Barnacle Cement Protein: An Efficient Bioinspired Corrosion Inhibitor

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Abstract

To prevent corrosion damage in aggressive environments such as seawater, metallic surfaces are usually coated with corrosion inhibitors typically made of organic molecules. Unfortunately, these inhibitors generally exhibit environmental toxicity, affecting living organisms and leaving harmful chemicals in natural habitats. Thus, there is a strong need to develop greener corrosion inhibitors that are chemically and mechanically robust but do not leach toxic chemicals. Here, we show that the recombinant protein rMrCP20 from the adhesive cement protein of the barnacle *Megabalanus rosa* efficiently protects AH36 steel against corrosion under high salt conditions mimicking the marine environment. We reveal that these anti-corrosion properties are linked to the protein's biophysical properties, namely its strong adsorption to surfaces combined with its interaction with free Fe ions released by steel substrates, which form a stable layer that increases the coating's impedance and delays corrosion. Our findings highlight the synergistic action of rMrCP20 in preventing corrosion and provide molecular-level guidelines to develop alternative green corrosion inhibitor additives.

Introduction

Corrosion damage is a ubiquitous issue for metallic structures submerged in the sea, as the high-salt environment causes surface material erosion and crevice corrosion by dissolution of the metal ions during complex electrochemical and anaerobic corrosion processes.\textsuperscript{1–3} The calculated rates of steel corrosion below sea level are between 0.08–0.14 mmyr\textsuperscript{-1} and 0.07–0.21 mmyr\textsuperscript{-1} in splash and tidal zones,\textsuperscript{3} while the global cost of corrosion is estimated to be \textit{ca.} US$2.5 trillion per year in 2013.\textsuperscript{4} The useful lifespan of metals becomes significantly reduced when exposed to saltwater, requiring frequent maintenance, which is especially costly for petroleum, offshore construction and sea transport industries. By implementing existing anti-corrosion protection methods, the cost could be reduced by 15–35%, or as much as US$875 billion annually.\textsuperscript{4} Among these strategies, the addition of chemical compounds to the medium in contact with the surface, called “corrosion inhibitors”, is one of the most convenient methods to reduce the corrosion rate of steels. Most efficient commercially-used inhibitors are organic compounds, such as azole derivatives,\textsuperscript{5,6} Schiff bases,\textsuperscript{7–9} phenolic compounds,\textsuperscript{3} amine derivatives,\textsuperscript{10,11} thio-compounds\textsuperscript{11,12} and pyrimidine derivatives.\textsuperscript{11,13} These inhibitors usually contain electronegative atoms, heterocyclic compounds containing polar functional groups and conjugated double bonds in their molecular structures, through which they absorb on metal surfaces by forming an adhesive film that restricts the exposure of the metal to corrosive media. Although these synthetic compounds exhibit good corrosion inhibition performance, a global concern is related to health and environmental issues due to the toxic reagents used in their synthesis processes and the release of toxic additives to the environment post-application.\textsuperscript{14} Therefore, there is a pressing need to engineer new and eco-friendly alternatives to current anti-corrosion additives.

Here, we report our findings on the anti-corrosion properties of a recombinant protein, rMrCP20, derived from the natural adhesive cement protein (MrCP20) of a common marine biofouler, the barnacle
Megabalanus rosa (M. rosa). Barnacles are sessile crustaceans that strongly adhere to solid immersed substrates. Blackwood et al.\textsuperscript{15} and Eashwar et al.\textsuperscript{16} have reported that barnacle attachment to stainless steel substrates in seawater leads to more substantial corrosion events under dead barnacles, while crevice corrosion of steel substrates underneath live barnacles was less than 4%. Barnacles have been demonstrated to adhere strongly to metal substrates\textsuperscript{17} and to minimize corrosion events where they attach. Hence, drawing inspiration from the structural adaptation of M. rosa and these reported observations, we posited that the adhesive protein MrCP20 may help prevent steel corrosion. Our team has previously unveiled the secondary and tertiary structure of rMrCP20\textsuperscript{18} and identified that its underwater adhesive properties stem from its high content of cysteines (Cys) and charged amino acid residues. Herein, we show that rMrCP20 protein exhibits concentration-dependent anti-corrosion properties, which we demonstrate is linked to its adhesive characteristics in conjunction with its ability to capture Fe ions released from the steel surface. Corrosion inhibition effect of rMrCP20 was demonstrated through time-resolved observations of corrosion on AH36 steel coupon samples, whereas the iron weight loss from the corrosion events were measured via inductively coupled plasma optical emission spectroscopy (ICP-OES). A series of electrochemical studies provided insights into the increased impedance, corrosion inhibition mechanism and redox reactions of the protein, while small angle X-ray scattering (SAXS) confirmed the protein’s oligomeric conformation at the optimal corrosion inhibition concentration. Quartz crystal microbalance with dissipation monitoring (QCM-D) and nanoindentation studies indicate that rMrCP20 strongly adhered to steel substrates, with the presence of Fe ions resulting in compaction of the nanoscale protein film. Finally, Fourier Transformed Infrared Spectroscopy (FTIR) in conjunction with molecular dynamics (MD) simulations identified the main molecular interactions between rMrCP20 and Fe ions, in particular indicating that Fe\textsuperscript{3+} ions form ionic bridges and coordination bonds with negatively-charged side chains residues and histidine residues of rMrCP20, respectively.

Results And Discussion

Anticorrosion Studies

rMrCP20 was first expressed and purified using the same protocol as previously described.\textsuperscript{18} To examine the corrosion-inhibition properties of rMrCP20 protein, epoxy-embedded polished AH36 steel coupons were immersed in a pH 8.3 buffer containing 150 mM NaCl and 20 mM Tris(hydroxymethyl)aminomethane (Tris), in the absence or presence of rMrCP20 at different concentrations (see Materials and Methods). Figures 1a,b illustrate representative pictures of AH36 coupons at various time points and their respective immersion solutions after 24 h, at a range of rMrCP20 concentrations from 0 to 10 mg/mL. In the absence of rMrCP20, the coupons underwent severe corrosion, with surfaces covered with rust and other corrosion products, and the respective buffer solution turning into intense brown color. At a low concentration of ca. 0.1 mg/mL, rMrCP20 was previously found to accelerate the corrosion of steel coupon surfaces.\textsuperscript{19} At increasing concentration of rMrCP20, corrosion was reduced considerably and little to no corrosion was observed on coupons incubated in 5 mg/mL and 10 mg/mL of proteins (Fig. 1a), while their respective buffer solutions post-incubation had a light brown
Randomized pitting corrosion events were observed across the coupon surfaces for corroded samples, with oxide products adhering to surfaces, as shown by representative scanning electron microscopy (SEM) images in Fig. 1c. Further analysis was performed using ImageJ software to estimate the corroded area and the results are shown in Fig. 1d. In the absence of rMrCP20, on average 91% of AH36 coupon surfaces was corroded after 24 h. However, at increasing protein concentration surface corrosion was reduced drastically, down to 2% at 10 mg/mL, thereby demonstrating a concentration-dependent corrosion inhibition effect of rMrCP20 on AH36 steel.

Weight loss measurements are routinely employed to evaluate the effectiveness of metal corrosion inhibitors. Fe content from the incubation solutions and respective coupon surfaces were measured by Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) to reflect the total weight loss for each sample at concentrations of rMrCP20 from 0.1 mg/mL to 10 mg/mL, and to calculate the inhibition efficiency using Eqs. (1)–(2).

\[
\text{CR} = \frac{8.76 \times 10^4 \Delta m}{\rho A T} \quad \text{(Eq. 1)}
\]

\[
\eta(\%) = \theta \times 100 \times \left(1 - \frac{CR_1}{CR_0}\right) \times 100 \quad \text{(Eq. 2)}
\]

where \( CR \) is the corrosion rate (\text{mm yr}^{-1}), \( \Delta m \) is the total weight loss (g), \( \rho \) is density of the metal samples (7.86 g cm\(^{-3}\)), \( A \) is the surface area (cm\(^2\)), and \( T \) is the exposure time (h). \( \eta \) is the corrosion inhibition efficiency (%), \( \theta \) is degree of surface coverage, while \( CR_0 \) and \( CR_1 \) are the weight losses of the coupons in the absence and presence of rMrCP20, respectively. The corrosion rate calculated via Eq. (1) decreased significantly with increasing the concentration of rMrCP20 (Supplementary Table 1), with the lowest corrosion rate of 0.12 mm yr\(^{-1}\) and a high corrosion-inhibition efficiency \( \eta \) 88.48% (Fig. 1e). The observed enhanced corrosion-inhibition efficiency is attributed to the strong adhesive properties of rMrCP20, whereby protein adsorption to the coupon surfaces –and hence the degree of coupon surface coverage– increased at higher protein concentrations.

### Electrochemical Studies

To further investigate the impedance brought about by different concentrations of rMrCP20, electrochemical impedance spectroscopy (EIS) measurements were performed for AH36 samples after 24 h incubation in buffer solutions containing various concentration of rMrCP20 protein. A protein concentration of 5 mg/mL was selected as the optimal concentration for further characterization as it exhibited good corrosion inhibition performance, whereas increasing the amount of protein to 10 mg/mL did not significantly affect the results.

Figure 2a-c show the Nyquist plots (Fig. 2a), Bode modulus (Fig. 2b) and phase angle representations (Fig. 2c) from EIS measurements. The Nyquist plots show single semi-circle loops from high to mid-frequency range, indicating that the corrosion of AH36 was controlled by a charge transfer process (Fig. 2a). Tailing was observed at the lower frequency range of each Nyquist plot, and these
imperfections can be attributed to the roughness and heterogeneity of the AH36 working electrode,\textsuperscript{24,26} distribution of active center,\textsuperscript{26,27} adsorption of inhibitor molecules\textsuperscript{28} and accumulation of corrosion products on the working electrode.\textsuperscript{27,28} The diameter of the Nyquist plot is directly correlated to the impedance of the system, hence providing a suitable comparison of the resistance of the Fe substrate exposed to different concentrations of \(\textit{r} \text{MrCP20}\). The diameters increased at higher concentration of \(\textit{r} \text{MrCP20}\), suggesting that \(\textit{r} \text{MrCP20}\) adsorbed onto AH36 surfaces increases the charge transfer resistance and hence imparts corrosion resistance properties.

Bode modulus plots (Fig. 2b) provide a more comprehensive representation of the impedance across the range of frequencies measured, while phase angle plots (Fig. 2c) indicate the phase shift across the range of frequencies and the plausible components in the respective equivalent electrical circuits (EEC). At higher protein concentrations, the Bode modulus plot showed an increase in impedance values at low frequencies, where corrosion events are usually observed, while the phase angle plots showed an increase in the maximum phase angle value. The higher impedance is attributed to the formation of more homogenous \(\textit{r} \text{MrCP20}\) protein films at higher protein concentrations. Furthermore, the diameter of the Nyquist semicircles also increased with exposure time at higher \(\textit{r} \text{MrCP20}\) concentration (Supplementary Fig. 1), which is caused by the formation of a mixed surface layer of corrosion products (iron oxide) and adsorbed proteins.\textsuperscript{28} The evolution of the surface film most likely involved complexation between \(\textit{r} \text{MrCP20}\) and Fe ions (Fe\textsuperscript{2+} and Fe\textsuperscript{3+}), which blocked the charge transfer path. This observation also highlights the stability of the adsorbed \(\textit{r} \text{MrCP20}\) molecules on the steel surface.

To further interpret the EIS results, an equivalent electrical circuit (EEC) was chosen to extract the relevant electrochemical parameters (Fig. 2a inset). In the circuit, \(R_s\) represents the solution resistance, \(R_{ct}\) the charge transfer resistance, \(CPE_{dl}\) and \(CPE_f\) the constant phase elements of the double layer and protein film respectively, and \(R_f\) the resistance of the adsorbed \(\textit{r} \text{MrCP20}\) film. The double layer capacitance (\(C_{dl}\)) values were calculated from the expression:\textsuperscript{23}

\[
C_{dl} = \frac{Y_0 \omega^{n-1}}{\sin\left[n\left(\frac{\pi}{2}\right)\right]} \quad \text{(Eq. 3)}
\]

where \(Y_0\) is the CPE constant, \(n\) is the CPE exponent, \(\omega\) is the angular frequency \((\omega = 2\pi f_{\text{max}})\) in rad/s, and \(f_{\text{max}}\) is the frequency at which the imaginary component of the impedance is highest. The corrosion inhibition efficiency (\% \(\eta\)) was calculated using Eq. (4):\textsuperscript{29}

\[
\eta_{EIS} \% = \frac{R_{ct}^0 - R_{ct}'^0}{R_{ct}^0} \times 100 \quad \text{(Eq. 4)}
\]

where \(R_{ct}^0\) and \(R_{ct}'^0\) are the charge transfer resistances in the presence and absence of \(\textit{r} \text{MrCP20}\), respectively. The values of the electrochemical parameters derived from the Nyquist plots are listed in Fig. 2d and demonstrate that adding \(\textit{r} \text{MrCP20}\) to the corrodent led to a reduction in \(C_{dl}\) and an increase in \(R_{ct}\) and \(R_f\), which became more apparent as the \(\textit{r} \text{MrCP20}\) concentration increased. As the concentration
of MrCP20 increased from 1 to 5 mg/mL, the $R_{ct}$ value increased from 926 Ω cm$^2$ to 4721 Ω cm$^2$, while the corresponding $\eta$ value increased from 19.8–84.3%. The value of phase shift ($n$) for CPE$_{dl}$ also increased from 0.79 without MrCP20 to 0.85 in the presence of 5 mg/mL MrCP20, suggesting a decrease in the heterogeneity of the coupon surface arising from adsorption of MrCP20. $C_{dl}$ for the control sample was 757 µF cm$^{-2}$, which was significantly higher than the corresponding value for the system incubated in MrCP20, indicating growth in the electrical double layer thickness. These results suggest that the adsorption of MrCP20 at the metal/electrolyte interface impedes charge transfer due to greater resistance. Hence, at higher concentration of MrCP20, the AH36 steel surface is better protected against corrosion.

To obtain further qualitative understanding of the corrosion reactions, potentiodynamic polarization measurements (PDP) measurements were carried out on AH36 samples after 24 h incubation in the respective solutions, and the polarization curves are shown in Fig. 2e. The polarization curves present two opposing reactions, the anodic branch corresponding to Fe dissolution and the cathodic branch due to hydrogen evolution. The obtained polarization parameters, namely corrosion potential ($E_{corr}$), corrosion current density ($i_{corr}$), anodic and cathodic Tafel slopes ($\beta_a$ and $\beta_c$) measured by the Tafel extrapolation method are presented in Fig. 2f. The corrosion rate ($CR$) and corrosion inhibition efficiency ($\eta_{PDP}$) of MrCP20 was calculated using Eqs. (5–6):29

$$\eta_{PDP} (\%) = \left(1 - \frac{i_{corr}^0}{i_{corr}'}\right) \times 100 \text{ (Eq. 5)}$$

$$CR = 3.17E^{-9} \times \frac{M}{nF\rho A} i_{corr} \text{ (Eq. 6)}$$

where $i_{corr}^0$ and $i_{corr}'$ are the corrosion current densities in the presence and absence of MrCP20, respectively, $3.17E^{-9}$ is conversion factor, the ratio $M/n$ is the equivalent weight, $F$ (96485 C mol$^{-1}$) is the Faraday constant, $\rho$ ($7.86gcm^{-3}$) the density of the metal samples, and $A$ is the area of the sample ($cm^{-3}$). As shown in Fig. 2e, both the anodic and cathodic $i_{corr}$ decreased in the presence of MrCP20, and $E_{corr}$ shifted towards the cathodic direction, which became more pronounced as the concentration of MrCP20 increased. In addition, Fig. 2f shows the shift in $\Delta E_{corr}$ values to less than 85 mV, which indicates that MrCP20 is a mixed-type inhibitor$^{30,31}$ with a stronger effect on the cathodic reaction than on the anodic reaction. This result was complemented with cyclic voltammetry (CV) measurements (Supplementary Fig. 2). Since the peaks of the redox reactions measured by CV were not shifted at different concentrations of MrCP20, the reaction mechanism appeared to remain consistent. In addition, the peak currents observed in CV scans for both the oxidation and reduction reactions progressively decreased with the addition of MrCP20, further confirming the lowered occurrence of redox reactions, and hence the corrosion inhibition properties of MrCP20. Furthermore, the peak currents from CV measurements remained constant in the presence of MrCP20 with repetitive scans, whereas in the absence of the protein the peak currents were much greater for the second scan.
As shown in Fig. 2f, $i_{\text{corr}}$ decreased from 28.5 $\mu Acm^{-2}$ without rMrCP20 down to 4.0 $\mu Acm^{-2}$ with 5 mg/mL of rMrCP20. The lower values of $i_{\text{corr}}$ in the presence of rMrCP20 can be attributed to protein adsorption onto the sample’s surface and hence to the formation of a protective layer, which hinders the electron transfer process at the interface and reduces the rate of corrosion reactions. Figure 2f also illustrates the effect of rMrCP20 on the Tafel slopes values $\beta_c$, which were independent of the rMrCP20 concentration, indicating that the presence of rMrCP20 can suppress the corrosion process by blocking reaction sites without affecting the kinetics of the cathodic reactions. 32 Meanwhile, a gradual decline in $\beta_a$ was observed with increasing rMrCP20 concentration, from 267 mV dec$^{-1}$ in buffer to only 110 mV dec$^{-1}$ in the presence of 5 mg/mL rMrCP20, indicating a change in the iron dissolution mechanism. Iron dissolution is attributed to the formation of metal-ion protein complexes as the protein interacts with the steel surface, which was further investigated by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), QCM-D and MD simulations (discussed below). Accordingly, the corrosion-inhibition efficiency improved at increased rMrCP20 concentration, from 24% (1 mg/mL) to 86% (5 mg/mL), respectively. In summary, the corrosion inhibition values $\eta$ obtained from all three methods (weight loss, EIS and PDP measurements) are consistent (Supplementary Fig. 3) and all indicate enhanced corrosion-inhibition activity as the concentration of rMrCP20 increased.

**Adsorption of rMrCP20 and Metal ion – Protein Interaction studies**

QCM-D measurements were carried out on Fe sensors to examine the adsorption properties of rMrCP20, followed by subsequent addition of FeCl$_3$ analyte as a source of free Fe$^{3+}$ to investigate the Fe$^{3+}$/adsorbed protein layer interactions. The changes in resonance frequency ($\Delta f$) and dissipation ($\Delta D$) of the sensor surface were measured simultaneously. Figure 3a shows the results obtained for the 5th overtone as a function of time for the following steps: flowing of rMrCP20, rinsing, flowing of FeCl$_3$ solution and second rinsing (see Materials and Methods). To allow direct comparison of the $\Delta f$ and $\Delta D$ shifts measured at different harmonics, the net shifts of the 5th and 11th overtones are displayed in Figs. 3b and c. Upon injection of rMrCP20, an exponential increase in $\Delta f$ intensity was measured (Fig. 3a), followed by a plateau, indicative of a rapid initial adsorption of rMrCP20 onto the Fe sensor surface followed by saturation at the surface. $\Delta f$ recorded was ca. -40.0 Hz and -39.2 Hz upon adsorption saturation for the 5th and 11th overtones, respectively. The significant decrease in $\Delta f$ was accompanied by a proportional increase in $\Delta D$ to 1.62 x 10$^{-6}$ and 1.55 x 10$^{-6}$ for 5th and 11th overtones, respectively. Fast initial adsorption rates suggests that rMrCP20 has a high affinity for the Fe sensor surface, which can be driven by complementary electrostatic and hydrophobic adsorption. 33–35 The changes in $\Delta f$ and $\Delta D$ due to rMrCP20 binding to the Fe sensor surface were generally similar amongst different overtones, indicating that the protein formed a compact and rigid layer. 33 While the inversely proportionate changes in $\Delta D$ and $\Delta f$ denote the strong binding kinetics of rMrCP20 on Fe substrate, $\Delta D$ decreased gradually while $\Delta f$ plateaued, suggesting that the adsorbed protein layer more tightly packed over time. The changes of $\Delta f$ was similar at different rMrCP20 concentrations, indicating that rMrCP20 formed a mono-
adlayer (Supplementary Fig. 4c). Upon rinsing, a slight decrease in $\Delta f$ intensity was detected due to the removal of excess unbound proteins, but most proteins remained strongly bound to the Fe surface.

The introduction of FeCl$_3$ induced large shifts in the QCM-D signals, splitting the response across the different harmonics (Supplementary Figs. 4a, b). The increase in $\Delta f$ intensity indicates significant binding interaction between the analyte and adsorbed protein layer, which can occur via formation of Fe$^{3+}$ metal-protein complexes and electrostatic interactions. The proportional increase in $\Delta D$ implies a loosen packing of the adlayer, which allows more ingress of water molecules that remained trapped within the adlayer. Upon the final rinsing step to remove any loosely bound material, only a slight decrease in $\Delta f$ intensity was observed, suggesting that Fe$^{3+}$ ions were strongly bound to the pre-adsorbed $\alpha$MrCP20 layer, whereas the slight decrease in $\Delta D$ indicated stiffening of the surface layer.

The mode of interaction between $\alpha$MrCP20 and the metal surface can be deduced from adsorption isotherms, which were obtained by adapting the results from weight loss measurements (Supplementary Table 1) to determine the adsorption characteristics of $\alpha$MrCP20 on AH36 steel in buffer solution. The degree of surface coverage ($\theta$) as function of concentration ($C$) of the protein was fitted into various adsorption isotherm models, including Langmuir, Temkin, Freundlich, Flory–Huggins, Frumkin and El-Awady to identify the best fit based on the obtained $R^2$ values. The best fit was obtained for the Freundlich model ($R^2 = 0.96$, Supplementary Table 2), which is generally applicable for multilayer adsorption events on heterogeneous surfaces, with the assumption of a large number of different types of binding sites acting simultaneously. The linear form of the Freundlich model can be written as:

$$log \theta = \frac{1}{n} log C_{inh} + log K_{ads} \quad (7) \quad (Eq. 7)$$

where $K_{ads}$ represents the Freundlich adsorption capacity (L/mg) and $n$ describes the heterogeneity of the system related to the adsorption intensity. A larger $n$ value connotes a more heterogeneous system, and $n > 1$ suggests a favorable adsorption process. Freundlich constants $K_{ads}$ and $n$ values were $1.33 \times 10^{-2} \text{L/mg}$ and 2.18, respectively. The value of $K_{ads}$ was then used to calculate the standard free energy of adsorption ($\Delta G_{ads}^0$):

$$\Delta G_{ads}^0 = -RT \ln \left(1 \times 10^6 K_{ads}\right) \quad (Eq. 8)$$

where $R$ is the universal gas constant, $T$ is the absolute temperature, and $10^6$ is the concentration of water molecules expressed in ppm. The calculated $\Delta G_{ads}^0$ value of -23.52 kJ/mol indicates spontaneous interaction between the protein and the surface of AH36 coupons, and is within the range for mixed adsorption involving both physisorption driven by electrostatic interactions as well as chemisorption caused by charge sharing or charge transfer from the protein molecules to the metal surface.

To determine the thickness of the protein and metal-protein layers, we carried out nanoindentation (Fig. 4a) on samples prepared similarly to QCM-D studies but on gold sensor substrates. Samples were equilibrated in buffer, and a cube-corner tip was used to indent the samples via a displacement-controlled
indentation of 200 nm in depth. Figure 4b shows the surface roughness profile of the samples and Fig. 4c the histogram of the average layer thickness, indicating significant difference between samples. The layer thickness was inferred from the slope change during the loading step of indentation cycles, as illustrated on load vs. displacement curves shown in Fig. 4d. The presence of a layer ca. 74 nm thick was consistently observed on the substrate when hydrated and was attributed to a thin oxide layer. Upon adsorption of rMrCP20, an indentation depth of ca. 82 nm was measured before the tip contacted the substrate, indicating that the protein formed a layer of ca. 8 nm (subtracting the thickness of the oxide layer). Subsequent indentation of the protein layer after interaction with FeCl₃ showed a layer of thickness ca. 79 nm, indicating that the protein layer became slightly more compact to ca. 5 nm. The results corroborate with the QCM-D results, where the adsorbed rMrCP20 layer became more compact and increased in density upon interacting with FeCl₃.

To assess the effect of Fe ions on rMrCP20 secondary structure and identify the type of binding interactions between Fe ions and the protein's side chains, ATR-FTIR spectroscopy measurements were conducted (Fig. 5). With rMrCP20 kept at 5 mg/mL concentration, different concentrations of FeCl₃ were added as indicated in Fig. 5a. The most significant changes in the spectra were observed at the protein:FeCl₃ molar concentration ratio of 1:60. At low FeCl₃ concentration, minimal changes were observed compared to rMrCP20-only spectra. The amide I band was further deconvoluted to obtain a semi-quantitative estimate of the secondary structural content, as shown in Fig. 5c. The initial β-sheet content of rMrCP20 protein was ~53% at 5 mg/mL. At the 1:60 protein:FeCl₃ concentration ratio, the amide I maximum of rMrCP20 spectra shifted from 1641 cm⁻¹ to 1631 cm⁻¹ (Fig. 5b), indicating a transition towards anti-parallel β-sheet structures. Similarly, the appearance of 1553 cm⁻¹ in amide II confirm that FeCl₃ at high concentrations induced changes in the protein's conformation. The amide III peaks at 1262 cm⁻¹ and 1296 cm⁻¹ were likewise more noticeable at high FeCl₃ content.

New peaks were noticeable at higher concentrations of FeCl₃, indicating that new bonds formed between the protein and Fe³⁺. The appearance of 800 cm⁻¹ and 910 cm⁻¹ bands were assigned to the stretching of aromatic -CH groups and C = C, respectively, attributed to their interaction with Fe³⁺. The distinct splitting of the band centered at 1043 cm⁻¹ to 1038 cm⁻¹ and 1053 cm⁻¹ can be attributed to chelation of Fe³⁺ ions by the imidazole side chain of histidine (His). The band at 1403 cm⁻¹, assigned as S = O bond appeared in the presence of Fe³⁺, and was attributed to the oxidation of thiol functional groups of cysteine residues. The appearance of 1138 cm⁻¹, 1262 cm⁻¹ and 1296 cm⁻¹ bands were assigned to new interactions involving the protein’s side chain C-OH, C-O/-CH₃ and amide bond C = O functional groups, respectively. The intensity of the COO⁻ stretching band of acidic residues at 1398 cm⁻¹ observed for rMrCP20 decreased, concurrently with the appearance of a peak at 1437 cm⁻¹ upon introduction of high FeCl₃ concentrations, is assigned to the formation of ionic bridges or coordination bonds between the carboxyl side chains and Fe³⁺.
Small angle x-ray scattering and MD simulation studies of rMrCP20 and Fe ions

Small angle x-ray scattering (SAXS) was used to determine the radius of gyration ($R_g$) of rMrCP20 protein in our first steps towards developing a mechanistic model for the protein's adsorption to substrate surfaces (Supplementary Fig. 5a,b,c). The oligomeric state of rMrCP20 was obtained from SAXS experiments. SAXS spectra of rMrCP20 were collected at 1.3, 4.1, and 6 mg/mL, respectively (Supplementary Fig. 5a). The scattering pattern of rMrCP20 at protein concentration of 1.3 mg/mL had a low signal to noise ratio and thus, no data pre-processing was performed with this dataset. $R_g$ values using Guinier approximation of rMrCP20 at protein concentration of 4.1 and 6 mg/mL (Supplementary Table 3) showed a slight increase in molecular size, suggesting that the shift is most likely due to interparticle interaction between rMrCP20 molecules. To exclude the concentration-dependent effect, the data of rMrCP20 at protein concentration of 4.1 mg/mL was used for further data analysis. The overall structural parameters are recorded in Supplementary Table 3. Based on the Porod-volume ($V_p$) and DAMMIF-excluded volume ($V_{ex}$), determined from the scattering pattern at protein concentration of 4.1 mg/mL, the molecular weight (MW) of rMrCP20 was calculated to be 42 $\pm$ 4.2 and 45 $\pm$ 4.5 kDa, respectively (Supplementary Table 3). Knowing the calculated monomeric MM based on the protein sequences is 21 kDa, the data indicate that rMrCP20 adopts a dimeric state in solution at 4.1 mg/mL.

To investigate the distribution of Fe ions interacting with the protein and to calculate the number of Fe ions bound to each residue, MD simulations were performed for rMrCP20 protein dimer in the presence of a high Fe ion concentration. Two types of initial structures were considered. One is the NMR structure of rMcP20 (PDB: 6LEK), another one is the structural model built by AlphaFold 2.\(^{44}\) SAXS data showed rMrCP20 adopts a dimeric state in solution. Thus, the dimeric forms of the both models were compared with the SAXS profile, and one of these dimers from AlphaFold 2 models showed a best fit, giving a $\chi^2$ value of 1.76 (Supplementary Fig. 5d-e). This dimeric model was selected for subsequent MD simulation.

From the MD, the largest cluster structure with 54 close-contacting Fe ions around the protein dimer and the inset highlighting the interactions of Fe ions at rMrCP20 interface are shown in Figs. 6a and b. The average number of Fe ions bound to the residues is shown in Fig. 6c. The high RMSD obtained in MD simulation may reflect the intrinsic flexibility of the dimers in the presence of Fe ions, which predominantly interact with the negatively charged residues Asp and Glu, binding up to more than 4 Fe ions, as shown in Fig. 6c. These Fe-protein interactions, especially those on the chain interfaces, may help to enhance protein-protein interactions, as experimentally observed from the spontaneous aggregation of rMrCP20 upon addition of FeCl$_3$ (see optical micrograph in Supplementary Fig. 6). The results corroborate the FTIR data (Fig. 5a), notably the significant shift of the carboxyl band at high FeCl$_3$ concentration attributed to the interaction of aspartic acid (Asp) and glutamic acid (Glu) side chains with Fe ions, occurring in conjunction with changes in the protein’s secondary structural conformation.

**Proposed multimodal anti-corrosion mechanism**
MrCP20 protein is highly negatively charged, comprising clusters of charged amino acids with a large number of 32 Cys residues that contribute to binding interactions with substrates, thereby allowing barnacles to adhere to charged surfaces such as metals.\textsuperscript{18,45} The thiol side chain on Cys residues is easily deprotonated at seawater pH, leading to a negatively charged sulfhydryl group\textsuperscript{46}, which then binds to charged surfaces ions via electrostatic interactions\textsuperscript{47} to form more stable thiolate-metal ion complexes.\textsuperscript{48} The side chains of Asp, Glu and His residues are also deprotonated and negatively charged at seawater pH, hence favoring electrostatic interactions with cations. A multimodal corrosion inhibition mechanism is proposed (Fig. 7) to explain our findings. Corrosion of metal surfaces often occurs on or adjacent to grain boundaries due to the different electrode potentials of the grain and boundary, making them susceptible to redox reactions that lead to intergranular corrosion in the presence of electrolytes,\textsuperscript{49} while other forms of corrosions such as uniform, crevice and pitting corrosions also occur concurrently at the substrate-electrolyte interface\textsuperscript{50,51}. MrCP20 protein readily adsorbs onto metal substrates, filling grain boundaries and coating the substrate surface thereby preventing the substrate's direct contact with seawater. The protein adheres strongly to the metal substrate through iron-protein interactions, thereby blocking the charge transfer path and functioning as a protective coating to inhibit different forms of corrosion.

**Conclusion**

MrCP20 protein exhibits a concentration-dependent anti-corrosion property to effectively inhibit corrosion of AH36 steel substrates in solution at MrCP20 concentrations above 5 mg/ml. The natural adhesive protein was shown to adsorb rapidly onto substrate surfaces, forming a relatively homogeneous layer that increases the impedance of the coating and reduces the propensity for spontaneous corrosion. The protein layer readily incorporates Fe ions, predominantly through electrostatic interactions with negatively charged residues at seawater pH conditions, concurrent with conformational changes in the protein's secondary structures, resulting in a thin proteinaceous film that enhances impermeability against seawater, thereby shielding the underlying substrate against aggressive corrosive ions. Our findings indicate that the barnacle adhesive protein MrCP20 has promising potential for further scaling-up development into an effective and green anti-corrosion additive for steels in the marine environment. Alternatively, the study provides molecular-level guidelines to develop artificial protein-based corrosion inhibitors inspired by the primary structure of MrCP20.

**Materials And Methods**

Preparation of steel coupons. AH36 steel coupons cut to 10 x 10 x 2 mm were purchased from Ebenezer Excel Engineering, Singapore. Chemical composition (in wt %) of the AH36 material was Nb 0.03, Cr 0.03, Mn 0.2 and the balance Fe. The coupons were embedded in cold mount epoxy (EpoFix, Struers), while for electrochemical studies, coupons were welded with an electrical wire before embedding in epoxy. Prior to all experiments, grinding was performed manually with a series of grit paper (# 800 to # 2000) to expose a surface area of 1 cm\textsuperscript{2} and then polished to a mirror-like finish using a diamond suspension (Ø = 3 µm,
Struers) to alleviate the effects of surface roughness on protein absorption. The samples were then washed with Milli-Q water (18.2 MΩ cm, Millipore™), cleaned with ethanol in an ultrasonic bath, washed again with Milli-Q water and finally dried with nitrogen gas.

Time-resolved study of corrosion on AH36 coupon. AH36 steel samples were immersed in a buffer solution with different rMrCP20 protein concentrations (0.1, 0.5, 1, 3, 5 and 10 mg/mL) and incubated at ambient temperature, on an orbital shaker set at 150 rpm for 24 hr. AH36 steel samples were removed at stipulated time points for visual inspection, and subsequently removed after 24 h for collecting corrosion-products on the coupons and from buffer solution. Experiments were triplicated, using freshly prepared AH36 steel coupons.

Weight loss measurements. To quantify the total Fe mass loss after incubation, both Fe content from corrosion products bound to sample surfaces and Fe content dissolved in incubation solutions were collected and measured using inductively coupled plasma optical emission spectrophotometer (ICP-OES) (PerkinElmer® Optima™ 8300). Corrosion products bound to the sample surfaces were removed with 10 min of ultrasonication in 5 mL of 1% HCl solution containing 100 mM hexamethylenetetramine (Sigma-Aldrich) for preventing additional Fe dissolution from the coupons. 1 ml of the respective Fe solutions were dissolved separately in 9 ml of 10% HNO₃, then further diluted by 10x in Milli-Q water and filtered through 0.45 µm membrane prior to ICP-OES measurements. Calibration for Fe concentration standard curve was first performed using 5, 10, 25, 50 and 100 ppm standard solutions, and the wavelength for metal detection was Fe 238.86 nm. All experiments were performed in triplicates.

Electrochemical measurements. Electrochemical measurements including electrochemical impedance spectroscopy (EIS) and potentiodynamic polarization (PDP) were conducted using PGSTAT302N Autolab (Metrohm) with NOVA 2.1 software. The conventional three-electrode system was used, in which the reference and working electrodes were saturated Ag/AgCl and AH36 steel with a single side of 10 x 10 mm exposed square area, respectively. A platinum sheet of 20 x 10 mm was chosen as the counter electrode as the area needs to be larger than the exposed working electrode to guarantee uniformity of the electrochemical reaction. The working electrode was exposed to buffer solution containing various rMrCP20 concentrations. All electrochemical measurements were performed at room temperature and repeated in triplicate.

Prior to measurements, the working electrode was incubated in a test solution for 30 minutes under stagnant condition until a stable open circuit potential (OCP) was attained. EIS measurements at different time intervals were conducted with a perturbation amplitude of 10 mV at OCP over a frequency range of 0.01–10000 Hz. The NOVA 2.1 software was employed for analysis of the EIS spectra. Nyquist and Bode plots were drawn by fitting the impedance data using equivalent circuit.

Potentiodynamic polarization (PDP) experiments were carried out after 24 hours of exposure, in the potential range from –0.25 V versus OCP to -0.4 V versus Ag/AgCl with the sweep rate of 10 mV/min.
The corrosion potential ($E_{\text{corr}}$) and corrosion current density ($i_{\text{corr}}$) were calculated by extrapolating the linear Tafel segments of the anodic and cathodic curves.

Quartz Crystal Microbalance with dissipation monitoring, QCM-D. The adsorption of $\alpha$MrCP20 on Fe substrate and its interaction with Fe$^{3+}$ were analyzed using Quartz Crystal Microbalance (QCM) with Dissipation monitoring (Q-Sense E4 QCM, Sweden), connected with IPC peristatic pump (Ismatec SA, Switzerland). The iron-coated quartz crystals with a fundamental frequency of 4.95 MHz were purchased from Q-sense (QSX 319, Biolin Scientific). All QCM sensor crystals were cleaned according to a standard protocol whereby the surface is immersed in 1% Hellmanex III (Sigma-Aldrich) solution for 30 minutes, then ultrasonicated in 99% ethanol (Merck) for 10 minutes, followed by rinsing with Milli-Q water and finally dried with nitrogen gas. These measurements were carried out at a constant flow rate of 100 µL/min and the temperature of the measuring cell was controlled at 22 °C. The resonant frequency of the oscillator ($f$) and the energy dissipation value ($D$) were recorded simultaneously as a function of time. The system baseline was established for 1 hour using the buffer solution without protein. After stabilization, 23 µM of $\alpha$MrCP20 solution was injected and the adsorption took place for 45 minutes. The cell was then rinsed with buffer until stable frequency and dissipation values were attained. 100 µM of FeCl$_3$ solution was injected and the adsorption of FeCl$_3$ on pre-adsorbed protein layer took place for 35 minutes. Buffer was then re-introduced to wash out the weak bound complexes.

**Attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR).** Samples were prepared at 1 and 5 mg/mL $\alpha$MrCP20 protein concentration with a series of FeCl$_3$ concentrations in separate vials of 100 µL volume and incubated at ambient temperature for 1 hour, by dipping the vials in liquid N$_2$ for 5 minutes and lyophilized immediately. Attenuated total reflection fourier-transform infrared (ATR-FTIR) spectroscopy measurements were performed on a Bruker Vertex 70 (Massachusetts, USA) instrument equipped with a PIKE Technologies MIRacle attenuated total reflection (ATR) Diamond-ZnSe 3-reflection accessory and a LN$_2$ cooled MCT detector. Scans were obtained at ambient temperature over the range of 4000 to 750 cm$^{-1}$ with a resolution of 2 cm$^{-1}$, averaged over scans obtained at 20 kHz for 1 minute. All spectra processing were performed on OPUS 6.5, in the sequence of water vapor subtraction, background subtraction of buffer, baseline correction, and min-max normalization using amide I band. Amide I band was deconvoluted by secondary derivation, with peak fitting performed using 100% Gaussian curves with individual FWHM kept relatively consistent. The deconvoluted peaks were then assigned to the respective secondary structures. [40,51-53]

**Nanoindentation.** QCM sensors (same as above) were first cleaned with ethanol and Milli-Q water, then dried with nitrogen gas. Each sensor was then incubated separately in 5 mL of 5 mg/mL $\alpha$MrCP20 protein solution for 50 min in 12-well plates, followed by 5 mL of buffer for 50 min. Half of the sensors were then incubated in 4 mL of 50 mM FeCl$_3$ for 40 min, followed by 4 mL of buffer for 40 min. All incubations were performed on a shaker. Nanoindentation was performed with a Hysitron TI 950 Triboindenter (Minnesota, USA), using a Berkovich (cube-corner) indentation tip. The tip was calibrated with a fused quartz standard sample of $E = 69.9$ GPa ± 10%. Sensors were attached to magnetic stainless steel specimen discs and
placed on a standard stage, then tip to optic calibrations were performed prior to lifting the tip 100 mm above sample and adding a drop of water on the sample, allowing the tip to submerge and equilibrate. After 30 minutes, indentations were performed with displacement-controlled setting of 200 nm depth. Load–hold–unload indentation function setup was applied for all indents, with segment intervals of 5 s (load), 2 s (hold), and 5 s (unload). At least 30 indents were obtained for each sample. Slope tangents were obtained separately from contact with sample layer and substrate, and intersections of the tangents indicated the sample layer thickness. The average for each sample group was shown and standard deviation indicated via error bars. Statistical significance between groups were acquired via two-sample t-test assuming unequal variances. P-value of $P < 0.0001$ (***) and $P < 0.001$ (****) represented significance difference between two groups.

**Field Emission Scanning Electron Microscopy – Energy Dispersive X-ray (FESEM-EDX).** AH36 coupons were adhered to sample stubs with adhesive carbon tape, and copper tape. Without coating, imaging was performed using JEOL JSM-FESEM 7800F PRIME (Massachusetts, USA), at SEI-mode, 20 kV, and 92 µA emission current. EDX maps were acquired at a 20 keV, 1024 resolution, with fixed duration of 3 frame counts, and process time 4, and pixel dwell time of 20 µs.

**SAXS Data Collection and Analysis.** SAXS data for MrCP20 were collected with a Xenocs Nano-inXider SAXS instrument equipped with a microfocus sealed-tube X-ray source (Cu, 30 W, 40 µm focus) and a Dectris Pilatus 3hybrid pixel detector. The X-rays are filtered through the two-dimensional single-reflection multilayer optics and collimated by a three-pinhole system. The sample-to-detector distance was set at 0.94 m, and the sample chamber and X-ray paths were evacuated prior to usage. This setup covers a range of momentum transfer of $0.08 < q < 4 \text{ nm}^{-1}$ [$q = 4\pi\sin(\theta)/\lambda$, where $2\theta$ is the scattering angle]. SAXS experiments of MrCP20 were carried out at room temperature in the buffer composed of 20 mM Tris-HCl, pH 8.3 and 150 mM NaCl, using the low-noise flow cell. The protein concentrations used were 1.3, 4.1 and 6 mg/ml. The data were collected for 45 min, and for each measurement a total of nine frames at 5 min intervals were recorded. The scattered X-rays detected by a two-dimensional area detector were converted to one-dimensional scattering using the built-in SAXS software (Xenocs).

All the data processing steps were performed using the program package PRIMUS. The scattering of the buffer was subtracted from the data. The experimental data obtained were analyzed for aggregation using a Guinier plot. The forward scattering, $I(0)$ and the radius of gyration, $R_g$, were computed using the Guinier approximation, which assumes that at very small angles ($q < 1.3/R_g$), the intensity is represented as $I(q) = I(0) \times \exp[-(qR_g)^2/3]$. These parameters were also computed from the extended scattering patterns using the indirect transform package GNOM, which provides the distance distribution function, $P(r)$, and hence the maximal particle dimension, $D_{max}$ and the radius of gyration, $R_g$. The hydrated volume, $V_p$, which was used to estimate the molecular mass of the protein, was computed using the Porod invariant. The theoretical scattering profiles were generated from the atomic resolution structure of MrCP20 (PDB ID: 6LEK state 1) by considering both monomeric and dimeric structures, and
the simulated models generated in this study. All generated theoretical scattering profiles were evaluated against the experimental scattering profile using CRYSOL.\textsuperscript{58}

**Molecular dynamics simulations. Initial Structure:** AlphaFold2 server\textsuperscript{59} was used to predict the structure of rMrCP20 in monomeric and dimeric forms. One of the dimeric form was found to be better consistent with the SAXS data in that the theoretical scattering profile of the dimeric structure calculated using CRYSOL\textsuperscript{55} fitted well with the experimental profile ($\chi^2 = 1.76$). Thus, this dimeric structure was taken as the initial structure for the following molecular dynamics simulation.

The rMrCP20 dimer was represented by AMBER99SB-ILDN force field.\textsuperscript{60} The system were solvated with 161,769 TIP3P\textsuperscript{61} water molecules, 802 Fe$^{3+}$ ions and 2354 chloride ions were added to neutralize the system. The system was put in a cubic box of 173 Angstroms with periodic boundary condition. The molecular dynamics (MD) simulation used GROMACS\textsuperscript{62} 5.1.2 software. The LINCS\textsuperscript{63} algorithm was used to constrain bonds between heavy atoms and hydrogen to enable a timestep of 2 fs. A 1.2 nm cutoff was used for Van der Waals interaction and short-range electrostatic interactions calculations, and Particle Mesh Ewald method was implemented for long range electrostatic calculations. Simulation temperature was maintained at 300K using a V-rescale thermostat\textsuperscript{64} and 1bar pressure using Parrinello-Rahman\textsuperscript{65} barostat. A production run of 100 ns was obtained.

### Declarations

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**Figures**
Figure 1

Time-resolved study of corrosion on AH36 coupon. a Representative pictures of AH36 coupons showing the concentration-dependent anti-corrosion effect of rMrCP20 at immersion time intervals of 0, 1, 3, 7 and 24 h, and after cleaning the coupons with 1% HCl. b Respective buffer solutions after 24 h of coupon immersion. c Field emission scanning electron microscope (FESEM) images and overlay of energy dispersive X-ray spectroscopy (EDX) map of pitting corrosions before and after removal of corrosion products on surface of coupons, with a pitting of ca. 300 mm diameter outlined. d ImageJ analysis to determine percentage corroded area of AH36 coupons with standard deviations represented by error bars.
Weight loss of AH36 over a 24 h incubation period and corresponding inhibition efficiency at increasing concentrations of rMrCP20.

**Figure 2**

Electrochemical impedance and potentiodynamic polarization (PDP) spectra after 24 h immersion in buffer solution in the absence and presence of rMrCP20 protein at concentrations of 1, 3 and 5 mg/mL.
Nyquist plots with inset displaying the plots at higher frequencies between $Z'$ 0 to 1500 W and equivalent circuit diagram as inset. **b** Bode modulus plot. **c** Phase angle plot. **d** Table summaries of electrochemical impedance parameters for AH36 in buffer solution, at various concentrations of rMrCP20 after 24 hours immersion. **e** PDP curves in the potential range from -0.25 V versus OCP to -0.4 V versus Ag/AgCl with the sweep rate of 10 mV/min in aerated condition at 25 °C. **f** PDP parameters after 24 hours immersion.
QCM-D measurements tracking real-time adsorption of 0.5 mg/mL rMrCP20 on Fe substrate and subsequent interaction with Fe$^{3+}$. a QCM-D frequency ($D_f$) shifts (left axis) and dissipation ($D_D$) shifts (right axis) for 5th overtone as a function of time. The inset displays an illustration of protein structural changes upon adsorption and interaction with Fe$^{3+}$. b, c Changes in frequency (b) and dissipation (c) obtained from the 5th and 11th overtones at different steps in QCM-D measurements.

Figure 4

Nanoindentation of rMrCP20 layer formed on substrate. a Illustration of indentation carried out with a cube-corner tip. b Scanning probe microscopy images of sample surfaces. c Bar plot of average values of layer thickness obtained by nanoindentation on substrate, substrate with adsorbed rMrCP20 layer, and substrate with rMrCP20 layer after interaction with FeCl$_3$. d Load-displacement (loading and unloading) curves with insets showing magnified views of the loading portion of indentation curves, where the change in slope was used to detect the substrate surface and infer the thickness of the protein film.
Figure 5

FTIR-ATR investigation of the effect of FeCl$_3$ on rMrCP20 protein structure. 

a ATR-FTIR spectra of rMrCP20 and FeCl$_3$ at different molar concentration ratios, illustrating the spectral changes and bond assignments.

b Shift in amide I band, indicating a transition of the protein secondary structure transiting towards anti-parallel β-sheets at higher FeCl$_3$ concentrations.

c Semi-quantitative secondary structure based on amide I band deconvolution.
Figure 6

MD simulation of \( \alpha \)-MrCP20 dimers in the presence of Fe ions (orange spheres). a Representative largest cluster structure of the protein dimer with each chain represented by different colors, illustrating the distribution of close-contacting Fe ions (orange spheres). b. Zoom in view of Fe ions at the \( \alpha \)-MrCP20 interface. c Plot of average number of Fe ions bound to the residues, with color-coded protein sequence indicated below the plot.
Figure 7

Proposed multimodal corrosion inhibition action of rMrCP20 protein on steel substrates, due to the adhesive property of the protein and its ability to spontaneously bind with free Fe ions.

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