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ARTICLE TYPE

Antimicrobial surface grafted thermally responsive PNIPAM-co-ALA nano-gels

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Thermally responsive Poly(N-isopropylacrylamide) co-allylamine (PNIPAM-co-ALA) nano-gels were synthesised and grafted onto non-woven polypropylene. Silver nitrate was incorporated into the nano-gels in their expanded state and
10 **their antimicrobial properties tested. Bacterial growth was measured before and after the Lower Critical Solution Temperature. Below the LCST, bacteria grew, above bacterial growth was prevented or retarded.**

There is increasing interest in designing films that can respond
15 to their environment, with thermal, pH, ionic-strength and other effects all being probed as potential triggers for changes in a films morphology. One of the most well know systems is poly(N-isopropylacrylamide) (PNIPAM) – sodium acrylate co-polymers which shows a thermally induced morphological change at 34°C.[1]
20 Other co-polymers including allylamine (ALA) have been investigated, and it has been shown that the choice of co-polymer allows the LCST (lower critical solution temperature) of such polymer gel systems to be controlled.[2] Much of the interest in these systems has been in developing drug delivery vehicles for
25 anti-cancer and other drugs.[3]

Constructing antimicrobial surfaces using metals such as silver as an antimicrobial has been extensively investigated by many researchers over recent years,[4, 5] with many products containing supposed antimicrobial activity due to silver on the market. There
30 is a specific need to produce wound dressings which can fight infection and to this end a number of 'silver activated' dressings are on the market including Acticoat[TM] from Smith & Nephew and many others. The difficulty is that such dressings are not always effective and there are concerns that releasing silver into a
35 wound, especially a large wound such as a burn might delay healing.[6, 7] Once solution to at least some of these problems might be to design a thin film system that can be integrated into a dressing that only releases its antimicrobial payload following stimulation from either bacteria in an infected wound, their
40 secreted virulence factors, change in wound pH or temperature since this would keep the antimicrobial substance such as silver solution or other substance spatially separated from delicate tissue unless it was actually required.[8,9] PNIPAM has been suggested as a matrix for of antibiotics and antimicrobials, but we
45 have not found any discussion of the utilisation of the increased wound temperature as the 'trigger' for antimicrobial release using this type of chemistry.[10 11]

This communication describes the synthesis of thermally responsive PNIPAM-co-ALA nano-gels that have been loaded
50 with silver nitrate and grafted to non-woven polypropylene. This grafting approach is achieved via amide coupling of the free amines in the nano-gels to plasma deposited maleic anhydride.[12]

The hydrogel nano-gels were synthesised by precipitation polymerisation. *N*-isopropylacrylamide, allylamine (10% molar
55 ratio), ethylene-glycol diacrylate (cross linker 1% molar ratio) were dissolved in 115 ml deionized water along with 37 mg of sodium dodecylsulfate (SDS) as a surfactant, freeze thawed three times and bubbled with N_2. Following removal of oxygen sodium persulfate in water was added as an initiator and the solution
60 heated to 70°C for 4 hours. The gels were then purified by dialysis in deionized water for 7 days with a yield of 31%. Silver nitrate was incorporated into the nano-gels by adding to a solution of 50 mmol dm^{-3} silver nitrate, then heating to 50°C and then cooling to 20°C before re-swelling in the silver nitrate
65 solution for 4 hours. The excess silver solution was removed by filter centrifugation 15 minutes at 10,000 rpm then washed in deionized water before a final re-centrifuge. The nano-gel size and LCST was measured using dynamic light scattering (Malvern Instruments), and found to be 220 nm +/- 10 nm below 34°C and
70 72 nm +/- 12 nm at 37°C and above (figure 1), as the polymer de-swelled with increasing temperature.
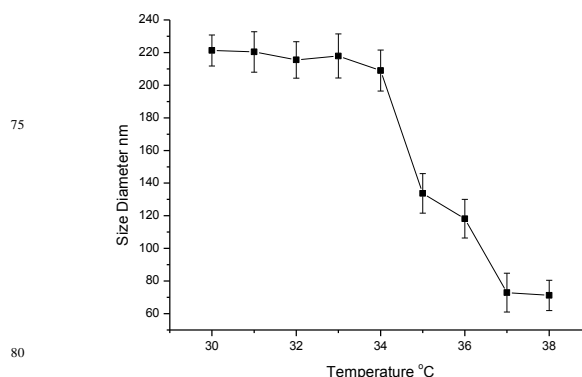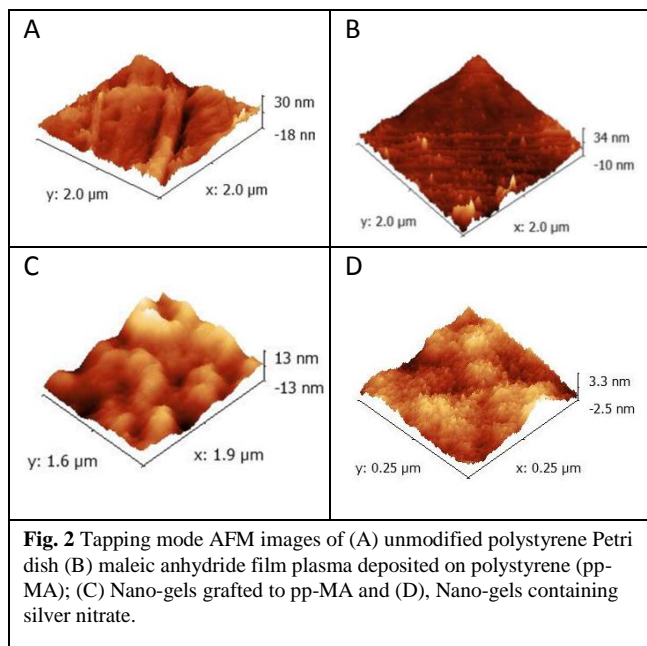

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Fig.1 Diameter of nano-gels containing silver nitrate measured as a function of temperature. LCST at 34.5°C.

In order to attach the nano-gels to non-woven polypropylene and polystyrene Petri dishes, the fabric were washed in
85 isopropanol for 5 minutes and dried before placing in a lab built 13.56 MHz plasma reactor.[13] Approximately 100 mg of maleic anhydride powder (Sigma) was degassed by freeze–thaw action under reduced pressure. All plasma reactions were run at 50W, using a pulsed square wave: 1   msec on, 40 msec off in order to
90 create a coherent film, but retain anhydride group functionality in the film. Immediately following formation of the maleic anhydride film (pp-MA) on fabric / polystyrene, the PNIPAM-ALA nano-gels containing silver nitrate were grafted to the pp-MA via amine nucleophilic attack from the AA to the anhydride
95 group on the film, forming amide linkages. The nano-gels were

left for 24 hours to graft before washing to remove excess material. Tapping mode Atomic Force Microscopy and TEM was used to image the gels on polystyrene pp-MA coated Petri dishes (AFM) and coated copper grids (TEM see ESI). FT-IR was used to follow the surface modification at each stage and shows clearly the maleic anhydride on the fabrics after plasma polymerisation with a peak 1785cm⁻¹ corresponding to the C=O) (ESI). When the nano-gels were grafted, the free amines react with the carbonyl functional groups to form the amide and the peak shifts to 1644 cm⁻¹.

Fig. 2 Tapping mode AFM images of (A) unmodified polystyrene Petri dish (B) maleic anhydride film plasma deposited on polystyrene (pp-MA); (C) Nano-gels grafted to pp-MA and (D), Nano-gels containing silver nitrate.

AFM of the unmodified polystyrene dish (figure 2A); pp-MA coated polystyrene dishes (figure 2B); the grafted nano-gels without silver nitrate in their dry state (figure 2 C) and the nano-gels containing silver nitrate (figure 2D) show the morphology of the grafted gels compared with the substrate. The image resolution was limited by the AFM tip diameter of 10 nm. Globular gel structures can be observed on the surface and a change in morphology on adding silver nitrate, probably due to a degree of silver nanoparticle formation within the gel following silver reduction, although this was not quantified. Measurements made in the gels expanded phase at ca. 24° C.

The antimicrobial properties and the temperature dependant release were tested against two clinically relevant strains of bacteria, *S. aureus* MSSA476 and *P. aeruginosa* PAO1 against both the silver nano-gels in solution and the grafted silver nano-gels. In each microbiology assay the bacteria was taken from an overnight culture grown in LB media, optical density at 600 nm was measured and the required starting inoculums prepared via dilutions in LB media. The growth of the bacteria in the presence of the silver nano-gels was assessed at two different temperatures one below the LCST at 28°C and one above the LCST at 37°C. Starting cultures of $1x10^3$ CFU/ml were added to a solution of silver nano-gels and either incubated at 28°C or 37°C. Every hour a sample was taken and a dilution series plated out on agar Petri dishes every hour for a period of 5 hours. The plates were incubated over night at 37°C and the colonies with all measurements performed in triplicate. For both *S. aureus* and *P. aeruginosa* there is a reduction in the growth of bacteria in the presence of the silver nano-gels at 37 °C compared to the control. However at 28°C there is (virtually) no difference in the growth

of bacteria after 5 hours, although the total growth was lower than the control at 37°C as both *S. aureus* and *P. aeruginosa* have evolved to grow most efficiently at this higher temperature (figure 3). Controls were grafted nano-gels which did not contain silver nitrate.
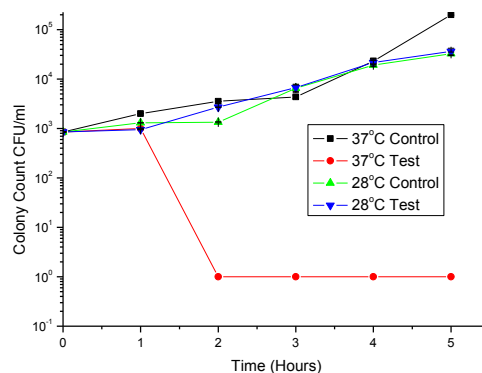
(A)   Test system with *Pseudomonas aeruginosa* PAO1
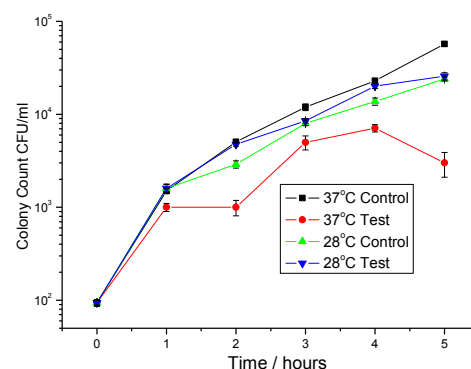
(B)   Test System with *Staphylococcus aureus* MSSA 476

Fig. 3: Real time measure of bacterial growth of (A) *P. aeruginosa* PAO1 and (B) *S. aureus* MSSA 476 with silver nano-gel (Test) and Control at 28° C and 37° C. Note, no error bars on 37°C test system for *P. aeruginosa* as no colonies were actually observed, suggesting complete killing on all replicates.

The antimicrobial properties of the silver nano-gels grafted to fabrics were also tested using a ring of inhibition type assay against both strains. *S. aureus* and *P. aeruginosa* at $1x10^8$ CF ml⁻¹ was added to an agar Petri dish and spread over the surface to create a lawn of bacteria. 3x3cm sized fabrics were placed onto the surface of the bacteria and left overnight (18 h) either at 37°C or 28°C (figure 4). At 37°C the fabrics show greater clearing of bacteria than the control as expected, however at 28°C no clearing was observed.

The results discussed in this communication suggest that utilising the much studied thermal responsive swelling / collapse of PNIPAM-co-AA copolymer might be an effective way of adding functionality to wound dressing systems where an antimicrobial response *only* subsequent to infection is required. The antimicrobial release trigger here being the fact that infected wounds / skin have a significantly elevated temperature compared

with healthy surrounding tissue, with a 2010 study of infected leg ulcers showing a mean temperature elevation of 3.6 °C compared with healthy skin 32˚ C.[14]
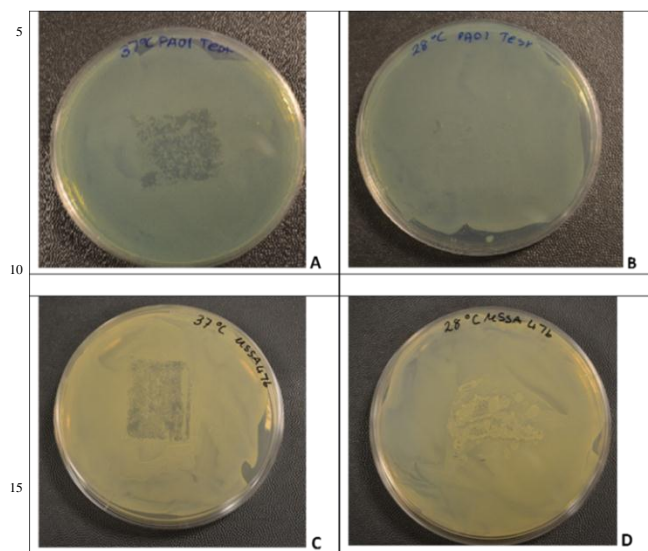

Fig.4 Bacterial lawns of *P. aeruginosa* grown with silver nano-gel containing fabric at 37°C (A) and 28 °C (B). *S. aureus* at 37°C (C) and 28 °C (D).

These findings suggest that thermal triggering may have utility in designing infection responsive wound dressings, and that the utility of well-known PNIPAM based polymers may be even greater than previously considered.

Notes and references

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