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ARTICLE

Layer-by-Layer Assembly of Polyoxometalate-Pyrene-Decorated Fluorescent Microspheres for Suspension Immunoassay of *Listeria Monocytogenes*

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Fluorescence microsphere suspension array technique has been developed as a promising tool for molecular detection of *Listeria monocytogene* (*LM*), which is a dangerous food borne pathogen that can cause severe food intoxication in both humans and animals. In such a technique, fluorescent microspheres as the key matrix for the detection are often decorated with neutral organic dyes, which usually suffer from instability, easy photobleaching and loss from the matrix. Herein, we design and synthesize a new organic-inorganic hybrid POM-pyrene compound $[N(C_4H_9)_4]_2[V_6O_{13}\{(OCH_2)_3CNH\text{-}CH_2\text{-}C_{16}H_9\}_2]$ (**1**), which is employed as a negative-charged fluorescent material to prepare new type of fluorescent microspheres by virtue of layer-by-layer electrostatic self-assembly method. As a result, such POM-pyrene-decorated fluorescent microspheres possess obvious luminescent property, superior photo-stability and long-term stability. Therefore, the POM-pyrene-decorated fluorescent microspheres are conjutated by monoclonal anti-*LM* antibodies, and introduced into the fluorescence microsphere-based suspension immunoassay system for the detection of *LM* in food, which exhibits a high sensitivity. The lowest limit of detection (LOD) of *LM* in artificially contaminated meat is down to 6 $CFU \cdot g^{-1}$ without cross-reactivity with other pathogens. Furthermore, the suspension immunoassay based on PS@POM-pyrene microspheres shows excellent specificity to *LM*, good repeatability and high efficiency in contrast to the conventional culture-based method.

1. Introduction

Listeria monocytogenes (*LM*) is a well-known food borne pathogen which can cause severe food intoxication in both humans and animals. [1] *LM* has been internationally recognized as an indicator of health monitoring. Many countries currently have imposed a low tolerance policy for the presence of *LM* in food. [2-4] Thus, efficient approaches for a timely and accurate detection of *LM* contamination are highly important for the microbial safety of food. Currently, three kinds of techniques have been developed to detect *LM*, including the traditional culture-based identification method, the nucleic acid-based method and the immuno-based diagnostic technology. The laboratory culture-based identification method is a standard mode but usually time-consuming, labor-intensive. [5, 6] The nucleic acid-based qualitative and quantitative analysis is now widely used as a high sensitive and specific method, but the false positive results are sometimes obtained because this method does not distinguish dead or alive pathogens [7a] and the amplification step of PCR[7b]. Furthermore, the false negative signal can also be obtained due to the PCR inhibitors.[7c,8] Immunological methods are based on the principle of antigen-antibody reaction with the advantages of simple operation and fast response, representing currently an important method for the detection of foodborne pathogenic bacteria. However, some of the immunological methods for example the enzyme-linked immunosorbent assay (ELISA) based on solid-phase immunoassay are easily subject to matrix interference of background, affecting the detection sensitivity and specificity. [8,9a,9b] In this research field, a new immunological method based on fluorescence microsphere suspension array technique has recently been developed due to its various advantages such as convenient operation, great reliability, high throughput screening and superior sensitivity in microbial detection. [10-12] In such suspension array system, the fluorescent microspheres are generally decorated by a series of organic dyes. However, these organic dyes are subjected to several impediments, for instance, instability, easy photo-bleaching, and broad emission wavelengths. Furthermore, the fluorescent microspheres are usually prepared by solvent swelling method based on physical adsorption, thus, the organic dyes are easy to leak from the microsphere matrix. Therefore, the exploration of new type of fluorescent materials with good luminescent property and photo-stability as well as new convenient ways to prepare fluorescent microspheres are currently key issues to develop immunological method to detect *LM*.

Layer-by-layer (LBL) electrostatic self-assembly technique has recently been explored as a convenient method to functionalize the surfaces of various materials[13], which could be also used to prepare fluorescent microspheres. [14] A brief preparation process may

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involve the alternate introduction of cationic and anionic polymers as well as charged luminescent dye molecules on the surface of the microspheres based on the strong electrostatic forces, leading to stable LBL self-assembly shell with fluorescent property. A key problem of this method is that many conventional fluorescent dyes are neutral molecules, which cannot be introduced into the LBL shell. Thus, the design and exploration of new type of charged fluorescent dye molecules become an important issue. Polyoxometalates (POMs) are one type of unique inorganic metal-oxo clusters composed of early transition metal elements (Mo, W, V, Nb etc) in their highest oxidation states. Especially, the rich oxygen surface of POMs can be decorated by organic, organometallic and metal-organic groups, representing one type of ideal precursors to design and synthesize new organic-inorganic hybrid molecules with desired functionalities. [15-17] In this aspect, POMs have ever been covalent-bonded with organic luminescent dye molecules, and these new type of hybrid luminescent molecules usually display various negative charges, chemically tunable luminescent activity, and high photo-stability. [18-20] The most important of all, such charged luminescent molecules possess inherent advantages to be easily introduced into the LBL self-assembly shell, providing an ideal type of precursors to prepare luminescent microspheres by the use of LBL assembly method.

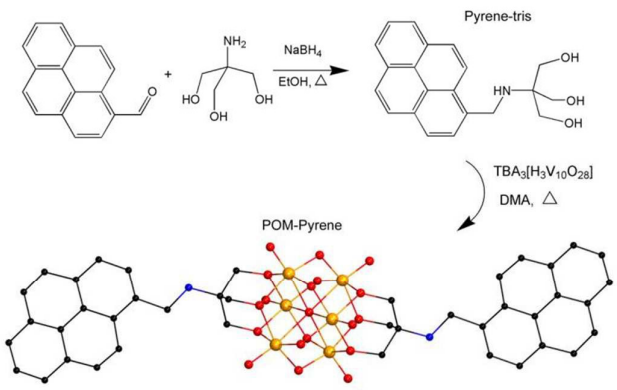
Based on aforementioned consideration, we design and synthesize a new organic-inorganic hybrid POM-pyrene compound $[N(C_4H_9)_4]_2[V_6O_{13}\{(OCH_2)_3CNH\text{-}CH_2\text{-}C_{16}H_9\}_2]$ (**1**). In compound **1**, the inorganic Lindqvist-type polyoxovandate anion is covalently connected with the luminescent pyrene groups via the organic Tris(hydroxymethyl)-aminomethane (Tris) bridges. The hybrid molecular material displays two negative charges and is charge-balanced by two tetrabutylammonium (TBA) cations. Luminescent measurement suggests that compound **1** exhibits excellent photo-stability and fluorescent property. Then, compound **1** is employed as a negative-charged dye molecule to prepare new type of fluorescent microspheres by the use of LBL assembly technique for the first time. The as-obtained polystyrene (PS) microspheres covered with the POM-pyrene-containing fluorescent shell is introduced into the suspension array system for the detection of *LM* contamination based on the flow cytometry (FCM) technique. [21-23] The relevant experimental results suggest that such a new detection system provides a convenient, time-saving and high sensitive way to detect *LM* for the safety of food.

2. Results and discussion

2.1 Synthesis, structure and luminescence of POM-pyrene 1

2.1.1 Synthesis of POM-pyrene 1

The functionalization of POMs with organic luminescent groups based on covalent bonds is a feasible strategy to prepare negative charged luminescent hybrid molecules. During the preparation, it is important to select a suitable organic bifunctional linker that can join POM units and organic luminescent groups together. Tris(hydroxymethyl)-aminomethane (Tris) has been extensively used in POM chemistry as a bifunctional organic linker.[24,25] In such a molecular bridge, the aminomethane group can combine with an organic functional group through easy aldimine condensation reaction, while the Tris(hydroxymethyl) groups can be directly



Scheme 1 Schematic view of the synthetic route of POM-pyrene **1**

connected on the surface of POM units. Lindqvist $\{V_6O_{19}\}$ POM is one of suitable POM fragments for the combination with organic Tris ligands.[24] Herein, we use Tris ligands to connect the Lindqvist $\{V_6O_{19}\}$ unit and luminescent pyrene groups together, forming a new organic-inorganic hybrid compound $[N(C_4H_9)_4]_2[V_6O_{13}\{(OCH_2)_3CNH\text{-}CH_2\text{-}C_{16}H_9\}_2]$ (POM-pyrene **1**). Compound **1** was synthesized in two steps, as shown in Scheme 1. Firstly, pyrene-1-carboxaldehyde is reacted with Tris ligand in hot ethanol solution and reduced by $NaBH_4$ to form $(HOCH_2)_3CNH\text{-}CH_2\text{-}C_{16}H_9$) (pyrene-Tris). Then, the pyrene-Tris is reacted with decavanadate $[N(C_4H_9)_4]_3[H_3V_{10}O_{28}]$ in hot DMA solution under dry nitrogen atmosphere. During the reaction process, the $\{H_3V_{10}O_{28}\}$ polyoxoanion is transferred into $\{V_6O_{19}\}$ fragment, which is stabilized by two tri-dentate Tris(hydroxymethyl) groups by sharing the O atoms between $\{V_6O_{19}\}$ units and Tris linkers. Brown block crystals suitable for the single-crystal X-ray diffraction were isolated in a week from a cooling and saturated solution of **1** in DMF with the ambient temperature of 5 °C. Increasing the temperature or the concentration of the solution just led to tiny microcrystals of **1**. The crystal structural data and additional physical measurements of POM-pyrene 1 were shown in supporting information (FigS1 to S8 and Table S1,S2).

2.1.2 Luminescent property of POM-pyrene 1

The luminescent properties of pyrene-1-carboxaldehyde precursor and the POM-pyrene compound **1** were investigated in the solid state at room temperature. As shown in Fig. 1a, the pyrene precursor exhibits one strong emission band at 495 nm upon excitation at 343 nm. Upon similar excitation (346 nm), compound **1** exhibits maximum emission band observed at 400 nm (Fig. 1b). Generally, the emission band of free pyrene precursor is attributed to the intraligand π-π* transitions [26]. In this case, the high energy emission (400 nm) of **1** which exhibits a blue-shift of 95 nm relative

to that of the free pyrene ligand (495 nm). Considering that the pyrene is still

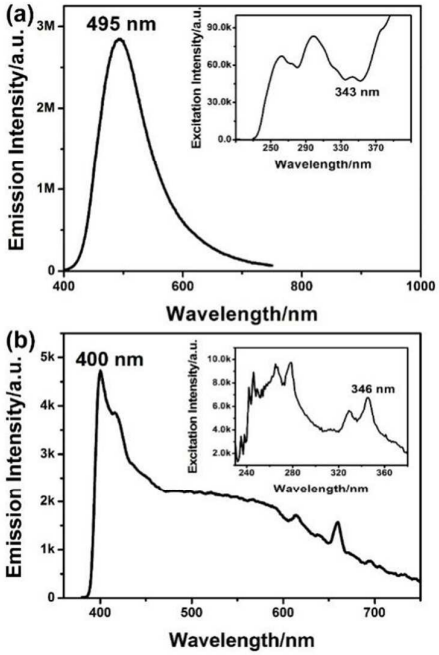
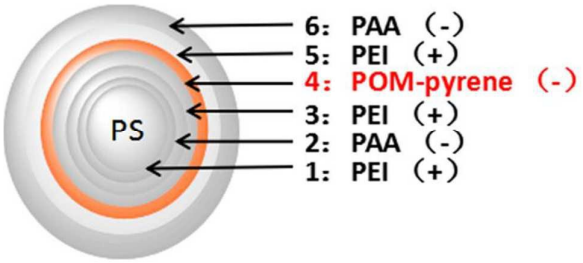


Fig. 1 Solid-state photoluminescence spectra of **(a)** free pyrene-1-carboxaldehyde precursor under 343 nm excitation, and **(b)** POM-pyrene **1** under 346 nm excitation.

the luminescence-active center in the hybrid molecule **1**, it is thus presumed that the introduction of POM units into the pyrene groups might increase the distance between adjacent pyrene planes, which effectively improve the energy gap between HOMO and LUMO of pyrene groups, leading to the blue-shift behaviour in such POM-pyrene hybrid molecule [25].

2.2 Preparation and characterization of POM-pyrene-decorated PS microspheres (PS@POM-pyrene)

POM-pyrene **1** exhibits good luminescent property with negative charges, representing a suitable type of luminescent molecules for the preparation of fluorescent microspheres by the use of LBL electrostatic self-assembly technique. In this case, the polystyrene (PS) microspheres were used as the matrix (P_0) and alternately coated by six positive and/or negative functional layers with PEI, PAA and POM-pyrene **1**, forming a six-layered shell PS@ PEI/PAA/PEI/POMs-pyrene/PEI/PAA (or shorten as PS@POM-pyrene (as shown in Scheme 2). During the preparation of the final product P_6 as well as the intermediates P_1-P_5, the introduction of different functional layers were was monitored by the zeta potential measurements, since the zeta potentials were changed upon coating materials with different charges on the surface of the microspheres (Fig. 2). The luminescent properties of the PS matrix (P_0) and the final PS@POM-pyrene product (P_6) were compared based on the measurements of laser scanning confocal microscopy, fluorescence spectroscopy and flow cytometry (FCM). As shown in Fig. 3, the white color of P_0 (Fig. 3a) changed into the light yellow color of P_6 (Fig. 3b) when the POM-pyrene was decorated on the PS microsphere. The whole P_6 products show deep-blue luminescence under UV light irradiation (Fig. 3d,f), but the P_0 shows no distinguishable luminescence (Fig. 3c,e). The fluorescence intensity value of P_0 and P_6 were measured by fluorescence spectroscopy and FCM, as shown in Fig. 4a and Fig. 4b respectively. It is observed that the fluorescence intensity of P_6 was significantly increased in contrast to those of P_0, confirming that POM-pyrene was successfully decorated on the PS microspheres with relatively strong emission. Moreover, the photo-stability of the final product P_6 was investigated after the sample was kept at room temperature for six months without protecting from light. As shown in Fig. 4a, the luminescent emission band of P_6 after keeping for six months showed comparable intensity in contrast to that of freshly prepared P_6, suggesting that the photo-stability of P_6 is sufficient enough for the general application in the fluorescence microsphere-based suspension immunoassay.



Scheme 2 Formation of six-layered shell by LBL electrostatic self-assembly technique. The composite microsphere is labelled with PS@PEI/PAA/PEI/POMs-pyrene/PEI/PAA or PS@POM-pyrene or **P_6**

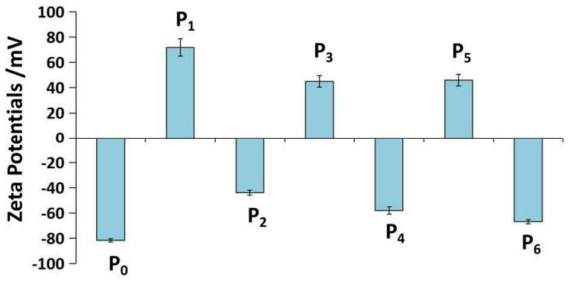


Fig. 2 Zeta potential measurements of P_0-P_6

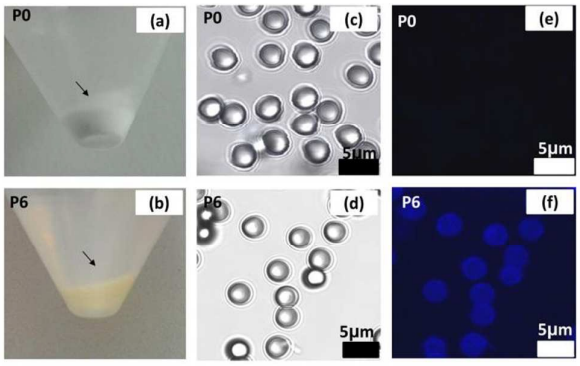


Fig.3 (a) and **(b)** photo images, **(c)** and **(d)** laser scanning confocal microscopy images in bright field, and (e) and (f) laser scanning confocal microscopy images in UV irradiation at 355 nm of P_0 and P_6, respectively.

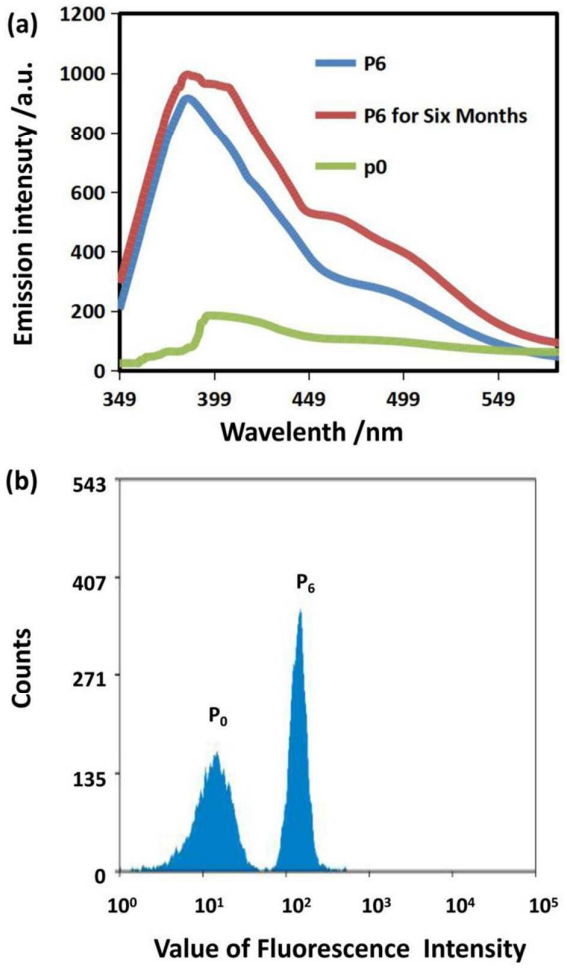


Fig. 4(a) Luminescent spectra of the PS matrix (P_0), the freshly prepared PS@POM-pyrene product (P_6) and P_6 after keeping for six months without protecting from light. **(b)** Flow Cytometry (FCM) analysis of the PS matrix (P_0) and PS@POM-pyrene product (P_6)

2.3 Biofunctionalization of PS@POM-pyrene microspheres with anti-*LM* mAbs

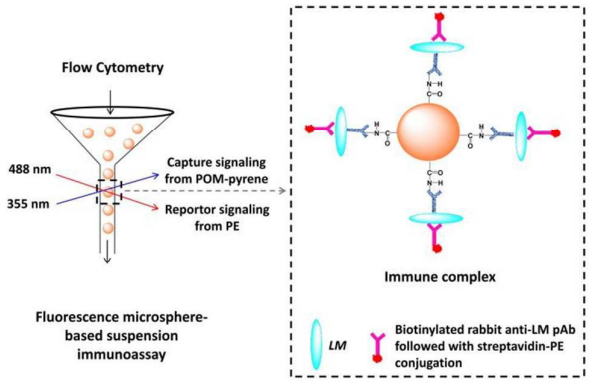
During the preparation of PS@POM-pyrene microspheres, we aim to coat the PS matrix with fluorescent dye and multiple carboxyl groups simultaneously, since the presence of –COOH group can greatly facilitate the interaction with abundant amino groups on the biomolecules for example the monoclonal antibody. In this case, the outermost layer of the final product **P_6** is coated with polyacrylic acid (PAA), which comprises rich carboxyl groups and suitable for the connection of biomolecules. Therefore, the anti-*LM* mAbs as the specific functional biomolecules were introduced into the PS@POM-pyrene microspheres (**P_6**) based on a two-step carbodiimide reaction route (as shown in Scheme S1). [27] As a result, the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres can be used as a new type of fluorescence probes in the *LM* detection immunoassay.

In order to check that mAbs have been successfully loaded on the microspheres, the commercial goat anti-mice antibody and SAPE were used for the fluorescence detection (Scheme S2). As shown in Fig. S9a, the median fluorescence intensity (MFI) values changed when different amounts of mAbs were added in the binding assay. The MFI values were increased when more mAbs were coupled on the surface of the microspheres. When 56 μg mAb was used to couple with 10^6 PS@POM-pyrene microspheres, the maximum MFI value was obtained. Therefore, the anti-*LM* mAbs bioconjugated **P_6** prepared by conjugating 10^6 PS@POM-pyrene microspheres with 56 μg mAb are used as the protocol for the following fluorescence microsphere-based suspension immunoassay.

2.4 Fluorescent microsphere-based suspension immunoassay

The binding abilities to *LM* Ag for the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres were tested in a sandwich immunofluorescence assay, as shown in Scheme 3. In such testing system, the formation of an immune complex usually includes the molecular recognition between anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres and the targeted *LM* antigen, followed by the visualization of a fluorescent streptavidin-PE conjugation by the use of a biotinylated detector (the rabbit anti-*LM* polyclonal antibodies).

To carry out fluorescent microsphere-based suspension immunoassay for *LM* detection, the microspheres were incubated with cellular samples containing *LM* Ag (n=5) or no *LM* Ag (PBS only, n=5) and the immunodiagnostic complex with fluorescently labelled streptavidin-PE can be detected by FCM with both the fluorescence of PE and POM-pyrene (Fig. S10). The MFI was shown in Fig. S9b. *LM* Ag samples incubated with anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres showed high MFI value, while the MFI value of PBS samples was almost not detected.



Scheme 3 Schematic view of the fluorescent microsphere-based suspension immunoassay (Inside the box: Schematic view of the formation of an immune complex)

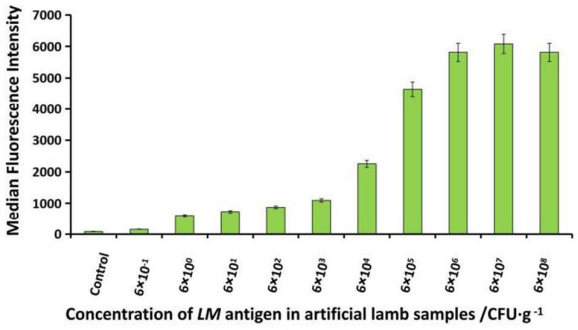


Fig. 5 MFI vs. concentration of *LM* antigen in lamb sample, showing the sensitivity of PS@POM-pyrene microsphere-based suspension immunoassay.

In order to accomplish accurate immunoanalysis for *LM* detection with the designed suspension immunoassay system and to test the *LM* detection in practical tissue samples, we further analyzed artificially *LM*-contaminated meat samples with different known concentrations of *LM* from 0.6 CFU·g-1 to 6×10^8 CFU·g^{-1}, which have been pre-enriched for 12 h. The results showed that anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres coupled with *LM* complex had different fluorescence intensity of PE and allowed the *LM*-positive samples to be distinguished from the *LM*-negative samples. It is noteworthy that when the lowest 6 CFU of *LM* Ag were added into 1 g lamb, the MFI value of anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres was still twice higher than the background fluorescence, indicating a positive reaction (Fig. 5). Therefore, the sensitivity of anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres used to detect Ag in pre-enriched artificially *LM*-contaminated meat samples was approximately 6 CFU·g^{-1}.

To examine if the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres react with pathogens other than *LM*, more than 10 bacterial Antigens which can be distingushed by standard culture-based method were applied in the suspension immunoassay. The results show that only the *LM* antigen can be accurately identified by anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres with a significantly high MFI value, confirming that the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres showed excellent specificity to *LM* without any cross-reactivity with other pathogens (Fig. 6).

The repeatability of such microsphere-based suspension immunoassay were also investigated. In the experiment, two *LM* samples were tested in triplicate for three times . The repeatability coefficient of variation (CV) of each samples were 2.12%, 2.24% and 2.18%, respectively, (Table S3) indicating that microsphere-based suspension immunoassay is highly repeatable.

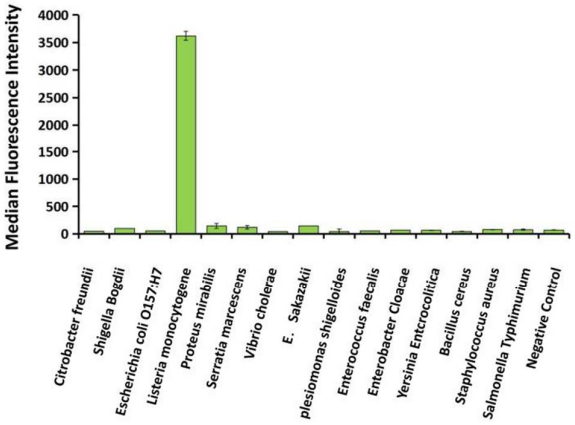


Fig. 6 Specificity of suspension immunoassay based on PS@POM-pyrene microspheres

Table 2. (a) Fluorescent microsphere-based suspension immune assay and (b) the standared culture-based method used for detecting *LM* Ag from Lamb samples

		(a)		Total
		+	—	
(b)	+	22	0	22
	—	0	26	26
	Total	22	26	48

Based on such experimental results, the accuracy of the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres-based suspension immunoassay was quite similar to that of the conventional culture-based method. For the 48 artificial lamb

samples, both approaches identified 22 samples as positive samples whereas 26 were negative (as shown in Table 2). However, the conventional culture-based method took more than 7 days to accomplish the assays, while microsphere-based suspension immunoassay was finished within a 24-h period. It is thus obvious that the anti-*LM* mAbs bioconjugated PS@POM-pyrene microspheres represents an efficient approach for detecting *LM* in food substances.

3. Conclusions

In summary, we design and synthesize a new POM-pyrene hybrid luminescent compound. Such negative-charged functional dye molecules are used to prepare new type of fluorescence microspheres by virtue of layer-by-layer self-assembly technique for the first time. The as-prepared fluorescent microspheres show good luminescent property, excellent photo-stability and long-term stability. Thus, such POM-pyrene decorated microspheres (labelled with PS@POM-pyrene) are employed in a fluorescent microsphere-based suspension array system for the detection of *LM*. Before measurements, the PS@POM-pyrene microspheres are bioconjugated with monoclonal anti-*LM* antibody to form the desired biomolecular probes. Such microspheres possess high sensitivity to detect Ag in pre-enriched artificially *LM*-contaminated meat samples. The lowest limit of detection (LOD) of *LM* content is ca. 6 $CFU \cdot g^{-1}$. Furthermore, the suspension immunoassay based on PS@POM-pyrene microspheres exhibit superior specificity to *LM*, good repeatability and high efficiency in contrast to the conventional culture-based method. This work may pave a new way to prepare new stable and low-cost fluorescent microspheres for the development of immunoassay technique to test *LM*. Moreover, the design and synthesis of new type of negative-charged POM-based hybrid luminescent compounds at the molecular level may be extended to preparing various POM-organic fluorescence compounds based microspheres, which can detect more pathogens depending on the availability of specific monoclonal/polyclonal antibodies and various POM-organic fluorescence microspheres using the fluorescent microsphere-based suspension array system. This work is ongoing in our research team.

4. Materials and methods

4.1 chemicals and reagents

All chemicals were of reagent grade and used without further purification. The precursor decavanadate $[TBA]_3[H_3V_{10}O_{28}]$ [28] and Tris-pyrene ($(HOCH_2)_3CNH\text{-}CH_2\text{-}C_{16}H_9$) [29] were synthesized based on literature procedures. Elemental analyses (C, H and N) were conducted on a Perkin-Elmer 240 C elemental analyzer, and V element was determined with a Leaman inductively coupled plasma (ICP) spectrometer. FT/IR spectra were recorded from KBr pellets in the range 4000-400 cm^{-1} on an Alpha Centaur FT/IR spectrometer. UV-vis spectra were obtained with a UV-7502C spectrometer (DMF as solvent) at 300 K. NMR spectra was recorded with an AS-500(500 MHz, ^1H NMR) spectrometer at room temperature. Fluorescence was recorded with a VARIAN fluorescence spectrophotometer. Polyacrylic acid (PAA) solution, N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccini mide (NHS) were all purchased from Sigma-Aldrich. Poly(ethylene imine) (PEI) solution was purchased from Aladdin Industrial. R-Phycoerythrin Conjugate (SAPE) and Pierce EZ-Link® Biotinylation Kits were ordered from Thermo Fisher Scientific Inc. HRP-labeled goat anti-mouse IgG (H+L) and biotin-labeled goat anti-mouse IgG (H+L) were obtained from Huamei (Beijing, China). Rabbit anti-*LM* polyclonal antibodies were prepapred by ourselvies and the biotin-labeled detection rabbit anti-*LM* polyclonal antibodies were prepared by the use of EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo, USA) according to the manufacturer's instruction.

4.2 Synthesis of POM-pyrene 1

The POM-pyrene hybrid compound **1** was prepared under a dry nitrogen atmosphere with dried solvents under anhydrous conditions. The preparation details and relevant physical characterizations are as follows: Decavanadate $[TBA]_3[H_3V_{10}O_{28}]$ (1.505 g, 0.89 mmol) and Tris-pyrene (0.90 g, 2.67 mmol) are placed in a Schlenk tube under nitrogen atmosphere. When anhydrous dimethylacetamide (DMA) (30 mL) is injected in the Schlenk tube, the resulting orange solution was stirred and heated in the dark under nitrogen at 85 ℃ for 48 h. Then, the green-brown solution was cooled to room temperature and filtered. An orange solid powder was obtained. The solid was washed with acetonitrile (1 mL) and diethyl ether (10 mL), and dried to get the compound **1** as an orange power (0.8 g, 33% yield based on vanadium). When the powder was dissolved in hot DMF solution and diffused by ether, orange block crystals of **1** were isolated in two weeks (0.26 g, 11% yield based on vanadium). Elemental analysis (%) calcd for $C_{74}H_{108}N_4O_{19}V_7$: C 53.43, H 6.55, N 3.37, V 18.38; found (%): C 53.29, H 6.17, N 3.31, V 18.52. Selected IR(KBr pellet, cm^{-1}): 2958, 2929, 2870, 1482, 1378, 1059, 945, 811, 795, 718, 581. ^1H NMR(d_6-DMSO, 500M, 298K): δ 8.43 (d, J = 9.0 Hz, 1H), 8.27 (t, J = 7.0 Hz, 2H), 8.24 – 8.22 (m, 2H), 8.14 (d, J = 8.0 Hz, 3H), 8.05 (t, J = 7.5 Hz, 1H), 5.07 (s, 6H), 4.52 (d, J = 7.0 Hz, 2H), 3.17 – 3.14 (m, 8H), 1.57 – 1.54 (m, 8H), 1.34 – 1.27 (m, 8H), 0.93 (t, J = 7.5 Hz, 12H). ^{13}C NMR(d_6-DMSO,500M,298K) : δ 136.1, 131.23, 130.9, 130.3, 128.8, 127.9, 127.8, 127.6, 127.2, 126.6, 125.4, 125.1, 124.6, 124.5, 124.3, 85.2, 58.0, 52.2, 43.5, 23.6, 19.7, 14.0.

4.3 Bacteria culture

The *LM* strain (ATCC19111, American Type Culture Collection ,USA) used in this study was cultured in *LM* culture medium. Other bacteria including *Citrobacter freundii* (ATCC8090), *Shigella Bogdii* (ATCC9207), *Escherichia coli O157:H7* (ATCC25922), *Proteus mirabilis* (JL08017), *Serratia marcescens* (ATCC13880), *Vibrio cholerae* (JL080118) , *E. Sakazakii* (JL08106), *Enterococcus faecalis* (ATCC14506),*Enterobacter Cloacae* (ATCC33420),*Yersinia Enterocolitica* (ATCC23715), *Bacillus cereus* (Isolate), *Staphylococcus aureus* (26001-25), *Salmonella Typhimurium* (ATCC13311) and *plesiomonas shigelloides* (ATCC14030) were cultured in Lubria-Bertani broth at 37 ℃ with shaking at about 250rpm. The concentrations of bacteria were determined on the plate count agar using plate counting[30].

4.4 Mice anti-*LM* monoclonal antibody (mAb) Preparation

The anti-*LM* mAb was produced using the hybridoma technology according to established protocols [31,32] with minor modifications. The mAb was purified by protein affinity chromatography [33] and stored at -20 ℃ for use.

4.5 LBL assembly of POM-pyrene-decorated PS microspheres (PS@ POM-pyrene)

Polystyrene (PS) microspheres were obtained from Bangs Laboratories, Inc. with the size of 3 μm in diameter (labelled with $\mathbf{P_0}$). The PS@POM-pyrene microspheres were prepared by the layer-by-layer electrostatic self-assembly method [34,35]. During the preparation, the coating process was monitored by the UV-vis spectra. In a typical experiment, $\mathbf{P_0}$ (1 mg) was dissolved in 5 mL of H_2O by ultrasonic dispersing technique to yield the PS suspension. Then, the PS microspheres were sequentially activated by coating with polyelectrolytes PEI, PAA, PEI, POM-pyrene, PEI and PAA, respectively, so as to obtain a POM-pyrene- decorated six-layered shell. The coating procedures were as follows. In the first step, 1 mL of PEI solution (PEI/1 M NaCl is 1:19 in v:v ratio) was added into the $\mathbf{P_0}$ suspension. The mixture was stirred at room temperature for 30 min. Then, the mixture was alternately centrifuged (10000 rpm, 5 min) and washed with H_2O for three times in order to remove the excess PEI chemicals. The final centrifuged deposits were dispersed into 5 mL of H_2O to yield $\mathbf{P_1}$. In the second step, 1 mL of PAA solution (PAA/1 M NaCl is 1:9 in v:v ratio) was added into $\mathbf{P_1}$ suspension and stirred at room temperature for 30 min. Then, the mixture was alternately centrifuged (10000 rpm, 5 min) and washed with H_2O for three times in order to remove the excess PAA chemicals. The final centrifuged deposits were dispersed into 5 mL of H_2O to yield $\mathbf{P_2}$. The third step repeated the first step again, and another layer of PEI was coated on the microspheres to yield $\mathbf{P_3}$. In the fourth step, the anionic POM-pyrene hybrid fluorescent compound **1** (1 mg) was dissolved in 5 mL of MeCN and added into the $\mathbf{P_3}$ suspension. After stirred at room temperature for 30 min, the mixture was alternately centrifuged (10000 rpm, 5 min) and washed with H_2O for three times in order to remove the excess POM-pyrene chemicals. The final centrifuged deposits were dispersed into 5 mL of H_2O to yield $\mathbf{P_4}$. In the fifth and sixth step, another PEI ($\mathbf{P_5}$) and PAA ($\mathbf{P_6}$) layers are alternately coated on the microsphere. The introduction of extra two layers not only played the role in stabilizing the POM-pyrene molecular fluorescent material on the microspheres, but also provided a PAA negative charge-modified surface of microspheres, which is easy to further link with anti-*LM* mAb functional groups for the *LM* detection. The final POM-pyrene-modified fluorescent microspheres were stored at 4 °C. The six-layered shell can be expressed as PS@PEI/PAA/PEI/POM-pyrene/PEI/PAA (PS@POM-pyrene).

4.6 Preparation of anti-*LM* mAb-bioconjugated PS@POM-pyrene microspheres

In a phosphate-buffered saline (PBS), the anti-*LM* mAbs were covalently bonded with the carboxylate groups on the surface of PS@POM-pyrene microspheres by the use of a typical two-step carbodiimide reaction (Scheme S1). Firstly, the carboxylate group (originated from PAA) on the surface of PS@POM-pyrene microspheres were activated for 20 min in 160 μL 0.1 M monobasic sodium phosphate (pH 6.9) containing 10 μL EDC (50 mg/mL) and 10 μL NHS (50 mg/mL). The activated microspheres were washed 3 times with PBS (pH 7.3) and collected by centrifugation. Secondly, the as-prepared microspheres were introduced into 500 μL PBS containing anti-*LM* mAbs at 37 °C for 2 h. Then, anti-*LM* mAb-conjugated PS@POM-pyrene microspheres were collected by centrifugation and washed with 500 μL PBS containing 0.5% Tween 20 (PBST) for three times in order to remove excess anti-*LM* mAb.

To detect whether the surface functionality of PS@POM-pyrene microspheres could be used for bioconjugation with anti-*LM* mAbs successfully and satisfactorily, the flow cytometric analysis was performed and Median Fluorescence Intensity value (MFI) was obtained after the as-prepared microspheres are labelled with goat anti-mouse biotin-labelling IgG and streptavidin-PE based on the specific molecular recognition (Scheme S2). The detailed protocol is as follows: The above collected resulting anti-*LM* mAb-conjugated PS@POM-pyrene microspheres were incubated with 100 μL goat anti-mouse biotin-labeling IgG (1:500) at 37 °C for 1 h. After the samples were washed twice with 300 μL PBST, the as-prepared microspheres were further incubated with 100 μL streptavidin-PE (10 μg/mL) at 37 °C for 0.5 h. Then, the samples were washed by 300 μL PBST, and the final labelled microspheres were re-suspended in 500 μL PBS per well, and analyzed with the MoFlo XDP High Speed Cell Sorter and Analyzer (Beckman-coulter). The analysis results were recorded as MFI values.

To achieve a better coupling, concentrations of anti-*LM* mAbs should be optimized. Different amounts of anti-*LM* mAbs (0, 14, 28, 56 and 112 μg) were tested for conjugation with 10^6 PS@POM-pyrene Microspheres. Different anti-*LM* mAb-conjugated PS@POM-pyrene microspheres were collected and corresponding MFI values were obtained by flow cytometric analysis. For anti-*LM* mAb-conjugated PS@POM-pyrene microspheres, best coupling may be achieved based on the amount of anti-*LM* mAbs when the highest MFI value was detected.

4.7 Fluorescence microsphere-based suspension immunoassay

LM detection is based on the specific molecular recognition between *LM* antigen (Ag) and anti-*LM* mAb-bioconjugated PS@POM-pyrene microspheres. The basic principle for the detection of the binding of *LM* antigen (Ag) and anti-*LM* mAb-bioconjugated PS@POM-pyrene microspheres are shown in Scheme 3. The fluorescence microspheres were incubated with cellular samples containing *LM* Ag (n=5) or no *LM* Ag (PBS only, n=5) for 1 h at 37 °C. Following two washes with 300 μL of PBST, samples were incubated with 100 μL biotin-labelled rabbit anti-*LM* polyclonal IgG for 1.0 h at 37 °C. After two washes, the mixture was incubated with 10 μg/mL streptavidin-PE for 0.5 h at 37 °C. The mixture was then washed with 300 μL PBST, re-suspended in 500 μL PBS per well, and analyzed by the use of MoFlo XDP High Speed Cell Sorter and Analyzer. If the MFI value of a sample was greater than twice of its background value, the particular sample was defined as a positive reaction. For cross-reactivity experiments, heat-killed bacteria such as *Citrobacter freundii, Shigella Bogdii* and *E.coli O157*were used.

For testing the repeatability, 2 different *LM* samples were checked in triplicate. Individual experiments were repeated three times.

To test the usefulness of our immunoassay for *LM* detection in food samples, fresh ground lamb was purchased from a local market. 1 g ground lamb was mixed with 10 mL *LM* medium (6×10^9 CFU·mL^{-1}) in a sterile 15-mL tube. The mixture was serially diluted in 10 times to reach different concentrations until a CFU of 0.06 in *LM* medium. All the diluted samples were mixed by vortex and cultured in 37 °C overnight. Heat-killed samples were prepared in the same way for the microsphere-based suspension immunoassay. 48 artificial meat samples were prepared and used in a comparative experiment involving the microsphere-based suspension immunoassay and the conventional culture based-method.

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Table of Contents

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Keywords: Polyoxometalate, Layer-by-layer, Fluorescence Microsphere, Immunoassay, *Listeria Monocytogenes*

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Title: Layer-by-Layer Assembly of Polyoxometalate-Pyrene-Decorated Fluorescent Microspheres for Suspension Immunoassay of *Listeria Monocytogenes*