UNIVERSITATEA DE MEDICINĂ ŞI FARMACIE „CAROL DAVILA”, BUCUREŞTI

ŞCOALA DOCTORALĂ

DOMENIUL MEDICINĂ

The particularities of nanotechnological applications on voice prostheses in view of improving vocal rehabilitation and quality of life in laryngectomized patients

REZUMATUL TEZEI DE DOCTORAT

Conducător de doctorat:

PROF. UNIV. DR. ANGHEL ION

Student-doctorand:

CACIANDONE (CĂS. CACIANDONE-CRÎNGUREANU)

MARA

ANUL

2024

Content

Introduction

Voice rehabilitation of laryngectomized patients has long been a challenge for bothe patients and medical professionals worldwide. Although there are various methods of recovery of phonatory function have been developed, including the use of the esophageal or the laryngophone, the current standard is represented by the use of voice prostheses. To insert the voice prostheses it is necessary to create surgically a communication between the trachea and the esophagus, either simultaneously with the laryngectomy intervention or after a period of time. Being used for over 40 years, voice prostheses have been constantly improved both from the point of view of configuration and the materials of which they are composed.

Despite these advances, there are a number of disadvantages associated with the use of voice prostheses. The main limitation is represented by the degradation over time of the silicone or polyurethane of which the prostheses are composed, which thus become dysfunctional and require more frequent replacement.

The most common cause for the replacement of the voice prostheses is trans-prosthetic leakage, generated by the development of bacterial, fungal or mixed biofilm, especially on the esophageal flange of the prosthesis, which impairs the proper functioning of the prosthesis.

The structure of the voice prostheses is based on elastic polymers (usually silicone) which predisposes the rapid colonization of microorganisms on the surface of the device and their further development under the shelter of self-generated extracellular polymeric matrix. This behavior provides them with protection against both host defense mechanisms and antimicrobial therapy.

The scientific objectives of this thesis are represented by the analysis of the replacement frequency of voice prostheses, the study of the microbial and polymicrobial profile of the biofilm that colonizes the replaced prostheses, as well as the identification and proposal of methods to fight against this phenomenon based on the use of nanotechnology. This research also aimed to evaluate if the frequency of replacement of voice prostheses is dependent on factors related to the patient, such as his age or existing comorbidities, or on characteristics of the prosthesis (such its type or diameter) or even on micro-environmental factors (such as exposure of voice prosthesis to a biologically active environment that favors biofilm development).

Specifically, the hypothesis of the influence of biofilm composition on the frequency of prostheses replacement was tested. Regarding the impact on the patient' quality of life, the

emotional and functional dynamics of the patients were evaluated, by means of a 10-item questionnaire and by comparing their responses over time.

For a comprehensive study, it was analyzed using the scanning electron microscopy technique the morphology of the biofilm colonizing the surface of a dysfunctional voice prosthesis. In the case study carried out, the topographical characteristics of biofilm were highlighted by assessing its accumulation on the four main areas of the prosthesis, namely the esophageal flange, the body of the prosthesis, its lumen and the tracheal flange.

In the general part of the work, there are presented general consideration regarding: laryngeal neoplastic pathology and total laryngectomy intervention, the history of phonatory rehabilitation methods, with an emphasis on the evolution of voice prostheses in this framework, the structure of biofilms, methods of evaluating and combating them, and distinctive aspects of advances in the field of nanotechnology on fighting the colonization of biofilms on the surface of medical devices in general and, in particular, on voice prostheses.

In order to achieve the proposed objectives for this study, we analyzed a group of 12 patients that underwent total laryngectomy and opted for the use of a voice prostheses for the phonatory rehabilitation. The observational study brings together parameters related to the moment of insertion of the voice prostheses, the frequency of replacement of the prostheses, as well as the assessment of the microbiological composition of the secretions collected from the surface of the voice prostheses and the implantation site (tracheoesophageal fistula).

The personal contribution part of the thesis also includes the study of different strategies to inhibit the development of biofilm on voice prostheses based on nanotechnology applications. Although the issue raised explores in depth a challenge associated with the use of voice prostheses and may seem a niche one, the literature provides an appreciable amount of information on the subject. The methods of preventing the development of biofilm rely on three levels of approach: the prophylactic treatment of the silicone composing the voice prostheses (for example by probiotics administration), the thorough understanding of the adaptative behavior of biofilms to the laryngopharyngeal environmental conditions and the modification of the surfaces from a physicochemical point of view, by applying certain compounds such as coating with different metals or nanomaterials.

The surface modification strategy was a point of interest of the present work and, for this purpose, we studied, identified and evaluated the effectiveness of the anti-biofilm proprieties of some specific nanostructures.

The first study presents, in Chapter 6, the manufacture of coatings based on nanostructures of magnetite, polyethylene glycol and a biologically active molecule (polymyxin B). By applying this nanostructure on the surface of voice prostheses, it was obtained the inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Magnetite nanoparticles were synthesized and functionalized by co-precipitation technique, followed by their deposition as thin coatings using the MAPLE technique. The obtained nanostructures and coatings were characterized by techniques such as X-ray diffraction (XRD), thermogravimetric analysis and differential scanning calorimetry (TGA-DSC), transmission electron microscopy with selected area diffraction (TEM-SAED), infrared spectroscopy in Fourier transform (FT-IR), infrared microscopy (IRM), scanning electron microscopy (SEM), and also by biological testing, consisting of cell viability tests and anti-biofilm tests. Modification of the voice prosthesis surface by applying the Fe_3O_4@PEG/PM nanocomposite led to a reduction in the number of CFU up to four orders of magnitude in the case of *S. aureus* strains, respectively up to five times in the case of *P. aeruginosa* strains.

The second study, presented in chapter 7, explored the functionalization of a magnetic nanostructure with streptomycin, respectively neomycin and their incorporation into the polymer-based spherical structure. Cell viability and antimicrobial tests emphasized the biocompatibility of these compounds with diploid human cells and highlighted their antimicrobial effect on Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus*) opportunistic bacteria.

Being located a crosspoint between otorhinolaryngology and microbiology, the topic is of interest both for clinical practice and for scientific research, being the foundation for additional research, with extension to fields related to medicine, such as medical bioengineering.

5. Evaluation of the particularities of a group of laryngectomized patients with voice prostheses

The purpose of the retrospective descriptive observational study brought together the identification of the main reason for the replacement of voice prostheses and the evaluation of the composition of the biofilm developed on the prosthesis implantation site, in the tracheoesophageal puncture site, as well as on the surface of the phonatory prosthesis. The focus point of this research was the microbiological assessment of the samples collected from the dysfunctional prostheses, after their removal, as well as from the tracheoesophageal puncture site. The samples collected from the latter location will be referred to in the continuation of the work as "wound secretion". The samples were tested to identify the pathogenic agents, bacterial or fungal that colonize the mentioned locations, and to provide the antibiogram, respectively the antifungigram, and the results were compared with those in the specialized literature.

The including criteria for selecting the patients in the study were as follows:

• patients hospitalized in the ENT Department of the Central Military Emergency University Hospital "Dr. Carol Davila", starting October 2018 to May 2023, with the diagnosis of pharyngo-laryngeal neoplasm operated on (total laryngectomy);

• lack of contraindications for surgery/anesthesia;

• the patients signed the informed consent according to the Declaration of Helsinki.

Following the application of these criteria, twelve patients were included that were diagnosed with advanced laryngeal neoplasm and underwent total laryngectomy. All patients in the selected group opted for the insertion of the voice prosthesis as a method of vocal rehabilitation post-total laryngectomy. Thus, a total of 54 interventions were carried out to insert the voice prosthesis or to replace the used prosthesis with a new phonatory device, the procedures being carried out in the period October 2018 – May 2023.

In five of these patients, the voice prostheses were inserted for the first time during the course of the study (41.66%). Correspondingly, in the case of the other seven patients, the interventions were represented by the removal of the used prostheses and their replacement with a new phonation device, i.e. reinsertion of the phonation device, carried out at variable time intervals, dependent either on subjective factors, such as the patient's compliance, or especially on objective factors such as the functional degradation of the phonatory device, as well as the occurrence of complications.

It should be noted that all voice prostheses were first inserted secondary to the laryngectomy intervention in the phonatory rehabilitation plan, at approximately one year post-intervention, mostly after completing the radiotherapy.

The studied parameters in the selected group of patients were:

- The therapeutic means used in patients and the evaluation of the impact on the quality of life by ensuring optimal functionality;

- Degree of phonatory rehabilitation;

- The development of local infections or on the prosthesis - fungal, bacterial or mixt;

- The design, synthesis and evaluation of the microbial anti-adhesive properties of the tested nanostructures;

- Rejection of the prosthetic device;

- Associated inflammatory and infectious pathology at the broncho-pulmonary level.

The mentioned parameters are completed by general data such as age, sex, living environment, histopathological form of neoplasm, main diagnosis at discharge and associated diagnoses (which increase the complexity of the case history), length of hospitalization and account/patient, as well as specific analyses.

The null hypothesis is that the voice prostheses exploitation time is not influenced by the composition of the biofilm that colonizes them, this being indirectly analyzed through the collected secretions. In a case study, the biofilm was visualized and characterized in detail using the scanning electron microscopy technique.

For this purpose, we separated the replacement intervals into four categories: under 4 months, 4-6 months, 7-11 months, over 12 months and examined the particularities of each group regarding the composition of the microorganism colonies on the prostheses and in the wound, such as and other parameters regarding age, comorbidities, type of phonatory prosthesis used and its diameter.

5.2. General presentation of study group

Among the patients, 91.67% were men (n=11), respectively 8.33% were women (n=1), the ratio between men and women being 11:1.

At the time of diagnosis with advanced laryngeal neoplasm, the patients were aged between 46 and 67 years, with a mean age of 59.25 years (standard deviation 6.25; CI: [55.28, 63.22]) and a median age of 61.50 years. At the time of the prosthetic fitting/replacement

intervention, the patients were aged between 46 and 73 years, with a mean age of 63.81 years (standard deviation 7.10; CI: [61.88, 65.75]) and a median age of 64 years.

5.3. Results

5.3.1. Duration of operation of phonatory prostheses

The replacement of the voice prosthesis was performed between 1 and 45 months from the date of its insertion, on average after 8.07 months (standard deviation 6.38; CI: [6.10, 10.04]), the median of the replacements being at 6 months.

In almost half of the cases the replacement was performed between 4 and 6 months, and in more than a third of the cases between 7 and 12 months. Thus, the device was replaced:

- In less than 4 months after installation: in 4.69% of cases (n=2),
- Between 4 – 6 months: in 48.84% of cases (n=21),
- Between 7 – 12 months: in 37.21% of cases (n=16),
- After more than 12 months: in 9.30% of cases (n=4).

Next, we evaluated whether there is a statistically significant association between the age of patients and the frequency of separate replacement in the four mentioned groups, the answer being that such an association cannot be established (p value=0.244).

5.3.2. The reasons for the replacement of voice prostheses

In the majority of analyzed cases, the cause that led to the replacement of the voice prosthesis was represented by trans-prosthetic leakage, due either to the accumulation of biofilm on the flanges of the phonatory prosthesis, or due to blockage of its lumen. Obstruction of the lumen of the prosthesis is the main factor that led to the deterioration of the function of the voice prosthesis in 85% of cases (46 of the replaced prostheses). In 4 cases, the cause was secondary to changes of the dimensions of the tracheoesophageal fistula (7.5% of the cases), while in 4 other cases the leaks were peri-prosthetic. No pulmonary or systemic infectious complications were recorded.

Microbiological analysis of secretions from the tracheoesophageal fistula (wound) and from phonatory prostheses

Following the microbiological analysis of the secretions from the wound, bacterial colonization was confirmed in 66.67% of the analyzed cases (n=24). The results confirmed the

colonization of the wound predominantly with a single species of bacteria (in 75% of cases, n=18), followed by the simultaneous presence of two bacterial species (in 20.83% of cases, n=5), and, less often, with three species of bacteria (in 4.17% of cases, n=1). No statistically significant association was noted between the interval of prosthesis replacement and the presence of bacteria (p=0.394).

Correspondingly, the microbiological analysis of the secretions from the phonatory prostheses confirmed the presence of bacteria in 52.78% of the analyzed cases (n=19). The secretions were predominantly populated with a single bacterial species (in 84.21% of cases, n=16), with two bacterial species at the same time (in 10.53% of cases, n=2), and more rarely with three bacterial species (in 5.26% of cases, n=1).

Furthermore, fungal colonization of the wound was discovered in 69.44% of the analyzed cases (n=25). In most cases, colonies of a single species were identified (in 76% of cases, n=19), but associations of two fungal species were also encountered nearly frequently (in 24% of cases, n=6). No statistically significant association was noted between patient age and the presence of fungi in wound secretions (p=0.559).

Accordingly, the assessment of the fungal colonization of the prostheses identified the presence of fungi in 77.78% of the analyzed cases (n=28). In most cases, the samples were populated with a single species of fungi (in 78.57% of cases, n=22), but associations of two species of fungi were also encountered frequently (in 21.43% of cases, n=6). No statistically significant association was noted between the replacement interval of the prosthesis and the presence of fungi (p=0.313).

On the collected samples from the voice prostheses, were identified seven bacterial species and five fungal species, these pathogens being found both in the form of monospecies colonies and grouped in polymicrobial associations. Correspondingly, ten bacterial species and also 5 fungal species were identified in the wound, which colonized the fistula tract either in the form of mono- or poly-species bacterial or fungal cultures, or by association in mixed polymicrobial colonization.

5.3.3. Analysis of samples collected from the tracheoesophageal puncture site

Depending on the type of culture identified when analyzing the samples collected from the wound, the following distribution was obtained within the 4 established intervals of replacement:

• Replacement after less than 4 months: the wounds showed bacterial cultures in 100% of cases (n=2),

• Replacement after 4-6 months: 31.58% of cases presented exclusively bacterial cultures (n=6), 26.32% exclusively fungal cultures (n=5), 36.84% mixed bacterial + fungal cultures (n=7), respectively 5.26% did not show microbial growth (n=1);

• Replacement after 7-12 months: 15.38% of cases presented bacterial cultures (n=2), 46.15% fungal cultures (n=6), respectively 38.46% mixed bacterial + fungal cultures (n=5);

• Replacement after more than 12 months: all cases (100%) showed mixed bacterial + fungal cultures (n=2).

Next, we investigated further the method of association of the species of microorganisms in the secretions that were collected from the wound, distributed according to the replacement interval of the prosthesis (as illustrated in Fig.5.1.):

• Replacement after less than 4 months: all secretions (100%) showed bacterial monoculture (n=2);

• Replacement after 4-6 months: 26.32% of cases presented bacterial monoculture (n=5), 21.05% fungal monoculture (n=4), 15.79% mixed cultures, composed of a bacterial and a fungal species (n=3), 15.79% of two bacterial and one fungal species (n=3), 5.26% cultures of one bacterial and two fungal species (n=1), 5.26% cultures of several bacteria (n=1), 5.26% cultures of more fungi (n=1), respectively 5.26% did not develop bacterial/fungal cultures (n=1);

• Replacement after 7-12 months: 7.69% of cases presented bacterial monoculture (n=1), 30.77% fungal monoculture (n=4), 23.08% mixed cultures, with one bacterial and one fungal species (n=3), 7.69% with two bacterial and one fungal species (n=1), 7.69% with one bacterial and two fungal species (n=1), 7.69% cultures of more than two bacterial species (n=1), respectively 15.38% cultures of more than two species of fungi (n=2);

• Replacement after more than 12 months: 50% of cases showed cultures consisting of one bacterial and one fungal species (n=1), respectively 50% cultures of one bacterial and two fungal species (n=1). No statistically significant association was noted between the outcome of wound secretions and the interval of prosthesis replacement. (p=0.635)

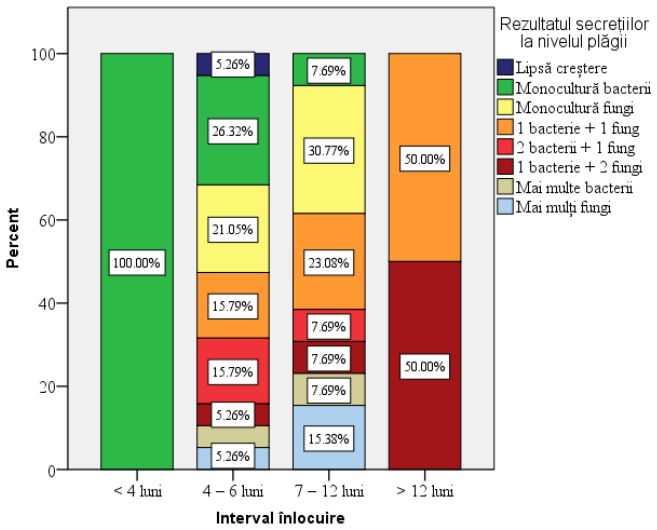

Fig. 5.1. Distribution of cases according to the result of secretions at the level of the wound and the interval of replacement of the prosthesis

Microbial and polymicrobial wound cultures

Focusing on the bacterial colonization of the wound, a third of the analyzed samples from a microbiological point of view confirmed the presence of *Pseudomonas aeruginosa* (in more than 3 out of 10 cases). Also, *Staphylococcus aureus* (in 1 out of 6 cases) was rather frequently encountered, whereas *Klebsiella pneumoniae* was encountered in about 1 in 10 cases.

Regarding fungal colonization of the wound, the presence of *Candida tropicalis* was confirmed in more than a quarter of the cases (in 1 out of 4 cases), but Candida krusei was also frequently encountered (in more than 1 out of 5 cases), *Candida albicans, Candida glabrata*, or other *non-albicans Candida* species (each of these in more than 1 in 10 cases).

In order to clarify the possibility of a significant association between the composition of secretions at the level of the wound and the frequency of replacement of the voice prosthesis, we examined at a greater degree the distribution of the identified microorganisms in the exploit intervals of the prostheses, but without obtaining statistically significant associations between the specific presence of bacterial strains, respectively fungi and the replacement interval of the prostheses.

5.3.4 Analysis of secretions collected from phonatory prostheses

Likewise, we evaluated the nature of the samples collected from the surface of the phonatory prostheses according to the pathogen, separately for the four named intervals of voice prostheses replacement, as well as the manner of association of the identified species divided into bacterial and fungal monocultures or different associations between bacterial and fungal species, related to the intervals of replacement.

Regarding the nature of the biofilm collected from the voice prostheses, we obtained the following results:

• Replacement after less than 4 months: the prostheses showed bacterial cultures in 50% of cases (n=2), respectively in 50% of cases fungal cultures (n=1)

• Replacement after 4-6 months: 26.32% of cases presented exclusively bacterial cultures (n=5), 36.84% exclusively fungal cultures (n=7), respectively 36.84% mixed bacterial + fungal cultures (n=7);

• Replacement after 7-12 months: 7.69% of cases presented bacterial cultures (n=1), 53.85% fungal cultures (n=7), respectively 38.46% mixed bacterial + fungal cultures (n=5);

• Replacement after more than 12 months: prostheses (n=2) showed an exclusively fungal culture, respectively a mixed, bacterial and fungal culture.

Depending on the pathogen identified in the microbial or polymicrobial cultures, the following associations were identified in the secretions from the voice prostheses The results are graphically presented in Fig 5.2.), relative to the replacement intervals:

• Replacement after less than 4 months: 50% of secretions showed bacterial monoculture (n=1), respectively 50% fungal monoculture (n=1);

• Replacement after 4-6 months: 21.05% of cases presented bacterial monoculture (n=4), 15.79% fungal monoculture (n=3), 31.58% mixed cultures with a bacterial species + a fungal species (n=6), 5.26% polymicrobial mixed cultures with two bacterial species + one fungal species (n=1), 5.26% cultures of several bacteria (n=1), respectively 21.05% cultures of several fungi (n=4);

• Replacement after 7-12 months: 7.69% of cases presented bacterial monoculture (n=1), 46.15% fungal monoculture (n=6), 23.08% mixed cultures consisting of a bacterial and a fungal species (n=3), 7.69% cultures of two bacterial species and one fungal species (n=1), 7.69% cultures of 1B+2F (n=1), respectively 7.69% cultures of several fungi (n=1);

• Replacement after more than 12 months: 50% of the cases presented a fungal monoculture (n=1), respectively 50% presented a mixed culture composed of a bacterial and a fungal species (n=1). No association was noted statistically significant between the result of secretions at the level of the prosthesis and the interval of its replacement. (p=0.130)

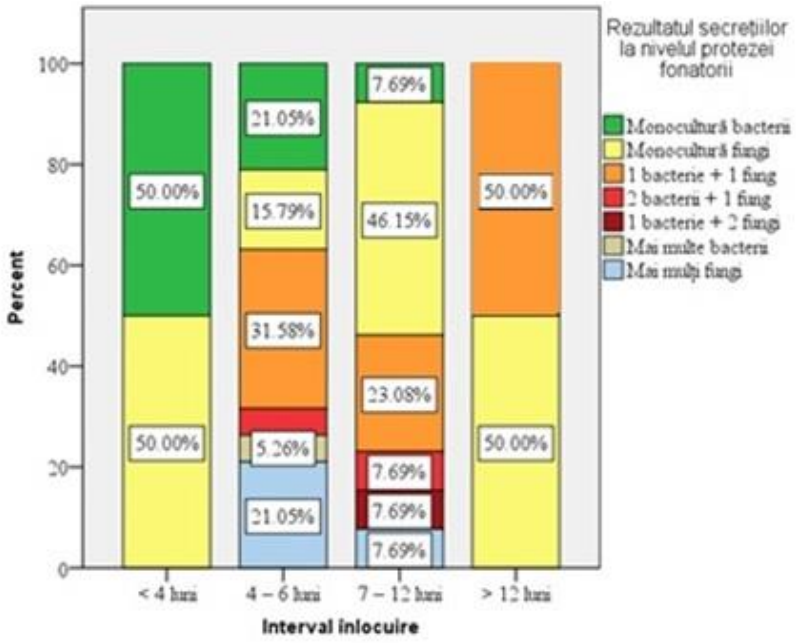

Fig. 5.2. Distribution of cases according to the result of secretions at the level of the prosthesis compared to the interval of its replacement

Microbial and polymicrobial cultures on phonatory prostheses

Regarding the bacterial composition of the secretions collected from the phonatory prostheses, the most frequently identified bacterial species were *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus* and *Klebsiella pneumoniae*. Regarding the fungal composition of the secretions from the wound, Candida Tropicalis was detected in almost a third of the cases, but *Candida krusei* (in more than 1 in 4 cases), *Candida albicans, Candida glabrata*, and other species were also encountered of Candida non-albicans (each of these in more than 1 in 10 cases).

Microbial and polymicrobial profiles of collected sample secretions

We investigated further the obtained results by assessing the way of organization of the most frequently identified microorganisms in the form of monocultures or in associations with

other strains (polymicrobial) or classes of microorganisms (mixed), both for the samples from the fistula, and especially for those from the phonatory prostheses.

A Chi-square test was applied to test the correlation between the nature of the biofilm on the prosthesis and its replacement interval. There is a statistically significant association between the nature of the biofilm and the frequency of prosthesis replacement, $\chi 2=21.773$, $p=0.010$, and the coefficients Phi (0.778) and Cramer's V (0.449) reveal that the connection between the two variables is one of strong intensity ($p= 0.010$).

This statistical test, together with the graphical representation, indicates that there is a strong trend of association between microbial colonization and shorter replacement intervals of phonatory prostheses. It is also noted that the nature of the biofilm is particular to each interval, with fungal predominance as the interval increases.

5.3.7. The association between the type of phonatory prosthesis and the duration of operation

Another aspect we evaluated is the association between the type of phonatory prosthesis and the duration of operation. A Chi-square test was applied to test the correlation between the type of prosthesis used and its replacement interval. There is a statistically significant association between the type of prosthesis and the frequency of prosthesis replacement, $\chi 2=17.775$, $p=0.007$, and the coefficients Phi (0.684) and Cramer's V (0.484) reveal that the connection between the two variables is one of strong intensity ($p =0.007$).

The average time of exploitation of Provox Vega prostheses was 6.08 months, with a median of replacements at 6 months, while, in the case of Blom Singer Classic prostheses, the lifespan of the device was 12.50 months, with a median of replacements at 9.50 months. For Provox 2 prostheses, the average duration of use was 10.40 months, with a median of replacements at 10 months.

5.3.8. Assessment of the impact on patients' quality of life

In order to assess the influence of the use of the voice prosthesis on the quality of life of laryngectomized patients, 9 of the 12 patients answered the items of the specific VR-QOL (Voice Related Quality of Life) questionnaire. The questionnaire included 10 questions regarding the difficulties faced by the patients, focusing on changes in the psycho-social sphere. Items 1, 2, 3, 6, 7 and 9 correspond to the physical-functional domain, while items 4, 5, 8 and 10 explore the

socio-emotional area. Responses were measured on a 5-point Likert scale, where 1 meant that the situation presented in the question did not apply to the patient at all, and 5 meant that it applied to him very much. The questionnaire was applied at the first and last intervention since they were enrolled in the study, moments hereafter referred to as T_0 and T_1.

A significant reduction of the score obtained between the two moments of the application of the questionnaire was noted, the impact on the patients' quality of life being much greater immediately after fitting the prosthesis than later on, when they adapted their lifestyle integrating the vocal rehabilitation by using voice prosthesis as their new status.

Applying the Wilcoxon signed-rank test indicated that the patients registered a statistically significant decrease in the score obtained at the end of the study (time T_1) compared to the score obtained at the beginning (time T_0) ($Z= -2.552$, $p=0.011$). The decrease in the score indicates that, as time went on, the patients no longer consider themselves as affected (by the difficulties presented in the questionnaire) as they were at the beginning. Also, the calculation of a Spearman correlation coefficient indicated that there is a strong tendency for this decrease in score to be greater as more time passes ($rs=0.714$, $p=0.031$).

6. *In vitro* Study 1- The use of polyethylene glycol-coated and functionalized magnetic nanoparticles to modulate microbial biofilms on speech prostheses

6.1. Introduction

In the context of the development of resistance of microorganisms to conventional antimicrobial agents, the need to create improved alternatives for combating treatment-recalcitrant biofilms is emerging. An interesting option is based on the exploitation of the advantages marked by the advances in nanotechnology and their use for the development of innovative bioactive surfaces with antibiofilm properties. Specifically, coating medical devices and implants with a layer composed of a nanostructure with antimicrobial properties represents an advantageous therapeutic option.

A substantial number of studies carried out with the starting point from these considerations have led to magnetic nanoparticles, in general, and to magnetite ones, in particular. These iron oxide magnetic nanoparticles have a number of favorable properties, including: availability, versatility, low cost, superparamagnetism, high saturation field, high electrical and thermal resistance, biocompatibility, are environmentally friendly and biodegradable, non-toxic to the human body, with intrinsic antimicrobial potential, and last but not least, the possibility of functionalization.

However, magnetite nanoparticles have the disadvantage of being unstable in air, showing a tendency to oxidize to maghemite after prolonged exposure. Furthermore, untreated magnetite nanoparticles cannot form stable fluids and are prone to agglomeration after production. To overcome this drawback, magnetite nanoparticles required in biomedical applications are generally protected by coatings composed of various biocompatible materials, such as natural polysaccharides, inert synthetic materials, and organic acids with varied structures.

With the aim of identifying compounds with combative properties on the bacterial biofilms on the phonatory prostheses, we carried out a study following which we present the obtaining of a material composed of magnetite, polyethylene glycol (PEG) and polymyxin B that has antimicrobial therapeutic applications. In particular, we report the synthesis and characterization of functionalized magnetite nanoparticles and the fabrication of thin magnetite-based coatings. These innovative nanostructured coatings were compositionally, morphologically and biologically characterized using various physicochemical techniques such as X-ray diffraction (XRD), thermogravimetric analysis and differential scanning calorimetry (TGA-DSC),

transmission electron microscopy with selected area diffraction (TEM-SAED), Fourier transform infrared spectroscopy (FT-IR), infrared microscopy (MRI), scanning electron microscopy (SEM), but also biological, by applying cell viability tests and tests anti-biofilm.

6.2. Material and methods

6.2.1. Preparation of functionalized nanoparticles and polymer-based coatings

Magnetite nanoparticles functionalized by coating with PEG were synthesized by a co-precipitation method, which involved the prior preparation of two solutions, one based on iron precursors and the second containing polyethylene glycol, ammonium hydroxide and deionized water. The synthesis process resulted in a quantity of 0.9 g of magnetite, which was then separated into two parts: one that was not interfered with, while the second was mechanically mixed with 0.05 g of polymyxin in one mL of chloroform.

The coatings formed at a laser fluence of 400 mJ/cm2 were deposited using the MAPLE technique on 1 x 1 cm glass samples, on a section through a phonatory prosthesis and doubly polished silicon (1 0 0) substrate. They were cleaned according to an internal laboratory procedure.

6.2.5. Evaluation of antibiofilm at 24 h and 48 h

The antibiofilm properties were tested by exposing the synthesized structures to bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

To test the effect of nanostructure-coated surfaces on biofilm development, samples were sterilized by exposing each side to UV radiation for 20 min. Next, each fragment of thus sterilized material was placed, individually, on a 6-well culture plate, followed by the addition of 2 mL of nutrient substrate and, consecutively, the inoculation of 50 μL of bacterial suspension corresponding to a McFarland density of 0.5 (approximately 1.5 x 108 (colony forming units)/mL). The plates prepared as described were left to incubate for 24 h at 37 °C. Afterwards, the materials were washed with phosphate buffered saline (TFS) and the culture medium was replaced with a fresh one to ensure biofilm development. The plates were incubated for 24 h and 48 h, respectively, after which the specimens were again washed with TFS solution and placed in test tubes containing 1 mL of TFS. The tubes were vigorously mixed for 30 seconds to detach the cells from the biofilm. The obtained cell suspensions were diluted and seeded on plates with solid culture medium to obtain and quantify the number of colony forming units/mL (CFU/mL).

6.2.6. Biocompatibility assessment

The biocompatibility property of the synthesized materials was studied by testing on murine osteoblasts. Samples were sterilized by UV exposure for 20 min on each side and placed in 6-well culture plates, followed by addition of cells at a density of 4 × 10 4 cells/cm^2. An Olympus phase-contrast inverted microscope was used to visualize the cells after 24 hours of incubation.

The MTT assay was applied to assess cell viability in the presence of the tested materials. Biocompatibility research is based on the principle of the reduction of the yellow salt (MTT) by metabolically active cells to the dark blue color specific for formazan crystals. The reduction coefficient is proportional to the number of viable cells, being, for this reason, an indicator for cell integrity. Spectrophotometry measurements of absorbance were made at a wavelength of 595 nm using a Thermo Scientific Appliskan plate reader.

The concentration of nitric oxide (NO) released by the cells in the culture medium was determined by the Griess method.

6.3. Results

Examining the anti-adherent properties of modified phonatory prostheses using nanoparticles against *Staphylococcus aureus* biofilm led to favorable results, as illustrated in Fig. 6.a). After an interval of 24 h, respectively 48 h, the control samples of cell cultures present a high degree of UFC/mL, namely $1.3x10^9$ CFU/mL, respectively $1.4x10^{11}$ CFU/mL. Comparatively, the modification using nanostructures of the surfaces of the phonatory prostheses led to a reduction in the number of CFU/mL by up to four orders of magnitude. Specifically, in the case of Fe_3O_4@PEG/PM coatings, a value of $1.4x10^5$ CFU/mL was recorded in the case of biofilms at 24 h of incubation and, correspondingly, of $1.1x10^7$ CFU/mL for biofilms after 48 h. These results support the anti -pronounced adherence to the development of *Staphylococcus aureus* biofilms.

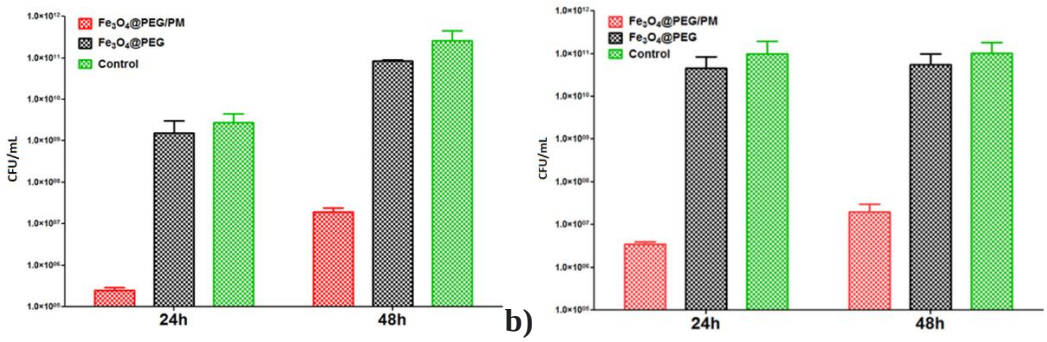

Fig 6. Assessment of the biofilm development of **a)** *S. aureus*, respectively **b)** *Pseudomonas aeruginosa* at 24 h and 48 h of incubation, respectively in the presence and absence of PEG-based and functionalized thin coatings.

Regarding the tests on *P. aeruginosa* biofilms, the control values of CFU/mL were similar at 24 h and 48 h, being around the value of approximately 10^{11} CFU/mL. These results reflect the increased affinity of P. aeruginosa strains for colonizing the surfaces of medical devices, which is why this opportunistic pathogen is associated with numerous nosocomial infections, which raises difficulties in terms of treatment. Moreover, compared to the analyzes carried out on *Staphylococcus aureus* strains, a lower inhibitory effect of both nanostructures is noted. Specifically, in the case of Fe_3O_4@PEG, the CFU/mL values were reduced by an order of magnitude after 24 h, quantifying $1.7x10^{10}$ CFU/mL, respectively $1.8x10^{10}$ CFU/mL after 48 h. On the other hand, regarding Fe_3O_4@PEG/PM coatings showed a more pronounced inhibitory effect, this nanostructure managed to reduce the number of P. aeruginosa CFU by up to five orders of magnitude, reaching the value of $1.5x10^{6}$ CFU/mL after 24 h of incubation of the biofilm and at $1.2x10^{7}$CFU/mL, at 48 h. The results are presented graphically in Fig. 6.b).

The sample containing Fe_3O_4@PEG showed no significant changes in the number of viable cells and the level of nitric oxide compared to the control samples, which confirms the biocompatibility of the compound. Similarly, the sample based on Fe_3O_4@PEG/PM showed satisfactory biocompatibility. It can be appreciated that the toxicity of polymyxin was reduced in the presence of the PEG polymer used to functionalize the magnetite nanoparticles.

Chapter 7. *In vitro* study - Magnetite nanoparticles functionalized with therapeutic agents (streptomycin/neomycin) to enhance antimicrobial properties on speech prostheses

7.1. Introduction

Aminoglycoside antibiotics have recognized effectiveness against Gram-positive, Gram-negative and Mycoplasma germs, having various applications in the treatment of infectious pathologies. However, their intense antimicrobial activity is overshadowed by the occurrence of side effects, which places them as last-line antibiotics. Thus, alternative combination therapies need to be identified to overcome these limitations.

Magnetite (Fe_3O_4), poly-lactic co-glycolic acid (PLGA), and chitosan (CS) structures have expressed encouraging results in various synergistic therapies, such as controlled and targeted treatment of infections (*Staphylococcus aureus, Pseudomonas aeruginosa*, *Candida albicans*), of hyperthermia and targeted delivery of antineoplastic drugs. The in vitro study presented in Chapter 7 describes the fabrication of magnetite-based materials to deliver the antibiotics streptomycin and neomycin encapsulated in PLGA-CS-Fe_3O_4@NEO and PLGA-CS-Fe_3O_4@STR polymeric biospheres, with the aim of using them to fight against biofilms that colonize prosthetic devices.

After synthesis, the obtained materials were examined both biologically, compositionally and morphologically by the following methods: X-ray diffraction (XRD), thermogravimetric analysis with differential scanning calorimetry (TGA-DSC), scanning electron microscopy (SEM), transmission electron microscopy with selected area electron diffraction (TEM-SAED), Fourier transform infrared spectroscopy (FT-IR), cell viability and antimicrobial assays.

7.2. Material and methods

7.2.1. Synthesis of Fe3O4@STR and Fe3O4@NEO nanoparticles and polymer-coated spheres

Antibiotic-functionalized magnetite nanoparticles were obtained by the co-precipitation method, using Fe^{2+} and Fe^{3+} in a ratio of 1:2M, according to literature references, and PLGA/CS/Fe_3O_4@STR and PLGA/CS microspheres /Fe_3O_4@NEO were prepared using the solvent evaporation method.

7.2.3. Biological characterization

7.2.3.1 Cell cultures

Human lung cell cultures were used for the in vitro tests. These cell cultures were kept at a temperature of 37°C, in a humid atmosphere with 5% CO_2, provided by the use of Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum. Visualization of cells and monitoring of different growth stages was possible using an Olympus IX71 phase-contrast inverted microscope.

7.2.3.2 Cell viability and toxicity tests

Cell viability after exposure to magnetite-containing nanoparticles and microspheres was assessed by the MTT [(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl) tetrazolium bromide] assay. The cells were incubated for 24 h in a culture medium in the absence of magnetite-based synthesis compounds (sample-control), as well as in their presence. After removing the culture medium, the cells were washed with a phosphate buffered saline solution. MTT solution (1 mg/mL) was then added, and the cells were further incubated at 37°C for two hours in the dark. Following its removal, an equal volume of isopropanol was added by pipetting to solubilize the formazan crystals. Absorption spectrophotometric measurements were performed at a wavelength of 595 nm with a microplate reader.

The viability of the MRC-5 cell culture line was also visualized using an Olympus inverted fluorescence microscope and the LIVE/DEAD® Cell Viability Assay Kit. Synthetically, after a 24-h incubation period with the nanoparticles, the cells were washed using warmed phosphate buffered saline and incubated with the ethidium-calcein mixture for 30 minutes at 37 °C. After a further washing process with phosphate buffered saline, the cells were prepared for imaging exploration.

The determination of the concentration of nitric oxide (NO) in the culture medium collected after exposure for 24 hours to nanoparticles was carried out using a Griess reagent and a sodium nitrate ($NaNO_2$) standard curve. This was used as a reference for evaluating the increase in NO as a consequence of the cytotoxic effect that triggers inflammation and cell death.

The level of lactate dehydrogenase (LDH) released in the culture media was quantified by applying a specific in vitro toxicity assessment kit, TOX7, centered on LDH. The culture medium was collected after 24 h of cell growth in the presence of the tested samples.

Absorbance was recorded with a microplate reader in the beam wavelength of 490 nm. Statistical analysis was performed on three replicates of each sample by unpaired Student's (t) test, and differences were considered statistically significant at values of $p < 0.05$.

7.2.3.3 Antimicrobial evaluation

The evaluation of the antibacterial properties of the nanostructures was carried out by exposure to the bacterial species of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The determination of the minimum inhibitory concentration (MIC) of the synthesized spheres and nanoparticles was carried out by a quantitative method based on a series of binary microdilutions in liquid medium, distributed in 96 wells of the culture plate. An amount corresponding to the concentration of 1000 μg/mL was added to the first well of each row. Using a micropipette, binary dilutions were made up to a final concentration of 0.05 μg/mL. After completing the microdilutions, 15 μL of 0.5 McFarland density suspension was added to each well. The seeded plates were incubated for 24 h at 37 °C and, after incubation, the MIC value for each sample was determined by visual inspection as the lowest concentration at which no microbial growth was observed (translated as no turbidity). The value was confirmed by recording the specific absorbance of the microbial cultures in a 600 nm beam using a spectrophotometer.

7.3. Results

7.3.3. Results of the biological evaluation

7.3.3.1. Cell viability

Regarding the biological characterization of the synthesized materials, the percentage of metabolically active cells was evaluated after 24 h by the MTT assay performed on human lung fibroblasts. No significant changes were detected for Fe_3O_4@STR and Fe_3O_4@NEO nanoparticles compared to the control sample, the number of viable cells exposed to these nanostructures being 96% of the value of the untreated ones.

However, an 11% increase in cell viability was observed for the PLGA/CS/PVA-Fe_3O_4@NEO microspheres compared to the untreated control. The results were calculated as the mean ± standard deviation of three replicates and expressed relative to the control cells (the result $p<0.05$ compared to the control sample). The resulting effect is statistically significant and

can be correlated with the enhanced biocompatibility of the polymer used, as well as with the potential to stimulate cell proliferation.

The biocompatibility of these samples was further confirmed by measuring the levels of NO and LDH released. The results obtained on cell growth upon exposure for 24 h to the functionalized nanoparticles were similar to those obtained for the untreated control sample. This exploration demonstrates that the synthesized microspheres and nanoparticles had no inflammatory or toxic effect on cell membrane integrity.

Following the use of fluorescence microscopy, it was revealed, through the specific staining of viable and dead cells, that PLGA/CS/PVA-Fe_3O_4-STR microspheres generate minimal changes in the number of viable cells compared to the control samples.

7.3.3.2. Results of antimicrobial testing

The antimicrobial effect was studied by a standardized test, with the aim of establishing the minimum inhibitory concentration (MIC) of the antibiotic-functionalized nanostructures against the tested pathogens. The results revealed differences in the MIC values of the compounds obtained, depending on the incorporated antibiotic and the microbial species evaluated. Microspheres containing Streptomycin (STR) showed lower MIC values on the strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* tested. The enhanced antimicrobial potential is due to a good absorption and release by the obtained nano-sized microsystem.

8. Personal contributions and conclusions

In the personal contributions part of the work, on the one hand, we followed the characterization of a group of laryngectomized patients who underwent phonation rehabilitation by fitting and periodically changing vocal prostheses, and, on the other hand, we conducted two studies focused on the identification of some nano-structures with combative properties against biofilms by applying them to phonatory prostheses.

Exactly, within the descriptive observational study, we evaluated general variables such as the patient's age, sex, the histopathological type of neoplasm that led to total laryngectomy intervention, as well as particular aspects of each case regarding the type of phonatory prosthesis fitted, the replacement interval of the voice prostheses for each patient, and, especially, bacterial or fungal colonization both locally, at the level of the tracheoesophageal fistula, and at the surface of vocal prosthesis. We studied the composition of the microbial and polymicrobial cultures, as well as the microbial and polymicrobial profiles, respectively for both the secretions collected from the insertion site of the phonatory prostheses, and for those collected from the recovered prostheses. More specifically, we studied the frequency with which germs were identified in the mentioned secretions, as well as their association modalities, and the distribution of pathogens according to the replacement intervals of the phonatory prostheses. We analyzed these data with the aim of detecting the potential factors that would influence the exploitation time of the phonatory prostheses. We also explored the occurrence of complications related to the fitting of the prostheses and the eventuality of the installation of inflammatory or infectious pathology at the broncho-pulmonary level.

The two studies that complete the personal contributions part of the thesis aimed at highlighting the particularities of nano-technology applications in fighting biofilms, with the particular aim of extending the operating life of phonatory prostheses.

Thus, the first study focused on the synthesis of magnetite nanoparticles coated with polyethylene glycol and functionalized with polymyxin B. They were obtained by the co-precipitation method and characterized by different methods. The efficiency of these structures in combating biofilms is based on the nano dimensions, in the range of 5 - 9 nm, the well-expressed crystallinity property, the enhanced biocompatibility, the reduced toxicity, and

especially on the inhibitory effect on the development of the biofilm. The evaluation from the biological point of view also marked a favorable impact that encourages the use of the materials synthesized in this study for the development of structures with combative anti-biofilm properties.

The second study has as its starting point the design of an innovative system, based on magnetite, for the distribution of streptomycin and neomycin antibiotics, encapsulated in polymeric biospheres of poly-lactic co-glycolic acid and chitosan functionalized with previously mentioned antibiotics, which led to the synthesis of PLGA-CS-Fe_3O_4@NEO and PLGA-CS-Fe_3O_4@STR structures.

Four magnetite-based nanocomposites were synthesized by co-precipitation and ultrasonication techniques. Both the obtained nanomaterials and the functionalized magnetite nanoparticles have spherical appearance and dimensions in the nano range, which increases their versatility in anti-biofilm applications. The lack of toxicity on human cells and the low CMI values on the bacterial species frequently found in the composition of biofilms that colonize the phonatory prostheses represent valuable advantages of these structures. Moreover, the incorporation of nanoparticles into polymer microspheres enhanced their biocompatibility, maintaining the optimal antimicrobial effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Specifically, covering the layers of streptomycin, respectively neomycin with these polymeric structures leads to overcoming the toxic effects determined by the use of these aminoglycosides. Efficient in the targeted and controlled delivery of transported drugs, the obtained materials stand out as performing candidates for the prophylaxis and treatment of infections in the field of otorhinolaryngology, and especially for combating biofilms on phonatory prostheses.

Conclusions

1. Vocal rehabilitation by using a vocal prosthesis is the method of choice for the substitution of lost function after total laryngectomy. Although there are other rehabilitation methods, such as the laryngophone or the use of the esophageal voice, vocal rehabilitation by fitting a voice prosthesis represents the standard and the most used technique.

2. In contrast to the indisputable advantages of fitting a speech prosthesis, there are also a number of significant disadvantages for the long-term impact on the patient's quality of life.

3. The average exploitation time of the voice prosthesis in the studied group was 8 months, the results being comparable to the data from the specialized literature. The lifespan of the prosthetic device is not dependent on the type of colonization, bacterial or fungal.

4. The main disadvantage is the need for frequent replacement of the device. The main cause for that is represented by trans-prosthetic leakage caused by biofilm build-up on the flanges and inside the voice prosthesis, which leads to physical damage to device’s material and, consequently, to its functionality.

5. There is a statistically significant association between the presence of microbial colonization and the duration of operation of the prostheses, but there is no significant influence between the specific types of strains in the composition of these secretions and the replacement frequency.

6. The main bacterial species identified in the samples collected from phonatory prostheses are, in this order: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Accordingly, the predominant fungal species are: *Candida tropicalis, Candida krusei* and *Candida albicans.*

7. The microbiological profile of the secretions collected from the phonatory prostheses was represented by fungal monocultures in the proportion of approximately 1/3 of the cultures, followed by mixed cultures between a bacterial and a fungal species, in 1/4 of the cases, and by bacterial monocultures in 1/5 of the cases.

8. There is a statistically significant association between the type of phonatory prosthesis used in the vocal rehabilitation plan and the duration of use of the prosthesis.

9. The development of biofilm on phonatory devices occurs independently of factors such as the patient's age, comorbidities, the type of device used. The colonization of phonatory prostheses with biofilm, regardless of its nature (bacterial/fungal/mixed), is favored by the

structure based on elastic polymers of the phonatory buttons, as well as by optimal environmental conditions provided by exposure to esophageal microflora, to various fluids, saliva, debris feeding, increased humidity, suitable temperature.

10. The morphology of the biofilm is particular to each interval analyzed, with the increase of fungal colonization over time.

11. Inhibiting the development of bacterial biofilm on speech prostheses is possible by modifying the surfaces of these devices using nanotechnology. Specifically, the results are promising regarding the impact on the lifespan of the voice prostheses, which can be extended by coating the devices with magnetite nanoparticles functionalized with different therapeutic agents (namely antibiotics).

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List of published scientific papers

1. PEG-Functionalized Magnetite Nanoparticles for Modulation of Microbial Biofilms on Voice Prosthesis - publicat în *Antibiotics* 2022, *11*(1), 39; https://doi.org/10.3390/antibiotics11010039 Indexat PubMed, Factor de impact 4,63

2. Magnetite Nanoparticles Functionalized with Therapeutic Agents for Enhanced ENT Antimicrobial Properties - publicat în *Antibiotics* 2022, *11*(5), 623; https://doi.org/10.3390/antibiotics11050623, Indexat PubMed Factor de impact 4,63

3. Biofilm assessment on voice prosthesis used for vocal rehabilitation in laryngectomized patients - publicat în *Romanian Journal of Medical Practice*, 2022, Vol. 17, No. 1 (86); *DOI: 10.37897/RJMP.2022.1.6* Indexat Scopus

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