CARBON NANODOTS COATED WITH OLIGONUCLEOTIDES AS FLUORESCENT HYBRIDIZATION PROBES FOR DNA MICROARRAY

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ABSTRACT. In this article we report an easy preparation of single stranded oligonucleotides conjugated to highly-fluorescent carbon quantum dots as efficient hybridization probes for DNA microarray. These nanotools enable fast detection and quantification of nucleic acid molecules. The main advantage of carbon nanodots (C-dots) over conventional organic dyes is their photobleaching resistance, which improves the signal intensity and thus lower amount of DNA material can be detected. In addition, the surface passivation of C-dots with ethylenediamine leads to the enhancement of their quantum yield and photoluminescence intensity, which is of great importance because, for many bioassays, the genetic amount is extremely limited. In the current report, nitrogen-doted C-dots were used as effective nanoquencher agents for fluorescent hybridization DNA microarray. Their bio-compatibility and stability make them promising fluorescent probes or DNA markers for bio-labeling and bio-imaging applications.

Keywords: Carbon quantum dots, DNA microarray, hybridization probes.

ВЪГЛЕРОДНИ НАНОТОЧКИ ПОКРИТИ С ОЛИГОНУКЛЕОТИДИ, КАТО ФЛУОРЕСЦЕНТНИ ХИБРИДИЗАЦИОННИ СОНДИ ЗА ДНК МИКРОЧИПОВЕ

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РЕЗЮМЕ. В настоящия доклад е представено приготвянето на едноверижни олигонуклеотиди конюгирани към високо-флуоресцентни въглеродни квантови точки, като ефикасни хибридизационни сонди за ДНК микрочипове. Тези нано-инструменти позволяват бързо откриване и количествено опредделяне на нуклеинови киселини. Основното предимство на въглеродните квантови точки (C-dots) спрямо конвенционалните органични багрила е тяхната устойчивост към фото-избелване, което подобрява интензитета на сигнала и по този начин може да се детектира по-малки количества ДНК материал. В допълнение, повърхностното пасивиране на C-dots с етилендиамин води до повишаване на квантовия им добив и респективно интензитета на фотолуминесценция, което е от голямо значение, тъй като за много биологични анализи генетичното количество е изключително ограничено. В представения доклад въглеродните точки дотирани с азот са използвани, като ефективни нано агенти за загасване на флуоресценцията при хибидизационните ДНК микрочипове. Тяхната био-съвместимост и стабилност ги прави обещаващи флуоресцентни сонди или ДНК за приложения, като био-маркиране и био-изобразяване.

Ключови думи: Въглеродни квантови точки, ДНК микрочипове, хибридизационни сонди.

1. Introduction

The rapid development of highly sensitive, cheap and easy to perform systems for effective detection of specific nucleic acids, aptamers, and oligonucleotides is necessary due to their widespread biotechnological applications (Gresham et al., 2006; Li et al., 2011; Qiang et al., 2014). The recently increased availability of various nanostructures has created interest in their use for the purposes of biotechnological DNA microaaray application. The efforts are focused on the fabrication of homogeneous fluorescence assays based on fluorescence resonance energy transfer or quenching mechanism for oligonucleotides detection (Zhang et al., 2017). It is interesting to note that these techniques generally compromise the use of a photosensitizer and quencher molecules attached to the opposite terminals of oligonucleotide probe. In the absence of the target analytes, the organic dyes are in close proximity and the emission is suppressed. However, upon the occurrence of specific recognition reaction with the target molecules (in the case with complementary oligonucleotide this reaction is hybridization), the quencher agent becomes physically separated from the photosensitizer molecule and thus visible emission is released. Nevertheless, a basic disadvantage of the conventional organic fluorescent sensor is the requirement for careful selection of a photosensitizer-quencher pair in order to ensure optimum efficiency (Li, et al., 2011). Examples of efficient nanoquenchers could be those with gold nanoparticles, carbon nanotubes, metal-organic frameworks, graphene oxide, etc. One of the main advantages of these nanomaterials is their cytotoxicity behavior (Liao et al., 2011; Kumarathasan et al.,

2015). Therefore, the development of new efficient nanoparticles is needed to replace the above nanomaterials.

Carbon quantum dots (C-dots) are potential candidates as nanoquencher agents for fluorescent hybridization DNA microarray. They have unique properties, like resistance to photo-bleaching, small and well-defined size, biocompatibility, low cytotoxicity, and relatively high quantum yield (Roy et al., 2015). In addition, C-dots possess high water solubility, which allows homogeneous sensing assay. This property is of particular importance for the DNA microarray detection of nucleic acids. The purpose of the current report is to develop the C-dots coated with oligonucleotides as fluorescent hybridization probes for DNA microarray. The nitrogen dotted fluorescent C-dots play a role as nano-quenchers in the detection processes.

2. Experimental Procedures

2.1. Materials.. Citric acid, ethylenediamine, sodium carbonate, and *p*-Phenylene diisothiocyanate were purchased from Wako. Amino-terminated DNA oligonucleotides with the following sequencing 5' Fluorescein-$(CH_2)_6$-AAA AAA AAA AAA AAA AAA AAA-CH_2-CH_2-SH 3' (or FAM-probe) and 5' TTT TTT TTT TTT TTT TTT TTT TTT-CH_2CH_2-N_2H 3' were obtained from Sigma-Aldrich. The used water was ultrapure obtained by Milli-Q ion exchange column (Millipore). Gold nanoparticles with 20 nm of diameter were prepared by standard protocol of Greg Herman (Bioconjugation Techniques, Third Edition).

2.2. Synthesis of Carbon nanodots (C-dots) *by microwave-assisted pyrolysis*. C-dots were prepared by the microwave-assisted pyrolysis method (Loukanov et al., 2016), where 1 g of citric acid was dissolved in 10 ml Milli-Q water. Then 200 μL of 1,2-ethylenediamine was added to the prepared solution and the reaction mixture was exposed to microwave irradiation for about 3 min (in 600 W microwave oven). After the occurred microwave pyrolisys, a yellowish brown gum was formed in the Becker glass. This gum was used in the next step without any preliminary purification.

2.3. Preparation of modified nucleotides. FAM-probe-SH templates were immobilized on the surface of 20 nm gold nanoparticles by following a standard protocol (Dharanivasan et al., 2016). The carbon nanodots were conjugated to 5' TTT TTT TTT TTT TTT TTT TTT TTT-CH_2CH_2-N_2H 3' oligonucleotide via *p*-phenylene diisothiocyanate linkage. For that purpose, 200 mg C-dots were dissolved in 100 mL of 50 mM carbonate buffer with pH 9.5, containing 150 mM NaCl. At this alkaline pH, the amino groups from C-dots surface remain unprotonated. Then, 40 mg of *p*-phenylene diisothiocyanate was added, and the reaction mixture was stirred for 30 min at room temperature. The modified carbon nanodots were purified from the reaction mixture by simple centrifugation with acetone. Thiocyanate modified C-dots were mixed with amino-terminated oligonucleotides in carbonate buffer with pH 9.5 at room temperature. After 30 min of incubation, the modified oligonucleotides were separated from the unreacted C-dots by gel electrophoresis.

2.4. Fluorescence quenching and detection of the hybridized DNA oligonucleotides. A 40 nM Tris-HCl buffer solution was prepared to study the fluorescence quenching reaction. Aliquots of FAM-probe-immobilized on gold nanoparticles and C-dot labeled oligonucleotides were added to the buffer solution and the reaction mixture was incubated for 20 min at ambient temperature and for 30 min at 50 °C. After the incubation, the fluorescence of the reaction solution was measured.

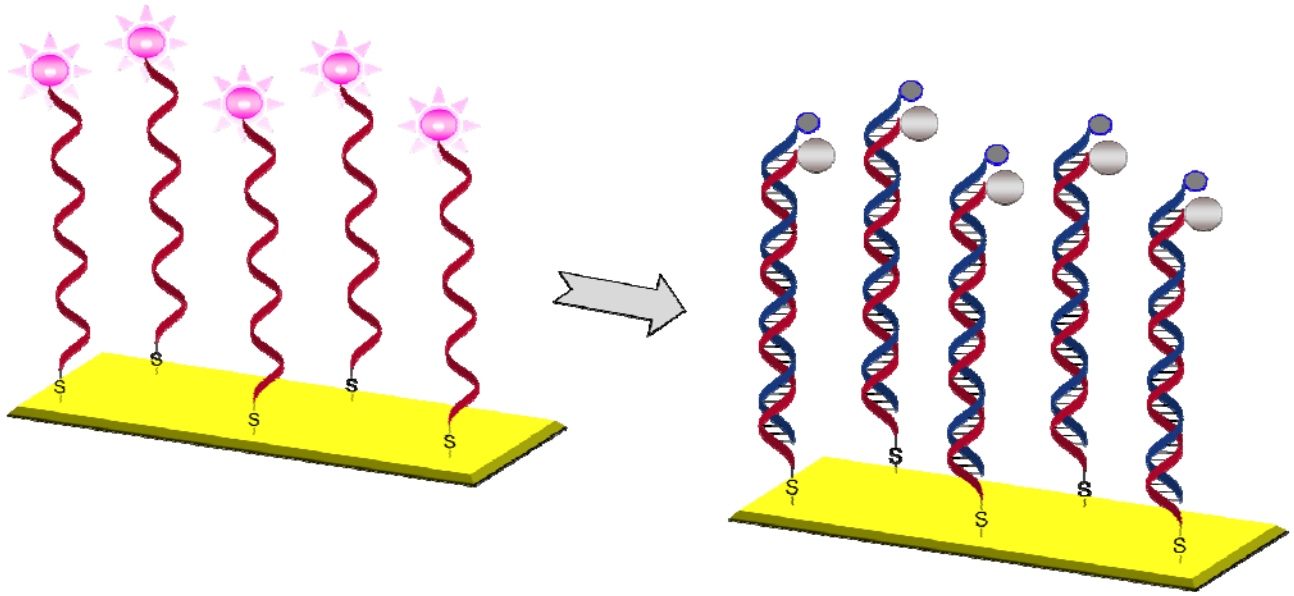

Fig. 1. Schematic illustration of the DNA microarray fluorescent based detection by quenching of fluorescein emission with carbon nanodots

2.5. Analysis and characterization of the nanomaterials and oligonucleotides hybridization. Jasco UV-VIS absorption spectrophotometer (model № V-570) and Jasco analytical spectrofluorometer ((model № FP-6300) were used for the absorption and fluorescence measurements respectively. High resolution X-ray photoelectron spectroscopy (XPS) was performed on an ESCAProbe P spectrometer equipped with a monochromatic aluminum X-ray radiation source (Omicron

Nanotechnology LTD., Taunusstein, Germany). Fourier transform infrared spectroscopy (FT-IR) spectra were recorded using a Tensor II spectrometer (Bruker) with Platinum ATR.

3. Result and discussion

3.1. Principles of C-dots fluorescence-based detection. The general principle of DNA array fluorescence detection in the current report is illustrated in Figure 1. The fluorescein modified oligonucleotide chains were immobilized on the surface of gold nanoparticles. In contact with their complementary oligonucleotides in the buffer, a reaction of hybridization occurred and double stranded DNA (dsDNA) structure was formed. An interaction between dsDNA and the hydrophobic aromatic core of C-dots might have occurred trough π-π stacking. The difference in the electrostatic and hydrophobic properties of FAM-probe and dsDNA leads to a different propensity to adsorb onto the surface of C-dots. Such effect has an impact on the sensitivity and selectivity in the DNA detection microarray. To understand deeply the properties of carbon nanodots, their characterization was performed with X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FT-IR).

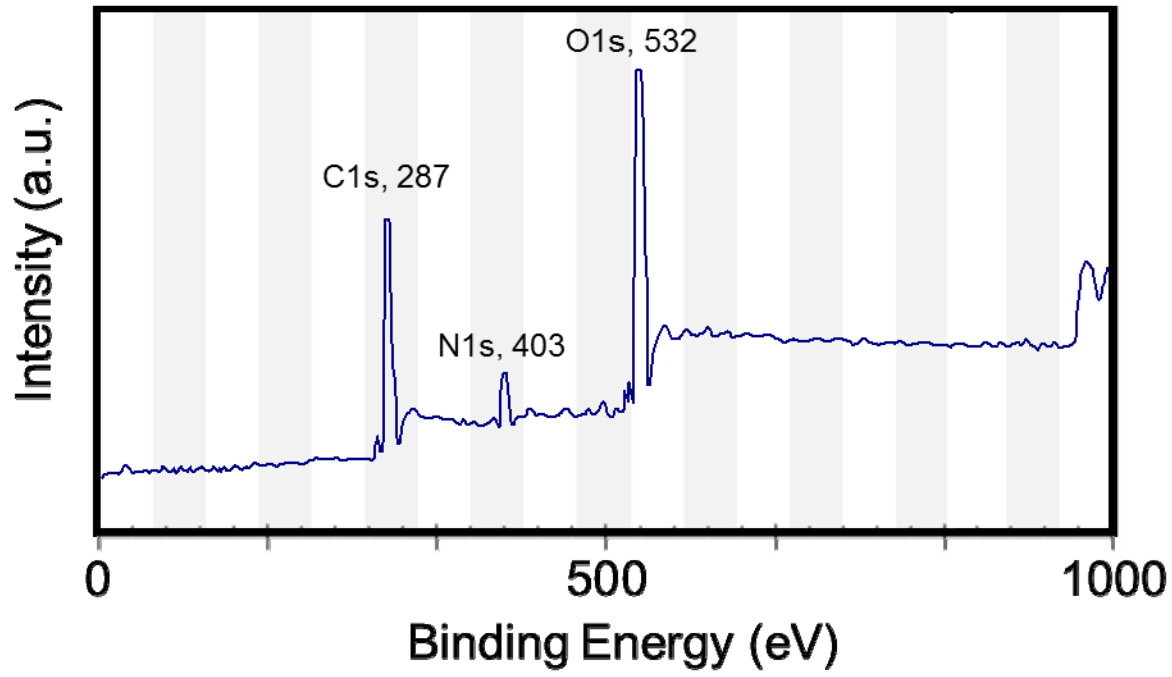

Fig. 2. X-ray photoelectron spectroscopy (XPS) spectrum of carbon nanodots prepared by microwave-assisted pyrolysis

3.2. X-ray photoelectron spectroscopic analysis. XPS is a sensitive and semiquantitive analytical method for studying the nanoparticle surface. It also provides qualitative information for the surface elemental composition and the bonding arrangements of the synthesized carbon nanodots. Hence, survey scans, from a binding energy of 1000 to 0 eV were recorded for C-dots, as shown in Fig. 2, in order to find their overall elemental composition. The binding energy for carbon is 287 eV (C1s), for nitrogen 403 eV (N1s) and for oxygen 532 eV (O1s). As illustrated in the figure carbon-to-oxygen (C/O) ratios of 2.12 and 1.39 was obtained. The values highlight the presence of carboxylic groups and hydrophobic graphitic regions on the surface of C-dots. In fact, the prepared C-dots are also soluble in organic solvents such as acetone, ethanol, methanol, EtOAc and DMSO. However, the high resolution scans revealed that the C-dots also contained C=C, C–C, C–O, C=O and O–C–O peaks on their surface, which might correspond to hydrophilic groups as carboxyl, hydroxyl and esters. A more detailed analysis of the surface chemical groups of C-dots can be achieved by IR spectroscopy.

3.3. FT-IR analysis of the carbon nanodots. As shown in Fig. 3, the absorption band at 3000~3500 cm^{-1} is associated to the combination of stretching vibrations of amino (H_2N–), hydroxyl (HO–), and alkyl (C–H) groups. The band at 3420 cm^{-1} corresponds to N–H; the stretching and bending vibrations of CH_2 and CH_3 groups are at 2975, 2927, 2854, 1460 and 1380 cm^{-1} respectively. The groups C=O and C=N have stretching vibrations at 1720 and 1660 cm^{-1}. There is N–H deformation vibration at around 800 cm^{-1}. The asymmetric and symmetric stretching vibration of C–O–C are 1095 and 1020 cm^{-1}.

3.4. Sensing of complementary oligonucleotide. In order to validate the mechanism described in Fig. 1, the fluorescence behavior of FAM-probe under different experimental condition was investigated. From Fig. 4 it is demonstrated that, in the absence of carbon nanodots, the FAM-probe exhibits strong fluorescence emission (blue line). However, after hybridization with oligonucleotide modified with carbon nanodot, a significant quenching of the fluorescence occurred (red line). This indicates that the presence of C-dot can quench the fluorescent dye effectively. In buffer solution with FAM-probe oligonucleotides and high concentration of C-dots as a control experiment, the quenching effect is much higher in comparison with the hybridization microarray experiment.

Subsequently, another calibration experiment with various concentration of oligonucleotides modified with C-dots was

performed to evaluate the range of detection by this method. With the increase of the concentration of modified oligonucleotide, enhancement of the quenching was observed. This can be attributed to the fact that, with more complementary C-dots-oligonucleotides added, a higher degree of DNA hybridization occurs and more dsDNA were obtained in the solution. The range of detection is established to be within the range of about 0.45 – 500 nM. It is interesting to note that independent studies with carbon nanoparticles and nanospheres were done and the determined range of detection was between 33 – 300 nm (Li et al., 2011).

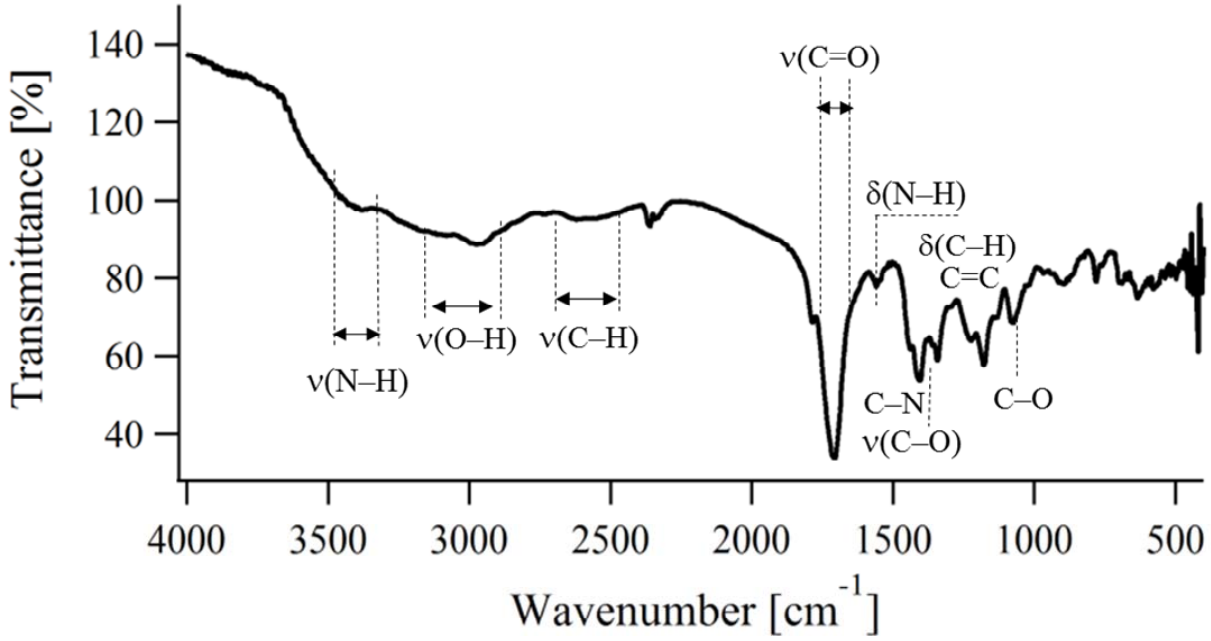

Fig. 3. Fourier transform infrared spectroscopy (FT-IR) spectrum of carbon nanodots prepared from citric acid and ethylenediamine by microwave-assisted pyrolysis

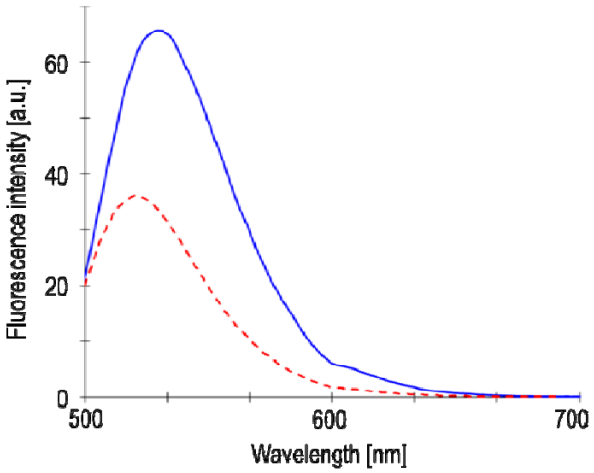

Fig. 4. Fluorescence emission spectra of FAM-probes in Tris-HCl buffer (blue solid line), FAM-probes hybridized with C-dots-oligonucleotides in Tris-buffer (red dotted line)

To investigate the selectivity performance of the proposed detection DNA microarray, single nucleotide polymorphism analysis was performed. This is an analysis in which a single nucleotide that occurs at a specific position in the nucleic chain might be exchanged with another nucleotide. Thus, the two possible variations are said to be alleles for this base. In our control experiment, the enhancement of the fluorescent signal was achieved when C-dots-oligonucleotide with one thymine base was exchanged with cytosine and introduced in the microarray analysis. It suggests that the hybridization did not occur completely in the case of single nucleotide polymorphism analysis.

4. Conclusion

We have developed a novel application of carbon quantum dots as a nanoquencher for DNA microarray analysis. Various nucleic acids can be detected by applying this analytical sensing technique. The linear dynamic range of detection is found to be 0.45 – 500 nM. The conclusions from this study act as the foundation for future works on utilizing this technique for fluorescent detection of essential biomolecules in the practical biotechnology.

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