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Dense Layer of Bacteriophages Ordered in Alternating Electric Field and Immobilized by Surface Chemical Modification as Sensing Element for Bacteria Detection

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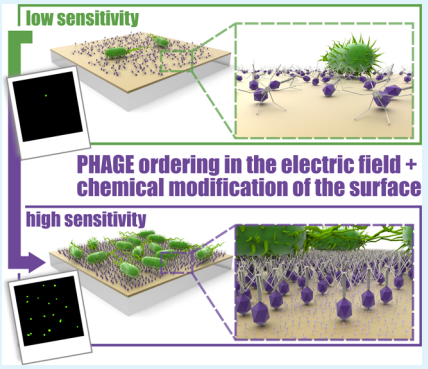
Supporting Information

ABSTRACT: Faster and more sensitive environmental monitoring should be developed to face the worldwide problem of bacterial infections. To remedy this issue, we demonstrate a bacteria-sensing element that utilizes dense and ordered layers of bacteriophages specific to the given bacteria strain. We combine (1) the chemical modification of a surface to increase the surface coverage of bacteriophages (2) with an alternating electric field to greatly increase the number of properly oriented bacteriophages at the surface. Usually, in sensing elements, a random orientation of bacteriophages results in steric hindrance, which results in no more than a few percent of all receptors being available. An increased number of properly ordered phages results in the optimal performance of phage receptors, manifesting in up to a 64-fold increase in sensitivity and a limit of detection as low as 100 CFU mL^{-1}. Our sensing elements can be applied for selective, sensitive, and fast (15 min) bacterial detection. A well-studied pair T4 bacteriophage—bacteria *Escherichia coli*, was used as a model; however, the method could be adapted to prepare bacteriophage-based sensors for detection of a variety of bacterial strains.



KEYWORDS: *T4 bacteriophages, orientation, alternating, electric field, surface modification, biosensor, nonlinear electrophoresis*

INTRODUCTION

The threat of bacterial infections concerns not only healthcare but also other areas, for example, the food or environmental industries. The European Centre for Disease Prevention and Control reported that only in Europe, 4.1 million patients are affected by healthcare-associated infection each year.[1] In the majority of cases, serious repercussions can be avoided, thanks to the fast and reliable detection of bacteria. Unfortunately, classical methods, although cheap and straightforward, require up to 72 h to produce a reliable output. Aid could be found in the development of sensors for bacterial detection, which utilizes bacteriophages as sensing elements. Bacteriophages are specialized viruses, whose hosts are bacteria. Phages undergo evolution; thus, they are highly efficient and specific in capturing bacteria. As there is an abundance of different bacteriophage types, it is possible to design biosensors detecting almost all bacterial strains. Additionally, their low preparation cost, stability in a wide range of temperature and pH, and, in many cases, tolerance of proteases[2] and organic solvents[3] make phages advantageous as natural sensing elements for bacterial detection.

The idea of using phages for sensing has been gaining increasing interest among scientists.[4−10] Until now, improvements in phage-based biosensors have occurred mainly due to an increase in the number of phages within the sensing elements. First, physisorbed layers of phages were used. For example, Olsen et al. showed the possibility of the detection of *Salmonella typhimurium* with a limit of detection of around 100 cells mL^{-1}.[11] However, the authors utilized filamentous phages isolated from a phage library, which is a very difficult, expensive, and time-consuming procedure. Another approach replaced physisorption with more sophisticated chemical binding by chemical modification of a surface. Comparing to that on bare gold, Singh et al. increased the number of deposited T4 phages by a factor of 5−7 using dextrose- and sucrose-covered surfaces and 37 times due to modification of the surface with cysteamine and glutaraldehyde.[12] However, this manifested in only a 4- and 12-fold increase in the sensitivity of detection of *Escherichia coli* bacteria, respectively. These results indicate significant deviation from linearity in the improvement of the sensitivity of phage-based sensors with increasing phage surface coverage. This was due to the random orientation of virions at the surface. As receptor-binding proteins (RBPs) are present only at the end of

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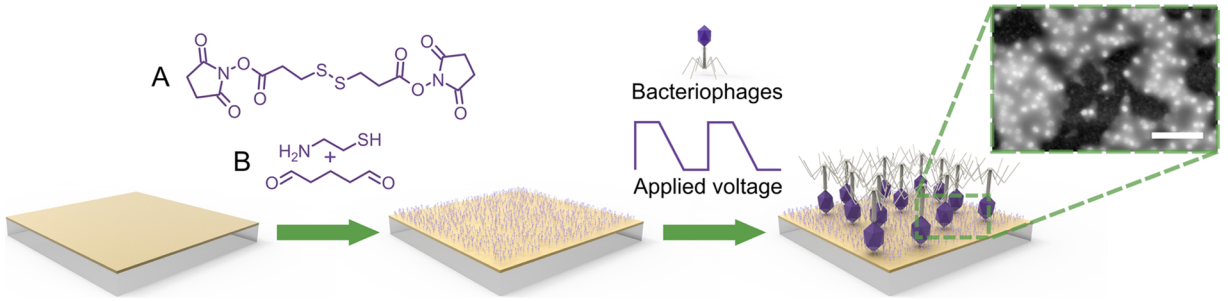

Figure 1. Scheme for fabricating dense sensing layers of ordered phages. At first, the gold surface was modified with (A) dithiobis(succinimidyl propionate) (DTSP) or (B) cysteamine and subsequently glutaraldehyde. The second step consisted of the deposition of phages in an alternating electric field. The magnification box shows the atomic force microscopy (AFM) image of the densely packed sensing layer of ordered bacteriophages. Scale bar corresponds to 1 μm. For the third step of the sensing element preparation protocol (not shown), the surface between the virions was covered with an inert protein, casein, to prevent unspecific binding of bacteria to the surface.

the tail spike of the T4 phage, such random orientation of the phages caused the majority of RBPs to be unavailable due to steric hindrance.[13] Moreover, a horizontal alignment of the virions is favored by entropy. In such an orientation, RBPs are not able to participate effectively in the bacteria capturing process. Of course, an increase in the number of randomly oriented virions results in a statistically higher amount of available receptors. Naidoo et al. estimated a "jamming" surface concentration of T4 phages to be 19 phages μm^{-2}, which gave the highest affinity for capturing bacteria.[14] Above this value, an increase in phage surface coverage does not result in the higher sensitivity of sensing elements. To overcome this limit, virions should be positioned in a tail-up orientation, that is, an arrangement in which the majority of RBPs is involved in the detection process. Proper alignment of virions also increases the theoretical maximum density of phage surface coverage to the geometrical limit, which is around 100 phages μm^{-2}.

Tolba et al. showed that T4 bacteriophages might be oriented by sophisticated and laborious genetic modifications.[15] Capsids were biotinylated due to the fusion of the small outer capsid protein and a biotin carboxyl carrier protein. Recombinant phages were oriented at the surface of streptavidin-coated magnetic beads. Another approach was demonstrated by Han et al.[16] and Anany et al.,[17] who described the orientation of phages due to electrostatic interactions. These cases are, however, inconclusive because the authors did not provide evidence for an improvement in the characteristics of the biosensors due to proper arrangement. Also, the genetic modification might render phages inactive.

In our recent paper, we provided evidence that bacteriophages might be oriented in a constant electric field due to the nonzero permanent dipole moment of the virions.[18] The majority of bacteriophages share this intrinsic property (as well as general structural design); thus, such an approach is not restricted to only model systems (T4 bacteriophage—*E. coli*). We achieved a 4-fold increase in captured *E. coli* cells in the case of ordered versus that of randomly oriented virions without a significant change in the surface coverage of the T4 bacteriophages. The improvement in sensitivity was solely due to the proper alignment of the virions, which was obtained mostly due to electrostatic interactions within the electrical double layer (EDL), that is, in close proximity to the surface.

Here, we provide proof for the combined effect of increased phage surface coverage and proper orientation of virions in an alternating external electric field. The procedure consists of two steps: (1) the chemical modification of the surface in one of two different ways and (2) the deposition of phages in an alternating electric field (Figure 1). Chemical immobilization of the bacteriophages allowed the synthesis of densely packed layers. Simultaneously, a properly designed alternating electric field enabled the effective orientation of the phages. The combination of these steps resulted in a significant increase in the number of available RBPs and a decrease in steric hindrance causing an up to 64-fold increase in sensitivity of the sensing elements with only a 2.5-fold increase in phage surface coverage. Alternating pulses were specially designed to facilitate the process.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Aldrich (Poznan, Poland) and used without additional purification. All growth media, buffers, and solutions were prepared according to guidelines provided in our previous work.[18]

Phage Preparation and Bacteria Culture. The final concentration of phages in TM buffer, utilized for phage deposition, was $1.11 \times 10^{11} \pm 0.15 \times 10^{11}$ PFU mL^{-1}. For more details on the preparation and purification of the phages, please refer to the Supporting Information.

E. coli BL21(DE3) (obtained from the Institute of Biochemistry and Biophysics in Warsaw, Poland) was used as a target analyte for the T4 phages. The strain harbored plasmids coding green fluorescence protein (GFP) and providing resistance to chloramphenicol and kanamycin. Bacteria were prepared according to the standard procedures. Chloramphenicol (25 mg) and kanamycin (50 mg) were added to 1 L of LB media. Isopropyl β-D-1-thiogalactopyranoside was added to LB media to a final concentration of 1 mM to induce the production of the GFP protein. At the beginning, a single colony from an agar plate was inoculated into LB medium for overnight culturing (37 °C, 200 rpm). The bacteria were cultured to obtain suspensions of OD_{600} = 0.40. The culture was then centrifuged (4000 rpm, 4 min) and resuspended in a greater amount of physiological saline. Dilutions ranged from 4 to 1000 times.

Enterobacter aerogenes PCM 1832 (purchased from Polish Collection of Microorganisms, Wroclaw, Poland) was used as a nonspecific strain to evaluate the selectivity of the prepared sensing layers of T4 phage. Bacteria were stained with SYTO RED 62 to be visible as red objects in confocal microscopy. More details are provided in the Supporting Information.

Solid Supports and Chemical Modification. T4 bacteriophages were deposited onto solid substrates. Glass plates (1 mm × 8 mm × 25 mm) covered first with a 5 nm layer of titanium and subsequently with a 150 nm layer of gold were used. The gold layer was hardened using an argon canon. Substrates were obtained from the Institute of Electronic Materials Technology (Warsaw, Poland). Prior to use, substrates were gently wiped and washed thoroughly with water and ethanol. Afterward they were sonicated sequentially in acetone, ethanol, isopropanol, and twice in deionized water for 5 min in each solvent. These substrates were

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used as the support for T4 layers (samples denoted as "bare gold") or were further subjected to chemical modifications.

Modification with DTSP. The DTSP modification procedure was adapted from research by Arya et al.[19] Clean solid substrates were immersed in a 2 mg mL^{-1} solution of DTSP in acetone for 20 h at room temperature. Then, the modified substrates were washed with acetone and rocked for 15 min in acetone to get rid of unreacted substrate. The as-modified plates were used for phage deposition within 1 h after modification and washing (samples denoted as "DTSP").

Modification with Cysteamine and Glutaraldehyde. The utilized procedure was reported by Singh et al.[12] Clean plates were exposed to a 50 mM solution of cysteamine hydrochloride for 20 h at 40 °C. Afterward, substrates were rinsed thoroughly with acetone and water and activated by incubating in a 2% (v/v) solution of glutaraldehyde in water for 1 h at room temperature. The substrates were then washed with a large amount of water and used for phage deposition within 1 h (samples denoted as "CA + GA").

Deposition of Phages in the Electric Field. The details of the setup used for generation of the electric field are given in our previous work and in the Supporting Information. The deposition cell was designed and manufactured by us according to blueprints provided elsewhere.[18] The cell consisted of three deposition chambers, which were capacitors filled with bacteriophage suspension. The plates of the capacitors were (1) the gold surface of the sensor and (2) a copper electrode. Because of the 200 μm PTFE separator insulating the copper electrode, there was no faradaic current in the system. The setup allowed for full control over the phage deposition procedure.

Phage suspension (160 μL) in TM buffer was placed in the deposition chamber of the deposition cell. Signal wire was connected to the gold surface of the plate on which the phages were deposited. Bacteriophages have a negatively charged head and positively charged fibers; thus, a positive voltage was applied to the gold plates to achieve the desired orientation. A ground was connected to the insulated copper electrode. The distance between electrodes was 1 mm. No potential was applied in the case of the control samples. Deposition of the phage-based sensing layers lasted for 30 min. Phages that did not bind to the surface were rinsed with a large excess of TM buffer. Then, the plates were immersed in a 5% suspension of skim milk in TM buffer for 30 min to block any surface unoccupied by phages with the inert protein casein and to avoid nonspecific binding of bacterial cells.

Analysis of the Number of Deposited Phages and Phage Deactivation. A plaque counting method was used to evaluate the influence of electric field and surface modification on the activity of the studied T4 bacteriophages. For more details please refer to the Supporting Information.

Analysis of Sensing Layers. The prepared biosensors were immersed in a bacteria solution for 15 min. Confocal microscopy was used to evaluate the efficiency of the studied sensing elements. For each sensing layer, at least 10 images in random positions were taken, which were analyzed and the number of bacteria per 1 mm^2 for each sample was determined.

For each set of phage deposition conditions, we performed at least three independent experimental runs. For the control, we always prepared two types of plates covered with (1) randomly oriented phages and (2) only casein (to check the possibility of nonselective deposition of bacteria on casein). Corresponding error bars were provided. The results were normalized with respect to randomly oriented phages. A more detailed description is provided in the Supporting Information.

Instrumentation. Detailed information about the instrumentation used is provided in the Supporting Information.

RESULTS AND DISCUSSION

We aimed to develop a method that could be easily adaptable for creating sensors based on bacteriophages for the detection of any desired strain of bacterium. We decided to use an intrinsic property of virions to achieve higher sensitivity and a lower limit of detection.[20] Therefore, we studied T4 bacteriophage together with its host bacteria *E. coli* as a model system. T4 bacteriophage belongs to *Caudovirales* order, which contains a vast majority of all known bacteriophages. *Caudovirales* share a common structural design, that is, genetic information (dsDNA) is stored in a capsid, to which a tail with fibers is attached.[21] Such asymmetry of the structure results in a permanent dipole moment of the virion. As a result, phages are susceptible to an electric field, which allows for orientation of the virions in an electric field. This property has already been utilized by Shurdov and Gruzdev,[22] who studied the tertiary structure of DNA within bacteriophage λ by observing the changes in fluorescence of acridine orange-stained virions oriented in the electric field. We aimed to develop a method for the preparation of chemisorbed and ordered layers of bacteriophages.

The virion of bacteriophage T4 is composed of a 110 nm × 80 nm icosahedral head (capsid) and a 98 nm contractile tail ended with fibers. dsDNA of length 168.9 kbp is stored in the capsid.[23−25] Depending on the conformation of the fibers, the measured values of the permanent dipole moment of T4 bacteriophages varies from 20 000 to 200 000 D.[26−29] Baran and Bloomfield showed that tail fibers are highly positively charged and the head−tail structure is negatively charged.[30] Such a distribution of charges was developed in the process of evolution as an improvement, facilitating the infection of the host bacterium. Positively charged fibers are electrostatically attracted to bacteria, which have a negatively charged surface.[30]

Orientation of Phages in the Alternating Electric Field. The device for creating sensing layers of phages deposited in an external electric field was in fact a capacitor filled with a bacteriophage suspension. The plates of this capacitor were the gold surface of the solid support and an isolated copper electrode (Figure S2). By proper design of the experimental setup, we deliberately ruled out the risk of faradaic current to avoid deactivation of the phages and uncontrolled redox reactions. This resulted in a limited effect of the electric field, which spread only within Debye length from the electrode. This distance was in the order of only a few nanometers, as phages were suspended in a chosen buffer of relatively high ionic strength. When a constant electric field was applied, the electric field within the phage suspension was quickly suppressed by the formation of an EDL. The orientation of the phages was mostly due to the electrostatic attraction between the capsids and the surface and electrostatic repulsion between the fibers and the surface. We demonstrated this effect recently, showing a 4-fold increase in efficiency of a bacteriophage-based sensing layer, in which T4 phages were ordered and the phage surface coverage was not affected by application of the constant electric field.[18] Here, we aimed to further increase the sensitivity of phage-based biosensors by facilitating the process of orientation of the virions by limiting the screening of the applied field. There was only very limited possibility to increase the Debye length of the studied system, as virions are unstable in low osmotic strength solutions.[31] Therefore, we decided to utilize an alternating electric field, which allowed the EDL to spread during interpulse periods.

At the beginning of each pulse, the field spread across the whole suspension until the EDL formed and screened the external field. The specific time constant, which describes the process of formation of the EDL and screening of the electric field by ions, is comparable with the time in which ions travel through the Debye length driven by electrophoretic forces. In our case, the estimated time constant was 2.6 μs (detailed calculations provided as Supporting Information). This prediction was in good agreement with other published results.[32] After this time, the external electric field was screened and

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remained effective only within Debye length, which in the studied system was a few nanometers.

To be sure that the system relaxed completely (i.e., that the EDL spread), we assumed that the time between pulses (i.e., with 0 V applied) should be comparable with the time of formation of the EDL. Simultaneously, the interpulse time cannot be too long as it should be much shorter than the time constant of the rotational diffusion of virions to avoid defocusing of the phages. The rotational diffusion coefficient of the T4 phages varies from 273 to 453 s^{-1}, depending on the conformation of the fibers of the virions. These values correspond to a rotational time constant (i.e., time of defocusing) of between 1.1 and 1.8 ms.[33]

Higher frequencies (shorter pulses and less time between pulses) corresponded to more events of active orientation of the phages by the effective field and limited defocusing of the phages. Lower frequencies allowed for exploitation of the whole length of each pulse in the most effective way to focus phages along the lines of the field and for the EDL to relax completely during interpulses periods, but increased defocusing. These contradicting time scales should be balanced. We judiciously chose 20 μs as the duration time of each pulse as well as the time between pulses, as it enabled a good compromise between all of the crucial factors.

In the case of a rectangular voltage waveform, at the beginning of each pulse an electric field spread across the solution. However, the drop in the applied potential to zero at the end of the pulse also generated an electric field, but directed in the opposite direction (Figure 2). This resulted in a zero net effect on virion orientation. To overcome this, we designed a voltage waveform that allowed the creation of a nonzero effect on the orientation of the phages. We exploited the nonlinear electrophoretic effect, which has been observed for a number of different particles, for example, polystyrene latex and aluminum oxide particles, and even yeast cells.[34] When the electric field is strong enough, that is, 100 V cm^{-1} and higher, which was the case in our system, the dependence between electrophoretic velocity and applied electric field is nonlinear.

$$\nu_{eph} = \mu_{eph}E + \mu^{(3)}_{eph}E^3 \quad (1)$$

where ν_{eph} is the electrophoretic velocity, μ_{eph} is the field-independent electrophoretic mobility coefficient, $\mu^{(3)}_{eph}$ is the nonlinear electrophoretic mobility coefficient, and E is the strength of the electric field. We designed a trapezoidal voltage waveform that allowed a substantial difference to be obtained between the values of effective electric field with a sharp increase and slow drop of the applied potential at the beginning and at the end of the pulse, respectively (Figure 2). Due to this nonlinear electrophoretic effect, phages were oriented at the desired position along the lines of the electric field. To ensure a proper difference in the electric fields, we decided that a slow drop of potential at the end of the pulse should last 20 μs. Together with 20 μs of pulse of maximal applied potential and 20 μs without any applied potential, this resulted in a 60 μs period, which corresponded to a frequency of 16.67 kHz. All exploited types of voltages and the resulting electric fields are presented in Figure 2. Changes in the electric field upon application of different voltages were calculated based on eq S5.

To check the validity of our hypothesis, we first prepared a set of sensing layers consisting of phages deposited with the use of rectangular pulses of electric field (10 V, various frequencies) (details in Figure S3). Utilization of such a waveform did not improve the efficiency of bacteria capturing in comparison to a constant electric field. The effect was only due to electrostatic interactions with the surface. As a control we also tested the trapezoidal waveform of frequency of 1 Hz (333 ms without an applied potential between the pulses), which allowed phages to defocus by rotational diffusion during interpulse periods. Again, no differences were observed in comparison to results from the square waveform and constant electric field proving that mainly electrostatic interactions between the surface (polarized positively) and capsids (negative pole of the dipole) dominated when the net focusing of the phages in the bulk was negligible. Utilization of the designed (10 V, 16.67 kHz) trapezoidal voltage waveform resulted in a 2.5-fold increase in sensitivity in comparison to that using the square waveform and constant electric field. When compared to that of the randomly oriented phages, the increase upon application of the trapezoidal waveform of properly adjusted frequency was around 10-fold (Figure 2).

We also confirmed our previous findings that 10 V was the most effective applied voltage for orientation of the phages (for details, see Figure S4). This value of applied voltage seemed to balance the electrophoretic force, which oriented the phages (higher potential was more favored), against unwanted phenomena like deactivation of the phages and increases in charge density of the EDL (higher potential was less favored).

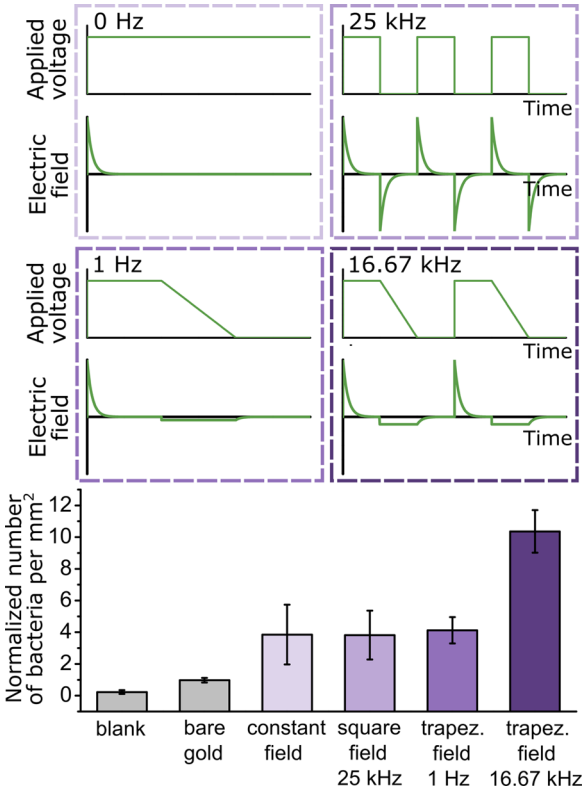

Figure 2. Comparison of sensitivity (normalized number of caught bacteria) of phage-based sensing layers prepared using various sets of parameters of the electric field. In all cases, the applied voltage was 10 V. Upper panels present all of the applied voltages and the resulting electric fields.

Combining Chemical Modification and Alternating Electric Field. We decided to follow two protocols of surface modification. Singh et al. developed a method in which the gold surface was covered with cysteamine and then glutaraldehyde.[12] Arya et al. utilized the self-assembly of monolayers of DTSP.[19]

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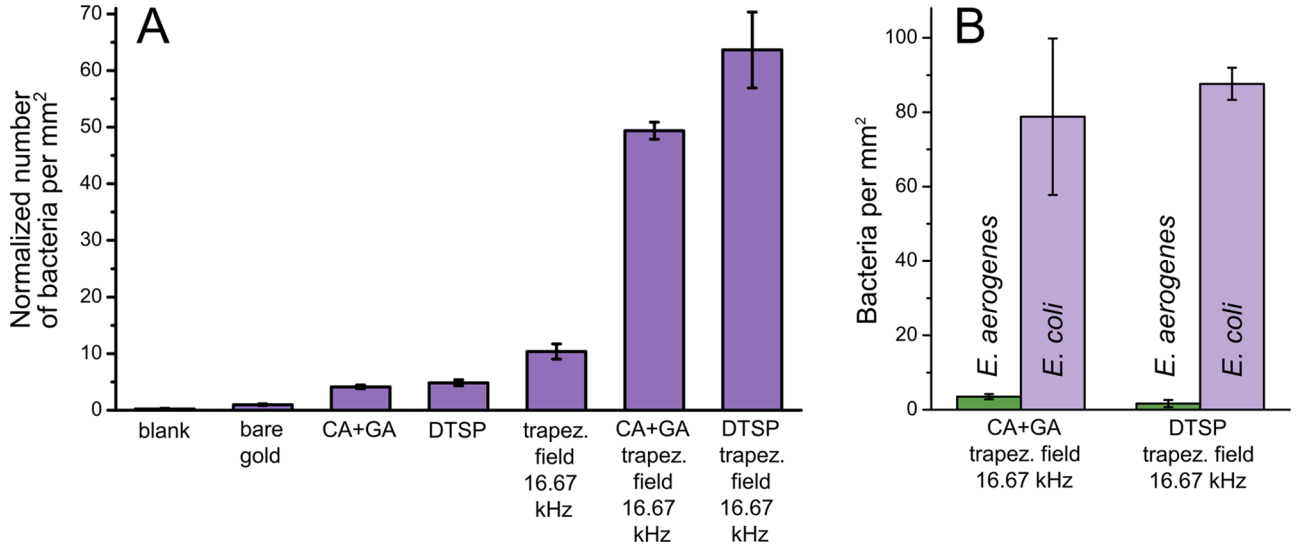

Figure 3. (A) Sensitivity (normalized number of caught bacteria) of phage-based sensor prepared with chemical modifications of the surfaces and applied trapezoidal voltage waveform. Combined effect of both methods resulted in up to a 64-fold increase in sensitivity. (B) Number of bacteria caught from tap water spiked with two species of bacteria: *E. coli* and *E. aerogenes*. Concentrations of bacteria were 4×10^5 and 5×10^5 CFU mL^{-1}, respectively. Great selectivity was assured by the natural properties of the bacteriophages.

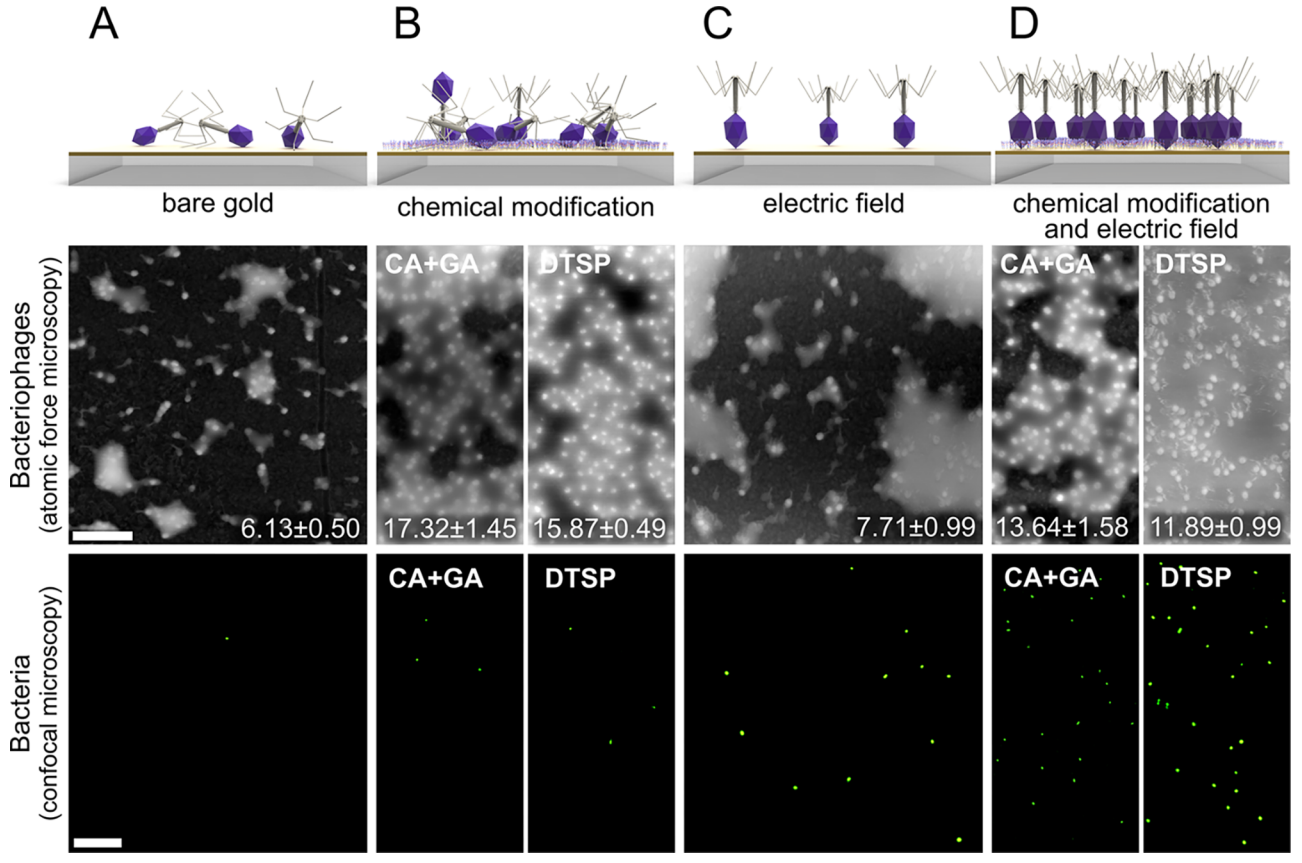

Figure 4. Schematic illustrations and corresponding AFM images of layers of phages deposited on (A) bare gold or in various conditions: (B) with chemical modifications of the surface (with cysteamine and glutaraldehyde (CA + GA) or DTSP), (C) in an electric field from trapezoidal voltage (16.67 kHz, 10 V), and (D) a combination of thereof. Numbers on AFM images correspond to surface coverage of phages in each layer in phages μm^{-2}. The presented values are means obtained from analysis of at least three samples. Corresponding confocal microscopy images show surface of sensing layers with caught bacteria. Each green spot depicts single bacterium. Scale bars on AFM and confocal microscopy pictures correspond to 1 and 50 μm, respectively.

The reported most effective surface coverage of T4 bacteriophages reached 18 phages μm^{-2} (method of Singh et al.) and around 20 phages μm^{-2} (method of Arya et al.; based on Figure 3c in ref 19). It should be noted that in the case of DTSP, a lower number of T4 phages (20 phages μm^{-2}) performed better in capturing bacteria compared to that with the maximal obtained

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surface coverage of around 30 phages μm^{-2} (based on Figure 3d in ref 19). This confirmed a jamming effect due to clustering of the virions at high surface coverage. The jamming coverage for the T4 phages was estimated by Naidoo et al. as 19 phages μm^{-2}.[14]

To further increase the sensitivity of the prepared sensing layers, we combined the chemical immobilization of phages with orientation using an electric field. We expected to exploit the combined effect of both procedures and thus to create densely packed layers of properly ordered phages, which were not sterically restricted. We first modified surfaces with DTSP or a combination of cysteamine and glutaraldehyde (CA + GA), and then deposited the phages in an alternating electric field of trapezoidal waveform of an applied potential of adjusted parameters (10 V and 16.67 kHz).

We obtained a 64-fold and 50-fold increase in the number of captured bacteria (i.e., sensitivity) due to the combination of alternating electric field (trapezoidal, 10 V and 16.67 kHz) and DTSP or CA + GA, respectively, when compared to that of the randomly oriented phages on a bare gold surface (Figure 3A). The application of an alternating electric potential alone resulted in around a 10-fold increase (for both the bare gold and chemically modified surfaces), whereas chemical immobilization alone increased the number of caught bacteria by 4−5 times. To further investigate the reasons for the combined effect of both studied factors, we used AFM to analyze the layers of phages after the deposition process in various conditions. We also calculated the surface coverage of phages within each layer (Figure 4). Both methods of chemical modification significantly increased the surface coverage of the phages. The electric field caused only a slight change in phage coverage. This proved that chemical modification increased the number of phages on the surface and therefore increased the number of caught bacteria, whereas the electric field increased the number of caught bacteria only by proper orientation of the phages. For comparison, we showed representative images acquired by means of confocal microscopy of the sensor surface with caught bacteria with GFP inside.

To compare the results, we analyzed ratios Δ between the increase in the number of phages and corresponding increase in the number of caught bacteria according to the formula

$$\Delta = \frac{\Delta_{\text{bacteria}}}{\Delta_{\text{phages}}} \tag{2}$$

Each ratio was calculated as a comparison of results obtained for the phage-based sensing elements prepared with chemical modifications of the surface (CA + GA or DTSP) versus results obtained for bare gold, that is,

$$\Delta_{\text{phages}} = \frac{\text{coverage}_{(\text{modification})}}{\text{coverage}_{(\text{bare gold})}} \tag{3}$$

$$\Delta_{\text{bacteria}} = \frac{\text{normalized number of bacteria}_{(\text{modification})}}{\text{normalized number of bacteria}_{(\text{bare gold})}} \tag{4}$$

For the randomly oriented phages (no electric field), we obtained two values, 1.50 ± 0.39 and 1.92 ± 0.31, for modifications with CA + GA or DTSP, respectively. Ratios larger than 1 indicate some cooperativity of the phages. This is the number of deposited phages needed to effectively capture a single bacterium. If the coverage is lower compared to this critical value, the efficiency of the sensing element is restricted. For layers of phages oriented in the field (both bare gold and modified surfaces), Δ values were much higher, that is, 2.69 ± 0.59 and 3.98 ± 0.90, respectively. This again proved that application of the electric field caused orientation of the phages in a tail-up head-down position, which reduced steric hindrance. Without ordering in the electric field, the increase in surface coverage resulted in an increase in steric hindrance. Due to the applied electric field, the number of available RBPs increased not only by increasing the surface coverage, but also by proper alignment of the virions, which facilitated bacterial recognition and greatly limited steric hindrance. The values of Δ for the ordered samples prepared with chemical modifications versus those of the randomly oriented phage layers on bare gold were 22.72 ± 4.73 and 33.59 ± 7.28 for CA + GA and DTSP, respectively, proving the combined effect of both factors. Detailed calculations of ratios are provided in the Supporting Information.

We also estimated that only 3−10% of receptors in the randomly oriented layers of phages was actually available to the bacteria in the samples and contributed to the detection process. To estimate these values, we assumed a perfect alignment of phages upon application of the electric field (assumption that 100% of RBPs were available) and analyzed the increase in the number of captured bacteria upon application of the electric field. We considered two regimes of phage surface coverage: low coverage (without steric hindrance) and high coverage (significant steric hindrance). Extensive discussion on this issue is provided in the Supporting Information.

Analysis of Selectivity and Limit of Detection. We analyzed the selectivity of the prepared phage-based sensing elements. We prepared real samples contaminated with two different species of bacteria. We compared the number of caught bacteria from tap water spiked with similar concentrations of *E. coli* BL21(DE3) (host bacteria) and *E. aerogenes* PCM 1832 (nonspecific bacteria) (Figure 3B). *E. aerogenes* bacterium was judiciously chosen for comparison, as it belongs to the same *Enterobacteriaceae* family as *E. coli*. To be able to distinguish signals coming from two different species, we stained bacteria *E. aerogenes* with the red fluorescent dye SYTO RED 62. For experiments, we used layers with T4 phages deposited in the electric field generated by the trapezoidal waveform of applied potential combined with chemical modifications of the surface. The concentration of *E. coli* was around 4×10^5 CFU mL^{-1}, whereas for *E. aerogenes*, it was around 5×10^5 CFU mL^{-1}. We proved the high selectivity of the phage-based biosensors and their usefulness in testing real samples, which is in good agreement with other published studies.[19] Comparison of the confocal microscopy images of the prepared spiked tap water and representative images of the sensing layer with caught bacteria are provided in the Supporting Information. The obtained results are in line with our previous findings on phage-based flow cytometry probes for bacterial detection.[35]

For evaluation of the limit of detection, we used only sensing layers with the highest sensitivity, that is, phages chemically immobilized on the DTSP-modified surface in the trapezoidal electric field (10 V, 16.67 kHz). We analyzed the number of bacteria caught for samples with different bacteria concentrations. The obtained data are presented in Figure 5. We estimated that the limit of detection was around 100 CFU mL^{-1}.

As published data indicate that an electric field might influence viruses, we showed that the utilized electric field did not deactivate bacteriophages and the decrease in the number of phages in the suspension was solely due to the deposition of the

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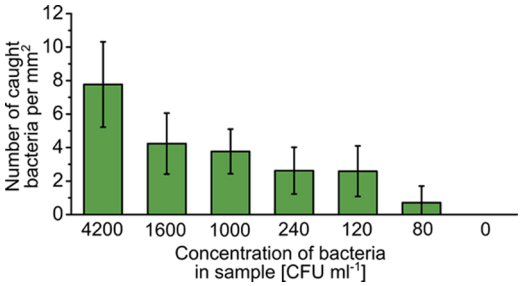

Figure 5. Analysis of limit of detection of prepared layers with the highest sensitivity, i.e., phages chemically immobilized on the DTSP-modified surface in the trapezoidal electric field (10 V, 16.67 kHz). Estimated limit of detection was around 100 CFU mL^{-1}.

phages. For more details please refer to the Supporting Information.

CONCLUSIONS

We demonstrated the combined effect of an increase in the number of T4 bacteriophages and their proper geometrical orientation on the sensitivity of a model sensing element for *E. coli* bacteria detection. Activation of the surface by utilization of CA+GA or DTSP resulted in a 4-fold and 5-fold improvement in sensitivity, respectively. Separate experiments proved that the proper arrangement of phages obtained by the orientation of virions in an alternating electric field improved sensitivity by 10-fold. Combined together these two approaches allowed for around a 50- to 64-fold increase in the number of captured *E. coli* cells by the T4 bacteriophage-based sensing element depending on the method of surface modification. This synergetic effect arose from the fact that ordering of phages at the chemically modified surface enabled dense layers of advantageously oriented phages to be obtained. Improvements introduced by us resulted in a limit of detection of around 100 CFU mL^{-1}, that is, in the range of the best phage-based sensors described to date.

We analyzed the process of orientation of the bacteriophages and proposed new characteristics of the applied electric field. Phages, as colloidal unsymmetrical particles with a permanent dipole moment, can interact and as a result be arranged with an electric field. In our previous report, we demonstrated a 4-fold increase in sensitivity upon deposition of phages in a constant electric field.[18] We took under consideration two contradicting time scales and we chose a frequency that allows efficient orientation of the bacteriophages. A specifically designed shape and certain duration times of pulses allowed for a further 2.5-fold increase, and thus 10-fold increase in sensitivity compared to that of the randomly oriented phages. Such increases in sensitivity were obtained without significant changes in phage surface coverage.

From the analysis of the obtained results, we concluded that in the randomly oriented phage-based sensing layers only a minority (few percent) of receptors was available for bacterial detection (detailed discussion in Supporting Information). Thus, the proper arrangement of phages is crucial for the development of such biosensors.

We obtained a superior sensing layer that allowed bacterial detection in 15 min. The method proposed by us can be used for the preparation of phage-based biosensors for the detection of almost every strain of bacteria, as T4 has the general structure that is common for the majority of bacteriophages. The proposed procedure can be also exploited to form ordered structures of other colloidal particles, molecules, proteins, or organisms with a nonzero dipole moment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b03497.

Methods of preparation of bacteriophage T4 and bacterium *E. aerogenes*, analysis of the number of deposited phages and phage deactivation, method of analysis of sensitivity of prepared phage layers, details about instrumentation, scheme of system for deposition of bacteriophages in the electric field, calculations of time constant, and the electric field in the prepared system, analysis of sensitivity of sensing layers prepared with square voltage with different frequencies, optimization of voltage in trapezoidal voltage waveform, extended discussion about steric hindrance in phage-based sensing layers, and confocal microscopy images of spiked tap water compared with those of the sensing layer with caught bacteria from the sample (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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