Novel N-halamine Biocidal Polymers for Water Purification

by

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Declaration

I declare that the work in this study is my own work and any paraphrased or summarised statements from the work of other researchers were referenced.

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Abstract

Novel heterocyclic biocidal polymers (N-halamines and poly Quat’s) were prepared for disinfection purposes. They were designed to increase bioactive sites and to stabilize the attached halogen ions to the N-halamine polymers. Two methods were used in their preparation; reacting linear polymers (polyacrylonitrile and polyethylacrylate) with uramil and co-polymerizing novel azo monomers (based on uramil) with diisocyanates (toluene-2,4-diisocyanate and tolylene-2,6-diisocyanate). The resulting polymers were halogenated to form N-halamine polymers and acidified to form poly Quat’s. The biological activity of the prepared monomers and polymers was evaluated. The optimum halogenation conditions for N-halamine polymers preparation were determined to improve the biological activity.

The particle size of the N-halamines was modified as follows; loading uramil to modified silica gels, 2-Cyano-functionalized and 3-(Isocyanato)propyl-functionalized silica gels, and by blending N-halamine polymers with sodium alginate followed by cross-linking with calcium chloride. Optimum preparation conditions and recycling possibilities of the alginate beads were determined. The biological activity of both modified silica and beads was evaluated.

A low cost version of the N-halamine polymers was prepared by cross linking polyepichlorohydrin with m-phenylenediamine followed by a reaction with cyanuric acid. A multi-filtration system based on this polymer and its halogenated form was designed to produce halogen-free water. The system is formed from three columns; sand, halogenated
polymer and non-halogenated polymer columns. The system regenerability and life-time was determined. A new design for a water purification system (multi-filtration), based on this polymer, was suggested and has potential for development at pilot scale. Some questions have been raised regarding the practicality of the main aim of this project - the potential for using halogenated polymers in water disinfection for water supply. However, a range of other applications have been suggested during the course of the thesis.

A new theory about the mode of action of N-halamine polymers has been created based on a combination of factors; contact, release and changing the nature of the bacterial medium. The theory was supported with microbiological and metabolomics evidence.
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Education. And finally I would like to thank my family (my wife Mona, my parents and my brother) for supporting me during this study.
Abbreviations and units

EPA: Environmental Protection Agency.

Poly Quat's: Poly quaternary ammonium salts.

FTIR: Fourier Transform Infrared.

NMR: Nuclear Magnetic Resonance.

LCMS: Liquid Chromatography Mass Spectrometry.

SEM: Scanning Electron Microscope.

TGA: Thermal gravimetric analysis.

GPC: Gel Permeation Chromatography.

PBS: Phosphate Buffered Saline.

PCA: Principle Components Analysis.

BPI: Base Peak Intensity Chromatogram.

OPLS: Orthogonal Projection to Latent Structures Analysis.

OD: optical density.

PEG: Polyethyleneglycol.

DMF: N,N-Dimethylformamide.

DMSO: Dimethylsulphoxide.

MilliQ water: Ultrapure Laboratory Grade Water.

Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cGMP: Cyclic Guanosine monophosphate.

ppm: parts per million.
\(d\): density.

\(v/v\): volume/volume.

\(w/w\): weight/weight.

\(\mu l\): microlitre.

\(ml\): millilitres.

\(mmol\): millimole.

\(^\circ C\): degree Celsius.

\(g\): gram.

\(mesh\): number of meshes in a square inch.

\(cfu/ml\): colony forming units per millilitre.

\(cfu/g\): colony forming units per gram.

\(g\): rcf = Relative Centrifugal Field.

\(Rpm\): revolutions per minute.
Materials and Instrumentation

Materials

The following materials have been used in this study; all chemicals were used as obtained from suppliers without extra purification:

Barbituric acid (98%), tin (granular, purum), resorcinol (>98%), m-phenylenediamine (>99%), nitric acid (reagent grade, fuming, >90%), sodium nitrite (ACS reagent, >97%), polyacrylonitrile (average MW 150,000, typical), polyethylacrylate (average MW 95,000 by GPC in toluene), toluene-2,4-diisocyanate (95%), toylene-2,6-diisocyanate (97%), bromine (reagent grade), iodine (ACS reagent, >99%), 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 labeling: 1.2 mmol/g loading), calcium hypochlorite (technical grade), cyclic guanosine-1',3'-monophosphate (cGMP) (>98%, HPLC), perchloric acid (ACS reagent, 70%), potassium bromide (photographic grade), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes, 99.5%), sodium hypochlorite (reagent grade, available chlorine 10%-15%), gelatine (solid, from cold water fish skin), formaldehyde (37% in water), glutaraldehyde (27% in water), sodium alginate (powder), cyanuric acid (98%) and triethylamine (>99%)

were supplied by Sigma Aldrich Chemicals, UK.

Polyepichlorohydrin (average MW ~700,000, GPC), sodium hydrogen carbonate (>99%), sodium hydroxide (>95%, pellets), hydrochloric acid (37%), potassium permanganate
(>99%), absolute ethanol (ACS reagent, 99.5%), acetonitrile (HPLC grade, 99.9%), sulphuric acid, starch (extra pure, Across Organics), sodium thiosulphate pentahydrate (crystalline), potassium iodide (granular, free flowing), N,N-dimethylformamide (99.99%) and methanol (analytical grade reagents) were supplied by Fisher Chemicals, UK.

Nutrient Broth and Nutrient Agar were supplied by Oxoid, Ltd, UK.

Cultures of *Staphylococcus aureus* and *Escherichia coli* were obtained from the Faculty culture collection. Primary cultures on nutrient agar slopes were stored at 4°C. Subcultures on nutrient agar plates were stored at 4°C.

**Instruments**

The analysis was performed at University of Surrey. The following instruments have been used in this study

**Infra Red (FTIR)**
- Perkin Elmer System 2000 FTIR (Fourier Transform Infrared, PerkinElmer LAS Ltd, UK) (32 scans).

**Nuclear Magnetic Resonance (NMR)**
- Bruker DRX-500NMR with Ultra Shield Bruker magnet (11.7467 Tesla magnetic field strength) was used for solution $^1$H NMR (16 scan) and $^{13}$C NMR (1024-10000 scans) (Bruker BioSpin Ltd, UK).
- Bruker AV-300NMR with Oxford magnet (7.04925 Tesla magnetic field strength) was used for solid $^{13}$C NMR (10000 scans) (Bruker BioSpin Ltd, UK).

**Liquid Chromatography - Mass Spectrometry (LCMS)**

- ACQUITY TQD UltraPerformance LC (Waters Ltd, UK).
- Q-Tof Premier MICROMASS mass spectrometer (Waters Ltd, UK).
- Column: ACQUITY UPLC BEH C18 2.1x100mm 1.7µm.
- Pre-column: VanGuard™ Pre-column, 2.1x5mm, ACQUITY BEH C18 1.7µm.
- Conditions; Column = C18 (BEH), Solvent A = water and 0.1% formic acid, Solvent B = acetonitrile and flow = 0.2ml/min.
- Run gradients: 0mins = 100% A - 0% B, 5mins = 100% A - 0% B, 25mins = 10% A - 90% B, 35mins = 10% A - 90% B, 36mins = 100% A - 0% B and 45mins = 100% A - 0% B.

**Scanning Electron Microscope (SEM)**

- EMITECH K575X sputter coater (Peltier cooled) for plating with gold under argon in an angle shape (different directions at 45° angle, 5 nm layer thickness).

**Elemental Analysis**

- CE440 elemental analyser (Exeter analytical Inc., UK).
Thermal gravimetric analysis (TGA)
- TGAQ500 thermal gravimetric analyser (TA instruments, UK).
- Conditions: Ramp, under nitrogen. Rate = 10°C/min up to 1000°C.

Sonication

Centrifugation
- Eppendorf centrifuge 5415R (Eppendorf Ltd, UK).
- Heraeus, Function line labofuge 400 (Heraeus Instruments, Germany).
- Beckman J2-21M/E centrifuge (Beckman coulter Ltd, UK).

Freeze-Drying
- Modulyo Freeze dryer (Edwards Ltd, UK).

Statistical Analysis
- MarkerLynx (Waters Ltd, UK).
- SIMCA-P+ (MKS Instruments UK ltd - Umetrics UK).
Chapter 1

Introduction, Review and Aim
1.1. Introduction

If moisture and nutrition are available micro-organisms can grow and some of these are harmful to humans, animals and plants. These micro-organisms may be present in water, air, clothes and on surfaces. Their transfer to living beings could cause many diseases. Drinking water, as an example, can be contaminated with different types of micro-organisms such as *Escherichia coli*, *Staphylococcus aureus*, coliform bacteria and *Cryptosporidium*, table 1.1 [1]. The level of the first three should not exceed 0 per 100 ml because of their harmful effects. Special action should be taken in the case of *Cryptosporidium*, table 1.1, contamination due to its resistance to chlorine [1], according to Water Supply (Water Quality) Regulations (2000) and their amendment (2007) (UK regulatory standard) [1].

To prevent contamination or infection with micro-organisms to water or surgical instruments, sterilization or disinfection should be applied [2]. Sterilization can be achieved using heat, incineration, boiling, autoclaving and radiation [2, 3]. This results in killing many micro-organisms including spores [2, 3]. Disinfection can be performed using chemicals and in this case the micro-organisms may be killed but not all spores [2, 4]. The chemicals used in the disinfection process are called antimicrobial agents [2, 5]. Any substances, or mixtures of substances, that are used to kill or inhibit the growth of harmful microorganisms (bacteria, viruses, or fungi) are called antimicrobial agents [2, 5]. There are 300 different active ingredients in antimicrobials present on the market as sprays, liquids, powders, and gases [2]. The registered number of these antimicrobial
agents exceeds 8000 types, registered by the United States Environmental Protection Agency (EPA); 50% of them are used to control the microorganisms in hospitals and similar environments [2]. They have been divided into; non-public health products (used for infected animals and any environment away from humans) and public health products (used to control infectious microorganisms) [2]. Public health antimicrobial products are of different types such as; bactericidals (bactericides or cidals, used to kill bacteria), bacteriostats or static agents (inhibitors to bacterial growth) and fungicides (used to kill fungi) [2]. Generally chemical substances designed to kill micro-organisms are called biocides.

From small molecules to polymers

Many chemicals, because of their biocidal action, have been used for decades for disinfection purposes such as: acids, bases, aldehydes, alcohols, quaternary ammonium salts, heavy metals, phenolic compounds, chloramines, sulphur containing compounds, stabilized chemical mixtures, ozone, peroxides and halogens [2, 5].

Acids can kill bacteria if pH < 3 after prolonged exposure time while bases have some effect only on Gram-positive cocci, rods, spore-formers, and some viruses [2]. Alcohols are effective against bacteria and algae and have limited effect on viruses and endospores [2, 5]. Chloramines are used in water treatments to improve the taste and odour but their action is very slow as disinfectants [2, 5]. Quaternary ammonium salts were considered as detergents and used as disinfectants for skin [2]. Heavy metals were one of the oldest disinfectants in use (used by ancient Egyptians); such as mercuric
chloride and silver nitrate [2, 5]. Halogens, especially chlorine, are considered as a group, the most effective disinfectants produced on a commercial level [2, 5].

In addition to the required application, the choice of a biocide depends on several factors such as: cost, potency (scope of kill), persistence, speed of kill, concentration, odour, corrosiveness, chemical instability, environmental toxicity, biodegradability and human sensitivity [6].

Many of these chemicals are no longer in use as biocides because of their effect on human health or due to poor efficacy; such as disinfecting swimming pools with chlorine. Chlorine has been used as a water disinfectant since the early 1900s [7-9] [10] but using it alone in disinfecting swimming pools is dangerous as it can dissipate easily when the water temperature is increased, resulting in water contamination [6]. Therefore, it is used with some heterocyclic compounds that can stabilize it in water such as cyanuric acid [6], oxazolidinones and imidazolidinones [11, 12]. Low concentrations of these heterocyclic compounds were used to stabilize the halogens in water. In addition, they were loaded with halogen and used as disinfectants such as trichlorocyanuric acid [6] and the different halogen derivatives of oxazolidinones and imidazolidinones [11, 12].

Polymers have also been used as disinfectants. The polymer itself can be used as a disinfectant or it can be used as a carrier for bioactive ions or molecules to deliver to the contaminated medium. These polymers are called biocidal polymers.

Biocidal polymers have been used as disinfectants in several applications such as water filters, air filters, surface coats, cooling towers, textiles, medical tools and air conditioning systems. They have the advantages of being stable for a long time and their degradation time is very long [13-16]. They have some limitation in their applications if
they are water-soluble, such as sterilization of drinking water, and if they are water insoluble the production costs are another issue in applying these polymers on a wide scale such as disinfecting water for a town (large population) due to their high production costs [17]. However, producing these high molecular weight compounds in their insoluble form is safer than using soluble biocides (with high or low molecular weight).

In the following review the types of biocidal polymers, structure, mode of action and biological activity will be discussed to investigate the ability of improving some types of water insoluble biocidal polymers to be used commercially in some applications such as water treatment.

Table 1.1: Examples of different types of micro-organisms which will be discussed during the review section and their effect on humans, animals and plants.

<table>
<thead>
<tr>
<th>Micro-organism Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>A major cause of hospital-acquired infection of surgical wounds and infections associated with indwelling medical devices. <em>S. aureus</em> causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release superantigens into the blood stream [18].</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>It is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, and intra-abdominal infections [19].</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>It is not considered a human pathogen; it may contaminate food but rarely causes food poisoning [20].</td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td>A fungus causing smut or black mould of several fruits and vegetables. It is less likely to cause human disease than some other <em>Aspergillus</em> species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur [21].</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Fusarium oxysporum</strong></td>
<td>It is a fungus that causes fusarium wilt disease in more than a hundred species of plants. It causes vascular wilt, yellows, corm rot, root rot, and damping-off to the infected plant [22].</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>It can cause diarrhoea, urinary tract infections, respiratory illness and pneumonia. These bacteria are found in the gut of humans and animals [1].</td>
</tr>
<tr>
<td><strong>Candida albicans</strong></td>
<td>It is a diploid fungus (a form of yeast), it can cause oral and genital infections in humans [20].</td>
</tr>
<tr>
<td><strong>Shigella boydii</strong></td>
<td>It is a Gram-negative bacteria which can cause dysentery in humans through oral infection [20].</td>
</tr>
<tr>
<td><strong>Giardia lamblia</strong></td>
<td>It is a flagellated protozoan parasite that colonises and reproduces in the small intestine, causing giardiasis [23].</td>
</tr>
<tr>
<td><strong>Clostridium perfringens</strong></td>
<td>It is a type of bacterium which produces spores and can be present in the gut of warm-blooded animals. Spores are particularly resistant to disinfection using chlorine and their presence in drinking water can be used to indicate a previous occurring contamination [1].</td>
</tr>
<tr>
<td><strong>coliform bacteria</strong></td>
<td>These bacteria are widely distributed in the environment and provide an insensitive measure of the microbiological quality</td>
</tr>
</tbody>
</table>
of the water supply [1].

| Cryptosporidium | It is a parasitic organism which can cause severe gastroenteritis. It has a very high resistance to chlorine disinfection [1]. |
| Entamoeba invadens | It is a very significant protozoan pathogen affecting several reptile taxa [24]. |
1.2. Review of biocidal polymers

Biocidal polymers are a class of polymers in which the polymer itself can be used as a disinfectant or as a source of disinfection species. Biocidal polymers are used for many applications of disinfection technologies such as water purification systems, surface coats, medical tools, textiles, air purification systems, air conditioning systems, and similar applications [17].

Biocidal polymers are divided into three main types:

1- Charged Polymer:
   a) Polymers containing quaternary nitrogen atoms (poly Quat’s) [25], Scheme 1.1 and 1.2.
   b) Polymers containing phosphonium salts (polymeric phosphonium materials) [26-33], Scheme 1.3.

2- Polymers with bioactive functional groups [34, 35], Scheme 1.4.

3- Polymeric delivery systems:
   a) Polymers delivering bioactive ions:
      i) Halogen ions:
         I) Polymers contain halogenated sulphonamide group [Halogenated poly(styrene-co-divinylbenzene)sulphonamide polymers] [36, 37], Scheme 1.6.
         II) N-Halamine biocidal polymers [13-16, 38], Scheme 1.5, 1.7 and 1.8.
      ii) Metal ions [39-43].
1.2.1. Charged Polymers

Most biocidal charged polymers are polycations such as poly Quat’s and polymeric phosphonium materials. Both of the previous two examples carry positive charges, due to the presence of the quaternary nitrogen atom in the poly Quat’s and the phosphonium group in the polymeric phosphonium materials. They are water-soluble in some cases. Therefore, they are unsuitable for some disinfection applications such as drinking-water disinfection. They have been modified to be water-insoluble by cross-linking or by increasing their molecular weight. In this case they are used as emulsions or suspensions in water.

It has been suggested that the mode of action of these two types of biocidal polymers depends on surface adsorption [45]. The polymer carries positive charges and bacteria at physiological pH carry a negative charge [45]. The polycations diffuse through the cell wall to bind to the cytoplasmic membrane resulting in disruption; releasing K⁺ ions and the constituents of the cytoplasmic membrane which leads to cell death [45]. The molecular weight of the polymer plays a very important role in the biological activity of these polymers, the lower the molecular weight of the polymer the more effective [46, 47].

1.2.1.1. Poly quaternary ammonium salts (Poly Quat’s)

Poly Quat’s are an important class of charged biocidal polymers prepared mainly by generating positive charges on the polymer represented by the quaternary nitrogen
atom. Elmer et al (1969), one of the first workers in this field, succeeded in adding a quaternary ammonium salt (such as alkyl dimethyl benzyl ammonium saccharinate) to a polyurethane pre-polymer (prepared by mixing poly(ethylene butylene) adipate with methylene bis(4-phenyl isocyanate); the resulting crude polymer had biocidal properties and was used in millable rubber stocks (millable gums) (it provides the highest abrasion resistance of any rubber, synthetic or natural), castings, sealants, adhesives, coatings, and thermoplastics [25]. These kinds of blended polyurethanes with quaternary ammonium salts were modified to form films to be used as surface coats [48, 49]. Films and finished fabrics were prepared by reacting quaternary ammonium salts such as (triethoxysilylpropyl)dimethyloctadecylammonium chloride with polyurethanes rather than blending [50]. These films exhibited a high biocidal activity against Staphylococcus aureus, table 1.1, in laboratory tests (agar plates and shake flasks methods) [50].

Similar polymers containing quaternary nitrogen atoms in cyclic structures have been developed [51]. The cyclic structures can be six-membered rings such as pyridine rings [51], pyrithione rings [52], Scheme 1.1a, or guanidine rings [47, 53-55], Scheme 1.1b. The rings can be also five-membered rings containing the quaternary nitrogen atoms such as pyrroolidine rings [56], Scheme 1.1c, hydantoin rings, Scheme 1.1d [57] or oxazoline rings [58], Scheme 1.1e.

Other types of poly Quat's have been developed as acyclic structures containing the quaternary nitrogen atoms in the main chain of the polymer, Scheme 1.1f [59] or as polymers containing quaternary nitrogen atom side chain, Scheme 1.2a. The latter showed growth inhibition of Klebsiella pneumoniae and Staphylococcus aureus, table 1.1, in laboratory tests [60, 61].
Poly Quat’s have been prepared in dendritic architectures, which showed biological power 100 times over the ordinary quaternary ammonium compounds against *E. coli*, table 1.1. The effectiveness of these series of dendritic biocides depended on the length of alkyl chains used in the quaternization and dendrimer size (generations) [62-64], Scheme 1.2b.

![Scheme 1.1: Structures of some poly Quat’s and rings used in their preparation.](image)

Polymers were modified using an acidic medium to convert them to their ammonium salt which gives the ability to dissolve in water to some extent and to generate positive charge which enables the polymer to have some biological power [7].
This type of material has been prepared by grafting acrylonitrile on starch or cellulose followed by a reaction with 2-aminothiazole and then acidified to generate the quaternary ammonium salt with the required positive charges [7], Scheme 1.2c.

Poly Quat’s were prepared as a form of nanoparticles [65] that can be blended with other polymeric compounds for dental purposes. They were blended to form a composite in combination with medical articles, medical devices [66] or as a surface coat [67].

![Chemical Structures](image)

Scheme 1.2: Structures of some other types of poly Quat’s.
1.2.1.2. Phosphonium Polymers

The second well-established category of charged biocidal polymers is constituted by polymers containing phosphonium groups, also known as polymeric phosphonium materials, Scheme 1.3a.

\[
\begin{align*}
\text{(a)} & & \text{(b)} \\
\text{A} & = \text{Cl}^-, \text{BF}_4^-, \text{ClO}_4^-, \text{PF}_6^- \\
\text{R} & = \text{alkyl groups} \\
\text{While } R & \text{ is alkyl group} \\
\text{And } X' & \text{ is Cl, BF}_4^-, \text{ClO}_4^-, \text{PF}_6^- \\
\text{Polymeric phosphonium materials} & \text{ Monomeric phosphonium materials} \\
[26-31, 68] & [26-31, 68]
\end{align*}
\]

Scheme 1.3: Structures of phosphonium salts; polymeric and monomeric.

The main method for preparing these polymers has been based on preparing monomers containing phosphonium salts; such as preparing a mixture of trioctyl-(3 and 4)-vinyl-benzylphosphonium chloride, Scheme 1.3b. These monomers were copolymerized with different vinyl monomers which effectively suppressed the growth of *Aspergillus niger* and *Flavobacterium peregrinum*, table 1.1, in laboratory tests. It was found that the antibacterial activity of the polymeric phosphonium salts is affected by the structure of the counter anions [26-31, 68]. The activity was low for a counter anion which tends to form a tight ion-pair with phosphonium ion, while it was high for those facilitating ionic dissociation to free ions [26-31, 68]. Furthermore, the biological activity depends on the molecular weight of the salt, the antibacterial activity increased with increasing the molecular weight [26-31, 68]. In spite of the fact that some other
researchers, as described before, indicated that the lower the molecular weight the higher the biological action [46, 47] which is more logical as the polymer in this case can easily penetrate the bacterial cell wall. These phosphonium monomers were grafted over poly(propylene) films by surface photo-grafting; exhibiting high antibacterial activity against \textit{S. aureus} and, particularly, \textit{E. coli}, table 1.1 [26-31, 68]. These monomers have also been used in preparing biocidal polyester films with bacteriostatic properties [26-31, 68] and grafted onto cellulosic materials to be used in water- and air-filters. The grafted monomers were prepared with different alkyl chains as substituents and their biological activity depended on the structure of these substituents [32, 33, 69-71]. Phosphonium salts have been mixed with poly(methyl methacrylate) and hot-pressed to give a moulding with a good surface that can be useful as an antiseptic agent [72].

Using the same pathway, a combination of poly Quat’s and poly phosphonium materials were designed by preparing polytributyl(4-vinylbenzyl)-phosphonium chloride and polytributyl(4-vinylbenzyl)ammonium chloride and their co-polymers with different ratios. The antibacterial activity of these co-polymers with different cationic centres increased with increasing the phosphonium monomer units in the polymers rather than the quaternary ammonium salts units [32, 33, 69-71].

The phosphonium polymers were modified to be water-insoluble by cross-linking the phosphonium monomers during the polymerization or co-polymerization process using divinylbenzene. The final cross-linked polymer exhibited good biological activity against \textit{S. aureus}, \textit{E. coli}, \textit{Bacillus subtilis}, \textit{Aspergillus flavus}, \textit{Fusarium oxysporum} and \textit{Candida albicans}, table 1.1, in laboratory tests [73]. The co-polymerization was carried out with aliphatic and aromatic monomers [73]. Some of the prepared phosphonium
polymers could be useful as impact modifiers, adhesives, polymer-bound catalysts, and flame retardants, beside their biocidal applications [74]. These phosphonium salts polymeric materials were used directly or as a blend with other polymeric matrices [75].

1.2.2. Biocidal polymers with bioactive functional groups

This type of biocidal polymer is based in its mode of action on the presence of bioactive functional groups linked to the aromatic parts of the polymer such as hydroxyl groups, carboxylic groups and halogens; these can disturb the cell-wall membrane of bacteria [34, 35], Scheme 1.4a.

These polymers have been prepared by introducing organic compounds that contain bioactive functional groups into the polymer structure [35]. The compounds with bioactive functional groups were connected to the polymer via amide, Scheme 1.4 a, c, and d [34, 35, 76], or ester linkages [77], Scheme 1.4e.

These bioactive polymers can be completely synthetic such as using Poly(styrene-co-maleic anhydride) as a carrier for active agents containing amino or hydroxyl groups like p-hydroxy aniline, Scheme 1.4c [78] and have shown biological activity that relates to the number of hydroxyl groups in the structure, Scheme 1.4d [76]. Or they can be semi-synthetic by loading some of these organic compounds that contain bioactive function groups (e.g. benzaldehyde derivatives) onto some natural product polymers like chitosan, Scheme 1.4b [34].
<table>
<thead>
<tr>
<th>(a)</th>
<th>(b)</th>
</tr>
</thead>
</table>
| **X** = OH, Cl or OCH₃  
**Y** = OCH₃ (if X=OH) or H  
Biocidal polymer with bioactive functional groups [34,35] | **X** = OH, Cl or OCH₃  
**Y** = OCH₃ (if X=OH) or H  
Chitosan modified with bioactive functional groups containing monomer [34] |

<table>
<thead>
<tr>
<th>(c)</th>
<th>(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(styrene-co-maleic anhydride) modified with a bioactive functional group containing monomer [78]</td>
<td>Biocidal polymer with multi-bioactive functional groups [76]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A compound containing bioactive functional group connected to a polymer through ester linkage [77]</td>
</tr>
</tbody>
</table>

Scheme 1.4: Structures of different types of polymers with bioactive functional groups.
1.2.3. Polymeric delivery systems (controlled release systems)

This type of biocidal polymer depends for its mode of action on delivering bioactive ions or molecules (controlled release action). These can be halogen ions from N-halamine biocidal polymer and halogenated poly(styrene-co-divinylbenzene)-sulphonamide polymers or metal ions such as silver ion controlled release systems [17, 36, 37, 39]. Bioactive molecules can be bioactive heterocyclic rings [79].

1.2.3.1. Polymeric systems delivering bioactive ions

This category of biocidal polymers depends for their mode of action on delivering bioactive ions such as halogen ions (X+) [80] or metal ions (such as silver ions) [39-43] to the bacterial cells. This review will focus on halogen-ion delivering systems.

Halogen, mainly chlorine, have been used directly for a long time in sterilization of drinking water. However, using halogens has posed two problems; first, chlorine is not stable for prolonged periods of time in water, if this has to be stored. Secondly, its levels cannot be adjusted easily; therefore the levels may increase uncontrollably.

Other ways were developed to stabilize the halogen and to adjust its concentration. So some heterocyclic compounds were used as stabilizers for halogens in water which will be reviewed in the following section.

1.2.3.1.1. Heterocyclic species as halogen stabilizers

Some heterocyclic compounds have been developed to stabilize halogen in water, to prevent its evaporation with time and to deliver it to the micro-organisms when they are present. For example, a compound such as 3-chloro-4,4-dimethyl-2-oxazolidinone,
Scheme 1.5c, which proved to be as effective as Cl₂, was developed and its slow release of positive Cl provides prolonged bactericidal activity [81]. The oxazolidinone derivative, Scheme 1.5c, showed high disinfecting power and did not decompose in water to any hazardous products. By comparing oxazolidinone derivatives, Scheme 1.5c, with Ca(OCl)₂ as biocidal agents it was found that the former exhibited high disinfecting power [82]. Bromine was also used instead of chlorine to form 3-bromo-4,4-dimethyl-2-oxazolidinone which showed higher reactivity but lower stability than oxazolidinone derivative, Scheme 1.5c, however still more powerful and stable than Ca(OCl)₂ [83].

Using the same reasoning, a new series of compounds was developed based on imidazolidinone, Scheme 1.5d-g, [84] and these showed high disinfecting power against different micro-organisms such as *Staphylococcus aureus*, *Shigella boydii*, *Entamoeba invadens* and *Giardia lamblia*, table 1.1, in laboratory tests. The imidazolidinone series of biocidal compounds contains compounds like: 1,3-dibromo-4,4,5,5-tetramethyl-2-imidazolidinone, Scheme 1.5f, 1-bromo-3-chloro-4,4,5,5-tetra-methyl-2-imidazolidinone, Scheme 1.5g, and 1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone, Scheme 1.5e. The order of reactivity of these series of compounds is 1.5f>1.5g>1.5e which means that the compound containing more bromine showed higher disinfecting power [84] and the reverse order of N-X stability [85] (where X= Cl or Br). By comparing compounds from the imidazolidinone series, Scheme 1.5d-g, and the oxazolidinone series, Scheme 1.5c, it was noticed that halogenated imidazolidinones showed higher disinfecting power than similar oxazolidinones [86, 87]. The mode of action of the halogenated heterocyclic compounds is based on direct transfer of chlorine or bromine atoms from the organic compound to the bacterial cell by contact [88]. These two groups of compounds
imidazolidinones and oxazolidones) were used successfully in medicine, anti-biofouling, and in the food industry [11, 12]; for example they were used widely in the egg-processing industry due to their high disinfecting power against S. enteritidis (on the surfaces of egg shells), Table 1.1 [89].

However, using organic compounds as a stabilizing agent still has some hazardous effects. The probability of decomposition of these compounds to result in toxic products is still an issue; while using them in drinking water will affect at least the taste, if not human health, even if used at very low concentrations. Therefore, using polymers in this field has become very interesting because polymers can be tailored to be high molecular weight, N-X stable (X = halogen ion), for prolonged periods of time and can be modified to be water-insoluble which makes them safer. This led to development of the first class in this category which is polymers containing halogenated sulphonamide groups, such as halogenated poly(styrene-co-divinylbenzene)sulphonamide polymers, able to deliver halogen ions.
While $X$ is Cl or Br  
N-halamine biocidal polymer based on five-membered heterocyclic ring [13-16]  

While $X$ is Cl or Br  
N-halamine biocidal polymer based on six-membered (triazinediones) heterocyclic ring [13-16]  

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While $X$ is Cl or Br  
N-halamine biocidal polymer based on five-membered heterocyclic ring [13-16]  

Oxazolidinone/imidazolidinone N-halamine derivatives [81-88]  

| (c), X=Cl, Y=O, R=H  
(d), X=Br, Y=O, R=H  
(e), X=Cl, Y=NCl, R=Me  
(f), X=Br, Y=NBr, R=Me  
(g), X=Cl, Y=NBr, R=Me |
| (h) X and Y=H  
(i) X and Y=Cl  
(j) X and Y=Br |

Hydantion containing N-halamine biocidal polymer [13-16]  

| ![Chemical structure](image)| ![Chemical structure](image) |

Oxazolidinone containing vinyl monomer [113-115]  

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Scheme 1.5: Monomeric and polymeric N-halamine biocides.
1.2.3.1.2. Halogenated poly(styrene-co-divinylbenzene)sulphonamide polymers

These kinds of polymers are able to deliver halogen ions, can be modified to different types and may be soluble in water, or not, depending on the modification which can be explained by its general structure, Scheme 1.6:

![Chemical structure](https://example.com/structure.png)

\[ \text{CH}_2-\text{CH}_2 \text{SO}_2-N^X \]

\[ X = \text{Cl or Br} \]

\[ R = \text{H, Na, Cl or Alkyl group} \]

Scheme 1.6: General structure for Halogenated sulphonamide polymers.

From scheme 1.6, if R is Na the polymer will be in the form of the sodium salt which gives it moderate water-solubility; while if it is an alkyl group, or chlorine, it becomes slightly insoluble but this depends on the polymer molecular weight. This polymer has been converted to the cross-linked form (insoluble form) using divinylbenzene during the polymerization process in order to affect the bacteria only by chlorine delivering. This work which began in 1978 by Emerson and co-workers, succeeded in preparing polymers containing mono- and dichlorosulfonamide groups based on divinylbenzene-styrene co-polymer [37]. The resulting polymer succeeded in killing 99% of bacteria in river water [37]. When the chlorine content of the polymer decreased the polymer could be recharged with halogen again [90]. These polymers were also shown to behave as oxidants [91].
1.2.3.1.3. N-halamine biocidal polymers

Based on the halogenated poly(styrene-co-divinylbenzene)sulphonamide polymers, N-halamine biocidal polymers were designed by constructing five or six-membered heterocyclic rings on the polymer [11, 17, 80, 85, 86, 92-106], Schemes 1.5a and b. The constructed heterocyclic rings have amide or imide functional groups to be halogenated in most of the reported examples [11, 17, 80, 85, 86, 92-106] but it can include all nitrogen containing compounds [107] in cyclic or acyclic structures [108]. The biological activity depends on the halogen atoms attached to the polymer and were shown to be rechargeable, like the sulphonamide polymers [11, 17, 80, 85, 86, 92-106]. A small amount of HOX, hydrolyzed by water, was also reported by some researchers; this increased the biological activity of the polymer [8, 9, 17]. Analogue polymers have also been prepared incorporating the heterocyclic rings on the polymer backbone directly [7-9]. The presence of the halogen on the heterocyclic ring (cyclic structures) provided more stability to N-X bond, compared to their presence in aliphatic chains, as in poly(styrene-co-divinylbenzene)sulphonamide polymers or acyclic N-halamine polymers. The stability of the halogen attached to the heterocyclic rings of the polymer was increased by introducing methyl groups as substituents to the rings [11, 17, 80, 85, 86, 92-106]. N-halamine polymers were prepared in water-insoluble form by cross-linking or increasing the molecular weight [11, 17, 80, 85, 86, 92-106]. N-halamine biocidal delivering systems can be used safely in sterilizing drinking-water and some applications like: disinfecting water supplies, swimming pools, hot tubs, industrial water systems, cooling towers, spacecraft, waste-water treatment plants, military field units, camping expeditions, air conditioning systems and other sanitizing applications [11, 89, 101-106].
The mode of action of N-halamine biocidal polymers has been suggested, as indicated by most efforts in this field to depend on contact between the polymer and the bacteria which results in halogen exchange, killing the bacteria [11, 38, 89, 101-106, 109]. However, some other workers in this field described the mode of action of these polymers as a form of halogen-ion release [16]. These polymers are very stable, do not decompose in water to form toxic products, or release halogen until contact with the bacteria occurs [11, 89, 101-106] as reported by some researchers while some others stated that these polymers leached small amounts of free chlorine into flowing water [8, 9, 17]. These different opinions about the mode of action and halogen release amount to water encouraged us to perform a separate study for this point.

The first work reported in this field was performed by constructing a five- or six-membered heterocyclic ring on poly-4-vinylacetophenone to form a new heterocyclic polymer which is chlorinated to form N-halamine polymer [94]. Therefore, N-halamine polymers can be classified based on the heterocyclic ring size to five-membered containing polymers such as dichlorohydantoins (Scheme 1.5i) or six-membered containing polymers such as trichlorotriazinediones (Scheme 1.5b), and dichloropyrimidinones (Scheme 1.5k). They were synthesized based on different types of polymers such as polystyrene, poly(methylvinylketone), and polymethacrylamide.

Polymers, based on five-membered heterocyclic ring (hydantion) such as poly-1,3-dichloro-5-methyl-5-(4'-vinylphenyl)hydantoin, Scheme 1.5i, were prepared by treating polystyrene with acetyl chloride to give poly-4-vinylacetophenone followed by a reaction with KCN and ammonium carbonate then chlorination, Scheme 1.5i. Hydantion based N-halamine polymer, Scheme 1.5i, exhibits outstanding potential as a biocide for a
broad variety of applications including potable water and air disinfection [94] due to its effective pH and temperatures ranges and ready regenerability [92]. However, it would not be useful to disinfect large bodies of water, such as city treatment plants, due to its cost and the large amount of material that would be necessary [92]. In addition to the evaluation of the hydantion-based polymers against bacteria they were evaluated for their efficacies in inactivating rotavirus in flowing water in a biocidal filter application using its N-chloro and N-bromo derivatives, Scheme 1.5i and j [110]. Both polymers were effective in inactivating rotavirus and the N-bromo derivative providing a higher biological activity than the N-chloro derivative [110].

Most of the N-halamine polymers are water insoluble but they have been modified to be water soluble by treating polyethyleneglycol (PEG) with imidazolidinone-containing monomers. This polymer was chlorinated using 10% sodium hypochlorite and a solution containing 1000 ppm of it inactivated the bacterium \textit{S. aureus}, table 1.1, within a 10 min contact time [102], Scheme 1.5l.

1.2.3.1.4. N-halamine surface coats

Using N-halamine biocidal polymers as surface coats is one of the most important applications of these polymers. N-halamines have been used as biocidal surface coats in hospitals, water stations and food industry: they were modified for use as emulsions in water to produce coatings which, once chlorinated, act as contact disinfectants [11, 111, 112]. The N-halamine modified surfaces required "relatively brief" contact times to several minutes to show biological activity [11, 111, 112]. The latexes can be formed by co-polymerization of the N-halamine monomer with other monomers in water with the
aid of a surfactant, or by chemical grafting N-halamine monomer onto an emulsified polymer backbone, followed by chlorination. The chlorinated coatings have been applied to fabrics and can be synthesized in granular form by applying them on glass and fibres [11, 111, 112].

Some oxazolidinone monomers were developed to be co-polymerized with other monomers to form N-halamine polymers upon halogenation such as 4-(alkylacryloxyethyl)-4-ethyl-2-oxazolidinones, Scheme 1.5m [113-115]. These oxazolidinone polymers are inexpensive to be synthesized and are efficient biocides in their granular form if used as surface-coat disinfectants [113-115]. They can be coated on glass, plastic, and fibrous materials, which gives them considerable potential as disinfectants [113-115]. Also, the 4-(acryloxyethyl)-4-ethyl-2-oxazolidinone homopolymer can be prepared and coated on glass or textile [101].

N-halamine polymers were used to produce bioactive surface-coats using N-halamine siloxane monomers. These surface-coats were used to prevent odour caused by bacterial generation of ammonia in an experimental simulation of a urine-soaked nappy pad [116]. They also confer antimicrobial protective functions on textiles and non-woven filter media for air and water [116]. The siloxane monomers were mixed with some quaternary ammonium salts to render the siloxane co-polymers some water solubility, to be used as a coated surface over cotton fibres, which showed biocidal efficacy against S. aureus and E. coli, table 1.1 [112]. It was determined that both N-halamine and Quat’s functional groups were effective against S. aureus, table 1.1, but only the N-halamine units were effective against E. coli, table 1.1 [112]. The co-polymers should be useful for applications for which aqueous media is preferred over organic solvents to be used during
coating procedures [112], Scheme 1.8a.

N-halamine surface coats can be prepared as a form of N-halamine epoxide coatings [111] such as N-halaminehydantoinyl epoxide [105].

N-halamine biocidal polymers have been proposed as additives for polyurethane films and coats by connecting the terminals of these polymers with the N-halmine polymers or with the heterocyclic rings only [117, 118].

1.2.3.1.5. N-halamine textiles

Producing bioactive fibres and textiles is very important especially in the medical field such as producing clothes and textiles for workers and patients. N-Halamine cotton and fibres have been prepared by grafting N-halamine monomers onto cotton by introducing the monomer directly into the cotton fibres or cellulose [98, 119] or grafting the cotton first and then reacting it with heterocyclic compounds [7, 8, 120]. For example, cotton has been grafted by acrylonitrile monomer followed by a reaction with cyanuric acid [7, 8, 120], Scheme 1.7a, or by connecting the heterocyclic compound to the cotton fibres in presence of polycarboxylic acids as a cross-linking agent [121]. This idea has also been used in grafting polysaccharides and cotton [120].

Using the same method siloxane ethers were used to load the heterocyclic N-halamine monomers to textiles, wood, silica gel and medical devices by reacting the monomers first with the siloxanes and reacting the product with the polymer [103]. Some silicate monomers can be grafted as well to the surfaces of the cotton [104], cellulose [122] and silica gel particles [123].

Heterocyclic vinylic compounds have been grafted onto textiles [96]. The grafted
N-halamine textiles exhibited potent antibacterial properties and showed to be durable and regenerable [97]. These vinylic compounds can be acyclic aliphatic compounds such as acrylamide; cotton fibres and textiles were grafted with acyclic aliphatic compounds which on halogenation obtained a bioactive character [99]. Similar to cyclic halamines, the resulting acyclics were able to provide durable and rechargeable biocidal functions. However, acyclic halamine structures can be hydrolysed easily compared to the cyclic halamines [124], Scheme 1.7b. The method of grafting was modified by spraying the amine- or amide-containing monomer and initiator solution onto the textile, or dipping the textile (e.g., cotton, polyester) in the monomer/initiator solution which resulted in polymerization with grafting to the textile fibres [93].

Synthetic fibres have been prepared using N-halamine monomers for example; biocidal cyclic N-chloramine moieties were covalently bonded to Nylon 6,6 [125], Scheme 1.7c and polyester fabrics [109, 126], Scheme 1.7d.

Some high performance fibres, including Nomex® and Kevlar® were chlorinated to be used as N-halamine biocidal polymers, Schemes 1.7e and f [80]. Although Nomex® was chlorinated without any significant decomposition, Kevlar® decomposed under the same chlorination conditions [127] so Kevlar® was coated with polymethacrylamide to increase its ability to be chlorinated [108].

**1.2.3.1.6. Improving the particle size of the N-Halamine biocidal polymers**

In some applications the particle size is very important such as in water filters, so as not to restrict the water flow. Particles were developed by polymerizing 3-triethoxysilylpropyl-5,5-dimethylhydantoin, Scheme 1.8c, on the surface of sand particles
to produce an adhered film that, upon chlorination with diluted sodium hypochlorite bleach, becomes biocidal, Scheme 1.8d. The biocidal efficacy of this coated sand was very high against the bacterial pathogens *S. aureus* and *E. coli*, Table 1.1; complete inactivation was observed within 1 min of contact for the former bacterium and in the interval of 1-5 min for the latter [128]. Silica gel particles can be used instead of the sand particles using the same monomer, 3-triethoxysilylpropyl-5,5-dimethylhydantoin [129], Scheme 1.8b.

Controlling particle size can be performed by converting the polymer into beads by cross linking the N-halamine monomers during the emulsion polymerization of the N-halamine monomers or by constructing the heterocyclic rings directly on polymer beads [14, 15].
1.2.3.1.7. Biological power increase and other uses of N-halamine polymers

To increase the biological power of N-halamine polymers, dendritic structures (poly Quat’s and N-halamine) have been prepared [130]. The biological activity of the dendritic structure is higher than that of normal linear polymers and such dendrimers have been used for the capture and neutralization of biological and chemical warfare agents [130].

There are some other uses of N-halamine polymers than using them as biocides
such as using them as oxidizing agents for organo-sulfur compounds [131].

\[
\begin{align*}
\text{(a) N-halamine/Quat's Siloxane [112]} & \quad \text{(b) N-halamine modified Silica gel [129]} \\
\text{(C) 3-Triethoxysilylpropyl-5,5-dimethylhydantoin [128]} & \quad \text{(d) N-halamine modified Sand [128]}
\end{align*}
\]

Scheme 1.8: N-halamine siloxanes and silica gels.

1.2.3.2. Polymers delivering bioactive molecules

A final class of biocidal polymers; based on their mode of action, delivering organic bioactive molecules that can act as “antibiotics” so their mechanism of action is considered as a controlled release mechanism [132].

The released molecules can be five-membered heterocyclic rings, Schemes 1.9a and b, such as 5-nitrofurylacrolein, which were released to the medium from functionalised poly vinyl alcohol by the action of moisture, Scheme 1.9a [132]. Other polymers have been prepared using \(N\)-(4-sulfamido-\(N\)-(5-methyl-3-isoxazolyl)-phenyl)maleimide monomer, Scheme 1.9b, which upon polymerization has some
antifungal activity on A. niger and C. albicans, table 1.1. These polymer-drug conjugates showed superior anti-microbial activity over the monomer at all concentrations [133]. The mode of action of this polymer may be a controlled release of the five-membered isoxazole ring to the medium.

The five-membered heterocyclic ring can be fused with benzene rings like vinyl acryl monomers with azole moieties, 2-hydroxy-3-(5-methyl-1,3,4-thiadiazol-2-y1)thiopropyl methacrylate, Scheme 1.9c [134]. The biological activity of this polymer was higher than that of corresponding monomer [134]. The mode of action of this polymer might be due to the release of the benzoimidazole ring.

The rings in the bioactive molecules can be modified to six-membered rings like 1-ethyl-6-fluoro-7-(4-(2-hydroxy-3-)2-methylacryloyloxy)propyl)piperazin-1-yl)-4-oxo-1,4-di-hydroquinolin-3-carboxylic acid monomer, Scheme 1.9d [135]. Polymerizing the latter monomer produces a drug delivery system releasing norfloxacin, a known inhibitor of bacterial DNA gyrase, preventing cell growth [135].

Bioactive molecules can be grafted over fibers so the fiber can work as controlled release systems for these molecules. For example, acrylic acid was grafted onto polyamide fibers. The resultant fibers, containing carboxylic groups in their structure, were additionally modified with penicillin, neomycin, and gentamycin to obtain antimicrobial fibers [136].

Bioactive molecules have been grafted onto wool to develop novel bioactive materials for external biological uses: prepared by using dye-like antibiotics (doxycycline and ciprofloxacin) which are highly active against Gram-negative and Gram-positive
bacteria, they are also the only antibiotics approved as a treatment for anthrax (*Bacillus anthracis*, table 1.1) [79].

In other examples, the bioactive species was connected to the polymer matrix through metal ions, *via* a complexation reaction, rather than the usual connection through covalent bonds; like neomycin, which coupled cellulose through Cu(II), Fe(III) or Zn(II) [137].

![Polymer delivering 5-nitrofurylacrolein](image1.png)

Polymer delivering 5-nitrofurylacrolein [132]

![N-(4-sulfamido-N-(5-methyl-3-isoxazolyl)-phenyl)maleimide](image2.png)

N-(4-sulfamido-N-(5-methyl-3-isoxazolyl)-phenyl)maleimide [133]

![2-Hydroxy-3-(5-methyl-1,3,4-thiadiazol-2-yl)thiopropyl methacrylate](image3.png)

2-Hydroxy-3-(5-methyl-1,3,4-thiadiazol-2-yl)thiopropyl methacrylate [134]

![1-Ethyl-6-fluoro-7-(4-(2-hydroxy-3-)2-methylacryloyloxy)propyl(piperazin-1-yl)-4-oxo-1,4-di-hydroquinolin-3-carboxylic acid](image4.png)

1-Ethyl-6-fluoro-7-(4-(2-hydroxy-3-)2-methylacryloyloxy)propyl(piperazin-1-yl)-4-oxo-1,4-di-hydroquinolin-3-carboxylic acid [135]

Scheme 1.9: Controlled release polymers and monomers for delivering bioactive molecules.
1.3. Conclusion

From this review it can be seen that polymers have been used for disinfection purposes successfully. Using polymers may be safer than using normal biocides. Different types of polymers were developed to work as biocides.

Most of the polymers prepared have been developed with a low number of bioactive centres through the polymer repeating unit. For example; most of the prepared N-halamine biocidal polymers mentioned above are capable of halogenation by a maximum 2-3 available positions for halogens per repeating unit of the polymer [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129].

The stability of the N-X bonds in the N-halamine biocidal polymers was improved by introducing methyl groups on the heterocyclic rings attached to the polymer [11, 82, 84-86, 88, 89, 102-104, 106, 116].

The mode of action of N-halamine biocidal polymers is still unclear. Most of the research on this subject refers to killing by contact only [11, 82, 84-86, 88, 89, 102-104, 106, 116] while others explain their mode of action as a release effect of the halogen from the polymer [16].

The particle size of the N-halamine polymers depends usually on the size of the starting materials used in their preparation [14, 15].

Most of the prepared biocidal polymers are expensive in their production which retards their use in big projects like sterilization of drinking water on a large scale for a big population [92].
1.4. Aim of work

Based on the previous review this study aimed to:

a) Improve the biological activity of some types of biocidal polymers such as poly Quat's and N-halamine biocidal polymers by increasing the number of bioactive sites per polymer repeating unit.

b) Improve the stability of the halogens attached to the N-halamine polymers by modifying the polymer's design to have strong electron donating groups substituted to the heterocyclic rings of the polymers.

c) Improve the particle size of the N-halamine polymers by developing novel modified silica gels that can stabilize the attached halogen using strong electron donating groups.

d) Produce N-halamine polymers with different bead-sizes by using sodium alginate as a matrix for water-insoluble polymers.

e) Produce novel N-halamine polymers using commercial low cost chemicals in order to reduce the production costs of this type of polymers for potential use in a wide range of applications. In addition, to investigate the ability of using these polymers in water disinfection systems (drinking water).

f) Investigate the mode of action of N-halamine polymers to elucidate the mechanisms involved.
Chapter 2

Preparation of novel heterocyclic polymers to be used in disinfection technology
2.1. Introduction

In this part of the work novel heterocyclic polymers were prepared. These were
designed to form bioactive polymers by loading them with halogen (chlorine, bromine
and iodine) to form the N-halamine derivatives or by quatermarization with diluted HCl to
form poly Quat’s. They were designed to carry more halogen and more positive charges
than similar N-halamine polymers described in the literature and to give more stability to
the halogen attached to the polymer than that in the literature [11, 82, 84-86, 88, 89, 102-
104, 106, 116].

The polymers were prepared in two different ways; by reacting uramil with
polymers directly and by preparing uramil-derived monomers followed by co-
polymerisation to form polyureas and polyurethanes. The structure of the prepared
polymers was determined using \(^1\)H NMR, \(^{13}\)C NMR, FTIR and elemental analysis.

2.2. Experimental

2.2.1. Preparation of polymers

2.2.1.1. Preparation of 5-aminobarbituric acid (Uramil) (1)

Fuming nitric acid (72 ml, \(d\ 1.52\)) was added to a round-bottomed flask
surrounded by an ice bath to keep the temperature below 40°C. Barbituric acid (50 g, 0.39
mol) was added over two hours to avoid any rise in temperature. The mixture was stirred
during the addition and the stirring continued further for one hour after addition. Water (215 ml) was added with stirring and the mixture cooled below 10°C using an external ice bath. The yellow solid material was filtered, washed with cold water copiously and dried at 60-80°C for three hours. The product was crystallized by dissolving in boiling water (450 ml). The hot solution was filtered and the filtrate was left overnight to collect the crystalline product. The crystalline product was filtered and dried for three hours at 110-115°C to form anhydrous 5-nitrobarbituric acid. Drying at this temperature gave 47 g (70%) anhydrous 5-nitrobarbituric acid. The compound started to decompose at 176°C which was the same as in the literature [138].

Anhydrous 5-nitrobarbituric acid (38 g, 0.22 mol) was dissolved in concentrated hydrochloric acid (300 ml). The solution was heated in a water bath until the material dissolved. Granulated tin (125 g) and concentrated hydrochloric acid (200 ml) were added gradually over 30 min. The solution was heated until the yellow colour of 5-nitrobarbituric acid disappeared and a white solid material formed instead. Concentrated hydrochloric acid (1500 ml) was added and the heating continued until the white material dissolved again, in the presence of a small amount of charcoal to remove any colour in the solution. The solution was filtered hot and the filtrate kept at 0°C overnight to collect uramil crystals. The first crop of uramil was collected by filtration and the filtrate was concentrated by evaporating 50% of the solvent and left overnight to collect the second crop. Uramil was dried under vacuum over concentrated sulphuric acid for five hours. The final product was 23 g (73%) and the melting point of the product was greater than 400°C, Scheme 2.1 [138].
Analysis, FTIR (KBr): $v_{\text{max}}$ (cm$^{-1}$) 1610 (C=O, imide), 1695 (C=O, amide), 3230 (N-H, imide) and 3460 (N-H amine). $^1$H NMR (DMSO, 500 MHz): 2.9 (s, 2H, NH$_2$), 3.2 (s, 2H, 2NH imides) and 7.9 (s, 1H, ring CH). $^{13}$C NMR (DMSO, 125 MHz); ppm 33.8 (CH, ring), 123 (C=O, amide) and 160 (C=O, imide).

\[
\text{Barbituric acid} \xrightarrow{\text{fuming nitric acid}} \text{5-nitrobarbituric acid} \xrightarrow{\text{Tin/Conc. HCl}} \text{H}_2\text{N}N\text{O}
\]

Scheme 2.1: Preparation of uramil.

2.2.1.2. Preparation of poly(N-iminouramil)ethylene (2) (series 1)

Polyacrylonitrile (0.50 g, 0.01 mol, average MW 150,000) and uramil (5-aminobarbituric acid) (1) (1.4 g, 0.01 mol) were heated in 30 ml N,N-dimethylformamide/absolute ethanol (2:1, v:v) at 120°C in the presence of sodium hydroxide (0.40 g, 0.01 mol) for 48 hours. The reaction mixture was cooled and added gradually to a flask containing 50 g ice and pH adjusted to 7 using 0.1M HCl. The reddish brown solid product was filtered, dried and weighed, which produced 1.5 g (73% yield), Scheme 2.2.

Analysis, FTIR (KBr): $v_{\text{max}}$ (cm$^{-1}$) 1603 (C=O, imide), 1690 (C=O, amide), 1498 (C=N), 1218 (C-N), 3200 (N-H, imide) and 3421 (N-H amine). $^1$H NMR (DMSO, 500 MHz): $\delta$ 2.04 (b.s, 2H, CH$_2$), 2.9 (s, 2H, NH$_2$), 2.1 (b.s, 1H, CH), 3.2 (s, 2H, 2NH imides), and 7.9 (s, 1H, ring CH). $^{13}$C NMR (DMSO, 125 MHz); ppm 28.6 (CH), 28.3 (CH$_2$), 33.5 (CH, ring), 36.4 (C-N), 121 (C=O, amide) and 163 (C=O, imide). Elemental
analysis, found (%): C, 42.0; H, 4.1; N 27.8. Calc. for C\textsubscript{7}H\textsubscript{8}N\textsubscript{4}O\textsubscript{3} (\%): C, 42.9; H, 4.1; N, 28.6.

The same reaction was performed using polyethylacrylate (1.0 g, 0.01 mol, average MW 95,000) instead of polycrylonitrile to prepare 5-polyacrylamidobarbituric acid (7) (series 2) and the final red solid product was filtered, dried and weighed, which produced 1.2 g (61% yield), Scheme 2.3.

Analysis, FTIR (KBr): $\nu_{\text{max}}$ (cm$^{-1}$) 1690 (C=O, amide, side chain), 1625 (C=O, imide), 1666 and 1680 (2C=O amide, ring amide), 3113 (NH, imide) and 3447 (NH, amide). $^1$H NMR (DMSO, 500 MHz): $\delta$ 1.2 (b.s, 2H, CH$_2$), 1.5 (b.s, 1H, CH), 5.4 (s, 1H, no 1), 7.1 (s, 1H, no. 2), 10.4 (s, 1H, no. 3) and 4.1 (s, 1H, no. 4), Scheme 2.3. $^{13}$C NMR (DMSO, 125 MHz): ppm 13.8 (CH$_2$), 28 (CH), 118 (ring CH), 150 (C=O, d), 163 (C=O, a), 164.9 (C=O, b) and 172 (C=O, c), Scheme 2.3. Elemental analysis, found (%): C, 42.4; H, 21.4; N, 3.3. Calc. for C\textsubscript{7}H\textsubscript{7}N\textsubscript{3}O\textsubscript{4} (\%): C, 42.6; H, 21.5; N, 3.6.
Scheme 2.2: Preparation of poly(N-iminouramil)ethylene and its halogenation and acidification, series 1.
Scheme 2.3: Preparation of 5-polyacrylamidobarbituric acid and its halogenation and acidification, series 2.
2.2.1.3. Diazotization of uramil

Uramil (1.40 g, 0.01 mol) was dissolved in concentrated sulphuric acid (5 ml). The temperature was kept at 0°C using an external ice bath. A cold solution of \( \text{NaNO}_2 \) (0.69 g of \( \text{NaNO}_2 \), 0.01 mol + 10 ml water) was added drop wise to the uramil solution with stirring to form the uramildiazonium salt (12), Scheme 2.4.

2.2.1.4. Preparation of 1,3-diamino-4-azo(5-barbituric acid)benzene (13)

\( m \)-Phenylenediamine (1.1 g, 0.01 mol) and sodium hydroxide (2.8 g, 0.07 mol) were dissolved in water (10 ml). The temperature was kept at 0°C using an external ice bath. This cold solution was added gradually to the uramildiazonium salt (12) with stirring. The resulting deep red solid product was filtered, dried and weighed, which produced 2.4 g (90% yield), Scheme 2.4.

Analysis, FTIR (KBr): \( \nu_{\text{max}} \) (cm\(^{-1}\)) 3330 (NH, imide), 3392 (NH, NH\(_2\) near azo group), 3429 (NH, NH\(_2\) group away azo group), 1603 (C=O, imide), 1680 (C=O amide), 2942 (CH aliphatic) and 1411 (N=N) \([\text{139, 140}]\). \(^1\)H NMR (DMSO, 500 MHz): \( \delta \) 5.4 (s, 2H, NH\(_2\) away azo group), 1.4 (s, 2H, NH\(_2\) near azo group), 6.1 (s, 2H, 2NH imide), 10.3 (s, 1H, ring CH) and 6.9-7.5 (m, 3H, benzene ring). \(^{13}\)C NMR (DMSO, 125 MHz): ppm 31 (ring CH), 175.9 (C=O, amide), 220 (C=O, imide) and 96.8, 107.8, 116.9, 124.5, 145 and 150 (aromatic). Elemental analysis, found (%): C, 45.1; H, 3.6; N, 32.9. Calc. for \( \text{C}_{10}\text{H}_{10}\text{N}_6\text{O}_3 \) (%): C, 45.8; H, 3.8; N, 33.1.
Scheme 2.4: Diazotization of uramil and its coupling with m-phenylenediamine and resorcinol.
2.2.1.5. Preparation of poly[(1,3-diamino-4(5-azobarbituric acid)benzene)-co-
(tolylene-2,6-diisocyanate)] (polyurea) (14) (series 3)

Monomer (13) (2.6 g, 0.01 mol) and tolylene-2,6-diisocyanate (1.7 g, 0.01 mol) were heated in N,N-dimethylformamide (30 ml) at 90°C for 5 hours. The reaction mixture was cooled and methanol (50 ml) was added. The brown product was filtered, washed copiously with methanol, dried and weighed, which produced 4.1 g (93% yield), Scheme 2.5.

Analysis, FTIR (KBr): \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1639 (C=0, imide), 1680 (C=0, amide), 3436 (N-H, broad band), 1118 (C-N) and 1472 (N=N) [139, 140]. \(^1\)H NMR (DMSO, 500 MHz): \( \delta \) 2.2 (s, 3H, CH\(_3\)), 4.2 (s, 1H, NH, no.1), 5.9 (s, 1H, NH, no. 3), 4.8 (s, 1H, NH, no. 2), 6.7 (s, 2H, 2NH imide), 7-8.3 (m, 6H, aromatic), 6.5 (s, 1H, NH, no.4) and 9.2 (s, 1H, heterocyclic ring CH), Scheme 2.6. \(^{13}\)C NMR (DMSO, 125 MHz): ppm 12.4 (CH\(_3\)), 49 (heterocyclic ring CH), 100.3, 110, 111.3, 111.7, 113, 118, 121, 123, 125, 137, 144 and 146 (aromatic), 162 (C=O, imide), 152 (C=O, a), 153 (C=O, b) and 174 (C=O, heterocyclic ring amide), Scheme 2.6. Elemental analysis, found (%): C, 52.1; H, 3.5; N, 24.8. Calc. for C\(_{19}\)H\(_{18}\)N\(_6\)O\(_5\) (%): C, 52.3; H, 3.6; N, 25.7.
Scheme 2.5: Preparation of poly[(1,3-diamino-4(5-azobarbituric acid)benzene)-co-(tolylene-2,6-diisocyanate)] (polyurea) and its halogenation and acidification, series 3.
Scheme 2.6: Numbering the hydrogen and carbon atoms in polyurea (14).

The same reaction was performed using toluene-2,4-diisocyanate to prepare poly[(1,3-diamino-4(5-azobarbituric acid)benzene)-co-(tolulene-2,4-diisocyanate)](polyurea) (19) (series 4), Scheme 2.7.

Analysis, FTIR (KBr): \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1609 (C=O, imide), 1650 (C=O, amide), 3446 (N-H, broad band), 1136 (C-N) and 1547 (N=N) [139, 140]. \(^1\)H NMR (DMSO, 500 MHz): \( \delta \) 2.1 (s, 3H, CH\(_3\)), 4.8 (s, 1H, NH, no.1), 6.7 (s, 1H, NH, no. 3), 7.0 (s, 1H, NH, no. 2), 7.3 (s, 2H, 2NH imide), 7.9-8.8 (m, 6H, aromatic), 7.7 (s, 1H, NH, no.4) and 9.1 (s, 1H, heterocyclic ring CH), Scheme 2.8. Elemental analysis, found (%): C, 52.2; H, 3.4; N, 24.9. Calc. for C\(_{19}\)H\(_{16}\)N\(_4\)O\(_3\) (%): C, 52.3; H, 3.6; N, 25.7.
Scheme 2.7: Preparation of poly[(1,3-diamino-4(5-azobarbituric acid)benzene)-co-(tolylene-2,4-diisocyanate)] (polyurea) and its halogenation and acidification, series 4.

Scheme 2.8: Numbering the hydrogen and carbon atoms in polyurea (19).
2.2.1.6. Preparation of 1,3-dihydroxy-4(5-azobarbituric acid)benzene (24)

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 uramildiazonum salt (12). The dark purple product was filtered, washed copiously with cold water, dried and weighed, which produced 2.6 g (99% yield), Scheme 2.4.

Analysis, FTIR (KBr): \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1603 (C=O, imide), 1705 (C=O, amide), 1411 (N=N) [139, 140], 3100 (N-H), 3432 (OH) and 2942 (CH aliphatic). \(^1\)H NMR (DMSO, 500 MHz): \( \delta \) 1.3 (s, 1H, OH near azo group), 5.4 (s*, 1H, OH away azo group), 6.2 (s, 2H, 2NH imides), 6.9-7.2 (s, 3H, aromatic) and 10.2 (s, 1H, heterocyclic ring CH). \(^13\)C NMR (DMSO, 125 MHz): ppm 49 (heterocyclic ring CH), 102.4, 103, 105, 106, 129 and 150.3 (aromatic), 158.3 (C=O, amide) and (C=O, imide). Elemental analysis, found (%): C, 45.1; H, 2.9; N, 20.9. Calc. for C\(_{10}\)H\(_8\)N\(_4\)O\(_5\) (%): C, 45.5; H, 3; N, 21.2.

2.2.1.7. Preparation of poly[(1,3-dihydroxy-4(5-azobarbituric acid)benzene)-co-(tolylene-2,6-diisocyanate)] (polyurethane) (25) (series 5)

Monomer (24) (2.6 g, 0.01 mol) and tolylene-2,6-diisocyanate (1.7 g, 0.01 mol) were heated in N,N-dimethylformamide (30 ml) for 5 hours at 90°C. The reaction was cooled and methanol (50 ml) was added. The brown product was filtered, washed copiously with methanol, dried and weighed, which produced 3.8 g (86% yield), Scheme 2.9.

Analysis, FTIR (KBr): \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1640 (C=O, imide), 1700 (C=O, urethane group), 1660 (C=O, heterocyclic ring amide), 3429 (N-H), 1135 (broad band for C-O & C-N bonds), 1471 (N=N) [139, 140] and 2920 (CH aliphatic). \(^1\)H NMR (DMSO, 500
MHz): δ 2.2 (s, 3H, CH₃), 4.8 (s, 1H, NH no.1), 4.2 (s, 1H, NH no.2), 6.8 (s, 2H, 2NH imide), 10.5 (s, 1H, heterocyclic ring CH) and 7.0-8.4 (s, 6H, benzene rings), Scheme 2.10. ¹³C NMR (DMSO, 125 MHz): ppm 11.4 (CH₃), 49 (heterocyclic ring CH), 109.9, 111.6, 113.2, 116, 117.9, 118.5, 120, 121, 125, 137.3, 137.7 and 146 (aromatic), 150 (C=O, ring amide), 185 (C=O, imide), 163.0 (C=O, b) and 153.2 (C=O, a), Scheme 2.10. Elemental analysis, found (%): C, 51.7; H, 3.2; N, 18.1. Calc. for C₁₉H₁₆N₈O₇ (%): C, 52.1; H, 3.2; N, 19.2.
Scheme 2.9: Preparation of poly[(1,3-dihydroxy-4(5-azobarbituric acid)benzene)-co-
(tolyene-2,6-diisocyanate)] (polyurethane) and its halogenation and acidification, series 5.
The same reaction was performed using toluene-2,4-diisocyanate to prepare poly[(1,3-dihydroxy-4(5-azobarbituric acid)benzene)-co-(tolulene-2,4-diisocyanate)] (polyurethane) (30) (series 6), Scheme 2.11.

Analysis, FTIR (KBr): $\nu_{\text{max}}$ (cm$^{-1}$) 1617 (C=O, imide), 1639 (C=O, amide), 1712 (C=O, urethane), 3417 (N-H, imide), 3458 (N-H, amide), 3550 (OH), 110 (C-N & C-O) and 1457 (N=N) [139, 140]. $^1$H NMR (DMSO, 500 MHz): $\delta$ 2.0 (s, 3H, CH$_3$), 6.4 (s, 1H, NH, no. 1), 7.1 (s, 1H, NH, no. 2), 6.9 (s, 2H, imide), 7.4-8.5 (m, 6H, aromatic), and 9.4 (s, 1H, heterocyclic ring CH), Scheme 2.12. $^{13}$C NMR (DMSO, 125 MHz): ppm 12 (CH$_3$), 49 (heterocyclic ring CH), 106, 110, 113, 114, 115, 116, 119, 119.6, 120, 121, 123 and 126 (aromatic), 128 (C=O, ring amide), 151 (C=O, imide), 154 (C=O, b) and 160 (C=O, a), Scheme 2.12. Elemental analysis, found (%): C, 51.8; H, 3.2; N, 18.5. Calc. for C$_{19}$H$_{16}$N$_8$O$_7$ (%): C, 52.1; H, 3.2; N, 19.2.

All prepared polymers are insoluble in water and most organic solvents. The non-modified polymers dissolve partially (less than 30%) in DMSO by soaking for 3-10 days. The partially dissolved polymers were filtered to carry out the analysis. However, MW (molecular weight) characterization was not possible due to the need to completely
dissolve the material in low-viscosity solvents for gel permeation chromatography (GPC).

Scheme 2.11: Preparation of poly[(1,3-dihydroxy-4-(5-azobarbituric acid)benzene)-co-(tolylene-2,4-diisocyanate)] and its halogenation and acidification, series 6.
2.2.2. Halogenation process

The prepared heterocyclic polymers were halogenated to form N-halamine derivatives as follows: polymer was dispersed in sodium hydroxide solution and the halogen (chlorine, bromine or iodine) was added gradually until neutralization to pH 7, table 2.1. The mixture was stirred for a further hour during which the temperature was kept below 5°C using an external ice bath. The resulting product was filtered, washed copiously with chlorine-free water, dried and weighed [8, 9]. The resulting polymers were completely water insoluble. The amounts of polymers and sodium hydroxide used in the preparation of each N-halamine polymer and the final yield are illustrated in table 2.1. The halogenation process was followed using FTIR spectroscopy [8, 120] and the halogen/g content was determined using iodometric titration [38, 141], table 2.2.
Table 2.1: Amounts of polymers and sodium hydroxide used in the preparation of the N-halamine polymers and the final yields.

<table>
<thead>
<tr>
<th>Product.</th>
<th>Reactant (weight, quantity)</th>
<th>Sodium hydroxide (g)</th>
<th>Final yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (chlorinated)</td>
<td>2 (1.96 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>2.0 g (67%)</td>
</tr>
<tr>
<td>4 (brominated)</td>
<td>2 (1.96 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>2.7 g (63%)</td>
</tr>
<tr>
<td>5 (iodinated)</td>
<td>2 (1.96 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>3.1 g (54%)</td>
</tr>
<tr>
<td>8 (chlorinated)</td>
<td>7 (1.97 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>1.5 g (44%)</td>
</tr>
<tr>
<td>9 (brominated)</td>
<td>7 (1.97 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>2.0 g (39%)</td>
</tr>
<tr>
<td>10 (iodinated)</td>
<td>7 (1.97 g, 0.01 mol)</td>
<td>1.6 (0.04 mol)</td>
<td>2.5 g (36%)</td>
</tr>
<tr>
<td>15 (chlorinated)</td>
<td>14 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.5 g (74%)</td>
</tr>
<tr>
<td>16 (brominated)</td>
<td>14 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.6 g (61%)</td>
</tr>
<tr>
<td>17 (iodinated)</td>
<td>14 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.8 g (62%)</td>
</tr>
<tr>
<td>20 (chlorinated)</td>
<td>19 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.6 g (89%)</td>
</tr>
<tr>
<td>21 (brominated)</td>
<td>19 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.7 g (71%)</td>
</tr>
<tr>
<td>22 (iodinated)</td>
<td>19 (0.44 g, 0.001 mol)</td>
<td>0.28 (0.007 mol)</td>
<td>0.9 g (70%)</td>
</tr>
<tr>
<td>26 (chlorinated)</td>
<td>25 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.4 g (66%)</td>
</tr>
<tr>
<td>27 (brominated)</td>
<td>25 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.6 g (67%)</td>
</tr>
<tr>
<td>28 (iodinated)</td>
<td>25 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.7 g (64%)</td>
</tr>
<tr>
<td>31 (chlorinated)</td>
<td>30 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.5 g (82%)</td>
</tr>
<tr>
<td>32 (brominated)</td>
<td>30 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.7 g (78%)</td>
</tr>
<tr>
<td>33 (iodinated)</td>
<td>30 (0.44 g, 0.001 mol)</td>
<td>0.20 (0.005 mol)</td>
<td>0.8 g (73%)</td>
</tr>
</tbody>
</table>
Table 2.2: FTIR characterization and halogen content of the N-halamine polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Bond</th>
<th>$v_{\text{max}}$ (cm$^{-1}$)</th>
<th>Halogen content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (chlorinated)</td>
<td>N-Cl</td>
<td>593</td>
<td>101 ±12</td>
</tr>
<tr>
<td>4 (brominated)</td>
<td>N-Br</td>
<td>688</td>
<td>114 ±18</td>
</tr>
<tr>
<td>5 (iodinated)</td>
<td>N-I</td>
<td>802</td>
<td>116 ±22</td>
</tr>
<tr>
<td>8 (chlorinated)</td>
<td>N-Cl</td>
<td>784</td>
<td>40 ±16</td>
</tr>
<tr>
<td>9 (brominated)</td>
<td>N-Br</td>
<td>680</td>
<td>68 ±15</td>
</tr>
<tr>
<td>10 (iodinated)</td>
<td>N-I</td>
<td>583</td>
<td>93 ±32</td>
</tr>
<tr>
<td>15 (chlorinated)</td>
<td>N-Cl</td>
<td>763</td>
<td>190 ±11</td>
</tr>
<tr>
<td>16 (brominated)</td>
<td>N-Br</td>
<td>618</td>
<td>198 ±10</td>
</tr>
<tr>
<td>17 (iodinated)</td>
<td>N-I</td>
<td>777</td>
<td>201 ±26</td>
</tr>
<tr>
<td>20 (chlorinated)</td>
<td>N-Cl</td>
<td>619</td>
<td>187 ±14</td>
</tr>
<tr>
<td>21 (brominated)</td>
<td>N-Br</td>
<td>618</td>
<td>191 ±12</td>
</tr>
<tr>
<td>22 (iodinated)</td>
<td>N-I</td>
<td>689</td>
<td>206 ±20</td>
</tr>
<tr>
<td>26 (chlorinated)</td>
<td>N-Cl</td>
<td>600</td>
<td>160 ±17</td>
</tr>
<tr>
<td>27 (brominated)</td>
<td>N-Br</td>
<td>712</td>
<td>178 ±22</td>
</tr>
<tr>
<td>28 (iodinated)</td>
<td>N-I</td>
<td>789</td>
<td>193 ±30</td>
</tr>
<tr>
<td>31 (chlorinated)</td>
<td>N-Cl</td>
<td>634</td>
<td>176 ±11</td>
</tr>
<tr>
<td>32 (brominated)</td>
<td>N-Br</td>
<td>683</td>
<td>183 ±25</td>
</tr>
<tr>
<td>33 (iodinated)</td>
<td>N-I</td>
<td>722</td>
<td>197 ±23</td>
</tr>
</tbody>
</table>
2.2.3. Quaternarization process

The polymer was dispersed in water (10 ml) and HCl (1M) added. The temperature was kept below 10°C using an external ice/water bath and the mixtures were stirred for 1 hour. Methanol (50 ml) was then added to the solution to precipitate the dissolved polymer. The resulting product was filtered, washed copiously with methanol, dried and weighed [7, 120]. The amounts of polymers, and the volumes of HCl used are given in table 2.3.

Table 2.3: Amounts of polymers and HCl used in the preparation of poly Quat's and the final yields.

<table>
<thead>
<tr>
<th>Product</th>
<th>reactant (weight, quantity)</th>
<th>1M HCl quantity</th>
<th>Final yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2 (1.96 g, 0.01 mol)</td>
<td>30.1 ml</td>
<td>2.1 g (70%)</td>
</tr>
<tr>
<td>11</td>
<td>7 (1.97 g, 0.01 mol)</td>
<td>30.1 ml</td>
<td>2.0 g (70%)</td>
</tr>
<tr>
<td>18</td>
<td>14 (0.44 g, 0.001 mol)</td>
<td>6.30 ml</td>
<td>0.5 g (83%)</td>
</tr>
<tr>
<td>23</td>
<td>19 (0.44 g, 0.001 mol)</td>
<td>6.30 ml</td>
<td>0.4 g (67%)</td>
</tr>
<tr>
<td>29</td>
<td>25 (0.44 g, 0.001 mol)</td>
<td>4.90 ml</td>
<td>0.5 g (74%)</td>
</tr>
<tr>
<td>34</td>
<td>30 (0.44 g, 0.001 mol)</td>
<td>4.90 ml</td>
<td>0.5 g (74%)</td>
</tr>
</tbody>
</table>
2.3. Results and discussion

N-halamine biocidal polymers and poly Quat's are very important types of biocidal polymers. N-halamine biocidal polymers have been used in disinfection technology for more than a decade [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. To prepare this type of polymer a heterocyclic ring is introduced into the polymer backbone or side chain. The heterocyclic ring contains amide or imide function groups which can be halogenated [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. In this work the heterocyclic polymers were prepared by reacting the ready-prepared 5-aminobarbituric acid ring (1) (uramil) with a polymer, Schemes 2.1 and 2.2. Uramil contains three main positions available for halogenation which gave the opportunity to prepare multi-halogen biocidal polymers (MHBP). This increases the number of available halogens per monomer unit compared with existing N-halamine polymers [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. The positions in uramil available for halogenation are imides, rather than amides. Halogenated imide groups are considered to have potential as biocides as the imide group can easily release free halogen [16, 112, 142]. For example the dissociation constant for the imide group in trichlorocyanuric acid is from $1.6 \times 10^{-12}$ to $8.5 \times 10^{-4}$, for amide halogen is $1.6 \times 10^{-8}$ in 1,3-dichloro-2,2,5,5-tetramethylimidazolidine-4-one and for amino halogen is $<10^{-12}$ for 1-chloro-2,2,5,5-tetramethylimidazolidine-4-one [142]. This indicates that the amine halamine bond is the most stable but results in a less bioactive polymer as halogen is not easily released in aqueous media [142]. The imide halogen exhibits the lowest stability but offers the best
biological action while amide halogens have intermediate stability and moderate biological activity; enough for effecting disinfection [16, 142]. The presence of the methyl groups on the heterocyclic rings in 1,3-dichloro-2,2,5,5-tetramethylimidazolidine-4-one and 3-chloro-4,4-dimethyl-2-oxazolidinone increases the stability of the halogens attached to the polymer by increasing the electron density over the ring, and this makes the hydantion based polymers the best halamine biocidal polymers currently on the market [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. We took this into consideration to design a new type of halamine polymer containing imide groups where the stability of halogen attached to the polymer improved by increasing the electron density over the ring.

In polymer (2) (Scheme 2.2) prepared by reacting uramil with polyacrylonitrile; the resulting structure is a resonance hybrid between enamines and full ring delocalization. The resonance driven by the electron pairs of the amino group will result in increasing the electron density over the heterocyclic ring which was expected to stabilize the nitrogen-halogen bond, making the release of halogen more difficult. The same effect was expected with polymer (7) (Scheme 2.3), in this case the OH group of the amidol form being the resonance driving force.

The monomers (13, 24) (Scheme 2.5) were designed following a similar reasoning. In this case we expect that by coupling the heterocyclic ring over m-phenylenediamine or resorcinol the electron density over the heterocyclic ring has been increased. We expect that this results in increasing the stability of the halogen-nitrogen bond on the ring. After co-polymerization to form the polyurea and polyurethane, the
nitrogen and the oxygen lone electron pairs still have the ability to resonate with the heterocyclic ring creating stability.

Thus, by making use of uramil and the selected polymer backbones, we aimed to develop a new series of water insoluble N-halamine biocidal polymers, exhibiting a good balance of stability-biocidal activity and containing multi-available positions for halogenation (4 or more). N-halamine biocidal polymers currently available on the market depend on only one available position for halogenation based on hydantion systems [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. Some previous examples of N-halamine biocidal polymers have been reported based on 2 and 3 available halogenation positions, using six membered heterocyclic rings [7-9].

As explained above, the novel prepared polymers were designated to have more available positions for halogenation. Reacting uramil with polyacrylonitrile gave polymer (2) (Scheme 2.2) with expected 5 available positions for halogenation while using polyethylacrylate instead of polyacrylonitrile gave polymer (7) (Scheme 2.3) with 4 expected available positions for halogenation. The halogenation of polymer (7) was a highly exothermic reaction, unlike the halogenation of (2). In spite of careful temperature control and slow addition of halogen to polymer (7) the halogenation yield was very low. A plausible explanation is that some amide bonds connecting the heterocyclic ring to the polymer were hydrolysed during halogenation. This is supported by FTIR data which shows the presence of carboxylic acid OH group in polymer (8) (2423-3510 cm\(^{-1}\)) (Scheme 2.3) and the low halogen content measured, compared with similar polymers (polymers 8-10, table 2.2).
Monomers (13, 24) (Scheme 2.5), prepared by coupling uramildiazonium salt with resorcinol and \( m \)-phenylenediamine, were considered as novel azo-dyes. These two monomers were co-polymerized with tolylene-2,6-diisocyanate and toluene-2,4-diisocyanate to form polyurethanes (25, 30) and polyureas (14, 19). In polyurethanes (25, 30) there are 5 available positions for halogenation and in polyureas (14, 19) there are 7 positions for halogenation.

At the same time, the presence of heterocyclic rings containing nitrogen atoms in all prepared polymers skeletons increases the possibility of quaternarization to form poly Quat’s. The prepared heterocyclic polymers were quaternarized using diluted HCl to form the corresponding quaternary ammonium salts. The prepared polymers (series 1-6) contain 4-8 possible available positions for quaternarization. These possibilities increased the positive charges number on the polymer which supports the biological activity of the prepared poly Quat’s. However, in spite that using HCl in quaternarization is a simple commercial way but the prepared polymers in this case are very sensitive to the change in pH.

Modifying the prepared heterocyclic polymers by converting them to N-halamines or poly Quat’s supports their application in disinfection technologies. The biological action of the prepared N-halamine polymers is based on halogen delivering to the micro-organisms cells [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. Poly Quat’s biological action is based on adsorbing the bacterial cells to the polymer as the bacteria at certain physiological state can carry negative charge while the polymers carry positive charges [45].
Based on these facts the prepared polymers (N-halamine and poly Quat’s) have the potential to be used in disinfection technologies with advantages over similar polymers in the market. These advantages are: 1. The polymers contain more available positions for halogenation and quaternarization resulting in expected powerful biological action, 2. The stability of halogens attached to the N-halamine polymer has been improved to keep a good balance between the stability and biological activity.

At the same time, the prepared heterocyclic polymers without modification can work as bioactive agents if they have dissolved in the suitable solvents. All the prepared polymers are partially soluble in DMSO. This small amount of dissolved polymer was evaluated for biological activity in chapter 3 in addition to the biological evaluation for the modified forms of these polymers (N-halamine and Poly Quat’s) in their insoluble forms. The biological activity of the novel prepared azo-monomers (13, 24) was also included in this study (in their soluble form) to investigate their potential as antiseptics or antimicrobial agents.

2.4. Conclusion

Novel biocidal polymers (N-halamine and poly Quat’s) were prepared. The number of the available positions for the halogenation and quaternarization was increased and the stability of the halogen attached to the N-halamine polymer was improved.
Chapter 3

Microbiological evaluation and optimizing halogenation conditions
Part 1

Bacteriological evaluation of the prepared polymers and monomers

3.1.1 Introduction

The aim of this part is to investigate and quantify the biological activity of the novel prepared polymers and monomers. The biological activity was evaluated against examples of Gram-positive \textit{(Staphylococcus aureus)} and Gram-negative \textit{(Escherichia coli)} bacteria using three different methods; 1- agar plate assay (screening), 2- a stirred flask method and 3- water purification columns on a laboratory scale.

1- Agar plate assay was performed for all prepared polymers and monomers; N-halamine and poly Quat's polymers (in their insoluble form), the unmodified heterocyclic polymers (in their partially soluble form) and the prepared azo-monomers (in their soluble form). N-halamine polymers and poly Quat's were classified into groups (series 1-6) to investigate the effect of increasing the number of bioactive sites per polymer repeating unit on biological activity.

2- Using the stirred flask method, the biological activity of the halogenated derivatives (chlorinated, brominated and iodinated) of one of the prepared N-halamine polymers (25), Scheme 3.2, was quantified by determining their biological effect on bacterial growth and viability of \textit{E. coli} and \textit{S. aureus} with and without released halogen quenching.
3- The chlorinated form of this polymer (26), Scheme 3.2, was evaluated in water purification filters on a laboratory scale. Some low cost monomers were used in preparing alternative derivatives to polymer (25) to reduce the production costs.

3.1.2. Experimental

3.1.2.1. Biological activity of the prepared polymers

3.1.2.1.1. Agar plate assay (screening)

Nutrient agar (Oxoid) was prepared (250 ml), held molten at 50°C and 1.0 ml of a 24hr nutrient broth culture of either Staphylococcus aureus or Escherichia coli was added as inoculum. The seeded agar was poured into square assay plates (24 x 24 cm); two plates for each type of bacterium, Gram-positive or Gram-negative. Thirty-six, 5 mm wells were cut into the agar according to a square pattern (chessboard). Small amounts of each polymer (0.03 g) were placed in a well in the middle of each square. The experiment was performed in replicate, three wells per polymer. The plates were incubated for 24 hours at 37°C and the inhibition zones around the polymers were recorded. All the prepared polymers, including halogenated polymers and quaternarized salts were examined for biocidal activity in their insoluble form. Unmodified polymers (non-halogenated polymer in their insoluble form) were used as controls [7-9, 120].

The unmodified heterocyclic polymers (non-halogenated polymers) (2, 7, 14, 19, 25 and 30), Schemes 3.1 and 3.2, as well as monomers (13 and 24) were examined in their partially soluble or soluble form by dissolving in dimethylsulphoxide (DMSO) and
the solvent was used as a control. For each polymer 0.01 g was dissolved in 1 ml DMSO (the samples were soaked in DMSO for more than 10 days) and 0.03 ml from the prepared solution was added to the wells in the plates, the experiment was performed in replicate, three wells per polymer or monomer [73].

3.1.2.1.2. Effect of the halogenated polymers (chlorinated, brominated or iodinated) on bacterial growth and viability

A culture of *E. coli* was prepared by inoculating one bacterial colony from a nutrient agar plate into 20 ml of nutrient broth in a Universal bottle and incubating for 24 hr at 37°C. From the bacterial suspension 0.1 ml was transferred to a 20 ml Universal bottle containing 10 ml fresh medium. A further five Universals were prepared so the total number was six; three were used in testing the effect of the polymer on bacterial growth and the other three to test the effect of the polymer on the viability of the bacteria.

To study the effect of the polymer on the rate of growth of *E. coli*, 0.5 g of the halogenated polymer (chlorinated, brominated or iodinated) was added to the first bottle while 0.5 g from the control polymer (non-halogenated) was added to the second bottle to act as a polymer control and the third was left as a bacterial control without polymer. The three bottles were stirred at 37°C and sampled at timed intervals for viable count.

To study the effect of the polymer on the viability of *E. coli*, the other three bottles were incubated for 17 hr at 37°C, and the number of bacteria determined by viable count. Then 0.5 g of the halogenated polymer was added to one; 0.5 g of the control polymer (non-halogenated) was added to the second, to act as a polymer control and the third vessel was left as a bacterial control. The three bottles were stirred at room
temperature and samples from each culture taken at regular time intervals for viable count.

The viable counts were performed in both experiments using the “Miles and Misra technique”, Figure 3.1 [143]. The same procedure was repeated to investigate the effect of the halogenated polymers on a Gram-positive bacterium (S. aureus).

Figure 3.1: Example of the viable counts using the “Miles and Misra” method.

3.1.2.1.3. Effect of the halogenated polymers (chlorinated, brominated and iodinated) on bacterial viability under released halogen quenching

The previous experiment was repeated (polymer effect on bacterial viability section), however, during the viable counts 0.05 ml of 0.5M sodium thiosulphate was added to each decimal dilution to quench any released halogen which may evolve during the reaction between the polymer and the bacteria.
3.1.2.2. Effect of the non-halogenated polymer on the liquid medium

Non-halogenated polymer, 0.5 g, (non-halogenated 25, Scheme 3.2) was added to each of two Universal bottles each containing 10 ml of sterile liquid medium. One of these was stirred at ambient temperature, and the other was stirred at 37°C, for 17 hr.

The polymer was allowed to settle in each vessel and 5 ml of the overlaying broth removed to a fresh, sterile, Universal bottle. Bacterial suspension, 0.05 ml, (either *E. coli* or *S. aureus* prepared as described above) was added to inoculate them and growth of the cultures followed by viable count during incubation at 37°C. Bacterial suspension, 0.05 ml, was used to inoculate 5 ml of sterile liquid medium as a bacterial control and a viable count performed at the same time intervals as the incubated Universal bottles.

3.1.2.3. Evaluation of polymer (26) (chlorinated) in water-filter columns

Polymer (26), Scheme 3.2, 1.0 g, was loosely packed in a glass tube (15 cm length and 1 cm diameter) to a height of 4 cm. Two columns were prepared containing the original polymer (non-chlorinated form, 25, Scheme 3.2) which acts as a control, one for the *S. aureus* and the other for the *E. coli*. Two columns contained the N-halamine polymer (26), Scheme 3.2, one for *S. aureus* and the other for *E. coli*, therefore four columns in total. Bacterial suspension, prepared by inoculating one bacterial colony in 20 ml of liquid medium (Nutrient Broth, Oxoid) and incubated for 17 hours at 37°C, was passed through the column and the output recycled through it again. Before recycling, 0.1 ml from the passed liquid was sampled for viable count. Five cycles were performed for each column. The number of viable cells in the original bacterial suspensions was determined before passing the bacteria through the column. In addition, the turbidity of
the liquid before and after passing through the columns was determined spectrophotometrically at 540 nm [8, 9].

3.1.3. Results and discussion

3.1.3.1. Biological activity investigation using agar plate assay

The biological activity of the prepared polymers (N-halamine polymers and poly Quat's) as well as the non-modified polymers (heterocyclic polymers) and monomers was examined using the agar plate assay.

3.1.3.1.1. Insoluble polymers

In this investigation the polymers were evaluated in their insoluble form (as solids). N-halamine polymers have the ability to exchange halogen with the bacterial cells although present in an insoluble form [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. Poly Quat’s are able to adsorb to the bacterial cells due to the positive charges generated on its surface [45] as discussed chapters 1 and 2.

Tested against Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria, the polymers were divided into three categories: the first group is the polymer without any modification (water insoluble polymers) including polymers (2), (7), (14), (19), Scheme 3.1, (25) and (30), Scheme 3.2, which acted as controls. The second category contains the water insoluble N-halamine biocidal polymers resulting from chlorination, bromination and iodination of the first category and this includes polymers (3), (4), (5), (8), (9), (10), (15), (16), (17), Scheme 3.1, (26), (27), (28),
(31), (32) and (33), Scheme 3.2. The third category contains the quaternarized forms (with limited water solubility), which resulted from acidification of the first category and this includes polymers (6), (11), (18), (23), Scheme 3.1, (29) and (34), Scheme 3.2. The polymers resulting from modifications to polymer (2), (7), (14), (19), Scheme 3.1, (25) and (30), Scheme 3.2, were considered as series 1, 2, 3, 4, 5 and 6 respectively, Schemes 3.1 and 3.2.

Tested by the agar plate method described earlier, zones of inhibition of bacterial growth around each polymer were recorded after incubation at 37°C for 24 hours (tables 3.1 and 3.2). Values given are the means of triplicate inhibition zone diameters, example in Figure 4.4 (chapter 4, see page 131).
Scheme 3.1: Series 1-4 of prepared biocidal polymers (N-halamines and poly Quat’s).
Scheme 3.2: Series 5 and 6 of prepared biocidal polymers (N-halamines and poly Quat's).
Table 3.1: Inhibition zone diameters (mm) around different polymers on agar plates containing Gram-negative bacteria (*E. coli*).

<table>
<thead>
<tr>
<th></th>
<th>series 1</th>
<th>series 2</th>
<th>series 3</th>
<th>series 4</th>
<th>series 5</th>
<th>series 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chlorinated</td>
<td>7 ±1</td>
<td>0</td>
<td>18 ±1</td>
<td><strong>29 ±1</strong></td>
<td><strong>24 ±1</strong></td>
<td><strong>28 ±1</strong></td>
</tr>
<tr>
<td>Brominated</td>
<td>18 ±1</td>
<td>8 ±1</td>
<td>22 ±1</td>
<td><strong>27 ±1</strong></td>
<td><strong>22 ±1</strong></td>
<td><strong>29 ±1</strong></td>
</tr>
<tr>
<td>Iodinated</td>
<td>9 ±1</td>
<td>6 ±1</td>
<td>15 ±1</td>
<td>15 ±1</td>
<td>18 ±1</td>
<td>10 ±1</td>
</tr>
<tr>
<td>Poly-quats</td>
<td>15 ±2</td>
<td>8 ±1</td>
<td>6 ±1</td>
<td>13 ±1</td>
<td>16 ±1</td>
<td>13 ±1</td>
</tr>
</tbody>
</table>

Table 3.2: Inhibition zone diameters (mm) around different polymers on agar plates containing Gram-positive bacteria (*S. aureus*).

<table>
<thead>
<tr>
<th></th>
<th>series 1</th>
<th>series 2</th>
<th>series 3</th>
<th>Series 4</th>
<th>series 5</th>
<th>series 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chlorinated</td>
<td>6 ±1</td>
<td>0</td>
<td>19 ±1</td>
<td>23 ±1</td>
<td><strong>28 ±1</strong></td>
<td><strong>28 ±1</strong></td>
</tr>
<tr>
<td>Brominated</td>
<td>14 ±1</td>
<td>10 ±1</td>
<td>20 ±1</td>
<td>21 ±1</td>
<td><strong>24 ±1</strong></td>
<td><strong>25 ±1</strong></td>
</tr>
<tr>
<td>Iodinated</td>
<td>9 ±1</td>
<td>8 ±1</td>
<td>18 ±1</td>
<td>31 ±2</td>
<td>19 ±1</td>
<td>20 ±1</td>
</tr>
<tr>
<td>Poly-quats</td>
<td>15 ±1</td>
<td>8 ±1</td>
<td>7 ±1</td>
<td>11 ±1</td>
<td>21 ±1</td>
<td>19 ±1</td>
</tr>
</tbody>
</table>

From tables 3.1 and 3.2, the controls (the original polymers without modification) did not show any biological activity; these polymers are not soluble in water and do not release any effective ion that can diffuse into the medium. The second category, N-halamine biocidal polymers, showed significant inhibition zones. These polymers are also water-insoluble; implying their mode of action is the release into the medium of soluble...
halogen-containing ions that are lethal to the bacterial cells. Producing wide inhibition zone around the polymers up to 28 mm in some examples indicates that the mode of action of these polymers (N-halamine polymers) is not by contact only [11, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145] as described in literature but ions can be released from the polymer to the medium.

The action of the third category (Poly Quat’s) depends on the solubility of the polymer itself. The conversion of the polymer to its quaternary salt increases its solubility (to a limited extent) so it can diffuse through the medium. In addition, the non-soluble part of the polymer (poly Quat’s) is able to adsorb the bacterial cells due to the presence of positive charges on the polymer [45].

Polymer (7), Scheme 3.1, and its modified polymer (8), Scheme 3.1, (chlorinated) did not show any bactericidal activity while the other modified forms of it showed low biological activity (series 2). This is may be due to halogenation of this polymer being a highly exothermic reaction which resulted in hydrolysis of some heterocyclic rings leading to a decrease in its biological power.

From tables 3.1 and 3.2 it was clear that the N-halamine polymers in series 3-6 show greater biological activity than series 1 and 2; perhaps because the number of available positions for the halogen in series 3-6 is greater than that in series 1 and 2, which is reflected in the amount of halogen loading on the polymer, table 2.2.

The biological activity of the quaternized forms is similar; especially series 1 which becomes very similar to series 3-6, and may be because the repeating unit formula-weight of the poly-Quat’s in series 1 is very low compared to the available positions for acidification on its surface.
3.1.3.1.2. Monomers and partially soluble polymers

Heterocyclic polymers (unmodified polymers) including polymers (2), (7), (14), (19), Scheme 3.1, (25) and (30), Scheme 3.2, as well as the monomers (13 and 24), were also tested for bioactivity by dissolving them in an organic solvent and using this solvent as a control, table 3.3. The unmodified heterocyclic polymers are partially soluble in DMSO so the small dissolved part was used to evaluate their biological activity for potential use as antifouling agents. The prepared azo-monomers (13 and 24) were examined for biological activity in their soluble form to evaluate the potential of their use as antiseptics or antimicrobial agents.

Table 3.3: The inhibition zones (mm) resulting from the dissolved control polymers and monomers.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (control)</td>
<td>5 ±1</td>
<td>5 ±1</td>
</tr>
<tr>
<td>2 (series 1 control)</td>
<td>9 ±1</td>
<td>7 ±1</td>
</tr>
<tr>
<td>7 (series 2 control)</td>
<td>8 ±1</td>
<td>9 ±1</td>
</tr>
<tr>
<td>13 (monomer)</td>
<td>8 ±1</td>
<td>6 ±1</td>
</tr>
<tr>
<td>14 (series 3 control)</td>
<td>9 ±1</td>
<td>9 ±1</td>
</tr>
<tr>
<td>19 (series 4 control)</td>
<td>10 ±1</td>
<td>9 ±1</td>
</tr>
<tr>
<td>24 (monomer)</td>
<td>9 ±1</td>
<td>8 ±1</td>
</tr>
<tr>
<td>25 (series 5 control)</td>
<td>10 ±1</td>
<td>8 ±1</td>
</tr>
<tr>
<td>30 (series 6 control)</td>
<td>10 ±1</td>
<td>7 ±1</td>
</tr>
</tbody>
</table>
DMSO was used as control in most cases except monomer (24), Scheme 3.2, which was dissolved in distilled water. DMSO inhibition zone diameter was 5 mm for both *E. coli* and *S. aureus*.

From table 3.3 it was clear that all the prepared polymers and monomers show some, but limited, biological activity perhaps indicating a potential use for them as antifouling agents and as antiseptics.

From the previous results it can be seen that all the halogenated polymers (N-halamine) and poly Quat’s showed significant biological activity, even outstanding in some cases which supports their use in the different applications of disinfection technology. The biological activity is directly proportional to the number of available positions for halogenation per repeating unit of the polymer. Increasing the number of available positions for halogenation per repeating unit of the polymer increases the biological activity of the polymer. The shape and width of the inhibition zones around the polymer indicates that there is a release of ions from the polymer to the bacterial media which suggests that the mode of action of the N-halamine polymers is not by contact only.

The non-modified polymers as well as the monomers may also be used as bioactive materials by dissolving them in suitable solvents.

Further biological evaluation was applied for one of the prepared N-halamine polymers because the agar plate assay cannot give full quantitative information for the biological activity or the rate of bacterial killing by the polymers. The biological power of the halogenated derivatives of polymer (25), Scheme 3.2, was quantified to investigate their availability to be used in different disinfection purposes.
3.1.3.2. Effect of the halogenated polymers on bacterial growth-rate and viability

3.1.3.2.1. Growth rate

In order to identify the effect of the prepared N-halamine polymers on bacterial growth bacterial cells were grown in the presence of the halogenated polymers. No bacterial growth was recorded in the presence of any of the halogenated polymers. Figure 3.3 shows that *S. aureus* does not grow in the presence of chlorinated polymer (26), Scheme 3.2, or in the presence of the polymer control (non-halogenated polymer) (25), Scheme 3.2; which, as *S. aureus* is not motile, may be due to adsorption onto the polymer surface. From Figure 3.2, *E. coli* also did not grow in presence of chlorinated polymer (26), Scheme 3.2. However, unlike *S. aureus* it grew in presence of the control polymer (non-halogenated polymer) but at a different rate and to a lower final population than the *E. coli* control. This may be due to differences in the motility and surface composition of the two types of bacteria.

The same behaviour was recorded for brominated polymer (27) (Scheme 3.2), Figures 3.4 and 3.5, and iodinated polymer (28) (Scheme 3.2), Figures 3.6 and 3.7. The halogen exchange between the halogenated polymer and the bacterial cells used in inoculating the medium may result in the cells death and stopped the growth. In addition, the possibility of halogen exchange between the halogenated polymers and the medium constituents such as protein may result in changing the nature of nutrition around the cells resulting in stopping the cells growth. The interaction between the halogenated polymers and the medium constituents will be discussed in details in chapter 6.
As shown in Figures 3.2 and 3.3 the non-halogenated polymer (25), Scheme 3.2, inhibits the growth of *S. aureus* and has some limited effect on the growth of *E. coli*. A plausible explanation may be the removal of critical nutrient broth components by the polymer through adsorption onto its surface. To investigate this possibility, fresh broth was treated with polymer (25), Scheme 3.2, at different temperatures, the polymer removed by allowing it to settle, then removing and using the overlying liquor. The isolated medium was inoculated with a fresh culture of *S. aureus* or *E. coli* and the bacterial growth followed by viable counts.

Figures 3.8 and 3.9 show that treating the broth with non-halogenated polymer (25), Scheme 3.2, adversely affects the rate of bacterial growth, even after removal of the polymer. This suggests that there may be a dual mode of action, one by direct contact polymer-bacteria (possibly there is significant bacterial adsorption in the case of *S. aureus*) and another by affecting the broth composition (through nutrient adsorption onto the polymer).
Figures 3.2 and 3.3: Log. plot of viable counts (cfu/ml) vs time of *E. coli* and *S. aureus*, respectively, grown in the presence of the chlorinated polymer (26), the control polymer (25) and without polymer (bacterial control).

3.2, *E. coli*

3.3, *S. aureus*

Where: T = Test (bacteria + chlorinated polymer 26), BC = Bacterial control (no treatment) and PC = Polymer control (bacteria + non-halogenated polymer 25).
Figures 3.4 and 3.5: Log. plot of *E. coli* and *S. aureus* viable count (cfu/ml) vs time grown in the presence of the brominated polymer (27), the control polymer (25) and without treatment (bacterial control).

3.4, *E. coli*

![Graph showing log no. of bacterial colonies cfu/ml vs time (hr)](image)

Where: T = Cells treated with brominated polymer, PC = Cells treated with the control polymer (non-halogenated polymer) and BC = Bacterial control (no treatment).

3.5, *S. aureus*

![Graph showing log no. of bacterial colonies cfu/ml vs time (hr)](image)
Figures 3.6 and 3.7: Log no. of *E. coli* and *S. aureus* viable count (cfu/ml) vs time during growth in nutrient broth; in the presence of the iodinated polymer (28), the non-halogenated polymer (25) and without treatment (bacterial control).

3.6, *E. coli*

![Graph of E. coli growth](image)

3.7, *S. aureus*

![Graph of S. aureus growth](image)

Where: T = Cells treated with the iodinated polymer, PC = Polymer control, cells treated with non-halogenated polymer, BC = Bacterial control, untreated cells.
Figures 3.8 and 3.9: Log plot of viable count (cfu/ml) vs time of *S. aureus* and *E. coli*, respectively, grown in liquid medium previously stirred with the non-halogenated polymer (25).

3.8, *S. aureus*

![Figure 3.8](image)

3.9, *E. coli*

![Figure 3.9](image)

Growth of *S. aureus* (*S*<sub>25</sub>) *E. coli* (*E*<sub>25</sub>) in medium pre-treated with the non-halogenated polymer at 25°C (or, *S*<sub>37</sub> and *E*<sub>37</sub> at 37°C). *S*<sub>c</sub> and *E*<sub>c</sub> are the respective control grown in untreated nutrient broth.
3.1.3.2.2. Viability

The effect of the halogenated polymers on bacterial viability was investigated by treating pre-grown cultures with the halogenated polymer. The chlorinated polymer (26), Scheme 3.2, achieved a 3 log reduction in the bacterial population in 7 minutes while no bacterial colonies were detected after 15 minutes (equivalent to a 9 log reduction) in the case of S. aureus, Figure 3.11. A similar behaviour was recorded with E. coli, a 3 log reduction was recorded in the first 7 minutes while no colonies were detected after 15 minutes (equivalent to a 10 log reduction), Figure 3.10. The brominated polymer (27), Scheme 3.2, behaves similarly to the chlorinated polymer (26), Scheme 3.2, for E. coli, Figure 3.12, and also for S. aureus but with a 9 log reduction in 15 minutes, Figure 3.13. The iodinated polymer (28), Scheme 3.2, showed the highest power of sterilization, Figure 3.14, showing a 10 log reduction in the population of E. coli after 7 min following contact with the bacteria. S. aureus behaves similarly; no colonies were detected after 7 min contact between the iodinated polymer (28), Scheme 3.2, and the bacteria, Figure 3.15.

To determine the rate of killing by the iodinated polymer (28), Scheme 3.2, the amount of polymer in contact with the bacteria was reduced; the experiment was repeated, but using 0.25 g of polymer in contact with 10 ml of bacterial suspension and the time intervals were reduced to detect any viable bacterial colonies early in the culture. For E. coli, a 5 log reduction in the bacterial population was achieved in 1 min and no viable colonies detected (equivalent to 10 log reduction) after 5 min contact time. The results for S. aureus were unequivocal, the rate of killing by the iodinated polymer could not be determined due to the powerful effect of the iodine ions from the polymer; no
viable colonies were detected after 1 min contact time (equivalent to 9 log reduction), Figures 3.16 and 3.17 respectively.
Figures 3.10 and 3.11: Log plot of viable counts (cfu/ml) vs time of E. coli and S. aureus, respectively, in nutrient broth after stirring with the chlorinated polymer (26), control polymer (25) and without polymer (bacterial control).

3.10, E. coli

3.11, S. aureus

Where: T = Test (bacteria + chlorinated polymer 26), BC = Bacterial control (no treatment) and PC = Polymer control (bacteria + non-halogenated polymer 25).
Figures 3.12 and 3.13: Log no. of colonies (cfu/ml) vs time of *E. coli* and *S. aureus*, after exposure to the brominated polymer (27), the non-halogenated polymer (25) and without treatment (bacterial control).

3.12, *E. coli*

3.13, *S. aureus*

Where: T = Cells treated with brominated polymer, PC = Cells treated with the control polymer (non-halogenated polymer) and BC = Bacterial control (no treatment).
Figures 3.14 and 3.15: Log no. of *E. coli* and *S. aureus*, respectively, vs time after treating the bacterial cells with; the iodinated polymer (28), (0.5 g/10 ml bacterial suspension) the non-halogenated polymer and without treatment (bacterial control).

3.14, *E. coli*

![Graph showing log no. of bacterial colonies/cm^3 vs time (hr) for E. coli.](image)

3.15, *S. aureus*

![Graph showing log no. of bacterial colonies/cm^3 vs time (hr) for S. aureus.](image)

Where: T = Cells treated with the iodinated polymer, PC = Polymer control, cells treated with non-halogenated polymer and BC = Bacterial control, untreated cells.
Figures 3.16 and 3.17: Log no. of *E. coli* and *S. aureus*, respectively, at timed intervals after stirring the bacterial cells with; iodinated polymer (28) (0.25 g/10 ml bacterial suspension) and without treatment (bacterial control).

3.16, *E. coli*

Where: T = Cells treated with the iodinated polymer and BC = Bacterial control, untreated cells.

3.17, *S. aureus*
The previous results indicated that the halogenated polymers have affected the bacterial viability of both Gram-positive and Gram-negative bacteria due to the halogen exchange between the polymer and the cells. The effect of the iodinated polymer is more than that of the brominated and chlorinated derivatives due to the lower stability of N-I bond than that of N-Cl or N-Br. These results encourage applying these polymers for disinfection purposes and indicated that the polymer design kept a balance between the stability and biological activity. In spite of the presence of strong electron donating groups stabilizing the halogen attached to the polymer, the polymer showed good biological activity.

However, these results did not help in clarifying the mode of action of these polymers, was it contact or release? Stirring the polymer with the bacterial cells may result in halogen exchange by both mechanisms. The halogen can exchange directly by the direct collision between the bacterial cells and the polymer particles or indirectly by releasing halogen ions to the medium followed by diffusion to the cells. During the investigation of the effect of halogenated polymers on bacterial viability, removing polymer results in stopping its action by direct contact. But if the polymer has released any halogen ions to the medium the effect will continue after removing polymer.

Assuming that the halogenated polymer has released some ions to the medium, stopping the effect of these ions will produce different results from what was reported before. To demonstrate this assumption, the same experiment (effect of halogenated polymer on bacterial viability) was repeated with quenching the free halogen released from the polymer after polymer removal.
To quench the released halogen ions, sodium thiosulphate was added to the collected samples for viable counts (immediately prior to counting). The chlorinated polymer (26), Scheme 3.2, achieved a 2 log reduction in the *E. coli* population in 40 minutes and no viable colonies were detected after 1.5 hr (9 log reduction). Similarly the chlorinated polymer achieved a 1 log reduction in 40 minutes for the *S. aureus* population and no bacterial colonies were detected after 1.5hr (9 log reduction), Figures 3.18 and 3.19 respectively. The brominated polymer (27), Scheme 3.2, showed greater activity, achieving a 4 log reduction in the *E. coli* population in 40 minutes and no viable colonies detected after 1.5 hr (9 log reduction) while it achieved a 4 log reduction in the *S. aureus* population in 15 minutes and no viable colonies detected after 40 minutes (9 log reduction), Figures 3.20 and 3.21 respectively. For the Iodinated polymer (28), Scheme 3.2, the experiment was performed with a 1 g : 40 ml ratio between the polymer weight and the bacterial suspensions due to the high biocidal power of the iodinated polymer. In spite of this reduced quantity of polymer, no *E. coli* or *S. aureus* colonies were detected at 7 min (9 log reduction), Figures 3.22 and 3.23 respectively.
Figures 3.18 and 3.19: Effect of the chlorinated polymer (26) on the viability of *E. coli* and *S. aureus* respectively under chlorine quenching.

3.18, *E. coli*

3.19, *S. aureus*

Where: T = Treated cells with the halogenated polymer and BC = Untreated cells (bacterial control).
Figures 3.20 and 3.21: Effect of the brominated polymer (27) on the viability of *E. coli* and *S. aureus* respectively under bromine quenching.

3.20, *E. coli*

3.21, *S. aureus*

Where: T = Treated cells with the halogenated polymer and BC = Untreated cells (bacterial control).
Figures 3.22 and 3.23: Effect of the iodinated polymer (28) on the viability of *E. coli* and *S. aureus* respectively under iodine quenching.

3.22, *E. coli*

![Graph 3.22 showing the effect of iodinated polymer on the viability of *E. coli*](image)

3.23, *S. aureus*

![Graph 3.23 showing the effect of iodinated polymer on the viability of *S. aureus*](image)

Where: T = Treated cells with the halogenated polymer and BC = Untreated cells (bacterial control).
From these results (with and without quenching) it was clear that the most powerful biocidal effect is exhibited by the iodinated polymer. Without halogen quenching the rate of killing of the chlorinated and brominated polymers are very similar, while with halogen quenching, the brominated polymer shows a more powerful effect on each type of bacteria (Gram-positive and Gram-negative). This may be related to the stability of the halogen on the polymer as discussed before; I-N bonds have the lowest stability therefore the halogen can be easily exchanged between the polymer and bacteria as explained before. The Cl-N exhibits the lowest biological power but is the most stable bond which supports its use in disinfection purposes.

The difference in the killing rates of the halogenated polymers with and without halogen quenching (a lower rate with quenching) indicates that halogen species (released from the polymers) are involved in the biocidal action; suggesting the mechanism of killing is not by contact alone. This dual mechanism of killing (contact + release of halogen species) will be discussed in chapter 6.

3.1.3.3. Determination of the killing rate of chlorinated polymer (31), Scheme 3.2, under quenching conditions and in comparison with chlorinated polymer (26), Scheme 3.2

Due to the high production costs of the N-halamine biocidal polymers, which restricts applying this type of polymer on a large scale, commercial toluene-2,4-diisocyanate was used instead of tolylene-2,6-diisocyanate to reduce the production costs of this polymer design. From Figure 3.24, chlorinated polymer (31) achieved a 6 log reduction in the *E. coli* population in 40 minutes and no bacterial colonies were detected
after 40 minutes (equivalent to 9 log reduction). For *S. aureus*, Figure 3.25, the chlorinated polymer (31) achieved a 6 log reduction in 40 minutes and no colonies detected after 90 minutes (equivalent to 9 log reduction). From Figures 3.24 and 3.25 it was clear that using toluene-2,4-diisocyanate (polymer 31) instead of tolylene-2,6-diisocyanate (polymer 26) gave very similar results. A complete study on producing more commercial low cost N-halamine polymers will be discussed in detail in chapter 5.
Figures 3.24 and 3.25: Effect of chlorinated polymer (31) on the viability of *E. coli* and *S. aureus* respectively under chlorine quenching.

3.24, *E. coli*

![Graph 1](image1.png)

Where: T = Cells treated with the chlorinated polymer and BC = Cells without treatment (bacterial control).

3.25, *S. aureus*

![Graph 2](image2.png)
3.1.3.4. Chlorinated polymer (26), Scheme 3.2, evaluation in water filters

This experiment investigated the potential of using one of the halogenated polymers (26) in water filters on a laboratory scale. Most of the biological evaluation for N-halamine polymers described in the literature was based on using columns in quantifying the biological activity of the N-halamine polymers [17, 92, 94]. However using columns alone is not enough because of the expected filtration effect. For this reason, we have quantified the biological activity of the N-halamine polymers using different methods such as agar plate assay and a stirred flask method as described above to have a complete picture of the biological power of the polymer before investigating columns.

Figures 3.26 and 3.27 show the effect of the control polymer (25) contained in a 4cm length and 1cm diameter column on suspensions of *S. aureus* and *E. coli*. After 3 cycles no viable colonies of *S. aureus* were detected in the eluate, Figure 3.26. Whereas for *E. coli*, although there was an initial reduction in the population, this recovered and there was no significant reduction overall in the bacterial population over the 5 cycles. This suggests that the control polymer is merely acting as a filter, allowing passage of the motile *E. coli* but not exhibiting any biocidal action, Figure 3.27.

Results from columns containing the chlorinated polymer (26) are shown in Figures 3.28 and 3.29 and table 3.4. After one passage through the column the populations of both types of bacteria were reduced to a non-detectable level (equivalent to an 8 log reduction).

This was also reflected in the spectrophotometric measurements (table 3.4); the negative values after 5 cycles are due to a bleaching effect on the broth medium. Further
incubation of the samples post-passage confirmed the biocidal effect and demonstrates the potential application of the polymer in water filters. The results obtained by this polymer are very similar to those reported in the literature on similar columns [17].

The previous data indicated that the prepared polymers and monomers have the potential to be used in disinfection technologies and the design of the novel prepared N-halamine polymers kept the balance between the stability and biological activity. The biological activity of the prepared N-halamine polymers was quantified using the agar plate method and stirred flasks method rather than using columns only. This demonstrates a good biological effect without any filtration possibilities that can be generated by columns. The difference in the biological action with and without quenching indicates that the mode of action of these polymers can be by release in addition to the contact effect and not by contact only as stated in most of the reported work in the literature [11, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145], further investigations into the mode of action of N-halamine polymers were conducted in chapter 6.
Figures 3.26 and 3.27: Log viable count (cfu/ml) of *S. aureus* and *E. coli* recovered from the eluate after each passage through the column containing control polymer.

3.26, *S. aureus*

![Graph showing log viable count (cfu/ml) of *S. aureus*](image)

3.27, *E. coli*

![Graph showing log viable count (cfu/ml) of *E. coli*](image)
Figures 3.28 and 3.29: Log viable counts (cfu/ml) of *S. aureus* and *E. coli* respectively after each cycle through the column containing chlorinated polymer (26).

3.28, *S. aureus*

![Graph showing log viable counts of S. aureus](image)

3.29, *E. coli*

![Graph showing log viable counts of E. coli](image)
Table 3.4: Absorbance (at 540 nm, read against a nutrient broth blank) and viable counts before and after perfusing columns of non-halogenated and halogenated polymers.

<table>
<thead>
<tr>
<th></th>
<th>Non-halogenated polymer</th>
<th>Halogenated polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(control)</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>0.41</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>0.30</td>
<td>-0.01</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>0.04</td>
<td>(Nd)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>1.33</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>0.46</td>
<td>0.03</td>
</tr>
<tr>
<td>(Viable counts (cfu/ml))</td>
<td>(2.6 x 10^9)</td>
<td>(1.8 x 10^9)</td>
</tr>
<tr>
<td></td>
<td>(8.5 x 10^7)</td>
<td>(3.1 x 10^9)</td>
</tr>
<tr>
<td></td>
<td>(8.4 x 10^8)</td>
<td>(Nd)</td>
</tr>
<tr>
<td></td>
<td>(Nd)</td>
<td>(Nd)</td>
</tr>
<tr>
<td></td>
<td>(2.5 X 10^9)</td>
<td>(Nd)</td>
</tr>
<tr>
<td></td>
<td>(7.5 X 10^7)</td>
<td>(Nd)</td>
</tr>
</tbody>
</table>

Nd = Non-detected.

3.1.4. Conclusion

The prepared polymers (N-halamine and poly Quat’s) showed high biological activity which supports their use in disinfection technologies. Similarly, the non-modified polymers (heterocyclic polymers) in their partially soluble form, as well as the prepared azo-monomers, showed good biological activity which supports the ability to use them as antifouling agents or antiseptics.
The derivatives of one of the prepared N-halamine polymers showed high biological effect on the viability and growth rate of Gram-positive and Gram-negative bacteria. A difference in the biological effect of the N-halamine polymers on bacterial viability with and without quenching of the released halogen ions indicates that the mode of action of the N-halamine polymers can be produced by ion release in addition to the contact.

One of the chlorinated derivatives of the N-halamine polymers was used successfully in water filters on a laboratory scale.

An alternative low cost monomer was used in polyurethane preparation without significant change in the biological activity of the polymer.

The non-halogenated polymer (25) showed some effect on the bacterial medium which then inhibits the bacterial growth.
Part 2

Optimising the halogenation conditions for the production of N-halamine polymers

3.2.1. Introduction

In this part of the work the optimum halogenation conditions of the N-halamine polymers were determined to achieve the best biological activity by increasing the halogen load on the polymer. Some researchers have used chlorine gas at 0°C [17, 92, 94] while others have used sodium hypochlorite at ambient temperature [14, 15, 98, 112, 128, 129] without any study of the possibilities of changing the conditions to improve the halogen load.

Sodium hypohalate (NaOX, where X is Cl, Br or I) was used in this study rather than using halogen gas; for commercial reasons as well as to reduce the danger resulting from using gases.

Three parameters were investigated; temperature, sodium hypohalate (NaOX, where X is Cl, Br or I) concentration and halogenation time. The polymer samples prepared under these different conditions were examined against examples of Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria. The study was performed using the halogenated derivatives of an example from the prepared polymers (polyurethane 25), Scheme 3.3.
3.2.2. Experimental

3.2.2.1. Chlorination of polymer (25) under different conditions

Chlorination conditions were changed methodically in order to determine optimal conditions. Three parameters were tested:

First, the effect of chlorination times: 5 conical flasks were prepared and each one contained 1 g of the non-chlorinated polymer, 5 ml of 10% (w/w) NaOCl solution and 5 ml water. Each flask was then stirred for a different time interval at room temperature; for 30 min, 1 hr, 2 hr, 4 hr and 6 hr and the product from each experiment filtered, washed copiously with halogen-free water and dried.

Second, the effect of chlorination temperature was determined; a set of 4 flasks was prepared and each one (containing 1 g of the non-chlorinated polymer, 5 ml of 10%, w/w, NaOCl solution and 5 ml water) was stirred for 1 hr at a different temperature; 0°C,
25°C, 30°C and 45°C. The product from each experiment was filtered, washed copiously with halogen-free water and dried.

Third, the effect of NaOCl concentration: again 4 conical flasks were prepared, this time each one contained 1 g of the non-chlorinated polymer and 10 ml of NaOCl solution. The NaOCl concentration was changed for each flask; 2.5%, 5%, 7.5% and 10% (w/w). Each flask was stirred for 1 hr at room temperature and the product from each experiment filtered, washed copiously with halogen-free water and dried under vacuum.

The experiment was repeated using sodium hypobrominate and sodium hypoiodinate to investigate suitable conditions for bromination and iodination. Sodium hypobrominate was prepared by dropping bromine over a solution of 10% (w/w) sodium hydroxide (in an ice bath to avoid raising the temperature) until it reached pH 7; a similar protocol was used to prepare 10% (w/w) sodium hypoiodinate.

The halogen load to the polymer was determined using iodometric titration [38, 141], table 3.5.
Table 3.5: The halogen load on the polymers prepared at different halogenation conditions.

<table>
<thead>
<tr>
<th></th>
<th>Chlorine (ppm)</th>
<th>Bromine (ppm)</th>
<th>Iodine (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Halogenated at different temperatures</strong> (NaOCl 5% for 1 hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5°C</td>
<td>107 ±0.7</td>
<td>169 ±12</td>
<td>208 ±20</td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>120 ±0.4</td>
<td>184 ±4.3</td>
<td>222 ±1</td>
</tr>
<tr>
<td>35°C</td>
<td>70 ±0.7</td>
<td>176 ±1.8</td>
<td>231 ±2.9</td>
</tr>
<tr>
<td>45°C</td>
<td>64 ±2.2</td>
<td>154 ±2.9</td>
<td>101 ±5.7</td>
</tr>
<tr>
<td><strong>Halogenated at different times</strong> (NaOCl 5%, ambient temperature)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>112 ±1.7</td>
<td>154 ±1.2</td>
<td>92 ±1</td>
</tr>
<tr>
<td>1 hr</td>
<td>122 ±2.5</td>
<td>169 ±11.4</td>
<td>208 ±2.9</td>
</tr>
<tr>
<td>2 hr</td>
<td>130 ±20</td>
<td>186 ±2.2</td>
<td>212 ±1</td>
</tr>
<tr>
<td>4 hr</td>
<td>141 ±1.5</td>
<td>190 ±0.7</td>
<td>218 ±2.9</td>
</tr>
<tr>
<td><strong>Halogenated at different concentrations of NaOX</strong> (1 hr at ambient temperature)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 % (w/w)</td>
<td>78 ±0.7</td>
<td>110 ±2.2</td>
<td>162 ±1</td>
</tr>
<tr>
<td>5.0 % (w/w)</td>
<td>118 ±0.7</td>
<td>172 ±2.9</td>
<td>208 ±2.9</td>
</tr>
<tr>
<td>7.5 % (w/w)</td>
<td>189 ±0.7</td>
<td>198 ±1.8</td>
<td>226 ±5.8</td>
</tr>
<tr>
<td>10 % (w/w)</td>
<td>210 ±0.9</td>
<td>218 ±3.2</td>
<td>231 ±3.0</td>
</tr>
</tbody>
</table>
3.2.2.2. Biological activity of the N-halamine polymers: varying halogenation parameters during the halogenation process

Bacterial suspensions were prepared by inoculating nutrient broth (10 ml) using a single bacterial colony followed by incubation at 37°C for 17 hours. The bacterial suspension (0.1 ml) was used to inoculate fresh bacterial suspensions in Universal vessels and they were incubated at 37°C for 17 hours. One of these vessels was used as bacterial control (either Gram-positive or Gram-negative) while each of the others was treated with 0.25 g of each polymer sample prepared at different time intervals. The vessels were stirred at room temperature, and viable counts performed at timed intervals.

The experiment was repeated to test the biological activity of polymer samples prepared at different chlorination temperatures and using different NaOCl concentrations. Brominated and iodinated polymers were examined similarly.

3.2.3. Results and discussion

3.2.3.1. Halogenation time

Halogenation time intervals of 0.5, 1, 2, 4 and 6 hr were examined using 5% (w/w) NaOCl at ambient temperature. For the chlorinated polymer (26), increasing the halogenation time up to 2 hours increased the biological activity of the polymer against E. coli; while after 4 hours the biological activity remained constant, Figure 3.30. The same effect was noticed for S. aureus, Figure 3.31. Similar behaviour was observed in the case of bromination, Figures 3.32 and 3.33, and iodination, Figures 3.34 and 3.35. Bromination and iodination were performed for up to 4 hours only. For the brominated
polymer, the biological activity increased with increasing bromination time, up to 2 hours; while after 2 hours of halogenation the biological activity remained constant for both *E. coli* and *S. aureus*. A similar pattern was recorded for the iodinated polymer. The quantity of the iodinated polymer was reduced to 0.06 g during this experiment. Conditions for bromination and iodination were the same as for chlorination (5% NaOX at ambient temperature, where $X = \text{Br or I}$).
Figures 3.30 and 3.31: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with the chlorinated polymer prepared at different chlorination times.

3.30, *E. coli*

![Graph showing the log number of *E. coli* colonies at different chlorination times.]

3.31, *S. aureus*

![Graph showing the log number of *S. aureus* colonies at different chlorination times.]

Where: BC = bacterial control (no treatment), 1 - 6 hr = samples halogenated for 1 - 6 hr.
Figures 3.32 and 3.33: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with brominated polymers brominated at different bromination periods.

3.32, *E. coli*

![Graph of log no. of *E. coli* colonies](image)

3.33, *S. aureus*

![Graph of log no. of *S. aureus* colonies](image)

Where: BC = bacterial control (no treatment), 1 - 6 hr = samples halogenated for 1 - 6 hr.
Figures 3.34 and 3.35: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with the iodinated polymers samples prepared at different iodination periods.

3.34, *E. coli*

Where: BC = bacterial control (no treatment), 1 - 6 hr = samples halogenated for 1 - 6 hr.
3.2.3.2. Halogenation temperature

The halogenation process in this case was performed using 5% NaOX (Where X =Cl, Br or I) for 1 hr. Note, the iodinated polymer load was decreased to 0.1 g.

For the chlorinated polymer, it can be seen that increasing the temperature of halogenation from 0°C to room temperature increases the biological activity against *E. coli*, while above room temperature the biological activity decreased. This may be because, above ambient temperature NaOCl becomes unstable and chlorine gas evolved from solution rather than reacting with the polymer; which can decrease the halogen load on the polymer, Figure 3.36, table 3.5. The same behaviour was seen for *S. aureus*, Figure 3.37.

For *E. coli*, the biological activity of the brominated polymer increases with increasing temperature until ambient temperature, and then stays constant until 35°C. After 35°C the biological activity begins to decrease, Figure 3.38. The same behaviour occurred with *S. aureus* but the biological activity in this case decreased above ambient temperature, Figure 3.39. There were similar results in case of the iodinated polymer, for both *E. coli* and *S. aureus*, Figures 3.40 and 3.41.
Figures 3.36 and 3.37: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with the chlorinated polymer prepared at different chlorination temperature (°C).

3.36, *E. coli*

3.37, *S. aureus*

Where: BC = bacterial control (no treatment) and RT = ambient temperature.
Figures 3.38 and 3.39: Log no. of bacterial colonies of *E. coli* and *S. aureus* colonies respectively after contacting with the brominated polymer prepared at different temperature (°C).

3.38, *E. coli*

3.39, *S. aureus*

Where: BC = bacterial control (no treatment) and RT = ambient temperature.
Figures 3.40 and 3.41: Log number of *E. coli* and *S. aureus* colonies respectively after the contact with iodinated polymers prepared at different temperatures (°C).

3.40, *E. coli*

![Graph](image1)

Where: BC = bacterial control (no treatment) and RT = ambient temperature.

3.41, *S. aureus*

![Graph](image2)
3.2.3.3. Halogen concentration

Samples of polymer prepared using different sodium hypochlorite concentrations (2.5, 5.0, 7.5 and 10% w/w) were placed in contact with the bacteria (at ambient temperature) and viable counts performed at timed intervals (the counting plates were incubated at 37°C overnight). From Figures 3.42 and 3.43 for *E. coli* and *S. aureus* respectively, polymer chlorinated by 10% (w/w) NaOCl effected complete sterilization in just 5 min. The samples were halogenated at ambient temperature for 1 hr. Increasing the concentration of sodium hypochlorite increased the biological activity of the polymer due to halogen load increasing, table 3.5.

The same behaviour was seen for both bromination (Figures 3.44 and 3.45) and iodination (Figures 3.46 and 3.47) for both types of bacteria. The amount of iodinated polymer was reduced to 0.02 g.
Figures 3.42 and 3.43: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with the chlorinated polymer prepared with different NaOCl concentrations.

3.42, *E. coli*

3.43, *S. aureus*

Where: BC = bacterial control and 2.5 - 10% = samples prepared using 2.5 - 10% NaOCl.
Figure 3.44 and 3.45: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with brominated polymers brominated with different NaOBr concentrations.

3.44, *E. coli*

3.45, *S. aureus*

Where: BC = bacterial control and 2.5 - 10% = samples prepared using 2.5 - 10% NaOCl.
Figure 3.46 and 3.47: Log no. of *E. coli* and *S. aureus* colonies respectively at timed intervals from the contact with iodinated polymers iodinated with different NaOI concentrations.

3.46, *E. coli*

![Graph of log no. of E. coli colonies at timed intervals](image)

3.47, *S. aureus*

![Graph of log no. of S. aureus colonies at timed intervals](image)

Where: BC = bacterial control and 2.5 - 10% = samples prepared using 2.5 - 10% NaOCl.
The previous results indicated that the biological activity of the N-halamine polymers can be changed by changing the preparation conditions. Performing the halogenation at room temperature for 1-2hr using 10% sodium hypohalate increases the biological activity of the prepared N-halamine polymers by increasing the halogen load onto the polymer, table 3.5. The iodinated polymer showed the maximum biological power under all conditions due to the low stability of N-I bond in comparison with N-Br and N-Cl which increases the ability of releasing more iodine.

It can be seen also from these results that halogenation using sodium hypochlorite under the optimum conditions achieves better results than that of the polymer prepared using chlorine gas at 0°C, part 1 of chapter 3. This indicates that using sodium hypochlorite can produce powerful N-halamine polymers using the optimum halogenation conditions, in addition to its economic value and the safety.

Determining the optimum halogenation conditions of N-halamine polymers supports the commercial production of these polymers by producing a polymer with high biological power to be used for a long time without re-halogenation.

3.2.4. Conclusion

The optimum halogenation conditions for polymers are: 10 ml of (10%, w/w) NaOX solution (where X = Cl, Br or I) for 2 hours at ambient temperature per gram of polymer. Using NaOCl under these conditions gives a more powerful effect than using chlorine gas at 0°C. Using NaOX not only reduces the costs but also the dangers associated with gas.
Chapter 4

Modifying the particle size of N-halamine biocidal polymers
4.1. Introduction

The particle size of N-halamine polymers was modified by attachment to carrier particles for potential future applications such as air and water filters so as not to restrict the flow-rate of water or air. In addition, the aim was to produce particles in different sizes to be used in suitable applications. The size modification was performed using two methods; by blending polymers (25) and (26) with sodium alginate followed by cross-linking with calcium chloride to form beads, and by loading uramil to modified silica gels. The biological activity of the prepared beads and modified silica were evaluated against examples of Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria. The optimum conditions for bead formation are described.

4.2. Experimental

4.2.1. Preparation of 2-iminouramil-functionalized and 3-(N-barbiturourea)propyl-functionalized silica gels (36 and 41 respectively) and their halogenation

2-Cyano-functionalized silica gel (1.14 g, 0.01 mol –CN based on carbon content) was suspended in N,N-dimethylformamide (DMF, 30 ml). Uramil (2.86 g, 0.02 mol) and triethylamine (60 μl) were added. The mixture was stirred at 120°C for 24 hours. The product was filtered hot, washed with hot DMF (100°C, 100 ml) and dried under vacuum, Scheme 4.1.
Analysis: FTIR (KBr): $\nu_{\text{max}}$ (cm$^{-1}$), 1701, 1668 and 1611 (C=O, heterocyclic ring), 1540 (C=N), 2942 (CH), 3124 (NH), 3435 (OH), 1100 (C-O and C-N). Solid state $^{13}$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.

Scheme 4.1: Preparation of 2-iminouramil-functionalized silica gel and its halogenation.

The same method was applied to 3-(N-barbitourourea)propyl-functionalized silica gel. 3-(isocyanato)propyl-functionalized silica gel (using 1.3 g, 10 mmol –CN based on carbon content) and uramil (2.86 g, 20 mmol) were refluxed together at 120°C in DMF (30 ml) in presence of triethylamine (60 µl). The product was filtered hot, washed with hot DMF (100°C, 100 ml) and dried under vacuum, Scheme 4.2.
Analysis: FTIR (KBr): $v_{\text{max}}$ (cm$^{-1}$), 1697, 1660 and 1611 (C=O, heterocyclic ring), 2943 (CH), 3124 (NH), 3432 (OH), 1100 (C-O and C-N). Solid state $^{13}$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 the previous reactions were also performed successfully using sodium hydroxide instead of triethylamine and the solvent (DMF, 30 ml) was replaced by a mixture of DMF and absolute ethanol (2:1).

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

The halogenation process was followed using FTIR spectroscopy [8, 120] and the halogen/g content was determined using iodometric titration [38, 141]. The values are given in table 4.1.

Table 4.1: FTIR characterization and halogen content of novel N-halamine biocidal modified silica gels.

<table>
<thead>
<tr>
<th>Modified silica</th>
<th>Bond</th>
<th>(v_{\text{max}}) (cm(^{-1}))</th>
<th>Halogen content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 (chlorinated)</td>
<td>N-Cl</td>
<td>804</td>
<td>68 ±3</td>
</tr>
<tr>
<td>38 (brominated)</td>
<td>N-Br</td>
<td>802</td>
<td>82 ±10</td>
</tr>
<tr>
<td>39 (iodinated)</td>
<td>N-I</td>
<td>784</td>
<td>102 ±0</td>
</tr>
<tr>
<td>42 (chlorinated)</td>
<td>N-Cl</td>
<td>799</td>
<td>74 ±0</td>
</tr>
<tr>
<td>43 (brominated)</td>
<td>N-Br</td>
<td>798</td>
<td>90 ±40</td>
</tr>
<tr>
<td>44 (iodinated)</td>
<td>N-I</td>
<td>804</td>
<td>105 ±10</td>
</tr>
</tbody>
</table>

The surface of N-halamine biocidal modified silica gels was studied using SEM (scanning electron microscope), Figure 4.1. The dried silica particles were plated with gold followed by SEM imaging.
4.2.2. N-halamine-alginate biocidal beads

Sodium alginate (2.0 % w/w) was dissolved in water (10 ml). A suitable polymer (see below), (2.5%, w/w), was added and the mixture was stirred for 30 min. The blend was added drop wise to a solution of calcium chloride (100 ml, 6% w/w CaCl\textsubscript{2}), Scheme 4.3. The beads were filtered and dried at 45°C for 24 hours.

The blended polymers with alginate were;

1- Pre-halogenated polymer (26) (Scheme 4.4) (beads 1), Figure 4.2.
2- Non-halogenated polymer (25) (Scheme 4.4) (beads 2), Figure 4.2. Beads 2 halogenated after their formation by soaking the beads (1 g) in sodium hypochlorite (10% w/w, 10 ml).

Scheme 4.3: Expected complexation product between the alginate and calcium ions.

Scheme 4.4: Structures of N-halamine polymers blended with sodium alginate.
The alginate based beads were characterized using FTIR, SEM and TGA analysis. **Beads 1**, FTIR (KBr): $\nu_{\text{max}}$ (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/d$T_{\text{max}}$), 60, 120, 210, 280, 300, 460, 700 and 1000. **Beads 2** (before halogenation), FTIR (KBr): $\nu_{\text{max}}$ (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/d$T_{\text{max}}$), 80, 140, 220, 340, 400, 450, 550, 700 and 1000. SEM images for the beads are shown in Figure 4.3. The dried beads were used for SEM imaging without plating with gold.
Figure 4.2: Photographs of the beads.

<table>
<thead>
<tr>
<th>Beads 1, containing pre-halogenated polymer, before drying:</th>
<th>Beads 2, containing non-halogenated polymer, before drying and halogenation:</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Beads 1, containing pre-halogenated polymer, before drying" /></td>
<td><img src="image2.png" alt="Beads 2, containing non-halogenated polymer, before drying and halogenation" /></td>
</tr>
<tr>
<td>Beads 1 after drying:</td>
<td>Beads 2 after drying without halogenation:</td>
</tr>
<tr>
<td><img src="image3.png" alt="Beads 1 after drying" /></td>
<td><img src="image4.png" alt="Beads 2 after drying without halogenation" /></td>
</tr>
<tr>
<td>Beads 1 after drying diameters (scale in mm)</td>
<td></td>
</tr>
<tr>
<td><img src="image5.png" alt="Beads 1 after drying diameters (scale in mm)" /></td>
<td></td>
</tr>
<tr>
<td>Sodium alginate beads (no blend) before drying:</td>
<td>Sodium alginate beads (no blend) after drying:</td>
</tr>
<tr>
<td><img src="image6.png" alt="Sodium alginate beads (no blend) before drying" /></td>
<td><img src="image7.png" alt="Sodium alginate beads (no blend) after drying" /></td>
</tr>
</tbody>
</table>
4.2.3. Biological activity of the beads and modified silica

4.2.3.1. Agar plate (screening)

The experiment was performed only for the modified silica gels. The method was performed as described earlier, chapter 3. The study was performed in 14 cm agar plates (3 per sample). Small amounts of each type of modified silica gel (0.03 g) were placed in the well in the middle of the plate, Figure 4.4.
4.2.3.2. Effect of the modified silica gel and blended alginate beads on bacterial viability

The biological activity of the modified silica and beads was quantified by studying their effect on bacterial viability.

For the alginate beads: bacterial suspension (*E. coli* or *S. aureus*) was prepared by inoculating 10 ml of nutrient broth in Universal bottles. The suspension was incubated at 37°C for 17 hours and 0.1 ml of this bacterial suspension was used to inoculate 5 different Universal bottles each containing 10 ml of fresh nutrient broth. The Universal vessels were incubated at 37°C for 17 hours treated with the following; **beads 1** (0.5 g), halogenated form of **beads 2** (0.5 g), non-halogenated form of **beads 2** (0.5 g) (control), sodium alginate beads (control) (0.5 g) and the fourth was used as a bacterial control. The viability was followed by counting at time intervals.
For the modified silica: the same method was used by treating three universal bottles with the following; halogenated modified silica (0.5 g) in the first bottle, non-halogenated modified silica gel (control) (0.5 g) in the second bottle and one of them was used as a bacterial control without treatment. The viability was followed by counting at timed intervals.

4.2.4. Beads re-halogenation

The optimum conditions for beads preparation were determined for recycling.

4.2.4.1. Changing the nature of the cross-linker

Different cross-linkers were studied:

4.2.4.1.1. Changing the quantity of calcium chloride

Using calcium chloride was the most successful method of preparation. The beads were formed by dropping the polymer (2.5%, w/w) / alginate (2.0%, w/w) mixture (in 10 ml distilled water) to a solution of calcium chloride (100 ml). The experiment was repeated using different calcium chloride baths with different calcium chloride ratios 2, 4, 6, 10, 20 and 40% (w/w). These ratios were used with and without curing. Curing was performed by heating the beads in the calcium chloride bath at 40°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.
4.2.4.1.2. Using gelatine with calcium chloride

Gelatine was used in different ratios 1-3% (w/w) with calcium chloride (10% w/w). Polymer (5%, w/w) / alginate (3%, w/w) 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 baths containing different ratios of gelatine. The beads were filtered, washed with distilled water and dried.

4.2.4.1.3. Aldehydes; formaldehyde and gluteraldehyde

Aldehydes, formaldehyde and gluteraldehyde, with different ratios (1, 2, 4, 8 and 10% w/w) were added during bead preparation as cross-linkers and the preparation method modified as follows: sodium alginate (2.0%, w/w) was dissolved in distilled water (10 ml). The aldehyde (formaldehyde or gluteraldehyde) was added [147]. The mixture was stirred for 1 hr, polymer (25 or 26) (2.5 %, w/w) was added 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/w) and the experiment was repeated but still no beads formed.

4.2.4.2. Changing the ratio of sodium alginate

Sodium alginate ratio was changed (1, 2, 3 and 4% w/w) during mixing with the polymer (25 or 26, 2.5% w/w), in 10 ml distilled water, followed by dropping into calcium chloride bath (10% w/w, 100 ml). The beads formed in each experiment performed using different alginate ratios were filtered, washed with distilled water and dried.
4.2.4.3. Changing the polymer ratio

The polymer (halogenated and non-halogenated) (25 and 26) ratio, (2, 3 and 5\% w/w), was changed to achieve the maximum load of polymer to the beads. The blend of polymer / alginate (3\%, w/w) (in 10 ml distilled water) in each case was dropped into calcium chloride bath (40\%, w/w) (100 ml). The beads were filtered, washed with distilled water and dried.

The biological activity of the prepared beads under different conditions was quantified by investigating their effect on *E. coli* and *S. aureus* viability as described before.

4.2.5. Swelling behaviour of the beads

Beads, 1 and 2, prepared under previous different conditions, (0.05 g) were soaked in tap water and distilled water in two different Universal bottles for 24 hours. The beads were filtered and the surface water was dried with paper tissues. The weight of the polymer was determined after the soaking period. The swelling ratio was calculated using equation 4.1.

Equation 4.1: Determination of swelling ratio.

\[
\% \text{ Swelling} = \frac{\text{Polymer weight after soaking} - \text{Polymer weight before soaking}}{\text{Polymer weight before soaking}} \times 100.
\]
4.3. Results and discussion

In order to improve the particle size of N-halamine biocidal materials uramil was loaded to modified silica gels. 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 reaction to cyano and isocyanate groups on silica under basic conditions which was performed successfully using triethylamine or sodium hydroxide as catalysts.

The structure of the resulting products still retained the stability of the halogen on the polymer compared to similar structures in chapter 2. The structures of the modified silica from the same type in the literature contain heterocyclic rings with substituted methyl groups (Dimethylhydantoin) [112, 116, 122, 123, 128, 145]. These methyl groups stabilize the halogen attached to the heterocyclic ring as electron donating group [112, 116, 122, 123, 128, 145]. For the novel prepared modified silica in this study, stronger electron donating groups have been used to increase the stability of the halogen attached to the heterocyclic ring; such as the amino group in modified silica (36) and the amide group in modified silica (41).

The structure of the resulting modified silica gel was studied using FTIR, solid state $^{13}$C NMR and elemental analysis. The FTIR data of modified silica (36) 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 solid state $^{13}$C NMR spectrum 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 (41); 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 solid state \(^{13}\)C NMR the carbonyl peaks appear at 153, 154, 161 and 162 ppm. Therefore, FTIR and solid state \(^{13}\)C NMR showed that the loading of the uramil to the modified silica gels was successful.

The SEM study was performed to investigate the particle size diameter and to prove that the particles were not damaged during the reactions, Figure 4.1. From Figure 4.1, it was noticed that the silica particles still keep their average diameter but smaller particles were scratched. This may be caused by stirring which can be avoided by shaking instead. However, the small fragments that separated from the silica particles will not restrict applying these particles in some applications such as water filters.

The halogenation of the modified silica gels was carried out using NaOX (X= Cl, Br and I) and the biological activity of the resulting N-halamine modified silica gels was investigated using agar plates, table 4.2.
Table 4.2: Inhibition zone diameters (mm) resulting from the halogenated modified silica gels (well diameter 5 mm).

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 (control)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>37 (chlorinated)</td>
<td>11 ±1</td>
<td>11 ±1</td>
</tr>
<tr>
<td>38 (brominated)</td>
<td>14 ±1</td>
<td>18 ±1</td>
</tr>
<tr>
<td>39 (iodinated)</td>
<td>17 ±1</td>
<td>24 ±1</td>
</tr>
<tr>
<td>41 (control)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>42 (chlorinated)</td>
<td>11 ±1</td>
<td>14 ±1</td>
</tr>
<tr>
<td>43 (brominated)</td>
<td>17 ±1</td>
<td>21 ±1</td>
</tr>
<tr>
<td>44 (iodinated)</td>
<td>26 ±1</td>
<td>28 ±2</td>
</tr>
</tbody>
</table>

From table 4.2, it can be seen that all the halogenated derivatives for both modified silica gels (36 and 41) have a biological effect on both Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria. The effect on S. aureus is greater than that on E. coli and the biological activity of the N-halamine modified silica gel derivatives (42, 43 and 44) is more than those derived from (36) (ie: 37, 38 and 39) due to presence of more available positions for halogens in (41).

The biological activity of the halogenated derivatives of modified silica gel (41) was quantified by determining their effect on bacterial viability.

The chlorinated modified silica (42) succeeded in performing a 3 log reduction in 7 hours for E. coli and a 4 log reduction for S. aureus in the same time period, figure 4.5 and 4.6 respectively. The brominated modified silica (43) performs a 4 log reduction in
the viability of both *E. coli* and *S. aureus* in 7 hours, Figures 4.7 and 4.8 respectively. The most powerful effect was achieved by iodinated modified silica. It performed a 9 log reduction in 15 min for both *E. coli* and *S. aureus*, Figures 4.9 and 4.10 respectively.

From the previous results it can be seen that the halogenated modified silica succeeded in reducing the bacterial viability of both Gram-positive and Gram-negative bacteria. As expected from previous results, the iodinated modified silica has the maximum biocidal power. Based on these results the particle size was increased whilst keeping moderate biological activity. The 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 strong electron donating groups attached to the heterocyclic ring the modified silica showed a moderate biological action which keeps the balance between the stability and biological activity. This stability may have reduced the biological power of the modified silica in comparison with similar types reported in the literature [142] but it can enable using this modified silica for a long time without re-halogenation as the silica will not lose the halogen easily. The biological activity of the modified silica is lower than that of the powder N-halamine biocidal polymer, described in chapter 2, as the number of functional groups on silica is lower which affects the number of heterocyclic rings that can be loaded to silica, resulting in low biological action. In addition, the N-halamine biocidal polymers in powder form have more surface area which increases the ability to contact with the bacteria.
Figure 4.5 and 4.6: Effect of chlorinated heterocyclic modified silica gel (42) on the viability of *E. coli* and *S. aureus* respectively.

4.5, *E. coli*

4.6, *S. aureus*

BC is the bacterial control, PC is the non-halogenated heterocyclic modified silica gel (control) and T is the chlorinated heterocyclic modified silica gel.
Figure 4.7 and 4.8: Effect of brominated heterocyclic modified silica gel (43) on the viability of *E. coli* and *S. aureus* respectively.

### 4.7, *E. coli*

[Graph showing the effect of brominated heterocyclic modified silica gel on the viability of *E. coli* over time (hr).]

### 4.8, *S. aureus*

[Graph showing the effect of brominated heterocyclic modified silica gel on the viability of *S. aureus* over time (hr).]

BC is the bacterial control, PC is the non-halogenated heterocyclic modified silica gel (control) and T is the chlorinated heterocyclic modified silica gel.
Figure 4.9 and 4.10: Effect of iodinated heterocyclic modified silica gel (44) on the viability of *E. coli* and *S. aureus* respectively.

4.9, *E. coli*

4.10, *S. aureus*

BC is the bacterial control, PC is the non-halogenated heterocyclic modified silica gel (control) and T is the chlorinated heterocyclic modified silica gel.
4.3.1. Sodium alginate beads matrix

The final particle size of the N-halamine modified silica depends on the particle size of the silica used as a starting material (2-Cyano-functionalized and 3-(isocyanato)propyl-functionalized silica gels). But in some applications such as air filters there is a need for bigger particles. Increasing the size of the starting silica (2-Cyano-functionalized and 3-(isocyanato)-propyl-functionalized silica gels) may result in reducing the biological activity of the product because the number of the functional groups, which are used to react with uramyl, placed on the particles’ surfaces will decrease. Most of the modified silica and beads reported in the literature are facing the same problem as they depend on the starting beads or silica size to produce the new particles which are produced with the same size as the starting particles [14, 15].

Therefore a new method was described to obtain particles with larger sizes. This new method can be used in producing different size particles because the particle size will depend on the dropping mechanism (dropper used). This can control the size of the produced beads to produce big or small size particles based on the required application.

This method is based on blending N-halamine polymer powders (25 or 26) with sodium alginate and cross-linking it by dropping to a calcium chloride bath to form insoluble particles. In this case, the particle size depends on flow of blend used so it can be changed to obtain any required size. At the same time the amount of the bioactive polymer in the particle matrix can be increased to improve the biological activity. This type of matrix has been used previously as a control-release system for releasing water-soluble antibiotics [146, 147]. This method was modified to be used with insoluble polymers. The biological activity in this case depends on the halogen ion release from the
beads or the contact with outer surface. These particles can be re-halogenated, which enhances the commercial value of the product.

Beads were prepared in two different ways; mixing chlorinated polymer (26) directly with sodium alginate, beads 1, and mixing non-halogenated polymer (25) with sodium alginate followed by chlorination, beads 2.

The beads were characterized using FTIR, TGA and SEM. FTIR and TGA this 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 beads 1 showed shift to lower values rather than beads 2 (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 [148]. Sodium alginate and the blended polymer showed peaks of decomposition of their main chain from 200-300°C [149]. But still a shift to lower value was noticed due to the presence of the halogenated polymer in beads 1.

The beads were examined by SEM, Figure 4.3, as well as photo imaging. The polymer showed a good spreading through the matrix, Figure 4.2.

The effect of beads 1 and 2 on bacterial (E. coli and S. aureus) viability was quantified. During the biological activity three controls were used; non-halogenated form of beads 2, sodium alginate beads without polymer and bacterial control (no beads).

**Beads 1** achieved a 9 log reduction in 3 hours for E. coli while **beads 2** achieved 1 log reduction in 5 hours, Figure 4.11. For S. aureus, **beads 1** achieved a 9 log reduction in 5 hours while **beads 2** only 1 log reduction in 5 hours, Figure 4.12.
Figures 4.11 and 4.12: biological effect of the beads matrix on the viability of *E. coli* and *S. aureus* respectively.

4.11, *E. coli*

4.12, *S. aureus*

BC bacterial control, AC sodium alginate beads as a control, PC beads 2 without halogenation as a control.
These results indicate that beads 2 have low biological activity because they need more halogenation time so the halogen can react with the polymer particles inside the beads. These data suggest that the best way to prepare the beads is the method used for preparing beads 1.

Beads 1 showed better biological action than the modified silica but lower than the polymer powder itself (chapter 3) because the ions take longer to diffuse out of beads to affect the cells. At the same time the contact effect between the polymer particles and the cells will be low as the cells can contact the outer surface particles while the particles in the middle of the beads can work only by release. In addition, the effective amount of polymer in the beads is lower than that used directly in the case of polymer powder evaluation.

4.3.2. Beads re-halogenation:

As indicated above beads preparation was successful and beads 1 showed good biological activity in comparison with the modified silica. To enable beads re-halogenation the optimum conditions for bead preparation were identified.

Calcium chloride ratio was changed; the polymer was blended with sodium alginate and dropped into different baths containing different concentrations of calcium chloride. The beads were formed with different ratios of calcium chloride 2, 4, 6, 10, 20 and 40% (w/w) with and without curing. The curing was performed at 40°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 ratio, with or without curing, decreases the swelling behaviour of the beads (tables 4.3 and 4.4) which affects the biological activity but it increases the beads' re-halogenation possibility. Raising calcium chloride ratio over 10% (w/w), with or without curing, reduces the biological activity of the beads (beads 1 and 2) while using this ratio (10% calcium chloride), with curing, keeps a good balance between biological activity and re-halogenation, Figures 4.13 and 4.14. This ratio, with curing, enables bead re-halogenation up to 3 times without any damage to the beads. From Figure 4.13, the beads prepared with 10-20% (w/w) calcium chloride with curing, achieved a 3 log reduction in 5 hours for *E. coli* while beads formed with 40% (w/w) calcium chloride with curing did not show good biological activity. Similar behaviour was noticed with *S. aureus* but with 40% (w/w) calcium chloride the beads achieved 1 log reduction, Figure 4.14.
Figures 4.13 and 4.14: Effect of changing the cross-linking agent (calcium chloride) (with curing) on the biological activity of the beads (beads 1) against *E. coli* and *S. aureus* respectively.

4.13, *E. coli*

![Graph showing the effect of different cross-linking agents on *E. coli*]

4.14, *S. aureus*

![Graph showing the effect of different cross-linking agents on *S. aureus*]

BC is the bacterial control.
A comparison was performed between the biological activity of cured and non-cured beads prepared using 10% (w/w) calcium chloride. It was noticed that the non-cured beads show more biological activity because without curing, the beads can swell more which gives a better chance for ion release. It was noticed that for *E. coli* the cured beads achieved a 3 log reduction in 5 hours compared to a 4 log reduction in the case of non-cured beads, Figure 4.15. The same behaviour was noticed for *S. aureus* but the non-cured beads achieve a 6 log reduction in 5 hours, Figure 4.16.

Some other types of cross-linker were used instead of salts, such as Aldehydes. Aldehydes were stirred first with sodium alginate before polymer addition [147]. The mixture was stirred and the beads should form at a certain stage of stirring as described in the literature [147]. Unfortunately the beads did not form, maybe because the polymer is water insoluble which affects the bead formation. This method was applied to form beads to work as a matrix for water soluble compounds [147]. Several trials were done using different ratios of aldehydes (1, 2, 4, 8 and 10% w/w) but no insoluble material was formed from these quantities. In addition, the polymer ratio was decreased from 2.5% to 1% and then 0.05% (w/w) but still no beaded insoluble material was formed. So, the aldehydes failed completely in generating this type of bead (which contains water insoluble polymer).
Figures 4.15 and 4.16: Comparing the biological activity of the cured and non-cured forms of beads 1 against *E. coli* and *S. aureus* respectively.

4.15, *E. coli*

![Graph comparing biological activity of cured and non-cured forms of beads 1 against *E. coli*.](image)

4.16, *S. aureus*

![Graph comparing biological activity of cured and non-cured forms of beads 1 against *S. aureus*.](image)

BC is the bacterial control.
Sodium alginate ratio was changed (1, 2, 3 and 4 % w/w) to investigate its action on the formed beads. Increasing the ratio of sodium alginate increases the viscosity, makes the "dropping" very difficult, but develops good re-halogenation characters to the beads. Increasing the alginate ratio increases cross-linking possibilities which supports re-halogenation potential of the beads. These different ratios of alginate were mixed with the N-halamine polymer (2.5% w/w) and dropped into a calcium chloride bath (10% w/w) with and without curing.

Increasing the ratio of the polymers to bead (2, 3 and 5% w/w) increases the biological activity of the beads (both beads 1 and 2). The beads were prepared by dropping into a bath containing 40% (w/w) calcium chloride as a cross-linker with curing overnight at 40°C and sodium alginate ratio was increased to 3%. For *E. coli*, it was noticed that beads 1 achieved a 3 log reduction in 5 hours while beads 2 achieved a 2 log reduction in 5 hours, Figure 4.17. For *S. aureus* it was noticed that beads 1 achieved a 4 log reduction in 5 hours while beads 2 achieved a 2 log reduction in 5 hours, Figure 4.18. Increasing the polymer ratio increases the biological activity of the prepared beads in spite of cross-linking with high calcium chloride ratio (40%) in comparison with results reported using same calcium chloride ratio and low polymer ratio (2.5%), Figures 4.13 and 4.14.

Gelatine was used with ratios 1-3% (w/w) with calcium chloride (10% w/w). It was noticed that using gelatine makes the beads more spherical and supported beads re-halogenation as it can share in the complexation with the calcium ion. But using gelatine with beads 1 resulted in losing some halogen by exchanging it between the polymer and gelatine during the beads formation which reduces the biological activity of the beads.
From the previous data, the best conditions for the bead formation is by mixing 3% (w/w) sodium alginate with 5% (w/w) N-halamine biocidal polymer (26) and cross-linking with a solution containing 10% (w/w) calcium chloride followed by curing at 40°C overnight.

4.3.3. 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 4.3 and 4.4, to investigate the amount of swelling during some applications such as water filters. From tables 4.3 and 4.4, the non-cured beads can swell more than the cured increasing the ratio of calcium chloride decreases the swelling. The swelling behaviour was studied with increasing polymer ratio. It was noticed that increasing the polymer ratio increases the swelling behaviour even with curing, table 4.5.

Tables 4.3 - 4.5 showed that the optimum beads preparation conditions enable moderate swelling behaviour which is very important in water filters to enable the interaction between the bacterial cells and the N-halamine polymer inside the beads.
Figures 4.17 and 4.18: Effect of increasing the polymer ratio to 5% (w/w) on the biological activity of the beads on *E. coli* and *S. aureus* respectively.

4.17, *E. coli*

![Graph showing the effect of increasing polymer ratio on E. coli](image)

4.18, *S. aureus*

![Graph showing the effect of increasing polymer ratio on S. aureus](image)

BC is the bacterial control.
Table 4.3: Swelling ratio of the non-cured beads prepared with different ratios of calcium chloride.

<table>
<thead>
<tr>
<th>Calcium chloride ratio (%, w/w)</th>
<th>Swelling ratio of <strong>Beads 1</strong> without curing (%, w/w)</th>
<th>Swelling ratio of <strong>Beads 2</strong> without curing (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>Tap water</td>
</tr>
<tr>
<td>4</td>
<td>111.8</td>
<td>94.3</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>20</td>
<td>3.9</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>1.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 4.4: Swelling ratio of the cured beads prepared with different ratios of calcium chloride.

<table>
<thead>
<tr>
<th>Calcium chloride ratio (%, w/w)</th>
<th>Swelling ratio of <strong>Beads 1</strong> with curing (%, w/w)</th>
<th>Swelling ratio of <strong>Beads 2</strong> with curing (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>Tap water</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>11.5</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4.5: Swelling ratio of the beads with curing with 20% (w/w) calcium chloride with different ratios of polymer.

<table>
<thead>
<tr>
<th>Polymer ratio (%, w/w)</th>
<th>Swelling ratio of Beads 1 with curing (%, w/w)</th>
<th>Swelling ratio of Beads 2 with curing (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>Tap water</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>21.6</td>
<td>16.6</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>22</td>
</tr>
</tbody>
</table>

The previous data indicate the possibility of producing blended beads with different sizes and a good biological activity. Producing beads in different sizes can support many applications by increasing the flexibility of producing the required size based on the required application. This will not restrict the size of the product particles to the size of the starting beads as reported in some research work [14, 15] or as noticed in the produced modified silica in this study. In spite of the fact that the beads have a bigger size than the prepared modified silica gel but it shows better biological action due to the presence of the powder N-halamine polymer blended with the beads. Optimising beads’ preparation conditions supports beads re-cycling.

Producing the beads in different sizes as a blend with water-insoluble polymers is a new method of bead production containing water-insoluble polymers, or drugs that can release bioactive species. Most beads of the same type in the literature are based on blending water soluble bioactive compounds with alginate [146, 147]. The mode of
action of the beads prepared in this study is based on ion release from the water-insoluble polymer and on contact between the bacterial cells and the outer surface of the beads.

4.4. Conclusion

The particle size was “improved” by loading bioactive heterocyclic rings to modified silica gels and by preparing beads containing bioactive polymer powder. Bead formation resulted in generating bioactive beads with different sizes. The best conditions for the bead formation is by mixing 3% (w/w) sodium alginate with 5% (w/w) N-halamine biocidal polymer (26) and cross-linking them with a solution contain 10% (w/w) calcium chloride followed by curing at 40°C overnight. Preparing the beads as beads 1 (blending pre-halogenated polymer with alginate) is better than beads 2 (blending the beads with non-halogenated polymer followed by halogenation) as the halogenated polymer was well dispersed in the beads.
Chapter 5

Evaluation of a novel commercial N-halamine biocidal polymer

in a water purification system on a laboratory scale
5.1. Introduction

The production costs are one of the main problems that restrict applying N-halamine polymers over a large scale as most of chemicals used in their preparation are expensive [11, 17, 80, 86, 92, 94, 100, 102, 103, 105, 111, 112, 122-126, 128, 129, 144, 145]. The only commercial products available in the market are the N-halamine textiles [96, 104, 119, 125, 126].

The aim of this part of the work was to reduce the production costs of N-halamine polymers by producing a low cost N-halamine polymer. For this purpose, a novel commercial cross-linked N-halamine biocidal polymer was prepared with large size particles and evaluated for use in water filters on a laboratory scale.

In addition, a water purification station containing this polymer was quantified on laboratory scale. Life-time and regenerability of the station were studied to optimise its working conditions. The ability of the non-halogenated polymer to trap free halogens was determined to evaluate its use as a halogen trap in water purification stations.

5.2. Experimental

5.2.1. Preparation of a novel commercial N-halamine biocidal polymer; cross-linked polyepicyanuriohydridin (PPC)

Polyepichlorohydrin (2 g, 0.02 mol, MW 700,000) was dissolved in DMF (30 ml). m-Phenylenediamine (0.2 g, 10% w/w) and sodium hydrogen carbonate (0.3 g) were
added and the reaction was heated at 120°C for 24 hr. Cyanuric acid (3.2 g, 0.025 mol) and sodium hydrogen carbonate (1.9 g) were added and the heating was continued for 24 hr. The resulting gel was added to water and washed with hot water (80°C, 100 ml) to remove any cyanuric acid contaminating the polymer. The polymer was cut into the required diameter granule (1-2 mm), Scheme 5.1.

Analysis: FTIR (KBr): $\nu_{\text{max}}$ (cm$^{-1}$), 1739, 1702, 1646 (C=O, heterocyclic ring), 1589 (C=N), 1260 (C-N and C-O), 2842, 2992 (CH aliphatic), 3210 (NH), 3434 (OH).
Solid state $^{13}$C NMR, 30-40 (aliphatic part), 155, 162 (C=O, heterocyclic ring).

The reaction was performed without cross-linking (linear polymer) to support the analysis. The following data was obtained for the linear polymer:

FTIR (KBr): $\nu_{\text{max}}$ (cm$^{-1}$), 1736, 1702, 1646 (C=O, heterocyclic ring), 1587 (C=N), 1260 (C-N and C-O), 2839, 2962 (CH aliphatic), 3130 (NH), 3428 (OH). $^1$H NMR (DMSO, 500 MHz): $\delta$ 2.5 (CH$_2$-N, singlet), 3.6 (CH$_2$-CH, broad band, aliphatic part), 10.1 (NH, broad singlet). $^{13}$C NMR (DMSO, 125 MHz): ppm 45 (CH$_2$), 69 (CH), 79 (CH$_2$-N), 155, 162 (C=O).
Scheme 5.1: Preparation of cross-linked polyepicyanuriohydrin (PPC) and its halogenation.
(PPC) (46) was chlorinated using sodium hypochlorite (10% w/w). The polymer (1.0 g) was soaked in sodium hypochlorite (10% w/w, 15 ml) and 5 ml distilled water overnight. The product was filtered, washed with 100 ml distilled water and dried. The amount of halogen load on the polymer was determined using iodometric titration [38, 141], 115ppm ±20.

5.2.2. Biological activity of the chlorinated cross-linked polyepicyanuriohydrin (Cl-PPC) (47)

The biological activity of (Cl-PPC) was evaluated against bacteria (E. coli and S. aureus) by stirring 1 g polymer (Cl-PPC) with 10 ml bacterial suspension (prepared as described before). The bacterial viability was followed by counting at timed intervals.

5.2.3. (PPC) (46) swelling behaviour

(PPC) (0.05 g) was soaked in tap water, distilled water and saline solution (1% w/w) in three different Universal bottles for 24 hours. The weight of the polymer was determined after the soaking period and the swelling was calculated as in equation 5.1:

Equation 5.1: Swelling ratio determination.

\[
\text{% Swelling ratio} = \frac{[(\text{Polymer weight after soaking} - \text{Polymer weight before soaking})]}{\text{Polymer weight before soaking}} \times 100
\]
5.2.4. (Cl-PPC) (47) Re-cycling

(PPC) (46) (1 g) was soaked in 20 ml sodium hypochlorite (7.5% w/w) overnight. The polymer was filtered, washed with distilled water (100 ml) and dried at 45°C for 24 hours. The amount of halogen on the polymer was determined using iodometric titration [141]. The polymer was heated in 20 ml sodium thiosulphate (0.01M) at 45°C for 1 hour then filtered, washed with halogen-free water and dried at 45°C for 24 hours. The polymer was re-halogenated using sodium hypochlorite then the halogen was removed with sodium thiosulphate (the process was repeated 4 times, charging with halogen then discharging with sodium thiosulphate) and in each case the amount of halogen was determined. After the fourth cycle the biological activity of the polymer was studied against bacteria (*E. coli* and *S. aureus*) using method described above.

5.2.5. Evaluation of (Cl-PPC) (47) in water filters on laboratory scale

(Cl-PPC) (10 g) (1-2 mm granules diameter) was packed into a 20 ml glass syringe as a model column. Bacterial suspension (*E. coli* and *S. aureus*) (10 ml) was perfused through the column. The viability was followed by counting before and after perfusion. The suspension was re-perfused again (for 10 cycles) through the column and bacterial viability was determined after each cycle. A column containing the non-halogenated polymer (PPC) was used as a control.

5.2.6. Determination of (Cl-PPC) (47) life time in water filters

The previous method (5.2.5) was repeated. Bacterial suspension recycling through the column was increased to 12 times. Each 12 cycles considered as one run. The second
run was performed with a fresh bacterial suspension. Three runs were performed for *E. coli* and four runs for *S. aureus* in two separated columns; one for *E. coli* and the other for *S. aureus*.

5.2.7. Non-halogenated polymer (PPC) (46) columns as a reverse column to the halogenated polymer column (Cl-PPC) (47)

Halogenated (Cl-PPC) and non-halogenated polymers (PPC) (10 g each) were packed into two different columns. Bacterial suspension (*E. coli*) was perfused through the halogenated polymer columns followed by the non-halogenated one. Bacterial viability was followed before and after perfusion through each column. Each experiment (run) was repeated 4 times using fresh bacterial suspension each time. During each run, 10 cycles were performed through the columns.

5.2.8. Sand as water filter

Sterilized sand was packed into 3 different glass column (30 g each) (20 ml glass syringe was used as model column). The columns were challenged with different bacterial suspension concentrations of *E. coli* (up to $10^{10}$, $10^{7}$ and $10^{4}$ cfu/ml) (one concentration per column). The viability was determined before and after perfusion through each column.
5.2.9. Determination of the quality of a water purification station on laboratory scale

A station (on a laboratory scale) was designed from 3 columns; sand (30 g), N-halamine biocidal polymer (Cl-PPC) \((47)\) (15 g) and non-halogenated polymer (PPC) (15 g) \((46)\) column, and challenged with 5 different runs of bacterial suspensions \((E. coli)\), Scheme 5.2. Each run was performed using a fresh bacterial suspension with bacterial concentration up to \(10^3\) cfu/ml. The same columns were used for each run to determine the maximum bacterial removed by the station. The counts were performed before and after perfusing through each column.

5.2.10. The regenerability of the water station

The previous experiment \((5.2.9)\) was repeated twice to investigate the regenerability of the station. The station was washed with 100 ml sodium hypochlorite 5\% (w/w) per column to kill any bacteria from the first experiment followed by washing with sterile halogen-free water (100 ml water per column). The halogenated polymer \((47)\) column was refreshed by filling it with sodium hypochlorite 10\% (w/w) overnight to reload the polymer with halogen. The halogenated polymer \((47)\) column was rewashed with distilled water (until no halogen was released the column). After cleaning and washing, the second experiment was performed with fresh bacterial suspension. The same precautions were followed in each recycling stage.
Based on this laboratory scale station, Scheme 5.2, a suggested large scale design for a water purification station based on multi-filtration technology was suggested for future work, Scheme 5.3. The large scale station is formed from three main columns; sand (unit 1, Scheme 5.3), halogenated polymer (unit 2, Scheme 5.3) and non-halogenated polymer (unit 3, Scheme 5.3). It was supported with sodium hypochlorite source for washing the columns, during recycling, to kill any bacterial cells still alive in the columns (unit 4, Scheme 5.3). The same tank will be used to re-halogenate the halogenated polymer in unit 2. Sodium hypochlorite waste (unit 5, Scheme 5.3) can be recycled by warming to separate the chlorine from the water. Chlorine (unit 6, Scheme 5.3) can be reacted again with sodium hydroxide to form sodium hypochlorite which can

Scheme 5.2: A schematic diagram for the water purification station (evaluated on laboratory scale).
be used again in unit 5. In addition, the water freed from the chlorine can be neutralized (unit 7, Scheme 5.3) and used again for washing proposes or dissolving sodium hydroxide (unit 8, Scheme 5.3), after filtration from any possible bacterial residue, Scheme 5.3.
Scheme 5.3: A suggested design for a large scale application of water purification system based on multi-filtration technology.
5.3. Results and discussion

A new synthetic pathway was created to prepare a commercial N-halamine biocidal polymer. The polymer was prepared by cross-linking polyepichlorohydrin with \( m \)-phenylenediamine (10\%, w/w ratio) in the presence of sodium hydrogen carbonate and N,N-dimethylformamide as a solvent. Cyanuric acid was added with additional amount of sodium hydrogen carbonate to produce a new cross-linked heterocyclic polymer (PPC) (46). Attaching the nitrogen atom of the heterocyclic ring to the CH\(_2\) group of polyepichlorohydrin stabilizes the halogen on the heterocyclic ring as an electron donating group. The costs of polyepichlorohydrin and cyanuric acid are very low in comparison with the costs of the raw materials of N-halamine biocidal polymers reported in the literature [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129].

(PPC) (46) was halogenated by soaking in sodium hypochlorite overnight to give the polymer the required time to swell and react with the hypochlorite. The polymer was produced in large granules up to 5 mm which facilitates its use in water filters and similar applications required a large particle size. The structure of (PPC) was determined using FTIR and solid state \(^{13}\)C NMR.

The biological activity of Cl-PPC (47) was studied by determining its effect on \textit{E. coli} and \textit{S. aureus} viability. Cl-PPC (47) was stirred with bacterial suspension and the viability was followed by counting at timed intervals. It was seen that the halogenated polymer (Cl-PPC) achieved a 9 log reduction in 1.5 hour for both \textit{E. coli} and \textit{S. aureus},
Figures 5.1 and 5.2, while no effect was reported for the non-halogenated polymer. These results indicated that (Cl-PPC) (47) can be used as a new N-halamine biocidal polymer.

The biological power of this polymer is 50% of the power of the chlorinated powder polymers prepared in chapter 2 such as N-halamine polymer (26). Polymer (47) (1 g) achieved similar biological power as 0.5 g of polymer (26) due to the difference in the surface area. The polymer in powder state has more surface area enabling it to have more contact with the bacterial cells. But still the production costs of polymer (47) is very low in comparison with the polymers prepared in chapter 2 or in the literature [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129] as well as the difference in the particle size.
Figures 5.1 and 5.2: Effect of (Cl-PPC) (47) on *E. coli* and *S. aureus* viability, respectively.

5.1, *E. coli*

![Graph showing the effect of Cl-PPC on *E. coli* viability](image)

5.2, *S. aureus*

![Graph showing the effect of Cl-PPC on *S. aureus* viability](image)

BC is the bacterial control, PC is the non-halogenated polymer (polymer control) and T is the halogenated polymer (Cl-PPC).
The swelling behaviour of the polymer was determined in different media to investigate the range of granule-size increase during the applications especially in water filters, table 5.1. The swelling ratio of the polymer can affect the water flow-rate.

Table 5.1: Swelling ratio of (PPC) (46) in different media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>% swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>26.3</td>
</tr>
<tr>
<td>Tap water</td>
<td>21.6</td>
</tr>
<tr>
<td>Saline (1% w/w)</td>
<td>20.9</td>
</tr>
</tbody>
</table>

From table 5.1, the swelling behaviour in saline solution is lower than that in distilled and tap water. The swelling ratio is not more than 27% which is very important to reduce the spaces between the particles to ensure a good contact between the polymer particles and water. This expansion ratio supports the idea of ion-exchange between the polymer and the medium which increases the biological power of the polymer.

To increase the economic value of N-halamine polymer (47), the recycling possibilities have been investigated. N-halamine polymers can be recycled by re-halogenation. The polymer was loaded with halogen and unloaded 4 times followed by determining the biological activity to investigate the biological power of the polymer after recycling several times. The amount of chlorine was determined using iodometric titrations [38, 141] after each cycle of polymer charging with halogen, table 5.2.
Table 5.2: Halogen load to polymer (PPC) (46) after each stage of halogenation and dehalogenation.

<table>
<thead>
<tr>
<th>Run number</th>
<th>Halogen content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st run</td>
<td>115 (± 2.0)</td>
</tr>
<tr>
<td>2nd run</td>
<td>101 (± 2.3)</td>
</tr>
<tr>
<td>3rd run</td>
<td>102 (± 3.3)</td>
</tr>
<tr>
<td>4th run</td>
<td>74 (± 5.3)</td>
</tr>
</tbody>
</table>

From table 5.2 it is clear that the polymer lost some of its ability to be charged with halogen after the first run and this loss increased after the fourth run. Due to this loss the biological activity of the polymer was determined again after the fourth cycle. From Figures 5.3 and 5.4, Cl-PPC (47), after the fourth recycling, achieved a 9 log reduction against *E. coli* and a 8 log reduction against *S. aureus* in 5 hours respectively. These results indicate that the polymer is still bioactive after 4 cycles of charging and discharging with halogen which increases the economic value of the polymer.

(Cl-PPC) (47) was applied in water filters on laboratory scale. Bacterial suspension of *E. coli* was perfused through the halogenated polymer column for 10 cycles and the viability was determined before and after these cycles. It was found that after 10 cycles of perfusing 10 ml bacterial suspension contains 4.3 X 10⁹ cfu/ml through the halogenated polymer column (Cl-PPC); there was no bacterial detection. The non-halogenated polymer (PPC) column succeeded in performing 1 log reduction as a filtration effect. The halogenated polymer succeeded in disinfecting the sample perfused through it which may encourage its use in water filters. The biological results achieved
by this polymer (47) in water filters are very close to those in the literature with similar polymers [150]. Polymer (26) prepared in powder form (chapter 2) showed a powerful effect during the evaluation of polymers against bacterial viability so it was used with lower amounts in smaller columns during its evaluation in water filters (chapter 3). In spite of this, polymer (26) showed good biological action with lower amounts than polymer (47) but still the production cost of (47) is lower, with low restriction to the water flow-rate.
Figures 5.3 and 5.4: Effect of (Cl-PPC) (47) (after the fourth run of charging with halogen) on the bacterial viability of *E. coli* and *S. aureus* respectively.

5.3, *E. coli*

5.4, *S. aureus*

BC is the bacterial control while T is the chlorinated polymer (Cl-PPC).
Polymer (47) showed good biological action in water filters so the life time of this column was investigated to determine the maximum number of bacteria that can be removed by this column. Different runs were performed through the column; each run with fresh bacterial suspension including 12 cycles per run. The runs were stopped as soon as the biological effect of the column decreased. From table 5.3, E. coli, the column succeeded in removing: 2.3X10^{10} cfu/10ml in the first, 10 X 10^{9} cfu/10ml in the second and 5 X 10^{9} /10ml in the third run through the column. So the column kills around 3.8 X 10^{10} cfu/30ml in three runs. After the third run the effect of the polymer begin to decrease. The particle size of the polymer used in this column was 1-2 mm and the rate of flow was 35 sec/cycle.
Table 5.3: Viable counts after each cycle of perfusing bacterial suspension (E. coli) through the columns in three different runs.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>BC (cfu/ml)</th>
<th>Run 1 (cfu/ml)</th>
<th>Run 2 (cfu/ml)</th>
<th>Run 3 (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.3 X 10⁹</td>
<td>2.3 X 10⁹</td>
<td>1.8 X 10⁹</td>
<td>1.8 X 10⁹</td>
</tr>
<tr>
<td>1</td>
<td>2.3 X 10⁹</td>
<td>1.8 X 10⁹</td>
<td>1.4 X 10⁹</td>
<td>1.6 X 10⁹</td>
</tr>
<tr>
<td>2</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>1.5 X 10⁹</td>
<td>1.5 X 10⁹</td>
</tr>
<tr>
<td>3</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>1.4 X 10⁹</td>
<td>1.4 X 10⁹</td>
</tr>
<tr>
<td>4</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>1.3 X 10⁹</td>
<td>1.3 X 10⁹</td>
</tr>
<tr>
<td>5</td>
<td>2.4 X 10⁹</td>
<td>0</td>
<td>1.3 X 10⁹</td>
<td>1.4 X 10⁹</td>
</tr>
<tr>
<td>6</td>
<td>2.4 X 10⁹</td>
<td>0</td>
<td>1.2 X 10⁹</td>
<td>1.4 X 10⁹</td>
</tr>
<tr>
<td>7</td>
<td>2.4 X 10⁹</td>
<td>0</td>
<td>1.1 X 10⁹</td>
<td>1.3 X 10⁹</td>
</tr>
<tr>
<td>8</td>
<td>2.4 X 10⁹</td>
<td>0</td>
<td>1.0 X 10⁹</td>
<td>1.2 X 10⁹</td>
</tr>
<tr>
<td>9</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>9.4 X 10⁸</td>
<td>1.2 X 10⁹</td>
</tr>
<tr>
<td>10</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>9.4 X 10⁸</td>
<td>1.2 X 10⁹</td>
</tr>
<tr>
<td>11</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>8.6 X 10⁸</td>
<td>1.1 X 10⁹</td>
</tr>
<tr>
<td>12</td>
<td>2.3 X 10⁹</td>
<td>0</td>
<td>8.0 X 10⁸</td>
<td>1.3 X 10⁹</td>
</tr>
</tbody>
</table>

From table 5.4, S. aureus, the column succeeded in removing; 1.5 X 10¹⁰ cfu/10ml in the first, 1.1 X 10¹⁰ cfu/10ml in the second, 7.7 X 10⁹ cfu/10ml in the third and 2.5 X 10⁹ cfu/10ml in the fourth run. So the column removes 3.6 X 10¹⁰ cfu/40ml in four runs. After the fourth run the killing power of the column polymer begin to decrease.
Table 5.4: The viable count after each cycle from perfusing the bacterial suspension, *S. aureus*, through the columns in four different runs.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>BC (cfu/ml)</th>
<th>Run 1 (cfu/ml)</th>
<th>Run 2 (cfu/ml)</th>
<th>Run 3 (cfu/ml)</th>
<th>Run 4 (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.5 \times 10^9$</td>
<td>$1.5 \times 10^9$</td>
<td>$1.1 \times 10^9$</td>
<td>$8.7 \times 10^8$</td>
<td>$8.6 \times 10^8$</td>
</tr>
<tr>
<td>1</td>
<td>$1.5 \times 10^9$</td>
<td>$2.1 \times 10^8$</td>
<td>$8.4 \times 10^8$</td>
<td>$7.6 \times 10^8$</td>
<td>$6.3 \times 10^8$</td>
</tr>
<tr>
<td>2</td>
<td>$1.5 \times 10^9$</td>
<td>$3.9 \times 10^3$</td>
<td>$8.0 \times 10^8$</td>
<td>$6.5 \times 10^8$</td>
<td>$6.8 \times 10^8$</td>
</tr>
<tr>
<td>3</td>
<td>$1.5 \times 10^9$</td>
<td>0</td>
<td>$1.5 \times 10^8$</td>
<td>$5.8 \times 10^8$</td>
<td>$6.6 \times 10^8$</td>
</tr>
<tr>
<td>4</td>
<td>$1.5 \times 10^9$</td>
<td>0</td>
<td>$1.5 \times 10^8$</td>
<td>$6.6 \times 10^8$</td>
<td>$7.8 \times 10^8$</td>
</tr>
<tr>
<td>5</td>
<td>$1.7 \times 10^9$</td>
<td>0</td>
<td>$1.8 \times 10^8$</td>
<td>$4.7 \times 10^8$</td>
<td>$7.6 \times 10^8$</td>
</tr>
<tr>
<td>6</td>
<td>$1.7 \times 10^9$</td>
<td>0</td>
<td>$1.3 \times 10^8$</td>
<td>$8.1 \times 10^8$</td>
<td>$7.2 \times 10^8$</td>
</tr>
<tr>
<td>7</td>
<td>$1.7 \times 10^9$</td>
<td>0</td>
<td>$8.1 \times 10^7$</td>
<td>$5.2 \times 10^8$</td>
<td>$8.0 \times 10^8$</td>
</tr>
<tr>
<td>8</td>
<td>$1.7 \times 10^9$</td>
<td>0</td>
<td>$7.5 \times 10^6$</td>
<td>$7.8 \times 10^8$</td>
<td>$7.7 \times 10^8$</td>
</tr>
<tr>
<td>9</td>
<td>$1.6 \times 10^9$</td>
<td>0</td>
<td>$6.7 \times 10^8$</td>
<td>$7.1 \times 10^8$</td>
<td>$5.9 \times 10^8$</td>
</tr>
<tr>
<td>10</td>
<td>$1.6 \times 10^9$</td>
<td>0</td>
<td>$3.8 \times 10^5$</td>
<td>$1.1 \times 10^8$</td>
<td>$6.6 \times 10^8$</td>
</tr>
<tr>
<td>11</td>
<td>$1.6 \times 10^9$</td>
<td>0</td>
<td>$7.5 \times 10^5$</td>
<td>$7.6 \times 10^7$</td>
<td>$5.5 \times 10^7$</td>
</tr>
<tr>
<td>12</td>
<td>$1.6 \times 10^9$</td>
<td>0</td>
<td>$3.9 \times 10^5$</td>
<td>$1.0 \times 10^8$</td>
<td>$6.1 \times 10^8$</td>
</tr>
</tbody>
</table>

The previous data indicate that the N-halamine polymer (47) is able to achieve complete disinfection for bacterial suspensions with a high bacterial concentration of either *E. coli* or *S. aureus* before its decay. These results demonstrate the potential of recycling through the columns several times.
5.3.1. Halogenated and non-halogenated polymers combination

The non-halogenated polymer (46) (PPC) was used as a trap for the halogen after the main column which contains the halogenated polymer (47) (Cl-PPC). The bacterial suspensions were perfused through the halogenated polymer (47) column followed by the non-halogenated (46). The released halogen from the halogenated polymer column may be trapped by the non-halogenated polymer column. At this stage the two columns may be used instead of each other which may increase the economical value of the columns.

It was observed that although the first column has been deactivated, halogenated polymer column (47) (Cl-PPC), the non-halogenated polymer (PPC) (46) column did not gain any biological activity, table 5.5. This may be explained on the basis that the halogenated polymer may lose its biological power before losing all the halogen attached to it so the amount of halogen passed to the second column may not enough to activate it. At the same time not all halogen reaches the non-halogenated polymer as most of the halogen was delivered to the bacterial cells. These results restrict the idea of reversing the columns. However, it was seen that no halogen was detected in the water after passing through the second column which supports another idea of designing a station containing the non-halogenated column after the halogenated one to produce water-free halogen.

A water station was created on a laboratory scale, Scheme 5.2, formed from three types of column; sand, halogenated polymer and non-halogenated polymer columns. The sand was used to regulate the number of bacteria going to the main column (halogenated polymer column) and at the same time to stop any residue or solid contents reaching the second column.
Sand was evaluated alone as a filter for bacterial suspensions and it was seen that it can filter out some bacterial cells at different bacterial concentrations, table 5.6.

The bacterial concentrations used in the station were reduced ($10^3$ cfu/ml). Different runs were performed through the station using fresh bacterial suspensions each time, table 5.7. It was seen that the bacterial cells were detected after the third run. In the fourth run there was some disinfection effect while from the fifth cycle the biological activity of the station was decreased, table 5.7.

Table 5.5: Counts before and after perfusing bacterial suspensions through the halogenated and non-halogenated columns.

<table>
<thead>
<tr>
<th></th>
<th>Counts Before perfusing through the columns (cfu/ml)</th>
<th>Counts after the halogenated polymer column (cfu/ml)</th>
<th>Counts after the non-halogenated polymer columns (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First run</td>
<td>4.3 X 10^9</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Second run</td>
<td>4.3 X 10^9</td>
<td>1.1 X 10^9</td>
<td>8.8 X 10^8</td>
</tr>
<tr>
<td>Third run</td>
<td>5.9 X 10^9</td>
<td>3.3 X 10^9</td>
<td>2.8 X 10^9</td>
</tr>
<tr>
<td>Fourth run</td>
<td>5.9 X 10^9</td>
<td>1.4 X 10^9</td>
<td>1.4 X 10^9</td>
</tr>
</tbody>
</table>
Table 5.6: Bacterial viability before and after each bacterial suspension perfusing through sand column.

<table>
<thead>
<tr>
<th>Sand column number</th>
<th>Viability before perfusing through the column (cfu/ml)</th>
<th>Viability after perfusing through the column (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{st}) column</td>
<td>1.6 \times 10^10</td>
<td>8.8 \times 10^6</td>
</tr>
<tr>
<td>2(^{nd}) column</td>
<td>1.6 \times 10^7</td>
<td>3.5 \times 10^4</td>
</tr>
<tr>
<td>3(^{rd}) column</td>
<td>1.6 \times 10^4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 5.7: Bacterial counts after each run from a station formed from three columns (sand, halogenated polymer and non-halogenated polymer).

<table>
<thead>
<tr>
<th>Before perfusing through the column (cfu/ml)</th>
<th>After perfusing through the column (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1(^{st}) column</td>
</tr>
<tr>
<td>1(^{st}) run</td>
<td>1.5 \times 10^3</td>
</tr>
<tr>
<td>2(^{nd}) run</td>
<td>1.5 \times 10^3</td>
</tr>
<tr>
<td>3(^{rd}) run</td>
<td>1.5 \times 10^3</td>
</tr>
<tr>
<td>4(^{th}) run</td>
<td>1.5 \times 10^3</td>
</tr>
<tr>
<td>5(^{th}) run</td>
<td>1.5 \times 10^3</td>
</tr>
</tbody>
</table>

No halogen was detected in the samples after perfusing through the station. These results indicated that the station succeeded in performing a significant disinfection without any halogen release in the outlet (the non-halogenated polymer (46) succeeded in removing the released halogen from the halogenated polymer column).
The regenerability of the station was investigated to increase the economic value. The station was cleaned after losing the biocidal power by washing the columns with sodium hypochlorite (10% w/w) followed by washing with sterile distilled water. The halogenated column was charged with halogen by filling the column with sodium hypochlorite and keeping it closed overnight, followed by washing with sterile distilled water. This cleaning and charging procedure was followed to recycle the station. It was seen that the station was fully effective after repeating the charging process 3 times, table 5.8, and the station worked actively until the fifth run in each experiment, table 5.8. This may be due to polymer drying; no drying was performed to the polymer, polymer drying results in losing some of the halogen load. No halogen was detected in the station outlet at any regeneration stage. These results indicate that the station can be regenerated and used several times without any halogen release in the outlet. Washing the third column (non-halogenated polymer column) with hypochlorite is not enough to fully charge it with halogen but it is enough to take any cells that passed to it. Fully charging of this polymer with halogen required soaking overnight to achieve the required biological effect.

The previous results indicated that the halogenated polymer (47) is able to disinfect the bacterial suspension in water stations on a laboratory scale. The polymer and the station can be recycled for use. No halogen passed from the station which encourages using this kind of station in producing halogen-free clean water.

This system can be used as a multi-stage filtration system for water purification on large scale to avoid chemical addition to water, Scheme 5.2. Most current projects are using chlorine to disinfect water which increase the health risk by the presence of halogen
in water [151]. Using such multi-stage filtration systems support bacterial removal without any halogen release.

A design for a complete system for water biological purification using multi-filtration system on a large scale is suggested. The system is formed from 3 main columns for water disinfection; sand, halogenated polymer and non-halogenated polymer (units 1, 2 and 3, Scheme 5.3). As soon as the biological action of the main column (halogenated polymer column) decreased the water-flow through this station can be stopped and a recycling process can be applied. The system can be supported by a sodium hypochlorite tank (unit 4, Scheme 5.3) which can be used to wash the sand and non-halogenated polymer columns to kill any bacterial cells contaminating them. In addition, the same tank would be used to re-halogenate the main column (halogenated polymer column, unit 2 Scheme 5.3) by filling the column with sodium hypochlorite and closing it overnight. The station can be washed with halogen-free water to remove any adsorbed halogen. Washing with water must be continued until no free halogen is released into the water outlet. At this stage the station is ready to be re-used. The system is supported with recycling units for sodium hypochlorite and water recycling to reduce the costs. The collected waste can be warmed to evaporate chlorine (unit 5, Scheme 5.3). Separating chlorine from water supports re-using both of them (water and chlorine). Chlorine can be re-dissolved in sodium hydroxide solution to prepare sodium hypochlorite to support unit 4 for washing and halogen re-charging. Water, freed from halogen, should be filtered to remove any contaminating residue followed by neutralization so it can be used again for washing purposes or for dissolving sodium hydroxide for sodium hypochlorite production. To re-use the water for washing in unit 8 it must stay sterile so the tubes and
the containing tanks should be prepared from N-halamine polymers to keep the water clean until re-use again. This system must be monitored in order to detect any bacterial level in the water outlet in addition to recycled water. The level of halogen must be followed as well, to be sure that the water is completely free of halogen.

This suggested system would introduce a complete system supporting the customers with water free halogen by applying this system on a wide scale and transferring the water to customers in tubes manufactured from N-halamine polymers.

This system can be supported with extra column for removing metal ions that may contaminate the water to construct a complete water purification system. Trials will be conducted in future work to achieve this goal by applying and quantifying this suggested system.
Table 5.8: Bacterial counts after each run from the station.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Run no.</th>
<th>Before passing through the column (cfu/ml)</th>
<th>After passing through the column (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st column (sand)</td>
<td>2nd column (N-halamine polymer)</td>
</tr>
<tr>
<td>1st</td>
<td>1st run</td>
<td>1.5 X 10^3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2nd run</td>
<td>1.5 X 10^3</td>
<td>3.5 X 10^3</td>
</tr>
<tr>
<td></td>
<td>3rd run</td>
<td>1.5 X 10^3</td>
<td>3.4 X 10^3</td>
</tr>
<tr>
<td></td>
<td>4th run</td>
<td>1.5 X 10^3</td>
<td>2.9 X 10^3</td>
</tr>
<tr>
<td></td>
<td>5th run</td>
<td>1.5 X 10^3</td>
<td>3.3 X 10^3</td>
</tr>
<tr>
<td>2nd</td>
<td>1st run</td>
<td>3.4 X 10^4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2nd run</td>
<td>3.4 X 10^4</td>
<td>1.1 X 10^4</td>
</tr>
<tr>
<td></td>
<td>3rd run</td>
<td>3.4 X 10^4</td>
<td>1.2 X 10^4</td>
</tr>
<tr>
<td></td>
<td>4th run</td>
<td>3.4 X 10^4</td>
<td>1.1 X 10^4</td>
</tr>
<tr>
<td></td>
<td>5th run</td>
<td>3.4 X 10^4</td>
<td>1.1 X 10^4</td>
</tr>
<tr>
<td>3rd</td>
<td>1st run</td>
<td>3.3 X 10^7</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>2nd run</td>
<td>3.3 X 10^7</td>
<td>1.2 X 10^7</td>
</tr>
<tr>
<td></td>
<td>3rd run</td>
<td>3.3 X 10^7</td>
<td>1.2 X 10^7</td>
</tr>
<tr>
<td></td>
<td>4th run</td>
<td>3.3 X 10^7</td>
<td>2.9 X 10^7</td>
</tr>
<tr>
<td></td>
<td>5th run</td>
<td>3.3 X 10^7</td>
<td>5.0 X 10^7</td>
</tr>
</tbody>
</table>

Note: each run was performed with a fresh bacterial suspension.
5.4. Conclusion

A novel cross-linked bioactive N-halamine polymer was prepared using low cost chemicals in different size granules. The polymer structure was determined using FTIR and solid $^{13}$C NMR. The polymer was used successfully in water filters on a laboratory scale. The polymer was recycled up to 4 times which increases its economic value. The biological life-time of the polymer was determined, the polymer disinfected 30 ml of *E. coli* suspensions contain $3.8 \times 10^{10}$ cfu/30ml in three runs and 40 ml of *S. aureus* suspensions contain $3.6 \times 10^{10}$ cfu/40ml in four runs. The polymer was used successfully in a water purification station on a laboratory scale. The station life-time and regenerability were determined; the station was recycled 3 times. The station was designed to have a special column containing the non-halogenated polymer to act as a trap for halogen-released from the main column (halogenated polymer column); no halogen was detected in the water outlet from the station. Sand was used to regulate the number of bacteria delivered to the main column. A water purification system based on multi-filtration technology was suggested to be applied on a large scale using this type of polymer.
Chapter 6

N-halamine biocidal polymers: specificity of mechanism of action
Part 1

Microbiological investigation

6.1.1. Introduction

The aim of this chapter is to investigate the biological mode of action of the synthesised N-halamine polymers (prepared in chapter 2, Scheme 6.1) in microbial cultures. It has been stated in the literature that the biological mode of action of N-halamine polymers is based on direct contact between the polymer and bacterial cells, without halogen release [7-9] or by halogen release into the media [16]. During this work a new theory has been proposed describing the biological mode of action of N-halamine polymers as a combination of mechanisms such as contact, release and changing the nature of the medium. The objectives of this study are to provide evidence that the N-halamine polymer can affect the bacteria by each of these mechanisms individually.
6.1.2. Experimental

6.1.2.1. Determination of the amount of halogen released from the chlorinated polymer during contact with water, nutrient broth and bacterial suspensions

Chlorinated polymer (26), 0.5 g, was stirred with 10 ml of chlorine-free water. The polymer was filtered, dried and the halogen load onto the polymer was determined before and after the experiment using iodometric titration [38, 141] to determine the amount of halogen released from the polymer to the water. The experiment was repeated using nutrient broth and bacterial suspensions (E. coli and S. aureus) instead of chlorine-free water.

6.1.2.2. Investigation of halogen release as a mode of action

N-halamine biocidal polymer (26) (0.5 g) was packed into a semi-permeable membrane sac (nominal molecular weight cut-off 12-14 KDa). The membrane was
immersed into a Universal bottle containing bacterial suspension (S. aureus or E. coli) (10 ml) grown for 17 hours, Figure 6.1. The bacterial viability was followed for 9 hours by counting at timed intervals using the method described in chapter 3.

A dialysis tube (nominal molecular weight cut-off 12-14 KDa) was used as a package for the polymer. The dialysis tube was prepared as follows; the tube (10-20 cm length) was boiled with a mixture of 1 mmole ethylenediaminetetraacetic acid disodium salt and 2% w/w sodium hydrogen carbonate (20 ml) for 10 min. The tube was removed and cleaned by boiling with distilled water (20 ml) for 10 min. The cleaning process was repeated twice followed by sterilization in a glass bottle.

Figure 6.1: N-halamine polymer (26) contained within a semi-porous membrane sac, suspended in a culture of E. coli.

6.1.2.3. Investigation of contact as a mode of action

Bacterial suspension (E. coli or S. aureus) was prepared by growing one bacterial colony in 50 ml nutrient broth in a 250 ml bottle for 17 hour at 37°C. The cells were
harvested, by centrifugation (3500g) and washed with sterile saline solution twice followed by freeze-drying.

The polymer was compressed into a small disk using KBr as a matrix to hold the polymer particles. Two disks containing the halogenated polymer (26) were prepared and a pre-weighed amount of the dried bacteria was placed between the two disks. Two other disks were prepared as a control, containing the non-halogenated polymer (25), and a pre-weighed amount of bacteria was placed between the two disks. Another control was performed using the bacterial powder itself without polymer disks. The disks were left in contact with the bacteria for 3 hours. The bacterial powders were removed from the disks, weighed, re-suspended in 10 ml of fresh nutrient broth and a viable count performed, Figure 6.2.
Figure 6.2: Polymer disks in contact with bacterial powder; the upper plate is the halogenated polymer while the lower is the control (non-halogenated polymer); (a) disks closed on bacterial powder while (b) disks opened and bacterial powder is shown on the disk.

6.1.2.4. Investigation of halogen exchange with the bacterial medium as a possible mode of action

N-halamine chlorinated polymer (26) (0.3 g) was stirred in nutrient broth (15 ml) for 3 hours at ambient temperature. The polymer was removed by centrifugation at 3500g. The supernatant was collected (10 ml) and divided into two parts (5 ml each): 1. Inoculated with *E. coli* suspension (0.05 ml), pre-grown at 37°C for 17 hours, 2. Inoculated with *S. aureus* (0.05 ml), prepared under the same conditions. Growth was followed by counting (as described in chapter 3) at timed intervals and counts compared to a bacterial control (*E. coli* or *S. aureus*) grown in fresh media.
Over the last 20 years N-halamine biocidal polymers have been used in many applications such as in water filters but their mode of action has remained unclear. Most of the work in this field has explained the bactericidal action by direct contact-only [7-9] or through halogen release-only into the media [16].

In this study it is suggested that the mode of action cannot be explained on the basis of only one mechanism, but by a combination of mechanisms operating simultaneously. These mechanisms can be a combination of:

1- Direct contact between polymer and cells.
2- Release of halogen from polymer to bacteria.
3- Halogen exchange between the polymer and bacterial medium constituents (e.g. medium protein), which then affect bacterial growth and viability.

To confirm this new model, experiments were designed which could provide evidence of these modes of action in isolation.

To demonstrate the direct contact effect freeze-dried bacterial cells were placed between two disks of the halogenated polymer, (26) to measure the direct effect of the polymer on the cells without the presence of liquid media necessary to mediate free-halogen release. Following the contact experiment the lyophilized bacterial cells, including controls, were recovered and levels of viable bacteria determined. They were recorded as cfu/g (colony forming units per gram) of freeze-dried material and the results recorded as a % recovery - calculated using Equation 6.1:
Equation 6.1: Recovery calculation.

\[ \text{% Recovery} = \left( \frac{\text{no. of bacteria after 3hr contact time}}{\text{original no of bacteria}} \right) \times 100 \]

The original number (before contact with the polymer) of the bacterial cells was determined by suspending a weighed amount of the dried cells in nutrient broth followed by a viable count, Figures 6.3 and 6.4.
Figures 6.3 and 6.4: Recovery of viable cells from freeze-dried material in contact with halogenated (26) and non-halogenated (25) polymers compared to a bacterial control.

6.3, *E. coli*

![Graph showing recovery percentages for *E. coli*.]

6.4, *S. aureus*

![Graph showing recovery percentages for *S. aureus*.]

BC= Bacterial control, PC= Polymer control (non-halogenated polymer disks) and T= Tested polymer (halogenated polymer disks).
The experiment was repeated three times, Figures 6.3 and 6.4. The extreme range of values seen is due to the variable distribution of bacterial cells through the solid residue after freeze-drying. However, in all the three experiments the chlorinated polymer has reduced the bacterial viability of both *E. coli* and *S. aureus* which indicates that the biocidal action of the N-halamine polymer can be produced by contact, Figures 6.3 and 6.4.

To provide evidence of bactericidal action without contact (halogen release), the polymer was contained within a semi-porous membrane (dialysis tubing) permeable to soluble small species (nominal molecular weight cut-off 12-14 KDa), and placed within a bacterial culture. Since the polymeric species are totally insoluble and unable to permeate the membrane, any bactericidal effect would indicate release of active species, such as released-halogen. For *E. coli* (Figure 6.5) it was noticed that the contained polymer effected a 9 log reduction, and for *S. aureus* a 5 log reduction, in 9 hours (Figure 6.6). The effect on *E. coli* being more pronounced than on *S. aureus*. Figures 6.5 and 6.6 clearly show bactericidal action without direct contact of polymers and cells. When the polymer is placed in water, it should spontaneously release halogen according to Equation 6.2. Any species that can react with the free-halogen will upset this equilibrium causing further release. Thus the polymer would show controlled-release of the dissolved halogen species; much more advantageous commercially than the use of free-hypochlorite, or similar species which have to be suitably dosed. This behaviour has been referred to as ‘bio-response’ [16] (a controlled-release action). The ions can be released to the bacterial cells directly or to the medium constituents such as protein. The halogen can be released to the medium protein converting it to a form of N-halamine
polymer which affects the bacterial cells and reduces the amount of nutrition suitable for the bacterial cells.

Figure 6.5 and 6.6: Effect of the halogen-release from the packaged polymer in semi-permeable membrane on the bacterial viability of *E. coli* and *S. aureus* respectively.

**6.5, *E. coli***

![Graph showing bacterial viability of E. coli over time](image)

**6.6, *S. aureus***

![Graph showing bacterial viability of S. aureus over time](image)
Equation 6.2: Release equilibrium equation.

\[
\text{H} - N - X + \text{H}_2\text{O} \rightleftharpoons \text{NH} + \text{HOX}
\]

From the previous results it can be seen that the polymer can affect the cells by contact and release.

In addition, more experiments were designed to prove that the polymer can affect the bacterial medium as well as the bacterial cells. To demonstrate that there is a halogen exchange between the polymer and bacterial medium constituents, the amount of delivered chlorine from the polymer to distilled water, medium and bacterial suspensions (\textit{E. coli} and \textit{S. aureus}) was calculated. The halogen exchange was calculated by determining the amount of chlorine loaded on the polymer before and after stirring in water, or nutrient broth with and without bacteria, table 6.1.

Table 6.1: The amount of delivered chlorine from the chlorinated polymer (1 g polymer : 20 ml bacterial suspension).

<table>
<thead>
<tr>
<th></th>
<th>Amount of released chlorine$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.19 ppm ± 0.05</td>
</tr>
<tr>
<td>broth medium</td>
<td>3.9 ppm ± 0.12</td>
</tr>
<tr>
<td>\textit{E. coli} (1.7 \times 10^9 cfu/ml) + broth medium</td>
<td>8.5 ppm ± 0.32</td>
</tr>
<tr>
<td>\textit{S. aureus} (3.1 \times 10^8 cfu/ml) + broth medium</td>
<td>5.9 ppm ± 0.24</td>
</tr>
</tbody>
</table>

(1 g : 20 ml) is the ratio between the weight of polymer and the liquid.

Note: $^a$ Determined as difference between original chlorine content in the polymer and chlorine content after contact with different media.
From the results in table 6.1 it is clear that the amount of halogen delivered to the nutrient broth medium is more than that to water, which indicates that there is exchange of halogen with nutrient medium resulting in medium protein's halogenation as a possible explanation. This disturbed the conditions suitable for bacterial growth which result in cell death. The halogen exchange between the polymer and the nutrient broth resulted in the spectrophotometer reading moving to negative values during the investigation of the ability of using the same polymer (26) in water filters (chapter 3, table 3.4). At the same time the amount of delivered halogen was increased in the presence of bacteria which shows that the polymer can exchange the halogen with the bacterial cells directly, by contact or release, consuming more halogen.

To provide more evidence that the mode of action may be due to changing the nature of nutrients within the cultural medium, growth of *E. coli* and *S. aureus* was followed both in fresh broth and in nutrient broth pre-treated by exposure to chlorinated polymer (26) for 3 hours, and subsequent removal of the polymer prior to inoculation of the medium.

Figures 6.7 and 6.8 shows that the bacteria failed to grow in the medium treated with the halogenated polymer, even after the polymer has been removed, demonstrating that the polymer has effected some change on the nature of the nutrients in the media, possibly through halogen transfer. The medium protein can receive halogen and work as an N-halamine polymer as the protein contain NH amide that can be halogenated by the action of the N-halamine polymer or any halogenation source to act as an N-halamine polymer.
The results indicate that the mode of action of these polymers is not by a single mechanism such as contact only [7-9, 11, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145], or halogen release only as stated in the literature [16], but by a combination of factors; contact, halogen release and transfer of halogen to medium components.
Figures 6.7 and 6.8: Growth of *E. coli* and *S. aureus* respectively in a medium pre-treated with chlorinated polymer (26).

6.7, *E. coli*

[Graph showing bacterial growth over time]

6.8, *S. aureus*

[Graph showing bacterial growth over time]

Where: EC and SC = *E. coli* and *S. aureus* bacterial controls, respectively. ET and ST = *E. coli* and *S. aureus* bacterial growth, respectively, in a pre-treated medium with the halogenated polymer.
6.1.4. Conclusion

A combined mode of bactericidal action by N-halamine polymers has been proposed based on direct contact, halogen release and halogen transfer to medium components.
Part 2

Metabolomics investigation

6.2.1. Introduction

The science of metabolomics is a study of the chemical “fingerprints” resulting from specific cellular processes [152]. These fingerprints are unique and can be generated on the basis of the low molecular weight metabolites involved in the cellular process [152]. They do not provide specific data about individual metabolites but an instantaneous snapshot that can be used to characterize the cell physiology [153].

To create these “fingerprints” cellular metabolites are extracted, separated and detected [153, 154]. A number of chromatographic methods are used in metabolite separation such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE). The metabolite detection is performed using nuclear magnetic resonance (NMR) or mass spectroscopy (MS) [153-155].

The aim of this part is to investigate the actual mode of action of N-halamine biocidal polymer using metabolomics to support the previous hypothesis in part 1 of this chapter that the mode of action of these polymers is a combination of release, contact and changing the nature of the medium. These data will be used in a future work to identify the actual mechanism of bacterial death by halogen ions as there are many explanations
of halogen action reported in the literature but the actual mechanism is still not clear. N-halamine polymer (47) (described in chapter 5) was used in this study, Scheme 6.2.

![Chemical structure of N-halamine polymer](image)

Scheme 6.2: N-halamine polymer used in investigating the mode of action of N-halamine polymers using metabolomics.

### 6.2.2. Experimental

#### 6.2.2.1. Determination of bacterial growth behaviour

Nutrient broth (50 ml) was inoculated using a single bacterial colony (S. aureus or E. coli) and incubated at 37°C. The bacterial growth was followed by determining the optical density (OD) at different time intervals using a spectrophotometer (wavelength = 540 nm). The relationship between time and optical density was determined.
6.2.2.2. Examination of changing hypochlorite concentrations on bacterial growth, viability and metabolites

Seven conical flasks (250 ml) each containing nutrient broth (50 ml) were inoculated with 0.1 ml of bacterial suspension (S. aureus or E. coli, previously grown for 17 hours at 37°C) and incubated at 37°C for 17 hours. One of these flasks was used as a bacterial control while the others were treated with different sodium hypochlorite concentrations (0.002%, 0.004%, 0.008%, 0.012%, 0.016% and 0.02% w/w). The bacterial viability was followed by viable counts (method described in chapter 3) at timed intervals.

Similar procedures were followed to determine the effect of different hypochlorite concentration on bacterial growth. The hypochlorite treatment was performed before incubating the flasks at 37°C. Growth was followed by determining the optical density (OD) using a spectrophotometer (wavelength = 540 nm) at timed intervals. The growth was followed until OD = 0.4 for E. coli and for OD = 0.8 for S. aureus, while the cells were still actively growing. The experiment was performed with and without pH control. To control the pH the experiment was repeated using nutrient broth prepared using phosphate buffer to control pH at 7.

The same method was used in determining the metabolite changes with hypochlorite concentration changes using FTIR as a metabolite detection method [154, 156]. The hypochlorite treatments were performed before incubating the suspensions and the growth was stopped by transferring the bacterial suspensions from each flask to 50 ml plastic vials held on ice. The metabolites were extracted as follows: bacterial samples (E. coli or S. aureus) were harvested by centrifugation (10 min at 3500g) at 4°C, washed
twice with fresh ice-cooled nutrient broth. The collected cells were re-suspended in 1 ml distilled water and lysed by adding 2 volumes of hot ethanol (50°C) [157]. Excess ethanol was removed by boiling for 30 min. Debris was removed by centrifugation in an Eppendorf centrifuge (10,000 rpm) for 10 min at 4°C. Samples were freeze-dried and the resulting powder investigated by FTIR [154, 156].

6.2.2.3. Determination of the metabolite changes with changing hypochlorite concentrations using LCMS

Bacterial suspensions were prepared as described above in 2 conical flasks (250 ml). One of the flasks was used as a bacterial control while the other was treated with sodium hypochlorite (0.008% w/w). The flasks were incubated at 37°C until OD = 0.4 for *E. coli* and OD = 0.8 for *S. aureus*. Growth was stopped by transferring the bacterial suspension to 50 ml plastic vials held in ice. The metabolites were extracted as follows (for both *E. coli* and *S. aureus*): the bacterial suspension was centrifuged for 10 min (3500g at 4°C). The harvested cells were washed twice with pre-cooled phosphate-buffered saline (pH 7.4) and re-suspended in 2 ml pre-cooled phosphate-buffered saline. The suspension was sonicated to lyse [155] the cells, centrifuged (10,000g for 10 min at 4°C) and the supernatant retained. Ice-cold methanol 4:1 [158] v/v was added, the mixture vortexed for 30 seconds, held at 4°C for 30 min and centrifuged at 10,000g for 10 min at 4°C. The supernatant was retained and freeze-dried. The precipitate was re-suspended in cold sterile “MilliQ” water (150 µl) and 100 µl transferred to LCMS vials for analysis.
6.2.2.4. Comparing the effect of N-halamine biocidal polymer (47) with the sodium hypochlorite effect on bacterial viability

Five universal bottles containing nutrient broth (10 ml each) were inoculated with 0.1 ml of *E. coli* bacterial suspension, previously grown for 17 hours at 37°C, and incubated at 37°C until OD 0.6 (while the cells were actively growing). The bottles were pre-treated with the following; chlorinated polymer (47) (0.8 g), non-halogenated polymer (46) (0.8 g) (polymer control), halogenated polymer (47) (0.8 g) in semi-porous membrane, sodium hypochlorite (0.008 % w/w) and the last bottle was used as a bacterial control. The vessels were stirred and growth followed by viable counts at timed intervals. The experiment was repeated using 0.9 g of polymer and changing the sodium hypochlorite concentration to 0.01 % (w/w) to find the weight of the polymer that can produce the same effect as sodium hypochlorite in concentrations between 0.008% - 0.01% w/w.

6.2.2.5. Determination of the metabolite changes induced by halogenated polymer using LCMS, ^1^H NMR and FTIR (bacterial cells lysed with perchloric acid)

Bacterial suspensions were prepared as described above in 5 different bottles (100 ml bottles, each containing 30 ml nutrient broth). The bottles were treated with the following: chlorinated polymer (47) (3 g), non-halogenated polymer (46) (3 g) (polymer control), chlorinated polymer (47) in semi-porous membrane (3 g), NaOCl (0.008%, w/w) and the last bottle was used as a bacterial control. All vessels were stirred and 3 samples were taken (5 ml per sample) at different time intervals (15, 30 and 60 min). Another bottle was used as a control without bacteria or treatments (nutrient broth only).
The metabolites were extracted as follows: bacterial samples (5 ml) were taken using pre-cooled syringes (-50°C) containing the quenching fluid (15 ml) (60% v/v aqueous methanol containing 70 mM Hepes). The samples were centrifuged for 5 min at 10,000g and 4°C. The supernatant was removed (contains the extracellular metabolites) and stored at -20°C. To the remaining cell pellet a methanol-water mixture containing 0.5 mole cGMP was added (500 μl). The cells were re-suspended by vortexing. Perchloric acid (35%; 2 ml) added, and the cells frozen at -80 °C. Samples were thawed and centrifuged at 10,000g and 4°C for 30 min to remove proteins and cell fragments. The clear supernatant was transferred to a fresh vial and neutralized by adding 1 ml of 5 M potassium carbonate solution. The samples were centrifuged at 10,000g and 4°C for 5 min to remove the precipitated perchlorates. The supernatant was stored at -20°C and freeze-dried [159].

FTIR investigation: part of the freeze-dried samples was used for FTIR (pre-weighed). ¹H NMR investigation: the rest of the sample was dissolved in D₂O (1ml), vortexed and then centrifuged at 10,000rpm at 4°C. The supernatant was transferred to NMR tubes for analysis.

LCMS investigation: the liquid solution used for NMR investigation was freeze-dried and the solid was extracted with acetonitrile/ammonium acetate buffer solution (9:1) (v/v). The samples were vortexed and then centrifuged at 10,000rpm at 4°C.
6.2.2.6. Determination of the metabolite changes induced by the halogenated polymer (47) using LCMS (bacterial cells lysed with sonication)

Nutrient broth (160 ml) was inoculated with *E. coli* (0.1 ml of bacterial suspension, previously grown for 17 hours) and incubated until OD = 0.6. The bacterial suspension was transferred to 4 different bottles (40 ml each). The bottles were treated with 4 different treatments; halogenated polymer (47) (T) (4 g) and non-halogenated polymer (46) (polymer control, PC) (4 g), NaOCl (0.008%, w/w) while some cells were left without treatment (bacterial control, BC).

The samples were collected at different times intervals; 0, 10, 30, 60 min (2.5 ml each), treated with 2.5 ml ice-cold methanol, kept on ice and centrifuged to collect the bacterial cells, 10,000g at 4°C. The pellets were re-suspended in 2.5 ml ice-cold buffer solution (PBS), sonicated 3 times (30 sec. each) [155] separated by equal cooling periods and centrifuged (10,000g at 4°C). The supernatant was collected. Each sample (500 μl) was extracted with 500 μl cold acetonitrile [160]. The rest of the samples were frozen (-20°C). The samples were vortexed for 30 sec., left on ice for 30 min. and centrifuged for 10 min, 10,000rpm at 4°C. The supernatant was collected, freeze-dried and kept at -20°C.

To prepare the samples for analysis, the dried samples were re-suspended in water (250 μl) and 160 μl was transferred to LCMS sample vials for analysis.
6.2.3. Results and Discussion

This section aims to investigate the actual mode of action of N-halamine biocidal polymer using metabolomics to support the previous hypothesis in part 1 of this chapter that the mode of action of these polymers is a combination of release, contact and changing the nature of the medium. At the same time the data were used to attempt to show that halogen-containing disinfectants (N-halamine polymers and sodium hypochlorite) have an effect on bacterial metabolites and to differentiate between their mechanisms (halogen bound to polymer and free halogen). These data will be used in a future work to identify the actual mechanism of bacterial death by halogen ions as there are many explanations of halogen action reported in the literature [161-168], but the actual mechanism is still not clear.

The bacterial cells were challenged with halogenated polymer (47) directly and contained in a semi-porous membrane. The metabolites of the bacterial cells were extracted in each case with different methods and analysed using different techniques (FTIR [154, 156], $^1$H NMR [154, 155, 169] and LCMS [154, 155]). The detected data was analysed using PCA (principle component analysis) [169, 170] to show the differences in the bacterial metabolite profiles recorded with FTIR, $^1$H NMR and LCMS data.

To use metabolomics in identifying the actual mechanism of biological action of N-halamine polymers, the effect of halogenated compounds on the bacterial metabolites
was explored using sodium hypochlorite as a model of one of the halogenated compounds.

At the same time, some other factors were determined to support the study such as:

a) Bacterial growth rate, to identify the best time of harvesting cells. The cells should be collected during logarithmic growth. The relationship between the optical density and time (Figure 6.9) shows that the best time to collect the bacterial cells is at optical density 0.5 - 1.8 for *E. coli* and *S. aureus* while the cells are still actively growing (log phase).

b) Determination of sodium hypochlorite effect on bacterial viability and growth, to identify a suitable sodium hypochlorite concentration that affects the bacterial growth without killing cells.

The bacterial cells were treated with different sodium hypochlorite concentrations and the bacterial viability of *E. coli* and *S. aureus* was followed by counting at timed intervals using the method described in chapter 3, Figures 6.10 and 6.11. For *E. coli* (Figure 6.10), it can be seen that the bacterial cells begin to die using 0.02% (w/w) NaOCl while for *S. aureus* (Figure 6.11) the cells start to die using 0.016% (w/w) NaOCl.
Figure 6.9: The optical density during the bacterial growth of *E. coli* and *S. aureus*.

Where OD is the optical density (nm).
Figure 6.10 and 6.11: The effect of different concentrations (% w/w) of NaOCl on the bacterial viability of *E. coli* and *S. aureus* respectively.

6.10 *E. coli*

The effect of different NaOCl concentrations on bacterial growth was studied by growing the bacteria in the presence of different concentrations of sodium hypochlorite. The bacterial growth was followed by determining the change in optical density each 30
min until OD = 0.4 for \textit{E. coli} and 0.8 for \textit{S. aureus} (Figures 6.12 and 6.13) (the figures show the time required to reach to the required OD with changing hypochlorite concentration). From Figures 6.12 and 6.13, growth retardation started from treating cells with 0.008\% (w/w) sodium hypochlorites for both \textit{E. coli} and \textit{S. aureus}. The same behaviour was noticed under controlled pH using buffered media (Figure 6.14) (the figure shows the time required to reach to the required OD with changing hypochlorite concentration). The pH was determined at the end of the growth to be sure that there was no change in the pH value, table 6.2.
Figure 6.12 and 6.13: Effect of different sodium hypochlorite concentrations (% w/w) on time interval required to obtain OD = 0.4 for *E. coli* and 0.8 for *S. aureus*.

6.12, *E. coli*

![Graph showing time required for OD = 0.4 for different concentrations of sodium hypochlorite for *E. coli*.

6.13, *S. aureus*

![Graph showing time required for OD = 0.8 for different concentrations of sodium hypochlorite for *S. aureus*.](image-url)
Figure 6.14: Effect of different sodium hypochlorite concentrations on time interval required to obtain OD = 0.4 for *E. coli* in buffered medium.

Table 6.2: The values of pH after 24 hours of *E. coli* growth in buffered medium.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC (cells with no treatment)</td>
<td>7.78</td>
</tr>
<tr>
<td>Cells treated with 0.002 % (w/w)</td>
<td>7.96</td>
</tr>
<tr>
<td>Cells treated with 0.004 % (w/w)</td>
<td>7.97</td>
</tr>
<tr>
<td>Cells treated with 0.008 % (w/w)</td>
<td>7.91</td>
</tr>
<tr>
<td>Cells treated with 0.016 % (w/w)</td>
<td>7.77</td>
</tr>
<tr>
<td>Cells treated with 0.02 % (w/w)</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Note: The pH of the medium before starting the experiment was 6.76.

The previous data indicate that the sub-lethal effective concentration of sodium hypochlorite on bacterial growth is in the range from 0.004-0.008% (w/w).

To confirm this and to prove that these concentrations can result in changes in bacterial metabolite, the effect of this range of sodium hypochlorite concentrations
(0.002, 0.004, 0.008, 0.012, 0.016 and 0.02%, w/w) on metabolites was determined. FTIR was used to investigate the changes in metabolite levels [154, 156].

No significant changes were recorded in the FTIR spectrum in the region from 500-4000 cm$^{-1}$ for the metabolites treated with different sodium hypochlorite concentrations but the following main peaks were recorded as characteristic peaks for the extracted metabolites of *E. coli*: 3280 cm$^{-1}$ for OH and NH, 2200-3400 cm$^{-1}$ for OH carboxylic acid, 2952 cm$^{-1}$ for CH aliphatic, 1654 cm$^{-1}$ for C=O, at 1520 cm$^{-1}$ for C=C and C=N, 1112 cm$^{-1}$ C-O and C-N.

The following main peaks were recorded for *S. aureus*: 3350 cm$^{-1}$ for OH and NH, from 2400-3400 cm$^{-1}$ for OH of carboxylic group, at 1624 cm$^{-1}$ for C=O, at 1400 cm$^{-1}$ for C=C or C=N, 1050 cm$^{-1}$ for C-O and C-N.

PCA was used to identify any changes in the metabolites FTIR with changing the hypochlorite concentration. PCA was performed on one component (first principle component) to compare the metabolomic profiles of treated cells (with different sodium hypochlorite concentrations) and non-treated cells.

A difference in the metabolomics profile was seen, Figures 6.15 and 6.16. The PCA indicated that there is an effect for the sodium hypochlorite on bacterial metabolites extracted from both *E. coli* and *S. aureus* and this effect increased with increasing the sodium hypochlorite concentration forming a linear relationship (linear profile), Figures 6.15 and 6.16. The cluster representing the metabolites treated with 0.02% (w/w) of NaOCl (maximum treatment) showed the maximum difference from the bacterial control for *E. coli* while treating *S. aureus* with 0.016 and 0.02% (w/w) showed similar effect.
Figure 6.15: A comparison between cells (*E. coli*) treated with different sodium hypochlorite concentrations and non-treated cells, PCA.

![Graph showing PCA analysis for E. coli](image1)

Where: PCI = first principle component and ▲, *, +, X and Δ = samples treated with 0.002, 0.004, 0.008, 0.012, 0.016 and 0.02% w/w NaOCl respectively while ■ = bacterial control.

Figure 6.16: A comparison between cells (*S. aureus*) treated with different sodium hypochlorite concentrations and non-treated cells, PCA.

![Graph showing PCA analysis for S. aureus](image2)
To support the FTIR data, further investigations were performed using other detection methods such as LCMS. The work was focused on one hypochlorite treatment (0.008%, w/w) as it was seen before that this concentration can affect the bacterial growth without killing the cells. The protocol applied to extract the metabolites was changed, as well as the method of cell-lysing. The cells were lysed by sonication rather than hot ethanol as some of the heat sensitive metabolites can be affected by rising temperature. PCA for LCMS data obtained for both *E. coli* and *S. aureus* (in replicates) was performed (Figures 6.17 and 6.18) to detect any changes in metabolite profile. Figures 6.17 and 6.18 show that there is a difference between the metabolite profile of cells treated with sodium hypochlorite and non-treated cells for both *E. coli* and *S. aureus*. PCA of LCMS has supported that of FTIR indicating that there is an effect for the halogenated compounds on bacterial metabolites.

Many explanations have been recorded in the literature regarding the reason for bacterial death or change by sodium hypochlorite [161-168]. Some researchers relate this to; DNA change [161], amino acids, and protein halogenation [162], ATP loss [163], enzymes inactivation [163], electron transport systems inactivation [163], cell membrane disturbance and nucleotides oxidation [163] and similar factors, but no clear mechanism was reported.

No research work was performed previously to investigate the mechanism of cell death by the action of halogenated compounds using metabolomics. The work in the current study can be used as a base to investigate the mechanism of cells death by the action of halogenated compounds using metabolomics. At the same time the previous results have encouraged applying this work to N-halamine biocidal polymers to
investigate their mode of action and to compare their mechanism of action (polymer bound halogen) with sodium hypochlorite mechanism (free halogen).
Figure 6.17 and 6.18: PCA of metabolites LCMS of *E. coli* and *S. aureus* respectively, treated with sodium hypochlorite (0.008%, w/w) compared to non-treated cells.

6.17, *E. coli*

Class 1: *E. coli* cells without treatments, + Class 2: *E. coli* cells treated with sodium hypochlorite, PC1 and PC2: first and second component.

6.18, *S. aureus*
An experiment was performed using different quantities of N-halamine polymer (47) to investigate its effect on bacterial viability and to determine the least amount of polymer required to have a sub-lethal effect on the bacterial cells. The polymer (47) was used directly with the cells and packaged inside a semi-porous membrane to apply the two mechanisms of action (direct and indirect) to show whether there is an effect due to halogen released through the membrane.

The bacteria were grown first until OD = 0.6 and then treated with: N-halamine polymer (47), packaged N-halamine polymer (47), non-halogenated polymer (46) (polymer control) and sodium hypochlorites in addition to a bacterial control (cells without treatments). The bacterial viability was followed by counting at timed intervals. Two different groups of data were obtained by using two different amounts of polymer (0.8 and 0.9 g) with and without the semi-porous package (the amounts were chosen based on previous results, chapter 5). Also two different concentrations of sodium hypochlorite were used 0.008 and 0.01% w/w.

From Figure 6.19 it can be seen that 0.8 g polymer gives a similar effect to sodium hypochlorite effect (0.008% w/w) especially in the first 60 min (60 min was selected to be the maximum contact time).
Figure 6.19: Comparison between the biological action of N-halamine biocidal polymer (47) (with and without semi-porous membrane) and hypochlorite effect.

Where: BC = bacterial control, PC = polymer control, T1 & T2 = halogenated polymer (0.8 and 0.9 g polymers), TS1 & TS2 = halogenated polymer in semi-porous membrane (0.8 and 0.9 g polymers) and TH1 & TH2 = sodium hypochlorite (0.008 and 0.01% w/w).

Using this amount of polymer (0.8 g) the effect of halogenated polymeric compounds on bacterial metabolites as well as the mode of action of this type of polymer was investigated. The bacterial cells were treated with halogenated polymer (47), halogenated polymer (47) packaged in semi-porous membrane, non-halogenated polymer (46) and sodium hypochlorite. Samples were collected at different time intervals, to investigate the effect of “challenging time” on the metabolites, and extracted with acetonitrile after lysing the cells by two different methods; with perchloric acid and sonication.

Using different methods of lysing cells (perchloric and sonication) and extraction can confirm the concept with different protocols. The extraction was performed at
different time intervals; 10, 30 and 60 min for cells lysed with perchloric acid and 0, 10, 30 and 60 min for cells lysed with sonication. LCMS, $^1$H NMR and FTIR were used to detect bacterial metabolites extracted from cells lysed with perchloric acid while LCMS was used to detect the metabolites extracted from cells lysed with sonication. PCA were used to analyse these data to create the required metabolomic profiles.

The effect of N-halamine polymer on bacterial metabolite can be detected by comparing metabolite profiles, generated by PCA, of cells treated with the halogenated polymer and non-treated cells. Figures 20-23, showing the PCA of different types of data (LCMS, $^1$H NMR and FTIR), indicate that there is a difference in the metabolite profile between cells metabolites treated with halogenated polymer directly and non-treated cells which prove that N-halamine polymers has an effect on bacterial metabolites. The difference was recorded using both the two different extraction protocols. It can also be seen that the clusters separation in the comparison of metabolites extracted from cells lysed with perchloric acid is greater than that of metabolites extracted with cells lysed with sonication. PCA was performed using the first two components. This change in the metabolites profile resulted from exchanging the halogen between the halogenated polymer and bacterial cells.
Figure 6.20: PCA of LCMS data of *E. coli* metabolites extracted from cells treated with N-halamine polymer directly, compared to non-treated cells, after 30 min treatment. Cells lysed with perchloric acid.

![Figure 6.20](image)

Figure 6.21: PCA of $^1$H NMR data of bacterial cells metabolites extracted from cells treated with N-halamine polymer directly, compared to non-treated cells (60 min treatment). Cells lysed with perchloric acid.

![Figure 6.21](image)

- Class 1: BC, cells without treatment, ▲ or ○ Class 2: T, cells treated with the halogenated polymer, PC1 and PC2: first and second component.
Figure 6.22: PCA of FTIR data of *E. coli* metabolites extracted from cells treated with N-halamine polymer directly compared to non-treated cells, (30 min treatment). Cells lysed with perchloric acid.

![Figure 6.22: PCA of FTIR data of *E. coli* metabolites extracted from cells treated with N-halamine polymer directly compared to non-treated cells, (30 min treatment). Cells lysed with perchloric acid.](image)

Figure 6.23: PCA of LCMS data of *E. coli* metabolites extracted from cells treated with N-halamine polymer directly compared to non-treated cells (60 min treatment). Cells lysed with sonication.

![Figure 6.23: PCA of LCMS data of *E. coli* metabolites extracted from cells treated with N-halamine polymer directly compared to non-treated cells (60 min treatment). Cells lysed with sonication.](image)

- Class 1: BC, cells without treatment, ▲ Class 2: T, cells treated with the halogenated polymer, PC1 and PC2: first and second component.
The metabolites profile of cells treated indirectly (through a semi-porous membrane) was compared with that of non-treated cells to prove that the mode of action of N-halamine polymers can proceed through a halogen release mechanism and not by contact only as stated by Worley S.D. et al for about 20 years [11, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145]. Figures 6.24-6.27 show a difference in the metabolites profile between metabolites extracted from cells treated with halogenated polymer in a package and non-treated cells. These differences were reported by different analysis methods and different cell-lysing methods which prove that this polymer affects the cells through ion release and not by contact only as stated in the literature [11, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145].

This change in the bacterial metabolites profile can result through two possible halogen release mechanisms:

1- Direct release to the medium followed by halogen delivering to bacterial cells.

2- Halogen release to medium components (e.g. protein). As soon as the protein receives halogen from the N-halamine polymer it can act as a halogenated polymer which increases the harmful effect on bacterial cells. Protein contains amide groups which can be halogenated easily by the action of released halogen ions.
Figure 6.24: PCA of LCMS data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells (30 min treatment). Cells lysed with perchloric acid.

![Figure 6.24: PCA of LCMS data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells (30 min treatment). Cells lysed with perchloric acid.](image1)

Figure 6.25: PCA of $^1$H NMR data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells (60 min treatment. Cells lysed with perchloric acid.

![Figure 6.25: PCA of $^1$H NMR data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells (60 min treatment. Cells lysed with perchloric acid.](image2)

- Class 1: BC, cells without treatment, ▲ or ○ Class 2: TS, cells treated with the packaged halogenated polymer, PC1 and PC2: first and second component.
Figure 6.26: PCA of FTIR data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells, after 30 min treatment. Cells lysed with perchloric acid.

Class 1: cells without treatment, Class 2: cells treated with halogenated packaged polymer, PC1 and PC2: first and second component.

Figure 6.27: PCA of LCMS data of bacterial metabolites extracted from cells treated with N-halamine polymer (packed in semi-porous membrane) compared to non-treated cells, (60 min treatment). Cells lysed with sonication.

Class 1: cells without treatment, ▲ Class 2: cells treated with halogenated packaged polymer, PC1 and PC2: first and second component.
However, still there is a possibility for the exchange of halogen between the bacterial cells and halogenated polymers through direct contact. Comparing the metabolites extracted from cells treated with N-halamine polymers directly (without membrane) and indirectly (inside a semi-porous membrane) may show that an additional effect happens by contact between the polymer and the cells. This can contradict the hypothesis that the mode of action of the N-halamine polymers is based on halogen release only, as stated by some researchers [16].

Figures 6.28-6.30 shows cluster-separation and differences in the metabolites profile between cells treated with the N-halamine polymer directly and indirectly which support the hypothesis that there is an additional action resulting from the direct contact between the polymer and bacterial cells. Figure 6.31 (showing PCA of metabolites LCMS data of cells lysed with sonication) did not show good separation between the clusters. So another type of statistical analysis was performed for these data (OPLS, orthogonal projection to latent structures analysis) to investigate the difference in the metabolites, Figure 6.32. OPLS showed cluster-separation proving that there is a difference between metabolites extracted from cells treated directly and indirectly with N-halamine polymers.

The contact can also happen between the polymer particles and medium protein. This can result in the same effect described before, of changing the medium protein; resulting in changing the nature of nutrients around the cells.
Figure 6.28: PCA of LCMS data of bacterial metabolites extracted from cells treated with N-halamine polymer directly and indirectly (packed in semi-porous membrane), 30 min treatment. Cells lysed with perchloric acid.

Figure 6.29: PCA of $^1$H NMR data of bacterial metabolites extracted from cells treated with N-halamine polymer directly and indirectly (packed in semi-porous membrane), 60 min treatment. Cells lysed with perchloric acid.

- Class 1: T, Cells treated with the halogenated polymer, ▲ or ◦ Class 2: TS, Cells treated with the halogenated packaged polymer, PC1 and PC2: first and second component.
Figure 6.30: PCA of FTIR data of bacterial metabolites extracted from cells treated with N-halamine polymer directly and indirectly (packed in semi-porous membrane), 10 min treatment. Cells lysed with perchloric acid.

![PCA of FTIR data](image)

Class 1: T, cells treated with the halogenated polymer, ▲ Class 2: TS, cells treated with the halogenated packaged polymer, PC1 and PC2: first and second component.

Figure 6.31: PCA of LCMS data of bacterial metabolites extracted from cells treated with N-halamine polymer directly and indirectly (packed in semi-porous membrane), 60 min treatment. Cells lysed with sonication.

![PCA of LCMS data](image)
Figure 6.32: OPLS for LCMS data of cell metabolites extracted from cells treated with N-halamine polymer directly and indirectly (packed in semi-porous membrane) after 60 min treatment. Cells lysed with sonication.

Class 1: T, cells treated with the halogenated polymer, Class 2: TS, cells treated with the halogenated packaged polymer, T(1)P: first component, T(2)O: first orthogonal component.

The challenge time with the N-halamine polymer results in a further effect on the bacterial metabolites. Figure 6.33 shows that the clusters positions have been changed by increasing the challenge time with the halogenated polymer. If the challenge time increased, more halogen can be exchanged between the polymer and the bacterial cells resulting in further changes in the bacterial metabolites profile. Similar results were reported before using sodium hypochlorite, Figures 6.15 and 6.16.
Figure 6.33: PCA of LCMS data of bacterial metabolites treated with N-halamine polymer (packed in semi-porous membrane) at different time intervals. Cells lysed with perchloric acid.

- Class 1: 10 min samples, + Class 2: 30 min samples, ▲ Class 3: 60 min samples, PC1 and PC2: first and second component.

The previous individual comparisons between each two treatments alone have been used to prove our suggested theory corners about the mode of action of N-halamine polymers individually. At the same time they were used to prove that the halogenated compounds have an effect of bacterial metabolites.

PCA analysis for all treatments at certain contact time supports the previous individual comparisons and shows the difference in the halogen delivery method by different halogenated compounds (polymer bound and free halogen). FTIR and LCMS data of the metabolites extracted from cells treated with different halogenated compounds (cells lysed with perchloric acid) were used as model examples while LCMS data were used to create some biomarkers.
Figure 6.34 shows FTIR-PCA analysis for metabolites extracted from bacterial cells treated with halogenated polymer (47) (with and without semi-pours membrane), non-halogenated polymer (46) and sodium hypochlorite. This analysis enabled the resolution of the various treatments in PCA space, and samples exposed to halogenated polymer (47) were clearly distinguishable (Figure 6.34, class 3). Interestingly, PCA was able to discriminate between cells exposed to non-halogenated polymer and the control (Figure 6.34, classes 1 and 2). Since cell viability was unaffected by exposure to non-halogenated polymer as described before, we speculate that the resolution of these samples must have been due to a secondary effect (i.e. other than cytotoxicity) consistent with the influence of the minor principal component (PC2) on the separation of these samples.
Figure 6.34: Principal components analysis of the FTIR spectra of cell lysates of cultures exposed, for 30 minutes, to various bactericidal treatments.

Class 1: untreated control culture; Class 2: culture treated with non-halogenated polymer; Class 3: culture treated with halogenated polymer; Class 4: culture treated with the halogenated polymer enclosed in a semi-permeable membrane; Class 5: culture treated with sodium hypochlorite. Points with the same symbol represent replicates.

Figure 6.35 shows LCMS-PCA analysis resulted in overlapping plots of the spectra from cultures treated with polymer (47) enclosed in the semi-permeable membrane (Figure 6.35, class 4) and cultures treated with sodium hypochlorite (Figure 6.35, class 5). This is presumably more reflective of the in vivo situation than the clustering obtained with FTIR, since no qualitative difference between the effects of enclosed polymer and free hypochlorite would be expected. Cultures treated with enclosed polymer were assumed to be affected by hypochlorous acid, formed by the chlorine ions released to water, which can affect the cells by a similar mechanism as sodium hypochlorite (Equation 6.3), possibly reflecting the greater potential of LCMS for providing distinguishing data, derived from resolving the samples into a series of
fractions. Treatments with both halogenated and non-halogenated polymer resulted in overlapping clusters (Figure 6.35, clusters 2 and 3), providing further evidence for an effect on the metabolome, derived from the polymer itself. Differentiation using FTIR spectra is based on a comparison of the intensity of functional groups within a population of metabolites, whereas LCMS-based differentiation derives from differences in molecular masses. This may help explain why LCMS appears to provide more effective differentiation of the different treatments when PCA is applied. Using this assumption, LCMS data were used for the identification of potential biomarkers.

Equation 6.3: HOCl formation due to chlorine ions release from the N-halamines

\[
\begin{align*}
\text{C} & \quad \text{N} \quad \text{Cl} + H_2O \\ 
\text{C} & \quad \text{N} \quad \text{H} + \text{HOCl}
\end{align*}
\]
Figure 6.35: Principal components analysis of the LCMS spectra of cell lysates of cultures exposed, for 30 minutes, to various bactericidal treatments.

Class 1: untreated control culture; Class 2: culture treated with non-halogenated polymer; Class 3: culture treated with halogenated polymer; Class 4: culture treated with the halogenated polymer enclosed in a semi-permeable membrane; Class 5: culture treated with sodium hypochlorite. Points with the same symbol represent replicates.

Some potential biomarkers were identified from the LCMS data. In particular a peak was detectable corresponding to a molecular mass of 101.1 after 30 min exposure to all of the experimental treatments but not in the untreated control, Figure 6.36. It is unlikely that this metabolite is a chlorinated derivative of an existing metabolite as it was also detected in cultures treated with the non-halogenated polymer so it presumably indicates significantly elevated levels of a metabolite with a molecular mass of 101.1 such as valerate or isovalerate.
Figure 6.36: Comparison between the intensity of the ion peak of a compound with molecular mass of 101.1 at different treatments.

Where: BC is the bacterial control, PC non-halogenated polymer, T halogenated polymer, TS halogenated polymer in a bag and TH is the sodium hypochlorite.

It was also observed that the exposed halogenated polymer, specifically resulted in increased levels of some ionic species such as the compound represented by the ion peak at molecular mass 172 (consistent with glyceraldehyde-3-phosphate), Figure 6.37. The other treatments such as exposure to the halogenated polymer contained by the semi-permeable membrane reduced the concentration of this metabolite to non-detectable levels. A hypothesis which may explain this finding is that HOCl is able to activate the DNA repairing enzyme (poly-ADP-ribose polymerize) which consumes sufficient NAD⁺ to interfere with ATP synthesis. Reducing the level of ATP, in this way, may explain the reduction in glyceraldehyde-3-phosphate.
Figure 6.37: Comparison between the intensity of the ion peak of a compound with molecular mass 172 at different treatments.

Where: BC is the bacterial control, PC non-halogenated polymer, T halogenated polymer, TS halogenated polymer in a bag and TH is the sodium hypochlorite.

All potentially chlorinating treatments resulted in a reduction in the metabolite corresponding to a molecular mass of 242 (consistent with thymidine), Figure 6.38. Halogenation of thymidine would, presumably, inhibit DNA synthesis, resulting in bactericidal activity.

The effect of the non-halogenated polymer on bacterial growth was previously reported (chapter 3). This effect could be due to attachment of bacterial cells to the polymer surface. However, the studies reported here are consistent with an effect of the non-halogenated polymer on the metabolome. The non-halogenated polymer contains amide bonds which, in theory, would be able to complex with metal ions exchanged through the cell wall. It also has the potential to consume hydrogen ions easily which
could disturb the equilibrium between intra- and extra-cellular protons, potentially having a deleterious effect on numerous metabolic processes. In chapter 2 some poly quaternary ammonium salts were prepared with similar structures by acidification, demonstrating the bactericidal activity of hydrogen ions.

Figure 6.38: Comparison between the intensity of the ionic species with a molecular mass of 242 at different treatments.

Where: BC is the bacterial control, PC non-halogenated polymer, T halogenated polymer, TS halogenated polymer in a bag and TH is the sodium hypochlorite.

The previous results indicated that the halogenated polymer enclosed in a semi-porous membrane has an effect on the bacterial metabolites consistent with our assertion that the halogenated polymer is able to affect the cells by halogen release and not by contact only as assumed by other researchers for 20 years [11, 101-105, 111, 112, 122,
123, 125, 126, 128, 129, 144, 145]. At the same time, the distinguishable difference between the clusters of metabolites treated with the halogenated polymer directly and the enclosed halogenated polymer indicated that there is an additional effect from the polymer per se. This also contrasts with the assertion that the mode of action of this type of polymer derives solely from halogen release into the aqueous environment [16]. These results support our previous hypothesis that the mode of action of N-halamine polymers is a combined effect of contact, release and changes in the nature of medium around the cells.

These results encourage further efforts to explain the actual mechanism of the bacterial cells’ death by the action of halogen containing compounds as well as the free halogen. In addition, they suggest that an investigation on the effect of the non-halogenated heterocyclic polymers on the ionic equilibrium through the cell could explain the mechanism of action of these compounds.

6.2.4. Conclusion

Although the bactericidal effect of halogens has been known for > 100 year, it had not, hitherto, been characterized by metabolomics investigation. Moreover, the systematic identification of biomarkers had not been undertaken. Halogen containing compounds had an effect on bacterial metabolites as well as the non-halogenated heterocyclic polymers. The effect of sodium hypochlorite on the bacterial metabolome was quantitatively dependent on the hypochlorite concentration. The mode of action of N-halamine polymer is now proved to reflect a combination of contact, release and,
possibly, a modification of the nature of the medium surrounding the cells. We hypothesise that the non-halogenated polymers as well as the non-halogenated repeating units in the N-halamine polymers may affect the metabolome, perhaps by disturbing the ionic equilibrium between intra- and extra-cellular positive charges.
Chapter 7

Summary, Final discussion, Final conclusion and Future work
7.1. Summary and Final discussion

Polymers have been used for decades in disinfection applications such as producing medical tools, water filters, surface coats, air filters and similar applications. There are different types of biocidal polymers; charged polymers (ammonium or phosphonium salts), polymer delivering systems (delivering ions or molecules) and polymers containing bioactive functional groups such as phenolic OH. Reviewing research results reported in the literature about biocidal polymers indicates that:

- The biological activity of some types of biocidal polymers can be improved.
- The production costs of these kinds of polymers should be reduced to use on a large scale.
- The particle size of the biocidal polymers is fixed and usually depends on the starting material particle size used in their preparation.
- The mode of action of some kinds of biocidal polymers such as N-halamine polymers is not clear.

Based on these facts, novel polymers were prepared by loading heterocyclic ring (Uramil, 5-aminobarbituric acid) to some linear polymers such as Polyacrylonitrile and polyethylacrylate. At the same time novel polyureas and polyurethanes were prepared by copolymerizing some novel prepared azo monomers (based on Uramil) with toluene-2,4-diisocyanate and tolylene-2,6-diisocyanate. The novel prepared polymers were converted to their bioactive forms by halogenation (chlorination, bromination and iodination) to form N-halamine biocidal polymers or by acidification to from poly Quat's. The
structures of the prepared monomers and polymers were determined using different spectroscopic techniques (FTIR, $^1$H NMR and $^{13}$C NMR) and elemental analysis.

The novel prepared N-halamine and poly Quat's biocidal polymers were designed to have more available positions for halogenation and quaternization than the polymers of the same type in the literature [14, 15, 25, 80, 96-98, 101, 112, 116, 125, 126, 128, 129] and to improve the stability of the halogens on the N-halamine polymer by introducing stronger electron donating groups to the heterocyclic ring. The stability of the halogen attached to the N-halamine polymers reported in the literature is based on the presence of methyl group as electron donating group [14, 15, 80, 96-98, 101, 112, 116, 125, 126, 128, 129]. The electron donating groups introduced in this study supports the stability of the halogen attached to the polymer to maintain the biological action for longer. The presence of a number of nitrogen atoms in the prepared polymers repeating unit supports their acidification to prepare poly Quat's containing multi-positive charges and increasing the biological action. Converting polymers to poly Quat's by diluted HCl is a very simple commercial way but the polymer in this case will be very sensitive to pH changes. So using this type of polymer will be restricted to some applications, e.g. antifouling agents, in which there is not much change in the pH value.

The biological activity of the prepared polymers was studied and most of them have shown a good disinfecting power in an agar plate screening assay to both Gram-positive and Gram-negative bacteria. The agar plate method was applied to the halogenated polymers and poly Quat's in their insoluble form while the monomers and the non-halogenated polymer were evaluated in their soluble and partially soluble forms to investigate their biological action for potential uses in disinfection purposes. It was
seen that with increasing the number of bioactive sites in the polymer repeating unit, the biological activity of the polymer increased. A good balance between stability and biological activity was achieved by these novel designs of N-halamine polymers.

The effect of different derivatives of one of the prepared polymers (25) (chlorinated, brominated and iodinated) on bacterial growth and viability was quantified. No bacterial growth was detected in the presence of any of these halogenated derivatives while viability was affected by their presence. Investigating the effect of these polymers on bacterial viability showed that the biological activity of the brominated (27) and chlorinated (26) derivatives was very similar, both achieved a 3 log reduction in 7 min contact time with bacteria (E. coli or S. aureus) and no colonies were detected after 15 min, using 0.5 g polymer; while the iodated polymer gives the highest effect by performing a 5 log reduction for E. coli in 1 min using 0.25 g polymer and no S. aureus colonies were detected after 1 min. The previous data were recorded without quenching the released halogen ions from the polymer. With released halogen quenching, the biological power is lower than that without quenching; which gives an indication that the mode of action of the polymer on bacteria is not by contact alone but can happen by release which contradicts reports in the literature that the mode of action can be by one of these two mechanisms only [11, 16, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145]. The biological power order is iodinated > brominated > chlorinated derivatives with or without halogen quenching. The results of brominated polymers begin to show a more powerful effect than the chlorinated polymer with released halogen quenching while they were very close without quenching.
An effect for the non-halogenated polymer (25) on bacterial growth was reported. The non-halogenated polymer inhibited \textit{S. aureus} growth. This may be due to \textit{S. aureus} adsorption to the polymer (25) (\textit{S. aureus} is not motile) and non-halogenated polymer (25) can separate some of the nutrient broth protein by hydrogen bonding which perhaps affects the amount of nutrient available to bacteria.

A separate study was performed to investigate the effect of non-halogenated polymer (25) on medium constituents. The bacteria were grown in nutrient broth pre-treated with non-halogenated polymer (25). It was seen that the bacterial growth was retarded in comparison with cells grown in fresh nutrient broth. These data indicate that the non-halogenated polymer (25) inhibits the bacterial growth by changing the nature of nutrition in the bacterial medium.

One of the prepared N-halamine polymers (chlorinated derivative) (26) was used in water filters on a laboratory scale. The filter succeeded in disinfecting bacterial suspensions perfused through it showing similar results to reports in the literature [17]. The biological activity of the prepared polymers was quantified using the stirred flasks method in addition to columns due to the expected filtration effect in the columns. Using stirred flasks shows the actual biological activity of the polymer in disinfecting the bacterial cells.

Toluene-2,4-diisocyanate was used instead of tolylene-2,6-diisocyanate in preparing the polyurethanes used in this study to reduce the production costs. One of the new prepared polymers (31) showed good biological activity close to that prepared using tolylene-2,6-diisocyanate (26), indicating that commercial materials can be used in producing this type of polymer.
The optimum halogenation conditions for preparing N-halamine polymers were determined to improve the halogen load to the polymer due to the shortage of data in the literature about the best halogenation conditions. The studied factors were the halogenation time, halogen concentration and halogenation temperature. It was found that the optimum conditions to halogenate 1 g of polymer can be produced by stirring the polymer (25) at ambient temperature with 10 ml 10% sodium hypochlorite for 2 hours. In this study sodium hypochlorite was used directly in the halogenation process to apply the same conditions that a customer can use if this work was commercialized.

The previous data showed that the biological power of the polymer was improved as well as the stability of the halogen attached to the polymer surface. But the particle size of the prepared polymers was still very small which can restrict some applications that require good flow rate. The particle size of the prepared N-halamine was modified by loading the heterocyclic ring (Uramil) onto modified silica gels (2-Cyano-functionalized silica gel and 3-(isocyanato)propyl-functionalized silica gel). The structures of the modified silica gels were studied using FTIR, solid state $^{13}$C NMR, SEM and elemental analysis. The novel prepared modified silica gels were halogenated (chlorinated, brominated and iodinated) and the biological activity was determined. The modified silica gels have recorded good biological activity but lower than the corresponding polymer powders (26, 27 and 28) prepared using the same heterocyclic ring; due to the high surface area of the powder as well as the high number of bioactive sites on the powder. The stability of the halogen attached to the silica particles has been improved by introducing electron donating groups to the heterocyclic rings such as amino groups. This stability leads to lower biological action in comparing the novel modified silica with
similar structures reported in the literature [142] but it enables using this modified silica longer without halogen recharging as the silica will not lose the halogen easily.

Due to low biological action of the modified silica gels a new method was created to produce N-halamine polymer beads in different sizes with good biological activity. Most of the large-size biocidal polymer particles reported in the literature based their size on the size of starting material used in their preparation [14, 15, 112, 145] while using sodium alginate easily enables changes in particles size [146, 147].

N-halamine biocidal beads were prepared by blending polymers prepared as N-halamine powders (25) or (26) with sodium alginate followed by cross-linking with calcium chloride. This new method enabled highly bioactive N-halamine polymers to be used in producing different sizes beads. The beads size is based on the dropper diameter used in dropping the alginate-polymer blend to the cross-linker bath. The structure of the beads was studied using FTIR, SEM and thermal analysis.

The biological activity of the prepared beads was determined and the optimum preparation conditions for their preparation were characterized. It was found that calcium chloride is the best cross-linker used in bead formation and it is better to blend pre-halogenated polymers with alginate rather than using non-halogenated polymers followed by halogenation; curing with calcium chloride as well as increasing the alginate ratio increases the recycling possibilities of the beads while increasing the polymer ratio increases the biological activity. The optimum conditions for preparing the beads are by blending pre-halogenated polymer (26) (5%, w/w) with sodium alginate (3%, w/w) followed by dropping into a calcium chloride bath (100 ml, 10% w/w) with curing over night. The biological activity of the prepared beads is more than that of modified silica
and close to that reported in the literature [142] without curing. With curing, the biological activity decreased but the ability of beads re-halogenation increased. At the same time it is lower than that of the polymer powder (26) itself as the surface area of the polymer powder is more than that of the beads and the mode of action of the beads will depend mainly on the ion release rather than contact. The contact can happen with the outer surface of the beads while the inner particles work with release only as there is no possibility of contact with bacterial cells. This new method enables blending water-insoluble polymers with a sodium alginate matrix to work by ion release. Sodium alginate has been used before for constructing beads that release water soluble drugs and bioactive compounds [146, 147].

One of the main problems of applying N-halamine polymers on a large scale is the production costs. To reduce the production costs of N-halamine biocidal polymers, a novel cross-linked N-halamine biocidal polymer (47) was prepared by cross-linking polyepichlorohydrin with m-phenylenediamine followed by a reaction with cynuric acid. This novel cross-linked polymer was prepared with low-cost chemicals which may encourage its industrial use on a large scale. The polymer structure was studied using FTIR and $^{13}$C NMR. The biological activity of the novel commercial polymer was studied against E. coli and S. aureus. The polymer was used in water filters on laboratory scale. The regenerability as well as the life-time of the polymer in water filters was determined. The polymer disinfected 30 ml of E. coli suspensions contain $3.8 \times 10^{10}$ cfu/30ml in three runs and 40 ml of S. aureus suspensions contain $3.6 \times 10^{10}$ cfu/40ml in four runs. A water purification station was created from three kinds of columns; sand, halogenated polymer (47) and non-halogenated polymer columns (46), and evaluated
against bacterial suspensions. The station was regenerated several times successfully and its life-time was determined. Non-halogenated polymer (46) column was used in combination with the halogenated one (47) to trap the halogen released from the first column to produce halogen-free water. The sand column was used to regulate the number of bacterial cells entering the halogenated column. Using this station as a multi-filtration system enables supporting clean water, free of halogen. Most of systems in the market use chlorine directly to disinfect water while using such systems will reduce the level of halogen going to customers. Reducing the production costs of N-halamine polymers can support constructing large scale multi-filtration systems for water purification. A design for a large scale system was suggested to in order to investigate the possibility of applying these polymers in future.

The mode of action of the prepared polymers was studied and a new theory for the mode of action of N-halamine polymers on bacterial cells was created. The new theory relates the mode of action of this type of polymers to a combination of different factors; contact, release and changing the nature of the nutrients around the cells. Halogen delivering to the medium can also be by contact or release as the polymer can exchange halogen with the medium protein by direct contact or by halogen releasing to medium followed by delivering to protein. The protein in this case can act as an N-halamine polymer as the NH amide in its structure converted to NCI. These new hypotheses are supported by microbiological and metabolomic evidence.

From microbiological point of view the new theory was supported as follows:
Polymer (26) was pressed into disks and placed in contact with freeze-dried bacterial cells. A change in bacterial viability was noticed after recovering the cells which supports the hypothesis that the polymer has an effect by contact only.

The polymer was packaged into a semi-porous membrane to prevent any contact between the polymer and cells. Effects on bacterial viability were recorded which supports the hypothesis that the polymer can kill by release. The release can be to the cells directly or to the medium protein to work as N-halamine polymer.

The bacterial cells were grown in a pre-treated nutrient broth medium with N-halamine polymer. The cells failed to grow which support the hypothesis that the N-halamine polymers can change the nature of medium around the bacterial cells resulting in their death. Halogen exchange between the N-halamine polymer and medium protein can result in converting it to N-halamine polymer.

The amount of delivered halogen from the polymer to water, nutrient broth and bacterial suspensions was determined. It was found that the amount of delivered halogen was increased in the presence of nutrient broth and increased even more in the presence of bacterial suspensions. These results indicate that the presence of nutrient broth increased the amount of delivered halogen; which indicates that there is a halogen exchange between the polymer and the medium constituents. In the presence of bacterial cells the amount of delivered halogen was increased more than in the presence of nutrient broth alone, indicating that some halogen was consumed by the cells, resulting in more halogen release from the polymer. These results indicate that there is exchange between the polymer and the nutrient broth.
From a metabolomics point of view: the bacterial cells were treated with the N-halamine polymer directly and indirectly (polymer packaged in semi-porous membrane) followed by metabolites extraction. The bacterial cells were lysed by different methods using perchloric acid or sonication. The resulting metabolites were detected with FTIR, $^1$H NMR and LCMS. PCA was performed for the data and profiles were generated for the metabolites.

By comparing the profiles of cells treated with the halogenated polymer indirectly (in semi-porous membrane) with non-treated cells it was proved that there is an action for the polymer with release. Similarly, the difference in metabolites profile between cells treated directly and indirectly with the halogenated polymer indicated that there is an additional effect by direct contact between the polymer and the cells or between the polymers and the medium protein followed by delivering halogen to the cells (changing the nature of medium protein).

Based on these results a new theory was stated based on a combination of factors as a mode of action of the N-halamine polymers; contact, release and changing the nature of bacterial cells medium. This new theory contradicts what has been stated in the literature [11, 16, 101-105, 111, 112, 122, 123, 125, 126, 128, 129, 144, 145]. At the same time the metabolomics data indicates that there is an effect for the non-halogenated polymer on bacterial metabolites. This may be because of the ability of the non-halogenated polymer to react with hydrogen ion which may result in the ionic equilibrium around the cell wall. Some biomarkers were suggested using LCMS data to be proved in a future work.
7.2. Final conclusions

1- The biological activity of N-halamine and poly Quat’s biocidal polymers has been improved as has the stability of the halogen attached to the N-halamine polymers.

2- The biological activity of the prepared polymers was quantified.

3- The particle size of N-halamine polymers was modified and a method for producing them in different sizes was developed.

4- An N-halamine biocidal polymer with low production cost was prepared and evaluated in water purification station on a laboratory scale.

5- A new suggested design for a multi-filtration system was suggested to produce water free halogen over a wide range.

6- The mode of action of N-halamine polymer was studied, from microbiological and metabolomics points of view, and a new theory was created on the basis of a combination of factors effect (control, release and changing the nature of the medium).

7- An effect for the non-halogenated polymer on bacterial metabolites was detected and some biomarkers were suggested for cells treated with different treatments; halogenated polymer (directly and indirectly), non-halogenated polymers and sodium hypochlorite.
7.3. Future work

The outcome of this study encourages future work in this field as follows:

1- Commercial evaluation for the low cost polymers prepared in this study to be used on a large scale as well as the suggested purification system.

2- Finding suitable applications to commercialize the prepared polymers.

3- Finding other possibilities to reduce the production costs of N-halamine polymers.

4- A full study for the relation between halogen stability and release. Determining the effect of increasing the donating power of substituted function groups to the heterocyclic ring in the polymer on the halogen stability.

5- Studying the interaction between the protein and the polymers (halogenated and non-halogenated).

6- Identifying the actual mechanism of the bacterial death with the action of halogenated compounds using metabolomics.

7- Investigating the effect of the non-halogenated polymers on bacterial metabolites.
Chapter 8

References


polyacrylamide by reacting it with benzoate esters and benzaldehyde derivatives.

36. Emerson, D. W.; Emerson, R. R.; Joshi, S. C.; Sorensen, E. M. T.; James, E.,
Polymer-bound sulfonilhydrazine functionality. Preparation, characterization,
(1979), 44, 4634.

(styrene-divinylbenzene). Preparation, characterization, and bactericidal action.

S., Effect of Alkyl Derivatization on Several Properties of N-Halamine

J. P., Antimicrobial activity and biocompatibility of polyurethane and silicone
catheters containing low concentrations of silver: a new perspective in prevention
187.

40. Bechert, T.; Boswald, M.; Lugauer, S.; Regenfus, A.; Greil, J.; Guggenbichler, J.

41. Gabriel, M. M.; Sawant, A. D.; Simmons, R. B.; Ahearn, D. G., Effects of silver


157. Loret, M. O.; Pedersen, L.; Francois, J., *Revised procedures for yeast metabolites extraction: application to a glucose pulse to carbon-limited yeast cultures, which reveals a transient activation of the purine salvage pathway*. Yeast (2007), 24, 47.


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