Ultrasensitive FRET-based aptasensor for interleukin-6 as a biomarker for COVID-19 progression using nitrogen-doped carbon quantum dots and gold nanoparticles

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Abstract
A label-free and specific FRET-based interleukin-6 (IL-6) aptasensor was developed using a DNA aptamer modified with nitrogen-doped carbon quantum dots (NCDs) and gold nanoparticles (AuNPs) as a donor-quencher pair. The assayed target was capable of disrupting the donor–acceptor assemblies yielding a concentration-related fluorescence recovery of NCDs (λ em = 445 nm and λ ex = 350 nm). By designing two different probes, the interaction of DNA aptamers with IL-6 protein was studied using FRET efficiency. It appeared that the sensing probes showed slightly different sensing profiles. One of the aptasensors showed a linear response of 1.5–5.9 pg/mL for IL-6 with a coefficient of determination of $R^2 \geq 0.99$ and a detection limit of 0.82 pg/mL (at S/N = 3). The experimental results indicated that the biosensor can be applied to determine IL-6 in human serum (with recovery of 95.7–102.9%). Due to the high sensitivity, excellent selectivity, and simplicity of the procedure, this strategy represents a promising alternative for IL-6 sensing in clinical applications.

Keywords Aptasensor · Fluorescence · FRET · IL-6 · Gold nanoparticles · Nitrogen-doped carbon quantum dots

Introduction
Elevated levels of interleukin-6 (IL-6) as a multi-functional cytokine have been documented in a variety of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, glomerular nephritis, and so forth [1–6]. COVID 2019 is spreading and emerging rapidly all over the world [7], and numerous studies have shown an increase in the level of IL-6 in COVID-19 hospitalized patients and its association with the severity of the disease and mortality [8, 9]. The normal value of IL-6 in the biological fluid of healthy adults ranges from 5 to 25 pg/mL; however, abnormal levels (e.g., up to 1000 pg/mL) can indicate serious health problems including chronic infections or various cancer [10]. A rapid and sensitive detection of molecular biomarker in trace amounts is very essential and valuable for detecting cancer in the early stages and treating a patient. This is challenging because the cytokine concentration in body fluids is low, normally in the Pico molar range [11], and cytokine assays may suffer interference from heterophilic antibodies, rheumatoid factors, and specific or nonspecific cytokine-binding proteins [12].

Nucleic acid aptamers are target-binding DNA or RNA obtained by an amplification-selection process called SELEX method [13]. The unique binding capability of the aptamers to specific targets like thrombin, interferon-γ, ATP, and AMP make them utilized for biomedical and molecular recognition [14–17]. So far, several methods by aptamer have been developed for IL-6 detection. Hahn’s group reported an IL-6R-binding RNA aptamer, named AIR-3, obtained by in vitro
selection from an RNA library. AIR-3 had a high affinity for human IL-6R, although AIR-3 did not inhibit the interaction of IL-6R with IL-6 [18]. Meyer et al. developed a short RNA aptamer, named AIR-3A, which adopted a parallel G-quadruplex structure and retained all necessary characteristics for high affinity and selective recognition of recombinantly produced IL-6R and as well as the native IL-6R presented on the cell surfaces [19]. Muhammad et al. presented a versatile approach for selective and sensitive detection of IL-6 cytokine in serum using the aptamer-based SERS biosensor [20]. Nanomaterials also have been used in several biological applications [21, 22]. A label-free aptasensor was presented for interleukin-6 based on impedimetric measurement of a gold nanoparticle/aptamer-modified electrode in artificial sweat [23]. Spiridonova et al. used aptamer immobilized on the MNPs to develop a nano-sensor immunoassay system [24]. The affinity of obtained DNA aptamers to the IL-6 was characterized by surface plasmon resonance. Giorgi-Coll et al. developed a colorimetric aptasensor for the detection of interleukin-6. The optical assay was based on the aggregation of AuNPs coated in two complimentary “sandwich-style” aptamers, each with different IL-6 target moieties. The binding of IL-6 to the complimentary aptamer pair causes the aggregation of the corresponding functionalized nanoparticles [25].

In recent years, the Förster resonance energy transfer (FRET) phenomenon has been utilized exclusively in the field of biomolecule detection because of its advantages in probing molecular interactions in real-time, being relatively simple and easy to use, and having high sensitivity and spatial resolution [26, 27]. The effectiveness of FRET process is extremely sensitive to the distance between donor and acceptor.

To the best of our knowledge, there is no report of FRET-based aptasensor for IL-6 detection. Motivated by this, here we designed a FRET-based aptasensor with high sensitivity and selectivity for in vitro IL-6 detection using NCDs as an energy donor and AuNPs as a quencher. Attachment of AuNPs-aptamer to NCDs completely quenched the NCDs’ emission through FRET interactions. However, in the presence of the target, the configuration of the aptamer was changed causing NCDs to split and move out. Therefore, the AuNPs were away from the NCDs’ surface, and the fluorescence of NCDs was restored.

**Experimental**

**Material**

Sodium hydroxide, ethylenediamine, phosphate salt buffer, citric acid, (98%, suitable for fluorescence), and hydrogen tetrachloroaurate(III) hydrate were obtained from Merck (≥ 99%, Merck Co., Darmstadt, Germany). IL-6 and quinine sulfate were purchased from Sigma-Aldrich (Sigma-Aldrich Co., Saint Louis, Missouri, USA). Two sequences of single-strand DNA aptamers (Table 1) were designed based on the previous studies [28] and synthesized by DynaBio and Tacapo Zist Company (Iran). The reagents and chemicals were applied as received with no purification.

**Instruments**

Cary Eclipse spectrofluorimeter were used to carry out all fluorescence emission intensity measurements (Varian Inc., Mulgrave, Australia). The absorption spectra were recorded by the Varian Cary 400 scan UV–Vis spectrophotometer. FT-IR spectra were obtained using a Fourier transform infrared (FT-IR) spectrometer (Tensor 27, Bruker). The dynamic light scattering (DLS) and zeta potential measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments, U.K). The morphologies and sizes of AuNPs and NCDs were characterized by the high-performance TEM (HRTEM, Zeiss-EM10C-100 kV, Germany) operating with the 200 kV operating voltage. Before microscopic imaging, the samples were coated and then air-dried on the copper grid (200 mesh).

**Preparation of AuNPs functionalized DNA aptamer (AuNP-Apt)**

Gold nanoparticles were synthesized by the classical Turk-ievich (see Sect. 1 in supporting information for detail) [29].

The prepared AuNPs in 10 mM PBS at pH 7.4 were mixed with 1.5 equivalents of the thiolated oligonucleotide for 24 h at room temperature. Subsequently, the mixture was purified by ultracentrifugation (32,000 g) after washing with buffer to yield the gold nanoparticle-DNA aptamer conjugate.

**Synthesis of carbon quantum dots by hydrothermal method**

NCDs were synthesized using one-pot hydrothermal technique according to our previous report (see Sect. 2 in supporting information for detail) [30].

**Table 1** The sequences of the ssDNA aptamer

| Sample name | Sequence (5'-3') |
|-------------|-----------------|
| 1           | SH-CTTCCAACGC   |
|             | CTGCTATTG       |
|             | TCACTCTTT       |
|             | AGT-3'          |
| 2           | SH-(CH2)6-TGG   |
|             | TGGATGCGCG      |
|             | CAGTCGGCG       |
|             | ACA-3'          |
Rhodamine 6G and Quinine hemisulfate have been used for quantum yield (QY) measurement of the quantum dots by our group (see Sect. 2 in supporting information for detail) [31–34].

Preparation of aptasensor

The FRET-based aptasensor was provided by dispersing 100 µL of aptamer-coupled gold nanoparticles (AuNP–Apt) into the 500 µL of PBS, and then 100 µL of NCD was added. Afterward, the centrifuge process (15,000 g) was used for the purification of the sensing probe (AuNP–Apt/NCD). Finally, the sensing probe was resuspended into the buffer solution.

One hundred microliters of the IL-6 solution with the different concentrations was added into the aliquots of the sensing probe (AuNP–Apt/NCD). After 1-h incubation at 37 °C, the fluorescence signal data were collected. According to Förster’s theory, FRET efficiency, $E$, is given by [35, 36]:

$$E = \left( 1 - \frac{F_{\text{probe}}}{F_{\text{recovery}}} \right) \times 100$$

where $F_{\text{probe}}$ is the fluorescence intensity of the probe and $F_{\text{recovery}}$ is the fluorescence intensity after the addition of the target.

IL-6 Detection in human serum

The quantitative detection of IL-6 was carried out in the human serum. The sample was twenty times diluted with PBS (pH 7.4). Then, the same technique as that in the standard solution was applied. The sensing probe was incubated in serum samples spiked with IL-6 at different concentrations followed by measuring of the fluorescence intensity.

Result and discussion

Characterization of AuNPs

AuNPs were synthesized by the reduction of HAuCl$_4$ at 100 °C. In this technique, citric acid acts as an efficient reducing as well as a coating agent on the surface of gold nanoparticles. The formation of AuNPs was confirmed by the UV–Vis spectrum. Not only the red color of the sample but also the size-dependent localized surface plasmon resonance (SPR) absorption, located at ~520 nm, demonstrates the successful formation of AuNPs (Fig. 1A-a) [29, 37]. The absorption maxima red-shifted upon modification with aptamer, indicating refractive index change on the surface of the AuNPs (Fig. 1A-b). The particle concentration of the AuNPs (12 nmol L$^{-1}$) was determined using an extinction coefficient of 6.15 × 10$^7$ L mol$^{-1}$ cm$^{-1}$ at 450 nm [38].

The TEM image, DLS analysis, and FT-IR spectra of the AuNPs were shown in the Fig. 1B, C, and D, respectively (see Sect. 3 in supporting information).

Characterization of NCDs

In continuation of our studies for the preparation of fluorescence NCD and considering their potential as a fluorescence probe, herein, we synthesized NCD according to our previous reports [39–41]. To investigate the optical properties of NCD, fluorescence spectra and UV–Vis absorption spectra analyses were carried out. Figure 2A indicates that NCD aqueous solution possesses optical absorption spectra in the UV–Vis region (black line), with a shoulder and a maximum peak at 235 and 330 nm, respectively, which can be related to the $\pi$-$\pi^*$ (C = C band) and $n$-$\pi^*$ (C = O band) transitions [42–44]. Furthermore, the fluorescence spectrum of NCD (blue line) exhibited an emission maximum at 445 nm ($\lambda_{ex} = 350$ nm), as shown in Fig. 2A.

Figure 2B shows the dependence of fluorescence emission of NCD on the excitation wavelength. By increasing excitation wavelength, the intensity of fluorescence emission initially increased and then steadily decreased. As the excitation wavelength changed from 300 to 400 nm, the emission wavelength exposed red-shift from 440 to 457 nm. This property is ascribed to the confinement energy observed in NCD through the energy of the band gap [45]. The HRTEM image and FT-IR spectra of NCD were shown in the Fig. 2C and D, respectively (see Sect. 4 in supporting information).

The quantum yields of the NCD were computed by the slope method through measuring and regressed the emission ($\lambda_{em} = 445$ nm) and absorbance ($\lambda = 350$ nm) of NCDs (Figure S1A) and compare it with the quinine hemisulfate (Figure S1B). Based on the results, the quantum yield of 5% was calculated for the NCDs. Consequently, the prepared NCDs consist of EDA and citric acid achieved without further surface modification showed inherently superior and comparable quantum yield to other carbon quantum dots synthesized from the other precursors using hydrothermal method [46].

Preparation of aptasensor

In the FRET process, NCDs transferred their energy to the quencher AuNPs, and thus, their fluorescence was quenched. It is essential for the FRET process that the donor emission spectrum overlaps the absorption spectrum of the acceptor which expresses a large influence on measured transfer efficiency [33]. In Figure S2A, the absorption spectrum of the AuNPs overlaps with the emission spectrum of NCDs. Consequently, in our FRET system, this spectral overlap guaranteed that the NCD and AuNP mixture is an appropriate candidate for fluorescence quenching.
The emission intensity of the NCD fluorescence peak (Figure S2B-a) significantly decreases in the presence of Apt-AuNP, representing the formation of NCD-Apt-AuNP conjugate (Figure S2B-b). However, the fluorescence emission was recovered to 50% of the initial value (NCD) in the presence of 1.5 pg mL⁻¹ IL-6 (Figure S2B-c).

Moreover, the zeta potential was utilized as an evidence of preparation of the probe by measuring the electronic charge on the nanoparticles surfaces in various environments. The zeta potential of the citrate-coated NCD was primarily $-3.53 \pm 0.5$ mV (Figure S2C). However, in the presence of the Apt-AuNPs (5 µM), the attending NCDs to the aptamer produced a significant reduction of the zeta potential to $-22.2 \pm 0.8$ mV, which may be attributable to the negative nature of the added DNA to the constructs. These data confirmed the successful preparation of the biosensor probe.

**Detection of IL-6 using aptasensor**

The principle of IL-6 detection by the FRET pair of NCDs and AuNPs was schematically described in Fig. 3. The thiol-modified aptamers (Table 1) capable of target recognition were conjugated to the surface of AuNPs through Au–S chemistry. Some studies have showed that carbon particles are able to attach with DNA probe via a $\pi-\pi$ stacking corridor [47] which makes NCDs hybridized with AuNPs-aptamers. For comparison between the band gap energy of the NCDs and the localized surface plasmon resonance (LSPR) energy of the AuNPs, a theoretical expected value for the band gap of the synthesized NCDs was also calculated. Because quantum confinement affects the band gap energy of particles less than 10 nm in size, the effective mass approximation model in below equation has been used to estimate the band gap of the prepared sample [48]. Size-dependent fluorescence of the quantum dots is related to
their size-dependent band gap of these artificial atoms, and
quantum confinement increases the band gap. As the elec-
tron–hole distance approaches and becomes smaller than the
Bohr radius, the electron–hole transition is confined to the
quantum size which results in an increasing band gap and
a blue shift in fluorescence. The shift in the band gap that
results from quantum confinement of the electron–hole pair
(exciton) in a quantum dot with radius $r$ can be [42]:

$$\Delta E_g = \frac{\hbar^2 r^2}{2 \varepsilon r^2} \left( \frac{1}{m_e^*} + \frac{1}{m_h^*} \right) - \frac{1.78e^2}{\varepsilon r} - 0.248E_{\text{Ry}}$$

where $E_{\text{Ry}}$, the Rydberg energy, is size dependent and can be
ignored except for quantum dots with small dielectric con-
stant ($\varepsilon$). It is assumed that the CQDs are epitaxial graphene
and $m_e^*$ and $m_h^*$ to be 0.19 $m_e$ and 0.25 $m_e$, respectively ($m_e$ =
free electron mass). Also in the absence of quantum confine-
ment, the gap energy is zero. The dielectric constant of gra-
phene is extreme, and thus, the second term in the equation
can be ignored. Thus, the band gap of 2.23 eV was calcu-
lated for 2.5 nm CQDs. On the other hand, the wide $\sim$ 520-
nm LSPR peak of the AuNPs shows the resonance energy
of $\sim$ 2.36 eV (at the peak maxima) that well matches the band
gap of the CQDs for radiation less energy transfer. Thus, this
FRET-based sensor involves the energy transfer between the
donor/acceptor pairs. In the absence of IL-6, the AuNPs that
play the acceptor role are efficiently nearby the donors
(NCDs), resulting in the decrease of emission intensity of
the NCDs that arises from their extremely overlapping spec-
tra. The interaction of IL-6 with the detection probe causes
the aptamers to attach preferentially to IL-6, resulting in
structural and conformational changes that cause dissocia-
tion of the NCDs from the aptamers, so that the fluorescence
emission recovers [49]. The increase of the fluorescence
emission is relative to the IL-6 amounts.

To evaluate the detection range, sensitivity, and
limit of detection (LOD) of the designed aptasensor for
IL-6, different concentrations of the IL-6 were mixed
with the detecting probe solution and then incubated
at room temperature for 10 min. Subsequently, the cor-
responding emission spectra (350–600 nm) were docu-
mented. Fluorescence emission intensity of different IL-6
amounts is presented in Figure S3A. The results showed
a good linear relationship between emission intensity and

![Fig. 2 Characterization of the synthesized NCDs. A The UV–Vis
spectrum (black) and fluorescence emission spectrum with 350 nm
excitation wavelength (blue) of NCDs; B fluorescence emission spec-
tra of NCDs with different excitation wavelengths in the range of
300–400 nm; C HRTEM image of NCDs; and D FTIR spectrum of
NCD](image)
IL-6 concentrations in the range of 1.5 to 5.9 pg mL$^{-1}$ ($R^2 = 0.994$) at emission wavelength of 445 nm (Figure S3B). The high sensitivity and also the linearity over a wide concentration range of the IL-6 makes our proposed aptasensor comparable or even superior than those presented in other literature (Table S1). The calculated LOD was 0.84 and 0.82 pg mL$^{-1}$ (3σ) for probe a and b, respectively. The aptasensor can be applied for quantitative recognition of IL-6 and, hence, emerged as an encouraging candidate for greatly sensitive assays utilized in clinical laboratory tests.

### Specificity of aptasensor

The specificity was investigated as a considerable method validation parameter to evaluate sensor response. The specificity of biosensor for IL-6 (1.5 pg/mL) detection in the presence of interfering proteins and cytokines (PBS as control, BSA, and TNF-a) was tested ($n = 3$), and no significant signal changes, confirming the specific detection of IL-6. Non-specific binding molecules such as bovine serum albumin (BSA), PBS sample, and TNF-a gave similar signal with no substantial variation in sensor response.

Additionally, the developed aptasensor was used to detect IL-6 in human serum, with the recovery results shown in Table 2. The results showed that IL-6 recoveries in human serum were between 95.7 and 102.9%, while the RSDs were from 3.9 to 6.7%.

The proposed aptasensor has compared with different fluorescent IL-6 sensors in electronic supporting information (Sect. 5 in electronic supporting information). Table S1 shows satisfactory of this work in comparison with published articles. Nonetheless, we believe this research provides a promising starting point for the future development of highly sensitive, real-time IL-6 detection. However, there is also a problem with the proposed probe. Patients with severe sepsis may have a high concentration above 500 pg/mL that is above the linear dynamic range and needs to be solved. Also, the red shift of the excitation and emission wavelength should be considered in order to prevent their screened off by UV absorbers that results in weaken signal.

| Sample | IL-6 spiked (pg/mL) | Founded (pg/mL) | RSD ($n = 3$, %) | Recovery (%) |
|--------|---------------------|-----------------|-----------------|--------------|
| 1      | 2.5                 | 2.4 ± 0.1       | 4.9             | 97.6         |
| 2      | 3.5                 | 3.6 ± 0.2       | 6.7             | 102.9        |
| 3      | 4.7                 | 4.5 ± 0.2       | 3.9             | 95.7         |

Table 2 Determination of IL-6 concentration in spiked human blood serum sample
Conclusions

The low abundance of IL-6 and increasing relevance as a prognostic marker for infections such as rheumatoid arthritis, inflammatory bowel disease, and glomerular nephritis make it an ideal model analyte. Elevated levels of IL-6 probably threaten the vital factors and have a crucial role in immune-mediated acute lung injury in COVID-19 patients. Herein, a low-cost FRET-based aptasensor for IL-6 sensing was developed using AuNPs and NCDs. This aptasensor performed well both in aqueous buffer solution and human blood serum samples and have high potential for utilization in diagnosis, treatment, and immunization of biomedicine, but the main limitation of the aptasensor is for determination of IL-6 in patients in severe sepsis that may have a high concentration above 500 pg/mL and also the UV excitation and emission wavelength of the probe that can be screened of by the UV absorbers.

Supplementary Information  The online version contains supplementary material available at https://doi.org/10.1007/s00604-022-05570-5.

Declarations

Conflict of interest  The authors declare no competing interests.

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