ESI for:

Nanoparticle-Based Mobile Biosensors for the Rapid Detection of Sepsis Biomarkers in Whole Blood

Alejandra Alba-Patiño,[1] Steven M. Russell,[1,*] Marcio Borges,[1] Nicolás Pazos-Pérez,[3] Ramón A. Álvarez-Puebla,[3,4] Roberto de la Rica.[1,2*]

[1] Multidisciplinary sepsis group, Balearic Islands Health Research Institute (IdISBa), Son Espases University Hospital, S Building, Carretera de Valldemossa 79, 07120 Palma de Mallorca, Spain.

[2] Department of Chemistry, University of the Balearic Islands, Carretera de Vallemossa km 7.5, 07021, Palma de Mallorca, Spain.

[3] Department of Physical and Inorganic Chemistry and EMaS, Universitat Rovira i Virgili, Carrer de Marcel.lí Domingo s/n, 43007 Tarragona, Spain.

[4] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain

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Supplementary materials and methods

1. Synthesis of PVP-coated nanoparticles

Gold(III) chloride trihydrate (99.9 %, $HAuCl_4 \cdot 3H_2O$), polyvynil pirrolidone (PVP, MW 25000), trisodium citrate dihydrated (99.9 %), ethanol (99.5%) and N,N-dimethylformamide (DMF, 99.9 %) were purchased from Sigma-Aldrich (Germany). All reactants were used without further purification. Milli-Q water (18 $M\Omega\ cm^{-1}$) was used in all aqueous solutions, and all the glassware was cleaned with aqua regia before the experiments.

Synthesis of gold seeds: spherical gold nanoparticles of approx. 15 nm diameter were produced by a modification of the well-known Turkevich method [1–3]. Briefly, 100 mL of water were heated to boil and an aqueous solution of sodium citrate (2.5 mL, 0.1 mM) was added. One minute after the addition, 198 μL of a 0.127 M $HAuCl_4$ were added into the boiling solution under vigorous stirring. A condenser was used to prevent the evaporation of the solvent and the mixture was allowed to react for 1 hour under continuous boiling and stirring. During this time, the color of the solution gradually changed from colorless to purple to finally become deep red.

Transfer and concentration of the gold seeds: The 15 nm Au particles were transferred into ethanol using PVP as a phase transfer through a modification of the Graff method, which renders them covered with PVP [4,5]. The as prepared gold nanoparticles (100 mL) were added drop-wise under vigorous stirring to an aqueous PVP solution (100 ml, 4 mM) and left under stirring at room temperature for 24 hours. Finally, the Au nanoparticles were centrifuged (twice at 10000rpm, 35 min), and the particles were redispersed in an ethanoic PVP solution (15 mL, 4 mM) to achieve a final Au^0 concentration of 1 mM.

Spherical gold nanoparticles growth: Au NPs of approx. 45 nm were prepared using a seed mediated approach [5,6]. To this end, a growth solution containing PVP in EtOH (150 mL, 4 mM) was prepared. Next, 550 μL of $HAuCl_4$ (0.127 M) aqueous solution were added, and immediately after to initiate the growth, 4 mL of the Au seeds in PVP/EtOH were injected under vigorous stirring. The solution was left undisturbed overnight to assure the completion of the overgrowth process. Finally, the particles were centrifuged

(twice at 7000rpm, 10 min) and redispersed in 190 mL DMF to achieve a final Au^o concentration of 3.75 $x10^{-4}$ M.

2. Synthesis of PAH- and citrate-coated nanoparticles

Gold(III) chloride hydrate (99.995 %) and sodium citrate tribasic dihydrate (99%) were purchased from Sigma-Aldrich (Germany). Poly(allylamine hydrochloride) (PAH) was purchased from Alfa Aesar. All reactants were used without further purification. Milli-Q water (18 MΩ cm^{-1}) was used in all aqueous solutions, and all the glassware was cleaned with aqua regia before the experiments.

PAH-coated nanoparticles were prepared by bringing to a boil a solution containing 33 mg of gold chloride in 190 mL of Milli-Q water and adding 19 mg of PAH dissolved in 10 mL of water under agitation with a magnetic stirrer (Final volume 200 mL). The growth of gold nanoparticles was visually confirmed by the generation of red-burgundy color. After 10 minutes the solution was let to dry at room temperature. The resulting PAH-covered nanoparticles are unstable (the aggregate easily after centrifugation) and therefore PAH around the nanoparticles was substituted for SH-PEG-NH_2 for the subsequent covalent attachment of proteins as indicated in the main manuscript.

Citrate-capped nanoparticles were obtained by bringing to a boil a solution containing 0.5 mM gold chloride and adding sodium citrate to a final concentration of 0.75 mM under agitation with a magnetic stirrer (Final volume 250 mL). The solution was boiled for 10 min after the color appeared. The citrate around the nanoparticles was substituted for SH-PEG-COOH for the subsequent covalent attachment of proteins as indicated in the main manuscript.

3. Development of an app for real-time densitometry with an augmented reality guidance system

Table S1. Algorithms from the OpenCV library (Java) used to automate image processing steps and a description of their function in the app. Full source code can be found at GitHub, and full documentation on the algorithms can be found at OpenCV.org.

General Pre-processing Steps	
cvtColor with COLOR_RGB2GRAY	Changes color space from RBG to Gray Scale
GaussianBlur	Applies a slight blur to the image to aid in edge detection
AR Guide Box Steps	
Imgproc.circle	A whited-out circle that acts as a max distance cap in the guide box
Imgproc.rectangle	Draws a fixed position guide box superimposed on the camera view
Biosensor Recognition Steps	
threshold with THRESH_OTSU	Making the image black and white to highlight edges of objects
Imgproc.Canny	Applies Canny edge detection algorithm to segment edges of objects
RETR_EXTERNAL and CHAIN_APPROX_SIMPLE	Finds outside edges of connected lines
arcLength and approxPolyDP	Checks for four-sided quadrilateral with 89-91 degree angles
Biosensor Image Remapping Steps	
getPerspectiveTransform	Calculates perspective transform from four pairs of corresponding points
warpPerspective amd INTER_LINEAR	Applies the perspective transformation with inter-linear interpolation
Colorimetric Signal Segmentation Steps	
GaussianBlur	Reduces noise for the adaptive threshold to work well
adaptiveThreshold with ADAPTVE_THRESH_GAUSSIAN_C	Masks out the background substrate leaving only the signal pixels
THRESH_BINARY	Prepares the mask to count total number of signal pixels
Pixel Measurement Steps	
getRectSubPix	Calculates the pixel intensities at the subpixel level for improved accuracy
Imgproc.rectangle	Draws AR boundaries around pixel measurement areas
AR Data Visualization Steps	
putText	real-time AR display of results for the background and the signal mean pixel densities

Table S2. Steps performed by the user for densitometric analysis with the scanner-based method versus the app-based method

Steps for scanner-based densitometry	**Steps for smartphone-based densitometry with our app**
1. Place assay in scanner	1. Place assay on a neutral background
2. Choose color space and resolution	2. Align camera using AR guide box
3. Scan the image	3. Hover smartphone until progress bar is full
4. Upload image to ImageJ	
5. Invert colors	
6. Zoom in to locate signal	
7. Choose the region of interested (ROI) where the signal is located	
8. Use histogram function for pixel quantification in the ROI	
9. Choose the ROI where the background signal is located	
10. Use histogram function in background ROI	
11. Record the values for each ROI	
12. Subtract background from signal	
13. Record results	

The app subsitutes hardware to fix the angle and position of the phone with the AR guidance system shown in Figure S1. With the AR guidance system, the user aligns the real-world view of the immunoassay within a square frame digitally superimposed on the smartphone screen (Figure S1a-S1c). This guides the user to position the smartphone within the appropriate distance and angle range to ensure reproducible measurements. Variations in lighting conditions are compensated for by means of gray card calibration [7]. When these imaging parameters are within the correct range, the app automatically segments the paper substrate from the surrounding visual context, locates the colored spot generated by the immunoassay, and starts calculating the pixel intensity while at the same time subtracting the background signal as calculated from four points around the region of interest (Figure S1d). This can be followed in real time with a progress bar that advances as the measurements are taken (Figure 1d(i) and Video S1). Any changes in imaging conditions that could result in inconsistent results trigger the app to stop taking measurements, therefore causing the progress bar to stop. This informs the user that the smartphone needs to be realigned with the AR guidance system to be within the accepted range of imaging conditions, at which point the measurements resume. When the app has acquired 50 valid measurements, the data set is evaluated to identify and remove outliers, and the average increase in pixel intensity with respect to the background signal is displayed. The whole process takes a few seconds (Video S1).

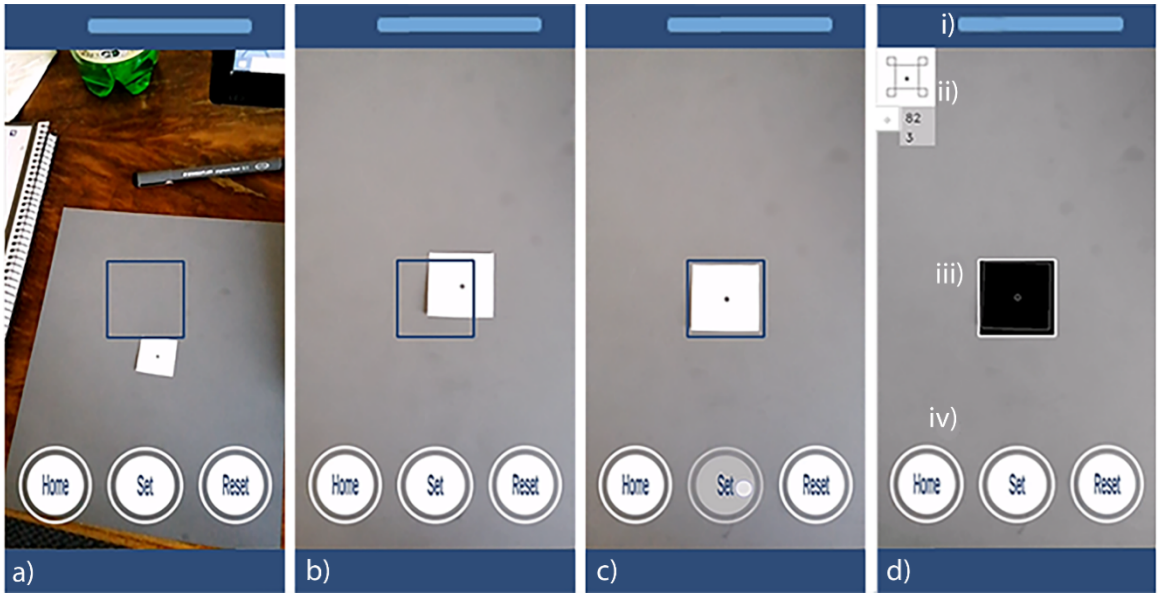

Figure S1. Screenshots of the AR guidance system ((a) to (c)) and the measurement screen (d). The guide box is augmented in a fixed position on top of the real-world view as seen through the camera of a smartphone. The user moves the smartphone from a general view of their environment (a), towards the paper biosensor (b), until the biosensor fits inside the guide box as close to the inner edge as possible (c). After tapping the Set button, the Measurement screen (d) appears. In this screen, the progress bar (i) fills up as valid data points are collected. A complete data set contains 50 data points. The data points are measured in the Analysis Zone (ii) where the pixel intensities of the background and the signal are quantified. The paper biosensor is segmented from the real-world environment when it is found in the Detection Zone (iii) and remapped to the Analysis Zone. The user can switch between setting the Detection Zone and Resetting the Guide Box, as well as returning to the Home screen by tapping on the Navigation Buttons (iv).

Additional Figures

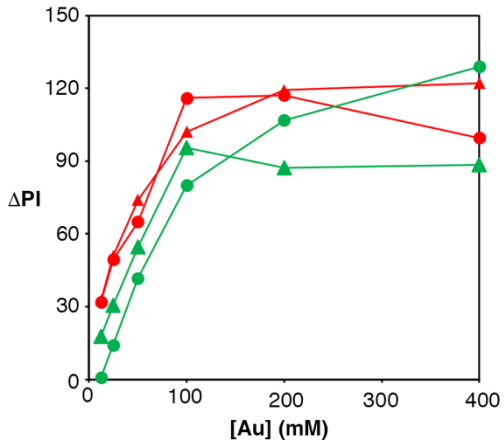

Figure S2. Specific signal obtained by subtracting the pixel intensity generated by biosensors modified biotinylated BSA from biosensors modified with non-biotinylated BSA when using amine-coated nanoprobes and unmodified paper (red dots), amine-coated nanoparticles and PAH-modified paper (red triangles), carboxylate-modified nanoparticles and unmodified paper (green dots) and carboxylate-modified nanoparticles and paper modified with PAH and PSS (green triangles). The highest specific signals are obtained with a nanoparticle concentration of 400 mM ([Au]) and amine-coated nanoparticles combined with PAH-modified paper or carboxylate-modified nanoparticles combined with unmodified paper.

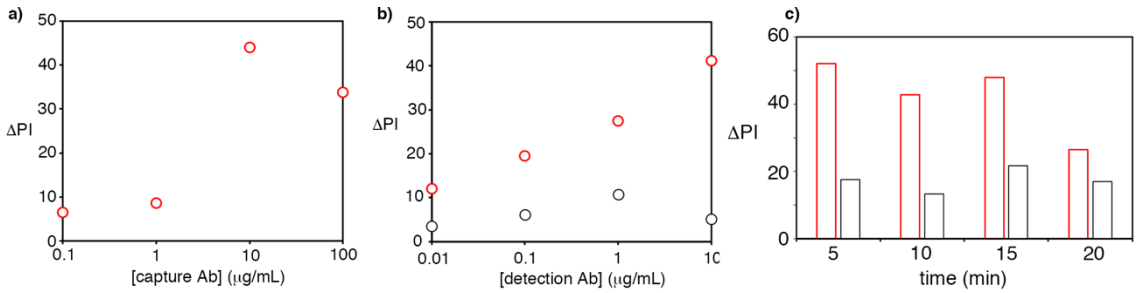

Figure S3. Optimization of immunoassay parameters; a) Immunoassay performed with paper biosensors modified with different concentrations of capture antibody (best condition 10 μg mL^{-1}); b) Immunoassays performed with different concentrations of detection antibody when the biosensors were modified with (red dots) or without (black dots) capture antibodies (best specific signal with 10 μg mL^{-1}); c) Immunoassays performed with different incubation time with the detection antibody and biosensors modified with capture antibodies (red) or control biosensores without capture antibody (black); increasing the incubation times does not result in higher specific signals.

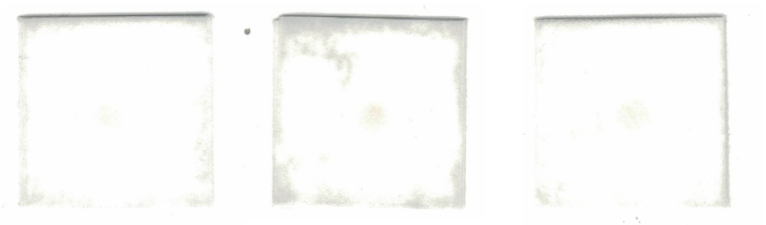

Figure S4. Scanned images of wet paper biosensors after adding a drop of blood and performing the washing protocol described in Materials and Methods (4 times with 1mL of PBST). The average pixel intensity in the region of interest is 4 ± 2, which is lower than the average signal of the blank in Figure 6c (15 ± 4), therefore demonstrating that the color of the matrix is not a major source of interference with the proposed biosensors.

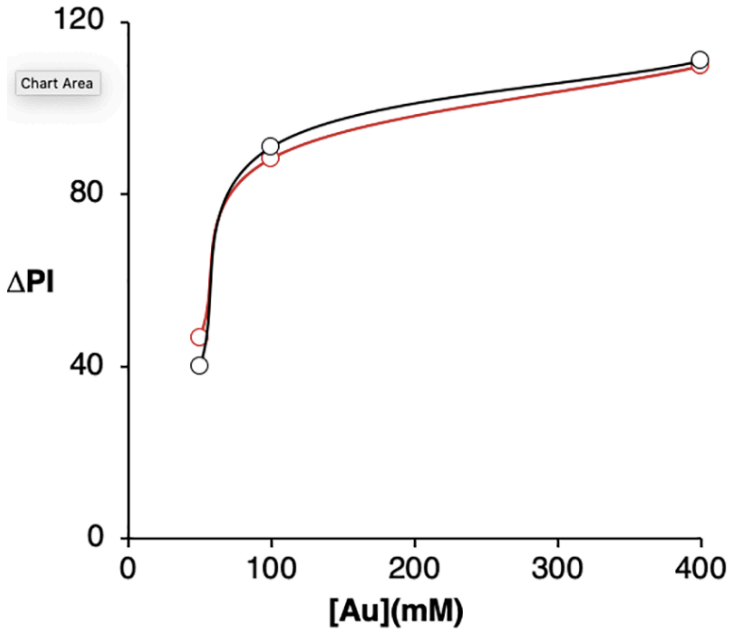

Figure S5. Stability of avidin-modified nanoparticles over time. Recently made nanoparticles (black dots) were compared with nanoprobes that were two months old (red dots, kept at 4ºC without any additional preservatives). The colorimetric signal ΔPI was obtained by subtracting the signal obtained with control biosensors modified with BSA from the signal obtained with biosensores modified with biotin-BSA. Both responses are very similar at the different nanoparticle concentrations assayed, which demonstrates that the avidin-modified nanoparticles are stable when kept for two months at 4ºC.

Table S3. Time required for each step of the assay.

Step	Time (seconds)
Incubation with sample (capture step)	300
Incubation with primary antibody	300
Incubation with nanoprobes	300
Wash steps	10 (each)
Mobile detection	Under 5

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