FINAL SCIENTIFIC REPORT
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(June 2012 - September 2016)

Project name:
BIOLOGICALLY INSPIRED SYSTEMS FOR ENGINEERED STRUCTURAL AND FUNCTIONAL ENTITIES

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Current report contains exclusively, as references, the publications of the group with acknowledgments for PNII-ID-PCCE-2011-2-0028 project (Contract no. 4/2012)

The project focused on two major directions: (i) *functional morphology mimicking*, to elucidate and exploit the relationship between biological structures and their functions, and (ii) *engineering the nano-scale biomolecular specific interactions and self-organization*, to reproduce living-matter mechanisms, studying, in a chemical approach (**to build structural and functional entities, able to mimic extra- and intra-cell components involved in specific mechanisms of living-matter, such as gene trafficking and extracellular matrix guided remodelling**). The expected benefits of the studies are to be associated to **individualized / personalized medicine**, but also to the **genetic engineering** and **genetic cell reprogramming** scientific fields. ***The "philosophy" of the project consists in applying advanced chemical conjugation methods to obtain new biomimetic (macro)molecular systems able to substitute or to complement functional architectures specific to the living-matter***.

The following four major **goals** were considered:

1. **A histone-mimetic "artificial nucleosome"**, fluorimetric traceable, able to act as a non-viral gene delivery system, with a packaging capacity of about 2 ÷ 5 kb (kilobases).
2. **A non-viral mimetic vehicle** (including hydrogels) for deliberately and controlled introduction of **special active compounds into cells**, based on polymers and polymeric adducts.

3. **An osseous callus-type biomimetic mineralization system with gene transfection ability**, immune-evasive and able to carry biochemical or pharmaceutical species, based on natural and synthetic macromolecules with cell recognition domains, active as a substrate for *ex-vivo* cell culturing **and potentially injectable in bone injured sites, in order to guide the cell-driven tissue restoration.**
   This goal represents an immediate application of the first two.
4. **Developments in investigative methods used in gene therapy or drug delivery systems.**

The first and the third goals involved new and innovative approaches in packaging and vehiculating one or several DNA segments, large enough to carry the required information to induce significant cell transformation. The second goal involved new and innovative approaches in loading, vehiculating and releasing drugs.

The considered targets of mimicking were: (i) a **"synthetic" nucleosome**, a polyplex-type ensemble able to support the biomimetic way of DNA packaging and trafficking, (ii) a **gene and drug transfection vehicle that emulates viral mechanisms using non-viral constituents**, acting as a versatile carrier optionally operable in magnetic field, and (iii) a **controlled mineralized substitute of the bone callus**, applicable in guided bone fracture healing as an adjuvant for extracellular matrix reinforcing.

The project was multidisciplinary, involving high expertise in chemistry, biochemistry, physics and biology, ensured by the project partners.

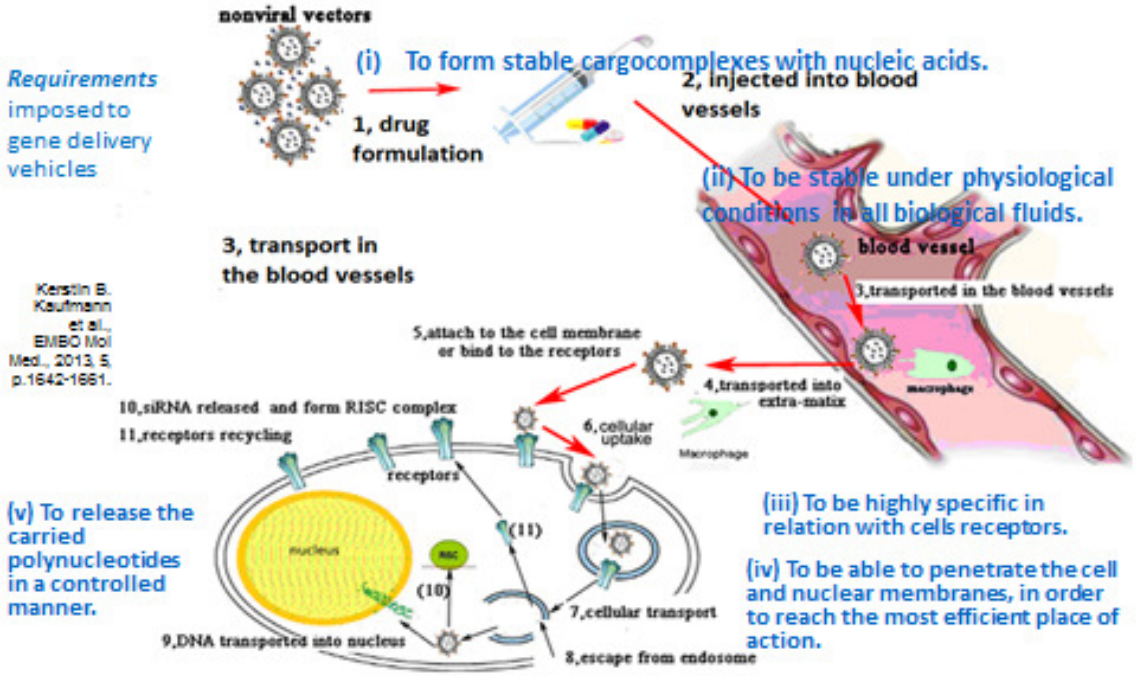
Objective 1. A histone-mimetic "artificial nucleosome"

In this context, *the project was focused on bio-mimicking at molecular level, by means of controlled conjugation reactions between chemical species with tailored reactivity, to attain the design and the obtaining of reproducible three-dimensional molecular architectures, able to become functional in a biological milieu*. The particulate nanoentities named *polyplexes* were generated by the ionic interactions between anionic DNA and multi-cationic structures or poly-cationic macromolecules. To deliver intact DNA segments, polyplexes must be able to pass unaltered through five barriers, outside and inside the target cells, namely (i) the extracellular matrix/environment traps, (ii) the cell membrane, (iii) the endocytotic and lysosomal environment, (iv) the nucleus membrane and (v) the chromatin crowded milieu (Scheme 1). More, they must release the transported DNA in a triggered mode, inside cell nucleus. It should be mentioned that the conjugation reactions are essential tools for modifying, improving and controlling the physical-chemical properties of macromolecular species of biochemical interest.

O1.1. Non-viral vectors obtained by Constitutional Dynamic chemistry with gene transfection ability

J.M. Lehn (Nobel Prize in supramolecular chemistry) in the book "Constitutional Dynamic Chemistry", Ed. M. Barboiu, 2012 (Topics Curr. Chem, 322, p1-32) explained that the new paradigm Constitutional Dynamic Chemistry (CDC) is in fact a Bridge from

Supramolecular Chemistry to Adaptive Chemistry, covering both the molecular and supramolecular levels. CDC occurs when the molecular entities (containing covalent bonds) could form and break in a reversible mode (allows a continuous change) by reorganization and exchange of building blocks.



Scheme 1. *Requirements imposed to gene delivery vehicles.*

In this context, researchers who work at the interface of biomaterials, gene therapy and drug delivery took into consideration the CDC paradigm and have identified several design parameters for the non-viral vectors to perform optimum delivery of biologically active material into cells.

Due to the huge variety of the DNA targets, the rational design, known as a suitable strategy to design non-viral vectors, became limited to a low number of components, deliberately positioned and fixed in specific positions on the vector backbone and thus should be completed by Dynamic combinatorial - DC approaches. Dynamic approaches allow for the simple generation of large chemical libraries from sets of building blocks, based on reversible exchanges between the components. Moreover, the molecular and supramolecular libraries can adapt to the system constraints, for example, allowing selection events driven by molecular recognition and self-assembly or in response to external stimuli.

Considering all this aspects, dr. M. Barboiu proposed a Dynamic Constitutional Strategy (DCS) for the construction of Dynamic Constitutional Frameworks (DCF) for DNA recognition based on 1,3,5-benzenetrialdehyde, poly-(ethyleneglycol)-bis(3-amino-propyl)-terminated (Mn 1500 $gmol^{-1}$), and Girard's reagent T, monoprotonated N,N-dimethylethylene amine, or aminoguanidine hydrochloride, that are the building blocks subjected to conceive DCFs by using the amino-carbonyl/imine reversible chemistry (Figure 1).[1] Progress has been

made, including our group also, towards achieving gene delivery, though the design principles for the materials and non-viral vectors that produce efficient delivery.[2]

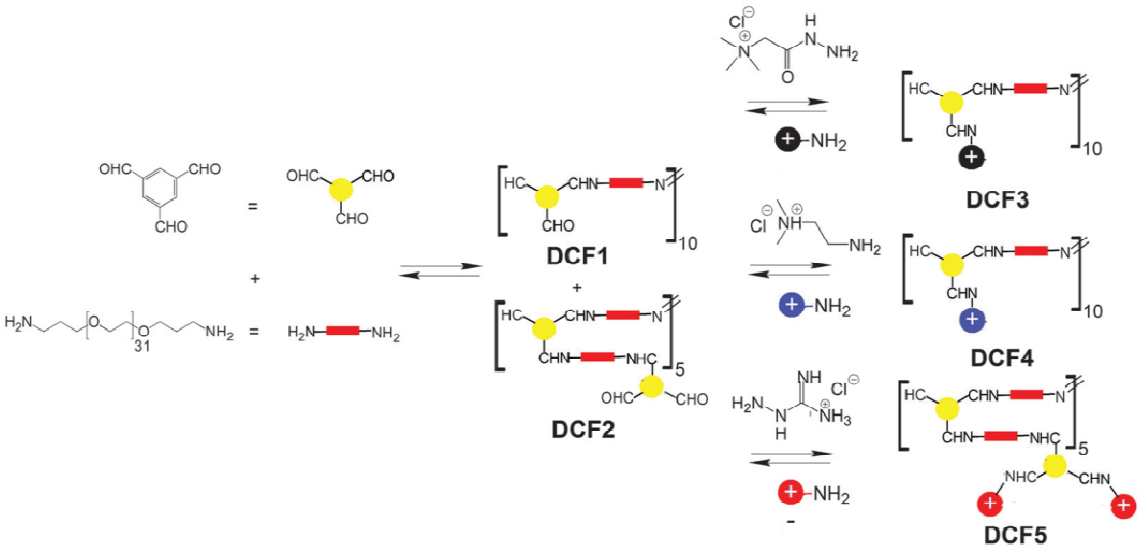


Figure 1. *Schematic representation of the synthesis of obtained DCFs resulting in the formation of DCF1:DCF2, 1:1, mol:mol. Further treatment of this mixture with positively charged heads, generates the DCF3-5 frameworks.*

This study revealed that the DNA binding is affected by cross-linked compact guanidinium framework DCF5 (at an impressive lower N/P ratio of 1 even for longer DNA strands), while the linear Ammonium frameworks DCF3 and DCF4 showed no binding properties (Figure 1). This sheds light on the dominant coiling versus linear DNA binding behaviours, closer to the histone wrapping DNA binding mechanism.

On the other hand, it is known that for successful transfection vectors must evade the immune system and be transported to the cell microenvironment for internalization, typically into an endosome, from which the vector must escape prior to being degraded as the endosome transitions into a lysosome. Thus vectors need to be designed with endosomal escape moieties or degradable components to facilitate dissociation of the nucleic acid from the vector. In this context, polyethyleneimine (PEI) and its derivatives are among the most studied polymeric materials for gene delivery and it's known for its ability of PEI to promote gene transfection *in vitro* and *in vivo.* The theoretical ratio of primary, secondary and tertiary amino groups in bPEI has been calculated as 1:2:1, respectively, and there is a close relationship between the pH of PEI and the positive charge density on PEI. Since the solution pH is an important factor in oligonucleotide complexation on bPEI, we have performed studies to model and optimize the complexation process depending on solution pH and bPEI/oligonucleotide ratio.[3] To unveil the mechanism of polycomplex formation, a molecular dynamic simulation was performed at the atomic-scale. Starting from an initial separation of ~40 Å between macromolecules, dsDNA and B-PEI approached each other and formed a stable complex after 12 ns (Figure 2). Under the determined optimal conditions, a maximal value of the binding efficiency was obtained experimentally, i.e. 99.96%.

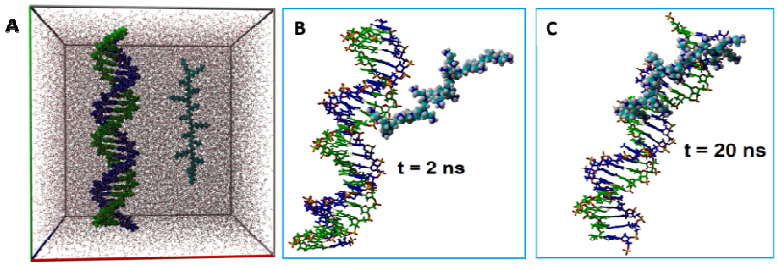


Figure 2. *Rendering of macromolecules dsDNA and B-PEI in a simulation cell with explicit water molecules (solvent): (**A**) initial equilibrated structures. Snapshots from the trajectory showing the interactions between dsDNA and B-PEI with formation of the polyplex at pH 5.8 and different simulation times: (**B**) t = 2 ns; (**C**) t = 20 ns; (water molecules are omitted).*

Despite the extensive popularity, the use of PEI *in vivo* and *in vitro* gene delivery is limited because of its low colloidal stability and its considerable cytotoxicity. To enhance stability and biocompatibility of PEI polyplexes, they can be combined with PEG; however, PEGs shield the positive charges of PEI, which often has the undesired effect of decreasing transfection efficiency. In the next study, 1,3,5-benzenetrialdehyde connectors 1, poly-(ethylene glycol)-bis(3-aminopropyl) terminated PEG (Mn ~ 1500 $gmol^{-1}$), and branched PEI (bPEI, 800 Da) building blocks were used to conceive DCFs for DNA recognition and binding (Figure 3 A).[4] The prepared DCF polyplexes are able to act as gene nanovectors, by forming stable polyplexes with dsDNA. Depending on the type and amount of associated DNA and on the molar ratio of bPEI/PEG, polyplexes have dimensions ranging between 40 and 125 nm (figure 3A). All tested vectors were capable of transfecting DNA into HeLa cells and demonstrated low cytotoxic levels; even at an N/P = 200 cell viability is over 90% relative to untreated control cells (Figure 3B, C). We can conclude that the presence of the PEG component and a moderate amount of b-PEI in DCFs are both important in the construction of highly transfecting and cyto-friendly polyplexes.

The simplicity of the synthetic strategy presented here can be easily used to self-generate dynamic constitutional networks presenting relative DNA/cell membrane synergistic affinities for the systematic rationalization of active delivery systems. Subsequently, This strategy leaves the possibility of DNA systems to self-select and self-generate the most adapted carrier for their own active and optimal transfection. In this respect, we have proposed a second generation of DCFs containing additional hydrophobic entities, besides core and charged units, to facilitate the self-assembly and adaptability of the DCFs. The proposed strategy was built on the screening of the DCFs constructed from PEG, squalene and bPEI with the core centres (Figure 4A).[5] These components adaptively generate multivalent polyplexes with variable sizes that DCFs and to increase their transfection (Figure 4B). The presence of squalene moiety in newly-prepared DCFs had indeed a crucial role influencing the transfection data and the biocompatibility.

Tested dynamic vectors showed considerable transfection on HeLa cells (at N/P ratio 50 and 200) and proved low cytotoxic levels. More interestingly, the overall size of the polyplexes is changeable depending on the ratio between the polyplex and the transfected DNA. These findings provide insights in the identification, via self-fabrication, of the multivalent adaptive dynamic vectors carrying out functional groups for optimal DNA binding, membrane penetration and transfection functions.

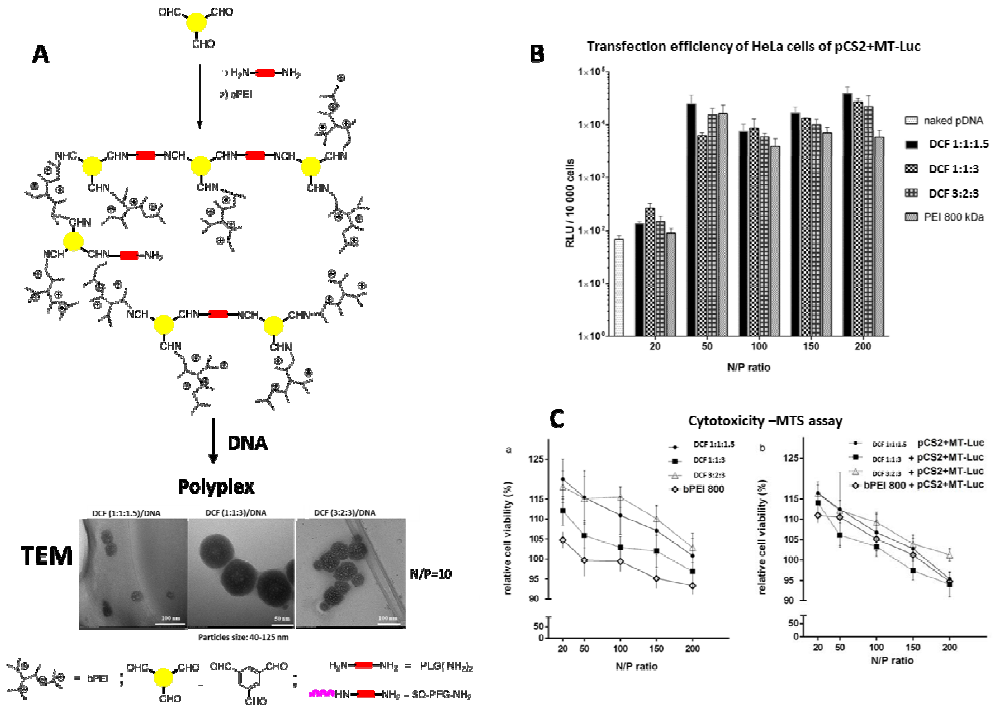


Figure 3. *A) Schematic representation of the synthesis of DCFs, B) Transfection efficiency at different N/P ratios measured at 48 hours. C) Cytotoxicity profiles of DCFs (a) and their respective DCFs /PEI/pCS2+MT-Luc polyplexes (b) based on the MTS assay.*

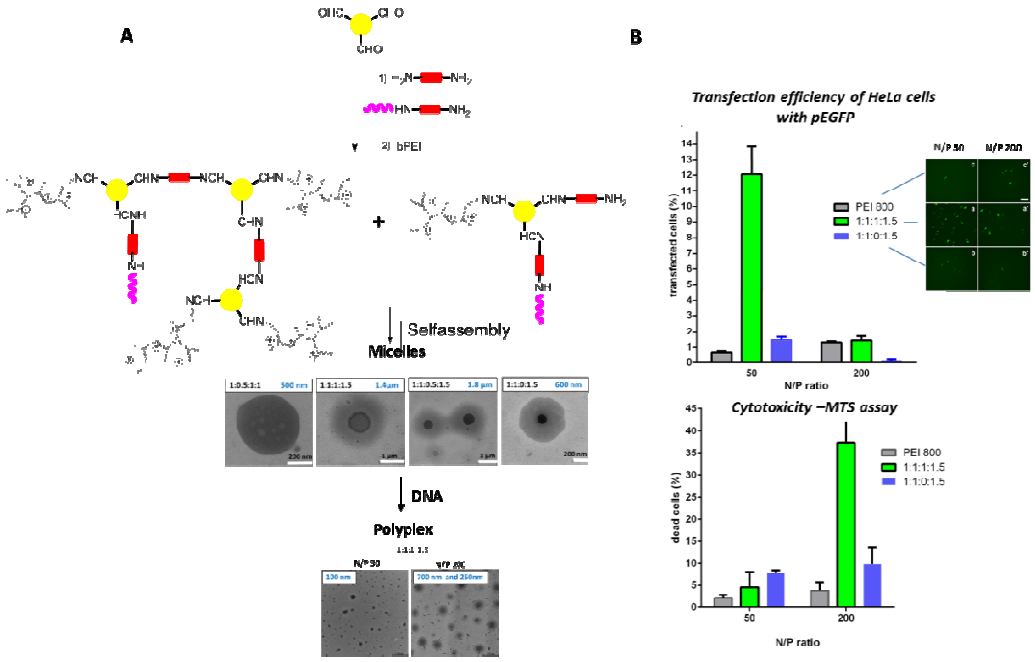


Figure 4. A) *Schematic representation of the synthesis of DCFs,* ***B)*** *eGFP gene transfection efficiency determined by flow cytometry,* ***C)*** *Cytotoxicity profiles of polyplexes based on MTS assay.*

It was taken into consideration, also, to obtain hydrophobic-hydrophilic systems with the structural blocks connected *via* imine bond (Figure 5). As hydrophobic structural blocks were used polypropyleneglycol and siloxane oligomers, and as hydrophilic blocks: polyethyleneimine, spermine and polyethyleneoxide. The morphological analysis confirmed the targeted design and the synthesized compounds proved transfection efficiency comparable with that of commercial reagents (Figure 5).[6]

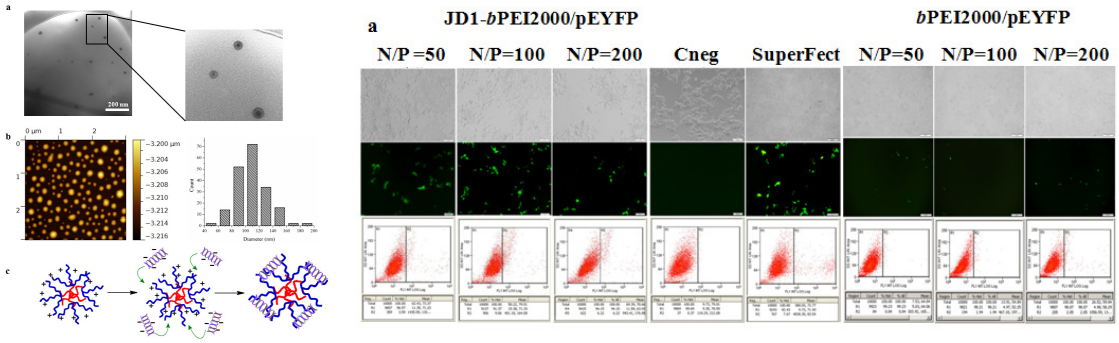


***Figure 5**. TEM and AFM images of the non-viral vectors based on polypropyleneglycol and siloxane oligomers, and as hydrophilic blocks: polyethyleneimine, spermine and polyethyleneoxide. Transfection efficiency for different N/P ratios.*

Summarizing the results we have developed in recent years in the field of the design and preparation of non-viral vectors for gene therapy, we strongly believe that the strategies we have chosen in the development of the core-like flexible vectors and Dynamic Constitutional Approach have strong potential in preparation of efficient and selective non-viral vectors. These directions could and will be further continued to provide cost-effective alternatives with high target selectivity comparing to the existing alternatives.

O1.2. Non-viral vectors based on polyrotaxane structures, as gene delivery systems

Since J. M. Lehn has defined the principles of supramolecular chemistry, for which he received the Nobel Prize in 1987, supramolecular chemistry became a central theme in chemistry, physics and the biological sciences. Supramolecular chemistry is a "dynamic chemistry" due to the lability of the interactions, responsible to connect the molecules by non-covalent intermolecular forces, resulting ensembles with other properties than initially ones.

In this idea, the present work have aimed to achieve a gene vehicle with a complex architecture, capable to accomplish a number of requirements, such as the following: to prove a convenient DNA packaging, transport and release, to exhibit a good biocompatibility and clearance, low cytotoxicity and a high transfection efficiency.

All these aims have been satisfied through synergistic interaction of α,ω-bis-propargyl-poly(ethylene oxide) of 1100 Da (PEG_{1100}) with acrilated β-cyclodextrin (acriloyl-β-CD) with acryl unit/CD molar ratio of 3/1 and the propargyl groups of PEG were reacted with 1-(3-brompropyl)silatrane, forming polyrotaxane structures. Later acriloyl groups of β-CD were reacted with primary amine groups of branched polyethylenimine of 2 KDa

(PEI_{2000}), via Michael addition, giving a polycationic conjugate with the main role to condense nucleic acids (Figure 6). The branched PEI molecules, mainly those having molecular weights exceeding 25 kDa, have proved an excellent transfection yield, but with the drawback of a poor cytocompatibility. By contrast, PEI of low molecular weight (e.g. PEI_{2000}) has shown proper cell viability, while causing a significant reduction in the rate of transfection. In this manner a substantial number of PEI_{2000} chains have been brought together to take part in the formation of a single macromolecular entity when a cumulative polycationic segment reached a molecular weight in the range of optimum effectiveness regarding gene transfer, higher than 25 kDa. Moreover by using as end-capped the silatrane azide, as a bulky groups to prevent the slipping out of the β-CD molecules from PEG chain, helps to obtain more antitumoral and antimicrobial effects. The polyrotaxane structure consisted in a non-viral vector with a length of 30 nm and a cross section of around 10 nm, being much closed with histone dimensions; these aspects were demonstrated by molecular dynamics (MD) simulations (Figure 7). *Results of Molecular Dynamics* shown dsDNA: PEI interactions and the minor and the major groves of DNA are not changed by its interaction with PEI macromolecules (Figure 8). This results was confirmed by circular dichroism analyses (Figure 8).

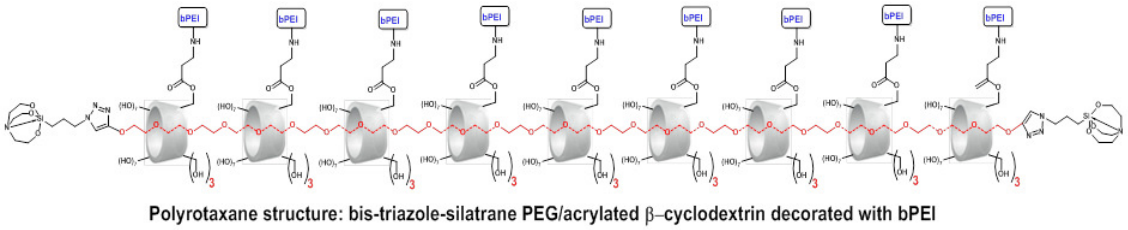


Figure 6. *The polyrotaxane structures.*

Figure 9 shows a comparison between the aggregate and the nucleosome/histone core structures. For the histone core the negatively charged arginine, lysine and histidine residues are represented as van der Waals spheres.

A cellular viability higher than 95 % was obtained for both uncomplexed polyrotaxane-PEI and its polyplexes with pCS2+MT-Luc (Figure 10).

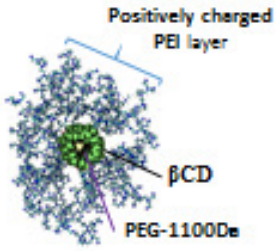


Figure 7. *In silico modelling of the polyrotaxane structure.*

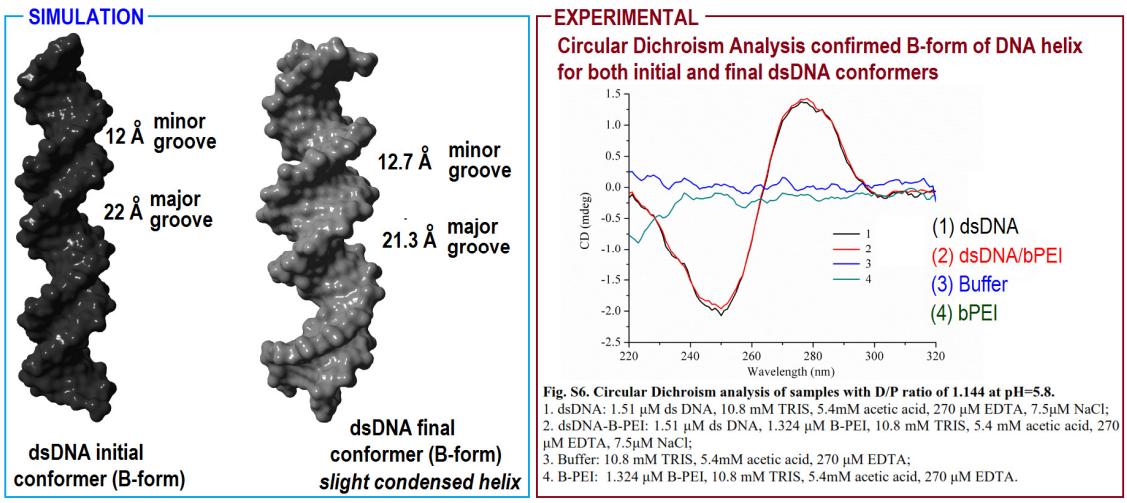


Figure 8. *Molecular Dynamic Simulation of dsDNA and experimental results by Circular Dichroism before and after interaction of dsDNA with PEI.*

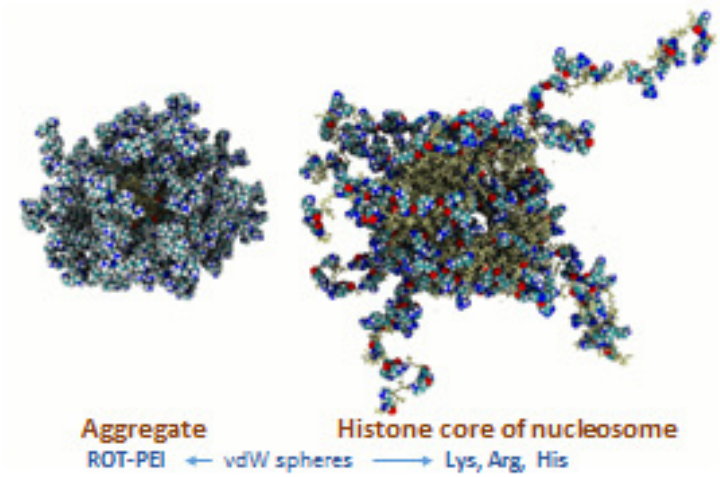


Figure 9. *Representation of polyrotaxane/dsDNA aggregate and histone core of nucleosome.*

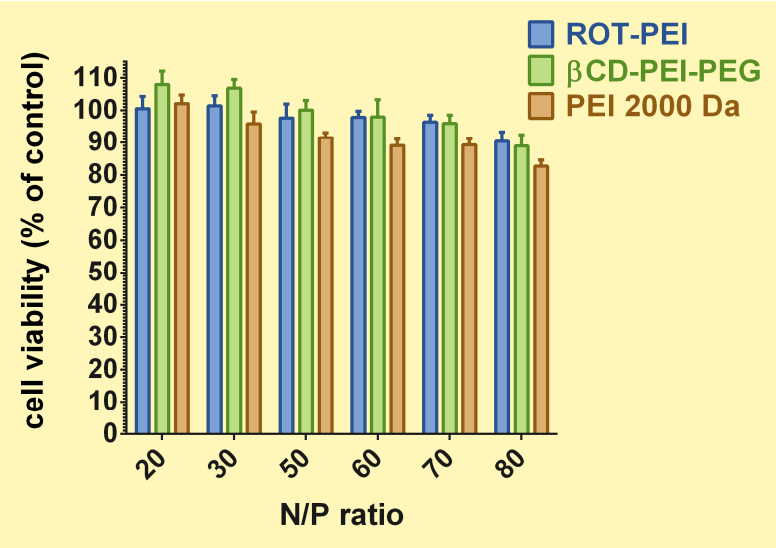


Figure 10. *Cytocompatibility of the carriers.*

The transfection efficiency of the polyplexes based on the synthesized conjugates was determined by two complementary techniques: the fluorescence microscopy, to qualitatively evaluate the abundance of transfected cells (Figure 11), and by the quantification of transfection ability of polyplexes (Figure 12). The used polyplexes are carrying Luciferase gene (pCS2+MT-Luc) able to encode green fluorescent protein (eGFP). HeLa cells were transfected with polyplexes made by combining pCS2+MT-Luc plasmid with polymeric carriers.

As it can be observed from Figures 11 and 12 for all tested N/P ratios, ROT-PEI polyplexes manifested the highest transfection efficiency.

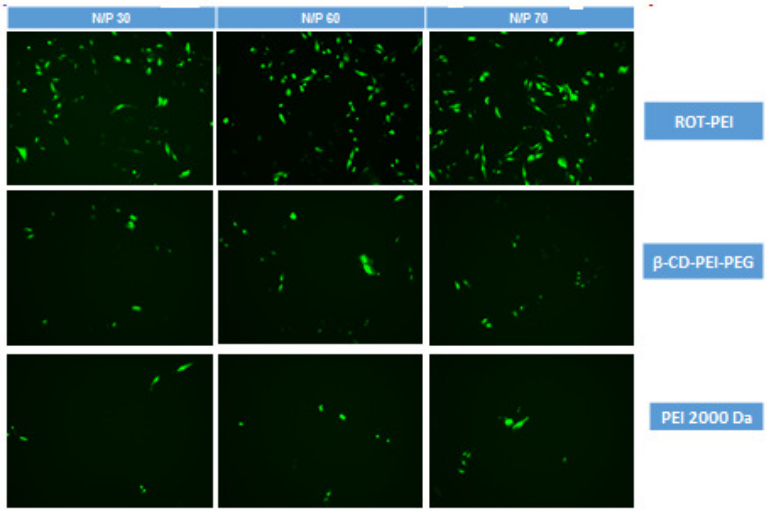


Figure 11. *Transfection of Hela cells with reporter gene eGFP by non-viral vector/pCS2+MT-Luc polyplexes (ROT-PEI: polyrotaxane-PEI; β-CD-PEI-PEG conjugate).*

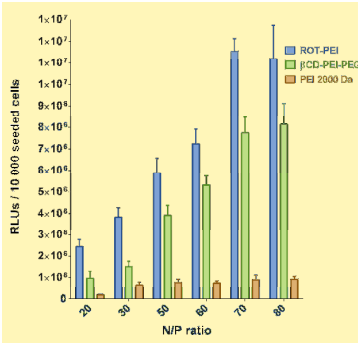


Figure 12. *Quantification of transfection ability of polyplexes, carrying Luciferase gene.*

O1.3. Non-viral vectors with covalent bonds and gene transfection ability.

Several transfection vehicles have been synthesized by covalent coupling of a number of hyperbranched PEI chains (Mw 2 kDa) with core molecules having different degrees of hydrophobicity (Figure 13).[7–9] In the resulting conjugated compounds the polycationic PEI moieties are externally disposed, surrounding the hydrophobic core molecule, adopting a dendrimer like structure. As central molecules there have been used: fullerene C60, 2,4,6,8-tetramethyl-2,4,6,8-tetrakis cyclotetrasiloxane (cD_4^H) and β-cyclodextrin (β-CD). The influence of PEGylation on transfection yield has been also studied.The synthesized compounds are presented as following:

1) **C60-PEI.** The synthesis have been carried out by direct amination of C60 molecule, via a nucleophilic addition mechanism. The reaction took place in chloroform, under nitrogen atmosphere, at ambient temperature and lighting, for 5 days. The molar ratio C60 : PEI was of 1 : 10, but after solvent removal and purification by dialysis, the molar ratio between the same components in final product was found of 1 : 3,5 (from TGA, XPS data). The reaction progress was evaluated using UV-Vis spectroscopy, tracing the disappearance of the peak at 330 nm. The covalent binding of PEI to C60 have been demonstrated by ^{13}C-NMR and FTIR spectroscopy, both emphasizing modifications in PEI spectra, mainly attributed to the conversion of primary amines in secondary ones.
2) **C60-PEG-PEI.** The synthesis was carried out in two steps, first of them consisting in the conjugation of mPEG-NH2·HCl (Mw 2 kDa) to fullerene, followed by PEI grafting, both of which beeing achieved via hydroamination reactions. The reaction took place in a mixture containing toluene and chloroform of 3 : 1 volume ratio, keeping the same conditions as those described for C60-PEI. The molar ratio between the reactants C60 : PEG : PEI was of 1 : 2 : 10, and the molar ratio of the same components in final products was found of 1 : 0.9 : 2.5. Each step took 5 days, after which the solvent removal and dialysis purification have been performed before freeze drying.
3) **cD_4^H-AGE-PEI.** In the first stage, the cD_4^H-AGE intermediate compound containing 4 oxirane reactive groups has been synthesized by hydrosililation of allyl glycidyl ether (AGE) with cD_4^H, in toluene, under nitrogen atmosphere, in the presence of Karstedt's catalyst, at 40°C, for 72 hours. The final product have been obtained by the reaction between cD_4^H-AGE and PEI, in DMSO, at 80°C, in the presence of isopropanol, for 40 hours. The dialysis purification have been followed by a freeze drying step.
4) **β-CD-PEG-PEI**. β-CD was firstly subjected to the reaction with acryloyl chloride, taking place in anhydrous DMF and under inert atmosphere, on an ice bath for 2 h, to be then continued at ambient temperature for another 5 days. The molar ratio β-CD : acryloyl of 1 : 7 (with an excess of 1,5%) was found to ensure a maximal substitution degree. The acrylated product, recovered by precipitation in a buffer solution (pH 7) and then by vacuum drying, was further functionalized with mPEG-NH2·HCl and PEI via Michael additions of primary amines to double bonds belonging to acryloyl groups. The reaction was conducted in methanol, at 65°C, for 72 h. The purification has been done by dialysis against ultrapure water, and then

followed by lyophilization. The structure has been proven by ^{1}H and ^{13}C RMN and FTIR spectroscopy.

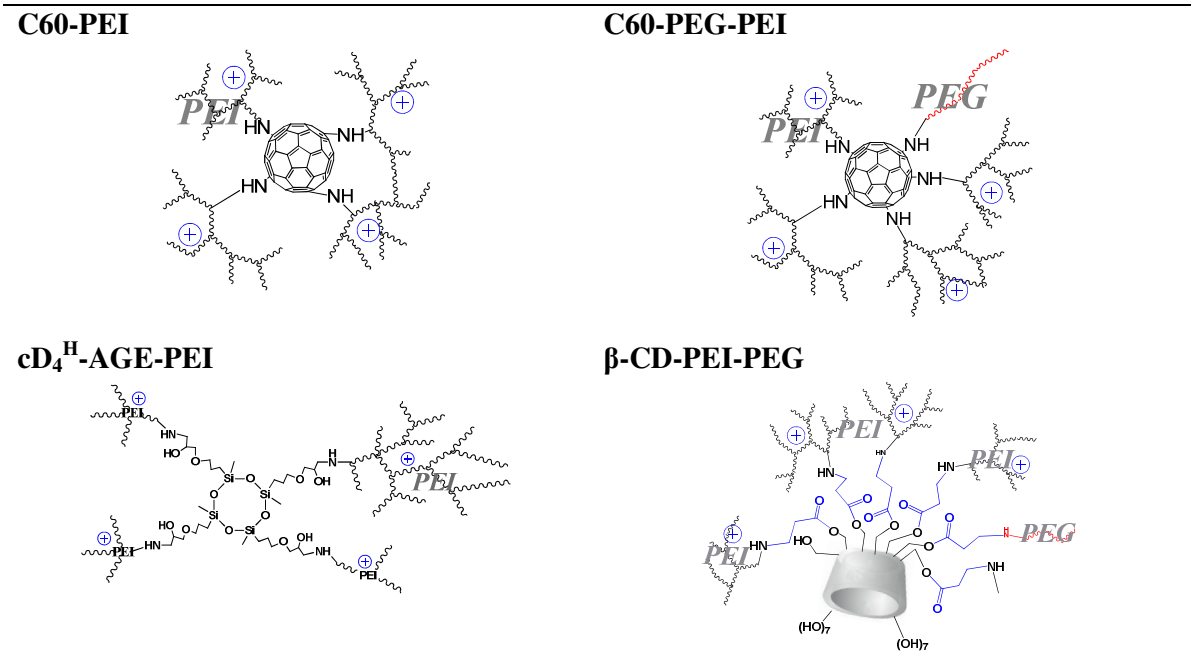


Figure 13. *The comparative chemical structures of C60-PEI, C60-PEG-PEI, cD_4^H-AGE-PEI and β-CD-PEI-PEG transfection vehicles.*

Molecular modeling was achieved by PM3 semiempirical molecular orbital calculations in order to optimize the geometry of the conjugates. Figure 14a illustrates the conformation of cD_4^H-AGE-PEI vehicle, which emphasizes an asymmetric structure, with a tendency to distinctly expose the positive charged (the polycation) and the uncharged (the siloxane ring and the linker AGE moiety) domains, belonging to C1 molecular point group symmetry. According to PM3 calculations, a considerable dipole moment of 8.3 Debye has been obtained. Figure 14b represents the molecular modeling of β-CD-PEG-PEI vector, built using oligomeric PEI and PEG chains, leading to a mass of 4.68 kDa vector. The structure of β-CD-PEG-PEI was optimized by minimizing its potential energy at the level of molecular mechanics, using YASARA field.

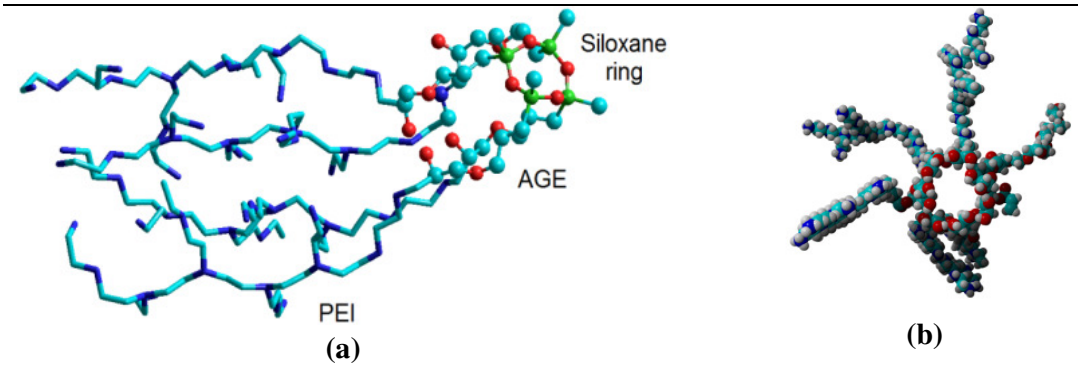


Figure 14. *The molecular structure of cD_4^H-AGE-PEI (a) and β-CD-PEG-PEI (b) conjugates obtained by molecular modeling.*

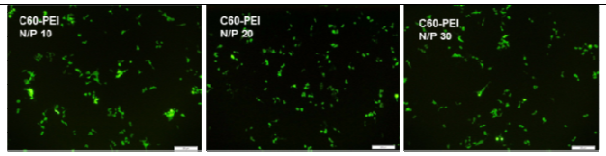
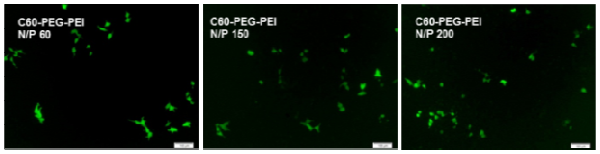
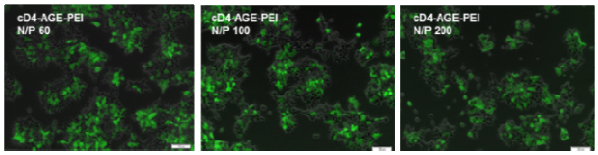
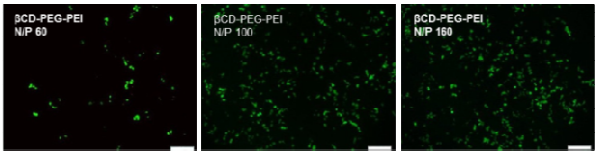
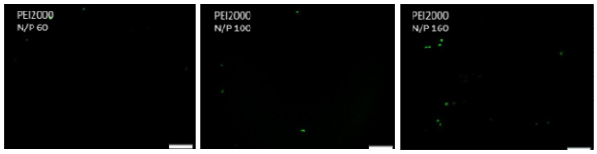
Transfection agent	Fluorescence microscopy images	Transfection yield
C60-PEI		25% (N/P 30)
C60-PEG-PEI		6% (N/P 200)
cD_4^H-AGE-PEI		38% (N/P 200)
β-CD-PEI-PEG		14% (N/P 160)
PEI (2 kDa)		~4% (N/P 160)

Figure 15. *The transfection efficiency of C60-PEI/pEYFP, C60-PEI-PEG/pEYFP, cD_4^H-AGE-PEI/pEYFP and β-CD-PEI-PEG/pEYFP on HEK 293T cells, as compared to PEI (2kDa).The right column indicates the maximum percent of transfected cells, achieved by flow cytometry, and the corresponding N/P ratios.*

The obtained DNA vehicles were subjected to *in vitro* biological tests to evaluate the **transfection efficiency** on HEK 293T cells. The cells were cultured in the presence of polyplexes prepared by complexation of polycationic edifices with plasmidial DNA (pEYFP). The transfected cells expressed the fluorescent protein encoded by pEYFP, therefore the transfection results have been evaluated both qualitatively by fluorescence microscopy (Figure 15), and quantitatively using flow cytometry assay. Each of the four tested vehicles show an N/P ratio corresponding to a maximum transfection yield, expressed as % transfected cells, ranging from 6% in C60-PEG-PEI to 38% in cD_4^H-AGE-PEI.

The **cytotoxicity** tests, performed using MTT method, indicate the viability of HEK 293T cells after incubation with media containing the polyplexes, having the same N/P ratios as those from transfection efficiency experiments. According to cell viability results (Figure 16), one can observe that all DNA vehicles exhibit a high cyto-friendly behavior. The percentage of viable cells obtained at maximum N/P ratio for each vector was found of about 90% for C60-PEI and cD_4^H-AGE-PEI, and 160-180% for β-CD-PEI-PEG.

C60-PEI	**C60-PEG-PEI**

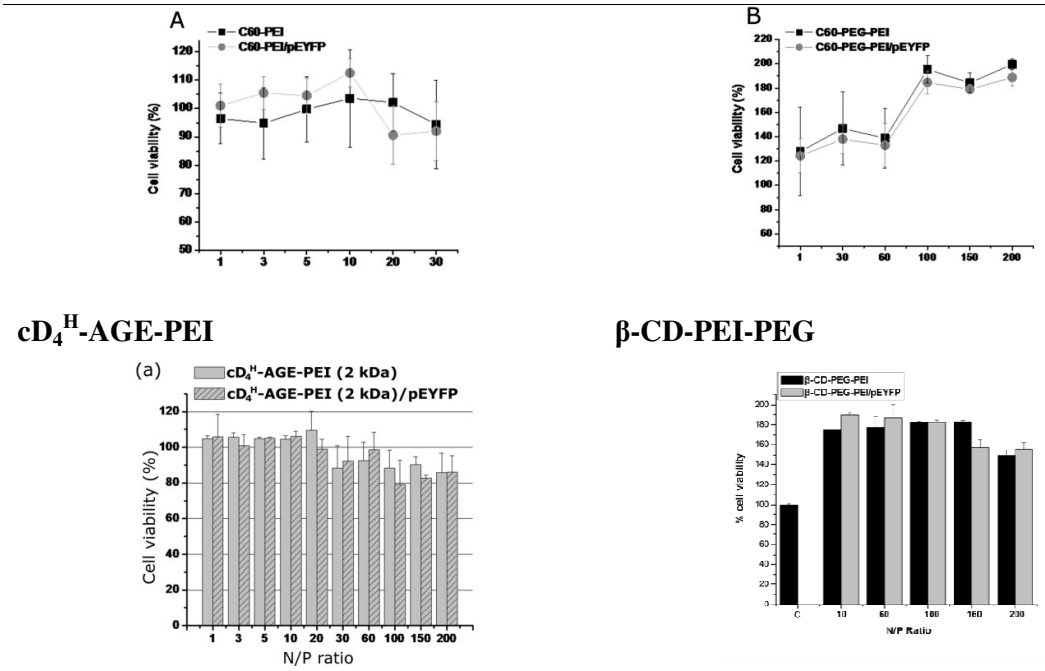


Figure 16. *The viability of HEK 293T cells in the presence of C60-PEI, C60-PEI-PEG, cD_4^H-AGE-PEI, β-CD-PEI-PEG and their polyplexes with pEYFP, at different N/P ratios.*

Conclusions:

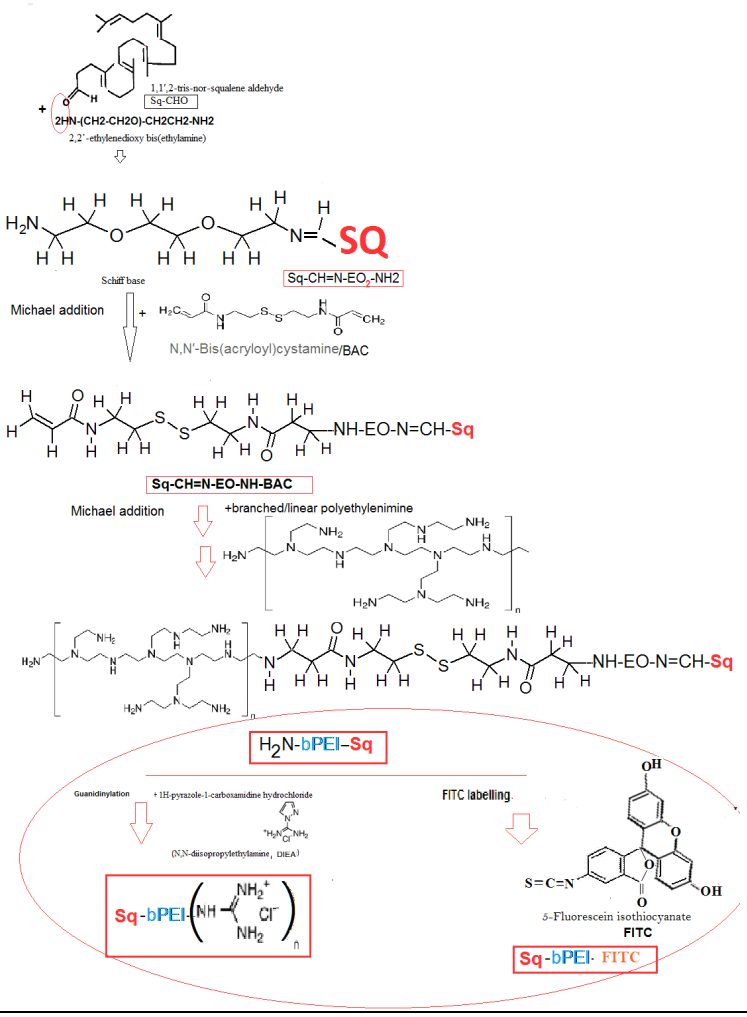
Among the synthesized vehicles, cD_4^H-AGE-PEI has been proved to be the most attractive regarding transfection efficiency, with a transfection yield higher than 38% at an N/P ratio of 200. As evidenced by molecular modeling calculations, the mentioned vehicle adopt an asymmetric conformation, which separately displays the polycation and the hydrophobic domains, facilitating the penetration of cell membranes. The PEGylated products exhibit a lower transfection efficiency of only 6% and 14% in C60-PEG-PEI and β-CD-PEI-PEG polyplexes, respectively, since PEG chains cover the nanoparticles, preventing the plasmid packaging with the benefit of a lower cytotoxicity. Thus, increasing N/P ratio produced a significant cellular proliferation, so that for N/P higher than 100, the cell number became almost double. Using the C60-PEI vehicle, a transfection yield of 25% has been achieved at small N/P ratios, of 20 – 30, for which the cell viability was still appropriate (~90%).

O1.4. Design, Synthesis and Characterization of Squalene/bPEI gene delivery vector, obtained by both reaction mechanisms: Constitutional Dynamic Chemistry and covalent bonds

Here a squalene/bPEI (Sq-PEI) based amphiphile was developed and characterized, envisaging its application in transfection.

To fulfil the requirements associated to the overcoming of the challenging high known number of biological barriers related to a transfection process specific moieties were included in the envisaged conjugate design. Mild preparation conditions (room temperature, usual solvents or aqueous reaction medium) were used, according to the reaction presented in

scheme 2.[10] The successful development of the final and intermediates Sq-based compounds was confirmed by spectral characterization (^{1}H-NMR, ^{13}C-NMR, FTIR).



***Scheme 2**. Sq-PEI based carrier synthesis strategy.*

O1.5. Polyplex inclusion in the vesicle formed by polysiloxane surfactants

Preliminary study for polyplex inclusion in vesicles formed by polysiloxan surfactants in order to increase their stability and to improve bioactives delivery control from the combined system also were done.[11–13]

Magnetite and chromite nanoparticles were used for the study. Their stable dispersions in water were obtained by physical methods using very low concentrations of the investigated surfactants. Resulted systems characterization by DLS, TEM, cryo-TEM and EDX evidenced the generation of various types of morphology with dimensions in the 20-200 nm range: individual particles, vesicle-like aggregates or composite particles.

The evaluation of surfactants and complex hybrid nanoparticles in respect to biocompatibility by MTT method gave good results (Figure 17).

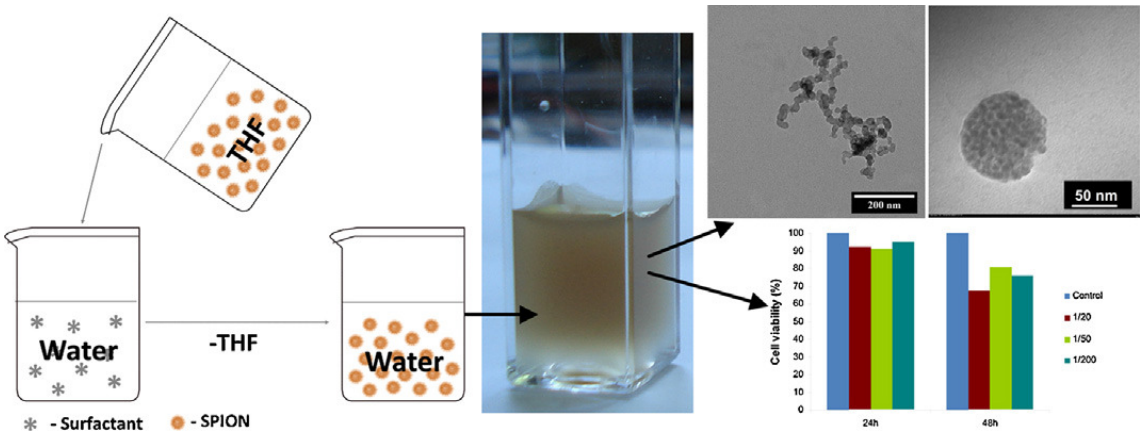


Figure 17. *Aqueous dispersion of metal oxide nanoparticles formed using siloxane surfactants and related TEM and biocompatibility data.*

Superparamagnetic iron oxide nanoparticles (SPION) and nystatin (used as a bioactive compound model) could be encapsulated together within the hydrophobic wall of the surfactant vesicles formed by S2, yielding stable colloidal systems.

In **conclusion** multifunctional nanoparticulate materials were prepared and characterized aiming the possible future use in combined non-viral gene carrier/scaffold systems intended for bone regeneration:

- Stable, spherical biopolymers based micro-/nanocapsules with inner porous AteCol or AteCol/DMSHA wall were obtained by combining classical double emulsion method with LbL technique.
- The wall permeability, particles size and size distribution, surface functionality and even nanoparticle type (spheres or capsules) was found to be easily controlled by varying the preparation parameters: formulation of the biopolymers/PCL cross-linker initial mixture; ratio of used solvents; ratio of PCL-DI to initial polymer mixture; surfactant and stabilizer concentrations; conditions applied for nanoparticles further surface functionalization (nature of the reactive polymer, its ratio in respect to polymer nanoparticles covered with PCL-DI, reactants concentration in the system, pH, temperature); reactants addition order; stirring rate in every step.
- The modification of the nanocapsules surface was confirmed by FTIR, SEM, TEM, DLS (change of size, size distribution, ξ-potential) investigations, and the grafting efficiency were quantified by fluorescence spectroscopy and fotocolorimetry measurements.
- According FTIR registration the protein native form was preserved.
- Reproducible method of PEI/PLL covered NPs (with 1.5 wt% and 5.1 wt% per g NPs, respectively), with a mean diameter of ~100-120 nm, as recommended for gene delivery purpose, was proved.
- From the agarose gel electrophoresis data the optimum N/P value was 3.5 for the PLL covered NPs and >6.5 for PEI covered NPs, similar to literature data for neat PLL and PEI. Thus, their compacting ability of DNA was not affected by inclusion in the NPs formulation.

- The proposed particles design offers advantages, which give rise to a high versatility (controlled size, chemistry and biodegradation rate depending on the envisaged application), recommending them as systems able to mediate drug delivery or gene transfection as so, or by their inclusion in injectable or preformed scaffolds (i.e. combined transfection systems).

Colloidal stable, hybrid, multifunctional particles with dimensions lower than 200 nm could be obtained using tromethamol-modified and carboxylate siloxanes surfactants:

- By a simple procedure based on the micellization ability of polysiloxanes derivatives in water, magnetic metal oxides (iron oxide or iron–chromium oxide) could be included in nanoparticles covered with siloxane of different morphology: individual particles, vesicle-like aggregates or composite particles.
- The hybrid nanoparticles generation was confirmed by TEM, completed by cryo-TEM and EDX investigations, as well as by DLS and Zeta potential measurements.
- The colloidal stability of the resulted dispersions, the nanoparticles dimensions and morphology was found to be dependent on NP-surfactant pair.
- Nystatin and magnetite nanoparticles (SPION) was proved to can be both encapsulated with the efficient siloxan-based surfactants in hybrid NPs, the drug being located within the hydrophobic wall of surfactant vesicles.
- The cytotoxicity testing (MTT method) of both, surfactants and metal oxides/surfactant hybrid NPs proved their biocompatibility, although the initial dodecylamine and oleic acid coated inorganic nanoparticles were cytotoxic.

Notably, the highly safe, versatile, stable, and easy-to-use nano-sized engineered constructs, were obtained by simple preparative methodologies based on the combination of classic and modern techniques, such characteristics making them an ideal choice for application in gene delivery or synergetic systems for drug/gene delivery or bioactive delivery/medical imagistics.

O1.6. Biosinthesis of exopolysaccharides, as precursors for gene delivery systems

The lactic acid bacteria are capable to biosynthesize extracellular biopolymers with different chemical structures dependent on the culture medium composition. For this end, a new culture medium composition was made, that is capable to induce lactic acid bacteria strain to produce a high amount of exopolisaccharides (EPS) biosynthesis (up to 26.6 g of freeze-dryer exopolysaccharides/L culture medium). The strain was isolated from yellow corn flour from Portugal and identified by 16S rDNA sequence as *Weissella confusa*. The extracted and purified exopolysaccharides were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR (HSQC), HPLC, GPC and TGA analysis. The HPLC analysis demonstrated only glucose in the exopolysaccharides structure. By NMR and FTIR analysis it was confirmed a dextran structure, with 96.8 % α-(1→6) bonds and 3.2 % α-(1→3) branch linkages. Also, the GPC analysis of purified exopolysaccharides revealed two fractions with a high molecular weight (3.3×10^5 and 1.6×10^7 g mol/L, respectively) and two fractions with a low molecular weight

(4.4×10^2 and 1.6×10^2 g mol/L, respectively). The thermogravimetric analysis showed two steps for exopolysaccharides degradation; the most important degradation peak is registered at 288°C with a weight loss of 65.9%. Taking into account the higher amount of EPS obtained by fermentation process and the natural culture medium compositions, this compound is obtained by an inexpensive procedure in a very short time and it can be attributed many applications (medical, pharmaceutical, alimentary).

Moreover, the obtained EPS structure can be suitable for functionalization in order to obtain a new class of transfection core.[14]

In the same time, a total of 52 yeasts strains were isolated and cultivated in two different culture media, namely, yeast peptone glucose medium and yeast acetaldehyde-diacetyl medium in order to obtain high amounts of diacetyl and acetaldehyde. These flavours can be used in alimentary industry as a substitute to chemical compounds. The initial screening of the strains was based on the qualitative reaction of the acetaldehyde with Schiff's reagent (violet colour) and diacetyl with Brady's reagent (yellow precipitate). The fermented culture media of 10 yeast strains were subsequently analysed by gas chromatography to quantify the concentration of acetaldehyde and diacetyl synthesized. Total titratable acidity values indicated that a value of 5.5ºSH, of the culture medium at basic pH, was more favourable for the acetaldehyde biosynthesis using strain D15 (*Candida lipolytica*; 96.05 mg L^{-1}acetaldehyde), while a total titratable acidity value of 7ºSH facilitated diacetyl flavour synthesis by strain D38 (*Candida globosa*; 3.58 mg L^{-1}diacetyl).[15]

Objective 2. A non-viral mimetic vehicle for deliberately and controlled introduction of special drugs into cells, based on polymers and polymeric adducts.

O2.1. Intelligent polymers for controlled delivery of biologically active molecules

Stimuli-sensitive or ‘‘intelligent’’ polymers are fascinating materials able to respond to small changes of the environment parameters by changing their inherent properties. The ability to respond to external stimuli is actually one of the most important features of living systems. Consequently, not surprisingly, the most considered applications of these materials are in medicine and pharmaceutical area as drug, gene and radionuclide delivery systems as special architecture adapted for targeted delivery and triggered release of biologically active compounds. Among stimuli-sensitive polymers, those sensitive to pH and temperature were used in this project because in the human body, these physico-chemical parameters change in different body compartments and under pathological conditions, acting as triggering agents. Here, poly(N-isopropylacrylamide) (poly(NIPAAm)) was used as thermosensitive polymer because in aqueous solution, it displays a lower critical solution temperature (LCST) at around 32 ºC. In order to increase the LCST towards the body temperature, poly(NIPAAm) was copolymerized with hydrophilic monomers with different functional groups such as hydroxyethylacrylamide (HEAAm),[16] methacrylic acid (MA),[17] maleic acid (MAc),[18,19] functionalized β-cyclodextrin (β-CD).[20] Then, the “intelligent” polymers were assembled or self-assembled in macroscopic hydrogels or micrometric or nanometric assemblies with

controllable properties such as phase transition temperature, size distribution, swelling/deswelling rates, hydrophilic/hydrophobic surface, charge density for an efficient delivery system.

In some cases, it has been synthesized porous hydrogels with double sensitivity, at pH and temperature, by copolymerization of NIPAAm with methacrylic or maleic acid in the presence of a cross-linker (N,N'-methylenebisacrylamide or by creating semi-interpenetrated networks.[21]

In physiological fluids (PBS, pH = 7.4), the carboxylic groups of MA are ionized and the hydrogel loses the thermosensitivity. Remarkably, when the carboxylic groups in the ionized state interact electrostatically with certain biologically active compounds, the hydrogel regains its thermosensitivity, collapses and releases a certain dose of drug (Figure 18). In this case, the pH-sensitive co-monomer (MA) acts as a biosensor and the temperature-sensitive co-monomer (NIPAAm) acts as a drug delivery component. This dual system may represent the basement of a new generation of drug delivery devices.

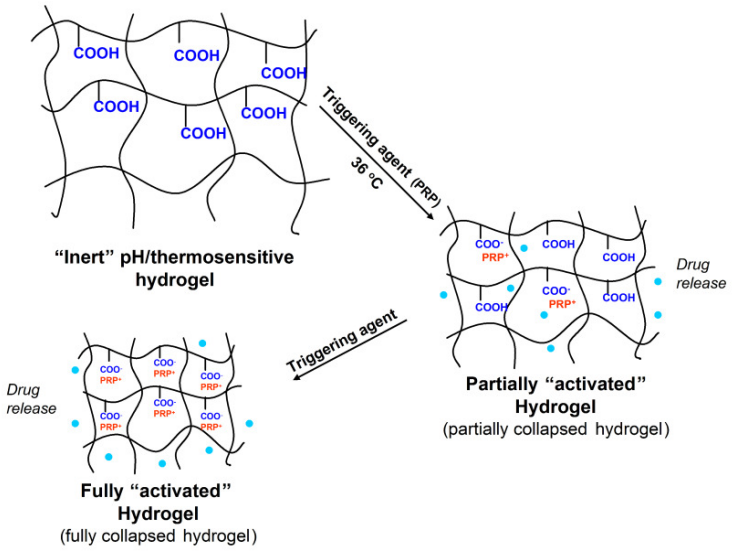


Figure 18. *Schematic representation of the principle of operation of pH/thermosensitive poly(NIPAAm-co-MA) microgels in the presence of the triggering agent, under simulated physiological conditions.*

In another approach, NIPAAm was copolymerized with vinyl derivatives of β-CD with the aim to obtain materials sensitive to temperature (microgels) able to retain selectively biologically active compounds. Moreover, these microgels are biodegradable because the CD was in such a manner functionalized, thus to have more than one polymerizable group per CD molecule. Due to the micrometric dimension and advanced porosity, these microgels display a sharp phase transition at physiological pH and temperature. Therefore, the response rate to small changes of the physiological parameters is very high. These hydrogels are able to release the drugs included in the hydrophobic cavity of the CD in a pulsed manner by an "ON-OFF" mechanism (Figure 19).

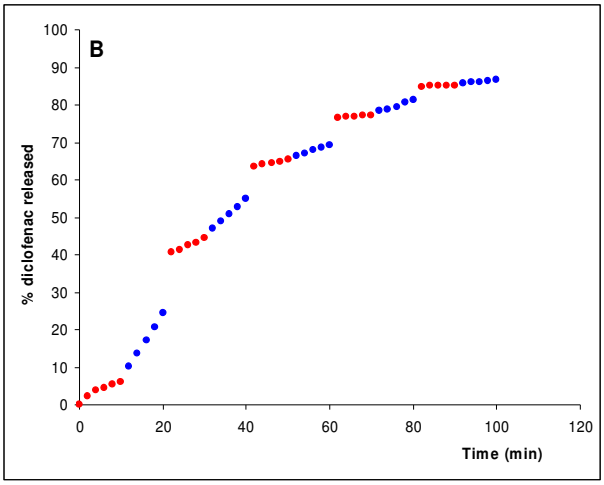


Figure 19. *Effect of temperature cycling (32 ℃) (•) and 40 ℃ (•) on kinetics of diclofenac release from poly(NIPAAm-co-β-CD) microgels under pseudo-physiological conditions.*

For biomedical applications that require particules with sub-micron dimensions, it has been synthesized, by precipitation polymerization method, nanoparticles sensitive to temperature based on NIPAAm and HEAAm,[22] or poly(NIPAAm) grafted on pullulan.[23] The phase transition has been determined by dynamic light scattering (DLS), UV-Vis and ^{1}H-NMR spectroscopy. It was proved that nanoparticles have a VPTT close to the human body temperature, therefore they can be used for biomedical applications. It has been shown that nanoparticles are spherical and monodisperse. The release rate of propranolol was deeply influenced by temperature: below the VPTT the microgels are in the swollen state and the drug is quickly released while above the VPTT the microgels are collapsed and the drug is slowly released.

It is well-known that the use of cyclodextrins (CDs) for controlled delivery of drugs is largely presented in the literature. Here, CD was used as protecting agent[24] or immobilized within dextran microspheres and CD-dextran complexes were packed in a glass column and then, the retention time of different drugs and drug model compounds was determined by liquid chromatography.[25]

Then, the release profiles of drugs and of drug model compounds (indole, 3-nitrophenol, p-hydroxybenzoic acid, diclofenac), characterized by different values of the retention time (high, moderate or low), were investigated. It was proven that the release rates were quite high even for drugs that exhibit very high retention time (high association equilibrium constant). Moreover, the volume of the release fluid strongly influences the rate of drug release. As a whole, “the sink conditions” must be continuously maintained, since at each drug concentration in the release medium, equilibrium occurs between the free and the CD-bound drug.

O2.2. Inclusion complexes of propiconazole nitrate with substituted β-cyclodextrins. Synthesis and characterization

As part of the first study covering Romania with regard to species distribution and the azole susceptibility pattern of fungal clinical isolates,[26,27] the antifungal properties of the inclusion complex of propiconazole nitrate with β-cyclodextrin (β-CD/PCZH-NO_3) was tested against a collection of 551 clinical yeast isolates received from hospitals throughout the country, alongside two triazoles, fluconazole (FLC) and voriconazole (VOR), and three echinocandins, caspofungin (CAS), micafungin (MCA) and anidulafungin (ANI), antifungal agents with extensive clinical use. The commercial formulation of VOR, where it is complexed with an enhanced cyclodextrin, sulfobutylether-β-CD (SBE7-β-CD/VOR), was used during the tests.

Antifungal testing and minimal inhibitory concentration (MIC) interpretation was performed by following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antifungal activity of our complex was generally similar to that of the VOR complex (Figure 1A) and that of the echinocandins (Figure 20B).

The next step in the study of vectors for enhancing PCZH-NO_3 efficacy was the preparation of three new inclusion complexes, in which the parental β-CD was replaced with three of its derivatives, namely sulfobutylether-β-CD (SBE7-β-CD), β-CD sulfated sodium salt (β-CD-SNa) and monochlorotriazinyl-β-CD (MCT-β-CD).[28]

Docking and molecular dynamics simulations (with water as an explicit solvent) of the inclusion complexes of PCZH-NO_3 with β-CD and SBE-β-CD were performed. The computational studies suggested the coexistence of different types of inclusion complexes, depending on the PCZH-NO_3 moiety that enters in the cyclodextrin cavity. The most energetically favorable conformation is with the aromatic ring of the dichlorophenyl moiety embedded in the cavity at the sugar ring level.

The experimental compound was particularly active, and superior to the VOR complex, against *C. glabrata* isolates (Figure 21A), which are known to often have a reduced susceptibility to azoles, and against many isolates with resistance to fluconazole (Figure 21B).

The inclusion complexes were then prepared by freeze-drying. The complexation and the information offered by the computational studies were confirmed by nuclear magnetic resonance spectroscopy (^{1}H-NMR), 2D rotating frame Overhauser effect spectroscopy (ROESY) NMR and differential scanning calorimetry (DSC). NMR titration-measured association constant values highlight that PCZH-NO_3 form the most stable inclusion complex with SBE7-b-CD, probably due to the interactions of the dioxolanyl and triazolic cycles with the glycosidic oxygens or the $–SO_3^-$ groups of the cyclodextrin. Additionally, the titration experiments provided data regarding the ratio complexation between the two components, which is 1:1 for all cases.

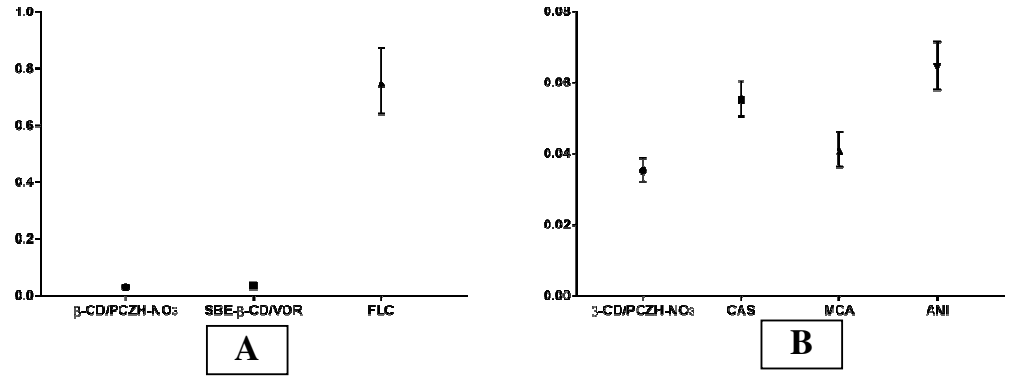


Figure 20. *β-CD/PCZH-NO_3 vs. clinically used antifungal agents – the geometric means of the MIC values, with 95% confidence intervals:* ***[A]*** *β-CD/PCZH-NO_3 vs. clinically used triazoles;* ***[B]*** *β-CD/PCZH-NO_3 vs. clinically used echinocandins.*

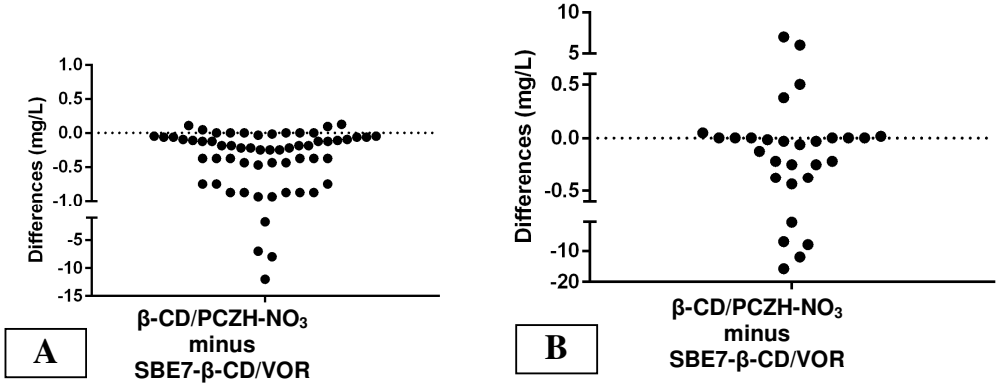


Figure 21. *β-CD/PCZH-NO_3 vs. SBE7-β-CD/VOR – absolute values of β-CD/PCZH-NO_3 minus SBE7-β-CD/VOR differences (mg/L):* ***[A]*** *antifungal activity against C. glabrata;* ***[B]*** *antifungal activity against non-krusei Candida FLC-resistant isolates.*

The assessment of the antifungal properties of the new inclusion complexes,[29] performed by following the same EUCAST guidelines, showed that, in the majority of cases, the minimal inhibitory concentrations (MIC) were in agreement with differences confined within the accepted ± 1 log2 dilution interval (Figure 22A). This suggested that the nature of the cyclodextrin does not significantly influence the *in vitro* behavior of PCZH-NO_3 towards fungal cells.

The cytotoxicity was also assessed on normal human dermal fibroblasts - NHDF (PromoCell) by performing the commercial CellTiter 96®AQueous One Solution Cell Proliferation Assay (Promega). The nonlinear regression curves (Figure 3B) showed the inclusion complex of PCZH-NO_3 with the parental β-CD to be more toxic than the complexes with the three β-CD derivatives. The half maximal inhibitory concentrations (IC_{50}) were two to three orders of magnitude higher than the concentrations required for antifungal activity.

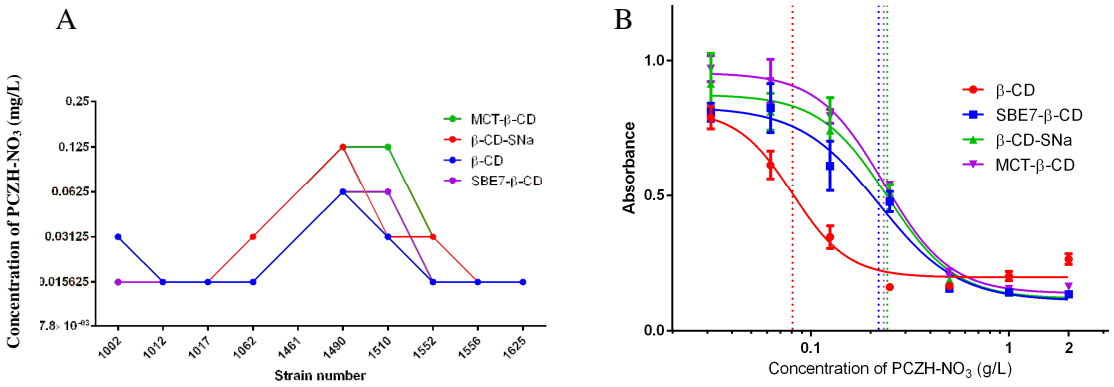


Figure 22. *The biological activity of the inclusion complexes.*
[A] *MIC agreement between the four tested inclusion complexes based on PCZH-NO_3 and β-CD or its derivatives against C. albicans isolates;* ***[B]*** *The cytotoxicity of the four inclusion complexes based on PCZH-NO_3 and β-CD or its derivatives - nonlinear fit of the dose response curves. The dotted lines indicate the IC_{50} values.*

The lack of significant differences in the antifungal susceptibility tests and the differences in cytotoxicity between the complex with the parental β-CD and the complexes with β-CD derivatives suggest that the type of cyclodextrin may be more important for the interaction of the compounds with the infected host than it is for the actual antifungal activity.

O2.3. New hybrid materials based on layered double hydroxides (LDHs) as drug delivery systems

Layered double hydroxides (LDHs) represent a class of materials characterized by layered structure, in which the lamellae are positively charged and stability of the structure is ensured by the anions which connect adjacent layers in an electrostatic way. LDHs are biocompatible materials, and can be explored as matrix for biomolecules/drugs storage and controlled release systems. Considering this, we focused on obtaining new hybrid materials based on LDHs able to act as effective drug delivery systems. Tramadol hydrochloride (TrH) has been chosen as model drugs, being intercalated in a ZnAl nitrate LDH matrix by anionic exchange method.[30] TrH was used in different amounts 0.1, 0.4 and 0.8 g for each 2 g ZnAlLDH. The precursors and hybrid materials were characterized by XRD, FTIR, RAMAN, EDX and SEM.

The obtained results point out the presence of the TmH in the interlamelar space in the case of ZnAlLDH_TrH 0.4 and ZnAlLDH_TrH 0.8 when a larger amount of TrH was used for the ion exchange. The FTIR and RAMAN spectra points out the absence of vibration bands characteristic to nitrate ions, which also confirms their replacement with TrH anions. In the other cases was observed the partial TmH intercalation and a significant absorption on the surface of ZnAlLDH. This study suggests that TmH can be intercalated into ZnAlLDH, and the new hybrid materials could open interesting perspectives for obtaining the drug reservoirs and controlled release systems.

O2.4. Nanomagnetite Particles and Their Cytotoxic Effect on Cancer Cells

The aim of these studies were to design, to construct and to characterize a complex carrier of nanoparticulate type, which consists of a magnetite core coated with polysiloxane[31] or heparin,[32] able to be loaded with lipase or Rhein, an antitumor drug (Figure 23). The tests using 10 μM, 20 μM and 30 μM equivalent free Rhein, as well as those using Rhein-loaded nanoparticles, showed that the viability of tumor hepatocytes is significantly reduced (to approximately 10%) by 30 μM Rhein loaded nanoparticles, and the Rhein conjugates exhibit a better antitumor activity as compared with the same concentration of free Rhein.

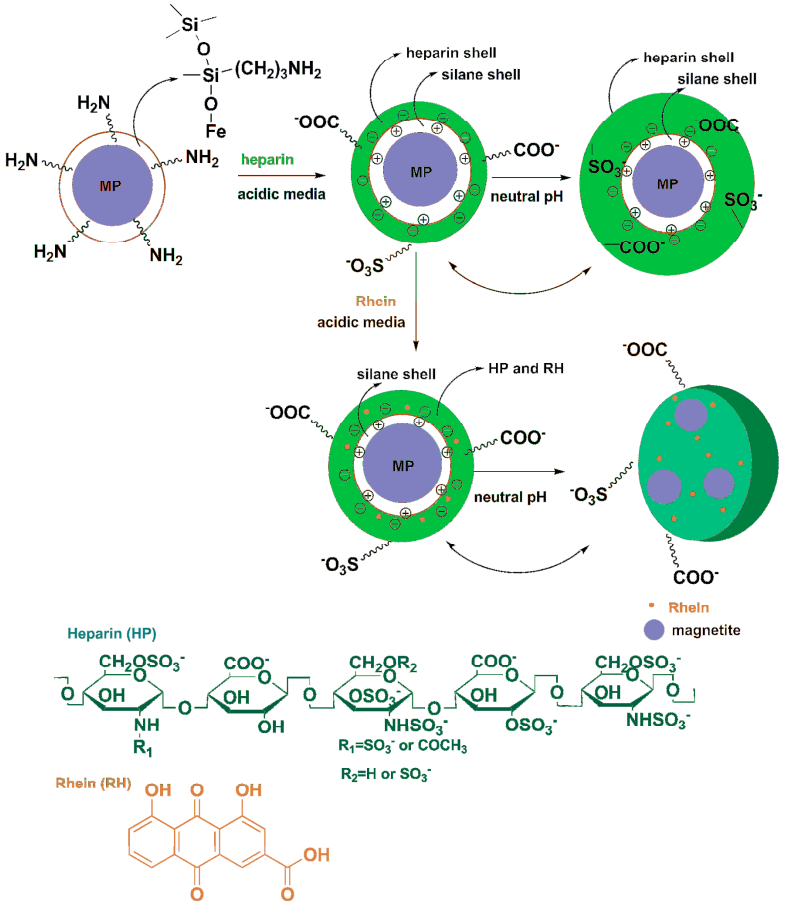


Figure 23. *The principle of MP-HP-RH nanoparticles preparation.*

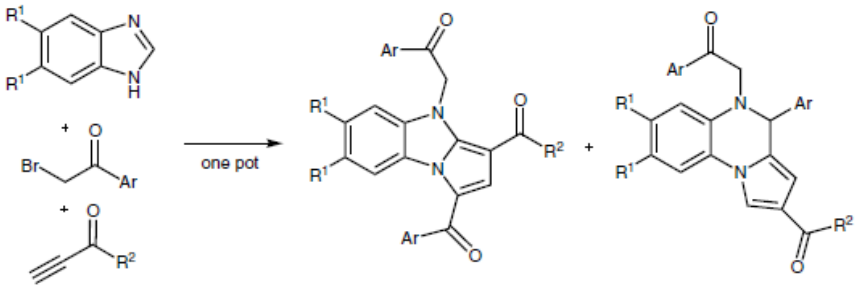
O2.5. . NMR investigations for biological samples

Our research is an original contribution to the "one pot" multicomponent synthesis of pyrroloquinoxalines and pyrrolobenzimidazoles. Thus, during the research activities we developed synthetic pathways for several compounds in the two above mentioned series.

These two heterocyclic series are widely reported and investigated mainly due to their biological properties. Pyrrolo[1,2-a]benzimidazole derivatives (Scheme 3) are known as antitumor agents, and are useful for treating central nervous system disorders. The

pyrrolo[1,2-a]quinoxaline system is found in various bioactive compounds that possesses a broad spectrum of biological activities, including anti-tuberculosis, antiparasitic, and central dopamine antagonist activities.

The structures of the synthesized compounds have been extensively studied by instrumental techniques, particularly by 1H, 13C, and 15N NMR spectroscopy, using less common two-dimensional techniques, as well as X-ray crystallography.



Scheme 3. *One-pot reaction of pyrrolo[1,2-a]benzimidazole derivatives*

We investigated the reaction mechanisms and we proposed synthetic conditions which favor either one or the other representative of the two heterocycles series mentioned above. In order to better characterize these reactions we have also synthesized the intermediates of the multicomponent reactions (benzimidazolium salts) by different synthetic pathways. For some of these intermediates, in addition to structural properties, their inclusion capabilities in cyclodextrines have been also investigated.

The research results have been partially published in a series of papers and other manuscripts are in preparation.[33–38]

Objective 3. A callus-type biomimetic mineralization system with gene transfection ability, immune-evasive and able to carry biochemical or pharmaceutical species, based on natural and synthetic macromolecules with cell recognition domains, active as a substrate for *ex-vivo* cell culturing and potentially injectable in bone injured sites, in order to guide the cell-driven tissue restoration.

O3.1. Development and characterization of injectable structured composites, able to load and transport nucleic acids

We established an easy procedure to preparation of hydrogels based on formation of G4 architecture of guanosine grafted on polyvinyl alcohol boric acid (PVAB) through boric ester dynamic link. We showed that introduction of β-cyclodextrin (βCD) to the hydrogels changes its properties, acting as an adjuvant, due to formation of inclusion complexes with guanosine (Figure 24). Hydrogels were prepared in straightforward fashion, starting with grafting of guanosine on polyvinyl alcohol boric acid (PVAB) in presence of lithium

hydroxide (LiOH) *via* boric acid-diol complexation resulting in the formation of dynamic covalent crosslinks in compound (Figures 24 and 25) which is free-flowing solution and by adding potassium chloride the formation of hydrogel occurs.

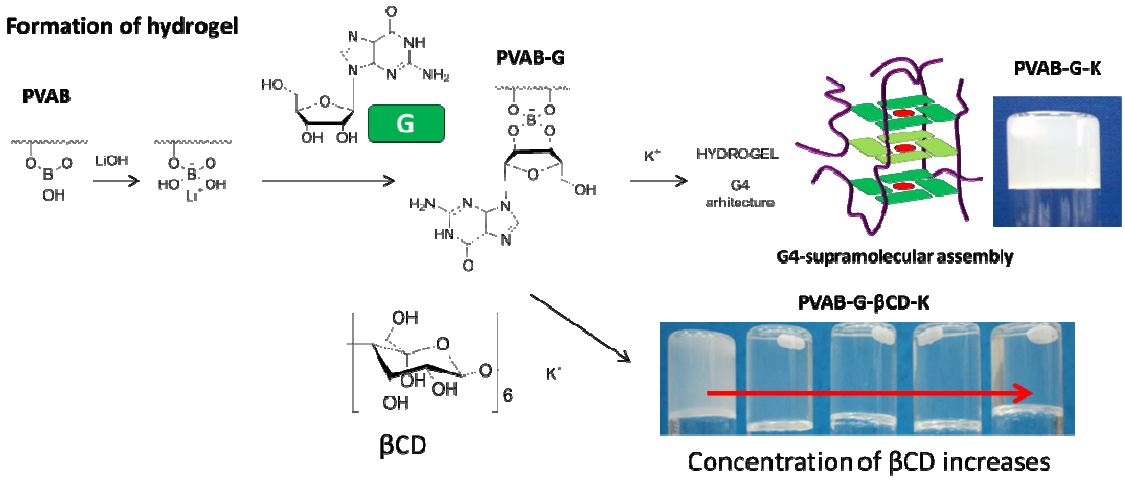


Figure 24. *Schematic representation for the formation of hydrogels.*

^{11}B-NMR spectroscopy has been applied to characterize formation of borate esters between PVAB and guanosine. By ^{1}H-NMR was proved the formation of inclusion complexes between guanosine and βCD.

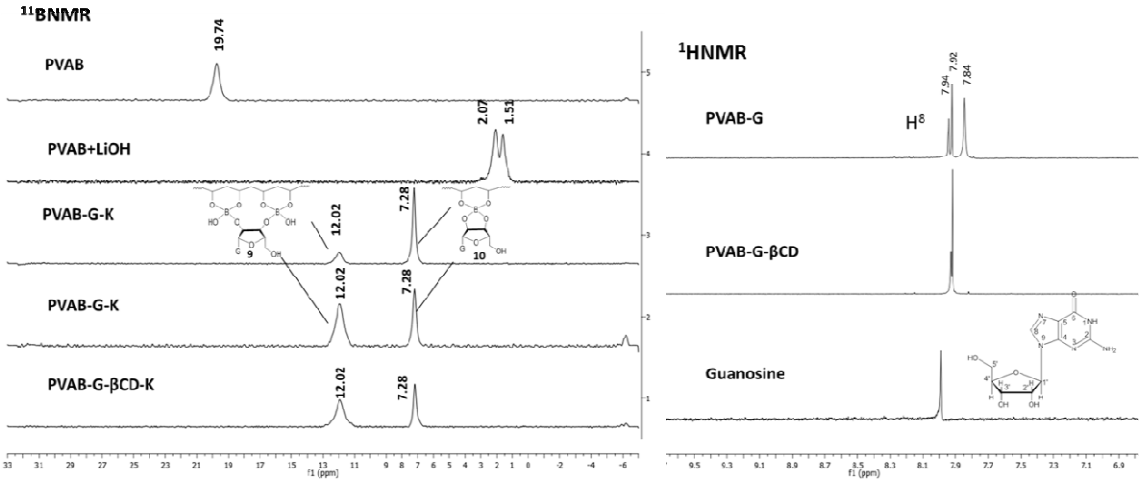


Figure 25. *^{11}B-NMR and ^{1}H-NMR in D_2O for the obtained compounds.*

PVAB-G forms a supramolecular ordered hierarchical polymer architectures only driven by presence of cation like K^+, Na^+ or Ba^{2+}.

Circular dichroism (CD) spectroscopy (Figure 26) is often used to identify G-quadruplex structures and in particular to distinguish all-parallel structures from antiparallel structures. In general, a peak around 260 nm and a trough around 240 nm imply the presence of a parallel G-quadruplex structure. We observed the G-quartet formation for PVAB-G-K and PVAB-G-βCD-K and not for PVAB-G. Presence of the positive bands in the

region of 290 nm could indicate that there is a well-defined stacking of the G-quartets within the hydrogel structure.

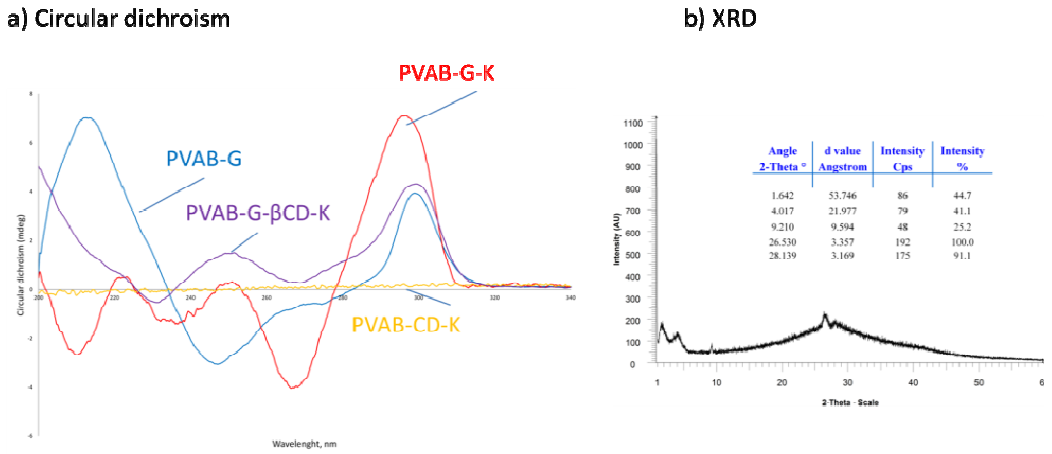


Figure 26. a) *Circular dichroism data.* ***b)*** *X-Ray Diffraction spectrum of PVAB-G-K.*

We have extended characterization of the hydrogel by using X-ray diffraction to obtain molecular-level evidence for G4-quartet formation and stacking of G4-quartet units (Figure 3). XRD data obtained from a freeze-dried sample of hydrogel PVAB-G-K showed a significant peak at $2\theta \approx 26.5°$ (d = 3.3 Å), which is in line with the π–π stacking distance between two planar G4-quartets. A signal at $2\theta \approx 4.01°$ with a corresponding distance of 21.97 Å, coordinated with the width of a single G4-quartet.

Presence of guanosine in obtained gels showed a hydrogel characteristic structure while analyzing by SEM (Figure 27). Presence of K^+ changed the morphology of obtained PVAB-G-K and PVAB-G-βCD-K gels to more dense and compact structure.

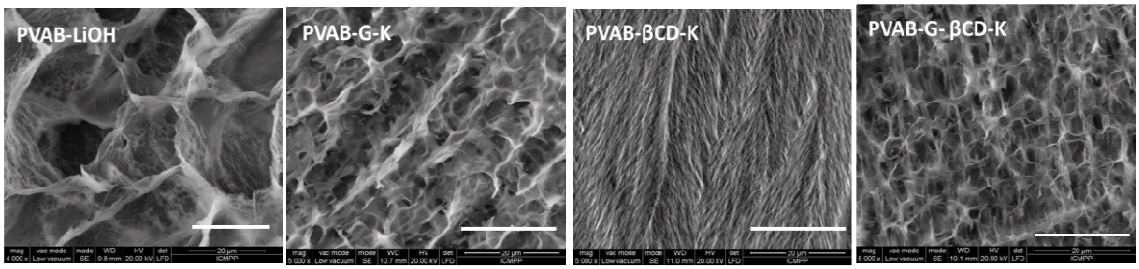


Figure 27. *SEM analysis (scale bar 20 µm).*

Dynamic oscillatory testing is a recognized way to reveal interesting data about microstructure of the materials and to correlate it with the macroscopic behavior (Figure 28). Thus, parameters like complex modulus (G*), storage and loss moduli (G' and G"), loss factor (tan δ=G"/G'), complex viscosity (η*) or phase angle (δ) can be determined obtaining an insight in the internal structure of the sample within the limits of the linear viscoelastic domain.

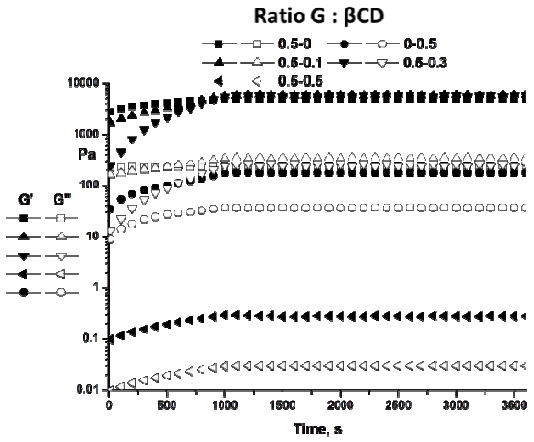


Figure 28. *Time test for the synthesized gels.*

For all samples G' increases fast and reaches a constant plateau after less than 700 s, an indication of the completion of gelation.

In conclusion was:

- Established an easy protocol for preparation of hydrogels based on G-quartet assembly
- Proved the formation of boronic esters by ^{11}BNMR
- Proved the formation of G-quartet/quadruplex assembly (Fluorescence, CD, XRD)
- Established the mode of interaction of βCD with hydrogel components, *i.e.* formation of inclusion complex with guanosine
- Studied some hydrogel properties (rheology, SEM). It is clear that the ratio of G to β-CD has a clear influence on the rigidity and stability of the resulted hydrogel. The softer gel is obtained for PVAB-G-βCD-K (0.5:0.5) while the hardest and most stable structure is characteristic for PVAB-G-K. All these results make us to conclude that these hydrogels can be modulated as injectable hydrogels.

O3.2. Hydrogels based on chitosan cinammaldehyde by dual crosslinking

Another important approach of this research direction is constituted by the obtaining of hydrogels from chitosan and cinammaldehyde by dual crosslinking attributed to the competition between the grafting of cinammaldehyde on the chitosan chains by imine bonding and self-organization of the newly formed imine units, resulting in the producing of some hydrophobic clusters which play the role of multibinder crosslinkers (Figure 29.a). The hydrogels have a sponge like morphology, with pore diameter controlled by the variation of the molar ration of the functional groups (Figure 29.b,c). Rheological investigation revealed elastic properties and a high swelling degree.[39] Preliminary experiments demonstrated that hydrogels favored the proliferation of the osteoblasts, having potential as scaffolds in bone regeneration.

The self-ordering capability of the imine units was demonstrated on a series of low molecular weight model compounds, by single crystal X-ray diffraction (Figure 30).[40] The studies were extended to the synthesis of some benzoate compounds with liquid crystalline

properties and antimicrobial activity, in order to be used for bio-PDLC composite obtaining for biomedical applications as artificial irises for aniridia treatment (Figure 30c,d).[41]

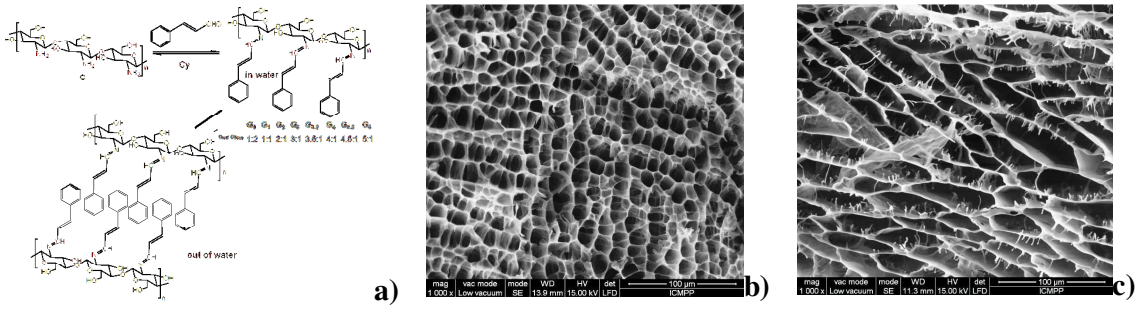


Figure 29. *Hydrogel obtaining demonstrated by NMR analysis and SEM images of two xerogels with different crosslinking degree*

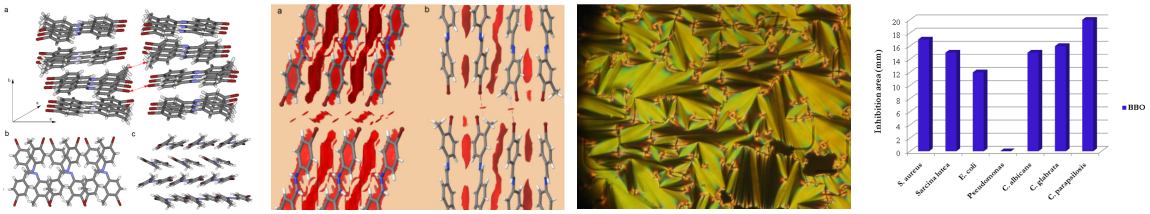


Figure 30. *Compact supramolecular packing of the imine model compounds as demonstrated by single crystal X-ray diffraction, the shape texture and antimicrobial properties of a representative benzoate compound*

O3.3. Preparation and *in vitro* evaluation of a substrate with transfection abilities, as substitute of hard tissue extracellular matrix (ECM)

The main study directions were devoted to:

(1) the development of a biomimetic scaffold (Figure 31);

(2) selection of the 3 non-vectors synthesised by our group (Figure 31);

(3) combination after selection of the 3 components to obtain a complex system, acting for substrate mediated gene transfer, current state considered a realistic alternative to accelerate clinical transfer by simplifying the preparation methodology of non-viral gene-delivery vectors and by improving performances.

The following steps were involved:

A. Organic matrix preparation and evaluation

a) selection, synthesis and purification of the components of the organic matrix: *natural components of ECM: protein (purified atelocollagen (AteCol) or thiolated collagen/atelocollagen, recommended for injectible formulations) and polysaccharides (hyaluronic acid or its derivative dimethylsilanediol hyaluronate- DMSHA, gellan); **synthetic component – a bifunctional reactive derivative of poly(ε-caprolactone), i.e. a diisocyanate (PCL-DI) or dimethacrylate (PCL-DA) -acting as a cross-linker and

degradation rate controller, able to impart increased mechanical resistance, stability and forming ability;[10]

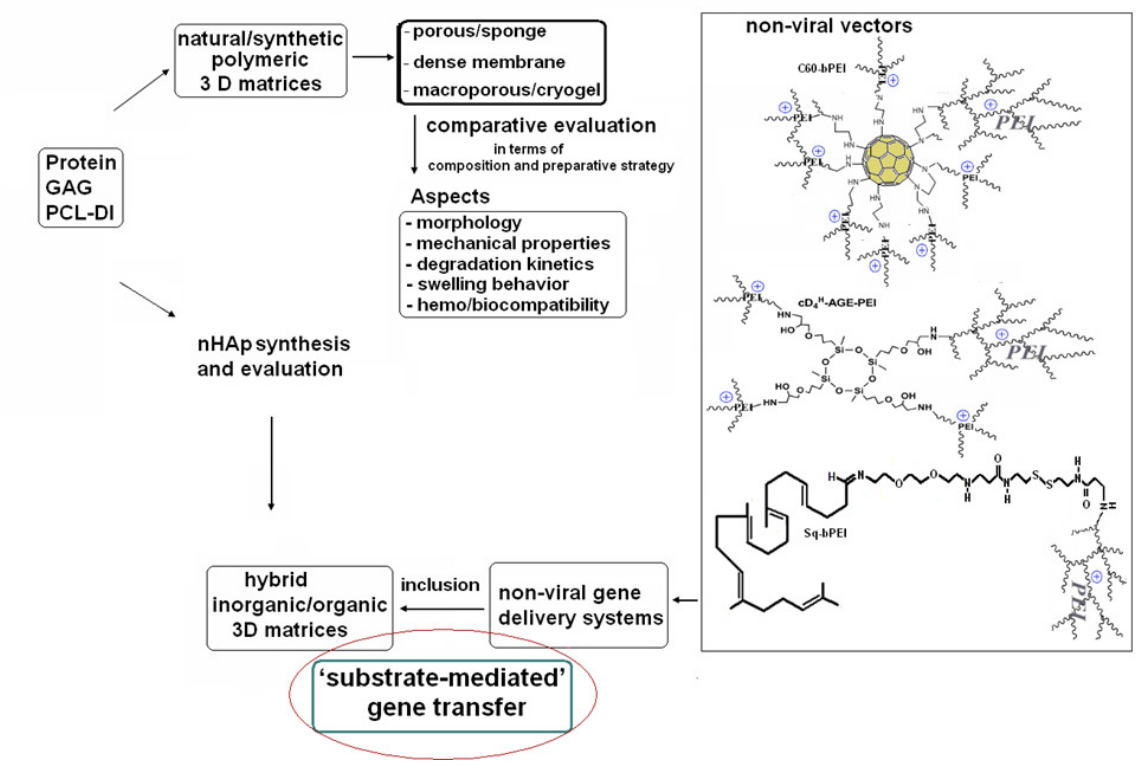


Figure 31. *Schematic representation of design and synthesis of biomimetic scafold.*

b) studies on specific interactions between formulations components, in order to reach stability and reproducibility of the final hydrogel, by choosing the appropriate preparation parameters (pH, components ratio, total concentration);

c) application of different procedures for the preparation of 3D structures based on the selected components and products evaluation in terms of chemical, mechanical, thermal, dielectric and biological properties, swelling and degradation behavior, with respect to the chemical composition (formulation) and specific morphology determined by synthesis protocol, purification facilities;[42–45]

d) selection of the appropriate formulation and preparation conditions making use of the structure- preparative protocol-properties relation, considering the application domain requirements – a macroporous 3D matrix (obtained by cryogelation) with a weight percent ratio of 100:10:10 AteCol:DMSHA:PCL-DI, respectively.[46–49]

B. Development of synthesis procedure for hydroxyapatite with appropriate composition, size (nano-sized), geometry, crystallinity and functionality and its inclusion in the formulation of inorganic/organic biocomposites (as a form of bioactive, bioresorbable biomaterial, representative for the 3rd/4th generation of biomaterials for bone regeneration and repair)

a) electric field assisted synthesis of nano-hydroxiapathite (nHAp) by co-precipitation in the presence of biomacromolecules;[50,51]

b) synthesis and characterization of surface functionalized nHAp by wet precipitation in the presence of cationic additives;[52]

c) inclusion of synthesized nHAp in the formulation of hybrid inorganic /organic cryogels (Figure 32), composition selection;[53]
d) preparation and evaluation in terms of morphology, stability, biocompatibility of hybrid biocomposites cellulose acetate/montmorillonite- silica.[54]

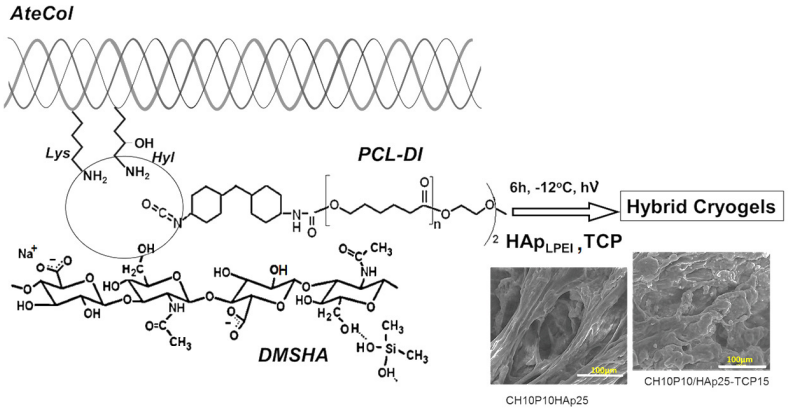


Figure 32. *Preparation of hybrid inorganic/ organic 3D structures.*

C. Inclusion of selected non-viral gene delivery vectors in preformed scaffolds
a) selection of new non-viral gene delivery vectors and their performances evaluation (DNA compacting ability, transfection efficiency, citotoxicity) (Figure 31);
b) inclusion of the selected non-viral gene delivery vectors, as polyplexes, in the multifunctional hybrid matrix;
c) study of the gene-delivery kinetics from the complex combined system;
d) comparative evaluation of the citotoxicity and transfection efficiency of the combined systems based on different selected non-viral gene delivery vectors relative to the known conventional vector (polyethylenimine/PEI, 25 kDa) and the simple, neat polyplex or used plasmid.[10,55]

As conclusions for these studies:
1) Biomimetic multifunctional hybrid inorganic/organic matrices based on functionalized nHAp and natural/synthetic polymers (AteCol/DMSHA/PCL), able to efficiently include gene-delivery systems were prepared.
2) New non-viral gene delivery vectors with improved citocompatibility and high transfection efficiency, based on combination of polyethylenimine (cationic polymer acting as non-viral transfection vector with high DNA compaction ability) with squalene/Sq-PEI, polysiloxan cycle/D4-AGE-PEI or fullerene/C60-PEI were selected.
3) The combined system matrix/gene delivery component is capable of acting as a reservoir that can supply in time the genetic material and determine a sustained expression of a particular protein (coded by the delivered plasmid) in the cells cultured in the presence of matrices. The expression of protein can be observed until about one month, when it is observed an accelerated degradation of the matrix. Also, in the case of embedding the polyplexes PEI 25 kDa/pEYFP, the matrix reduces the toxicity of the branched cationic polymer PEI 25 kDA, while obtaining an increased gene transfection in cells.

4) In the case of C60-PEI immobilization in the matrix walls (inclusion in cryogel feed formulation) , the polyplexes obtained with a plasmid encoding a variant of green fluorescent protein (pEYFP) at an N/P=30 determine the expression of protein encoded by the plasmid in about 40% of cells incubated in the presence of matrix/polyplexes for 3 days. Cells expressing the fluorescent protein can be observed for periods of time up to 13 days of culturing in the presence of matrix C60-PEI/pEYFP. The degradation of matrix occurs gradually, and at 17 days of incubation an advanced degradation is observed.

5) No obvious cytotoxicity of the neat matrix or of the matrix/polyplexes systems was noticed on HEK 293T cells, no matter the C60-PEI vector was immobilized in the walls or included in the pores (after incubation with polyplex dispersion).

Thus new combined systems scaffold/non-viral gene delivery vector for bone regeneration and treatment were developed. The selected formulation offers improved performances in terms of toxicity reduction, insuring long term expression and increased stability of the transfection system.

O3.4. The preparation of chitosan derivatives by grafting therapeutic aldehydes, exploiting the principles of dynamic covalent chemistry to obtain complex supramolecular architectures.

In this sense, nanostructured films with self-defense properties against microorganisms were obtained, with moderate hydrophilicity proper to the biocompatible materials, fact which recommend them for biomedical applications including the indwelling implants.[56,57] Among these films, those based on vanillin demonstrated fungicidal activity against *Candida albicans* strains and a slow release of the antifungal vanillin due to the compact packing at nanometric level. From this reason, the vanillin-imino-chitosan films were proposed as thin coatings of the medical instruments, especially of those used for the treatment of the immune-deficient patients (Figure 33).[58]

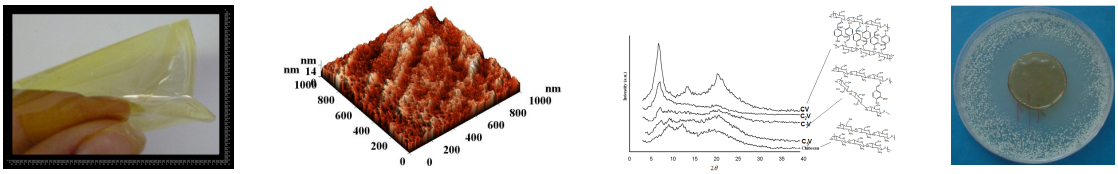


Figure 33. *Representative images of a vanillin-imino-chitosan film:* ***a)*** *self-standing film;* ***b)*** *nanostructured surface of the film as exhibited by AFM;* ***c)*** *the compact packing of the film evidenced by WXRD;* ***d)*** *antifungal activity of the film against C. albicans.*

O3.5. The design of experiments applied for preparation of clay aerogel polymer composites with highly porous internal structure

Clay-based aerogels have attracted an attention owing to their highly porous internal structure and vast applications. In order to investigate the possibility of preparation and biomedical application of natural highly porous materials, we investigated the design of experiments applied for preparation of clay aerogel polymer composites.[59] Three design

variables have been chosen for the proposed experimentation system, i.e. the amounts of montmorillonite (MMT), polyvinyl alcohol (PVA) and sodium dodecyl sulfate (SDS). The major objective for optimization was to maximize the porosity of the materials and performances of materials for sorption. Applying different calculated ratios of reagents, the aerogel sorbent prepared under the optimal conditions of 2.109% w/v PVA, 2.678% w/v MMT and 0.210% w/v SDS bestowed the best sorption performance. Additionally, various synthetic approaches to tune the hydrophobicity of the obtained highly porous materials to enlarge their application have also been performed. All of the prepared clay aerogel polymer composites have been characterized by means of scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) technique.

Objective 4. Developments in investigative methods used in gene therapy or drug delivery systems.

O4.1. A study on electrospray mass spectrometry of fullerenol $C_{60}(OH)_{24}$

It is demonstrated here that electrospray mass spectrometry is a perfectly suitable method to study fullerenol $C_{60}(OH)_{24}$ in pure water and in the presence of aqueous ammonia solution in the negative and positive ionization modes.[60] Fragmentor voltage and capillary voltage were optimized in order to obtain a good signal stability and the best peak intensity distribution for the fullerenol $C_{60}(OH)_{24}$ in both negative and positive modes. While the predominant base peak observed for $C_{60}(OH)_{24}$ in the negative ionization mode was $[M–H]^-$ at *m/z* 1127, those observed in the positive mode were multiply charged $[M\text{-}H_2O+4H]^{4+}$ at *m/z* 279 and $[M\text{-}12H_2O+2NH_3+6H]^{6+}$ at *m/z* 158. In the negative ionization mode, fullerenol readily lose not only H_2O but also lose OH• and H• radicals giving rise to ion species that contain long-living fullerenoxyl radicals and/or short-living fullerenyl radicals, respectively.

O4.2. Multilayer lectin-glyconanoparticles architectures for QCM enhanced detection of sugar-protein interaction

Biological membranes contain dense areas of carbohydrate that has a significant role in cell-cell recognition processes through multivalent binding of lectins. Assuming that higher level in the local density of carbohydrates leads to an improvement in activity has been reported numerous multivalent artificial systems containing various nanosystems (fullerenes, carbon nanotubes, nanoparticles and vesicle) generating multivalent carbohydrate nanoplatforms.

For this purpose, were prepared glycoNPs-lectin composite multilayers organized through specific carbohydrate-lectin multivalent recognition.[61] Our strategy relies on specific very weak sugar-lectin interactions. Evidence is presented that such multivalent interactions between gold glycoNPs and concanavalin A (ConA) can be efficiently used for the construction of multilayered composite architectures and for important enhancement of QCM detection and quantification of carbohydrate–lectin interactions. Con A was immobilised on QCM quartz crystals by combinations of a non-specific hydrophobic interaction and by

recognition of mannan polysaccharide. Specific adsorption through mannoside recognition is a useful method for ConA immobilization and can be accomplished with self-assembled hydrophobic monolayers or adsorbed films of high specificity.

Also, were created new multilayer architecture by successive deposition, layer by layer, of glyconanohybrids based on molybdenum oxide {Mo132} / mannoside $(NH_4)_{42-n}$**2**$_n$**1a**) or glycoside $(NH_4)_{42-m}$**3**$_m$**1a** and ConA, based on multivalent sugar-lectin interactions.[62] These architectures can be easily prepared and quantified using QCM, which detects the adsorption mass at sensor surface on the basis of the reciprocal piezoelectric effect.

The glyconanocapsules obtained specifically interact with lectins and self-assemble in multilayer hybrid architectures only if their external multivalent carbohydrate presentation and lectin recognition sites are compatible. The "biomimetic hybrid multilayers" described here are stable under a continual water flow and they may serve as artificial networks for a greater depth of understanding of various biological mechanisms, which can directly benefit the fields of chemical separations, sensors or storage-delivery devices.

O4.3. Sensor devices, as catalytic supporters, for surface enhanced Raman spectroscopy analysis

One-dimensional Single-Walled Carbon Nanotubes (SWNTs)/nanoparticle hybrid materials, in which SWNTs are often used as scaffolds for the assembly of nanoparticles, are of great importance due to their vast applications in sensor devices, as catalytic supporters, surface enhanced Raman spectroscopy analysis, cancer cell imaging, and thermal therapy. In order to discover new and facile methods to position metal nanoparticles on SWNTs, we involved specially designed synthetic DNA strand as both dispersing agent for SWNTs and suitable template to metal nanoparticle synthesis (Figure 34).[63]

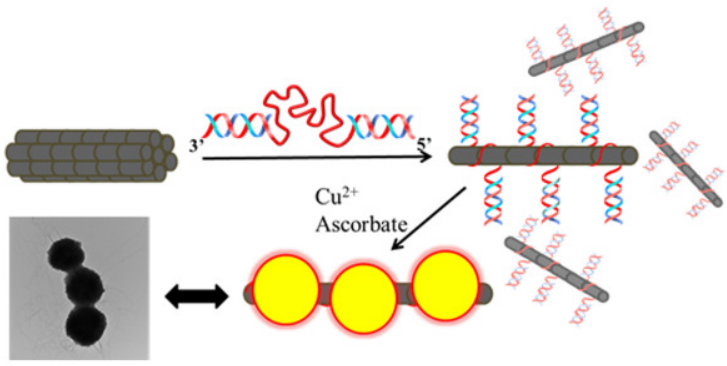


Figure 34. *Dispersion of SWNT bundle by ssDNA containing short dsDNA regions to form SWNT/CuNPs in solutions of* Cu^{2+} *and sodium ascorbate.*

Involved DNA sequence, containing a single-stranded domain for the dispersion of carbon nanotubes and double-stranded domains for the selective growth of CuNPs, was successfully utilized. The final SWCNT/CuNP hybrids were characterized using fluorescence spectroscopy and transmission electron microscopy. Thus prepared hybrid materials a suitable for biomedical applications involving cell transfection and imaging.

O4.4. Sequential pulsed laser deposition technique for the obtaining of high quality Al-doped ZnO (AZO) films with uncommon (110) orientation on amorphous substrate.

ZnO and impurity doped ZnO films have been intensively studied due to their large potential application demonstrated for organic light emitting diodes, solar cells, surface acoustic wave devices, piezoelectric transducers and gas sensing devices. For some applications, thin films of nonpolar zinc oxide may be better suited.

In this context, we proposed the sequential pulsed laser deposition technique for the obtaining of high quality Al-doped ZnO (AZO) films with uncommon (110) orientation on amorphous substrate. This technique consists in alternating laser ablation of two metallic targets (i.e., zinc and aluminum) at room temperature in various experimental conditions (for different dopant concentrations and oxygen deposition pressures). The dependence of the structural, optical and electrical properties with dopant concentration and oxygen deposition pressure was investigated systematically. We note a transition from the (002) preferential orientation of crystallites to an uncommon (110) orientation due to a combined effect of doping concentration and deposition pressure decreasing. For constant deposition pressure of 5 Pa the film crystallinity is changed from preferential (002) to polycrystalline when increasing dopant concentration. For the maximum dopant concentration that we have investigated (i.e., 4.4% at.) structural properties of AZO films are changed from a polycrystalline phase to a (110) preferential orientation when the deposition pressure decreases. This uncommon growth mode is accompanied by a change of the morphology from a densely packed granular structure to a more rarefied one (Figure 35). Moreover, the band gap widens up to 3.88 eV and the electrical resistivity drops to 5.4×10^{-2} Ωcm. The structural changes were attributed to two mechanisms: a first one, responsible for the (002) phase suppression as a consequence of aluminum ion bombardment during the doping process and, a second one, in charge with (110) phase growth as the diffusion rates of zinc and oxygen atoms are affected by the dopant incorporation and by the decrease of deposition pressure.[64]

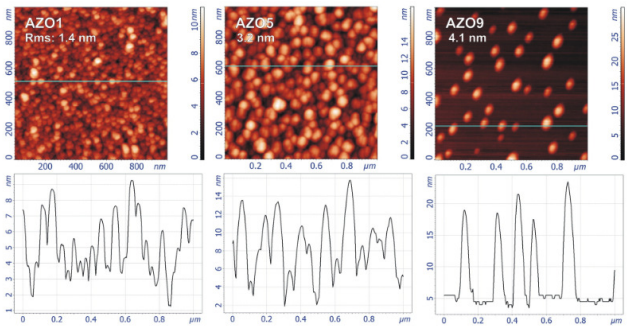


Figure 35. *AFM images recorded for samples with increasing dopant concentration at constant oxygen deposition pressure of 5 Pa and with increasing deposition pressure at constant doping concentration of 4.4% at.*

O4.5. Using different non-invasive methods (FTIR, XPS, ADX, SEM) to investigate the degradation mechanism that occur in fingernails by psoriasis.

In the development and optimization of non-invasive diagnostic, non-invasive techniques, i.e. ATR-FTIR, XPS, EDX, and SEM can be used. By using these methods we demonstrated how the molecular structures of keratin and cystine of nails are damaged due to the presence of psoriatic disease.[65]

O4.6. Development of testing protocols by electrochemical methods of nanoscale systems useful in transfection

In the context of design and preparation of some non-viral vectors, electrochemical techniques have been used in the sequential characterization of synthesized compounds and/or to evaluate the formation of certain structures, by emphasizing the disappearance of redox peaks attributed to certain functional groups from precursors or the appearance of new peaks specific to the newly formed complex.

One research direction in this framework was to evaluate the imine bond formation in order to obtain supramolecular architectures with biomedical applications. The self-ordering capability of the imine units was electrochemically investigated on a series of low molecular weight Schiff base compounds with bromine end groups (presented at O3.2.).

Another important approach in this research direction was to evaluate the effective formation of inclusion complexes between cyclodextrins and different compounds with certain biomedical or pharmacological purposes. The main interest in cyclodextrins lies in their ability to form inclusion complexes with a wide variety of hydrophobic guest molecules. Application of electrochemical techniques to the characterization/detection of the inclusion complexes is enabled only when the guest molecule is electroactive, emphasizing either a subtle shift of the redox potential characteristic to guest molecule or a decrease of the diffusion-limited currents resulting from a change of the diffusion coefficient. By using appropriate chemical architecture, the toxicity of viologens (1,1′-di(hydroxycarbyl)-4,4′-bipyridinium) was proved to be significantly reduced by host–guest complexation, which turns them into candidates of pharmaceutical interest.[1] Among other techniques, the inclusion complex was validated also by cyclic voltammetry showing that the interaction of the bipyridil moiety of guest viologen with the β-cyclodextrin host causes pronounced changes in their redox behavior (Figure 36).

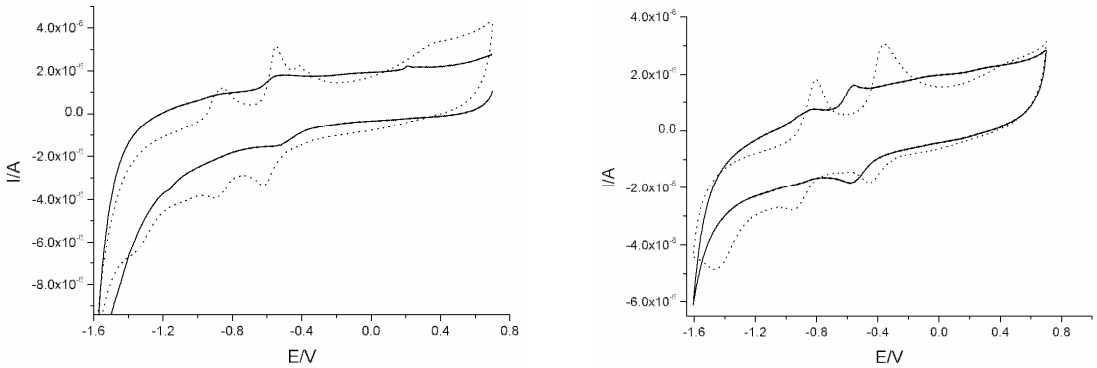


Figure 36. *Comparative electrochemical behavior of bPy2+PDMS (dotted lines) and β-CD/bPy2+PMDS (solid lines), during the first voltametric cycle (a), and after 10 successive cycles (b).*

In the formation of inclusion complexes, the inner diameter of the cavity of CDs and the size of the guest molecule play an important role and these factors have been studied by cyclic voltammetry in the context of inclusion complexes of thiotriazinone (TTZ) with α-cyclodextrin and β-cyclodextrin.[2] The chemistry of 1,2,4-triazinone ring derivatives has

attracted an increasing amount of attention due to their structures and diverse applications in antibacterials, antidepressants, antiviral drugs, pesticides and herbicide dyes. Figure 37 shows only a slight alteration of redox peaks when mixing TTZ with α-CD, indicating a partial inclusion of TTZ molecule into the cavity of α-CD. The association of β-CD with TTZ resulted in remarkable modifications of the electrochemical signals characteristic to TTZ indicating that interactions between CD and TTZ were well-established with the redox center of TTZ located inside the host cavity.

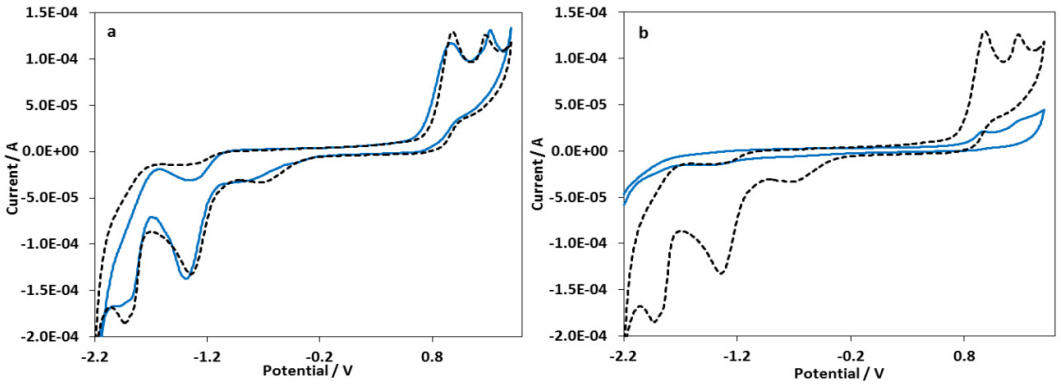


Figure 37. *Electrochemical activity of 20 mM TTZ as free dissolved in solution (dotted line) and as inclusion complex (solid line) with α-CD (a) and β-CD (b) in 0.1 M NaClO4 supporting electrolyte.*

The selective complexation process was also studied in the context of this project, when investigating the capability of crown ether-based structures to bind metal and organic cations from aqueous solution.[3] Electrochemical studies have demonstrated that a graft copolymer p(HPMA-NAS-18C6) obtained by reacting the succinimide rings of N-(2-hydroxypropyl)-methacrylamide-co-N-acryloxysuccinimide copolymer p(HPMA-NAS), with 2-aminomethyl-18-crown-6 (18c6), is able to bind lead cations (Pb^{2+}) from aqueous solution and can efficiently be applied in the development of electrochemical sensor for Pb^{2+} sensitive detection (Figure 38).

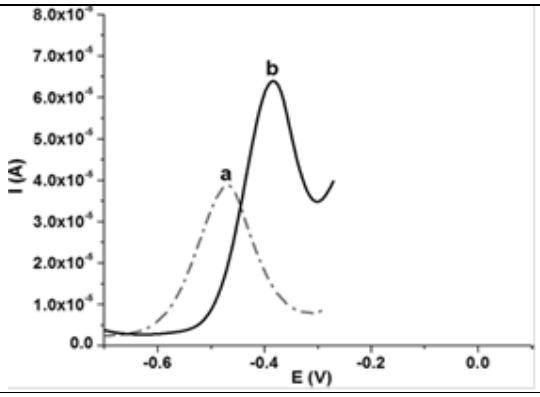


Figure 38. *DPASV response of (a) bare Au electrode and (b) p(HPMA-NAS-18C6) modified electrode to 3 ppm Pb^{2+} in Tris 0.1 M.*

In the context of controlled synthesis of different metal nanoparticles for medical applications, electrochemical deposition has proved to be a powerful tool for the fabrication of nickel–cobalt alloy nanoparticles onto carbonic materials.[4] The electrodeposition process of Ni-Co nanoparticles with different sizes and distributions on different canbon materials used as support (graphene, fullerene and carbon nanotubes) was investigated. It has been shown

how the morphology and composition of the resulting Ni-Co nanoparticles are affected by changing the operating conditions, further reflected in their electrochemical activity and electrocatalytic acitvity toward glucose detection (Figure 39).

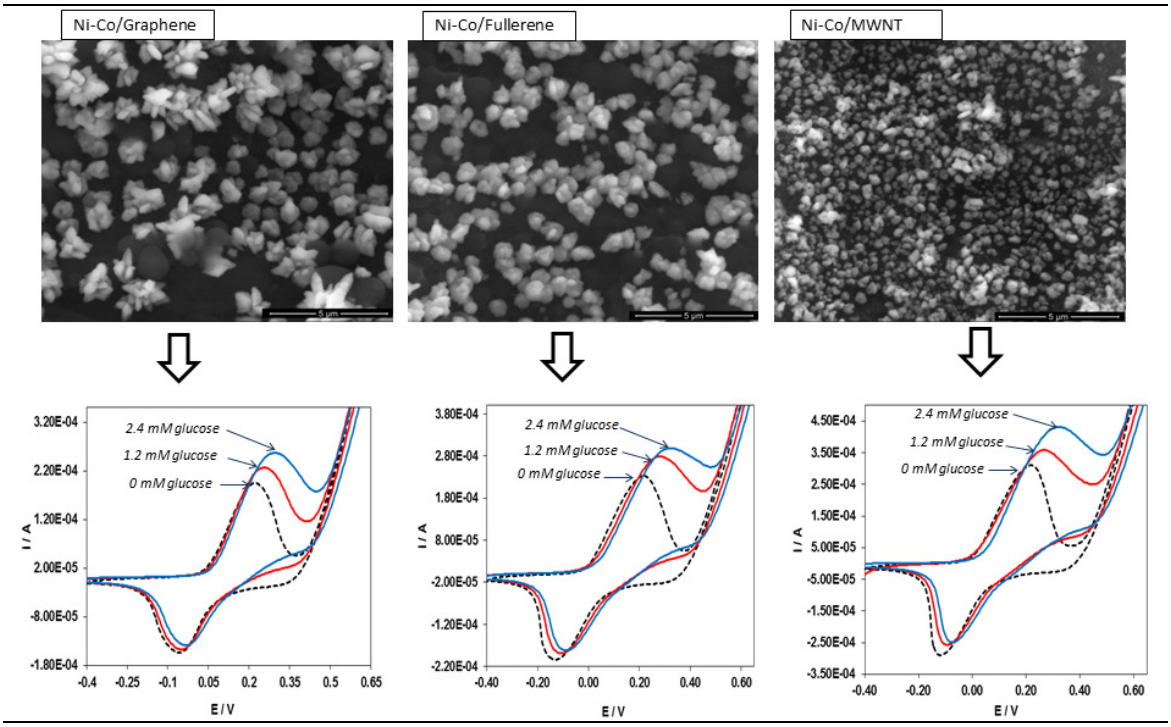


Figure 39. *Ni−Co nanoparticles deposited on graphene, fullerene and CNT modified electrode: morphology and their corresponding electrocatalytic response towards glucose.*

Another important research direction in the context of established objective of the project is the evaluation of DNA attachment on the systems used in transfection. The most used electrochemical technique, such as cyclic voltammetry, could evaluate the attachment of DNA to the surface of micelles by highlighting the disappearance of peaks characteristic to micellar structures and / or appearance to new ones specific to the complex formed. The ability of self-assembled micellar structures to attach DNA was evaluated by comparatively studying their electrochemical behavior before and after interaction with DNA, taking into account that guanine and adenine are the only nucleic acid bases that can be oxidized and the electrochemical detection of DNA in biological samples is based on the oxidation of guanine and/or adenine. At this stage, the sensitivity is very low since the concentration of guanine containing DNA is low and experimental studies are in progress in order to improve the sensitivity and reproducibility of guanine oxidation, by a smart labeling with electroactive compounds (data not published yet).[5]

Project breviary

1. Interdisciplinarity

The present highly *multidisciplinary project* will be developed by a team comprising seven research groups, in order to cover the involved different area of competence. Table 1 summarizes the team composition and argues the *groups complementarity*.

Table 1. *The composition of the project team, and the groups complementarity.*

Code	Research group affiliation	Research leader	Area of expertise (the main directions)
CO	"Petru Poni" Institute of Macromolecular Chemistry – *Nanobioconjugates* Research Group	Dr. Mariana Pinteală *Project Manager*	- fullerene functionalization, polymer synthesis, physical-chemical investigation, organic synthesis, polymer conjugation
P1	"Petru Poni" Institute of Macromolecular Chemistry – *Molecular Design* Research Group	Acad. Bogdan C. Simionescu *Scientific Manager*	- *in silico* design of molecular and supramolecular architectures, physical-chemical properties modelling, chemical structure validation
P2	"Petru Poni" Institute of Macromolecular Chemistry – *Self Assembling Systems* Research Group	Dr. Mihail Bărboiu	- synthesis of advanced (macro)molecular systems, producing of well-defined supramolecular aggregates, functional (macro)molecular structures and systems
P3	"Petru Poni" Institute of Macromolecular Chemistry – *Biosystems Physical-Chemistry* Research Group	Dr. Adrian Salic	- biomolecules physical-chemistry, biomolecules - polymers interactions, functional biomolecular systems, proteomics, (poly)peptide derivatization
P4	"Nicolae Simionescu" Institute of Cellular Biology and Pathology	Acad. Maya Simionescu	- *in vitro*, *ex-vivo*, *in-vivo* tests involving DNA isolation, characterization, trafficking
P5	"Gheorghe Asachi" Technical University of Iaşi	Prof. dr. Geta David	- polymer functionalization, (bio)chemical conjugation, organic-inorganic composites, scaffolds for tissue engineering
P6	"Costin D. Neniţescu" Centre of Organic Chemistry – Romanian Academy, Bucharest	Dr. Florina Dumitru	- advanced (bio)chemical investigations - synthesis of building blocks with self-assembly features and for fullerene derivatization

2. Resources and budget

The project benefited of the research infrastructure of all involved institutions. Four types of studies were performed: (i) computational chemistry, (ii) chemical synthesis, (iii) physical-chemical, biochemical and biophysical imagistics and investigation, (iv) cell culturing and molecular biology.

The total budget of the project: 6 999 150 lei
The budget distribution as a whole per budget categories:

Salaries: 59.02 %
Inventors: 21.46 %
Motilities: 9.15 %
Overheads: 10.37%

The quality of the human resources: 52 persons (26 under 35 years old):

Researchers (including senior researchers): 17 persons
Masters and PhD students: 15 persons
Post-doc positions: 17 persons
Administrative: 3 persons.

The logistic support was an important key for the project success. In this respect, the main costs were only related to the reagents and equipment associated consumables and extensions, especially electronic parts, sophisticated glassware and kits.

3. Results:

Published papers:
Accepted papers:

References

(1) Catana, R.; Barboiu, M.; Moleavin, I.; Clima, L.; Rotaru, A.; Ursu, E.-L.; Pinteala, M. Dynamic Constitutional Frameworks for DNA Biomimetic Recognition. *Chem. Commun.* **2015**, *51*, 2021–2024.

(2) Clima, L.; Rotaru, A.; Cojocaru, C.; Pinteala, M.; Simionescu, B. C. Polymer Engineering Focusing on Drug/Gene Delivery and Tissue Engineering. *5 Th IEEE Int. Conf. E-Health Bioeng. - EHB 2015* **2015**, 248–249.

(3) Clima, L.; Ursu, E. L.; Cojocaru, C.; Rotaru, A.; Barboiu, M.; Pinteala, M. Experimental Design, Modeling and Optimization of Polyplex Formation between DNA Oligonucleotides and Branched Polyethylenimine. *Org. Biomol. Chem.* **2015**, *13*, 9445–9456.

(4) Turin-Moleavin, I.-A.; Doroftei, F.; Coroaba, A.; Peptanariu, D.; Pinteala, M.; Salic, A.; Barboiu, M. Dynamic Constitutional Frameworks (DCFs) as Nanovectors for Cellular Delivery of DNA. *Org. Biomol. Chem.* **2015**, *13*, 9005–9011.

(5) Clima, L.; Peptanariu, D.; Pinteala, M.; Salic, A.; Barboiu, M. DyNAvectors: Dynamic Constitutional Vectors for Adaptive DNA Transfection. *Chem. Commun.* **2015**, *51*, 17529–17531.

(6) Marin, L.; Ailincai, D.; Calin, M.; Stan, D.; Constantinescu, C. A.; Ursu, L.; Doroftei, F.; Pinteala, M.; Simionescu, B. C.; Barboiu, M. Dynameric Frameworks for DNA Transfection. *ACS Biomater. Sci. Eng.* **2015**, *2*, 104–111.
(7) Uritu, C. M.; Varganici, C. D.; Ursu, L.; Coroaba, A.; Nicolescu, A.; Dascalu, A. I.; Peptanariu, D.; Stan, D.; Constantinescu, C. A.; Simion, V. Hybrid Fullerene Conjugates as Vectors for DNA Cell-Delivery. *J. Mater. Chem. B* **2015**, *3*, 2433–2446.
(8) Uritu, C. M.; Calin, M.; Maier, S. S.; Cojocaru, C.; Nicolescu, A.; Peptanariu, D.; Constantinescu, C. A.; Stan, D.; Barboiu, M.; Pinteala, M. Flexible Cyclic Siloxane Core Enhances the Transfection Efficiency of Polyethylenimine-Based Non-Viral Gene Vectors. *J. Mater. Chem. B* **2015**, *3*, 8250–8267.
(9) Ardeleanu, R.; Calin, M.; Maier, S. S.; Dascalu, A. I.; Cojocaru, C.; Uritu, C. M.; Nicolescu, A.; Silion, M.; Peptanariu, D.; Pinteala, M. Transfection-Capable PEGylated-Cyclodextrin-Containing Polycationic Nanovectors. A New Synthesis Pathway. *Beilstein J. Org. Chem.* **2016**, *sent for publication*.
(10) David, G.; Vasiliu, T.; Clima, L.; Peptanariu, D.; Balan, M.; Simionescu, B. C. Squalene/Polyethyleneimine – Based Non-Viral Gene Delivery Systems. *Lucru* **2016**.
(11) Racles, C. Polydimethylsiloxane–indomethacin Blends and Nanoparticles. *AAPS PharmSciTech* **2013**, *14*, 968–976.
(12) Racles, C.; Mares, M.; Sacarescu, L. A Polysiloxane Surfactant Dissolves a Poorly Soluble Drug (Nystatin) in Water. *Colloids Surf. Physicochem. Eng. Asp.* **2014**, *443*, 233–239.
(13) Racles, C.; Iacob, M.; Butnaru, M.; Sacarescu, L.; Cazacu, M. Aqueous Dispersion of Metal Oxide Nanoparticles, Using Siloxane Surfactants. *Colloids Surf. Physicochem. Eng. Asp.* **2014**, *448*, 160–168.
(14) Petrovici, A. R.; Rosca, I.; Stoica, I.; Silion, M.; Nicolescu, A.; Dodi, G.; Simionescu, N.; Varganici, C. D.; Ivanov, I.-C.; Pinteala, M. Biosynthesis of Exopolysaccharides by Weissella Confusa in a New Culture Medium. *Rom Biotech Lett* **2016**, *in press*.
(15) Rosca, I.; Petrovici, A. R.; Brebu, M.; Stoica, I.; Minea, B.; Marangoci, N. An Original Method for Producing Acetaldehyde and Diacetyl by Yeast Fermentation. *Braz. J. Microbiol.* **2016**.
(16) Fundueanu, G.; Constantin, M.; Asmarandei, I.; Bucatariu, S.; Harabagiu, V.; Ascenzi, P.; Simionescu, B. C. Poly (N-Isopropylacrylamide-Co-Hydroxyethylacrylamide) Thermosensitive Microspheres: The Size of Microgels Dictates the Pulsatile Release Mechanism. *Eur. J. Pharm. Biopharm.* **2013**, *85*, 614–623.
(17) Constantin, M.; Bucatariu, S.; Harabagiu, V.; Popescu, I.; Ascenzi, P.; Fundueanu, G. Poly (N-Isopropylacrylamide-Co-Methacrylic Acid) pH/Thermo-Responsive Porous Hydrogels as Self-Regulated Drug Delivery System. *Eur. J. Pharm. Sci.* **2014**, *62*, 86–95.
(18) Fundueanu, G.; Constantin, M.; Bucatariu, S.; Ascenzi, P. pH/Thermo-Responsive poly(N-Isopropylacrylamide-Co-Maleic Acid) Drug Delivery System with a Sensor and an Actuator. *Polymer* **2016**, *submitted*.
(19) Tarabukina, E. B.; Simonova, M. A.; Bucatariu, S.; Harabagiu, V.; Fundueanu, G.; Filippov, A. P. Behavior of Thermo-and pH-Responsive Copolymer of N-Isopropylacrylamide and Maleic Acid in Aqueous Solutions. *Int. J. Polym. Anal. Charact.* **2016**, *21*, 11–17.
(20) Constantin, M.; Bucatariu, S.; Ascenzi, P.; Simionescu, B. C.; Fundueanu, G. Poly (NIPAAm-Co-β-Cyclodextrin) Microgels with Drug Hosting and Temperature-Dependent Delivery Properties. *React. Funct. Polym.* **2014**, *84*, 1–9.
(21) Asmarandei, I.; Fundueanu, G.; Cristea, M.; Harabagiu, V.; Constantin, M. Thermo-and pH-Sensitive Interpenetrating Poly (N-Isopropylacrylamide)/Carboxymethyl Pullulan Network for Drug Delivery. *J. Polym. Res.* **2013**, *20*, 1–13.

(22) Bucatariu, S.; Fundueanu, G.; Prisacaru, I.; Balan, M.; Stoica, I.; Harabagiu, V.; Constantin, M. Synthesis and Characterization of Thermosensitive Poly (N-Isopropylacrylamide-Co-Hydroxyethylacrylamide) Microgels as Potential Carriers for Drug Delivery. *J. Polym. Res.* **2014**, *21*, 1–12.
(23) Constantin, M.; Bucatariu, S.; Stoica, I.; Fundueanu, G. Smart Nanoparticles Based on Pullulan-G-poly(N-Isopropylacrylamide) for Controlled Delivery of Indomethacin. *Int J Biol Macromol* **2016**, *accepted*.
(24) Bucatariu, S.; Constantin, M.; Ascenzi, P.; Fundueanu, G. Poly (Lactide-Co-Glycolide)/Cyclodextrin (Polyethyleneimine) Microspheres for Controlled Delivery of Dexamethasone. *React. Funct. Polym.* **2016**, *107*, 46–53.
(25) Constantin, M.; Bucatariu, S.; Harabagiu, V.; Ascenzi, P.; Fundueanu, G. Do Cyclodextrins Bound to Dextran Microspheres Act as Sustained Delivery Systems of Drugs? *Int. J. Pharm.* **2014**, *469*, 1–9.
(26) Minea, B.; Nastasa, V.; Moraru, R.; Kolecka, A.; Flonta, M.; Marincu, I.; Man, A.; Toma, F.; Lupse, M.; Doroftei, B. Species Distribution and Susceptibility Profile to Fluconazole, Voriconazole and MXP-4509 of 551 Clinical Yeast Isolates from a Romanian Multi-Centre Study. *Eur. J. Clin. Microbiol. Infect. Dis.* **2015**, *34*, 367–383.
(27) Mares, M.; Minea, B.; Nastasa, V.; Bostanru, A.; Toma, V.; Cristea, V. C.; Murariu, C.; Pinteala, M.; Marincu, I.; Doroftei, B. In Vitro Inhibitory and Fungicidal Activity of Echinocandins against a Collection of Clinical Yeast Isolates from Romania. *Eur. J. Clin. Microbiol. Infect. Dis.* **2016**, *submitted*.
(28) Marangoci, N. L.; Minea, B.; Nicolescu, A.; Neamtu, A.; Varganici, C. D.; Rosca, I. Inclusion Complexes of Propiconazole Nitrate with Substituted β-Cyclodextrins: Synthesis, Characterization and in Silico Assessment. *J Mater. Sci Eng* **2016**, *5*, 72.
(29) Rosca, I.; Minea, B.; Peptanariu, D.; Marangoci, N.; Fifere, A.; Mares, M. Inclusion Complex of Propiconazole Nitrate with Substituted β-Cyclodextrin S II: In Vitro Assessment of Antifungal Properties. *J Mater. Sci Eng* **2016**, *5*, 71.
(30) Alexandrica, M. C.; Silion, M.; Timpu, D.; Popa, M. I. A Study on the Intercalation Process of Tramadol Hydrochloride in ZnAl - Nitrate Layered Double Hydroxides. *Rev. Roum. Chim.* **2016**, *61*, accepted.
(31) Durdureanu-Angheluta, A.; Ignat, M.-E.; Maier, S. S.; Pricop, L.; Coroaba, A.; Fifere, A.; Pinteala, M.; Chiriac, A. Lipolytic Biocatalyst Based on Recyclable Magnetite-Polysiloxane Nanoparticles. *Appl. Surf. Sci.* **2014**, *292*, 898–905.
(32) Durdureanu-Angheluta, A.; Uritu, C. M.; Coroaba, A.; Minea, B.; Doroftei, F.; Calin, M.; Maier, S. S.; Pinteala, M.; Simionescu, M.; Simionescu, B. C. Heparin-Anthranoid Conjugates Associated with Nanomagnetite Particles and Their Cytotoxic Effect on Cancer Cells. *J. Biomed. Nanotechnol.* **2014**, *10*, 131–142.
(33) Nicolescu, A.; Deleanu, C.; Georgescu, E.; Georgescu, F.; Iurascu, A.-M.; Shova, S.; Filip, P. Unexpected Formation of Pyrrolo [1, 2-a] Quinoxaline Derivatives during the Multicomponent Synthesis of Pyrrolo [1, 2-a] Benzimidazoles. *Tetrahedron Lett.* **2013**, *54*, 1486–1488.
(34) Nicolescu, A.; Balan, M.; Georgescu, E.; Georgescu, F.; Ursu, L.; Simionescu, B. C.; Filip, P.; Deleanu, C. Benzimidazolium-Cyclodextrin Inclusion Complexes. *Rev. Chim.* **2013**, *64*, 451–455.
(35) Georgescu, E.; Nicolescu, A.; Georgescu, F.; Teodorescu, F.; Marinescu, D.; Macsim, A.-M.; Deleanu, C. New Highlights of the Syntheses of Pyrrolo [1, 2-a] Quinoxalin-4-Ones. *Beilstein J. Org. Chem.* **2014**, *10*, 2377–2387.
(36) Georgescu, E.; Nicolescu, A.; Georgescu, F.; Shova, S.; Teodorescu, F.; Macsim, A.-M.; Deleanu, C. Novel One-Pot Multicomponent Strategy for the Synthesis of Pyrrolo [1, 2-a] Benzimidazole and Pyrrolo [1, 2-a] Quinoxaline Derivatives. *Synthesis* **2015**, *47*, 1643–1655.

(37) Georgescu, E.; Nicolescu, A.; Georgescu, F.; Teodorescu, F.; Shova, S.; Marinoiu, A. T.; Dumitrascu, F.; Deleanu, C. Fine Tuning the Outcome of 1, 3-Dipolar Cycloaddition Reactions of Benzimidazolium Ylides to Activated Alkynes. *Tetrahedron* **2016**, *72*, 2507–2520.
(38) Georgescu, E.; Nicolescu, A.; Georgescu, F.; Shova, S.; Simionescu, B. C.; Deleanu, C. Contributions to Syntheses of pyrrolo[1,2-A]benzimidazole Derivatives via 1,3-Dipolar Cycloaddition Reactions. *Rev. Roum. Chim.* **2016**, *61*, 283–290.
(39) Marin, L.; Moraru, S.; Popescu, M.; Nicolescu, A.; Zgardan, C.; Simionescu, B. C.; Barboiu, M. Out-of-Water Constitutional Self-Organization of Chitosan–Cinnamaldehyde Dynagels. *Chem. Eur. J.* **2014**, *20*, 4814–4821.
(40) Marin, L.; Harabagiu, V.; van der Lee, A.; Arvinte, A.; Barboiu, M. Structure-Directed Functional Properties of Symmetrical and Unsymmetrical Br-Substituted Schiff-Bases. *J. Mol. Struct.* **2013**, *1049*, 377–385.
(41) Ailincai, D.; Marin, L.; Shova, S.; Tuchilus, C. Benzoate Liquid Crystals with Direct Isotropic–smectic Transition and Antipathogenic Activity. *Comptes Rendus Chim.* **2016**, *19*, 556–565.
(42) David, G.; Simionescu, B.; Maier, S.; Balhui, C. Micro-/Nanostructured Polymeric Materials: Poly (ε-Caprolactone) Crosslinked Collagen Sponges. *Dig J Nanomater Biostruct* **2011**, *6*, 1575–1585.
(43) Lefter, C.-M.; Maier, S. S.; Maier, V.; Popa, M.; Desbrieres, J. Engineering Preliminaries to Obtain Reproducible Mixtures of Atelocollagen and Polysaccharides. *Mater. Sci. Eng. C* **2013**, *33*, 2323–2331.
(44) Maier, V.; Lefter, C. M.; Maier, S. S.; Butnaru, M.; Danu, M.; Ibanescu, C.; Popa, M.; Desbrieres, J. Property Peculiarities of the Atelocollagen–hyaluronan Conjugates Crosslinked with a Short Chain Di-Oxirane Compound. *Mater. Sci. Eng. C* **2014**, *42*, 243–253.
(45) Simionescu, B. C.; Neamtu, A.; Balhui, C.; Danciu, M.; Ivanov, D.; David, G. Macroporous Structures Based on Biodegradable Polymers—candidates for Biomedical Application. *J. Biomed. Mater. Res. A* **2013**, *101*, 2689–2698.
(46) Diaconescu, R.; Simionescu, B. C.; David, G. Control and Prediction of Degradation of Biopolymer Based Hydrogels with Poly (Ɛ-Caprolactone) Subunits. *Int. J. Biol. Macromol.* **2014**, *71*, 147–154.
(47) David, G.; Cristea, M.; Balhui, C.; Timpu, D.; Doroftei, F.; Simionescu, B. C. Effect of Cross-Linking Methods on Structure and Properties of Poly (ε-Caprolactone) Stabilized Hydrogels Containing Biopolymers. *Biomacromolecules* **2012**, *13*, 2263–2272.
(48) Balhui, C.; David, G.; Drobota, M.; Musteata, V. E. Dielectric Characterization of Biopolymer/Poly (ϵ-Caprolactone) Hydrogels. *Int. J. Polym. Anal. Charact.* **2014**, *19*, 234–244.
(49) David, G.; Fundueanu, G.; Pinteala, M.; Minea, B.; Dascalu, A.; Simionescu, B. C. Polymer Engineering for Drug/Gene Delivery: From Simple towards Complex Architectures and Hybrid Materials. *Pure Appl. Chem.* **2014**, *86*, 1621–1635.
(50) Maier, S. S.; Pinteala, M.; Simionescu, B. Procedeu Pentru Controlul Caracteristicilor Particulelor de Hidroxiapatită Sintetizată În Prezenţa Biomacromoleculelor. *OSIM Bucur.* **2013**, *cerere de brevet A / 00710/ 27.09.2013*.
(51) Maier, S. S.; Pinteala, M.; Maier, V.; Simionescu, A.-B. Instalaţie Pentru Controlul Sintezei Particulelor de Hidroxiapatită Prin Intermediul Câmpului Electric Aplicat În Regim Capacitiv. *OSIM Bucur.* **2016**, *cerere de brevet 2016*.
(52) David, G.; Sacarescu, L.; Timpu, D.; Vasiliu, T. A Comparative Study of Functionalized Nano-Hydroxyapatite. In; IEEE, 2015; pp. 1–4.
(53) Timpu, D.; Simionescu, B. C.; Sacarescu, L.; Vasiliu, T.; David, G. Functional Hydroxyapatite Synthesis and Possible Applications- a Comparative Study. *Lucru* **2016**.

(54) Corobea, M. C.; Muhulet, O.; Miculescu, F.; Antoniac, I. V.; Vuluga, Z.; Florea, D.; Vuluga, D. M.; Butnaru, M.; Ivanov, D.; Voicu, S. I. Novel Nanocomposite Membranes from Cellulose Acetate and Clay-silica Nanowires. *Polym. Adv. Technol.* **2016**.
(55) Constantinescu, C. A.; Stan, D.; Uritu, C. M.; Pinteala, M.; Simionescu, M.; Calin, M. Dynamin-Dependent Endocytosis Is the Main Pathway Involved in DNA Transfection of HEK 293T Cells by Siloxane/PEI-DNA Polyplexes. In; Bulletin of RSCB: Oradea, Romania, 2016; p. 59.
(56) Marin, L.; Ailincai, D.; Mares, M.; Paslaru, E.; Cristea, M.; Nica, V.; Simionescu, B. C. Imino-Chitosan Biopolymeric Films. Obtaining, Self-Assembling, Surface and Antimicrobial Properties. *Carbohydr. Polym.* **2015**, *117*, 762–770.
(57) Ailincai, D.; Bejan, A.; Titorencu, I.; Drobota, M.; Simionescu, B. C. IMINO-CHITOSAN DERIVATIVES. SYNTHETIC PATHWAY AND PROPERTIES. *Rev Roum Chim* **2014**, *59*, 385–392.
(58) Marin, L.; Stoica, I.; Mares, M.; Dinu, V.; Simionescu, B. C.; Barboiu, M. Antifungal Vanillin–imino-Chitosan Biodynameric Films. *J. Mater. Chem. B* **2013**, *1*, 3353–3358.
(59) Rotaru, A.; Cojocaru, C.; Cretescu, I.; Pinteala, M.; Timpu, D.; Sacarescu, L.; Harabagiu, V. Performances of Clay Aerogel Polymer Composites for Oil Spill Sorption: Experimental Design and Modeling. *Sep. Purif. Technol.* **2014**, *133*, 260–275.
(60) Silion, M.; Dascalu, A.; Pinteala, M.; Simionescu, B. C.; Ungurenasu, C. A Study on Electrospray Mass Spectrometry of Fullerenol C60 (OH) 24. *Beilstein J. Org. Chem.* **2013**, *9*, 1285–1295.
(61) Mahon, E.; Mouline, Z.; Silion, M.; Gilles, A.; Pinteala, M.; Barboiu, M. Multilayer Lectin–glyconanoparticles Architectures for QCM Enhanced Detection of Sugar–protein Interaction. *Chem. Commun.* **2013**, *49*, 3004–3006.
(62) Barboiu, M.; Mouline, Z.; Silion, M.; Licsandru, E.; Simionescu, B. C.; Mahon, E.; Pinteala, M. Multivalent Recognition of Concanavalin A by {Mo132} Glyconanocapsules—Toward Biomimetic Hybrid Multilayers. *Chem. Eur. J.* **2014**, *20*, 6678–6683.
(63) Ursu, E.; Clima, L.; Hejesen, C.; Rotaru, A.; Pinteala, M. DNA-Mediated Copper Nanoparticle Formation on Dispersed Single-Walled Carbon Nanotubes. *Helv. Chim. Acta* **2015**, *98*, 1141–1146.
(64) Coman, T.; Ursu, E. L.; Nica, V.; Tiron, V.; Olaru, M.; Cotofana, C.; Dobromir, M.; Coroaba, A.; Dragos, O.-G.; Lupu, N. Improving the Uncommon (110) Growing Orientation of Al-Doped ZnO Thin Films through Sequential Pulsed Laser Deposition. *Thin Solid Films* **2014**, *571*, 198–205.
(65) Coroaba, A.; Pinteala, T.; Chiriac, A.; Chiriac, A. E.; Simionescu, B. C.; Pinteala, M. Degradation Mechanism Induced by Psoriasis in Human Fingernails: A Different Approach. *J. Invest. Dermatol.* **2016**, *136*, 311–313.

[1] N. Marangoci, S.S. Maier, R. Ardeleanu, A. Arvinte, A. Fifere, A.R. Petrovici, A. Nicolescu, V. Nastasa, M. Mares, S.A. Pasca, R.F. Moraru, M. Pinteala, A. Chiriac, "Low Toxicity β-Cyclodextrin-Caged 4,4′-Bipyridinium-bis(siloxane): Synthesis and Evaluation", *Chemical Research in Toxicology,* **27(4), 2014, 546-557**

[2] A. Arvinte, N. Marangoci, A. Nicolescu, M. Pinteala, „Electrochemical evidence for inclusion complexes of thiotriazinone with cyclodextrins" RSC Advances 6, 2016, 82817 - 82823

[3] I.-M. Simionca, A. Arvinte, M. Pinteala, "Crown ether-based structures for sensitive electrochemical detection", *High Performance Polymers* 27(5), **2015**, 669-675

[4] A. Arvinte, F. Doroftei, M. Pinteala, "Comparative electrodeposition of Ni-Co nanoparticles on carbon materials and their efficiency in electrochemical oxidation of glucose" *J Appl Electrochem* 46, **2016,** 425–439

[5] Arvinte A et al. *under investigation*