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ORIGINAL PAPER

A fluorometric assay for oxytetracycline based on the use of its europium(III) complex and aptamer-modified silver nanoparticles

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Abstract

An ultrasensitive assay is described for the determination of oxytetracycline (OTC) at nanomolar levels. The method is using silver nanoparticles (AgNPs) that were first modified with OTC-binding aptamer and then exposed to the OTC-Eu(III) complex. The pink fluorescence of the OTC-Eu(III) complex on the AgNPs is almost completely quenched. On addition of OTC, it will compete with the OTC-Eu(III) complex for binding to the aptamer on the AgNPs. The OTC-Eu(III) complex is released and becomes strongly fluorescent, with excitation/emission peaks at 385/620 nm. The resulting assay was validated in terms of linearity and linear range, sensitivity, selectivity, detection limit and accuracy. Under optimum conditions, response is linear in the 10 to 500 nM OTC concentration range, and the limit of detection is 1.9 nM. The method was applied to the determination of OTC in spiked milk and tablets samples, and it gave satisfactory results.

Keywords Antibiotic · OTC-Eu^{3+} complex · Recovery assay · Fluorescence quenching method · Triton X-100 · Milk analysis

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Introduction

Oxytetracycline (OTC) is the most commonly used veterinary antibiotic against Gram-negative and Gram-positive microorganisms [1, 2]. The broad-spectrum antimicrobial OTC prevents the formation of proteins within the bacterial cell by binding to the 30S ribosomal subunit. OTC is employed for the treatment of infective illnesses of animals and as animal growth promoter. Its accumulation in food such as milk, meat and egg causes dangerous effects to human health [3, 4]. In the animals, a high percentage of the ingested antibiotics is either aggregated in tissues or excreted into the environment and only a small amount of the antibiotics is metabolized. So, it is necessary to find effective and simple methods to identify the OTC in contaminated food products [5].

Among optical aptasensors, fluorescent aptasensors have received tremendous attention, because of their advantages, including rapid analysis, high sensitivity and simplicity of recognition, easy operation and little damage to sample [6, 7].

Europium, a rare earth metal, is the reactive member of lanthanide series [8]. Eu(II) and Eu(III) are two

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oxidation states of europium and there is no more stable oxidation state than Eu(III) [9]. Eu(III) emits red light, whilst composition and structure of host affects the luminescence of Eu(II). Under UV light, deep-red luminescence can be obtained [10, 11]. This element is utilized in the construction of fluorescent glass. Fluorescence of europium is employed to investigate biomolecular interactions in drug-discovery screens. Furthermore, europium is also utilized in the anti-counterfeiting phosphors in euro banknotes [11].

Fluorescence quenching is decreasing of fluorescence intensity of fluorescent substance by different molecular interactions, including collisional interactions (dynamic quenching), formation of complex in the ground-state (static quenching), excited state reactions, molecular rearrangements or energy transfer [12]. Factors such as the nature of quencher and fluorophore, solvent polarity, and amount of quencher are effective in the quenching of fluorescence [12]. In the metal nanoparticles, the decreasing in amount of the radiative to non-radiative decline rate and the quantum yield of fluorophore can lead to the quenching of the fluorescence intensity of dye. In general, energy transfer occurs between metal NP (energy acceptor) and dye (energy donor) in a way like to FRET but this energy transfer is explained by nanomaterial surface energy transfer (NSET). The physical principles and the interval between the nanoparticle and the dye molecule (FRET: < 100 Å and NEST ~ 200 Å) in FRET and NEST are different. The dipole–dipole interaction in FRET and the dipole-metal surface interaction in NSET is considered [13, 14]. Silver nanoparticles (AgNPs) are one of the most important and most applied nanomaterials. AgNPs are used as either quenchers or enhancers of fluorescence of organic dyes in which the quenching or increasing of fluorescence depends on size and shape of AgNPs and the interval between the organic fluorophore and AgNPs [13].

Short single-stranded oligonucleotides, recognized as aptamers, are prepared by the systematic evolution of ligands by exponential enrichment (SELEX) technique. They bind to their cognate targets with high affinity, specificity and selectivity via their unique three-dimensional (3D) conformations [15, 16]. Aptamers have many advantages, such as structural flexibility and stability, great thermal stability, non-toxicity and non-immunogenicity, easily produced and modified. Due to these advantages, aptamers are used to develop fluorescent, colorimetric, and electrochemical aptasensors [16–18].

Rare-earth complexes especially Eu(III) complexes are considering as clean energy conversion substances utilizing ligands for effectual photo-excited energy conversion. Moreover, combination of lanthanide ions with ligands such as tetracycline leads to a strong luminescence [19]. In other hand, lanthanide complexes are suitable and efficient alternatives to organic fluorophores because they have advantages, such as high quantum yield, excellent solubility, long lifetime (μs to ms), large Stokes shift (200–300 nm) and narrow emission spectrum (under 10 nm bandwidth) [20, 21]. Herein, a novel method was presented for specific and selective determination of OTC based on Aptamers–conjugated AgNPs and OTC-Europium ion (Eu^{3+}) complex. The method was based on competition between oxytetracycline and OTC-Eu^{3+} complex to interact with aptamer, and corresponding difference in their fluorescence intensity.

Materials and methods

Materials

An OTC aptamer (Apt), 5′-CGA CGC ACA GTC GCT GGT GCG TAC CTG GTT GCC GTT GTG T –Thiol-3′ [22], was purchased from Bioneer (South Korea). Oxytetracycline (OTC) was purchased from Biological Product Institution of Chinese Medicine and was directly dissolved in deionized water. During the experiment, stock solutions (1.00×10^{-4} mol L^{-1}) were diluted with deionized water to obtain working solutions of OTC and europium ion (Eu^{3+}). Sodium chloride and sodium citrate trihydrate, sodium Borohydride, triton X-100, hydrochloric acid, tetracycline, cefixime, cefalexin, Tris (hydroxymethyl)-amino methan were purchased from Merck (Germany).

All prepared solutions including stock and working were stored at 0–4 °C. All reagents were used without further purification.

Methods

Preparation of the OTC-Eu^{3+} complex

OTC can strongly bind to Eu^{3+}. Initially, optimal conditions for the formation of the complex between OTC and europium ion was investigated, and then this complex was used for the development of the method. For the complex formation: 2.00 mL of OTC (5.00×10^{-7} mol L^{-1}), 2.00 mL of Eu^{3+} (5.00×10^{-7} mol L^{-1}), 2.00 mL of triton X-100 (10% *v*/v) and 1.00 mL of Tris–HCl (0.10 mol L^{-1}, pH 8) buffer were mixed thoroughly at room temperature and the fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 385/620 nm) was measured using the Synergy H4 microplate reader (BioTek, USA).

Preparation of silver nanoparticles

AgNPs was synthesized according to the previous method [23]. In summary, 5.0 mL $AgNO_3$ (1 mM) was added drop-by-drop (about 1 drop/s) into 15.0 mL $NaBH_4$ (2 mM) solution in an ice bath (0 °C). A magnetic stirrer was used to mix the solution vigorously. To prevent the aaggregation of AgNPs, 0.03 g sodium citrate tri-hydrate was added to the final solution. The AgNPs was stored in a dark bottle at 4 °C for further use.

Preparation of the aptamers-conjugated AgNPs

1 μL aptamer (25 μM) was added to 45 μL of AgNPs solution. Then 10 μL of 10% (*v*/v) Tween 80 in water was added and incubated at room temperature for 24 h. To remove the unconjugated aptamers, the solution was centrifuged for 15 min at 8500 g and washed twice with deionized water.

Preparation of real samples

Milk samples were diluted 80 folds with deionized water and were spiked with two concentrations of OTC (250 and 450 nM final concentration). For preparation of tablet solution, 3 tablets of OTC (100 mg, Razac Pharma. Co, Iran) were weighed, powdered and solubilized to make solution of 500 nM of OTC. For detection of OTC, 100 μL OTC-Eu^{3+} complex (500 nM) and 2 μL Aptamers-conjugated AgNPs were mixed with 98 μL of milk or tablet samples. Then, at room temperature, the amounts of OTC in the samples were calculated via the measurement of fluorescence intensity.

Result and discussion

Characterization of AgNPs

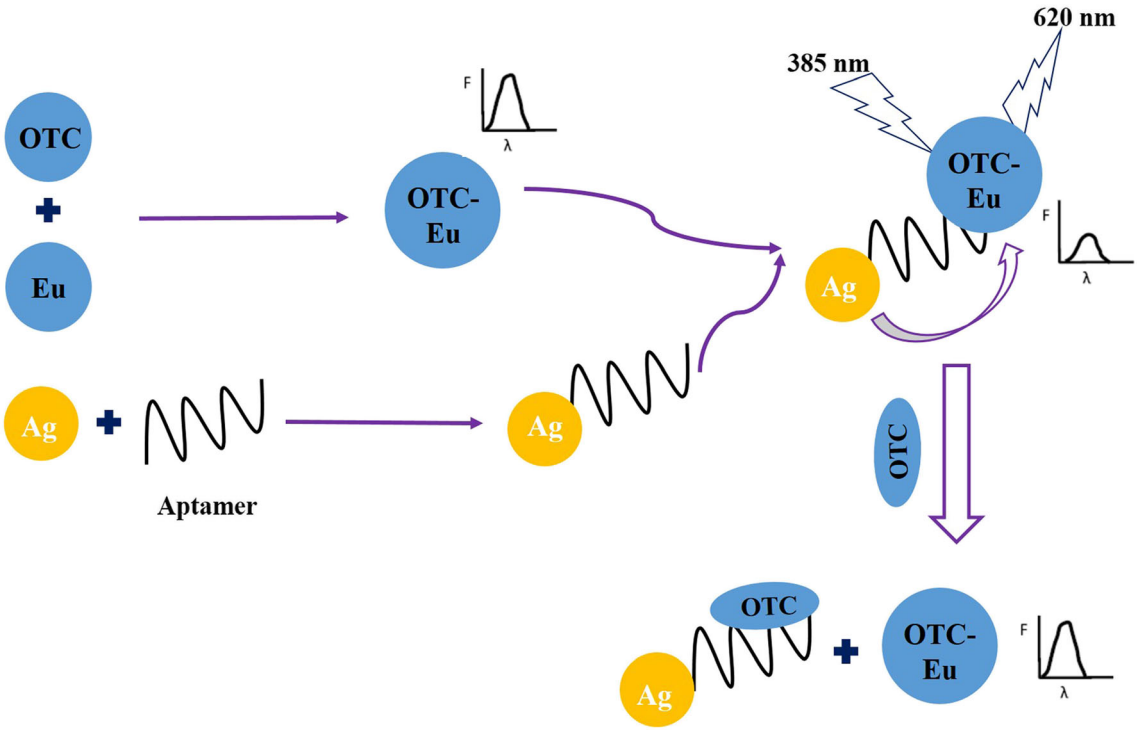
Particle size of 33.1 ± 6.1 nm and zeta potential of −26.4 ± 2.6 mV was obtained from particle size analyzer (the sample volume was 1 mL). Moreover, the average size of the prepared silver nanoparticles, as obtained by TEM analysis (Fig. S1a), was about 20 nm. Fig. S1b shows the UV–Vis spectrum of the silver nanoparticles with absorption maximum wavelength of 400 nm.

Gel electrophoresis

A 2.5% agarose gel electrophoresis was utilized to verify aptamer binding to AgNPs. As indicated in Fig. S2, no aptamer band was seen in lane 2 due to the covalent conjugation of aptamer to AgNPs.

Sensing scheme

The assay strategy is shown in Scheme 1. First, the optimum condition was obtained for the formation of OTC-Eu^{3+} complex, then, this complex and Aptamers-conjugated AgNPs were used for detection and quantification of OTC. The Eu^{3+} has an appropriate level of energy and permits the transmission of intramolecular energy from the lowest triplet level of the complex to a 4f level of the europium ion. Therefore, upon absorption of light, the OTC was excited and then after intersystem crossing to the triplet state, transferred the excitation energy to Eu^{3+} ion [24]. Moreover, to avoid quenching effect of coordinated water molecules upon the fluorescence of OTC-Eu^{3+} complex, surfactants such as Triton X-100 was used because micellar environment leads to the protection of the OTC-Eu^{3+} complex against non-radiative processes [25].



Scheme 1 Schematic illustration of the fluorescent method for OTC detection based on use of its europium(III) complex and silver nanoparticles

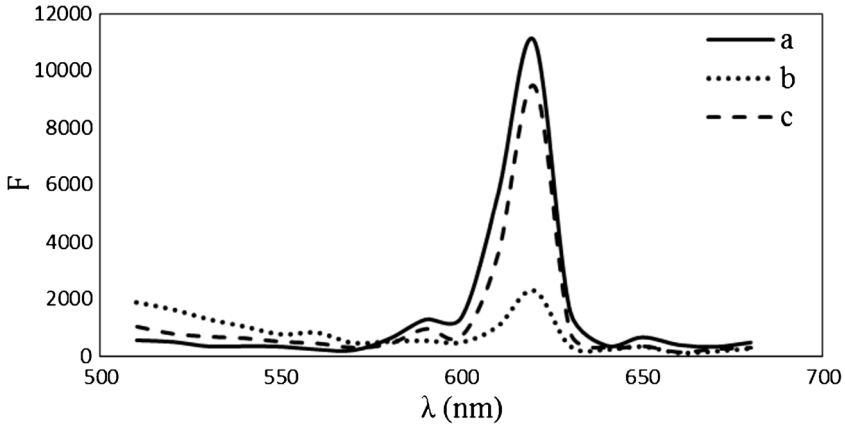


Fig. 1 Fluorescence emission spectra: curve (**a**) OTC-Eu^{3+} complex, curve (**b**) OTC-Eu^{3+} complex + Aptamers-conjugated AgNPs, curve (c) OTC-Eu^{3+} complex + Aptamers-conjugated AgNPs + OTC (250 nM)

In the presence of Aptamers-conjugated AgNPs, the fluorescence intensity of OTC-Eu^{3+} complex decreased because of binding of aptamers on the surface of AgNPs to OTC in the complex. As, AgNPs quench the fluorescence of OTC-Eu^{3+} complex due to the close proximity between AgNPs and OTC-Eu^{3+} complex. Upon the addition of OTC to this mixture, both OTC-Eu^{3+} complex and OTC compete for binding to Aptamers-conjugated AgNPs, so that fluorescence intensity increases. As OTC concentration increases, free OTC-Eu^{3+} complex increases and as a result the fluorescence intensity enhances. In Fig. 1, the curve (a) displays the fluorescence emission spectrum of the OTC-Eu^{3+} complex. Following the addition of Aptamers-conjugated AgNPs, the decrease in fluorescence intensity was observed (curve b). Finally, curve c shows increase in fluorescence intensity after addition of OTC (final concentration: 250 nM).

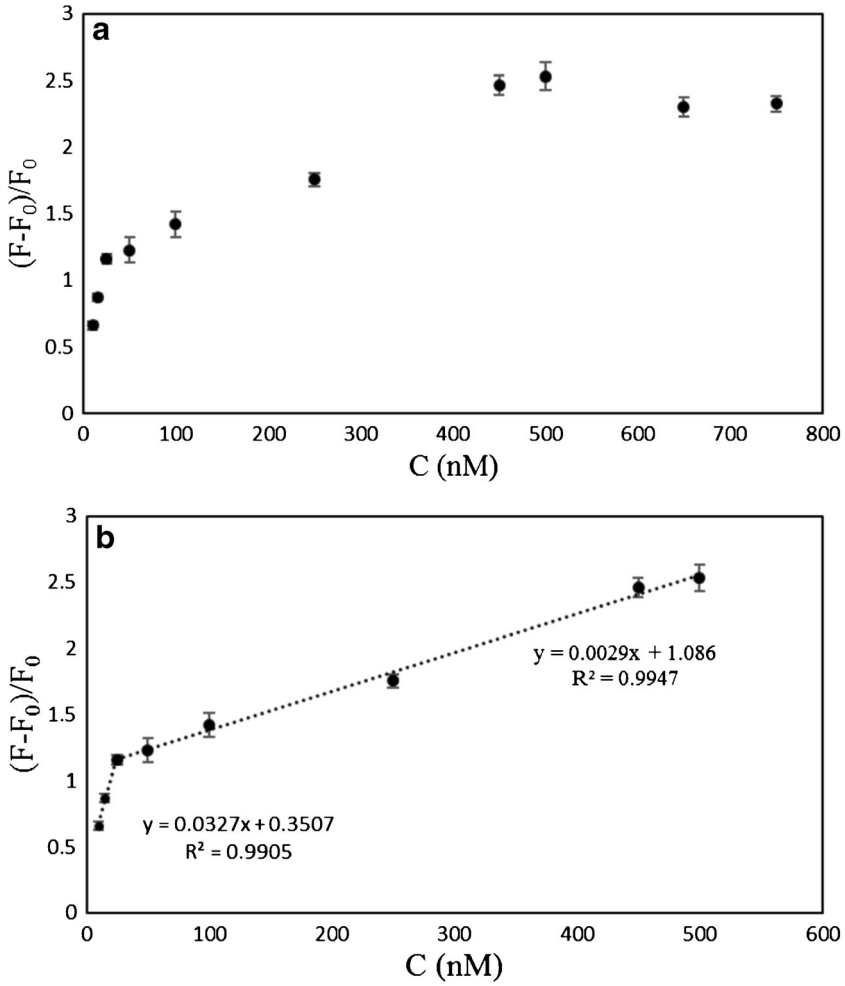


Fig. 2 **a** Relative fluorescence intensity of the assay as a function of OTC concentration. **b** Calibration plot of the assay for OTC ($\lambda_{ex}/\lambda_{em}$ = 385/620 nm, pH 8, [OTC-Eu^{3+}] = 500 nM, 5 min). The error bars illustrate standard deviation for five replicates

Table 1 Comparison of the method for determination of OTC with other previously reported works

Type of sensor	Linear range (nM)	LOD (nM)	Applicability	Reference
Aptamer-based cantilever array sensors	1–100	0.2	Not tested in real sample	[26]
Aptamer-based fluorescent	0–250	1.67	Milk	[27]
Electrochemical aptasensor	22–1300	21	Blood serum, urine	[28]
Enzyme-linked aptasensor	–	27	Milk	[29]
Photoelectrochemical aptasensor	4–150	0.9	Tablet	[30]
Direct electrochemical sensor	800–40,000	120	Chicken feed, fish, chicken, shrimp	[31]
Chemilumiometry	50–8000	30	Tap water, honey	[32]
SERS aptasensor	4.6×10^{-5} -0.46 pg/ml	4.35×10^{-6} pg/ml	Fishmeal	[33]
Colorimetric aptasensor	10^{-9}-100	1 ag/ml	Milk	[34]
Aptamer-based fluorescence	10–500	1.9	Tablet, milk	This study

Optimization of method

The following parameters were optimized: (a) pH value; (b) Mole ratio of the OTC-Eu^{3+} complex and (c) Reaction time. Respective data and Figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) Best pH value was 8 (Fig. S3); (b) Optimal mole ratio of the OTC-Eu^{3+} complex was 1:1 (Fig. S4); (c) Optimal reaction time on formation of the OTC-Eu3+ complex was 5 min (Fig. S5).

Linearity range and detection limit of OTC

100 μL OTC-Eu^{3+} complex (500 nM) and 2 μL Aptamers-conjugated AgNPs were incubated with a range of OTC, 0–750 nM final concentration, and the volume was adjusted to 200 μL with no farther incubation. Then, fluorescence intensities, $\lambda_{ex}/\lambda_{em} = 385/620$ nm, were measured using the Synergy H4 microplate reader. The fluorescence intensity of OTC-Eu^{3+} complex was displayed as $(F - F_0)/F_0$. F and F_0 were the fluorescence intensities with and without OTC, respectively. Data are means ±SD, $N = 5$. In this method, AgNPs quenched the fluorescence intensity of OTC-Eu^{3+} complex.

Figure 2a displays the relative fluorescence intensity of the method at 620 nm at various concentrations of OTC. For this assay, as exhibited in Fig. 2b, the plot of the relative fluorescence value versus OTC concentration in the range from 10 to 500 nM showed two linear dynamic ranges with different slopes. The linear regression equations were $Y = 0.0327X + 0.3507$ and $Y = 0.0029X + 1.086$ with r^2 of 0.9905 and 0.9947, respectively. According to IUPAC definition (3 times the standard deviation in blank/slope), the limit of detection (LOD), was obtained to be 1.9 nM.

Some of available sensors for determination of OTC have been demonstrated in Table 1. Compared to other sensors, the method indicated a satisfying LOD and linear range.

Selectivity of the fluorescent assay

Selectivity of assays is important for their practical applications. Specificity of the analytical method was evaluated by

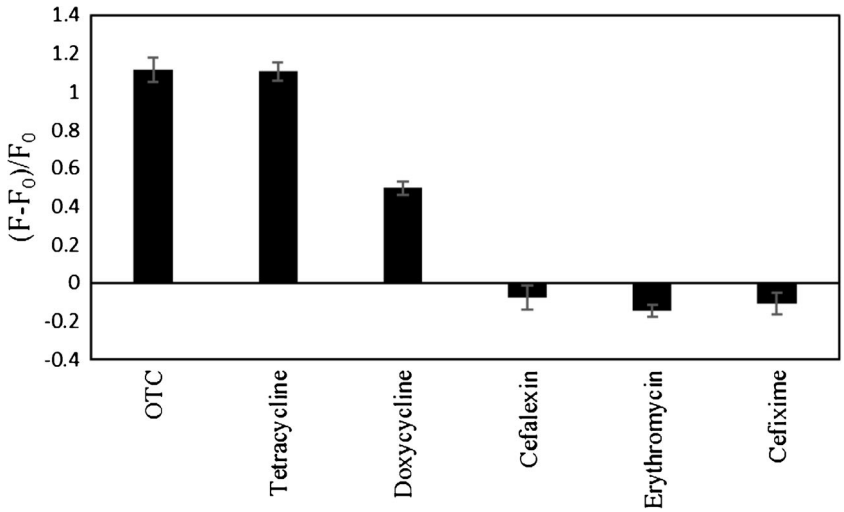


Fig. 3 Efficiency of the relative fluorescence intensity of the assay in the presence of various drugs for analysis of selectivity assay. The error bars illustrate the standard deviation for five replicates

Table 2 Results for the detection of OTC in real samples using the analytical approach. Data are mean ± standard deviation for five replicates

Sample	Added(nM)	Found (nM)	Recovery (%)	RSD (%)
Milk (low fat)	250	245.5	98.2	1.1
Milk (low fat)	450	421.7	93.7	1.5
Milk (Full Fat)	250	234.8	93.9	2.5
Milk (Full Fat)	450	440	98.0	2.4
Tablet	–	264.9	105.6	3.5

measuring the relative fluorescence intensity $(F\text{-}F_0)/F_0$ in the presence of several structurally related compounds such as tetracycline and doxycycline, and non-structurally related compounds like cefixime, cephalexin and erythromycin. Figure 3 shows that, in comparison to OTC, almost no signal changes are caused by cefixime, cephalexin and erythromycin. However, tetracycline and doxycycline increase the response as they belong to the same family of drugs (Fig. S6).

Detecting OTC in real samples

To further validate the performance of the method in real sample, the detection of OTC in two types of milk and tablet were carried out. Table 2 represents the analysis results of OTC spiked in milk and OTC in tablet samples through the suggested method. The data indicated that the suggested fluorescent approach is highly accurate and repeatable for the determination of OTC. So, it was shown that the suggested aptamer-based method for OTC detection had many considerable advantages, like short detection time, high sensitivity, good specificity and excellent reproducibility, which presents a new way for detection of OTC in environmental and biological samples.

Conclusion

In summary, a new and sensitive fluorescent assay was proposed for determination of OTC based on OTC-Eu^{3+} complex as a fluorescence probe and AgNPs as a quencher. We studied the optimum conditions for OTC-Eu^{3+} complex. Under the optimal conditions, OTC increases the fluorescence intensity of OTC-Eu^{3+} complex. The increase is proportional to the concentration of OTC. The assay indicated good selectivity toward OTC and antibiotics which belonged to tetracyclines family. Besides, this approach was successfully utilized for determination of OTC in milk and tablet samples. However, the assay indicated two linear dynamic ranges with different slopes for OTA, making the measurement of the target in unknown samples a little difficult.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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