2013

An Investigation of the Biochemical Properties Of Tetrazines as Potential Coating Additives

Swarna Jaiswal

Technological University Dublin, swarna.jaiswal@tudublin.ie

Brendan Duffy

Technological University Dublin, brendan.duffy@tudublin.ie

Luke O'Neill

Technological University Dublin, Luke.oneill@tudublin.ie

See next page for additional authors

Follow this and additional works at: <https://arrow.tudublin.ie/cenresart>

Part of the [Biomaterials Commons](#), and the [Microbiology Commons](#)

Recommended Citation

Jaiswal S. et al.' (2013) An investigation of the biochemical properties of tetrazines as potential coating additives'. *Materials Science and Engineering: C*, Vol. 33(4): pp. 1925-34. doi: 10.1016/j.msec.2012.12.094.

This Article is brought to you for free and open access by the Crest: Centre for Research in Engineering Surface Technology at ARROW@TU Dublin. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@TU Dublin. For more information, please contact arrow.admin@tudublin.ie, aisling.coyne@tudublin.ie, gerard.connolly@tudublin.ie.
Funder: Technological University Dublin

Authors

Swarna Jaiswal, Brendan Duffy, Luke O'Neill, and Patrick McHale

An investigation of the biochemical properties of tetrazines as potential coating additives

Swarna Jaiswal^{1,2}, P.C. Rajath Varma², Luke O'Neill³, Brendan Duffy^{2*} and Patrick McHale¹

¹School of Biological Sciences, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland

²Centre for Research in Engineering Surface Technology (CREST), FOCAS Institute, Dublin Institute of Technology, Dublin 8, Ireland

³FOCAS Institute, Dublin Institute of Technology, Dublin 8, Ireland

*Corresponding author: Dr. Brendan Duffy; Tel: +353-1-402-7964;

E-mail: brendan.duffy@dit.ie

Abstract

1,2,4,5-Tetrazine and its 3,6-disubstituted derivatives are currently used for a range of industrial and medical applications as they exhibit particular coordination chemistries, characterized by electron and charge transfer phenomena. The aim of the present work is to synthesise two tetrazine derivatives, namely 3,6-dihydrazino-1,2,4,5-tetrazine (DHDTZ) and 1,2,4,5-tetrazine dicarboxylic acid (DCTZ), and determine their antibacterial, antioxidant and anticorrosion characteristics as additives in a sol-gel coating on SS316L steel. The structure of the tetrazines was confirmed by NMR and FTIR while the surface morphology of bacterial cells in their presence was observed by AFM. Their ability to inhibit corrosion on 316L stainless steel was electrochemically determined using a potentiodynamic scanning (PDS) technique. The corrosion inhibition results showed that the acidic DCTZ provided the best corrosion protection. The concentration-dependent antioxidant capacity of the tetrazines was confirmed by both DPPH radical scavenging activity and FRAP assays, showing higher activity for DHDTZ than DCTZ. Furthermore, a DHDTZ doped sol-gel solution was prepared and curing parameter (temperature and time) was optimised for coating on microtitre wells and stainless steel panel. The antibacterial activity of the coated surfaces against *P. aeruginosa* ATCC 27853 and the biofilm forming bacteria *S. epidermidis* CSF 41498 was determined. DHDTZ showed significantly higher antibacterial activities with MIC as low as 31 ppm compared to 250ppm for DCTZ.

Keyword: Antibacterial, Anticorrosion, Coating, Sol-gel, Tetrazine

1. Introduction

Environmental and clinical surfaces that anchor bacteria can become sources of healthcare associated infections (HCAIs). Bacteria, including opportunistic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, can survive on surfaces outside the human host, over a range of temperatures for long time periods [1]. Meticillin-resistant *S. aureus* (MRSA) are known to contaminate healthcare facility items such as pens, uniforms, gowns, blood-pressure cuffs, mattresses, pillows, chairs and bed frames [2, 3] and can persist on surfaces even after steam cleaning and chlorine biocide treatment. In addition, moist environments will favour surface bacterial proliferation [4] especially in hospital environments where pathogens can be transmitted by contact.

Stainless steel is a commonly used material for fixtures and medical devices due to its strength, high wear resistance and chemical stability. When used as an *in vivo* medical device material, stainless steel can be exposed to a range of aggressive electrolytes, such as chlorides, and free radicals which can induce oxidative pitting corrosion on exposed susceptible components [5]. Traditionally phosphates or citrates were commonly used to treat surface to improve their inherent corrosion resistance [6] however more recently nitrogen rich organic compounds such as triazoles have been used for surface treatments [7]. Organic heterocyclic compounds with π -bonds in particular display good corrosion inhibition properties due to the availability of sacrificial electrons provided by the π -orbitals [8]. In some cases the N-containing heterocyclic compounds and their derivatives, such as benzotriazole derivatives, 1,3,4-thiadiazole derivatives, have excellent synergistic antibacterial and anticorrosion properties.

The sol-gel process can be used to form nano structured inorganic films (typically 200nm to 10 μ m in overall thickness) that are more resistant than metals to oxidation, corrosion, erosion and wear while also possessing good thermal and electrical properties [9]. The technology has gained popularity very recently for the preparation (at relatively low temperature) of porous coatings with desirable surface chemistries, chemical durability [10] and thermal stability [11] especially for biomedical applications [12, 13]. Sol-gels have also been used as carrier mediums for antibacterial agents including metals (including complexes) [14] and organic actives [15].

1,2,4,5-tetrazines (s-Tetrazines) are 6-membered aromatic heterocyclics with four nitrogen atoms symmetrically arranged on the ring. Tetrazine derivatives are known to be biologically active with antitumor, antibacterial, antifungal and anti-malarial activities [16, 17]. They have been widely used in pesticides, herbicides and as anti-inflammatory drugs. Some, such as 3,6-bis(2-methoxyphenyl)-1,2-dihydro-1,2,4,5-tetrazine (2-MDHT), have been shown to act as effective corrosion inhibitors for mild steel immersed in an acidic medium [18]. The excellent π acceptor capacity of tetrazines (for π back-donation from low-valence metals) with poor σ basicity enables them the formation of stable metal-metal bridging structures. Indeed, they can readily be reduced to stable 1,4-dihydro derivatives [19], allowing control over the degree of complexation. To date there have been limited studies of the biological properties of some 3,6-dihydrazino-1,2,4,5-tetrazine (DHDTZ) and 1,2,4,5-tetrazine dicarboxylic acid (DCTZ) and in particular, their use in antibacterial surface coating by the sol-gel process.

The aim of the present work is to synthesise two tetrazine derivatives, namely 3,6-dihydrazino-1,2,4,5-tetrazine (DHDTZ) and 1,2,4,5-tetrazine dicarboxylic acid (DCTZ), and determine their antibacterial, antioxidant and anticorrosion characteristics in solution. Their antibacterial activity

and antibiofilm was also measured as additives in a sol-gel coating on SS316L steel against *P. aeruginosa* ATCC 27853 and the biofilm forming bacteria *S. epidermidis* CSF 41498.

2. Experimental

2.1. Tetrazine Synthesis

Tetrazine derivatives were synthesized using a reported method [20] and their structures were confirmed by nuclear magnetic resonance spectroscopy (NMR) (^1H and ^{13}C) and Fourier transform infrared spectroscopy (FTIR) techniques. NMR was performed at room temperature on liquid samples employing a Bruker 400 MHz spectrometer. FTIR spectra were measured in the 4000-400 cm^{-1} range with a resolution of 1cm^{-1} and recorded on a Perkin-Elmer Spectrum instrument in transmission mode using a potassium bromide (KBr) disc.

2.2. Biological studies

The antibacterial activities (minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and bacterial growth kinetics) of the tetrazine derivatives, DHDTZ and DCTZ, were assessed using a microdilution broth method [21]. The effect of DHDTZ on bacterial morphology was assessed by using atomic force microscopy on glass slides.

2.2.1. Bacterial strains.

Stock culture and wild type (WT) clinical and environmental bacterial isolates were used in this study. These included the Gram positive *S. aureus* ATCC 25923, *Staphylococcus epidermidis* CSF 41498, methicillin-resistant *S. epidermidis* (MRSE) WT-3, MRSA ATCC 43300 and the Gram negative *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, multidrug resistant (ampicillin, gentamicin and ceftazidine) *Enterobacter* WT 6, *P. aeruginosa* ATCC

27853 and meropenem-resistant *P. aeruginosa* WT 2. All test bacteria were grown, sub-cultured and maintained on Mueller-Hinton agar (LAB M) and stored at 4 °C. A single colony from a culture grown overnight on Mueller-Hinton agar (MHA) was inoculated into Mueller-Hinton broth (MHB) and incubated aerobically at 37 °C for 24 h. The OD₆₀₀ of the culture was adjusted to 0.132 (corresponding to 1×10^8 CFU ml⁻¹) using sterile MHB and further diluted 1 in 100 to give a final working concentration of 1×10^6 CFU ml⁻¹.

2.2.2. Kinetic measurement of bacterial growth.

The growth kinetics of the bacteria, in the presence of the tetrazines, were examined by measuring the OD_{600nm} of the suspensions in the microtiter wells at 30 minute intervals over 24 hours with a 30 second agitation before each measurement. Growth curves were recorded using the Gen5 reader control and data analysis software (micro plate spectrophotometer, Biotek). Kinetic parameters such as specific growth rate (μ) and lag time (λ) for each growth curve were calculated. The increase in lag time was defined as the time required for the culture with the test compound to record an increase in OD₆₀₀ of 0.10 minus the corresponding time for the positive control without test compound [22]. The $\mu_{\max}$ was determined from the regression slope from the linear portion of the log plot during early exponential growth phase [22].

2.2.3. Assessment of bacterial damage by chemical assay.

The release of malondialdehyde (MDA) from test bacteria was measured as an indicator of damage to their membranes caused by the tetrazine compounds. MDA was quantified based on its reaction with thiobarbituric acid (TBA) to form a pink MDA-TBA adduct [23], which shows absorbance at 532 nm. Overnight broth cultures (1 ml) of Gram negative (*P. aeruginosa* ATCC

27853) and Gram positive (*S. epidermidis* CSF 41498) bacterial cells were treated with equal volumes of tetrazine compounds at their respective MBCs, while samples without tetrazines were used as controls. An aliquot (1 ml) of the suspension was mixed with 10% (w/v) trichloroacetic acid (TCA, 2 ml) and the pellets were removed after centrifugation at 11,000 rpm for 35 minutes. The supernatant was centrifuged for an additional 20 minutes to ensure that the cells and precipitated proteins were completely removed. Freshly prepared TBA solution (0.67% w/v, 3 ml) was added to the supernatant. The samples were incubated in a boiling water bath for 10 minutes and then cooled. The absorbance of the test and control solutions was measured at 532 nm using a UV-vis-NIR spectrophotometer (Perkin Elmer Lambda 900).

2.2.4. Study of the effect of DHDTZ treatment on bacterial morphology.

DHDTZ treated and untreated bacterial cultures were visualized using atomic force microscopy (Asylum MFP-3D BIO) in the tapping mode to avoid moving bacterial cells as well as to minimize any physical damage to the cells by the AFM tips. A bacterial culture (10^6 CFU ml⁻¹) was treated with the MBC level of DHDTZ in equal volumes and incubated for 24 hours at 37 °C. Bacterial cells were pelleted by centrifugation at 3,000 rpm for 2 min and resuspended in distilled water for analysis. Treated and untreated samples were placed on 1 cm² clean glass slides and allowed to dry in air at room temperature. For AFM analysis a maximum scan range of 20 μm × 20 μm with a typical resonant frequency of 300 KHz was used.

2.3. Antioxidant capacity analysis

In the present study the DPPH radical scavenging capacity assay (DPPH RSC) and the ferric reducing ability of plasma (FRAP) assay were used to estimate the total antioxidant capacity

of the DHDTZ and DCTZ. These methods were performed according to reported protocols [24, 25] For the DPPH RSC ascorbic acid (Asc) was used as a reference compound while Trolox and Asc (Sigma–Aldrich) were used as standards for the FRAP assay.

2.4. Electrochemical studies - Potentiodynamic Scanning (PDS)

The electrochemical data as obtained using a Solartron SI 1287/1255B system comprising of a frequency analyser and potentiostat. Potentiodynamic scanning was performed using an electrochemical cell (PAR K0235 Flat Cell) with an exposed area of 0.78 cm^2 in phosphate buffered saline (PBS) where the SS 316L metal acted as a working electrode, a silver/silver chloride (Ag/AgCl) electrode was used as a reference electrode and platinum mesh as a counter electrode. All scans were acquired in the region from -0.8 V to $+0.8 \text{ V}$ vs. open circuit potential (E_{oc}), with a scan rate 1 mV.s^{-1} at room temperatures ($20 \pm 2 \text{ }^\circ\text{C}$).

2.5. Preparation of sol-gels

(a) *Stock (blank) sol-gel*: The two separate sol-gels were prepared from the respective precursors (MTEOS and TEOS) having been mixed with ethanol for 5 min followed by the gradual addition of nitric acid and water as described previously [26] . A blank, additive free sol-gel, serving as a control, was prepared by mixing 2:1 ratio of methyl triethoxysilane (MTEOS) and Triethoxysilane (TEOS) sol gel. The mixture was stirred continuously for 24 h.

(b) *Tetrazine (DHDTZ) doped sol-gels*: Different quantities of a DHDTZ powder were doped into aliquots of stock sol-gel to give doping concentrations of 1, 2 and 3% (w/w) of tetrazine with respect to the final coatings weight (allowing for solvent loss and curing). The modified sol-gel solutions were stirred for 24 h to complete the reactions.

2.6. Coating procedure

The sol-gels were applied as coatings on 24 well microtitre plates (Nunc, Denmark). Sol-gels (100 μ l) were dispensed into the wells and cured in a vacuum tight oven at the 70°C for 2h. The sol-gels were spin coated onto the stainless steel panels (3×2cm) and glass slide (1cm²) (specially coating instrument systems-spin coat G3P-8) at 1000 rpm with 5 sec RAM and 1 min dwell time and cured at 70°C for 2h. All coatings were tested to determine antibacterial activity.

2.7. Morphology of coating by SEM

The tetrazine (DHDTZ) doped sol-gel coated stainless steel panel were analysed by scanning electron microscopy (SEM) in order to observe the homogeneous coating. The coated sample was mounted on an aluminum stub and sputter coated with a platinum/palladium layer with a thickness of about 10 nm (Cressington 208HR sputter coater) for imaging purposes. The morphology of coating was analysed using a Hitachi SU70 SEM operating at 3 KeV at different magnifications.

2.8. Antibacterial activity of coating surface

2.8.1. Screening of samples by microtiter well coating method

The antibacterial activity of different concentration (1, 2, 3 % (w/w)) of DHDTZ-doped sol-gel coatings against the test bacteria, *P. aeruginosa* ATCC 27853 and *S. epidermidis* CSF 41498, was determined using the microtiter plate method. Test bacteria (250 μ l) from the 10⁶ CFU ml⁻¹ suspensions and 250 μ l of MHB medium were added to all test wells. A blank well containing the respective cured DHDTZ-doped sol-gel coating with sterile MHB (500 μ l) and a negative

control well containing the blank sol-gel with the bacterial suspension (500 μ l) were used. The microtiter plates were incubated for 24 h at 37°C.

The antibacterial activities of the different coatings were determined by visual analysis and find minimum incorporated inhibitory concentration (MIIC) and minimum incorporated bactericidal concentration (MIBC). The coating with the lowest tetrazine concentration that completely inhibited bacterial growth was considered the MIIC. The MIBC was determined by the modified imprint method where the well contents (10 μ l) were sub cultured onto MHA plates. The MIBC was considered to be the lowest concentration that produced no visible bacterial growth on the MHA plate after 24 h at 37°C. All experiments were performed with triplicates and repeated at least twice.

2.8.2 Microbial adhesion procedure

S. epidermidis CSF 41498 (400 μ l, 10^6 CFU ml⁻¹) was pipetted onto the test (DHDTZ doped sol-gel, 1% (w/w)) and blank (MTEOS+TEOS sol-gel) coated stainless steel panels (3 $\times$ 2cm). The panels were incubated for 24 h at 37°C. The coated surface of the panels was pressed on the surface of MHA plates and then placed into MHB (20ml). Plates and broths were incubated overnight at 37°C and a visual inspection carried out.

3. Results and Discussion

3.1. NMR and FTIR analysis

¹H-NMR data (in D₂O): 8.78 ppm (2 H, s, COOH) and ¹³C NMR (in D₂O): 155.2 ppm, 158.8 ppm (-COOH) confirmed the structure of DCTZ. The structure of DHDTZ was established by ¹H-NMR data (in DMSO): 4.25 ppm (4 H, br s, 2 NH₂), 8.39 ppm (2 H, br s, 2 NH) and ¹³C

NMR (in DMSO): 163.3 ppm (quaternary C). The FTIR data of DCTZ showed bands at 1660 cm^{-1} (-C=O stretch), 1284 cm^{-1} (-C-O), 1188 cm^{-1} (=C-N stretches), whereas the spectrum of DHDTZ showed bands at 3295, 3220, 3153, 3025 cm^{-1} (N-H broad stretches), 1639, 1539 cm^{-1} (NH_2 stretches), 1313 cm^{-1} (N-H asymmetric stretches), 1297, 1171 cm^{-1} (=C-N stretches), 1110, 1053, 1005 cm^{-1} (C-N stretch).

3.2. Biological evaluation

3.2.1. Effect of tetrazines on bacterial growth

The MICs and MBCs of the tetrazines for the test bacteria are given in Table 1. The activity of DHDTZ was higher than DCTZ. The MIC of DHDTZ for all test organisms was in the range 31-63 ppm compared to 250-500 ppm for DCTZ. The MICs and MBCs were almost identical for DCTZ, whereas for DHDTZ the MBC value was one fold (2^1) higher than the MIC value. The results showed that DHDTZ exhibited a 4 times higher antibacterial activity than DCTZ against most of the test bacteria, including the antibiotic resistant strains.

3.2.2. Effect of different concentrations of DHDTZ and DCTZ on lag phase (λ) and maximum specific growth rate (μ_{max}) of test bacteria

Bacterial growth can be depicted generally by a growth curve which includes a lag phase where bacteria adapt themselves to the growth medium and conditions. During this period, individual bacteria mature and grow, but are not yet unable to divide. The differentiation between the lag phases of all bacteria including the resistant strains in the presence of the both tetrazines was most apparent at lower concentrations (Tables 2 & 3). Conversely the maximum specific growth rate of all bacteria in the presence of DHDTZ was almost similar to DCTZ. The extension of the lag phase is probably the most widely used parameter to describe the inhibitory effects of

antimicrobial compounds. A linear positive correlation (not shown) was obtained between the lag phases with the increasing doses of tetrazines for all test strains. With respect to μ -max an inverse relationship was observed, where a gradual reduction in μ -max was detected as the concentration of tetrazines increased. Thus, the data obtained from the increase in λ and decrease in μ -max studies confirmed that sub-MIC concentrations (highest concentration below the MIC) of tetrazines also inhibited bacterial growth.

3.2.3. Kinetic growth curve

The effect of DHDTZ on the bacterial growth over 24 h at 30 min time interval was examined. Fig. 1 and 2 show bacterial growth of Gram positive and Gram negative bacterium respectively in the presence or absence of the DHDTZ compound. Bacterial growth in the absence of test compounds (control) was rapid with a short lag phase and high specific growth rate. The presence of different concentrations of DHDTZ resulted in variable levels of growth inhibition against all test organisms. A dose of 31 ppm of DHDTZ completely inhibited growth of all test organisms except for *Enterobacter* WT 6 over 24 hours.

The lag phase of the Gram negative bacteria (Fig.1) increased from 10 to 16.4 hours in the presence of DHDTZ at sub MIC concentrations. *P. aeruginosa* ATCC 27853 (Fig. 1a) exhibited a lag phase of 10.4 hours at a sub-MIC concentration (16 ppm) of DHDTZ while growth of the antibiotic resistant *P. aeruginosa* WT 2 (Fig. 1b) was delayed for 12.5 hours. Growth of *E. cloacae* and multi-antibiotic resistant *Enterobacter* WT6 was delayed for 11.6 and 16.2 hours at 16 ppm and 31 ppm respectively (Fig. 1c, d).

In the case of Gram positive bacteria the lag phase increased from 4.5 hours to between 7.5 and 18.3 hours at sub-MIC values of DHDTZ (Fig. 2). At the sub MIC value of 8 ppm DHDTZ growth of *S. epidermidis* CSF 41498 was delayed for 18.5 hours (Fig.2c). Growth of *S. aureus*

ATCC 25923, MRSA and MRSE was delayed for 7.53, 8.05 and 8.25 hours respectively at 16ppm.

3.2.4 Assessment of bacterial damage by chemical assay.

The damage caused by the tetrazines to the bacterial cell wall/membrane was measured by determining the release of MDA. By monitoring the position and absorbance of the MDA peak maximum, typically around 530nm. Fig. 3a and 3b show the release of MDA from cells treated with the tetrazine (DHDTZ and DCTZ) at their MBCs value (31 ppm and 250 ppm respectively). DHDTZ treated *S. epidermidis* and *P. aeruginosa* show higher release of MDA when compared to DCTZ treated and untreated cells, along with a shift in the $\lambda_{\max}$. These results confirm the greater activity of DHDTZ against the test bacteria with a blue shift indicating that potential oxidative processes are occurring.

3.2.5. Effect of DHDTZ on microbial morphology.

Atomic force microscopy has been used to study the morphology of animal, human and bacterial cells [27]. AC mode AFM microscopy was used to examine the effect of the DHDTZ (at MBC) on Gram positive and Gram negative bacterial surface morphology. Untreated Gram negative *P. aeruginosa* appear as intact rods by AFM (Fig.4a). DHDTZ treatment at MBC (31 ppm) significantly changed the morphology of the bacterium as the outer membrane developed many shallow pits of irregular shape (Fig.4b). The topographical cross sections show the variation of height and size of selected treated and untreated bacterial cells. An irregular curve in treated samples shows the damage to the cells with respect to the control. In the case of *S. epidermidis*,

DHDTZ (31 ppm) treatment caused intact spherical cells (Fig 4c) to degrade and lyse to form irregularly condensed masses (Fig 4 d).

3.2.6. Possible mechanism for antibacterial action of tetrazine compounds.

The mechanism underlying the bactericidal effect of tetrazines is not known. The current work demonstrated that DHDTZ had higher antibacterial activity than DCTZ. The pKa value of DHDTZ is higher than DCTZ as the amino functionality is more electropositive in solution than the DCTZ carboxyl functionality [28, 29]. This may enable DHDTZ to interact more effectively with the negatively charged bacterial surface via electrostatic forces. This interaction could disrupt the outer (Gram negative) and/or plasma membranes (Gram positive, Gram negative), leading to leakage of intracellular components (as shown earlier by MDA release) and cell death. Hamouda and co-workers [30] have shown that the charge of an antimicrobial compound can be crucial for its activity. If the antimicrobial compound had the same charge as the bacterial-cells, repulsive forces could dominate and prevent intimate contact.

3.3. Antioxidant capacity analysis

The tetrazine derivatives were evaluated for their antioxidant capacity using both the DPPH radical scavenging and FRAP assays. The antioxidant capability of the tetrazine determines its ability to combat reactive oxygen species which cause rapid damage to living cells.

In the DPPH assay free radicals are scavenged by the test compound (antioxidant). The remaining DPPH and EC₅₀ value is determined. EC₅₀ value is the concentration of antioxidant that is required to reduce 50% of radicals formed. The lower the EC₅₀ value, the higher is the antioxidant capacity [31]. Fig. 5 shows the DPPH scavenging ability of Asc, DHDTZ and DCTZ at various concentrations (3.12, 6.25, 25 and 12.5 ppm) After a 30 min reaction, DPPH was

reduced up to 46.62% by DCTZ, 8.42% by the reference ascorbic acid and 3.75% by DHDTZ at 25 ppm. Fig.5a indicating that DHDTZ solute has the highest antioxidant activity. The EC_{50} , values for the ascorbic acid, DHDTZ and DCTZ were 18.7 ± 2.0 ppm, 6.7 ± 0.5 ppm and 222.9 ± 21.7 ppm respectively (Fig 5b). Therefore, DHDTZ was found to be very potent as a DPPH free radical scavenger with a 3 times higher activity compared to the reference compound, ascorbic acid.

The FRAP assay measures the ability of an antioxidant compound to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), to form an intense blue Fe^{2+} -TPTZ complex with an absorption maximum at 593 nm. The experimental results (Fig 5c.) showed that the 25 ppm of DHDTZ and DCTZ were capable of reducing Fe^{3+} . FRAP values were estimated from a calibration curve of the standards, Trolox and Asc (Fig 5d). The reducing ability of DHDTZ was about 8 times higher than that of DCTZ and reference compound.

3.4. Electrochemical studies-PDS

Polarisation methods, such as potentiodynamic scanning, are often used for laboratory based corrosion testing as they provide useful information on the corrosion mechanisms, corrosion rate and susceptibility of specific materials to corrosion in designated environments. The potentiodynamic polarization behaviour of steel (SS 316 L) when exposed to PBS solution with and without organic inhibitors (DCTZ, DHTDZ) was studied to compare the anticorrosion effects of the two compounds.

The corrosion parameters such as corrosion current densities (I_{corr}) and corrosion potential (E_{corr}) were estimated by the Tafel method within the range of $OCP \pm 1$ mV [32]. The polarization resistance is calculated using Stern-Geary equation

$$I_{corr} = B/R_{pol} \quad (\text{Eqn 1})$$

where R_{pol} is the polarisation resistance and B is the proportionality constant for the particular system which is calculated from the slopes of the anodic (β_a) and cathodic (β_c) Tafel regions as shown by Eqn. 2.

$$B = \frac{\beta_a \cdot \beta_c}{2.303(\beta_a + \beta_c)} \quad (\text{Eqn 2})$$

The stainless steel 316 L samples were characterized with E_{corr} and I_{corr} values of -0.245 V and $5.15 \times 10^{-8} \text{ A.cm}^{-2}$ respectively, when exposed to PBS solution. The E_{corr} value increased to +0.245 V and the I_{corr} value increased by a logarithmic order of 1 when SS 316 L was exposed to PBS solution containing 250 ppm DCTZ (Fig.6). A lower current density in the anodic branch of the polarization curve and higher polarization resistance ($R_p = 8.5 \times 10^5 \Omega \cdot \text{cm}^2$) for 250 ppm DCTZ in PBS solution indicates the anodic ($M \rightarrow M^{n+} + ne^-$, M =metal) process is inhibited. This can be attributed to deposition of the inhibitor molecules on the alloy as a result of interaction between the inhibitor and the metal surface especially the Fe, Cr, Ni and Mo phases that can form oxides which effectively seals the surface against further corrosive reaction. However higher I_{corr} ($1.10 \times 10^{-6} \text{ A.cm}^{-2}$) and lower R_p ($2.8 \times 10^3 \Omega \cdot \text{cm}^2$) values were found when SS 316 L was exposed to 250 ppm DHDTZ in PBS solution. This is probably due the fact that the DHDTZ may exist in either its neutral form or as a cation in PBS medium. In such cases, DHDTZ adsorption on SS316L is not as strong as that of the acidic DCTZ.

3.5. Morphology of coating surface by SEM

The surface morphology of the 2% DHDTZ sol-gel (2:1 molar ratio of MTEOS/TEOS) coating was characterized by SEM in Fig. 7. The coated surface appears to be porous and homogenous

(Fig. 7a). The presence of surface depressions, of 200 – 400 nm diameter, on the surface is unusual and not normally seen for such sol-gel coatings. Such features would act as potential release sites for the tetrazines and enable their rapid elution in the presence of bacteria.

3.6. Antibacterial activity of coating surface

3.6.1. Screening of samples by microtiter well coating method

The results for microtitre wells containing sol-gel coatings doped with different concentrations of DHDTZ against *P. aeruginosa*. Fig.8 showed no bacterial growth visible in all tested incorporated concentrations (1%, 2% and 3% (w/w)) whereas there was growth (turbidity) in the control wells (MTEOS /TEOS sol-gel + culture). These results agreed with the modified imprint method and determined MIBC values by sub culturing onto the MHA. There was no growth observed on the agar plate at the incorporated concentrations of DHDTZ in contrast to the control wells has growth on agar plate. Thus, the MIIC value $\leq 1\%$ and the MIBC value $\leq 1\%$ were observed. Similar pattern of results was also obtained for *S. epidermidis* which has not been shown.

3.6.2. Microbial adhesion procedure

The adhesion procedure established the survival of bacteria on the surface of a blank and doped sol-gel coating (1% DHDTZ) on SS316L. A comparison of the control and DHDTZ treated stainless steel panels following 24 h contact with *S. epidermidis* CSF 41498 shows in (Fig.9). After 24 h of exposure to the colonies the sol-gel coated panels were pressed on the agar, after 24 h incubation at 37 °C the agar in contact the blank sol-gel showed strong colony growth while the DHDTZ doped sol-gel did not (Fig 9a). This result was confirmed by the turbidity method,

where panels were immersed in a vial of MHB for 24 h at 37 °C. The turbidity of the medium in the presence of the blank sol-gel panel contrast with the transparent medium for the equivalent DHDTZ doped sol-gel (Fig 9b).

These results indicate that there was a significant reduction in bacterial colonies on the DHDTZ tetrazine doped sol-gel coated panel when compared to the equivalents over a 24h period. From a number of reviews on surface contamination and current antimicrobial coatings technologies published [33, 34]. It has been seen that one common strategy to inhibit surface colonization is to make it antibacterial. This is either achieved by direct coating or impregnation with antibacterial agents for active release into the neighboring environment. Therefore, one of the main benefits of tetrazine impregnation is the ability to prevent the formation of bacterial growth in environmental condition.

4. Conclusion

The biochemical properties of two tetrazine compounds, DHDTZ and DCTZ, are presented. DHDTZ had a higher antibacterial activity than DCTZ against all the test bacteria. Gram positive bacteria were more sensitive to the compounds than Gram negative bacteria, possibly due to different cell surface characteristics. An increase in lag time and decrease in maximum specific growth rate were observed for all test bacteria in the presence of the tetrazine derivatives. A concentration-dependent antioxidative capacity was confirmed using two different antioxidant assay systems. DHDTZ had a higher total reducing power and scavenging activity when compared to DCTZ. The corrosion inhibition property of the two compounds suggest that DCTZ acts as better corrosion inhibitor on SS 316 L alloy when exposed to PBS medium. The microtitre well coating and microbial adhesion methods confirmed that DHDTZ doped sol-gel

coated stainless steel panel shows no bacterial growth on the surface compared to the blank coated panel. These results indicate that DHDTZ doped sol-gel coatings can be applied to environmental surfaces where bacterial colonization may occur. Results also indicate that the tetrazine family has potential to be used as anticorrosive agents with efficient antioxidant characteristics.

Acknowledgment

The authors would like to gratefully acknowledge Mr. Michael Whelan for SEM assistance. The authors wish to acknowledge the Dublin Institute of Technology (Dublin, Ireland) for funding under the ABBEST scholarship programme and Enterprise Ireland under the ARE Programme and Sol ECoat (TD-07-346).

Ethical approval: Ethical approval was received from the Dublin Institute of Technology (DIT) Ethics Committee (Internal DIT code Ref 52/09).

References

1. S.J. Dancer, *Lancet Infection*, 8 (2008) 101-113
2. S. Oie, I. Hosokawa, A. Kamiya, *Journal of Hospital Infection*, 51 (2002) 140-143.
3. S. Bures, J.T. Fishbain, C.F.T. Uyehara, J.M. Parker, B.W. Berg, *American Journal of Infection Control*, 28 (2000) 465-471.
4. D. Dorjnamjin, M. Ariunaa, Y.K. Shim, *International Journal of Molecular Sciences*, 9 (2008) 807-820.
5. N. Silvestry-Rodriguez, K.R. Bright, D.C. Slack, D.R. Uhlmann, C.P. Gerba, *Applied and Environmental Microbiology*, 74 (2008) 1639-1641.

6. K. Bewilogua, G. Bräuer, A. Dietz, J. Gäbler, G. Goch, B. Karpuschewski, B. Szyszka, *CIRP Annals - Manufacturing Technology*, 58 (2009) 608-627.
7. Y. Ahmad, A. Mogoda, A. Gadallh, *International Journal of Electrochemical Science*, 7 (2012) 4929-4941.
8. N.F. Atta, A.M. Fekry, H.M. Hassaneen, *International Journal of Hydrogen Energy*, 36 (2011) 6462-6471.
9. P.C.R. Varma, J. Colreavy, J. Cassidy, M. Oubaha, B. Duffy, C. McDonagh, *Progress in Organic Coatings*, 66 (2009) 406-411.
10. P.C.R. Varma, J. Colreavy, J. Cassidy, M. Oubaha, C. McDonagh, B. Duffy, *Thin Solid Films*, 518 (2010) 5753-5761.
11. P.C.R. Varma, B. Duffy, J. Cassidy, *Surface and Coatings Technology*, 204 (2009) 277-284.
12. S. Pandey, S.B. Mishra, *Journal of Sol-Gel Science and Technology*, 59 (2011) 73-94.
13. R. Gupta, A. Kumar, *Biomedical Materials*, 3 (2008) 034005-034020.
14. S. Jaiswal, K. Bhattacharya, M. Sullivan, M. Walsh, B. S. Creaven, F. Laffir, B. Duffy, P.McHale, *Colloids and Surfaces B: Biointerfaces*, 102 (2013) 412– 419.
15. H. Böttcher, K-H. Kallies, H. Haufe. *Journal of Sol-Gel Science and Technology*, 8 (1997) 651-654.
16. A. Borkowski, M. Szala, D. Wolicka, *Chemistry and Ecology*, 27 (2011) 57-68.
17. D. Nhu, S. Duffy, V.M. Avery, A. Hughes, J.B. Baell, *Bioorganic & Medicinal Chemistry Letters*, 20 (2010) 4496-4498.
18. L. Elkadi, B. Mernari, M. Traisnel, F. Bentiss, M. Lagrenee, *Corrosion Science*, 42 (2000) 703-720.
19. W. Kaim, *Coordination Chemistry Reviews*, 230 (2002) 126-138.

20. D.L. Boger, J.S. Panek, M. Patel, *Organic Synthesis* 70 (1992) 79-92
21. S. Jaiswal, B. Duffy, A.K. Jaiswal, N. Stobie, P. McHale, *International Journal of Antimicrobial Agents*, 36 (2010) 280-283.
22. P. Nobmann, A. Smith, J. Dunne, G. Henahan, P. Bourke, *International Journal of Food Microbiology*, 128 (2009) 440-445.
23. S. Singh, P. Patel, S. Jaiswal, A. Prabhune, C. Ramana, B. Prasad, *New Journal of Chemistry.*, 33 (2009) 646-652.
24. A. K. Jaiswal, G. Rajauria, N. Abu-Ghannam, S. Gupta, *Journal of Food Biochemistry*, 36 (2012) 344-358.
25. I.F.F. Benzie, J. Strain, *Analytical Biochemistry*, 239 (1996) 70-76.
26. S. Jaiswal, P. McHale, B. Duffy, *Colloids and Surfaces B: Biointerfaces*, 94 (2012) 170-176.
27. P.C. Braga, D. Ricci, *Antimicrobial Agents and Chemotherapy*, 42 (1998) 18-22.
28. H. Busscher, R. Bos, H. Van der Mei, P. Handley, in: N.W. Baszkin A (Ed.) *Physical chemistry of biological interfaces*. Marcel Dekker, 2000, pp. 431-458.
29. C.C. Ezabadi IR, Zoumpoulakis P, Geronikaki A, Sokovic M, Glamocilija J, Cirić A., *Bioorganic & medicinal chemistry*, 16 (2008) 1150-1161.
30. T. Hamouda, J. Baker Jr, *Journal of Applied Microbiology*, 89 (2000) 397-403.
31. S. Gupta, A.K. Jaiswal, N. Abu-Ghannam, *Journal of Food Processing and Preservation* 36 (2012) 412-422.
32. E.C. David, M.A. Hiskey, *Journal of Pyrotechnics*, 7 (1998) 711-714.
33. K. Page, M. Wilson, I.P. Parkin, *Journal of Materials Chemistry*, 19 (2009) 3819-3831.
34. A. Kugel, S. Staflien, B.J. Chisholm, *Progress in Organic Coatings*, 72 (2011) 222-252.

Table 1 Minimum inhibitory concentrations (MICs, ppm) and Minimum bactericidal concentrations (MBCs, ppm) of DCTZ and DHDTZ for Gram negative and Gram positive, antibiotic-resistant and susceptible organisms.

Organic Compounds	Organisms									
	Gram negative						Gram positive			
	<i>E. coli</i> ATCC 25922		<i>P. aeruginosa</i> ATCC 27853		<i>E. cloacae</i> ATCC 13047		<i>S. aureus</i> ATCC 25923		<i>S. epidermidis</i> CSF 41498	
DCTZ	MIC 500	MBC 500	MIC 250	MBC 250	MIC 250	MBC 250	MIC 125	MBC 250	MIC 250	MBC 250
DHDTZ	63	125	31	63	31	31	31	31	31	31

Organic Compounds	Antibiotic resistant Organisms							
	Gram negative				Gram positive			
	<i>P. aeruginosa</i> WT 2		<i>Enterobacter</i> WT 6		MRSA ATCC 43300		MRSE WT 3	
DCTZ	MIC 250	MBC 250	MIC 500	MBC 500	MIC 125	MBC 125	MIC 125	MBC 125
DHDTZ	31	63	63	125	31	31	31	31

		Gram negative				Gram positive			
Sample	Conc. (ppm)	<i>P. aeruginosa</i> ATCC 27853		<i>E. cloacae</i> ATCC 13047		<i>S. aureus</i> ATCC 25923		<i>S. epidermidis</i> CSF 41498	
		λ	μ_{max}	λ	μ_{max}	λ	μ_{max}	λ	μ_{max}
DCTZ	0	6.2 ± 0.1 ±	6 0.0 5 0	3.3 ± 0.8 ±	0 0 0 0	4.2 ± 0.1 ±	0 0 4 0	4.50 ± 0.1 ±	0 5 0
		1	.	.	.	.	.	.	.
			0	0	0	0	0	3	0
			2	1	1	1	2	0	1
	31	8.0 ± 0.1 ±	6 0.0 5 0	3.4 ± 0.7 ±	6 0 0 0	6.0 ± 0.1 ±	0 3 0	5.52 ± 0.1 ±	0 2 0
		1	.	.	.	.	.	.	.
			0	0	0	0	0	2	0
			1	1	1	2	1	8	2
	63	8.3 ± 0.1 ±	8 0.0 3 0	4.2 ± 0.7 ±	8 0 0 0	10. ± 0.1 ±	3 0 2 0	12.0 ± 0.1 ±	3 0 0 0
		1	.	.	.	.	.	.	.
			0	0	0	0	0	3	0
			1	1	2	2	1	0	1
	125	11. ± 0.1 ±	0 0.0 2 0	6.3 ± 0.7 ±	2 0 0 0	14. ± 0.1 ±	0 0 2 0	16.4 ± 1.0 ±	7 0 7 0
		5	.	.	.	.	.	.	.
			0	0	0	0	0	0	0
			2	2	1	5	1	7	3
	250	N - 0 -	N - 0 -	N - 0 -	N - 0 -	N - 0 -	N - 0 -	NG - 0 -	
		G		G		G			
	0	6.1 ± 0.1 ±	9 0 6 0	3.3 ± 0.1 ±	2 0 0 0	3.3 ± 0.1 ±	1 0 3 0	3.20 ± 0.1 ±	0 5 0
		.	.	.	.	.	.	.	.
		0	0	0	0	0	0	2	0
		2	1	2	1	2	1	1	1
	8	8.6 ± 0.1 ±	0 0 1 0	6.0 ± 0.8 ±	3 0 0 0	5.2 ± 0.1 ±	9 0 2 0	18.5 ± 0.0 ±	0 6 0
		.	.	.	.	.	.	.	.
		0	0	3	0	0	0	3	0
		3	1	1	1	3	1	2	1

DHDT 16	10. ± 0.1 ±	11. ± 0.7 ±	7.5 ± 0.1 ±	NG - 0.0 ±
Z	4 0 1 0	5 0 0 0	3 0 1 0	2 0
	. .	. .	. .	.
	3 0	0 0	3 0	0
	0 2	1 2	1 1	1
31	N - 0 -	N - 0 -	N - 0 -	NG - 0 -
	G	G	G	

Table 2 Effect of different concentrations of DCTZ and DHDTZ on the lag time (λ , hours) and maximum specific growth rate (μ max, hrs⁻¹) of Gram negative and Gram positive bacteria.

NG: no growth.

Table 3 Effect of different concentrations of DCTZ and DHDTZ on the lag time (λ , hrs) and maximum specific growth rate ($\mu_{\max}$, hrs⁻¹) of Gram negative and Gram positive antibiotic-resistant bacteria.

Sample	Conc (ppm)	Antibiotic resistant Gram negative				Antibiotic resistant Gram positive			
		<i>P. aeruginosa</i> WT 2		<i>Enterobacter</i> WT 6		MRSA ATCC 43300		MRSE WT 3	
		λ	$\mu_{\max}$	λ	$\mu_{\max}$	λ	$\mu_{\max}$	λ	$\mu_{\max}$
DCTZ	0	8.14 ± 0.2	0.7 ± 0	3.5 ± 0.2	1.0 ± 0	4.3 ± 0.1	0.7 ± 0	3.2 ± 0.1	0.2 ± 0
		0	0	0	0	0	0	0	0
		2	1	2	1	1	1	3	1
	31	9.24 ± 0.2	0.5 ± 0	3.1 ± 0.2	0.0 ± 0	7.0 ± 0.1	0.6 ± 0	6.5 ± 0.1	0.1 ± 0
		0	0	0	0	0	0	0	0
		1	2	2	3	2	1	4	4
	63	10.0 ± 0.2	0.2 ± 0	4.1 ± 0.1	0.9 ± 0	12. ± 0.1	0.5 ± 0	11. ± 0.1	0.0 ± 0
		0	0	0	0	0	0	0	0
		1	1	1	2	1	1	2	2
	125	12.5 ± 0.2	0.0 ± 0	4.4 ± 0.1	0.9 ± 0	N - 0 -	G -	N - 0	-
		0	0	0	0				
		5	1	1	1				
	250	NG - 0 -		11. ± 0.1	0.5 ± 0	N - 0 -	G	N - 0 -	-
				0	0				
				1	0				

500	NG - 0 -				N - 0 -				N - 0 -				N - 0 -			
	G				G				G				G			
0	6.6	±	0.2	±	4.4	±0.0	0.1	±	3.4	±	0.1	±	5.5	±	0.2	±
			0	5	0		4	1	9	0		3	0	7	0	
			.		.					.		.			.	
			0		0				0		0		0		0	1
			2		1				1		1		2		1	
8	11.1	±	0.2	±	4.0	±0.0	0.1	±	6.4	±	0.1	±	7.1	±	0.2	±
			2	0	0		8	1	5	0		0	0	4	0	
			.		.					.		.			.	
			0		0				0		0		0		0	0
			2		2				1		1		3		2	2
DHD 16	12.5	±	0.1	±	10.	±0.0	0.1	±	8.0	±	0.1	±	8.2	±	0.1	±
TZ			0	8	0		1	1	4	0		5	0	2	0	
			.		.					.		.			.	
			0		0				0		0		0		0	0
			1		1				2		1		2		1	2
31	NG	-	0	-	16.	±0.0	0.1	-	N	-	0	-	N	-	0	-
							2	1	1							
											G				G	
63	NG	-	0	-	N	-	0	-	N	-	0	-	N	-	0	-
											G				G	

NG: no growth.

Figure Captions

Fig. 1. Growth curves (24 h) of Gram negative bacteria (a) *P. aeruginosa* ATCC 27853, (b) *P. aeruginosa* WT 2, (c) *E. cloacae* ATCC 13047 and (d) *Enterobacter* WT 6 in the presence of different concentrations (8, 16, 31 and 63 ppm) of DHDTZ.

Fig. 2. Growth curves (24 h) of Gram positive bacteria; (a) *S. aureus* ATCC 25923, (b) MRSA ATCC 43300, (c) *S. epidermidis* CSF 41498 and (d) MRSE WT 3 in the presence of different concentrations (8, 16, 31 and 63 ppm) of DHDTZ.

Fig. 3. Estimation of release of malondialdehyde (MDA) from (a) *S. epidermidis* CSF 41498 and (b) *P. aeruginosa* ATCC 27853 in the presence and absence of tetrazine compounds at their MBC concentrations.

Fig. 4. AFM images and cross-section profiles showing the effect of DHDTZ at its MBC value on morphology of *P. aeruginosa* ATCC 27853 (a) before and (b) after exposure and *S. epidermidis* CSF 41498 (c) before and (d) after exposure.

Fig. 5. Comparative antioxidant capacity of DHDTZ and DCTZ by DPPH and FRAP assays (a) Remaining DPPH in the presence of different concentrations of DHDTZ, DCTZ and Asc, (b) EC₅₀ value of Asc, DHDTZ and DCTZ analyzed by DPPH method, (c) FRAP values of DCTZ and DHDTZ (μM of Trolox TE/ μg and μM of AscE/ μg of compound), (d) Calibration curves of Trolox and Asc for the estimation of FRAP value.

Fig. 6. Potentiodynamic scans on SS 316 L exposed to PBS solution with 250 ppm DCTZ, 250 ppm of DHDTZ and without inhibitor. In the inset: corrosion current densities (I_{corr}), corrosion potentials (E_{corr}) and polarization resistance R_p estimated by Tafel analysis of the scans.

Fig. 7. SEM images of the 2% DHDTZ doped sol-gel (2:1 ratio of MTEOS/TEOS) coating on stainless steel panels.

Fig. 8. Antibacterial activity of DHDTZ doped (concentrations 1%, 2% and 3% w/w) sol-gel coated microtitre wells against *P. aeruginosa* ATCC 27853. (a) MIIC, and (b) MIBC determined by modified imprint method. Blank wells contain DHDTZ + medium and control wells (MTEOS + TEOS sol-gel) and bacterial culture. Experiments were performed in triplicate represented by numbers (1, 2, and 3).

Fig. 9. Comparison of the imprints of blank (MTEOS +TEOS) and DHDTZ-doped sol-gel coated stainless steel panels on MHA (37 °C, 24 h) following 24 h contact with *S. epidermidis* CSF 41498. (a) imprints on MHA, (b) MHB containing panels (37 °C, 24 h): control vial shows turbid medium in the presence of blank coated panel (MTEOS+TEOS sol-gel) and test shows transparent medium in the presence of DHDTZ doped sol-gel coated panel

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

Graphical Abstract

Highlights:

- Tetrazine derivatives showed strong antibacterial activity against bacterial pathogens.
- Electrochemical studies confirmed the anticorrosion property of the compounds.
- Significant antioxidant activity was exhibited by the compounds.
- Tetrazine doped sol-gel coating inhibited bacterial growth on 316L stainless steel
- Tetrazine derivatives can be used as a potential hygiene coating additive.