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Construction of Fluorescence Sensing Platform on the Basis of Carbon Nitride Nanosheet for the Detection of Interferon-γ

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Abstract

Purpose: Interferon-γ (INF-γ) is a cytokine that participates in the immune reaction of the body. Its level of secretion can reflect the immune response condition after the body is infected by pathogens, which is a significant indication of clinically-related diseases. Therefore, it is of great significance in application to develop a fluorescence biosensor to inspect INF-γ with rapidness, high sensitivity and high practicability. **Method:** The fluorescence sensor is made on the basis of the two-dimensional nano-material namely Carbon Nitride Nanosheet (CNNS) and the Aptamer probe to identify INF-γ (Apt@INF-γ). CNNS can quickly quench the Cy5 fluorescent dye modified on the Apt@INF-γ probe due to the Photoinduced Electron Transfer (PET), but when the INF-γ exists, Apt@INF-γ specifically identifies and combines it. The complex of Apt@INF-γ and INF-γ is away from CNNS, which can effectively block the fluorescent signal of Apt@INF-γ being quenched by CNNS. **Result:** The sensitive detection of IFN-γ protein can be achieved through the application of CNNS/Apt@INF-γ fluorescence sensing platform. In this method, the intensity of the fluorescent signal is positively correlated with the concentration of IFN-γ, of which the liner response range is 0.5 - 100 ng/mL and the limit of detection is 0.303 ng/mL. In addition, this fluorescence sensing platform has the advantages of high specificity, simple operation and low costs. It can inspect the content of IFN-γ in clinical serum samples without interference. The actual recovery rate of serum samples is 97.11% - 106.96%. **Conclusion:** Therefore, the CNNS/Apt@INF-γ sensing platform is expected to be implemented in the actual clinical detection, also conducive to developing a universal fluorescence biosensor to inspect other target materials.

Keywords

Carbon Nitride Nanosheet, Aptamer, Interferon-γ, Fluorescence Sensing

Analysis

1. Introduction

The Interferon-γ (IFN-γ) is a kind of soluble homologous protein with activity and a molecular weight of 16.5 KDa. As the only Type II interferon, it is a cytokine secreted by T ancillary cells, cytotoxic T cells, natural killer cells and macrophages under the stimulus of certain endogenous and exogenous factors, featuring the functions of anti-virus, anti-tumor, cell proliferation resistance and immune regulation [1] [2] [3]. IFN-γ participates in the immune reaction of the body caused by the infection of many pathogens. Therefore, the level of IFN-γ can be used to evaluate the immune response conditions of cells to pathogens. In clinical practice, the change in IFN-γ level is indicated in the diagnosis of infectious diseases such as tuberculosis [4] [5] [6], AIDS [7] and brucellosis [8]. It can also be used for the auxiliary diagnosis and disease course monitoring of diseases such as liver cancer [9], cervical cancer [10] and breast cancer [11].

Carbon Nitride Nanosheet (CNNS) is an emerging functional nano-material, which can be used in multiple fields, for example, biosensing, drug transport and catalysis application. As a two-dimensional nano-material, CNNS has many advantages, including stable structure, simple preparation and good biocompatibility [12] [13]. Due to its large surface area, CNNS can absorb various biomolecules. Compared with dsDNA, CNNS expresses a stronger affinity than that of ssDNA. CNNS has the capacity of fluorescence quenching [14] [15], which has a great prospect in the application of biological detection. Compared with other nano-material, the preparation of CNNS is of low cost, which makes it conducive to practical implementation. Most research on CNNS focuses on the development of optimized synthesis methods and the fluorescence property, and the research on its fluorescence quenching property is relatively less. Its application research needs to be further developed.

The IFN-γ level in the serum of clinical patients has been implemented into the detection of the process of related diseases, so the detection of IFN-γ level is of great importance. At present, the frequently used method to inspect IFN-γ level is Enzyme-Linked Immunosorbent Assay (ELISA) [16] [17] [18], which has low stability, long time-consuming and large sample size needed. The current research designs a simple and sensitive detection by developing a fluorescence sensor with the aptamer Apt@IFN-γ modified by the anthocyanin fluorescent dye (Cyanine 5.5, Cy5) as the identifying component and the signal probe and CNNS as the fluorescence quenching agent to construct a new type of fluorescence sensing platform CNNS/Apt@IFN-γ together to inspect Interferon-γ. In the sensing process, CNNS is to conduct fluorescence quenching as the receptor. The large amount of π-π on the surface of CNNS accumulates to make it easy to absorb the aptamer, then leads to the photoinduced electron transfer, causing

the fluorescence quenching of Cy5. When the target molecule exists, the aptamer combines with the specificity of the target molecule, then the spatial structure of the aptamer changes, leading to the aptamer being away from CNNS. The transfer of optoelectronics is blocked, and the fluorescence of Cy5 can be expressed normally, which realizes the fluorescence sensing. The current research will inspect the property of this sensing strategy.

2. Materials and Methods

Human recombinant IFN-γ protein, Bovine Serum Albumin (BSA), D-Galactoallic (Gal) and Tris-HCl buffer (20 mM Tris, 5 mM KCl, 5 mM$MgCl_2$, 100 mM NaCl, pH 7.4) are purchased from Sangon Biotech (Shanghai) Co., Ltd.; Interferon-α (IFN-α), Interferon-β (IFN-β) and Tumor Necrosis Factor (TNF) are purchased from R&D systems (Minneapolis, USA); Fetal Bovine Serum (FBS) is purchased from gibco Company (Grand Island, USA) the water used in the experiments is all ultra-purified water; the IFN-γ aptamer sequence marked by Cy5 fluorescent dye is as follows: 5'-Cy5-GGG GTT GGT TGT GTT GGG TGT TGT GTC-3', composed by Sangon Biotech (Shanghai) Co., Ltd. All samples are reacted under the temperature of 37˚C. The fluorescence spectrophotometer (RF-6000, Shimadzu) is used to conduct fluorescence detection.

2.1. Methods

2.1.1. Preparation of CNNS

CNNS was synthesized based on our previous method [19]. Melamine molecules were polymerized at high temperature to form g-C3N4, and then g-C3N4 was processed with ultrasonic treatment in water and centrifugation at 6000 rpm to remove accumulated block-shaped g-C3N4. Take the supernatant and conduct centrifugation at 15000rpm to obtain the sediment CNNS. Dissolve CNNS again into the ultra-purified water, then prepare CNNS with the concentration of 1 mg/mL as the reserve solution and store it at 4˚C for the later use. Before using it, process it with ultrasonic treatment for 20 min in advance to ensure its good dispersibility.

2.1.2. Optimization of Experimental Conditions

1) Optimization of the Concentration of CNNS: In order to determine the best fluorescence quenching effect of this sensing method, fix the concentration of the aptamer probe Apt@IFN-γ as 50 nM, then react the Apt@IFN-γ probe and the CNNS with different concentration (0, 10, 20, 40, 60, 80, 100 and 120 μg/mL) at the temperature of 37˚C. Use the fluorescence spectrophotometer to measure the fluorescence intensity and observe the fluorescence quenching conditions. This is repeated for 3 times in parallel for each sample.

2) Optimization of Quenching Time: Incubate 50 nM Apt@IFN-γ and the CNNS with the concentration of 60 μg/mL for a certain time (1, 2, 4, 6, 8, 10, 15, 30, 40, 60 and 120 min) under the temperature of 37˚C. After the incubation, use the fluorescence spectrophotometer to measure the fluorescence intensity and

observe the fluorescence quenching conditions. This is repeated for 3 times in parallel for each sample.

3) Optimization of the Reaction Time of Apt@IFN-*γ* and IFN-*γ*: Incubate 50 nM Apt@IFN-*γ* and 50 ng/mL IFN-*γ* for different durations (0, 10, 20, 30, 40, 50 and 60 min) under the temperature of 37˚C separately. After the incubation, add the CNNS with the concentration of 60 μg/mL into the reaction system and conduct incubation with fluctuation for 10 min under the temperature of 37˚C, then use the fluorescence spectrophotometer to measure the fluorescence intensity and observe the best reaction time of the sensing platform. This is repeated for 3 times in parallel for each sample.

2.1.3. Fluorescence Analysis of the Sensing System

1) Feasibility Analysis: The current research needs to discuss the changes of fluorescence sensing signals with different ingredients (Apt@IFN-*γ*, CNNS, IFN-*γ*, CNNS + Apt@IFN-*γ* and CNNS + Apt@IFN-*γ* + IFN-*γ*). After the incubating reaction according to the sensing scheme, record the emission spectrometry within the range of 655 - 750 nm under the excitation of 648nm, observe the fluorescence expression conditions and analyze the feasibility of CNNS/Apt@IFN-*γ* sensing strategy.

2) Sensitivity Analysis: Conduct quantitative detection on IFN-*γ* to explore the detection sensitivity of this sensing method. Firstly, use Tris-HCl buffer to prepare the IFN-*γ* stock solution with the concentration of 1.5 μg/mL. Dilute the Apt@IFN-*γ* marked by Cy5 in the Tris-HCl buffer preliminarily to 150 nM as the reserve solution. Under the best experimental conditions, add the IFN-*γ* reserve solution with different volumes (1, 4, 8, 16, 20, 60, 120, 200, 300, 400 and 600 μL) and fully mix it with 1 mL Apt@IFN-*γ* (150 nM), then add Tris-HCl to 2 mL and incubate it with fluctuation under the temperature of 37˚C for 40 min. After the incubation, add 0.18 mL CNNS mother liquor (1 mg/mL) and then use Tris-HCl buffer to fix the volume to 3 mL. Continue the reaction of the solution for 10 min. Finally, use the fluorescence spectrophotometer to measure the fluorescence intensity F, and record the fluorescence intensity of the inspected samples without IFN-*γ* as F_0. Each concentration is measured in parallel for 3 times. Record the emission spectrometry within the range of 655 - 750 nm under the excitation wavelength of 648 nm. Use x coordinate to represent the concentration of IFN-*γ* and y coordinate to represent the fluorescence intensity of the system and draw a standard curve, then calculate and analyze the liner range of the sensing detection and the lower limit of detection.

2.1.4. Specificity Detection

In order to examine the specificity to for the IFN-*γ* detection of this sensing platform of CNNS/Apt@IFN-*γ*, prepare the IFN-*γ* with the concentration of 50 ng/mL and the IFN-*α*, IFN-*β*, TNF, Gal and BSA with the concentration of 100 ng/mL as the samples to be tested. In addition, mix 50 ng/mL of IFN-*γ* and 100 ng/mL of IFN-*α*, IFN-*β*, TNF, Gal and BSA separately to prepare the mixed so-

lution as the sample to be tested, and prepare the blank control group without any protein molecules. Add Apt@IFN-γ (150 nM) probe solution with concentration of 1mL into 1mL of sample to be tested separately and fully mix them, then conduct incubation with fluctuation under the temperature of 37˚C for 40 min. After the incubation, add 0.18 mL CNNS (1 mg/mL) solution and 0.82 mL Tris-HCl buffer and fully mix them for 10 min incubation reaction. Use fluorescence spectrophotometer to measure the fluorescence intensity under the excitation wavelength of 648 nm and the mission wavelength of 667 nm. Each sample is repeatedly measured in parallel for 3 times, then compare and analyze the fluorescence intensity in the detection system with different molecules.

2.1.5. Detection of Samples

This is to examine the performance of CNNS/Apt@IFN-γ fluorescence sensing platform in the actual detection of serum samples. Add IFN-γ with three concentrations (0.5, 10 and 30 ng/mL) separately into 10% FBS under optimal conditions to prepare the samples to be tested. After the incubating reaction under the sensing scheme, detect the fluorescent signals. Conduct parallel experiments 5 times for the samples with each concentration, and set a sample without IFN-γ as the blank sample. Calculate the recovery rate to evaluate the performance of this sensing system in the actual detection serum samples.

3. Result and Discussion

3.1. Experiment Principle

The detection principle of the CNNS/Apt@IFN-γ fluorescence sensing platform constructed by the current research is as shown in **Figure 1**. CNNS absorbs the Apt@IFN-γ due to the conjugation of π-π. Because that the reduction of spatial distance between CNNS and Apt@IFN-γ after the absorption will generate interaction and cause electron transfer and energy transmission. With CNNS as the energy receptor and the Cy5 modified on the Apt@IFN-γ probe as the fluorescence donor, the Photoinduced Electron Transfer (PET) occurs between the two objects. Such process is the electron transfer of Cy5 under optical excitation to the conduction band of CNNS causing the fluorescence quenching. When the IFN-γ exists, after the specificity binding between Apt@IFN-γ and IFN-γ, the spatial constructure of Apt@IFN-γ changes. When the CNNS fluorescence quenching agent is added, the binding material of Apt@IFN-γ and IFN-γ will not be absorbed by CNNS. The Apt@IFN-γ probe dissociated in the solution but not binded with the IFN-γ of object target expresses the sturcture of single-stranded DNA, which will be absorbed by CNNS to cause the fluorescence quenching of the Cy5 fluorophore modified on the aptamer probe. As the concentration of IFN-γ grows, the fluorescent signal released by Apt@IFN-γ will be increased, so as to realize the quantitative analysis of IFN-γ. The method constructed by the current research will provide a potential new type of sensing platform for the clinical detection of IFN-γ.

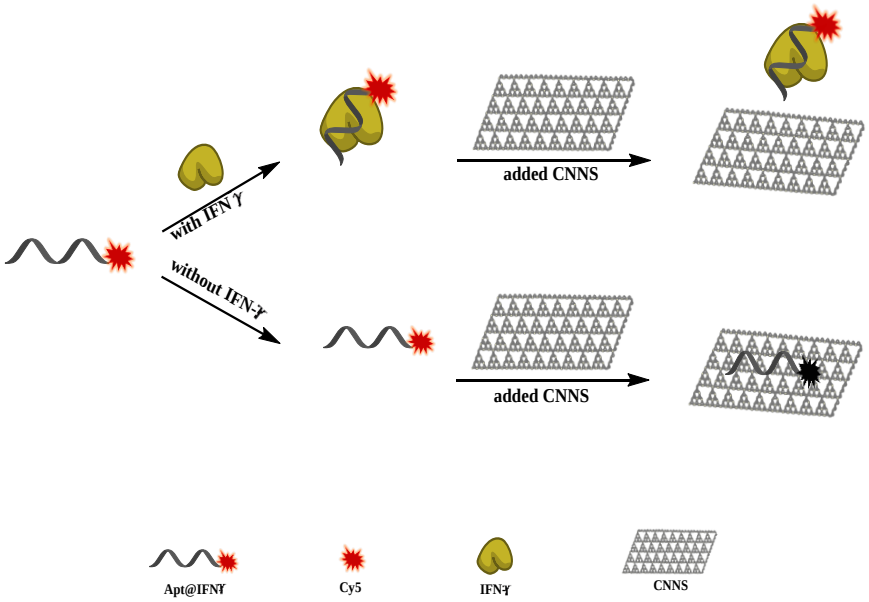

Figure 1. Schematic illustration of the principle of the method for IFN-*γ* detection based on fluorescence quenching of carbonitride nanosheets.

3.2. Feasibility Analysis

We have evaluated the feasibility of CNNS/Apt@IFN-*γ* as the fluorescence sensing platform to inspect IFN-*γ*. Conduct fluorescent detection by using Apt@IFN-*γ*, CNNS and IFN-*γ* as the separate ingredients of samples to be tested. As shown in **Figure 2**, the ingredient of the Apt@IFN-*γ* sample is the recognition probe modified by Cy5. Its detection result shows that a strong fluorescent signal appears at the position of 667 nm. While the detection results of CNNS and IFN-*γ* samples show that the fluorescent signal at the position of 667 nm is almost zero, which indicates that the quenching agent CNNS and the IFN-*γ* ingredient of the target molecule will not interfere the sensing platform to conduct fluorescent detection for the specificity of IFN-*γ*. The fluorescent detection result of CNNS + Apt@IFN-*γ* sample indicates that CNNS, as the fluorescence quenching agent, can effectively quench the fluorescence on the aptamer probe after being binded with Apt@IFN-*γ*. The background signal of this constructed sensing platform is low, which is conductive to improve the accuracy of the IFN-*γ* detection. The CNNS + Apt@IFN-*γ* + IFN-*γ* sample is the target object with complete ingredients of the sensing system, and the detection result indicates that with the existence of a certain concentration of molecular target, a strong fluorescence emission peak appears at the 667 nm position of the emission spectrometry. Therefore, the sensing platform of CNNS/Apt@IFN-*γ* can take advantage of the fluorescence sensing signal depended by the target molecule concentration to monitor the target molecule of IFN-*γ*, which means that this method is feasible.

3.3. Optimization of Experimental Conditions

To determine the best detection performance of the CNNS/Apt@IFN-*γ* sensing

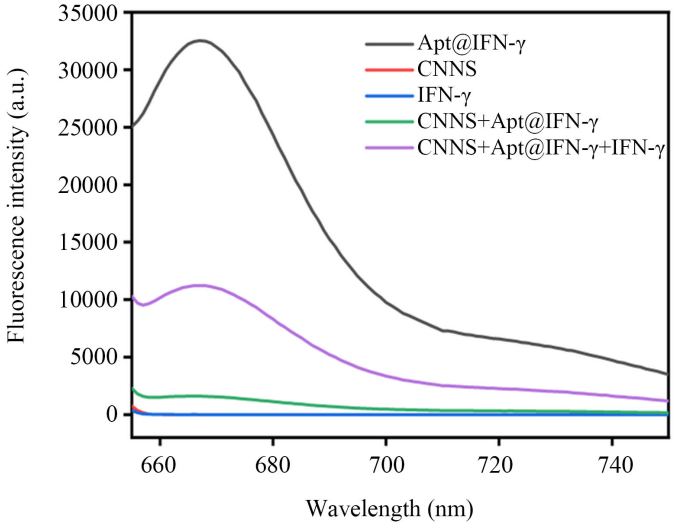

Figure 2. Fluorescence emission spectra under different conditions: Apt@IFN-γ, CNNS, IFN-γ, CNNS + Apt@IFN-γ and CNNS + Apt@IFN-γ + IFN-γ (with the presence of 50 ng/mL IFN-γ). The concentrations of Apt@IFN-γ and CNNS were 50 nM and 60 μg/mL, respectively.

platform, we optimize the concentration of CNNS, the quenching time and the reacting time between the probe and the IFN-γ of target objects. With CNNS as the fluorescence quenching agent, whether the fluorophore Cy5 on the probe can be effectively quenched by CNNS is the key to the feasibility of this sensing method. The result is as shown in **Figure 3(A)**. We fix the Apt@IFN-γ with the concentration of 50 nM. When we add the CNNS with different concentrations, the fluorescent signals of Apt@IFN-γ aptamer is weakened constantly as the increase of the CNNS concentration. When the CNNS concentration reaches 60 μg/mL, the fluorescence quenching effect of the reaction system becomes gradually stable. As shown in **Figure 3(B)**, the fluorescence quenching efficiency of CNNS can reach 95.2%. In terms of the quenching reaction time, as shown in **Figure 3(C)**, when the CNNS is added into the fluorescent probe, the fluorescent signals can be quenched quickly. When the reaction time reaches 10 min, the fluorescence quenching system becomes stable and the quenching performance remains stable when the reaction time reaches 120 min. The best reaction time of Apt@IFN-γ and IFN-γ is as shown in **Figure 3(D)**. When the reaction time of Apt@IFN-γ and IFN-γ reaches 40 min, the inspected fluorescent signal intensity reaches to the highest level and the fluorescence intensity remains unchanged when the reaction time reaches 60 min, which indicates that the detection signal of this sensing platform has the good stability. Therefore, the best experimental condition of this sensing strategy is that the reaction time of the aptamer probe and the target object reaches 40 min. To ensure the lowest background signal, the concentration of CNNS shall be 60 μg/mL and the quenching time is 10 min. Such conditions will be used in subsequent experiments.

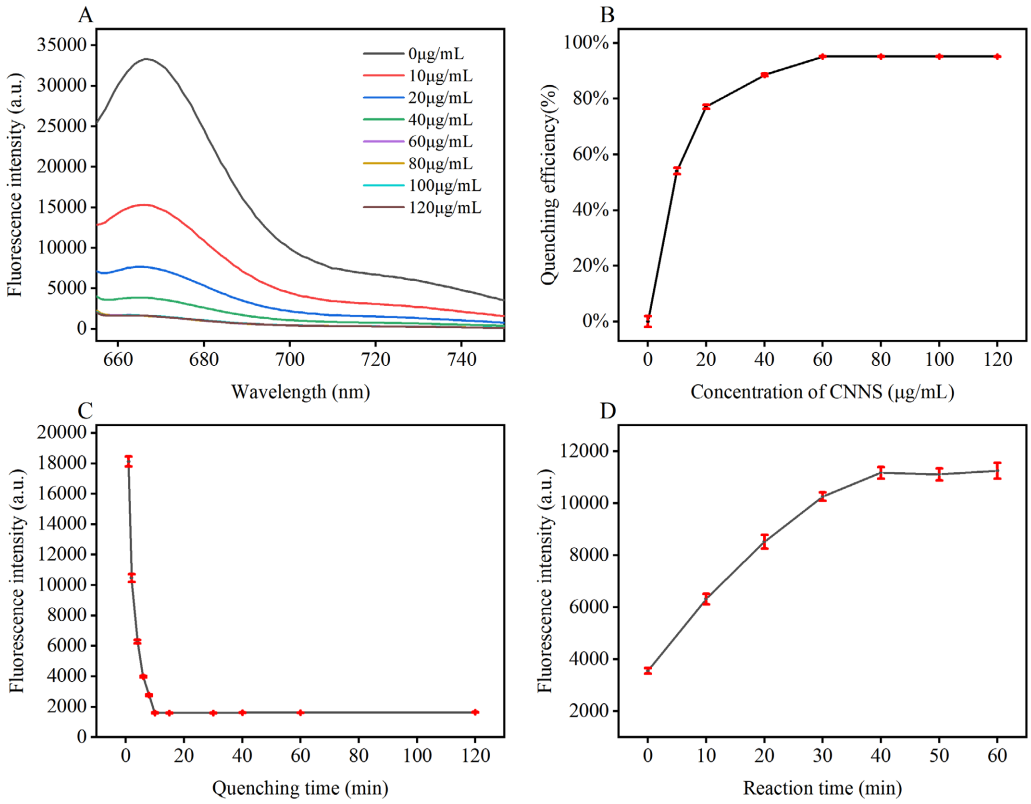

Figure 3. (A) The optimization of CNNS concentration. The concentration of Apt@IFN-γ was 50 nM. (B) The quenching efficiency of CNNS. (C) The fluorescence quenching time. The concentrations of Apt@IFN-γ and CNNS were 50 nM and 60 μg/mL, respectively. (D) Reaction time of 50 nM Apt@IFN-γ and 50 ng/mL IFN-γ, and then added 60 μg/mL CNNS to continue reaction for 10 min.

3.4. Sensitivity Analysis

With the best reaction conditions, we further explore the relations between the fluorescent signal intensity and the IFN-γ concentration in this reaction system. Adjust the excitation wavelength to 648 nm to measure the fluorescence emission spectrometry within the range of 655 - 750 nm and record the value of fluorescence intensity at the 667 nm position of mission wavelength. The result is as shown in **Figure 4**. When the IFN-γ concentration is within the range of 0.5 - 100 ng/mL, the value of fluorescence intensity ΔF ($\Delta F = F - F_0$) is positively correlated with the concentration of IFN-γ. The linear regression equation is $\Delta F = 192.823X + 74.074$, and the correlation $R^2 = 0.9945$. As the increase of IFN-γ concentration, the fluorescent signal intensity of the sensors is significantly enhanced. The Limit Of Detection (LOD) can be calculated by the ratio of the standard deviation of fluorescence intensity of three-time blank sample solution to the slope of the standard curve, which is 0.303 ng/mL. Therefore, this sending method can be applicable for the quantitative detection of IFN-γ. Compared with the research of other IFN-γ detection method, although the electrochemical detection method has a lower LOD, but has a longer pretreatment time and more steps [20] [21]. ELISA, as a traditional detection method, features a good

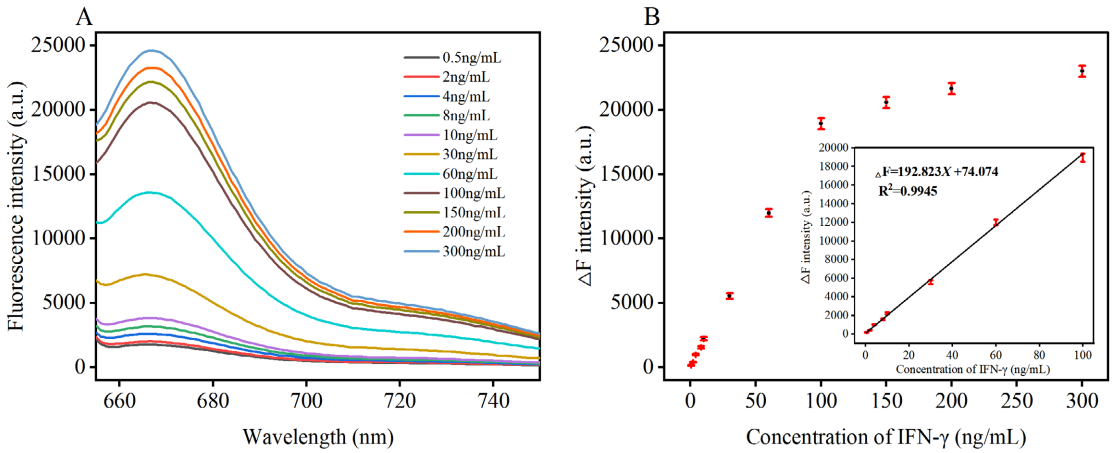

Figure 4. (A) Fluorescence emission spectra of IFN-*γ* with different concentrations. (B) Standard curve of fluorescence intensities against the concentrations of IFN-*γ*. The concentrations of Apt@IFN-*γ* and CNNS were 50 nM and 60 μg/mL, respectively. Reaction on 37˚C for 40 min, and then added 60 μg/mL CNNS to continue reaction for 10 min.

sensitivity, but has cumbersome operation, long time-consuming, high costs and deviations of detection results between experimental lots [18] [22]. This CNNS/Apt@IFN-*γ* sensing platform has the advantages of simple and fast operation, which can also meet the clinical application requirement.

3.5. Specificity Analysis

Selectivity is the key issue to develop a diagnostic detection method. The selectivity examination result of the constructed CNNS/Apt@IFN-*γ* sensors is as shown in **Figure 5**. When the target molecule IFN-*γ* exists, a strong fluorescent signal expression is detected by this sensing platform, while in actual conditions, there are some other molecules in the serum, such as IFN-*α*, IFN-*β*, TNF, Gal and BSA. When these molecules exists in the reaction system, there is no obvious fluorescent signal response detected by this sensing platform, while in the mixed solution, the CNNS/Apt@IFN-*γ* sensing platform can detect the fluorescent signal expressed by the existed IFN-*γ* in a relatively accurate way, which is very close to the fluorescent signal intensity expressed by the single IFN-*γ* ingredient. These results indicate that the CNNS/Apt@IFN-*γ* sensor can detect the content of IFN-*γ* with specificity, and its fluorescent response to IFN-*γ* is much higher than that to other interfering molecules. Even though various kinds of interfering molecules exist simultaneously with IFN-*γ*, the sensor can still maintain sufficient sensitivity and accuracy, and this fluorescence sensor features good distinguishing performance, which means that the CNNS/Apt@IFN-*γ* sensor developed by the current research is of high selectivity and of potential possibility in the application of complex sample analysis.

3.6. Sensing Application in Serum Samples

Clinical serum is the preferred sample to inspect IFN-*γ*, also detection sample with the easy access clinically. The IFN-*γ* in serum has the potential to become

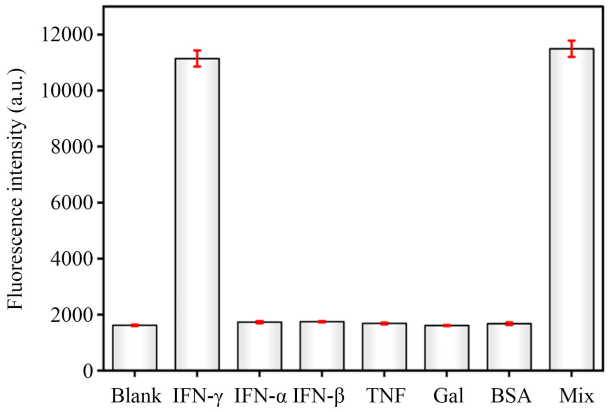

Figure 5. The selectivity of the Fluorescence assay. The concentration of IFN-*γ* was 50 ng/mL, nonspecific molecules were all 100 ng/mL and the concentration of mixed liquid: IFN-*γ* was 50 ng/mL, IFN-*α*, IFN-*β*, TNF, Gal and BSA were all 100 ng/mL. Blank sample was without IFN-*γ*.

Table 1. Determination results of IFN-*γ* in Serum samples (n = 5).

Sample	IFN-*γ* Spiked (ng/mL)	AFB1 Detected (ng/mL)	Recovery (%)	RSD (%)
Serum	0.5	0.535	106.96	1.00
	10	10.308	103.08	5.21
	30	29.134	97.11	3.97

the biomarker for the detection of infectious diseases such as latent tuberculosis. Therefore, under the best experimental conditions, we further verify the practicability of this method in the determination of serum samples. The determination result of the constructed sensor is as shown in Table 1. We add IFN-*γ* with different concentrations in the 10% serum samples. According to the analysis, the recovery rate is 97.11% - 106.96% and the relative standard deviation is 1.00% - 5.21%. This result indicates that the reproducibility of the constructed sensor is good, and can meet the actual application requirement. This method is applicable to the analysis of real biological samples with interference. The CNNS/Apt@IFN-*γ* sensor can detect the serum samples, which are easily available clinically, to monitor the changes in IFN-*γ* content.

4. Conclusion

IFN-*γ* is a significant part of the body to perform its immune functions. The detection of IFN-*γ* content is conducive for us to understanding the immune response conditions of the body to the pathogen stimulus, which can be an important indicator for disease diagnosis and disease process monitoring. At present, the quantitative detection method of IFN-*γ* is conducted through immunological methods. ELISA [16] [17] [23] and Enzyme-Linked Immunospot Assay (ELISPOT) [24] [25] are the current clinically existing methods to detect IFN-*γ*. The reagent

of these methods has several problems such as bad stability, difficulty in storing, lot-to-lot deviations, high costs and long time-consuming detection. In the current research, we combine the fluorescence quenching property of CNNS and its affinitive absorbability to ssDNA with the selectivity of Apt@IFN-γ to develop a fluorescence biosensor for the detection of IFN-γ. Compared with the classic method of ELISA, the sensing method has characteristics such as fewer steps, simple operation, fast detection and low costs. The fluorescence of Apt@IFN-γ marked by Cy5 can be quenched by CNNS through PET under the situation without IFN-γ, and can form the composite of Apt@IFN-γ and IFN-γ under the existence of IFN-γ, of which the fluorescence cannot be effectively quenched. We use the prepared sensing method and detect a good linear relation between the fluorescence intensity and the level of IFN-γ within the range of 0.5 - 100 ng/mL, and the LOD is 0.303 ng/mL. This method achieves the detection of IFN-γ with sensitivity and specificity, which is applicable to serum samples. This method is conducive to the development and application of Carbon Nitride nanosheet material in disease diagnosis, and predicts the potential capability of the sensing platform constructed by CNNS and Apt@IFN-γ in terms of the analysis methodology of clinical samples.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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