SCIENTIFIC REPORT

Dendrimer-carbon nanostructure conjugates as drug delivery support
Phase 4 - period december 2015 – september 2016

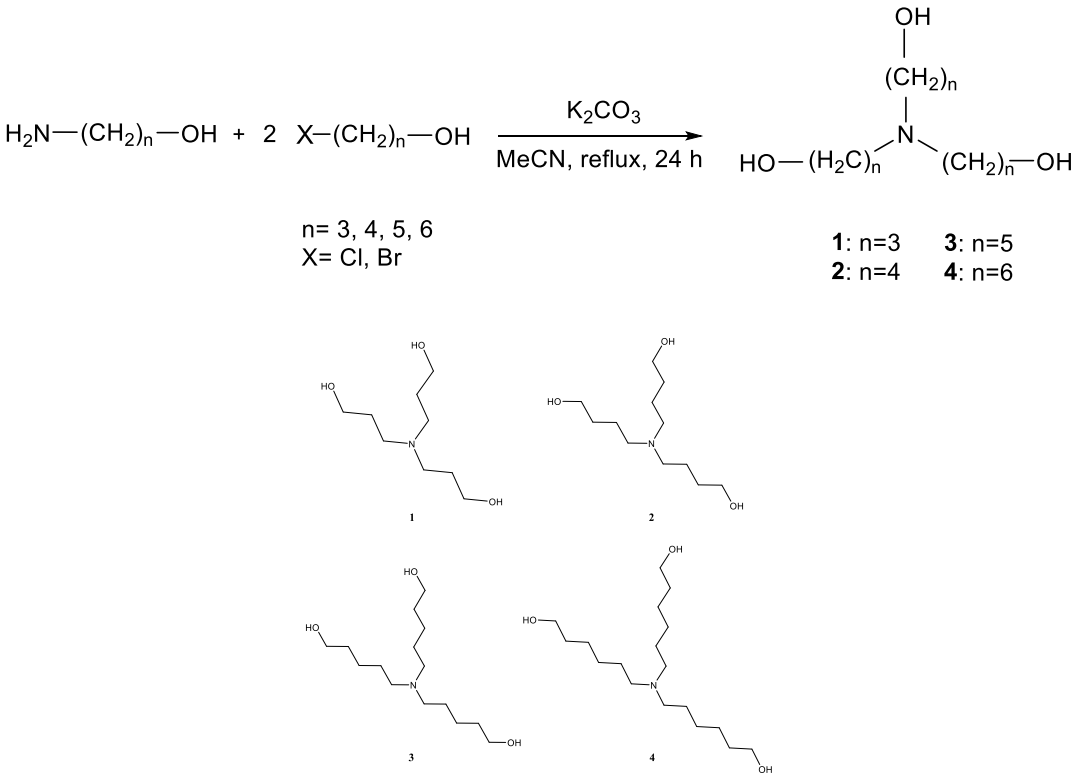
Faza VI. Cell wall penetration and cellular toxicity testing of dendrimer-carbon nanostructure complexes

Testing of penetration capability of dendrimer-carbon nanostructure complexes

1. Synthesis of novel trialcanolamine derivatives and their use in catalysis

1.1. Synthesis of trialcanolamines

In this step the chemical synthesis of aliphatic aminoalcohol derivatives with the corresponding chloro- and bromo-hydroxyalcane derivatives (substituted in the terminal positions of the carbon chain) was accomplished. The reaction products obtained were symmetric trihydroxyamines with different carbon chain length (Scheme 1)



Scheme 1. Synthesis of trialcanolamines

Experimental

In a 25 ml round bottom flask under continuous stirring 1 eqv of aminoalcohol was dissolved in 10 ml acetonitrile and 2.1 eqv of the corresponding halogenated alcanol was added. Potassium carbonate was added in exces to the reaction mixture in order to neutralize the chlorhidric or the bromhidric acid resulted as side product. The reaction was carried out under reflux conditions for 24 hours. After reaching room temperature, the mixture was filtered in order to remove the formed salts. The filtrate was collected and the solvent evaporated using a rotavapor. The obtained reaction products were light yellow colored viscous liquids.

Characterization of the obtained products (tripropanolamine (**1)**, tributanolamine (**2**), tripentanolamine (**3)**, trihexanolamine (**4**)) was realized by mass spectrometry (MS, Agilent/Technologies 6320).

Results

In the following figures are presented the mass spectra of the obtained compounds **1-4.**

Tripropanolamine exact mass is 191.15 g/mol while tributanolamine exact mass is 233.19 g/mol. The MS spectra reveal the corresponding peak for [M+1] at *m/z* 192.16 for compound **1** and at *m/z* 234.20 for compound **2** (Figure).

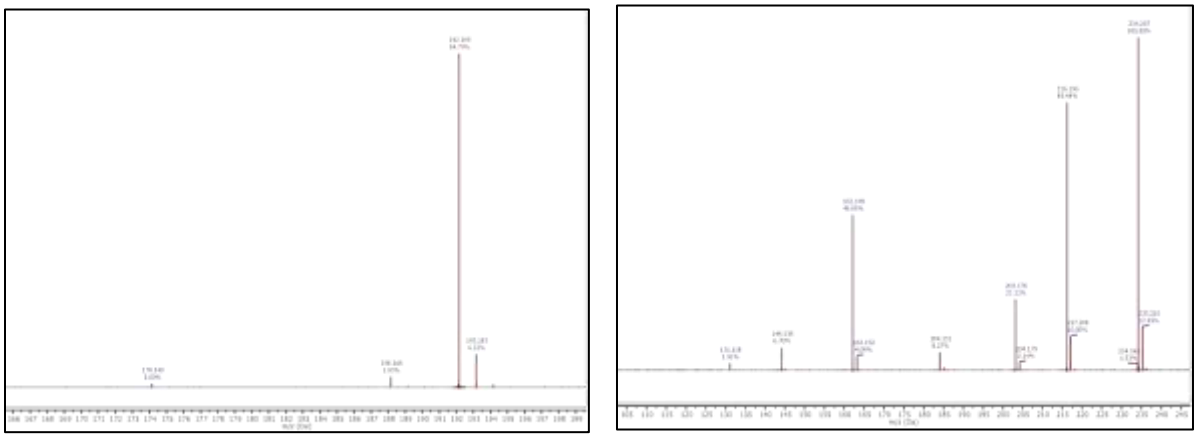


Figure 1.Tripropanolamine and tributanolamine mass spectra

In the MS spectra of tripentanolamine the peak at *m/z* 276.25 value correspond to [M+1], being in concordance with the compound exact mass (275.24 g/mol). In case of compound **4** the MS characterization also confirmed the molecule exact mass (317.29 g/mol) by the peak at *m/z*

318.30 value (Figure 2)

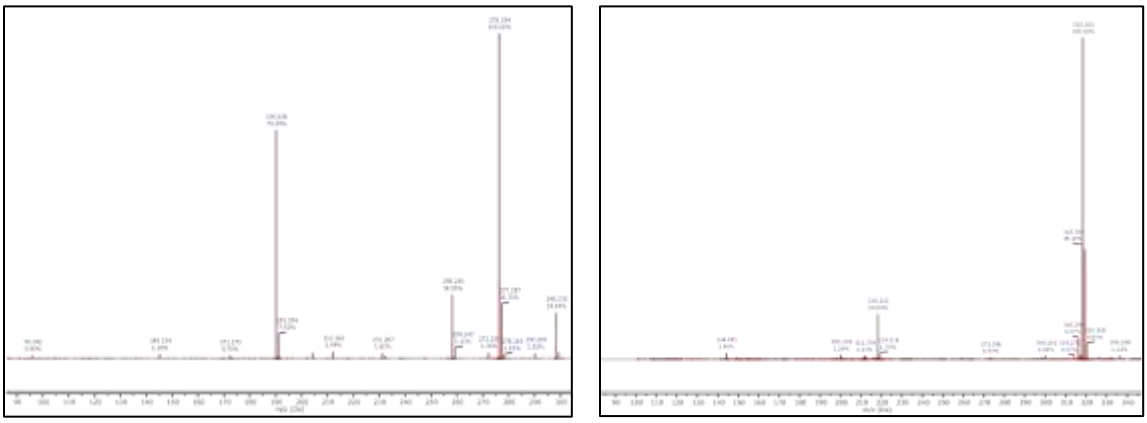


Figure 2. Mass spectra of tripentanolamine and trihexanolamine

1.2. Trialcanolamines in homogenous catalysis

In this phase the possibility of using trialcanolamines as support for the Cu^0 nanoparticles was tested in order to obtain liquid phase metallic catalysts with application in homogenous catalysis.

Copper (0) nanoparticles are used as catalysts in various reduction reactions. According to literature data, the reduction of *p*-nitrophenol (PNP) with sodium borohydride to *p*-aminophenol (PAP) in presence of metallic nanoparticles is considered a modell reaction of heterogenous catalysis. Due to the distinct phase of the reaction media and catalyst, the reaction time is longer.

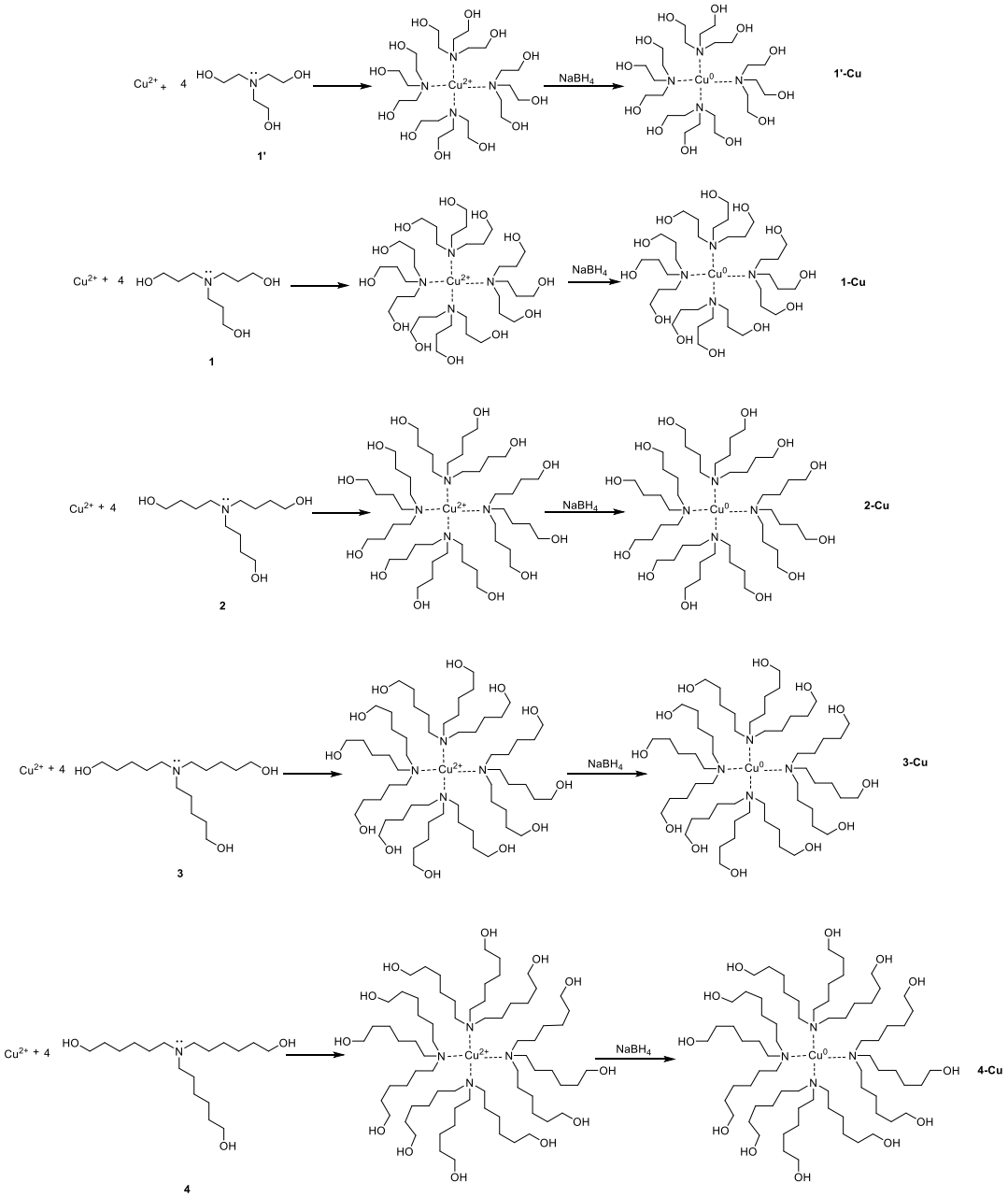
Preparation of the catalyst

In the first part of the research, a metanolic 10 *w/v %* $CuCl_2 \cdot 2H_2O$ solution was prepared, representing the source of Cu^{2+} nanoparticles. In order to test if the compounds 1-4 are suitable as support for copper (0) nanoparticles with application in homogenous catalysis, the trialcanolamine-Cu^0 catalysts were obtained according to the following scheme (Scheme). Also the commercially available triethanolamine (**1'**) was tested with this purpose.

Experimental

In a 25 ml round bottom flask containing 5 ml of Cu^{2+} solution trialcanolamine (**1'-1-4,** 0.4 mmol) dissolved in methanol (1 ml) was added dropwise under continuous rigurous stirring. The

resulted mixture was refluxed for 24 hours, obtaining the trialcanolamine-Cu^{2+} species. The mixture was left to reach room temperature and then the reducing agent ($NaBH_4$, 4 mmol) was added portionwise to reduce the Cu^{2+} ions to Cu^0 which present catalytic activity. The solid dark colored Cu^0 particles (which were formed due to the reduction of the Cu^{2+} not bonded to the tertiary nitrogen atom of the dendrimer) were removed via vacuum filtration using a PTFE membrane (porosity 0.2 μm). The resulted green filtrate represented the liquid state catalyst, noted further as **1'-Cu, 1-Cu, 2-Cu, 3-Cu, 4-Cu**, which activity was tested in the reduction of the PNP.



Scheme 2. Obtaining **1'-Cu, 1-Cu, 2-Cu, 3-Cu, 4-Cu catalysts**

Catalytic activity testing

The catalytic activity determination was realized via UV-visible spectrophotometric measurements (Agilent 8453 spectrophotometer). For this purpose a quartz cuvette was used with 1 cm path length and 3 ml volume. Aqueous solutions of PNP (1.2 mM) and $NaBH_4$ (15 mM) were prepared. The sample contained 1620 µl ultrapure water, 1000 µl $NaBH_4$ solution and 180 µl PNP solution. The catalyst volume was 10 µl in each case.The sample was stirred in order to facilitate the homogenization after the addition of the catalyst. Spectra were recorded at 5 s intervals.

The aqueous solution of PNP presents a maximum absorbance line at 318 nm wavelength. When $NaBH_4$ is added, the absorbance maximum appears red shifted at 401 nm. In the UV-vis spectra the product (PAP) appears at 300 nm wavelength.

The figure listed below illustrate the color changing of the sample solution after catalyst addition. Also it can be observed that, the Cu^0 nanoparticles coordinated to the N atom of the dendrimer are released due as the catalytic reaction took place and can be collected via filtration and reused as solid phase catalysts in heterogenuous catalysis.

Figure 3. Reaction media before and after the catalyst addition

In the following figures are presented the recorded UV-vis spectra for each catalyst and also the efficiency of the catalysts is compared.

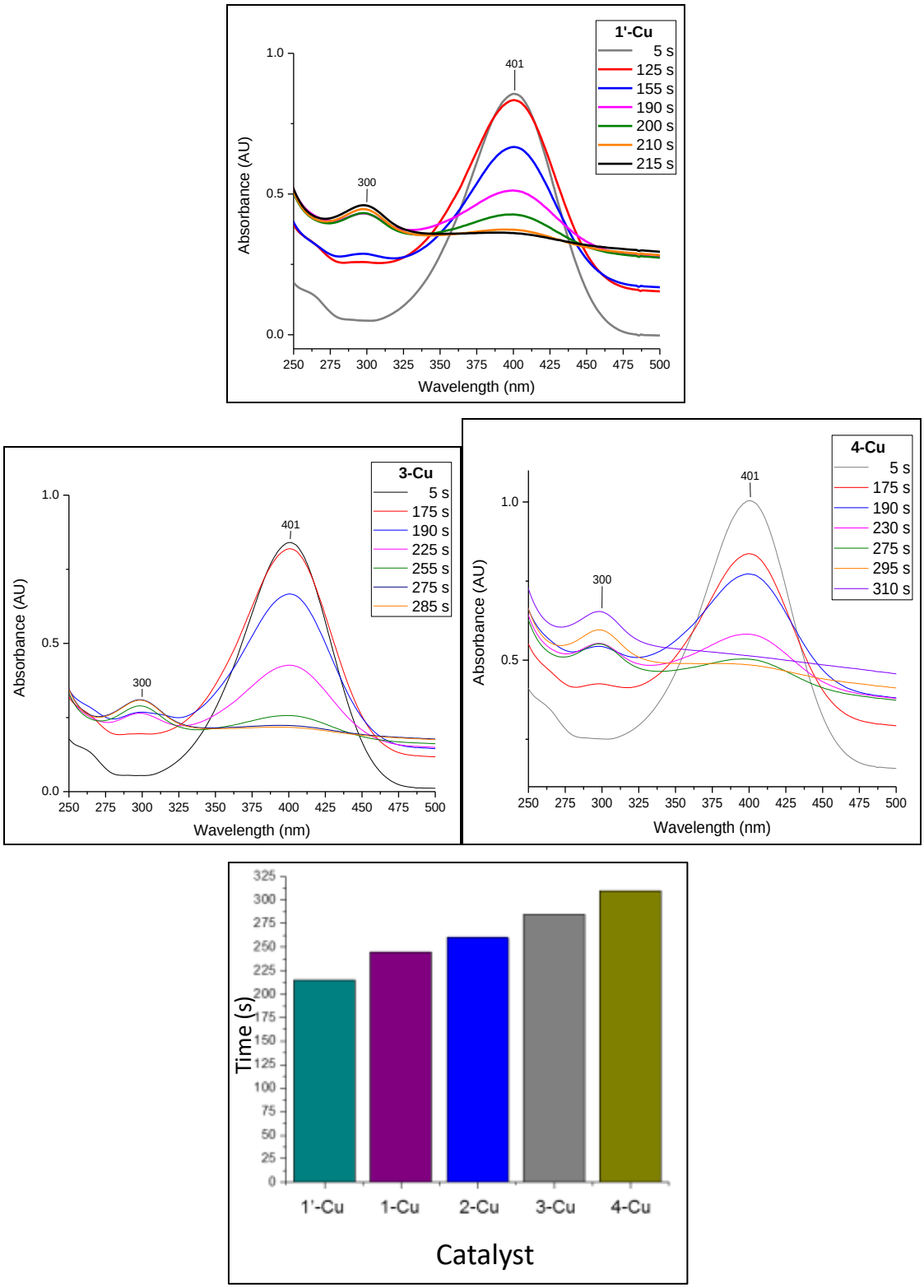
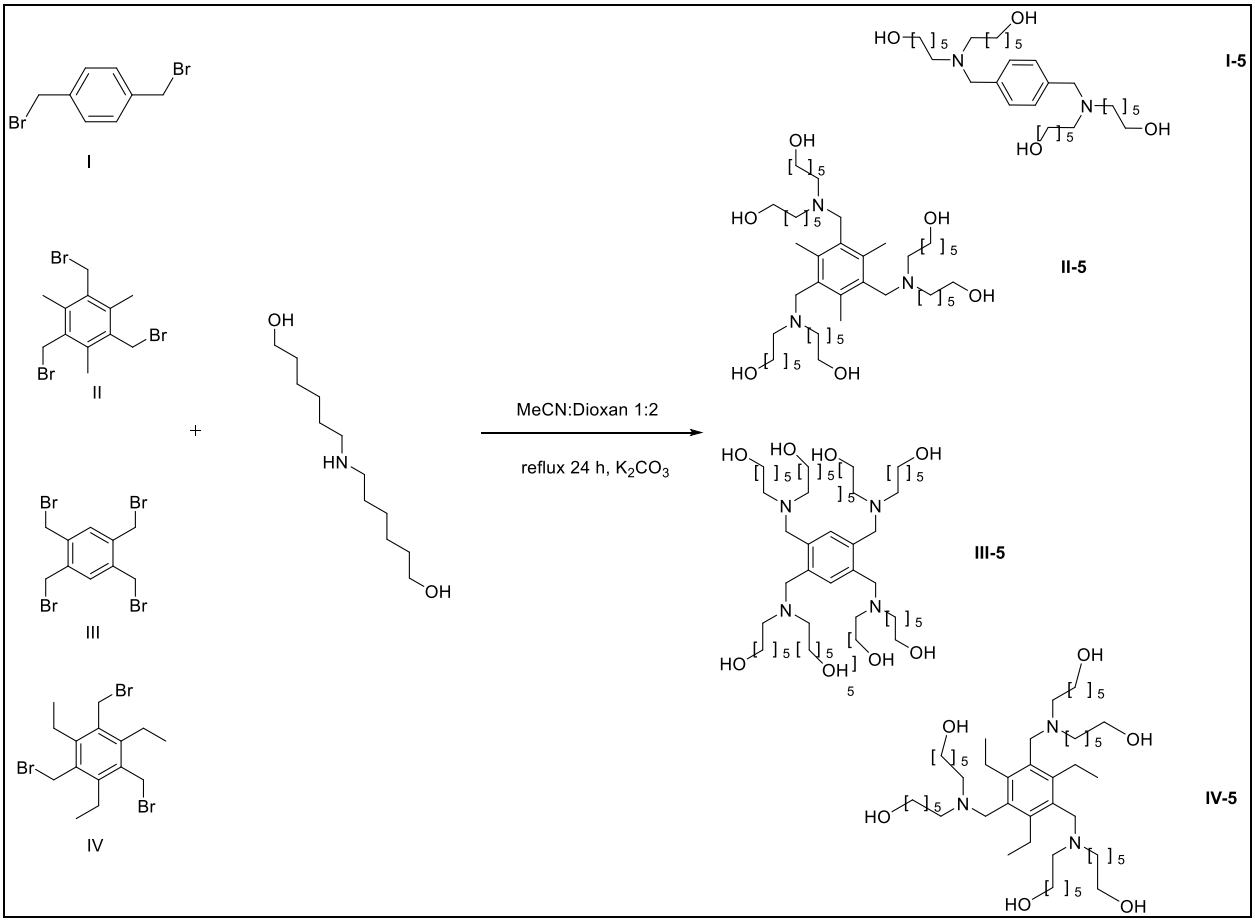


Figure 4. UV spectra for used catalysts.

2. Synthesis and characterization of zero generation dendrimers

In this stage the synthesis of zero generation dendrimers was accomplished using dihexanolamine as dendron according to the following scheme (Scheme).



Scheme 3. Synthesis of aromatic core zero generation dendrimer

Experimental

In a 25 ml round bottom flask under continuous stirring the aromatic core (**I-IV**, 1 eqv) was dissolved in 15 ml of acetonitrile:dioxane 2:1 mixture. Dihexanolamine (2.2 eqv) and K_2CO_3 (3 eqv) were added and the resulted mixture was refluxed for 24 hours. After reaching room temperature, the resulted salt (KBr) was removed by vacuum filtration. The filtrate was collected and the solvent was evaporated on rotary evaporator; a yellow viscous liquid resulted as product. The reaction was monitored by thin layer chromatography (TLC Silica gel 60 F_{254}). The obtained

dendrimers were characterized by the following methods: mass spectrometry MS (Agilent/Technologies 6320), ^{1}H-NMR (Bruker 600 MHz) and ^{13}C-NMR (Bruker 150 MHz) spectroscopy.

Dihexanolamine was synthesized and characterized by spectrometric (MS) and spectroscopic methods (^{1}H-NMR, ^{13}C-NMR). The figure presents diethanolamine mass spectra. The molecular ion is present at *m/z* 218.21 value, being in concordance with the exact mass of the molecule (217.20 g/mol).

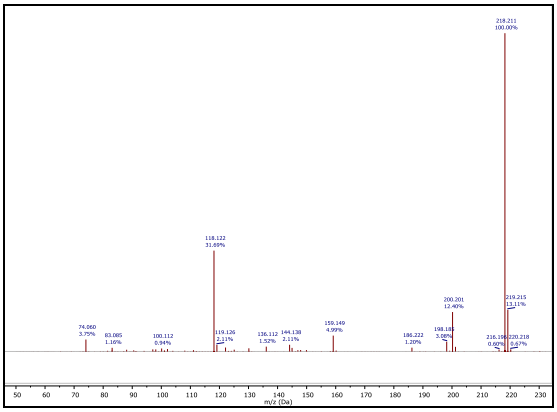


Figure 5. Mass spectra of dihexanolamine

The appearance of the characteristics peaks in NMR spectra confirm the tructure of the molecule. In the following figures are presented the ^{1}H-NMR (D_2O, 600 MHz, 298 K) and ^{13}C-NMR (CD_3OD, 151 MHz, 298 K) spectra of the compound.

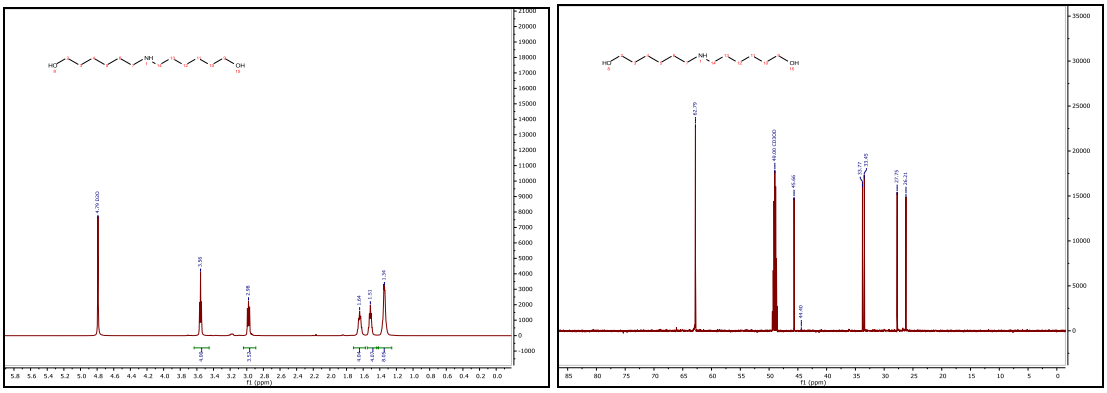


Figure 6. NMR spectra of dihexanolamine

In the following figures is presented the ^{1}H-NMR ($CDCl_3$, 600 MHz, 298 K) spectra and the mass spectra of compound **I-5** (Mw= 536.46 g/mol). Resuls confirm the structure of the

dendrimer.

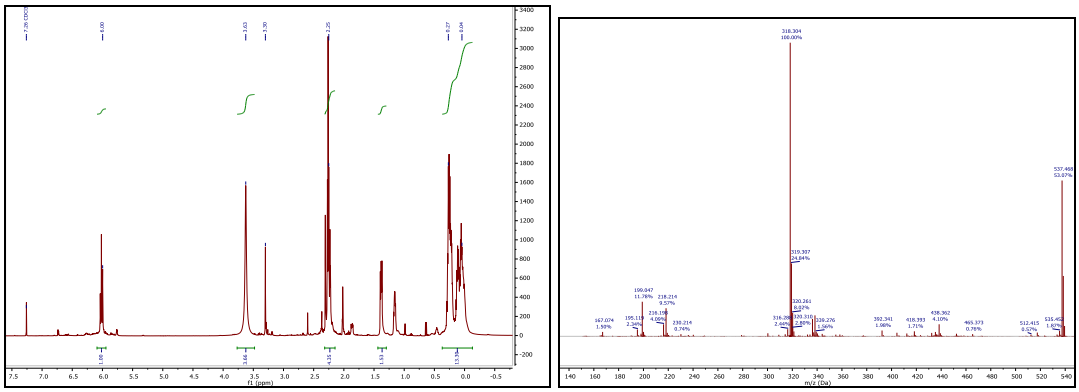


Figure 7. ^{1}H-NMR spectra and mass spectra of compound **I-5**

3. Catalytic activity study of some dendrimers

In this stage the utility of the synthesized zero generation dendrimers was tested as support for Cu^{2+} ions to be redused *in situ* to Cu^{0} nanoparticles, which present cataltytic activity in various reduction reactions.

For this purpose the studied reaction was the hydrogenation of *p*-nitrophenol with sodium borohydride in presence of the Cu^{0} nanoparticles coordinated by the tertiary nitrogen atoms of the zero generation dendrimers. The tested dendrimers were the following:

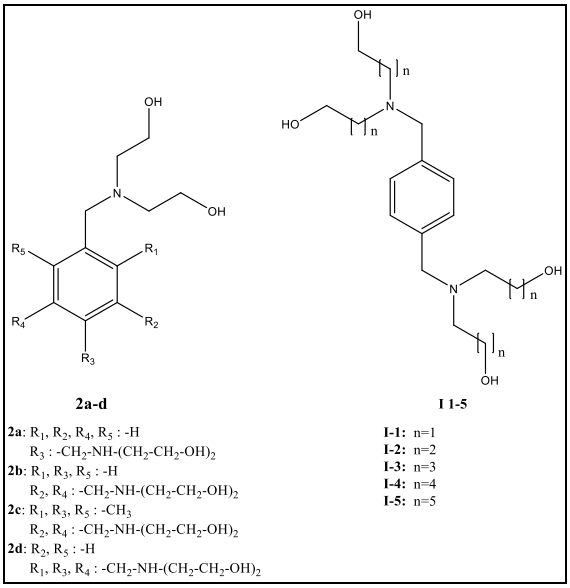
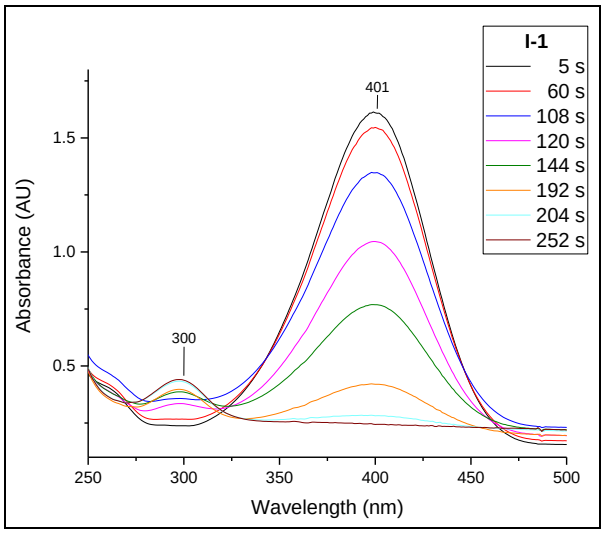
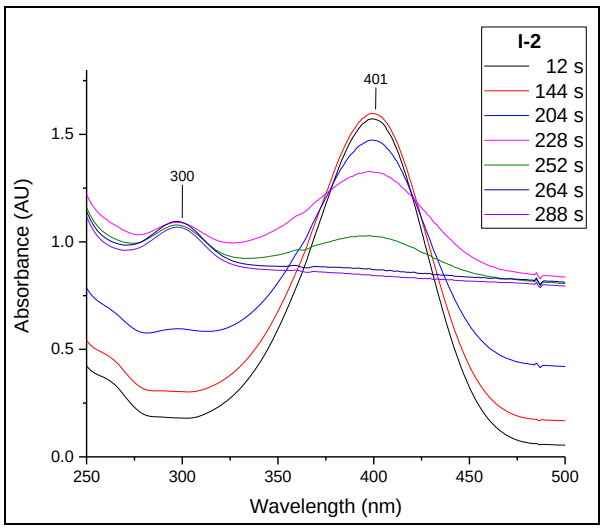
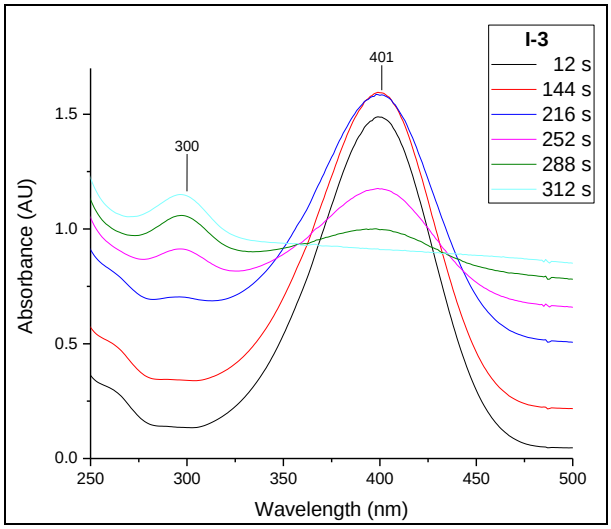


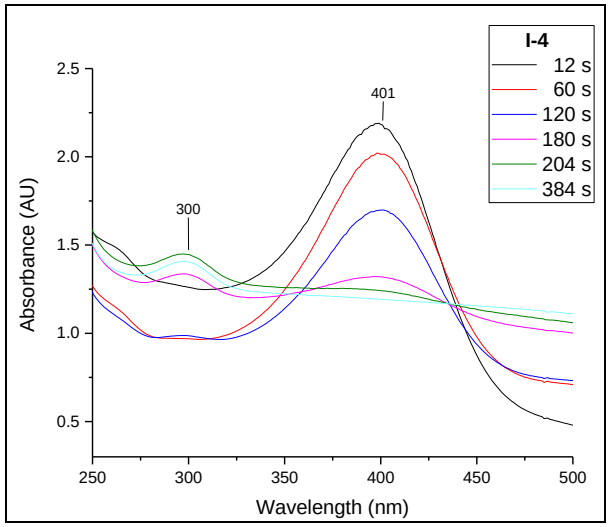
Figure 8. Zero generation dendrimers tested for catalysis

Obtaining catalysts and spectrophotometric determinations were realized according to the procedure descibed in case of trialcanolamine testing. The UV-vis spectra were recorded at 12 s intervals. The figures illustrate the catalytic activity of the compounds **I 1-Cu – I 5-Cu**.









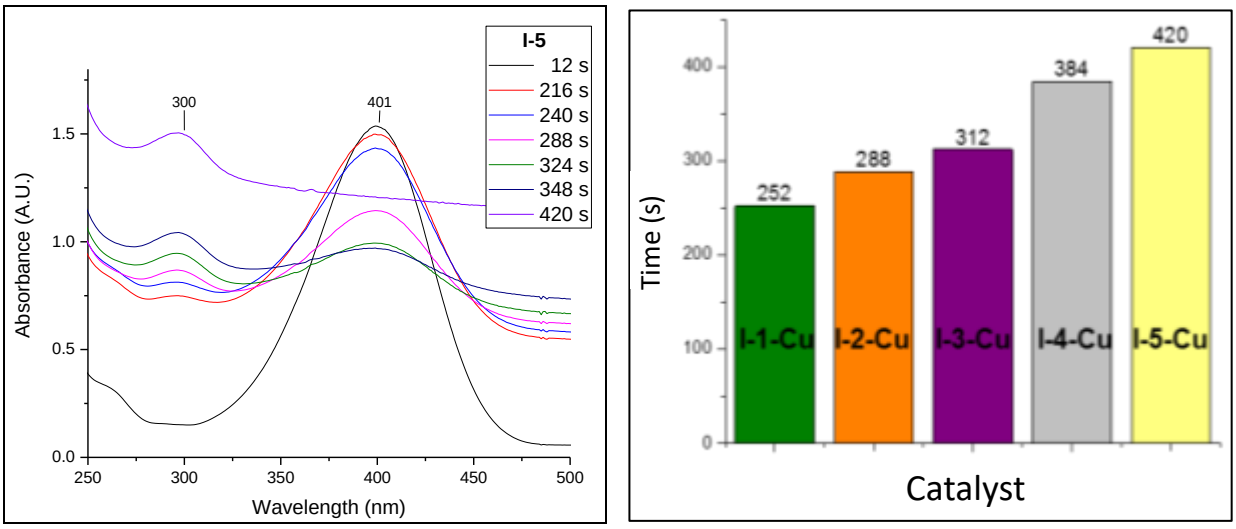


Figure 9. Catalytic activity of **I-1-Cu, I-2-Cu, I-3-Cu, I-4-Cu, I-5-Cu** catalysts and comparison

The figure 10 listed below present the spectrophotometric determination results in case of the catalysts obtained from compound **2a-d.**

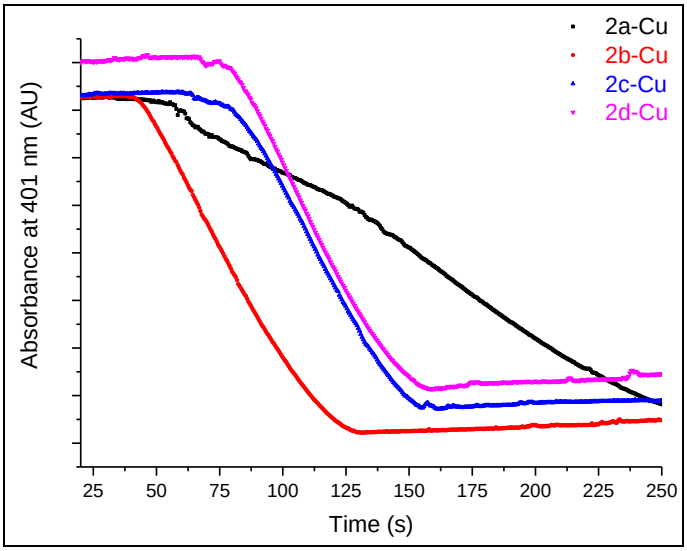


Figure 10. Catalytic activity of **2a-Cu, 2b-Cu, 2c-Cu, 2d-Cu** catalysts

4. Synthesis, characterization and biologic activity testing of carbon nanotubes derivatized with dendrimers

In this phase of the research the functionalization of single walled- (SW), double walled- (DW) and multi walled (MW) carbon nanotubes with dendrimers was accomplished. The used structures (dendrimers and linkers) for carbon nanotube derivatization were the following:

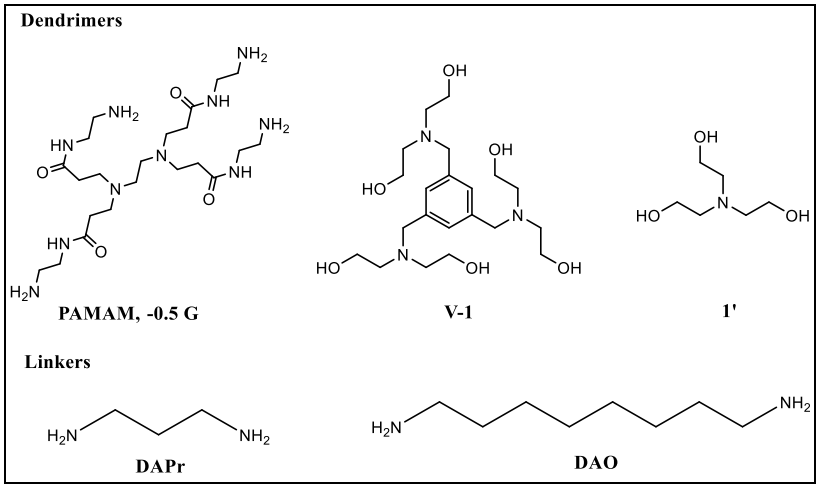
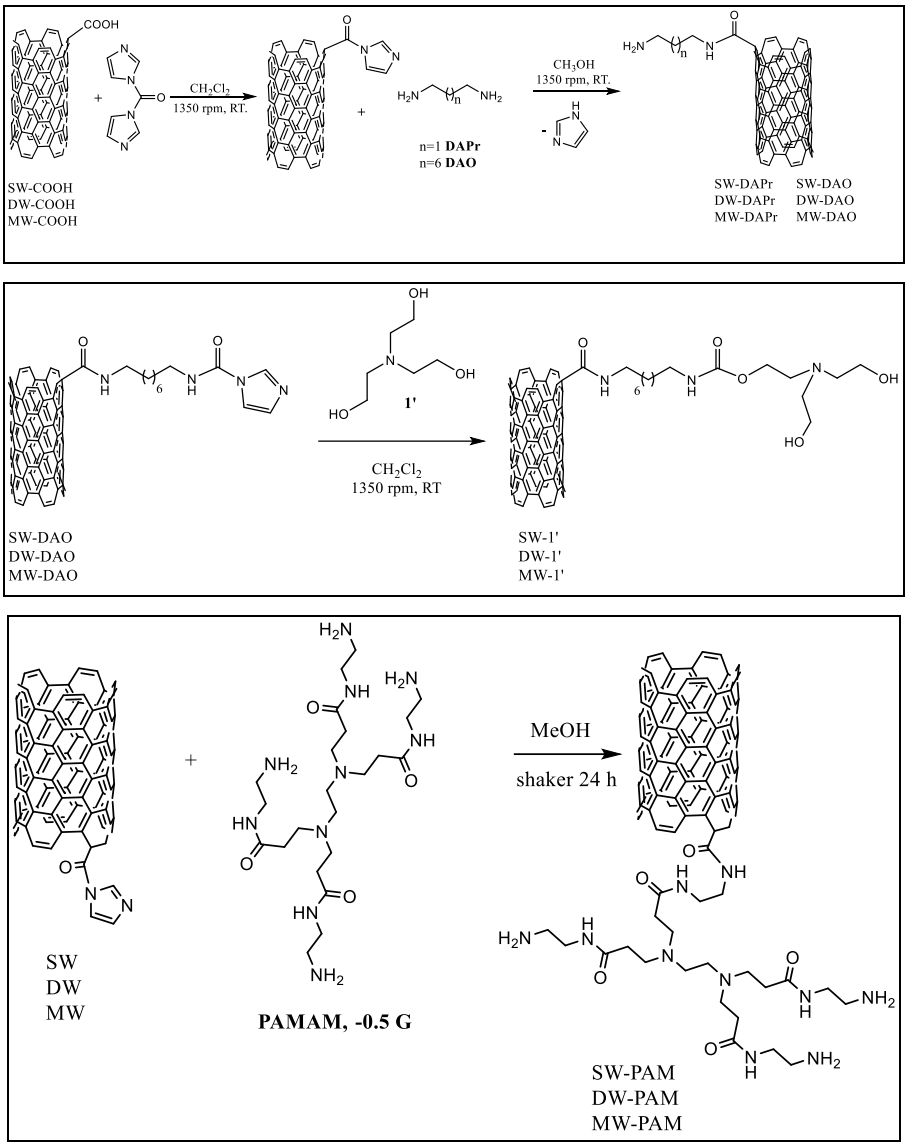


Figure 11. Dendrimers and linkers used for carbon nanotubes functionalization

Carbon nanotubes functionalized with carboxyl groups were covalently derivatized with 2 linkers(1,3-diaminopropane and 1,8-diaminooctane) using carbonyldiimidazole (CDI) as activating agent for the –COOH groups.

Carbon nanotubes were functionalzied according to the following scheme:



Scheme 5. Methods to obtaining functionalized carbon nanotubes.

Experimental

In a 50 ml Falcon tube de carboxyl-derivatized carbon nanotubes (SW-COOH, DW-COOH, MW-COOH, 200 mg) were suspended in and sonicated for 30 minutes. The activating agent (500 mg) dissolved in dry dichloromethane (5 ml) was added to the suspension. The resulted mixture was sonicated for 20 minutes and left overnight on shaker at 1350 rpm rotation speed. The activated carbon nanotube species were separated from the suspension by vacuum filtration over a PTFE membrane (porosity 0.22 um), washed several times with dry dichloromethane untill the complet removal of the exces CDI and formed imidazole. The procedure was monitored by thin

layer chromatography using dichloromethane:methanol 9:1 eluent mixture, R_f=0.54.

After filtration the activated carbon nanotubes were suspended in methanol (10 ml) using a 14 ml Falcon tube and sonicated for 20 minutes. To the resulted suspension linkers (DAPr, DAO, 200 mg) and dendrimer (PAMAM) dissolved in methanol (3 ml) were added and left overnight on shaker at 1350 rpm at room temperature.

The nanotubes functionalized with linker type structures were separated from the suspension by vaccum filtration over PTFE membrane filter (porosity 0.22 um), washed with methanol until the complete removal of the unreacted linker and dried.

The resulted nanostructures were characterized using different techniques: elemental analysis, STEM-EDX analysis, IR spectroscopy. Also the biological toxicity of several synthetized nanostructures was tested. In case of carbon nanotubes functionalized with PAMAM dendrimer also the catalytic activity was tested when the functionalized species were used as support for the Cu^0 nanoparticles coordinated by the tertiary N atoms of the dendrimer

The elemental analysis results are summarized in the following table:

Table 1. Elemental analyses results.

Sample	N%	C%	H%	Sample	N%	C%	H%	Sample	N%	C%	H%
SW-COOH	*0,23*	91,72	1,20	*DW-COOH*	*0,18*	89,29	0,96	*MW-COOH*	*0,19*	86,49	1,25
SW-DAPr	1,07	90,31	1,21	DW-DAPr	1,24	91,73	1,33	MW-DAPr	1,11	90,45	1,38
SW-DAO	1,04	92,94	1,54	DW-DAO	0,99	94,01	1,49	MW-DAO	1,12	90,50	1,57
SW-PAM	3,13	89,59	2,07	DW-PAM	2,75	92,26	1,88	MW-PAM	1,75	89,26	1,78
SW-1’	1,45	93,24	1,62	DW-1’	1,23	89,39	1,98	MW-V-1	1,36	89,50	1,89

The STEM-EDX analysis findings were in good agreement with the elemental analysis results. In the figures 12 are illustrated the analysis data of the STEM-EDX determinations.

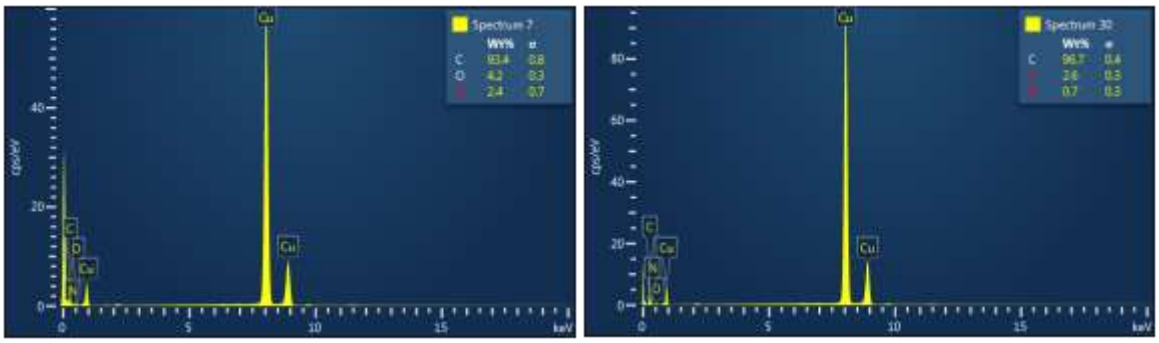


Figure 12. STEM-EDX analysis of the DW-V-1, DW-1’ carbon nanostructures

5. Catalytic activity testing

In this step the catalytic activity testing of the Cu^0-PAMAM dendrimer functionalized nanotubes as catalysts in heterogenous catalysis was accomplished. Three catalysts were obtained and used in solid phase in catalysis. Also their activity was compared with the activity of the Cu^0 nanoparticles not coordinated to the nanotube-dendrimer support.

The spectrophotometric determination results are presented in the following figures.

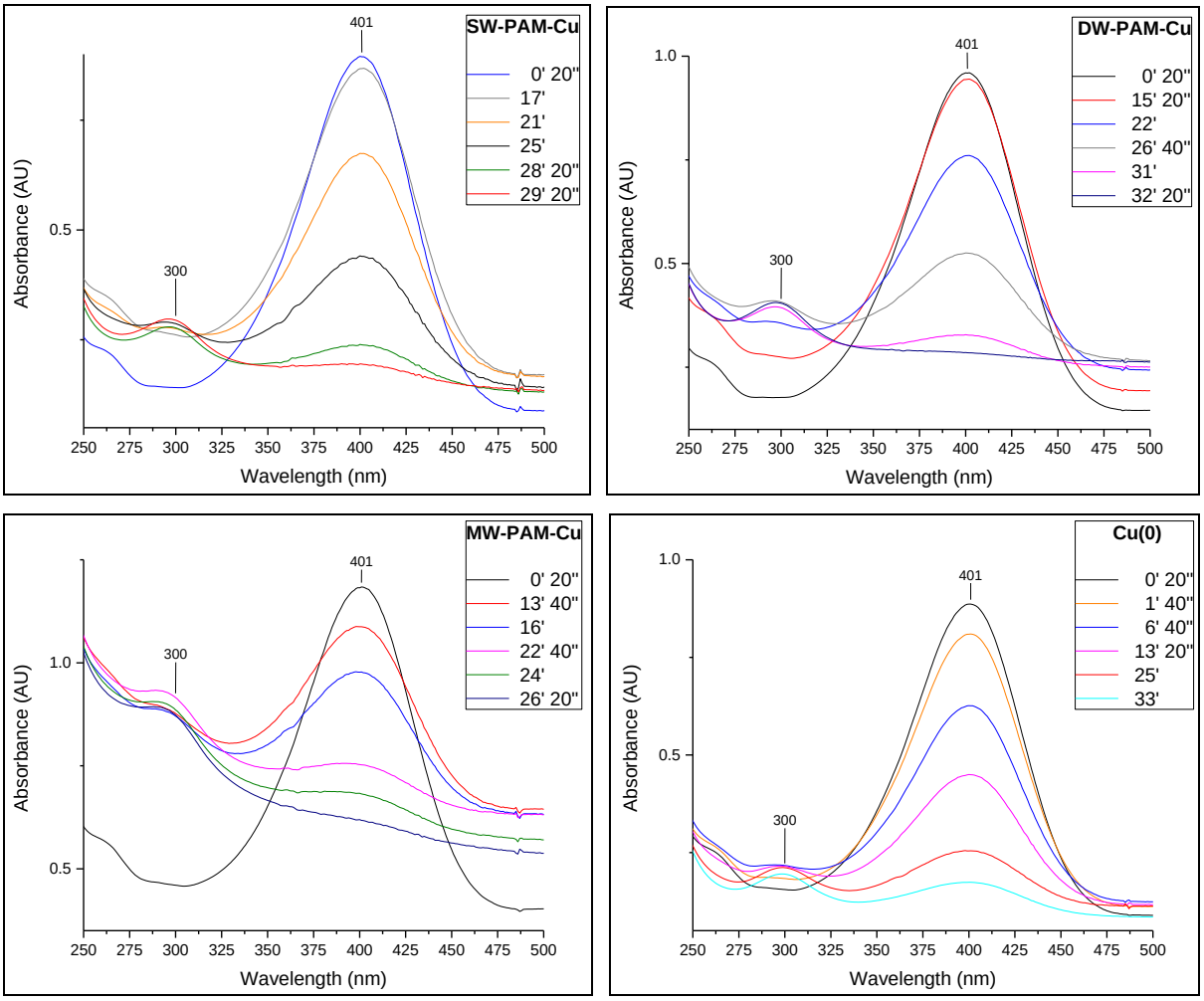


Figure 13. UV-VIS spectra of the catalytic activities.

6. Biological activity testing

In this phase the biological activity of the obtained functionalized carbon nanostructures was accomplished. The carbon nanotubes functionalized with dendrimer and linkers were tested. For this purpose a 0.05 % Triton X-100 surfactant aqueous solution was prepared. 10 mg of

functionalized nanostructure was suspended in 10 ml surfactant solution and sonicated for 30 minutes. The prepared samples were stored in the fridge until testing and resonicated for 10 minutes before added to the cell media.

The test were conducted on the HaCaT cell line (human keratinocyte), which were cultivated in the media consisting of 88 % DMEM (Dulbecco's Modified Eagle Medium), 10 % fetal calf serum (FCS), 1 % penicilline/streptomycina and 1 % L-glutamine.

7. Testing of penetration capability of dendrimer-carbon nanostructure complexes

In this phase the cellular wall penetration capacity of the double walled carbon nanotubes functionalized with compound **1'** was tested.

For this purpose the HaCaT cells were grown on round glass blades and treated with DW-1'. The blades were fixed in a solution of 2.5 % glutaraldehyde in 100 mM phosphate buffer for 1 hour and washed repeatedly with 0.15 mM phosphate buffer as it follows: first washing cycle of 30 minutes and the next three of 60 minutes each. For post fixation the blades were left for 45 minutes at 4 ºC in a 2 % osmium tetraoxide in 100 mM phosphate buffer solution. After this treatment, the blades were washed with 0.15 mM phosphate buffer in two cycles of 15 minutes. Dehydration was performed stepwise with ethanol in different concentrations: 30 % ethanol for 15 minutes, 50 % ethanol for 15 minutes and 70 % ethanol for 30 minutes at de 4 ºC, followed by dehydration process performed at room temperature under the following parameters: 80 % ethanol for 30 minutes, 90 % ethanol for 15 minutes and 100 % ethanol for 15 minute in three cycles.

The encapsulation in epon 812 type resin was made stepwise involving infiltration in 1:2, 1:1, 2:1 epon 812-ethanol mixture and in pure epon 812 for 1 hour. The recipient caps were left open overnight. The encapsulation mixture was prepared right before used under the niche consisting of 5 ml solution A (mixture of 66 ml epon 812 in 100 ml dodecenyl succinic anhydride), 5 ml solution B (mixture of 100 ml epon 812 in 84 ml methane-isobenzofuran-1,3-one) and 150 µl polymerization accelerator. The samples were placed in plastic capsules filled with the encapsulation mixture and were kept in the oven at 50 º C for 2-3 days to polymerize. The blades were removed by thermal shock under liquid nitrogen.

The modeling of the blocks was performed under a binocular, using a special block modeling blade, cutting trapezoid shapes with equal lateral edges located at 45 º from the large base. Ultramicrotom Leika UC6 was used for the ultrafine cutting. The sections obtained were 70-

90 nm thick, deposited on copper grides covered in formvar and carbon and were visualized using a transmission electron microscope (TEM) JEOL JEM-1010 with tungsten filament and equipped with Camera MegaViewIII CCD (Soft Imaging System GmbH). The images were recorded at the Electronic Microscopy Center of Babes-Bolyai University in Cluj-Napoca.

The following figures illustrate the captured pictograms of the sections. The controll represents the cells without carbon nanotubes encapsulated into the resin while the sample contains the treated cells with carbon nanotubes encapsulated into the epon 812 resin.

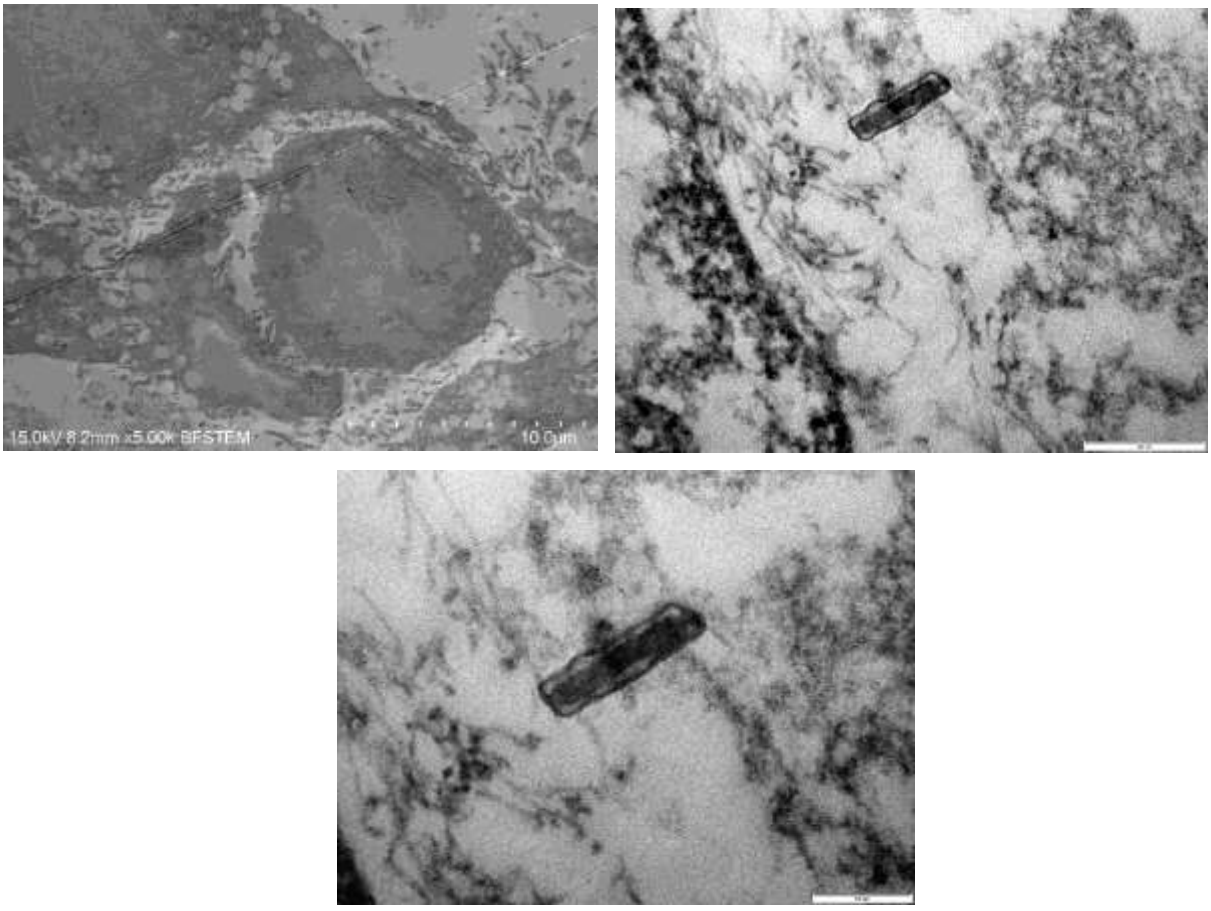


Figure 14. TEM images: controll (left) and sample (right)

From figure 15 can be observ a small interaction between cells trated with dendrimeric compounds (figura 15).

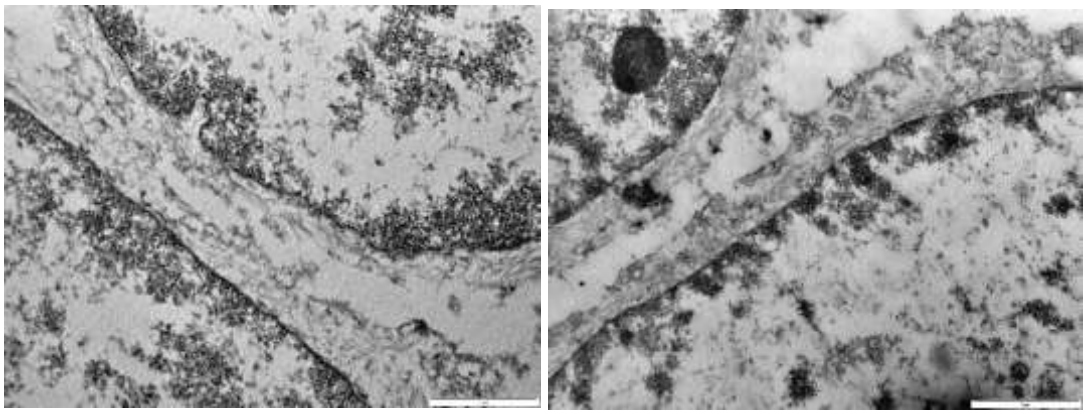


Figura 15. Interactiuni la nivel celular.

Also, it can be seen agglomerations of compound inside the cell, which confirms the permeability of chemicals and their usability in the transport of biologically active compounds.

Consequently, TEM microscopy allows viewing and tracking dendritic compounds, proving the ability of the cell wall penetration for functionalized carbon nanostructures.

Testing the cellular toxicity and analyses of dendrimer-carbon nanostructure complexes

Testing the cellular toxicity and analyses of dendrimer-carbon nanostructure complexes

Cell viability determination was performed using the MTT colorimetric assay. It is a quantitative test, based on the reduction of the yellow water soluble salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, which are violet crystals, insoluble in water. The cells were incubated for 24 hours in plates with 96 wells and functionalized carbon nanostructures were added in different concentrations. After a new incubation cycle, the MTT was added (10 ul/well, 5 mg/ml solution) and the samples were incubated for 90 minutes at 37 ºC (humidity conditions ~90 %, 5 % CO_2 content). The resulted formazan crystals were solubilized by adding a solvent (40 mM HCl, 0.1 % Triton X-100 in isopropanol).

Wells containing only cell medium culture were used as reference and as poositive controll (CA) were considered those wells which contained cell media culture and HaCaT cell line without nanomaterial added.

Cell vialbility was evaluated by absorbance measuring at 550 nm wavelength value. The reference wavelength used was 630 nm. Each sample absorbance was recorded 5 times and the

average absorbance value was taken into consideration when plotting.

Since the formed quantity of formazan is liniar dependent of the viabile cell number, the viability of the cells can be expressed as:

$$\text{Viability \%} = (A_P * 100)/ A_{CA}$$

, where A_P is the sample absorbance

A_{CA} is the positive control absorbance

In the following figures the cytotoxicity test results are illustrated.

The results are showing, that the lowest toxicity presented the functionalized double walled carbon nanotubes. Those functionalized with compound **1'** and with PAMAM dendrimer do not affect the studied cell line in low concentrations. The more toxic nanostructures turned out to be the functionalized single walled carbon nanotubes, which present high toxicity over 60 ug/ml concentration. The derivatives of the multi walled carbon nanotubes presented the lowest toxicity even in high concentrations.

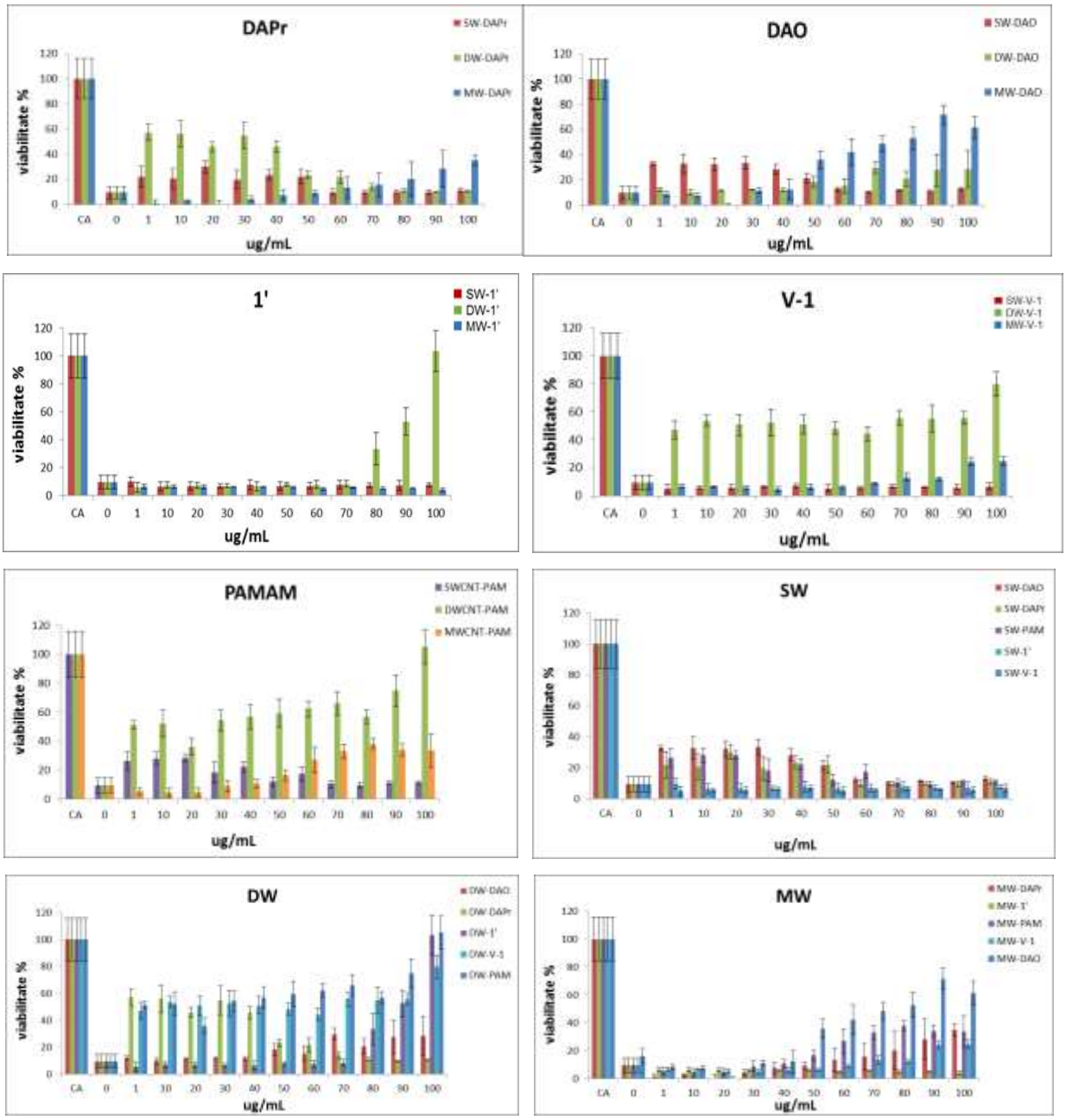


Figure 16. Cytotoxicity tests results.

The results of this phase have materialized in these ISI and international conferences:

1. A.M. Iranmanesh, M. Saheli, M. V. Diudea, Counting polynomials in the crystal network flu (CMedDu), *Studia Universitatis Babes-Bolyai Chemia*, *Babes-Bolyai Chemia*, 61(1), 115-126, **2016**
2. L.C. Nagy, M.V. Diudea, Ring signature index, *MATCH Commun. Math. Comput. Chem.*, 77(2), **2016**.
3. M. Saheli, A. Loghman, M.V. Diudea, CLUJ and related polynomials in bipartite hypercube hypertubes, *Studia Universitatis Babes-Bolyai Chemia*, *Babes-Bolyai Chemia*, 61(3), 485-493, **2016**
4. Sipos TA, Füstös ME, Katona G, Synthesis and characterization of derivatized carbon nanostructures *XXII. International Conference on Chemistry* **2016**. November 3-6, Cluj-Napoca, Romania. WEB: www.emte.ro.