Supporting Information

Ca^{2+}@Cu-CD nanoprobe for dual detection of glycine and Ex-vivo glycine imaging

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Contents in the supporting information:

reproducibility of Ca@Cu-CD/GCE; (C) DPV profiles obtained for 0.1M Gly on Ca@Cu-CD/GCE showing the stability of the sensor for 20days

Figure S12 The mean interaction plot of the larval crawling speed of (a) Control and (b,c, and d) 20, 40, and 80 μg/ml, respectively, in all treated groups.

Figure S13 Climbing assay in each nanocomposite treated setup comparable with control flies; behavioural abnormality was not significantly found with increases up to 40 μg/ml concentration

Figure S14 No Significant differences were observed in touch sensitive score of treated flies at 20,40 and 80 μg/ml as compared to control.

Figure S15 Light/dark preference assay of larva, Petri dishes. (a) Control and (b−d). 20, 40, and 80 μg/ml doses of the hybrid nanocomposite treated larval groups, respectively, (e) Graph of the light/dark preference test (N = 15 (60 larvae) per time point).

Characterization

A UV-VIS spectrophotometer (Shimadzu 2650) and a spectrofluorometer (Quantamaster, Horiba-PTI) were used to acquire UV-visible absorption and fluorescence spectra. Particle size and zeta potential data were obtained using a zeta sizer Nano ZS 90, Malvern instrument. Fourier Transform Infrared Spectroscopy (FTIR) (IRAffinity-1S, Shimadzu, spectrophotometer) was used to identify the surface functional groups. The morphology of the synthesised samples was determined by using a scanning electron microscope (JEOL JSM-6480 LV) and distribution of elements in the prepared sample were investigated using a Transmission electron microscope (FEI, Tenchi G2 TF30-ST). The surface composition of Ca@Cu-CD was examined by X-ray photoelectron spectrum (XPS) recorded by (PHI 5000 VersaProbe III). A Bruker Multimode-8 atomic force microscope (AFM) was used to examine the surface topography and sample roughness. A Bruker Multimode-8 atomic force microscope (AFM) was used to examine the surface topography and sample roughness. The average fluorescence lifetime was calculated by using a Horiba Jobin von TCSPC (time-correlated single-photon counting technique). Using a spectrophotometer with an integrating sphere and the software Felix GX 4.1.2, the absolute photoluminescent quantum yield is calculated.

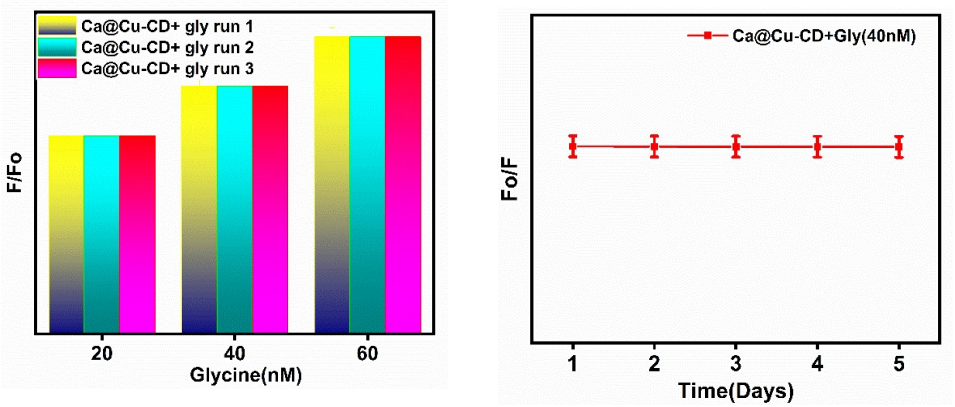
Glycine detection in real sample

Human serum of three different person were collected from a CWS Hospital, Rourkela. The human serum centrifuged at high speed (1000 rpm) for 2 min. The supernatant was collected, and different concentrations of glycine were added to 3.0 mL of the supernatant to prepare a series of test samples. The fluorescence detection of human serum samples was conducted under the same conditions, except that the glycine standard was replaced by glycine-containing samples. The fluorescence spectra of glycine-spiked systems were measured after incubation

for 3 min. The content of glycine in samples was calculated from the plotted linear relationship between glycine and F_0/F.

Repeatability and stability study of glycine detection

The reproducibility of the system was investigated by using the relative standard deviation (RSD) of several measurements. To evaluate the repeatability, a series of three solutions of Ca@Cu-CD with a constant concentration of (100µM) were prepared and glycine detection were carried out on the same day. The calculated RSD% value was 0.48% indicating good repeatability of assay using the Ca@Cu-CD probe. In addition, the stability of the proposed measurement was investigated over five days using for a particular concentration of glycine (40 nM) solution. The calculated RSD% value was 1.12%, which stability of the proposed sensing protocol. The stability studies of the Ca@Cu-CD has been mention in below.



Physical behavioural study of *Drosophila* in presence of Ca@Cu-CD

Experimental procedure

Larva crawling behavior Assay

The crawling assay was done with six third instar larva from each treatment concentration (20 µg/ml,40 µg/ml,80µg/ml of Ca@Cu-CD nanocomposite and control.[1] Larva from different treatment vials were collected and washed by using 1X PBS to remove the food particles. A crawling plate was made using 2% agar poured in a petri plate to provide a surface for larval crawling.[2] An agar plate was made for initial acclimatization of larva to crawling surface. One by one, the larvae were picked to the centre of a different agar plate and a graph paper was placed beneath to trace the path. Meanwhile, the photo was taken (SAMSUNG M51). The time taken by each larva to reach the periphery of the petri plate was measured, and that time was divided by 1 min to calculate the crawling speed. On the agar gel, the larvae left a trailing impression/mark of their travelled path. A black marker was used to sketch the larvae's crawling routes, and their average speed per second was then plotted.

Climbing Assay

Climbing is an innate behavior of Drosophila. Drosophila always tries to climb vertically against gravity, so they showed negative geotactic behavior. Adult fruit flies locomotory behavior was evaluated using this same technique as in a reported protocol.[3] 5-days old flies

(40 adult flies) were moved to the climbing apparatus from three distinct concentrations.[4,5] Flies were taped gently to the bottom of the vial, and the duration of 10 s to climb 16 cm of the tube was recorded. All concentrations of the nanocomposite and control were tested nine times using this methodology. Percentages of total flies were used to determine the number of flies in each group that successfully climbed the mark of 16 cm in the time of 10s.

Touch sensitivity Study

The central nervous system, different body segments and neuromuscular junctions works in a coordinated manner to produce a sensation i.e., referred to as touch. The brain of drosophila contain central pattern generators (CPG) is considered as the main source of this stimuli. The movement is possible due to oscillatory network even in the absence of the external sensory input. However, without a feedback loop from the peripheral nervous system (PNS), the larva's body segments expansion and contraction occurs in an uncoordinated manner. In the late embryonic stage, the signals from the CPG initiates peristatic movement and it remains throughout the larval stage. The CPG signals the chordotonal organ of the PNS for locomotion as well as sensation.[6] Therefore, any sensory damage in the larvae will obstruct the larvae to respond to the stimuli. From this assay, larval behaviour is studied and the neuronal defect can be scored. The exact procedure is followed for isolating the larva, washing and acclimatization in the agar plate environment. For providing mechanical stimuli to larvae, toothpick was glued with a soft eyelash. This was used in gently pricking the thoracic segments of the larvae. The responses of the larvae were recorded and noted. This was scored according to Dhar et. Al. 2020.[7]

Larval light/dark Preference Assay

This experiment is used to detect an early photoreceptor deficiency using the approach described by Sabat et al. 2016.[8] A Petri dish was divided into four quadrants, with the opposite quadrant being colored black (two quadrants are black). Then, 1% agarose was added and let to set. Fifteen third instar larvae from both the control and treatment vials were kept in the dark for 6 h before the experiment began. The larvae were placed on the agar plate, and the lid with the same marking as the Petri plate was closed. The Petri dish was illuminated uniformly, and the larvae were given 5 min to move freely between the dark and light sections. After 5 min, we removed the lid and images were captured. Each batch of larvae performed the test three times, and the experiment was conducted in five sets.[3,8]

Result and Discussion

Crawling Assay

The crawling behavioral test is a more practical assay to explore the neuronal abnormalities in an early stage of larva for the neuronal mechanosensory investigation. The crawling behavior of third instar larvae was studied in the *Drosophila* model. The neuronal toxicity caused by the nanocomposite exposure can disrupt the coordinated crawling of larvae. The healthy larvae move in a straight line, whereas the abnormal ones zigzag and sometimes slow down. Thus, the crawling assay is preferable for identifying abnormalities in gene expression that might result in fatalities during the pupal and adult stages. In the crawling assay, no distinct curve or

turn has been recorded for the control larvae. There was no significant crawling path change for the treatment concentrations of 20 μg/ml and 40 μg/ml, whereas a significant change was seen in 80μg/ml concentration. In the control vial of larvae, 0.904 ± 0.003 were able to cover the distance in mm/s, whereas 0.839 ± 0.165 were able to cover the distance in 20 μg/ml, 0.804 ± 0.142 in 40 μg/ml and 0.406 ± 0.094 in 80 μg/ml. The crawling speed of third instar larva clearly indicate that the treatment of 20 μg/ml and 40μg/ml larva significantly covers the same distance in mm/s comparable to control larva, whereas the crawling speed of 80μg/ml treated third instar larva was significantly reduced and shows more zig-zag and slow motion mainly indicating neuronal defects or changes. The larvae tracking paths and the crawling speed plot is demonstrated in Figure S12.

Climbing Assay

The climbing experiment describes the behavioral changes that occur in flies in response to gravity. The number of flies that could ascend to 16 cm in the 10 s is used to analyze this test. In due order, the number of flies that could climb up to 16 cm was normalized to 100%. The assay was performed 9 times (N = 9) for each concentration, including control. In the control flies vial, 75.55 ± 4.80 were able to climb, whereas 79.22 ± 14.97 in 20 μg/ml, 82.44 ± 16.44 in 40μg/ml, and 85.00 ± 9.60 in 80 μg/ml were able to climb up to the 16 cm mark. The result of the climbing assay is plotted in a graph shown in Figure S13. The climbing ability was seen nonsignificant up to 40μg/ml of treated flies compared to the control flies of the setup.

Touch Sensitivity Test

In our experiment, we found that there is no significant change in touch sensation in the treatment concentrations of 20μg/ml,40μg/ml,80μg/ml. For 20μg/ml nanocomposite treatment, the larva touch sensitivity score was 2.68±0.20, which was practically identical to the control group score that was 2.91 ±0.27(the scores for both groups were more than 2 but below 4, indicating that larvae mostly retract and then move but sometimes turn 90^0). Similarly, at 40μg/ml and 80μg/ml concentration the touch sensitivity score was 2.93 ± 0.38 and 2.85±0.39 respectively (the scores for both groups were in between 2 and 3, indicating that the larvae hold their movement before moving forward) as shown in figure S14.

Larval Light-Dark preference assay

The larva's light preference test was done to look for any early defects in the light-sensing neurons. In this experiment, the percentage of larvae attracted to light decreased as the concentration of nanocomposite treatment increased. The control group's percentage of larvae attracted to light was 38.66 ± 7.77%. There were 46.66 ± 8.43 in 20 μg/ml ,56 ± 8.00 in 40 μg/ml, 59.99 ± 14.60 of light-sensitive larvae in 80μg/ml as shown in Figure S15. However, light was avoided or dark was preferred by 61.33 ± 7.77% larvae from the control group, 53.33 ± 8.43 in 20μg/ml, 44 ± 8.00 in 40 μg/ml, 40.004 ± 14.60 in the case of 80 μg/ml hybrid nanocomposite-treated larval groups.

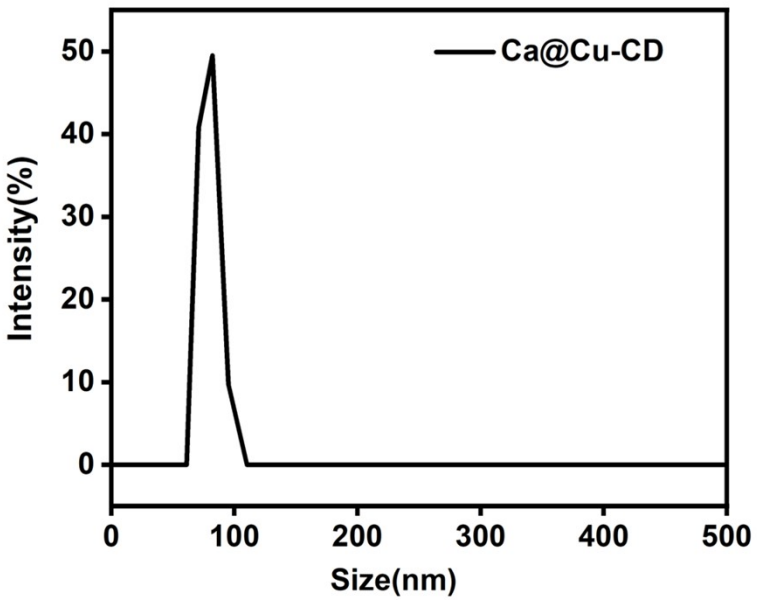


Figure S1 Hydrodynamic size of Ca@Cu-CD

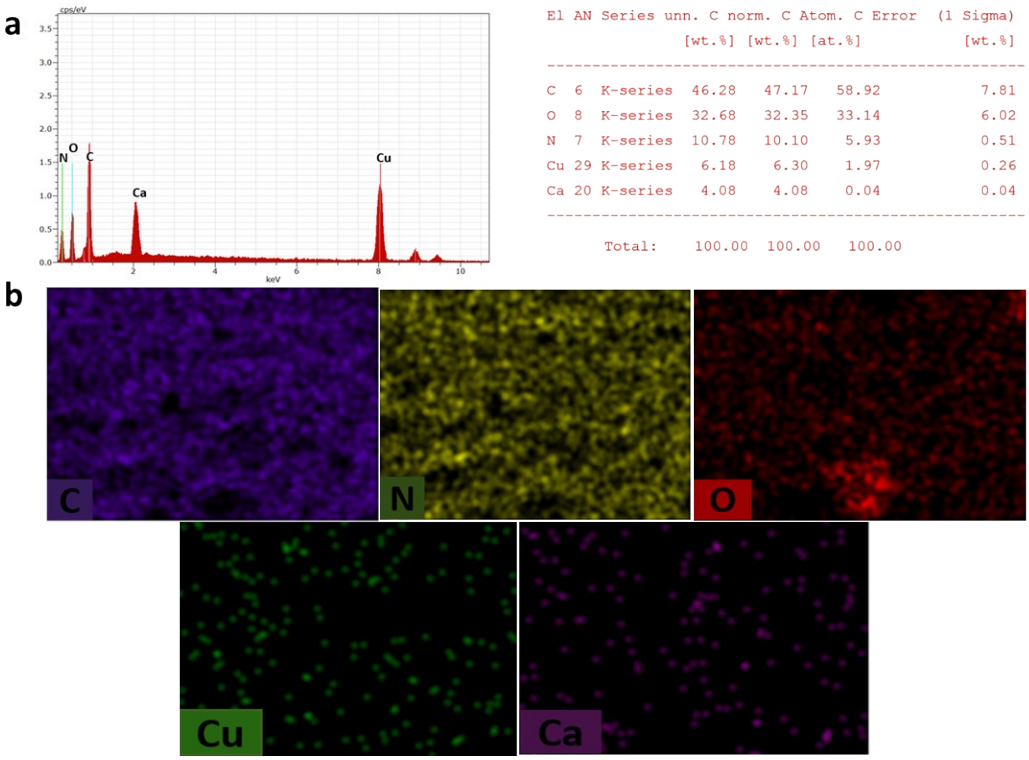


Figure S2 EDAX and elemental mapping of Ca@Cu-CD

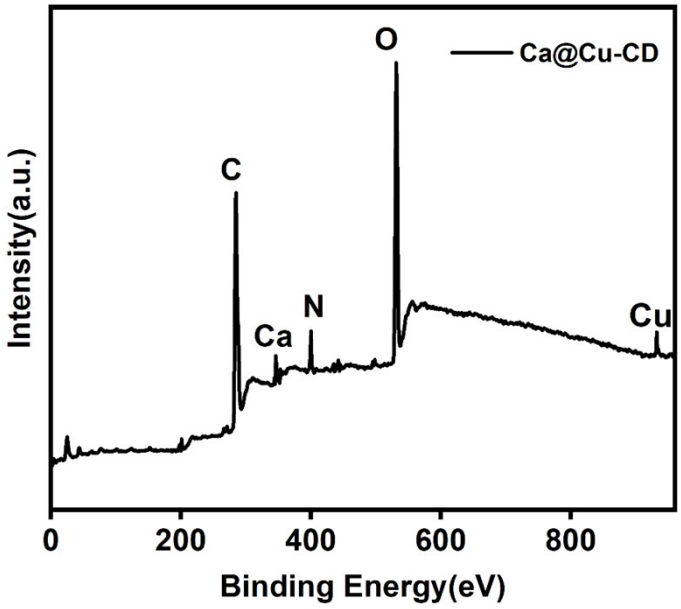


Figure S3 XPS Survey Peak of Ca@Cu-CD

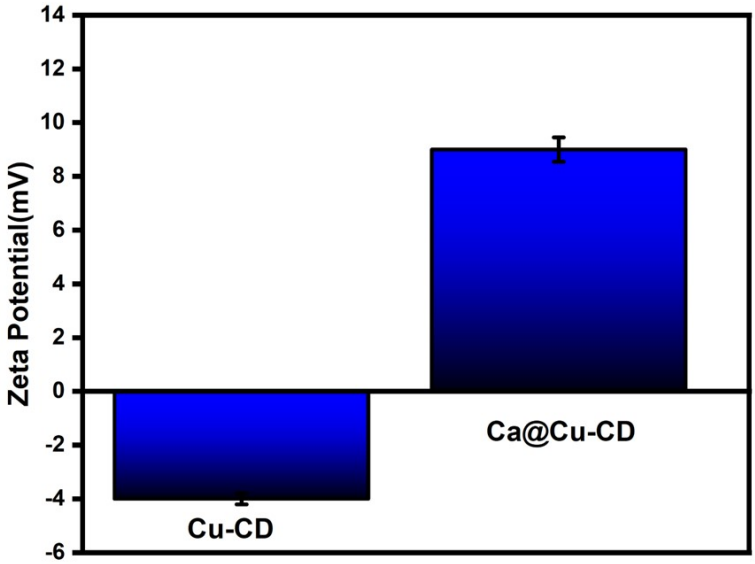


Figure S4 Zeta Potential of Ca@Cu-CD

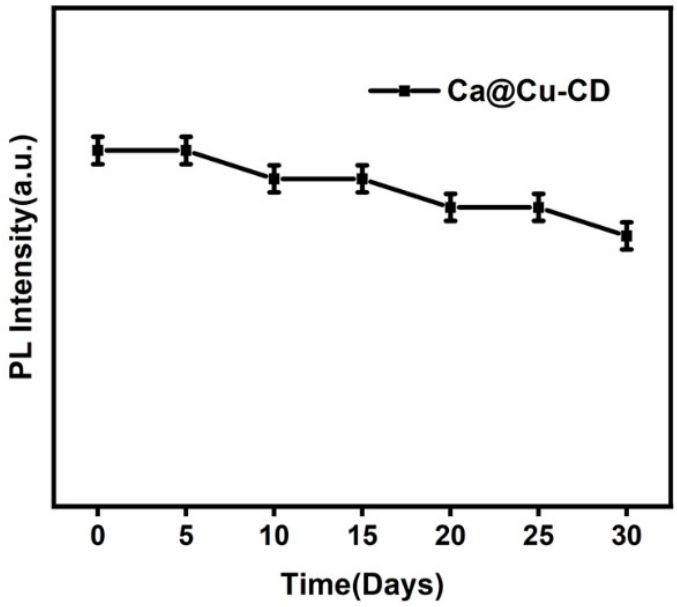


Figure S5 FL stability of Ca@Cu-CD

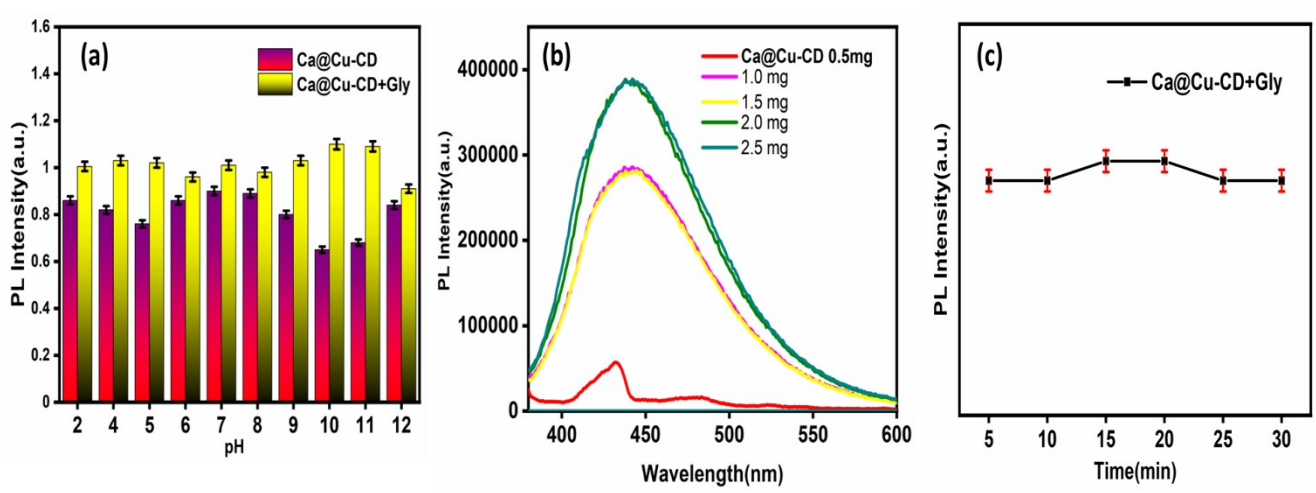


Figure S6 (a) pH of the Ca@Cu-CD, pH of Ca@Cu-CD after the addition of glycine(b) FL with increasing conc. of the probe (c) Time dependent PL emission after addition of glycine

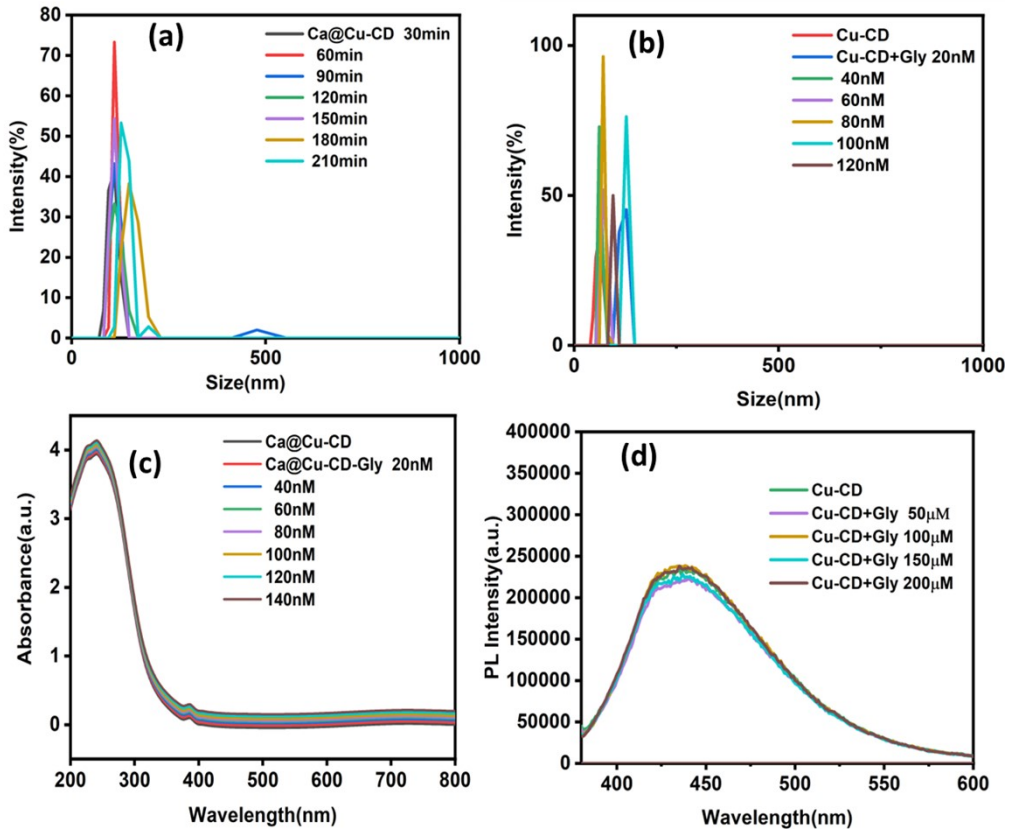


Figure S7 (a) Hydrodynamic size of Ca@Cu-CD with different time intervals(b) Hydrodynamic size of Cu-CD after glycine addition (c)change in UV absorbance of Ca@Cu-CD in the presence of glycine (d)FL Intensity of Ca@Cu-CD with change in concentration of glycine

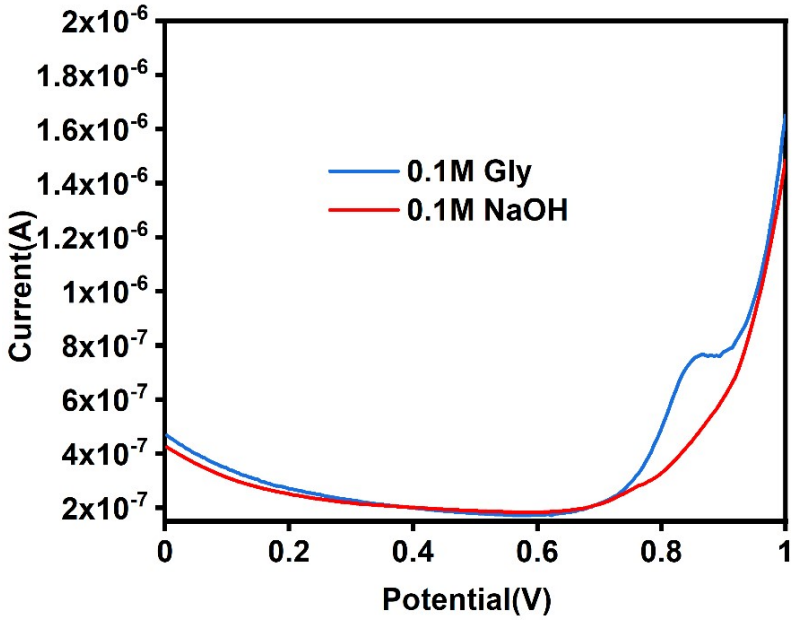


Figure S8 The DPV responses of 0.1 M Gly in 0.1 M NaOH

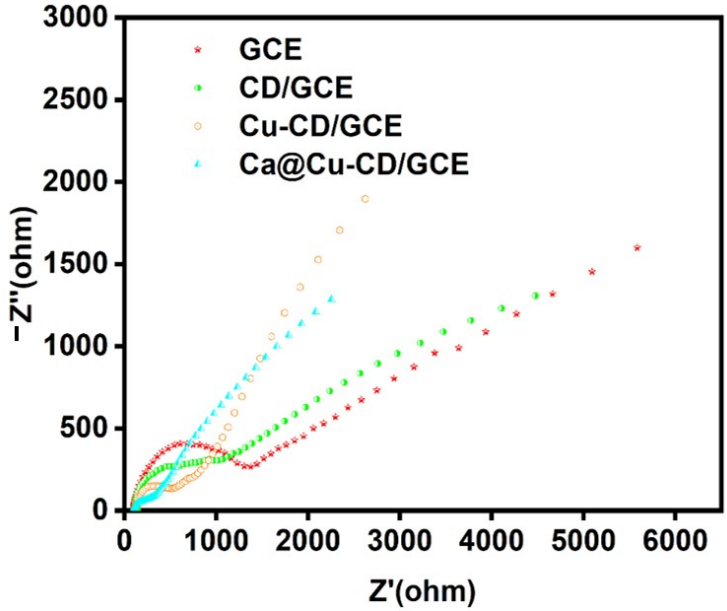


Figure S9 Electrochemical impedance spectra (EIS) of bare GCE, CD/GCE, Cu-CD/GCE, Ca@Cu-CD/GCE

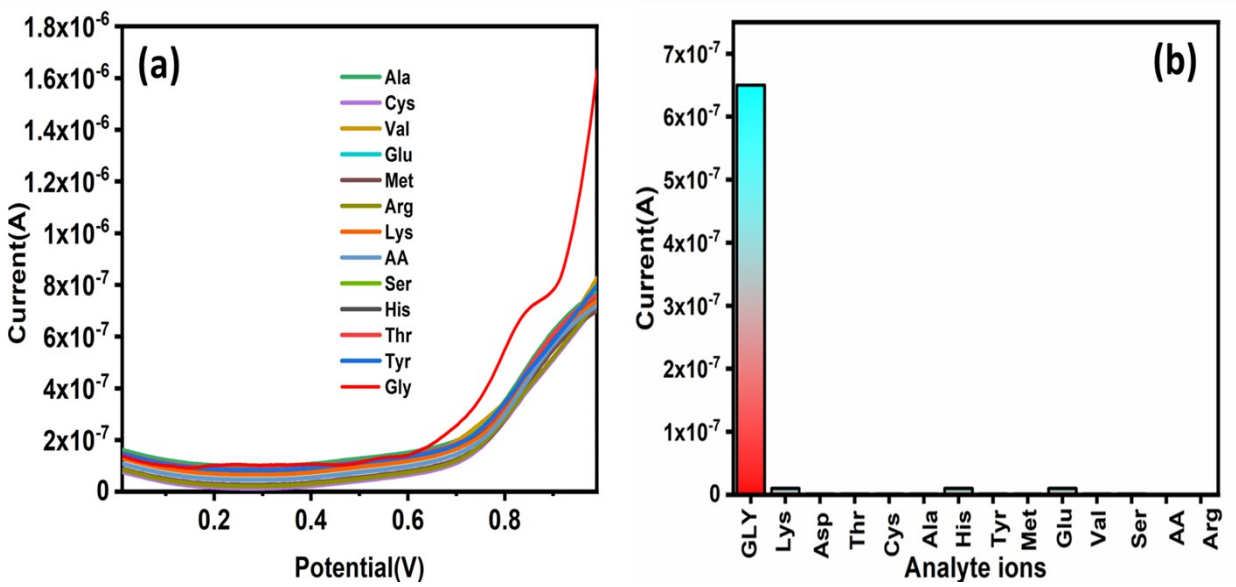


Figure S10 (A) and (B) Selectivity studies of Ca@Cu-CD/GCE towards Gly in the presence of various analyte species

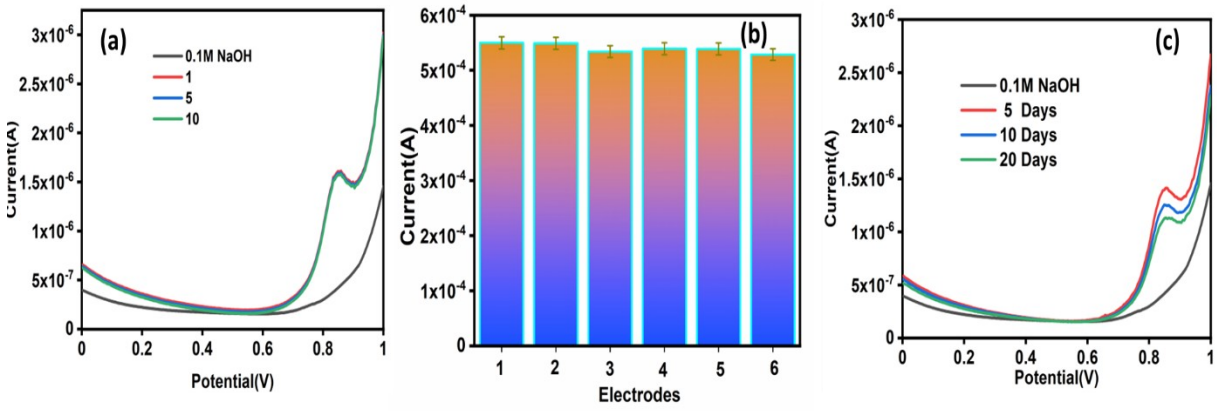


Figure S11 The DPV curves after immediate washing for three times showing the reusability of the sensor; (B) The peak currents of five independently coated electrodes showing the reproducibility of Ca@Cu-CD/GCE; (C) DPV profiles obtained for 0.1M Gly on Ca@Cu-CD/GCE showing the stability of the sensor for 20days

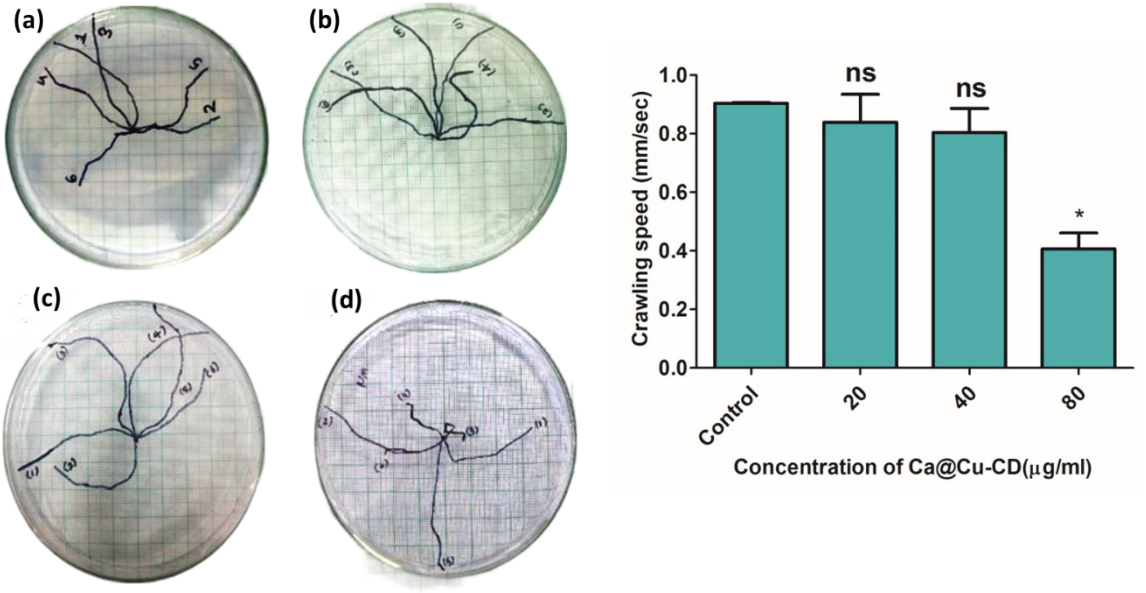


Figure S12 The mean interaction plot of the larval crawling speed of (a) Control and (b,c, and d) 20, 40, and 80 µg/ml, respectively, in all treated groups.

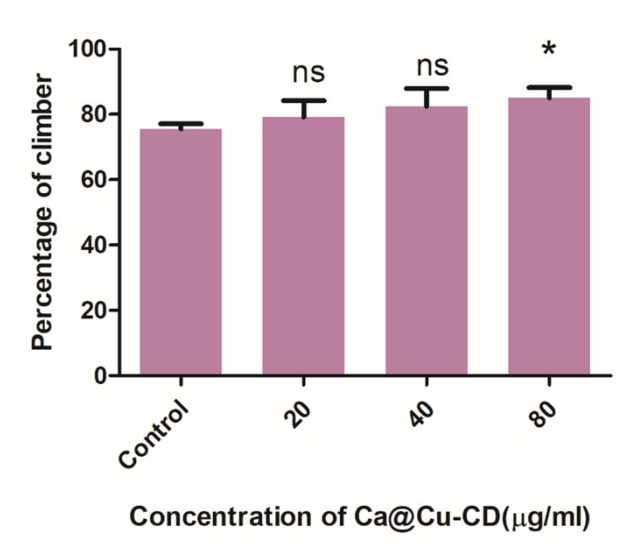


Figure S13 Climbing assay in each nanocomposite treated setup comparable with control flies; behavioural abnormality was not significantly found with increases up to 40 μg/ml concentration

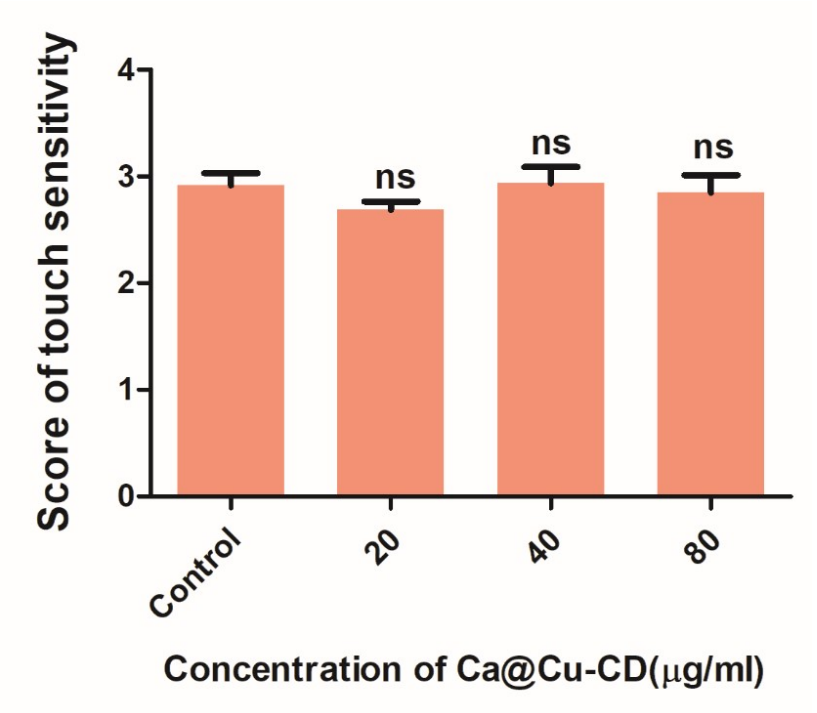


Figure S14 No Significant differences were observed in touch sensitive score of treated flies at 20,40 and 80 μg/ml as compared to control.

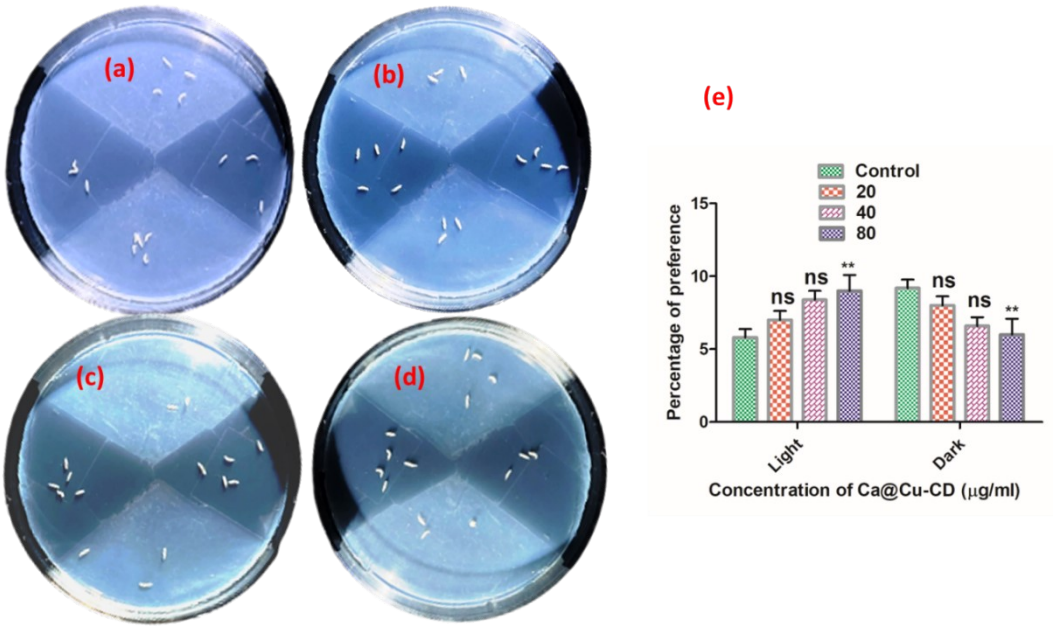


Figure S15 Light/dark preference assay of larva, Petri dishes. (a) Control and (b–d). 20, 40, and 80 μg/ml doses of the hybrid nanocomposite treated larval groups, respectively, (e) Graph of the light/dark preference test (N = 15 (60 larvae) per time point).

Table-S1 Change in hydrodynamic size of Ca@Cu-CD on addition of different concentrations of glycine

Ca@Cu-CD (mg/ml)	Glycine (nM)	Particle size (nm)
2	0	83
2	20	127
2	40	146
2	60	197
2	80	263
2	100	411
2	120	551
2	140	641

Table S2- Freeze thaw condition (-70°c to room temperature)			
	Cycle-1(nM)	Cycle-2(nM)	Cycle-3(nM)
Serum-1	21.25	21.35	21.50
Serum-2	41.45	41.65	41.55
Serum-3	61.53	61.71	61.79

Table -S3 A comparison of the sensor performance with similar work

Material	Detection method	LOD	LDR	Ref
AuCuNC	**Fluorescence**	**5.8 µM**	**600−5000**	**9**
NiO NPs/GCE	**Amperometry**	**0.9 µM**	**0.1 ~1.2 mM**	**10**
flavoenzyme glycine oxidase	**Electrochemical**	**16.5 ± 2.9 µM**	**-**	**11**
RuHCF/rGO PIG electrode	**Electrochemical**	**0.4 µM**	**-**	**12**
N-CQDs/Cu	**Fluorocence**	**10 nM**	**2.33-28.48 µM**	**13**
AuCuNC@N-GQD/GCE	**EC & fluorescence**	**0.01; 1 µM**	**0.01−1000; 5−1000 µM**	**14**
CQD/Sn^{2+}	**fluorescence**	**0.76 µM**	**5–1000 µM**	**15**
Ca@Cu-CD	**Fluroscence & EC**	**17.2 &4.1 nM**	**5-495nM ;1nM - 10µM**	**this work**

LOD Calculation in electrochemical sensing

LOD Calculation LOD = 3S/M

S - The standard deviation of the current responses of blank

M -Slope of the linear plot

LOD Calculation in fluorescence sensing

LOD = 3 SD/ṕ

SD refers to standard deviation and

ṕ slope of linear regression curve

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