FLUORECENTLY LABELED BIONANOTRANSPORTERS OF NUCLEIC ACID BASED ON CARBON NANOTUBES

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We propose an approach to the design of a new type of hybrids of oligonucleotides with fluorescein-functionalized single-walled carbon nanotubes. The approach is based on stacking interactions of functionalized nanotubes with pyrene residues in conjugates of oligonucleotides. The amino- and fluorescein-modified single-walled carbon nanotubes are obtained, and their physico-chemical properties are investigated. The effect of the functionalization type of carbon nanotubes on the efficacy of the sorption of pyrene conjugates of oligonucleotides was examined. The proposed non-covalent hybrids of fluorescein-labeled carbon nanotubes with oligonucleotides may be used for the intracellular transport of functional nucleic acids.

1. Introduction

Carbon nanotubes (CNTs) possessing chemical passivity and compatibility with biomacromolecules and cells are investigated actively in different fields of nanobiotechnology and biomedicine. The wide variety of both covalent and non-covalent functionalization methods have been described [1]. The application of CNTs as transporters of biologically active compounds into living cells requires the presence of reporter groups in their structure to carry out the monitoring of transfection during both *in vitro* and *in vivo* experiments. The presence of fluorophore residues linked to the CNT surface could provide us the possibility to observe the nanocomplexes by confocal microscopy.

2. Experimental Part

Commercial carboxylic acid functionalized single-walled carbon nanotubes (SWNT-COOH, Sigma-Aldrich, 652490) were used in this work.

Functionalized nanotubes and their hybrids with oligonucleotides were characterized by UV-Vis, fluorescent, and infrared spectroscopies, Raman spectroscopy, thermogravimetric and elemental analyses, transmission and scanning electron microscopies. A fluorimeter Cary Eclipse (Varian Inc., USA), spectrophotometer Shimadzu UV-vis-2100 (Shimadzu, Japan), electron microscopes LEO 1430 (LEO, Germany) and JEM 2010 (JEOL, Japan), confocal microscope Cell Observer SD (Zeiss, USA), IR-spectrometer Scimitar FTS 2000 (Digilab, Australia), Raman spectrometer T64000 (Horiba Jobin Yvon, Italy), CNT-analyzer Carlo Erba 1106 (Carlo Erba, Italy), and thermoanalyzer TG 209 F1 (NETZSCH, Germany) were used for these purposes.

Hexamethylenediamine and PAMAM G3.0 functionalized SWNTs (SWNT-HMDA and SWNT-PAMAM) were prepared by analogy with [2,3]. Fluorescein-labeled carbon nanotubes, SWNT-HMDA-FITC and SWNT-PAMAM-FITC, were obtained by analogy with [4,5]. Oligonucleotides were synthesized within the solid-phase phosphoramidite method on an automatic synthesizer ASM-800 (Biosset, Russia). Conjugates of oligodeoxyribonucleotides and oligo(2′-O-methylribonucleotides) with pyrene residues attached to the 5′-phosphate group directly or via a hexa(ethyleneglycol) phosphate linker were synthesized as described previously [6] and used for the preparation of hybrids with SWNT. The structure of the conjugates was proved by MALDI-TOF mass spectrometry, UV and fluorescent spectroscopies.

For the preparation of hybrids, the functionalized SWNTs were dispersed in a 5×10^{-6} M water solution of 5′-pyrene conjugate of oligonucleotide by ultrasonication during 30 min. The concentrations of functionalized SWNT were varied within 2.5–250 μg/ml.

3. Results and Discussion

Here, we propose an approach to the preparation of non-covalent hybrids of oligonucleotides with fluorescent single-walled carbon nanotubes based on the stacking interactions of pyrene conjugates of oligonucleotides with the surface of nanotubes. This approach does not require severe conditions at the stage of a modification of CNTs and is remarkable due to the relative simplicity of the synthesis of pyrene conjugates and a higher den-

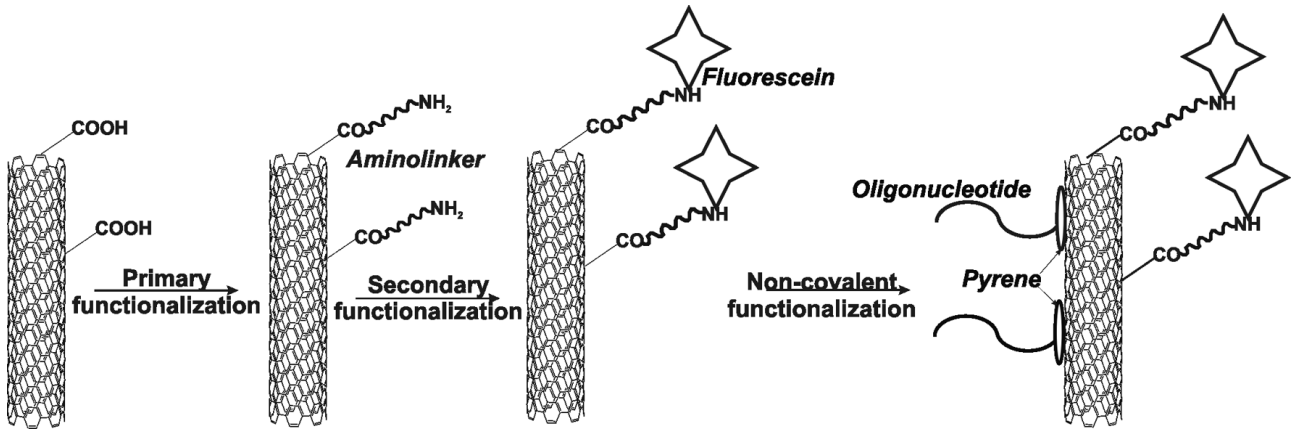

Fig. 1. Strategy of SWNT multifunctionalization

sity of CNT functionalization as compared with other methods.

We have developed the multifunctionalization strategy including primary covalent SWNT functionalization by the aminolinker introduction, secondary covalent SWNT functionalization by the coupling of fluorescein isothiocyanate (FITC) to amino groups, and the non-covalent attachment of 5′-pyrene conjugates of oligonucleotides on the SWNT surface (Fig. 1).

Two types of amino-modified SWNT bearing hexamethylenediamine residue (SWNT-HMDA) or polyamidoamine dendrimer G3.0 (SWNT-PAMAM) were prepared. The Kaiser test [7] was employed to quantify the amount of amino groups after the primary functionalization step (0.11–0.26 mmol/g). The fluorescein-labeled SWNTs (SWNT-HMDA-FITC and SWNT-PAMAM-FITC) were obtained, by using these amino-modified SWNTs. Amino- and fluorescein-modified nanotubes were characterized by infrared spectroscopy, thermogravimetric analysis, elemental analysis, Raman spectroscopy, and transmission and scanning electron microscopies.

T a b l e 1. The data of thermogravimetric and elemental analyses of functionalized SWNTs

Functionalized SWNT	Element content, %			Weight loss upon heating up 400 °C, %
	C	H	N	
SWNT-COOH	89.8	0.7	–	7.7
SWNT-HMDA	79.5	2.5	4.7	27.8
SWNT-PAMAM	56.2	5.5	14.6	42.5
SWNT-HMDA-FITC	70.7	3.0	3.5	46
SWNT-PAMAM-FITC	63.6	2.9	5.7	44

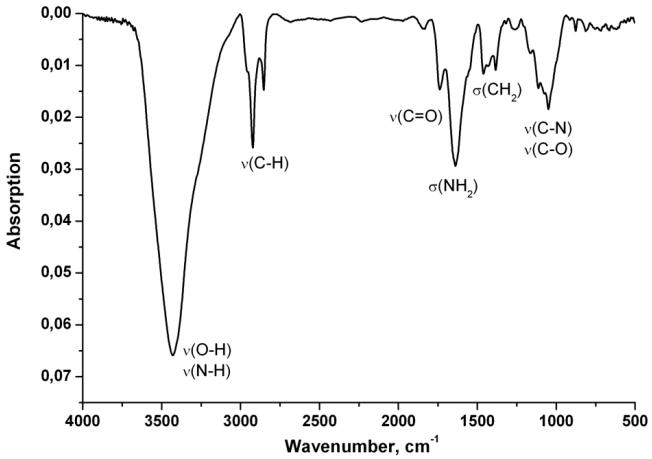

Fig. 2. FTIR spectrum of SWNT-PAMAM-FITC

The FTIR spectra of aminomodified (SWNT-HMDA and SWNT-PAMAM) and fluorescently labeled (SWNT-HMDA-FITC и SWNT-PAMAM-FITC) carbon nanotubes show characteristic vibration modes corresponding to ν(N–H) in amino groups and ν(C=O) and ν(C–N) in amide groups. The FTIR spectrum of SWNT-PAMAM is presented in Fig. 2 as an example.

Thermogravimetric and elemental analyses confirmed the presence of HMDA, PAMAM, and FITC residues on the surface of functionalized SWNTs (Table 1).

The maxima in the region of 260–290 nm (corresponded to SWNTs) and 495 nm (corresponded to fluorescein) were observed in the electronic absorption spectra of fluorescein-labeled SWNTs. The Raman spectra of functionalized SWNTs display the D band ($\omega_{\rm D}$ = 1300 cm^{-1}), G band ($\omega_{\rm G}$ = 1500–1550 cm^{-1}), and the radial breathing mode (RBM) band ($\omega_{\rm RBM}$ = 158 cm^{-1}). The diameter was calculated according to [8] and was equal

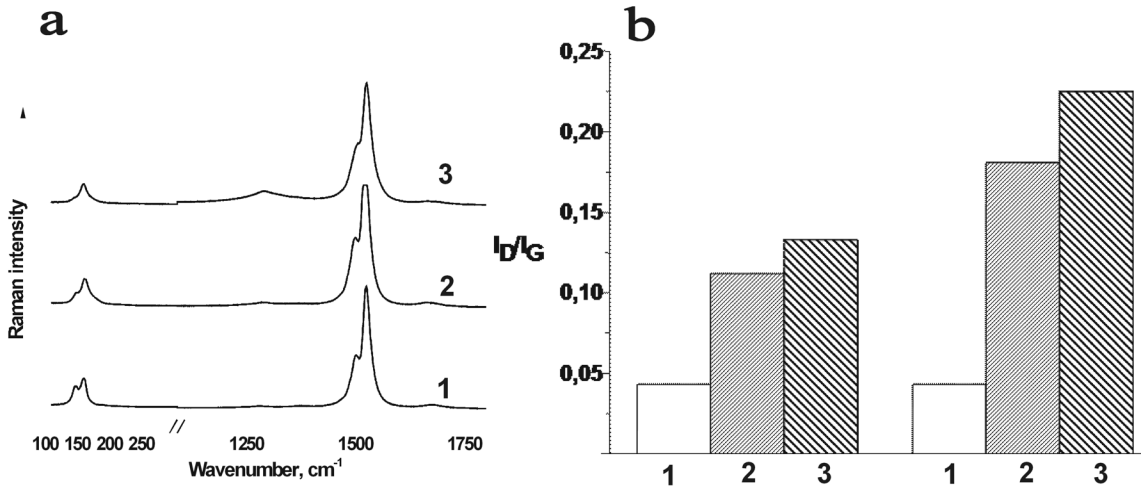

Fig. 3. Raman spectra of functionalized SWNTs (*a*) and I_D/I_G ratios (*b*). *1* – SWNT-COOH, *2* – SWMT-HMDA, *3* – SWNT-HMDA-FITC

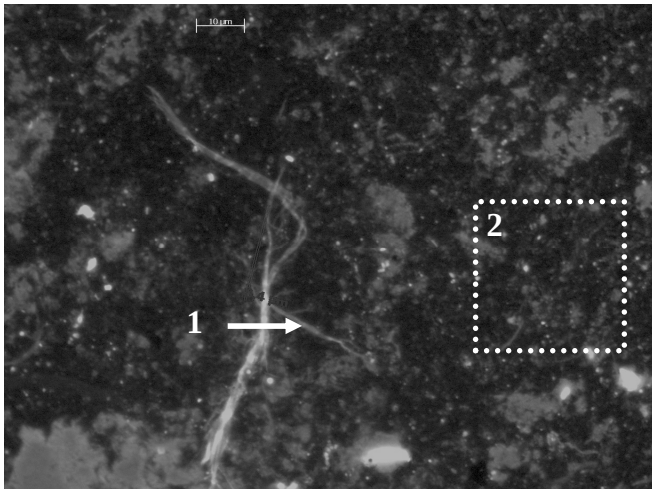

Fig. 4. Confocal microscopy of SWNT-HMDA-FITC. *1* – bundle of nanotubes; *2* – individual nanotubes

to 1.71 nm. The ratio I_D/I_G grows upon the functionalization that can be considered as an indirect confirmation of the organic molecule attachment to the SWNT surface (Fig. 3).

The individual SWNTs with length up to 1.5 μm and their aggregates were observed, by using SEM. The additional organic groups were visualized as bulky structures on the ends of SWNTs. The presence of PAMAM and PAMAM-FITC groups on the SWNT surface was confirmed by TEM. The possibility of the visualization of fluorescently labeled SWNTs by confocal microscopy was demonstrated (Fig. 4).

5′-Pyrene conjugates of oligodeoxyribonucleotides and oligo(2′-O-methylribonucleotides) (Fig. 5) were purified by PAAG electrophoresis and immobilized on functionalized SWNTs.

The fluorescent properties of the obtained hybrids were investigated. The increasing amount of SWNTs in a solution reduced the fluorescence intensity of pyrene groups because of the fluorescence quenching upon the interaction with nanotubes [9]. We used this phenomenon to elaborate the method of quantitative estimation of the amount of oligonucleotides fixed on the SWNT surface. This method may be applied for the concentration of nanotubes in a solution up to 50 mg/ml.

The presence of bulky functional groups on the nanotube surface may affect the rate and the completeness of the sorption of pyrene residues. The isotherms (25°C) of the absorption of 5′-pyrene modified oligonucleotides on the nanotubes surface are plotted in Fig. 6.

It is shown that the rates of oligonucleotide sorption are slightly different in the cases of modified and unmodified SWNTs. The complete sorption of oligonucleotide (90-95%) was achieved when the concentration of SWNTs was about 50 μg/ml irrespective of the type of nanotube functionalization. The presence of a hexaethyleneglycol linker between the pyrene residue and an oligonucleotide decreases the oligonucleotide sorption efficiency, if the concentration of SWNTs is less than 50 μg/ml. The capacity of a carbon nanotube was amounted to be about 100 μmol/g.

The data on non-covalent hybrids of oligonucleotides with functionalized SWNTs were obtained, by using high-resolution electron microscopy. TEM im-

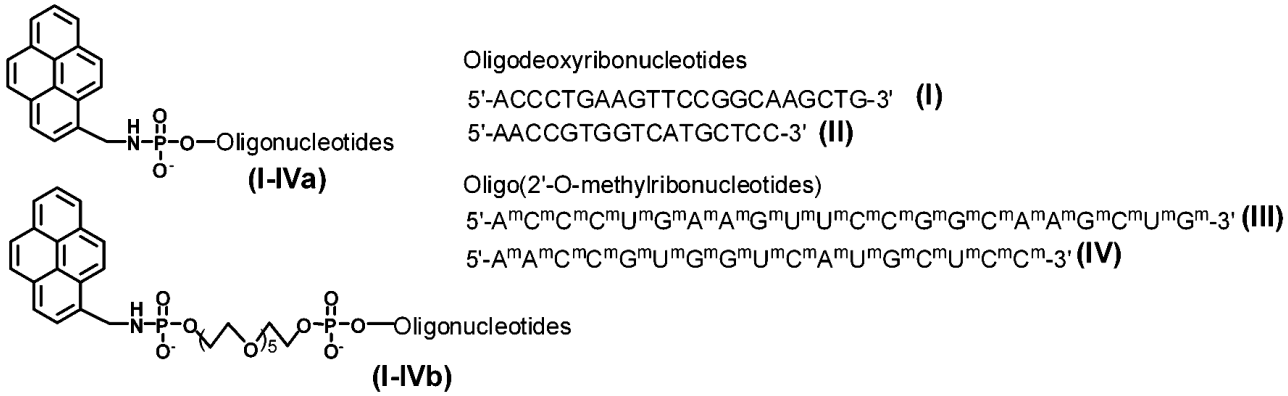

Fig. 5. 5′-Pyrene-modified oligonucleotides used for the preparation of hybrids

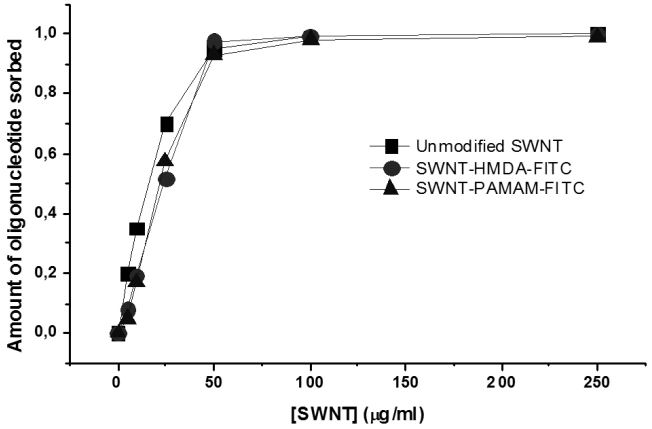

Fig. 6. Isotherms of the 5′-pyrene conjugate (**Ia**) absorption on the surface of functionalized nanotubes. Conditions: 25 °C, oligonucleotide concentration 5×10^{-6} M

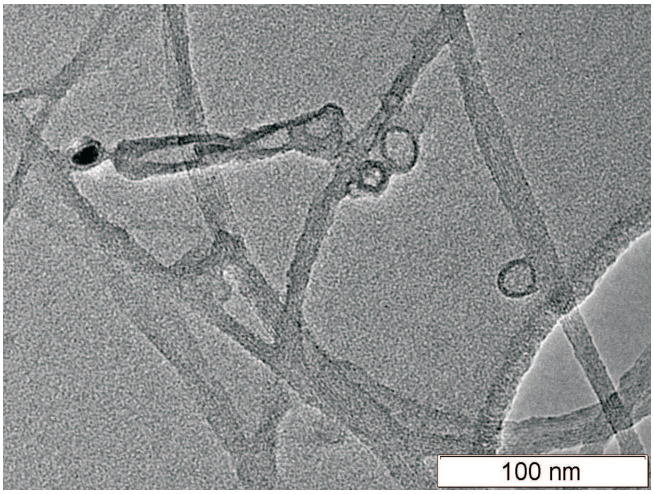

Fig. 7. TEM-image of the non-covalent hybrid of 5′-pyrene conjugate (**IIa**) with SWNT-HMDA-FITC

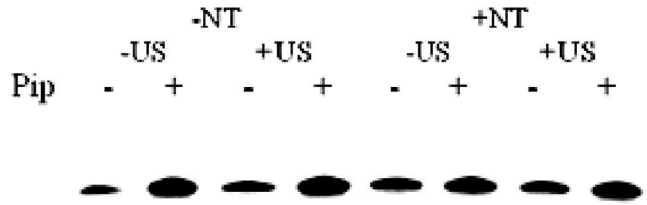

Fig. 8. Stability of oligonucleotide **(Ia)** during preparation of their hybrids with functionalized SWNTs (NT) and ultrasonication (US). Pip – piperidine treatment

ages demonstrated the simultaneous presence of a functional group (FITC, PAMAM, PAMAM-FITC) and an oligonucleotide in the structure of hybrids. Oligonucleotides were visualized as folded nanosized structures on the SWNT surface (Fig. 7).

The investigation of the desorption of 5′-pyrene conjugates of oligonucleotides from the CNT surface was performed. Pyrene conjugates of oligonucleotides were displaced from the CNT surface by methylene blue that caused the precipitation of carbon nanotubes. The increase of the desorbed 5′-pyrene conjugate of oligonucleotides in supernatant upon a rise of the methylene blue concentration was observed by the gel electrophoresis assay. These data confirmed the non-covalent nature of hybrids.

The stability of oligonucleotides during the preparation of hybrids was studied. The gel electrophoresis assay displayed no strand breaks in oligonucleotides after the ultrasonication in the presence of SWNT-COOH (Fig. 8). The absence of apurinic/apyrimidinic (AP) sites in oligonucleotides attached to SWNT-COOH was demonstrated by the piperidine treatment. The reversed phase HPLC analysis of hydrolyzates of the same oligonucleotides did not detect any products of nucle-

oside oxidation. These data proved the stability of oligonucleotides during the formation of hybrids.

4. Conclusions

The results obtained demonstrate the feasibility of the proposed approach for the design of hybrids of oligonucleotides with CNTs. Such non-covalent hybrids may be applied to making the bionanotransporters of functional nucleic acids (siRNA, NA-enzymes, aptamers, *etc.*)

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ФЛУОРЕСЦЕНТНО МІЧЕНІ
БІОНАНОТРАНСПОРТЕРИ НУКЛЕЇНОВИХ
КИСЛОТ НА ОСНОВІ ВУГЛЕЦЕВИХ НАНОТРУБОК

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Р е з ю м е

У даній роботі ми пропонуємо новий підхід до створення гібридів олігонуклеотидів з флуоресцентно міченими одностінними вуглецевими нанотрубками. Використаний нами підхід заснований на стекинг-взаємодії залишків пірену в складі піренільних кон'югатів олігонуклеотидів з поверхнею функціоналізованих нанотрубок. Було отримано аміно- і флуоресцеїн-модифіковані одностінні вуглецеві нанотрубки і вивчено їх фізико-хімічні властивості. Досліджено ефект впливу типу функціоналізації вуглецевих нанотрубок на ефективність сорбції піренільних кон'югатів олігонуклеотидів. Запропоновані в роботі нековалентні гібриди флуоресцентно мічених вуглецевих нанотрубок з олігонуклеотидами в перспективі можуть бути використані як транспортери функціональних нуклеїнових кислот у клітини.