Assessment of bio-based materials for enhanced signal detection of sporozoites using csELISA

Diego Gomez-Maldonado

Auburn University

Haley Stephens

Auburn University

Alice C. Sutcliffe

CDC: Centers for Disease Control and Prevention

Maria Andrea Camarano Eula

Auburn University

Iris Beatriz Vega Erramuspe

Auburn University

Ellen M. Dotson

CDC: Centers for Disease Control and Prevention

Maria Soledad Peresin

Auburn University

Sarah Zohdy (✉ sarahzohdy@gmail.com)

Auburn University

Research Article

Keywords: Malaria, Plasmodium, chitosan, cellulose nanocrystals, QCM-D, circumsporozoite, paper-based assays

Posted Date: January 28th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1082751/v1>

License: (cc) (i) This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Assessment of bio-based materials for enhanced signal detection of sporozoites using csELISA

Diego Gomez-Maldonado^{1,2*}, Haley Stephens^{1*}, Alice C. Sutcliffe³, Maria Andrea Camarano Eula¹, Iris Beatriz Vega Erramuspe^{1,2}, Ellen M. Dotson³, Maria Soledad Peresin^{1,2}, Sarah Zohdy^{1,4†}.

¹School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States, ²Forest Products Development Center, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States, ³Centers for Disease Control and Prevention, Atlanta, GA, United States, ⁴College of Veterinary Medicine, Auburn University, Auburn, AL, United States

*These authors contributed equally to this work

† Corresponding author, current address: President's Malaria Initiative (PMI), US Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

Background:

Malaria is responsible for over 435,000 deaths annually, with most cases occurring in sub-Saharan Africa. Detecting the presence of *Plasmodium* spp. sporozoites (spzs) in the salivary glands of *Anopheles* vectors of the parasites using the circumsporozoite enzyme-linked immunosorbent assay (csELISA) is an important malaria surveillance method. The addition of bio-based materials have shown the potential to improve the adsorption and binding of target antigens and thus can improve the sensitivity and detection of analytes in immunoassays. Here, we evaluate the use of two bio-based polymers, chitosan, and cellulose nanocrystals (CNC), as antibody carriers and substrate coating on 96-well plates and on a paper substrate to determine whether detection of *Plasmodium falciparum* (Pf), *P. vivax* VK210 (Pv210) and *P. vivax* VK247 (Pv247) can be improved through assay modification.

Methods:

Modified csELISA assays were conducted using chitosan and CNC as either an antibody carrier or a well coating on 96-well plates (ultra-low- and high-affinity) and results read using standard spectrophotometry of 96-well plates and a quantitative image-based color analysis using photographs of paper plate assays. Changes in frequency and dissipation, resulting from the adsorption of antibodies to model films in a quartz crystal microbalance with dissipation monitoring (QCM-D), were followed to understand better the interactions between the bio-based materials and assay proteins.

Results:

The csELISA performed on high-binding well-plates showed that chitosan, used either as an antibody carrier or well coating, resulted in the greatest increase in detection for Pv210 and Pv247 positive recombinant proteins, increasing absorbance readout values up to 6x for Pv210 and up to 5x for Pv247. On paper csELISA (PcsELISA), chitosan as an antibody carrier yielded the greatest increase in detection sensitivity for all three *Plasmodium* species when color intensity of positive recombinant proteins was compared to blanks. Compared to controls, chitosan as a carrier resulted in a ~2.5-fold increase in color intensity for Pf, a ~4-fold increase for Pv210, and a ~2-fold increase for Pv247. QCM-D showed a preferred interaction between the assay antibodies and chitosan surfaces, most likely driven by electrostatic interactions.

Conclusion:

The addition of bio-based materials, mainly chitosan, as shown by QCM-D interactions, absorbance readout values, and image-based color analysis, increased the color intensity of positive samples run through csELISA in all systems, allowing for clearer detection of *Plasmodium* spp visually, using a spectrophotometer and quantitative color intensity. Furthermore, the adaptation of a PcsELISA coated with chitosan using positive recombinant proteins shows potential as a cost-effective alternative assay platform as it reduced reagent volumes by 80% and assay run time from seven hours to one hour.

Keywords: Malaria, *Plasmodium*, chitosan, cellulose nanocrystals, QCM-D, circumsporozoite, paper-based assays.

Background

Human malaria is a life-threatening disease caused by *Plasmodium*, a group of apicomplexan parasite species that is vectored by female *Anopheles* mosquitoes (1). Detecting the presence of *Plasmodium* spp. sporozoites in the salivary glands of *Anopheles* is an important malaria surveillance method, which helps measure the intensity of malaria transmission and evaluate intervention methods to assess programmatic impact (2).

The current gold standard for sporozoite detection in infected mosquitoes is the enzyme-linked immunosorbent assay (ELISA) (3). Specifically, the circumsporozoite (cs) sandwich ELISA utilizes a capture and detection antibody complex to identify the circumsporozoite protein, the major surface protein of the sporozoite (4), and has been used globally as an entomological indicator for the detection of infectious mosquito vectors.

The csELISA provides high sensitivity and specificity for *Plasmodium* detection (5). Despite these advantages and the relatively low cost associated with csELISAs when compared to molecular techniques, there are some disadvantages. Assays are run in 96-well plates or tubes, requiring large volumes of sample and reagents for optimal performance. Due to cross-reactivity, non-specific binding of proteins to the capture antibodies may also result in false positives (6), requiring an additional boiling step for confirmation. The csELISA technique is time-consuming, requiring more than seven hours to complete, with multiple incubation steps of 30 minutes to two hours. Additionally, a microplate reader is traditionally required to measure the colorimetric readout (7), and these devices can cost up to \$20,000 USD without taking into consideration maintenance and potential future software upgrades.

To circumvent some of these drawbacks, paper-based alternatives to traditional 96-well plates have been developed as a base immunoassay, including adaptations of ELISAs. Paper-based ELISAs offer a suitable alternative in terms of cost reduction, ease of use, and conservation of assay reagents and samples (8). Paper-based assays eliminate the need for a plate reader, and alternatives such as cellphone imaging and color analysis via photo editing software can be used for objective colorimetric analysis. Since reagents

are deposited directly onto the paper substrate rather than into the well of a 96-well plate, the surface area of the reaction is reduced, thereby decreasing the volume of antibodies, samples and other reagents used (9). Despite the numerous advantages that come with paper-based assays, there are limitations, notably in those that use a peroxidase-based reaction as colorimetric indicator. Due to differences in capillary forces among the reagents and analytes, uneven distribution over the test zone can result in heterogeneous color formation, making correct interpretation of the results difficult (10).

To overcome this lack of homogeneity and improve binding affinity between the antibodies and the test zones, as well as to act as coating agent smoothing the surfaces (and thus capillary forces), bio-based materials can be used as additives in the process. Some of these bio-based materials have been demonstrated to improve biomolecule stability and immobilization on the well walls (11). The goal of this work was to assess if the addition of these materials can improve assay sensitivity and reduce detection limits when compared to traditional 96-well plate based ELISAs.

One such bio-based material is cellulose. This polymer is formed by glucose monomer links in β (1 $\rightarrow$ 4)-D-glucopyranose conformation, and it is the most abundant polysaccharide on earth, present in the cell walls of plants and wood, found in tunicates and algae, and is also produced by some bacteria (12). Fibrils are packed in two alternating forms within the cellulose macrostructure: a tight crystalline form, where cellulose chains are highly ordered, and an amorphous one. Traditional processing of wood results in lignocellulosic pulp, which is the raw material to produce paper (13). However, after an acid hydrolysis step, the pre-treated lignocellulosic pulp can be processed to selectively remove the amorphous portions and produce cellulose nanocrystals (CNC) (14).

Like cellulose, chitosan has also shown potential as a bio-based material for assay improvement. Chitosan is a polycationic linear polysaccharide which is easily obtained from chitin, the second most abundant natural polysaccharide after cellulose. Chitosan is produced by the partial deacetylation of chitin under alkaline conditions, which is a structural component of the exoskeleton of arthropods and cell walls of fungi and yeast (15-17). This biodegradable, nontoxic and biocompatible bio-based material has

demonstrated a key role in paper-based assays, producing uniform color signals at the detection spot when used as a substrate (18, 19).

Here, the chemistry of bio-based materials was integrated with the well-established csELISA to determine whether this could potentially improve the detection of sporozoites of *P. falciparum*, *P. vivax* 210 and *P. vivax* 247. To better understand the interactions of the bio-based materials with the antibodies used in the assays, Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) was used to analyze the adsorption of the assay antibodies to thin films of the bio-based materials, where frequency and dissipation shifts were used to quantify the adsorption capability as an indicator of the strength of the interactions.

Methods

Materials and reagents

Two bio-based materials were selected based on their binding properties to antibody proteins: chitosan (85% deacetylated, Alfa Aesar, Cat. N°: J64143) and cellulose nanocrystals (CNC, 10.7% wt., 2016-FPL-CNC-098, Process Development Center, The University of Maine). All capture and detection antibodies, positive controls (recombinant proteins), and substrates were obtained from BEI Resources (Cat no. MRA-890; MRA-1028K)(20). These recombinant proteins have been used in other studies adapting the csELISA to other assay platforms, such as multiplex bead-based immunoassays (21). The *P. falciparum* positive control antigen is derived from recombinant protein R32tet₃₂ produced in *Escherichia coli* (Smith Kline and French Laboratories, USA) (22). The *P. vivax*210 and *P. vivax*247 positive control antigen, PvCSPv1, is a recombinant fusion protein that includes cs regions of both *P. vivax*210 and *P. vivax*247 produced in *Pichia pastoris* (Protein Potential, USA) (23). All antibodies and positive controls were received lyophilized and were rehydrated to the concentrations indicated in the kit instructions. These recombinant proteins were used as positive controls and will be referred to as “recombinant proteins” from this point onward.

Capture antibodies were anti-sporozoite monoclonal antibodies (mAb) against three target antigens: regions of the *Plasmodium falciparum* (Pf), *P. vivax-210* (Pv210), and *P. vivax* 247 (Pv247) circumsporozoite (cs) proteins. Detection antibodies were obtained already conjugated to horseradish peroxidase (HRP). Stock concentrations of Pf, Pv210, and Pv247 recombinant proteins were 100 pg/μL, 9100 pg/μL, and 4550 pg/μL, respectively. Working recombinant protein solutions were made by diluting the stock solutions in blocking buffer to 2 pg/μL, 182 pg/μL, and 91 pg/μL for Pf, Pv210 and Pv247, respectively. The blocking buffer was a solution of 0.5% casein in phosphate-buffered saline (PBS, pH 7.4), and the wash buffer was a solution of 0.05% Tween 20 in PBS, pH 7.4. Bio-based materials were used in assays as either well-coating or antibody-carrier or in combination on the same plate. The csELISA protocol used was obtained from BEI Resources (20).

Testing of bio-based materials on high- and ultra-low binding 96-well plates

Circumsporozoite ELISA was performed on four types of clear polystyrene 96-well microplates. The four plates were divided into two categories: high binding plates (Corning 3366, round bottom, and MaxiSorp™/Nunc-Immuno, flat bottom), and ultra-low binding plates (Corning® 7007, round bottom and Corning® 3474, flat bottom). Absorbance readings of all plates were performed using a SpectraMax 190 spectrophotometer system from Molecular Devices (Silicon Valley, CA, U.S.). Both chitosan and CNC were tested as well-coating materials for the plates and as antibody carriers. When used as antibody carriers, the polymers were mixed with the antibodies in solution before adding to the plates.

Preparation of bio-based materials for well coating

For well-coating solutions, a 0.1% chitosan solution and a 0.1% CNC suspension were prepared in 1% acetic acid and water, respectively. All suspensions and solutions were prepared with Milli-Q water (Millipore Inc., 18.2 MΩ cm). Suspensions of CNC were sonicated for 5 minutes at 25% amplitude, with the pulser set in five seconds intervals of three second “on” and two seconds “off,” while maintaining the suspension in an ice bath, prior to use. Wells of the 96-well polystyrene plates were filled with 200 μL of the bio-based material, covered with a lid and incubated for 30 minutes at room temperature. Thereafter, the solution, or suspension, was aspirated and the wells were rinsed as follows: i) wells coated with chitosan

0.1% were rinsed one time with 200 μ L of 1% acetic acid and two times with 200 μ L MilliQ-water; ii) wells coated with 0.1% CNC were rinsed one time with 200 μ L of PBS-Tween (0.05%) and two times with 200 μ L MilliQ-water. After that, the plates were left covered with plate lids overnight at room temperature (ca. 20 °C) to air dry.

Preparation of bio-based materials for antibody carriers

A 1% chitosan solution was prepared in 1% acetic acid and then diluted to 0.1% with PBS, pH 7.4. CNC suspensions were diluted in PBS, pH 7.4 to obtain a 0.1% concentration and sonicated, as previously described. The Pf, Pv210 or Pv247 stock capture antibodies were rehydrated as described (20), and added to 5 mL of the prepared bio-based material solution (in the place of PBS). Antibody-bio-based material solutions were then vortexed and incubated at room temperature for one hour before use. The remainder of the protocol was followed as described in the MR4 csELISA protocol (20).

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

To better understand the interactions occurring between the bio-based materials and the csELISA antibodies, adsorption of the Pf, Pv210 and Pv247 antibodies on model films was performed in a QSense Analyzer from Biolin Scientific (Västra Frölunda, Västra Frölunda, Sweden). Model films were prepared over polyethyleneimine (PEI) precoated gold quartz sensors (5 MHz base resonance) by spin coating 180 μ L of 0.1% solutions of the bio-based materials at 3000 rpm for 1 min in a WS-650MZ-23NPPB spin coater by Laurell Technologies Corporation (North Wales, PA, U.S.). For measurements, the sensors were first stabilized on PBS buffer (pH 7.4) at 25 °C with a flow of 50 μ L/min. Then, 4 μ L/mL of Pf or 8 μ L/mL of Pv210/Pv247 antibody solutions were poured into the channel and allowed to interact with the surfaces until no changes were detected. Finally, a rinse with PBS was performed to remove the excess material. Data analysis was performed using with DFind Software from Biolin Scientific (Västra Frölunda, Sweden) using the Composite Sauerbrey model and the software's predetermined density values.

Development of paper ELISA template

A paper-mimic of 96-well plates (Figure 1) was created on chromatography paper (Whatman Grade 1 Chr cellulose chromatography paper, from Cytiva, at Marlborough, MA, U.S.). Circular zones 6.5 mm in

diameter, resembling the wells of 96-well ELISA well plates, were drawn onto the paper with pencil. With a commercial liquid wax solution, wells were then outlined using a fine-tip paintbrush. To create a hydrophobic well-barrier, the “plate” was heated on a hot plate at 100°C for three minutes to allow for complete penetration of the wax through the paper matrix. Chitosan and CNC were then evaluated on the paper ‘plates’ in different combinations as antibody carriers and well-coating. Recombinant proteins and bio-based materials were prepared as described above. For well-coatings, 100 µL of bio-based material solution or suspension was pipetted onto paper wells and allowed to dry overnight. For bio-based material antibody carrier solutions, 10 µL was pipetted onto each well.

Paper cs-ELISA protocol adaptation

All incubation steps from the csELISA protocol (20) were reduced to 15 minutes. Excess reagent was removed after 15 minutes by placing a Kimwipe (Kimtech Science Kimwipes Delicate Task Wipes product code #34155) on the underside of the paper plate, which wicked liquid through. To prevent leaking of the wells or mixing of the well contents on the underside of the paper, the paper-plate was suspended by placing it on an empty reagent reservoir. All volumes were reduced to 20% of the volume outlined in the csELISA protocol (20). Working sample and antibody solution volumes were reduced from 50 µL to 10 µL, blocking and wash buffers were reduced from 200 µL to 40 µL, and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was reduced from 100 µL to 20 µL. Following the addition of ABTS, plates were allowed to incubate for 15 minutes before a cellphone camera was used to take a photograph of the entire paper-plate, to create an image to assess the resulting color change.

For the recombinant proteins tested, dilutions were made according to the original stock concentration, a two-fold dilution of the stock concentration, the reported working solution concentration, and a ten-fold dilution of the working concentration. Pf: 100 pg/µL (stock), 50 pg/µL, 2 pg/µL (working), and 0.2 pg/µL. Pv210: 9.1 ng / µL, 4.55 ng / µL, 182 pg/µL, and 18.2 pg/µL. Pv247: 4.55 ng / µL, 2.275 ng / µL, 91 pg/µL, and 9.1 pg/µL.

CYMK Analysis for Paper

A calibration curve was prepared to convert the observed color change into numerical data. Briefly, photos taken 15 minutes after adding ABTS were uploaded into photo editing software (Adobe Photoshop CC 2019) and analyzed using the Cyan-Yellow-Magenta-Black (CYMK) color processing. The average color of each well was obtained by selecting the well-borders and applying a filter (Filter -> Blur -> Average) that takes the average color of a selection and replaces the original selection with the color average (Figure 1). The CYMK color values were then recorded for each selection and plotted to compare color intensity changes associated with the pure recombinant protein concentrations (Figure 1). As the concentration of the recombinant proteins increased, greater differences in the percent color intensity were seen, which were associated with a more visible difference in color between concentrations.

Results

Bio-based materials on high- and ultra-low-binding 96-well plates

Pf

For assays assessing Pf antibodies, none of the CNC or chitosan combinations as antibody carriers or plate coatings on high-affinity binding and ultra-low binding plates displayed a significant improvement in the detection of Pf recombinant proteins at the working concentration (2 µg/µL). For high-binding plates (Corning 3366 and MaxiSorp™), the control wells with the recombinant proteins exhibited the greatest absorbance values compared to the wells with samples where the bio-based materials were used. On ultra-low binding plates (Corning® 7007 and Corning® 3474), the absorbance values for all samples, including control wells without bio-based material used, were very low (Figure 2).

Pv210

In the case of Pv210, the use of chitosan enhanced the detection sensitivity of the recombinant proteins on high-binding and ultra-low binding plates. When Pv210 recombinant proteins were tested at the working concentration (182 µg/µL), assays performed with chitosan as well-coating or antibody-carrier showed absorbance values up to six times higher than those from the control wells (without chitosan as a

well-coating or antibody carrier) (Figure S1). Moreover, on high-affinity binding plates, the absorbance values of Pv210 from wells where chitosan was used as a well-coating, antibody carrier or when used at the same time with CNC as a carrier at recombinant protein concentration of 18.2 $\mu\text{g}/\mu\text{L}$ were comparable to those with Pv210 at 182 $\mu\text{g}/\mu\text{L}$ in wells where bio-based materials were not used as a well-coating or antibody carrier. Thus, using chitosan, as well-coating or antibody increased the sensitivity of recombinant protein concentration detection by 10-fold (Figure **Error! Reference source not found.2**).

Circumsporozoite ELISA control assays (no bio-based materials as antibody carriers or well-coatings) performed on ultra-low binding plates for Pv210 yielded no increase in absorbance values when recombinant protein was present over the dilution series, compared to blanks (blocking buffer only) (Figure 2), indicating that little to no antibody binding occurred on the well surfaces. Nevertheless, the absorbance values obtained when chitosan was used either as an antibody carrier or well-coating were between 1.2 and 1.7x higher than those observed for Pv210 on control wells of high-affinity binding plates.

For Pv210 assays performed with CNC as a well-coating, results more heavily depended on the specific plate type, and did not yield an increased absorbance value over the recombinant protein or when the chitosan was used alone either as an antibody carrier or well-coating. With CNC applied as a well-coating with chitosan as an antigen carrier on the high-binding plate a two-fold increase in absorbance was observed when compared to controls.

Pv247

The results for Pv247 on high-affinity binding plates showed a similar trend to that observed for Pv210 (see Figure 2), with up to a five-fold increase in absorbance values when compared to controls when chitosan was used as an antibody carrier or well-coating. Chitosan, either as a well-coating or antibody carrier, was also capable of reducing the working concentration, and lowering the detection limit, of the Pv247 recombinant protein up to five-fold without a significant reduction in sensitivity for assays carried out on both high-binding plates (Figure 2).

Adsorption of antibodies on model film in QCM-D

QCM-D followed the adsorption of the capture antibodies onto model films of the bio-based materials to better understand the affinity between them. Flow concentrations of the antibodies were 2 $\mu\text{g}/\mu\text{l}$ for Pf, 182 $\mu\text{g}/\mu\text{l}$ for Pv210, and 91 $\mu\text{g}/\mu\text{l}$ for Pv247 in PBS and flow of 50 $\mu\text{L}/\text{min}$. The graphs of results are shown in Figure 3, and a summary of the relevant values is presented in Table 1. Table 1 also presents the shifts after washing with PBS, as these values are the irreversible mass attached to the surface of the thin films. With Pf, Pv210 and Pv247 antibodies, chitosan presented higher frequency drops than CNC, indicating a better interaction between the antibodies and chitosan over that of CNC. The Pf antibody appeared to interact more strongly with both materials contrary to the observed performance in 96-well plates. The Pf antibody was the only antibody of the three with multiple layers adsorbed, as demonstrated by the multiple changes of slope in the frequency and dissipation plots (Figure 3B). These multilayers may reduce the available sites for antibody binding with the antigen. For Pf, chitosan had 2.3-fold higher adsorption of the antibodies when compared to CNC adsorption, while for Pv210 and Pv247 the frequency and mass shifts were similar for both chitosan and CNC.

Table 1: Data extracted from QCM-D of irreversible adsorbed antibodies on model films of cellulose nanocrystals (CNC) and chitosan surfaces.

	Pf			Pv210			Pv247		
Model film	ΔF [Hz]	ΔD [ppm]	$\Delta m_{\text{Sauerbrey}}$ [ng/cm ²]	ΔF [Hz]	ΔD [ppm]	$\Delta m_{\text{Sauerbrey}}$ [ng/cm ²]	ΔF [Hz]	ΔD [ppm]	$\Delta m_{\text{Sauerbrey}}$ [ng/cm ²]
CNC	-10.25 ± 0.03	0.51 $\pm$ 0.01	180.3 $\pm$ 0.2	-6.28 $\pm$ 0.03	0.22 $\pm$ 0.01	117.0 ± 0.2	-4.20 $\pm$ 0.02	0.11 $\pm$ 0.01	79.1 ± 0.2
Chitosan	-23.93 ± 0.02	1.77 $\pm$ 0.01	394.8 $\pm$ 0.1	-7.30 $\pm$ 0.02	0.13 $\pm$ 0.01	136.0 ± 0.2	-4.34 $\pm$ 0.02	0.35 0.01	77.2 ± 0.2

Bio-based materials as antibody carriers and well-coatings with the Paper csELISA

CYMK data was compared between seven different assays for three antibodies: chitosan well-coating and CNC antibody carrier combination, CNC well-coating and chitosan antibody carrier combination, chitosan well-coating, CNC well-coating, chitosan antibody carrier, CNC antibody carrier, and a control (no antibody nor carrier and no bio-based well-coating).

Pf

For *Pf* recombinant proteins tested at the working concentration (2 µg/µL), the combination of chitosan as an antibody carrier and CNC as a well-coating yielded an approximately 4.5-fold increase in CMYK color intensity (with blank/background values subtracted) compared to controls (no antibody carrier or well-coating). Similar increases were observed from the other two CNC assays, with CNC as an antibody carrier or well-coating alone. Chitosan as an antibody carrier alone provided an approximately 2-fold increase in color intensity when compared to controls (no antibody-carrier or well-coating; Figure 4 and S2).

When ten-fold dilutions were performed, CNC as a well-coating and chitosan as an antibody carrier continued to perform best at lower concentrations, followed closely by CNC as either a well-coating or antibody carrier (Figure 4).

Pv210

At the recombinant protein working concentration, the top three performing bio-based polymer assays involved an antibody carrier (Figure 4). Two of them had chitosan as the antibody carrier, yielding a 4-4.5-fold increase in color intensity when compared to controls (no antibody carrier or well-coating). Chitosan as an antibody carrier alone increased color intensity approximately 4-fold, while chitosan as an antibody carrier in combination with CNC as a well-coating and CNC as an antibody carrier alone provided an approximate 4.5-fold increase in color intensity.

At the point following the working concentration (first dilution of antigens *Pv210* tested), similar increases for chitosan as an antibody carrier in combination with CNC as a well-coating as well as CNC as

a well-coating alone were observed, with CNC as a well-coating providing the greatest increase in intensity. Chitosan as an antibody carrier alone performed better than controls (no antibody carrier or well-coating) but did not perform as well as the first two which involved CNC.

Pv247

For the Pv247 recombinant protein assayed at the working concentration, the same three assays that performed optimally with Pv210 antibodies and recombinant proteins performed the best. (Figure S2). Chitosan as an antibody carrier alone yielded the greatest increase in color intensity, yielding an approximate 2-fold increase in color intensity when compared to controls (no antibody carrier or well-coating; Figure 4). Chitosan as an antibody carrier in combination with CNC as well-coating and as CNC as a well-coating alone performed similarly, yielding slightly less than a 2-fold increase of color intensity when compared to controls (no antibody carrier or well-coating).

When ten-fold dilutions were performed, a similar trend was observed as for the other antigens: CNC as a well-coating performed the best at lower concentrations, with chitosan as an antibody carrier and the combination of chitosan as an antibody carrier with CNC as a well-coating performing just below.

Discussion

In this study, two bio-based polymers, chitosan and CNC, were used to modify the csELISA and any resulting differences in detection capabilities were assessed. The use of low quantities (<0.1% wt. of the solutions used) of both bio-based materials was shown to improve detection signals. Chitosan as an antibody carrier performed the best overall in improving detection on polystyrene plates regardless of high- or ultra-low binding affinity. The favored interaction of chitosan with the antibodies was also observed on the QCM-D, where the surface it generated showed higher affinity for the antibodies. These results are likely related to the favorable interactions between the positively-charged amino groups of the chitosan and the negatively charged antibodies (24). Furthermore, due to its chemical structure, chitosan can generate

electrostatic interactions and hydrogen bonding with different polymers, which may explain the increased affinity observed when used with the commercially treated plates.

Thus, these results support the addition of chitosan as either a well-coating or antibody carrier in the csELISA to help increase the intensity of the signal when detecting Pv210 and Pv247 proteins. This may be particularly useful when determining the presence of sporozoites in field collected mosquitoes, where the number of sporozoites can be highly variable (25).

Furthermore, the csELISA for malaria parasite detection in mosquitoes was adapted to a paper format using the same bio-based materials as well-coatings and antibody carriers, thus combining the paper-based csELISA, which reduced the volumes of reagents and samples necessary, with the improvements observed using the bio-based materials.

Combining bio-based materials with paper-based assays provided advancements regarding the detection and visual readability of adapted csELISAs using cost-effective materials. The data presented here can be used to further develop and improve paper-based assays. Figure 5 shows photos taken of PcsELISAs after 15 minutes of drying and after drying overnight. Despite the higher intensity values seen in assays with CNC as a well-coating, further optimization of bio-based materials may be needed to obtain consistently reproducible results. In assays where CNC was used as a well-coating (Fig 5. c-1), reagents tended to leak through the hydrophobic barriers, false positives were common, and overall color intensity was greater. This made results harder to distinguish using the naked eye. Using CNC with a different paper substrate, for example, nitrocellulose or nanocellulose coated papers, may provide a viable alternative to prevent reagent leaking while maintaining color intensity. Color homogeneity, which can best be seen following overnight drying (Fig. 5 x-2), was improved for both chitosan and CNC when compared to the control (no antibody carrier or well-coating) (Fig. 5 a-1). The chitosan antibody carrier assay (Fig. 5 b-1) showed a much clearer, even distribution of color throughout the entire test well. The change in color intensity as recombinant protein concentration increased was easily visible to the naked eye for assays with chitosan as antibody carrier. This is an important consideration, particularly if plate readers are not available. Here, the chitosan antibody carrier paper-based csELISA was shown to have the most potential

to be further explored for future use and scalability. This is due to consistent quantitative colorimetric data and clear visual results. Chitosan as an antibody carrier does not require the additional overnight incubation step that is necessary to coat paper ‘plates’. Future work evaluating the use of paper-based csELISAs will reveal whether this method can be implemented for more economical and efficient detection of sporozoites in field-collected mosquitoes in resource-limited settings.

The more favorable use of chitosan as an antibody carrier rather than a well-coating is likely related to the more efficient interaction between the antibodies and chitosan when put in contact with the solution. Specifically, more active points of chitosan can interact with the antibodies when mixed in solution instead of those sites interacting with the paper or plate surfaces. This effect of groups exposed would also affect the surface available to interact with the antibodies when being applied as the coating, as the surface area of the bio-based materials will become more compact during the adsorption and drying (26). Furthermore, the improved detection of the recombinant proteins when using the CNC as a well-coating and chitosan as an antibody carrier can also be explained by the increase in surface area that the CNC coating induced on the paper csELISA, which can be measured by the changes in rugosity and porosity of the paper (27). This change in surface area could also increase the interaction between the chitosan and the cellulosic surfaces - either the paper by itself, or the paper coated with CNC - in the presence of PBS, as the greater salt content promotes adsorption between the two bio-based materials, chitosan and paper (cellulose)(28).

Conclusions

For Pv210 and Pv247, results of the experiments conducted on 96-well plates showed that chitosan improved the detection of the recombinant proteins, as demonstrated by the higher absorbance values observed when compared to controls (no antibody carrier or plate coating). This is potentially due to a higher affinity between the chitosan and antibodies than the antibodies with the well surfaces. This improved interaction was also demonstrated by QCM-D, where adsorption of the antibodies was higher on the chitosan model films. Experiments with PcsELISAs showed the same trend, where the use of chitosan

as either an antibody carrier or well-coating increased intensity values when compared to the controls (no antibody carrier or well-coating). The use of CNC as a well-coating with PcsELISAs also improved the signal further when compared with the test zones with no bio-based material, possibly by increasing the available surface area for antibody-substrate interactions. Unlike the addition of chitosan which changed the charge of the wells in polystyrene plates, CNC is a 3-D material which added volume and area, another benefit to paper-based assays when compared to polystyrene plates.

The bio-based materials tested, particularly chitosan, both as antibody carriers and well-coatings, have demonstrated their feasibility in improving the detection of the sporozoite proteins from *P. falciparum*, *P. vivax* 210 and *P. vivax* 247 when used as an additional step in csELISA. Furthermore, this work demonstrated the capability of chitosan and CNC to not only improve detection in traditional polystyrene plates (both high- and ultra-low affinity binding), but also with paper-based csELISAs. Although further development of PcsELISAs is needed, this could be a more convenient assay with regard to cost, availability, scalability, time, and ease-of-use.

Abbreviations

CNC: Cellulose nanocrystals

csELISA: Circumsporozoite enzyme-linked immunosorbent assay

PcsELISA: Paper circumsporozoite enzyme-linked immunosorbent assay

spz: Sporozoites

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data are made available in the manuscript and supporting documentation.

Competing interests

The authors declare no competing interests.

Disclaimer

The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the views of the CDC. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the US Department of Health and Human Services.

Funding

This work was supported by a CDC innovation fund (iFund) award and the School of Forestry and Wildlife Sciences and the Office of the Vice President for Research (OVPR) intramural grant program (IGP) award at Auburn University. Laboratory support was provided by the US Centers for Disease Control and Prevention Malaria Research and Reference Reagent and Resource Center (MR4) and BEI Resources for providing the *Plasmodium falciparum* and *P. vivax* 210 and 247 Sporozoite ELISA Reagent Kits.

Author's contributions

AS, ED, SP, SZ conceived the study and participated in its design and coordination. HS, DGM, ACE conducted the laboratory work. HS, DGM, ACE analyzed the data. HS, DGM, AS, and SZ wrote the manuscript and created figures. DGM, HS, AS, ACE, IBVE, ED, SP, SZ participated in data analysis and interpretation. All authors read and approved of the final manuscript.

Acknowledgements

The team is grateful to the Auburn University IGP program for providing support and collaborative opportunities for this work. The authors also thank

Authors' information

Diego Gomez-Maldonado present address: Forest Products Development Center in the School of Forestry and Wildlife Sciences at Auburn University.

Haley Stephens present address: Department of Biological Sciences, College of Science and Mathematics at Auburn University.

Haley Stephens and Diego Gomez-Maldonado contributed equally to this work.

School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States

Haley Stephens, Diego Gomez-Maldonado, Andrea Camarano Eula, Iris Beatriz Vega Erramuspe, Soledad Peresin, and Sarah Zohdy

Forest Products Development Center, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States

Diego Gomez-Maldonado, Iris Beatriz Vega Erramuspe, Soledad Peresin

Centers for Disease Control and Prevention, Atlanta, GA, United States

Alice Sutcliffe, Ellen Dotson

411

412

References

1. Arrow KJ, Panosian C, Gelband H, Institute of Medicine (U.S.). Committee on the Economics of Antimalarial Drugs., National Academy of Sciences (U.S.). Saving lives, buying time economics of malaria drugs in an age of resistance. Washington, DC: National Academies Press,; 2004.
2. Fontenille D, Meunier JY, Nkondjio CA, Tchuinkam T. Use of circumsporozoite protein enzyme-linked immunosorbent assay compared with microscopic examination of salivary glands for calculation of malaria infectivity rates in mosquitoes (Diptera : culicidae) from Cameroon. *Journal of Medical Entomology*. 2001;38(3):451-4.
3. Lequin RM. Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clinical Chemistry*. 2005;51(12):2415-8.
4. Coppi A, Natarajan R, Pradel G, Bennett BL, James ER, Roggero MA. The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *J Exp Med*. 2011. p. 341-56.
5. Gan SD, Patel KR. Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay. *Journal of Investigative Dermatology*. 2013;133(9):E10-E2.
6. Durnez L, Van Bortel W, Denis L, Roelants P, Veracx A, Trung HD, et al. False positive circumsporozoite protein ELISA: a challenge for the estimation of the entomological inoculation rate of malaria and for vector incrimination. *Malar J*. 2011;10:195.
7. Sia SK, Linder V, Parviz BA, Siegel A, Whitesides GM. An integrated approach to a portable and low-cost immunoassay for resource-poor settings. *Angewandte Chemie-International Edition*. 2004;43(4):498-502.
8. Cheng C-M, Martinez AW, Gong J, Mace CR, Phillips ST, Carrilho E, et al. Paper-Based ELISA. *Angewandte Chemie-International Edition*. 2010;49(28):4771-4.

- 435 9. Maxwell EJ, Mazzeo AD, Whitesides GM. Paper-based electroanalytical devices for accessible diagnostic
436 testing. *Mrs Bulletin*. 2013;38(4):309-14.
- 437 10. Evans E, Moreira Gabriel EF, Benavidez TE, Tomazelli Coltro WK, Garcia CD. Modification of microfluidic
438 paper-based devices with silica nanoparticles. *Analyst*. 2014;139(21):5560-7.
- 439 11. Carrilho E, Martinez AW, Whitesides GM. Understanding Wax Printing: A Simple Micropatterning Process
440 for Paper-Based Microfluidics. *Analytical Chemistry*. 2009;81(16):7091-5.
- 441 12. Pandey JK, Takagi H, Nakagaito AN, Kim H-J. Handbook of polymer nanocomposites : processing,
442 performance and application. Volume C, Polymer nanocomposites of cellulose nanoparticles.
- 443 13. Smook G. Handbook For Pulp and Paper Technologists (The SMOOK Book), Fourth Edition: TAPPI
444 PRESS; 2016.
- 445 14. Moon RJ, Martini A, Nairn J, Simonsen J, Youngblood J. Cellulose nanomaterials review: structure,
446 properties and nanocomposites. *Chemical Society Reviews*. 2011;40(7):3941-94.
- 447 15. Kumar M, Muzzarelli RAA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical
448 perspectives. *Chemical Reviews*. 2004;104(12):6017-84.
- 449 16. Younes E, Haas GP, Dezso B, Ali E, Maughan RL, Kukuruga MA, et al. LOCAL TUMOR-IRRADIATION
450 AUGMENTS THE RESPONSE TO IL-2 THERAPY IN A MURINE RENAL ADENOCARCINOMA. *Cellular*
451 *Immunology*. 1995;165(2):243-51.
- 452 17. Younes I, Rinaudo M. Chitin and chitosan preparation from marine sources. Structure, properties and
453 applications. *Marine Drugs* March 2015. p. 1133-74.

- 454 18. Kumar M. A review of chitin and chitosan applications. *Reactive & Functional Polymers*. 2000;46(1):1-27.
- 455 19. Gabriel EFM, Garcia PT, Cardoso TMG, Lopes FM, Martins FT, Coltro WKT. Highly sensitive colorimetric
456 detection of glucose and uric acid in biological fluids using chitosan-modified paper microfluidic devices. *Analyst*.
457 2016;141(15):4749-56.
- 458 20. Wirtz R. *Plasmodium vivax* Sporozoite ELISA Reagent Kit. In: Control CFD, editor.: American Type
459 Culture Collection (ATCC); 2017. p. 1-12.
- 460 21. Sutcliffe AC, Irish SR, Rogier E, Finney M, Zohdy S, Dotson EM. Adaptation of ELISA detection of
461 *Plasmodium falciparum* and *Plasmodium vivax* circumsporozoite proteins in mosquitoes to a multiplex bead-based
462 immunoassay.
- 463 22. Young JF, Hockmeyer WT, Gross M, Ballou WR, Wirtz RA, Trosper JH, et al. Expression of *Plasmodium*
464 *falciparum* circumsporozoite proteins in *Escherichia coli* for potential use in a human malaria vaccine. *Science*.
465 1985;228(4702):958-62.
- 466 23. Sim KL, Hoffman S, Arevalo M, Herrera S, inventorsUnique DNA and polypeptide sequences based on the
467 circumsporozoite protein of *Plasmodium vivax*. US2006.
- 468 24. Orelma H, Filpponen I, Johansson L-S, Laine J, Rojas OJ.
- 469 25. Beier JC, Onyango FK, Koros JK, Ramadhan M, Ogwang R, Wirtz RA, et al. Quantitation of malaria
470 sporozoites transmitted in vitro during salivation by wild Afrotropical *Anopheles*. *Medical and Veterinary*
471 *Entomology*. 1991;5(1):71-9.
- 472 26. Israelachvili JN. *Intermolecular and surface forces*. 3rd ed. Burlington, MA: Academic Press; 2011. xxx, 674
473 p. p.

- 474 27. Salas C, Nypelö T, Rodriguez-Abreu C, Carrillo C, Rojas OJ. Nanocellulose properties and applications in
475 colloids and interfaces. *Current Opinion in Colloid & Interface Science*. 2014;19(5):383-96.
- 476 28. Mishima T, Hisamatsu M, York WS, Teranishi K, Yamada T. Adhesion of β -d-glucans to cellulose.
477 *Carbohydrate Research*. 1998;308(3):389-95.
- 478
479

480

481

Figures

Figure 1

Workflow showing conversion of cellphone photograph to numerical color data. CMYK changes in each well were averaged to one single value, which was then identified as the color intensity difference percentage. This color intensity value was then plotted against antigen concentration to determine the relationship between the two.

Figure 2

ELISA results for Pf, Pv210 and Pv247 run on high-binding (MaxiSorp™) and ultra-low binding(Corning® 3474) plates. **The conditions tested were chitosan as antibody carrier or well-coating (Chi 0.1% coating), chitosan as an antibody carrier (Chi 0.1% carrier) and chitosan as well-coating and cellulose nanocrystal as a carrier (Chi 0.1% coating / CNC 0.1% carrier).** The samples and controls were run at four concentrations: the standard cs-ELISA recombinant protein working solution concentrations and three 10-fold dilutions. The working solution concentrations were: 2 $\mu\text{g}/\mu\text{l}$ for Pf, 182 $\mu\text{g}/\mu\text{l}$ for Pv210 and 91 $\mu\text{g}/\mu\text{l}$ for Pv247. The absorbance was measured at 405 nm. Samples and controls were run in triplicate and each assay was repeated three times. Bars represent SD, n=9.

Figure 3

QCM-D results of adsorption of Pf, Pv210, and Pv247 antibodies in model film surfaces of cellulose nanocrystals (CNC) and chitosan (Chi). The surfaces were analyzed over precoated gold sensors with PEI with 0.1% CNC or chitosan solutions at 3000 rpm. Flow concentrations of the antibodies were **2 $\mu\text{g}/\mu\text{l}$ for Pf, 182 $\mu\text{g}/\mu\text{l}$ for Pv210, and 91 $\mu\text{g}/\mu\text{l}$ for Pv247** in PBS and flow of 50 $\mu\text{L}/\text{min}$.

Figure 4

Paper csELISA results for all antibody carrier and well-coating assays. Recombinant proteins were tested at concentrations as follows: Pf at 0 pg/μL, 0.2 pg/μL, 2 pg/μL, 50, pg/μL, and 100 pg/μL, Pv210 at 0 pg/μL, 18.2 pg/μL, 182 pg/μL, 4500 pg/μL, and 9100 pg/μL, and Pv247 at 0 pg/μL, 9.1 pg/μL, 91 pg/μL, 2275 pg/μL, and 4550 pg/μL. Data were plotted and converted to log-scale as a graph of color intensity difference versus concentration of the recombinant protein. The change in color intensity for each concentration was calculated as the difference between the average CMYK value and the corresponding blank (in percentage).

Figure 5

Photographs of paper-plate assays were taken using a cellphone camera after drying for 15 minutes, and overnight. Significant differences can be seen in the distribution of color and overall well homogeneity between the three different assays. Both bio-based material assays showed a notable improvement over the control, with the chitosan carrier assay showing the clearest visible difference in increasing antigen concentration.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [csELISAmanuscriptsupportinginformation.docx](#)