UNIVERSITY POLITEHNICA OF BUCHAREST
Doctoral School of Materials Science and Engineering
DEPARTMENT OF METAL MATERIALS SCIENCE, PHYSICAL METALLURGY

SUMMARY OF THE DOCTORAL THESIS
Contributions regarding the development of new Materials in the category of antimicrobial acrylic cements used for fixing hip endoprostheses

Author: Eng. IVANOV ALINA (ROBU)

Scientific coordinator: Univ. Prof. Habil. Dr. Eng. Antoniac Vasile Iulian

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Bucharest 2022

Contents of the doctoral thesis summary

Thanks

This thesis would have been impossible without the help, support and guidance of the wonderful people who, through their way of being, contributed to my formation as a human being, instilling in me the desire to complete it. I thank them once again for all those life lessons offered over time and for the example they set for me.

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ABSTRACT

We performed a statistical analysis of the clinical results of the use of BHR-type prostheses in an orthopedic clinic, to identify the causes of their failure. We took extracted prostheses samples and analyzed them from the interface of bone cement - metallic biomaterial point of view. The results showed that the failures were based on several possible causes, including the type of cement used, poor preparation technique and improper use of bone cement. As a result, we performed a second experimental study, namely the in vitro comparative analysis of commercial cements used to fix the analyzed hip endoprostheses following the chronology of surgery which showed that the handling process influences the structure and properties of bone cements. Subsequently, a case study and experimental research was performed for the selection of an essential oil with optimal antimicrobial properties, usable in the development of new bone cements. The conclusion was that peppermint oil is the most effective and has the right properties to be used in the development of new antimicrobial bone cements. We obtained new bone cements with antimicrobial properties, by modifying the inorganic component of a commercial PMMA-based cement, with standard viscosity, using as antimicrobial agents, silver nanoparticles embedded in a ceramic glass and hydroxyapatite impregnated with peppermint oil and gentamicin, we performed the morphological and structural characterization, the evaluation of the properties and the evaluation of the antimicrobial and biocompatibility activities of the experimental samples of newly obtained bone cements.

The findings highlight that the properties analyzed and the performance of bone cements with peppermint essential oil and silver nanoparticles against tested pathogens suggest that these antimicrobial additives appear promising to be used in clinical practice against bacterial infections.

Keywords*: hip endoprostheses, PMMA-based bone cement; peppermint essential oil; silver nanoparticles; gentamicin; antimicrobial properties, BHR*

INTRODUCTION

Presentation of the topic: Contributions regarding the development of new materials in the category of antimicrobial acrylic cements used for fixing hip endoprostheses

In the doctoral thesis the focus was on evaluating the clinical biofunctionality of acrylic cements used to fix hip stents, and the development of new biomaterials in the category of acrylic cements with antimicrobial activity, using antimicrobial additives (nanoparticles silver, essential oils, antibiotics).

Total hip arthroplasty also generates significant failures that require revision interventions. These can be reduced by efficiently collecting data obtained through research and comparing implant types and using reproducible measurements.

Following the literature study, we performed a statistical analysis of the clinical results regarding the use of BHR (Birmingham Hip Resurfacing) stents in an orthopedic clinic to identify the causes of their failure. We took explants from patients with hip restoration arthroplasty and analyzed them from the point of view of the interface of bone cement with metallic biomaterial. The analyzes showed that the failures are not due to the execution of the prosthesis or its principle of operation, but to several aspects such as: the type of cement used, poor preparation technique and improper use of bone cements. As a result, we performed a second experimental study, namely the in vitro comparative analysis of commercial cements used to fix the hip stents analyzed following the chronology of surgery, to evaluate the factors that influenced the chemical composition and structure of each bone cement sample under specific intraoperative conditions. . From the obtained results we can say that the cement handling process (mixing stage, waiting stage, working stage) has an important role on the structure and mechanical properties of bone cements and it is recommended to avoid manual mixing for low viscosity bone cements.

Given that the widespread use of antibiotics has led to the development of mechanisms that allow bacteria to survive in antibiotic environments, we conducted a case study and conducted experimental research for the selection of an essential oil with optimal antimicrobial properties, usable in development of new bone cements. After the physico-chemical characterization and the antimicrobial effect and later the comparison of the properties of three essential oils resulted in the fact that peppermint oil is the most effective and has the right properties to be used in the development of new antimicrobial bone cements.

Subsequently, we obtained new bone cements with antimicrobial properties, by modifying the inorganic component of a commercial cement based on PMMA, with standard viscosity, using as antimicrobial agents, silver nanoparticles embedded in a ceramic glass (2% and 4%), respectively hydroxyapatite impregnated with peppermint oil and gentamicin. Thus, three compositions of new bone cements were obtained, on which, together with a standard commercial cement and a cement in which 0.5% gentamicin was added, we performed the following studies, namely morphological and structural characterization, property evaluation and evaluation antimicrobial and biocompatibility activities of experimental samples of newly obtained bone cements. Three standard microbial strains were used to evaluate the antimicrobial effect: a Gram-negative strain (*Pseudomonas aeruginosa* ATCC 27853), a Gram-positive strain (*Staphylococcus aureus* ATCC 25923), a yeast strain (*Candida albicans* ATCC 10231), and biocompatibility. by evaluating cell proliferation by the tetrazolium salt (MTT) test.

SYNTHETIC PRESENTATION OF THE CHAPTERS OF THE DOCTORAL THESIS

Chapter 1. Current state of research on acrylic cements used in orthopedic surgery for fixing hip endoprostheses

The hip joint has an essential role in the body, the locomotion not being possible without it. Bone cements are used to secure artificial joints, such as hip prostheses or knee prostheses. This material fills the space between the implant and the joint and is introduced as a fluid mixture, which hardens over time.

PMMA cement was used as a cementing material to fix the femoral component and the acetabular component and to distribute the contact stresses between the implant and the bone over a large area.

Currently, the most commonly used bone cements can be classified into two categories: acrylic bone cements and calcium phosphate bone cements.Cimentul PMMA este unul dintre cele mai rezistente materiale folosite în ortopedie, având un rol extrem de important în activitatea endoprotetică, precum și în alte intervenții reconstructive.

Antibiotic therapies, either by systemic administration or by prophylactic use of antibiotic-containing bone cements, are other treatment modalities that have contributed to the growing success of joint replacement surgery. Due to the high local concentration of an antibiotic around the stent, the use of antibiotic bone cements in their composition has great advantages compared to systemic antibiotic therapy [1].

The widespread use of antibiotics has led to the development of genetic and biochemical mechanisms that allow bacteria to survive in antibiotic environments. This is a cause for concern regarding the efficacy of antibiotics commonly used in the composition of bone cements, especially the efficacy of gentamicin.

At the moment, from a commercial point of view, the market segment related to bone cements is divided into two categories: bone cements with antibiotic and bone cements without antibiotic. The addition of hydroxyapatite in bone cements has advantages (increases bioactivity and biocompatibility) but also disadvantages (decreases elasticity, tends to form agglomerations in cement). Although calcium phosphate has excellent prospects for bone conductivity, resorption, and biocompatibility to promote bone regeneration [2–4], there are many shortcomings regarding calcium phosphate itself. Limited mechanical strength, high brittleness and curing time are the most important deficiencies of the material, in addition to obtaining a weighted degradation rate, so that it can only be used in areas without very high pressure [4].

Chapter 2 Objectives of the doctoral thesis

The main objective of the research in this doctoral thesis was the development, characterization and testing of new biomaterials in the category of acrylic bone cements used to fix hip stents with antimicrobial activity, using antimicrobial additives (silver nanoparticles, essential oils, antibiotics).

To achieve this goal, several steps were taken:

I. Carrying out a complex synthesis on the current state of research on acrylic cements used in orthopedic surgery for fixing hip stents

II. Carrying out a statistical analysis of the clinical results regarding the use of BHR type prostheses, in an orthopedic clinic, in order to identify the causes of their failure and performing a comparative in vitro analysis of the commercial cements used in the previous study.

III. Physico-chemical characterization and antimicrobial effect of three essential oils with a clearly defined purpose of demonstrating possible antibacterial properties, which could prove to be usable in the development of new bone cements

IV. Obtaining new bone cements with antimicrobial properties.

V. Morphological and structural characterization, evaluation of properties and evaluation of antimicrobial and biocompatibility activities of experimental samples of newly obtained bone cements

Chapter 3 Methods and equipment used in experimental research

3.1. Materials used and obtaining experimental samples

The experimental samples were obtained by modifying the inorganic component of a commercial cement based on PMMA, with standard viscosity, respectively the commercial cement "Fix 1" produced by Groupe Lépine. As antimicrobial agents, to strengthen the inorganic component of bone cement, silver nanoparticles embedded in a ceramic glass (2% and 4%) were used, respectively hydroxyapatite impregnated with peppermint oil and gentamicin, obtaining 3 compositions of new bone cements. . To study their antimicrobial effect, bone cement samples with the chemical composition shown in Table 3.2 were investigated.

Table 3.2. Composition of samples from experimental antimicrobial bone cements

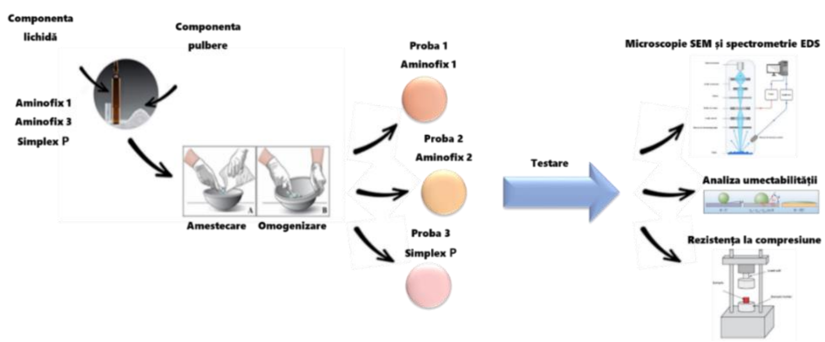
Sample coding	**Composition**		**Content of antimicrobial additive**
	Powder	**Liquid**	
GM	PMMA, BPO, $BaSO_4$	MMA, BMA, DmpT, HQ	0,5% gentamicin
HUM	PMMA, BPO, $BaSO_4$, HaP	MMA, BMA, DmpT, HQ	10% peppermint essential oil
AM1*	PMMA, BPO, $BaSO_4$	MMA, BMA, DmpT, HQ	2% silver nanoparticles
AM2*	PMMA, BPO, $BaSO_4$	MMA, BMA, DmpT, HQ	4% silver nanoparticles

**silver nanoparticles used as an antimicrobial agent are encapsulated in a ceramic glass matrix*

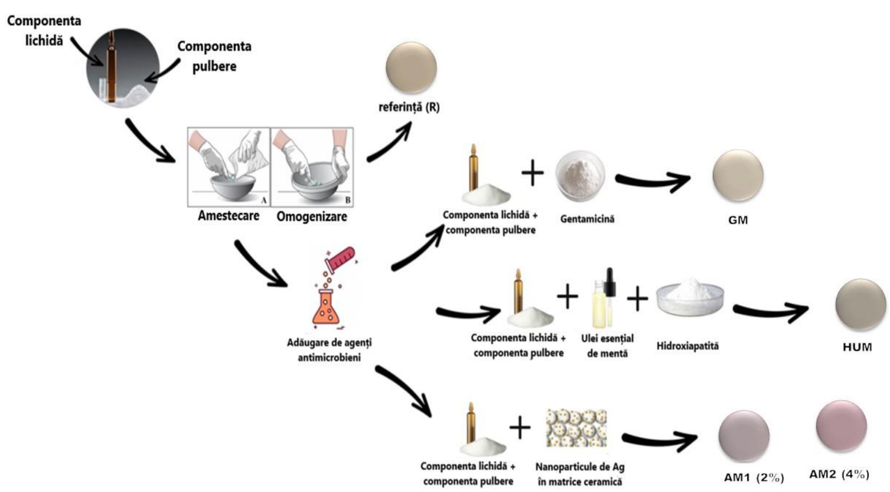
All three essential oils come from the same manufacturer, namely SC HOFIGAL IMPORT - EXPORT SA.

3.1. Methods and equipment used in experimental research

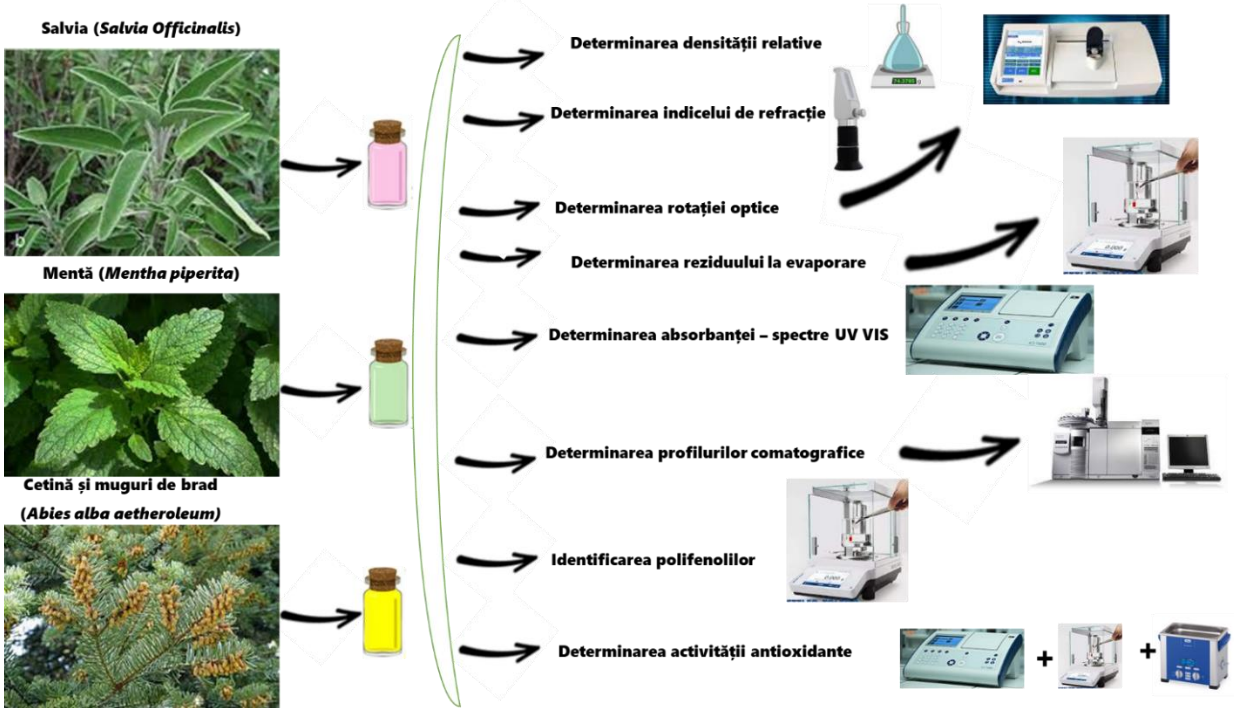
For the in vitro comparative analysis of commercial cements (Aminofix 1, Aminofix 3, Simplex P) used in clinical practice for fixing BHR type hip stents at Colentina Clinical Hospital Bucharest, morphological and structural characterization and properties evaluation were performed.



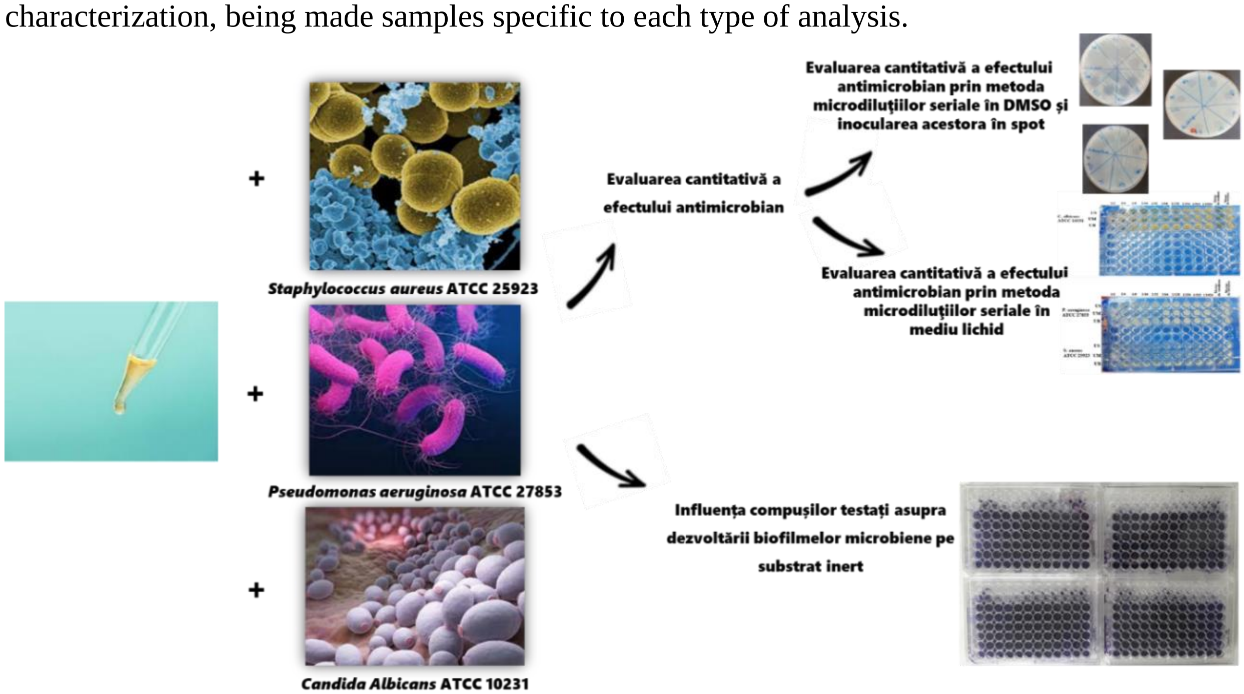
The figure below shows the process of obtaining experimental samples of bone cement, to which antimicrobial agents have been added.



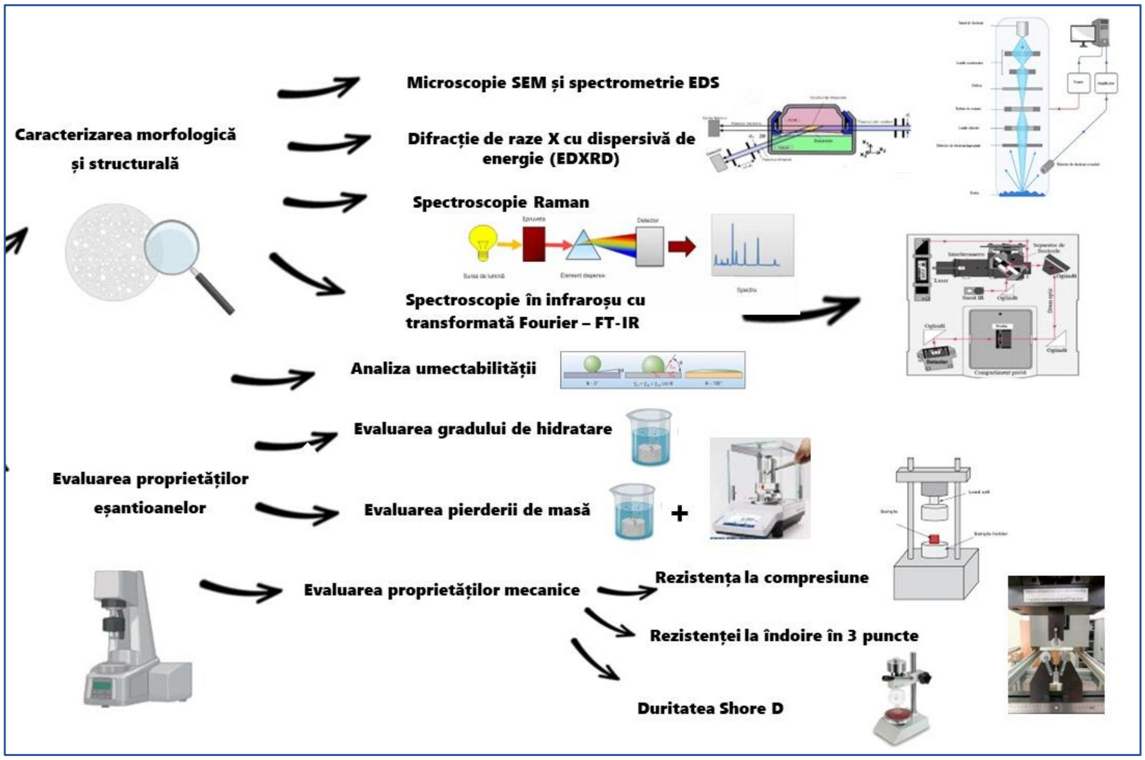
For the selection of an essential oil with optimal antimicrobial properties, usable in the development of new bone cements, we performed the physico-chemical characterization and the antimicrobial effect.



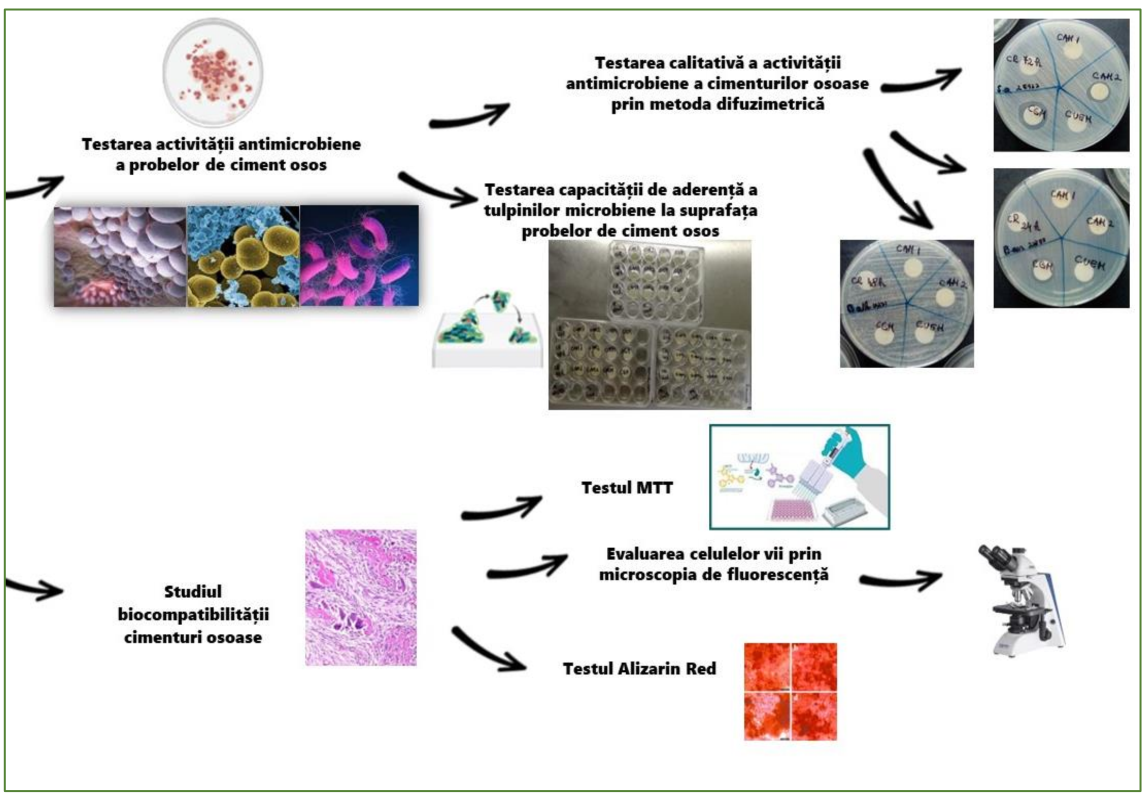
The resulting experimental samples were prepared for morphological and structural characterization, being made samples specific to each type of analysis.



After obtaining the experimental samples of newly obtained bone cements, we performed the following study, namely their morphological and structural characterization, evaluation of properties, evaluation of antimicrobial and biocompatibility activities.



Caracterizarea morfologică și structurală
Microscopie SEM și spectrometrie EDS
Difracție de raze X cu dispersivă de energie (EDXRD)
Spectroscopie Raman
Spectroscopie în infraroșu cu transformată Fourier – FT-IR
Analiza umectabilității
Evaluarea proprietăților eșantioanelor
Evaluarea gradului de hidratare
Evaluarea pierderii de masă
+
Evaluarea proprietăților mecanice
Rezistența la compresiune
Rezistenței la îndoire în 3 puncte
Duritatea Shore D



Testarea activității antimicrobiene a probelor de ciment osos
Testarea calitativă a activității antimicrobiene a cimenturilor osoase prin metoda difuzimetrică
Testarea capacității de aderență a tulpinilor microbiene la suprafața probelor de ciment osos
Studiul biocompatibilității cimenturi osoase
Testul MTT
Evaluarea celulelor vii prin microscopia de fluorescență
Testul Alizarin Red

Chapter 4. Case study and experimental research on the influence of bone cements used and the technique of cementing BHR-type hip stents on their failure in clinical practice

4.1. Presentation of the problem and the working protocol used to solve it

Failure of hip prostheses is a problem that requires further investigation and analysis.

Initially, a statistical study of the use of BHR prostheses in Romania was performed. The use of this type of system has been followed for the last 10-14 years at the Colentina Clinical Hospital, on 300 hips. This statistical study resulted in an average age of patients of 45 years, who were aged between 16-76 years, with a use of 33% of them for women. Due to the fact that BHR stents are intended especially for male patients, young and with a very good bone stock, its use in women has decreased its success rate. In this statistical study, the pathological specificity of the patients for whom the endoprosthesis was used was followed, the general failure rate being approximately 1-4%. Most cases of failure are caused by poor surgical techniques, especially in terms of surgical approach, implant orientation and notches of the cortex of the femoral neck.

Given that little is known about the impact of cementation techniques on the clinical outcomes of hip reconstruction, and recovery analyzes of failed hip reconstructions show large variations in cement mantle thickness and penetration into the femoral head, the types of orthopedic cement used were analyzed. in the stents studied. Doctors used orthopedic cements from the following manufacturers Stryker Howmedica Osteonics and Groupe Lepine. To make a comparative analysis of the three types of commercial bone cements, frequently used to fix BHR type systems, they were prepared in the operating room and the samples were mixed and placed in molds simultaneously with the introduction of cement and the pressing of prostheses in the body. by the surgeon. Cement handling was performed similarly to the surgeon. Subsequently, an analysis of the handling properties, mechanical properties, structure and composition on samples was performed to evaluate the factors influencing the properties and structure of each bone cement prepared in intraoperative conditions.

The results show that poor handling can destroy bone cement with very good properties. Therefore, it is recommended, as far as possible, to avoid manual mixing.

4.2. Identification of the causes of failure of BHR type hip stents by microscopy analysis

Three failed hip prostheses were collected from the Colentina Clinical Hospital in Bucharest and were analyzed from the point of view of the interface of bone cement with metallic biomaterial.

Using the system assigned to DeLee and Charnley and Gruen [5,6], who divided the acetabulum into three equal zones, we created a sketch (Figure 4.6.) To establish the main areas of interest when applied to the cross section of a BHR. explanted (main areas of interest: Zone 1 - upper zone; Zone 2 - intermediate zone; Zone 3 - radial zone).

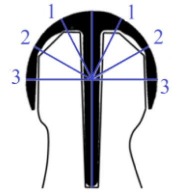


Figure 4.6. Regions of interest (1-3) used for the analysis of cement thickness and penetration

Measurements of the cement mantle and the degree of penetration were made. Using the "Image J" program, the dimensions of the cement mantle thickness in the 3 areas of interest, the circumference and the perimeter of the acetabular cup were measured.

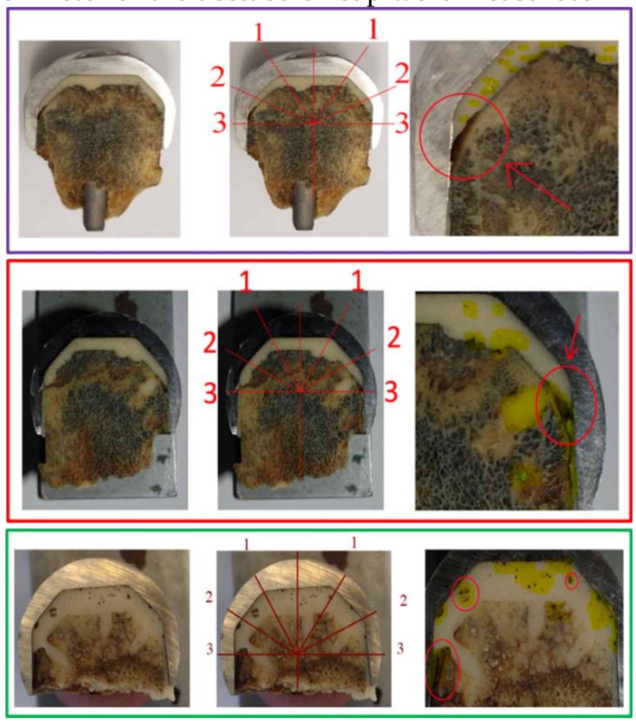
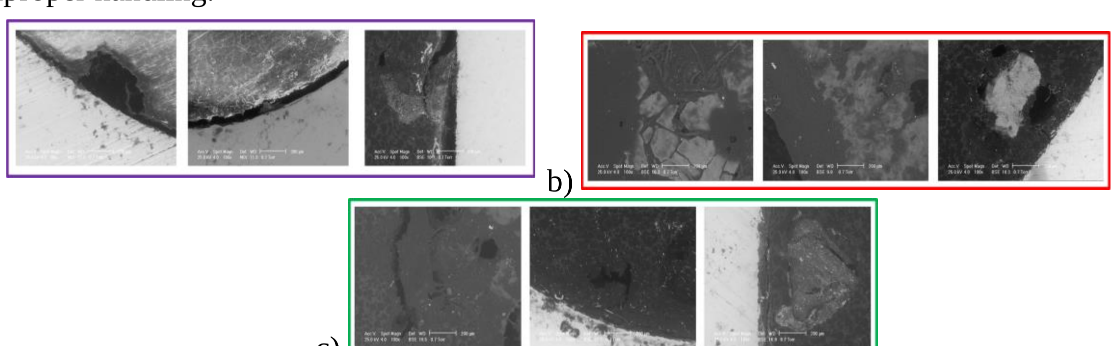


Figure 4.7. Longitudinal sections by BHR hip stents of samples 1, 2 and 3 (left), reporting areas of interest 1-3 to the longitudinal section (middle) and highlighting defects (right)

Zone 1 is overloaded and the bone is too far from the acetabular cup. In sample 2, the non-uniformity with which the cement mantle is applied is visible to the naked eye, and its thickness is inadequate. In sample 3, the orthopedic cement mantle far exceeds the optimal dimensions in some areas. Air bubbles formed due to improper mixing, and the cement penetrated the bone. The much too thick layers of cement highlighted have values of about 14 mm, compared to 2 mm according to the literature. We can assume that the penetration of orthopedic cement into the bone is caused by its low viscosity. By enlarging the image (right) we notice gaps and metal debris.
Following the scanning electron microscopy (SEM) investigations, all the samples show: lack of adhesion of the cement-metal interface, defects in the bone cement, lack of adhesion to the cement mantle, lack of homogeneity of the bone cement with agglomerations of material probably due to improper handling.



a) b) c)

Figure 4.10 Representative SEM images of existing non-uniformities at the bone cement interface - prosthesis in samples 1 (a), 2 (b) and 3 (c) - at 100X magnification

The analyzes showed that the failures are not due to the execution of the prosthesis or its principle of operation, but to several aspects such as: patient selection, type of cement used, poor preparation technique and improper use of bone cements, surgical techniques weak, especially in terms of surgical approach, implant orientation, and notches of the femoral neck cortex.

4.3. In vitro comparative analysis of commercial cements (Aminofix 1, Aminofix 3, Simplex P) used in clinical practice for fixing BHR-type hip stents at an orthopedic clinic

Failure of hip prostheses is a problem that requires further investigation and analysis. To determine whether the failure of the analyzed BHR-type hip stents can be related to the cementation technique, we performed a comparative analysis of three types of commercial bone cements, which were prepared in the operating room - under conditions similar to those used in explants. analyzed. The samples were mixed and placed in specially prepared specimens, simultaneously with the introduction of cement and the pressing of prostheses in the body by the surgeon. Cement handling was performed similarly to and simultaneously with the surgeon. In this doctoral dissertation, an analysis of the handling properties, mechanical properties, structure and composition on samples was performed to evaluate the factors that influence the final properties and structure of each bone cement in intraoperative conditions. The results show that poor handling can destroy bone cement with very good properties. Therefore, it is recommended, as far as possible, to avoid manual mixing.

4.3.1. Experimental results of SEM scanning electron microscopy and EDS spectrometry

The SEM analysis was performed to assess the structure of the experimental samples and to reveal the presence of potential agglomerations that could affect its homogeneity. The elemental composition of the samples was determined by EDS analysis. SEM images showed typical microstructures for PMMA-based cements: granules in polymer powder, polymerized monomer matrix, radiopacifying element, in this case, barium sulfate ($BaSO_4$) and pores.

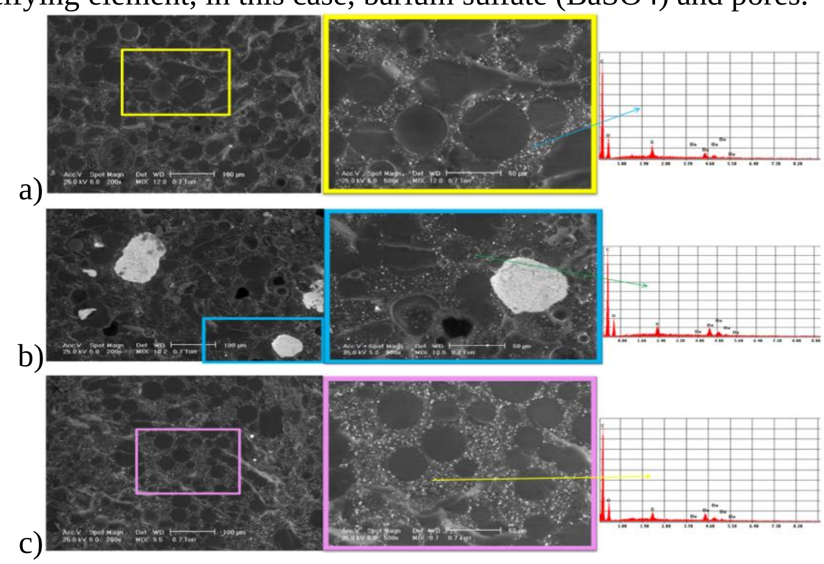


Figure 4.15. Representative SEM images and corresponding EDS spectra of sample 1 (a), sample 2 (b) and sample 3 (c) investigated

In contrast, for sample 2, the SEM images show agglomerations of the radiopacifying element due to improper mixing of the cement components. The most homogeneous structure was observed for sample 3. The EDS analysis confirmed the composition of commercial bone cement and highlights the presence of C and O in the two polymer phases (majority elements) and Ba and S in the composition of the radiopacification element.

As a first conclusion, we recommend centrifugal mixing for this type of cement and avoiding manual mixing of the cement. Regarding the clinical significance of the results presented in this section, we can mention that mixing is a very important step in the preparation of bone cement. If the bone cements are not mixed well, agglomerations could appear in the structure with a strong influence on the mechanical properties.

4.3.2. Experimental results on the wettability of cements

The hydrophilic / hydrophobic state of the materials provides information about its biocompatibility because the surface properties strongly influence the biomaterial-tissue interface. A hydrophilic surface reflects a good wettability and adhesion, so a better osseointegration. The results obtained indicate that the sample surfaces were hydrophilic. An optimal value of the contact angle for cell adhesion is approximately 55 °, the closest value measured in this research being obtained in sample 3 (55.1 °) followed by sample 1 and sample 2. Images of the corresponding contact angle for each investigated sample is presented in and in Figure 4.16.

Figure 4.16. Images with liquid droplets on the surface of the materials: sample 1 (yellow), sample 2 (blue), sample 3 (pink)

The contact angle values for the investigated samples were very close to each other ranging between 55 and 61 degrees. The results obtained indicate that the sample surfaces were hydrophilic. A hydrophilic surface reflects good wettability and adhesion, so better osseointegration. An optimal contact angle value for cell adhesion is approximately 55 ° [7,8], the closest value measured in this research being obtained in sample 3 (55,1 °) followed by sample 1 and sample 2.

4.3.3. Experimental results on the mechanical properties of cements

Determination of mechanical resistance to compression

The appearance of the samples before and after performing the compression test is shown in Figure 4.17. For each investigated bone cement we used three samples (except for sample 1 for which 4 samples were used), each having the shape of a cylinder.

Figure 4.17. Image of samples 1 (yellow), 2 (blue) and 3 (pink), before and after the compressive strength test (before compression - left, after compression - right)

Following the compression test, the software automatically generates the comparative diagram for all tested samples (Figure 4.19).

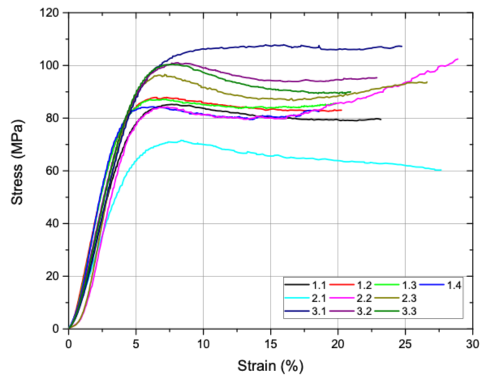
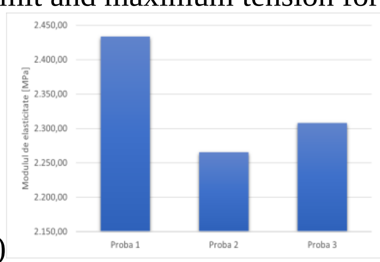


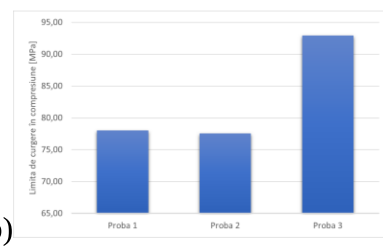
Figure 4.19. Comparative diagram for compression test

From figure 4.19 we can observe, following the compression test, a ductile behavior for all the investigated samples. The numerical values after the compression test performed on the investigated samples are presented in Table 4.9.

From the values obtained for the compressive strength it was observed that all the investigated bone cements met the minimum value established by the ASTM F451 standard (70 MPa). The best value was recorded by sample 3, an average of 92.94 MPa, followed by sample 1 (78.81 MPa) and sample 2 (77.55 MPa). Regarding the modulus of elasticity of the investigated samples, the values are close. The average modulus of elasticity of samples 1 is 2433.75 ± 263.68 MPa. For samples 2, the modulus of elasticity has an average value of 2265.47 ± 582.60 MPa, and for samples 3, the average value was 2308.03 ± 32.15 MPa. The maximum stress is the highest value of the stress recorded during the test and has similar values for the two types of cement manufactured by the same manufacturer (~ 85 MPa).

We made comparative graphs with the values obtained of the modulus of elasticity, flow limit and maximum tension for the three types of orthopedic cement (Figure 4.20).

a) 

b) 

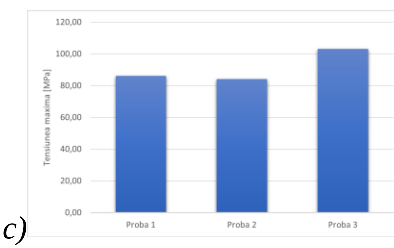
c) 

Figure 4.20. Comparative graph for modulus of elasticity (a), compressive flow limit (b) and maximum stress (c)

Stereomicroscopy determinations for orthopedic cements were performed for the three types of samples whose appearance showed radical material defects following mechanical tests. In the image obtained for the first sample (Figure 4.21.a) the presence of porosities is easily noticed. Also, following the mechanical compression tests, a crack formed in the middle area. For the area marked in the image with 2, a defect formed in the preparation of the cement is noticed.

Figure 4.21. Stereomicroscopy image for sample 1 (a), sample 2 (b) and sample 3 (c)

For the second sample, (Figure 4.21.b), a branching crack formed parallel to the direction of stress. Cracks perpendicular to the direction of mechanical stress have also formed. There is also a breaking area with a "stepped" appearance, similar to the appearance of breaking by cleavage. The low viscosity of this type of cement amplifies the formation of cracks. This image highlights the breaking area with a “river valley” appearance that continues with the crack in the material and circular holes are observed. Figure 4.21.c shows the porous aspect of the sample and the aspect obtained by the sample as a result of the barrel effect accompanied by areas of flow of the material. The barrel effect occurs as a result of the mechanical compression test. The material deforms in the crack area.

4.4. Conclusions

The investigated samples showed a structure and morphology typical for acrylic bone cements, with a tendency to form agglomerations, in the case of sample 2, due to improper mixing of the cement. The wettability measured by determining the contact angle indicates a hydrophilicity of the investigated samples. From the point of view of mechanical properties, all the investigated samples have optimal values according to the ASTM F451 standard, the best compressive strength being obtained for sample 3.

From the obtained results we can say that the cement handling process (mixing stage, waiting stage, working stage) has an important role on the structure and mechanical properties of bone cements and it is recommended to avoid manual mixing for low viscosity bone cements.

Chapter 5. Case study and experimental research on the selection of an essential oil with optimal antimicrobial properties, usable in the development of new antimicrobial bone cements

Given the need to discover and develop new ways to combat postoperative infections and starting from the fact that nature is the best generator of solutions we performed the physico-chemical and antimicrobial characterization of a number of 3 essential oils to determine the potential in relation to the existing need.

The three essential oils tested in this doctoral thesis were:

- **US - Sage essential oil - Salvia officinalis**
- **UM - Mint essential oil - Mentha piperita aetheroleum**
- **UB - Fir essential oil - Abies alba aetheroleum**

All three oils come from the same manufacturer, respectively SC HOFIGAL IMPORT - EXPORT SA.

5.1. Physico-chemical characterization of the tested essential oils

Essential oils are essentially natural oils, obtained by distillation and having a distinctive odor, of the plant or other sources from which it is extracted. Many components of the essential oils of the same plant can be extracted from different parts of plants with completely different properties. They are produced using different methods, with steam distillation being the most common method, while other methods such as mechanical extraction, solvent extraction or supercritical fluid extraction are less used. The main components of these oils are terpenoids, followed by diterpenes and aromatic compounds.

To date, there are not enough studies that can give us a clear idea of how essential oils work. Given the complexity of their chemical composition, everything suggests that this mode of action is complex and it is difficult to identify the molecular path of action. It is very likely that each of the components of essential oils has its own mechanism of action.

The purpose of these analyzes was to evaluate the antimicrobial effect of three essential oils (fir, mint and sage essential oil) on the ability to grow, multiply and generate monospecific biofilms of three standard microbial strains: a Gram negative strain *(Pseudomonas aeruginosa ATCC27853*), a Gram-positive strain (*Staphylococcus aureus ATCC25923*), a yeast strain (*Candida albicans ATCC10231*).

For each of the essential oils analyzed was determined: relative density, refractive index, optical rotation, evaporation residue, solubility, UV-VIS spectra, chromatographic profile by GC-MS, polyphenols were identified and the results are presented in tables and figures next:

Table 5.1. Relative density of studied essential oils:

Sample	Sage essential oil	Peppermint essential oil	Fir essential oil
Relative density	0,890-0,908	0,900 – 0,916	0,855 – 0,875

Tabelul 5.2. Refraction index of studied essential oils:

Sample	Sage essential oil	Peppermint essential oil	Fir essential oil
Refraction index	1,456-1,466	1,457 – 1,467	1,475 – 1,481

Tabelul 5.3. Rotația Optical rotation of studied essential oils:

Sample	Sage essential oil	Peppermint essential oil	Fir essential oil
Optical rotation	-26,0 º - -10,0°	-30° - -10°	-15° - +5°

Tabelul 5.4. Evaporation residue of studied essential oils:

Sample	Sage essential oil	Peppermint essential oil	Fir essential oil
Evaporation residue	max. 1,5%	max. 1,5%	max. 1,5%

Solubility. Essential oils are generally lipophilic and immiscible with water. They can be diluted with pure solvents such as alcohol and polyethylene glycol, and for topical applications it is safest to be diluted in fatty oils. Essential oils are generally soluble in organic solvents such as ethyl alcohol, acetone, hexane, chloroform, benzene, ether, etc.

Absorbance - UV-VIS spectra

a b c.

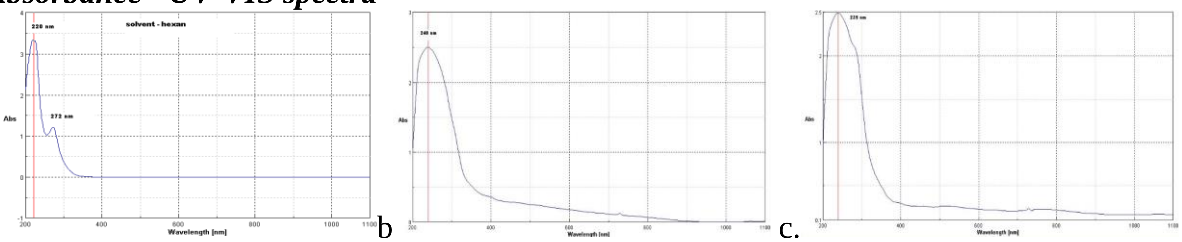


Figure 5.1. Spectra of sage (a), peppermint (b) and fir (c) essential oils in hexane

The chromatographic profiles of the three analysis oils are presented in figure below:

a b c

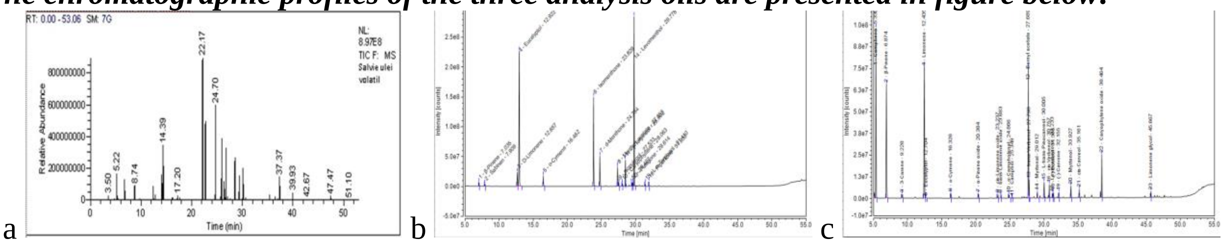


Figure 5.2. Chromatographic profiles of the three essential oils of sage (a), mint (b) and fir (c)

Identification of polyphenols. For all the studied oils, the appearance of blue coloration was registered, which indicates the presence of polyphenols..

5.2. Evaluation of the antimicrobial effect of the tested essential oils

Qualitative evaluation of the antimicrobial effect by the method of serial microdilutions in DMSO and their inoculation in the spot

The reading of the results was performed by assessing the clarity of the inhibition area and noting the working dilution until which the appearance of this area was observed.

The arrangement of dilutions of essential oil on the surface of the solid medium previously inoculated at the preset temperature highlighted the appearance of some inhibition zones that allowed the establishment of the MIC value. Thus, for the Gram-positive strain Staphylococcus aureus ATCC 25923 the MIC value was that corresponding to the dilution 1/4 for sage and fir essential oil and 1/8 for peppermint oil (figure 5.3 A, B, C).

A. B. C.

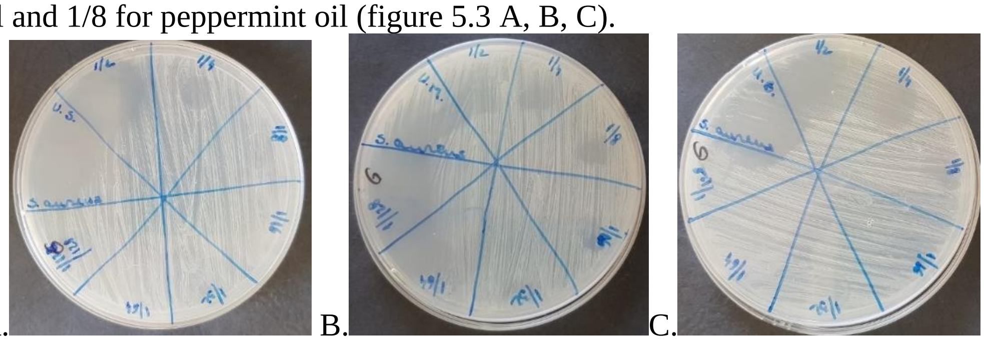


Figure 5.3 Appearance of the zone of inhibition manifested by the different dilutions of essential oils against the Gram-positive strain Staphylococcus aureus ATCC25923 (A-sage oil, B-peppermint oil, C-fir oil)

For Gram negative strain Pseudomonas aeruginosa ATCC 27853 the MIC value was that corresponding to 1/128 dilution for sage and fir essential oil and 1/128 + for peppermint oil (figure 5.4 A, B, C).

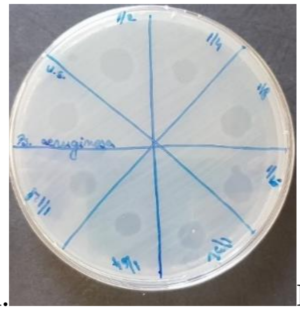
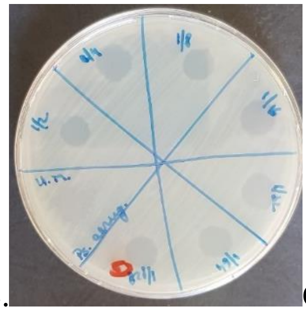
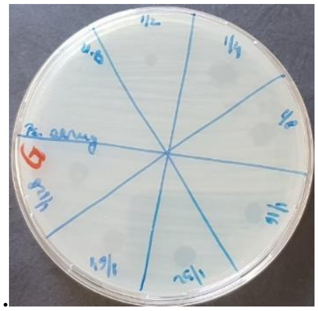
A.  B.  C. 

Figura 5.4 Aspectul zonei de inhibiție manifestată de diferitele diluții de esențiale esențiale față de tulpina Gram negativă Pseudomonas aeruginosa ATCC27853 (A-ulei de salvie, B-ulei de mentă, C-ulei de brad).

For the yeast strain *Candida albicans* ATCC102310 the CMI value was the one corresponding to the 1/128 dilution for all the essential oils tested (figure 5.5 A, B, C), being the most sensitive strain of the 3 studied.

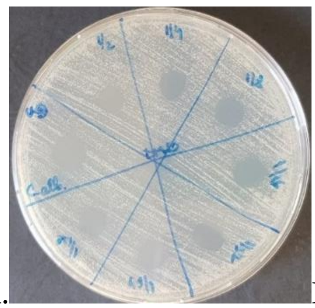
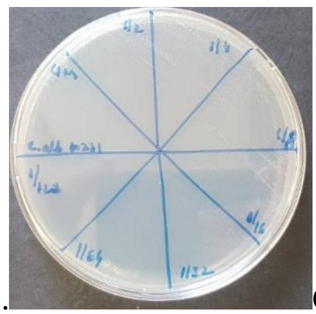
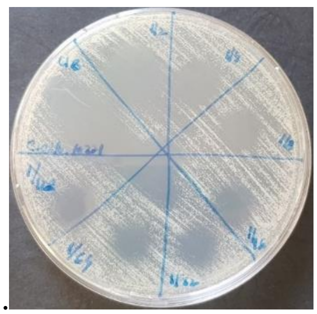
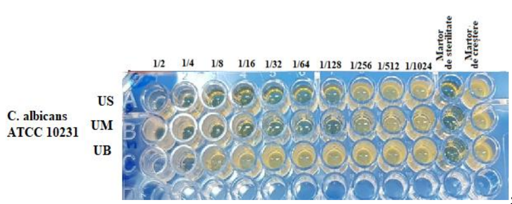
A.  B.  C. 

Figure 5.5 Appearance of the zone of inhibition manifested by the different dilutions of essential oils against the yeast strain Candida albicans ATCC 10231 (A-sage oil, B-peppermint oil, C-fir oil)

Quantitative evaluation of the antimicrobial effect by the method of serial microdilutions in liquid medium

To determine the values of the minimum inhibitory concentration (MIC) represented by the smallest amount of essential oil capable of inhibiting the growth and multiplication of microbial cells, after incubating the plates at the preset temperature for one day, the results obtained by macroscopic observation and spectrophotometric reading were analyzed. 620 nm.



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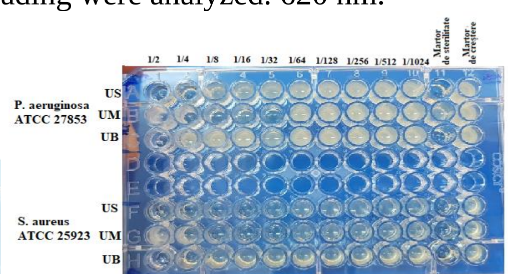


Figure 5.6 Scheme for performing binary dilutions the series of tested compounds and the appearance of microbial growth after the incubation stage, in order to determine the MIC values.

The dilution of the compound corresponding to the last well in which the development of the culture was no longer observed represented the CMI value for the respective compound (figure 5.6). In subsequent wells, including the growth control well, the environment was cloudy due to the multiplication of microbial cells. The sterility control well did not show bacterial growth, the liquid content being clear and transparent. The test results performed in liquid medium are not entirely consistent with the test results on solid medium, probably because the tested essential oils were not miscible with the liquid medium in which the binary serial dilutions were performed (Table 5.8.).

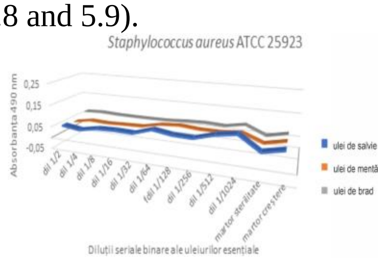
Table 5.8. MIC values obtained by the method of binary serial dilutions in liquid medium

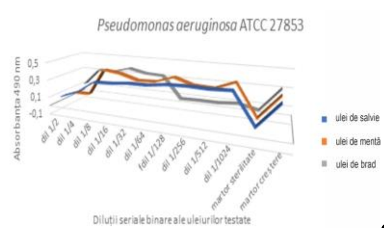
Bacterial strain / essential oils	**MIC value expressed as dilutions**		
	Sage essential oil (US)	**Peppermint essential oil (UM)**	**Fir essential oil (UB)**
***Staphylococcus aureus* ATCC 25923**	dil. 1/4	dil. 1/8	dil. 1/4
***Pseudomonas aeruginosa* ATCC 27853.**	dil. 1/128	dil .1/128	dil. 1/128
***Candida albicans* ATCC 10231**	dil. 1/128	dil. 1/128	dil. 1/128

Therefore, for this category of samples, the determination of MIC values should be performed by several alternative methods that allow a more accurate assessment of the effectiveness of antimicrobial activity.

Study of the influence of the tested compounds on the development of microbial biofilms on inert substrate

Testing the properties of essential oils to inhibit the adhesion of bacterial cells to the inert substrate and the generation of monospecific biofilms allowed to establish the values of the Minimum Biofilm Eradication Concentration (CMEB) [9–11] These values are represented by the highest oil dilutions that inhibited adhesion and are represented in the graphs below (figures 5.7, 5.8 and 5.9).

a. 

b. 

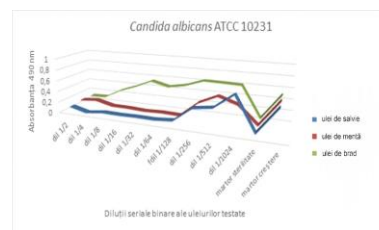
c. 

Figure 5.7 Graphical representation of CMEB values for Gram-positive strain Staphylococcus aureus ATCC 25923, absorbance values at 490 nm (a), for Gram-negative strain Pseudomonas aeruginosa ATCC 27853, absorbance values at 490 nm (b) and for the yeast strain Candida albicans ATCC 10231 values determined by absorbance at 490 nm (c)

For the most part, the CMEB values are similar to the CMI, with the exception of fir essential oil which seems to have stimulated the adhesion of the yeast strain at dilutions greater than 1/32 (Figure 5.10).

5.3. Conclusions resulting from the physico-chemical and antimicrobial characterization of the three essential oils analyzed

As a result of the physico-chemical and antimicrobial characterizations of the three essential oils analyzed, it turned out that the best option for modifying the inorganic component of a commercial cement based on PMMA, with standard viscosity, is *Mentha piperita* peppermint essential oil.

Chapter 6 Characterization and testing of new bone cements with antimicrobial properties

6.1. Morphological and structural characterization

6.1.1. Experimental determinations of scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDS)

The results of the scanning electron microscopy determinations coupled with the energy dispersion X-ray spectroscopy are shown in the following figures.

The SEM image for the reference sample (figure 6.1.) Shows a homogeneous structure in which four phases are highlighted: granules from the polymer powder; pores; the polymerized monomer and the radiopaque element, in this case barium sulphate (BaSO4). The granules in the polymer powder are dark circular surfaces in which the pores are included. The polymerized monomer and barium sulfate form a matrix that surrounds the polymer granules. The bright white dots are due to the radiopaque element (BaSO4).

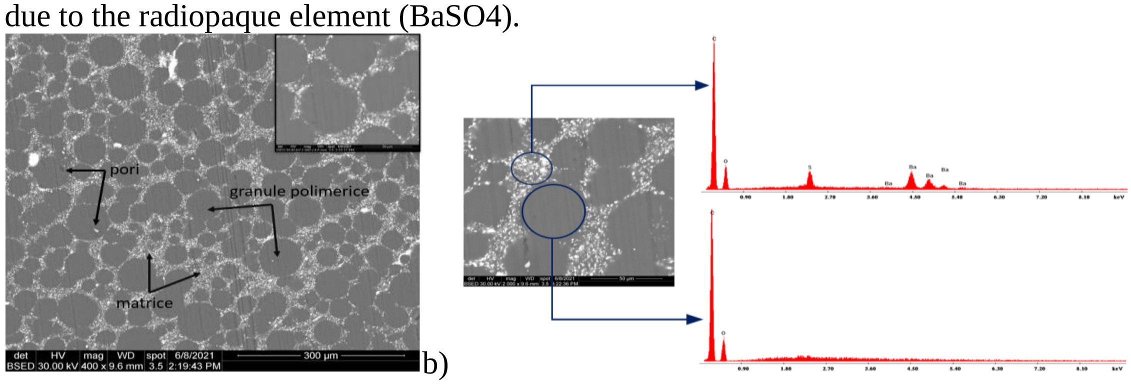
a)  b)

Figure 6.1. SEM image of the reference sample R (400 X) a) and Representative SEM image (2000 X) and EDX analysis performed on different areas for the reference sample R

Local elemental analysis performed by energy dispersive X-ray spectroscopy (EDS), shown in Figure 6.2., Highlights the presence of the majority elements, C and O, of the two polymer phases (pre-polymerized granules and polymerized monomer) and the elements Ba and S from the composition of the radiopaque element (barium sulphate present in the polymerized monomer matrix).

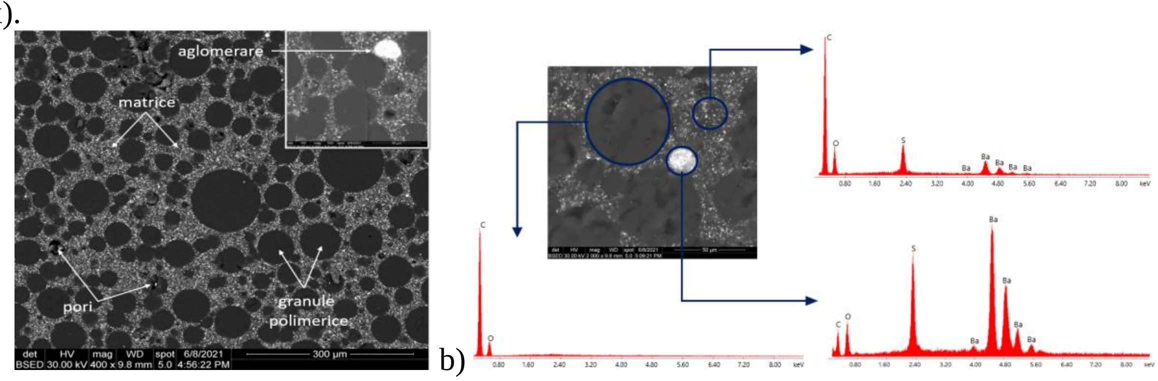
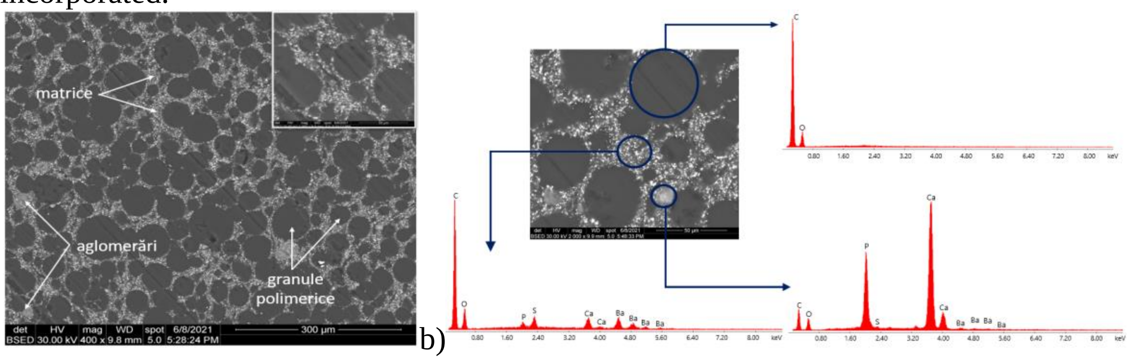
a)  b)

Figure 6.3. SEM image of the GM sample (400 X) a) and representative SEM image (2000 X) and EDX analysis performed on different areas for the GM sample

The SEM image for the GM sample containing gentamicin shows a relatively homogeneous structure and the presence of agglomerations of the radiopaque element (as it results from the EDS spectrum, made in that area - figure 6.3 b.), Which appeared after an inadequate homogenization process. Also, in figure 6.3., The four specific phases of a bone cement based on PMMA are highlighted: granules from the polymer powder; pores; the polymerized monomer and the radiopaque element.

The local elemental analysis also highlights in this case the presence of the majority elements, C and O, from the two polymer phases (pre-polymerized granules and polymerized monomer) and the elements Ba and S from the composition of the radiopaque element (barium sulfate present in the monomer matrix). polymerized).

The results of the SEM analysis for the HUM sample are shown in Figure 6.5. A relatively homogeneous structure is observed and the presence of small agglomeration areas of hydroxyapatite in the polymerized monomer matrix (as shown in the EDS spectrum in Figure 6.6.). Along with these areas of structural inhomogeneities, one can also observe the granules from the polymer powder and the polymerized monomer matrix in which the radiopaque element (BaSO4) is incorporated.

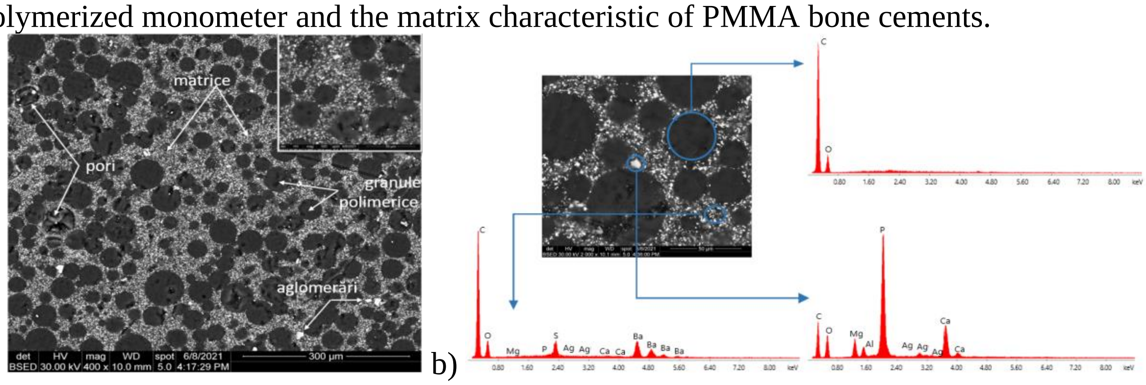


a) b)

Figure 6.5. SEM image of the HUM sample (400 X) a) and Representative SEM image (2000 X) and EDX analysis performed on different areas for the HUM sample b)

In the corresponding EDS spectra (figure 6.6.), The presence of the majority elements, C and O, of the two polymer phases (pre-polymerized granules and polymerized monomer), of the Ba and S elements in the composition of the radiopaque element and of the P elements is highlighted. and Ca from the composition of hydroxyapatite. Because peppermint essential oil consists of organic compounds from the class of terpenes and alcohols (chemical elements: C, O and H), the EDS spectra do not show the presence of other chemical elements.

The SEM results for the AM1 sample containing 2% silver nanoparticles embedded in a ceramic glass matrix (Figure 6.7.) Show small areas where agglomerations of the antimicrobial agent have formed in the polymerized monomer matrix. It also highlights the pores, the pre-polymerized monometer and the matrix characteristic of PMMA bone cements.



a) b)

Figure 6.7. SEM image of sample AM1 (400 X) a) and representative SEM image (2000X) and EDX analysis performed on different areas for sample AM1

In the EDS spectra (figure 6.8.) The presence of the majority elements, C and O, of the two polymer phases (pre-polymerized granules and polymerized monomer), of the elements Ba and S in the composition of the radiopacizing element and of the elements Ag, P, Like Mg in the composition of the antimicrobial agent.

Unlike the AM1 sample, in the SEM images obtained for the AM2 sample (4% antimicrobial agent), (figure 6.9.) The formation of larger agglomerations is observed.

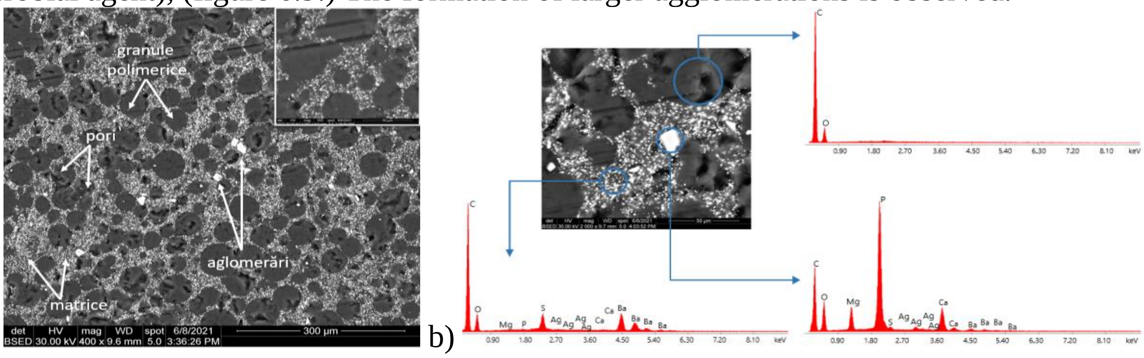


Figure 6.9. SEM image of the AM2 sample (400X) a) and Representative SEM image and EDX analysis performed on different areas for the AM2 sample

The EDS spectra highlight the presence of elements in the composition of bone cement: C, O, Ba, S, Ag, P, Ca and Mg (figure 6.10).

The SEM analysis revealed that, after hardening, all the analyzed bone cements showed the typical microstructure of PMMA-based cements, in which four phases are highlighted: granules from the polymer powder; pores; polymerized monomer; and the radiopacifying element, in this case, barium sulphate (BaSO4) [12,13]. SEM images show a good dispersion of barium sulfate and antimicrobial additives in the PMMA-PBMA matrix (poly (methyl methacrylate) -poly (butyl methacrylate) matrix). A slight tendency of antimicrobial agents to form agglomerations can be observed, especially in bone cement samples containing 4% silver nanoparticles (AM2 sample). The EDS analysis supports this observation and highlights the presence of the majority elements, C and O, of the two polymer phases, Ba and S respectively in the composition of the radiopacifying element and Ag, Mg, P, and Ca from HAp and silver nanoparticles incorporated in a ceramic glass matrix.

6.1.2. Experimental determinations of infrared spectroscopy with Fourier transform – FTIR

Figure 6.11 represents the FT-IR spectra of PMMA-based bone cements that have been investigated experimentally.

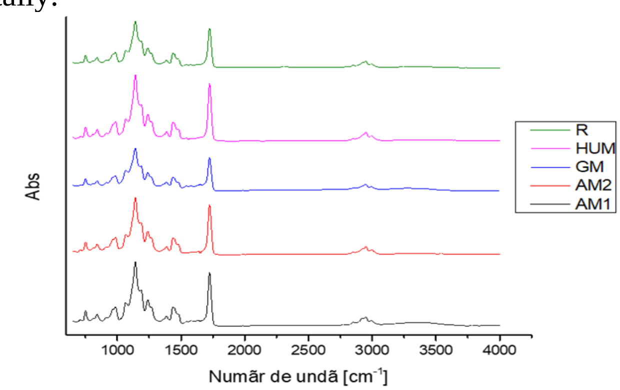


Figure 6.11 FT-IR spectra of bone cements

The FT-IR spectra recorded for the experimental samples highlight the characteristic bands for poly (methyl methacrylate) and poly (butyl methacrylate), polymers in the composition of the investigated cements.

At 752 cm-1, the characteristic bands of the CH groups in the aromatic ring present in benzoyl peroxide (BPO) and N-N-dimethyl-p-toluidine (components of the polymerization reaction initiation system) were highlighted. Following the FT-IR investigations, it is confirmed that all experimental cements are in the category of PMMA-based acrylic cements.

6.1.3. Experimental determinations of Raman spectroscopy

Figure 6.12. shows Raman spectra collected on samples R, GM, HUM, AM1 and AM2. All models were collected with a red laser source (785 cm-1). The second-order basic polynomial correction was applied as a post-acquisition process to eliminate the fluorescence contribution.

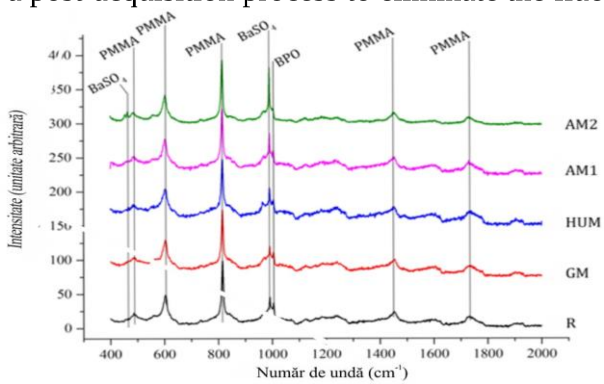


Figure 6.12 Raman models collected on studied samples - made of PMMA-BaSO4-BPO as major components. The most intense strips were labeled and assigned to that component of bone cement

The spectra represented have many similarities, due to the similar composition of the different samples, which are prepared with the same solid components (PMMA, BaSO4, BPO).

By comparison, only minor differences in spectra were detected and consist of differences in the relative intensities of the specific bands.

The most relevant refer to samples AM1 and AM2 with relatively higher signals of the band from 990 cm-1 assigned ν1 (SO4) from BaSO4 and sample R with a higher intensity associated with the band attributed to the vibration of the romance cycle (C6H6) from 1000 cm-1 of the BPO component.

6.1.4. Experimental determinations of energy dispersion X-ray diffraction (EDXRD)

The figure below graphically represents the EDXRD spectra collected on samples R, GM, HUM, AM1 and AM2 as diffracted intensity as a function of the scattering angle 2θ. The most intense Bragg reflections, those belonging to the PMMA component, were labeled with corresponding Miller indices. The fluorescence contributions of Ba are visible near the 30 ÷ 40 ° region.

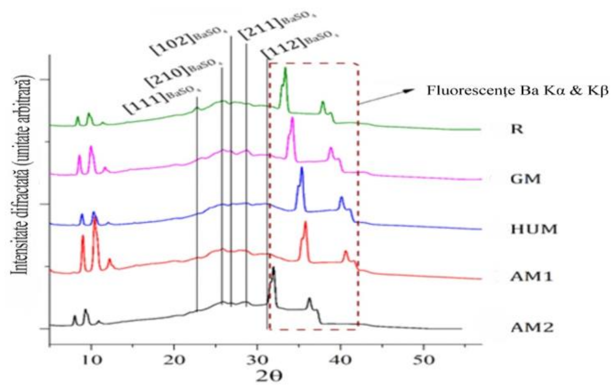


Figure 6.13 EDXRD spectra collected on the analyzed samples.

6.2. Evaluation of the properties of experimental samples of bone cements

6.2.1. Experimental results for the evaluation of wettability properties by determining the contact angle

Figure 6.14 shows the aspects of the drops formed on the surfaces of the samples from the investigated bone cements. To study the hydrophilic or hydrophobic behavior of the samples, but also the free energy of the surface for testing, 3 types of liquids were used, namely: water, di-iodomethane (DIM) and ethylene glycol (EG). Surface wettability behavior is influenced by both the type of sample and the type of liquid used, as can be seen in Figure 6.15.

The average values of the measured contact angles are centralized in Table 6.3.

Table 6.3 Values of contact angles of characterized bone cements

	R	GM	HUM	AM1	AM2
Contact angle, Θ [°], water	70,85	85,10	58,05	55,13	66,86
Contact angle, Θ [°], DIM	27,07	40,26	31,17	50,75	40,63
Contact angle, Θ [°], EG	57,23	50,05	50,12	52,85	45,94

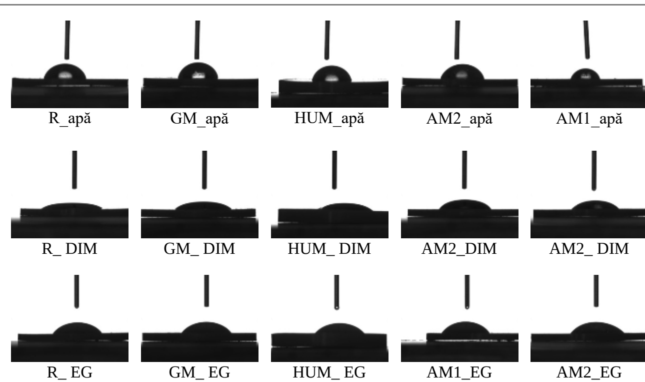


Figure 6.14 Appearance of droplets formed on the surface

In the figure below is represented by a statistical graph the dispersion of the values of the contact angles recorded for each sample and each liquid.

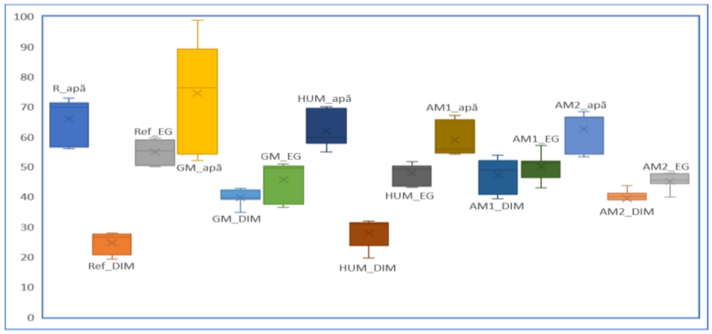


Figure 6.15 Diagram of values of contact angles tested with water, di-iodomethane (DIM) and ethylene glycol (EG)

The contact angle values for the investigated samples, when the liquid used for the test was water, are quite dispersed, the lowest value being the AM1 sample (55.13°), followed by the HUM sample with 58.05°, AM2 66,86°, the reference sample R 70.85°, and the highest value is the GM sample 85.10°. The standard value for determining the contact angle of PMMA is approximately 69 degrees. The most hydrophobic character can be observed in the case of the GM sampleProbele HUM, AM1 și AM2 prezintă un caracter mai hidrofil, care este cauzat de componentele ceramice (hidroxiapatită și sticlă ceramică) care au fost adăugate cu agenții antimicrobiene respectivi.

6.2.2. Experimental results regarding the determination of the hydration degree

The degree of hydration (water absorption capacity) is an important property of materials that influence the behavior of bone cement. Moreover, water can more easily penetrate into a porous, heterogeneous structure at the interface area between the polymer and the filler. The more heterogeneous areas of the material structure represent the numerous spaces through which water can accumulate. The figure graphically represents the evolution of the degree of hydration (Ha) evaluated in distilled water at a temperature of 37 ° C over a period of 21 days, for all experimental samples.

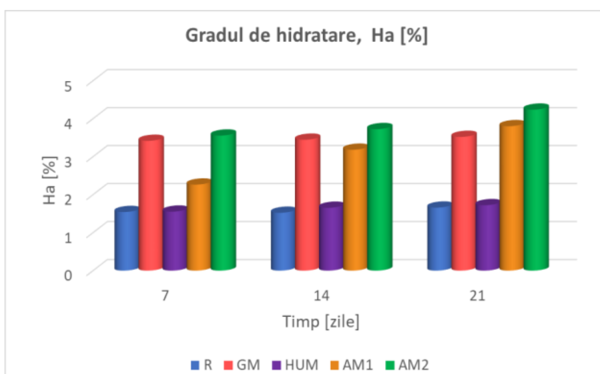


Figure 6.16 Variation of hydration over a period of 21 days in distilled water, at 37 ° C, for experimental samples

6.2.3. Experimental results for determining the degradation rate

The figure shows the variation of the degradation rate over a period of 28 days in simulated physiological fluid (SBF) at a temp. of 37 ° C, for the investigated bone cements.

There is an increase in the degradation rate for samples containing gentamicin and peppermint essential oil, while the reference sample has a constant degradation rate over time. In the case of samples containing silver nanoparticles, up to 28 days no degradation process is observed, but only a continuous absorption process.

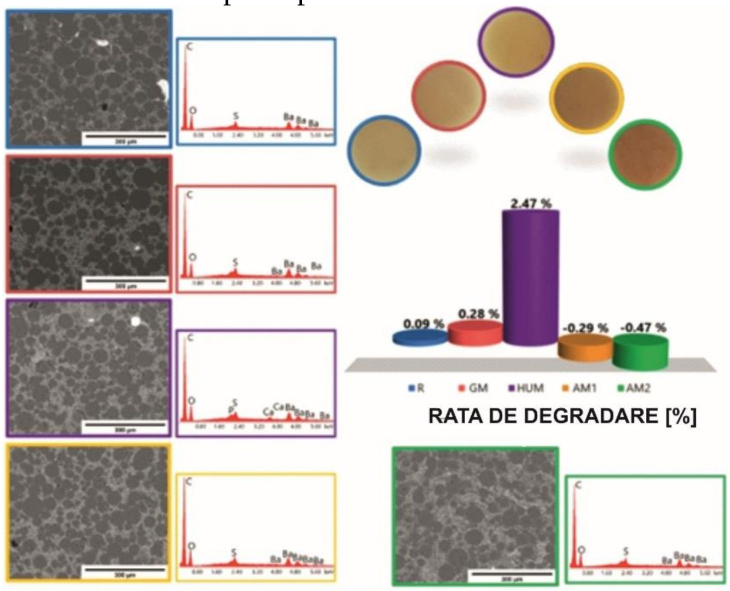


Figure 6.18 Morphological aspects revealed by SEM and elemental analysis of the surface by EDS of PMMA experimental bone cements after 28 days in SBF

The results recorded for the degree of hydration and the rate of degradation are consistent with the results obtained when assessing the wettability of surfaces by determining the contact angle.

6.2.4. Experimental study on the determination of mechanical properties

Experimental determination of bending strength

A bone cement with a high resistance to bending allows a very thin thickness of the bone cement mantle and is therefore ideal for minimally invasive treatment options, but also for hip or knee prostheses.

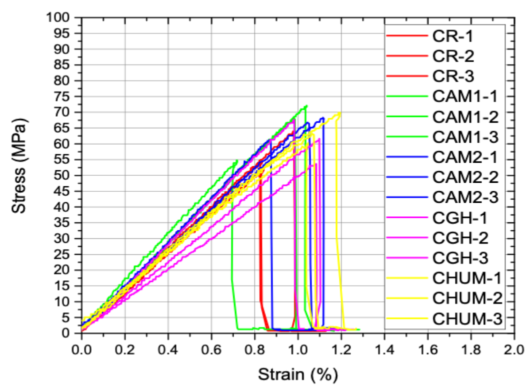


Figure 6.25 Comparative diagram of bending tests

The average values of bending resistance show that the addition of antimicrobial agents produces an increase: by 3.7% for GH samples; 9.5% for HUM samples; 10.5% and 11.4% for AM1 and AM2 samples. Apparently, the bending strength results obtained are not strongly influenced by the addition of antimicrobial additives, all values recorded being above the limit required by International Standard ISO 5833, at least 50 MPa.

Experimental determination of compressive strength

Regardless of the antimicrobial agent added, the mechanical behavior was largely similar in the elastic region. Apparently, compared to the reference sample (R) the addition of gentamicin

(GH sample) does not influence the modulus of elasticity of bone cement, while the addition of silver nanoparticles (samples AM1 and AM2) leads to a slight decrease of about 4% and 5% of its values. The addition of peppermint oil to hydroxyapatite shows a stronger decrease of about 9%.

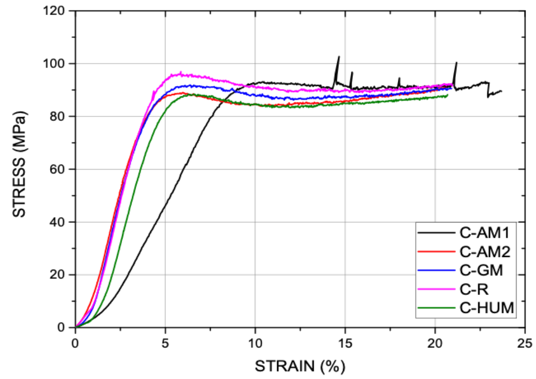


Figure 6.26 Diagram with compression test results for all experimental samples

Despite the fact that the addition of antimicrobial agents to the compression test leads to a decrease in mechanical properties, this decrease is of an acceptable amplitude (maximum 12%) and the strength values for the investigated bone cement still meet the minimum requirements of ASTM F451. (ie a strength of at least 70 MPa).

Experimental determination of Shore D hardness

An essential characteristic of bone cements is hardness. In the tested samples, this parameter changes depending on the additives as follows: starting from R (average Shore D hardness of 85.5 ◦ShD) - which also has the highest determined value followed by the bone cement sample with gentamicin, of whose addition decreases by one unit the average shore D hardnesses determined (84.5◦ShD).

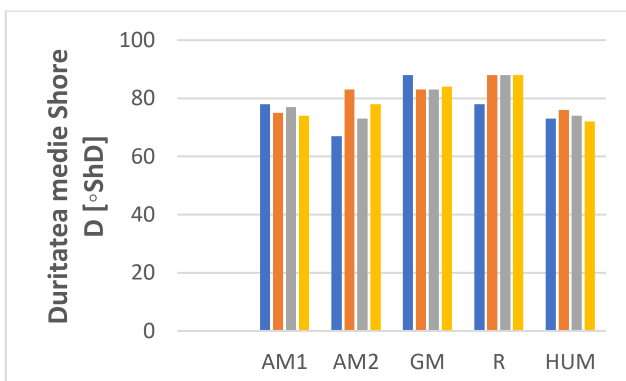


Figure 6.27 Graph of Shore D hardnesses determined on experimental bone cements

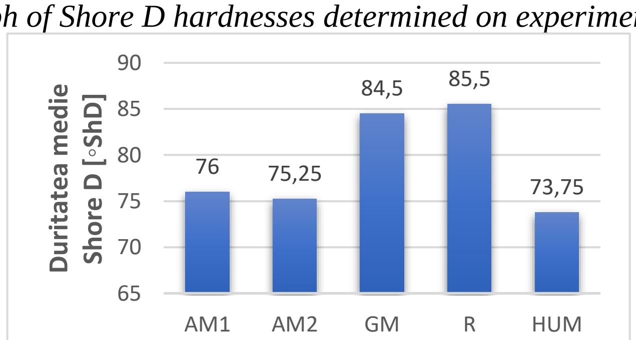


Figure 6.28 Average Shore D hardness determined on the five experimental bone cements

At a considerable difference (76 ◦ShD) we find the average of the sample values with a content of 2% silver particles followed immediately by the one with a content of 4% Ag (75.25 ◦ShD). The biggest influence on the hardness of the new bone cements is the addition of 10% peppermint oil (73.75◦ShD), which decreases by 11.75 units the hardness, compared to the reference sample (85.5 ◦ShD).

The bottom line is that each of the additives influenced the hardness of the bone cements, but they all fall within existing hardness standards.

6.3. Evaluation of antimicrobial activity and biocompatibility of experimental bone cement samples

6.3.1. Experimental results of qualitative determination of antimicrobial activity by diffusimetric method

Experimental tests showed that the sample of bone cement loaded with gentamicin (GM) expressed a clear zone of inhibition only against bacterial strains, being used as a positive control for bone cement samples loaded with other different antimicrobial agents. At the same time, the HUM sample and the AM2 sample showed their inhibitory effect, generating clear areas of inhibition with diameters compared to those of the GM sample: 25 mm for Staphylococcus aureus ATCC 25923 and 20 mm for Pseudomonas aeruginosa ATCC 27853 (Figure 6.29).

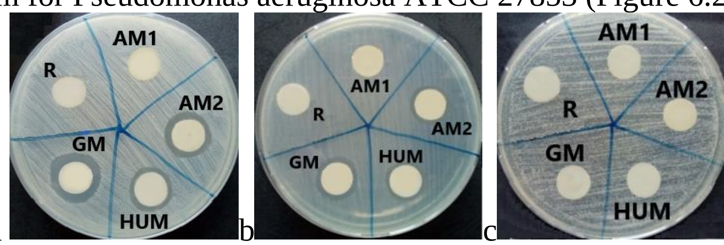


Figure 6.29 Evaluation of antimicrobial properties for experimental PMMA bone cements: (a) growth inhibition zone for Gram-positive strain Staphylococcus aureus ATCC 2592; (b) growth inhibition zone for Gram-negative strain Pseudomonas aeruginosa ATCC 27853, c) growth inhibition zone for Candida albicans strain ATCC 10231, after 72 hours of incubation

The lowest sensitivity was expressed by the fungal strains of Candida albicans ATCC 10231. In this case, all tested samples did not show any area of inhibition (Figure 6.29).

6.3.2. Experimental results for determining the adhesion capacity of microbial strains to the surface of bone cement samples

Quantitative evaluation of the ability of the selected strains to adhere and generate biofilm on the surface of the tested samples was performed using the viable cell counting method. CFU / mL values were determined for biofilms developed at 24, 48 and 72 hours, on the inert substrate represented by the bone cement samples.

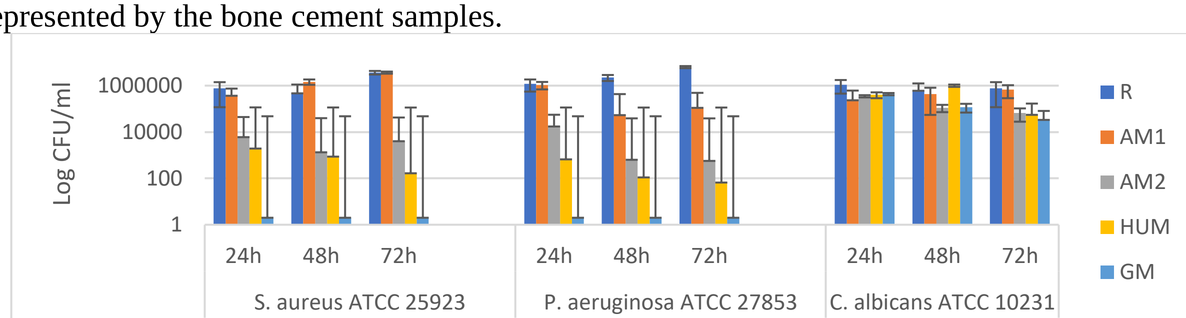


Figure 6.30 Graphical representation of CFU / ml values evaluating the degree of microbial biofilm development on the surface of the tested bone cement samples

For the bone cements investigated by us, the results show that, although the bacterial cells adhered poorly to the surface of the bone cement after 24 hours of incubation, they could not develop a mature biofilm, and the decrease in CFU / mL values after 48 was not significant.

In contrast, the ability of the Candida albicans strain to adhere to and generate biofilm was NOT significant, recording the highest values of CFU / mL.

6.3.3. Experimental results for determining the biocompatibility of bone cements

The results of the MTT test show that the bone cement samples tested stimulated cellular metabolism, with a significant increase in proliferation compared to the control sample (CTRL).

The MTT test showed that all the bone cements tested did not have a cytotoxic effect, the viability values being higher compared to the control sample. Fluorescence images showed that MG-63 cells were viable and no dead cells or cell fragments were observed. Moreover, the cells formed filaments to move and make contact with neighboring cells, suggesting that MG-63 cells had an active phenotype.

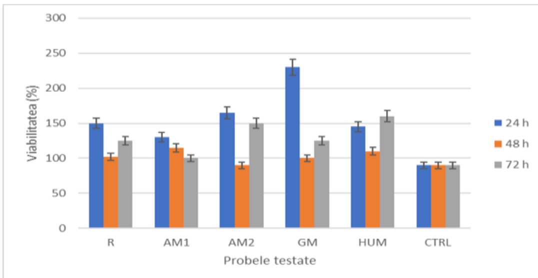


Figure 6.31 MTT test showing the viability of MG-63 cells in the presence of experimental PMMA bone cements after 24, 48 and 72 hours.

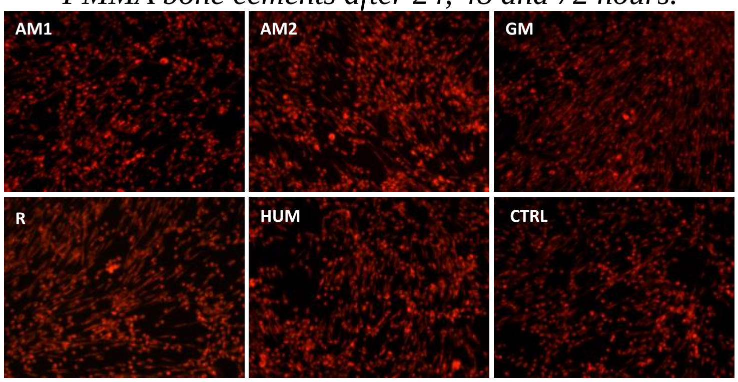


Figure 6.32. Fluorescence images of MG-63 cells stained with CMTPX fluorophore in the presence of experimental bone cements.

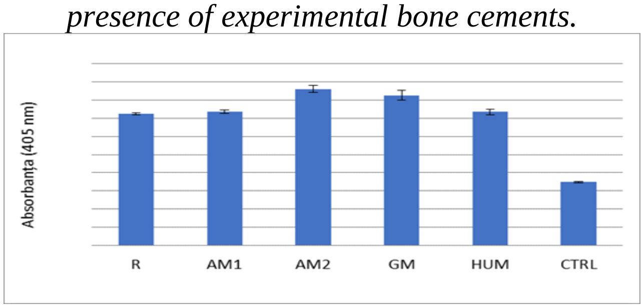


Figure 6.33. Alizarin Red test showing the osteogenic potential of experimental bone cements on MG-63 cells

The osteogenic potential of bone cements on MG-63 cells was quantified using the Alizarin Red test. After 21 days, in the presence of bone cements, MG-63 cells increased their osteogenic potential. This was demonstrated by an increase in calcium deposits in all samples compared to the control sample (CTRL).

6.3.4. Conclusions on the antimicrobial and biocompatibility assessment of experimental bone cement samples

The antimicrobial properties were demonstrated for GM, HUM and AM2 samples, which generated clear areas of inhibition against *Staphylococcus aureus* ATCC 25923 I ATCC 27853. It was observed that not only the type of antimicrobial agent is important, but also the amount used.

Staphylococcus aureus and *Pseudomonas aeruginosa* are known to be clinically relevant pathogens associated with bone infection and intrinsic drug resistance, respectively. Regarding the biocompatibility assessed by the MTT test using human MG-63 cells, all cements are biocompatible without any cytotoxicity effect.

The performance of bone cements with peppermint essential oil and silver nanoparticles against these two pathogens suggests that these antimicrobial additives appear promising to be used in clinical practice against bacterial infections.

Cap.7. Conclusions, own contributions and future directions

7.1. Conclusions and own contributions

The paper entitled "Contributions to the development of new materials in the category of antimicrobial acrylic cements used to fix hip stents" is an analysis of current studies and concerns of scientific research on obtaining new bone cements used to fix prostheses.

The first conclusion that emerged from the theoretical and experimental analyzes performed, it can be said that the information gap at the demarcation line between medical and engineering research has begun to be filled.

The obtained results and their interpretation allowed the conclusion of the contributions of the doctoral thesis, a series of them being original contributions. Theoretical research and experiments have been directed so as to make major contributions both fundamentally and practically, namely:

1. Contributions on the appropriateness of addressing the topic in the content of research conducted worldwide and nationally on antimicrobial acrylic cements used to fix hip stents.
2. Contributions regarding the establishment of the experimental program and the research methodology in accordance with the standardized methods, aligned with the requirements of the European Union and the unconventional methods.
3. Contribution to the processes that take place at the interface cement - implant - tissue.
4. Contributions regarding the establishment of the theoretical and applied possibilities of realization and modification of the characteristics of polymeric biomaterials.
5. Contributions regarding the production of antimicrobial acrylic cements.

Conclusions and general assessments

From the analysis of the present doctoral thesis, the following conclusions and general assessments result:

1. Total hip arthroplasty (THA) restores the functionality and mobility of patients suffering from degenerative hip joint disease. The total hip stent aims to take over the biomechanical function of the joint when it is severely affected. Fixation of the femoral component and the acetabular component to a hip stent can be achieved by using bone cement (cemented prosthesis) or by pressing on the bone (uncemented prosthesis).

2. Although in 1870 Themistokles Gluck fixed a total ivory knee prosthesis using plaster cement and rosin, the first use of bone cement in orthopedics is widely attributed to the famous English surgeon John Charnley, who in 1958 used it for total hip arthroplasty of a metal femoral prosthesis. This has been an important milestone in the progress of orthopedic surgical procedures. Charnley was also the first to realize that PMMA can be easily used to fill the medullary canal and is easy to model on bone morphology.

3. Bone cement helps to fill small bone defects or to fix bone components on orthopedic or dental prostheses. PMMA cement is one of the most resistant materials used in orthopedics, having an extremely important role in endoprosthetic activity, as well as in other reconstructive interventions. PMMA, together with various additives, gives the mixture a set of physical and chemical properties. Currently, the most commonly used bone cements can be classified into two categories: acrylic bone cements and calcium phosphate bone cements.

4. Acrylic bone cement is currently the most widely used biomaterial for fixing stents used in arthroplasty and has a high performance due to its range of properties. PMMA-based bone cements are two-component systems, comprising a solid phase (a polymer powder) and a liquid phase (a liquid monomer).

5. When a synthetic biomaterial is implanted in the body without any immune function, there is always the risk of infection and bone cement is not excluded. An implanted stent is particularly sensitive to the growth of bacteria on its surface, as microorganisms can multiply as an adverse reaction of the body's immune system to a foreign body, by primary contamination due to non-sterile materials or by hematogenous spread, where the infection comes from a distant place and is transferred to the implant area. As the bacteria reproduce, they form a protective biofilm, which has a low sensitivity to antibiotics.

6. The use of antibiotic bone cements reduces the incidence of infection especially if used in combination with oral antibiotic administration. In contrast, the clinical efficacy of antibiotic-releasing bone cements is not universally accepted due to the fact that long-term exposure to low doses may be a cause for the development of antibiotic resistance. Many bacteriological, physical and chemical factors should be considered when selecting an antibiotic, such as antibacterial spectrum, good bactericidal effect at low concentrations, low allergenicity, insignificant effect on cement performance, chemical and thermal stability, water solubility due to small molecular size and good release rate of antibiotics from bone cement.

7. The widespread use of antibiotics has led to the development of genetic and biochemical mechanisms that allow bacteria to survive in antibiotic environments. This is a cause for concern regarding the efficacy of antibiotics commonly used in the composition of bone cements, especially the efficacy of gentamicin. Thus, the problem of the reduced effectiveness of antibiotics in the composition of bone cements has created the need to investigate new antimicrobial agents to be introduced into bone cement. Silver nanoparticles provide unique chemical and physical properties that greatly enhance the antimicrobial effects of silver. Silver ions inactivate enzymes vital to bacteria and deactivate the mechanism of bacterial DNA replication. Clinical application is limited by the difficulty of incorporating and dispersing silver nanoparticles into acrylic bone cements. However, studies are contradictory regarding the influence of the addition of silver nanoparticles in acrylic-type bone cements. It is certain, however, that as the concentration of silver nanoparticles increases, the mechanical strength of bone cements decreases. In order to establish the optimal

concentrations to correspond to an adequate long-term antimicrobial activity, but also to maintain the mechanical properties necessary to perform the clinical function, additional studies are needed.

8. There are several factors related to the mixing technique, the presence of blood, body fats or fluids or possible delamination produced when the cement is introduced into the bone cavity, which will affect the volume and inter-facial microstructure of the cements and, consequently, their behavior. mechanic. The presence of porosity in bone cements strongly depends on the mixing technique used. Techniques used to reduce porosity include mechanical or ultrasonic mixing, cement pressurization, mixture centrifugation and vacuum mixing.

9. The main additives commonly found in bone cements are radiopaque elements, such as BaSO4 and ZrO2 and antibiotics. These radiopaque agents have been shown to have a significant effect on the mechanical properties of acrylic-type bone cements, which also influence the morphology of the cement. The addition of antibiotics reduces their mechanical properties, although this influence depends very strongly on the amount of antibiotic added. Other additives have been investigated in order to improve the mechanical properties of the analyzed cements, by strengthening the cement matrix with hard particles, such as ceramic glass particles, either with carbon fibers or nanotubes, or by adding inorganic bone or matrix. demineralized bone (hydroxyapatite or other calcium phosphates), which could improve both the mechanical properties of cement and the bone-cement interaction, creating a direct link between these bioactive particles and the surrounding bone.

7.2. Own contributions and possibilities to capitalize on research

The experimental research carried out brought a series of original contributions obtained through their theoretical interpretation. The original contributions and the most important results obtained will be presented:

1. A complex synthesis of scientific documentation was carried out, which led to many new results and interpretations, many of which were innovative and original.
2. For the first time in our country, correlations were made between intra-operative conditions, the type of hip stent, the characteristics of the bone cement used and its handling for fixing prostheses.
3. In order to obtain acrylic cements with antimicrobial additives usable for fixing the femoral components of the total hip stents, the elaboration flow was established.
4. A case study and experimental research related to the selection of an essential oil with optimal antimicrobial properties, usable in the development of new antimicrobial bone cements, was performed.
5. The flow for obtaining bone cement was designed, verified through our own experimental research.
6. The results obtained from the experimental research carried out, corroborated with the studies carried out on the basis of information from the literature, allowed the formulation of some original aspects regarding the concepts of biocompatibility and biofunctionality.
7. Starting from the statistical data provided by the main producers of cements for hip prostheses and their computerized processing, an original database was created which was used to develop the model of its selection depending on the applicability.
8. A statistical analysis of the clinical results on the use of BHR prostheses in an orthopedic clinic was performed to identify their causes of failure.
9. In vitro comparative analysis of commercial cements used in the previous study (Aminofix 1, Aminofix 3, Simplex P) was performed following the chronology of surgery, to assess the factors that influenced the chemical composition and structure of each bone cement sample under specific intraoperative conditions.

10. The physico-chemical characterization of three essential oils and the antimicrobial effect of three essential oils were performed with the clearly defined purpose of demonstrating possible antibacterial properties, which could prove usable in the development of new antibacterial bone cements.
11. Characterization was performed with the clearly defined purpose of demonstrating possible antibacterial properties, which could prove to be usable in the development of new antibacterial bone cements.
12. New bone cements with antimicrobial properties were obtained by modifying the inorganic component of a commercial viscosity cement based on PMMA, with standard viscosity, using as antimicrobial agents, silver nanoparticles embedded in a ceramic glass (2% and 4%) , respectively hydroxyapatite impregnated with peppermint oil and gentamicin, being obtained 3 compositions of new bone cements:

- bone cement with hydroxyapatite impregnated with 10% peppermint essential oil and the HUM experimental test resulted,
- bone cement with 2% silver nanoparticles embedded in a ceramic glass and the experimental sample AM1
- bone cement with 4% silver nanoparticles embedded in a ceramic glass and the AM2 experimental sample resulted

13. Morphological and structural characterization and evaluation of the properties of new bone cements with antimicrobial properties were performed.
14. The characterization of the evaluation of the antimicrobial and biocompatibility activities of the experimental samples of newly obtained bone cements was performed.
15. The experimental samples were analyzed by SEM coupled with EDS and FT-IR spectroscopy, for the morphological and structural characterization of the studied bone cements. Also, the wettability properties of the surface were evaluated by determining the contact angle and the study of the degradation of the samples in simulated physiological environment (SBF) was performed. The SEM analysis revealed that, after hardening, all the analyzed bone cements showed the typical microstructure of PMMA-based cements and a good dispersion of barium sulphate and antimicrobial additives in the PMMA-PBMA matrix is observed, and the EDS analysis supports this observation and highlights the presence of the majority elements, C and O, from the two polymer phases, Ba and S respectively from the composition of the radiopacifying element and Ag, Mg, P, and Ca from HAp and silver nanoparticles incorporated in a ceramic glass matrix.
16. Experimental determinations of Raman spectroscopy and Experimental determinations of energy dispersion X-ray diffraction (EDXRD) confirmed that the new bone cements do not have significant structural changes.
17. The evaluation of the properties of the experimental bone cement samples showed that the addition of antimicrobial agents did not induce structural changes in the newly developed PMMA bone cement samples. All experimental specimens had a structure and morphology typical of PMMA bone cements, with a slight tendency to form agglomerates, especially in PMMA bone cement samples containing 4% silver nanoparticles.
18. Moisture measured by the contact angle was decreased by the addition of antimicrobial agents, except for gentamicin-modified PMMA bone cements. All additives used kept the contact angle values within the desired limits and showed good cell adhesion.
19. Exposure of the experimental samples of PMMA bone cement to water and SBF solution did not significantly affect their structure. The addition of antimicrobial agents did not significantly affect the degree of hydration and degradation of PMMA bone cements.
20. Antimicrobial properties have been demonstrated for GM, HUM and AM2 samples, which generated clear areas of inhibition against Staphylococcus aureus ATCC 25923 and Pseudomonas

aeruginosa ATCC 27853. It was observed that not only the type of antimicrobial agent is important, but also the amount used. .

21. Staphylococcus aureus and Pseudomonas aeruginosa are known to be clinically relevant pathogens associated with bone infection and intrinsic drug resistance, respectively.

22. Regarding the biocompatibility assessed by the MTT test using human MG-63 cells, all cements are biocompatible without any effect of cytotoxicity.

23. The performance of bone cements with peppermint essential oil and silver nanoparticles against these two pathogens suggests that these antimicrobial additives are promising for use in clinical practice for fixing hip prostheses, protecting against bacterial infections.

It is mentioned that the experimental part was performed mainly within the Faculty of Materials Science and Engineering, Department of Materials Science and Physical Metallurgy, but experimental determinations were performed in other laboratories as well. Among them are the experimental research carried out in the laboratories of the University of Bucharest, Faculty of Biology, Department of Microbiology.

Without claiming to exhaust the theoretical and experimental research in the analyzed field, the paper makes a modest theoretical and practical contribution, opening at the same time new perspectives for future research.

7.3. Future directions of research

Future research will aim to conduct studies to determine the mechanical strength of the investigated bone cements; the study of the chemicals released following the degradation of the investigated materials, in order to establish the effect it may have on the human body and the study of biological properties by performing in vitro and in vivo biocompatibility tests on animals.

Also, future research will focus on obtaining new bone cements with antimicrobial properties by adding other additives or by modifying the amounts to improve the properties:

- bone cement with hydroxyapatite impregnated with oregano essential oil (this is widely recognized for their antimicrobial activity, but also for its antiviral and antifungal properties, but also for its antioxidant, anti-inflammatory, antidiabetic properties) [14–23].
- bone cement with hydroxyapatite impregnated with 5, 15, 20 or 25% peppermint essential oil
- bone cement with modification of the amount of silver nanoparticles embedded in a ceramic glass in quantities different from those presented in this doctoral thesis, respectively with 1% and 2% Ag

7.4. Dissemination of the results obtained

Articles:

1. First author article - *Additives Imparting Antimicrobial Properties to Acrylic Bone Cements* / **MATERIALS (Q1) - https://www.mdpi.com/1996-1944/14/22/7031#cite**

Robu, A.; Antoniac, A.; Grosu, E.; Vasile, E.; Raiciu, A.D.; Iordache, F.; Antoniac, V.I.; Rau, J.V.; Yankova, V.G.; Ditu, L.M.; Saceleanu, V. Additives Imparting Antimicrobial Properties to Acrylic Bone Cements. *Materials* **2021**, *14*, 7031. https://doi.org/10.3390/ma14227031

2. First author article – *Bone Cements Used for Hip Prosthesis Fixation: The Influence of the Handling Procedures on Functional Properties Observed during In Vitro Study* / **Materials (Q1) - https://www.mdpi.com/1996-1944/15/9/2967#cite**

Robu, A.; Ciocoiu, R.; Antoniac, A.; Antoniac, I.; Raiciu, A.D.; Dura, H.; Forna, N.; Cristea, M.B.; Carstoc, I.D. Bone Cements Used for Hip Prosthesis Fixation: The Influence of the Handling Procedures on Functional Properties Observed during *In Vitro* Study. *Materials* **2022**, *15*, 2967. **https://doi.org/10.3390/ma15092967**

3. Article - *Failure analyses of a non-cemented hip prostheses faild duet o the stem fracture* / **revista University Politehnica of Bucharest Scientific Bulletin series B – Chemistry and materials science**
Milea, G.C.; Necsulescu, D.A.; Ghiban, B; Stere, A; **Robu, A**; Bujor, C; Ene, R; Forna, Failure analyses of a non-cemented hip prostheses faild duet o the stem fracture, *revista UPB Scientific Bulletin series B – Chemistry and materials science*, **2022**, 84, 2, 175-186. **https://www.scientificbulletin.upb.ro/rev_docs_arhiva/rez8c4_562063.pdf**

4. Review - *Magnesium-Based Alloys Used in Orthopedic Surgery* / **MATERIALS (Q1) - https://pubmed.ncbi.nlm.nih.gov/35161092/#:~:text=Magnesium%20(Mg)%2Dbased%20alloys,nondegradable%20metals%20implants%20in%20orthopedics**
Antoniac, I; Miculescu, M; Manescu, V; Stere, A; Quan, PH; Paltanea, G; **Robu, A;** Earar, K. Magnesium-Based Alloys Used in Orthopedic Surgery, *Materials,* **2022***, 15, 1148,* **https://doi.org/10.3390/ma15031148**

5. **Article** - *Fluoride Treatment and In Vitro Corrosion Behavior of Mg-Nd-Y-Zn-Zr Alloys Type* / **MATERIALS (Q1) – https://www.mdpi.com/1996-1944/15/2/566**
Quan, PH; Antoniac, I; Miculescu, F; Antoniac, A; Manescu, V; **Robu, A**; Bita, AI; Miculescu, M; Saceleanu, A; Bodog, AD; Saceleanu, V, Fluoride Treatment and In Vitro Corrosion Behavior of Mg-Nd-Y-Zn-Zr Alloys Type, 2022, 15, 2, 566, **10.3390/ma15020566**

International conference participation:
1. Aurora ANTONIAC*, Iulian ANTONIAC, Elena GROSU, Iuliana Mihaela DELEANU, **Alina ROBU***, Anca Daniela RAICIU, *Swelling properties and water vapor permeability of some compositions based on biopolymers and essential oils*, International Conference on Innovative Research, ICIR EUROINVENT, IASI, May, 26-27, 2022

2. Lavinia DRAGOMIR, **Alina ROBU**, Aurora ANTONIAC*, Iulian ANTONIAC, Alina VLADESCU, Horatiu DURA, Norin FORNA, Mihai Bogdan CRISTEA and Ioana CARSTOC, *Biodegradation Evaluation of some Magnesium-based Alloys type Mg-Zn-Zr-Ag before and after Synthetic Hydroxyapatite Coating using RF-Magnetron Sputtering,* International Conference on Innovative Research, ICIR EUROINVENT, IASI, May, 26-27, 2022

Book chapters:

1. Book chapter: Printech Publishing House, Bucharest, ISBN 978-606-23-1306-7, Materials used in medicine - interdisciplinary aspects, editor Antoniac Iulian; Chapter: bone cements used in orthopedics, Author: Alina Robu, pages 83-123
2. Book chapter: Printech Publishing House, Bucharest, ISBN 978-606-23-1306-7, Materials used in medicine - interdisciplinary aspects, editor Antoniac Iulian; Chapter Perspectives in transdermal therapeutic systems Authors: Grosu Elena, Antoniac Aurora, Antoniac Iulian, Raiciu Anca Daniela, Robu Alina, pages 1-39

The present summary summarizes in a concise form the contents of Chapters 1-7. The numbering of chapters, subchapters, figures and tables corresponds to that of the thesis. The significant bibliographic references used in the thesis summary are presented.

List of figures from the entire doctoral thesis

List of tables from the entire doctoral thesis

List of abbreviations from the entire doctoral thesis

AFS - Sterile physiological water
BHR - Birmingham Hip Resurfacing
BMA - Butyl methacrylate
BPO - Benzoyl peroxide
CDHA - Non-stoichiometric or calcium-deficient hydroxyapatite
CMEB - Minimum eradication concentration of biofilms
CMI - Minimum inhibitory concentration
CPC - Calcium phosphate cements
DCP - Coinage
DCPD - Bruşită
DmpT - Dimethyl-β-toluidine
EDS - X-ray spectroscopy with energy dispersion
EDXRD - X-ray diffraction with energy dispersion
EtO - Ethylene oxide
FT-IR - Fourier transform infrared spectroscopy)
HQ - Hydroquinone
HV - High viscosity cement
LPPO II - Second generation lipophosphonoxins
LV - Cement with low viscosity
MC - Mineralized collagen
MCP - Anhydrous monocalcium phosphate
MCPM - Monocalcium phosphate monohydrate
MMA - Methyl methacrylate
MRSA - Methicillin-resistant Staphylococcus aureus
MRSE - Methicillin-resistant Staphylococcus.epidermidis
MTT - Metabolism of tetrazolium salts (SERVA Electrophoresis GmbH)
PBMA -Butyl polymethacrylate
PBS - Phosphate buffered saline
PC - Phosphatidylcholine
PHA - Precipitated hydroxyapatite
PI - Propidium iodide
PMMA- Polymethyl methacrylate
SBF - simulated physiological fluid
SEM - Scanning electron microscopy
THA - Total hip arthroplasty

THR - Total replacement of the hip joint
TTCP - Tetracalcium phosphate
UHMWPE - Ultra high molecular weight polyethylene
ZTA - Hardened zirconia with alumina

Selective Bibliography

1. Lewis, G. *Properties of Acrylic Bone Cement: State of the Art Review*; John Wiley & Sons, 1997; Vol. 38;.
2. Pina, S.; Oliveira, J.M.; Reis, R.L. Natural-Based Nanocomposites for Bone Tissue Engineering and Regenerative Medicine: A Review. *Advanced Materials* 2015, *27*, 1143–1169.
3. Fukuda, N.; Tsuru, K.; Mori, Y.; Ishikawa, K. Fabrication of Self-Setting β-Tricalcium Phosphate Granular Cement. *Journal of Biomedical Materials Research - Part B Applied Biomaterials* **2018**, *106*, 800–807, doi:10.1002/jbm.b.33891.
4. Xu, S.; Liu, J.; Zhang, L.; Yang, F.; Tang, P.; Wu, D. Effects of HAp and TCP in Constructing Tissue Engineering Scaffolds for Bone Repair. *Journal of Materials Chemistry B* **2017**, *5*, 6110–6118, doi:10.1039/c7tb00790f.
5. Johnston, R.C.; Fitzgerald, R.H.; Harris, W.H.; Poss, R.; Müller, M.E.; Sledge, C.B. Clinical and Radiographic Evaluation of Total Hip Replacement. A Standard System of Terminology for Reporting Results. *J Bone Joint Surg Am* **1990**, *72*, 161–168.
6. Wang, H.V.; Johnston, S.R.; Fellow, P.; Rosen, D.W. *DESIGN OF A GRADED CELLULAR STRUCTURE FOR AN ACETABULAR HIP REPLACEMENT COMPONENT*;
7. Ma, Y.; Cao, X.; Feng, X.; Ma, Y.; Zou, H. Fabrication of Super-Hydrophobic Film from PMMA with Intrinsic Water Contact Angle below 90°. *Polymer (Guildf)* **2007**, *48*, 7455–7460, doi:10.1016/j.polymer.2007.10.038.
8. Brugnara, M.; Volpe, C. della; Siboni, S.; Zeni, D. Contact Angle Analysis on Polymethylmethacrylate and Commercial Wax by Using an Environmental Scanning Electron Microscope. *Scanning* **2007**, *28*, 267–273, doi:10.1002/sca.4950280504.
9. Josse, J.; Valour, F.; Maali, Y.; Diot, A.; Batailler, C.; Ferry, T.; Laurent, F. Interaction Between Staphylococcal Biofilm and Bone: How Does the Presence of Biofilm Promote Prosthesis Loosening? *Frontiers in Microbiology* **2019**, *10*, doi:10.3389/fmicb.2019.01602.
10. Cotar, A.I.; Grumezescu, A.M.; Andronescu, E.; Voicu, G.; Ficai, A.; Ou, K.L.; Huang, K.S.; Chifiriuc, M.C. Nanotechnological Solution for Improving the Antibiotic Efficiency against Biofilms Developed by Gram-Negative Bacterial Strains. *Letters in Applied NanoBioScience* **2013**, *2*, 97–104.
11. Tan, H.; Peng, Z.; Li, Q.; Xu, X.; Guo, S.; Tang, T. The Use of Quaternised Chitosan-Loaded PMMA to Inhibit Biofilm Formation and Downregulate the Virulence-Associated Gene Expression of Antibiotic-Resistant Staphylococcus. *Biomaterials* **2012**, *33*, 365–377, doi:10.1016/j.biomaterials.2011.09.084.
12. Cavalu, S.; Antoniac, I.V.; Fritea, L.; Mates, I.M.; Milea, C.; Laslo, V.; Vicas, S.; Mohan, A. Surface Modifications of the Titanium Mesh for Cranioplasty Using Selenium Nanoparticles Coating. *Journal of Adhesion Science and Technology* **2018**, *32*, 2509–2522, doi:10.1080/01694243.2018.1490067.
13. Cavalu, S.; Ratiu, C.; Ponta, O.; Simon, V.; Rugina, D.; Miclaus, V.; Akin, I.; Goller, G. *IMPROVING OSSEOINTEGRATION OF ALUMINA/ZIRCONIA CERAMIC IMPLANTS BY FLUORIDE SURFACE TREATMENT*; Vol. 9;.

14. Leyva-López, N.; Gutiérrez-Grijalva, E.; Vazquez-Olivo, G.; Heredia, J. Essential Oils of Oregano: Biological Activity beyond Their Antimicrobial Properties. *Molecules* **2017**, *22*, 989, doi:10.3390/molecules22060989.
15. Brdjanin, S.; Bogdanovic, N.; Kolundzic, M.; Milenkovic, M.; Golic, N.; Kojic, M.; Kundakovic, T. Antimicrobial Activity of Oregano (Origanum Vulgare L.): And Basil (Ocimum Basilicum L.): Extracts. *Advanced technologies* **2015**, *4*, 5–10, doi:10.5937/savteh1502005B.
16. Karadayı, M.; Yıldırım, V.; Güllüce, M. *Antimicrobial Activity and Other Biological Properties of Oregano Essential Oil and Carvacrol*; 2020;
17. Evangelopoulo, G.; Solomakos, N.; Ioannidis, A.; Pexara, A.; R Burriel, A. A Comparative Study of the Antimicrobial Activity of Oregano, Rosemary and Thyme Essential Oils against Salmonella Spp. *Biomedical Research and Clinical Practice* **2019**, *4*, doi:10.15761/BRCP.1000197.
18. Leonelli Pires de Campos, A.C.; Saldanha Nandi, R.D.; Scandorieiro, S.; Gonçalves, M.C.; Reis, G.F.; Dibo, M.; Medeiros, L.P.; Panagio, L.A.; Fagan, E.P.; Takayama Kobayashi, R.K.; et al. Antimicrobial Effect of Origanum Vulgare (L.) Essential Oil as an Alternative for Conventional Additives in the Minas Cheese Manufacture. *LWT* **2022**, *157*, 113063, doi:10.1016/j.lwt.2021.113063.
19. Brđanin, S.; Bogdanović, N.; Kolundžić, M.; Milenković, M.; Golić, N.; Kojić, M.; Kundaković, T. *Advanced Technologies 4(2) ANTIMICROBIAL ACTIVITY OF OREGANO (Origanum Vulgare L.) AND BASIL (Ocimum Basilicum L.) EXTRACTS*; 2015;
20. Simirgiotis, M.J.; Burton, D.; Parra, F.; López, J.; Muñoz, P.; Escobar, H.; Parra, C. Antioxidant and Antibacterial Capacities of Origanum Vulgare l. Essential Oil from the Arid Andean Region of Chile and Its Chemical Characterization by Gc-Ms. *Metabolites* **2020**, *10*, 1–12, doi:10.3390/metabo10100414.
21. Fournomiti, M.; Kimbaris, A.; Mantzourani, I.; Plessas, S.; Theodoridou, I.; Papaemmanouil, V.; Kapsiotis, I.; Panopoulou, M.; Stavropoulou, E.; Bezirtzoglou, E.E.; et al. Antimicrobial Activity of Essential Oils of Cultivated Oregano (*Origanum Vulgare*), Sage (*Salvia Officinalis*), and Thyme (*Thymus Vulgaris*) against Clinical Isolates of *Escherichia Coli* , *Klebsiella Oxytoca* , and *Klebsiella Pneumoniae*. *Microbial Ecology in Health & Disease* **2015**, *26*, doi:10.3402/mehd.v26.23289.
22. Lu, M.; Dai, T.; Murray, C.K.; Wu, M.X. Bactericidal Property of Oregano Oil Against Multidrug-Resistant Clinical Isolates. *Frontiers in Microbiology* **2018**, *9*, doi:10.3389/fmicb.2018.02329.
23. Coccimiglio, J.; Alipour, M.; Jiang, Z.-H.; Gottardo, C.; Suntres, Z. Antioxidant, Antibacterial, and Cytotoxic Activities of the Ethanolic *Origanum Vulgare* Extract and Its Major Constituents. *Oxidative Medicine and Cellular Longevity* **2016**, *2016*, 1–8, doi:10.1155/2016/1404505.