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PAPER

Specific detection of *Campylobacter jejuni* using the bacteriophage NCTC 12673 receptor binding protein as a probe

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Campylobacter jejuni is found in the intestines of poultry, cattle, swine, wild birds and pet animals and is the major cause of foodborne gastroenteritis in developed countries. We report the use of the receptor binding protein (RBP) of Campylobacter bacteriophage NCTC 12673 for the specific capture of *Campylobacter jejuni* bacteria using RBP-derivatized capturing surfaces. The Gp48 RBP was expressed as a glutathione S-transferase-Gp48 (GST-Gp48) fusion protein and immobilized onto surface plasmon resonance (SPR) surfaces using glutathione self-assembled monolayers (GSH SAM). Bovine serum albumin (BSA) was used to block any non-specific binding. Glutathione SAM leads to an oriented attachment of the protein, resulting in a two- to three-fold improvement of bacterial capture when compared to dithiobis(succinimidyl propionate) (DTSP) SAM-based unoriented attachment. The specificity of recognition was confirmed using *Salmonella enterica* subsp. *enterica* serovar Typhimurium as a negative control, which indeed showed negligible binding. The detection limit of the RBP-derivatized SPR surfaces was found to be 10^2 cfu/ml. Finally, GST-Gp48 was also immobilized onto magnetic beads that were successfully used to capture and pre-concentrate the host pathogen from suspension.

Introduction

Fast, sensitive and accurate identification and detection of bacterial pathogens has become an area of tremendous interest for food, water and public health safety. Conventional methods usually rely on culture-based biochemical assays, which are accurate but can be laborious and time consuming. Thus, the last decade has seen sustained efforts towards the development of alternate pathogen detection technologies.[1,2] Quartz crystal microbalance,[3,4] flow cytometry,[5,6] mechanical resonators,[7,8] amperometry,[9] and surface plasmon resonance (SPR)[10,11] have been explored as potential transduction platforms for such applications. SPR has been used extensively for the detection of pathogens, their toxins and spores using DNA,[12] RNA,[13] polyclonal[14,15] and monoclonal antibodies[10,11] as probes.

Antibodies are the most commonly used biological probes for the detection of bacterial pathogens. They however suffer from temperature and pH dependent instability and thus have a short shelf-life.[16] Therefore, bacteriophage-based technologies has been looked upon as a robust alternative biological probe for such recognition. Bacteriophages are bacterial viruses, which recognize and bind to specific receptors on their host surface in order to initiate infection. Bacteriophages use their receptor binding protein (RBP) for such receptor recognition and binding.[17] This recognition is highly specific and is therefore exploited for bacterial typing. Such high levels of specificity also make bacteriophage technologies highly promising as probing elements in pathogen biosensing platforms. Whole phages have been used for such detection in conjunction with different transduction approaches.[18–22] In previous reports, we have shown that chemically immobilized T4 phages act as an effective probe for capturing *E. coli* K12 onto surfaces[23] and successfully extended their use to SPR-based detection approaches.[24]

However, use of whole phage suffers from two main drawbacks.[25] First, the drying of surface-immobilized whole-phages severely impairs their capture efficiency. Secondly, overexposure to the surface-immobilized phages leads to bacterial lysis and eventual destruction of the captured pathogen being detected. We therefore recently reported the use of the phage receptor binding protein as an alternative probe for the capture and detection of the bacterial host.[25] We have demonstrated that the use of RBPs overcomes the shortcomings of the whole-phage approach and can be successfully employed for sensitive and selective detection of the pathogen. In addition, genetic engineering allows us to express desired tags on RBPs to facilitate their surface immobilization and also provides freedom to tailor

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their affinity and binding properties. Thus, RBPs offer several advantages over antibodies as well as whole-phage based approaches.

Our initial proof-of-concept involved the SPR-based detection of *Salmonella* using the P22 phage RBP as molecular probe. In the work presented here, we have used a recently described RBP[26] (Gp48) of phage NCTC 12673 for the capture of *Campylobacter jejuni* using RBP-functionalized microbeads. *C. jejuni* is indeed one of the most prominent foodborne pathogens. It is found in the intestines of poultry, cattle, swine, wild birds and pet animals and is the major cause of gastroenteritis in both developed and developing countries.[27] An estimated 2.1 to 2.4 million cases of human campylobacteriosis is reported every year in the US making *C. jejuni* the most common bacterial foodborne pathogen causing illness.[28,29] Therefore, developing a detection platform for *C. jejuni* is of prime importance for food and water safety monitoring. The RBP employed here was specifically expressed as a glutathione-S-transferase (GST) Gp48 fusion protein. The GST tag was utilized for the oriented immobilization of the proteins onto surfaces using glutathione SAM. Such RBP-functionalized beads could be used as part of a pre-concentration strategy aimed to extract the target pathogen from complex food matrices. Use of phage RBPs would leverage the natural resilience of phage-based technologies when compared to antibodies.

In this study, initial assessments of capture specificity were performed using surface plasmon resonance. These RBPs were then immobilized onto capturing beads, which were then successfully used for the specific capture and pre-concentration of the pathogen from solution. Pre-concentration is indeed an important step in the analysis of cells (or metabolites) in large food samples.[30] Biosensor platforms use a very low sample volume and thus cells from food samples have to be concentrated to low volumes for their analysis. Our results therefore show that bacteriophage RBPs can be leveraged in both pre-concentration and detection steps for the monitoring of pathogenic bacteria.

Experimental

Materials

Dithiobis(succinimidyl propionate) (DTSP), glutathione (reduced) (GSH) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA) and were used as received. SPR gold chips were purchased from GWC technologies, USA and were washed sequentially in acetone, isopropanol, ethanol and MilliQ® water for 5 min each prior to use. Phosphate buffered saline (PBS) solution was prepared by mixing one BupH phosphate buffered saline pack to 500 ml of MilliQ® water yielding a solution of 0.1 M phosphate and 0.15 M NaCl (pH 7.2). PBS wasfurther diluted to 0.01 M before use. Tween-20 was obtained from MP Biomedicals, Inc. (OH, USA). Bacteria were fluorescently labeled using SYTO 12 BC stain purchased from Invitrogen (Carlsbad, CA, USA).

Tosyl-activated Dynabeads® M-280 was purchased from Invitrogen, US and was washed in PBS prior to their use. These magnetic beads are 2.8 μm in size and have been pre-functionalized to actively bind to proteins/peptides. The concentration of the bead solution was 2×10^9 beads/ml in the supplied suspension, which was diluted to a working concentration of 10^6 beads/ml for all experiments. All other solvents, chemicals and reagents were analytical grade and were used without further purification.

The gold substrates were fabricated using piranha cleaned 3″silicon (1 0 0) substrate by sputtering a 5 nm thick chrome adhesion layer followed by 25 nm thick gold layer. The sputtered substrates were diced into 5 mm × 7 mm rectangular chips using a dicing saw machine (Disco DAD 321). The chips were sonicated in acetone for 10 min (Branson® Ultrasonics 1510, 40 khz frequency) followed by subsequent washes in isopropanol, ethanol and MilliQ® water for 5 min each prior to their use.

Luria-Bertani (LB) medium was purchased from Quelabs (Montreal, QC, Canada) and was prepared by dissolving 25 g of the LB powder in 1 l of distilled water. LB-agar medium was prepared by adding 6 g of agar in 400 ml of LB media. Nutrient broth (NB) powder was purchased from Difco™ (MD, USA) and was prepared by dissolving 8 g powder in 1 l of distilled water.

Expression and purification of GST-Gp48 and GST proteins

The details of the production of the RBPs of bacteriophage NCTC 12673 has been described elsewhere.[26] Briefly, Gene 48 was amplified from the *C. jejuni* bacteriophage NCTC 12673 DNA (GeneBank ID: GU296433) using PCR. The appropriate PCR product was ligated into a *Sma*I (Fermentas) cut pGEX 6P-2 vector (GE Healthcare). This plasmid encodes the N-terminal glutathione-S-transferase (GST) tag and was used to produce a GST-Gp48 fusion protein. Overnight blunt ligation was performed at room temperature in the presence of *Sma*I. The correct orientation and product integrity were confirmed by sequencing of the insert (Molecular Biology Services Unit, University of Alberta). GST-Gp48 protein was expressed in *Escherichia coli* BL21 cells (Invitrogen) transformed with the pGEX 6P-2 plasmid containing gene *48* and purified as a soluble 170 kDa protein according to Kropinski *et al.*[26] Cells were grown at 30 °C to an $OD_{600} = 0.5$, induced with 0.1 mM IPTG and incubated overnight at 30 °C with shaking at 150 rpm. Cells were harvested, resuspended in PBS with 1 mM DTT and disrupted by sonication (Branson Sonifier S450A, 5 pulses of 2 min at 20% power) in the presence of 5 mM EDTA (Fermentas) and ProteoBlock protease inhibitor cocktail (Fermentas). Cell debris was removed by centrifugation at 27000 g for 30 min, soluble fraction was filtered through the 0.22.m filter (Millipore) and subjected to glutathione-agarose affinity chromatography (Sigma-Aldrich) according to manufacturer's instructions. Elution buffer contained PBS with 10 mM reduced glutathione (Sigma-Aldrich) and its pH was adjusted to 8.0 using NaOH. The resulting GST-Gp48 protein was additionally purified by ion-exchange chromatography using MonoQ (GE Healthcare) column and AKTA Explorer FPLC system (GE Healthcare). Linear gradient of 0–500 mM NaCl in 50 mM potassium phosphate (pH 8.0) was used; the major peak was collected and dialyzed against PBS. The protein concentration was determined by measuring the absorbance of protein solution at 260 and 280 nm. GST was also expressed and purified as a separate protein to be used in a control experiment. pGEX 6P-2 vector without an insert was used for this purpose. All other expression and purification procedures were the same as described above for GST-Gp48 protein.

Preparation of bacterial cultures

Campylobacter jejuni subsp. *jejuni* strain 11168H from the National Collection of Type Cultures (NCTC, Health Protection Agency Culture Collections, UK) was subcultured onto Mueller-Hinton (Difco) agar plates for 18 h at 37 °C under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2). Cells were removed from the surface of agar plates, resuspended in PBS buffer, washed three times with PBS and used in GST-Gp48 binding experiments. *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 19585 from the American Type Culture Collection (ATCC, USA) was grown by streaking onto a Nutrient agar plate and incubated overnight at 37 °C. Single colonies from the plate were inoculated into 3 ml LB media and were grown overnight at 37 °C in a shaker to obtain an overnight bacterial culture. Then, 1 ml aliquots of bacterial culture were centrifuged in a bench-top Eppendorf centrifuge at 13000 rpm for 1 min and cells were resuspended in 1 ml PBS for future use in binding experiments.

Immobilization of the GST-Gp48 protein onto gold surface

All the immobilization steps described have been performed at room temperature unless a different temperature condition has been stated. All gold substrates used for the experiments were sonicated sequentially in acetone, isopropyl alcohol, ethanol and water for 5 min each to clean the surface prior to use. The washed gold substrates were incubated in a 2 mg ml^{-1} solution of glutathione in PBS or 2 mg ml^{-1} solution of DTSP in acetone for 1 h on an orbital shaker at 1000 rpm. The GSH SAM substrates were washed twice in PBS for 5 min each to wash away excess reagent from the surface. The DTSP SAM substrates were washed in acetone, isopropanol, ethanol and PBS for 5 min each. The functionalized substrates were incubated in a 5 μg ml^{-1} solution of GST-Gp48 protein in PBS for 1 h on an orbital shaker at 1000 rpm to ensure homogenous mixing. The protein-functionalized substrates were washed with 0.05% Tween-20 PBS solution for 5 min followed by two washes in PBS for 5 min each. The DTSP-protein sample was also washed in 10% ethanolamine for 10 min to block all the free succinimidyl groups. The thoroughly washed substrates were incubated in a 1mg ml^{-1} solution of BSA in PBS for 1 h to block any free substrate surfaces. The BSA blocked surface was washed with PBS twice for 5 min each and these substrates were used for bacterial capture studies.

Analysis of bacterial binding on GST-Gp48 immobilized surfaces

The protein-immobilized substrates were exposed to host or non-host bacteria (10^9, 10^8 and 10^7 cfu/mL) for 20 min. The substrates were subsequently washed with 0.05% PBS Tween-20 for 5 min followed by 2 similar washes in PBS for 5 min each. The substrates were fixed with a 2% solution of glutaraldehyde in MilliQ® water for 1 h. The fixed substrates were washed twice with MilliQ® water for 5 min followed by sequential incubation in 60, 70, 80, 90 and 100% ethanol for 5 min each. Finally, the substrates were dried overnight at 42 °C and imaged using the Hitachi S-4800 scanning electron microscope (SEM). The bacterial binding density was enumerated from the SEM images using the cell counter plugin of ImageJ software (NIH, USA). The standard deviation indicated for each data set was obtained from counting 8 images for each experiment.

SPR spectroscopic analysis

SPR analysis was performed on gold-coated SF-10 glass substrates to study real time functionalization and bacterial capture. The surface functionalization method was duplicated on the SPR substrates to obtain protein-immobilized substrates, which were used for concentration dependent bacterial capture studies. The RBP immobilized substrates were dried under filtered N_2 flow and loaded into the flow chamber with a prism assembly for continuous monitoring of binding events in the form of SPR spectra. SPR studies were carried out on a SPR instrument from GWC technologies (USA) in the Kretschmann configuration. The SPR instrument was operated at a wavelength of 830 nm. Refractive index matching solution ($n = 1.72$) was used for making contact between the SPR chip and prism. The SPR instrument was calibrated with different concentrations of ethanol in water (data not shown). It was observed that a change of 9 SPR pixels corresponds to a change of 0.006 in the refractive index of the surface.

The baseline for the RBP immobilized substrates was established by flowing PBS for 300 s. Following this, the desired concentration of host or non-host bacteria in PBS was introduced into the SPR flow cell at the speed of 100 μl/min. The flow of the bacteria was carried out for 25 min. The SPR flow cell was again flushed with PBS to remove any loosely bound or unbound bacteria. The change of SPR angle with changing concentration of bacteria indicates its interaction with the RBPs on the substrate's surface. All the washing steps during SPR analysis were carried out at a flow rate of 200 μl/min.

Immobilization of RBPs on magnetic beads

10^6 tosyl-activated beads were suspended in PBS and washed twice with cycles of centrifugation at 5000 rpm prior to their use. The beads were then incubated with 5 μg ml^{-1} of the GST-Gp48 protein for 3 h to facilitate protein immobilization. The protein-functionalized beads were centrifuged down at 5000 rpm for 1 min followed by two washes in PBS for 5 min each to remove excess/unbound protein. These beads were further incubated in BSA for 30 min to block any free surfaces and prevent any nonspecific binding of the bacteria. The beads were then centrifuged down at 5000 rpm and were again washed twice in PBS for 5 min each. The 10^9 cfu/ml host as well as non-host bacteria suspended in PBS were stained with 1 μl ml^{-1} concentration of SYTO 12 green fluorescent dye for 5 min. The stained bacteria were washed twice in PBS resuspended in PBS prior to their exposure to the RBP functionalized beads. The beads were finally incubated with stained 10^9 cfu/ml bacteria for 20 min to facilitate capture. The beads were centrifuged at a lower speed of 2000 rpm to pellet only the beads but not the free bacteria. They were washed twice in PBS for 5 min each prior to their analysis. All centrifugation steps were performed using a bench-top Eppendorf centrifuge. An Olympus IX81 microscope (Tokyo, Japan) equipped with a FITC filter and a Roper Scientific Cool-Snaps HQ CCD camera (Duluth, GA, USA) was used to record the fluorescence images of the captured bacteria.

Results and discussion

Bacterial capture studies by the GST-Gp48 protein on gold substrates

DTSP SAM-based unoriented as well as GSH SAM-based oriented protein immobilized gold substrates were checked for host bacterial capture efficiency. Various events in the RBP immobilization and subsequent bacterial capture are shown schematically (Fig. 1). The GSH SAM based immobilization leverages the GST tag of the GST-Gp48 protein and thus provides a definite orientation of the protein on the surface (Fig. 1, top panel). On the contrary, the succinimidyl group of DTSP SAM reacts with free amine ($-NH_2$) groups of the GST-Gp48 fusion protein and therefore results in its random binding on the surface as illustrated in Fig. 1 (bottom panel). Both approaches were checked for their bacterial capture efficiency. All the other immobilization parameters in the two approaches were kept constant and the bacterial capture was enumerated using scanning electron microscopy images.

Surfaces functionalized with the two different approaches were exposed to 10^9 cfu/ml of the *C. jejuni* host in PBS for 20 min. Fig. 2A and 2B show scanning electron micrographs of the resulting surfaces. It is clear that the GST SAM- as well as DTSP SAM-based GST-Gp48 immobilized protein result in a very high density of captured bacteria, which are too numerous to count (TNTC). The SEM images show a uniform coverage of host bacteria on the surface of the RBP functionalized substrates. It was also observed that the bacteria tend to agglomerate at this concentration, which would result in erroneous capture density calculations. However, high magnification images give a visual indication that the GSH SAM-based protein-immobilized surface (Fig. 2C) captures a higher bacterial density than the DTSP SAM-based protein surface (Fig. 2D). The two functionalization methods were thus tested with lower bacterial concentrations to obtain a quantitative estimate of capture. The GSH and DTSP SAM-based protein functionalized surfaces were exposed to 10^8 and 10^7 cfu/ml concentration of host bacteria in PBS. The DTSP SAM-based protein immobilized surfaces gave a capture density of 3.51 ± 0.21 and 1.39 ± 0.34 bacteria/100 μm^2 respectively for 10^8 and 10^7 cfu/ml concentration of bacteria. In contrast, we obtained capture densities of 7.58 ± 0.28 and 3.39 ± 0.36 bacteria/100 μm2 on exposure of 10^8 and 10^7 cfu/ml concentration of bacteria respectively on the GSH SAM-based protein immobilized surfaces. Thus, there is a 2-fold and 3-fold increase in capture for the two different bacterial concentrations using an oriented immobilization of the RBP. We therefore concluded that RBP protein immobilization using GST tags was more suitable for developing a biosensor platform for *C. jejuni* detection and thus this method was extended to SPR substrates for all further experiments.

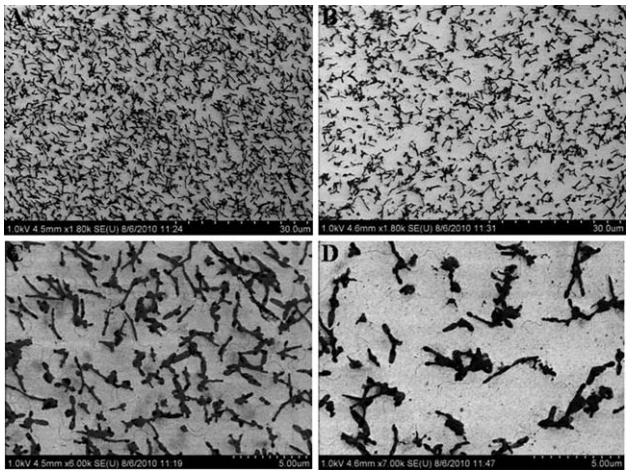


Fig. 2 Representative SEM images of Campylobacter jejuni capture by immobilized Gp48 (A) GSH SAM based GSTGp48 immobilization and (B) DTSP SAM based GST-Gp48 immobilization. The high magnification images show a marked difference in host bacterial capture for the (C) GSH SAM-GST-Gp48 and (D) DTSP SAM-GST-Gp48 sample.

The selectivity of recognition was checked using different controls. Bovine serum albumin (BSA) was used as a protective layer to prevent any non-specific attachment of the bacteria to the substrate surface. *Salmonella* was used as a non-specific bacterial control and was captured at densities of 0.45 ± 0.12 and 0.63 ± 0.24 bacteria/100 μm^2 for the unoriented and oriented protein surface respectively. This confirms that the recognition of the Gp48 protein is specific toward its host bacteria. The protein-functionalized surfaces were also checked for any non-specific binding of the bacteria alone. BSA protected bare gold surfaces, BSA protected GSH SAM surfaces and BSA protected GSH-GST surfaces showed a capture density of 0.25 ± 0.05, 0.05 ± 0.02 and

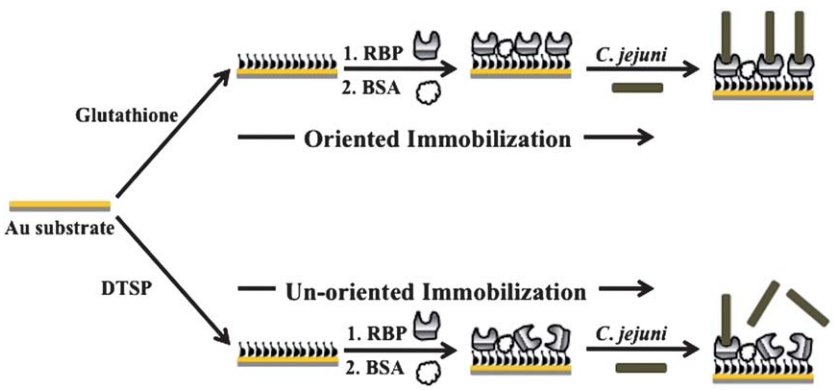


Fig. 1 Schematic showing the various surface functionalization steps and bacterial capture on the substrate's surface. The top panel shows events in oriented immobilization of GST-Gp48 protein using GSH SAM while the lower panel shows an unoriented immobilization.

0.11 ± 0.01 bacteria/100 μm^2 respectively when exposed to the 10^9cfu/ml concentration of host bacteria in PBS. The controls indicate that there is insignificant binding of the host bacteria to the BSA protected surfaces in the absence of the Gp48 protein. Thus, BSA protective layers efficiently prevent any non-specific interaction of the host bacteria to the surface and the observed bacterial binding in the presence of the RBPs is solely due to their specific recognition ability. All the bacterial capture densities are listed in Fig. 3 along with the SEM images of the control experiments.

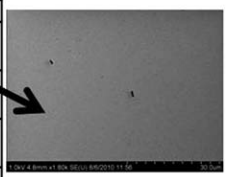
Real time SPR analysis of functionalization events and bacterial capture

All the surface functionalization steps were monitored in real time at room temperature using SPR. The SPR sensorgram for the process is shown in Fig. 4A. All experiment samples were injected into the chamber at a flow rate of 100 μl min^{-1} while all washing steps were performed at a flow rate of 200 μl/min. The baseline for the gold substrate was recorded by flowing PBS for 300 s. Following this, a 2 mg ml^{-1} solution of glutathione-PBS was injected into the SPR chamber to facilitate GSH SAM formation on the gold substrate. It was observed that SAM formation is achieved in 600 s following which the PBS was flushed in for 300 s to wash away the excess of glutathione from the surface. The SPR intensity decreases from 15 pixels to approximately 4 pixels during the wash suggesting that a large amount of loosely bound glutathione is removed from the surface. A 5 μg ml^{-1} solution of GST-Gp48 protein in PBS was then put into the SPR chamber to allow its immobilization onto the surface. The protein immobilization curve saturates in 2500 s with a change in SPR intensity by 60 pixels. The surface was again flushed with PBS for 300 s, which did not show any change in the SPR intensity confirming that all the protein binds specifically to the glutathione monolayer and that its interaction to the bare gold surface was negligible. Then, 1 mg ml^{-1} solution of BSA in PBS was put into the chamber to block the free gold surface to prevent any non-specific interaction with the bacteria. The BSA signal reaches an equilibrium in 400 s with an intensity change of 52 pixels and then decreases sharply in the subsequent washing step with PBS. We attribute the sharp increase and decrease in signals due to the difference in refractive index between a 1 mg mL^{-1} solution of BSA and the PBS solution. In addition, some weakly bound BSA is likely removed by the PBS rinse resulting in an overall signal increase of 15 pixels.

A 10^9 cfu/ml concentration of host *C. jejuni* was then injected into the chamber to allow its capture onto the surface. We observed a rapid increase in the SPR intensity, which indicated the binding of the bacteria to the immobilized Gp48 protein. The bacterial binding curve saturates in nearly 20 min with an increase in the SPR intensity of approximately 35 pixels. The surface was finally washed again by flowing PBS, which showed a slight decrease in the SPR signal intensity due to removal of any loosely bound bacteria on the surface. The results from this study indicate that the bacterial binding requires approximately 20 min to saturate and the same time scale was followed for the subsequent studies.

SPR analysis of capture specificity and sensitivity

The Gp48 protein immobilized SPR substrates were tested for their sensitivity towards the host bacteria. Fig. 4B shows the sensorgrams for the capture of bacteria for a number of different concentrations. The baseline for the substrates was established by flowing PBS solution for 300 s at a speed of 100 μl/min. Following this, specific concentrations of host bacteria in PBS were injected into the SPR chamber for 1500 s to allow them to bind to the substrate. The substrates were finally flushed with PBS at a flow rate of 200 μl min^{-1} to remove any loosely bound/unbound bacteria from the surface. The double arrow dotted line in Fig. 4B shows the time when PBS was injected into the SPR chamber. The washing results in a small decrease in the



Cell type	Surface type	Cells per 100 μm2
C. jejuni (10^9cfu/mL)	Au/BSA	0.25 ± 0.05
C. jejuni (10^9cfu/mL)	Au/GSH/BSA	0.05 ± 0.02
C. jejuni (10^9cfu/mL)	Au/DTSP/GST-Gp48	TNTC[1]
C. jejuni (10^9cfu/mL)	Au/GSH/GST-Gp48	TNTC
C. jejuni (10^8cfu/mL)	Au/DTSP/GST-Gp48	3.51 ± 0.21
C. jejuni (10^8cfu/mL)	Au/GSH/GST-Gp48	7.58 ± 0.28
C. jejuni (10^7cfu/mL)	Au/DTSP/GST-Gp48	1.39 ± 0.34
C. jejuni (10^7cfu/mL)	Au/GSH/GST-Gp48	3.39 ± 0.36
S. Typhimurium (10^9cfu/mL)	Au/DTSP/GST-Gp48	0.63 ± 0.24
S. Typhimurium (10^9cfu/mL)	Au/GSH/GST-Gp48	0.45 ± 0.12
C. jejuni (10^9cfu/mL)	Au/GST	0.11 ± 0.01

Fig. 3 The table shows the enumerated bacterial density for different samples and control experiments. The respective SEM images for control experiments are shown and indicated by the arrows. [1]TNTC = Too numerous to count.

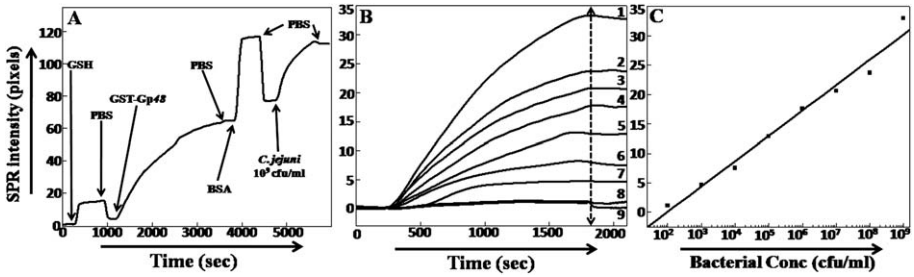


Fig. 4 SPR analysis of bacterial binding to Gp48 functionalized substrates (A) The SPR curve showing intensity changes during various surface functionalization steps, protein immobilization and subsequent host bacterial capture on gold-coated SF-10 glass substrates. (B) SPR curves showing the intensity change when the GSH SAM-GST-Gp48 substrates were exposed to different concentrations of the host bacteria. Curves 1–8 show intensity change response for C. jejuni concentrations of 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu/ml, respectively while curve 9 corresponds to the change on exposure to a 10^9 cfu/ml concentration of the Salmonella control. (C) Plot showing the SPR response *vs.* log bacterial concentration (cfu/ml).

SPR intensity signal suggesting that most of the bacteria are tightly bound to the Gp48 protein on the surface. A 10^9 cfu/ml concentration of bacteria gave an SPR intensity change of 33 pixels which matches well with the change we observed previously (Fig. 4A). We also studied the capture using 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu/ml concentrations of host bacteria in PBS. The SPR intensity increase was recorded as 23.69, 20.65, 17.59, 12.85, 7.53, 4.69 and 1.16 respectively (Fig. 4 B, Curves 2–8). The result shows a clear dependence of the signal intensity on the concentration of the host bacteria. We were able to obtain a distinguishable signal for bacterial concentrations as low as 10^2 cfu/ml. The Gp48 modified SPR substrate was also exposed to 10^9 cfu/ml concentration of non-host bacteria (Fig. 4B, Curve 9). The non-host bacteria shows a slight increase in the SPR intensity equivalent to 1.15 pixels which was similar to the signal obtained from exposing the substrates to 10^2 cfu/ml concentration of the host bacteria (Fig. 4B, Curve 8). However, the washing of control sample removes nearly all the non-host bacteria that is non-specifically attached to the surface, which decreased the SPR signal back to the baseline level. This experiment validates the fact that the recognition offered by the Gp48 RBP is highly specific and selective. Thus, bacteriophage RBPs could be used as an excellent probe for sensitive and selective detection of *C. jejuni*.

Fig. 4C shows a plot of the SPR signal changes on exposure of RBP modified substrates to host bacteria of different concentration. The SPR signal plotted against the log value of the host bacterial concentration shows an increase in signal with increasing concentration of bacteria as seen from the response curves (Fig. 4B). The SPR response change could be fitted into a straight line ($R^2 = 0.99$) and thus can be directly correlated to the concentration of the bacteria.

Bacterial binding analysis using capturing beads

We have also demonstrated that tosyl-activated Dynabeads® M-280 could be functionalized with the RBPs to facilitate specific host bacterial capture for subsequent pre-concentration using external magnetic fields. *Salmonella* was used as a control to demonstrate specificity of bacterial capture by the beads. The bacterial capture analysis was performed using fluorescence microscopy and the host as well as non-host bacteria were stained using SYTO 12 BC green fluorescent dye prior to imaging. The RBP functionalized BSA protected beads were exposed to stained host bacteria and analyzed under the microscope to confirm the capture of bacteria on the surface (Fig. 5A). The fluorescence image shows that the host bacteria were captured successfully on the beads surface and each fluorescent dot on the surface corresponds to one captured bacterium. We also observed a strong aggregation of the beads after exposure to the host bacteria. This was expected since one bacterium could bind to more than one bead simultaneously, resulting in the aggregation observed in the fluorescence image. The beads themselves were clearly seen under the fluorescence microscope, which could be either due to some interaction of the fluorescence dye with the bead's surface or due to auto-fluorescence. The magnetic beads indeed show auto-fluorescence and it was confirmed by observing them under the fluorescence microscope without exposure to any fluorescent dye (data not shown).

As a control, the exposure of RBP functionalized beads to non-host *Salmonella* does not show any bacterial capture (Fig. 5B). However, we do observe some aggregation of the particles in this sample as well which could have resulted due to cross binding of the GST-Gp48 protein or BSA during the surface functionalization process. However, the extent of aggregation was much less compared to what was observed on exposure to the host bacteria (Fig. 5A). We therefore successfully demonstrated that the RBPs could be exploited to specifically and selectively capture host bacteria onto magnetic beads that can be concentrated by applying an external magnetic field. This would enable us to pre-concentrate low bacterial loads from a food sample for subsequent detection. These preliminary results are being followed up with detailed studies of the application of these magnetic beads in pre-concentrating the host

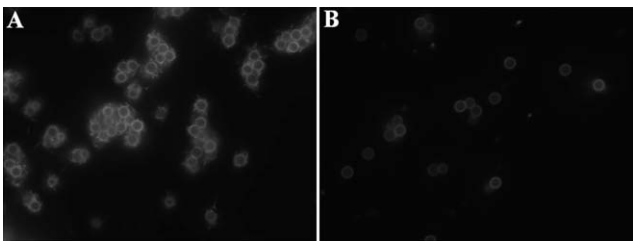


Fig. 5 Fluorescence image of bacterial capture by Gp48 functionalized beads (A) C. jejuni bacteria captured on the GST-Gp48 protein immobilized magnetic beads (B) exposure of GST-Gp48 protein immobilized magnetic beads to control Salmonella bacteria does not show any binding. All images were recorded at 160X magnification.

bacteria from a sample source for eventual detection using appropriate transducing platforms.

Conclusions

We have demonstrated that genetically engineered receptor binding protein, Gp48, of NCTC 12673 phage could be exploited as a molecular probe for the sensitive and selective detection of *C. jejuni* on gold surface based transduction platforms. The present study demonstrates the applicability of using Gp48 in SPR based bacterial detection but could be easily extended to any other biosensor detection platform. The Gp48 protein was expressed as a fusion with a GST tag to facilitate its purification. We have successfully shown that this tag can also be exploited to immobilize the fusion protein onto a GSH SAM coated surface. In addition, our results suggest that the GST tag based immobilization of Gp48 protein provides a preferred orientation on the surface which improves the subsequent host bacterial capture compared to DTSP SAM based random orientation. Bovine serum albumin was used as a surface protective layer and a series of control experiments using BSA protected gold surfaces, BSA protected GSH SAM surfaces and BSA protected GSH SAM-GST surfaces shows negligible binding of the host bacteria in the absence of Gp48 protein. Additionally, *Salmonella* was used as a negative control, which showed insignificant bacterial binding on the Gp48 immobilized surface.

The GSH SAM based immobilization methodology was extended onto gold coated SF10 glass substrates and the real time surface functionalization and bacterial capture was studied using SPR. RBP functionalized SPR substrates were subsequently used to demonstrate a sensitive and selective detection of host *C. jejuni* at concentrations as low as 10^2 cfu/ml. We also demonstrate that the RBPs could be successfully immobilized onto magnetic beads and could be exploited to pre-concentrate the bacteria from a large sample volume. We aim to pursue this work and extend it into real food samples in the future. Bacteriophage receptor binding proteins thus show tremendous promise as biological probes to develop a robust and stable biosensor platform for pathogen detection.

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