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Antibacterial Strategies from the Sea: Polymer-Bound Cl-Catechols for Prevention of Biofilm Formation

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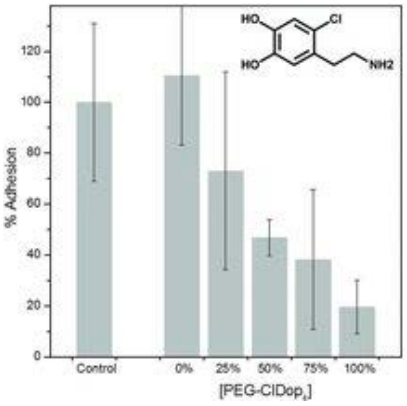
Inspired by the amino acid 2-chloro-4,5-dihydroxyphenylalanine (Cl-DOPA), present in the composition of the proteinaceous glue of the sandcastle worm Phragmatopoma californica, a simple strategy is presented to confer antifouling properties to polymer surfaces using (but not releasing) a bioinspired biocide. Cl-Dopamine is used to functionalize polymer materials and hydrogel films easily, to prevent biofilm formation on them.



Natural strategies to prevent bacterial colonization offer great promise for the development of effective, biocompatible, and environmentally friendly non-fouling coatings. Inspired by the amino acid 2-chloro-4,5-dihydroxyphenylalanine (Cl-DOPA) present in the composition of the proteinaceous glue of the sandcastle worm *Phragmatopoma californica*, we present a simple strategy to confer antifouling properties to polymer surfaces using (but not releasing) a bioinspired biocide. Taking advantage of the catechol reactivity, Cl-dopamine was easily incorporated into hydrogels or co-deposited as a thin coating. Polymer-bound Cl-catechol groups effectively prevented attachment of bacteria, while they showed no toxicity to attached cells. The antifouling performance of Cl-dopamine depended on the flexibility of the polymer chain to which it was attached (i.e., the accessibility of the Cl-catechol groups to interact with the bacterial membrane) and it was concentration dependent. The simplicity, low cost, and flexibility of this strategy promises wide application for antibacterial coatings of biomedical devices of almost any kind.

Bacterial biofilms are an important source for health problems and disease: tooth decay, biofouling of implants and biomedical devices, chronic infections etc.[1] Different coating strategies have been investigated in recent years to reduce biofilm formation.[2] Passive coatings mainly rely on ammonium-functionalized polymers, poly(ethylene glycol) (PEG) and zwitterionic polymers, which prevent the attachment of bacteria. Active strategies rely on the release of an active compound into the biological medium (i.e., silver or antibiotics) that kill suspended bacteria. Both approaches have also been combined in single coatings.[3,4] While passive strategies are preferred in terms of biocompatibility and environmental issues, active strategies are often more effective. However, long term use of biocides leads to environmental pollution and supports the development of resistant microbial strains. Nature has developed fascinating strategies over millions of years to prevent harmful bacterial colonization on living tissues.[5] Plants and animals have adaptively developed a vast array of defense mechanisms in response to an ever-present pathogen pressure. These natural strategies have inspired anti-biofilm coatings containing or releasing antimicrobial peptides, antibiotics, quorum-sensing inhibitors, essential oils, and bacteriolytic enzymes.[5] However, the poor bioavailability, poor proteolytic stability, and in some cases cytotoxicity of these compounds have limited their practical applications in medical materials and devices.

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Biofilms are also ubiquitous in the marine world. The surfaces of ship hulls or underwater pipelines are rapidly conquered by films of small micro-organisms (bacteria, fungi, algae) which mediate subsequent attachment and growth of mussels, polychaete worms, or barnacles. These biofilms contribute to early corrosion and higher fuel consumption during displacement due to increased hydrodynamic drag. Little is known about the non-fouling strategies that the sea organisms themselves use for protection against bacteria film formation. Recent studies on the sandcastle worm, *Phragmatopoma californica*, a marine polychaete, have found Cl-DOPA in the proteinaceous glue, which it uses for cementing grains and constructing its tube-like shelter.[6] The aminoacid 4,5-dihydroxyphenylalanine (DOPA) is a well known component in marine glues, able to mediate adhesion underwater by co-ordinating with metal oxide surfaces and by crosslinking and solidifying the protein components via quinone oxidation and self-reaction.[7–11] The electron-withdrawing Cl-substituent in the catechol ring lowers the pK_as of the phenolic OH groups ($pK_{OH1} \approx 8.3$ and $pK_{OH2} \approx 12.7$) and lowers the oxidation potential of the catechol unit. As a consequence, Cl-DOPA shows a higher affinity for co-ordination to the surface and is less prone to self-polymerize. It has also been suggested (though not yet proved) that the role of the chlorinated DOPA in the cement could relate to the prevention of microbial fouling and degradation. In fact, chlorocatechols in solution are well known to be toxic and are considered as dangerous environmental pollutants.[12,13] However, the toxicity of chlorocatechols has been related to their ability to interact and diffuse across biological membranes and, therefore, the potential toxicity of polymer-bound chlorinated catechols remains unclear. It is also important to note that halogenated secondary metabolites of marine algae have also shown antibacterial activity.[14] In the following, we demonstrate that 2-Cl-dopamine retains the high underwater reactivity of mussel-inspired materials[15,16] and, in addition, prevents biofilm formation on derived materials and coatings. We provide a simple, flexible and effective biocompatible antifouling strategy using (but not releasing) a marine bioinspired biocide.

Cl-dopamine was easily incorporated into hydrogels and coatings in three different ways: i) crosslinking of Cl-dopamine-functionalized star-PEG under oxidative conditions; ii) one-pot Cl-dopamine functionalization and gelation of natural biopolymers;[16] and iii) coating of glass surfaces by codeposition of dopamine and Cl-dopamine mixtures.[17] In the first case, a 4-arm-star poly(ethylene glycol) was end-functionalized with Cl-dopamine (PEG-ClDop$_4$) or dopamine (PEG-Dop$_4$). The two PEGs were mixed in different ratios in the presence of an oxidant and were spin-coated onto glass substrates to obtain thin hydrogel films (≈250 nm in dry state, ≈2.5 μm in swollen state) with different concentrations of Cl-dopamine. The Cl-dopamine and dopamine units reacted (Supporting Information, Figure S1) and generated the crosslinking points of the network. ^{1}H-NMR spectroscopy confirmed the presence of multiple oxidation and reaction intermediates during the gelation process (Supporting Information, Figure S2a). Under our conditions, ca. 59% of the Cl-catechol groups reacted to form network points, as estimated from ^{1}H-NMR spectroscopy measurements (Supporting Information, Figure S2b). The films were stable and the film thickness did not change after incubation in a buffer at different pHs or in a bacteria culture medium for several days (Supporting Information, Figure S10).

In the second case hyaluronic acid (Hyal), alginate (Alg), or gelatin (Gel) was simply mixed with Cl-dopamine and the corresponding thin hydrogel films (Hyal-ClDop, Alg-ClDop, or Gel-ClDop) were deposited onto the substrates by immersion, following a recently reported one-pot reaction strategy.[16] This method allows the incorporation of the catechol derivative in almost any polymer matrix by simultaneous alkaline pH-induced self-polymerization and the reaction of the catechols with functional groups on the polymer backbone. Films of 8–15 nm thickness were obtained (Supporting Information, Table S1). The analogous gels crosslinked with dopamine were also obtained for control experiments (Hyal-Dop, Alg-Dop, and Gel-Dop). In the third case, glass substrates were immersed in alkaline solutions of Cl-dopamine and dopamine in different ratios to obtain thin polydopamine and poly(Cl-dopamine) coatings (pDop and pClDop).[17] UV spectroscopy of coated quartz slides demonstrated the presence of the polycatechol layer (Supporting Information, Figure S3). The film thickness decreased with increasing Cl-Dop concentration from ≈24 nm for the pure pDop to 10–13 nm for the pure pCl-Dop films (Supporting Information, Figure S4). These results evidence the lower redox potential of the Cl-susbstituted catechol and, consequently, its weaker tendency to self-polymerize.

The presence of functional catechol units in the crosslinked films was confirmed by UV spectroscopy after incubation with 9-anthraceneboronic acid (An-B(OH)$_2$). Boronic acids form complexes with catechol hydroxyls by the formation of boronate esters at basic pH. These complexes dissociate at acidic pH. **Figure 1** shows the UV spectrum of a PEG-ClDop$_4$ film after incubation with An-B(OH)$_2$ at pH 7.4. The appearance of the bands at 320–390 nm reveal the formation of the boronate ester. These bands disappeared after washing the film at pH 2.5, demonstrating the reversibility of the complex with available catechol units in the film. The density of accessible Cl-catechol groups in the film was calculated from the absorbance value (see Supporting Information for details) to be Γ_{362} = 1.3×10^{15} molecules cm^{-2}. Therefore, the estimated concentration of accessible Cl-catechol groups in the swollen PEG-ClDop$_4$ gel (ca. 2.5 μm thickness) was ≈9 mM. Similar concentrations of catechol groups were found in the PEG-Dop$_4$ gel. Polycatechol coatings and (Cl)-dopamine-modified Gel, Hyal, and Alg hydrogel films showed significantly lower absorbance values after incubation with An-B(OH)$_2$. The concentration of catechol groups in the swollen film in these cases was below the detection limit of the method (i.e., it is lower than 1 mM).

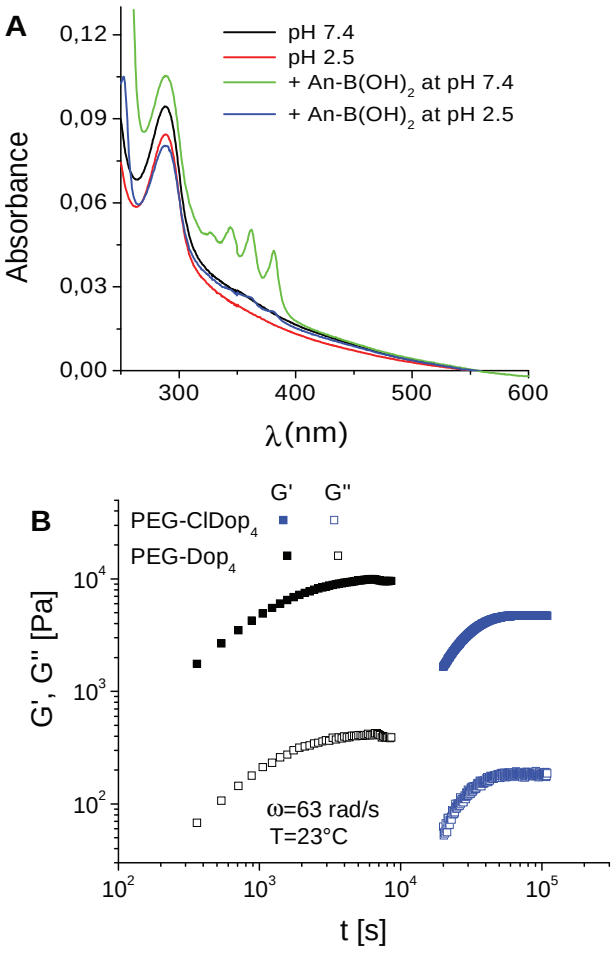

Figure 1. Characteristics of PEG-ClDop$_4$ films. a) UV–vis spectra of a PEG-ClDop$_4$ film spin-coated on a quartz substrate after washing at pH 7.4 (black) and at pH 2.5 (red), incubation with 9-anthraceneboronic acid and washing at pH 7.4 (green) and subsequent washing at pH 2.5 (blue). B) Evolution of the shear and loss moduli (G', G'') during crosslinking of PEG-Dop$_4$ and PEG-ClDop$_4$.

The mechanical properties of the PEG-ClDop$_4$ and PEG-Dop$_4$ crosslinked films were measured by piezorheology.[18] This is a relevant property of the coatings to be taken into account when comparing their antibacterial activity, since bacterial attachment is known to depend on substrate rigidity amongst other factors.[19] PEG-ClDop$_4$ and PEG-Dop$_4$ solutions were mixed with the oxidant and placed between two shear plates with a gap of 100 μm and sealed with an immiscible oil to avoid water evaporation during the measurement. The shear modulus (G') of the gels during crosslinking was measured and the final properties

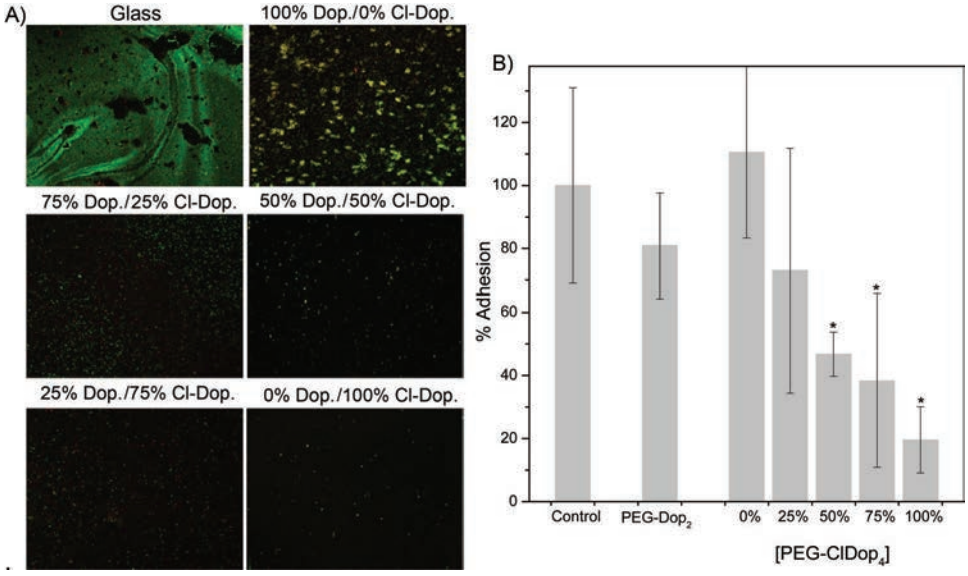

Figure 2. Bacteria attachment on PEG-ClDop$_4$/Dop$_4$ films. A) Representative microscopic images of fluorescently stained *E. coli* attached to Cl-dopamine-modified PEG hydrogels after incubation for 24 h. Living bacteria were stained with Syto9 (green) and dead bacteria with propidium iodide (red). B) Percentage of adherent *E. coli* to Cl-dopamine-modified PEG hydrogels with respect to a glass slide or the primer (PEG-Dop$_2$) after incubation with bacterial suspension for 1 h. Results were obtained from three independent experiments. ∗Significant differences ($P < 0.05$) compared with the control (glass slide).

of the crosslinked gels (corresponding to ≈59% crosslinking degree, as estimated from the ^{1}H-NMR spectroscopy measurements) were compared. Despite differences in the gelation kinetics (slower for PEG-ClDop), both gels showed similar mechanical properties after crosslinking, with shear moduli of 6 and 10 kPa for the crosslinked PEG-ClDop$_4$ and PEG-Dop$_4$, respectively. (Figure 1B and Supporting Information, Figure S5). Therefore, possible differences in bacterial assays between Cl-dopamine and dopamine-functionalized films cannot be attributed to differences in the substrate rigidity.

Bacterial attachment is the first prerequisite for biofilm formation. Therefore, we tested the antifouling properties of the Cl-dopamine containing films by evaluating attachment of the gram negative bacterium *Escherichia coli* and comparing these results to bacterial attachment to dopamine-modified analogues. **Figure 2**A shows representative microscopic images of the fluorescently stained bacteria attached to the PEG-ClDop/Dop hydrogels. A strong decrease in bacteria density at the surface (up to 80%, Figure 2) was observed with increasing concentration of Cl-dopamine fraction in the gel, indicating a clear inhibitory effect on bacteria attachment of the Cl-dopamine functionalisation. The dopamine-modified PEG gel did not prevent bacteria attachment. Note that the estimated concentration of Cl-dopamine units in the PEG-ClDop swollen gel was 9 mM, significantly higher than the concentration of free Cl-dopamine in solution required to kill 80% bacteria (1 mM, Supporting Information, Figure S6). Our results show that polymer-bound Cl-dopamine at a concentration of ≈9 mM confers effective antifouling characteristics to the PEG films.

Figure 3A shows the results of the bacterial attachment assay in Cl-dopamine- and dopamine-modified alginate, hyaluronic acid, and gelatin. A drastic reduction in the bacterial density on the surface was observed for the Cl-dopamine-modified gels, demonstrating the great potential of Cl-dopamine to prevent biofilm formation on relevant biomaterials by simple mixing in a one-pot method.

Interestingly, similar tests on the pure pClDop coating did not show significant reduction of bacteria adhesion. However, coatings obtained by simultaneous co-deposition of Cl-Dop and Dop showed up to 40% reduction in bacteria adhesion. It is worth noting that the antibacterial effect in the polycatechol films was significantly weaker than in the hydrogel films. We attribute this result to the different rigidities of the films. The self-polymerized catechol matrix is more rigid than the hydrogel matrix and, therefore, Cl-catechol groups might not be able to effectively interact with the bacterial membrane. This interaction has been demonstrated to be crucial for the biocide effects of Cl-catechols in solution.[12,13]

Agar diffusion tests were performed in order to determine if the nonfouling effects were due to leakage of Cl-catechol from the coatings in the solution. No inhibition zone was detected on any substrate (Supporting Information, Figure S7), indicating that the Cl-dopamine was irreversibly bound to the polymer and the bacterial repellence of the films was only due to polymer-bound Cl-catechol. Note that a minimum concentration of 1 mM (= 0.25 mg mL^{-1}) Cl-dopamine in solution is required for a 84% decrease in bacterial concentration in 2 h (Supporting Information, Figure S6). We also evaluated if polymer-bound Cl-catechols were able to kill bacteria in solution by dynamic contact assays. For this purpose, the concentration of bacteria in solution after incubation with Dop- or Cl-Dop-modified hydrogels was measured. No differences in the bacteria concentration

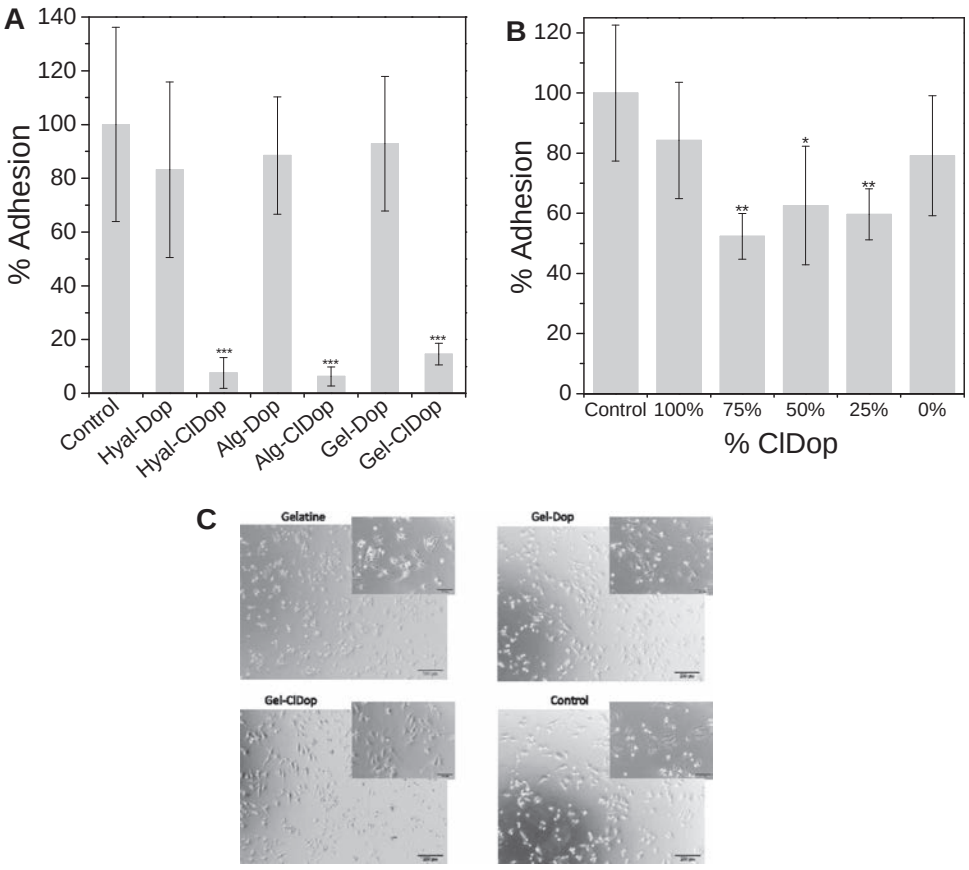

Figure 3. Percentage of adherent *E. coli* to: A) Cl-dopamine- and dopamine-modified alginate, hyaluronic acid, and gelatin films. B) pClDop and pDop coatings after 1 h exposure to bacterial suspension. Results were obtained from three independent experiments. Significant differences: ∗: $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$, compared with the control (glass cover slide). C) Microscopic pictures (4× and 10×) of HeLa cells after incubation with Gel-Dop- and Gel-ClDop-modified substrates. The results on gelatine and the glass slides are also shown as controls.

were detected (Supporting Information, Figure S8), indicating that polymer-bound Cl-dopamine effectively prevented biofilm formation but did not kill bacteria.

In order to assess the biocompatibility of the Cl-dopamine-modified films, we incubated the adherent mammalian cancer cell line, HeLa cells, with Dop- and Cl-Dop-modified gelatine films. No qualitative differences in the attached cells were observed by microscopy (Figure 3C). A PrestoBlue viability assay confirmed that the cell viability was similar in all of the substrates and in the control (Supporting Information, Figure S9A), indicating that the polymer-bound Cl-dopamine modification does not have any toxic effects on attached cells. We also incubated HT1080 cells with the eluent from PEG-$ClDop_4$ modified substrates in order to demonstrate that the films did not release small amounts of Cl-Dop to the solution that could harm cells (note that a concentration of 20 μM of Cl-Dop in solution is already cytotoxic).[20] The PrestoBlue viability assay confirmed no toxicity effects in the cells (Supporting Information, Figure S9B). These results demonstrate that the toxicity of polymer-bound Cl-Dop was selective with bacteria and did not harm cells.

In conclusion, Cl-dopamine combines the underwater reactivity of the catechol unit (well known from mussel-inspired DOPA-containing polymers) and adds non-fouling properties (presumably like in the sandcastle worm) to the derived materials. Cl-Dop functionalization presents several advantages over available methods for preventing biofilm formation: i) Cl-catechols are readily available (e.g., waste from the paper industry); ii) they can be simply reacted with almost any polymeric film or coating during deposition under water without the need of additional synthetic steps; and iii) polymer-bound Cl-dopamine is nontoxic for cells. In summary, Cl-dopamine modification is a cheap and generic antifouling method that can be applied to almost any material; it is environmentally friendly, it does not generate resistance to antibiotics, and it is not cytotoxic. Cl-dopamine functionalization opens new possibilities for new-generation antimicrobial coatings with wide use in biomedical applications. Our results also provide important experimental evidence for the role of halogenated catechols in the adhesive proteins of marine animals.

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