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High capture efficiency of lectin surfaces for *Cryptosporidium parvum* biosensors

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Note: work was performed at 1, the authors are now at 2 and 3 respectively.

HIGHLIGHTS

- Characterisation of lectin immobilisation strategies
- Use of lectins for high efficiency pathogen capture
- Lectin immobilisation better under static conditions

ABSTRACT

Detection of waterborne pathogens, such as *Cryptosporidium*, is essential for ensuring drinking water safety. Biosensors are one emerging technology aiming to improve the existing time-consuming and expensive monitoring approach. Surface functionalisation is a key aspect of biosensor development and here we propose that lectins offer an excellent alternative to antibodies offering a higher capture efficiency for oocysts. We also demonstrate that lectin immobilisation using a protocol of 11-mercaptoundecanoic acid followed by EDC-sulfo-NHS results in superior performance, compared to three other common surface functionalisation protocols, achieving an 86% capture efficiency. We also determine that capture efficiency with lectin surfaces is enhanced by the use of static conditions, in contrast to previous work with antibodies where surface capture was improved by convective conditions. These results will be highly useful in guiding the development of new biosensor systems for protozoan pathogen detection.

Keywords: immobilisation protocols; capture efficiency; biorecognition; lectins; Cryptosporidium

INTRODUCTION

Cryptosporidium parvum (*C. parvum*) is a zoonotic waterborne protozoan parasite that causes the disease cryptosporidiosis [1]. The transmission of cryptosporidiosis is accomplished through the parasites robust, long-lived oocysts whereas infection with *C. parvum* is a result of the parasites sporozoites [2]. The main symptom of cryptosporidiosis is diarrhea but cryptosporidiosis can also cause vomiting, fever, nausea, abdominal discomfort and respiratory problems [3]. *C. parvum* is infectious in very low concentrations, approximately 1-10 oocysts [4], and sporozoites can infect the host straight after they are excysted from the oocysts [2]. *Cryptosporidium* can become fatal when infects immuno-compromised people, young children or the elderly [1, 3, 5]. It has been responsible for several waterborne outbreaks of disease, the biggest of which was recorded in Milwaukee, Wisconsin, USA in 1993 [3]. In this case, approximately 400,000 people were infected, and in 69 of those cases symptoms were fatal [6]. There have also been recent outbreaks in Australia [7], the UK [8] and Sweden, with a dramatic rise in the number of outbreaks over the last decade [9]. High levels of endemic disease are suspected [10]. The parasite is robust, surviving for a long time in water, shows resistance to standard methods of water treatment [4, 11], e.g. chlorination, and its presence does not correlate well with indicator organism monitoring. Therefore, many water companies perform direct monitoring of *Cryptosporidium* [12].

Existing, regulatory approved monitoring methods are time-consuming and manpower intensive, e.g. the manual microscopy counting of oocysts [12]. Several different new approaches to detection have been proposed [13], including optical methods, impedance and other electrical techniques, molecular methods and biosensors. One advantage of the biosensor approach is portability, which would allow on-site testing of raw waters or at water treatment works after initial sample processing. Further advantages include the potential for automation and the possibility to be operated by non-specific personnel [14-16]. QCM [17],

SPR [18] and cantilevers [19] have all been investigated to detect *Cryptosporidium*, currently reaching a detection limit of 100 oocysts/mL.

All of the *Cryptosporidium* biosensor studies have utilised antibodies as the biological recognition element. We have previously studied how the immobilisation protocol for the antibodies impacts upon oocyst capture efficiency, recommending protein G under convective conditions with a minimum antibody concentration of 20µg/mL [20].

While antibodies offer high selectivity there are several drawbacks to their utilisation as biosensor biorecognition elements, including their expense and the difficulty of sensor regeneration. Recently, lectins are emerging as an interesting alternative as biorecognition elements for biosensors. A wide variety of low-cost lectins are commercially available offering high stability in standard buffers, high surface densities due to their smaller size and the ability to discriminate between bacteria at the sub-species level [21-23]. Lectin biosensors have been applied to molecular detection, e.g. on SPR for pesticides [24] or the NanoMonitor system detecting glycan biomarkers [25], as well as to the detection of cancer cells [26] and bacteria. Lectin based magnetoelastic sensors have achieved detection limits of 60 cells/mL [27] and QCM devices have shown species discrimination [28] as well as *E. coli* quantification [22, 23, 29] and subspecies identification [30].

While it is well-know that optimisation of immobilisation protocols is essential for enhanced biosensor performance with antibodies due to factors such as antibody orientation [20], there has been less work on the impact of surface immobilisation protocols for lectins. Serra et al found that avidin-biotin linkers performed better than direct adsorption to the surface, especially when bacteria were added to the biotin-lectin containing solution [29], whereas other authors have used different chemical cross-linking approaches [21]. In this paper we propose that lectins offer excellent alternative for *Cryptosporidium* biosensors and determine the optimal immobilisation protocols for lectin based oocyst biosensors.

MATERIALS AND METHODS

Cryptosporidium parvum immobilization

C. parvum and other reagents

Viable *C. parvum* oocysts were obtained from Creative Science Company, Moredun Research Institute. The lectins, concavalin A (conA) and biotin-labeled conA as well as 11-mercaptoundecanoic acid (11-MUA), avidin, cysteamine, glutaraldehyde, 1-ethyl-3(dimethylaminopropyl)-carbodiimide (EDC), sulfo-N-hydroxysuccinimide (sulfo-NHS) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich. Glass coverslips were purchased from Fisher Scientific. For the staining Crypto-a-glo and No-Fade mounting medium were obtained from Waterborne Inc, with MeOH and DAPI from Sigma.

Preparation of *C. parvum* and sample surface

Glass coverslips were sputter-coated with a 10nm chromium layer and a 40nm gold layer. Gold-coated glass samples were cleaned with a mixture of hydrogen peroxide (30%), ammonia (25%), deionized water (volume ratio 1:1:5) at 75 °C for 5 minutes. Every immobilization protocol was performed three times under static and convective conditions respectively. A flow cytometer device (Becton Dickinson, FACSCalibur 83) was utilized in order to produce vials of *C. parvum* solution that contained 100 oocysts each.

Immobilization protocols

Immobilization of oocysts to gold surface with no cross-linker application

Gold-coated samples were covered with a solution containing 15ml 0,1M acetate buffer pH 5 mixed with 1 mM Mn^{2+}, 1 mM Ca^{2+} and 2mg conA for 1 hour, dried in a flow of nitrogen and immersed in a *C. parvum* solution for 1 hour. In the control sample ConA was replaced by PBS. After each step was complete, samples were rinsed with PBS (10mM pH 7.4) in order to remove any unbound reagents.

Immobilization of oocysts to gold surface with application of cysteamine- glutaraldehyde

Gold-coated glass samples were incubated with a 12mM cysteamine solution for 12 hours and a 2.5% v/v glutaraldehyde solution for 30 minutes. Afterwards they were immersed in a conA (1mg/ml) solution for 1 hour and finally covered in a *C. parvum* solution for another hour. Cysteamine was replaced by PBS in the control sample. After each step was complete, samples were rinsed with PBS (10mM pH 7.4) in order to remove any unbound reagents.

Immobilization of oocysts to gold surface with application of 11-MUA, EDC-sulfo-NHS

Gold-coated glass samples were incubated with a 1mM 11-mercaptoundecanoic acid solution (11-MUA) and kept in a dark place for at least 24 hours, dried on a stream of nitrogen and immersed in an EDC-sulfo-NHS solution (volume ratio 1:1) for 1 hour. Samples were then incubated with conA (1mg/ml) for 1 hour and immersed in a *C. parvum* solution for 1 hour. 11-mercaptoundecanoic acid was replaced by PBS in the control sample. After each step was complete, samples were rinsed with PBS (10mM pH 7.4) in order to remove any unbound reagents.

Immobilization of oocysts to gold surface with application of biotin-avidin

Gold-coated samples were immersed in a 15ml 0.1M acetate buffer pH 5 containing 1 mM Mn^{2+}, 1 mM Ca^{2+} and 1mg of avidin solution, rinsed with PBS and immersed in a new acetate buffer solution containing 2mg of biotin-labeled ConA for 1 hour respectively. Afterwards samples were incubated with a *C. parvum* solution for 1 hour and rinsed again. Avidin was replaced by PBS in control samples. After each step was complete, samples were rinsed with PBS (10mM pH 7.4) in order to remove any unbound reagents.

Figure 1 summarizes the different protocols tested for the immobilization of *C. parvum*.

Exposure to *C. parvum*

The EPA1623 protocol was used for staining samples. Briefly, the samples were air-dried, methanol added and left to dry again. Next DAPI was added for about 1 min and then, followed by rinsing before Crypto-a-glo was put on every sample and left to incubate for 1 hour. Afterwards, the samples were rinsed twice with PBS and a drop of non-fade medium (approximately 45µl) was put on every sample. Lastly, the samples were ready for observation under the microscope (Inverso Epi-Fluor, CETI, Medline Scientific) and enumeration of oocysts.

RESULTS AND DISCUSSION

Four different functionalization protocols for the immobilization of con A on gold surfaces were compared by evaluating subsequent capture efficiency for *C. parvum*. Table 1 presents the results from the different immobilization protocols under different conditions. Optimal incubation times and temperatures as found in the literature were applied for each protocol respectively. In the first protocol no cross-linker was applied and con A surface coating took place by physiosorption. In this case, con A binds with the thiol groups on one side and on

the other with *Cryptosporidium parvum*. In the second protocol, cysteamine/glutaraldehyde was used since cysteamine has the ability to form SAM layers which then bind with glutaraldehyde. Glutaraldehyde binds through covalent bonds with con A. The third protocol was avidin/biotin-labeled con A, as previously tested on biosensors by Serra et al [29]. Avidin is a protein that has the ability to bind through sulfur bonds with the surface and also with vitamin biotin, in this case, biotin-labeled conA. The final protocol, tested the efficiency of 11-MUA combined with EDC and NHS. 11-MUA is a cross-linker that acts by creating SAM layers, which bind with EDC and sulfo-NHS. EDC converts the carboxyl groups of the SAM layers to amine groups and produce an intermediate. This intermediate first becomes stable through sulfo-NHS and then binds with con A.

The results detailing the capture efficiency of the differently prepared lectin surfaces, under static conditions, are shown in Figure 2A. Compared to the control samples there is no statistically significant difference ($p=0.5$) between the physiosorbed con A surfaces or with the avidin-biotin linkage whereas for the other two cross-linkers the difference in capture efficiency of oocysts compared to the control was statistically significant at even $p=0.001$. Therefore, it is clear that the choice of an appropriate cross-linker is essential for optimal functioning of a *Cryptosporidium* lectin biosensor. Physisorbtion most likely results in a lower level of surface coverage of con A and no control over orientation/binding site availability is obtained which will also act to reduce the capture efficiency.

A comparison of all the different protocols with each other, under static conditions (Figure 2A) reveals that at $p=0.5$ there is no statistically significant difference between the physiosorption approach and avidin cross-linking or between the avidin and cysteamine cross-linking methods. However, even at $p=0.001$ the enhancement of capture efficiency when using cysteamine compared to direct physisorption is statistically significant as is the improvement of binding observed for 11-MUA over all other protocols. This indicates that the use of the lectin immobilization protocol incorporating 11-MUA would be the best choice for creating a *Cryptosporidium* oocyst biosensor. Potential explanations for this effect include surface coverage/density of con A and/or arrangement of the lectin at the surface.

Our previous work [19] has shown that immobilization of antibodies and incubation with oocysts, both under convective conditions, results in a higher capture efficiency than in the static case. This is attributed to more effective sample delivery to the surface and therefore improved performance. Therefore, it might be expected that operating the lectin

immobilization and subsequent oocyst capture under a convective set-up would further improve the capture efficiencies noted above. However, the convective case (Figure 1B) presents significantly lower capture efficiencies for all protocols, and in this case only the 11-MUA protocol is better than the controls.

Comparing the static and convective cases directly it seems that operating in static conditions is more effective for capture efficiency. Figure 2 indicates a comparison of static and convective conditions for each immobilization protocols. It is clear that the data points lie on the side of the static conditions, i.e. with a slope lower than one which would be observed if the flow conditions had no impact, showing this set-up is more efficient. Lectin binding and pathogen capture has a lower affinity than antibody capture and relies on the formation of multiple interactions; therefore, it is possible that the use of convective conditions agitates the sample preventing the effective formation of multiple bonds to confer stability. Indeed, when comparing to antibodies [20], the best results reported here for the 11-MUA immobilization protocol and static conditions, exceed the capture efficiency demonstrated by the best antibody immobilization protocol. The recovery rate is 86% versus around 50%, a considerable improvement. This higher capture efficiency may trade-off against specificity in complex samples, but a multiplex system incorporating several lectins to confer selectivity could compensate for this [30].

CONCLUSIONS

We propose that lectins offer an excellent alternative biorecognition element for protozoan pathogen biosensors. Comparing four different protocols it is clear that immobilizing conA with 11MUA and EDC-sulfo-NHS results in the highest capture efficiency. Furthermore, static conditions are optimal for biosensor immobilization and oocyst exposure since significantly worse results were obtained for every immobilization protocol, except physiosorption, which was in any case poorly performing, for convective conditions. This is in contrast to antibody coated surface performance and is most likely due to the lower affinity of lectin binding, requiring multiple interactions for a strong attachment. This condition could result in a trade-off between capture efficiency and throughput, depending upon the volumes to be tested. The advantage though of the lectin approach is that higher capture efficiencies were demonstrated than with antibodies, which highlights the high potential of lectins in waterborne pathogen biosensors. Furthermore, lectins could maybe even find other

applications, e.g. replacing antibodies in IMS, depending upon selectivity, which remains to be investigated.

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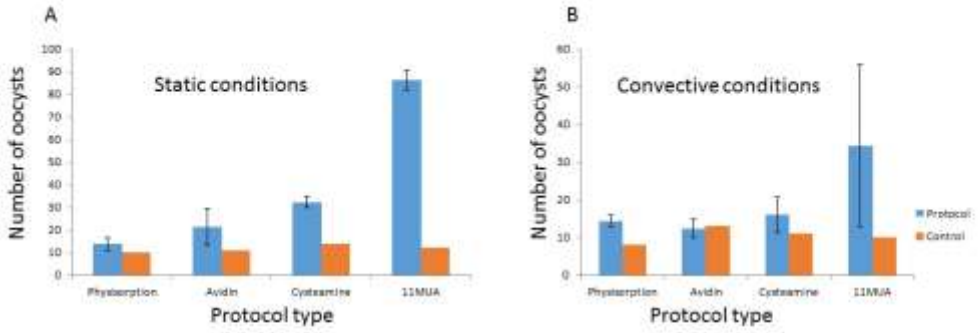

Figure 1: A) Oocyst capture efficiency of con A surfaces with the lectin immobilised by different protocols under static conditions. The result for each protocol is an average of 3 measurements with the y axis being the number of oocysts counted on the surface following incubation with 100 oocyst counted samples. Controls were undertaken for each protocol (red bar in the graph for each experiment). B) Oocyst capture efficiency of con A surfaces with the lectin immobilised by different protocols under convective conditions. The result for each protocol is an average of 3 measurements. Controls were undertaken for each protocol.

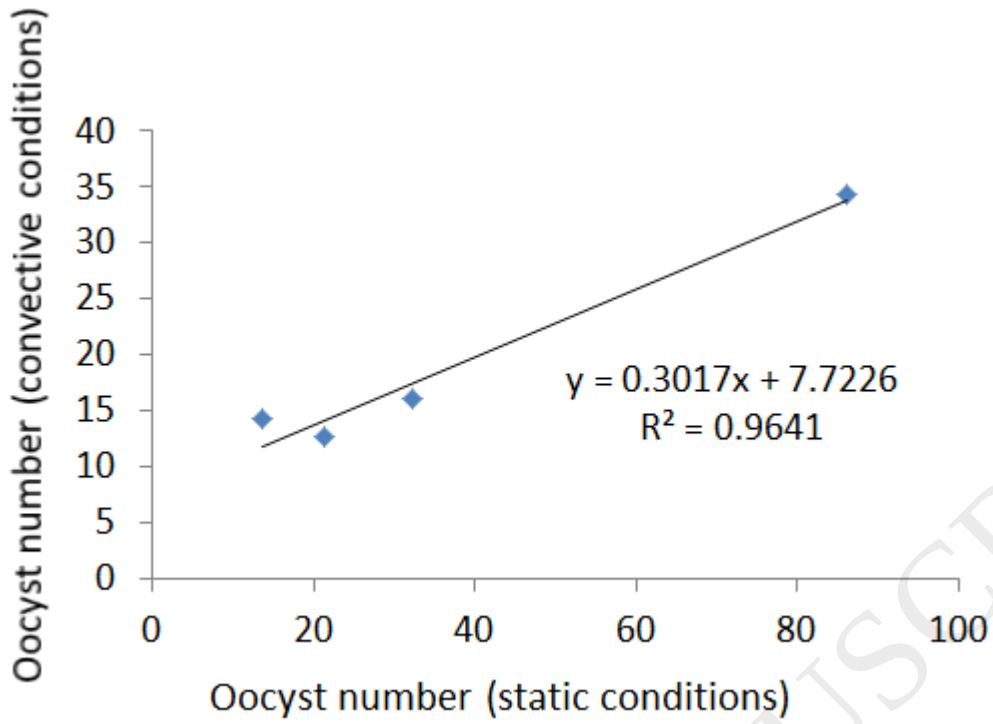

Figure 2: Plot of convective conditions (y-axis) against static conditions (x-axis) for the four different immobilisation protocols. As the slope is less than 1 this indicates that the oocyst capture was better in the static protocols.

Table 1: Average recovery rates with the different lectin immobilization methods, under different conditions.

TABLE 1:

Protocol	**Static Conditions**			**Convective Conditions**		
	Average recovery rates (%)	**Standard deviation**	**Control**	**Average recovery rates (%)**	**Standard deviation**	**Control**
Physisorption	13.6	3.05	10	14.3	1.52	8
Cysteamine/ Glutaraldehyde	32.3	2.51	11	16	4.58	11
Avidin/Biotin	21.3	8.14	14	12.6	2.08	13
11-MUA, EDC/NHS	86.3	4.50	12	34.3	21.50	10