Electrochemical features of the interaction between (R)/(S)-methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate and DNAs

Aili Liu1,2*, Xiaomei Dong2, Huile Jin2, Shun Wang2* & Weizhong Jiang1*

1College of Materials Science and Engineering, Donghua University, Shanghai -201 620, China
2College of Chemistry and Materials Engineering, Wenzhou University, Wenzhou -325 035, Zhejiang, China

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Cyclic voltammetry was exploited to understand the interaction between chiral methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate molecules (denoted as (R)- or (S)-5FUPPM) and double-stranded (ds) or G-quadruplex (G4) DNAs. Using Fe(CN)63− as the redox mark, experiments illustrate that the anodic peak of Fe(CN)63− oxidation decreases as the concentration of 5FUPPM in the solution is increased. Calculation with the Langmuir equation yields the binding equilibrium constant of 9.1 × 103 for the dsDNA and (S)-5FUPPM and of 3.3 × 103 for dsDNA and (R)-5FUPPM, suggesting that (S)-5FUPPM has more favorable interaction with dsDNA. On the other hand, the calculated binding constant between G4-DNA and (S)-5FUPPM is 1.6 × 105, as opposed to 3.2 × 104 between G4-DNA and (R)-5FUPPM. The results suggest that the binding selectivity of (R)-5FUPPM with DNAs, defined with (K_{G4-DNA}/K_{dsDNA}), is about 5 times larger than that of (S)-5FUPPM and DNAs, which is consistent with their inhibition rate on tumor cell. The information achieved here suggests that electrochemical characterization can provide useful information for the screening of new drug candidates.

Keywords: Cyclic voltammetry, DNA, Gold electrode, Intercalation, (R)/(S)-5FUPPM

The electrochemical method provides a simple, yet effective approach to characterize properties of various interfaces,1−3 in which interactions between two different substances can be investigated via fixing one of the two reagents onto a substrate that is designated as a working electrode. The selection of the working electrode varies widely, ranging from metal to coated glasses.4−6 This research employed gold (Au) as the working electrode, where DNA molecules, one of the two reagents to be studied, were deposited on Au through self-assembly. The other reagent that interacts with the DNA molecules is G-quadruplex ligands. G-quadruplex ligands have been proposed to be selective anticancer agents by acting as telomerase inhibitors7−9 and/or transcriptional repressors of c-MYC oncogene.10−13 They comprise a stack of G-tetrads, the planar association of four guanines in a cyclic Hoogsteen hydrogen bond.

A drawback that many G-quadruplex ligands are facing in drug application is their non-specific cytotoxicity, which is believed to arise from their interactions with duplex DNAs.14-16 Ideally, G-quadruplex ligands bind exclusively to their target G-quadruplex. Therefore, understanding the binding selectivity is essential to the development of G-quadruplex ligands for their therapeutic applications. Among G-quadruplex ligands known, 5-Fluorouracil (5-FU) has been increasingly employed alone or in combination with other cytotoxic drugs and hormones in the treatment of various types of tumors such as breast, colorectal and gastric cancers.17,18 In order to improve their selectivity and reduce the incidence of toxicity such as in the bone marrow, gastrointestinal tract, central nervous systems, etc., many derivatives of 5-FU have been developed.20,21 In this research cyclic voltammetry was employed to understand interactions between G-quadruplex and duplex DNAs and aminophenol derivatives of (R)/(S)-methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate (denoted as (R)- or (S)-5FUPPM) that were synthesized in our lab. The results illustrate that the binding selectivity of (R)-5FUPPM with DNAs is about 5 times larger than that of (S)-5FUPPM.

Experimental

Instruments

Cyclic voltammetry (CV) measurements were carried out with AUTOLAB PGSTAT30 electrochemical workstation (Metrohm AG) controlled by a personal
A conventional three-electrode system was employed in the measurements using a bare or DNA coated gold electrode (d=2 mm) as the working electrode, a saturated Ag/AgCl electrode as the reference electrode and a Pt plate as the counter electrode. Studies were performed at ambient temperature (22±2 °C). All potentials reported in the following were referred to the Ag/AgCl electrode. Unless otherwise stated, electrolyte solutions were thoroughly degassed with N2 and kept under an N2 blanket during the experiments. Melting points of the (R)- or (S)-5FUPPM were recorded on a Digital Melting Point ApparatusWRS-1B and are uncorrected. Thin layer chromatography (TLC) was performed using precoated silica gel 60 GF254 and column chromatography was performed using silica gel (300 – 400 mesh). IR spectra were taken on an EQUINOX-55 instrument. 1H NMR and 13C NMR spectra were recorded on a AVANCE-300 instrument using tetramethysilane (TMS) as an internal standard and DMSO-d6 as the solvent.

**Chemicals**

Double-stranded DNA (dsDNA) and G4-quadruplex DNA (G4-DNA) were purchased from Shanghai Chemical Reagents Company (China); their base sequences are as follows:

dsDNA: 5'-SH-(CH2)6-CGCGAATTCCGCG-3'
G4-DNA: 5'-SH-(CH2)6-(TTAGGG)4-3'

All DNA stock solutions (100 mM) were prepared with Tris–HCl buffer solution (pH 6.8) and were kept frozen before its usage. In a typical experiment, 5-fluorouracil derivatives and DNAs were dissolved in 5.0 mM Tris–HCl buffer solution (pH 6.8) that also contained 5.0 mM NaCl as the supporting electrolyte. All other chemicals and solvents were purchased from Aldrich and Fluka. Deionized water (18.22 MΩ-cm) was used for the preparation of all solutions.

**Synthesis of (R)-(S)-5FUPPM**

The starting material, 5-fluorouracil-1-acetic acid (5-fluoro-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-1-acetic acid) was prepared from 5-FU and bromoacetic acid, according to a method published earlier. Then 3.76 g of 5-fluorouracil-1-acetic acid was mixed with 3.24 g of 1-hydroxybenzotriazole (BtOH) in 60 mL DMF solution. After adjusting the temperature to 0°C, 30 mL of DMF solution that contained 6.18 g of dicyclohexyl carbodiimide (DCC) was added slowly. Afterward, the reaction continued at room temperature for 6 h, and then 4.31 g of L-Phenylalanine methyl ester hydrochloride (L-MBS) and 2.8 mL of triethylamine (TEN) were added to the above solution. After stirring for 5 h, a white solid was obtained. After filtration, the filtrate was concentrated under reduced pressure and the residue was separated by column chromatography to collect (R)-FUPPM. The yield of the product (R)-FUPPM is about 55% and the melting temperature is found around 191°C.

1H NMR (DMSO-d6, 300MHz) δ: 11.84 (s, 1H), 8.73 (d, J=7.5 Hz, 1H), 7.94 (d,J=6.9 Hz, 1H), 7.20~7.32 (m, 5H), 4.47~4.52 (m,1H), 4.31 (s, 2H), 3.59 (s, 3H), 2.89~3.06 (m, 2H); 13C NMR (DMSO-d6, 300 MHz) δ: 171.69, 166.87, 157.74 (d, J = 25.6 Hz), 149.72, 139.32 (d, J = 226.9 Hz), 136.99, 131.10 (d, J = 33.6 Hz), 129.26, 128.43, 126.77, 53.95 (NCH), 52.03 (OCH3), 49.39 (NCH3), 36.93; IR (KBr) ν: 3423(N-H), 3072 (ArH), 1706 (C=O), 1234 (C=O−C), 700 cm−1.[α]D10 = 12.7 (c 1.0, DMF).

The preparation of (S)-FUPPM is similar to the procedure of (R)-FUPPM synthesis, except that L-MBS was replaced by D-Phenylalanine methyl ester hydrochloride (D-MBS). The characterization of the white solid yielded a melting temperature of about 191°C. 1H NMR (DMSO-d6, 300 MHz) δ: 11.83 00 (s, 1H), 8.72 (d, J = 7.6 Hz, 1H), 7.93 (d, J = 6.8 Hz, 1H), 7.19~7.30 (m, 5H), 4.30~4.50 (m, 1H), 4.30 (s, 2H), 3.59 (s, 3H), 2.93~3.00 (m, 2H); 13C NMR (DMSO-d6, 300 MHz) δ: 171.67 (C−O), 166.86 (C−O), 157.63 (d, J = 25.6 Hz), 149.71, 139.32 (d, J = 226.9 Hz), 136.98, 131.08 (d, J = 33.6 Hz), 129.25, 128.42, 126.76, 53.94 (NCH), 52.01 (OCH3), 49.38 (NCH3), 36.93; IR (KBr) ν: 3307 (N−H), 3072 (ArH), 1709 (C=O), 1669 (C=C), 1551, 1447 (−CH=−), 1380 (C=−H of CH3), 1242 (C−O−C), 700 cm−1.[α]D10 = -12.7 (c 1.0, DMF).

Molecular structures of (R)- and (S)-5FUPPM are shown in scheme 1.

**Scheme 1 — The chemical structure of (R)- and (S)-5FUPPM**
Preparation of the working electrode

Au electrodes were first polished carefully with 1.0, 0.3 and 0.05 µm alumina slurry and then cleaned ultrasonically in acetone, ethanol, and water, respectively, for 10 min. The freshly cleaned electrodes were scanned over the potential range of 0.0 to +1.5 V (vs. Ag/AgCl) in 0.5 M H₂SO₄ until a constant voltammogram was obtained. Afterward, they were polarized at 0 V for 3 min. Finally, the Au electrodes were rinsed with deionized water and were modified immediately by transferring 20 µL of 0.5 mM DNA solution onto its surface. The Au electrode was then left overnight to let DNA solution air-drying. The DNA-covered Au electrodes were then soaked in sterile water for 4.0 h before being rinsed with deionized water to remove those loosely adsorbed DNAs. The thus-obtained DNA-covered Au electrodes are denoted as Au/DNA in the following context. The effective surface area of the unmodified Au electrode was estimated from cyclic voltammograms by integrating the cathodic peak of the reduction of the Au oxide layer in 0.5 M H₂SO₄.

Results and Discussion

Figure 1 shows CVs of 5.0 mM Fe(CN)₆³⁻ at (a) a bare Au and (b, c) Au/DNA electrodes, respectively. There is a pair of well-defined redox peaks at the bare Au electrode (curve a) with the peak-to-peak separation (ΔEₚ) of 94 mV. After the DNA coating, there was an obvious decrease in the peak amplitudes (curves b and c), indicating that the DNA film has acted as an inert blocking layer to reduce the total active sites of the Au electrode and to hinder the diffusion of ferricyanide toward the electrode. CVs of the same DNA electrode remained stable after 20 scans in the Tris-HCl buffer solution, suggesting the stability of the DNA-coated electrode.

Figure 2 presents CVs of bare Au electrode in a solution of (a) 0.43 mM (R)-5FUPPM in Tris-HCl buffer solution, (b) 0.43 mM (S)-5FUPPM in Tris-HCl buffer solution, and (c) 5.0 mM K₃Fe(CN)₆. The supporting electrolyte was 5.0 mM KCl. Notably, no redox peak was attained with (R)-5FUPPM or (S)-5FUPPM, suggesting that those two substances do not undergo electrochemical reaction within the parameter ranges investigated in this research. To investigate the interaction of (R)-5FUPPM with dsDNA, G4-DNA, in (Fig. 3A) the dsDNA-covered Au electrodes were scanned in a buffer solution containing 5.0 mM Fe(CN)₆³⁻ probe molecule, in which different amounts of (R)-5FUPPM were added into the test solution. These experiments showed that the peak current of the probe molecule decreased as (R)-5FUPPM concentration was increased. Please note that results in (Fig. 2) have confirmed that (R)-5FUPPM in the electrolyte solution does not undergo any electrochemical reaction. Therefore, the reduced electroactivity of Fe(CN)₆³⁻/Fe(CN)₆⁴⁻ shall be attributed to the changes of surface property, which clearly depend on (R)-5FUPPM concentration. A similar scenario was seen in (Fig. 3B), where the working electrode was G4-DNA modified Au electrode.

Figure 4 presents CVs of 5.0 mM Fe(CN)₆³⁻ probe molecule in the presence of different amounts of interactions of (S)-5FUPPM with dsDNA, and
G4-DNA were tested in (Fig. 4), where 5.0 mM Fe(CN)$_6^{3-}$ was used as the redox probe molecule while the concentration of (S)-5FUPPM was adjusted. Similar to the scenario seen in (Fig. 3), the more (S)-5FUPPM was added into the test solution, the stronger the peak current was reduced. This suggests that the interaction between (S)-5FUPPM and the DNA molecules alters the DNA film, making Fe(CN)$_6^{3-}$ harder to penetrate through it. In both (Figs. 3 & 4) the anodic peak potential shifted toward a negative direction as the result of increasing 5FUPPM concentration. Similar behavior has been suggested arising from electrostatic interaction.$^{27}$

The above CVs showed that the higher the amounts of (R)- or (S)-5FUPPM added, the lower the anodic peak current became. When the concentration of (R)- or (S)-5FUPPM reached 4.3 mM, the peak current no longer decreased (i.e. Δ$I$ reach maximum). Following the Langmuir equation$^{28}$

\[
\frac{1}{\Delta I_p} = \frac{1}{\Delta I_{p,\text{max}}} + \frac{1}{\Delta I_{p,\text{max}} k c}
\]

where $k$ is the equilibrium constant of the interaction between (R)/(S)-5FUPPM and the DNA film, $c$ is the concentration of (R)/(S)-5FUPPM, $\Delta I_p$ is the current drop and $\Delta I_{p,\text{max}}$ stands for the maximum current drop(Fig. 5) plots $1/\Delta I_p$ as a function of $1/c$. A good linear relationship is achieved between the reciprocal of the current drop and that of the (R)/(S)-5FUPPM concentration. The binding equilibrium constant $k$ is obtained from the slope of the plot in (Fig. 5). Table 1 lists the binding constants of (R)/(S)-5FUPPM with dsDNA and G4-DNA. The selectivity of (R)/(S)-5FUPPM was defined as the ratio of the $k_{\text{G4-DNA}}/k_{\text{dsDNA}}$ and are calculated accordingly. These data clearly demonstrate that molecular structure has a significant influence on their interactions with DNAs, in which the selectivity of (R)-5FUPPM is 5 times larger than that of the (S)-5FUPPM.
4. Conclusion

This study investigated interactions of (R)/(S)-5FUPPM with dsDNA and G4-DNA with cyclic voltammetry technique. A potential concern is whether these molecules can independently interact with the redox marker. Given that (R)/(S)-methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate and redox marker are both in the solution phase and the concentration of redox marker is more than 10 times higher than (R)/(S)-methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate, if there is a strong interaction between these two reagents, there should not be such a significant change in the CVs. Therefore, we believe that there is either no or rather weak interactions between (R)-(S)-methyl-2-(5-fluorouracil-1-acetamide)-3-phenylpropionate and the redox marker.

It is known that the drug activity, such as cancer inhibition rate, is related to their interactions with G4-DNA, whereas the toxicity arises from their interaction with normal dsDNA. Our experiments show that while both (R)/(S)-5FUPPM interact with G4-DNA and dsDNA strongly, (R)-5FUPPM exhibits a larger binding constant with G4-DNA. We would like to point out that (R)/(S)-5FUPPM have recently been investigated for their anticancer activities against human Bel-7402 cell lines. The antitumor activities in vitro for these compounds were evaluated by the sulforhodamine B (SRB) method for BEL-7402 cells. The results of these assays show that (R)-5FUPPM exhibits a moderate to the good inhibiting effect on the growth of the BEL-7402 cells at different concentrations of $10^{-4}$ to $10^{-8}$ M, whereas (S)-5FUPPM exhibits inhibition only at high concentration. Such a trend is the same as the binding constants of (R)/(S)-5FUPPM with G4-DNA seen in this research. This study demonstrates that the electrochemical method is a sensitive technique to reflect and differentiate interactions between DNAs film and a chemical reagent. More systematic and thorough research is needed to validate such a correlation for screening potential drug candidates.

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Reference

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