RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

1   **Electrochemical detection of cholesterol based on competitive host–guest**

2   **recognition using a β-cyclodextrin/poly(*N*-acetylaniline)/graphene-modified**

3   **electrode**

4

5   Long Yang[a, 1], Hui Zhao[b,1], Shuangmei Fan[a], Genfu Zhao[a], Xin Ran[a], Can-Peng Li[a,]*

6

7

8   [a] School of Chemical Science and Technology, Yunnan University, Kunming 650091,

9   PR China.

10   [b] Laboratory for Conservation and Utilization of Bio-resource, Yunnan University,

11   Kunming 650091, PR China.

12

13

14

15

16

17

18

19   [1] These authors contributed equally to this work.

20   * Corresponding authors.

21   Fax or Tel: 86-871-65031119. E-mail: lcppp1974@sina.com (C.-P. Li);

22

1

23   **Abstract:** A sensitive and selective electrochemical approach for cholesterol sensing

24   based on a competitive host–guest recognition between β-cyclodextrin (β-CD) and

25   signal probe (Methylene blue)/target molecule (cholesterol) using

26   β-CD/poly(*N*-acetylaniline)/graphene (β-CD/PNAANI/Gra)-modified electrode was

27   developed. Due to the host–guest interaction, MB molecules can enter into the

28   hydrophobic inner cavity of β-CD, and the β-CD/PNAANI/Gra modified glassy

29   carbon electrode displays a remarkable anodic peak. In the presence of cholesterol,

30   competitive interaction to β-CD occurs and the MB molecules are displaced by

31   cholesterol. This results in a decreased oxidation peak current of MB. As MB is a well

32   known redox probe and hence can be easily detected using differential pulse

33   voltammetery technique. A linear response range of 1.00 to 50.00 μM for cholesterol

34   with a low detection limit of 0.50 μM (*S/N*=3) was obtained by using the indirectly

35   method. The proposed method could be successfully utilized to detect cholesterol in

36   serum samples, and may be expanded to analysis of other non-electroactive species.

37   Besides, the host–guest interaction between cholesterol and β-CD was studied by

38   molecular modeling calculations, which revealed that the complexation could reduce

39   the energy of the system and the complex of 2:1 host–guest stoichiometry had the

40   lowest ΔE value of -10.45 kcal/mol. The molecular docking studies suggested that

41   hydrogen bonding, electrostatic interactions, and hydrophobic interactions should be

42   the major driving forces for the formation of the inclusion complex.

43

44   **1. Introduction**

45   Cholesterol is a vital component in cells and tissues of humans, playing a

46   functional role in construction of cell membranes or serving as a biosynthetic

47   precursor of bile acids, vitamin D, steroid hormones etc. [1] The normal level of total

48   cholesterol in healthy human serum is ~200 mg dL^{-1}. [2] Excess cholesterol in blood

49   serum forms plaques in the arteries of blood vessels which prevent the blood

50   circulation and cause cardiovascular diseases. [3] Thus the levels of total cholesterol in

51   serum and food are major parameters for diagnostic treatment. Over various analytical

52   methodologies, the electrochemical approach of different kinds of cholesterol

53   biosensing platforms has received great attention in past few decades as reviewed by

54   Arya et al. [4] Most of the cholesterol biosensors reported till date are based on the

55   detection of electrooxidation of hydrogen peroxide produced during the catalysis of

56   cholesterol by cholesterol oxidase. [5] This requires a high anodic potential that can

57   induce simultaneous oxidation of other electrochemically active species present in

58   samples leading to false positive signals. Besides, detection selectivity in these

59   methods relies on the use of cholesterol selective enzymes which are expensive,

60   unstable, and prone to denaturation. [3] Therefore, an alternative, simple, and cost

61   effective method for the sensitive and selective detection of cholesterol is highly

62   desirable.

63   The concept of indicator displacement assay (IDA) has received considerable

64   interest with the development of supramolecular chemistry, which exploit the

65   potential of artificial receptors, particularly macrocyclic hosts, for its promising

66   applications in molecular recognition and sensing. [6,7] The sensing principle of IDA

67   relies on the competition between a test substance and an indicator for the same

68   binding site on the host. [8] When an analyte is added to a solution containing

69   host·indicator complex, the analyte displaces the indicator from the binding site.

70   Upon displacement of the indicator, a change in signal is observed. Cyclodextrins

71   (CDs) as the typical macrocyclic molecules have toroidal shapes with hydrophobic

72   inner cavities and hydrophilic exteriors. [9] The interesting characteristics can enable

73   them to bind a wide variety of hydrophobic guest molecules to form stable host–guest

74   complex in their hydrophobic cavity. [10] The host–guest interaction between β-CD and

75   cholesterol has been used for cholesterol extraction from bioenvironment such as food,

76   cell membranes, blood serum and cultured cells. [3] This suggests that β-CD can be an

77   alternative to cholesterol enzyme for selective recognition of cholesterol. However,

78   this interaction does not produce any signal change. In addition, cholesterol is one of

79   the non-electroactive species, resulting its direct electrochemical determination is very

80   difficult. Alternatively, designing a competitive host–guest recognition system by

81   selecting an electroactive probe as signal indicator might have promising applications

82   for the analysis of non-electroactive species. Mondal and Jana [3] have recently carried

83   out fluorescent detection of cholesterol using graphene -β-CD (Gra-β-CD) hybrid

84   system, where the optical detection of cholesterol was carried out using rhodamin 6G

85   (R6G) dye as a fluorophore. Although the competitive host–guest interaction system

86   has been widely applied in the fluorescent sensing filed, [3,11-15] it is rarely investigated

87   for electrochemical sensing applications except few researchers contributed to this

88   area. [16,17]

89 Herein, a non-enzymatic electrochemical approach for cholesterol sensing based

90 on a competitive host-guest recognition between β-CD and signal probe/target

91 molecules using β-CD/poly(*N*-acetylaniline)/Gra (β-CD/PNAANI/Gra)-modified

92 electrode was proposed. Methylene blue (MB) and cholesterol were selected as the

93 probe and target molecules, respectively. Gra was chosen here considering that it can

94 enhance the electrode conductivity and facilitate the electron transfer. The

95 introduction of PNAANI film is used to steadily immobilize β-CD and to avoid the

96 non-specific adsorption of MB on the Gra film by a π–π stacking interaction. Due to

97 the host–guest interaction, MB molecules can enter into the hydrophobic inner cavity

98 of β-CD, and the β-CD/PNAANI/Gra modified glassy carbon electrode displays a

99 remarkable anodic peak. In the presence of cholesterol, competitive interaction to

100 β-CD occurs and the MB molecules are displaced by cholesterol. This results in a

101 decreased oxidation peak current of MB. As MB is a well known redox probe and

102 hence can be easily detected using differential pulse voltammetery (DPV) technique.

103 Agnihotri et al. [17] reported an electrochemical detection of cholesterol using

104 Gra-β-CD hybrid as the sensing matrix, while the host–guest interaction in their work

105 did not occur on the electrode surface (in an electrochemical cell). Compared with this

106 previous report, the host–guest interaction in the present work occurred completely on

107 the β-CD/PNAANI/Gra-modified electrode surface, which is less expensive as an

108 electrochemical cell needs 3 mL materials. And the non-specific adsorption of MB on

109 the Gra film was avoided by the PNAANI film. The proposed method could be

110 successfully utilized to detect cholesterol in serum samples, and exhibited a promising

111   application in practice. In addition, the host–guest interaction between cholesterol and

112   β-CD was investigated by molecular modeling calculations.

113

114   **2. Materials and methods**

115   **2.1. Chemicals**

116   Graphite oxide was purchased from Nanjing XFNANO Materials Tech Co., Ltd.

117   (Nanjing, China). Cholesterol, MB, β-CD, and *N*-acetylaniline (NAANI) were

118   obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of

119   analytical grade. Phosphate buffer (PBS, 0.1 M pH 7.0) was used as working solution.

120   All aqueous solutions were prepared with deionized water (DW, 18 MΩ cm).

121

122   **2.2. Apparatus**

123   Electrochemical impedance spectroscopy (EIS) and DPV experiments were

124   performed with a CHI 660E Electrochemical Workstation from Shanghai Chenhua

125   Instrument (Shanghai, China) and conducted using a three-electrode system, with the

126   modified GCE as working electrode, a platinum wire as the counter electrode, a

127   saturated calomel electrode (SCE) as the reference electrode. The morphologies of the

128   prepared samples were characterized by a QUNT200 scanning electron microscopy

129   (SEM, USA). UV–visible spectra were analyzed in a U-2001 Hitachi (Tokyo, Japan)

130   UV spectrophotometer. Fourier transform infrared (FTIR) study was performed over

131   the wavenumber, range of 4000–400 cm^{-1} by a Thermo Fisher SCIENTIFIC Nicolet

132   IS10 (Thermo Fisher, Massachusetts, USA) FTIR impact 410 spectrophotometer

133   using KBr pellets. Raman spectra were obtained on a 400F PERKINELMER Raman

134   spectrometer (USA) with 514.5 nm wavelength incident laser light.

135

136   **2.3. Molecular docking**

137   The crystal structures of β-CD (ID: IHEGEI) and cholesterol (ID: CHOEST21)

138   were obtained from Cambridge Crystallographic Data Centre (CCDC) and optimized

139   using molecular dynamics simulation with the Gaussian 03 program. Both the

140   optimized structures were used as a starting structure in the docking study.

141   AutoDock4.2 with Lamarckian Genetic Algorithm (LGA) was used for docking study.

142   An initial population of 150 individuals with a maximum number of energy

143   evaluations of 25,000,000 and a maximal number of generations of 27,000 were used

144   as an end criterion. An elitism value of one was used and a probability of mutation

145   and crossing-over of 0.02 and 0.8 was used, respectively. We have defined the

146   conformational search space implementing an $60 \times 60 \times 60$ grid and 0.375 Å spacing

147   between each point in such a way that it covered both the external surface and the

148   internal cavity of the β-CD. A total of 50 docking runs were carried out. At the end of

149   each run, the solutions were separated into clusters according to their lowest RMSD

150   and the best energy score value based on an empirical free energy function. Clustering

151   was performed on the docked complexes with a cut-off of 2 Å. From the docking

152   calculations, the lowest energy conformation was selected as the cholesterol/β-CD

153   binding mode, and the binding free energy of the cholesterol/β-CD complex was

154   calculated by using the semi-empirical method PM3.

155

2.4. Preparation of the Gra

156

157   The graphite oxide was exfoliated into graphene oxide (GO) sheets by

158   ultrasonication at room temperature for one hour. The as-obtained yellow-brown

159   aqueous suspension of GO was stored at room temperature and used for further

160   experiment. Compared with the traditional procedure using highly toxic hydrazine as

161   reductant, ascorbic acid (AA) was used as reducing agent to prepare Gra in DW at

162   room temperature. In a typical experiment, 50 mg of AA was added into 10.0 mL of

163   0.5 mg mL^{-1} GO aqueous suspension and stirred for 48 h at room temperature. After

164   centrifuging and washing with DW for three times, the resulting Gra material was

165   obtained by freeze-drying.

166

2.5. Preparation of the modified electrodes

167

168   Glassy carbon electrode (GCE, 3 mm in diameter) was polished with 0.3 and 0.05

169   μm Al$_2$O$_3$ powder respectively and subsequently sonicated in ethanol and DW to

170   remove the adsorbed substance and dried in air. The Gra was dissolved in DW at a

171   concentration of 0.5 mg mL^{-1} with the aid of ultrasonic agitation for 20 min, resulting

172   in a homogeneous suspension. To prepare the Gra modified electrode, 5 μL of the Gra

173   suspension was dropped onto the electrode surface and dried at room temperature.

174   The obtained electrode was noted as Gra/GCE. In order to prepare the

175   PNAANI/Gra/GCE, the Gra/GCE was held at a constant voltage of 1.0 V for 1 min in

176   a mixture solution containing 0.1 M NAANI and 1.0 M HClO$_4$ aqueous solution, and

8

177   then swept from −0.2 to 1.0 V for 25 cycles at a scan rate of 100 mV s^{-1}. Finally, by

178   electro-oxidation of the PNAANI/Gra/GCE at a constant potential of 1.2 V for 12 min

179   by amperometric i–t curve technique in a mixture solution containing 0.05 M β-CD

180   and 0.1 M LiClO$_4$ in DMSO, the β-CD/PNAANI/Gra/GCE was obtained successfully.

181   For comparison, a similar procedure was used to prepare PNAANI/Gra/GCE

182   and β-CD/PNAANI/GCE.

183

184   **2.6. Electrochemical measurements**

185   DPV was applied in 0.1 M pH 7.0 PBS from −0.7 to 0.7 V with a pulse amplitude

186   of 0.05 V and a pulse width of 0.05 s. EIS was recorded in the frequency range from

187   10^{-1} to 10^5 Hz with an amplitude of 5 mV using 2.0 mM $[Fe(CN)_6]^{3-/4-}$ redox couple

188   (1:1) with 0.1 M KCl as supporting electrolyte. All the measurements were carried out

189   at room temperature. As cholesterol has very low solubility in water, 1.0 mM stock

190   solution of cholesterol was prepared in ethanol and diluted to different concentrations

191   by 0.1 M pH 7.0 PBS for further use. Before electrochemical measurements, the

192   β-CD/PNAANI/Gra/GCE was incubated with 1.0 mM MB solution (in 0.1 M pH 7.0

193   PBS) for a definite time, and rinsed gently with ultrapure water. Then, the electrode

194   was further incubated with different concentrations of cholesterol solution. After that,

195   the electrode was rinsed gently with ultrapure water and the current response of the

196   MB-bound β-CD/PNAANI/Gra/GCE were investigated by DPV in 0.1 M pH 7.0

197   PBS.

198

3. Results and Discussion

3.1. Design strategy of the electrochemical sensor

Scheme 1 illustrates the assay protocol of the proposed electrochemical sensor based on the competitive host–guest interaction between β-CD and MB (signal probe)/cholesterol (target). MB molecules can enter into the inner cavity of β-CD due to the host–guest interaction, and the MB-bound β-CD/PNAANI/Gra/GCE displays a remarkable oxidation peak due to MB. However, in the presence of cholesterol, competitive association to the β-CD occurs and the MB molecules are displaced by cholesterol. This results in a decrease of the oxidation peak current of the MB probe.

3.2. Molecular docking

In recent years, widespread use has been made of computer aided molecular modeling to rapidly and simply obtain a three dimensional image of the most likely structure of the inclusion complex. Typically, the more negative the binding energy is, the stronger interaction is between the host and guest molecules. As listed in **Table 1**, the lowest binding free energy (ΔE) was –6.68 kcal/mol for the host–guest complex of cholesterol and β-CD with 1:1 stoichiometry calculated by PM3 method. The lowest energy docked conformation for 1:1 complex of cholesterol and β-CD, shown in **Fig. 1A**, reveals that the B-, C-, and D-rings of cholesterol molecule inserted into β-CD cavity due to hydrophobic interactions, however, the cyclohexanol part (A-ring) and the alkyl chain of the cholesterol molecule located just outside the β-CD host. The A-, B-, C-, and D-rings of cholesterol were indicated in **Scheme 2**. Among all the

221 docking conformations of cholesterol and β-CD, two other conformations capture our

222 attention. One is the cyclohexanol part (A-ring) that inserted into the cavity of β-CD,

223 the other is the alkyl chain of the cholesterol that inserted into β-CD cavity. Thus, we

224 speculated that one cholesterol molecule may bind with two β-CD molecules to form

225 a 1:2 inclusion complex. So we have further studied the interaction between one

226 cholesterol and two β-CD molecules. The crystal structure of two adjacent β-CDs (ID:

227 WISRIZ) was obtained from Cambridge Crystallographic Data Centre (CCDC) and

228 also optimized using molecular dynamics simulation with the Gaussian 03 program.

229 The docked conformation was displayed in **Fig. 1B**, indicating that the cyclohexanol

230 (A-ring) and the alkyl chain of cholesterol molecule inserted into the cavities of two

231 β-CD molecules, respectively. The ΔE calculated by PM3 method was −10.45

232 kcal/mol, which is much lower than that that of a 1:1 inclusion complex, indicating

233 that a 1:2 guest–host binding mode is more stable. Analysis of host–guest interaction

234 as obtained from the docking studies reveals that hydrogen bonding, electrostatic

235 interactions, and hydrophobic interactions are the predominant driving forces of the

236 1:2 guest–host complex. Firstly, the hydroxyl on A-ring of the cholesterol molecule

237 formed hydrogen bonding with the hydroxyl of β-CD and the bond length is 2.0 Å.

238 Secondly, as shown in **Fig. 1C**, the surface of cholesterol molecule is mainly

239 negatively charged, while the internal cavities of the two adjacent β-CDs are mainly

240 positively charged. Thus, strong electrostatic interactions formed between cholesterol

241 molecule and the two β-CDs. Thirdly, as revealed in **Fig. 1D**, strong hydrophobic

242 interactions also formed between cholesterol molecule and the two β-CDs. The

243   cyclohexanol and the alkyl chain groups of the cholesterol molecule inserted into the

244   hydrophobic cavities of two β-CD molecules, respectively. Recently, Cheng et al. [18]

245   proposed a similar 1:2 cholesterol/β-CD complex structure based on the 2D [1]H

246   ROESY results. This result is in accordance with that of our obtained from molecular

247   docking.

248

249   **3.3. Characterization of the β-CD/PNAANI/Gra/GCE film**

250   The reduction of the GO was characterized by UV–visible spectroscopy, FTIR

251   spectroscopy, and Raman spectroscopy, respectively. Initially, UV–visible

252   spectroscopy was used to study the reduction of GO. As shown in **Fig. 2A**, the GO

253   shows a strong absorption at 230 nm and a shoulder at 300 nm, which correspond to

254   the π–π* transition of the aromatic C=C bond and the n–π* transition of the C=O

255   bond, respectively. After reduction, the peak at 230 nm shifts to 260 nm indicating the

256   restoration of the π-conjugation network of the Gra. The disappearance of the peak at

257   300 nm reflects the effect of deoxygenation. To further illustrate the formation of Gra,

258   FTIR spectra were employed to investigate the reduction of GO. **Fig. 2B** shows the

259   FTIR spectra of the GO and Gra. As observed, the GO displays several characteristic

260   bands at approximately 3425, 1724, 1617, 1213, and 1052 cm^{-1}, which are caused by

261   the stretching vibrations of –OH, C=O, C=C, C–O–C, and C–O, respectively. After

262   reduction, the band at 1724 cm^{-1} corresponding to the C=O stretch vanishes, and the

263   bands at 3425 and 1213 cm^{-1} decreases dramatically, confirming that the GO was

264   reduced to Gra by AA. Raman spectroscopy is one of the most widely used techniques

265   to characterize the structural and electronic properties of Gra including disordered and

266   defective structures, defect density, and doping levels. **Fig. 2C** shows the typical

267   Raman spectra of GO and Gra. As expected, GO displays two prominent peaks at

268   1360 and 1602 cm^{-1} corresponding to the D and G bands, respectively. The Gra shows

269   two prominent peaks at 1355 and 1589 cm^{-1}, corresponding to the breathing mode of

270   k-point phonons of A_{1g} symmetry (D band) and the E_{2g} phonons of C sp^2 atoms (G

271   band) of Gra, respectively. The intensity ratio of the D band to the G band (I_D/I_G) is

272   clearly higher when compared with that of GO (0.97 vs. 0.78), suggesting a decrease

273   in sp^2 domains and a partially ordered crystal structure of Gra induced by AA

274   reduction.

275   Because the PNAANI and β-CD were immobilized on the Gra/GCE by

276   electro-polymerization electro-oxidation, respectively. It is difficult to characterize by

277   FTIR. Thus, the Gra/GCE, PNAANI/Gra/GCE, and β-CD/PNAANI/Gra/GCE films

278   were investigated using SEM. As shown in **Fig. 3A**, the microstructure image reveals

279   that after reduction the Gra material consists of randomly aggregated thin, wrinkled

280   sheets closely associated with each other. It is noted that after polymerization of

281   NAANI on the surface of Gra/GCE, a layer of PNAANI was densely covered on the

282   surface of Gra/GCE (**Fig. 3B**). Besides, it can be seen from **Fig. 3C** that a layer of

283   β-CD thin films was uniformly covered on the surface of PNAANI/Gra/GCE,

284   indicating that the β-CD/PNAANI/Gra/GCE film was successfully obtained.

285

286   **3.4. Electrochemical characterization of the modified electrodes**

287   EIS was performed at the potential of 0.1 V and the frequency ranges was from

288   10^1 to 10^5 Hz, using 2.0 mM $[Fe(CN)_6]^{3-/4-}$ redox couple (1:1) with 0.1 M KCl as

289   supporting electrolyte. The value of the electron-transfer resistance (R_{ct}) of the

290   modified electrode was estimated by the semicircle diameter. **Fig. 4A** illustrates the

291   EIS of the bare GCE, Gra/GCE, β-CD/PNAANI/GCE, and β-CD/PNAANI/Gra/GCE.

292   Obviously, the bare GCE exhibited a semicircle portion and the value of R_{ct} was

293   estimated to be 750 Ω. While the R_{ct} decreased remarkably at Gra/GCE, indicating

294   that Gra had good conductivity and improved obviously the diffusion of ferricyanide

295   toward the electrode interface. In the case of β-CD/PNAANI/GCE, its R_{ct} further

296   increased to 1500 Ω due to the poor conductivity of β-CD, suggesting that large

297   amount of β-CD molecules were successfully immobilized on the surface of

298   PNAANI/GCE. Furthermore, the R_{ct} of the β-CD/PNAANI/GCE is much larger than

299   that of the β-CD/PNAANI/Gra/GCE, although the introduction of β-CD results in the

300   increase of the semicircle diameter. These results indicate that the Gra film can

301   significantly accelerate the interfacial charge transfer between the electrochemical

302   probe $[Fe(CN)_6]^{3-/4-}$ and the modified electrode, which will be beneficial to the good

303   analytical performance of the electrode.

304   **Fig. 4B** shows the DPV responses of different electrodes in 0.1M pH 7.0 PBS

305   after incubation with 1.0 mM MB. On the Gra/GCE (**curve a**), an oxidation peak was

306   observed due to the non-specific adsorption of MB on Gra via π–π stacking. However,

307   there is no oxidation peak of MB on the PNAANI/Gra/GCE (**curve b**), indicating that

308   the adsorption of MB was restrained by PNAANI film. Furthermore, on the

309   β-CD/PNAANI/Gra/GCE (**curve d**), the oxidation peak current of MB increases

310   obviously due to the good molecular recognition property and high enrichment

311   capability of β-CD compared to all the other electrodes. As a comparison, the DPV

312   response of MB on the β-CD/PNAANI/GCE was also investigated (**curve c**). It is

313   noted that the peak current is smaller than that on the β-CD/PNAANI/Gra/GCE.

314   These imply that the large specific surface area and good electron transfer property of

315   Gra are important to improve the electrochemical performance of the modified

316   electrode. In general, it can be concluded that the introduction of Gra film is

317   beneficial to enhancing the electron transfer of the electrochemical sensor, and the

318   PNAANI film can avoid successfully the non-specific adsorption of MB on Gra film

319   via π–π stacking interaction.

320

321   **3.5. Feasibility of the electrochemical sensor**

322   To demonstrate the assay feasibility of the proposed sensor, DPV response of the

323   β-CD/PNAANI/Gra/GCE was investigated in 0.1 M pH 7.0 PBS. As can be seen from

324   **Fig. 4C**, no detectable signal (**curve a**) is observed for the β-CD/PNAANI/Gra/GCE

325   in 0.1 M pH 7.0 PBS due to the absence of the redox mediator MB. After incubated in

326   1.0 mM MB solution for 25 min, the MB-bound β-CD/PNAANI/Gra/GCE was then

327   tested in 0.1 M pH 7.0 PBS and an obvious oxidation peak of MB (**curve b**) can be

328   observed at about –0.1 V. When the β-CD/PNAANI/Gra/GCE was first incubated in

329   1.0 mM MB solution for 25 min and further incubated in 10 μM cholesterol solution

330   for 30 min, then tested in 0.1 M pH 7.0 PBS, a decreased oxidation peak (**curve c**)

331   was obtained due to competitive association of cholesterol/MB to the β-CD occurs.

332   This is because cholesterol has higher binding affinity to β-CD cavity due to its

333   hydrophobic nature. This suggests that the MB molecules present inside the

334   β-CD/PNAANI/Gra/GCE host can be replaced by cholesterol and the MB-bound

335   β-CD/PNAANI/Gra/GCE can be used to sensitively detect cholesterol by the

336   competitive electrochemical sensing strategy.

337

338   **3.6. Optimization of experimental conditions**

339   Several control experiments were carried out to determine the optimum reaction

340   conditions. **Fig. 5A** shows the effect of the potential cycle number of the

341   electrodeposition of PNAANI film on the DPV peak current of the

342   PNAANI/Gra/GCE in 0.1 M pH 7.0 PBS after incubation in 1.0 mM MB solution. It

343   is noted that the oxidation peak current of MB decreases with an increase of the cycle

344   number and reaches about 0 at 25 cycles, indicating that the surface of Gra/GCE is

345   covered completely by PNAANI film and that the PNAANI/Gra/GCE can

346   successfully avoid the non-specific adsorption of MB on the surface of Gra by π–π

347   stacking interactions. Therefore, a cycle number of 25 was selected for further study.

348   Moreover, the effect of the electro-oxidation time of β-CD on the DPV peak current

349   of the β-CD/PNAANI/Gra/GCE was also investigated (**Fig. 5B**). Due to the

350   host-guest interaction between MB and β-CD, it was found that the peak current of

351   MB increases with the increase of the deposition time and a maximum is observed at

352   12 min. This suggests that the immobilized amount of β-CD is at a maximum at 12

353   min. Thus, this time was selected as the optimum electro-oxidation time for β-CD on

354   the PNAANI/Gra/GCE. In addition, the incubation time of the modified electrode in

355   MB solution is one of the key parameters that will affect the response performance of

356   the electrode. **Fig. 5C** shows that the oxidation peak current of the electrode increases

357   with the increase of the incubation time and reaches a maximum at 25 min for MB

358   incubation. Therefore, 25 min is selected as the optimum incubation time for the

359   as-prepared electrode in MB solution. Similarly, the effect of the incubation time of

360   the modified electrode in cholesterol solution was also studied. **Fig. 5D** shows that the

361   oxidation peak current of the electrode decreases with the increase of the incubation

362   time and reaches about 0 at 30 min for cholesterol incubation. Therefore, 30 min is

363   selected as the optimum incubation time for the as-prepared electrode in cholesterol

364   solution.

365

366   **3.7. Quantitative analysis of the electrochemical sensor toward cholesterol**

367   Under optimal conditions, DPV was used to determine the concentrations of

368   cholesterol because it is a highly sensitive and low-detection limit electrochemical

369   method. **Fig. 6A** shows the DPV curves of electrochemical signal on the MB-bound

370   β-CD/PNAANI/Gra/GCE under different concentrations of cholesterol solution. The

371   oxidation peak currents of MB decreased with increased cholesterol concentrations.

372   **Fig. 6B** shows the corresponding calibration curve for cholesterol quantification. The

373   peak currents were proportional to the cholesterol concentrations between 1.00 and

374   50.00 μM with a detection limit of 0.50 μM (S/N=3). The corresponding regression

375   equation was calculated as ΔI (μA) = 0.154C (μM) + 0.588 with correlation

376   coefficients of 0.998. Detection limit was less than 1.0 μM which was quite low and

377   satisfactory with respect to other recently reported articles. **Table 2** illustrates few of

378   the recent literatures on cholesterol sensing platforms, through both enzymatic and

379   non-enzymatic sensing routes. The detection limit and sensitivity of the present

380   sensing strategy is comparatively better than the reported ones.

381

382   **3.8. Selectivity, reproducibility, and stability**

383   As we know, human blood serum contains many more biocomponents like salts,

384   amino acids, carbohydrates, lipids etc., those can interfere with cholesterol detection

385   and hamper the selectivity of the electrochemical sensor. Therefore, we have tested

386   interference from common molecules present in human blood serum and found very

387   negligible interference. As shown in **Fig. 6C**, some salts, carbohydrates, protein,

388   anionic surfactant, etc. including glucose, AA, bovine serum albumin (BSA), sodium

389   dodecyl sulphate (SDS), NaCl, KCl, and, MgCl$_2$ showed negligible interference even

390   at the concentration of 2.0 mM, compared to cholesterol detected for only 30 μM

391   concentration. Six equal MB-bound β-CD/PNAANI/Gra/GCEs were used to evaluate

392   the fabrication reproducibility of the present method for cholesterol detection. The six

393   modified electrodes exhibited similar signals with a relative standard deviation of

394   3.9%, indicating satisfactory reproducibility. Additionally, a long-term stability

395   experiment was performed intermittently (every 5 days) and used to examine the

396   stability of the MB-bound β-CD/PNAANI/Gra/GCE. The constructed sensor was

397 stored in a refrigerator at 4 °C when not in use. Initial responses of over 94.3% and

398 85.6% remained after storage for 15 and 30 days, respectively, indicating an

399 acceptable stability of the MB-bound β-CD/PNAANI/Gra/GCE.

400

401 **3.9. Real sample analysis**

402 The proposed method was used to detect cholesterol in serum samples using

403 standard addition methods to evaluate the feasibility of the MB-bound

404 β-CD/PNAANI/Gra/GCE for real sample analysis. The serum sample was diluted

405 fifty times with 0.1 M pH 7.0 PBS. Results showed recoveries ranging from 98.4% to

406 105.2% and RSDs ranging from 2.6% to 4.5% (**Table 3**). The results demonstrated

407 that this method can be extended for cholesterol detection in blood.

408 The proposed sensing platform may also be expanded to wide and potential

409 applications in biological and environmental samples. It is worthy note that, as an

410 oligosaccharide, β-CD is more stable than cholesterol selective enzymes (mostly

411 oxidase) under complex conditions. Thus the present sensing platform seems to be

412 more suitable for analysis of practical cholesterol samples than traditional

413 enzyme-based biosensor.

414

415 **4. Conclusions**

416 In conclusion, based on a competitive host-guest interaction between β-CD and

417 signal probe/target molecules, a new electrochemical approach for cholesterol sensing

418 using β-CD/PNAANI/Gra-modified electrode was developed. Due to the good

419 electron transfer property of the Gra, the excellent inhibiting ability of PNAANI film

420 for the non-specific adsorption of MB, and the excellent host–guest recognition of

421 β-CD, the developed β-CD/PNAANI/Gra/GCE displays excellent analytical

422 performance for the electrochemical detection of cholesterol: the linear response

423 range is 1.00–50.00 μM and the LOD is 0.50 μM (S/N=3). In addition, the developed

424 electrochemical sensing platform is important as it does not use any enzyme or

425 antibody for detection of cholesterol efficiently with outstanding selectivity over the

426 common interfering species. Besides, the host–guest interaction between cholesterol

427 and β-CD was studied by molecular modeling calculations, which revealed that the

428 complexation could reduce the energy of the system and the complex of 2:1

429 host–guest stoichiometry had the lowest ΔE value of –10.45 kcal/mol. The molecular

430 docking studies suggested that hydrogen bonding, electrostatic interactions, and

431 hydrophobic interactions should be the predominant driving forces for the formation

432 of the inclusion complex.

433

434 **Acknowledgements**

438

439 **References:**

440   1   N. Batra, M. Tomar, V. Gupta, *Biosens. Bioelectron.*, 2015, **67**, 263–271.

441   2   J. Motonaka, L. R. Faulkner, *Anal. Chem.*, 1993, **65**, 3258–3261.

442   3   A. Mondal, N. R. Jana, *Chem. Commun.*, 2012, **48**, 7316–7318.

443   4   S. K. Arya, M. Datta, B. D. Malhotra, *Biosens. Bioelectron.*, 2008, **23**,

444   1083–1100.

445   5   U. Saxena, M. Chakraborty, P. Goswami, *Biosens. Bioelectron.*, 2011, **26**,

446   3037–3043.

447   6   F. Biedermann, V. D. Uzunova, O. A. Scherman, W. M. Nau, A. D. Simone, *J.

448   Am. Chem. Soc.*, 2012, **134**, 15318−15323.

449   7   F. Biedermann, M. Vendruscolo, O. A. Scherman, A. D. Simone, W. M. Nau, *J.

450   Am. Chem. Soc.*, 2013, **135**, 14879−14888.

451   8   G. Ghale, W. M. Nau, *Acc. Chem. Res.*, 2014, **47**, 2150−2159.

452   9   Y. Guo, S. Guo, J. Ren, Y. Zhai, S. Dong, E. Wang, *ACS Nano*, 2010, **4**,

453   4001–4010.

454   10 G. B. Zhu, X. Zhang, P. B. Gai, X. H. Zhang, J. H. Chen, *Nanoscale*, 2012, **4**,

455   5703–5709.

456   11 F. Biedermann, W. M. Nau, *Angew. Chem. Int. Ed.*, 2014, **53**, 5694 –5699.

457   12 C. Li, J. Feng, H. X. Ju, *Analyst*, 2015, **140**, 230–235.

458   13 G. Ghale, V. Ramalingam, A. R. Urbach, W. M. Nau, *J. Am. Chem. Soc.*, 2011,

459   **133**, 7528–7535.

460   14 J. M. Chinai, A. B. Taylor, L. M. Ryno, N. D. Hargreaves, C. A. Morris, P. John

461   Hart, A. R. Urbach, *J. Am. Chem. Soc.*, 2011, **133**, 8810–8813.

462   15 X. Mao, D. Tian, H. Li, *Chem. Commun.*, 2012, **48**, 4851−4853.

463 16 G. B. Zhu, L. Wu, X. Zhang, W. Liu, X. H. Zhang, J. H. Chen, *Chem. Eur. J.*,

464   2013, **19**, 6368 – 6373.

465 17 N. Agnihotri, A. D. Chowdhury, A. De, *Biosens. Bioelectron.*, 2015, **63**,

466   212–217.

467 18 Y. Cheng, P. Jiang, S. Lin, Y. Li, X. Dong, *Sens. Actuators B*, 2014, **193**,

468   838–843.

469 19 L. Zhu, L. Xu, L. Tan, H. Tan, S. Yang, S. Yao, *Talanta*, 2013, **106**, 192–199.

470 20 C. Z. Zhao, L. Wan, L. Jiang, Q. Wang, K. Jiao, *Anal. Biochem.*, 2008, **383**,

471   25–30.

472 21 N. Ruecha, R. Rangkupan, N. Rodthongkum, O. Chailapakul, *Biosens.*

473   *Bioelectron.*, 2014, **52**, 13–19.

474 22 A. Ahmadalinezhad, A. C. Chen, *Biosens. Bioelectron.*, 2011, **26**, 4508–4513.

475 23 A. K. Giri, A. Sinhamahapatra, S. Prakash, J. Chaudhari, V. K. Shahi, A. B.

476   Panda, *J. Mater. Chem. A*, 2013, **1**, 814–822.

477 24 A. Umar, R. Ahmad, S. W. Hwang, S. H. Kim, A. Al-Hajry, Y. B. Hahn,

478   *Electrochim. Acta*, 2014, **135**, 396–403.

479

480 **Figure captions:**

481

482 **Scheme 1.** Illustration of the strategy of the proposed electrochemical sensor based on

483 the competitive host–guest interaction between β-CD and MB (signal

484 probe)/cholesterol (target).

485

486   **Scheme 2.** The chemical structure of cholesterol.

487

488   **Fig. 1.** Lowest energy cholesterol/β-CD docked complex for 1:1 **(A)** and 2:1 **(B)**

489   host–guest stoichiometry (left is the side view, right is the top view). The electrostatic

490   forces **(C**, left is the bottom view, right is the top view; red represents the strongest

491   positively charged, blue represents the strongest negatively charged**)** and hydrophobic

492   forces **(D**, left is the side view, right is the bottom view; brown represents the

493   strongest hydrophobic, blue represents the strongest hydrophilic**)** of cholesterol/β-CD

494   docked complex for 2:1 host–guest stoichiometry.

495

496   **Fig. 2.** UV–vis absorption spectra **(A)**, FTIR spectra **(B)**, and Raman spectra **(C)** of

497   GO and Gra.

498

499   **Fig. 3.** SEM images of Gra/GCE **(A)**, PNAANI/Gra/GCE **(B)**, and

500   β-CD/PNAANI/Gra/GCE **(C)**.

501

502   **Fig. 4. (A)** EIS characterization of GCE, Gra/GCE, β-CD/PNAANI/GCE, and

503   β-CD/PNAANI/Gra/GCE. using 2.0 mM $[Fe(CN)_6]^{3-/4-}$ redox couple (1:1) with 0.1 M

504   KCl as supporting electrolyte. **(B)** DPV responses of the Gra/GCE (a),

505   PNAANI/Gra/GCE (b), β-CD/PNAANI/GCE (c), and β-CD/PNAANI/Gra/GCE (d)

506   in 0.1M pH 7.0 PBS after incubation with 1.0 mM MB. **(C)** DPV response of the

507 β-CD/PNAANI/Gra/GCE in 0.1 M pH 7.0 PBS **(a)**, incubated in 1.0 mM MB solution

508 for 25 min **(b)**, and further incubated in 10 μM cholesterol solution for 30 min then

509 tested in 0.1 M pH 7.0 PBS **(c)**.

510

511 **Fig. 5.** Effects of the cycle number of the CV deposition of PNAANI **(A)** and the

512 electro-oxidation time of β-CD **(B)** on the DPV peak currents of the

513 PNAANI/Gra/GCE in 0.1 M pH 7.0 PBS after incubation in 1.0 mM MB solution.

514 Effects of incubation time on the DPV peak currents of the β-CD/PNAANI/Gra/GCE

515 in 0.1 M pH 7.0 PBS after incubation in 1.0 mM MB solution **(C)** and further

516 incubated in 50 μM cholesterol solution **(D)**.

517

518 **Fig. 6. (A)** DPV curves of the proposed sensing platform under different

519 concentrations of cholesterol. **(B)** Calibration curves for the determination of

520 cholesterol using the proposed sensor. The error bars represent the standard deviations

521 of three parallel tests. **(C)** Interference studies using different species in the developed

522 cholesterol detection method, using DPV and keeping all the parameters constant. The

523 cholesterol concentration is 30 μM against the concentration of all other substances,
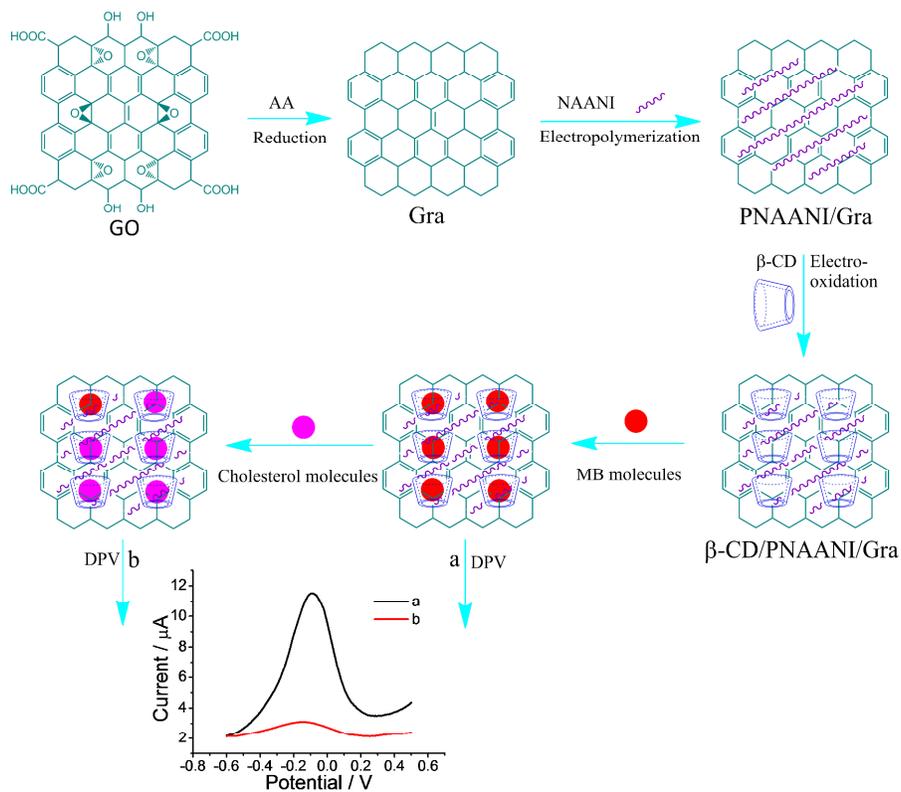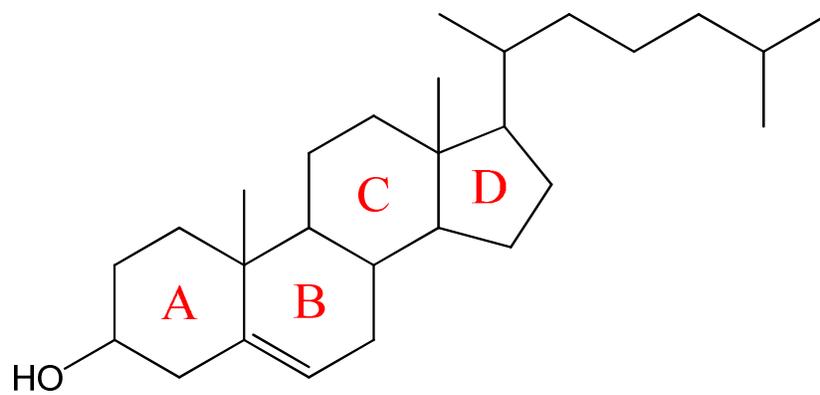
524 which is kept at 2.0 mM.

525

526

527

528

Figures:

GO

Gra

PNAANI/Gra

AA
Reduction

NAANI
Electropolymerization

β-CD | Electro-oxidation

β-CD/PNAANI/Gra

MB molecules

Cholesterol molecules

DPV b

a DPV

Scheme 1.

Yang et al.
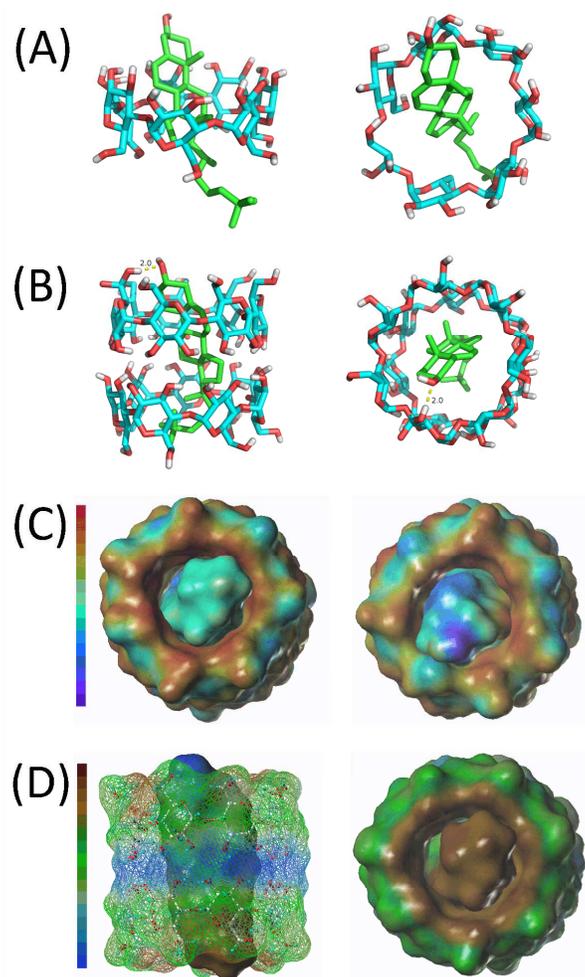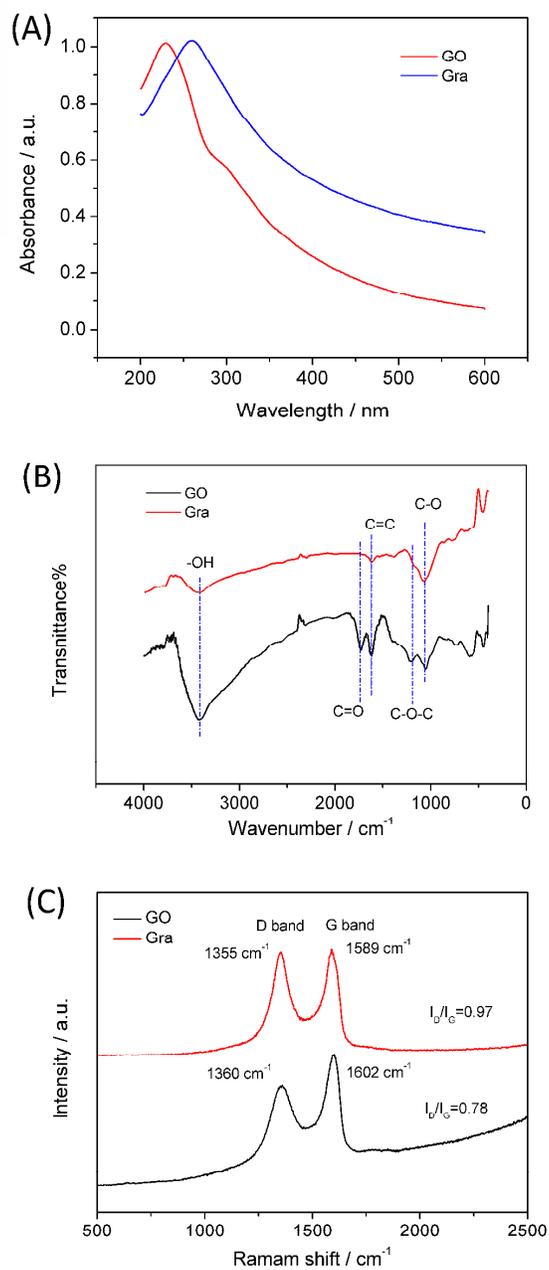
Scheme 2.

Yang et al.

(A)

(B)

(C)

(D)

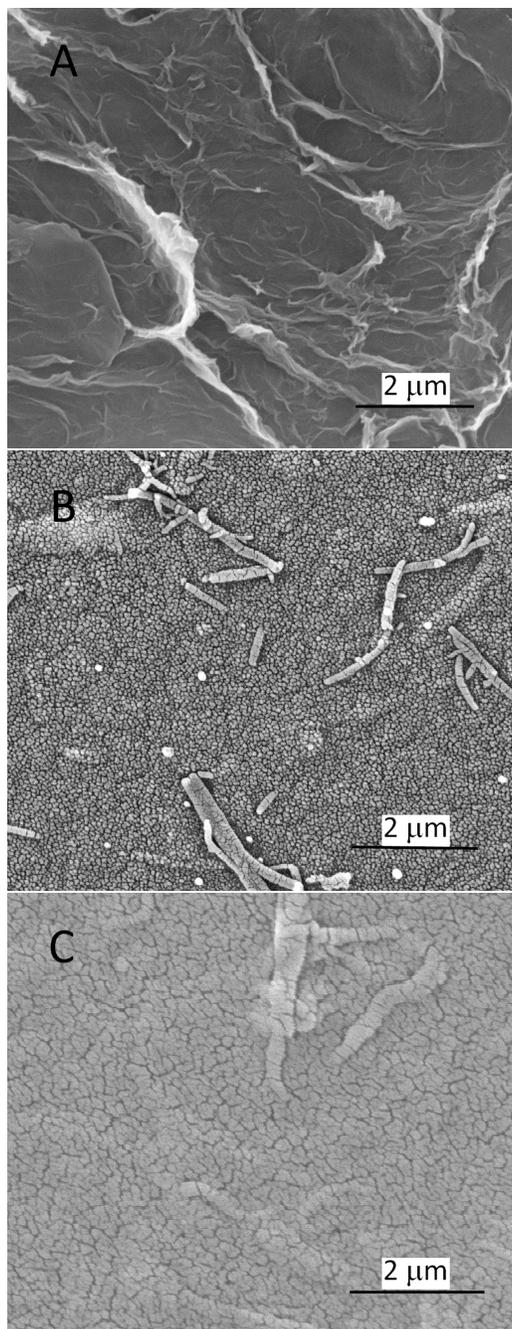**Fig. 1.**

Yang et al.

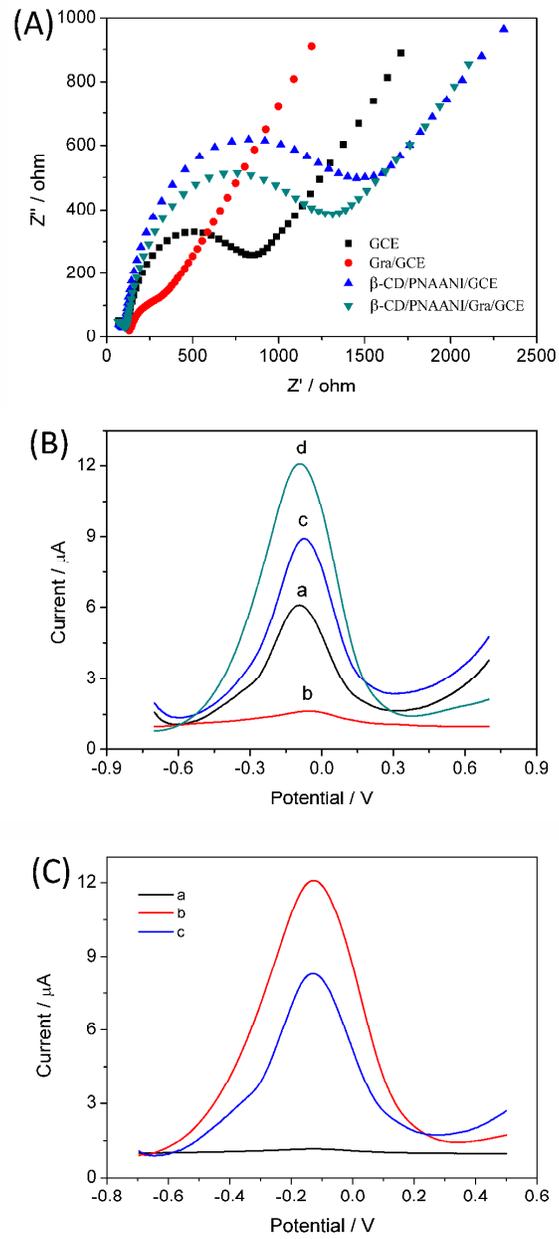Fig. 2.

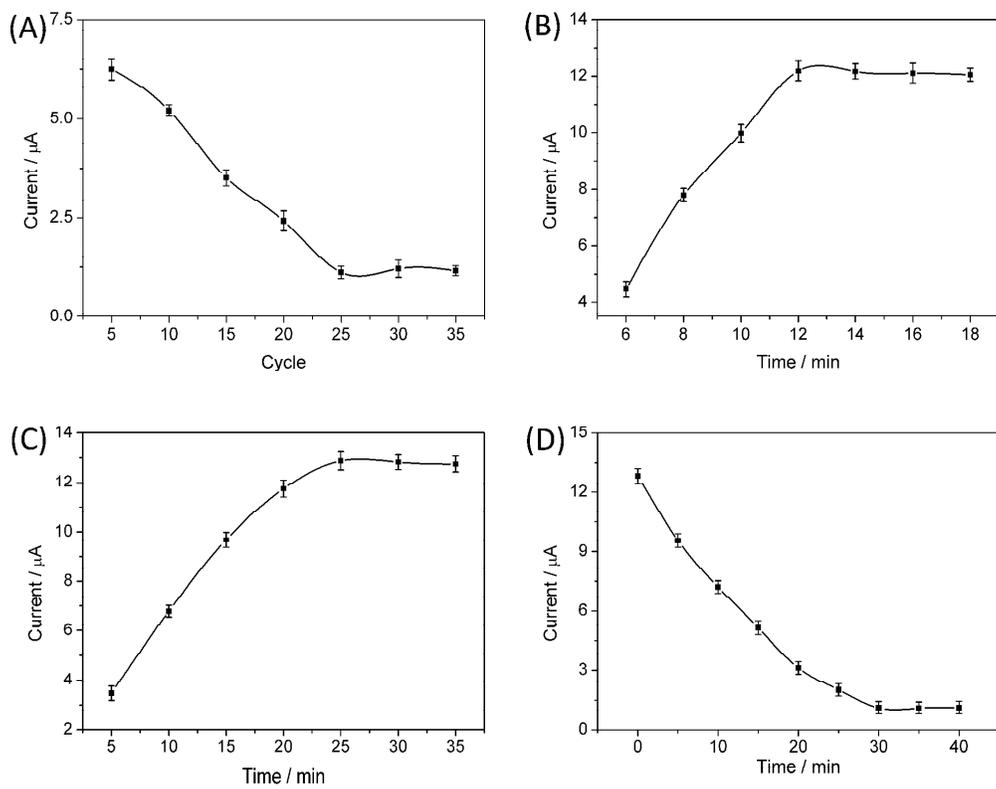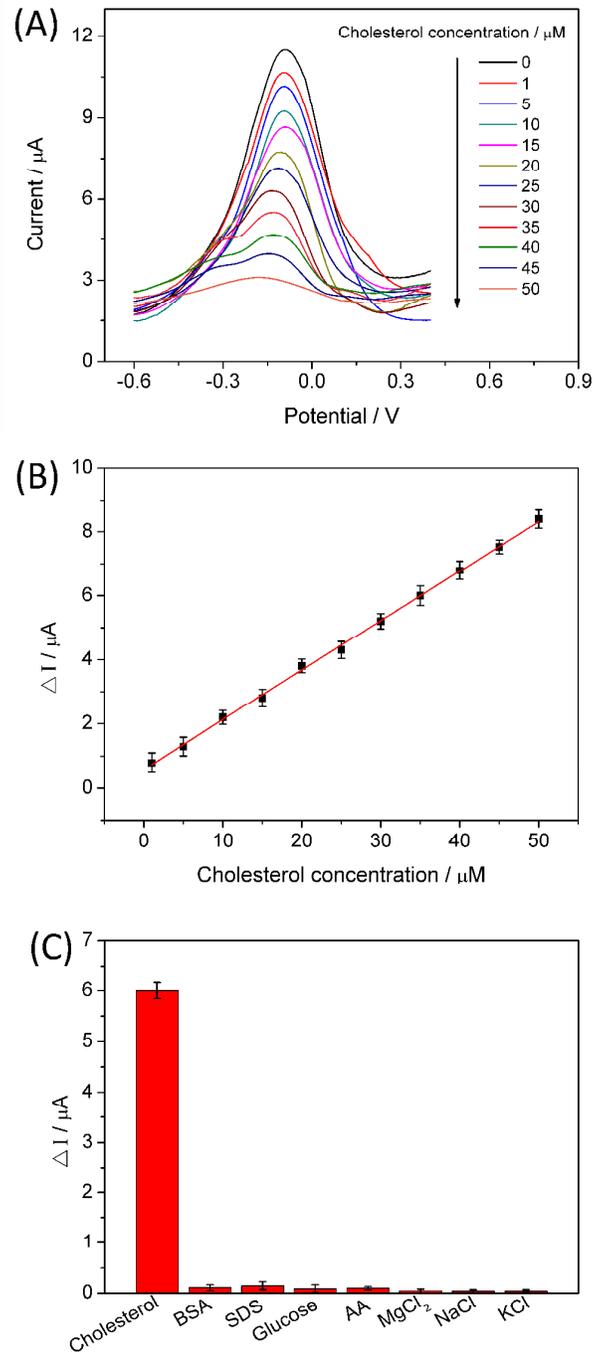Yang et al.

Fig. 3.

Yang et al.

Fig. 4.

Yang et al.

Fig. 5.

Yang et al.

Fig. 6.

Yang et al.

Table 1

The interaction energy between cholesterol and β-CD for 1:1 stoichiometry calculated by PM3 method.

System	Cluster rank	Number in cluster	ΔE (kcal/mol)
Cholesterol/β-CD	1	23	−6.68
	2	7	−6.45
	3	8	−6.45
	4	4	−6.39
	5	2	−6.32
	6	1	−6.32
	7	2	−6.25
	8	2	−6.24
	9	1	−5.71

Table 2

Comparison of the present work with other recent literatures, using various electrode or matrix for cholesterol sensing.

Electrode or Matrix	Method	Liner range (µM)	LOD (µM)	Ref
Nafion/ChOx/GNPs-MWCNTs/GCE	DPV	10.0–5000.0	4.3	19
Chit–Hb/Chit–ChOx	amperometry	10.0–600.0	9.5	20
ChEt–ChOx/ZnO–CuO/ITO/glass	CV	500.0–12000.0	500.0	1
ChOx/PANI/PVP/Graphene	amperometry	50.0–10000.0	1.0	21
ChOx/Nano-ZnO/ITO	CV	130.0-10360.0	13.0	22
ChOx/ZnO(T)/CT/GCE	CV	400.0–4000.0	200.0	23
Nafion/ChOx/−Fe_2O_3/Ag	CV	100.0–8000.0	18.0	24
AuE/dithiol/AuNPs/MUA/ChOx	CV	40.0–220.0	34.6	5
Grp/β-CD/Methylene Blue	DPV	1.0–100.0	1.0	17
Grp/β-CD/Rhodamine 6G	Fluorescence	5.0–30.0	5.0	3
β-CD/PNAANI/Gra/GCE	DPV	1.0–50.0	0.50	This work

Table 3

Determination of cholesterol in human serum samples (*n*=3).

Sample	Added (µM)	Founded (µM)	RSD (%)	Recovery (%)
1	5.0	4.92±0.22	4.47	98.4
2	10.0	10.15±0.35	3.45	101.5
3	20.0	21.03±0.54	2.59	105.2

Tables

Yang et al.

33