SCIENTIFIC REPORT

Dendrimer-carbon nanostructure conjugates as drug delivery support
Phase 1 – period: December 2011– December 2012

Phase II. Design, computational optimization and synthesis of dendrimers and functionalized carbon-nanostructures

WP2.1. Modeling of carbon nanostructure-dendrimer conjugates

In order to find optimal structures in terms of stability, the geometry optimization of nanostructures containing one or more substituents has been performed.

Computational method.

The structures were preoptimized with the PM6 semiepirical method, the obtained geometries were reoptimized at the Hartree-Fock level of theory (HF) using 3-21G* basis set and subsequently with 6-31G (d, p) and using the density functional theory (DFT) using the hybrid B3LYP functional with 6-31G(d, p) basis set. To verify if the obtained geometries correspond to a minimum, the harmonic vibrations were calculated at the corresponding level of theory; absence of negative frequencies confirms that these structures are a local minimum. The calculations were performed using Gaussian 09 program rev B01. Aromaticity of the structures was evaluated using the (nucleus independent chemical shift) NICS aromaticity descriptor NICs, calculated at the theoretical level B3LYP / 6-31G (d, p). The index was evaluated at the center of individual rings (NICS0) and at 1A distance above and below the ring (NICS1 / NICS-1), also at the functionalized fullerene core.

Study of multiple addition regioselectivity to fullerene C_{60}.

Multiple addition regioselectivity on bonds between two hexagons [6,6] was investigated on C_{60} fullerenes, where the number of functional groups varies between n = 1-6. As the number of regioisomers is very high, for example addition to monoadduct is possible in nine different positions, for this study bulky functional groups (ex. malonic acid) were chosen; and because of steric considerations the cis addition is excluded. Bonds [6,6] where addition can take place are highlighted in Figure 1 (dark blue). The hexakisaduct which could result as the final product has a tetrahedral symmetry (*T* or T_h depending on the symmetry group). To study the stability of structures the total energy (thermodynamic stability) and HOMO-LUMO gap energy (kinetic stability) was considered, results are presented in Table 1. It can be seen that the total energy per heavy atoms decreases with increasing number of functional groups, but it should be noted that the energy difference between regioisomers is very small. For example, in the case of bisaducts isomer (2) with C_1 symmetry is more stable compared to (2b) with only 1.16kcal / mol. The situation is similar in tris and tetra adducts, thus it can be concluded that multiple addition is not thermodynamically controlled.

Table 1. Single point calculation results obtained at the B3LYP/6-31G(d,p) level of theory: total energy (E_{tot} in au), total energy divided by the number of heavy atoms (E_{tot}/N în au), and the HOMO-LUMO energy gap (E_{gap} în eV).

#	Structure	E_{tot} (au)	E_{tot}/N	E_{HOMO}	E_{LUMO}	E_{gap} (eV)
(**0**)	$C_{60} - I_h$	-2286.1741	-38.1029	-5.987	-3.225	2.763
(**1**)	$C_{60}C(COOH)_2 - C_2$	-2702.5977	-40.3373	-5.763	-3.178	2.585
(**2a**)	$C_{60}(C(COOH)_2)_2 - C_1$	-3119.0221	-42.1489	-5.677	-3.088	2.589
(**2b**)	$C_{60}(C(COOH)_2)_2 - D_2$	-3119.0203	-42.1489	-5.618	-3.129	2.489
(**3a**)	$C_{60}(C(COOH)_2)_3 - C_2$	-3535.4444	-43.6475	-5.577	-3.046	2.531
(**3b**)	$C_{60}(C(COOH)_2)_3 - C_2$	-3535.4454	-43.6475	-5.538	-2.998	2.540
(**3c**)	$C_{60}(C(COOH)_2)_3 - C_3$	-3535.4477	-43.6475	-5.809	-2.786	3.023
(**4a**)	$C_{60}(C(COOH)_2)_4 - C_1$	-3951.8704	-44.9076	-5.741	-2.740	3.001
(**4b**)	$C_{60}(C(COOH)_2)_4 - D_2$	-3951.8668	-44.9076	-5.432	-2.962	2.470
(**5**)	$C_{60}(C(COOH)_2)_5 - C_2$	-4368.2934	-45.982	-5.654	-2.651	3.003
(**6**)	$C_{60}(C(COOH)_2)_6 - T$	-4784.7135	-46.909	-5.662	-2.194	3.469

WP2.2. QSAR/QSPR study. Drug design

Fullerene reactivity varies with the increase in the number of adducts, which is reflected in the difference in HOMO-LUMO gap energy values. Therefore in case of the monoadduct the energy gap is smaller compared to C_{60} fullerene and continues to decrease in case of fullerenes with two functional groups. C_{60} fullerenes with 3-6 functional groups has higher gap with 0.24-0.7 eV. This can be explained by the fact that mono and bisadduct breaks the symmetry of fullerene C_{60} and lowers the stability which is due to the sphericity of the molecule. Successive additions increase the local aromaticity of certain hexagons (see Figure 1, marked hexagonal rings, with an octahedral spatial arrangement). To confirm this observation, in rings of interest the NICS aromaticity descriptor was calculated. Preferential addition positions are best reflected by the analysis of the HOMO-LUMO orbitals. In Annex 1 the frontier orbitals are shown; analyzing the orbital coefficients it becomes clear that the additions are allowed in positions marked in Figure 1. The bond lengths in case of C_{60} fullerene are 139.5 pm for [6.6], and 145.3 pm for [5,6] type bonds. In case of the hexakisaduct these bonds in the highlighted hexagons in Figure 1 are 143 pm / 139.5 pm for the [5.6] / [6,6] bond types. Therefore the bond lengths are very close the possibility of a local delocalization can occur.

The increase in aromaticity is reflected in the NICS values, which in case of the considered hexagons decrease from NICS0=–2.42 in case of pristine fullerene to the value of –9.83, while the values measured in at the center of the structure (global aromaticity) are -2.675 and -11.266, for C_{60} and the hexakisaduct, respectively. Therefore after the addition by the formation of aromatic hexagons the structure is stabilized. By the comparison of trisaduct regioisomers it can be observed that kinetically the most stable isomer (3c) is the one where an aromatic hexagon is isolated by the functional groups (NICS0 = -8.827). The NICS values measured in the center of the functionalized fullerene have been correlated with the total energy divided by the number of atoms.

It can be observed in Figure 2 that there exists a very good linear relationship if the structures are separated in two sets, where in the first set the less aromatics regioisomers are included, while the second set includes the adducts with a high number of functional groups, which have the maximum number of aromatic hexagons. Thus it can be concluded that the stability of the structure depends very much on the number of aromatic hexagonal rings resulting from consecutive additions.

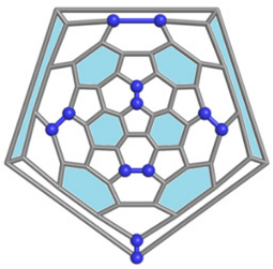


Figure 1. Preferential positions for multiple additions on [6,6] type bonds in case of fullerene C_{60}. The aromaticity of the highlighted hexagons increases with the number of functional groups; these cycles have an octahedral arrangement.

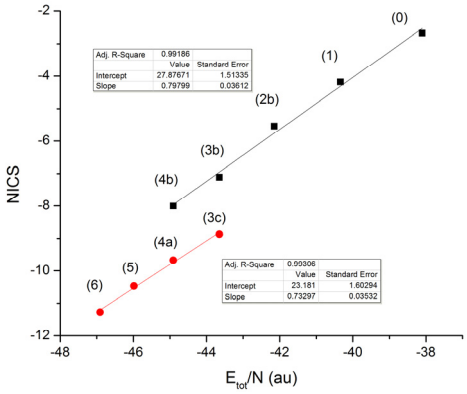
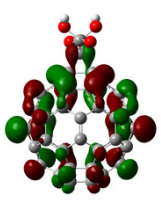
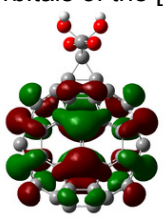


Figure 2. Graphical representation of the measured NICS values at the fullerene center as function of the total energy per number of heavy atoms (a.u.).

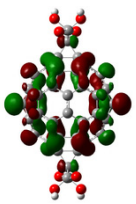
Annex 1. Frontier molecular orbitals of the [6,6] cycloadducts $C_{60}(C(COOH)_2)_n$ n=1–6.



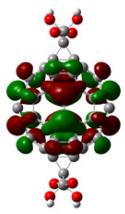
(**1**) HOMO



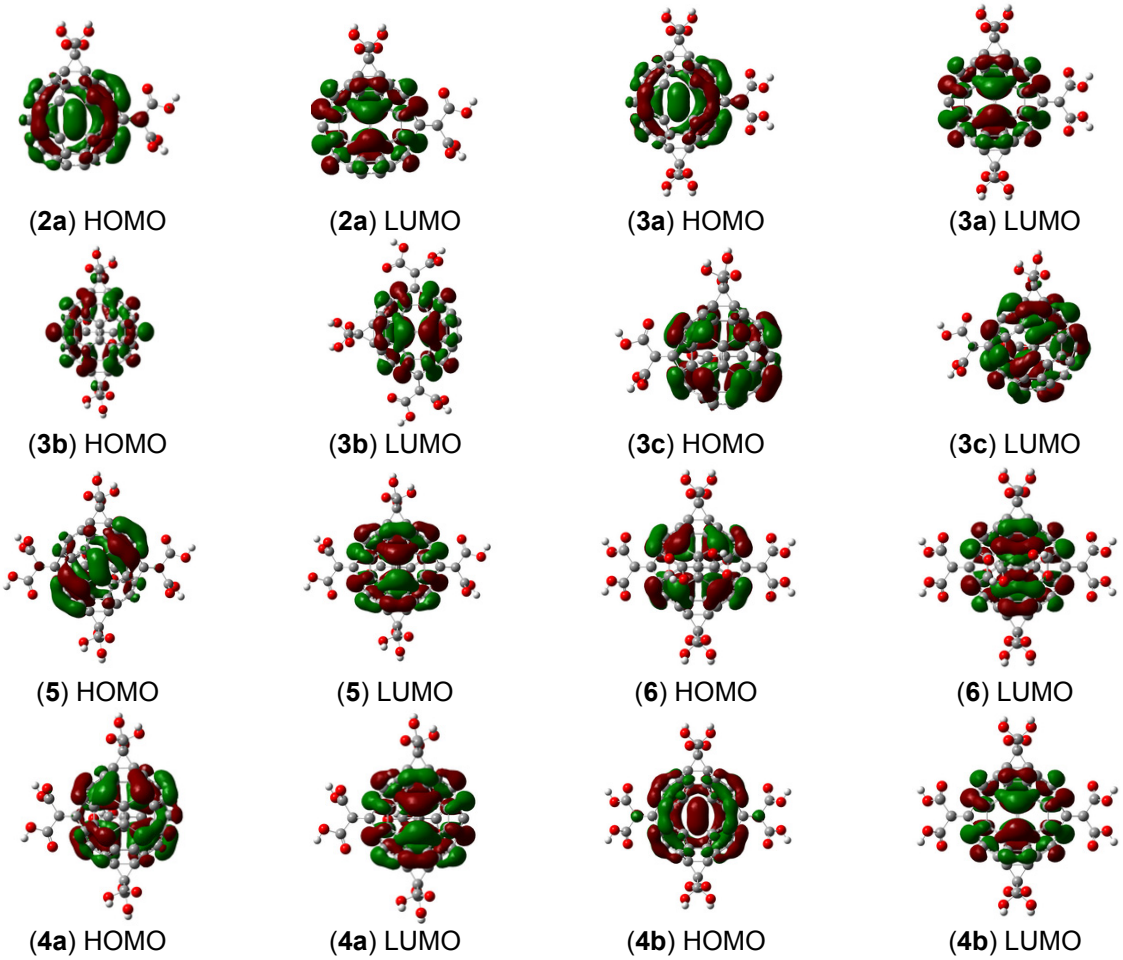
(**1**) LUMO



(**2b**) HOMO



(**2b**) LUMO



WP2.3. Synthesis and physico-chemical characterization of functionalized dendrimers and carbon nanostructure

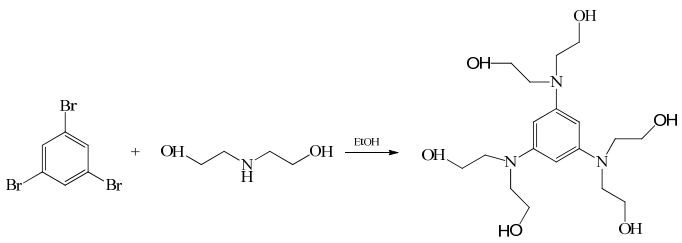
In order to build transport type molecular structure was started from the idea to the realization of dendritic structures (core) following the external surface functionalized with amino and hydroxyl to attach carbon nanostructures functionalized by a chain-type spacer.

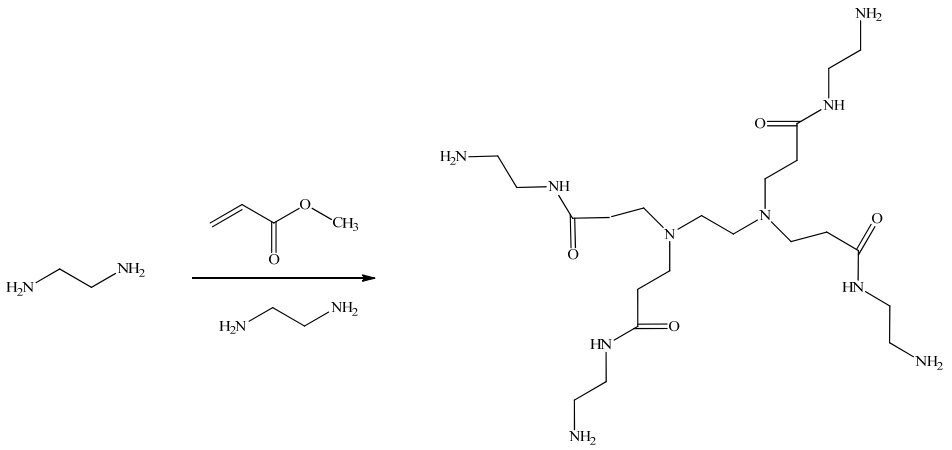
Functionalizing carbon nanostructures (fullerenes and nanotubes) was achieved by several methods:

1. reaction in the presence of microwaves
    a. In the presence of catalysts bis (cyclopentadienyl) hafnium (titanium)
    b. In the presence of sodium nitrite
2. Reactions at reflux
    a. In the presence of catalysts bis (cyclopentadienyl) hafnium (titanium)
    b. In the presence of sodium nitrite
    c. in the presence of oxidizing mixture

Two types of dendritic structures were considered, one having the starting structure tribromide 1,3,5-benzene and ethylene diamine for the second.

Building 1,3,5-tribromo benzene and PAMAM type dendrimers.





Procedure. A mixture of 1,3,5-tris (bromomethyl) benzene (100 mg) and diethanolamine (103mg, 3.5 eq.) in excess of ethanol was refluxed for 12h. The resulted mixture was concentrated under reduced pressure. The product was re-precipitated from -18^{o}C, making a transparent white substance, which is filtered and dried under vacuum.

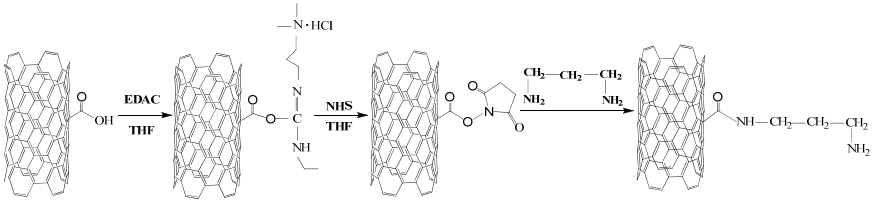
Functionalization of carbon nanotube with OH and COOH groups

To obtain functionalized product with hydroxyl groups was used the following method: suspend the carbon nanotube with a single wall / multi-walled (10mg, Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences) in a solution of potassium hydroxide (50ml 10M KOH, Mw = 56.1056 g / mol, ρ = 2.044 g / cm^3) for one hour in the ultrasonic bath (transonic 460 / H, AUSTRIA ELMA, 100W, 40kHz). The resulting suspension was heated at 100°C for a further hour. Vacuum filtration membrane (PTFE, 0.2 μm pore), washed with doubly distilled water (3x15 mL) and allowed to dry in the oven. Hydroxylated product is characterized by FT-IR (FTIR BRUKER EQUINOX 55, 7500-370cm-1, resolution> 0.5cm-1, Michelson interferometer type detector DLATGS).

Oxidation of carbon nanotubes with a single wall / multi-wall is achieved by suspension (20mg, Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences) in a mixture (20mL (1: 3)) of sulfuric acid (H_2SO_4, 98%, Mw = 98.08g / mol, ρ = 1.84g / cm^{-3}) and nitric acid (HNO_3, 70%, Mw = 63.01g / mol, ρ = 1.3913g / cm^{-3}) then leave for 8 hours in a ultrasonic bath (transonic 460 / H, AUSTRIA ELMA, 100W, 40kHz). The resulting solution was centrifuged to remove the unreacted acid mixture. The product thus obtained is left to dry under vacuum and is characterized by FT-IR spectroscopy (FT-IR EQUINOX 55 BRUKER, 7500-370cm^{-1}, resolution> 0.5cm^{-1}, Michelson interferometer type detector DLATGS).

Functionalization of carbon nanotube and fullerene with alkyl amidic groups

The method consists of the following steps: carbon nanostructures functionalized with COOH groups was suspended in dichloromethane (35mL, CH_2Cl_2, Mw = 84.93g / mol, ρ = 1.33g / cm^{-3}) in a 50 mL flask fitted with tube calcium chloride ($CaCl_2$, Mw = 110.98 / mol, ρ = 2.15 / cm^{-3}) and leave in the ultrasonic bath (transonic 460 / H, AUSTRIA ELMA, 100W, 40kHz) at room temperature. Add 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (20 mg, EDAC, $C_8H_{17}N_3Cl$, Mw = 191.7g / mol) and N-hydroxysuccinimide (15 mg, NHS, $C_4H_5NO_3$, MW = 115.09g / mol) and sonicate for one hour at room temperature. Subsequently add diamino-alkanes (ex.1,4-diaminobutane, 9μL, $C_4H_{12}N_2$, Mw = 88.15g / mol, ρf = 0.877g / mL) and sonicate the resulting mixture for two hours, it was the optimal reaction time confirmed by elemental analysis. Before filtration 15mL of THF added to the reaction mixture to facilitate its vacuum filtration membrane (PTFE, 0.2 μm pore), then wash with THF (3x10mL) and let it dry in the oven.



Structure characterizations

The resulted products were characterized by IR, RAMAN, ^{1}H-RMN, elemental analysis, MALDI-TOF-MS, SEM and TEM microscopies.

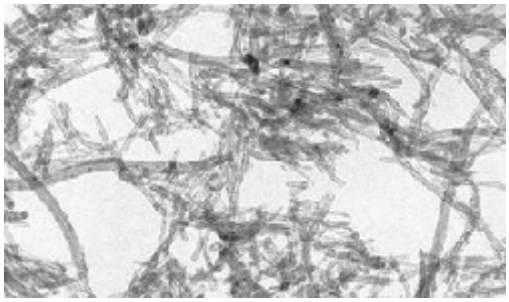


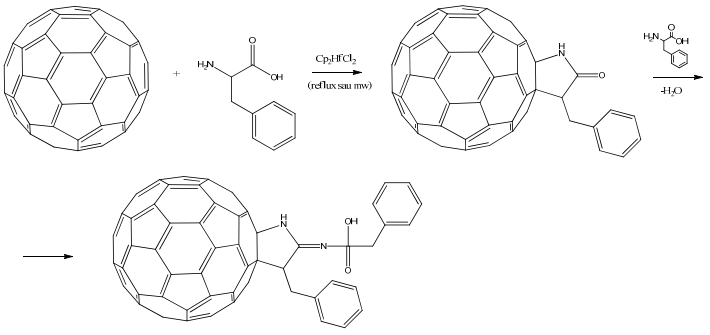
Figure 3. COOH functionalized multi wall nanotube (M-COOH)

Fullerene functionalization with amino acid

Fullerene functionalization was performed with different amino acid (alanine, phenylalanine, nitro phenylalanine, serine, phenylglycine, aspartic acid in different conditions (reflux, oven). General method consists of:

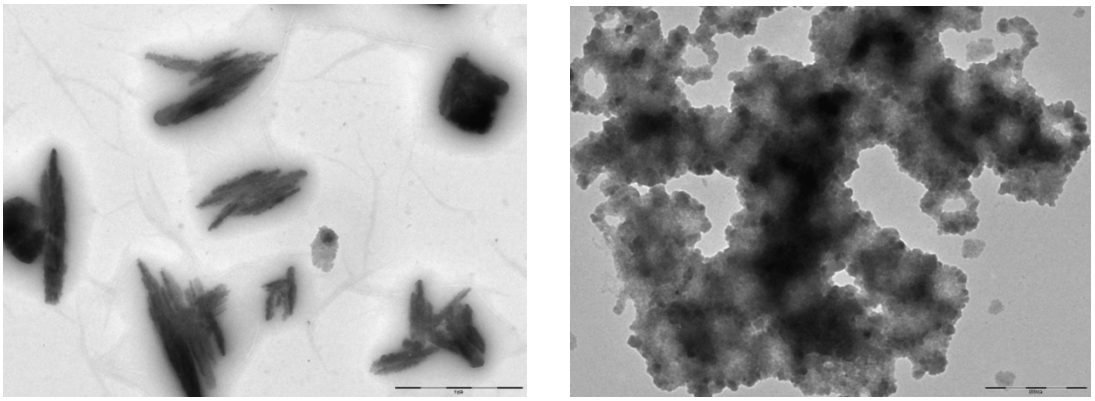
Procedure: In a round bottom flask of 50 mL was dissolved fullerene (100 mg, C60, 0.1389 mmol, Mw = 720.64 g / mol) in toluene (or dichlorobenzene) (30 ml, C_7H_8, MW = 92.14 g / mol, ρ = 0.865 g / ml). To the resulting mixture was added the amino acid (D, L-alanine, 24.7 mg, ALA, $C_3H_7NO_2$, 0.2778 mmoles, MW = 89.09 g / mol) and the catalyst bis (cyclopentadienyl) hafnium (or titanium) (IV) (25 mg, Cp-H $C_{10}H_{10}Cl_2Hf$, Mw = 379.58 g / mol) then let stirring at reflux for 24h afford (1) -C60-ALA-H-rf. In a round bottom flask of 50 mL was dissolved fullerene (100 mg, C60, 0.1389 mmol, Mw = 720.64 g / mol) in toluene (30 ml, C_7H_8, MW = 92.14 g / mol, ρ = 0.865 g / ml). To the resulting mixture is added to the amino acid (D, L-alanine, 24.7 mg, ALA, $C_3H_7NO_2$, 0.2778 mmoles, MW = 89.09 g / mol) and sodium nitrite (26.1 mg, NN, $NaNO_2$, MW = 69.00 g / mol) and a small amount of hydrochloric acid (1 ml, HCl, MW = 36.46 g / mol, ρ = 1.2 g / ml) then allowed to stir at reflux for 24 hours.

Processing method: the resulting mixture was evaporated to dryness using a rotary evaporator (Bruker). The solid was allowed to sonicate for 10 minutes in the ultrasonic bath with acetone (2x40 ml, C_3H_6O, MW = 58.08 g / mol, ρ = 0.7899 g / ml) and then filtered under vacuum through membrane (PTFE porous 0.2 µm), washed with water (2x20 ml H_2O, MW = 18.01 g / mol, ρ = 1 g / ml) and again with acetone (10 ml) to remove the amino acid of the catalyst unreacted then allowed to dry in the oven. For separation of unreacted product the mixture was purification and separation by silica gel column chromatography using toluene as eluent. The first fraction mauve-purple which is separate unreacted fullerene C60 fullerene functionalized then collect fractions. Their color varies from dark purple-brown-yellowish brown. Impurities are removed they also remain on the column to the start point. After collecting the solvent fractions are evaporated to dryness in a rotary evaporator and the product is obtained as solid.



Structures characterization

Products were characterized by IR, RAMAN, ^{1}H-RMN, elemental analysis, MALDI-TOF-MS, SEM and TEM microscopies.



TEM micrograph of C_{60} and functionalized C_{60} with alanine.

WP2.4.Optimization of reaction conditions

Optimizing method for obtaining the functionalized nanostructures was done in order to obtain high yields and high purity complex structures. In the process of optimizing the following parameters were considered: reaction time, reaction temperature and solvent followed. Reactions were repeated for different mixtures of solvent, such toluene, 1,2-dichlorobenzene, DMF, THF.

WP2.5.Testing the cellular toxicity of functionalized dendrimer and carbon nanostructure

Nanostructures obtained were assayed for toxicity to determine the range of concentrations usable without causing cell apoptosis. Testing cytotoxicity of fullerenes and carbon nanotubes was performed on tumor cell line A549. Human lung carcinoma cell line (A549) was purchased from the Cell Line Service, Germany. A549 was established in 1972 by DJ Giard, from a woman with lung adenocarcimon. An important feature of this line is the tumor that is overexpressed epidermal growth factor receptor.

Sample preparation. The cells were grown in Dulbecco's modified Eagle's medium (Lonza) supplemented with 2 mM L-glutamine, Pen / Strep 100 U / ml and 10% FCS, maintained in a humidified incubator (37°C, 5% CO2). Testing the cytotoxic effect of carbon nanotubes functionalized fullerenes and test kit was performed using cellular cytotoxicity Cytotoxic 96 (R) Non-Radio Cyto (Millipore) kit that cause mitochondrial dehydrogenase enzyme activity. They convert reagent WST-1 (water soluble tetrazolium) to formazan. The amount of formazan produced by the viable cells can be quantified by measuring the absorbance using a spectrophotometer plates. The 5x103 / well cells were cultured in 96-well plates and allowed 24 hours to adhere to the substrate and then the culture medium was changed to culture medium containing different concentrations of nanotubes / functionalized fullerenes and simple. The nanotubes were resuspended in water to give a stock solution of 20mg / ml in which serial dilutions were made in culture medium. After 24 hours the media was removed and the nanoparticles in each well were added 90ul 10ul average + reagent WST-1. After incubation for 30 minutes at 37C, the absorbance was determined at a wavelength of 450nm to 650nm with reference. In Figure 1 we can see that starting concentration 100 micrograms / ml nanotubes all 3 induced significant decrease in cell viability. Instead, the concentrations tested, simple and functionalized fullerenes not significantly affect cell viability were A549 (Figure 2).

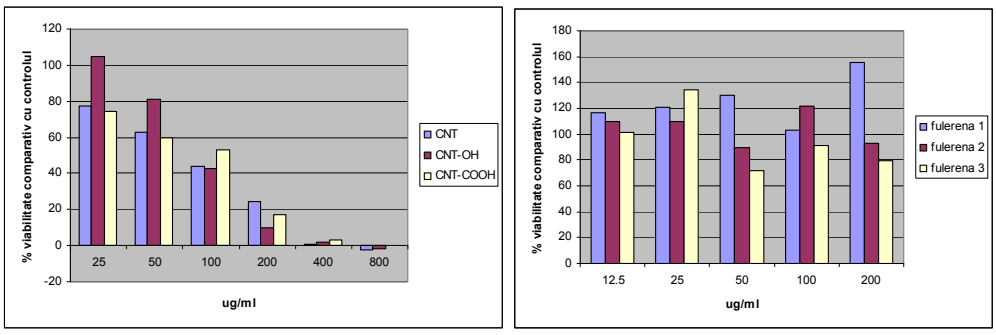


Figure 1. Cytotoxicity test results for carbon nanotube and fullerene derivatives.

WP2.6.Results dissemination: articles, communications

In conclusion, carbon nanostructures functionalization has been achieved with hydroxyl, carboxyl, amino acids and aminoalkyl groups. Such structures were characterized by IR, Raman, MS, ^{1}H-NMR, ATR, SEM and TEM.

Phase II results have materialized in these ISI articles:

1. A. Iranmanesha, M.V. Diudea, Cluj-Tehran Index, *MATCH Commun. Math. Comput. Chem.*, 69 (**2013**) 121-130.
2. B. Szefler, M.V. Diudea, Polybenzene multitori, *Cent. Eur. J. Chem.*, 10(6), 2012, 1779-1785.
3. Mircea V. Diudea, B. Szefler, Nanotube junctions and the genus of multi-tori, *Phys. Chem. Chem. Phys.*, 2012, 14, 8111–8115
4. M.V. Diudea, Centric connectivity index by shell matrices, 2012 (accepted)
5. M. Saheli, A. Iranmanesh, B. Szefler, M.V. Diudea, Omega polynomial in CQ crystal networks, 2012 (accepted)
6. M.E. Füstös , N.A. Dima, G. Katona, Functionalization of C_{60}-fullerenes, XVIII International Conference on Chemistry, November 22-25, 2012, Baile Felix.