

Research Article

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Synthesis of functionalized carbon nanotubes for fluorescent biosensors

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Abstract: Despite the development of pharmacy, there are still incurable diseases for which the medicine has not been found yet. Because many diseases are asymptomatic in their first stage of development, often early detection is the crucial factor in combating them. The article describes the process of synthesis of carbon nanotubes (CNTs) which can be useful in medical diagnostics. CNTs were synthesized by chemical vapor deposition. The obtained material was subjected to functionalization – attaching fluorescent markers. In order to check the usefulness of the obtained structures in diagnostics, their fluorescent properties were examined. The results of fourier transform infrared spectroscopy thermogravimetric analysis and scanning electron microscopy prove that, after proper functionalization, CNTs could be used as fluorescents markers.

Keywords: functionalization, biosensors, fluorescence, carbon nanotubes, chemical vapor deposition

Abbreviations

CNTs carbon nanotubes
CVD chemical vapor deposition

CCVD catalytic-chemical vapor deposition
TGA thermogravimetric analysis
FTIR Fourier transform infrared

1 Introduction

Nanotechnology is a discipline dealing with nano-scale objects, i.e., at the level of individual atoms and molecules [1]. It creates many opportunities in various specializations, from the military, through architecture, to medicine, which in recent years has attracted a lot of interest [2]. Along with the development of nanotechnology, many nanomaterials have been developed and applied. One of them are carbon nanotubes (CNTs). Due to their unique properties, they attract an increasing attention [2,3]. The strength and size of CNTs make them ideal candidates to replace many less durable components [3]. Today, CNTs find a wide range of applications in electronics, mechatronics, and optics. They are also extremely strong and lightweight construction material, so they can be used for the production of tennis rackets or bicycle frames [1,2].

CNTs also find a number of medical applications [4,5]. After proper functionalization (surface modification), it is possible to give them new biocompatibility properties. CNTs have been shown to be useful as new drug delivery systems [6]. CNTs, functionalized with macromolecules (antibodies, proteins, DNA) as well as low-molecular drugs, can be carriers transporting the molecule directly to the pathologically changed tissues [7,8]. Initially, the possibility of using such carriers in cancer therapy and treatment of viral infections was studied. Currently, the possibility of using CNTs as carriers in immunotherapy or gene therapy has been also proven [1,3,4].

Continuous progress in research to improve medical imaging technology has resulted in the development of a new field called fluorescent imaging. Nanoparticles can be successfully used in the diagnosis of various diseases

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(including cancer) as innovative contrast factors. It is believed that carbon nanotube imaging may be more effective than standard contrast factors [2,5]. This is due to the small size of nanotubes, their ability to accumulate in the pathologically changed area and optical properties as well as the ease of modification of CNTs' surface. The use of ligand-coated CNTs enables the detection of pathological changes at the cellular level, which makes it possible to detect cancerous changes earlier and increases the probability of a complete recovery of patients because of early disease detection [6].

The research objective was to investigate the possibility of attachment of structurally diverse fluorophores on the surface CNTs. It is expected that structural diversity (total charge, different fluorescence characteristics) could be used in medical diagnostics, after proper functionalization. This may open the way for further investigation that could successfully contribute to the development of novel diagnostic technique. It is believed that this method would be noninvasive and allow to detect the disease at an early stage of development (at the cellular level).

The nanotubes were functionalized with three fluorescent markers: rhodamine B, 5(6)-carboxyfluorescein, and 4-(1-pyrenyl)-butyric acid. The choice of these fluorescent markers was based on the fact that they can provide different models for the study of modified CNTs' interactions with the cell membrane and, moreover, their different structures (inert, acidic, and base fluorophore) should also determine the different degree of affinity to the component of cell membrane. This article only describes the first phase of research on the possibility of using modified CNTs as fluorescent markers. The aim was to investigate the possibility of attaching the markers to the surface of the nanotubes with the use of reagents/methods determining the highest biocompatibility of modified nanomaterials.

The article includes: methodology that describes precisely the whole process of CNTs preparation; results discussion that consists of examination and characterization of obtained material, and conclusion that comments on conducted research.

2 Methodology

The process of fluorescent CNTs' preparation consisted of several steps. First, CNTs were synthesized using chemical vapor deposition method. Then, the obtained material had to be purified. Next step was functionalization,

which included three stages: oxidation, incorporation of amines, and fluorescent dyes attachment. All the steps are shown on the process flow diagram on Figure 1.

2.1 CNTs preparations

CNTs were synthesized by catalytic-chemical vapor deposition (CCVD) [4,9,10]. The apparatus used in the synthesis process was a three-zone horizontal furnace, allowing temperature control of each zone. The scheme of the system is presented in Figure 2. I zone (300°C) is the catalyst solution evaporation zone and II zone (300°C) and III zone (825°C) are deposition zones. The process carrier gas was argon.

Inside the furnace a quartz tube was placed, inside which (in the deposition zone) a silicon substrate covered with nanometer SiO₂ layer was placed. The implementation of the synthesis process required ensuring the initial conditions by creating an neutral atmosphere. For this purpose, the flow of two gases, argon and helium, was switched on. The gas flow rate was 1 SLPM (Standard Liter Per Minute). After 10 min, the furnace was turned on. When the temperature was reached, the helium flow was switched off and argon flow reduced to 0.5 SLPM and hydrogen flow was switched on (0.2 SLPM). System was equipped with a precise substrate dosing system – Medima S2 pump. As a substrate of the reaction, ferrocene–xylene solution (2 g/L) was used. Ferrocene (Fe(C₅H₅)₂) was a source of carbon and iron, which was a catalyst for reaction, and xylene (C₈H₁₀) was a source of carbon. The mixture was administered from the outside at the inlet velocity of 9.5 mL/h [10].

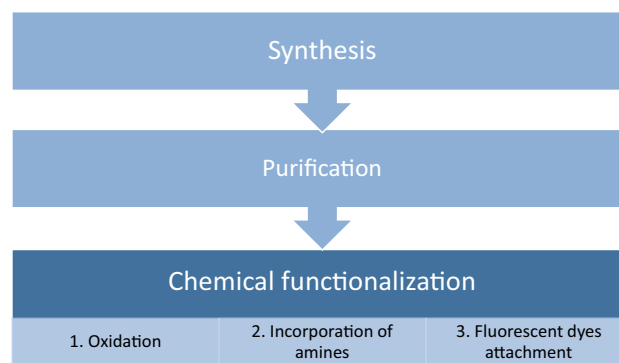


Figure 1: Process flow diagram.

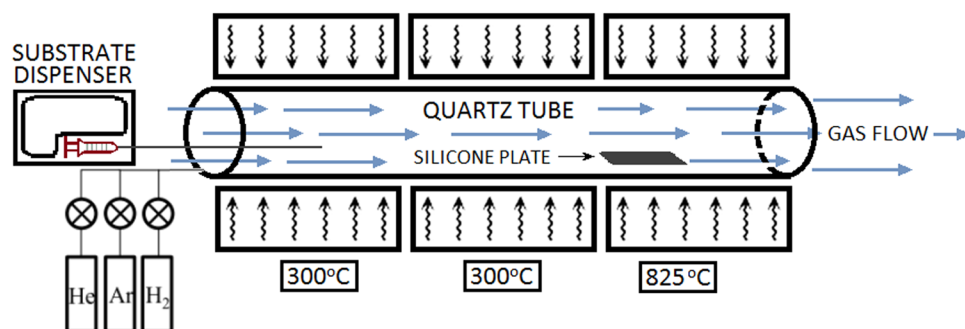


Figure 2: Scheme of CVD CNTs synthesis.

2.2 Purification

CNTs must be properly purified prior to the process of functionalization. In the first stage of purification, CNTs were annealed at 450°C for 2 h in order to remove amorphous carbon. The choice of temperature resulted from the fact that, in 450°C, the amorphous carbon should be removed without losses for the CNTs [11].

In the next stage of purification, the CNTs were treated with 5 M HCl to remove the residues of metal catalyst. The chemical purification procedure was carried out 4 times, controlling the presence of iron chloride in the solution using $K_4[Fe(CN)_6]$ (potassium hexacyanoferrate(II)). In the presence of iron ions, potassium hexacyanoferrate (in an inert or slightly acidic medium) forms a dark blue, amorphous sediment, called Prussian blue.

After removal of the remaining metallic catalyst. The material then had to be washed several times with Milli-Q water to achieve pH 7. The nanotubes were then suspended in water and lyophilized.

2.3 Chemical functionalization

All commercially available reagents were from Sigma-Aldrich. Solvents and inorganic acids were supplied by Avantor Performance Materials Poland SA. Triazine coupling reagents were obtained in accordance with procedures developed at the Institute of Organic Chemistry of the Lodz University of Technology [12,13].

FT-IR analysis: Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) was performed using a Thermo Scientific Nicolet iS50 Fourier transform infrared (FTIR). DRIFT spectra were collected in the range of 400–4,000 cm^{-1} . The powders were mixed with KBr, ground to a fine powder, and then placed in the spectroscopy measuring chamber.

2.3.1 Oxidation

The purified CNTs were oxidized using a mixture of HNO_3 and H_2SO_4 (1:3, v/v) [11]. The CNTs (50 mg) were suspended in a mixture (200 mL) 1:3 (v:v) of HNO_3 (65%) and H_2SO_4 (95%) and pre-sonicated for 1 h. Then, the suspension was intensively stirred and heated at 65°C for 5 h. In the next stage, the mixture was diluted with Milli-Q water (300 mL). The oxidized CNTs were filtered and washed with water until pH 7. Finally, they were suspended in water (20 mL) and lyophilized. Finally, 42.3 mg of CNTs was obtained

FTIR: $\nu = 3,560, 3,504, 3,437, 2,965, 2,920, 2,863, 1,720, 1,712, 1,698, 1,629, 1,227, 1,194, 1,057, 1,038 \text{ (cm}^{-1}\text{)}$.

2.3.2 Incorporation of amines onto the surface of pre-functionalized carbon nanotubes

The oxidized CNTs (50 mg) were suspended in DMF (5 mL). The mixture was sonicated for 15 min [11] before the addition of 4-(4,6-dimethoxy-[1,3,5]-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMT/MMB/ BF_4^- ; 1.64 g, 5 mmol) and *N*-methylmorpholine (NMM; 0.55 mL, 5 mmol) in DMF (5 mL) [14]. The mixture was sonicated for 1 h at 10°C. Then 1,4-diaminobutane (0.881 g, 10 mmol) was added to the suspension. The mixture was stirred vigorously for 12 h at room temperature. The functionalized CNTs were then filtered and washed three times with DMF (5 mL), with H_2O –DMF (1:1; 5 mL), with H_2O (5 mL), and with DMF (5 mL) to remove by-products of the reaction and excess reagents. The modified CNTs were dried through lyophilization. The process of incorporation of amide derivative on the surface of CNTs is shown in Figure 3.

FTIR: $\nu = 3,650, 3,570, 3,438, 2,963, 2,931, 2,867, 2,248, 2,211, 1,628, 1,595, 1,584, 1,483, 1,465, 1,435, 1,363, 1,217, 1,026, 905 \text{ (cm}^{-1}\text{)}$.

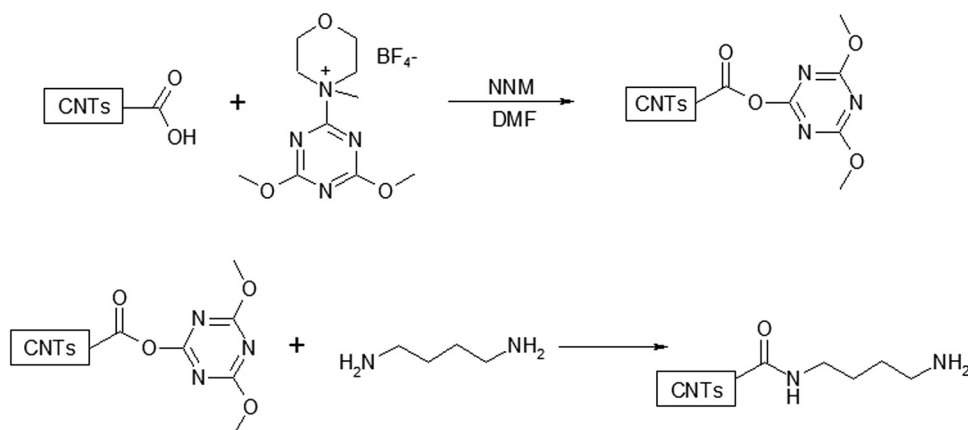


Figure 3: Synthesis of CNTs modified with 1,4-diaminobutane via amide bond.

2.3.3 Fluorescent dyes attachment

In all cases, 50 mg functionalized CNTs containing free amino groups on their surface were used to attach the fluorophores. Before reaction, modified CNTs were dispersed in DMF/NMM mixture (1:1) and then cooled to 288 K. Parallel fluorophore activation was carried out to obtain the corresponding triazine esters of rhodamine B, 5(6)-carboxyfluorescein, 4-(1-pyrenyl)butyric acid [14].

Activation of rhodamine B (general procedure): In intensely stirred and cooled in the water-ice bath solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium toluene-4-sulfonate (DMT/NMM/TosO[−]) (0.826 g, 2 mmol) in DMF (5 mL), rhodamine B (0.958 g, 2 mmol) and NMM (0.11 mL, 1 mmol) were added. The reaction was carried out for 1 h.

Activation of 5(6)-carboxyfluorescein: DMT/NMM/TosO[−] (0.826 g, 2 mmol), 5(6)-carboxyfluorescein (0.753 g, 2 mmol), and NMM (0.22 mL, 2 mmol).

Activation of 4-(1-pyrenyl)butyric acid: DMT/NMM/TosO[−] (0.826 g, 2 mmol), 4-(1-pyrenyl)butyric acid (0.577 g, 2 mmol), and NMM (0.11 mL, 1 mmol).

Pretreated modified CNTs (50 mg) were added to the solutions of the triazine esters of fluorescent markers. Stirring was continued for 12 h at room temperature. The functionalized CNTs were then filtered and washed three times with DMF (5 mL), with H₂O–DMF (1:1; 5 mL), with H₂O (5 mL), and with DMF (5 mL) to remove by-products of the reaction and excess reagents. The modified CNTs were dried through lyophilization. The process of incorporation of fluorescent dyes on the surface of CNTs is shown in Figure 4. Fluorescent markers are shown in Figure 5.

FTIR of CNTs modified with 4-(1-pyrenyl)butyric acid: $\nu = 3,680, 3,443, 2,927, 2,859, 1,714, 1,630, 1,590, 1,564, 1,465, 1,431, 1,362, 1,209, 1,023$ (cm^{−1}).

FTIR of CNTs modified with 5(6)-carboxyfluorescein: $\nu = 3,433, 2,930, 2,858, 1,660, 1,630, 1,570, 1,465, 1,436, 1,363, 1,218, 1,190, 1,046, 1,017, 996$ (cm^{−1}).

FTIR of CNTs modified with rhodamine B: $\nu = 3,585, 3,448, 2,933, 2,866, 2,247, 1,747, 1,624, 1,557, 1,465, 1,440, 1,364, 1,230, 1,170, 1,028$ (cm^{−1}).

3 Results and discussion

After the thermal treatment process, 30% weight loss was observed, which indicates the formation of a significant amount of amorphous carbon deposits. Contaminants that were the remains of the catalyst after the CVD process represented 23% of the weight of CNTs. Diameter of obtained CNTs is near to 40 nm. Maximum length is around 200 nm. Figure 6 shows SEM images of obtained CNTs.

Chemical functionalization of CNTs: In order to introduce appropriate ligands to the surface of the carbon nanomaterial, it was necessary to carry out preliminary modifications (pre-functionalization). Therefore, the purified CNTs were subjected to an oxidation reaction in order to introduce carboxylic groups on their surface, the presence of which was necessary for further functionalization.

In the process of further functionalization, carboxyl group was transformed into amide derivatives with 1,4-diaminobutane. The choice of diamine resulted from the fact that the presence of reactive primary amine group allows further modification of CNTs' surface, and at the same time, the amide bond formed on the surface of CNTs is characterized by significant stability that seems to be necessary due to further application of modified CNTs.

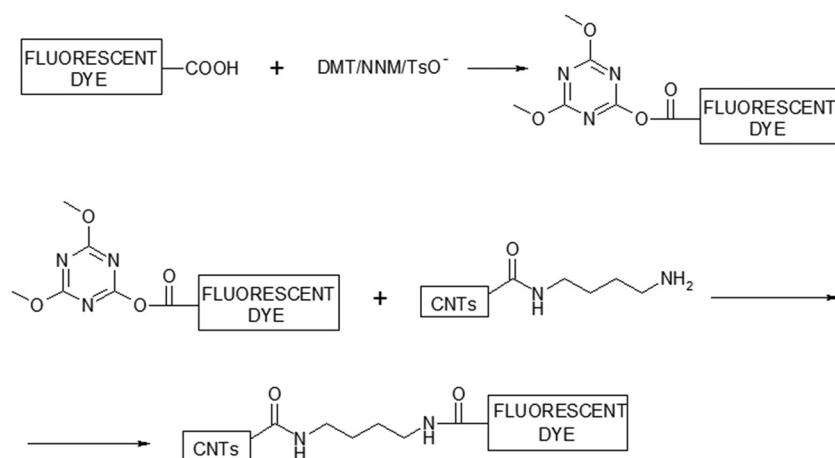


Figure 4: Synthesis of MWCNTs with fluorescent dye.

Modified CNTs were used as substrates in the reaction with various fluorescent markers: rhodamine B, 5(6)-carboxyfluorescein, and 4-(1-pyrenyl)butyric acid.

All applied fluorescence markers contain in their structure a carboxylic group, which enables the formation of a permanent amide bond with the amine group derived from 1,4-diaminobutane attached to CNT.

The structures of materials prepared at all stages of chemical modification have been confirmed based on FTIR tests (Figure 7).

Analyzing the obtained FT-IR spectra, characteristic peaks corresponding to the functional groups introduced in the different stages of chemical modification were observed.

CNTs after the oxidation reaction: The presence of the band in the range $3,000\text{--}3,500\text{ cm}^{-1}$ corresponds to stretching vibrations of OH groups and in addition the present of signals in range $1,695\text{--}1,725\text{ cm}^{-1}$ corresponds to stretching vibrations $\text{C}=\text{O}$ in the carboxylic acids.

On the FT-IR spectrum CNTs functionalization of 1,4-diaminobutane are present peaks of C-H deformation vibrations of alkyl groups in the range $1,350\text{--}1,370\text{ cm}^{-1}$ and signals of bending vibration of the alkyl C-H groups of 1,4-diaminobutane in the range $1,430\text{--}1,480\text{ cm}^{-1}$. On the presence of alkane groups CH_2 provides band

$2,850\text{--}2,970\text{ cm}^{-1}$ corresponds stretching vibration band. The peak $1,628\text{ cm}^{-1}$ corresponds to the $\text{C}=\text{O}$ group in the amide group. The presence of band $1,200\text{--}1,400\text{ cm}^{-1}$ corresponds to the stretching vibration of C-N.

Analyzing the spectra of CNTs after attachment of fluorescent dyes, one can observe an increase in intensity of absorbance values for stretching vibrations of carbonyl groups $\text{C}=\text{O}$ in the range $1,600\text{--}1,700\text{ cm}^{-1}$ and increase of absorbance in the range $1,430\text{--}1,480\text{ cm}^{-1}$ characteristic for alkane groups. A decreased intensity of absorbance was observed for range $3,000\text{--}3,500\text{ cm}^{-1}$, which may indicate a decrease in the number of NH_2 groups.

In the next stage, the thermal stability tests of the obtained materials were carried out (Figure 8).

Figures 8–12 show thermograms TGA for CNTs after individual stages of chemical modification. Thermograms 10–12 refer to the CNTs with attached fluorescent dyes. CNTs functionalized as fluorescent markers contain carboxylic groups, what is proven by weight loss in the temperature range $450\text{--}500$. Weight loss in the temperature range $300\text{--}400$ indicates the presence of amine groups. In addition, thermograms show that the maximum decomposition rate of CNTs (which corresponds to the oxidation of the nanotubes) before modifications is higher

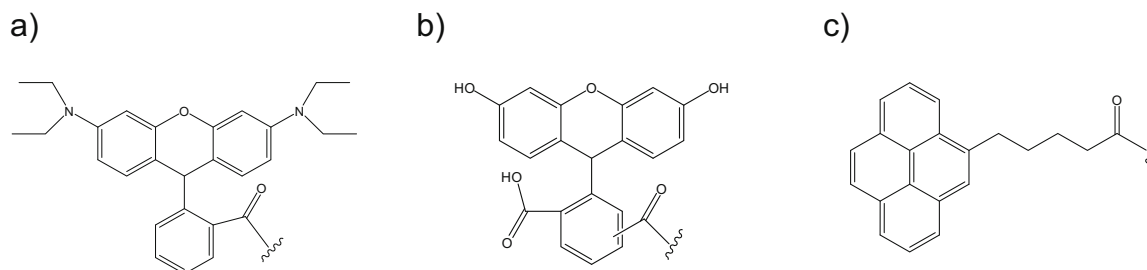


Figure 5: Fluorescent markers: (a) rhodamine B, (b) 5(6)-carboxyfluorescein, (c) 4-(1-pyrenyl)butyric acid.

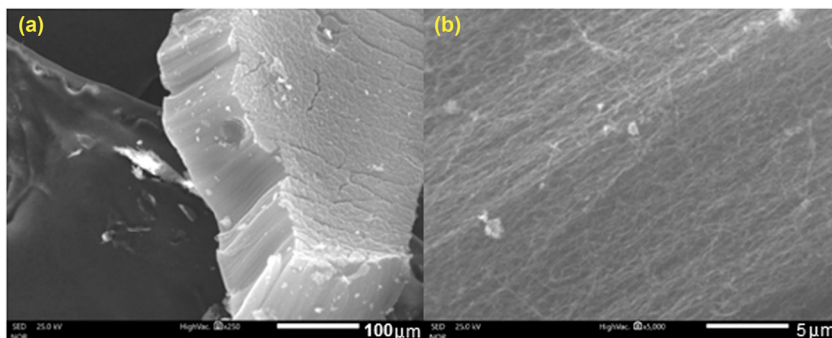


Figure 6: SEM images of unmodified CNTs: (a) magnification $\times 250$; (b) magnification $\times 5,000$.

than when fluorescent markers are attached (637°C – unmodified CNTs, 652°C – CNTs after oxidation, $580\text{--}600^{\circ}\text{C}$ – CNTs with fluorescent markers) (Figure 13).

According to EDS results, obtained markers consisted of carbon, oxygen, and nitrogen. There were also minor contaminants in the form of iron, which were catalyst residues from the CVD synthesis process (Table 1).

4 Conclusion

The article presents the results of the efficient modification of CNTs which may allow for their applications in medical diagnostics. As a result of chemical functionalization, three fluorescent acidic, base, and neutral dyes (5(6)-carboxyfluorescein, rhodamine B, and 4-(1-pyrinyl)-butanoic acid) were attached to the surface of CNTs. The

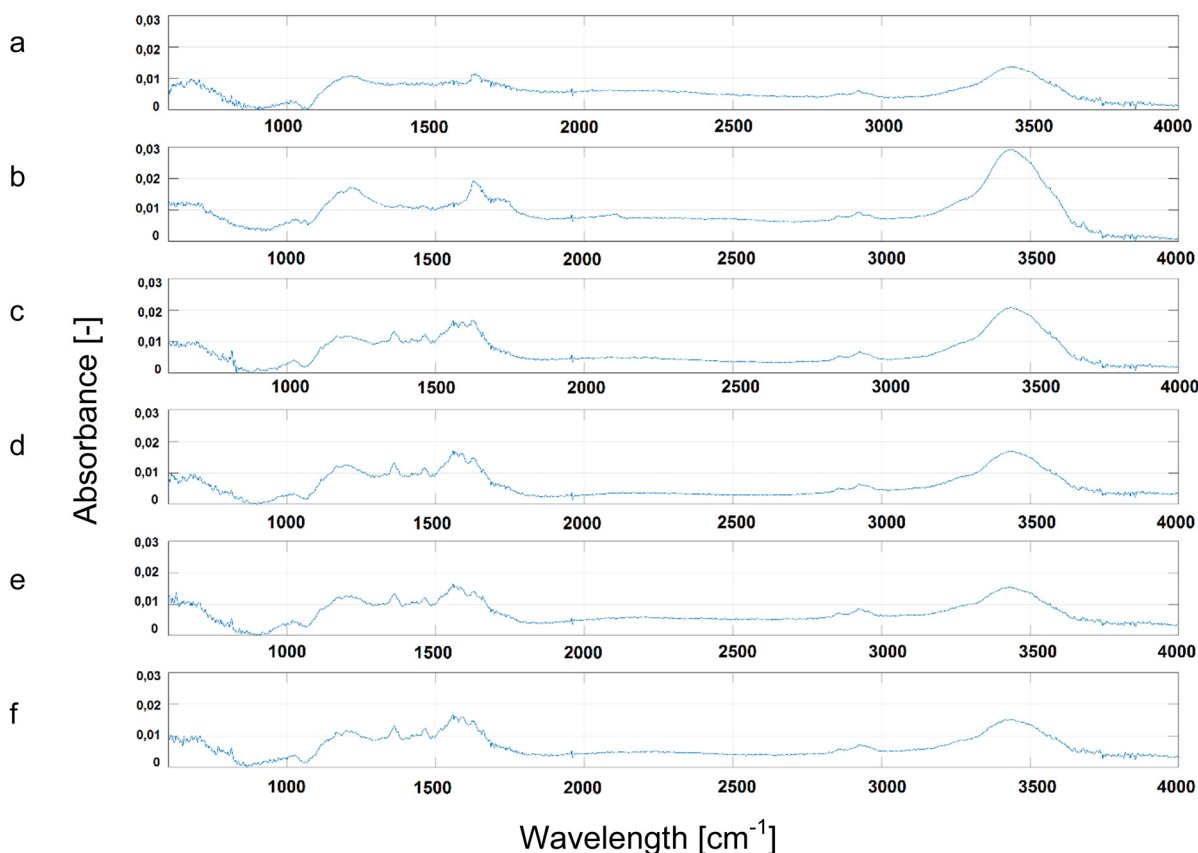


Figure 7: FTIR spectra for CNTs after each stage of chemical modification: (a) unmodified CNTs after purification processes; (b) modified carbon CNTs after oxidation reaction; (c) modified CNTs after amidation reaction with $\text{NH}_2\text{--}(\text{CH}_2)_4\text{--NH}_2$; (d) modified CNTs after acylation with 4-(1-pyrinyl)-butanoic acid; (e) modified CNTs after acylation with 5(6)-carboxyfluorescein; (f) modified CNTs after acylation with rhodamine B.

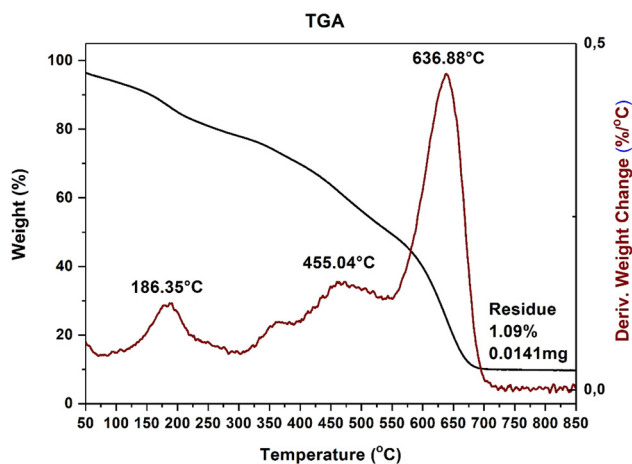


Figure 8: TGA thermogram for unmodified CNTs after purification processes.

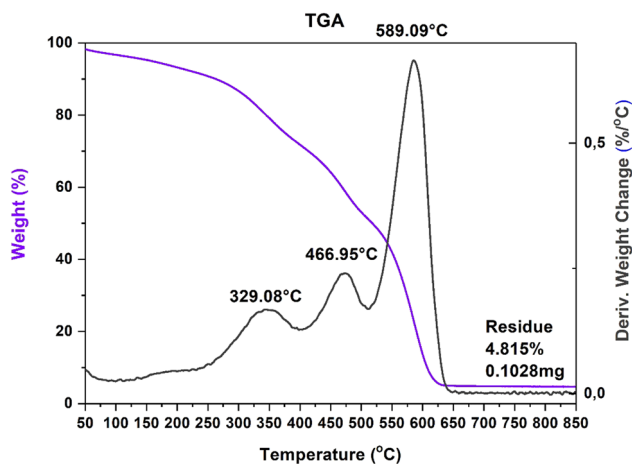


Figure 11: TGA thermogram for modified CNTs after acylation with rhodamine B.

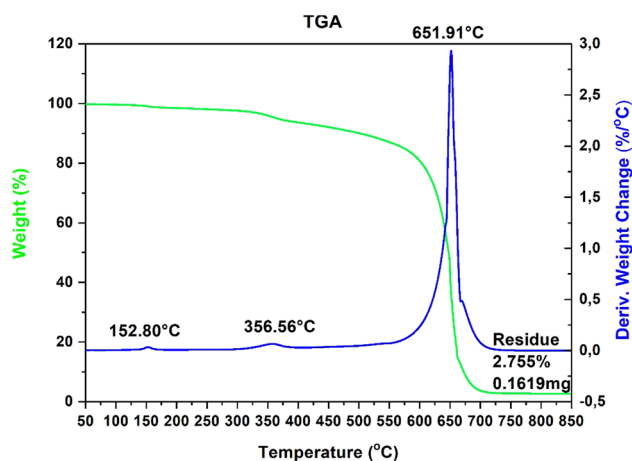


Figure 9: TGA thermogram for modified carbon CNTs after oxidation reaction.

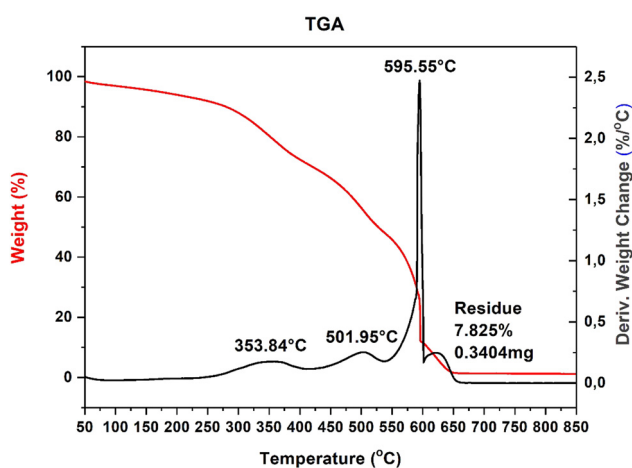


Figure 12: TGA thermogram for modified CNTs after acylation with 5(6)-carboxyfluorescein.

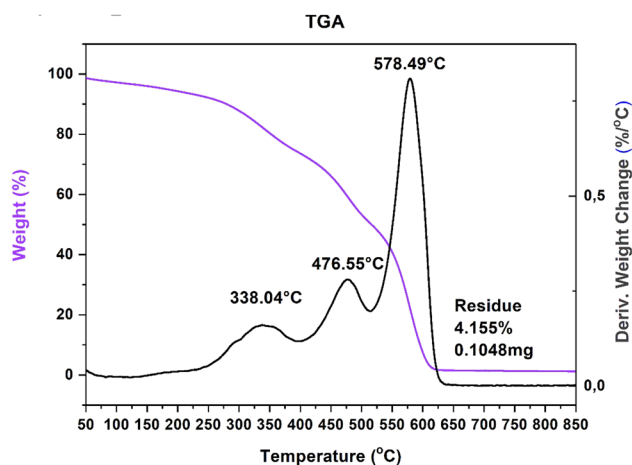


Figure 10: TGA thermogram for modified CNTs after acylation with 4-(1-pyrinyl)-butanoic acid.

effectiveness of all stages of chemical functionalization was evaluated by FTIR spectroscopy and thermogravimetric analysis. The obtained results prove that the modification of CNTs passed successfully and the fluorescent markers were attached to their surface.

The obtained CNTs can be used in the future as fluorescent biosensors useful in medical diagnostics, because after being coated with appropriate ligands they can enable to locate and (thanks to their fluorescent properties) detect pathological changes at the cellular level, allowing for early detection of disease. Such material may constitute a modern solution in cancer diagnostics. Medical imaging with the use of nanotubes may give better results than imaging with standard contrast agents. It is also possible to monitor the effectiveness of treatment

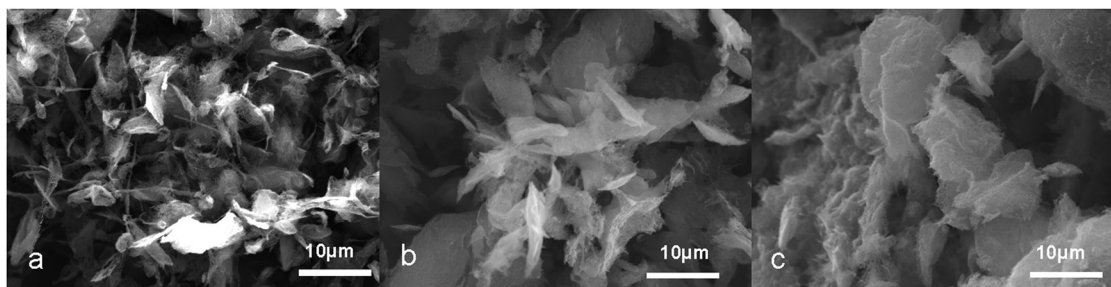


Figure 13: SEM images (magnification $\times 2,500$) for CNTs after acylation with fluorescent dyes: (a) 4-(1-pyrinyl)-butanoic acid; (b) 5(6)-carboxyfluorescein; (c) rhodamine B.

Table 1: EDS results for CNTs with fluorescent dyes

Element	Mass%	Atom%
CNTs with 4-(1-pyrinyl)-butanoic acid		
C	83.84	87.99
O	14.88	11.72
Fe	1.28	0.29
Total	100.00	100.00
CNTs with 5(6)-carboxyfluorescein		
C	71.71	76.66
N	12.47	11.43
O	14.44	11.59
Fe	1.38	0.32
Total	100.00	100.00
CNTs with rhodamine B		
C	67.58	72.37
N	17.54	16.11
O	14.11	11.34
Fe	0.77	0.18
Total	100.00	100.00

using this imaging method. Early detection of cancer and the simultaneous use of targeted therapy with the ability to monitor treatment efficacy is a significant step towards improving treatment efficiency.

Conflict of interest: The authors declare no conflict of interest regarding the publication of this paper.

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