UNIVERSITY OF BUCHAREST
FACULTY OF CHEMISTRY

SUMMARY OF PH. D. THESIS

CONTRIBUTIONS ON USING COLLAGEN AS SUPPORT IN PHARMACEUTICAL FORMULATIONS FOR WOUND TREATMENT

Ph. D. adviser
Prof. dr. Minodora Leca

Ph. D. student
Lavinia Mincan (Brăzdaru)

2015

CONTENT

INTRODUCTION

Biomaterials used for treatment of wounds have to allow the development of cells and tissue regeneration, to be biodegradable and bioresorbable and the antigenic character must be absent.

Type I fibrilar collagen, carrying out all the above conditions, is the most used natural biomaterial. Moreover, it forms composites with many natural and synthetic polymers, as well as with ceramics.

Collagen based biomaterials are widely used as medical devices, scaffolds for tissue regeneration, artificial implants, supports for delivery of drags, growing factors or cells. It is used also in genetic therapy. It is also an active principle, functioning as haemostatic and dressing for wound healing. Being a protein, it is a substrate for bacteria too. Consequently, to be used for wound healing it must be associated with antibiotics and/or antiseptics.

The objective of thesis is the preparation of collagen-based biomaterials – hydrogels and porous matrices – usable as topic delivery systems for the antimicrobial compounds tannic acid and chlorhexidine digluconate, included individually or as mixtures, intended for healing of wounds of different etiologies.

The dressings must have optimum elasticity to facilitate the intimate covering of wounds, have to adhere to wounds but to be removed without difficulty to avoid tissue damage, must protect from the microbes from the environment, must be permeable for water, to prevent dehydration or accumulation of fluids, have to prevent the formation of excessive granular tissue, must be obtained with any dimension or thickness, must be non-antigenic and non-toxic, must be sterilizable, must be reservoirs of antibiotics and must protect against mechanical actions.

Tannic acid, vegetable product, reduces the pain, produces rapid hemodynamic stabilization, limits secondary infection and diminishes cicatricial tissue. It is also crosslinking agent for collagen.

Chlorhexidine digluconate, antiseptic compound administrated topically, binds on the walls of bacterial cells, having the unique property of substantivity. Moreover, it destroys fungi and their spors. Systemic absorption and toxicity are reduced, heling of wounds is more rapid, and compatibility with antibiotics is good.

Consequently, it is expected that the collagen-based biomaterials containing tannic acid and chlorhexidine digluconate be useful for curing of wounds of different etiologies.

Chapter 1 presents the role of collagen in cell growing and tissue regeneration, characteristics of biomaterials, methods for collagen crosslinking, its role and of porous dressings in chronic wound healing.

Chapter 2 contains structure, properties and using of tannic acid in medicine.

Chapter 3 presents structure and properties of chlorhexidine dicluconate as well as the mechanism of acting to destroy bacteria.

Chapter 4 describes matherial and methods used to characterize hydrogels and matrices.

Chapter 5 starts with characterization of the initial collagen hydrogel and continues with that of the diluted ones, with 0.9, 1.1 and 1.3% collagen and pH 3.8 and 7.4;1.1% was selected for preparation of biomaterials. Characterization of hydrogels with pH 3.8 and 5, 10 and 15% tannic acid, with pH 3.8 or 7.4 and 1.82, 4.55 and 9.09% chlorhexidine digluconate and of those containing the mixtures of the two components and having pH 3.8 from the point of viwes: structural – by FT-IR and UV-DC and rheologic – by stationary method to determine the flowing properties and dynamic to establish the elastic ones is discussed in paragraphs 5.3-5.5.

Chapter 6 includes characterization of porous matrices obtained by lyophilization of hydrogels from previous chapter by FT-IR – to investigate the preservation of collagen native structure and SEM – to establish matrices' morphology, which determines the delivery and allow the growing and migration of cells. The degrees of crosslinking of collagen was appreciated by water absorption and resistance to digestion with collagenase from *Clostridium histolyticum*.

Chapter 7 presents the investigation of *in vitro* delivery of tannic acid from the matrices contaning tannic acid as well as from those containing all the mixtures between the two antimicrobials in phisiological conditions, using saline phosphate buffer solution as delivery medium and a modified USP device („sandwich" device). Concentration of delivered tannic acid was determined by UV spectrofotometry. When the matrices contain only tannic acid, the highest amount is delivered by the matrix containing 10% and the lowest by that containing 5%, while when they contain mixtures of antimicrobials – by that containing the minimum amounts of tannic acid and chlorhexidine digluconate (5.00 and1.82% respectively), that is by the less crosslinked one.

Chapter 8 contines he general conclusions.

1. BIOMATERIAL CHARACTERISTICS OF TYPE I NON-DENATURED COLLAGEN

Biomaterials must allow the development of cells, produce tissue regeneration, be slightly antigenic or non-antigenic, biodegradable, and bioresorbable.

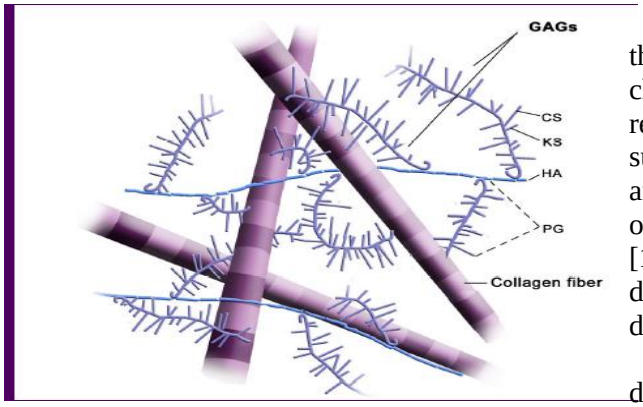
Type I collagen, the most important natural biomaterial due to its remarkable biocompatibility, reduced antigenity, [6,7] possibility to control the biodegradation [7] and formation of composites with many natural and synthetic polymers as well as with ceramics is applied in medicine, pharmacy, cosmetics, tissue engineering and orthogenesis. [8,9]

To be accepted as biomaterial, the collagen structure must be as closed as possible on that of the native collagen, of triple helix, characteristic to collagen molecule.

The collagen-based biomaterials may have different forms: hydrogels, membranes, porous matrices, fibers, tubes or composite materials. [15, 16]

1.1. Role of collagen in cellular development and tissue regeneration

Collagen, as such or associated with other components of the extracellular matrix (ECM), has an important role in the physiology and behaviour of conjunctive tissue cells. ECM consists of a complex mixture of proteins: glicosaminoglycans (GAG) and their proteoglycans (PG), adhesive glycoproteins (laminin, fibronectin, tenascein, nidogen) and fibrous proteins (collagens and elastine), [17] as can be seen in Figure 1.1.



Figurae 1.1. Scheme of MEC: HA – hyaluronic acid, CS – condroitin sulphate, KS – keratin sulphate, PG – proteoglicans, GAG – gligozaminoglicans

Collagen is a family of proteins characterized by the repeating of the sequence Gly-X-Y into α-peptide chain (Gly – glycine residue, X, Y – other amino acid residue). Capacity to bind compound makes it useful as support for delivery of drugs, growing factors and cells, and its anchoring function contributes to the formation of scaffolds for tissue repairing and regeneration. [17,21,22] It contributes also to local deposition and delivery of growth factors, plays a role during organ development, wound healing and tissue reaper. [18, 25]

Its qualities of biomaterial for cellular development and tissue repair are supported also by its particularities: biodegradability, low immunogenicity and possibility of its isolation in pure state on a large scale, which makes it indispensible for medicine, pharmaceutical, cosmetics or food industry.

1.2. Antigenic character

The collagen immunogenicity is very poor compared to other proteins. The major antigenic determinants, placed into the telopeptide regions of tropocollagen molecule, [33-35] can be eliminated. But collagen contains other two types of antigenic determinants: the triple helix and the sequence of amino acids from α chains situated on fibril surfaces. [36-40] The lower immunogenicity of collagen from fibrils compared with that from tropocollagen is due to the reduction of the access of antigenic determinants during the formation of fibrils. [41,42]

The antigenicity of telopeptides from tropocollagen is due to their amino acid composition, considerably different from that of triple helices, they containing low amounts of tyrosine.

The collagen is the best characterized protein antigen, the antigenic determinants mediating the formation of humoral anticorps being clearly delimitated. [36,38,39,60] Obtained in aseptic conditions it present special problems and at physiological ionic strength and pH tends to aggregate at 37^{o}C, in such condition the stable form being the statistical coils.

The places responsible of antigenicity, found in the short regions of tropocollagen chains, are vulnerable to the protease; they are also crosslinking places. Localization of antigenic activity into telopeptides gives the possibility to investigate the fibrillogenesis, haemostatic control and pathologic defects of collagen.

1.3. Biodegradability

Collagen, having protein structure, is biodegradable. It is degraded in tissues by catabolic processes including degradation with specific collagenase and phagocytosis. Collagenase has the unique capacity to break the α-chains in a single place. Splitting only the main chain, biodegradation with collagenase allows the evaluation of the degree of crosslinking. The amount of tripsine measures the extent of denaturizing. The fibrils are degraded from exterior, while the inside molecules become progressively accessible. [74]

Degradation of collagen *in vivo* is a complex process, its particularities depending on collagen type. Degradation *in vitro* is stimulated by incubation with bacterial collagenase, catepsine, pepsin or tripsine. Such tests allow comparison of similar materials; correlation with *in vivo* degradation is difficult. [89-92]

1.4. Control of biodegradability by crosslinking

Absorption and biodegradation velocity are adjusted by the supplementary chemical crosslinking of collagen, [81, 93-98] performed to increase the biomaterial life time and its biological stability.

The native and denatured collagen is crosslinked by physical and chemical treatments.

1.4.1. Crosslinking by physical methods

The crosslinking methods not involving chemical compounds reduce the cytotoxicity risks.

Ultraviolet radiations produce free radicals, concentrated especially into the aromatic rings of tyrosine and phenylalanine, but prolonged irradiation produces collagen denaturizing and strongly crosslinked biomaterials can not be obtained. [100] Depending on the presence or absence of water gamma radiations produce different effects: breaking of chains in its absence and crosslinked in its presence. [100]

Thermal dehydration is performed by heating of solid biomaterials in vacuum at 110^{o}C and 100 torr for some days, [101] conditions eliminating water from molecules and producing chain bonding.

1.4.2. Chemical crosslinking

Chemical crosslinking has the following advantages: reduces the biomaterial antigenity, improves mechanical and biological stability and, in some forms of treatments, reduces calcification. [101,109] The treatment reduces material imunogenity [107] and increases resistance to enzymatic degradation [50], but increases cytotoxicity. [104,108] The most used agents are aldehydes. Carbodiimids crosslink collagen without interposition of the agent; consequently the collagen does not contain foreign fragments. [109,110]

Crosslinking by ionic bonds is an alternative to the chemical one. It does not generate potential toxic residues. Polyelectrolyte as kitosan, for example, forms ionic bonds between their amine groups and carboxyl groups of collagen giving complexes that increase mechanical resistance. [113]

1.5. Role of collagen in wound management

The main function of types I-III collagen is of scaffold for conjunctive tissues. At the beginning of healing type III collagen deposits, while the amount of type I collagen increases during the process. [114]

Type I collagen, besides its role of main component of the cicatricial tissue, has a key role in: [114] control of inflammatory response at injury and reparation, influencing the cellular mitogenesis, differentiation and migration; protein synthesis into ECM; synthesis and deliberation of cytokines and grows factors; interaction between the enzymes remodeling ECM, including matriceal metalloproteinase.

1.5.1. Collagen in healing of chronic wounds

The above interactive process is degraded in chronic wounds. [128] Infection is usually the reason of chronicity. It varies from microbial colonization increasing to critical contamination and suprainfection.

The extrinsic (diabet or smoking) and intrinsic (oxygen pressure and excessive inflammatory response) factors can affect directly the collagen metabolism and slow down the healing. [114]

1.5.2. Collagen dressings in wound management

Collagen dressings are advantageous for wound treatment due to its functions in healing: [114,131] inhibition/deactivation of matriceal proteinases; increasing of fibroblasts production and permeation; contribution in taking over and biodisponibility of fibronectine; help in protection of leucocytes, macrophages, fibroblasts, epithelial cells; assistance in maintaining of chemical and thermic micro-medium.

Porous dressings are efficient in healing of wounds of different etiologies. [133-135] Using of collagen materials are not only clinically efficient, but also from economic point of view. [136-139]

R e f e r e n c e s

6. M. Chvapil, Reconstituted collagen, in "Biology of collagen", ed. A. Viidik şi J. Vuust, Academic Press, London, **1980**.
7. K.P. Rao and T. Josep, Collagen graft copolymers and their biomedical applications, în "Collagen Biotechnology", ed. M. E. Nimni, CRC Press, Boca Raton, **1988**.
8. G. Goissis, L. Piccirili, J.C. Goes, A. M. De Guzzi Plepis şi D.K. Das-Gupta, Anionic collagen: polymer composites with improved dielectric and rheological properties, Artificial Organs 22**,** 203-209, **1998**.
9. S. Omura, N. Mizuki, S. Horimoto, R. Kawabe şi A. Fujita, A newly developed collagen/silicone bilayer membrane as a mucosal substitute: a preliminary report, British J. Oral Maxillofacial Surg. 35, 85-91, **1997**.
15. V. Trandafir, şi M.G. Albu, Achievement and Perspectives in the collagen-based biomaterials, International Symposium Advanced Materials and Processes for Ecological Manufacturing of Leathers, Bucureşti, Nov. 2nd, **2007**.
16. V. Trandafir, Z. Vuluga, V. Danciu, M.G. Albu, R. Truşcă şi C. Iancu, Collagen-based composite aerogels for medical applications, Int. Symp. Adv. Mater. Processes Ecol. Manufacturing of Leathers, Bucureşti, November 2, **2007**.
17. D. Schuppan, M. Schmidt, R. Somasundaram, R. Ackermann, M. Ruehl, T. Nakamura şi E.O. Riecken, Collagens in the liver extracellular matrix bind hepatocyte growth factor, Gastroenterology 114, 139-152, **1998**.
18. Y. Yamaguchi, D.M.Mann şi E. Ruoslathi, Negative regulation of transforming growth factor-β by the proteoglycan decorin, Nature 346, 281-284, **1990**.
21. S.R. Frenkel, B. Toolan, D. Menche, M.I. Pitman şi J.M. Pachence, Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair, J. Bone Jt. Surg. 79-B, 831-836, **1997**.
22. S. Wakitani, T. Kimura, A. Hirooka, T. Ochi, M. Yoneda, N. Yasui, H. Owaki şi K. Ono, Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel, J. Bone Jt. Surg. 71-B, 74-80, **1989**.
25. E.D. Hay, Extracellular matrix, J. Cell Biol. 91, 205-223, **1981**.
33. F.O. Schmitt, L. Levine, M.P. Drake, A.L. Rubin, D. Pfahl şi P.F. Davison, The antigenicity of tropo-collagen, Proc. Natl. Acad. Sci. USA 51, 493-497, **1964**.
34. P.F. Davison, L. Levine, M.P. Drake, A. Rubin şi S. Bump, The serologic specificity of tropocollagen telopeptides, J. Exp. Med. 126, 331-346, **1967**.
35. C. Steffen, R. Timpl şi I. Wolff, Immunogenicity and specificity of collagen. V. Demonstration of three different antigenic determinants on calf collagen. Immunol. 15, 135-144, **1968**.
36. D. Michaeli, G.R. Martin, J. Kettman, E. Benjamini, D.Y. Leung şi B.A. Blatt, Localization of antigenic determinants in the polypeptide chains of collagen, Sci. *166*, 1522-1524, **1969**.
37. H. Furthmayr, W. Beil şi R. Timpl, Different antigenic determinants in the polypeptide chains of human collagen, FEBS Lett. 12, 341–344, **1971**.
38. H. Lindsley, M. Mannik şi P. Bornstein, The distribution of antigenic determinants in rat skin collagen, J. Exp. Med. 133, 1309-1324, **1971**.
39. R. Timpl, W. Beil, H. Furthmayr, W. Meigel şi B. Pontz, Characterization of conformation independent antigenic determinants in the triple-helical part of calf and rat collagen, Immunol. 21, 1017-1030,**1971**.
40. H. Furthmayr şi R. Timpl, Immunochemistry of collagens and procollagens, Int. Rev. Connect. Tissue Res. 7, 61-99, **1976**.
41. K.S. Weadock, E.J. Miller, E.L. Keuffel şi M.G. Dunn, Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions, J. Biomed. Mater. Res. 32, 221-226, **1996**.
42. M.D. Harriger, A.P. Supp, G.D. Warden şi S.T. Boyce, Glutaraldehyde crosslinking of collagen substrates inhibits degradation in skin substitutes grafted to athymic mice, J. Biomed. Mater. Res. 35, 137-145, **1997**.
50. K.R. Meade şi F.H. Silver, Immunogenity of collagenous implants, Biomater. 11, 176-180, **1990**.
60. R. Timpl, P.P. Fietzek, H. Furthmayr, W. Meigel şi K. Kűhn, Evidence for two antigenic determinants in the C-terminal region of rat skin collagen, Fed. Eur. Biochem. Soc. (FEBS) Lett. 9, 11-17, **1970**.
74. H.G. Welgus, J.J. Jeffrey, G.P. Stricklin, W.T. Roswit şi A.Z. Eisen, Characteristics of the action of human skin fibroblast collagenase on fibrillar collagen, J. Biol. Chem. 255, 6806-6813, **1980**.
81. M.E. Boon, J.M. Ruijgrok şi M.J. Vardaxis, Collagen implants remain supple not allowing fibroblast ingrowth, Biomater.16, 1089-1093, **1995**.
89. A.M. Diamond, S.D. Gorham, D.J. Etherington, J.G. Robertson şi N.D. Light, The effect of modification on the susceptibility of collagen to proteolysis: I. Chemical modification of amino acid side chains, Matrix 11, 321-329, **1991**.
90. J. Megerman, E. Reddi. G.J. L'Italien, D.F. Warnock şi W.M. Abbott, A laboratory model to quantitate the resistance of collagen vascular grafts to biodegradation, J. Biomed. Mater. Res. 25, 295-313, **1991**.
91. I.V. Yannas, J.F. Burke, C. Huang şi P.L. Gordon, Correlation of in vivo collagen degradation rate with in vitro measurements, J. Biomed. Mater. Res. 9, 623-628, **1975**.
92. T. Okada, T. Hayashi şi Y. Ikada, Degradation of collagen suture in vitro and in vivo, Biomater. 13, 448-454, **1992**.
93. R.F. Oliver, R.A. Grant, R.W. Cocs şi A. Cooke, Effect of aldehyde crosslinking on human dermal collagen implants in the rat, Br. J. Exp. Path. 61, 544-549, **1980**.
94. N. Olmo, J. Turnay, J. Herrera, J.G. Gavilanes şi A. Lizarbe, Kinetics of in vivo degradation of sepiolite-collagen complexes: effect of glutaraldehyde treatment, J. Biomed. Mater. Res. 30, 77-84, **1996**.

95. L.H.H. Olde Damink, P.J. Dijkatra, M.J.A. van Luyn, P.B. van Wachem, P. Nieuwenhuis şi J. Feijen, In vitro degradation of dermal sheep collagen crosslinked using a water-soluble carbodiimide, Biomater. 17, 679-684, **1996**.
96. L.H.H. Olde Damink, P.J. Dijkatra, M.J.A. van Luyn, P.B. van Wachem, P. Nieuwenhuis şi J. Feijen, Changes in the mechanical properties of dermal sheep collagen during in vitro degradation, J. Biomed. Mater. Res. 29, 139-147, **1995**.
97. L.H.H. Olde Damink, Structure and properties of crosslinked dermal sheep collagen, Ph. D. Thesis, University of Twente, The Netherlands, **1992**.
98. K. Anselme, H. Petite şi D. Herbage, Inhibition of calcification in vivo by acyl azide crosslinking of a collagen-glycosaminoglycan sponge, Matrix 12, 264-273, **1992**.
100. B. Chevallay şi D. Herbage, Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy, Med. Biol. Eng. Comput. 38, 211-218, **2000**.
101. K.S. Weadok, R.M. Olsen şi R.H. Silver, Evaluation of collagen crosslinking techniques, Biomater. Med. Dev. Artif. Org. 11, 294-318, **1984**.
104. I. Rault, V. Frei, D. Herbage, N. Abdul-Malak şi A. Huc, Evaluation of different chemical methods for cross-linking collagen gel, films and sponges, J. Mater. Sci.-Mater. Med. 7, 215-221, **1996**.
107. K.R. Meade, şi F.H. Silver, Immunogenicity of collagenous implants, Biomater. 11, 176-180, **1990**.
108. A. Jayakrishnan şi S.R. Jameela, Glutaraldehyde as a fixative in bioprosthetic and drug delivery matrices, Biomater. 17**,** 471-84, **1996**.
109. H.M. Powell şi S.T. Boyce, EDC cross-linking improves skin substitute strength and stability, Biomater. 27, 5821-5827, **2006**.
110. H.M. Powell şi S.T. Boyce, Wound closure with EDC cross-linked cultured skin substitutes grafted to athymic mice, Biomater. 28, 1084-1092, **2007**.
113. F. Berthod, G. Saintigny, F. Chretien, D. Hayek, C. Collombel şi O. Damour, Optimization of thickness, pore size and mechanical properties of a biomaterial designed for deep burn coverage, Clin. Mater. 15, 259–265, **1994**.
114. A. Rangaraj, K. Harding şi D. Leaper, Role of collagen in wound management, Wounds UK 7, 54-63, 2011.
128. T.A. Mustoe, K. O'Shaughnessy şi O. Kloeters, Chronic wound pathogenesis and current treatment strategies: a unifying
131. D. Brett, A review of collagen and collagen-based dressings, Wounds 20, 347-353, **2008**.
133. M.P. Curran şi G.L. Plosker, Bilayered bioengineered skin substitute (Apligraf). A review of its use in the treatment of venous leg ulcers and diabetic foot ulcers, Biodrugs 16, 439-455, **2002**.
134. H.P. Ehrlich, Understanding experimental biology of skin equivalent: from laboratory to clinical use in patients with burns and chronic wounds, Am. J. Surg. 187, 29S-33S, **2004**.
135. K.G. Harding, T. Kreig şi S.A. Eming, Efficacy and safety of the freeze-dried cultured human keratinocytes lysate, LymphoDerm 0.9%, in the treatment of hard-to-heal venous leg ulcers, Wound Rep. Regen. 13, 138-147, **2005**.
136. M. Ehrenreich şi Z. Ruszczak, Update on tissue-engineered biological dressings, Tissue Eng. 12, 1-18, **2006**.
137. D.R. Baillie, P. Stawicki, N. Eustance, D. Warsaw şi D. Desai, Use of human and porcine dermal-derived bioprostheses in complex abdominal wall reconstructions: a literature review and case report, Ostomy Wound Management 53, 30-39, **2007**.
138. K.S. Jehle, A. Rohatgi şi MK Baig, Use of porcine dermal collagen graft and topical negative pressure on infected open abdominal wounds, J. Wound Care 16, 3637, **2007**.
139. D.M. Parker, P.J. Armstrong, J.D. Frizzi şi J.H. North, Porcine dermal collagen (Permacol) for abdominal wall reconstruction, Curr. Surg. 63, 255-258, **2006**.

2. TANNIC ACID

The tannins, widely spread in vegetable products, can be hydrolysable and condensed. [1]

Hydrolysable tannins are esters of sugars, especially glucose, and of phenolic carboxylic acids, as gallic and ellagic. The model compound is tannic acid (TA) – 1,2,3,4,6-penta-O-galoyl-D-glucose. [2]

2.1. Structure and properties

TA is the commercial form of tannin. [5] It is a pentagalloyl glucose – the central part of molecule – esterified at hydroxyl groups with gallic acid (Figure 3,1). It is hydrolysable tannin. [6, 7]

TA molecular formula is $C_{76}H_{52}O_{46}$ and its mass is 1701.2 g.mol^{-1}. [4]

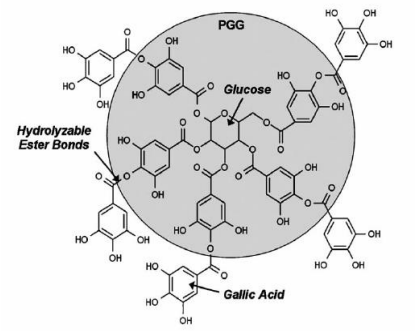


Figure 3.1. Structure of tannic acid

Tannic acids has the following physical properties: white, yellowish or slightly brown amorphous powders, inodorous or with slight characteristic odor and strongly astringent taste, very soluble in water – 2850 g/L, insoluble in alcohol and acetone, almost insoluble in benzene, chloroform and ether, decompose without melting above 200ºC and is slightly acids, the pH values ranging between 3 and 6.

Due to the free phenolic hydroxyl groups they form strong hydrogen bonding with proteins and carbohydrates, resulting complexes. [8] In addition, hydrophobic interactions may contribute to the formation of complexes. [9]

2.2. Using of tannic acid in medicine

In ‘20 TA was introduced to treat grave burns and soon became standard therapy for patients with burns, [10] due to the significant reduction of toxemia degree and massif decrease of death rate. [11] Its using presents some other advantages: local precipitation of proteins, which reduces or eliminates pain; prevention of plasma loss; limitation of secondary infection and diminution of amount of cicatricial tissue.

Antimicrobial activity of TA is well documented: inhibits increasing of many fungi, yeasts, bacteria and viruses. [14-17] TA presents also some other benefic effects: antimutagenic and anticarcinogenic activity, [14] induction of apoptosis in animal cell; [18] involving into hialuronidaze system, producing its destructions in the same way Echinacea does and intensifying its effect, defending the cells from viral invasion; [19] antioxidant action [18] and inhibition of collagenase activity from *Clostridium histolyticum*, preventing degradation of collagen from ECM. [20]

Collagen biodegradability is reduced by stabilization with crosslinking agents. It improves mechanical properties and reduces enzymatic and thermal degradation, controlling the biomaterial life. [24]

Considering the TA structure and the multitude of functional groups of collagen, hydrogen bonds, ionic interactions, hydrophobic and covalent bonds may form between the two components. [27]

TA is the tannin having the highest affinity for collagen. [35, 36] Having different hydroxyl and carboxyl groups, hydrogen bonds form in different points, giving supplementary stability to collagen. [38]

Type I collagen crosslinked with TA presents the following advantages: good enzymatic stability, higher biocompatibility and better promptness in healing of wounds compared with the native one. [41]

Given its property to inhibit the action of collagenase in ECM, [19] TA is a valuable crosslinking agent, presenting reduced cytotoxicity, as well as antimicrobial and anti-inflammatory properties. [41]

R e f e r e n c e s

1. E. Haslam, ”Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action”, Cambridge University Press, Cambridge, 1998.
2. J.D. Reed, Nutritional toxicology of tannins and related polyphenols in forage legumes, J. Anim. Sci. 73, 1516-1528, **1995**.
5. E. Haslam, ”Chemistry of Vegetable Tannins”, Academic Press, London, 1966.
6. I. Mueller-Harvey, Analysis of hydrolysable tannins, Animal Feed Sci. Technol. 91, 3-20, **2001**.
7. J.C. Isenburg, D.T. Simionescu şi N.R. Vyavahare, Tannic acid treatment enhances biostability and reduces calcification of glutaraldehyde fixed aortic wall, Biomater. 26, 1237-1245, **2005**.
8. E. Haslam, „Plant Polyphenols-Vegetable Tannins”, Revisited, Cambridge University Press, Cambridge, 1989.
9. H.I. Oh, J.E. Hoff, G.S. Armstrong şi L.A. Haff, Hydrophobic interaction in tannin-protein complexes, J. Agric. Food Chem. 28, 394-399, **1980**.
10. E.C. Davidson, Tannic acid in the treatment of burns, Surg. Gynecol. Obstet. 41, 202-221, **1925**.
11. S.B.A. Halkens, A.J.J. Van den Berg, M.J. Hoekstra, J.S. Du Point şi R.W. Kreis, The use of tannic acid in the local treatment of burn wounds: intriguing old and new perspectives, Wounds 13, 144-158, **2001**.
14. A. Scalbert, Antimicrobial Properties of Tannins, Phytochem. 30, 3875-3883, **1991**.
15. K.-T. Chung, T. Y. Wong, C.-I Wei, Y.-W. Huang şi Y. Lin, Tannins and human health: a review, Critical Rev. Food Sci. Nutr. 38, 421-464, **1998**.
16. M.M. Covan, Plant Products as Antimicrobial Agents, Clin. Microbiol. Rev. 12, 564-582, **1999**.
17. S.M. Colak, B.M. Yapici şi A. Yapici, Determination of antimicrobial activity of tannic acid in pickling process, Rom. Biotechnolog. Lett. 15, 5325-5330, **2010**.
18. N.S. Khan, A. Ahmad şi S.M. Hadi, Anti-oxidant, pro-oxidant properties of tannic acid and its binding to DNA, Chem.-Biol. Interact. 125, 177-189, **2000**.
19. http://www.appliedhealth.com/nutri/page8477.php
20. G. Krishnamoorthy, P.K. Sehgal, A.B. Mandal şi S. Sadulla, Studies on collagen-tannic acid-collagenase ternary system: Inhibition of collagenase against collagenolytic degradation of extracellular matrix component of collagen, J. Enzyme Inhib. Med. Chem. 2011 Aug 8; 21823836, (doi:10.3109/14756366.2011.596834).
24. R. Lanza, R. Langer şi J. Vacanti, ”Principles of Tissue Engineering” ed. 2-a, Elsevier, San Diego, 2000.
27. B. Madhan, V. Subramanian, J. Raghava Rao, B. Unni Nair şi T. Ramasami, Stabilization of collagen using plant polyphenoles: Role of catechine, Int. J. Biol. Macromol. 37, 47-53, **2005**.
35. Y. Fueaesaku, V. Mizuhira şi H. Nakamura, The new fixation method using tannic acid for electron microscopy and some observations of biological specimens, Proceedings of the 4th International Congress on Histochemistry and Cytochemistry, ed. T. Takeuchi, K. Ogawa şi S. Fujita, 155, 1972.
36. K. M. Meek, The use of glutaraldehyde to preserve reconstituted collagen for electron microscopy, Histochem. 73, 115-120, **1981**.
38. B. Madhan, P. Thanikaivelan, V. Subramanian, J. Raghava Roa, B.U. Nair şi T. Ramasami, Molecular mechanics and dynamics studies on the interaction of gallic acid with collagen like peptides, Chem. Phys. Lett. 346, 334-340, **2001**.
41. V.Natarajan, N.Krithica, B.Madhan şi P.K. Sehgal, Wound healing potential of tannic acid crosslinked collagen scaffolds, 63rd Indian Pharmaceutical Congress, 16-18 December 2011, Bengaluru, India, lucr. AIII-54.

3. CHLORHEXIDINE DIGLUCONATE

3.1. Structure and properties

Chlorhexidine (CH), introduced in clinical practice in 1954, [1] is a bisquanide containing two 4-chlorophenilene rings and two biguanide groups connected by a hexamethylenic chain. [2]

White crystalline powder, having the formula $C_{22}H_{30}Cl_2N_{10}$, molar mass 505.45 g/mol, melting temperature 134-136^{o}C, CH is poorly soluble in water (0.8 g/L la 20^{o}C) and can not be used as such for obtaining of collagen materials. Being a base, it is stable as a salt: [3] digluconate (CHDG), diacetate and dihydrochloride. The most used in clinical practice is CHDG, especially for skin antisepsis. [4]

Antiseptics destroy the microorganisms or inhibit their growing on living tissues [7, 8] and are administrated topically. CHDG fulfill generally the conditions imposed for and ideal antiseptic.

CHDG is active over a wide pH range, between 5 and 8, which includes the skin physiological pH. It is a strong base at this pH and presents the highest activity. [16] Dissociation produces dications with the positive charges on nitrogen atoms situated at the ends of hexamethylenic bridge. [17,18] Cationic character makes possible binding on bacterial cell walls of, [19] presenting the unique property of substantivity. [3]

3.2. Mechanisms of antibacterial action

Being soluble in water and strong base at the physiological pH of skin, CHDG is completely dissociated at this pH, eliberating CH dications. [20] As they are lipophylic, interact with phospholipids and lipopolysugars from the membranes of bacterial cells al low concentrations, favouring the entrance into their cell. [21] Antimicrobial efficacy is consequently due to interaction of CH dications with phosphate and carboxyl groups from the walls of microbial cells. [22] Thus the osmotic equilibrium is altered, the permeability of cellular walls increased and dications penetrate bacteria, [3] perturbing the metabolic processes. CH dications indirectly affect the enzymatic function of dehydrogenase and ATP-ase from the cellular walls. [21] CHDG is bacteriostatic at low concentrations and bactericide at high ones.

CHDG is an efficient antifungal agent, able to destroy not only the fungi, but also their spores. [8] It is not a sporicid, but forestalls spores development, inhibiting their effect, but not germination. [23]

R e f e r e n c e s

1. A.D. Russell, Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria, J. Appl. Microbiol. 92 Suppl, 121S-135S, **2002**.
2. G. Greenstein, C. Berman şi R. Jaffin, Chlorhexidine: an adjunct to periodontal therapy, J. Periodontol. 57, 370-376, **1986**.
3. Z. Mohammadi, Chlorhexidine gluconate, its properties and applications in endodontics, Iranian Endodontic J. 1, 113-125, **2008**.
4. T.J. Karpanen, T. Worthington, E.R. Hendry, B.R. Conway şi P.A. Lambert, Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of Staphylococcus epidermidis, J. Antimicrobial Chemotherapy 62, 1031-1036, **2008**.
7. S.S. Block, Definition of terms, în "Disinfection, Sterilization, and Preservation", ed. S.S. Block, Lippincott Williams & Wilkins, Philadelphia, 2001.
8. G. McDonell şi A.D. Russell, Antiseptics and disinfectants: activity, action, and resistance, Clin. Microbiol. Rev. 12, 147-179, **1999**.
16. http://www.burmester-pharma.de/deutsch/analyse_datenblatt/a1929.pdf
17. A. Albert şi E.P. Sargeant, "Ionization Constants of Acids and Bases", Methuen, London, 1962.
18. C.G. Jones, Chlorhexidine: Is it still the gold standard?, Periodontology 15, 55-62, **2000**.
19. W.J. Loesche, Chemotherapy of dental plaque infections, Oral Sci. Rev. 9, 65-107, **1976**.
20. G. Greenstein, C. Berman şi R. Jaffin, Chlorhexidine: an adjunct to periodontal therapy, J. Periodontol. 57, 370-376, **1986**.
21. B. Athanassiadis, P.V. Abbott şi L.J. Walsh, The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics, Aust. Dent. J. 52, S64-S82, **2007**.
22. B.P. Gomes, S.F. Souza, C.C. Ferraz, B.P. Teixeira, A.A. Zaia, L. Valdrighi şi F.J. Souza-Filho, Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine root dentine in vitro, Int. Endod. J. 36, 267-275, **2003**.
23. A.D. Russell şi M.J. Day, Antibacterial activity of chlorhexidine, J. Hosp. Infect. 25, 229-238, **1993**.

4. MATERIALS AND METHODES

4.1. Materials

Type I fibrilar collagen was supplied by the Textile and Footwear Research and Development National Institute, Leather and Footwear Research Institute branch, INCDTI-ICPI Bucharest.

CHDG (20% aqueous solution) was purchased from Fargo, Germany.
TA was bought from Sigma-Aldrich, Germany.
GA (25% aqueous solution) was bought from Merck, Germany.
Type I collagenase from *Clostridium histolyticum* was purchased from Sigma-Aldrich, Germany.

4.2. Methods of analysis

Collagen hydrogels were characterized by rapid physical-chemical methods, FT-IR, UV-CD, stationary and dynamic rheology and porous matrices by FT-IR, SEM and water absorption. The matrices with mixtures of AT and CHDG were subjected to enzymatic degradation and evaluation of delivery of TA.

4.2.1. Rapid physical-chemical methods

Rapid methods for characterization of the initial collagen hydrogels include determination of contents of: dry substance, ash, total nitrogen, proteic substance, fatty mater and pH.

4.2.2. FT-IR spectroscopy

FT-IR was used to identify the functional groups, analysis of the tertiary structure of collagen and emphasizing of interaction with other compounds (IR 6000 instrument with MKII Golden Gate Single reflection ATR system, Jasco), spectral range 4000-200 cm^{-1}).

4.2.3. Circular dichroism

UV-CD was used to emphasize the triple helical structure of collagen from hydrogels. UV-CD spectra were obtained using a Jasco J-810 spectropolarimeter, within the range 250-195 nm.

4.2.4. Scanning electron microscopy

Matrices were characterized morphologically by scanning electron microscopy (SEM). Recording of images was done using a Hitachi S-2600N microscope with 4 nm resolution (at 25 kV, in high vacuum when the detector for secondary electrons was used) and acceleration tension from 0,5 to 30 kV.

4.2.5. Rheological methods

Hydrogel viscosities, their destructuration and restructuration were determined by stationary rheology and viscoelastic behaviour by dynamic rheology.

Stationary rheological determinations were made using Haake VT 550 reoviscozimeter equipped with MV1 sensor system for medium viscosities and RheoWin 4 Thermo Fischer Scientific software.

Dynamic measurements were done with Micro Fourier Transform Rheometer MFR, GBC, Australia, functioning under squeezing flowing regime.

4.2.6. Absorption of water

Appreciation of crosslinking degrees of matrices and their capacity to absorb biological fluids was done by water absorption at room temperature.

4.2.7. Enzymatic degradation in vitro

Resistance to digestion with collagenase was studied in physiological conditions, by incubation of samples in 1µg/mL collagenase solution in saline phosphate buffer solution with pH 7.4 at 37^0C, using collagenase from *Clostridium histolyticum*, activity 125 U/mg. Degradation was followed for 9 days.

4.2.8. Evaluation of drug delivery

Amounts of TA delivered by matrices were determined using a modified USP device with "sandwich" cells. TA concentration was measured by UV spectrophotometry, at 276 nm.

5. CHARACTERZATION OF INIŢIAL COLLAGEN HYDROGELS, CONTAINING TANNIC ACID, CHLORHEXIDINE DIGLUCONATE AND THEIR MIXTURES

Hydrogels – semisolid colloid systems having aqueous disperse phase and solid disperse medium, [1,2] are defined as tridimensional networks of hydrophilic polymers able to absorb wide amounts of water

or biological fluids. [3-5] Soft consistency and high content of water make their properties similar to tissues. [8] Permeable for the dissolved molecules, they are convenient supports for drug delivery. [9-11]

Consistent collagen solution/gels are tridimensional networks of fibrils randomly interwoven by hydrogen bonds, ionic interactions and hydrophobic forces. Given the high number of hydrophilic groups they include huge amounts of water [13] and have weak mechanical properties. Their improving is done by crosslinking. But the chemical agents produce cytotoxicity and reducing of biocompatibility. [14, 16-20]

The hydrogel properties are determined by the integrity of molecules from fibrils. Thus the hydrogels must be very pure and their molecules have native conformation. Viscosity is very important, influencing the crosslinking density, drug delivery velocity and pores' size.

5.1. Initial hydrogel

Initial hydrogel, extracted from raw calf skin by basic and acid treatments, [23] was characterized by purity – by the so called rapid methods and by the integrity of triple helices of molecules from fibrils.

5.1.1. Rapid methods of analysis

The rapid methods of analysis include determinations of contents of: dry substance (evaporation at 105°C until constant mass) – 2.67%, total nitrogen (Kjeldahl method) – 0.47/17.6*, proteic substance – from the total nitrogen (multiplying by 5.67 – factor of transformation of nitrogen in protein) – 2.64/98.88*, ash (calcination at 600-800°C until constant mass) – 0% and fats (extraction with petroleum ether and evaporation till constant mass) – 0%, plus pH. The marked values are recalculated for dry substance.

5.1.2. Characterization by circular dichroism

UV-CD spectrum of type I collagen looks like that of poly-L-proline II: presents an intense minimum at 200 nm and less intense wider maximum between 220 and 225 nm, assigned to triple helix conformation [24-27] (Fig.5.1).

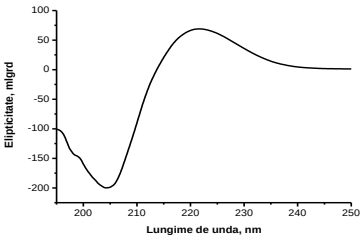


Figure 5.1. UV-CD collagen spectrum

Spectrum of collagen from the initial hydrogel, with concentration 0.35% in acetic acid 0.1 M la 22°C, using 0.2 mm quartz cuvette and a slit of 400 μm, has the minimum at 204 nm and maximum at 221 nm, according to literature. [24-27] Zero point elipticity is 213.5 nm, and Rpn – 0.35, higher than reported for similar conditions (0.10-0.13) [27]. This can be due to a more advanced helical packing or to a higher collagen concentration. The characteristics of spectrum demonstrate that the triple helical conformation of collagen molecules was not affected and can be used for obtaining of biomaterials.

5.2. Selecting of concentration of collagen hydrogel for preparation of biomaterials

Amphoteric polyelectrolyte, collagen has positive net charge in acid medium, negative in basic one and isoelectric range 4.5-5.5. [28] Hydrogel consistency and its interaction with other compounds depend on pH: viscosity, interactions and stabilities at shear are maximum at pH 2.5-3.5 and at about 7.5.

To select the optimum concentration, hydrogels with 0.9, 1.1 and 1.3% (mass/volume) collagen and pH 3.8 and 7.4 were prepared.

The rheograms obtained for acid hydrogels at 23°C are represented in Figure 5.2.

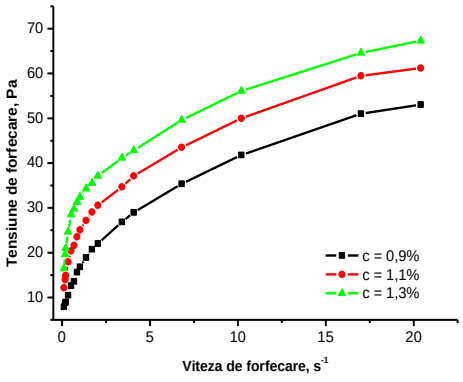


Figure 5.2. Rheograms for acid hydrogels having

The hydrogels have pseudoplastic behaviour and η^* increases with collagen concentration.

The hydrogels having pH 7.4 behave similarly, but η^* values are lower.

The rheograms show that for 0.9% the slope reduces at rate of 17 s^{-1} at both pHs, which means that the hydrogels destructurate slightly. The same thing happens at pH 7.4 for 1.1% hydrogel.

The parameters describing the rheological behaviour of hydrogels were determined with the most used models for describing of pseudoplastic behaviour of fluids:

- Ostwald-de Waele, [31] for fluids with no

flow limiting stress:

$$\tau = K.\ \dot{\gamma}^{n} \quad (5.1)$$

where τ is the shear rate, K – consistency index and n – flowing index and

- Herschel-Bulkley: [32]

$$\tau = \tau_o + K.\dot{\gamma}^{n} \quad (5.2)$$

where τ_o is the stress from which the flowing starts or the flowing limiting stress.

The hydrogels having τ_o values, the parameters were calculated using relation (5.2). The obtained values are given in Table 5.2, together with those of viscosities at zero shear rate, η_o.

The values η_o increase with collagen concentration at both pHs and are higher at a given concentration for acid hydrogels. Consistency inices, of 6.5-7.3 times higher for the acid ones, increase with collagen concentration, and the flowing ones decrease. The sub-unitary values of flowing indices confirm the pseudoplastic behaviour of hydrogels and increasing of pseudoplasticity with increasing collagen concentration.

Table 5.2. Rheological parameters for hydrogels having shown concentrations and pHs

Hydrogel concentration, %/ rheologic parameter	η_o, Pa	K, Pa.s^n	n
	pH 3,8		
0,9	1,553	16,321	0,394
1,1	1,857	23,641	0,314
1,3	2,504	29,835	0,259
	pH 7,4		
0,9	0,414	2,496	0,591
1,1	0,839	3,513	0,541
1,3	1,567	4,106	0,521

Stationary rheological behaviour shows that the most convenient concentration of the initial from the point of view of stability under the action of shearing forces and consistency is 1.1%, both. So the viscosity of slightly basic hydrogel is only half of the acid one, increasing of concentration is not justified by contribution to viscosity.

5.3. Hydrogels containing tannic acid

TA binding rapidly and firmly on collagen in acid medium, hydrogels with pH 3.8 were prepared.

To establish the optimum amount of TA for crosslinking, 5, 10, and 15% TA was introduced into the 1.1% collagen hydrogels, reported to the amount of collagen from hydrogel.

The hydrogel containing 5% TA is more opalescent and viscous compared with reference; the one with 10% looks similar, but opalescence and viscosity are higher. Fragility increases, stirring producing fragmentation, but at rest becomes again homogenous. At 15% opalescence and viscosity increase, a slight non-homogeneity appears, fragmentation increases and the time to regain the initial appearance is longer.

The hydrogels were characterized structurally – by FT-IR and UV-CD – and rheological.

5.3.1. Characterization by FT-IR spectroscopy

Non-denatured type I collagen is composed of 20 amino acid residues connected by C-N bonds and consists of tripeptide oligomeric sequences Gly-X-Y, [33] (Gly – glycine residue, X and Y – residues of any oter amino acid). This constitutes the **primary structure of** collagen chain.

Each third position of α-polypeptide chain is occupied by glycine, while proline – Pro and hydroxiproline – Hyp form 1/3 from the amino acids residues; Gly-Pro-Hyp sequences, characteristic to collagen, are frequent. Inflexibility of Pro and Hyp stiffen the chain and make impossible the formation of alpha helix, but favours the spontaneous formation of a helix twisted to the left, of polyproline II type (PP II). [34] The conformation is not stabilized by hydrogen bonds, the groups forming the bonds being in unfavorable positions. Stability is produced by the steric repulsions of pyrolidine rings from Pro and Hyp. [34] The helix contains three amino acids residues per step, is more extended than alfa and excludes long range interactions. The arrangements of primary structures form the **secondary structures** of segments, and for collagen of the whole chain. [35]

Three helices twisted to left are twisted to the right, around a commune central axis, forming the triple helix characteristic to native collagen molecule (tropocollagen), conformation called **tertiary structure**, [36-40] stabilized by the hydrogen bonds between chains. Tertiary structures represent the way of auto-assembling of secondary ones into the three dimensional protein conformation of molecule.

The following bands were identified into FT-IR spectrum of collagen: [42] 1640 and 1660 cm^{-1} – very intense, due mainly to stretching vibrations of amide C=O bond, to which the hydrogen bonds coupled with stretching vibrations C-N also contribute, referred to as ***amide I***; 1500-1600 cm^{-1} – intense, given by

N-H oscillation vibrations (60% contribution [43, 44]) coupled strongly with C-N stretching from amine, called ***amide II***; bands in the region 1400-1200 cm^{-1}, assigned to stretching of C-N coupled with in plane oscillations of N-H from amide bonds, called ***amide III***. Other bands assigned to amide groups are: 3289 cm^{-1} – N-H stretching coupled with hydrogen bonds – ***amide A*** and 2920 cm^{-1} – shoulder, given by the symmetrical stretching of CH_2 – ***amide B***. Weaker bands are finding at: 1450 cm^{-1} – CH_2 oscillation, 1260 cm^{-1} – N-H oscillation coupled with C-N stretching, 1078 şi 1021 cm^{-1} – C-O stretching and 804 cm^{-1} – stretching of molecule skeleton.

Amide I and II bands being produced by the absorption given by triple helices of collagens, [47] can be used to establish the preservation of the native structure.

To determine the preservation of native conformation or amount of triple helices present the following are used: (a) ratios of absorbance of bands amide III and CH_2 oscillation from 1450 cm^{-1}, $\mathbf{A_{III}/A_{1450}}$ – value 1 or higher shows intact triple helices and lower ones denatured collagen; (b) differences between the wave numbers of bands amide I and II, $\mathbf{(\nu A_I - \nu A_{II})}$ – values higher than 100 cm^{-1} indicating the presence of denatured collagen. The ratios of absorbance of bands amide I and A, $\mathbf{A_I/A_A}$, reflect the extent of crosslinking – the higher the value the more advanced the crosslinking – [48] are also useful.

FT-IR spectrum of collagen from the initial hydrogel within the range 1700-1000 cm^{-1} is presented in Figure 5.8 by the orange colour. The spectra of the hydrogels containing TA are given also in the Figure.

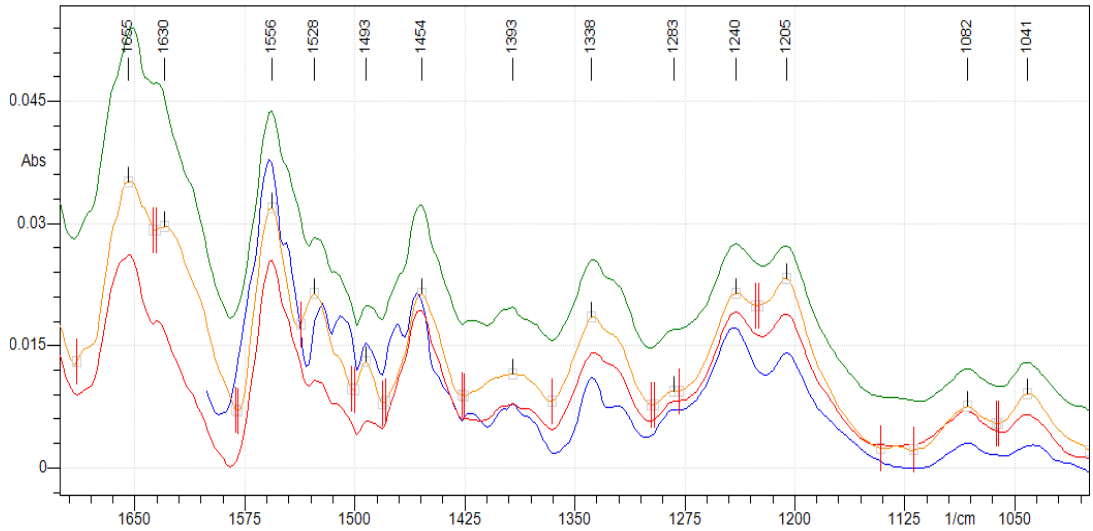


Figure 5.8. FT-IR spectra of collagen hydrogels: reference and with 5%; 10% and 15% TA

The Figure shows weak and wide bands for the reference hydrogel, due to the low concentration of collagen. Amide I band is find at 1655 cm^{-1}, with a prominent shoulder at 1630 cm^{-1} and weaker ones on both sides, amide II at1556 cm^{-1} – more intense and 1528 cm^{-1} – weaker, with shoulders at lower wave numbers, amide III at 1240 and 1205 cm^{-1}; of CH_2 group at 1454 cm^{-1}, with shoulders on both sides, in agreement with literature [49] (Table 5.4).

Table 5.4. Wave numbers of amide I-III bands, of CH_2 oscillation and $(\nu A_I - \nu A_{II})$ values for reference hydrogel and those with TA

TA, %	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2 stretching, cm^{-1}	$(\nu A_I - \nu A_{II})$, cm^{-1}
0	1655	1556	1240	1454	99
5	1655	1556	1242	1458	99
10	1655	1558	1240	1456	97
15	1657	1556	1240	1456	101

The base lines of bands being difficult to trace, only the differences $(\nu A_I - \nu A_{II})$ were used. The value 99 cm^{-1} shows that the reference does not contain denatured collagen.

TA FT-IR bands are different from those of collagen, excepting that from 1447 cm^{-1}, close to that of CH_2 group, but it is weak.

The bands amide I-III and 1454 cm^{-1} have the same wave number as for the native collagen, but increasing of TA concentration modifies amide II band: intensity of that from 1528 cm^{-1} decreases and a shoulder appear at 1545 cm^{-1}.

The differences $(\nu A_I - \nu A_{II})$ have values lower than 100 cm^{-1}, excepting 15% TA which is 101 cm^{-1}. Therefore collagen-TA interactions do not affect the characteristic bands of collagen.

5.3.2. Characterization by UV-CD spectroscopy

The UV-CD spectrum of collagen hydrogel with pH 3,8 is presented in Figure 5.10 (black colour).

Comparing it with that of the initial collagen some differences can be find: (a) minimum is flattened; (b) maximum seems to be sharper, but this is due the larger scale from Figure 5.10; (c) minimum is displaced towards lower wavelengths; (d) maximum is find to higher wave lengths; (e) point of zero elipticity has a higher wavelength; (f) Rpn is high, 3,54 times higher than that obtained from spectrum from Figure 5.1 of the initial collagen.

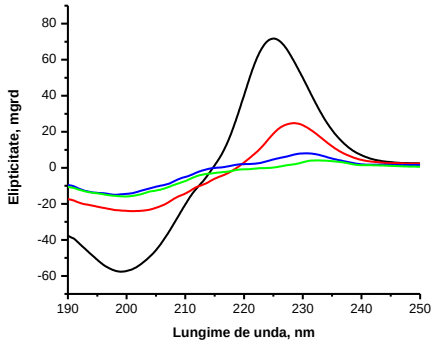


Figure 5.10. UV-CD spectra of the reference hydrogel and of those with TA: 5%; 10% şi 15%

Such a high Rpn value was not found in literature, but only spectra for diluted solutions are reported, under 0.15%, for which the value range between 0.12 and 0.15. [26, 53, 54]

To establish the reasons for which a spectrum having the form and characteristics from Figure 5.10 was obtained, a study on the effect of collagen concentration and opening of spectropolarimeter slit on characteristics of UV-CD spectrum was undertaken, the hydrogel used for preparation of the biomaterials being more concentrated.

5.3.2.1. Effect of collagen concentration on UV-CD spectrum

Aqueous solutions of molecular collagen were used, with concentrations 0,1-1,0% in 0,1 M acetic acid, slit opening 400 μm and cell thickness 0,2 mm. The obtained results are presented in Figure 5.11.

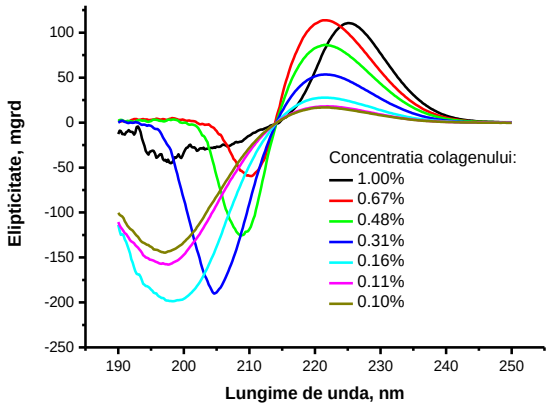


Figura 5.11. Effect of increasing collagen concentration on UV- CD spectrum; slit opening – 400 μm

Increasing of collagen concentration increases significantly the intensity of maximum, but is position is preserved, while minimum displaces significantly towards higher wavelength, modifying Rpn values. The point of zero elipticity is common, at 214 nm, that is it does not depend on concentration. The wavelengths of maxima are very close for concentrations 0.10- 0.67% (221.2-221.6 nm) and becomes 226 nm for 1%. The intensity of maximum increases linearly with concentration between 0.10 and 0.67%, but between 0.67 and 1.00% the decrease is slighter. Intensities of minima vary as follows: increase with collagen concentration from 0.10 at 0.16%, decrease a bit between 0.16 and 0.31%, more between 0.31 and 0.67% and very slow between 0.67 and 1.00%. Their positions displace sensible towards lower λ with increasing dilution between 0.67 and 0.10%; at 1% the peak is larger and its right position more difficult to be established.

The following dependence on concentration results for Rpn from the combination of variations of positive and negative peaks: slight increase between 0.10 and 0.31% (from 0.15 to 0.25); higher increase between 0.31 and 0.48% (from 0.25 to 0.70); even higher increase between 0.48 and 0.67% (from 0.70 to 1.9); after 0.67% the increase is reduced again, reaching finally the value 2.4.

5.3.2.2. Effect of slit opening

The characteristics of UV-CD spectrum are altered by opalescence of system. Collagen solutions are slightly turbid, effect accentuated by concentration. Using very narrow cuvetts and large slit openings the effect is diminished, but sometimes the dispersive factor can not be reduced sufficiently.

Slit opening has no effect on maximum and point of zero elipticity at collagen concentrations under 0.67%, but at 1% it decreases slightly, with no change of position. The forms, intensities and wide of minimums are strongly affected for the first five concentrations: the elipticities decrease with increasing concentration. The form of spectrum modifies significantly at 1% and the same thing is expected at higher concentrations. Consequently the UV/CD spectrum has the form specific for collagen only at low concentrations. [24-27] Intensity of the negative peak decreasing and that of the positive one increasing with rising of collagen concentration, Rpn increases and can exceed unity. Thus, the value 1.24 obtained for 1.1% collagen hydrogel can be considered correct.

Consequently the ***existence of the positive and negative peaks at the wavelength specific for collagen, irrespective of the values of intensities*** may be suggested as a criterion for existence of the triple helix conformation of molecules from fibrils for concentrated collagen solutions. The presence of triple helices can be confirmed by dilution, but only when it is possible.

Coming back to the effect of TA on collagen, the characteristics of spectra given by the hydrogels containing TA are given in table 5.5

Table 5.5. Wavelengths, elipticities, zero elipticity points and Rpn values for hydrogels H1-H4

Hydrogel	Minimum		Maximum		Points of zero elipticity, nm	Rpn
	λ, nm	θ, mdeg	λ, nm	θ, mdeg		
H1	199.0	-57.7	225.0	71.7	214.8	1.24
H2	200.8	-24.0	228.6	24.8	218.8	1.03
H3	198.2	-15.0	230.6	8.0	215.2	0.53
H4	199.8	-15.9	232.2	4.1	224.6	0.26

Figure 5.10 shows that all the minima and maxima are more flattened for the hydrogels containing TA, Rpn values are lower especially at large amounts of TA, and zero elipticity points are displaced towards higher values compared to those of collagen. Such changes being proofs for partial denaturizing of collagen, [53, 54] it can be think that the hydrogels contain denatured collagen. But the differences (νA_I - νA_{II}) lower than 100 cm^{-1} data confirm the proposed criterion for the native collagen conformation.

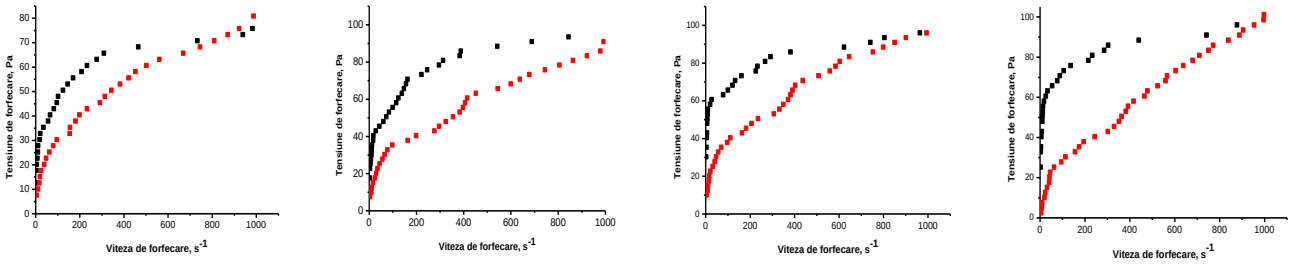
Considering the effect of turbidity on spectrum, the changes seen for the hydrogels containing TA can be assigned to increasing of opalescence, hypothesis supported also by the very close spectra of hydrogels containing 10 and 15 % AT.

What is sure is the fact that increasing of TA concentration displaces slightly the minimum towards higher values, decreases visibly the intensity of maximum, increases its wavelength and decreases Rpn.

5.3.3. Characterization by stationary rheological behaviour

Stationary rheological behaviour allows determination of consistency and plasticity of disperse systems, [55] properties allowing predicting of hydrogels behaviour on application on skin, of technique of application and of behaviour at the application place. Viscosity determines the displaying on skin, kinetics of delivery of active compounds contained and stasis time.

The rheograms recorded for the reference hydrogel and those containing TA when the shear rates were increased and decreased, on a large range of rates to establish the shearing sensitivity, rates at which restructuration begins and regaining of structure are presented in Figures 5.16-5.19.



a b c d

Figure 5.16-5.19. Rheograms of hydrogels: a – reference and with b –5%, c – 10% and 15% TA

The rheogram of reference shows flowing at low stress, ideal elastic behaviour at low shears, than pseudoplastic, destructing at about 10 s^{-1} and continuing at higher stresses, abrupt reducing of slope at about 300 s^{-1}, then again ideal plastic behaviour. Reduced resistance to shear is due to low concentration of collagen. Reducing of share rates the rheogram is placed under the previous one, meaning that the structure is not regained completely. Hysteresis loops appear, showing that the hydrogel is thixotropic.

The effect of crosslinking on rheological behaviour is emphasized in Figures 5.17-5.19.

The consistency of hydrogels, their resistance at shearing and thixotropy increase with amount of TA. Restructuration is reduced for 15% TA, suggesting the surpassing of amount needed for crosslinking.

Determination of η_0 by linearization of dependences apparent viscosity-shear rate shows the presence of two lines for each hydrogel, intersecting at 10 s^{-1}. This means that destructing start at the same rate for each hydrogel. The higher slopes at higher rates demonstrate the increase of pseudoplasticity. η_0

values determined from lines obtained at low shear rates and are given in Table 5.6. The hydrogel with 10% TA is the most viscous, supporting the hypothesis that 15% exceeds the amount for crosslinking.

The influence of TA on flowing capacity was emphasized by flowing indices, calculated with Ostwald-de Waele equation, (Table 5.6.).

Table 5.6. Dynamic viscosities and flowing indices for samples H1-H4 obtained with increasing and decreasing shear rates

Hydrogel	Increasing shear rates		Decreasing shear rates	
	η_0, Pa.s	n	η_0, Pa.s	n
H1	9.80	0.43	3.80	0.46
H2	1.86	0.35	3.73	0.50
H3	2.55	0.42	5.37	0.44
H4	19.17	0.41	0.98	0.79

The hydrogel restructuration, determined from rheograms recorded reducing the shear rates (Table 5.6), shows η_0 values smaller than those obtained increasing them. Hydrogel containing 15% TA restructures the slowest. The flowing indices are in agreement with viscosity values.

5.3.4. Characterization by dynamic rheological behaviour

Dynamic rheological measurements have no effect on structure of the systems subjected to determinations. The values of storage or elasticity moduli, G', and loss or viscosity ones, G", at low deformation amplitude as the material response be linear viscoelastic, allow the determination of elastic and viscous contributions to viscoelastic behaviour: large G' values indicate the preponderance of elastic properties, while large G" – prevalence of viscous ones. Their values allow the distinction between non-crossilnked and crosslinked systems: both moduli are high and the curves giving their dependence on frequency are almost parallel for highly crosslinked systems; [55] for non-crosslinked no relation exists.

The dependences of G' and G" on ω from Figure 5.22 show: (a) G' > G" for all the hydrogels, that is their behaviour is preponderantly elastic; (b) the differences of moduli at a given frequency increase with TA amount; (c) both increase practically linearly with ω; (d) the lines representing the moduli dependences are parallel for each hydrogel, excepting the reference one, showing that it is crosslinked very slight; (e) hydrogel with 10% TA has the highest G' şi G" values (is the most elastic and viscous), which means that it has the highest crosslinking degree; (f) hydrogel with 15% TA is less elastic and less viscous, that is less croslinked than that with 10%, because of the slight denaturizing of collagen produced by the excess of TA. Consequently, the most suitable amount of TA to crosslink the collagen from hydrogel is 10%.

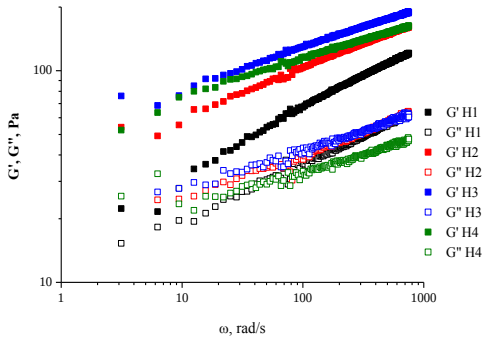


Figura 5.22. Dependences of G' şi G" on ω for hydrogels H1-H4

The apparent viscosities, η*, at a given frequency, ν, are given by the corresponding G"/ν ratios multiplied by 2π. Values thus obtained depend linearly on angular frequencies and the slopes depend on TA amount: reference hydrogel has the lowest slope, it increases a little for 5 and 10% TA and is situated between the previous two at 15%. Extrapolating at 1 rad/s (0.16 Hz) the following values were obtained: 19.08 Pa.s for the reference, 39.80 Pa.s for the hydrogel containing 5% TA, 56.09 Pa.s for that with 10% and 52.83 Pa.s for 15%. The value for the last hydrogel is lower than that for 10% because of the excess of TA, which probable affects slightly the native collagen.

The η_0 values are much higher than those obtained from stationary measurements.

The highest value was obtained for the hydrogel with 10% TA, as in the case of dynamic viscosities, which destructs less under the action of shear stress. This means that it is the most consolidated. The results support the hypothesis of slight denaturizing produced by the excess of TA.

5.4. Hydrogels containing chlorhexidine digluconate

Such hydrogels were prepared with acid – 3,8 and slight basic pH – 7,4, [56, 57] CHDG being active at pH 5-8, [58] with maximum activity at the physiologic pH of skin. [59] The following amounts of CHDG reported to the amount of collagen from hydrogel were introduced: 1.82, 4.55 and 9.09%. The hydrogels having sight basic pH are too fluid and glutaric aldehyde (GA) as crosslinking agent was

introduced to increase consistency. Three series of hydrogels were prepared: (a) with pH 3.8, (b) with pH 7.4 and (c) pH 7.4 and 0.15% GA. They were characterized by the methods used for those containing TA.

5.4.1. Characterization by FT-IR spectroscopy

FT-IR spectra of the reference hydrogels with pH 3.8 and 7.4, of that having the last pH but crosslinked with 0.15% GA and of hydrogels containing CHDG are presented in Figure 5.25.

Increasing of pH reduces significantly the intensities of all bands due to a slight phase separation in slight basic hydrogels, which reduces collagen concentration; crosslinking with GA produce no change.

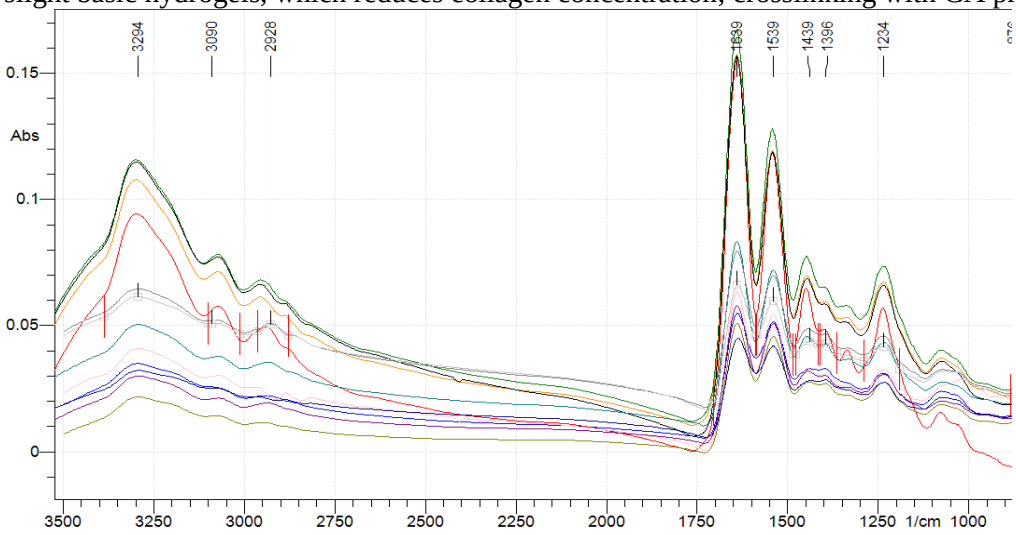


Figure 5.25. FT-IR spectra of hydrogels having pH 3.8 and: 0, 1.82, 4.55, 9.09% DGCH; pH 7.4 and: 0, 1.82, 4.55, 9.09% DGCH; pH 7.4, 0.15% AG and: 0, 1.82, 4.55, 9.09% CHDG [56,57]

Comparing CHDG spectrum with that of collagen, commune bands are seen only within the range 3750-2750 cm^{-1} and amide I band. The ratios A_{III}/A_{1450} can be used to establish the effect of CHDG on conformation of collagen. The ratios A_I/A_A, measuring the extent of crosslinking, can be also calculated.

The wave numbers obtained for the acid hydrogels are presented in Table 5.7.

Table 5.7. Wave numbers of bands amide I-III and A, CH_2 oscillation, ratios A_{III}/A_{1450} and A_I/A_A and differences of frequencies amide I and II for acid hydrogels

CHDG, %	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2, cm^{-1}	Amide A, cm^{-1}	A_{III}/A_{1450}	A_I/A_A	(νA_I - νA_{II}), cm^{-1}
0	1639	1543	1238	1450	3298	0.96	1.38	96
1.82	1641	1541	1234	1448	3298	1.88	1.29	100
4.55	1639	1543	1234	1446	3298	2.15	1.28	96
9.09	1639	1542	1234	1446	3298	1.21	1.29	97

The wave number of bands is preserved in the presence of CHDG. The bands of hydrogels containing 4.55 şi 9.09% CHDG superpose. Consequently CHDG interacts slightly with collagen.

A_{III}/A_{1450} ratios range between 0.96 (reference) and 2.15 (hydrogel with 4.55% DGCH); differences (νA_I - νA_{II}) are under 100 cm^{-1}, demonstrating that no hydrogel contains denatured collagen.

A_I/A_A ratios show that the reference is less crosslinked. CHDG reduces it a bit, due to the binding of CH dications on carboxyl groups of collagen. Their number being very low at pH 3.8, the crosslinking is very weak. Thus, CHDG does not affect collagen conformation, but reduces slightly crosslinking. [56, 57]

5.4.2. Characterization by UV-CD

UV-CD spectra were recorded only for the reference hydrogel and those containing 4.55% CHDG (Figure 5.27). [56, 57]

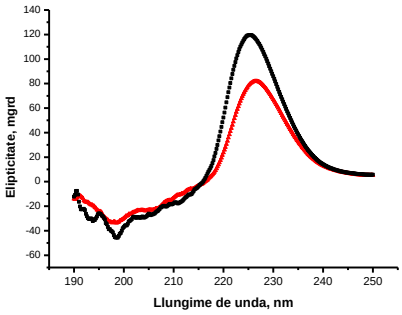


Figure 5.27. UV-CD spectra for hydrogels with pH 3,8 and: ▪ – 0; ▪ – 4.55% DGCH

The reference hydrogel spectrum is very similar to that of 1% collagen solution from Figure 5.10, but the negative peak is better outlined and more intense and the Rpn ratio is higher. This can be explained by the reducing of flexibility of collagen molecules due to their supramolecular association in fibrils.

Correlating with the results obtained by FT-IR it can be concluded that the acid medium favours association.

Introduction of CHDG reduces the intensities of both peaks – more of the positive peak and less of the negative one – and consequently reduces the Rpn value. CHDG distorts collagen slightly or produces the increasing of molecules' flexibility. Considering also the results obtained by FT-IR, it can be concluded that CHDG reduces slightly the supramolecular association, by its interposing between fibrils, increasing thus the flexibility at the molecular level. The micro zones consisting of fibrils crosslinked with CHDG, which are more fluid, alters a little the hydrogel structure creating the aspect of slight discontinuity.

5.4.3. Characterization by stationary rheologic behaviour

The rheograms recorded for the reference acid hydrogel and the three amounts of CHDG at low shear rates, ranging between 0.1 and 20.4 s^{-1}, are presented in Figure 5.30. [56, 57]

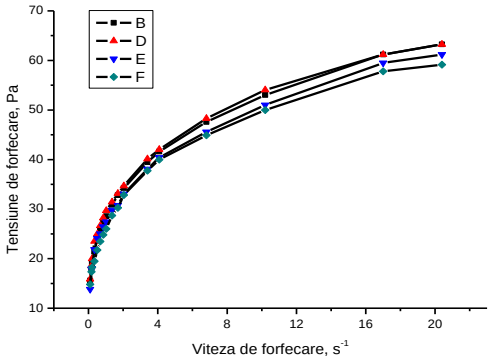


Figure 5.30. Rheograms of acid hydrogels containing: B – 0; D – 1,82; E – 4,55; F – 9,09% CHDG

Introducing 1.82% CHDG the viscosities increase slightly at low shear rates, which means that collagen is slightly crosslinked with CH dications. Shear rates higher than 10.2 s^{-1} destruct the hydrogel. Increasing the amount of CHDG, the rheograms are placed under that of reference. Consequently the increasing of amount of CHDG decreases viscosities: the weight of crosslinking with dications increases with CHDG concentration and crosslinking by hydrogen bonds decreases. The last type of interactions assures the consistency of collagen hydrogels. [56,57]

CHDG affects similarly the dynamic viscosities. The hydrogel with 1.82% CHDG is the most viscous, followed by reference and by that with 4.55%. The rheological parameters were calculated with Herschel-Bulkley equation (Table 5.10).

Table 5.10. Rheological parameters for reference acid collagen hydrogels and with CHDG

CHDG concentration, %/ rheologic parameter	τ_o, Pa	K, Pa.s^n	n
0	0.986	27.582	0.272
1.82	0.982	28.411	0.263
4.55	0.921	26.644	0.273
9.09	0.959	25.704	0.276

The obtained values show that CHDG does not affect τ_o and flowing indices. [56, 57] The above finding demonstrates that collagen hydrogel becomes discontinuous when the CHDG concentration increases.

5.4.4. Characterization by dynamic rheologic behaviour

Dependences of G' şi G" on frequency is represented in Figure 5.33 for acid reference hydrogels and those containing CHDG. [56, 57]

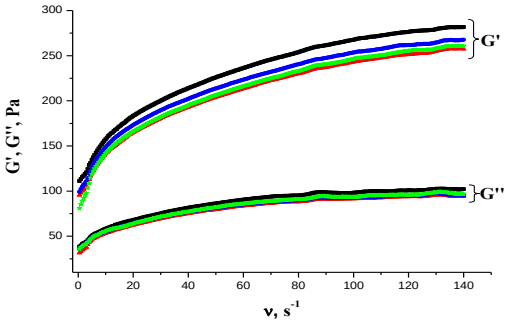


Figure 5.33. Dependences of G' and G''on ν for acid hydrogels: ■ – 0, ▲ – 1.82, ■ – 4.55 şi ■ – 9.09% DGCH

Moduli increase with frequency for all the hydrogels and decrease when CHDG concentration increases. G' values are 2.5 to 2.9 times higher than G'', which means that elasticity prevails on viscosity.

Table 5.11. Viscosity of reference hydrogel and of those containing CHDG

CHDG %	Viscosity, Pa.s	
	$\nu = 0{,}5\ s^{-1}$	$\nu = 140\ s^{-1}$
0	12.188	0.116
1.82	10.047	0.107
4.55	11.487	0.108
9.09	11.468	0.110

Differentiation of curves for the used amounts of CHDG is a proof of slight crosslinking of collagen with CH dications, interactions also emphasized by the other methods. The viscosities at minimum (0.5 s^{-1}) and maximum (140 s^{-1}) frequency calculated with equation (5.3) are given in Table 5.11.

At the lower frequency CHDG produces first a slight decrease of viscosity, than a slight increase. When the frequency is maximum the value are very close and lower than that of the reference.

5.5. Hydrogels containing tannic acid and chlorhexidine digluconate

TA was not used as crosslinking agents for collagen hydrogels containing CHDG.

Secondary polyphenolic metabolite, TA has antimicrobian properties both *in vivo* and *in vitro*. [60]. Introduced in collagen hydrogels, it functions as crosslinking agent and antimicrobial.

Crosslinking the collagen fibrils by physical forces, TA can diffuse at the wound surface. The two compounds may interact at pH 3.8 by hydrogen bonds and ionic forces, because of the very low number of carboxylic groups existing in collagen at such a pH. They can act individually or synergistically.

Collagen hydrogels with TA and CHDG and composition from Table 5.12 were prepared. [61]

Table 5.12. Compositions and abbreviations of hydrogels with pH 3.8 containing TA and CHDG

TA, %	5	10	15	5	10	15	5	10	15
CHDG, %	1.82	1.82	1.82	4.55	4.55	4.55	9.09	9.09	9.09
Abreviation	H5	H6	H7	H8	H9	H10	H11	H12	H13

The hydrogels are more opalescent than those with TA. Opalescence and viscosities increase both with TA and CHDG concentration. Hydrogels' homogeneity also decreases, but – kept at 4°C – suffer no syneresis even after three months. Starting with 4.55% CHDG the hydrogels include air bubble that can not be eliminated because of the high viscosity. They affect hydrogel characterization.

5.5.1. Characterization by FT-IR spectroscopy

FT-IR spectra of hydrogels with 1.82% CHDG and 5, 10 and 15% TA are presented in Figure 5.36.

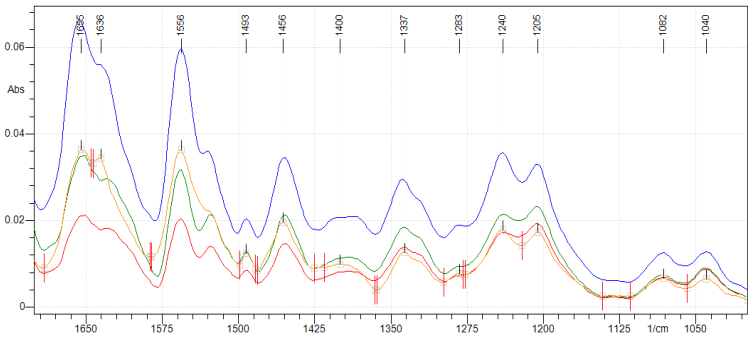


Figure 5.36. FT-IR spectra of hydrogels containing 1.82% DGCH reference and with: 5%; 10% şi 15% TA

Very similar with those of acid hydrogels containing only TA and having bands practically at the same wavelength, the spectra show that collagen-TA interaction is higher than collagen-CHDG.

The band wavelengths and differences required for characterization of collagen are given in Table 5.13.

The differences are lower than 100 cm^{-1} for hydrogels P5 and P6 and 101 cm^{-1} for P7. Thus, no hydrogel contains denatured collagen.

Table 5.13-5.15. Wave numbers of bands amide I-III, CH_2 stretching and values of differences (νA_I - νA_{II}) for hydrogels H1 and H5-H13

Hydrogel	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2 stretching, cm^{-1}	$(\nu A_I - \nu A_{II})cm^{-1}$
H1	1651	1556	1238	1454	95
H5	1655	1556	1240	1456	99
H6	1651	1556	1240	1454	95
H7	1657	1556	1240	1454	101
H8	1659	1556	1240	1454	103
H9	1651	1556	1240	1454	95
H10	1651	1556	1240	1454	95
H11	1657	1556	1242	1456	101
H12	1653	1556	1240	1454	97
H13	1651	1556	1240	1456	95

Spectra of hydrogels H8-H10 look similar to previous ones, which means that interactions do not depend significantly on amount of CHDG. The bands have the same ν values, excepting amide I for the hydrogel with 5% TA, displaced towards higher ν values, but even this contains no denatured collagen.

The bands amide I and II preserve their forms and positions for the last series, but amide II splits up: a new band appears for 10% TA at 1541 cm^{-1} and its intensity increases with TA concentration. It is assigned to interactions of –OH groups of TA with amide ones of CHDG, [63,64] that become perceptible at higher concentrations of both compounds. The CH_2 band also modify: a weak shoulder at 1470 cm^{-1} for 10% TA can be seen, more intense for 15%, assigned to interactions of hexamethylene bridges of CHDG with the hydrophobic groups of TA.

The wave numbers of the bands for hydrogels H1 and H11-H13 from Table 5.13-5.15 show that the value of the difference is 101 cm^{-1} only for P11. Consequently the hydrogels are free of denatured collagen.

FT-IR spectra demonstrates that the band amide II splits at higher concentrations of TA and CHDG, a new band appear at 1541 cm^{-1} and a shoulder on CH_2 group band at 1470 cm^{-1}, that is TA-CHDG interactions appear; none of hydrogels contains denatured collagen and they can be used as biomaterials.

5.5.2. Characterization by UV-CC spectroscopy

UV-CD spectra for the hydrogels containing TA and CHDG are presented in Figures 5.39-5.41.

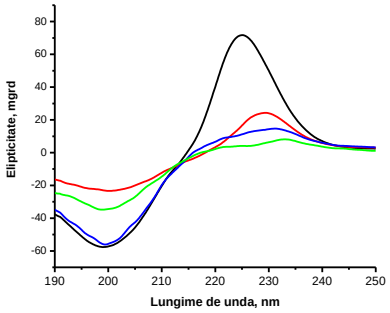


Figure 5.39. UV-CD spectra for hydrogels H1 and H5-H7

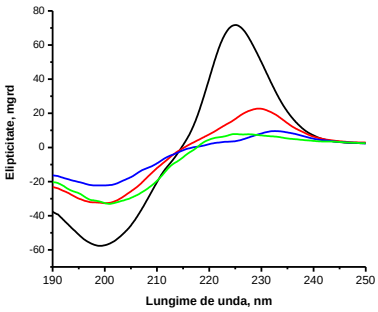


Figure 5.40. UV-CD spectra for hydrogels H1 and H8-H10

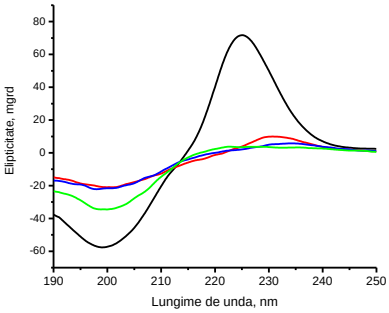


Figure 5.41. UV-CD spectra for hydrogels H1 and H11-H13

Their aspects are similar with those of hydrogels with TA (fig. 5.9), as in the case of FT-IR spectra, but some differences can be seen. Characteristics of spectra from Fig. 5.39-5.41 are given in Tab. 5.16-5.18.

Table 5.16-5.18. Wavelength, elipticities, zero elipticity points and Rpn values obtained for hydrogels H1and H5-H13 from Table 5.12.

Hydrogel	Minimum		Maximum		Zero elipticity point	Rpn
	λ, nm	θ, mdeg	λ, nm	θ, mdeg		
H1	199.0	-57.7	225.0	71.7	214.8	1.03
H5	200.2	-23.4	229.4	24.3	218.2	1.04
H6	199.4	-56.1	231.2	14.7	215.8	0.26
H7	198.8	-34.8	233.0	8.1	217.2	0.23
H8	200.0	-32.6	229.6	22.8	215.2	0.70
H9	200.0	-22.2	232.6	9.6	216.8	0.43
H10	201.0	-33.0	225.0	7.9	217.6	0.24
H11	201.0	-21.0	230.6	9.9	221.6	0.47

H12	200.6	-21.6	234.0	5.8	220.0	0.27
H13	199.8	-34.5	228.6	3.7	217.2	0.11

Differences for 1.82% CHDG can be seen starring with 10% TA: λ of minimum, its wide and elipticity are very close to those of reference; maximum is less outlined, λ closer to that of hydrogel with 10% TA and elipticity almost double; zero elipticity points are close and Rpn half of the hydrogel with TA, due to the increasing of intensity of minimum. All the above are produced by the increase of turbidity and slight phase separation. Intensities of minimum and maximum decrease for 15% TA, but Rpn values remain very close. When CHDH concentration was increased to 4.55% the spectra from Figure 5.40 were obtained: minimum wavelength is very close to that of the reference, but their heights are different and intensity is the highest for the hydrogel with 15% AT. Maximum displaces toward higher λ when TA concentration increases, excepting that with 15% TA, which regain the value for the reference; the heights decrease continuoasly. Zero elipticity point increases with TA concentration and Rpn values decreases. The decreasing of intensities of both peaks, of Rpn value and displacing towards red of zero elipticity points are considered evidences for partial denaturizing of triple helices. [53, 54] But heir responsible of these changes are opalescence, slight phase separation and air bubble. Considering the above proposed criterion for the native conformation of collagen in concentrated collagen solutions/hydrogels, it can be concluded that they do not contain denatured collagen.

Spectra from Figure 5.41 are very close for 5and 10% TA, while for 15% the maximum is more flattened. The values from the Table 5.16-5.18 show that the differences of minima are very small for hydrogels with 5 and 10% TA, but λ of maxima is the highest for hydrogel with 10% TA and its intensity is 1.7 lower than of hydrogel with 5%. Zero elipticity points are close and Rpn decreases with TA amount.

If the criterion proposed in paragraph 5.3.2 for the presence of native conformation of collagen is accepted, it can be asserted that no hydrogel contains denatured collagen, as FT-IR also shows.

5.5.3. Characterization by stationary rheological behaviour

The hydrogels containing 1.82% CHDG and the three amounts of TA are more opaque and more non-homogenous that those containing only TA. The most transparent and homogenous is that with 5% TA, very similar with reference. Viscosity at rest seems to increase significantly when TA amount increases.

The rheograms obtained when the shear rates are first increased within the range 0-100 s^{-1}, in which destruction is insignificant, are presented in Figure 5.42 for the reference hydrogel and H5-H7.

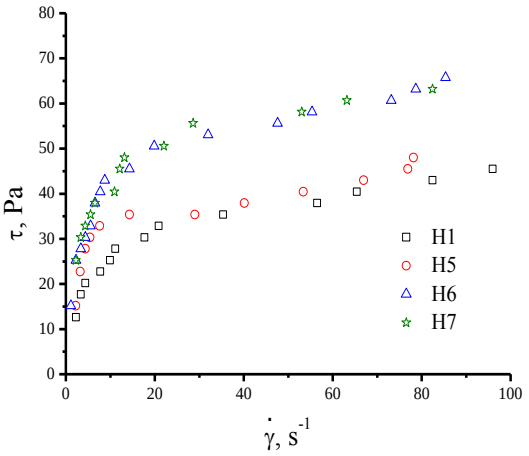


Figure 5.42. Rheograms of hydrogels H1 and H5-H7 obtained with increasing shear rates

The Figure demonstrates the following: [61] TA increases viscosities by crosslinking; τ_o values are low, that is they flow easily; they behave ideal plastic at low shear rates, than pseudoplastic; destruction of structure stars at 10 s^{-1} and ceases after 20 s^{-1}; after 20 s^{-1} all have the same viscosities.

Linearization of dynamic viscosities gives also two lines for each hydrogel, which intersect at 10 s^{-1}.The obtained η_0 values are given in Table 5.19.

The dynamic viscosities of hydrogels with CHDG and TA are higher than that of the reference, but lower than the corresponding ones containing TA due to interaction AT-DGCH, and increase with TA amount, which shows that 15% TA is not an excess.

The rheograms obtained with reducing shear rates show that hydrogel with cu 1.82% CHDG and 5% TA – the most homogenous – restructures the most rapidly, followed by the one with 10%.

The dynamic viscosities are significantly reduced compared with that obtained with increasing and decrease when TA concentration increases: the more crosslinked hydrogels need more time to regain the structure. The flowing indices vary with viscosities conversely.

Hydrogels with 4.55% CHDG look very similar to those from the previous series, but are more viscous at rest and contain air bubbles. Hydrogel with 10% TA is the most viscous, followed by H8. Viscosities become very close at rates exceeding 20 s^{-1}.

Linearization of viscosities shows also two lines for each hydrogel, intersecting at 10 s^{-1} too. The dynamic viscosities (Table 5.19-5.21) are lower than for 1.82% CHDG, because a larger part of TA is

consumed and collagen is less crosslinked. They increase for 5 and 10% TA, but decrease for 15%, probably because of the higher amount of TA-CHDG associates having lower viscosity, which interpose between fibrils. Thus, the hypothesis is supported by the close values of viscosities of the other hydrogels.

Table 5.19-5.21. Dynamic viscosities and flowing indices for hydrogels H1 and H5-H13 obtained with increasing and decreasing shear rates

Hydrogel	Increasing of shear rates		Decreasing of shear rates	
	η_0, Pa.s	n	η_0, Pa.s	n
H1	9.80	0.43	3.80	0.46
H5	10.14	0.63	7.55	0.40
H6	15.21	0.48	4.45	0.46
H7	20.72	0.30	3.89	0.47
H8	15.51	0.47	8.02	0.39
H9	16.66	0.55	7.91	0.39
H10	11.93	0.49	1.64	0.62
H11	21.17	0.28	6.62	0.41
H12	14.85	0.42	5.07	0.41
H13	9.11	0.70	3.82	0.51

The flowing indices are given in Table 5.19-5.21. They decrease with TA concentration increase.

The hydrogels with 5 and 10% TA restructure more when the shear rate are decreased; that with 15% TA regains its structure less than reference, due to the greater number of TA-CHDG associates.

The dynamic viscosities are 2 to 7.5 times lower than that obtained with increasing shear rates (Table 5.19-5.21). The low value of hydrogel P10 is supported by the high value of flowing index from the same Table, which shows that the hydrogel containing 15% TA flows most easy.

Hidrogels with 9.09% are DGCH the most opalescent, the less homogenous, seem the most viscous at rest and contain the most and the biggest air bubbles.

At the minimum shear rates η* values increase in the order: H13 < H12 < H11; this relation continues until about 10 s^{-1}. Above this value H13 becomes more viscous and the values for the others become close. This can be explained by the orientation of TA-CHDG associates in the direction of stress, which favours the interaction between fibrils. The contribution of air bubble must be added to this: they can not leave the space between sensor cylinders at low shear rates, while at high rates a part leaves the hydrogel due to its lower viscosity. The values in Table 5.19-5.21 show that, indeed, H13 has the lowest viscosity and H11 the highest.

When CHDG concentration is 9.09% the hydrogels with 5 and 15% CHDG recover the best.

Consequently stationary rheological behaviour suggests the formation of TA-CHDG associates at high concentrations of components, less viscous than the rest of hydrogel, which produces the aspect of non-homogeneity.

5.5.4. Characterization by dynamic rheological behaviour

Dependences of storage and loss moduli on angular frequencies are given in Figures 5.51-5.53.

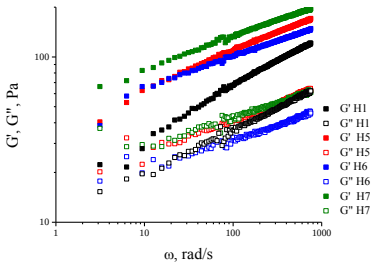


Figure 5.51. Dependences G' and G" on ω for hydrogels H1 and H5-H7

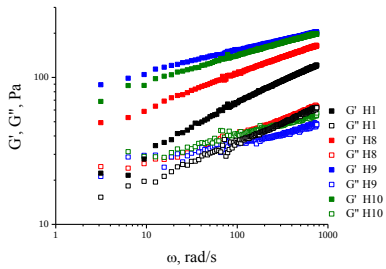


Figure 5.52. Dependences G' and G" on ω for hydrogels H1 and H8-H10

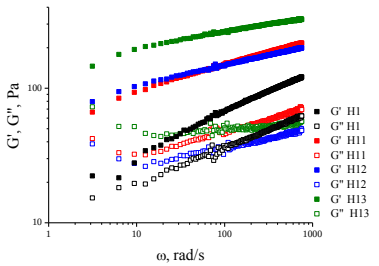


Figure 5.53. Dependences G' and G" on ω for hydrogels H1 and H11-H13

Comparing with the data for hydrogels containing TA, similitude and differences are finding.

Similitude: G' for hydrogels with TA are higher than that of the reference; all the G' values are a little higher than G" on the whole ω range; both moduli increase linearly with ω; differences between moduli at a given frequency are the higher the higher the amount of TA; lines giving the dependences of G' are parallel with those for G" for each hydrogel, as expected for crosslinked systems, excepting for H1, for which the values are close at low ω.

Differences: the hydrogel with 15% TA has the highest values G' and G", not the one with 10% as in the case the CHDG is absent (a part from TA is consumed by CHDG and it is not in excess compared to

the amount requires for crosslinking); the hydrogel with 10% TA flows even easier than the reference one, especially for ω higher than 100 rad/s. In this case the optimum amount of TA is 15%.

Increasing the amount of CHDG at 4.55% the dependences on TA concentration and ω from Figure 5.52 are obtained for G' and G". The values are a little higher than for the previous series, the lines giving the dependence of G' and G" on ω remain parallel for each hydrogel and the values for 10 and 15% TA are close, that is CHDG contributes also to crosslinking. Crosslinking is optimum both for 10 and 15%.

The maximum amount of CHDG gives the dependence of moduli on frequency from Figure 5.53. The hydrogel containing 9.09% CHDG and 15% TA (H13) has the highest elasticity; those containing 5 and 10% TA have lower and closed elasticities. The lines giving the dependences of moduli on ω remain parallel for the hydrogels with 5 and 10% TA, but for 15% the parallelism disappears, probably because of the larger amount of TA-CHDG agglomerates, emphasized also by the stationary rheological behaviour.

The dependences of viscosities on ω for each series of hydrogels are strictly linear and they decrease with increasing of ω practically in the same way in each series. The values obtained by extrapolation at the frequency of 1 rad/s from Table 5.22 varies within the series as in the case of those obtained by stationary measurements: increase with the amount of TA, but are much higher than those obtained from stationary measurements, due to the fact that the hydrogels are not destructed.

Table 5.22. Dynamic viscosities of hydrogels H5-H13 at the angular frequency of 1 rad/s

Hydrogel	H5	H6	H7	H8	H9	H10	H11	H12	H13
η, Pa.s	41.6	43.4	58.2	41.0	81.2	66.7	66.0	76.9	58.6

CHDG increases the viscosities of hydrogels containing the same amount of TA, excepting the one with 15%, for which increases at first, than decreases for 9.09%, because of the great number of TA-CHDG associates, which hinder crosslinking with TA and – being less viscous – reduce viscosity.

R e f e r e n c e s

1. „The American Heritage Dictionary of the English Language", ed. A 4-a, updatată în 2009, Houghton Mifflin Company.
2. "Collins English Dictionary" ed. 6-a completată şi neprescurtată, Harper Collins Publishers, 2003.
3. N.A. Peppas şi A.G. Mikos, Preparation methods and structure of hydrogels, în "Hydrogels in Medicine and Pharmacy", ed. N. A. Peppas, vol. 1, CRC Press, Boca Raton, 1986.
4. L. Brannon-Peppas, Preparation and characterization of crosslinked hydrophilic networks, în "Absorbent Polymer Technology", ed. L. Brannon-Peppas şi R.S. Harland, Elsevier, Amsterdam, 1990.
5. N.A. Peppas, P. Bures, W. Leobandung şi H. Ichikawa, Hydrogels in pharmaceutical formulations, Eur. J. Pharm. Biopharm. 50, 27-46, **2000**.
8. B.D. Ratner şi A.S. Hoffman, Synthetic hydrogels for biomedical applications, în "Hydrogels for Medical and Related Applications", ed. J.D. Andrade, ACS Symposium Series, No. 31, American Chemical Society, Washington, DC, 1976, 1-36.
9. N.A. Peppas şi R. Langer, New challenges in biomaterials, Science 263, 1715-1720, **1994**.
10. K. Park, "Controlled Release: Challenges and Strategies", American Chemical Society, Washington, 1997.
11. N.A. Peppas, Hydrogels and drug delivery, Curr. Opin. Coll. Int. Sci. 2, 531-537, **1997**.
13. E.A. Abou Neel, U. Cheema, J.C. Knowles, R.A. Brown şi S.N. Nazhat, Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties, Soft Matter 2, 986–992, **2006**.
14. V. Charulatha şi A. Rajaram, Influence of different crosslinking treatments on the physical properties of collagen membranes, Biomaterials 24, 759-769, **2003**.
16. A. Jayakrishnan şi S.R. Jameela, Glutaraldehyde as a fixative in bioprosthetic and drug delivery matrices, Biomater., 17**,** 471-84, **1996**.
17. H.M. Powell şi S.T. Boyce, EDC cross-linking improves skin substitute strength and stability, Biomater., 27, 5821–5827, **2006**.
18. H.M. Powell şi S.T. Boyce, Wound closure with EDC cross-linked cultured skin substitutes grafted to athymic mice, Biomater. 28, 1084–1092, **2007**.
19. W.M. Elbjeirami, E.O. Yonter, B.C. Starcher şi J.L. West, Enhancing mechanical properties of tissue-engineered constructs via lysyl oxidase crosslinking activity, J. Biomed. Mater. Res. 66A, 513-521, **2003**.
20. W. Friess, Collagen – biomaterial for drug delivery, Eur. J. Pharm. Biopharm. 45, 113-361, **1998**.
23. M.L. Tiffany şi S. Krimm, The effect of temperature on the circular dichroism spectra of polypeptides in the extended state, Biopolymers 11, 2309-2316, **1972**.
24. M.G. Venugopal, J.A.M. Ramshaw, E. Braswell, D. Zhu şi B. Brodsky, Electrostatic interactions in collagen-like triple-helical peptides, Biochem. 33, 7948-7956, **1994**.
25. Y. Feng, G. Melacini, J.P. Taulane şi M. Goodman, Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-Pro-Hyp sequences. 2. Synthesis and conformational analysis by circular dichroism, ultraviolet absorbance, and optical rotation, J. Am. Chem. Soc. 118, 10351-10358, **1996**.
26. U. Freudenberg, S.H. Behrens, P.B. Welzel, M. Muller, M. Grimmer, K. Salchert, T. Taeger, K. Schmidt, W. Pompe şi C. Werner, Electrostatic interactions modulate the conformation of collagen I, Biophys. J. 92, 2108-2119, **2007**.
27. M. Leca, V. Trandafir şi M.G. Albu, Preparation and characterization of some collagen gels and matricers, „Ovidius" Annals of Medicine Sci. – Pharmacy 1, 84-92, **2003**.

28. M.G. Albu şi M. Leca, Rheological behaviour of some cross-linked collagen hydrogels for drug delivery use, European Cells & Materials, 10, 8, **2005**.
29. H. A. "A handbook of elementary rheology", Institute of Non−Newtonian Fluid Mechanics, University of Wales, 2000.
30. J. A. M. Ramshaw, N. K. Shas şi B. Brodsky, Gly-X-Y tripeptide frequencies in collagen: a contest of host-guest triple-helical peptides, J. Struct. Biol. 122, 86-91, **1998**.
34. K. Kar, P. Amin, M. A. Bryan, A. V. Persikov, A. Mosh, Y.-H. Wang şi B. Brodsky, Self-association of collagen triple helix peptides into higher order structures, J. Biol. Chem. 281, 33283-33290, **2006**.
35. IUPAC, Compendium of Chemical Terminology, Internet ed., http://goldbook.iupac.org/ T06282 html
36. G. N. Ramachandran, Structure of collagen, Nature 174, 269-270, **1954**.
37. A. Rich şi F. H. Crick, Nature 176, 915–916, **1955**.
38. P. M. Cowan, S. McGavin şi A. C. North, Nature 176, 1062-1064, **1955**.
39. A. Rich şi F. H. Crick, The molecular structure of collagen, J. Mol. Biol. 3, 483-506, **1955**.
40. G. N. Ramachandran, Structure of collagen at the molecular level, în "Treatise on collagen" vol. 1, "Chemistry of collagen", ed. G. N. Ramachandran, Academis Press, London, 1967.
42. C. Petibois, G. Gouspillou, K, Wehbe, J-P. Delage şi G. Deleris, Analysis of type I and IV collagens by FT-IR spectroscopy and imaging for a molecular investigation of skeletal muscle connective tissue, Anal. Bioanal. Chem. 386, 1961-1966, **2006**.
43. C. Palpandi, P. Ramasamy, T. Rajinikanth, S. Vairamani and A. Shanmugam, Extraction of Collagen from Mangrove Archeaogastropod *Nerita* (*Dostia*) *crepidularia* Lamarck, 1822, Am-Euras. J. Sci. Res. 5, 23-30, **2010**.
44. N.F. Mohd Nasir, M.G. Raha, N.A. Kadri, S.I. Sahidan, M.Rampado and C.A Azlan, The Study of Morphological Structure, Phase Structure and Molecular Structure of Collagen- PEO 600K Blends for Tissue Engineering Application**,** Amer. J. Biochem. Biotechnol. 2, 175-179, **2006**.
47. A. Barth, Infrared spectroscopy of proteins, Biochim. Biophys. Acta 1767, 1073-1101, **2007**.
48. Z. Garcia, H. Naik, R., Collighan, M. Griffin, J. C. R. Cabello şi A. Pandit, In-vitro characterization of an enzymatically cross-linked collagen-elastin like polymer scaffold, 8th World Biomaterials Congress, Amsterdam, May 28 – June 1, 2008.
49. K. Belbachir, R. Noreen, G. Gouspillou, C. Petibois, Collagen types analysis and differentiation by FTIR spectroscopy. Anal. Bioanal. Chem. 395, 829-837, **2009**.
53. E. M. Brown, R. L. Dudley, A. R. Elsetinow, A conformational study of collagen as affected by tanning procedures, J. Amer. Leather Chem. 92, 225-233, **1992**.
54. D. Jenness, C. Sprecher şi W. C. Johnson Jr., Circular dichroism of collagen, gelatin and poly(proline) II in the vacuum ultraviolet, Biopolymers 15, 513-521, **1976**.
56. D. Şulea, M. V. Ghica, M. Micutz, M. G. Albu, **L. Brăzdaru**, T. Staicu, M. Leca şi L. Popa, Characterization an *in vitro* release of chlorhexidine digluconate comprised in type I collagen hydrogel, Rev. Roum. Chim. 55, 543-551, **2010**.
57. M. Leca, D. Şulea, M. V. Ghica, M. Micutz, M. G. Albu, **L. Brazdaru**, T. Staicu şi L. Popa, Collagen hydrogels containing chlorhexidine digluconate: Characterization and in vitro drug release**,** Proc. 3rd Int. Conf. ICAMS 2010, September 16-18, Bucharest, 205-210.
58. DGCH pH "Basic and Clinical Pharmacology", Miscellaneous Antimicrobial Agents; Disinfectants, Antiseptics, & Sterilants, cap. 50, McGraw-Hill Comp., http://basic-clinical-pharmacology.net/chapter50.htm
59. http://www.burmester-pharma.de/deutsch/analyse_datenblatt/a1929.pdf
60. M. Murphy Cowan, Plant Products as Antimicrobial Agents, Clin. Microbiol. Rev. 12, 564-582, **1999**.
61. S. K. Filoche, K. Soma şi C. H. Sissons, Antimicrobial effects of essential oils in combination with chlorhexidine digluconate, Oral Microbiol. Immunol. 20, 221-225, **2005**.
62. A. Albert şi E.P. Sargeant, "Ionization Constants of Acids and Bases", Methuen, London, 1962.
63. **L. Brazdaru**, M. Leca, M. Micutz, M. G. Albu şi T. Staicu, Stationary and dynamic Rheological behaviour of some collagen hydrogels for wound management containing mixtures of chlorhexidine digluconate and tannic acid, Proc. 4th Int. Conf. ICAMS 2012, September 27-29, Bucharest, 237-242.
64. **L. Brăzdaru**, M. Micutz, T. Staicu, M. Albu, D. Şulea, M. Leca, Structural and rheological properties of collagen hydrogels containing tannic acid and chlorhexidine digluconate intended for wound dressings, C. R. Chim., 18, 160-169, **2015**.

6. CHARACTERIZATION OF POROUS MATRICES CONTAINING TANNIC ACID, CHLORHEXIDINE DIGLCONATE OR THEIR MIXTURES

Collagen matrices are largely applied in medicine: scaffolds in tissue engineering, [6] haemostatic and protection material for wounds and the new formed tissue, [6, 7] systems for inclusion and delivery of cells, proteins, nucleic acids and drugs. [8-11]

6.1. Matrices containing tannic acid

6.1.1. Characterization by FT-IR spectroscopy

Spectra of the reference and of matrices containing 5, 10 and 15% TA are given in Figure 6.1.

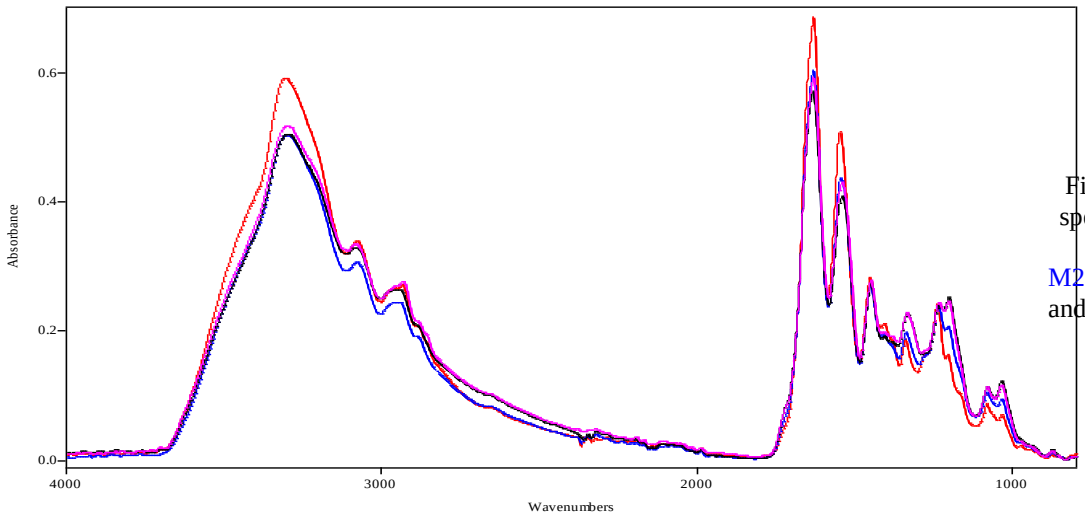


Figure 6.1. FT-IR spectra of matrices with: M1 – 0; M2 – 5; M3 – 10 and M4 – 15% AT

FT-IR spectrum of the reference matrix presents all the collagen bands, [18] excepting the shoulder at 2920 cm^{-1} (amide B), [20] which is superposed with another shoulder at 2957 cm^{-1}.

The ν vales for the reference and matrices containing TA and (νA_I-νA_{II}) are given in Table 6.1.

The ν vales of bands amide I and II for the reference matrix are a little lower than for hydrogel, but the others are the same. The following bands from complex band amide III are observed in the reference spectrum: 1338 cm^{-1} – very weak, 1277 cm^{-1} – shoulder, 1237 and 1203 cm^{-1} – shoulders, according to literature. [22-24] The value (νA_I - νA_{II}), of 84 cm^{-1}, shows the absence of denatured collagen.

Figure 6.1 reveals that TA produces the following modifications: (a) Intensities of bands amide A decrease, due to the bonding of TA on collagen and reducing of number of hydrogen bonds between fibrils.

(b) Sub bands of the band amide III are found at 1336-1332, 1278-1276, 1235-1231 and 1202-1200 cm^{-1} and their intensities modify with increasing of TA concentration: 1335 cm^{-1} increases slightly its intensity, but they are the same for 10 and 15%; intensity of shoulder of reference at 1277 cm^{-1} decreases

Table 6.1. Wavenumbers of bands amide A, I-III, CH_2 stretching and differences (νA_I - νA_{II}) for matrices with specified TA concentrations

TA, %	Amide A, cm^{-1}	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2 stretching, cm^{-1}	(νA_I - νA_{II}), cm^{-1}
0	3301	1629	1545	1237	1451	84
5	3292	1630	1542	1235	1448	88
10	3291	1630	1539	1232	1447	91
15	3291	1631	1539	1231	1447	92

and disappears in the matrix with 15% TA. The reference band at 1237 cm^{-1} displaces towards lower ν, but its intensity is preserved. Shoulder intensity at 1203 cm^{-1} increases: it becomes a band at 5% TA, for 10% its intensity is comparable with that at 1237 cm^{-1}, and for 15% is more intense. Transformation of shoulder at about 1200 cm^{-1} in band and increase of its intensity are due to crosslinking with TA. (c) Intensities of reference bands at 1080 and 1031 cm^{-1} modify: the second, less intense, becomes more intense than the first for 10 and 15% TA. Differences (νA_I - νA_{II}) increase with TA concentration, but are lower than 100 cm^{-1}.

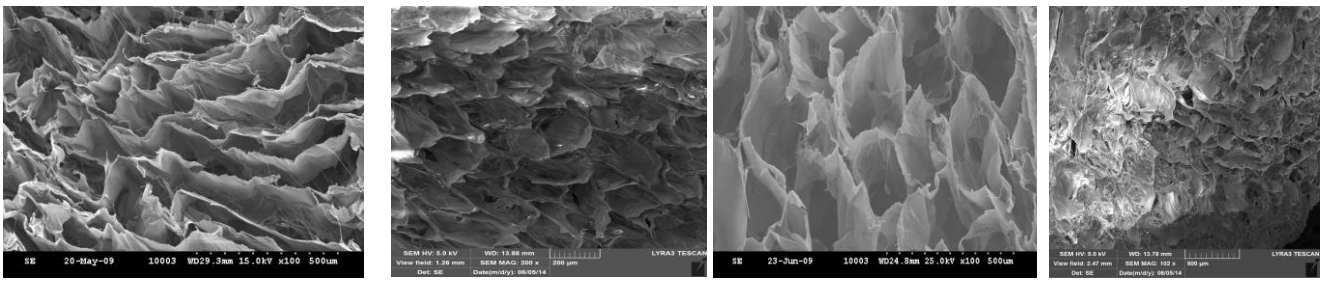
The slight displacing of bands amide A, II and III towards lower wave numbers, increasing of intensity of band from 1200 cm^{-1} from the complex band amide III and decreasing of intensity of band amide I when TA is introduced can be associated with collagen crosslinking by such forces.

6.1.2. Characterization by SEM

SEM, emphasizing the agglomeration of fibrils and changing of pore size, allow the establishing of crosslinking extension. SEM images of reference matrix and of those with TA are given in Figures 6.3a-d.

The Figure 6.3a shows lamellar matrix for reference, with elongated pros crossed by collagen fibrils, 100-500 µm length and 50-150 µm wide. There are also annular pores, with dimensions varying from 100 to 200-230 µm, interconnected by collagen fibers and fibrils about 0.3-8.0 µm thick.

The lamellar structure is absent into the hydrogel containing 5% TA (Figure 6.3b), pores are annular and flattened, with dimensions of 40-220 µm. The difference of morphology is due to higher viscosity of the hydrogel containing 5% TA, which produces larger ice crystals.



a – 100X b – 200x c – 100x d – 100x

Figure 6.3. SEM images 100x of matrices: a – reference and with b – 5%; c – 10%; d – 15% TA

When TA concentration is double the pores become annular, their large sizes are 350-500 µm and small ones 100-250 µm and matrices contain large compact zones of collagen, due to crosslinking with TA.

The aspect of matrices with 15% TA is non-homogenous (Figure 6.3d) with pores and compact zones of collagen, determined by the more advanced crosslinking. The small pores and compact zones support the affirmation that TA in excess affect slightly the collagen conformation.

6.1.3. Characterization by water absorption

The remarkable capacity of collagen matrices to absorb water is given by the great number of hydrophilic groups: amide, carboxyl and hydroxyl. [30] Swelling in aqueous media is very important for their using as dressings, providing the hydration of tissue surface and delivery of the drug. Swelling with biological fluids is the first obligatory step in matrix degradation.

The results for water absorption, expressed as ratio of mass of absorbed water and that of initial matrix (g/g), are presented in Figure 6.5 for matrices M1-M4, with a detail inside. [18]

The highest amounts of water are absorbed by the reference matrix (curve B). It absorbs vey much within the first 70 min, becoming more and more gelatinous, less between 70 and 100 min, and in 120 min it loses the integrity. Water absorbed by the matrix obtained from the hydrogel containing 5% TA is by far more reduced. Being crosslinked, it does not loose its integrity, but transforms in a continuous bulky gelatinous mass. Absorption is pretty high within the first 200 min, and than decreases gradually.

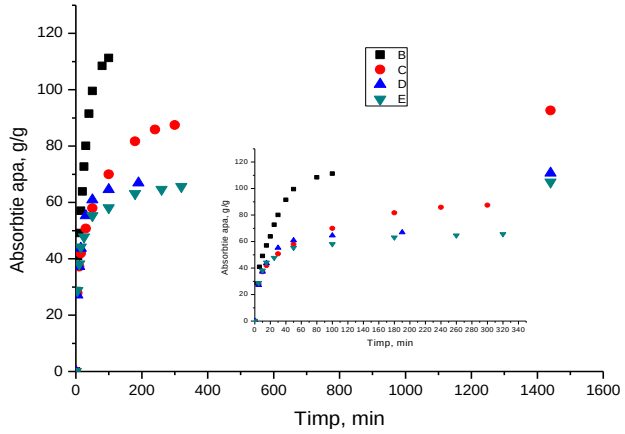


Figure 6.5. Water absorbed within 24 h by matrices containing TA: B – 0; C – 5; D – 10; E – 15%

Increasing the amount of TA at 10% water absorption decreases more and after 100 min the difference between it and reference becomes significant.

The absorption curve for the matrix containing 15% TA is very close to the previous one, suggesting that the degrees of crosslinking are close and demonstrating that 10% TA is enough for collagen crosslinking.

6.1.4. Characterization by digestion with collagenase

Collagenase, the most used enzyme for *in vitro* digestion of collagen, [34] splits the chains, leaving the intermolecular bonds intact. This is why the method is used to evaluate the degree of crosslinking.

Digestion was done in physiological conditions – saline phosphate buffer solution with pH 7.4 and 37ºC. [18] The matrices with TA serve as references for those containing TA and CHDG.

The matrices that do not contain TA disappear in about 30 min. If they contain 5% TA the integrity and form is preserved for about 240 min, than disintegrate in highly swollen fragments. Doubling the amount of TA matrices are undivided for 24 h, and the fragments formed by further disintegration are bigger than for the previous matrices. For 15% TA the matrices resist for 9 days, the last day of observation. Samples preserve the initial form, but their volume is much higher. [18]

6.2. Matrices containing chlorhexidine digluconate

6.1.1. Characterization by FT-IR spectroscopy

FT-IR spectra of the reference and of matrices containing CHDG obtained from hydrogels having pH 3.8 and 7.4, the last in the absence and presence of GA, are presented in Figure 6.6. [16]

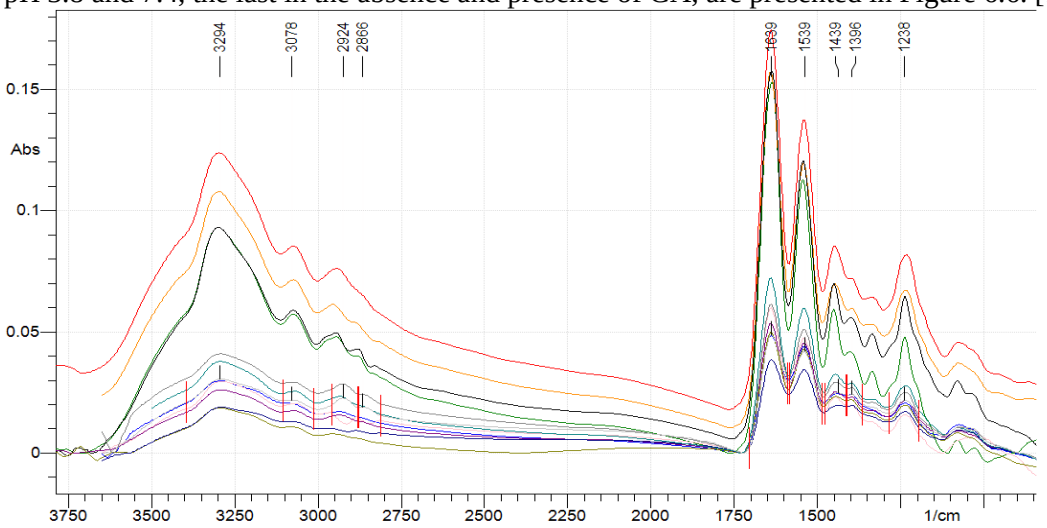


Figure 6.6. FT-IR spectra of matrices obtained from: acid hydrogels with 0, 1.82, 4.55 and 9.09% CHDG; weakly basic hydrogels with 0, 1.82, 4.55 and 9.09% CHDG; weakly basic and 0.15% GA with 0, 1.82, 4.55 and 9.09% CHDG

FT-IR spectra are grouped in two classes: with intense bands –matrices resulted from acid hydrogels and pretty weak and very close – matrices obtained from slight basic hydrogels.

Matrices obtained from acid hydrogels, discussed farther, have very close intensities for bands amide I and II are, but different for amide III. The values of characteristics of spectra are given in Table 6.2.

The Table demonstrates that CHDG does not modify the positions of collagen bands.

CHDG increases the ratios A_{III}/A_{1450}, that is it stabilizes the helices. The values much higher than unity must not be taken as a measure of consolidation of triple helices, the bands being large and the errors for the base lines high. But the tendency of increasing of ratios with increasing amount of CHDG is real.

Table 6.2. Wavenumbers of bands amide I-III, A and CH_2 stretching, ratios A_{III}/A_{1450} and A_I/A_A and differences (νA_I - νA_{II}) for the reference and containing CHDG matrices

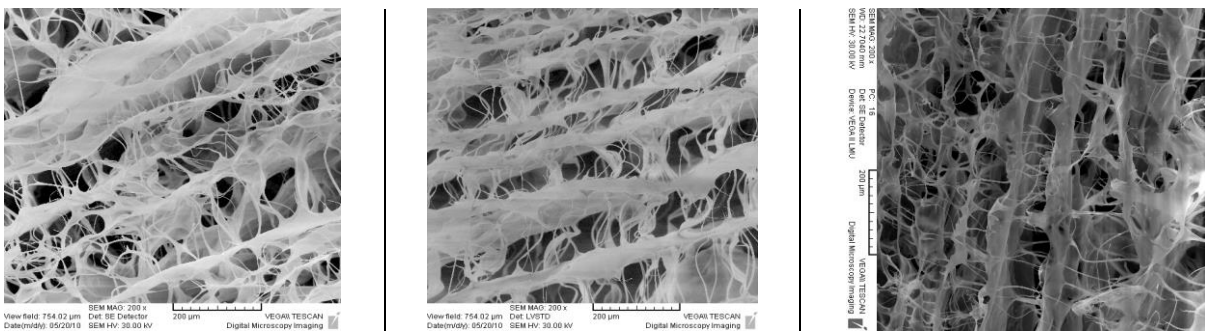
DGCH, %	Amide A, cm^{-1}	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2 stretching, cm^{-1}	A_{III}/A_{1450}	A_I/A_A	(νA_I - νA_{II}), cm^{-1}
pH 3,8								
0	3293	1639	1542	1234	1446	1.98	1.27	97
1,82	3300	1637	1543	1238	1450	2.00	1.37	94
4,55	3298	1639	1542	1234	1446	2.08	1.36	97
9,09	3298	1637	1543	1238	1450	2.15	1.37	94

The ratios A_I/A_A, measure of crosslinking, increase slightly with CHDG concentration, due to slight crosslinking of collagen with CH dications and forming of micro zones crosslinked by weaker forces.

Decreasing of intensity of band amide I can be due to the slight increase of interactions by hydrogen bonds in collagen, that is to a slight crosslinking, shown also by the slight increase of ratios A_I/A_A.

7.2.2. Characterization by SEM

SEM images of the reference matrix and of those resulting from the acid hydrogels containing 1.83 and 4.55% CHDG are presented in Figure 6.7a-c. [16]



a | b | c

Figure 6.7. SEM images 200x of matrices obtained from acid hydrogels: a – reference; b – with 1.82 % and c – with 4.55% CHDH

Introduction of 1.82% CHDG gives the image from Figure 6.7b: the matrix is lamellar, but the lamellae are thicker and the distances lower, resulting pores of lower size. The thickening of lamellae are due o the crosslinking of fibrils with CH dications. Increasing the CHDG concentration to 4.55% modifies morphology more – Figure 6.7c: lamellae are closer, and thin lamellae and small pores prevail.

6.3. Matrices containing tannic acid and chlorhexidine digluconate

6.3.1. Characterization by FT-IR spectroscopy

Spectra of matrices are presented and discussed at constant amounts of CHDG.
FT-IR spectra of matrices M5- M7 are presented in Figure 6.11. [18]

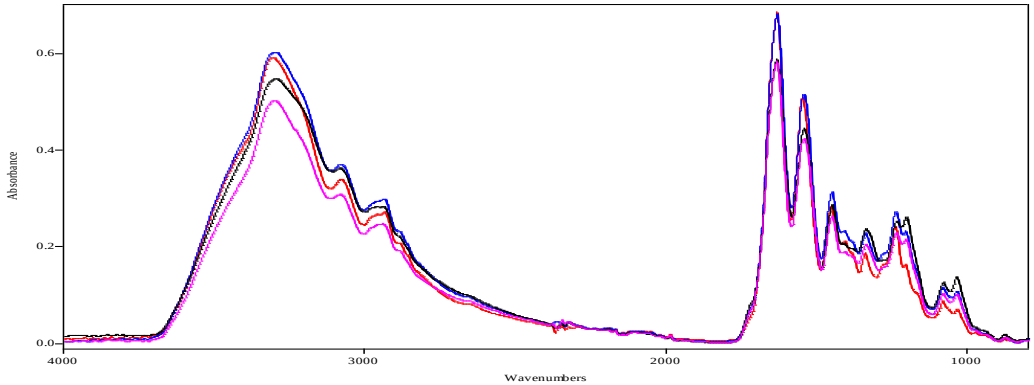


Figure 6.11. FT-IR spectra of matrices with 1.82% CHDG and: P1 – 0; P5 – 5; P6 – 10 and P7 – 15% TA

Wave numbers of bands amide A, I-III and CH_2 and differences (νA_I - νA_{II}) are given in Table 6.3.

Table 6.3.-6.5. Wave numbers of bands amide A and I-III, CH_2 stretching and differences (νA_I - νA_{II}) for matrices containing all the combination between the three amounts of TA and CHDG

Matrix	TA, %	Amide A, cm^{-1}	Amide I, cm^{-1}	Amide II, cm^{-1}	Amide III, cm^{-1}	CH_2 stretching, cm^{-1}	(νA_I - νA_{II}), cm^{-1}
M1	0	3301	1629	1545	1237	1451	84
M5	5	3294	1629	1541	1236	1448	88
M6	10	3296	1631	1539	1234	1447	92
M7	15	3292	1630	1539	1233	1447	91
M8	5	3299	1631	1538	1233	1447	93
M9	10	3299	1631	1538	1235	1448	93
M10	15	3299	1631	1537	1231	1447	94
M11	5	3306	1631	1539	1237	1449	88
M12	10	3303	1632	1539	1235	1448	92
M13	15	3297	1631	1536	1234	1447	91

Spectra of matrices containing 1.82% CHDG and 5, 10 şi 15% TA are very similar to that of matrix M1 in the frequency range 4000-1400 cm^{-1}, in which the bands amide A, B, I and II as well as CH_2 stretching are find. Slight decrease of intensities and slight displacing towards lower frequency of band amide A can be seen only for 10 and 15% TA. Intensities of bands modify in the range 1400-1200 cm^{-1}, but their positions remain the same: shoulder at 1203 cm^{-1} becomes band and its intensity increases with TA amount, as for matrices with TA. They are a little more intense compared with the last ones, probably due to the blocking of a small part of TA by CHDG, which reduces crosslinking. Changing of band intensities with TA amount in the range 1120-1000 cm^{-1} are similar to those for matrices containing only TA. The above results demonstrate that TA interacts stronger with collagen than with CHDG, and a part of it interacts also with CHDG. Differences (νA_I - νA_{II}) are the same as for matrices containing TA, that is they do not contain denatured collagen.

At 4.55% CHDC changes are the same when TA concentration increases, but the decreasing of intensities are higher, especially when the matrix contains 10% TA – Table 6.3.

Differences (νA_I - νA_{II}) are a little higher compared to the previous matrices, but all remain lower than 100 cm^{-1}, which means that they do not contain denatured collagen.

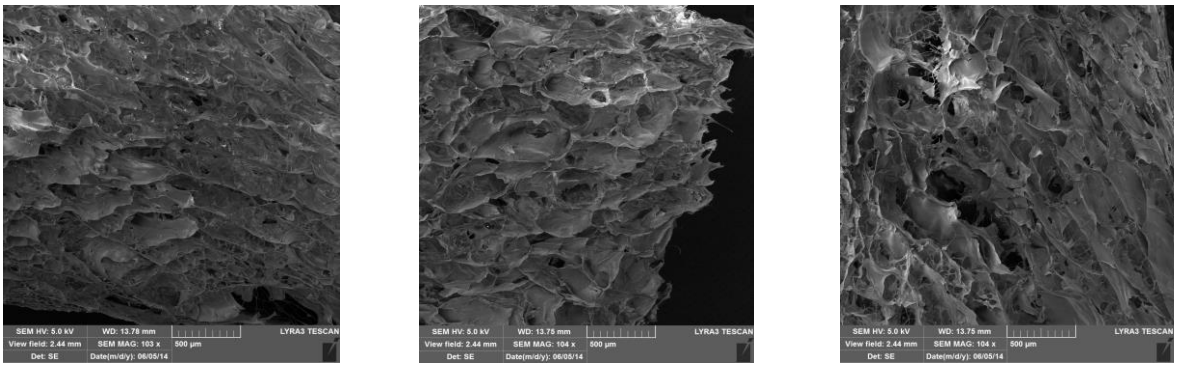
Changing brought by doubling the amount of CHDG (9.09%) are similar with the previous ones. As a characteristic, the intensities of all the bands are very close when the matrices contain 10 and15% TA.

The frequency of bands for matrices containing 9.09% CHDG is given also in Table 6.3-6.5. Differences (νA_I - νA_{II}) show that matrices M11-M13 contain no denatured collagen.

Concluding, the collagen matrices containing all the combinations between 5, 10 and 15% TA and 1.82, 4.55 and 9.09% CHDG contain no denatured collagen and they can be used as wound dressings.

6.3.2. Characterization by SEM

SEM images of matrices with 1.82% CHDG and 5, 10 and 15% TA are presented in Fig. 6.14.a-c.



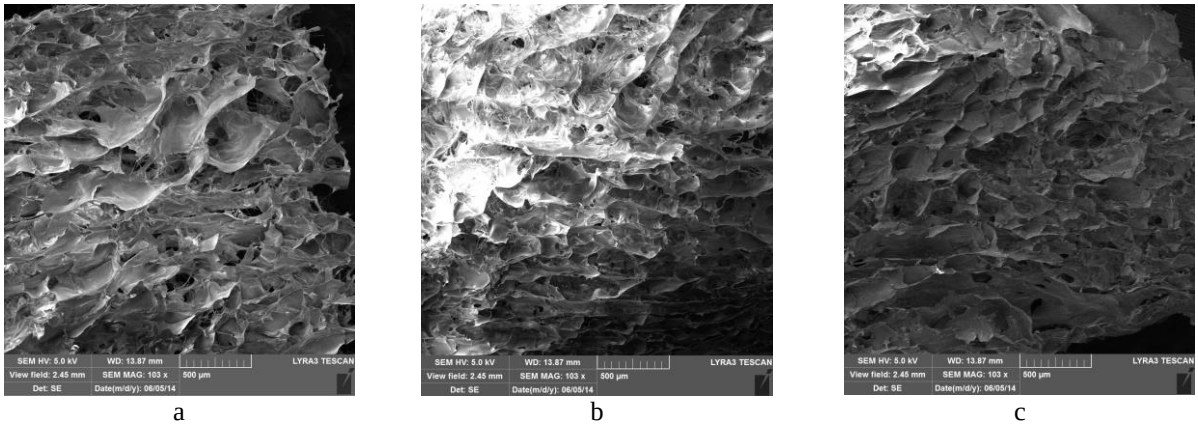
a b c

Figure 6.14. SEM images 100 x of matrices containing 1.82% CHDG and: a – 5, b – 10 and c – 15% TA

Compared with those containing TA, they have smaller and less regular pores.

The aspects of matrix surfaces containing 5.05% CHDG and TA are similar to previous ones and present the same tendency of slight decreasing of pore size with TA amount. The matrices become more compact when TA concentration increases.

The amount of 9.09% CHDG gives the matrices having the images from Figure 5.16.



a b c

Figure 6.16. SEM images 100x of matrices containing 9.09% DGCH and: a – 5, b – 10 and c – 15% AT

So the aspect is not modified compared with the previous ones, pore sizes are less uniform and, at the last two concentrations of TA, they are smaller, because of the stronger crosslinking.

Concluding, the most suitable ratio between the two antimicrobials from the point of view of size and uniformity of pores is 10% TA/5.05% CHDG, conclusion detached also from other properties.

6.3.3. Characterization by absorption of water

The **c**urves giving the amounts of water absorbed by the matrices obtained from the hydrogels containing 1.82% CHDG and the three amounts of TA within 24 h are presented in Figure 6.14. [18]

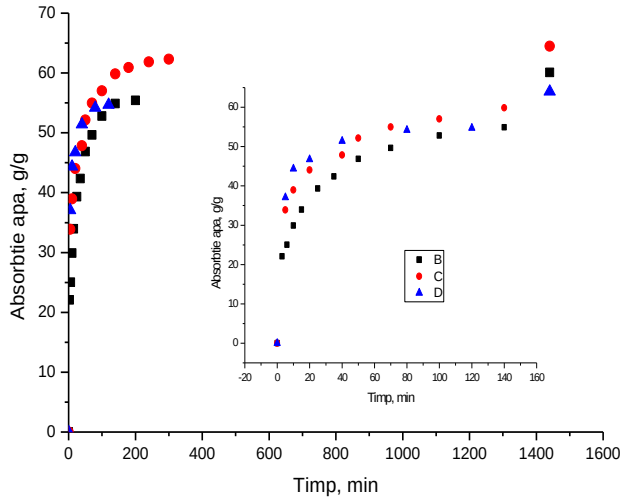


Figure 6.14. Amounts of water absorbed by matrices with 1.82 % CHDG and: ■ – 5; ● – 10 and ▲ – 15% TA

The matrix containing 10% TA absorbs the highest amount of water, excepting the first 40 min when that with 15% TA absorbs a little more, and not that with 5%, which is less crosslinked.

Compared to absorptions of matrices with TA, series in which it decreases with TA concentration increasing, when 1.82% CHDG is present the above order is not respected: the matrix with 10% TA absorbs the maximum amount within 24 h, but it is lower than when the matrix does not contain CHDG. Consequently CHDG reduces the hydrophilicity of matrices crosslinked with TA.

When the hydrogels from which the matrices were obtained contain 4.55% CHDH, the absorption curves look similar, but the absorbed amounts are higher, comparable with those of matrices containing TA. The highest amount is absorbed by the matrix with 10% TA, followed by that with 5%. The explanation for increasing of matrices hydrophilicity for 4.55% CHDG is that this is the optimum amount of CHDG to be combined with TA.

Increasing the amount of CHDG at 9.09% preserves the appearance of curves, but the absorption depend inversely on the amount of TA under 120 min: the most water is absorbed by the matrix with 15% TA, followed by that with 10%, and the less that with 5% TA. Between 4 and 24 h the order of absorption is: matrix with 15% TA absorbs more tan that with 5%, while that with 10% absorb the lowest amount. All the matrices are less hydrophobic than those with 4.55% CHDG, but more hydrophobic than those with 1.82%. The difference of absorptions after 24 h is small.

Consequently it can be said that the most convenient amount of CHDG to be combined with the three amounts of TA seems to be 4.55%, and among them, the combination 10% TA-5.05% CHDG.

6.3.4. Characterization by digestion with collagenase

The simultaneous presence of the two antimicrobials in the collagen matrices increases spectacularly the resistance to digestion with collagenase. Thus, all the matrices resist 9 days (the last day of observation), excepting that with 5% TA and 1.82% CHDG, which – in the last day – are fragmented into 2 or 3 pieces. This demonstrates once more that CHDG crosslinks also collagen, or at least it binds the collagen fibrils, increasing thus resistance to digestion. The matrices with 9.09% CHDG and 5 and 10% TA preserve their form, while the edges of those containing 15% TA are eroded a little. This is in accordance with the previous observations that 15% TA surpasses the amount required for crosslinking and the excess affects collagen. [18]

References

6. M. Geiger şi W. Friess, Collagen sponge implants: applications, characteristics and evaluation: Part II, Pharm. Tech. Europe, 14, 58-66, **2002**.
7. M. Geiger şi W. Friess, Collagen sponge implants: applications, characteristics and evaluation: Part I, Pharm. Tech. Eur., 14, 48-56, **2002**.
8. V. Glattauer, J. F. White, W.B. Tsai, C.C. Tsai, T.A. Tebb, S.J. Danon, J.A. Werkmeister şi J.A. Ramshaw, Preparation of resorbable collagen-based beads for direct use in tissue engineering and cell therapy applications, J. Biomed. Mater. Res., A 92, 1301-1309, **2010**.
9. F. Takeshita, N. Hokaiwado, K. Honma, A. Banas şi T. Ochiya, Local and systemic delivery of siRNAs for oligonucleotide therapy, Methods Mol. Biol., 487, 83-92, **2009**.
10. F. Takeshita şi T. Ochiya, Therapeutic potential of RNA interference against cancer, Cancer Sci., 97, 689-696, **2006**.
11. A. Sano, M. Maeda, S. Nagahara, T. Ochiya, K. Honma, H. Itoh, T. Miyata şi K. Fujioka, Atelocollagen for protein and gene delivery, Adv. Drug Deliv. Rev., 55, 1651-1677, **2003**.
15. M.V. Ghica, M.G. Albu, L. Popa, M. Leca, **L. Brăzdaru**, C. Cotruţ, şi V. Trandafir, Drug delivery systems based on collagen-tannic acid matrices, Rev. Roum. Chim., 54, 1103-1110, **2009**.
16. D. Şulea, M.G. Albu, M.V. Ghica, **L. Brăzdaru**, M. Leca şi L. Popa, Characterization and in vitro release of chlorhexidine digluconate contained in type I collagen porous matrices, Rev. Roum. Chim., 56, 65-71, **2011**.
17. **L. Mincan**, M.G. Albu, M. Leca şi R. Sîrbu, Izolation of type I fibrilar collagen and characterization of collagen matrices, Timişoara Medical J. 55 Suppl., 212-214, **2005**.

18. **L. Mincan**, M. Micutz, M.V. Ghica, M.G. Albu, M. Leca, C. Chelaru, **C**haracterization of collagen matrices containing tannic acid and chlorhexidine digluconate and tannic acid delivery, Int. J. Pharm., sent for publication.
20. C. Petibois, G. Gouspillou, K, Wehbe, J-P. Delage şi G. Deleris, Analysis of type I and IV collagens by FT-IR spectroscopy and imaging for a molecular investigation of skeletal muscle connective tissue, Anal. Bioanal. Chem., 386, 1961–1966, **2006**.
22. R.J. Jakobsen, L.L. Brown, T.B. Hutson, D.J. Fink şi A. Veis, Intermolecular interactions in collagen self-assembly as revealed by Fourier transform infrared spectroscopy, Sci., 220, 1288-1290, **1983**.
23. K.J. Payne şi A. Veis, Fourier transform IR spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies, Biopolymers, 27, 1749-1760, **1988**.
24. A. George şi A. Veis, FTIRS in H2O demonstrates that collagen monomers undergo a conformational transition prior to thermal self-assembly in vitro, Biochem., 30 2372-2377, **1991**.
30. K. Pal, A.K. Banthia, D.K. Majumdar, Polymeric Hydrogels: Characterization and Biomedical Applications – A mini review, Design. Monomers Polymers, 12, 197-220, **2009**.
34. R. Mayne şi R. Burgeson, „Structure and Function of Collagen Types", Academic Press, N.Y., 1987.

7. DELIVERY OF TANNIC ACID FROM MATRICES

Due to physical, chemical and immunological properties, biocompatibility, absence of toxicity, weak antigenity, capacity of recovery and haemostatic action collagen is a support for many drugs. [10-13]

TA binds with collagen by hydrogen bonds, [24] electrostatic forces, [25] hydrophobic interactions [26] and covalent bonds. [27] Crosslinking can be modulated by the amount of TA introduced. [28]

Supposing that the collagen matrix is a homogenous polymeric support, the drug – dissolved or suspended – can be free or bound. The matrix being porous, the free drug diffuses and is released immediately, while the one partially immobilized is released steadily, depending on the diffusion of the biologic fluid as well as on the progress of swelling and matrix erosion. The prolonged delivery is favoured by the porosity and the tridimensional structure of the matrix. [32, 33] The kinetics of delivery is influenced also by the chemical treatment applied to matrix, as well as on modification of its porosity or density. [34]

The kinetic equation describing the delivery of drugs has the general form:

$$m_t/m_\infty = kt^n \qquad (7.1)$$

where m_t is the amount of drug delivered at time t, m_∞ – total amount of drag contained by support, m_t/m_∞ – fraction of delivered drug, k – kinetic constant and n – delivery exponent, indicating the mechanism of delivery: (a) if n has the value 0.5 the drug diffusion rate is much lower than that of relaxation of support, delivered amount is proportional to square root from releasing time and delivery is governed by the Fick law; the model which describes the behaviour was elaborated by Higuchi; (b) when n is equal to 1 the drug diffusion rate is much higher than that of relaxation of support, delivered amount is proportional with releasing time and is described by the zero order model; (c) if n ranges between 0.5 and 1 the drug diffusion rate is of the same order of magnitude as that of relaxation of support, delivery is based not only on diffusion but is associated with other mechanisms, and the model describing it was elaborated by Ritger-Peppas. [30, 35, 36] For porous supports that do not swell the value of n is lower than 0.5 and an extension of Peppas model is used, called the power law model, for which n ranges between 0 and 1.

Releasing of TA was determined *in vitro* in physiological conditions: saline phosphate solution with pH 7.4 as delivery medium and 37^0C. TA interacting wit CHDG by hydrogen bonds and hydrophobic forces, [37] the delivery from the two categories of matrices must be discussed separately.

7.1. Delivery from matrices containing tannic acid

TA **c**oncentration was measured by UV spectrophotometry, from the values of absorbance of maximum from 276 nm, using the calibration curve previously drawn. [38]

The amounts delivered by the matrices obtained from the hydrogels containing the three amounts of TA, expressed as percentages from the total amount introduced, are presented in Figure 7.2.

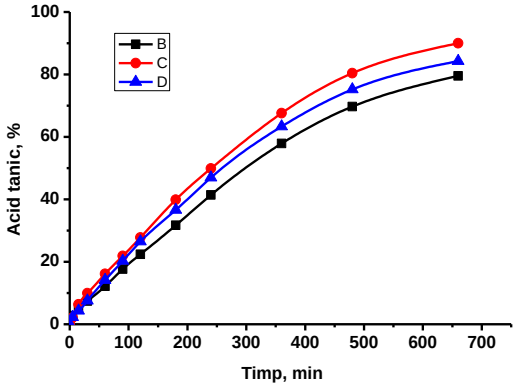


Figure 7.2. TA cumulative delivery curves from the collagen matrices with: B – 5; C – 10 and D – 15% TA

The curves have the same appearance: until about 240 min the delivered amount increases practically linearly with time, than it is reduced and after 500 min tends to a plateau. The highest amount is delivered by the matrix containing 10% TA, which support the hypothesis that 10% TA is enough to crosslink the collagen. The excess denatures it and increases the number of functional groups; thus TA is more strongly bound on collagen and less is delivered.

Delivery data were introduced in the models describing delivery kinetics, and the values obtained for correlation coefficients are given in Table 7.1.

The values show that data are the best correlated by the Ritger-Peppas model, as expected for porous matrices, for which – besides diffusion of drug and relaxation of support – other phenomena, as swelling of matrix interfere in delivery. The firs order model can be valid only during the first minutes.

The kinetic parameters k and n calculated using the Ritger-Peppas model are presented in Table 7.2, together with the maximum delivered amounts measured (at 660 min) – c_m.

Table 7.1. Values of correlation coefficients obtained using mentioned models

TA, %	Higuchi Model	Zero order model	Ritger-Peppas model
5	0.9700	0.9702	0.9924
10	0.9794	0.9568	0.9916
15	0.9775	0.9562	0.9902

Table 7.2. Kinetic parameters calculated with Ritger-Peppas model for matrices with TA

% AT	k, min-^{n}	n	c_m, %
5	0,711	0,734	79,54
10	1,113	0,685	90,00
15	0,961	0,698	84,28

The Table shows that the values of delivery coefficients are very close for the matrices containing 10 and 15% TA, probably due to the close values of the crosslinking degrees. Consequently some other phenomena taking place when the matrices are introduces into the delivery solution contribute also to delivery, the most important being swelling, which probable controls the delivery. The results are in accordance with those obtained by absorption of water, which are very close for these two matrices.

7.2. Delivery from matrices containing tannic acid and chlorhexidine digluconate

The delivery of TA from matrices containing all the combinations between TA and CHDG was determined similarly, and the delivered amounts expressed analogous. Correlation coefficients were calculated with the same equations and delivery parameters with Ritger-Peppas, which also describe the best delivery. Curves presenting regularities at constant TA concentrations, they are discussed separately.

7.2.1. Delivery from matrices with 5% tannic acid

Cumulative delivery curves of TA from matrices with 5% TA and DGCH are given in Figure 7.3.

The regions of linear increase of amounts of TA with time in the first 240 min are replaced by exponential ones. The higher the percentage of CHDG, the lower the amount of TA delivered, becoming considerably smaller for the matrix with maximum amount of CHDG. The results are in agreement with dynamic viscosities of hydrogels from which the matrices were obtained. This suggests that diffusion of TA

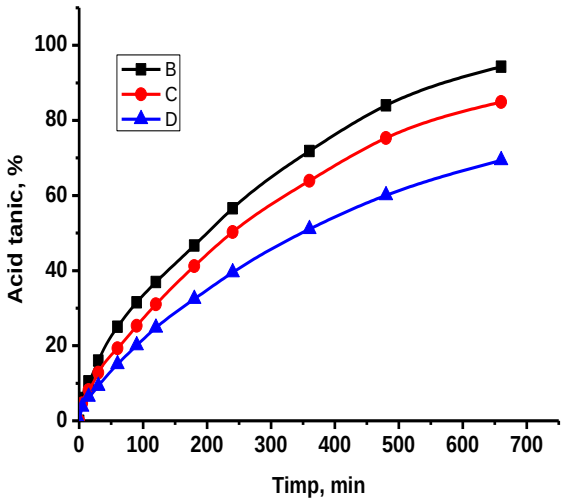


Figure 7.3. TA delivery cumulative curves from matrices with 5% TA and B – 1.82, C – 4.55 and D – 9.09% DGCH

through the hydrogel formed by swelling of matrices play an important role in its delivery.

The values of correlation coefficients for kinetic equations are close to unity for Ritger-Peppas equation too, as can be seen from Table 7.3.

Table 7.3. Correlation coefficients calculated using specified models for matrices with 5% AT and CHDG

% DGCH	Higuchi model	Zero order model	Ritger-Peppas model
1,82	0,9960	0,9329	0,9974
4,55	0,9919	0,9424	0,9959
9,09	0,9911	0,9506	0,9973

Table 7.4. Kinetic parameters given by Ritger-Peppas model for matrices containing 5% AT and CHDG

% DGCH	k, min^{-n}	n	c_m, %
1,82	2,548	0,562	94,28
4,55	1,789	0,601	84,89
9,09	1,289	0,618	69,40

Delivery exponents from Table 7.4, calculated with Ritger-Peppas equation, are lower than for the matrix with the same amount of TA, with very close values for those containing 4.55 and 9.09% CHDG.

The maximum amounts of TA, given in the same Table, decrease with increasing of CHDG amount, due to the increase of hydrogels viscosities when amount of CHDG is higher (Tables 5.19-5.21).

7.2.2. Delivery from matrices with 10% tannic acid

Cumulative delivery curves of TA from the matrices containing 10% TA are given in Figure 7.4.

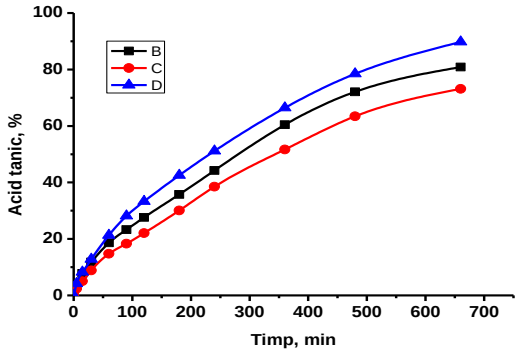


Figure 7.4. TA delivery curves for matrices with 10% TA and: B – 1.82, C – 4.55 and D – 9.09% DGCH

Table 7.5. Values of correlation coefficients obtained for matrices with 10% TA and 1.88, 4.55 and 9.09% CHDG

CHDG, %	Higuchi model	Zero order model	Ritger-Peppas model
1.82	0.9876	0.9547	0.9957
4.55	0.9810	0.9685	0.9968
9.09	0.9939	0.9444	0.9977

Table 7.6. Kinetic parameters obtained with Ritger-Peppas model for matrices with 10% AT and DGCH

% DGCH	k, min^{-n}	n	c_m, %
1.82	1.405	0.630	80.85
4.55	0.862	0.689	73.15
9.09	1.878	0.600	89.74

Delivery curves are placed similar with the previous ones only for 1.82 and 4.55% CHDG, while that for 9.09% is placed above the first two. Therefore the highest amounts of TA are delivered by the matrix containing the most CHDG, followed by that with 1.82%. The positions of curves are in agreement with viscosities of hydrogels from which matrices were obtained, the most viscous being the hydrogel with 4.55% CHDG. Thus, diffusion of TA through the hydrogel formed by swelling plays important role.

The values of correlation coefficients obtained with the kinetic models are closest to unity for Ritger-Peppas model also, as can be seen from Table 7.5.

Delivery coefficient has the highest value for matrix with 4.55% CHDG (Table 7.6).

7.2.3. Delivery from matrices with 15% tannic acid

Cumulative delivery curves of TA from the matrices containing 15% TA are given in Figure 7.5.

The Figure shows that the higher the amount of CHDG the higher the amount of TA delivered. Delivered amounts are in agreement with hydrogels' viscosities from which matrices were obtained.

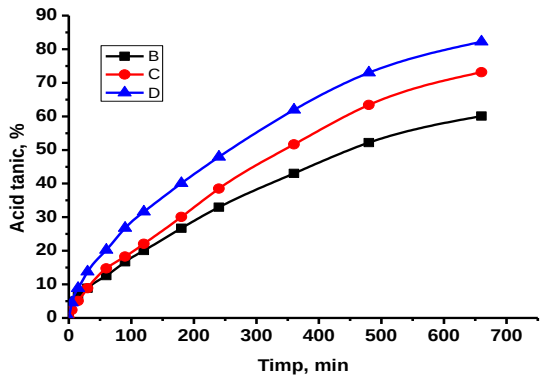


Figure 7. TA delivery cumulative curves from matrices with 15% TA and: B – 1.82, C – 4.55 and D – 9.09% DGCH

Table 7.7. Correlation coefficients obtained for matrices with 15% TA and CHDG

% DGCH	Higuchi model	Zero order model	Ritger-Peppas model
1,82	0,9852	0,9633	0,9963
4,55	0,9920	0,9419	0,9959
9,09	0,9952	0,9387	0,9976

Tabelul 7.8. Kinetic parameters obtained with Ritger-Peppas model for matrices with 15% AT and DGCH

% DGCH	k, %/min^n	n	c_m, %
1,82	1,041	0,628	60,06
4,55	1,434	0,601	68,33
9,09	2,002	0,577	82,21

Correlation coefficients have the highest values calculated with Ritger-Peppas model (Table 7.7).

Values of delivery coefficients, calculated with Ritger-Peppas model, are given in Table 7.8.

The amounts of TA delivered increase with increasing of CHDG concentration, TA binding on the antibacterial compound being weaker than on collagen, as specified above.

References

10. C. H. Lee, A. Singla şi Y. Lee, Biomedical application of Collagen, Int. J. Pharmaceutics, 221, 1-22, **2001**.
11. H. C. Goo, Yu-S. Hwang, Y. R. Choi, H. N. Cho şi H. Suh, Development of collagenase-resistant collagen and its interaction with adult human dermal fibroblasts, Biomat. 24, 5099-5113, **2003**.
12. J. M. Pachence, Collagen-based devices for soft tissue repair, J. Biomed. Mater. Res. A 33, 35-40, **1996**.
13. R. K. Panduranga, Recent developments of collagen-based materials for medical applications and drug delivery systems, J. Biomater. Sci. Polym. Ed. 7, 623-645, **1995**.
25. K. M. Meek şi J. B. Weiss, Differential fixation of poly(L-arginine) and poly(L-lysine) by tannic acid and its application to the fixation of collagen in electron microscopy, Biochim. Biophys. Acta., 587, 112-120, **1979**.
26. W. D. Loomis, Removal of phenolic compounds during isolation of plant enzymes, Methods Enzymol., 13, 535-563, **1969**.
27. B. Madhan, V. Subramanian, J. Raghava Rao, B. Unni Nair şi T. Ramasami, Stabilization of collagen using plant polyphenoles: Role of catechine, Int. J. Biol. Macromol., 37, 47-53, **2005**.
28. B. Madhan, C. Muralidharan şi R. Jayakumar, Study on the stabilisation of collagen with vegetable tannins in the presence of acrylic polymer, Biomater., 23, 2841-2847, **2002**.
30. N. A. Peppas, P. Bures, W. Leobandung şi H. Ichikawa, Hydrogels in pharmaceutical formulations. Eur. J. Pharmaceutics and Biopharmaceutics, 50, 27-46, **2000**.
32. Z. Ruszczak şi W. Friess, Collagen as a carrier for on-site delivery of antibacterial drugs. Adv. Drug Delivery Rev., 55, 1679-1698, **2003**.
33. D. G. Wallace şi J. Rosenblatt, Collagen in drug delivery and tissue engineering, Adv. Drug Delivery Rev., 55, 1631-1649, **2003**.
34. M. Grassi şi G. Grassi,. Mathematical modelling and controlled drug delivery: matrix systems, Current Drug Delivery, 2, 97-116, **2005**.
35. S. Li, Y. Shen, W. Li, X. Hao, A common profile for polymer-based controlled releases and its logical interpretation to general release process, J. Pharm. Pharmaceut. Sci. 9, 238-244, 2006.
36. H. Teles, T. Vermonden, G. Eggink, W. E. Hennink, şi F. A. de Wolf, Hydrogels of collagen-inspired telechelic triblock copolymers for the sustained release of proteins, J. Controlled Release, 147, 298-303, **2010**.
37. **L. Mincan**, M. Micutz, M.V. Ghica, M.G. Albu, M. Leca, C. Chelaru, Characterization of collagen matrices containing tannic acid and chlorhexidine digluconate and tannic acid delivery, Int. J. Pharm., sent for publication.
38. M. V. Ghica, M. G. Albu, L. Popa, M. Leca, **L. Brăzdaru**, C. Cotruţ şi V. Trandafir, Drug delivery systems based on collagen-tannic acid matrices, Rev. Roumaine Chim., 54, 1103-1110, **2009**.

8. GENERAL CONCLUSIONS

The rapid methods of characterization show that initial hydrogel contains sufficiently pure collagen to be used for preparation of biomaterials, and UV-CD spectrum that the native conformation is preserved.

The optimum concentration of collagen in hydrogel to prepare biomaterials – hydrogels and porous matrices – determined from stationary rheological properties, is 1.1%, the hydrogel having both convenient consistency and stability under the action of shear forces.

Characterization of hydrogels

Obtaining of collagen hydrogels containing the antimicrobial compounds tannic acid or/and chlorhexidine digluconate, intended for healing of dry wounds, having the characteristics:

- **native conformation of collagen molecules;**
- **sufficient consistency to be applied and remain on wound;**
- **sufficient elasticity to be stretched on wound without breaking.**

1. **Hydrogels containing TA**. **FT-IR spectra** show change of amide II band due to collagen-TA interactions: increasing of TA concentration decreases intensity of band at 1528 cm^{-1} and a shoulder appears at 1545 cm^{-1}, assigned to crosslinking. TA in used concentrations does not denatured collagen.

Intensities of minima, maxima and Rpn values from **UV-CD spectra** decrease visibly when TA concentration increases, but the wavelengths remain practically unchanged, which signify that the hydrogels do not contain denatured collagen. It was demonstrated that the high Rpn value obtained for hydrogels are due to high concentration of collagen and hydrogel opalescence and not to denaturizing. The **criterion for recognizing the native structure of collagen in concentrated solution/hydrogels** was established: **the existence of maximum and minimum at wavelengths specific to collagen, irrespective of the values of intensity, that is of Rpn values**.

The hydrogels remain **pseudoplastic** and **thixotropic** but are more viscous, properties increased by TA concentration. The hydrogel containing 10% TA has the most consolidated structure.

Elasticity prevails, characteristic increased by TA. Both moduli increase linearly with angular frequency and the line giving their variation are parallel, confirming crosslinking with TA. The most elastic and viscous is the hydrogel containing 10% TA, tat is the most crosslinked. 15% TA exceeds the amount required by crosslinking and affects a bit collagen.

2. Hydrogels containing CHDG. CHDG does not modify the positions of collagen **FT-IR** bands, conclusion supported by the differences between the wave numbers of bands amide I and II (< 100 cm^{-1}). The ratios $A_{III}/A_{1450} > 1$, that is the denatured collagen is absent. A_I/A_A ratios decrease, which means that crosslinking by hydrogen bonds is reduced slightly due to ionic interactions of carboxyl groups with CH dications.

UV-CD. CHDG reduces the maxima and modifies less the minima, does not denatured collagen but produces a slight phase separation, more reduced in acid medium, because of the lower number of dissociated carboxyl groups at pH 3.8. Collagen is not denaturized.

The hydrogels have **pseudoplastic behaviour with limiting flowing stress**. Dynamic viscosities decrease slightly with increasing of CHDG concentration, consistency indices are a little reduced in each series, and flowing indices are a bit increased.

Elastic and **viscous** contributions depend on CHDG concentration and pH: in acid medium both moduli decrease slightly when CHDG is introduced and elastic component prevails; in weakly basic medium the values are higher for both moduli.

3. Hydrogels containing TA and CHDG. TA-CHDG interactions in acid medium are identified in **FT-IR spectra** only at the maximum concentration of CHDG: the band amide II splits and a new band at 1541 cm^{-1} appears. A shoulder on the methylene stretching band can also be seen. The intensities of both increase when TA concentration is raised. Values $A_{III}/A_{1450} > 1$ and differences between the wavenumbers of bands amide I and II < 100 cm^{-1}, so they do not contain denatured collagen

UV-CD Spectra look very similar to those of hydrogels containing TA. Flattening of maxima and minima are increased by CHDG concentration, but their positions and zero elipticity points displace insignificantly. The minimum Rpn value – 011 – was obtained for the hydrogel containing the maximum amounts of both components.

Stationary rheology suggests formation of TA-CHDG associates, especially at high concentrations of both components. Viscosity of hydrogel containing 15% TA is lower than that of the one with, probable because of the associates forming between TA and CHDG. Chlorhexidine dications form with collagen associates with lower viscosity, which create the aspect hydrogels non-homogeneity.

Dynamic rheology shows that the hydrogels are preponderantly elastic. The two components increase with TA concentration. The hydrogel containing maximum amounts of TA and CHDG has maximum elasticity and that with 9.09% CHDG and 5% TA maximum viscosity.

Characterization of matrices

Obtaining of porous collagen matrices containing the antimicrobial compounds tannic acid or/and chlorhexidine digluconate, intended for healing of moist wounds, having the characteristics:

- **native conformation of collagen molecules;**

- **pore size able to assure growing and migration of epithelial cells;**
- **good capacity to absorb biological fluids;**
- **satisfactory resistance to degradation.**

1. Matrices containing TA. **FT-IR spectra** show slight displacing of bands amide A and II towards lower wave numbers, increasing of band intensity at 1200 cm^{-1} from the complex band amide III and the decrease of intensity of band amide I when TA is introduced, changing associated with crosslinking of collagen by TA by hydrogen bonds. The differences of wave numbers of bands amide I and II increase when TA concentration increases, but remain lower than 100 cm^{-1}, that is TA does not denatured collagen.

SEM. Lamellar morphology characteristic to collagen matrices disappears, the pores are annular and relatively elongated, their size vary within large limits, increase with TA concentration, having compact collagen zones as a result of crosslinking. Pore sizes are suitable for development and migration of cells.

Amounts of water absorbed decrease with increasing of TA percentage, from about 90 g/g for the matrix with 5% AT to 65 g/g for that with 15% in 24 h. The values are very close for 10 and 15% TA, suggesting similar crosslinking degrees. Consequently 10% TA is enough for collagen crosslinking

Digestion with collagenase from *Clostridium histolyticum* in physiological conditions shows that matrice resistance increases with rise of TA content; those with 10 and 15% TA are undivided after 9 days.

2. Matrices containing CHDG. CHDG does not modify the of **FT-IR** bands of collagen. Ratios $A_{III}/A_{1450} > 1$ and wave number differences of bands amide I and II < 100 cm^{-1} show that they do not contain denatured collagen. Ratios A_I/A_A suggest that CHDG reduces slightly crosslinking by hydrogen bonds, due to crosslinking with chlorhexidine dications.

SEM. The lamellar morphology is preserved for 1.82% CHDG obtained from acid hydrogel, while for the other concentrations it is not. If prepared from slight basic hydrogels, pores and fibril agglomerates are larger, collagen-CHDG interactions being stronger. Pores allow growing and migration of cells.

3. Matrices containing TA and CHDG. **FT-IR spectra** show that collagen-TA interactions are stronger than collagen-CHDG. The differences between wave numbers of bands amide I and II < 100 cm^{-1}. Consequently they do not contain denatured collagen and can be used as dressings for wound healing.

SEM. Pores are smaller, more elongated and irregular when both components are present; matrices contain agglomerates that increase with component concentrations. Consequently CHDG crosslinks also collagen, producing denser tridimensional networks. Their sizes allow cell development and migration.

Absorption of water is reduced by increasing TA concentration, but CHDG has also a slight decreasing effect, that is it crosslinks also collagen. Absorption capacity remained acceptable.

Digestion of matrices demonstrates that presence of the two antimicrobials increases highly resistance to digestion with collagenase, meaning that both components crosslink collagen. Only the matrix containing the lowest amounts of antimicrobials – 5% TA and 1.82% CHDG – does not resist, presenting 2-3 fragments in the 9^{th} day. Consequently the both component contribute to collagen crosslinking.

Delivery of tannic acid from matrices

It was determined using a modified USP device (sandwich).

Matrices containing TA. Cumulative amounts of delivered TA increase linearly with time within the first 4 h, than the slope decreases steadily, tending to a plateau. Delivery is described by the Ritger-Peppas model. This demonstrates that, besides diffusion, some other phenomena contribute to delivery, the most important and probable being swelling. The largest amount is delivered by the matrix containing 10% TA and the lowest by that with 5%.

Matrices containing TA and CHDG. The linear portions disappear from the delivery curves, they being exponential over the whole range of time on which the measurements were made (11 h). Delivery kinetics is described also by the Ritger-Peppas model, proving that swelling of matrices contribute to delivery.