Experimental and theoretical study of DNA oxidation

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The electrochemical oxidation of DNA and bases at a glassy carbon electrode were investigated using cyclic and square-wave voltammetry analysis. The predicted oxidative potential for DNA and bases using DFT method (B3LYP) is in agreement with the experimental ones.The DNA oxidation ascribes to N-protonated guanine and adenine. The calculated oxidative peaks indicate that the initial potential of DNA oxidation is around 0.432V for N-protonated guanine and 0.368V (vs.hydrogen standard electrode) at pH 7.00, and DNA as well as bases in acidity solution are more stable than those in alkalescent solution.

DNA damage caused by reactive oxygen species includes a large variety of lesions spanning from base and sugar damage to DNA breaks and DNA-protein cross-links, and is associated with cancer. More than 60 different base lesions have been identified, DNA oxidation is a dangerous factor leading to cancer[1]. Why does DNA oxidize easily? And how many is the initial potential oxidating DNA?

DNA oxidation is commonly investigated using electrochemistry. Electrochemists have done a lot of work on the electrochemical reactions of nucleic acids, and among

the nucleic acid constituents only bases were shown to undergo reduction and/or oxidation at electrode surfaces[2–7].The electrochemical mechanisms of guanine and adenine oxidation in solution have been also thoroughly investigated, however, there have been few mechanistic studies of its oxidation at electrodes [8–10], and it is difficult to study the oxidation process of bases using eletrochemistry.

Quantum chemistry is a basic and reliable theory to study chemistry, so the reaction mechanisms of DNA and bases at a electrode are discussed using eletrochemistry and quantum chemistry here.

Suppose $O_1 + nH^+ + ne^- = R_1$ is an irreversible redox couple.According to Laviron's equation [11-13].

$$Ep_1 = E_1^{o'} + \frac{RT}{\alpha_1 nF}\ln\frac{RTk_{s1}}{\alpha_1 nF}\frac{RT}{\alpha_1 nF}\ln v$$

Where a_1 is the transfer cofficient, Ep_1 the peak potential, ks_1 the standard rate constant of the surface reaction, v the scan rate and $E_1^{o'}$the formal potential.When the peak of potential for $O_1,nH^+/R_1$ at a electrode appers, $\frac{di}{dt} = 0$, $[O_1]=[R_1]$, Nernst's equation is

$$\begin{aligned} Ep_1 &= E_1^{o'} + \frac{RT}{F}\ln\frac{[H^+][O_1]}{[R_1]} \\ &= E_1^{o'} + \frac{RT}{F}\ln[H^+] \\ &= E_1^{o} + K \end{aligned}$$

Where E_1^o is the standard potentional, F the Frarday constant (96.485C.mol^{-1}), i the current, t the time and K the scaled factor. K of any redox couple at same condition is a constant. Thus

$$E_1^{o} = Ep_1 - K$$

If another irreversible redox couple as a reference electrode is $O_2 + nH^+ + ne^- = R_2$, the standard potentional $E_2{}^o$, $E_2{}^o = Ep_2 - K$, is also obtained.

The batteriy reaction of two half reactions in gas state is written as:

$$O_1 + R_2 = O_2 + R_1 \qquad (1)$$

The transformed Gibbs free energy ($\Delta rG_1{}^o$) of reaction (1) is calculated as

$$\Delta rG_1{}^o\,(298.15K) = \sum G^o{}_{product} - \sum G^o{}_{reactant}$$

The transformed Gibbs free energy (ΔrG^o) of reaction in solution is calculated

$$\Delta rG^o\,(298.15K) = \sum (G^o + G^o aq)_{product} - \sum (G^o + G^o aq)_{reactant}$$

$$= \Delta rG^o{}_1 + \Delta rG^o{}_{aq}$$

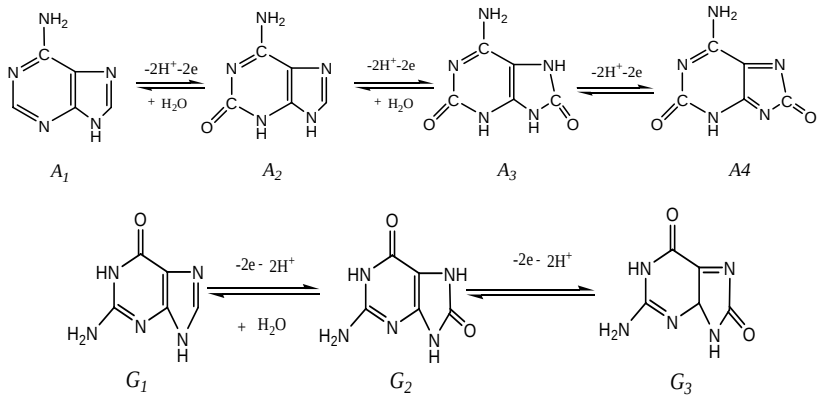
where G^o represents standard Gibbs free energies of product and reactant in gas state at 298.15K and 1atm, and $G^o{}_{aq}$is energies of solvation in water at 298.15K and 1atm.

Thus the predicted peak potential (Ep_2) is calculated as

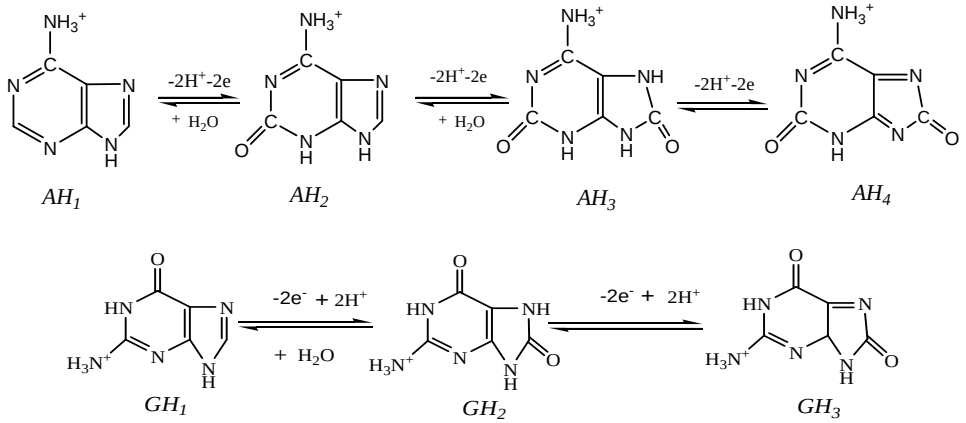
$$\Delta rG^o(298.15K,1atm) = -nF(E_2{}^o - E_1{}^o)$$

$$= -nF(Ep_2 - E_{P1})$$

The mechanism for guanine and adenine are [14-15]:



The reaction mechanisms of N-protonated guanine and adenine are also designed as



AH_1 AH_2 AH_3 AH_4

GH_1 GH_2 GH_3

Here p-Hydrogenquione(*p-BQ*)/phenol(*Phen*) as a referrence electrode is

$$p\text{-}BQ_{(g)}+4H^{+}{}_{(g)}+4e^{-}====Phen_{(g)}+H_2O_{(g)}$$

The batteries reactions in gas state is written as:

$$G_3,4H^{+}/G_1:G_{3(g)}+Phen_{(g)}====p\text{-}BQ(g)+G_1(g)$$

$$G_3,2H^{+}/G_2:2G_{3(g)}+Phen_{(g)}+H_2O_{(g)}\ ===2G_{2(g)}+p\text{-}BQ_{(g)}$$

$$G2,2H^{+}/G1:2G2_{(g)}+Phen_{(g)}===2G1_{(g)}+BQ_{(g)}+H_2O_{(g)}$$

$$A_4,2H^{+}/A_3:2A4_{(g)}+H_2O_{(g)}+Phen_{(g)}===\ 2A_{3(g)}+p\text{-}BQ_{(g)}$$

$$A_3,2H^{+}/A_2:2A_{3(g)}+Phen_{(g)}===\ 2A_{2(g)}+p\text{-}BQ_{(g)}+\ H_2O_{(g)}$$

$$A_3,4H^{+}/A_1:A3_{(g)}+Phen_{(g)}===\ A_{1(g)}+\ H_2O_{(g)}\ +p\text{-}BQ_{(g)}$$

$$A_2,2H^{+}/A_1:2A_{2(g)}+Phen_{(g)}===\ 2A_{1(g)}+\ H_2O_{(g)}+p\text{-}BQ_{(g)}$$

$$A_4,6H^{+}/A_1:2A_{4(g)}+3Phen_{(g)}===\ 2A_{1(g)}+3p\text{-}BQ_{(g)}+\ H_2O_{(g)}$$

$$A_4,4H^{+}/A_2:A_{4(g)}+Phen_{(g)}===\ A_{2(g)}+p\text{-}BQ_{(g)}$$

Where $A_{1(g)}$, $A_{2(g)}$, $A_{3(g)}$,$A_{4(g,}$ $G_{1(g)}$, $G_{2(g)}$, $G_{3(g)}$, $p\text{-}BQ_{(g)}$, $Phen_{(g)}$ and $p\text{-}BQ_{(g)}$ represent A_1, A_2, A_3 ,$A_{4,}$ G_1, G_2, G_3,*p-BQ*, *Phen* and *p-BQ* in gas state, respectively.

The batteries reactions in gas state of N-protonated guanine and adenine is also calculated as above method.

The CV curves of phenol are shown in Figure1, the peaks, at + 0.566, 0.681 and

0.959V, corresponds to phenol oxidation in pH 9.00, 7.00 and 3.00 phosphate buffer solution, respectively.The peak of phenol oxidation shifts to positive direction with pH decrease.

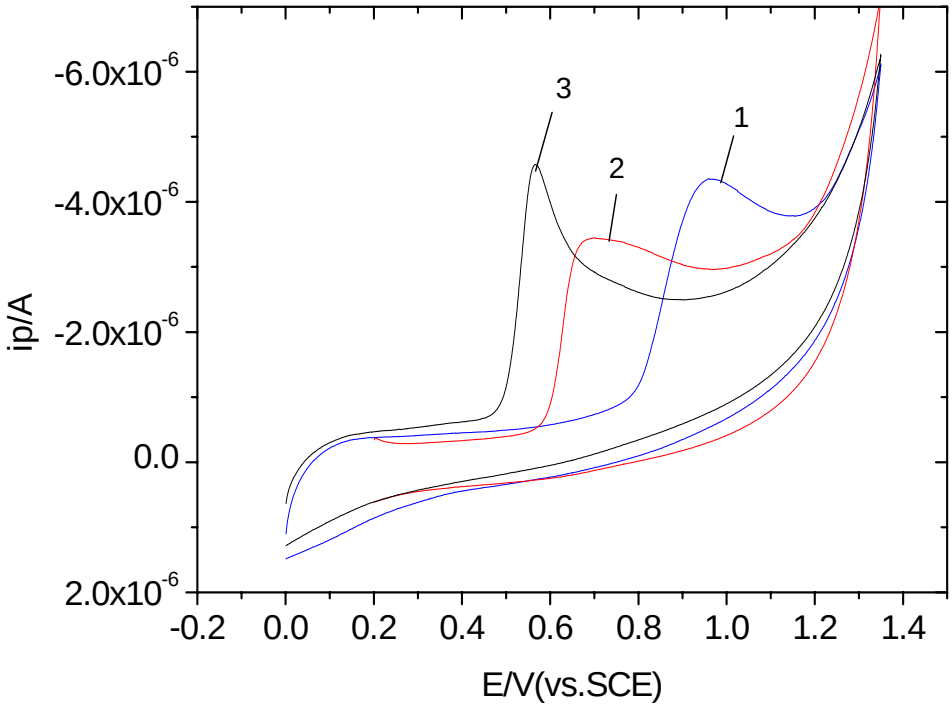


Figure 1 | CV curve of phenol at pH 9.00,7.00 and 3.00. 1, 1 mmol.L^{-1} phenol in pH 3.00 phosphate buffer solution; 2, 0.5 mmol.L^{-1} phenol in pH 7.00 phosphate buffer solution;3, 1 mmol.L^{-1} phenol in pH 9.00 phosphate buffer solution.

The peaks of adenine and guanine in the cyclic voltammetry (CV) curves are weak, only the Square-wave voltammetry (SWV) curves are obtained. The SWV curves of adenine are shown in Figure 2(a), the peak, at +1.066 and 1.332, corresponds to adenine oxidation in pH 9.00 phosphate buffer solution, and the peaks, 1.142 and 1.304V, corresponds to adenine oxidation in pH 7.00 and 3.00 phosphate buffer solution, respectively.The oxidative peak for adenine shifts to the negative direaction with pH values increase.

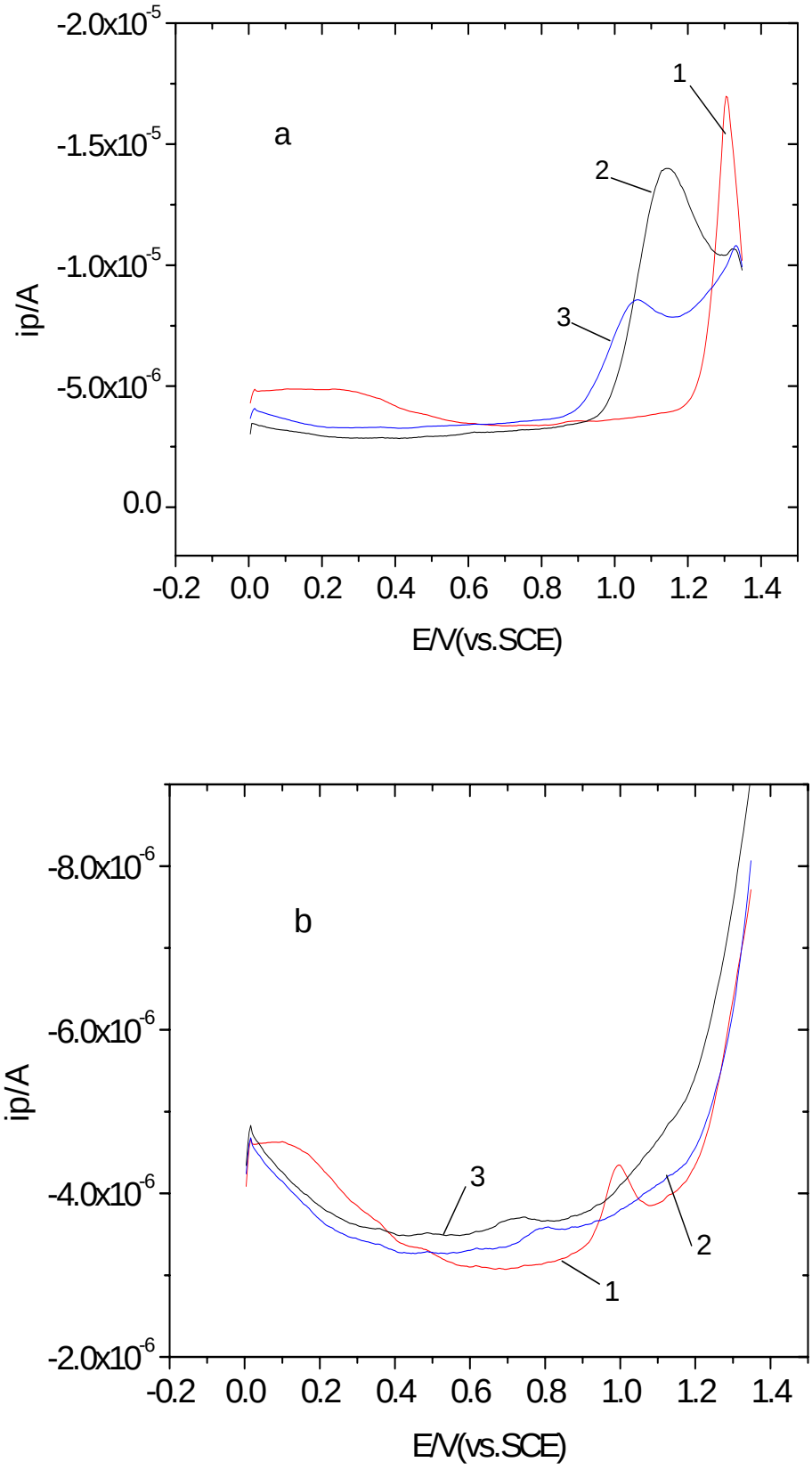
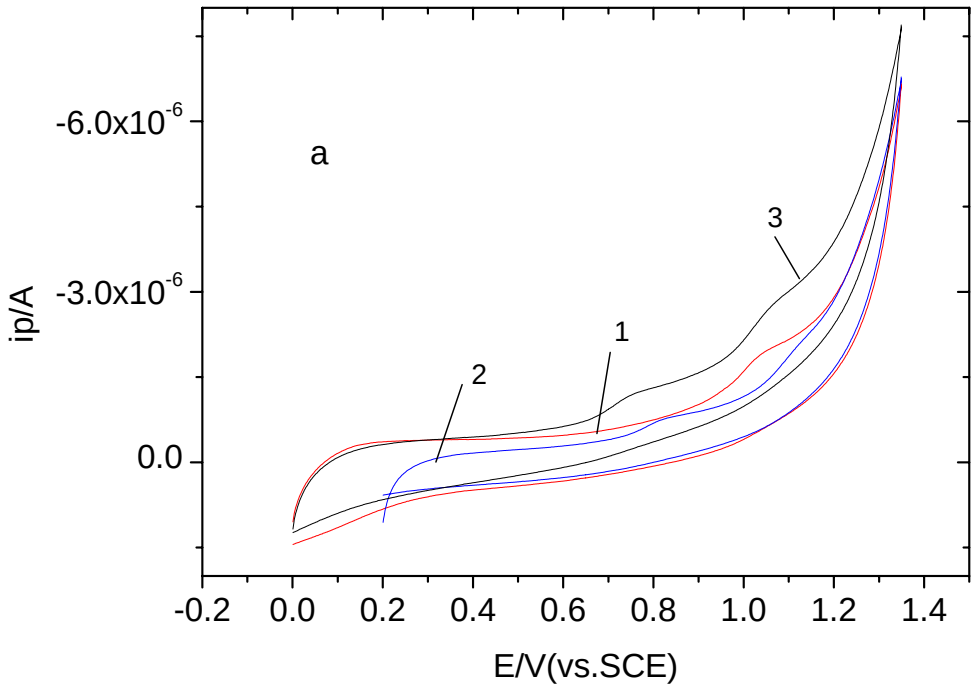


Figure 2 | SWV curve of 2 mmol.L^{-1} guanine and adenine at pH 9.00,7.00 and 3.00. **a**:1, pH 3.00; 2, pH 7.00; 3,

pH 9.00.**b**:1, pH 3.00; 2, pH 7.00;3, pH 9.00.

The SWV curves of guanine are shown in Figure 2(b), the peak, at +0.718, 0.796 and 0.996V, corresponds to guanine oxidation in pH 9.00, 7.00 and 3.00 phosphate buffer solution, respectively. The oxidative peak for guanine also shifts to the negative direaction with pH increase.

The CV curves of DNA are shown in Figure 3(a), the peaks, at +0.748 and 1.064, corresponds to DNA oxidation at pH 9.00, and ascribe to the oxidation of guanine and adenine, respectively. The peaks, at 0.818 and 1.115V corresponds to DNA oxidation at pH 7.00; and the peak ,at 1.039V, corresponds to guanine oxidation at pH 3.00. These results is inagreement with the previous work[16-18]. The oxidative peak for the adenine of DNA in pH 3.00 phosphate buffer solution is not found due to high background current.



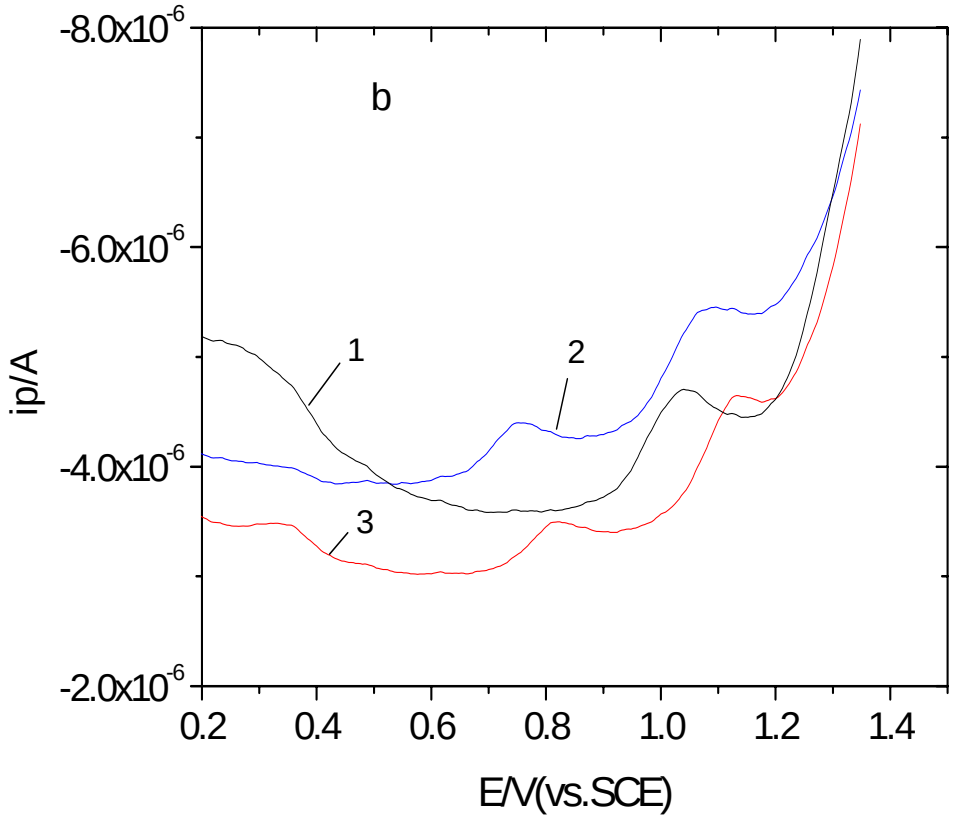


Figure 3 | CV and SWV curve of 120.0mg.L^{-1} DNA at pH 9.00,7.00 and 3.00. a:1,CV curve of DNA in pH 3.00 phosphate buffer solution; 2, CV curve of DNA in pH 7.00 phosphate buffer solution;3, CV curve of DNA in pH 9.00 phosphate buffer solution.b: a:1,SWV curve of DNA in pH 3.00 phosphate buffer solution; 2, SWV curve of DNA in pH 7.00 phosphate buffer solution;3, SWV curve of DNA in pH 9.00 phosphate buffer solution.

The SWV curves of DNA are shown in Figure 3(b), the peaks, at +0.750 and 1.082, corresponds to DNA oxidation at pH 9.00, and the peaks, at 0.817 and 1.133V, corresponds to DNA oxidation at pH 7.00, and the peak at 1.040V corresponds to guanine oxidation in DNA at pH 3.00. The oxidative peak for DNA in SWV curves is consistent with those in CV.

The Gibbs free energies in gas state (G^o), the energies of solvation (G^o_{aq}), the transformed Gibbs free energy (ΔrG_1^o) in gas state and the transformed Gibbs free

energy (ΔrG^o) in water are shown in Table1. Under the same experimental condition the predicted oxidation potentials vs.SCE at B3LYP/6-311+G(g,p) level close to those at B3LYP/6-31G(d) and B3LYP/6-31G(d,p) level.

Table 1 Gibbs free energies in gas state (G^o), energies of solvation (G^o_{aq}), the transformed Gibbs free energy (ΔrG_1^o) in gas state, the transformed Gibbs free energy in water(ΔrG^o),calculated peak potential(Cal.) and experimental peak potential (Exp.)

		G^o	G^o_{aq}	$\triangle rG_1^o$	$\triangle G_{aq}^o$	$\triangle rG^o$		Cal.			Exp.	
	Basis set	(Hartree)	($KJ.mol^{-1}$)	($KJ.mol^{-1}$)	($KJ.mol^{-1}$)	($KJ.mol^{-1}$)		(V)			(V)	
pH							3.00	7.00	9.00	3.00	7.00	9.00
G_1	6-31G(d)	-542.465974	-123.3518									
	6-31G(d,p)	-542.481206	-124.8984									
	6-311G+(d,p)	-542.629613	-144.5862									
G_2	6-31G(d)	-617.711322	-122.1396									
	6-31G(d,p)	-617.728521	-123.8954									
	6-311G+(d,p)	-617.904991	-145.2550									
G_3	6-31G(d)	-616.462058	-161.5988									
	6-31G(d,p)	-616.473044	-162.6020									
	6-311G+(d,p)	-616.644466	-186.8460									
GH_1	6-31G(d)	-542.774557	-412.7332									
	6-31G(d,p)	-542.791924	-415.1576									
GH_2	6-31G(d)	-618.014284	-428.6172									
	6-31G(d,p)	-618.033523	-431.3760									
GH_3	6-31G(d)	-616.737670	-462.6842									
	6-31G(d,p)	-616.750010	-462.9768									
phen	6-31G(d)	-307.389033	-27.0028									
	6-31G(d,p)	-307.402641	-26.9192									
	6-311G+(d,p)	-307.483538	-33.2310									
p-BQ	6-31G(d)	-381.396835	-16.9708									
	6-31G(d,p)	-381.403080	-17.5560									
	6-311G+(d,p)	-381.507836	-27.0864									
H_2O	6-31G(d)	-76.405457	-26.8774									
	6-31G(d,p)	-76.416683	-25.7488									
	6-311G+(d,p)	-76.454820	-32.3950									
A_1	6-31G(d)	-467.237418	-61.9894									
	6-31G(d,p)	-467.250947	-62.9090									
	6-311G+(d,p)	-467.372816	-71.2690									
A_2	6-31G(d)	-542.460531	-134.9304									

	6-31G(d,p)	-542.475590	-135.9754						
	6-311G+(d,p)	-542.623901	-158.8818						
A_3	6-31G(d)	-617.694471	-162.5602						
	6-31G(d,p)	-617.711718	-164.5666						
	6-311G+(d,p)	-617.887777	-196.1256						
A_4	6-31G(d)	-616.474650	-97.7702						
	6-31G(d,p)	-616.484753	- 98.6480						
	6-311G+(d,p)	-616.655788	-116.9564						
AH_1	6-31G(d)	-467.567362	-290.1338						
	6-31G(d,p)	-467.582871	-291.8476						
AH_2	6-31G(d)	-542.786442	-359.1874						
	6-31G(d,p)	-542.804273	-361.2356						
AH_3	6-31G(d)	-618.012403	-432.2538						
	6-31G(d,p)	-618.031536	-432.1798						
AH4	6-31G(d)	-616.756533	-391.8750						
	6-31G(d,p)	-616.768975	-393.8396						
G_3/G_1	6-31G(d)			-30.7656	48.2790	17.6134	0.9136	0.635	0.520
	6-31G(d,p)			-22.5819	47.0668	24.4849	0.896	0.618	0.503
	6-311G+(d,p)			-24.7978	48.4044	23.6066	0.898	0.620	0.505
G_3/G_2	6-31G(d)			-264.8421	115.8278	-149.0143	1.345	1.067	0.952
	6-31G(d,p)			-248.6611	112.5252	-136.1359	1.312	1.034	0.919
	6-311G+(d,p)			-237.6813	121.7216	-115.9597	1.259	0.981	0.866
G_2/G_1	6-31G(d)			203.3108	-19.2698	184.0410	0.482	0.204	0.089
	6-31G(d,p)			203.4973	-18.3916	185.1057	0.479	0.201	0.086
	6-311G+(d,p)			188.0856	-24.9128	163.1728	0.536	0.258	0.143
GH_3/GH_1	6-31G(d)			-117.3310	59.9830	-57.3480	1.108	0.830	0.715
	6-31G(d,p)			-111.1978	57.1824	-54.0154	1.099	0.821	0.706
GH_3/GH_2	6-31G(d)			-408.4569	105.0434	-303.4135	1.745	1.467	1.352
	6-31G(d,p)			-395.8781	98.3136	-297.5645	1.730	1.452	1.337
GH_2/GH_1	6-31G(d)			173.7950	14.9226	188.7176	0.470	0.192	0.077
	6-31G(d,p)			173.4825	16.0512	189.5337	0.468	0.190	0.075
A_4/A_1	6-31G(d)			119.7254	74.7802	194.5056	0.791	0.513	0.398
	6-31G(d,p)			130.2563	73.8188	204.0751	0.783	0.505	0.390
	6-311G+(d,p)			100.3729	77.4136	177.7865	0.805	0.527	0.412
A_4/A_3	6-31G(d)			-110.2526	-92.6706	-202.9232	1.485	1.207	1.092
	6-31G(d,p)			-98.9446	-92.7124	-191.6570	1.456	1.178	1.011
	6-311G+(d,p)			-87.8387	-119.7988	-207.6375	1.497	1.219	1.104
A_4/A_2	6-31G(d)			16.5853	-27.1282	-10.5429	0.986	0.708	0.593
	6-31G(d,p)			22.9049	-27.9642	-5.0593	0.972	0.694	0.579
	6-311G+(d,p)			19.9249	-35.7808	-15.8559	1.000	0.722	0.607
A_3/A_2	6-31G(d)			143.4074	38.4142	181.8216	0.488	0.210	0.095
	6-31G(d,p)			144.7543	40.7968	185.5511	0.478	0.200	0.085
	6-311G+(d,p)			127.6886	48.2372	175.9258	0.503	0.225	0.110
A_3/A_1	6-31G(d)			114.9811	83.7254	198.7065	0.444	0.166	0.051

	6-31G(d,p)	114.6004	85.2720	199.8324	0.441	0.163	0.048			
	6-311G+(d,p)	94.1058	98.6062	192.7120	0.460	0.182	0.067			
A_2/A_1	6-31G(d)	86.5549	129.0366	215.5592	0.401	0.123	0.008			
	6-31G(d,p)	84.4466	129.7472	214.1938	0.404	0.126	0.011			
	6-311G+(d,p)	60.5230	148.9752	209.4982	0.416	0.138	0.023			
AH_4/AH_1	6-31G(d)	-132.6429	206.7010	74.0581	0.895	0.617	0.502			
	6-31G(d,p)	-120.2269	206.3248	86.0979	0.885	0.607	0.492			
AH_4/AH_3	6-31G(d)	-299.5354	-43.8482	-343.3836	1.849	1.571	1.456			
	6-31G(d,p)	-285.8592	-41.5687	-327.4276	1.807	1.529	1.414			
AH_4/AH_2	6-31G(d)	-99.0102	42.7196	-56.2906	1.105	0.827	0.712			
	6-31G(d,p)	-93.8274	41.9672	-51.8602	1.093	0.815	0.700			
AH_3/AH_2	6-31G(d)	101.5097	129.2876	230.7973	0.361	0.083	-0.032			
	6-31G(d,p)	91.9637	125.5028	217.4665	0.396	0.118	0.003			
AH_3/AH_1	6-31G(d)	83.4436	125.2746	208.7182	0.418	0.140	0.025			
	6-31G(d,p)	82.8161	123.9466	206.7627	0.423	0.145	0.030			
AH_2/AH_1	6-31G(d)	65.3776	121.2618	186.6394	0.475	0.197	0.082			
	6-31G(d,p)	67.4281	122.3904	189.9188	0.467	0.189	0.074			
DNA(CV)								1.039	0.818	0.748
									1.115	1.064
DNA(SWV)								1.040	0.817	0.750
									1.133	1.082
G(SWV)								0.996	0.796	0.718
A(SWV)								1.304	1.142	1.066
										1.332
Phen(CV)								0.959	0.681	0.566

The experimental and calculated potentials vs. saturated calomel electrode (SCE of 0.2416V at 25℃) are shown in Table 1. From Table 1 the calculted oxidative peaks of 0.707 V at pH 9.00, 0.822V at pH 7.00 and 1.098V at pH 3.00 for N-protonated guanine at B3LYP/6-31G(d,p) level and 0.715 V at pH 9.00, 0.830V at pH 7.00 and 1.108V at pH 3.00 at B3LYP/6-31G(d) level for N-protonated guanine are consistent with the experimental peak of 0.718V at pH 9.00, 0.796V at pH 7.00 and 0.996V at pH 3.00, respectively,indicated that the experimental oxidative peak of 0.718 V at pH 9.00, 0.796V at pH 7.00 and 1.096 at pH 3.00 ascribes to the couple of GH_3/GH_1. The calculted oxidation peaks of 1.092 V at pH 9.00, 1.207V at pH 7.00 and 1.485V at pH

3.00 for adenine at B3LYP/6-31G(d) level, 1.011 V at pH 9.00, 1.178V at pH 7.00 and 1.456V at pH 3.00 for adenine at B3LYP/6-31G(d,p) level and 1.104 V at pH 9.00, 1.219V at pH 7.00 and 1.497V at pH 3.00 for adenine at B3LYP/6-311G +(d,p) level are in agreement with the experimental peaks of 1.066V at pH 9.00, 1.142V at pH 7.00 and 1.304V at pH 3.00, respectively, indicated that the oxidative peaks of 1.073 V at pH 9.00, 1.188V at pH 7.00 and 1.466V at pH 3.00 ascribe to the couple of A_4/A_3, the experimental peak of 1.332V at pH 9.00 is inagreement with the calculated potential of 1.456V at B3LYP/6-31G(d) level and 1.414V at B3LYP/6-31G(d,p) level for the couple of AH_4/AH_3 at pH 9.00, indicated the method for predicting the oxidative peak for a couple electrode at a electrode is reliable. In our previous works B3LYP/6-31G(d,p) for predicting reversible potential at a electrode is a reliable method [19 -21].The products, GH_2 and A_2 ,A_3 ,strongly absorbed on the electrode, undergoe hydrolysis, and the hydrolysised products such as 7,8-dihydro- 8-hydroxyguanine (8-OH-Gua) can also oxidiaze at a a glassy carbon microelectrode[22-23]. The calculated and experimental oxidative peaks indicate that the peaks for DNA and bases oxidation shift to postive direaction with the decrease of pH, indicated that DNA and bases in acidity solution are more stable than those in alkalescent solution.

In human body, some similar products of DNA oxidation are found, guanines are easily oxidized giving rise to two major products 2,6-diamino-4-hydroxy- 5-formamidopyrimidine (FapyGua) and 8-OH-Gua. 8-OH-Gua is eventually further oxidized to produce guanidinohydantoin and spiroiminodihydantoin.The primary products formed from adenine are 7,8-dihydro-8-hydroxyadenine (8-OH-Ade) and

4,6-diamino-5- formamidopyrimidine (FapyAde). Damage to pyrimidines includes thymine glycol, 5-hydroxycytosine and dihydrouracil [1]. These products are indicator of DNA mutation.

The predicted oxidation peak of GH_2/GH_1, GH_3/GH_2,A_2/A_1 *and* A_3/A_2 in pH 7.00 phosphate buffer solution at B3LYP/6-31G(d,p) level is 0.190,1.452,0.126 and 0.200V vs.SCE or 0.432,1.694, 0.368 and 0.442V vs. hydrogen standard electrode, respectively. From Figure 2 the experimental peak of GH_3/GH_1 is weaker than that of A_4/A_3 due to the higher oxidation potential of GH_3/GH_2, indicated that these reactions occur slowly, and undergo higher transitions. Although the peaks of GH_2/GH_1, GH_3/GH_2,A_2/A_1 *and* A_3/A_2 in the CV and SWV are not observered the initial potential of N-protonated guanine and adenine oxidation in DNA is around 0.432 and 0.368V vs. hydrogen standard electrode at pH 7.00, respectively, indicating that the factor of catalyzed these reactions of DNA bases is dangerous，or the potential of a couple electrode higher than this level is also dangerous.

Method: Adenine, guanine and calf thymus DNA were obtained from Sigma and used without further purification. The 0.1M phosphate buffer solution containing 0.1M NaCl (PBS) was used as the supporting electrolyte solution and adjusted to the desired pH by adding 1 M NaOH or HCl solution, pH=7.00. Water used was doubly distilled and sterilized in an autoclave. Cyclic voltammetry (CV) was performed using

a CHI 660B electrochemical workstation (CHI, USA). The frequency was varied over the range 105±1 Hz, with an amplitude of 1 mV. A three-electrode cell was used in the measurements, a glass carbon disk electrode (GCE, 3mm in diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference and a platinum wire counter electrode. Square-wave voltammetry analysis (SWV) was also performed with the following parameters: potential range between +0.1 and +1.3V; frequency = 15 Hz; step potential = 4mV; amplitude = 25mV.A GCE was prepared for the experiments by polishing to gain a mirror-like appearance, first with fine wet emery papers (grain size 4000) and then with 1.0mm and 0.3mm alumina slurry on micro cloth pads (Buehler, USA). After the initial polishing, the GCE were resurfaced using 0.05mm alumina slurry. First, the GCE were sonicated in the water twice then in 1:1 (v/v) isopropyl alcohol and acetonitrile mixture for 10min.All CV and SWV experiments were carried out at 25℃.

According to the molecular structure and symmetry, the initial geometries of p-BQ and phenol, adenine, guanine and derivatives, are optimized at same level. All the calculations were performed by the DFT method (B3LYP)[24] with 6-31G(d), 6-31G(d, p) and 6-311+G(d,p) using the GAUSSIAN 03 suite of programs[25]. All optimized geometries were further examined through vibrational frequency analysis. The molecule structure for calculating energies of solvation is optimzed in water, and the energies of solvation at 298.15K and 1atm were calculated by the Polarized Continuum (overlapping spheres) model (PCM) of Tomasi and coworkers for analytic energies at the same level .[26-28]

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