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BIOMATERIALS FOR MEDIATION OF CHEMICAL AND BIOLOGICAL WARFARE AGENTS

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■ **Abstract** Recent events have emphasized the threat from chemical and biological warfare agents. Within the efforts to counter this threat, the biocatalytic destruction and sensing of chemical and biological weapons has become an important area of focus. The specificity and high catalytic rates of biological catalysts make them appropriate for decommissioning nerve agent stockpiles, counteracting nerve agent attacks, and remediation of organophosphate spills. A number of materials have been prepared containing enzymes for the destruction of and protection against organophosphate nerve agents and biological warfare agents. This review discusses the major chemical and biological warfare agents, decontamination methods, and biomaterials that have potential for the preparation of decontamination wipes, gas filters, column packings, protective wear, and self-decontaminating paints and coatings.

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INTRODUCTION

Recent events have shown that the use of chemical and biological weapons is more than just an abstract threat. The March 1995 terrorist release of sarin in the Tokyo subway system and the post-September 11 anthrax attacks in the United States reveal the ease with which these weapons can be utilized. A great deal of effort has been expended over the past few years to develop mechanisms to mediate the effects of such attacks. New initiatives to educate, train, and equip emergency personnel have been instigated, and physician networks are in place to detect the first signs of a mass attack so that resources can be mobilized in a timely manner. In addition, considerable effort is being expended to devise methods and materials that will neutralize both chemical and biological agents at the initial stages of an attack and, thus, to minimize the effects of the attack.

The most important factor in limiting the effects of an intentional release of a chemical or biological agent is to quickly identify the agent and minimize exposure to the population. Because early detection is so important, research into the development of devices capable of detecting and identifying chemical and biological agents has been an ongoing effort. These efforts are the subject of recent reviews (1, 2).

Another tactic that can be used to mediate the effects of a chemical or biological attack is to instigate in situ decontamination of the agent. The difficulty with this approach is that by the time the attack is detected, it is usually too late to significantly mitigate the effects. Further, most of the agents are unstable or are quickly dissipated into the environment.

One solution to this situation is the development of biomaterials with the ability to decontaminate chemical and biological agents. Such materials would be able to act as passive devices that could be deployed at sites determined to be likely targets of attack. The use of self-decontaminating coatings or fabrics could minimize the initial effects of a chemical or biological attack. The current state-of-the-art development of these new types of materials is the focus of this review.

CHEMICAL WARFARE AGENTS

Properties

Chemical warfare (CW) agents are divided into two major classes depending on their fate in humans and mammals (3). The first category includes the nerve agents sarin (GB), soman (GD), tabun (GA), and VX (Figure 1). These organophosphorus (OP) esters irreversibly block the enzyme acetylcholine esterase. The second category consists of the vesicant and blister agents Lewisite and sulfur mustard or mustard gas (Figure 2). Raw sulfur mustard and purified sulfur mustard are commonly referred to as H and HD, respectively (4).

Although not considered as part of CW weapons, the nitrogen mustard derivatives that comprise ethylbis(2-chloroethyl)amine (HN1), methylbis(2-chloroethyl)

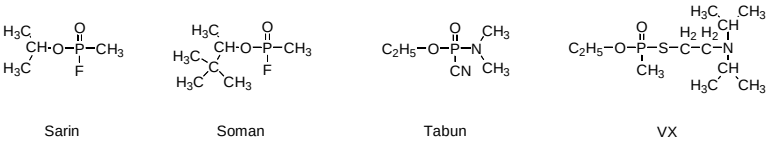

Figure 1 Organophosphorus-based nerve agents.

amine (HN2), and tris(2-chloroethyl)amine (HN3) are potent vesicant and blister agents (Figure 2). Their physico-chemical and toxicological properties have been previously studied and reviewed (5).

The CW agents are found in the liquid state under ambient conditions and exhibit variable physical and chemical properties, including viscosity, density, volatility, vapor pressure, water solubility constants, and Henry's law constants (Table 1; References 4 and 5). The vapor pressure and volatility increase in the order VX < GA < HD < GD < Lewisite < GB. Unlike GA and GB, HD, Lewisite, VX, and GD are associated with low water solubility. VX is the most persistent CW agent, as given by its low volatility and Henry's law constants. Moreover, it is the most resistant to water hydrolysis and hence to degradation in aqueous media (5). Despite their low water solubilities, HD and Lewisite are rapidly hydrolyzed in water (5, 6).

HD is a strong alkylating agent and is known to react with several biomolecules, including proteins, DNA, RNA, and other compounds of physiological importance, including inorganic and organic phosphate (6, 7). The earliest signs and symptoms resulting from systemic poisoning with HD include vomiting and nausea (8). HD induces delayed symptoms, including eye and skin lesions and injury of the respiratory tract (8). The duration of latent period following intoxication is variable depending on the HD dose (8). Eye and skin injuries appear usually within minutes to hours after exposure, whereas incapacitating respiratory damages develop within days after intoxication (8, 9). According to several studies on mustard gas

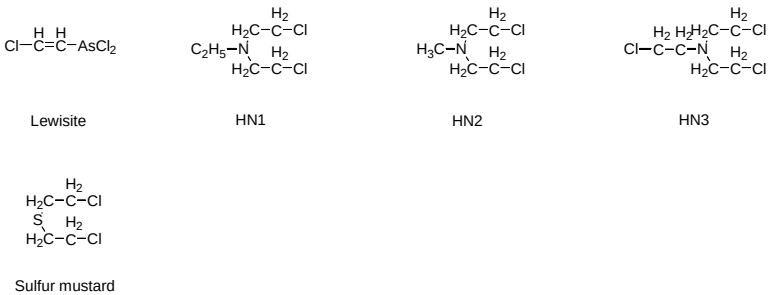

Figure 2 Main vesicant and blister chemical warfare agents.

TABLE 1 Summary of the physical properties of major chemical warfare agents. Reproduced from Munro et al. (5)

CW agent	Lewisite	GA	GB	GD	HD	VX
Formula	$C_2H_2AsCl_3$	$C_5H_{11}N_2O_2P$	$C_4H_{10}FO_2P$	$C_7H_{16}FO_2P$	$C_4H_8Cl_2S$	$C_{11}H_{26}NO_2PS$
Molecular weight (g/mol)	207.35	162.1	140.1	182.2	159.08	267.4
Boiling point (°C)	190	220–246	158	198	215–217	298, decomposes
Melting point (°C)	−18	−50	−56	−42	13–14	−39, calculated
Density at 25°C (g/ml)	1.89 (20°C)	1. 073	1.102 (20°C)	1.022	1.27 (20°C)	1.008 (20°C)
Vapor pressure at 25°C (mmHg)	0.58	0.07	2.10 (20°C)	0.40	0.11	0.0007
Volatility (mg/m^3)	4,480	610	22,000	3,900	920	10.5
Vapor density (air = 1)	7.1	5.6	4.9	6.3	5.5	9.2
Water solubility (g/L)	0.5	98 (25°C)	Miscible	21 (20°C)	0.92	30
Henry's law constant ($atm \times m^3/mol$)	3.2×10^{-4}	1.52×10^{-7}	5.4×10^{-7}	4.6×10^{-6}	2.1×10^{-5}	3.5×10^{-9}
Physical appearance	Liquid colorless (pure), amber/ brown (aged)	Oily liquid Colorless to brown	Liquid colorless	Liquid colorless	Oily liquid Clear/pale yellow, black if impure	Oily liquid, light amber/amber

Abbreviations: Lewisite [dichloro(2-chlorovinyl)arsine]; GA, tabun (ethyl-N-N-dimethylphosphoroamidocyanidate); GB, sarin (isopropyl methylphosphonofluoridate); GD, soman (pinacolylmethylphosphonofluoridate); HD, sulfur mustard (2,2′-dichlorodiethyl sulfide); VX [S-2-(diisopropylamino)ethyl O-ethyl methylphosphonothioate].

factory workers, exposure to HD significantly increases the risk of skin and lung cancers (10). Death often results from secondary respiratory infections, especially bronchitis and bronchopneumonia, as well as lung edema, respiratory insufficiency, and cardiovascular failure (7, 10). Some of the intermediates formed during the degradation of HD are characterized by high toxicities (6).

Little information is available on Lewisite toxicity and health effects. Lewisite is a potent inhibitor of enzymes. Inhibition may result from the interaction of Lewisite arsenic groups with enzyme cysteine residues (11). An indirect route of action relies on the binding of Lewisite to compounds such as dihydrolipoic acid, which is a cofactor for several enzymes (12). Inhibition further proceeds by the association of these particular enzymes to the resulting Lewisite/cofactor complex (12). The overall resulting effect is cell death (11). Health effects, such as eye irritation and skin pain, occur immediately after exposure (11). Other symptoms, including sneezing, lachrymation, coughing, and salivation, appear rapidly after exposure (13). Eye and skin damages and lesions in the upper respiratory tract may be delayed for several hours after exposure. Death may result from respiratory failure (13, 14).

Nerve agents act by binding the esterase enzymes, especially acetylcholine esterase (AChE), present in the blood and at the neuromuscular junctions in the peripheral and central nervous systems (15). Nerve agents are also known to directly interact with tissue, ion channels, and receptors, including nicotinic and muscarinic receptors (15, 16). AChE regulates the level of the neurotransmitter acetylcholine (ACh) by degrading it. Nerve agents associate with AChE via phosphorylation, which results in enzyme inhibition and leads to excess ACh accumulation. The ACh buildup within the nervous system is mainly responsible for the health signs and symptoms developed after exposure to nerve agents (17). The AChE/nerve agent adduct undergoes a process called ageing, which involves the alkylation of the phosphonyl group attached to the inhibited enzyme, and leads to the irreversible binding of the nerve agent to the enzyme (18). Once aged, the enzyme cannot be reactivated. The propensity of the AChE/nerve agent complex to chemically age varies from one nerve agent to another. The half-lives of AChE/GA, AChE/GB, and AChE/GD adducts are 46 h, 5 h, and 1.3 min, respectively (19). The ageing process takes place at a much slower rate with the AChE/VX system (20).

Exposure to nerve agents can lead to low and moderate intoxications by oral, dermal, and (or) inhalation routes. Each route is associated with a specific sequence of health effects, which depends on factors such as exposure duration and nerve agent concentration (21). The symptoms following a high-level exposure to nerve agents by any of the modes previously mentioned develop within minutes to hours and include miosis, nausea, tightness in the chest, vomiting, increased sweating and salivation, lachrymation, headaches, weakness, fasciculation, muscular and abdominal cramps, diarrhea, blurring of vision, anxiety, and tremor (22, 23). Death can occur through the paralysis of respiratory muscles, severe depression of the central nervous system, and increased bronchial secretion (24). Delayed neuropathy

results from the nerve agent–induced inhibition of neuropathy target esterase and usually appears within 7 to 14 days after exposure (25). Severe poisoning by nerve agents has been reported to induce long-term physiological, psychological, and neurological effects, such as cerebral impairment and cardiac malfunctioning resulting from myocardial damage, as well as posttraumatic stress disorder and differences in intellectual and motor skills (24). Most products of nerve agent degradation exhibit low-to-moderate toxicities (5, 26).

Medical Therapy for Chemical Warfare Agents Intoxication

Pyridostigmine bromide, which exhibits reversible anticholinesterase activity, is a standard drug used in preventive treatments of nerve agent poisoning (27). The strategy relies on the ability of pyridostigmine bromide to shield AChE from the action of nerve agents. Once introduced in the presence of AChE, pyridostigmine bromide binds reversibly to the enzyme active site. The effects of subsequent exposure to nerve agent are largely limited as the enzyme active site is blocked. The antidotal efficiency of the spontaneously reactivating AChE inhibitor decreases gradually after administration, as it progressively dissociates from AChE and is eliminated by the body (27). However, the process is slow when compared with the hydrolysis of unreacted nerve agent in vivo (27). To ensure optimum protection, pyridostigmine has to be administered orally on a daily basis every 8 to 14 h (21). A major disadvantage of pyridostigmine is that it poorly penetrates the blood-brain barrier and, hence, only provides protection to the peripheral nervous system. Consequently, it is necessary to use it in combination with a postexposure therapy. Another major drawback of pyridostigmine is that it indirectly induces the accumulation of acetylcholine at the cholinergic synapses as a result of AChE inhibition. The ensuing side effects are similar to those induced by nerve agent contamination, although less intense (28).

A conventional strategy for the treatment of intoxication to nerve agents consists in administrating cholinolytic drugs, such as atropine, to reduce the health effects provoked by the accumulation of acetylcholine at the synaptic cleft. Atropine inhibits muscarinic effects, including parasympathetic miosis, salivation, overstimulation, and bradycardia (18). Because atropine has little effect at nicotinic sites, it is systematically employed in combination with a drug from the oxime group, such as pralidoxime chloride and 2-PAM, which suppress muscular weakness and prevent respiratory paralysis (29, 30). Oximes act by disrupting the AChE/nerve agent adduct. Consequently, the initial activity of AChE is regenerated upon release of the nerve agent. Given the inability of oximes to reactivate AChE once chemical ageing has occurred, it is essential to administer them early after nerve agent intoxication (31). This problem is of major concern, especially in the case of intoxication with GD, for which ageing is extremely rapid [the half-life of AChE/GD is about 2 min (30)]. In addition to restoring the hydrolysis of acetylcholine, highly effective bis-pyridinium Hagedorn oximes, such as HI-6 and HLö-7, have been shown to induce non-AChE-reactivating effects, which could

be of importance for survival after GD intoxication (30, 32). The anticonvulsive additive, diazepam, is administered to limit seizure and resulting brain damage (33–35). Although powerful, this combinatorial therapy does not succeed in preventing some of the nerve agent–induced effects, including postexposure incapacitation and performance deficits (36). Moreover, despite the use of anticonvulsive agents, brain damage may still occur (36).

Research has been conducted on the development of biological scavengers as an alternative therapeutic approach to the conventional treatment described above. Their mode of action is to protect the blood AChE from inhibition by sequestrating or hydrolyzing the nerve agent (37). The first category of bioscavengers are enzymes, which exhibit strong affinity for nerve agents and are inhibited by them, such as carboxylesterases (CaEs), antibodies, and cholinesterases (ChEs), including fetal bovine serum AChE (FBS-AChE), equine serum butyrylcholinesterase (EqBChE), and human serum butyrylcholinesterase (HuBChE) (38–40). Full protection against several lethal doses of nerve agents was achieved in animal models, such as mice, rats, and rhesus monkeys, when ChE was administered prophylactically (41). As only physiologically insignificant nerve agent–induced effects were observed, no postexposure treatment based on atropine or oximes was necessary (41). It is worth mentioning that in the absence of nerve agent intoxication, the exogeneous ChE induced a major increase in the AChE activity of pretreated animals without provoking any physiological or neurological effect. CaE, which is present in high concentrations in the plasma of rodents, provides them with a natural protection against intoxication with G nerve agents (GA, GB, and GD) (37). Therefore, it constitutes another potential bioscavenger. However, CaE reacts poorly with VX and could not afford any protection against this particular nerve agent (38). Promising attempts have also been made to develop monoclonal antibodies against nerve agents (42–44).

The second category of bioscavengers corresponds to enzymes and catalytic antibodies capable of hydrolyzing nerve agents. Interesting results have been obtained on the development of catalytic monoclonal antibodies and may lead to efficient treatments (45–47). The ability of nerve agent–degrading enzymes administered exogeneously to detoxify nerve agents and OP pesticides in vivo has been demonstrated in the particular cases of human paraoxonase (PON) and the bacterial phosphotriesterase from *Pseudonomas diminuta* using mammalian models (45). Available enzymes with anti-OP activity suffer the disadvantage that they exhibit high Michaelis constants and low turnover numbers. To become appropriate catalytic bioscavengers for human protection, their catalytic efficiency needs to be greatly enhanced. Moreover, enzymes from bacterial sources are less attractive than those from mammalian origin, as they are more likely to induce immune response (48). In an effort to generate enzymes with the appropriate characteristics, research has been conducted on the alteration of enzymes such as HuBChE and PON by site-directed mutagenesis (40, 48). Encouraging results have been obtained toward the ultimate goal of isolating mutants with OP acid anhydride hydrolase activity and capable of spontaneous reactivation (40).

Pre- and postexposure treatments against HD contamination, which were reviewed exhaustively by Papirmeister et al. (49), are based on chemical scavengers, nicotinamide adenine dinucleotide (NAD^+) level stabilizers, and antiinflammatory drugs. When administered prior to or after exposure to HD, the scavenger sodium thiosulfate affords protection against systemic intoxication. Unfortunately, sodium thiosulfate is inappropriate for auto-injection, as it is only effective in high doses. Moreover, because it penetrates poorly through the cell membrane, its action is limited mainly to extracellular areas. Other scavengers, such as thiophosphonates, dithiocarbamates, glutathione, and N-acetyl-L-cysteine, have also been reported to reduce lethality, although they exhibit lower protective efficacy as compared with sodium thiosulfate. Antiinflammatory drugs, such as the steroid dexamethasone and the antihistamine promethazine, provide protection against lethality when used as postexposure treatments. Only a few drugs are potent agents against locally mediated intoxication by HD. Precursors of NAD^+, such as nicotinamide, are effective for the treatment of dermal lesions resulting from local intoxication, whereas steroids show moderate efficacy against dermal and respiratory lesions. Scavengers are ineffective for protection against HD.

The main antidotes for treating intoxication with Lewisite are the British-anti-Lewisite (BAL), which is to dithiol 2,3-dimercapto-1-propanol, and the compound 2,3-dimercaptopropanol glucoside, also known as BAL-INTRAV (17). The use of less toxic BAL analogs, such as meso-2,3-dimercaptosuccinic acid and 2,3-dimercapto-1-propanesulfonic acid, has also been reported (4).

BIOLOGICAL WARFARE AGENTS

Although the list of potential biological agents reads like a medical microbiology text, only a small fraction of the pathogenic organisms are considered real threats. The agents considered to be the most likely to do serious damage to population centers are reviewed in a series of articles by the Working Group on Civilian Biodefense (50–53). From the extensive list of potential agents, the Working Group has focused on three bacterial agents, *Francisella tularensis* (50), *Yersinis pestis* (51), and *Bacillus anthrasis* (52), as those most likely to cause disease and deaths in such numbers as to cripple a city or region. The Working Group has also focused on smallpox and a small collection of hemorrhagic fever viruses as the most likely viral agents (53, 54). Finally, the group has reviewed the possible use of botulism toxin in a bioterror/biowarfare attack (55). Some of the features of these agents are summarized in Table 2.

As can be seen in Table 2, the likely biological threats cover a wide range of types and activities. Botulism toxin, one of the most toxic substances known, behaves in a manner similar to the chemical agents in that it acts quickly and is not a disease organism. It has a short environmental half-life and its effects are confined to those in direct contact with the material. The selected viral agents are all highly pathogenic with high mortality rates. Even though many of the viral agents are transmissible from person to person, only smallpox is thought to be highly

contagious. The most highly contagious of the biological agents is tularemia (50). Although the death rate due to tularemia is low (about 1.4%), the large number of cases that would occur in a deliberate release makes it a credible threat. Plague in the pneumonic form, as would occur from aerosol inhalation, is highly contagious and very quickly establishes disease. The high mortality rate from plague makes it an especially dangerous weapon. However, it is anthrax that is considered the greatest of the biological threat agents (52).

Anthrax can be acquired through three routes. The disease can initiate through skin contact with spores, through ingestion of contaminated food, or by inhalation of airborne spores. The spores of *B. anthracis* are environmentally stable, being able to persist as infective units for years under proper conditions. Numerous countries have initiated programs to produce weaponized anthrax spores and delivery systems. The credibility of the anthrax threat and the difficulty in mounting an adequate response were brought to the public's attention with the attacks of 2001 (52). Because of its persistence in the environment, anthrax has been a driving force for the development of self-decontaminating biomaterials for mediation of biological warfare agents.

DECONTAMINATION OF STOCKPILED NERVE AGENTS

Chemical Means

Large stockpiles of CW agents, including GB, GD, HD, and VX, are stored around the world. In the United States, these chemical stockpiles need to be destroyed as stipulated by public law 99-145 and public law 104-484 (56). CW agent decontamination is performed, to a large extent, via incineration by the Army under the Chemical Stockpile Disposal Program. Alternative technologies have been developed and include, for example, acid- or base-catalyzed hydrolysis, supercritical water oxidation, and electrochemical oxidation and reduction (57). Chemical detoxification of HD is usually conducted in hot water (90°C) under vigorous mixing (56). Final disposal to the environment is enabled by combining the neutralization process with a posttreatment of the hydrolysate, which relies on incineration or biodegradation (56, 58). Volatile organic compounds (VOCs) are destroyed by photodegradation and photooxidation (56). Alkaline mixtures, such as hypochlorite-containing solutions and the decontamination solution 2 (DS-2), which is composed of diethylenetriamine, ethylene glycol monomethyl ether, and sodium hydroxide, are also potent media for HD decontamination (5, 59, 60). Other alternative oxidative technologies involve the use of nontoxic and noncorrosive oxidizers, such as hydrogen peroxide and potassium molybdate (61, 62). Unlike HD, VX hydrolysis does not proceed in neutral-to-basic media and requires basic conditions (63). The general procedure relies on the neutralization of VX via treatment with aqueous NaOH at 90°C, followed by the oxidation of the resulting hydrolysate using a hypochlorite solution (5). Decontamination can also be performed by oxidation in the presence of aqueous bleach or N,N'-dichloroisocyanurate (5, 63). The

TABLE 2 Biological agents that pose a high potential threat

Agent	Agent type	Effects	Treatment	Transmission
Botulism toxin	Neurotoxin most likely as an aerosol or directly added to foods	Inhibits neurotransmitter release resulting in paralysis with death from airway obstruction	Supportive	No person-to-person transmission
Smallpox	Viral	The virus is acquired by inhalation. After 7–17 days incubation, signs of infection become apparent as rash and pustule formation. Death usually occurs in the second week of infection	Supportive	Highly contagious High virus titer in the saliva leads to aerosol transmission Very fast spread through a susceptible population
Hemorrhagic fever viruses	Group of viruses including Marburg/Ebola, Lassa fever, Rift Valley fever, Yellow fever, and New World arenaviruses, among others	A diverse group but in general a high fever is common. Also often accompanied by bleeding and intravascular coagulation and other hemorrhagic complications	Supportive therapy often including administration of antiviral drugs, especially Ribavirin	Ebola, Marburg, Lassa, and New World arenaviruses can be transmitted person to person; the others are not
Francisella tularensis (tularemia)	Bacterial Small gram-negative coccobacillus, nonmotile, non-spore-forming.	Disseminated infection with target organs including lungs, lymph nodes, spleen, liver, and kidney. Fever and body aches incapacitating within 1–2 days of aerosol exposure. High morbidity but low mortality rates among healthy patients	Antibiotic therapy	No human-to-human transmission

Yersinis pestis (plague)	Bacterial Small gram-negative bacillus Nonmotile and non-spore-forming	Weaponized plague would be expected to be of the pneumonic type. Infection within 1–6 days of exposure, fever, cough, gastrointestinal effects (nausea, vomiting, abdominal pain). Death occurring quickly after symptom onset in up to 25% of cases	Antibiotic therapy	Highly contagious, probably by aerosol transmission
Bacillus anthracis (anthrax)	Bacterial Gram-positive spore-forming bacillus	Three forms: cutaneous, gastrointestinal, and inhalational. Cutaneous anthrax: cutaneous lesions untreated can lead to systemic disease Gastrointestinal: nausea, vomiting, bloody diarrhea, sepsis, high mortality rates Inhalational: fever and chills, drenching sweats, fatigue, nausea and vomiting, sepsis, high mortality rates	Antibiotic therapy	No known person-to-person transmission

proposed posttreatment involves supercritical water oxidation or biodegradation (5). GB and GD are readily detoxified at room temperature in alkaline aqueous media (60). The hydrolysis is usually followed by biodegradation of the neutralization products (64). Similarly, GA hydrolysis in water is highly effective, especially at alkaline pH (5). GA detoxification is also expected to proceed in the presence of sodium hypochlorite, as well as DS-2 (5, 65), with methanol-potassium hydroxide as another possible decontaminant (5). Lewisite is efficiently detoxified by oxidants such as hydrogen peroxide and iodine (66). Lewisite neutralization also occurs rapidly in the presence of alkalies and NaOH with mild heating (5). Environmentally friendly oxidizers, such as hydrogen peroxide, have also been tested and shown to be effective for the detoxification of G agents (GA, GB, and GD) and VX (67).

Biological Means

Due to the strong public opposition to disposal methods such as incineration, there has been considerable effort to design more environmentally benign methods of OP decommissioning. Furthermore, methods such as incineration and the toxic, corrosive, and flammable nature of many decontamination solutions are not amenable for use over large areas or on sensitive equipment or personnel. For these reasons, there has been vast interest in using biological means of bioremediation for the detoxification of OP compounds (68–70).

A number of enzymes exist that are capable of degrading a wide range of OP compounds (69, 70). The first OP-degrading enzyme was observed by Mazur, who reported the hydrolysis of DFP to hydrofluoric acid and diisopropylphosphoric acid by enzymes found in the tissues of humans and rabbits (69, 71). Since this report, a wide number of bacteria and eukaryotic organisms have been shown to possess the ability to degrade OP compounds (69, 71). OP-degrading enzymes have been isolated from a variety of sources, including microorganisms, insects, plants, mammals, birds, and squid (71–78). Bacteria with OP-degrading capabilities have been identified in *Flavobacterium*, *Pseudomonas*, *Bacillus*, and *Anthrobacter*. In addition, OP-degrading activity has been found in the fungi *Phanaerochaete chrysosporium* and *Trichoderma viride* and in algae and cyanobacteria (69, 70).

Considerable research has focused on the isolation of OP-hydrolyzing enzymes and the cloning and sequencing of the genes that code for them. This information has been successfully used for the introduction of these genes into other organisms, for site-directed mutagenesis (79–84), for directed evolution studies (85, 86), and for the development of protein:protein fusions (87, 88). The enzymes responsible for OP degradation are ideal for biocatalysis because they do not require expensive cofactors, are stable over a wide range of temperatures and pH, and generally have broad substrate specificity. The nomenclature and classification of phosphoric triester hydrolases can be rather bewildering and the reader is referred to a number of reviews that describe in more detail the many types of OP- and CW-degrading

enzymes (69, 70, 89, 90). Nevertheless, these enzymes are very diverse and the exact physiological substrate is not known for most of them (89). They have been classified somewhat sporadically as the phosphotriester hydrolases (EC 3.1.8), which are further broken down into two basic categories based on their substrate specificity: (*a*) the aryldialkylphosphatases (EC 3.1.8.1), which cleave P-O, P-S, and P-N bonds; and (*b*) the diisopropylfluorophosphatases (DFPases, EC 3.1.8.2), which cleave P-F and P-CN bonds.

The most thoroughly characterized enzyme is the organophosphohydrolase (OPH) from *Pseudomonas dismuta* MG (72). Native OPH is a metalloenzyme, requiring zinc for catalytic activity, and has broad substrate specificity. OPH has been shown to degrade pesticides, such as paraoxon, parathion, coumaphos, and diazinon, as well as nerve agents such as GB, GD, and VX (79, 80). For some substrates, such as paroxon, OPH catalyzes the hydrolysis at a rate very close to the diffusion-controlled rate (91); however, for substrates such as VX, hydrolysis may occur at 0.1% of this rate (79). The DFPase enzymes isolated from squid (74, 84, 92, 93, 94) and the OP anhydrolase (OPAA) (69, 89) have also gained much attention because they can efficiently catalyze the hydrolysis of DFP, GD, GB, and GA.

For the detoxification of nerve agents and pesticides, bacteria and fungus are attractive because of the ease of large-scale production by fermentation. A number of applications using resting cells for the decontamination of OPs have been reported. One process used the resting cells of *Flavobacterium* sp. for the hydrolysis of coumaphos, which was followed by UV-ozonation to completely degrade the pesticide (95). Methods using mixed culture or engineered organisms that can grow on OP toxins and their degradation products, such as p-nitrophenol, have been investigated (96–98). These direct inoculation processes are desirable because they are low cost and have few environmental side effects. They do, however, tend to be slow, require frequent feeding, may require buffering and pH control, and are subject to interference by native flora (70). Furthermore, the catalytic rates of OP hydrolysis by free cells can be limited due to substrate diffusion through the cell wall/membrane (99, 100). Mulchandani and coworkers have circumvented this problem by developing recombinant strains of *Escherichia coli* that express OPH that is anchored on the cell surface (100, 101). Hong et al. also used a cryoimmobilization technique that was reported to improve substrate diffusion rates across the cell wall (99). The application of immobilized whole cells in stirred tank reactors is desirable if the immobilized biocatalyst can be shown to be reusable, maintain stability for long periods of time, and resist chemical and mechanical damage (70). Wild and coworkers have used a number of bacteria cryoimmobilized in poly(vinyl alcohol) for the degradation of paraoxan (99), coumaphos (102), neutralized GB (103), and thiodiglycol (104). Mulchandani and coworkers have investigated pesticide detoxification using *E. coli* with surface-expressed OPH adsorbed on nonwoven polypropylene fabric (105) and specifically adhered to cellulose using *E. coli* with surface-expressed cellulose-binding domain (106).

A number of applications involving purified OP-hydrolyzing enzymes or cell free extracts have also been reported. At least one report has investigated the use

of free enzymes in aqueous phase for degradation of OP toxins (107, 108). Because most OP compounds have limited solubility in water, Russell and coworkers developed techniques using microemulsions to solubilize and concentrate the hydrophobic compounds (109, 110). OPH and OPAA have also been shown to be stable in fire-fighting foams, which can effectively decontaminate large surfaces without solvents and toxic solutions (68, 111–113). Paraoxon vapors have also been hydrolyzed using solid lyophilized OPH in a gas phase bioreactor (114).

A number of applications have been developed using immobilized enzymes. A few of these will be discussed in more detail in the section on biomaterials for decontamination of chemical weapons.

BIOMATERIALS

Biomaterials Against Chemical Weapons

Since the discovery of OP-degrading enzymes, there has been interest in the application of these enzymes not only for the decontamination of waste waters and nerve agent stockpiles, but also for the protection of farmers and military personnel. Significant research has gone toward the development of clothing for the protection of individuals against exposure to nerve agents or the preparation of coating materials for resistance to chemical agents. Current protective clothing relies on an adsorptive polyurethane layer impregnated with activated carbon for OP adsorption. This type of clothing must be carefully disposed of because it lacks the ability to detoxify itself; furthermore, the adsorptive layer adds extra weight to the clothing. Special chemical agent–resistant coatings (CARC) that prevent the penetration of chemical agents through the coating polymer layer are also required for military vehicles (115). The development of enzyme-containing polymeric materials will enable the preparation of a wide variety of self-decontaminating clothing and surfaces.

The preparation and purification of enzymes can be costly; therefore, it is imperative to immobilize an enzyme in order to maximize productivity. Effective immobilization methods allow for the preparation of an immobilized enzyme that retains most of its native activity, maintains high operational stability in its working environment, and maintains high storage stability. Since the late 1970s, a number of processes have been investigated for immobilizing OP-degrading whole cells and enzymes. Many methods have been used for developing biomaterials for application in biosensors and bioelectronics (1, 2). Because there are already a number of reviews discussing this rather large body of work, we focus on the preparation of biomaterials for the use in detoxification and as protective barriers.

Much attention has been focused on preparing materials for use in the decontamination of OP-contaminated aqueous waste streams and for the detoxification of chemical weapon stockpiles. The first reports of an immobilized enzyme preparation were by Munnecke in 1977 and 1979 (116, 117). In these experiments, a crude cell extract from a mixed culture, capable of degrading nine organophosphate insecticides, was bound to glass beads via an azide-coupling method. The

enzyme-bead preparations were then used in packed columns and fluidized beds. The beads maintained good stability (about 50% of initial activity during discontinuous use over 180 days). However, the enzyme preparation was susceptible to deactivation by solvents and unknown inhibitors in the aqueous waste stream (117).

Caldwell & Raushel (118, 119) immobilized *Pseudomonas diminuta* OPH by adsorption onto trityl agarose and by covalent attachment using glutaraldehyde onto various nylon supports, including filters, membranes, tubing, and packings. They found that the trityl agarose column was not practical due to enzyme leaching from the support (118). On the other hand, covalent attachment of OPH to nylon membranes showed no leakage and no loss of activity after four weeks of operation. Havens & Rase investigated the effect of support chemistry and configuration on the performance of immobilized OPH for OP detoxification (120). The supports (of varying particle size and porosity) were packed into columns and compared for their ability to attach enzyme and maintain stability. The authors found that a N-hydroxysuccinimide (NHS)-activated support had the highest activity and a carbonyl diimidazole support had the highest stability (66.9-day half-life). It was suggested that the differences between the support materials were caused by variations in support charge, porosity, or spacer length.

More recently, Mulchandani and coworkers have looked at the immobilization of *E. coli* with surface-expressed OPH onto cotton (87, 106). By cloning and surface expression of a cellulose-binding domain, they were able to very efficiently (and irreversibly) immobilize the bacteria onto cellulose fabrics and filters. This technique provides a unique alternative to immobilized enzymes because enzymatic purification is not required. Furthermore, there is no need to worry about the immobilization chemistry deactivating the biocatalyst. They were further able to show that the nonviable cells were reusable and that they lost less than 10% of their OP degrading ability after 45 days (87, 106).

Grimsley et al. also investigated the use of cellulose for the covalent immobilization of OPH using glutaraldehyde (121). Cotton is a highly absorptive material, and in combination with nerve agent–degrading enzymes, would be appropriate for use as decontaminating towelettes, gauze, swabs, bandages, and wound dressings (121). A cotton towelette was reported to degrade 0.12 mg paraoxon/min/cm^2 of fabric and should be able to degrade gram-size quantities of nerve agent in a few minutes (121). However, long-term stability may be a concern because a dry fabric with trehalose added as a stabilizer lost nearly 30% of its initial activity after 35 days (121).

Recent advances in materials synthesis using enzymes have allowed the preparation of a variety of bioplastics and enzyme-polymer composites for use as reactive monoliths, foams, fibers, wipes, and coatings (122–124). These polymers involve the incorporation (usually covalent) of the biological material directly into the polymer. The enzyme may actually participate in the reaction, and via the reactive functionalities on the enzyme surface, form multiple covalent attachments with the polymer. This ensures retention of the enzyme in the polymeric material. Furthermore, as described below, enzymes prepared in this way maintain

considerable stability under normally denaturing conditions. A number of methods have been used to prepare bioplastic or enzyme-polymer composite materials with OP-degrading enzymes and these are discussed below.

Enzyme-containing polyurethanes are ideal matrices due to their ease of preparation, the large range of polymer properties that can be prepared, and the ability for multipoint covalent attachment of the enzyme to the polymer. These bioplastics are prepared by reacting a polyurethane prepolymer that contains multiple isocyanate functionalities with an aqueous solution containing the enzyme. Both foams and gels can be prepared depending on the reactivity of the isocyanate (125). Foaming occurs during the reaction of the isocyanates with water to form carbamic acid, which degrades to carbon dioxide and an amine. If carbon dioxide is generated at a fast rate, it will generate a porous foam structure. Amines react with the isocyanate to crosslink the polyurethane matrix. Any enzyme that is present in the aqueous solution can participate in the polymer synthesis via the lysine residues on the surface, effectively creating an enzyme-containing polymer network with multi-point attachment (125, 126). Havens & Rase were the first to investigate the incorporation of OPH into a polyurethane sponge (127); this approach has also been studied extensively in our laboratory (127–131).

A detailed kinetic analysis of OPH incorporated into polyurethane foams showed that no internal or external diffusion limitations exist in aqueous media. Furthermore, more than 50% of activity retention was observed with a modest increase in the K_M from 0.047 mM to 0.124 mM (127). The multipoint covalent attachment of the enzyme-polyurethane affords very high stability, increasing enzyme half-live from 1.8 days for soluble enzyme in buffer at ambient conditions to 278 days for the immobilized preparation (128). The enzyme-containing polyurethane also had increased thermostability at 50°C, increased resistance to proteolytic attack, and increased resistance to buffered bleach solutions when compared to soluble enzyme (128, 132). LeJeune et al. also prepared polyurethane foams containing AchE, reporting that 90% of available enzyme activity was retained within the polymer during synthesis. The AchE-foams were highly active after storage for two full years (132, 133).

Drevon & Russell described the incorporation of the nerve agent–degrading enzyme DFPase into polyurethane foams (130). The activity of the DFPase in the bioplastic was shown to be limited by internal diffusion. However, some return of activity was achieved by the addition of Pluronic surfactants during the immobilization process, which brought the catalytic efficiencies up to 67% of the soluble enzyme. Thermostability assays of DFPase-polyurethanes revealed that the immobilized DFPase follows biphasic deactivation kinetics, quickly losing nearly 90% of its activity, followed by the formation of a hyperstable active form of the enzyme. This finding was supported by studying the inactivation of PEG-DFPase using circular dichroism, which suggested that the hyperstable form of the enzyme has an increased content of β-sheet.

In an extension of the work using polyurethane foams, Drevon et al. prepared water-borne polyurethane coatings containing DFPase (134). Water-borne polyurethane coatings are prepared by reaction of an aqueous polyester-based

polyol dispersion with a water-dispersible aliphatic isocyanate. Film formation occurs at room temperature by the evaporation of water. As this takes place, crosslinking occurs by condensation between the hydroxyl groups on the polyols and the polyisocyanates. This is an ideal matrix for enzyme immobilization because no solvent is required and the amine functionalities on the enzyme can react with the isocyanates (134).

When DFPase was added to a water-borne polyurethane preparation, it was shown to be homogeneously distributed throughout the coating matrix and coupled irreversibly to it (134). The enzyme-containing coating hydrolyzed DFP at high rates in buffered medium, retaining approximately 39% of its intrinsic activity. Furthermore, DFPase was very stable in the coatings, losing only 40% activity after 100 days of storage at room temperature. DFPase also exhibited enhanced thermostability, exhibiting biphasic deactivation kinetics at 65°C, similar to what was seen with DFPase in polyurethane foams (131, 134).

Gill & Ballesteros have investigated *P. dimunita* organophosphate hydrolase immobilized in sol-gel polymers and enzyme-polymer composite materials (135, 136). They developed a sol-gel encapsulation technique that employs poly(glyceryl silicate) (PGS) rather than the conventional poly(methyl silicate) (PMS). When they compared the efficiencies of OPH immobilized in sol-gel materials with OPH immobilized in polyurethane foam, they observed high activity retention in the PGS-derived sol-gel (94%) and polyurethane foam (68%), whereas the sol-gel prepared using PMS had an activity retention of only 28%. All three preparations had good stability over 700 h at 40°C, with the PGS sol-gel performing best after long time periods (135).

In a more recent work, Gill & Ballesteros investigated the properties of OPH-silicone biocomposites (136). The biocomposites were first prepared by adsorption of the enzyme to poly(hydroxymethylsiloxane) or by covalent attachment of the enzyme to silica activated with 3-isocyanatopropyltriethoxysilane. These enzyme-powders were used as activated fillers in room-temperature vulcanizable (RTV) silicone polymers. These protein-silicone biocomposites can be prepared with a wide range of physical properties and can be formed into biocatalytic sheets, thick films, granulates, and solid foams (137). The activity retention of OPH in OPH-silicones ranged from 49% at very high enzyme loading to 67% at low enzyme loadings. When used as packing in continuous reactors in the presence of 10% v/v isopropanol, the silicone polymers were, in general, able to handle larger OP fluxes than OPH-polyurethane. Furthermore, silicone composites had better physical stability (especially in the presence of high solvent concentrations) than polyurethanes, which swelled slightly and began to disintegrate after long-term operation.

Biomaterials Against Biological Weapons

DECONTAMINATING SURFACES FOLLOWING A BIOLOGICAL ATTACK Decontamination of areas exposed to most of the biological threat agents is not considered a priority activity (50–55). Botulism toxin in the environment will decay at a rate of

about 1% per minute, and the current recommendation is to avoid contaminated areas for hours to days to allow the inactivation of the toxin (55). The viral agents are all very sensitive to environmental exposure (53, 54). The original agents used in the attack will have been inactivated by environmental factors before any disease is detected. The persistence of the viral agents in patient contaminated linens varies, but these materials are easily decontaminated by hot water and bleach. The situation with *Yersinia pestis* is similar to that of the viral agents. The *Y. pestis* is very sensitive to sunlight and heat. Persistence of the plague bacilli in the environment is thought to be less than 1 h after the dissipation of the primary aerosol (51). The situation is only slightly worse for *Francisella tularensis*. The bacteria may survive for extended periods in cold moist environments, but is quickly killed by heat and desiccation (50). There is little danger from secondary dispersal of the organisms once they have settled onto a surface. Surfaces are easily decontaminated with a 10% bleach solution.

In contrast to the other agents, *B. anthracis* spores demonstrate long-term persistence in the environment. Because of the particle size and density of weaponized anthrax spores, there is considerable tendency for re-aerosolization of spores after the original aerosol has settled to surfaces. This was found to be the case in the Senate office building following the 2001 attacks (52). In the case of anthrax, therefore, anything that diminishes the risk of re-aerosolization will reduce the risk of infection. The recommendation for thorough decontamination following an anthrax attack (52) could, in effect, be met by a self-decontaminating surface.

SELF-DECONTAMINATING SURFACES A long-standing approach to the production of biocidal surfaces has been to synthesize materials that release antimicrobial materials into the environment (see, for example, References 138–140). This approach is useful if the goal is to develop materials that will be used for indwelling medical devices whose working lifetime will not exceed the time required for all of the antibiotic to leach from the material. The drawbacks to these materials when they are considered for mediation of bioterror/biowarfare attacks is that they will become ineffective over relatively short time periods and that they will leach potentially toxic materials into the environment. A much better solution to this problem is materials with significant antimicrobial activity, which do not release toxic compounds into the environment. These types of materials would have a much longer useful lifetime when deployed as passive barrier devices.

The current trend in biocidal materials for surfaces is to prepare polymers that are modified with quaternary amine groups (141–144). Quaternary ammonium salts are well known as having biocidal activity (143). The use of soluble versions of these compounds or of entrapped compounds has all of the same problems as do the controlled release materials described above. By preparing surfaces with covalently bound quaternized ammonium, the problem of environmental release of toxic material is minimized (142). Surface modification can be performed with a variety of materials and can be applied to different surfaces. The use of polyurethane supports for the quaternized ammonium salts allows the coating of a number of

different surfaces (141, 142). Other polymer backbones can be used as supports allowing for a number of different materials (142, 145). Because the active material does not leach from the surface, the material could be prepared for long-term use and multiple exposures.

SELECTIVE RESPONSE TO BIOWEAPONS The biocidal materials prepared with quaternary ammonium salts are able to kill bacteria on contact. The major drawback of such materials is that they are not selective in their activity. A material that can selectively kill only those organisms that are a real danger would have the advantage that it could couple the killing activity with a specific detector. Generating a material that would specifically and simultaneously bind, kill, and detect anthrax would be ideal for a passive barrier. Work in very different areas has begun to point the way to the creation of such materials.

The bacteriophage, viruses that infect bacteria, are quite host specific, with some infecting only specific strains within a bacterial species. A protein is produced as part of the viral replication cycle that will digest the bacterial cell wall to allow the progeny phage to escape the cell. These proteins, called lysins, are often species specific and therefore fit the criteria for a material that can selectively destroy a threat organism. Such a protein has recently been purified that specifically kills only *B. anthracis* (146). This protein will lyse vegetative cells of *B. anthracis* and, by measuring ATP from the resulting release of cytoplasm, the presence of the bacteria can be detected (146). Because all bacteria are infected by bacteriophage, a similar protein can be found for any bacterial strain. A material with immobilized lysin and a mechanism to capture specific bacteria could fulfill the requirements for an ideal passive barrier.

We have been working on the preparation of polyurethane coatings that contain immobilized antibodies (R.R. Koepsel & A.J. Russell, manuscript submitted). Antibodies have the ability to specifically bind a target antigen. We have shown that this ability is maintained when the antibody molecules are immobilized. These materials can bind and hold an organism that would then be attacked by a lysin or other biocidal substance, thus providing an additional layer of selectivity.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent terrorist events have shown that chemical and biological attacks are more than just an academic possibility. Because chemical and biological agents pose such a deadly threat, enormous efforts will need to be focused on the protection of military and civilian personnel. Current decontamination protocols, including the use of incineration and chemical decontamination solutions that may be ideal for decommissioning chemical stockpiles or decontaminating large areas after chemical or biological exposure are not effective for the protection of personnel. The development of a new generation of biomaterials is required that can simultaneously act as a barrier, a reporter, and a decontaminator for both chemical and

biological weapons. Such materials should be active on contact with an agent and retain activity over long periods of time.

Several current lines of research are pursuing the development of self-decontaminating materials that offer a passive means of protecting personnel and equipment from chemical and biological agents. Currently these materials do not have the ability to sense the presence of the agent. New materials that are selective and specific are on the horizon. With future advances in biotechnology and material science, we can expect these "smart materials" to play an integral role in personal protections. These types of materials will find uses not only in the defense industry, but in the biomedical, food, and chemical industries as well. The merging of biotechnology with materials science offers a plethora of possibilities for the design of advanced materials that are only now being investigated.

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