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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ABSTRACT

Faster and more sensitive environmental monitoring should be developed to face the worldwide problem of bacterial infections. To remedy this issue we demonstrate bacteria sensing element, which utilizes dense and ordered layers of bacteriophages specific for given bacteria strain. We combine 1) chemical modification of the surface to increase the surface coverage of bacteriophages 2) with 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 hindrances, which cause that no more than few percent of all receptors is available. Increased number of properly ordered phages results in optimal performance of phage receptors, what manifests in up to 64-fold increase of sensitivity and limit of detection as low as 100 CFU mL^{-1}. Our sensing elements can be applied for selective, sensitive and fast (15 min) bacteria detection. Well studied pair T4 bacteriophage – *Escherichia coli* was used as a model, however the method could be adapted to prepare bacteriophage-based sensors for detection of variety of bacterial strains.

INTRODUCTION

The threat of bacterial infections concerns not only healthcare, but also other branches of industry, e.g. food or environmental industries. The European Centre for Disease Prevention and Control reports that only in Europe 4.1 million patients are affected by health care-associated

infection each year.[1] In majority of cases a serious repercussions can be avoided thanks to fast and reliable detection of bacteria. Unfortunately, classical methods, although cheap and straightforward, require up to 72 h to obtain reliable output. An aid could be found in development of sensors for bacteria detection, which utilize bacteriophages as sensing elements. Bacteriophages are specialized viruses, which 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 the biosensors detecting almost all bacterial strains. Additionally, low preparation cost, stability in 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 bacteria detection.

The idea of utilization of phages for sensing is gaining increasing interest among scientists.[4–10] Until now improvements of the phage-based biosensors were introduced mainly due to increase in number of phages within the sensing elements. First, physisorbed layers of phages were used. For example Olsen et al. showed the possibility of detection of *Salmonella typhimurium* with limit of detection of around 100 cells mL^{-1}.[11] However, Authors utilized filamentous phages isolated from phage library, which is very difficult, expensive and time-consuming procedure. Another approach replaced physisorption with more sophisticated chemical biding due to chemical modification of the surface. Comparing to bare gold Singh et al. increased a number of deposited T4 phages by factor of 5 to 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 4- and 12-fold increase of the sensitivity of detection of *Escherichia coli* bacteria, respectively. These results indicated significant deviation from the linearity in improvement of the sensitivity of phage-based sensors with increase of

phage surface coverage. This was caused by random orientation of virions at the surface. As receptor binding proteins (RBPs) are present only at the end of the tail spike of T4 phage, such random orientation of phages caused that majority of RBPs were unavailable due to steric hindrance.[13] Moreover, the horizontal alignment of the virions is favored by entropy. In such orientation RBPs are not able to participate effectively in bacteria capturing process. Of course, increase of number of the randomly oriented virions results in statistically higher amount of available receptors. Naidoo et al. estimated "jamming" surface concentration of T4 phages as 19 phages μm^{-2}, which gives the highest affinity to capture bacteria.[14] Above this value increase in phage surface coverage does not result in higher sensitivity of sensing elements. To overcome this limit, virions should be positioned in tail-up orientation, i.e. arrangement in which majority of receptor binding proteins is involved in detection process. Proper alignment of virions increases also theoretical maximum of density of phage surface coverage to 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 small outer capsid protein and 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 claimed the orientation of phages due to electrostatic interactions. These cases are however inconclusive, because Authors did not provide evidence for improvement of the characteristics of biosensors due to proper arrangement. Also the genetic modification might render phages inactive.

In our recent paper we provided the evidence that bacteriophages might be oriented in the constant electric field due to non-zero permanent dipole moment of virions.[18] Majority of the

bacteriophages share this intrinsic property (as well as general structural design), thus such approach is not restricted to only model systems (T4 bacteriophage – *Escherichia coli*). We achieved the 4-fold increase of captured *E. coli* cells in case of ordered versus randomly oriented virions without significant change in surface coverage of T4 bacteriophages. The improvement of sensitivity was solely due to proper alignment of virions, what was obtained mostly due to electrostatic interactions within electrical double layer (EDL), i.e. in close proximity to the surface.

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

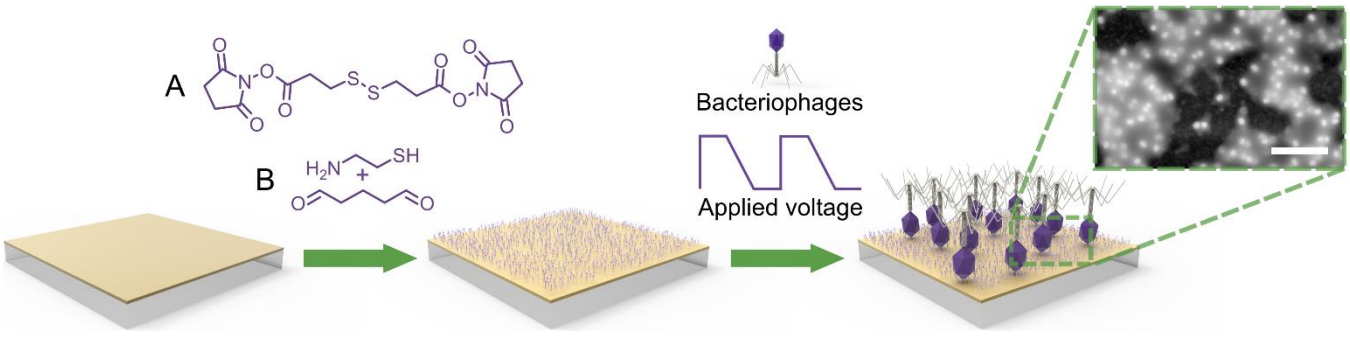

Figure 1. Scheme for fabricating dense sensing layers of ordered phages. At first gold surface was modified with **(A)** dithiobis(succinimidyl propionate) (DTSP) or **(B)** cysteamine and subsequently glutaraldehyde. Second step consisted of deposition of phages in the alternating electric field. Magnification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

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]

Phages preparation and bacteria culturing

Final concentration of phages in TM buffer, utilized for phage deposition, was $1.11\times10^{11} \pm 0.15\times10^{11}$ PFU mL^{-1}. For more details on preparation and purification of phages please refer to Supporting Information.

Escherichia coli BL21(DE3) (obtained from the Institute of Biochemistry and Biophysics in Warsaw, Poland) was used as a target analyte for T4 phages. The strain harbored a plasmids coding green fluorescence protein (GFP) and providing resistance to chloramphenicol and kanamycin. Bacteria were prepared according to the standard procedures. 25 mg of chloramphenicol and 50 mg of kanamycin were added to 1 L of LB media. Isopropyl β-D-1-

thiogalactopyranoside (IPTG) was added to LB media to final concentration of 1 mM to induce the production of the GFP protein. At the beginning, the single colony from 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 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 non-specific strain for T4 phage to evaluate selectivity of prepared sensing layers. Bacteria were stained with SYTO RED 62 to be visible as red objects in confocal microscopy. More details are provided in Supporting Information.

Solid supports and chemical modifications

T4 bacteriophages were deposited onto solid substrates. 1 × 8 × 25 mm glass plates covered first with 5 nm layer of titanium and subsequently with 150 nm layer of gold were used. The gold layer was hardened using 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. Afterwards the

y were sonicated sequentially in acetone, ethanol, isopropanol, and twice in deionized water for 5 minutes in each solvent. Such substrates were used as support for T4 layers (samples denoted as "bare gold") or were further subjected to chemical modifications.

Modification with dithiobis(succinimidyl propionate) (DTSP)

DTSP modification procedure was adapted from research of Arya et al.[19] Clean solid substrates were immersed in 2 mg mL^{-1} solution of DTSP in acetone for 20 h at room

temperature. Then modified substrates were washed with acetone and rocked for 15 min in acetone to get rid of unreacted substrate. Such modified plates were used for phage deposition within 1 h after modification and washing (samples denoted as "DTSP").

Modification with cysteamine and glutaraldehyde

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

Deposition of phages in the electric field

The details on setup used for generation of electric field is 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 a capacitors filled with bacteriophage suspension. The plates of capacitors were 1) gold surface of the sensor and 2) copper electrode. Because of the 200 μm PTFE separator insulating copper electrode there was no faradaic current in the system. The setup allowed for full control over the phage deposition procedure.

160 μL of phage suspension in TM buffer was placed in deposition chamber of the deposition cell. Signal wire was connected to gold surface of the plate on which phages were deposited. Bacteriophages have negatively charged head and positively charged fibers, thus

positive voltage was applied to gold plates to achieve desired orientation. Ground was connected to insulated copper electrode. The distance between electrodes was 1 mm. No potential was applied in case of control samples. Deposition of phage-based sensing layers lasted for 30 minutes. Phages which did not bound to the surface were rinsed with a large excess of TM buffer. Then plates were immersed in 5 % suspension of skim milk in TM buffer for 30 min to block surface unoccupied by phages with inert protein casein and to avoid nonspecific binding of bacterial cells.

Analysis of number of deposited phages and phage deactivation

Plaque counting method was used to evaluate the influence of electric field and surface modification on activity of studied T4 bacteriophages. For more details please refer to Supporting Information.

Analysis of sensing layers

Prepared biosensors were immersed in bacteria solution for 15 min. Confocal microscopy was used to evaluate the efficiency of studied sensing elements. For each sensing layer at least 10 images in random positions were taken, which were analyzed and 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. As control we prepared always two types of plates covered with: 1) randomly oriented phages and 2) only casein (to check the possibility of non-selective deposition of bacteria on casein). Corresponding error bars were provided. The results were normalized in

respect to randomly oriented phages. More detailed description is provided in Supporting Information.

Instrumentation

Detailed information about used instrumentation is provided in Supporting Information.

RESULTS AND DISCUSSION

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

Virion of bacteriophage T4 is composed of 110 × 80 nm icosahedral head (capsid) and 98 nm contractile tail ended with fibers. dsDNA of length of 168.9 kbp is stored in the capsid.[23–25] Depending on the conformation of the fibers the measured values of 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 distribution of charges was developed in the process of evolution as an improvement facilitating the infection of the host bacteria. Positively charged fibers are electrostatically attracted to the bacteria, that have negatively charged surface.[30]

Orientation of phages in the alternating electric field

The device for creating sensing layers of phages deposited in the external electric field was in fact a capacitor filled with bacteriophage suspension. Plates of such capacitor were gold surface of the solid support and 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 phages and uncontrolled redox reactions. This resulted in limited effect of the electric field, which spread only within Debye length from the electrode. This distance was in order of only few nanometers, as phages were suspended in proper buffer of relatively high ionic strength. When constant electric field was applied the electric field within the phage suspension was quickly suppressed by formation of electrical double layer (EDL). The orientation of phages was mostly due to the electrostatic attraction between capsids and the surface and electrostatic repulsion between fibers and the surface. We demonstrated this effect recently - the 4-fold increase of efficiency of 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 virions by limiting the screening of the applied field. There was only very limited possibility to increase the Debye length of studied system, as virions are unstable in

low osmotic strength solutions.[31] Therefore, we decided to utilize alternating electric field, which allowed the EDL to spread during inter-pulse periods.

At the beginning of each pulse the field spread across the whole suspension until the electrical double layer was formed and screened the external field. The specific time constant, which described the process of formation of electrical double layer and screening of the electric field by ions, is comparable with time in which ions travel through 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 a good agreement with other published results.[32] After this time the external electric field was screened and remained effective only within Debye length, which in studied system was few nanometers.

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

Higher frequencies (shorter pulses and less time between pulses) corresponded to more events of active orientation of phages by effective field and limited defocusing of 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 EDL to relax completely during inter-pulses periods, but increased defocusing. These contradicting time scales should be balanced. We

judiciously chose 20 μs as duration time of each pulse as well as time between pulses, as it constituted good compromise between all the crucial factors.

In case of rectangular voltage waveform, at the beginning of each pulse there was electric field spreading across the solution. However, the drop of the applied potential to zero at the end of the pulse also generated electric field, but directed oppositely (Figure 2). This resulted in zero net effect of virion orientation. To overcome this we designed voltage waveform that allowed creating non-zero effect of orientation of phages. We exploited non-linear electrophoretic effect, that was proved for number of different particles, e.g. polystyrene latex and aluminum oxide particles and even yeast cells.[34] When electric field is strong enough, i.e. 100 V cm^{-1} and higher, which was a case in our system, the dependence between electrophoretic velocity and applied electric field is non-linear:

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

where ν_{eph} is electrophoretic velocity, μ_{eph} is field-independent electrophoretic mobility coefficient, $\mu^{(3)}_{eph}$ is non-linear electrophoretic mobility coefficient and E is strength of the electric field. We designed trapezoidal voltage waveform, which allowed obtaining substantial difference between values of effective electric field between sharp increase and slow drop of the applied potential at the beginning and at the end of the pulse, respectively (Figure 2). Due to the non-linear electrophoretic effect, phages were oriented at the desired position along the lines of the electric field. To ensure proper difference in electric fields, we decided that slow drop of the 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 60 μs period, which corresponded to frequency of 16.67 kHz. All exploited types of voltages and resulting electric

fields are presented in Figure 2. Changes in the electric field upon application of different voltages was calculated based on Equation S5.

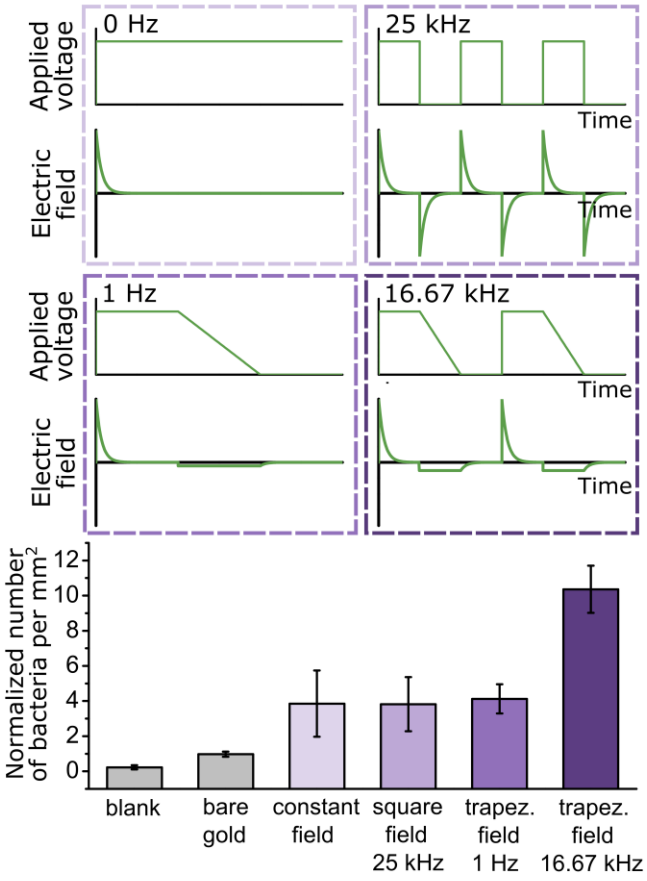

Figure 2. Comparison of sensitivity (normalized number of caught bacteria) of phage-based sensing layers prepared using various sets of parameters of the electric field. In all cases applied voltage was 10 V. Upper panels present all the applied voltages and the resulting electric fields.

To check validity of our hypothesis we first prepared a set of sensing layers consisting of phages deposited with use of rectangular pulses of electric field (10 V, various frequencies) (details in Figure S3). Utilization of such waveform didn't improve efficiency of bacteria capturing in comparison to constant electric field. The effect was only due to electrostatic interactions with the surface. As control we also tested trapezoidal waveform of frequency of 1

Hz (333 ms without applied potential between the pulses), which allowed phages to defocus by rotational diffusion during inter-pulses periods. Again, no differences were observed in comparison to square waveform and constant electric field proving that mainly electrostatic interactions between surface (polarized positively) and capsids (negative pole of the dipole) played role, when net focusing of phages in bulk was negligible. Utilization of designed (10 V, 16.67 kHz) trapezoidal voltage waveform resulted in 2.5-fold increase of sensitivity in comparison to square waveform and constant electric field. When compared to randomly oriented phages the increase upon application of 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 for orientation of phages (for details see Figure S4). This value of the applied voltage seemed to balance the electrophoretic force, which oriented phages (higher potential was more favored), against unwanted phenomenon like deactivation of phages and increase of charge density of electrical double layer (higher potential was less favored).

Combining chemical modification and alternating electric field

We decided to follow two protocols of surface modification. Singh et al. developed the method, in which the gold surface is covered with cysteamine and then glutaraldehyde.[12] Arya et al. utilized self-assembly monolayers of dithiobis(succinimidyl propionate) (DTSP).[19] The reported most effective surface coverages 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. 11). It should be noted that in case of DTSP less T4 phages (20 phages μm^{-2}) performed better in capturing bacteria comparing to maximal obtained surface coverage of around 30 phages μm^{-2}

(based on Figure 3d in Ref. 11). This confirmed a jamming due to clustering of the virions at high surface coverage. The jamming coverage for T4 phages was estimated by Naidoo et al. as 19 phages μm^{-2}.[14]

In order to further increase sensitivity of prepared sensing layers, we combined chemical immobilization of phages with orientation using electric field. We expected to obtain 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 combination of cysteamine and glutaraldehyde (CA+GA) and then deposited phages in the alternating electric field of trapezoidal waveform of applied potential of adjusted parameters (10 V and 16.67 kHz).

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

acquired by means of confocal microscopy of sensor surface with caught bacteria having green fluorescent proteins (GFP) inside.

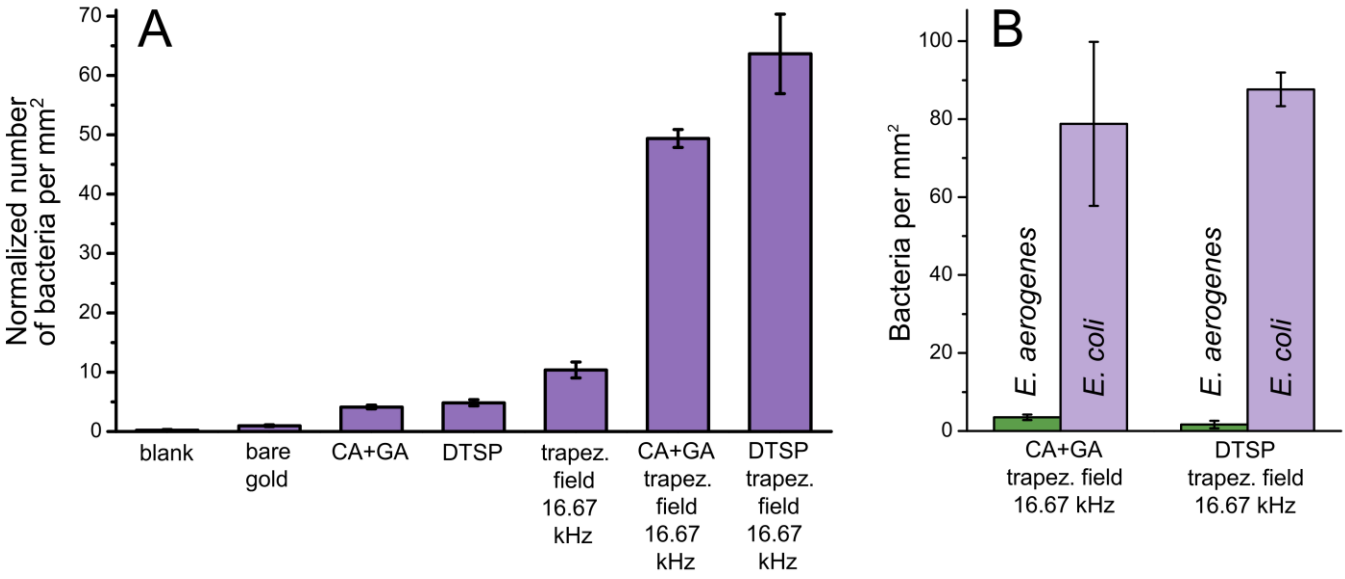

Figure 3. (A) Sensitivity (normalized number of caught bacteria) of phage-based sensor prepared with chemical modifications of the surfaces and applied trapezoidal voltage waveform. Combined effect of both methods resulted in up to 64-fold increase in sensitivity. **(B)** Number of bacteria caught from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

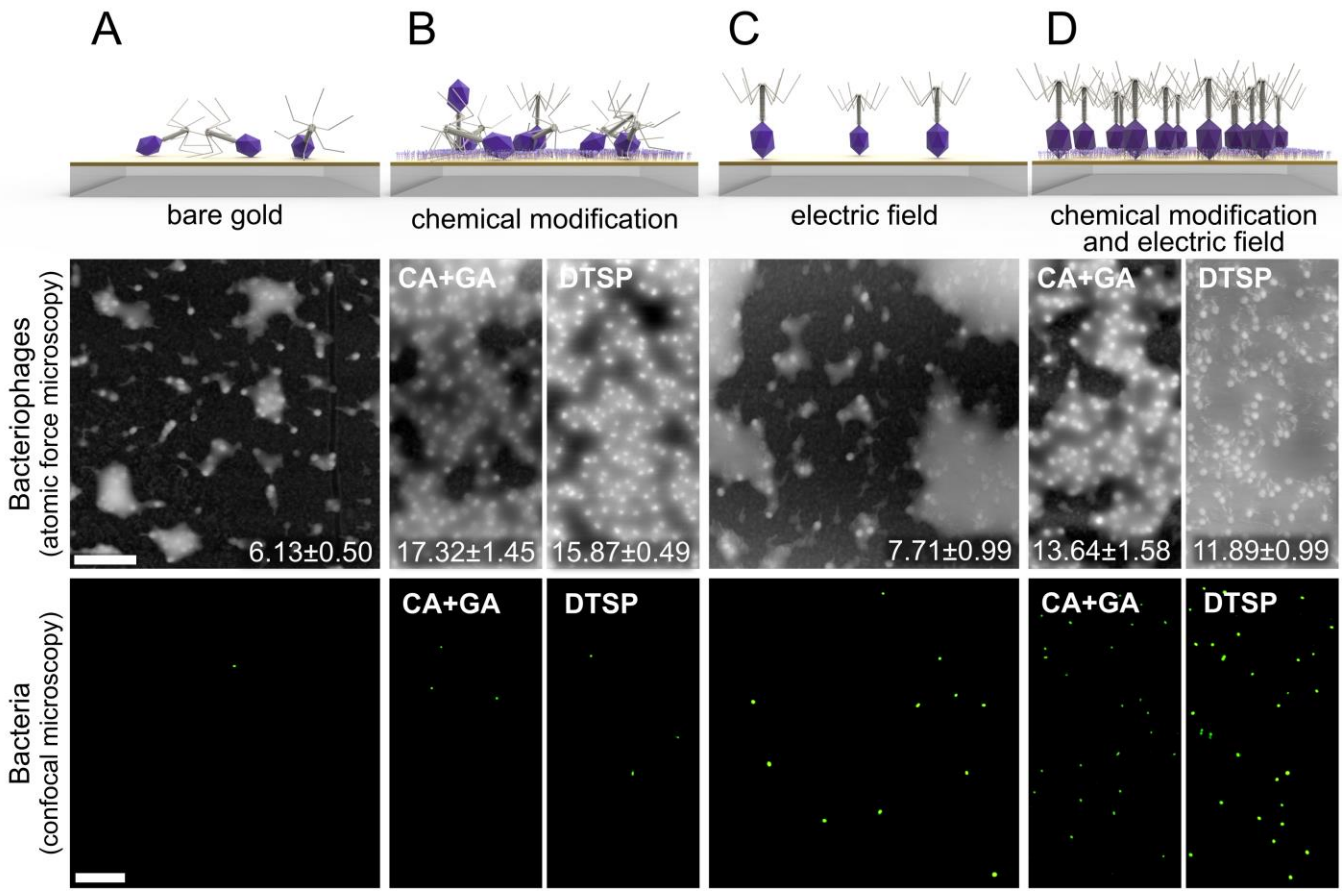

Figure 4. Schematic illustrations and corresponding atomic force microscopy (AFM) images of layers of phages deposited on **(A)** bare gold or in various conditions: **(B)** with chemical modifications of the surface (with cysteamine and glutaraldehyde (CA+GA) or dithiobis(succinimidyl propionate) (DTSP)), **(C)** in electric field from trapezoidal voltage (16.67 kHz, 10 V) and **(D)** 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 bacteria. Scale bars on AFM and confocal microscopy pictures correspond to 1 µm and 50 µm, respectively.

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

$$\Delta = \frac{\Delta_{\text{bacteria}}}{\Delta_{\text{phages}}} \quad (2)$$

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

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

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

For 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. There is a number of deposited phages needed to effectively capture single bacteria. If the coverage is lower comparing 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, i.e. 2.69 ± 0.59 and 3.98 ± 0.90, respectively. This again proved that application of the electric field caused orientation of phages in tail-up head-down position, what enabled to avoid steric hindrance. Without ordering in the electric field the increase of surface coverage resulted in increase of steric hindrances. Due to applied electric field, the number of available RBPs increased not only by increasing of the surface coverage, but also by proper alignment of virions, which facilitated bacteria recognition and greatly limited steric hindrances. Values of Δ for ordered samples prepared with chemical

modifications versus randomly oriented phage layers on bare gold were 22.72 ± 4.73 and 33.59 ± 7.28 for CA+GA and DTSP, respectively, proving combined effect of both factors. Detailed calculations of ratios are provided in Supporting Information.

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

Analysis of selectivity and limit of detection

We analyzed the selectivity of prepared phage-based sensing elements. We prepared real sample contaminated with two different species of bacteria. We compared number of caught bacteria from tap water spiked with similar concentrations of *Escherichia coli* BL21(DE3) (host bacteria) and *Enterobacter aerogenes* PCM 1832 (non-specific 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 red fluorescent dye SYTO RED 62. For experiments we used layers with T4 phages deposited in the electric field generated by trapezoidal waveform of applied potential combined with chemical modifications of the surface. Concentration of *E. coli* was around 4×10^5 CFU mL^{-1}, whereas *E. aerogenes* was around 5×10^5 CFU mL^{-1}. We proved high selectivity of phage-based biosensors and their usefulness in testing

real samples, what is in good agreement with other published studies.[19] Comparison of confocal microscopy images of prepared spiked tap water and representative images of sensing layer with caught bacteria are provided in Supporting Information. Obtained results are in line with our previous findings on phage-based flow cytometry probes for bacteria detection.[35]

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

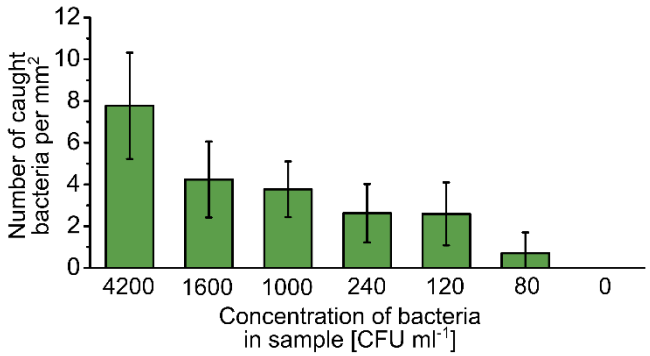

Figure 5. Analysis of limit of detection of prepared layers with the highest sensitivity, i.e. phages chemically immobilized on DTSP-modified surface in trapezoidal electric field (10 V, 16.67 kHz). Estimated limit of detection was around 100 CFU mL^{-1}.

As published data indicate that electric field might influence viruses, we showed that utilized electric field didn't deactivate bacteriophages and decrease of number of phages in the suspension was solely due to deposition of phages. For more details please refer to Supporting Information.

CONCLUSIONS

We demonstrated the combined effect of increase in number of T4 bacteriophages and their proper geometrical orientation on the sensitivity of the model sensing element for *E. coli* bacteria detection. Activation of the surface by utilization of CA+GA or DTSP resulted in 4-fold and 5-fold improvement of sensitivity, respectively. Separate experiments proved that proper arrangement of phages obtained by orientation of virions in alternating electric field gave 10-fold increase. Combined together these two approaches allowed for around 50- to 64-fold increase in number of captured *E. coli* cells by 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 to obtain dense layers of advantageously oriented phages. Improvements introduced by us resulted in limit of detection of around 100 CFU mL^{-1}, i.e. in a range of best phage-based sensors described to time.

We analyzed the process of orientation of bacteriophages and proposed new characteristics of applied electric field. Phages, as colloidal unsymmetrical particles with permanent dipole moment, can interact and as a result be arranged with the electric field. In our previous report we demonstrated 4-fold increase of sensitivity upon deposition of phages in constant electric field.[18] We took under consideration two contradicting time scales and we chose frequency that allow to orient bacteriophages efficiently. Specifically designed shape and duration times of the pulses allowed for further 2.5-fold increase and thus 10-fold increase comparing to 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 randomly oriented phage-based sensing layers only minority (few percent) of receptors were available for bacteria detection (detailed discussion in Supporting Information). Thus proper arrangement of phages is crucial for development of such biosensors.

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

ASSOCIATED CONTENT

Supporting Information. Methods of preparation of bacteriophage T4 and bacteria *Enterobacter aerogenes*, analysis of 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 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 hindrances in phage-based sensing layers and confocal microscopy images of spiked tap water compared with sensing layer with caught bacteria from such sample are provided as Supporting Information.

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Author Contributions

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

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