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Supplementary Information

Haptoglobin Electrochemical Diagnostic Method for Subclinical Mastitis Detection in Bovine Milk

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Buffer composition

The composition of buffer solutions comprised 0.1 M Tris at pH 7.2, 1 mM of $MgCl_2$, and 0.1% BSA for incubations (incubation buffer); 0.1 M Tris at pH 7.2, 1 mM of $MgCl_2$, and 0.05% Tween-20 for washings (washing buffer); and 0.1M Tris at pH 9 supplemented with 1 mM of $MgCl_2$ for the enzymatic reaction and the electrochemical measurements. All buffer solutions were prepared with milli-Q water.

Synthesis of magnetic nanoparticles

Firstly, 1.25 g of chitosan was dissolved in 250 mL of 1% (v/v) acetic acid solution under stirring conditions and heated at 45 °C for 60 minutes. In addition, $FeCl_3$ (6.48 g) and $FeSO_4 \cdot 7H_2O$ (5.56 g) were dissolved in 250 mL of water in an ultrasonic bath for 15 minutes. The $Fe^{2+/3+}$ solution was then dripped into the stirred and heated chitosan solution for 30 minutes. Finally, the previous solution was degassed and dropwise added to the heated (70 °C) and stirred (1000 rpm) 1.25 M NaOH solution (600 mL) under a N_2 atmosphere. After finishing, the solution was stirred for another 45 minutes and chitosan-modified magnetic nanoparticles (MNPs@Chi) were magnetically collected and thoroughly washed with water, water/ethanol (1:1 v/v%), and ethanol three times each. After that, the MNPs@Chi nanoparticles were dried at 70 °C for 24 hours.

FT-IR characterization (*from Figure 2b*)

The nonmodified MNPs show the distinctive bands centred at ca. 3300 cm^{-1} and 1640 cm^{-1} due to the hydroxyl (-OH) vibrations, and the strong peak at about 600 cm^{-1} due to Fe-O stretching vibrations [1–3].

The chitosan spectrum exhibits the characteristic absorption bands at 3450 cm^{-1} (O-H stretching), 2880 cm^{-1} (CH-stretching), 1653 cm^{-1} (Amide I), 1585 cm^{-1} ($-NH_2$ bending), and 1320 cm^{-1} (Amide III). Moreover, the absorption bands found at ca. 1156 cm^{-1} (anti-symmetric stretching of the C-O-C bridge) and 1032 cm^{-1} (skeletal vibrations involving the C–O stretching) are distinctive of the saccharide structure [4].

The PANI spectrum displays characteristic absorption peaks at around 3400 and 3200 cm^{-1} (N–H stretching modes), at ca. 2910 cm^{-1} (asymmetric C–H stretching vibrations), and an important absorption band in the range of wave numbers from 2000–1600 cm^{-1} related to the benzenoid ring. The peaks at 1690 and 1648 cm^{-1} correspond to the C=N stretching mode for imine. The peaks at 1540 and 1430 cm^{-1} were assigned to C=C stretching vibration for quinoid and benzenoid rings, respectively. The peak at 1290 cm^{-1} is attributed to C-N stretching for benzenoid ring, and the peaks at 1240 and 1100 cm^{-1} are the characteristic polaron and bipolaron form of emeraldine salt type polyaniline. Finally, the peak at 900 cm^{-1} was assigned to the C–H plane bending vibration [5,6].

Optimization of biofunctionalization of the MNPs@Chi/PANI with Hemoglobin

pH optimization: The biofunctionalization with Hb was tested at three different pHs, including acidic (pH 5.5), neutral (pH 7.2), and basic (pH 9.6) reaction conditions. Once Hb was conjugated in the three conditions, these particles were used in the electrochemical detection assay in the absence of Hp (negative) and presence of 00.34 µg mL^{-1} Hp (positive). The signal:background ratio (S/B) was used to select the optimum pH that gives the highest sensitivity to the sensing strategy.

Hb concentration: Different concentrations of Hb, ranging from 1 to 32 g L^{-1}, were used for their functionalization on the magnetic particles. The optimum concentration was chosen according to the S/B values.

Blocking agent: Unreacted aldehyde groups of glutaraldehyde molecules used for haemoglobin immobilization need to be blocked (neutralized) since they could interact with the amino groups of the antibodies used for the labelling. This interaction contributes to the nonspecific binding of the reagents to the magnetic particles, increasing the background signal and thus decreasing the sensitivity of the bioassay and increasing the limit of detection (LOD). In order to minimize the unspecific binding, four blocking agent (2% BSA, 5% nonfat dry milk, 5% ethanolamine (adjusted to pH 7.4), and 0.5 M glycine) were evaluated by incubating the MNPs@Chi/PANI-Hb for 2 hours at 37°C.

Concentration of MNPs@Chi/PANI-Hb: This parameter is important since a limited number of particles would result in an inappropriate capture of the target molecule in the sample. In order to do this, four concentrations of MNPs@Chi/PANI-Hb (40, 100, 200, and 400 µg) were used for Hp detection.

Stability of MNPs@Chi/PANI-Hb

The stability of the MNPs@Chi/PANI-Hb was investigated by monitoring the concentration of Hp over a period of 40 days using DPV under optimized conditions (Figure S1). The stock of MNPs@Chi/PANI-Hb was stored at 4°C in PBS with a pH of 7.4 when not in use.

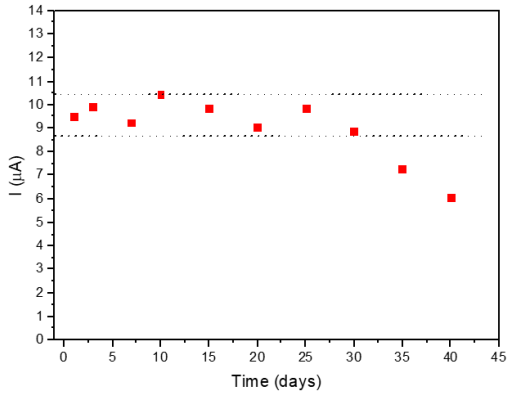

Figure S1. Stability of MNPs@Chi/PANI-Hb. Each measurement was performed in triplicate with 0.34 µg mL^{-1}. The upper and lower dashed lines correspond to the (Mean ± SD)$_{Day\ 1.}$

Optimization of electrochemical determination of haptoglobin using MNPs@Chi/PANI-Hb

Different parameters of the electrochemical bioassay were evaluated. The reaction time of target recognition by haemoglobin as well as the time of labelling with the antibodies was optimized in order to reduce the total assay time. Both isolation and labelling incubations were evaluated for different times (10, 20, 30, and 40 min). **Figure S2a** shows the electrochemical response for each condition, showing the signal in the presence (positive) and absence (negative) of haptoglobin. It was found that the shorter the incubation time, the lower the current intensity achieved. Based on these results, the incuba-

tion time was defined as forty-five minutes. Longer times were not tested because, although they could slightly increase the sensitivity, they affect the simplicity and speed of the assay. Furthermore, the sensitivity of the bioassay is more than adequate for the normal values of Hp in cow's milk.

The concentration of the labelling antibody was also optimized to ensure that the Hp captured on the magnetic particles was efficiently labelled and that the reagent was not the limiting reactant, thereby affecting the assay sensitivity. The electrochemical detection of Hp was performed with four different Ab-ALP concentrations (0.2, 1, 5, and 10 µg mL^{-1}). **Figure S2b** shows the signals obtained for each Ab-ALP concentration in the presence and absence of Hp. As can be seen, increases in the concentration of labelling antibodies greater than 1 µg mL^{-1} did not produce an increase in the analytical signal, demonstrating that the labelling of the sample was complete at concentrations greater than 1 µg mL^{-1}. Thus, the optimum Ab-ALP concentration was fixed at 2 µg mL^{-1}.

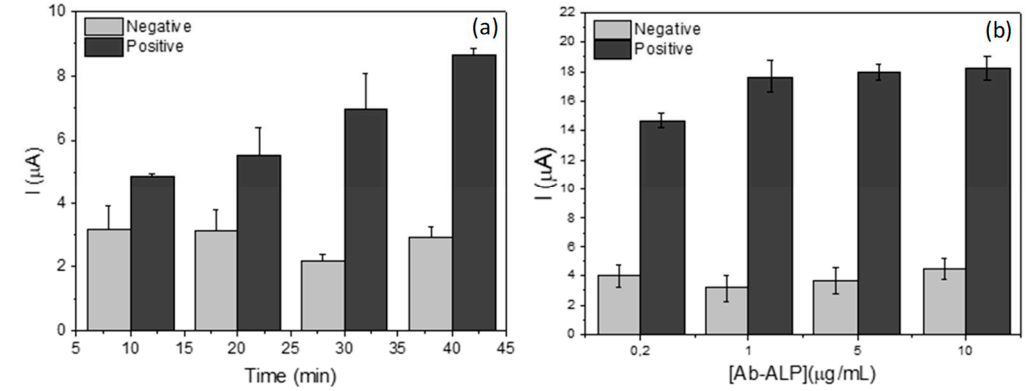

Figure S2. Evaluation of the incubation times and the secondary antibody concentration. (a) Incubation steps of target isolation and labelling for 10, 20, 30, and 40 min each. (b) Labelled antibody (Ab-ALP) optimization evaluated from 0.2 to 10 µg mL^{-1}. Tests were performed with 0.34 µg mL^{-1} (positive) and without (negative) Hp solution. In all cases, the concentration of reagents was 200 µg MNPs-Chi@PANI-Hb and 1 µg mL^{-1} Ab1.

Optimization of enzymatic reaction parameters

Parameters such as substrate concentration, enzymatic reaction time, evaluation of non-specific adsorption have also been studied. First of all, the nonspecific absorption studies were performed in order to confirm that the analytical response only correspond to the ALP-labelled haptoglobin. **Figure S3a)** there is only analytical response when the sample is incubated with both antibodies, indicating minimal nonspecific binding of the antibodies to other molecules or assay components.

Subsequently, the concentration of the enzymatic substrate was evaluated from 0.6 to 15 mM of naphthyl phosphate (**Figure S3b**). The substrate concentration chosen for all subsequent assays was 5mM, as it has the highest sensitivity (highest S/B ratio as shown in the Table S1), since at higher substrate concentrations, the background signal increases.

Table S1. Optimization of the enzymatic substrate. Current obtained for the four naphthyl phosphate concentration in presence (1 µg mL^{-1} **)** and absence (negative control) of haptoglobin.

	Naphthyl phosphate (mM)			
	0.6	**1.7**	**5**	**15**
Negative control†	1.830	2.560	1.100	2.119
1 µg mL^{-1}†	7.175	6.668	9.606	11.107
S/N ratio	3.9	2.6	**8.7**	5.2

Moreover, the enzymatic reaction time was also evaluated. Figure S3b) shows the current obtained when the ALP was incubated with 1-naphthyl phosphate from 1 to 24 min. The oxidation peak current exhibits an initial increase during the early minutes of the enzymatic reaction and reaches a steady-state after longer incubation times (around 10 minutes). In order to optimize the analysis time, a 5-minute enzymatic reaction time was determined as the optimal choice.

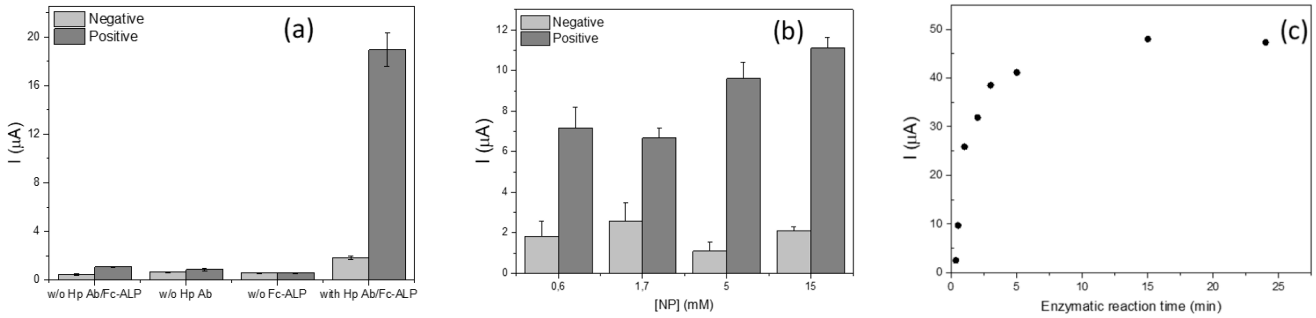

Figure S3. Enzymatic reaction optimization. (a) Specific and nonspecific binding of the antibodies to the components of the assay in presence and absence of the target; (b) Reaction progression of 1-naphthyl hydrolysis by alkaline phosphatase enzyme; (c) Enzymatic substrate optimization (1-naphthyl concentration) evaluated in presence and absence of Hp (1 μg mL^{-1}).

Calibration curve for the electrochemical Hp detection

Five different standard solutions were prepared by diluting bovine haptoglobin reference solution in 0.1 M Tris buffer at pH 7.2 supplemented with 1 mM of magnesium chloride and 0.1% BSA. The Hp dynamic range was evaluated from 0.02 to 0.25 μg mL^{-1}.

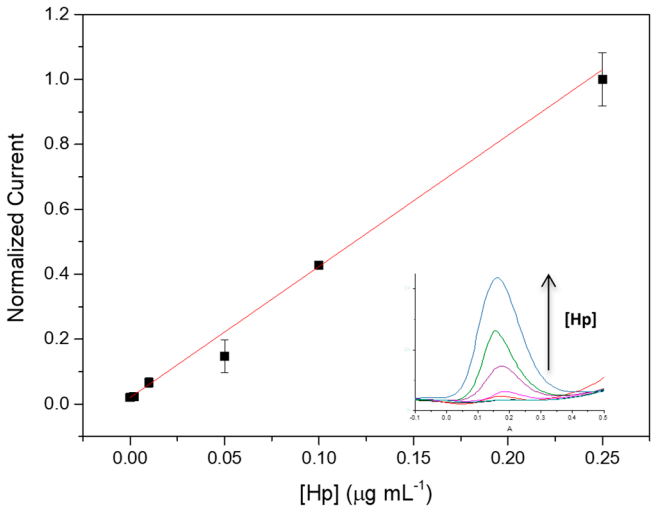

Figure S4. Calibration curve for haptoglobin in Tris buffer by the electrochemical determinations (n=3).

Parameter definitions for qualitative validation

The Contingency Table S2 is based on the calculation of probability for bioassay that give a two-category classification: positive or negative, acceptable or unacceptable, etc. Then, the qualitative method result is compared with the results obtained using the gold standard or a confirmatory method. From this table, it is possible to calculate some performance parameters, such as false positive, false negative, sensitivity, and specificity rates.

Table S2. Design of the contingency table and organization of the validation information for estimating quality parameters of the assay.

	Real positive sample	**Real negative sample**	
Positive by the method	True positive (tp)	False positive (fp)	**tp +fp**
Negative by the method	False negative (fn)	True negative (tn)	**fn + tn**
Total	**tp + fn**	**tf + tn**	**N**

Sensitivity is "the ability of a method to detect truly positive samples as positive".

Sensitivity rate "is the probability, for a given concentration, that the method will classify the test sample as positive, given that the test sample is a 'known' positive". It can be calculated as:

Sensitivity rate = tp / (tp + fn)

where tp are truly positive test samples, and fn are false negative test samples.

Specificity is defined as "the ability of a method to detect truly negative samples as negative". Specificity rate "is the probability, for a given concentration, that the method will classify the test sample as negative, given that the test sample is a 'known' negative". It can be expressed as:

Specificity rate = tn / (tn + fp)

where tn are truly negative test samples, and fp are false positive test samples.

Accuracy (also named analytical accuracy [7]) defined as "the ability of a method to classify correctly a sample as negative or positive". It can be expressed as:

Accuracy = (fp + fn)7 (tn + fn + tp +fp)

where fp are false positive test samples, fn are false negative test samples, tn are truly negative test samples, and tp are truly positive test samples.

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