Fabrication of a novel fluorescent polyacrylonitrile electrospun nanofiber for DNA-based optical biosensing of microRNA-21

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Keywords: fluorescent nanofiber, CdSe/ZnS quantum dots, polyacrylonitrile, microRNA-21, DNA probe, electrospinning

Abstract
In this study, an optical platform based on fluorescent nanofiber was developed for detection of microRNA-21 as a biomarker of cancerous cells. The fluorescent polyacrylonitrile nanofiber was fabricated using electrospinning method. The surfaces of nanofiber were treated by NaOH to convert nitrile groups to carboxy moieties. Activation of these carboxyl groups by EDC-sulfo NHS coupling agents was done and then the covalent bonding between COOH groups of nanofiber and –NH2 groups of amino-probe was formed. The results of SEM, XRD, FTIR, and fluorescence microscopy confirmed that the process of fluorescent nanofiber synthesis was successfully performed. Hybridization of probe and microRNA-21 showed that the capture efficiency of this fluorescent nanofiber was 1 pmol μl⁻¹ and these nanofibers have the potential to be used as optical sensors for detection of microRNA-21.

Introduction
MicroRNAs (miRNAs) are a class of endogenous, small and single-stranded noncoding RNAs of 18 to 25 nucleotides in length [1]. There are more than 1000 microRNAs in the human genome that have potential regulatory role in numerous biological processes such as early evolution, cell differentiation, proliferation and apoptosis [2, 3].

The study of microRNAs structure and function show that several microRNAs are abnormally expressed in cancerous specimens. In addition, functional differences between various types of tumors and stages of cancer are associated with microRNA expression [4–7]. These short non-coding RNAs have both oncogenic and tumor-suppressor potentials. MicroRNA-21 (Mir-21) is one of the most important microRNAs that is overexpressed in several tumors such as cholangiocarcinoma [8, 9], glioblastoma [3], multiple myeloma cells, and breast cancer [10, 11]. According to several studies, it has been proved that Mir-21 can be used as a novel biomarker for diagnosis and prognosis of some types of cancer [4, 5].

In recent years, several methods have been developed for analysis and detection of microRNAs, including northern blotting, in situ hybridization, quantitative Reverse-Transcription-Polymerase Chain Reaction (qRT-PCR), oligonucleotide microarrays and deep-sequencing [12], however, microRNAs unique features such as small size, low abundance and sequence similarity between family members, make these techniques time-consuming, complicated and costly with low sensitivity [13–15]. Given these considerations, development of more sensitive and cost-effective methods is essential. In this regard, as a novel diagnostic method, various nanotechnology-based approaches, including nanobiosensors, are being introduced and represent highly sensitive, simple and cost-effective features compared to the techniques mentioned for the detection of microRNAs [6, 7].

The most important feature of a nanobiosensor is the immobilization of a bioreceptor on a relatively high surface area platform, in order to increase sensor sensitivity and selectivity. One of the best platforms for nanobiosensors is the polymer nanofiber that could be simply fabricated by electrospinning with high throughput production [16–18], high surface to volume ratio [19, 20] and porosity, easy functionalization and affordable price [21, 22]. High surface-to-volume ratio and porosity of nanofibers lead to the immobilization of...
more probes, which will increase the sensitivity of the diagnostic system. Chemical nature of polymer nanofibers provide the possibility of functionalization with various chemical agents and thus different biological receptors, especially DNA probes can be easily immobilized at the surfaces of nanofibers. It is also possible to prepare multifunctional electrical, magnetic and optical nanofibers using other nanoparticles which provide a better response time and higher sensitivity than conventional sensors.

Polyacrylonitrile (PAN) nanofiber has potential applications in a variety of scientific fields [13, 17]. PAN is a stable organic polymer that is wildly applied for generating nanofiber by electrospinning due to its proper physical properties, such as flexibility and solubility in many solvents.

Although significant progress has been achieved in the synthesis of fluorescent nanofiber for different applications, to the best of our knowledge there is no report for production of fluorescent nanofiber with QDs and application of this platform for detection of microRNAs. Fluorescent Quantum dots are a new class of nanomaterials with specific photophysical properties and well established tools in biomedical imaging applications and optical biosensors. The linear relationship between changes in QD fluorescence in the presence of different concentration of analytes cause widely use of QDs in chemical and biosensing configurations. However, the application of these sensing principles in practical devices requires the immobilization of functionalized QDs in standard sensing platforms such as polymeric nanofibers.

Therefore, in this study, fluorescent PAN nanofiber applying hydrophobic CdSe/ZnS QDs was fabricated using electrospinning method and after functionalization of PAN surfaces, a designed specific probe for Mir-21 was attached to the surfaces of PAN nanofiber. Then this optical platform was characterized and used for specific detection of Mir-21.

Materials and methods

Materials

Polyacrylonitrile (PAN) polymer (Mw: 100,000 g mol⁻¹) was received from Polyacryl Company (Iran). Dimethylformamide (DMF, 99%) was purchased from Daejung (Korea). CdSe/ZnS quantum dots (QDs) were obtained from Plasmachem (Germany). NaOH (99.0%) and chloroform (99.8%) was purchased from Merck (Germany), Mir-21 with sequence of UAGCUUAUCAGAUGUUAG (Molecular weight = 7004.1 g/mole; HPLC purified), the sequence of UGGCUCAGUUCAGCAGGAACAG as a control and amino-probe with the complementary sequences to the Mir-21 with sequences of 5’-H2N-C5- UCAACAUCAGUCGUAAGCUSA-3’ were purchased from Exiqon (USA). Ethyl-3-(3- dimethyl amino propyl) carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) was obtained from Sigma-Aldrich (USA).

All other chemicals and reagents were in analytical grade. Deionized water was used for preparation of solutions and washing samples.

Methods

Fabrication of fluorescent PAN nanofiber (FPAN)

In order to prepare the fluorescent nanofiber, a homogeneous solution of PAN in DMF at concentration of 13 wt.% was made. The hydrophobic CdSe/ZnS QDs were diluted in chloroform and then the desired volume of QDs was dispersed in PAN to prepare a fluorescent spinning solution of PAN-QDs. The mixture was stirred at room temperature for 20 min.

One ml of PAN-QDs colloidal solution was electrospun using a syringe pump with a feed rate of 0.2 ml h⁻¹. A 20 kV voltage was applied between the syringe needle (gauge 23) and plate collector using a high-voltage power supply. The distance between needle and collector was adjusted to 15 cm. Final fluorescent nanofiber was collected on an aluminum plate.

Functionalization of fluorescent PAN nanofiber

To convert the nitrile groups of PAN nanofiber to carboxyl groups, 1 cm² piece of FPAN was immersed in 100 ml of 2 N NaOH aqueous solutions at 60 °C for 5 min the membrane was removed and thoroughly washed with deionized water. Then the hydrolyzed nanofiber was dried at room temperature.

Subsequently, the EDC-sulfo NHS coupling reagents were used for activation of carboxylic acid groups on the surface of fluorescent PAN nanofiber. Therefore, carboxyl functionalized fluorescent nanofiber was immersed in 1 ml solution of 0.02 M EDC and shaken for 30 min. Then, 1 ml solution of 0.1 M NHS was added to the nanofiber surface and shaken for another 30 min.

For the conjugation of amino-probe to the surface of fluorescent nanofiber, different concentrations of probes (0.1, 0.25, 0.5, 1 pmol µl⁻¹) in PBS was added to the surface of nanofiber and was shaken for 3 h at room temperature.
The fluorescent nanofiber was characterized with scanning electron and fluorescence microscopy. Functional groups on the surface of functional nanofiber were analyzed with FTIR. The phase and composition of nanofiber were obtained using XRD technique.

**Hybridization process between Mir-21 and the probe**

After characterization of fabricated fluorescent platform, hybridization process between probe conjugated nanofiber and Mir-21 was performed using four concentrations of Mir-21 (0.1, 0.5, 1, 2 pmol μl⁻¹). The PBS solution of microRNA was added to the surface of fluorescent nanofiber and the nanofiber was shaken for 30 min and washed three times with deionized water.

**Results and discussion**

The schematic representation of this study was demonstrated in figure 1. After electrospinning of fluorescent PAN solution, the surfaces of nanofiber was treated by NaOH to obtain carboxyl functionalized nanofiber. These carboxyl groups were activated by EDC- sulfo NHS coupling reagent to prepare the nanofiber for conjugation of amino-probe. After characterization of fluorescent nanofiber by SEM, XRD, FTIR and fluorescence microscope, the targeted microRNA and probe was hybridized and further analysis was performed for evaluation of optical nanofiber performances.

First, different concentrations of PAN in DMF solvent were electrospun to obtain optimal concentration of polymer solution to fabricate uniform nanofiber without any bead in the structure.

In order to study the surface morphology, average size and size distribution of nanofiber, the nanofiber was analyzed by Scanning Electron Microscope (SEM, MIRA3 TESCAN SEM). Based on the typical SEM image, the nanofiber obtained from 12 wt.% PAN solution was beaded (figure 2(A)-inset). The nanofiber fabricated by 13 wt.% PAN solutions was uniform in size and morphology with the diameter of 250 nm and no bead was seen along the fibers (figure 2(A)). As a result, 13 wt.% PAN solutions were selected as the appropriate concentration for further experiments. There was no difference between PAN nanofiber and FPAN in morphology that confirmed the embedding of QDs in PAN nanofiber figure 2(B). SEM image was carried out after functionalization and immobilization of probe to the surfaces of PAN nanofiber. It showed that the diameter of nanofiber was increased from 250 nm to 370 nm which is the confirmation of Mir-21 probe conjugation to the surfaces of nanofiber (figure 2(C)).

For producing the fluorescent nanofiber, the solution containing 13 wt.% PAN and QDs (PAN-QDs) was electrospun again. Figure 2(D) showed the polymeric solution of PAN containing QDs under UV excitation. The homogeneous red color of the PAN-QDs indicates that the QDs are homogenously dispersed in the solution. The electrospun nanofibers then were visualized under an Olympus fluorescence microscope. Figures 2(E) and (F) are related to the bright field and fluorescent images of fluorescent nanofiber, respectively. They showed the successful fabrication of fluorescent nanofiber with high intensity and brightness.
Figure 2. SEM images of (A) PAN, (B) PAN-QD, (C) FPAN-Mir nano fiber; the inset in figure A shows the beaded nano fiber in 12% w/v concentration of PAN; (D) The polymeric solution of PAN and PAN-QD under UV excitation; (E), (F) Bright field and fluorescent images of fluorescent nano fiber.

Figure 3. (A) FT-IR spectrum of (a) PAN, (b) PAN-QD, (c) PAN-QD-NaOH, (d) FPAN-probe and enlarged spectra of the characteristic peaks for PAN-QD-NaOH and FPAN-probe. (B) XRD patterns of (a) PAN, (b) FPAN-Mir; (C) 1.5% agarose gel electrophoresis of the probe residual solution in concentrations of (1) 0.1, (2) 0.25, (3) 0.5, (4) 1 pmol/μl; (D) 1.5% agarose gel electrophoresis of the Mir residual solution in concentrations of (1) 0.1, (2) 0.5, (3) 1, (4) 2 pmol/μl; (M) 100 bp molecular marker.
Fourier transform infrared spectroscopy (FTIR) analysis was performed using a Jasco, model 6300 spectrometer to approve the different functionalization steps. Figure 3(A) illustrated the FTIR spectra of the PAN nano-fiber (figure 3(A)-a), FPAN (figure 3(A)-b), NaOH modified PAN nano-fiber (figure 3(A)-c) and probe conjugated PAN nano-fiber (figure 3(A)-d).

The infrared spectra of PAN nano-fiber (figure 3(A)-a) showed significant absorbance peaks at 2930, 2241 and 1736 cm\(^{-1}\) that were assigned to C=O functional groups, C–H stretches and nitrile groups of PAN chains, respectively. No significant change was observed after electrospinning of PAN-QDs. This confirmed that QDs were embedded in the nano-fiber and were not connected to the fiber surfaces (figure 3(A)-b).

To conjugate the amino-probe to the surfaces of nano-fiber, the nano-fiber was treated with sodium hydroxide; consequently a number of the C≡N groups were converted to COOH. The small peaks of at 1580 cm\(^{-1}\) and 3300 cm\(^{-1}\) demonstrated the partial conversion of nitrile group to the carboxylic acid on the nano-fiber surfaces (figure 3(A)-c).

The peaks at 2412 cm\(^{-1}\), 3362 cm\(^{-1}\) and 1778 cm\(^{-1}\) confirmed the successful conjugation of the specific amino-probe to the surfaces of carboxylated nano-fiber. These results validated the step by step process of Mir-21 detection platform fabrication (in figure 3(A)-d) [23, 24].

Moreover, x-ray diffraction patterns were obtained from PW 1800 Philips XRD to determine phase composition of nano-fiber. The peak appeared at 2\(\theta\) = 16° showed the crystalline nature of PAN in figure 3(B)-a; the major peaks at 31, 45, 56, 66, and 75 were assigned to the crystalline structure of DNA probe and Mir-21. They approved the immobilization of DNA probe on the surfaces of nano-fiber (6 figure 3(B)-b) [25].

Furthermore, the number of probes that could be attached to the surface of nano-fiber was optimized by agarose gel electrophoresis using different concentration of probe. After performing the conjugation process of probe and nano-fiber, the remaining solution on the surfaces of nano-fiber was collected and loaded in to the wells of 1.5% agarose gel. The band at lane 4 in figure 3(C) represented that the maximum concentration of the probe which can be attached to the PAN/QDs nano-fiber is 0.5 pmol \(\mu\)l\(^{-1}\).

After hybridization, the residual solution was analyzed with agarose gel electrophoresis, to estimate the capture efficiency of the fluorescent nano-fiber. The electrophoresis result showed that 0.5 pmol \(\mu\)l\(^{-1}\) of probe can hybridize with 1 pmol \(\mu\)l\(^{-1}\) of microRNA, thus the detection limit of this optical nano-fiber is 1 pmol \(\mu\)l\(^{-1}\) (figure 3(D)).

In order to evaluate the fluorescent nano-fiber efficiency in hybridization and detection of targeted microRNA, the fluorescent nano-fiber hybridized with different concentration of microRNA was observed separately under a fluorescence microscope. As shown in the figure 4, fluorescent intensity of nano-fiber was
Table 1. The performance comparison of some assay for mir-21 detection.

| No. | Type of nanoparticle | Method          | Speed (min) | LOD    | References |
|-----|----------------------|-----------------|-------------|--------|------------|
| 1   | AuNP/MoS2/GCE        | Electrochemical | >60         | 0.086 fM | 26         |
| 2   | AuNPs/capture probe/TB | Electrochemical | >60         | 78 aM  | 27         |
| 3   | MoS2/Thi/AuNPs       | Electrochemical | >50         | 0.26 pM | 28         |
| 4   | DNA1/Fe3O4NPs/Thi and DNA2/Fe3O4 NPs/FC | Electrochemical | >240        | 0.46 pM | 29         |
| 5   | PolyT/CuNPs          | Fluorescence detection | >180        | 100 fM | 30         |
| 6   | CaF2: Yb, Ho@SiO2    | FRET            | >60         | 500 nM | 31         |
| 7   | FPAN                 | Fluorescent     | 30          | 1 pM   | This study |

NP: nanoparticle; GCE: Glassy Carbon Electrode; Thi: Thionine; FC: Ferrocene Carboxaldehyde; TB: Toluidine Blue; FRET: Fluorescence Resonance Energy Transfer.

decreased after hybridization of probe and microRNA on the surfaces of nanofiber. By increasing the microRNA concentration from 0.1 pmol μl⁻¹ to 1 pmol μl⁻¹, the fluorescent of QDs was decreased and quenching of QDs was occurred at 1 pmol μl⁻¹ of mir-21 (figures 4(A)–(C)). This decrease might be due to the energy transfer between the aromatic rings of nitrogenous bases and QDs that confirmed the successful performance of optical nanofiber in detection of target microRNA. A comparison between fluorescent intensity of the nanofibers treated with 1 pmol μl⁻¹ of Mir-21 and the control sequence (figure 4(D)) indicated that the designed probe hybridized specifically to the Mir-21 sequences and the fluorescent of these nanofibers changes only in the presence of this microRNA. This data confirmed the specificity of the designed platform for detection of Mir-21.

In table 1, the limit of detection and speed of optical platform designed in this work were compared with other reports. The synthesized fluorescent nanofibers provided a simple, rapid, high sensitive and specific platform for Mir-21 detection due to placing QDs in nanofiber structure using a selective probe, while the methods used in other cases, despite the high sensitivity, are time consuming and complicated.

Conclusion

MicroRNAs play an important role in the tissues development and maintenance. Their identification and control in blood and tissues can provide theranostic applications for a wide range of diseases, such as metabolic diseases, hormonal regulation, muscle function and cancers.

Due to the low concentration and small size of microRNAs, their sensitive and specific identification in biological samples has become one of the challenges faced by researchers. To overcome these problems, nanobiosensors have been introduced. In this study, a fluorescent nanofiber platform was successfully fabricated for specific detection of Mir-21.

We combined both polymeric properties of PAN such as a surface and fluorescent properties of quantum dot simultaneously to introduce a new platform that can be used for detection of microRNA in biological applications. High surface-to-volume ratio and porosity of PAN nanofibers as an immobilization platform in combination with QDs fluorescent provide a better response time and higher sensitivity than conventional sensors. The use of fluorescent nanofibers with high surface to volume ratio and porosity as immobilization platform increased the efficiency and sensitivity of this detection method. This platform was provided an affordable, high sensitive and specific, simple and rapid test for detection of Mir-21. However, further optimization of its specificity and functionality in tissue cultures and clinical samples are required in order to find applications in the field of biomedical science.

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