Use of glycerol as an optical clearing agent for enhancing photonic transference and detection of *Salmonella typhimurium* through porcine skin

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Abstract. The objective of this study was to evaluate glycerol (GLY) and GLY + dimethyl sulfoxide (DMSO) to increase photonic detection of transformed *Salmonella typhimurium* (*S. typh*-lux) through porcine skin. Skin was placed on 96-well plates containing *S. typh*-lux, imaged (5 min) using a CCD camera, and then completely immersed in PBS, GLY, DMSO, GLY+DMSO in a dose- and time-dependent manner and re-imaged (5 min). The percent of photonic emissions detected (treated or untreated skin relative to no skin controls) was used for analysis. Treatment for 4 h with 50% GLY-PBS and 50:30:20% GLY:DMSO:PBS increased photonic detection compared to untreated skin, 100% PBS, or 30:70% DMSO:PBS. Treatment with 50% GLY in the presence of 20 and 40% DMSO (v/v with PBS) increased photonic detection compared to 50% GLY alone and in the presence of 10% DMSO: 50% GLY (v/v with PBS). Data indicate that GLY and GLY +DMSO are effective optical clearing agents on porcine skin (2–3 mm thick) when treated for 4 h to increase detection of emitted photons. Clearing agents such as GLY have the potential to minimize effects of porcine skin tissue as one of the photon transmittance barriers (i.e., skin, fat, muscle, and visceral tissues) *in vivo*. © *2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2363366]

Keywords: bioluminescence; *Salmonella typhimurium*; porcine skin; glycerol; optical clearing.

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1 Introduction

It is estimated that 2 to 4 million cases of salmonellosis occur in the U.S. each year, which accounts for $1 billion annually in direct and indirect medical costs. The food industry continues to have growing concerns regarding *Salmonella* exposure, especially antibiotic-resistant strains of *S. typhimurium* appearing in animal and human health critical control points.[1] To date, most strategies have focused on post-slaughter evaluation of meat products; however, pre-slaughter intervention points are being developed that recognize the role the live animal plays as host to bacterial pathogens.

Many pathogens in the food animal gastrointestinal tract are difficult to diagnose, and surveys from fecal cultures on farms is presently the pre-harvest detection method of choice since the degree of fecal shedding is directly correlated with the incidence of carcass contamination. However, microbial shedding is sporadic and culture methods are time-consuming and provide little information on the internal genesis of bacterial pathogenicity that may lead to increased bacterial shedding. To evaluate disease exposure and pathogenicity in food animals, a more efficient research model could be developed. This technology could be utilized for pre- and post-harvest food safety issues, which today are a national concern. A possible proposal would be the use of photon-emitting bacteria (e.g., *Salmonella typhymurium*-lux) to model stress involvements in pathogenicity and improve research methods over conventional methods since natural (not transformed) *Salmonella* are not photon-emitting bacteria. This technology, which has been applied primarily in small animal models,[2–4] could identify additional control points and provide targets for direct intervention in the transmission of specific food-borne pathogens in harvested food animal species, as is currently being developed for swine models.[5]

However, an obstacle to the *in vivo* imaging process using bioluminescent bacteria is the fact that light attenuation by absorption and scattering in tissue is significant. In fact, others have shown that a bioluminescence signal is reduced by 2 to 9 orders of magnitude (depending on wavelength) when the luminescence source is located 1 cm deep in the tissue.[6] In this context, both scattering and absorption contribute to signal attenuation whereas spatial resolution is primarily compromised by light scattering.[6] One photon transmittance barrier that could be altered to increase photon detection is skin tissue. Optically cleared neonatal porcine skin could potentially increase the sensitivity of detection of photon-emitting bacte-

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ria in the gastrointestinal tract, resulting in improvements in the research model. In more mature and large swine, the optical clearing agents could be utilized on visceral tissue during laparoscopic procedures to improve the sensitivity of detection of photon-emitting bacteria. Several previous investigations have observed optical improvements using hyperosmotic chemicals, such as glycerol, DMSO, polypropylene glycol, and polyethylene glycol (in mouse skin;[7] hamster skin;[8] human skin;[9] and porcine stomach[10] tissues), as determined by spectrophotometric methods for obtaining transmittance and diffuse reflectance measurements. Other proposed mechanisms for light scattering reduction in tissue as a result of optical clearing are index matching of agent with tissue components,[11,12] and molecular/structural modification of collagen.[13]

The objectives of this study were to use glycerol as an optical clearing agent to (1) cause an improvement in luminescence light transmission through porcine skin, and (2) evaluate the change in optical properties (absorption coefficients and reduced scattering coefficients) of porcine skin.

2 Materials and Methods

2.1 *Tissue Preparation*

Market-type swine ($\sim$110 kg) and mature (pregnant) gilts ($\sim$154 kg) were available for collection of porcine skin immediately following slaughter at the Mississippi State University abattoir. Once the carcass was scalded, the hair was removed and the carcass was hung and eviscerated. Scalded, dehaired pig skin was utilized for these studies due to abattoir regulatory compliance guidelines that prohibit the removal of unprocessed skin prior to scalding and dehairing as part of normal carcass processing procedures. Diffusional and optical properties of pig skin can be influenced by the scalding/hair removal process, as preliminary data from our laboratory demonstrate that control (i.e., not treated with optical clearing agents), scalded, dehaired skin exhibits a three-fold greater photonic transference compared to native skin not scalded and dehaired. However, in the presence of the optical clearing agent glycerol (4 h treatment), native skin had a slightly greater ($P<0.05$) photonic transference than processed skin at 50% glycerol treatment compared to untreated controls, but did not differ ($P>0.10$) for 100% glycerol-treated skin (i.e., photonic transference in the presence of glycerol changed proportionally among skin types with 100% glycerol; data not shown). These preliminary data would suggest that the processing of the pig skin prior to *ex vivo* experiments does not augment photonic transference compared to native skin when contrasting optically cleared (glycerol-treated) skin to companion untreated controls. For studies, a 25 cm $\times$ 25 cm area of tissue was removed with a knife from each side of the shoulder, which included layers of skin, fat, and muscle. Muscle and fat tissue layers were removed from skin using a scalpel blade. The skin was then cut into 30 mm $\times$ 30 mm pieces to cover a minimum of 6 wells on a black 96-well plate. The skin pieces were stored at 4 °C in a humid environment up to 96 h to facilitate experimental paradigms. The thickness of skin pieces was measured prior to each experiment with a caliper. A second set of skin from the pregnant gilts was prepared in the same manner and stored in water-moist gauze at 4 °C for 24 h until analysis of skin optical properties by UV-VIS-NIR spectrophotometry (described ahead).

2.2 *Bacterial Plate Preparation*

Salmonella typhimurium (ATCC #14028; Manassas, VA) transformed with plasmid pAKlux1 (*S. typh*-lux) was utilized for generation of photons and imaging. The plasmid (11,904 bp) is a broad-host-range cloning vector maintained at a medium copy number, is mobilizable, is compatible with numerous plasmid replicons, and contains the lux operon.[14] The lux operon is a cluster of genes (lux CDABE) isolated from a nematode symbiont bacterium *Xenorhabadus luminescens* (luciferase).[15] This operon also encodes the biosynthetic enzymes for the proper substrate. A colony of *Salmonella typhimurium* was used to inoculate 5 ml of sterile Luria Broth (LB) and grown with vigorous aeration overnight at 37 °C. A flask containing 50 ml LB broth was then inoculated with 1.5 ml of cells from an overnight inoculum and grown with vigorous aeration at 37 °C until optical density (O.D; at 550 nm, 0.8 was reached using a spectrophotometer 8453 (Agilent Technologies, Palo Alto, CA), which, as determined from culture on Brilliant Green agar plates, equates to $\sim 1 \times 10^7$ colony-forming units (CFU) of bacteria. The flask of cells was maintained on ice for 15 min until harvesting. Cells were harvested by centrifugation at 3000 rpm ($1700 \times g$) for 10 min at 4 °C. The cell pellet was then resuspended in washing buffer (WB; 25 ml sterile ice-cold 10% v/v glycerol), following which a sequence of 3 cycles of centrifugation and resuspension in washing buffer was conducted. The electroporation apparatus (Gene Pulsar II; Bio-Rad, Hercules, CA) was set to 2.5 kV, 25 μF, and 200 ohms. Forty μl of competent cells were added to an ice-cold 2-mm gap cuvette with 1 μl of plasmid. The cuvette was loaded into the instrument chamber and the pulse applied. The cuvette was removed, and 450 ml of LB broth were added and mixed by inversion. The cells were transferred to a 1.5 ml eppendorf tube and incubated for 1 h with moderate shaking at 37 °C. After incubation, 200 to 500 μl of transformation culture were plated on Brilliant green ampicillin agar plates for selection. The Berthold/Nightowl camera with a 25 mm and $f=0.95$ camera lens was positioned 245 mm above the imaging target (96-well plate format) using WinLight 32 version 2.51.11901 software (Berthold Technologies, Oak Ridge, TN). All photon counting was conducted following 5-min acquisition periods and single frame accumulation with 1×1 binning and low gain. The plate was divided into 8 sample areas using 6 wells per area. Three wells per area were filled with 100 μl of sterile LB, and 3 wells per sample area were filled with 100 μl of *S. typh*-lux-LB broth. The plate was maintained on a plate warmer at 37 °C inside the Nightowl imager during imaging. In accordance with previous investigations monitoring luminescence measurements via a photomultiplier photometer at 560 nm for *E. coli* transformed with the plasmid pCGLS-1,[15] the images of the 96-well plate were analyzed at a 560-nm wavelength with uniform areas created for each well on the plate.

2.3 *Optical Clearing Agents and Imaging Procedure*

For each experiment, the control black 96-well plate containing sterile broth and *S. typh*-lux-LB broth was imaged for

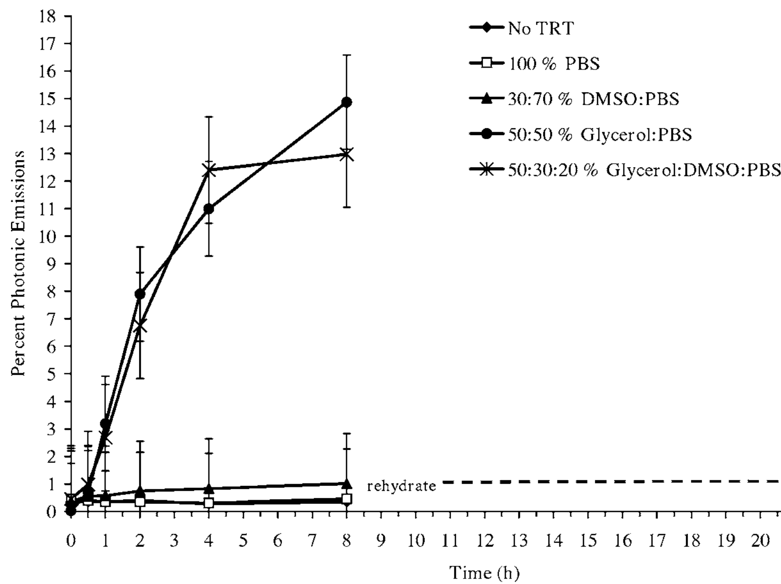

Fig. 1 Time course of glycerol alone (50:50% Glycerol:PBS) or with DMSO (50:30:20% Glycerol:DMSO:PBS) as an optical clearing agent in market-weight porcine skin (3-mm thick) relative to 100% PBS (dilution vehicle), 30:70% DMSO:PBS, or no treatment (No TRT). Skin was then rehydrated with 100% PBS and re-imaged 16 h later (24 h post-initiation of treatment).

5 min. The skin was then placed over the sample sections of the plate and imaged again for 5 min. The skin was completely immersed for the appropriate time (0.5 to 24 h depending on the experimental paradigm, described ahead), the plate was re-imaged for 5 min without skin, and then the treated skin was placed over the sample sections and imaged for 5 min. An analysis report of the images was created using WinLight 32 to evaluate the 96-well plate grid areas for photons/pixel/s. The raw data were corrected for background by averaging the wells containing sterile LB broth only for each sample section and then subtracting the value from each well containing the *S. typh*-lux-LB broth for each sample section. The background subtracted data were then used to calculate percent photonic emissions [i.e., (skin or treated skin/no skin control) $\times$ 100 %] and used for statistical analysis in each experiment (described ahead).

Initially, a time course design was developed to evaluate the optical clearing abilities of glycerol (GLY) on market-weight porcine skin treated with 100% PBS (phosphate buffered saline vehicle used for dilutions), 30:70% DMSO:PBS, 50:50% GLY:PBS, 50:30:20% GLY:DMSO:PBS, and no treatment that paralleled the agents used by other investigators studying tissue clearing in porcine stomach tissue using near-infrared spectroscopy.[16] The plate was imaged again for 5 min without treated skin and then again for 5 min with treated skin placed over the sample sections of the plate after immersion in the specified treatments for 0.5, 1, 2, 4, and 8 h. Immediately after the 8 h imaging sequence, the skin was immersed in PBS for 16 h and re-imaged at 24 h post-treatment. A second time course, treatment at 0.5, 1, 2, 4 h and rehydration with PBS for 8 h for re-imaging at 12 h post-treatment, similar to that described for market-weight porcine skin. was developed to evaluate mature gilt porcine skin treatments, with treatments as follows: 50:50% GLY:PBS, 100% PBS, 30:70% DMSO:PBS, 50:30:20% GLY:DMSO:PBS, 100% GLY, and no treatment.

Skin immersed for 4 h did not differ ($P > 0.10$) from immersion for 8 h (Fig. 1) in the initial time course experiment; therefore, subsequent investigations evaluated different concentrations of GLY as an optical clearing agent by immersing the skin for 4 h only. A dose-response design using various concentrations of GLY and PBS was formulated as follows: 5:95% GLY:PBS, 10:90% GLY:PBS, 25:75% GLY:PBS, 50:50% GLY:PBS, 75:25% GLY:PBS, 100% GLY, and no treatment. Following this, a dose-response design using various concentrations of GLY and DMSO brought to volume with PBS was developed as follows: 50:50% GLY:PBS, 90:10% GLY:DMSO, 80:20% GLY:DMSO, 70:30% GLY:DMSO, 60:40% GLY:PBS, 50:50% GLY:PBS, and no treatment. A final targeted treatment design using GLY:DMSO:PBS was performed by immersing skin tissue for 4 h in the following treatments: 50:50% GLY:PBS, 50:30:20% GLY:DMSO:PBS, 30:70% DMSO:PBS, 100% PBS, and no treatment (Table 1). As a measure of variation among replicates in relation to treatment effects, the coefficients of variation (CV) were calculated among studies for similar treatments (see Table 1) and were 34.7 and 23.7% for the intrareplicate CV for pre- and post-treatment, respectively, and 31 and 35% for the inter-replicate CV for pre- and post-treatment, respectively.

2.4 *Optical Properties Measurement*

A UV-VIS-NIR spectrophotometer (Perkin-Elmer, Lambda 900; Boston, MA) with an integrating sphere attachment was used to measure total transmission (T_t) and diffuse reflectance (R_d) for a set of porcine skin treated with GLY as the optical clearing agent. The skin sample thickness was measured for each sample with an accuracy of approximately 30 μm (i.e., better than 1% accuracy for a given measurement). At each wavelength between 350 nm and 800 nm (stepsize=5 nm), both T_t and R_d were measured and corrected for baseline mea-

Table 1 Combinations and Ratios of Glycerol, Phosphate, Buffered Saline (PBS), and Dimethyl Sulfoxide (DMSO) as Treatments on 3-mm-thick Porcine Skin for Evaluating Optical Clearing Properties of Glycerol and Photonic Transference Through Porcine Skin.

Optical clearing agent ratio and combinations	Pre-treatment mean percent photonic emissions±SE[a]	Post-treatment mean percent photonic emissions±SE[a]	Post-treatment mean percent photonic emission change±SE[a]
Glycerol:PBS Ratio			
5:95% glycerol:PBS	1.50±0.13[a]	1.10±0.21[b]	−0.40±0.12[b]
10:90% glycerol:PBS	1.67±0.08[a]	1.25±0.09[b]	−0.42±0.08[b]
25:75% glycerol:PBS	1.54±0.15[a]	4.30±0.47[c]	2.75±0.39[c]
50:50% glycerol:PBS	2.51±0.44[a]	9.19±0.85[d]	6.68±0.59[d]
75:25% glycerol:PBS	1.92±0.30[a]	8.54±0.75[d]	6.62±0.53[d]
100% glycerol	2.18±0.35[a]	7.83±0.96[d]	5.65±0.68[d]
No treatment	1.91±0.21[a]	1.90±0.70[a]	0.01±0.14[a]
Glycerol:DMSO Ratio			
90:10% glycerol:DMSO	0.43±0.05[a]	10.77±1.27[cd]	10.35±1.25[cd]
80:20% glycerol:DMSO	0.47±0.05[a]	13.76±1.17[b]	13.29±1.14[b]
70:30% glycerol:DMSO	0.40±0.03[a]	12.78±1.04[bc]	12.37±1.02[bc]
60:40% glycerol:DMSO	0.42±0.08[a]	14.38±1.12[b]	13.96±1.08[b]
50:50% glycerol:DMSO	0.38±0.06[a]	12.30±1.13[bcd]	11.93±1.10[bcd]
50:50% glycerol:PBS	0.37±0.03[a]	10.36±1.03[d]	9.98±1.02[d]
No treatment	0.39±0.04[a]	0.47±0.07[a]	0.08±0.06[a]
Glycerol:DMSO:PBS			
50:50% glycerol:PBS	0.34±0.04[a]	4.91±0.77[b]	4.58±0.75[b]
50:30:20% glycerol:DMSO:PBS	0.26±0.03[a]	6.48±0.92[b]	6.22±0.91[b]
30:70% DMSO:PBS	0.31±0.43[a]	0.50±0.08[a]	0.20±0.07[a]
100% PBS	0.24±0.03[a]	0.22±0.03[a]	−0.02±0.01[a]
No treatment	0.32±0.04[a]	0.24±0.05[a]	−0.07±0.05[a]

Treatment by immersion for 4 h. Superscripts with different letters in each column designate values that differ ($P < 0.05$).
[a]Photonic emissions were generated by *Salmonella typhimurium*-lux bacteria.

surements and normalized to a standard. Also, at each wavelength, the values of the absorption coefficient (μ_a) and the reduced scattering coefficient (μ_s) were calculated from the spectrometer data using an inverse adding doubling program developed by Prahl in 1993[17] that takes into account the thickness decrease of approximately 15% as a result of soaking in an optical clearing agent. On average, the thickness decreases by 15%, but since the optical properties are calculated on a sample-by-sample basis, using the corresponding thickness measurement for each, the optical properties are expected to deviate no more than 1% based on the thickness measurements. The porcine skin anisotropy factor (g) was assumed to be 0.93 based on published values[18] and is assumed to remain unchanged although this has not been definitively determined in the literature. Four samples of skin were measured using digital calipers to within ±30 μm prior to treatment, after immersion for 1 h in 100% GLY and after rehydration in PBS for 1 h.

2.5 *Statistical Analysis*

The percentage of photonic emissions was calculated from 3 treated wells compared to 3 untreated wells within the skin specimen for each treatment. A total of 8 treated/untreated replicates was performed for each experiment, and data within skin treatment and across skin treatments were normalized to

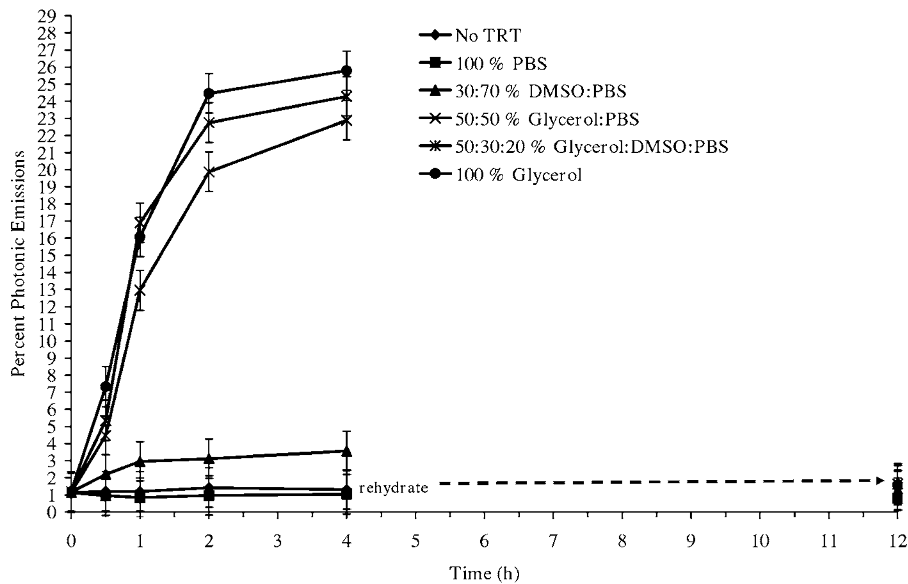

Fig. 2 Time course of glycerol alone (100% and 50:50% GLY:PBS) or with DMSO (50:30:20% Glycerol:DMSO:PBS) as an optical clearing agent in mature gilt porcine skin (3-mm thick) relative to 100% PBS (dilution vehicle), 30:70% DMSO:PBS, or no treatment (No TRT). Skin was then rehydrated with 100% PBS and re-imaged 8 h later (12 h post-initiation of treatment).

companion controls (i.e., no skin) and pooled for analysis ($n=24$ wells per treatment for each experiment). The ANOVA procedure revealed if significant differences occurred between treatments within an experiment followed by least significant difference for comparison of means within an experiment. The time-dependent experiments (repeated over time, within skin specimen imaging, in the presence or absence of optical clearing agents) were analyzed as a repeated measures ANOVA using the mixed procedure of SAS, and individual time-point means were compared ($P<0.05$) using the method of least-square means (SAS 9.1, Cary, NC).

3 Results

After immersion for 0.5, 1, 2, 4, and 8 h, market-weight shoulder porcine skin (3-mm thick) immersed in 50% GLY did not differ from immersion in 50:30% GLY:DMSO (v/v in PBS; $P>0.10$) in the percentage of photonic emissions detected (Fig. 1). However, both treatment groups exhibited a greater ($P<0.05$) percent photonic emission detected than no treatment, 30% DMSO (v/v in PBS), or 100% PBS at 2, 4, and 8 h post-treatment (Fig. 1). Also shown in Fig. 1, the percentage of photonic emissions detected did not differ ($P>0.10$) from the no treatment or treatment groups when rehydrated by soaking in PBS for 16 h at 24 h post-treatment. Mature gilt shoulder porcine skin (3-mm thick) treated with 100% GLY, 50:50% GLY:PBS and 50:30% GLY:DMSO (v/v in PBS) exhibited greater ($P<0.05$) percentage of photonic emissions detected than no treatment, PBS, and DMSO alone after treatment for 1, 2, and 4 h (Fig. 2). Also shown in Fig. 2, the percentage of photonic emissions detected did not differ ($P>0.10$) from the no treatment or treatment groups when rehydrated by soaking in PBS at 12 h post-treatment.

Market-weight shoulder porcine skin (3-mm thick) treated for 4 h with varying ratios of GLY:PBS (50:50, 75:25, and 100:0%) increased ($P<0.05$) the percentage of photonic emissions detected (9.19±0.85%, 8.54±0.75%, and 7.83±0.96%, respectively) compared to no treatment and 5:95% GLY:PBS (1.90±0.70%, and 1.10±0.21%, respectively). The percentage of photonic emissions detected was not different ($P>0.10$) for skin pieces imaged for 5 min without treatment (Table 1).

Market-weight shoulder porcine skin (3-mm thick) treated for 4 h with altering the ratios of DMSO (10, 20, 30, 40, and 50%; Table 1) demonstrated that addition of DMSO at 20% and 40% (13.76±1.17% and 14.38±1.12%, respectively) with GLY (v/v in PBS) increased ($P<0.05$) the percentage of photonic emissions detected compared to 90:10% GLY:DMSO (10.77±1.27%) and 50:50% GLY:PBS alone (10.36±1.03%). Another evaluation of skin immersed for 4 h in 50:50% GLY:PBS alone and 50% GLY in combination with 30% DMSO (v/v in PBS), (4.91±0.77% and 6.48±0.92%, respectively), resulted in an increase ($P<0.05$) in the percentage of photonic emissions detected compared to no treatment, PBS, and DMSO alone (0.24±0.05%, 0.22±0.03%, and 0.50±0.08%, respectively). The percentage of photonic emissions detected was not different ($P>0.10$) for skin pieces imaged for 5 min without treatment (Table 1).

The percent reflectance (Fig. 3(a)) decreases (three- to four-fold) and the total percent transmission (Fig. 3(b)) increases (five- to six-fold) due to glycerol immersion in the spectral range of interest (500 to 600 nm; detection of luciferase is at 560 nm). After 1 h of rehydration with PBS, the percentage of reflectance increases (one- to two-fold) toward the pre-treated values while the percentage of transmission decreases (two- to three-fold) toward the pre-treated values. The plots for the average ($n=4$) values for μ_a (Fig. 3(c)) and μ_s (Fig. 3(d)) as a function of wavelength are described for skin without treatment, with treatment for 1 h with 100% GLY, and then following rehydration with PBS for 1 h. The

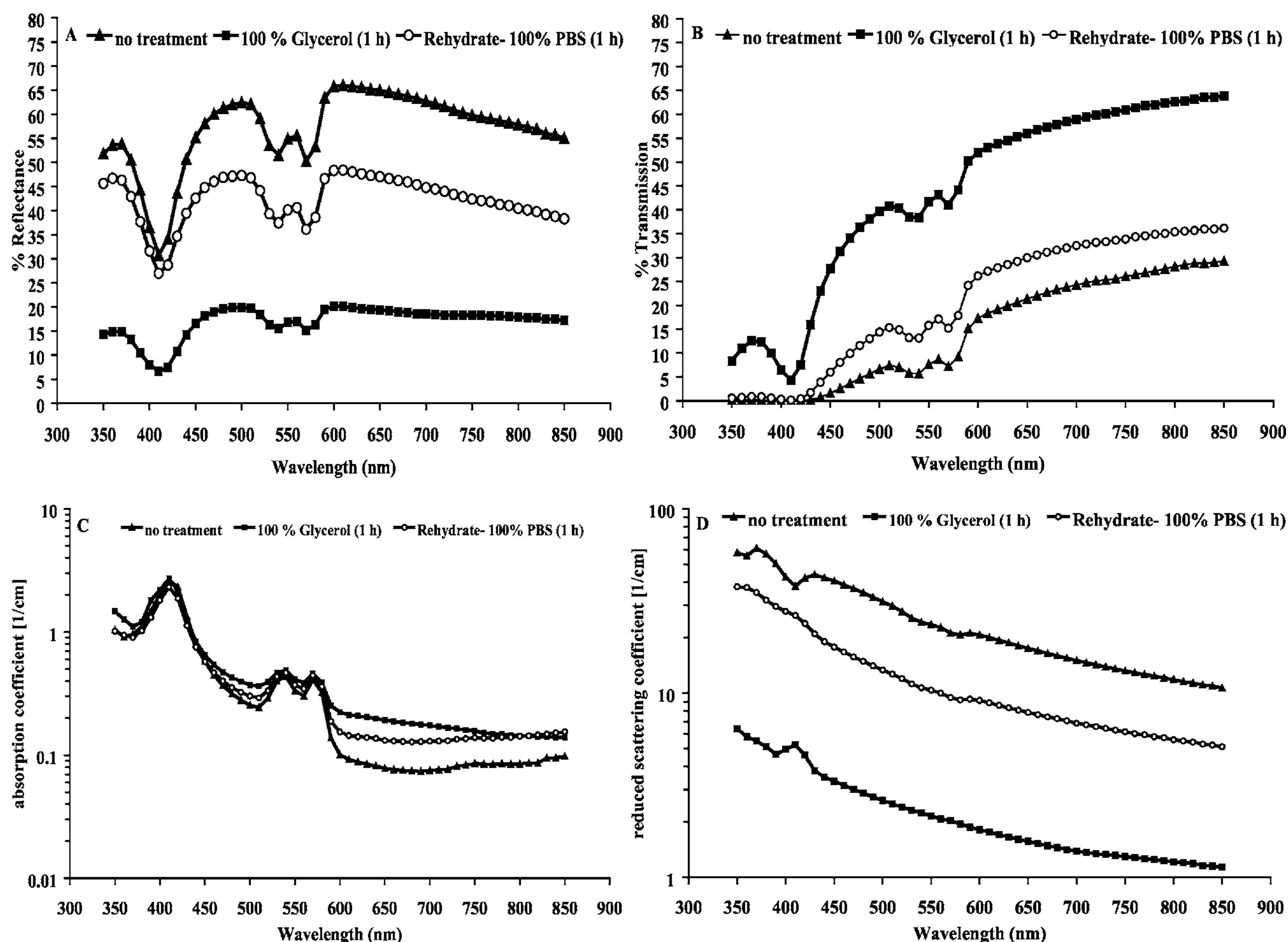

Fig. 3 Panel A: Percent reflectance as function of wavelength for no treatment, 100% glycerol (1 h), and rehydrated in 100% PBS (1 h). In the spectral range of interest (500 to 600 nm) the diffuse reflectance decreases three- to four-fold due to glycerol immersion. Panel B: Percent transmission as function of wavelength for no treatment, 100% glycerol (1 h), and rehydrated in 100% PBS (1 h). In the spectral range of interest (500 to 600 nm), the total transmission increases five- to six-fold due to glycerol immersion. Panel C: Absorption coefficient (μ_a) as function of wavelength for no treatment, 100% glycerol (1 h), and rehydrated in 100% PBS (1 h). These data were obtained from the reflection and transmission values along with sample thickness. Curves represent average values over four measurements. Panel D: Reduced scattering coefficient (μ_s) as function of wavelength for no treatment, 100% glycerol (1 h), and rehydrated in 100% PBS (1 h). These data were obtained from the reflection and transmission values along with sample thickness. The anisotropy factor (g) was assumed to be 0.93. Curves represent average values over four measurements.

absorption coefficient (μ_a) does not change significantly ($P > 0.10$) over the wavelength range of interest (450 to 600 nm) as a result of either the clearing or the rehydration process. In contrast, the reduced scattering coefficient (μ_s) changes by approximately one order of magnitude over the entire wavelength range. After rehydration in PBS, the reduced scattering coefficient restores toward its original value. However, after rehydration with PBS for 1 h, the thickness remains decreased by only 5% from the original thickness. Thickness measurements were accurate to within 30 μm for any given sample. On average for all samples, the thickness came back to within 5% of the pre-treatment thickness with an accuracy of ±1%.

4 Discussion

In this study, we have evaluated total transmission, diffuse reflectance, absorption coefficient, and reduced scattering coefficient of light in untreated and optically cleared porcine skin. In addition, we also investigated glycerol and DMSO as optical clearing agents that have the potential to improve the sensitivity of detection of photons through porcine skin. Within the spectral range of interest (500 to 600 nm), the addition of glycerol caused the absorption coefficient to remain the same while decreasing reflectance, increasing transmission, and reducing the reduced scattering coefficient of light while improving the sensitivity of detection of photonic emissions from transformed *Salmonella* through porcine skin compared to untreated porcine skin. These results are similar to the significant improvements in light transmittance (29%) and the decrease in diffuse reflectance (31%) observed previously on swine gastric tissue using a combination of 50% glycerol and 30% DMSO in which the synergistic clearing effect is thought to be due to the membrane permeability and carrier effect of DMSO.[16] Similar improvements of brightness and spatial resolution through optical coherence tomography and CCD detection after using 50% GLY when the chemilu-

minescence target is covered by biological tissue with a thickness of either 1 or 3 mm has also been reported previously.[19,20] Our results concerning the improvement in the sensitivity of detecting photons through porcine skin treated with 100% GLY and 50% GLY:50% PBS are similar to the results previously mentioned by other researchers. In a two-photon microscopy of *ex vivo* human dermis, a high concentration of GLY is similarly effective in improving image contrast and penetration depth.[21] Similarly, a significant and stable optical clearing effect also results in rat skin 8 to 10 min post-injection using 75% GLY in distillate water.[22] In our studies, porcine skin treated with 100% GLY, 50% GLY:50% PBS, and a combination of 50% GLY: 30% DMSO: 20% PBS, results in small improvements of photon detection after immersion for 30 min and 1 h. However, greater improvements were made with increasing detection of photons after immersion for 2 and 4 h in treatments. As processed porcine skin was used in these studies (i.e., scalded and dehaired as per normal swine harvesting procedure), it should be noted that the optical properties of porcine skin and the actions of GLY as an optical clearing agent may differ when applied *in vivo* and require further study. Nevertheless, it appears that GLY and DMSO as optical clearing agents improved the sensitivity of detection of photonic light emitted by transformed (lux) *Salmonella typhimurium* through porcine skin.

It is believed that the primary mechanism of reduced scattering is through index matching between the ground substance (i.e., amorphous intercellular substances) and collagen.[11] Collagen makes up nearly 70 to 80% of the dry weight of skin and is assumed to account for a majority of light scattering within the skin. Another mechanism related to index matching is through dehydration, which results in water loss from the interstitial space. The components become tightly packed and increase the concentration of glycosaminoproteins, leading to an increased refractive index.[12] The index of refraction for water, tissue, and glycerol, respectively, are 1.33, 1.45, and 1.47 based on other studies.[6,8,9] Using GLY on the porcine stomach, previous studies have suggested that optical clearing is strongly correlated with the water desorption within tissue; therefore, the water content present in tissue affects optical clearing.[10,13] Moreover, trends in water loss of porcine stomach tissue were similar to the change of transmittance and diffuse reflectance in porcine skin immersed in combinations of propylene glycol, DMSO, and oleic acid.[10] Therefore, the clearing capability of an agent in porcine skin is likely correlated well with its dehydration effect.[10] Researchers described a hyperosmotic chemical agent as having the ability to decrease and then increase cell volume, which is indicative of permeable substances.[12] After GLY application to hamster skin, the improved visualization *in vivo* of subdermal blood vessels was attributed to a reduction in light scattering in the dermis and subsequent increase in light depth penetration. However, the observed optical and morphological effects were reversed upon hydration of skin with phosphate-buffered saline[8] and agree with our results of rehydrating treated porcine skin (GLY and DMSO) by soaking in PBS. With respect to penetration and skin depth, a previous study demonstrated that a 15-min topical treatment with 80% GLY and 50% DMSO to palmar skin of human volunteers resulted in an increase in imaging depth to 1.1 mm,[23] which parallels our study results following immersion of 3-mm-thick porcine skin for a minimum of 30 min in the hyperosmotic chemical agent (GLY+DMSO), which improved photonic detection two-fold.

The evaluation of various bandwidths using spectroscopy has previously been reported to increase transmission and decrease reflectance through mouse skin post-treatment with glycerol.[7] Moreover, within the spectral range of interest, the absorption coefficient remained constant while the reduced scattering coefficient decreased by approximately one order of magnitude. However, after rehydration, both transmission and reflectance values returned to within 10% of untreated values.[7] Our results also indicate a return to a similar quantitation of detected photons than that detected prior to any treatments after rehydrating the treated skin tissue with PBS. Following GLY application to rodent dermis, the tissue has been reported to become optically transparent with an increase in transmission of two orders of magnitude within the wavelength of 400 to 700 nm using an ultraviolet-visible spectrophotometer; yet the tissue turbidity returned to normal following PBS application.[22] Researchers have also found that DMSO has the ability to rapidly improve the penetration depth of light but does not enhance image contrast and is attributed to the fast diffusion characteristics of DMSO into tissue (porcine stomach) as studied with optical coherence tomography.[10] Similarly, our results indicated an improvement in the detection of photons when treating with GLY in combination with DMSO; however, while numerically greater in all cases, the effect was not always statistically greater than GLY alone (e.g., with 10% DMSO).

5 Conclusion

An assessment of the optical properties of porcine skin suggests that the addition of hyperosmotic chemical agents (i.e., glycerol) results in no change in absorption of photonic light, decreases photonic reflectance, increases photonic transmittance, and reduces photonic scattering. These qualities of optical clearing agents (glycerol and DMSO) improved the sensitivity of detection of photonic light emitted by transformed (lux) *Salmonella typhimurium* through porcine skin. Specifically, glycerol exhibited a dose dependence with a substantial increase in photonic detection at concentrations greater than 25% glycerol with immersion. Similar increases in photonic detection occurred after 4- and 8-h treatments with glycerol, as well as a partial return to pre-treatment values for treated skin rehydrated after soaking in PBS. To this end, glycerol and glycerol+DMSO are potential effective optical clearing agents for increasing photonic detection through porcine skin as a means to enhance the sensitivity of biophotonic models directed toward the detection of transformed (photon-emitting) bacterial pathogens *in vivo*, *ex vivo*, or *in situ*.

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