Macroscopic N-halamine biocidal polymeric beads

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ABSTRACT: The particle size of N-halamine biocidal polymers was methodically modified forming beads of different sizes by blending water-insoluble N-halamine polyurethane with sodium alginate as the matrix, and loading heterocyclic rings onto modified silica gels. The biological activity of the prepared beads and halogenated modified silica derivatives were evaluated against examples of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. The recycling possibilities and the optimum preparation conditions of the blended beads were investigated; blending pre-halogenated polyurethane (5%, w/v) with sodium alginate (3%, w/v) followed by cross-linking with $CaCl_2$ (10%, w/v) at 40^{o}C are the optimum preparation conditions for the alginate beads.

Keywords: N-halamine; beads; silica; bacteria.

INTRODUCTION

N-halamine polymeric materials have been studied extensively over the last two decades.[1-20] Prepared by constructing heterocyclic rings containing amide or imide groups on polymeric supports,[1-20] these polymers have to be halogenated to exert biocidal action.[1-20] For such applications, N-halamine polymers have been prepared as powders [1,14,15] and as defined particles;[4-6] their production in definite particle size is very important for certain applications, such as their use in water filters. Suitable particle sizes can be obtained by using polymeric beads in their preparation [4-6], by preparing monomers and polymerizing them to form the beads, [4-6] and by grafting heterocyclic monomers onto silica particles,[12] Scheme 1a.

In this work N-halamine biocidal polymeric materials were produced in a definite particle size by preparing novel heterocyclic modified silica gels with particle sizes in the range 200-400 mesh, designed to increase the number of sites for halogenation; allowing a higher halogen loading for biocidal action, compared to the current modified silica gels reported in the literature, Scheme 1a.[12] At the same time the design improved the stability of the halogen attached to the heterocyclic ring by incorporating stronger electron donating groups than those suggested in the literature.[12]

In a different approach, sodium alginate was used to generate N-halamine polymers of different size particles, rather than the restricted size described previously in the literature.[4-6,12] Sodium alginate has been used previously to generate a matrix for the controlled release of water-soluble drugs and has been modified to carry a positive charge to act as bioactive material by adsorbing bacteria onto its surface.[21,22] In this work sodium alginate beads were prepared by blending water-insoluble N-halamine biocidal polymers

with sodium alginate as a matrix followed by cross-linking with calcium chloride. An N-halamine biocidal polymer with 5 available positions for halogenation was chosen to exert the biological activity of the generated beads,[1,17,18] Scheme 1b.

As well as the optimum conditions for bead preparation, the possibility of re-halogenation was investigated to improve the biological properties and prolong the life-time of the particles.

Biological activity of the prepared silica and beads was evaluated against examples of Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*).

EXPERIMENTAL

Materials

Barbituric acid, granulated tin, resorcinol, fuming nitric acid, sodium nitrite, toluene-2,6-diisocyanate, sodium alginate, bromine, iodine, 2-Cyano-functionalized silica gel (200-400 mesh, extent of labelling: 1.5-2.0 mmol/g per 7% carbon loading), 3-(Isocyanato)propyl-functionalized silica gel (200-400 mesh, extent of labelling: 1.2 mmol/g loading), calcium hypochlorite, gelatine, formaldehyde, gluteraldehyde and triethylamine were supplied by Sigma Aldrich Chemicals, UK. Sodium hydroxide, absolute ethanol, sulphuric acid, starch, sodium thiosulfate, potassium iodide, calcium chloride, N,N-dimethylformamide (DMF) (99.99%) and methanol (analytical grade reagents) were supplied by Fisher Chemicals, UK. Nutrient broth and nutrient agar were supplied by Oxoid. Cultures of *Staphylococcus aureus* and *Escherichia coli* were obtained from the faculty culture collection. Primary cultures on nutrient agar slopes and subcultures on nutrient agar plates were stored at 4^{o}C. All chemicals were used as obtained from suppliers without extra purification.

Preparation of 2-iminouramil-functionalized (2) and 3-(N-barbiturourea)propyl-functionalized (6) silica gels and their halogenation.

2-Cyano-functionalized silica gel **(1)** (1.14 g, 0.01 mol based on carbon load) was suspended in N,N-dimethylformamide (DMF) (30 ml). Uramil (2.86g, 0.02 mol) and triethylamine (600 µl) were added. The mixture was stirred at 120^{o}C for 24 hours. The product was filtered hot, washed with hot DMF (100 ml) and dried, (Scheme 2).

Analysis: FTIR (KBr): ν_{max} (cm^{-1}), 1701 and 1668 (C=O, heterocyclic ring), 1540 (C=N), 2942 (CH), 3124 (NH), 3435 (OH), 1100 (C-O and C-N). Solid ^{13}C NMR, 6-10 (aliphatic part carbons), 86 (CH of the ring), 151 (C=N), 152 and 162 (C=O). Elemental analysis; found (%, w/w): C, 12.3; H, 1.1; N, 6.4, calculated (%, w/w): C, 14.6; H, 1.7; N, 8.5.

The same procedure was followed to prepare 3-(N-barbiturourea)propyl-functionalized silica gel **(7)**. 3-(Isocyanato)propyl-functionalized silica gel (**6**) (using 1.3 g, 0.01 mol based on the carbon load) and uramil (2.86 g, 0.02 mol) were refluxed together at 120^{o}C in DMF (30 ml) in presence of triethylamine (600 µl). The product was filtered hot, washed with hot DMF (100 ml) and dried, Scheme 3.

Analysis: FTIR (KBr): ν_{max} (cm^{-1}), 1697, 1660 and 1611 (C=O), 2943 (CH), 3124 (NH), 3432 (OH), 1100 (C-O and C-N). Solid ^{13}C NMR, 7, 23, 40 (aliphatic part carbons), 86 (CH of the ring), 153, 154, 161, 162 (C=O). Elemental analysis; found (%, w/w): C, 13.1; H, 1.4; N, 7.1, calculated (%, w/w): C, 14.3; H, 1.6; N, 8.4.

Both of these reactions were performed equally successfully using sodium hydroxide instead of triethylamine and a mixture of DMF and absolute ethanol (2:1) as the solvent instead of pure DMF.

Halogenation of the novel modified silica gel was performed using NaOX (X = Cl, Br or I) and chlorination performed using commercial sodium hypochlorite (10%, w/v); by soaking the modified silica gel (1 g) in water (10 ml) and sodium hypochlorite (10 ml, 10% w/v) with stirring at ambient temperature for 1 hour. Bromination and iodination were performed similarly using sodium hypobrominate and hypoiodinate prepared by adding bromine or iodine to a sodium hydroxide solution (10%, w/v) gradually until pH 7.

The halogenation process was followed by FTIR spectroscopy [1] and the halogen/g content was determined using iodometric titration.[8] The values are given in Table 1.

The surface of the N-halamine biocidal modified silica gel was examined using SEM (scanning electron microscopy), Figure 1.

Preparation of N-halamine biocidal beads

N-halamine polyurethane **(14)** was prepared according to the methodology reported earlier.[1,17,18]

Diazotization of uramil

Uramil [23] **(11)** (5-aminobarbituric acid) (1.40 g, 0.01 mol) was dissolved in concentrated sulphuric acid (5 ml). The temperature was kept at 0^{o}C using an external ice bath. A cold solution of $NaNO_2$ (0.69 g of $NaNO_2$, 0.01 mol + water, 10 ml) was added drop-wise to the uramil solution with stirring to form the uramil diazonium salt **(12),**[1,17,18] Scheme 4.

Preparation of 1,3-dihydroxy-4(5-azobarbituric acid)benzene (13)

Resorcinol (1.1 g, 0.01 mol) and NaOH (5.5 g, 0.14 mol) were dissolved in water (20 ml) and added gradually to cold uramil diazonium salt **(12)**. The dark purple product that

precipitated was filtered, washed copiously with cold water, dried and weighed, producing 2.6 g (99% yield), [1,17,18] Scheme 4.

Polyurethane polymer preparation

Monomer **(13) (**2.6 g, 0.01 mol) and toluene-2,6-diisocyanate (0.01 mol) were heated in DMF (30 ml) for 5 hours at 90^{o}C. The reaction was cooled and 50 ml of methanol added. The brown product was filtered, washed copiously with methanol, dried and weighed,[1,17,18] Scheme 4.

Polyurethane chlorination

Polymer **(14)** (1 g) was stirred in NaOCl (10%, w/v) for 2 hours at ambient temperature. The resulting product was filtered, washed with chlorine-free water and dried,[17,18] Scheme 4.

Alginate bead Formation

Sodium alginate was dissolved in water (10 ml) to 2.0 % w/v. A suitable polymer (see below) was added, (2.5%, w/v), and the mixture was stirred for 30 min. The blend was added drop-wise to a solution of calcium chloride (100 ml, 6% w/v $CaCl_2$), Scheme 5. The beads were filtered and dried at 45^{o}C for 24 hours.

The blended polymers with alginate were;

1- Pre-halogenated polymer **(15)** (Scheme 4) (**AB1**), Figure 2.
2- Non-halogenated polymer **(14)** (Scheme 4) (**AB2**), Figure 2. **AB2** halogenated after their formation by soaking the beads (1 g) in sodium hypochlorite (10% w/v, 10 ml) for 30 min.

The alginate based beads were characterized using FTIR, SEM and TGA analysis. **AB1,** FTIR (KBr): ν_{max} (cm^{-1}), 1593 (broad band, C=O), 2319-3634 (OH carboxylic), 3342

(OH), 3030 (CH aromatic), 2917 (CH aliphatic), 3141 (NH), 1071 (C-O), 1023 (C-N), 1549 (C=N), 658 (N-Cl) and 1412 (N=N). TGA: T_o (dm/dT_{max}), 60, 120, 210, 280, 300, 460, 700 and 1000. **AB2** (before halogenation), FTIR (KBr): ν_{max} (cm^{-1}), 1612 (broad band, C=O), 2433-3678 (OH carboxylic), 3334 (OH), 3024 (CH aromatic), 2949 (CH aliphatic), 3251 (NH), 1126 (C-O), 1112 (C-N), 1550 (C=N) and 1469 (N=N). TGA: T_o (dm/dT_{max}), 80, 140, 220, 340, 400, 450, 550, 700 and 1000. SEM images for the dried beads are shown in Figure 3.

Determination of the biological activity of the prepared beads matrix and modified silica gels.

Agar plate method

This experiment was performed only for the modified silica gels. Nutrient agar (Oxoid) was prepared (250 ml), held molten at 50^0C and 1.0 ml of a 24hr nutrient broth culture of either *Staphylococcus aureus* or *Escherichia coli* was added as an inoculum. The seeded agar was poured into plates; two plates for each type of bacterium, Gram-positive and Gram-negative. Wells, 5 mm diameter were cut into the agar, and small amounts of each type of modified silica gel (0.03 g) were placed in the well in the middle of the plate. The experiment was performed in triplicate, three plates for each polymer.[1] Plates were incubated for 24 hours at 37ºC and the inhibition zones around the well were recorded, Figure 4.

Stirred flask method

The biological activity of the modified silica gels and alginate beads was determined by studying their effect on bacterial viability. For the alginate beads: bacterial suspension (either *E. coli* or *S. aureus*) was prepared by inoculating 10 ml of nutrient broth in a

universal bottle. The culture was incubated at 37^{o}C for 17 hours and 0.1 ml of this suspension used to inoculate 5 different universal bottles each containing 10 ml of fresh nutrient broth which were incubated at 37^{o}C for 17 hours. Individual cultures were treated as follows; **AB1 (**0.5 g), **AB2** (0.5 g), **AB2** halogenated form (0.5 g), sodium alginate beads (control, no blended polymer) and the fifth was used as a bacterial control. Viability was followed by the "Miles and Misra" method [24] at different time intervals.[17] For the modified silica gel the same method was applied. Three universal bottles were used; the first treated with halogenated modified silica gel (0.5 g), the second treated with non-halogenated modified silica gel (control, 0.5 g) and the third was used as a bacterial control. The viability was followed as above.

Bead regeneration

Optimum conditions were determined for "recycling" (re-halogenating) the beads.

Changing the nature of the cross-linker

Changing the ratio of calcium chloride

Using calcium chloride was the most successful preparation method; the beads were formed by dropping the polymer (2.5%, w/v)/alginate (2.0%, w/v) mixture in 10 ml distilled water into a solution of calcium chloride (100 ml). The experiment was repeated using different calcium chloride concentrations 2, 4, 6, 10, 20 and 40% (w/v). These concentrations were used both with and without curing. Curing was performed by heating the beads in the calcium chloride bath at 40^{o}C for 12 hours while non-cured samples were stirred in calcium chloride for 1 hr at ambient temperature. The beads were filtered, washed with distilled water and dried.

Using gelatine with calcium chloride

Gelatine was used in different concentrations 1-3% (w/v) with calcium chloride (10% w/v). Polymer (5%, w/v)/alginate (3%, w/v) mixture (in 10 ml distilled water) was dropped into a bath containing the mixture of calcium chloride and gelatine. The experiment was repeated using different ratios of gelatine. The beads were filtered, washed with distilled water and dried.

Aldehydes; formaldehyde and gluteraldehyde

Aldehydes, formaldehyde and gluteraldehyde, with different ratios (1, 2, 4, 8 and 10% w/v) were added during bead preparation as cross-linkers and the preparation method modified as follows: Sodium alginate was dissolved in distilled water (2.0% w/v, 10 ml). The aldehyde (formaldehyde or gluteraldehyde) was added [22]. The mixture was stirred for 1 hr, polymer (**14** or **15**) was added to 2.5 %, w/v and stirring continued for 30 min. The beads did not form so the polymer ratio was decreased from 2.5% to 1% and then to 0.05% (w/v) and the experiment was repeated but still no beads formed.

Changing the ratio of sodium alginate

Sodium alginate ratio was changed (1, 2, 3 and 4% w/v) during mixing with the polymer (**14** or **15**) (2.5% w/v), in 10 ml distilled water, followed by dropping into a calcium chloride bath (10% w/v, 100 ml). The beads were filtered, washed with distilled water and dried.

Changing the polymer ratio

The polymer (halogenated and non-halogenated) **(14** and **15)** ratio was changed, (2, 3 and 5% w/v), to achieve the maximum load of polymer on the beads. The blend of polymer/alginate (3%, w/v, in 10 ml distilled water) in each case was dropped into

calcium chloride bath (10%, w/v, 100 ml). The beads were filtered, washed with distilled water and dried.

The biological activity of the prepared beads under different conditions was quantified, as the effect of the beads on bacterial viability; determined as described before (using stirred flasks method) [17] for both *E. coli* and *S. aureus*.

Swelling behaviour of the beads

Beads, **AB1** and **AB2**, prepared under different conditions, (0.05 g) were soaked first in tap water and then distilled water in two different universal bottles for 24 hours each. The beads were filtered, the surface water absorbed with paper tissues, and the swelling ratio was calculated using equation 1.

Equation 1: Determination of swelling ratio.

% Swelling = [(Polymer weight after soaking – Polymer weight before soaking) / Polymer weight before soaking] X 100.

RESULTS AND DISCUSSION

In order to improve the particle size of N-halamine biocidal materials, uramil was reacted with two different types of modified silica gels; 2-Cyano-functionalized silica gel (200-400 mesh) and 3-(Isocyanato)propyl-functionalized silica gel (200-400 mesh). The reaction was an addition to the cyano or the isocyanate groups loaded on silica under basic conditions which was performed successfully using triethylamine or sodium hydroxide as catalysts. The resulting products retained the stability of the halogen on the polymer.

Structures of modified silica of the same type in the literature contain heterocyclic rings with substituted methyl groups (Dimethylhydantoins).[12] These methyl groups, as electron

donating groups, stabilize the halogen attached to the heterocyclic ring.[12] The novel modified silica in this study has stronger electron donating groups that have increased the stability of the halogen attached to the heterocyclic ring, such as the amino group in modified silica **(2)** and the amide group in modified silica **(7)**.

The FTIR data of modified silica **(2)** shows the disappearance of the cyano group signal (2216 cm^{-1}) of 2-cyano-functionalized silica gel and the appearance of the carbonyl group signals of the heterocyclic ring at 1701 and 1668 as well as the NH signal at 3124 cm^{-1}. The ^{13}C NMR indicates the appearance of the carbonyl carbon signals of the heterocyclic ring at 152 and 162 ppm while the CH carbon of the heterocyclic ring gives a signal at 86 ppm.

Similar results were obtained for modified silica **(7)**; from the FTIR, the carbonyl peaks of the heterocyclic ring and the urea side chain appear at 1697, 1660 and 1611 cm^{-1} while the NH appears at 3124 cm^{-1} and from ^{13}C NMR the carbonyl peaks appear at 153, 154, 161 and 162 ppm. Therefore, FTIR and ^{13}C NMR showed that the loading of the uramil to the modified silica gels was successful.

SEM was performed to investigate the particle size diameter, and to demonstrate that the particles were not damaged during the reactions, Figure 1. It can be seen that the silica particles still keep their average diameter but smaller particles were scratched. However, the small fragments that separated from the silica particles will not restrict use of these particles in some applications, such as water filters.

Biological activity of the N-halamine modified silica gels was investigated using an agar plate technique, Table 2.

From table 2, it can be seen that all the halogenated derivatives of both modified silica gels **(2** and **7)** have an inhibitory effect on both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The effect on *S. aureus* is generally greater than that on *E. coli* and the biological activity of the N-halamine modified silica gel derivatives (**3**-**5**) is lower than those derived from **(7)** (i.e.: **8**-**10**).

The biological activity of the halogenated derivatives of modified silica gel **(7)** was quantified by determining their effect on bacterial viability. Chlorinated modified silica (**8**) succeeded in achieving a 3 log reduction in viability in 7 hours for *E. coli* and a 4 log reduction for *S. aureus* in the same time period, Figures 5a and 5b respectively. The brominated modified silica **(9)** achieved a 4 log reduction in the viability of both *E. coli* and *S. aureus* in 7 hours, (Figures 5c and 5d respectively). The most powerful effect was achieved by iodinated modified silica. It achieved a 9 log reduction in 15 min for both *E. coli* and *S. aureus*, (Figures 5e and 5f respectively).

From the previous results it can be seen that halogenated modified silica succeeded in reducing the viability of both Gram-positive and Gram-negative bacteria. As expected from previous results,[1,17] the iodinated modified silica has the maximum biocidal power. These results also show it was possible to improve particle size whilst keeping moderate biological activity. Stability of the halogen attached to the heterocyclic ring of silica was improved by using a heterocyclic ring supported with stronger electron donating groups. In spite of the presence of these strong electron donating groups, attached to the heterocyclic ring, the modified silica showed moderate biological action which maintains the balance between stability and biological activity. In comparison with similar types reported in the literature [4-6] this stability may be reflected in reduced biological power of

the modified silica. However, it may prolong the active life of the modified silica without re-halogenation as the silica will not easily loose the halogen.

Biological activity of the modified silica is lower than that of the powdered N-halamine biocidal polymer, prepared by our group;[1,17,18] as the number of function groups on silica is lower, which affects the number of heterocyclic rings that can be loaded onto silica. In addition, the N-halamine biocidal polymers, in powder form, present a greater surface area which would improve contact with the bacteria.

Sodium alginate beads matrix

The final particle size of the N-halamine modified silica is dependant on the particle size of the silica used as a starting material (2-Cyano-functionalized and 3-(Isocyanato)propyl-functionalized silica gels). But for some applications there is a need for larger particles. Increasing the size of the starting silica however, may result in reducing the biological activity of the product because the number of the functional groups on the particle surface, which are used to react with uramil, will decrease. Most of the modified silicas, and other beads reported in the literature, have the same problem.[4-6,14]

A new method has been described here, based on blending N-halamine polymer powders **(14** or **15)** with sodium alginate followed by cross-linking with calcium chloride, to obtain larger-size insoluble particles. This method can be used to produce different sized particles because the particle size will depend on the dropper used; enabling production of a range of sizes depending on the required application. At the same time, the bioactive polymer content in the particle matrix can be increased to improve the biological activity. Similar matrices have been used previously as control-release systems for releasing

water-soluble antibiotics.[21,22] The method was modified to be used with insoluble polymers; the biocidal activity in this case depends on halogen ion release from the beads or contact with the outer surface of the beads.

Two methods of preparing beads were compared; mixing chlorinated polymer **(15)** directly with sodium alginate, (**AB1**) and mixing non-halogenated polymer **(14)** with sodium alginate followed by chlorination, (**AB2)**.

The resulting beads were characterized using FTIR, TGA and SEM. FTIR and TGA confirmed the presence of both sodium alginate and N-halamine polymers in the blend. Characteristic signals for the heterocyclic polymers appeared in the FTIR such as the azo group (1425 cm^{-1}), NH (3230 cm^{-1}) and carbonyl group (1601 cm^{-1}). The N-Cl signal appeared in the FTIR spectrum at 658 cm^{-1}. TGA peaks for **AB1** showed a shift to lower values compared to **AB2** (non-halogenated) because the burning rate of the halogenated polymer is faster than that of the non-halogenated polymer due to the conversion of NH to N-Cl.[25] For example, the water signal which appears from 80-100^{o}C has moved down to 75-85^{o}C. Sodium alginate and the blended polymer showed peaks of decomposition of their main chain from 200-300^{o}C.[26] These peaks appear for both of them when the TGA is performed for each one separately. The beads were examined by SEM, Figure 3, as well as photo imaging, Figure 2.

The effect of **AB1** and **AB2** on bacterial (*E. coli* and *S. aureus*) viability was quantified. During the biological activity three controls were used; **AB2** (non-halogenated), sodium alginate beads (without blended polymers) and bacterial control (no beads).

AB1 achieved a 9 log reduction within 3 hours for *E. coli* while the halogenated form of **AB2** achieved 1 log reduction in 5 hours, Figure 6a. For *S. aureus*, **AB1** achieved a 9 log

reduction in 5 hours while **AB2** (halogenated form) only 1 log reduction in 5 hours, Figure 6b.

The results indicated that **AB2** (halogenated form) has low biological activity, due to the longer halogenation time required to enable halogen penetration to the polymer particles. These data suggest that the best way to prepare the beads is that used for preparing **AB1** i.e.: mixing chlorinated polymer directly with sodium alginate.

AB1 showed better biological action than the modified silica but lower than the polymer powder itself [17] perhaps because the ions take longer to diffuse out of the beads. The beads release the same quantity of ions as calculated but over a longer time period - 6 hours to release the same amount of halogen as released by the powder.[17]

At the same time, the contact effect between the polymer and the cells will be low as the cells can only contact the outer surface of the beads. Moreover, the effective amount of polymer in the beads is lower than that used directly in the case of polymer powder evaluation.

Re-halogenation

To enable bead re-halogenation, the optimum conditions for bead preparation were identified. Polymer was blended with sodium alginate and added drop-wise to baths containing different concentrations of calcium chloride: to yield beads with different ratios of calcium chloride content 2, 4, 6, 10, 20 and 40% (w/v), cured and non-cured. Curing was performed at 40^{o}C for 12 hours while non-cured samples were stirred in calcium chloride for 1 hr after drop-formation at ambient temperature.

It was noticed that increasing calcium chloride content, with or without curing, decreases the swelling behaviour of the beads (Tables 3 and 4), which in turn affects the biological

activity; however, it increases the possibilities of beads re-halogenation. Raising calcium chloride ratio over 10% (w/v), with or without curing, reduces the biological activity of the beads (**AB1** and **AB2**) while using this ratio (10% calcium chloride) with curing maintains a good balance between biological activity and re-halogenation, Figures 7a and 7b. This ratio (10% w/v) with curing, enables re-halogenation up to 3 times without any damage to the beads which had been noticed with re-halogenation if the beads had been prepared using a low calcium chloride ratio and without curing. From Figure 7a, the beads prepared with 10-20% (w/v) calcium chloride (with curing) achieved a 3 log reduction in 5 hours for *E. coli* while beads formed with 40% (w/v) calcium chloride with curing did not show good biological activity. Similar behaviour was noticed with *S. aureus* and with 40% (w/v) calcium chloride the beads achieved 1 log reduction, Figure 7b.

Similar results were obtained when comparing the biological activity of cured and non cured beads prepared using 10% (w/v) calcium chloride, Figures 8a and b. The non-cured beads show more biological activity perhaps because without curing, the beads swell more enabling ion release.

Other types of cross-linkers, such as Aldehydes,[22] were investigated instead of salts. Unfortunately beads did not form. Several trials were carried out using different ratios of aldehydes (1, 2, 4, 8 and 10% w/v) but no insoluble material was formed.

Sodium alginate concentration was varied (1, 2, 3 and 4 % w/v) to investigate the effect on the formation of beads. Increasing the ratio of sodium alginate increases the viscosity, making the formation of “drops” difficult, but enhances the re-halogenation character of the beads by increasing the cross-linking possibilities. These different concentrations of

alginate were mixed with the N-halamine polymer (2.5% w/v) in water (10 ml) and dropped into a calcium chloride bath (10% w/v) with and without curing.

Increasing the ratio of the polymers (2, 3 and 5% w/v) increases the biological activity of the beads (both **AB1** and the halogenated form of **AB2)**. The beads were prepared by dropping into a bath containing 40% (w/v) calcium chloride as a cross-linker with curing overnight at 40^{o}C and the sodium alginate ratio was also increased to 3%. It was seen that **AB1** achieved in 5 hours a 3 log reduction against *E. coli* while **AB2** (halogenated form) achieved a 2 log reduction against the same bacterium, Figure 9a. For *S. aureus* it was noticed that **AB1** achieved a 4 log reduction in 5 hours while **AB2** (halogenated form) achieved a 2 log reduction in the same time period, Figure 9b. Increasing the polymer ratio increased the biological activity of the beads despite cross-linking at high concentration of calcium chloride (40%) compared to results reported using the same calcium chloride ratio with low polymer concentration (2.5%), Figures 9a and 9b.

Gelatine was also investigated at concentrations of 1-3% (w/v) with calcium chloride (10% w/v). Using gelatine enabled a more spherical shaped bead to develop and supported re-halogenation process as it can share in the complexation with the calcium ion. However, using gelatine with **AB1** may result in losing halogen through exchange between the halogenated polymer and gelatine during bead formation - which may reduce the biological activity of the beads.

From the previous data, the best blend for bead formation is: 3% (w/v) sodium alginate and 5% (w/v) N-halamine biocidal polymer **(15),** cross-linked with calcium chloride 10% (w/v) followed by curing at 40^{o}C overnight.

The swelling behaviour of the beads

The swelling behaviour of the beads was determined in tap and distilled water for cured and non-cured beads, Tables 3 and 4. From tables 3 and 4, non-cured beads swell more than cured and increasing the concentration of calcium chloride decreases swelling. At the same time increasing the polymer ratio increases swelling behaviour, even with curing, Table 5.

The previous data indicate the possibility of producing blended beads of different sizes and good biological activity which can support many applications requiring flexible flow rate. Although the beads are larger than the prepared modified silica gel, they showed better biological action due to the presence of the blended N-halamine polymer powder. Optimising bead preparation supports bead re-cycling. Producing beads as a blend with water insoluble polymers provides a new method of bead production containing water insoluble polymers and of different sizes that can release bioactive ions (halogen). Additional effects may be explained on the basis of contact between the bacterial cells and the outer surface of the beads. Bioactive beads of large size and good swelling behaviour may encourage employing this type of bead in water filters in the future without restricting the water flow-rate.

CONCLUSIONS

Particle size was improved by loading bioactive heterocyclic rings onto modified silica gels and preparing beads containing bioactive polymer powder. Bead formation generated bioactive beads of different sizes that release bioactive ions rather than water soluble molecules. The best conditions for bead formation was mixing 3% (w/v) sodium alginate with 5% (w/v) N-halamine biocidal polymer **(15)** and cross-linking them by dropping

into calcium chloride solution (10% w/v) followed by curing at 40^{o}C overnight. Blending pre-halogenated polymer with alginate (**AB1**) is better than blending the beads with non-halogenated polymer followed by halogenation **(AB2**).

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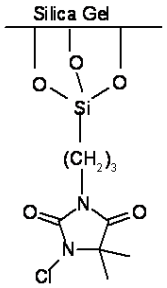
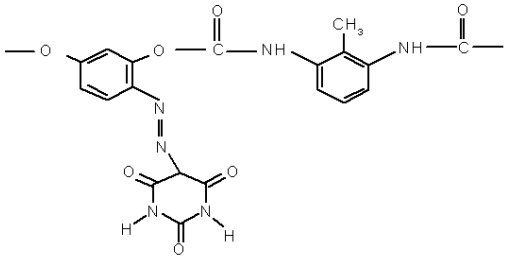
Scheme 1 a) An example of modified silica with heterocyclic rings, b) N-halamine polymer to be blended with sodium alginate.

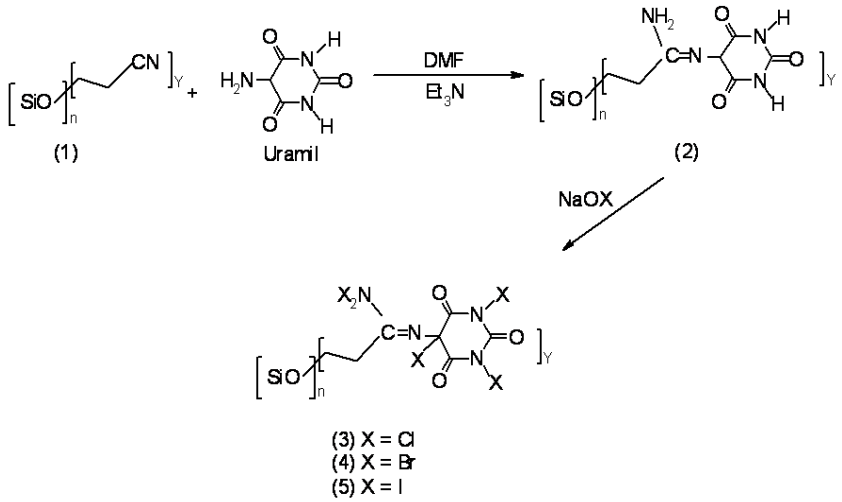


(a)

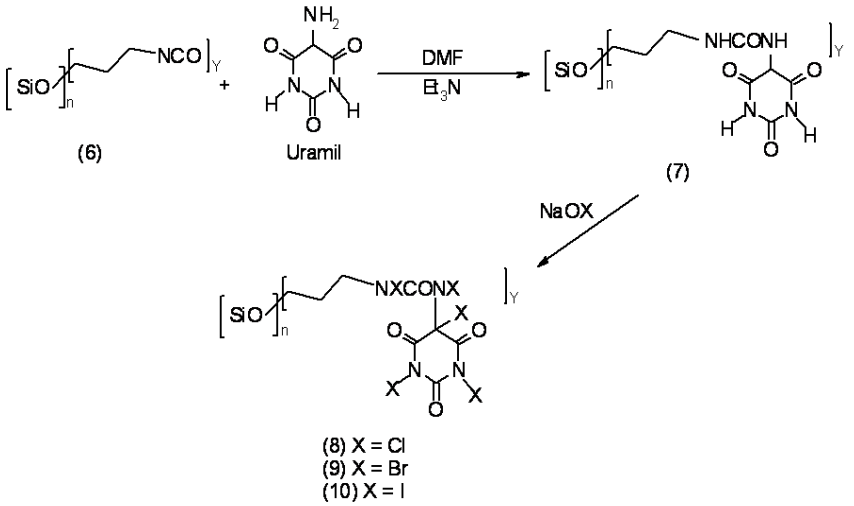


(b)

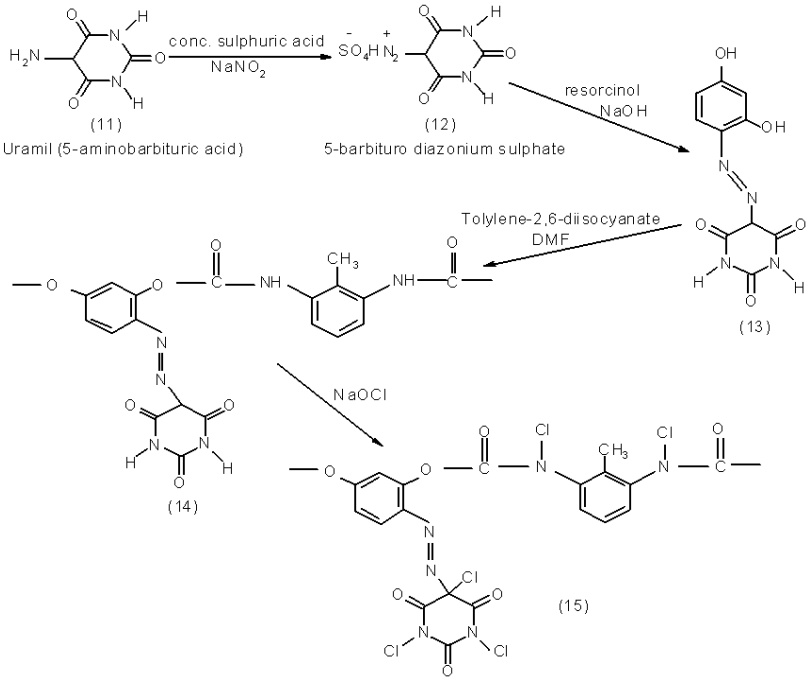
Scheme 2 Preparation of 2-iminouramil-functionalized silica gel and its halogenation.



Scheme 3 Preparation of 3-(N-barbiturourea)propyl-functionalized silica gel and its halogenation.



Scheme 4 Preparation of the N-halamine polyurethane and its chlorination.



Scheme 5 Expected complexation product between the alginate and calcium ions.

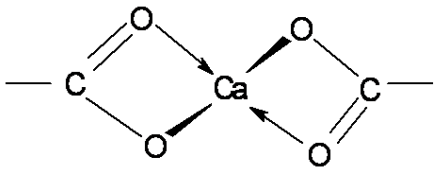


Figure 1 Modified silica gel particles SEM before and after halogenation; (a) and (b): silica gel particles before reaction while (c) and (d): silica gel particles after halogenation.

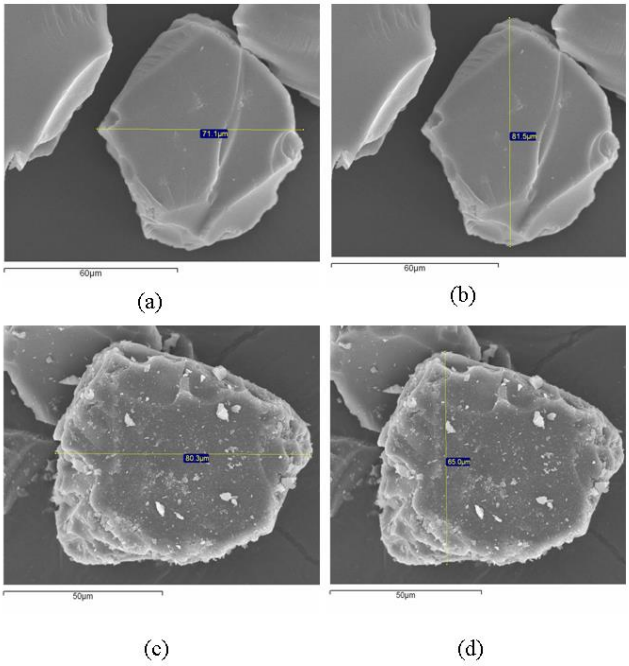


Figure 2 Photographs of the alginate beads in their hydrated and dried status; (a) **AB1** before drying, (b) **AB2** before drying and halogenation, (c) **AB1** after drying and (d) **AB1** after drying (scale in mm).

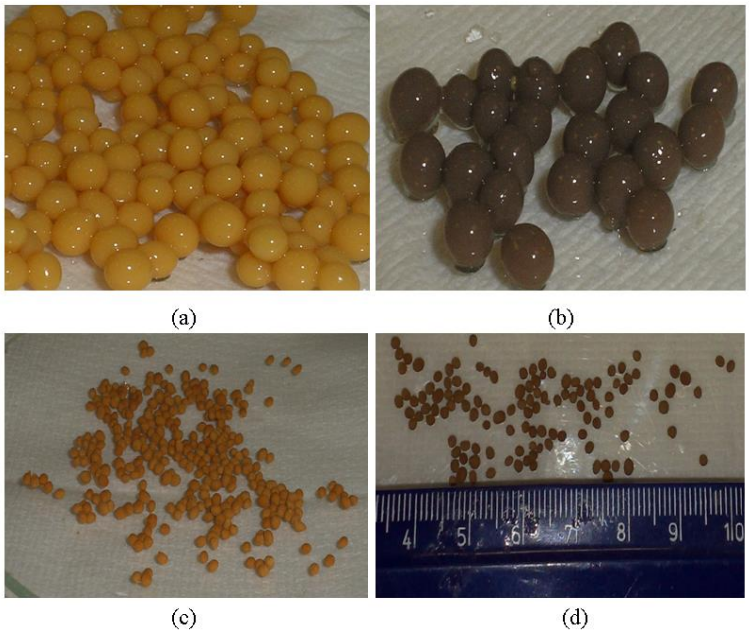


Figure 3 SEM of the beads before and after curing; (a) **AB1** non-cured, (b) **AB1** cured, (c) **AB2** cured and (d) Close-up of **AB1** cured.

Note: Beads were cured by heating in calcium chloride at 40^{o}C after formation.

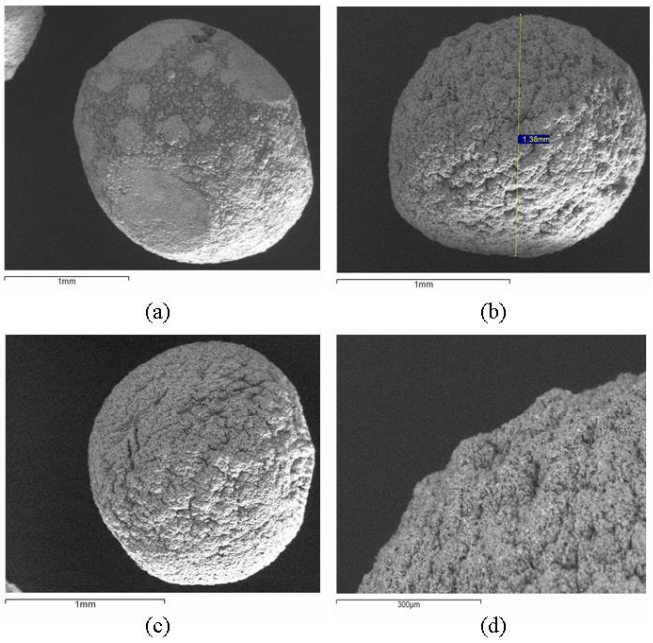


Figure 4 Inhibition zones resulting from one of the N-halamine biocidal modified silica gels, in triplicate.

Figure 5 Effect of halogenated [chlorinated (a and b), brominated (c and d) and iodinated (e and f)] heterocyclic modified silica gels (**8-10**) on *E. coli* and *S. aureus* viability, respectively. BC is the bacterial control, PC is the non-halogenated heterocyclic modified silica gel (control) and T is the halogenated heterocyclic modified silica gel.

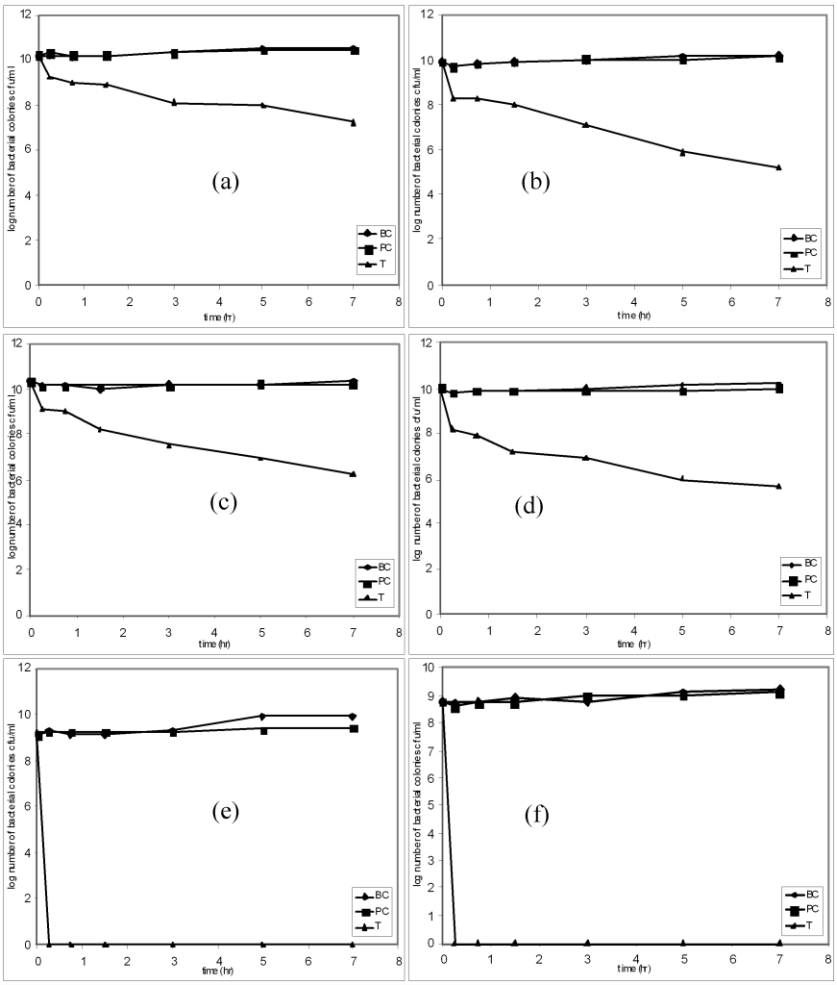


Figure 6 biological effect of the beads matrix on (a) *E. coli* and (b) *S. aureus*. BC bacterial control, AC: sodium alginate beads as a control, PC: **AB2** (non-halogenation form) as a control, Beads 1: **AB1** and Beads 2: **AB2** (halogenated form).

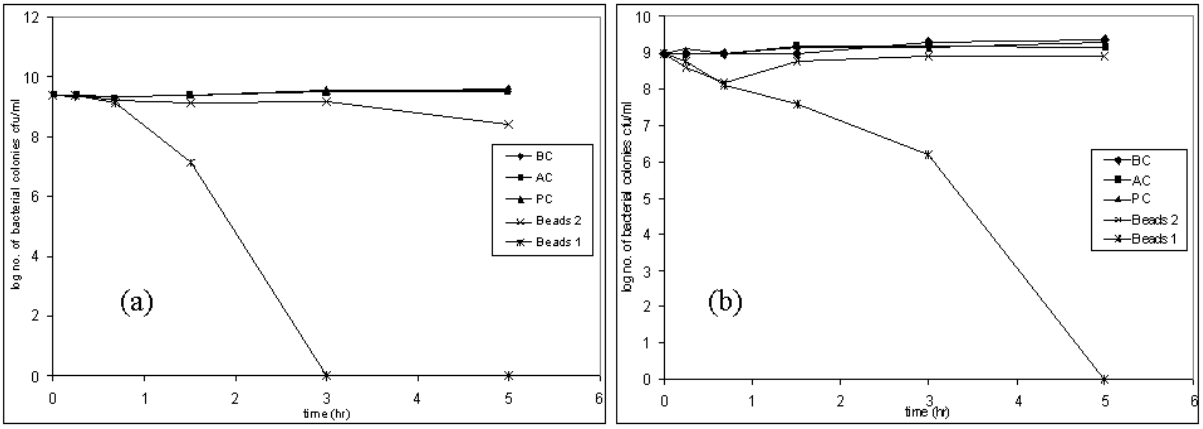


Figure 7 Effect of changing the cross-linking agent (calcium chloride) (with curing) on the biological activity of the beads (**AB1**) against (a) *E. coli* and (b) *S. aureus*. BC is the bacterial control.

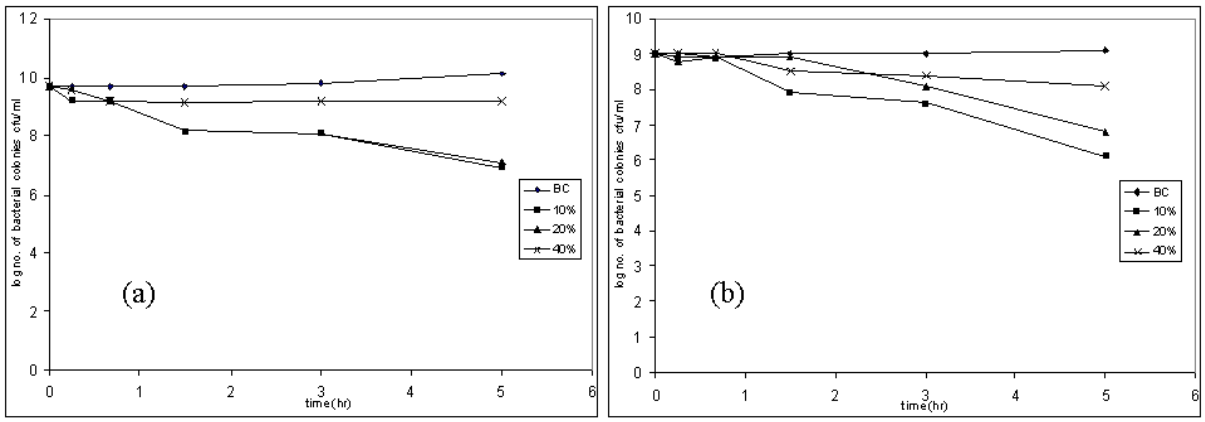


Figure 8 Comparing the biological activity of the cured and non cured forms of **AB1** against (a) *E. coli* and (b) *S. aureus*. BC is the bacterial control.

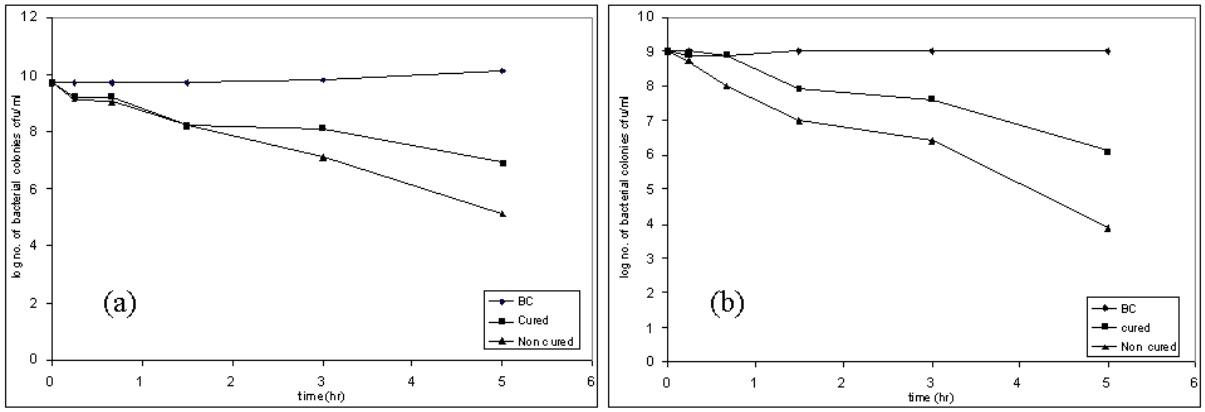


Figure 9 Effect of increasing the polymer ratio, up to 5% (w/w), on the biological activity of the beads against (a) *E. coli* and (b) *S. aureus*. BC is the bacterial control, Beads 1 is **AB1** and Beads 2 is **AB2** (halogenated form).