NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY **POLITEHNICA** BUCUREŞTI
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PHD THESIS

Summary

Spongy materials based on collagen, piroxicam and ciprofloxacin for surgical applications

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The present work started from an idea regarding the level of local healing required in any surgical specialty. Starting from this idea and together with Prof. Ileana Rău , this doctoral thesis was born and developed.

I would like to thank her especially for all the help offered unconditionally, for the initiation and support of the present project, but also for the careful guidance, support and encouragement to complete this project.

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KEY WORDS SUMMARY

Collagen; Piroxicam; Ciprofloxacin; Functional biomaterials; Enzymatic degradation; Drug realease; Antimicrobial activity; Cytotoxicity;

INTRODUCTION

The research carried out during the doctoral study focused on the synthesis of new collagen-based materials, materials in which a drug was embedded. The drugs considered were piroxicam – a non-steroidal anti-inflammatory drug from the oxicam class and ciprofloxacin – an antibiotic from the fluoroquinolone class. These materials were spongy in nature and represent an important source of biocompatible material with the human body, also helping to restore and heal traumatized tissue, having the benefit of being absorbed, and releasing medicinal substances locally.

Following the literature study, it was found that the use of such materials represents an adjuvant method in surgical treatments, as spongy materials can store drugs and help local tissue regeneration through collagen.

The work is structured in two parts and consists of eight chapters. The first part includes 3 chapters and are dedicated to the bibliographic study, while the second part includes 5 chapters and is presenting the original contributions.

In chapter 1 various surgical specialties are presented in which sponges containing anti-inflammatory and antibiotic with a role in regeneration and acceleration of local healing can be applied. Various organic and inorganic materials that can be used as biomaterials in the medical field are also presented, including the presentation of certain medicinal substances applied systemically or locally.

Chapter 2 consists in deepening the elements, properties, biomimetic and characteristics of collagen materials, as well as highlighting their applicability in the medical-surgical field.

Chapter 3 describes the principles and kinetic methods of mathematical analysis of drug release, as well as some indicative examples.

Chapter 4 describes the reagents, apparatus, and methods used to obtain and characterize drug-loaded collagen sponges.

In chapter 5 are presented the analyzes that were carried out for collagen sponges with piroxicam, respectively optical measurements, enzymatic degradation experiments, water absorption and *in vitro* drug release, with applications in the veterinary field.

Chapter 6 presents the analyzes that have been carried out for collagen sponges with piroxicam, namely optical measurements, spectral measurements, enzymatic degradation, water absorption and *in vitro* drug release experiments, with applications in the medical field.

Chapter 7 describes the analyzes that were carried out for collagen sponges with ciprofloxacin, respectively optical measurements, spectral measurements, biological effects on some pathogenic microorganisms, the cytotoxic study on normal cells with *in vitro* testing, enzymatic degradation experiments, water absorption and *in vitro* drug release, biological effects on some pathogenic microorganisms, with applications in the medical field.

Chapter 8 describes preliminary tests by enzymatic degradation and water absorption experiments for antibiotic and anti-inflammatory collagen sponges with potential applications in various surgical specialties, such as oral surgery or head and neck surgery or other surgical specialties.

The present thesis ends with the presentation of general conclusions, original contributions, further development perspectives, list of published works and conferences, as well as bibliographic references.

The present thesis includes 23 figures, 13 tables and 166 bibliographic references.

The results of this work were capitalized through 3 publications of which 1 article in the UPB Bulletin and 2 articles in ISI rated journals. Also, the results were presented at 1 international conference.

In this summary, both the pagination in the table of contents and the numbering of the chapters, sub-chapters, figures, tables and bibliographic references are kept the same as in the thesis.

TABLE OF CONTENTS

CHAPTER 1. MATERIALS USED IN ORO – MAXILLO – FACIAL SURGERY

1.1.2. ORO - MAXILLO - FACIAL SURGERY

Oro-maxillo-facial surgery (OMF) represents a surgical branch of medicine that deals with the surgical treatment of the cervico-facial soft parts, viscerocranium bones, salivary glands, temporo-mandibular joint, craniofacial malformations, with plastic reconstructive in head and neck surgery and also with the surgical treatment of dento-periodontal diseases. For this reason, doctors specializing in Oro-Maxillo-Facial Surgery hold a double license, being graduates of both the Faculty of General Medicine and the Faculty of Dental Medicine **[1-3].** Oral surgery represents only a small part of Oro-Maxillo-Facial Surgery, this being limited only to interventions in the oral cavity.

In head and neck surgical specialties, plastic surgery, as well as in other surgical specialties, various organic and inorganic materials are used materials that are biocompatible with the human body. In addition to these materials, various drugs such as antibiotics, antiseptics or anti-inflammatories can also be used and they can be applied

locally or systemically. All these combined elements have as final goal the improving of the quality of life after surgerical interventions and represent an adjuvant method for the healing and recovery process of the body at local level.

CHAPTER 2. COLLAGEN

2.2. PROPERTIES OF COLLAGEN

Collagen is one of the most abundant fibrous proteins that has many mechanical functions, especially in mammals, in all its defining forms. It presents itself as a polymer that can be characterized by a pronounced hydrophilicity, by variable ionic character, by a diversified functionality, being able to be involved in several systems that interact with various macromolecular or micromolecular compounds **[36, 37, 43-47].**

2.3. MATERIALS BASED ON COLLAGEN

Collagen-based materials can be made from a wide variety of molecular structures, in microstructures or nanostructures that can be found in the form of hydrogel (for a controlled release of the various components that can be embedded), collagen membranes (can be used in the therapeutic process of dialysis or for the storage of medicinal substances), sponges (which have a very important role in various therapeutic acts such as the dressing for burns or varicose ulcers, due to their hemostatic or tissue substitute role), collagen fibers (can be used to reinforce membranes and sponges etc.) **[37].**

CHAPTER 3. CONTROLLED RELEASE KINETICS OF LOCALLY DRUGS ADMINISTERED

3.1. INTRODUCTION

Regardless the route of administration, the key factor for the success and reliability of any formulation depends on the bioavailability of the drug, thus defining the amount and the rate with which it is released from the pharmaceutical form, thus becoming available for absorption and consequently for the pharmacodynamic effect **[74].**

Among the topically administered systems, there are liquid pharmaceutical forms, spongy matrices (collagen sponges), membranes, fibers, multiparticulate systems (micro- and nanoparticulate), intensively studied in recent years and in continuous development. The main purpose of topical formulations is to improve the therapeutic activity of the substance and to overcome the problems related to solubility, state of aggregation, bioavailability, biodistribution, lack of selectivity and even to reduce the negative side effects of the drug. **[81, 83, 84].**

3.2. KINETIC MODELS OF MATHEMATICAL ANALYSIS

Various mathematical models are used to model the drug release kinetics from pharmaceutical formulations such as hydrogels or sponges and establish its transport mechanism.

Many mathematical models tend to describe significantly controlled kinetic release dynamics and the phenomenon involved, thus saving time and money. These mathematical

systems enable the optimization of drug release and estimate the effect of the proposed parameters on kinetic release **[80, 85].**

RESEARCH OBJECTIVES

The main objective of the thesis was to obtain new collagen-based materials functionalized with a non-steroidal anti-inflammatory - piroxicam and/or an antibiotic from the fluoroquinolone class - ciprofloxacin. This main objective was achieved by fulfilling the following specific objectives:

- Preparation of spongy collagen-based materials for veterinary applications and their characterization by:
  - Optical measurements
  - Water absorption experiments
  - Enzymatic degradation experiments
  - *In vitro* drug release
- Preparation of spongy materials based on collagen and piroxicam for medical applications and their characterization by:
  - Optical microscopy
  - Spectral measurements
  - Water absorption experiments
  - Enzymatic degradation experiments
  - *In vitro* drug release kinetics
- Preparation of materials based on collagen and ciprofloxacin with potential oral surgical applications and their characterization by:
  - Water absorption experiments
  - Enzymatic degradation experiments
  - *In vitro* release of ciprofloxacin
  - *In vitro* biological effects of collagen sponges and ciprofloxacin on some pathogenic microorganisms
  - Cytotoxic study of collagen-based materials on normal cells – *in vitro* tests
- Preparation of materials based on collagen, ciprofloxacin and piroxicam with potential oral surgical applications – preliminary tests and their characterization by:
  - Water absorption experiments
  - Enzymatic degradation experiments.

CHAPTER 4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. DRUGS

For the studies carried out, a non-steroidal anti-inflammatory drug - Piroxicam and an antibiotic from the fluroquinolone class - Ciprofloxacin were used.

4.1.2. COLLAGEN

For the present study a fibrilar type I collagen extracted from bovine derma at National Institute of Leather and Footwear Research was used. The used gel contained 2.82% collagen.

4.2. METHODS

4.2.1. PREPARATION OF SPONGIOUS MATERIALS BASED ON COLLAGEN

The concentration of the collagen gel was adjusted from a concentration of 2.82% and an acidic pH to a concentration of 1% and a pH of 7.4 using 1 M sodium hydroxide. The drug solution (piroxicam, ciprofloxacin, or mixtures of piroxicam and ciprofloxacin) was added to the collagen gel in order to obtain certain concentrations of the drug compared to dry collagen. The obtained gel was cross-linked with a glutaraldehyde solution so that the concentration of glutaraldehyde relative to the dry collagen was 0.5%.

Tables 4.1, 4.2 and 4.3 show the systems studied based on collagen, piroxicam and/or ciprofloxacin.

Table 4.1 Systems studied based on collagen and piroxicam

Sample code	Concentration, % in relation to dry collagen
P1	1
P2	1,5
P3	2
P4	50
P5	80

Table 4.2 Systems studied based on collagen and ciprofloxacin

Sample code	Concentration, $g_{drug}/g_{collagen}$
C1	0,5
C2	0,75
C3	1

Table 4.3 Systems studied based on collagen, piroxicam and ciprofloxacin

Sample code	Concentrations, $g_{drug}/g_{collagen}$
PC 1	0,15 piroxicam; 0,05 ciprofloxacin
PC 2	1,5 piroxicam; 0,5 ciprofloxacin

Cross-linked gels were kept for 24 hours at a temperature of 40C and then lyophilized using Lyophilizer (Delta 2-24 LSC, Martin Christ, Germany). The gels were transferred to Petri dishes (figure 4.4) where they were initially frozen at - 40^0C for 12 hours. In the following 8 hours at a temperature of - 40^0C and a pressure of 0.1 mbar, the actual lyophilization took place. Then the temperature was increased in steps as follows, up to 10^0C over a period of 8 hours, then to 20^0C over a period of another 8 hours and to 30^0C over a

period of another 8 hours while maintaining the pressure at 0.1 mbar. The final lyophilization took 4 hours: 1 hour at 30^0C and 0.001 mbar and another 3 hours until the temperature reached 35^0C. After another 48 hours of freeze-drying, the collagen-based sponges were obtained.

Figure 4.4 Collagen-based gels transferred into Petri dishes before lyophilization

CHAPTER 5. COLLAGEN-BASED SPONGES FOR VETERINARY APPLICATIONS

In the last decades, periodontal and peri-implantation research on animals has been focused on different types of treatments. Oral diseases are very common in small and large animals such as cats, dogs, horses, cattle and other ruminants and should receive much more attention in veterinary practice, as there are diseases specific to the oral cavity that pose a great risk to health these animals. The present study is focused on the synthesis and characterization of collagen-based sponges with piroxicam in view of their use in animal treatment. Results on the characterization of these new collagen-based materials are presented and discussed in view of their possible oral applications in mammals such as felines and other animals **[67, 68, 72, 73, 157].**

5.2. WATER ABSORPTION EXPERIMENTS

The water absorption results of collagen sponges without or with different concentration of piroxicam are shown in Figure 2. As expected, in time, the water content in sponges is increasing and the presence of the drug has as consequence the decreasing of the absorbed water content. For example, after 24h P4 sponge has absorption of 34% compared to collagen sponge C (57%) and P5 sponge (22%). However, after 24h, the sample containing the highest drug content disintegrated, while the P4 sponge continued to absorb water until 168h and then disintegrated. **[157].**

5.4. *IN VITRO* DRUG RELEASE OF THE MEDICINE

The kinetic experimental data were graphically illustrated as cumulative drug release (%) versus time (Fig. 5.3).

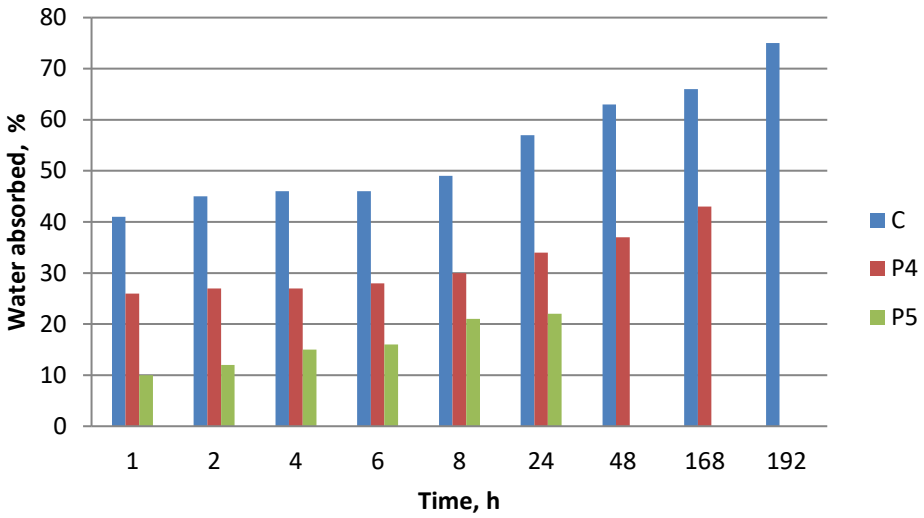


Figure 5.2. Water absorption in collagen sponges: C – collagen sponge, P4 – collagen sponge with 50% piroxicam, P5 – collagen sponge with the highest piroxicam content (80%)

From Figure 5.3, a rapid drug release effect is obvious in the first hour, recording a value of about 31% for sponge P4 and 41% for sponge P5, respectively, followed by a gradual piroxicam delivery in the next 10 hours of experiment up to 69.96% (formulation P4) and 79.83% (formulation P5). It can be noticed that a higher content of piroxicam led to an increase of drug release percentage about 1.14 times [157].

The biphasic drug release behavior is beneficial for alleviating and controlling local inflammation and pain specific to oral diseases of various etiologies **[23, 157].**

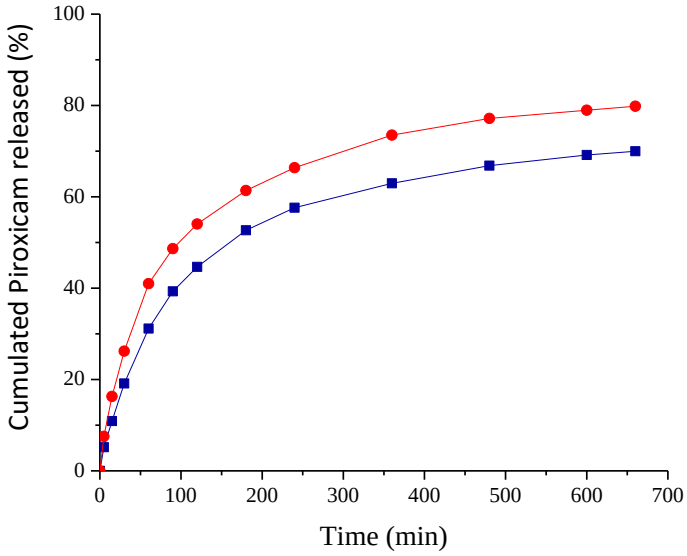


Figure 5.3. Cumulative release profiles of piroxicam from collagen sponges as a function of time; ■ – collagen sponge with 50% piroxicam and • – collagen sponge with 80% piroxicam

5.4. PARTIAL CONCLUSIONS

The present study led to the following conclusions:

- The collagen solution with or without piroxicam were chemical cross-linked with glutaraldehyde and prepared in the form of sponges by lyophilization process;
- The optical microscopy images displayed a fibril structure with interconnected pores in all obtained sponges but with piroxicam aggregations in samples C2 and P5;
- The collagen sponge with no piroxicam content absorbed an important quantity of water, and when drug was added into the collagen sponge composition the water absorption rate decreased. It can be concluded that the water absorption for the studied sponges is decreasing with the drug dose increasing;
- A high content of drug results in the sponge disintegration. It cannot be stated that the samples are totally degraded in the sense of mass loss, they disintegrate and practically fibers of collagen with piroxicam are presented in the solution (PBS with collagenase);
- The kinetic studies indicated a byphasic drug delivery, favorable for ensuring an adequate piroxicam concentration at the application site to manage the inflammation and consecutive pain associated with different oral disease;
- These preliminary results suggest that such kind of sponges with high dose of piroxicam could be used for different treatments in big animals like equines and bovines **[157].**

CHAPTER 6. COLLAGEN AND PIROXICAM BASED SPONGES FOR MEDICAL APPLICATIONS

Starting from the idea of the special benefit of the interference between collagen-based materials and drug beneficial properties, this work was focused on the synthesis and characterization of new collagen-piroxicam materials. These new collagen-based materials present a good water absorption, and the piroxicam release suggests a biphasic drug release profile whereas the obtained values for the release exponent revealed a complex release mechanism including swelling, diffusion, and erosion.

Collagen sponges with piroxicam were prepared for this study as described in Chapter 4. The concentrations of piroxicam were 1%, 1.5% and 2% relative to collagen **[158].**

6.1. CHARACTERIZATION OF SPONGOUS MATERIALS

6.1.2. SPECTRAL CHARACTERIZATION

The presence of the piroxicam in the collagen sponges was certified also by the IR spectra Table 6.1 lists the assignments for the main absorption peaks obtained for the recorded infrared spectra. As expected, the IR spectra were similar because the peak characteristics for the main vibrations are the same for collagen and piroxicam. However, some wavenumber changes were present **[158].**

Table 6.1. FTIR spectra peak position and assignments for collagen sponges

Assignment	Wavenumber (1/cm)			
	Collagen sponge	*Collagen* + 0.5% piroxicam sponge	*Collagen* + 1% piroxicam sponge	*Collagen* + 1% piroxicam sponge
N-H stretch (ν_{NH}) of amide A **[159, 160];** Hydrogen bonding of the N-H group with a carbonyl group of the peptide chain **[159]**	3540	3569	3552	3550
CH_2 asymmetrical stretch (ν_{asCH_2}) by amide B band **[159, 160]**	3017	3008	3016	3007
C-O stretching vibration (ν_{CO}) **[159];** Amide I C=O stretching **[160, 161]**	1681	1681	1680	1680
Hydrogen bond between N-H stretch (ν_{NH}) and C-O (ν_{CO}) **[159]**	1607	1606	1608	1610
Helical structure of collagen **[159]** Stretching vibration of the C-N group **[159]** C=N stretching of piroxicam **[161]** CH_2 deformation (δ_{CH_2}) **[162]**	1494	1499	1499	1496
CH_3 bend (δ_{CH_3}) **[162]** CH_2 bending vibration (δ_{CH_2}) **[160]**	1443	1441	1443	1443
CH_2 group wagging vibration **[159]**	1376	1379	1387	1380
NH deformation of amide III (δ_{NH}) **[162]**	1274	1274	1273	1275
Hydrogen bonding of N-H bending **[159, 160]**	1241	1237	1240	1240
NH deformation of amide III (δ_{NH}) **[162]** S=O asymmetric stretching **[161]**	1191	1192	1189	1190
Ester bond **[159]**	1113	1113	1115	1113
$\nu_{(C\text{-}O)}$, $\nu_{(C\text{-}O\text{-}C)}$ of carbohydrate moieties (collagen) **[162]**	1062	1063	1062	1063
Skeletal stretching vibrations **[160]**	899	899	900	900

6.2. WATER ABSORPTION EXPERIMENTS

Figure 6.2 shows the results obtained for the water absorption experiments. Collagen matrices should exhibit adequate water absorption abilities, allowing biological fluids to penetrate the spongy structure and, consequently, drug diffusion into the network polymer network. The data obtained suggest that the mass of piroxicam does not affect the water absorption capacity At the same time, as expected, the water absorption is increasing in time, being 100% after 24 hours **[158].**

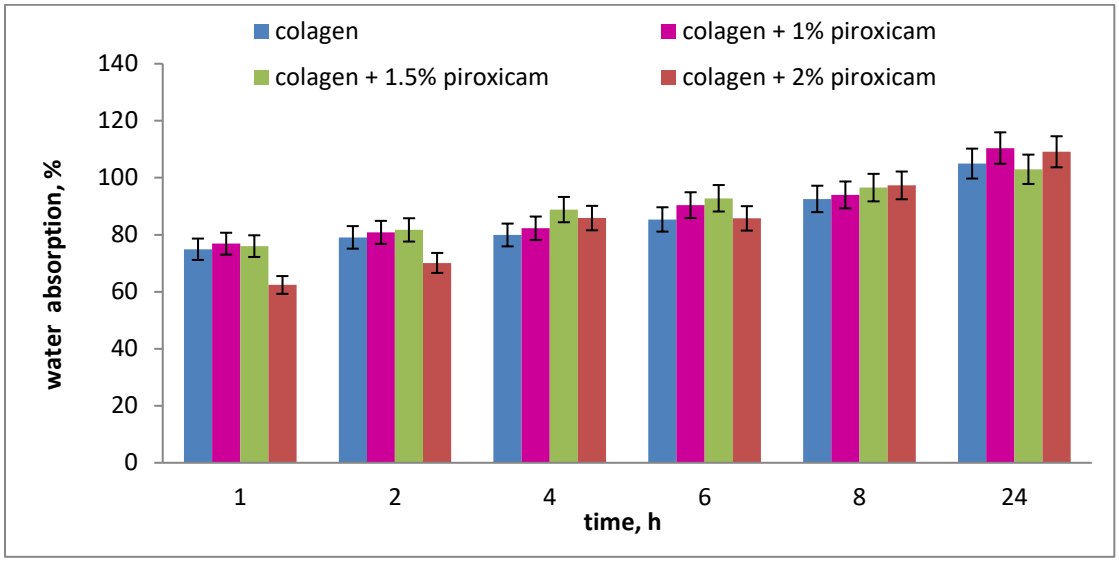


Figure 6.2. Water absorption in sponges containing piroxicam

6.3. ENZYMATIC DEGRADATION EXPERIMENTS

Collagen enzymatic degradation experiments, as shown in Figure 6.3, revealed that the addition of piroxicam resulted in decreased degradation of the sample. However, after more than 8 hours in collagenase solution, the samples disintegrate, even if they are not completely degraded. Degradation of sponges is relatively slow in the begining (5 – 10% in the first two hours). After 8 hours and before disintegration, around 30% of the samples are degraded suggesting that such kind of materials could be useful in oromaxillofacial surgery **[158]**.

All analyses indicate that the designed collagen sponges present adequate properties as drug release supports. For this reason the collagen-piroxicam sponges were further analyzed from the kinetic point of view **[158].**

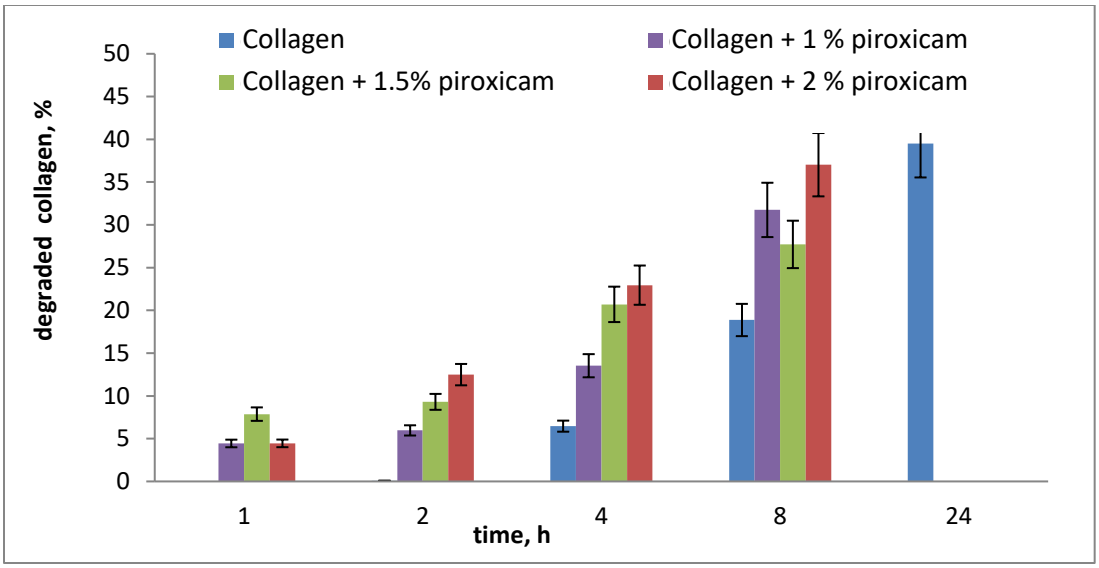


Figure 6.3. Enzymatic degradation of piroxicam sponges

6.4. *IN VITRO* DRUG RELEASE KINETICS

From Table 6.2 it can be seen that the power law model describes well the drug release (correlation coefficient > 0.98, being higher than the ones specific to Higuchi and zero-order models) **[158].**

The piroxicam release from the designed formulations showed an anomalous drug transport kinetic mechanism, the values obtained for the release exponent being smaller than 0.5. **[158].**

Figure 6.4 reveals typical biphasic drug release profiles, with an important piroxicam burst release effect in the first 30 minutes for the collagen matrices with 1%, 1.5%, and 2% piroxicam, ensuring an inflammation rapid diminution, followed by a prolonged release over the next hours of experiments. The obtained values for the release exponent (<0.5) revealed a complex release mechanism including swelling, diffusion, and erosion **[52, 115, 158].**

Table 6.2. Kinetic parameters and correlation coefficients specific to power law model, correlation coefficients specific to Higuchi and zeroorder models, and drug release percentage.

Collagen sponge (% piroxicam)	The rate constant ($1/min^n$)	The release exponent	The correlation coefficient			Drug release percentage (%)
			The power law model	The Higuchi model	Zero Order Model	
1	0,11	0,39	0,9811	0,9716	0,8761	87,44
1,5	0,12	0,38	0,9860	0,9755	0,8822	96,24
2	0,10	0,41	0,9892	0,9831	0,9009	91,33

Corroborating drug release results with those of collagenase degradation, it can be affirmed that before the sponge disintegration the drug is almost completely released. Moreover, depending on where these materials will be used, the fact that they absorb water suggests that they can absorb oral liquids or sanguinolent liquids and thus reducing the inflammation and the hematoma risk **[158].**

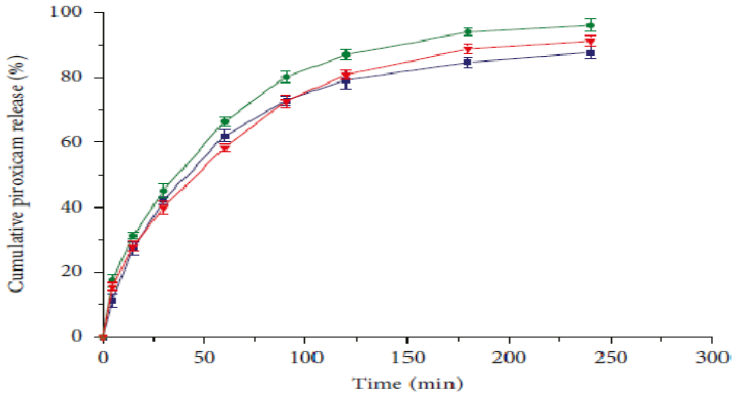


Figure 6.4. Cumulative release profiles of piroxicam from collagen matrices as a function of time: ■ – 1% piroxicam,●- 1.5% piroxicam,▼ – 2% piroxicam

6.5. PARTIAL CONCLUSIONS

This study presents new collagen-based materials for biomedical applications, especially in dentistry. These new materials, spongy collagen-based materials, obtained by the lyophilization method, were characterized by various techniques such as optical microscopy, IR spectroscopy, water absorption, enzymatic degradation and estimation of "*in vitro*" release kinetics **[158].**

The results obtained concerning the hydrolysis and enzymatic degradation showed the inhibitor role of the cross-linking agent, while the piroxicam presence determines an increasing sponge degradation under collagenase leading, at the end, to the sponge disintegration.

The kinetic profiles determined for piroxicam release suggest biphasic drug release profiles whereas the obtained values for the release exponent (<0.5) revealed a complex process.

CHAPTER 7. MATERIALS BASED ON COLLAGEN WITH CIPROFLOXACIN FOR POTENTIAL ORAL SURGICAL APPLICATIONS

In this chapter, the characterization of a new collagen-based material is presented. This material was obtained in a spongy form and was functionalized with an antibiotic, ciprofloxacin. The targeted applications of these kind of materials concern the post-operative prophylaxis. The *in vitro* tests (antimicrobial, cytotoxic, drug release) showed that

sponges with a concentration of 0.75 g of ciprofloxacin per gram of collagen could be beneficial for the desired applications **[163].**

7.1. WATER ABSORPTION EXPERIMENTS

Figure 7.1. presents the water absorption capacity of the synthetized materials. It can be seen that in time, the water quantity absorbed is increasing for all samples, but the water up-take is depending on the drug concentration. For samples with smaller drug concentration (0.50% and 0.75%) the water absorption is similar, even smaller, to that of collagen sponges (without drug). For the sponges with the highest drug concentration studied this water up-take is higher at all the times, this behavior being due to probably the drug which absorb more water than collagen **[163].**

The increased fluid retention in the porous structures indicates that a large amount of biological fluid can be absorbed when such supports are in contact with a surgical wound. This sponge can adhere easily and favors the formation of new regenerated tissues **[163].**

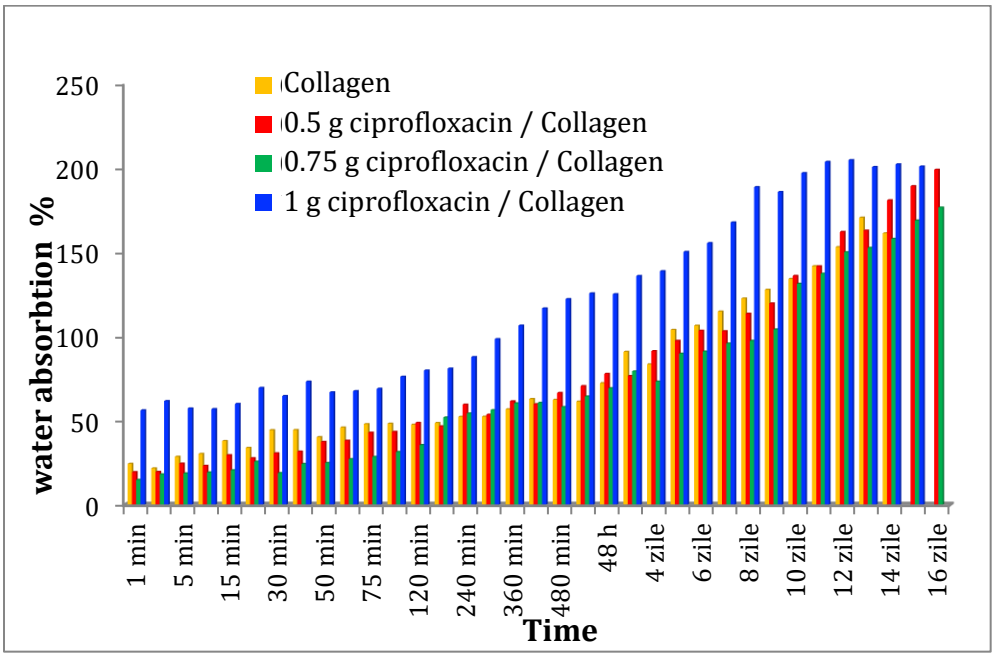


Figure 7.1 Water absorption behavior for the studied samples

7.2. ENZYMATIC DEGRADATION EXPERIMENTS

Figure 7.2 shows the study of the behavior of sponges in terms of enzymatic degradation. As expected, the degradation increases in time and the presence of ciprofloxacin favors the degradation. It can be seen that sponges containing ciprofloxacin are 50% degraded after 24 hours, compared to collagen-only sponges (approximately 15% degraded). After 24 hours, the sponges containing ciprofloxacin disintegrate **[163].**

The *in vitro* biodegradation results showed that a balanced degradation rate is obtained, targeted to get an adequate drug release with direct consequences on treatment effciency and improved patients compliance **[163].**

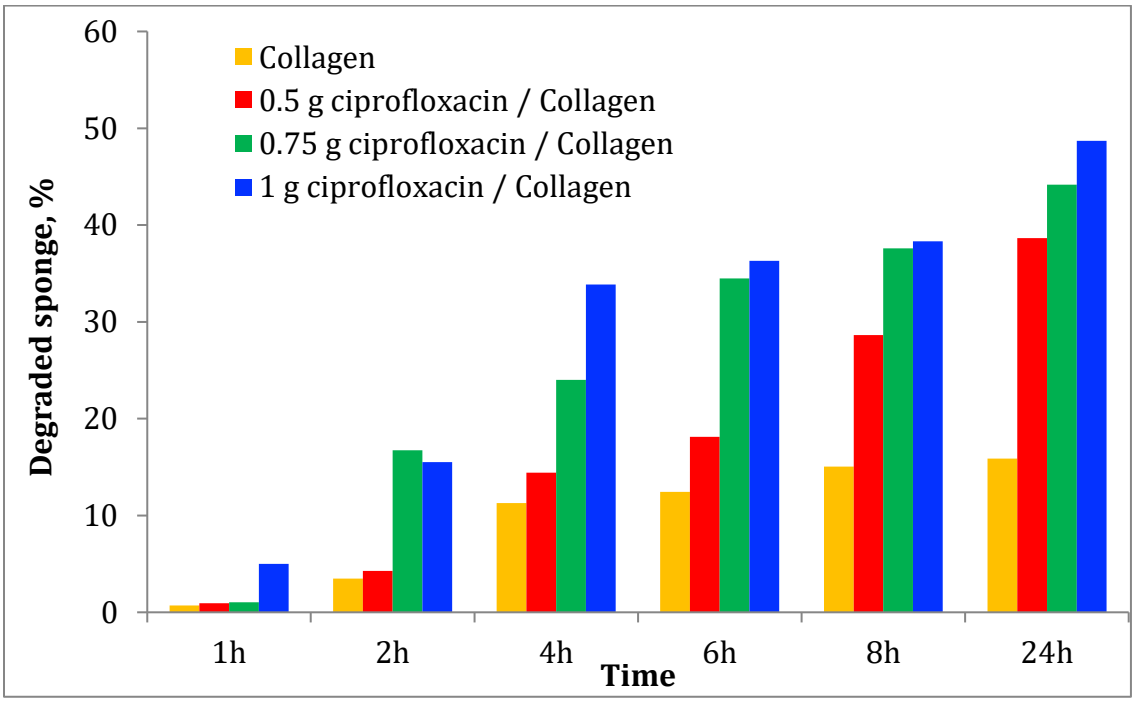


Figure 7.2. Enzymatic degradation behavior for the studied materials.

7.3. *IN VITRO* RELEASE OF CIPROFLOXACIN

A critical parameter to consider for the formulation and evaluation of ciprofloxacin-based collagen sponge material designed to prevent and control infection associated with head and neck surgery is the drug release kinetics, monitored by the influence of drug content on kinetic patterns **[163].**

The fraction of ciprofloxacin released at each time point was computed as percentage of the total drug from collagen sponge **[163].**

Figure 7.3 illustrates similar kinetic profiles for all three formulations characterized by an initial burst release in the first 2 h followed by a progressive and prolonged drug release for a longer period up to 24 h **[163].**

The sponge with 0.50 g ciprofloxacin/g collagen exhibited a rapid release (43.15%), followed by the sponge with maximum level of ciprofloxacin (34.26%), while the formulation with medium drug concentration leads to the smallest burst effect (27.45%). It can be remarked that this release is 1.6 times faster for ciprofloxacin concentration of 0.5% in comparison with 0.75%. The quite pronounced burst release effect could be due to the large sponges water absorption in the first 120 min **[163].**

The cumulative antibiotic released percentage after 24 h is of 81.63% for the sample containing 0.50% ciprofloxacin, while the collagen supports having a higher drug content showed 61.70%, respectively 69.86% of ciprofloxacin release within the same period of time (Table 7.1). **[163].**

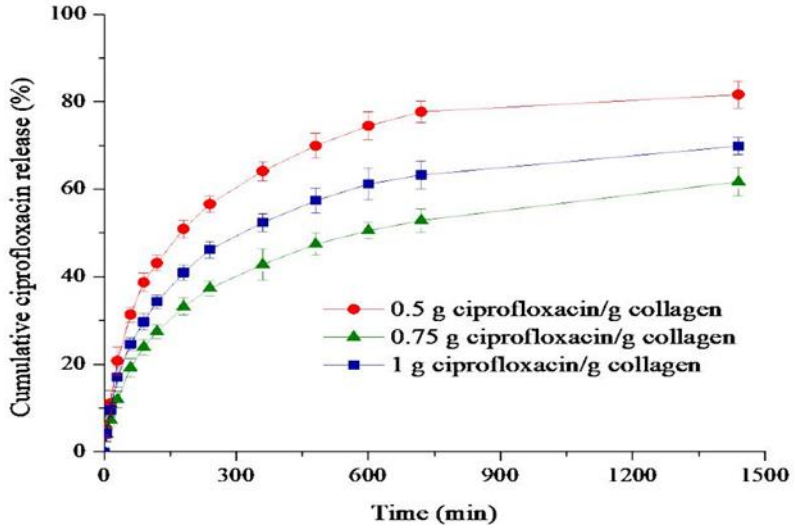


Figure 7.3 Time-dependent cumulative release profiles of ciprofloxacin from collagen matrices

Table 7.1. Correlation coefficients (R) for Higuchi, Zero-order, and Power law kinetic models; kinetic parameters specific to the Power law model and cumulative ciprofloxacine released percentage.

Ciprofloxacin sponges (CPX) – collagen (C) g CPX/g C	Correlation coefficient, R			The rate constant, k ($1/min^n$)	The release exponent, n	Drug released (%)
	Model Higuchi	Zero Order Model	The Power Law Model			
0,5	0,9448	0,7961	0,9762	0,084	0,33	81,63
0,75	0,9716	0,8502	0,9875	0,044	0,37	61,70
1	0,9572	0,8194	0,9820	0,063	0,35	69,86

Ciprofloxacin release patterns were further evaluated to establish the kinetic mechanism by checking the *in vitro* experimental data with the Peppas law model and its particular cases, Higuchi (n = 0.5) and zero order (n = 1), the values of the corresponding correlation coefficients being presented in table 7.1 **[163].**

The values of correlation coefficients R between 0.9762 and 0.9875 indicate a better verification of the power law model and demonstrate that such a model could be used to

describe the mechanism of ciprofloxacin release from collagen sponges. Furthermore, release exponent values ranging from 0.33 to 0.37 reveal a non-Fickian drug transport mechanism involving multiple steps **[163]:**

- an initial resorption of the drug retained on the surface of the sponge,
- absorption of the release medium in the porous structure, hydration of the polymers and swelling of the sponge, these two stages corresponding to the sudden release effect, followed by
- the diffusion of the drug retained in the polymer network during the lyophilization process simultaneously with the progressive degradation of the release support, this stage being correlated with the prolonged and sustained release of drugs.

7.4. *IN VITRO* BIOLOGICAL EFFECTS OF COLLAGEN SPONGES AND CIPROFLOXACIN ON SOME PATHOGENIC MICROORGANISMS

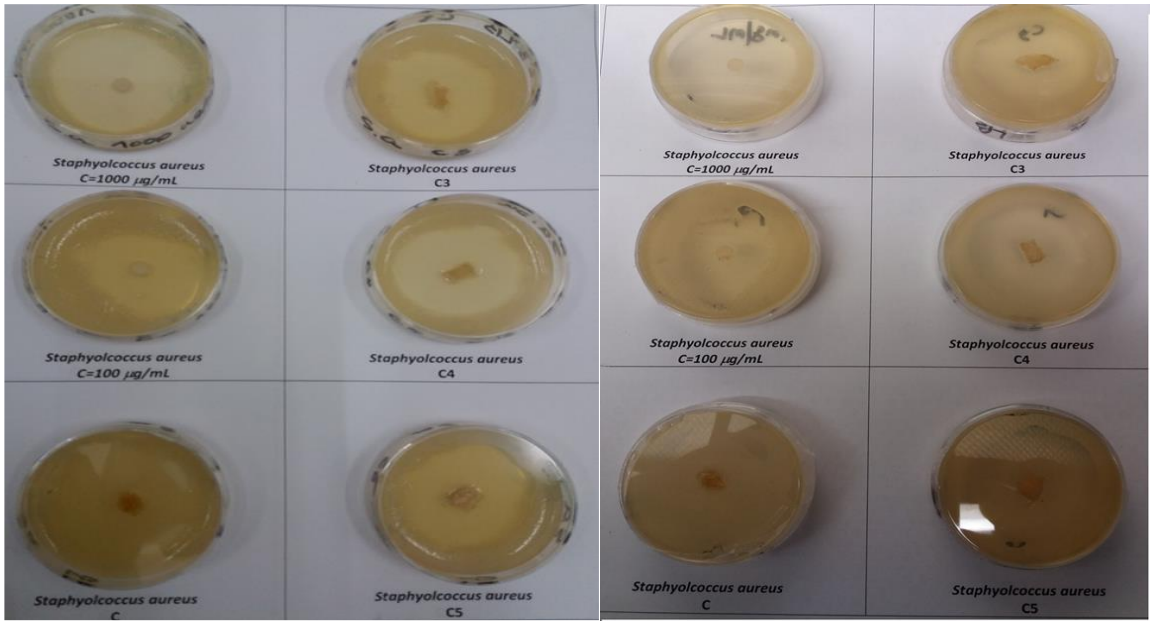
Table 7.2 shows the results obtained for collagen-based materials tested against different pathogens. From this table it can be seen that for *Escherichia coli* the best results were obtained for the sponge containing 1g ciprofloxacin/g collagen, the result being similar to that obtained for solutions of 1000 g/mL ciprofloxacin. Similar results were obtained for the other collagen materials – ciprofloxacin showing that *E. coli* is sensitive to the tested products **[163].**

Important results (Figure 7.4) were also obtained when the synthesized materials were tested against *Staphylococcus aureus*. It is known that ciprofloxacin is not an antibiotic with activity against gram-positive microorganisms. However, the studied microorganism is sensitive to the active substance, the best result being obtained for the sample of 1g ciprofloxacin / g ciprofloxacin (inhibition diameter of 44.5 ± 2.22 mm), the inhibition diameter being greater than that observed for ciprofloxacin 100 g/mL. The results obtained during this study confirm that the mechanism of the active substance (ciprofloxacin) is the inhibition of DNA synthesis. The positive results obtained in the case of gram-positive microorganisms (*Staphylococcus aureus*) may be due to the fact that the research was done with microbial strains from standardized collections, without mutations that can give resistance to the tested antibiotic. These results are similar to those obtained by F. Puoci **[134],** underlining the fact that collagen-based materials with ciprofloxacin could be used for the desired applications **[163].**

In the case of the (fig. 7.5), the best results are obtained in the case of sample C5, the results being similar to those obtained when a solution of ciprofloxacin with a concentration of 1000 μg/mL is tested. In the case of samples C4 and C3, similar results were obtained. Consequently, based on the results obtained, it is estimated that the studied microorganism is sensitive to the tested products **[163].**

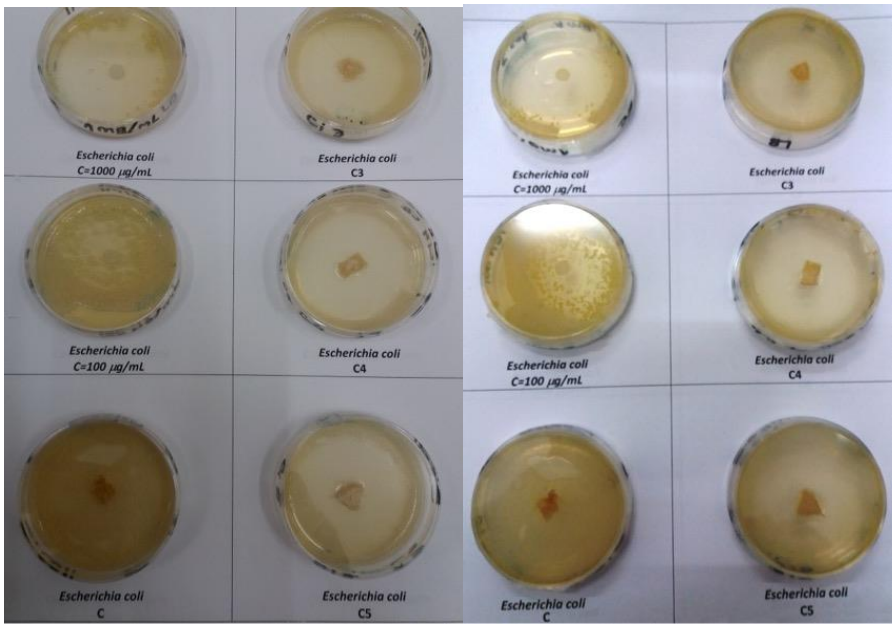
Table 7.2. Inhibition diameter of the tested sponges against different pathogens.

Sample	Inhibition diameter, mm			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Candida parapsilopsis*
100 μg ciprofloxacin/mL	35.0 ± 1.75	0	0	0
1000 μg ciprofloxacin/mL	42.5 ± 2.12	44.0 ± 2.20	0	0
Collagen sponge (C)	10.5 ± 0.52	0	0	0
Collagen sponge and ciprofloxacin (0,5 g ciprofloxacin / g collagen) (C3)	37.0 ± 1.85	42.0 ± 2.10	0	0
Collagen sponge and ciprofloxacin (0,75 g ciprofloxacin / g collagen) (C4)	40.0 ± 2.00	42.0 ± 2.10	0	0
Collagen sponge and ciprofloxacin (1 g ciprofloxacin / g collagen) (C5)	38.5 ± 1.92	44.5 ± 2.22	0	0



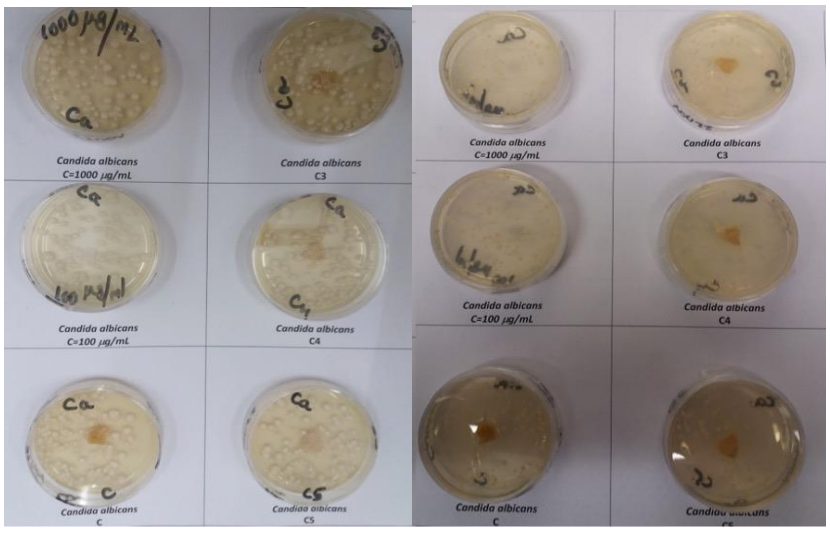
(a) (b)

Figure 7.4 Effect of collagen-based materials and ciprofloxacin on *Staphylococcus Aureus*. (a) – front view and (b) – back view – for code example, see table 7.2.



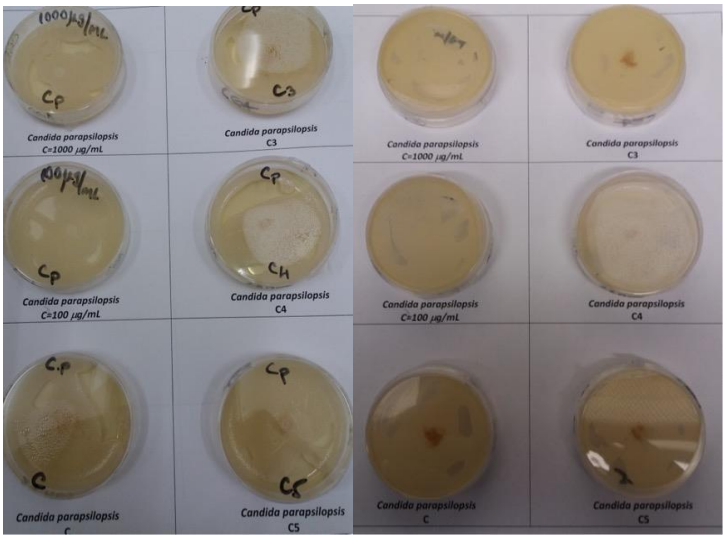
(a) (b)

Figure 7.5 Effect of collagen-based materials and ciprofloxacin on *Echerichia coli*. (a) – front view and (b) – back view – for code example, see table 7.2.



(a) (b)

Figure 7.6 Effect of collagen-based materials and ciprofloxacin on *Candida parapsilosis*. (a) – front view and (b) – back view – for code example, see table 7.2.



(a) (b)

Figure 7.7 Effect of ciprofloxacin – collagen-based materials on *Candida albicans*. (a) – front view and (b) – back view – for code example, see table 7.2.

7.5. CYTOTOXIC STUDY OF COLLAGEN BASED MATERIALS ON NORMAL CELLS – *IN VITRO* TESTS

The results revealed that in case of the cell line tested after 6 h and 24 h of exposure a PI higher than 1 is obtained suggesting that the cells are stimulated by the sponges. After 48 h of exposure the PI is decreasing and becomes closer to 1 when the cells were treated with ciprofloxacin-based materials. Moreover, in the case of the sample containing 1g ciprofloxacin/g collagen at 48 h the PI decrease was significant (PI = 0.77) showing that this sponge become cytotoxic if it is used more than 24 h for these cells **[163].**

Table 7.6 HUVEC proliferation at different time exposure to ciprofloxacin-based materials

Sample	Proliferation index (PI)		
	6 h	24 h	48h
100 μg ciprofloxacin /mL	1.17 ±0.0234	1.21 ± 0.0182	1.08 ± 0.0216
Collagen sponge	1.34 ± 0.0268	1.29 ± 0.0194	1.23 ± 0.0246
Collagen sponge with ciprofloxacin (0,5 g ciprofloxacin /g collagen)	1.46 ± 0.0292	1.48 ± 0.0222	0.94 ± 0.0188
Collagen sponge with ciprofloxacin (0,75 g ciprofloxacin /g collagen)	1.56 ± 0.0312	1.36 ± 0.0204	1.04 ± 0.2080
Collagen sponge with ciprofloxacin (1 g ciprofloxacin /g collagen)	1.24 ± 0.0248	1.16 ± 0.0174	0.77 ± 0.0154

7.6. PARTIAL CONCLUSIONS

The present study showed that materials based on collagen and ciprofloxacin can be synthesized in the form of a sponge. The obtained results suggest that such sponges absorb a significant amount of water, these materials can be used in head and neck surgery, because they can degrade quite quickly, approximately 40-50%, in the first 24 hours. Moreover, for the same time interval, the cumulative percentage of ciprofloxacin release was between 61.70-81.63%, the kinetic profiles showing a biphasic allure aimed at preventing and controlling local infection associated with head and neck surgery, avoiding invasion or subsequent bacterial proliferation **[163].**

Solutions of the active substance (Ciprofloxacin) in concentrations of 1000 g/mL inhibit the development of the studied bacteria (at this concentration, the studied bacteria show the phenomenon of sensitivity).

At an active substance concentration of 100 □g/mL, the studied bacteria showed a phenomenon of resistance, their development not being influenced by the presence of the active substance – Ciprofloxacin.

In the case of Candida sp. none of the tested materials inhibited the growth of the tested microorganisms. For this reason it is appreciated that Candida sp. shows resistance to all materials tested.

At the same time, these materials highlighted the bacteriostatic effect, by inhibiting the development of gram-negative bacteria such as Escherichia coli and gram-positive bacteria such as Staphylococcus aureus (Staphylococcus sp. from international commercial collection, which does not show the phenomenon of chemoresistance).

Other important finding is that these materials present even a stimulating e effect on normal HUVEC cell line and thus they do not present cytotoxic e ect for exposure times less than 24 h. Due to the fact that the sponges with 1g of ciprofloxacin/g collagen show a cytotoxic e effect on the HUVEC cell line after 24 h exposure it can be concluded that *in vitro* an optimum e ect (antimicrobial and not cytotoxic) is obtained for the sponges with 0.50 g ciprofloxacin/g collagen and 0.75 g ciprofloxacin/g collagen. Corroborating this aspect with the drug release experiment and the physical-chemical results a collagen sponge containing 0.75 g ciprofloxacin for 1 g of collagen could be recommended for in *vivo* tests in order to confirm the findings presented in this paper.

CHAPTER 8. MATERIALS BASED ON COLLAGEN, CIPROFLOXACIN AND PIROXICAM WITH POTENTIAL ORAL SURGICAL APPLICATIONS - PRELIMINARY TESTS

Considering the results obtained for spongy materials based on collagen functionalized with ciprofloxacin and piroxicam, new collagen-based materials were designed to contain both ciprofloxacin and piroxicam, taking into account the fact that the two drugs are complementary and a synergistic effect is disired.

Thus, following the procedure presented in chapter 4, sponges containing both ciprofloxacin and piroxicam were prepared. The collagen-based sponges contained 0.05 g

ciprofloxacin/g collagen and 0.15% piroxicam (sample PC 1), respectively 0.5 g ciprofloxacin/g collagen and 1.5% piroxicam (sample PC 2).

In the beginning this preliminary study was only focused on water absorption and sponge degradation experiments in the presence of collagenase.

8.1. WATER ABSORPTION EXPERIMENTS

Figure 8.1 compares the results obtained in the water absorption test experiments. It is observed that the addition of the drug leads to a significant increase in water absorption compared to the collagen matrix.

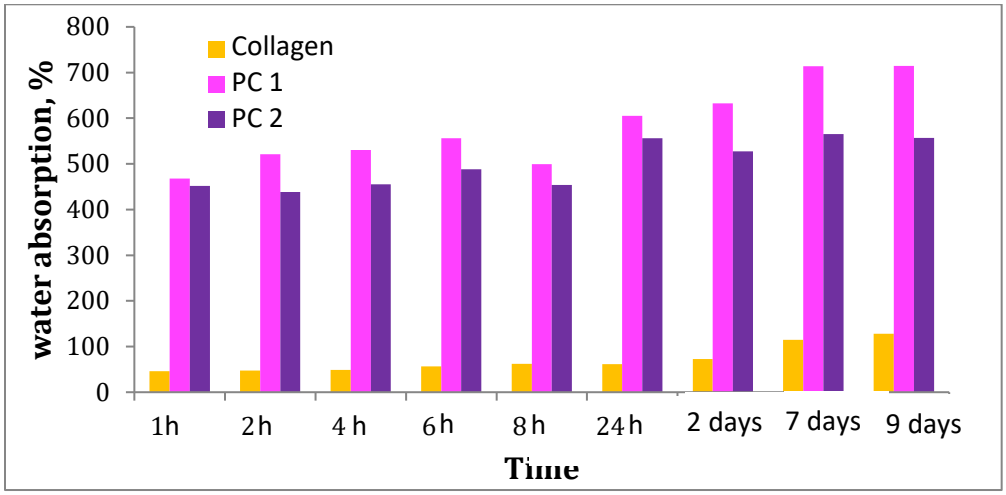


Figure 8.1 Water absorption for samples containing both drugs compared to a collagen sponge

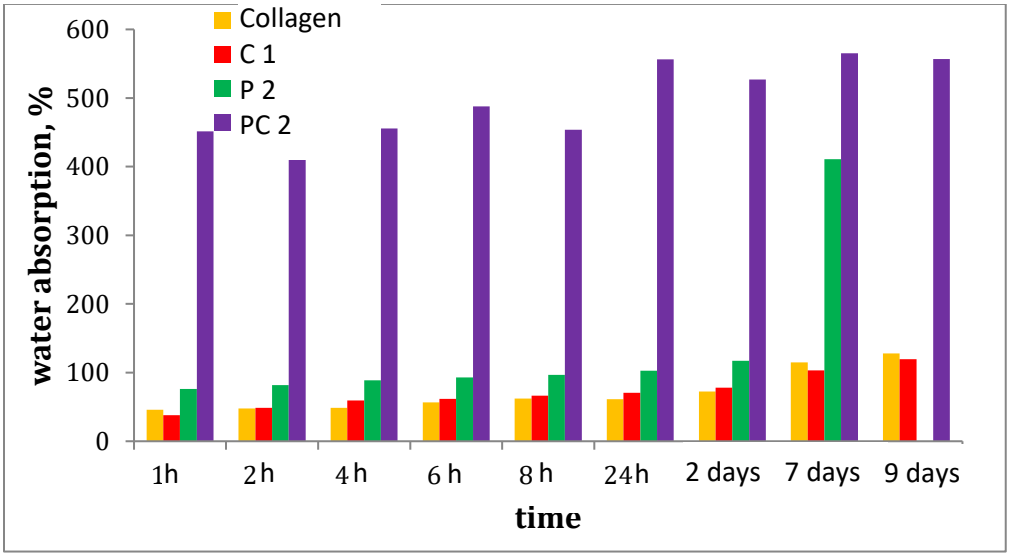


Figure 8.2 Comparison of water absorption for samples containing the same concentration of ciprofloxacin or piroxicam C1 – collagen sponge containing 0.5 g ciprofloxacin/g collagen; P2 – collagen sponge containing 1.5% piroxicam; PC2 – collagen sponge containing 0.5 g ciprofloxacin/g collagen and 1.5% piroxicam

By comparing the percentage of water absorbed by the sponge containing both drugs with the percentage of water absorbed by the sponges functionalized with only one drug, a synergistic effect can be observed. If when the sponge contains only one drug the percentage of absorbed water is similar (with a slight increase in the case of the sponge with piroxicam) while the sponge containing both ciprofloxacin and piroxicam absorbs about 5 times more water.

8.2. ENZYMATIC DEGRADATION EXPERIMENTS

Enzymatic degradation experiments showed that by adding drugs the degradation of the sponge occurs faster, a higher concentration of the drug leading to faster degradation (figure 8.3). Immersion of samples containing ciprofloxacin and piroxicam in collagenase-containing solution led to results suggesting that in the first 8 hours there is a competition between water absorption and sponge degradation, the absorbed water compensating for the degraded collagen mass. In addition in the first hours, the sponge containing both drugs absorbs more water than the degraded sponge mass. After 24 hours, the sample containing the highest amounts of drugs shows a more pronounced degradation. It should be noted that both ciprofloxacin and piroxicam sponge samples disintegrated only after 72 hours.

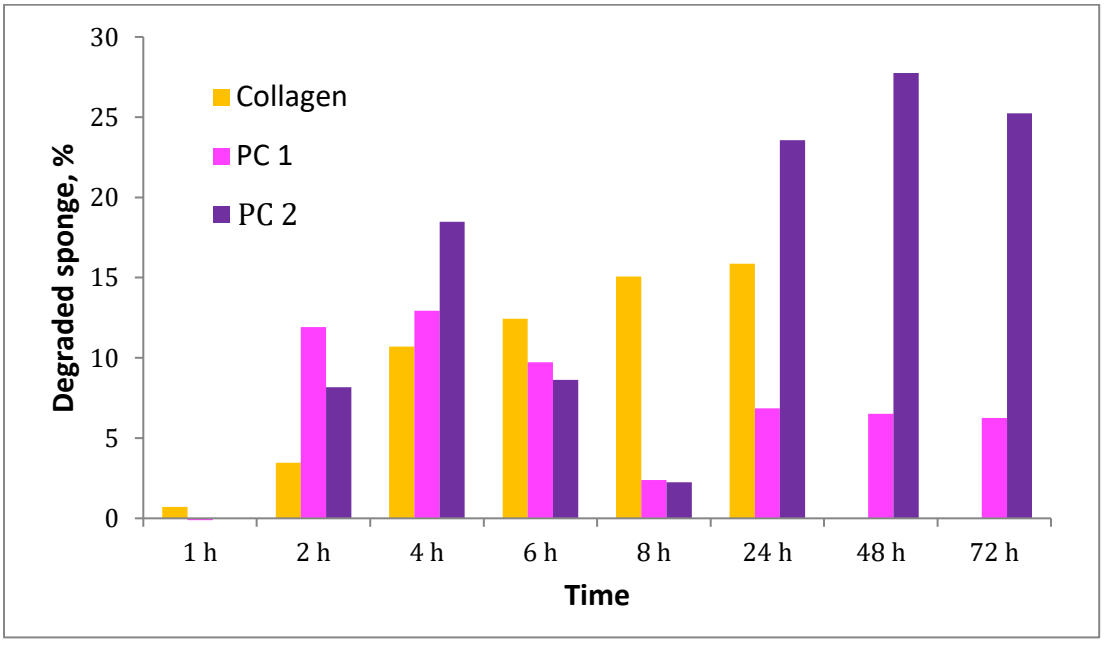


Figure 8.3 Enzymatic degradation for samples containing both drugs compared to a collagen sponge

If the comparison of the behavior with the action of collagenase (figure 8.4) is regarded, it can be stated that even in this case the joining of the two drugs leads to a synergistic effect. Generally, the presence of ciprofloxacin or piroxicam has the effect of

accelerating degradation. In addition, the presence of piroxicam causes at the same time the disintegration of the spongy matrix after 8 hours of immersion. However, when the two drugs are joined in a sponge, the degradation of the matrix in the presence of collagenase is slowed down, and the matrix disintegrates after 6 days.

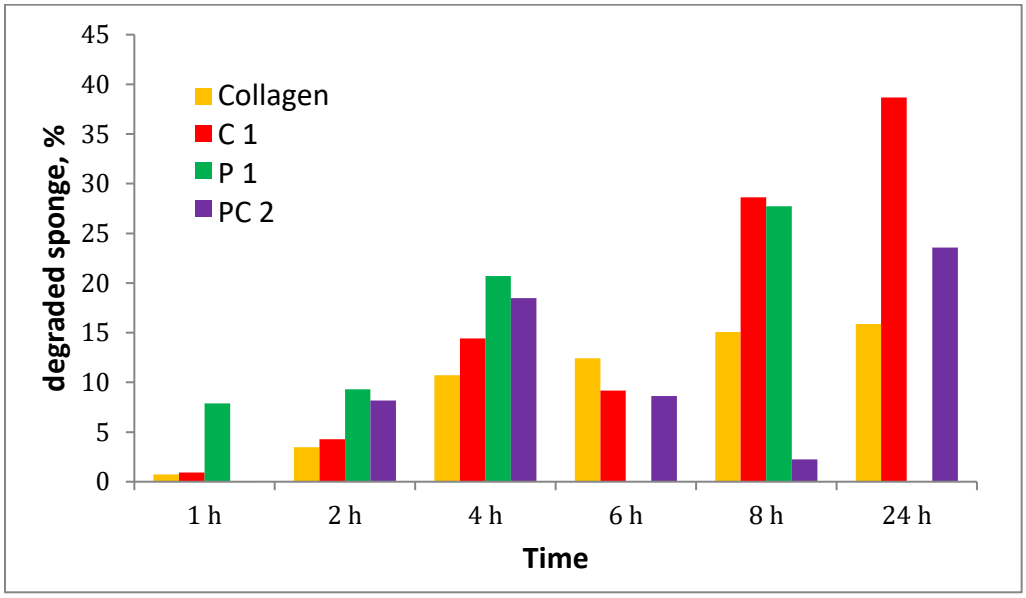


Figure 8.4 Comparison of enzymatic degradation for samples containing the same concentration of ciprofloxacin or piroxicam C1 – collagen sponge containing 0.5 g ciprofloxacin/g collagen; P2 – collagen sponge containing 1.5% piroxicam; PC2 – collagen sponge containing 0.5 g ciprofloxacin/g collagen and 1.5% piroxicam

8.3. PARTIAL CONCLUSIONS

The study carried out on collagen-based materials functionalized with ciprofloxacin and piroxicam showed that the addition of the two drugs has the effect of improving the water absorption properties, respectively the resistance to collagenase action.

The large amount of water absorbed, 5 times more than in the case of the material containing only ciprofloxacin or only piroxicam, leads to swelling of the sponge, a fact that could be beneficial for applications in the field of oro-maxillo-facial surgery.

This study was to be completed with antimicrobial activity, cytotoxicity and drug release tests. Considering the results obtained on materials containing only one of the drugs, it is expected that the new materials will also present antimicrobial activity and cytotoxicity similar to those obtained in the case of materials containing only ciprofloxacin.

CONCLUSIONS

C.1. GENERAL CONCLUSIONS

Worldwide, the pain is an important and problematic symptom after any kind of surgery, regardless the type of surgery, the size of the wound, or the patient's associated pathology. Current research in medicine are based on the development of any potential cures to address the patient's needs, the main goal being to reduce and to relief the pain, the inflammation and the sensation and enhance the recovery process. All these aspects will contribute to improving the patient's quality of life, this being one of the main reasons for using a variety of drugs in the treatment of chronic and acute pain.

Infections or superinfections may occur before, during or after surgery. Before surgery, in most cases, there are traumatic factors or superinfected formations not treated in time that cause infections, while during the surgical intervention, pathogenic factors can appear by contaminating the wound. Postsurgery superinfection may occur due to poor local hygiene and in some rare cases there may be a negative or allergic reaction of the body or there may be seromas.

In this doctoral thesis, collagen-based materials and their functionalization with ciprofloxacin and piroxicam were studied. The purpose of this study was to combine the properties and implicitly the benefits of the collagen with two medicinal substances, namely an antibiotic and an anti-inflammatory, ciprofloxacin and piroxicam, drugs that can be released locally, at the surgical site for more effective local tissue regeneration and a optimal local healing.

Several tests were performed in order to determine the viability of these antibiotic and anti-inflammatory collagen sponges, the beneficial interaction between these elements, the collagen-based spongy material, the antibiotic – ciprofloxacin, the anti-inflammatory – piroxicam and the optimal time of resorption or degradation of these compounds in the human body.

The aim of this research was therefore the design, the creation, the realization and the characterization of these spongy materials based on collagen, anti-inflammatory and antibiotic, these materials being created to have a beneficial role in the healing process and with applications in different surgical specialties, particulary in Oro-Maxillo-Facial Surgery.

The results obtained during the doctoral stage led to the following conclusions:

- It is possible to obtain by lyophilization spongy materials with different concentrations of anti-inflammatory, antibiotic or a combination of these two drugs;
- Depending on the anti-inflammatory concentration, the obtained materials can find applications in human or veterinary medicine.

For materials with a high piroxicam content, the main conclusions are:

- The optical microscopy images showed a fibrillar structure with interconnected pores in all the sponges obtained but also piroxicam aggregations given the high drug content;

- The collagen sponge without piroxicam absorbed a significant amount of water, and when the drug was added to the composition of the collagen sponge, the amount of water absorbed decreased. It can be concluded that water absorption for the studied sponges decreases with increasing the drug dose;
- A high drug content leads to disintegration of the sponge. It cannot be stated that the samples are totally degraded in the sense of mass loss, they disintegrate and practically the collagen fibers with piroxicam are present in the solution (PBS with collagenase);
- Kinetic studies indicated a biphasic drug release, favorable for providing an adequate concentration of piroxicam at the site of application to manage the inflammation and the consequent pain associated with various oral diseases;
- These preliminary results suggest that such high-dose piroxicam sponges could be used for various treatments in large animals such as horses and cattle.

The study focused on the synthesis and characterization of new collagen-based materials with lower concentrations of piroxicam demonstrated that these new materials may have biomedical applications, particularly in dentistry. These new materials, collagen-based spongy materials obtained by freeze-drying method, were characterized by various techniques such as optical microscopy, IR spectroscopy, water absorption, enzymatic degradation and *in vitro* kinetic release. The main conclusions of this study are:

- The results obtained regarding enzymatic hydrolysis and degradation showed the inhibitory role of the cross-linking agent, while the presence of piroxicam causes an increase in the degradation of the sponge in the presence of collagenase, which ultimately leads to the disintegration of the sponge.
- The kinetic profiles determined for the release of piroxicam suggest a biphasic drug release profile, while the values obtained for the release exponent (< 0.5) revealed a complex release mechanism including swelling, diffusion and erosion.
- The results obtained suggest that in the near future piroxicam-collagen-based biomaterials could easily represent a viable and modern solution for the treatment of various types of wounds that occur in oro-maxillo-facial surgery, combining the best properties of collagen and non-steroidal anti-inflammatory.

The study carried out on spongy materials functionalized with ciprofloxacin showed that it is possible to synthesize, in the form of a sponge, materials based on collagen and ciprofloxacin. The obtained results suggest that:

- Such sponges absorb a significant amount of water, these materials can be used in head and neck surgery, because they can degrade quite quickly, approximately 40-50%, in the first 24 hours;
- For the same time interval, the cumulative percentage release of ciprofloxacin was between 61.70-81.63%, the kinetic profiles showing a biphasic allure aimed at

preventing and controlling local infection associated with head and neck surgery, avoiding bacterial invasion or subsequent proliferation;

- Active substance solutions (Ciprofloxacin) in concentrations of 1000 μg/mL inhibit the development of the studied bacteria, (at this concentration the studied bacteria manifest the phenomenon of sensitivity);
- At an active substance concentration of 100 μg/mL, the studied bacteria show a phenomenon of resistance, their development not being influenced by the presence of the active substance – Ciprofloxacin;
- in the case of *Candida sp*. none of the tested materials inhibited the growth of the tested microorganisms. For this reason, it is estimated that *Candida sp*. shows resistance to all tested materials;
- these materials have a bacteriostatic effect, by inhibiting the development of gram-negative bacteria such as *Escherichia coli* and gram-positive bacteria such as *Staphylococcus aureus* (*Staphylococcus sp.* of international commercial collection, which does not show the phenomenon of chemoresistance);
- these materials also show a stimulatory effect on the normal HUVEC cell line and therefore do not show a cytotoxic effect for exposure times of less than 24 hours. Due to the fact that the sponges with 1g ciprofloxacin/g collagen show a cytotoxic effect on the HUVEC cell line after 24 h of exposure, it can be concluded that *in vitro* an optimal effect (antimicrobial and not cytotoxic) is obtained for the sponges with 0.50 g ciprofloxacin/g collagen and 0.75 g ciprofloxacin/g collagen;
- Corroborating the drug release experiment and the physical - chemical results, a collagen sponge containing 0.75 g of ciprofloxacin per 1 gram of collagen could be recommended for in vivo tests to confirm the results presented in this thesis.

The study carried out on collagen-based materials functionalized with ciprofloxacin and piroxicam allowed the following conclusions to be obtained:

- The joining of the two drugs has the effect of improving the water absorption properties, respectively the resistance to collagenase action.
- The large amount of water absorbed, 5 times more than in the case of the material containing only ciprofloxacin or only piroxicam, leads to swelling of the sponge, a fact that could be beneficial for applications in the field of oro-maxillo-facial surgery.
- This study has to be completed with antimicrobial activity, cytotoxicity and drug release tests. Considering the results obtained on the materials containing only one of the drugs, it is expected that these materials also present antimicrobial activity and cytotoxicity similar to those obtained in the case of materials containing only ciprofloxacin.

It can be concluded that the spongy materials synthetized and characterized in this thesis can find applications in various surgical interventions, with the preservation of the basic properties and the advantage of generating new characteristics by combining collagen

with a non-steroidal anti-inflammatory and an antibiotic. These materials can have a triple active role: local hemostasis by absorption of fluids, acceleration of the healing process and drugs reservoir.

C.2. ORIGINAL CONTRIBUTIONS

This doctoral work brings original contributions in obtaining collagen-based materials, materials functionalized with a non-steroidal anti-inflammatory drug – piroxicam and/or an antibiotic from the fluoroquinolone class – ciprofloxacin.

The elements of originality that stand out in this thesis are the following:

- obtaining of new spongy materials (collagen - piroxicam) that can be used in pain management for big animals;
- obtaining of new spongy materials (collagen - piroxicam) with effective properties in terms of water absorption, enzymatic degradation and drug release, materials that can find applications in various branches of surgery;
- obtaining of new spongy materials (collagen - ciprofloxacin), materials that have proven higher antimicrobial and cytotoxic properties compared to the antibiotic itself;
- obtaining of new spongy materials that contain both anti-inflammatory and antibiotic drugs.

C.3. PROSPECTS FOR FUTURE DEVELOPMENT

The results obtained during the doctoral stage open new directions of research considering the applications of these materials. Thus, for the development of these materials and for their capitalization, the present studies must be continued with *in vivo* studies.

The preliminary study carried out on the materials containing both piroxicam and ciprofloxacin will be completed with antimicrobial activity, cytotoxicity and drug release tests, as well as with *in vivo* studies.

PAPERS

1. **Daniel – Cristian Ioan**, I. Rău, G.T. Tihan, R.G. Zgarian, M.V. Ghica, M.G. Albu Kaya, E.C Dinu-Pirvu, Piroxicam-Collagen-Based Sponges for Medical Applications, International Journal of Polymer Science, 2019, Article Number: 6062381 I.F. (2019) = 1,646
2. **Daniel – Cristian Ioan**, I. Rău, M. G. Albu Kaya, N. Radu, M. Bostan, R. G. Zgârian, G. T. Tihan, C.-E. Dinu-Pîrvu, A. Lupuliasa, M.-V. Ghica, Ciprofloxacin-Collagen-Based Materials with Potential Oral Surgical Applications, Polymers 2020, 12 (9), pp. 1 – 13, art. No. 1915, I.F. (2020) = 4,329
3. **Daniel – Cristian Ioan**, I. Rău, M. Albu-Kaya, R. Zgârian, G. Tihan, C. Dinu-Pîrvu, L. Popa, M. Ghica, Collagen based sponges for veterinary applications, U.P.B. Sci. Bull., Series B 2024, 86, (1), pp 135 – 144, I.F. = 0,5

Cumulative I.F.= 6,475

SCIENTIFIC CONFERENCES ATTENDANCE

Daniel – Cristian Ioan, G. T. Tihan, R. G. Zgârian, M. V. Ghica, M. G. Albu-Kaya, C. Dinu-Pîrvu, I. Rău, RICCCE Conference 20th Romainian International Conference on Chemistry and Chemical Engineering, „New collagen-piroxicam based materials for oral applications" Poster, 2017.

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