3rd International Conference
"Advanced Composite Materials Engineering"
COMAT 2010
27- 29 October 2010, Brasov, Romania

COLLAGEN-BASED HYBRID POLYMER NETWORKS

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Abstract: *A series of biodegradable polymeric scaffolds was prepared by using a combination of natural (collagen, hyaluronic acid) and synthetic polymers (poly(ε-caprolactone)) in various compositions. Short-range and long-range crosslinking, physical and chemical crosslinking methods were both applied, including UV irradiation, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxy-succinimide system, sylanols and a bifunctional poly(ε-caprolactone) derivative. In order to evaluate the crosslinking efficiency the resulted porous sponges or membranes were characterized by Fourier transform infrared (FT-IR) spectroscopy, thermal analysis (DSC), measurements of the relative permittivity ε' and of the dielectric loss ε" over a large frequency and temperature range. The morphology and swelling behavior of the products were studied evidencing formulation/preparation procedure-related differences.*
Keywords: *collagen, poly(ε-caprolactone), hyaluronic acid, crosslinking, functional polymers*

1. INTRODUCTION

Collagen, as the most common extra cellular matrix (ECM) protein component and the main component of most connective tissues in the animal body, offers advantages such as biocompatibility, low toxicity and natural abundance, in addition to well-documented structural, physical, chemical and immunological properties, being one of the natural polymers preferentially envisaged for biomedical, pharmaceutical and cosmetically fields, often in relation with tissue engineering strategies [1]. Most of them have been favorably applied to healing of skin defects. The disadvantages of using collagen as an effective biomaterial, especially for tissue repair, are related to its low biomechanical stiffness and rapid biodegradation. Many efforts have been made to stabilize collagen. Crosslinking is usually used to improve the mechanical performances (strength and durability) [2]. The recipe and the conditions of crosslinking are highly important in producing collagen based scaffolds with appropriate mechano-physical, chemical, biological properties and controlled porosity. Different physical and chemical crosslinking methods were developed [3, 4]. The main physical methods, i. e. ultraviolet (UV) or dehydrothermal (DHT) crosslinking, have the advantage of cleaness but may induce partially denaturation of collagen fibers. The chemical methods are generally divided into two categories, *bi-functional* (i.e. aldheydes, isocyanates, multifunctionally activated synthetic hydrophilic polymers and mixtures thereof) *and amide-type* (i.e.,1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxy-succinimide - EDC/NHS system). However some drawbacks are associated with the use of different crosslinking systems/procedures, such as: biocompatility decrease due to the potential toxic effect of residual molecules and/or compounds released when the biomaterial is exposed to biological environments (glutaraldehyde), a decrease in elasticity and toughness accompanying the increase in tensile strength (limited zerolength crosslinks - EDC/NHS system), low coupling efficiency (EDC), possible collagen substrate denaturation and uncontrolled modification (only surface modification, instead of bulk collagen – UV and laser treatment), not enough good control on the final porosity. That's why recently the use of hybrid crosslinking systems was investigated [5], opening new routes for materials, that optimally meet the criteria for improved performances required in biomedical applications. Most of recent research shift also to the development of new collagen-based or collagen-like structures with improved properties, such as hybrid biomaterials (i.e. collagen/synthetic polymer, collagen - glycosaminoglycans) or recombinant collagens, which are biointeractive/biomimetic and will allow seamless host–graft integration. They can be introduced in such hybrid biomaterials formulations yielding polymer mixtures, composites, interpenetrating polymer networks etc. Multifunctionally activated synthetic hydrophilic polymer having two or more functional groups capable of reacting with primary amino groups on collagen molecules are often used to form a crosslinked collagen matrix

[6]. One synthetic biodegradable polymer of highest interest for biomedical applications is poly(ε-caprolactone) (PCL), because of its low cost, sustained biodegradability, availability at low molecular weight, and excellent drug permeability [7]. Recently the collagen:PCL biocomposite membranes were investigated for support of fibroblasts and keratinocytes in tissue engineered skin replacements [8]. It was proved that these materials are able to provide a further range of applications in regenerative medicine, i.e. as support matrices for dermal and epidermal skin cells to produce bi-layer skin substitutes and models.
Mucopolysaccharides are also acting as long range crosslinking agents, giving usually rise to the improvement of the mechanical properties of the resulting composite material. In this context, our interest envisaged the preparation and characterization mainly of the collagen modified with hyaluronic acid (HA), originating from its unique properties as matrix in the field of tissue engineering, such as: antithrombogeneity, ability to influence cellular functions (migration, adhesion and proliferation), hydration of ECM and binding of effector molecules (e.g. growth factors).
Our goal was to develop hybrid biomaterials that combine the bioactive features of natural polymers, like collagen and glycosaminoglycans (GAGs) such as hyaluronic acid, and physical characteristics of the synthetic ones. Considering the recent successful use of hybrid crosslinking systems [5], here short- and long-range crosslinking, physical and chemical methods were combined and applied to the different collagen-based compositions and their efficacy was comparatively evaluated. UV irradiation, EDC/NHS system, sylanols and a bifunctional PCL derivative are between them. Silanols were recently introduced, especially in cosmetics formulations, for their peculiar abilities in binding polysaccharides and glycoproteins, together with their biological activity (cytostimulation, tissue restructuration, cytoprotection, metabolic modulation), characteristics of interest for tissue engineering (i.e. skin substitutes). Photocrosslinking and the crosslinking with EDC are viable strategies for non-toxic collagen-based biomaterial preparation. Special interest was devoted to the evaluation of the efficacy of poly(ε-caprolactone) diisocyanate (PCL-DI) as a long-range crosslinker in combination with short-range crosslinking methods and reagents.

2. METHODS AND RESULTS

2. 1. Experimental procedures

Materials. The details of preparation of type I atelocollagen (AteCol) can be found in ref. [9]. Methylsilanetriol stabilized on marine collagen hydrolysate microparticles (MSHC) and dimethylsilanediol hyaluronate (DMSH) solution (DSHCN, 0.6-0.8wt%) were kindly supplied by the company EXSIMOL S. A. M. (Monaco). Poly(ε-caprolactone) glycols (PCL, Mn-2000), 4,4'- methylenebis-(cyclohexyl isocyanate) (H12MDI) Triton X-100, polyvinylpyrrolidone K 15, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccin-imide (NHS), 2,4,6- trinitrobenzenesulfonic acid solution (TNBS, 1M) and dimethyl-sulfoxide (DMSO) were purchased from Fluka (Germany). All other solvents (ethanol, acetone) and reagents of analytical grade, commercially available, were used without further purification. Bidistilled water was used for the formulations preparation.
Scaffolds preparation and crosslinking. PCL-DI was prepared by reacting vacuum dried PCL with excess H12MDI, in bulk, at 70 °C for 5h, with stirring, under inert atmosphere (nitrogen). Unreacted H12MDI was removed under high vacuum. The product was characterized by spectral (^{1}H-NMR and FT-IR) and analytical methods.
9 spongy scaffolds and 5 dense membranes of different formulations were prepared, with different ratios of AteCol and PCL, with and without dimethylsilanediol hyaluronate. These were subjected or not to short-range crosslinking methods like: (1) UV irradiation (Osram HBO 200 W super pressure mercury lamp, 14.6 W, Iv = 1100 cd) for different periods of time (10, 20, 30 or 70 min) or (2) EDC/NHS crosslinking.
Briefly, porous noncrosslinked AteCol matrices (references) were obtained after lyophilization of an acidic dispersion (0.8% (w/v), pH ~2). For AteCol-DSHCN compositions the appropriate amount of DSHCN was dropping blended with the AteCol dispersion, followed by stirring for homogenization. The compositions with MSHC were prepared in a similar manner, the microparticles being added slowly, in little amounts to the stirred AteCol dispersion. For the formulations containing PCL the calculated PCL-DI amounts, in the appropriate volume of solvent (2:3 v/v acetone:DMSO) containing 4 wt% (relative to PCL-DI) stabilizer (Triton-X 100), were added by dropping to the dispersions (AteCol or AteCol-DSHCN, respectively), the mixtures were homogenized 10 min by stirring and 5 min by sonnication. After deaeration the mixtures were frozen (-20 °C) and lyophilized with a CHRIST freeze dryer, Alpha 1-4 LSC type. The resulted sponges were irradiated according the mentioned procedure.
For comparison, samples of AteCol and AteCol-DSHCN lyophilized sponges were crosslinked by using EDC/NHS system in ethanol/water mixture (EDC:NHS:collagen carboxylic acid groups 10:10:1, ethanol mole concentration of 0.13) according to a recently developed alternative [10]. After reaction for 14 h at 4ºC, the

scaffolds were washed with saline (15 min), water (3x5min), 0.1 m Na_2HPO_4 (pH 9.1) for 30 min, phosphate buffer (3x30 min), water (3x5 min), citrate buffer (3x30 min). Part of the resulted dispersion was mixed with an appropriate amount of PCL-DI in acetone-DMSO. The prepared mixtures were degassed to remove the bubbles, frozen at -20 ºC and lyophilized.

For film preparation, the AteCol (1.8 wt%) and AteCol-DSHCN-PCL dispersions (PCL in acetone) were cast on the dish (covered with a thin film of polyvinyl pyrrolidone) after deaeration or/and centrifugation (5000rpm, 10 min), respectively, and then dehydrated by slow drying in an desiccator under vacuum, to obtain an appropriate film.

Characterization. The ^{1}H-NMR and FTIR spectra were obtained on an Avance DRX400 (Bruker) spectrometer working at 400 MHz and a Vertex 70 (Bruker) spectrophotometer, respectively. DMSO-d_6 was used as a solvent and TMS as internal standard. FT-IR spectra were recorded with the resolution of 4 cm^{-1} in the 400–4000 cm^{-1} range.

To assess the degree of collagen crosslinking, the free amino group content was determined spectrophotometrically (λ= 345 nm, SPECORD 200 Analytic Jena instrument) after reaction of the non-crosslinked amino groups of the samples with TNBS, according to an assay described by Bubnis et al. [11]). The concentration of the reacted amine groups was calculated using the following equation [11, 12]:

$$[NH_2] = (A\ V)/(\varepsilon\ l\ m) \quad (1)$$

where $[NH_2]$ denotes the reacted amine group content [in mol/g of collagen gel]; ε, the molar absorption coefficient of trinitrophenyl lysine ($1.46\ 10^4$ l mol^{-1} cm^{-1}); A, the absorbance; V, the volume of the solution [mL]; l, the path length [cm]; and m, the weight of the sample [mg]. The free amine group contents were calculated by assuming that the uncrosslinked collagen gel has 100% free amine groups [10, 13].

The denaturation temperature (Td), indicative for the degree of matrix crosslinking, was measured using differential scanning calorimetry (DSC) with a Mettler 851 system. Samples were heated from -20°C to 140°C at a heating rate of 3°C/min, in nitrogen atmosphere, with an empty aluminum pan as the reference. The denaturation temperature was determined as the peak value of the corresponding endothermic phenomena.

The porosity values of the composite sponges were measured by liquid displacement, similar to a published method [14]. Methylene chloride was used as the displacement liquid.

Measurements of the complex permittivity ε* (ε *= ε ′-j ε ″) and conductivity σ (σ=2πf ε_0 ε ″) were carried out using an dielectric and impedance analyser CONCEPT 40 (Novocontrol Technologies, Germany). over the frequency range of 10^{-2} Hz–10^6 kHz, from -100 to 100 °C.

The surface and cross-section morphologies of scaffolds were observed directly by a scanning electron microscope (SEM - Quanta 200 apparatus, working in low vacuum mode) without sputter coating by conducting matter.

The swelling capacity studies were performed at room temperature by immersing the weighed lyophilized samples of 2 x 2 x 0.3 cm in bidistilled water. At specified time intervals the samples were taken out of the water, blotted to remove surface water and weighed. The mass swelling ratio (SR) is calculated by the relation:

$$SR\ (g\ g^{-1}) = (Ws–Wd)/Wd \quad (2)$$

where Wd is the weight of dry sample, and Ws is the weight of swollen one. The equilibrium swelling ratio (ESR) is the ratio after equilibrium swelling. The experimental plot was obtained from average of three samples.

2.2. Results and discussions

The prepared collagen-based compositions are listed in Table 1. Depending on the PCL content and the sample thickness, the products apearence can be varied from semi-transparent to non-transparent.

In order to evaluate the PCL-DI efficiency, the degree of crosslinking was estimated by the free amino groups analysis, DSC and FT-IR measurements. Figure 1 shows the FTIR spectra of collagen before and after the crosslinking with contribution also of PCL-DI. The characteristic bands of collagen are situated at 3350, 3081, 1650, and 1540cm^{-1}, denoted as amide A, B, I and II. After crosslinking *via* PCL-DI the intensity of these bands is increasing, and the signal ascertained to isocyanate groups (2262 cm^{-1}) is vanishing, and disappear after irradiation, indicating the decrease of the amount of -NH_2 group in collagen and -N=C=O in the mixture, by an UV facilitated coupling. The IR absorption ratio of amide III band (1240 cm^{-1}) and that from 1450 cm^{-1} (A_{III}/A_{1450}), remain very close to unity for all matrices, confirming that the triple helical structure of collagen remained unaltered by the addition of PCL-DI, at least for low amounts in the final collagen-based matrices. However, the signals situated at 2930, 1724 and 1017 cm^{-1}, assigned to the methylene groups in the polymer matrix, the ester carbonyl and CH_2O- stretching in PCL chain are diminishing by water rinsing after longer irradiation periods (Fig. 1d), aspects associated with PCL chain photodegradation and fragmentation, occurring for more than 15 min irradiation, as concluded from further investigations. The DSC, -NH_2 titration data and dielectric properties investigations confirmed also the crosslinking efficiency for different formulations (Table 2, Figure 2).

Table 1: Collagen based materials formulation

Sample	AteCol	Second component		Crosslinking method		
	wt %	Nature	wt %	EDC/NHS	UV	PCL derivative wt %
1.0	100	-	-	-	no	0*
1.1					no	0 5 15 30
1.2					yes	0 5 15 30
2.0	100	-	-	yes	no	0
2.1					no	2
2.2					yes	2
3.0	30	MSHC	70	-	no	0 1
3.1					yes	0 1 20
4.0	100	DMSH	1	-	no	0
4.1				no	yes	2
4.2				yes	yes	2
5.0*	100	DMSH	1		no	2 15
5.1*					yes	2 15
6.0*	100	DMSH	10		no	2 15
6.1*					yes	2 15

* - dense films

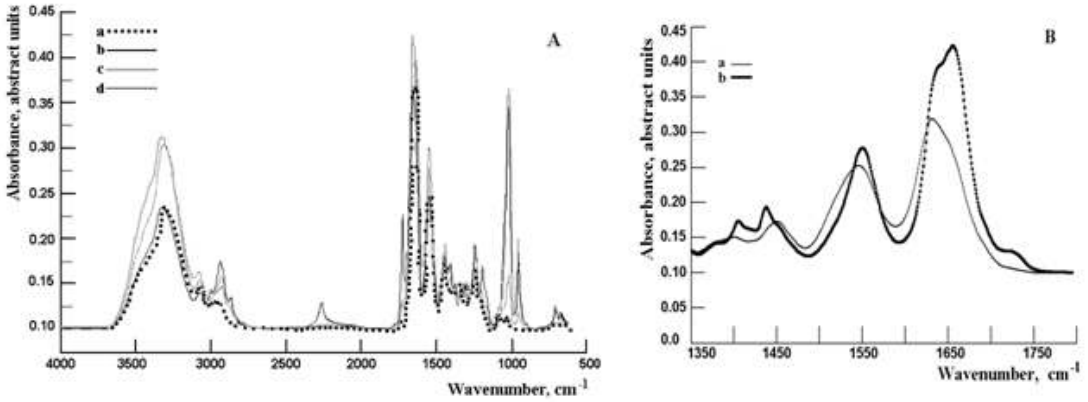

Figure 1. Effect of UV irradiation and of PCL-DI content in hybrid material formulation on the structure of collagen-based sponges. Typical integral and detailed FT-IR spectra for the samples: (A) a-1.1.0, b-2.1, c-2.2 (30 min UV irradiation), d-water rinsed 2.2 and (B) a) -1.1.0 and b)-2.2 (30 min UV) Samples code – according to Table 1

Table 2. Characteristics of the prepared colagen sponges

No	Sample Code	Td ºC	Tm ºC	Enthalpy of denaturation J g^{-1}	Porosity %	Pore size µm	Crosslinking degree %
1	1.1	70.78		-279.66	98.4	154/190	-
2	1.2.2	77.15	49.92 peak	-136.21	94.0	78/110	40.0
3	1.2.3	77.55	48.47, shoulder	-295.64	93.9	80/120	51.2
4	1.1.4	72.22	49.39; 53.03, peaks	-285.27	94.0	60/70	15.35
5	1.2.4	78.67	47.15, shoulder	-255.96	91.2	100/152	51.8
6	3.0.1	80.91		-274.87	94.9	74/74	11.9
7	3.1.3	83.91	55.99	-130.99	91.9	65/72	66.3
8	2.0	72.74		-318.30	95.6	49/150	66.1
9	2.1	75.47		-358.8	92.8	255/325	690
10	2.2	75.65		-260.2	94.9	354/376	69.6
11	4.0	92.77		-142.77	98.3	79/90	30
12	4.1	97.44		-131.30	93.7	60/65	44

The measurements of the dielectric properties revealed distinct relaxation processes at negative temperatures and around 40 °C in wet PCL crosslinked sponge, associated with PCL Tg and collagen denaturation. Dried AteCol sponge presents a large peak around 60 °C. It was assumed that PCL acts also as a plasticizer. High differences are observable between dried and wet AteCol samples, due to loosely bound water.

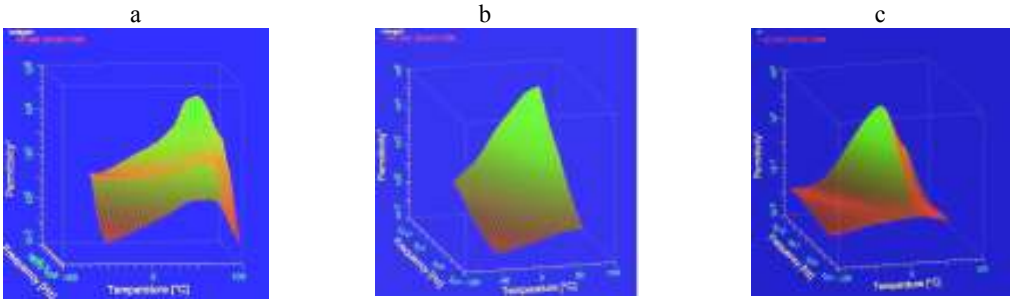

Figure 2. Temperature dependencies of the dielectric loss at various frequencies for dry (a) and wet (b) unmodified atelocollagen (samples 1.0 and 1.1.0, Table 1) and (c) crosslinked atelocollagen (sample 2.2 – 30 min UV irradiation)

Figure 3 shows that the prepared collagen-based materials exhibit mainly heteroporous morphology with inter-connected pores, ranging from 50 to 300μm (Table 2). Such pore size gradient through the scaffold can help oxygen and nutrients to diffuse toward the cells and waste products to drain out of the matrix, whereas the pore interconnec-tivity can promote cell migration and angiogenesis. Fibrillar structure of collagen is changing due to fibrils ensheeting in PCL connecting walls. More uniform/controlled pore size may be obtained by inclusion of silanol stabilized collagen microparticles. Fibrous structures are more pronounced at low PCL content, while integrally lamellar aggregates appear where PCL amount increases. Pore walls increase, and thus mechanical strength of collagen gels is expected to increase too. A lamellar structure with smaller pores is obtained by using EDC/NHS system. The partial photodegradation of PCL bridges at high UV irradiation periods (>20 min) yields the increase of matrix porosity and pore interconnectivity (Fig. 3, c, g and d, h). Meanwhile, the correlation between PCL content and the short-range crosslinking with network structure implies a relative control of the polymer networks properties.

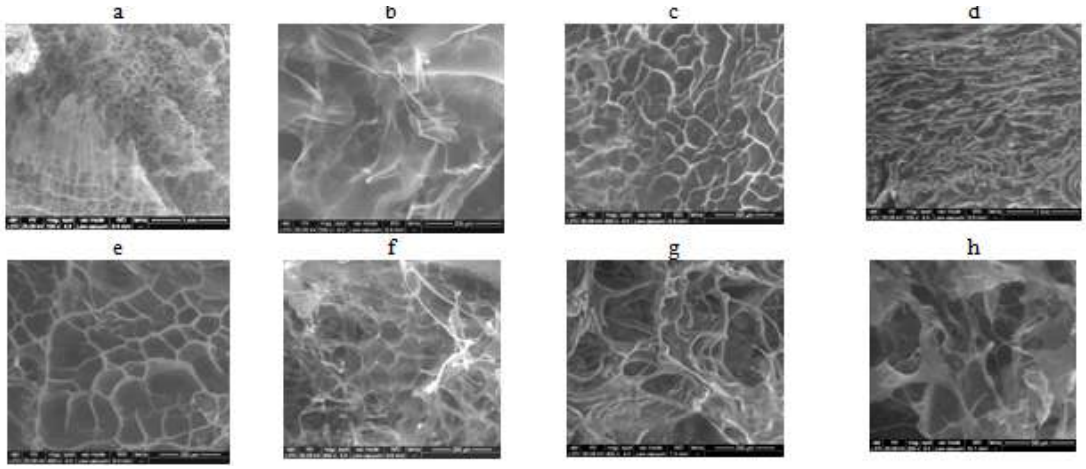

Figure 3. Typical microphotographs for uncrosslinked (a) AteCol (sample 1.1.0) and (b) AteCol-DMHS composite (sample 4.0) and crosslinked collagen–based sponges: (c)-1.1.2, (d)-2.0, (e)-3.1.3, (f)-4.1, (g)-1.2.4., (h)-2.2. f-h: UV irradiation for 30 min

Fig. 5 A, B presents the swelling kinetics for different matrices formulations and UV irradiation intervals. ESR values increase by including HA in the recipe and by increasing its amount, by decreasing the proportion of PCL-DI (Figs. 1a,b), or by increasing the UV irradiation period over 20 min. The last effect sustains the earlier observation on a possible photodegradation of PCL chains at higher UV irradiation durations. However, a low PCL concentration (≤1%) makes crosslinking difficult, yielding sponge integrity depreciation. In practice, hydrogels with high water uptake capacity and fast equilibrium swelling are preferred because of their higher permeability and biocompatibility.

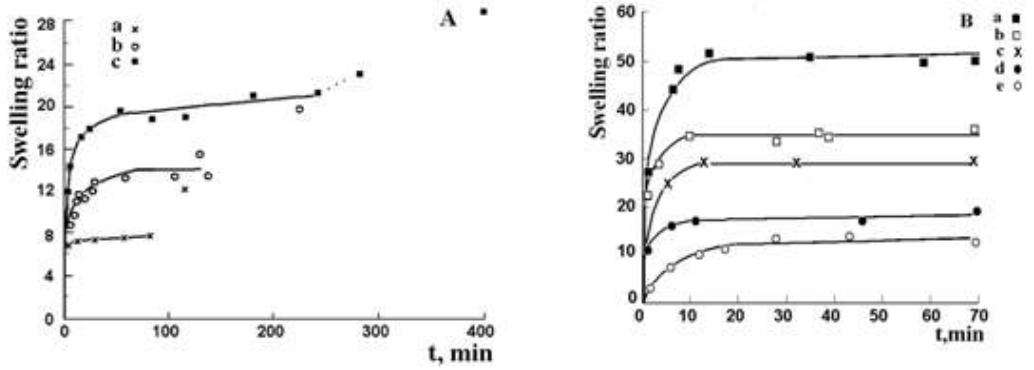

Figure 5. Influence of scaffold composition and UV irradiation time on dynamic swelling behavior.
(A) sample 3.1.2 (Table 1) after a)-0, b)-30 and c)-70 min UV irradiation
(B) samples a)-4.1, b) 4.2, c) 2.2, d) 2.0, e) 2.1 UV irradiation for samples 2.2, 4.1, 4.2 (Table 1) - 30 min

3. CONCLUSIONS

The results confirm that PCL-DI can be incorporated in the collagen cross-linking process, in order to enhance the structural stability and to modulate the properties of the resulting substrate. The biocompatible crosslinking systems allow the formation of defined hybrid collagen–HA-PCL scaffolds, with improved control on composition and structural features, and hence suitable for biomedical purposes (tissue engineering, wound dressing etc.).

ACKNOWLEDGEMENTS: The research work has been financially supported by the Romanian Ministry of Education and Research, within ANCS grant no. ID-318 / 2008, Contract no. 675 / 2009.

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