Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Preferential disruption of *E. coli* biofilm *via* ratiometric detection and targeting of extracellular matrix using graphene oxide conjugated red emitting fluorescent copper nanoclusters

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1. Experimental Section

S01 Materials. Iron (III) chloride hexahydrate, potassium chloride, lead (II) nitrate, silver nitrate, zinc chloride, rhodamine 101, sodium azide, hexadecyl trimethylammonium bromide (CTAB), crystal violet, sulfuric acid, nitric acid, rose bengal (RB), hydrazine hydrate, N-Phenyl-1-naphthylamine (NPN), 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$. RNAse, 2',7'-dichlorofluorescein diacetate (DCFDA), lipopolysachharides (LPS) of *E. coli* O26:B6, tert-butyl hydroperoxide (TBHP) and dialysis tubing cellulose membrane were purchased from Merck Sigma-Aldrich, USA. Sodium hydroxide, aluminium chloride hydrous, copper (II) sulphate pentahydrate and cadmium chloride monohydrate were purchased from SDFCL, India. Sodium bicarbonate, sodium acetate, sodium bromide, sodium sulphate, calcium chloride anhydrous, magnesium chloride hexahydrate, manganese chloride tetrahydrate, sodium chloride, nickel (II) sulphate hexahydrate, orthophosphoric acid (PA), Phosphate buffer saline (PBS 10X), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Luria broth (LB) and Mueller Hinton broth (MHB), agar and bovine serum albumin were purchased from HiMedia, India. Sodium carbonate anhydrous, sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate and cobalt (II) nitrate were purchased from SRL, India. 11-245 kDa protein marker, Live/dead cell assay kit (bacteria) and singlet oxygen sensor green (SOSG) reagents were purchased from Gibco, Invitrogen. Graphite powder was purchased from TCI chemicals, India. Tissue culture coated 6 and 96 well plates were obtained from Eppendorf. BODIPY™ TR Cadaverine was purchased from ThermoFischer Scientific. Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*, MTCC-3196) and Gram-negative bacterium *Escherichia coli* (*E. coli*, MTCC-43) were procured from Microbial Type Culture Collection and Gene Bank, CSIR-IMTECH, Chandigarh.

S02 Characterization Methods. The absorption and fluorescence spectra of NMs were recorded in NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific™) and Varian

Cary Eclipse fluorescence spectrophotometer (Agilent), respectively. The morphological studies were done using Tecnai T20 twin, TEM 200 kV (FEI, Netherlands). Hydrodyanamic diameter and zeta potential values were measured using Zetasizer Nano ZS (Malvern Instruments). FTIR spectra were recorded on IRAffinity-1S Fourier transform infrared spectrophotometer (Shimadzu). X-ray powder diffraction (XRD) and Raman spectroscopy was carried out using SmartLab 9kW rotating anode X-ray diffractometer (Rigaku Corporation) and Horiba- Lab RAM HR evolution, respectively. XPS and TGA analysis was done by taking ~30 mg of powdered sample in Prevac XPS and Netzsch (STA449F1) analyser (heating in a range of 20-600°C). Fluorescence microscopy studies were done using Carl Zeiss LSM 510 META. HPLC analysis was done by using Waters HPLC system. CD spectroscopy was performed by using Jasco-ASCO-815 spectropolarimeter. Different bacterial, biofilm related assays and growth inhibition measurements were done by using Synergy Microplate Reader, Biotek. UV-Vis and fluorescence studies were done using 3 ml of each of the sample solution, whereas 1 ml of each sample solution was used for DLS/zeta studies. TEM studies were performed by directly placing a drop of the selected NMs on carbon coated copper grid. FTIR, XPS, TGA/DSC and XRD studies, Raman was performed using dried samples (~30 mg).

Synthesis of GO. Graphene oxide copper nanocomposite i.e., Cu@GO@CTAB was synthesized by following four sequential steps. Initially, graphene oxide (GO) was produced using modified Hummer's method from pure graphite powder. For this, ~500 mg of graphite powder was mixed with 20 mL sulfuric acid (H_2SO_4). After mixing, sodium nitrate ($NaNO_3$) was added in equal w/w ratio to the graphene powder. Then, ~1 mg/mL of potassium permanganate ($KMnO_4$) was added slowly to the reaction mixture. After 1 h of normal mixing, the temperature was raised to ~35°C, followed by adding ~50 mL of double distilled water. Then, the complete reaction mixture was heated at ~95°C for 6 h following stirring overnight at room temperature. After that, the reaction mixture was collected and neutralized by washing

via repeated centrifugation at 5000 rpm for 5 mins. The dark brown coloured material was obtained and designated as GO.

Synthesis of GO@CTAB: In the second step of synthesis, GO@CTAB was prepared by mixing GO (10 mg/mL) and cetyltrimethylammonium bromide (CTAB) (1.8% w/w). This reaction mixture was sonicated for 1 h and dialyzed against double distilled water for 24 h and the isolated solid material was designated as GO@CTAB.

***Stability analysis*.** The time dependent fluorescence stability studies of the NMs were analysed by spectrophotometric studies over a period of 30 days while keeping at 4°C. Similarly, fluorescence spectra at different temperature ranging from 10-70˚C were measured and pH dependent fluorescence studies were done by diluting the samples at different pH *viz*., 5.5, 6.8, 7.4 and 9.5.

Spectrophotometric and microscopic studies in presence of high NaCl concentrations. The concentration of NaCl in human blood is ~154 mM. Thus, to confirm the fluorescence stability of prepared NMs in biological system, (0.5 mg/mL) were titrated with different NaCl concentrations *viz*., 0.1, 0.15, 0.25, 0.250, 0.500, 0.750 and 1M. Finally, the fluorescence intensity was recorded at two different wavelengths viz., ~474 and ~645 nm (for Cu@GO@CTAB). Also, the SEM and TEM imaging was also performed for both GO@CTAB and Cu@GO@CTAB NM after the synthesis and suspending them in 1000 mM NaCl for 24 h.

***Metal ion interference studies*.** Interference of chelating metal ions (cations and counter anions) on fluorescence intensity of the synthesized NMs was monitored by titrating the NMs (0.5mg/mL) against 100 µM of 15 different metal ions *viz*., Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^{+}, Cd^{2+}, Pb^{2+} and Al^{3+} in the ratio of 1:1 (v/v). Furthermore, the interference of chelating counter anions of sodium *viz*., OH^{-}, N_3^{-}, HCO_3^{-},CO_3^{2-}, $H_2PO_4^{-}$ CH_3COO^{-} was also studied. The fluorescence intensity was measured at two different wavelengths viz., ~474 and ~645 nm (for Cu@GO@CTAB).

Table S1. Different reducing agents used for the synthesis of CuNC

Sr. No	Reducing Agent	Color Change	Absorption (nm)	Fluorescence (nm)	Particle size=nm (PDI) (Zeta potential)
1	Imidazole	Bluish Green	278	NA	-
2	Ethylene diamine (EDA)	Purplish Grey	291	NA	-
3	Indole diacetic acid (IDA)	Sky blue	267	NA	-
4	Nitrilotriacetic acid (NTA)	milky Sky blue	278	NA	-
5	Ornithine	Brownish Yellow	560	330/453 (164 FI)	56±7 (0.521)
6	Histidine	Brownish Yellow	434, 560	370/464 (92 FI)	121±15 (0.380)
7	**Hydrazine Hydrate**	**Brownish Yellow**	**280**	**400/650 (400 FI)**	**10.70±1.3 (0.369) (-14.0 ±2.1 mV)**

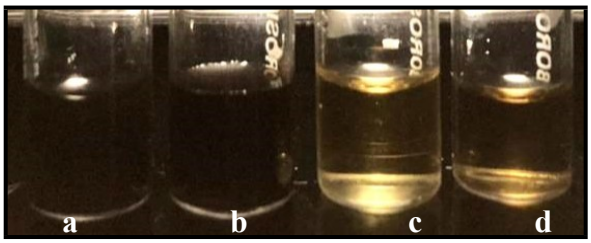

Figure S01. Photographic image of GO, GO@CTAB, CuNC and Cu@GO@CTAB (a-d) after one month storage.

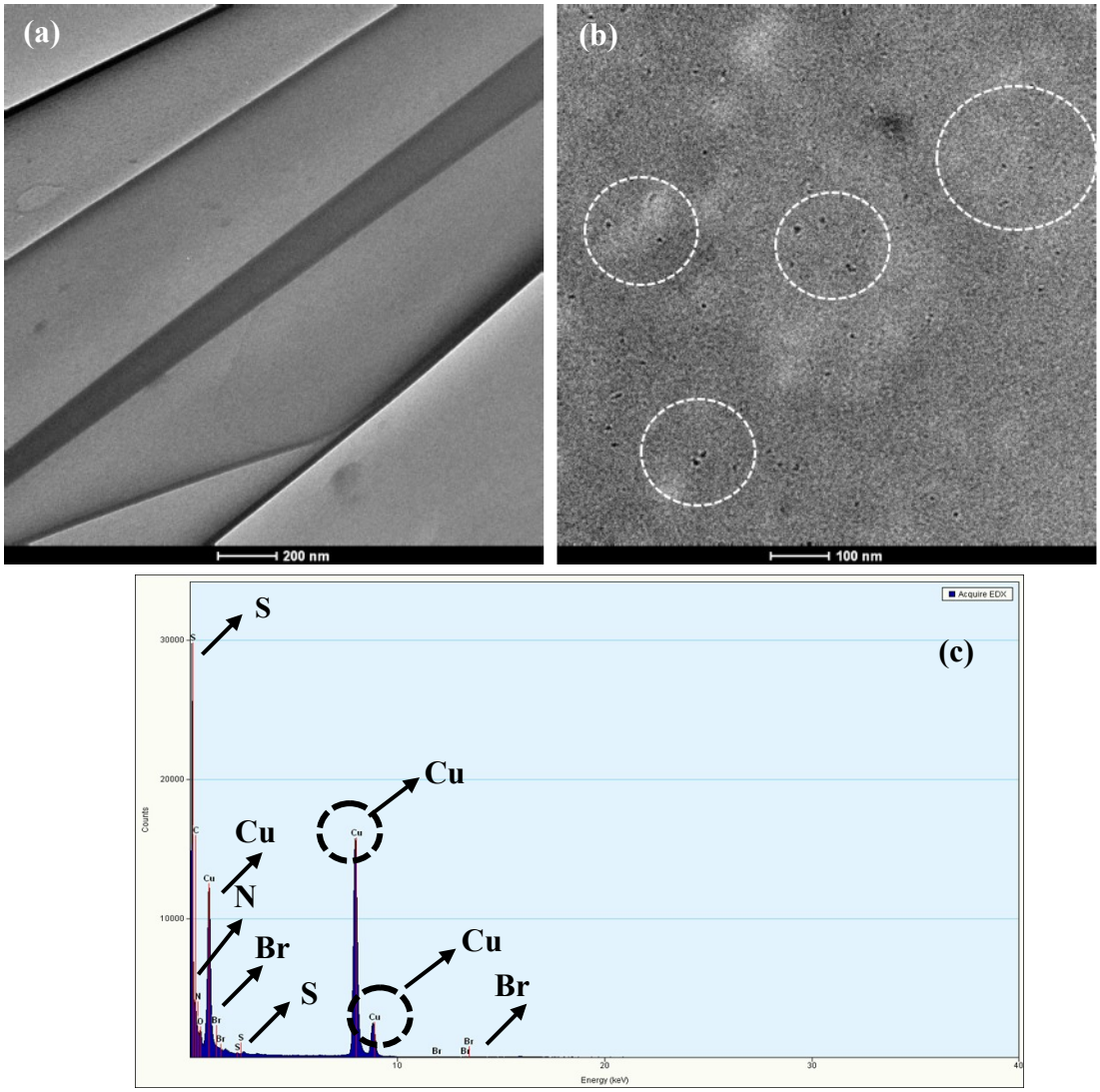

Figure S02. TEM micrographs for (a) GO nanosheets, (b) CuNC and (c) EDAX pattern for Cu@GO@CTAB.

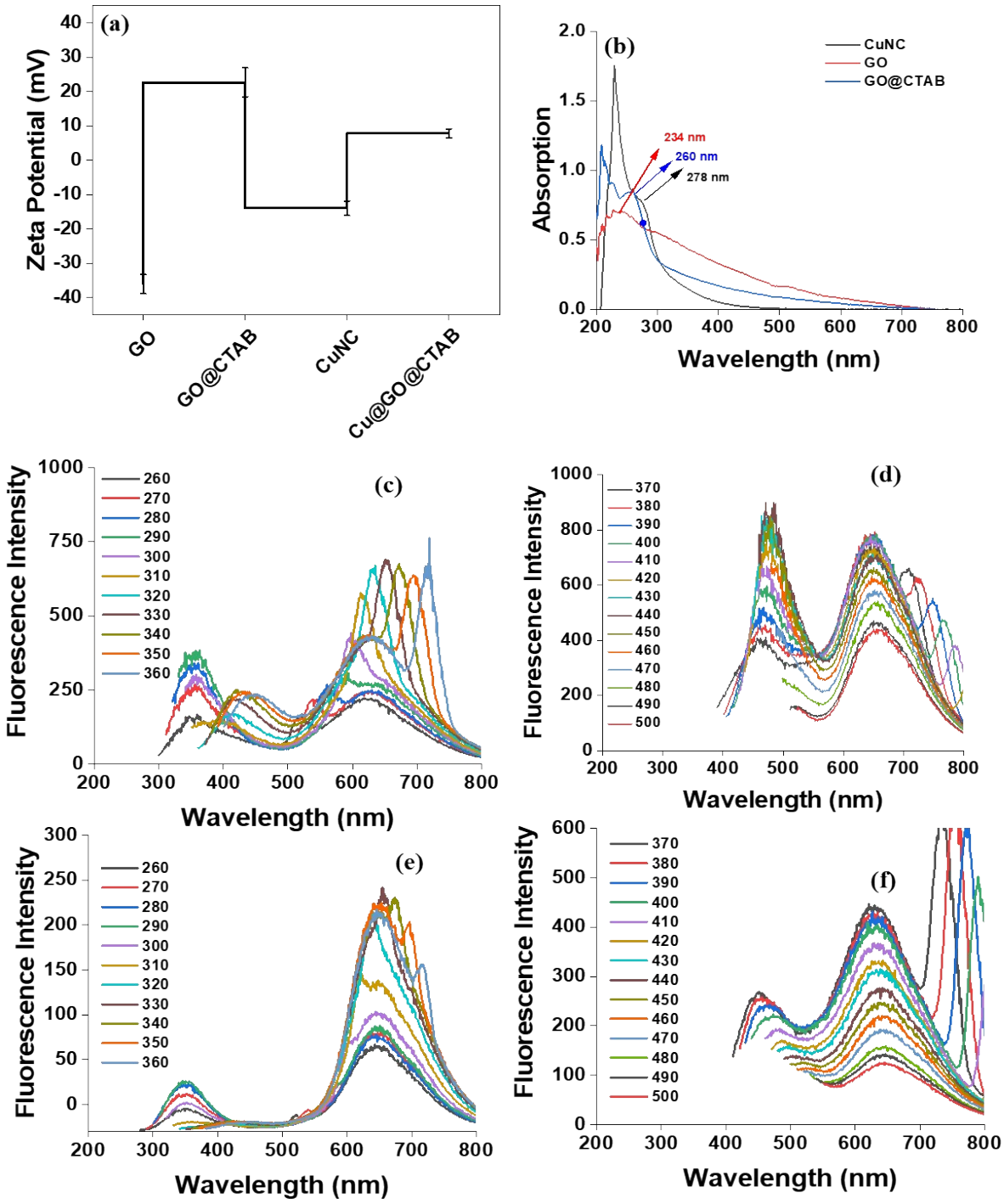

Figure S03. Spectroscopic characterization**.** (a) Zeta potential measurement for all NMs, (b) UV-Vis spectra for GO, GO@CTAB and CuNC, (c, d) fluorescence emission profile of CuNC and (e, f) fluorescence emission profile of Cu@GO@CTAB when excited from 260 to 500 nm.

Table S2 Quantum yield (QY) calculations

Sample	Solvent	Refractive Index	Ex/Em (nm)	Average QY (%)
Rhodamine 101	Ethanol	1.32	450/595	100
CuNC	Water	1.33	400/478, 648	12.6±1.5
Cu@GO@CTAB	Water	1.33	400/474, 645	2.9±0.3

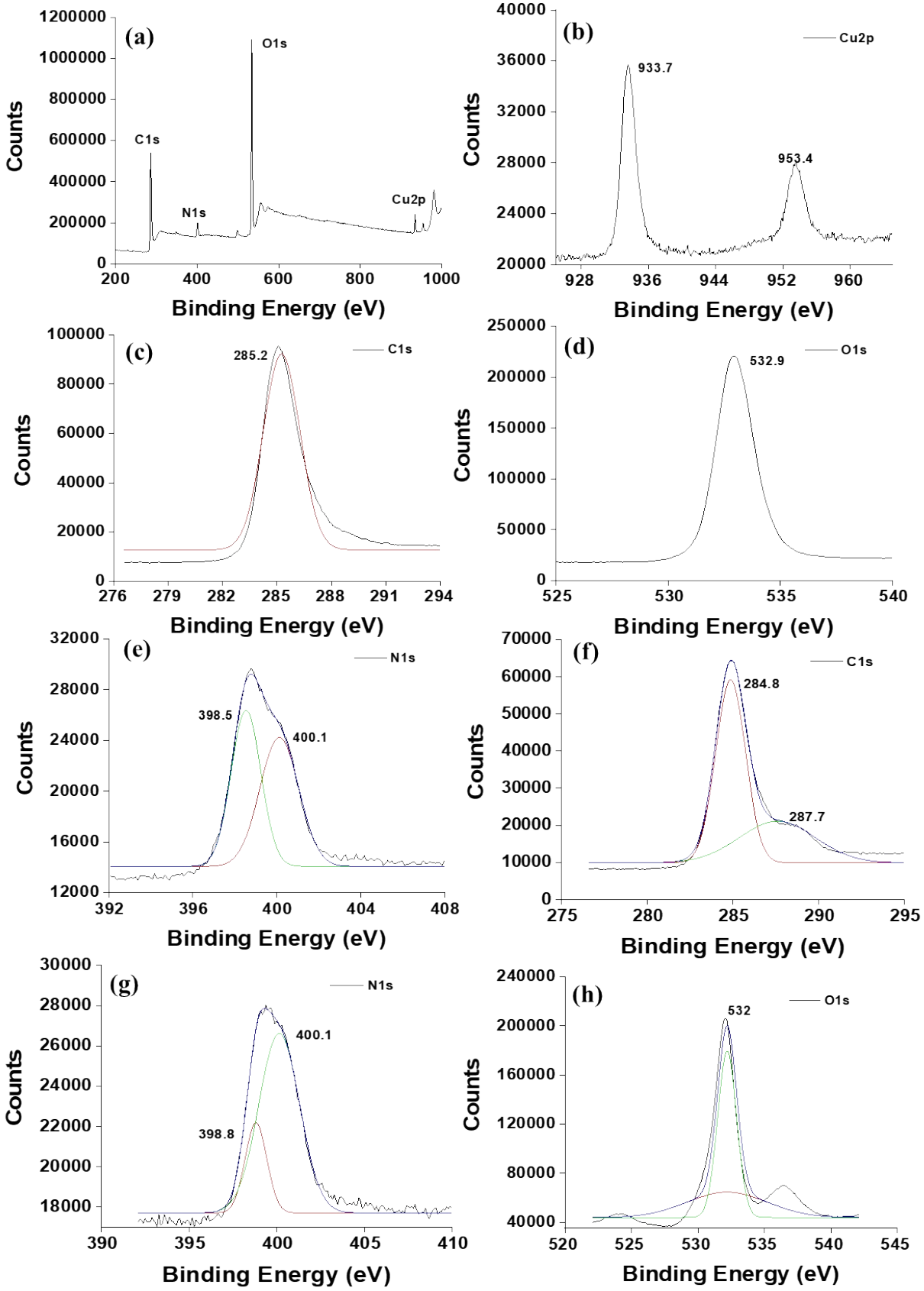

Figure S04. XPS spectrum of (a-e) CuNC and (f-h) Cu@GO@CTAB.

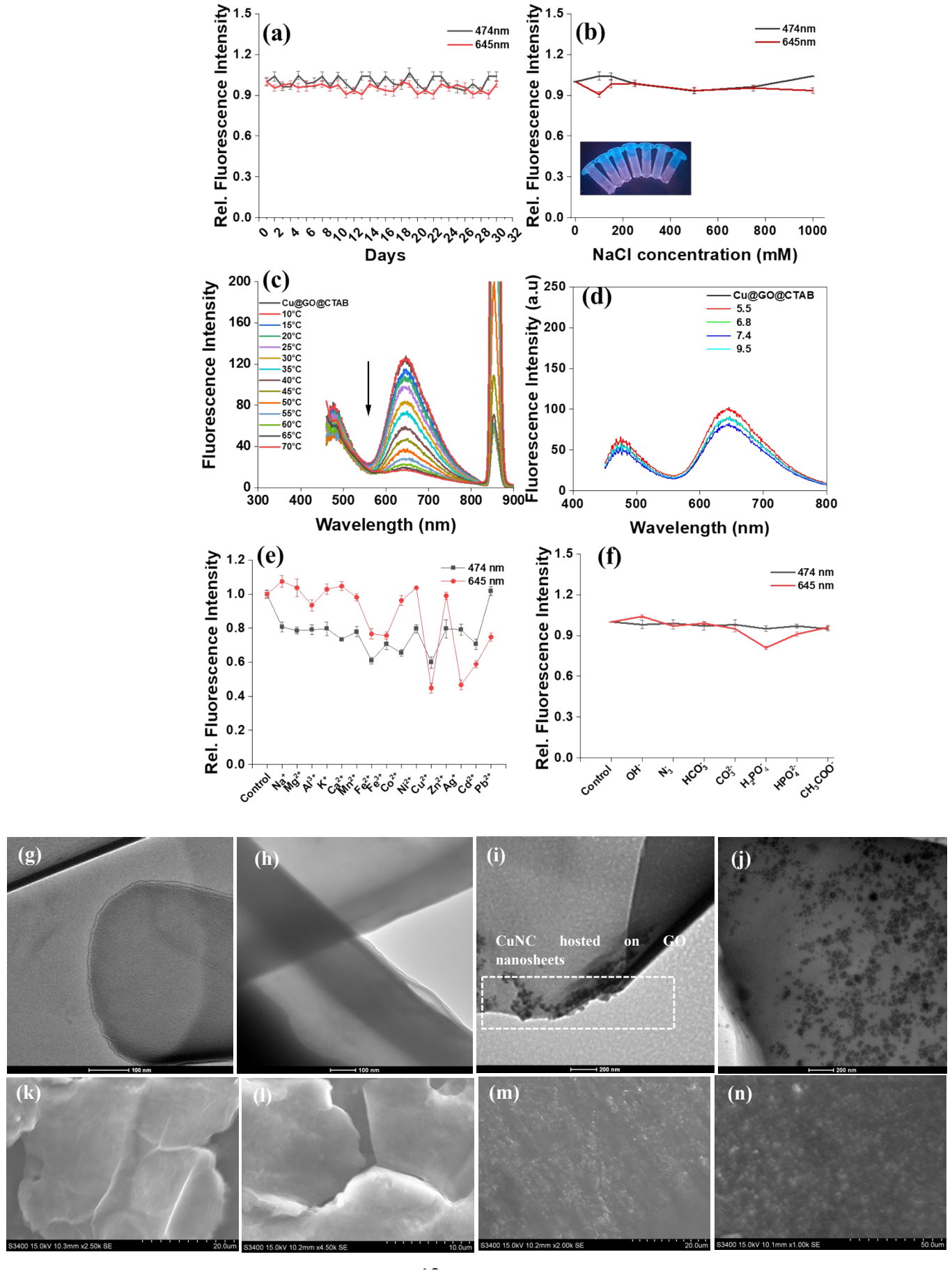
(a)
474nm
645nm
Rel. Fluorescence Intensity
Days
(b)
474nm
645nm
Rel. Fluorescence Intensity
NaCl concentration (mM)
(c)
Cu@GO@CTAB
10°C
15°C
20°C
25°C
30°C
35°C
40°C
45°C
50°C
55°C
60°C
65°C
70°C
Fluorescence Intensity
Wavelength (nm)
(d)
Cu@GO@CTAB
5.5
6.8
7.4
9.5
Fluorescence Intensity (a.u)
Wavelength (nm)
(e)
474 nm
645 nm
Rel. Fluorescence Intensity
Control
(f)
474 nm
645 nm
Rel. Fluorescence Intensity
Control
(g)
(h)
(i)
CuNC hosted on GO nanosheets
(j)
(k)
(l)
(m)
(n)

Figure S05. Physicochemical stability evaluation of prepared Cu@GO@CTAB NM (a) Kinetic (30 days), (b) varying NaCl concentrations, (c) thermal, (d) varying pH conditions, (e, f) cations and counter anions of sodium. TEM micrographs of GO@CTAB [g (after synthesis), h (after suspending in 1 M NaCl concentration for 24 h)] and Cu@GO@CTAB [i (after synthesis), j (after suspending in 1 M NaCl concentration for 24 h)] and; in the lower lane showing the SEM images of GO@CTAB [k (after synthesis), l (after suspending in 1 M NaCl concentration for 24 h)] and Cu@GO@CTAB [m (after synthesis), n (after suspending in 1 M NaCl concentration for 24 h)], respectively.

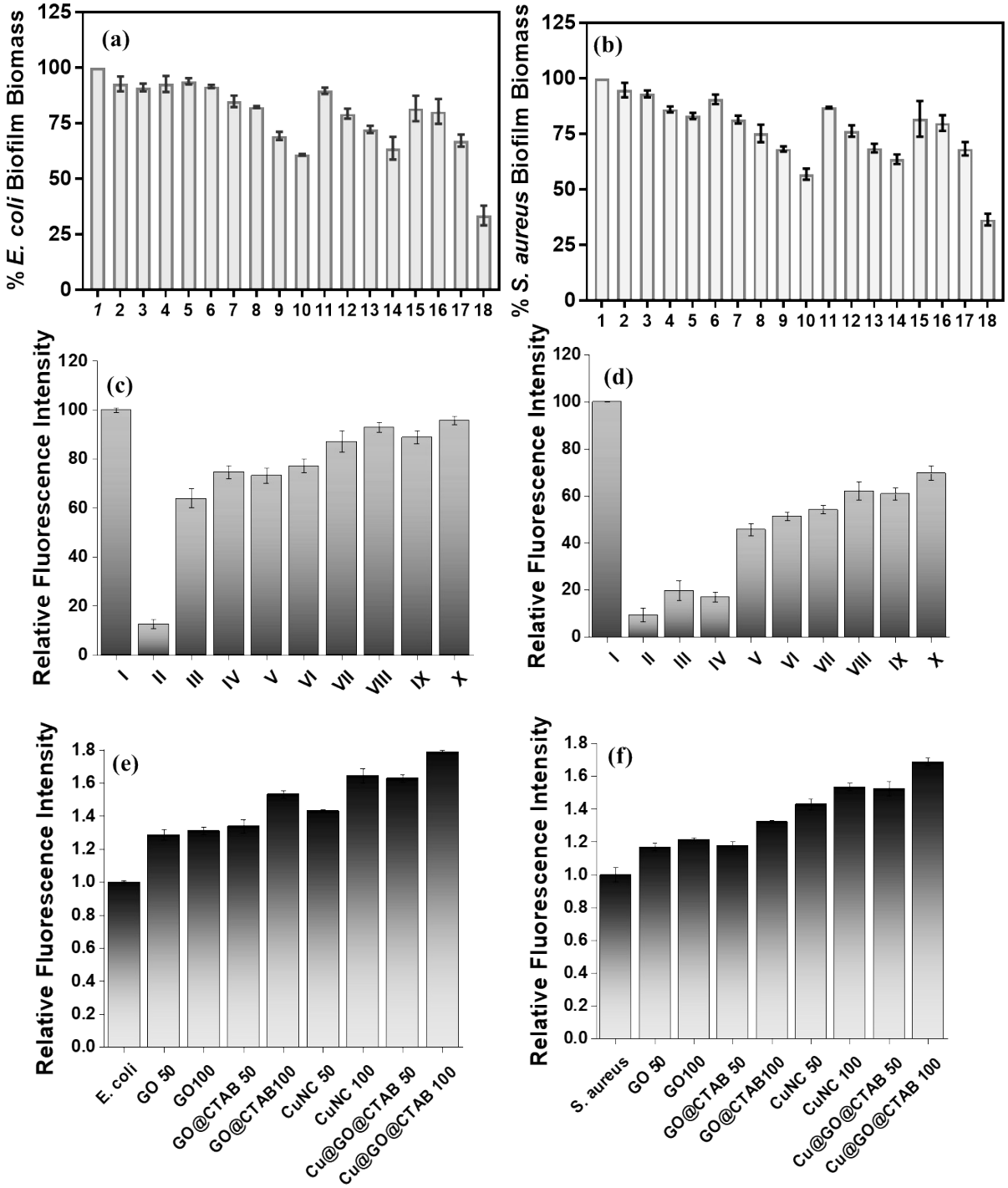

Figure S06: Antibiofilm activity evaluation of the developed NMs and controls; (a, b) *E. coli* and *S. aureus* biofilm biomass analysis by crystal violet assay (1-control; 2, 3, 4, 5- GO; 6-CTAB (1.8%); 7, 8, 9, 10-GO@CTAB; 11, 12, 13, 14-CuNC and 15, 16, 17, 18-Cu@GO@CTAB at 10, 25, 50, 100 µg/mL, respectively), (c, d) reactive oxygen species generation assay (I-Hydrogen peroxide control (500 µM), II-control, III-GO 50, IV-GO 100, V-GO@CTAB 50, VI-GO@CTAB 100, VII-CuNC-50, VIII-CuNC 100, IX-Cu@GO@CTAB 50 and X-Cu@GO@CTAB 100 µg/mL for *E. coli* and *S. aureus*, respectively, (e, f) singlet oxygen generation assay for *E. coli* and *S. aureus*, respectively.

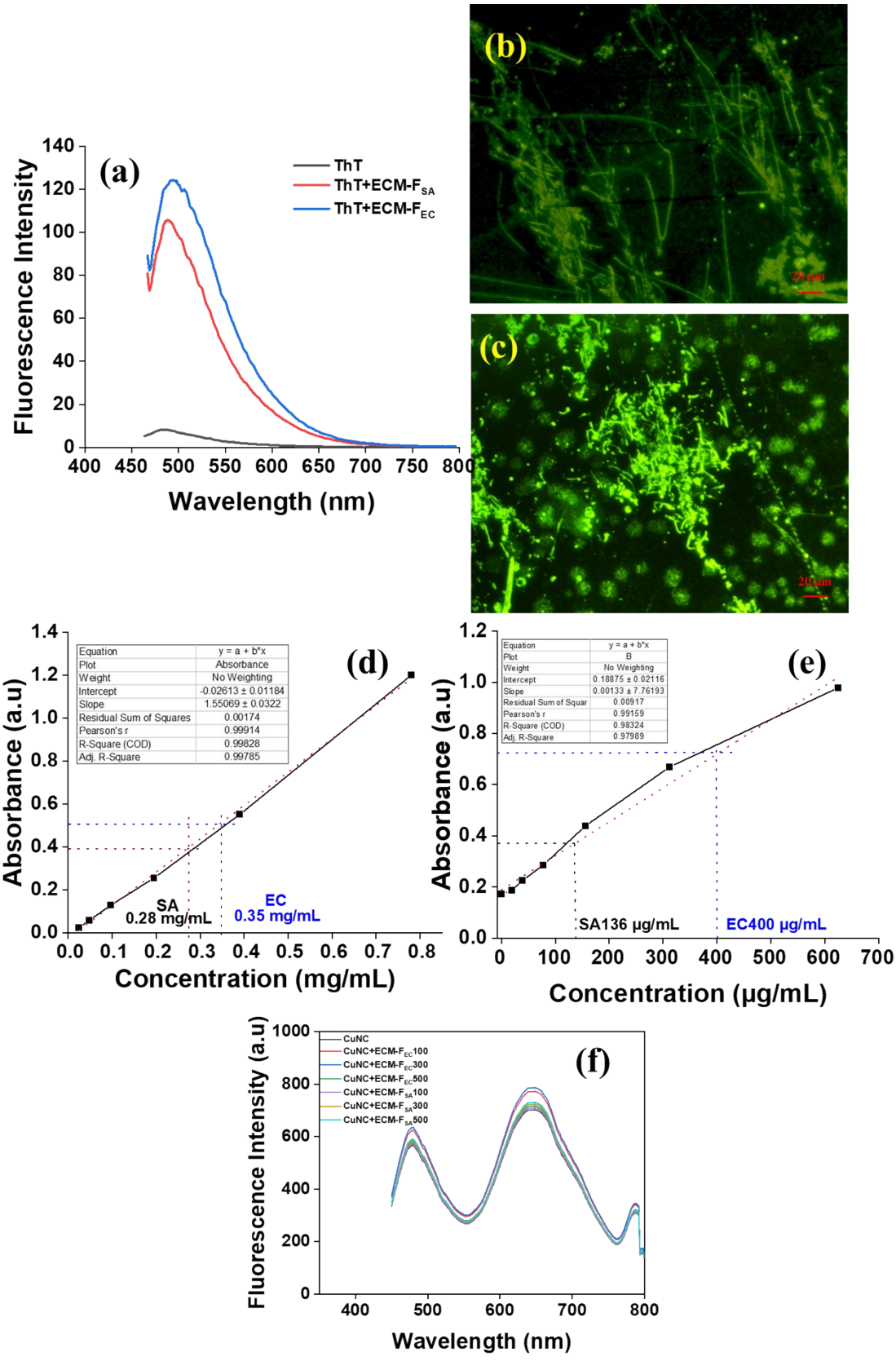
(a)
ThT
ThT+ECM-F$_{SA}$
ThT+ECM-F$_{EC}$
Fluorescence Intensity
Wavelength (nm)
(b)
20 μm
(c)
20 μm
(d)
Equation y = a + b*x
Plot Absorbance
Weight No Weighting
Intercept -0.02613 ± 0.01184
Slope 1.55069 ± 0.0322
Residual Sum of Squares 0.00174
Pearson's r 0.99914
R-Square (COD) 0.99828
Adj. R-Square 0.99785
SA
0.28 mg/mL
EC
0.35 mg/mL
Absorbance (a.u)
Concentration (mg/mL)
(e)
Equation y = a + b*x
Plot B
Weight No Weighting
Intercept 0.18875 ± 0.02116
Slope 0.00133 ± 7.76193
Residual Sum of Squar 0.00917
Pearson's r 0.99159
R-Square (COD) 0.98324
Adj. R-Square 0.97989
SA136 μg/mL
EC400 μg/mL
Absorbance (a.u)
Concentration (μg/mL)
(f)
CuNC
CuNC+ECM-F$_{EC}$100
CuNC+ECM-F$_{EC}$300
CuNC+ECM-F$_{EC}$500
CuNC+ECM-F$_{SA}$100
CuNC+ECM-F$_{SA}$300
CuNC+ECM-F$_{SA}$500
Fluorescence Intensity (a.u)
Wavelength (nm)

Figure S07. ECM isolation studies and composition evaluation (a) ThT assay (b, c) ThT fluorescence microscopy study for ECM-F_{EC} and ECM-F_{SA}, (scale bar is 20 μm) (d) polysaccharide quantification by phenol sulfuric acid assay and (e) protein quantification *via* BCA assay (f) corresponds to fluorescence emission behavior of CuNC when treated with varying concentrations of ECM-FEC and ECM-FSA.

Limit of detection for ECM-F_{EC}

Regression Statistics	
Multiple R	0.978586264
R Square	0.957631077
Adjusted R Square	0.947038846
Standard Error	22.84023184
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	47164.12857	47164.12857	90.40881956	0.000682912
Residual	4	2086.704762	521.6761905		
Total	5	49250.83333			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	40.86666667	21.26309867	-1.921952548	0.126985762	-99.90249289	18.16915956	-99.90249289	18.16915956
X Variable 1	0.519142857	0.054598597	9.50835525	0.000682912	0.36755285	0.670732865	0.36755285	0.670732865

SE of intercept (Standard error)	**21.26309867**
SD of intercept (Standard deviation)	**52.07332864**
LOD	**331.1020896**
LOQ	**1003.339666**

Figure S08. Calculation for LOD and LOQ for ECM-F_{EC} through linear regression method.

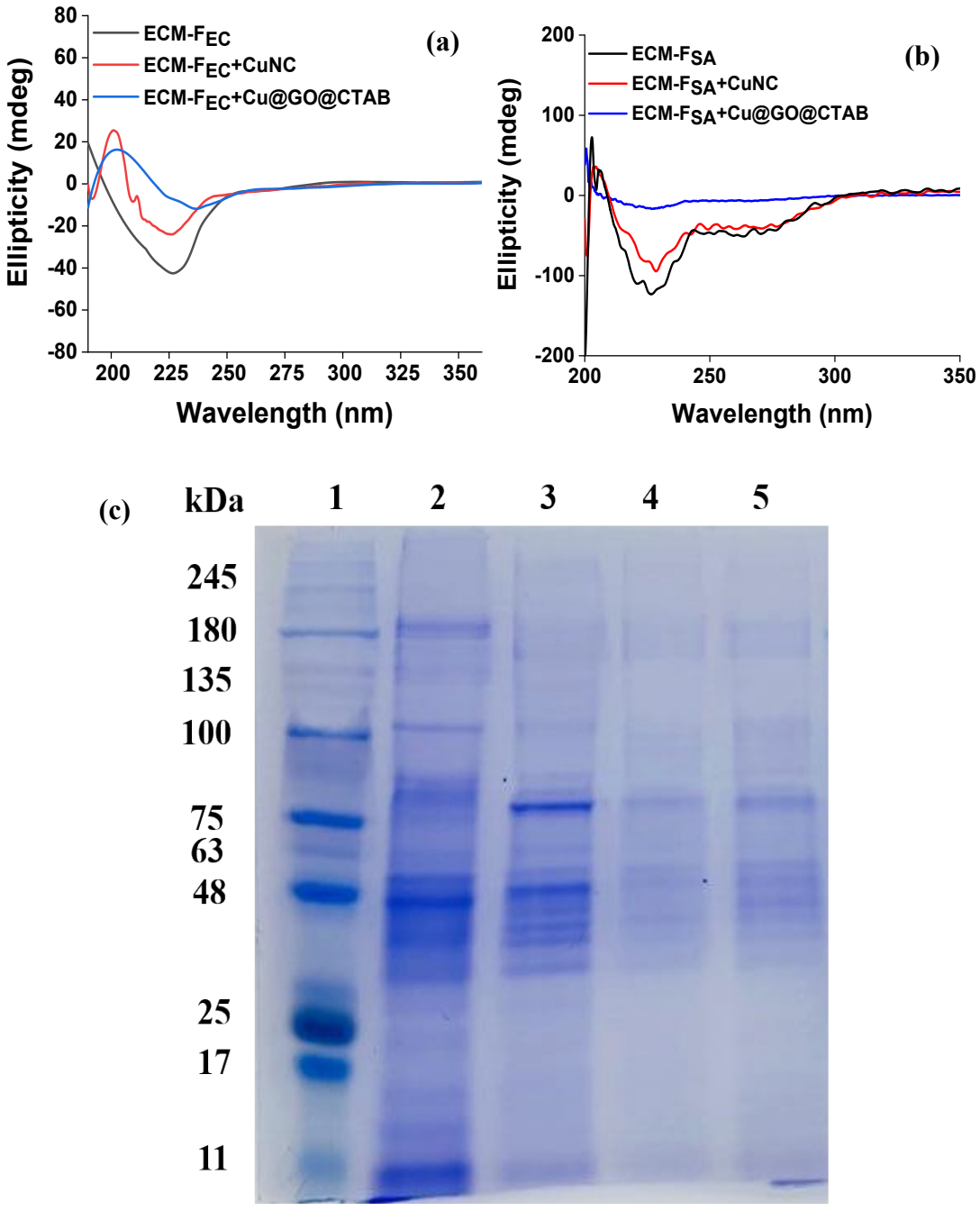

Figure S09. CD spectroscopy-based interaction of ECM-F_{EC} and ECM- F_{SA} with NMs (a, b) and SDS-PAGE (12%) analysis of (1) marker, (2) ECM-F_{EC}, (3) ECM-F_{SA}, (4) ECM-F_{EC}+Cu@GO@CTAB and (5) and ECM-F_{SA}+Cu@GO@CTAB (c).

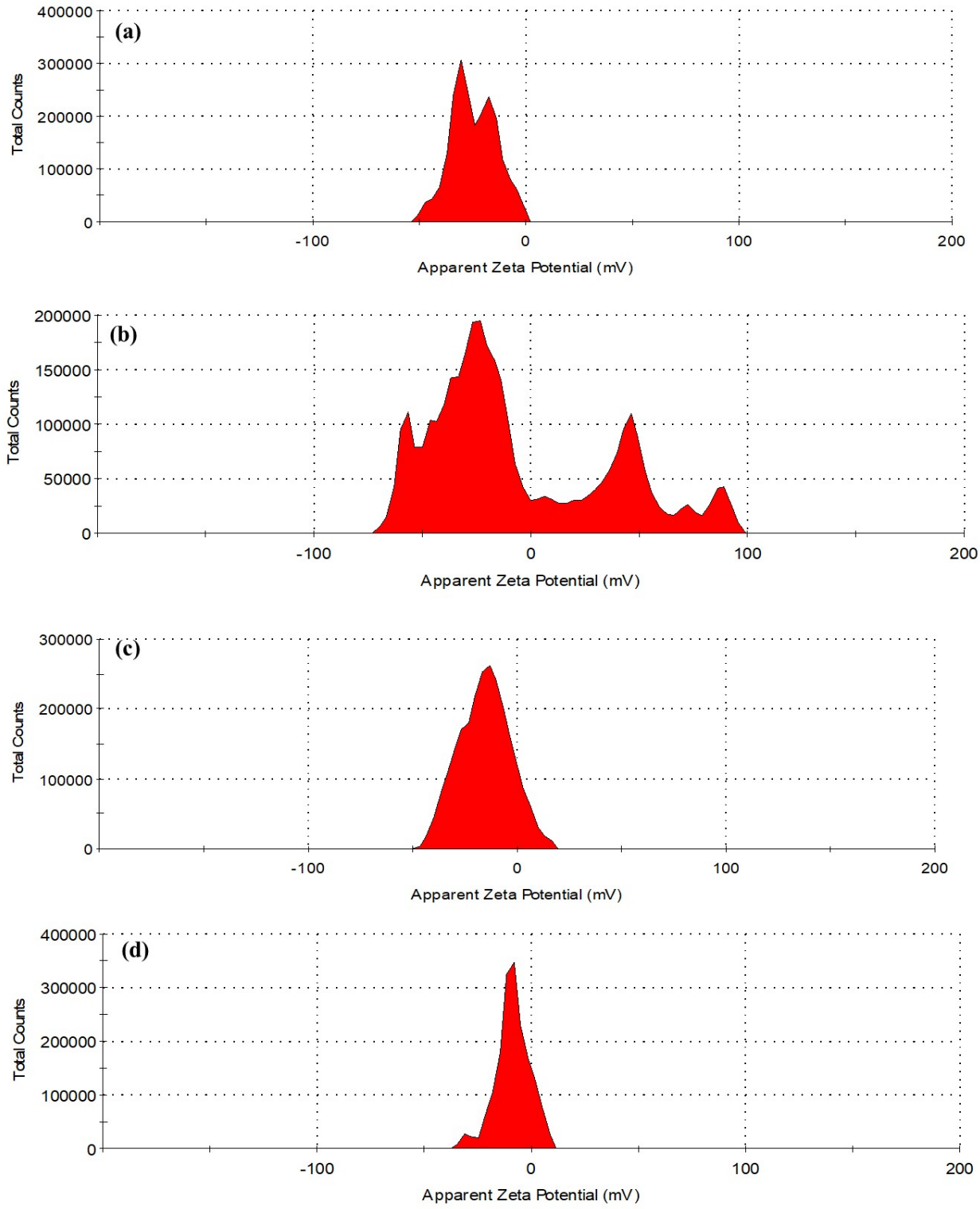

Figure S10. Zeta potential measurement for (a) ECM-F_{EC}, (b) ECM-F_{EC} treated with Cu@GO@CTAB, (c), ECM-F_{SA} (d) and ECM-F_{SA} treated with Cu@GO@CTAB.

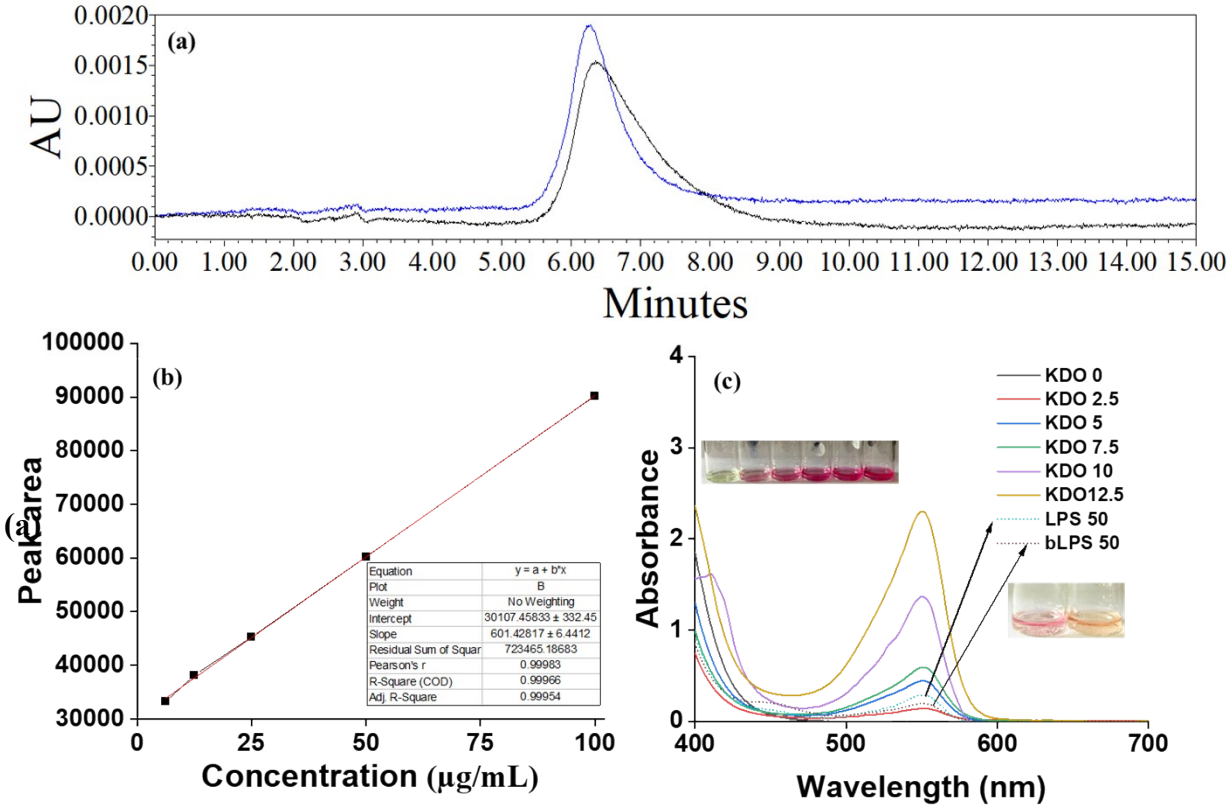

Figure S11. HPLC study. (a) Chromatogram of LPS (blue line), bLPS (black line), (b) standard curve for LPS at 210 nm, (c) KDO assay for evaluating purity of bLPS and inset showing the color change profile revealing the amount of KDO present in the samples.

(a) Limit of detection for LPS

Regression Statistics	
Multiple R	0.986440549
R Square	0.973064956
Adjusted R Square	0.967677947
Standard Error	6.403345443
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7406.41447	7406.4147	180.63172	4.08223E-05
Residual	5	205.014163	41.002836		
Total	6	7611.42851			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	121.9263456	4.604166421	26.48174163	1.43535E-06	110.090959	133.7617	110.091	133.76173
X Variable 1	4.28470255	0.318803885	-13.43993204	4.08223E-05	-5.104214026	-3.46519	-5.10421	-3.465191

SE of intercept	**4.604166421**
SD of intercept	**12.17802018**
LOD	**9.478176087**
LOQ	**28.42675113**

(b) Limit of detection for bLPS

Regression Statistics	
Multiple R	0.981803175
R Square	0.963937474
Adjusted R Square	0.957927053
Standard Erro	12.99492347
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	27082.66	27082.66678	160.3776	1.48587E0-5
Residual	6	1013.208	168.8680361		
Total	7	28095.87			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	206.824	7.58621	27.2631	1.60945E-07	188.261651	225.387274	188.261651	225.3872743
X Variable	7.111577	0.56155	-12.66403	1.48587E-05	-8.48565889	-5.73749703	-8.48565889	-5.737497032

SE of intercept	**7.58621**
SD of intercept	**21.456864**
LOD	**10.1**
LOQ	**30.6**

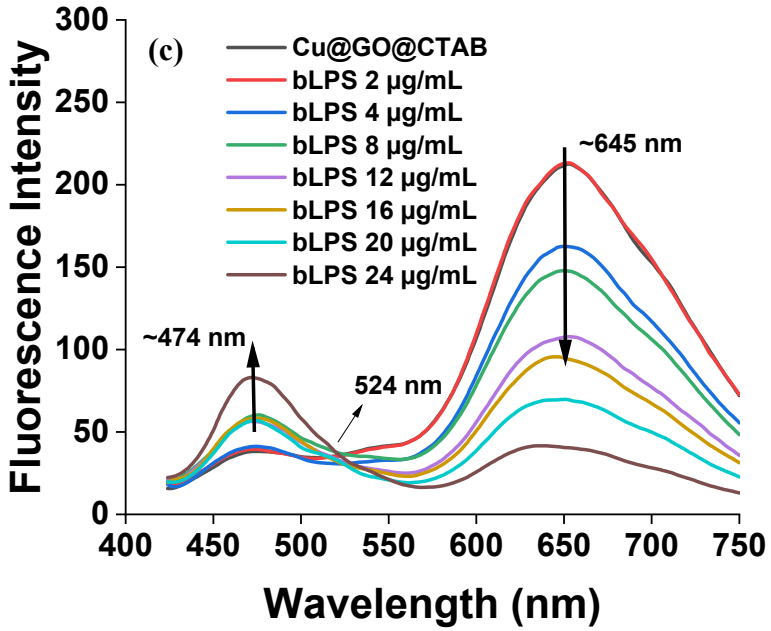

Figure S12. Calculation for LOD and LOQ for (a) LPS and (b) bLPS through linear regression method and (c) shows fluorescence spectroscopy for Cu@GO@CTAB NM titration with bLPS (2-24 µg/mL).

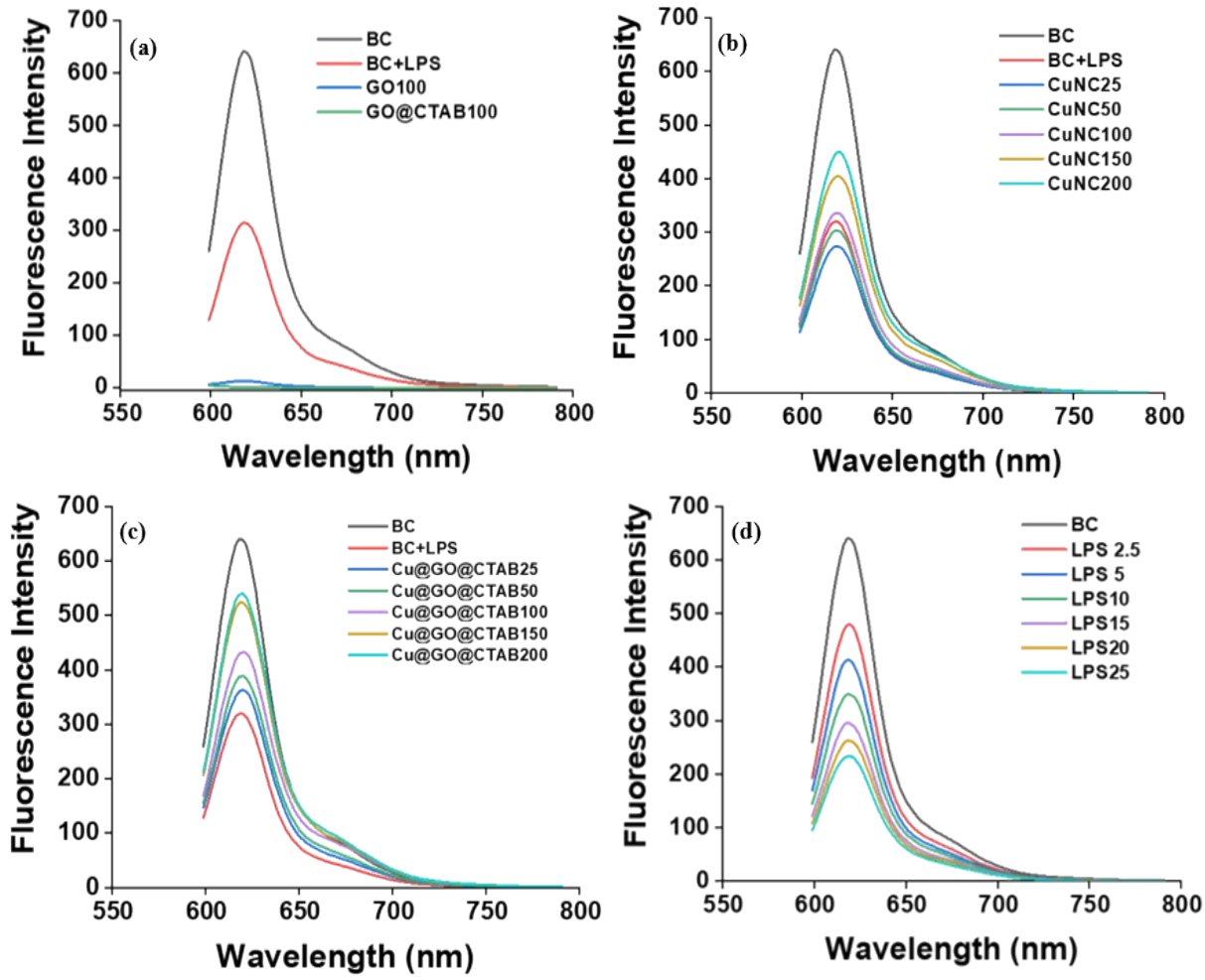

Figure S13. Bodipy- cadaverine (BC) LPS displacement assay (a) BC interaction with LPS and in presence of GO 100, GO@CTAB 100, (b) with CuNC, (c) with Cu@GO@CTAB and (d) with varying concentrations of LPS (2.5 – 25 µg/mL).

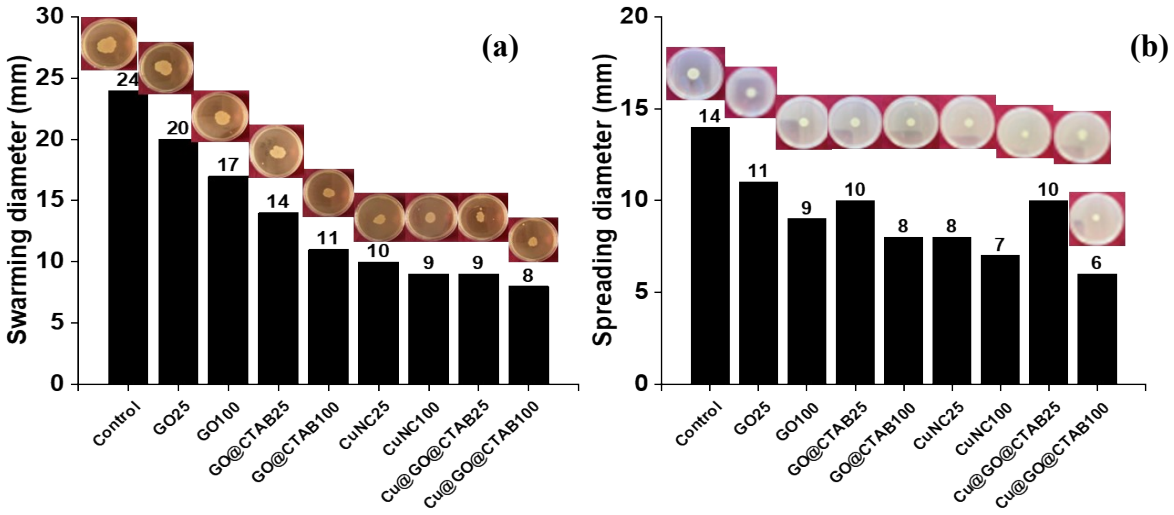

Figure S14. Motility assay (a) swarming assay for *E. coli* and (b) spreading assay for *S. aureus*.

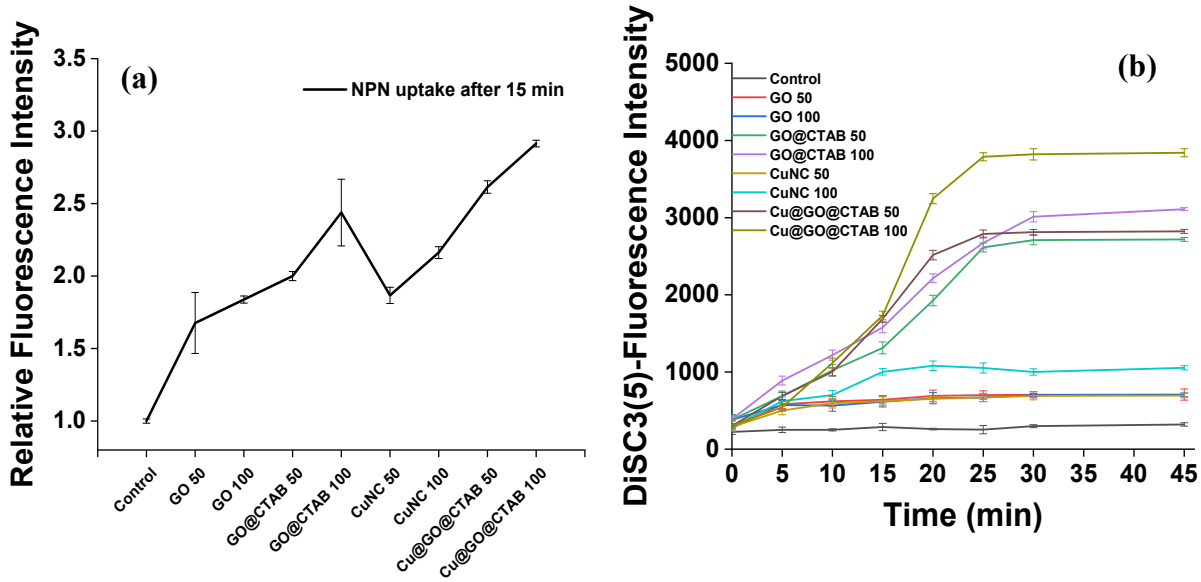

Figure S15. Membrane integrity assay. (a) NPN assay and (b) $DiSC_3(5)$- assay for *S. aureus*.

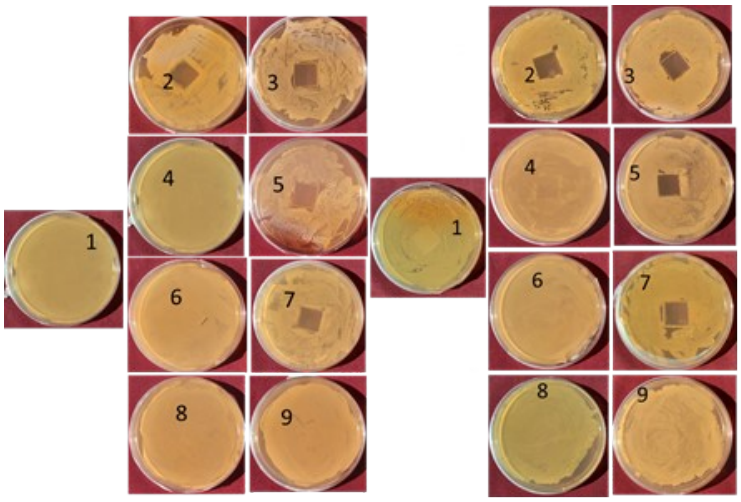

Figure S16. Antibacterial coating studies of different NMs (1) *E. coli* and *S. aureus* (LHS & RHS respectively), (2, 3) Cu@GO@CTAB at 50 and 100µg/mL, (4, 5) CuNC at 50 and 100µg/mL, (6, 7) GO@CTAB at 50 and 100µg/mL, (8, 9) GO at 50 and 100µg/mL.