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Microscopic determination of tetracycline based on aluminum-sensitized fluorescence of a self-ordered ring formed by a sessile droplet on glass slide support

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Abstract

A fluorescent microscopic determination of trace amount of tetracycline is reported based on the aluminum-sensitized fluorescence effect of a self-ordered ring formed by a sessile droplet on glass slide support. Since the evaporative loss of the solvent from the edge wedge of the droplet that is spotted on a hydrophobic-treated glass slide, an outward capillary flow of the interior solvent of the droplet occurs. The resulted outward capillary flow then carries the solute to the perimeter of the droplet spot where the solute accumulates to form a fluorescent self-ordered ring (SOR). Depending on the spotted volume of the aluminum–tetracycline chelate solution, different size of SOR with the outer diameter (o.d.) less than 1.1 mm and the ring belt width less than 21.6 μm can be obtained. Data analysis for the imaged SOR by using a digitalized CCD camera showed that the chelate molecule across the fluorescent SOR belt section follows a Gaussian distribution, and the maximum fluorescent intensity (I_{max}) was found to be proportional to tetracycline content. When a 0.1 μl droplet was spotted on the solid surface, tetracycline in the range of 7.5–800.0 fmol (or 7.9×10^{-8} to 800.0×10^{-8} mol l^{-1}) can be detected, and the limit of detection can reach 0.8 fmol (or 7.9×10^{-9} mol l^{-1}). With present method, the contents of tetracycline in capsule, tablet, urine and fresh milk were satisfactorily detected with the recoveries of 97.0–106.5% and RSD of 1.2–4.2%, correspondingly.
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Keywords: Tetracycline; Aluminum; Self-ordered ring (SOR) technique; Fluorescent microscopic analysis

1. Introduction

Fluorescent detection methods have led to major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity [1]. One of the most exciting aspects of fluorescence technologies is their ability to support decreasing sample sizes down to the single-molecule detection level [2,3], which in turn provides the opportunity for miniaturization and high-throughput screening [4,5]. For example, fluorescence microscopy, when coupling digital imaging acquisition and dried spot

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technique, has been widely used in biochemical studies including the total quantification of nucleic acid and protein, DNA sequencing, PCR product analysis, and determination of trace amounts of fluorescent drugs, medicines and other fluorescent analytes [6–9].

Dried spot analysis, for example on the solid support such as thin film substrate including octadecylsilanized silica and poly(vinyl chloride) plate [9,10], has many advantages since it can form a nearly perfect ring system, and has a good reproducibility, allowing the technique to approach the detection capabilities of inductively coupled plasma mass spectrometry (ICPMS) [11] and digitally imaging facilities such as CCD camera [12]. By spotting a tiny amount of a liquid sample onto a thin film substrate, where it is subsequently dried into a small solid residue, the analytes are concentrated prior to analysis and the background arising from the sample substrate is further reduced[8]. On the other hand, the dried spot technique procedure removes differences in matrix chemistry from different solutions [11]. However, the sensitivity improvement for the spot analysis chiefly focuses on how to reduce the background contribution from the thin film substrate on the glass slide support [13].

The evaporation of a sessile droplet on a substrate and then leaving ring like stains are not particular to coffee, but also is a general phenomenon in nature such as mineral rings left on washed glassware, banded deposits of salt on the sidewalk during winter, and enhanced edges in water color paintings [14,15]. Lately, there has been growing importance concerning this simple phenomenon. When a droplet of dilute solution containing dispersed solutes is spotted onto a hydrophobic solid surface, the evaporation loss of solvent from the edge of the drop should be replenished by the solvent from the interior of the spotted drop in order to keep the edge of the drop pinned, an outward capillary flow occurs and then carries the dispersed solutes of the spotted drop outward which deposit along the edge of the droplet spot. The deposition of the solutes forms a self-ordered ring (SOR) [10,16,17]. Reports can be found for the formation of mesoscopic ring structures on solid surfaces by using a variety of materials including porphyrin derivatives, nano-particles and carbon nanotubes [18]. High-throughput automatic DNA mapping methods and arrays of DNA spots for gene expression analysis based on drying droplets have been developed [19,20]. Our studies have showed that the proposed spot technique, when coupling with fluorescent microscopic imaging technique, can be used for highly sensitive determination of trace amounts of fluorescent drugs, medicines and other fluorescent analytes.

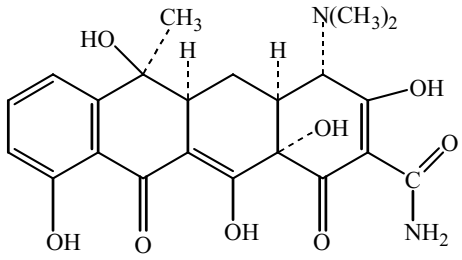

Fig. 1. Chemical structure of tetracycline.

Tetracycline is an important member of antibiotics (Fig. 1). Due to the wide applications in clinical practice and veterinary medicine, animal nutrition and as feed additives [21], it has coincidentally produced a rising concern regarding the presence of residues from such agents in the tissue, serum, urine and food supply. Thus, the quantification of tetracycline is of importance. Tetracycline reacts with di- or trivalent metal ions to form highly fluorescent chelates and the fluorescence intensity of the chelates under acidic, neutral or alkaline conditions are stronger than free tetracycline although it shows strong fluorescence under basic conditions [22]. Extensive literatures were available and numerous methodologies have been developed for analytical purposes, including spectrofluorimetry [23], spectrophotometry [24], chromatography [21,25], polarography [26], and microorganism technology [27]. These methods, however, have suffered from the disadvantages of (i) limited sensitivity and lower tolerance level of coexisting foreign substances [23,24,26]; (ii) time consuming and poor precision and specificity [27]; and (iii) using of poisonous organic solvents [21]. In routine monitoring of trace amounts of tetracycline in pharmaceutical formulations as capsule, human urine, fresh milk, the methods should be rapid, precise, economical in cost and time, and friendly to the environment. Thus herein we developed a sensitive method of trace amount tetracycline using aluminum-sensitized fluorescence

with the SOR technique, and only consumed nanoliter to microliter samples, supplying a possibility that monitors the pharmacological studies in tissues or cells.

2. Experimental

2.1. Apparatus

The SOR formed on the surface of glass slide was observed under an Olympus IX70 inverted microscope system, equipped with a 100-W mercury arc lamp and a BV mirror cube unit with excitation filter of 400–440 nm, barrier filer of 475–800 nm, the dichroic mirror of DM 455 nm, the 4× objective (N.A. 0.10) and 10 × objective (N.A. 0.25) (Olympus, Tokyo, Japan). The SOR image was captured by employing a Cohu 4910 series cooled CCD (Cohu, CA, USA) coupled with Scion Image software package for Windows. An MVS-1 vortex mixer (Beide Scientific Instrumental Ltd., Beijing, China) was used to blend the solutions in 0.5 ml micro-tubes. The Origin 5.0 software package was used for the linear and Gaussian fits.

2.2. Reagents

Tetracycline stock solution (1.00×10^{-3} mol l^{-1}) was prepared by dissolving appropriate amount of hydrochloride salt of tetracycline (Amresco Inc., OH, USA) in doubly distilled water and kept in a refrigerator at 4 °C. Further dilute standard solutions were freshly prepared by appropriate dilution with water before use. 1.00×10^{-3} mol l^{-1} aluminum ion stock solution was prepared by dissolving $KAl(SO_4)_2 \cdot 12H_2O$ with 0.1 mol l^{-1} HCl (Chongqing Reagent Plant, Chongqing, China). 0.6% (w/v) solution of poly(vinyl alcohol)-124 (PVA-124, polymerization degree is 1700–2400, and hydrolysis degree (saponification) is 98–99 mol%; Shanghai Chemical Reagent Co., Shanghai, China) in water, and a 4.5% (v/v) solution of dimethyl dichlorosilane (DMCS, The First Chemical Reagent Plant of Shanghai, Shanghai, China) in toluene were used. Tris–HCl solution (pH 5.2) was used as reaction medium. All reagents were of analytical-reagent grade without further purification. Doubly distilled water was used throughout.

2.3. Preparation of hydrophobic glass slides

Glass slides were cleaned and pretreated mainly according to reference [28]. In order to clear off the oil impurities on the surface, the slides were first ultrasonically washed with detergent and then with 18.0 mol l^{-1} H_2SO_4 saturated with $K_2Cr_2O_7$, respectively. After washed with doubly distilled water and acetone, the glass slides were then dried by nitrogen flow, and immersed in 4.5% (v/v) DMCS in toluene at room temperature. The residue of the DMCS–toluene was removed at last by immersing in $CHCl_3$ and in acetone, respectively, and then dried with nitrogen gas flow.

2.4. Pretreatment of samples

Capsules and tablets of tetracycline hydrochloride were purchased from Beiling Pharmaceutical Ltd. (Chongqing, China) and Southwest Pharmaceutical Industrial Ltd. (Chongqing, China), respectively. According to reference [29], the tablets were pounded into powders. About 0.25 g of the fine powder and content of capsules were accurately weighed and directly dissolved in 0.01 mol l^{-1} HCl, respectively, and diluted to 250 ml with doubly distilled water. The filtrate were further diluted 10-fold with doubly distilled water and used as analyte of samples. The fresh human urine and milk samples were 25- and 250-fold diluted with doubly distilled water, respectively before general analysis procedures.

2.5. Procedure

Typically, appropriate working solutions of tetracycline, or sample solutions, 60 μl of 1.00×10^{-4} mol l^{-1} aluminum ion solution and 100 μl Tris–HCl solution were made up to 0.5 ml micro-tube and then vortex-mixed. After standing 30 min, 75 μl of 0.6% (w/v) PVA-124 solution was added and the mixture was diluted with doubly distilled water to 0.5 ml, and mixed thoroughly. Then 0.1–1.0 μl of the mixture was spotted on the surfaces of the pretreated glass slides. The glass slides were immediately transferred to a 70 °C oven for 3–5 min, then observe and measure the formed SOR image under Olympus IX 70 inverted microscope system, and the observed SOR image was captured by employing a Cohu 4910 series cooled

CCD coupled with Scion Image software package for Windows.

3. Results and discussion

3.1. Features of SORs

Fig. 2A shows a typical planform of the yellow-green fluorescent SOR image of the aluminum–tetracycline chelate formed on a DMCS pretreated glass slide, and Fig. 2B displays the digital distributed presentation of the chelate molecules through the ring center. In Fig. 2A, the o.d. (2R) of the ring is ca. 1.1 mm if 0.5 μl chelate solution was spotted on the glass slide, and

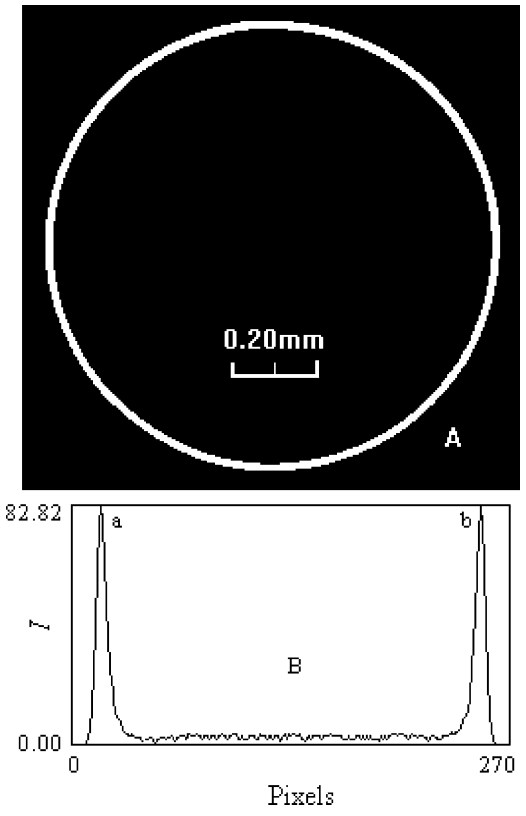

Fig. 2. A typical planform of the yellow-green SOR image of aluminum–tetracycline chelate formed on a DMCS pretreated glass slide (A) and the fluorescence intensity of chelate molecules through the ring center (B). Spotted solution: tetracycline, 4.0×10^{-6} mol l^{-1}; Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA, 0.09% (w/v); pH, 5.2. Droplet volume, 0.5 μl. A 4 × objective was used. The o.d. (2R) is 1.1 mm, and the SOR belt width (2δ) is 21.6 μm.

the SOR belt width (2δ) is 21.6 μm. Both the size of the ring (2R) and the ring belt width (2δ) will increase with increasing droplet volume. In Fig. 2B, it can be seen that the SOR is symmetrical and the fluorescence intensity in and out of the SOR is near to zero. Namely, the contribution from the background is very small, which is a great improvement compared to the traditional dried spot method using thin film substrate such as octadecylsilanized silica and poly(vinyl chloride) plate [9,10]. By fitting the fluorescence intensity data of Fig. 2B, we found that the distribution of chelate molecules across the SOR belt section follows a Gaussian function (Fig. 3, $\chi_a^2 = 13.15$, $\chi_b^2 = 10.98$), and the two σ-values, half bandwidth of the Gaussian curve where the fluorescence intensity is $0.607I_{max}$, are much close to each other (Fig. 3, $\sigma_a = 2.407$ pixels, ca. 11.55 μm; $\sigma_b = 2.440$ pixels, ca. 11.71 μm when 4 × object was used). This Gaussian distribution of the molecules on the SOR is identical to the report of other authors [30], and the deposition process depends on the initial concentration of the solution and the profile of the shrinking droplet. As Fig. 4 shows, the SOR belt width (2δ) will slightly increase with increasing initial chelate concentration under the same droplet volume, but the o.d. of the ring is changeless.

3.2. Relationship between the radius of SORs and the volume of the droplet

A small aqueous drop (0.x μl) spotted onto a hydrophobic substrate can be regarded as a segment before the solvent evaporation occurs, and the radius (R) of the spotted drop on the substrate can be expressed as [31,32]

$$R = \left\{ \frac{6}{\pi tg(\theta/2)[3 + tg^2(\theta/2)]} \right\}^{1/3} V^{13} = KV^{13} \quad (1)$$

where R is the radius of the spotted drop on the substrate. Due to the hydrophobic features of the DMCS pretreated surfaces, the contact line is pinned to its initial position and the R-values will not change with the evaporation of the solvent [17]. So, R is, in fact, the outer radius of the SOR, θ the contact angle of the drop with the substrate, which is decided by the hydrophobic features of the substrate that was pretreated with DMCS and the properties of the solution. The greater the contact angle θ is, the smaller the size of the SOR will be. V is the volume of the droplet.

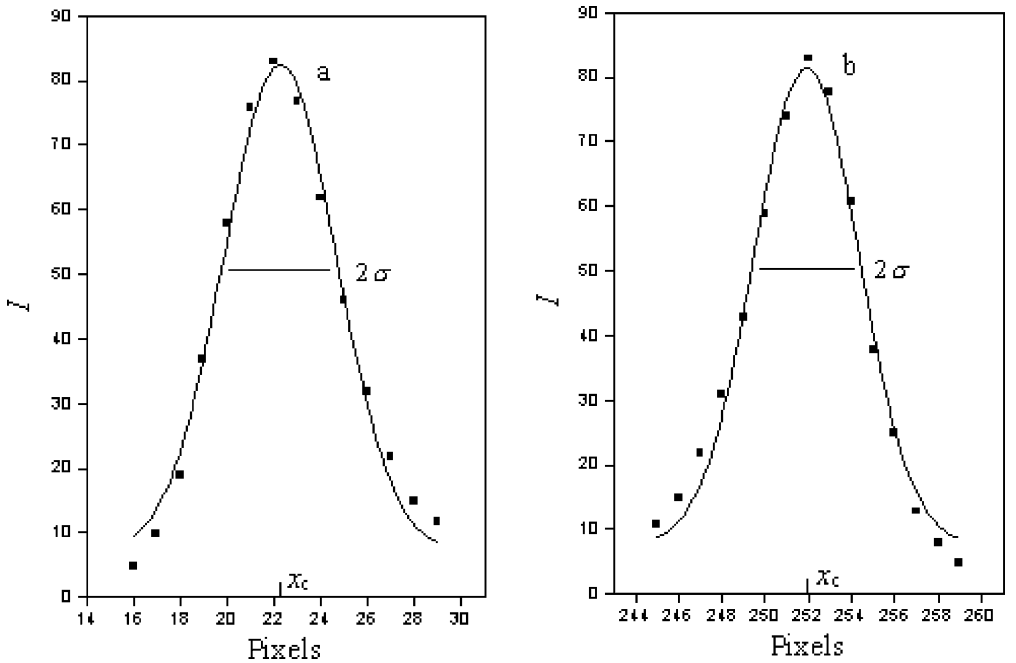

Fig. 3. Gaussian fits of peak a and b in Fig. 2B. Square symbol is original data, Straight line without symbol is Gaussian fit data. Peak 'a': $I = 6.94 + (94.66/\sqrt{\pi/2})e^{-0.086(x-22.26)^2}$, ($\chi_a^2 = 13.15$, $\sigma_a = 2.407$ pixels, about 11.55 μm), Peak 'b': $I = 7.35 + (93.01/\sqrt{\pi/2})e^{-0.084(x-251.9)^2}$, ($\chi_b^2 = 10.98$, $\sigma_b = 2.440$ pixels, about 11.71 μm).

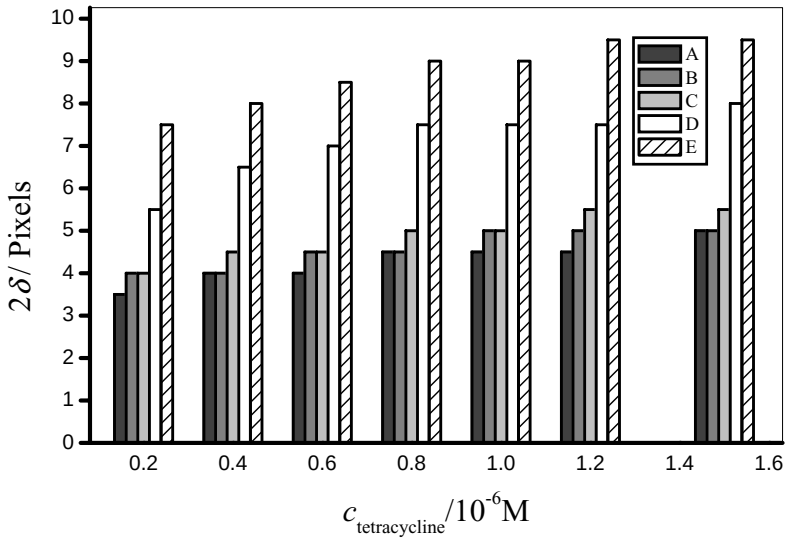

Fig. 4. Dependence of SOR belt width (2δ) on the content of tetracycline. Spotted solution: Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. A 4 × objective was used for all droplets. Droplet volume (μl): (A) 0.1; (B) 0.3; (C) 0.5; (D) 1.0; (E) 1.5.

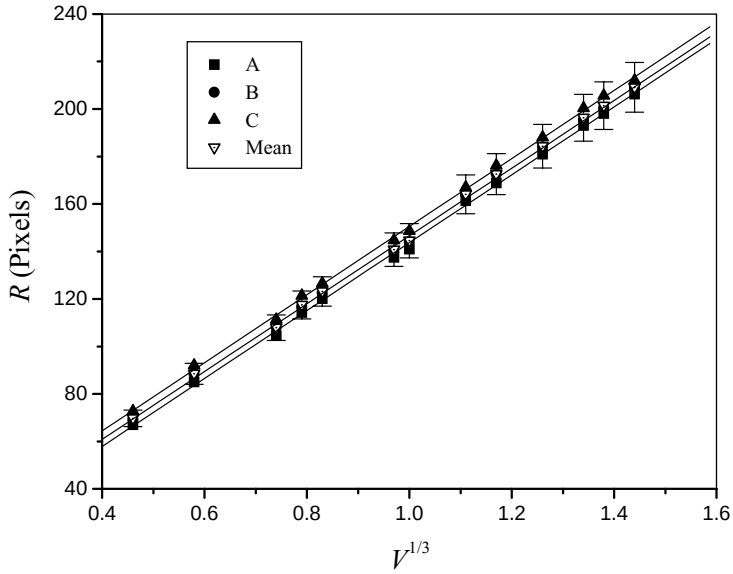

Fig. 5. Relationship between the outer radius of SORs and the volume of the droplet. Spotted solution: tetracycline, 4.0×10^{-6} mol l^{-1}; Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. A 4 × objective was used for all droplets. A, B and C refer to three different batch pretreated glass slides. Linear regress equations: (A) $R = 0.7010 + 143.0V^{1/3}$ ($r = 0.9995$); (B) $R = 3.769 + 142.8V^{1/3}$ ($r = 0.9995$); (C) $R = 7.078 + 143.4V^{1/3}$ ($r = 0.9996$). Error bars were obtained at 5% level of the average value of three outer radius data of SOR formed by using same droplet volume on the three different pieces of glass glides.

Eq. (1) shows that the radius of the SOR is proportional to $V^{1/3}$ if K is a constant. Fig. 5 shows Eq. (1) is perfectly obeyed in the range of spotting 0.1–3.0 μl droplet solution. In addition, it can be seen that the results by using three pieces of glass slide are identical, and the reproducibility is very good, indicating the hydrophobic pretreatment procedures of the glass slides with DMCS are reasonable.

3.3. Optimization of the general procedure

First, the effect of pH on chelate of aluminum–tetracycline was studied. The assay showed that the fluorescence intensity of SOR depended on the reaction medium of the spotted solution. After having tested the buffer solutions, including HAc–NaAc, Britton–Robinson, Tris–HCl, and hexahydropyridine–HCl and hydropyridine–HCl, we found that the maximum fluorescence intensity of SOR (I_{max}) is the strongest only when using Tris–HCl solution of pH 5.2. As Fig. 6 shows that optimal pH values are in the range of 4.9–5.5 and any pH values out of this range make the fluorescence intensity of SOR diminish, especially at pH 7.0 the fluorescence intensity almost decreases to zero, which is possibly due to the formation of aluminum hydroxide.

We found that the formed chelate of aluminum ion with tetracycline have strong fluorescence after the droplet evaporated. Fig. 7 indicates that the aluminum-sensitized fluorescence is constant when the concentration of aluminum ion is equal to tetracycline. The 1:1 molar ratio of aluminum and tetracycline can be proved also by keeping the total concentration of aluminum and tetracycline as 8.0×10^{-6} mol l^{-1} while the concentrations of the two components are changed simultaneously (Fig. 8). The result is in agreement with the results of other reports [23,33]. However, in a practical situation, the concentration of tetracycline acting as an analyte is unknown, and an excess of aluminum ion must be used. In this assay, we kept the aluminum ion concentration is 1.2×10^{-5} mol l^{-1}.

We and other authors [10,35] have reported that PVA-124 plays an important role in the SOR formation since it could modify the properties of a solid surface by adhering onto the hydrophobic substrates, and it is

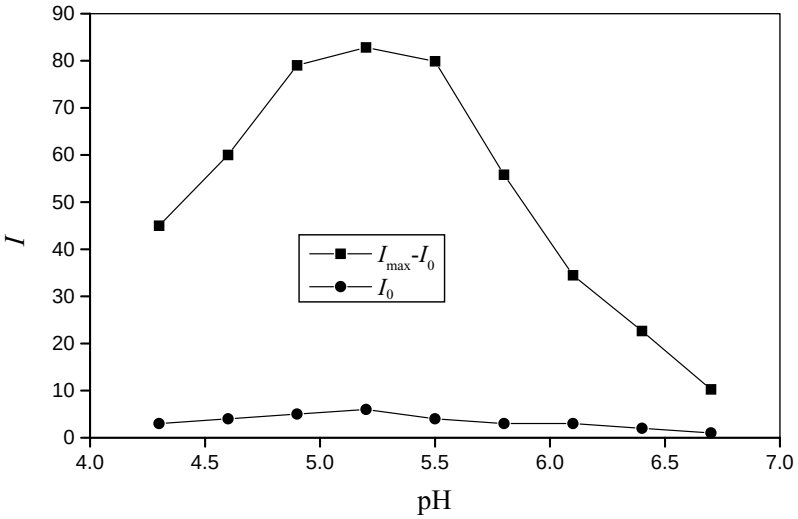

Fig. 6. The pH effect of the spotted solution on the fluorescence emission of SOR. I_0 was the fluorescence intensity of the formed SOR without tetracycline. Spotted solution: tetracycline, 4.0×10^{-6} mol l^{-1}; Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v). Droplet volume, 0.5 μl. A 4 × objective was used.

the most effective ring-forming assistant among several kinds of water-soluble polymers [10,34,35]. The formation of an SOR is strongly dependent on the concentration of PVA. If the concentration of PVA-124 were too small, the ring-like deposit that contains almost all the solute is not formed on the hydrophobic substrate. If its concentration were too large, however, the σ-values of the Gaussian curve would increase, accompanying the decrease of I_{max} values [35]. One possible reason is that high viscosity in the solution with increasing PVA-124 can also modify the deposition by preventing the drop from attaining an equilibrated droplet shape, and some part of the solutes may be included in the high viscous PVA-124 membrane,

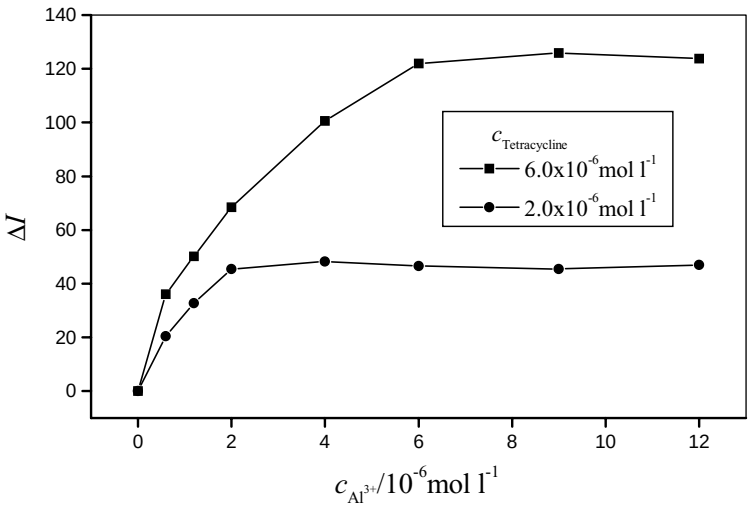

Fig. 7. Influence of aluminum ion concentration. Spotted solution: PVA-124, 0.09% (w/v); pH, 5.2. Droplet volume, 0.5 μl. A 4 × objective was used.

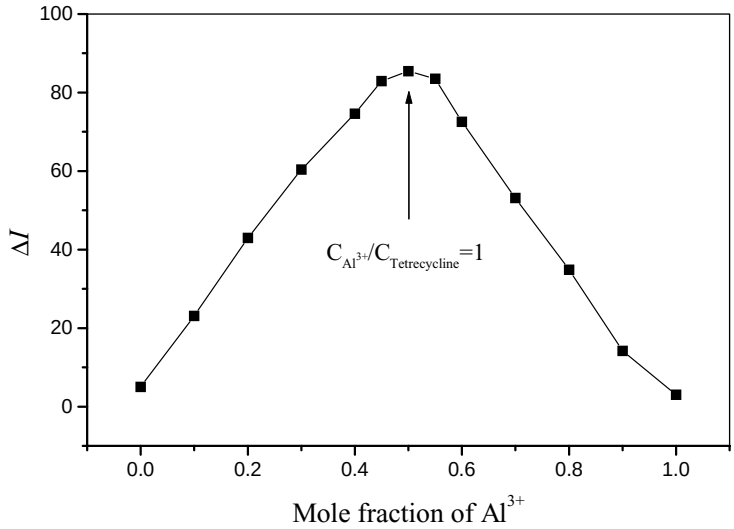

Fig. 8. Molar ration of aluminum–tetracycline in the SOR. Spotted solution: Al^{3+} and tetracycline concentrations were changed simultaneously by keeping the total concentration of Al^{3+} and tetracycline at 8.0×10^{-6} mol l^{-1}; pH, 5.2. Droplet volume, 0.5 μl. A 4 × objective was used.

which keeps the solute from transporting along the outward capillary flow of the solvent [16]. Fig. 9 shows the effect of PVA-124 on the fluorescence intensity. In this assay, we choose the appropriate concentration of PVA-124 in the solution is 0.09% (w/v). In addition, it was found that suitable oven temperature was 70 °C for the evaporation of the solvent in the spotted droplet at our laboratory humidity.

Table 1
Influence of coexisting foreign substances[a]

No	Substances	Concentration (mol l^{-1})	Change of I_{max} (%)	No	Substances	Concentration (mol l^{-1})	Change of I_{max} (%)
1	Ba^{2+}, Cl^-	4.8×10^{-4}	+11.1	18	ctDNA	3.8	+3.5
2	Ca^{2+}, Cl^-	4.0×10^{-4}	+9.2	19	Sucrose	5.0×10^{-4}	−10.9
3	NH_4^+, Cl^-	5.0×10^{-3}	−9.4	20	Lactose	5.0×10^{-4}	−11.2
4	Na^+, Cl^-	5.0×10^{-2}	−2.9	21	Maltose	5.0×10^{-4}	−9.8
5	K^+, Cl^-	5.0×10^{-3}	−4.2	22	L-Lys	2.5×10^{-5}	−6.3
6	Mg^{2+},SO_4^{2-}	4.0×10^{-5}	−4.8	23	Starch	0.2	−6.8
7	Zn^{2+}, NO_3^-	4.1×10^{-5}	+10.9	24	L-Gly	2.5×10^{-5}	−2.8
8	Co^{2+}, Cl^-	9.9×10^{-6}	−7.4	25	L-Phe	5.0×10^{-5}	−9.1
9	Fe^{3+}, Cl^-	5.0×10^{-6}	−10.2	26	L-Ser	5.0×10^{-5}	−9.0
10	Cu^{2+}, SO_4^{2-}	2.0×10^{-6}	−4.2	27	SDBS	1.0×10^{-6}	+7.7
11	Ni^{2+}, SO_4^{2-}	3.8×10^{-6}	−6.5	28	SDS	1.0×10^{-6}	+7.9
12	Cr^{3+}, Cl^-	4.0×10^{-6}	−8.3	29	CTMAB	1.0×10^{-6}	−5.5
13	Mn^{2+}, SO_4^{2-}	3.0×10^{-6}	10.0	30	TritonX-100	1.0×10^{-4}	+8.9
14	PO_4^{3-}	1.0×10^{-5}	−6.6	31	Urea	1.0×10^{-4}	−8.7
15	Glucose	5.0×10^{-4}	−10.3	32	Lactonflavin	1.0×10^{-6}	−7.7
16	BSA	10	+7.9	33	Nicotinamide	3.3×10^{-6}	−8.0
17	HAS	7.5	+5.8	34	Calcium Pantothenate	5.0×10^{-5}	−9.9

[a] Concentration of starch and TritonX-100 are expressed as mg ml^{-1} and % (v/w), BSA and ctDNA are μg ml^{-1}, respectively. Spotted solution: tetracycline, 4.0×10^{-6} mol l^{-1}; Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. Droplet volume: 0.5 μl. A 4 × objective was used.

3.4. Influence of coexisting foreign substances

Table 1 lists the tolerance concentration of coexisting foreign substances in the spotted solution with the tolerance level of 10%. Coexisting foreign substances were tested including proteins, DNA, metal ions, amino acids, water-soluble vitamins, and glucide. Both common metal ions in urine, such as Na^+, K^+, Ca^{2+}, NH_4^+, and glucide, urea can be allowed with high concentration level above 1.0×10^{-4} mol l^{-1}. Whereas, substances including Mg^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Cr^{3+}, Mn^{2+}, PO_4^{3-}, amino acids, lactonflavin, nicotinamide, calcium pantothenate can be allowed with concentration level above 1.0×10^{-6} mol l^{-1}, about 10–100 fold higher than the allowed levels reported by other authors [33]. Biological molecules such as bovine serum albumin (BSA) and calf thymus DNA (ctDNA) can be allowed at 10 and 3.8 μg ml^{-1}, respectively, which supplies a good permission for the assay of tetracycline in body fluid samples.

From these data, we can see that the proposed method can be established in the presence of high concentration coexisting foreign substances and has good selectivity. It can be applied to the direct determination of trace amounts of tetracycline in medicine materials, fluid and milk without prior separation of interfering species. In addition, the excipients usually added in the preparation of capsules and tablets, such as lactose, glucose and starch, did not interfere with the results of tetracycline [36]. Thus, it is not necessary to consider other interference in the coexisting medium.

3.5. Calibration curves

Fig. 3 has proved that the variation of fluorescence intensity across the SOR belt section obeys a Gaussian distribution when the volume of the droplet is at 0.x μl level. Based on that, we establish quantitative relation between the maximal fluorescence intensity ($I_{max,}$ located in the center of the ring belt section) and the amount of the fluorescent materials (m) [13]

$$I_{max} = \frac{K_2}{4\pi\sigma K_1 (KV^{13} - \delta)} m = \xi m \quad (2)$$

where K_1 is an integral constant related to δ and σ; K_2 is a constant related to the emission properties of the fluorescent materials. As K is related to the assembly conditions of SOR according to Eq. (1), ξ is related to the emission properties of the fluorescent materials, evaporative velocity of the solvent, the drop volume, and the properties of solution. As Eq. (2) shows, I_{max} is proportional to the amount of the fluorescent materials in the solution if ξ is a constant by controlling the reaction medium, PVA-124 concentration in spotted solution and the evaporation velocity of the solvent at normal humidity in an oven. Table 2 displays the analytical parameters for the determination of tetracycline by using different droplet volumes. From which we can see when the droplet volume is 0.1 μl, the present SOR method can be used to determine tetracycline in the range of 7.5–800.0 fmol (or 7.9×10^{-8} to 800.0×10^{-8} mol l^{-1}), and the limit of determination reaches 0.8 fmol (or 7.9×10^{-9} mol l^{-1}) with three-fold of signal-to-noise

Table 2
Analytical parameters of SOR method[a]

Droplet volume (μl)	Linear range ($\times 10^{-14}$ mol per ring)	Linear regress equation (m, mol per ring)	Correlation coefficient (r, $n = 6$)	LOD (3σ, fmol per ring)
0.1	0.8–80.0	$\Delta I = -1.3 + 2.2 \times 10^{14}\, m$	0.9994	0.8
0.2	1.0–100.0	$\Delta I = -1.0 + 1.6 \times 10^{14}\, m$	0.9993	1.0
0.3	2.9–480.0	$\Delta I = -0.5 + 4.8 \times 10^{13}\, m$	0.9995	2.9
0.5	3.6–500.0	$\Delta I = 3.0 + 4.0 \times 10^{13}\, m$	0.9996	3.6
1.0	3.9–6000.0	$\Delta I = 4.0 + 3.8 \times 10^{13}\, m$	0.9993	3.9

[a] Spotted solution: Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. A 10 × objective was used for observing SOR by spotting 0.1 and 0.2 μl solutions. A 4 × objective was used for that of other SORs. Above data can also be expressed as the tetracycline concentration in the spotted solution by dividing the volume of the droplet. For example, the linear range of 0.1 μl can be expressed as 7.9×10^{-8} to 800.0×10^{-8} mol l^{-1}, and the LOD is 7.9×10^{-9} mol l^{-1}.

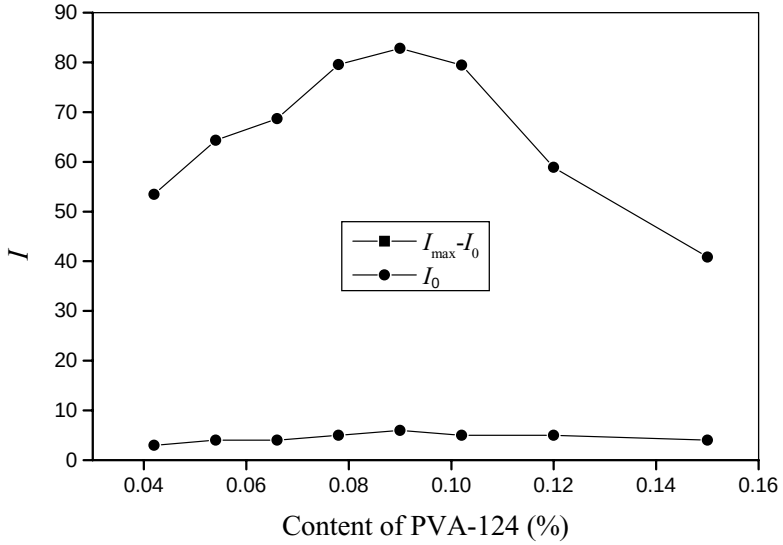

Fig. 9. Effect of PVA-124 content on the fluorescence emission of SOR. Spotted solution: tetracycline, 4.0×10^{-6} mol l^{-1}; Al^{3+}, 1.2×10^{-5} mol l^{-1}; pH, 5.2. Droplet volume, 0.5 μl. A 4 × objective was used.

ratio ($S/N = 3$). In addition, the slopes of the regression equations decrease with increasing droplet volume (under same objective), showing the experimental results are identical to theoretical expression in Eq. (2).

3.6. Determination of tetracycline hydrochloride in samples

The proposed method was applied to the determination of tetracycline hydrochloride in capsules and

Table 3
Determination results of tetracycline hydrochloride in Capsule and Tablet[a]

Samples	Content of tetracycline hydrochloride (g per granule or piece)	Average (g per granule or piece, $n = 5$)	RSD (%, $n = 5$)	Recovery (%, $n = 5$)
Capsule	0.237, 0.239, 0.241 0.243, 0.245	0.241	1.2	97.33–101.67
Tablet	0.235, 0.236, 0.240 0.243, 0.245	0.240	1.7	97.00–102.3

[a] Reference value of tablet or capsule sample was 0.25 g tetracycline hydrochloride per granule or piece supplied by the suppliers. Capsule was purchased from Beiling Pharmaceutical Ltd. (Chongqing, China), and Tablet from Southwest Pharmaceutical Ltd (Chongqing, China). Spotted solution: Al^{3+}, 1.20×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. A 4 × objective was used. Droplet volume: 0.5 μl.

Table 4
Determination of recovery in practical urine and fresh milk samples[a]

Samples	Added tetracycline ($\times 10^{-6}$ mol l^{-1})[b]	Found tetracycline ($\times 10^{-6}$ mol l^{-1})[b]	Recovery (%)[b]	RSD (%)[b]
Human urines	3.00	2.96–3.14	101.7	2.3
	2.00	1.95–2.08	100.5	2.5
Fresh milks	0.100	0.097–0.105	101.0	3.0
	0.200	0.198–0.220	106.5	4.2

[a] Urine samples were diluted 25-fold dilution, while fresh milk samples were diluted 250-fold.
[b] Five measurements were made ($n = 5$). Spotted solution: Al^{3+}, 1.2×10^{-5} mol l^{-1}; PVA-124, 0.09% (w/v); pH, 5.2. A 4 × objective was used. Droplet volume: 0.5 μl.

tablets, which were in good agreement with reference values with the recoveries of 97.0–102.3% and RSD of 1.2 and 1.7%, correspondingly (Table 3). To test the present assay, we determined the tetracycline concentrations in human urine and fresh milk samples. These samples did not undergo any pretreatment except dilution with water. The maximum levels of tetracycline in human urine and in milk samples are about 1×10^{-8} to 1.7×10^{-5} mol l^{-1} [33] and 0.1 μg ml^{-1} [21], respectively. As Table 4 shows that the determination results of these practical samples are very satisfactory, and can be made at the recoveries of 100.5–106.5% and RSD of 2.3–4.2%, indicating the method is reliable and practical.

4. Conclusions

From above descriptions, we can see that SOR technique has advantages. Firstly, the assay has a high sensitivity since the solutes can be concentrate along the perimeter of the droplet spot and because the contribution from the background in SOR technique is very small compared to other solid support such as thin film substrate including octadecylsilanized silica and poly(vinyl chloride) plate [9,10], that is greatly beneficial to the sensitivity improvement. Secondly, this method is based on solid deposition, and the matrix effect in aqueous solution is greatly avoided. Finally, only nanoliter to microliter samples were required, so this method can be used for precious sample analysis. Therefore, the SOR technique plays an important role in decreasing the interference of foreign components and can find wide applications in the determinations of biological samples in which the contents of coexisting substrates are very high. We believe the SOR technique, if combining to a well integrated mapping system, statistical analysis system or a robot as the spotting engine to fully automate image collection, processing, and map construction, will become sufficiently general for various biochemical analysis and other analysis.

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