



OPEN Surface plasmon resonance detection of anti-cancer drug flutamide by graphitic carbon nitride/chitosan nanocomposite

Hassan Nasiri[✉] & Karim Abbasian[✉]

The g-C₃N₄/CS biosensor was designed, fabricated, and tested using compounds such as glucose, urine, lactose, and flutamide at a molarity of 10 μ M, which could demonstrate a high sensitivity of 200 μ m⁻¹ for flutamide. Powerful effective medium theory and FDTD simulation were used to predict the most favorable mode and plasmonic properties of a graphite carbon nitride and chitosan nanocomposite. The research also explores the characteristics of surface plasmon resonance exhibited by the nanocomposite as the chitosan content is adjusted. Subsequent simulations are conducted on nanocomposites incorporating a thin layer and a modified gold structure. The intricate simulations ultimately reveal the optimal combination, tested under three different pH conditions (6.2, 7.2, and 8). In acidic conditions, the kinetic profile yielded a $K_D = 3.45 \times 10^{-7}$, surpassing the K_D value of a thin film. The Au significantly outperformed the alternative material. The biosensor demonstrated linear behavior across a wide concentration range from 1 to 150 μ M, achieving a detection limit of 120 nM with its high sensitivity.

Keywords SPR sensor, Flutamide biosensor, Graphitic-carbon nitrite-chitosan

A significant improvement in our ability to regulate diseases due to the quick diagnosis of these conditions has been seen in recent years^{1,2}. The well-known surface plasmon resonance (SPR) is one useful tool for researching molecule interactions^{3,4}. The technique is used in drug discovery⁵, clinical diagnostics⁶, and other domains as a transducer in affinity sensors⁷. One can use the label-free approach for SPR imaging or integral measurements^{8,9}. The several uses of the method, including the detection of electrochemical processes such as ion adsorption and nucleation, have been covered in a great deal of papers and reviews^{10,11}. 4-nitro-3-trifluoromethyl-isobutylanilide, or flutamide (FLT), is a non-steroidal anti-androgen that controls the release of testosterone^{12,13}. It is commonly prescribed for managing prostate cancer, with a typical dosage of 250 mg taken three times daily to restrict testosterone production by prostate cancer cells^{14,15}. FLT is also used in screening assays and identifying endocrine disruptors. Side effects of FLT may include gynecomastia and tenderness, and the drug typically becomes bioavailable within 1 to 2 h after consumption^{16,17}.

Much research has focused on FLT sensing from many perspectives. For example, Yiran Luo et al. used an electrocatalyst formed by a composite of carbon particles and tungsten disulfide for the electrochemical detection of flutamide¹⁸ with a low sensitivity for environmental application. Also, Afshan Mehrabi et al. by cyclic voltammetry and differential pulse voltammetry tried the electrochemical behavior of the FLT as its selectable sensing¹⁹. In another work, rGO nanosheets covered molybdenum tungsten electrodes claiming real-time detection of FLT with the lowest detection limit 20. The fluorescent property of nitrogen/sulfur co-doping of carbon quantum dots was utilized for FLT determining in the environment and pharmaceutical tablets²⁰. By studying the works for FLT sensing, one infers that the challenge is wide with its details in applications variety, sensing conditions such as selectivity, sensing range, linearity, and so on.

Two-dimensional materials like graphene, graphene oxide, and graphitic carbon nitride (g-C₃N₄) have attracted a lot of attention from the scientific community recently because of their electrical and optical characteristics as well as their potential as substrates for composite matrices. Because of its great chemical and thermal durability, g-C₃N₄, a polymeric semiconductor with an optical band gap of 2.7 eV, has been demonstrated to have advantageous physical and chemical characteristics as well as a high reduction potential^{21–24}. Like graphite, g-C₃N₄ is primarily composed of carbon and nitrogen with sp² hybridization²⁵.

Faculty of Electrical and Computer Engineering, University of Tabriz, Tabriz, Iran. ✉email: h.nasiri@tabrizu.ac.ir; k_abbasian@tabrizu.ac.ir

It is a member of the class of carbon nitrides that are distinguished by their distinct covalent bonding between atoms²⁶. Because g-C₃N₄ contains heptazine and tri-s-triazine rings, it is thought to be the most stable type of carbon nitride^{27,28}. Its structure consists of tri-s-triazine units organized in a π -conjugated planar layered framework, which offers remarkable resistance to acidic and alkaline conditions and thermal stability up to 600 °C. Due to the strong condensation capacity of the tri-s-triazine rings, which are joined by weak van der Waals forces between layers, g-C₃N₄ is an indirect semiconductor with exceptional photocatalytic and electrocatalytic capabilities^{13,29}. Conversely, chitosan is a biopolymer formed from chitin that is non-toxic, biocompatible, bioactive, and biodegradable³⁰. It is used in a wide range of sectors including water treatment, paper, textiles, food, medicine, and agriculture due to its special properties^{31,32}. Furthermore, chitosan is a well-liked option for developing sensors and biosensors due to its superior film-forming properties^{33,34}. Also, it is a prime contender for biological material receptors due to its amino bonding and capacity to fortify composite structures^{35,36}. SPR sensors are highly sensitive to minute changes in molecule concentration, as they rely on alterations in the refractive index of the near-surface environment. Unlike fluorescent techniques that necessitate the labeling of molecules³⁷, SPR can detect biological interactions without the need for additional materials, saving time and money. Additionally, SPR sensors offer real-time monitoring of interactions, allowing for the observation of dynamic changes over time³⁸. They also require small sample volumes, making them suitable for applications with limited or costly samples. Furthermore, this non-destructive method can be applied to living or sensitive samples without causing harm³⁹.

The study utilized the effective medium theory to simulate a composite material incorporating graphitic carbon nitride through the surface plasmon resonance method. By altering the composition to include chitosan, the final composite was examined using SEM, FT-IR, and XRD techniques. The composite was then tested by kinetic parameter under varying pH conditions, with the most optimal linear response achieved at a pH of 6.2, demonstrating a sensitivity of 200 μm^{-1} .

Method and designing

Material

For the purpose of making phosphate buffer solution (PBS), sodium phosphate dibasic (Na₂HPO₄), sodium phosphate monobasic (NaH₂PO₄), and urea (CH₄N₂O, 98%) were acquired from Dr. Tajalli comp. Flutamide (C₁₁H₁₁F₃N₂O₃) was acquired from Taiwan's Sigma Aldrich. No purification was performed; all reagents were used exactly as received.

The study utilized double-distilled and deionized water, with chitosan supplied by Sabz Gostaresh Azin Turkan in Maragheh, Iran. The synthesis of g-C₃N₄ involved the bottom-up method, where small particles were used to create complex structures⁴⁰. In contrast, the top-down method involved breaking down large materials into smaller particles and thin nanosheets⁴¹. The bottom-up approach included techniques such as ionic liquid, supramolecular pre-assembly, and hydrothermal methods to synthesize g-C₃N₄ sheets on a large scale through thermal polymerization or carbonization of small organic compounds like melamine, cyanamide, Dicyanamide, or urea. Dante et al. obtained g-C₃N₄ by pyrolyzing melamine cyanurate at 650 °C for 50 min under atmospheric conditions for glucose sensing⁴². The top-down approach utilized chemical exfoliation and ultrasonic exfoliation methods, with chemical exfoliation being preferred for its efficiency and ability to adjust the g-C₃N₄ structure easily for large-scale production^{43,44}.

Device of analysis

Using Cu K α radiation ($\lambda = 1.54 \text{ \AA}$), XRD (XPRT-PRO, Pan Analytical B.V., The Netherlands) was used to analyze the crystal structure of the produced sample. FTIR analysis of the functional group presence was conducted with a JASCO FT-IR 4600LE spectrometer. The SEM (JSM-6510) was used to examine the microstructure and surface morphology. Utilizing energy-dispersive X-ray spectroscopy (EDS) to examine the chemical composition. Moreover, we also used the Bionavis SPR 210 device for kinetic testing.

Modification of biosensor

The g-C₃N₄/CS/Au SPR sensing medium was made by carefully following a methodical process. First, 3 ml of CS was dissolved in 3 ml of 1% acetic acid solution. Next, this solution was supplemented with an extra 3 mg of g-C₃N₄. The mixture was ultrasonically agitated for 5 h to guarantee a consistent dispersion throughout. Next, using the spin coating process, a small amount (25 μL) of the resultant blend was carefully placed onto a gold layer and allowed to dry entirely⁴⁵. The outcome was the successful development of an exquisitely precise and elegant g-C₃N₄/CS /Au SPR sensing medium (see Fig. 1a).

Optimization of biosensor

To enhance the sensitivity of the biosensor, the effective medium theory was applied to a composite of graphitic carbon nitride and varying percentages of chitosan (10%, 20%, and 30%)⁴⁴. Surface plasmon resonance and resonance angle changes were utilized due to their high sensitivity to surface alterations. The biosensor was simulated using the FDTD method⁴⁶, showing different states such as modified surface in five scenarios⁴⁷, as illustrated in Fig. 1b.

Result

Surface measurement

The sample had sheet-like structures, as seen by the subsequent SEM image (Fig. 2a), with CS particles linked to g-C₃N₄ sheets that had a diameter of around 30 nm. The elements C, N, and O were found in the composite, with weight percentages of 37.89%, 56.07%, and 6.04%, respectively, according to an

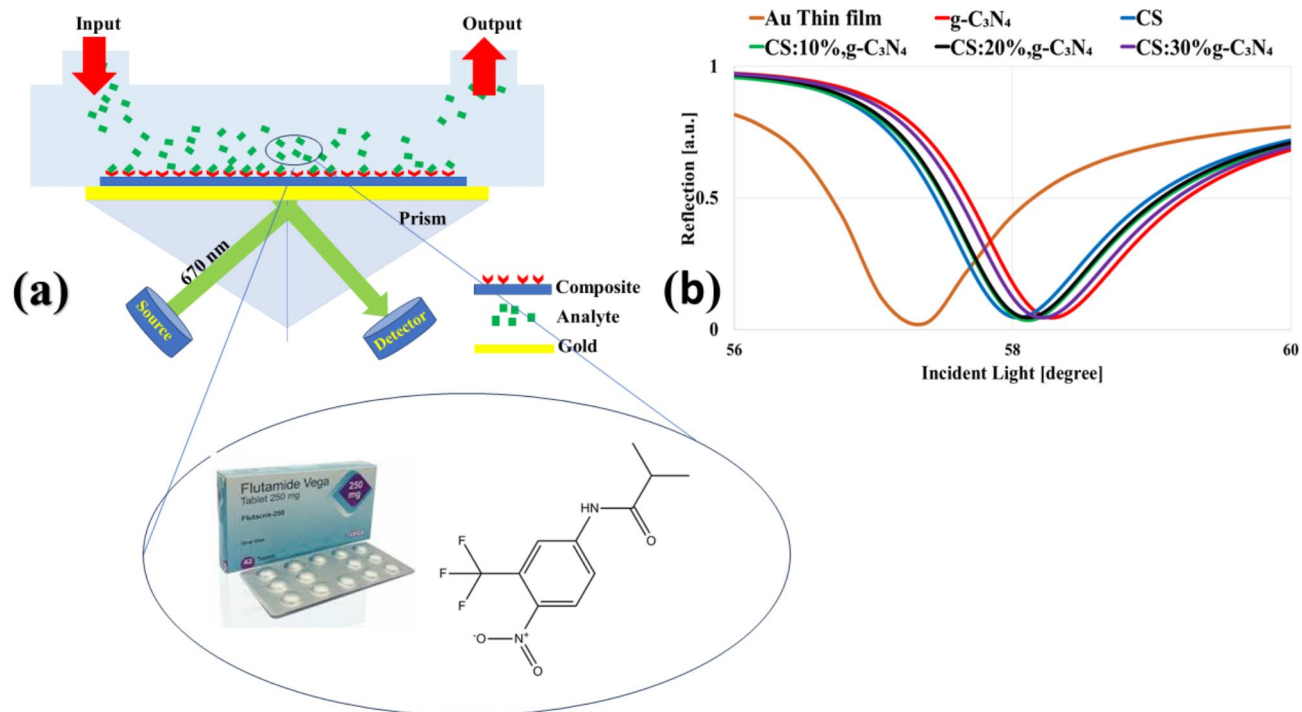


Fig. 1. Schematic of experimental (a) and SPR diagram of EMT in various CS in composite (b).

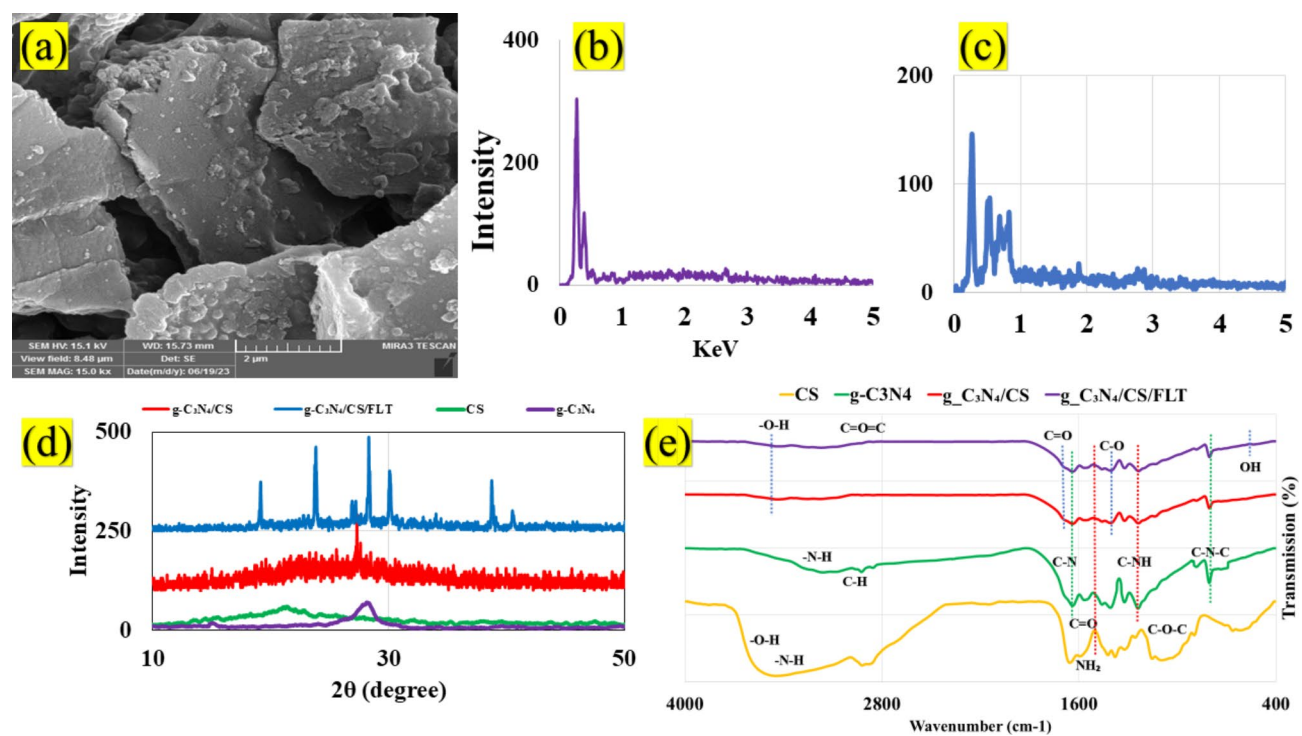


Fig. 2. (a) FESEM of g-C₃N₄/CS composite, (b) EDS of composite, (c) EDS of composite after injection of FLT, (d) XRD pattern of g-C₃N₄/CS, g-C₃N₄/CS/FLT, CS and g-C₃N₄ composite, and (e) FTIR of g-C₃N₄/CS/FLT (purple), g-C₃N₄/CS (red), g-C₃N₄ (green) and CS (yellow).

elemental analysis (Fig. 2b). Also, after injecting flutamide onto the composite, EDS was prepared, which is shown in Fig. 2c. The results showed that the presence of F and O atoms in the flutamide structure was clearly seen in EDS. In the measured 2θ range, the XRD pattern of chitosan exhibited a broad diffraction peak at 2θ around 21° that is characteristic fingerprint of semi-crystalline chitosan. The peaks at 12.8° and 27.6° in g-C₃N₄ are in good accordance with those reported in literature. The more intense peak at 27.6° is attributed to (002) plane due to stacking of layers in conjugated aromatic system. The less intense peak at 12.8° , however, seems well to the layered packing structure of tri-s-triazine units, assigning to (100) plane in JCPDS 87–1526 card. XRD pattern of drug loaded nanocomposite provides some typical peaks of Flutamide at 2θ of 9° to 25.3° but not all of them, representing well dispersion of drug particles within the nanocomposite matrix (see Fig. 2d)^{47,48}. The FTIR spectra of the g-C₃N₄, CS, g-C₃N₄/CS and g-C₃N₄/CS/FLT samples are depicted in Fig. 2e. The distinctive peaks of CS and g-C₃N₄ were identified in the g-C₃N₄/CS sample⁴⁹. The CS sample exhibited strong absorption peaks at 1024 and 1068 cm⁻¹ attributed to the C-O stretching vibration of alcoholic hydroxyl groups⁵⁰. The presence of N-H breathing modes in the 3000–3600 cm⁻¹ range suggests the presence of surface N-H or NH₂ groups. Peaks in the 1200–1600 cm⁻¹ region indicate the stretching vibration of the aromatic CN heterocycle, while bands at 810 cm⁻¹ are linked to the respiratory vibration of the heptazine units. XRD analysis confirmed the successful combination of g-C₃N₄ and CS, as shown in Fig. 2d and f alongside the FTIR data^{34,51}.

Optimization of the flow rate

Initially, we assessed the performance of the biosensor when exposed to different concentrations of FLT (0.1, 1, and 10 μ M). At FLT concentrations of 1 and 10 μ M, there was a change in the surface plasmon resonance angle, and no reaction was observed compared to 0.1 μ M⁵². To address the mass transport limitation (MTL) effect in analytical studies, flow rate optimization was deemed necessary. MTL occurs when an analyte binds to the immobilized ligand faster than it diffuses to the biosensor's surface, impacting kinetic parameters^{53,54}. Various flow rates of FLT were tested to optimize this parameter. The response curve for Ka was consistent across all flow rates except for 10 μ L min⁻¹. A smoother exponential curve and reduced signal were observed at a flow rate of 10 μ L min⁻¹. However, there was no significant difference in the reaction between flow rates of 30 μ L min⁻¹, 40 μ L min⁻¹, and 50 μ L min⁻¹ (1 μ M). Similarly, there was no notable difference in the dissociation rate with flow rates ranging from 30 to 330 s. A slower flow rate appeared to allow FLT to remain on the composite surface longer for binding, as evidenced by a longer dissociation time indicating interaction between FLT and the composite material. A flow rate of 10 μ L min⁻¹ was ultimately chosen for further testing due to its strong RU signal and minimal MTL.

Assessment of the kinetic parameters

Figure 3 shows the interactions between the composite and various concentrations of FLT. It was observed that at higher concentrations of FLT, the association process occurred more rapidly in the optimized conditions (pH 6, 25 °C) resulting in k_a and k_d values of 1.27×10^3 (Ms)⁻¹ and 4.39×10^{-4} s⁻¹, respectively. A low K_D value of 3.45×10^{-7} indicated a strong affinity of the FLT-specific g-C₃N₄/Cs for FLT. Our findings suggest that the biotinylated composite is suitable for clinical evaluation in blood serum. The values of K_D and K_A were used to calculate ΔG_b using specific Equations^{55,56}.

$$A + B \xrightleftharpoons[k_d]{k_a} AB$$

$$\Delta G_b = -RT \cdot \ln K_A$$

The universal gas constant (R) is 8.314 J.K⁻¹ mol⁻¹, where K_A represents the affinity constant ($1/K_D$) and T is the temperature in Kelvin. At a temperature of 298 K, the value of ΔG_b for FLT and its specific composite interaction was -30.486 kJ mol⁻¹. The negative ΔG_b value suggests that the interaction between FLT and the specific g-C₃N₄/CS is spontaneous⁵⁷.

Figure 3 illustrates that the biosensor performs better under acidic conditions compared to other environments. The improved linear relationship is attributed to the absorption of hydrogen protons by the active groups in the g-C₃N₄ structure at pH 6.2, resulting in the formation of hydrogen groups. This transformation enhances the photocatalytic activity of g-C₃N₄ by facilitating the reaction with water to produce hydrogen and oxygen⁵⁸. Conversely, in a neutral environment, the active groups in the g-C₃N₄ structure absorb hydroxide electrons and convert them into hydroxide groups^{59,60}. This alteration diminishes the photocatalytic efficiency of g-C₃N₄ as the hydroxylated groups compete with the primary catalytic groups, hindering the photocatalysis process.

Calibration curve for FLT

The range of detection for the SPR-based biosensor designed for clinical diagnosis of FLT was evaluated by testing concentrations from 1 to 150 μ M. The flow rate remained constant at 10 μ L min⁻¹, and the experiments were carried out at a temperature of 25 °C. Each concentration was injected four times to create a calibration curve for FLT concentrations. The study was performed in phosphate buffer solutions with varying pH levels (7.2, 6.2, and 8). The linear regression equations for pH 7.2 and pH 8 were $y = 0.0002x + 0.0042$ ($R^2 = 0.933$) and $y = 0.0001x + 0.0048$ ($R^2 = 0.902$), respectively. The buffer at pH 6.2 ($y = 0.0002x + 0.0035$, $R^2 = 0.9839$) was identified as the optimal flow buffer. At higher FLT concentrations, the system response rose linearly; concentrations beyond 150 μ M resulted in a plateau, mainly because the biosensor surface's active sites were saturated. With a linear range of 1 to 150 μ M and a calculated Limit of Detection (LOD) of 0.12 μ M, this sample

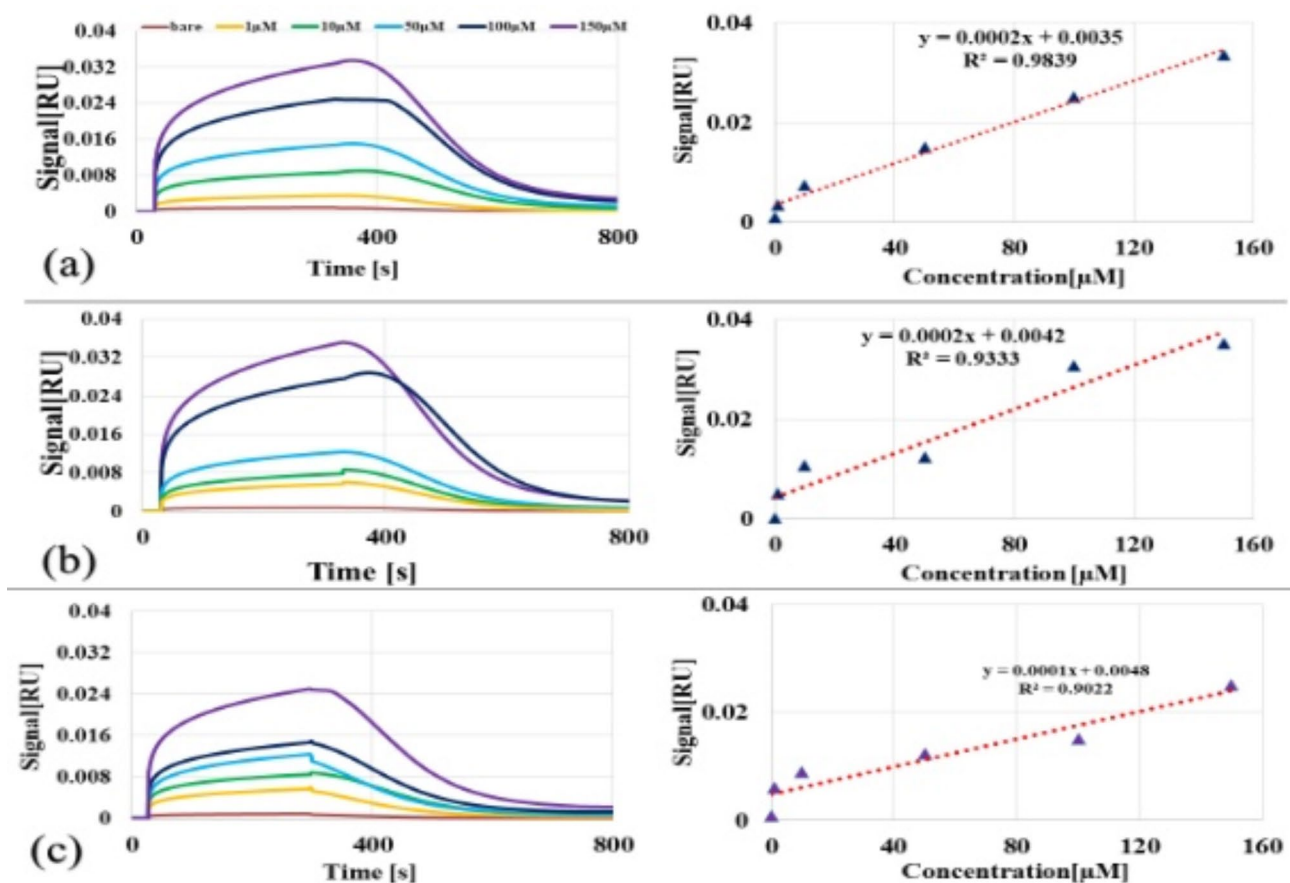


Fig. 3. Response of biosensor (injection rate: $10 \mu\text{L min}^{-1}$, $T = 25^\circ\text{C}$) in (a) pH=6.2, (b) pH=7.2 and (c) pH=8.

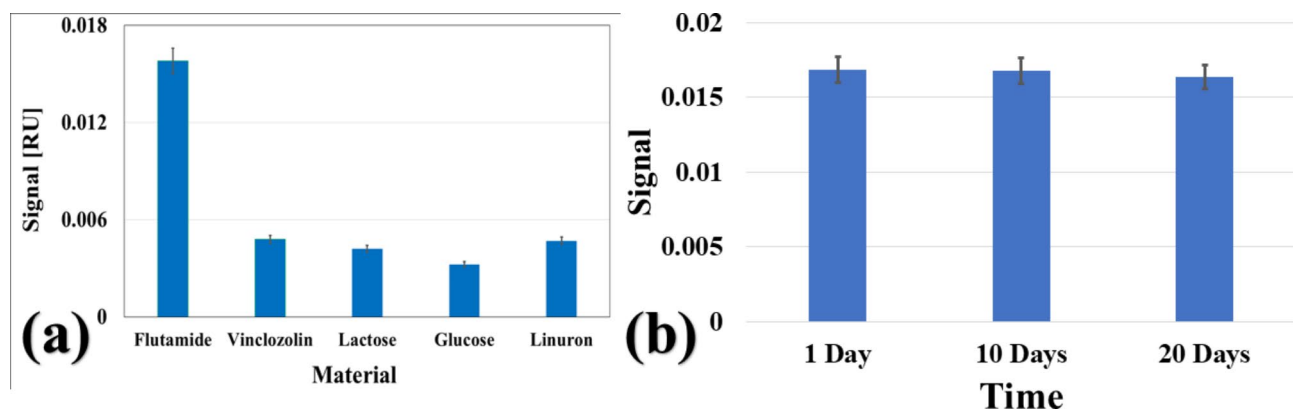


Fig. 4. Selectivity (a) and stability (b) of FLT biosensor.

is appropriate for use in clinical settings. All experiments have been performed 5 times and the average results are reported.

Selectivity and stability of the biosensor

The $\text{g-C}_3\text{N}_4/\text{CS}$ biosensor was tested using compounds such as glucose, urine, lactose, and flutamide at a molarity of $10 \mu\text{M}$ to evaluate its performance. Figure 4 displays the results, showing that the flutamide sensor produced a stronger signal compared to other compounds that were more susceptible to interference. The stability of the biosensor was also assessed at different times (1, 10, and 20) throughout the day at room temperature (25°C) in a controlled environment. The results, depicted in Fig. 4 as an average of 3 measurements, revealed that the biosensor experienced less than a 3% reduction in performance over the course of the test, which was conducted

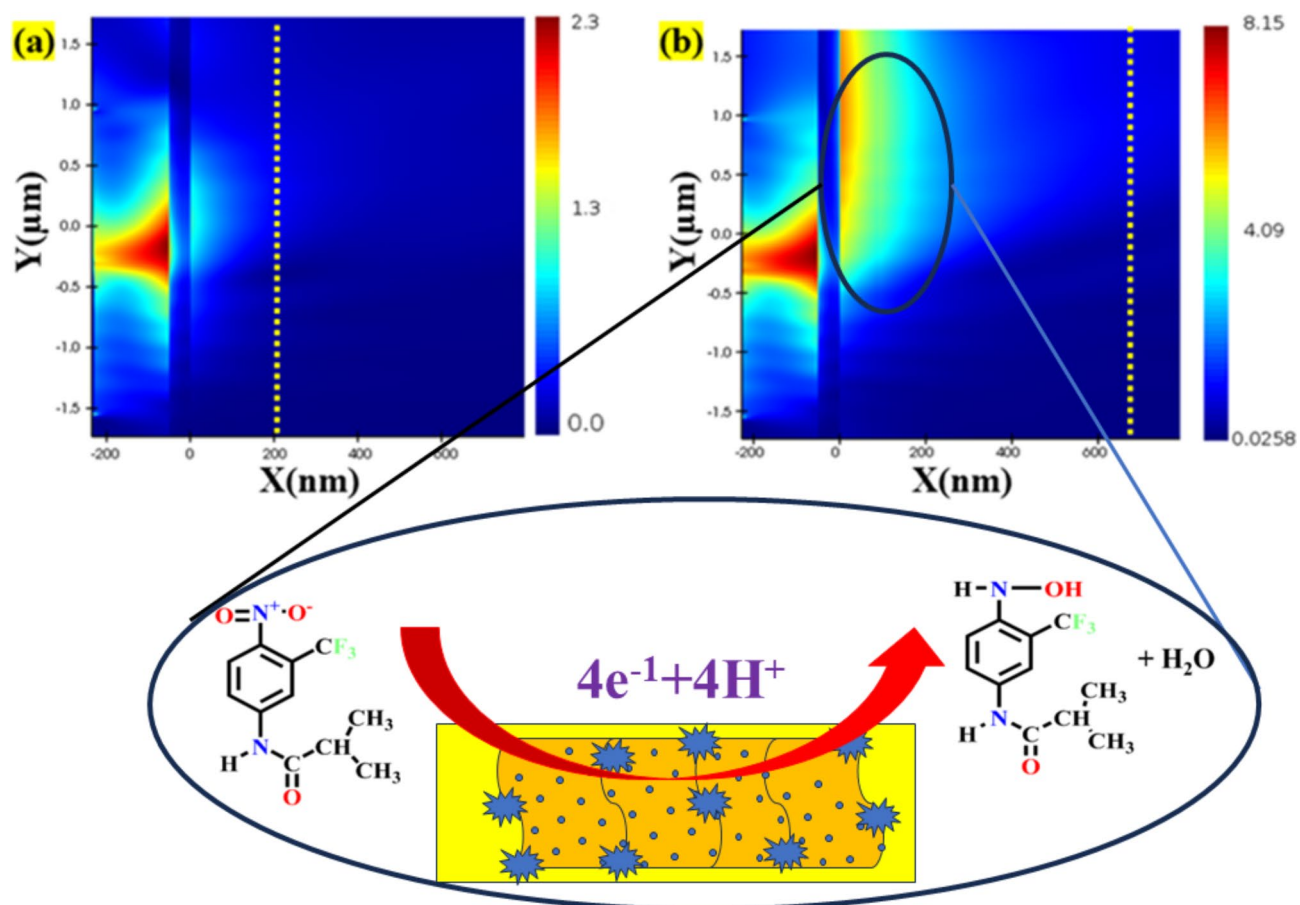


Fig. 5. Evanescent fields of (a) gold thin layer and (b) g-C₃N₄/CS modified gold chip and mechanism of detection.

Sensing layer	Detection mechanism	Linear range [μ M]	Limit of detection [nM]	References
BiVO ₄ -rGO/CE-BN	Electrochemical	0.04–102.2	0.011	⁶³
Bi ₂ O ₃ /h-BN	Electrochemical	0.04–87	9	⁶⁴
CuNCs	Fluorescent	0.10–100	61.82	⁶⁵
g-C ₃ N ₄ /CS	SPR	1–150	120	This work

Table 1. Comparison of g-C₃N₄/CS biosensor to previews research.

with 3 repetitions. The stability of the composite can be attributed to the stability of graphitic carbon nitride and chitosan, as well as the conditions of the measurement environment^{61,62}.

Ganesh et al. demonstrated that Flutamide can be reduced on exfoliated g-C₃N₄¹³. The reduction of the FLT on g-C₃N₄ is facilitated by the evanescent field generated by the SPR. This field can influence the analyte up to 700 nm and trap it for reduction on g-C₃N₄. The evanescent field for thin films of gold and modified gold thin films is illustrated in Fig. 5. It was observed that with a thin layer of gold, the evanescent field can reach the analyte up to 200 nm. However, when a modified gold layer is used to sensing of analyte, the evanescent field can extend up to approximately 700 nm. This indicates that the field in the analyte environment, along with the trapping of Flutamide on the sensing substrate, enhances the reduction potential.

Also, in the following, the results of the designed biosensor are compared with previous research and are displayed in Table 1.

Real sample detection

The real sample was prepared using the gravity filtration procedure. The selective filter utilized was 0.45 micron. Without pausing, the river water moved steadily through the filter. We used the HPLC method to measure the FLT of the original sample, which was measured in five cases. The average of the measurements was 45.3 μ M, which was raised to 100 μ M by adding FLT to the sample (TDS: 590). diluted solutions were made using $M_1V_1 = M_2V_2$, and the results are shown in Table 2.

	Added [μM]	Found [μM]	Recovery	RSD%	SD
River water	5	4.97	99	3.59	0.17
	25	26.3	105.2	4.16	1.038
	50	48.8	97.6	5.1	2.587
	100	99.7	99.7	4.85	4.82

Table 2. Detection of FLT in real sample.

Conclusion

The purpose of the work was to improve the performance of an SPR biosensor employing g-C₃N₄/CS, a composite of g-C₃N₄ and CS. To precisely identify FLT, the researchers optimized the composition and flow rate of analytes by theoretical and experimental assessments. When exposed to FLT solution, the SPR sensor with the g-C₃N₄/CS thin film outperformed the unmodified gold chip with better signal fluctuations. Additionally, the sensor's sensitivity increased by 200, allowing it to identify FLT at a low concentration of 0.12 μM . The potential of g-C₃N₄-based biosensors for biological applications is demonstrated by this work.

Data availability

Data will be available upon reasonable request by corresponding author: Hassan Nasiri (h.nasiri@tabrizu.ac.ir).

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Declarations

Competing interests

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Ethical approval

Not included.

Additional information

Correspondence and requests for materials should be addressed to H.N. or K.A.

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