A Reduction of Settlement Probability of *Chlorella Vulgaris* on Photo-
Chemically Active Ceramics with Hierarchical Nano-Structures

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Abstract

Biofouling is defined by the adsorption of biomolecules or microorganisms on technical surfaces, which are causing adverse negative effects on the functionality (decrease of ship speed) and safety (infections of implants) of quite a number of industrial products. Conventional, anti-biofouling is done by coating a technical surface with highly poisonous tin-organic, which have already been banned for environmental protection. Therefore, the developing of biologically benign coatings becomes a long-term pursue for the industry. Here, we study the *Chlorella vulgaris* settlement on self-disinfecting titanium dioxide surfaces with three different micro-structures: a flat surface, a light harvesting surface with nano-structure and a hierarchical surface structure, spanning This document is the accepted manuscript version of the following article:


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over 5 orders of magnitude (from 0.1 nm to 10 μm). These titanium dioxide surfaces were prepared by Ostwald ripening. This sample manufacturing process gains new catalytic properties as a self-cleaning effect, especially for the light harvesting surface with nano-structure (bulk metallic glass). *Chlorella vulgaris* dispersions were growing in glass flasks together with the different surface samples over the full time of the experiments. Therefore, this study was made as part of a laboratory scale test. It was found, that bulk metallic glass structures made by Ostwald ripening is showing the highest catalytic and at the same time the best self-cleaning effects. Additionally, the *Chlorella vulgaris* settlement probability was found to depend on the Wenzel roughness. The surfaces with a high Wenzel roughness were the one with the lowest *Chlorella vulgaris* settlement. A semi-field test is proving the comparable antibiofouling performance of our surfaces with existing polymeric or sharkskin like structures on the timescale of one month.

**Keywords:** antibiofouling; Wenzel roughness; settlement points; *Chlorella vulgaris*; self-cleaning

1. **Introduction**

Technical surfaces which are immersed into aqueous media get almost instantaneously covered by a layer of proteins and followed by adsorption of microscopic life forms (e.g. cells, algae, bacteria).[1] This phenomena is defined as biofouling, which describes the adsorption on technical surfaces of molecular, single microscopic species, bacteria slime, large algae, layers of crustae (barnacles, mussels), sponges, and more.[2]
Biofouling often causes severe hindrances on the functionality of technical and biological system, such as inability to utilize sensors, reduced speed of ships, or infections in case of contaminated implants for humans or animals.[2–4] In the past, most technical aquatic surfaces were coated with tin-organic coatings.[1] These coatings are releasing continuously poison due to perpetual self-polishing and simply poisoning in this way all life forms in the aquatic vicinity of the coating.[1] Therefore, these tin-organic coatings were banned recently and replaced by copper (another heavy metal) containing coatings. However, the banning of all self-polishing surface coatings for aquatic applications is currently under a political debate.[2] In biomedical application, silver might be a promising option for antibacterial implant applications.[5] The marine and freshwater industry have no other option as to find safe alternatives for existing anti-biofouling systems. One of these alternatives are natural biocides, which secreted by aquatic plants and are under study currently.[6] Synthetic organic coatings are not able to synthesize own chemicals or store biochemical components for a long time, due to decreasing activities of different proteins.[6] Their application is therefore limited by the required self-leaching coatings, fast fouling and high costs.[6] Another approach is to reduce the adhesion strength of a microscopic object, which provides successful to some extent, especially when the ship moves fast, drag can wash life forms away.[7] Low elastic moduli were also reported to reduce fouling adhesion strength, especially in combination with a surface energy of ~23 mN, because extremely low surface energy supports adhesion of microalgae based on hydrophobic effect.[8] This
effect was only successful on fast moving vessels to be able to release the fouling, otherwise the surface was still strongly fouled on longer timescales.[9]

Recently, nano-heterogeneous surfaces has been proved to be able to reduce adhesion and even prevent attachment of proteins and viruses.[10] This approach is useful for a limited amount of proteins with known structure, but is unreliable for marine or freshwater applications due to the vast diversity of available proteins. Many other proteins can still coat and inactivates the system.[11] Amphiphiles, dentrites and peptites are also able reduce biofouling; but further tests with larger species variations in long term studies are still missing.[12,13] Liquid infused surfaces show significant antifouling capability on short timescales, however the liquid seeped out of the structures, causing significant fouling on timescale of weeks.[14] The famous sharkskin and rice leaf surface structures were designed to reduce fouling attachment points,[15–17] so that living microorganisms were unable to move into the voids, this mechanism is quantified by roughness feature sizes (Wenzel roughness) and microalgae settlement probability.[18,19] Since biological life form sizes span over several orders of magnitude (3 – 4), an antibiofouling structure spanning over this whole size range is necessary.[1,2,15] The idea for a Wenzel roughness of a technical surface beyond that of a shark skin is explored in this work on hierarchical titanium dioxide structures made by Ostwald ripening. Another approach combined biological low elastic modulus gels in combination with alginate particles for structured surfaces and copper to release poison.[20] However, this study achieved due to the low particle size only limited
advantage over glass slides, while the high dispersion forces of copper caused on short
timescales increased biofouling.[20]

The idea of using titanium dioxide (TiO₂) for hierarchical technical surfaces in the field
of anti-biofouling is not new. Many creative approaches were studied and reported over
the time, as the following research examples are showing. Patrocinio et al. reported a
TiO₂/WO₃ thin film surface produced by layer-by-layer with a photocatalytic self-
cleaning effect.[21] Jalvo et al. also used the photocatalytic self-cleaning effect of TiO₂
as a disinfection possibility using the UV part of the sunlight.[22] Xu et al. reported in
their study the difference for the wettability and self-cleaning properties between a
Cassie and a Wenzel surface of hierarchical surfaces made with TiO₂-Polymer
nanocomposites.[23] TiO₂ nano-particles were also coated on limestone for the
investigation as self-cleaning stone surfaces on the construction side, which was
reported by Quagliarini et al. in 2012.[24] Padmanabhan et al. published a review article
about the actual status of TiO₂ based self-cleaning surfaces and their applications.[25]

In this study, we focus on hierarchical Wenzel surfaces for marine applications. The
self-cleaning features of TiO₂ are combining these hierarchical Wenzel surfaces made
by Ostwald ripening. This combination is gaining new anti-biofouling features of
hierarchical TiO₂ surfaces by significantly decreasing of the contact area for the
attachment of living species, like algaes. Therefore, we compare the settlement
probability of Chlorella vulgaris on flat, needle like and hierarchical micro-nano sisal
structured titanium dioxide structures bulk metallic glass (BMG) produced by Ostwald
ripening. The structural hierarchy is spanning over 5 orders of magnitude (from 0.1 nm to 10 μm). These surfaces are able to harvest light and perform oxygen radical based self-cleaning and disinfection,[26] which results in low settlement probabilities of *Chlorella vulgaris* on them (Fig. 1). The hierarchical sisal structures is showing the lowest attachment. However, inorganic fouling poses a larger thread for hierarchical sisal structures than biofouling, as it increases attachment side by orders of magnitude. All titanium surfaces exposed lower *Chlorella vulgaris* attachment, compared to non-radical producing glass references surfaces.

![A) BMG with biofoul settlement. B) Almost complete removal of bacteria and algae settlement of a BMG sample over mechanical energy, driven by tide or a moving ship. The force vector of the mechanical energy is given by $\vec{F}$.](image)

Fig. 1. Utilized antifouling structures. A) BMG with biofoul settlement. B) Almost complete removal of bacteria and algae settlement of a BMG sample over mechanical energy, driven by tide or a moving ship. The force vector of the mechanical energy is given by $\vec{F}$.

2. Methods and Materials

2.1 Materials and Sample production

The bulk metallic glass (Ti$_{40}$Zr$_{25}$ – BMG) substrates were produced according to reference.[27] In brief: binary alloy ingots were prepared by arc melting pure elemental metals of Ti and Zr with a purity of 99.5% (Sigma, St. Louis, USA) in a water-cooled copper crucible and introduced in a Ti-gettered argon atmosphere. To achieve
compositional homogeneity, the alloys were re-melted at least four times and accompanied with magnetic stirring of the molten alloy. The Ti-Zr alloy as precursor and matrix was cut from the ingot via electric spark cutting. Ti-Zr alloy, flat Ti, Ti needle and Ti with added Zr powder were all exhibiting a size of $20 \times 20 \times 1 \text{ mm}^3$. Ti-Zr alloy, Ti platelets for Ti needle growth and Ti plates with added Zr powder were polished with waterproof SiC paper with (#240, #800, #2000). Sample cleaning were proceeded by ultrasound sonication in ethanol for 30 minutes, followed by rinsing with ultrapure water.

To gain titanium needles as well as the sisal structures, the sandpaper polished and sonicated samples were immersed into 5M NaOH in Teflon coated autoclaves and heated for 12 – 16 hours up to 140° C. The growth of needle like structures on pure Ti and nano-sisal like structures in case of Ti-Zr alloys is due to the Oswald ripening process as described in reference.[27] Ultrapure water was produced from a Purelab Classic (Elga Labwater, Lane End, High Wycombe, UK). The shark skin like structure, hydrophobic PDMS and hydrogel structure (negatively charge terminated PEM) were all produced according to reference.[11] As mentioned before, samples served here only as references, the timelines of biofouling performances of these samples can be observed in reference.[11]

2.2 *Chlorella vulgaris* adsorption and sample evaluation

All samples were placed into separate Erlenmeyer flasks (500 mL) and immersed into 50 mL of *Chlorella vulgaris* dispersion. *Chlorella vulgaris* were grown in a special
growth solution (for composition of the growth solution, see supporting information (SI) table S2). Both, Chlorella vulgaris and the growth solution (sold as Chlorella vulgaris growth solution) were obtained as Freshwater Algae Culture Collection (FACHB-collection) from the Institute of Hydrobiology, Wuhan, P.R. China. Water evaporating from the flasks was replaced with ultrapure water to prevent changes in ionic strength. After 2 weeks the whole Chlorella vulgaris dispersion was replaced.

A hemocytometer (Shanghai Qiujing, Shanghai, P.R. China) was utilized to determine the Chlorella vulgaris concentration at the start of experiments. Starting concentration of Chlorella vulgaris was 2.9 × 10^7 cells/mL. Chlorella vulgaris were exposed to white light stemming from a red/blue diode array (type 2835, Ling Dong, Shenzhen, P.R. China) at a light flux of 8.3 mW m⁻² (measured via a Thorlabs PM100D, Dachau, Germany). Samples were investigated at day 1, 2, 4, 8, 16, 24, 32, whereby the sample was removed from the Chlorella vulgaris dispersion, rinsed with ultrapure water and observed in reflection mode with a 10 × and 40 × objective to determine Chlorella vulgaris coverage. The Chlorella vulgaris grow solution and sample dispersion has been shaken at every day. An Olympus BX-51 bright-field microscope (Olympus, Tokyo, Japan) was utilized to acquire reflection micrographs of the BMG, flat Ti, Ti needle and Ti-Zr powder sample, as well as Chlorella vulgaris settlement on these. For this purpose, the samples were taken from the Chlorella vulgaris dispersion, which resulted in aeration for every sample for about 5 – 10 minutes per day. The Chlorella vulgaris settlements on the bright-field reflection micrographs were evaluated with
ImageJ v1.52v. Scanning electron microscope (SEM) micrographs were acquired by a Hitachi SU-8010 (Hitachi, Tokyo, Japan) with an acceleration voltage of 10 and 20 kV and a working distance of ~10 mm. Electron dispersive X-ray (EDX) measurements were gained with the same device with an acceleration voltage of 30 kV.

One-sided ANOVA (Origin 9.1.0 SR2) was utilized to determine the statistical significance of the gained results. The Wenzel roughness ($R_{\text{Wenzel}}$) for all samples were calculated based on the Wenzels approach.[18,28] With the three-dimensional rough surface area ($S_{\text{Actual}}$), two-dimensional plane surface area ($S_{\text{Geometric}}$), surface area of the sample type ($S_{\text{Structure}}$) and the optimal two-dimensional surface area ($S_{\text{Optimal}}$):

$$R_{\text{Wenzel}} = \frac{S_{\text{Actual}}}{S_{\text{Geometric}}} = \frac{S_{\text{Structure}}}{S_{\text{Optimal}}}$$

(1)

It is worth mentioning, that small roughness flat Ti and smooth glass were considered to have a Wenzel roughness of 1.

The complete calculation of the surface areas and Wenzel roughness ($R_{\text{Wenzel}}$) for each sample is shown and explained in the support information (SI, chapter 1, Fig. S1 and table S1).

2.3 Antibiofouling test with river water in semi-field test environment

Lots of artificial surfaces perform splendid in laboratory tests but fail in field tests, like large shark skin structures in the range of hundreds of micrometers, which repel barnacles but supported the settlement of Ulva-Linza, smaller larvae or other algae [18,29,30]. To determine the real antibiofouling performance, repeatable conditions
over a long time must be fulfilled. This is difficult to perform with natural rivers, and conditions, especially as the light changes due to weather conditions, and the river often changes in water level further compromising the amount of impacting light and especially water depth and present life forms. In some cases, the samples might not touch water at all if the water level falls significantly. For this reason, a semi-field test was performed utilizing a fish tank and river water. This test river water (Majia Go, Harbin, P. R. China) in a fish tank with pump induced water flow of 5.5 ± 0.4 cm/min, light flux 2.5 mW/cm for 8 hours per day was chosen, which is a comparable condition to reference.[11] Evaporating water was refilled with distilled water while the sample now was hanging 90° compared to laying like in case of Chlorella vulgaris test.

3. Results and Discussion

3.1 Investigation of produced structures

The Oswald ripening process was successfully utilized to create needle like structures on the originally smooth titanium plates (Fig. 2A). A pure smooth titanium plate exhibits after Oswald ripening a nano-needle covered surface (Fig. 2B). The addition of zirconium powder to the sodium hydroxide containing solution adds randomly distributed nano-sisals as a surface feature (Fig. 2C). Utilizing titanium-zirconium alloy or BMG instead of pure titanium sheets allows the establishment of a regular and dense nano-sisal coverage (Fig. 2D).

Determining the contact area of an average Chlorella vulgaris of each type of sample,
the glass, as well as the pure titanium plate exhibit a large contact area in the range of ~12 µm². This sample is followed by the needle and titanium zirconium powder enriched samples, which both scored in the range of 2.3 – 2.45 µm² (Fig. 2E). The BMG structure displayed different properties like orders of magnitude lower contact areas due to sharper needles (Fig. 2E), which exhibit 1 atom at the needle top, as stated in reference.[27] On the contrary, the Wenzel roughness of these structures is the highest for the Oswald ripened BMG nano-sisal structure, and lowest for the flat samples (Fig. 2F). It is worth of note, that the investigated Ti-needle, BMG and Ti-Zr nano-sisal structure are containing powder samples exhibit Wenzel roughness orders of magnitude above those previously reported.[18]

Fig. 2. Utilized antifouling structures and properties of produced samples. A) Original titanium plate, B) Oswald ripened Ti nano-needles, C) random nano-sisal like structure for zirconium powder containing solution upon Oswald ripening a titanium plate, D) dense nano-sisal structure on Oswald ripened titanium-zirconium alloy (BMG – bulk metallic glass). The scale bars are 5 µm. E)
Determined contact area of an average *Chlorella vulgaris* on corresponding sample; F) Wenzel roughness factor ($R_{\text{Wenzel}}$) of flat titanium plates (Ti Plate), Oswald ripened Ti-needle containing sample (Ti Needle Structure), Oswald ripened titanium plate with added Zr powder (Ti-Zr Random Structure), Oswald ripened Ti-Zr alloy featuring nano-sisals. For E and F, the plotted values are to find in SI table S1.

### 3.2 Antibiofouling properties of different Wenzel roughness containing structures

Exposing the produced structures and a pure glass slide to *Chlorella vulgaris*, all titanium-based structures performed order of magnitude better than a clean glass slide highlighting the power of the photocatalytic self-cleaning of titanium dioxide for antibiofouling. Significant differences ($\alpha < 0.05$) were determined between Ti-Zr and flat Ti sample, whereby the flat Ti sample displayed same coverage as Ti-needle (Fig. 3A and 3B). The only structure which is exhibiting biofouling values which were exhibiting constantly low biofouling compared to other samples is the BMG sisal structure (Fig. 3A and 3B). Compared to smooth glass, the biofouling of the nano-sisal structure was on the timescale of one month decreased by factor $\sim 330$, while it was for smooth titania factor 10. This is pointing out the significant influences of self-cleaning and structure based antifouling properties.
Fig. 3. *Chlorella vulgaris* biofouling level of surfaces: A) on original Ti, Oswald ripened titania, Oswald ripened TiZr and Oswald ripened BMG containing self-disinfecting surfaces, B) logarithmic displayed zoom into of A) without glass slide sample, C) in relation to contact area for titania containing surfaces, D) in relation to the Wenzel roughness for titania containing surfaces.

Correlating the contact area and the Wenzel roughness with the average biofouling value of one month, a clear relation of the fouling level with both values can be determined (Fig. 3C and 3D). Although low contact areas for *Chlorella vulgaris* down to 0.5 µm² for Ti-needle and Ti-Zr did not influence the fouling level significantly. However, very low contact areas as in case of BMG caused a significant reduction in biofouling (Fig. 3C and 4). This low contact area of the dense sisal-structures stems from an extremely high Wenzel roughness, which is orders of magnitude above any previously reported value.[18] The fact, that Wenzel roughness in the range of 0 – 11.8 does not show any significant decrease in biofouling is due to the too high contact area.
It is noted, that the slightly increased biofouling after 2 weeks of immersion for the Ti-Zr sisals in Fig. 3 and 4 (and SI Fig. S2) is caused by rapidly increasing inorganic fouling, mainly due to CaCO₃. This effect is explained with ongoing highly efficient mineralization (oxidation of organic matter, also containing calcium (e.g. *Chlorella vulgaris*, Fig. 4)) close to the surface, while the negatively charged surface attracts positively charged calcium ions. This supports inorganic fouling, passivating the surface and increasing contact area (Fig. S3). It is worth of note, that the passivated surface also reduced self-cleaning and self-disinfecting properties of titanium-based surfaces. Due to the high performance of self-disinfecting coatings, the glass slide was not used as a reference in further tests.

### 3.3 Semi-field test with natural species variety
The BMG structure shows in case of 50% decreased light flux in a simulated field test a decreased inorganic and organic fouling compared to the *Chlorella vulgaris* only test. It is noted, that in this case a shear flow existed and the samples were rotated by 90° compared to the previous settlement test. These results are in agreement with results of polymeric thin films in reference.[11] The biofouling rate which includes in this case also dead *Chlorella vulgaris* (determined by color) was for BMG 2.5% (Fig. S4), surpassing the values of hydrophilic PDMS, hydrophobic PDMS (a standard fouling release coating), PDMS based sharkskin and in line with negatively charged hydrophilic gel films (Fig. S5 and S6). Ionic concentration of river water compared to *Chlorella vulgaris* dispersion (35 mg/L vs. 3.6 g/L, SI table S2-S4) is differing by order of magnitude and was found to affect the formation of inorganic fouling which contributes the main part in Fig. 3 and Fig. S4. In addition, the constant water flow in our fishtank is expected to reduce the local ion concentration, and flushing potential biofoulants away. A lower light flux, although it decreases the rate of biofoulant oxidation, decreases also the amount of low mobility negatively charged oxonium ions on the surface. This effect can be explained by the fact, that protons are highly mobile and leave surface more quickly. Reduced light flux is therefore reducing the charge-based calcium ion diffusion to the surface. This decreases the inorganic fouling, keeping the attachment sites for the biofoulants low. Biofouling species have due to the permanent water flux less time to release protein-based glue.[7] In addition, the present hydrodynamic drag is flushing them away. Therefore, the species are less likely to be
attached. It is noted, that BMG is in this case again the structure which is at least fouled compared to pure titanium Ti-needle or Ti with added Zr powder. This result also proves that the Wenzel roughness is able to reduce the attachment of biofouling species.

An important reasons for the low performance of Ti plates in Fig. S4 is the presence of inorganic and dead *Chlorella vulgaris*. If one negates this “dead” biofouling component, one gets different results (Fig. 5A). In this case, a flat Titanium surfaces achieve the highest performance with only 0.01% living *Chlorella vulgaris* and other algae on the surface (determined by color). This value is followed by BMG with 0.1% living biofouling, followed by Ti-needle structure with 0.3% and Ti-Zr with 2%. These results are showing, that the biofouling of living species does not necessarily correlate with the Wenzel roughness, but most likely on the contact area which the surface and the biofoul have, as it causes more effective radical transport and therefore biofoul killing (Fig. 5B).

Comparing only the living species on BMG and flat titanium with the one of gel films or shark skin like structures, one sees differences in the order of magnitude (Fig. S5). These differences are explained with the different interactions, which is in case of gel films based on elastic modulus, ions and surface charges.[31,32] In addition, the PEM surfaces we are using here for comparison are not emitting radicals and exhibit self-polishing, but not self-cleaning properties, severely affecting settlement properties of cells and biofoulants.[11,33]
Fig. 5. A) Comparison of biofouling degree on various titanium containing surfaces in a test with river water. B) Logarithmic display of A).

4. Conclusion

Technical surfaces based on Titanium dioxide (TiO$_2$) can be utilized as self-cleaning and self-disinfecting antibiofouling surfaces. In addition to this effect, significant improvement compared to this self-disinfection approach is achieved based on contact area minimization characterized by the high Wenzel roughness, mainly due to hierarchical structure formation in case of (bulk metallic glass) BMG. In the actual literature, lower Wenzel roughness were reported for different technical hierarchical TiO$_2$ surfaces.[21,23] The fact, that not only single species but also multiple species from a natural river sample were to a large extent unable to settle on the dense nanosial structure, shows that it is able to repel multiple life forms and life form sizes not only one type in an artificial environment, especially when exposed to flow and in an orientation, that does not support gravitational aided sedimentation on it. Moreover, BMG is showing a good self-cleaning process over the time period of one month, due to the very high Wenzel roughness and low contact area. Other research groups are reporting problems with a crusting layer from the dead Chlorella vulgaris rests on the
TiO₂ surfaces with lower Wenzel roughness and higher contact area, which are lowering the catalytic self-cleaning effect of the TiO₂ surfaces dramatically at a short time period.[22] The focus on other studies in the field is about the poising of *Chlorella vulgaris* with TiO₂ containing structures, which is can be a problem for the environment.[34][35] An effective modification of technical TiO₂ surfaces by Ostwald ripening to gain sisal-structures to lower the contact areas for *Chlorella vulgaris* is not an environmental problem, as we could show in this study. This is due to the effect, that light based oxygen radical creation supports the antibiofouling properties. Due to negative titanate surface charge, the sisal-structures are prone to inorganic fouling, increasing attachment sites and thus inactivation and biofouling occur over the scale of several months even in case of BMG. It was also determined, that water with low ionic strength is causing decreased inorganic fouling on the sisal structure, as the inorganic fouling and hence surface passivation and contact area increase is reduced.

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6. **References**


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**Graphical Abstract:**