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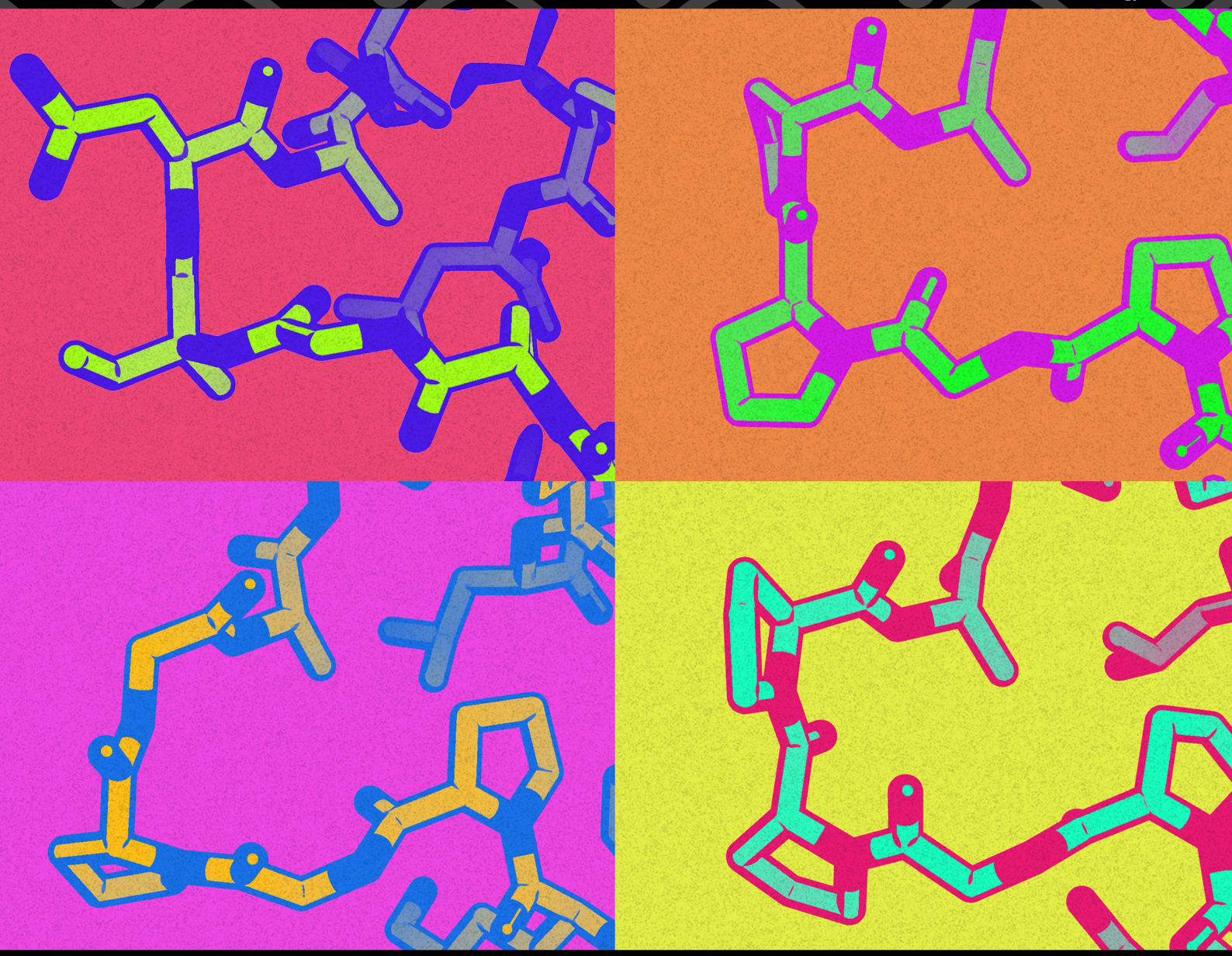

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Boronic Acid Moieties Stabilize Adhesion of Microalgal Biofilms on Glassy Substrates: A Chemical Tool for Environmental Applications

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Photosynthetic organisms such as diatoms microalgae provide innovative routes to eco-friendly technologies for environmental pollution bioremediation. Living diatoms are capable to incorporate *in vivo* a wide variety of chemical species dispersed in seawater, thus being promising candidates for eco-friendly removal of toxic contaminants. However, their exploitation requires immobilization methods that allow to confine microalgae during water treatment. Here we demonstrate that a biofilm of *Phaeodactylum tricornutum* diatom cells grown on the surface of a glassy substrate bearing boronic acid protruding moieties is stably anchored to the substrate resisting mechanical stress and it is suitable for removal of up to 80% metal ions (As, Cr, Cu, Zn, Sn, Pb, Sb) in a model polluted water sample. Control experiments also suggest that stabilization of the biofilm adhesion occurs by interaction of boronic acid surface groups of the substrate with the hydroxyl groups of diatoms extracellular polysaccharides.

Introduction

Heavy Metals (HMs) are contaminants distributed worldwide not only in areas directly affected by anthropogenic activities but also in globe remote regions, since they persist in both water and soil matrices, they are easily transported by air and marine currents, eventually jeopardizing the whole trophic web.[1–2] HMs mainly accumulate in marine sediments, together with organic pollutants,[3,4] at the water-seabed interface, or inside the seabed, where they are not firmly immobilized.

Depending on environmental conditions, heavy metals can affect benthic organisms and microbial populations,[1–5] negatively interacting with enzymes, cell membranes, and DNA in biological systems. They have cytotoxic, cancerogenic, and mutagenic effects at values higher than tolerated threshold levels.[6]

Literature shows a series of conventional methods for HMs removal from contaminated soil and water samples, mainly based on ion exchange, reduction or oxidation processes, electrochemical treatments, membrane-based filtration, or chemical precipitation techniques.[7] Nevertheless, most methods are expensive, time-consuming, they require the use of undesirable synthetic xenobiotics and produce toxic sludges that must be disposed. On this ground, bioremediation by living microorganisms, such as bacteria, yeasts, microalgae, or fungi, is currently gaining great interest as a cheap and green alternative technology for sensing and removal of a variety of pollutants, including heavy metals, in contaminated water and lands.[8,9]

In particular, photosynthetic microalgae provide multiple benefits, being both viable bioremediating microorganisms and natural sources of biochar,[10] a biosorbent non-living material suitable for wastewater filtration and decontamination.[11–13]

Among microalgae, diatoms are unicellular photosynthetic organisms that dominate phytoplankton, living in suspension or substrate-adhesion both in freshwater and marine habitats. They exist in more than 100.000 species with different size (from 10 to 200 μm) and shapes.[14] With respect to other species, such as green and red microalgae, diatoms bear an impressive capability to adapt and survive to environmental condition changes (***e.g.*** salinity, pH, temperature),[15] thus being suitable candidates for wastewater bioremediation.

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Their resistance to harsh conditions is mainly related to the presence of mesoporous biosilica shells, known as frustules, that protect diatoms from ultraviolet dangerous light or viruses. Frustules also represent a source of biosilica-based materials for various applications, obtainable by *in vivo*[16,17] or *in vitro* chemical modification approaches of diatoms shells.[18–22]

Moreover, diatom species with substrate adhesion properties, such as *Phaeodactylum tricornutum*, secrete exopolysaccharides (EPSs) intimately bound to frustules, that host symbiotic bacteria and act as a protective barrier against unfavourable external conditions.[23] Hence, the suitability of such species as environmentally remediating microorganisms with respect to microalgae suspended in seawater is related both to their resistance and to the natural tendency to grow as stable biofilms on supports. Indeed, immobilization[24] of microorganisms such as diatoms, on surfaces of proper substrates represents a convenient approach to develop eco-friendly systems for seawater or seabed bioremediation, since it allows (*i*) to boost and stabilize microalgal biological activity, (*ii*) to control parameters relevant to diatoms density and healthiness, (*iii*) to easily remove the biofilm after its decontaminating action and (*iv*) to recover the resulting biomass for further treatments or applications *in vitro*.

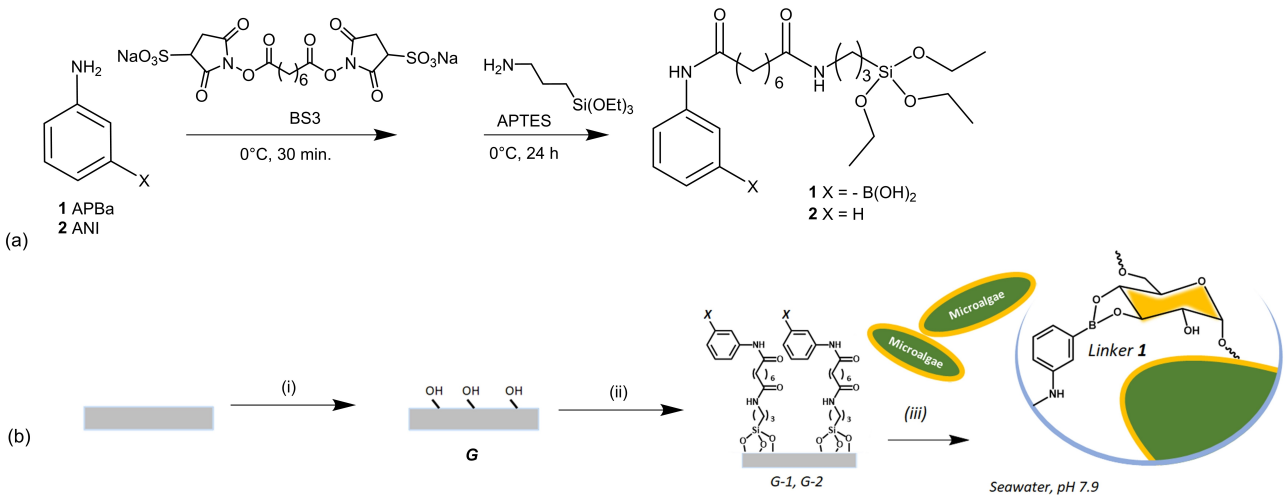
Literature shows that straightforward, cheap, and harmless protocols of immobilization of microorganisms are mainly based on their physical adsorption to the substrate surface by weak electrostatic interaction.[25] Nevertheless, they often suffer from cell detachment.

Conversely, methods based on covalent binding would be more efficient in terms of adhesion stability. Few examples of these methods have been reported so far, such as the immobilization of yeast cells on glass surfaces by imine-forming reactions of a glutaraldehyde bifunctional linker with the amino groups on both substrate's and cells' surfaces.[26] However, glutaraldehyde was found to be toxic and to affect metabolic pathways of organisms. Among alternative covalent binding approaches, the condensation reaction of peripheral boronic acid moieties on functionalized substrate surfaces with 1,2- or 1,3-diol groups of external polysaccharides of living cells deserves great interest. Indeed, it requires straightforward and mild experimental conditions (pH 7–8) and it is a pH-dependent reversible process that eventually allows a controlled detachment of cells and recycle of substrates. Such a reversible reaction, leading to cyclic boronate ester complexes, has been exploited so far to isolate diol-containing biomolecules such as glycoproteins,[27] enzymes or nucleosides by affinity chromatography on boronic functionalized cellulose beads[28] or polymeric monoliths.[29] Few examples have been reported so far, in which the covalent interaction between boronic acids and vicinal diols was investigated to immobilize yeasts[30] or targeted cancer cells[31,32] on boronic functionalized substrates, availing of the presence of carbohydrates or sialic acid on cells membranes. Here we report for the first time that the surface functionalization of glassy substrates with tailored synthetic organic molecules bearing pendant boronic acid groups is an effective approach to stabilize adhesion of biofilms of diatoms microalgae, such as *Phaeodactylum tricornutum*, by boronate forming condensation reaction with EPS diol groups on cell membranes. We also demonstrate the capability of immobilized diatoms to capture and remove a series of heavy metal ions from seawater model samples, this paving the way to new eco-friendly manageable systems for environmental pollution remediation based on living microorganisms.

Results and Discussion

Chemical design of the organic linker and glassy substrate functionalization

The molecular structure of the organic linker **1** (Scheme 1a) was designed to bear two different peripheral triethoxysilyl and



Scheme 1. a) Synthesis of linkers **1** and **2**. b) Surface functionalization of glassy substrates with the linkers and immobilization of cells (*i*): activation of substrates with a 1 : 2 mixture of sulphuric acid (98% m/V) and hydrogen peroxide (30% V/V) in water at 90 °C for 1 h; (ii) condensation with **1** or **2** in water and toluene (1 : 100 V/V) for 24 hours at 40 °C; (*ii*) adhesion experiments in seawater with living microalgae.

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phenylboronic acid units, suitable for the condensation reactions of **1** with the silanol groups on the glass slide surface and the 1,2-diol groups in exopolysaccharides of diatoms cell membranes, respectively. The organic linker also bears aliphatic chains (C_6 and C_3 alternated with amide moieties) that confer flexibility to the structure to favour the interaction of pending phenyl boronic acid groups with the cells surface, overcoming possible steric hindrance around EPS vicinal diols reactive sites and avoiding the collapse of the living cells on the substrate.[33,34] The synthetic pathway to **1** is straightforward and it consists in a one-pot process involving two consecutive condensation reactions between the bifunctional (sulfosuccinimidyl)suberate ester (BS3) with the amino groups of 3-aminophenylboronic acid (APBa) and 3-(aminopropyl)triethoxysilane (APTES) (Scheme 1a).

The isolated organic linker **1** was characterised by GC-MS, ^{1}H, ^{13}C NMR, and FT-ATR spectroscopies (see Experimental).

The linker molecules were then grafted to the glass slide, after a preliminary surface treatment with a mixture of sulphuric acid and hydrogen peroxide (piranha solution, see Experimental) to activate its reactive silanol groups (step *i* in Scheme 1b). The grafting reaction was carried out dissolving **1** (10 mM) in a mixture of water and toluene (1 : 100 V/V) and stirring at 40 °C for 24 h in the presence of the immersed glass slide (step *ii* in Scheme 1b).

The reaction was monitored observing the reduction of the absorption peak intensity at 317 nm, diagnostic of the presence of unreacted free **1** in solution. After 3 h, a 40 ± 5 % absorbance reduction was observed (Figure 1a), corresponding to 6.03 mM of free unreacted **1** that remained constant in the next hours, as the evidence that the grafting process was terminated. This allows to estimate ~4 mmol of **1** grafted to the glassy surface (see Supporting S1 for calibration).

After washing with toluene, ethanol, and bidistilled water, and drying at room temperature, the functionalized substrate G-**1** (Scheme 1b) was characterized by FT-ATR spectroscopy (Figure 1b).

Comparison of FT-ATR spectrum of G-**1** with the spectra of the bare glass (G) and the free **1** (Figure 1b) evidenced the presence of (*i*) a broader signal at wavenumbers higher than 3000 cm^{-1} with symmetrical and asymmetrical stretching signals at 2850–2931 cm^{-1} of suberate aliphatic C–H bonds, (*ii*) aromatic overtone signals at 2300 cm^{-1}, (*iii*) C=O stretching and N–H bending signals at 1740–1625 cm^{-1} belonging to the amide bond, (*iv*) a weak B–O stretching signal around 1320 cm^{-1},[35] (*v*) Si–C and Si–O stretching signals at 1270 and 1070 cm^{-1}, respectively.

Raman spectroscopy was performed as a further characterization of **1** deposited on a silicon wafer, in 80–600 cm^{-1} and 600–1900 cm^{-1} ranges, where vibrational bands corresponding to bonds that involve B, C, O, H, N, and Si atoms are observed (Figure 1c). B–C and B–O signals appeared as sharp and intense peaks. Signals at ~350 cm^{-1}, ~800 cm^{-1}, and ~900 cm^{-1} were

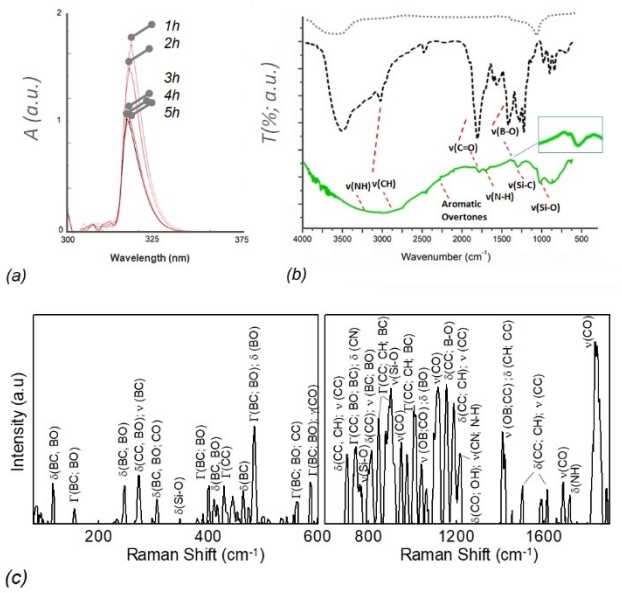

Figure 1. (a) Kinetics of the grafting reaction as function of reduction of UV-vis absorption peak of **1** at 317 nm in a mixture of water and toluene (1 : 100 V/V) over time. (b) FT-ATR spectra of bare glass G (light grey), the molecular linker **1** (dark grey), and **1**- functionalized glass G-**1** (green). (c) Raman spectrum of **1** on silicon wafer in 80–600 cm^{-1} and 600–1900 cm^{-1} ranges. Vibrational modes are also reported as Γ (torsion), δ (bending), ν (stretching) and γ (wagging), and assigned to specific chemical bonds.

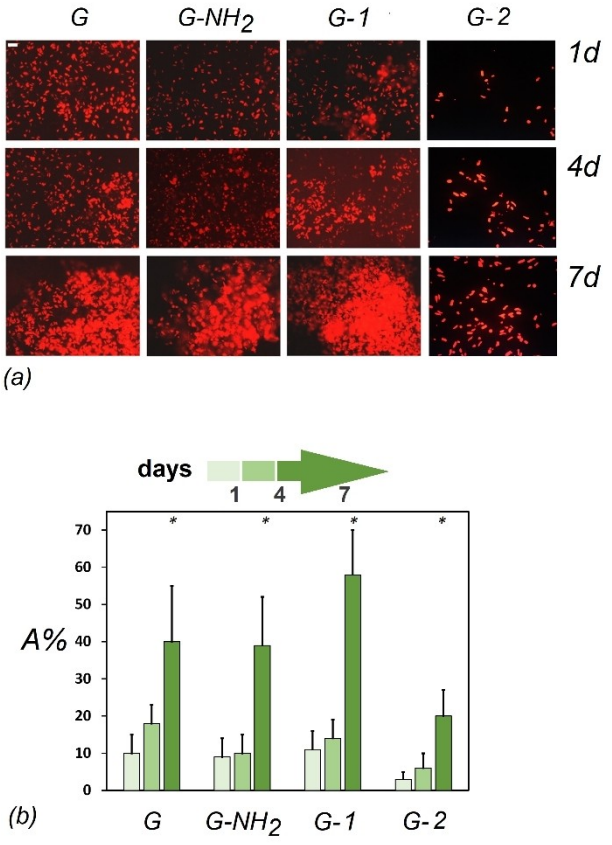

Figure 2. (a) Bidimensional Fluorescence Microscopy images of *P. tricornutum* cells adhering on bare glass (G), and glass slides functionalized with APTES (G-NH_2) and **1** (G-1); (scale bar: 20 μm). (b) Adhesion percentage parameter (A%) recorded, analyzed, and validated *via* Image J. Results were considered statistically significant with $p < 0.05$.

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attributed to Si–O deformation and stretching, while C–O and C=O stretching sharp peaks were observed at ~1100 and ~1840 cm^{-1}, respectively. C–C and C–H aliphatic and aromatic vibrations were evident in the 600–1900 cm^{-1} range, while bonds involving N, H, and C (C–N and N–H) mainly gave rise to bands at ~750 cm^{-1}, 1220 $cm^{-1,}$ and 1620 cm^{-1}.[36–38]

Biological assays

After a preliminary *in vitro* daily microalgal propagation, *Phaeodactylum tricornutum* cells growth and adhesion were investigated on three different substrates: bare glass (G), glass slides functionalized with APTES (G-NH_2) and **1** (G-**1**). Tests were performed after 1-, 4- and 7-day time intervals, observing the autofluorescence of chloroplasts by bidimensional fluorescence microscopy, as proof of viability of the living cells during their growth and adhesion (see Experimental). The increase of adherent cell density was evident and significant from the 1st to the 7th day (Figure 2a), with the observation of clustering effects, most evident from the 4th to the 7th day. The surface functionalization with **1** slightly increased the intrinsic biological aptitude of *Phaeodactylum tricornutum* cells to adhere to substrates (Figure 2b, with related estimation by the ImageJ analyser software).

Functionalization was observed to influence the biofilm resistance rather than the spontaneous cells adhesion capability, as observed by mechanical stress tests carried out after 7 days using two different methods. In the first test, the medium was subjected to flushing under centrifuge field, inside conical falcon tubes, with the substrates fixed at their basal ground. In the second method, G, G-$NH_{2,}$ and G-**1** substrates coated with cell biofilms were subjected to 1 h incubation inside the seawater medium under stirring. After the stress experiments, a decrease in the number of adhered cells was observed (Figure 3a), with the best resistance and highest adhesion retention estimated by Image J for the biofilm coating G-**1** (Figure 3b).

To evaluate if the resistance to stress and stability of the biofilm adhesion on G-**1** could be due to a stable interaction between the linker's boronic acid groups and diatoms' EPS diol groups, a control experiment was performed using the linker **2**. This molecule only differs from **1** since it lacks the boronic acid moiety. Indeed, **2** was obtained by the same synthetic procedure described for **1**, using aniline instead of 3-aminophenylboronic acid (see Experimental and Scheme 1).

Upon grafting **2** to a glass slide, the resulting G-**2** substrate was subjected to the diatom biofilm growth under the same experimental conditions reported for G, G-$NH_{2,}$ and G-**1**. The functionalization with **2** reduced the affinity of cells with the glass slide, likely due to the reduction of polarity of the substrate in contact with polar exopolysaccharides of cell membranes. Moreover, in the absence of boronic acid moieties, the biofilm was not resistant to both stress experiments, with a reduction of the adhesion percentages of 4% in test 1 and 1.5% in test 2. This outcome is likely due to the hydrophilic

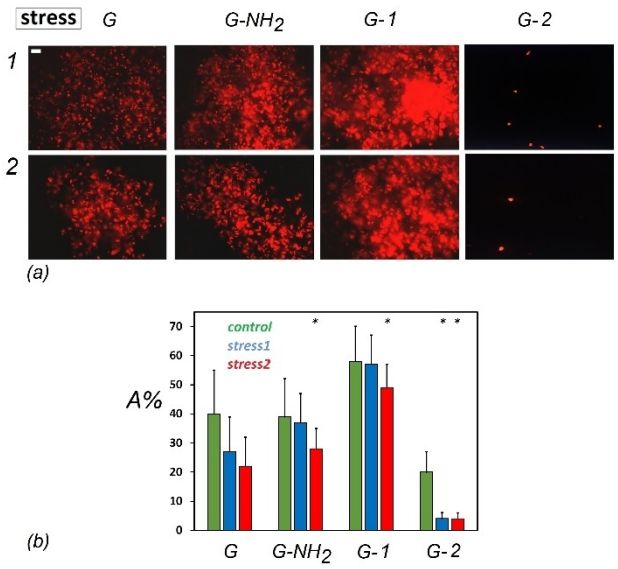

Figure 3. (a) Bidimensional Fluorescence Microscopy images of self-fluorescent *P. tricornutum* cells adhering to G, G-NH_2, G-**1**, G-**2** substrates before and after the stress tests 1 and 2;); (scale bar: 20 μm). (b) Adhesion percentage parameter (A%) recorded, analyzed, and validated *via* Image J after stress 1 and stress 2 experiments. Results were considered statistically significant with $p < 0.05$.

nature of *Ph. tricornutum* cell surfaces, inhibiting cell interaction with hydrophobic substrates.

Conversely, a stable interaction of the diol groups of EPSs in diatoms cell membranes with boronic acid moieties is supposed to have a real key role in the biofilm adhesion stability on G-**1**.

As a further proof of the important role of this kind of interaction, a series of control experiments were carried out treating G, G-$NH_{2,}$ and G-**1** with a FITC-dextran luminescent polymer probe bearing vicinal diol reactive sites. As expected, a stable grafting of the polymer was observed only for the G-**1** substrate bearing the boronic acid functional groups on the surface (see Supporting S2).

The proof that it is possible to avail of EPS diol moieties abundant on diatoms surface to stably immobilize these microalgae on boronic acid functionalized substrates is a significant result to overcome the issue of scarcity of shell external silanol groups of living microalgae that are less convenient reactive sites for cells immobilization.[39,40]

Bioremoval of Heavy Metals from seawater

The G-**1** substrate coated with diatoms biofilm after a 7-day growth was kept for 24 h in immersion in a aqueous solution containing a series of heavy metal ions (See Experimental) to investigate the biofilm capability to incorporate and remove HMs. The analysis of HM concentration before and after the experiment was performed *via* inductively coupled plasma mass spectrometry (ICP-MS). Preliminary calibration was carried out with a standard multi-metal solution in the 0.02-300 ppb range. Histograms in Figure 4 refer to HM concentrations in

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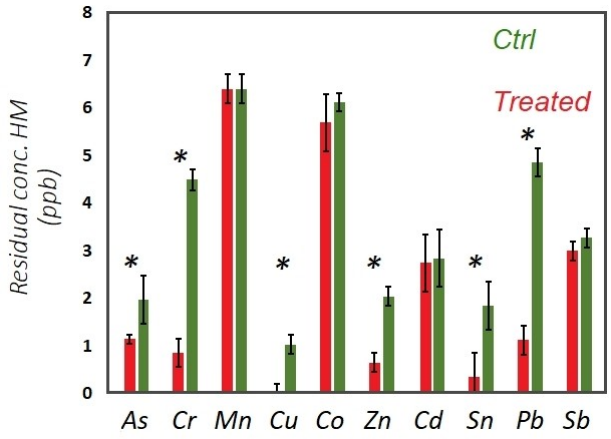

Figure 4. HMs bioremediation with *Ph. Tricornutum* coated G-**1** after 7-days growth: histograms of concentration (ppb) before (green) and after (red) 24 h incubation. Results were considered statistically significant with $p < 0.05$.

solution before (green color) and after (red color) 24 h immersion of the biofilm coated G-**1** substrate.

Except for Mn, Co, and Cd, the microalgal biofilm on G-**1** efficiently reduced the concentration of As, Cr, Cu, Zn, Sn, Pb, and Sb, as a known capability of *P. tricornutum* to biosorb and bioaccumulate these metals. Indeed, the bioremediating action for Cr (VI) with enhanced lipidic and biomass production of *Phaeodactylum tricornutum*, was already reported.[35]

Biosorption is mainly supposed to occur due to (*i*) a plethora of functional groups on the cell walls surface, such as carboxyl and amino groups, acting as metal ion chelating units,[12] (*ii*) the presence of porous biosilica shells surface suitable for HM adsorption with respect to the walls of other cells, (*iii*) the biosorbing suitability of exopolysaccharides exposed on diatoms cells surface.[15]

Our results are also consistent with the data of bioremediation by other microorganisms, such as local marine bacteria removing 50–70% of HMs (especially As and Cd) from the Mediterranean Tyrrhenian coast, or local lactic acid bacteria efficiently removing ~100% of Ni^{2+}, Cr^{2+}, Cd^{2+} and Pb^{2+} from the Alexandrian Mediterranean seacoast.[5,41–42] However, the use of diatoms microalgae like *P. tricornutum* is more profitable than acidophile bacteria, since diatoms are more resistant to a wide range of climate conditions and nutrients availability and they boast better stability and growth/adhesion capacity.

Moreover, the immobilization approach provided herein is very straightforward with respect to alternative more sophisticated, time and cost-consuming methods, such as the electrospinning approach to improve cells' adhesion.

Despite the physiological limitations related to living-based hybrid systems, the proposed approach can be potentially integrated in industrial processes, since diatoms culture are easily scalable. In fact, *Phaeodactylum tricornutum* has high biomass productivity (~250 mg L^{-1} per day), cell growth kinetic rate higher than that of 90% green microalgae, as well as continuous EPS production. *P. tricornutum* algal cultures are also resistant to serial changes of volume culture media, starting from 0.1 L inoculum up to 250 L final culture volumes. These features are important in view of the possible design of tanks made of diatom biofilms adhered to glass walls for seawater bioremediation.

Finally, diatoms are photosynthetic microalgae fixing carbon dioxide upon sunlight absorption, thus making the approach even more profitable since it allows to combine seawater bioremediation with atmospheric CO_2 capture.

Conclusion

In conclusion, we have demonstrated that it is possible to improve the stability of adhesion of a biofilm of *Phaeodactylum tricornutum* diatoms microalgae by a straightforward approach based on the surface fuctionalization of glass substrates with a tailored organic linker bearing boronic acid moieties capable to interact with vicinal diols of exopolysaccharides on diatoms surface. This work proves the concept that chemical functionalization of substrates is a promising tool to favour the immobilization of living microorganisms keeping them viable and capable to exert their physiological bioremediating function to incorporate exogenous compounds, including contaminants like heavy metals removed from seawater solutions. Further investigation will be extended to the treatment of marine sediment samples, in view of the possibility to explore the potential of our immobilization strategy as a route to new scalable, manageable, and eco-friendly living microalgal based systems for water and seabed *in situ* bioremediation of the marine ecosystem.

Experimental Section

General information

Bis-3-sulfo-*N*-hydroxysuccinimide ester (BS3), 3-(aminopropyl)triethoxysilane (APTES), 2-aminophenylboronic acid (APBa), aniline, fluorescein isothiocyanate-dextrane (FITC-Dextrane, 40 MW), glass surfaces, hydrogen peroxide, sulfuric acid, bidistilled water were purchased from Sigma Aldrich. FTIR-ATR (Fourier Transformed Infrared-Attenuated Total Reflectance) spectra of bare and functionalized substrates were performed by Perkin Elmer Spectrum Two Spectrophotometer equipped with A 2×2 mm diamond crystal (4000–400 cm^{-} range with a 2 cm^{-} resolution)

Raman spectra were recorded by a LabRAM HR (Horiba-Jobin Yvon, Montpellier, France) spectrometer with 532 nm excitation laser under ambient conditions. Low laser power (< 1 mW) was used to avoid heat-induced modification or degradation of the sample due to focused laser light during spectrum acquisition. The collection time was longer than 200 sec. The excitation laser beam was focused through a 50X optical microscope (spot size ~1.3 μm, working distance 1 cm). The spectral resolution was 1 cm^{-1}.

Raman measurements were used to detect vibrational bonds between B, C, O, and H and obtain a fingerprint of the molecule.

UV-Vis Shimadzu 2401 PC spectrophotometer was used to record the absorption spectra of the free linker in solution during the functionalization of the substrate. An Axiomat, Zeiss microscope

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(Oberkochen, Germany; passband $\lambda_{exc} = 540$ nm, passband $\lambda_{emi} > 590$ nm) and TRITC filter were used to observe the autofluorescence of chloroplasts during experiments of cells growth and adhesion on substrates.

ICP-MS spectra were performed by an iCAP-Q ICP-MS instrument from Thermo Scientific. The instrument was fed with Argon gas and connected to 2% nitric acid fill for the preset flush. All data were obtained after the definition of a HM type calibration line: response in a multi-metal 0.02–300 ppb range (***i.e.*** 0.02, 0.2, 2, 20, 100, 300 ppb).

Synthesis of the linker 1

In a three-neck round-bottom flask, 2-aminophenylboronic acid (APBa, 0.48 mmol) and bis-3-sulfo-N-hydroxysuccinimide ester (BS3, 0.4 mmol) were dissolved in a mixture (5 ml) of dry CH_2Cl_2 and acetone (9:1 vol) at 4 °C. After 30 min, 3-(aminopropyl)triethoxysilane (APTES, 0.48 mmol) was added and the mixture was stirred at 4 °C for 24 h. The reaction was monitored by thin-layer chromatography using a mixture of hexane:ethyl acetate (7:3 vol) as the eluent. After 24 h, the mixture was filtered to remove insoluble by-products. Water and dichloromethane were added to the mixture and the product was separated in the organic phase. After drying with anhydrous sodium sulphate, dichloromethane was evaporated at reduced pressure, yielding 65% of **1** (151 mg) as a yellow solid that was used without further purification.

^{1}H NMR (500 MHz, CD_3CN): δ 7.66 (s, 1H), 7.61 (s, 2H), 7.30–7.28 (m, 1H), 4.64 (d, J = 7.2 Hz, 2H), 4.36 (q, J = 7.0 Hz, 6H), 3.67 (dd, J = 13.0, 6.9 Hz, 2H), 2.67–2.63 (m, 4H), 2.20–1.98 (m, 6H), 1.88–1.84 (m, 4H), 1.75 (dd, J = 9.0, 5.0 Hz, 9H), 1.17–1.12 (m, 2H).

^{13}C NMR (500 MHz, CD_3CN, δ): 8.1, 18.1, 23.4, 26.4, 29.2, 30.3, 36.1, 42.2, 59.9, 117.1, 122.1, 124.8, 129.8, 148.6, 173.3, 182.0 ppm.

FTIR (KBr): ν = 3452, 2933, 2870, 2090, 1658, 1568, 1450, 1407, 1279, 1108, 1045, 788, 647, 503 cm^{-1}.

MS (EI, 70 eV) *m/z* (%): 496 [M^+], 428 (51), 340 (100), 325 (68), 251 (6), 207 (5), 179 (4), 147 (7), 74 (35).

Synthesis of the linker 2

In a three-neck round-bottom flask, aniline (0.47 mmol) and bis-3-sulfo-N-hydroxysuccinimide ester (BS3, 0.41 mmol) were dissolved in a mixture (5 ml) of dry CH_2Cl_2 and acetone (9:1 vol) at 4 °C. After 30 min, 3-ammino-propyl-triethoxy-silane (APTES, 0.47 mmol) was added and the mixture was stirred at 4 °C for 24 h. The reaction was monitored by thin-layer chromatography using a mixture of hexane:ethyl acetate (7:3 vol) as the eluent. After 24 h, a filtration was performed to remove organic insoluble products. After the addition of water and CH_2Cl_2, the product was extracted in the organic phase. The solvent was distilled at reduced pressure, thus isolating **2** in 58% yield (122 mg) as a yellow solid. The product was characterized *via* ^{1}H-NMR, ^{13}C-NMR, and FT-IR and used without further purification.

^{1}H NMR (500 MHz, DMSO) δ 7.03–6.94 (m, 2H), 6.55 (d, *J* = 8.0 Hz, 2H), 6.51–6.44 (m, 1H), 3.76–3.69 (m, 2H), 3.45 (q, *J* = 7.0 Hz, 6H), 2.97 (dt, *J* = 45.6, 7.4 Hz, 1H), 2.52 (dd, *J* = 6.7, 3.0 Hz, 1H), 2.33–2.13 (m, 1H), 2.03 (t, *J* = 7.4 Hz, 1H), 1.54–1.33 (m, 4H), 1.28–1.15 (m, 2), 1.17–1.08 (m, 4H), 1.06 (t, *J* = 7.0 Hz, 9H), 0.52 (dd, *J* = 9.7, 7.1 Hz, 2H).

^{13}C NMR (500 MHz, CD_3CN, δ): 7.7, 18.1, 19.1, 23.3, 26.9, 29.1, 36.2, 40.1, 41.0, 44.3, 56.8, 58.1, 59.7, 114.1, 116.2, 117.2, 129.4, 149.5, 173.2 ppm.

FTIR (KBr): ν = 3454, 2953, 2860, 2095, 1647, 1573, 1260, 1150, 1041, 790, 657, 512 cm^{-1}.

Chemical functionalization of glass substrates

Glass substrates (1 cm × 2 cm) were firstly subjected to the activation of silanol surface groups by treatment with a 1:2 mixture of sulphuric acid (98% m/V) and hydrogen peroxide (30% V/V) in water at 90 °C for 1 h. Substrates were then washed with water and respectively reacted with the ligands APTES, **1** or **2** (10 mM final conc.) in a mixture (10 mL) of water and toluene (1:100 V/V) for 24 hours at 40 °C. After several washing steps in toluene, acetone, ethanol, and water, the functionalized slides were dried under vacuum and used for experiments of microalgal growth and adhesion, as well as for the control experiments with the FITC dextran polymer reported in Supporting S2.

Growth and adhesion assay of *Phaeodactylum tricornutum* on glass substrata

After *in vitro* daily propagation,[43] *Phaeodactylum tricornutum* cells were seeded on glass substrates and cells growth was monitored after 1, 4, and 7 days observing the autofluorescence of chloroplasts of single or clustered cells[44] by a TRITC filter *via* epifluorescence microscopy. Cells adhering to substrates at different times were recorded using at least 15 digital images per sample acquired through a CCD camera (Leica DC100, Leica Microsystems, Germany; Meyer Instruments, Houston, TX, USA) and the adhesion percentage parameter (A%) was calculated with the Image J image analysis software.

Stress tests and adhesion assays of *Phaeodactylum tricornutum* diatom biofilms on glass substrates

After the 7 days adhesion experiments, substrates coated with diatom biolfilms were subjected to two stress tests. Stress 1 refers to flushing of medium under centrifuge field (2500 rpm 20') using conical falcon tubes, while stress 2 consists in the use of a 1 h incubation of populated substrates into microalgae medium under stirring (1000 rpm). After both experiments, cells still adhering to substrates were recorded using at least 15 digital images per sample acquired through a CCD camera (Leica DC100, Leica Microsystems, Germany; Meyer Instruments, Houston, TX, USA) and the adhesion percentage parameter (A%) was again calculated with the Image J analysis software.

Simultaneous experiment of heavy metals biosorption

The G-**1** substrate samples coated with diatoms biofilm after a 7-day growth were prepared in triplicate and kept for 24 h in immersion in a aqueous solution (fresh seawater, after autoclave sterilization and ultrafiltration treatment), containing a series of 10 ppb As, Cr, Mn, Cu, Co, Zn, Cd, Sn, Pb, Sb heavy metal ions as nitrate salts, from a 1000× stock in 5% nitric acid (Certified Reference Material EPA6010, CPA Chem).

Statistical validation

Microscopy images were processed using an ImageJ analyzer software (National Institute of Health, New York, NY, USA). A Mann-Whitney U test was used to validate differences recorded among adhesion parameters values during culturing and after stress tests and remediation experiments. Data were collected after experi-

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ments performed in triplicate. Results were considered statistically significant with $p < 0.05$.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: boronic acid · heavy metals · polysaccharides · surface organic chemistry · diatoms microalgae · bioremediation

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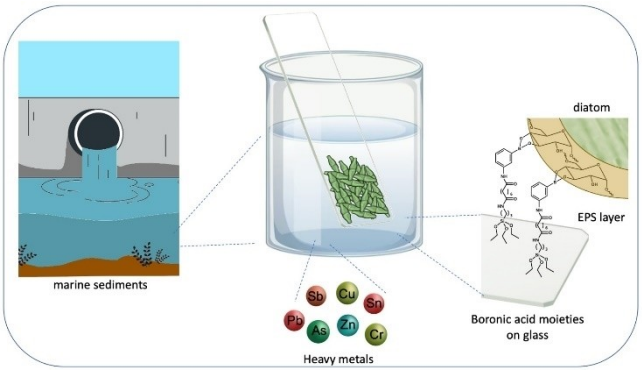

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1 – 8

Boronic Acid Moieties Stabilize Adhesion of Microalgal Biofilms on Glassy Substrates: A Chemical Tool for Environmental Applications

A biofilm of diatoms microalgae is stably anchored on glassy substrates by covalent boronic acid-diol condensation: it efficiently bioremediates seawater from heavy metals.

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