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(54) **BIOLOGICAL ACTIVE COATING COMPONENTS, COATINGS, AND COATED SURFACES**

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(73) Assignee: **REACTIVE SURFACES, LTD.**

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(57) **ABSTRACT**

Disclosed herein are novel coatings and paints comprising a biomolecule composition, wherein the biomolecule composition comprises a phosphoric triester hydrolase. Also disclosed herein are methods of detoxification of a surface contaminated with an organophosphorus compound by contacting the surface with such a coating or paint. Also disclosed herein are novel coating and paint components derived from microorganisms.

BIOLOGICAL ACTIVE COATING COMPONENTS, COATINGS, AND COATED SURFACES

[0001] This application claims the benefit of Provisional Patent Application Entitled "Bioactive Protein Paint Additive, Paint, and Painted Various," Ser. No. 60/409,102, filed Sep. 9, 2002, incorporated herein in its entirety by reference.

BACKGROUND OF THE INVENTION

[0002] A. Field of the Invention

[0003] The present invention relates generally to the field of biological molecules as components of coatings conferring an activity or other advantage to the coating proteinaceous molecule related to the biological molecule. More specifically, the present invention relates to proteins as such components of coatings. In one specific regard, the present invention relates to protein compositions capable of organophosphorus detoxification, and methods of reducing organophosphorus compounds on surfaces. More specifically, the present invention relates to coatings such as paints that degrade organophosphorus compounds such as pesticides and chemical warfare agents. The present invention further relates to paint and coating compositions and methods of their use to detoxify organophosphorus chemical warfare agents.

[0004] B. Description of the Related Art

[0005] Organophosphorus compounds ("organophosphate compounds" or "OP compounds") and organosulfur ("OS") compounds are used extensively as insecticides and are highly toxic to many organisms, including humans. OP compounds function as nerve agents. The primary effects of exposure to these agents are very similar, including inhibition of acetylcholinesterase and butyrylcholinesterase, with the subsequent breakdown of the normal operation of the autonomic and central nervous systems (Gallo and Lawryk, 1991).

[0006] Over 40 million kilograms of OP pesticides are used in the United States annually (Mulchandani, A. et al., 1999a). The number of people accidentally poisoned by OP pesticides has been estimated to be upwards of 500,000 persons a year (LeJeune, K. E. et al., 1998). Depending on the toxicity to the organism (e.g., humans), repeated, prolonged and/or low-dose exposure to an OP compound can cause neurotoxicity and delayed cholinergic toxicity. High-dose exposure can produces a fatal response (Tuovinen, K. et al., 1994).

[0007] Arguably of greater danger to humans, however, is the fact that some of the most toxic OP compounds are used as chemical warfare agents ("CWA"). Chemical warfare agents are classified into G agents, such as GD ("soman"), GB ("sarin"), GF ("cyclosarin") and GA ("tabun"), and the methyl phosphonothioates, commonly known as V agents, such as VX and Russian VX ("R-VX" or "VR"). The most important CWAs are as follows: tabun (O-methyl dimethylamidophosphorylcyanide), which is the easiest to manufacture; sarin ("isopropyl methylphosphonofluoridate"), which is a volatile substance mainly taken up through inhalation; soman ("pinacolyl methylphosphonofluoridate"), a moderately volatile substance that can be taken up by inhalation or [illegible] or aerosol; and VX ("O-ethyl S-diisopropylaminomethyl methylphosphonothioate") and its isomeric analog R-VX ["O-isobutyl S-(2-diethylamino)-methylphosphonothioate, R-VX or VR"], both of which can remain on material, equipment and terrain for long periods, such as weeks, with R-VX being an especially persistent substance. All CWAs are colorless liquids with volatility varying from VX to sarin. VX is an involatile oil-like liquid, while sarin is a water-like, easily volatilized liquid. By addition of a thickener (e.g., a variety of carbon polymers), soman or other more volatile agents may be made to be less volatile and more persistent.

[0008] The CWAs are extremely toxic and have a rapid effect. Such agents enter the body through any of the following manners: inhalation, direct contact to the skin with a gas or with a contaminated surface, or through ingestion of contaminated food or drink. The poisoning effect takes longer when the agents enter through the skin, but is much faster when they are inhaled because of the rapid diffusion in the blood from the lungs. These toxins are fat-soluble and can penetrate the skin, but take longer to reach the deep blood vessels. Because of this, the first symptoms may not appear for 20-30 minutes after initial contact with a contaminated surface. This increases the danger for personnel entering a contaminated area, because the contamination may not be detected for 30 minutes or more (depending on concentrations) after the contaminated area is entered.

[0009] The first and most important method of protection from nerve agents is to prevent exposure. For military personnel and other first responders, masks and full body protective gear are available, but this equipment has certain drawbacks. Impermeable suits and even some air permeable suits are bulky and hot. The equipment inhibits free movement and tasks are harder and take longer to complete. In addition to those factors, hard physical work in these suits this may cause heat stress or even collapse. There may also be long delays before decontamination can be completed so the protective gear must be worn for long periods. This makes for a marginally acceptable first defense against a chemical warfare agent attack. Decontamination is also time-consuming so the equipment must often be destroyed and new equipment provided. It is also difficult to provide everyone with such protective equipment in the general population, and the effectiveness of such equipment diminishes during use. Tasks requiring detailed work using fingers and hands such as keystrokes on a keyboard, or pushing buttons on phones or equipment can be severely hampered by such bulky protective gear.

[0010] In addition to direct contact with a gaseous agent during an attack, surfaces that are exposed to the gas retain their toxicity for long periods of time. The OP nerve agents are soluble in materials such as paint, plastics, and rubber, allowing agents to remain in those materials and be released over long time periods. Nerve agents with thickening agents are even more persistent and difficult to decontaminate from a painted surface such as a wall, vehicle, or even a computer keyboard. It is understood that on painted metal surfaces, soman may persist for from one to five days, and that the less volatile VX may persist for 12 to 15 days. Under certain [illegible]

are less exposed to the environment can be especially difficult to decontaminate. Decontamination also requires detection, which is often not possible, and so resources and time may be wasted treating uncontaminated surfaces.

[0011] Historically, most approaches to chemical agent decontamination have focused on the treatment of surfaces after chemical exposure, whether real or merely suspected, has occurred. There are several current methods of decontamination of surfaces. One method is post-exposure washing with hot water with or without addition of detergents or organic solvents, such as caustic solutions (e.g., DS2, bleach) or foams (e.g., Eco, Sandia, Decon Green). Additional types of methods are anapplication of use of intensive heat and carbon dioxide applied for sustained periods, and incorporation of oxidizing materials (e.g., TiO_2 and porphyrins) into coatings that, when exposed to sustained high levels of UV light, degrade chemical agents (Buchanan, J. H. et al., 1989; Fox, M. A., 1983). Chemical agent resistant coatings ("CARCs") have been developed to withstand repeated decontamination efforts with such caustize and organic solvents. However, the resulting "decontaminated" materials are often still contaminated. Moreover, many decontamination procedures aerosolize contaminants on surfaces to be cleaned. In addition, it is often hard to clean certain kinds of surfaces such as those with rough texture, or with deep crevasses and other hard to reach areas that must often "self-decontaminate."

[0012] Although each of these approaches can be effective under specific conditions, a number of additional limitations exist. Caustic solutions degrade surfaces, create personnel handling and environmental risks, and require transport and mixing logistics. Additionally, alkaline solutions, such as a bleaching agent, is both relatively slow in chemically degrading VX OPs and can produce decontamination products nearly as toxic as the OP itself (Yang, Y.-C. et al., 1990). While foams may have both non-specific biocidal and chemical decontamination properties, they require transport and mixing logistics, may have personnel handling and environmental risks, and are not effective on sensitive electronic equipment or interior spaces. CARCs have been shown to become porous after sustained UV light exposure that can create a sponge effect that may actually trap chemical agents and delay decontamination. Moreover, these approaches are not well suited for decontamination of convoluted surfaces. Decontamination with heat and carbon dioxide presents logistical requirements and does not allow rapid reclamation of equipment. UV-based approaches can be costly and have logistical requirements, including access to UV-generating equipment and power, as well as the production of toxic byproducts of degradation (Yang, Y.-C. et al., 1992; Buchanan, J. H. et al., 1989; Fox, M. A., 1983).

[0013] One attempted solution to the problem of surface contamination has been to provide paints with shedding ("chalking") properties such as an acrylic surface that may shed, or at least not be penetrated by a CWA, making decontamination easier. This has been unsatisfactory solution, however, because the area remains contaminated and there is no way to know if the surface is or is not poisonous. In addition, shedding coatings over existing painted surfaces nated surfaces, and in many instances may require washing despite the shedding characteristic.

[0014] Various enzymes have been identified that detoxify OP compounds, such as organophosphorus hydrolase ("OPH"), organophosphorus acid anhydrolase ("OPAA"), and DFPase, which detoxifies O,O-diisopropyl phosphorofluoridate ("DFP"). A number of civilian (e.g., Texas A&M University, private sector), and military laboratories [e.g., the Army research facilities at Edgewood (SBCCOM)] have worked on enzyme-based detection or decontamination systems for OP compounds. Various approaches taken in such laboratories include dispersion systems or immobilization systems of one or more OP degrading enzymes for use in detection or decontamination of OP compounds, as well as for convenience of handling of the enzyme preparation.

[0015] Sensors of OP compounds using an OP compound degrading enzyme have been described primarily for the detection of OP pesticides. OP compound sensors have been described that detect pH changes upon OP compound degradation using recombinant *Escherichia coli* cells expressing OPH cryoimmobilized in poly(vinyl)alcohol gel spheres (Rainina, E. I. et al., 1996). Endogenously expressed OPH from whole Flavobacterium sp. cells or cell membranes have been described as immobilized to glass membrane using poly(carbamoyl sulfonate) and poly(ethyleneimine) to produce a sensor of pH changes due to OP compound degradation (Gaberlein, S. et al., 2000a). OP compound sensors have been described that detect pH changes upon OP compound degradation using recombinant *Escherichia coli* cells, expressing OPH cytosolically or at the cell surface, that were fixed behind a polycarbonate membrane (Mulchandani, A. et al., 1998a; Mulchandani, A. et al., 1998b). An OP compound sensor has been described that detects optical changes upon OP compound degradation using recombinant *Escherichia coli* cells, expressing OPH at the cell surface, that were admixed in low melting point agarose and applied to membrane that was affixed to a fiber optic sensor (Mulchandani, A. et al., 1998c).

[0016] An OP compound sensor has been described that detects pH changes upon OP compound degradation using purified OPH chemically cross-linked with bovine serum albumin by glutaraldehyde on an electrode's glass membrane and covered with a dialysis membrane (Mulchandani, P. et al., 1999). Such chemically cross-linked OPH has been placed on a nylon membrane, and the membrane affixed to a fiber optic sensor to detect optical changes upon OP compound degradation (Mulchandani, A. et al., 1999a). Purified OPH has been immobilized by glutaraldehyde to glass-beads having aminopropyl groups in the construction of an OP compound degradation sensor (Mulchandani, P. et al., 2001a). An OP compound sensor has been described that detects optical changes upon OP compound degradation using recombinant Moraxella sp. cells, expressing OPH at the cell surface, that were admixed in 75% (w/w) graphite powder and 25% (w/w) mineral oil and placed into an electrode cavity (Mulchandani, P. et al., 2001b). Purified OPH was attached to silica beads by glutaraldehyde or N-γ-maleimidobutyrylozy succinimide ester linkages, and the beads placed as a layer on a glass slide to construct a sensor (Singh, A. K. et al., 1999). Purified OPH has been

compound cleavage by decreased fluorescence (Rogers, K. R. et al., 1999). Purified OPH has been immobilized by placement within a poly(carbamoyl sulfonate) prepolymer that was allowed to polymerize on a heat-sealing film in the construction of a sensor (Gaberlein, S. et al., 2000b). A purified fusion protein comprising OPH and a FLAG octapeptide sequence was immobilized to magnetic particles (Wang, J. et al., 2001). Additional sensors using OPH have been described (Mulchandani, A. et al., 2001).

[0017] Different OP compound degrading enzyme compositions have been described, primarily for the detoxification of OP pesticides (Chen, W. and Mulchandani, A., 1998; LeJeune, K. E. et al., 1998a). A parathion hydrolase enzyme degrading cell extract has been immobilized onto silica beads and porous glass (Munnecke, D. M., 1979; Munnecke, D. M., 1978). OPH has also been immobilized onto porous glass and silica beads (Caldwell, S. R. and Raushel, F. M., 1991b). Purified OPH has been mixed with fire fighting foams in an attempt to create a readily dispersible decontamination composition (LeJeune, K. E., and Russell, A. J., 1999; LeJeune, K. E. et al., 1998b). Purified OPH has been incorporated into micelles in an OP compound degradation device (Komives, C. et al., 1994). Purified OPH has been encapsulated in a liposome for use in OP compound degradation (Pei, L. et al., 1994; Petrikovics, I. et al., 1999). OPH enzyme supported by glass wool in a biphasic solvent and gas phase reactor for OP compound detoxification has been described (Yang, F. et al., 1995). Purified OPH has also been immobilized onto trityl agarose and nylon (Caldwell, S. R. and Raushel, F. M., 1991a). Recombinant *Escherichia coli* cells co-expressing OPH and a surface expressed cellulose-binding domain have been immobilized to cellulose supports (Wang, A. A. et al., 2002). Partly purified OPH, acetylcholinesterase or butyrylcholinesterase has been incorporated into polyurethane foam sponges (Havens, P. L. and Rase, H. F., 1993; Gordon, R. K. et al., 1999). Partly purified or purified OPH has been incorporated into solid polyurethane foam (LeJeune, K. E. and Russell, A. J., 1996; LeJeune, K. E. et al., 1997; LeJeune, K. E. et al., 1999). Recombinant *Escherichia coli* cells expressing OPH have been immobilized in a poly(vinylalcohol) cryogel (Hong, M. S. et al., 1998; Efremenko, E. N. et al., 2002; Kim, J.-W. et al., 2002). Purified OPH has been immobilized in polyethylene glycol hydrogels (Andreopoulos, F. M. et al., 1999). Recombinant *Escherichia coli* expressing OPH at the cell surface has been immobilized to polypropylene fabric by absorption of the cells to the fabric (Mulchandani, A. et al., 1999b). Purified OPH was immobilized to mesoporous silica by Tris-(methoxy)carboxylethylsilane or Tris-(methoxy)aminopropylsilane (Lei, C. et al., 2002). A fusion protein comprising OPH and a cellulose-binding domain has been immobilized to cellulose supports (Richins, R. D. et al., 2000). Sonicated *Escherichia coli* cells expressing a fusion protein comprising OPH, a green fluorescent protein, and a polyhistidine sequence as an affinity tag, have been attached to a nickel-iminodiacetic acid-agarose bead resin (Wu, C.-F. et al., 2002). A fusion protein comprising OPH and a polyhistidine sequence as an affinity tag has been attached to a chitosan film (Chen, T. et al., 2001). A purified fusion protein comprising an elastin-like polypeptide and OPH has

[0018] In addition to OPH, other OP compound enzyme compositions have been described. Purified OPAA has been encapsulated in a liposome for use in OP compound degradation (Petrikovics, I. et al., 2000a; Petrikovics, I. et al., 2000b). Purified OPAA has been mixed with fire fighting foams, detergents, and a skin care lotion in an attempt to create a readily dispersible decontamination composition (Cheng, T.-C. et al., 1999). Purified squid-type DFPase has been encapsulated in erythrocytes for use in OP compound degradation (McGuinn, W. D. et al., 1993). Purified squid-type DFPase has been coupled to agarose beads (Hoskin, F. C. G. and Roush, A. H., 1982). Purified squid-type DFPase has also been incorporated into a polyurethane matrix (Drevon, G. F. et al., 2002; Drevon, G. F. et al., 2001; Drevon, G. F. and Russell, A. J., 2000).

[0019] U.S. Patent Publication No. US 2002/0106361 A1 discusses a marine anti-fungal enzyme for use in a marine coating. However, the substrate for the enzyme was incorporated into the marine coating, and the enzyme was in a marine environment as the organism from which it was obtained. Immobilized enzymes in an latex are discussed in the April, 2002 edition of "Emulsion Polymer Technologies," by the Paint Research Association website http://www.pra.org.uk/publications/emulsion/emulsion highlights-2002.htm.

[0020] However, to date, there has been limited success in using these and other approaches to harness the potential of these enzymes in systems that can be readily and cost effectively used in field-based military or civilian applications. Thus, despite the current understanding of the various OP compound degrading compositions and techniques, whether based on caustic chemicals or enzymes, there is a clear and present need for compositions and methods that can readily be used in OP compound degradation. This is particularly true for the detoxification of OP chemical warfare agents. In particular, compositions and methods are needed that will detoxify surfaces contaminated with OP compounds.

SUMMARY OF THE INVENTION

[0021] The present invention provides compositions and methods for their use as components of surface treatments such as coatings. More specifically, the present invention provides compositions and methods for incorporating biological molecules into coatings in a manner to retain biological activity conferred by such biological molecule.

[0022] The present invention provides compositions and methods capable of effective decontamination of OP compounds, as well as prophalactic protection of buildings, equipment, and personel that contact such objects, from OP compounds, including CWAs. As they relate to detoxification of OPs, the compositions and methods disclosed herein differ substantially from prior efforts, which focus on enzymatic detoxification of chemical compounds by application of a decontamination composition or method to the site of contamination after contact with the chemical compound. The preferred embodiments of the present invention represent a paradigm shift in chemical decontamination. They demonstrate usable compositions and methods for prophy-

phylactic treatment with the coatings of the invention are a preferred embodiment, such a coating can also be used to coat a surface after contamination occurs. A preferred coating comprises a paint. Specifically, a paint comprising a preferred enzyme composition of the present invention degrades an organophosphorus compound, including a chemical warfare agent, into a significantly less toxic compound. Another preferred coating comprises a clear coat, a textile treatment, a wax, elastomer, or a sealant.

[0023] Further, the present disclosure is the first composition of which Applicant is aware that comprises a bioactive molecule such as an enzyme composition that retains activity after being admixed with paint. In addition, it still retains activity after the paint is applied to a surface, and renders the surface bioactive.

[0024] In light of these and additional disclosures herein, it is now possible to produce paints and other coatings that detoxify chemical compounds for extended periods. Remarkably, a preferred enzyme composition of the present invention remained stable in the coating for an extended period of time (e.g., months) at ambient conditions. It is contemplated that the extended period of activity may further comprise time periods in excess of a year. In particular, stabilized embodiments of the enzyme composition are designed to enhance the life time of the biomolecule composition in a coating and on a surface. In a preferred embodiment, the invention comprises at least one polymer-based compound that provides prophylactic protection and continuous detoxification of an organophosphorus nerve agent.

[0025] In certain embodiments, it is contemplated that the compositions and methods of the present invention may be used to produce self-decontaminating surfaces that remain active for extended periods. In a specific aspect, it is contemplated that such a self-decontaminating surface will not need additional decontamination compositions or methods to effect decontamination, thereby minimizing logistical requirement. In a particular aspect, it is contemplated that the compositions and methods of the present invention may be easily applied in advance of, during or after exposure to a chemical compound. In another preferred aspect, the compositions and methods of the present invention will be used in conjunction with existing other decontamination compositions and methods. In a preferred facet, the compositions and methods of the present invention are applied in advance of, during or after a field-based military operation to protect troops against a chemical warfare agent.

[0026] A recombinant phosphoric triester hydrolase may be produced using specific expression vectors in a variety of host cells. In the practice of the present invention, any of the described cells, nucleic acid sequences, genes, gene fragments, vectors and transcriptional or translational signals may be used, or any others that are known in the art. In preferred facets, the enzyme is grown in bacterial, fungal, plant (e.g., corn), or insect cells. In preferred embodiments, an expression vector includes an opd DNA fragment in the correct orientation and reading frame with respect to the promoter sequence to allow translation of the opd gene or gene fragment. In a specific aspect, the expression vector

embodiments the enzyme is an OPAA enzyme. In other preferred embodiments, the enzyme is a DFPase. Examples of the cloning and expression of exemplary opd gene and gene fragments are described (McDaniel, S. et al., 1988; McDaniel, 1985; Wild, J. R. et al., 1986; each incorporated herein in its entirety by reference).

[0027] In general embodiments, a biomolecule of the present invention refers to a compound comprising one or more chemical moieties normally produced by a living organism such as an amino acid, a nucleic acid, a sugar, a lipid, or a combination thereof. The invention provides a coating comprising a biomolecule composition, wherein the biomolecule composition comprises an active biomolecule. A biomolecule typically has a function in or upon a living organism, such as binding another molecule, catalyzing a chemical reaction, or a combination thereof. Specific examples of such activity by a biomolecule include an antibody binding an antigen, a cell receptor binding a ligand, an enzyme binding a substrate, a transport protein may bind a ligand, etc. In some aspects, binding a ligand may be a desired activity such as, for example, to sequester an undesired molecule, such as a toxin, to the biomolecule. Often, a biomolecule's activity further comprises a specific chemical reaction in addition to a physical/chemical affinity for another molecule. For example, an enzyme may accelerate a chemical reaction upon the bound substrate, a cell receptor may change conformation and/or become enzymatically active or inactive toward a second substrate, a transport protein may mitigate the movement of a molecule, etc. In another example, a biomolecule may comprise a ligand that induces or inhibits such activity in an enzyme, a cell receptor, a transport protein, and the like.

[0028] An "active biomolecule" refers to biomolecule that retains these types of properties in a coating of the present invention. The ability to confer bioactivity to a coating provides numerous uses in addition to the preferred bioactivity of detoxification of OP compounds.

[0029] Further, disclosed herein is the discovery that an active biomolecule may function in a coating in environments differing from the native environment of the organism from which it was obtained or derived. For example, an active biomolecule may retain activity in a coating under conditions of temperature, salinity, pH, moisture, or a combination thereof that differ from the organism's native environment.

[0030] The ability of a biomolecule to retain its function as an active biomolecule in a composition of the present invention, particularly a coating, may be detected and measured by any technique known to one of ordinary skill the art, including the various assays described herein.

[0031] A further disclosure of the present invention is the preparation of an active biomolecule with a limited number of processing and/or purification steps from other cellular molecules of the organism in which it was produced. Typically, the organism is a microorganism. The combination of an active biomolecule and such cellular molecules is known herein as a "biomolecule composition." It is contemplated that the inclusion of such cellular molecules may provide a protective or stabilizing effect for the activity of the active

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