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Durable Visible Light-activated Antiviral Coatings for Fabrics Used for Personal Respirator such as the N95 Mask

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Abstract:

The FDA has recognized growing threats from infectious diseases in the hospital and community. As a precaution, to themselves and patients, caregivers wear personal protective equipment (PPE) such as respirator masks to minimize contact transmission onto facial skin or airborne inhalation of pathogenic organisms. Case-control studies conducted in Beijing and Hong Kong showed that wearing masks was independently associated with protection from SARS in a multivariate analysis. However, a study performed by the CDC *showed definitive evidence of the transmission of virus particles from PPE to other people and surfaces in a hospital setting.*

Our goal was to develop an economical and manufacturable coating for personal protective equipment which will inactivate more than 99.9% of a challenge inoculum of influenza virus within one hour under normal conditions of use. Our approach was to immobilize [coat] photoactive dyes onto surfaces [filtration media]. These dyes when exposed to visible light catalyze the production of singlet oxygen from air [oxygen] and are known to inactivated viruses and bacteria.

The preferred coating was developed from a suitable combination of dyes, attachment methods and filtration media. We determined that the most cost effective and active dye to use was Rose Bengal, a potent antiviral and bacterial agent. Literature methods used to modify Rose Bengal for immobilization to acrylate polymers proved to be unreliable, so an alternate synthetic pathway was developed. Rose Bengal was synthetically modified to attach either an acrylate or styry monomer to the dye and polymerized with other acrylate monomers to form a multifunctional acrylic polymer. This polymer contained reactive side chains which aided permanent bonding to nylon filtration media. Coating [dyeing] was achieved in a conventional fabric "dyeing" which can be scaled to full size manufacturing. The dye incorporated polymer has been synthesized in efficient 25 gallon batches and further scale-up should be straight forward. d further. This research enhanced the efficiency of the antiviral effect and permits development of cost-effective methods for commercial manufacture of the devices [N95 masks] while retaining marked antiviral properties.

List of Terms and Abbreviations

FLU	influenza A
LAAM	Light activated antimicrobial material
OTC	Over the counter
PDI	Photodynamic Inactivation
PPE	Personal protective equipment

SECTION 1. This section is limited to two pages. Please provide a concise, cogent Section 1 that will be understood by a broad audience.

Highlights/Significant Findings.

1. N95 masks can be assembled from antimicrobial filter material [outer covering] and retain NIOSH required properties of filtration and breathability. This finding was independently confirmed by Nelson labs.
2. The antimicrobial filter material was shown to be fully biocompatible. This finding was independently confirmed by WuXi Apptec.
3. Antimicrobial filter material can be produced using these polymers in a large scale, conventional cloth dyeing process.
4. Antimicrobial polymers can be manufactured [synthesized] in an economical manner using methods that are typical of large scale polymer production.
5. Antimicrobial dyes that produce singlet oxygen and that are attached to a polymer retain high levels of antiviral and antibacterial activity. This is significant because previously these dyes had been shown only to be active in free solution.
6. Antimicrobial active is related to the % incorporation of dye into the polymer. Maximum dye incorporation does not necessarily lead to higher activity. "Tuning" the amount of antimicrobial dye in the polymer leads to higher activity.
7. Two methods were explored for the incorporation [attachment] of antimicrobial dye into the polymer. One method involved attaching a synthetically modified dye to a preformed polymer. This method was synthetically difficult and the resulting dye-polymer was difficult to solubilize for the coating process. The alternate attachment method involved polymerizing the dye into the polymer as it was formed. In addition, during the polymerization, other modified acrylate monomers can be added to the polymerization mixture that increases the polymer's solubility and ability to crosslink to the surface of the filter material. This led to permanently dyed material.

Translation of Findings.

The progress and developments described above show that filter materials can be modified to incorporate antimicrobial compounds that are effective when immobilized. These developments have not been used in the workplace yet, but the technology should lead to improved worker safety when the user is exposed to infectious agents such as influenza and other viruses that are spread through aerosols and contact with contaminated surfaces.

Outcomes/Relevance/Impact.

The research supported in this Phase I grant was directed towards taking an interesting academic finding, incorporation of photoactive dyes into filter materials for antiviral activity, and reducing the concept to a practical industrial process. The key breakthrough was developing a polymerization method that allowed incorporation of several different monomers into the polymer at the same time. Through careful selection of the monomers used, several different properties could be incorporated into the polymer at one time; antimicrobial activity, solubility, and reactivity to the surface that

the polymer will be coated. This is an enabling technology that can be used to incorporate other useful activities into coating materials.

SECTION 2

Scientific Report.

2.1 Transmission of infectious diseases

The transmission of infectious disease occurs by two principal means: (1) airborne droplets containing viruses or bacteria (a sneeze); and (2) contact transmission, that is, the physical transfer of microorganisms from a biological source (blood or other liquid, skin) to another person (from a person's hand to another person) or from a surface (counter, table, respirator mask, clothing, carpet or hospital curtain) to another surface (a person's skin, mouth or eye). Transmission of viruses such as respiratory syncytial virus (RSV), influenza, and the noroviruses is common in hospitals and other areas where people congregate.

Influenza spreads rapidly throughout the world in seasonal epidemics [5]. The World Health Organization estimates that influenza epidemics cost the US economy $71-167 billion per year and that 250,000-500,000 people die every year from influenza epidemics [1]. Influenza enters the body through the nose or throat. Groups most at risk are health care workers, hospital patients, and the young and geriatric population. In closed settings, [e.g., hospitals, child-care centers, military barracks, college dormitories, nursing homes] infections spread rapidly.

The influenza virus readily undergoes genetic changes that complicate our ability to fight the virus [6]. New, multiple measures are needed to decrease infection rates. Influenza and other respiratory viral infections declined in Hong Kong during the 2003 SARS epidemic when public health interventions included closing schools, swimming pools, and other public gathering places, canceling sports events and disinfecting taxis, buses, and public places. A high percentage of people wore masks in public and washed hands frequently and, in general, much less social mixing occurred [2]. Case-control studies conducted in Beijing and Hong Kong showed that wearing masks in public was independently associated with protection from SARS in a multivariate analysis; one study found a dose-response effect, although the retrospective questionnaire design limited the ability to draw firm conclusions [3,4].

2.2 Relevance of N95 masks and an antimicrobial coating.

As a precaution, to themselves and patients, caregivers wear personal protective equipment (PPE) such as respirator masks to minimize contact transmission onto facial skin or airborne inhalation of pathogenic organisms. The mask traps organisms but in doing so becomes a disseminator of infectious particles. The caregiver may often adjust the mask with their hands for comfort and to keep a good air seal on the face. They also may remove the mask for interim activities (paperwork, drinking fluids, eating, and breaks). Each time the hands (gloved or not) touch a contaminated mask or piece of clothing, they become a potential vector for contact transmission. The Centers for Disease Control has recently published a paper with *definitive evidence of the transmission of virus particles from PPE to other people and surfaces in a hospital setting* [7].

Influenza is only one of many viruses that are spread by aerosolized droplets when infected people cough or sneeze. Therefore, a new, low cost approach to preventing the spread of viral infections in general, and influenza in particular, would be extremely beneficial. During an epidemic or pandemic, infection rates could be reduced if a simple method existed to interrupt contact transmission. Although high efficiency air filters can provide some level of defense, they are expensive and difficult to breathe through due to a relatively high-pressure drop across the filter. **WHO** [8] **recommends the use of personal protective masks that are at least as effective as NIOSH N95 mask/respirators to limit transmission in highly infectious environments**. The FDA guidance document (July 2007) recommends N95 masks for Medical primary care: first responders, hospitals, clinics, urgent care, elderly care, childcare, animal husbandry as well as OTC non-emergency uses in home and business, stockpiling for seasonal outbreaks, OTC emergency stockpiling (governments) and also for consumer protection during food processing and handling.

NIOSH estimates that 90 million N95 filtering faceplate respirators will be needed to protect workers in the healthcare sector during a 42-day outbreak, likely requiring reuse of respirators. They suggest that research opportunities exist for development of simple, efficacious decontamination methods that can be used without negative effects on respirator integrity and reducing risks associated with handling a respirator that has been used for protection against a viral threat [9]. Using our patented technology, LaamScience believes it can achieve this goal.

There are several antimicrobial N95 masks on the market utilizing iodine, silver or tryclosan, however none of these are approved by the FDA and these approaches are not likely to inactivate viruses. The FDA Antimicrobial Medical Devices Advisory Meeting in May, 2007 acknowledged growing threats in the hospital and community from infectious diseases. Their cautions and concerns on improvements included the concern that antimicrobial compounds may leach off or have toxic side effects, may not kill microorganisms in a short enough time to be useful, among other characteristics. The physical characteristics of the Light Activated Anti Microbial (LAAM)-coating for N95 masks address these concerns.

2.3 Photodynamic Inactivation (PDI)

Photodynamic therapy has been shown to be able to inactivate many enveloped viruses in culture, including HIV [10,11], HSV [11-13], influenza [10], vesicular stomatitis virus [VSV] [10,11,14], Sendai [10], cytomegalovirus [11] and suid herpes virus [13] through the production of singlet oxygen, $^1O_2$. Lenard and colleagues [10] investigated the mechanism of inactivation of enveloped viruses with Rose Bengal or hypericin and found that $^1O_2$ cross-linked protein G on the surface of VSV, thus inhibiting viral fusion. Other viruses that they tested are presumed to be inactivated by a similar mechanism. Virus inactivation was proportional to the intensity of light used. Both dyes are known to produce $^1O_2$ on exposure to light and both were ineffective in the dark. Thermal decomposition of poly[1,4-dimethyl-t-vinylnaphthalene-1,4-endoperoxide] produces $^1O_2$ and also inactivated enveloped viruses, confirming that $^1O_2$ was the active material [14].

Photodynamic inactivation [PDI] of microorganisms requires the presence and interaction of three nontoxic components: photosensitizer, light, and oxygen [15]. The

initiating step of the photosensitizing mechanism is the absorption of a light photon by the sensitizer, causing a promotion of the molecule from its ground state to the unstable excited singlet state with a half-life in the range of 10^{-6} to 10^{-9} sec [16].

The problem of bacterial multidrug resistance might be overcome by photosensitization, as this induces damage and cytotoxic events in a wide variety of microorganisms by a variety of mechanisms. Both, gram-positive bacteria such as S. aureus or Deinococcus radiodurans and gram-negative bacteria such as Acinetobacter baumannii, which represent a significant problem in hospitals, are highly sensitive to this treatment [17,18]. Several photo-sensitizers inactivate both enveloped and non-enveloped viruses [19,20], Because of the multi-target mechanism of action, photosensitization may be a good method to accomplish inactivation of a wide range of organisms, provided that the ambient light is adequate.

Chemistry of LAAM Fabric

The LSI fabric surface [respirator mask] is designed to be self-decontaminating. This is accomplished by permanently, covalently bonding a thin, biocompatible, non-leachable dye coating to the exterior of the mask by a standard textile-finishing process. The coating is antimicrobial by virtue of producing singlet oxygen upon absorbing visible light. Singlet oxygen is the active antimicrobial agent.

Rationale for choice of Rose Bengal: Rose Bengal was chosen as the initial coating for the LaamScience N95 respirator mask because of 1] the proven safety for clinical applications, 2] literature reports of broad spectrum activity against a wide range of microorganisms, 3] ready availability, and 4] acceptable cost. Polyacrylic acid [anchor polymer] is bound to the fabric surface and is linked to Rose Bengal [photoactive dye] via an amine group [chemical linker; Fig. 1a below; [21].

Rose Bengal is a photosensitive dye in widespread use in the evaluation of ocular surface diseases, including herpes simplex virus. This dye penetrates living cells [22] and is used clinically in ophthalmology solutions to stain the cornea and conjunctiva to determine the extent of eye damage in dry eye. It also has been cleared as Robengatrope, for use in liver function studies. It has been given orphan product status as a photosensitizing agent for treatment of metastatic melanoma by Provecta Pharmaceuticals [Knoxville, TN]. A master file on this agent [#15176] is held by Sigma-Aldrich.

Several features of photoinactivating agents are critical to their safety for human use. Singlet oxygen has a short life span [four microseconds in water [23,24] and 70 microseconds in air [25] and a short radius of action [20 nanometers in cells [25], 200 nanometers in water and 3 micrometers in air] and can, therefore, exert its effects only on microbial components that are in close proximity [on the surface of] to the photosensitizer [26,27]. PDI is a relatively fast process; within 1 hour of treatment, most bacterial cells are unable to accomplish cell division [26] and this is followed by inhibition of DNA and protein synthesis. Photoinactivation by Rose Bengal proceeds primarily by a type II [singlet oxygen mediated] mechanism [28,29]. Brooks et al. [30] showed that this dye possess the potential for potent antiviral activity against extracellular virus. Rose Bengal treatment of virus suspensions typically produced 4-to 5-log decreases in virus plaque-forming unit [PFU] titer. Intracellular virus was found to

be markedly resistant to photoinactivation [30]. In a mouse model of acute primary HSV keratitis, topical application of Rose Bengal reduced surface virus titers by 1 million-fold and intracellular virus in homogenates only 32-fold [31]. Nanomolar concentrations of free **Rose Bengal** photoinactivated the enveloped viruses HIV and VSV [11].

Rationale for Polymer: Most commercial fibers are either natural fibers, cellulosic or protein fibers, or they are synthetic. Natural fibers have a large number of chemically reactive groups on their surfaces whereas most synthetic fibers [e.g. the SpectraMax nylon fabric used for the outer layer of the LSI masks] have few reactive groups. Grafting of a polymer to the surface of synthetics allows much larger surface coverage since multiple dye compounds can be attached to each polymer molecule [32]. The method used as a basis for the LaamScience technology employs a polymer [polyacrylic acid] with many potential graft sites [carboxyl groups] to maximize the surface coverage, grafted to the surface to amplify the number of reactive sites. Small molecules, i.e., the dye compounds, with the desired surface properties are grafted to this surface polymer. This allows considerable control over the surface properties of fibers.

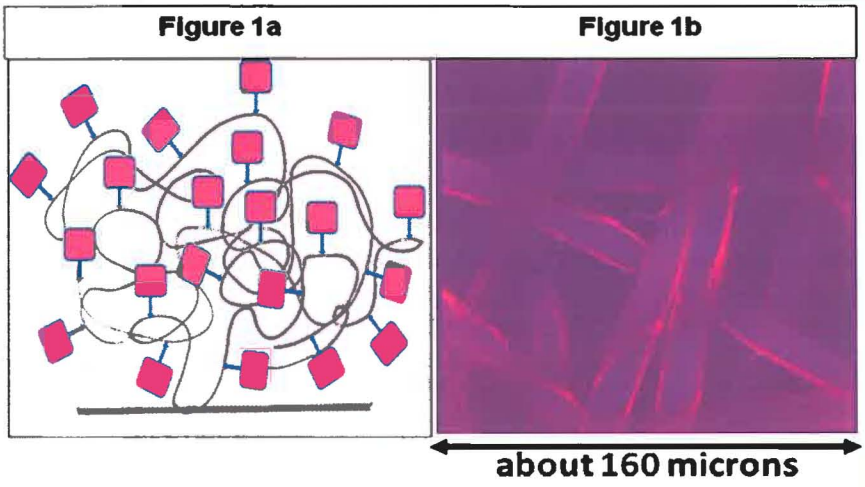

Figure 1a. Dye-Linker-Polymer (dye compound) Basic Structure. Solid line: Fabric surface; thin curved line: Polyacrylic acid; Pink boxes: Rose Bengal; Blue arrows: covalent linker [208 Thompson, K. 2005].

Figure 1b. Transmission Electron microscopy of Nylon 24 gsm coated with Rose Bengal PAA conjugate. Red fluorescence from Rose Bengal.

Rationale for Choice of PAA as Graft Agent: Polyacrylic acid [PAA] is a 'finding of no significant impact' [FONSI] food additive that acts as a stabilizer and thickener, and can be used as an indirect food additive. It also is found in intraocular lenses and as an inactive ingredient in some pharmaceuticals such as Lidoderm and Finacea. Tobiesen and Michielsen [33] grafted poly-[acrylic acid] [PAA] to the surface of nylon. The PAA acts as an amplifier, converting a single graft site to approximately 1000 potential graft sites that are available for the attachment of dye compounds.

2.4 Summary of the specific aims of the Phase I grant: The original Specific Aims of the Phase I research included a feasibility study to determine if candidate dyes can be linked to carriers and retain high levels of antimicrobial activity when attached to filtration media. We demonstrated initial feasibility of this technology against a variety of microorganisms, including influenza virus [H1N1], cowpox, *Staphylococcus aureus*, and *methicillin-resistant S. aureus* [MRSA]. Research focused on developing a simple and cost-effective method for large-scale production. Specifically, the research plan was to

quantify singlet oxygen generation by each potential coating mixture [dye concentrations/combinations with carrier polymer] and to show direct comparison of singlet oxygen generation to influenza virus inactivation rate.

Phase I Milestones:

1. Choose the most cost-effective dye-carrier combinations that retain the highest level of singlet oxygen production and antiviral activity

2. Choose the most efficient and scalable textile attachment method to retain maximum singlet oxygen production and antiviral activity

3. Ensure that the chosen coatings are stable and effective [maintain required virus inactivation rate] under likely conditions of use [room temperature and humidity, normal light conditions]

2.5 Results of Phase I research

Summary: In the original application, we planned to test a number of dye polymer [dye-carrier] combinations and attachment methods before testing stability and efficacy. We improved the preparation of the dye-polymer conjugate [**Conjugate II**] with a new process that polymerizes Rose Bengal styrene with acrylic acid and other polymerizable groups. **Conjugate II** is more active than **Conjugate I** to killing bacteria and inactivating virus. Further antimicrobial activity optimization and manufacturing process improvements will be the focus of Phase II. Use of **Conjugate II** solves several of our technical hurdles. We have reduced the number of steps needed to produce a conjugate and this method allows easy addition of other functionality such as solubility, reactivity [crosslinking], and increased activity. A pilot line designed to mimic large scale manufacturing will help us develop a fully scalable process.

Since our data on the first Rose Bengal dye-polymer conjugate [**Conjugate I**] showed good initial efficacy for decontamination of bacteria and viruses, we additionally pursued studies of virus and bacterial killing for data leading to the submission of a 510K device (Class II) application to the FDA for a N95 anti-microbial coated mask. The application was submitted in October, 2008, according to our business plan schedule. Results of some of these studies are included (4.4.5). These studies completed on mask properties were planned, in part, for the Phase II portion of this SBIR. The results presented below indicate that **Conjugate I** used to make fabric produced good killing of bacteria and viruses in a light- and time-dependent manner. Data on stability and efficacy are also included. LaamScience provides evidence herein that the speed of microorganism inactivation or kill on titers of inocula that would be encountered in the hospital setting is sufficiently fast to warrant use on an N95 mask worn in such settings.

Reviewers of the Phase I application were concerned about photobleaching and filtration efficiency. These studies were additionally pursued and the results are included below (sections 4.4.6.5 and 4.4.8). The initial Rose Bengal dye-polymer coated fabric was submitted for cytotoxicity, skin irritation and skin sensitization studies and show a high degree of safety. By performing these studies on a fabric coated with our dye-polymer, we have made great strides toward eliminating risk moving to manufacturing scale up.

2.6 Preparation of Dye-Polymer Conjugates

2.6.1 Preparation of Conjugate I: Rose bengal-5-(3-aminopropylcarbamoyl)pentyl poly(acrylic acid) with 5% loading on 450,000 MW poly(acrylic acid) [**Conjugate I**] was synthesized in five steps; 6-Bromopentanoic acid was converted to the corresponding acid chloride by treatment with oxalyl chloride; this acid chloride was reacted with (3-aminopropyl)carbamic acid *tert*-butyl ester to yield the intermediate bromo compound which was then reacted with Rose Bengal to yield an ester which the Boc protecting group was then removed by treatment with hydrogen chloride in dioxane to give rose bengal-5-(3-aminopropylcarbamoyl)phenyl ester hydrochloride. **Conjugate I** was prepared by reaction of poly(acrylic acid) with rose bengal-5-(3-aminoi-propylcarbamoyl)-phenyl ester hydrochloride in the presence of DMTMM in water, Figure 2.

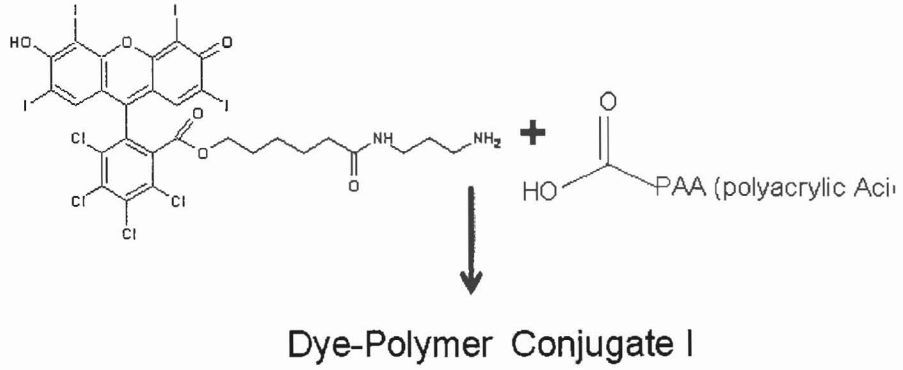

Figure 2. Preparation of Rose Bengal Polyacrylic Acid by Attachment

2.6.2 Preparation of Conjugate II: Rose Bengal with 2.5% loading incorporated into a 50,000 MW acrylate polymer [**Conjugate II**]. A monomer mix was prepared by heating and mixing 2.2 g of propylene glycol, 5.3 g of Rose Bengal styrene monomer (CV-4844), 4.2 g of hydroxyethyl methacrylate (HEMA), 4.2 g of AAE-10 [surfactant], 2.9 g of KH-10 [polymerizable surfactant], 264 g of acrylic acid, and 4.2 g of itaconic acid. Monomer mixture was metered into to a rapidly stirring aqueous solution of catalyst [2.7 g of ammonium persulfate (APS) and 2.7 g of sodium meta bisulfate (SMBS)] under a nitrogen blanket. The reactor is allowed to reach 65° C and stabilize. The monomer mix finished approximately 90 minutes after it started and the batch was cooled to below 45° C and the bright red product was filtered through a 100 micron filter. Final yield was 2 liters of soluble acrylate polymer, 12% solids, 2.5% Rose Bengal (Figure 3).

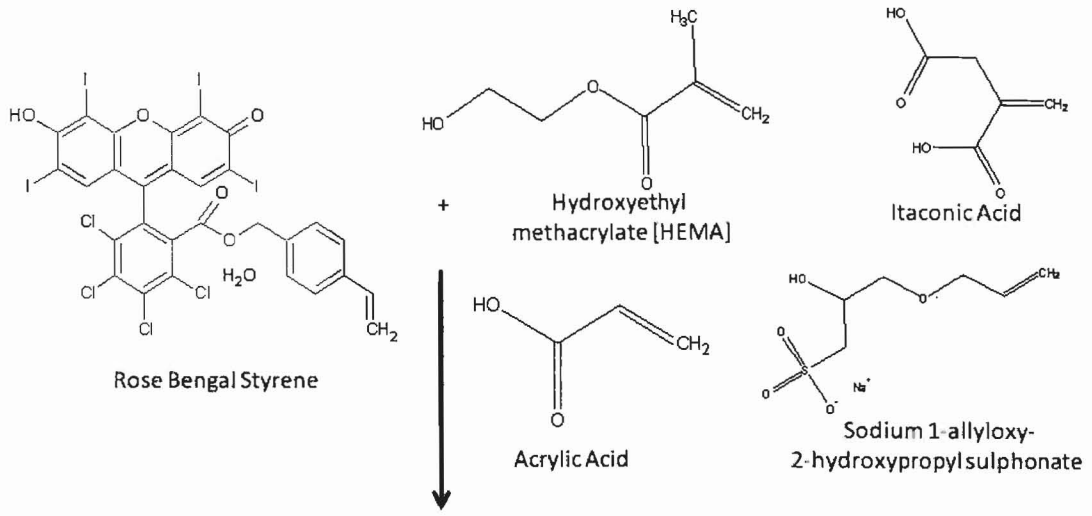

Figure 3. Preparation of Rose Bengal Polyacrylic Acid by Polymerization

2.6.3 Preparation Dye-Polymer Conjugate Coated Fabric: 20 liters of 0.08% w/w aqueous solution of a rose bengal dye polymer [**Conjugate I or II**] was prepared by diluting a 10.8% solid rose bengal photodynamic polymer [1:8] in cold water. This solution was applied [padded] continuous onto a 1.0 osy [ounce / square yard], 20 inch wide, non-woven nylon to yield an 85% pickup [w/w of dye polymer solution to fabric weight] of solution. Fabric with dye polymer solution was immediately passed through an 3 meter, 175° C forced air drying oven at a speed of 2 meters / minute, wherein the fabric surface temperature reached 172° C.

Bench Testing/Performance Characteristics of Fabric: Assay Performance: LaamScience chose times and light intensities to reflect general conditions of use in the surgical suite. Levels are described in 'lux', the SI unit for lighting, which is equal to one lumen per square meter. Hospital lighting conditions generally range from 20 to 1000 lux in patient rooms, with hallways in the 1000 lux range and surgical suites in the 30,000 to 100,000+ lux [1].

2.6.4 Comparison of Rose Bengal to Eosin. The antimicrobial activites of Rose Bengal and Eosin conjugates, type II, were compared. The solution quantum yield of Rose Bengal and Eosin in solution is 0.995 and 0.67, respectively and the production of singlet oxygen by the fabrics mirrored the solution activity. The Rose Bengal fabric had bacterial and viral kills of greater than 4 logs while eosin dyed fabric, even produced with dye bath concentration two fold greater than the Rose Bengal, 0.08% versus 0.04% respectively, had lower activity. This supports our use of Rose Bengal fabric for our Conjugate II optimization studies.

2.6.5 Measurement of Color Intensity/Consistency: We now use the Hunter ColorFlex 45/0 [Hunter Associates Laboratories, Reston, VA] spectrophotometer to determine fabric color intensity. The Hunter colorimeter measures the reflected color of opaque solids using a 45°/0° measurement geometry [the illumination source shines on the sample at an angle 45° from the perpendicular to the sample surface. Each reading

produces a value for "L*" [white/black], "a*" [red/green], and "b*" [yellow/blue]. Averaging multiple readings with rotation between readings minimizes measurement variation associated with non - uniformity or texture. The instrument is calibrated using white and black standards, and the sample to be measured is centered under the sample port and backed using a non-calibrated white tile, which provides a consistent neutral background. Five separate readings are taken of each sample, using five different areas of the sample rotated (preferably at 90°).

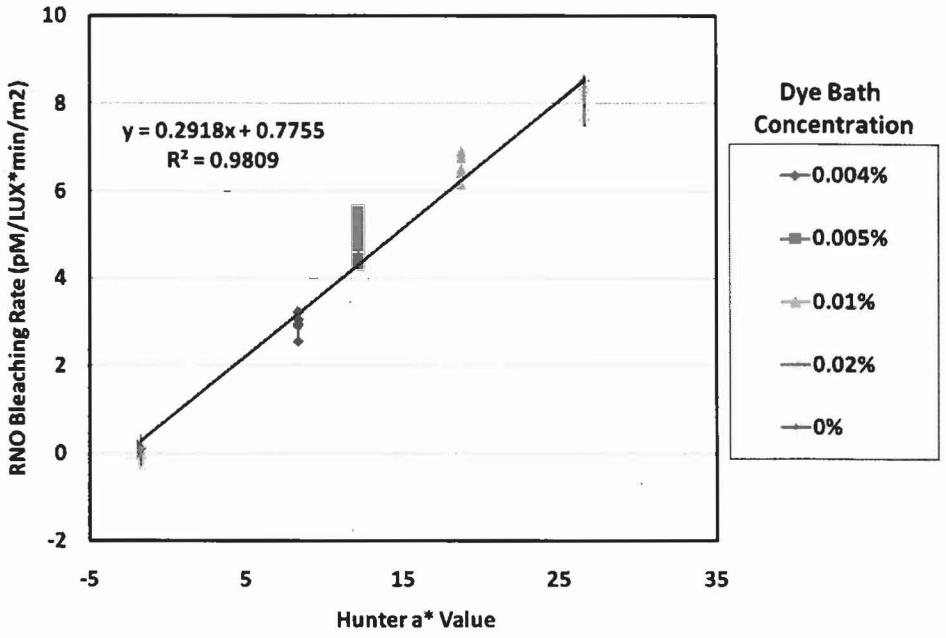

Figure 4. Relationship of Singlet Oxygen (RNO Assay) to Hunter a* Value

Figure 4 shows singlet oxygen generated is linearly proportional to the amount of Rose Bengal dye on the fabric as measured by the Hunter a* Value. Fabrics used in these studies that were prepared from **Conjugate I** which, due to its limited solubility, were coated from isopropanol solutions. Soluble **Conjugate II** was also applied to fabric with aqueous solutions and we observed comparable color density on the fabric (not shown).

Results: The LaamScience fabric antimicrobial properties are derived from the coating of Rose Bengal – polyacrylic acid [**Rose Bengal Conjugate I or II**]. The visible spectrum of Rose Bengal has absorbance maxima at 535 and 565 nm and the reflectance spectrum of Rose Bengal-dyed fabric also clearly shows these maxima (not shown). There was, as expected, a direct inverse correlation of dye concentration on the fabric with reflectance. Results show that the higher concentration of dye leads to both increased color intensity (Hunter a* Value) and increased singlet oxygen production by the RNO assay in picomoles/lux-min/m^2.

Hunter Color Intensity/Consistency Summary: Acceptance criteria for testing future lots of Rose Bengal-coated masks for the Hunter a* values were generated by combining data from the within-lot variation study [40 samples from one lot of Rose Bengal-coated fabric], the lot to lot study from three lots of N95 masks made with lots of Rose Bengal-coated fabric manufactured by the same process [75 samples], room temperature stability study [45 'Time 0' samples],and other mask study measurements [6 samples].

The mean a* value for all studies is 12.05 with a standard deviation of 0.492. Confidence limits [95%] of a* value measurements were +95% of 13.03 and -95% of 11.07. More than 95% of the a* values fell within these limits. Only one value from the within-lot variation study fell slightly below the -95% confidence limit.

2.6.7 RNO Assay [Singlet oxygen generation assay]: To obtain a quantitative, direct measure of the activity of various LAAM fabrics, a singlet oxygen assay based on earlier reports [35-37] was optimized for the LAAM technology. The rate of change in dimethyl-p-nitrosoaniline [RNO] concentration is directly related to light intensity [lux], irradiation time, and fabric color [density]. Singlet oxygen reacts directly with histidine to produce a transannular peroxide that bleaches RNO; the reaction of singlet oxygen with RNO causes a decrease in color monitored spectrophotometrically [35, 36, 37]. The concentration range of detection for RNO on the spectrophotometer at 440 nm is from 5 to 40 micromolar. LAAM fabric samples of 16 cm^2 significantly reduce RNO concentrations in the presence of 50 mM histidine. To obtain the rate of concentration change per moles per lux-min/m^2 the delta RNO concentrations were divided by the lux x incubation time and multiplied by factors to normalize for the volume and area of fabric tested.

Results: The RNO assay for singlet oxygen production showed a strong positive correlation with increasing dye bath concentration and also with Hunter a* value [Fig. 4]. The dynamic range of the method was from 1.9 to 10 pM/lux*minute/m^2; coefficient of correlation [R^2] was 0.95. The performance of the RNO assay was assessed using the following procedures; Within Day Precision of the RNO Assay, Operator to Operator Variation, Total Imprecision (Day to Day Variation) of the RNO assay, Effect of Irradiation Time and Light Intensity on RNO Assay with Rose Bengal-coated Fabric, and Effect of Time and Light Intensity on Precision of the RNO Assay with Rose Bengal-coated Fabric. Acceptance Limits for the RNO assay for manufactured fabric (QC) were developed from a compilation of these test results (not shown).

2.6.8 Bacterial Assays: LAAM Rose Bengal-coated fabric produces singlet oxygen when irradiated with visible light. Singlet oxygen can damage and kill some types of bacteria. A modification of ASTM E 2149 was used to measure the bactericidal effects of the singlet oxygen produced by the fabric. Bacteria were inoculated onto the LAAM fabric which was then irradiated with visible light. The bacteria were then extracted from the fabric and diluted in saline. The viability of the diluted bacteria was determined by plating them onto a solid nutrient medium and incubating them in an environment that is favorable to growth. Growth was evidenced by the development of discrete colonies which could be counted.

Results: Studies analogous to the ones for the RNO assay were performed to determine the relationship of irradiation time, light intensity, and singlet oxygen generation to antibacterial activity on Rose Bengal-coated fabric. A direct linear relationship was observed between increasing irradiation time or light intensity and bacterial kill [Fig. 5]. The effect of increasing light intensity (lux) or irradiation time on Rose Bengal-coated fabric on the decrease in Viable *S. aureus* was assessed. Assays were performed as in Figure 5. Both increasing light and time resulted in linear decreases in viable bacteria. (Not shown).

These data can be used to predict a probable Minimum Effective Concentration [MEC; the concentration of singlet oxygen needed to produce a 3 log decrease in bacteria concentration] for S. aureus with Rose Bengal-coated fabrics. The singlet oxygen MEC for is reached by exposing fabric to 14,000 lux for 60 minutes. The corresponding singlet oxygen production rate, as measured by the RNO Assay, is 1.48 picomoles/Lux-min/m^2.

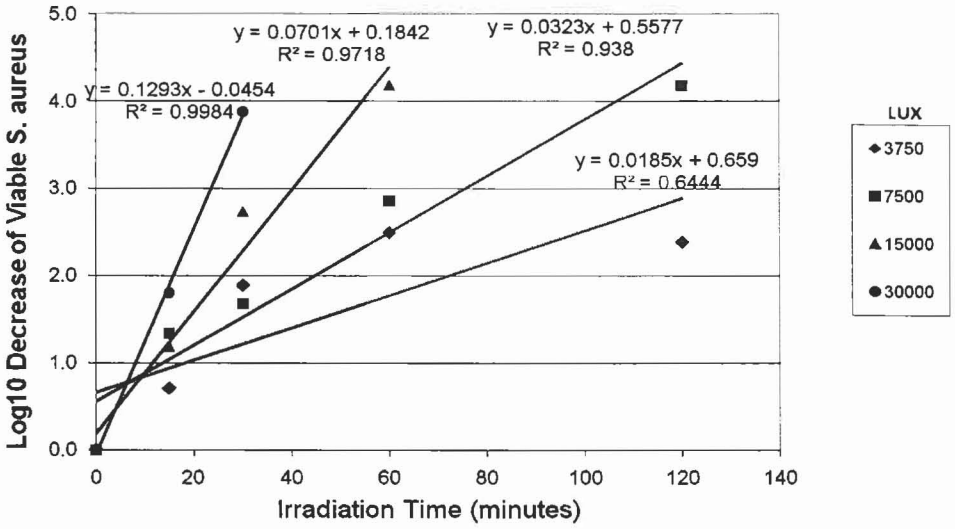

Figure 5. Effect of irradiation time of Rose Bengal-coated fabric on decrease in viable S. aureus [Conjugate I]. Results are shown for irradiation at 3,750 (diamonds), 7,500 (squares), 15,000 (triangles) and 30,000 (circles) lux. Increasing irradiation time resulted in increased bacterial kill.

2.6.9 Bacterial Assays: Comparison of Conjugate I and II Coated Fabric: Nylon fabric was coated with either Rose Bengal Conjugate I or II and cured as described above. The concentration of Conjugate I used was 0.01% which was the maximum soluble concentration that could be obtained in aqueous solution. A 4 log kill of S. aureus was observed with Conjugate I with light intensities of 10,000 Lux, but observed kill fell off rapid with decreasing light. Because Conjugate II was formulated with polymerizable surfactants it had a much higher Use of Conjugate II at 0.08 and 0.06% dye concentrations gave fabric with a marked increase in S. aureus kill; the fabric coated at .08% showed a 2 log increase in kill at 2500 Lux over that for Conjugate I coated fabric. This shows that by changing the conjugate formulation that higher coating concentrations can be achieved which result in more efficacious fabric, especially in lower light conditions (Figure 6).

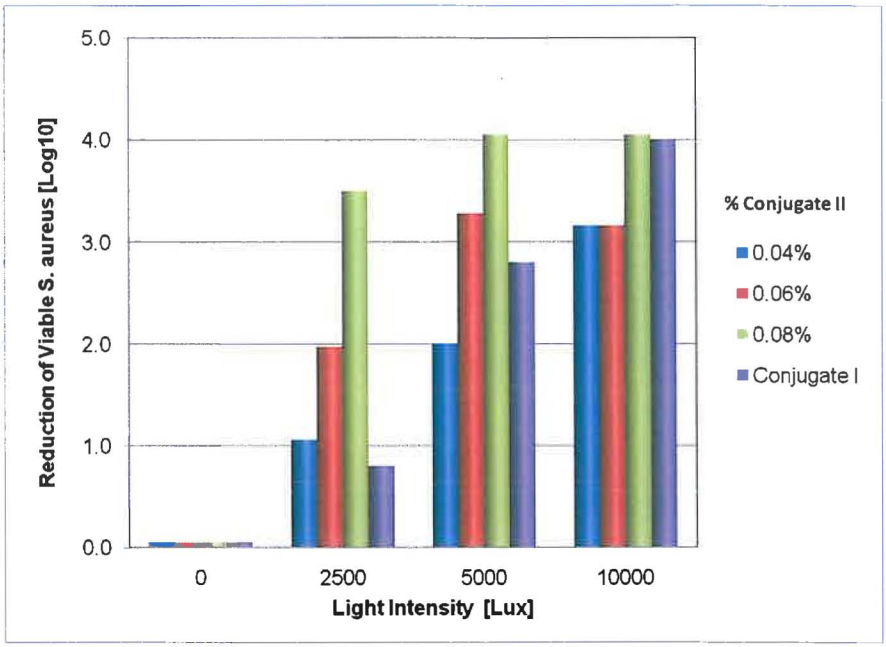

Figure 6. Light Intensity versus Antibacterial Activity
Log Reduction of STAPH by nylon Coated with Conjugate I or II

2.7 Virus Inactivation

2.7.1 Viral Assays: One of the main 2008 IOM Draft PPE Action Plan recommendations was to assess the viability of virus on respirators. From this document: 'Although respirators serve to protect the wearer, concerns exist that viruses remaining on the respirator transform it into a fomite that may serve as a vehicle for infection of the wearer, or others, through handling or reaerosolization.' LaamScience used a variety of assays to measure the residual activity of viruses exposed to the LAAM fabrics under different conditions [light intensities, light sources, time]. The following section describes initial experiments with Influenza type A viruses.

$TCID_{50}$ Assays: These assays are used as an alternate method to plaque assays to measure virus titer. These are typically used for virus strains like influenza that do not produce clear plaques. The $TCID_{50}$ is the quantity of a pathogenic agent [virus] required to produce a cytopathic effect [CPE] in 50% of inoculated cell cultures, where cultures demonstrating any CPE are considered positive. CPE are visible morphologic changes in virus-infected cells affecting intracellular structures and cell functions, e.g. chromosomal aberrations, viral inclusion bodies, membrane permeability, and protein synthesis which are distinct and different from morphology of control cells.

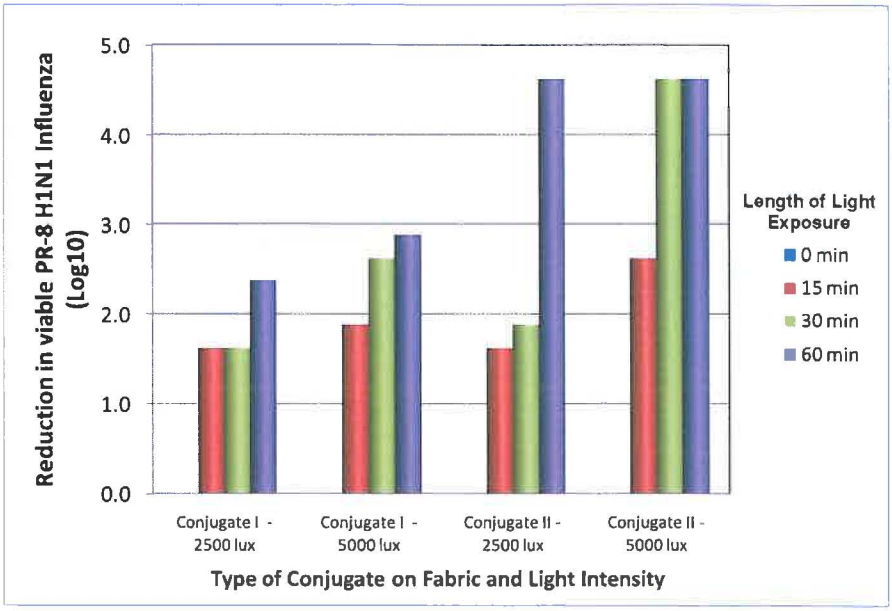

Figure 7. Effect of Irradiation Time and Light Intensity on Virucidal Activity of Conjugate I and II Coated Fabric [Lots L2 and #1177.026]

Results [Fig 7]: In a set of experiments analogous to those run for the bacterial MEC estimation, Rose Bengal Conjugate I or II coated mask fabric was treated with Influenza A/PR8/34 at various light intensities and irradiation times to estimate the MEC of singlet oxygen for this virus strain. Influenza A/PR8/34 at a $TCID_{50}$ titer of 1.75×10^5 was added to four-cm^2 pieces of mask fabric, which were irradiated at the indicated times and light intensity. Fabric was extracted to remove virus and the extracts were serially diluted and used to infect MDCK cells in a 96-well plate as described above. Wells were observed for CPE each day for four days. In general, reduction in virus titer was directly correlated to light intensity and irradiation time.

For Conjugate I coated fabric a light irradiation of either 2500 or 5000 lux for 60 minutes gave less than a 3 log reduction in viable Influenza PR-8, while the Conjugate II coated fabric gave over a 4 log reduction in viable Influenza under the same conditions. A light intensity of either 2500 lux for 30 minutes or 5,000 lux for 60 minutes was chosen for further experimentation for determination of actual MEC for the Conjugate II fabric.

2.8 Additional testing for NIOSH certification:

2.8.1 Within-Lot Variation where the mask fabric samples consistently inactivated at least 99.9% of either strain of the virus under these conditions.

2.8.2 Lot-to-Lot Variation where mask fabric samples from 3 lots consistently inactivated at least 99.9% [three logs] of the WSN or PR8 virus when tested with 10^6 PFU. These results are consistent with the mask color intensities and RNO generation rates.

2.8.3 Leach testing: Spectrophotometric and Bacterial: All three fabric lots chosen for mask fabrication had low leaching properties [absorbance < 0.006], low

antibacterial activity of the leachate, and high intrinsic antibacterial activity when directly tested on *S. aureus*.

2.9 Antimicrobial activity spectrum [Completed on Conjugate I Coated Fabric]

2.9.1 Staphylococcus aureus [*S. aureus*; nonresistant strain]: Three lots of masks with Conjugate I coated fabric were tested for antibacterial activity against *S. aureus* in three separate runs on three different testing days under the conditions listed above. In all cases, each mask sample produced >99.9% kill for this strain of *S. aureus*.

2.9.2 Methicillin-resistant *S. aureus* [MRSA]: Three lots of masks with Conjugate I coated fabric were tested for antibacterial activity against MRSA in three runs on three separate testing days. Conjugate I coated fabric is effective against MRSA at 14,000 lux and 1 hour irradiation time. Accelerate aging does not decrease antibacterial activity against MRSA.

2.9.3 Antibacterial Activity with Different Light Sources [Tungsten and Florescent]: The LAAM Rose Bengal treated fabric must perform with all sources of artificial lighting including tungsten and fluorescent. In one experiment, the effect of two different light sources on bacterial inactivation by LAAM Conjugate I coated fabric was examined. Eight replicate samples were tested at different positions under a full spectrum compact fluorescent fixture. These were compared to three replicates run with a tungsten light source. These values were compared to non-irradiated fabric samples. Results: Samples irradiated with both the tungsten and fluorescent lights produced > 99 % reduction in viable S. aureus. Non-irradiated Rose Bengal-coated and non-coated fabric showed no growth whereas the irradiated non-coated fabric control showed bacterial growth. Rose Bengal-coated fabric should show equivalent effectiveness using illumination from different standard light sources.

2.9.4 Accelerated Aging Accelerated aging was done at AppTec. Packaged masks [Conjugate I coated fabric] from each lot were stored in Environmental Specialties chambers set to 55C, 70 to 80% RH in the dark for the indicated time periods. Testing was done by Hunter a* measurement, RNO assay and *S. aureus* culture assays. Control samples that had been stored under normal room conditions of temperature and RH were run as controls in the assay with samples that had been aged for two, four, and eight weeks. Linear regression slope [mean colorimetric intensity and singlet oxygen production rate] produced with each lot did not vary by more than 0.1 over the course of the study, once the dye compound on the mask had cured. Stability of the mask coating also was verified by culture assay with *S. aureus.* The data to date would indicate a probable stability of at least two years with storage at room temperature. Accelerated aging will continue, with test dates every four weeks, to 32 weeks, to obtain a probable long-term shelf-life of 5 years.

2.9.5 Photobleaching: One possible problem associated with the use of such coatings is "photobleaching" of the photosensitizers, which can result from degradation of the photosensitizer by the singlet oxygen generated. However, in earlier reported studies [2] when the photosensitizer-containing coating was exposed to seven cycles of alternating light and dark periods [16 h of light and 8 h of darkness], no reduction in its bactericidal activity was detectable; a 5.7 log10 reduction in the viable count of a suspension of S. aureus [2.3 x 10^6 CFU/ml] was obtained after 6 h of illumination. These findings suggest

that photobleaching, at least in the short term, would not be a problem. The Mann-Whitney U test was used to compare the number of survivors in the various suspensions with the number of survivors from the control samples. A P value of <0.05 was considered statistically significant. In none of the experiments did exposure of the microbial suspensions to the coatings in the dark result in a significant reduction in the viable count of the suspension.

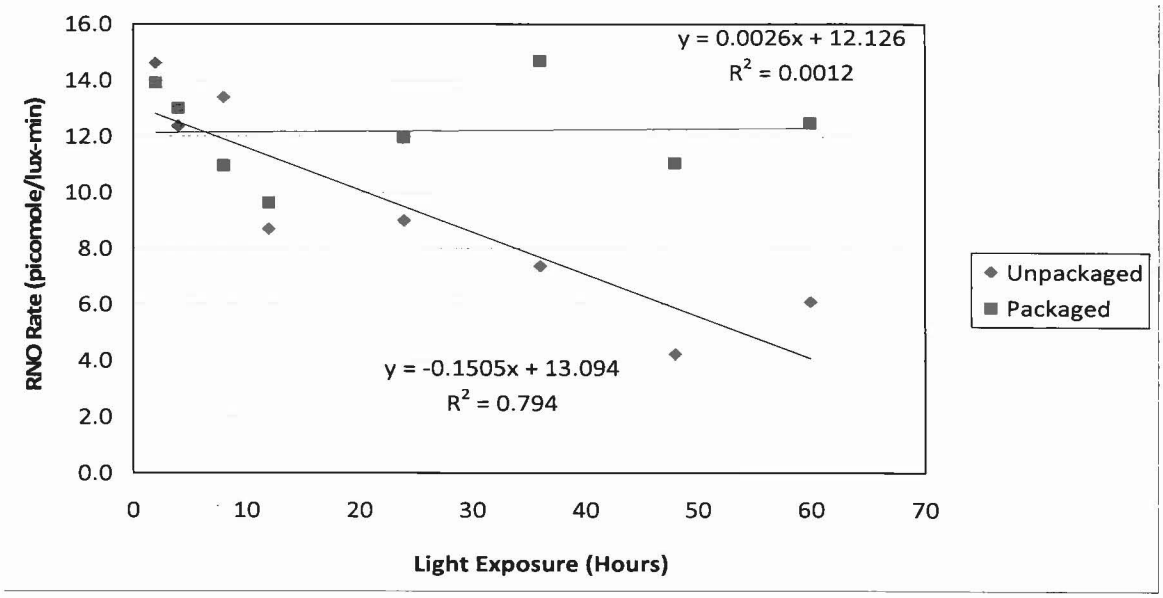

Figure 8. Photobleaching of wrapped and un-wrapped LSI masks [RNO rates after irradiation of Lot 2 masks at 14,000 lux for the indicated time] Wrapped and non-wrapped masks containing the Rose Bengal-coated fabric were exposed to seven cycles of alternating light [approximately 14,000 lux] and dark periods [16 h of light and 8 h of darkness].

Acceptance criteria: There should be no reduction in Hunter a* assay results, or any decrease in activity against the test organisms, of the packaged masks.

Results: Existing mask packaging is sufficient to protect the mask from photobleaching for at least three days [Figure 8]. Long-term stability and antimicrobial activity studies of the LSI mask under high-light conditions are ongoing. The new foil-lined packaging will be used to protect the masks. The unwrapped mask cannot be used for longer than 12 hr under these lighting conditions without compromise of its antimicrobial activity.

2.10 Safety Testing under GLP conditions.

2.10.1 In vitro toxicity *In vitro* toxicity of Conjugate I mask fabric to mammalian cells was evaluated by WuXi AppTec, Inc [St. Paul, MN]. Leachable extracts were allowed to diffuse to L-929 Mouse Fibroblast cells which were employed for these assays by a method compliant with the requirements specified in (ISO 10993-5:1999). Results: The mask fabric was scored at '0' and is considered non-toxic.

2.10.2 Skin Irritation and Sensitization The dermal irritation and sensitization potential of Conjugate I mask fabric was tested as required by regulation of medical device biocompatibility at WuXi AppTec, Inc [St. Paul, MN]. **Primary Skin Irritation:** The dermal irritation potential of the coated mask fabric was tested on the shaved skin of rabbits at WuXi AppTec, Inc [StPaul, MN]. Clinical Observations: None of the animals on study showed abnormal clinical signs during the 72 hour test period. Dermal Observations: There were no significant dermal reactions observed at the test sites on the rabbits at the 60 minute, 24, 48 and 72 hour observation *periods.* **Repeated patch dermal sensitization test:** This test was designed to evaluate the allergenic potential or sensitizing capacity of a test article. This test was used as a procedure for the screening of contact allergens in guinea pigs and extrapolating the results to humans, but does not establish the actual risk of sensitization in humans. Clinical Observations: None of the animals in the study showed abnormal clinical signs during the test period. The severity was calculated as '0' at each time point.

Administrative Note: These animal studies were not funded as part of the current SBIR, but the results are included to demonstrate the safety of the fabric in cytotoxicity and sensitivity studies.

2.10.3 NIOSH/Physical integrity

In addition to RNO generation and microbicidal studies performed at LaamScience, a number of physical parameters were tested by Nelson Labs [Salt Lake City, UT] to independently demonstrate compliance with NIOSH standards. *The LSI mask met all specifications for certification as an N95 NIOSH Respirator.* Including: Standard Test Method for Resistance of Surgical Mask to Penetration by Synthetic Blood [ASTM F 1862, No blood penetration was observed]; Standard Test Method for NaCl Particulate Aerosol Penetration [42 CFR part 84.181 and NIOSH procedures RCT-APR-STP-0057, 0058, and 0059, Average initial resistance to airflow was 7.8 mm H_2O]; Standard Test Method for Bacterial Filtration Efficiency [ASTM F2101, BFE was 99.8% or greater]; Standard Test for Differential Pressure [Delta-P, pressure drop across the mask met specifications. The differential pressure was an average of 5.3 mm H_2O/cm^2. This would rate on the comfort scale as hot]; Standard Flammability Test Method [16 CFR 1610, All parameters met specifications. LSI masks meet the specification for Type A, Class I, no flame spread]; Standard test method for Inhalation/Exhalation Resistance [42 CFR Part 84.180, APR-STP 007 [inhalation] and RCT-APR-STP-003 [exhalation], All parameters for the test and controls exceeded specifications [inhalation average 4.6 mm H_2O; exhalation 6.3 mm H_2O]. The lower inhalation/exhalation resistance [as compared to the predicate Gerson 2130 mask] should make this mask more comfortable to wear.]

2.11 Technology Developed: The LaamScience mask is a combination medical mask /NIOSH-certified N95 Respirator. A medical mask covers the user's nose and mouth and provides a physical barrier to fluids and particulate materials. A medical respirator is fitted to the user's face, forming a seal that provides a physical barrier to fluids, particulate materials, and aerosols. The masks are individually-wrapped in opaque, peel-open packages for maximum stability and are provided nonsterile. The masks are for single use only and are disposable.

The masks are designed to be self-decontaminating. This is accomplished by permanent bonding of a thin, biocompatible, non-leachable coating to the exterior of the mask by a normal textile-finishing process. The coating on the outer layer of the mask generates an antimicrobial agent [singlet oxygen] in the presence of light to reduce microbial contamination on the surface of the mask. This agent is intended only to reduce the potential for passive, contact transmission of viable organisms from the mask to other surfaces, particularly during donning, doffing and adjustment of the mask, and is not intended to have any effect on the user.
Indications for use: The Mask is designed to cover the users' nose and mouth to protect personnel from the transfer of microorganisms, body fluids and particulate material. The masks are indicated for use in any procedure or situation where there is a risk of such transfer. The masks are for single-use only.

The singlet oxygen produced by the mask coating inactivates Influenza A, *S. aureus* and MRSA by three to four logs within one hour of virus or bacteria contact with the mask surface under light levels typical of hospital settings. The mask is not intended for prolonged contact with intact skin; the coated layer of the mask is the outermost layer that is unlikely to come into contact with the wearer's skin. Nevertheless, the mask was evaluated for biocompatibility of the coated fabric per FDA-modified Use of International Standard ISO-10993, Biological Evaluation of Medical Devices Part-1: Evaluation and Testing. The required tests for this level include cytotoxicity, sensitization, and irritation. The coated mask fabric, as well as saline and oil extracts of the coated fabric, showed no toxicity in any test, thereby ensuring the safety of the product for its intended use.

2.11 Current status of the product. The LaamScience N95 Respirator and Surgical Mask is under development. It has been tested by LaamScience, Nelson Laboratories, and WuXi AppTec to NIOSH standards in accordance with 42 CFR Part 84 and meets CDC guidelines for TB control. The mask met all prescribed challenge test criteria of 95% efficiency or better for bacterial filtration efficiency, particulate filtration efficiency, breathing resistance, differential pressure. The masks are resistant to synthetic blood per ASTM F 1862 and are nonflammable per CPSC CS-191-53.

2.12 How the company has benefitted. LaamScience has benefitted from the program by adding personnel to perform these studies needed to submit the N95 with SERQET to the FDA for 510K approval. An application for a bridge loan has been submitted to the North Carolina Biotechnology Center (pending). Partnerships with North Carolina textile manufacturers and hospitals are underway for the advancement of the manufacturing process and clinical trials for efficacy in the reduction of the spread of communicable diseases.

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None

Materials available for other investigators.
None