Living Cells of Staphylococcus aureus Immobilized onto the Capillary Surface in Electrochromatography: A Tool for Screening of Biofilms

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800294f · Source: PubMed

Anal. Chem. **2008,** *80,* 5103–5109

Living Cells of *Staphylococcus aureus* Immobilized onto the Capillary Surface in Electrochromatography: A Tool for Screening of Biofilms

Jie Chen,[†] Adyary Fallarero,[‡] Anni Määttänen,[§] Malena Sandberg,[‡] Jouko Peltonen,[§] Pia M. Vuorela,[‡] and Marja-Liisa Riekkola*,[†]

Laboratory of Analytical Chemistry, Department of Chemistry, P.O. Box 55, University of Helsinki, FIN-00014 Helsinki, Finland, and Department of Biochemistry and Pharmacy, and Laboratory of Paper Coating and Converting, Faculty of Technology, Åbo Akademi University, FIN-20500 Turku, Finland

Microorganisms attach to nonliving surfaces in many natural, industrial, and medical environments, enveloped within extracellular polymeric substances. The result is a biofilm. Biofilms are reported to exist in 65−80% of bacterial infections refractory to host defenses and antibiotics therapy and are regarded as a central problem in present-day medical microbiology. Understanding of the parameters governing the interaction of antimicrobials with biofilms is thus of great interest in any attempt to increase biocide efficacy. In this work, study was made of the feasibility of using open tubular capillary electrochromatography (CEC) in bacterial biofilm studies with living cells. *Staphylococcus aureus* was selected as model bacterium. First, *S. aureus* was shown, under various conditions, to form a biofilm on the inner wall of a fused-silica capillary coated with poly(L-lysine). Optimal conditions for biofilm formation, such as bacterial concentration, growing time, and the stability of the ensemble, were preliminarily defined with conventional 96-microtiter well plates. Continuous flushing of the capillary with fresh cells meant that no growth medium was needed. The presence of biofilm in the capillary was confirmed by atomic force microscopy. Interactions between *S. aureus* biofilms and different antibiomicrobial agents were studied by capillary electrochromatography. The effect of five antibiotics (penicillin G, oxacillin, fusidic acid, rifampicin, vancomycin) on biofilms was examined in terms of retention factors and reduced mobilities of the antibiotics. The antibiotic susceptibility profile for *S. aureus* is similar as the result of minimal inhibitory concentrations registered on the 96-microtiter well plates for both planktonic and biofilm cells. The results show, for the first time, that bacterial biofilms can be studied by CEC. The technique allows highly efficient and easy characterization of interactions between *S. aureus* biofilms and potentially active antimicrobial compounds under different conditions. Reagent and cell consumption are minimal.

The discovery that biofilms represent the natural form of bacterial life outside laboratory conditions represents a milestone in modern medical microbiological research and is having a major impact on pharmaceutical approaches to find more efficient biocide drugs.[1] It has now been established that the natural way for bacteria to live is in highly organized, multicellular complexes referred to as biofilms, which behave in quite a way different from independent, free-floating cells. Biofilm formation occurs through sequential steps beginning with organisms adhering to an available surface and to each other. By clustering together, bacteria form thick layers featuring complex structures and a metabolism different from that in cell suspensions.[2] From the biomedical and pharmaceutical perspective, probably the most important feature of bacterial biofilms is their increased resistance to many antimicrobial agents as compared with their planktonic (suspension) counterparts. This feature has been linked to their multifactorial origin.[3] The sessile mode of bacterial life also protects biofilm-embedded microbes from host phagocytic clearance, greatly limiting the ability of the host to deal with the infection.[4] In addition, biofilms may harbor other pathogenic organisms and may even allow an exchange of resistance plasmids.[5] Biofilms thus represent a highly persistent form of bacterial infection that is notoriously difficult to eradicate.

*Staphylococcus aureu*s is a Gram (+) bacterium regarded as a leading cause of nosocomial infections and the etiologic agent of a wide range of diseases. Among the diseases are endocarditis, osteomyelitis, toxic shock syndrome, food poisoning, and skin infections.[4] Diseases caused by the colonization of indwelling

* To whom correspondence should be addressed. E-mail: marja-liisa.riekkola@helsinki.fi.

[†] University of Helsinki.

[‡] Department of Biochemistry and Pharmacy, Åbo Akademi University.

[§] Laboratory of Paper Coating and Converting, Faculty of Technology, Åbo Akademi University.

(1) Lasa, I. *Int. Microbiol.* **2006**, *9*, 21–28.
(2) Nishimura, S.; Tsurumoto, T.; Yonekura, A.; Adachi, K.; Shindo, H. *J. Orthop. Sci.* **2006**, *11*, 46–50.
(3) Tenover, F. C. *Am. J. Med.* **2006**, *119* (6A), S3–S10.
(4) Jabra- Rizk, M. A.; Meiller, T. F.; James, C. E.; Shirtliff, M. E. *Antimicrob. Agents Chemother.* **2006**, *50* (4), 1463–1469.
(5) Donlan, R. M.; Costerton, J. M. *Clin. Microbiol. Rev.* **2002**, *15* (2), 167–193.

10.1021/ac800294f CCC: $40.75
Published on Web 05/28/2008

medical devices are particularly problematic since they provide a route to overcome the body's barrier defenses and a surface for bacteria growth on. *S. aureus* biofilms are widely present on implanted biomedical devices, accounting together with *Staphylococcus epidermidis* for more than half of the infections associated with prosthetic devices.[6] *S. aureus* biofilms are typically refractory to host defenses and antibiotics and have a marked persistence in the host organism.[4]

The standard approved procedures, which challenge planktonic cell suspensions with an antimicrobial agent, do not provide good results when it comes to modeling biofilm formation and evaluating antimicrobial susceptibility. As an alternative, recent years have seen the development of a variety of biofilm model systems.[5,7,8] Nevertheless, the Clinical and Laboratory Standards Institute (CLSI) does not appear to have an approved method for assessing antibiofilm effectiveness.[9,10] Fluid dynamics is an important factor known to influence biofilm formation in natural environments. For example, biofilms formed under high shear, turbulent flow, have been proved to be stronger, more stable, and more strongly attached than their low-shear counterparts.[11] There is thus an imperious need of developing new methods in which the testing of antibiofilm activity can be conducted. The requirements for such techniques include the possibility to predict the susceptibility of the biofilm-associated organisms in vivo with reasonable reliability and simplicity and at reasonable cost. As we shall show, capillary electrochromatography (CEC) offers a viable approach.

Capillary electrochromatography belongs to the family of capillary electromigration techniques. In CEC, the movement of the mobile phase through a capillary, filled, packed, or coated with a stationary phase, is achieved by electroosmotic flow. The retention of analytes is due to a combination of electrophoretic migration and chromatographic retention.[12] So far, capillary electromigration techniques have been successfully applied to the separation, identification, and characterization of mixtures of both living and dead bacteria.[13–18] No capillary electromigration technique studies are available for living bacterial biofilms. In this work, we studied the potential to immobilize living biofilm-forming bacteria onto fused-silica capillaries in CEC. The presence of biofilm on the capillary inner surface was confirmed by atomic force microscopy (AFM). The novel living cell immobilized capillary electrochromatographic system was employed for the screening of *S. aureus* biofilms. In addition, the effect of different antimicrobial agents on the biofilms formed on the capillaries was evaluated. The new technique features highly efficient and easy characterization of interactions between *S. aureus* biofilm and potentially active antimicrobial compounds as well as minute reagent and cell consumption.

EXPERIMENTAL SECTION

Materials. Dimethyl sulfoxide (DMSO) and hydrochloric acids (1 and 0.1 M) were from Oy FF-Chemicals Ab (Yli-Ii, Finland). Tryptic soy broth (TSB), Mueller-Hinton broth and tryptic soy agar (TSA) were obtained from Fluka, while 3-aminopropyltriethoxysilane (APTES), fusidic acid (MW = 516.71), oxacillin (MW = 401.44), penicillin G (MW = 334.40), rifampicin (MW = 822.94), vancomycin (MW = 1449.25), phosphate-buffered saline (PBS), crystal violet, and poly(L-lysine) (MW = 150 000−300 000, 0.1% w/v) were from Sigma-Aldrich.

Instrumentation. Electrophoretic measurements were made with a Hewlett-Packard 3DCE system (Agilent, Waldbronn, Germany) equipped with a diode array detector and an air-thermostating capillary. The data acquisition rate was 100 Hz, and the response time of the detector 0.1 s. Uncoated fused-silica capillaries were from Composite Metal Services Ltd. (Worcestershire, UK). Dimensions were 50 μm i.d. and 375 μm o.d. The length of the capillary to the detector was 30 cm with a total length of 38.5 cm. A Lauda Ecoline Re-104 water bath (Lauda-Königshofen, Germany) was used to control the temperature of the autosampler. A Universal SPM system Ntegra Prima (NT-MDT, Zelenograd, Russia) equipped with a replaceable scanner (SC100NTF) was used for the AFM studies. AFM imaging on the capillaries were taken after forming biofilms using a flow cell (Friedrich & Dimmock) coupled to a peristaltic pump (Watson Marlow).

Sample and Buffer Preparation in CEC. The water, buffer, and broth samples were sterilized by autoclaving at 121 °C for 15 min. The concentration of background electrolyte (BGE) solution was 8 mM (ionic strength 20 mM), with the pH adjusted to the desired value with 1.0 M sodium hydroxide. A pK_a value of 7.2 for phosphoric acid was used for calculations of ionic strength of the BGE. The 1.0 mg/mL stock solutions of antibiotics were prepared in sterile water, except for the susceptibility studies in 96-microtiter well plates in which sterile Mueller-Hinton broth was utilized. The final concentration of antibiotic samples was 100 μg/mL in corresponding BGE solution. Phosphate buffer was used as BGE solution for pH value 7.4 and acetate buffer for pH values 6.0−5.0.

Methods. *Culture of Staphylococcus aureus.* The *S. aureus* methicillin-susceptible strain ATCC 25923 was provided for each coating. Culturing was done in the Division of Pharmaceutical Biology at the Faculty of Pharmacy, University of Helsinki, Finland. TSB-bacterial suspensions were prepared by culturing the refreshed strain on TSB at 37 °C, 200 rpm, for 4 h, until a concentration of 10^8 colony-forming units (cfu)/mL was achieved, as assessed by turbidity measurement (595 nm) and TSA counts. For all the experiments conducted in the CEC system, BGE-bacterial suspensions were prepared by centrifuging TSB suspensions at 2500*g* for 5 min, resuspending the bacterial pellet in BGE,

(6) Fluckiger, U.; Ulrich, M.; Steinhuber, A.; Döring, G.; Mack, D.; Landmann, R.; Goerke, C.; Wolz, C. *Infect. Immun.* **2005**, *73* (3), 1811–1819.
(7) Ceri, H.; Olson, M. E.; Stremick, C.; Read, R. R.; Morck, D.; Buret, A. *J. Clin. Microbiol.* **1999**, *37* (6), 1771–1776.
(8) Pitts, B.; Hamilton, M. A.; Zelver, N.; Stewart, P. S. *J. Microbiol. Methods* **2003**, *54*, 269–276.
(9) Noguera D. R.; Pizarro G. E.; Regan J. M. In *Microbial biofims*; Ghannoum, M., O'Toole, G. A., Eds.; American Society for Microbiology, ASM Press: Washington, DC, 2004.
(10) Saginur, R.; St. Denis, M.; Ferris, W.; Aaron, S. D.; Chan, F.; Lee, C.; Ramotari, K. *Antimicrob. Agents Chemother.* **2006**, *50* (1), 55–61.
(11) Pereira, M. O.; Kuehn, M.; Wuertz, S.; Neu, T.; Melo, L. F. *Biotechnol. Bioeng.* **2002**, *78* (2), 164–171.
(12) Riekkola, M. L.; Åke Jonsson, J.; Smith, R. M. *Pure Appl. Chem.* **2004**, *76*, 443–451.
(13) Desai, M. J.; Armstrong, D. W. *Microbiol. Mol. Biol. Rev.* **2003**, *67* (1), 38–51.
(14) Berthod, A.; Rodriguez, M.; Armstrong, D. W. *Electrophoresis* **2002**, *23*, 847–857.
(15) Rodriguez, M. A.; Armstrong, D. W. *J. Chromatogr., B* **2004**, *800*, 7–25.
(16) Szumski, M.; Klodzinska, E.; Buszewski, B. *J. Chromatogr., A* **2005**, *1084*, 186–193.
(17) Lantz, A. W.; Bao, Y.; Armstrong, D. W. *Anal. Chem.* **2007**, *79*, 1720–1724.
(18) Michael, A.; Rodriguez, Andrew, W.; Lantz; Daniel, W. *Anal. Chem.* **2006**, *78*, 4759–4767.

and repeating twice the centrifugation step in which no significant loss of bacteria was registered.

Establishment of Conditions for Biofilm Formation Using 96-Microtiter Well Plates. Biofilm formation by *S. aureus* was first studied using flat-bottomed, polystyrene 96-microtiter well plates (Nunclon Δ surface), to establish the optimal bacterial concentration and growing time, which could then be applied to the CEC system. To find the optimal conditions for the bofilm formation, bacterial aliquots (250 μL/well) at different concentrations (10^1-10^7 cfu/mL in TSB) were seeded into plates, and 37 °C, 200 rpm at times ranging from 4 to 24 h, were applied. Afterward, bofilm formation was quantitatively assessed by crystal violet staining as in Kolari et al.[19] Assay quality control parameters indicated for screening procedures (screening window coefficient, Z' factor; signal-to-background, S/B, and signal-to-noise, S/N) were calculated according to Zhang et al.[20] and Bollini et al.[21] Repeatability of the assay signal was also assessed by registering well-to-well, plate-to-plate, and day-to-day variations. Effect of DMSO on destroying or preventing biofilm formation was also established by exposing bacteria to DMSO concentration ranging from 0.001 to 20%.

Establishment of Antimicrobial Susceptibility Profile of *S. aureus* Biofilms Using 96-Microtiter Well Plates. Susceptibility of the biofilms formed on the microplates to the five antibiotics was measured as minimal inhibitory concentration (MIC), and the values were compared with the MIC obtained for planktonic cultures grown under the same conditions. Stock solution of all antibiotics were prepared in sterile Mueller-Hinton broth and diluted in TSB using 2-fold serial dilutions in plates (from 0.12 ng/mL to 1024 μg/mL). Determination of the MIC for both plantonic and biofilm bacteria was done by using standard techniques according to CLSI as in Saginur et al.[10]

Establishment of Conditions for Bacterial Attachment Using Silica Plates. As coating agents, APTES, 4% (v/v in acetone), and poly(L-lysine), 0.0001 or 0.01% (w/v in sterile H_2O), were used following the protocols of Dhamodharan and McCarthy[22] and Rindorf et al.[23] Coated or uncoated silica plates were incubated with TSB-bacterial suspension (10^8 cfu/mL) using the conditions described above, and biofilm formation was also quantified by crystal violet staining, in a protocol modified to be carried out in a 5-mL total volume.

Atomic Force Microscopy Visualization of Bacterial Biofilm Formation on Silica Plates and on the Outside Surface of the Fused-Silica Capillary. Bacterial biofilms formed on silica pieces (squares of 6.25 cm^2) and on the outside wall of fused-silica capillaries were studied by AFM visualization. Five silica samples were included in the study: silica pieces coated with poly(L-lysine) 0.01% (w/v) alone, coated silica pieces where biofilms were formed during 0 and 18 h, and coated silica pieces that were exposed to TSB during 0 and 18 h. In samples of 0 h, the bacterial suspension or TSB was added to the silica and immediately removed. For tests on capillaries, the outer polyimide layer of the capillary was burned off, after which the outer surface was cleaned through acid preconditioning and coated with poly(L-lysine). The capillary was then exposed in a flow cell to BGE-bacterial suspensions (instead of TSB). During the biofilm formation, the flow cell was kept under sterile conditions. Owing to the continuous flushing of the capillary with fresh planktonic cells, no growth (nutrient) medium was needed. The protocols for forming biofilm on the capillary at 0 and 18 h were similar to those used for the CEC system, and for the control samples, only BGE solution was pumped through the flow cell. Uncoated rectangular silicon cantilevers (MikroMasch, model NSC15/No Al) were used for imaging. All the images (512 × 512 or 1024 × 1024 pixels) were captured using the intermittent contact mode (high-amplitude region) in ambient conditions (RH = 25−40% and T = 23 ± 3 °C). A damping ratio of ∼0.50−0.76 and a line frequency of 0.65−1.56 Hz was used for imaging. The images were processed with the scanning probe image processor (Image Metrology) software to remove possible slope or noise from the images.

Capillary Coating with Poly(L-lysine). Poly(L-lysine) was used to shield negatively charges, as the positively charged amino groups bind to the negatively charged capillary surface, offering positive charges for the bacteria to bind to. Before coating, the fresh capillary was subjected to hydrochloric acid pretreatment consisting of the following steps: 20-min flush with 1 M HCl, 10-min flush with 0.1 M HCl, and 20-min flush with sterile water at a pressure of 940 mbar. After acid preconditioning, the capillary was flushed for 30 min with sterile phosphate buffer saline at 940 mbar and for 30 min with poly(L-lysine) (1:10 v/v diluted in BGE) at 50 mbar, and finally the capillary was flushed once again with sterile PBS for 15 min to remove the extra poly(L-lysine) not bound to the capillary surface.

Capillary Coating with *S. aureus* and Formation of Biofilm. After acid preconditioning and coating of the capillary with poly(L-lysine), the capillary was treated for 17−20 h with bacteria. Briefly, the capillary was flushed for 30 min with *S. aureus* at low pressure (50 mbar) and left to stand filled with bacteria for 30 min. The whole 60-min treatment was repeated for a total of 17−20 times.

Effect of Antibiotics on Biofilm Formation. Antibiotics were either injected into the biofilm-coated capillary by applying a pressure of 50 mbar for 3 s, or the biofilm coating was treated with the antibiotics (penicillin and fusidic acid). In the treatment, the capillary was flushed for 2 min with the antibiotics, and then the antiobitics were left standing for 2 h.

Electroosmotic Flow (EOF) Measurements. EOF marker DMSO was used as an indicator for surface charge changes during the formation of biofilm. EOF was also monitored in the antibiotic/biofilm interaction studies by carrying out six successive runs. The EOF marker was injected by applying a pressure of 50 mbar for 3 s. Voltage was ±20 kV. The temperature of the capillary and autosampler was fixed at 37 °C. Before every injection, the coated capillary was flushed for 2 min with BGE. The time of the runs ranged from 5 to 20 min depending on the mobility of the EOF marker.

Measurement of Colony-Forming Units. The bacterial cell concentrations before and after biofilm formation were monitored

(19) Kolari, M.; Nuutinen, J.; Salkinoja-Salonen, M. S. *J. Ind. Microbiol.* **2001**, *27*, 343–351.
(20) Zhang, J. H.; Chung, T. D. Y.; Oldenburg, K. R. *J. Biomol. Screening* **1999**, *4* (2), 67–73.
(21) Bollini, S.; Herbst, J. J.; Gaughan, G. T.; Verdoorn, T. A.; Ditta, J.; Dubowchik, G. M.; Vinitsky, A. *J. Biomol. Screening* **2001**, *7* (6), 526–530.
(22) Dhamodharan, R.; Mc Carthy, T. J. *Macromolecules* **1999**, *32*, 4106–4112.
(23) Rindorf, L.; Jensen, J. B.; Dufva, M.; Pedersen, L. H.; Høiby, P. E.; Bang, O. *Opt. Express* **2006**, *14* (18), 8224–8231.

as follows: inlet vial content (sample marked as no. 1) and outlet vial content (sample marked as no. 2) were collected after 18 × 60 min treatment. Excess bacteria in the capillary after 18 treatments were removed by applying a 15-min BGE flush and collecting as sample no. 3. After 2-h treatment with antibiotics in the capillary, detached bacteria (sample marked as no. 4) were collected by applying a 15-min BGE flush. For each sample, appropriated different cell dilutions were prepared in TSB, and 100 μL was transferred to TSA plates and incubated overnight at 37 °C. Cfu/mL values were calculated on the basis of the TSA counts.

Statistical Analysis. Results of the experiments conducted in the 96-well microplates are given with the means and standard deviation of means (SD) of at least eight replicates. Results were processed with Microsoft Office Excel 2003 SP2 (for Windows) and SYSTAT software (version 11.00 for Windows). In the case of paired comparisons, the unpaired t of Student's test was used. $p < 0.05$ was always considered as statistically significant.

RESULTS AND DISCUSSION

Establishment of Conditions for Biofilm Formation Using 96-Microtiter Well Plates as a Reference Model. One of the simplest methods to study biofilm development involved the inoculation of bacteria into microtiter wells containing an appropriate growth medium. Different factors can thus be studied within a single test date.[24–27] A negative feature of the method is the discontinuous flow,[24] though in some studies this has been circumvented by adding a flow cell that allows the continuous flow of fresh nutrients into a chamber. The chamber is then equipped with a window to allow direct viewing of the biofilm under a microscope without disturbing the community. In this work, conventional 96-microtiter well plates were used to define preliminary conditions for capillary electrochromatographic studies (bacterial concentration, growing time) and clarify the stability of the ensemble. Because *S. aureus*, like most bacteria, is moderately negatively charged at neutral pH, it will adhere to hydrophobic or only slightly negatively charged surfaces such as polystyrene microtiter plates.[25] For detection of the biofilm formation, quantitative crystal violet staining was applied, followed by solvent extraction of the bound dye and measurement of spectrophotometric absorption.

The two critical factors of cell concentration and incubation time were studied first. In agreement with the result of Giacometti et al.,[27] a significant biofilm formation ($p < 0.005$, when compared to control wells) was found to occur at 10^6 cfu/mL of seeding concentration after 17 h of growing, and this value was selected for further experiments. In relation to the growing time, a detailed time-course study of biofilm formation was conducted, in which quality control parameters (S/N, S/B, Z') were used as indicators for optimal assay conditions (Figure 1). Optimal biofilm formation was shown to occur between 12 and 18 h (higher Z' values), although biofilms were also found to form at longer incubation times without refreshing of the growth medium. No significant biofilm formation was detected when the incubation lasted less than 4 h (Figure 1, inset). Apparently, at least 6−7 h are required for biofilm formation of *S. aureus*, and even 24-h incubation has not resulted in nutrient deprivation effects.[24–28]

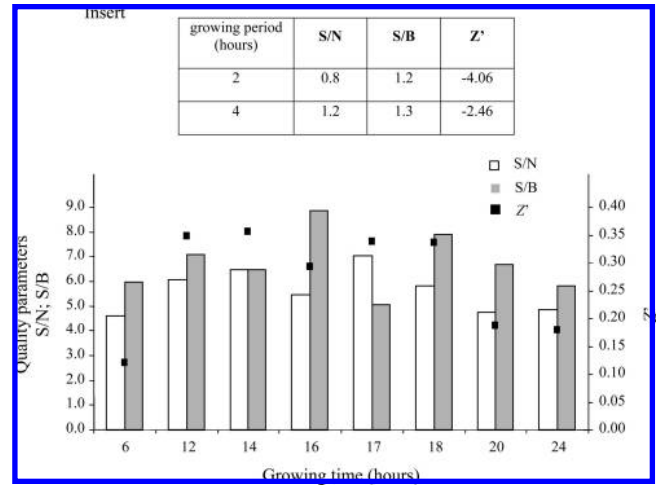

Figure 1. Effect of growing time on *S. aureus* biofilm formation using 96-microtiter well plates. Bacterial suspensions (10^6 cfu/mL) in TSB were allowed to form biofilms on 96-microtiter well plates for periods of 2, 4 (inset, table), and 6−24 h (figure). Quality parameters (S/N; S/B, Z') were used to evaluate the optimal incubation conditions.

Table 1. Performance of the *S. aureus* Biofilm Screening Assay in 96-Microtiter Well Plates, Measured with Quality Control Parameters and Variability Measures[a]

parameters	
Z'	0.47
S/B	15.6
S/N	6.2
separation band	1.3
well-to-well repeatability (CV, %)	14.8
plate-to-plate variability (CV, %)	3.2
day-to-day repeatability (CV, %)	1.5
DMSO tolerability (%)[b,c]	≤5

[a] Bacterial suspensions (10^6 cfu/mL in TSB were plated and allowed to grow for 18 h in 96-microtiter well plates. Quality control parameters were calculated for wells containing bacterial biofilm (maximal signal) or TSB (background signal) as proposed by Zhang et al.[20] and Bollini et al.[21] [b] The effect of DMSO on biofilms measured during the 18 h of biofilm formation (prevention effect). [c] The effect of DMSO on biofilms measured during the 2-h exposure after the formation (destruction effect).

Once the optimal conditions for *S. aureus* biofilm formation on microtiter plates were established, data supporting the adequate performance of this assay was collected (Table 1). This is standard procedure for the development of a screening assay.[29] The calculated Z' value fell within the acceptance limits for cell-based methods[20] and confirmed the feasibility of the assay for separating the signal due to bacteria from the background signal and thus for differentiating between biologically active and inactive compounds. The correct performance of the assay was also confirmed

(24) Amorena, B.; Gracia, E.; Monzón, M.; Leiva, J.; Oteiza, C.; Perez, M.; Alabart, J. L.; Hernández-Yago, J. *J. Antimicrob. Chemother.* **1999**, *44* (1), 43–55.
(25) Gross, M.; Cramton, S. E.; Götz, F.; Peschel, A. *Infect. Immun.* **2001**, *69* (5), 3423–3426.
(26) Stepanovic, S.; Vukovic, D.; Jezek, P.; Pavlovic, M.; Svabic- Vlahovic; M, *Eur. J. Clin. Microbiol. Infect. Dis.* **2001**, *20*, 502–504.
(27) Giacometti, A.; Cirioni, O.; Ghiselli, R.; Orlando, F.; Mocchegiani, F.; Silvestri, C.; Licci, A.; De Fusco, M.; Provinciali, M.; Saba, V.; Scalise, G. *Antimicrob. Agents Chemother.* **2005**, *49* (10), 4042–4045.
(28) Olson, M. E.; Ceri, H.; Morck, D. W.; Buret, A. G.; Read, R. R. *Can. J. Vet. Res.* **2002**, *66*, 86–92.
(29) Tierno, M. B.; Johnston, P. A.; Foster, C.; Skoko, J. J.; Shinde, S. N.; Shun, T. Y.; Lazo, J. S. *Nat. Protoc.* **2007**, *2* (5), 1134–1144.

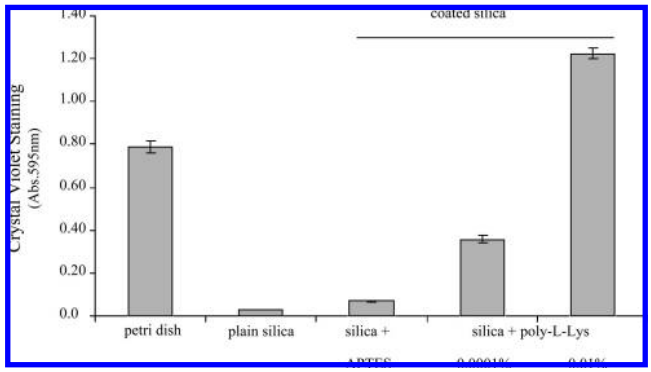

Figure 2. Effect of coating of silica plates on *S. aureus* biofilm formation. Bacterial suspensions (10^6 cfu/mL) were plated and allowed to grow for 18 h on plain silica plates and on plates coated with APTES or poly(L-lysine). Biofilm formation on plain polystyrene Petri dishes (i.d. 6 cm) was used as a control.

in the low well-to-well, plate-to-plate, and day-to-day variations of the strongest signal. In addition, the tolerance of assay for DMSO up to 5% (Table 1) was compatible with the use of DMSO as EOF marker in the CEC studies.

Establishment of Conditions for Bacterial Attachment and Biofilm Formation on Silica Plates. Biofilm formation is a three-step process that requires the primary adhesion of bacteria to a substrate surface followed by the formation of various cell layers.[1] For the formation of biofilms in fused-silica capillaries, the first step involved the binding of *S. aureus* to the silica surface, which under the conditions of the study was negatively charged. Experiments were thus first conducted to assess the ability of the bacteria to form biofilm on silica plates.

No biofilm was formed on uncoated silica surfaces (Figure 2), probably because unlike polystyrene, silica confers a highly negatively charged environment in which *S. aureus* cannot spontaneously bind. In this regard, it has been extensively shown that direct interaction of bacteria and surfaces is dependent on interionic forces (among other factors), so that biofilm formation is not favored when bacteria and surfaces are similarly charged.[25] This finding suggested the need for a positively charged coating agent, and indeed, coating with APTES and poly(L-lysine) (creating positive charges through covalent and ionic bonding, respectively) increased *S. aureus* binding to the silica surface (Figure 2). Biofilm formation was more extensive on silica plates coated with poly(L-lysine), and the effect was shown to be dependent on the reagent concentration (Figure 2).

Further evidence of the ability of *S. aureus* to form biofilm on silica plates coated with poly(L-lysine), 0.01%, under the indicated experimental conditions was provided by AFM (Figure 3). Silica sample treated with bacteria for 18 h in TSB showed clear structured bacterial aggregations of more than 300−500 nm, confirming the formation of biofilm. AFM images of other bacterial biofilms[30,31] are similar, but the thickness of the biofilm layer varies due to the physical properties of the bacterial strain.

Poly(L-lysine) Coating in Capillary Electrochromatography. On the basis of the results obtained with silica plates, poly(L-lysine) was chosen for the immobilization of the negatively

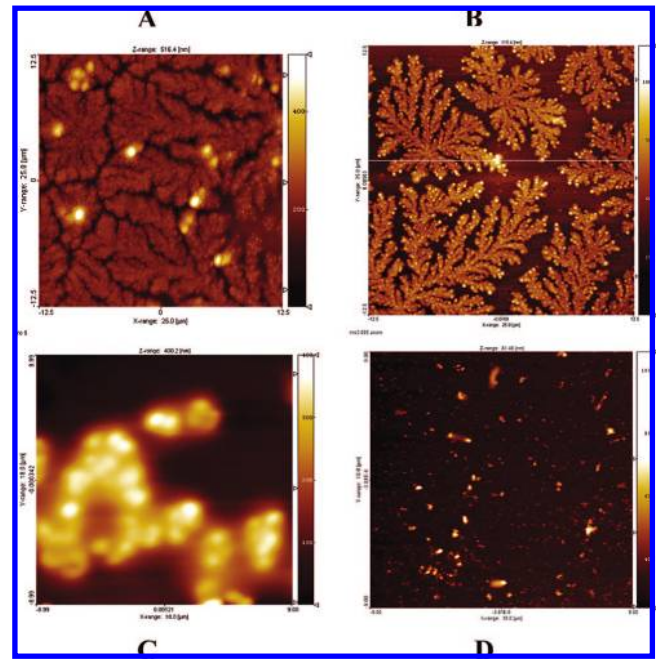

Figure 3. Visualization of *S. aureus* biofilms formed on silica plates and on the outside wall of fused-silica capillaries by AFM (tapping mode). Bacterial suspensions (10^6 cfu/mL) in TSB or BGE were plated and allowed to grow for 18 h on silica plates or on the outer wall of a capillary after coating with poly(L-lysine) 0.01%. Images A and C correspond to bacteria grown for 18 h on the coated silica and capillary, respectively, while B and D show the control results, where TSB and BGE were left to stand for 18 h on the silica and capillary, respectively.

charged capillary inner surface. Poly(L-lysine) provides positively charged amino groups, which form an excellent platform for the binding of planktonic bacteria. It is seen clearly from Figure S-1 (Supporting Information) that the biofilm coating changed the capillary inner wall into negatively charged surface.

Different concentrations of poly(L-lysine) were tested in capillary electrochromatography. It was found that even though 0.0001% (w/v) poly(L-lysine) was sufficient to assist the formation of the biofilm, the EOF was not reversed. Thus, higher concentrations, 0.01 and 0.05% (w/v) poly(L-lysine), were tested. Because 0.01% (w/v) poly(L-lysine) provided more a stable surface, it was selected for the coating of the fused-silica capillaries. The stability of the coating was evaluated by measuring the mobility of the EOF during six consecutive runs. The RSD of the EOF mobility was 2.73%.

Stability of Biofilm Formation. The immobilization of living bacteria as biofilm on a fused-silica capillary wall represents a new approach for study of the effect of antibiotics on biofilm. A prerequisite for such a study is the stability of the coating. We evaluated the stability of the biofilm on the capillary wall in a way similar to the stability of the poly(L-lysine) coating, by measuring the mobility of the EOF during successive runs. After preconditioning of the capillary and coating with poly(L-lysine) as described in the Experimental Section, the capillary was flushed for 30 min with *S. aureus* at low pressure (50 mbar) and left to stand filled with the bacterial solution for 30 min. The whole 60-min treatment was repeated 17−20 times corresponding to 17−20 h. The excess bacteria were then flushed out with BGE solution, and the stability of the biofilm was

(30) Öztürk, O.; Sudagidan, M.; Türkan, U. *J. Biomed. Mater. Res. A* **2006**, *81* (3), 663–8.
(31) Jonas, K.; Tomenius, H.; Kader, A.; Normark, S.; Römling, U.; Belova, L. *BMC Microbiol.* **2007**, *7* (70), 1–9.

Table 2. Effect of Coating Time on EOF Mobility in Capillaries Coated with *S. aureus*[a]

coating time (h)	EOF mobility (10^{-4}cm^2/(V s)) (n = 6)	RSD of EOF (%)
17	4.71	0.75
18	5.64	0.17
19	4.78	1.50
20	4.35	0.26

[a] In coating time, 1 h corresponds to one 60-min sequence of treatment with *S. aureus*.

Table 3. Cell Concentration as Colony Forming Units (cfu) Measured in Six Capillaries at Different Stages of Experiments by the Procedure Described in the Experimental Section[a]

	capillary					
no.	I	II	III	IV	V	VI
1	3.3×10^4	7×10^4	2.4×10^4	28×10^4	8.8×10^4	220×10^4
2	2.7×10^2	1.7×10^2	0.07×10^2	49×10^2	14×10^2	220×10^2
3	1.7×10^2	6.5×10^2	0.33×10^2	47×10^2	1.8×10^2	160×10^2

[a] Samples no. 1 and no. 2 are solutions collected from the inlet and outlet of capillaries, respectively, after 18 h (18 × 60 min treatment). Sample no. 3 represents the bacteria left in the capillary after 18-h coating, collected during 15-min flushing with BGE.

determined by measuring the EOF mobility in the capillary during six consecutive runs.

As can be seen from Table 2, the EOF mobility in the capillary does not vary markedly with coating times from 17 to 20 h. This is in agreement with the formation of biofilm by *S. aureus* observed on the 96-well microplates. RSD values of the EOF mobilities were nevertheless lowest with 18 h coating, and this was selected as optimal coating time.

It is also important to know the lifetime of the biofilm in the capillary. For these studies, the EOF mobilities were measured at pH 7.4 and 37 °C on two successive days, with 12 consecutive runs on each day. The capillary was left filled with BGE solution overnight, at 37 °C. The average RSD of the EOF mobilities for 24 successive runs over the two-day period was 2.1%, confirming that the capillary was stable for at least two days. In addition, a low average RSD of 0.35% for the EOF mobilities of 40 consecutive runs carried out with the same capillary indicated a high stability of the biofilm surface.

To determine the reproducibility of the coating, five capillaries were coated by the same procedure (see Experimental Section). The average RSD of the EOF mobility in the five capillaries was 4.3%. Every EOF mobility value was an average of six successive runs. CFU measurements (Table 3) showed that the bacteria were alive during the coating procedure (samples no. 1 and no. 2, numbering explained in Experimental Section) after 18 h. Sample no. 2 was decreased by 10^2-fold in comparison to the suspension (sample no. 1) applied into the capillary. Also sample no. 3, which was obtained after the removal of excess free floating bacteria by flushing the capillary for 15 min with BGE, showed the same level of decrease. The difference stayed the same (10^2-fold) even after 10 times higher bacterial concentrations were applied into the capillary (see capillary VI in Table 3). The results indicated that bacteria had attached to form biofilms inside the capillary.

The effect of pH (values from 5.0 to 7.4) on biofilm stability was investigated by measuring the EOF mobilities (results not shown here). The biofilm stability was better at pH 7.4 (RSD 0.76%) than at lower pH (RSD 7.2%- 9.9%).

The outer wall of the capillary, subjected to the same conditions for biofilm formation as used in the CEC studies, was visualized by AFM. Images obtained (Figure 3) showed again bacterial attachment and aggregate formation that was not seen in the control images.

Effect of Antibiotics on Biofilm. It has repeatedly been established that bacterial biofilms are more resistant to many antimicrobial agents than are planktonic cells, and this increase in resistance is probably multifactorial. The antiobiotic susceptibility profile obtained in microtiter plates proved that *S. aureus* biofilms were chemoresistant, as MIC_{50} biofilms increased up to 1000 times when compared to planktonic MIC_{50} values. The effective antibiotics were rifampicin, fusidic acid, penicillin, and oxacillin, with biofilms MIC_{50} values of 3.1×10^{-2}, 6.3×10, 13.0×10^{-2}, and 25.0×10^{-2} μg/mL, respectively. Biofilms were found to be less susceptible to vancomycin (biofilm MIC_{50} value, 1 μg/mL).

Our interest in this work was to assess the potential of capillary electrochromatography for the study of interactions between antimicrobial agents and biofilm.

The interactions between antibiotics and biofilm were investigated by injecting the antibiotics into a capillary immobilized with living biofilm, and first retention factors and then reduced mobilities were calculated. The electropherograms of EOF and antibiotics on uncoated capillary and on capillary coated with biofilm are presented in Figure S-2 (Supporting Information). The retention factor is a useful measure of the interaction of an analyte with the coating

$$k' = \frac{t_m - t_{eo}}{t_{eo}} \quad (1)$$

where migration times of the analyte (t_m) and an unretained component (t_{eo}) are used to describe the ratio of the time the analyte stays in the stationary phase to the time the analyte is in the mobile phase. Equation 1 can be used for calculation of the retention factor of an uncharged compound. For charged analytes, Rathore and Horvath[32] have introduced k'' as a measure of retention due to reversible binding of the analyte to the CEC stationary phase. For both uncharged and charged analytes

$$k'' = \frac{t_m(1 + k''_e) - t_{eo}}{t_{eo}} \quad (2)$$

where the velocity factor $k''_e = u_{ep}/u_{eo}$ (u_{ep} is the electrophoretic mobility of the analyte in the uncoated capillary and u_{eo} is the electroosmotic mobility in the coated capillary). Wiedmer et al.[33] transformed eq 2 into a more convenient form (3):

$$k' = t_m\left(\frac{1}{t_{eo}} + \frac{1}{t_{m'}} - \frac{1}{t_{eo'}}\right) - 1 \quad (3)$$

(32) Rathore, A. S.; Horváth, C. *Electrophoresis* **2002**, *23*, 1211–1216.
(33) Wiedmer, S. K. M.; Jussila, R.; Hakala, M. S.; Pystynen, K.-H.; Riekkola, M.-L. *Electrophoresis* **2005**, *26*, 1920–1927.

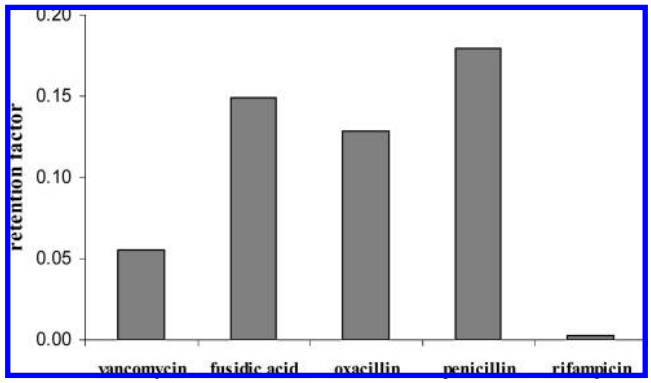

Figure 4. Retention factors for antibiotics on biofilms. Running conditions: fused-silica capillary, total length 38.5 cm, length to detector 30 cm, inner diameter 50 μm, outer diameter 375 μm; capillary temperature 37 °C; sample injection 3 s at 50 mbar; running voltage 20 kV; UV detection 214 and 280 nm.

where $t_{m'}$ and $t_{eo'}$ are, respectively, migration times of the analyte and the EOF in the uncoated capillary. We used eq 3 for the measurement of the retention factors for antibiotics. The migration times of antibiotics studied ($t_{m'}$) and EOF ($t_{eo'}$) in the uncoated capillary was determined by capillary electrophoresis. The migration times of five antibiotics were also measured on biofilm-coated capillaries. From the retention factors k', the retention of the antibiotics by the biofilm was seen to decrease in the following order (Figure 4): penicillin > fusidic acid > oxacilin > vancomycin > rifampicin.

The RSDs of the retention factors for these antibiotics are in the range from 0.25 to 0.9%.

Kenndler[34] has introduced the reduced mobility to express the effect of the EOF on all separation parameters relevant for capillary electrophoresis. The reduced mobility μ_i is given by

$$\mu_i = \frac{\mu_{\mathrm{eff},i}}{\mu_{\mathrm{eff},i} + \mu_{\mathrm{eo}}} \qquad (4)$$

where μ_{eff} is the effective electrophoretic mobility of the analyte and μ_{eo} is the EOF in the coated capillary. We calculated the reduced mobility to explore the relative interactions of antibiotics with uncoated and biofilm-coated capillaries. As can be seen in Figure 5, all antibiotics interact more strongly with biofilm than with uncoated capillaries. Penicillin has the strongest interaction with the biofilm, and vancomycin the weakest. The order of the interactions between antibiotics and biofilm calculated as reduced mobility (penicillin > fusidic acid > oxacilin > vancomycin > rifampicin) follows the order calculated on the basis of retention factors. We should notice the fact that mobility is expressed by the net migration velocity while the retention factor reveals interactions between analytes and the stationary phase. The results together show not just the susceptibility of the biofilm but also the integration of contributions by many factors including net charge, velocity, and size of the antibiotic.

(34) Kenndler, E. J. *Microcolumn Sep.* **1998**, *10*, 273–279.

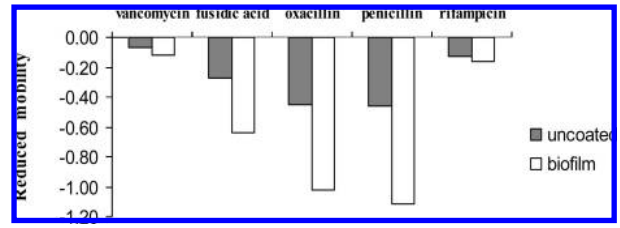

Figure 5. Reduced mobilities of antibiotics on uncoated capillary surfaces and biofilms. Running conditions: fused-silica capillary, total length 38.5 cm, length to detector 30 cm, inner diameter 50 μm, outer diameter 375 μm; capillary temperature 37 °C; sample injection 3 s at 50 mbar; running voltage 20 kV; UV detection at 214 and 280 nm.

Since the interaction time between antibiotics and biofilms was very short when the antibiotics were injected, two of antibiotics, penicillin and fusidic acid, were also left to stand for 2 h, before their effect on the biofilm was studied by EOF and cfu measurements (Results not shown.). No notable differences were found in the EOF after the injection of antibiotics and after a 2-h treatment of the capillaries with antibiotics, and cfu measurements confirmed that planktonic cells were still viable after the 2-h treatment. However, the cfu values revealed that increase in the concentrations of penicillin and fusidic acid from 10 (cfu values of sample no. 4 were 0 and 50, respectively, indicating intact biofilm inside the capillary) to 100 μg/mL (cfu values of sample no. 4 were 3.0×10^2 and 4.0×10^2, respectively) in the 2-h treatment of biofilms resulted in more detached cells, thus indicating that antibiotic compounds at higher concentrations started to destruct the biofilm.

CONCLUSIONS

A novel electrochromatographic technique involving coating of open tubular fused-silica capillaries with living planktonic cells was developed for biofilm studies. The technique allows highly efficient and easy characterization of interactions between biofilms and potentially active antimicrobial compounds under different conditions, e.g. continuous flow. Reagent and cell consumption is minimal. Our preliminary results show that viable bacterial biofilms can be studied by capillary electrochromatography.

ACKNOWLEDGMENT

We express our thanks to Dr. Kari Kreander for his assistance. Financial support was provided by the Research Council for Natural Sciences and Engineering, the Academy of Finland under Grant 116288 (M.-L.R.), and Borg's Foundation at Åbo Akademi University (P.M.V.).

SUPPORTING INFORMATION AVAILABLE

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

Received for review February 12, 2008. Accepted April 18, 2008.

AC800294F