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(54) Title: POLYMER INHIBITORS OF QUORUM SENSING

(57) Abstract: This invention relates to polymer inhibitors of quorum sensing, methods for their preparation and use of such polymers in the prevention and treatment of bacterial infections and in the manufacture of shaped articles having an increased resistance to bacterial infections.

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POLYMER INHIBITORS OF QUORUM SENSING

Field of the invention

This invention relates to polymer inhibitors of quorum sensing, methods for their preparation and use of such polymers in the prevention and treatment of bacterial infections and in the manufacture of shaped articles having an increased resistance to bacterial infections.

Background of the invention

Since the 1930s, antibiotics have played a key role in the treatment of bacterial infections. However, over the last fifty years, as resistance to antibiotics has developed, it has become increasingly difficult to treat certain bacterial infections, in particular those propagated by multiple resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA).

A main focus of attention in the development of new antibiotic agents has been in the development of agents that prevent bacterial reproduction. Although considerable success has been achieved using this approach, the success is sometimes short lived, as it is relatively easy for a population of resistant bacteria to develop.

An alternative approach to the limitation of the spread of a pathogenic bacterial infection is to attempt to interfere with the virulence determinants that contribute to pathogenesis. It is known that many Gram-positive and Gram-negative bacteria communicate via the production and sensing of small, diffusible signal molecules, to coordinate virulence determinant production. Accordingly, this event, termed quorum sensing, is a potential therapeutic target. Agents capable of interfering with quorum sensing may in turn inhibit virulence and thus control infection, by blocking cell-to-cell communication. An example of such an approach is the inhibition of quorum sensing using halogenated furanone molecules that appear to inhibit the expression of virulence factors in certain pathogenic systems.

There is a need for new and alternative approaches to inhibiting the spread of bacterial infections which coordinate virulence through quorum sensing. Surprisingly, we have now found such an approach.

According to a first aspect of the invention there is provided a polymer adapted to sequester a quorum sensing signalling molecule.

According to a further aspect of the invention, there is provided the use of a polymer adapted to sequester a quorum sensing signalling molecule for the preparation of a medicament for the prevention or treatment of a bacterial infection which coordinates virulence by means of said quorum sensing molecule.

According to a further aspect of the invention, there is provided the use of a polymer adapted to sequester a quorum sensing signalling molecule for the preparation of shaped articles.

According to a further aspect of the invention, there is provided the polymer adapted to sequester a quorum sensing signalling molecule for coating an article.

According to a further aspect of the invention, there is provided an article coated with a polymer adapted to sequester a quorum sensing signalling molecule.

According to a further aspect of the invention, there is provided a method of preparing a polymer adapted to sequester a quorum sensing signalling molecule which comprises template directed polymerisation of functional monomers in the presence of the quorum sensing molecule or an analogue or derivative thereof, thereby producing a polymer formed from an array of said monomers, whereof at least part of said polymer is complementary to at least part of said quorum sensing signalling molecule.

Brief description of Drawings

Figure 1 shows the structures of typical quorum sensing signal molecules.

Figure 2 shows the structure of *N*-(3-oxo-hexanoyl)-DL-homoserine lactone (AHL).

Figure 3 shows the minimised structure of AHL.

Figure 4 shows a virtual library of functional monomers.

Figure 5 shows the complex of MBAA (left), DEAEM (right) with AHL template.

Figure 6 shows the complex of itaconic acid (left), EGMP (middle) and acrylamide (right) with AHL template.

Figure 7. Screening of the polymers for affinity towards the AHL (peak wavelength- λ= 201 nm).

Figure 8 shows the chromatogram showing the complete adsorption of 100 ng of AHL by 100 mg of itaconic acid-based polymer.

Figure 9 shows the effect of polymers on growth

Figure 10 shows the effect of polymers on bioluminescence.

Detailed Description of the Invention

Quorum sensing signalling molecules that may be sequestered by polymers according to the invention include those molecules illustrated in Figure 1. In particular, we prefer the quorum sensing signalling molecule to be an N-acylhomoserine lactone (AHL). The AHL molecule may be racemic (DL) or chiral. We particularly prefer AHL molecules which contain additional carbonyl group in the acyl chain, in particular in the 3 position, relative to the carbonyl of the acyl group. An especially preferred AHL molecule is N-(3-oxo-hexanoyl)homoserine lactone, which is also known as N-(β-ketocaproyl)-homoserine lactone.

By "sequester" we mean that the polymer is able to remove, at least in part, the quorum sensing signalling molecule from a use environment, e.g. from a body fluid or fluid material coming into contact with a body fluid or a body part. Other terms comparable with "sequester" are: absorbent, ligand or binding agent.

We prefer the polymer to be a synthetic polymer, particularly a polymer prepared by chain polymerisation of a reactive monomer or oligomer containing an activated double bond.

Preferably the polymer adapted to sequester a quorum sensing signalling molecule is water soluble, e.g. with a water solubility of at least 1 μg/ml or is dispersible in water, under physiological conditions, e.g. a pH of about 7.4 and an ionic concentration of about 50 mM. In certain circumstances, where the polymer is intended for use in a more acidic environment, e.g, the gut, then the polymer can be adopted to be water soluble or water dispersible at that pH.

The ability of the polymer to sequester quorum sensing signalling molecules makes it useful as a medicament, particularly in the prevention of bacterial infections which are mediated by quorum sensing signalling molecules, especially those in which AHL signalling molecules play a part. Thus we provide a method of treating or preventing a bacterial infection in which virulence is coordinated by means of a sensing molecule which comprises administering to a patient suffering from such an infection a therapeutically effective amount of the polymer adapted to sequester a quorum sensing signalling molecule.

Pathogenic bacteria that may be treated using the polymers according to the invention may be either Gram-negative or Gram-positive, and include *Pseudomonas aeruginosa, Burholderia cepacia, chromobacterium violaceum, Yersinia pestis, Yersinia enterocolitica, Yersinia pseudotuberculosis, Aeromonas hydrophilia, Brucella melitensis, Bacillus subtilis, Streptococcus pneumoniae, Enterococcus faecalis, E. coli, H. pylori, Neisseria meningitides, Porphyromonas gingivalis, Proteus mirabilis, S. typhimurium, Streptococcus pyrogenes, S. aureus,* etc.

When used as a medicament the polymer may be take the form of pharmaceutical compositions in a form suitable for topical administration to the lung include aerosols, e.g. pressurised or non-pressurised powder compositions;

compositions in a form suitable for oesophageal administration include tablets, capsules and dragees;

compositions in a form suitable for administration to the skin include creams, e.g.

oil-in-water emulsions or water-in-oil emulsions;

compositions in a form suitable for administration intravenously include injections and infusions; and compositions in a form suitable for administration to the eye include drops and ointments.

According to the invention there is also provided a pharmaceutical composition comprising, preferably less than 80% and more preferably less than 50% by weight of, a polymer according to the invention, in a mixture with a pharmaceutically acceptable diluent or carrier.

Examples of such diluents and carriers are:

for tablets and dragees - lactose, starch, talc, stearic acid;

for capsules - tartaric acid or lactose; and

for injectable solutions - water, alcohols, glycerin, vegetable oils.

Although such a composition may contain a minor proportion of the polymer, compared to the excipients, it is within the scope of this invention to have compositions which are entirely formed from the polymer, which may be presented in a form suitable for administration to, or application to, a body surface or orifice.

When the polymer is to be administered to the lung it may be inhaled as a powder which may be pressurised or non-pressurised. Pressurised powder compositions of the compounds of formula I may contain a liquified gas propellant or a compressed gas. In non-pressurised powder compositions the active ingredient in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable carrier comprising particles of up to, for example, 100 μm in diameter.

Suitable inert carriers include, e.g. crystalline lactose.

For the above mentioned uses the doses administered will, of course, vary with polymer employed, the mode of administration and the treatment desired. However, in general, satisfactory results are obtained when the polymer is administered at a daily dosage of from about 1μg to about 20 mg per kg of animal body weight, preferably given in divided doses 1 to 4 times a day or in sustained release form.

For man the total daily dose is in the range of from 70 μg to 1,400 mg and unit dosage forms suitable for administration comprise from 20 mg to 1,400 mg of the compound admixed with a solid or liquid pharmaceutical diluent or carrier.

The polymers according to the invention have the advantage that they are less toxic, more efficacious, are longer acting, have a broader range of activity, are more potent, produce fewer side effects, are more easily absorbed or have other useful pharmacological properties, compared to other bacterial therapies, such a antibiotics. In particular, the polymers are much less likely to give rise to resistance.

When the polymer adapted to sequester a quorum sensing signalling molecule is used for the preparation of shaped articles, those shaped articles may be any medical device or apparatus which is likely to come into contact with bacterial infections in which virulence is mediated by quorum sensing signalling molecules. Such shaped articles include in-dwelling stents and catheters, joint replacements, wound dressings, sutures, membranes, filters, implants, prosthetics etc.

As necessary, the characteristics of the functional monomers from which the polymer is made may be selected to suit the production methods of the shaped article. For example, the polymer may be constructed so that it can be moulded, e.g., blow or injection moulded, spun, e.g. into a mesh, fabric or thread or cast.

When the a polymer adapted to sequester a quorum sensing signalling molecule is used for coating an article, the articles may be the same as those mentioned above for shaped articles. In particular, the polymer according to the invention may be used to coat catheters and stents. The coating may be applied in solution, e.g, an aqueous solution and then the solvent removed by drying or evaporation.

Alternatively, the reactive monomer may be applied to a surface in solution, e.g. in the manner of a paint or varnish, and polymerised *in situ*. Surfaces that may be treated included those that may be prone to being covered, at least partially, in a biofilm, for example the walls of buildings, in particular toilet walls and the walls of operating

theatres. Other surfaces that may be treated include the hulls of boats and surface of supports of marine structures, e.g oil rigs, where coating may help prevent fouling.

The components of polymers under present invention, such as monomers or oligomers can be grafted to the surface of articles by e.g. UV, chemical or plasma treatment, by adsorption, entrapment or by conjugation.

Another form for polymer application is a solid or powdered article or e.g. pressed powdered material.

Methods for making polymer according to the invention are conventional in the art of polymer technology, and are described, for example, in WO 01/30856, WO 01/55235 and Piletsky et al, Analyst 126(10) 1826 – 1830 (2001).

Suitable polymers may be made by template directed polymerisation, that is by polymerisation of functional monomers in the presence of the quorum sensing molecule or an analogue or derivative thereof. This tends to produce a polymer formed from an array of said monomers in which at least part of said polymer is complementary to at least part of said quorum sensing signalling molecule. The resulting polymer may be used either with the template in place, or more preferably, with the template removed. Template molecules may be removed by conventional washing techniques, e.g., dialysis.

Alternatively, the polymer may be made from appropriately selected functional monomers, without any template, by conventional polymerisation techniques known *per se*. Complementarity of the resulting polymer is then a consequence of the nature of the starting functional monomers and any cross-linking agents that may be used.

In either process, the selection of the functional monomers is crucial to the performance of the polymer intended to sequester the pre-determined quorum sensing signalling molecule.

Functional monomers may be selected using the techniques described in WO 2006/067431, which relates to a procedure for computer aided rational molecular design.

In general, factors which are important in selecting monomers include selecting those functional monomers, that is molecules with an activated double bond, which contain additional functionality, which is able to bind with high affinity to key distinguishing features of the quorum sensing signalling molecule.

Typically, monomers are identified by their ability to form specific directional hydrogen bonds with the quorum sensing signalling molecule, e.g. by donating or accepting hydrogen bonds. Where the quorum sensing signalling molecule has a hydroxyl or carbonyl function, then this can sequestered by a hydrogen bond donating group in the monomer, e.g. by an amino or hydroxyl group. Where the quorum sensing signalling molecule has a hydroxyl group, then this can be sequestered by a monomer having a carbonyl or imine group.

Other forms of interaction that may be important in selecting monomers that can selectively recognise quorum sensing signalling molecule include electrostatic interactions, van der Waal's forces and shape complementarity.

Electrostatic interactions may be important in selecting monomers intended to form polymers adapted to sequester quorum sensing signalling molecule having charged amino acids as part of their structures. Such functional monomers would have charges complimentary to for example, the positive charges in lysine, arginine or histidine residues (that is by having a positive charge) or the negative charges in aspartate or glutamate residues (that is by having a negative charge).

Van der Waal's charges can be brought in selecting functional monomers for the preparation of polymers for sequestering quorum sensing signalling molecule containing aromatic residues, particularly aromatic amino acid residue, for example, phenylalanine, tyrosine and tryptophan. In these case, the functional monomer may

contain a complimentary aromatic residue, for example a phenyl or naphthalene residue.

In certain case, selectivity in binding may be brought about by the functional monomer having a complimentary shape to one or more portions of the quorum sensing signalling molecule. In such circumstances, the fit of the quorum sensing signalling molecule into the monomer should be optimised, in accordance with shape complimentarity analysis. Shape complementarity analysis may be made using the program SC (http://www.ccp4.ac.uk/ccp4i_main.html). We particularly prefer polymers that that bind to quorum signalling molecules with a good shape complementarity, that is with a S_c greater than 0.50, more preferably greater than 0.55, particularly greater than 0.60.

The selection of appropriate monomers and polymers can be made also by screening of polymer libraries.

In certain cases, the complimentarity of the polymer may be masked or protected in the functional monomer, to prevent interference with the polymerisation of the monomer. As such, hydroxyl groups in the monomer may be protected by a protecting group, which then may be removed after polymerisation, by methods which do not affect the integrity of the polymer. Such protecting groups are well known to the person skilled in the art and include benzyl groups, which may be removed by hydrogenation in acetic acid and acetyl, which may be removed by hydrolysis, either in acid or in basic solution.

Functional monomers may include monomers of some or all of the following types: vinyl monomers, allyl monomers, acetylenes, acrylates, methacrylates, aminoacids, nucleosides, nucleotides, carbohydrates, phenols, heterocycles, aniline and other aromatic amines, and derivatives of any of the preceding compounds, including in particular, monomers tailored to interact by some shape or bonding interaction with the chosen quorum sensing signalling molecule.

Where the polymer is prepared by template polymerisation, preferable monomers are those which interact non-covalently with the template. Preferable monomers are those that may be polymerised through a radical mechanism. Co-monomers may be included, in particular cross linking agents, eg EGDMA (ethylene glycol dimethacrylate). A porogen may also be included in the polymerisation system, eg dimethyl formamide.

Suitable functional monomers include those set out in Figure 4.

Examples of carrying out the invention

Computational screening of a functional monomer library towards N-(3-oxo-hexanoyl)-DL-homoserine lactone (AHL)

The structure of the target compound for this study is the *N*-acyl homoserine lactone (AHL) shown in Figure 2, in which the acyl group is derived from 3-oxo-hexanoic acid. This compound has a number of key recognition elements, in particular the lactone ring is able to accept hydrogen bonds, e.g. from one or more amino- and hydroxyl groups. The amide group is able to donate a hydrogen bond from the –NH- function and accept a hydrogen bond to the =O function of the group. In addition the 3-oxo group is able to accept a hydrogen bond. The directional nature of hydrogen bond at these signature recognition sites and the spatial requirements for strong bonding allow the design of polymers which can selectively sequester these AHL in the presence of other signalling molecules.

Other factors, such as shape, size and van der Waals forces may also play apart in determing the ability of a polymer to sequester a chosen signalling molecule. These considerations can better be appreciated in light of the computer modelling study described below. However, the primary structural features that can be identified in the AHL molecule include the lactone functionality, the secondary amide group and the β-dicarbonyl function.

The molecular modelling of monomer:template interactions for the target analytes, AHL was carried out using the computer-aided rational design technique (WO

01/55235, WO 2006/067431 and Piletsky et al., Analyst 126(10) 1826 – 1830 (2001) pioneered by Cranfield University as a generic protocol for MIP preparation. The protocol is based on the simulation of a complex formation between selected monomers and template in a monomer mixture, using a Silicon Graphics workstation running UNIX (IRIX 6.5) as the hardware, and SYBYL 7.0 ™ as the industry standard software system. The rational design of MIPs is described for the template AHL. The screening was carried out using a database of 21 of the most commonly used functional monomers and the results discussed.

The rational design protocol

The rational design of MIPs was carried out on a Silicon Graphics Octane workstation running the IRIX 6.5 operating system. The workstation was configured with two 195 MHz reduced instruction set processors, 1 Gb memory and a 20 Gb fixed drive. The system was used to execute the software packages SYBYL 7.0 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA).

The rational design protocol involves 3 steps.

1. Design of molecular model of AHL to be screened
2. Design of functional monomer database
3. Screening using a LEAPFROG™ algorithm

These three steps give a very good indication as to the best monomer(s) for polymer preparation in a given solvent.

Design of molecular model of AHL to be screened

AHL was modelled by calculating. the charges for each atom on the template and the structures refined using molecular mechanical methods. Energy minimisation was performed to a value of 0.001 kcal/mol and this model was then used for the design of a MIP (Figure 3).

The oxygen atoms are shown in red and the nitrogen atoms are shown in dark blue. The white atoms are carbon and the light blue atoms are hydrogens. This structure was charged with the Gasteiger-Huckel approximation method, and refined by the

molecular mechanics method applying an energy minimisation using the MAXIMIN2 command.

Design of Functional Monomer Database

The Cranfield library that was designed for the screening of AHL contains 22 of the most commonly used functional monomers, these being acidic, basic or neutral molecules. These monomers possess polymerisable residues and residues capable of interacting with a template through electrostatic, hydrophobic van der Waals forces, and dipole-dipole interactions. Preferred monomers are those that are able to interact with the template through non-covalent interactions and that can be polymerised through a radical mechanism. Most of the monomers used for MIP preparation are described extensively in the literature as a result of their ease of polymerisation by temperature conditions or photo-initiation, availability and cost. The charges for each atom of each monomer are calculated and the structures of the monomers refined using molecular mechanical methods in a similar manner to that of the AHL. The structures of the monomers in the database are shown in Figure 4.

Screening using a LEAPFROG™ algorithm

The database of monomers was screened against the charged and neutral forms of the templates. These monomers were also charged depending on whether nitrogen or a carboxylic acid group was present in order to reflect possible scenario during the polymerisation or rebinding conditions. Using the LEAPFROG™ algorithm for the screening of AHL resulted in a table ranking the monomers with the highest binding score (kcal/mol), as the best candidates for polymer preparation was generated. Typically, 60,000 iterations was needed to make a full examination of the binding of the monomers with AHL. The results from this were examined and the empirical binding score evaluated. The binding energies are shown in Table 1.

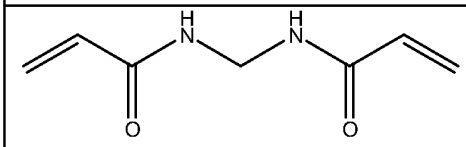
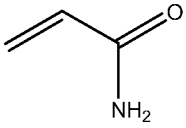
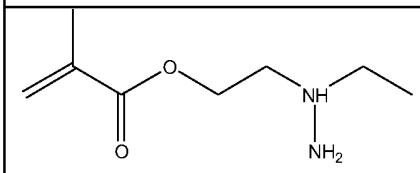
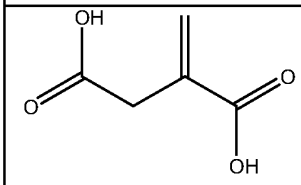
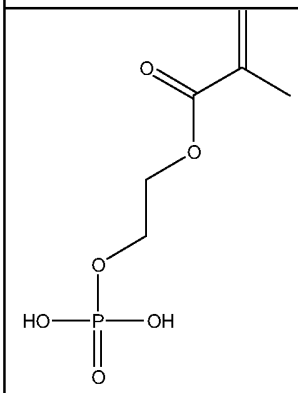
	1:SLN	2:BINDING
 LPF_11	BISACRYLAMIDE	-28.23
 LPF_6	ACRYLAMIDE	-27.32
 LPF_13	DEAEM+	-26.83
 LPF_8	ITACONIC ACID	-24.93
 LPF_9	EGMP	-18.92

Table 1. Binding Energy Table of charged AHL.

The monomers giving the highest binding scores represented the best candidates for polymer preparation forming the strongest complexes with the template. The top five highest binding monomers were identified as acrylamide, MBAA (methylene

bisacrylamide), DEAEM (N,N-diethylaminoethyl methacrylate), itaconic acid and EGMP (ethylene glycol methacrylate phosphate) (Figures 5 and 6).

The Leapfrog results from the modelling exercise clearly show that it should be possible to design polymers for AHL.

Polymer synthesis

Based on the computational modelling five monomers were selected for polymer preparation: MBAA, DEAEM, itaconic acid, EGMP and acrylamide. 10 g of the polymer contained 2 g of corresponding functional monomer, 8 g of cross-linker (EGDMA) and 100 mg of 1,1-azobis (cyclohexanecarbonitrile) as an initiator. Itaconic acid-based polymer was prepared as above but with ratio 3 g of itaconic acid per 7 g of cross-linker. 10 ml of dimethylformamide was used as a solvent/porogen. The polymer was prepared by thermo-polymerization at 80 °C for 12 h. After synthesis polymer was ground, washed with methanol and sieved to obtain the fractions with size 38-125 μm.

SPE protocol

100 mg of each polymer fraction was packed in the empty 1-ml SPE cartridges between two polypropylene frits. In order to perform the screening of the polymers at the physiological conditions AHL was dissolved in Phosphate Buffered Saline (PBS), pH 7.4. Cartridges were conditioned with 2 ml of water and loaded with 1 ml of AHL solution (1 mg/ml). The eluent was collected and evaluated using spectrophotometer at wavelength 201 nm (Figure 7). The height of the peak was used for quantification of AHL adsorption.

Based on the SPE screening it was found that acrylamide, MBAA and itaconic acid demonstrated the highest retention of AHL from 1 mg/ml solution (100%, 91% and 80%, correspondingly).

Quantification of *N*-(3-oxo-hexanoyl)-DL-homoserine lactone using HPLC-MS-MS

In order to quantify more precisely the adsorption of AHL all following studies were made using HPLC-MS-MS. HPLC separation was conducted using a Waters 2975 HPLC system equipped with Luna C18(2) column (150x3 mm, 3 μm, Phenomenex). N-

(3-oxo-hexanoyl)-DL-homoserine lactone (Sigma, K4355) was received from University of Kent.

Mobile phase: water (A), methanol (B) in a binary system with 0.1% of formic acid as an additive. **The elution gradient**: for 10 min- solution A (water/0.1% formic acid), then linear gradient from 0 to 70% of solution B from 10 to 30 min, return to 100% A solution at 30 min and washing before next injection for 10 min (total time- 40 min). In order to minimize the salt content which could get into the mass-spectrometer the measurement of the sample was recorded between 25 to 30 min, all other time liquid was diverted to the waste using "solvent delay" option. The flow rate was 0.2 µl/min; the injection volume - 10 µl; the column temperature - +20 °C. 102 m/z fragment of AHL (Morin et al. 2003) was detected by mass spectrophotometer Micromass Quatro Micro (Waters, UK) with an ESI interface in positive ion mode.

The MS parameters were following: desolvation gas - 850 L/h, cone gas-50 L/h, capillary 4.5 kV, cone-25 V, CE- 20, source temperature- +120 °C, desolvation temperature- +350 °C, collision energy - 25 V, multiplier - 650 V. Peak with retention 26 min was quantified (Figure 8).

Determination of binding capacity of itaconic acid-based polymer using HPLC-MS-MS

100 mg of the IA-based polymer with fraction size 38-125 µm were packed in the 1-ml SPE tubes. All experiments were done in duplicate. During the loading of the sample the flow rate was controlled; recommended flow- no more than 0.5 ml/min.

SPE protocol:

- Pre-conditioning of the cartridges: 2 ml of HPLC quality water
- Loading: 1 ml of the sample containing Phosphate buffered saline (PBS, pH 7.4) spiked with 100 ng/ml of AHL, 10 µg/ml of AHL and 1 mg/ml AHL*

*1 mg/ml sample was diluted 100 times for the LC-MS measurement

The quantification of the samples showed that IA-based polymer adsorbed 100 % of AHL from 100 ng/ml sample (1 μg of AHL per g of polymer); 99.8% -from 10 μg/ml sample (99.8 μg of AHL per g of polymer); 99.43% from 1 mg/ml sample (9.94 mg of AHL per g of polymer).

MIP synthesis

Based on the results of molecular modelling and polymer screening, MIP and corresponding blank polymers based on itaconic acid were prepared. The polymer composition was following: 50 mg of AHL, 152 mg of itaconic acid, 810 mg of EGDMA, 1 g of DMF and 10 mg of initiator (1,1-azobis (cyclohexanecarbonitrile)). The blank polymer was prepared similarly but without template (AHL). Polymer mixture was degassed using argon and kept on ice and polymerized for 30 min with UV (Cermax lamp, light intensity- 0.015 W/cm^2). After the polymerization reaction was completed, the vials were incubated in the oil bath at 80 ºC for 12 hours for thermo-annealing.

MIP and blank polymers were ground and sieved. Fractions between 45 and 106 μm were collected and washed in methanol. In order to remove AHL from the MIP, Soxhlet extraction of the polymer for 3 days was performed.

Conclusions:

1. Three polymers based on acrylamide (AA), N, N′-methylene bis acrylamide (MBAA) and itaconic acid (IA) were found to be very efficient for adsorption of the *N*-(3-oxo-hexanoyl)-DL-homoserine lactone.
2. Itaconic acid- based polymer was selected as a leading polymer for adsorption of *N*-(3-oxo-hexanoyl)-DL-homoserine lactone.
3. Two methods of *N*-(3-oxo-hexanoyl)-DL-homoserine lactone quantification were optimised: spectroscopic (for 0.1- 1 mg/ml concentrations of AHL) and HPLC-MS-MS (for 100 ng- 1000 ng/ml concentrations of AHL).
4. The testing showed that IA-based polymer could adsorb quantitatively *N*-(3-oxo-hexanoyl)-DL-homoserine lactone from Phosphate Buffered Saline solution, pH 7.4 at very broad range of concentrations- from 100 ng/ml (100% adsorption) to 1 mg/ml (99.4% adsorption).

5. Extra work was done on preparation of MIP with AHL as a template and corresponding Blank polymer. The fraction between 45 and 106 μm was collected and washed in order to remove the template and non-reacted monomers. Some quantity of the MIP was left without washing for the testing of possibility of controlling microorganisms growth by releasing of AHL in addition to its sequestering.

Inhibition experiments

The target for the present study was an N-acylated homoserine lactone (AHL) – namely DL- N(3-oxo-hexanoyl) homoserine lactone (3oC6-AHL) - the cognate signal molecule for the quorum sensing system regulating bioluminescence in *Vibrio fischeri*. Polymers were designed and synthesised at Cranfield and supplied to Kent as follows:

Polymer 1 – Blank polymer prepared without template
Polymer 2 – Polymer prepared with template and template removed
Polymer 3 – Polymer prepared with and still containing template

Template = 3oC6-AHL..

Aim

A preliminary study was undertaken to investigate whether

(i) Any of the polymers were able to sequester the signal molecule (3oC6-AHL) and attenuate bioluminescence *in vitro*.

(ii) Any of the polymers caused inhibition of growth to ensure that attenuation of bioluminescence was due to sequestration of signal molecule and not due to toxicity of polymer or non-specific binding of nutrients.

Methods.

V. fischeri ATCC 7744 was used throughout the study. A single colony was inoculated into NB + 2%NaCl and incubated overnight with reciprocal shaking at 200rpm and 25°C. For each of the incubations 0.25ml of an overnight culture was inoculated into 10ml NB + 2% NaCl containing, where required, 50 mg of the polymer. Medium containing polymer was left overnight at 4°C to maximise the opportunity for any non-

specific adsorption of medium components to occur. All cultures were incubated at 25°C in 15 ml Falcon tubes with constant inversion.

Results

Figure 9 shows the effect of polymers on growth.

Dataset for above

	Absorbance (595nm)							
Time	**C1**	**C2**	**1A**	**1B**	**2A**	**2B**	**3A**	**3B**
0	0	0	0	0	0	0	0	0
3	0.41	0.38	0.37	0.36	0.33	0.38	1.7	1.7
5	0.55	0.52	0.51	0.48	0.48	0.48	1.85	1.85
7	0.74	0.69	0.67	0.67	0.67	0.68	1.87	2
9	0.89	0.89	0.69	0.8	0.8	0.8	2.26	2.22
24	1.18	1.17	1.11	1.09	1.08	1.11	1.77	1.79

Figure 10 shows the effect of polymers on bioluminescence.

Dataset for figure 10:

Time (h)	Light							
	C1	**C2**	**1A**	**1B**	**2A**	**2B**	**3A**	**3B**
0	0	0	0	0	0	0	0	0
3	90	91	38	44	328	374	796	798
5	512	386	60	92	1730	1763	2942	2890
7	985	850	80	130	2786	3026	4766	4982
9	1430	1346	112	186	3468	3488	5706	5694
24	238	226	47	48	566	580	1873	1985

Following the conclusion of the experiment all cultures were plated on to NB + 2% NaCl to ensure no contaminants were present. All grew identically and no contaminants were visible after 96h incubation at 25°C.

Comments

(i) None of the polymers inhibited growth, whether by toxicity or sequestration of nutrients, to *V. fischeri.*

(ii) Polymer 3 dispersed much better in the medium giving rise to much higher background absorbancy in comparison to 1 & 2. It should be noted that the polymers alone did not give rise to any endogenous fluorescence.

(iii) Polymer 1 completely attenuated bioluminescence in *V. fischeri* and, as outlined in (i), this cannot be due to toxicity or non-specific adsorption of nutrients otherwise growth would have been inhibited. Therefore, it is reasoned that polymer 1 specifically sequesters the signal molecule – 3oC6-AHL.

(iv) To different degrees, 3oC6-AHL leaches out of polymers 2 and 3 giving rise to the early (within 3h) induction of bioluminescence in these cultures.

Activated charcoal was added to the *V. fischeri* cultures as outlined above for the polymers. Whilst no bioluminescence was shown it was demonstrated that no growth occurred – ie. activated charcoal was shown to be a non-specific adsorbant that sequestered nutrients causing inhibition of growth.

Claims

1. Polymer adapted to sequester a quorum sensing signalling molecule.
2. Use of a polymer according to claim 1 for the preparation of a medicament for the prevention or treatment of a bacterial infection which coordinates virulence by means of said quorum sensing molecule.
3. Use of a polymer according to claim 1 for the preparation of shaped articles.
4. Use of a polymer according to claim 1 for coating an article.
5. Article coated with a polymer according to claim 1.
6. Method of preparing a polymer according to claim 1 which comprises template directed polymerisation of functional monomers in the presence of the quorum sensing molecule or an analogue or derivative thereof, thereby producing a polymer formed from an array of said monomers, whereof at least part of said polymer is complementary to at least part of said quorum sensing signalling molecule.

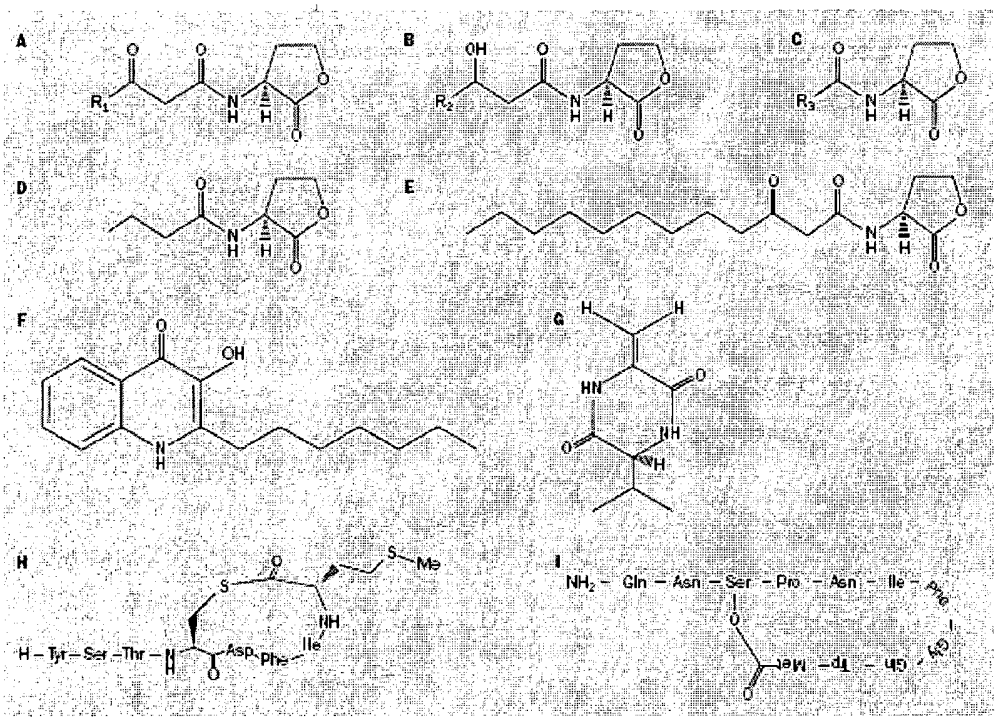


Figure 1. Structures of quorum sensing signal molecules. Generic structures for the N-acylhomoserine lactone family of signal molecules, produced by Gram-negative bacteria, with either (A) -oxo, (B) –hydroxy, or (C) no substituent at the 3 carbon position of the acyl side chain are shown. R=remaining number of carbons within the acyl side chain. For the P aeruginosa AHLs shown, N-butanoylhomoserine lactone (C4-AHL) (D) and N-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-AHL) (E), R3 is CH3(CH2)2 and R1 is CH3(CH2)8. The "pseudomonas quinolone signal olecule" (PQS), 2-heptyl-3-hydroxy-4-quinolone (F), and the diketopiperazine (DKP) cyclo(_Ala-L-Val) (G) are also produced by P aeruginosa. The quorum sensing peptides produced by the Gram-positive bacteria S aureus (Group I) (H) and E faecalis (the gelatinase biosynthesis-activating pheromone, GABP) (I) are also shown.

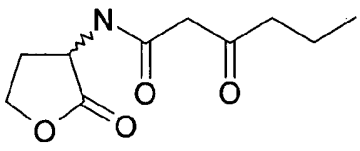


Figure 2 Structure of *N*-(3-oxo-hexanoyl)-DL-homoserine lactone (3oC6-AHL).

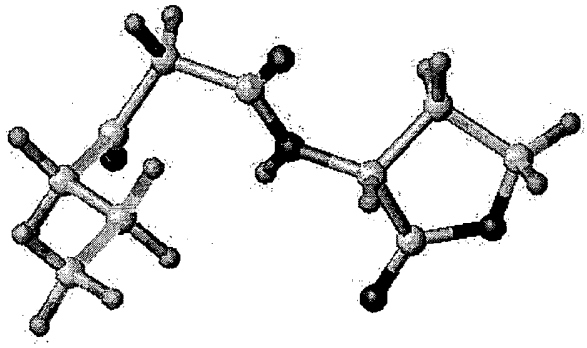


Figure 3: The minimised structure of 3oC6-AHL.

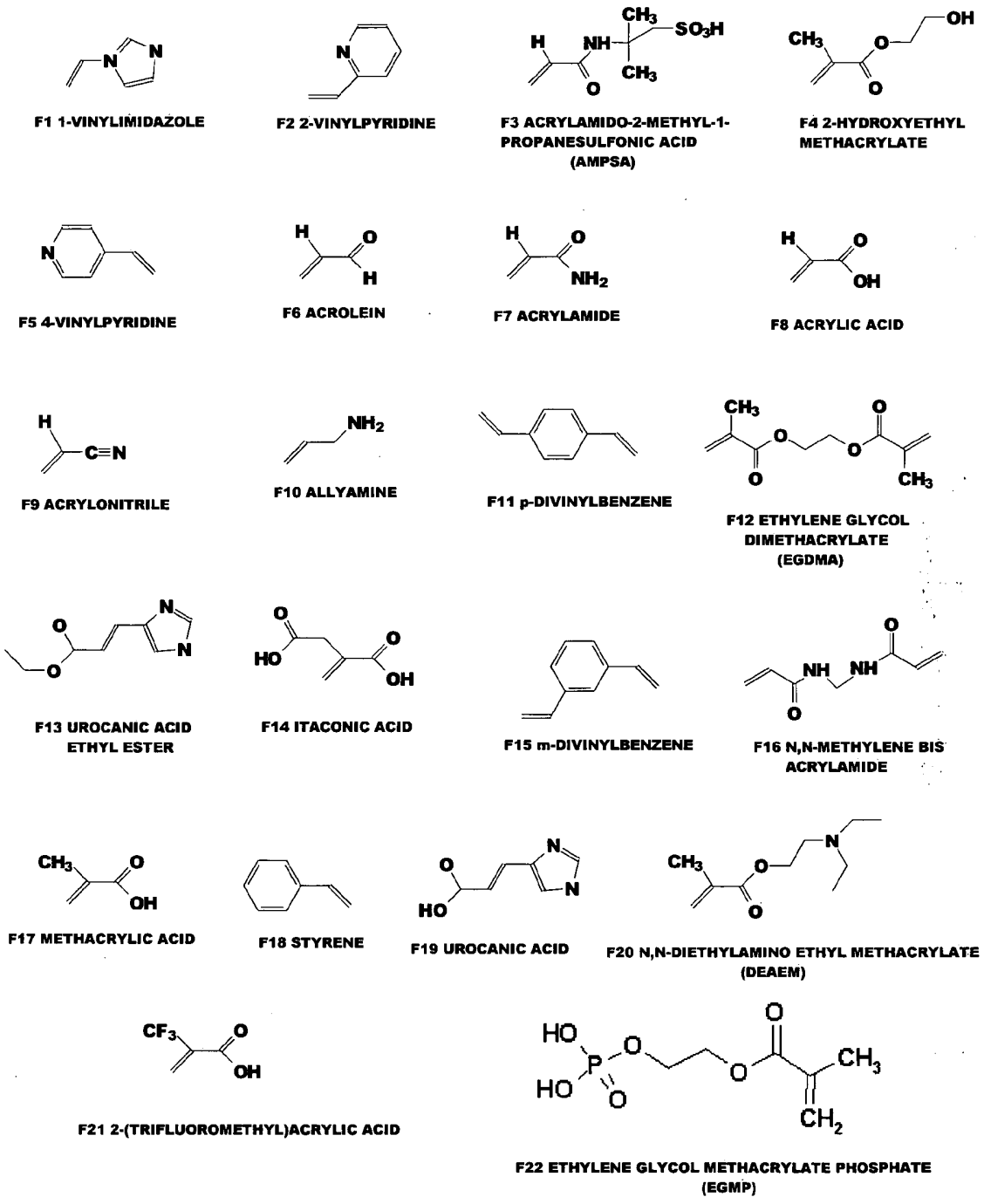


Figure 4 A virtual library of functional monomers.

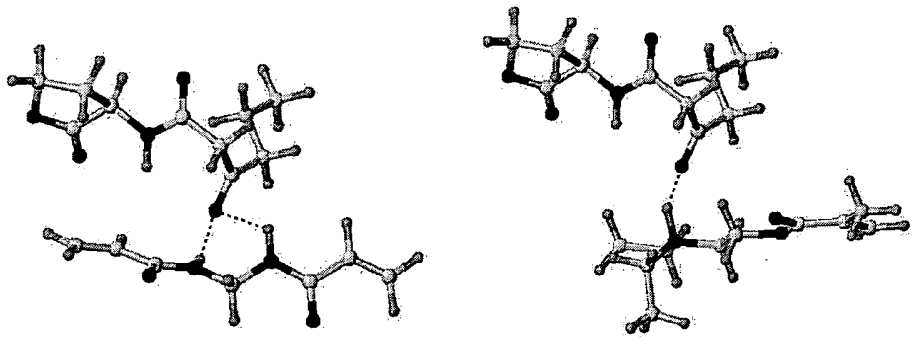


Figure 5 The complex of MBAA (left), DEAEM (right) with AHL template.

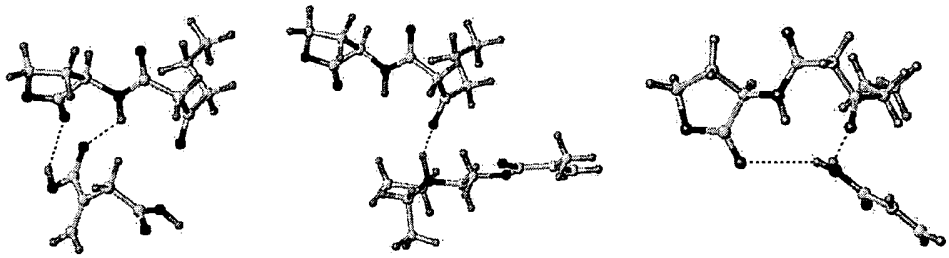


Figure 6 The complex of itaconic acid (left), EGMP (middle) and acrylamide (right) with 3oC6-AHL template.

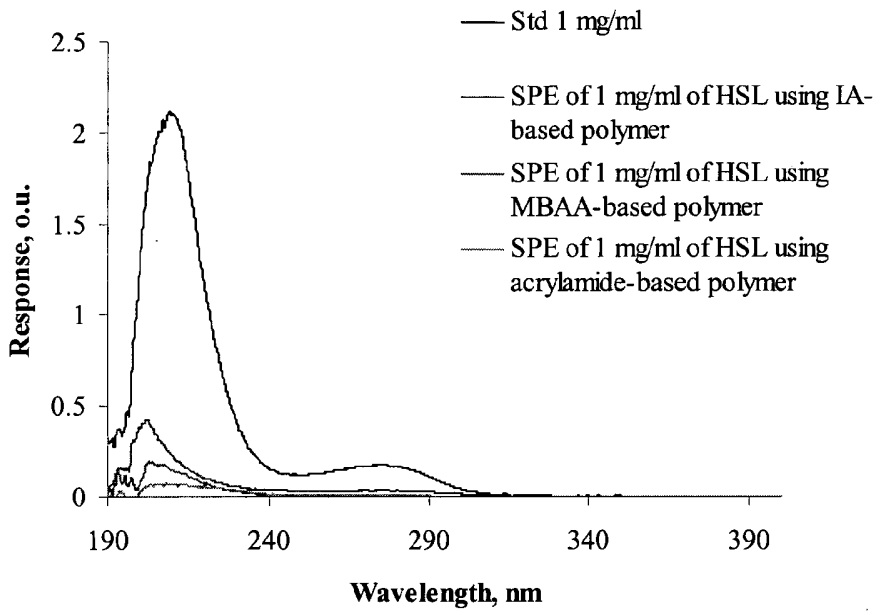


Figure 7 Screening of the polymers for affinity towards the 3oC6-AHL (HSL) (peak wavelength- λ= 201 nm).

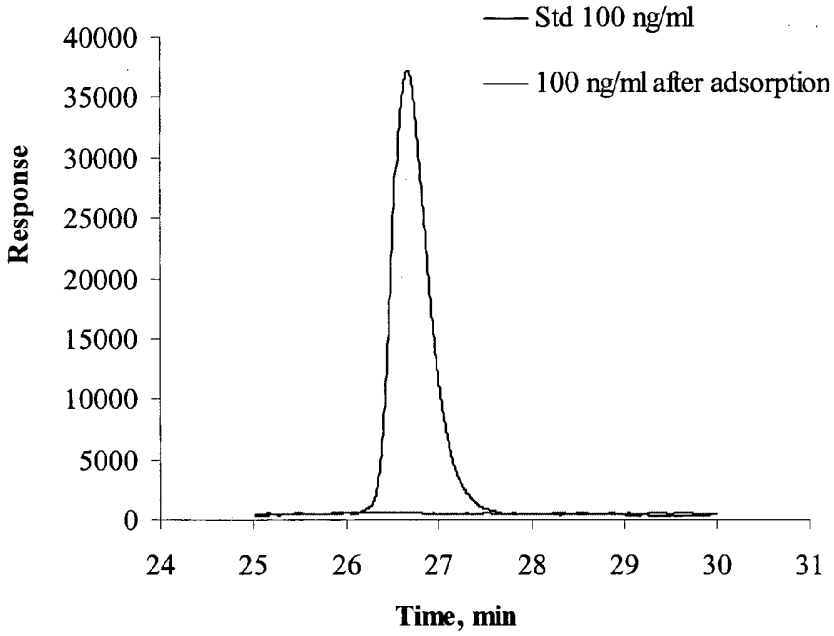
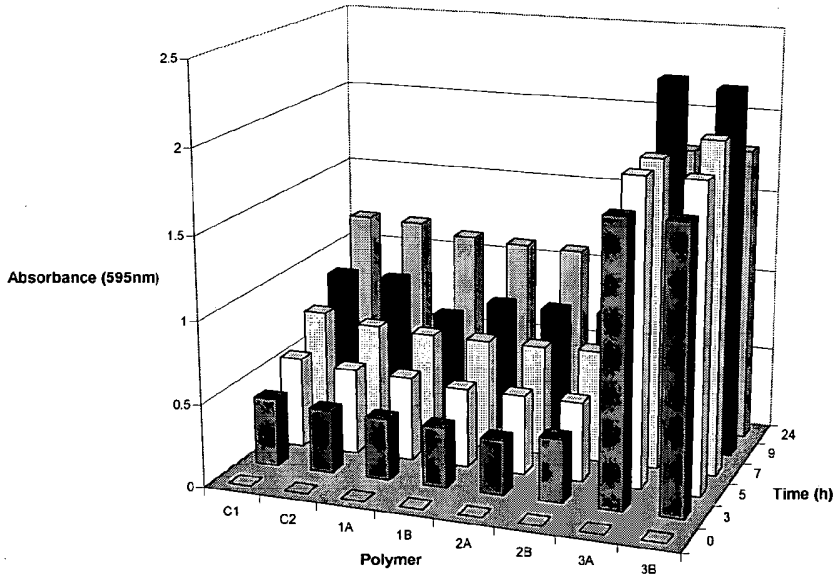
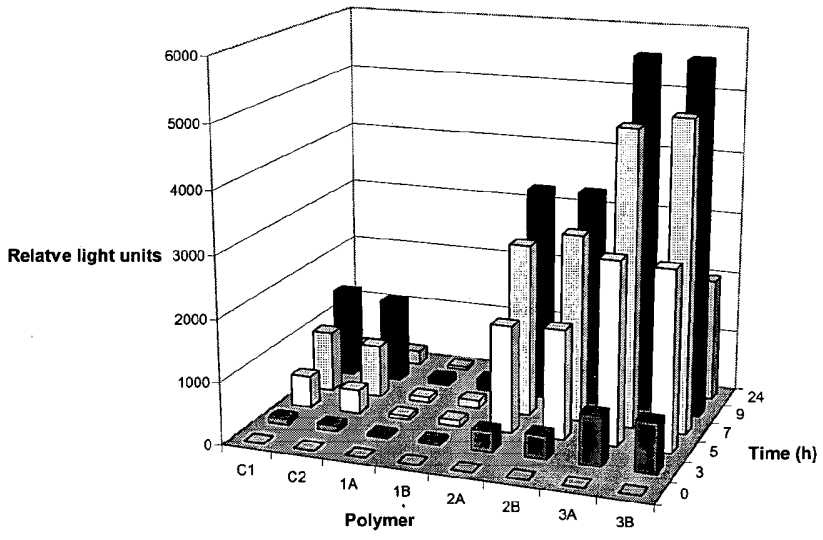


Figure 8 The chromatogram showing the complete adsorption of 100 ng of 3oC6-AHL by 100 mg of itaconic acid- based polymer.



Key – C1 & C2 are control duplicates in the absence of any polymer. Polymer 1,2 and 3 are as outlined above (see Inhibition Experiments) with each done in duplicate. Growth was monitored for 9 hours then left overnight to give 24h datapoint.

Figure 9 The effect of polymers on growth



Key – C1 & C2 are control duplicates in the absence of any polymer. Polymer 1,2 and 3 are as outlined above (see Inhibition Experiments) with each done in duplicate.

Figure 10 The effect of polymers on bioluminescence.