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ANTITUMORAL THERANOSTIC PLATFORMS BASED ON CARBON DOTS AND POLYMER MATRICES

Research grant: PN-III-P1-1.2-PCCDI-2017-0083
Financial contract: 37PCCDI/2018
Project Manager: Acad. Bogdan C. Simionescu

Scientific report
Stage I (2018)

The activities of 2018 stage for all the three component projects have been accomplished. Thus, in the framework of P1 project there were carried out studies on the experimental adaptation of imide derived Carbon Dots as synthesis precursors, preconditioning procedures being tested (purification and recrystallization procedures for ensuring granulations compatible with the automatic system of solid dosage used in the process). Studies on the antitumoral activity of Imide derived Carbon Dots were focused in this stage on the types derived from the thermal processing of precursors such as N-Hydroxyphthalimide and N-Hydroxysuccinimide. The studies conducted by means of a new experimental configuration allowed discerning the process phases, their optimization and the setting of parameters for each stage individually. In the framework of P2 project, the experiments run to produce imide derived Carbon Dots gels with anti-tumoral features resulted in the selection of the following polymers: sodium alginate, carboxymethyl cellulose, guanosine and crosslinked polyacrylic acid. The biocompatibility, mucoadhesivity and biodegradability characteristics of the selected polymers allow the use of the prepared gels in the biomedical field. In both projects, the biological actions for Imide derived Carbon Dots (P1) in 2D and 3D cell cultures, and the gel embedded version (P2) were evaluated. The range of concentrations between the toxic effect and the detectable minimum impact in several human non-tumor cell lines was assessed, establishing a value of 50 μg / ml (5%), which is consistent with the pharmacological interest for Carbon Dots in NHF and NHS. The impact on some cancer lines with various lesional and histological biochemical features (representative for human neoplasms, the extensive target of clinical and research interest: breast, lung, glioblastoma, colon etc.) has been evaluated. Both cytotoxic and developmental profile of various tumors in vitro, differential effects were found. The degree of proliferation, the fraction of apoptotic suicide and the metabolic activity (important parameters of cancer aggressiveness) were modulated in different proportions, apparently in relation to the cancer cell profile.
Within the P2 project, critical cell culture systems were designed in order to evaluate gelified nanoformulations. All stages of the process as well as the additional stages (preparation of the synthesis reactor, removal of Carbon Dots dispersion, elimination of the pyrolysis residue etc.) were determined in detail within P3 project. The micro-pilot installation for synthesizing Imide derived Carbon Dots, and automation and interfacing elements were designed.

(b) Scientific and technical description

Component project: 1 (P1)

Activity 1.1 - *Studies on the experimental adaptation of synthesis precursors to the requirements specific to the biomedical field (advanced purification, sterilization etc.)*

The preparation process of Imide derived Carbon Dots nanostructures used as antitumoral theranostic platforms involves the use of some commercially available imidic organic compounds (N-Hydroxyphthalimide - NHF, N-Hydroxysuccinimide - NHS, Succinimide - SI, Maleimide - MI, N-Hydroxynaphthalimide - NHN). In order to be used as Carbon Dots synthesis precursors for biomedical applications, a series of preconditionings of these compounds (purification and recrystallization to provide granulations compatible with the system of solid dosage used in the process) are required.

Activity 1.2 - *Preparation of Carbon Dots from Imidic precursors (NHS, NHF, SI); physicochemical characterization; optimization of structure and synthesis parameters;*

In this stage, the studies on the antitumoral activity of Imide derived Carbon Dots have been concentrated on the types derived from the thermal processing of precursors N-Hydroxyphthalimide (NHF), N-Hydroxysuccinimide (NHS) and Succinimide (SI). The use of imidic Carbon Dots as theranostic platforms of investigation and anti-tumoral treatment involves their preparation under high-reproducibility conditions of the pyrolytic process, able to produce Carbon Dots nanostructures with identical physico-chemical characteristics from one batch to another. The thermal decomposition process of the precursors is discontinuous, and it was necessary to design an experimental configuration that allows for a good reproducibility of the processes involved in the production of Imide derived Carbon Dots. In order to use them in the medical field as antitumoral theranostic platforms, it is necessary to select an experimental configuration that allows both the high reproducibility of the morpho-structural characteristics of Carbon Dots from one batch to the other, and the automation of the process as well as ensuring the requirements of specific microbiological content. The investigations conducted with this new experimental configuration allowed the proper identification of the process phases, their optimization as well as setting the parameters for each stage. A number of interesting issues regarding the setting of operating parameters have been highlighted. The experimental configuration resulting from the performed investigations allows the preparation of imide derived Carbon Dots with high reproducibility from one batch to another.

Activity 1.3 –*In vitro evaluation on the selected cell lines of the photoluminescence of theranostic platforms based on imide derived Carbon Dots in order to establish the diagnostic protocol*

In the first phase of this in vitro evaluation activity of photoluminescent emission, a validation series of photoluminescence properties (fluorochromic activity) was started - in parallel with the measurement of the main behavior targeted by the grant, namely the cytolithic action. It was found a significant cytolytic activity (cyto-inhibitory / cytomodulatory) differentiated for the chemical formulations of this step, and the existence of a potential of a molecular reporter of fluorochromic type. For both nanostructures (NHF and NHS, respectively), we have found useful performances, at the limit of detection by standard methodologies (conventional fluorescence microscopy - Zeiss Observer Z1 microscopic platform), and in relation to biologically useful cytoinhibitors. We are convinced that under these conditions it would be worthwhile assessing the photoactivation property (intrinsic to many fluorochromes) as an additional (unforeseen) objective in the grant proposal. The biological parameter for establishing the diagnostic protocol measured as an indicator of the biological effect of CDots (NHF and NHS) was the cell viability on multiple representative normal human normal tumor lines, as well as a human murine mammary line corresponding to the human tumor breast line, testing the antitumor effects on the animal model (Table 1). The cell viability was measured by two methods: MTT (measurement by absorbance) and Promega (fluorescence measurement). MTT is an indicator of the level of cellular metabolic activity; the second test evaluates the ability of living cells to convert from the non-fluorescent (resazurin) form to the fluorescent form (resorufin) a redox indicator, which is lost in the dead cells.

Table 1. Effect of C-Dots with NHF and NHS in different cell types

Tip de celule	Tip de C-Dots	
	NHF	NHS
MDA-MB231 (Human breast adenocarcinoma)	++	+
A549 (Lung carcinoma)	++	+
A375 (Human malignant melanoma)	++	-
RPMI8226 (Plasmacytoma; Myeloma)	++	-
HT29 (Colorectal adenocarcinoma)	-	++
4T1 (Mouse breast cancer)	-	++
L363 (Human malignant melanoma)	-	++
U87 (Glioblastoma astrocytoma - human brain)	-	-
HMLE (Human mammary epithelial cells)	-	-

++$p<0.005$, + $p<0.05$, - no effect

Overall, we consider that the effects of CDots are achieved through multiple and different biochemical mechanisms differentiated in various types of malignancy. Thus, for each cell line we measured and compared the absorbance / fluorescence signals for standardized metabolites as a *bona fide* indicator of viability under the two conditions: the cell population exposed only to solvent (PBS) versus cell population exposed to nominal concentration of nano CDot compound. In

conclusion, with these results we achieved all the indicators of this first stage and we have all the arguments to deepen this research plan in the configuration initially proposed, going to the following phases to investigate the potential for biological interactions of CDots.

Component project: 2 (P2)

Activity 1.4 - *Selection of mucoadhesive polymeric materials (polysaccharides and / or synthetic polymers) and preparation methods for obtaining gel-like bio-composites including Imide derived Carbon Dots*

Hydrogels based on natural and synthetic polymers represents a field of great interest for a wide range of biomedical applications, from drug delivery to tissue engineering, due to their hydrophilic properties and their biocompatibility potential. However, such gels are often fragile, opaque and devoid of self-healing ability, thus significantly limiting their application in various biomedical fields. In this activity, a variety of natural polysmers (polysaccharides) was investigated to obtain gel bio-composites. By working with the human body, the selection of polymers has been made taking into account the need to meet certain constraints, including biocompatibility, lack of toxicity, and biodegradability. This is the reason why the following types of natural polymers have been chosen: chitosan, sodium alginate, carboxymethyl cellulose, xanthan, gelan, carrageenan, guanosine. To improve biocompatibility as well as gel properties and water retention capacity, some of these were accomplished by combining natural polymers with a biocompatible synthetic polymer, or crosslinked polyacrylic acid (also called Carbomer Ultrez 10) commonly used as a stabilization agent in various applications in pharmaceutical and cosmetic industries. Supramolecular hydrogels are a new class of non-covalent crosslinked materials, representing a perfect blend of the benefits of synthetic hydrogels and supramolecular polymers. After the preliminary tests of gels polymer preparation, the following polymers were selected: sodium alginate, carboxymethyl cellulose, guanosine and cross-linked polyacrylic acid. The biocompatible, mucoadhesive and biodegradable properties of the above-mentioned polymers recommend the materials based thereon for the use in the biomedical field, particularly in the intracellular provision of drug molecules.

Activity 1.5 – *Obtaining of gel biocomposites, morpho-structural characterization, luminescent emission and optimization of preparation parameters*

A series of gel materials with different concentrations based on sodium alginate (AS) or carboxymethyl cellulose (CMC) and Ultrez Carbomer 10 were prepared. All of the materials prepared have gel properties, being very viscous, i.e. they have an opalescent color in in the case of use of Carbomer, yellow in the case of AS and transparent for CMC, BDBA-K, BDBA-Ba, BDBA-Mg gels (Figure 1). After testing the rheological properties of the obtained materials, the best formulations resulted in Carb-F2, AS-F5, CMC-F3 and BDBA-Mg, these being taken into account for loading with Carbon Dots prepared from N-Hydroxyphthalimide obtained according to the

protocol reported by Stan et al. The chemical structure of the gels was highlighted by spectral method, and Raman spectroscopy respectively.

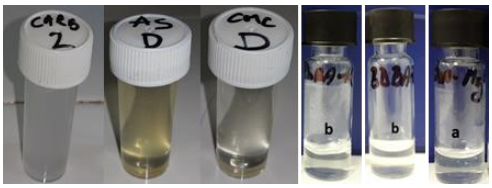

Fig. 1. Gel samples of Carb-F2, AS-F5, CMC-F3, BDBA-K, BDBA-Ba, BDBA-Mg

Figure 2 shows the images of the newly prepared gel materials, in terms of formulations loaded with CDots, namely Carb-F4, AS-F6, and CMC-F6 excited under UV at the wavelength of 365 nm. Based on these images, it was visually noticed that the CMC-F4 formulation presents a much stronger emission compared to those of Carb-F4 and AS-F6 formulations.

Fig. 2. Images of gels based on Carb, AS, and CMC, respectively (a) in absence and (b) loaded with Carbon Dots excited under UV

Activity 1.6 - *In vitro evaluation on selected the cell lines of luminescent emission of theranostic platforms based on Imide derived Carbon Dots in order to establish the diagnostic protocol*

The behavior of these nanoparticles embedded in gel may be of interest for human topical applications. The gels were tested in the ongoing synthesis procotol of the mentioned partner (Figure 3) on two aggressive murine lines of breast cancer (4T1, left diagram) and murine melanoma (B16, right diagram) respectively in 2D cultures. The viability assessment was performed in 72 hours. In relation to the viability of the control culture (the black columns near the ordinate axes) we noticed that the gel itself produces toxic effects much more significant in the case of K-based formulations, this toxicity augmenting the contribution of NHF-type particles (5% for each of the situations). Therefore, our conclusion at this stage is that both the structure and some other issues of the formulation of the categories of gels require optimization in order to be considered for oncological use. The images in Figure 3 include fluorescence measurements of viability (Cell Titer-Blue Cell Viability Assay Promega) which indicates significant impairment of the A375 human melanoma line (result consistent with the 2D evaluation).

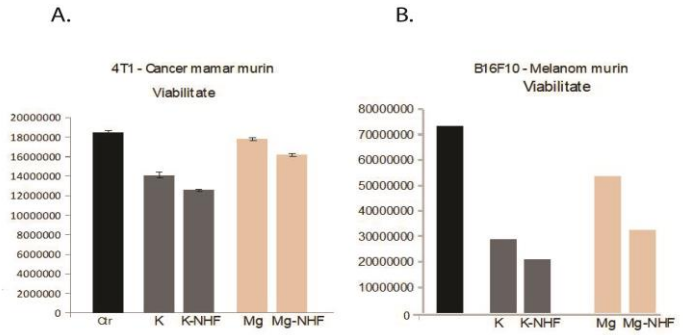

Fig. 3. Efects of K and Mg gels over cell viability in murine breast cancer (4T1 – fig. A.) and murine melanoma (B16F3 – fig. B. skin cancer)

Figure 4 shows the corresponding 3D morphological culture imaging revealing a significant detrimental impact undern gel culture conditions in the following experimental set-up. Figure 4a represents the 3D control culture in matrigel, Figure 11b - 3D culture in matrigel with 5% NHF in the culture supernatant and Figure 11c - 3D in matrigel with 5% NHF embedded in commercial gel. The cell line of this evaluation was A375 (human melanoma). The impact of the embedment of NHF-type nanoparticles in gel (commercial) reveals a potentiation of toxic activities beyond the level provided in case of NHF-particles in solution.

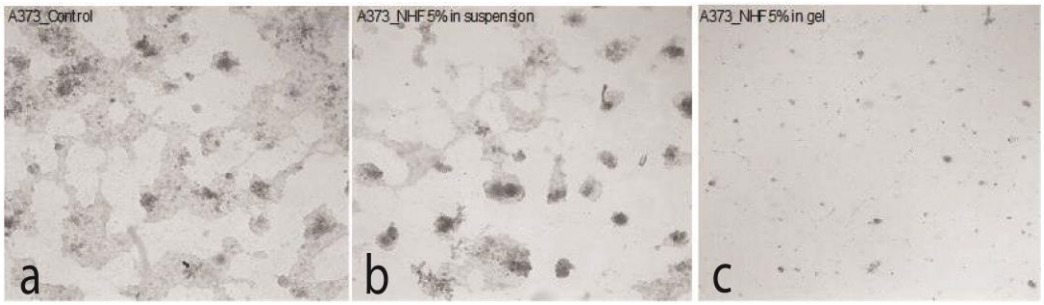

Fig. 4. Efects of gels with NHF 5% in human melanoma (A375), various culture conditions

Thus, both the viability (Cell Titer-Blue Cell Viability Assay, Promega) and mitochondrial activity (fluorescence evaluation using MitoTracker Red, Molecular Probe) are much less intense - the diagrams describing the MDA-MB231 and 4T1 lines.

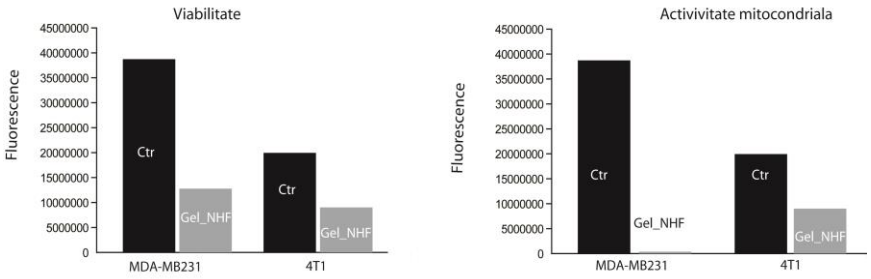

Fig. 5. Efects of NHF 5% embedded in commercial gel over MDA-MB231 and 4T1 lines viability (left) and mitochondrial activity (right).

The magnitude of the fluorescence signal in culture of this commercial gel (with NHF) differs between the two lines, even in the control culture, due to these lines have different biological characteristics. Fluorescence scan indicates similar results.

Component project: 3 (P3)

Activity 1.7 - *Identification of synthesis steps for imide derived CDots and operating parameters*

In the framework of P1 project, the concept of the experimental lab-scale installation represents the basis of study for P3 project and subsequently the achievement of micropilot system for preparation of imide derived Carbon Dots for medical applications. Due to the obtaining process of Carbon Dots is discontinuous, the detection of all working phases, of the parameters specific to each stage and their optimization were necessary. Studies have also aimed at selecting alternatives that can provide the basis for process automation. It is worth mentioning that due to the application scope it is necessary to keep within close limits the physical and chemical configuration of Carbon Dots produced in each individual batch. These requirements can be satisfied by the detailed determination of all phases of the pyrolytic process, of the additional stages (preliminary preparation of the synthesis reactor, evacuating the Carbon Dots dispersion after the completion of the process, removal of the pyrolytic residues etc.). In addition to the synthesis micro-reactor, the experimental configuration involves control systems for temperature, flow rate, liquid and powder dosing, centrifugation and vacuum drying systems.

Activity 1.8 - *Configuration of a pyrolytic processor of imide precursors, identification of technical solutions to control the process parameters*

The technical solution obtained from the experimental evaluations involves the use of a reaction vessel made of quartz or ceramic material due to both the high temperatures at which the main sequence of the pyrolytic process has to be conducted and the sudden quench step of the reaction mass involving the vessel resistance at repeated thermal shocks. The reaction vessel is equipped with a series of liquid injection / exhaust elements. The micro-reactor jacket allows the temperatures required for each stage. Removal of residues after each batch / cleaning of the microreactor is performed by introducing / discharging some solvents, the process being also assisted ultrasonically. Carbon Dots dispersion evacuation and waste disposal are performed by means of digitally controlled micro-pumps. The introduction / dosing of various liquids throughout the process is accomplished by means of digitally controlled microelectro-valves. All systems and devices will be interfaced to a computer provided with custom control software.

(c) Results dissemination

The results obtained during Stage I of the project were valorized by submitting to publication two scientific papers, the registration at O.S.I.M. of a patent request, respectively the presentation of an oral communication in the fremawork of a scientific event.

Papers sent to publication in ISI journals

1. Corina Savin, Catalina A. Peptu*, Corneliu S. Stan, Crina Tiron, Bogdan C. Simionescu, Maricel Danu, Carbon dots in carbomer gel - New composite with antitumoral activity, *Pharmacological Reports*.

2. Crina Tiron, Corneliu S. Stan*, Adrian Tiron*, Catalina A. Peptu, Florin E. Zugun, Bogdan C. Simionescu, Imide derived Carbon Dots – A New Promissing Approach in Cancer Treatment, *Journal of Pharmaceutical Sciences*.

Patent request submitted to O.S.I.M.

Corneliu S. Stan, Petronela Horlescu, Bogdan C. Simionescu, Cătălina A. Peptu, Sorin Ibănescu, Coordinative compounds of Gd(III) and Mn(II) usable as obtaining precursors of Carbon Dots nanostructures, A/00704/21.09.2018.

Works communicated in scientific conferences

Adina Coroabă, Gabriela Pricope, Bogdan Craciun, Anca E. Chiriac, Mariana Pinteală, "**Non-invasive techniques for the investigation of dermatological diseases**", *First Balkan Conference on Medical Mycology and Mycotoxicology*, - Balkan Fungus 2018, September 13-15, 2018, Timișoara, România

(d) Presentation of the structure of research and technological services offering the web-link in the Erris platform

One of the objectives of the project consisted in diversifying the offer of specialized R & D services offered to both universities and research institutes as well as to the industrial and economic environment. In this respect, taking into account the budget allocated for the first stage of the project, all the partners in the consortium have acquired the equipment necessary to carry out the project activities. Thus, the coordinating institution acquired a Hermle Labortechnick Z446 high-speed laboratory centrifuge (15,000 rpm) as well as a Quanta FI integration sphere needed to determine absolute quantum yields and chromatic parameters, an important accessory for an already available fluorescence spectrometer Horiba Jobin Yvon Fluoromax 4P. The partner P1 has improved its infrastructure by purchasing computers, an analytical balance, and a mechanical stirrer. Partner P2 has purchased a bioassay pre-treatment module, whereas Partner P3 has purchased a CO_2 incubator. All these equipments were added on the ERRIS platform on the pages of each institution:

https://erris.gov.ro/Centrul-de-Cercetare-POLIMER
https://erris.gov.ro/ICMPP
https://erris.gov.ro/INSTITUTUL-REGIONAL-DE-ONCOL
https://erris.gov.ro/INSTITUTUL-NATIONAL-DE-CERCE-31

(e) Research jobs supported by the program, including the newly employed human resource

The running of the project allowed full-time employment of 9 new researchers, of which 3 researchers were nominated in the project team in the submitted project proposal, whereas 6 researchers filled the vacant positions established through the research contracts. Two other researchers will be considered for the vacant posts within P1 partner starting with January 2019.

(f) Presenting the use / improvement of existing competencies / resources at the consortium level (checks)

Partners P1, P2, and P3 provided sums to valorize the skills in their field of activity. For this purpose, the amounts were used by means of A1 checks, taking into account that all the institutions in the consortium are from Iași. A series of analyzes were carried out in agreement with the objectives of the project and the contracts established between the involved institutions.

References

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