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Antifouling performances of macro- to micro- to nano-copper materials for the inhibition of biofouling in its early stages

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Copper has been known to possess antimicrobial properties since as far back as the Phoenician era where ship hulls were copper sheathed to prevent the inevitable effects of biofouling. As a consequence of evolving scientific research and development, the realisation of novel materials and agents has enabled new scientific branches – such as nanotechnology. In this paper we investigate the performance of different forms of copper (macro, micro and nano) for application as antifouling materials. Samples are deployed in SmartBay Ireland for four weeks and analysed for evidence of biofouling. It was found that copper in its nano form, produced the greatest antifouling effectiveness in both PDMS and sol–gel matrices.

Introduction

Almost all underwater equipment and structures are prone to the overwhelming effects of a process known as biofouling. Biofouling crudely consists of a two-stage process (i) microfouling: whereby microorganisms, diatoms, among other algae, spores and cyprids attach to a surface leading to (ii) macrofouling; where larger grazing organisms such as barnacles, muscles, macroalgae and hydroids further attach to the surface. This process largely contributes to a range of different problems: loss of manoeuvrability, loss of speed and efficiency, increased fuel consumption and thus carbon footprint, compromise in data readings, reduction in efficiency where overall cost of ownership is driven up – all obstacles for the end user in maintenance, efficiency and costs. Bulk copper has been used for centuries for the protection of underwater hulls of numerous marine craft. Owing to this natural biocide property, copper is routinely applied to boat's surfaces – usually in the form of copper and copper based compounds, whereby it is generally agreed that cupric copper (Cu^{2+}) is most responsible for the toxicity of copper.[1]

Nanocomposites of inorganic materials in polymer matrices have attracted a great deal of attention owing to their wide application as biosensors, optical devices, and catalytic and antimicrobial platforms.[2] For such a synthesis to be realized, different nanocomposite approaches have been developed such as the incorporation of pre-synthesised nanoparticles into a polymer matrix[3] with the use of common reagents such as sodium borohydride, polyethylene glycol and various protecting agents. Nanoparticles can also be embedded within a polymer matrix using physical and chemical vapour deposition, and indeed, sol–gel synthesis.[3] Using these approaches can often be laborious and cumbersome relying on specialist chemicals and preparations.

Polydimethylsiloxane (PDMS) is an elastomer that has been routinely owing to its many useful properties such as high flexibility, ease of moulding, low cost, non-toxic nature and its chemical inertness – all desirable when deploying under marine environment conditions.[4] Metal nanoparticle containing thin films and polymer matrices have both shown enhanced mechanical properties, multifunctionality and separations as well as more recently, antimicrobial and antifouling properties – particularly with nanoparticle doped matrices.[3] All of these methods often require long multi synthetic steps. More recently, methods have focussed on producing one-step nanoparticle syntheses, in particular Chen *et al.*, have synthesised gold nanoparticle–PDMS composite polymers through direct immersion into a gold chloride solution. These films were then further functionalised with enzymes – highlighting yet another application for these promising routes of material generation.[5]

The research presented in this article compares the nano, micro and macro form of copper used as an antifouling material. We report a simple, reproducible synthesis for copper nanoparticles and directly dope them (pre-synthesised nanoparticles and pre-purchased microparticles) into 2 very different polymeric matrices for comparison; PDMS and sol–gel against the bulk macro form. The materials are currently used as optically clear, marine rugged materials for sensing applications and thus are relevant to for anti-biofouling applications. The

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materials were deployed in a long term, high-fouling marine study (Summer 2012) and were also benchmarked against the best performing commercial antifouling paints.

Materials and methods

Reagents

Two commercial antifouling paints (Cruiser Uno and Waterways – International Paints), copper microparticles (2 μm) were purchased from Kehoe Marine Ltd, Ireland. PDMS (Sylgard 182) was purchased from Dow Corning, Ireland. Sodium borohydride ($NaBH_4$), copper(II) sulfate ($CuSO_4$: 98%), ethane-1,2-diol ($C_2H_6O_2$: 99%), polyvinylpyrrolidone (PVP: $M_w \sim 40\,000$), trimethoxymethylsilane (TMPMS), isobutyltrimethoxysilane (IBTMOS), sodium hydroxide (NaOH) and Folin–Ciocateu phenol, were all purchased from Sigma Aldrich, Ireland and used without any further purification steps.

Nanoparticle synthesis

Nanoparticles were each synthesised according to a polyol reduction method previously published in Chapman *et al.*, 2011. In short, a 4 mM solution of $NaBH_4$ was made up in ethane-1,2-diol (pH 7.0) as a stock reducing agent at ambient temperature. A 5 mM solution of metal salt was prepared with ethane-1,2-diol prior to any reflux synthesis. A protecting agent of PVP was made up to 0.1% concentration. All reactants were introduced in the following order and refluxed at 160 °C for a 24 h period; metal salt (15 mL), $NaBH_4$ (2.5 mL), PVP (2.5 mL). The nanoparticles were then centrifuged for 10 min at 12 000 rpm at ambient temperatures. The supernatant was poured off and methanol was added (10 mL) to wash the nanoparticles. This step was repeated three times where the pellet was re-dispersed in the PDMS and sol–gel polymers accordingly.

Sol–gel synthesis

A stock sol–gel solution was prepared by adding 36 mL ethanol, 12 mL of trimethoxymethylsilane (TMOMS), 12 mL isobutyltrimethoxysilane (IBTMOS) under constant stirring (10 min) in a Schott glass bottle that had been previously treated with methanol and acetone (1 : 1). Following this, 36 mL of Milli-Q water was added and left to stir vigorously for 5 min, 2 mL of 1 M solution of hydrochloric acid in Milli-Q water was added drop wise for 3 min and left to stir for 2 h until the solution was of a viscous consistency. The washed nanoparticle pellet was weighed (0.001 g) and then re-suspended in the sol–gel, which was then mixed thoroughly for homogeneity. Following this, separately, copper microparticles were then weighed (0.01 g) and also introduced into the sol–gel matrix.

Material preparation

Experiments were performed using a Sylgard 182 elastomer owing to its versatile mechanical, chemical and dielectric properties. It also has a number of other common uses, including biomedical matrix selection, aerospace sealant and as a soft lithography material. Sylgard 182 is a platinum catalyzed vinyl addition silicone polymer purchased from Dow Corning, Ireland. The product consists of a 10 : 1 mixture of Sylgard 182 base resin and curing agent consisting of polydimethylhydrosiloxane (PMHS) and polydimethylsiloxane (PDMS). The base resin comprises of ~65% vinyl end capped PDMS and ~35% soluble silica filler (50% SiO_2, 45% trimethyl-end-capped PDMS and 5% vinyl-end-capped PDMS) plus ~6 ppm chloroplatinic acid adduct as the polymerizing catalyst was weighed (0.001 g) and then re-suspended in the PDMS, which was then mixed thoroughly for homogeneity. Following this, copper nano and microparticles (100 mg) were then doped in the same manner for the sol–gel and PDMS matrix. All of the materials tested and preparation are demonstrated in Table 1.

Material deployment

A long-term material deployment study was used to investigate the overall effectiveness of each of the materials. A test site in Galway Bay, Ireland was used (SmartBay Buoy: GPS: 53.253353, −9.044422), where the internal structure of a marine buoy was retrofitted to hold deployment frames containing the test materials, shown in Fig. 1.

Material characterisation

Wettability & surface roughness. A series of contact angle measurements were taken using a sessile drop method (Artray and Navitar camera FTA32 video 2.0 datalog software). Measurements were taken through the analysis of sitting drops upon each of the materials through automatic drops of HPLC grade water ($n = 9$) with a range of 10–2000 mN m^{-1}, resolution 0.2%, accuracy ±1%. The drop was released from a 25-gauge 10 mL hypodermic syringe needle where all contact angles are quoted in degrees (°). Each sample was then photographed for each polymer treatment at ambient room temperature.

Atomic Force Microscopy (AFM) was performed using a commercial AFM (Dimension 3100 AFM using a Nanoscope IIIa controller, equipped with a phase imaging extender Digital Instruments); this was operated in tapping-mode atomic force microscope (TM-AFM), using standard silicon tips (Tap300,

Table 1 Illustrating different method preparation for sol–gel and PDMS matrices for marine deployment test

Sol–gel preparation		PDMS preparation	
Cu-NPs	100 mg polyol synthesised nanoparticles doped in 50 mL sol–gel	Cu-NPs	100 mg polyol synthesised nanoparticles doped in 50 mL sol–gel
Cu-μPs	100 mg of pre-purchased doped in 50 mL sol–gel	Cu-μPs	100 mg of pre-purchased doped in 50 mL PDMS

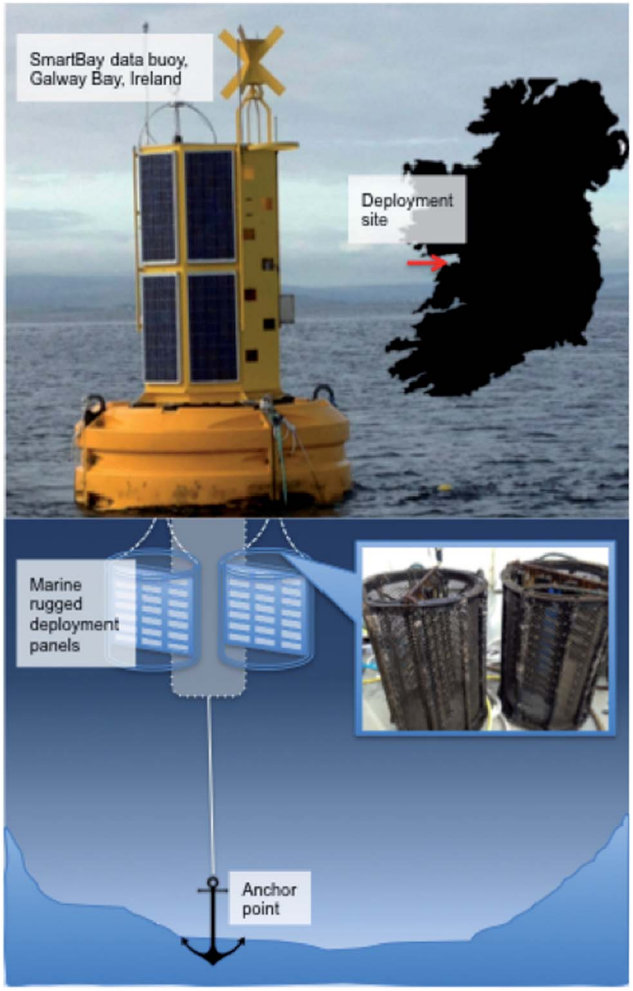

Fig. 1 Schematic showing SmartBay Ireland, Galway bay buoy deployment site, deployment rig and test panels.

Budget Sensors, Romania) with 42 N m^{-1} nominal spring constant and 330 kHz nominal resonance frequency. All images were recorded in air at room temperature at a scan speed of 1–2 Hz. The background slope was resolved using first or second order polynomial functions. No further filtering was performed.

Mass assessment. The materials were weighed both before and after the 4 week study, thus measuring the changes in mass through the duration of the study (% mass change). Samples, following exposure, were then rinsed with Milli-Q water to ensure non-adhered material was removed *i.e.* particulate matter, and allowed to dry until the weight remained constant. A subtraction of weight of the glass substrate plus the sample before and after was carried out where a subtraction or addition of weight was measured, giving an overall mass percentage change.

Glycocalyx (slime test). Glycocalyx production was evaluated using a series of fixation and staining techniques. Slime production was measured on the test material that was attached to the surface only. The slides were washed twice with 1 mL of sterile Milli-Q water prior to staining. The slime was initially reacted with Carnoy's solution (absolute ethanol, chloroform and glacial acetic acid in a 6 : 3 : 1 ratio) for a 30 min period. The solution was then decanted and a 0.1% toluidine blue was added to stain the biofilm present on the polymers for 1 h. The excess stain was decanted off and the polymers were rinsed twice with Milli-Q water (3 mL). NaOH (0.2 M) was added and heated to 85 °C for 1 h. Each sample was allowed to cool to room temperature and optical density was measured at 590 nm on a UV-vis spectrometer.[6]

Surface characterisation (microscopic). Surface analysis of adhered biomaterial, cells and matter of each of the samples was carried out using a Hitachi S3400 scanning electron microscope (SEM). Accelerating voltages of 5–7 keV with secondary (SE) electron detection was used. Working distance of 5 mm was used. Samples were prepared ($n = 3$), by cutting samples and directly attaching them to commercially available carbon tabs (Agar, UK).

Surface characterisation (macroscopic). Visual characterisation was carried out using a Cannon S340 digital camera. Images were taken both before and after the study, 7 days. Camera height was placed approximately 10 cm from the thin film surface and macro setting was used to achieve high resolution.

Protein adsorption assay. Protein determination was performed using a modified Lowry assay. Using commercial reagents as specified in Reagents: 40 mL of modified Lowry reagent powder was made up in Milli-Q water in a separate manner Folin–Ciocalteu's phenol solution was diluted in Milli-Q water (1 : 5) and remained stored until required at room temperature (RT). The glass-coated samples were rinsed with Milli-Q water prior to analysis and then added to a 50 mL sample tube. The tubes were then filled with ultra-pure water and sonicated for 5 min (which was found to give a maximum protein extraction from the slide). Following this, centrifugation was applied at 3000 rpm for 5 min at RT (again this was optimised for a maximum extraction of protein) to remove particulates that might have been suspended in the solution following sonication. An aliquot of the sample (0.5 mL) was taken and added to a 7 mL centrifuge sample tube, where 0.50 mL of Lowry reagent solution was added; this was mixed and incubated for 20 min at room temperature. Following this 0.25 mL of Folin–Ciocalteu's phenol solution was added to the sample tube and was shaken with incubation for 30 min at room temperature. The absorbance was measured using UV-vis at 750 nm ($n = 9$).[7]

Carbohydrate adsorption assay. Determination of total carbohydrate content was carried out according to a modified method first prepared by Dubois *et al.*[8] A 5% w/v phenol solution was made up in Milli-Q water and sulfuric acid 95–97% was prepared and stored until required. The glass-coated samples were rinsed with Milli-Q water prior to analysis and then added to a 50 mL sample tube. The tubes were then filled with ultra-pure water and sonicated for 5 min. Following this, centrifugation was applied at 3000 rpm for 5 min at RT to remove particulates that might have been suspended in the solution following sonication. An aliquot of the sample (0.5 mL) was taken and added to a 7 mL centrifugation sample tube where 0.5 mL phenol solution and 2.5 mL sulfuric acid was added and mixed immediately. The sample tube was then incubated for 10

min at room temperature and following this 15 min at 30 °C. After 5 min at room temperature the absorbance was measured at 480 nm for the determination of acidic carbohydrates and or at 490 nm for the determination of neutral carbohydrates ($n = 9$).

Results

Nanoparticles in materials

Copper nanoparticles were successfully synthesised using a previously developed polyol reduction method published in Chapman *et al.*, 2010. The nanoparticles were characterised using UV-visible spectroscopy, where characteristic absorption bands were observed, Fig. 2.

Synthesised nanoparticles were studied initially using ultraviolet-visible (UV-vis) absorption spectroscopy using a double beam spectrophotometer (Cary 50) in the wavelength from 200–100 nm. Fig. 2(a) shows the UV-vis spectra of the copper colloid. The absorption bands for Cu nanoparticles have been reported to be in the range of 300–600 nm depending largely on size and morphology.[9] In this case, a local maximum of the optical absorbance occurs at 357 nm, which is directly attributed to the surface plasmon resonance of the copper nanoparticles. In using PVP in the nanoparticle reaction, the surfactant has the ability to form an absorption layer at the surface of the copper nanoparticle to prevent aggregation. This is achieved through increasing the repulsion force between the particles. Fig. 2(b) shows a field emission micrograph for colloidal copper nanoparticles synthesised, where a size distribution pie chart (Fig. 2(c)) illustrates average size per frequency range. It was found that the particles are in the size range of 50–100 nm with irregular spherical morphology. In synthesising such nanoparticles, copper presents a problem due to ease of oxidation. Even a very thin oxide layer can significantly alter the physical and chemical properties. In order to prevent this, PVP is deliberately used as a joint aggregation and anti-oxidation protective polymer. Addition of antioxidants, such as ascorbic acid, is also another way of protecting by markedly decreasing the overall rate of oxidation for copper – however, its stability as an agent for aggregation is debateable.

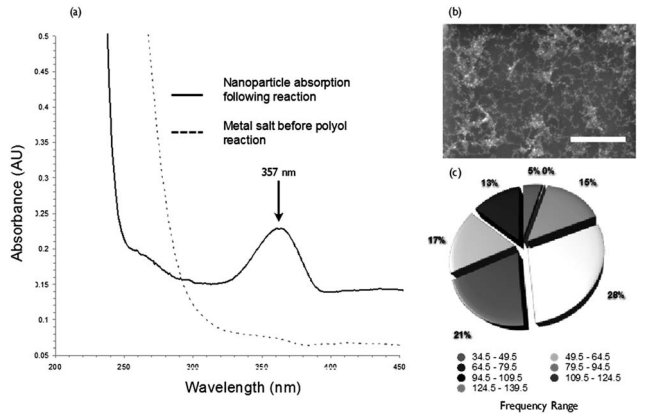

Fig. 2 Nanoparticle characterisation using (A) UV-visible spectroscopy, (B) FESEM and (C) particle size distribution chart (scale bar = 500 nm).

Following the synthesis and characterisation of the nanoparticles, it is necessary to "clean-up" the sample. This can be achieved by repeatedly, centrifuging and washing with ethanol or similar solvent. The recovered pellet can then be directly doped into the specific matrix. In this case, PDMS and a synthesised sol–gel were used as specific doping matrices. Pellets of copper nanoparticles were introduced into the sol–gel and PDMS, respectively. The same was then performed for copper microparticles for both matrices. Wettability of both materials was investigated by measurement of static contact angles using ultra pure water, Fig. 3.

Structural and topographic analysis revealed surface morphology for each of the polymer matrices used in this study: PDMS and sol–gel. PDMS revealed smooth wavy channels, exhibiting low wettability values. Both commercial antifouling paints have low wettability (WW – 14.54 ± 2.1°, CU – 13.91 ± 4.3°), where the sol–gel doped materials are similar to these. PDMS exhibits slightly higher wettability, demonstrating an average *ca.* 20° between the commercial antifouling paints and sol–gels, respectively. Similarly, Hemmilä *et al.* have

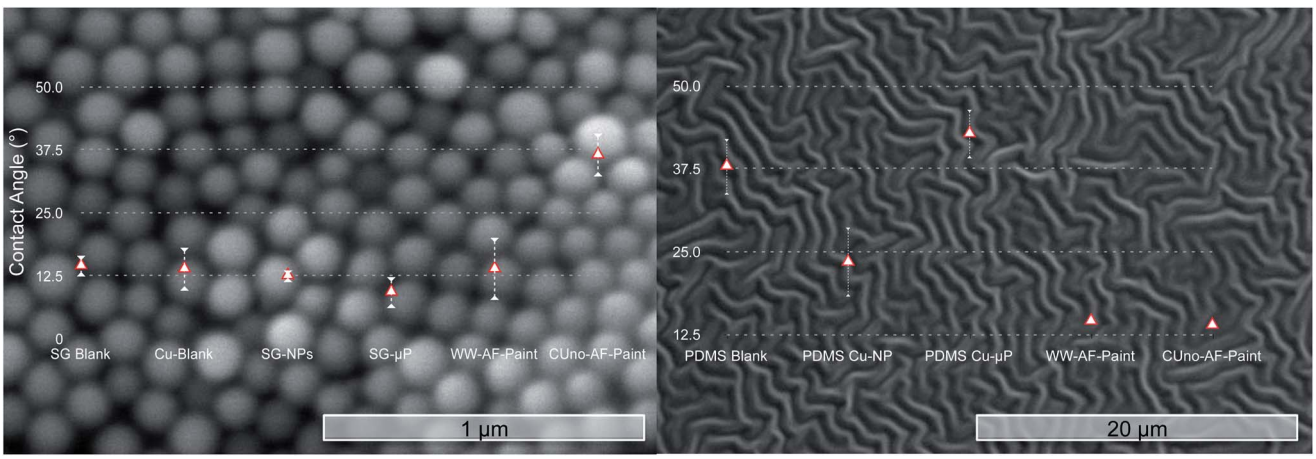

Fig. 3 Scanning electron micrograph for (left) sol–gel morphology and (right) PDMS with relative contact angles overlaid for each of the materials. Surface roughness for sol–gel (rms = 182 nm) and PDMS (rms = 258 nm).

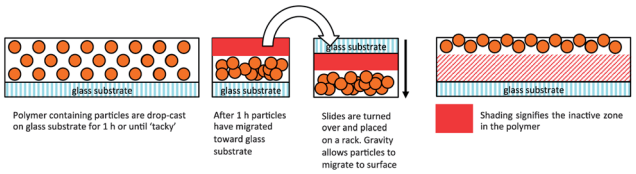

Fig. 4 Drying procedure for the copper particles in each polymer. Shaded zone indicates inactive polymer zone.

characterised PDMS using both SEM and contact angle analysis. The authors have also achieved wettability values in the range of *ca.* 30°,[10] where similar wavy surface topographies were shown. When copper nanoparticles were introduced into both PDMS and the sol–gel matrices, a notable shift in wettability was observed. In PDMS, the copper nanoparticles were found to increase the wettability (23.45 ± 5.2°), where the copper microparticles have decreased the wettability (42.81 ± 3.7°). This slight increase in contact angle compared to the blank can be attributed to the drying procedure of this sample. The samples were dried until a tacky consistency was observed; at this point samples were turned upside down for the final drying step. This was to ensure all particles migrated as close to the surface of the polymers rendering them surface active. In the case of PDMS and the sol–gel, this caused the microparticles to move to the surface of the sample as demonstrated in Fig. 4. A previous experiment (not shown) demonstrated that the particles had dried under the surface of the material and thus demonstrated similar properties to the blank matrix they were doped within. Therefore, it was necessary to overcome this with the proposed drying method.

This was an important step in order to render the surface active at the water–polymer interface. In doing so, a material where the active ingredients are at the surface and not suspended deep in the polymer matrix is created.

Biofouling assessment

A series of assays were developed and used to assess the antifouling performance of the materials and the commercial antifouling paints following their immersion in the marine environment. These tests quantify certain biochemical signatures and components of biofilm and the overall biofouling process characterised by the adhered material to the test materials.

Slime and mass assessment

A measurement of slime and mass can be applied as a simple quantitative tool for determining biofilm adhesion to any substrate. A slime assay is routinely used as a qualitative and quantitative assay in the detection of an important biochemical attributed to biofilm forming organisms known as glycocalyx.[11] A slime detection assay was applied and shown in Fig. 5, where it was found that the macro copper samples have the most slime attachment *vs.* mass. Copper nanoparticles, when doped into a sol–gel, have shown least slime levels (4.56 ± 0.13 AU) when plotted with mass (0.11 ± 0.02%). Similarly, the copper nanoparticles have also been the best performing antifouling dopants for PDMS, where least slime and mass for this polymer group were also observed. The commercial antifouling paint (WW-AF-Paint 4.71 ± 0.43) has also demonstrated a good anti-slime sorption result from this assay. For the sol–gel, copper materials the following levels of effectiveness for slime sorption have been observed: SG-NPs > SG-μPs > Cu-blank. Where the best result has been observed for copper nanoparticles, followed by copper microparticles, followed by the macro (copper blank).

Numerous studies have already shown that most copper present in the environment is not present as the cupric ion.[12] Nanoparticles have been showing researchers new ways to deliver antimicrobial effects; in particular silver is already well established. Although, only a few studies have been reported for the effects of copper nanoparticles, they have shown that copper nanoparticles have a promise as bactericidal agents. In a study be Aruoja *et al.*, copper nanoparticles were compared to the bulk metal for toxicity on a marine test organism, *Pseudokirchneriella subcapitata* where they demonstrated that the bulk copper samples were not as toxic to the organism when compared to the nanoparticle form. This is also true of the study here, where we have demonstrated that the nanoparticle doped samples are delivering improved antifouling response in each of the assays reported herein.[13] Separately, Yoon *et al.* have already reported the antibacterial effects of silver and copper nanoparticles using

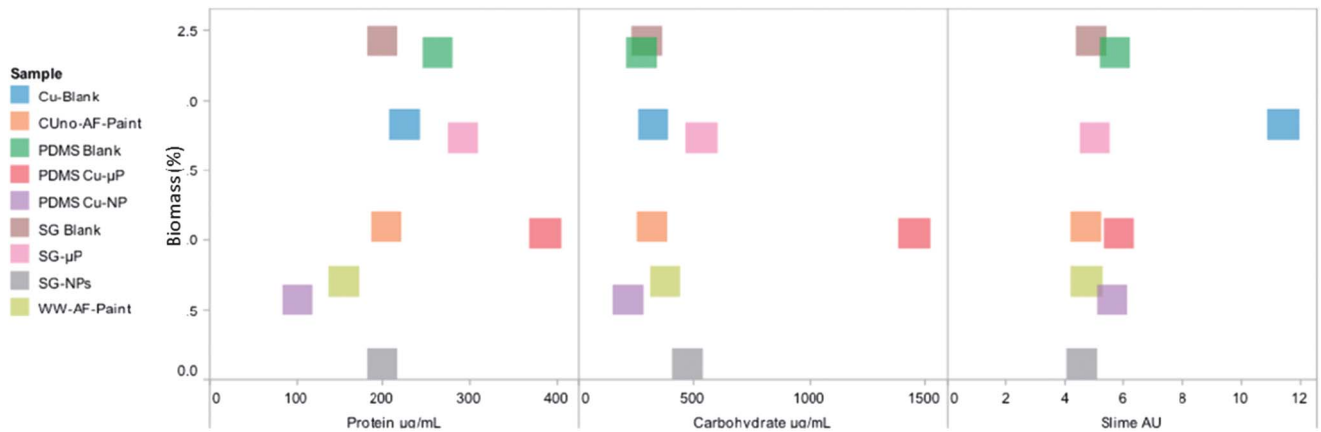

Fig. 5 *XY* box plot showing protein, carbohydrate and slime adsorption *vs.* percentage mass increase for each of the antifouling materials ($n = 9 \pm 1$ SD).

strains of *Escherichia coli* and *Bacillus subtilis*, where copper had shown superior antibacterial activity compared to silver nanoparticles.

Carbohydrate adsorption

Carbohydrate adsorption is an important measurement in determining the biochemical composition of the biofouling material on the surface of each of the test materials. Carbohydrates are known to be a contributing biochemical in EPS[14] and thus a smaller biochemical response indicates less biofouling. In using this assay, quantitative measurements can be realised, and thus the level of biofouling proliferation can be determined. The copper nanoparticle doped sol–gel material has again proved to be effective in resisting biofouling adsorption.[15,16] Comparing the carbohydrate adsorption to mass for each material, the nanoparticle samples have routinely demonstrated least carbohydrate adsorption and mass value. The commercial WW-AF-Paint (154.4 ± 32.13 μg mL^{-1}) was the best performing commercial paint in this study. The nanoparticle samples have again demonstrated excellent resistance to EPS attachment, and biofouling in this assay. Generally, the results from best to worst performing for carbohydrate adsorption were as follows: PDMS Cu-NP > WW-AF-Paint > SG Cu-NP > CU-AF-Paint > PDMS Cu-μP > SG Cu-μP > Cu blank > PDMS blank, SG blank.

Protein adsorption

Protein adsorption was similarly quantified on each of the test materials, Fig. 5. Consistently, the nanoparticle sample has been best performing throughout. Protein adsorption has also material demonstrated that nano-copper in the sol–gel matrix was the best performing material, where 201.23 ± 21.13 μg mL^{-1} and percentage mass of 0.12% in the SG-NP sample was achieved.

Numerous studies have been performed to elucidate the mechanisms of antibacterial or antifouling action of nanoparticles. It is difficult to distinguish between the effect of particle and the ions that are released by the nanoparticles.[17] Although this study was not designed to distinguish between the effect of ion and particle, it is quite clear that nanoparticles have demonstrated more potent antifouling effects when compared to the micro and macro forms of copper. Cioffi *et al.*, have demonstrated that significant antimicrobial activity was due to ions present in addition to a nanoparticle[18] whereby demonstrating a dual effect: where nanoparticle and ion operate together in a given matrix. This could also be a reason for the nanoparticle doped samples showing advanced antifouling effects throughout this study. The exact mode of action of nanoparticle is also debated through literature. However, the most dominant argument comes from the fact that nanoparticles are able to enter a cell wall and cause DNA damage or cell wall rupture[19] again this is consistent with our own results where nanoparticle samples have all performed better than the micro and macro copper samples. This further supports that nanoparticle and ionic properties of the sample outperform the ionic argument alone.

Conclusions

Copper has been shown to exhibit different levels of antifouling potency when doped into materials in its 3 forms; nano, micro and macro. Consistently the nano form of copper has demonstrated best antifouling responses in each of the assays for overall biofouling characterisation. A benchmark using the best performing commercial antifouling paints also show the merit of the materials.

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