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# Preparation of Luminol-doped Nanoparticle and Its Application in DNA Hybridization Analysis

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The dye-doped silica nanoparticles can be used as nanobiosensors that are able to recognize and detect specific DNA sequence. In this paper, spherical nanosized luminol/$SiO_2$ composite particles have been synthesized with reverse micells via hydrolysis of tetraethyl orthosilicate (TEOS) in the microemulsion. The nanoparticles were modified with chitosan and used to label DNA, forming the DNA probe which was used to hybridize with target DNA immobilized on a PPy modified Pt electrode. The hybridization events were evaluated by electrogenerated chemiluminescence (ECL) measurements and only the complementary sequence could form a double-stranded DNA (dsDNA) with DNA probe and give strong ECL signals. A three base mismatch sequence and a non-complementary sequence had almost negligible responses. Due to the large number of luminol molecules inside silica nanoparticles, the assay allows detection at levels as low as $2.0\times10^{-12}$ mol/L of the target DNA. The intensity of ECL was linearly related to the concentration of the complementary sequence in the range of $5.0\times10^{-12}$—$1.0\times10^{-9}$ mol/L.

**Keywords** luminol, silica nanoparticle, ECL, DNA

## Introduction

The analysis of DNA sequence and DNA mutant detection play fundamental roles in the rapid development of molecular diagnostics and in the anticancer drug screening.

Therefor many detection techniques of DNA sequence have been developed in recent years. These techniques mainly depend on the nucleic acid hybridization[1] and their sensitivities are related to the specific activity of the label linked to the DNA probe. The degree of hybridization of probe to its complementary DNA sequence in sample is translated into a useful electric signal, which is a measurement of the amount of that specific sequence in the sample.

Traditional DNA probes labeled with radioisotopes[2,3] were not widely applicable to a routine environment due to the complex procedures and a potential hazard to analysts. Various novel techniques such as fluorescence,[4] electrochemistry[5-7] have been developed. Biotin,[8] digoxigenin,[9] and fluorescent dyes[10] are popular reagents for labeling DNA.

Electrogenerated chemiluminescence (ECL) is named electrochemiluminescence, which combines chemiluminescence (CL) and electrochemistry and allows the detection of the analytes at low concentration over a wide range. In comparison to other methods, ECL has a large number of additional advantages, such as high sensitivity and selectivity, rapid and convenient operation and relatively simple instrumentation system.[11] So ECL is becoming more and more important in many fields.[12] Tris (2,2′-bipyridyl) ruthenium (Ⅱ) (TBR) is an excellent ECL substance and has been used as a label in DNA analysis. However, TBR is too expensive to be widely applied.

Great attention has been paid to luminol which is one of the most efficient ECL active substances. The ECL reaction of luminol allows the detection of a wide range of analytes, such as $H_2O_2$,[13] enzymes[14] and many metal ions.[15,16] However, it is difficult to be labeled directly to a DNA strand because of the limitation of its chemical structure. The development of nanotechnology gives a promising future to chemistry and biomedical fields.[17-19] Core/shell nanoparticles have been used to develop a new class of nanobiosensors that is able to recognize and detect specific DNA sequence in a homogeneous format.[20] In this work, we chose luminol as core and synthesized luminol-doped silica nanoparticles (luminol/$SiO_2$) via reverse microemulsion. The luminol/$SiO_2$ was then modified with chitosan, a natural cationic polymer, and used to label ssDNA through forming a tight complex between DNA and chitosan. As a result luminol labeled ssDNA probe was obtained in this way. The DNA probe labeled with luminol was used to hybridize with the target ssDNA immobilized on

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the electrode and the hybridization event was detected by ECL measurement. The results showed that this method was more sensitive than other methods attributed to the high luminescence efficiency of luminol and the large number of luminol molecules inside silica nanoparticles. The intensity of ECL was linearly related to the concentration of the complementary sequence in the range of $5.0\times10^{-12}$—$1.0\times10^{-9}$ mol/L and as low as $2.0\times10^{-12}$ mol/L ssDNA could be detected.

## Experimental

### Apparatus

All ECL measurements were carried out using a Laboratory-constructed ECL system shown in Figure 1. A Model 173 potentiostat (Princeton Applied Research. PAR, EG ΣG) and an XFD-8A waveform generator (Ningbo Radio Factory, Ningbo, China) were used for giving and controlling waveforms and potential. The CL emission was detected and amplified using a 9901 luminometer (Rongsheng Electronic Equipment CO., Shanghai, China) and the output signal which is directly related to the ECL light intensity was recorded using a Chart Recorder (Dahua Instrument Co, Shanghai, China). The three-electrode system was used. A Pt plate (4 mm×10 mm) fixed on black plexiglass was used as a working electrode. A saturated Ag/AgCl was used as a reference electrode and a platinum Pt wire as an auxiliary electrode. All ECL reactions were carried out in a 1 mL cell placed directly in the front of photomultiplier tube.

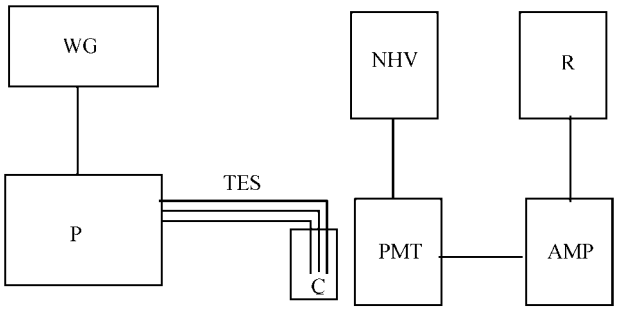


**Figure 1** The schematic diagram of apparatus used for ECL measurement. WG: waveform generator; P: potentiostat; TES: three electrode-system; C: cell; PMT: photomultiplier tube; AMP: amplifier; R: recorder; NHV: negative high voltage.

A CHI 630 electrochemical analyzer (CHI Instruments Inc. USA) and a cary 50 ultraviolet-visible spectrophotometer (Volan Cary Co., USA) were employed.

### Reagents

All reagents were of analytical-reagent grade or better and distilled deionized water was used for the preparation of solutions.

Luminol was purchased from Shanxi Normal University (Shanxi, China). The stock solution was prepared by dissolving 17.7 mg of Luminol in 1.0 mL of 0.5 mol/L NaOH and diluting with water to 100 mL to obtain the concentration of $1.0\times10^{-3}$ mol/L. The stock solution was kept in 4 ℃ freezer. The working solution was obtained by diluting the stock solution with water to the required concentration. Chitosan oligomer was purchased from Aldrich company (US). The 0.1% (*m/m*) chitosan working solution was prepared by dissolving chitosan in 1.0% (*V/V*) acetid acid. Tetraethyl orthosilicate (TEOS) and trimethoxysilylpropyldiethylenetriamine (DETA) were purchased from United Chemical Technologies (Bristol, PA).

A 24-base synthetic terminal 5′-$PO_4^{3-}$-oligonucleotide probe ($PO_4^{3-}$-5′-GAG CGG CGC AAC ATT TCA GGT CGA-3′), its fully complementary sequence (5′-TCG ACC TGA AAT GTT GCG CCG CTC-3′), a three-base mismatched sequence (5′-TCG *T*CC TGA AA*C* GTT GCG CC*T* CTC-3′) and a non-complementary sequence (5′-GAC TAA GAC CCG AAA AAT TAA GAG -3′), were all obtained from Shenggong bioengineering Co. Ltd. (Shanghai, China). The oligonucleotide stock solution was prepared using water, which was kept frozen. Pyrrole was kindly offered by Shanghai chemical factory (Shanghai, China). PBS buffer (1.5 mol/L NaCl＋10 mmol/L sodium phosphate buffer, pH＝6.8) was used for the hybridization and washing solution. $H_2O_2$ (24 mmol/L) and $Na_2CO_3$-$NaHCO_3$ buffer (0.2 mol/L, pH＝10.0) were also used.

### Procedure

**Synthesis of luminol-doped silica nanoparticle** The luminol-doped silica nanoparticle (luminol/$SiO_2$) was synthesized according to the literature.[21,22] 7.5 mL of cyclohexane, 1.8 mL of *n*-hexanol, 1.77 mL of TritonX-100, 340 μL of $1.0\times10^{-3}$ mol/L of luminol solution were mixed and stirred for 0.5 h at room temperature, forming a uniform W/O microemulsion. Then 100 μL of TEOS and 60 μL of $NH_3$•$H_2O$ (28—30 wt%) were added to the microemulsion. Hydrolysis of TEOS within the miroemulsion was initiated by adding $NH_3\cdot H_2O$. The reaction was allowed to continue for 24 h. After the reaction was completed, luminol-doped silica nanoparticles (luminol/$SiO_2$) were isolated with acetone followed by centrifuging and washing with ethanol and water several times to remove surfactant molecules and free luminol thoroughly.

**Modification of luminol/$SiO_2$** 100 μL of chitosan solution were added to 1 mL of dispersed luminol/$SiO_2$ nanoparticle solution and reacted for 0.5 h at room temperature (25 ℃). The silica nanoparticles could be modified with chitosan through the bond between amino group of chitosan and hydroxyl group of the silica nanoparticles as well as the attraction of static electricity. The chitosan modified silica nanoparticles were thoroughly rinsed with deionized water to remove excess chitosan.

**Preparation of ssDNA/luminol/$SiO_2$ probe** 2 OD of ssDNA (about 66 μg) was diluted in 1.0 mL of

water, 50 μL of which was added to the modified nanoparticles and the reaction was continued for 120 min at room temperature. The final product was washed, centrifuged and suspended in PBS (pH＝6.8) buffer, either used immediately for test or stored at 4 ℃ for later usage.

**Immobilization of ssDNA on PPy/Pt electrode** As described in previous literature,[23] the PPy/Pt electrode was immersed into an acetate buffer solution (0.2 mol/L, pH＝5.0) containing $1.0\times10^{-9}$ mol/L target ssDNA (complementary sequence, noncomplementary sequence or three-base mismatched sequence), while applying a potential at ＋0.5 V (vs. Ag/AgCl) for 200 s. After that the electrode was rinsed with 1.0 mol/L KCl and water respectively to remove the nonspecific adsorption of DNA.

**Hybridization of luminol/$SiO_2$/ssDNA probe to target oligonucleotide** The electrode immobilized with target ssDNA was immersed into a stirred hybridization solution containing luminol/$SiO_2$/ssDNA probe for 30 min at 40 ℃. After that, the electrode was thoroughly washed with the same buffer and was then employed as working electrode for the ECL measurement.

**ECL measurement** 1.0 mL of $Na_2CO_3$-$NaHCO_3$ buffer and 50 μL of $H_2O_2$ with 24 mmol/L were added to the ECL cell. The electrodes were immersed into the cell that was placed in the dark chamber. The chamber was closed and the shutter was opened. ＋1.0 V (vs. Ag/AgCl) potential was exerted on the working electrode and the ECL signal was recorded. The height of the peak was used for quantification.

## Results and discussion

### Optimization of the conditions for ECL of luminol

Luminol can give strong ECL in alkaline solution when an appropriate potential was exerted. The ECL intensity can be greatly enhanced by the addition of $H_2O_2$. We examined the factors which influenced the ECL of luminol and optimized the conditions. They are: ＋1.0 V (vs. Ag/AgCl) potential; $Na_2CO_3$-$NaHCO_3$ buffer (pH＝10.0) and $1.2\times10^{-3}$ mol/L $H_2O_2$.

### Characterization of luminol/$SiO_2$ nanoparticles

Tan[21] synthesized $Ru(bpy)_3^{2+}$-doped silica nanoparticles using water-in-oil microemulsion. We prepared nanoparticles using luminol instead of $Ru(bpy)_3^{2+}$. According to the literature,[24] the molar ratio of water to TEOS (*H*) and the molar ratio of water to TritonX-100 (*R*) can influence the size of the nanoparticles. We prepared nanoparticles of different sizes by adjusting the values of *H* and *R* and finally chose *H*＝43 and *R*＝7. Under these conditions the particles with the average size of 60 nm were produced as shown in Figure 2. The ECL experiment indicated that the luminol/$SiO_2$ nanoparticles could give strong ECL emission but $SiO_2$ nanoparticles without luminol gave no emission, which proved that luminol was doped in $SiO_2$ nanoparticles and luminol/$SiO_2$ nanoparticles were successfully produced. The nanoparticles were kept stable for two months at room temperature.

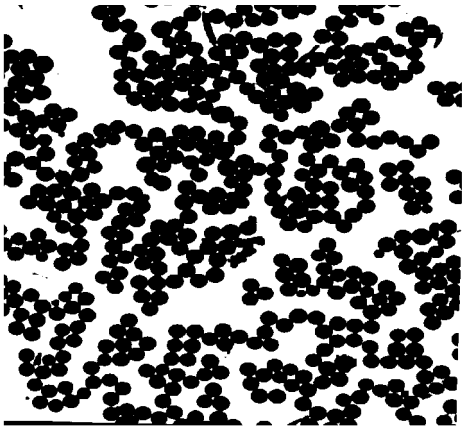

**Figure 2** TEM micrographs of luminol-$SiO_2$ nanoparticles formed in the TEOS/cyclohexane/water system at 10,000 × magnification.

### The modification of luminol-doped silica nanoparticle with Chitosan

We modified the luminol/$SiO_2$ nanoparticles produced above with chitosan. The modified nanoparticle and the unmodified nanoparticle were dropped on the Pt electrodes respectively. After being dried in air the electrodes were washed thoroughly and used for ECL measurement. Only the electrode with modified nanoparticles gave ECL signal. The result can be explained with the fact that chitosan could be attracted to Pt electrode and could not be removed from electrode easily. This proved that chitosan had been modified to luminol/$SiO_2$ nanoparticles.

### Preparation of luminol/$SiO_2$/ssDNA probe

According to the literature,[25,26] ssDNA can bind with chitosan via forming a tight chitosan-ssDNA complex. We added ssDNA solution to chitosan-modified luminol/$SiO_2$ nanoparticle solution. After acting for 120 min, the particles were centrifuged and separated. The supernatant was used for the UV measurement. The supernatant absorbance of DNA at 260 nm was much weaker than that before acting with particles. The result showed that most of ssDNA had been connected to particles and luminol/$SiO_2$/ssDNA probe was obtained. The probe was also applied to hybridize with its complementary sequence immobilized on the electrode. After that the electrode was used for ECL measurement. Strong ECL was observed, which indicated that ssDNA had been connected to luminol/$SiO_2$ particles. The results above proved that the luminol/$SiO_2$/ssDNA probe was successfully prepared. The immobilization schematic diagram of ssDNA on the chitosan-modified nanoparticle is showed in Figure 3.

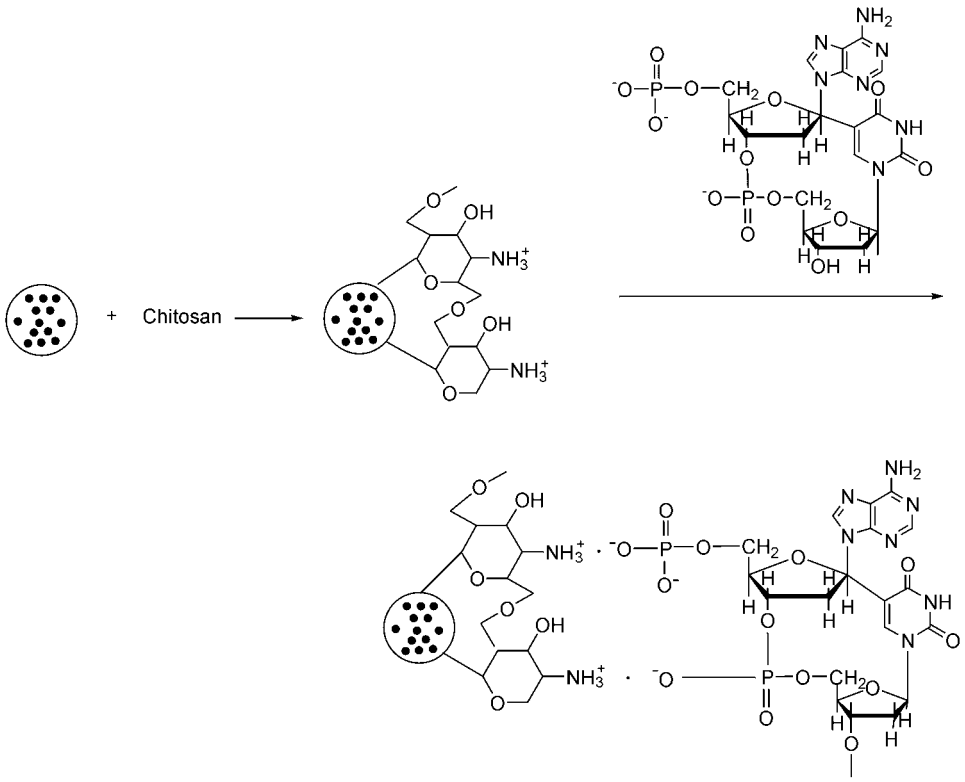


**Figure 3** Immobilization of ssDNA at chitosan modified silica nanoparticles.

## Optimization of the conditions for hybridization

It was reported that oligonucleotide-functionalized nanoparticles exhibit unique hybridization selectivity and effective discrimination ability between different target sequences.[27] And the hybridization is sensitive to the hybridization conditions, such as temperature and ionic strength. Here adjusting different temperatures and ionic strength, as shown in Figure 4 and Figure 5, we have optimized the conditions for hybridization and succeeded in detecting DNA sequence and discriminating three-base mismatch in 24-base DNA sequence.

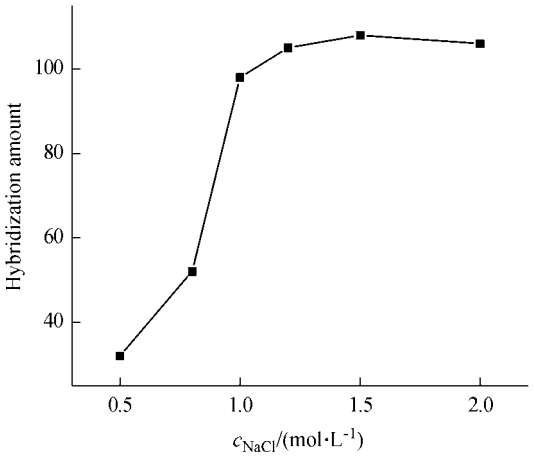


**Figure 4** Effect of NaCl concentration on the hybridization reaction of luminol/$SiO_2$/ssDNA probe with its complementary sequence in PBS solution. ssDNA concentration: $10^{-9}$ mol/L; hybridization time: 30 min; hybridization temperature: 40 ℃. The ECL conditions: +1.0 V (vs. Ag/AgCl) potential; $Na_2CO_3$-$NaHCO_3$ buffer (pH=10.0) and $1.2\times10^{-3}$ mol/L $H_2O_2$.

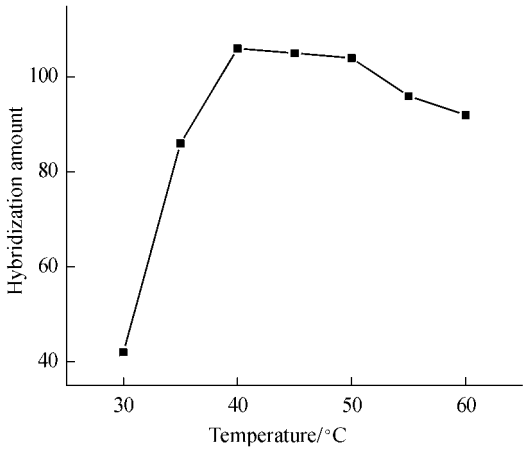


**Figure 5** Effect of temperature on the hybridization reaction of luminol/$SiO_2$/ssDNA probe with its complementary sequence in PBS solution. DNA concentration: $10^{-9}$ mol/L; Hybridization time: 30 min. The ECL conditions are the same as those in Figure 4.

## The recognition of Target oligonucleotide and ECL measurement

Different ssDNA sequences (complementary sequene, noncomplementary sequence and three-base mismatched sequence) were immobilized on the PPy/Pt electrode. The electrode was immersed in the luminol/$SiO_2$/ssDNA probe solution of PBS at 40 ℃ for 30

min. The electrode was washed thoroughly to remove the nonspecificly adsorpted ssDNA and then used as working electrode for the ECL measurement. As shown in Figure 6, only complementary sequence gave significant ECL emission. The noncomplementary sequence had negligible responses, which were equivalent to that of the blank measurement (without DNA on the electrode). Although three-base mismatched sequence had slight emission, it still could be identified with complementary sequence. This proved that the prepared luminol/$SiO_2$/ssDNA probe in this work was highly selective to the target DNA sequence and could be used to identify the DNA sequence with three-base mismatch. Figure 7 showed that the ECL intensity had a linear response with the concentration of complementary sequence in the range of $5.0\times10^{-12}$—$1.0\times10^{-9}$ mol/L. The regression equation is $I_{ECL}=19.5\ \lg\ c_{DNA}+248$. The detection limit was evaluated to be $2.0\times10^{-12}$ mol/L based on a signal to noise ratio of 3.

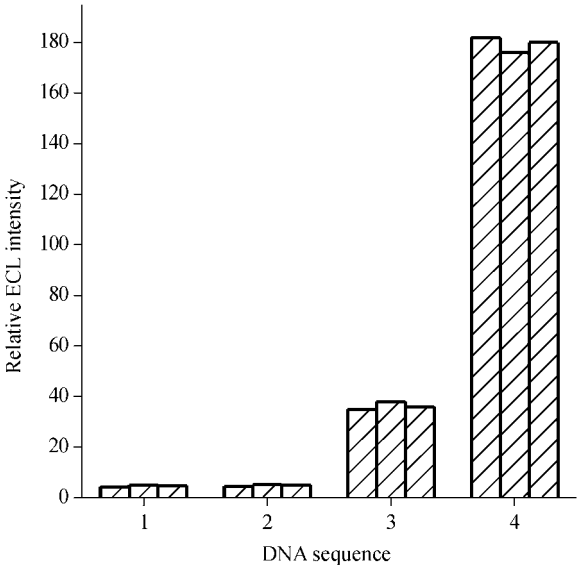


**Figure 6** Comparison of hybridization event of 24-base oligonucleotide of different sequence ($1.0\times10^{-9}$ mol/L) with luminol-$SiO_2$-DNA probe. 1, blank measurement; 2, noncomplementary sequence; 3, three-base mismatched sequence; 4, complementary sequence; (hybridization solution: PBS; hybridization time: 30 min.; hybridization temperature: 40 ℃).

## Conclusion

Luminol-doped silica nanoparticles of uniform sizes have been synthesized via reverse micelle for the first time. They were modified with chitosan and used for connecting with ssDNA. Then the ECL active substance luminol was labeled to DNA. The luminol/$SiO_2$/ssDNA probe was used for the recognition and determination of target DNA. The results showed that the probe had a good linear response to its complementary sequence in the range from $5.0\times10^{-12}$ to $1.0\times10^{-9}$ mol/L. The detection limit was $2.0\times10^{-12}$ mol/L. Due to the excellent ECL character of luminol and large number of luminol molecules inside silica nanoparticles, this method displayed higher sensitivity. It provided a simple, sensitive and inexpensive method for gene analysis and chip technology.

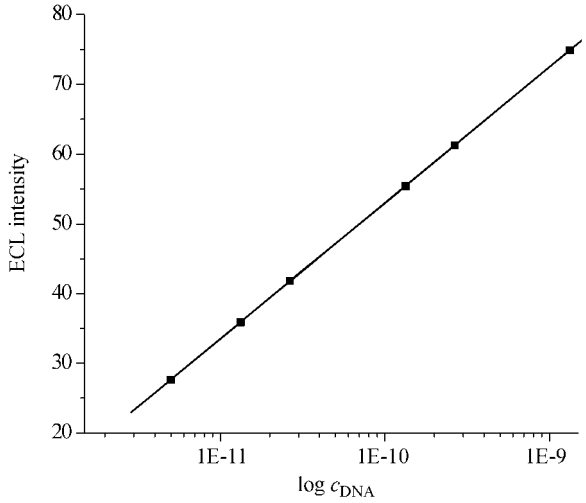


**Figure 7** Plot of ECL intensity vs. the logarithm of the concentration of complementary sequence The ECL conditions: +1.0 V (vs. Ag/AgCl) potential; $Na_2CO_3$-$NaHCO_3$ buffer (pH=10.0) and $1.2\times10^{-3}$ mol/L $H_2O_2$.

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