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Multifunctional coatings created using an antimicrobial polymer as a platform for titania precipitation on cotton

June S. Lum,[1] Stephen S. Salinas,[1,2] Shaun F. Filocamo[1]
[1]Development and Engineering Center, US Army Natick Soldier Research, Natick, Massachusetts
[2]Massachusetts Institute of Technology, Cambridge Massachusetts
Correspondence to: S. F. Filocamo (E-mail: shaun.f.filocamo.civ@mail.mil)

ABSTRACT: Titania precipitation on cotton has been achieved using a commercially available antimicrobial polymer, Reputex 20. The cotton swatches precipitated with titania retain antimicrobial activity, and we have also shown the ability to encapsulate diisopropylfluorophosphatase (DFPase), an enzyme capable of breaking down organofluorophosphates. Cotton swatches are easily prepared and precipitation occurs at room temperature in aqueous solutions at neutral pH. Both the antimicrobial properties of Reputex 20 and the hydrolytic activity of the DFPase enzyme are retained after titania precipitation, generating a cotton material exhibiting multifunctional properties. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43199.

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INTRODUCTION

Soldiers in the field are exposed to various threats such as bacterial infections, chemical contamination, and environmental uncertainties. While materials are available to deal with each of these individual threats, multiple layers would be required for the soldier to have all of these protections. This would increase the load affecting the Soldier's mobility, and potentially negatively mitigating any benefit. The ability to combine protective functionalities into one material or coating would be advantageous for improving the protection of the Soldier at a significantly lower load cost. However, most textile treatments are designed to address one issue at a time (e.g., moisture wicking, insect repellent, or antimicrobial). Multifunctional materials are desired for textile modification and studies are underway using the use of metal oxide-based coatings.

Recent studies have illustrated methods to modify textiles with metal oxides, which have the potential to provide novel functionality to the fabric. Some recent research into nanoparticle cotton modification has illustrated the incorporation of titanium oxide nanosols,[1–4] zinc oxide nanostructures[5] or CdSe and CdTe nanowires onto the fiber surfaces.[6] Focusing on the widespread use of titania based materials, the applications that would be advantageous on fabric surfaces include: self-cleaning properties,[3,7,8] UV-protection,[2,3,9,10] hydrophobicity[1] and bacteriocidal properties with the incorporation of silver particles.[8,11,12] However, most of the reported procedures for applying titania involve solvo-thermo processes, which are unfavorable for the incorporation of environmentally sensitive materials such as enzymes. A recent report of applying Ag-doped TiO_2 nanosols as a coating to cotton fabrics at relatively low-temperatures still required heating and curing treatments of 120 – 150°C.[8] Other methods to incorporate titania nanoparticles into textiles for biocidal activity utilize highly acidic conditions[13] or UV light.[14] Some of these nanoparticle treatments are not as susceptible to degradation due to standard textile manufacturing conditions such as high temperatures and caustic reagents. However, most of these materials cannot provide many of the advantages that biologically derived materials have to offer.

Biological materials such as enzymes are attractive for targeted protection because they have been naturally engineered with high specificity and selectivity. Unlike many synthetic decontaminating agents, enzymes tend to be reusable, have high turnover rates, and generally are considered nontoxic. Incorporation of these types of materials into fabrics has the potential to provide continuous protection against harmful threats, such as chemical nerve agents. One specific example is diisopropylfluorophosphatase (DFPase), which was first isolated from the ganglion of *Loligo vulgaris*.[15,16] DFPase is capable of cleaving a P—F bond in diisopropylfluorophosphate, a highly toxic organophosphate, as well as hydrolyzing other toxic reagents known to be used in chemical warfare, such as sarin, soman, and tabun (Scheme 1).[16,17]

A simple solution is required to combine the broad utility of metal oxides such as titania with the specificity and selectivity

of biologics that maximizes functionality and protection while minimizing load. There are few industrial techniques that use mild conditions (e.g., aqueous, neutral pH, ambient temperature, and pressure) to impart novel functionality onto fabric surfaces, such as cotton. Reputex 20 is a commercially available antimicrobial polymer that is easily applied to cotton and linen fabrics. The main active antimicrobial ingredient is polyhexamethylene biguanide (PHMB—Figure 1) and is commonly found in basic consumer products such as swimming pool sanitizers, contact lens solutions, wet wipes, and antidandruff shampoos. The source of PHMB antibacterial activity is the biguanide functional group, which disrupts the bacterial cell membrane. PHMB has also been extensively researched as an additive to surgical scrubs to prevent the spread of bacterial infections.[18] Reputex 20 can be simply applied as a thin coating to cotton textiles by padding, thereby incorporating an antimicrobial function to the fabric. Reputex 20 is a nitrogen-containing polymer, and one can take further advantage of these reactive handles to create coatings of metal oxides using mild, bioinspired reaction conditions to encapsulate biological materials.

Biomimetic sol–gel processes have also been identified for precipitating TiO_2 nanostructures using peptides which are made up of amine-containing residues.[19] Elaborating upon the knowledge that N-containing histidine residues precipitate titania, block copolymers such as poly(hydroxylated isoprene-*b*−2-vinylpyridine) have been designed and used to precipitate titania.[20] Thin coatings of metal oxides such as titania are receiving attention due to the ease of production and the possibility of encapsulation (and stabilization) of biological species.[21,22] Encapsulation of environmentally sensitive enzymes, such as DFPase, has been achieved and allows for the incorporation of DFPase within a metal oxide coating.[23] This enzyme is fairly stable and a good model enzyme to work with for exploring initial metal oxide encapsulation studies onto fabric-based substrates. This report details the direct precipitation of a titania matrix onto cotton in the presence of DFPase using the reactive handles on Reputex 20. It was shown that under mild reaction conditions, multiple reactive functionalities can be incorporated *in situ* to impart protective capabilities (e.g., biological and chemical decontamination) onto the fabrics. This illustrates the potential for this technique to provide novel combinations of functions to passive surfaces, while also exploring the impacts that each added functionality may have on another.

EXPERIMENTAL SECTION

Materials

The recombinant enzyme, DFPase was purchased from Codexis (proteinaceous mixture containing approximately 40% enzyme) and used without further purification. The polymer, commercially known as Reputex 20 Microbiocide solution [20% w/w PHMB or poly(iminoimidocarbonyliminoimidocarbonyliminohexamethylene hydrochloride)] was acquired from Lonza. Titanium(IV) (bisammonium lactato) dihydroxide (TBALD—50 wt % solution in water), diisopropylfluorophosphate (DFP), piperazine-*N*,*N*′-*bis*(2-ethanesulfonic acid) (PIPES), Trizma base, and Borax were purchased from Sigma-Aldrich. Mannitol Salt Broth

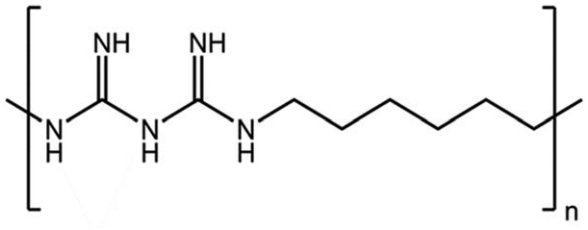


Figure 1. Structure of PHMB, the active antimicrobial ingredient in Reputex 20.

(Himedia) and Agar (Acros) were used to prepare mannitol salt agar plates and soft agar for overlay studies. Dey-Engley (DE) broth was obtained from BD Falcon, and nutrient broth was obtained from Sigma Aldrich. *Staphyloccocus aureus* strain 27217 was obtained from American Type Culture Collection (ATCC). Ultrapure sodium dodecyl sulfate (SDS) solution (10%) was obtained from Life Technologies and diluted with water prior to use for initial laundering studies. Fluoride standard (0.1 M) and the bichichoninic acid (BCA) protein assay kit were obtained from Fisher Scientific and used as directed. Protein Assay Dye Reagent Concentrate for the Bradford assay was obtained from Bio-Rad. Deionized (DI) water was obtained using a Thermo Scientific Barnstead Smart2Pure water purification system.

Methods

Cotton Fabric Preparation. Cotton fabric pieces were "scoured" in an aqueous 2% w/v Borax solution (heated to between 80°C and 90°C) for 1 h to remove any residual chemicals (sizing) from the manufacturer. Swatches were then wrung out and placed in a heated DI water bath for 5 min to rinse out the Borax. Cotton pieces were then laid out to air-dry overnight. Larger cotton pieces were cut into manageable swatches (1″ × 1″, 2″ × 1″, or 2″ × 2″) using a pneumatic dye cutter. Reputex 20 was deposited onto substrates by immersing the swatches in aqueous solutions of 2.5% Reputex 20 for 15 min. Depositions onto swatches occurred inside petridishes and solutions were agitated on a mini shaker. Swatches were then removed with tweezers and placed in an oven at 120°C for 15 min. Dried swatches were then rinsed briefly for 5 min in DI water to remove excess polymer and laid out to dry on the bench top.

Titania Precipitation and DFPase Encapsulation. Reputex 20-coated swatches were wet in 25 m*M* Tris HCl buffer (pH 7.5) in disposable 25-mL petridishes and titanium(IV) (bisammonium lactato) dihydroxide (50% TBALD) was introduced to yield a final concentration of 1% TBALD. Petridishes were placed on an orbital shaker set at 250–300 rpm for 1–6 h at room temperature (RT). Afterward, swatches were removed from precipitation solutions and washed four times with DI water. Swatches were left to dry on paper towels at RT overnight. If enzyme needed to be introduced, varying concentrations of protein/DFPase solutions (protein concentration range of 5–25 mg/mL) made using Tris buffer were added for *in situ* precipitation. To evaluate another encapsulation method of introducing enzyme, swatches could also be soaked in 5 mL of protein/DFPase solutions for 15 min (excess solution dabbed off), before being placed in a 1% solution of TBALD in Tris buffer. The washing

steps after precipitation for "soaked" swatches remained the same. Washing steps are necessary to remove any excess "adsorbed" DFPase and excess reactants such as TBALD.

BCA or Bradford Assay. Swatches were soaked for 15 min in DFPase solutions prepared in Tris buffer at a concentration of 5 mg/mL. Excess enzyme solution was removed by placing swatches on the insides of falcon tubes. Tubes were then spun down at 300 rpm for 60 s. Centripetal force helped remove excess protein solution and was also collected for protein assays. DFPase-soaked swatches were then placed in 1% TBALD solutions to allow precipitation. Protein analysis via BCA Method or Bradford Assay were conducted on the protein solutions before and after soaking to determine the amount of protein absorbed by the cotton swatches. Samples were run in triplicate and values reported are averaged.

Fluoride Assay for DFPase Activity. The Orion Dualstar pH/ISE meter system with the fluoride half-cell ISE (94-09BN) and sure-flow single-junction reference electrode (90-01) from Thermo Fisher Scientific were used to obtain the fluoride concentration data for DFPase activity measurements. Tests were performed in small pyrex dishes. Each dish was rinsed in DI water and dried before use. The electrodes were immersed in the dish and cotton swatches were soaked for 3 min in 7 mL of PIPES buffer (25 m*M*, pH 7.5) before measurements were initiated with the addition of 3 mL of 0.01 *M* DFP solution. The fluoride concentration of the resulting solution was measured for 3 min, and data was collected from the fluoride meter (linked by HyperTerminal software) at intervals of 5 s. Data were collected on three separate swatches from each set and averaged. Initial rates are calculated as M/s after converting fluoride meter data from ppm to molarity.

Scanning Electron Microscopy and Energy-Dispersive X-ray Spectroscopy. Images were collected on a Zeiss EVO 60 scanning electron microscope (SEM) equipped with a tungsten filament and Energy Dispersive Spectroscopy (EDS) measurements were used to analyze elemental composition on swatches. EDAX genesis software was used to analyze the spectra obtained from EDS. Swatches were cut down to fit onto SEM studs and coated with gold–palladium using a Balzers Med 010 sputter coater set to 30 mA for 180 seconds. SEM images and energy-dispersive X-ray (EDX) spectroscopy spectra were taken at 1000× magnification to determine the extent of polymer and titania coating.

Antimicrobial Test Methods. *S. aureus* (ATCC 27217) was inoculated into nutrient broth (37°C) and grown to achieve an optical density (OD) of one at 600 nm. For AATTCC 147, five streaks of bacteria culture were applied across a mannitol salt plate using a sterile loop and cotton swatches (precipitation time was held at 2 h) were laid across the streaks. For the bacterial lawn overlay study, cells were diluted tenfold using nutrient broth and 20 mL were spiked into 7 mL of mannitol soft agar (kept warm at 55°C). The soft agar was then poured over cotton swatches laid on mannitol salt agar plates.

Escherichia coli (BZB2116—Pugsley Strain[24]) was inoculated into nutrient broth (37°C) and grown to achieve an OD of one at 600 nm. Bacterial lawn overlay studies were done with diluted cells; 20 mL were spiked into 7 mL of nutrient soft agar (kept warm at 55°C). The inoculated soft agar was then poured over cotton swatches laid on nutrient plates.

Plates were covered and allowed to solidify at RT. All plates were then stored overnight in a Shel Lab General Purpose Series incubator set to 37°C for 16–20 h. All samples were run in triplicate. Zones of clearing (ZOC) were evaluated and images captured using the camera in the Q-Count Instrument (Spiral Biotech).

Quantitative antibacterial activity against *S. aureus* and *E. coli* was evaluated using AATCC Test Method 100. Immediately following precipitation of swatches, three control swatches and sets of three treated swatches, with varying titania precipitation times, were inoculated. For inoculation, *S. aureus* (ATCC 27217) or *E. coli* (BZB2116) was grown from frozen glycerol stocks in 10 mL of nutrient broth and incubated for 6 h on a 37°C shaker. Once an OD of one had been achieved (approximately 10^7 CFUs), the culture was diluted by a factor of 10 using nutrient broth. 100 mL of the diluted culture was used to inoculate each of the precipitated swatches. Cotton swatches were inoculated in sterile plastic petridishes, and then placed in an incubator at 37°C for 18 h. Swatches were then placed into 5 mL of DE neutralizing broth and vortexed for 5 min to allow bacteria to fall off into solution. Serial dilutions up to 10^{-4} for controls and 10^{-3} for precipitated samples were created using 20 m*M* sodium phosphate buffer (pH 7.2). Solutions were then tested using TEMPO selective *S. aureus* media (TEMPO STA) or selective *E. coli* media (TEMPO EC) according to manufacturer's instructions.

After quantitative antimicrobial fabric tests (AATCC 100) were performed, the bacterial counts were determined using a TEMPO system. The TEMPO system and TEMPO STA and TEMPO EC supplies were purchased from the manufacturer, BioMérieux. The TEMPO system has been validated by the AOAC Research Institute as a Performance-Tested Method.[25] TEMPO STA or TEMPO EC are automated tests for the enumeration of gram-positive staphylococci (*S. aureus*) or gram-negative bacterium (*E. coli*), respectively, based on the format of the TEMPO MPN procedure (miniaturized card containing 48 wells across three different dilutions for the automatic determination of the MPN[26]). The system fills the card, and after 24–27 h incubation at 37°C, automatically reads the card and calculates the MPN as CFU/mL.

Lab-Scale Laundering Conditions for 1″ × 1″ Cotton Swatches. Initial studies on the durability of the titania-Reputex 20 coatings were evaluated using a modified bench-top experiment. Cotton swatches were prepared as described previously with a titania precipitation time of 4 h and dried in air overnight. Scoured cotton swatches and Reputex 20-coated swatches were studied along for comparisons. The 1″ × 1″ swatches were subjected to three different conditions: water only washing, 1% SDS washing, and 1% SDS washing followed by a 1 h heating cycle at 120°C. During the washing phase, individual swatches were placed into 5 mL of solution (DI water or 1% SDS) contained in a 50 mL Falcon tube and vortexed on high (speed 10) for 10 min. Each swatch was then transferred to another tube

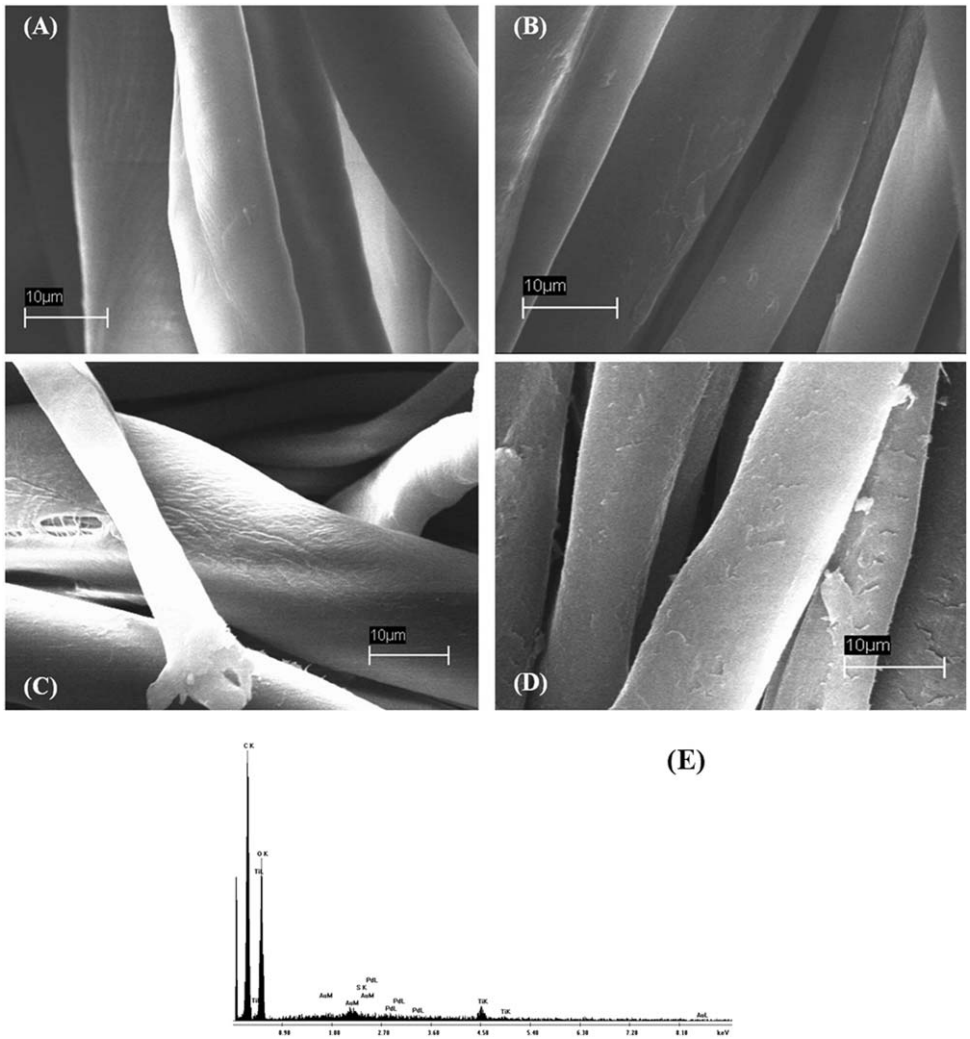


Figure 2. SEM images of cotton swatches (A) scoured cotton control, (B) Reputex 20-coated cotton, (C) Reputex 20/titania-precipitated cotton, (D) Reputex 20/Titania/encapsulated DFPase on cotton. (E) EDX spectrum of Reputex 20-coated cotton swatch precipitated with TBALD and DFPase.

containing 10 mL of DI water, and vortexed at the same speed for 10 min to "rinse" the fabric squares. Three separate rinse cycles were performed after washing, and one set of SDS-treated swatches were laid to dry in an oven set to 120°C. All swatches were left out on the bench top at room temperature for 2 h prior to overlay studies. Overlay studies were done in duplicate and retained antimicrobial function (zone of clearing) was evaluated against *S. aureus* using mannitol-based soft agar and mannitol salt agar plates.

RESULTS AND DISCUSSION

Precipitation and DFPase Encapsulation

Titania deposition using the polymer, Reputex 20, was successful and SEM and EDX confirmed the presence of titanium on the cotton swatches (Figure 2). The SEM images show there is a slight gradual increase in the surface roughness of the cotton filaments with the application of Reputex 20 and accordingly with the titania precipitation. SEM of scoured cotton swatches [Figure 2(A)] show very little surface features, while precipitated Reputex 20/titania/DFPase cotton filaments show roughening of the surface and flaking consistent with a coating [Figure 2(D)]. Reputex 20 contains the active ingredient: PHMB. PHMB contains multiple nitrogen-containing functionalities, which is responsible for the condensation and precipitation of titanium dioxide from an aqueous solution of TBALD. Previous studies using amine modified Sephadex beads[23] and protamine[27] successfully demonstrated the need for nitrogen-containing functionalities to generate titania. Recent examples of nitrogen-containing compounds that precipitate titania include poly(hydroxylated isoprene-*b*−2-vinylpyridine) block copolymers,[20] and peptide-derived biomineralization.[28] Reputex 20 is applied via padding[18,29] and adheres directly to the fibers of the swatch, and the titania coating is deposited on top of the antimicrobial polymer after precipitation. Due to the cationic nature of PHMB in Reputex 20, ionic, and hydrogen bonding are believed to be the driving forces for cotton attachment.[30] A layering approach is adopted to build the functionalities onto the textiles. The mild precipitation, initiated by Reputex 20, proceeds quickly at room temperature, and a precipitation time of approximately 2 h was needed to generate enough titania

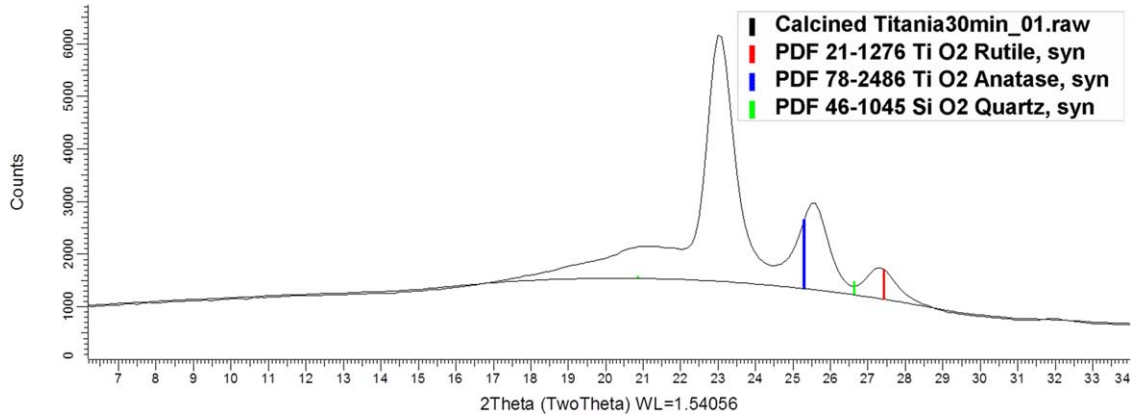


Figure 3. XRD spectrum for calcined titania sample with anatase (blue) and rutile (red) peaks referenced. The diffraction pattern for quartz is also labeled in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

coating to register a titanium peak using EDX spectroscopy [Figure 2(E)]. There was a slight difference in the amount of titanium present when precipitation times were extended beyond 2 h, but the difference is within the range of standard deviation. Increasing the precipitation time did not correlate to higher rates of DFPase activity, however, changes in antimicrobial activity were realized for increased titania coating (see further). The titania deposited is an amorphous form since the precipitation is completed at room temperature, and XRD was studied to confirm that a titania-based material can be made to encapsulate the DFPase. A lysozyme-mediated synthesis of titania and silica was reported at room temperature to generate amorphous metal oxides that retained antibacterial properties.[31] A mild precipitation procedure was used to insure that DFPase remained active during and after titania deposition.

X-ray Diffraction Studies

Since the precipitation conditions were mild, the resulting swatch sample most likely contained amorphous titania. X-ray Diffraction (XRD) studies were used to confirm titania precipitation from a Reputex 20 solution (2.5% v/v). Titania powders obtained from solution studies were collected and dried at room temperature. The room-temperature dried samples did not display any peaks corresponding to crystalline forms of titania after blank subtraction. The lack of crystalline peaks for the room temperature precipitation is not surprising as we propose amorphous titania is being deposited onto the cotton swatches. To increase the crystallinity for XRD, some of the powdery solid (generated at room temperature) was treated to calcination at 700°C (inside quartz XRD tubes) for 24 h to remove excess polymer and other organic residue. The XRD spectrum (Figure 3) of calcined titania samples showed two peaks that match the powder diffraction patterns for synthetic forms of both anatase and rutile phases of titania. The XRD studies confirmed that amorphous titania material was generated using Reputex 20 during room temperature precipitation studies and that if desired, titania crystallinity could be improved with heating. Cotton swatches with precipitated titania and DFPase were not heated due to the instability of the enzymatic species, and the activity was studied after encapsulation.

DFP Hydrolysis and DFPase Assay Studies

The enzyme DFPase, is readily encapsulated within the titania matrix, and coated cotton swatches were tested for activity. The fluoride release is measured over a period of 3 min and Table I lists the rate data obtained for the hydrolysis of DFP after the DFPase encapsulated cotton swatches are equilibrated in buffer solution. After the *in situ* precipitation time of 1 h, the average rate of F^- generation was 5.75×10^{-7} M/s, and for 2 h, the hydrolysis rate was 5.30×10^{-7} M/s, and for 6 h, the rate was 4.45×10^{-7} M/s. There is a slight trend in loss of activity and increase in standard deviation for the hydrolysis rates when the *in situ* precipitation time is increased from 1 to 6 h. As the precipitation time is increased for *in situ* studies, two possible reasons for lower rates can be considered. First, previous studies have shown that the amount of titania coating deposited increased as the precipitation time increases. The increased deposition of titania would account for the higher standard deviations in relative rates as precipitation time was changed to 6 h. As the titania coating grows, the enzyme is further embedded within the oxide matrix, which would also reduce the diffusion rate of DFP substrate to the DFPase active site. Second, the DFPase enzyme might be inactivated with the prolonged exposure to TBALD solution during the lengthier precipitation studies. Control studies were performed by exposing protein/DFPase solutions to the same concentration of TBALD and also to

Table I. Relative Initial Rates of DFP Hydrolysis Obtained from Solution Studies.

Solution condition (protein mass)	Average initial rate × 10^{-7} (M/s)	Relative decrease
Protein only (0.01 mg)	40.8	n/a
1% Reputex 20 solution (0.01 mg)	20.2	2.0
1% TBALD (0.01 mg)	3.95	10.3
1% Reputex 20 and 1% TBALD (precipitate formed)	1.12	36.4

Protein concentrations are based on the amount of dissolved enzyme.

Table II. Average initial Rates for Precipitated Swatches after Soaking in PIPES Buffer to Determine Protein Desorption

Swatch precipitation studies after initial soaking to determine protein desorption					
Protein concentration (mg/mL)	Ppt time (h)	Method	Average initial rate $\times 10^{-7}$ (M/s)	Drying time (RT; days)	Relative decrease
25	2	*In situ*	2.68	5	2
25	2	*In situ*	2.13	8	2.5

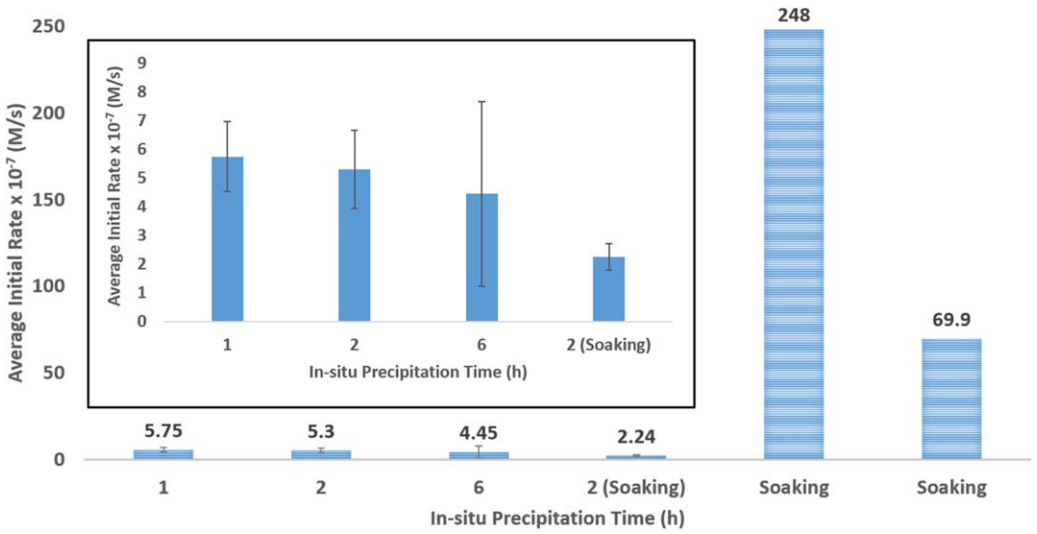


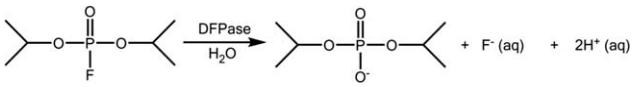
Figure 4. Relative initial rates of DFP hydrolysis (F^-generation measured in ppm and converted to M/s) obtained from swatch studies. Inset shows enlargement of rates from *in situ*-precipitated swatch studies. Protein concentrations were held at 25 mg/mL for precipitation studies and 5 mg/mL for adsorbed cotton swatches. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Reputex 20 used in precipitation studies. Studies using a reduced concentration of protein/DFPase were measured to examine the effects of introducing a solid surface into the solution-based assays to determine if adsorption to cotton swatches lowered hydrolysis rates.

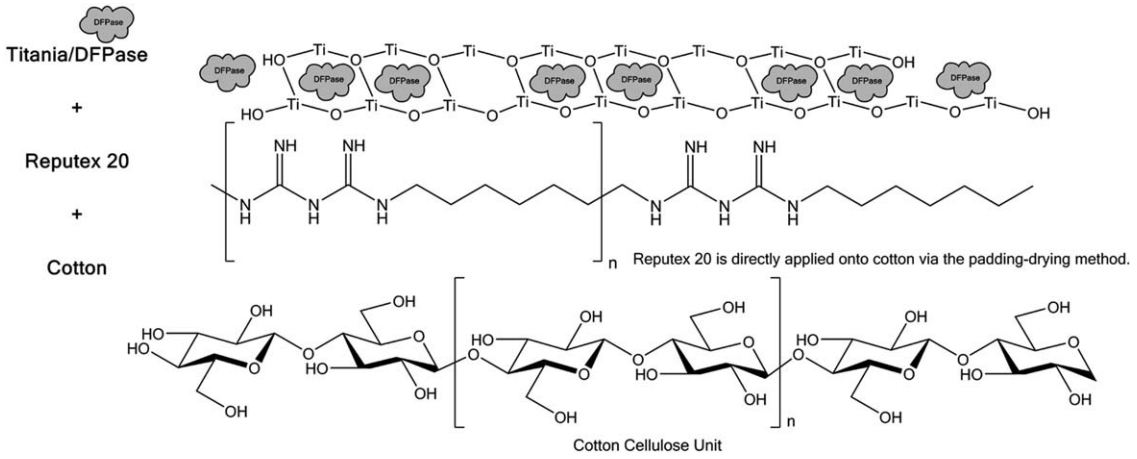
In the solution studies of DFPase, decreases in rates of fluoride release correlated to exposure of the enzyme to Reputex 20 and to TBALD. Similarly, there was a twofold decrease in hydrolysis rate when the DFPase is stirred with 1% Reputex 20 solution and a tenfold decrease when solution studies contained 1% TBALD solution (Table I). Recent studies showed a 15-fold decrease in substrate turnover when AChE enzyme was exposed to cationic polymer used in inkjet-printed sol–gel coatings on paper.[32] Even in the absence of cotton swatches, when precipitation is initiated *in situ*, the rate decreases by a factor of 36× relative to unchanged DFPase, which could correlate inactivity to TBALD exposure and the encapsulation of the DFPase into a solid matrix. With further encapsulation of any remaining active enzyme, the ability of DFP to reach the DFPase active site is also slowed relative to DFPase in solution. Soaking cotton swatches containing Reputex 20 in the buffer solution while DFP is introduced does not significantly change the rate of F^- release in solution, which means there are no absorption effects from having the cotton fabric matrix present (data not shown).

The swatch DFPase F^- assay results can be compared to the rates of F^- generated by adsorbed protein onto cotton swatches. The average initial rate for swatches soaked with 5 mg/mL protein was determined to be 248 $\times$ 10^{-7} M/s. The DFPase contained within the titania matrix is proposed to be released at a much slower rate when comparing to protein adsorbed onto cotton swatches. When the protein is just adsorbed to the fabric, the increased rate of DFP hydrolysis would just correspond to a single-use and burst release of DFPase enzyme. We propose the increased presence of DFPase when encapsulated within a metal oxide layer over just adsorption onto cotton.[32] To determine the effect of protein desorption into solution, adsorbed cotton swatches, and precipitated swatches were soaked in PIPES buffer solutions (these solutions were analyzed using a micro-BCA kit to determine protein concentration released—see further) and dried once prior to DFP hydrolysis studies.

The initial DFP hydrolysis rate for adsorbed cotton swatches post-protein desorption studies was reduced from 248 $\times$ 10^{-7} to 70 $\times$ 10^{-7} M/s (an average rate decrease of 3.5 times).



Scheme 1. Enzyme hydrolysis of diisopropylfluorophosphate (DFP) by DFPase.



Scheme 2. Proposed chemical scheme of DFPase incorporated into layers of titania-Reputex 20 and coating interactions with cellulose units of cotton.

However, for titania-precipitated swatches, the hydrolysis rate decrease is not as pronounced after the initial soaking to determine protein release. Table II lists the change in initial rates for dried-precipitated swatches and shows that the decrease in rate is only 2–2.5 times less than the average rate for swatches that were not presoaked and dried prior to assay studies (Figure 4: 2 h titania precipitation time, initial hydrolysis rate of 5.3×10^{-7} M/s). The smaller change in hydrolysis rate for titania-precipitated swatches supports our hypothesis that DFPase remains present longer compared to enzyme-adsorbed swatches. Also, prolonged storage of the dried-precipitated swatches for over a week did not dramatically impact the DFP hydrolysis rate.

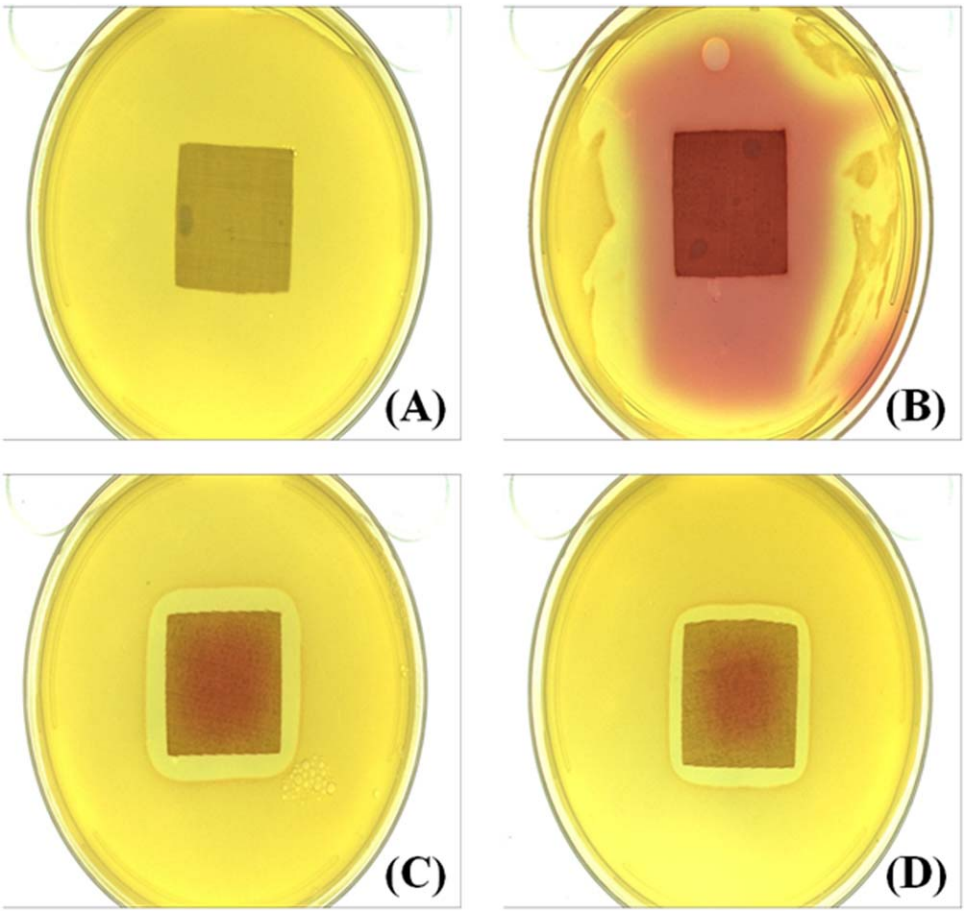


Figure 5. Cotton swatches overlaid with *S. aureus* 27217 on mannitol salt agar plates: (A) scoured cotton control, (B) Reputex 20-coated cotton, (C) Reputex 20/titania-precipitated cotton (D) Reputex/titania-encapsulated DFPase on cotton. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

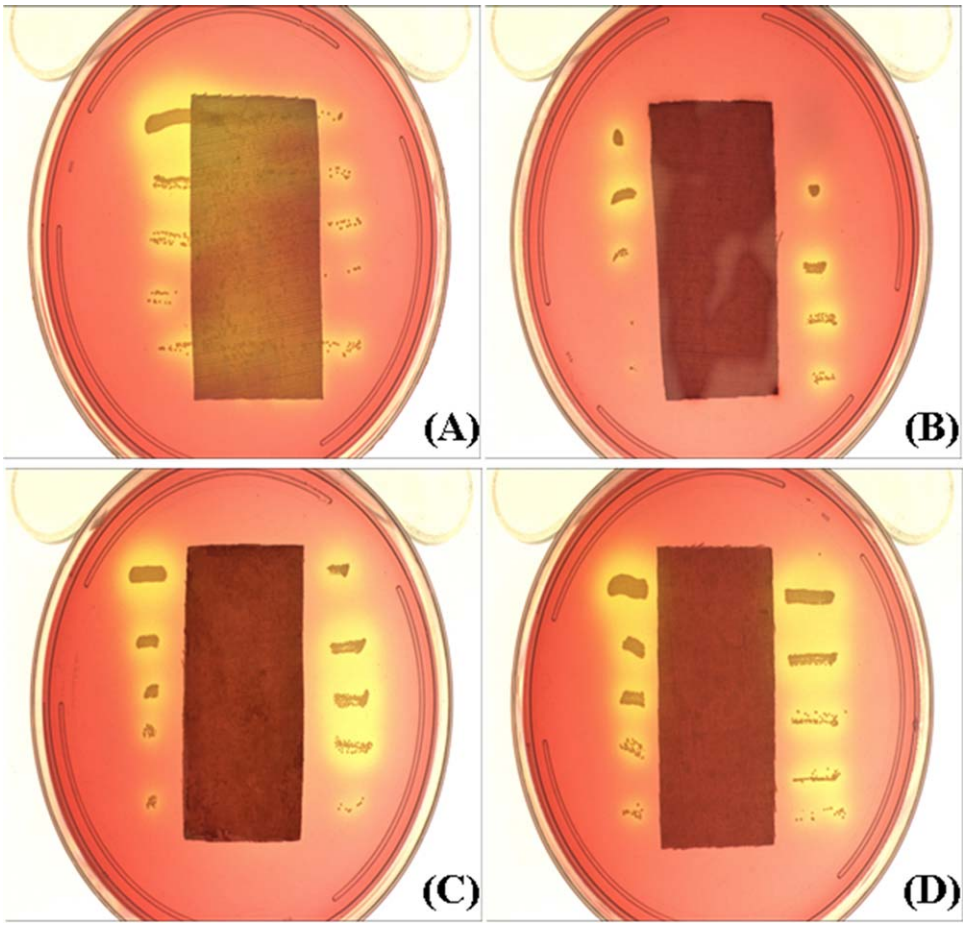


Figure 6. AATCC 147 streak overlay test results with *S. aureus* on mannitol salt soft agar plates. (A) Scoured only cotton, (B) Reputex 20-treated cotton, (C) Reputex/titania-precipitated cotton, (D) Reputex/titania/DFPase-precipitated cotton swatch. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The decrease in hydrolysis rate is about 26-fold less for precipitated swatches compared to adsorbed swatches. Hydrolytic activity is extrapolated to confirm that active protein/DFPase is encapsulated within the titania matrix on the swatches. BCA measurements on the PIPES solution of soaked/adsorbed cotton swatches accounts for an average concentration of 26.3 μg/mL of protein. For precipitated swatches, the protein concentration was too low and beyond the concentration range for using the micro-BCA kit. This decreased amount of protein released into solution correlates to the lower rates of F^- activity for precipitated swatches compared to adsorbed swatches. All swatch studies confirm the presence of DFPase after titania precipitation and drying.

The method of precipitation also affects the rate of fluoride release in solution. Comparing the 2-h precipitation time, the *in situ* method is over two-times faster than the "soaking" method. When a swatch is soaked in the protein/DFPase solution, excess solution is removed before placing the "soaked" swatch in fresh 1% TBALD buffer solution. This effectively limits the protein/DFPase that is present during titania precipitation. *In situ* precipitation preserves a high concentration of DFPase in solution throughout the precipitation, providing a steady source of protein or enzyme for encapsulation. The reduced relative rates for presoaking could also be related to the DFPase enzyme being located beneath the generated titania coating, while *in situ* precipitation results in more active enzyme being incorporated throughout the coating and layers of titania (Scheme 2).

The concentration of protein soaked into the Reputex/cotton swatches was determined using BCA. The amount of protein removed from solution prior to precipitation conditions was determined to be approximately 6 ± 1% per square inch swatch. The approximate concentration of protein per square inch of cotton material is calculated to be 8.8 μg/mL. BCA assays after

Table III. *S. aureus* Reduction Results from AATCC 100 Test Method for TiO_2-Reputex 20-Coated Cotton Fabric Swatches

	Swatch type	Percent kill (%)	Log kill
1	Scoured cotton	Control	n/a
2	Reputex 20-coated	99.9	3.58
3	TiO_2-Reputex 20, 1 h	87.09	0.89
4	TiO_2-Reputex 20, 2 h	87.09	0.89
5	TiO_2-Reputex 20, 4 h	61.39	0.41
6	TiO_2-Reputex 20, 6 h	No reduction	No reduction

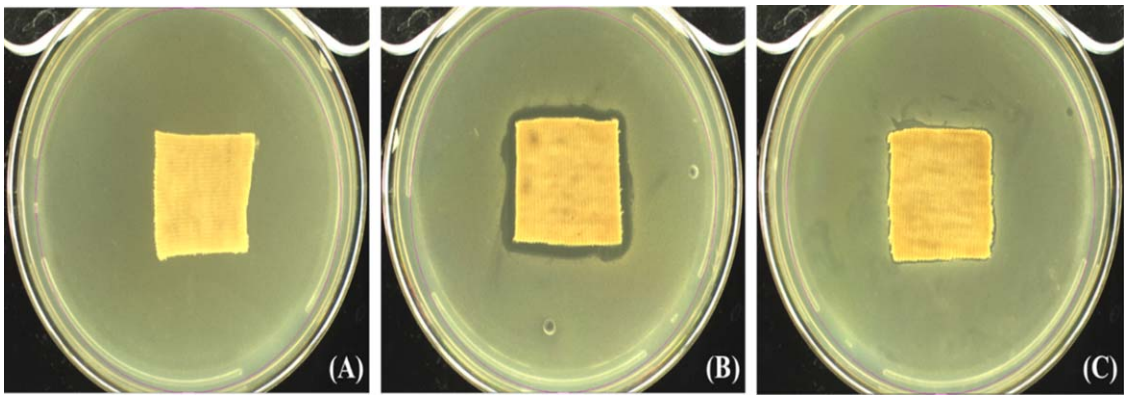


Figure 7. Overlay results for antimicrobial effects against *E. coli*. (A) Scoured cotton—no zone of clearing; (B) Reputex 20-coated cotton—clear zone of clearing; (C) TiO_2-Reputex 20-treated cotton—small zone of clearing. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in situ precipitations could not be determined due to the interference of excess amount of metal oxide present in the solutions. The amount of "soaked" DFPase (encapsulated) is proposed to be less than the amount of protein determined, since the supplied enzyme material was provided as a mixed proteinaceous salt with 40% DFPase purity and contained crude cell debris which increases the overall protein concentration. Retained DFPase activity is encouraging after either precipitation treatment and demonstrates that the precipitation conditions are mild enough to keep DFPase active during encapsulation. Reduced enzymatic activity has been previously documented for DFPase when immobilized within coatings or encapsulated.[23,33,34] Another possible reason for lower enzyme activity was discovered to be the active ingredient, PHMB, in Reputex 20 and the mode of antimicrobial action. PHMB is electrostatically attracted to the negatively charged cell wall of bacteria and disrupts activity by displacing divalent cations necessary to the structure of the cell membrane.[35] The crystal structure of DFPase contains two Ca^{2+} cations, one for structural integrity and the other to maintain the active site for DFP/substrate binding.[16,36] Solutions of protein shaken with 1% Reputex 20 resulted in the production of some insoluble material, based on BCA analysis; approximately 20% protein remained in solution. Further modifications could be introduced to shield the biological entity to insure more stability during precipitation.

Antimicrobial Studies

Reputex 20 has been used previously as an antimicrobial solution to treat cotton fabric[37] or as an additive in nonwoven surgical fabrics.[18] Antibacterial activity is driven by the biguanide functional groups in PHMB, which disrupts the bacterial cell membrane.[18] To the best of the authors' knowledge; this report is the first time that the Reputex 20 polymer solution has been used to precipitate titania from solution. The important thing to note is that after precipitation, the antimicrobial polymer is still active against *S. aureus*, and all Reputex-treated fabric swatches still displayed zones of clearing (ZOC) for overlay studies (Figure 5). Streak tests (AATCC 147) are presented in Figure 6. As expected, the untreated scoured cotton [Figure 5(A)] did not show a ZOC against *S. aureus* and even shows growth underneath the cotton swatch area. All Reputex 20-treated swatches [Figures 5(B–D)] preserve an area underneath the swatch where *S. aureus* has not grown supporting retained biocidal activity of the antimicrobial polymer. Titania-precipitated swatches generated using another polymer treatment do not display antimicrobial activity (data not shown). The ZOC qualitatively shrinks with the addition of titania and further reduces with the encapsulation of protein/DFPase within the titania matrix. Precipitation seems to reduce the activity of Reputex 20, probably due to the Reputex 20 treated fibers being coated by precipitated titania, therefore, allowing less Reputex 20 to leach from the fabric. However, significant biocidal activity is still retained for swatches with titania precipitated from Reputex 20 [Figure 5(C)]. Reputex 20 has been used to apply antimicrobial coatings to hospital gowns in studies involved in durability studies,[18] and we propose that the Reputex 20 layer remains durable after being coated by precipitated titania. Streak tests with larger swatches also demonstrated biocidal activity against *S. aureus*. As the coverage increases from Reputex 20 coated [Figure 6(B)] to titania precipitated/DFPase encapsulated [Figure 6(D)], the antimicrobial area around the cotton swatch shrinks, which confirms decreased leaching of Reputex 20 with precipitation. Streak testing correlates well to the reduction of ZOC in overlay studies with *S. aureus*. Successive overlays would be needed to demonstrate durability of the polymer coating after multiple exposures to *S. aureus*.

Experiments determining quantified biocidal activity against *S. aureus* and *E. coli* were completed for titania-coated swatches using the standardized AATCC 100 test method, an assessment designed to measure the activity of antimicrobial fabrics. Swatches were precipitated using the described protocol and various precipitation times were studied to examine the effect of increased titania coating. Tests were run in-triplicate and results are documented in Tables III (*S. aureus*) and IV (*E. coli*). The results show a clear downward trend in antimicrobial activity against *S. aureus* as the titania precipitation time increases from

Table IV. *E. coli* Reduction Results from AATCC 100 Test Method for TiO_2-Reputex 20-Coated Cotton Fabric Swatches

Swatch type	Percent kill (%)	Log kill
Scoured cotton	Control	n/a
Reputex 20-coated	99.00	2.00
TiO_2-Reputex 20, 1 h	99.00	2.00
TiO_2-Reputex 20, 2 h	99.00	2.00
TiO_2-Reputex 20, 4 h	98.74	1.90
TiO_2-Reputex 20, 6 h	97.44	1.59

1 to 6 h. There is an increase in the amount of titania deposited on the fabric swatches and the Reputex 20 resides below the titania-coating layer. The lower antimicrobial activity is attributed to a decrease in leaching of the antimicrobial polymer, which is supported by the *S. aureus* overlay studies (Figures 5 and 6).

Reputex 20 also demonstrates effectiveness against gram-negative organisms and overlay studies were completed to determine the antimicrobial activity against *E. coli*. The results are depicted in Figure 7 for scoured cotton, Reputex 20-coated cotton, and TiO_2-Reputex 20-coated cotton. As described earlier for *S. aureus*, there is a qualitative decrease in the size of the ZOC around the cotton swatch as the titania coating is added. Results for *E. coli* correlate well with the results from the *S. aureus* studies. The smaller ZOC for titania-coated samples is proposed to be due to a reduction in leaching of the antimicrobial polymer. A protective effect can be envisioned for titania coating preventing the Reputex 20 from being released all at once. Data from quantitative studies for *E. coli* (Table IV) supports reduced leaching of the antimicrobial polymer with the applied titania coating. Both the Reputex 20-coated swatches and titania-precipitated swatches were highly effective at reducing *E. coli* growth, maintaining 99% reduction up to a precipitation time of 2 h. The activity only drops to 97% after a precipitation time of 6 h, when the titania coating reduces the amount of antimicrobial polymer released, however, the titania does not prevent leaching of the Reputex 20 and still maintains reduction of bacterial growth for *E. coli*.

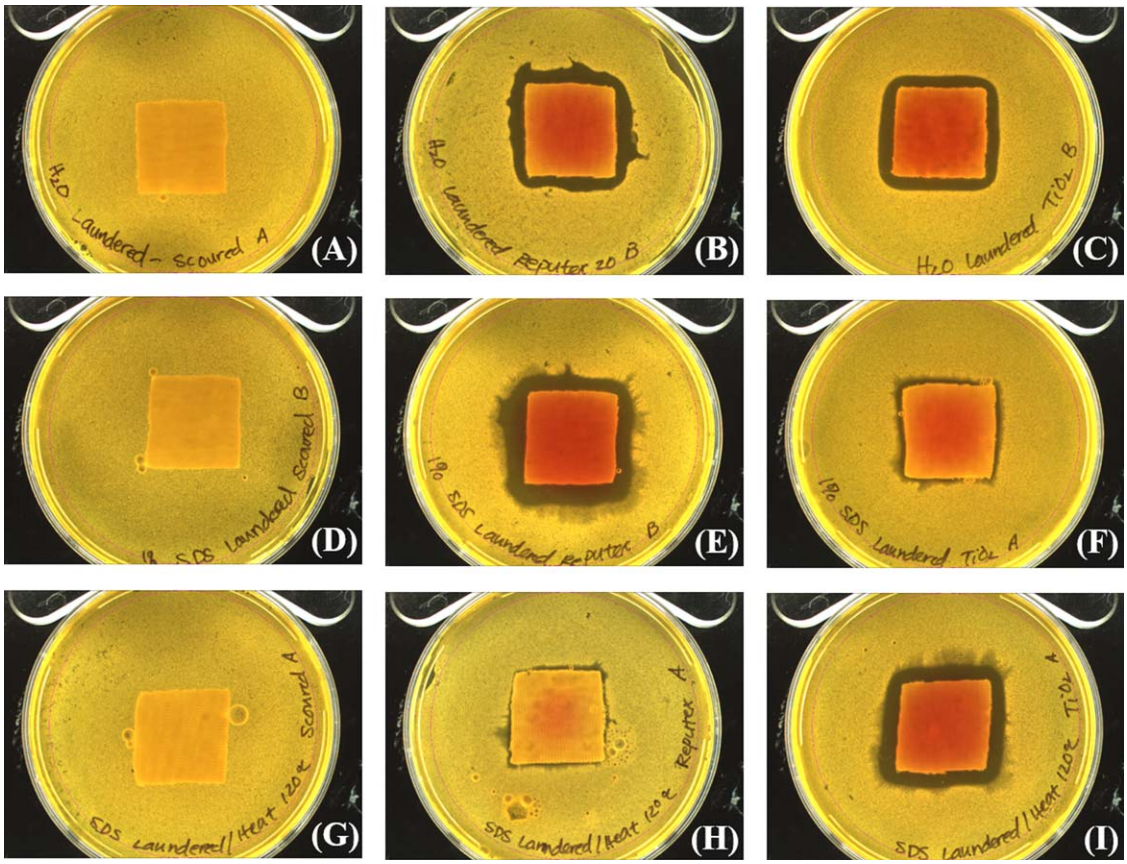


Figure 8. Overlay results for antimicrobial effects against *S. aureus*. (A) Scoured cotton-washed in water only—no zone of clearing; (B) Reputex 20-coated cotton-washed in water only—retained zone of clearing; (C) TiO_2-Reputex 20-treated cotton-washed in water only—retained zone of clearing; (D) Scoured cotton-washed in 1% SDS solution—no zone of clearing; (E) Reputex 20-coated cotton-washed in 1% SDS solution—retained zone of clearing; (F) TiO_2-Reputex 20-treated cotton-washed in 1% SDS solution—retained smaller zone of clearing; (G) Scoured cotton-washed in 1% SDS solution and heated—no zone of clearing; (H) Reputex 20-coated cotton-washed in 1% SDS solution and heated—small zone of clearing; (I) TiO_2-Reputex 20-treated cotton-washed in 1% SDS solution and heated—retained zone of clearing. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Most treatments for fabrics have not yet proven successful at preventing the loss of activity after multiple washings.[38] One recent example with cotton fabric has been reported, and microencapsulation of berberine was shown to retain biocidal activity even after 20 washes.[39] Another documented example of immobilized enzymes on cotton fabrics exhibited laundering durability and antimicrobial activity after 30 washes, however, the enzymes were applied to ester-crosslinked or copper-chelated cotton samples.[40] Durability studies and laundering experiments determining the protective effects of our applied metal oxide coating technology for added antimicrobial functionalities are being investigated. Antimicrobial efficacy against *S. aureus* was tested after preliminary laundering studies (3 washes) on the 1″ × 1″ swatches and the results are depicted in Figure 8. Laundering conditions were mimicked using a benchtop vortex machine and drying temperatures of 120°C were examined by laying swatches in an oven for 1 h. Figure 8(B, C) shows little effect from just water laundering on the treated swatches, and 1% SDS solution does not completely wash away the Reputex 20 or titania coating [Figure 8(E, F)], and zones of clearing are still maintained. However, when heat is applied after SDS laundering, there is slight reduction in the ZOC against *S. aureus* for Reputex 20-treated cotton swatches [Figure 8(H)]; and when titania is present, the laundering and heat effects are not as detrimental and a larger inhibition zone is present [Figure 8(I)]. As mentioned previously, we propose a protective effect created by the titania coating, which reduces the leaching of Reputex 20 and helps maintain the antimicrobial polymer even during washing studies. We have demonstrated that initial washing does not completely remove the Reputex 20 polymer system and further laundering studies investigating more washes and larger scaled experiments are currently underway.

CONCLUSION

We have demonstrated the room temperature preparation of a multifunctional coating material applied to cotton substrates with the incorporation of DFPase into a titania matrix precipitated by a commercially available antimicrobial polymer, Reputex 20. The antimicrobial polymer retains biocidal activity and clearly displays zones of clearing against *S. aureus* and *E. coli*. By retaining near-neutral pH, enzymatic encapsulation of DFPase is achieved during titania precipitation onto the antimicrobial cotton swatches. The DFPase enzyme remains functional and hydrolyzes DFP, releasing fluoride ions. The rate of F^- ion release for encapsulated enzyme is reduced compared to free enzyme, but still allows for steady chemical decontamination of DFP. The two functionalities, antimicrobial protection, and chemical decontamination are independently preserved after titania precipitation, validating the mild precipitation procedure. Antimicrobial activity was validated after initial washing studies (3 washes), which further supports the protective effects of the titania coating.

This simple method can be adapted to study other enzymes capable of biological or chemical decontamination. Dual-functionality was shown with these cotton swatches and future work will be on modifying the technique to add other functionalities, such as self-cleaning or UV-protection. With the correct manipulation of conditions, the titania platform could be tailored to add functionality to the multifunctional material. The approach here demonstrates the ease of incorporating multiple functionalities directly onto cotton fabrics, with the use of a commercially available antimicrobial polymer as a facile platform for titania preparation. The compatibility of different functionalities needs to be addressed, as it was unintentionally discovered that the antimicrobial polymer Reputex 20 may have reduced the activity of the DFPase enzyme. There are other antimicrobial polymers that are currently being investigated for use in fabric studies and methods will be explored to determine more favorable options to generate compatible multifunctional materials. These multifunctional materials and coatings could be applied to other surfaces relevant for making useful products. The studies reported here pave the way toward exploring biofriendly approaches for creating lightweight multifunctional cotton fabric material useful for protecting deployed Soldiers.

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