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Short communication

Comparison of immobilization strategies for *Cryptosporidium parvum* immunosensors

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ABSTRACT

Cryptosporidium is a waterborne protozoan parasite, which is problematic for the water industry due to widespread environmental presence, low infectious dose and resistance to chlorine disinfection. To replace the existing, slow regulatory monitoring procedure, immunosensors have been proposed. The performance of such sensors is often limited by the antibody immobilization. The aim of this article was to determine the immobilization protocol offering the highest oocyst capture efficiency. Four methods were tested both under static and convective conditions: physisorption, cysteamine–glutaraldehyde linkage, 3-aminopropyl-triethoxysilane functionalization and protein G linkage. The protein G protocol was shown to present the highest recovery rates in both conditions. For the protein G protocol, different antibody concentrations were tested and it was concluded that there is little difference in the recovery rates when the concentration of IgG antibody is higher than 20 μg/mL. Additionally, operation under convective rather than static conditions increased the recovery for every protocol. These results can be useful to inform the design of future biosensor systems, using antibodies as the detection element, for *Cryptosporidium*.

1. Introduction

Cryptosporidium parvum and *Cryptosporidium hominis* are protozoan parasites that can cause the illness cryptosporidiosis, if consumed by humans [1]. There are 18 other species, but the above two are responsible for the majority of human disease outbreaks [2]. The sporozoites of this parasite infect the gastrointestinal tract causing several symptoms, the most common of which being diarrhoea; cryptosporidiosis can also lead to nausea, vomiting, weight loss, or fever [3]. This disease, triggered by consumption of as little as 10 oocysts [4], can be fatal, especially for immunocompromised people [5]. It is estimated that around 60,000 cases occur annually in the UK, with the most common means of infection being waterborne oocysts [6]. *Cryptosporidium* is ubiquitous in the environment and is resistant to standard water treatment systems [7]; therefore this pathogen represents a major problem for the water industry.

Monitoring of the water supply for the presence of *Cryptosporidium* oocysts is a regulatory requirement and the detection of as little as one oocyst per 10 L can trigger the issuing of a boil water notice [8]. Several protocols have been utilized to detect oocysts in water samples such as conventional microscopy, immunological assays, flow cytometry, fluorescent in situ hybridization (FISH), fingerprinting, PCR, PCR-based sequencing and restriction fragment length polymorphism (PCR-RFLP), electrophoretic mutation scanning, real-time PCR coupled to high resolution melt (HRM) analysis and U.S. Environmental Protection Agency (EPA) Method 1623 [9]. All techniques require analysis of a large volume (up to 1000 L) of water and sample preparation (e.g. filtration, centrifugation, immunomagnetic separation) is a critical part of the process. In this paper, however, we are interested in the detection method. Most of the above techniques have the ability to identify a very small number of oocysts, with PCR-RFLP offering the potential for viability and species discrimination [9].

Nevertheless FISH presents limitations concerning the detection of non-viable oocysts, flow cytometry presents low ability to detect oocysts in general and inability in species discrimination both in labelled and in unlabelled samples [1,9], PCR is very time-consuming and EPA 1623 is sensitive to a number of parameters like silica size and concentration [10]. Moreover, those techniques need expensive laboratory equipment, highly trained staff and expensive reagents [11].

An alternative approach to the detection of oocysts is biosensors. Biosensors are devices that include a transducer which collaborates with a biological material in order to detect an analyte, where the measured signal corresponds to the quantity of the analyte [12]. Furthermore, they present an ideal portable format [13], they are comprehensive [14], can be operated by non-specific personnel [15] and present potential for incorporation in automated systems [16]. There are several different biosensor techniques such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM),

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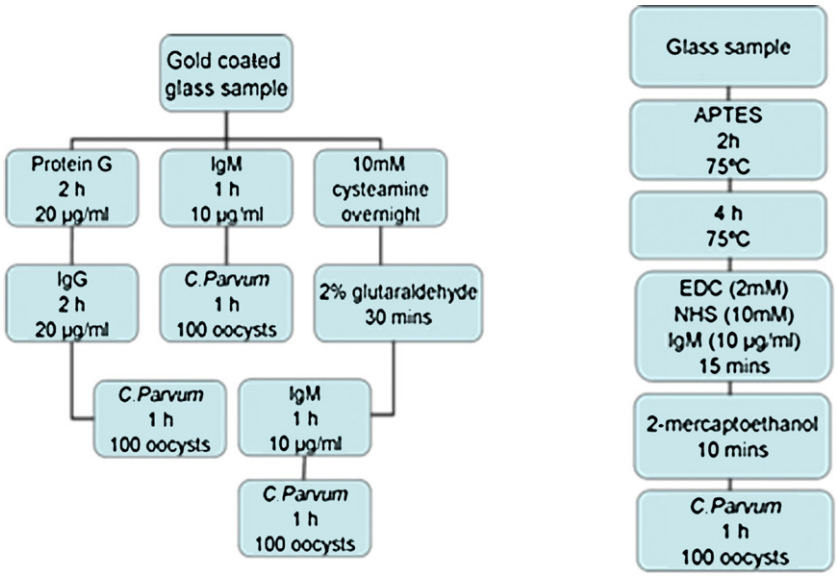

Fig. 1. Overview of the different immobilization protocols.

lead zirconate titanate (PZT) which have been trialled for the detection of this parasite with very promising initial results [11,17–19].

All the *Cryptosporidium* biosensor studies have used antibodies as the biological recognition element. However, in addition to the different sensor technologies, these studies have also utilized different antibodies and immobilization strategies; therefore comparison between these studies is difficult. The main disadvantage of immune-based biosensors is the low capture efficiency of surface-immobilized antibodies. This is strongly influenced by the antibody immobilization protocol, which controls surface coverage, orientation and the preservation of activity [20]. However, there has been little work to characterize which surface recognition strategy and protocol is the most efficient for the development of *Cryptosporidium* biosensors, though Rony Das in his thesis has discussed the use of antibody fragments [21].

The purpose of this work was to investigate a number of immobilization chemistries and identify the best one in relation to the subsequent oocyst binding and to make recommendations on biosensor operation. These results will be of great use in the development of the right type of biosensor for the detection of this problematic waterborne pathogen.

2. Materials and methods

2.1. Reagents

Viable *C. parvum* oocysts were obtained from Creative Science Company, Moredun Research Institute. Phosphate buffer saline (PBS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), cysteamine, protein G, sulfo-*N*-hydroxy-sulfosuccinimide (sulfo-NHS), 3-aminopropyl-triethoxysilane (APTES), glutaraldehyde, hydroxylamine, 2-mercaptoethanol, diamidino-2-phenylindole (DAPI), ethanol, methanol (MeOH), dimethyl-sulfoxide (DMSO), hydrogen peroxide and sulphuric acid were all purchased from Sigma–Aldrich. Mouse monoclonal antibody immunoglobulin M (IgM), Crypto-a-glo and No-fade mounting medium were obtained from Waterborne Inc. and goat polyclonal antibody immunoglobulin G (IgG) was obtained from Abcam.

All solutions were prepared with PBS to give final concentrations of IgM (10 μg/mL), IgG (20 μg/mL), protein G (20 μg/mL), glutaraldehyde (2%), cysteamine (10 mM), sulfo-NHS (10 mM), EDC solution (2 mM), 2-mercaptoethanol (20 mM) and APTES (0.4%). Samples of 100 oocysts of *C. parvum* were counted using a flow cytometer (Becton Dickinson, FACSCalibur 83).

2.2. Immobilization of antibodies followed by oocysts

Prior to every immobilization protocol, the glass coverslips (13 mm diameter, from Fisher) were sputter-coated with a 10 nm chromium layer and a 40 nm gold layer. The gold-coated samples were then cleaned with sulphuric acid:hydrogen peroxide (volume ratio 3:1) for 5 min followed by ethanol for 5 min and afterwards rinsed with PBS. Every experiment was performed three times, both under static and non-static conditions. The non-static conditions were obtained by placing the samples in a plate shaker (IKA® KS 4000 I control) at 60 rpm. An overview of the immobilization protocols is shown in Fig. 1 and details are given below.

2.2.1. Immobilization of antibodies directly to gold surface (physisorption)

The gold-coated glass samples were incubated with IgM for 1 h, rinsed twice with PBS and finally left exposed to 100 oocysts for 1 h. The control samples were incubated with PBS instead of antibody.

2.2.2. Immobilization of antibodies to cysteamine–glutaraldehyde functionalized gold surface

The gold-coated glass samples were left overnight in a cysteamine solution with nitrogen bubbling through. Samples were immersed in glutaraldehyde for 30 min which acted as a linking agent and subsequently incubated with IgM for 1 h. Samples were then exposed to 100 oocysts for 1 h. The control samples were incubated with PBS instead of cysteamine. Samples were rinsed twice with PBS after every immobilization step.

2.2.3. Immobilization of antibodies to protein G functionalized surface

Samples were incubated with protein G for 2 h and then incubated with IgG for 2 h. Finally, they were exposed to 100 oocysts for 1 h. The control samples were incubated with PBS instead of antibody. Samples were rinsed twice with PBS after every immobilization step.