The Effects of Cationic Surfactants on Marine Biofilm Growth on Hydrogels

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Abstract

A method for the quantification of biofilm formation on hydrogel protective coatings for optical sensors and cameras has been developed using fluorescein diacetate (FDA) hydrolysis. In conjunction with these measurements the release of the fouling resistant cationic surfactants benzalkonium chloride, tallowbenzyldimethylammonium chloride and dicocodimethylammonium chloride was measured, using high performance liquid chromatography (HPLC), to enable correlation to be made between release and biofilm resistance and thus determine the active lifetime of such coatings. Results indicate that the twin-chained material, dicocodimethylammonium chloride, produced superior biofouling resistance as, at the 12 week time point, little fouling was detected on this coating. The hydrogel containing the long-chained tallowbenzyldimethylammonium chloride (mainly C16 and C18 chains) was the next best fouling resistant material, withstanding biofilm formation for 9 weeks. This correlates with the fact that each of these materials had an extremely slow to zero release rate, due to their irreversible binding to the hydrogel over the experimental timescale. Incomparison the shorter chained benzalkonium chloride (mainly C12 and C14), showed signs of biofilm formation at the 3 week time point.

Keywords: biofilm; surfactants; fluorescein diacetate; marine; optical sensors

Introduction

Biofilm formation on marine underwater structures, vehicles, sensors etc. is an ongoing problem leading to macrofouling and surface corrosion. Microfouling occurs in any situation where there is a solid/liquid, liquid/gas or solid/gas interface (Wimpenny, 1996). Biofilms form in many areas and in most cases their presence is unwelcome. A marine biofilm is commonly composed of bacteria, diatoms, protozoans, microalgae and macroalgae (Anderson, 1995). Surfaces are initially colonized by bacteria and diatoms creating a 'slime ' layer, the biofilm, which is highly hydrated. When established the biofilm is able to confer a defence for the organisms within it thus making the task of biofilm resistant materials more difficult (Costerton & Lashen, 1984).

The use of marine monitoring equipment has increased in recent years and long-term monitoring from remote buoys has necessitated biofilm resistant strategies to be developed in order that accurate data can be collected. Research into formation of biofilms on marine underwater sensors and camera lenses has shown that after 1–2 weeks in temperate waters a typical biofilm would result in useless data and poor camera images (Kerr *et al*, 1998). At present, work to create a transparent fouling resistant coating for marine optical sensors and cameras continues.

Hydrogel coatings containing cationic surfactants have been shown to prevent biofouling formation on sensors and cameras increasing their underwater deployment time for up to 20 weeks (Cowling *et al*., 1998). Cationic surfactants possess two characteristics, a hydrophillic head and a hydrophobic tail thus making them useful in many areas of industry. Their antimicrobial properties are utilized in products such as eyedrops, mouthwashes and laundry agents. Their surface active properties are important as lubricators, constituents for polishes and in corrosion inhibition (Linfield, 1970). It is however, their dual properties that make them useful for biofouling resistance.

In order to understand the relationship between release of cationic surfactants from the hydrogel and biofilm formation it was necessary to find a method for quantitative biofilm determination. The hydrogels used were poly- (hydroxyethyl methacrylate) with an equilibrium water content (E.W.C.) of 40%. Current methods for testing biofouling resistant chemicals either test the response of a test organism, usually a diatom, to the substance under investigation (Callow & Willingham, 1996; Wigglesworth-Cooksey & Cooksey, 1996) or estimate biofilm metabolic or physiological activities by targeting either the heterotrophic or autotrophic component. These methods generally require removal of the biofilm from the substrate, which results in considerable loss in precision. Staining techniques followed by light microscopy are not useful as the hydrogel is also stained, making counting of bacteria impossible. Biofouling accumulation can also be measured by UV transmission (Parr *et al*., 1998; Marrs *et al*., 1999). Such methods are however, only capable of investigating small areas at a time which, results in many measurements having to

be taken on each sample. The methods described above are limited as they do not realistically model a biofilm population which is, at the initial stages, a mix of bacteria (heterotrophic) and algae (autotrophic). In addition, these methods are time consuming. A quick and reliable estimation of biofilm metabolic activity was required to measure the effectiveness of potential fouling resistant hydrogels in the marine environment.

Fluorescein diacetate (3',6'-diacetyl-fluorescein) (FDA) hydrolysis has been used to assess microbial activity in marine (Gumprecht *et al*., 1995; Poremba, 1995) and freshwater sediments (Battin, 1997), activated sludge (Fontvielle *et al*., 1992) as well as in pure cultures of bacteria (Schnürer & Rosswall, 1982) and algae (Gilbert *et al*., 1992). This colourless compound is hydrolysed by both free and membrane bound enzymes (Stubberfield & Shaw, 1990) releasing a coloured end product, fluorescein, which can be measured by spectrophotometry. Only recently has this method been applied to biofilm estimation on surfaces (De Rosa *et al*., 1998). This work follows the development of marine biofilms on submerged, surfactant treated hydrogels using FDA hydrolysis and monitors the release of cationic surfactants from the hydrogel. The advantages of the FDA method are the whole sample can be measured, the biofilm remains attached to its substrate, it can be used on opaque samples where light transmission microscopy is not useful and it measures over a wide range of biofilm thickness'. The method provides a more accurate and sensitive estimation of biofilm activity than others previously detailed and is easy and rapid to perform.

Materials and methods

Preparation of the hydrogels

The hydrogels used were transparent and contained 40% water. They were prepared in 250 mm x 250 mm poly-methyl methacrylate (PMMA) moulds to a thickness of 1–2 mm then stored in distilled water until required. The details of their preparation can be found elsewhere (Smith, pers. comm.; Refojo, 1966).

Cationic surfactants

The three cationic surfactants used were quaternary ammonium compounds, their structures are shown in Figure 1. The surfactants were benzalkonium chloride (BAC) (Aldrich), tallowbenzyldimethylammonium chloride (DMHTB-75™) (Akzo Nobel) and dicocodimethylammonium chloride (2C-75™) (Akzo Nobel). The hydrogels were loaded with surfactant by soaking them in 5% w/v solutions for 14 days (EEC MAST II, pers. comm.). In the case of the DMHTB-75™ material it was loaded at the hydrogel production stage due its relative insolubility. Previous work had shown (Smith, pers. comm.) that release from hydrogels soaked in DMHTB-75™ to be the same as those with DMHTB-75™ included at the preparation stage. However, hydrogels prepared with DMHTB-75™ included at the production stage were found to be more reproducible.

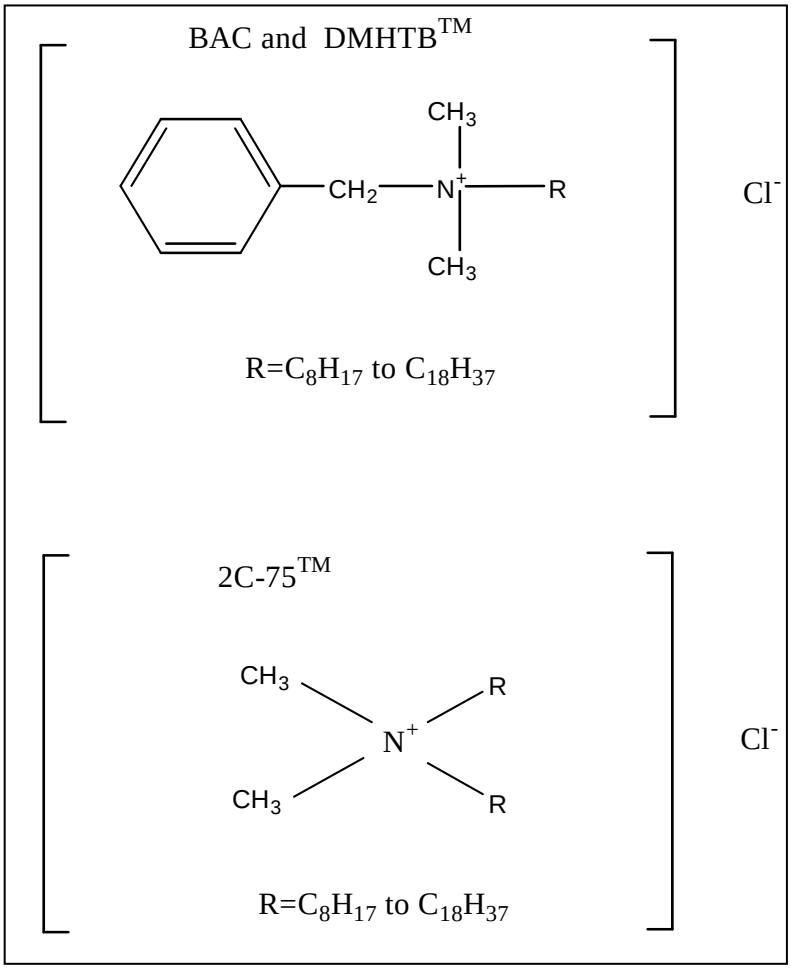

Figure 1. Structures of cationic surfactants. Benzalkonium chloride (BAC) tallowbenzyldimethylammonium chloride (DMHTB-75™) and dicocodimethylammonium chloride (2C-75™).

Marine exposure trials

The trials were carried out at the University Marine Biological Station (UMBS) which is on the island of Cumbrae in the Firth of Clyde on the West Coast of Scotland. The hydrogels were held on a PMMA frame. There were 16 samples exposed, four containing benzalkonium chloride, four containing DMHTB-75™, four containing 2C-75™ and four unloaded hydrogels. They were arranged in a Latin-square formation. The exposed area of each hydrogel sample was 60 mm x 80 mm. The rack was suspended from Keppel Pier in a vertical orientation to a depth of 3 m in the sea. Figure 2 shows the layout of the test coatings. Keppel Pier is 30–40 m from the shoreline. The trial began in July 2000 and was run for a period of 12 weeks. Samples for both surfactant quantification and biofilm detection were taken at, 3, 6, 9 and 12 weeks. Quantitative analysis of cationic surfactant content was also carried out on the BAC and the DMHTB-75™ at zero time. The sea temperature in this area during the period of the trials ranged from 13 °C to 15 °C.

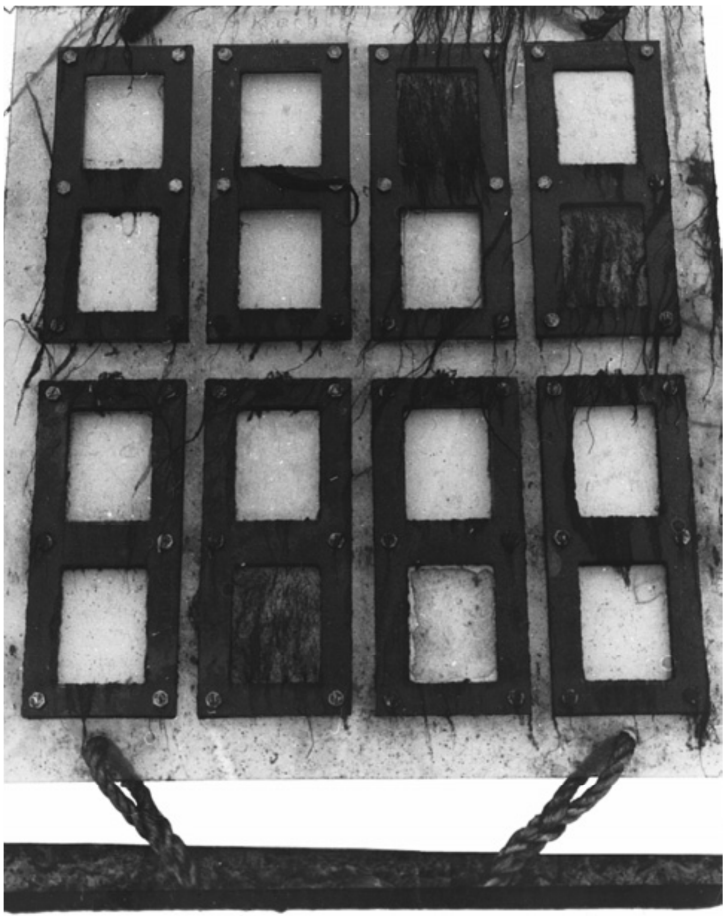

Figure 2. Hydrogel coatings in PMMA (poly-methyl methacrylate) frame at 6 week time point. The samples are in a Latin-square formation. Row 1 is benzalkonium chloride, dicocodimethylammonium chloride, unloaded hydrogel and tallowbenzyldimethylammonium.

Quantitative analysis

The BAC and the DMHTB-75™ materials were quantitatively analysed using high performance liquid chromatography (HPLC) coupled with UV detection at 214 nm (Guilfoyle *et al*., 1990). The column used was a Techsphere 5CN, 25 cm x 4·6 mm (HPLC Technology), the flowrate was 2.00 ml min^{-1}, and the mobile phase was 80:20, acetonitrile and 0·06M NaH_2PO_4. The results were reported as weight percent of BAC of dry gel weight. A dry weight was calculated at each time-point to account for any physical changes in the gel during the timescale of the experiment. The 2C-75™ material was analysed using a method described by Huang (1987). The 2C-75™ is transparent in the UV range therefore a method of Indirect Photometric Chromatography (IPC) is used where the analyte displaces the UV active species, in this case p-toluenesulphonic acid, from the mobile phase. The column used was a Techshere 5CN, 25 cm x 4·6 mm (HPLC Technology), the flowrate was 1 ml min^{-1}, the mobile phase was 25:75, methanol and 5 mM p-toluenesulphonic acid and the wavelength was 260 nm. This results in a negative baseline, which represents the UV transparent species. Sample discs were taken using a cork borer with a diameter of 20 mm. Three were cut from each gel at each time point. The surfactants were extracted from the hydrogel using a method previously described. (EEC MAST II, pers. comm.).

Optimization of hydrogel dimensions, sampling and fluorescein diacetate method

A preliminary experiment was conducted to determine optimum surface area sampling size and length of submersion of hydrogels to obtain a measurable biofilm and also the optimum conditions for carrying out the FDA hydrolysis method.

Varying sizes of PMMA coupons were submerged for differing lengths of time in the sea at UMBS. The sample PMMA coupons were collected and transferred to sealed plastic containers with a small amount of seawater to prevent the biofilms drying out. The samples were then transported in a cool box to the laboratory where they were stored at 4 °C, to prevent growth from the time of sampling, and analysed within 24 h of collection. Marine biofilm estimation was performed as described below except differing lengths of incubation were used.

Estimation of marine biofilm activity using fluorescein diacetate (FDA)

Estimation of marine biofilm activity was carried out by a modification of the method based on the Adam and Duncan (2001) method. Four cores (30 mm diameter) were cut from each hydrogel section (60 x 80 mm) and placed into individual 60 ml glass powder jars. Fifteen millilitres of 60

mM potassium phosphate buffer pH 7.6 (8.7 g K_2HPO_4:1.3 g KH_2PO_4 made up to 1 l in deionized water) was added to each jar and 0.2 ml of 1000 µg fluorescein diacetate (3',6'-diacetyl-fluorescein, Sigma-Aldrich Co. Ltd) ml^{-1} acetone solution added to start the reaction. One jar from each treatment was retained as a blank without the addition of the FDA substrate. The lids were replaced on the jars and the jars then placed in an orbital incubator (Gallenkamp orbital incubator, 100 rev min^{-1}) at 10°C ±1 °C for 1 h. The following steps involving chloroform/methanol were carried out in a fume cupboard. Once removed from the incubator, the 30 mm diameter cores were taken out of the buffer/FDA solution and 15 ml of chloroform/methanol (2:1 v/v) added immediately to terminate the hydrolysis reaction. The lids were replaced on the jars and the contents shaken thoroughly by hand. The content of each jar was filtered (Whatman, No. 2) into 100 ml conical flasks and the filtrates measured at 490 nm on a spectrophotometer (Hitachi U-1100 spectrophotometer). The blank from each treatment was used to zero the spectrophotometer before reading the sample absorbency.

The concentration of fluorescein released during the assay was calculated using the calibration graph produced from 0–5 µg fluorescein ml^{-1} standards, which were prepared from a 20 µg fluorescein (fluorescein sodium salt, Merck-BDH, Analar) ml^{-1} standard solution by appropriate dilution in 60 mM potassium phosphate buffer pH 7.6.

Results

Application to marine biofilm detection

The results from the preliminary experiment indicated a surface area sampling size between 20 mm^2 and 50 mm^2 and a submersion period of >14 days was sufficient to allow development of a sizeable marine biofilm. The optimum length of incubation using the conditions described below was 1 h, which allowed a measurable amount of fluorescein to be released without the substrate becoming limiting. Rather than performing the analyses at 30 °C, which is the temperature at which maximum FDA hydrolysis occurs (Adam & Duncan, 2001) or any other arbitrary temperature, monthly average sea temperatures were obtained from UMBSM. A temperature of 10 °C was chosen to provide realistic environmental conditions for carrying out the analyses. This temperature was a mean of 12 months temperature recordings that included winter months, as it was decided to develop a method that would apply in all seasons. By keeping the incubation time short and the incubation temperature low, changes that could occur to the biofilm population would be minimized.

Biofilm FDA hydrolytic activity was measured, on each surfactant-treated hydrogel as well as the untreated hydrogel (blank), at the 3, 6, 9 and 12 week time points. The results given in Table 1 illustrate biofilm development on the hydrogels investigated over time, as indicated by an increase in the amount of fluorescein released during the course of the incubation.

Table 1. Biofilm activity

	µg fluorescein released h^{-1}			
hydrogel treatment	unloaded	BAC	2C-75™	DMHTB-75™
Sampling time (weeks)				
3	1.393 ± 0.448	0.053 ± 0.002	0.043 ± 0.003	0.066 ± 0.013
6	1.313 ± 0.372	1.374 ± 0.116	0.063 ± 0.003	0.124 ± 0.011
9	1.042 ± 0.135	1.568 ± 0.663	0.325 ± 0.154	0.342 ± 0.114
12	0.884 ± 0.125	1.149 ± 0.337	0.330 ± 0.054	1.447 ± 0.265

Average values ± standard error (SE), n = 3

The untreated hydrogel (blank) was quickly colonized by bacteria and the biofilm well established by the 3-week time point. At 3 weeks the blank hydrogel's FDA activity had reached a maximum of 1.393 µg fluorescein released h^{-1}, after which FDA activity was seen to decrease steadily to 0.884 µg fluorescein released h^{-1} at the 12-week time point. This reduction in activity, indicating a decrease in the amount of biofilm detected, is due to larger organisms, such as invertebrate larvae, grazing off bacteria on the biofilm as well as nutrient scavenging by algae, making it patchy (Anderson, 1995). A similar biofilm growth pattern was seen for the BAC treated hydrogel, except a lag phase of 3 weeks was seen before an increase in FDA activity was observed. FDA activity

increased from the 3-week time point to a maximum of 1.568 µg fluorescein released h^{-1} at 9 weeks. The activity then decreased rapidly, as seen in the blank hydrogel curve, due to bacterial grazing. The DMHTB-75™ and 2C-75™ treated hydrogels showed very different patterns of biofilm growth. The DMHTB-75™ hydrogel showed little FDA activity over the 3–9 week sampling period suggesting little biofilm growth had occurred. It was not until the 9-week time point that an increase in FDA activity was observed. This activity rose steeply to its highest measured value of 1.447 µg fluorescein released h^{-1} at 12 weeks. The 2C-75™ hydrogel maintained a low level of FDA activity throughout the course of the experiment. For the first 6 weeks an extremely low FDA activity of 0.053 µg fluorescein released h^{-1} (average value) was measured. This value rose to 0.327 µg fluorescein released h^{-1} on average, during weeks 9–12. This level of FDA activity was extremely low compared to the other hydrogels tested and indicates that little biofilm growth occurred on this hydrogel.

Biofilm formation on the various surfactant treated hydrogels was also visually assessed at each sampling time point. Figure 2 illustrates the various hydrogels held on a PMMA frame after the 6-week exposure time. The untreated (blank) hydrogels are easily recognizable by the extensive algal fouling present on the hydrogel surface. The other hydrogels retained their transparent appearance with little to no apparent fouling.

Release of surfactants

The hydrogels containing surfactants were quantitatively analysed using HPLC as described in the methods section. Table 2 shows the levels found. The amount of 2C-75™ found is greater relative to that found in the BAC and DMHTB™, although the soaking time was the same. This may be attributed to its twin structure. Figure 3 shows the release of BAC and DMHTB-75™ over the 12-week period. It can be seen that the BAC releases in a linear fashion over the period while the DMHTB-75™ is practically all retained in the hydrogel. BAC with its predominately C12 and C14 chains is water-soluble. It can therefore be contained mainly in the pore water of the hydrogel with a small amount becoming irreversible bound to the hydrophobic part of the hydrogel (Smith *et al.*, 2000). The DMHTB-75™ is mainly composed of C16 and C18 and is fairly insoluble at room temperature. As it is a much more hydrophobic material than BAC, it is likely to attach itself to the more hydrophobic parts of the hydrogel i.e. not the porewater. Such attachment would be irreversible, in this environment, which in addition to DMHTB-75™'s reluctance to release into the polar seawater environment would result in DMHTB-75™ being retained in the hydrogel.

The 2C-75™ is mainly composed of C12 and C14 chains. It is water soluble but separates into two layers when left standing. It is a twin-chained material and has a more hydrophobic

character than the single chained BAC and thus, it is able to attach by hydrophobic interaction to the non-water portions of the hydrogel. From the quantitative analysis over the 12 weeks of the study, 2C-75™ appears to be retained by the hydrogel thus suggesting, despite its water solubility, it is predominately held in the non-polar areas of the hydrogel.

Table 2 Average release of cationic surfactants.

	% cationic surfactant (wt/wt)		
hydrogel treatment	BAC	2C-75™	DMHTB-75™
Sampling time (weeks)			
0	11.163±0.094	Not done	12.622±0.273
3	8.898±0.5722	29.701±9.319	12.111±0.241
6	6.889±0.224	19.776±1.631	13.984±0.020
9	4.166±0.347	25.110±4.276	11.261±0.118
12	2.222±0.217	28.709±3.822	10.137±0.320

Average values ± standard error (SE), n = 3

Discussion

The estimation of biofilm growth provided by the FDA hydrolysis method correlated well with release rates of the fouling resistant cationic surfactants. The FDA hydrolysis results showed that the BAC-treated hydrogels had a sizeable biofilm forming by 3 weeks suggesting the active lifetime of this coating was short. This statement was verified by the release rate of BAC from the hydrogel. BAC content of the hydrogel decreased linearly over the course of the experiment from 11.16% w/w to approximately 2% w/w at the 12 week time point. The decrease in BAC content within the

hydrogel diminished the materials fouling resistance, allowing bacterial attachment and biofilm growth. The DMHTB-75™ hydrogel showed little biofilm formation until after the 9-week time point, where FDA hydrolytic activity rose sharply to the 12-week time point. Somewhere, shortly after the 9-week sample was taken, the release of DMHTB-75™ was sufficient as to allow biofilm growth to increase. The release rate of DMHTB-75™ from the hydrogel was extremely slow and the concentration of DMHTB-75™ was only slightly reduced over the course of the experiment. Initially the concentration of DMHTB-75™ was 12·5% w/w and by week 12 it had fallen to 10.2% w/w. The antifouling properties of the DMHTB-75™ surfactant appear to be lost at this point therefore the active lifetime of this coating is approximately 9 weeks. The DMHTB-75™ offers an extended period of biofouling resistance compared with the BAC and this must be attributed to its ability to modify the hydrogel surface i.e. its ability to remain in the hydrogel. However, even with the slow release rate, the material loses its ability to deter fouling. Finally, the FDA hydrolysis

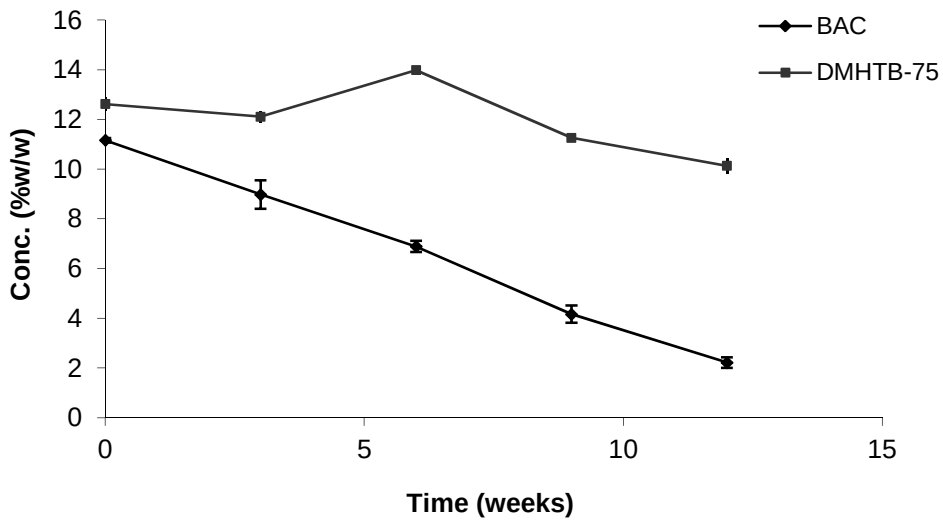

Figure 3. Release of benzalkonium chloride (BAC) and tallowbenzyldimethylammonium chloride (DMHTB-75™) from the hydrogel over the 12 week period. Standard error bars are given, n=3. BAC; DMHTB-75™.

results for the 2C-75™ hydrogel indicated little biofilm activity that again corresponded well with the results obtained from the release of 2C-75™ from the hydrogel. Almost no 2C-75™ was released from the hydrogel over the 12 week exposure period suggesting the level of antifouling resistance the hydrogel held at the start of the trial was the same as the resistance imparted after 12 weeks. This effect is purely surface action as no antimicrobial release was found with 2C-75™. The 2C-75™ is retained in the hydrogel enabling it to modify the surface which in turn extends the biofouling free lifetime of the coating. A slight increase in FDA activity was observed at the 9 week

time point, indicating a change in the biofilm but this increase was minimal. The active lifetime of 2C-75™ was >12 weeks and was the greatest of all the cationic surfactant hydrogel combinations tested.

Conclusions

The FDA method for determining biofilm activity on the hydrogel coatings is successful and rapid. The quantitative analysis of release of surfactant demonstrated that retention of the surfactant extends the lifetime of the coating. It can be postulated that the primary defence of DMHTB™ and 2C-75™ is a surface active phenomenon. BAC is releasing and can therefore thought to be acting as an antimicrobial until the levels of release become low and not effective.

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