SCHOOL OF ADVANCED STUDIES OF ROMANIAN ACADEMY
DOCTORAL SCHOOL OF CHEMICAL SCIENCES
PETRU PONI INSTITUTE OF MACROMOLECULAR CHEMISTRY, IAȘI
CHEMISTRY Field

ZWITTERIONIC MATERIALS WITH ANTIMICROBIAL PROPERTIES AND DRUG DELIVERY CAPABILITIES

SUMMARY OF DOCTORAL THESIS

PhD supervisor:
CS II DR: MARCELA MIHAI

PhD student:
TROFIN MARIN-AUREL

2023

CONTENTS

Thesis/Summary

INTRODUCTION

Polymers can be found in a multitude of forms, of different structures and sizes, from nanoparticles to membranes or fibers. Among them, polymeric microparticles stand out due to advantages such as: controllable size, drug adsorption and release capacity, chemical and physical stability, possibility of synthesis in large quantities and possibility of surface functionalization. In the biomedical field, microparticles are used for the delivery and controlled release of drugs/nucleic acids, in imaging and diagnostics, and in tissue engineering. Zwitterionic materials have been intensively studied in recent years due to properties such as biocompatibility, low toxicity and protein and bacterial non-adhesion. These materials have found numerous applications in the biomedical field, especially in the development of antimicrobial materials and drug carrier systems. In addition, due to their biomimetic character, zwitterionic materials do not trigger an immune response from the body upon contact with the physiological environment.

This study starts from the *hypothesis* that porous systems with drug loading and release capacity, obtained by suspension polymerization, can acquire new properties and functions if they are subjected to suitable polymer-analogous transformations. Thus, by introducing zwitterionic groups into the structure of some copolymers, they could acquire intrinsic antimicrobial activity, increased hydrophilicity, biocompatibility and resistance against protein and bacterial adhesion. In addition, these zwitterionic materials can be used in the development of hybrid materials (with zein) with the aim of improving antimicrobial activity and drug transport capacity.

In this context, the scientific objectives pursued in this doctoral thesis are:

1. **Synthesis of porous microparticles by the suspension polymerization method**, using the monomers N-vinylimidazole, glycidyl methacrylate, four crosslinking agents, and different porogenic agents;
2. **Investigating the influence of some synthesis parameters on the structure, synthesis yield and properties of the obtained microparticles**, with the gradual determination of the optimal synthesis conditions;

3. **Synthesis of zwitterionic materials by betainization of porous microparticles** using various betainization agents (sodium chloroacetate, acrylic acid, methacrylic acid, 1,3-propanesultone);
4. **Preparation of hybrid zwitterionic materials** (by zein grafting during/after suspension polymerization);
5. **Characterization of obtained materials** by infrared spectrometry, mercury porosimetry, dynamic water vapor sorption, thermogravimetric analysis, scanning electron microscopy, atomic force microscopy, water swelling studies and others;
6. **Carrying out drug sorption/release studies** (under simulated gastrointestinal conditions) by zwitterionic and hybrid zwitterionic microparticles using tetracycline hydrochloride and doxycycline hydrochloride as model drugs;
7. **Study of the intrinsic antibacterial activity** of zwitterionic and hybrid zwitterionic materials against some strains of bacteria (*Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae*).

The doctoral thesis entitled "Zwitterionic materials with antimicrobial properties and drug delivery capabilities" is composed of two parts:

Part I, entitled *Current state of knowledge*, comprises **Chapter 1** and is a thorough literature survey that accumulates general information on zwitterionic polymers, their classification (polyampholytes and polybetaines), the main synthesis methods (direct polymerization or polymer-analog transformations) and examples of biomedical applications, especially as antimicrobial materials or drug delivery systems. Also, this chapter includes a concise presentation of zein, a protein with a polyampholyte structure, which will later be used in the production of hybrid materials.

Part II is structured in five distinct chapters and summarizes the results obtained during the doctoral studies. The results describe the experimental route of the samples starting from synthesis, functionalization, characterization and up to the investigation of antibacterial properties and drug sorption and release studies. **Chapter 2** presents information on the materials and synthesis, transformation, and characterization methods used in this study.

Chapter 3 describes the preparation of porous microparticles based on N-vinylimidazole and glycidyl methacrylate, crosslinked with mono-/di-/triethyleneglycol dimethacrylate or divinylbenzene, not reported so far in the literature. The influence of some factors (related to

both the organic and the aqueous phase) on the synthesis yield and the specific surface area of the microparticles is investigated. Using the parameters proven to be optimal, microparticles were synthesized using various porogenic agents.

Chapter 4 presents the process of betainization and characterization of porous microparticles (presented in Chapter 3). In the case of zwitterionic microparticles, their degree of betainization was determined and the influence of this transformation on the morphology and porous structure of the microparticles was also analyzed. The structural changes induced by the introduction of zwitterionic groups were highlighted, among others, by semi-quantitative elemental analysis.

In **Chapter 5**, hybrid zwitterionic microparticles (with zein) were synthesized by two methods (grafting during/after the suspension polymerization process) through the ring-opening reaction between the epoxy ring in the glycidyl methacrylate structure and the free amino groups in the protein structure. These materials were subsequently betainized using three different betainizing agents.

Chapter 6 comprises, in the first part, studies on the sorption and release capacity of doxycycline and tetracycline by zwitterionic or hybrid zwitterionic microparticles. The studies continued with the evaluation of the antimicrobial activity of the microparticles (zwitterionic and hybrid zwitterionic) against Gram-positive and Gram-negative bacterial strains.

In **Chapter 7**, the general conclusions of the studies included in this doctoral thesis are presented. Also, each chapter ends with its own conclusion section. At the end of the thesis, there is a list of consulted and cited bibliography. **Annex 1** includes the dissemination activity of the results obtained during the doctoral internship, and **Annex 2** is made up of the scientific articles published during this period.

CHAPTER 3

POROUS MICROPARTICLES SYNTHESIS

This chapter includes the synthesis of porous microparticles using the suspension polymerization method, including the optimization studies aimed at obtaining the highest yield and specific surface area.

3.1. Suspension polymerization reaction

For the synthesis of porous microparticles, the monomers glycidyl methacrylate (GMA), N-vinylimidazole (NVI) and one of the four crosslinking agents were used: monoethylene glycol dimethacrylate (EGDMA), diethylene glycol dimethacrylate (DEGDMA), triethylene glycol dimethacrylate (TEGDMA) or divinylbenzene (DVB). The polymerization process follows a radical mechanism, and the general reaction, depending on the crosslinker used, can be found in Figure 3.1a (dimethacrylic crosslinker), respectively 3.1b (divinylbenzene crosslinker).

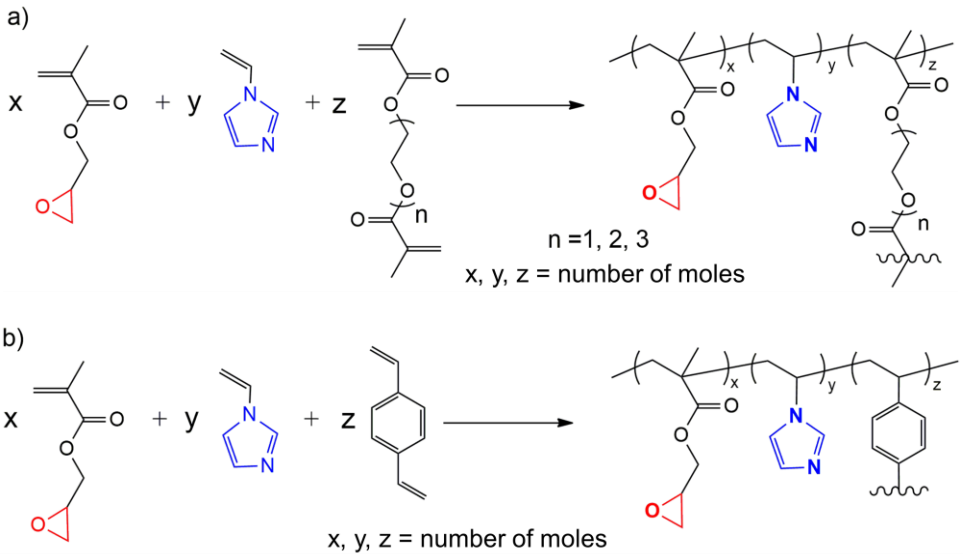

Figure 3.1. *Copolymerization reaction between GMA and NVI using a) dimethacrylic crosslinker or b) divinylic crosslinker* (Trofin et al., 2022; Trofin et al., 2023).

3.2. Establishing optimal synthesis conditions

The structure and morphology of the microparticles, as well as the synthesis yield, are influenced by a series of factors that may be related to the organic phase, the aqueous phase or the stirring conditions. Therefore, the influence of some parameters on the polymerization reaction was studied, with the aim of identifying the most suitable conditions.

3.2.1. Factors related to the organic phase

- *The molar percentage of NVI:* four types of microparticles were synthesized using different molar percentages of NVI: 60%, 45%, 30%, 20%. Within these syntheses, the parameters kept constant were: EGDMA as crosslinker in a molar percentage of 10%, stabilization system = PAV, GEL and $MgCl_2$, porogen = toluene, dilution D = 0.5 and stirring speed = 300 rpm. SEM images of the obtained microparticles can be found in Figure 3.3.

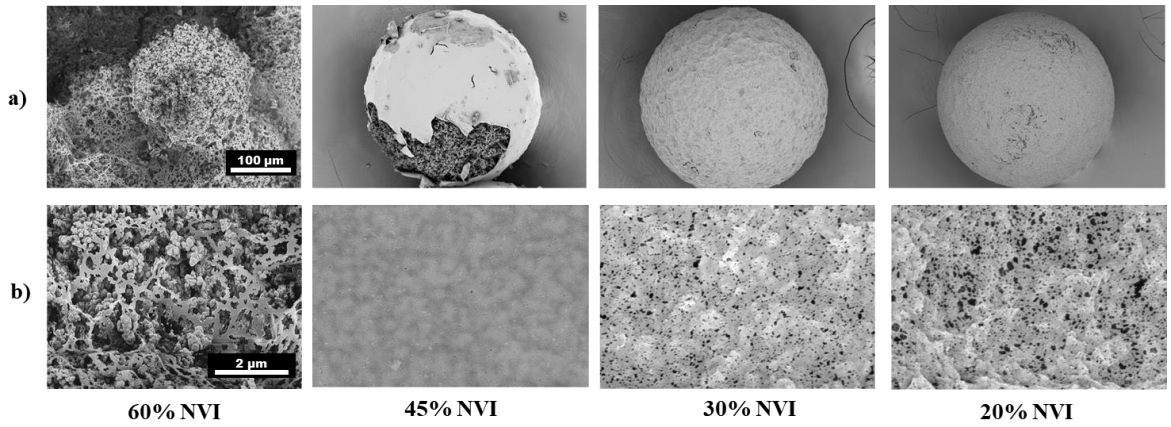

Figura 3.3. *The influence of the molar percentage of NVI on the morphology of porous microparticles a) general look; b) surface appearance, in the case of using the EGDMA as crosslinker in a molar percentage of 10%* (Trofin et al., 2023).

The molar percentage of NVI that provided the microparticles with the highest specific surface, respectively 30% NVI, was chosen for subsequent studies (Trofin et al., 2023).

- *Crosslinker chemical structure and degree of crosslinking:* microparticles were synthesized using the four crosslinking agents at 10% molear percent. With the exception of DEGDMA, the chosen crosslinking agents lead to the formation of spherical microparticles, and based on the specific surface area values, it was concluded that TEGDMA is the optimal crosslinking agent (15 m^2/g) compared to EGDMA (10 m^2/g) or DVB (8 m^2/g). Also, the yield and specific surface area increase with the degree of crosslinking. *Optimization studies on the monomers used and the ratio between them conclude that the molar ratio GMA:NVI:TEGDMA = 40:30:30 is optimal for obtaining microparticles with the highest specific surface area* (Trofin et al., 2023).
- *Dilution*: represents the ratio between the volume of the porogenic agent and the volume of the organic phase (Costa et al., 2020). Analyzing the values of the specific surface

area, yield and content in nitrogen and epoxy groups of the microparticles, it was decided that *the optimal dilution has a value of 0.5* (Trofin et al., 2023).

3.2.2. Factors related to the aqueous phase

- *Stabilization system composition:* syntheses were performed using five different stabilization systems, with the organic phase and stirring conditions being kept constant. The main stabilizing agent, polyvinyl alcohol (PAV), was used as such or in various combinations with gelatin (GEL) and inorganic salts. Table 3.5 shows the stabilization systems used and the properties of the porous structure of the resulting microparticles.

Table 3.5. *The influence of the composition of the stabilization system on the properties of microparticles* (Trofin et al., 2023).

RMM	**Stabilization system components**	**S_{sp} (Hg) (m^2/g)**	**VP (mL/g)**	**P (%)**	**d_m (nm)**
GMA:NVI: TEGDMA = 40:30:30	PAV	9.14	1.129	62.05	494
	PAV, GEL	33.91	0.962	71.86	113
	PAV, GEL, NaCl	38.46	1.190	50.73	124
	PAV, GEL, $MgCl_2$	48.32	1.314	45.75	109
	PAV, GEL, $CaCl_2$	34.83	0.953	46.51	110
RMM = molar ratio between monomers, S_{sp} (Hg) = specific surface area determined by mercury porosimetric technique, VP = pore volume, P = porosity, d_m = mean pore diameter.					

The microparticles with the highest specific surface area and the best yield (91%) are those synthesized using the stabilization system consisting of *PAV, GEL and magnesium chloride*.

- *Molar mass and degree of hydrolysis of PAV*: the optimal grade of PAV for the synthesis of porous microparticles is the one with Mw = 52650 g/mol and degree of hydrolysis of 98%.

3.3. Porous microparticles synthesis

Using the optimal conditions established during the optimization study, microparticles were synthesized using three different porogenic agents, the samples being encoded according to the agent used: $(GNT)_T$ (toluene), $(GNT)_A$ (n-butyl acetate) and $(GNT)_{TA}$ (mixture toluene: n-butyl acetate (1:1, v/v)). The specific surfaces and yields of these microparticles were: 91% and 48.32 m_2/g for $(GNT)_T$, 90% and 51.7 m_2/g for $(GNT)_{TA}$, 93% and 61.7 m_2/g for $(GNT)_A$. For comparative purposes, microparticles were synthesized using zein solvents as porogenic agents: dimethylsulfoxide (sample $(GNT)_{DMSO}$), and dimethylformamide (sample $(GNT)_{DMF}$) respectively.

CHAPTER 4
ZWITTERIONIC MICROPARTICLES SYNTHESIS AND CHARACTERISATION

4.1. Synthesis and characterization of porous zwitterionic microparticles

This chapter presents the synthesis and characterization of zwitterionic microparticles obtained by betainization of previously synthesized porous microparticles. For this purpose, the imine nitrogen atom in the structure of the repetitive units of NVI was transformed by substitution or addition reactions, into the corresponding tetra-substituted ammonium cation, representing the cationic part of the betaine group. Depending on the betainizing agent used, the anionic group is represented by the carboxylate (sodium chloroacetate, acrylic acid, methacrylic acid) or sulfonate (1,3-propanesultone) group (Figure 4.1).

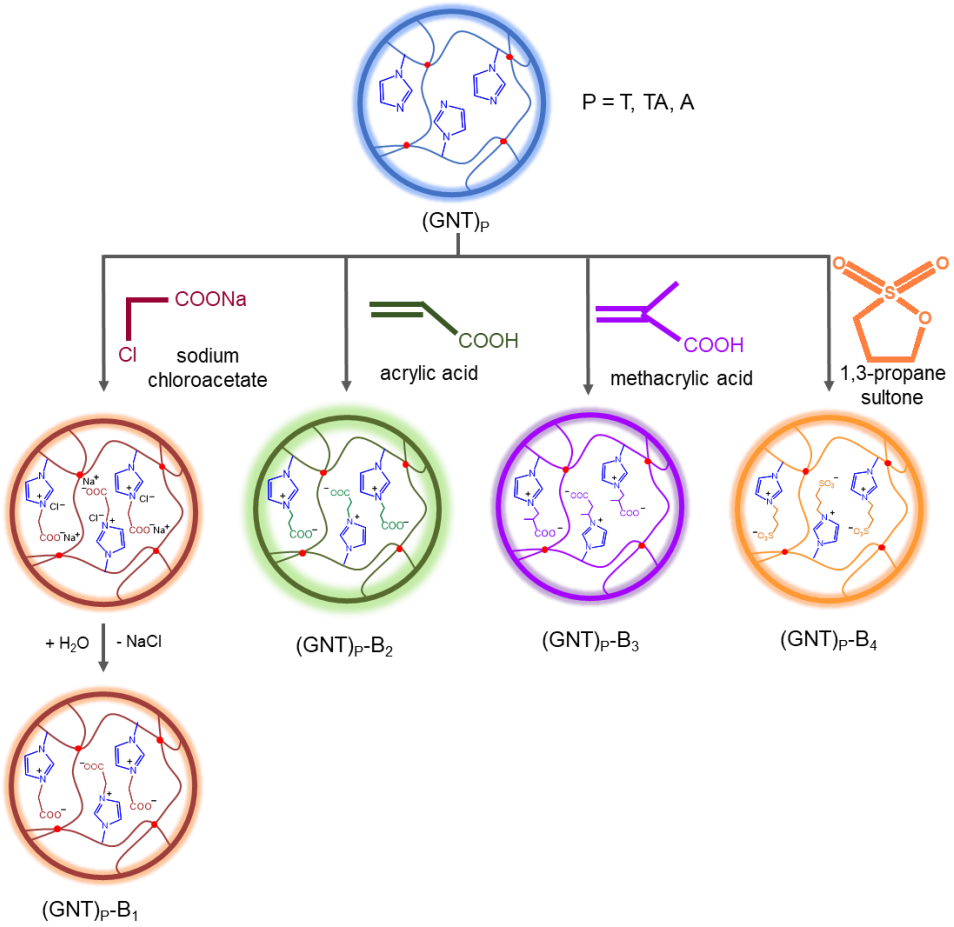

Figure 4.1. *Schematic representation of the structure of porous microparticles before and after transformation with different betainizing agents.*

Porous microparticles synthesized under optimal conditions (as demonstrated in Chapter 3), obtained in the presence of three different porogenic agents: $(GNT)_T$, $(GNT)_{TA}$, $(GNT)_A$, were subjected to the polymer-analog transformation process.

4.1.2. Analysis by FTIR-ATR spectroscopy

The FTIR-ATR adsorption spectra of the microparticles before and after betainization have a high degree of similarity, confirming that the betainizing agents do not significantly change the structure of the copolymer, and only react with the imidazole rings. The spectra of the zwitterionic microparticles show the characteristic bands for the carboxylate and sulfonate groups, respectively. The degrees of betainization were determined by reporting the vibration intensities corresponding to the quaternary (1560 cm^{-1}) and neutral (1553 cm^{-1}) nitrogen atoms (Table 4.1).

Table 4.1. *Betainization degrees obtained from FTIR analysis, for zwitterionic microparticles based on $(GNT)_T$, $(GNT)_{TA}$ and (GNT)A.*

Betainized sample	**Betainization degree**			
	sodium chloroacetate	**acrylic acid**	**methacrylic acid**	**1,3-propanesultone**
$(GNT)_T$	63.83	62.08	55.61	55.91
$(GNT)_{TA}$	76.21	70.33	67.33	58.48
$(GNT)_A$	90.2	84.24	80.98	60.49

SEM micrographs suggest that the betainization process does not change the spherical shape and porous morphology of the microparticles. The C/N and O/N atomic ratios, determined by EDAX analysis, increase upon transformation due to the newly introduced group consisting of a short hydrocarbon chain and a carboxylate group, which results in increased carbon and oxygen content. The results from the EDAX analysis are very close to the theoretical ones, being consistent with the values of the betainization degrees presented in Table 4.1. The analysis of the porous structure was carried out by atomic force microscopy and mercury porosimetry, resulting in the fact that the sample $(GNT)_A$-B_1 presents the highest values for both specific surface area (48.59 m^2/g) and average roughness (100.8 nm) .

Ion exchange capacities (Table 4.4) provide information on the density of cationic and anionic groups in the zwitterionic structure. The close values between the anionic and cationic exchange capacities suggest an equimolecular ratio between the acidic and basic groups thus confirming the chemical structures of the zwitterionic microparticles shown in Figure 4.1.

Table 4.4. *Ion exchange capacities (gravimetric and volumetric) for zwitterionic microparticles based on (GNT)A.*

Sample	Anionic exchange capacity		Cationic exchange capacity	
	CSA_g	**CSA_v**	**CSC_g**	**CSC_v**
$(GNT)_A$-B_1	2.70	1.49	2.61	1.41
$(GNT)_A$-B_2	2.53	1.28	2.40	1.15
$(GNT)_A$-B_3	2.39	1.06	2.29	0.95
CSA_g = gravimetric anion exchange capacity, CSA_v = volumetric anion exchange capacity, CSC_g = gravimetric cation exchange capacity, CSCv = volumetric cation exchange capacity.				

4.1.7. Swelling studies in water

The swelling behavior of zwitterionic microparticles can be influenced by the pH variation of the medium in which the swelling is carried out. It is known that poly(carboxybetaines) are sensitive to changing pH values (Racovita et al., 2021). Swelling ratios in media with pH values in the range 2-9 are shown in Figure 4.8. High swelling ratios at low pH are due to electrostatic repulsions that occur between neighboring cationic groups. With the increase in the pH values of the swelling medium, the carboxylic groups are gradually deprotonated, and the microparticles return from the cationic to the zwitterionic form, with the decrease in the degree of swelling at equilibrium. The values of the diffusion exponent *n* from the Korsmeyer-Peppas equation are in the range 0.205-0.222 indicating that the transport mechanism of water molecules is Fick type, being controlled mainly by diffusion through the pores of the polymer matrix (Figure 4.11).

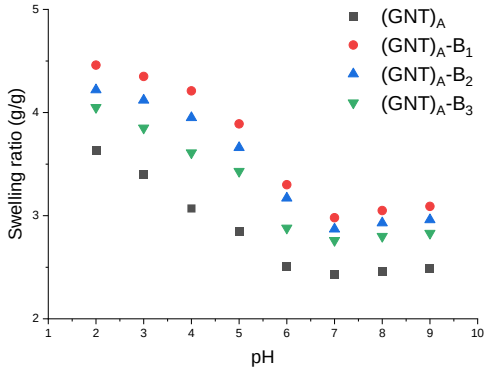

Figure 4.8. *Swelling ratios at equilibrium of $(GNT)_A$ and zwitterionic microparticles in aqueous solutions with different pH values.*

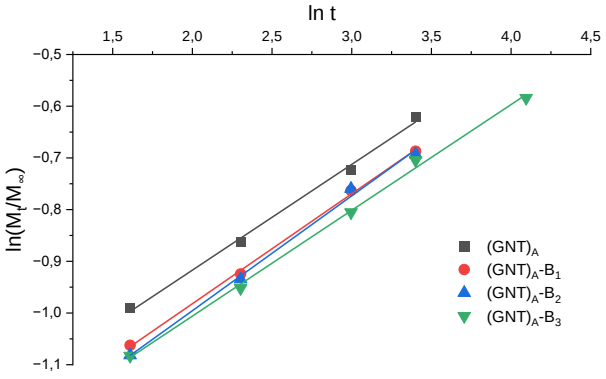

Figure 4.11. *Plot of ln(Mt/M∞) versus ln t for $(GNT)_A$ microparticles before and after betainization.*

CHAPTER 5
HYBRID ZWITTERIONIC MATERIALS

This chapter presents the synthesis of hybrid microparticles with zein, by grafting zein onto previously synthesized porous microparticles (Chapter 3) or by grafting zein during suspension polymerization. Subsequently, these microparticles were subjected to the betainization process with three different agents and structurally and morphologically characterized.

5.1.1.1. Zein grafting during suspension polymerization

Porous microparticles containing zein can be obtained by simultaneously carrying out the grafting and polymerization reactions, when a new carbon-nitrogen covalent bond is formed through the epoxy ring-opening reaction in the presence of the free amino group belonging to zein (from glutamine units) (Figure 5.2).

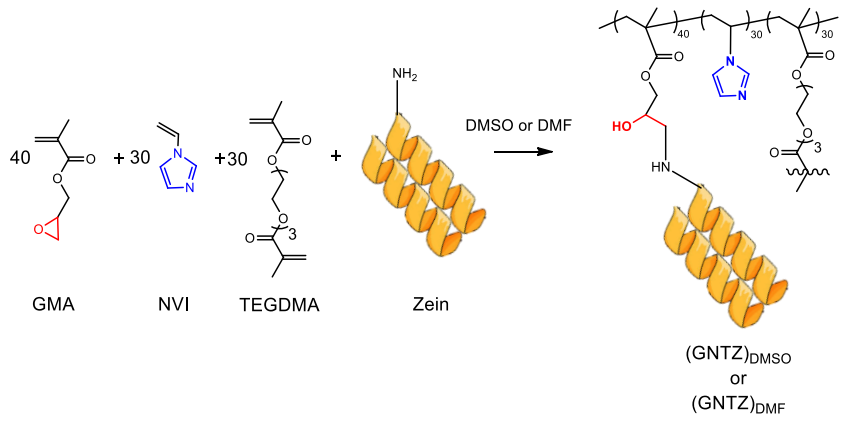

Figure 5.2. *Synthesis of hybrid zein microparticles by suspension polymerization.*

The obtained microparticles have a porous structure, numerous pores and asperities can being present on their surface. The yields of the hybrid porous microparticles synthesis reactions were 61.70% ($(GNTZ)_{DMSO}$ sample) and 50.96% respectively ($(GNTZ)_{DMF}$ sample). Considering the value of the reaction yield, only $(GNTZ)_{DMSO}$ microparticles will be considered in the following studies.

5.1.1.2. Post-polymerization zein grafting

For this process, the microparticles made using different porogenic agents and whose synthesis was presented in Chapter 3 were used: (GNT)T, (GNT)TA, (GNT)A. The grafting reaction of zein on porous microparticles, in basic pH, is shown in Figure 5.4:

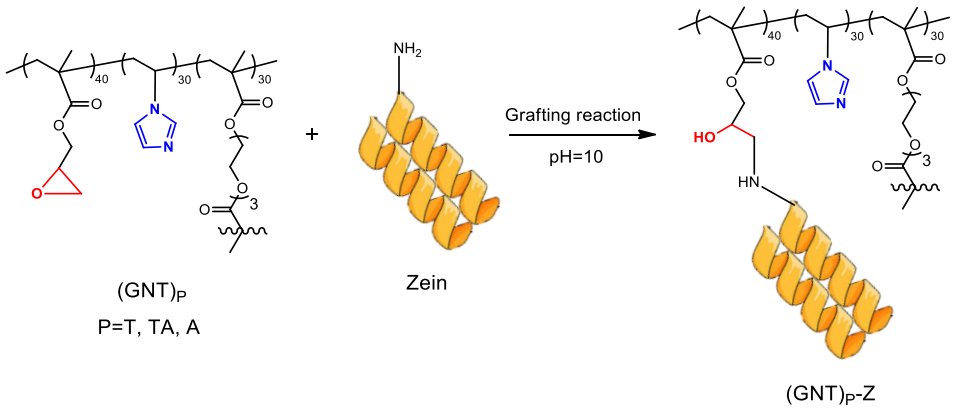

Figura 5.4. *Zein grafting reaction on the surface of porous microparticles.*

The highest amount of zein was grafted on the porous microparticles $(GNT)_T$-Z. This can be explained by the fact that $(GNT)_T$ microparticles have larger pore diameter values (d_m = 109 nm) compared to those of $(GNT)_{TA}$ (d_m = 97 nm) and $(GNT)_A$ microparticles (d_m = 94 nm), thus facilitating the access of zein to the epoxy groups in the structure of the crosslinked microparticles. In an optimization study, the optimal conditions for zein grafting on porous microparticles $(GNT)_T$ were established: solvent for zein - methanol 80%, concentration of zein solution - 3% (m/v), mass ratio microparticles: zein = 1:1(g/g), temperature – 60°C, reaction time – 5 hours and pH – 10. The microparticles obtained under these conditions were marked $(GNT)_T$-Z.

5.1.1.3. Betainization reaction of hybrid porous microparticles

Hybrid microparticles obtained both by suspension polymerization and those obtained by grafting zein onto $(GNT)_T$ microparticles were subjected to betainization with the three different betainizing agents: sodium chloroacetate, acrylic acid, and methacrylic acid. The introduction of zwitterionic groups into the structure of hybrid microparticles brings a series of advantages: increased hydrophilicity, variable properties depending on pH and a possible antimicrobial activity.

5.1.2. Analysis by FTIR-ATR spectroscopy

Analyzing the FTIR-ATR absorption spectrum of the $(GNTZ)_{DMSO}$ sample, obtained by suspension polymerization in the presence of zein, the specific bands of zein can be observed: amide I (1654 cm^{-1}), amide II (1538 cm^{-1}), amide A (3302 cm^{-1}), and ν(NH) (3300 cm^{-1}). The grafting of zein to the epoxy group during the suspension polymerization process does not affect the tertiary structure of the protein, only the flexible structures between the α-helices

being most likely involved in the reaction. Following the betainization process, a decrease in the specific zein signals and a slight increase in the intensity of the ν_{asym}(C=O) band from 1722 cm^{-1} is noted, probably due to conformational changes.

FTIR-ATR analysis of the hybrid microparticles obtained by grafting zein on $(GNT)_T$ porous microparticles, before and after betainization, highlights a number of aspects: the specific signals of zein in the spectrum of the sample $(GNT)_T$-Z are weaker compared to its counterpart, synthesized by adding zein from the suspension polymerization phase. After betainization it is observed that the band at 1655 cm^{-1} becomes wider due to the development of the ν_{asim}(COO-) sub-band, also there is an increase in absorption in the 1530-1580 cm^{-1} area and a new band appeared at 835 cm^{-1}, related to vibrations of the imidazolium cation.

Based on the FTIR-ATR spectroscopy results, the degrees of betainization were calculated, representing the percentage of imidazole nuclei that successfully participated in the betainization reaction (Figure 5.11).

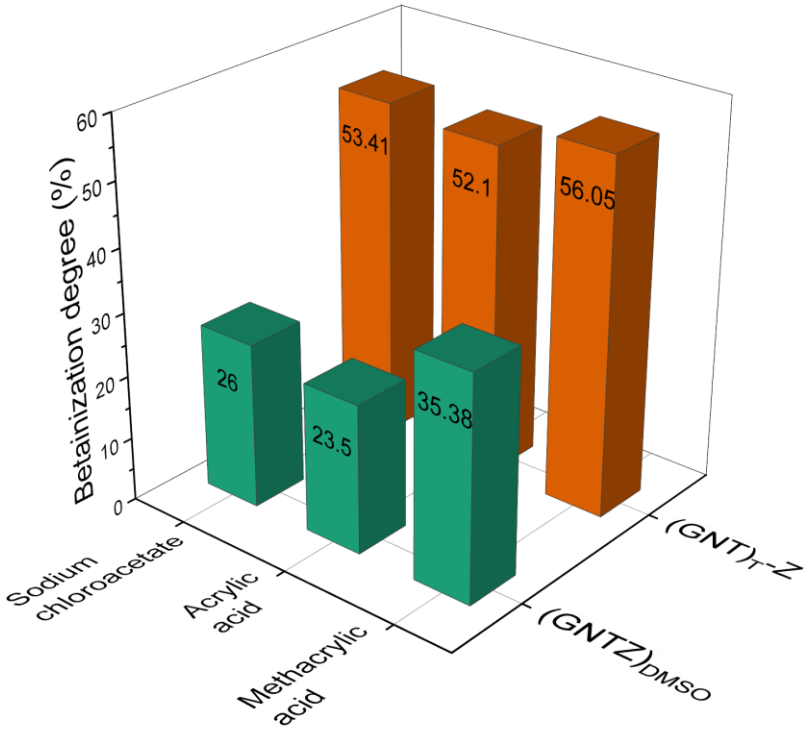

Figure 5.11. *Betainization degrees of zwitterionic hybrid microparticles based on* $(GNTZ)_{DMSO}$ *and* $(GNT)_T$*-Z.*

5.1.4. Surface morphology analysis by SEM microscopy

The obtained microparticles were analyzed using the scanning electron microscopy technique (Figure 5.13 and Figure 5.14).

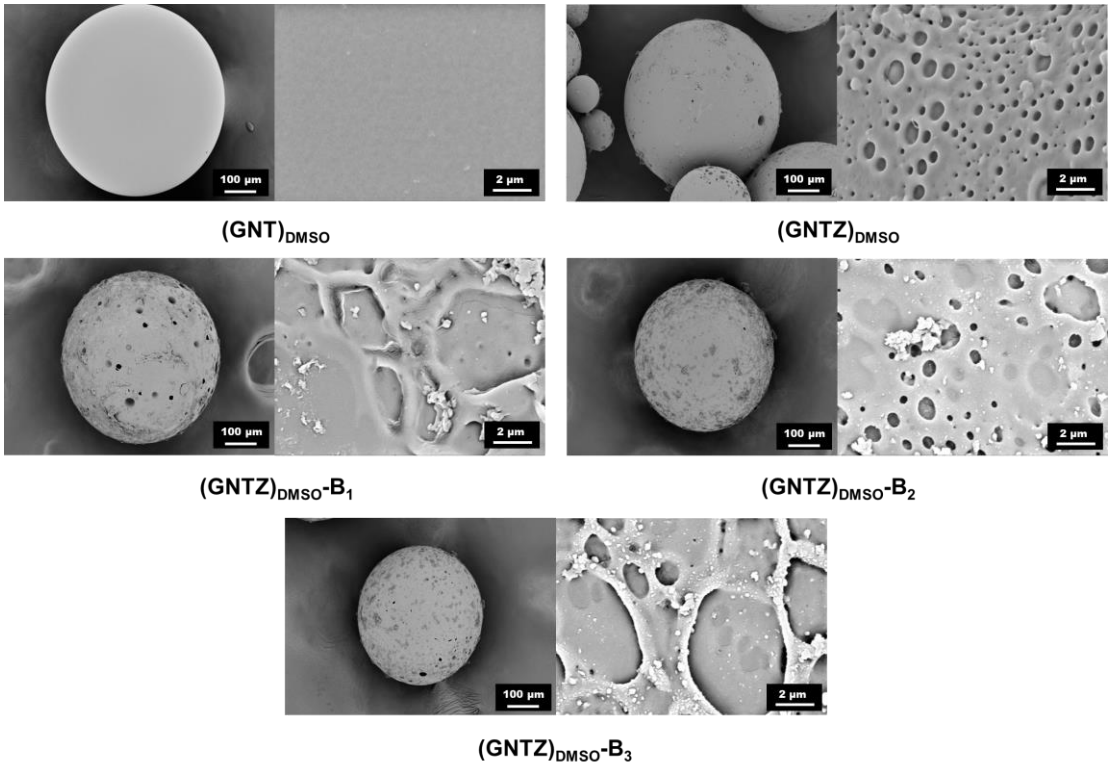

Figure 5.13. *SEM images of zwitterionic hybrid microparticles synthesized by zein grafting during polymerization.*

As observed in Figure 5.13, in the case of $(GNT)_{DMSO}$ microparticles, the zein solvent did not act as a porogenic agent, the microparticles surface being smooth and uniform. On the other hand, $(GNTZ)_{DMSO}$ microparticles, synthesized in the presence of zein, show numerous pores of different sizes. The betainization process changes the microparticles surface appearance, but does not affect their shape.

The grafting of zein on the surface of microparticles $(GNT)_T$ leads to the modification of it's surface morphology by covering the existing pores, with the formation of a dense shell that causes a decrease in the number and size of the pores (Figure 5.14). The subsequent betainization process does not have a significant impact on the surface texture of these microparticles, the porous structure being preserved regardless of the betainization agent used.

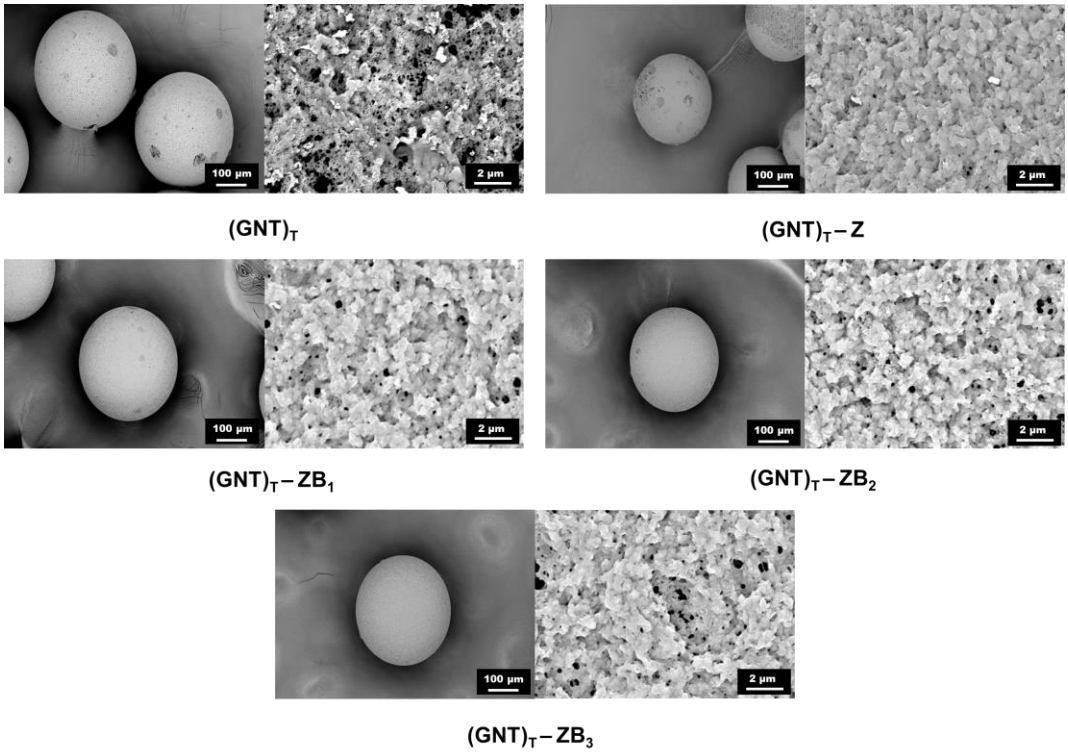

Figure 5.14. *SEM images of the microparticles synthesized by post-polymerization grafting of zein, before and after grafting/betainization.*

5.1.7. Characterization of the porous structure

The grafting process of zein, both during suspension polymerization and on already formed microparticles, leads to obtaining samples with relatively close values of specific surfaces and pore size. Betainization of hybrid microparticles causes a decrease in the specific surface regardless of their type. This behavior is more accentuated in the case of using acrylic or methacrylic acids as betainizing agents, which most likely cause a conformational rearrangement of the macromolecular chains.

5.1.8. Swelling studies of hybrid microparticles

The degrees of swelling depend primarily on the grafting method of the zein. When zein grafting is done directly from the suspension polymerization reaction, the microparticles are more hydrophobic (S_w = 116-132%) compared to those obtained by post-polymerization grafting (S_w = 223-261%). After betainization, the degree of swelling in water increases slightly, probably due to the presence of carboxylate groups in the structure of the zwitterionic hybrid microparticles, being, however, influenced by the degree of betainization and the type of betainization agent used. All these findings lead to the conclusion that grafting and betainization reactions have occurred.

CHAPTER 6
APPLICATIONS OF PRECURSOR, ZWITTERIONIC AND ZWITTERIONIC HYBRID MICROPARTICLES

This chapter presents the results obtained evaluating the drug loading and release capacity of zwitterionic and hybrid zwitterionic microparticles, as well as their antibacterial activity.

6.1. Zwitterionic microparticles-doxycycline systems

Doxycycline hydrochloride (DCH) was used for sorption studies on $(GNT)_A$, $(GNT)_A$-B_1, $(GNT)_A$-B_2 and $(GNT)_A$-B_3 microparticles. The optimal conditions for carrying out the sorption process are: pH 8.5, contact time 300 minutes, drug solution concentration $1 \cdot 10^{-3}$ g/mL and temperature 308 K. Zwitterionic microparticles show enhanced sorption of the drug, fact which can be associated with their higher degree of swelling (the degree of swelling varies in the order: $(GNT)_A$-B_1 > $(GNT)_A$-B_2 > $(GNT)_A$-B_3 > $(GNT)_A$). Also, kinetic studies suggest that the loading process occurs in two steps: diffusion through macropores followed by diffusion through mesopores. Thermodynamic studies suggest that DCH sorption is spontaneous and endothermic, and there is a physicochemical interaction between the drug and the polymer matrix.

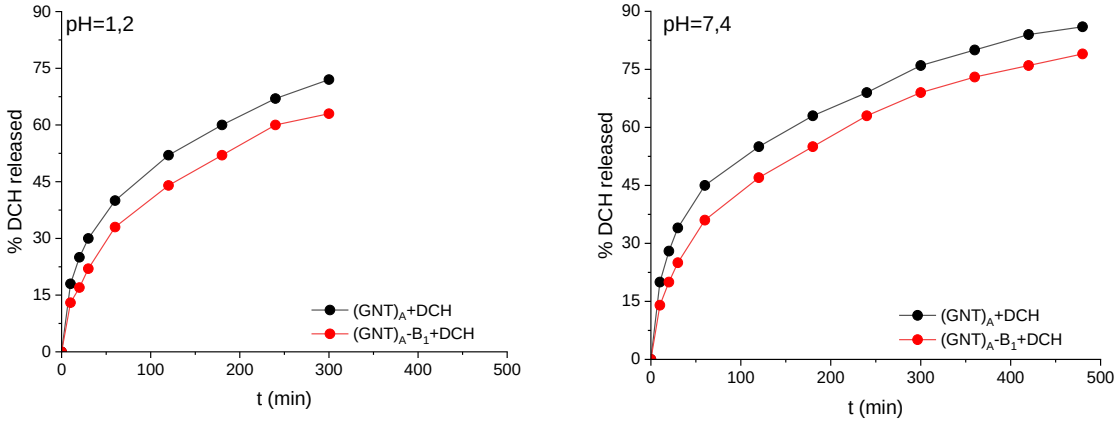

***Figure 6.12**. Release profile of DCH from $(GNT)_A$+DCH and $(GNT)_A$-B_1+DCH microparticles, at pH = 1,2 and pH = 7,4 expressed as a percentage.*

The release of DCH (Figure 6.12) was studied using four mathematical models (Higuchi, 1967; Kopcha et al., 2011; Korsmeyer et al., 1983; Talevi & Ruiz, 2021), concluding

that the release is slower in the case of zwitterionic microparticles and the release mechanism is mainly dictated by diffusion phenomena.

6.3. Zwitterionic hybrid microparticles- tetracycline systems

The ability of hybrid and zwitterionic hybrid microparticles to sorb tetracycline hydrochloride (TCH) was evaluated using different initial drug concentrations (Figure 6.21).

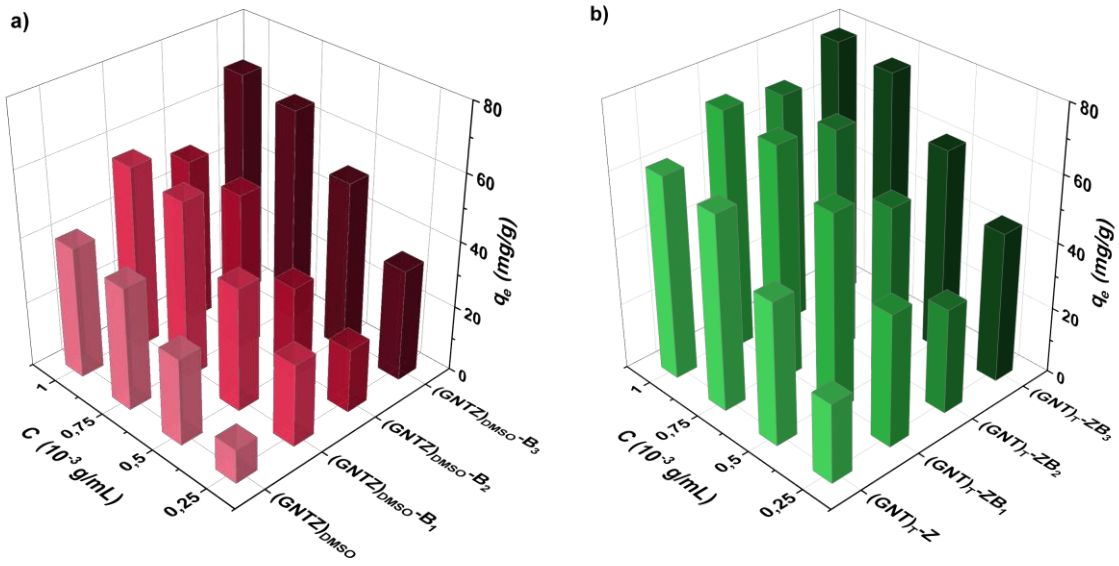

Figure 6.21. *Amount of TCH sorbed by hybrid and zwitterionic hybrid microparticles obtained by zein grafting a) during suspension polymerization or b) after polymerization, for several initial TCH concentrations.*

The sorption capacity increases with increasing initial concentration of the drug solution, and the zwitterionic hybrid microparticles have a higher loading capacity compared to their counterparts that have not been betainized, with the highest amounts of TCH found in the case of $(GNTZ)_{DMSO}$-B_3 microparticles (67 mg/g) and $(GNT)_T$-ZB_3 (78.56 mg/g), respectively.

The release capacity of tetracycline from the hybrid and zwitterionic hybrid microparticles was studied at pH = 1.2 for 2h then at pH = 7.4 until equilibrium was reached with the release curves illustrated in Figure 6.24. The graphical representations show that the release of TCH occurs at a lower speed in the case of hybrid microparticles, compared to the zwitterionic hybrid ones, which, due to a pronounced swelling, allow the rapid diffusion of the drug. The values of the diffusion exponent *n* calculated based on the Korsmeyer-Peppas model between 0.512 and 0.610 suggest that the release mechanism of tetracycline from hybrid/zwitterionic hybrid microparticles is a complex one, resembling a non-Fickian

diffusion. By applying the Kopcha model, it was concluded that the dominant drug release mechanism is diffusion, and the microparticles release the drug without undergoing erosion processes.

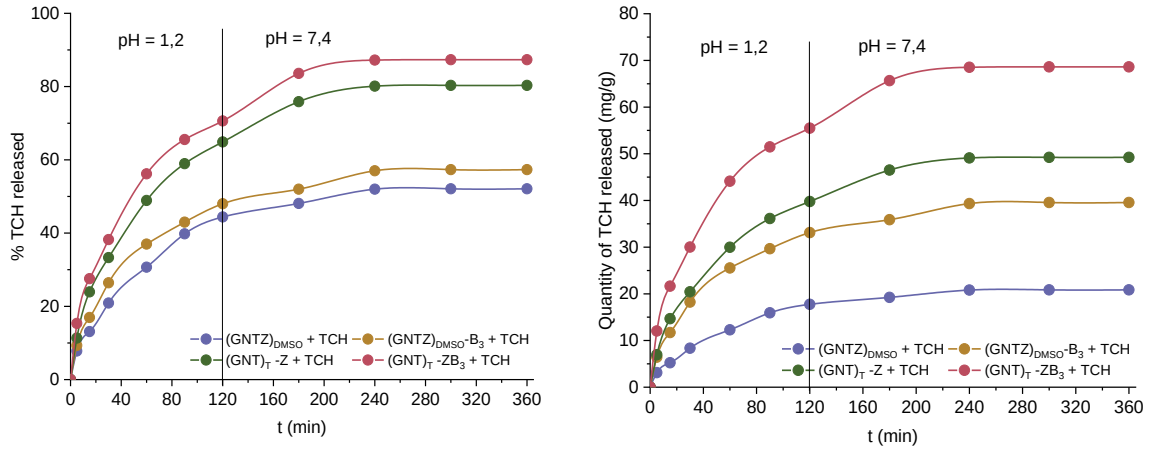

Figure 6.24. *Release curves of TCH from $(GNTZ)_{DMSO}$+TCH, $(GNTZ)_{DMSO}$-B_3+TCH, $(GNT)_T$-Z+TCH and $(GNT)_T$-ZB_3+TCH microparticles.*

6.4. Antimicrobial activity testing of synthesized microparticles

Antimicrobial activity of initial, zwitterionic and hybrid zwitterionic microparticles was tested against four bacterial strains, namely: Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*).

6.4.1. Antimicrobial activity of zwitterionic microparticles

Among the zwitterionic microparticles, sample $(GNT)_A$-B_1 was effective against *K. pneumoniae* and *S. aureus*. There are important differences between $(GNT)_A$-B_1, $(GNT)_A$-B_2 and $(GNT)_A$-B_3 microparticles in terms of antimicrobial activity against *K. pneumoniae* and *S. aureus*, although structurally they differ only by the length of the alkyl chain of the carboxybetaine unit. Since the presence of the $–CH_3$ group located between the cationic and the anionic group induces steric effects, it can be concluded that the antibacterial activity is enhanced when the zwitterionic structure contains the carboxylate group separated from the quaternary nitrogen atom by an alkyl chain of the shortest possible length.

6.4.2. Antimicrobial activity of hybrid and hybrid zwitterionic microparticles

Figure 6.27 shows the antimicrobial activity of both the hybrid microparticles obtained by suspension polymerization and the corresponding zwitterionic hybrid microparticles. $(GNTZ)_{DMSO}$-B_1, $(GNTZ)_{DMSO}$-B_2 and $(GNTZ)_{DMSO}$-B_3 microparticles were highly effective

against all tested bacterial strains (destroying up to 97% of bacterial cells), the antibacterial activity being clearly superior to the precursor and hybrid ones. It appears that the introduction of carboxybetaine-type zwitterionic groups contributes significantly increases antibacterial activity with a major negative impact on bacterial cell viability.

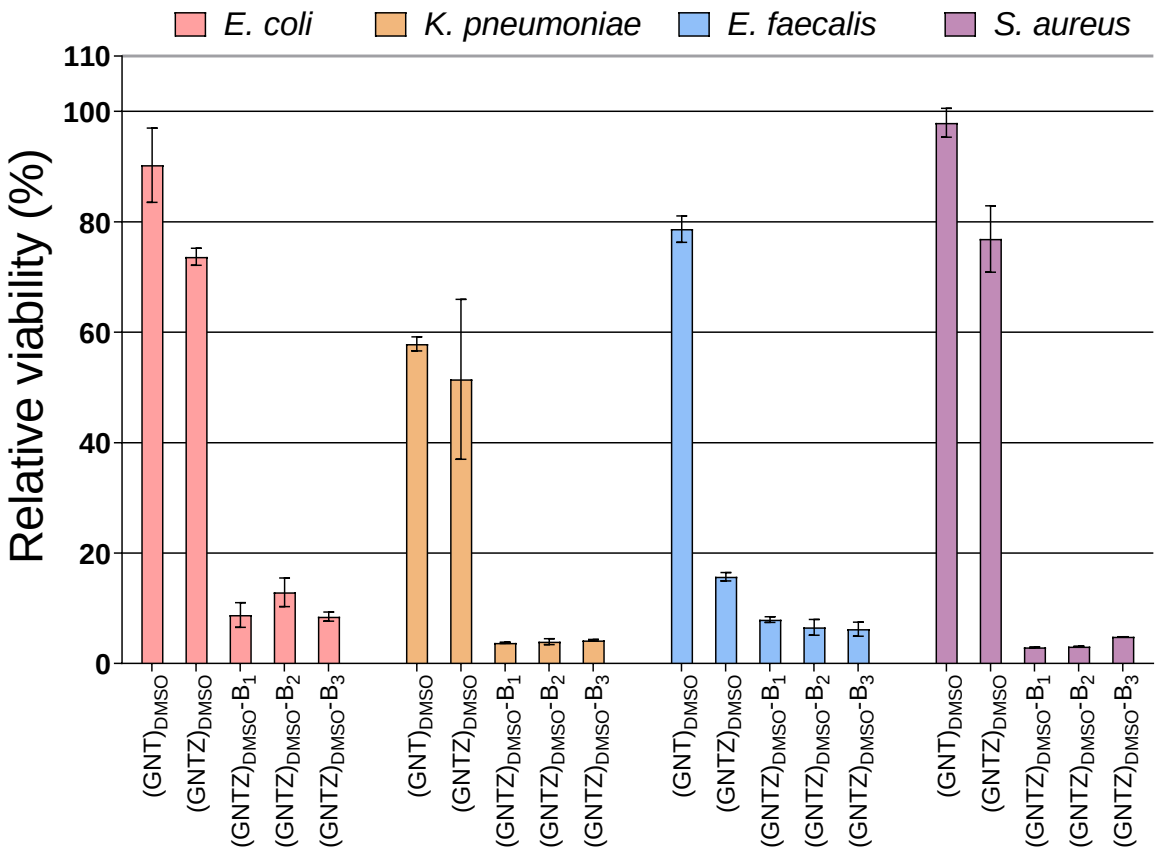

Figura 6.27. *Antimicrobial activity of suspension polymerization hybrid microparticles and corresponding zwitterionic hybrid microparticles against reference strains.*

The hybrid microparticles obtained by post-polymerization grafting of zein and the corresponding zwitterionic hybrids were effective against the bacterial strain *E. faecalis*, killing up to 90% of the bacterial cells. For *S. aureus* only sample $(GNT)_T$-ZB_1 was moderately effective, cell viability being 60%. For the other two bacterial strains tested, non-betainized microparticles had the weakest antibacterial activity, but $(GNT)_T$-ZB_1 microparticles were effective (killing up to 52% in *E. coli* and up to 65% in *K. pneumoniae*).

CHAPTER 7
GENERAL CONCLUSIONS

The original studies presented in this doctoral thesis conclude the following:

- Using the suspension polymerization method, porous microparticles based on N-vinyl imidazole, glycidyl methacrylate and one of the four crosslinkers were synthesized: ethylene glycol dimethacrylate, diethylene glycol dimethacrylate, triethylene glycol dimethacrylate and divinylbenzene.

- The influence of several parameters on the synthesis yield and the specific surface was evaluated, concluding that the best conditions for the synthesis of porous microparticles are: NVI percentage 30%, TEGDMA crosslinker, degree of crosslinking 30%, dilution 0.5, stabilization system composed of PAV, GEL and $MgCl_2$, PAV with molecular mass 52650 g/mol, degree of hydrolysis 88% and stirring speed inside the reactor of 300 rpm (Chapter 3, Subchapter 3.3.);

- Porous microparticles were synthesized under optimal conditions using as porogenic agents: toluene, $(GNT)_T$, n-butyl acetate, $(GNT)_A$, and a mixture both (1:1 v/v), $(GNT)_{TA}$, obtaining spherical microparticles with average diameters between 200 and 350 μm (Chapter 3, Subchapter 3.3.1.);

- The three types of microparticles were betainized using sodium chloroacetate, acrylic acid, methacrylic acid or 1,3-propanesultone. The highest degrees of betainization (determined by the analysis of FTIR spectra) were obtained in the case of sodium chloroacetate and the lowest in the case of 1,3-propanesultone (Chapter 4, Subchapter 4.1.1.);

- The obtained zwitterionic microparticles were characterized by FTIR spectroscopy, SEM-EDAX analysis, atomic force microscopy, mercury porosimetry, optical microscopy and swelling studies (Chapter 4, Subchapters 4.1.2.-4.1.6);

- Hybrid microparticles with zein were synthesized by two methods: grafting by adding zein in the organic phase during suspension polymerization, or post-polymerization grafting on porous microparticles presented in Chapter 3 (Chapter 5, Subchapter 5.1.1.1.-5.1. 1.2.).

- It has been shown that in order to graft as much zein as possible, the process must be carried out under the following conditions: 3% (m/v) zein solution in 80% (v/v) methanol,

temperature 60°C, reaction time of 5 hours and pH = 10 (to favor the opening of the epoxy cycle) (Chapter 5, Subchapter 5.1.1.2.).

- The two types of hybrid microparticles were subjected to the betainization process with sodium chloroacetate, acrylic acid and methacrylic acid (Chapter 5, Subchapter 5.1.1.3.). FTIR spectroscopy confirms that the zein grafting and betainization process proceeded successfully.

- The swelling capacity of the hybrid microparticles increases upon betainization and is mainly influenced by the degree of betainization and not by the nature of the betainizing agent used (Chapter 5, Subchapter 5.1.8.).

- Doxycycline loading on zwitterionic microparticles $(GNT)_A$-B_1, $(GNT)_A$-B_2, $(GNT)_A$-B_3 was carried out. The drug is best sorbed by microparticles at pH = 8.5, temperature of 308 K, contact time of 6 hours. The zwitterionic microparticles retain larger amounts of doxycycline compared to the $(GNT)_A$ sample, and of these, $(GNT)_A$-B_1 accumulates the highest amount of drug (Chapter 6, Subchapter 6.1.1.).

- Langmuir, Freundlich and Dubinin–Radushkevich isotherms are suitable for characterizing the sorption process of doxycycline and suggest that this is a process following an ion exchange mechanism (Chapter 6, Subchapter 6.1.3.).

- The release of doxycycline occurs faster in the case of $(GNT)_T$ microparticles and slower in the case of $(GNT)_A$-B_1 microparticles. The release is faster in an acidic environment due to the increased swelling of the microparticles.

- Hybrid zwitterionic microparticles were loaded with tetracycline hydrochloride, the highest amounts of TCH being sorbed in the case of $(GNTZ)_{DMSO}$-B_3 (67 mg/g) and $(GNT)_T$-ZB_3 (78.56 mg/g) microparticles. In the simulated physiological conditions $(GNT)_T$-ZB_3 microparticles provide the most efficient release (>80% of the amount of TCH initially sorbed).

- The antimicrobial activity of zwitterionic and hybrid zwitterionic microparticles was tested against strains of *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella pneumoniae*. The intrinsic antibacterial properties are enhanced by the presence of zwitterionic groups and zein. The best antibacterial activity is shown by zwitterionic hybrid microparticles based on $(GNTZ)_{DMSO}$, which reduce the viability of all bacterial strains by more than 80% (Chapter 6, Subchapter 6.4.1.).

DISSEMINATION OF RESULTS

The results presented in this doctoral thesis were the subject of 3 ISI indexed articles, 5 oral communications and 2 posters presented at international conferences:

ISI articles:

1. S. Racovita, **M.-A. Trofin**, D. F. Loghin, M.-M. Zaharia, F. Bucatariu, M. Mihai, S. Vasiliu, Polybetaines in biomedical applications, *International Journal of Molecular Sciences*, 22, 9321, 2021 (FI_{2022} = 5.6).
2. **M.-A. Trofin**, S. Racovita, S. Vasiliu, A.-L. Vasiliu, M. Mihai, Porous crosslinked zwitterionic microparticles based on glycidyl methacrylate and N-vinylimidazole as possible drug delivery systems, *International Journal of Molecular Sciences*, 23, 2022 (FI_{2022} = 5.6).
3. **M.-A. Trofin**, S. Racovita, S. Vasiliu, A. Bargan, F. Bucatariu, A.-L. Vasiliu, M. Mihai, Synthesis of crosslinked microparticles based on glycidyl methacrylate and N-vinylimidazole, *Macromolecular Chemistry and Physics*, 2300253, 2023 (FI_{2022} = 2.5).

Oral communications:

1. Zwitterionic porous microparticles containing betaine units with drug delivery capabilities, **M.-A. Trofin***, S. Vasiliu, S. Racovita, M. Mihai, *23rd International Conference of Materials, Methods & Technologies*, 19-22 August 2021, Burgas, Bulgaria.
2. Zwitterionic porous microparticles - Advanced materials as potential drug delivery systems, **M.-A. Trofin***, S. Racovita, S. Vasiliu, A.-L. Vasiliu, M. Mihai, *2nd ICMPP - Open door to the future. Scientific communications of young researchers*, 19 November 2021, Iași, Romania.
3. Porous crosslinked microparticles based on glycidyl methacrylate and n-vinylimidazole as precursors for advanced polymeric materials, **M.-A. Trofin***, S. Racovita, A.-L. Vasiliu, S. Vasiliu, M. Mihai, *12th International Conference on Materials Science & Engineering*, 9-12 March 2022, Brașov, Romania.
4. Release studies of tetracycline from zwitterionic crosslinked copolymer with carboxybetaine units, **M.-A. Trofin***, S. Vasiliu, S. Racovita, M. Mihai, *NextChem*

exploratory workshop –innovative and trans-sectorial technologies, 4th edition, 19-20 May 2022, Bucharest, Romania.

5. New hybrid crosslinked microparticles containing zein for drug delivery, **M.-A. Trofin***, S. Racovita, A.-L. Vasiliu, S. Vasiliu, M. Mihai, *20th Polymers National Symposium*, 5-8 July 2022, Velingrad, Bulgaria.

Posters:

1. Soft materials as zwitterionic porous microparticles with drug delivery capabilities, A.-L. Vasiliu, **M.-A. Trofin**, S. Vasiliu, Ș. Racoviță, D. F. Loghin, M. Mihai, *International Conference on Interfaces*, 21-25 September 2021, Pula, Italia.
2. New zwitterionic copolymer with carboxybetaine moieties as possible drug delivery system, **M.-A. Trofin** , S. Racovita , S. Vasiliu , A.-L. Vasiliu , D. F. Loghin, O. Boita , M. Mihai, *28th International Conference Progress in Organic and Macromolecular Compounds*, *Macro Iași*, 7-9 October 2021, Iași, Romania.

Other results related to this thesis, which were not included in this work, made the object of **2 ISI articles**, **6 oral communications**, and **3 posters** presented during international conferences:

ISI articles:

1. M.-M. Zaharia, C.-A. Ghiorghita, **M.-A. Trofin**, F. Doroftei, I. Rosca, M. Mihai, Multifunctional composites of zwitterionic resins and silver nanoparticles for point-of-demand antimicrobial applications, *Materials Chemistry and Physics*, 275, 125225, 2022 (FI_{2022} = 4.6).
2. M.-M. Zaharia, A.-L. Vasiliu, **M.-A. Trofin**, D. Pamfil, F. Bucatariu, S. Racovita, M. Mihai, Design of multifunctional composite materials based on acrylic ion exchangers and CaCO3 as sorbents for small organic molecules, *Reactive and Functional Polymers*, 166, 2021 (FI_{2022} = 5.1).

Other oral communications:

1. Nano-silver in-situ synthesis in zwitterionic beads, as antimicrobial inorganic/organic composites, M. Mihai, M.-M. Zaharia, C.-A. Ghiorghita, M. M. Bazarghideanu, **M.-A. Trofin***, *23rd International Conference of Materials, Methods & Technologies*, 19-22 August 2021, Burgas, Bulgaria.

2. Synthesis and characterization of quartz sand/polyethyleneimine composite particles with application in water cleaning, F. Bucatariu*, M.-M. Zaharia, L.-M. Petrila, **M.-A. Trofin**, M. Mihai, *11th International Conference on Environmental Engineering and Management*, 8-10 September 2021, Iași, Romania.
3. Daisogel/(polycation/polyanion) composite microparticles with affinity for emerging pollutants, M. Mihai, L.-M. Petrila, **M.-A. Trofin**, M.-M. Zaharia, F. Bucatariu, *11th International Conference on Environmental Engineering and Management*, 8-10 September 2021, Iași, Romania.
4. Synthesis of inexpensive and reusable ion exchange resins with high retention capacity toward heavy metal ions from Tarnița surface waters, M.-M. Zaharia*, F. Bucatariu, M. Ignat, **M.-A. Trofin**, M. Mihai, *11th International Conference on Environmental Engineering and Management*, 8-10 September 2021, Iași, Romania.
5. Zwitterionic grafted gellan: synthesis, solution and gel properties, M. Karayianni, **M.-A. Trofin***, M. Mihai, S. Pispas, *3rd ICMPP - Open door to the future. Scientific communications of young researchers*, 18 November 2022, Iași, Romania.
6. Sulfobetaine functionalized gellan gum: synthesis, solution and gel properties, **M.-A. Trofin***, M. Karayianni, S. Racovita, S. Vasiliu, S. Pispas, M. Mihai, *NextChem exploratory workshop –innovative and trans-sectorial technologies, 5th edition*, 22-23 May 2023, București, Romania.

Posters:

1. Synthesis and characterization of acrylic ion exchange resins and their environmental applications as sorbents, M.-M. Zaharia, A.-L. Vasiliu, **M.-A. Trofin**, M.-M. Bazarghideanu, C. Blegescu, M. Mihai, *28th International Conference Progress in Organic and Macromolecular Compounds*, 7-9 October 2021, Iași, Romania.
2. Electrostatic complexation between zwitterionic grafted gellan gum and zein, **M.-A. Trofin**, M. Karayianni, S. Vasiliu, S. Racoviță, M. Mihai, S. Pispas, *12th Conference of Hellenic Biomaterials Society*, 15-17 December 2022, Atena, Grecia.
3. Synthesis and solution properties of soluble zwitterionic derivatives of gellan gum, **M.-A. Trofin**, M. Karayianni, S. Racovita, S. Vasiliu, S. Pispas, M. Mihai, *37th Conference of European Colloid & Interface Society*, 3-8 September 2023, Napoli, Italia.

Research internships:

1. 25.10 - 07.11.2021, short research internship in the project "Porous zwitterionic microparticles with zein and betaine units with antimicrobial activity and drug delivery capabilities", Institute for Polymer Research, Dresden, Germany
2. 17.09 - 17.10.2022, short research internship in the project "Porous zwitterionic microparticles with zein and betaine units with antimicrobial activity and drug delivery capabilities", Physical and Theoretical Chemistry Institute, National Hellenic Research Foundation, Athens, Greece.

BIBLIOGRAPHY

Costa, L. C., Monteiro, R. C., Castro, H. M. A., Ribeiro, T. S., Oliveira, M. A., Torquato, E. C. C., Arcanjo, M. E., & Marques, M. R. C. (2020). Glycidyl Methacrylate-ethylene Glycol Dimethacrylate Copolymers with Varied Pore Structures Prepared with Different Reaction Parameters. *Materials Research*, *23*(3). https://doi.org/10.1590/1980-5373-mr-2019-0550

Higuchi, W. I. (1967). Diffusional Models Useful in Biopharmaceutics. *Journal of Pharmaceutical Sciences*, *56*(3), 315–324. https://doi.org/10.1002/jps.2600560302

Kopcha, M., Tojo, K. J., & Lordi, N. G. (2011). Evaluation of Methodology for Assessing Release Characteristics of Thermosoftening Vehicles. *Journal of Pharmacy and Pharmacology*, *42*(11), 745–751. https://doi.org/10.1111/j.2042-7158.1990.tb07014.x

Korsmeyer, R. W., Gurny, R., Doelker, E., Buri, P., & Peppas, N. A. (1983). Mechanisms of solute release from porous hydrophilic polymers. *International Journal of Pharmaceutics*, *15*(1), 25–35. https://doi.org/10.1016/0378-5173(83)90064-9

Talevi, A., & Ruiz, M. E. (2021). Baker-Lonsdale Model of Drug Release. In *The ADME Encyclopedia* (pp. 1–7). Springer International Publishing. https://doi.org/10.1007/978-3-030-51519-5_37-1

Trofin, M., Racovita, S., Vasiliu, S., Bargan, A., Bucatariu, F., Vasiliu, A., & Mihai, M. (2023). Synthesis of Crosslinked Microparticles based on Glycidyl Methacrylate and N-vinylimidazole. *Macromolecular Chemistry and Physics*. https://doi.org/10.1002/macp.202300253

Trofin, M.-A., Racovita, S., Vasiliu, S., Vasiliu, A.-L., & Mihai, M. (2022). Porous Crosslinked Zwitterionic Microparticles Based on Glycidyl Methacrylate and N-Vinylimidazole as Possible Drug Delivery Systems. *International Journal of Molecular Sciences*, *23*(23), 14999. https://doi.org/10.3390/ijms232314999