Analytical Methods

Rapid detection of dibutyl phthalate in liquor by a semi-quantitative multicolor immunosensor with naked eyes as readout

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Excessive intake of dibutyl phthalate (DBP) can cause serious health problem. Hence, it is necessary to develop some simple but sensitive method to monitor DBP level in foods. In this study, a multicolor competitive immunosensor for DBP detection has been proposed based on the etching of gold nanorods (GNRs) using naked eyes as readout. DBP was immobilized on the surface of wells by the physical absorption (called as solid-DBP) and used to capture the anti-DBP. The addition of target (called as added-DBP) can compete with the solid-DBP to combine with anti-DBP, leading to a little amount of anti-DBP immobilized on the plate. Then a horseradish peroxidase (HRP) labeled second anti-DBP was combined with anti-DBP to form the antigen-antibody-second antibody competition complex on the well surface. The amount of HRP immobilized on the plate was inversely proportional to the concentration of DBP in the sample. 3,3',5,5'-tetramethylbenzidine (TMB) was oxidized to produce the oxidized form of TMB (TMB^{2+}) at present of HRP, which can etch GNRs to different degrees within 90 s and results in the vivid color changing of the system because of the changing of localized surface plasmon resonance (LSPR) of GNRs and can be distinguished with the naked eye. The value of LSPR shift ($\Delta\lambda$) of GNRs was inversely proportional to the concentration of DBP in the range of 150 ~ 2700 μg L^{-1} (0.54 ~ 9.72 μM), the limit of detection was 76 ng L^{-1}. The concentration-dependent multicolor immunosensor was easier to achieve semi-quantitative or qualitative detection over the traditional system. The proposed multicolor immunosensor has been applied to detect DBP in liquor with satisfied results and provided a promising platform for on-site application in resource-poor regions.

Introduction

Dibutyl phthalate (DBP) is commonly used to improve the flexibility and stability of the polyvinyl chloride (PVC) production.[1, 2] Excessive intake of DBP can cause serious harm to the human health, such as abdominal obesity and insulin resistance.[3, 4] Many strict rules have been regulated to limit the amount of DBP in food. For examples, DBP should be absent in infant food and the quantity of migration from packing bag to food should be below 300 μg L^{-1} in European Union (EU).[5] The maximum residue of DBP is set to 300 μg L^{-1} in liquor by the Chinese National Standard.[6] Since PVC production has been used as transmission pipeline frequently during the process of liquors production, DBP in PVC production may migrate to the liquor and cause the contamination. So it is necessary to develop some simple but sensitive methods to detect DBP in liquor samples, especially for on-field detection (such as in resource limited area).

Many techniques, such as gas chromatography-mass spectra (GC-MS),[7-9] triple tandem mass spectrometry,[10] fluorescence,[11] gas chromatography -flame ionization detector （GC-FID）[12] and chemiluminescence[5] have been developed and applied to detect DBP with high sensitivity. Mostly of these methods required relative expensive and bulky equipment, and time-consuming steps, which was limited their application in the on-field detection. Enzyme-linked immunosorbent assay (ELISA)[13, 14] has been paid extensively attention owing to the special molecule recognition between antibody and its corresponding antigen. This technique had been coupled with many readout technologies, including electrochemistry,[15] fluorescence,[11] colorimetry[13] to develop simple but selective sensors for different targets. Among them, colorimetric has attracted growing attention owing to its inherent merits, such as low cost, simplicity, and rapid readability with naked eyes. The commercially available colorimetric immunoassay kits have been used for DBP detection on-field.[16] Only single colour changing presented in this method, and it is some time difficult to discern with the naked eyes if little colour difference presented. A spectrometer is needed to realize quantify detection, which limited their on-field applications. It is necessary to find some way to address this problem. The transition from the variation of monochromic intensity to multicolor changes may facilitate the

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improvement in accuracy as human eyes can distinguish 10 million of different colors.[17] Noble metal nanoparticles have been widely used to develop multicolour sensors for different targets since the solution colour of noble metal nanoparticles is highly dependent on their size, shape and composition [14, 18-27] Recently, our group develop some multicolor colorimetric sensors based on the regulation of the aspect ratio of gold nanorods (GNRs) and which in turn change the colour of the system.[28-31] Our early study indicated that TMB^{2+} can quantitatively etch GNRs and the system exhibit distinct colour changing (brown, green, blue, purple and pink and so on).[32] Compared with the early reported multicolour sensors based on metal nanoparticles, the proposed multicolor immunosensor was similar to the conventional colorimetric ELISA with the only difference in the colour display step: in the conventional ELISA an acidic solution is added to display a colour, whereas in our previous study a mixture solution consisting of acid and GNRs is added to display various colours.[32] Which is simple and chemical friendly. In this study, a competition colorimetric immunoassay was proposed for DBP detection for the first time, which combined the advantage of high specificity of the immune assay and the simple but sensitive of multicolor colorimetric detection. The affecting parameters, including CTAB concentration and incubated time were optimized to provide a good performance in sensitivity of the colorimetric immunosensor. The systems display multicolor changing and which can be easily distinguished by naked eyes and realize semi-quantitative analysis of DBP. The developed method has been used to detect the DBP in the liquor with high satisfaction.

Materials and methods

Regents and equipment

Enzyme-linked immunosorbent assay (ELISA) test kits and related experimental solutions were obtained from WDWK Biotechnology Co., Ltd. (Beijing, China). The ELISA kit contained 96-wells plate coated with DBP, a series of DBP standards, anti-DBP, anti-DBP- HRP and 20× wash buffer. TMB, substrate A (H_2O_2) and B (TMB,) solutions, ascorbic acid (Vc), dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-n-octyl phthalate (DNOP) and diallyl phthalate (DAP) were supplied by Aladdin (Shanghai, China). Cetyltrimethyl ammonium bromide (CTAB), gold (III) chloride tri-hydrate ($HAuCl_4 \cdot 3H_2O$), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), ascorbic acid hexane (C_6H_{14}) and sodium oleate (NaOL) were synthesized by Sinopharm (Shanghai, China). Methanol was bought from Fu Chen (Tianjin, China). Hydrochloric acid (HCl) was obtained from Lanxi Xuri (Zhejiang, China). All the other chemicals were of analytical grade. All the solutions were prepared with deionized water from DirectQ3 UV system (resistance 18.2 MΩ·cm, Millipore). The ultraviolet visible (UV-vis) spectrum of GNRs was determined by a micro-plate spectrophotometer (Multiskan GO, Thermo Scientific, USA) at room temperature. Transmission electron microscopy (TEM) images were performed on transmission electron microscopy (Tecnai G2 F20 S-TWIN, FEI, USA) to detect the surface morphology of GNRs. The images of the colorful wells were recorded by a Canon digital camera (Japan).

Synthesis of GNRs

GNRs were synthesized using a binary surfactant mixture.[33] The seed solution of GNRs was prepared as follow. Firstly, 0.6 mL of freshly prepared $NaBH_4$ solution (6 mM) in ice water was used to reduce $HAuCl_4$ (5 mL, 0.5 M), solution color turned from yellow to brown after vigorously stirring for 2 min and standing for 30 min at room temperature before using. A binary surfactant mixture containing CTAB and NaOL was used in GNRs growth solution. CTAB (1.4 g) and NaOL (0.2468 g) were dissolved in 100 mL of warm water, followed by being cooled to room temperature. $AgNO_3$ (7.2 mL, 4 nM) was added to the above solution before being stirred for 15 min. The solution presented colorless after $HAuCl_4$ (100 mL, 1 mM) was added to the above solution and continuously stirred for another 90 min. Then HCl (0.84 mL) was added to the wells to adjust the solution pH, followed by stirred for 15 min, Vc (0.5 mL 0.064 M) was mixed with the above solution before being added freshly prepared gold seed solution (160 μL). Lastly, solution was stirred for 30 s and kept static for 12 h. The UV-vis spectrums of the freshly synthesized GNRs were measured by a micro-plate spectrophotometer.

Construction of DBP standard colorimetric card

The standard colorimetric card was constructed by adding different concentrations of DBP according to the user guide of the ELISA kits. It should be noted the order of the addition of of DBP standard solution, anti-DBP, anti-HRP-DBP were important in measurement. A red shift of LSPR could be observed and multicolor was presented with the increasing of DBP concentration. LSPR peaks were directly measured by micro-plate spectrophotometer and the colourful wells were recorded by digital camera to construct DBP standard colorimetric card. Notably, GNRs required preprocess before using. The freshly prepared GNRs were centrifuged at 8000 rpm for 15 min to remove the supernatant; the precipitate was re-dispersed into CTAB solution with a final concentration of 0.1 M. The concentration of H_2O_2 and TMB were 1mM and 0.75 mM respectively. The pH of the solution was 7.00, 10 uL 5 M HCl was added to inactivate HRP. Each concentration had been detected for three times and the average values were used.

Detection of DBP in the liquor sample

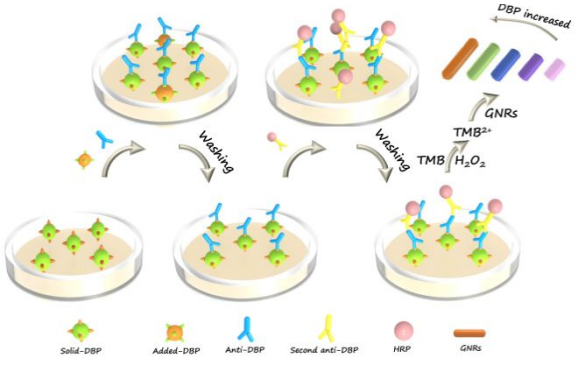
Erguotou from different makers were bought from local supermarket (Fuzhou, China) and were selected as the representative samples to confirm the potential application of the proposed immunosensor. Firstly, the DBP in liquor (5 mL) was extracted with 2 mL of cyclohexane under a vigorously shaking for 3 min, followed by standing for 10 min. The upper solution was dried with nitrogen current at room temperature and the extracted DBP was dispersed in methanol. The processed sample (50 μL) was introduced to wells for DBP detection; the steps were the same as that for the construction of standard colorimetric card. The obtained solution colour was compared

with the standard colour card to estimate the DBP concentration in the samples.

Results and discussion

Principle of the proposed multicolor immunosensor for DBP

Scheme 1 showed the principle of the proposed multicolor immunosensor for DBP detection. The strategy comprised three parts: competitively binding, oxidant production and colours presentation. The first step was to form a typical competition-format reaction. DBP was firstly immobilized on the surface of wells by the physical absorption (called as solid-DBP). The solid-DBP was utilized to capture the anti-DBP. The addition of DBP (called as added-DBP) competed with the solid-DBP to combine with anti-DBP, leading to a little amount of anti-DBP immobilized on the plate. The add-DBP, antibody, and add-DBP-antibody had not been modified on the surface of the plate can be washed easily. Then a HRP labelled second anti-DBP was combined with anti-DBP to form the antigen-antibody-second antibody competition complex on the well surface, the excessive amount of HRP labelled second anti-DBP had not been modified on the surface of the plate would be washed. So the amount of HRP immobilized on the plate was inversely proportional to the concentration of DBP in the sample. HRP then acted as a bridge linking immunization and colorimetric method. TMB was oxidized to TMB^{2+} by H_2O_2 at present of HRP.[34] GNRs were selectively shorted by TMB^{2+} from the ends while keeping the diameter nearly unaffected[35]. Large amount of TMB^{2+} will result in large degree of etching and the solution will display a colourful transition and which can be discerned with the naked eyes easily.[17] Based on this principle, a relationship between the amount of DBP in the sample and the colour of the system can be developed and used for semi-quantitative DBP detection. Therefore, the value of localized surface plasmon resonance shift ($\Delta\lambda$) of GNRs was inversely proportional to the concentration of DBP and can be used for quantitative DBP detection.



Scheme 1. The principle of the proposed immunosensor.

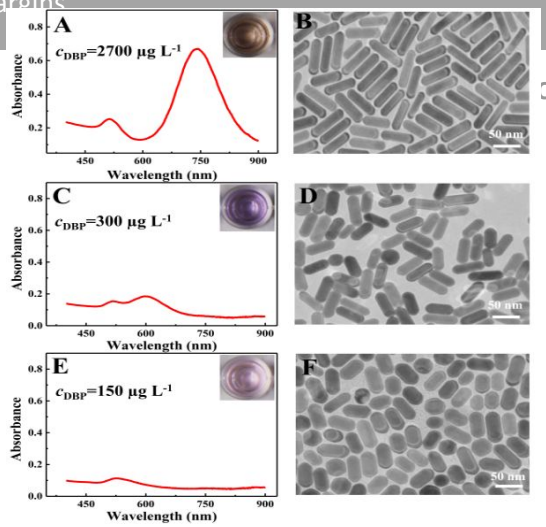


Figure 1. (A), (C) and (E) show the UV-vis spectrums, (B), (D) and (F) show the corresponding TEM images of GNRs in the presence of (A) and (B) 2700 μg L^{-1}, (C) and (D) 300 μg L^{-1} and (E) and (F) 150 μg L^{-1} DBP.

Simple experiments had been carried out to investigate the feasibility of the proposed immunosensor. Figure 1 was shown the LSPR peak, TEM images, and solution colours of the system at present of different amount of DBP. GNRs exhibited two separate surface plasmon resonances bands in the visible region, including the longitudinal plasmon band (LSP) and transverse plasmon band (TPB). At high added-DBP concentration (2700 μg L^{-1}), the LSP and TPB of GNRs were located at ~751 and ~525 nm, respectively, and the colour of the system is brown. The corresponding TEM image showed that the GNRs have an average aspect ratio of ~ 4.0 (Figure 1B). When DBP concentration changed to 300 μg L^{-1}, LSP was significant blue shifted to ~ 600 nm and peak intensities decreased as well, the colour of the system changed to purple (Figure 1C). The corresponding aspect ratio of GNRs decreased to ~ 3.0 (Figure 1D). When 150 μg L^{-1} DBP was added, the LSP disappeared and only the TPB presented, the colour of the system was pink (Figure 1E), indicating the transformation of GNRs into nanospheres. The corresponding aspect ratio was further decreased to ~ 1 (Figure 1F). These results verified that different concentrations of DBP would lead to multicolor presentation of the system and which confirmed the feasibility of the proposed multicolor immunosensor.

Optimization of the experimental conditions

The experimental conditions, such as concentration of CTAB, the incubation time between anti-DBP and DBP (both added-DBP and solid-DBP), and the reaction time between H_2O_2 and TMB, had been optimized to reach the best performance of the proposed system. $\Delta\lambda$ (the difference in LSPR peak of GNRs before and after the etching process) was chosen as the index to evaluate the effects of experimental conditions since the morphological of GNRs has a close relationship with the spectrum shift.[36] $AuBr^{2-}$-$(CTA)^{2+}$ complex was formed on the surface of GNRs to facilitate the etching of GNRs by TMB^{2+}. This is

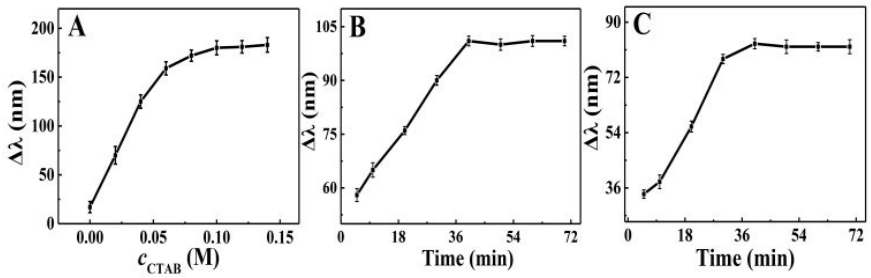


Figure 2. The effect of (A) CTAB, (B) the reaction time between anti-DBP and DBP and (C) the reaction time between H_2O_2 and TMB on LSPR shift.

attributed to the decrease in the oxidation potential of $AuBr^{2-}$-$(CTA)^{2+}$ / Au.[37] So the CTAB concentration has greatly effect on the performance of the system. As shown in Figure 2A, with the increase of CTAB concentration, the value of Δλ quickly increased and then reached a platform when the concentration of CTAB was over 0.1 M (Figure 2A). So 0.1 M CTAB was chosen for the further study. The amount of the labelled HRP on the plates strongly depended on the incubation time between anti-DBP and DBP. Lack of incubation time would reduce the sensitivity of the immunosensor. Figure 2B showed the relationship between Δλ and the incubation time between anti-DBP and DBP. The values of Δλ increased with the increase of the reaction time firstly and then reached maximum when the time was over 40 min, so 40 min was chosen as the optimized incubation time in the following study. The reaction time between H_2O_2 and TMB affected the amount of TMB^{2+} produced, so the reaction time has been optimized also. The value of Δλ increased rapidly at the initial stage and then reached a balance state after 40 min (Figure 2C). Therefore, 40 min was selected as the optimal reaction time for further study.

Calibration curve for DBP detection

The performance of the proposed competition multicolor immunosensor was evaluated under the optimized conditions. We investigated the concentration-dependence multicolor immunosensor by adding a series of DBP standard solution. The concentration of added-DBP standard solution varied from 150 to 2700 μg L^{-1}. UV-vis spectrums and colourful wells were recorded simultaneously. The added-DBP and the solid-DBP were competed to combine with anti-DBP; high concentration of added-DBP would result in a little amount of anti-DBP immobilized on the well of the plates, so a little amount of HRP could be immobilized on the plate through the selective reaction between DBP and HRP labelled second anti-DBP. Therefore, the amount of immobilized HRP was inversely proportional to the concentration of added-DBP. Different amount of HRP will result in different degrees of etching and the GNRs will become short with the decreasing of target concentration and the longitudinal peak of GNRs was blue shifted (Figure 3A). There has a good linear relationship between Δλ and lg c_{DBP} from 150 to 2700 μg L^{-1} (Figure 3B), the limit of detection was 76 ng L^{-1} (3 σ /slope). The equation can be presented as follow:

$$\Delta\lambda = -137.88 \lg c_{DBP} + 523.29, R^2 = 0.995$$

Where Δλ presents the difference in LSPR peak of GNRs before and after the etching process, c presents the concentration of added-DBP and R^2 is the regression coefficient. LSPR was red shifted with a vivid colour changing (such as pink, purple, blue, green and brown). Compared with the monochromic intensity changes from the traditional ELISA method, multicolour presentation from the proposed method could be discerned with the naked eyes easily (Figure 3C). EU has declared that the maximum reside of DBP in liquor should be below 300 μg L^{-1}.[38] We can obviously observe that were closely related to the concentration of added-DBP and the colours between safety (pink) and danger (purple, blue, green and brown) could be obviously distinguished with naked eyes. Hence, the proposed multicolor immunosensor could be used to evaluate DBP concentrations in the samples easily.

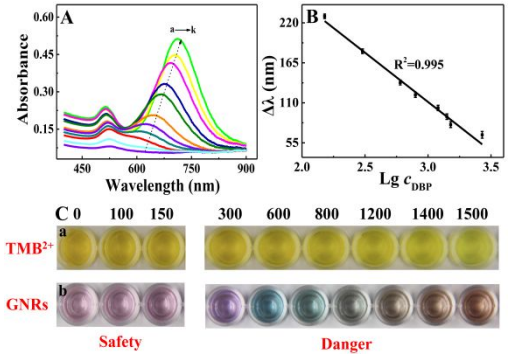


Figure 3. (A)UV-vis spectrums of GNRs (100 μL) reacted with TMB^{2+} (100 μL) after the addition of different concentrations of DBP. The concentration of DBP from (a) to (k): 0, 100, 150, 300, 500, 600, 800, 1200, 1400, 1500, 2700 μg L^{-1}, respectively; (B) The linear relationship between Δλ and lg c_{DBP}; (C) The colour changing of the system by (a) the traditional ELISA method and (b) the proposed method after the addition of different concentrations of DBP

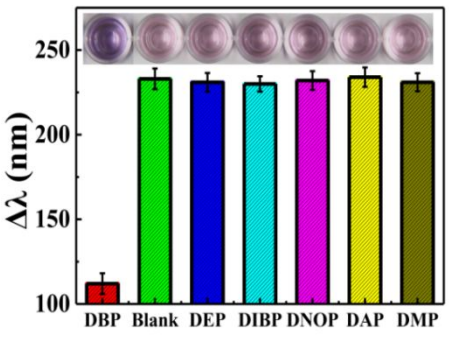


Figure 4. Selectivity of the multicolor colorimetric DBP immunosensor, LSPR shift of the GNRs after the addition of interferences and DBP the solution colours were shown inside the images.

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Table 1. Detection of DBP in liquor by the proposed method and the commercial assay kits.

Sample	Color	DBP kit assay* (μg L^{-1})	Calculated value** (μg L^{-1})	Spiked (μg L^{-1})	Color	DBP kit assay (μg L^{-1})	Calculated value (μg L^{-1})	Recovery	RSD
1		200.00	206.58	100		295.36	290.56	98.37 %	5.6%
2		218.78	209.47	100		314.06	301.24	95.92%	4.3%
3		210.09	208.11	100		302.02	297.00	98.28%	4.9%
4		227.43	205.42	100		315.37	304.17	96.45%	5.1%

*The concentrations of DBP were measured by the commercial DBP kit (DBP kit assay).
**The concentrations of DBP were calculated through the working curve.

Selectivity of the proposed immunosensor

Several compounds with similar structure, such as DEP, DIBP, DNOP, DAP and DMP, were selected as the potential interferences. The interferences mention above was separately added to the reaction system and the concentrations of interferences were 10-fold higher than that of DBP (300 μg L^{-1}). As shown in Figure 4, at present of the interference, Δλ was nearly the same with that of blank and the colour of the system was pink. But at present of the 300 μg L^{-1} of DBP, the Δλ has great changing with that at present of interference and the colour of the system changed to purple. The difference of colours of the system could be distinguished with the naked eyes easily. These results indicated the proposed immunosensor has high selectivity; this is probably attributed to high affinity between DBP and its corresponding antibody.

Detection of DBP in liquor samples

To further evaluate the real applicability of the proposed immunosensor, liquor of *Erguotou* from four makers were chosen as examples. The content of DBP in liquor can be accurately calculated through the standard curves and the concentration range can be estimated by standard card with naked eyes. The results were shown in Table 1. The colours of the system after the addition of different samples all showed pink, these outcomes indicated that the concentrations of DBP in the samples were between 150 to 300 μg L^{-1}. With the addition of DBP (100 μg L^{-1}) to the sample, the solution colour turned from pink to purple, indicating that the quantity of DBP was about ~ 300 μg L^{-1}. The concentration of DBP in liquor evaluated with the naked eyes was closed to the calculated value. Furthermore, both results were in good accordance with the values reached through the commercial available DBP assay kits. The standard addition recovery rates were in the range of 95.92 ~ 98.37 % and the RSD was range from 4.3 ~ 5.6 %. The solution colour between danger and safety can be obviously distinguished with the naked eyes. All results demonstrated the proposed immunosensor has a great potential in DBP detection on-field.

Conclusions

In summary, a multicolor immunosensor has been developed to determine the quantity of DBP in the liquor samples based on the etching of GNRs. LSPR was blue shifted and vivid multicolor were generated during the etching of GNRs. The proposed method combined the advantages of the high selectivity of the immunoassay and high sensitivity of vivid colour changing of the etching of GNRs by TMB^{2+}. The proposed biosensor displayed a concentration-dependent multicolor immunosensor that could be distinguished with the naked eyes easily and which has been applied to detect DBP in liquor samples with satisfied results. This multicolor assay displayed the advantages of simplicity and visualization, which could be used for on-field fast measurement after coupling with simple sample treatment.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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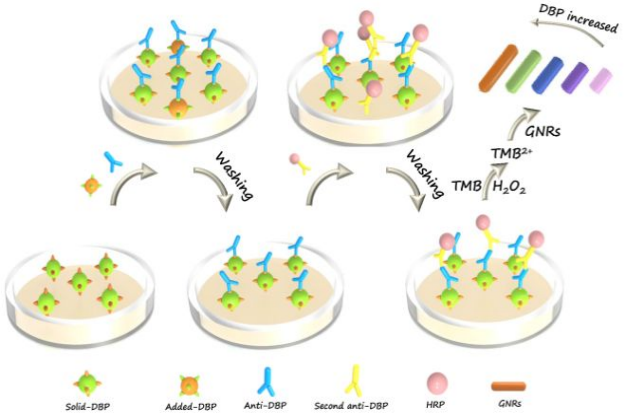

Rapid detection of dibutyl phthalate in liquor by a semiquantitative multicolor immunosensor with naked eyes as readout.