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Multifunctional tunable Cu₂O and CuInS₂ quantum dots on TiO₂ nanotubes for efficient chemical oxidation of cholesterol and ibuprofen†

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In this study, a CuInS₂/Cu₂O/TiO₂ nanotube (TNT) heterojunction-based hybrid material is reported for the selective detection of cholesterol and ibuprofen. Anodic TNTs were co-decorated with Cu₂O and CuInS₂ quantum dots (QDs) using a modified chemical bath deposition (CBD) method. QDs help trigger the chemical oxidation of cholesterol by cathodically generating hydroxyl radicals ([•]OH). The small size of QDs can be used to tune the energy levels of electrode materials to the effective redox potential of redox species, resulting in highly improved sensing characteristics. Under optimal conditions, CuInS₂/Cu₂O/TNTs show the highest sensitivity ($\sim 12\,530\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$, i.e. up to 11-fold increase compared to pristine TNTs) for cholesterol detection with a low detection limit (0.013 μM) and a fast response time (1.3 s). The proposed biosensor was successfully employed for the detection of cholesterol in real blood samples. In addition, fast (4 s) and reliable detection of ibuprofen (with a sensitivity of $\sim 1293\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$) as a water contaminant was achieved using CuInS₂/Cu₂O/TNTs. The long-term stability and favourable reproducibility of CuInS₂/Cu₂O/TNTs illustrate a unique concept for the rational design of a stable and high-performance multi-purpose electrochemical sensor.

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Introduction

Multifunctional nanomaterials integrated in a single device have gained considerable interest in the recent advancement of technology in biosensing applications for monitoring food

and water quality (environment applications), for the detection of disease biomarkers (healthcare applications), and in genomics.^{1,2} A variety of nanomaterials such as metal oxides (TiO₂, ZnO, MgO, *etc.*), metal sulphides (CdS), polymers and carbon-based materials (carbon nanotubes (CNTs) and gra-

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† Electronic supplementary information (ESI) available: Different stages of fabrication of a non-enzymatic biosensor, FESEM images of the cross-sectional view of CuInS₂/Cu₂O/TNT and Cu₂O/TNT electrodes decorated with CuInS₂ and Cu₂O QDs for different deposition cycles along with their corresponding QD size distribution. The schemes showing the variation in band-gaps of Cu₂O QDs and CuInS₂ QDs with particle sizes respectively, EDX spectra and elemental mapping of CuInS₂/Cu₂O/TNTs, and comparison of high-resolution XPS

spectra of Cu 2p for Cu₂O/TNT, CuInS₂/TNT, and CuInS₂/Cu₂O/TNT electrodes. Fitted curves of EIS spectra and the values of circuit parameters of TNTs, Cu₂O/TNTs, CuInS₂/TNTs, CuInS₂(4)/Cu₂O/TNTs, CuInS₂(6)/Cu₂O/TNTs, and CuInS₂(8)/Cu₂O/TNTs, fitting of the linear portion of the Mott-Schottky curves of TNTs, Cu₂O/TNTs, and CuInS₂/Cu₂O/TNTs, cyclic voltammetry of Cu₂O/TNT and CuInS₂/Cu₂O/TNT electrodes for varying the loading density of Cu₂O and CuInS₂ QDs (by keeping the Cu₂O QD deposition fixed to 10 cycles) respectively. Cyclic voltammetry response studies of the pristine TNTs as a function of cholesterol concentration. Linear fitting of the calibration curve of TNT, Cu₂O/TNT, and CuInS₂/TNT electrodes derived from their corresponding amperometric response for cholesterol detection. The effect of pH and temperature of the electrolyte on the current response of the CuInS₂/Cu₂O/TNT electrode for cholesterol detection, reproducibility and repeatability of the CuInS₂/Cu₂O/TNT electrode towards cholesterol, linear fitting of the calibration curve of TNT, Cu₂O/TNT, and CuInS₂/TNT electrodes for ibuprofen detection. The effect of pH and temperature of the electrolyte and reproducibility and repeatability of CuInS₂/Cu₂O/TNTs for ibuprofen detection. FESEM cross-sectional images of the used Cu₂O/TNTs, CuInS₂/TNTs, CuInS₂(4)/Cu₂O/TNTs, CuInS₂(6)/Cu₂O/TNTs, and CuInS₂(8)/Cu₂O/TNTs and comparison between the XRD patterns of the fresh and used CuInS₂/Cu₂O/TNTs. See DOI: <https://doi.org/10.1039/d4nr00422a>

phene) have been widely investigated as dual-analyte biosensing platforms for the detection of multiple biological analytes such as cholesterol, H_2O_2 , glucose, and pharmaceutical drugs.¹ In these applications, the surface of the biosensor was immobilized with two different enzymes for the selective detection of corresponding analytes.^{3–5} However, owing to the intrinsic instability and expensive nature of enzymes, these biosensors are far away from practical use in point-of-care applications. Non-enzymatic biosensors consisting of hybrid materials can be used as an alternative approach to overcome the above limitations. These sensors provide many beneficial features such as low cost, ease of operation, long shelf life, and resistance to variable sensitivity.^{6–8} In recent years, the incorporation of functional materials on the surface of matrix elements (TNTs), constituting a hybrid nanostructure, has attracted considerable attention.^{9–12} For instance, an electrode material with high catalytic activity and few charge recombination sites is desirable.¹³ In order to obtain this objective, electrodes can be decorated with a combination of functional materials (which, for example, are composed of a matrix material and two different catalysts, usually QDs) with high catalytic activity, which is highly promising.^{11,14,15} The higher electrochemical activity of QDs can firstly be attributed to their small size, which results in a large surface area. Moreover, the size of QDs can be tuned in the range of quantum confinement effects in such a way that a minimum resistance is encountered by electrons at the surface of the biosensor.¹⁶ Another benefit is that the comparable dimensions of QDs and bio-elements offer rapid electronic communication between the QDs and specific recognition sites of the biomolecule during the electrocatalytic process.¹⁷

The electrochemical performance of the non-enzymatic biosensor is mainly governed by the catalytic activity of the active material as well as the physiochemical properties of the substrate.¹⁸ Thus, the selection of the substrate material is as critically important as selecting the active material. Recently, anodic TNTs have gained considerable interest as substrate materials in biosensing due to their various intrinsic properties such as chemical stability, low cost, and excellent biocompatibility.^{19,20} One advantage of the self-organised nanotubular structure formed on a Ti foil *via* anodization is its good adhesion to the Ti substrate, which can greatly reduce and simplify the otherwise often tedious process of the modified electrode fabrication. Moreover, the well-aligned nanotubular structure has copious space for the loading of active materials.

Cholesterol detection is of scientific and technological importance due to its role in predicting ever-increasing coronary heart diseases and strokes.²¹ Numerous efforts have been devoted to the development of highly sensitive, efficient, and reliable cholesterol sensors for real-time detection.^{22,23} Among them, electrochemical sensing of cholesterol is highly promising for application in rapid and high-throughput biological assays.^{24,25} Limited reports are available on the non-enzymatic sensing of cholesterol using various electrode systems, hence providing a huge room for further improvement. For instance,

a macro-porous gold electrode modified with Pt nanoparticles (NPs) was utilized for enzyme-free cholesterol detection with a sensitivity of $226.2 \mu\text{A mM}^{-1} \text{cm}^{-2}$.²⁶ Another study reports²⁷ a sensitivity of $\sim 62.5 \mu\text{A mM}^{-1} \text{cm}^{-2}$ for a cholesterol biosensor composed of flower-like Cu_2S nanoplates grown on a Cu rod. Non-enzymatic cholesterol biosensors based on the deposition of Pt NPs on a CNT thin film achieved a sensitivity up to $8.7 \mu\text{A mM}^{-1} \text{cm}^{-2}$.²⁸ Recently, our group has reported a non-enzymatic cholesterol sensor using Cu_2O NP-decorated TNTs with a maximum sensitivity of around $6034.04 \mu\text{A mM}^{-1} \text{cm}^{-2}$, which is much higher than the values reported earlier.²⁹ It might be due to the fact that decorating TNTs with Cu_2O NPs leads to the formation of p–n junctions on the surface of TNTs, which suppress the electron–hole pair recombination. The sensitivity of the biosensor can further be increased by tailoring the morphology of Cu_2O QDs and using another suitable co-catalyst. CuInS_2 with a band gap of $\sim 1.5 \text{ eV}$ is an efficient co-catalyst. Most of the work reported till date exploits the optical properties of CuInS_2 QDs in many applications including environmental monitoring, optics, electronics, and energy conversion and storage.^{14,30–32} However, no report is available to explore the sensing aspects of CuInS_2 QDs for biological species.

The presence of pharmaceutical wastes in original forms or as metabolites in water streams is an emerging form of water pollution increasing the concern about on-site analysis of these drugs to check the water quality. Ibuprofen, a non-steroidal and anti-inflammatory drug, is a widely used, and serves as a first choice to treat fever and pain in humans.^{33,34} About 90% of this drug is metabolized by the liver in the form of hydroxyl and carboxyl species, while the remaining 10% of the drug is excreted *via* urine unaffected.^{35–37} The inevitable use of ibuprofen and its discharge in pharmaceutical industries, hospital effluents and human metabolites necessitates the development of novel analytical techniques, procedures and protocols for its detection in waste water.^{35–40} Owing to the smaller size of ibuprofen, nanomaterials (particularly QDs) as electrode materials can play a vital role in the analysis of ibuprofen and produce a significant response when it interacts with the surface of the sensor.

Herein, we report the synthesis of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ hybrid nanostructures as biosensing platforms for cholesterol and ibuprofen detection. Anodic TNTs were decorated with Cu_2O and CuInS_2 QDs by a modified CBD method to achieve more active sites at the surface of the electrode for electrochemical redox reactions and generate a maximum reduction current as a readout signal, thereby obtaining a sensor for cholesterol detection. The Cu_2O and CuInS_2 QDs help facilitate the chemical oxidation of cholesterol by cathodically generating $\cdot\text{OH}$. This approach of co-decoration with multiple functional QDs can effectively amplify the sensing response. The fabricated electrode was further employed for the detection of ibuprofen in waste water. The higher sensitivity of the biosensor for cholesterol and ibuprofen leads to the advancement of the biosensing technology towards dual-analyte biosensor in both diagnostic and environmental applications.

Experimental

Materials and methods

Titanium sheets of 0.1 mm thickness and 99.6% purity were procured from GoodFellow, England, UK. Highly pure ethylene glycol (EG), ammonium fluoride (NH_4F), copper sulfate (CuSO_4), copper(II) chloride (CuCl_2), sodium sulfide (Na_2S), indium fluoride (InF_3), sodium thiosulfate pentahydrate ($\text{H}_{10}\text{Na}_2\text{O}_8\text{S}_2$), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, USA. Other chemicals such as cholesterol, urea, ascorbic acid (A.A.), glucose, and L-cysteine were supplied by Sinopharm Chemical Regent Co. Ltd, China. The aforementioned chemicals were used as received without further purification and de-ionized (DI) water was used in all experiments.

Fabrication of non-enzymatic biosensors

Anodization of Ti foil. TNTs were directly grown on a Ti substrate *via* a facile two-step anodization process to obtain nanotubes with an open-top morphology. Prior to anodization, the Ti foils were successively immersed in acetone, ethanol, and DI water for 15 min each to clean their surface and then dried under a nitrogen (N_2) stream. Anodization was performed in a two-electrode electrochemical cell, where a Ti foil was used as the anode and a Pt sheet as the cathode that were immersed in an electrolyte containing EG, NH_4F (0.25 wt%) and DI water (5 wt%), as shown in Fig. S1(a).[†] During the first step, the Ti foil was anodized at 50 V for 4 h to obtain an initial layer of TNT array. The layer was then removed *via* sonication in water and a shiny surface of Ti substrate with a patterned morphology was achieved. The Ti substrate with a patterned morphology was then subjected to the second step of anodization using a fresh electrolyte under similar experimental conditions. The fabricated TNTs were finally annealed at 450 °C for 2 h to convert the as-anodized amorphous phase into the desired anatase phase.

Decoration of TNTs with Cu_2O QDs. The fabricated annealed TNT arrays were decorated with Cu_2O QDs by a sonication-assisted CBD method, as described in our previous report with slight modification.²⁹ Briefly, TNTs were sequentially dipped in two different solutions during continuous sonication, as shown in Fig. S1(b).[†] At first, TNTs were immersed in a mixture of 1.0 M CuSO_4 and 1.0 M $\text{H}_{10}\text{Na}_2\text{O}_8\text{S}_2$ (1 : 4) aqueous solution for 10 s at room temperature, subsequently washed with DI water and then immersed in 0.5 M NaOH for another 10 s at 70 °C. Finally, the modified TNT samples were rinsed with DI water to remove the residual salts. This four-step immersion and rinsing process is considered as one cycle. This process was repeated for 10 cycles to increase the loading density of Cu_2O QDs on TNTs.

Decoration of Cu_2O QDs/TNTs with CuInS_2 QDs. CuInS_2 QDs were deposited on Cu_2O QDs/TNTs *via* the same modified CBD process. Cu, In, and S ions were sequentially adsorbed onto the surface of Cu_2O QDs/TNTs, where they react with each other to form CuInS_2 QDs. For this purpose, the Cu_2O QD/TNT samples were successively immersed in three different aqueous solutions followed by washing with DI water to remove the unreacted precursors (Fig. S1(c)).[†] In the first step, the Cu_2O QD/TNT sample was immersed in a 0.10 M aqueous solution of

InF_3 under continuous sonication for 60 s and then rinsed with DI water. In the second step, the same sample was immersed in a 1.3 mM aqueous solution of CuCl_2 for 30 s followed by washing with DI water. Finally, the sample was immersed in a 0.14 M aqueous solution of Na_2S for 240 s and then rinsed with DI water. This three-step process was considered as one cycle. Different Cu_2O QD/TNT samples were loaded with different amounts of CuInS_2 QDs. For this purpose, the process was repeated for 2, 4, 6, and 8 aforementioned cycles. The samples were labelled $\text{CuInS}_2(2)/\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2(4)/\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNTs}$, and $\text{CuInS}_2(8)/\text{Cu}_2\text{O}/\text{TNTs}$. CuInS_2 QDs were also loaded on TNTs following the same procedure under similar conditions for comparison.

Characterization

A field emission scanning electron microscope (Hitachi FE-SEM S4800, Japan) was used to study the morphology of the samples. A high-resolution transmission electron microscope (HRTEM, Tecnai G2 F20 S-Twin, FEI, USA) equipped with an energy-dispersive X-ray (EDX) spectrometer was employed to obtain the detailed structural and elemental compositions of the samples. An X-ray photoelectron spectrometer (XPS, PHI 5600, US) and an X-ray diffractometer (XRD, X'pert Philips PMD, $\text{CuK}\alpha$ 1.54056 Å) were used to determine the chemical bonding and crystal structure of the samples, respectively.

Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) was performed using a Zahner (IM6EX, Germany) in the frequency range of 1 MHz to 10 mHz at an open circuit potential, while cyclic voltammetry (CV) and amperometry were performed using an electrochemical workstation (Biologic SP-300, France) with a three-electrode configuration. TNTs, $\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2/\text{TNTs}$, and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ were used as the working electrodes with an exposed area of 0.35 cm², a saturated calomel ($\text{Hg}/\text{Hg}_2\text{Cl}_2$) electrode as the reference electrode and a Pt foil as the counter electrode. All electrochemical experiments were conducted in a 0.1 M phosphate buffer solution (PBS) with a pH of ~7.5. For cholesterol sensing, a 5 mM stock solution was prepared by dissolving cholesterol in 2 ml of 2-propanol, which was then diluted in 0.1 M PBS as per requirement.

A stock solution of ibuprofen was prepared by weighing and crushing 10 tablets of commercial ibuprofen using a mortar and pestle. Then, 10.31 mg of ibuprofen powder was dissolved in 20 ml of methanol and filtered using a 20 µm pore size filter paper. The filtered solution was further diluted in methanol up to 100 ml to obtain a stock solution. In the experiment, 0.1 M PBS with pH 5.5 was used as an electrolyte for all electrochemical measurements.

Results and discussion

Scanning electron microscopy

Fig. 1(a–h) shows the typical top and cross-sectional view of FESEM images of the pristine TNTs, $\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2/\text{TNTs}$,

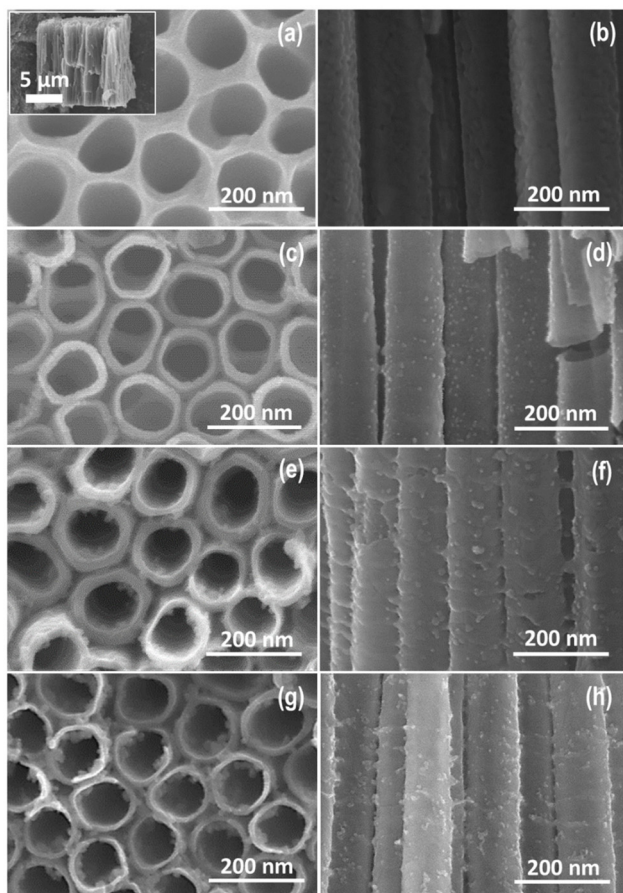


Fig. 1 FE-SEM images showing the top view and cross-sectional view of (a and b) pristine TNTs, (c and d) TNTs decorated with Cu_2O QDs, (e and f) TNTs decorated with CuInS_2 QDs, and (g and h) TNTs codecorated with CuInS_2 and Cu_2O QDs. The inset in (a) shows the film thickness of TNTs.

and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$. The FESEM images show that the pristine TNTs possess self-organised and vertically oriented nanotubular structures that are orderly grown on a Ti substrate, as shown in Fig. 1(a and b). It can be observed that the opening of the pristine TNTs is circular in shape with an inner diameter of ~ 100 nm and a wall thickness of ~ 15 nm. The nanotube length was found to be around $10\ \mu\text{m}$ (inset of Fig. 1a). The FESEM images of Cu_2O QDs/TNTs heterojunctions prepared for 10 CBD cycles are shown in Fig. 1(c and d). The Cu_2O QDs with an average size of about 5 nm can be seen on the top and inner surfaces of the nanotubes in addition to the interface of TNTs. The FESEM images of CuInS_2 QD-loaded TNTs for 6 CBD cycles are presented in Fig. 1(e and f). The uniformly dispersed CuInS_2 QDs with an average size of around 4–6 nm can be observed at the top as well as along the cross-sectional surfaces of the nanotubes. The top view of the hybrid $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNT}$ system is shown in Fig. 1g. Interestingly, in spite of the high loading density of QDs, the nanotube opening is not blocked. Fig. 1h depicts the cross-sectional view of TNTs loaded with both Cu_2O and $\text{CuInS}_2(6)$

QDs. It is clear from the images that the surface of TNTs has become much rough, as the amount of QDs on nanotubes has increased. These results also show that the sonication-assisted CBD method did not destroy the morphology of the vertically oriented nanotubes. Fig. 2(a–l) shows the FESEM images of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ hybrid system, along with the estimated QD size, for different loading cycles of CuInS_2 QDs while keeping the loading content of Cu_2O QDs fixed to 10 cycles. It can be seen that the CuInS_2 QD content increases with the increase in deposition cycles. Furthermore, the presence of QDs onto the inner surface and interface of TNTs can also be seen in the corresponding cross-sectional FESEM images (Fig. 2(b, e, h and k)). The bar diagrams in Fig. 2(c, f, i and l) show the distribution of QD sizes for $\text{CuInS}_2(2)/\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2(4)/\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNTs}$ and $\text{CuInS}_2(8)/\text{Cu}_2\text{O}/\text{TNTs}$ estimated from their corresponding cross-sectional SEM images. The average QD size increases from 4.9 nm to 9.5 nm with the increase in deposition cycles for the loading of CuInS_2 QDs. It is also evident that a particle size distribution with a QD size of about 7 nm is homogeneous for $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNTs}$ (Fig. 2i). It should be noted that these sizes fall in the range where the quantum size effects for CuInS_2 NPs can be observed.^{41,42} These results can also be confirmed from the high-resolution SEM images of the same samples, as shown in Fig. S2(a–d),† where the QD size distribution shows variation in the size of QDs from 5.4 to 9.2 nm. In the case of Cu_2O QD/TNT electrode system, the same procedure was followed to optimize the loading efficiency and size of the QDs. The FESEM cross-sectional images are provided in the ESI in Fig. S3(a–e)† along with the size distribution of the QDs. For 10 deposition cycles of Cu_2O , uniform and homogeneous distributions of Cu_2O QDs were observed with a particle size of ~ 5 nm. From these data along with electrochemical impedance and sensing results in subsequent sections, the deposition cycles of 10 (Cu_2O) and 6 (CuInS_2) represent an optimum condition for loading of Cu_2O and CuInS_2 QDs respectively.

Transmission electron microscopy

In order to get more detailed morphological and structural insights into the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$, HRTEM was performed. Fig. 3a and b shows the TEM images of the sample, demonstrating the successful deposition of uniformly dispersed Cu_2O and CuInS_2 QDs over the nanotube walls, as pointed by arrows. Fig. 3c shows the smooth morphology of the tube walls with a thickness of about 15 nm and a tube diameter of around 100 nm. The HRTEM images (Fig. 3d) show three different lattice spacings. A lattice spacing of ~ 0.35 nm is assigned to TNTs, which corresponds to the (101) plane of anatase TiO_2 . The QDs with a size of about 3 nm and a lattice spacing of 0.32 nm can be assigned to the (112) lattice planes of CuInS_2 . The other type of QDs with a comparable size of about 4 nm and a lattice spacing of 0.214 nm is ascribed to Cu_2O QDs with preferred orientation along the (200) crystal plane. These results can be further confirmed from the XRD data. Furthermore, the elemental mapping along with energy-

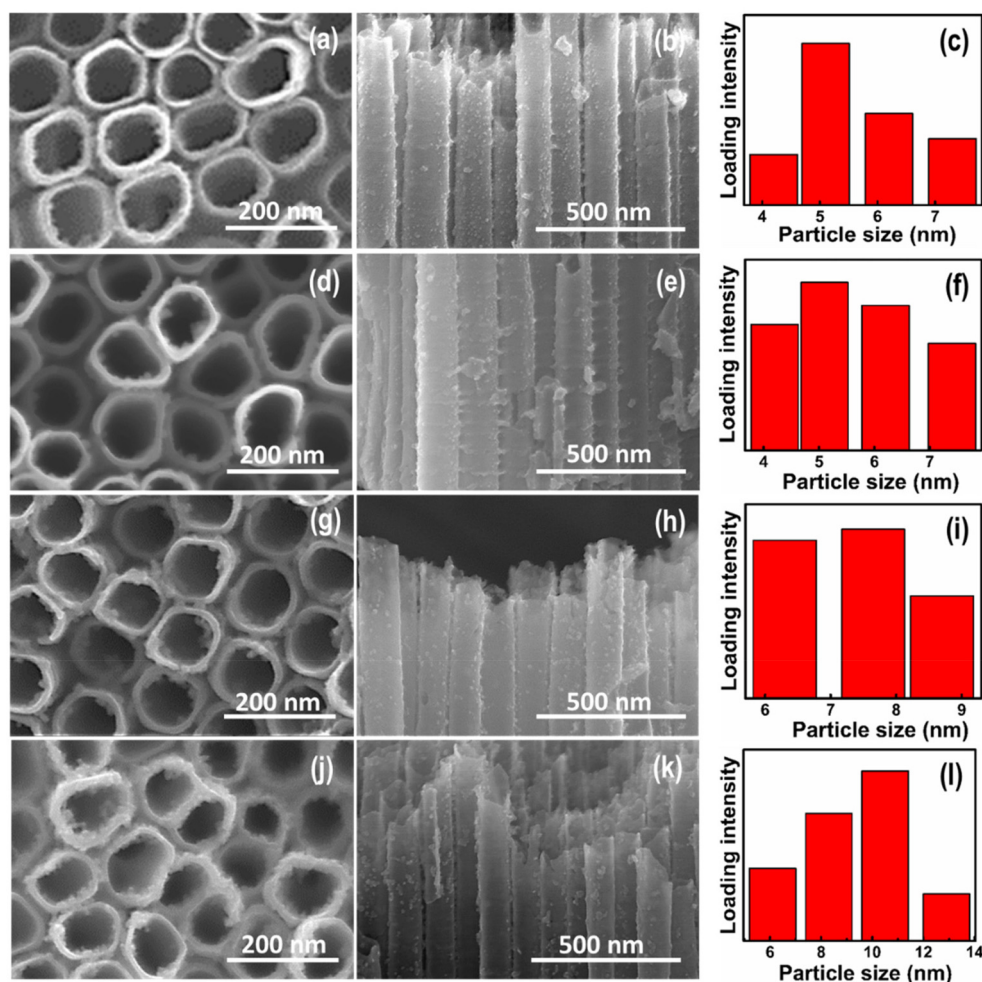


Fig. 2 FE-SEM images showing the top view, cross-sectional view and corresponding QD size distribution of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrodes decorated with CuInS_2 QDs for (a–c) 2 cycles, (d–f) 4 cycles, (g–i) 6 cycles and (j–l) 8 cycles while keeping the loading of Cu_2O QDs the same.

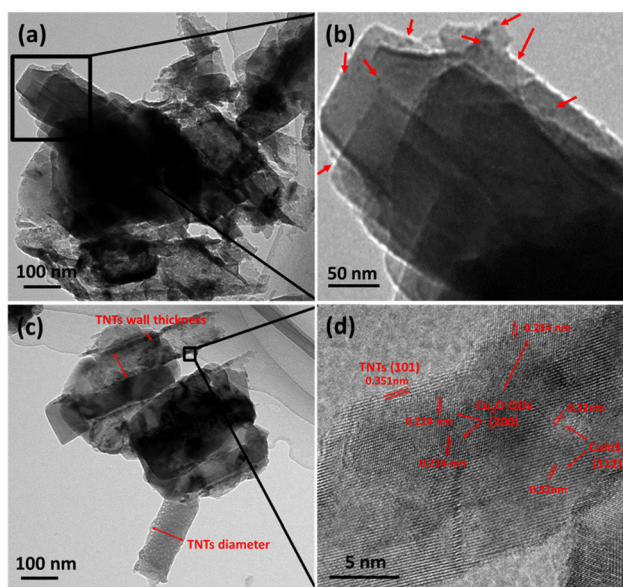


Fig. 3 (a–c) TEM and (d) HRTEM images of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$.

dispersive X-ray spectra of the same sample (from the top view with a scanning area of $2 \mu\text{m}$) for Ti, O, Cu, In, and S is shown in Fig. S4(a–f).† Fig. S4(a–e)† depicts the uniform distribution of Cu_2O and CuInS_2 QDs on the TNT matrix. Moreover, the presence of well-defined peaks of Ti, O, Cu, In and S are evident from Fig. S4f† and their atomic weight% illustrates the formation of a good stoichiometric hybrid system.

X-ray diffraction

The XRD patterns of the pristine TNTs, $\text{Cu}_2\text{O}/\text{TNTs}$, $\text{CuInS}_2/\text{TNTs}$, and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ are presented in Fig. 4. The main diffraction peak at about 25.27° can be indexed to the (101) anatase TiO_2 phase⁴³ (Fig. 4a), which agrees well with the literature (JCPDS card #01-071-1166). After the formation of heterojunctions due to the decoration of Cu_2O QDs on TNTs, an additional well-defined peak at 42.47° was observed that is attributed to the (200) plane of Cu_2O (Fig. 4b).^{44,45} This peak can be indexed to the cubic crystal structure according to JCPDS card #00-034-1354. The XRD pattern of CuInS_2 QDs decorated on TNTs is presented in Fig. 4d. In addition to the

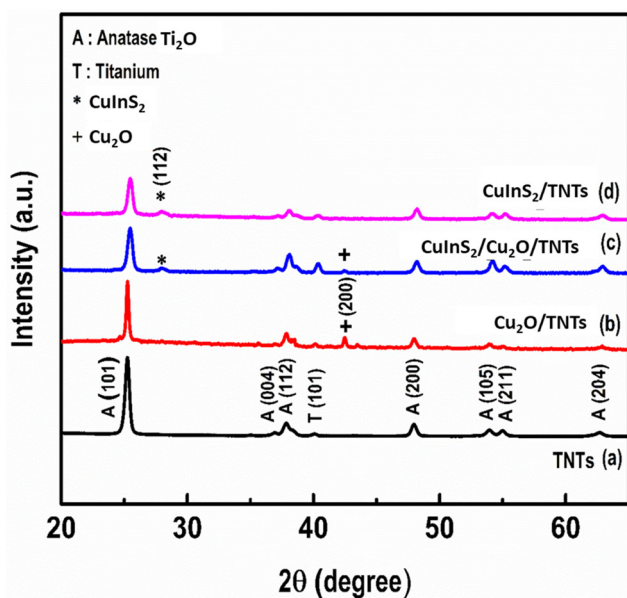


Fig. 4 XRD patterns of (a) pristine TNTs, (b) $\text{Cu}_2\text{O}/\text{TNTs}$, (c) $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ and (d) $\text{CuInS}_2/\text{TNTs}$.

typical peaks of anatase, a peak at 27.95° corresponding to the (112) crystal plane of CuInS_2 has been observed that can be indexed according to JCPDS card #85-1575. However, the lower peak intensity may be due to the relatively lower amount and smaller size of the QDs (Fig. 4d). Fig. 4c shows the XRD pattern of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ hybrid nanostructure. The diffraction peaks in the XRD pattern of the aforementioned composite system can be attributed to anatase TiO_2 , Cu_2O and

CuInS_2 QDs. Furthermore, it was found that the diffraction peak of Cu_2O located at around 42.47° was less prominent in the case of hybrid nanostructures (after decoration of Cu_2O QD/TNT with CuInS_2 QDs). This may be due to the deposition of CuInS_2 QDs on the Cu_2O QD/TNT sample. No obvious impurity peaks were observed, suggesting the high purity of the synthesized samples.

X-ray photoelectron spectroscopy

The chemical composition and valence state of the $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNT}$ system were investigated by XPS. Fig. 5(a–e) shows the high-resolution spectra of O 1s, Ti 2p, Cu 2p, In 3d, and S 2p respectively. The deconvoluted spectra of O 1s (Fig. 5a) consists of a main peak at a binding energy of around 529.9 eV, which corresponds to the oxygen atom in Ti–O–Ti and the small peak at ~ 531.3 eV can be attributed to surface hydroxyl ions.⁴³ In the case of core-level spectra of Ti 2p (Fig. 5b), the two peaks located at binding energies of about 458.7 eV and 464.4 eV can be assigned to $\text{Ti } 2p_{3/2}$ and $\text{Ti } 2p_{1/2}$ respectively which confirms the existence of the Ti^{4+} state in the material.⁴⁶ The XPS spectrum of Cu 2p is deconvoluted into four peaks (Fig. 5c), where the two main peaks can be attributed to the Cu $2p_{3/2}$ (931.6 eV) and Cu $2p_{1/2}$ (951.6 eV) spectra, corresponding to the Cu^{1+} state. The other two small peaks at 932.7 eV and 953.2 eV are due to the traces of Cu^{2+} within the sample, which could be due to the exposure of the sample to the atmosphere. It is also worth noting that the Cu 2p satellite peaks related to Cu^{2+} oxidation state,^{43,46} which are usually located at ~ 942 eV and 962 eV, did not appear in the spectra.⁴⁷ Therefore, it can be concluded that the majority of Cu^{2+} has been reduced to Cu^{1+} during the course of reactions, as shown in the ESI (Fig. S5†). At the initial stage of electrode fabrication

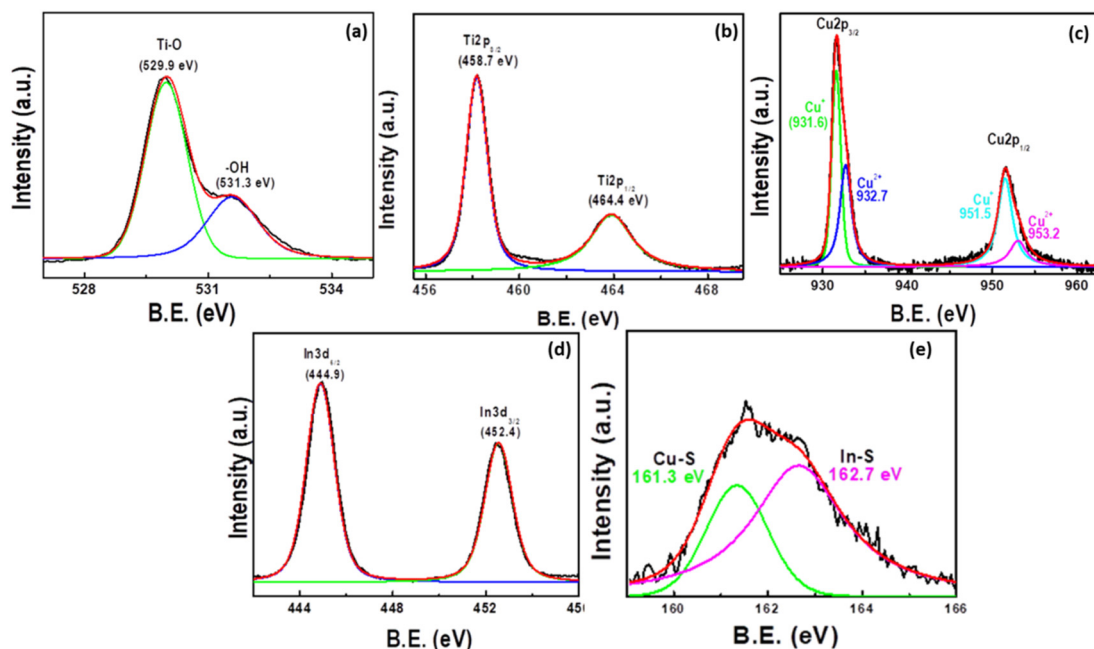


Fig. 5 High-resolution XPS spectra of $\text{CuInS}_2(6)/\text{Cu}_2\text{O}/\text{TNT}$ samples showing (a) O 1s, (b) Ti 2p, (c) Cu 2p, (d) In 3d and (e) S 2p states.

when only Cu₂O QDs were deposited on TNTs, a significant amount of Cu²⁺ is also present in the system, as indicated by the satellite peaks in Fig. S5a.† However, by depositing CuInS₂ QDs, the traces of Cu²⁺ are significantly reduced (Fig. S5c†). The In 3d_{5/2} and In 3d_{3/2} peaks (shown in Fig. 5d) were located at around 444.9 eV and 452.4 eV with a peak splitting of 7.5 eV, which agrees well with the reported values for In³⁺.^{48,49} The S 2p has doublet peaks of S 2p_{1/2} and S 2p_{3/2} due to spin-orbit coupling, which can be assigned to the S²⁻ oxidation state.^{48,49} The first peak of S 2p (Fig. 5e) is located at ~161.5 eV, which can be attributed to the Cu-S bond, while the other peak at ~162.6 eV is from In-S.³⁰ Based on the XRD, FESEM, HRTEM, and XPS results, it is concluded that Cu₂O and CuInS₂ QDs have been successfully deposited on TNTs by our facile sonication-assisted CBD method.

Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy was employed to evaluate the charge transport and transfer properties of the optimized electrode in a relevant electrolyte. Fig. 6a shows the Nyquist plots of the EIS for different electrode configurations in the frequency range of 1 MHz to 10 mHz at an open circuit potential. These plots consist of a semicircle region at higher frequencies that corresponds to the electron transfer limited process. The diameter of the semicircle gives the values of charge transfer resistance (R_{ct}) at the interface of the electrode. As shown in the figure, an abrupt decrease in the area of the semicircle is observed after decorating TNTs with Cu₂O QDs, indicating the improved electron transfer ability. Furthermore, the diameter of the semicircle decreases even more due to the deposition of CuInS₂ co-catalysts on Cu₂O/TNTs. The inset of Fig. 6a shows the magnified view of Nyquist plots at higher frequencies. To find the equivalent circuit for each curve, the fitting of the EIS data was performed using the ZView 2 software. The equivalent circuit for all samples is illustrated in Fig. 6c, and the fitted data are presented in Fig. S6(a-f).† For most of the samples, it is observed that the impedance data is suitably fitted using a series combination of R - C (R and C in parallel) and R -CPE units. Here CPE denotes the constant phase element, R denotes the electric charge transfer resistance and C denotes the double-layer capacitance.^{50,51} Fig. 6c(i) shows the equivalent circuit for TNTs. R_1 represents the charge transfer resistance at the TiO₂/electrolyte interface, CPE (also called non-ideal double-layer capacitance) is due to the diffusion of electrolytes within nanotubes, R_2 and C_1 correspond to the Ti/TNT interface and R_3 and C_2 for the Helmholtz layer.⁵⁰ Fig. 6c(ii) corresponds to the equivalent circuit of the Cu₂O/TNT and CuInS₂/TNT samples. R_1 and CPE₁ correspond to the TNT-electrolyte interface and R_2 and CPE₂ to the TiO₂/Cu₂O (or CuInS₂/TiO₂) junction. The third (R_3 , C_1) and fourth (R_4 , C_2) units correspond to the Cu₂O-electrolyte (or CuInS₂-electrolyte) interface and Helmholtz layer respectively. Fig. S6† also shows the fitting parameters for all the samples. It is observed that the decoration of Cu₂O and CuInS₂ on TNTs greatly decrease the values of charge transfer resistance. The decrease in the impedance

value observed in the Nyquist plots can be ascribed to the efficient catalytic activity of the co-catalyst and small (and adjustable) size of the QDs.¹⁶ Both CuInS₂ and Cu₂O QDs offer a higher interface area with suitable band positions for the efficient electron transfer, which strongly enhances the electron transfer kinetics of redox reaction at the surface of the hybrid material. It should be noted that the smallest area of the semicircle for the CuInS₂/Cu₂O/TNTs electrode indicates an efficient electrical network through CuInS₂ and Cu₂O QDs even for this complex hybrid structure. As a result of this configuration, the overall electro-oxidation rate can be greatly improved. The Nyquist plots for different loading cycles of CuInS₂ QDs are shown in Fig. 6b. The diameter of the semicircle decreases due to the increase in the loading of QDs up to 6 deposition cycles. A further increase in the deposition cycles subsequently increases the impedance. This enhancement in impedance can be attributed to the increase in the size of QDs and will be discussed in the section of cyclic voltammetry. The equivalent circuit in Fig. 6c(iii) shows the circuit units corresponding to the TNT-electrolyte, TNT-CuInS₂ and TNT-Cu₂O junctions in addition to the Helmholtz layer (fourth unit).

The Mott-Schottky (MS) technique was employed to measure the properties (*e.g.* carrier densities, flat band potential and conduction type) of the electrode material at the electrode-electrolyte interface. These measurements were performed in a 1 mM NaOH solution using a three-electrode electrochemical cell with Ag/AgCl (in saturated KCl) as the reference electrode, a platinum foil as the counter electrode, and TNTs, Cu₂O/TNTs and CuInS₂/Cu₂O/TNTs as the working electrodes. The carrier densities N_d in different heterostructures can be accurately measured using the following Mott-Schottky equation:

$$\frac{1}{C^2} = \left[\frac{2}{e\epsilon\epsilon_0 N_d A^2} \right] \left[V - V_{fb} - \frac{kT}{e} \right].$$

The + and - signs are used for n-type and p-type semiconductors respectively. C is the interfacial capacitance, ϵ is the dielectric constant of the semiconductor electrode (for TiO₂ its 76), ϵ_0 is the vacuum permittivity (8.854×10^{-12} F m⁻¹), e is the electron charge (1.603×10^{-19} C), V_{fb} is the flat band potential, N_d is the carrier density, A is the electrode area (here the measured area of working electrode is 1.0 cm²), V is the applied voltage, k_B is the Boltzmann constant (1.38×10^{-23} J K⁻¹), and T is the absolute temperature.

Fig. 7 shows the derived $1/C^2$ - V MS curves for TNT, Cu₂O/TNT and CuInS₂/Cu₂O/TNT electrodes. The carrier density N_d for TNTs, Cu₂O/TNTs and CuInS₂/Cu₂O/TNTs can be determined by plotting the tangent to the linear portion of the Mott-Schottky curve (as shown in Fig. S7a-c†) and using the following equation:

$$N_d = \frac{2}{e\epsilon_0\epsilon_{\text{TiO}_2}m}$$

where m is the slope of the Mott-Schottky curve. The slope of TNTs has the highest value leading to a carrier density of

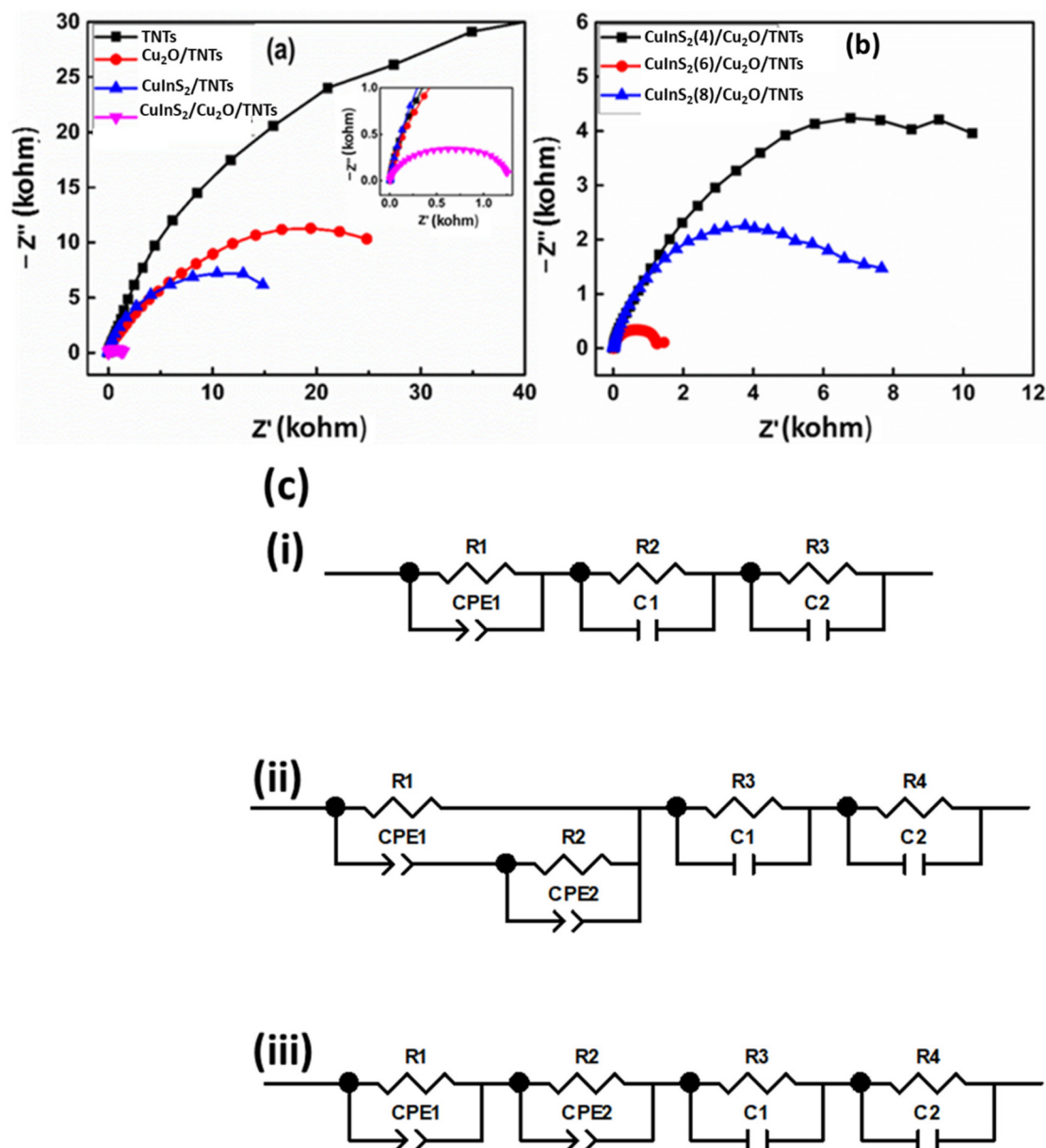


Fig. 6 (a) Electrochemical impedance spectroscopy (EIS) Nyquist plots for the prepared materials in 0.1 M PBS (pH 7.5). Inset: enlarged view for high frequencies. (b) Nyquist plots for $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ synthesized by varying CBD cycles for CuInS_2 QDs on $\text{Cu}_2\text{O}/\text{TNTs}$. (c) Equivalent circuits of (i) TNTs, (ii) $\text{Cu}_2\text{O}/\text{TNTs}$ and $\text{CuInS}_2/\text{TNTs}$, and (iii) $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ with different deposition cycles of CuInS_2 .

$0.275 \times 10^{19} \text{ cm}^{-3}$ (Table 1). The carrier concentrations for $\text{Cu}_2\text{O}/\text{TNTs}$ and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ are $0.399 \times 10^{19} \text{ cm}^{-3}$ and $0.92 \times 10^{19} \text{ cm}^{-3}$ respectively. All the samples show positive slopes ascribed to the n-type conduction of the electrode material.

The value of the flat band potential plays a significant role in determining the charge transfer process in heterostructures. The flat band potential (V_{fb}) can be obtained from the intercept of the liner portion of the Mott-Schottky curve on the x -axis. The values of the flat band potential for different samples are listed in Table 1. The flat band potential for TNTs is -0.68 , and is shifted towards a more negative potential by incorporating Cu_2O and CuInS_2 into TNTs. The flat band potential can also be used to determine the conduction (E_{CB})

or the valence band (E_{VB}) edges for TNTs. Once V_{fb} is known, E_{CB} can be calculated from the following relation:

$$E_{\text{CB}} = V_{\text{fb}} + kT \ln \left(\frac{N_{\text{d}}}{N_{\text{CB}}} \right)$$

where N_{VB} and N_{CB} are the effective density of states in the valence and conduction bands of TiO_2 . Once E_{CB} is determined, E_{VB} can be easily found provided and E_{g} (3.2 eV) is known. The calculated values of the band edges are converted into the NHE scale by

$$E_{\text{NHE}} = E_{\text{Ag/AgCl}} + E_{\text{Ag/AgCl}}^{\circ}$$

where $E_{\text{Ag/AgCl}}^{\circ}$ is 0.199.

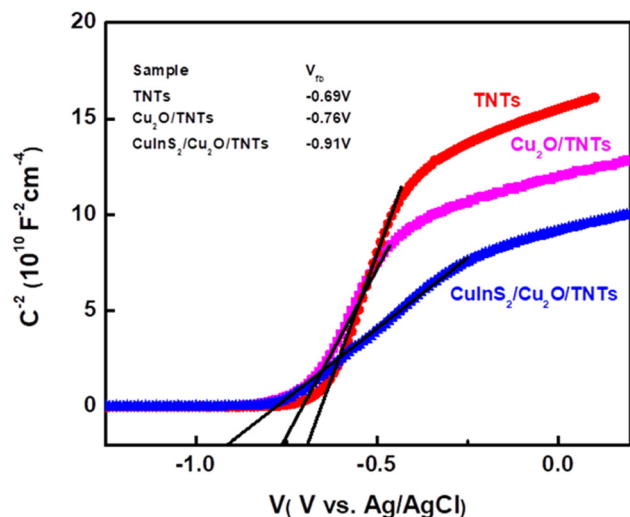


Fig. 7 Mott-Schottky curves and the respective flat band values of TNTs, Cu₂O/TNTs and CuInS₂/Cu₂O/TNTs.

Table 1 Mott-Schottky parameters calculated/extracted from the fitting of the linear portion of the curve of TNTs, Cu₂O/TNTs and CuInS₂/Cu₂O/TNTs

Sample	Slope (<i>m</i>)	Carrier density <i>N_d</i> (cm ⁻³)	Flat band potential <i>V_{fb}</i> (V)
TNTs	51.26×10^{10}	0.275×10^{19}	-0.69
Cu ₂ O/TNTs	35.35×10^{10}	0.399×10^{19}	-0.76
CuInS ₂ /Cu ₂ O/TNTs	15.31×10^{10}	0.92×10^{19}	-0.91

The calculated values of E_{CB} and E_{VB} for TNTs are -0.51 eV and 2.71 eV respectively. These values are in line with the values determined from the Brus model. However, in case of composite structures, the exact values of E_{CB} and E_{VB} for Cu₂O and CuInS₂ cannot be determined from the MS plots. Therefore, the cyclic voltammetry data and Brus model were used to estimate the band edge positions of Cu₂O and CuInS₂.

Cyclic voltammetry

A key aspect for the enhanced electrochemical and catalytic performance is the size-dependent variation in the electronic structure of the QDs and surface states, which may affect the charge transport properties of the biosensors. Therefore, the correlation between the electronic structure and the sizes of the QDs is highly desirable to obtain an optimum electrode configuration.

Cyclic voltammetry (CV) has been considered as an effective approach for finding the electronic band gap and energy band positions (*e.g.* conduction band (CB) and valence band (VB)); however, the interpretation of the surface states can be an intricate process. Charge transfer between QDs occur through electrons and holes, which manifests as reduction and oxidation peaks in CV. As the size of the QDs decreases, the reduction and oxidation peaks shift towards more negative and positive potentials respectively (due to quantum size-

induced variations in the band gap). Thus, the charge transfer across QDs can be tuned and investigated by observing the oxidation and reduction peaks with their intensity dependent on the alignment with redox levels in the electrolyte.⁵² For this reason, we investigated the influence of the QD size on the conduction band (E_{CB}) and valence band (E_{VB}) positions by utilizing the size-dependent voltammetric response, as shown in Fig. S8(a and b).† The investigations were carried out on Cu₂O/TNTs and CuInS₂/Cu₂O/TNTs electrode systems by varying the deposition cycles of the QDs. The size and loading density of both the QDs increase by increasing the deposition cycles. Both parameters can be estimated and extracted from the SEM micrographs, as shown in the ESI (Fig. S3(a–e)†) for the Cu₂O/TNT electrode system and in Fig. 2(a–l) for the CuInS₂/Cu₂O/TNTs electrode. In the case of Cu₂O/TNTs (Fig. S8a†), a pair of well-defined reduction (-0.42 V) and oxidation (-0.06 V) peaks along with an additional complementary reduction peak at -0.58 V can be observed. Ideally, this oxidation and reduction peak can be assigned to the electron transfer process through the E_{VB} and E_{CB} energy levels inside Cu₂O QDs. However, the true separation is of a more complex form given by the Marcus-Gerischer approach⁵³ as an overlay of the state distributions in the solid and liquid. It is evident from Fig. S8a† that the oxidation and reduction peaks gradually shift towards more positive and negative potentials respectively, while the redox peak separation is increased by decreasing the size of the QDs from 12.5 nm to 4 nm. This suggests that the size of the QDs plays a dominant role in the overall charge transfer. Fig. S8b† shows the CV response of the CuInS₂/Cu₂O/TNT electrode for different loading cycles of CuInS₂ QDs (by keeping the deposition of Cu₂O QDs fixed to 10 cycles). An additional reduction peak (-0.85 V) and a corresponding small oxidation peak (-0.6 V) appears along with the pair of previously observed redox peaks (for the Cu₂O/TNT electrode). However, the complementary reduction peak at -0.58 V (observed in Fig. S8a†) related to Cu¹⁺/Cu⁽⁰⁾ seems to merge with the reduction peak for Cu²⁺/Cu¹⁺, as evident from the peak broadening. Moreover, an obvious peak shifting appears, with the decrease in the QD size, for all redox peak potentials towards positive and negative values corresponding to oxidation and reduction peaks, respectively. In Fig. S8(b),† the magnitude of the redox peak current increases by increasing the loading of CuInS₂ QDs (up to 6 cycles) with an average CuInS₂ particle size of ~7.5 nm. However, a further increase in the deposition cycles increases the particle size up to 9.5 nm, which reduces the redox peaks current. These results show that the smaller and larger sized QDs exhibit much lower peak current intensities than the mid-sized/(optimum-sized) QDs. These results are in agreement with the understanding that the smaller and bigger QDs have more defect sites on the surface, due to a relatively short reaction time, compared to those with an intermediate size (likely to experience an improvement in surface defects).⁵⁴ The larger QDs are usually formed by the Ostwald ripening process, which can result in the generation of defects. Hence, the redox process can be influenced by the surface trap states. Moreover, the initial increase and then a

substantial decrease in redox current with the increase in QD size are ascribed to the energy band alignment and shielding of Cu₂O QDs by CuInS₂ QDs, which may reduce the synergistic effect of Cu₂O QDs, resulting in decreased electrochemical performance. Therefore, a balance between the loading density and size of the QDs along with the corresponding redox current density is required. Thus, the loadings of 10 Cu₂O QDs and 6 CuInS₂ QDs on the CuInS₂/Cu₂O/TNT electrode during deposition cycles were taken as optimum values because of the higher loading density and enhanced electrochemical activity. The band gap energies of various sizes of Cu₂O and CuInS₂ QDs were calculated to further elaborate the concept of quantum confinement. Our previous results showed that the onset values of the redox potential for both systems (Cu₂O and CuInS₂ QDs) may not reflect the actual positions of the VB and CB. Therefore, the Brus model has been utilized for calculating the band gap energy of QDs of various sizes according to the following equation:^{55,56}

$$E(R) = E_g^{\text{bulk}} + (h^2 \pi^2 / 2eR^2)(1/m_e + 1/m_h)$$

in this equation, R is the size of the QDs, E_g^{bulk} is the band gap energy of bulk Cu₂O (2.4 eV) or CuInS₂ (1.5 eV) QDs, h is Planck's constant, e is the charge of an electron, and m_e and m_h are the effective masses of the electron and hole of Cu₂O or CuInS₂ respectively. In the case of CuInS₂ QDs, $m_e = 0.16m_0$ (m_0 is the mass of electron) and $m_h = 1.3m_0$, while for the Cu₂O QDs, $m_e = 0.99m_0$ and $m_h = 0.58m_0$.

Furthermore, the VB and CB positions of Cu₂O and CuInS₂ QDs of different sizes were calculated individually using the following relations:

$$E_{\text{VB}} = \chi + 0.5E_g - E_c$$

$$E_{\text{CB}} = E_{\text{VB}} - E_g$$

where χ is the electronegativity of the QDs, E_g is the bandgap energy of the bulk material and E_{CB} and E_{VB} are the corresponding CB and VB energies respectively. These results are summarized in Table 2 and illustrated in Schemes S1 and S2† for Cu₂O and CuInS₂ QDs respectively. Scheme S1† shows the energy levels of Cu₂O QDs with different sizes compared to the energy levels of the matrix material (TiO₂). A gradual shift in the CB energies and valence band energies of these Cu₂O QDs was observed by decreasing the size of the QDs. This shifting

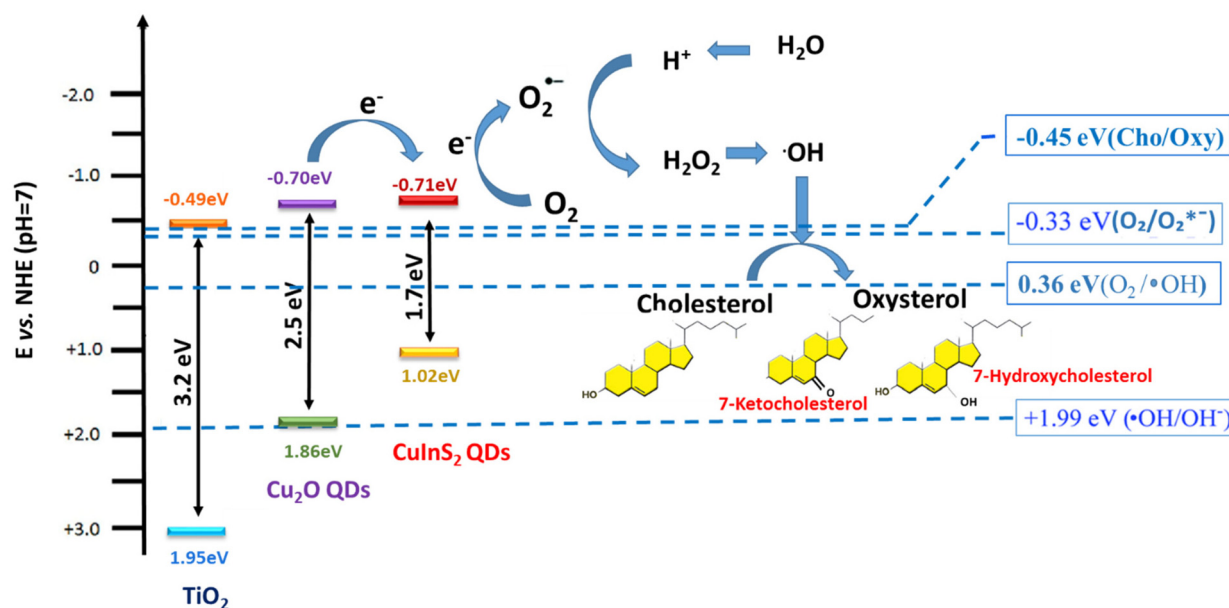
leads to an increase in the band gap energies from 2.43 eV to 2.73 eV by reducing the size of Cu₂O NPs to QDs (from 12.5 nm to 3.5 nm), which are in good agreement with the theory of quantum confinement effect. Scheme S2† shows the band gaps and band edge positions of different-sized CuInS₂ QDs. It exhibits that the band gap reduces from 2.0 eV to 1.6 eV by increasing the QD size in a range of 5.0–9.5 nm and nearly approaches the bulk value for a size of 9.5 nm.

The charge transfer mechanism in CuInS₂/Cu₂O/TNT biosensors is illustrated in Scheme 1. The band gap of anatase TNTs is about 3.2 eV, while the band gaps of 5 nm Cu₂O QDs and 7.5 nm CuInS₂ QDs are 2.5 eV and 1.7 eV respectively. It can be seen in Scheme 1 that the CB of CuInS₂ QDs lies higher than that of Cu₂O QDs and TiO₂, where the CB of all these three active electrode materials align in such a way to follow the order from higher to lower position as CuInS₂ > Cu₂O > TiO₂. The corresponding VB position of these materials follows the reverse order. As previously observed, the large CB energy offset will increase the electron injection rate from CuInS₂ to Cu₂O and then to TiO₂. However, applying a sufficiently negative potential causes the electrode to donate electrons to the reducing species in the electrolyte. Moreover, Scheme 1 further illustrates the proposed mechanism for the chemical oxidation of cholesterol. The initiation of cholesterol chemical oxidation was carried by the electro-reduction of adsorbed O₂ on the surface of the electrode. The electrode gets polarized at a sufficiently negative potential; generally known as the oxygen reduction reaction (ORR) that produces hydroxyl radicals (*OH).^{57–59} These hydroxyl radicals are critical to initiate the cholesterol chemical oxidation to produce oxysterols (7b-ketocholesterol, 7a-hydroxycholesterol and 7b-hydroxycholesterol).⁶⁰ In addition, the presence of Cu¹⁺ in CuInS₂ QDs is also evidenced in breaking the O–O bond of H₂O₂ to generate *OH radicals, that cause a dramatic increase in the reduction current.⁶¹ This may greatly improve the performance of the biosensing platform.

The order of decoration of TNTs with Cu₂O and CuInS₂ QDs also plays a significant role in the electrochemical performance of the biosensor. The cascade band gap alignment of TiO₂ with Cu₂O and CuInS₂ QDs show the reduced electron transfer recombination rate; however, by switching the decoration order from CuInS₂/Cu₂O/TNTs to Cu₂O/CuInS₂, QD/TNT may reduce the electrode conductivity.

Table 2 Effect of the size of Cu₂O and CuInS₂ QDs on their band gap and band edge positions

Sample	Electronegativity χ (eV)	Particle size (nm)	Conduction band energy E_{CB} (eV)	Valence band energy E_{VB} (eV)	Band gap energy E_g (eV)
Cu ₂ O/TNTs	5.3	3.5	−0.79	1.95	2.73
		5	−0.70	1.86	2.56
		6.5	−0.67	1.82	2.49
		8.5	−0.65	1.80	2.46
		12.5	−0.63	1.79	2.43
CuInS ₂ /TNTs	4.9	5	−0.82	1.19	2.02
		5.5	−0.74	1.11	1.85
		7.5	−0.71	1.02	1.73
		9.5	−0.66	0.98	1.64



Scheme 1 Schematic of cholesterol oxidation on CuInS₂/Cu₂O/TNTs.

Electrocatalytic oxidation of cholesterol by cathodically generated reactive oxygen species (ROS)

To investigate the electrocatalytic oxidation of cholesterol by the CuInS₂(6)/Cu₂O/TNT electrode, cyclic voltammetry was performed. Fig. 8a presents the CV scans of different electrode configurations in 0.1 M PBS recorded at a scan rate of 50 mV s⁻¹ by sweeping the potential from positive to negative values. Since the first sweep has been scanned in the negative direction (following the US convention), the working electrode increases its capacity to donate electrons to chemical species, thus obtaining a cathodic/reduction current (positive current), which is represented in the upper branch of cyclic voltammograms (CV). While the current density is obtained when the potential is scanned in the positive direction, the anodic/oxidation current is negative.

Fig. 8a shows no redox peak in the case of the pristine TNT electrode, whereas a pair of well-defined quasi reversible redox peaks were found for all other electrodes. For instance, Cu₂O QD-decorated TNTs show an abrupt increase in the current density along with reduction and oxidation peaks. The first reduction peak at a potential of ~-0.41 V corresponds to Cu²⁺/Cu¹⁺, while the second reduction peak at a potential around -0.50 V is attributed to the Cu¹⁺/Cu⁽⁰⁾ redox pair. In the lower half cycle, an oxidation peak is observed at -0.06 V that corresponds to the oxidation of Cu⁽⁰⁾ to Cu²⁺. In order to check the electrochemical response of the CuInS₂ QDs deposited on the pristine TNT, the CV of CuInS₂(6) QDs/TNTs was also performed (Fig. 8a, blue curve). The electrochemical activity of the CuInS₂ QD-loaded TNTs was higher than that of Cu₂O/TNTs, with the predominant redox peaks for the reduction and oxidation of CuInS₂. The reduction current reaches its highest value after the co-decoration of Cu₂O QDs/TNTs with CuInS₂

QDs. The composite CuInS₂(6)/Cu₂O/TNT electrode also shows well-defined redox peaks. The peak at -0.75 V corresponds to the reduction of CuInS₂ followed by the corresponding oxidation peak at -0.6 V. The other reduction peak observed at -0.34 V are attributed to the reduction of Cu²⁺ to Cu¹⁺. Conversely, the broad peak close to 0.1 V is due to the oxidation of Cu⁽⁰⁾ to Cu²⁺. Moreover, in the case of the CuInS₂/Cu₂O/TNT hybrid electrode, the current density is significantly higher than the other electrode configurations. This is due to the availability of higher catalytic sites as a result of QD deposition and suitable band gap alignment of the QDs with TNTs (Scheme 1). Fig. 8b shows the CV scans of the CuInS₂(6)/Cu₂O/TNT electrode in response to the increasing cholesterol concentration in the electrolyte. A dramatic increase in the reduction peak current density has been found with the addition of cholesterol in the electrolyte, which is due to the combined catalytic effect of Cu₂O and CuInS₂ QDs. The maximum reduction current density has been measured in response to the cholesterol concentration of 287.5 μM in the electrolyte. An increase in the current density with the increase in cholesterol concentration is due to its chemical oxidation by the electrochemically generated hydroxyl radicals produced by the composite electrode. For the pristine TNTs, no significant increase in oxidation and reduction current density was observed after the addition of cholesterol in the electrolyte (Fig. S9†), which shows that the TNT/Ti electrode exhibits less electrocatalytic activity towards the cholesterol chemical oxidation. Additionally, a voltammetric peak observed at -1.5 V corresponds to the oxygen reduction process.

The effect of different scan rates on the electrochemical responses of the CuInS₂(6)/Cu₂O/TNT electrode was recorded in 0.1 M PBS containing 0.25 mM of cholesterol (Fig. 8c). The redox peak current density increases by increasing the scan

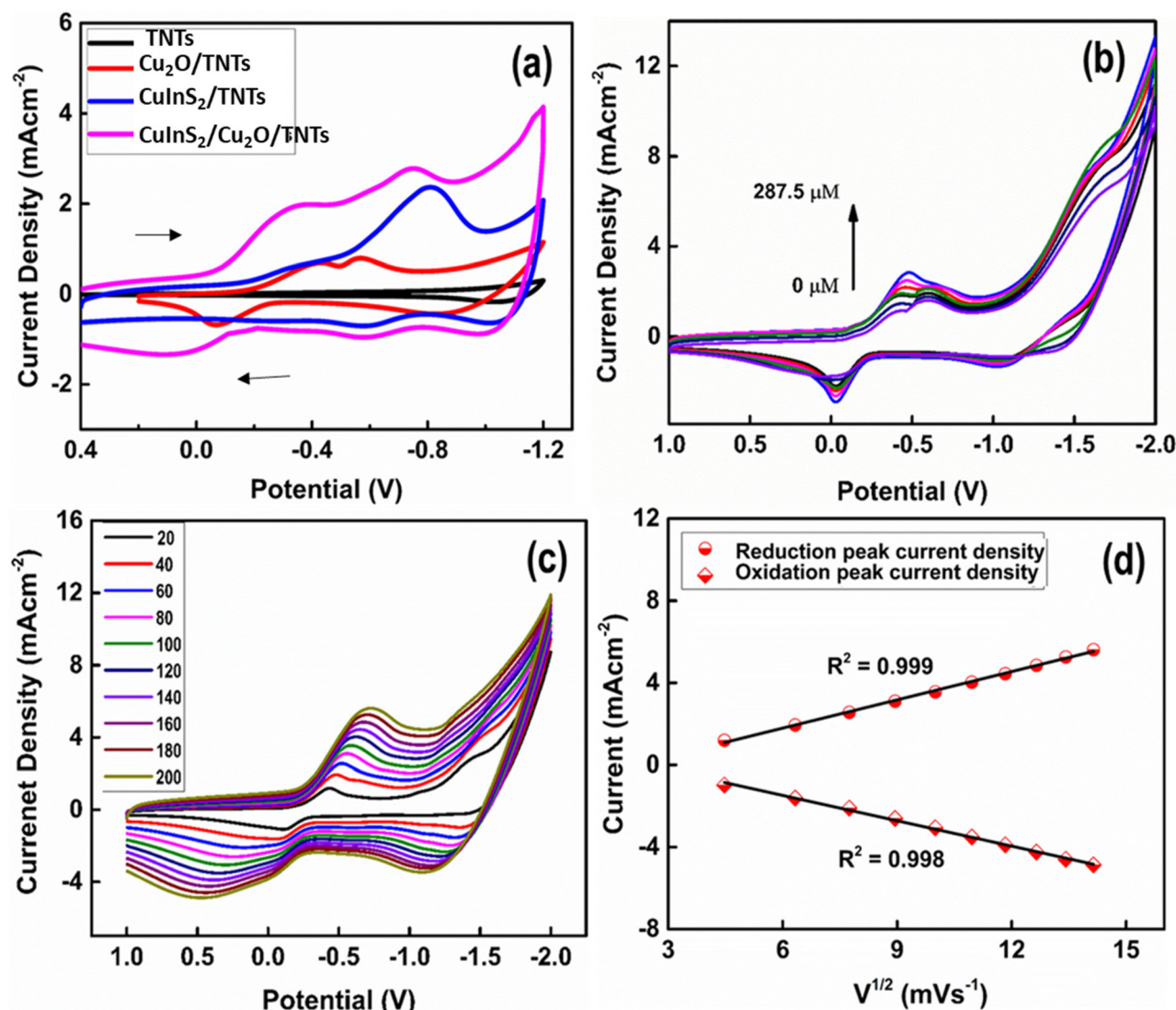


Fig. 8 (a) Cyclic voltammograms of pristine TNT, Cu₂O/TNT, CuInS₂/TNT and CuInS₂/Cu₂O/TNT electrodes in 40 ml of 0.1 M PBS obtained at 50 mV s⁻¹. (b) CV scans of the CuInS₂/Cu₂O/TNT electrode with the addition of different concentrations of cholesterol, and (c) as a function of scan rate (20–200 mV s⁻¹) in the presence of 250 μM cholesterol in 40 ml of 0.1 M PBS. (d) Plots of the oxidation and reduction peak currents versus root mean square of scan rate ($v^{1/2}$).

rates in the range of 20–200 mV s⁻¹, while a shift in the redox peak potential was observed as the scan rate increases. That is, the reduction peak shifts towards more negative potential and the oxidation peak towards positive potential. Fig. 8d shows that the relationship between the reduction and oxidation peak current densities and root mean square of the scan rate is linear with correlation coefficients (R^2) of about 0.999 and 0.998 respectively. The observed behaviour indicates that the chemical oxidation of cholesterol on the CuInS₂(6)/Cu₂O/TNTs electrode is diffusion controlled.

Amperometry

The amperometric curves of the pristine TNT, Cu₂O/TNT, CuInS₂/TNT, and CuInS₂(6)/Cu₂O/TNT electrodes were obtained for the successive addition of various concentrations of chole-

sterol under vigorous stirring conditions (Fig. 9a). For these measurements, a potential of -0.5 V was applied, which gave rise to reduction current. Upon the addition of different cholesterol concentrations, a rapid increase in the current density was observed. It was found that the reduction current of the CuInS₂(6)/Cu₂O/TNT electrode is much higher than that of the other electrode configurations, which is consistent with the CV results (Fig. 8a). The sequence through which the biosensors exhibit a higher current response is CuInS₂/Cu₂O/TNTs > CuInS₂/TNTs > Cu₂O/TNTs > TNTs. The CuInS₂/Cu₂O/TNT, CuInS₂/TNT, and Cu₂O/TNT electrodes achieved 95% of the steady-state current within ~1.3 s, 2.0 s, and 2.5 s, respectively. These values are much lower than the pristine TNTs (10 s). Fig. 9b shows the calibration curve of the CuInS₂(6)/Cu₂O/TNT electrode. It illustrates the linear dependence of the current

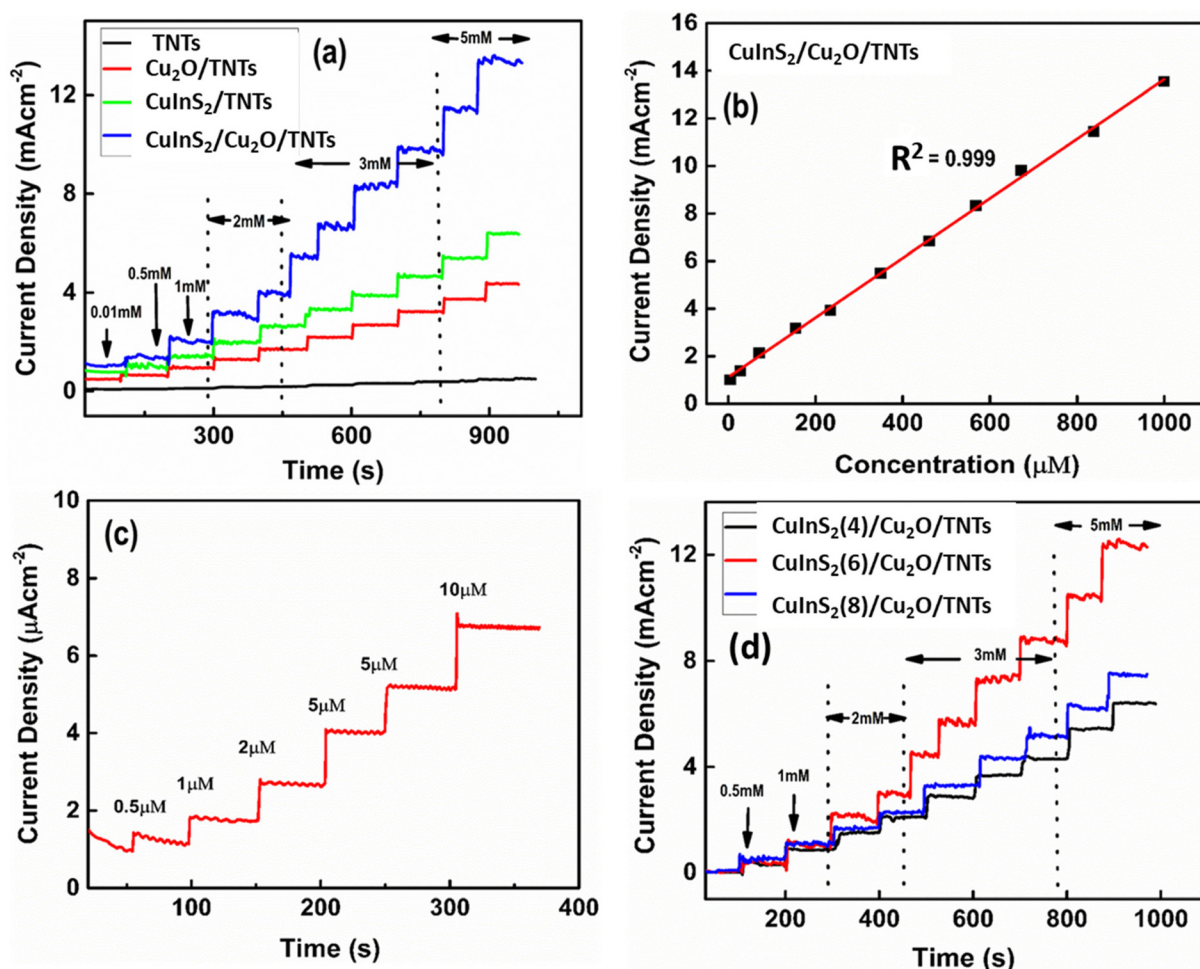


Fig. 9 (a) Amperometric response of TNT, Cu₂O/TNT, CuInS₂/TNT, and CuInS₂/Cu₂O/TNT electrodes at -0.5 V in 0.1 M PBS at pH 7.5 upon successive addition of different cholesterol concentrations. (b) Corresponding linear calibration curve of the CuInS₂/Cu₂O/TNT electrode. Amperometric response of CuInS₂/Cu₂O/TNTs (c) at low cholesterol concentrations and (d) for different deposition cycles of CuInS₂ QDs.

response on the cholesterol concentration. The calibration curves for all other electrodes are presented in the ESI (Fig. S10†). These calibration curves were derived from their corresponding amperometric responses by taking into account the dilution factor (due to 40 ml of electrolyte solution) for cholesterol concentration. It is worth mentioning that the linear range of cholesterol detection is much wider for the CuInS₂(6)/Cu₂O/TNT electrode (0.005 – 1 mM) compared to the other electrode configurations. Table 3 summarizes the characteristic parameters of all prepared electrodes obtained from their amperometric responses. The sensitivity of the CuInS₂(6)/Cu₂O/TNTs/Ti electrode is about $12\,530\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$, which is much higher than the values obtained for Cu₂O/TNTs ($4650\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$), CuInS₂/TNTs ($6960\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$), and pristine TNTs ($1125\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$). The amperometric data of the CuInS₂/Cu₂O/TNT electrode towards the low concentrations of cholesterol are presented in Fig. 9c. The limit of detection (LOD) of the hybrid electrode ($0.013\ \mu\text{M}$) is much lower than that of Cu₂O/TNT and CuInS₂/TNT electrodes, as given in Table 3. The low detection limit was obtained by

adding the lowest possible concentration of cholesterol, where a considerable rise in the current response was observed. These results demonstrate that the TNT electrode is electrochemically less active for the required redox reaction. Therefore, the decoration of TNTs with Cu₂O QDs enhances the cholesterol chemical oxidation process and greatly improves the response current. Moreover, the co-decoration of CuInS₂ QDs on the Cu₂O QDs/TNTs further amplifies the current response

Table 3 Comparison of the obtained parameters for different electrodes derived from the linear fitting of calibration plots of corresponding electrodes

S. no.	Electrode materials	LOD (μM)	Sensitivity ($\mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$)	Response time (s)	LDR (μM)
1	Pristine TNTs	4.8	1125	10	4.8–838
2	Cu ₂ O/TNTs	0.05	4650	2.5	27.5–671.4
3	CuInS ₂ /TNTs	0.025	6960	2	27.5–838
4	CuInS ₂ /Cu ₂ O/TNTs	0.013	12 530	1.3	4.8–999

and improves the response time and sensitivity. Fig. 9d illustrates the effect of deposition cycles of CuInS₂ QDs on the amperometric responses of the CuInS₂/Cu₂O/TNT electrodes. It is clear from the figure that the optimized content of CuInS₂ QDs with a corresponding maximum current response is obtained for 6 deposition cycles. The comparison of various important parameters related to the cholesterol sensor fabricated in this study (CuInS₂/Cu₂O/TNTs) with those reported in the literature is given in Table 4.^{25–28,62–69} It can be seen that the electrode reported here is superior in terms of sensitivity, linear range, response time, and detection limit. This is due to the availability of higher catalytic sites as a result of QD deposition and suitable band gap alignment of the QD with TNTs (Scheme 1).

Selectivity

The ability of the biosensor to differentiate between the different interfering species from the target molecule is one of the most important factors in sensing applications. Ascorbic acid, urea, glucose, H₂O₂, and L-cysteine can be the

common interfering species for the electrochemical oxidation of cholesterol. However, most of them are electrochemically active at positive potentials.^{70–74} Assuming that the concentration of cholesterol in human blood is much higher than the interfering biological species, the amperometric responses of the CuInS₂(6)/Cu₂O/TNT electrode towards the addition of different concentrations of cholesterol as well as interfering species were recorded (Fig. 10a). The current responses by these interfering species are negligible compared to the sharp current signal towards cholesterol, indicating the excellent selectivity of the proposed sensor for cholesterol detection. The possible reason for such a high selectivity can be the use of negative potential values where the aforementioned interfering species are not electrochemically active.

pH response

The pH value of the buffer solution has a strong impact on the electrochemical performance of the biosensor. Therefore, it is important to obtain an optimum value of pH at which the bio-

Table 4 Comparison of CuInS₂/Cu₂O/TNT electrodes with other modified electrodes as a cholesterol sensor

S. no.	Electrode materials	LOD	Sensitivity	Response time (s)	LDR	Ref.
1	Grp/ β -CD/methylene blue	1 μ M	0.01 μ A μ M ⁻¹	20	0.001–0.10 mM	62
2	Cu ₂ S NRS/CRIE	0.1 μ M	398 μ A mM ⁻¹ cm ⁻²	—	0.01–6.8 mM	27
3	1D porous tubular Ag/GCE	180 μ M	0.7 μ A mM ⁻¹ cm ⁻²	—	28–0.33 mM	63
4	Pt NPs/CNT	5 μ M	8.7 μ A mM ⁻¹ cm ⁻²	—	0.005–10 mM	28
5	Pt/Au	15 μ M	226.2 μ A mM ⁻¹ cm ⁻²	—	0.5–3.5 mM	26
6	ChO _x /MoS ₂ -AuNPs/GCE	0.26 μ M	4460 μ A mM ⁻¹ cm ⁻²	—	0.5–48 μ M	25
7	PTZ-ChO _x -modified SPE	2.3 μ M	33.1 mA M ⁻¹ cm ⁻²	—	0.015–0.15 mM	68
8	AuPt-Ch/GCE	10 μ M	90.7 μ A mM ⁻¹ cm ⁻²	7	0.05–6.2 mM and 6.2–11.2 mM	65
9	AuE/dithiol/AuNPs/MUA/ChO _x	34.6 μ M	9.02 μ A μ M ⁻¹	—	0.04–0.22 mM	66
10	CuInS ₂ /Cu ₂ O/TNTs/Ti	0.013 μ M	12 530 μ A mM ⁻¹ cm ⁻²	1.3	4.8–999 μ M	Present work

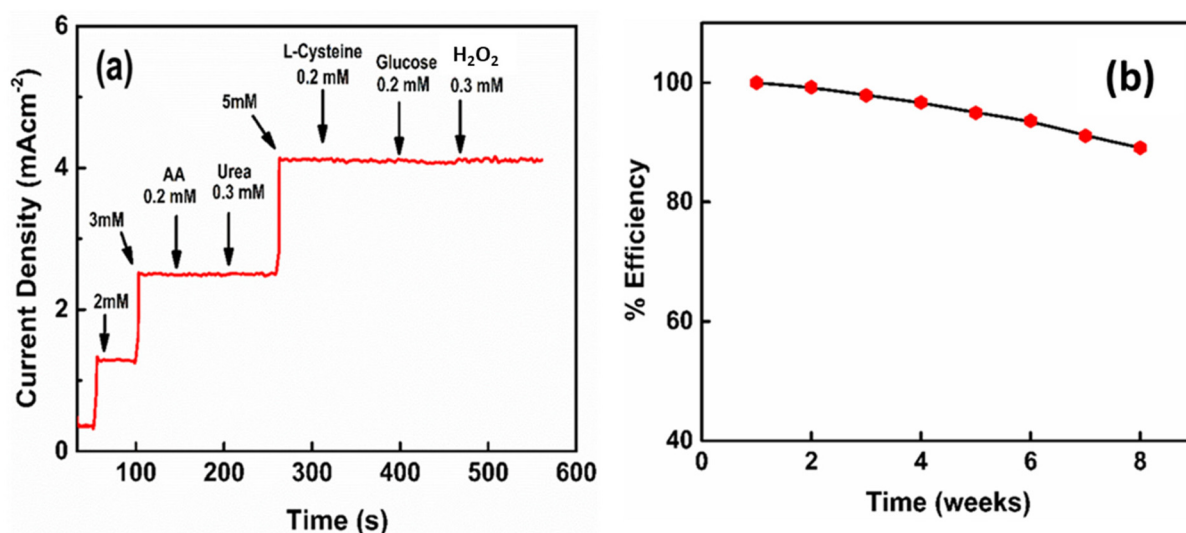


Fig. 10 (a) Amperometric response of the CuInS₂/Cu₂O/TNT electrode towards cholesterol with different interferents in 0.1 M PBS (pH 7.5). (b) Shelf life (stability) curve of CuInS₂/Cu₂O/TNTs as a function of time in weeks.

sensor exhibits its highest current response. For this reason, the pH of the assay solution was varied from 4 to 9. The corresponding responses of the biosensor were measured by CV in the presence of 0.25 mM cholesterol solution, as shown in Fig. S11a.† These results indicate that the electrochemical activity of the biosensor deteriorates in more acidic and basic media. As the pH of the electrolyte decreases to low values, the protonation of Cu₂O occurs that results in the formation of its sub-oxides. In the case of CuInS₂, the low pH values result in the formation of the predominant Cu₂S phase.⁷⁵ The current density increases with the increase in the pH value of the solution and the highest current response of the biosensor was measured at pH 7.5 due to the stability of the co-catalyst Cu₂O and CuInS₂ QDs. A further increase in the pH value increases the OH[−] in the solution which causes the formation of Cu and In hydroxides.^{76–78} Therefore, for the sensitive analysis of cholesterol, an optimum value of pH 7.5 was chosen for the electrochemical experiments.

Thermal stability of biosensors

One of the important factors influencing the practicality of the biosensor is its thermal stability. Fig. S11b† shows the current response of the biosensor in 0.1 M PBS containing 0.25 mM cholesterol in the temperature range of 20–70 °C. The reduction current is found to increase with the temperature owing to a higher reaction rate due to the increased diffusion of redox species. It is demonstrated that the proposed biosensor is highly stable over a temperature range with fast reaction kinetics.

CuInS₂/Cu₂O/TNT electrode shelf life

To evaluate the performance of the biosensor over a period of time, the current responses of the CuInS₂(6)/Cu₂O/TNT electrode for 0.25 mM cholesterol were measured on weekly basis for 2 months, as shown in Fig. 10b. After each measurement, the used electrode was thoroughly washed with DI water, dried and then stored under ambient conditions. The data collected from the repeated experiments depict that the CuInS₂(6)/Cu₂O/TNT electrode maintains 89% of its initial current values. This suggests the good long-term stability of the proposed electrode. Moreover, the reproducibility and repeatability of the biosensor was determined by measuring the current responses towards the addition of 0.25 mM cholesterol. Six samples were prepared under similar conditions for reproducibility measurements, and their amperometric responses were measured (Fig. S11c†). A relative standard deviation (RSD) value of 3.9% is obtained, which shows the high reproducibility of the biosensor. The repeatability of the biosensor was evaluated using a single electrode and taking 10 successive CV scans, as shown in Fig. S11d.† The RSD value of 2.8% demonstrates that the electrode was not poisoned by the redox species and can be used repeatedly for the detection of cholesterol.

Real-sample analysis

To evaluate the practicality and feasibility of the CuInS₂(6)/Cu₂O/TNT electrode in clinical diagnostics, the proposed elec-

trode was used to detect cholesterol in human blood serum samples using the standard addition method. Prior to assay, the serum samples were appropriately diluted with PBS (pH 7.5) to bring the cholesterol concentration in the linear calibration range. The cholesterol concentration in serum samples was calculated from the standard calibration curve. The cholesterol content values assessed with the proposed biosensor were compared with those measured by the commercially available cholesterol sensors, as presented in Table 5. The assay results for clinical serum samples are highly correlated with the reference values, with relative errors (REs) ranging from 0.4% to 3.2%. These results indicate that the proposed biosensor has great potential for practical use with good accuracy and precision.

Electrochemical detection of ibuprofen

CuInS₂/Cu₂O/TNT were further employed to study the quality of stream water. Ibuprofen was used as a water contaminant. The efficiency of the electrode as an electrocatalyst for the detection of ibuprofen was determined using CV and amperometry. Fig. 11a shows the cyclic voltammetry of the pristine TNT, Cu₂O/TNT, CuInS₂/TNT and CuInS₂/Cu₂O/TNT electrodes. The redox behaviour of the electrodes show similar results as evident from the previous section with the highest redox current attained for CuInS₂/Cu₂O/TNTs. The increase in the redox peak current is attributed to the combination of the synergic effect of Cu₂O and CuInS₂ QDs with TNTs. This fact can be due to the availability of more redox species (due to oxidation and reduction of Cu₂O and CuInS₂ QDs) for the electrochemical oxidation of ibuprofen. The smaller size of QDs contributes or accelerates the oxidation process. Fig. 11b shows the CV curve of the CuInS₂/Cu₂O/TNT electrode with the gradual addition of different concentrations of ibuprofen within the electrolyte. A proportional increase in the redox current is obtained by increasing the analyte concentration. The variation in the redox peak current over a range of scan rate is shown in Fig. 11c. It is evident from the figure that the variations in the scan rate show an increase in the redox peak current with a gradual shift in the peak positions towards more negative (reduction) and positive (oxidation) potentials. This shifting in the peak positions can be ascribed to the quasi irreversible process of ibuprofen oxidation at the surface of the electrode. Furthermore, the nature of the process can be determined by plotting the peak current with respect to the square root of scan rate (Fig. 11d). The linear fitting of the plot reveals the

Table 5 Comparison of cholesterol values detected in human blood serum using reference and proposed methods

Sample	Commercial sensor (μM)	Proposed sensor (μM)	REs (%)
1	123	119	3.2
2	232	236	1.7
3	465	463	0.4
4	769	783	1.8

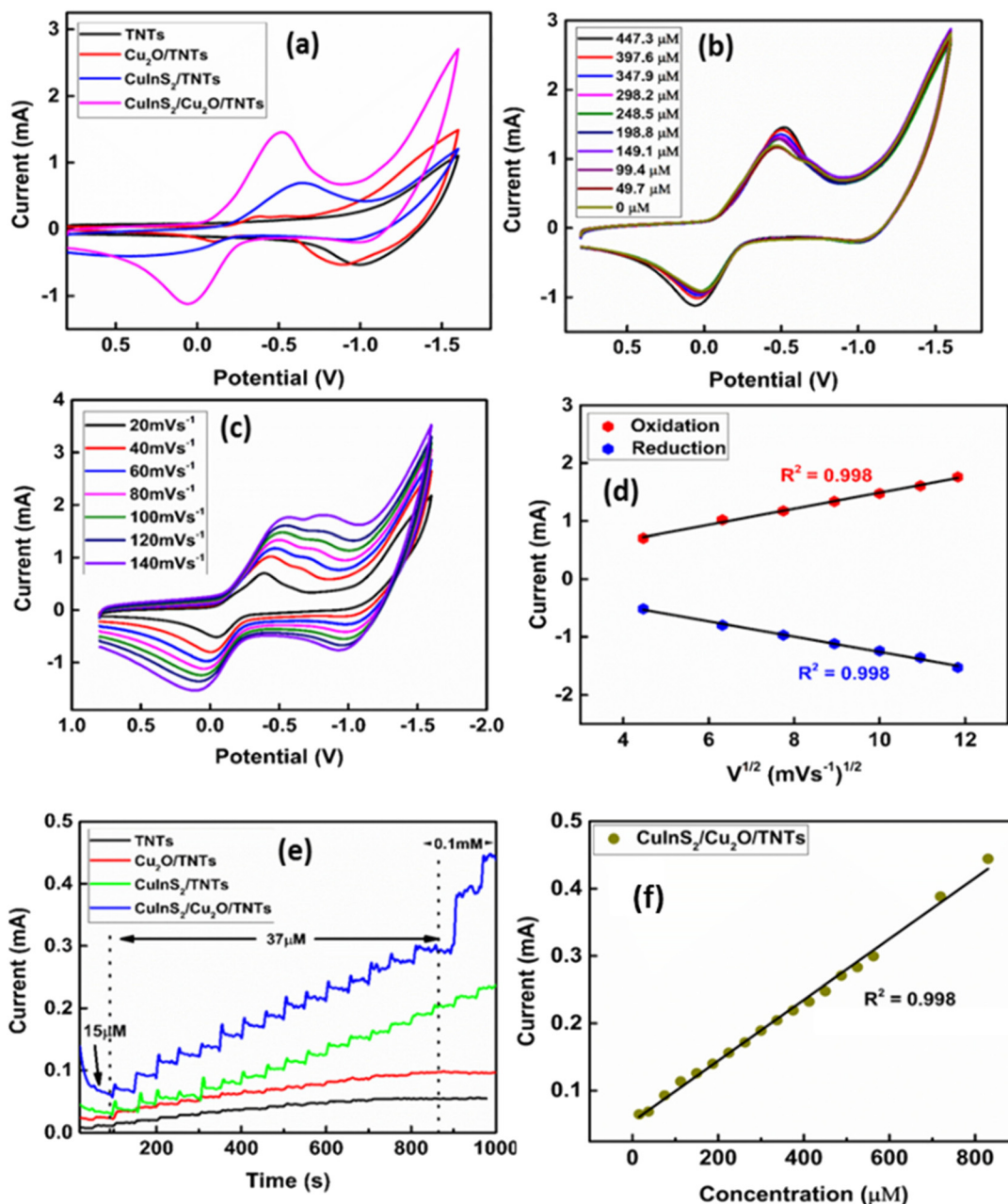


Fig. 11 (a) Cyclic voltammograms of pristine TNT, $\text{Cu}_2\text{O}/\text{TNT}$, $\text{CuInS}_2/\text{TNT}$, and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrodes in 40 ml of 0.1 M PBS (pH 5.5) obtained at 50 mV s^{-1} . (b) CV scans of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode with the addition of different concentrations of ibuprofen, and (c) as a function of scan rate ($20\text{--}140 \text{ mV s}^{-1}$) in the presence of $99.4 \text{ }\mu\text{M}$ ibuprofen. (d) Plots of the oxidation and reduction peak current vs. root mean square of scan rate ($v^{1/2}$). (e) Amperometric response of TNT, $\text{Cu}_2\text{O}/\text{TNT}$, $\text{CuInS}_2/\text{TNT}$, and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrodes at -0.5 V in 0.1 M PBS at pH 5.5 upon successive addition of different ibuprofen concentrations. (f) Corresponding linear calibration curve of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode.

diffusion-controlled oxidation process at the electrode surface.

Fig. 11e shows the amperometric response of the pristine TNT, $\text{Cu}_2\text{O}/\text{TNT}$, $\text{CuInS}_2/\text{TNT}$, and $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrodes at -0.5 V by gradually increasing the concentration of ibuprofen within the electrolyte. The $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode exhibits a rapid increase in the current response towards the addition of each ibuprofen concentration with a response time

of 4 s. The linear fitting of the calibration plot, derived from the amperometric data, for all electrodes is shown in Fig. 11f and Fig. S12(a–c)†. The $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode shows (Fig. 11f) a higher sensitivity up to $3.694 \text{ mA mM}^{-1} \text{ cm}^{-2}$ and a wider linear range of detection ($15\text{--}825 \text{ }\mu\text{M}$) compared to the $\text{CuInS}_2/\text{TNT}$ s, $\text{Cu}_2\text{O}/\text{TNT}$ s and TNTs (Fig. S12(a–c)†), which display rather lower values of sensitivities (summarized in Table 6).

Table 6 Comparison of the sensitivities of different electrodes for ibuprofen detection, derived from the linear fitting of the calibration plots of the corresponding electrodes

S. no.	Electrode materials	Sensitivity ($\mu\text{A mM}^{-1} \text{cm}^{-2}$)
1	Pristine TNTs	262.76
2	$\text{Cu}_2\text{O}/\text{TNTs}$	349.768
3	$\text{CuInS}_2/\text{TNTs}$	816
4	$\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$	1293

Proposed mechanism for electrochemical oxidation of ibuprofen

The electrochemical oxidation of ibuprofen can be suggested on the basis of naproxen oxidation as both are almost structurally equivalent molecules.³⁹ In the first step, the deprotonation of benzyl radicals and carboxyl radicals of ibuprofen was achieved *via* single electron oxidation. Second, the elimination of CO_2 from both radicals produces a common benzyl radical, which undergoes further oxidation resulting in benzyl cations. The reaction of this benzyl cation with H_2O produces alcohol, and a mixture of alcohols and ketones are produced as end products of other subsequent reactions.

Selectivity, stability and reproducibility of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ for ibuprofen detection

To evaluate the selectivity of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ for ibuprofen, a sequence of typical analgesic drugs (*e.g.* paracetamol, morphine, and hydroxyzine) as possible interferents were added besides ibuprofen and the current response was recorded, as shown in Fig. 12a. The amperometric response of these drugs due to non-specific oxidation is much weaker than the response current induced by ibuprofen, thus indicating the acceptable specificity of the sensor. In Fig. 12b, the stability

studies of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode show that only 11.6% activity is reduced for the electrode for a time period of two months with an interval of 7 days.

The pH of the buffer solution is optimized to obtain a higher electrochemical response *via* CV by varying the pH from 3.0 to 8.0, as shown in Fig. S13a.† The data are plotted as cathodic current *vs.* various values of pH. It shows that the peak current reduces by increasing the pH of the electrolyte and very less oxidation of ibuprofen occurs in basic media. Furthermore, the cathodic peak current is significantly high in acidic media. This result thus indicated that reasonable oxidation of ibuprofen is only possible in acidic media. Therefore, pH 5.5 is used for further experiments to keep it close to a natural pH value. The response of increasing temperature in the sensing behaviour of the electrode for ibuprofen detection is shown in Fig. S13b.† The electrode shows the obvious behaviour of increasing the response current due to the increases in the diffusion rate with the temperature. The reproducibility measurements with 5 different samples (Fig. S13c†), fabricated individually, show a narrow range of variation in response current (for 37 μM ibuprofen) with an RSD value of 1.1%. In addition, the biosensor shows a higher repeatability, exhibiting an RSD value of 1.4%, thus showing a slight difference in the response current after 10 cycles (Fig. S13d†). These results reflect that the current technique is within the error limit (within 5%) and reliable for analysis of ibuprofen.

Real-sample analysis of ibuprofen on $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$

Ibuprofen obtained from a pharmacy was used to prepare the stock solution. Here, instead of a buffer solution, lake water is used as an electrolyte. First, 5 mM of stock solution was introduced into an electrochemical cell by a standard addition method and the corresponding amperometric response

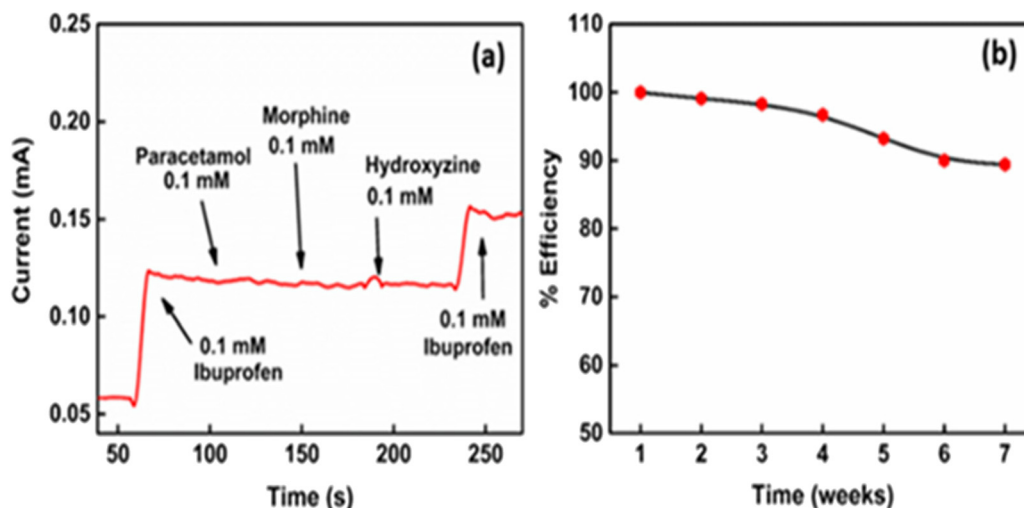


Fig. 12 (a) Amperometric response of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode towards cholesterol with different interferents in 0.1 M PBS (pH 5.5). (b) Shelf life (stability) curve of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ as a function of time in weeks.

Table 7 Comparison of ibuprofen values detected in lake water using the reference and proposed methods

Sample	Commercial sensor (μM)	Proposed sensor (μM)	REs (%)
1	298	291.3	2.3
2	476.2	461.5	3.1
3	655	649	0.9
4	812	820.7	1.1

current was measured. The relative error value was calculated by comparing the concentrations measured by the proposed method with that measured using a spectrometer, as presented in Table 7. The results revealed a close correspondence between both techniques, which shows that the proposed method can be used for the reliable detection of ibuprofen in on-site analysis of waste water.

The above-mentioned results indicate a higher electrochemical activity of $\text{CuInS}_2/\text{CdS}/\text{TNTs}$ towards cholesterol and ibuprofen. This outstanding performance of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode results from the uniform distribution of QDs. The CuInS_2 and Cu_2O QDs usually produce electrochemically active sites, as confirmed by CV. Both QDs provide pathways to the redox species on the surface of TNTs for efficient charge transport at the interface, as depicted from the reduced diameter of the Nyquist plot for $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$. Moreover, the higher redox peak current in CV and enhanced amperometric response of $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$ show the significance of the structural design of the biosensor.

Conclusions

An unconventional electroanalytical methodology for simple, selective, and reliable detection of cholesterol and ibuprofen was explored. TNTs as matrix materials were sequentially co-decorated with Cu_2O and CuInS_2 QDs. The hybrid electrode triggers the chemical oxidation of cholesterol by generating hydroxyl radicals ($\cdot\text{OH}$). Tuning the size of Cu_2O and CuInS_2 QDs makes the hybrid electrode more effective for cholesterol detection with a sensitivity of around $12\,530\,\mu\text{A}\,\text{mM}^{-1}\,\text{cm}^{-2}$ (for $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNTs}$), which is three times higher than that of the $\text{Cu}_2\text{O}/\text{TNT}$ electrode ($4650\,\mu\text{A}\,\text{mM}^{-1}\,\text{cm}^{-2}$) due to the rapid electronic communication and high surface reactivity. Furthermore, electrochemical analysis of the $\text{CuInS}_2/\text{Cu}_2\text{O}/\text{TNT}$ electrode for ibuprofen shows a higher sensitivity up to $1293\,\mu\text{A}\,\text{mM}^{-1}\,\text{cm}^{-2}$. The fabricated electrode showed long-term stability, excellent reproducibility, and low relative error values compared with the commercially available techniques, which verify that the proposed sensing platform provides a convenient multifunctional sensing technique.

Conflicts of interest

There are no conflicts to declare.

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