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Preparation and characterization of a clay–polyvinylpyridinium matrix for the removal of bacterial cells from water

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Polyvinylpyridinium salts were immobilized onto a clay matrix and were then tested for their antibacterial properties. The clay-polyvinylpyridinium matrix was prepared by the copolymerization of γ-methacryloxypropyltriethoxy silane bonded covalently to clay and 4-vinylpyridine and subsequent quaternization with benzyl halides. Suspension tests for antibacterial properties of the immobilized bactericide against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* demonstrated the high activity of the pyridinium salts that are bonded to the polyacrylate spacer. Advantageously, these insoluble clay-polymer bactericides could be applied without any contamination by the substrate.

1. Introduction

There has been considerable interest in recent years in the removal and destruction of microorganism from water during the disinfection process using both chemical and physical means. One route to remove these organic contaminants from the water is via adsorption techniques [1–3]. Unfortunately the use of soluble polymer biocides in watertreatment is hindered due to toxicity problems and the presence of a residual agent [4, 5]. This problem could be solved, if the bactericide were to be covalently bonded to the polymeric matrix or onto the surface of another insoluble material. Insoluble synthetic materials with antimicrobial activity have been used as insoluble disinfectants [6, 7]. The antimicrobial activity of cross-linked poly(vinylpyridinium halides) has been investigated by several research groups [8–10]. Several methods to develop a polymeric supporting material better able to remove microorganisms have been examined [11–14]. Recently, insoluble polymers based on cross-linked polystyrene–polyethyleneimine polymers [15, 16], on poly(glycidyl methacrylate)–tetra ethylene pentamine [17], on alumina supported poly(styrene-co-chloromethylstyrene) [18] and on sucrose methacrylate [19] have been investigated as potential media to remove bacteria from water. The adsorbed cells were found to be alive and could be cultured again after rinsing. The degree of cross-linking has a profound effect on the removal coefficient due to its effect on the swelling behaviour of the polymeric matrix in water [20]. In the case of cross-linked polymers carrying bactericides as pendant groups, the accessibility of the bactericidal groups as well as the swellability of the carrier in aqueous media are of great importance. The latter factor can be influenced by the type of spacer groups and the type and the cross-linking density of the polymer. The main objective of this research is the preparation of a novel clay-polymer matrix.

Many clay minerals have served as excellent solid supports. In our case bentonite clay, that has a natural abundance, ready availability and good swelling ability was chosen as the natural solid support to remove the organic pollutants from water. The preparation and characterization of a clay with accessible polyacrylate group was reported in our previous paper [21]. Using the appropriate combination of polyvinylpyridinium, spacer and clay, we were able to synthesize a novel clay-poly (acrylate-co-vinylpyridinium halides) in which the polymer was covalently bonded to the clay. The ability of clay-polyvinylpyridinium halides to capture bacterial cells depends on the chemical structure of the matrix. The degree of swelling was also found to enhance the capture of bacteria. In order to rationalize the effect of the structure of the insoluble pyridinium type clay polymer on the removal coefficient various types of polymers were used to remove *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aereus* from water. The removal coefficient proportionally increased with the pyridinium group content. This result indicated that the presence of clay-supported pyridinium salt sharply increased the removal coefficient of bacterial cells.

2. Experimental procedure

2.1. General methods

The following instruments were used to obtain spectral data; an At1 Unicam Mattson 1000 Fourier

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transform infra-red (FTIR) spectrometer, and a Mettler 2200 model Karl Fischer Coulometric Titrator. The differential thermal analysis (DTA) experiments described in this paper were performed on a Schimadzu Net Work System 50 instrument at a heating rate of 10 °C min^{-1} in an air atmosphere. The structural investigations performed on the composite materials were performed at room temperature using a Rigaku (System RadB) X-ray diffractometer equipped with a graphite monochromator and Mo radiation at a scan rate of 1 °C min^{-1}.

2.2. Chemicals

All the chemicals used in this work were supplied by Aldrich and they were used after proper purification. The 4-vinylpyridine was freshly distilled prior to use.

2.3. Hydrolysis of γ-methacryloxypropyl trimethoxysilane (A-174)

γ-methacryloxypropyl trimethoxysilane (97%, d = 1.05 g mL^{-1}, b.p. 98 °C/666.1 Pa, Aldrich) was used as received. The typical procedure for making up the sol-solution was as follows. A reaction mixture containing a solution of A-174 (2.48 g, 10 mmol) and isopropanol (26.4 g 440 mmol) was stirred for 2 h and then the mixture was hydrolysed with the required amount of water (1.35 mol H_2O per mol A-174) in isopropanol in the presence of an acid catalyst, hydrogen chloride (0.15 M) solution, which maintained the pH at 4. The required amount of water was determined by use of the Karl Fischer Coulometric Titrator and found to be 1.35 mol per silane. The mixture was stirred in the ambient atmosphere at room temperature until a clear sol solution was obtained. The hydrolyzate emitted methanol during the reaction which was removed under vacuum and characterized by gas chromatography. The solvent was removed via vacuum distillation and an oily residue was collected.

2.4. Preparation of polyacrylate-clay sol–gel materials

Bentonite from Çanakkale Clay Company (Turkey) was used in the reactions after it had been purified by sedimentation. The development of clay– A-174 materials using sol–gel techniques was carried out as a two-step process. Firstly, hydrolysed A-174 dissolved in tetrahydrofuran (15 ml) and distilled water (5 ml) was mixed with the clay in a closed vessel which was stirred overnight at room temperature. The pH of the solution was adjusted to 5.5 by bubbling CO_2 through it. A free radical copolymerization using 4-vinylpyridine was then performed in toluene at 80 °C with Azoisobutynonitrile (AIBN) (recrystallized from methanol) for 8 h to yield the polymer-clay composites in which the polyvinylpyridine (PVP) and polyacrylate were covalently bonded to the clay. The solid products were ground and subjected to further hydrolysis with the water. This was followed by condensation at 80 °C in the presence of acetic acid (25% solution) to produce a copolymer linked to the clay. The samples were then washed several times with dimethylformamide (DMF) in a Soxhlet apparatus to remove impurities. The samples were then dried in a vacuum oven at 75 °C for 12 h and at 100 °C for 24 h until the sample weight became essentially constant. The content of the vinylpyridine unit in the composite varied between 10–100%.

In order to obtain the clay matrix, a clay-A-174-PVP composition (5 g) was stirred with DMF (10 ml) at 50 °C, then benzylbromide or chloride (10 g, 0.045 mol) was added and the mixture was stirred and refluxed overnight. The product was filtered, repeatedly washed with water, ethanol, acetone and diethylether, and dried in a vacuum oven at 80 °C. The structure of the materials were investigated by Fourier transform infrared (FT-IR) spectroscopy differential thermal analysis (DTA) and X-ray diffraction (XRD).

2.5. Strains

Escherichia coli, ATCC 35218, *Pseudomonas aeruginosa*, USDA B771, and *Staphylococcus aureus*, ATTC 25293 were used in all the experiments. These organisms were subcultured every 3–4 weeks and routinely maintained on nutrient agar (Oxoid) at 4 °C.

2.6. Preparation of cell suspension

One loopful of each bacterium was inoculated into 10 ml of Luria Broth Base, Miller (LB) broth (Sigma) and incubated at 37 °C for 18–20 h. Then, the bacterial cells contained in 6.5 ml of the culture suspension were harvested by centrifugation and repeatedly washed with sterilized water. The collected cells were resuspended in 6.5 ml of sterilized water.

2.7. Contact of polymer with cells

All procedures were carried out under aseptic conditions. 100 mg of the clay-polymer matrix was placed in a 100 ml flask containing 19 ml of sterilized water. The matrix was wetted by agitation. Then 1 ml of the cell suspension prepared as previously described was transferred into the flask and agitated on a rotary shaker at 300 r.p.m. at 37 °C.

2.8. Measurement of the viable cell counts

The viable cell counts of the bacteria were measured using the surface spread plate technique, the disc diffusion technique and the agar dilution technique. Before sampling, the agitation of the contact suspension was stopped and the suspension was allowed to stand for 2–3 min until the clay matrix settled. 0.1 ml samples were taken from the flasks at each sampling time and serial dilutions were prepared with saline. 0.1 ml of the diluted sample was spread onto triplicate nutrient agar plates. The plates were incubated at 37 °C for 18–24 h and then the number of viable cells (colonies) were manually counted and the results were expressed as mean colony forming units per ml.

2.9. The content of pyridinium groups

The content of pyridinium groups in the matrix was determined in the following manner. The matrix was mixed for 1 h with an excess amount of 1 N sodium nitrate solution in order to convert the matrix from its halide form into a nitrate. Titration of the eluate with standard 0.1 N silver nitrate solutions, using eosin as an indicator, gave the amount of pyridinium groups contained in the matrix.

3. Results and discussion

The clay-polyvinylpyridinium halides were prepared (Fig. 1) by the condensation of γ-methacryloxypropyl trimethoxysilanols (A-174) with clay followed by copolymerization with 4-vinylpyridine in the presence of AIBN. The homopolymer and the divinylbenzene-cross-linked polyvinylpyridinium salts were prepared for use in comparative tests. Copolymerization of clay-A-174 with 4-vinylpyridine (VP) and subsequent quaternization with benzylbromide or chloride yielded the immobilized ammonium salts.

From theoretical considerations a monomolecular layer of A-174 on every clay particle is essential for optimal bonding. If the surface area of the clay and the specific wetting area of the A-174 are known then the quantity of the A-174 required for a monomolecular coating can be calculated from the following formula:

$$\text{Weight of (A-174)} = [\text{weight of clay} \times SA]/SW \quad (1)$$

Here, SA is the specific surface area of the clay in m^2g^{-1}, and SW is the specific wetting area of the A-174 in m^2g^{-1}. In practice, however, using the calculated quantity of silane does not achieve the optimum improvement in bonding. The most advantageous quantity can be either larger or smaller than the calculated value. The distribution of silane over the whole surface area of the clay is dependent on the activity of the surface and on the solvent used. We found that a rough value of the required quantity of silane was 0.2–1.5 wt % calculated on the clay. The quantity of silane used in the reaction was calculated to be: weight of (A-174) $= (5\text{ g} \times 7\text{ m}^2\text{g}^{-1}]/314\text{ m}^2\text{g}^{-1} = 0.11$ g.

To examine the effect of the structure of the insoluble clay-A-174-PVP matrix on the removal coefficient of bacteria showed that excellent antibacterial activity was obtained if the active pyridinium group content exceeds 0.9 mmol g^{-1}. Therefore BP-1 in which the active pyridinium group content is 1.05 mmol g^{-1} was chosen as a standard sample.

3.1. X-ray characterization

X-ray diffraction patterns of samples annealed at 200 °C measured on a Rigaku (system RandB) X-ray diffractometer equipped with a graphite monochromator and Mo radiation at a scan rate of 1 °C min^{-1} are presented in Fig. 2.

The patterns were indexed using the triclinic unit cell of bentonite and the results are listed in Table I. ($a = 0.5155$ nm, $b = 0.8959$ nm, $c = 0.7407$ nm,

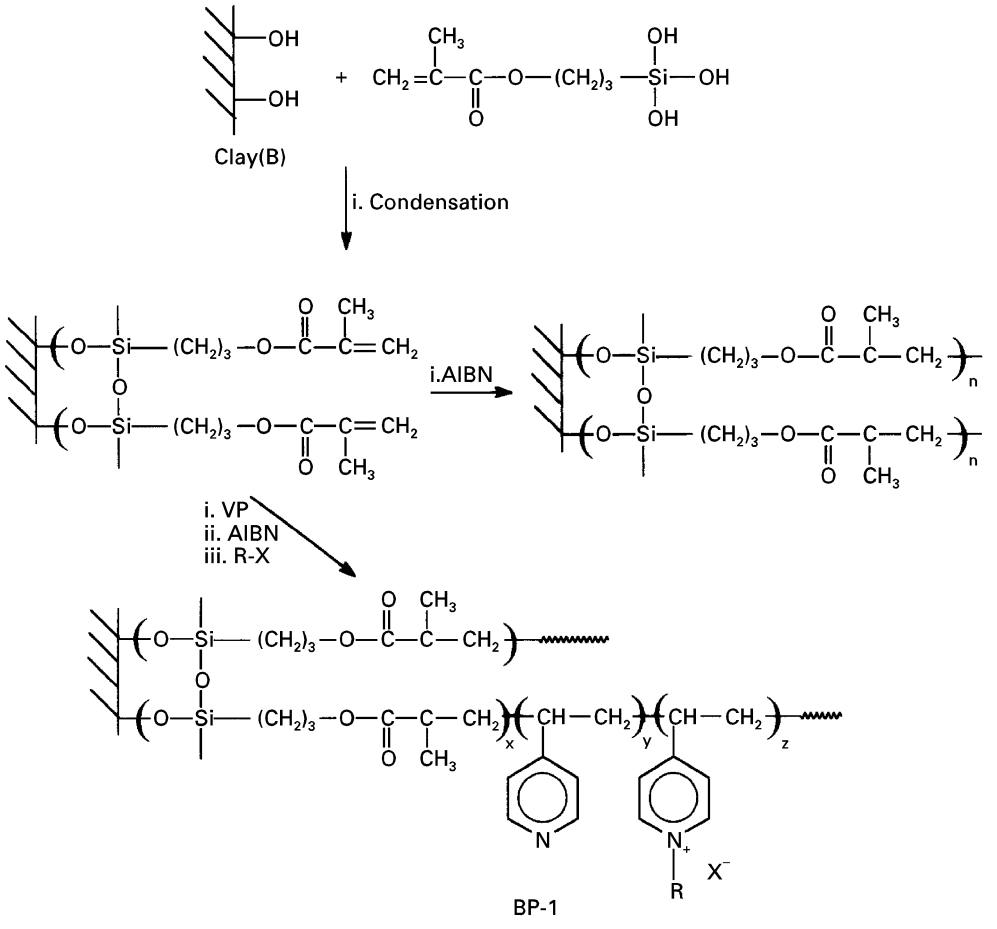

Figure 1 The synthetic route.

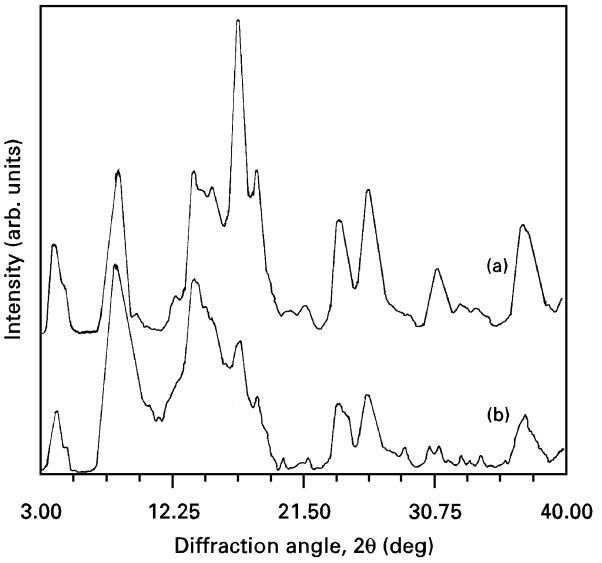

Figure 2 XRD diffraction patterns of the (a) bentonite (b) bentonite-A-174-VP.

$\alpha = 91.68^{\circ}$, $\beta = 104.87^{\circ}$, $\gamma = 89.94^{\circ}$) It is clear that A-174 has partially entered into the layer of bentonite, however covalent bonding did not occur with the atoms which form the 001 layer, instead covalent bonds were formed with the 002 layer atoms. Since the initial peak position does not change, then the unit cell dimension does not appear to be changed. Because of the highly symmetrical arrangement of the atoms in the layers and the relatively weak bonding between them, the layers can be displaced with respect to one another. The geometrical relationship between adjacent sheets of atoms in contact might remain the same, although the relationship between more distant atoms were changed.

The result of such imperfections is the absence of certain types of reflections. If for example, displacements occurred along both the *a* and *b* axes, the only *hkl* reflections that are possible are the basal (001) reflections.

The diffraction patterns of each sample prepared at different stoichiometry ratios were compared with the BP-1 and bentonite diffraction patterns. The crystallinity of the synthesized samples is probably a function of the interaction between PVP and Bentonite-A174 in the form of H-bonding between the –N groups of PVP and the surface hydroxyls of bentonite. Although the samples were not fully crystalline, diffraction peaks were observed in the measured XRD patterns displayed in Fig. 2.

To estimate the efficiency of polymer structure absorption onto bentonite, the X-ray patterns of samples containing different polymer: bentonite ratios were chosen. In general the determination of the bentonite group by XRD is simple, but the identification of the members of the group is more difficult. The prominent (001) and (002) basal reflections are usually sufficient for identification. It was evident that for BP-1 samples the (c) peak was no longer present which indicated that PVP was linked via covalent bonding to the layer that form (c) peaks. This linking did not take place with the (a) peak forming atoms that were present in all the examined samples. In all cases, the XRD patterns of the samples contained the characteristic peaks for bentonite, except for the (a) peak which was attributed to the non-bonding plane symmetry.

3.2. FT-IR characterization

It is well established in the literature that FT-IR spectra are sensitive to both structural and compositional variations in minerals. The absorption frequencies of bentonite, A-174-bentonite (Fig. 3) and A-174-bentonite-vinylpyridinium salts (Fig. 4) were measured. The absorption frequencies of bentonite at 3693, 3667 and 3623 cm^{-1} were attributed to –O–H bonds. The absorption frequency of –O–H bonds depends on the degree of association of these groups.

As a general rule, the –O–H groups that are an intrinsic part of the layered silicate structure and are weakly bonded to the silicate layer absorb in the frequency range of 3600–3700 cm^{-1}, whereas adsorbed water adsorbed IR radiation at the lower frequency of 3455 cm^{-1}. Bands at 3693, 3667 and 3623 cm^{-1} that remained even after a thermal treatment at 200 °C were correlated with structural –O–H

TABLE I Diffraction peak positions and indexing for Bentonite

No	Observed diffraction angle (deg)	Calculated diffraction angle (deg)	Interplanar spacing	I/I_0	*h*	*k*	*l*
1	5.70	5.69	7.183	27	0	0	1
2	9.97	9.89	4.077	100	1	1	1
3	11.37	11.40	3.580	66	0	0	2
4	12.16	12.17	3.346	87	0	1	2
5	14.30	14.22	2.865	34	1	2	1
6	16.31	16.34	2.506	46	1	3	1
7	17.44	17.47	2.346	39	1	3	1
8	20.55	20.58	1.991	29	1	3	2
9	22.45	22.52	1.816	30	2	2	3
10	24.53	24.56	1.666	34	2	4	0
11	26.54	26.53	1.545	31	1	1	4
12	27.55	27.54	1.491	29	1	5	2
13	29.86	29.87	1.380	29	0	4	4

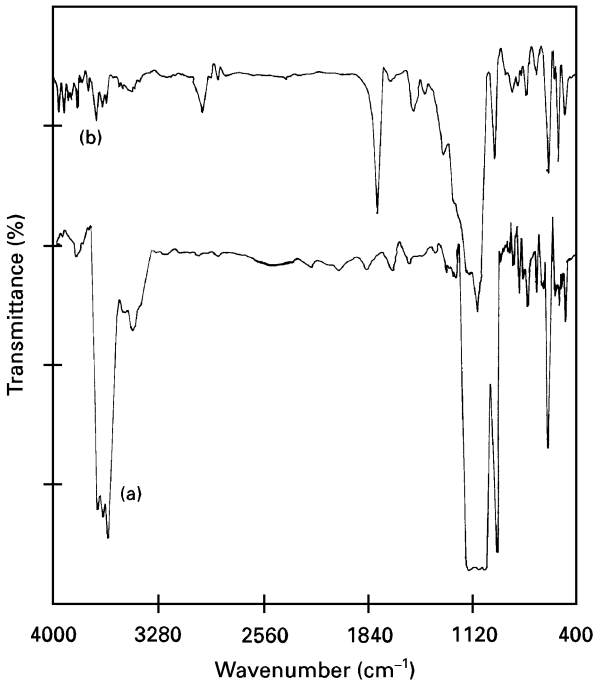

Figure 3 FT-IR spectra of (a) bentonite and (b) bentonite-A-174.

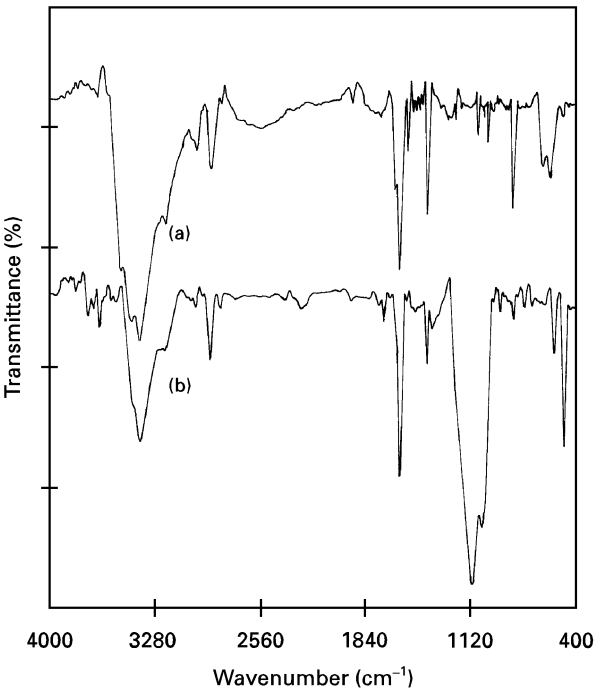

Figure 4 FT-IR spectra of (a) PVP and (b) BP-1 samples.

groups. The bands at 3623 and 3667 cm^{-1} were assigned to a bond between the basal hydroxyls of one sheet and the puckered oxygens of the next sheet, whilst the band at 3693 cm^{-1} was attributed to interlayer hydrogen bonding.

The sharp decrease in the –O–H frequency in sample BP-1 was attributed to covalent bonding between –O–H groups of the clay and the A-174. We do not claim that all the –O–H groups would be displaced however we note the sharp decrease in the peak associated with this type of bonding as shown in Fig. 1.

An FT-IR spectrum recorded on BP-1, after subtracting kaolinite as a reference, showed the presence of an Si–OH stretching frequency at 3700 cm^{-1}, an Si–O–Si frequency at 1041 cm^{-1} (broad), a peak at 1735 cm^{-1} that was attributed to C = O, peaks at 462 and 539 cm^{-1} were attributed to –Si–O–R, and finally –Si–O stretching frequencies at 919, 1041 and 1112 cm^{-1}.

3.3. DTA characterization

The DTA curves for bentonite and the BP-1 sample are presented in Fig. 5. Curves for bentonite were flat up to 511 °C showing a small water loss at 266 °C which was attributed to the loss of interlayer water. The size and the character of the DTA peaks depended on the nature of the adsorbed cation and on the pretreatment of the sample. Bentonite showed an intense endothermic peak starting at 461 °C and ending at 546 °C which was assigned to dehydration and to the loss of crystal structure, and also exothermic reactions due to the formation of new phases at elevated temperatures.

Organic materials usually show exothermic reactions when heated. It has been suggested in the literature that the nature of these reactions might be altered by interaction with the clay minerals. In the case of BP-1, two exotherms were observed. One was attributed to the glass transition temperature of the polymer, and the other was assigned to the melting of the organic matrix, a point that was verified by thermogravimetry, an approximately 40% weight loss occurred in this temperature region.

The DTA data can be correlated with the FT-IR spectra and XRD curves for B and BP-1 samples heated between 100–800 °C. The bands at 3600–3700 cm^{-1} assigned to –O–H groups in the lattice were seen to rapidly decrease in intensity between 500–600 °C, corresponding to the observed endotherm in the DTA curves. Coincident with the dehydration, a change in the spectra was evident in the 700–900 cm^{-1} region of the spectrum. In particular the Al–OH band at 910 cm^{-1} was lost, and the band

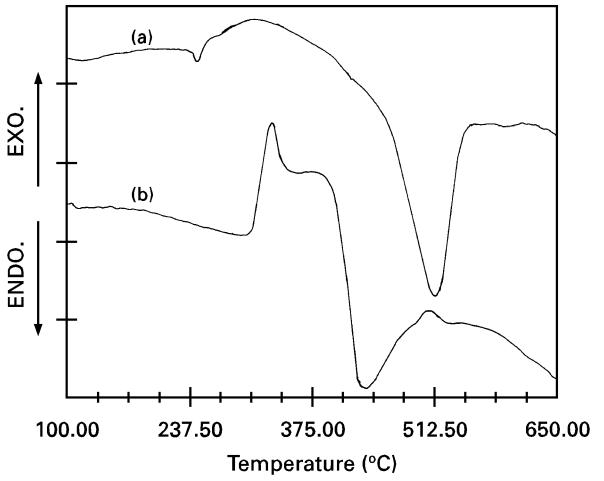

Figure 5 DTA curves of (a) bentonite and (b) BP-1 (Heating rate 10 °C min^{-1}).

at 1100 cm^{-1} broadened and merged into the main band at 1000 cm^{-1}. This change in the spectrum was due to a structural transformation.

3.4. Antibacterial activity of the insoluble bactericides

The antibacterial activity of the clay supported bactericides was tested by a suspension test, the disc diffusion technique and also the agar dilution technique. *Escherichia coli*, ATCC 35218, *Pseudomonas aeruginosa*, USDA B771, and *Staphylococcus aureus*, ATTC 25293 were used as the test organisms. The solid material to be investigated was suspended in water containing a bacteria culture of known bacterial counts. The suspension was shaken and samples were removed after different time intervals for count determination. During stirring at 37 °C without the matrix, there was no substantial change in the viable cell number of any of the test organisms. When the cells and the matrix were stirred together in water the viable cell number decreased. Figs 6 and 7 show that the viable cell number obviously decreased in the presence of the clay matrix. It should be noted that about 99–100% removal of the test organisms occurred in 0.08 h. In the many tests we performed during this investigation we always obtained a much higher absorption rate using the natural clay matrix.

We have also tested the bactericidial and bacteriostatic activity of the polymer using the disc diffusion technique, the agar dilution technique and also by spreading the polymer directly onto the bacterial culture. The effect of the clay matrix on the bacteria is listed in Table II.

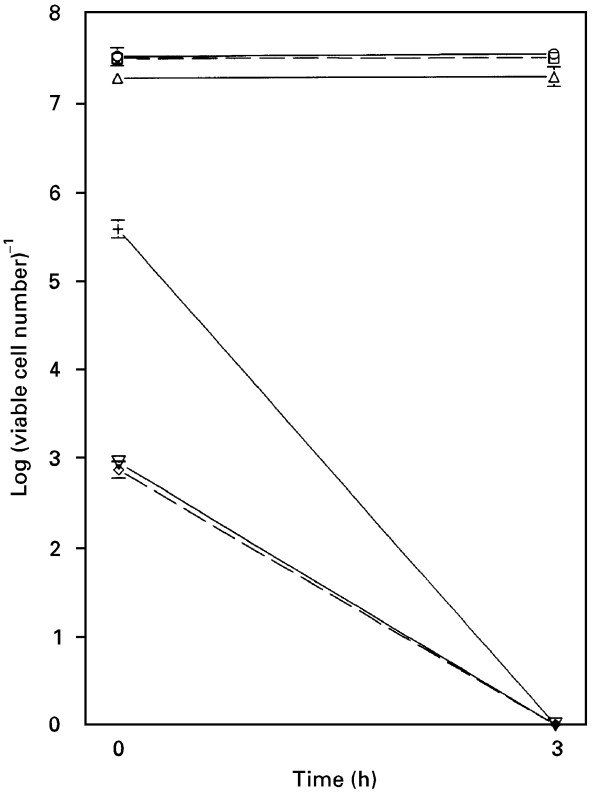

Figure 6 Removal of various bacteria by BP-1. Key: (○) *E. coli*, (△) *P. aeruginosa*, (□) *S. aureus* (all without the polymer) and (+) *E. coli*, (◇) *P. aeruginosa*, (▽) *S. aureus* (all with the polymer).

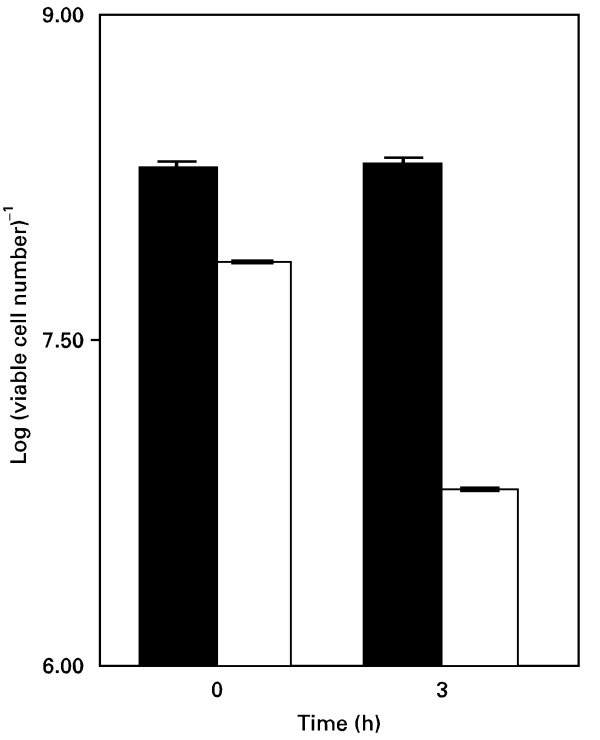

Figure 7 Removal of *E. coli* by PVP. Bars show the standard deviation of the mean.

TABLE II Effect of clay-polyvinylpyridinium matrix on the bacteria

Bacteria	Disc diffusion technique	Agar dilution technique	Spreading directly on bacteria
Escherichia coli	N	N	N
Pseudomonas aeruginosa	N	N	N
Staphylococcus aureus	N	N	N

N: No bactericidial and bacteriostatic activity (no inhibition of growth)

TABLE III Removal coefficients and removal percentage for *E. coli*, *P. aeruginosa* and *S. aureus* from aqueous media by the clay matrix

Bacteria	Initial viable cells (Cells per mL)	Removal coefficient (mL g^{-1} h)	Removal percentage after 3 h (%)
E. coli	3.43×10^7	47 684	100
S. aereus	1.9×10^7	106 310	100
P. aeruginosa	3.26×10^7	109 452	100

3.4.1. Removal coefficient

Table III lists the values of the removal coefficients obtained for the removal of various bacteria by the clay matrix. The removal coefficients were calculated using the following equation:

$$\text{Removal coefficient} = V/wt \log[N_0/N_t] \qquad (2)$$

where V is the volume of viable cell suspension, w is the dry weight of the matrix, t is the contact time, N_0 is the initial viable cell number (count) and N_t is viable cell number (count) after contact time t.

Another series of experiments were performed to elucidate if the clay matrix maintained the viability of the bacteria, or killed them on capture. A suspension of viable bacteria were passed through a glass column bed that was washed several times with saline. The polymer beads were then taken out and inoculated in a nutrient broth. The washing solution was also cultured, but no multiplication of the bacteria was observed in solution. However the clay matrix had captured the bacterial cells alive.

4. Conclusions

The results demonstrate that a pyridinium type polymer bonded to a clay containing a polyacrylate spacer group can be used to remove bacteria from contaminated water. It can be repeatedly used after rinsing without any contamination. The swelling of the clay in aqueous media, increases the effectiveness of the active groups. The investigation of the biocidal activity of a clay matrix containing active quaternary ammonium salts is in progress in our laboratory.

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