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Tuning the incorporation of electroactive metals into titanium phosphate nanoparticles and the reverse metal extraction process: Application as electrochemical labels in multiplex biosensing

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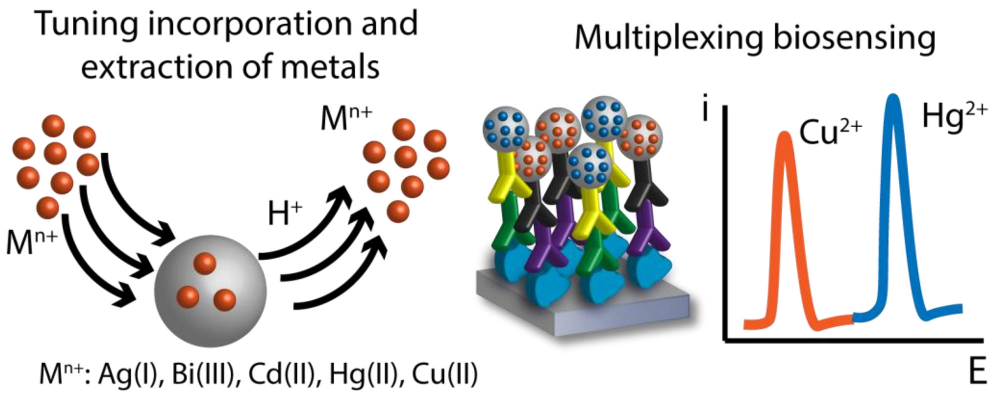
Graphical Abstract (for review)

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Titanium phosphate nanoparticles were modified with electroactive metals
- Metal amount introduced into the nanoparticles could be tuned
- Acidic media was successful to extract and detect the metals
- Cu and Hg were the most appropriate metals (sensitivity and selectivity)
- A multiplexing biosensor was developed with the modified nanoparticles

Tuning the incorporation of electroactive metals into titanium phosphate nanoparticles and their extraction for multiplexing electrochemical biosensing

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ABSTRACT

In this work, titanium phosphate nanoparticles were modified with different electroactive metals such as cadmium, bismuth, copper, silver and mercury by a cation exchange reaction. The amount of metal introduced into the nanoparticles depended strongly on the counter-ion used during the exchange reaction and the type of metal, so that nanoparticles with a high metallic load could be generated. For the detection of these metal-modified nanoparticles, the electrolytic medium used played an important role since the use of acid allows to extract a large part of the introduced metal by reverting the cation exchange reaction. The electrochemical detection of the nanoparticles was evaluated, being the nanoparticles modified with copper and mercury the most adequate in terms of sensitivity and selectivity with the aim of multiplexing detection. As a proof-of-concept, these nanoparticles were used as a detection label in a multiplexing electrochemical biosensor for the simultaneous detection of two analytes.

KEYWORDS: Nanoparticle; Titanium phosphate; Metal incorporation; Multiplexing; Biosensing

INTRODUCTION

Metal-based nanoparticles are being widely used as detection label in electrochemical biosensors[1–3] since they have interesting properties such as the possibility of biofunctionalization, multiple and easy synthesis methods, biocompatibility and, typically, electroactivity. The great variety of metallic nanoparticles available is very convenient for multiplexing assays[4,5], where the simultaneous detection of several analytes is carried out. Nanoparticles that can be loaded with different electroactive species[6], such as liposomes[7], apoferritins[8,9] or other nanoparticles[10,11] are also a constant resource in this field. Nevertheless, the development of novel or enhanced nanoparticles that can load a large amount of electroactive species is still a relevant research problem in order to improve the sensitivity of the detection in electrochemical biosensing. For instance, titanium phosphate nanoparticles (TiPNPs)[12,13] are very interesting because they have a porous structure and an amorphous coating of acid phosphates that provides them with a strong ionic-exchange functionality. However, these nanoparticles have only been modified with cadmium, lead or zinc for use as detection labels[14–17]. These metals are reduced at a quite negative potential, which can also reduce other concomitant species in the solution. Furthermore, the hydrogen evolution reaction usually occurs at a close potential (in acidic solutions) and could affect the efficiency of the metal electrodeposition on the electrode surface. Therefore, the evaluation and characterization of other electroactive metals introduced into these nanoparticles[18,19] may lead to better detection labels for electrochemical biosensors. In this work, titanium phosphate nanoparticles were modified with several electroactive metals such as cadmium, bismuth, silver, mercury and copper. The amount of metal introduced into the nanoparticles was tuned by changing the metal or the counter-ion used during the cation exchange reaction. The modification of the nanoparticles with metals that are reduced at more positive metals, allowed to use different electrolyte media able to extract the metals more efficiently than in previous studies reported in the literature. The capacity of multiplexing detection of these nanoparticles was evaluated by the development of a biosensor as a proof-of-concept.

MATERIALS AND METHODS

Apparatus and electrodes

Electrochemical measurements were conducted with μStat 8000 (DropSens) potentiostat/galvanostat interfaced to an Apple Macbook Air laptop and controlled by the DropView 8400 2.2 software. 8-channel screen-printed carbon electrochemical arrays (SPCEs) were purchased from DropSens (ref. 8X110). These devices, with a circular working electrode of 2.56 mm diameter, have been previously described[20]. 8-channel arrays were connected to the potentiostat through a specific connector, DRP-CAST8x. All measurements were carried out at room temperature and using an aliquot of 25 μL of the appropriate solution. All reported potentials are related to the silver pseudoreference screen-printed electrode.

Reagents and solutions

Cadmium nitrate, cadmium acetate, cadmium acetylacetonate, mercury acetate, copper acetate, silver nitrate, bismuth nitrate, bovine serum albumin fraction V (BSA), phosphoric acid(crystalline), docusate sodium salt(AOT), poly-(allylamine hydrochloride), glutaraldehyde, titanium(IV) butoxide were purchased from Sigma. Sulfuric acid (98%), acetic acid (100%), phosphoric acid solution (85%), dried ethanol, sodium hydroxide and hydrochloric acid were purchased from Merck. Neutravidin (NTV) was purchased from Fisher Scientific. Human tissue transglutaminase was purchased from Zedira. biotinylated goat anti-human IgA and IgG (anti-IgA-BT, anti-IgG-BT) were purchased from Life Technologies. Varelisa Celikey tissue transglutaminase IgA ELISA kit was purchased from Phadia. Ultrapure water obtained with a Millipore Direct Q5™ purification system from Millipore was used throughout this work. All other reagents were of analytical grade. Unless stated otherwise, 2 μL of a nanoparticle dispersion were employed for the modification of electrodes.

Synthesis of titanium phosphate nanoparticles modified with metals

The synthesis of titanium phosphate nanoparticles was carried out following a procedure found in the literature[12] slightly modified and previously reported[18]. For the synthesis of metal-modified titanium phosphate nanoparticles, an aqueous suspension (1 mL) of TiPNPs (40 mg/mL) was dispersed in 17 mL of a 10 mM aqueous solution of the appropriate metallic salt and the resulting mixture was stirred at 50 ˚C for 24 h. Then, the final mixture was centrifuged, the solid precipitate was washed three times with 10 mL of ultrapure water and the nanoparticles were dried under vacuum overnight.

Bio-functionalization of MTiPNPs with neutravidin and antibodies

For the bio-functionalization of MTiPNPs, a method previously reported was employed[21]. The neutravidin-biotin reaction was used for the conjugation of MTiPNPs-NTV with biotinilated anti-IgA and anti-IgG antibodies. Briefly, in a low-binding micro-tube, a 1:1 mixture (in PBS) of biotinylated antibody solution (5 µg/mL) and MTiPNPs-NTV (100 µg/mL) were left to incubate for 50 min under constant stirring. After the reaction, a small amount of BSA was added to these solutions (final concentration of 0.25% BSA), in order to minimize the possible non-specific adsorptions in the immunosensor.

Bioassay and electrochemical detection procedures

For the biosensor, modification of electrodes with the sensing element (transglutaminase) and the different steps were carried out by following a method previously developed in our group[22] but using PBS as buffer solution. The reaction with the secondary antibodies was performed using bio-functionalized MTiPNPs. The electrochemical detection was carried out with 25 µL of 0.1 M H_2SO_4 by square-wave anodic stripping voltammetry with a deposition step at -1 V for 60 s.

RESULTS AND DISCUSSION

Tuning the incorporation of metals into titanium phosphate nanoparticles

Titanium phosphate nanoparticles modified with metals (MTiPNPs) can be detected by electrochemical methods after the introduction of electroactive metals into their structure. TiPNPs modified with different electroactive metals were synthesized: cadmium, bismuth, copper, silver and mercury. These metals are easily measured by anodic stripping voltammetry, and the stripping processes usually occur at different potentials. In order to study the electroactivity of these nanoparticles, cyclic voltammetry was employed with an electrolyte valid for the different metals: 0.1 M pH 4.5 acetate buffer solution. The surface of the screen-printed working electrode was modified with an aqueous suspension of nanoparticles (2 µL of 2 mg/mL). **Figures 1A** and **1B** show the cyclic voltammograms obtained for all the MTiPNPs. Although, the cathodic processes are difficult to assign to the reduction of metals since the oxygen reduction reaction takes place at close potentials and could affect in the response obtained, the most interesting process is the anodic stripping. Most of the nanoparticles showed one good resolved stripping peak due to the oxidation of the previously reduced metal. For BiTiPNPs, no stripping process was observed initially. To confirm the presence of bismuth into the nanoparticles, a preconcentration step was applied (-1.4 V for 30 s), and then the scan was performed towards positive potentials, observing the stripping process. This fact suggests that a lower amount of bismuth is introduced into the nanoparticles or the extraction is more difficult than for other metals (probably due to the lower solubility of bismuth cations). For CdTiPNPs, the cyclic voltammogram was recorded up to -1.4 V in order to achieve the reduction and observe the stripping process that appears at more negative potentials. These studies show that the kind of metal introduced into the MTiPNPs can be tuned and that has a strong influence in the detection of the nanoparticles.

It is expected that the cation exchange reaction leading to the metallic nanoparticles follows the general mechanism of the **equation 1**. This mechanism suggests that a higher extraction of protons from the initial nanoparticles would lead to a higher amount of metal introduced into the nanoparticle structure. Therefore, if a salt with a weaker base counter anion is used, it should be able to bind more protons, and to induce a shift of the reaction to the products according to the Le Chatelier's principle.

$$TiPNP - H \ + \ M^{n+} \rightleftarrows TiPNP - M \ + \ nH^{+} \quad \textbf{(equation 1)}$$

The effect of two different anions on the electrochemical response of CdTiPNPs was evaluated by using cadmium nitrate and acetate salts during the cation exchange reaction. After obtaining the final product as described in the Experiment section, 2 µL of a 2 mg/mL aqueous dispersion of the nanoparticles were adsorbed into the working electrode, and square-wave anodic stripping voltammetry was used to carry out the detection of the metal from the nanoparticles (0.1 M pH 4.5 acetate buffer was used). **Figure 1C** shows that the highest signal was obtained for the CdTiPNPs synthesized with acetate, and the lowest signal was found for CdTiPNPs synthesized with nitrate, as expected theoretically. These results show that the amount of metal can be tuned by using different anions in the cationic exchange reaction, and a higher amount of metals would lead to a more sensitive detection using electrochemical techniques. In previous works reported in the literature, only nitrate was employed for the cation exchange reaction, which suggests that the detection of these nanoparticles would be less sensitive than for those synthesized in this work.

[FIGURE 1]

Enhancing the extraction of metals and detection of titanium phosphate nanoparticles

As the introduction of metals is carried out by a cation-exchange reaction, the extraction of metals and their detection could be enhanced by reversing this reaction. In order to study this process, several electrolytic media were evaluated for the detection of the different MTiPNPs. 2 µL of MTiPNPs aqueous solutions were added to the electrode surface and left to dry. Then, the cyclic voltammograms were registered and the stripping peak currents were compared. **Figure 2A** shows the significant differences obtained for the same nanoparticles in different media or for different nanoparticles in the same medium. As a general rule, acidic media provided the best results for the metallic extraction and electrochemical detection, even obtaining a significant signal for BiTiPNPs in HCl and HNO_3, which clearly demonstrates that acid media are able to extract more easily the metals from the nanoparticles. We have studied the cation exchange and the reverse process between TiPNPs and metals previously[23]. In this study, we showed that the metallic cations interact with the phosphate groups of the nanoparticles, and after acidic treatment, the metals are again extracted to the solution and the phosphate groups are recovered (as confirmed by the IR spectra). In previous works[14–17], acetate buffer was employed for the detection of the cadmium-based nanoparticles, and as we clearly demonstrate here, they are not the most appropriate metal or conditions to obtain a sensitive detection. Considering the peak currents (for sensitivity) and potentials (for selectivity in a multiplexing approach), H_2SO_4 was chosen as the electrolytic medium for the following experiments.

[FIGURE 2]

Multiplexing capabilities of metal-modified titanium phosphate nanoparticles

The multiplexing properties of MTiPNPs were evaluated by square-wave voltammetry using 0.1 M H_2SO_4 as the electrolytic medium. Electrode surface was modified with 2 µL of 0.5 mg/mL MTiPNPs of different binary mixtures (and individually to assign each stripping peak). **Figure 2B** shows the voltammograms of individual MTiPNPs and **Figure 2C** shows the voltammograms of several mixtures. Peak potentials of the stripping processes were in the following order: BiTiPNPs (-0.35 V), CuTiPNPs (-0.25 V), AgTiPNPs (-0.1 V) and HgTiPNPs (+0.2 V), while that a very small peak was observed for individual CdTiPNPs around -1 V (it increased in some of the binary mixtures (in presence of Hg and Bi)). Under these conditions, the stripping of BiTiPNPs/CuTiPNPs and AgTiPNPs/HgTiPNPs could not be resolved. Considering these facts and the magnitude of the peak currents, CuTiPNPs and HgTiPNPs were chosen for the multiplexing experiments because the stripping processes were perfectly resolved and a high peak current was obtained for both nanoparticles.

In order to evaluate the analytical performance of the Cu and Hg-modified titanium phosphate nanoparticles, voltammograms were recorded after the modification of the electrode surface with increasing concentrations of nanoparticle mixtures (2 µL solution). A potential of -1 V for 30 s was chosen as the optimal electrodeposition step. **Figure 3A** shows the voltammograms obtained for increasing concentrations of HgTiPNPs and CuTiPNPs. A working linear range between 0.01 and 0.3 µg/mL was obtained for CuTiPNPs, whereas a linear range between 0.01 and 0.4 µg/mL for HgTiPNPs was obtained. The response was linear according to the following equations: i_p (µA) = 133 (±5) [CuTiPNPs] (µg/mL) - 0.1 (±0.2), (R^2 = 0.992) and i_p (µA) = 193 (±1) [HgTiPNPs] (µg/mL) + 0.5 (±0.1), (R^2 = 0.9994), with estimated detection limits of 0.01 and 0.008 µg/mL, respectively. Clearly, the greater amount of metal introduced into these nanoparticles and the most effective detection allowed their determination at much lower concentrations than for previously evaluated AgTiPNPs[19] or CdTiPNPs[21], even with lower deposition times, leading to nanoparticles with very promising multiplexing properties. These results suggest that HgTiPNPs and CuTiPNPs could enhance significantly the detection in biosensing when these nanoparticles are used as labels in comparison to the typically used CdTiPNPs[14–17].

Copper and mercury-modified titanium phosphate nanoparticles were modified with neutravidin following a method previously reported in the literature[23], and applied, as a proof-of-concept, in a multiplexing biosensor for the simultaneous detection of anti-transglutaminase IgG and IgA antibodies. Most of the biosensing steps were similar to those described in a previous work[22], but CuTiPNPs and HgTiPNPs were bound to anti-IgG-BT and anti-IgA-BT, respectively, by the neutravidin-biotin interaction. A mixture of the positive and negative serum controls of two commercial ELISA kits for anti-tTG IgA and IgG detection was used as sample solution (1:1:2 ratio for controls and PBS). **Figure 3B** shows the voltammetric response obtained for 0, 7.3 and 45.5 U/mL concentrations of the analyte mixture, which demonstrates the good analytical performance of the multiplexing approach using CuTiPNPs and HgTiPNPs.

[FIGURE 3]

CONCLUSIONS

In this work, we have shown the possibility of tuning the amount of metal introduced into titanium phosphate nanoparticles by varying the type of metal and the counter-ion used during the cation exchange reaction. This fact has allowed to obtain nanoparticles with a great metallic load, and, therefore, with an excellent detection capacity for their use as detection label in electrochemical biosensors. In addition, it has been demonstrated that the electrolytic medium used in the detection step plays a crucial role for the metal extraction and its correct choice has allowed the very sensitive detection of the nanoparticles. These results have provided the basis for the development of a detection method for these metal-based nanoparticles, which were used successfully as detection label in a multiplexing electrochemical biosensor.

ACKNOWLEDGEMENTS

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FIGURES

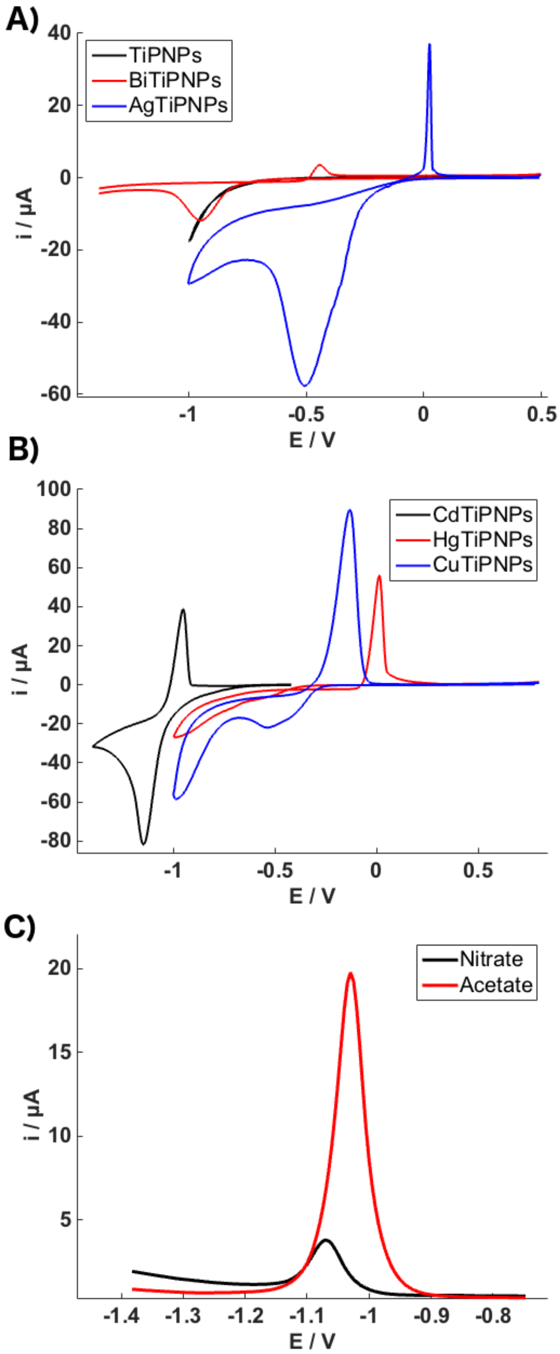

Figure 1. A) Cyclic voltammograms of TiPNPs, AgTiPNPs (between +0.5 and -1.0 V) and BiTiPNPs (between -1.4 V to +0.5 V with a deposition step at -1.4 V for 30 s). **B)** Cyclic voltammograms of CdTiPNPs (between -0.4 and -1.4 V), HgTiPNPs and CuTiPNPs (between +0.8 V and -1.0 V) in 0.1 M pH 4.5 acetate buffer. **C)** Square-wave voltammetry of CdTiPNPs synthesized by using a nitrate or acetate salt during the cation-exchange reaction.

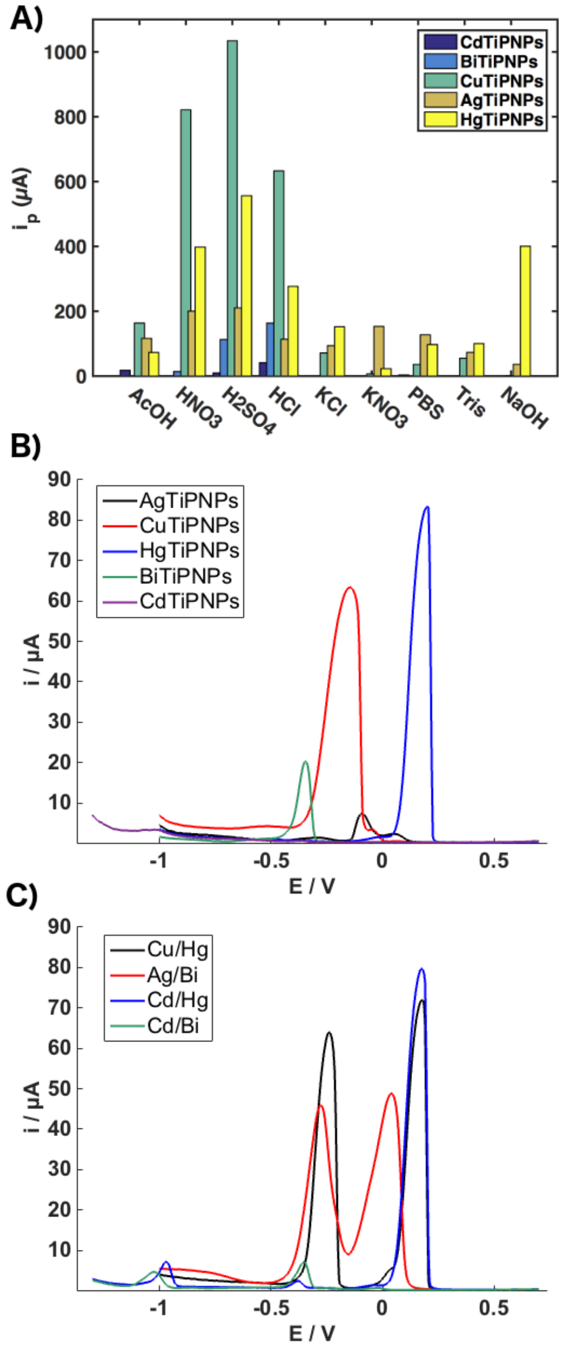

Figure 2. A) Peak currents of the anodic stripping process for the different MTiPNPs using different electrolytic media. Concentration of electrolytes was 0.1 M. **B)** Square-wave voltammetry of the different MTiPNPs in 0.1 M H_2SO_4. **C)** Square-wave voltammetry of several MTiPNPs binary mixtures in 0.1 M H_2SO_4.

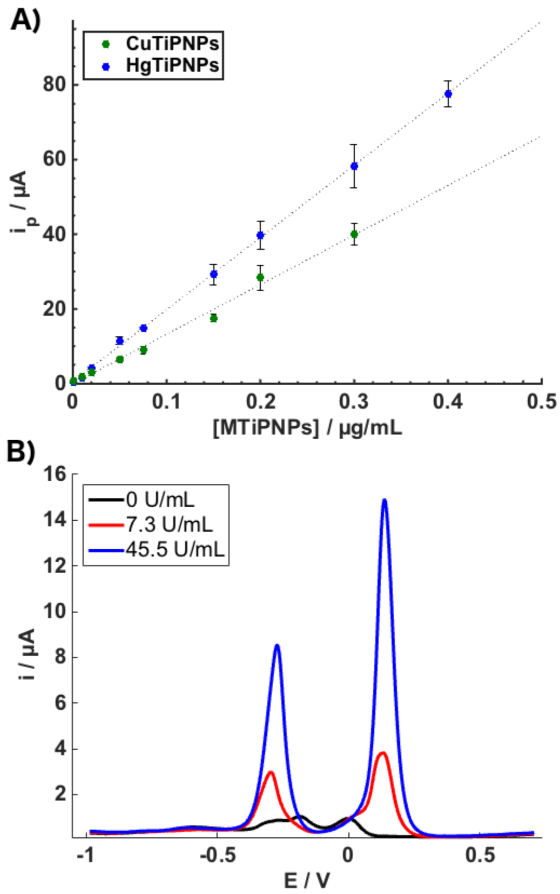

Figure 3. A) Calibration plots of the HgTiPNPs and CuTiPNPs. **B)** Electrochemical response of the proof-of-concept multiplexing biosensor using HgTiPNPs and CuTiPNPs as detection label.