Published in final edited form as:
Langmuir. 2012 January 17; 28(2): 1351–1359. doi:10.1021/la203348j.

Capture efficiency of *Escherichia coli* in fimbriae-mediated immunoimmobilization

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Abstract

Capturing pathogens on a sensor surface is one of the most important steps in the design of a biosensor. The efficiency of a biosensor at capturing pathogens has direct bearing on its sensitivity. In this work we investigated the capturing of *Escherichia coli* on substrates modified with antibodies targeting different types of fimbriae: K88ab (F4), K88ac (F4), K99 (F5), 987P (F6), F41 and CFA/I. The results suggest that all these fimbriae can be used for the efficient immobilization of living *E. coli* cells. The immobilization efficiency was affected by the purity and clone type of the antibody and the fimbriae expression level of the bacteria. For a specific fimbriae type, a higher immobilization efficiency was often observed with the monoclonal antibodies. Immunoimmobilization was utilized in an antibody microarray immersed in a mixed culture of pathogens to demonstrate the rapid and simultaneous label-free detection of multiple pathogens within less than an hour using a single test. The capture rate of living pathogens exceeds a single bacterium per 100×100 μm^2 area per half an hour of incubation for a bulk concentration of 10^5 cfu/ml.

Introduction

Bacterial pathogens are generally detected using either a polymerase chain reaction (PCR) or antibody-based techniques [1]. The PCR approach offers the accurate determination of pathogens at the genomic level, but requires a proper design of primers targeting specific genes [2]. Antibody-based techniques usually involve two events: capturing of the targeted pathogen on the sensor surface and follow-up signal generation. Efficient capturing is always desired, since it will facilitate converting captured pathogens into a detectable signal and, most importantly, a higher capture efficiency will result in a higher sensitivity (lower detection limit). Extensive research has been reported on the development of new detection methods that involve converting an already captured pathogen into an output signal by optical, electrochemical, mechanical or other means [3]. However, there has been little study of how to enhance the capture efficiency. One such example is the work by Rivas et al. evaluating the binding capacity and cross-reactivity of 200 different antibodies for the detection of environmental toxins [4]. In this work we focus on the factors in selecting antibodies that affect the efficiency of capturing living *E. coli* cells expressing different types of fimbriae.

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Supporting Information
Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

During the past decade considerable advances were made in detecting pathogens by coupling immunological techniques with chemical and electronic actuators based on chemoluminescence [5], electrochemical impedance [6], surface plasmon resonance (SPR) [7], quartz crystal microbalance (QCM) [8] and wave guides [8]. The majority of these techniques rely on the capture of pathogens on a substrate modified with antibodies raised against target pathogens. However, many researchers paid little attention to how the choice of antibodies affects the efficiency with which the pathogens are captured. Usually the antibodies used to capture pathogens are polyclonal antibodies raised against *whole* pathogens, such as anti-*E. coli*. Such antibodies in practice often provide a very low sensing efficiency for living bacteria, since only a small fraction of the immunoglobulins are specific against the bacterial surface antigens, while a large proportion of the immunoglobulins target the interior antigens, which are located within the interior regions of the bacterial cell and hence cannot be exploited for capturing living cells. In principle, the sensitivity of the biosensors in these reports could be enhanced by simply employing a specific antibody raised against a surface antigen.

Recently we demonstrated the immunoimmobilization of living bacterial cells through specific interactions between bacterial surface antigens and corresponding antibodies [9, 10]. Immobilized bacterial cells can easily be imaged on silicon or glass substrate without a need for labeling, making immunoimmobilization a label-free detection method. Various bacterial surface antigens can be used for the immobilization of living bacterial cells. Among the common surface antigens, fimbriae are particularly suitable candidates for immunoimmobilization because they protrude from the bacterial surface and do not undergo rotary motion as do flagella. Fimbriae are common bacterial surface antigens associated with many pathogenic bacteria. Some common fimbriae types identified for enterotoxigenic *E. coli* (ETEC) strains include K88 (F4) [11], 987P (F6) [12], K99 (F5) [13], F41 [14] and CFA/I [15]. The rapid identification of fimbriae type could assist the evaluation of potential threats caused by unknown pathogens. In this work we extend our investigation to wild-type *E. coli* strains expressing distinct types of fimbriae, and the results suggest that all the tested fimbriae types could lead to the efficient immobilization of living *E. coli* cells.

Experimental

Bacteria

Wild-type strains of *E. coli* were obtained from Dr. D. Francis at South Dakota State University, the *E. coli* Reference Center at Pennsylvania State University and Montana State University. The fimbriae type and relevant properties of these strains are listed below.

Two wild-type strains (H10407 and 3030-2) were genetically modified to express fluorescence proteins for the microarray experiment. Wild-type ETEC strain H10407 was transformed with plasmid pDsRed-Express (Clontech, Mountain View, CA) for the expression of red fluorescence protein. The fluorescent strain was named H10407-pDsRed. Wild-type *E. coli* strain 3030-2 was transformed with plasmid pQGgfp (laboratory construction) for the expression of green fluorescence protein, and the fluorescent strain was named 3030-2-GFP. The construction of H681-K99 has been described previously [16]. Strain 3.1012 was stained using a fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI). Cells were initially incubated in LB media, then pelleted by centrifuge from a 1-ml culture with a cell density of ~5×10^8 colony-forming units (cfu)/ml, re-suspended in 1 ml of PBS and stained with DAPI at a final concentration of 50 μg/ml for 15 min at room temperature. After the excess dye was washed off using PBS, the stained cells were mixed with other fluorescent cells and used for microarray experiments. The repeated washing of stained cells by centrifuge should be avoided, since the shear-force caused by the centrifuge could remove the fimbriae and thus reduce the immobilization efficiency.

All the bacterial species were obtained from frozen bacteria stock kept at −80°C on a Lysogeny broth (LB) plate and incubated at 37°C overnight. An LB liquid medium without antibiotics was then inoculated with the bacteria and shaken at 125 rpm at 37°C. The bacterial cells were harvested when the culture optical density at 600 nm (OD_{600}) reached 0.5–0.6, which corresponds to a CFU value of ~5×10^8 per ml.

Antibodies

All the antibodies used for this work are G-type immunoglobins (IgG). Antibodies against *E. coli* fimbriae were either prepared in our lab or purchased commercially (Novus Biologicals LLC, Littleton, CO; Abcam, Cambridge, MA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Veterinary Laboratories Agency (VLA), UK). All the polyclonal antibodies were further purified using a column filled with protein-G-modified resin to remove the non-immunoglobin serum proteins. For monoclonal antibodies this purification was omitted since they had been purified by the manufacturers. The antibodies were diluted with PBS buffer to concentrations between 10 μg/ml and 20 μg/ml for the modification of silicon or glass substrates with a full monolayer of antibody.

The polyclonal anti-CFA/I was prepared by immunizing a rabbit intramuscularly (i.m.) with purified CFA/I fimbriae proteins. Four weeks post immunization the rabbit was bled to varify for serum anti-CFA/I titers using ELISA. The serum was further purified using a protein G column to remove the non-immunoglobulin serum protein. The polyclonal anti-K99 was prepared similarly.

Modification of silicon substrate with antibodies

Silicon substrates were used for majority of the experiments. Briefly, pre-cleaned silicon chips were soaked in a solution of APTES in methanol (2%) for 15 min, followed by further incubation in a solution of BMPS in anhydrous acetonitrile (10 mM) for 30 min. Antibody solutions were deposited onto these activated substrates as small droplets (each droplet ~1 μl in volume) using a micropipette. The chips with antibody droplets were kept in a humidity chamber for ~1 hr at room temperature to allow the covalent linking of the antibody to the substrate. The excess antibody molecules on the chip surface were washed off with a copious amount of PBS buffer before inoculation with bacteria. Each chemical procedure associated with the modification of silicon substrate was verified by X-ray photoemission spectroscopy as described in our previous publication [10]. We found that an Ab concentration above a minimum level has little effect on immobilization efficiency, as long as the concentration is enough to form a monolayer of Ab on the substrate. This is understandable, because once the substrate surface is covered by a monolayer of antibody, the excess antibody molecules will be rinsed away by PBS buffer in the rinsing step. Our experiments show that an antibody concentration of ~6–14 μg/ml is suitable in most cases.

Immunoimmobilization of living E. coli

The immunoimmobilization of living bacteria followed the procedure reported earlier [9]. Detailed procedure was provided in the Supplementary Data.

Preparation of antibody microarray

The antibody microarray on glass slides was prepared using a microplotter (Bio-Rad, VersArray Chipwriter Compact System). Details are presented in the Supplementary Data.

Detection of multiple strains using an antibody microarray

An antibody microarray prepared above was incubated with a mixed culture of H10407-pDsRed, 3030-2-GFP and DAPI-stained 3.1012 for ~45 min under ambient conditions. The

excess and loosely attached cells were rinsed off using PBS buffer, and the microarray with living cells was observed using an epifluorescence microscope. In most experiments the three strains were mixed at an equal ratio to demonstrate the simultaneous detection of multiple bacterial species. In some experiments the strains were mixed at different relative ratios to study how the immobilization efficiency was affected by the cell concentrations in the mixed culture.

Results and discussion

Fimbriae expression

The expression of bacterial appendages such as fimbriae and flagella is usually investigated using electrophoresis-based methods (such as DSD-page and immunoblotting) and transmission electron microscopy (TEM) [25]. Recently AFM has been successfully applied to studying the structure of such filamentary appendages because it requires minimal sample preparation, has high spatial resolution, provides highly sensitive force measurement and has the versatility to be coupled with other analytical techniques [26–28]. The nine *E. coli* strains used in this work were imaged using an AFM system (Figure 1), and the expression of fimbriae was revealed unambiguously. For example, the K88ab and K88ac fimbriae appeared to be curly, while the 987P, F41, K99 and CFA/I fimbriae showed a rod-like morphology. The AFM imaging suggests that the expression of the fimbriae for 9.1360 is weak. Although K99 fimbriae were expressed for three strains, H681-K99, B41 and 9.1360, they were not visible for most 9.1360 cells imaged because of their low expression levels.

Antibody specificity and cross-reactivity

In our previous work, *S*. Typhimurium mutants were constructed to express CFA/I fimbriae as a model for studying immunoimmobilization [9, 10]. These *S*. Typhimurium mutants showed satisfactory immobilization efficiency through an interaction between CFA/I antigen (Ag) and the corresponding antibody (Ab). CFA/I is a native surface antigen that has been identified for many ETEC isolates [29], and this type of fimbriae is not expressed in wild types of *S.* Typhimurium. In this work we tested the immobilization efficiency of this Ab-Ag interaction for wild-type ETEC strain H10407, and efficient immobilization was observed, as shown in Figure 2A. Besides CFA/I, all the other fimbriae types tested in this work can lead efficient immunoimmobilization when the corresponding antibody was used. The results are shown in Figures 2,3&4. A dense monolayer of living cells was immobilized on silicon substrates by the following strain-antibody pairs: strain 3.1012 and anti-987P (Figure 2B), strain 263 and anti-K88ab (Figure 3A), strains 1836-2 and 3030-2 and anti-K88ac (Figures 3E&F), strain H681-K99 and anti-K99 (Figures 4A&E), and strain 2.0961 and anti-F41 (Figure 4D). Strain B41 expresses both K99 and F41 fimbriae, and its immunoimmobilization can be achieved with either Ab-Ag pair, as shown in Figures 4B,C&F.

A particular advantage of immunoimmobilization is that the specificity of Ab-Ag interactions ensures that a given bacterial strain can only be immobilized by antibodies against its surface antigens, and irrelevant antibodies will not be able to provide such immobilization. This is evidenced by an example shown in Figure 2C, in which a silicon substrate modified with anti-K99 showed no immobilization for 3.1012, a 987P expressing strain. While such Ab specific immobilization was commonly observed in our experiments for most tested strains, bacteria immobilization through non-corresponding antibodies did also occur, presumably because of antibody cross-reactivity. For example, strain 263 expressing K88ab fimbriae and strains 1836-2 and 3030-2 expressing K88ac fimbriae can be immobilized with either anti-K88ab or anti-K88ac with almost equal efficiency (Figure 3). The cross-reactivity in this case is most likely due to the limited specificity associated with

polyclonal antibodies (pAb), because different subtypes of K88 fimbriae share great similarity among their structures [11, 30, 31]. Similar cross-reactivity is also observed between pAb against CFA/I and strains expressing K99, H681-K99 and B41. At this time the exact reason for this cross-reactivity is unclear because the structures of K99 and CFA/I fimbriae are substantially different [13, 15]. It is possible that monoclonal antibodies against K88 or CFA/I fimbriae will present less cross-reactivity because monoclonal antibodies only recognize a single epitope of the antigen and thus have a better chance of differentiating distinct fimbriae. Monoclonal antibodies (mAb) against K88 fimbriae are not commercially available, and this hypothesis is yet to be tested.

Immobilization efficiency and the purity of antibodies

In immunoimmobilization experiments, bacterial cells are bound through antibody molecules covalently linked to the substrate surface. Therefore, the immobilization efficiency is correlated to the surface density of the antibody molecules on the substrate. For a given bacterium, the larger the number of antibody molecules available to bind the fimbriae within a unit area of the substrate, the stronger the overall binding of the cell will be, the more reliable the immobilization efficiency will be. This was confirmed in our previous work, in that a polyclonal antibody purified through a protein G column showed a higher immobilization efficiency while unpurified anti-serum often led to poor or even no immobilization [10]. This is because the large amount of non-immunoglobin proteins in antisera competes for the binding sites on the substrate surface, which reduces the surface density of the antibody molecules on the substrate.

Although purified pAb offered satisfactory immobilization for bacteria expressing CFA/I fimbriae, mAbs in general showed higher immobilization efficiencies than pAbs. Figure 4A–H compares the immobilization of strains H681-K99, B41 and 2.0961 on substrates modified with mAb and pAb against K99 and F41 fimbriae. Although both pAbs and mAbs provided immobilization of these strains to certain levels, it is clear that higher immobilization efficiencies were observed for mAbs, as evidenced by the high cell densities in Figure 4A–D relative to those shown in panels E–H. For example, the cell density in Figure 4A is roughly twice that shown in Figure 4E. Similar higher cell densities were observed for strains B41 and 2.0961 when mAb was used, implying a better immobilization efficiency for mAbs than pAbs against the same antigen. Although we did not compare mAbs vs. pAbs for all the fimbriae types listed in Table 1 because of the limited availability of mAbs, the results shown in Figure 4 suggest that for a given strain, mAbs generally offer higher immobilization efficiency than pAbs against the same surface antigen. This is understandable considering the facts a mAb recognizes only one epitope, so that a better specificity is often obtained and mAbs have a higher purity than pAbs because they are produced using different protocols than that used for pAbs [32]. The mAbs generally have a purity of more than 80% after purification using a protein G/A column, since there is no nonspecific immunoglobulin in the crude product. In contrast, pAbs contain many unrelated nonspecific immunoglobulins that do not recognize the antigen, and the estimated content of the related IgG for pAbs is rather low [33], even after purification through a protein G/A column. The low content of related IgG in pAbs means a low surface density of the related IgG molecules on the substrate, which reduces the immobilization efficiency.

In order to achieve higher immobilization efficiency, mAbs are preferred; however, they are often difficult to obtain. It is possible to obtain pAbs with high purity (> 80%) through affinity purification using a column filled with immunogen-modified resin, and such high-purity pAbs are expected to afford immobilization efficiencies similar to those of mAbs. However, this kind of purification is often very difficult to conduct because of the challenges associated with the preparation of immunogen-modified resin.

Immobilization efficiency and the antigen expression level

Since most antibodies were raised against the major units of the fimbria (the shaft), it is possible that one fimbrial filament could be bound by multiple antibody molecules. Although it remains unclear at this time how many antibody molecules can bind to a single fimbrial filament of a living cell, it is reasonable to hypothesize a single filament binding to a single antigen because of the geometrical constraints associated with Ab-Ag interactions. Therefore the immobilization efficiency will be proportional to the number of Ab-Ag binding pairs per unit area of the microorganism surface because the force required to break a single Ab-Ag binding (rupture force) remains similar for different ligand-receptor pairs, as revealed by single molecular force microscopy [34]. Therefore, both the density of the antibody on the substrate surface and the number of available antigens on the bacterial surface play critical roles in the immobilization efficiency. For a substrate with a given antibody density, a higher expression level of the targeted antigen will lead to more Ab-Ag binding pairs per unit area, and thus to a higher immobilization efficiency, until a monolayer of bacteria saturates the available antibody modified surface.

This hypothesis is supported by a comparison of the immobilization efficiencies of three strains that express K99 fimbriae at different levels: H681-K99, B41 and 9.1360. The AFM imaging revealed different K99 expression levels, with H681-K99 and 9.1360 showing the most and least abundant fimbriae, respectively (Figure 1B,C&I,). The immobilization experiment conducted using the same antibodies (mAb and pAb against K99) suggests a correlation between the fimbriae expression level and the immobilization efficiency: the most efficient immobilization was observed for H681-K99 (Figures 4A&E), while the poorest immobilization efficiency was observed for 9.1360 (Figures S1A&B). It should be noted that, if the expression level of fimbriae is too low, such as in the case of 9.1360, the immobilization efficiency does not show a significant difference between mAb and pAb (Figures S1).

The correlation between immobilization efficiency and antigen expression level is also supported by our work on *S*. Typhimurium. A *S*. Typhimurium strain, H72-pBAD-cfa, was constructed to allow the control of the CFA/I fimbriae level by adjusting the concentration of arabinose as the inducer. The CFA/I expression of this strain was reduced to a minimum when there was no arabinose added to the growth medium. The amount of CFA/I per cell increases as the result of an increase in the arabinose concentration in the growth medium. Under identical conditions (substrates, temperature and incubation time), only a small amount of cells were immobilized on the substrate when a bacterial culture containing no arabinose (0 μg/ml) was used, while much higher immobilized cell densities were observed for the same strain if harvested from a medium with a inducer concentration of ~ 0.3 μg/ml.

Label-free detection of multiple E. coli strains

The detection of pathogens using a combinatory approach of microarray techniques and antibody-based assays is not new to the field [4, 5, 35, 36]. However, many of these reports focus on the detection of a single bacterial strain [35] or require further treatment of the samples after the capturing of bacterial cells on the substrate [4, 5]. Furthermore, previous reports paid limited attention to antibody selection: typically antibodies against the whole bacteria were used. This work demonstrates that immobilization efficiency can be improved significantly and the rapid detection of multiple pathogenic strains can be achieved efficiently and reliably by coupling immunoimmobilization with the microarray technique. An example protocol is presented in Figure 5. A microarray-based sensor is first prepared by plotting multiple antibodies on a glass slide in such a way that the location of each spot is coded according to antibody specificity. Although only five antibodies (five colors) are shown on the microarray in Figure 5, more complex patterns can be easily prepared

depending on the antibody availability. After exposing this antibody microarray to unknown samples with potential bacterial contamination, such as milk, contaminated water or extracts from solid food, the targeted bacterial cells will be immobilized on the specific spots with the corresponding antibody and thus can be identified according to the location of these pre-assigned spots. An important advantage of this method is that immobilized cells are detected by direct visual inspection and hence no further chemical tagging or electronic sensing procedure is required.

In Figure 6 a successful application of this approach is demonstrated. Because of the limitations of fluorescent bacteria we focused on the detection of only three strains of *E. coli*, using a single antibody microarray as a proof-of-concept experiment. The microarray was prepared using a microplotting system with three pAbs, anti-CFA/I, anti-K88ac and anti-987P, as the inks. Each antibody was plotted as two rows separated from each other. The microarray was incubated for ~45 min under ambient conditions with a culture containing three fluorescent strains mixed at an equal ratio, H10407-RFP, 3030-2-GFP and 3.1012. In order to differentiate the cells after immobilization, bacteria were stained or genetically modified to show distinct fluorescence. An image of the mixed cells is shown in Figure 6A, and the sorted cells on the microarray are shown in Figure 6B, in which each antibody spot only recognizes cells with the corresponding fimbriae. Notice that the immobilized bacteria on each spot have a similar cell density. It should be noted here that the fluorescence was used as an additional indicator to identify the strains in order to evaluate the cross-reactivity. This immunoimmobilization is a label-free method and it will not be necessary to stain the cells in a practical setting: the bacterial strains will be identified based solely on the locations of the immobilized cells in the microarray. Because the bacteria are localized, it will be easy to extract the bacteria from the microarray spots for additional tests such as DNA sequencing to further verify the identifications.

One concern for such an assay is “how reliable is the bacterial identification?”. Under high optical microscope magnification, it was determined that nonspecific adsorption contributes to less than 5% of the total immobilized cells. An example of such high magnification images is shown in Figure 6C. The microarray was also tested using different batches of bacterial cultures to validate the reproducibility of the assay quantitatively. The detection of multiple bacterial strains was quantified by means of the number of immobilized cells per unit area as a function of cell concentration, incubation time and other factors. The results suggested that this microarray can be reliably used for the detection of multiple bacteria reliably and reproducibly. Detailed results of the quantitative study will be presented separately.

This assay should be considered primarily as a qualitative method, and estimations of bacterial concentrations in sample solutions based on cell densities on microarray spots should be made with caution. For a given Ab the cell density of the immobilized bacteria on a microarray spot is determined not only by the cell concentration of the culture but also by the incubation time. For example, a similar cell density was observed for all three strains when mixed non-equally but incubated for a longer duration (2hr) (Figure 6D). By comparing Figure 6B&D, it would be difficult to evaluate the mixing ratio based on the cell coverage density alone although identical microarrays were used in the two experiments. This feature can be used to boost the detection sensitivity for samples with a low bacterial concentration: the microarray can simply be incubated for an extended period.

Many factors contribute to the detection signal of the microarray, including concentration of the cells in the medium, incubation time, antibody purity and clone type. In this work cultures of a fairly high cell concentration (5×10^8 cfu/ml) were used to demonstrate the validity of the assay. However, the detection through immunoimmobilization can be readily

performed using samples with a much lower cell concentration, less than 10^5 cfu/ml. We have conducted extensive and systematic studies on rate of capture of living bacteria as functions of growth medium and bulk cell concentrations varying from ~1×10^8 cfu/ml to ~1×10^4 cfu/ml. In determining the rates of attachment of various bacteria we limited the count to the bacteria immobilized in the first 10 minutes of exposure to the bacterial medium (a) to avoid the saturation of the antibody-modified areas with bacteria and (b) to prevent the contribution to the rate of attachment from the regeneration of the bacteria. When the antibody-modified areas are close to saturation the competition for an antibody-modified area reduces the immobilization probability for a given bacterium. This becomes particularly important when the concentrations of cells in the bulk medium > ~ 5×10^6 cfu/ml. On the other hand, when one incubates the antibody-activated area for a long period of time the division of cells will contribute to the immunoimmobilization, which is shown clearly in Figure S3 in the Supplementary Data. In order to determine the rates of attachments, the reproducibility of these results for a number of different bacteria, and whether there is a relation between the rate of attachment and the bulk concentration of a given strain of bacteria we conducted a number of experiments with two bacterial strains (ETEC H10407 and Salmonella *enterica serovar* Typhimurium H72-pBBScfa mutant), in which we counted under an optical microscope the number of cells immobilized in a 100×100-μm^2 antibody-activated area in the first 10 minutes of exposure of this area to the bacterial medium. The results of these experiments are shown in Figure 7A&B: In all the experiments we observed a linear relationship between the cell capture rate and the cell concentration in the bulk. The capture rate, defined as the cell number inside a (100-μm)^2 area normalized to one minute of exposure time, increases linearly as the cell concentration goes up from ~3×10^3 cfu/ml to ~3×10^7 cfu/ml for the two different bacterial species. The slopes of these relationships and the standard deviations of the slopes are given in the caption of Fig. 7. As suggested by the slopes, the rate of attachment of Salmonella appears to be about six times higher than that of ETEC H10407. The reason for this might be that the polyclonal antibodies used in these experiments were raised against the CFA/I fimbriae isolated from the Salmonella mutant cited above, which appears to give a lower capture rate when used against the ETEC H10407 strain. More on this work is under preparation for publication elsewhere.

Conclusions

Our work has demonstrated that various types of fimbriae can be successfully targeted for the immunoimmobilization of living bacteria. The approach can, of course, easily be extended to other surface antigens for the detection of non-fimbriae bacteria; for example, antibodies against O antigens (LPS) and H antigens (flagella) can be used. The immobilization efficiency is affected by multiple factors, including the clone type and purity of the antibodies, the expression level of the bacterial surface antigens, the bacterial concentrations in the sample, the incubation time and perhaps the linker chemistry. Monoclonal antibodies are generally preferred over polyclonal antibodies when available because of their higher purity and specificity and low cross-reactivity. When combined with microarray techniques, immunoimmobilization can be used for the facile and simultaneous label-free detection of multiple pathogens as a rapid on-site response to microbial contaminations before systematic PCR-based analysis can be performed in a well-equipped facility. The detection of pathogens in this assay relies on the direct visual observation of living cells owing to the polished surface of the substrate, so no further labeling is required. On such polished surfaces a single bacterium within a 200×200 μm^2 field of view can easily be recognized. The observed capture rate of living pathogens approaches a single bacterium per 100×100 μm^2 area per half an hour of incubation for a bulk concentration of 10^5 cfu/ml.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by ONR Multidisciplinary University Research Initiative (MURI) grant N00014-10-1-0946, Montana State University funds supporting the Imaging and Chemical Analysis Laboratory (ICAL), National Institutes of Health Grants P20 RR020185 and R21 AI080960-01A1, an equipment grant from the M.J. Murdock Charitable Trust, and the Montana State University Agricultural Experimental Station. We thank Dr. Jean Starkey and Dr. Kate McInnerney for their help on the Bio-Rad system, and the *E. coli* Reference Center of Pennsylvania State University and Dr. D. Francis for *E. coli* strains, Ms. Sukriye Celikkol for her help in the immonoimmobilization experiments. The help of the ICAL staff, particularly Ms. Linda Loetterle, Ms. Laura Kellerman and Nancy Equall on handling bacteria and on AFM and SEM analysis is greatly appreciated.

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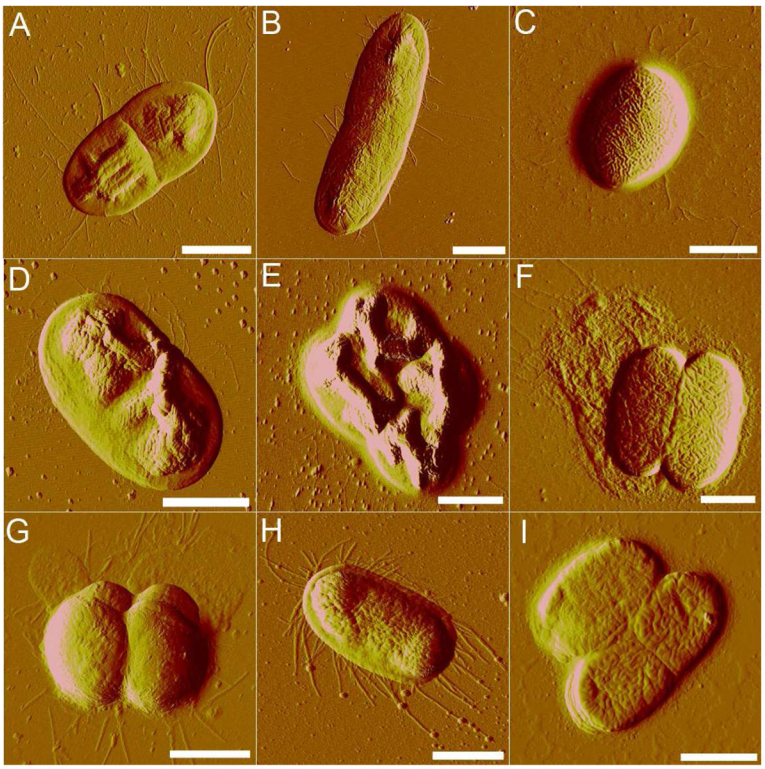

Figure 1.
AFM images of fimbriated *E. coli* strains: (A) H10407 with CFA/I fimbriae, (B) H681-K99 with K99 fimbriae, (C) B41 with K99 and F41 fimbriae, (D) 3030-2 with K88ac fimbriae, (E) 1836-2 with K88ac fimbriae, (F) 263 with K88ab fimbriae, (G) 3.1012 with 987P fimbriae, (H) 2.0961 with F41 fimbriae, and (I) 9.1360 with no visible fimbriae. Scale bar: 1 μm.

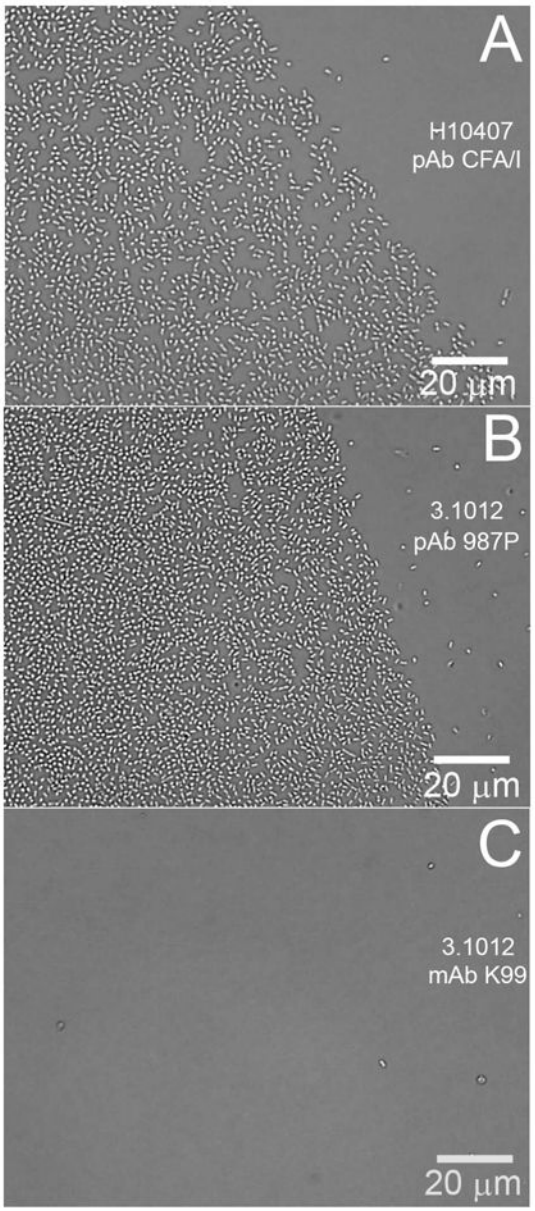

Figure 2.
Immunoimmobilization of (A) H10407 on a substrate modified with anti-CFA/I, (B) 3.1012 on a substrate modified with anti-987P, and (C) 3.1012 on a substrate modified with an irrelevant antibody, anti-K99.

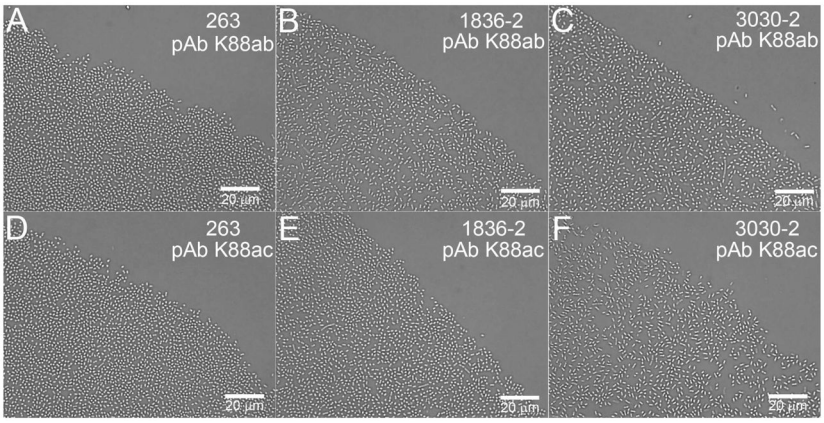

Figure 3.

Immobilization of *E. coli* strains expressing K88 fimbriae using antibodies against K88ab (top row) and against K88ac (bottom row): Panels (A), (B) and (C) show the immobilized bacterial strains (marked on each panel) on substrates modified with anti-K88ab, while panels (D), (E) and (F) show the same strains immobilized on substrates modified with anti-K88ac.

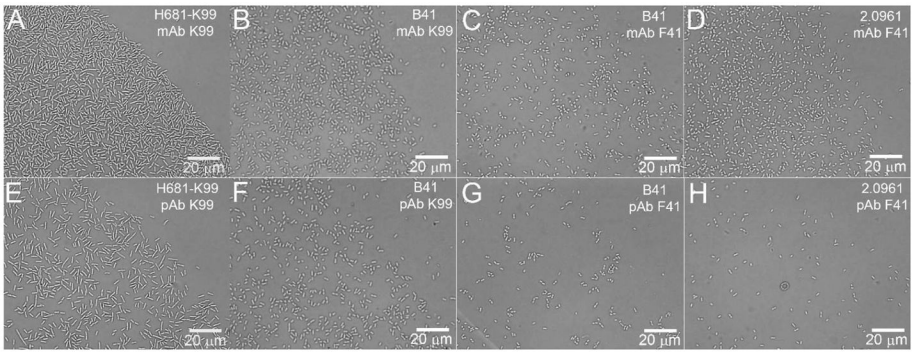

Figure 4.

Comparison of immobilization efficiencies between monoclonal and polyclonal antibodies. The strain names and the antibodies are noted on each panel. Higher immobilization efficiencies are observed for the monoclonal antibodies, shown in panels A–D, than for the polyclonal antibodies, shown in panels E–H.

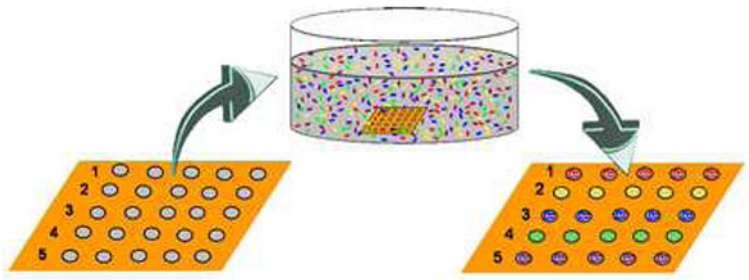

Figure 5.
Schematic representation of the rapid detection of multiple pathogens by combining microarray techniques and immunoimmobilization (see text for details).

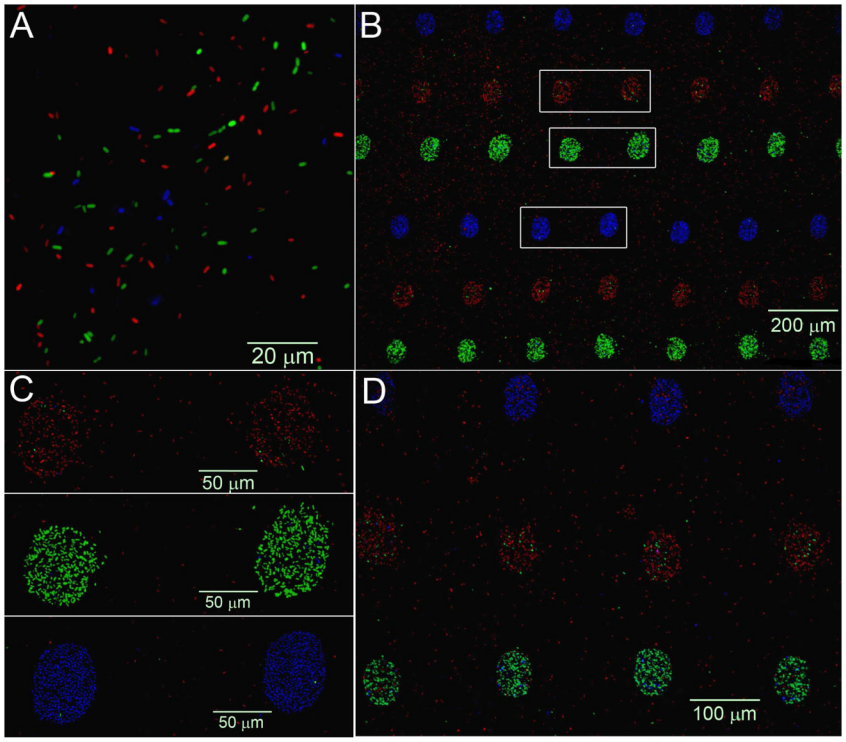

Figure 6.

Simultaneous detection of multiple strains of pathogens: (A) A fluorescence image of mixed cells of H10407-pDsRed (red), 3030-2-GFP (green) and 3.1012 (blue). (B) Live cells immobilized on a microarray prepared using anti-987P (blue color in the 1st and 4th rows from the top), anti-CFA/I (red color in the 2nd and 5th rows from the top) and anti-K88ac (green color in the 3rd and 6th rows from the top). The antibody microarray was incubated with cells mixed at equal concentrations (~5×10^8 cfu/ml). (C) The spots within the rectangular boxes in (B) are imaged at a higher magnification to inspect cross-reactivity. (D) A microarray identical to that used in panel (B) but incubated for 2 hr with a culture mixed at a ratio of 1:2:4 (3030-2-GFP: 3.1012:H10407-pDsRed).

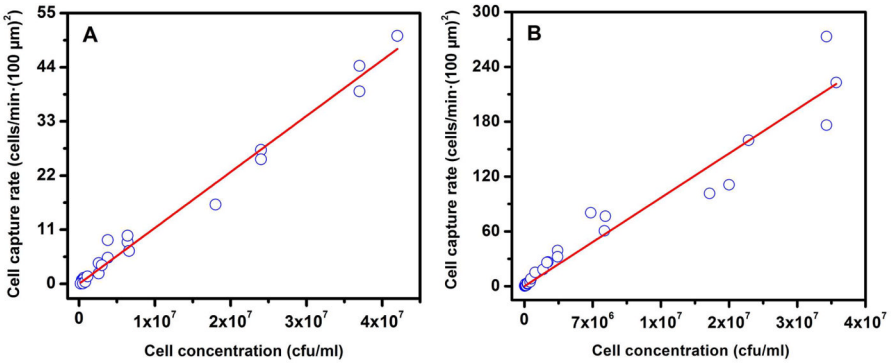

Figure 7.

These graphs show the rate of capture as a function of cell concentration. (A) This experiment was conducted by varying the cell concentration of ETEC H10407 from ~ 3×10^5 cfu/ml to 3×10^7 cfu/ml. For a given cell concentration the rate of capture was determined by counting the cells immobilized in the antibody-activated area (100×100 μm^2) in the first 10 minutes of bacterial incubation, which appears to increase linearly ($R^2 = 0.99$) with the cell concentration. The slope of this increase, shown in panel (A), is ~$1.2 \pm 0.1\times10^{-6}$ cells/(min·(100 μm)^2)/(cfu/ml). (B) This experiment was conducted by varying the cell concentration of Salmonella *enterica serovar* Typhimurium H72-pBBScfa from ~ 3×10^4 cfu/ml to 3×10^7 cfu/ml. For a given cell concentration the rate of capture increases linearly ($R^2 = 0.96$) with the cell concentration. The slope of this increase, shown in the figure, is $(6.9 \pm 0.3)\times10^{-6}$ cells/(min·(100 μm)^2)/(cfu/ml).

Table 1

E. coli strains tested in this work

Strain name	Fimbriae type	Relevant properties	Source	Reference
3030-2	F4 (K88ac)	Wild-type O157:K87:K88ac, LT^+, STb^+	SDSU	17, 18
263	F4 (K88ab)	Wild-type O8:K87:K88ab, LT^+, STb^+	SDSU	17, 19
1836-2	F4 (K88ac)	Wild-type $K88ac^+$; LT^-, ST^-, $astA^+$	SDSU	17, 20
H681-K99	F5(K99)	Parent strain *X*6212 asd^-, plasmid pMAK99-asd^+ expressing K99	MSU	16, 21, 22
9.1360	F5 (K99)	Wild-type $O64:K99^+$	PSU	-
3.1012	F6 (987P)	Wild-type $987P^+$	PSU	-
2.0961	F7 (F41)	Wild-type $O101:F41^+$	PSU	-
H10407	CFA/I	Wild-type $O78:H11:CFA/I:LT^+:ST^+$	MSU	23, 24
B41	K99, F41	Wild-type K99:F41, $StaP^+$	MSU	22

Table 2

Antibodies used in this work

Ab name	Cat. number	Clone type	Host	Source
anti-CFA/I	n/a	polyclonal	rabbit	MSU
anti-K99	n/a	polyclonal	rabbit	MSU
anti-K99	sc-66038	monoclonal (401)	mouse	Santa Cruz
anti-987P	NB100-62530	polyclonal	sheep	Novus
anti-K88ab	NB100-62532	polyclonal	sheep	Novus
anti-K88ac	NB100-62533	polyclonal	sheep	Novus
anti-F41	ab35291	polyclonal	sheep	Abcam
anti-F41	RAE0238	monoclonal	mouse	VLA