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ARTICLE TYPE

Preparation and Analysis of a new Bioorganic Metallic Material

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5 Biofouling on metal surfaces is one of the main reasons that increase ship drag. Many methods have already been used to reduce or remove it with moderate success. In this study, the synthetic peptide has been utilized to react with the 304 stainless steel aiming to generate a bioorganic stainless steel with a facile technique. After the reaction, the white matters were found on the surface of the treated stainless steel via SEM, whilst the nontreated stainless steel had not. Element analysis confirmed that excessive N
10 existed on the surface of the treated samples by integrated SEM-EDS facility, implying the presence of peptides binding on the surface of the bioorganic stainless steel. The FTIR spectra showed amide A, II peaks on the surface of the bioorganic stainless steel suggesting that either the peptides grafting onto the steel surface or polypeptide composition accumulated on the steel samples. XPS analysis of the treated steel demonstrated that there was nitrogen bonding on the surface and it was a chemically bond via a
15 previously unreported chemical interaction. The treated steel has a markedly increased contact angle ($65.7 \pm 4.7^\circ$ of water contact angle for nontreated steel in comparison to treated, $96.4 \pm 2.1^\circ$), which supported the observation of the wettability change of the surface, i.e. the decrease of the surface energy value after peptides treated. The changes of the surface parameters (such as, Sa, Sq, Ssk and Sku) of the treated steel by surface analysis have been observed.

20 1. Introduction

Ship and marine facilities' surface is easy to be fouled by marine fouling organisms.¹ These fouling organisms caused great harm and huge economic losses to the ordinary transportation and military equipment. Traditional antifouling paints releasing toxic
25 chemicals can prevent adhesion of fouling organisms effectively. However, they also will bring serious pollution to the marine environment. Currently, a few new techniques have been proposed for solving the problems of marine fouling in order to reduce bio-fouling of the ship.²⁻⁷

30 Experimental results show that homarine and its aqueous extract can inhibit the growth of the diatoms effectively, prevent barnacle larvae and marine benthic diatoms attaching to the surface of the ships. Application of anti-adhesion compounds could lead to the development of hull coatings.⁸⁻⁹ The research
35 indicated that the drag reduction via antifouling and the alteration of surface energy of the material surface is closely related. Fouling will become difficult or defaced desorption becomes very easy when the surface energy of the material is low or ultra-low, in turn, it achieves the effect of drag reduction.¹⁰

40 In the recent year, a new concept of bioorganic stainless steel has been proposed¹¹ in which synthetic peptide has made to react with the metallic material surface. It was reported such materials have a lower surface energy and difficult to be attached by fouling organisms. Biological peptides, such as silver binding
45 peptides, palladium bonded peptides, platinum bonded peptides,¹² can react with metals to generate new materials. The iron oxide binding peptide is the first polypeptide of connecting biological

peptide and metal. It is one of the synthetic peptide in the peptide library.¹³⁻¹⁵ Stainless steel is a very common metallic material, and
50 is widely used in various industries. A new bioorganic metallic material was obtained by the reaction between pili of microorganisms and steel.¹⁶⁻¹⁹ Wong *et al* obtained a material with low surface energy using polypeptide reacted with stainless steel.²⁰ The reaction activity of stainless steel with peptides can
55 be increased via adding dopamine.²¹ Later, another scholar studied the factors affecting the binding capacity to stainless steel.¹⁹ Elizabeth proved that peptide-steel reaction led to a formal or semi-formal organic-metal covalent bond formation because stainless steel shared electrons with the dithiocycloheptane of
60 peptide.¹¹ Some peptides with linear chain do not contain disulfide ring, but an indole group of L-tryptophan has a cyclic chain structure, and may share electrons with metal to generate a new material. L-histidine can be used in protein tag. The imidazole group located in residues of histidine as an electron donor can
65 form a coordinate bond by reacting with metal ions, which is immobilized on a matrix material. This group is likely to produce specific chelation with metal ion (Ni^{2+} , Cu^{2+} , Co^{2+} , etc.) which is fixed on the chromatography filler.²²

Previous studies on bioorganic stainless steel focused more
70 on the identification of favourable peptide by phage display technique or the identification of binding domain. However, the functional and surface property changes of these bioorganic stainless steels have not investigated or reported in details. This study fabricated one new bioorganic stainless steel, aiming to
75 reveal the alterations of the surface parameters and functions after reaction with the specifically selected peptide. The multiple

characterization assays in this study confirm that the bioorganic stainless steel has modified surface properties.

2. Materials and methods

2.1 Raw materials and sample preparation

The peptide, denoted as BioP, was synthesized by Shanghai Science Peptide Biological Technology Co., Ltd through solid phase peptide synthesis and purified by reversed-phase high-performance liquid chromatography (HPLC). The peptide have 12 amino acids (NLNPNTASAMHV) with the molecular weight of 1268.4 Figure 1 shows the spatial structure of the BioP.

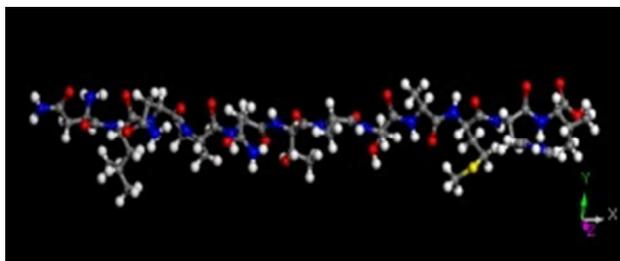


Fig.1 The spatial structure of the peptide

Grade 304 stainless steel discs ($\phi 10 \times 1$ mm, with constituent elements as following: C: $\leq 0.08\%$; Si: $\leq 1.00\%$; Cr: 18.00-20.00%; Mn: $\leq 2.00\%$; Ni: 8.00-11.00%; P: $\leq 0.035\%$; S: $\leq 0.030\%$ and negligible N)²³ were annealed at 1040°C for 1 hour. One surface of the discs were polished using sandpaper of increasing five grit sizes (Eagle Inc, Korean): 120#, 240#, 400#, 600# and 1200# and an aqueous slurry of 0.05 μ m colloidal silica. The polished samples were washed using dish washing detergent and distilled water, and then immersed in 95% (v/v) ethanol for 20 minutes on a shaker with slow shaking rate. Then these samples were washed with distilled water, and immersed in acetone for 5 min, rinsed with distilled water. They were placed into 12-well cell culture plates, and covered by 4 ml phosphate buffered saline (PBS, pH 7.4) containing 10 μ g /ml peptide for peptide reaction. The reaction plates were placed on the shaker, with the rate of 100 r/min and were incubated at room temperature for 1 hour. After the reaction, the samples were washed more than 6 times with distilled water until peptide was not detected in the wash liquor by a spectrophotometer and dried in the drying chamber. The treated samples are denoted as BioS in corresponding to reaction products with BioP.

2.2 Surface morphology analysis

The SEM equipped with E-1045 ion sputtering device (JSM-6300, JEOL, Japan) was used to obtain the images of the sample surfaces. All samples were coated with a very thin layer of gold before analysis to improve the image contrast. SEM data were collected under the accelerating voltage of 15 kV and lateral resolution of 3.0 nm. Five different locations of each sample were scanned and multiple images were collected.

2.3 Surface composition analysis

Infrared spectroscopy (NEXUS, USA) was used to determine chemical composition of the sample surfaces. The spectra ranged from 4000 to 800 cm^{-1} with resolution of 0.9 cm^{-1} were collected using the infrared spectrometer equipped with attenuated total reflection (ATR) accessory. Background spectrum was collected first and samples were pressed against the wafer of the ATR accessories with appropriate pressure ensuring a high signal to noise ratio. The spectra for each sample were collected by scanning 128 times. The spectra were processed by baseline correction, smoothing and normalization via the OMNIC software.

Phenom prox SEM equipped with EDS (Phenom, German) was used to conduct the element analysis of the sample surface. The instrument uses Quad backscattered electron detectors, which can give information on the compositions and morphology of the samples. The image acquisition device contains four image capture functions. The memory sample position function allows the selection of the best location automatically. Images obtained by choosing the "full mode" under 25000 magnification conditions, and distribution of elements at those points were obtained via tipping some points in the samples surfaces. To confirm the presence of specific element, the certainty values were set to greater than 90%.

X-ray Photoelectron Spectroscopy (XPS) (AXIS-ULTRA DLD-600W, Shimadzu-Kratos, Japan) used to examine the electronic state of the elements of the sample surfaces. The base pressure in the analytical chamber was lower than 7×10^{-8} . Monochromatic Al K α source was used at a power of 450W. The analysis spot was 300 \times 700 μ m. The resolution of the instrument was 0.48 eV for Ag 3d 5/2 peak. The scan step was 0.05 eV. XPS spectra were generated by XPS software equipped within the instrument.

2.4 Surface contact angle measurement and surface energy calculation

The static contact angles of the stainless steel sample surfaces under two liquids, water and glycerol²⁴, were measured at 25°C in air using a contact-angle meter (Data Physics Instruments CO., LTD, Filderstadt, German) based on the sessile drop technique. All the contact angles were determined by averaging 5 measurements for each of the sample. Surface energy of samples were calculated via Owens-Wendt-Rabel-Kaelble equation²⁵ (1):

$$(1 + \cos\theta)\gamma_L = 2 \left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+} \right) \quad (1)$$

Where θ is the contact angle formed by the liquid on the solid, γ_L is the surface tension of the liquid, γ_S^{LW} , γ_L^{LW} are Lifshitz-van der Waals component of solid and liquid respectively, γ_S^+ is Lewis acid component of solid, γ_L^+ is Lewis acid component of liquid, γ_S^- is base component of solid, γ_L^- is the base component of liquid.

2.5 Surface roughness analysis

The surface roughness was analyzed by using surface profile measuring instrument (Huazhong University of Science and Technology, Wuhan, China), which produced multiple surface parameters. The lateral and vertical resolutions for the surface

profile measuring instrument were 0.1 and 0.2 μm . The parameter data were collected with a diamond probe, which has resolution of 0.01 μm . The LISA (Laser Interferometer Space Antenna) rounded was debugged until its centre point was at (0, 0), adjusted the tip of the meter to make the tip just touched sample. The sampling length of X and Y direction were 4 mm, and sampling interval of X and Y direction were 0.2 μm . The surface morphology signals of the sample were sent to the signal processing system by the laser interferometric displacement sensor. Then the sample surface parameters were calculated.

2.6 Statistic calculation

In each experimental set, three specimens per group for two sample groups (Nontreated and BioS), were tested. Recorded data was averaged and represented as a mean value \pm SEM. Groups were compared using independent T-tests. $p \leq 0.05$ was considered statistically significant. In graphs, statistical significance was indicated at two levels: * $p \leq 0.05$, ** $p \leq 0.01$.

3. Experimental Results

3.1 Surface morphology and composition of the steel samples

After the surface polishment, all steel samples showed smooth and shining surface. After peptide treatment, there was no macroscopic alteration on surface appearance. Figure 2 shows the nontreated and treated samples.



Fig.2 Optical images of nontreated (a) and BioS (b).

3.1.1 SEM analysis

After the multiple step polishment procedure, the smooth, flat and homogeneous surface of the steel samples has been obtained with low variation across the samples as shown in SEM image (Fig.3 (a)).

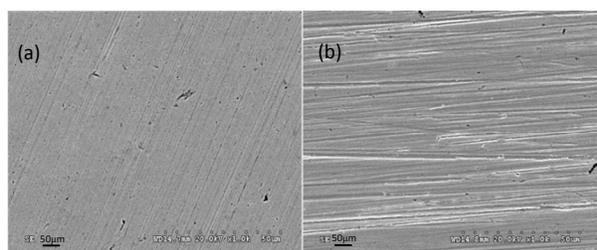


Fig.3 SEM images of nontreated (a) and BioS (b).

However, after the reaction with the BioP, all stainless steel surfaces presented a layer of white substance visible from the SEM images, whilst the original sample surface did not, as shown

in Fig.3(b). The layer was ascribed to the bonded peptide on the 304 stainless steel.

3.1.2 Infrared spectrum analysis

Infrared spectroscopic analysis was performed on stainless steel surfaces of nontreated and BioS (Fig.4).

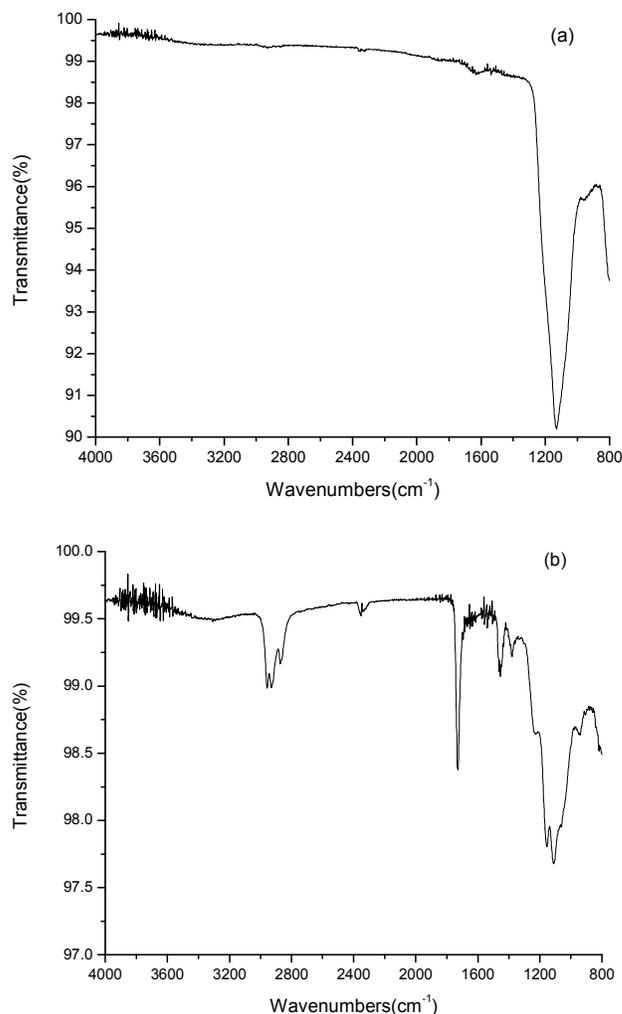


Fig.4 Infrared spectra of nontreated (a) and BioS (b).

The strong peak appeared in the nontreated sample spectrum (Fig.4a) in the 1100-1150 cm^{-1} region was the Si-O-Si vibration.^{26, 27} BioS spectra showed that surfaces of stainless steel treated with BioP exhibited some changes. Compared with the nontreated sample, a broad peak occurred in the infrared spectra of the peptides treated sample surfaces in the 3300-3500 cm^{-1} region, which proved the presence of amide A. There were 2 obvious and small peaks in the vicinity of 1610-1370 cm^{-1} in the BioS spectra. The result indicated that the peptides have aromatic C=C stretching vibration. Since peptides contained peptide bonds, i.e. CO=NR(H), the peaks appearing around 3000 cm^{-1} corresponding to V(C-H) (including saturated or unsaturated); the peak at 1680 cm^{-1} to ν (C=O); the C=O to the carbonyl group; 1450 cm^{-1} , 1380 cm^{-1} to an alkyl group; N-H bending of amide II were found at 1490-1620 cm^{-1} ; 1229-1301 cm^{-1} was C-N

stretching and NH bending, were indicators of presence of amide groups.

3.1.3 EDS analysis

SEM equipped with EDS was used to observe the surface topography of the sample and to detect the elements within the substances on the sample surface. The surface topography and element spectra of samples were shown in Figure 5. The higher magnification images in Figure 5 in comparison to those in Figure 3 clearly demonstrated the presence of new substance on the treated steel surfaces.

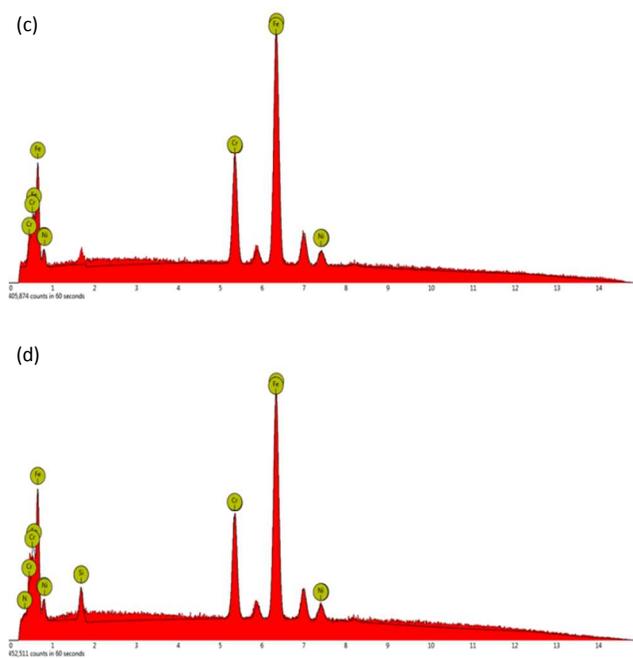
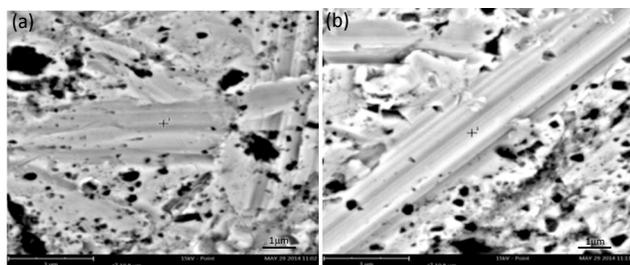


Fig.5 SEM- EDS of samples.

SEM of nontreated (a) and BioS (b), EDS of nontreated (c) and BioS (d). "+" in the SEM images: representative points on the surface covering with white substance used to get EDS value.

By the EDS detector, the surface spectroscopic analysis showed that the nontreated sample mainly contained iron, chromium and nickel, the concentrations of which were 72.4%, 18.2% and 7.7%, respectively; and chromium and nickel content ratio was about 18:8, a ratio in line with the proportion of chromium and nickel content of the 304 stainless steel (the formula is 0Cr18Ni9). Table 1 shows the summary of the

chemical composition of the steel samples.

The surfaces of the samples treated with peptide also contained iron, chromium and nickel. The content ratio was essentially the same as the nontreated stainless steel. However, the surfaces of the treated samples also contained N and Si. Element Si may come from residue chemicals after using the silicon containing sandpaper and silica slurry. Excessive nitrogen were detected only on the surfaces of samples treated with peptide over the nontreated specimen (max 0.08%). The content of N was about 0.9% by weight, which supported that the N element-containing substance was formed on the sample surface after treatment with the polypeptide.

Table 1 The chemical composition of the steel samples from SEM-EDS

Element	Nontreated	BioS
Fe (%)	72.4±0.2	71.8±0.1
Cr (%)	18.2±0.1	17.9±0.3
Ni (%)	7.7±0.2	7.4±0.2
Si (%)	1.6±0.1	2.2±0.3
N (%)	0.02±0.01	0.8±0.1

3.1.4 XPS analysis

To further identify the elements involving in the interaction and possible bonds in the samples, XPS analysis was used to examine the electronic state of the elements of the treated samples in comparison to 304 steel. Spectra analysis of elements of the iron, chromium, nickel, nitrogen, oxygen demonstrated that chromium 2p 1/2 and 2p 3/2 orbitals did not have effect on the bond formation, and no electron shifts were found. No significant changes were observed in the spectra of the oxygen 1s orbital and carbon 1s orbital. Electron shifts were found in the iron (Fig.6 (a)) and nickel (Fig.6 (b)) 2p 1/2 and 2p 3/2 orbitals, which showed that iron and nickel play an important role in the formation of bond. The 304 stainless steel contained negligible amounts of nitrogen, a classical nitrogen orbital was found on the surface of the 304 stainless steel. Lower peaks, near the location of the unbonded nitrogen 1s orbital (Fig.6 (c)) were detected on the treated sample surfaces. This peak did not match any known shifts in nitrogen, whilst nitrogen was confirmed present in the samples, thus, the peak suggests that nitrogen associated with the bonding which has not been previously described. Electrons can flow through five-ring structure of L-Proline, making it feasible that the five-ring structure could interact with surface electrons of steel by chemically binding with them. The similar intensity of nitrogen spectra between the nontreated and BioS samples might be resulted from two facts: XPS tested the emitted photoelectrons from very superficial surface (up to 2 nm depth) and the metal samples were not perfect flat; and the reaction products of peptide in BioS samples were not distributed homogenously. Thus, the tested points of BioS sample might have the nitrogen concentration close to the baseline level (nontreated sample), but the binding energy of BioS was considerably larger than that in nontreated sample, indicating new nitrogen element in BioS sample appeared. It is worth to mention that we rely on XPS to reveal the alteration of local chemical and physical environment on nitrogen, not for quantity measurement of nitrogen. Taking all

together, the difference between the electronic states of iron, nickel and nitrogen on the surface of the treated samples and 304 stainless steel confirm that the treated steel sample was a new material.

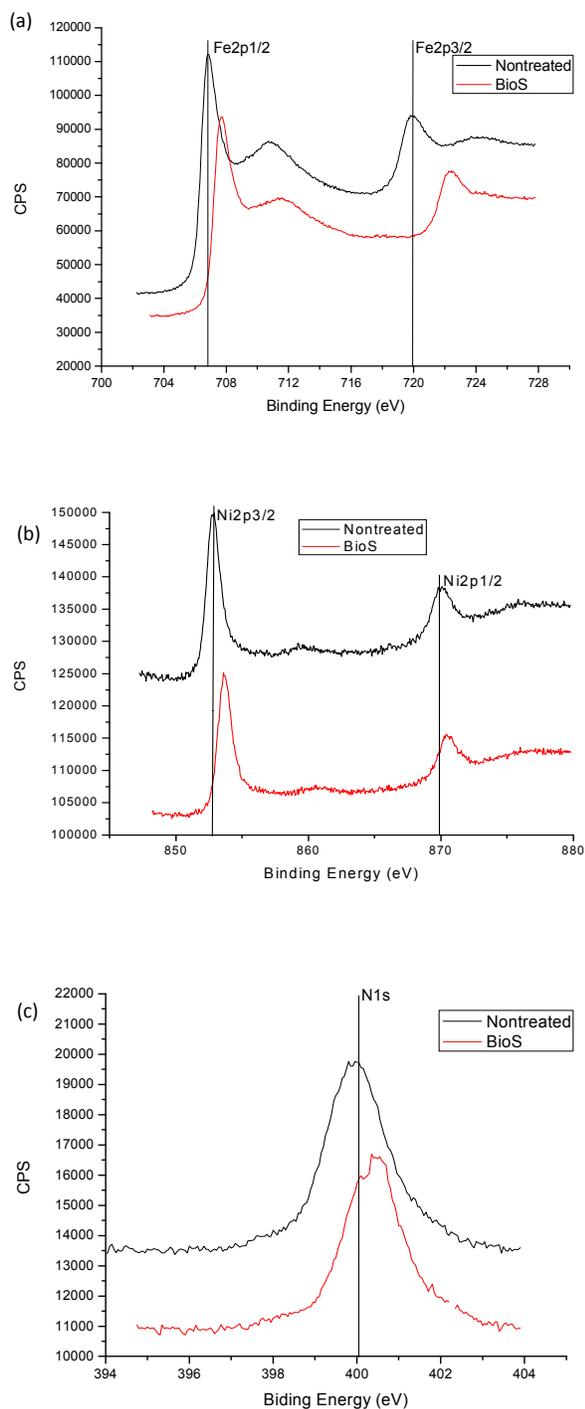


Fig.6 XPS spectra of elemental orbital shifts of nontreated and BioS.

(a)Fe; (b) Ni; (c) N.

3.2 Surface parameters of the steel samples

The chemical composition analysis confirmed the presence of new substances on the surface of the treated samples. However the effects of the substances on the surfaces' physical properties, especially the surface energy and roughness are unknown. The surface contact angle and roughness parameters were measured to detect the changes.

3.2.1 Contact angle

Contact angle which evaluates the hydrophobic performance of sample surfaces, was obtained by a contact angle measuring instrument. The data shown in Figure 6 demonstrated that the water contact angle of the treated sample surfaces significantly increased after peptide treatment.

The water contact angle of nontreated samples surface were around $65.7 \pm 4.7^\circ$, and the contact angles of BioS were $96.4 \pm 2.1^\circ$. The glycerol contact angle of original samples were $53.5 \pm 4.7^\circ$, and the contact angles of BioS were $83.5 \pm 1.2^\circ$. The value of the contact angle increased significantly after being treated with peptides.

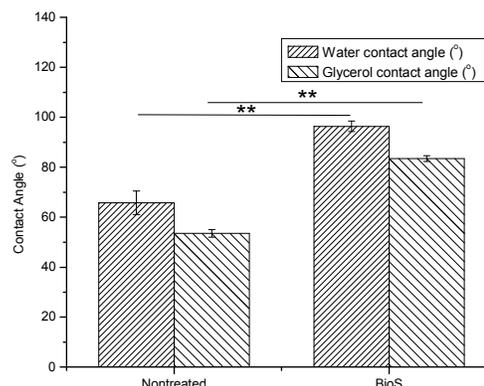


Fig.7 Contact angles of the steel samples' surface.***P<0.001.

Surface energy of samples were calculated via Owens-Wendt-Rabel-Kaelble equation (1) and the parameters shows in Table 2. The surface energy value of nontreated, BioS were 41.3, 25.0 mN/m respectively which demonstrated surface energy of 304 stainless steel decreased by reacting with peptides.

Table 2 Surface free energy constants for test liquids (in mJ / m²)

Liquid	γ_L	γ_L^{LW}	γ_L^+	γ_L^-
Water	71.8	21.8	25.5	25.5
Glycerol	64.0	34.0	3.92	57.4

3.2.2 Surface roughness

Surface roughness related parameters including surface roughness (Sa), surface root mean square deviation (Sq), skewness in the roughness profile (Ssk) and surface height distribution kurtosis (Sku) were measured, as summarized in Table 3. The detailed calculations of the parameters were derived from the equation (2)-(5).^{28,29}The data showed that the surface roughness value (Sa) of the samples increased after peptides treated in the BioS.

Surface root mean square deviation (Sq) was used to express the root mean square of surface roughness deviated from the reference plane. It was shown that the value of Sq increased after BioP treated. Skewness in the roughness profile (Ssk) is a measure of the symmetry of the amplitude distribution curve about the mean line. Skewness indicates whether the surface consists of mainly peaks or valleys or an equal combination of both, a negative Ssk value indicates a greater distribution of valleys about the mean line as the amplitude distribution curve is skewed above the mean line. It was found that the Ssk of the samples decreased after BioP treated. Surface height distribution kurtosis (Sku) is to describe amplitude distribution curve for the points of surface profile. Topography height of surface will be in the centre of the base plane when the value of Sku is greater than 3.0. It is better that the value of Sku is close to 3. The value of the samples was dropped after peptides treated.

$$Sa = \frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} |z(x_k - y_l) - u| \quad (2)$$

$$Sq = \sqrt{\frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} (z(x_k - y_l) - u)^2} \quad (3)$$

$$Ssk = \frac{1}{MNSq^3} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} (z(x_k - y_l) - u)^3 \quad (4)$$

$$Sku = \frac{1}{MNSq^4} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} (z(x_k - y_l) - u)^4 \quad (5)$$

Where u is the average height of the calculated region, M is the collected points in the x direction of the reference plane, N is the collected points in the y direction of the reference plane, x_k is the concavo-convex level in the x direction of the base surface, y_l is the concavo-convex level in the y direction of the base surface.

Table 3 Surface parameters of the steel samples

Number	Sa(μm)	Sq(μm)	Ssk	Sku
Nontreated	0.945±0.053	1.496±0.007	4.077±0.236	37.9±2.5
BioS	1.187±0.004	1.679±0.056	0.720±0.016	14.8±1.8

4. Discussion

A few peptides have been reported able to react with stainless steel.^{11,14,16}In this study, we selected BioP to react with steel samples and conducted multiple characterisation investigation of the treated samples. Although the peptide has been studied by Zuo and Vreuls,^{17,30}there was no systematic study on the treated steel samples. Reaction with the peptide at the mild conditions resulted in alteration of the metal surfaces' chemical and physical properties. A new substance was generated on the surface of the samples, which has been detected by multiple chemical and physical analysis assays. The white particulate substance was visible on SEM images, where the nitrogen element associating with the new white substance and the changed FTIR spectra and XPS spectra of the treated steel surface chemically confirmed the presence and composition of the new substance. The physical

property changes including the water contact angle and surface roughness parameters further supported the observations. In our study, the reacted steel samples have been washed vigorously before the characterisation. Thus, the physically trapping of peptide on the steel sample surface was low. All the results provide strong evidence that bioorganic metallic material has been formed on the treated steel samples.

SEM is a convenient and powerful technique to study surface topographic change, which has been used to study stainless steel surface modified by peptide.^{11,21} In combination with EDS analysis, we did not only observe the new substance on the treated steel surfaces, but also detected their composition using nitrogen as the marker for peptide grafting degree. All peptides have unique peptide bonds, i.e. CO=NR (H). The reaction extent of stainless steel and peptides can be reflected by N content.^{11,21}EDS is an accurate technique to determine the composition of the surface substance of the sample. In the treated steel sample surfaces, the Fe, Cr and Ni component proportion have been detected. Their concentrations were same as the component ratio of the original stainless steel. Element nitrogen on the surface proved that the stainless steel surface treated with the peptide comprised a novel nitrogen-containing substance, and the content is relatively stable (Table 1). The new steel sample generated by reacting between peptide and stainless steel can be classed as bioorganic stainless steel.

ATR-FTIR has been used to study the surfaces treated with peptide.^{31,32}Peptide bond, carbon-hydrogen bonds, carbon-oxygen bond, etc. were found on the surface of the treated samples in our study (Fig.4). There were 2 obvious and some small valley in the vicinity of 1610-1370 cm⁻¹ in the BioS spectra. The result indicated that the peptide has aromatic C=C stretching vibration. N-H bending of amide II were found at 1490-1620 cm⁻¹; 1229-1301 cm⁻¹ is C-N stretching and NH bending.³³These spectra suggested that the occurrence of a reaction between the steel and peptide generated a substance containing organic ingredients which may be obtained by joining peptide and free electrons of stainless steel. The treated steel material exhibited new spectral peaks which did not belong to original steel material. Hence, peptide has been physically and chemically bonded to the steel surfaces through the active groups and elements on the steel. The FTIR and XPS spectra of the treated sample supported the EDS results. However, the specific reaction mechanisms is complex which will be studied in subsequent experiments.

The contact angle is determined by the surface morphology and chemical substances.³⁴The generation of a new substance on the sample surface, is the main cause that led to the changes in the surface parameters. According to Young's equation,³⁵the surface energy of the samples reduced when the contact angles were increased which is consistent with the calculation results. Thus, contact angles changes of samples can be used to analyse the changes of surface energy of samples qualitatively. As can be seen from the Fig.7, the value of the contact angle increased significantly (p<0.001) in comparison to the nontreated sample. Thus, the hydrophobic properties of the steel surfaces increased. However, the improvement amount of contact angle or surface energy was not big, further investigations to generate new generation of antifouling materials is required. The increased in the sample contact angles could be partially due to the surface

topographic change after peptide bonding. The changes of contact angle of steel sample indicated that the reaction with BioP can make considerable influence on steel surface.

Surface morphology has a greater effect on contact angle compared to chemical composition.³⁴In this study, it was found that the sample surface became smoother after treating with peptide (Table 3). The changes of surface roughness parameters were mainly caused by the new substance generated on the surface of the sample. The steel sample contained numerous grain boundaries, and BioS has been shown to bind with higher adhesive force to grain boundaries compared with regions within grains.³⁶Majority of the reactions probably happened in the trough of the surface, and the complex structure of new substance on the surface led to the smoother surface after BioS bonded with stainless steel.

5. Conclusions

In summary, we successfully produced a new material via reacting between peptides and the 304 stainless steel, which also known as bioorganic stainless steel. The material has considerable different physical and chemical properties compared to the original sample. Bioorganic stainless steel has a higher contact angle than unmodified stainless steel, and the surface became smoother. The new bioorganic material has potential to further optimize into a new type of material for the construction of the hull, submarine pipelines, etc. to reduce drag caused by sea water because of its new surface properties.

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Notes and references

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