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Silica nanoparticles-assisted electrochemical biosensor for the rapid, sensitive and specific detection of *Escherichia coli*

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Supporting Information

ABSTRACT: To face the emerging issues raised by bacterial infections in biomedical, food and environmental contexts, the combination of nanotechnologies and biosensors characteristics is increasingly seen as a promising tool to develop rapid, highly sensitive and specific devices for reliable bacterial detection and quantification. We present here the design of an electrochemical immuno-biosensor, integrating non cytotoxic silica NPs, in a two-step spin coating process. Unlike biosensors that integrate a unique antibody-based layer without NPs, this new tool allows, through cyclic voltammetry measurements, the continuous and non-saturated detection of *E. coli* bacteria in only 5 min. and can reach up to 10^3 CFU/mL in 30 min. It offers great promises for industrial purposes as electrode functionalization is rather simple and its architecture can be tuned on demand to target a variety of micro-organisms, and the highlighted indicators (variations of current intensity, intensity ratios and/or difference of potentials) can easily be implemented in a screen displayed packaging.

Keywords - immunosensor, cyclic voltammetry, *E. coli*, nanoparticles

1. INTRODUCTION

The detection of pathogenic bacteria is of critical importance for health and safety issues in clinical diagnosis, food industry, water and environment quality, as well as struggle against potential bioterrorism[1]. Traditional and conventional methods for bacteria identification and detection are mainly based on culture and colony counting[2], polymerase chain reaction[3] and immunology tests[4]. Despite their high sensitivity, most of them require pre-enrichment of bacteria in samples and/or DNA-

amplification and, thus, tend to be laborious, time consuming and expensive. Consequently, many efforts have been made to reduce the cost and detection time while improving the reliability, sensitivity and robustness of new detection techniques. In this context, biosensors, inspired from immunology tests like ELISA one, have raised an incredible interest as they allow this detection in a single autonomous and integrated device[5–7]. Simultaneously, nanotechnologies have experienced, over the last decades, a fast and growing development, especially in biomedical fields[8–10]. Combining the physical properties and high surface-to-volume ratios of nanoparticles (NPs) to surface modification and composition of transducers, the search for highly sensitive and selective biosensors can be strongly renewed. Indeed, NPs have already been successfully applied to detect molecules of biological interest such as metallic ions, nucleic acids and proteins[11].

Nowadays, a worldwide threat concerns pathogens that can cause serious, even fatal, illnesses through the contamination of food, water or medical tools, such those induced by the well-known *E. coli* O157:H7, that is susceptible to even develop resistance to current antibiotic therapies[12,13]. Regarding this issue, research on biosensors for the detection and elimination of *E. coli* bacteria is fairly extensive not only because of the wide range of existing transducers but also because of the broad spectrum of NPs properties especially developed for this purpose[14]. Optical biosensors, essentially based on Surface Plasmon Resonance (SPR), and fluorescence measurements have been successfully applied to the detection of *E. coli*, with limits of detection (LOD) as low as 10^2 -

10^3 CFU/mL[15], similar to electrochemical and gravimetric biosensors[16]. Indeed, these LODs are strongly improved when integrating NPs as shown, for instance, by Joung *et al.* who designed an SPR biosensor for *E. coli*, based on gold NPs, and reduced its LOD by three orders of magnitude[17]. A milestone was reached with fluorescein-enriched silica NPs associated with magnetic Fe_3O_4 -NPs that allow detecting 3 CFU/mL within 75 min.[18]. Despite such a high sensitivity, optical tools require expansive setups and analyses of experts which are not really convenient for wide use. Gravimetric biosensors, mainly based on Quartz Crystal Microbalance, coupled with gold or iron oxide NPs are easier to use and can detect as low as 10^2 CFU/mL[19]. Compared to these tools, electrochemical biosensors appear to be the least restrictive, since they offer portability, low cost and easy manipulation. For instance, Settingerton and Alocilja took advantage of Cyclic Voltammetry (CV) to detect down to 6 CFU/mL within 1 h thanks to immuno-functionalized magnetic/polyaniline core/shell NPs[20].

In this work, we present the first results on the design of an electrochemical biosensor, in which the presence of silica-NPs, functionalized with specific polyclonal antibodies (Abs), is used to target and bind *E. coli*. Subsequently, the interaction inhibits the current response of the transducer, observed by CV. We highlight the existence of straightforward monitoring indicators for *E. coli* detection and quantification that can easily be screen-displayed on a future point of care device. Therewith, this new biosensor can be applicable to a wide variety of targeted organisms only by changing the bioreceptor grafted on the NPs. Although optimizations are still in progress in terms of NPs and bioreceptor concentrations, according to the expected LOD (in the range of 10^3 to 10^6 CFU/mL), the time of detection can be lowered to only 5 min., the best LOD of 10^3 CFU/mL being achieved in only 30 min. Such characteristics and tunable biosensor architecture pave the way for industrial purposes.

2. MATERIALS AND METHODS

2.1. Materials - Gram negative *E. coli* bacteria (MRE 162 strain) were a kind gift from the Centre d'Etudes du Bouchet, DGA (Direction Générale de l'Armement, France). Polyclonal antibodies (Abs) against *E. coli* were purchased from Thermo Scientific. Bovine Serum Albumin (BSA), ethanol, ammonium hydroxide, L-arginine, tetraethylorthosilicate (TEOS) and aminopropyl triethoxysilane (APS), used for silica nanoparticles (NPs) synthesis, were purchased from Sigma Aldrich. Polyelectrolytes, used as a sublayer for NPs fixation in the biosensor, respectively cationic poly (allylamine hydrochloride) (PAH, Mw = 56000) and anionic polystyrene sulfonate (PSS, Mw = 70000), were purchased from Sigma Aldrich.

$\text{K}_3\text{Fe}(\text{CN})_6$, $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ and phosphate buffered saline (PBS, 10 mM, 0.137 M NaCl, pH 7.4), used for cyclic voltammetry measurements, were also purchased from Sigma-Aldrich. Millipore ultrapure water (pH 5.5, resistivity > 18.2 M Ω .cm) was used for NPs cleaning and for preparing all the buffers.

2.2 Electrochemical measurements - Cyclic voltammetry (CV) measurements were performed with a 910 PSTAT mini from Metrohm and data were collected with the associated software PSTAT. Data analysis was controlled with a Matlab routine, estimating the oxidation and reduction currents and the associated potentials. Gold screen printed electrodes (4 mm in diameter), with carbon auxiliary and silver reference electrodes, also purchased from Metrohm, were used as sensors. Before any solution was applied, sensors were rinsed with ultrapure water and allowed to dry. Sensors were disposed off, after a set of bacteria detections. CV measurements were performed, at room temperature, in PBS in presence of 10 mM of $\text{K}_4\text{Fe}(\text{CN})_6$ redox probe to estimate the blocking properties of the bilayers versus electron-transfer process. CVs were carried out from -0.4 V to 0.6 V, at a rate of 100 mV/s, those presented in this work being acquired after the stabilization of the system.

2.3. Preparation of SiO_2 -NPs⁻ solutions - Synthesis of negatively charged home-made SiO_2 -NPs⁻ was carried out following synthesis developed by Hartlen *et al.*[21]. The first step consisted in synthesizing SiO_2 seeds. For this purpose, 100 mL of a 6 mM L-arginine water solution were introduced in a vial at 60°C, under magnetic stirring (150 rpm). After temperature stabilization, 10 mL of TEOS were slowly added so as to respect the interface. Seeds of about 30 nm in diameter were obtained after complete consumption of TEOS. The second step consisted in the growth of these seeds. Typically, 10 mL of SiO_2 seeds were introduced in a mixture of 455 mL of ethanol and 35 mL of ammonium hydroxide in water solution ($[\text{NH}_3] = 1$ M), under stirring (700 rpm). TEOS was then introduced in the amount calculated to get the targeted diameter (100 nm in this study), at a rate of 0.5 mL/h[22]. Surface modification was performed by adding APS (six equivalents assuming a rate of 2-3 $\mu\text{mol}/\text{m}^2$ of active OH groups at the surface of the silica particles) to the SiO_2 -NPs⁻ solution; the mixture was then stirred for 48 h in a water bath at room temperature, and then for 2 h at 100°C. Final SiO_2 -NPs⁺ were washed using ultrapure water and ethanol and three cycles of centrifugation (13,000 rpm for 20 min.). SiO_2 -NPs⁺ were finally suspended and kept in ultrapure water. They were sonicated before any experiment to prevent their aggregation.

2.4. Preparation of bacteria suspensions - *E. coli* cells were grown on LB agar plates and incubated for 16 h at 37 °C. After scraping, bacteria were suspended in PBS to obtain an optical density of 1. Serial dilutions were then

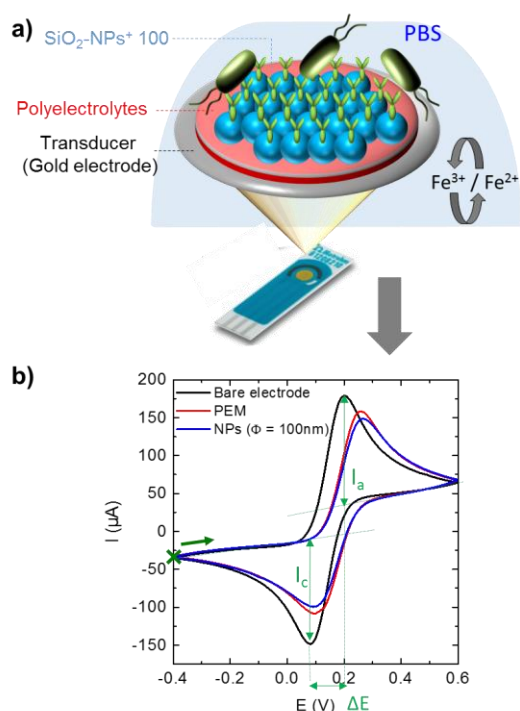
made to adjust their concentration. 10 μL of each dilution were then spread on the biosensor, previously functionalized by the appropriate systems, described in the “Results and Discussion” section. Before any CV, bacteria were incubated 5, 15 or 30 min. on the biosensor, and the surface was then rinsed with PBS, to establish the needed detection time.

2.5. Architecture of the biosensor - The architecture of the biosensor is briefly described here. Some details, mainly concerning optimization protocol, are described more precisely in the “Results and Discussion” section. After cleaning the sensor with ultrapure water, a first CV measurement was made on bare electrode that served as a control for the subsequent functionalization steps. A polyelectrolyte multilayer (PEM), constituted by the alternate deposition of PSS and PAH, was then formed by spin coating (45 s, 100 rpm, 200 s^{-2}) on the gold working electrode, by spreading alternatively 10 μL of each polyelectrolyte (5 g/L in ultrapure water). Between each layer, the sensor was rinsed with ultrapure water, and a CV measurement was made to follow the PEM construction. Then, when NPs were implemented into the biosensor, 10 μL of a $\text{SiO}_2\text{-NPs}^+$, at a concentration of 10 g/L, were attached through electrostatic interactions to the PSS ending layer of the PEM, by spin coating (20 s, 200 rpm, 200 s^{-2}) before being rinsed with ultrapure water. Finally, a drop of 7 μL of Ab, concentrated at 0.05 mg/mL in PBS, was deposited either on the PEM or on the NPs and left to incubate for 2 h at room temperature. Non-occupied sites were finally blocked with a 1 mg/mL solution of BSA for 30 min.

3. RESULTS AND DISCUSSION

3.1. Biosensor architecture - We here compare two types of biosensor for *E. coli* bacteria detection: one is implemented with NPs grafted with Abs whereas the other one lacks NPs and only exhibits Abs as a bioreceptor. This comparison allows assessing the role of NPs in biosensor performances. Both systems exhibit a similar initial architecture (Figure 1a, Figure S1): the transducer, a gold electrode, is first functionalized with a polyelectrolytes multilayer (PEM) in order to electrostatically immobilize the second layer of either NPs grafted with Abs or Abs alone. The PEM has already been studied in details in [23]. It is made of a spin-coated (45 s, 100 rpm) assisted Layer by Layer assembly of polystyrene sulfonate polyanions (PSS⁻) and poly(allylamine hydrochloride) polycations (PAH⁺): 6 ((PSS-PAH)₃ or PEM₆) or 7 ((PSS-PAH)₃-PSS or PEM₇) layers are alternatively deposited from a 5 g/L polyelectrolyte solution by electrostatic interactions with the ending layer adjusted according to the charge of the targeted system: PEM₇, ending with a PSS layer, allows immobilizing positively charged NPs whereas the PEM₆, ending with a PAH layer, allows the immobilization of

negatively charged bacteria or of the Abs through bonds with its carboxylate functions.



j'ai mis des points à la place des virgules

Figure 1. a) Schematic illustration of the architecture of the NPs-based biosensor designed for *E. coli* detection. On the gold electrode are successively deposited a polyelectrolyte multilayer (PEM), a layer of NPs (100 nm in diameter) welcoming the Abs specific to *E. coli*. In the absence of NPs, the Abs are directly deposited on the PEM. For bacteria detection, the biosensor is immersed in a PBS solution containing the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox couple for voltammetry measurements. b) Cyclic voltammograms of $\text{Fe}(\text{CN})_6^{3-/4-}$ performed after each functionalization step of the transducer.

Following this first functionalization, a self-assembled layer of Abs is formed either directly on the PEM or on a monolayer of NPs previously deposited on the PEM. In the former case, Abs are incubated on the transducer for 2 h at a concentration of 0.05 mg/mL, unbound sites being finally blocked with Bovin Serum Albumin (BSA). In the latter case, we used harmless positively charged silica NPs (coated with aminopropyltriethoxysilane (APS)) of 100 nm in diameter[24]. They were integrated through a spin-coated (20 s, 200 rpm) deposit from a 10 g/L solution. Abs were then deposited on NPs with the same protocol exposed above.

Then, the detection of bacteria is ensured through CV experiments (Figure 1b, Figure S1). From these voltammograms, various parameters are extracted and analyzed for quantification: the peak intensities for oxidation (I_a) and reduction (I_c) and the difference of potential between the

two peaks (ΔE) which are indicative of respectively the chemical and electrochemical stabilities of the redox couple in the medium.

Before any measurement in the presence of bacteria, the effect of these diverse layers added to the transducer were studied to unravel which modifications would be attributed to actual detection thereafter. Briefly, compared with voltammograms obtained on the bare electrode, those following the deposition of a PEM showed a slight decrease of both oxidation and reduction peak intensities (Figure 1b), as reported before[23]. This suggests a decrease in the kinetics of charge transfer at the electrode surface, due to the presence of a "screening" thin film of polyelectrolytes. Besides, adding NPs on the PEM did not induce significant differences in the subsequent voltammograms (Figure 1b), certainly due to the surface positive charges that (i) do not add supplementary resistance and that (ii) might favor charge transfer, the redox couple being negatively charged. Despite a decrease in the CV signal subsequent to these functionalizations, its shape remains unchanged and peak intensities are still largely enough for the ongoing bacteria detection.

3.2. *E. coli* bacteria detection - First, to test the robustness of our biosensors, we first had to eliminate any undesired non-specific detection. We therefore performed some controls by directly depositing *E. coli* bacteria on a PEM₆, i.e. in the absence of either NPs or Abs and recording the CV. The subsequent CV showed no significant variation as compared to CV obtained on the PEM₆ (see Figure S2). This shows that no detection took place through unspecific electrostatic interactions between the positively charged PAH last layer and the negatively charged bacteria. Consequently, thereafter, only the bio-recognition events will lead to a change in the CV characteristics.

Then, we compared the biosensor performance depending on the presence or the absence of NPs, *E. coli* being incubated at different concentrations on the functionalized transducer for 15 min. In the absence of NPs, when the Abs were implemented into the biosensor architecture, on a PEM₆, the CV showed simultaneously a decrease in oxidation-reduction peak intensities and of their ratio (Figure 2b-c blue stars) and an increase in the difference of potential (Figure 2d blue stars). The same trend was observed after incubation with bacteria at least at low concentration, both parameters stabilizing at concentrations higher than 4.10^7 CFU/mL. This suggests first that, logically, the Ab-bacteria antigen (Ag) specificity guarantees the recognition events and a minimal detection of *E. coli* bacteria. Second, the saturation of the signal at concentrations above 4.10^7 CFU/mL limits the quantification range of the biosensor, making it hardly possible. On the opposite, the combination of Abs deposited on a layer of silica-NPs, previously attached on a PEM₇, led to a progressive modification of the CVs following the same

trend on the whole range of bacteria concentrations: (i) the oxidation-reduction peak intensities and their ratio decreased continuously with the addition of increasingly concentrated bacteria suspensions (Figure 2a-c) while (ii) the difference of potential continuously increased (Figure 2d) (red squares). Such phenomena convey a slow-down in the kinetics of the charge transfer, due to an increased adsorbed mass at the electrode surface. These modifications suggest that *E. coli* bacteria are actually detected all over the tested range of concentrations [10^6 ; 10^9 CFU/mL]. Consequently, coupling Ab with NPs did induce a significant increase of efficiency in detecting *E. coli* bacteria, in a non-saturated and continuous way, as compared to the self-assembled system functionalized by Abs alone.

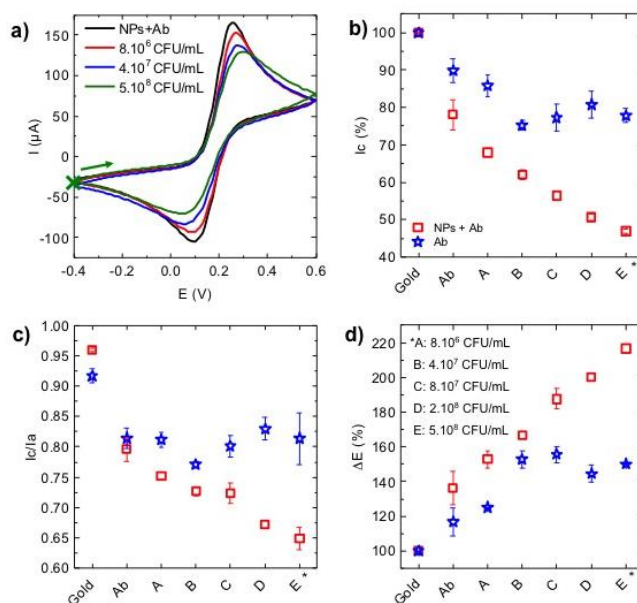


Figure 2. Influence of the presence of NPs in the electrochemical detection of *E. coli*. a) Representative cyclic voltammometry experiments based on the redox couple $\text{Fe}(\text{CN})_6^{3-/4-}$ performed after incubation of increasing concentrations of bacteria during 15 min. on the transducer functionalized by NPs/Ab. Variation with the concentration of *E. coli* of (b) the reduction peak intensity I_c , (c) the ratio between oxidation and reduction peak intensities I_c/I_a and (d) the difference of potential ΔE . Results are normalized to the values obtained on the bare gold electrode and thus expressed in %. Errors bars account for three independent measurements.

This study was also performed during shorter (5 min.) and longer (30 min.) incubation times in the presence of *E. coli* and identical linear trends were obtained (Figure 3). From such linear variations, it was thus possible to determine the sensitivity (slope of the linear regression) and the LOD (abscissa at the origin, in a first approximation) of the designed biosensors, from the intensity variations obtained between detected bacteria and Ab (Table 1 and Figure 3). Considering the initial and non-optimized Ab

concentration of 0.05 mg/mL, the silica-NPs assisted biosensor achieves a LOD of 8.10^5 CFU/mL within 15 min. With exactly the same experimental conditions, a decrease of the time detection to only 5 min. allowed anyway to accurately detect *E. coli* with a LOD of 1.10^6 CFU/mL (Table 1 and Figure 3).

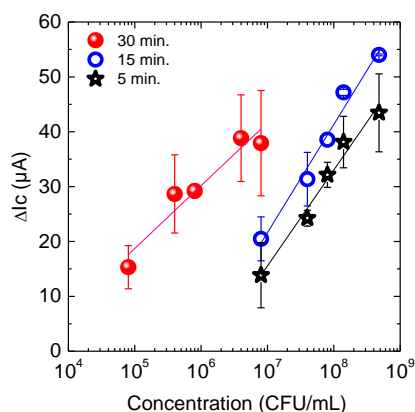


Figure 3. Variation of the reduction peak intensity obtained on the NPs-Abs assisted biosensors during the detection of various concentrations of *E. coli* bacteria and for three different times of detection (5, 15 and 30 min.). Error bars account for three independent measurements.

Detection Time	5 min.	15 min.	30 min.
Sensitivity ($\mu\text{A}/\text{CFU}/\text{mL}$)	18	20	12
LOD (CFU/mL)	1.10^6	8.10^5	2.10^3
Tested range (CFU/mL)	$8.10^6 - 5.10^8$	$8.10^4 - 8.10^6$	

Table 1. Performance of the biosensors integrating Abs grafted on NPs, depending on the detection time. Sensitivities and LOD are determined from the variations of I_c .

Though these LODs seem relatively high, it has to be underlined that (i) in various diseases, such as urinary infections, the significant threshold to qualify an infection is above 10^5 CFU/mL[25] pourquoi la ref est-elle passé en gras????and (ii) no usual detection technique is able to detect such a quantity in less than 15 min. Nevertheless, an optimization of this parameter was undertaken to meet the expected higher sensitivity. By increasing the time of detection to only 30 min., the Abs silica-NPs based biosensor was able to detect *E. coli* concentrations as low as 2.10^3 CFU/mL but, surprisingly with a lower sensitivity than the previous ones (Table 1 and Figure 3).

In the literature, lower LODs, of the order of tens to hundreds of CFU/mL, were obtained with electrochemical sensors incorporating magnetic NPs. They required however at least 1 h to reach this detection level[20,26]. At the same time, also using magnetic NPs, Varshney and Li reached a higher LOD of 10^5 CFU/mL within 35 min.[27]. Based on these reports, it seems that there is a

real need to systematically specify the biosensor efficiency not only in terms of sensitivity or LOD, but also in terms of required incubation time, all these parameters depending on the objective of the end-user who will pay more attention to the detection time while another one will expect a high sensitivity whatever the duration. In that sense, the NPs-based biosensors presented here offer nice perspectives.

Finally, a last parameter is important, namely the sensor specificity. To test this parameter, same experiments were performed with another strain of Gram negative bacteria, *Pseudomonas fluorescens*. This strain did not trigger any significant variations of the CV shapes, whatever their concentration (see Figure S4). Indeed, the oxidation-reduction peak intensities and the difference of potentials did not decrease more than 5%, even for the highest bacteria concentration, which is well below the modifications reported for *E. coli*. These observations showed that *Pseudomonas fluorescens* were not detected by this biosensor, in agreement with the specificity of the Abs towards some Ag O units of *E. coli* membrane lipopolysaccharides, which differ in *Pseudomonas* species. This confirms a first step in this biosensor specificity.

4. CONCLUSIONS

In summary, this study illustrates a proof of concept of a new type of NPs assisted biosensor. It first shows that a rather simple and time-saving functionalization might be applied to an electrochemical transducer for the detection of *E. coli*. Most of all, it emphasizes the undeniable contribution of biocompatible silica NPs to the biosensor sensitivity. Indeed, in the absence of NPs, bacteria are detected to a small extent, however no quantification is possible due to non-continuous and random variations in the CV characteristic parameters. On the contrary, when NPs are implemented into the biosensor, bacteria are not only detected in the tested ranges (from low to high amounts) but always in a continuous way. This increase in sensitivity is likely linked to the much higher specific area of the NPs that can be grafted with Abs, as compared to a plan substrate. It might also be associated with a better orientation of the bioreceptors on such APS modified NPs. The variations of indicators, such as I_c , I_a , I_c/I_a and ΔE , qualitatively and straightforwardly point to the actual detection of *E. coli*. In addition, based on the linear variations of intensities, the quantification of detected *E. coli* is fulfilled. Extensive optimizations are still in progress to achieve the Holy Grail of an ultra-sensitive and fast biosensor. For instance, the reported current performance can be highly enhanced by the control of the orientation (through EDC-NHS protocol) and concentration of Abs grafted on the NPs. Nevertheless, the initial optimizations presented in this work highlight the power of NPs integrated into biosensors as *E. coli* are specifically detected in a very short time (5 min.) and the detection is enhanced

to 10³ CFU/mL by simply adjusting the time of detection (30 min.) according to the expectations of potential industrial applications.

Supporting Information. Details about all results obtained for control, specificity and other experiments (Figures S1-S3).

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Declaration of interest

None.

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