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Article

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Antibacterial efficiency of surface-immobilized *Flavobacterium*-infecting bacteriophage

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Keywords: Phage therapy, surface adsorbed bacteriophages, antibacterial surfaces, aquaculture, virus material, biomaterial

Abstract: Control of bacterial diseases by bacteriophages (phages) is gaining more interest due to increasing antibiotic resistance. This has led to technologies to attach phages on surfaces to form a biomaterial that can functionally display phages that interact with bacteria, to carry out successful infection cycles. Such a material could be applied in many environments, where the target pathogens are expected. Although this approach has been applied successfully in a few studies already, the basis of the antibacterial effect by the immobilized phages is unclear, and the interpretation of the results depends on the study. Here, we studied the phage attachment density, their detachment rate and infectivity on five different surfaces: silicon, amine-treated silicon, gold, carboxylate-treated gold and crosslinker-activated carboxylate-treated gold. The density of attached phages varied between the different surfaces and was highest on the crosslinker-activated carboxylate-treated gold. To understand whether the antibacterial effect is caused by the attached or the detached phages, the strength of the immobilization was analyzed by performing 3-12 washing steps. The detachment rates differed between the materials, with the amine treated silicon surface generating the highest release of phages and maintaining the highest infectivity, even after extensive washing. On the other hand, covalent crosslinking seemed to interfere with the infectivity. Our results suggest that the detachment of the phages from the surface is a possible mechanism for the antibacterial effect. Furthermore, we introduce a measure of the infectivity by comparing the bacterial growth reductions produced by the phage-treated materials to the effect caused by a known number of free phages, resulting in a unit "Effective PFU/surface area", a comparable standard between different studies.

1. Introduction

Bacteriophages are viruses that parasitize bacteria to produce progeny, and due to this property, they can be used to control the growth and infectivity of the host. Although this approach, phage therapy, has been known already for a century, interest is increasing due to rising antibiotic resistance.[1] Because of their host-specific infectivity, phages can be used as specialized killers toward pathogenic bacteria in contrast to antibiotics, which generally have a wide impact on microorganisms.

Aquaculture i.e. fish farming is a growing food production industry because of the collapse of many natural fisheries.[2] Fish are grown in high densities, which increases the possibility of disease outbreaks that can cause high mortalities.[3] Usually, treatment of these diseases requires the use of antibiotics, which has been shown to lead to the increase of antibiotic resistance among environmental bacteria.[4,5] Columnaris disease caused by the *Flavobacterium columnare* is a bacterial disease in freshwater fish resulting in devastating epidemics at fish farms around the world.[6] It has been shown that phage therapy can be used to prevent columnaris disease in the laboratory environment by direct addition of phages in the water.[7] However, in a typical fish farm environment (net pens and flow-through systems) the water volumes can be extremely high, diluting or removing the added phages via the water flow. Therefore, immobilization of phages on a surface to provide a long-lasting antibacterial effect could be an optimal solution. But the question remains: Are surface attached phages any good? Furthermore, is the antibacterial effect caused by the attached or the detached phages? This information is central to understand the antibacterial effect of surface-immobilized phages.

Although an antimicrobial effect of immobilized phages has been demonstrated already in studies regarding food packaging[8,9] and health care equipment[10,11], there is very little consistency regarding the mechanisms of antibacterial effect of surface-immobilized phages. In these previous studies, neither the number of detached nor attached phages were reported, leaving the mechanism of phage infection unclear. Covalent crosslinking of phages to a surface has been found to increase the density and activity of phages in biosensor applications[12-15] and has been utilized also in antibacterial studies.[16-18] However, the mechanisms behind the results remain unclear. Covalently immobilized phages were found to cause the lysis of the bacteria on the surface,[16] and to have a higher antibacterial effect compared to physisorbed phages.[18] In contrast, Liana et al found that the infectivity of the chemically modified and T4-phage treated indium tin oxide surfaces was produced by the detached phages.[19] Also, Wang et al detected a higher infectivity on a plasma-treated surface without a covalent crosslinker than with it.[17]

To clarify the mechanisms involved with antimicrobial effects of immobilized phages, we studied the phage attachment density, the detachment rate and the infectivity of surface-immobilized *Flavobacterium*-infecting bacteriophages on five different surfaces: silicon, amine-treated silicon, gold, carboxylate-treated gold and crosslinker-activated carboxylate-treated gold. These specific surfaces were selected because they have been used in the previous immobilization studies and are known to result in different surface densities of phages.[20,21] To understand whether the antibacterial effect of these surfaces is caused by the attached or the detached phages, the strength of the immobilization was also analyzed by 3 - 12 washing steps. The amine treated silicon surface generated a high release of phages and maintained the highest infectivity, even after extensive washing, suggesting that detached phages are important. On the other hand, covalent crosslinking seemed to interfere with the phage infectivity. Furthermore, by using a combination of microbiological and imaging methods, we define a standard for reporting the antibacterial efficiency of phage-based biomaterials.

2. Materials and methods

2.1. Phage production

Phage FL-1 is originally isolated from a fish farm in Finland,[22] and it infects *Flavobacterium sp.* It was obtained from the +4 °C stock and its amplification was done using the standard double-layer agar method with a phage-bacteria ratio producing semi-confluent Shieh-agar[23] plates. After an overnight incubation, 5 ml of Shieh was added per plate, and plates were shaken overnight at +4 °C to elute the phages. The lysate was filtered with a 0.45 µm filter to remove the bacteria. The filtered lysate was then purified using an ÄKTAprime plus chromatography system with a quaternary-amine activated ion-exchange column QA-1 by BIA Separations (Suppl. Fig. S1, S2). The remaining salt was removed by a two-step buffer replacement with a cellulose dialysis tube in a 50 mM sodium phosphate buffer. Eventually, a pure phage solution with 8 x 10^{10} PFU/ml was obtained (PFU=Plaque forming unit).

2.2. Substrates

The five selected substrate surfaces for this study were: untreated crystalline silicon with a thin native oxide surface, amine-treated crystalline silicon (with native oxide), untreated gold, carboxylate-treated gold, and carboxylate-treated gold with a covalent crosslinker. Precut (5 mm x 5 mm) crystalline silicon substrates were purchased from Ted Pella, USA. (3-Aminopropyl)triethoxysilane (APTES) was used as the amine compound treating Si. The surface was prepared by first treating silicon substrates for 1 min with 100 W O_2 plasma in a reactive ion etcher (Oxford Plasmalab 80 Plus) to obtain hydroxyl groups to the silicon surface. A 1:2 mixture of APTES: EtOH (99.5%) was then added for 20 min at 50 °C, washed with 99.5% EtOH and DI (De-ionized) water, and baked at 108 °C for 50 min. The gold surface was prepared on a silicon substrate with the JEOL JFC-1100 sputter coater using about 1 kV energy, 10 mA current and 1 hour processing time. The carboxylate treatment for the gold consisted of an MUA (11-Mercaptoundecanoic acid) coating, done by submerging Au coated Si substrates to 10 mM MUA for 24 h, followed by washings with 99.5% EtOH and twice with sterile DI water. Additional crosslinker activation of the MUA-treated gold was done by preparing a 0.1 M EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and 0.4 M NHS (N-Hydroxysuccinimide) mixture to DI water, incubating the substrate in it at room temperature (RT) for 40 min and dip washing it twice with DI water.

2.3. Phage immobilization, detachment, and titration

All the immobilization experiments were conducted in a 50 mM phosphate buffer at pH 7.2. FL-1 bacteriophage solutions (200 µl) with 1.5 x 10^8 PFUs were pipetted on the substrates individually and left to adhere overnight at +4 °C under 90 RPM shaking. Next, the materials were washed six times in 1 ml 20 mM phosphate buffer (pH 7.2) by dipping to remove most of the unbound phages. After these baseline washes, phages were detached from the sample surfaces by first placing the samples into the well of a 24 well plate with 1 ml of phosphate buffer and shaking at 400 RPM for a set period of time. The buffer was then replaced and the shaking was repeated 3, 6, 10 or 12 times. The first detachment was done for 90 minutes and the following detachments for 20 - 30 minutes. In addition, some samples were washed four extra times by dipping after the last detachment treatment (12+4 samples). This extra wash was used to minimize the number of phages transferred from the detachment buffer. Fresh buffers were used for every sample at every step during the detachments. The number of detached phages was analyzed by titering. Plates were prepared by mixing 100 µl overnight grown *Flavobacterium sp.* and 2 ml Shieh with 0.7% low melt agarose tempered to 40 °C and poured over the Petri dish. After the agarose was solidified, a 10 µl droplet of raw or diluted detachment buffer was pipetted over it and incubated 48h, after which the plaques were counted. Three individual replicates were done for all samples and the standard error of the mean (SEM) was calculated.

Activation of phage carboxyl groups with the EDC crosslinker was studied by adding 1 mg of EDC and 1 mg of NHS to 1 ml of 2 x 10^9 PFU/ml FL-1 phage solution, resulting in 5 mM EDC and 9 mM NHS. The solution was incubated for 15 minutes at RT, and a dilution series was plated with the double layer agar method. A diluted crosslinker experiment was made with 50 µM EDC and 90 µM NHS.

2.4. Helium ion microscopy (HIM) and Transmission electron microscopy (TEM)

Samples were prepared for TEM using negative staining with 2% Phosphotungstic acid (PTA). Five microliters of purified FL-1 phage stock was pipetted on a Formvar-carbon-coated TEM-grid and let to adhere there for 2 min, 5 µl of staining was added and after 2 min, the excess was dried off. Imaging was done with a JEOL JEM-1400 HC Transmission Electron Microscope. Substrates with immobilized phages were prepared for HIM imaging by removing the excess buffer with a corner of a paper, and then air-drying them in ambient conditions. Samples were imaged with Zeiss Orion Nanofab using helium as the imaging gas. An acceleration voltage of 30 kV and aperture size of 10 µm were used, resulting in an ion current of 0.5 pA.

2.5. Infectivity measurements

Infectivity of the surfaces with immobilized phages was measured on a 24-well plate (Sarstedt, TC-plate 24 well, Standard, F). The substrate was placed in 1 ml of Shieh medium, and 50 µl of fresh overnight grown *Flavobacterium sp*. culture (2.5 x 10^6 CFU) was added. The culture was mixed for 15 seconds at 400 RPM and then incubated at RT without shaking for 48 h. Before addition of the bacteria, some substrates were shaken at 400 RPM for 5 sec, incubated for 5 min and moved to a second well (containing the Shieh medium), to study the effect of detached phages on bacterial growth. To compare the effect of immobilized phages to the one caused by free phages, infections with free phages with multiplicities of infection (MOI: s) between 1 and 0.0001 were performed by adding 10 µl of FL-1 serial dilutions to some wells. The optical density of the culture at the wavelength 590 nm was measured with Multiskan microplate photometer after 18, 27 and 48 hours, after the latent period of the phage, known to be 1-2 hours (Suppl. Fig. S3). A duplicate measurement was made at every time point and the average value was calculated. Three individual replicates were done for all the infectivity samples. The statistical significance of the results was analyzed with the Student's two-sample t-test.

3. Results and discussion

Purified FL-1 phage was imaged with TEM (Figure 1a). It is a myophage with an icosahedral head (diameter 55 nm) and approximately 100 nm long tail.

3.1 Attached phages

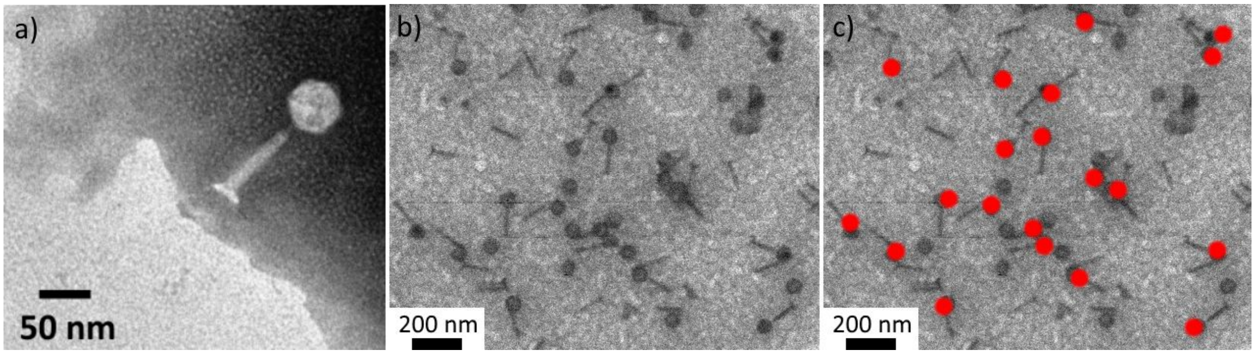

Figure 1. Phage FL-1 samples were imaged with TEM and HIM. a) TEM-image of phage FL-1 b) 12+4 detached Au+MUA+EDC sample imaged with HIM. c) Figure b with red dots marking the counted (intact) phage particles.

The number of attached phages for different surface treatments was analyzed by imaging the samples with HIM (Figure 1b). Four systematically selected areas (100 μm^2) per sample were imaged and only phage particles with an intact head and tail were counted (Figure 1c). More information is found in the Supplementary Table S1. Three individual replicates were done per treatment.

As expected, the density of attached phages varied between the surfaces (Figure 2). For each surface, no significant differences were found between 6 and 12+4 detachment treatments ($p>0.05$). After the full set of 12+4 detachment steps, silicon had clearly the lowest phage density. The silicon surface is covered with a native oxide, giving it the chemical properties of silicon oxide.[24] Silicon oxide surface is negatively charged in pH 7,[25] polar[24] and weakly polarizable, which means that neither electrostatic (as phages typically have negative effective charge at pH 7),[26] van der Waals (vdW), nor hydrophobic forces are likely responsible for the phage adsorption.

The density of phages on the APTES-treated silicon surface was about 100-fold compared to the untreated silicon after a full set of detachment treatments. This can be explained by the positive charge of the APTES-surface at pH 7.2 due to the protonated amine groups. Phage FL-1 has a negative effective charge at this pH, as demonstrated during the purification of the phage stock, when phages were bound to the amine treated surfaces of the monolithic column at pH 7.2.

The average phage density on the gold surface was about 50 phages/100 μm^2, even though gold has been shown to have a slight negative charge at pH 7.[27] The binding may have, however, been caused by high polarizability, which results in strong vdW and hydrophobic interactions.[28] Indeed, a previous study[21] on adsorption of non-tailed phages to SiO_2, gold, carboxyl, methyl, and amine-treated surfaces in different pH and ionic concentrations found that for some phages, adsorption to the gold surface was almost as high as to the amine-treated surface at pH 7. When the unfavorable electrostatic effect was shielded by increasing the ionic strength from 0.01 M to 0.1 M, adsorption increased even more. High adsorption of tailed phages on gold is reported in the literature with densities of 0.7,[29] 0.49[30] and 10.12 phages/μm^2,[31] with the first two of those comparable to our observation of 50 phages/100 μm^2.

The phage density on MUA treated gold surface was comparable to gold and APTES-coated silicon. A previous study found fewer attached phages on the APTES treated ITO-surface compared to the carboxylate-treated one,[20] suggesting planar surfaces have distinct adsorption dynamics compared to particulate surfaces, for which amine treatment resulted in strong adsorption.[32,33] This suggestion was not confirmed by our study. Although the phage density on the APTES is higher than on MUA in our measurements, the difference is not significant ($p=0.07$).

The highest density of all the five surfaces was found on the Au+MUA surface treated with EDC. Compared to untreated MUA, the density was significantly higher ($p<0.05$), which was the result of covalent immobilization by the carbodiimide chemistry.

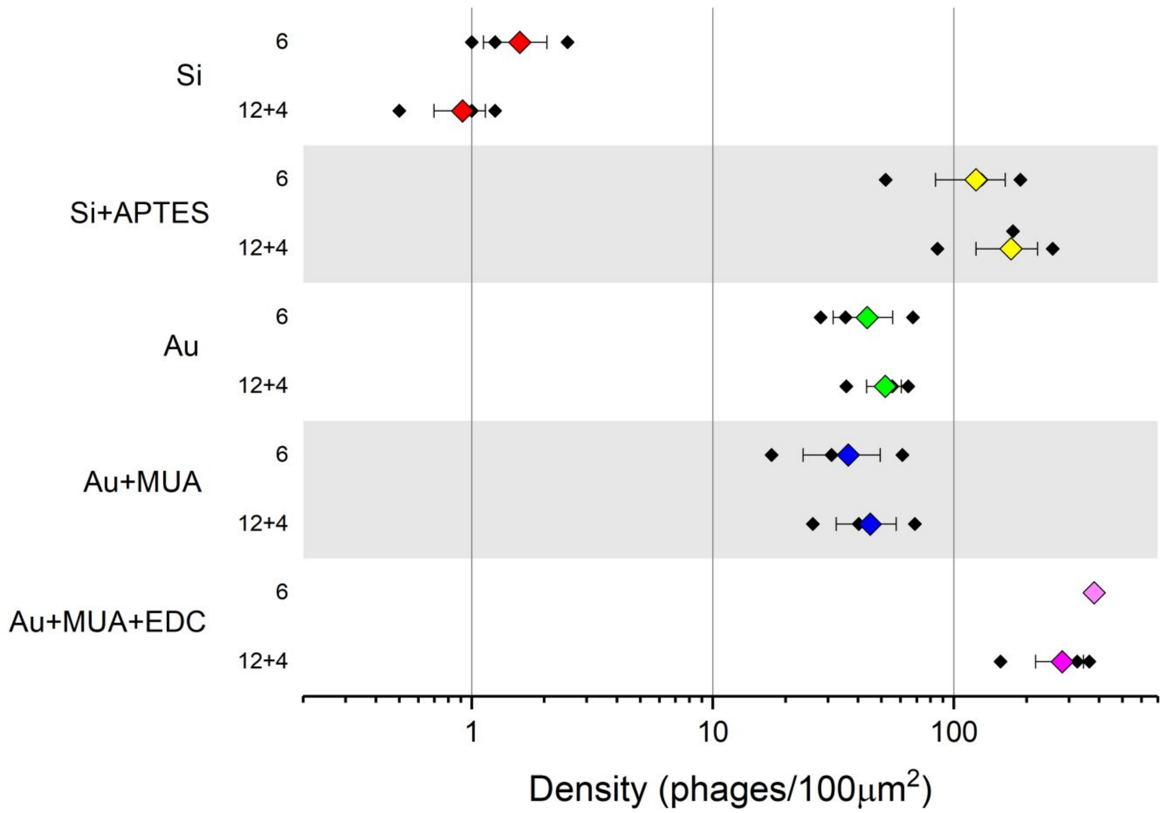

Figure 2. The average density of attached phages on the different surfaces calculated from the HIM images. The numbers after the name of the material are the number of detachment steps (6 or 12+4). Each replicate is shown by small black diamonds, the colored symbols + error bars show the mean ± SEM, (n=3). For the Au+MUA+EDC 6 sample only one image area was used.

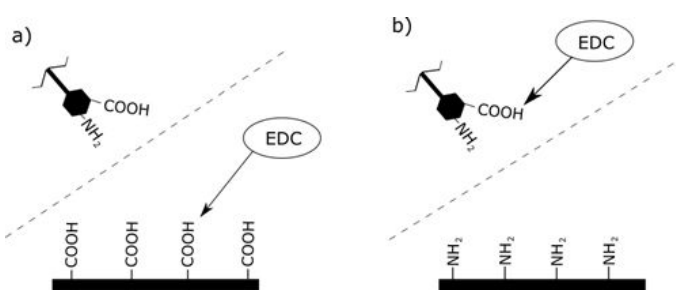

Figure 3. Different strategies for the covalent immobilization with the EDC a) Activation of the carboxylates on the MUA

surface with EDC. b) Activation of the carboxylates on the phage proteins with EDC.

In the literature, covalent crosslinking has been done either by activating the phage itself[12,34] or by activating the surface.[17] In our study, the EDC crosslinker was used to activate the carboxylates on the MUA surface prior to the reaction with the amines on the phage (Figure 3a). Because the APTES surface had a higher number of attached phages than the MUA surface, we were also interested in activating the carboxylate groups on the phage (Figure 3b). Preliminary experiments suggested that the infectivity of the surface was lost when using this approach. Therefore, we tested the infectivity of the phage after an EDC activation with the double layer method. Treatment of phages with 5 mM EDC and 9 mM NHS (concentration commonly used in the literature) reduced the infectivity (PFU´s) of the phage solution from $(1.2 \pm 0.2) \times 10^9$ PFU/ml to $(1 \pm 0.6) \times 10^3$ PFU/ml. When EDC was diluted 100-fold (50 µM EDC, 90 µM NHS), phage infectivity was reduced to $(4 \pm 2) \times 10^8$ PFU/ml. Therefore, activation of phages with the crosslinker was not pursued further. Some previous studies have discussed the possible harmful effects of phage activation with EDC,[16,34] but direct evidence of the loss of infectivity was not shown before. We note that EDC activation of phages is not location specific, which could prevent the phage from binding the host, or could crosslink the phages with each other, compromising the infectivity.

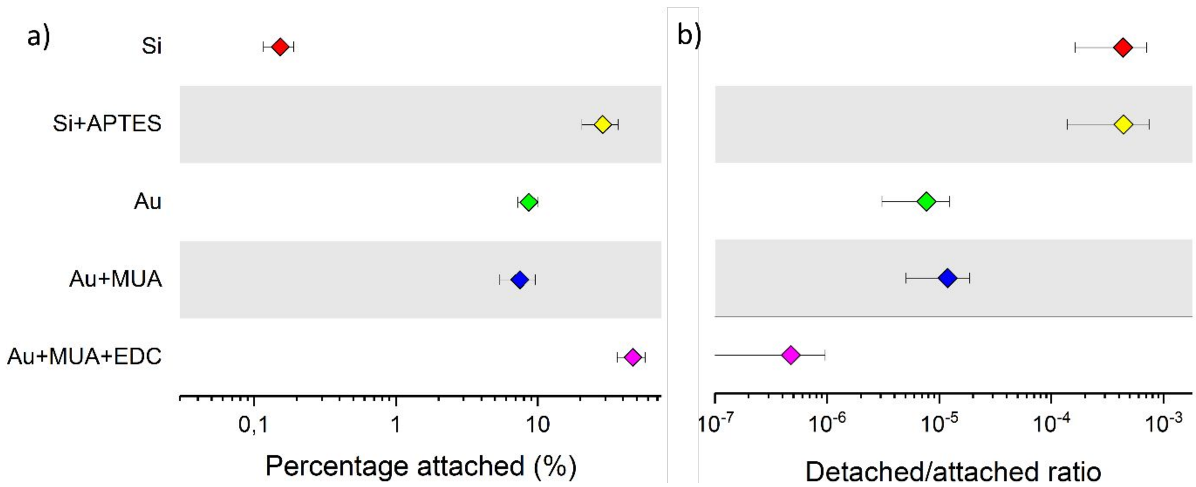

Figure 4. Phage attachment and detachment. a) Percentage of attached phages of the initial phage stock (1.5 x 10^8 PFU). b) The number of detached phages from detachment step 12 divided by the attached phages after 12+4 rounds. Errors were calculated by adding the relative uncertainties in quadrature. Mean ± SEM, (n=3).

To give an indication of the efficiency of phage attachment on different surfaces, we calculated the ratio of the total number of attached phages, calculated by multiplying the average phage density from HIM images with the total substrate area, to the number of PFUs in the purified phage stock used for the immobilization. For the Au+EDC+MUA surface, the percentage is high, over 40%, but for silicon about 0.1% (Figure 4a). It must be noted, however, that PFU of the initial stock was determined by plaque assay in which only the number of infective phages are counted. Imaging, on the other hand, sees all intact phage particles but their infectivity cannot be concluded. Thus, the estimate of phage attachment is actually an upper limit.

3.2. Detached phages

To study the release of phages from the surfaces, we titered the detachment buffers. After the first three rounds of detachment washes, the highest number of phages were released to the detachment buffer from the Si+APTES surface. For silicon, Au and Au + MUA, the number of detached phages decreased with the increase of the number of performed detachment steps. For the Si + APTES sample, the change was not significant ($p > 0.05$). After a full set of 12 detachment steps, APTES still has the highest release. However, the number of attached phages did not really seem to change on APTES along with the increase in the number of detachment steps (Figure 2). This apparent contradiction may be explained by the high number of phages still attached on APTES sample after 12+4 detachments compared to the number of detached phages, being only ~ 0.1 percent of the attached (Figure 4b).

Next, we estimated the strength of the phage binding on different surfaces, by studying the detached-attached ratios (Figure 4b). The ratio was lowest for the Au+MUA+EDC, (4×10^{-7}), suggesting strong binding. Indeed, EDC covalently crosslinks the surface carboxylates and the phage amines. For gold and gold+MUA, the result of relatively strong binding was not entirely expected, as hydrophobic and vdW interactions are usually considered weaker interactions compared to the electrostatic (as in APTES). In the literature, a higher detachment ratio 0.007% for gold with a tailed phage has been reported,[29] however, fewer washing steps (5 vs 18) were made.

Previous studies of the release of the surface immobilized phages are rare. Vonasek et al[35] found that amine treated cellulose releases a significant number of phages in the aqueous environment. Liana et al[19] studied infectivity of immobilized phages on COOH, NH_2, and CH_3 functionalized, and untreated ITO surfaces. All the studied surfaces had a similar number of detaching phages (10^6 PFU/ml), and they concluded that these phages produced the antibacterial effect measured. Our results suggest that the number of released phages varies between different surface treatments, and also with the number of detachment steps done.

3.3. Effective infectivity

Infectivity of the immobilized phage was studied by measuring the optical density of the host bacteria when exposed to the phage treated material.

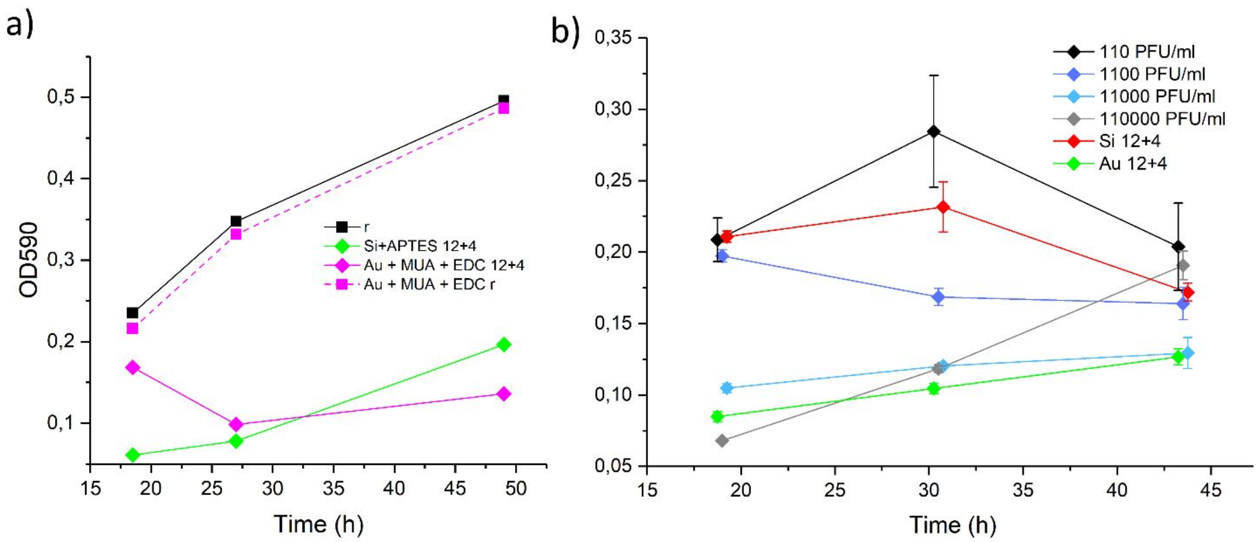

Figure 5. Effect of phage-treated surfaces and free phage on *Flavobacterium sp.* growth curves. a) Bacterial growth is reduced by the phage-treated surfaces (Si+APTES, Au+MUA+EDC) but not with phage-free surface (dashed line) or when no phage or surface is added (r, black line). b) Bacterial growth is reduced by both the phage-treated surfaces and added phage. Phage concentration 110 000 PFU/ml equals MOI 0.04 (with bacterial concentration of 2.5 x 10^6 CFU/ml). Mean ± SEM, (n=3).

An example of results for the antibacterial effect of phage-immobilized surfaces is presented in Figure 5a. First of all, the phage treated surfaces were able to significantly reduce the growth of the bacteria compared to the reference (no added phage), as expected. For the Si+APTES 12+4 sample, the bacterial growth reduction occurred already at the first time point (19 h), or even before, demonstrating high antibacterial efficiency. For the Au+MUA+EDC surface, the maximum in the growth reduction occurred at 27 hours. In a longer time scale, the turbidity of the culture increased again even with the phage treated surfaces, as a result of the growth of phage-resistant bacteria.[36]

Often, the efficiency of the phage-treated surfaces has been studied by comparing the growth of phage-exposed bacteria to growth of untreated bacteria, to calculate the reduction in the (logarithmic) colony forming unit (CFU). Another measure is the equivalent multiplicity of infection for the materials, which makes comparisons between different materials easier. There, the growth reduction caused by the immobilized phage is compared to different (known) MOIs of the free phage, and efficiency is presented as equivalent MOI.[17] However, here, we have developed the approach further and present infectivity as "effective infectivity" (PFU) per surface area. The benefit of this approach, compared to the equivalent MOI,[17] is that information on bacterial numbers is not required. In this study, an effective infectivity is computed by linear interpolation between the closest measured free phage reference treatment optical densities *OD1* and *OD2* corresponding to PFUs *P1* and *P2*, giving an Effective PFU for a measured optical density *ODSample* of a phage-treated material:

$$Effective\ PFU = P1 + \frac{P1 - P2}{OD2 - OD1} \times (OD1 - ODSample). \qquad (1)$$

Figure 5b demonstrates the experiments where the optical density of the Si and Au 12+4 surfaces is compared to the optical densities produced by known numbers of free phages. For example, for the silicon sample, the optical density of the sample falls between the values of 110 and 1100 PFUs/ml of added free phage at second and third time points, so those are the data points used in the interpolation. The first timepoint was left out from the calculation because it does not fall between any free phage data points. Similar calculations were conducted for every sample, interpolating the efficiency separately for all time points, which thus produced several measurement points for mean effective infectivity over time. Three individual replicates were done for all the samples and the standard error of the mean (SEM) was calculated. The fractional error of the free phage titer was 23 percent (phage stock titer $(6.2 \pm 1.4) \times 10^9$ PFU/ml) and it was added in quadrature. When using this approach for other phage-bacterium systems, it should be noted that the bacterial growth dynamics with phage differ between strains and species, and the Flavobacterium strain used here responds relatively slowly compared to e.g. *E. coli*, which may require shorter incubation times. In addition, it is important to have a free phage reference infection in the same conditions (simultaneously), to rule out the effects of culture conditions and e.g. the growth phase of the bacteria. The accuracy of the effective PFU can be increased by using more free phage references, for example 100, 200, 500 and 1000 PFU/ml instead of 100 and 1000 PFU/ml.

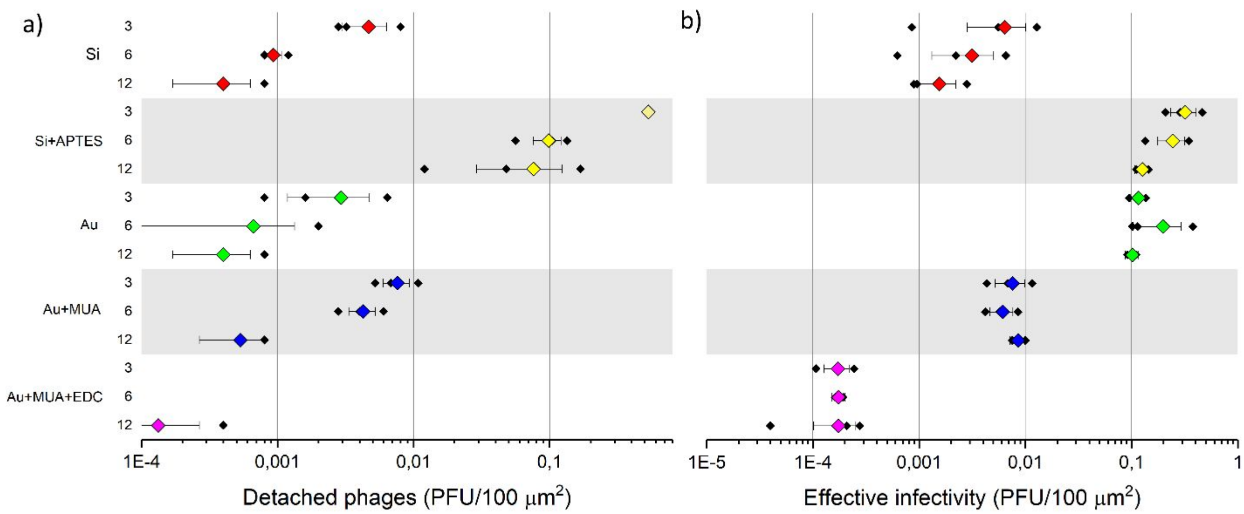

Figure 6. Phage detachment and effective infectivity of different surfaces with immobilized phage. Samples were dip-washed 6 times before detachments. The number after the name of the material indicates the number of detachment steps after dip washing. a) The number of infective phages in the detachment buffer per sample surface area. b) Average values of effective infectivity (defined in the text) of the phage treated materials per surface area. All 12-detachment step samples were washed an additional four times before infectivity tests. Colored symbols: Mean ± SEM, (n=3), black symbols: individual replicates.

When comparing the infectivity and the number of detached phages, common trends but also differences can be found (Figure 6). APTES with a high number of detached phages had a high infectivity but gold with low number of detached phages also had a high infectivity. APTES infectivity shows a trend of decrease with the detachment steps (3 to 12), but the change was not statistically significant ($p>0.05$). After all the 12+4 detachment steps, APTES had the highest infectivity, 26 percent higher than gold ($P<0.05$) and 160-fold compared to untreated silicon. The MUA treated gold surface had about ten times lower infectivity than untreated gold. EDC treated MUA has the lowest infectivity, about ten times lower than silicon.

Previously, Liana et al.[19] reported that all surfaces (carboxyl, amine, neutral) produced an equal antibacterial effect by the released phages. According to our results, the infectivity and the number of released phages varies between the surface treatments. Tawil et al.[18] found that covalent immobilization with MUA+EDC resulted in ten-fold growth reduction compared to plain gold. Their result is contradictory to our results which show over 100-fold infectivity of the plain Au compared to the MUA+EDC treated case

According to our results, the number of washes or detachment treatments does not have a significant effect on the infectivity of the surfaces. However, the number of detached phages varies between the detachment treatments. A long lasting infectivity is a desirable feature of the material for prolonged use in an aquatic environment. Within this study, the Si+APTES or gold surface is most suitable from that point of view.

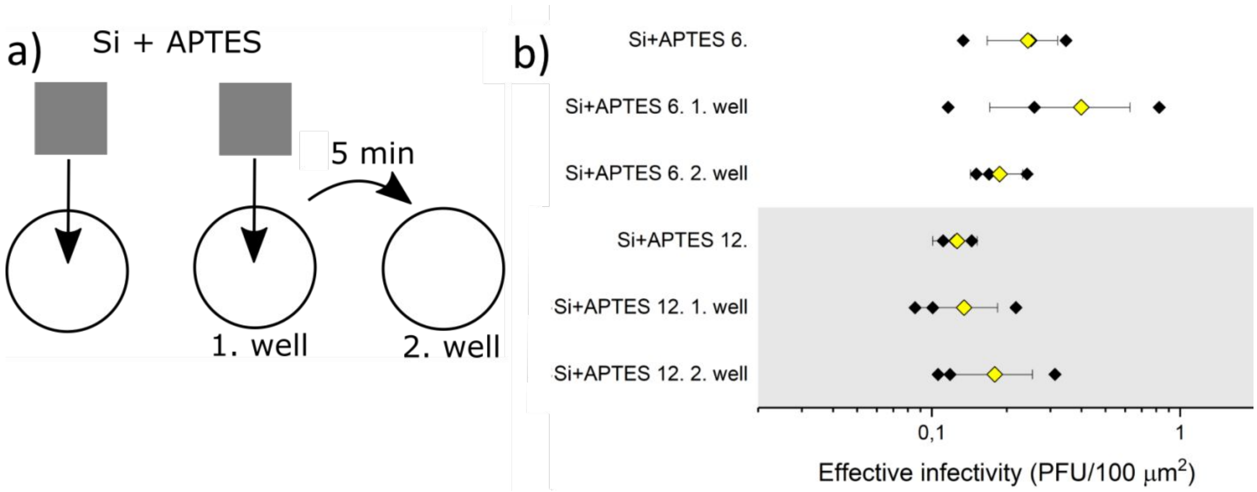

Figure 7. A well-change experiment was used to resolve the effect of the detached phages. a) Sample substrate was transferred to another sample well after 5 min incubation. b) The effect of the well change on the effective infectivity for the 6 and 12 times detached Si + APTES sample. Colored symbols: mean ± SEM, (n=3), black symbols: individual replicates

While the actual antibacterial effect is caused by the phage replication, one of the main questions in using surfaces with immobilized phages is whether the initial effect is caused on the surface by the attached phages or in the solution by the detached phages. To separate the effects, we performed an experiment, where a Si + APTES surface was transferred from one well to another before the addition of bacteria. The sample was placed in 1 ml of Shieh medium, shaken 400 RPM 5 sec, incubated 5 minutes and moved to another well (Figure 7a). We expect that if the antibacterial effect is caused by the detaching phages, we should see bacterial growth reduction in both of the wells. On the other hand, if the effect is caused by the attached phages, the antibacterial effect should be seen only in the second well, where the surface is present. We observed that infections occurred similarly in both wells (Figure 7b), suggesting that the antibacterial effect is mainly caused by detached phages for this surface.

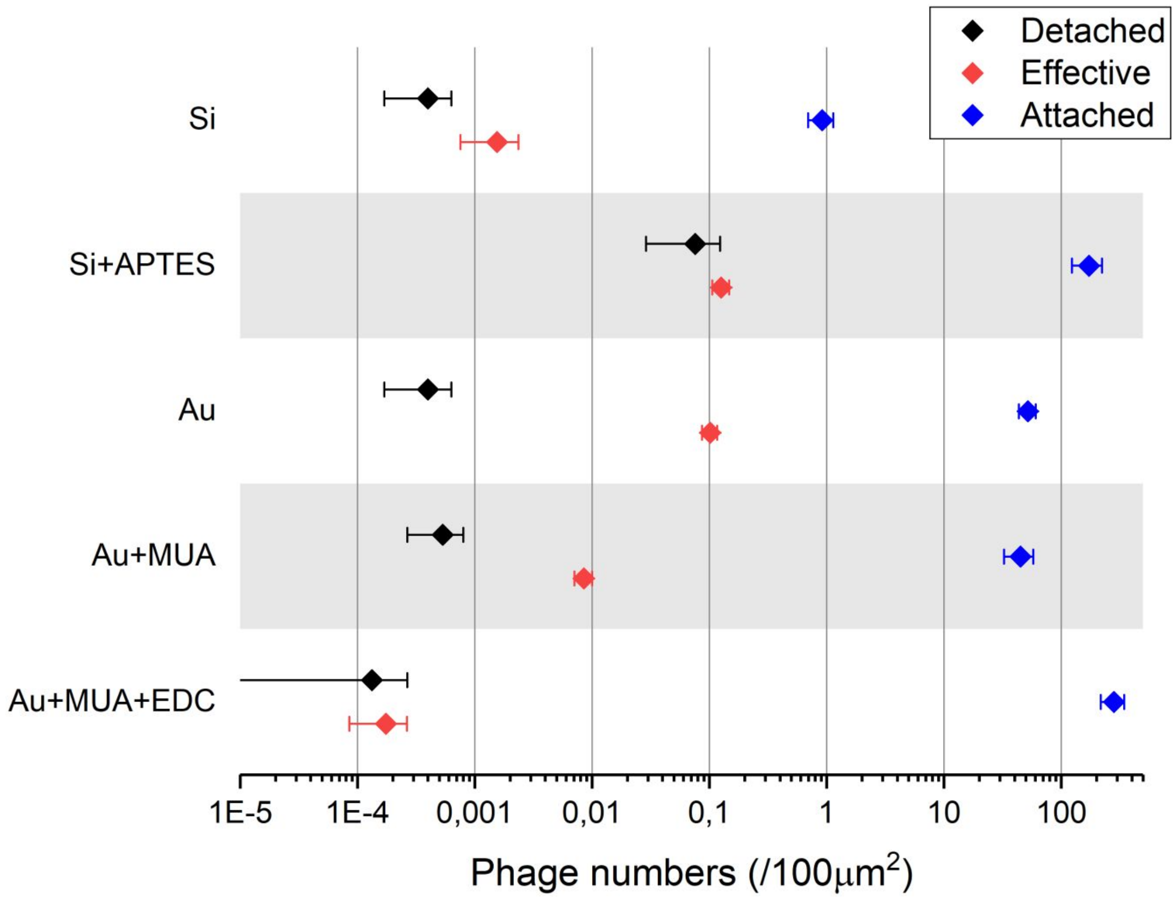

Figure 8. All three PFUs (attached, detached, effective infecting) for the 12 or 12+4 detached substrates. Mean ± SEM, (n=3),

As a final conclusion, the numbers of attached, detached and effectively infecting phages of all five materials are presented in Figure 8, after the full set of detachment and washing steps. Silicon and gold had equal number of detaching phages, but the number of attached and effective PFUs were hundredfold on gold vs silicon. This suggests that phages were attaching on the gold surface in such a way that they retained their infectivity. In the literature, when T4 phages were immobilized on the gold surface by physisorption, the attachment and the lysis of the bacteria on the surface were detected,[37,38] proving that infection on the surface is possible. However, it is worth to keep in mind that in real life scenarios like in aquaculture it is possible that infectivity is reduced by the presence of substances such as other organic material on the surface.[39]

In contrast, APTES-treated silicon had the highest infectivity and also the highest number of detaching phages. Additionally, it was confirmed by the well change experiment that detaching phages produced the effect. The EDC crosslinked surface had the highest number of attached phages, but the infectivity was lowest. The high number of attached phages did not result in high infectivity. It is possible that the infectivity was lost upon the chemical interaction with the EDC treated surface, or by the imperfect orientation of the phage. Unfortunately, it was not possible to resolve the orientation of the immobilized phages with the imaging methods used. In the literature, inequality between the infectivity and the number of covalently bound phages has been found before, with a suggestion that some phages are inactive or misoriented.[17]

Conclusions

We studied the attachment density, detachment rate and infectivity of *Flavobacterium*-infecting FL-1 bacteriophage on different surfaces: silicon, amine-treated silicon, gold, carboxylate-treated gold, and crosslinker-activated carboxylate-treated gold. It was found that detached phages could produce a significant antibacterial effect, especially on the amine-treated silicon. Therefore, when studying phage immobilization, one must be careful with what is causing the measured effect. Covalent cross-linking between the phages and surface, on the other hand, produced highest numbers of attached phages, but seemed to interfere with infectivity. Therefore, we suggest that the controlled release of antibacterial phage, i.e. the phage reservoir approach, might be preferable for practical applications. In the future, development of imaging methods capable of resolving the orientation of the immobilized phage could make a tremendous contribution to this field. We also introduced a novel, standardisable, way to quantify the antibacterial effects of surfaces, by comparing the antibacterial effect of the material surface to the effect caused by a known number of free phages.

This effective PFU/surface measure will make results from different studies comparable.

Supporting Information.

Purification data, data for density, growth curve. (Supplementary.docx)

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Author Contributions

The experiments were conceived by all authors and performed by M. L. 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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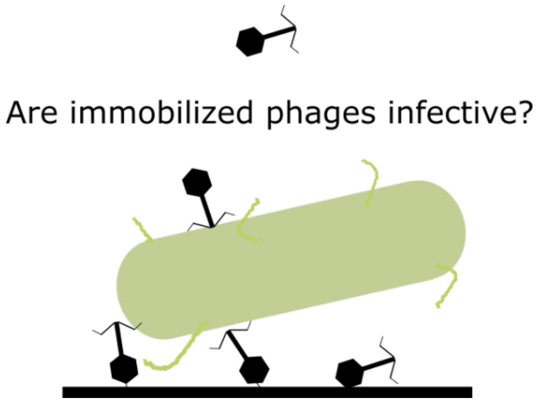
ToC figure


Are immobilized phages infective?