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ORIGINAL PAPER

A New Phosphorimetry for the Determination of Trace Alkaline Phosphatase Using Multi-wall Carbon Nanotubes and Its Diagnosis of Human Diseases

Jia-Ming Liu · Ying Rao · Li Chen · Xin-Xing Wang · Li-Ping Lin · Chang-Qing Lin · Li-Hong Zhang · Ying Ou-Yang

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Abstract Multi-wall carbon nanotubes (MWNTs) could be modified as water soluble MWNTs (it was called as MWNTs-A and MWNTs-B) by chemical methods. MWNTs-A and MWNTs-B could emit room temperature phosphorescence (RTP) signal on the surface of nitrocellulose membrane (NCM). A new solid substrate-room temperature phosphorimetry (SS-RTP) for the determination of trace alkaline phosphatase (ALP) was established based on the signal magnification effect of tween-80 and ALP on MWNTs-B's RTP intensity and the linear relationship between the content of ALP and the ΔI_P of the system. The linear range of this method was 0.0020–0.80 (fg spot^{-1}, sample volume: 0.40 μl spot^{-1}), the regression equation of working curve was $\Delta I_P = 0.8170 + 96.84 m_{ALP}$(fg spot^{-1}), correlation coefficient (r) was 0.9986. This method had high sensitivity (detection limit (LD): 1.4 ag spot^{-1}), good selectivity (Er≤± 5%, coexistence species were of no interference), high precision (RSDs were 4.4%-1.2%) and accuracy. It was applied to the determination of trace ALP in human serum and the diagnosis of human diseases. The results were tallied with those of enzyme-linked immunosorbent assay (ELISA). The mechanism of new SS-RTP for the determination of trace ALP was discussed, which laid the theory foundation for the analytical application of MWNTs in life science.

Keywords Multi-wall carbon nanotubes · Alkaline phosphatase · Solid substrate-room temperature phosphorimetry · Diagnosis of human diseases · Signal magnification

J.-M. Liu (✉) · L. Chen · X.-X. Wang · L.-P. Lin · Y. Ou-Yang
Department of Chemistry and Environmental Science,
Zhangzhou Normal Colleage,
Zhangzhou 363000, People's Republic of China
e-mail: zzsyliujiaming@163.com

Y. Rao
Department of Environment Science
and Engineering Fudna University,
200433 Shanghai, People's Republic of China

C.-Q. Lin · L.-H. Zhang
Department of Food and Biological Engineering,
Zhangzhou Institute of Technology,
Zhangzhou 363000, People's Republic of China

Introduction

According to the literatures, osteosarcoma [1], kidney disease [2], liver cirrhosis [3], metastatic neuroendocrine tumors [4] and other kinds of diseases will be caused if the content of ALP in human body is abnormal. Thus, searching for a new method with high sensitivity and accuracy to determine trace ALP, and studying the relationship between the content of ALP and human diseases have become a current research focus. There are many methods for the determination of ALP, such as surface-enhanced raman spectroscopy (LD: 1.0×10^{-8} g ml^{-1}) [5], electrochemical method (LD: 6.7×10^{-12} g ml^{-1}) [6], flow screen-printed amperometric method (LD: 7.0×10^{-12} g ml^{-1}) [7], potentiometric method (LD: 0.1 g ml^{-1}) [8], flow injection potentiometric method [9] and ELISA [10]. The maximal sensitivity of the methods mentioned above is pg level, and using the content of ALP to diagnose human diseases is rarely reported. Obviously, searching for a new method with high sensitivity and accuracy for the determination of ALP has important academic research value; meanwhile, it has significance in prevention and cure of human diseases.

In recent years, there are some references to the application of MWNTs in many fields, such as the determination of trace metallic ions by electrophoretic deposition [11], preparation of transmission electron microscope [12] and microsensor [13]. However, SS-RTP for the determination of trace bioactivity substance by MWNTs is rarely reported. There have been some reports on the determination of trace glucose (LD: 0.1 fg glucose spot^{-1}) [14] and mercury (LD: 0.3 fg mercury spot^{-1} [15], 0.2 fg mercury spot^{-1} [16]) by SS-RTP, indicating that SS-RTP has high sensitivity and wide application prospect. Whereas, can MWNTs emit RTP signal? Can a high sensitive SS-RTP be established for the determination of trace ALP and the diagnosis of human diseases using the luminescent property of MWNTs?

In this study we found that when MWNTs successively reacted with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2 in ultrasonic disintegrator at 25 °C, carboxyl reaction took place on the surface of MWNTs, and MWNTs was changed into MWNTs-B; MWNTs-B could emit weak RTP on the solid substrate of NCM, which limited its analytical application; tween-80 had sensitizing effect on the RTP signal of MWNTs-B; ALP could greatly enhance the RTP intensity of MWNTs-B, and the content of ALP was proportional to the ΔI_P of the system. According to the facts above, a new SS-RTP for the determination of trace ALP based on MWNTs-B spiked by tween-80 has been established. This research result not only exploits the analytical application of MWNTs in life science, but also promotes the development of MWNTs and SS-RTP research.

Experimental

Apparatus and reagents

Phosphorescent measurements were carried out on a Perkin-Elmer LS-55 luminescence spectrophotometer with a solid surface analysis apparatus (Perkin Element Corporation of U.S.). The instrument's main parameters were as follows: delay time: 0.1 ms; gate time: 2.0 ms; cycle time: 20 ms; flash count: 1; Ex. slit: 10 nm; Em. slit: 10 nm; scan speed: 1,500 nm min^{-1}. Other apparatus used were JEM-2000EX transmission electron microscope (TEM, Japan Electric Company), KQ-250B ultrasonic washing machine (Kunshan Ultrasonic Machine Company), AE240 electronic analytical balance (Mettler-Toledo Instruments Company Limited) and electrically heated thermostat (Tai-ke instruments Company, Beijing). A 0.50-μl flat head micro-injector (Shanghai Medical Laser Instrument Plant, China) was used to introduce the test solution of μl level and blank reagent.

Preparation of ALP (Sigma Company) working solution: 1.00 mg ml^{-1} ALP solution was prepared and then gradually diluted to 10.00 and 1.00 pg ml^{-1} with Na_2CO_3-$NaHCO_3$ buffer solution (pH=9.12). MWNTs (S.MWNTs-4060, Φ= 40–60 nm, L=1–2 μm; Shenzhen Nanotech Port Co., Ltd.) and 3.5% (V/V) tween-80 were also used in this experiment. All reagents were of A.R. grade except that ALP was primary standard reagent. The water used was prepared by thrice quartz sub-boiling distillation.

Filter paper, polyamide membrane (PAM), acetyl cellulose membrane (ACM) and NCM were purchased from Xinhua Paper Corporation (Luqiaosijia Biochemical Plastic Plant, Hangzhou, China). The paper sheers were pre-cut into wafers (Φ=1.5 cm).

Preparation of MWNTs-A and MWNTs-B

The structure of MWNTs was modified by chemical methods [17, 18] to prepare MWNTs-A and MWNTs-B, respectively. 0.10 g MWNTs, 6.00 ml concentrated H_2SO_4 and 3.00 ml concentrated HNO_3 (2:1, V/V) were added into beaker, and then reacted at 25 °C for 24 h in ultrasonic disintegrator. After filtrating (with 0.22 μm filter membrane), and washing for 3 times, the filtrate was collected and diluted to 100 ml with water, the water soluble MWNTs (MWNTs-A) was obtained. To improve the solubility of MWNTs and obtain more intense RTP signal of MWNTs, 0.10 g MWNTs, 6.00 ml concentrated H_2SO_4 and 3.00 ml concentrated HNO_3 (2:1, V/V) were added into another beaker, and reacted at 25 °C for 24 h in ultrasonic disintegrator. And then 8.00 ml concentrated H_2SO_4 and 2.00 ml of 30% H_2O_2 were added into the mixture, stirring for 30 min. After filtrating, washing for 3 times, the filtrate was collected and diluted to 100 ml with water, the water soluble MWNTs (MWNTs-B) was obtained.

Measurement of phosphorescence

Certain amount of ALP, 2.00 ml MWNTs-B and 2.00 ml tween-80 were added into a 25-ml colorimetric tube, diluted to 25 ml with water, mixed homogeneously, stored at 40 °C for 15 min, and then cooled by flowing water for 5 min. NCM was pre-cut into wafers (Φ=15 mm) and a ring indentation (Φ=4.0 mm) was made at the center of each sheet with a standard pinhole plotter. They were immersed in 1.00 mol l^{-1} Pb^{2+} solution for 10 sec and then dried at 90±1 °C for 2 min. Then, 0.40 μl of reagent was suspended on NCM by a 0.50-μl flat head micrometer syringe. The NCM wafers were dried at 90±1 °C for 2 min. At the same time, a reagent blank experiment was also conducted. The I_{P2} of test solution (MWNTs-B + tween-80 + ALP) and the I_{P1} of reagent blank (MWNTs-B + tween-80) were measured at the wavelength of 454/618 nm ($\lambda_{ex}^{max}/\lambda_{em}^{max}$). Then ΔI_P (= I_{P2}–I_{P1}) was calculated.

Results and discussion

TEM images of MWNTs and MWNTs-B

The TEM images of MWNTs and MWNTs-B were photographed by TEM (Fig. 1a–b). Results showed that MWNTs-B (treated with mixed acid for six times) had clean surface, integrated tubular, good uniformity and reproducibility compared with MWNTs.

Phosphorescence spectra

The phosphorescence spectra of MWNTs, MWNTs-A and MWNTs-B-tween-80-ALP were scanned by the experimental method described above, and shown in the Fig. 2a–b. When 1.00 mol l^{-1} Pb^{2+} was used as the ion perturber, MWNTs could emit weak RTP signal on the surface of NCM (λ_{em}^{max} = 650.4nm, I_P=20.6, Fig. 2a, curve B and Table 1). The λ_{em}^{max} of MWNTs was obviously different from that of NCM (λ_{em}^{max} = 585.0nm, Fig. 2a, curve A and Table 1). After treatment with H_2SO_4-HNO_3, MWNTs-A could emit RTP signal (λ_{em}^{max} = 630.8nm, I_P=35.5, Fig. 2a, curve C and Table 1), and the RTP signal was stronger than that of MWNTs (ΔI_P=14.9), with a blue shift from 650.4 nm to 630.8 nm compared with MWNTs (Fig. 2a, curve B and Table 1). The reason contributing to blue shift of λ_{em}^{max} and the enhancement of RTP signal of MWNTs-A might be similar to fluorescence changes reported in Refs. [19, 20]. There might be two results after being modified by H_2SO_4-HNO_3: first, MWNTs was cut short, which caused the energy spacing between the lowest empty orbital and highest occupied orbital of MWNTs to augment, the phosphorescence peak of MWNTs was shifted to shorter wavelength [19, 20]; second, the number of disfigurement of MWNTs was increased and the energy of excitation capture was strengthened, the RTP signal was also enhanced [17]. The RTP signal of MWNTs increased sharply after adding H_2SO_4 and H_2O_2 ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 453.5/623.5nm, I_P=60.1, ΔI_P=39.5, Fig. 2a, curve D and Table 1). The λ_{em}^{max} of MWNTs-B (Fig. 2a, curve D and Table 1) was blue shifted from 650.4 nm to 630.8 nm compared with MWNTs-A (Fig. 2a, curve C and Table 1). The reason might be that MWNTs were further cut short [19, 20]. According to the RTP signal of MWNTs-B was enhanced after being dealt with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2, it could evidently indicate that the water-solubility of MWNTs was enhanced.

In the presence of tween-80, the RTP intensity of MWNTs-B was enhanced ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 449.4/616.5nm, I_P=109.6, Fig. 2b, curve E and Table 1) after being heated at 40 °C for 15 min. The ΔI_P was 49.5, which indicated that tween-80 had an enlargement effect on the RTP intensity of MWNTs-B (it was 1.8 times larger than that without tween-80). When 50.00 pg ALP was added, the RTP intensity of MWNTs-B was sharply enhanced ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 453.5/618.1nm, I_P=189.9, Fig. 2b, curve G and Table 1), the ΔI_P was 80.4. Thus, 454/618 nm was chosen as the working wavelength for the determination of ALP.

Optimum measurement condition

Optimization of the volume and concentration of reagents

For the system containing 0.16 fg ALP $spot^{-1}$, the effects of volume and concentration of reagents on the ΔI_P were studied, respectively. Results showed that when the volume and concentration of reagents were as follows: 2.00 ml of 3.5% tween-80, 2.00 ml MWNTs-B and 0.10 g MWNTs, the ΔI_P of the system reached the maximum and remained stable (Table 2).

Selection of solid substrate

For the system containing 0.16 fg ALP $spot^{-1}$, the effects of different substrates, such as PAM, ACM, NCM and paper on the ΔI_P of the system were examined, the ΔI_P were 8.8 (RSD was 3.9%), 9.7 (RSD was 3.5%), 14.4 (RSD was 2.1%), 7.9 (RSD was 4.1%), respectively. Results showed that the ΔI_P of the system reached the highest (14.4) when NCM was used. Thus, NCM was selected as the solid substrate in this experiment.

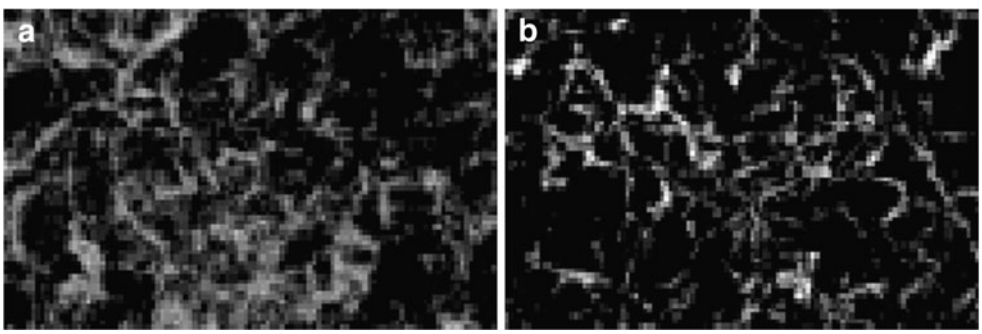

Fig. 1 **a** TEM image of MWNTs. **b** TEM image of MWNTs-B

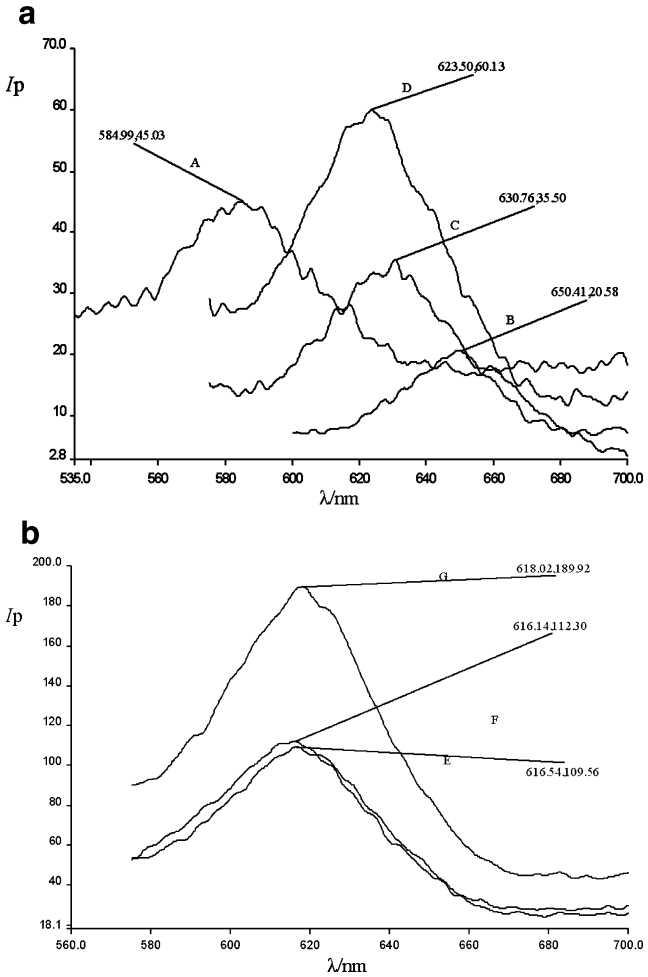

Fig. 2 **a** RTP spectra for MWNTs, MWNTs-A and MWNTs-B (Curves B, C and D are phosphorescence peaks of MWNTs, MWNTs-A and MWNTs-B, respectively. MWNTs-A was water-soluble MWNTs after being dealt with H_2SO_4-HNO_3. MWNTs-B was water-soluble MWNTs after being dealt with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2.). **b** RTP spectra for MWNTs-B-tween-80-ALP system (Curves E, F and G are phosphorescence peaks of MWNTs-B-tween-80, MWNTs-B-tween-80-50.00 pg ALP and MWNTs-B-tween-80-0.13 pg ALP, respectively.)

Selection of sensitizer

For the system containing 0.16 fg ALP spot^{-1}, the effects of different sensitizers, such as sodium laurycsulfonate, sodium dodecyl benzene sulfonate, cetyltrimethyammonium bromide, polyacrylamide, poly sodium acrylate, TrtionX-100 and tween-80 on the ΔI_P were examined, the ΔI_P were 11.7 (RSD was 2.8%), 11.0 (RSD was 3.1%), 8.3 (RSD was 4.0%), 9.9 (RSD was 3.7%), 10.8 (RSD was 3.3%), 11.2 (RSD was 2.9%) and 14.5 (RSD was 2.1%), respectively. Results showed that the ΔI_P of the system reached the highest when tween-80 was used. Thus, tween-80 was chosen as the sensitizer in this experiment. The ΔI_P of the system with tween-80 (14.5) was 2.6 times larger than that without it (5.4). It might be that tween-80 and ALP reacted to form the sensitized-micelle inclusion complex, which made the rigidity of MWNTs-B increase [14].

Effect of ion perturbation

For the system containing 0.16 fg ALP spot^{-1}, the effects of ions, such as Cu^{2+}, Ag^{+}, Pb^{2+} and I^{-} with the same concentration of 1.00 mol l^{-1} on the ΔI_P were examined, the ΔI_P were 8.6 (RSD was 4.2%), 12.8 (RSD was 2.4%), 14.3 (RSD was 1.6%) and 13.6 (RSD was 2.0%), respectively. Results showed that the ΔI_P of the system reached the maximum when Pb^{2+} was used. Thus, Pb^{2+} was selected as the ion perturber. The heavy atom effect of Pb^{2+} can greatly enhance the transition probability from the singlet state (S_1) to triplet state (T_1) of the luminescent particles, thus make the ΔI_P of the system increase sharply. When the concentration of Pb^{2+} were 0.20, 0.40, 0.50, 0.80, 1.00 and 1.20 mol l^{-1}, the corresponding ΔI_P values were 8.5 (RSD was 4.1%), 9.7 (RSD was 3.7%), 10.2 (RSD was 3.5%), 12.4 (RSD was 2.6%), 14.3 (RSD was 1.8%) and 13.8 (RSD was 2.5%), respectively. Results showed that the ΔI_P of the system reached the maximum when 1.00 mol l^{-1} Pb^{2+} was used. For MWNTs-B, the enhacing order of heavy atom was $Pb^{2+} > I^{-} > Ag^{+} > Cu^{2+}$, namely, as the mass of the heavy atom increased, the RTP signal enhanced.

Table 1 Phosphorescent property of MWNTs, MWNTs-A, MWNTs-B and MWNTs-B-tween-80-ALP system

No.	Sample	λ_{em}^{max} (nm)	I_P	ΔI_P	$\Delta\lambda_{em}^{max}$ (nm)
A	NCM	585.0	45.0		
B	NCM + 0.10 g MWNTs	650.4	20.6		
C	NCM + 2.00 ml MWNTs-A	630.8	35.5	14.9	19.0
D	NCM + 2.00 ml MWNTs-B	623.5	60.1	39.5	7.3
E	NCM + 2.00 ml MWNTs-B' + 2.00 ml tween-80	616.5	109.6	49.5	7.0
F	NCM + 2.00 ml MWNTs-B' + 2.00 ml tween-80 + 0.13 pg ALP	616.1	112.3		
G	NCM + 2.00 ml MWNTs-B' + 2.00 ml tween-80 + 50.0 pg ALP	618.1	189.9	80.4	

Table 2 Effects of volume and concentration of reagents on ΔI_P values

Reagents	Concentration or volume	The ΔI_P values of MWNTs-B-tween-80-ALP	Optimum values
tween-80 (%) RSD (%)	1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5	6.6, 7.9, 9.5, 10.2 , 14.4, 9.6, 7.6 4.1, 3.9, 3.7, 3.4, 3.0, 3.6, 3.8	3.5%
tween-80 (ml) RSD (%)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0	6.5, 7.8, 10.0, 14.3, 10.9, 9.6, 8.5, 7.0 4.2, 4.0, 3.5, 2.4, 3.1, 3.8, 4.0, 4.3	2.00 ml
MWNTs-B (ml) RSD (%)	0.10, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00	8.5, 10.7, 12.4, 13.1, 14.3, 12.9, 11.1 3.6, 3.1, 2.7, 2.4, 2.0,. 2.8, 3.0	2.00 ml
MWNTs (g) RSD (%)	0.010, 0.050, 0.10, 0.15, 0.20	7.4, 9.6, 14.5, 14.4, 14.4 3.8 ,3.5, 2.2, 2.3 , 2.4	0.10 g

Acidity for reaction

For the system containing 0.16 fg ALP spot^{-1}, the effects of different pH values on the ΔI_P of the system were examined. When the pH values were 0.94, 1.33, 1.52, 2.02, 4.10, 6.75 and 10.00, the corresponding ΔI_P values were 14.4 (RSD was 2.0%), 14.4 (RSD was 1.9%), 14.5 (RSD was 1.7%), 14.4 (RSD was 2.0%), 12.5 (RSD was 2.6%), 10.8 (RSD was 3.2%), 8.3 (RSD was 4.1%), respectively. Results showed when the value of pH was within the range of 1.52–2.02, the ΔI_P of the system reached the maximum and remained stable. We could see that carboxyl reaction could carry out successfully in concentrated acid condition. It caused the soluble MWNTs-B increased. Finally, the ΔI_P values reached the maximum.

Temperature and time for the reaction

For the system containing 0.16 fg ALP spot^{-1}, the effects of reaction time and temperature on the ΔI_P of the system were examined. When the reaction time were 5, 10, 15, 20, 25 and 30 (min), the ΔI_P values were 9.3 (RSD was 3.5%), 12.4 (RSD was 2.5%), 14.3 (RSD was 2.0%), 13.4 (RSD was 2.4%), 13.1 (RSD was 2.6%) and 9.0 (RSD was 3.7%), respectively. And when the temperature were 15, 30, 40, 50, 60 and 70 (°C), the ΔI_P values were 9.3 (RSD was 3.2%), 10.9 (RSD was 3.0%), 14.5 (RSD was 1.8%), 12.6 (RSD was 2.1%), 11.3 (RSD was 2.8%) and 10.1 (RSD was 3.0%), respectively. Results showed when the reaction time was 15 min and the reaction temperature was 40 °C, MWNTs successively reacted with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2 to form water soluble MWNTs-B, which caused the ΔI_P reached the maximum.

Oxygen and humidity

For the system containing 0.16 fg ALP spot^{-1}, when drying N_2 was passed into the system for 10, 20 and 30 min, the ΔI_P were 14.1 (RSD was 2.5%), 14.3 (RSD was 2.1%) and 14.6 (RSD was 1.6%), respectively. And when drying N_2 was not passed into the system for 10, 20, and 30 min, the ΔI_P were 14.2 (RSD was 2.4%), 14.5 (RSD was 2.1%), and 14.7 (RSD was 1.8%), respectively. Results showed that the system was stable whether drying N_2 was passed or not. Therefore, we would not pass drying N_2 to simplify the experiment.

Stability of the system

Under the optimal conditions above, for the system containing 0.16 fg ALP spot^{-1}, when the time were 5, 10, 15, 20, 25 and 30 min, the ΔI_P were 14.3 (RSD was 2.2%), 14.4 (RSD was 2.0%), 14.4 (RSD was 1.9%), 14.3 (RSD was 2.5%), 14.3 (RSD was 2.1%) and 14.4 (RSD was 1.8%), respectively. Based on these facts, it showed that the ΔI_P of the system stayed invariable and had good repeatability within 5–30 min.

Table 3 Linear range, RSD, LD, luminescent reagent and Ref. (0.0020 fg spot^{-1} and 0.80 fg spot^{-1} of ALP were measured in parallel for 7 times, respectively.) and LD (calculated by 3Sb/*k* which referred to the quotient between decuple of the blank reagent's standard deviation and the slope of the working curve. When the blank solution was measured repeatedly for 11 times, the I_P were 109.6, 109.5, 109.5, 109.5, 109.6, 109.5, 109.6, 109.6, 109.5, 109.5 and 109.5, respectively. Sb was 0.046.)

Method	Linear range (g ml^{-1})	RSD (%)	LD (g ml^{-1})	Luminescent reagent.	Ref.
SS-RTP by ALP enhancing MWNTs-B's RTP	5.0×10^{-15}–2.0×10^{-12} 2.0–800.0 (ag spot^{-1})	4.4–1.2	3.5×10^{-15} 1.4 (ag spot^{-1})	MWNTs-B	
Electrochemical method	2.0×10^{-11}–2.5×10^{-9}		6.7×10^{-12}	Tyrosinase	Ref. [6]
Affinity adsorption SS-RTP	5.0×10^{-15}–800.0×10^{-15}	3.9–3.1	1.1×10^{-15}	Rhodamine 6G	Ref. [21]
Affinity adsorption SS-RTP	5.0×10^{-15}–800.0×10^{-15}	3.6–4.9 3.1–4.7	1.0×10^{-15} 1.1×10^{-15}	Rhodamine 6G–dibromoluciferin	Ref. [22]

Table 4 Effects of coexistent ions or coexistent materials on ALP (0.40 pg ALP ml^{-1} and 0.40 pg ALP ml^{-1}-X μg (allowed concentration) coexistence materials and ions were parallelly determined by present method (n=6). Meanwhile, its relative error and allowed multiple (the quotient obtained between X μg allowed ions or materials and 0.40 pg ALP ml^{-1}) were calculated.)

This method				Ref. [22]
Coexistent ions or coexistent materials	Allowed concentration	Multiple	Realative error (%)	Multiple
K^+	120.0 μg	3.0×10^5	2.2	2.7×10^4
F^-	120.0 μg	3.0×10^5	3.9	2.7×10^4
Cl^-	120.0 μg	3.0×10^5	2.2	2.7×10^4
NO_2^-	120.0 μg	3.0×10^5	5.0	2.7×10^4
L-Alanine	40.0 μg	1.0×10^5	1.7	2.7×10^4
L-Threonine	120.0 μg	3.0×10^5	4.8	2.7×10^4
Br^-	36 μg	9.0×10^4	−3.7	2.7×10^4
SO_4^{2-}	36 μg	9.0×10^4	−4.5	2.7×10^4
$C_2O_4^{2-}$	36 μg	9.0×10^4	−3.8	8.0×10^3
SCN^-	36 μg	9.0×10^4	−4.8	8.0×10^3
Ni^{2+}	36 μg	9.0×10^4	2.7	8.0×10^3
Zn^{2+}	36 μg	9.0×10^4	1.9	8.0×10^3
Ti (IV)	36 μg	9.0×10^4	4.0	8.0×10^3
L-Proline	36 μg	9.0×10^4	3.0	8.0×10^3
L-Serine	36 μg	9.0×10^4	3.8	8.0×10^3
L-Leucine	72 μg	1.8×10^5	4.0	8.0×10^3
Ba^{2+}	32 μg	8.0×10^4	4.9	7.0×10^3
Cd^{2+}	32 μg	8.0×10^4	2.8	7.0×10^3
Bi^{3+}	32 μg	8.0×10^4	3.9	7.0×10^3
L-Valine	86 μg	2.2×10^5	2.5	7.0×10^3
L- Histidine	32 μg	8.0×10^4	1.9	7.0×10^3
L-Isoleucine	96 μg	2.4×10^5	2.5	
L-Cysteine	24 μg	6.0×10^4	4.0	
Fe^{3+}	20 μg	5.0×10^4	2.0	3.0×10^3
Fe^{2+}	20 μg	5.0×10^4	3.4	3.0×10^3
L-Arginine	60 μg	1.5×10^5	3.1	3.0×10^3
ClO_4^-	1.2 μg	3.0×10^3	−2.4	800
BrO_3^-	1.2 μg	3.0×10^3	−1.5	800
L-Glutamine	70 μg	1.7×10^5	2.5	800
L-Tryptophan	1.2 μg	3.0×10^3	3.5	800
Mg^{2+}	0.6 μg	1.5×10^3	1.5	600
L- Aspartic acid	90 μg	2.2×10^5	2.6	600
L-Phenylalanine	0.6 μg	1.5×10^3	4.2	600
L- L ysine	48 μg	1.2×10^5	2.4	400
Ca^{2+}	0.32 μg	0.8×10^3	2.5	
Hg^{2+}	0.32 μg	0.8×10^3	3.1	400

Working curve, sensitivity and precision

When the ALP concentrations in standard solution were 0.0050, 0.040, 0.20, 0.40, 0.60, 1.20, 1.60 and 2.00 (pg ml^{-1}) and the system was determined for 7 times by present method, the ΔI_P average values of systems were 2.7 (RSD was 4.4%), 3.9 (RSD was 4.2%), 8.5 (RSD was 3.8%), 14.3 (RSD was 2.0%), 22.8 (RSD was 1.8%), 46.9 (RSD was 1.6%), 61.4 (RSD was 1.4%) and 80.4 (RSD was 1.2%), respectively. Results showed that the content of ALP had a good linear correlation to the ΔI_p, and the regression equation of the working curve could be expressed as $\Delta I_p = 0.8170 + 96.84m_{ALP}$ (fg $spot^{-1}$, with the volume of 0.40 μl per spot, n=7), r=0.9986. The linear range, RSD, LD and

luminescent reagent of this method were compared with those of Refs. [6, 21, 22]. Results are listed in Table 3.

We could conclude that the sensitivity of this method was higher than that of reference [6], and the linear range was wider than that of Refs. [6, 21, 22]. Compared with references [21, 22], though the sensitivity of present method was lower, it was suitable for the determination of trace ALP in living creature. Besides, present method had several merits, such as simple and rapid operation, low analysis cost, and a new phosphorescence labelling reagent was also exploited, which provided a new way for the application of MWNTs.

Interference experiment

For the system containing 0.16 fg ALP spot^{-1}, the allowed concentration of coexistence ions and materials (Er≤±5%) were compared with the Ref. [22] (160.0 ag ALP spot^{-1}). The results are listed in Table 4.

From Table 4, we could see that the interference extent order of non-polar amino acid on ALP was: L-proline > L-alanine > L-leucine > L-valine > L-isoleucine. The interference extent order of polar amino acid on ALP was: L-cysteine > L-serine > L-Threonine. The interference extent order of acid and alkaline amino acid on ALP was: L-histidine > L-lysine > L-arginine > L-glutamine > L-aspartic acid, which indicated that the interference extent of acid amino acid was higher than that of alkaline amino acid. The interference extent order of aromatic amino acid was: L-phenylalanine > L-tryptophan. Thus, we could see that the allowed multiple of coexistence of present method was higher than that of Ref. [22], indicating that the selectivity of present method was better.

Table 5 ALP in human serum, RSD% and percent recovery obtained by present method and ELISA (n=8)

Serum	Obtained (ng ml^{-1})	RSD (%)	Added (ng ml^{-1})	Obtained (ng ml^{-1})	Recovery (ng ml^{-1})	Percent recovery (%)	ELISA (ng ml^{-1})
1	50.1	1.5	5.00	55.2	5.1	102	51.3
2	55.4	1.8	6.00	61.4	6.0	100	54.2
3	53.6	2.1	5.00	58.7	5.1	102	55.1
4	57.8	1.6	6.00	64.0	6.2	103	58.4
5	52.7	1.9	5.00	57.9	5.2	104	53.5
6	58.3	1.4	6.00	64.4	6.1	102	59.5
7	60.2	1.7	6.00	66.5	6.3	105	60.8
8	56.9	1.3	6.00	63.2	6.3	105	58.6
9	61.5	1.2	6.00	67.6	6.1	102	62.1
10	51.4	1.7	5.00	56.6	5.2	104	52.7
11	28.9	3.9	3.00	31.9	3.0	100	28.1
12	27.3	2.3	3.00	30.3	3.0	100	27.9
13	19.0	3.1	2.00	21.1	2.1	105	19.7
14	13.4	2.7	2.00	15.4	2.0	100	13.6
15	30.6	3.6	3.00	33.7	3.1	103	31.3
16	34.8	2.4	3.00	37.8	3.0	100	35.4
17	38.5	3.5	4.00	42.7	4.2	105	39.5
18	41.7	2.8	4.00	45.7	4.0	100	42.5
19	45.2	3.2	5.00	50.3	5.1	102	45.6
20	47.1	2.5	5.00	52.1	5.2	104	48.0
21	10.7	4.2	1.00	11.7	1.0	100	10.3
22	9.2	2.3	1.00	10.2	1.0	100	9.6
23	12.7	2.9	1.00	13.7	1.0	100	12,3
24	11.8	4.3	1.00	12.8	1.0	100	12.2
25	8.9	4.7	1.00	9.8	0.9	90.0	8.7
26	10.6	4.0	1.00	11.6	1.0	100	11.0
27	9.8	4.5	1.00	10.7	0.9	90.0	9.6
28	10.3	3.2	1.00	11.3	1.0	100	10.7
29	11.5	3.2	1.00	12.5	1.0	100	11.9
30	12.4	3.1	1.00	13.4	1.0	100	12.8

Analysis of samples

Serums from 30 persons were diluted with Na_2CO_3-$NaHCO_3$ buffer solution (pH=9.12) until the concentration of ALP reached the level of pg ml^{-1}. The ALP content of the samples was determined by the method described above, and a standard addition recovery rate experiment was also conducted. This method was compared with ELISA method (Zhangzhou Traditional Chinese Medical Hospital of Fujian in China), and the results are listed in Table 5.

From Table 5, we could see that results of this method were tallied with those of clinical detection and diagnosis by Zhangzhou Traditional Chinese Medical Hospital. We could conclude that the ALP content of serum 11–20 within 13.1–49.2 ng ml^{-1} was in the normal range, the ALP contents of serum 21–30 were lower than 13.1 ng ml^{-1}, and the ALP contents of serum 1–10 were higher than 49.2 ng ml^{-1}. According to the decrease of ALP content in liver patient's serum and the increase of ALP content in osteopathy patient's serum, we can diagnose that serum 21–30 might be the liver patients' serum; while serum 1–10 might be the osteopathy patients' serum. According to the results of clinical detection and diagnosis, treatment on patients 1–10 and 21–30 was done. After 4 weeks, the ALP content was determined by the method described above and the result was 13.1–49.2 ng ml^{-1}, which indicated that the ALP content in patients 1–10 and 21–30 were in the level of healthy people. Meanwhile, no diseases were found after B diasonagraph detection and computer-X-Ray-faultage-photography detection. Based on the facts above, we can use this method to determine trace ALP in human serum and diagnose human disease.

Mechanism of SS-RTP for determination of trace ALP

According to Ref. [17], when MWNTs successively reacted with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2, the carboxylation reaction was carried out, while the number of defect of MWNTs was increased, which made the capture of excitation energy and the fluorescence signal strengthened. The similar phenomenon was found in the analysis of phosphorescence.

In this paper, when MWNTs successively reacted with H_2SO_4-HNO_3 and H_2SO_4-H_2O_2, the RTP intensity was enhanced from I_P=20.6 (MWNTs) to I_P=35.5 (MWNTs-A) and I_P=60.1 (MWNTs-B), respectively, and λ_{em}^{max} had blue shift from 650.4 nm to 630.8 nm (MWNTs-A) and 623.5 nm (MWNTs-B), respectively. The enhancement of RTP signal showed that there was a carboxylation reaction of MWNTs occurring in the system, and made the defects and the water-solubility of MWNTs increased. The reason for the blue shift of λ_{em}^{max} of RTP signal from MWNTs-A and MWNTs-B might be that the MWNTs were cut short continuously [19].

In the presence of tween-80, there may be two results: 1. hydrophobic groups of tween-80 reacted with the hydrophilic groups of MWNTs-B to form the amphiphilic structure, reducing the surface tension of solution and changing the interface state of the system [23] to enhance the solubility of MWNTs-B. 2. Tween-80 was dispersed and adsorbed to the side-wall of MWNTs-B. The polyoxyethylene of tween-80 extended to MWNTs layer and densely covered on the surface of MWNTs to form a hydrophilic phase with certain thickness [24], which made the water-solubility of MWNTs enhanced, and ultimately led the RTP intensity of MWNTs to increase greatly.

In the system of tween-80-MWNTs-B, $-NH_2$ in ALP, which was similar to wheat germ agglutinin, could react with the free -COOH on the surface of MWNTs-B to form peptide bond [25], increasing the rigid structure of MWNTs-B and causing the RTP intensity of MWNTs-B to increase greatly (Fig. 2b, curve G). The ΔI_P was linear to the content of ALP. Thus, we could use the SS-RTP to determine trace ALP.

If the nanotubes are too large, they will not bind in the active site of ALP. However, the nanotubes (S.MWNTs-4060, Φ=40–60 nm, L=1–2 μm) were suitable to ALP in this experiment. The linear relationship between ALP and ΔI_P showed that the free $-COOH$ on the surface of MWNTs-B could successfully react with the $-NH_2$ in ALP, namely, MWNTs-B could bind in the active site of ALP.

Conclusion

Based on tween-80 enhancing MWNTs-B's RTP signal, we have proposed a new SS-RTP for the determination of trace ALP and a new technique for the diagnosis of human diseases, and have exploited the analytical application of MWNTs and SS-RTP in life science.

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