Anal. Chem. **2002,** *74,* 1805−1810

Arrays of Self-Assembled Monolayers for Studying Inhibition of Bacterial Adhesion

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This paper describes a simple and convenient method for the rapid screening of potential inhibitors of bacterial adhesion and for the quantitative evaluation of the efficacy of the inhibitors using arrays of self-assembled monolayers (SAMs) of alkanethiolates on gold that are presented on a 96-well microtiter plate. The SAMs present mixtures of α-D-mannopyranoside (a ligand that promotes the adhesion of uropathogenic *Escherichia coli* by binding to the FimH proteins on the tip of type 1 pili), and tri(ethylene glycol) moieties (organic groups that resist nonspecific adsorption of proteins and cells). The SAMs provide surfaces for studies of adhesion of uropathogenic *E. coli* to specific ligands; they also provide excellent resistance to nonspecific adhesion. Using arrays of mannoside-presenting SAMs, inhibitors of bacterial adhesion were easily screened by observing the number of bacteria that adhered to the surface of the SAMs in the presence of inhibitor. The potency of the inhibitor was quantified by measuring the percentage of inhibition as a function of the concentration of the inhibitor. The properties of SAMs, when combined with the convenience and standardization of a microtiter plate, make arrays of SAMs a versatile tool that can be applied to high-throughput screening of inhibitors of bacterial, viral, and mammalian cell adhesion and of strongly binding ligands for proteins.

This paper describes an experimentally convenient method to study inhibition of bacterial adhesion using arrays of self-assembled monolayers (SAMs) presented on a 96-well microtiter plate. Adhesion to the surface of host cells is an essential step in microbial colonization and the development of infections.[1−3] With the increasingly common medical problem of bacterial resistance against traditional antibiotics,[4] agents that can block microbial attachment to the host cell surface have become important as possible candidates for new classes of therapeutics for the treatment of infectious diseases.[5,6] The availability of efficient, controllable methods for evaluating the inhibitory activities of potential drug candidates will facilitate the development of therapeutics based on blocking adhesion.

Among the methods for evaluating the activities of potential inhibitors of bacterial adhesion, hemagglutination inhibition assays and solid-phase binding (ELISA-type) assays are commonly used. Hemagglutination inhibition assays are based on the aggregation of erythrocytes in the presence of lectins or other proteins that recognize ligands on the cell surface.[7] The complexity of the diverse carbohydrates presented on the surface of red blood cells often complicates agglutination, and results from hemagglutination inhibition assays are not always reproducible from laboratory to laboratory. In solid-phase binding assays, ligand or receptor (isolated proteins or cells) is usually adsorbed onto a surface noncovalently. The ligand or receptor density is difficult to control in this process, and bovine serum albumin (BSA) is often used to block the uncoated surface sites and to reduce nonspecific adsorption. Even when the ligands are attached covalently, ligand density can vary, and blocking may still be necessary.

Self-assembled monolayers (SAMs) of alkanethiolates on gold, in contrast, are surfaces that are structurally well defined at the molecular level; they offer substantial control over ligand density and environment.[8−10] Previous results from this laboratory have demonstrated that SAMs prepared from alkanethiols terminated in oligo(ethylene glycol) moieties effectively resist the nonspecific adsorption of proteins[11−13] and the nonspecific adhesion of mammalian cells.[14] Mixed SAMs consisting of alkanethiolates terminated in oligo(ethylene glycol) groups and alkanethiolates terminated with the ligand of interest, therefore, provide an excellent model surface with which to study biointerfacial problems.[15,16] SAMs prepared for studies of biomolecular recognition are usually supported on gold-coated glass slides. These SAMs on glass are convenient for surface plasmon resonance (SPR) studies[17,18] and for patterning of proteins and cells.[19,20] They are not, however,

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10.1021/ac011042o CCC: $22.00
Published on Web 03/16/2002

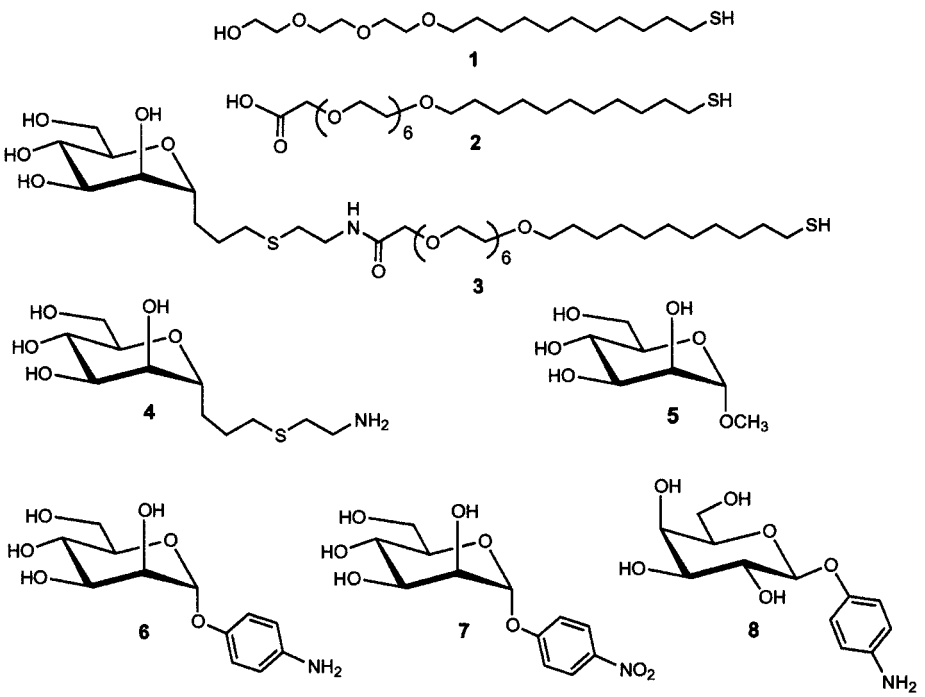

Figure 1. Chemical structures of the compounds used in this study.

the most suitable substrates when many samples of SAMs are required and manipulated, as in an inhibition assay. One of our objectives in this work was, therefore, to develop arrays of SAMs presented in a format that is convenient to manipulate and that is compatible with standard instrumentation for rapid, parallel screening.

Here, we describe arrays of SAMs presented on 96-well microtiter plates that allow the examination of biospecific adhesion of uropathogenic *Escherichia coli*, which is the leading cause of urinary tract infections.[21,22] Because *E. coli* attached to the bladder and urethra are exposed to fluid shear, adhesion is critical for the establishment of infection.[23] The adhesion of uropathogenic *E. coli* to the surfaces of epithelial cells on the inner wall of the bladder is mediated by the interactions between FimH adhesin on the tips of type 1 pili and α-D-mannopyranoside residues in oligosaccharides present on the host cell surfaces.[24−26] We demonstrate that these arrays provide a convenient tool with which to study bacterial adhesion and to screen potential inhibitors of bacterial adhesion.

EXPERIMENTAL SECTION

Materials. All materials and reagents were used as received. Methyl α-D-mannopyranoside (**5**), *p*-aminophenyl α-D-mannopyranoside (**6**), *p*-nitrophenyl α-D-mannopyranoside (**7**), *p*-aminophenyl *β*-D-galactopyranoside (**8**), 4,4′-azobis(4-cyanovaleric acid), 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and phosphate buffered saline (PBS: 10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) were from Sigma-Aldrich. Syto 13 (green fluorescent probe) was from Molecular Probes Inc. The flat-bottom 96-well polystyrene plate was from Corning. The glass-bottom 96-well polystyrene plate was from Whatman. Uropathogenic *E. coli* (laboratory strain RB 128, isolated from patients with acute pyelonephritis) used in our studies originally was a kind gift from Dr. H. Shaw Warren (Massachusetts General Hospital, Boston). Alkanethiols **1** and **2** (Figure 1) were prepared as described previously.[12,18]

Synthesis of 11-[(3-(α-D-Mannopyranosyl)propyl)thioethylaminocarbonyl Methoxy Hexa(ethoxy)]undec-1-yl-thiol 3. Compound **4** was prepared by reaction of 3-(α-D-mannopyranosyl)propene[27] with mercaptoethylamine [*hν* (254 nm), 4,4′-azobis(4-cyanovaleric acid), H_2O−MeOH (10:1), 10 h, 87%]. Coupling of **4** with $HO_2CCH_2(OCH_2CH_2)_6O(CH_2)_9CH{=}CH_2$ [EDC, 4-(dimethylamino)pyridine, *N*, *N*-diisopropylethylamine, DMF, 26%] followed by reaction with thioacetic acid [*hν* (254 nm), 4,4′-azobis(4-cyanovaleric acid), H_2O−MeOH (10:1), 10 h, 50%] and deprotection of the acetyl group (NaOMe, MeOH, rt, 2 days, 74%) provided **3**. Selected ^{1}H NMR data (500 MHz, CD_3OD): δ 4.00 (s, 1H, $NHCOCH_2O$), 3.85 (ddd, 1H, J = 2.5, 4.9, 10.2 Hz, H-1 αMan). ES-MS calcd for $C_{35}H_{72}NO_{13}S_2$ ($M + H^+$) 778.4. Found, 778.5.

Preparation of SAMs. The gold substrates were fabricated by electron-beam evaporation of an adhesion layer of titanium (10 nm), followed by a layer of gold (80 nm) onto the 96-well plates.

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A solution (60 μL) of alkanethiols **1** (2 mM in ethanol) and **2** or **3** (2 mM in ethanol) in varying ratios was added into the gold-coated wells and incubated for 14 h. Solutions were then removed, and the wells were washed three times with ethanol and dried under a stream of N_2. We assume that the relative mole fractions of these two thiols on the surface are the same as in the solution. For the immobilization of the mannoside ligand to the plate presenting mixed SAMs of **1** and **2**, the plate was sequentially incubated with a freshly prepared solution of EDC (0.2 M) and NHS (0.05 M) in Milli-Q-purified water for 10 min, in a solution of mannoside ligand **4** (2 mg/mL in 25 mM sodium phosphate buffer, pH 8.0) for 1 h, and finally, in sodium phosphate buffer (25 mM, pH 8.6) for 30 min. Between incubations, the plate was washed with Milli-Q-purified water.

Studies of Bacterial Adhesion. Uropathogenic *E. coli* were cultured and harvested as described previously.[28] Bacteria (10^7−10^8 cells/mL in PBS) were stained by preincubation with Syto 13 (green fluorescent probe) for 20 min. The stained bacterial suspension was then transferred to the 96-well microtiter plate presenting SAMs and incubated at room temperature for 30 min. The total number of bacteria in each well was in large excess over the number of potential bacteria that could bind to the surface, hence the exact amount of bacteria in suspension did not effect the experiments. The solutions containing bacteria were removed, and the wells were washed three times with PBS. The digital micrographs of the bacteria that adhered to the SAM surfaces were taken using a Leica fluorescent microscope connected to a CCD camera at 100× magnification with the focus at the central field of the well. The total number of the bacteria in the digital images was quantified as a measurement of the total fluorescent area using the Scion Image program (NIH). In each case, the entire digital image was analyzed.

Studies of Inhibition of Bacterial Adhesion. The bacteria stained with Syto 13 were incubated with **5**−**8** (2.5 mM in PBS) at room temperature for 1 h before being transferred to the 96-well microtiter plate presenting SAMs with $\chi(\mathbf{3}) = 0.001$. The bacteria are unable to use the inhibitors (**5**−**8**) as carbon sources, as indicated by no change in the bacterial population after a 16-h incubation (25 °C) in PBS containing 2.5 mM inhibitor. To determine the K_i^{SAM} of **6**, the bacteria were incubated for 1 h with **6** serially diluted four times between each well. The percentage inhibition was calculated as $\{[A(\mathrm{nI}) - A(\mathrm{I})]/A(\mathrm{nI})\} \times 100\%$ (A, total fluorescent area; nI, no inhibitor; I, with inhibitor). The data were fit using KaleidaGraph 3.0 (Synergy Software).

RESULTS AND DISCUSSION

Construction of Arrays of SAMs. A schematic representation of the preparation of arrays of SAMs is shown in Figure 2. The gold substrates were fabricated by electron-beam evaporation of an adhesion layer of titanium (10 nm), followed by a layer of gold (80 nm), onto 96-well microtiter plates (the flat-bottom 96-well polystyrene plate and the glass-bottom 96-well polystyrene plate yielded indistinguishable results). A photograph of a typical gold-coated plate is shown in Figure 3. Arrays of SAMs were prepared by adding 60 μL of a solution of alkanethiols (total concentration of 2 mM in ethanol) to these gold-coated wells. We used both a direct method[12,13] and a method based on a common reactive intermediate, *N*-hydroxylsuccinimidyl ester,[18] to prepare the SAMs presenting mannoside ligands above a continuous layer of tri(ethylene glycol) groups (Figure 2). In the direct method, the alkanethiol **3** terminated with α-D-mannopyranoside was synthesized chemically. The solution of **3** was mixed in varying concentrations with a solution of alkanethiol **1** terminated with a tri(ethylene glycol) group. These mixtures were then added to microwells to produce SAMs that had four mole fractions of **3** ($\chi(\mathbf{3})$): 0, 0.001, 0.01, or 0.1; we assume that the relative mole fractions of alkanethiols **1** and **3** on the surface and in the solution are the same, since earlier studies indicate that the composition of SAMs for similar species is well-described by the composition of the solution of thiols from which it is made.[11,18,29−32] In the common intermediate method, arrays of mixed SAMs consisting of **1** and **2** were first prepared using the direct method. The surface carboxylic group was then converted to the reactive *N*-hydroxylsuccinimidyl ester group using 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The mannoside ligand **4** was coupled to the surface through amide bond formation to produce the mannoside-presenting SAMs. We assume that the resulting mole fraction of **3** on the surface is equal to that of **2**, since the yield of coupling product using this common intermediate method is almost quantitative for SAM surfaces having $\chi(\mathbf{2}) < 0.1$ and amines of normal nucleophilicity.[18]

Biospecific Adhesion of Bacteria to the Surface of SAMs. The adhesion of bacteria to surfaces and host cells can occur by a number of mechanisms, both biospecific (carbohydrate−protein, protein−protein) and nonspecific (hydrophobic or electrostatic).[33] The nonspecific adhesion of bacteria often interferes with and complicates the study of biospecific adhesion. Surfaces that can resist the nonspecific adhesion of bacteria are, therefore, highly desirable for the study of biospecific adhesion of bacteria. In general, SAMs presenting oligo(ethylene glycol) moieties effectively resist nonspecific adsorption of proteins[11−13] and nonspecific adhesion of mammalian cells.[14] Our recent studies suggest, however, that the adhesion of microbial cells to the SAM surfaces is more variable and complex.[34] In this study, the SAM presenting only **1** terminated in a tri(ethylene glycol) group effectively resisted the adhesion of uropathogenic *E. coli*, because no bacteria adhered to the surface (Figure 4A). In contrast, the bacteria readily adhered to SAMs having $\chi(\mathbf{3}) = 0.001$ (Figure 4B,D) prepared using both the direct and the common intermediate methods, and maximal attachment was observed when $\chi(\mathbf{3}) = 0.01$ (Figure 4C). Although the binding between a pilus and a single mannoside is weak (the inhibition constant is in the millimolar range),[35] the polyvalent interactions[36] between multiple copies of the pili and multiple mannoside ligands allow the bacteria

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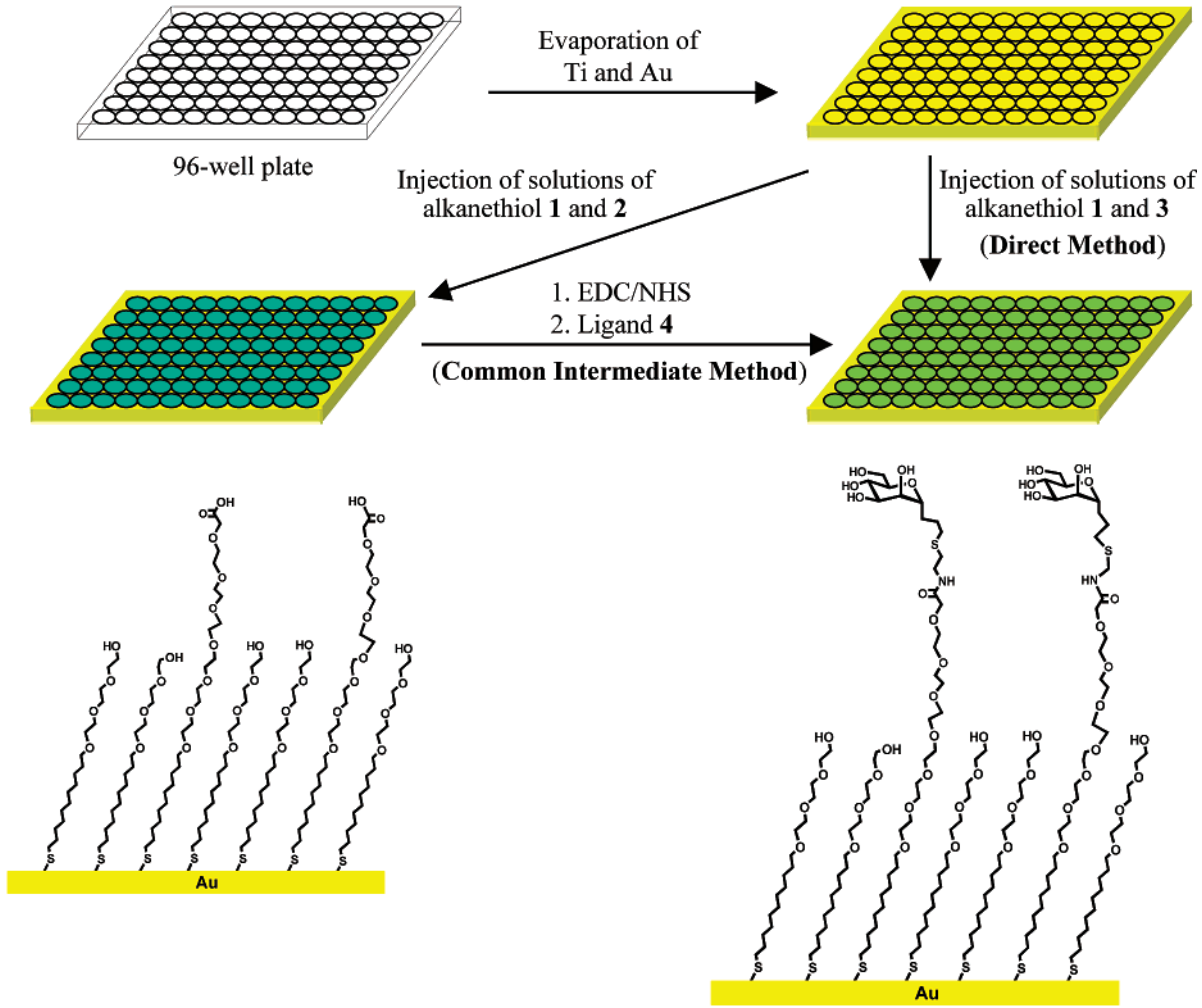

Figure 2. Schematic representation of the preparation of arrays of SAMs presenting mannoside ligand using both a direct method and a common intermediate method. The tri(ethylene glycol)-terminated thiol extends 2.2 nm from the surface of the gold; the mannoside-terminated thiol containing a hexa(ethylene glycol) group extends 3 nm. The oligo(ethylene glycol) moieties prevent the nonspecific adhesion of proteins and cells to the surface of the SAM.[11−14]

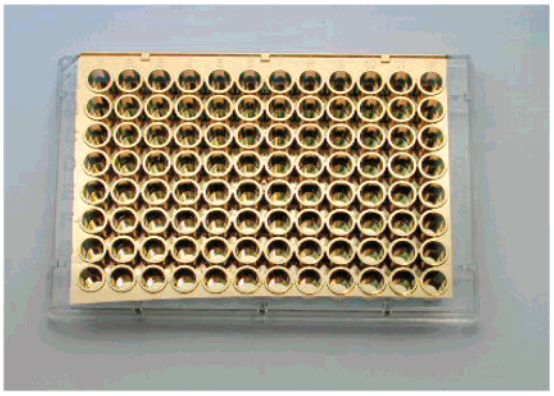

Figure 3. Photograph of the gold-coated 96-well microtiter plate.

to adhere strongly to the surface of mannoside-presenting SAMs. We estimate that there are 5×10^3 mannoside residues/μm^2 (assuming that the distance between two sulfur atoms on the gold is 0.5 nm) on the surface of a SAM having $\chi(\mathbf{3}) = 0.001$ and that each bacterium displays roughly 100−400 pili evenly distributed across its surface.[35,37] The polyvalent nature of the interaction between the bacterium and the SAM surface presenting mannoside ligands has been demonstrated in recent experiments using optical tweezers.[28]

Screening of Inhibitors of Bacterial Adhesion. Using arrays of SAMs having $\chi(\mathbf{3}) = 0.001$, we studied the inhibition of bacterial adhesion using four compounds: methyl α-D-mannopyranoside (**5**), *p*-aminophenyl α-D-mannopyranoside (**6**), *p*-nitrophenyl α-D-mannopyranoside (**7**), and *p*-aminophenyl *β*-D-galactopyranoside (**8**). The *E. coli* were fluorescently stained using a cell-permeable, intercalating dye. The stained bacteria were incubated with the potential inhibitors **5**-**8** (2.5 mM in PBS) for 30 min and then transferred into the wells coated with SAMs having $\chi(\mathbf{3}) = 0.001$. The total number of bacteria that adhered was quantified as the total area of fluorescent bacteria in the image analysis. As expected, **8** did not show any inhibitory effect (Figure 4E), since the type 1 pili adhesin is mannose-specific. In contrast, when the bacteria were incubated with the mannoside-based ligands **5**, **6**, or **7**, the amount of bacteria that adhered to surfaces presenting α-D-mannopyranoside ligand, **3**, was significantly reduced (Figs. 4F-H). The total number of the bacteria in the digital images was quantified as a measurement of the total fluorescent area. The

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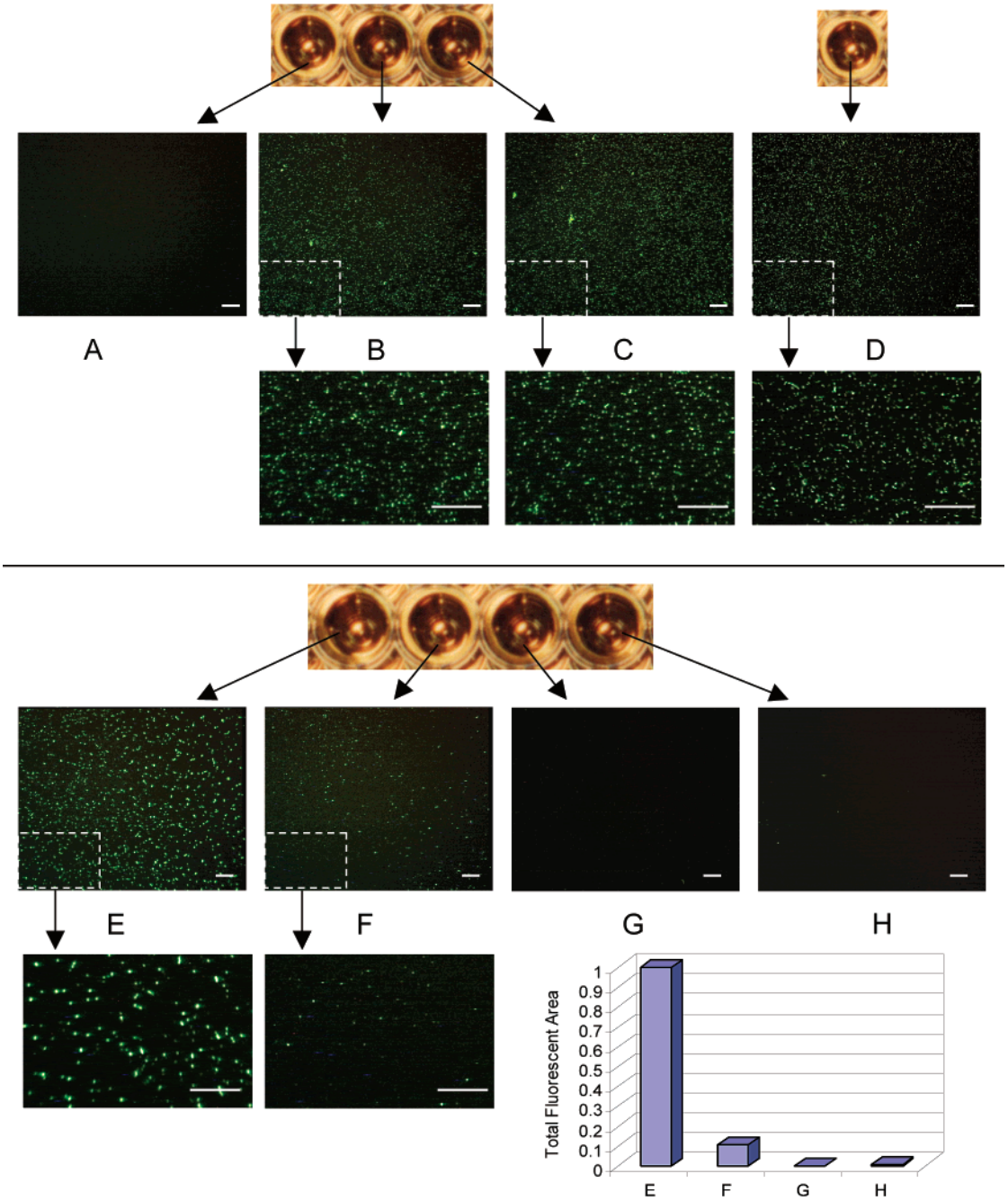

Figure 4. (Top) Fluorescent images of bacteria that adhered to the surfaces of SAMs prepared by either the direct method (A−C) or the common intermediate method (D): (A) SAM presenting only **1**, (B) SAM having χ(**3**) = 0.001 and χ(**1**) = 0.999, (C) SAM having χ(**3**) = 0.01 and χ(**1**) = 0.99, (D) SAM having χ (**3**) = 0.001 and χ (**1**) = 0.999. (Bottom) Fluorescent images of bacteria that adhered to SAMs having χ (**3**) = 0.001 and χ (**1**) = 0.999 after the bacteria were incubated with 2.5 mM of *p*-aminophenyl β-D-galactopyranoside **8** (E), methyl α-D-mannopyranoside **5** (F), *p*-aminophenyl α-D-mannopyranoside **6** (G), or *p*-nitrophenyl α-D-mannopyranoside **7** (H). The histogram shows the normalized total fluorescent area in images E−H. Scale bar: 100 μm.

histogram in Figure 4 shows normalized total fluorescent areas for corresponding images F−H. From the analyses of the images, one could easily tell that compounds **6** and **7** were more inhibitory than **5**, since there were still significant bacteria (~10%) adhered to the surface in the presence of **5**, and almost no bacteria adhered in the presence of **6** and **7**. These results are consistent with the fact that **6** and **7** are among the best of the known monomeric inhibitors for type 1 pili adhesion, possibly as a result of interaction between the phenyl group on **6** and **7** and a hydrophobic binding region in the FimH adhesin of type 1 pili.[35] The complete inhibition of bacterial adhesion to the SAM surface at high concentration of the potent inhibitors also confirms that the adhesion is biospecific.

Quantitative Evaluation of Inhibitory Potency. To determine if this adhesion assay would yield quantitative data, we examined the inhibition of bacterial adhesion with the inhibitor **6** at different concentrations. As in other 96-well-plate-based assays, the bacteria were incubated with a solution of **6** prepared by serial dilution and then transferred to the 96-well plate presenting SAMs having χ(**3**) = 0.001. Figure 5 shows the percentage of inhibition as a function of the concentration of **6**.

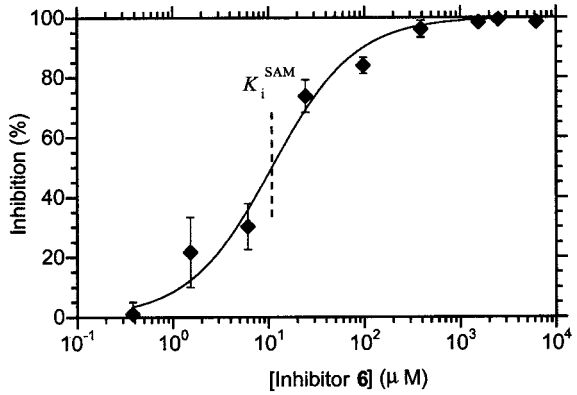

Figure 5. Curve showing the percentage inhibition of bacterial adhesion as a function of the concentration of **6**. The percentage of inhibition was calculated as $\{[A(\mathrm{nI}) - A(\mathrm{I})]/A(\mathrm{nI})\} \times 100\%$ (A, total fluorescent area; nI, no inhibitor; I, with inhibitor). The points represent the average of two sets of data, and the length of the error bar is the difference between each pair of data. The curve is a fit of a simple binding isotherm with K_i^{SAM} as an adjustable parameter. The K_i^{SAM} is defined as the concentration of inhibitor required to achieve 50% inhibition. For **6**, $K_i^{SAM} = 11 \pm 1.5\ \mu$M.

The data were fit to a simple binding isotherm with K_i^{SAM} as an adjustable parameter in eq 1. The K_i^{SAM} is defined as the value of [inhibitor] required to achieve 50% inhibition. The K_i^{SAM} for **6** determined from the inhibition curve is $11 \pm 1.5\ \mu$M, which is close to values (58[38] or 20 μM[39]) observed in hemagglutination inhibition assays. The value of K_i^{SAM}(**6**) is also comparable to the IC_{50} value ($49 \pm 17\ \mu$M) of **7** determined using ELISA.[40]

$$\%\ \text{inhibition} = \frac{[\text{inhibitor}]}{[\text{inhibitor}] + K_i^{SAM}} \qquad (1)$$

CONCLUSIONS

SAMs presented on gold-coated 96-well microtiter plates are much easier to manipulate and handle than those on glass substrates, and the 96-well plate format is compatible with standard instrumentation. Mixed SAMs consisting of alkanethiolates terminated in oligo(ethylene glycol) groups and in a mannoside ligand recognized by type 1 pili provide synthetic, biospecific surfaces that promote the adhesion of uropathogenic *E. coli* through polyvalent interactions between multiple copies of pili on the bacterium and multiple copies of mannoside ligands presented on the SAM surfaces. We have demonstrated that arrays of these SAMs presented on the 96-well format provide a useful tool for the screening of potential inhibitors of bacterial adhesion and for the quantitative evaluation of the efficacy of the inhibitors. We presume that the same techniques can be applied with virtually no modification to higher density microwell formats.

SAM-based assays in a microtiter plate format offer several advantages, as compared to the hemagglutination inhibition assay as commonly carried out in solution and the solid-phase binding assay conducted using conventional plates. First, we can form surfaces that interact biospecifically with cells with essentially no background signal, using mixed SAMs comprising alkanethiolates terminated with the ligand of interest and alkanethiolates terminated in oligo(ethylene glycol) or other moieties that resist nonspecific adhesion or adsorption.[41−44] These types of mixed SAMs allow us to eliminate the blocking step with BSA and give good definition of the surfaces at the molecular level. Second, it is easy to control the density of ligands on the surface of the SAM by varying the ratio of two alkanethiols in solution. Third, the method is simple, since only bacteria and the ligand that mediates the adhesion are essential in the assay; this simplicity comes by eliminating the need for other biological components, such as red blood cells, enzymes, or antibodies. Because there are readily available chemical methods (for example, the common intermediate approach) for introducing the ligands of interest to the SAM surface, many ligands could be easily immobilized on the surface, and the synthesis of many distinct alkanethiols is not required.

The assay method described here can be easily extended to study inhibition of viral and mammalian cell adhesion by using appropriate arrays of SAMs. Arrays of SAMs that present specific ligands for proteins can also be used to immobilize proteins and to study protein−ligand and protein−protein interactions with the use of readily available conjugation reagents and detection techniques in ELISA assays.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (GM30367) and relies on methods developed in work supported by DARPA. We used shared MRSEC facilities supported by NSF (DMR-9809363). We thank Dr. H. Shaw Warren (Massachusetts General Hospital, Boston) for providing us the strain of *E. coli*. X.Q. acknowledges a postdoctoral fellowship from the Natural Sciences and Engineering Research Council (NSERC) of Canada. S.J.M. is grateful to NIH for a postdoctoral fellowship.

Received for review September 28, 2001. Accepted January 24, 2002.

AC011042O

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