Electronic Supplementary Material (ESI) for Nanoscale Advances.

SUPPORTING INFORMATION

Pen Direct Writing of SERRS-based Lateral Flow Assays for Sensitive Detection of Penicillin G in Milk

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Synthesis of the immunogen for the production of anti-ampicillin antibodies (anti-P)

The immunogen was prepared by conjugating ampicillin to BSA, as schematised in Figure S1. The ampicillin-BSA conjugate was obtained as previously reported by *Cliquet et al.*[1] with minor modifications. Briefly, 15.5 mg of BSA and 24.0 mg of ampicillin (free base) were dissolved in 4.5 mL of phosphate buffer (0.1M, pH 7.0). 27 µL of glutaraldehyde (25%) were added dropwise to the mixture and let to react overnight at room temperature under stirring. The ampicillin-BSA conjugate was then purified from excess of ampicillin and by-products by gel filtration on PD-10 cartridge packed with Sephadex G-25 (VWR, Milan, Italy), using PBS as the eluent. The separation was accomplished according to manufacturer's instructions.

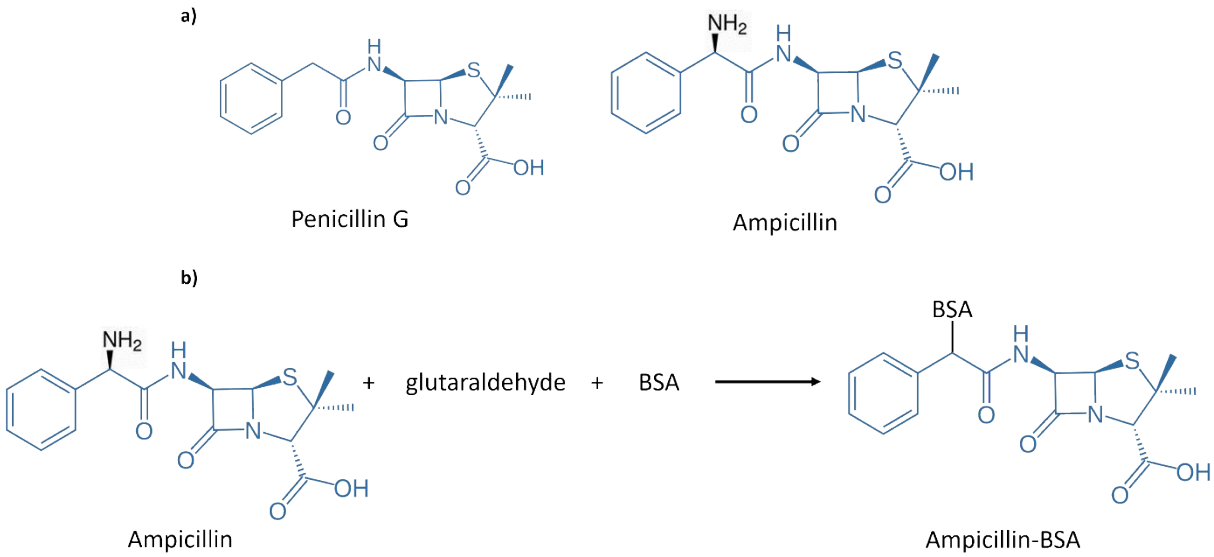

Figure S1: (a) Chemical structures of Penicillin G and Ampicillin. (b) Scheme of the synthesis of the immunogen used for the production of anti-ampicillin antibodies.

Details of custom chamber

A custom chamber has been designed in FreeCad (v 0.19), sliced in Ultimaker Cura (v. 4.13.1) and fabricated using a filament 3D printer (Creality Ender 3, 0.6 mm nozzle, 0.4 mm layer height, 0.45 mm initial layer height, 10% infill, 210°C print temperature, 60°C bed temperature, 60 mm/s print speed) using polylactic acid (Radionics, product number: 174-0002). This has been mounted with 9 cool white LEDs to keep a reproducible light on the strips, independent from the laboratory light conditions.

Figure S2: 3D-printed custom chamber for taking pictures of the LFIA strips with the same light conditions, prior assembly of the LEDs.

Pen writing optimization

As first step, various commercial pens listed in Table **S**1 were purchased, cleaned, and used for direct writing on different types of paper substrates.

A general procedure for cleaning the pens and incorporate the liquid "ink" was developed as follows. The initial commercial ink present in the pens was eliminated by pushing air through an empty syringe in the plastic ink reservoir tube. Residual ink from the tubes and nibs was then cleaned by immersion and sonication in isopropyl alcohol (IPA) for 24 h (or until no traces of inks were observed in IPA), followed by sonication in DI H_2O for 24 h. Finally, cleaned tubes and nibs were dried at room temperature. Cleaned pen tubes were filled by pushing functional inks (by a small syringe (no needle)) or a Gilson pipette, using a small tip to facilitate the insertion of ink in the plastic tube. Once the tube was completely filled, the metallic nib was reapplied in order to allow writing.

For gel pens an initial wash and sonication step in chloroform was performed prior the described IPA and H_2O washes.

A cleaning procedure was not necessary for the fountain pen, since it had a changeable and empty ink reservoir, and so it was directly filled likewise the other pens.

Table S1. List of commercial pens of different brands, nib dimensions and line width selected to use for pen-on-paper approach.

Pen	Brand	Line Width*
Crystal Bic 1.0 mm	BIC©	0.4 mm
Crystal original fine 0.8 mm	BIC©	0.35 mm
Crystal original medium 1.0 mm	BIC©	0.4 mm
Crystal large 1.6 mm	BIC©	0.6 mm
Felt-tip Pen	STABILO©	~ 1mm
Fountain Pen	PARKER©	~ 0.8 mm
Gel pen	CLASSMATES©	0.4 mm

*According to the manufacturer

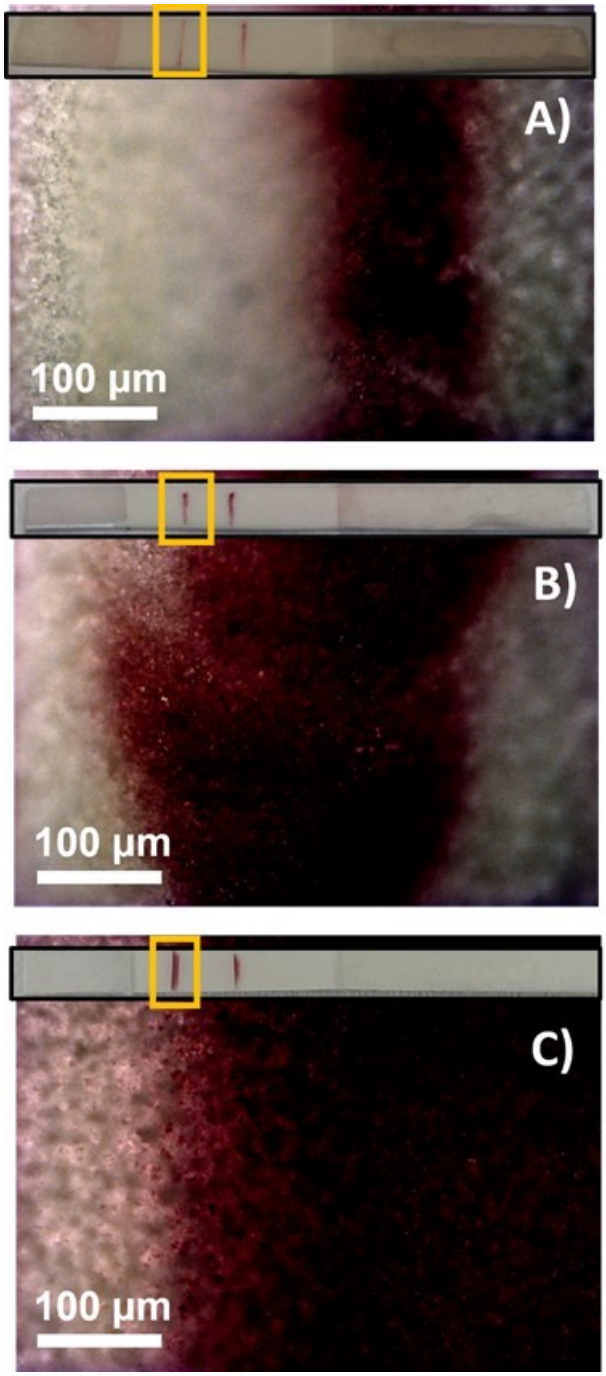

Figure S3: Digital photographs of LFIA strips and optical microscopy images of lines written manually by different commercial pens: A) BIC© Crystal 1.0 mm; B) Crystal large BIC© 1.6 mm; C) Parker© Fountain Pen.

After initial screening, three pens were chosen for easiness of cleaning procedure, method, and performance of writing: *Crystal Bic* 1.0 mm, *Crystal large* 1.6 mm and the fountain pen. These were used for deposition of lines on LFIA strips; The written strips were tested, and microscopy was used for looking at how the written lines were visible through attachment of the nanoparticles (Figure S3).

Figure S3a shows how the *Crystal BIC* 1.0 mm did not deposit functional materials on the entire width of the line and, as well as *Crystal large* 1.6 mm (Figure S3b), did not give continuous tract on the lines. This effect was probably due on the characteristics of a ballpoint pen, which dispenses ink over a metal ball at its point and in this case, the ball point was "skipping", leaving some gaps during the deposition. Instead, as shown in Figure S3c, a fountain pen yielded a more homogeneous deposition on the line. Its working mechanism allows to draw ink from the reservoir through a feed to the nib and deposit it on paper via both gravity and capillary action[2]. A fountain pen was therefore chosen for the development of the SERRS-LFIA test.

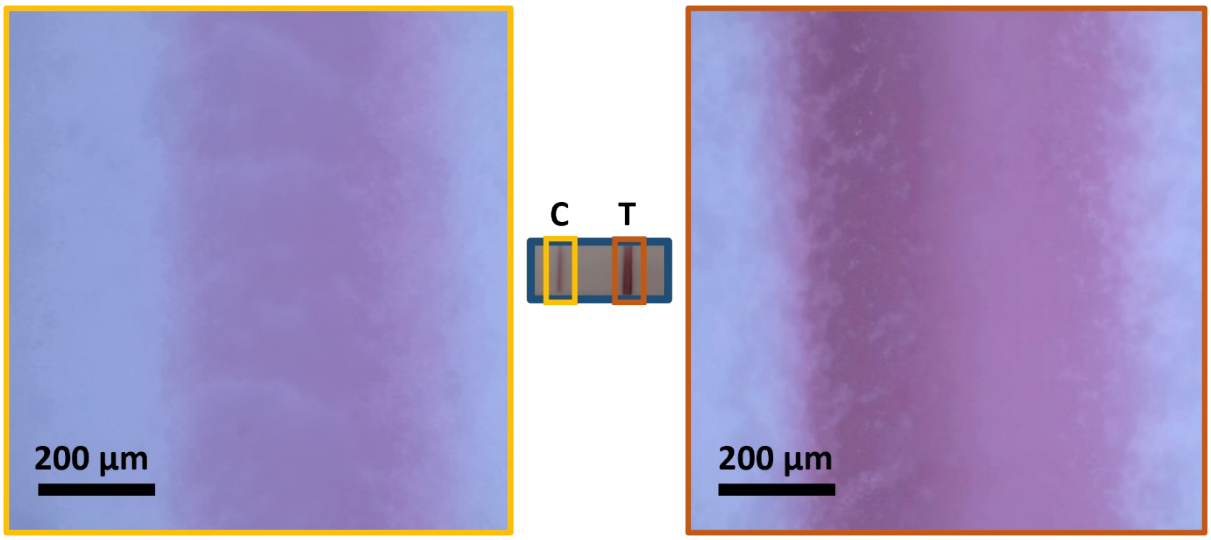

Figure S4: Digital photograph of LFIA strip and optical microscopy images of both test and control lines written manually by fountain pen on a developed strip. The images have been acquired after 15 min from the sample addition.

Optimization of SERRS-LFIA parameters

Different parameters have been assessed to optimize the LFIA test, such as the influence of the nitrocellulose membrane, the sample pad, the running buffer, the amount of deposited materials on the two lines and the pre-treatment of the milk sample. Various nitrocellulose membranes with different protein binding capabilities, pore sizes and thus capillarity speed rate were tested as these parameters influence the transport of the conjugate into the membrane allowing an optimal reaction time and homogeneity of the devices[3]. In particular, for this work the membrane CNPC-10 μm (Advances Microdevices LTD, India) with higher protein binding capability was chosen. Two different sample pads were tested and the best results, especially in terms of flow, were obtained using the glass fiber sample pad GFBR4 (Advances Microdevices LTD, India). Regarding the running buffer, TRIS-HCl (20 mM, pH 8), BB (20 mM, pH 8) and PB (20 mM, pH 7.4) with the addition of different additives were tested. Specifically, it was observed that the incorporation of a protein, such as BSA or casein, in the running buffer caused a decrease in intensity of the LFIA lines. Considering that this effect would be further enhanced by the presence of proteins in milk, it was decided not to add any proteins in the final buffer. A non-ionic detergent, such as Tween 20, was also tested in different percentage and finally used as 0.2% v/v. The concentration of both test and control line was object of study to improve the performances of the test; the final concentrations were 1 mg/ml of Penicillin-BSA and 0.7 mg/ml of secondary antibody on the two lines, respectively. Both lines were handwritten, by using the biomolecules' constituent of the test/control lines as inks. Using milk without pre-treatment did influence the test because of the complexity of the matrix. Specifically, the milk's main components are protein and fat. Proteins caused a decrease in the colour intensity of the assay lines, while fat altered the flow by increasing the viscosity and caused a block in the pores of the nitrocellulose membrane[4]. Therefore, to overcome protein interference and maintain a good sample flow, the milk matrix was diluted 1:1 using PB (20 mM, pH 7.4) containing 0.2% *v/v* Tween 20.

Optimization of anti-P-Au NPs

To optimise the quantity of conjugate anti-P-Au NPs, 9 different combinations of three optical densities (OD) by three quantities of antibody for conjugation on the nanoparticles were tested on a negative sample (only buffer).

As described above, the minimum amount of antibody to protect Au NPs from salt-induced aggregation (6 µg/ml) was identified by flocculation test. Nevertheless, considering the competitive format of the assay, it was decided to further optimise this value by investigating lower quantities of antibody (from 6 µg/ml to 2 µg/ml) and higher concentration of conjugate (OD 2 to 4), while maintaining constant the concentration on the test and control line. The results obtained are reported in Figure S4. In particular, an FF-DoE has been exploited by means of the software CAD. This was made by mapping the intensity of the test lines as a function of optical density and Ab quantity.

Finally, 4 µg/ml anti-P and a final OD 3 for the conjugate were used, since it was giving a good intensity on the test line, comparable to the higher Ab amount, though improving the competition, avoiding a signal saturation, decreasing the amount of Ab used, usually the most expensive component of the test.

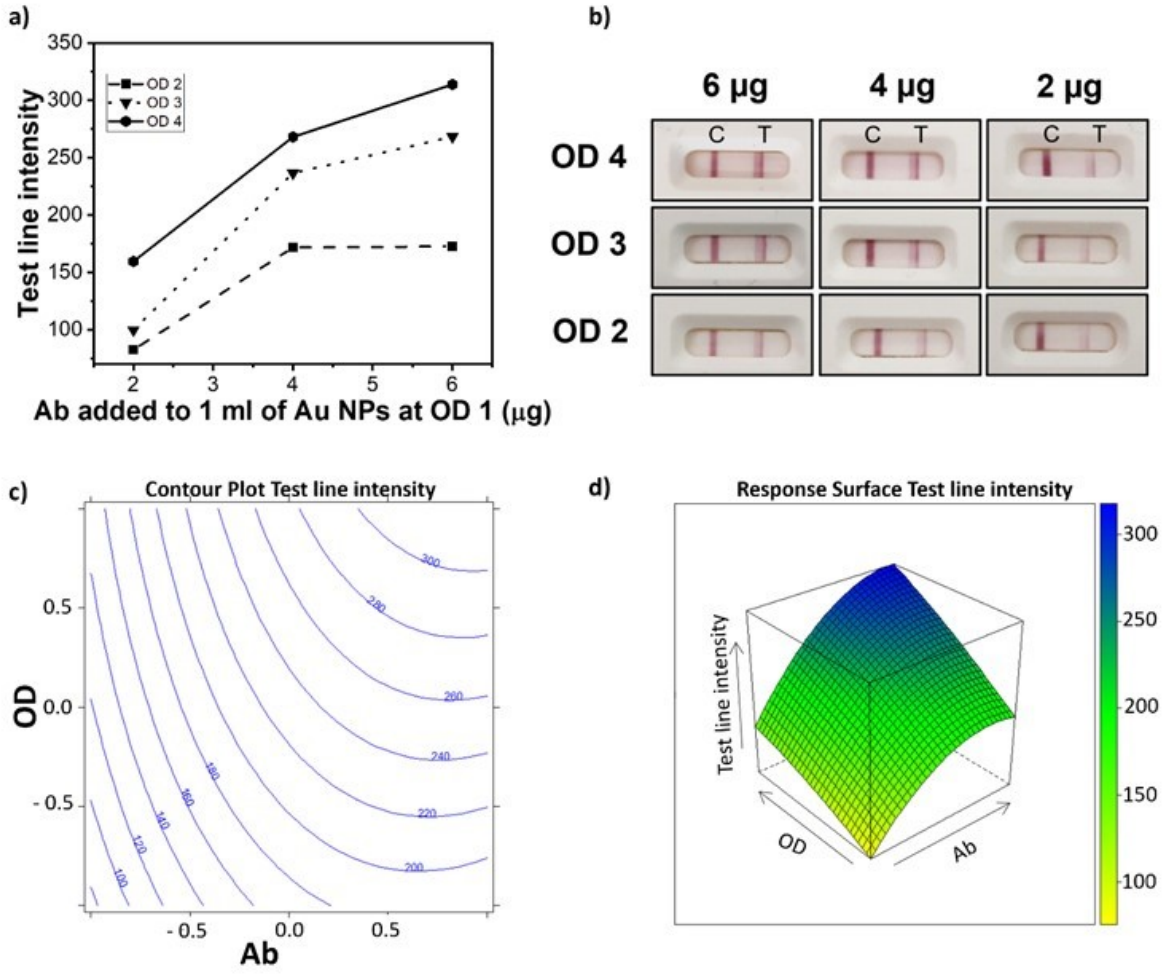

Figure S5: (a) Graphical representation of the optimisation of the conjugate, showing the intensity of the test line of 9 possible combination (matrix optical density by quantity of anti-P). (b) Photographical

images of the combination of three optical densities and 3 quantities of anti-P in presence of running buffer (negative sample). (c) Response surface and (d) Contour Plot of the test line intensity from the FF-DoE optimisation of the OD and Ab quantity of the test.

Coefficient of variation (CV) for SERRS-LFIA measurements

Table S2: CV for the SERRS analysis of the intensity of the reference peak for RhITC (1647 cm^{-1}) for Figure 6c of the manuscript (9 acquisition points).

Concentration (ppm)	CV
0	0.39
0.002	0.25
0.02	0.35
0.2	0.29
2	0.42
20	0.27
200	0.28
500	0.35

Inter-day reproducibility

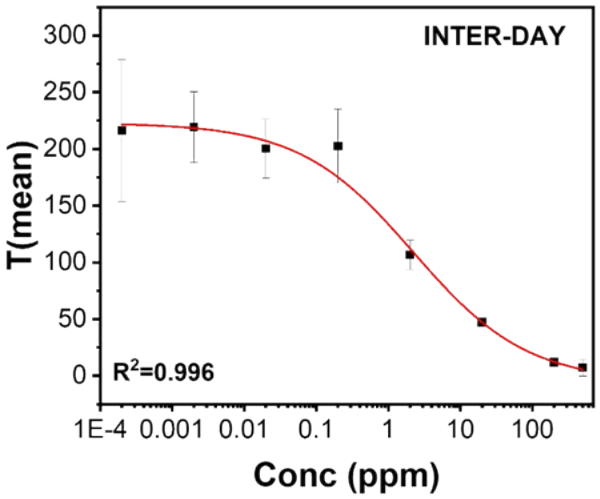

Figure S6: Inter-day reproducibility of the colorimetric response of the test line. The bars represent the error of three independent measurements.

Comparison with conventional LFIA test

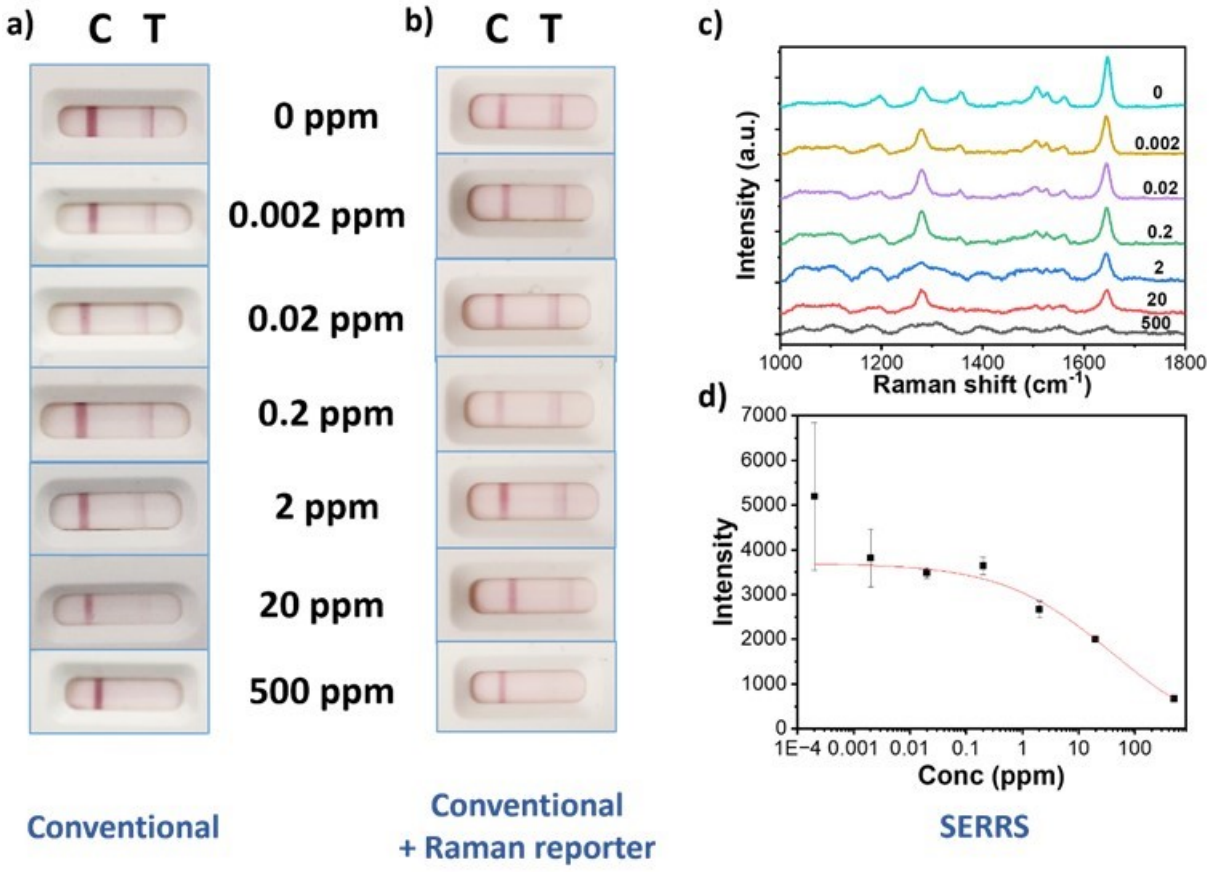

Figure S7: a) Visual readout of strips, obtained dispensing with the Biojet Quanti 3000 dispenser, at different concentration levels of penicillin G; b) visual readout of strips dispensed by means of the Biojet Quanti 3000 dispenser, using the conjugate RhITC-anti-P-Au NPs; c) SERRS spectra obtained for different levels of concentration of penicillin G; d) Calibration curve obtained considering the intensity of the reference peak for RhITC (1647 cm^{-1}) on test line as a function of Penicillin G concentration (the error bar represent just two SERRS measures).

Comparison with literature

Recently, few successful examples of SERS-LFIA assays have been proposed for detection of antibiotics in food and milk. For example, Shi *et al.* have developed a multiplex assay for simultaneous detection of neomycin and quinolones. The test showed accurate and highly sensitive detection with a substantial increase in the limit of detection (LOD) of the two antibiotics (27 and >300 times higher for neomycin and quinolones, respectively) compared to conventional LFIA tests [5]. Fan *et al.* showed duplex detection of tetracycline and penicillin in milk by using Au@Ag NPs, reaching LODs of 0,015 ng/mL and 0.010 ng/mL, respectively [6]. Tian *et al.* have used Raman reporter mediated-gap Au NR@Au NPs for the detection of enrofloxacin in food samples [7].

Table S3: Comparison between SERS-LFIAs for detection of antibiotics in literature.

Antibiotics	Matrix	Immunoprobe	LOD	Reference
Neomycin and Quinolone	Milk	Au NP-PAPT	0.37 pg/mL and 0.55 pg/mL	5
Tetracycline and penicillin	Milk	Au@Ag-DTNB and Au@Ag-MBA	0.015 ng/mL and 0.010 ng/mL	6
Enrofloxacin	Shrimp meat, swine meat and liver	$AuNR^{BDT}$@Au	0.12 pg/mL	7
Neomycin	Milk	Au NP-PAPT	0.216 pg/mL	8
Lincomycin	Buffer	Au NP-MBA		9
Chloramphenicol, Thiamphenicol, florfenicol	Chub	Au@Ag-MBA	0.36 ng/mL, 0.20 ng/mL, and 0.78 ng/mL	10
Penicillin G	Milk	Au NP-RhITC	vLOD: 20ppm IC_{10}-IC_{90}:0.03-97.5 ppm	Our work

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