containing 10% *Cystosteria Barbata*. The rheogram obtained for the same range of shearing speeds as well as for the prior system is presented in Fig. 13. The summary of the results obtained for the two gels containing *Ceramium Rubrum* alga extract is shown in Table 6.

TABLE VI
CHARACTERISTICS OF 0,6 % COLLAGEN GELS CONTAINING CERAMIUM RUBRUM EXTRACT

Content of extract in gel, % (g/100g gel)	Viscosity at zero shearing speed, mPa.s	Rheological behaviour/remarks
5	2386,59	Pseudoplastic
10	3065,66 2656,64	Pseudoplastic, with all the points Value without the final point

- All gels were introduced in the refrigerator, at a 4oC temperature, for maturation. They were stirred from time to time during the first four hours, after which they were left to rest for 12 hours minimum and then subjected to shearing.
- After maturation, all gels with a neutral *p*H have a much higher viscosity than those with *p*H 3, so the former could no longer be submitted to rheological measurements (they are destroyed due to shearing forces).
- All 0,6% collagen gels which have the *p*H 3 and which do not contain/contain 70% ethyl alcohol solution or hydro-alcoholic extracts from the three marine algae within the same solvent have a pseudoplastic rheological behaviour; as they present a decrease of apparent viscosities along with the increase of the shearing speeds.

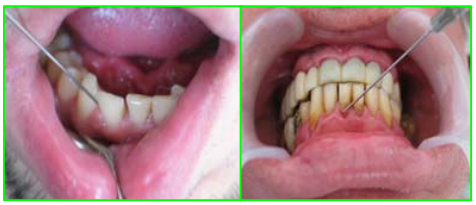

Fig. 14 The application of pharmaceutical formulation

The application of pharmaceutical formulation with controlled action in the oro-dental diseases is observed in the following images, where is visible the application of prepared gels in the periodontal pocket.

IV. Conclusions

- In the present work were obtained six gels with 0,6 % collagen containing 5% respectively 10 % hidro-alcoholic marine algae extracts from Black Sea Coast (*Cytoseria barbata, Ulvae lactuca, Ceramium rubrum*) and 0,2 % chlorhexidine digluconate.
- The obtained gels were characterised by rheological measurements and all present a pseudoplastic rheological behaviour and they have a decrease of apparent viscosities along with the increase of the shearing speeds.
- The collagenic gels were tested on periodontal diseases.
- Collagenic gels containing marine algae extracts and chlorhexidine salt could be used as pharmaceutical

formulae with optimum efficiency, having well profiled organoleptic properties.

REFERENCES

[1] Löe H. Oral hygiene in the prevention of caries and periodontal disease. *International Dental Journal*, **50** (3): 129-139,(2000).
[2] M. S. Lewis şi K. A. Piez, J. Biol. Chem. **239** (10), 3336 (1964).
[3] J. B. Parks, Biomaterials: Principle and Applications, CRC Press, Boca Raton, (2002).
[4] K. Yoshimura, M. Terashima, D. Hozan şi K. Shirai, J. Agric. Food Chem. **48** (10), 685 (2000).
[5] JP2003301144.
[6] JP2003092997.
[7] Y. Nomura, M. Yamano şi K. Shirai, J. Food Sci. **60** (6), 1233 (1995).
[8] P. Montero, C. Alvarez, M. A. Marti şi A. J. Borderias, J. Food Sci. **60**, 1 (1995).
[9] M.-T. Sheu, J.-C. Huang, G.-C. Yeh and H.-O Ho, Biomaterials **22**, 1713 (2001).
[10] J. B. Parks, Biomaterials: Principle and Applications, CRC Press, Boca Raton, 2002.
[11] K. S. Weadock, E. J. Miller, I. D. Bellincampi, J. P. Zawadsky and M. G. Duna, J. Biomed. Mater. Res. **29**, 1373 (1995).
[12] S. D. Gorham, N. D. Light, A. M. Diamond, M. J. Williams, A. J. Bailey, T. J. Wess and N. J. Leslie, Int. J. Biol. Macromol. **14**, 129 (1992).
[13] S. Yunoki, T. Suzuki and M. Takai, J. Biosci. Bioeng. **96**, 575 (2003).