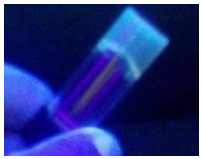
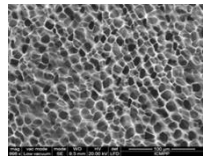
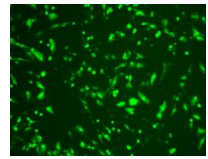
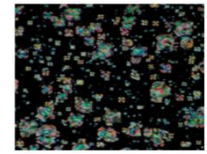
ROMANIAN ACADEMY
"Petru Poni" Institute of Macromolecular Chemistry, Iasi

COMPLEX SUPRAMOLECULAR STRUCTURES WITH BIOMEDICAL APPLICATIONS

– Abstract of the PhD thesis –

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No. 4929/14.X.2016

To .....

This is to inform you that on **October 31st 2016**, at **12:00**, the library of the „Petru Poni" Institute of Macromolecular Chemistry, Iasi will host the public defense of the PhD thesis entitled "**Complex supramolecular structures with biomedical applications**". The thesis was elaborated by Mrs. Daniela Ailincai, chemical engineer, to acquire the PhD degree.

The doctoral jury is composed of

PRESIDENT:

Dr. Valeria HARABAGIU, senior researcher "Petru Poni" Institute of Macromolecular Chemistry, Iasi

SCIENTIFIC SUPERVISOR

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Dr. Silvia DESTRI, Institute for Macromolecular Studies ISMAC, Milan, Italy

According to the Regulations on the organization and PhD thesis defense within the Romanian Academy, please find enclosed a summary of the PhD thesis. Your comments and appreciations will be highly appreciated. You are kindly invited to attend the public presentation of the PhD thesis.

DIRECTOR

Dr. Anton Airinei

ACADEMIA ROMÂNĂ
INSTITUTUL DE CHIMIE MACROMOLECULARĂ "PETRU PONI"
IAȘI

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During the preparation of this thesis, I received the intellectual, moral and affective support of wonderful people, to whom I want to dedicate a page of warmth thanks.

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Special thanks to my colleagues: ***Andrei, Manuela, Anda*** *and* ***Elena*** *for their unconditional support and for making this road a beautiful one, full of laughs and beautiful memories.*

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The support of ***my mother*** *and the personal example she gave me of devotement and work capacity, her love and encouragements made me stronger and gave me the desire to do everything better. For her love, attention and for the education she gave me, I want to thank her with all my heart!*

Thank you!

Iasi, October 2016

The PhD thesis entitled "**Complex supramolecular structures with biomedical applications**" has 205 pages organized in five chapters which include 23 tables, 94 figures, 11 schemes and 260 references. In the next pages, the most significant results of this PhD thesis will be presented. The figures and tables are numbered as in the thesis.

TABLE OF CONTENTS

INTRODUCTION

Supramolecular chemistry, also known as the "chemistry beyond the molecules" is based on the study of molecular recognition and highly ordered assembling, generated by nonconvalent interactions or reversible covalent ones.[1] In 1987, the Nobel Prize for Chemistry was awarded to Donald J. Cram, Jean-Marie Lehn and Charles J. Pedersen for "the development and use of molecules with highly specific and selective structural interactions".[2] This event meant the apparition and recognition of supramolecular chemistry as an important and self-standing field. Due to the fact that supramolecular systems are built from structural blocks which are linked together through noncovalent interactions, they can present sensitivity to external stimuli.[3] These stimuli can be light (for photosensitive materials), some chemical agents (for example metals for systems with metalo-selection)[4], temperature, pH or ionic strength.[5] Thus, by this characteristic of being stimuli responsive, the materials obtained by supramolecular chemistry present a highly adaptive character[6], particularity which enlarges even more their application field.

Even more fascinating is the fact that through supramolecular chemistry can be generated architectures which are very difficult to be obtained through the conventional way of classic covalent chemistry, which involve many synthetic steps and for which the purification is really difficult.

After only 50 years of existence, the supramolecular chemistry was largely used in different domains, including: the development of molecular machines[7], molecular sensors[8], in obtaining systems for gas sorption, chemical catalyzers, nanoreactors[9] or drug delivery systems[10]. Supramolecular chemistry is a discipline which transcends an important number of domains such as: organic chemistry, physical chemistry, coordinative chemistry, macromolecular chemistry, materials science, biological science and many others.

The objectives of researchers who work in the field of supramolecular chemistry involve many and diverse classes of molecules. The most important are: metallo-cycles and metallo-cages obtained through coordinative chemistry metal-ligand[11], nanoentities based on carbon, such as nanotubes, graphene and fullerenes and their supramolecular autoassemblies[12], molecular hosts which contain halogen bonding[13], liquid crystals[14], supramolecular hydrogels or nonviral vectors for gene therapy.[15]

From all the things mentioned up to now, it can be concluded that due to the reversible character of the interactions which govern the principles of supramolecular chemistry, this science has a dynamic character.

The reactions which may be used in dynamic chemistry can be divided in two categories: exchange reactions – which involves the substitution of one reagent with another, generating the same type of chemical bond, such as trans-esterification and disulfide exchange reaction and the second type, the so called forming reaction, which are based mainly on generating new chemical bonds, such as Diels-Alder or aldol reactions. In certain situations, the same chemical reaction can be included in both categories. For example, the Schiff base formation can be considered a forming

reaction, giving rise to imine linkages and in the same time, an exchange reaction if in the system exist two or more amines or aldehydes (trans-imination reaction).

More than this, in the field of the materials which are generated through dynamic covalent chemistry, the chemistry of Schiff base formation represent the most used pathway to the obtaining of supramolecular dynamic assemblies, such as: epitaxial crystals, microtubes, porous amorphous molecules, self-healing films and many others.

The PhD thesis entitled "Complex supramolecular structures with biomedical applications" is coordinated on three different directions of supramolecular chemistry, as follows: dynamic supramolecular structures based on chitosan, hydrophobic-hydrophilic nonviral vectors and PDLC composites (polymer dispersed liquid crystals), all of these constituting current and interesting topics in the field of biomaterials science.

The thesis is organized in two parts, the first one including literature data, while in the second one are described the original results, being structured in five chapters.

Chapter I presents literature data regarding the state of the art in the three different directions approached in the thesis, while the chapters II, III and IV represent the original part.

In the **Chapter II** is presented the obtaining and characterization of some dynamic supramolecular structures based on iminochitosan, being organized in two subchapters, according to the two types of described systems: (i) iminochitosan films and (ii) iminochitosan hydrogels. Both subchapters start with a brief introduction in which is highlighted the importance of the obtained systems and also an argumentation regarding the chosen approach to these aims and the used materials in their obtaining and last but not least, the novelty brought to the field. Both, the iminochitosan films and hydrogels (based on 2-formyl-phenyl-boronic acid (2-FPBA) and citral) have been characterized from the structural point of view, being evidenced the forming of the imine linkage, which is responsible of some morphological peculiarities, demonstrated by atomic force microscopy, scanning electron microscopy and wide angle X-ray diffraction. All the systems, obtained and characterized in detail, were tested, in order to establish their potential applicability in the biomedical field as covering materials for medical devices with antimicrobial properties - in the case of the iminochitosan films, or such as hydrogels with antifungal properties for the treatment of recurrent vulvovaginitis - in the case of the hydrogels based on chitosan and 2-FPBA. The hydrogels based on chitosan and citral will be intensely studied for being applied as drug delivery systems; in this sense, up to now was demonstrated their *in vivo* biocompatibility and also their ability to retain hydrophobic compounds.

Chapter III describes the synthesis and characterization of new nonviral vectors based on hydrophobic-hydrophilic imines for gene delivery, being organized in two main subchapters, according to the two different obtained systems: (i) polycationic systems with a hydrophobic core based on 1,3,5 benzenetrialdehyde and Jeffamine D (JD-PEI) and (ii) polycationic systems with a hydrophobic core based on 1,3,5 benzenetrialdehyde and siloxane (TAS-PEI). The aim of the study was the obtaining of

amphiphilic structures based on reversible imine linkages, which due to the imination and trans-imination processes would be able to self-organize in spherical structures similar to micelles: with a hydrophobic core and a hydrophilic shell. The obtained compounds have been characterized from the structural point of view by ^{1}H-NMR. The theoretical design has been confirmed by transmission electron microscopy, when the spherical morphology of the obtained compounds has been evidenced. The ability of the synthesized compounds to interact with DNA forming polyplexes was evaluated by agarose gel electrophoresis, at different values of the N/P ratio (the molar ratio between the nitrogen atoms from the nonviral vector structure and the phosphorus atoms in the nucleic acid structure). The polyplexes were characterized from the morphological point of view by transmission electron microscopy and atomic force microscopy and their colloidal stability was determined by Zeta potential measurements. Subsequently, biological tests on cells have been done, when the cytotoxicity of the polyplexes and their transfection efficiency were evaluated, tests which confirmed and indicated this method based on the use of the principles of dynamic covalent chemistry and the hypothesis of the amphiphilic compounds as being appropriate for the design and obtaining of efficient nonviral vectors for gene therapy.

Chapter IV includes the obtaining and characterization of new PDLC systems, based on polyvinylalcohol boric acid (PVAB) as a polymeric matrix and two different liquid crystals: a commercial nematogen (5CB); and a smectic liquid crystal (BBO), with antimicrobial activity, designed and obtained in our laboratory. The obtained PDLC systems were characterized by polarized optical microscopy, scanning electron microscopy and differential scanning calorimetry - techniques which allowed us to demonstrate the ability of PVAB to constrain the liquid crystal to form spherical droplets with narrow dimensional polydispersity. The surface characteristics of the PDLC films have been determined by water to air contact angle measurements and surface free energy calculations, indicating that the obtained systems may present potential bioapplications, the values of the water to air contact angle being close to that of the natural cornea (in the case of the BBO liquid crystal).

The thesis ends with a chapter which presents the experimental part (**Chapter V**), which includes the solvents and the reagents used for the synthesis of the compounds presented in the thesis, the experimental procedures and also the methods and the devices used for their characterization.

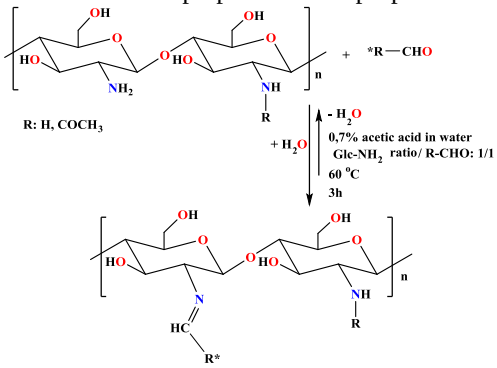
The thesis includes also the dissemination activities of the results obtained during the PhD studies: the list with the published scientific papers and also the list with the participations at different scientific manifestations, and the references used in the thesis.

CHAPTER II
Dynamic supramolecular structures based on chitosan

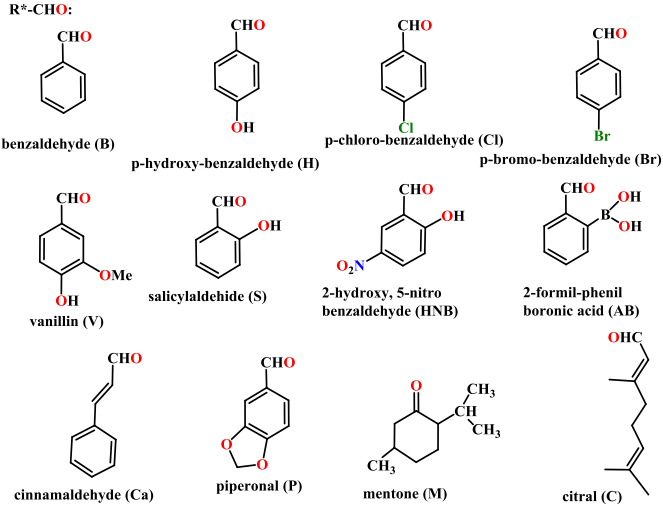
Chapter II had as objective the obtaining and characterization of new dynamic biomaterials based on chitosan, as films and hydrogels.

II.1 Biodynamic iminochitosan films

Twelve iminochitosan biopolimers (**CX**) have been obtained by the acid condensation reaction (Scheme 2) between amine groups of chitosan (**C**) and different monoaldehydes (**X**) (Scheme 3), investigating the effect of the reversibility of the imine linkage formation on the films preparation and properties.



Scheme 2. *The obtaining of the iminochitosan derivatives*



Scheme 3. *The chemical structures of the monoaldehydes used for the obtaining of the biopolymers and their corresponding codes*

FTIR spectroscopy and wide angle X ray diffraction (WXRD) demonstrated the structuring of the biopolymers into a three dimensional morphology, obtained through the alternation of layers of hydrophilic chitosan and hydrophobic imine units. The three dimensional architecture appeared to be facilitated by the solubility of the aldehyde in water, fact which gives more time to the imine linkages to participate in trans-imination processes. More than this, the dynamic mechanical analysis (DMA) data revealed that in the structure of the biopolimeric films remained small amounts of water even after they were dried in the oven, favoring the trans-imination processes and thus the ordering, which continued even in the dried films. The alternation of the hydrophilic/hydrophobic layers assured a very good balance of the hydrophilic/ hydrophobic entities at the surface and led to films with moderate wettability- a first desideratum in obtaining of biocompatible materials (Figure 18).

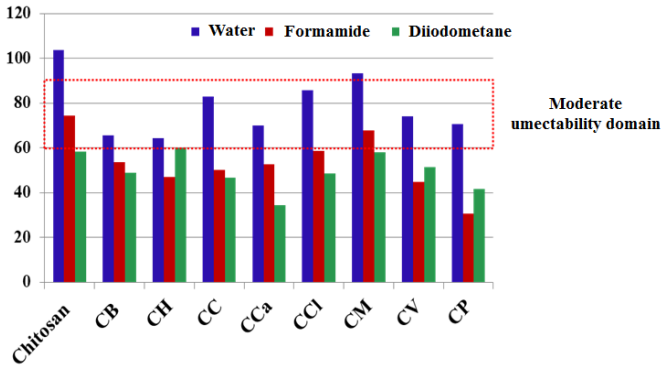
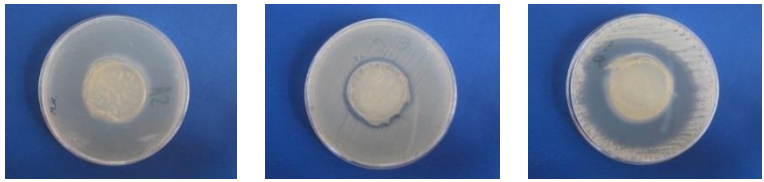
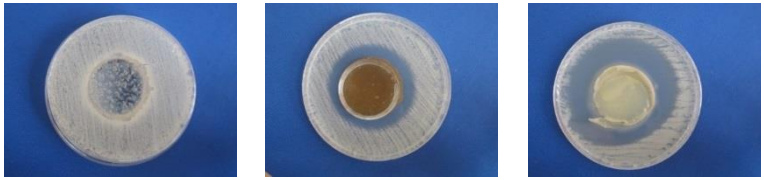


Figure 18. *Mean values of the contact angle of the iminochitosan films with water, formamide and diiodomethane*

All the iminochitosan films presented a nanostructured surface, such as was demonstrated by atomic force microscopy (AFM), which facilitates cells adhesions and proliferation. Moreover, the iminochitosan films presented self-defense properties, being able to inhibit the growth of the microorganisms on large areas around the films (Figure 19) and having real potential in preventing biofilm formation. This is a consequence of the presence of the reversible imine linkages which confer dynamic properties to the films, assuring a slowly release of the antimicrobial aldehyde in the microbiological environment.



a) **b)** **c)**



d) **e)** **f)**

Figure 19. *The antimicrobial activity of the biopolymer CCl against a) S. aureus, b) E. coli, c) C. albicans and the antifungal activity of d) chitosan, e) CP, f) CAB against C. albicans*

This study demonstrated the fact that the grafting of imine units on chitosan chains assisted by the dynamic covalent chemistry, offers interesting opportunities in the development of valuable biomaterials with tunable surface morphologies and structures.

II.2 Hydrogels based on iminochitosan

Hydrogels obtained using chitosan and 2-formyl-phenyl-boronic acid

Chitosan based hydrogels have been prepared using a carbonyl compound named 2-formyl-phenyl-boronic acid (2-FPBA) as crosslinking agent, compound which creates the premises of both chemical crosslinking – *via* imine bonding with the chitosan amine groups, and physical crosslinking – *via* hydrogen bonding of the OH groups of the boronic moieties, respectively. Hydrogels with different crosslinking densities were prepared using variable molar ratios between the chitosan amino groups and the aldehyde functionality of 2-FPBA (Table 6). Due to the fact that NMR spectroscopy revealed the increase in time of the imine linkage density, reaching a maximum in one week, hydrogels which were kept 7 days before lyophilization have been also characterized (marked with *). An interesting observation was the fact that the hydrogels crosslinked with a low amount of aldehyde (H3, H3.5, H3*, H3.5*) collapsed when they were heated at 75 ^{o}C, while those obtained using higher amounts of aldehyde (H1, H2, H2.5, H1*, H2*, H2.5*) kept their integrity when they were heated at 75 ^{o}C or even at higher temperatures. According to the literature, this indicated the predominance of physical interactions into the hydrogels with low amount of 2-FPBA and the predominance of chemical crosslinking in the case of the hydrogels with higher amount of 2-FPBA.

Table 6. *The codes and molar ratios of NH_2/CHO functionalities for the understudy hydrogels*

Code	**H1/ H1***	**H2/ H2***	**H2.5/ H2.5***	**H3/ H3***	**H3.5/ H3.5**	**H3.75/ H3.75***	**H4/ H4***
NH_2:CHO ratio	1:1	2:1	2.5:1	3:1	3.5:1	3.75:1	4:1

The hydrogels which were obtained mainly by chemically crosslinking presented a tridimensional supramolecular architecture based on the segregation of hydrophilic chitosan and hydrophobic aromatic iminoboronate, as was demonstrated by WXRD measurements.

The study of the rheological properties of the obtained systems revealed the fact that hydrogels can be obtained up to a molar ratio between NH_2/CHO functionalities equal to 3.75, data which are in agreement with the visual monitoring of the samples (Figure 28).

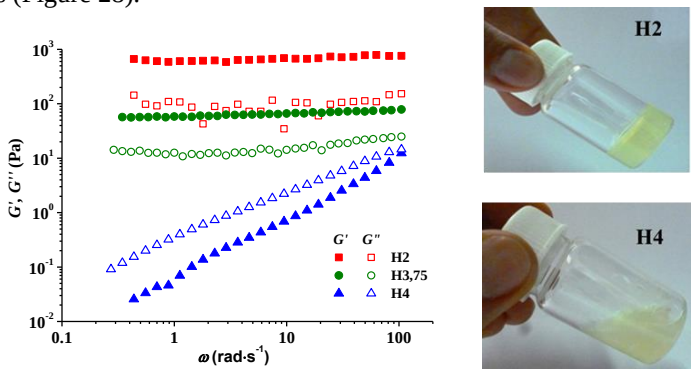


Figure 28. *The dependence of G' and G'' on the frequency for* ***H2****,* ***H3.75***** and* ***H4***** at 37 °C and 1 Pa and pictures of representative hydrogels*

The hydrogels presented high swelling ratios, between 10 and 110, depending on the crosslinking degree and also on the pH of the solution used for the swelling measurements. The antifungal activity of the hydrogels and of the hydrogels components was preliminary tested against two *Candida* strains: *C. glabrata* and *C. albicans* on both planktonic yeast and biofilm. The pure aldehyde killed the *Candida* yeast very fast, while the hydrogels due to the presence in their structure of the reversible imine linkages, and also due to the stabilization of the imine linkage by an intramolecular hydrogen bond, killed the *Candida* yeast with a much slower rate, proving the fact that the hydrogels are able to provide a sustained release of the antifungal aldehyde in the biomimetic medium (Figure 33).

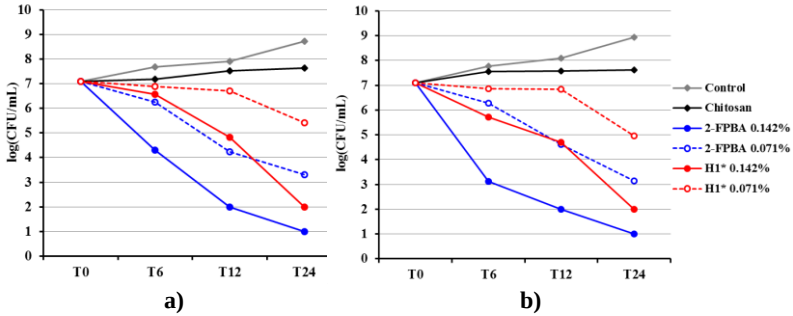


Figure 33. *The evaluation of the antifungal activity of the hydrogels on planktonic yeast of a) Candida albicans and b) Candida glabrata*

More than this, the hydrogels proved a very good antifungal activity against *Candida* biofilms too, the metabolic activity of the biofilm being reduced with more than 99.5% by the hydrogel, in comparison with only 7% by the chitosan used as a

reference, indicating these hydrogels as promising materials for the treatment of recurrent vulvovaginitis infections.

Hydrogels obtained using chitosan and citral

Further, we aimed to obtain hydrogels based on chitosan and citral, a monoaldehyde extracted from lemon oil, which is highly hydrophobic. Due to the antagonistic character of the two components: chitosan - which is hydrophilic and citral - which is hydrophobic, this monoaldehyde should be able to generate auto-assembled supramolecular hydrogels.

Different hydrogels have been prepared by varying the ratio between amine groups of chitosan backbones and aldehyde functionality of citral (Table 11).

Table 11. *The codes and molar ratios of NH_2/CHO functionalities for the understudy hydrogels*

Code	**C1/ C1***	**C2/ C2***	**C2.5/ C2.5***	**C3/ C3***	**C3.5/ C3.5**	**C4/ C4***	**C4.5/ C4.5***
NH_2:CHO Ratio	1:1	2:1	2.5:1	3:1	3.5:1	4:1	4.5:1

All the obtained hydrogels presented a layered supramolecular architecture and a porous morphology, such as was evidenced by WXRD and scanning electron microscopy (SEM) (Figure 38).

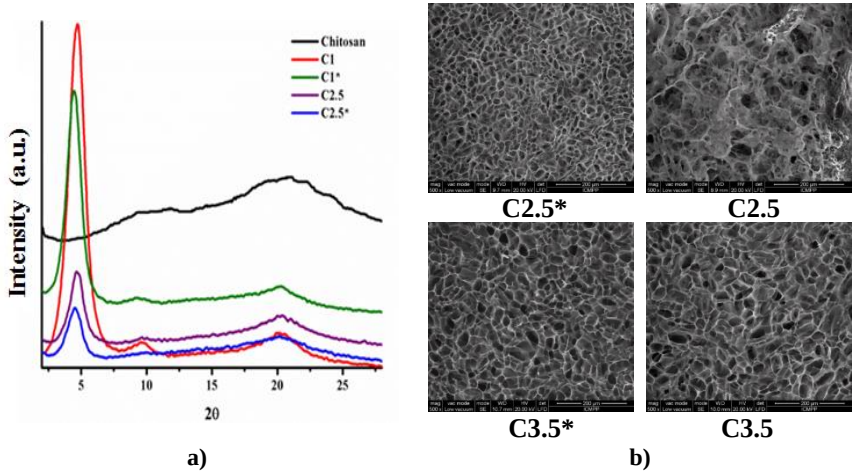
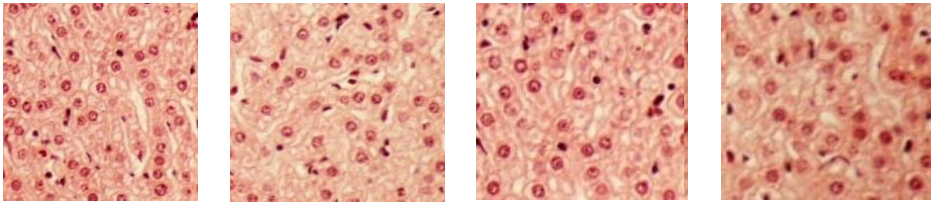


Figure 38. *a)WXRD profiles and b) SEM microphotographs of representative hydrogels*

The viscoelastic behavior of the hydrogels was investigated by rheological measurements, all samples presenting a thixotropic behavior, with reversible sol-gel transitions.

Due to the fact that these hydrogels were designed targeting applications in the biomedical field, such as drug delivery systems or scaffolds for tissue engineering, their *in vivo* biocompatibility was evaluated by determining the hematological,

biochemical and immune profile of the mice who received the understudy hydrogels. The obtained results didn't reveal any significant changes of the tested parameters in the case of the treated animals compared to those who received only physiologic serum, used as control (Figure 44), indicating these hydrogels as biocompatible.



a) **b)** **c)** **d)**

Figure 44. *a) Control group – hepatic section. Col HE x10 and treated group with b) C1, c) C2, d) C3. Col HE x 10*

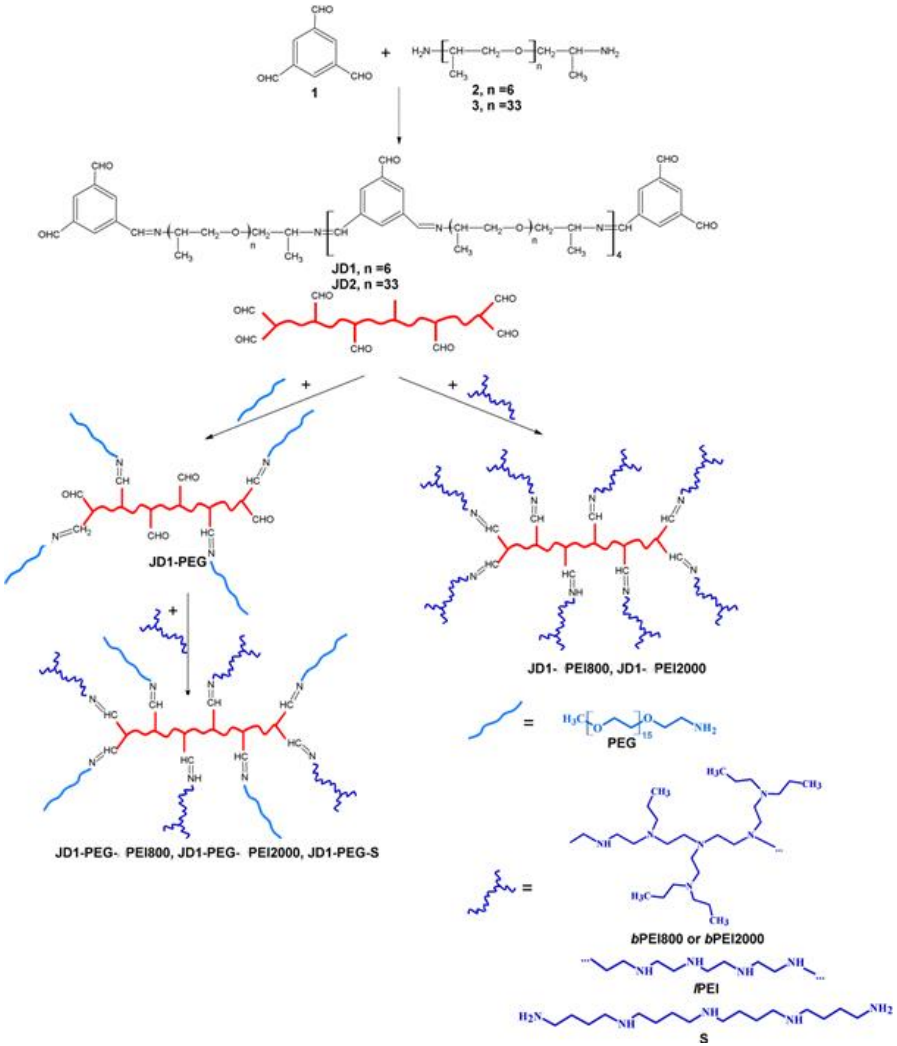
CHAPTER III
Nonviral vectors based on hydrophobic-hydrophilic imines

The objective of this study was the obtaining and characterization of new systems based on hydrophobic-hydrophilic entities, which due to their amphiphilic nature should be able to self-organize in spherical architectures, similar to dendrimers able to interact with DNA and to deliver it into the cell.

To avoid the difficult steps of synthesis and purification, which usually appear in dendrimers preparation, we chose as synthetic pathway the acid condensation reaction between amines and aldehydes. The self-assembling in spherical structures should be favored by the imination and trans-imination processes characteristic to the reversible imine linkage and also due to the natural tendency of energy minimization. In this context, exist the premises that, in an appropriate environment, the obtained compounds to give rise to spherical architectures with a large distribution of the functionalities on the surface which can allow strong interactions with DNA.

III.1 Dynamic systems with hydrophobic core based on trialdehyde and Jeffamine D

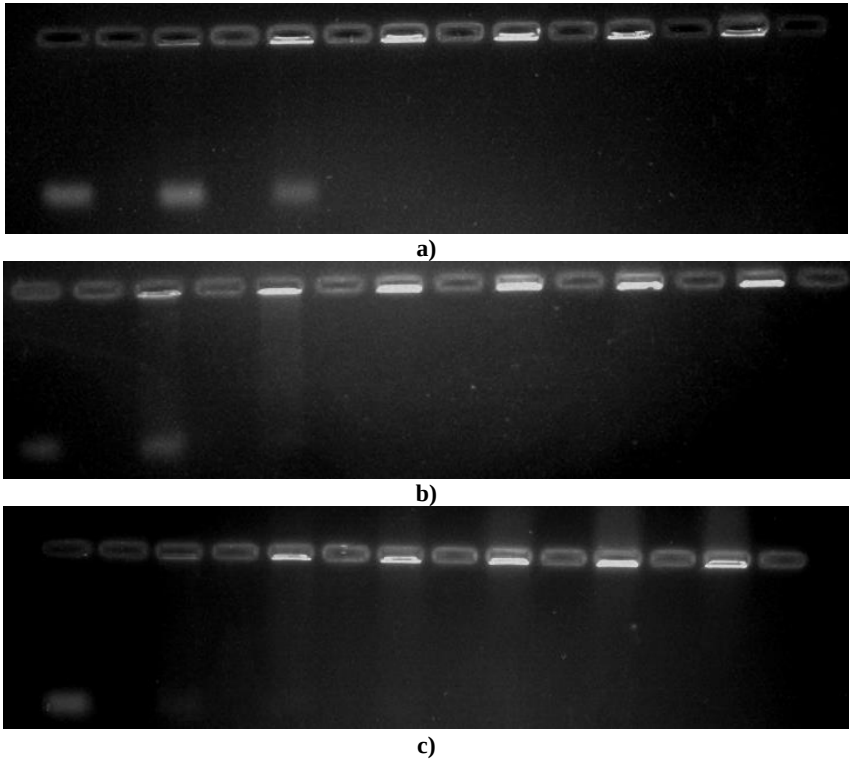
The first series of hydrophobic/hydrophilic compounds which mimic the dendrimers architecture has been synthesized by forming covalent, dynamic linkages starting from the following reagents: 1,3,5 benzenetrialdehyde and Jeffamine D (400 Da and 2000 Da respectively) – as hydrophobic structural entities and hyperbranched polyethylene imine (800 Da and 2000 Da respectively), (bPEI), linear polyethylene imine (lPEI), spermine and polyethylene glycol – as hydrophilic entities (Scheme 7).



Scheme 7. *Synthetic strategy: 1,3,5-benzenetrialdehyde and Jeffamine 400 or Jeffamine 2000 are combined to form hydrophobic JD1 and JD2; The hydrophilic parts have been connected to the hydrophobic core via imine bond formation between JD1 and hydrophilic PEG segments and/or cationic sites: spermine, S; linear polyethylenimine, lPEI; branched polyethylenimine, bPEI800, bPEI2000. The systems water solubile: JD1-bPEI800, JD1-bPEI2000, JD1-PEG-bPEI800, and JD1-PEG-spermin were used for further studies as DNA delivery vectors.*

Agarose gel electrophoresis was used to determine if the obtained water soluble compounds (***JD1-PEG-bPEI800, JD1-PEG-Spermine, JD1-bPEI800*** and ***JD1-bPEI2000***) are able to interact with DNA. The tests have been performed at different values of the N/P ratio (molar ratio between the nitrogen contained in the structure of the compounds and the phosphorus contained in the DNA). This technique

revealed that only ***JD1-PEG-bPEI800, JD1-bPEI800*** and ***JD1-bPEI2000*** reduced the electrophoretic mobility of DNA proving ability to form polyplexes, ability which correlates well with the higher number of nitrogen atoms present in their structure (Figure 51).



a)

b)

c)

Figure 51*. Agarose gel electrophoresis for a)* ***JD1-bPEI800****, b)* ***JD1-bPEI2000*** *for N/P= 0.5; 1; 3; 5; 10; 20 and c)* ***JD1-PEG-bPEI800****, for N/P= 1; 3; 5; 10; 15; 20, at pH=7.4; naked DNA was used as control*

The Zeta potential (ζ) has been investigated for the naked compounds and for their polyplexes using electrophoretic light scattering. It was evidenced that the polycationic ***JD1-bPEI800***, ***JD1- bPEI2000*** and ***JD1-PEG-bPEI800*** samples show, as expected, positive ζ-potential according to their „proton sponge" effect in buffer solution. The polyplexes present a negative Zeta potential at low N/P ratios, and become positive with the increase of the N/P ratios, reaching maximum values of +8, +13 and +7 mV, respectively for the N/P ratio equal to 200 at which a complete DNA binding was reached by agarose gel electrophoresis.

The size and morphology of the compounds and of their polyplexes was evaluated using transmission electron microscopy (TEM) and AFM. The TEM images proved the success of the theoretical design, all three compounds being able to self-assemble in spherical nanoentities. Figure 55 presents representative AFM images for

the polyplexes based on ***JD1-bPEI800*** and on ***JD1-bPEI2000***, at different N/P ratios. As it was observed by AFM, the formed polyplexes have also spherical shape, with nanometric size and narrow dimensional polydispersity. In the case of the polyplex of ***JD1-PEG-bPEI***, no architectures with regular shape could be observed.

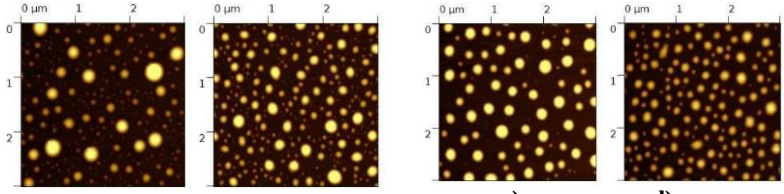
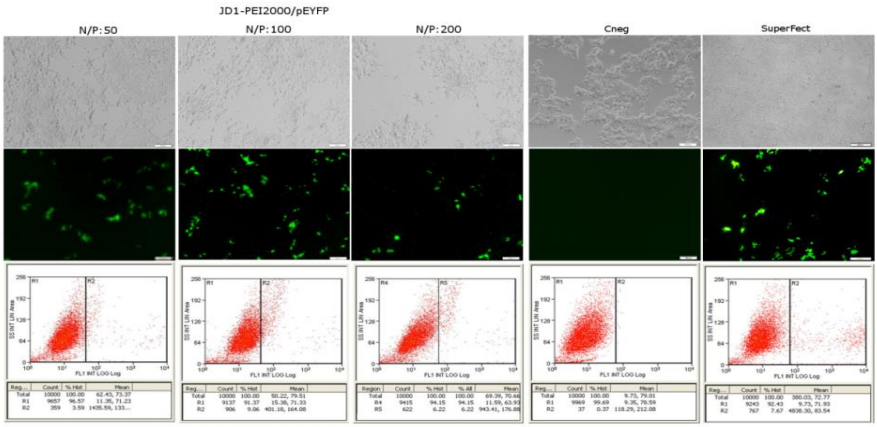


Figure 55*. Atomic force microscopy images for* ***JD1-bPEI 800*** *at N/P=50 and b) N/P=100 and for* ***JD1-bPEI 2000*** *at c) N/P=50 and d) N/P=100*

The cytotoxicity of the compounds and of their polyplexes evaluated on HEK 293T cells using the MTT assay, proved values higher than 80% of the cell viability N/P ratio lower than 100.

The ability of ***JD1-bPEI800***, ***JD1-bPEI2000*** and ***JD1-PEG-bPEI800*** to efficiently deliver DNA into HEK cells, has been followed by evaluating the expression of the fluorescent protein, YFP into the cells at 48 h after transfection with the polyplexes formed between the compounds and a plasmidic DNA which encodes a fluorescent yellow-green protein (pEYFP). The visualization by fluorescence microscopy and the quantitative analysis of transfection using flow cytometry revealed the percent of YFP-positive cells (Figure 58). The results were compared under the same experimental conditions to the commercial reagent SuperFect from Qiagen at its optimal conditions. Among the studied polyplexes, ***JD1-bPEI2000/pEYFP*** shows the higher gene transfection efficacy ~9% at N/P = 100 (a value of 1.2 fold higher than that obtained with the SuperFect commercial reagent).



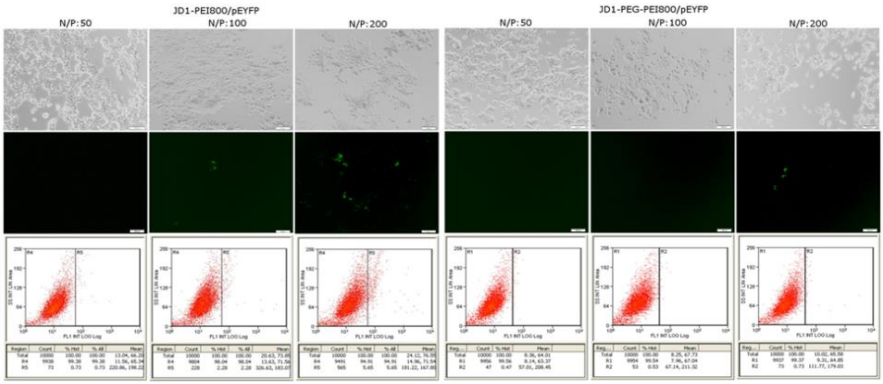


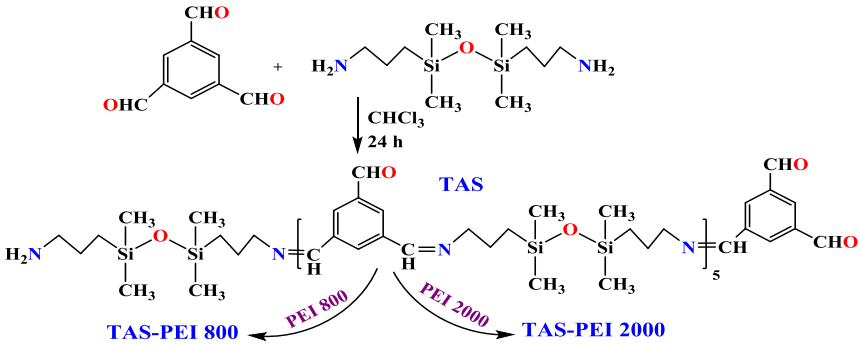
Figure 58. *In vitro pEYFP plasmid transfection assay. Bright-field (upper rows) and the fluorescence (middle rows) visualization of YFP protein expression in HEK 293T cells transfected with* ***JD1-bPEI2000/pEYFP*** *(top) and* ***JD1-bPEI800/pEYFP*** *(down) polyplexes at different N/P ratios. Scale bar 100 μm. Representative flow cytometry dot plots presenting side scatter versus YFP fluorescence (FL1) of transfected HEK 293T cells (lower rows)*

The described study brings an important contribution to the development of gene therapy field, presenting a new method to obtain nonviral vectors with minimum synthetic effort, using the principles of dynamic covalent chemistry.

III.2 Dynamic systems with hydrophobic core based on trialdehyde and siloxane

Taking into consideration the fact that the previously described design based on hydrophobic/hydrophilic imines proved efficiency in transfection, the study was continued using another hydrophobic core based on siloxane – a building block revealed by literature data to confer good transfection efficiency to the non-viral vectors containing it.

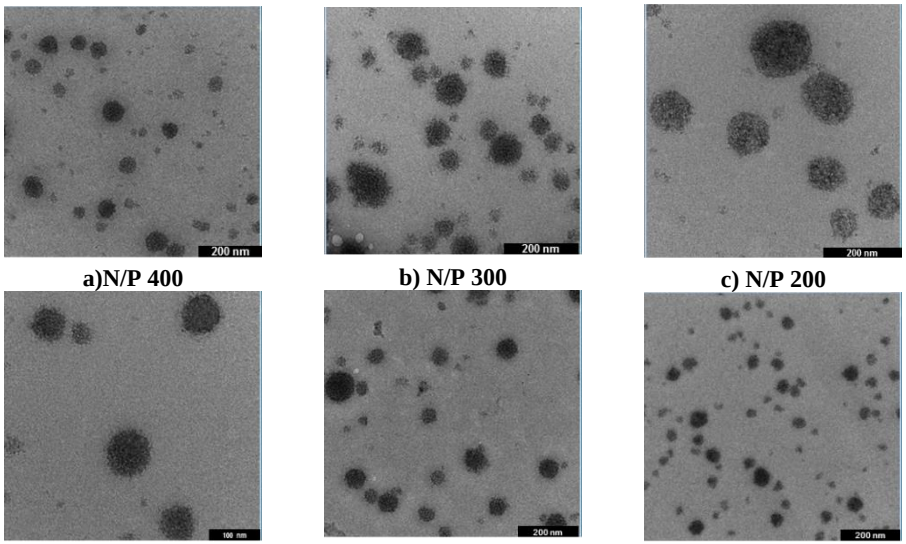
Having this in mind, a new core/shell structure was designed, using the dynamic chemistry of the imine linkage, based on 1,3,5 benzenetrialdehyde, 1,3-bis-(3-aminopropil)-1,1,3,3-tetramethyl siloxane as hydrophobic blocks and PEI 800 or PEI 2000 as hydrophilic blocks (Scheme 8).



Scheme 8. *The obtaining of nonviral vectors* ***TAS-PEI 800*** *and* ***TAS-PEI 2000***

The chemical structure of the obtained compounds has been confirmed by ^{1}H-NMR. To check the theoretical design, the synthesized compounds ***TAS-PEI 800*** and ***TAS-PEI 2000*** have been analyzed using TEM. In both cases, the forming of spherical structures with nanometric size of 20 nm in the case of the compound based on PEI 800 and 95 nm in the case of the compound based on PEI 2000, depending on the molecular weight of the used PEI was observed.

Agarose gel electrophoresis has been used to determine the ability of the synthesized compounds to interact with DNA, using at first a commercial salmon sperm DNA and further, a plasmidic one having 4830 base pairs. Both compounds ***TAS-PEI 800*** and ***TAS-PEI 2000*** proved to be able to reduce the electrophoretic mobility of DNA, being able to form polyplexes and can be used as nonviral vectors in gene therapy. The polyplexes have been further characterized from the dimensional and morphological points of view by TEM, technique which revealed the obtaining of spherical architectures with dimensions in the range 40-100 nm. Figure 66 contains representative images for the polyplexes based on ***TAS-PEI2000*** and ***TAS-PEI800*** at different values of the N/P ratios.



a)N/P 400 **b) N/P 300** **c) N/P 200**

d) N/P 30 **e) N/P 60** **f) N/P 400**

Figure 66. *TEM images for the polyplexes based on* ***TAS-PEI 800*** *(a, b, c) and* ***TAS-PEI 2000*** *(d, e, f) at different N/P ratios*

The cytotoxicity of the polyplexes based on ***TAS-PEI 800*** and ***TAS-PEI 2000*** has been evaluated on Hela cells at different values of the N/P ratio and the obtained data showed that all polyplexes present a low cytotoxicity which allows them to be used as nonviral vectors.

The transfection efficiency has been determined firstly qualitatively by fluorescence microscopy using the reporter gene GFP (green fluorescent protein) and secondly quantitatively by the luciferase test. Both methods indicated that the

transfection efficiency increases with the increase of the N/P ratios and has better values compared to the naked PEI used as control (Figure 68). Also, it was evidenced a higher transfection efficiency in the case of the PEI2000 based compound, in agreement with the results presented in the literature and also with our previous study.

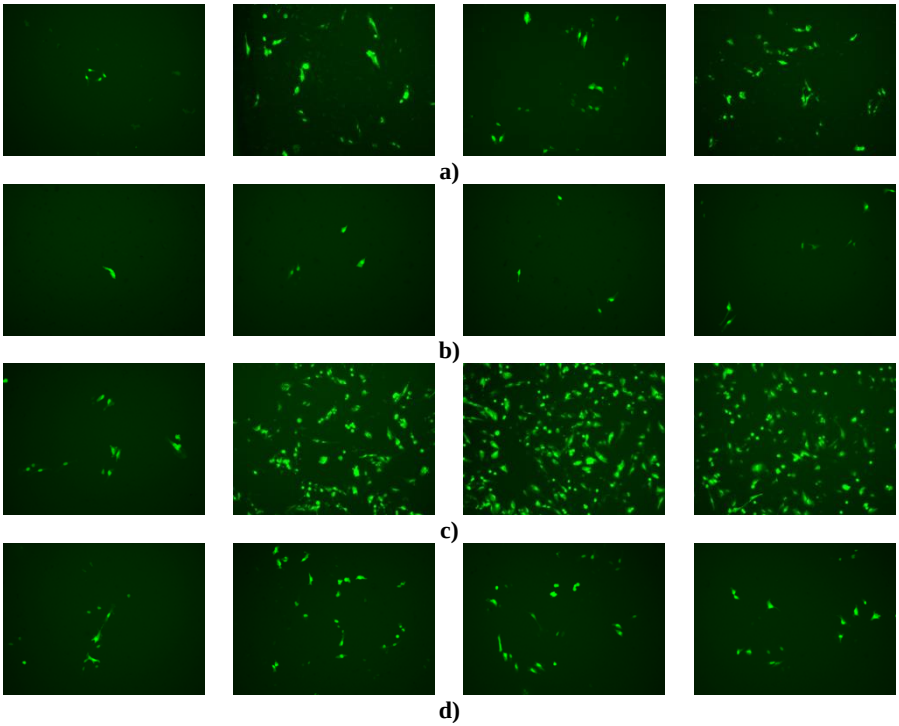


Figure 68. *The visualization of the expression of the GFP protein in the Hela cells, transfected with the polyplexes based on a)* ***TAS-PEI 800*** *and b)* ***PEI 800*** *at different N/P ratios: 100, 200, 400, 600 and c)* ***TAS-PEI 2000*** *and d)* ***PEI 2000*** *at different N/P ratios: 30, 60, 100, 200.*

This study showed once more that the method of using dynamic covalent chemistry in the synthesis of compounds which are going to be used as nonviral vectors in gene therapy is successful and can generate systems which can reach remarkable transfection efficiencies.

CHAPTER IV.
Polyvinyl boric acid – polymeric matrix in obtaining PDLC systems with potential bioapplications

The application of **PDLC** systems in the biomedical field such as artificial irises or biosensors for biological fluids involves the use of biocompatible polymeric matrixes.[16] The polymer which is usually used in these cases is the polyvinyl alcohol (PVA) due to its intrinsic properties: lack of toxicity, completely biodegradability, chemical stability and also film forming ability. Studies dedicated to the obtaining of **PDLC** systems with homeotropic anchoring of the liquid crystal into the polymeric matrix of PVA concluded that in order to reach this desideratum is necessary the use of a third component in the composite material: a surfactant.[17] Its role is to interact with the molecules of the liquid crystal, determining their perpendicular alignment on the polymer surface, changing by this the configuration of the director, the planar anchoring usually generated by PVA being transformed into a homeotropic one. But the use of a supplementary component complicates more the composite formulation and increase the manufacture cost due to the complexity of the involved processes.

In order to avoid this problem, we proposed the replacing of PVA/surfactant mixture with polyvinyl boric acid (**PVAB**) - a polymer which presents the ability to promote an electrostatic attraction with the dipole moment of the liquid crystal molecules, creating by this the premises of homeotropic anchoring.

In this context, two systems of **PDLC** composites have been designed and obtained: a first one using a liquid crystal with nematic mesophase: 4-cyano-4'-penthyl biphenyl (**5CB**) and a second system using a smectic liquid crystal buthyl-p-[p′-n-octyloxy benzoyloxy] benzoate (**BBO**).

The **PDLC** composites have been obtained by the encapsulation method, using different gravimetric ratios between the liquid crystals and **PVAB**.

All composites formed free standing, flexible films. Their structural properties, the droplets forming, their dimension, distribution and configuration, the anchoring effect and the morphological stability have been studied by polarized optical microscopy (POM), differential scanning calorimetry (DSC) and SEM. The surface properties of the composites have been also determined by measuring the contact angle and by calculating the surface free energy of the composites and of their components.

PDLC systems based on PVAB and 5CB

The investigations of the **PDLC** composites obtained using **5CB** liquid crystal have demonstrated that the polymeric matrix facilitates the homeotropic anchoring of the liquid crystal into the droplets, due to the interfacial attraction forces between the electron deficient boron atom of **PVAB** and the electron rich cyan group of the liquid crystal **5CB**. Due to the presence of the boron atom into the structure of **PVAB**, an appropriate ratio between the interfacial forces and the attraction forces is reached, leading to a uniform distribution and a narrow dimensional polydispersity of the droplets in the case of the composites with higher amount of liquid crystal 40% (Figure 71). The droplets diameter is around 4 μm, dimension which is ideal for

optoelectronic applications.

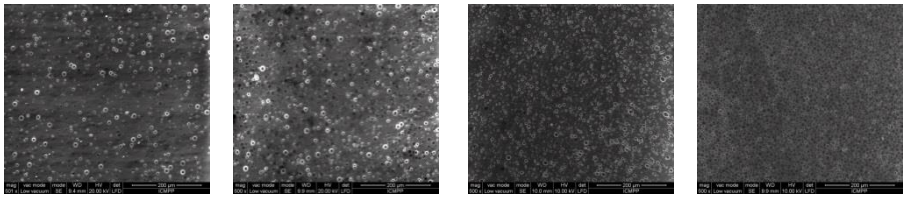


Figure 71. *SEM microphotographs of the PDLC films*

The immiscibility of the composite components **PVAB** with the liquid crystal prevents the loss of liquid crystal consumed as a plasticizer of the polymeric matrix, fact which brings two advantages: (i) the use of a smaller amount of liquid crystal to obtain a high content of microsized droplets and further a lower cost and (ii) the optical properties of the polymeric matrix are preserved in the **PDLC** composites. More than this, the contact angle measurements and the surface free energy calculations revealed a potential biocompatibility of the prepared composites.

This study proved that **PVAB** can successfully replace the PVA polymer when a homeotropic alignment of the liquid crystal into the droplets is desired.

PDLC systems based on PVAB and BBO

On the other hand, the obtaining of **PDLC** composites using a smectic liquid crystal and **PVAB** proved the ability of this polymeric matrix to constrain the growth of the smectic liquid crystal into spherical droplets with planar alignment and a mean diameter of 7 μm, an appropriate value for optoelectronic applications (Figure 95).

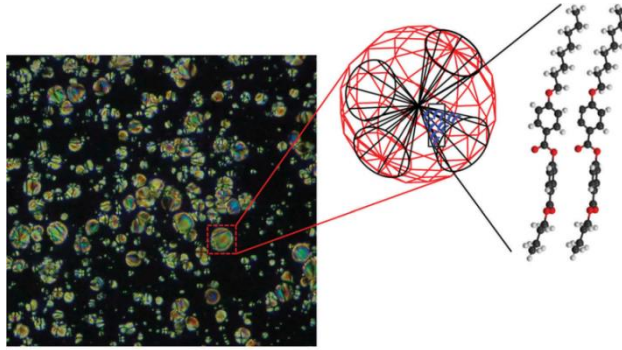


Figure 95. *POM image of a PDLC film and the topology of the Smectic A polygonal texture in a droplet*

Also in this case, it was observed the fact that the components, **PVAB** and **BBO** are completely immiscible, fact which constitutes a great advantage. The values of the surface free energy of the **PDLC** films are high enough to assure the capacity of the systems to respond at electric stimuli and to assure the cellular adhesion. More

than this, the hydrophilicity of the PDLC films can be tuned by controlling the morphology of the **PVAB** matrix, in such a manner that fulfills the requirements of the multiple biomedical applications.

It is very important to mention that the used smectic liquid crystal **BBO** has been especially designed to be used in bioapplications. In this sense, the liquid crystal had a smectic mesophase stable on a large range of temperature (69-28 ^{o}C), including the human body temperature, a direct transition isotropic - smectic and last but not least, it presents antimicrobial activity against many pathogenic agents (Figure 84).

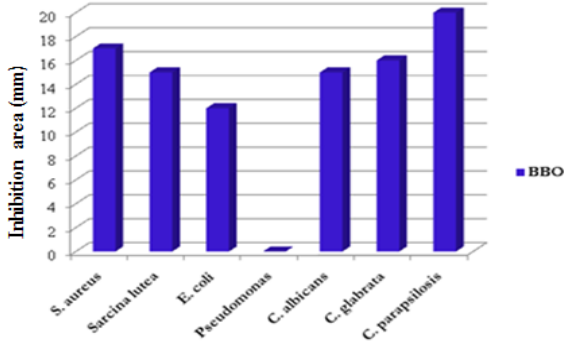


Figure 84. *The obtained inhibition areas using BBO liquid crystal against different microorganisms*

GENERAL CONCLUSIONS

The PhD thesis entitled "**Complex supramolecular structures with biomedical applications**" is structured in two parts: a literature study (Chapter I) and original contributions (Chapters II-IV) and it ends with the experimental part (Chapter V) and general conclusions.

The first chapter presents literature data regarding the three different directions addressed in the thesis: dynamic supramolecular structures based on chitosan, nonviral vectors based on hydrophobic/hydrophilic imines and PDLC composites.

The original results, organized in three chapters, have focused on the following directions:

- ✓ The obtaining and characterization of iminochitosan derivatives, as films and hydrogels
- ✓ The synthesis and characterization of nonviral vectors based on hydrophobic/hydrophilic imines
- ✓ The obtaining and characterization of PDLC systems obtained using PVAB as a polymeric matrix

The studies lead to the following conclusions:

1. Twelve iminochitosan biopolymers have been obtained by the acid condensation in aqueous solution of the amine groups of chitosan backbones and different monoaldehydes with antimicrobial properties.

- The FTIR spectroscopy revealed the forming of the imine linkage, and also the reorganization of the intra- and inter-molecular hydrogen bonds.
- All biopolymers presented a layered supramolecular architecture due to three ordering forces: hydrophilic/hydrophobic segregation, intermolecular hydrogen bonding between the chitosan chains and aromatic-aromatic interactions, as was demonstrated by WXRD.
- The film surface presented small values of the roughness, characteristic to smooth films and also small values of the phase contrast shift, indicating differences in terms of relief and not variations of the chemical compositions.
- All the films presented moderate wettability, making them promising candidates for being used as scaffolds in tissue engineering.
- The antimicrobial tests against *Candida albicans, Staphilococcus aureus* and *Escherichia coli* evidenced the fact that due to the imine forming reversibility, the films present dynamicity, being able to assure a slowly release of the aldehyde into the microbiological media.

2. Dual crosslinked hydrogels have been obtained using for the first time 2-formyl-phenyl-boronic acid (2-FPBA) as chitosan crosslinker in different ratios of amine and aldehyde functionalities.

- The synthetic pathway has been verified by synthesizing a model compound by reacting 2-FPBA with D-glucosamine - the structural unit of chitosan.
- FTIR and NMR spectroscopy performed on the model compound and on the hydrogels demonstrated that the formed imine is stabilized by an intra-molecular hydrogen bond.
- The NMR spectroscopy revealed the increase in time of the imine linkage density reaching a maximum in seven days
- Iminoboronate chitosan hydrogels present a layered tridimensional morphology, as was demonstrated by WXRD.
- The gelation limit was established at a molar ratio between amine/aldehyde functionalities equal to 3.75/1, by rheological measurements.
- The swelling ratio of the hydrogels, measured in three different solutions: water, acetate buffer and phosphate buffer presented values between 10-110, depending on the crosslinking density and on the pH of the media in which was evaluated the swelling process.
- The hydrogels proved strong antifungal activity against two *Candida* strains: *Candida glabrata and Candida albicans*, on both planktonic yeast and biofilm.

3. New hydrogels have been obtained based on chitosan and using for the first time citral as a crosslinking agent, in different ratios of amine and aldehyde functionalities.

- The synthetic pathway has been verified by synthesizing a model compound by reacting 2-FPBA with D-glucosamine - the structural unit of chitosan.
- The forming of imine linkages in the model compound and in the hydrogels has been evidenced by NMR and FTIR spectroscopy.
- The NMR spectroscopy revealed the increase in time of the imine linkage density reaching a maximum in ten days.
- Citral-chitosan based hydrogels present a layered tridimensional morphology, as was showed by WXRD.
- The gelation limit was established to correspond to a molar ratio between amine/aldehyde functionalities equal to 4.2/1, by rheological measurements.
- All hydrogels presented a thixotropic behavior.
- The swelling ratio of the hydrogels, measured in three different solutions: water, acetate buffer and phosphate buffer presented different values, depending on the crosslinking density and on the pH of the media in which was evaluated the swelling process.
- The *in vivo* biocompatibility tests demonstrated that the hydrogels are biocompatible.
- The hydrogels proved to be able to adsorb a fluorescent hydrophobic compound, fact which demonstrated their potential to be used for the delivery of hydrophobic drugs.

4. Different amphiphilic compounds have been synthesized by forming dynamic reversible imine linkages between a hydrophobic core (based on benzenetrialdehyde and Jeffamine D) and different hydrophilic shells: hyperbranched polyethylene imine with a molecular weight of 2000 Da and 800 Da, linear polyethylene imine, spermine and polyethylene glycol.

- The hydrophobic core was obtained as a linear chain with 8 aldehyde groups, as demonstrated by NMR spectroscopy and GPC.
- TEM performed on the amphiphilic compounds revealed their spherical morphology.
- The compounds with the hydrophilic shell based on PEI with the molecular weight of 800 Da or 2000 Da and the one with the hydrophilic shell containing PEG and PEI 800 proved a high ability to complex DNA, forming polyplexes, as was demonstrated by agarose gel electrophoresis.
- The polyplexes presented a spherical morphology evidenced by AFM, with nanometric size and narrow dimensional polidispersity.
- The viability of the HEK cells in the presence of the polyplexes remain very high (more than 80%), showing that the polyplexes are biocompatible.
- The transfection efficiency demonstrated that the compound based on PEI 2000 Da presents the highest transfection efficiency, even higher than a commercial transfection reagent, used as reference.

5. Two amphiphilic compounds have been synthesized by forming dynamic reversible imine linkages between a hydrophobic core (based on an aromatic trialdehyde and 1,3-bis-(3-aminopropil)-1,1,3,3-tetramethyl siloxane) and two different hydrophilic

shells: hyperbranched polyethylene imine with a molecular weight of 2000 Da and hyperbranched polyethylene imine with a molecular weight of 800 Da.

- The hydrophobic core was obtained as a linear chain with 9 aldehyde groups, as demonstrated by NMR spectroscopy and GPC.
- The amphiphilic presented spherical morphologies.
- Both compounds formed polyplexes with DNA, as was demonstrated by agarose gel electrophoresis.
- The polyplexes were nanosized spherical entities, as revealed by TEM.
- The viability of the Hela cells in the presence of the polyplexes remain very high (more than 90%), showing that the polyplexes are biocompatible and can be used as gene delivery systems.
- The qualitatively and quantitatively transfection efficiency tests indicated the obtained systems as efficient nonviral vectors for gene delivery.

6. New PDLC composites have been obtained using for the first time PVAB as a polymeric matrix and two liquid crystals: a commercial nematogen, 5CB and a smectic liquid crystal designed and synthesized in our laboratory, BBO.

- POM, SEM and DSC techniques revealed the ability of PVAB to generate microsized droplets.
- The values of the contact angle and of the surface free energy of the PDLC films indicated a potential biocompatibility of these systems, making them suitable for bioapplications.
- The smectic liquid crystal BBO presented antimicrobial activity against *Staphylococcus aureus* and *Sarcina lutea* - gram positive bacteria, *Escherichia coli* - gram negative bacteria and *Candida albicans*, *Candida glabrata* and *Candida parapsilosis* - fungi.

*The original results presented in the thesis were published as scientific articles in international and national journals (**7 scientific papers**) as follows*:

Articles published in recognized international journals (ISI ranked journals)

1. Luminita Marin, **Daniela Ailincai**, Elena Paslaru**,** Monodisperse PDLC composites generated by use of polyvinylalcohol boric acid as matrix, *RSC Advances*, **2014**, 4, 38397 -38404. (**ISI: 3,289**)
2. **Daniela Ailincai**, Andrei Bejan, Irina Titorencu, Mioara Dobrota, Bogdan C. Simionescu, Imino-chitosan derivatives. Synthetic pathway and properties, *Revue Roumaine de Chimie*, **2014**, 59, 385-392. **(ISI: 0,21)**
3. Luminita Marin, **Daniela Ailincai**, Mihai Mares, Elena Paslaru, Mariana Cristea, Valentin Nica, Bogdan C. Simionescu, Imino-chitosan biopolymeric films. Obtaining, self-assembling, surface and antimicrobial properties, *Carbohydrate Polymers*, **2015**, 117, 762-770. **(ISI: 4,2)**
4. **Daniela Ailincai**, Luminita Marin, Sergiu Shova, Cristina Tuchilus, Benzoate liquid crystal with direct isotropic-smectic transition and antipathogenic

activity, *Comptes Rendus Chimie*, **2015,** doi. org/10.1016/j.crci.2016.01.008. **(ISI: 1,798)**

5. **Daniela Ailincai**, Cosmin Farcau, Elena Paslaru & Luminita Marin, PDLC composites based on polyvinyl boric acid matrix – a promising pathway towards biomedical engineering, *Liquid Crystals*, doi:10.1080/02678292.2016.1172353 (**ISI: 2,244**)
6. **Daniela Ailincai**, Luminita Marin, Simona Morariu, Mihai Mares, Andra-Cristina Bostanaru, Mariana Pinteala, Bogdan C. Simionescu, Mihai Barboiu, Dual crosslinked iminoboronate-chitosan hydrogels with strong antifungal activity against Candida planktonic yeasts and biofilms, *Carbohydrate Polymers*, **2016**, 152, 306–316. (**ISI: 4,2**)
7. Luminita Marin, **Daniela Ailincai**, Manuela Calin, Daniela Stan, Cristina Constantinescu, Laura Ursu, Florica Doroftei, Mariana Pinteala, Bogdan C. Simionescu, Mihai Barboiu, Dynameric Frameworks for DNA Transfection, *ACS Biomaterials Science & Engineering*, **2016, 2, 104-111**.

Articles published in recognized international journals (ISI ranked journals) which are not included in the thesis

1. **Daniela Ailincai**, Helmut Ritter, Cyclodextrin-poly(ε-caprolactone) based nanoparticles able to complex phenolphthalein and adamantyl carboxylate, *Beilstein Journal of Nanotechnology*, **2014**, 5, 651–657. (**ISI:2,778**)

National and European projects – member in the team

1. Research assistant: "*Biologically inspired systems for engineered structural and functional entities*", PNII-ID-PCCE-2011-2-0028.
2. Research assistant: "*Diode electroluminiscente organice flexibile cu emisie in alb pentru iluminare*", PNII-PT-PCCA-2013-4-1861.
3. Research assistant:"*Multifunctional dynamic hydrogels with tuned morphology for biomedical applications*", PN-II-RU-TE-2014-4-2314.
4. Research assistant:*"SupraChem Lab"*, Horizon 2020 WIDESPREAD 2-2014: ERA Chairs.
5. Research assistant:" *New approaches in designing polymer surfaces with controllable pattern for applications in biomedicine and high technologies*", PN-II-RU-TE-2014-4-2976.

Mobilities during the PhD studies

1. At the **Institute of Nuclear Chemistry and Technology**, Warsaw, **Polland** in the framework of program **Erasmus+** *"Joint innovative training and teaching/learning program in enhancing development and transfer knowledge of application of ionizing radiation in materials processing".*

2. At the **University of Palermo**, **Italy** in the framework of the program **Erasmus+** *"Joint innovative training and teaching/learning program in enhancing development and transfer knowledge of application of ionizing radiation in materials processing".*

Participations at national and international conferences

a) Oral Communications

1. **Daniela Ailincai**, Luminita Marin, Dragos Peptanariu, Daniela Stan, Cristina Ana Constantinescu, Manuela Calin, Mariana Pinteala, Mihail Dumitru Barboiu, Synthese et caracterisation d'un nouveau transporteur non-viral d'ADN, a base de benztrialdehyde, Jeffamine D et polyethyleneimine (pei) ramifie, *8ème Colloque Franco-Roumain de Chimie Appliquée (COFrRoCA)*, **2014**, Montpellier, France.
2. **Daniela Ailincai**, Luminita Marin, Mihai Mares, Bogdan C. Simionescu, The synthesis and characterization of new imino-chitosan biopolymeric films with antimicrobial properties, *3ème Colloque Franco-Roumain de Chimie Médicinale*, **2014**, Iasi, Romania.
3. **Daniela Ailincai**, Non-Viral Gene Delivery Vectors based on Dynamic Hydrophobic – Hydrophilic Imines, *Seventh Cristofor I. Simionescu Symposium Frontiers in Macromolecular and Supramolecular Science*, **2015**, Iasi, Romania.
4. **Daniela Ailincai**, Bogdan C. Simionescu, Luminita Marin, Polymer dispersed liquid crystals based on polyvinylalcohol boric acid matrix, *International Conference on Materials Science, Applied Physics and Chemistry*, **2015**, London, Great Britain.
5. **Daniela Ailincai**, Mariana Pinteala, Mihai Barboiu, Luminita Marin, Chitosan iminoboronate hydrogels with antifungal activity, *ACS on Campus*, **2016**, Bucharest, Romania.
6. Daniela Ailincai, Mariana Pinteala, Bogdan C. Simionescu, Mihai Barboiu, Luminita Marin, Hydrogels based on chitosan and 2-formylphenyl boronic acid – promising materials for the treatment of Candida infections, *XII*th *French-Romanian Polymer Meeting*, **2016**, Sophia Antipolis, France.
7. Daniela Ailincai, Luminița Marin, Chitosan based hydrogels *via* iminoboronate motif as promising materials for the treatment of candidiasis, **2016**, A-XXXIV-a The National Conference of Chemistry, Căciulata, Romania.

b) Poster presentations

1. **Daniela Ailincai**, Mariana Pinteala, Bogdan C. Simionescu, Luminita Marin, Chitosan Iminoboronate Hydrogels – New Promising Materials for the Treatment of Candidiasis, *Eighth Cristofor I. Simionescu Symposium – Frontiers in Macromolecular and Supramolecular Science*, June, **2016**, Iasi, Romania.
2. Geta David, Adina Coroaba, Laura Elena Ursu, Dragos Peptanariu, **Daniela Ailincai**, Mariana Pinteala, Hybrid biopolymer/synthetic multilayer micro-/nanocapsules for drug/gene delivery, *4*th *International Conference on Multifunctional, Hybrid and Nanomaterials*, March, **2015**, Sitges, Spain.

3. **Daniela Ailincai**, Luminita Marin, Carmen-Mihaela Popescu, Supramolecular chitosan hydrogels via reversible imine linkage, *2nd EPNOE Junior Meeting*, October, **2016**, Sophia Antipolis, France

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