Registration number,
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of Macromolecular Chemistry

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SCIENTIFIC STAGE REPORT

PN-III-P4-ID-PCCF-2016-0050 Project. Contract no. 4/2018.

-- 2018 Stage --

Mimicking Living Matter Mechanisms by Five-dimensional Chemistry Approaches

Mimarea mecanismelor viului prin abordări ale chimiei supramoleculare, în cinci dimensiuni

Acronym: 5D-nanoP

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Contents

I. The project main objective

The generic aim of **5D-nanoP** project is to contribute to the emerging field of biomimetics, by supramolecular chemistry advanced approaches, in a strong multi- and inter-disciplinary context. As main objective, the project intends to develop a strategy to synthesize and test (macro)molecular entities of dynamic (re)organizing type, able to act as nanoplatforms of biologic and biomedical relevance.

II. The project frame

Mimicking of the living matter mechanism of cooperation by complementarity represents one of the most challenging tasks of supramolecular chemistry. The momentary solution consists in using particularly designed molecular unimers, endowed with the necessary amount of chemical information.

The **5D-nanoP** project is dedicated to interfacing the fundamental research area of constitutional dynamic chemistry with the practical approaches of medicinal chemistry and biomedical applications. In the spirit of a metaphor of Jean-Marie Lehn (Nobel Prize in Chemistry, 1987), the project aims to materialize the concept of 5D chemistry in designing, synthesizing, characterizing, and using molecules with conditional affinity, to build versatile supramolecular nanoplatforms able to vectorize compounds of pharmaceutical or biochemical relevance, all of them involved in physiologic and pathologic processes at cell- and tissue-level.

The project will add the layer of 5D chemistry over the backgrounds of molecular assembling line techniques, to produce particulate nanoplatforms, self-assemblable in the virtue of the chemical information stored by the designed unimer molecules. Two modern techniques of building dynamic chemical structures will be considered: (**i**) the use of self-immolative linkers, and (**ii**) the space stepwise and time phased assisted synthesis. In order to prove the applicability of the produced nanoplatforms, an ex vivo cell cultivation system will be developed, to emulate tissue/tumor niches.

Seven teams will be involved in the **5D-nanoP** project, to cover the main addressed research areas: (**i**) the *in silico* molecular design, (**ii**) the development of a unimers chemical library, (**iii**) the development of a molecular assembling line, (**iv**) the conjugation of the developed platforms with chemical species of biomedical interest, (**v**) the build of *ex vivo* emulating niches, and (**vi**) the bio-oriented assessment of the nanoconstructs efficacy.

III. Generic approaches within the 2018 stage of the project

The 2018 stage of **5D-nanoP** project is devoted to (**i**) ultimate documentary clarifications on the envisioned topics, (**ii**) general work organization from the point of view of the experimenting techniques, methods and protocols, and (**iii**) first general studies in the area of producing and testing (macro)molecular nanoplatforms having potential bio-medical applications. The main goal of the said stage consists in starting the collaboration between the project teams, within the frame of the assumed implementation plan, by means of:

- a common definition of the structure and functionality of the nanoplatforms to be developed;
- the general experimental strategies to be used during the synthesis and characterization of the nanoplatforms;
- a first / generic selection of the supramolecular-active unimers and of (macro)molecular segments involved as nanoplatform's building-blocks;
- the concept of testing the developed nanoplatforms from the points of view of their (**i**) chemical structure, (**ii**) supramolecular behavior (dynamic re-organization of their parts, *time and space scales of their existance*, structural integrity under real phyisical-chemical and biochemical conditions), (**iii**) functionality proving in simulated environments, and in real biochemical and cellular contexts.

The implementation plan of the future stages will be particularized starting from the findings and achievements resulted during the 2018 stage, obeying the assumed goals of the project.

The details of the implementation plan of Stage 2018 are summarized in Table 1.

Table 1. *Implementation plan of* ***5D-nanoP*** *– Stage 2018.*

Year	Stage	Main objective of Stage 2018	Activities and sub-activities carried out	Stage results and deliverables
2018	The single one	**Preliminary, documentary and experimental studies on the design and obtaining of nanoplatforms**	**A1.1.** Defining of bio- and physico-chemical principles for the selection of nanoplatform (macro)molecular constituents (unimmers, substrata, spacers, functional groups and segments), and of their *ex vivo* testing milieus and/or environments.	1. Research report. 2. Generic protocols of nanoplatforms synthesis and testing. 3. Four scientific papers. 4. Project workshops
			A1.2. Establishing of feasible synthesis pathways, and designing the protocols of synthesis and of physico-chemical testing for the nanoplatform components, and of the conditions of their functionality testing.	
			A1.3. Selection of precursors and of chemical adjuvants involved in nanoplatforms synthesis.	
			A1.4. Preliminary testing of chemical synthesis principles and pathways in nanoplatforms producing.	

IV. Defining the pragmatic goals of 5D-nanoP project

Generally speaking, **nanoplatforms are functional entities, compositionally and structurally defined at molecular level**. Their generic function is **to constitute the confined "scene" of particular highly-selective processes**.

In the field of bio-medical applications (and *from the point of view of bio-pharma practitioners' community*), three types of nanoplatforms can be defined:

- immobile / immobilized, irrigable „**assembling lines**", reversibly attached to rigid substrata in order to collectively act as micro-reactors for the (bio)synthesis and delivery of biochemically / pharmaceutically relevant compounds;
- soft, nanoparticulate, spatially-defined, **colloidally-free, or hydrogel-embedded structures** capable of functioning as spatial and temporary "headquarters" / locations of (bio)chemical reactions, or as portable and triggerable carriers;
- **lax-structured macromolecular edifices**, having docking sites and/or dockable segments, able to temporarily collect and confine molecular precursors and/or actuators of interest in site-guided interactions / reactions.

Each of them is versatile enough to could be *a priori* designed in terms of structure-to-function relation. Therefore, *nanoplatforms are considered to represent rudimentary nano-robots, well-defined at molecular level, and having reproducible local functionality*. Their **main drawbacks** consist in (**i**) the **uncontrollable real-time operation**, and (**ii**) the **uncertain fate, after end-of-service**, especially in biological milieus.

5D-nanoP project intends to make some steps towards reproducibly producing nanoplatforms applicable as tools in bio-medical research and, hopefully, in medical cure. **The distinctive approach of the project is related to the valorization of supramolecular mechanisms of directed and controlled (re)assembling of unimers** (molecules having complementary conformation, and the ability to generate functional molecular constructs by reciprocal docking; defined as building-blocks in producing supramolecular aggregates).

Figure 1 presents a generic type of functional nanoplatforms, such as those that would be developed through the project.

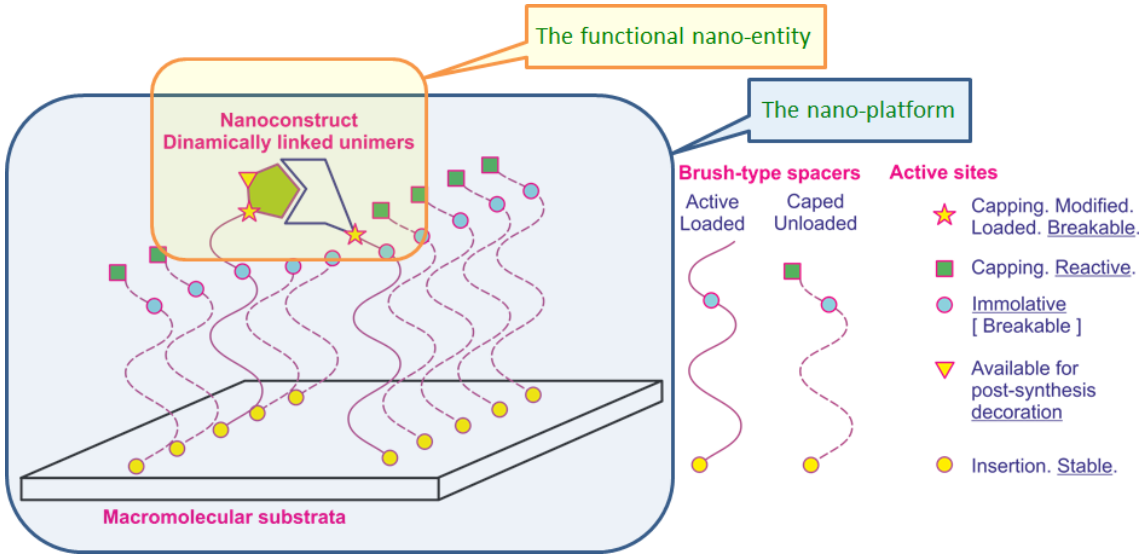


Figure 1. *A generic type of nanoplatforms. The overall structure of "assembling line" kind.*

The generic nanoplatform structure in Figure 1 is dedicated to the dynamic assembling of temporarily linked unimers on top of a brush-like substratum, in order to generate a functional (nano)construct able to be released in a triggered manner. Particularly, the "assembling line" kind of nanoplatforms favors the confinement of at least two distinctive types of unimer molecules, by generating a crowded (macro)molecular environment which statistically increases their reciprocal docking. Usually (but not necessarily), one of the unimers is pre-attached to the brush segments, and the other one is added and dosed through a carrier fluid, by the irrigation of the immobile nanoplatform.

The (macro)molecular design of such nanoplatforms take into consideration the crowding requirements of the specific unimers that will be used, by means of their (**i**) overall volume and mass, (**ii**) steric peculiarities, (**iii**) necessary rotational freedom during docking attempts, (**iv**) potential lateral chains and groups, (**v**) precise reactivity, hydrophilicity, and range of physical interactions, and (**vi**) physical-chemical affinity against the carrying fluid. The density of the brush segments and the amount of pre-attached unimer is correlated with the (imposed) yield of the supramolecular assembling processes, implicitly with the docking kinetics, and with the irrigation flow. A supplemental design constraint is defined by the requirement of final construct triggered detaching. In such a case, some rigorously cleavable linkages and/or molecular segments must be included, usually on the brushes chain.

5D-nanoP project will attempt to develop nanoplatforms (potentially) of "assembling line" type, able to produce and deliver (supra)molecular nano-constructs having relevant biochemical and/or pharmacological action. In this way, the membranes of cell organelles could be mimicked in a lucrative manner, by reproducing the pathways of the directed / assisted supramolecular assembling of biologic functional constructs.

V. Tasks assumed by the individual teams involved, at the project start

5D-nanoP project is benefitting from the expertise of seven research teams (a coordinator, **Co**, and five partners, **P1** to **P6**), chosen to ensure the complementarity imposed by the ambitious project goals. Table 2 resumes the main tasks of the involved teams.

Table 2. *The role of the teams involved in 5D-nanoP project, and their tasks during 2018 stage.*

Team	Leaders, role, and tasks
Co	***Team leader:*** **Professor Atto Laaksonen**, PhD (computational chemistry)
	Role in the project: Conducting *in silico* studies on nanoplatforms components, steric design, functionality, and fate. Assistance on sysnthesis design, and instrumental characterization.
	Specific task during 2018 stage: Selection of *in silico* investigation techniques, according the envisioned peculiarities of unimers and nanoplatforms.
P1	***Team leader:*** **Professor Claudiu T. Supuran**, PhD (pharmaceutical chemistry)
	Role in the project: Guiding the nanoplatforms design, synthesis, characterization and testing, according the project objectives. Selection of unimers. Design the investigation experiments and functionality tests.
	Specific task during 2018 stage: Testing enzyme systems for unimers pre-selection. Design and conduct stopped-flow experiments to evaluate enzyme systems efficacy.
P2	***Team leader:*** **Dr. Ioan Cianga** (macromolecular synthesis; conductive polymers)
	Role in the project: Design, synthesis, and characterization of nanoplatforms precursors and macromolecular support systems for testing and application.
	Specific task during 2018 stage: Quasi-exhaustive reviewing the subjects on conductive polymers synthesis, characterization and applications as versatile substrata. Designing protocols for nanoplatforms precursors production.

Table 2. *(Continue)*

Team	Coordinator, role, and tasks
P3	***Team leader:*** **Dr. Gheorghe Funduianu** (biomaterials with tailored properties)
	Role in the project: Production and characterization of tissue and tumor surrogates, as emulation environments for nanoplatforms testing.
	Specific task during 2018 stage: Documenting the techniques, methods and protocols for producing reproducible 3D structured soft fibrillar / porous cell culturing systems and biomaterials based on (bio)macromolecular compounds. Designing the strategy for tissue / tumor surrogates producing.
P4	***Team leader:*** **Dr. Maria Cazacu** (organic and macromolecular synthesis)
	Role in the project: Design, synthesis and characterization of functional ligands, including bio-degradable and bio-active segments, as parts of functional nanoplatforms.
	Specific task during 2018 stage: Documentary investigation of the subject of functional (macro)molecular entities and segments. A first selection of brush polymer candidates, and of the chemistry behind their synthesis, modification, and reactivity.
P5	***Team leader:*** **Dr. Maya Simionescu** (cell biology and pathology)
	Role in the project: Advanced testing of nanoplatforms functionality in relation with cell and tissue requirements. Proving the potential applications of nanoplatforms.
	Specific task during 2018 stage: The selection of the appropriate techniques, methods and protocols for *in vitro* and *ex vivo* testing of unimers, nanoplatforms, and nanoconstructs. Developing the investigation strategies at biological level.
P6	***Team leader:*** **Dr. Călin Deleanu** (advanced investigation of chemical compounds)
	Role in the project: Structure elucidation of the synthesized precursors, molecular segments, and nanoplatforms. Proofing and proving the physical-chemical characteristics of the used compounds and synthesized products.
	Specific task during 2018 stage: Elaboration of the main experimental protocols for structure elucidation and properties measurement for all the potential compounds used and synthesized during the project stages.

Figure 2 summarizes the steps to be performed in order to produce and test "assembling line" types of nanoplatforms, like those pictured in Figure 1, together with the involved teams. A massive implication of *in silico* studies is necessary in order to guide all the unimers selection and synthesis steps, and to elucidate the involved mechanisms, including those related to the functionality of the unimers, nanoplatforms and nanoconstructs.

The first step is dedicated to the compilation of a library of unimer candidates, followed by the rigorous selection of those that reproducibly obey the requirements of supramolecular assembling, both in simple biochemical systems, and in (macro)molecular crowded environments. This step is critical and highly chronophagous (time consuming). This is why, some enzyme involving systems will be considered in the attempts to emulate real biological / pharmacological environments, like those that valorize the inhibition of carbonic anhydrases. P1 team will lead the first step in unimers selection, by searching some carbonic anhydrase inhibitors prone to supramolecular assembling, in parallel with other (back up) candidates.

The second and the third steps will be leaded by teams P2 and P4, in order to develop the molecular components of the designed nanoplatforms, and to accurately demonstrate the reproducibility of both the involved processes, and the (supra)molecular architecture of them and of the produced nanoplatforms. Figure 3 depicts the large involvement of Co team, and the support of P6 team in the decisions to be taken. The synthesis of nanoplatforms (starting from and including the various developed segments) represents the second critical step to be circumvented under the guidance of molecular modeling, and based on instrumental analyses.

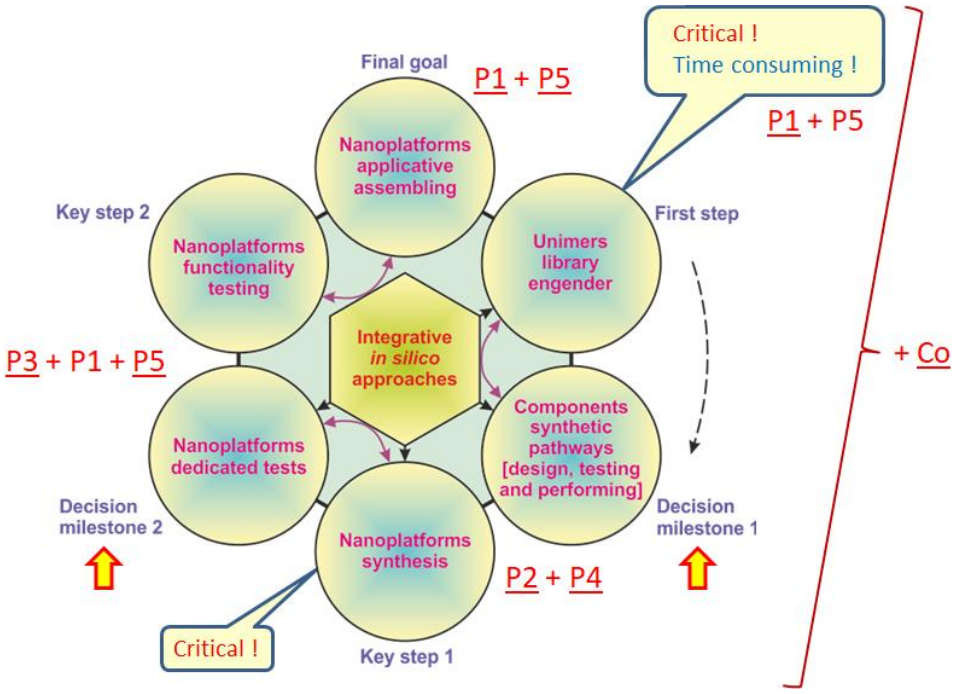


Figure 2. *The blueprint of the overall teams involvement in 5D-nanoP project envisioned steps.*

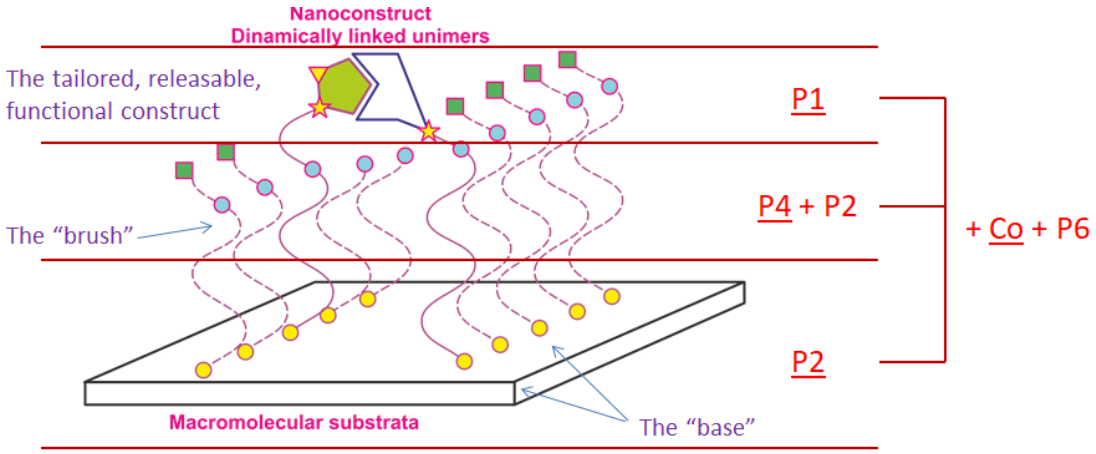


Figure 3. *Teams involved in the development of nanoplatforms components.*

Steps four and five are dedicated to nanoplatforms physical-chemical and functional testing. Based on the principles elaborated by P1 team, two types of (bio)macromolecular surrogates will be developed as realistic environments, mainly by the contribution of P3 team, and under the guidance of P5. Figure 4 describes the collaboration framework during the four and five steps. Especially in the fifth step, team P4 will intervene in guiding the selection of release triggering conditions of the supramolecular nanoconstructs. In parallel, P1 team will conduct the preliminary functionality tests, in order to establish the procedures of nanoplatforms usage, and to design the frames of real applications. Through the collaboration between P1 and P5 teams, the surrogate environments variously loaded with nanoplatforms will be tested against primary cultured cells, tumoral cells, and (likely) against some microorganisms, in order to prove the effects of the carried / released nanoconstructs.

The tissue / tumor surrogates will specifically include the biological / biochemical cues of both chemical and morphological types. In order to ensure the optimal density of necessary cues, biomacromolecules will represent the main fraction of the surrogates' composition.

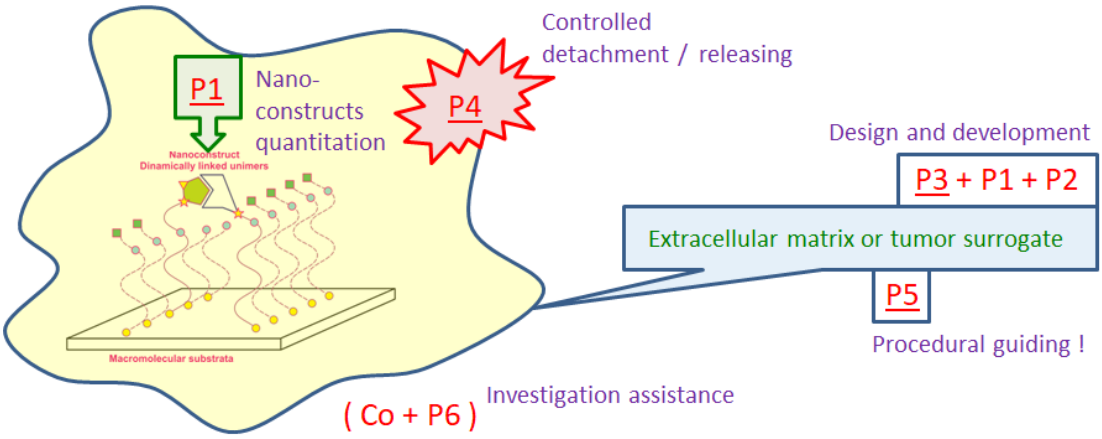


Figure 4. *The collaboration framework during the preliminary nanoplatforms testing.*

The final step of the project will be the responsibility of P1 and P5 teams, and will develop the "real world" biomedical applications of, and with the developed nanoplatforms, together with the usage of the nanoconstructs produced in real time. Figure 5 describes the facts of interest in the final functionality evaluation of nanoplatforms. Potentially, inhibitors of cancer-associated carbonic anhydrases will represent the applications of the developed nanoplatforms.

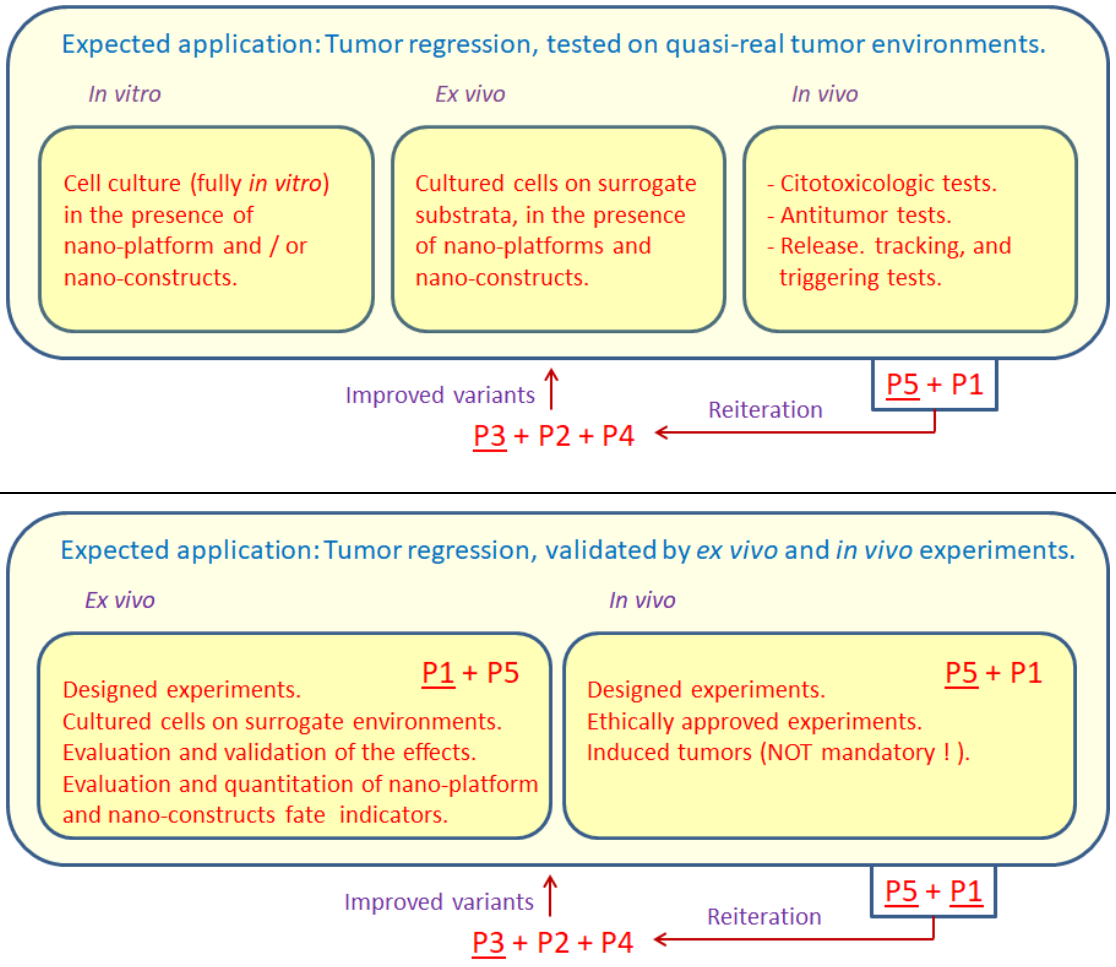


Figure 5. *A blueprint of the nanoplatforms expected applications testing.*

VI. The research results of 2018 stage

Two classes of results were obtained during the 2018 stage of 5D-nanoP project:

- the creation of a targeted documentary resource devoted to the subsequent stages of the project;
- preliminary experimental data regarding the choosing of feasible unimers that belong to the class of carbonic anhydrase inhibitors.

A. The documentary approaches were conducted over the six distinctive areas of interest in the 5D-nanoP project ((**i**) unimers selection, characterization, and use, (**ii**) macromolecular synthesis of nanoplatforms substrata, (**iii**) organic synthesis and characterization of particular segments, (**iv**) production of systems for *ex vivo* nanoplatforms testing, (**v**) computational chemistry and *in silico* techniques for functional (macro)molecules investigation, (**vi**) techniques and methods for biological / biomedical evaluation of nanoplatforms and nanoconstructs functionality), and are described in the following sections.

B. The experimental investigation of a class of carbonic anhydrases inhibitors has led to data that are the subject of a scientific article accepted for publication in *Journal of Enzyme Inhibition and Medicinal Chemistry* (ISI; 2017 Impact Factor: 3.638; Taylor & Francis Online; open access), in November 7, 2018, under the title: *Inhibition of bacterial α-, β- and γ-class carbonic anhydrases with selenazoles incorporating benzenesulfonamide moieties*. The particular studied inhibitors belong to a wider class, that of benzensulfonamides having antitumor activity (Sun, et al., 2017), and was used to test the stopped flow technique which will be further involved in the studies dedicated to the evaluation of unimers efficacy.

VI.1. General aspects on the approaches and applications of supramolecular chemistry

Supramolecular chemistry represents the ‘‘chemistry beyond the molecule’’ (Lehn, 1988). In contrast to molecular chemistry, which is predominantly based on the covalent bonding of atoms, supramolecular chemistry is based upon intermolecular interactions, i.e. on the association of two or more building blocks, which are held together by intermolecular bonds. The dynamic and reversible nature of the non-covalent interactions endows the resultant supramolecular architectures with excellent stimuli responsive features, and infinite possibilities (Zhang & Wang, 2011; Yang, et al., 2015; Dong, et al., 2015). Among various non-covalent interactions, including hydrogen bonding, π-π stacking interactions, host–guest interactions, electrostatic interactions, and charge-transfer interactions, host–guest interactions are attracting more and more attention, arising from their distinctive properties by introducing macrocylic hosts into supramolecular systems (Yu, et al., 2012; Xue, et al., 2015). Macrocylic molecules, such as crown ethers, cyclodextrins, calixarenes, cucurbiturils and pillararenes, usually have hydrophobic cavities in which the guests can be embedded (Yu, et al., 2015; Appel, et al., 2012). These magnificent macrocycles provide ideal platforms for the fabrication of supramolecular systems for biomedical applications. Within the current project we intend to develop, *inter alia*, several distinct research directions involving supramolecular approaches based on host–guest interactions, more precisely on cyclodextrin-based systems. Cyclodextrins (CDs), a class of cyclic oligosaccharides with six to eight D-glucose units linked by a-1,4-glucose bonds, are water-soluble, nontoxic, commercially available compounds with low price, and their structures are rigid and well defined. Most importantly, they possess a hydrophobic cavity that can bind various inorganic/organic/biological molecules and ions in both aqueous solution and the solid state, CDs are extensively studied as not only excellent receptors for molecular recognition but also convenient building blocks to construct nanostructured functional materials, especially bioactive materials.

We also proposed to develop a new membrane channel based on cyclodextrin tubes decorated with alkyl chains attached *via* amide, ester or ureic bonds. The development of the proposed supramolecular construct requires the completion of the following steps:

(i) preparation of pseudorotaxane structure composed of a short linear polymer chain (axle) and threading cyclodextrin molecules;

(ii) preparation of polyrotaxane by the addition of a blocking component to the axle in order to sterically hinder the de-threading of the cyclodextrin molecules;

(iii) intermolecular coupling of the threading cyclodextrin by the covalent attachment of a short linker;

(iv) isolation of the empty cyclodextrin molecular tubes by cleaving the blocking component;

(v) covalent attachment of alkyl chains on the available functional groups.

The construction of molecular cyclodextrin tubes have been reported. A simplified version of the process is presented in Figure 6 (Harada, et al., 1992; Harada, et al., 1993; Ichi, et al., 2001).

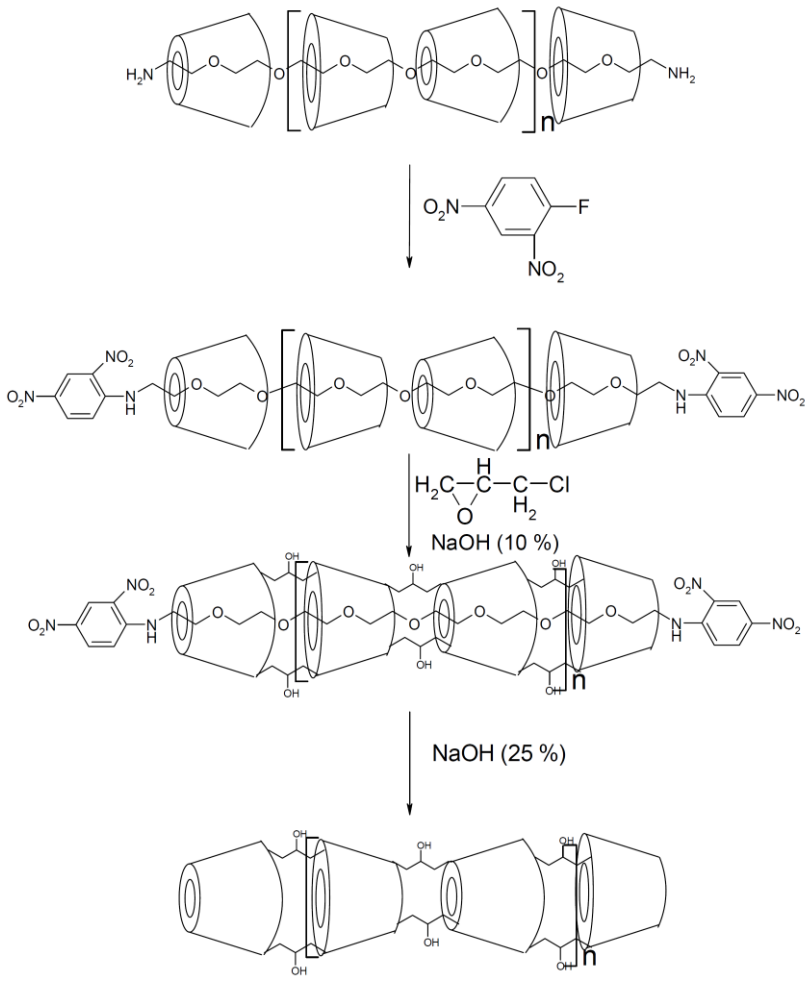


Figure 6. *Schematic representation of the preparation of cyclodextrin molecular tubes: blocking the pseudorotaxane (with 2,4-dinitrofluorobenzen), intermolecular coupling of threading cyclodextrin units and release of cyclodextrin molecular tubes respectively.*

We expect to explore experimentally and theoretically the formation of host-guest inclusion complexes of newly synthesized organic compounds (fluorophores / ligands) from our labs and investigate their biomedical applications including cell and/or cellular component

labeling (Pricope, et al., 2018), enzymatic activity inhibition or acceleration (Gray, et al., 2013; Kraskouskaya, et al., 2013), drug delivery systems, etc. Our preliminary results (Pricope, et al., 2018) have shown excellent outcomes on cellular components labeling by host-guest complexes and this research will be extended by:

- design, synthesis and screening of new pH sensitive fluorophores (or other small organic ligands), supplemented by theoretical studies (DFT / HF / semiempirical);
- experimental formation of inclusion complexes with host molecules (cyclodextrins); detailing the binding mechanism for host-guest complexes by molecular docking simulations;
- theoretical investigation (molecular docking and molecular dynamics [MD]) on the possibility for the interactions between inclusion complexes and selected enzymes / proteins (e.g., protein kinases); such approach will enable the detecting of binding sites (pockets), identification of the most probable complexes and further equilibration by MD simulations to assess the role of explicit water molecules in ligand binding and complex stabilization;
- experimental applications of the inclusion complexes in cell labeling and development of essays to support the theoretical results on enzyme activity inhibition or acceleration;
- explore new host molecules (calyxarenes) and investigate the host-guest interactions followed by applications in the above mentioned fields;
- for particular systems, additional computational and experimental research can be undertaken to reveal molecular interactions between ligands and other type of macromolecules (e.g. oligonucleotides, charbohydrates, etc.).

The theoretical and experimental research on the enzyme activity inhibition is an actual topic (and a completely new direction for us), which we expect to develop in the frame of the present project. Experimental and computational tools existing at our department are appropriate in these lines. For example, UV-Vis, fluorescence and circular dichroism spectroscopy techniques will be used to investigate experimentally the interaction between molecular/macromolecular entities. The available software programs that will be employed deal with Gaussian/GaussView (Gaussian 09. *Gaussian*. Inc., Wallingford CT, **2009**. Official Website: http://www.gaussian.com/) for DFT/HF quantum chemistry calculations; MOPAC (MOPAC2016, Version: 18.025W, Stewart Computational Chemistry, web: HTTP://OpenMOPAC.net; (Stewart, 2013)) coupled with GabEdit (Allouche, 2011) for semiempirical computations and YASARA-Structure (Krieger & Vriend, 2015; Krieger & Vriend, 2014); Official web-site of YASARA software: www.yasara.org) for molecular docking and molecular dynamics simulations.

Besides the host–guest interactions, it is expected that supramolecular approach will also be involve in the development of the platforms to design artificial enzyme models mimicking natural enzymes, which is a promising and active field that has been pursued by researchers for several decades (Marchetti & Levine, 2011; Steed & Gale, 2012). Although many attempts have been made to reproduce the structures and functions of enzymes, the complexity of enzymes via natural selection and evolution severely constrained the ability of researchers to replicate the enzymatic features. Therefore, it is a long-term goal for chemists to develop synthetic chemical equivalents to natural enzymes in terms of structure, catalytic efficiency, specificity, selectivity, etc. Beyond understanding the behavior of molecules composed of constituent atoms, the advent of supramolecular chemistry allows one to capture the collective behavior of organized ensembles of molecules. The features of enzymes, both substrate recognition and catalysis, were intrinsically managed by their supramolecular structures (Hammes-Schiffer & Benkovic, 2006; Merlo, et al., 2005; Benkovic & Hammes-Schiffer, 2003). Essentially, the combined complexity and cooperativity of enzymes can be adequately achieved by the dynamic assembly of supramolecular blocks with catalytic moieties and binding sites.

The term of nanozyme was initially introduced in 2004 by Manea et al. (Manea, et al., 2004) and Gao et al. (Gao, et al., 2007), and is currently attributed to all nanomaterials that possess an intrinsic enzyme-like activity (Wei & Wang, 2013; Yan, 2018). Most of them are represented by simple or combined metal/metal oxide nanoparticles, usually amended with various ligands to enhance and fine tune the catalytic behavior, although carbon-based (i.e. fullerene, graphene) and other types of nanozymes have been also reported (Garg, et al., 2015; Wang, et al., 2016; Lin, et al., 2014; Sun, et al., 2018).

Similar to the natural enzymes, nanozymes are nanosized, with irregular shapes, rich surface chemistry, and their activities may be characterized by slightly adjusting the same set of methods and procedures commonly used for protein-type catalysts due to the fact that both are active against the same categories of substrates (Zhou, et al., 2017; Jiang, et al., 2018). Moreover, nanozymes are active on biologically relevant substrates and metabolites within the common range of environmental and physiological conditions, providing an important link between nanomaterials and living systems (Wang, et al., 2016; Zhou, et al., 2017; Fathi Karkan, et al., 2017; Korschelt, et al., 2018; Li, et al., 2018).

Various modifications of the initial nanozymes by doping, alloying, core-shell or surface deposition (Bhagat, et al., 2018; Li, et al., 2018), as well as by physical, chemical or biological conjugation with (re)active, dynamic molecular / supramolecular structures at surface (Chen, et al., 2015; Liu & Liu, 2017; Fernandes, et al., 2017; Vranish, et al., 2018) may conduct to multifunctional integrated nanomaterials (Gao, et al., 2017; Wu, et al., 2018; Wu, et al., 2018) that are particularly attractive to design, synthesize, characterize and test within the framework of the 5D-nanoP project. Figure 7 depicts a potential nanozyme-based nanoplatform.

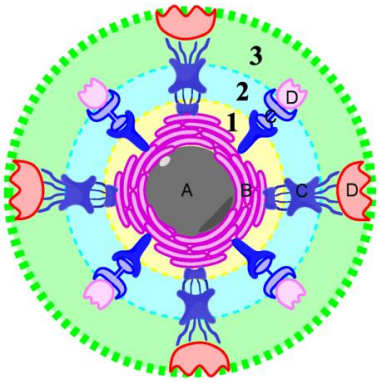


Figure 7. *Schematized nanoplatform architecture: stage 1 comprised from nanozyme core (A), nanozyme surface (B), and primary functionalization; stage 2 – labile linked component(s) (C); stage 3 – cell/molecular recognition segment (D).*

In the last decades, increasing effort has been directed toward potential applications of organosilanes as surface modifiers (Mansur, et al., 2002; Mansur, et al., 2005; Mansur, et al., 2000). Furthermore, flexibility with respect to terminal functionalities of the organic molecules allows control of the hydrophobicity or hydrophilicity of surfaces. The attachment is typically mediated by first silylating the surface followed by immobilization of biomolecules of interest (Guo, et al., 1994; Jang & Liu, 2009; Pasternack, et al., 2008). In this proposal, on the silanized support, having a chemically modified surface with amine, halogen or carbonyl groups, it is intended to develop *three-dimensionally cross-linked copolymer structures* with biological activity having one or more of the following properties:

- release in a controlled manner biologically active agents (biologic markers, drugs or enhancers);
- to retain from biological fluids the essential elements necessary for the diagnosis process (filters for biomarkers).

The aim of the proposed study is to optimize methods of modification of some solid supports, in the form of filters or membranes, accessible in specialized stores in the field of medical technology, and transforming them into functional platforms for biomedical applications (Marques, et al., 2013; Chen, et al., 2012; Mansur & Ancelmo Piscitelli Mansur, 2012; Mansur, et al., 2011). First of all, glassy filters attached to laboratory or medical syringes will be used (see picture below). These surfaces can be used to build interesting nano-level architectures. Starting from the solid glass-based support, it is aimed to develop a multifunctional organic / polymeric structure capable of changing its chemical / biochemical functionality by simple laboratory procedures.

The objective is to develop a sensitive, with interchangeable functionality by standardized laboratory methods, and cost effective *platform based on active compound attached to a market accessible inert supports, such as glassy or other type of filters or membranes with very large pores*. It is intended to develop polymeric, polyethylene and polysaccharides based copolymers capable of interacting with metallic nanoparticles (NPs) through chemical or physical bonds. NPs will have the role of increasing the active surface, on the one hand, and on the other hand they can actively participate in the valorisation of the final product by the intrinsic chemical properties of NPs, such as oxidation-reducing or physical properties, such as magnetic and thermodynamic properties under constraints. The active compounds will be attached to the NPs surfaces by simply absorbing into the polymer layer covering the core-shell NPs or by means of inclusion complexes with macrocycle-like molecules such as cyclodextrins and their derivatives. At this stage, it taken into account the advantage of the studies already reported on magnetite nanoparticles (MNP) in the field of interests (Durdureanu-Angheluta, et al., 2014; Lü, et al., 2009; Durdureanu-Angheluta, et al., 2012). Specifically, the inert support will be functionalized by silylating, after which copolymers based on polyethyleneimine and dextran will be grafted on the surface to modify the physical properties (flexibility, load, etc.) and on the branches and nodes of the network will be attached NPs. When it is desired to load into the macromolecular networks active compounds (drugs or ligands) fixed by *reversible linkage*, without chemical bonds, the polymeric network or NPs will be grafted with cyclodextrin derivatives able to form inclusion compounds.

It is envisioned in the current project that the convergence of dynamic and supramolecular chemistry, and medicine will contribute to the development of supramolecular medicine, an area focusing on the utilization of supramolecular chemistry/molecular assembly as a means to improve the practice of medicine. In a broader context, Supramolecular Medicine can be defined as the supramolecular formulation of diagnostic and therapeutic agents for the diagnosis, treatment, and prevention of disease (Cui & Xu, 2017). The unique and often advantageous properties of supramolecular materials have led to extensive exploration of their use in the fields of drug delivery, disease diagnosis and imaging, and regenerative medicine. On the medicine side, the emergence of nanostructure-based drug delivery systems has provided a new means for optimizing drugs' pharmacokinetic profiles for more effective therapies, and also new momentum for developing innovative strategies with which to battle cancer and other incurable diseases. Our input in the field will be generally based on our expertise in the dynamic combinatorial approach which will add and important issue into the field of Supramolecular Medicine.

Another possible approach is related with the dynamic theranostics, which could be developed for different types of cancer treatments, using nucleic acid therapy and fluorescent imaging. Theranostics deliver therapeutic drugs and diagnostic imaging agents at the same time within the same dose and have vast applications in medicine. First focus will be addressed to syntheses and developmental studies of the novel multifunctional materials as *imaging agent*, including linear or branched polymers, dynamers, hydrophil-hydrophob-aggregates (HHA), multifunctional organic molecules (Catana, et al., 2015; Turin-Moleavin, et al., 2015; Clima, et al., 2015) (Figure 8). These materials will be functionalized with imaging agents to promote one or more diagnostic imaging techniques (i.e. magnetic resonance imaging, nuclear imaging

(PET/SPECT/CT), and/or fluorescence imaging/optical imaging). Second focus will address nucleic acid therapy which holds promise in the treatment of both acquired and inherited diseases. Addressed nucleic acids can be: therapeutic gene, plasmids, antisense oligonucleotides, aptamers etc. For delivery of nucleic acids we aim to use nonviral vehicles based on synthetic polymers, dendrimers, dynamers, cell-penetrating peptides playing the role of *therapeutic agent.*

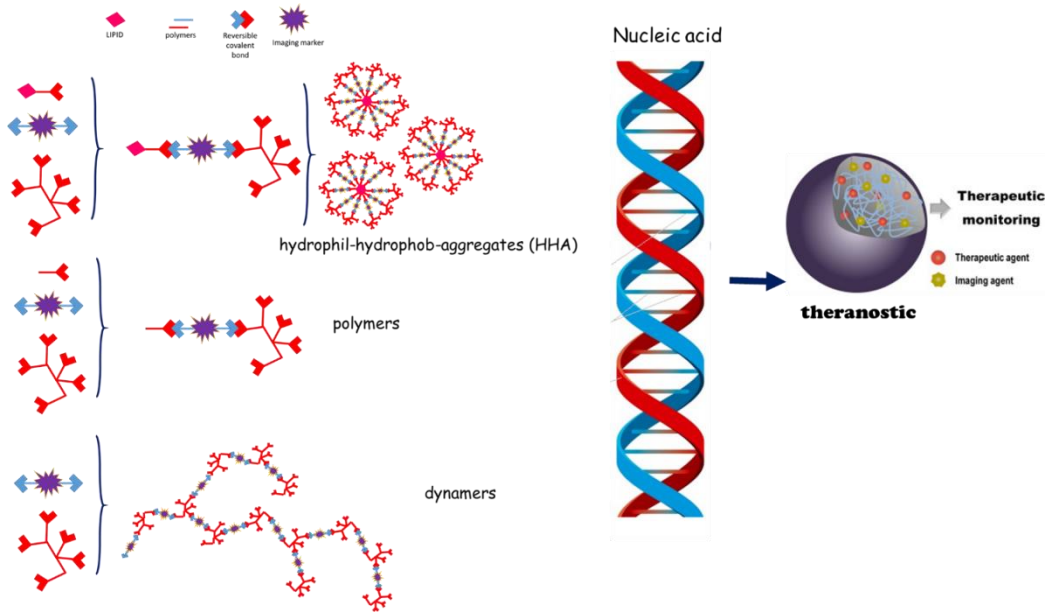


Figure 8. *General concept for preparation of dynamic theranostics.*

Constitutional dynamic chemistry and reversible covalent bonding to link active components may represent a new evolutional approach to produce chemical diversity. A specific advantage with constitutionally generated systems addresses the possibility to self-adjust to biological target species at a given time, in a certain environment at nanoscale dimensions.

VI.2. Unimers selection, characterization, and use

The process of unimers selection will be guided by two principles:

- identification, *a priori* evaluation, and comparative screening of the intrinsic (bio)chemical / pharmacologic functionality, considering their potential role within the overall nanoplatform functionality, both as an individual molecule, and as a component of supramolecular constructs; significant involvement of computational chemistry techniques is expected in these selecting steps;
- the mandatory presence, in their molecule, of particular groups or segments capable to be involved in, to mediate, or to trigger conjugation and / or supramolecular aggregation processes; in addition, selected unimers should maintain or potentiate their (bio)chemical / pharmacologic functionality during and after the mentioned processes, even if temporarily masking could be accepted.

Selected unimer candidates will constitute a compounds library, organized by the following criteria:

- their generic (bio)chemical / pharmacologic activity as individual molecules;
- their chemical reactivity, and physical-chemical properties;
- their ability to be involved in supramolecular interactions;
- the classes of reactions / processes to which they can participate without losing their pre-defined functionality.

In order to constitute the mentioned libraries, all candidate molecules will be assessed by *in silico* investigations, and then by instrumental analytic characterization. Besides, stability, solubility, and general reactivity tests will be performed. The final selected compounds will be consistently evaluated form the points of view of their *(cyto)toxicity*, *immunogenicity*, *biochemical inhibitory effects*, *secondary metabolic / systemic effects*, and *after-service fate* (kinetically reproducible and systematic bio-decomposition into non-aggressive products).

The first classes of potential candidates, identified based on literature data, for now, are:

- carbonic anhydrases inhibitors of clases XII, IX, or II (Supuran, et al., 2004; Supuran & De Simone, 2015; Supuran, 2008), among which:
  - cucurmin inspired sulfonamide (Ramya, et al., 2017);
  - coumarin derivatives (Lomelino, et al., 2016);
  - special types of polyamines and carboxilic acids (Singh, et al., 2018);
- immunomodulatory betulin triterpenes (Ghannadian, et al., 2013);
- glycosylated nucleobases (Seela, et al., 2009);
- dopamine as adjuvant of anticancer drugs (Sarkar, et al., 2008).

VI.3. Macromolecular synthesis of nanoplatforms substrata

Conductive polymers (CPs) represent an important resource in supramolecular chemistry. They are most commonly synthesized either via electro-chemical polymerization of the constituent monomers at the surface of an electrode (Heinze, et al., 2010; Bendrea, et al., 2013), or in the solution/solid state in the presence of a catalyst (e.g. an oxidant such as $FeCl_3$) (Toshima & Hara, 1995). To conduct electricity, conjugated polymers need to be oxidized or reduced; the processes of oxidation or reduction result in the backbone of the polymer being ionized, which necessitates the presence of counter ions that are commonly known as dopant ions (in analogy to the "doping" of inorganic semiconductors). The dopant ions can be introduced during or after the synthesis of the CPs, either via simple mixing or chemical immobilization of the dopant on the backbone of the polymer. In cases where the polymer and dopant interact purely through non-covalent interactions it is possible for low molecular weight dopants to leach out of the CP matrix, concomitant with a reduction in the conductivity of the material. This phenomenon is used in the case of CP-based drug delivery devices which function by proactively expelling the biologically active dopant from the material upon electrical stimulation into the biological milieu. Various classes and methods of CPs synthesis are largely described in monographs (Leclerc & Morin, 2010).

It is noteworthy that research on CPs for biomedical applications expanded greatly in the 1980s (Figure 9 summarizes some of their repeating units), when it was found that these materials were compatible with many biological molecules (Bendrea, et al., 2011; Guo, et al., 2013; Hardy, et al., 2013). Since then, their useful properties (like electrical, (Jakubiec, et al., 1998), electrochemical, (Svirskis, et al., 2010), optical (Wu & Chiu, 2013), actuation (Daneshvar & Smela, 2014)) were exploited in order to obtain advanced materials for biomedical applications like regenerative medicine, (Bendrea A.-D., 2011) (Bendrea, et al., 2011), diagnosis (Li & Liu, 2014) and therapy (Yang, et al., 2012).

Design principles and construction strategy for new CPs (nano)platforms

CPs allow control of chemical structure and film morphology in order to build specific properties into the material itself. Fore the present project, as CPs basic structures can be viewed as a blank canvas, to conceive tailored molecular designs starting from a wide library of polymeric structures, we schematically illustrate, in the Figure 10, how chemists conveniently have access to a wide palette of “brushes” to improve and modify these polymers accordingly with the need of each bioapplication. For the new proposed bio-nanoplatform a "bottom-up" strategy will be employed.

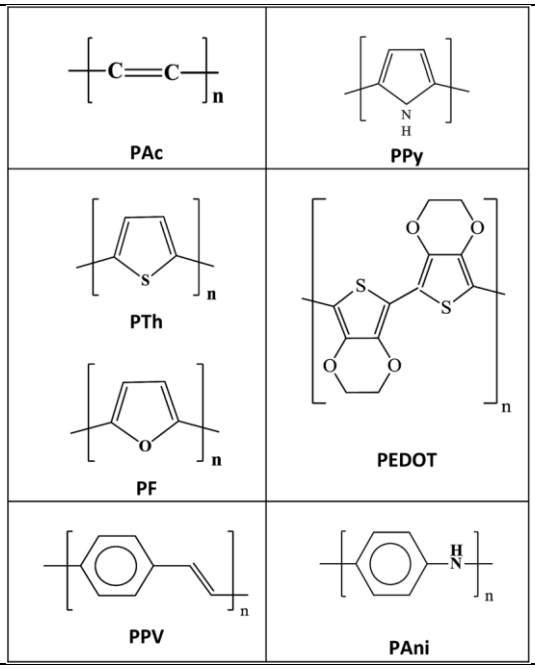


Figure 9. *Structural units formula of the mostly used CPs in bioapplications.*

Acronyms:
PAc polyacetilene;
PPy polypyrrole;
PTh polythiophene;
PF polyfuran;
PEDOT polyethylenedioxythiophene;
PPV polyphenylenevinylenes;
PAni polyaniline.

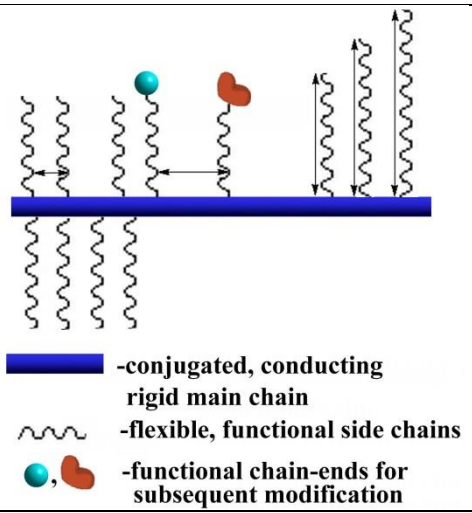


Figure 10. *General presentation of structural peculiarities of the new proposed CPs biomaterials.*

As can be seen from that figure, the synthesis of new CPs structure (shown in Schemes 1 and 2) are envisioned, keeping the "hairy-rod" branched architecture. Among the HR-CPs synthesis methods, the “macromonomer technique” will be employed (see for exemplification Scheme 3), due to its advantages related to well-defined grafting density and side-chain length, defect-free polymer structures and easy access to copolymer synthesis. Actually, this method enables the elaborate design, preparation and complete characterization of the side chains prior to the ultimate step-growth polymerization.

For this purposes new electroactive macromonomers will be prepared using post-polymerization chain-ends modification (structures 1 and 2 in Schemes 1 and 2) or controlled polymerization methods (ring-opening polymerization (ROP) or cationic ring-opening polymerization (CROP)-structure 4 in Scheme 2 and the structures in Scheme 3) for various biocompatible and/or biodegradable polymers, the initiation of which will be done with electroactive initiators. Depending on the reactions variable applied, the obtained molecular weight will me modulated in order to enable the variation of the grafted chains length in the final CPs.

Generally, as polymerization methods, chemical (oxidative polycondensation, Suzuki, Yamamoto polycondensation) or eletrochemical polymerization will be used (see Figure 11). Depending on the chosen polymerization method the side chain grafting density will be adjusted.

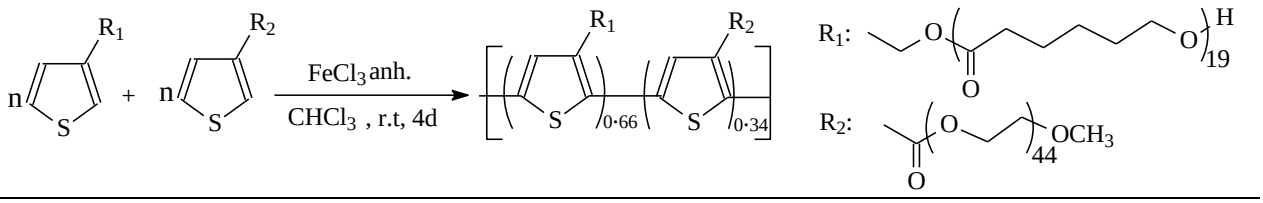
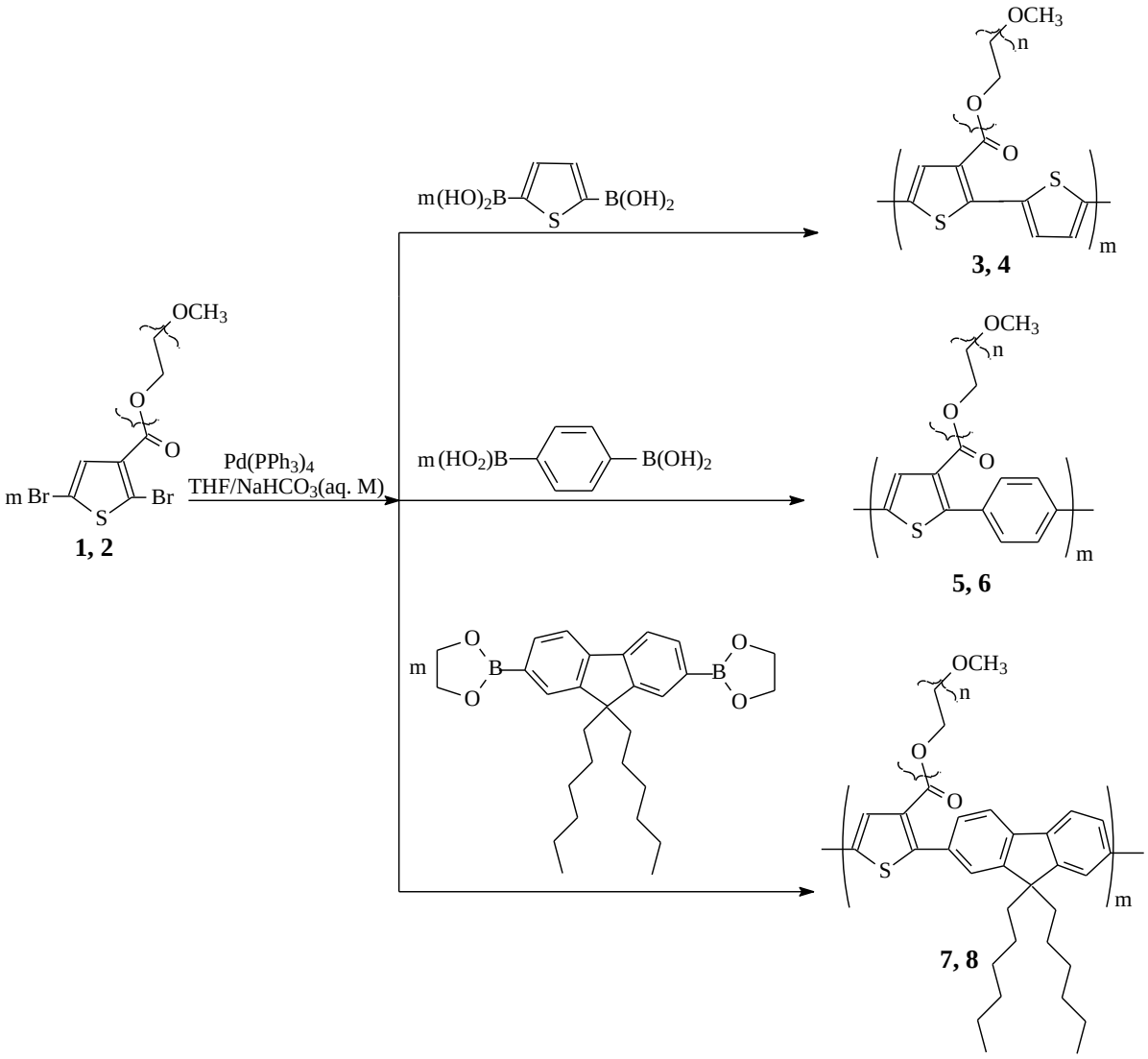


Figure 11. *The oxydative polycondensation pathway.*

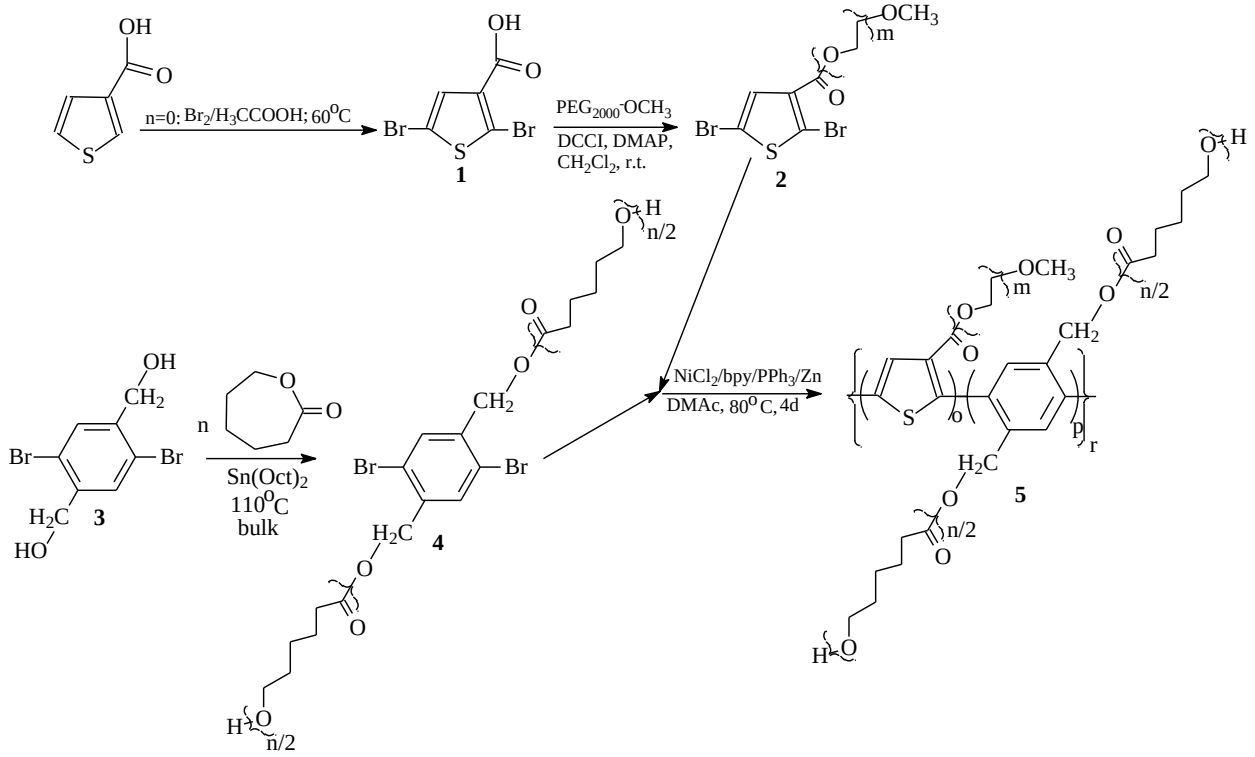


Scheme 1. *Synthesis pathway for fluorescent, amphiphilic and water self-dispersible new nanoplatforms designed for diagnosis and therapy.*

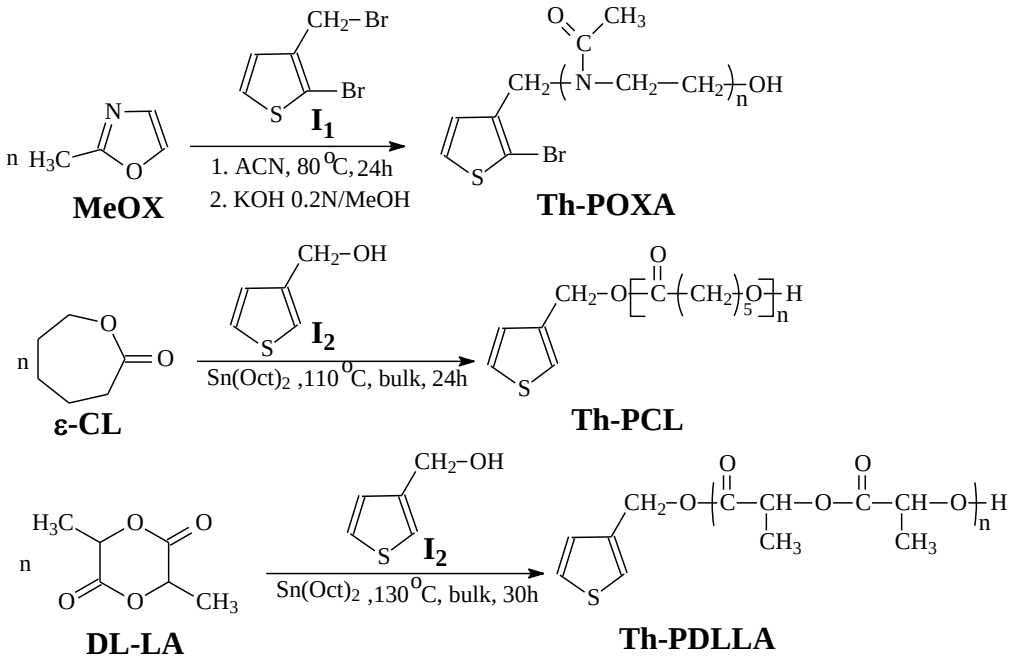
As can be seen in Scheme 1, the side chains grafting density can be also adjusted in the alternating CPs by the size and geometry of comonomers employed for Suzuki polycondensation.

As illustrated in Figure 10, the flexible, pendant side chains on the rigid conjugated main chain will be functional ones. Thus, they will be water-soluble and biocompatible (PEG or poly-2-methyl-2 oxazoline, (POXA)) or polar, hydrophobic, bicompatible and biodegradable as poly-ε-caprolactones (PCL) or poly-DL-lactide (PDLLA). The functional groups at the end of these flexible side chains will allow for the post-polymerization modification with various

biomolecules for biofunctionalization or will work as initiator for other monomers in order to obtain CPs with block-copolymers side chains. The attention will be driven toward hydroxyl functional end-group (see Scheme 3), which it is not so often used for connection with bioentities.



Scheme 2. *Amphiphilic, heterografted CPs with potential for tissue engineering applications.*

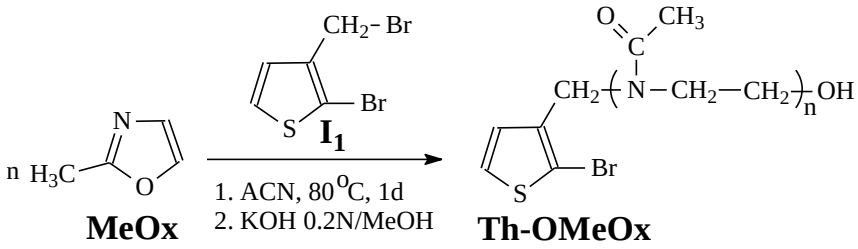


Scheme 3. *Synthesis of electroactive and photoactive amphiphilic or amphipolar biocompatible macromonomers.*

Mostly of the proposed designed structures are amphiphilic, non-ionic rod- coil construction, with propensity for self-assembling in selective and non selective solvents due to the electronic and geometric dissymmetry between the CPs-HR building blocks, structures able to generate complexity at the nanoscale. As conjugated main chains, polythiophenes (PTh) it will be investigated but also some PTh-derived copolymers, as those shown in Scheme 1, will be synthesized as well. It could be also possible to change Th with its derivative EDOT as the main component. Beside basic structural characterization of the obtained structures, a deep characterization will be performed as well including the prospective bioapplications.

New Syntesized Building Blocks and Materials

In the present study, we report about the design and synthesis of a new oligo(2-methyl-2-oxazoline)- based macromonomer which contains at one chain end a photo- and electroactive 2-bromo-substituted thiophene ring (Th-OMeOx in Scheme 4). Using commercially available thiophene, (2-bromo-3-(bromomethyl)thiophene), that possess at the 3-position the -CH2Br functionality, usefully acting as initiator for 2-methyl-2-oxazoline (MeOX) cationic ring opening polymerization, the new macromonomer was synthesized (Scheme 4) (Cirpan, et al., 2001; Cianga, et al., 2007).

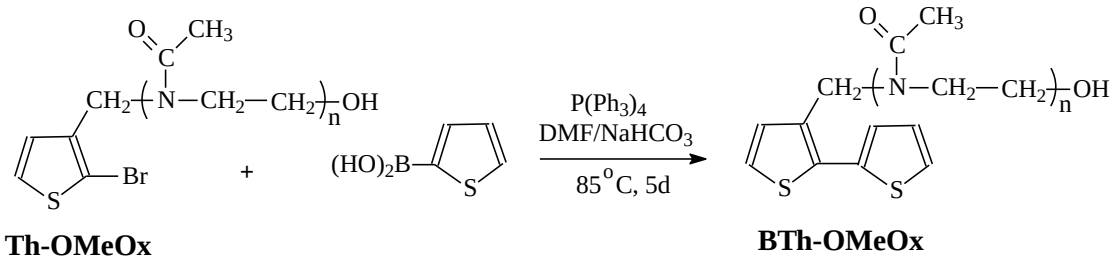


Scheme 4. *Synthesis pathway of macromonomer Th-OMeOx.*

As the polymerization reaction was quenched by the addition of KOH methanolic solution, the placing of the hydroxyl functionality at the opposite end of the OMeOx obtained chain was allowed. Th-OMeOx was designed as a geometric shape and electronic character dissymmetrical, amphipathic compound, with hydrophobic and aromatic thiophene at one extremity of the biocompatible, hydrophilic, aliphatic oligo(2-oxazoline). The presence of reactive bromine and hydroxyl groups in the structure, additionally enhance the amphiphilc balance of Th-OMeOx. Moreover, in solutions of selective solvents, it is possible to make use of hydroxyl end-functional groups to drive hydrogen-bonding-induced intermolecular π-π stacking of Th-OMeOx molecules succeeding in this way for photophysical properties modulation (Lam, et al., 2013).

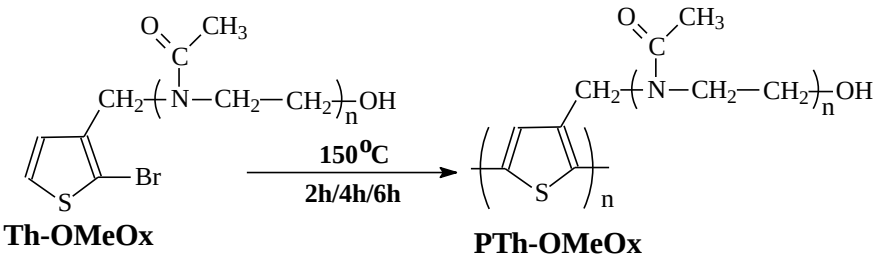
The presence of bromine functionality in the structure of Th-OMeOx allow for subsequent modification. The new reactions were performed in both solution and solid state.

1. In solution-synthesis of new bithiophene macromonomer (BTh-OMeOx) by Suzuki condensation (Scheme 5).



Scheme 5. *Synthesis of bithiophene macromonomer BTh-OMeOx obtained by Suzuki coupling.*

2. Self-Acid Assisted Polymerization (SAAP) in solid state of Th-OMeOx macromonomer (Scheme 6).



Scheme 6. Polymerisation of macromonomer **Th-OMeOx** in solid state.

Solid state polymerization (SSP) is an ideally attractive and environmentally sound procedure given that it can be implemented at relatively low operating temperatures, in which side reactions and thermal degradation should be insignificant, while requiring inexpensive and uncomplicated equipment (Pisuchpen, et al., 2017).

IV.4. Organic synthesis and characterization of particular segments

The chemistry used to produce biomolecule-biomaterial conjugates has been recently reviewed (Spicer, et al., 2018) and is summarized in Figure 12. It plays a key role in influencing bioactivity and construct performance. The best technique available in a certain scenario is highly dependent on the precise construct and must be carefully considered during material design.

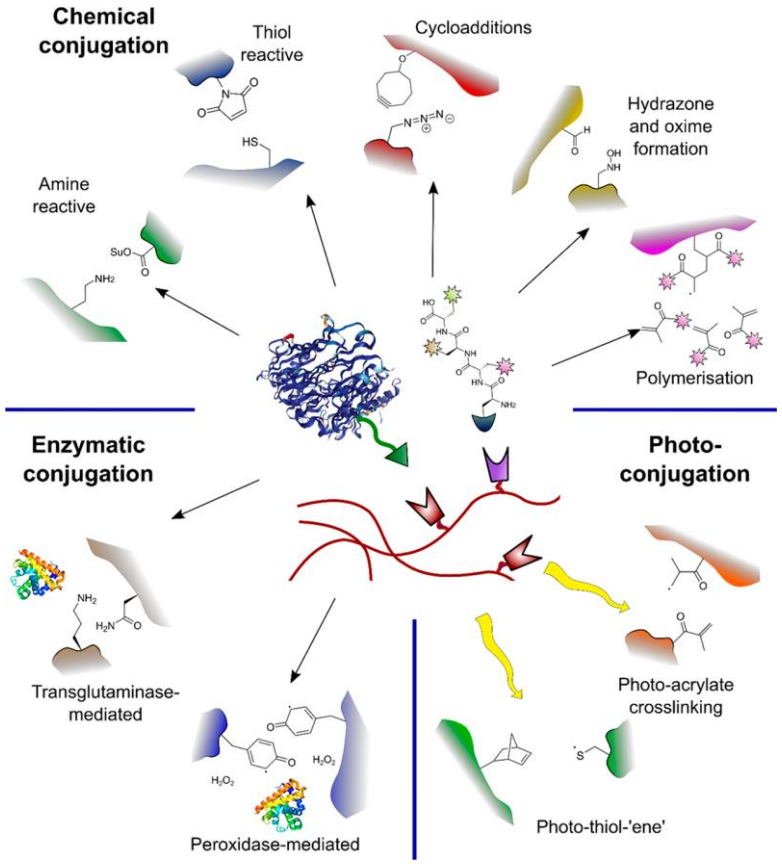


Figure 12. *Key covalent methods by which biomolecule-material conjugation can be achieved.*

One of the major challenges in the development of safe and effective antibody-drug conjugates (ADC) has been the generation of suitable chemical linkers between the cytotoxic drug and the monoclonal antibody. While the synthesis of linker chemistry is quite complex and several aspects must be critically balanced to guarantee efficacy, ultimately, the nature of the chemical linker being used shapes the release profile of the cytotoxin. The majority of antibody-drug conjugates currently in clinical development use only a limited number of chemical linkers, including hydrazones (release mechanism – designed for serum stability and degradation in acidic comportamnets within the cytoplasm), disulfides (release mechanism - designed to be cleaved through disulfide exchange with an intracellular thiol, such as glutathione), peptides (released mechanism – designed to be enzymatically hydrolyzed by lypozomal proteases such as cathepsin B) or thioether (release mechanism – nonreducible and designed for intracellular proteolytic degradation) bonds. Principally, these chemical linkers exploit the differences in intracellular pH, reduction potential or enzyme concentration to trigger the release of the cytotoxin in the cell (Nair, et al., 2014). One of the unique features of ADCs is that they offer a unique-targeted therapeutic strategy by combining the best features of both antibodies and small-molecule drugs to create a single moiety that is highly specific and cytotoxic. However, one of the biggest challenges in the development of antibody-drug conjugates is the generation of suitable linkers offering high drug-linker stability in circulation for the conjugation of antibody and drug.

Dynamic chemical devices involve morphological or constitutional modifications in molecular or supramolecular systems, induced by internal or external physical or chemical triggers (Barboiu & Lehn, 2002). Supramolecular polymers are defined as the entities generated by the polyassociation of molecular monomers bearing complementary binding groups capable of connecting through the usual non-covalent interactions implemented in supramolecular chemistry: electrostatic, hydrogen bonding, donor–acceptor, Van der Waals as well as metal ion coordination. Molecular dynamers are reversible covalent polymers, including entities based on various reactions such as: transesterification, transetherification, Diels–Alder reaction, [2+2] photodimerization, radical reaction and boronate ester formation (Lehn, 2005).

Thus, the interactions which are exploited to link the monomeric precursors can be varied from strong covalent bonds in polymers to dynamic linkages based on metallasupramolecular or dynamic covalent chemistry or even weak supramolecular forces, e.g. π-π interactions, halogen bonding or hydrogen bonding. Dynamic combinatorial chemistry (DCC) implements the reversible connection of the molecular components of a supramolecular entity, as well as the ability of the supramolecular species to exchange their components (Lehn, 1999).

The main links considered for attachment of ligands to nanoplatforms and nanoentities are thioethers, functional groups that involve a –C=N– or carbonyl unit, other groups with low stability. Some details are resumed below.

Thioethers

Reactions between thiols and various types of unsaturations or electrophiles, referred to as thiol-X chemistries (ten Brummelhuis & Schlaad, 2012) have been used extensively to modify everything from small molecules, polymers, and particles to macroscopic substrates (Figure 13). The success of these types of reactions is due to their "click chemistry" nature, as they are highly efficient and selective, produce no or few by-products, and can be conducted under mild reaction conditions (Brosnan & Schlaad, 2014). Numerous thiol-X reactions have been broadly classified as *click reactions* in which the thiol reacts via pathways as diverse as radical-mediated thiol-ene reactions, amine-catalyzed thiol-epoxy reactions, thiourethane-forming thiol-isocyanate reactions, and thiol-halide reactions, among others as illustrated in Scheme 7. They are radical-mediated processes that are initiated by heat in the presence of azo species such as 2,2′-azobis(2-methylpropionitrile) – AIBN – or by photochemical methods with or without photoinitiator (eg 2,4-dimethoxyacetophenone – DMPA-). The light initiation of the thiol-ene reaction is more advantageous because it is cytocompatible. Until recently, the thiol-ene coupling had been

predominantly employed in polymer and materials chemistry, but recent work has firmly established this reaction in the field of bioconjugation, where it has found applications in glycobiology, the synthesis of thioglycosides, the detection of thio-phosphorylated proteins, protein spin labelling, tandem application with native chemical ligation for the synthesis of S-modified peptides, lipidated peptides, the development of lipophilic amino sugar libraries, and the synthesis of stapled peptides. This reaction has also found wide application in biopolymers, for example the synthesis of glycol-microspheres (Healy, et al., 2016). This class of reactions is a much-used approach to the modification of biological and biomimetic moieties, this being a more biologically friendly approach to the modification of materials. These types of reactions do not require toxic catalysts such as those required in the copper-catalyzed Huisgen cycloaddition reaction between azides and alkynes, and they are tolerant to air and water (to a certain extent), which makes them very appealing to provide the facile modification of peptides and proteins. The use of thiol-X chemistry to modify peptides and proteins is not entirely new. Cysteine, a natural amino acid, bears a convenient primary thiol group, making it an ideal substrate for modification in peptides and proteins. This type of chemistry has allowed researchers to expand the application scope of these well studied materials (Brosnan & Schlaad, 2014).

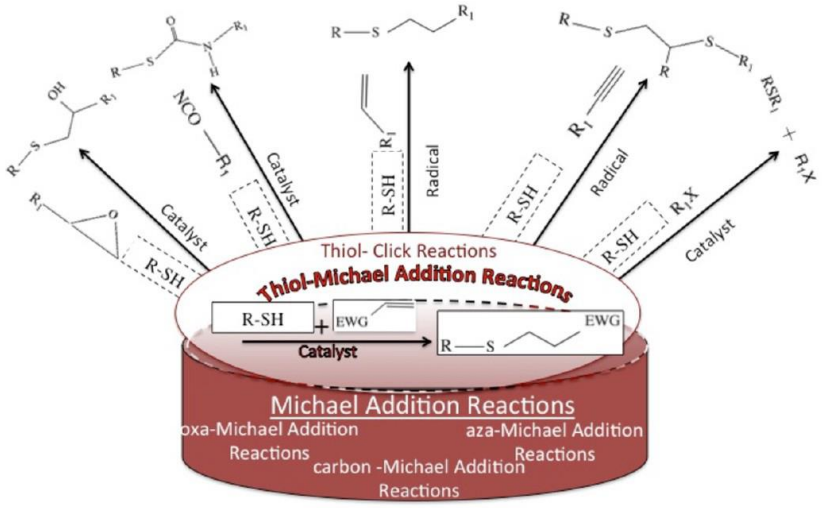


Figure 13. *Reactions that fall into the field outlined by the two powerful reaction methodologies: the powerful thiol-click reaction paradigm and Michael addition reactions* (Nair, et al., 2014).

Thioethers represents (8.8%) the third most exemplified constituent of the sulfur containing drugs (cimetidine, thiethylperazine, pergolide etc.) (Ilardi, et al., 2014). In the literature are presented a lot of cases were the stabilization by the introduction of thioether bridges shown promising results for peptides lacking a cyclic backbone or intramolecular crosslinks (Kluskens, et al., 2009). As the thioether bond is resistant to reduction and thiol–disulfide exchange, this type of bridge can be expected to yield peptides and proteins with higher *in vivo* stability compared to the disulfide-linked variants (Knerr, et al., 2011). Thioether crosslinks are generally more difficult to introduce into proteins than into short peptides, but incorporation can be achieved by enzymatic modification or chemical strategies such as alkylation of cysteine residues by crosslinking agents (Nilsson, et al., 2017).

Functional groups that involve a C=N or carbonyl unit, such as imines, esters or amides

Are of special interest because they may undergo disconnection/reconnection cycles (for example, trans reactions like imination, esterification and amidation). The most proeminent types used in DCC are boronic acid and imine condensation (Lehn, 1999).

Among the known reversible covalent reactions, amino/carbonyl condensations to give C=N products such as imines, hydrazones and oximes are particularly attractive in view of the very wide range of structural variations available, the easy synthetic accessibility, the control through conditions of yields, rates and reversibility, as well as their role and potential for

application in both biological/medicinal and materials sciences. In particular, the acylhydrazone functionality provides both dynamic character, through the reversibility of the imine-type C=N unit, and hydrogen bonding sites through the amide group, thus exhibiting double dynamic behaviour [Barboiu, 2002]. The dynamic behaviour of imines is of special interest in view of its central role in chemistry and biochemistry as well as in materials science. In particular, the reversible nature of imine bond formation makes it an attractive process for use in DCC because the (amine + carbonyl) condensation into imine-type compounds usually takes place under mild conditions. In general, imines can participate in three types of equilibrium-controlled reactions:

(1) hydrolysis, in which the imine reverts back to the precursors, that is, amine and carbonyl containing compound(s), on the addition of water;

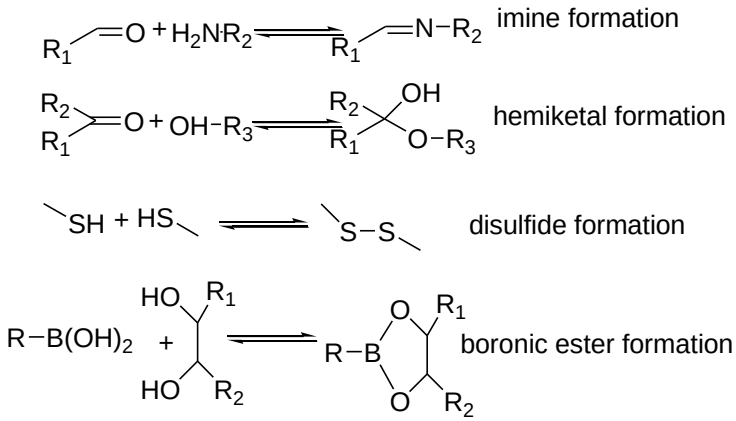
(2) transamination, in which, with the introduction of a second amine (or carbonyl-based molecule), the original imine may undergo an exchange of the amine residue to give a new imine;

(3) imine– imine exchange, in which, on the introduction of a second imine, the two imines can undergo a reaction whereby the amine components are exchanged.

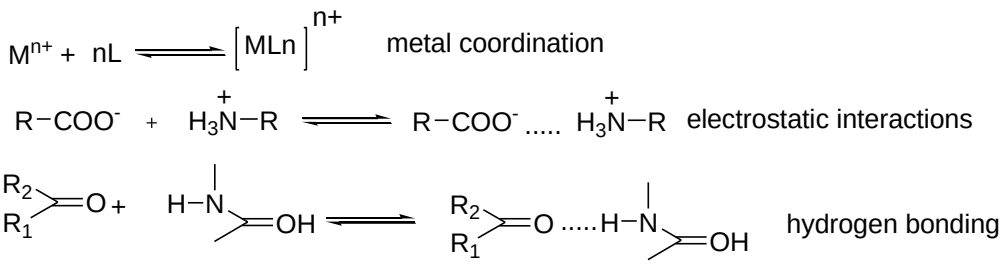
Dynamic exchanges that involve the C=N bonds in imines, hydrazones and oximes are the most widely used dynamic covalent reactions. They have been applied to the syntheses of complex 2D and 3D molecular architectures, covalent organic frameworks and the construction of self-sorting systems, rotary switches and molecular walkers and to study motional covalent dynamics (Ciesielski, et al., 2014).

A selection of potentially reversible reactions or interactions for the dynamic systems were summarized in Scheme 7.

Reversible Covalent Bond Formation



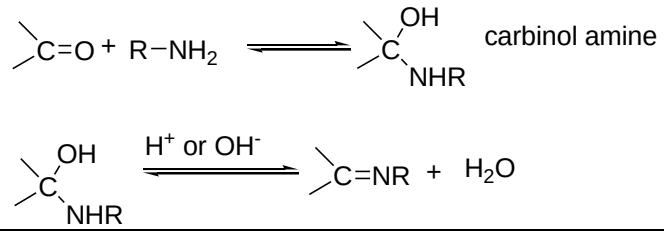
Reversible Interactions



Scheme 7. *Different dynamic linkages based on dynamic chemistry.*

Imine bond formation is an efficient reaction which involves the loss of H_2O when an amino and carbonyl group react to form a C=N bond either intra- or intermolecularly. Typically, the reaction is conducted by refluxing the starting material(s) under azeotropic conditions, often

in the presence of a catalytic amount of acid (Scheme 8). Adding H_2O to an imine results in recovery of the starting materials as a consequence of its hydrolysis



Scheme 8. *Mechanism of the imine bond formation/hydrolysis.*

Other groups with low stability

Candidate chemical groups for creating temporary bridges in the assemblies foreseen 5D-nanoP aproaches could be: anhydride, silyl-ether, and silyl-ester, all of them highly susceptible to biodegradation.

IV.5. Production of systems for ex vivo nanoplatforms testing

In order to make drug development less costly and more efficient, one of the strategies is to use model systems, that mimic closely the *in vivo* tumor (Westhouse, 2010). Tissue and tumor surrogate are used to *in vitro* measure the drug efficiency during the preclinical stages of drug development, or can be used to study tumor biology *in vitro*, by monitoring cancer cell proliferation, invasion, matrix remodelling, angiogenesis or metastasis (Taubenberger, 2014; Song, et al., 2014; Hickman, et al., 2014; Das, et al., 2015; Katt, et al., 2016). The two most important systems used today for testing chemotherapeutics are conventional two-dimensional (2D) systems (cell monolayers obtained by culture in plastic flasks) and three-dimensional (3D) tumor culture including spheroids, cellular multilayers, matrix embedding cultures, bioreactors and microfluidic devices. Despite its poor record as predictive of human cancer drug outcomes, 2D cell culture remains the most use in the early stages of drug screening and testing. Even if the cost and the complexity of 3D platforms have limited their adoption as an industry standard, their use for drug screening has significantly bridged the gap between 2D assay and animal studies (Cox, et al., 2015).

The most difficult problem in producing tissue / tumor surrogates consists in the rational choosing of macromolecular compounds to be used. The common types and their advantages and disadvantages are summarized in Table 3 (Ferreira, et al., 2018).

Table 3. *Advantages and disadvantages of different origin materials used for the production of scaffold-based 3D in vitro tumor models.*

Type	Origin	Examples	Advantages	Disadvantages
Natural	Mammalian	Collagen Matrigel Hyaluronan Gelatin Decellularized Matrix	• Contain *in vivo* similar domains (e.g., laminin, elastin, fibronectin) • Cellular adhesive properties • Recapitulate cells-ECM interactions present *in vivo* • Enzymatically degradable • Cell adhesion properties • High biocompatibility • Affordable	• Exact composition is unknown • Batch-to-batch variability • Limited level of control over matrix stiffness along time • May require further modification to simulate *in vivo* tissues ECM components • Fabrication methods can be cytotoxic
	Non-mammalian	Alginate Chitosan Silk-fibroin		

Table 3. *(Continue)*

Type	Origin	Examples	Advantages	Disadvantages
Synthetic	Chemical synthesis	Polyethylene glycol (PEG) Polylactic acid (PLA) Poly(ε-caprolactone) (PCL) Poly (lactic-co-glycolic acid) (PLGA)	• Good structural definition and chemically defined. • Highly tunable mechanical properties.	• Lack ECM-mimicking domains • Require further modification to increase bioadhesion and biocompatibility • Degradation can result in acidic by-products
Hybrid	Chemical modification	Alginate-RGD PEG-RGD PEG-fibrinogen	• Combine the ease of chemical modification and the presence of ECM-like domains	• High-costs • Representation of few ECM components

Surrogates based on collagen

Aiming the development of biomimetic materials as components in 3D systems with appropriate physico-mechanical and chemical stability, usually collagen functionalization is performed to achieve spatial and temporal control, by cross-linking. Thiol groups are often the choice for applications envisaging biomedical area, considering their high reactivity/ known chemistry allowing the obtention of complex systems based on simple reactions in mild conditions (nearly neutral pH, low temperatures, high selectivity, good yield). As an example, Michael addition reactions (thiol-ene, thiol-izocyanate, etc. – Figure 14) are the most used alternative for the synthesis of complex architectures.

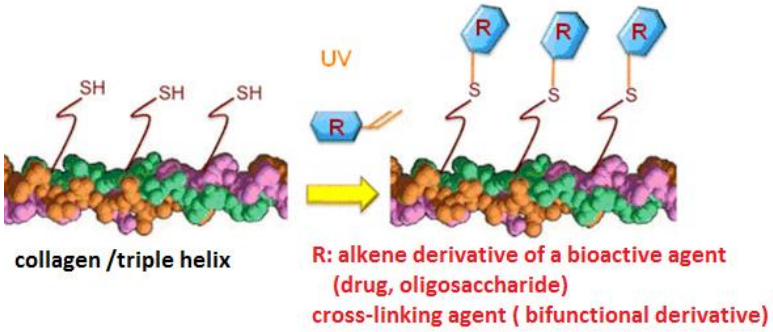


Figure 14. *Complex functionalization of collagen via thiol groups preliminary introduced in the protein structure.*

Collagen thiolation may be performed by different approaches, based on the presence of amino- and carboxyl groups in the protein structure (Figure 15). Cystamine is the most used source of mercaptoamine radicals (Nicolas & Bryson, 2005). Collagen may be or not pre-activated with water- soluble carbodiimide. The raw product is subjected to dialysis at the reaction end to remove any unreacted compound or side-reaction product. The product may be separated from the aqueous solution by lyophilization (with retention of disulfide groups), or can be subjected to reduction reaction (at pH about 9, in the presence of glycine and dithiothreitol, or dithiothreitol alone) in order to obtain the thiol derivative.

One of the methods suposes the use of –COOH groups from the aspartic or glutamic acid units from the protein chain in the reaction with mercaptoamine radical (Russo, et al., 2014; Wilson, et al., 2001). The functionalized collagen is further separated by modifying the pH (pH 2), followed by dyalisis against acid solution (0.012 N HCl), and lyophilization.

The collagen functionalization using cystamine supposes the direct mixing of the two reactants with addition of 1-etil-3-(3-dimethylaminopropyl)carbodiimide (EDC) as an activator. It is important to maintain the pH at 3, when collagen is still soluble. The final product may be

purified by dialysis, subjected to reduction, then dialysed again (Wilson, et al., 2001). The maximum of modified groups corresponds to the number of carboxylic groups (from the aspartic and glutamic acid units), of about 9-12%, thus allowing the obtention of soluble compounds with a controlled substitution degree, according the requirements of the envisaged application domain (i. e. 3 mol % from the aminoacids content, 0.33 mmol/g dried product respectively) (Nicolas & Bryson, 2005). To reach a higher functionalization degree one can apply the transformation of the free amine groups in carboxyl ones by reaction with succinic anhydride, followed by the reaction with different mercaptoamine compounds. For example, in the reaction with 2-mercaptoethylamine (Figure 16) the substitution degree may be of 34.7% (34.7 % thiol groups/100 COOH groups in Col-COOH (Xu, et al., 2015)), corresponding to an amount of 0.503 mmol SH/g collagen.

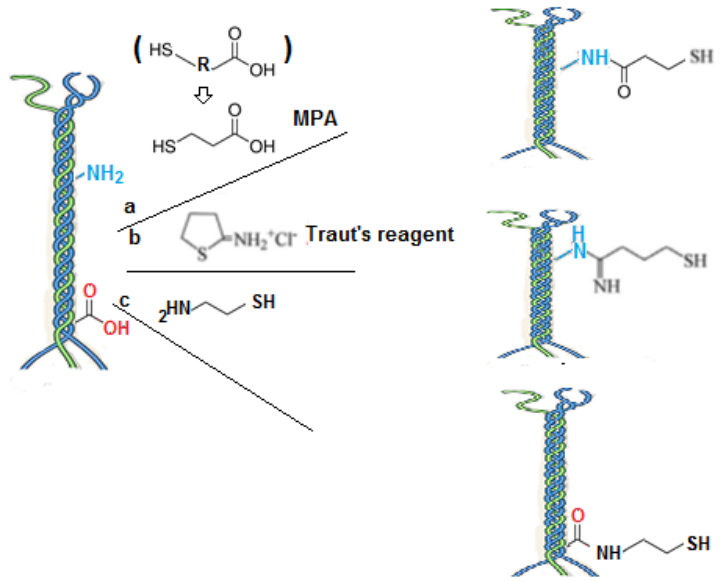


Figure 15. *Collagen thiolation alternatives.*

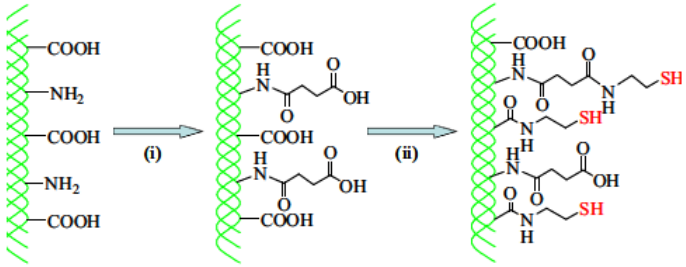


Figure 16. *Preparation of Col-SH. Conditions:*
(i) succinic anhydride, 0 °C, pH 9, 4h;
(ii) 2-mercaptoethylamine hydrochloride, EDC, NHS, N2, 24h.

Another approach for introduction of thiol groups, by using amine groups from a polymer substrate implies the use of a mercapto- carboxylic acid (i.e. thioglycolic acid or 3-mercaptopropionic acid) (Zhu, et al., 2012). The covalent binding is insured by forming amide bonds between the primary amine groups of the compound subjected to thiolation and the carboxyl groups of the mercapto-carboxylic acid, reaction mediated by the EDC/NHS system - carbodiimide (EDC or EDAC) and N–hydroxysuccinimide or N-hydroxysulfosuccinimide (NHS). The reaction may be performed in one step (one pot feeding) or in two steps: (i) activation of the mercapto-carboxylic acid; (ii) reaction with the substrate detaining primary amine groups (Figure 17). The last recent alternative uses DMF as reaction medium in order to avoid the hydrolysis of the O-acylisourea ester, unstable in aqueous media, yielding a higher

thiolation degree. The raw product requires the purification by dialysis followed by lyophilization.

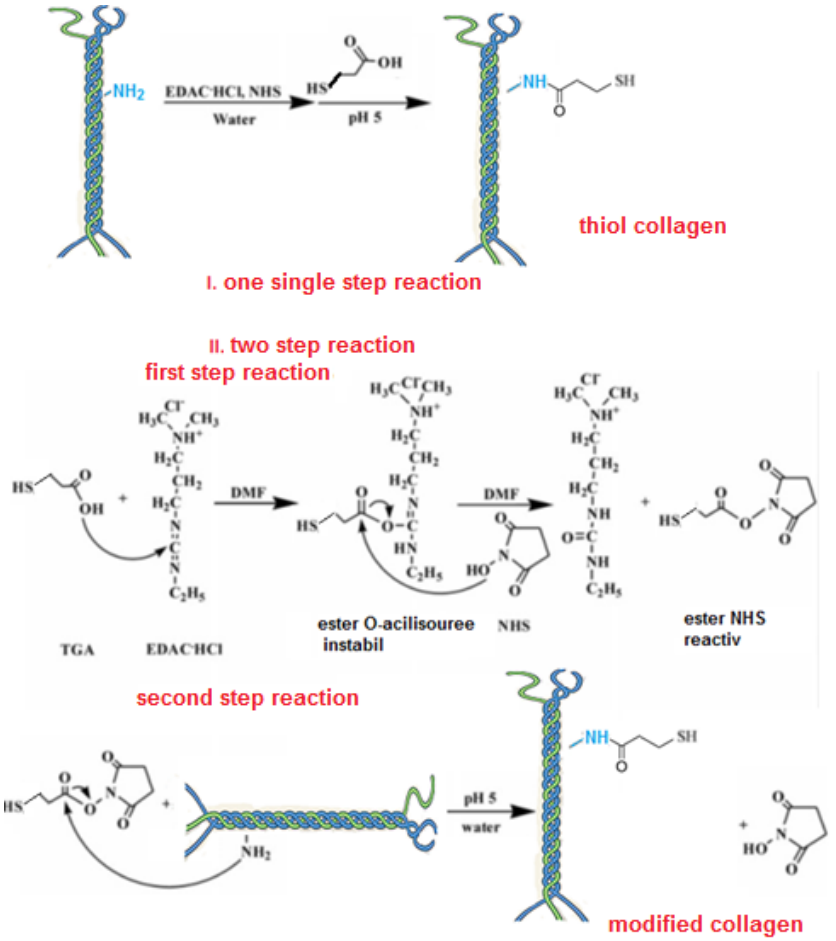


Figure 17. *Thiolation via reaction of primary amine groups of the substrate with mercapto-carboxylic acid (alternatives).*

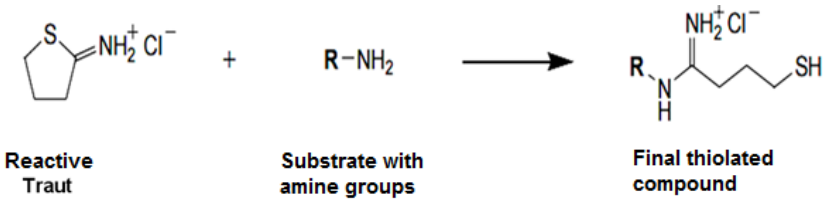


Figure 18. *Thiolation with Traut's reagent.*

The use of reagent Traut for thiolation implies the breaking of the S-C bond, opening of the Traut's reactive cycle, and its binding to the amine groups from the matrix subjected to functionalization (Figure 18). Reaction with Traut's reagent was applied for functionalization of collagen matrix (Shi, et al., 2011), or gelatin-siloxane nanoparticles (Balthasar, et al., 2005). The reaction took place directly, without any additive, (initiators, accelerators/activators), at ambient temperature (25 °C).

IV.6. Computational chemistry and *in silico* techniques for functional (macro)molecules investigation

The methods of computational chemistry are largely used in supramolecular chemistry, to study self assembling (Ashwanikumar, et al., 2018), biologic activity of particular compounds (Begum, et al., 2018), pharmacological mechanisms (Di Meo, et al., 2016), conformational

changes in complex molecular systems (Koehl & Delarue, 2018; Zheng, et al., 2019), constrained conformation and spatial extension of (bio)macromolecules (Beu, et al., 2019; Wang & Wang, 2019) etc.

Among smart nano-carriers for drug/gene delivery, supramolecular drug delivery systems (DDS) are rapidly gaining popularity due to their high modularity and a rich diversity of non-covalent interactions (multiple hydrogen bonding, hydrophobic interactions, host–guest recognition, π–π interactions, charge-transfer interactions, electrostatic interactions, metal-ligand coordination, etc) facilitating a great variety of self-assembled guest structures to host and deliver drugs (even several types) within one single platform (Webber & Langer, 2017; Hu & Wang, 2018). Their soft and dynamic weakly bound 3D structures, created from building blocks (unimers) by molecular recognition, offer a multitude of possibilities for stimuli-response mechanisms to unload the cargo when reaching the target cells. Supramolecular DDS can be designed to be more selective than conventional DDS as cancer cells have different environment and structure compared to healthy cells. They are superior to conventional polymeric nanoparticles as they can dynamically alter their structures and functions and undergo both conformational and phase transitions upon responding to intracellular (pH, redox agents as glutathione and their gradients and other chemo-stimuli, proteins, enzyme concentration, hormone levels etc) and external stimuli (light, heat and ultra-sound) in a controlled manner. Also their degradation and clearance mechanisms can be designed better from the beginning.

Cyclodextrins (CD), cucurbit(n)urils and calixarenes and other related systems have been so far the most successful supramolecular hosts being biocompatible with low toxicity and having a capacity to form easily inclusion compounds with a great variety of guests (small neutral, cationic and anionic molecules and polymers) that can be dissociated on many different types of stimuli in the vicinity of the target. Related supramolecular hydrogels with sol-to-gel pH-responsive behaviour have also attracted much interest as DDSs. Supra-molecular dendrimers are yet another category of widely used delivery systems. There are many more. At this moment we are in the process to choose the appropriate supramolecular molecules and chemistry.

Modeling in 5D

One of the aims of **5D-nanoP** project is to create a virtual in silico laboratory where to design, optimize and evaluate the chemical and supramolecular building blocks and assemble the molecular DDS systematically in a hierarchical way. Modelling is an important part of nearly every experimental component in the project in (**i**) predicting motifs and molecular systems, (**ii**) assisting in synthesis work for finding the right chemistry and conditions (**iii**) as well as interpreting results from characterization. The following four steps will be taken into consideration.

Step 1:

Supramolecular host molecules will be choosen (**i**) based on the earlier work of the project partners and on the scientific literature, and (**ii**) by performing "virtual screening" to predict suitable supramolecular motifs and structures based on concepts of crystal engineering, by means of synthons, as well as, classifying molecular shapes and Hirshfeld interaction surfaces in the Cambridge Structural Database (CSD) and topological methods (as implemented in the TOPOS software). To fine-tune the supramolecular structures for improved therapeutics, we will apply the computational scheme of Kulkarni et al. (Kulkarni, et al., 2016).

Step 2:

In order to describe the (macro)molecular constructs and the supramolecular structures, the physics-based particle models, "electronic", "atomistic" and "coarse-grained", will be used. This refers to "multi-scale modelling" to cover the length scales to build a realistic DDS. This requires quantum, classical and mesoscopic models and methods to be applied. In practise we solve Schrödinger and Newton equations for molecular systems. Models of the nanoplatforms will be built, together with polymers to which the supramolecular hosts are attached with weak

bonds to get detached upon stimuli after the drug is loaded (Kang, et al., 2017). This work will be first done on atomistic level, and then scaled-up by using coarse-grained (CG) models. The whole system will be considered in aqueous environment at physiological conditions, with ions and small molecules included. Important part of the work wil be dedicated to modelling biological diffusion processes.

Step 3:

Further, the barriers and how to penetrate them without destroying the DDS will be addressed. Much of this work will be done using the CG models, but also with hydrodynamical continuum models with a flow. Possibly, a pulsating flow in tubes corresponding blood vessels taking into account of the curved topologies will be had in view. This requires non-equilibrium simulations with Lees-Edwards sliding brick boundary conditions to include the shear flow.

Step 4:

Finally, the scenario on approaching the cancer cells will be, possibly, considered. As the physical and chemical conditions differ at the close vicinity of the cancer cell, the host in the supramolecular construct is expected to respond to the new environment and release the drug. To find the drug attributes relevant in physical, chemical and biological mechanisms after the drug is released, pharmacokinetic and mathematical modelling will bw considered (Prokop & Davidson, 2008; Liu, et al., 2012), to obtain rate constants for several key processes. Possibly we look into the degradation and elimination process of the remains of the DDS.

IV.7. Techniques and methods for the evaluation of nanoplatforms and nanoconstructs biological / biomedical functionality

The envisioned range of applications of the developed nanoplatforms/ nanoconstructs is related to cancer therapy. In this respect, **5D-nanoP** project aims to develop research tools of (macro)molecular type, and, possibly, therapeutic strategies using them.

The designed functionality of the supramolecular entities to be developed must be tested and proved by using biologic / biomedical techniques. Recent literature describes the minimally required techniques, methods and protocols needed to adequately investigate the potential therapeutic nanoplatforms / nanoreactors / nanovectors (Duo, et al., 2018; Peng, et al., 2018; Wang, et al., 2018; Mukerabigwi, et al., 2018; Dong, et al., 2018).

A set of such techniques and methods are considered to be used throughout the project, to test and evaluate all the precursors and the experimental products (nanoplatforms, nanoconstructs, carriers, and tissue / tumor surrogates). The minimal list, which is in expansion according the particular requierements, includes the following.

1. ***Cytotoxicity testing***, performed using appropriate human cells, including endothelial cells, smooth muscle cells, monocytes, fibroblasts, mesenchimal stem cells, special cell lines, purchased from specialized cell culture collections (e.g. ATCC, ECACC, CLS). As methods, MTT / XTT assay, and LDH are had in view (minimally).

2. ***Investigation of cell death mechanisms involved in precursors/ nanoplatforms / nanoconstructs cytotoxicity***, by the accurate evaluation of the mechanisms of cell death. Necrosis (accidental cell death) and apoptosis (normal or programmed cell death) mechanisms and effects will be considered. The methods to be used are annexin V / propidium iodide assay, and mitochondrial membrane potential assay.

3. ***In-vitro tests to determine the effect of precursors / nanoplatforms / nanoconstructs on cellular morphology***, by investigating the cells in bright field, and by Phalloidin / Hoechst staining, which is a useful tool for investigating the distribution of F-actin and nuclei in cells, using fluorescence microscopy.

4. ***The study of the intracellular signaling pathways activated by nanoplatforms in human cells***, by testing the phosphorylation of the three main signaling kinases, (**i**) mitogen-

activated protein kinases (MAPKs) p38, (**ii**) extracellular signal-regulated kinases 1/2 (ERK1/2), and (**iii**) c-Jun N-terminal kinase (JNK) in human skin cells (fibroblasts and epidermal cells) and human epithelial lung cells exposed to different nanoparticle concentration by Western blot (Pîrvulescu, et al., 2011).

5. ***The study of the inflammatory response following exposure of human cells to precursors / nanoplatforms / nanoconstructs***, by (**i**) quantifying the cytokines and chemokines production, and (**ii**) reactive oxigen species (ROS) production.

6. ***Blood compatibility after intravenous administration***, by evaluation of induced hemolysis and erythrocyte aggregation.

7. ***Cellular uptake of precursors / nanoplatforms / nanoconstructs***, by labeling them and by measuring the cellular fluorescence.

8. ***In vitro colonization of 3D macroporous matrices (scaffolds) with human cells***, in order to evaluate the cytocompatibility / cytotoxiciy of the developed tissue / tumor surrogates.

9. ***Histological / imunohistochemical assays on tissue / tumor surrogate cryosections***, to put in evidence cell progressive invasion, accommodation, proliferation, and evolution.

10. ***Quantitative real-time PCR***, to determine the expression of a certain gene in order to put in evidence phenotypic and genotypic deviations of the cells cultured on the surrogates, including in the presence of the developed precursors / nanoplatforms / nanoconstructs.

11. ***Western blot assay***, to quantify the level of specific protein expression by the cells cultured into the surrogates.

All the mentioned techniques and methods were documented, and the associate protocols were pre-tested in order to be prepared for the future tests.

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VII. Quantifiable results of the project, up to the current date

The **5D-nanoP** project delivered the quantifiable results which are summarized below.

1. The project WEB page

The significant details of **5D-nanoP** project are gathered at the WEB address:

http://www.intelcentru.ro/5D-nanoP

2. Published / accepted / send for publication papers

The following four scientific papers resulted during the fisrt stage of 5D-nanoP project. All of them are mentioning the project in the Acknowledgment section.

[1] Cioaca G., Pinteala M., Bacaita E.S., Oprea I., Crumpei Tanase I., Volovat S.R., Dragan V.S., Trocaru S., Anton C., Nonlinear Behaviors in Gene Therapy: Theoretical and experimental aspects, *Materiale Plastice*, 2018, 55(3), 340-343; http://www.revmaterialeplastice.ro (IF: 1.248) (Published).

[2] Angeli A., Pinteala M., Maier S.S., Del Preted S., Capasso C., Simionescu B.C., Supuran C.T., Inhibition of bacterial α-, β- and γ-class carbonic anhydrases with selenazoles incorporating benzenesulfonamide moieties, *Journal of Enzyme Inhibition and Medical Chemistry*, https://doi.org/10.1080/14756366.2018.1547287 (IF: 2.332) (Accepted).

[3] Ma C., Laaksonen A., Liu C., Lu X., Ji X.,The peculiar effect of water on ionic liquids and deep eutectic solvents, *Chem Soc Rev*, DOI: 10.1039/c8cs00325d (IF: 40.182) (Accepted).

[4] Iacob M., Racles C., Dascalu M., Tugui C., Lozan V.I., Cazacu M., Nanomaterials Developed by Processing Iron Coordination Compounds for Biomedical Application, *Journal of Nanomaterials* (IF: 2.207) (Open access, review. In revision.).

3. Book chapters

[1] Fossepre M., Leherte L., Laaksonen A., Vercauteren D.P., Understanding the Structure and Dynamics of Small Peptides and Proteins Through the Lens of Network Science, in *Biomolecular Simulations in Structure-Based Drug Discovery*, (Editors: Cervasion F.L. & Spiwok V.), Wiley-VCH Verlag (2018).

4. Participation in conferences / symposia

[1] Pinteala M., Nanoparticles in Theragnostics Approach: Synthesis, Structure, Particularities; Nuclear Medicine Days, *Iasi, Romania, 1-4 November 2018* (Invited Conference Presentation).

[2] Bucatariu S., Constantin M., Fundueanu G., *pH/temperature-sensitive microgels for self-regulated drug delivery systems*, 4-th International Conference on Chemical Engineering, Iasi, Romania, 31 October - 02 November 2018 (Oral Presentation).

[3] Racles C., Cazacu M., Zaltariov M., Iacob M., Butnaru M., Siloxane-based compounds with tailored surface properties for health and environment, *International Conference On Phosphorus, Boron And Silicon*, PBSi 2018, Barcelona, Spain, December 10-12 (Oral Presentation).

[4] Ailiesei L.G., Cianga L., Bendrea A.D., Hitruc E.G., Cianga I., *What link can NMR Spectroscopy have with AFM Microscopy? – Unraveling complex configurational and supramolecular organization in conjugated polymers*, Chem 2018 - Chemistry Faculty Conference, IASI, 25-26 October, 2018 (Oral communication).

5. Training sessions / invited trainers / internships

[1] Prof. Francesca Mocci, University of Cagliari, Italy, in "Petru Poni" Institute of Macromolecular Chemistry, 4 - 25 September 2018; Invited Trainer.

[2] Prof. Francesca Mocci, University of Cagliari, Italy, in "Petru Poni" Institute of Macromolecular Chemistry, 31 October - 4 December 2018; Invited Trainer.

[3] Dr. Anca Bendrea, Universitat Politecnica de Catalunia, Departament d'Enginerya Quimica, and Barcelona Research Center for Multiscale Science and Engineering (Prof. Carlos Aleman, Prof. Elaine Armelin, Barcelona; 30 October -9 November 2018; Training Session.

4. Dr. Pinteala Mariana, Dr. Maier Stelian S., Acad. Simionescu Bogdan C., University of Firenze, Italy, 4 September-12 September; Experimental approaches in the study of carbonic anhydrase inhibitors; Short Stays.

5.Dr. Marangoci N., Institute of Polymer Research, Dresden, Germany, 22-30 Noiembrie, 2018; Short Stay.

6. International Workshops

[1] Pinteala Mariana, **IMAGO-MOL Cluster Meeting**: *2nd Meeting of the Working Group on the Establishment of the National Center for Nuclear Medicine*. Oral Presentation, and signing of the Nuclear Medicine Consortium Memorandum, 03.11.2018.

7. Internal Workshops/ Work meetings

- Three working meetings to establish the communication pathways, and working strategies.
- Prof. Aatto Laaksonen, the project director, has held scientific meetings with each partner teams, leading to intermediate and final scientific reports.

VIII. Topics and prognoses for Stage 2019

Based on the extensive documentation approaches, and on some preliminary experimental results, the following commitments can be formulated for the next stage of the project.

1. Regarding the compounds prone to supramolecular assembling

- Reasoned screening of unimer candidates.
- Investigation of the ability to supramolecular assembilig of some particular inhibitors of carbonic anhydrases.
- *In silico* investigation of supramolecular assembiling ability, mechanisms, and, potentially, kinetics.

2. Regarding the nanoplatforms synthesis and physical-chemical evaluation

- Development and characterization of macromolecular supports of the following types:
  - conductive polymers with defined morphology;
  - silicon-based / silicon-including macromolecular compounds;
  - gelling (bio)macromolecules.

3. Regarding the tailored and reproducible functionalization of (bio)macromolecules

- Functionalization with reactive moieties (hydrophilic iminodiacetate groups: DMES, IMA, and hydrophobic trimethylsilane or tetramethyldisiloxane groups: AlCP, Sal).
- Design and synthesis of ligands containing both hydrophilic and lipophilic sequences capable of modifying their conformation and exposure of active / functional groups in dependence of the environmental polarity (pH); the latter could be internal (ether, ester, imine), or terminal (triazoles, -COOH, NH2, SH, etc.) groups.

4. Regarding the production of reproducible supramolecular systems

- Preparation of mesoporous silica *in situ* functionalized with organic groups suitable as substrates for attaching active principles.

5. Regarding the production of simple constituents of future tissue / tumor surrogates

- Design and development of solid 3D macroporous matrices mimicking extracellular matrices.
- Preparation of biodegradable hydrogels with controlled pore size and mechanical properties.
- Development of pH- and temperature-sensitive hydrogels based on (bio)macromolecules.

We certify that all the goals of 2018 stage of **5D-nanoP** project have been achieved.

December 3, 2018

Project Director,
Professor Aatto Laaksonen, PhD