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Synthesis of [N-bis(4-methyl benzene-diamino formyl)methyl phosphonic acid] and its europium (III) complex and its application as DNA electrochemical sensor

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The new tripod ligand [N-bis(4-methyl benzene-diamino formyl)methyl phosphonic acid] and its europium (III) complex ($Eu(pic)_3L$) have been synthesized and characterized by TG-DSC, IR spectroscopy, UV-vis spectroscopy, elemental and molar conductance analysis. The experimental results show that the ligand forms the 1:1 complex with europium picrate. The results of UV-vis spectroscopy and cyclic voltammetry indicate that there are intercalation interaction between DNA and $Eu(pic)_3L$. When the $Eu(pic)_3L$ is regarded as DNA hybridization probe and used in the study of DNA electrochemical sensor, no obvious electrochemical signal response could be found with $Eu(pic)_3L$ under the effect of modified electrodes single-strand DNA testing. However, obvious electrochemical signal response is found when it is under the effect of double-stranded DNA testing. It indicates that the biosensor can discriminate well between the complementary sequences from the base-mismatched and the non-complementary sequences. So $Eu(pic)_3L$ can be used as a biosensor to discriminate between the complementary sequences from the base-mismatched and the non-complementary sequences.

Keywords: Phosphorus-containing tripod ligand, Rare earth complex, DNA electrochemical sensor

The derivatives of tripod ligand have been paid great attention in the field of spectroscopy and synthesis because of their special structure, they were divided into three categories. Firstly, the ligand added to the group which has the ability of push-pull electronic and coordination on its basic frame[1]; Secondly, the ligand which has been added to the new coordination atom and group. This category of derivative is the product of the design which had been considered to enhance coordination properties[2]; Lastly, the ligand which has been designed easily to form hydrogen bond by adding the new coordination atoms[3]. Hence, the derivatives' application in the field of molecular recognition and supramolecular self-assembly has also received great attention by researchers[4]. The ligand which has been designed and synthesized belongs to the third category, it is a kind of asymmetric phosphorus tripod ligand which has great solubility in water and the ability to form hydrogen bond easily.

In recent years, studies on the interaction between DNA and metal or metal complex have been active research in electrochemical sensor's areas[5,6] because of complex's optical, electrical and other fine features which can identify ssDNA and dsDNA and improve its ability to bind with dsDNA by intercalation, for example, a study used 1,10-phenanthroline(phen)[7] and (3,2-a,3',2'-c)DPPZ[8] as a ligand. However, these coordination compounds are all charged positively, during inserting with double-stranded DNA, they also have nonspecific electrostatic interaction with single-stranded DNA.

The interaction between compounds and single-stranded DNA can impact the consequence of the electrochemical testing greatly[9]. During the experimental process, it was found that the coordination compound that has been synthesized this time was electrically neutral, it can eliminate the nonspecific electrostatic interaction between compounds and single-stranded DNA. Even better, the structure of this compound can be easily controlled, the chemical stability is better and the electrochemical activity is higher. Based on these properties, the interaction between DNA and the europium(III) compound of asymmetric phosphorus tripod ligand was studied. By using UV-vis spectroscopy, the application of the complex was investigated when it was regarded as DNA hybridization probe in the study of DNA electrochemical sensor. Because the compound can identify the base-mismatched sequence quickly when performing the electrochemical testing, it can be applied in the field of genetic testing, DNA damage detection and preliminary screening of anti-cancer drugs.

Experimental Section

Reagents and apparatus

All reagents used in this work were analytical reagent grade. Before use 2-aminopyridine, N-(phosphono methyl) iminodiacetic acid, THF, pyridine, benzene and Oxalyl chloride must be dried by adding CaH_2. ct-DNA, EDC and NHC were purchased from Aladdin Industrial (Shanghai) Corporation, Shanghai, China. The following DNA sequences were all purchased from Sangon Biotech (Shanghai) Co, Ltd.

S1—Single DNA sequence: 5'-NH_2-$(CH_2)_6$-TCCTG GTCCCACCTTTGC

S2—complementary sequence:5'-GCAAAGGTGGG ACCAGGA

S3—base-mismatched sequence: 5'-GCAAAGGTG AGACCAGGA

S4—three bases-mismatched sequence: 5'-GCAAAT GTGAGACAAGGA

S5—non-complementary sequence: 5'-GAGCAGC GTCGTCTTACA

In this work, the electrochemical studies involve interaction with DNA, the PBS (Na_2HPO_4-NaH_2PO_4 system) buffer solution involved were 0.01, 0.05 and 0.1 mol·L^{-1} respectively. According to its absorbance at 260 nm with a known molar extinction coefficient value of 6600 $M^{-1}\cdot cm^{-1}$, the concentration of ct-DNA was determined. On the other hand, the ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280}, was 1.8-1.9, indicating that the DNA was sufficiently free of protein. All solutions were prepared in triply distilled deionized water.

The infrared spectrum was recorded on a FIPR-8400S FT-IR spectrophotometer (Shimadzu) in the range of 400 - 4000 cm^{-1} using KBr pellets. Elemental analyses for C, H, and N were performed on an Elementar Vario EL elemental analyzer (Germany). ^{1}H and ^{13}CNMR spectra were taken in Chloroform-d at room temperature with a INOVA-400 MHz (Germany Bruker) instrument with chemical shift relative to tetramethylsilane. Thermogravimetric (TGA) were carried out on a DT-40 (Shimadzu) unit at a heating rate of 10°C·min^{-1} under a nitrogen atmosphere from 20° to 600°C. A CHI 650C electrochemical analyzer (China) in connection with a glassy carbon working electrode, a platinum auxiliary electrode, and an Hg/HgCl reference electrode was used for all electrochemical measurements.

Synthesis of the ligand and its europium (III) compound

As shown in Fig. 1, 50 mL benzene, 2 mL DMF and 6 g N-(phosphonomethyl) iminodiacetic acid hydrate were added into the three-necked flask with drying, condenser, and dropping funnel. After stirring well, 26 mL oxalyl chloride was dripped into the flask slowly by dropping funnel at 30°C. Then the temperature was raised, but it was controlled under 40°C and the reaction continued for 6-7 h. After this step, a kind of yellow viscous liquid was synthesized. It was dripped into another round flask at 0°C. In this flask, 4.3 g (40.18 mmol) *p*-toluidine was dissolved by 50 mL benzene, 80 mL THF and 6 mL pyridine in advance. After dripping, the reaction was kept for 10 h at 50°C, the solution turned yellow. The solvent was removed on a rotary evaporator, gradient to give the red-brown liquid. The ligand was obtained in a yield of 52%. The NMR data of product was as follow:

^{1}H NMR (Chloroform-*d*, 400 MHz): 2.32 (s 3H), 2.34 (s 3H), 3.51(s 2H), 3.63 (s 2H), 4.70 (s 2H) 7.16(d 2H), 7.19(d 2H), 7.41(d 2H), 8.69(s 1H); ^{13}CNMR (Chloroform-*d*, 100 MHz): 21.00 , 29.79, 57.66, 59.12, 119.48, 119.82, 129.68, 129.81, 134.45, 134.66, 135.26, 166.67, 168.84; 5 mL $Eu(pic)_3$ anhydrous ethanol solution (containing 0.2 mmol $Eu(pic)_3\cdot 6H_2O$) was dripped into 5 mL ligand anhydrous ethanol solution (containing 0.2 mmol ligand), the mixture was stirred for 8 h. Then $Eu(pic)_3L$ was gotten by the measure of centrifugal separation.

The interaction between DNA and $Eu(pic)_3L$

UV–vis spectra experiment

When using the UV-vis spectroscopy to study the interaction between $Eu(pic)_3L$ and DNA, 3 mL tris-NaCl solution was added into a quartz sample pool as a reference substance and 3 mL $Eu(pic)_3L$ (1×10^{-5}mol·L^{-1}) solution was added into the quartz sample pool. To increase the concentration ratio of DNA and $Eu(pic)_3L$ solution, the same volume of ct-DNA solution was added into the two pools every time which had been mentioned, separately. The absorption spectrum of the mixed solution was scanned at 200-500 nm.

Electrochemical experiment

The cyclic voltammetry (CV) of the DNA-$Eu(pic)_3L$ solution (1×10^{-4} mol·L^{-1}) was carried out at a scan rate of 50 mV/s, and the voltage of amperometric current versus time was fixed at 100 mV and the electro reduction current was measured at 100 s. The PBS (Na_2HPO_4-NaH_2PO_4 system) buffer

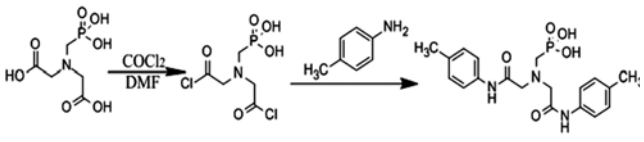

Fig. 1 — The synthetic route of the Ligand

solution involved in this work was 0.05 mol·L^{-1} (*p*H=6.86).

Application of Eu(pic)$_3$L as hybridization probe in DNA biosensors

Preparation and hybridization of DNA probes

Prior to hybridization detection, the DNA probe was immobilized to a GCE through a classical assembly method as described in reference[10]. The glassy carbon electrode was cleaned and oxidized by the measure of Electrolysis coulomb at 0.5V. EDC and NHC mixed solution at appropriate concentrations of 20μL were then added to each electrode for 2 h at room temperature. After rinsing with PBS buffer solution (0.01mol·L^{-1}, *p*H=7.2), to the treated electrode was added 10μL S1(10μM) solution on the surface. The preparation of the glassy carbon electrode modified by S1(S1/GCE) was finished when the S1 solution was dried naturally. In the same way, 20μL S2(20μM) solution was added on the surface of S1/GCE which had been rinsed, after being placed for 5 h at room temperature, the DNA hybridization probe was prepared successfully (S2-S1/GCE). The preparations of S3-S1/GCE, S4-S1/GCE and S5-S1/GCE were same as it.

Indicator binding and electrochemical measurements

Before measurements, the S1/GCE, S2-S1/GCE, S3-S1/GCE, S4-S1/GCE and S5-S1/GCE were immersed into PBS (*p*H=7.2) buffer containing Eu(pic)$_3$L solution (1×10^{-4}mol·L^{-1}) for absorption equilibrium. After rinsing with the deionized water, the electrodes were then transferred into a PBS (*p*H=7.2) solution without Eu(pic)$_3$L for differential pulse voltammetric (DPV) scans. The parameter conditions of DPV included a scan rate of 50 mV/s, a potential increment of 0.004V, a pulse amplitude of 0.05V, a pulse width of 0.05 s, a pulse period of 0.2 s and a potential range between -0.2V and -1.0V.

Results and Discussion

Analysis of composition and structure of the ligand and its europium (III) complex

Composition and solubility of the ligand and complex

The results of the element analysis of the complex and the ligand are shown in Table 1. It is seen that the ligand has coordinated with Eu (III) atoms. The complex (Eu(pic)$_3$L) was soluble in DMSO, DMF, water, methanol and ethanol, it was slightly soluble in chloroform and ethyl acetate and insoluble in ether and benzene.

FT-IR Spectra

The IR data of the complex and ligand (cm^{-1}) was shown in Table 2. Through these data, it was obvious that the –NH absorption peak of the ligand appeared at 3275 cm^{-1}. However, the –NH absorption peak of Eu(pic)$_3$L appeared at 3518 cm^{-1}. The frequency of the absorption peak was increased. It can be inferred that N atoms had coordinated with the ligand[11], and the coordination effect made the frequency of –C=O absorption peak which had been connected to –NH, to decrease. Moreover, the frequency of the –OH absorption peak increased from 3325 cm^{-1} to 3408 cm^{-1}, it was inferred that this kind of phenomenon was due to coordination effect. By the way, the –P=O absorption peak of the ligand disappeared from the spectra. The conclusion was drawn that O atoms had coordinated with the ligand.

In order to further determine the structure of ligand, Gaussian 03 program was used to calculate the ligand. Figure 2 showed the quantitative calculation atomic number of the ligand. Through the quantitative calculation, the total energy of the ligand was proved E_L=3.91×10^{6}KJ·L^{-1} and the stability of the ligand was very good. Figure 3 showed that the structure of the ligand was asymmetric, its three chains extended in the direction of three-dimensional space. Table 3 recorded the net charge distribution of the partial atoms of the ligand, O atoms of the ligand' s preferred coordination ability is better than N atoms[12].

Table 1 — The results of the element analysis of the complex and the ligand

Comp	N	C	H	M
L	10.35(10.37)	56.31(56.30)	5.93(5.93)	—
Eu(pic)$_3$L	13.49(13.53)	35.78(35.76)	2.05(2.09)	12.17(12.24)

Table 2 — The IR data of the complex and ligand (cm^{-1})

Comp	ν_{O-H}	ν_{N-H}	$\nu_{P=O}$	$\nu_{C=O}$
L	3325	3275	1519	1673
Eu(pic)$_3$L	3408	3518	—	1635

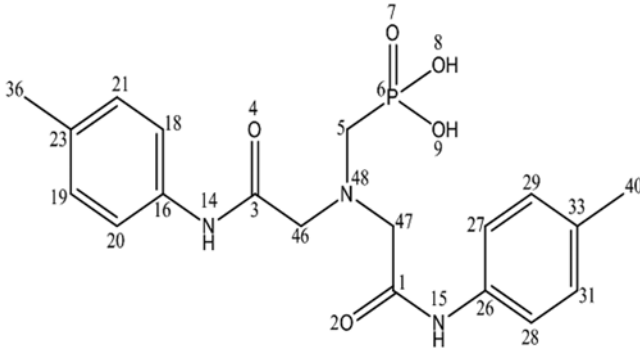

Fig. 2 — The atom number of the quantitative calculation ligand

Further investigation of coordination

Molar ratio method

For the purpose of learning more about the coordination compound, the method of mole ratio was used to determine the coordination ratio. The method of mole ratio is to scan the UV-vis spectra of the different concentrations mixed solution of the ligand and the $Eu(pic)_3$ at 350 nm. As shown in Fig. 4(A), the increase in Eu^{3+} concentrations led to the increase absorbance, it is the reflection of the formation process of the coordination compound. Further more, it was found that when the ratio of Eu^{3+} and the ligand is more than 1, the absorbance tends to be stable as shown in Fig. 4(B). This is a powerful evidence to prove that the coordination equilibrium of Eu^{3+} and the ligand has reached[13].

Thermal analysis

The curve of TG-DSC (20-600°C) was determined in nitrogen atmosphere, the velocity was 40 mL·min^{-1}, and the raising speed of the temperature was 10°C·min^{-1}. The results of thermal gravimetric (TG) and differential scanning calorimetry (DSC) of the $Eu(pic)_3L$ are presented in Fig. 5. There was no weightlessness and Thermal phenomenon for $Eu(pic)_3L$ before 200°C. The first loss in the range 200-300°C accounts for the release of the ligand, the second step occurs in the temperature range 300-600°C which corresponds to the loss of the pic^+. The weightlessness rate of the whole process was similar to the theoretical weightlessness rate. These proves that the coordination ratio of the ligand and the Eu^{3+} is 1:1.

According to the IR spectra, the data of the element analysis and the curve of TG-DSC, etc., the possible structure is shown in Fig. 6.

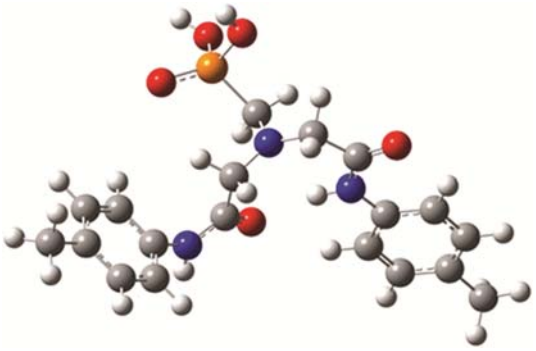

Fig. 3 — The Molecular structure of the ligand

The interaction between DNA and $Eu(pic)_3L$

UV–vis spectra experiment

Electronic absorption spectra were used to study the interaction between $Eu(pic)_3L$ and DNA, and the results are shown in Fig. 7. It can be clearly found that the complex exhibits a strong absorption peak at 350 nm. With the addition of increasing amounts of DNA, the peak decreases accordingly, suggesting that $Eu(pic)_3L$ has interacted with DNA[14]. Additionally, it has been well known that the shift of the absorption peak is related to the binding mode of the small molecules to DNA. A typical intercalation into base pairs usually results in significant hypochromism effects[15,16]. For the studied complex, a new complex was found between them, The mechanism of this

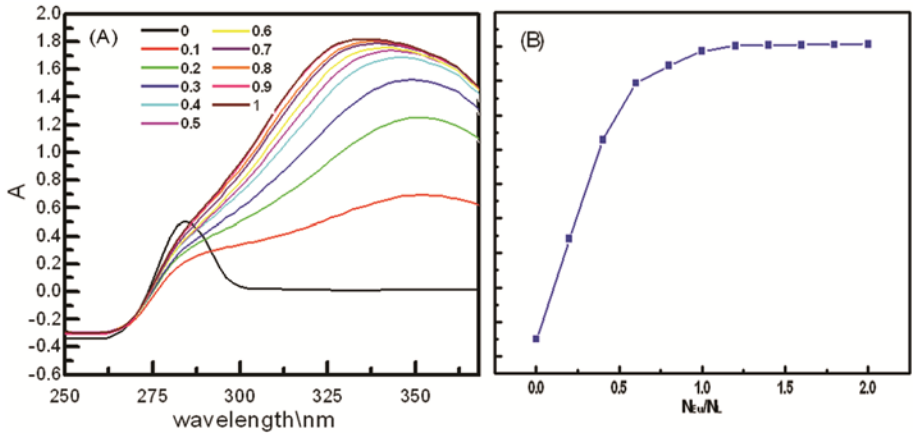

Fig. 4 — The UV-Vis of the ligand with complexes in 350 nm

Table 3 — The net charge distribution of the partial ligand

Tag	2	4	7	8	9	14	15	48
Atom	O	O	O	O	O	N	N	N
Charge	-0.235	-0.620	-0.862	-0.866	-0.867	-0.992	0.0.900	-0.005

process was the electron filled in the π orbital, decreased the transition probability of π-π* orbital. Moreover, the ligand which was inserted into the DNA, produced electronic accumulation with the base pairs on the DNA, made the characteristic absorption change, that is another evidence for the theory that they are interacted by the insert form[17].

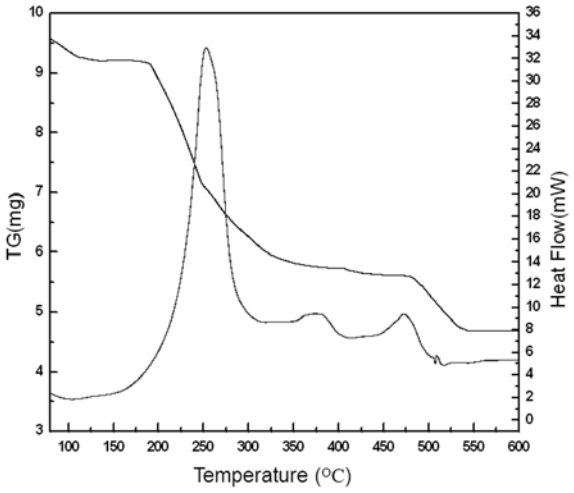

Fig. 5 — The TG–DSC curves of $Eu(pic)_3L$

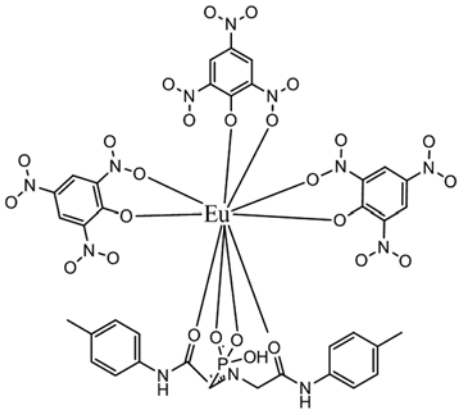

Fig. 6 — The structure of $Eu(pic)_3L$

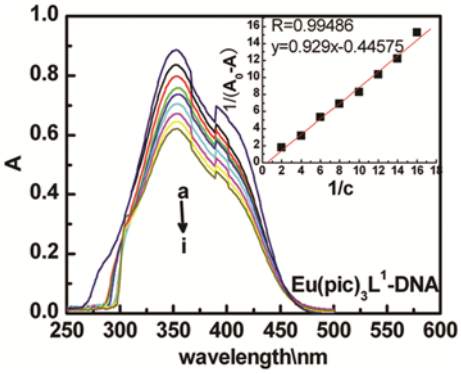

Fig. 7 — $Eu(pic)_3L$ of DNA under UV spectra of different concentration [a:$C_{Eu(pic)3L}$=1×10^{-5}mol•L^{-1} b-i :C_{DNA}=0.625, 1.25, 1.875, 2.5, 3.125, 3.875, 4.5, 5×10^{-6}mol•L^{-1}]

Cyclic Voltammetry

The electrochemistry of $Eu(pic)_3L$ was first investigated by CV technology. After the interaction between DNA and complex molecules which has an activity electric, the mode of DNA binding was determined according to the variation of the characteristics of redox peak current of complex molecules, it worked well when potential ranged from -0.6V from 0.6V. The CV curves of $Eu(pic)_3L$ at different scanning speed and the CV curve of the interaction between DNA and $Eu(pic)_3L$ are shown in Fig. 8 and Fig. 9, respectively. It was found that $Eu(pic)_3L$ presented a reversible redox process, which indicated the complex was a kind of electrochemically active material. It could be considered that rare earth ion Eu (III) was the center of electrochemically active component of, its electrode reaction corresponded to process of electron transfer that europium ion in the complexes occured with the applied potential varying, namely Eu(III) converted to Eu(III) process. The peak current of CV curves of $Eu(pic)_3L$ decreased

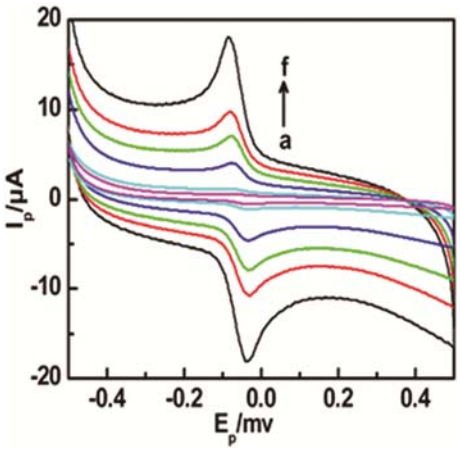

Fig. 8 — The CV curves of $Eu(pic)_3L$ at different scanning speed

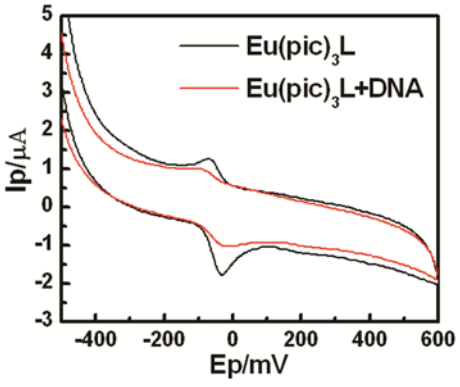

Fig. 9 — The CV curves of $Eu(pic)_3L$ with DNA [$C_{Eu(pic)3L}$=1×10^{-4}mol•L^{-1} a-f: 10, 200, 400, 600, 800, 1000 mV/s $C_{Eu(pic)3L}$=1×10^{-4}mol•L^{-1} C_{PBS}=0.01 mol•L^{-1}]

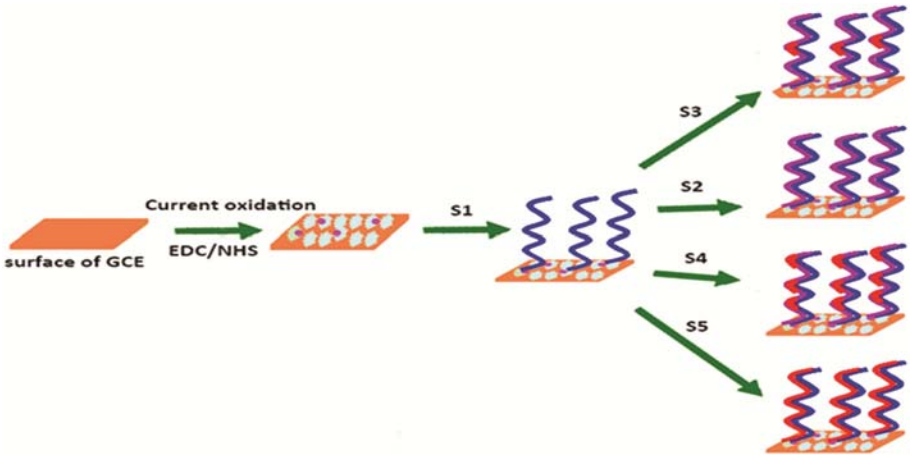

Fig. 10 — Diagram of the DNA biosensor with hybrid indicator

significantly after adding the DNA, that was because a new compound which was produced in the system, and the $Eu(pic)_3L$-DNA led to the decrease of the system's diffusion coefficient[18,19], peak current reduced may be due to bonding interaction which occurred when the complex molecules embedded DNA molecules, which lowered the free complex concentration in the solution, and the number of complex molecules which migrated to the surface of electrode, reduced per unit time, leading to current of redox peak to reduce, the positive shift of potential confirmed that the complexes bind to DNA by the mode of insertions at the same time. Cyclic voltammetry experiment showed that the intercalation between complex and DNA had taken place[20].

Application of $Eu(pic)_3L$ as hybridization probe in DNA biosensors

As mentioned above, $Eu(pic)_3L$ has the advantage to be applied in DNA hybridization probe sensor. The schematic diagram for the fabrication and detection procedures of the DNA biosensor are showed in Fig. 10.

The best enrichment time of 20 min was selected after testing the complex enrichment time. The electrochemical experiments, showed that the best effect was seen with was the concentration of complex as 1×10^{-4} mol·L^{-1}. Using DPV investigated S1/GCE, S2-S1/GCE, S3-S1/GCE, S4-S1/GCE and S5-S1/GCE that enriched by complex, selectivity of $Eu(pic)_3L$ as hybridization indicator was described. Figure 11 show the DPV peaks of complex at the capture probe (S1) modified electrode before (curve a) and after hybridization with the complementary sequence of S2 (curve e), one-base mismatched sequence of S3 (curve d), three-base mismatched sequence of S4 (curve c) and non-complementary

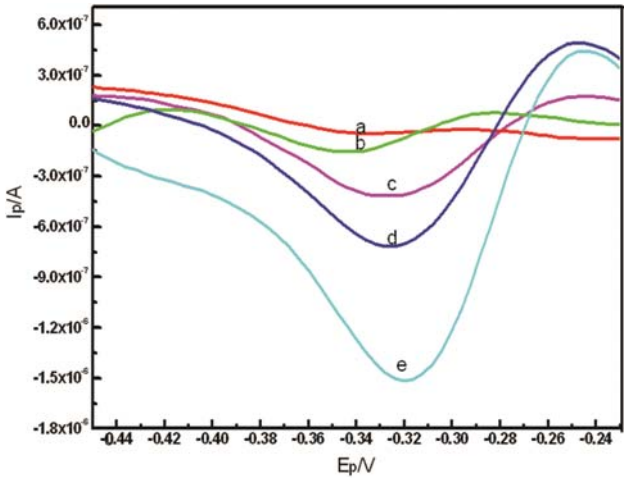

Fig. 11 — The DPV curves of $Eu(pic)_3L$ with DNA [a: Captureprobe sequence of S1; b: non-complemetary sequence of S5; c: three-base mismatched sequence of S4; d: one-base mismatched sequence of S3; e: complementary sequence of S2]

sequence of S5 (curve b). It is clear that on S5-S1/ GCE, only a small oxidation peak appeared, and this signal is very close to that on S1/GCE, suggesting that the hybridization event between S5 and S1 did not take place. However, when S1/GCE was hybridized with the perfectly complementary sequence of S2, it was found that the electrochemical response of the complex was enhanced remarkably. Alternatively, when S3 and S4 were hybridized, it was observed that the detected signals attenuate as compared with S2-S1/GCE, and the attenuated degree parallels with the mismatching number of the bases. This is because the double helix structure become more imperfect with the increase in the mismatch number, and therefore the intercalative sites for binding with complex also decrease[21]. These results indicate that the biosensor based on using complex as indicator presents an excellent hybridization specificity.

The above analysis indicate that the biosensor which had been used $Eu(pic)_3L$ as a DNA hybridization probe could work well to discriminate the complementary sequences from the base-mismatched and the non-complementary sequences. For example, this kind of biosensor can be applied in the field of Genetic testing, such as DNA damage detection. DNA damage detection was caused by harmful chemicals, physical radiation and ultrasonic degradation, it was a kind of damage towards the bases, the breaking of the DNA chain. The damage led to a series of adverse consequences such as DNA mutation and the change of DNA structure. The electrochemical testing towards DNA damage was divided into two categories. One is the method, which directly tests the lost of bases, the broken of the DNA chain, this method has taken the advantage of the electrochemical activity of DNA[22]. The other is to track the electrochemical signal response of chemicals which can form covalent bond with DNA. The method used in this paper belongs to the second one. The mechanism of the method is to build the assembly structure of double helix DNA on the surface of electrode. By taking the advantage of the special electronic structure of double helix DNA, this model can perform long-range electron transfer with the surface of electrode when the electrochemical activity insert molecule is present. This method follow a strict principle towards the bases of the double helix DNA, if there is a single-base mutation in the chain of double helix DNA molecule, the appearance of the electrical signal will be inhibited. That is, the change of the electrical signal which is caused by base-mutation will be detected quickly during testing the gene sequence. However, some kinds of genetic disorders and DNA damage were caused by base-mutation, which had been induced by some other factors[23]. According to this theory, the biosensor which were used $Eu(pic)_3L$ as a DNA hybridization probe can be used in a quick detection, it also can provide the basis of DNA damage detection and genetic disorders detection.

$Eu(pic)_3L$ was a kind of rare earth metal coordination compounds which had the biological activity such as antimicrobial properties, antitumor , antiviral and the insert interaction form with DNA. These properties determined that this kind of rare earth metal coordination compounds played an important role in the filed of biomedical research. The working mechanism of rare earth metal coordination compounds antitumor drugs is to combine with the tumor cells and then kill them. Studies had showed that the working mechanism mentioned above was similar to the mechanism for quinolones to kill bacteria[24]. The two kinds of drugs, both can inhibit the DNA and topoisomeraseII and interfere with the DNA replication, leading to the DNA damage. For this reason, the method which fixed single-strand DNA on electrochemical sensor to detect the electrical signal produced by the interaction between DNA and anti-tumor drugs in the DNA hybridization process, can effectively determine whether hybridization process had changed in the DNA sequence. These consequences truly provided a new measure to perform the anti-cancer dynamics research and pharmacology study. It is of great significance for implementing preliminary screening of anti-cancer drugs. As is known to all, the tumor cells has a better ability to recognize compounds with alkaline groups such as pyridine and amino than normal cells, so taking a systematical research about the biosensor which had used compounds like $Eu(pic)_3L$ as a DNA hybridization probe can help us to get useful law of screening anti-cancer drugs.

Because of the alkaline groups contained in the ligand, the targeting property towards tumor tissues of rare earth complex which had been designed and synthesized in this paper may be higher than others. Related work is under way. To date, most of DNA sensors applied research still stays in the experimental stage, the foundation to really get into the practical application domain is to improve the specificity of electrochemical sensors which had chosen rare earth metal coordination compound as a DNA hybridization probe. Moreover, to combine DNA electrochemical sensor with modern information technology and to realize the automation of DNA electrochemical sensors, is an important factor which is promoting DNA sensor applied research.

Conclusion

In this paper, a new kind of tripod ligand [N-bis(4-methyl benzene-diamino formyl)methyl phosphonic acid] and its europium complex have been synthesized and characterized. Its interaction with DNA is comprehensively studied using UV and electrochemical methods. The results show that the complex interacts with DNA via a specific groove binding mode, and $Eu(pic)_3L$ had good electrochemical property to interact with DNA. Based on this, the $Eu(pic)_3L$ has been utilized as a DNA hybridization probe to perform a electrochemical sensor study. As a DNA hybridization probe, it can accurately discriminate the complementary sequences from the base-mismatched and the non-complementary sequences by tracking the change of the electrochemical

signal response. The utility of the new electrochemical hybridization indicator provides a simple, rapid and highly specific detection of the target DNA sequences.

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