



# Speciality Chemicals Magazine

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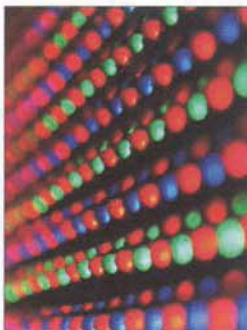
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## 28 Lights, camera, action



## 56 Catalan Chemspec

### In the next issues of Speciality Chemicals Magazine

- June**
- Outsourcing
  - Biotechnology
  - Electronic chemicals
  - Chiral chemistry
  - Chemspec Europe 2009 Show Preview & Edition
  - REACH special publication

- July/August**
- Pigments & colours
  - Catalysts
  - Separation technology
  - Low temperature chemistry
  - Outsourcing Special Publication

### Regulars

<b>Viewpoint</b>	<b>4</b>
<b>News</b>	<b>6</b>
<b>Forthcoming events</b>	<b>58</b>

### Pharmaceutical intermediates

<b>Fortress pharma</b>	<b>18</b>
One of the API sector's newer players has quietly emerged as a global contender. Andrew Warmington spoke with Aesica Pharmaceuticals	
<b>OxaPEGylation technology for API properties</b>	<b>22</b>
In the second of two articles, Dr Andreas Meudt and Dr Sebastian Würtz of Archimica show how oxa acids can be used in pharmaceutical applications	

### Contract research & toxicology

<b>The '3Rs' in reproduction &amp; developmental toxicity studies</b>	<b>24</b>
Ing. Chris de Ries of Notox gives an overview of current developments in regulatory reproductive toxicity assessment	
<b>Selecting a CMO: Criteria to consider</b>	<b>26</b>
Ed Price of PCI Synthesis looks at what an emerging pharma company should look for in CROs and CMOs	

### Sustainability

<b>Turn on, tune in</b>	<b>28</b>
Many chemicals firms are innovating for sustainability in batteries, insulation and lighting. Elisabeth Jeffries reports	
<b>Sustainable chemicals from renewable sources</b>	<b>32</b>
Mark J. Burk of Genomatica explores the meaning of sustainable chemistry and explains the firm's recent progress in the field	
<b>It's not just about us...</b>	<b>36</b>
Peter Cartwright of Dow Corning discusses what sustainability means to the company and the wider speciality chemicals industry	

### Peptides & proteins

<b>Peptide City USA</b>	<b>40</b>
A small city in Southern California is the home of most contract manufacturing of therapeutic peptides in the US. Andrew Warmington visited Bachem, Peptisyntha and the PolyPeptide Group in Torrance	
<b>Peptide antimicrobials</b>	<b>46</b>
Juan Carlo Carvajal of Reactive Surfaces introduces the ProteCoat technology	

### Organosulphur chemistry

<b>The chemistry of DMSO: 2007 in review</b>	<b>50</b>
Dr Artie McKim and Dr George Kvakovszky of Gaylord Chemical survey the chemical literature of 2007 with regard to dimethyl sulphoxide	

### Chemspec India review

<b>Go fourth to Mumbai</b>	<b>54</b>
Chemspec India returned in style in April. We report from the show floor	

### Chemspec Europe preview

<b>Chemspec Europe heads to Spain</b>	<b>56</b>
The first ever Chemspec Europe in Southern Europe is next month	

# Peptide antimicrobials

In the first of two articles, **Juan Carlo Carvajal** of **Reactive Surfaces** introduces the ProteCoat technology

One of the most promising new concepts in antimicrobial technology is the use of peptides as biopesticides. Such peptides have the potential to combine the environmentally friendly nature of biopesticides with the fast-acting and broad-spectrum nature of the more traditional chemical pesticides.

It is this potential that Reactive Surfaces has tapped in order to develop a new generation of antimicrobial additives for coatings with its ProteCoat product line. ProteCoat additives are efficacious biocides, which can be used alone or synergistically in combination with existing biocides to kill fungi and bacteria effectively, including the spore stages of some.<sup>1</sup>

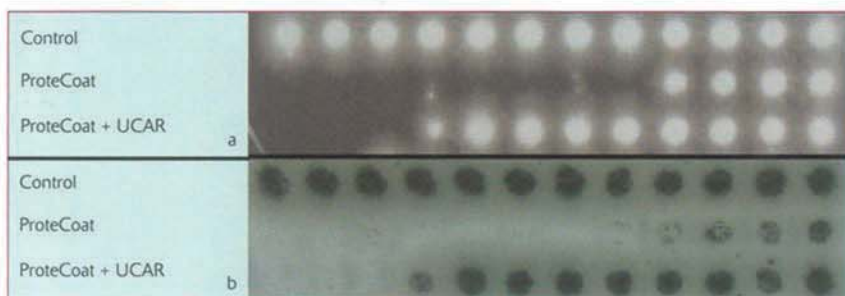
ProteCoat is a broad spectrum antimicrobial additive based on the emerging antimicrobial peptide (AMP) technology. We believe that it offers substantial advantages over the traditional biocidal and biostatic chemicals that are currently used in coatings.

ProteCoat additives are safe and biodegradable, work under mild conditions and are not associated with the production or release of hazardous compounds into the environment. As such, they bring a new technology to bear on the problem of biocides for coatings, offering protection without the adverse human health and environmental impact often associated with the biocides currently being used.

## Biological approach

The emergence of resistance to commonly used antibiotics and biocides has stimulated the search for new, naturally occurring bactericidal and fungicidal agents. AMPs are ubiquitous molecules that are found in most plants and animals. They represent the first line of defence against pathogens in living systems.

Built upon AMP technology, ProteCoat additives are designed to function in minute quantities against the vegetative and spore stages of target microorganisms. As with chemical biocides, the mode of action of is compatible with the components of the coating, such that the desired biocidal



**Figure 1 - Biocidal activity of ProteCoat challenged with fungal spores of *F. oxysporum* (a) & *A. nidulans* (b)**

activity is achieved. In addition, it was considered crucial that ProteCoat additives should not only work alone but should also work synergistically with existing biocides and other antimicrobial components of coatings.

Antimicrobial coating additives such as ProteCoat must exhibit four key properties, all of which the peptides used in ProteCoat achieve:

- Selective toxicity to discriminate between target microbial cells and non-target organisms
- Rapid killing, as the time needed for killing should be shorter than the doubling time of the target microorganism
- Effectiveness against a broad range of microorganisms
- A mechanism of action such that the target microbes cannot easily develop resistance<sup>2</sup>

More generally, in order to provide for selective toxicity and broad spectrum activity, ProteCoat additives are designed to target those features which are ubiquitous among microbial cells but do not exhibit toxic effects or cytotoxicity to mammalian cells. For rapid killing, the site of action targets the cell surface rather than the cell interior. ProteCoat can also affect the target membrane, even in the presence of a spore coat or another protective envelope.

## Broad-spectrum & selective

The microorganisms responsible for paint spoilage are not unique. Indeed, almost the whole spectrum of fungi, bacteria and yeast thrives in the coating environment, so efficient biocides must have broad-spectrum antimicrobial efficacy to offer the optimum preservation, as well as being selective. That ProteCoat achieves this can be attributed its ability to target the unique characteristics of the outer membranes of microbes.

Unlike multi-cellular animals, the membranes of microbes are organised in such a way that the outer region of the lipid bilayer, the region exposed to the environment, is composed of lipids with negatively charged groups. All AMPs share a common three-dimensional arrangement: they fold into molecules with one hydrophobic face and one charged face.

The mechanism of this class of AMPs begins with peptide-membrane interaction, followed by the displacement of lipids and the alteration of the membrane structure. Primarily due to this mode of action, ProteCoat can compromise a broad spectrum of microbes (Table 1).

The testing procedures developed include a minimal inhibitory concentration (MIC) comparison against a panel of microorganisms in broth, as well as with Ucar 451 from Dow Chemical. This is a styrene-acrylic emulsion polymer for coat-

**Table 1 - Minimum inhibitory concentrations of ProteCoat with micro-organisms**

Microorganisms	Host	Trait	MICs (ppm)*
<b>Bacteria</b>			
<i>Staphylococcus aureus</i>	Human pathogen	Gram-positive	79
<i>Enterococcus faecalis</i>	Human pathogen	Gram-positive	48
<i>Escherichia coli</i>	Human pathogen	Gram-negative	40
<i>Erwinia amylovora</i>	Plant pathogen	Gram-negative	20
<i>Erwinia carotovora</i>	Plant pathogen	Gram-negative	39
<i>Ralstonia solanacearum</i>	Plant pathogen	Gram-negative	44
<i>Bacillus atrophaeus</i>	Test strain	Gram-positive	5
<b>Fungi</b>			
<i>Aspergillus fumigatus</i>	Human pathogen	Aspergillosis	18
<i>Aspergillus parasiticus</i>	Human pathogen	Aflatoxin producer	64
<i>Ceratocystis fagacearum</i>	Plant pathogen	Oak wilt	13
<i>Fusarium oxysporum</i>	Plant & human pathogen	Opportunistic pathogen	14
<i>Fusarium sambucinum</i>	Plant pathogen	Dry rot	6
<i>Magaporthe grisea</i>	Plant pathogen	Rice blast	30
<i>Ophiostoma ulmi</i>	Plant pathogen	Dutch elm disease	8
<i>Rhizoctonia solani</i>	Plant pathogen	Wilt producing	39

\* - The peptide was challenged with 104 fungal spores/ml or 106 bacterial spores/ml

ings applications that has hydroxyl and carboxylic acid functional groups and a suitably high (45°C) glass transition temperature (T<sub>g</sub>).

Growth inhibition was determined by replicating each plate onto the appropriate growth media, followed by incubation for at least 24 hours, in the case of *Bacillus atrophaeus*, a gram-positive, spore-forming bacterium that is a common, less dangerous surrogate of *Bacillus anthracis*, the causative agent for anthrax.

Two fungi were incubated for 48-72 hours. These were: *Aspergillus nidulans*, a model organism and a less dangerous surrogate of *Aspergillus fumigatus*, a potentially deadly human pathogen and a major allergen; and, *Fusarium oxysporum*, the causal agent of vascular wilt disease in plants and an emerging opportunistic human pathogen.<sup>3</sup>

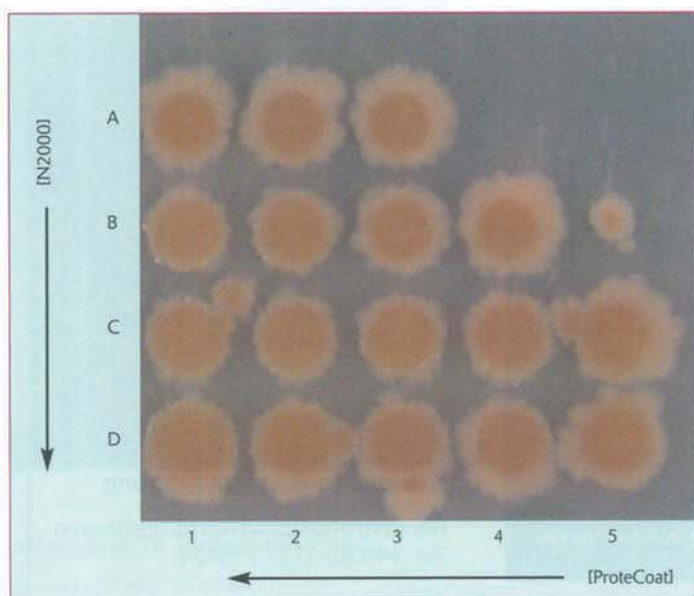
All three micro-organisms were tested in two environments: a broth in which the peptide was delivered and challenged with spores in a solution and when the peptide was formulated with the Ucar 451 latex, which was then painted onto the bottom of each well and subsequently challenged with spores delivered in a solution. In each case, MICs were determined by exposure to 10<sup>7</sup> spores/ml for each microorganism and two or three independent replicates for each.

The broth MIC was 59 ppm for *B. atrophaeus* and 63 for both fungi. The Ucar 451 MIC was not determined for *B. atrophaeus*; for both fungi, it was 625 ppm. Thus, the anticipated increase in MICs as ProteCoat was admixed with the coating did take place, significant antimicrobial biocidal activity against each of the target microorganisms was observed.

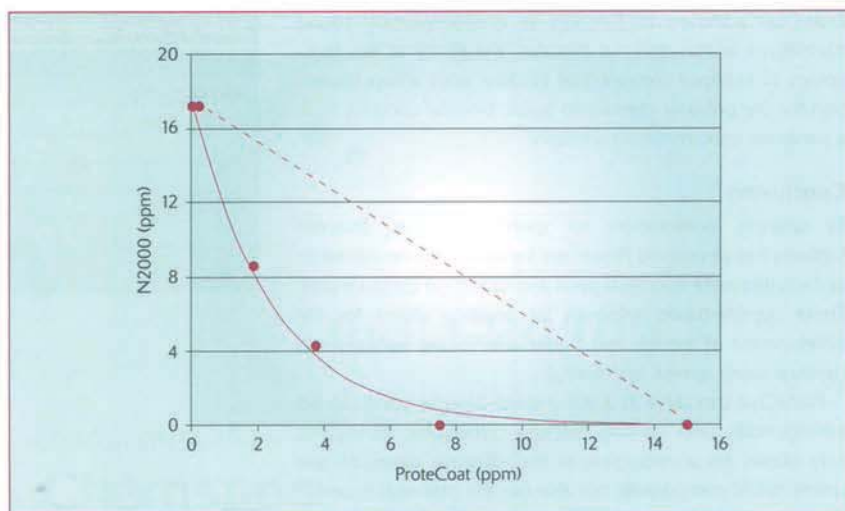
Figure 1 demonstrates the biocidal activity of ProteCoat alone and mixed with Ucar 451, compared with a control. In all cases, ProteCoat was challenged with 2 x 10<sup>6</sup> spores in a solution. The second and third rows are representative of a two-fold dilution series of ProteCoat, with an initial concentration of 1,250 ppm in the far left well, for three independent experiments. These results show that ProteCoat as a stand-alone antimicrobial additive in cured films exhibits broad-spectrum killing of both fungal spores and vegetative cells.

### Synergism with conventional biocides

The ability of AMPs to compromise the integrity of the cellular membrane raised the possibility that, in addition to rapid killing, the peptides in small amounts might have a synergistic effect on other biocides, potentially by increasing their



**Figure 2 - Synergism between ProteCoat & Verichem N2000 against *B. atrophaeus***



**Figure 3 - Synergism between ProteCoat & Verichem N2000 against *B. atrophaeus***

effective permeability. Biocide synergism experiments were conducted with three broad-spectrum biocides commonly used in waterborne coatings, augmented with ProteCoat.

These were: Rohm & Haas's Kathon LX 1.5% (2-methyl-4-isothiazolin-3-one), a common in-can preservative; Troy's Polyphase (3-iodo-2-propynyl butyl-carbamate), a common after-application iodo-type additive; and, Verichem N2000 (dodecylguanidine) from Verichem Products, which provides in-can and in-solution (e.g. in water cooling facilities) control against algae, bacteria, fungi and yeasts.

Each was used singularly in combination with ProteCoat. A two-dimensional, 'checkerboard' microdilution technique was used to characterise the interactions between ProteCoat and each of them.<sup>5</sup>

The term 'checkerboard' refers to the pattern of microtitre wells formed by multiple dilutions of the two biocides being tested in concentrations equal to, above and below their respective MIC. It consists of columns in which each well contains the same concentration of biocide A, being diluted along the x-axis, and rows in which each well contains the same concentration of biocide B, being diluted along the y-axis.

Figure 2 illustrates this for Verichem N2000 against *B. atrophaeus*. In this, the top right hand corner represents 100% growth inhibition by the biocides and the direction of the arrows indicates decreasing concentrations of each.

Biocide combinations were challenged with 2 x 10<sup>6</sup> spores and replicated after 24 hours on growth media. As can be seen, there are two dilutions along each axis, representing an approximately 3.5 to four-fold increase in efficacy of both N2000 (wells 5A and 5B) and ProteCoat (wells 4B and 5B).

Figure 3 shows the synergistic activity of the combined biocides in an isobologram, allowing for comparison with results expected in non-synergistic activity. On this figure, solid circles indicated the observed values and the solid line represents the best fit ( $R^2 = 0.978$ ), while the dotted line demonstrates what would be expected for an additive effect.

Table 2 shows the experimentally determined growth inhibition at a given biocide or ProteCoat concentration in percentage terms.  $E_o$ , or the expected percent growth inhibition for a 'non-synergistic' or additive combination, was calculated as previously described.<sup>5</sup> The observed percent growth inhibition for each combination is provided in the  $E_o$  column and the final column,  $E_o/E_e$ , is the observed-to-expected ratio; any ratio of >1.5 indicates synergism.<sup>4,5</sup>

These results clearly demonstrate that very low concentrations of ProteCoat additives can significantly increase the efficacy of a conventional biocide against spore-forms of both fungi and bacteria. Coupled with the stand-alone capability of

ProteCoat additives to function as environmentally sound alternatives to conventional biocides, the ability of this technology to enhance conventional biocides adds a new dimension for the polymer chemist to tweak biocidal cocktails to fit a particular contamination scenario.

**Conclusion**

By tailoring biomolecules for specific purposes, Reactive Surfaces has developed ProteCoat for coatings bioengineered to decontaminate microbial pests and biological contaminants. These peptide-based additives for coatings allows for the development of mould- and bacterial-inhibiting surfaces and coatings using 'green' technology.

ProteCoat can serve as a stand alone biocide, yet it can act synergistically with existing biocides. Synergistic activity not only allows for a reduction in the effective doses of two antimicrobial compounds, but also has the potential to revive the use of biocides to which pathogens have developed resistance. This synergism can be expanded to include other biocides, such as metals, natural products and enzymes.<sup>6</sup>

Cocktails of biocides, including AMPs, are predicted to have not only synergistic effects but also to be 'tunable' to

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**Table 2 - Synergistic activity of ProteCoat in combination with Verichem N2000**

Microorganism	% growth inhibition				
	ProteCoat	N2000	E <sub>c</sub>	E <sub>o</sub>	E <sub>o</sub> /E <sub>c</sub>
<i>B. atrophaeus</i>	0% (0.47)	55% (8.75)	55%	75.6%	1.37
	0% (0.93)	55% (8.75)	55%	94%	1.70
	0% (1.87)	55% (8.75)	55%	100%	1.88
<i>F. oxysporum</i>	15% (0.45)	21% (1.1)	33%	55%	1.64
	44% (0.9)	21% (1.1)	56%	92%	1.63
	44% (0.9)	0% (0.54)	44%	80%	1.80

particular coatings and micro-organisms. ProteCoat can be used in-process, in-can, in-film. It can be admixed, layered or added in or as a topcoat to the coating.

Since ProteCoat is a naturally occurring polymer of amino acids, it can be fermented and produced on a commercially feasible basis. This technology, we believe, provides polymer chemists with a new arsenal of effective, safe, environmentally-sound biocide options.

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