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Hemolysis as a Rapid Screening Technique for Assessing the Toxicity of Native Surfactin and a Genetically Engineered Derivative

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Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/ep.12444

If biosurfactants are to achieve their promise in environmental oil-spill remediation, their toxicity to marine life must be assessed. A killifish larvae assay is commonly used as a measure of toxicity but is difficult and nonlinear in response. Red blood cell (RBC) hemolysis has also been the basis for assays of some surfactant levels. Here we present a modified sheep RBC suspension assay and compare its response to that of the fish assay for surfactin and its genetically modified variant fatty-acyl-glutamate (FA-Glu). This is the first report of hemolytic activity as a property of FA-Glu. The method's potential for screening for toxicity against marine organisms is demonstrated. © 2016 American Institute of Chemical Engineers Environ Prog, 00: 000–000, 2016

Keywords: hemolysis, biosurfactant, bioassay, toxicity, green chemistry

NOVELTY/SIGNIFICANCE

It was determined that FA-Glu, a genetically engineered derivative of surfactin, can cause hemolysis and that this could serve as a measure of potentially toxic effects of FA-Glu. The sensitivity of RBCs to hemolysis by the surfactants parallels that of fish larvae used in determining toxicity of surfactants to marine organisms. Hemolysis is thus a simple, rapid, and useful prescreening technique for determining the toxicity of surfactants toward fish.

INTRODUCTION

Biosurfactants are surface-active substances produced by microorganisms. Chemically, they can consist of sugars, fatty acids, and amino acids [1–3]. They have useful surface activity, physiological, biocidal, and physicochemical properties, and, as a result, have received renewed attention in recent years [4]. Their biodegradability makes them environmentally friendly [5], and as they are produced by fermentation of renewable feedstocks [6,7], they are not linked to uncertain supplies of petrochemicals.

Bacillus sp. produce an assortment of cyclic lipopeptide surfactants. One such example is surfactin (for structure, see Supporting Information, Figure S1.) which consists of a 12–17 carbon β-hydroxy fatty acid joined via an amide linkage to the amino-terminal glutamic acid residue of a heptapeptide (L-Glu/L-Leu/D-Leu/L-Val/L-Asp/D-Leu/L-Leu) [8]. The carboxy-terminal leucine is esterified to the β-hydroxyl group of the fatty acid to form a lactone. Surfactin is a well-characterized biosurfactant and has potential in detergents, pharmaceuticals, cosmetics, food products, and oil recovery. It is also known to have antiviral activity [9], and thus has potential for use in preventing viral infections.

Scientists at Modular Genetics, Inc. (Woburn, MA) genetically modified *Bacillus subtilis* OKB105 to produce a substance termed *Fatty-Acyl-Glutamate,* or FA-Glu (for structure, see Supporting Information, Figure S1) [10]. FA-Glu has a 12-17 carbon β-hydroxy fatty acid; however, it lacks the heptapeptide moiety, and instead has only a single glutamic acid residue. Similar to surfactin, it has physicochemical properties with potential applications [3]. The modification was aimed at producing a surfactant with higher solubility and lower toxicity than surfactin to reduce environmental impacts while retaining sufficient surfactant properties, as well as giving it potential for other uses, for example, in pharmaceuticals, food, and personal care products.

An important consideration for commercializing a biosurfactant is toxicity. A specific case is the potential for using a biosurfactant for oil-spill remediation. If used for this purpose, it must be less toxic to fish and other aquatic organisms than currently used surfactants. Methods for assessing biosurfactant toxicity include bioassays using bacteria, fungi, and brine shrimp [11,12]. Vollenbroich *et al.* (1997) describe a cytotoxicity assay for surfactin using human and animal cell lines [13]. Another method examines the effects of biosurfactants on germination and elongation of plant seedlings [11,12]. An established method for determining toxicity for marine organisms [3] uses larval marine and estuarine fish species such as the inland silverside (*Menidia beryllina)* and sheepshead minnow (*Cyprinodon variegatus)* as accepted screening organisms for toxicity [14,15]. Unfortunately, these fish larvae potentially demonstrate differential responses to changes in salinity which could indicate altered susceptibility

Additional Supporting Information may be found in the online version of this article.

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to toxicants over a wide range of test salinities as encountered from coastal to offshore conditions [16]. Under laboratory conditions, Gulf killifish (*Fundulus grandis*) embryos have been successfully incubated and hatched at salinities between 0 and 80% [17]. This species is widely distributed in river, estuarine, and coastal marshes of the Gulf of Mexico [18] and tolerates a wide range of environmental salinities [17,19]. The fish grow quickly at the 7 − 8-day-old larval stage, enabling detection of toxic effects that are less readily seen in older animals.

Although larval estuarine and marine fish have been used to evaluate the toxicity of pollutants [16,20,21], fish bioassays have drawbacks, as collecting and hatching the fish eggs, and culturing and feeding the larvae and juveniles posthatch can be cumbersome and time consuming. For larval bioassays, fish must be microscopically examined daily for up to 4 days to determine viability. Even though culture techniques have been developed for this species [22] and are widely used, often only a relatively small number of fish are available for testing. For these reasons, obtaining sufficient data for results to be statistically significant can also be difficult. The Gulf killifish specifically represents a larval aquatic species with a wide environmental salinity tolerance. For this reason, it was chosen to determine if parallel responses exist between a fish acute toxicity bioassay and a hemolysis assay. As surfactin and FA-Glu have potential for oil-spill remediation, efforts were made to improve the fish assay commonly used to assess marine toxicity.

An assay that does not suffer from these drawbacks would be useful in assessing biosurfactant toxicities. Previous investigators have demonstrated that surfactin is capable of lysing red blood cells (RBCs) and have used this phenomenon to develop agar plate [23–25] and spectrophotometric assays [9,26,27]. In our laboratory, we have confirmed these observations and have modified this approach to develop a rapid and more quantifiable spectrophotometric method for measuring RBC hemolysis by surfactin and FA-Glu, and show that the hemolytic titers of the surfactants closely parallel their toxicities toward *F. grandis* fish larvae.

MATERIALS AND METHODS

Chemicals

Defibrinated sheep RBCs were obtained from Becton-Dickinson as a suspension and stored at 4°C until needed. All other chemicals used were reagent grade. Buffer A: 50 m*M* Tris, pH 7.2, with 0.04% NaN_3 and 10 m*M* $MgCl_2$. Buffer B: 50 m*M* Tris, pH 7.2, w/0.04% NaN_3, 0.85% NaCl and 10 m*M* $MgCl_2$. Where necessary, Buffer A was supplemented with NaCl over the range from 0 to 40 ppt (parts per thousand).

Red Blood Cell Preparation

An aliquot of RBC suspension was added to a disposable 15 mL centrifuge tube containing 4 volumes of Buffer B, mixed by gentle inversion, and the cells recovered by centrifugation at 4°C for 5 min at 1500*g*. The supernatant was discarded. The recovered cell pellet (∼1.2 mL) was washed twice by 1:10 v/v dilution with Buffer B, mixed by inversion, and recovered by centrifugation as above. The final cell pellet was suspended in Buffer B to produce a 10% (v/v) suspension that was stored on ice until needed.

Surfactant Preparation

Purified surfactin and FA-Glu for the suspension hemolysis assays were kindly provided by Modular Genetics, Inc., Woburn, MA. The surfactin for the agar assay was prepared by chloroform precipitation of surfactin from the collapsed foam fraction of surfactin captured from fermentation broth by foam fractionation. Surfactants were weighed out on an analytical balance, then added to Buffer B, followed by addition of sufficient dilute NH_4OH to dissolve the surfactant. Surfactant solutions were stored at −20°C until needed.

Hemolysis-Based Toxicity Protocol

Agar-plate assays were performed in Petri dishes containing 0.5% Bacto agar supplemented with sheep RBC. The agar was added to 50 m*M* phosphate buffer (pH 7.00) containing 0.04% NaN_3 and 0.85% NaCl. The mixture was heated to boiling to dissolve the agar, and then cooled to 50°C. Sterile RBC suspension was aseptically mixed with the agar (2.5% (v/v) RBC) and the mixture was poured into 10-cm-diameter glass Petri dishes and allowed to solidify.

For hemolysis in RBC agar, 5-mm-diameter discs were cut from Whatman 3MM paper and carefully placed on the surface of the agar plates. Surfactant solutions (5–15 μL) were added to the discs with a micropipet. Surfactant-free solutions served as controls. All plates were incubated at 4°C. Diameters of the clear zones resulting from hemolysis were measured from digital photographs of the plates.

Suspension hemolysis assays were performed in 1.5 mL microcentrifuge tubes. Buffer A containing varying concentrations of NaCl was pipetted into the tubes, followed by predetermined volumes of either surfactin or FA-Glu. Amounts of surfactant added were scaled to provide similar percentages of the reported critical micellar concentrations, which are 20 and 350 ppm for surfactin and FA-Glu, respectively [3]. Final volumes were 1.4 mL. To this was added 100 μL of 10% v/v washed RBC suspension. The tubes were capped, then mixed on an inversion mixer at room temperature for 60 min during which time the RBCs were lysed, releasing hemoglobin (Hb). After incubation, the tubes were spun at 2000*g* for 5 min at room temperature and the cell pellets were discarded. Hemoglobin in the supernatants was measured spectrophotometrically at 415 nm, the absorbance peak for the heme group. Controls consisted of tubes containing Buffer B plus RBCs without surfactant. The A_{415} of Hb recovered from supernatants in tubes containing 100 μL of RBCs disrupted by osmotic shock in DI water was arbitrarily defined as 100% hemolysis, and used to calculate the % hemolysis of RBCs exposed to surfactants.

For FA-Glu, two replicates were performed at seven conditions; for surfactin, four replicates were performed at one condition and two replicates at another. Pooled estimates of the 95% confidence intervials were then obtained for each surfactant. The fraction hemolysis versus surfactant concentration curves for each salt level and each surfactant were fit by nonlinear regression with a two-parameter (for the 5 experimental surfactant levels) logistic function and the root mean square error from the fitted equation was calculated using JMP software (version 11, SAS Institute). This form (sigmoidal) was chosen to capture some features of a threshold response and an approach to complete hemolysis. The sigmoidal fits are available in Supporting Information.

Fish-Based Toxicity Protocol

The methods for this assay were described previously, where the toxicities of surfactin and FA-Glu were compared [3]. Salinity levels were comparable to those in this study, but, as will be seen, surfactant levels were lower.

RESULTS AND DISCUSSION

The ability of surfactin to lyse red blood cells is well documented [9,23–27] and was confirmed in this investigation. Figure 1 shows the correlation between the amount of surfactin and hemolysis zone diameters for a single set of experiments using RBC-agar plates. A near-linear increase in zone diameters with larger amounts of surfactin was

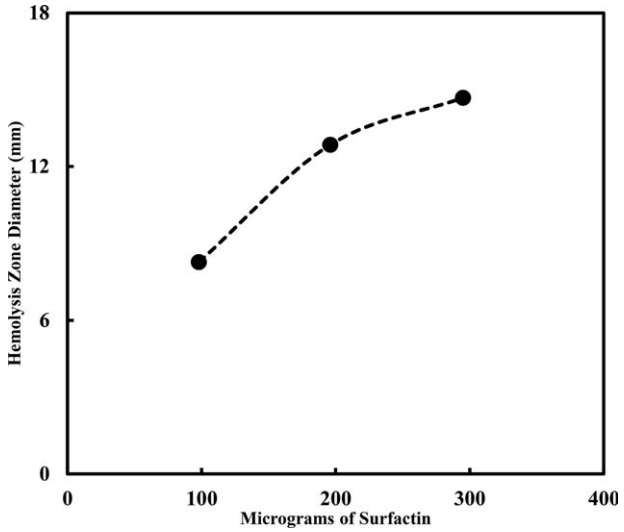

Figure 1. Effect of surfactin on RBCs immobilized in agar. Surfactin was purified by chloroform precipitation. These experiments were to demonstrate the application of the previously published method and were not replicated [23–25].

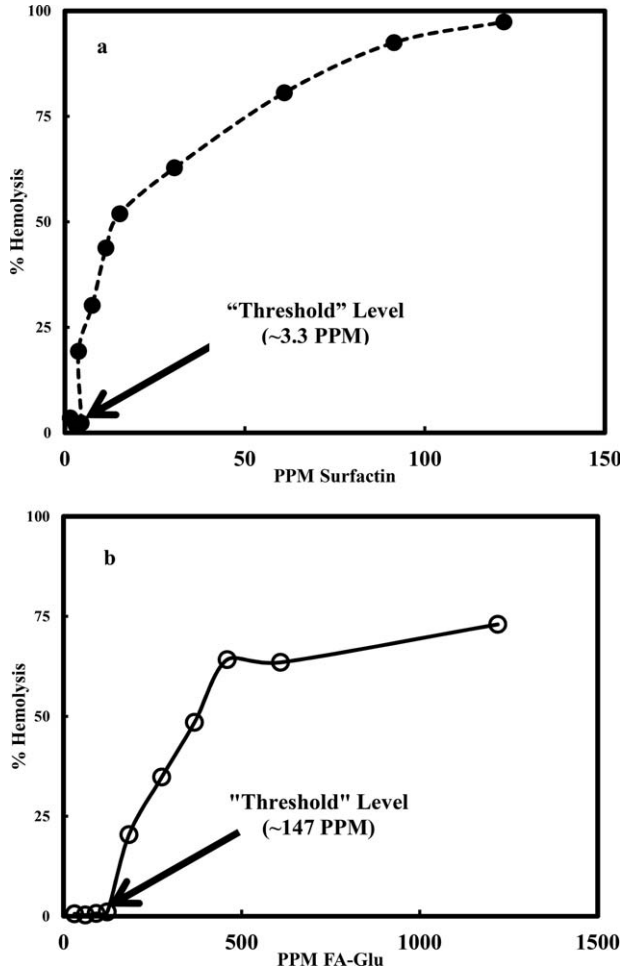

Figure 2. Effect of (a) surfactin and (b) FA-Glu concentrations on hemolysis of sheep RBCs in Buffer B [0.85% (8.5 ppt) saline]. For both surfactants, hemolysis occurs only after the surfactant concentration reaches a "threshold" value, below which little to no hemolysis is observed. Threshold values for surfactin and FA-Glu are ~3.3 and ~147 ppm, respectively. These experiments were not replicated.

observed, showing a clear dose response of RBCs to surfactin. However, the data also show that relatively high levels of surfactin are required to visualize the hemolytic response.

FA-Glu was also tested in RBC agar and found to produce hemolytic zones (data not shown). These were very faint and difficult to visualize; however, they provided evidence that FA-Glu has hemolytic properties.

Hemolysis in agar is a well-known technique for detecting surfactants and surfactant-producing microorganisms [23–25]. However, this approach has its limitations. Considerable time and effort are required to prepare blood agar plates, and long incubation times are required before hemolytic zones can be observed. Also, in this technique, accurate zone diameters as a function of surfactant dose can be difficult to determine, thereby reducing quantitative resolution. Moreover, when used in detecting surfactant-producing microorganisms, false-positive and -negative responses often result [23]. These, in addition to the relatively low sensitivity of the agar plate technique observed here, prompted development of a better method.

Figure 2 shows the dose response of the suspension hemolysis method for the two surfactants in normal saline (0.85%) over a wide range of concentrations. The hemolytic activity of FA-Glu confirms that initially detected using RBC agar. This is the first time that FA-Glu has been shown to have hemolytic properties.

Clearly, surfactin is a more potent hemolytic agent, as it causes RBC lysis at concentrations much lower than those required by FA-Glu. For both surfactants, there is a "threshold" concentration, below which no hemolysis is observed. Above this concentration, RBC lysis occurs. Surfactin and FA-Glu have critical micelle concentrations (CMC) of 20 and 350 ppm, respectively [3]. Hemolysis by both surfactants occurs well below these concentrations, indicating that hemolysis is not dependent on micelle formation by the surfactants. This is consistent with the observation that surfactin-induced leakage of 5(6)-carboxyfluorescein from unilamellar phospholipid vesicles occurs at concentrations well below surfactin's CMC [28].

Figure 2 also demonstrates that hemolysis can be used for quantitation of individual surfactants. With surfactin, the hemolysis profile is linear between ~4 and 15 ppm. Similarly, for FA-Glu, the useful, linear range for quantitation is from ~180 to 460 ppm. Only with surfactin (at ~120 ppm) was hemolysis complete, whereas the maximum hemolysis observed with FA-Glu was ~73%. The observation that further additions of surfactant are required to cause greater hemolysis shows that the interaction between the RBCs and the surfactants results in consumption of the latter, possibly due to irreversible binding of the fatty acid moieties of the surfactants with RBC membrane lipids.

Figure 2 also shows that the spectrophotometric assay is much more sensitive than the agar-plate method. With the former, hemolysis by surfactin occurs at <10 ppm, whereas with the agar-plate method, the smallest hemolytic zone required ~500 ppm of surfactin. Similar results were observed with FA-Glu as well.

A quantitative spectrophotometric assay for surfactin using hemolysis was developed by Moran *et al.* (2002). However, to maximize sensitivity, these investigators included 2.5 *M* ethanol in the assay mixture which likely imparted fragility to the RBC membranes. Moreover, this modification increased sensitivity such that a nearly all-or-none response was observed with little gradation following slight increases in surfactin concentration. The procedure described here does not suffer from this problem.

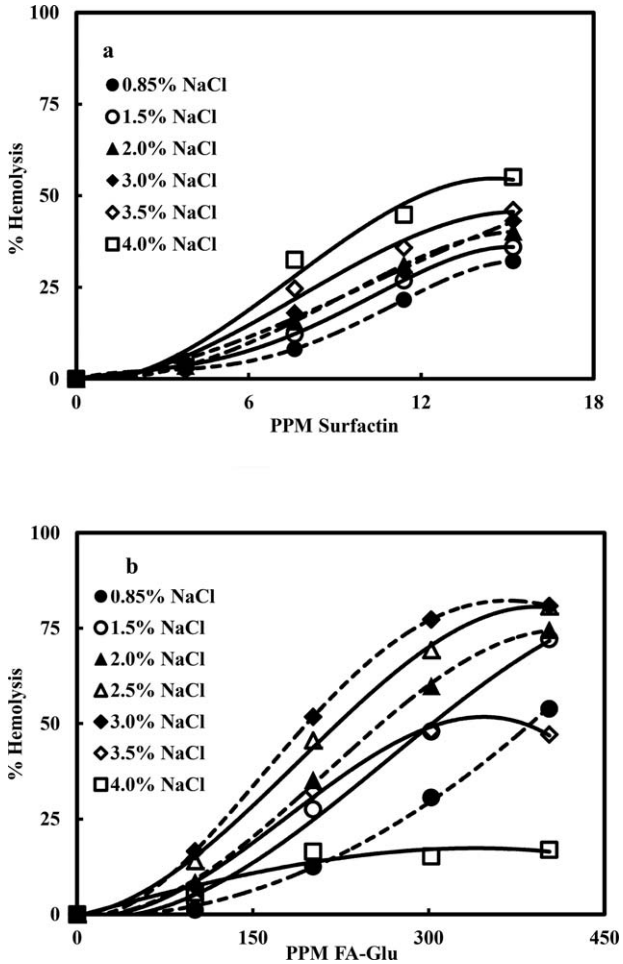

Figure 3. Effect of increasing salinity on hemolytic activity of (a) surfactin and (b) FA-Glu. The profiles shown are smooth curves that connect the points; plots of the regression fit to the two-parameter logistic function are available in Supporting Information (Figures S2 and S3). The pooled standard deviation of the hemolysis percentile was ±0.94 (d.f. = 4) for surfactin and ±0.78 (d.f. = 7) for FA-Glu, resulting in pooled 95% confidence intervals for the hemolysis percentiles of ±2.6 and ±1.8, respectively.

Compared with the agar-plate method, this approach is less labor intensive and requires less time. Moreover, it relies on a more precise spectrophotometric measurement of hemoglobin released from the RBCs by surfactant-induced lysis. In addition, the sensitivity provided by instrumental analysis should permit detection of lesser amounts of surfactant and improve quantitation.

To complement the fish assay as a toxicity test in marine environments, the hemolysis technique must also function effectively in solutions containing saline in concentrations usually found in estuaries as well as the open ocean. For this reason, the effect of salinity on each surfactant's hemolytic activity was tested over the range of 8.5–40 ppt NaCl.

Above 8.5 ppt saline, greater hemolytic activity was observed with each successive increase in salinity for surfactin (Figure 3a). At the highest saline concentration tested (40 ppt), the extent of hemolysis by surfactin was ~72% higher than that obtained with 8.5 ppt saline. Clearly, the hemolytic effect was potentiated by increasing salinity. With FA-Glu, this trend is only seen to a level of 25–30 ppt NaCl, beyond which level hemolysis decreases. The pooled 95% confidence intervals for hemolysis percentiles were ±2.6 and ±1.8 for surfactin and FA-Glu, respectively. When fit to the two-parameter logistic function (including the point (0,0)), the root mean square errors were 4.3 and 5.8 for surfactin and FA-Glu, respectively.

When added to RBCs, surfactin and FA-Glu disrupt the integrity of the RBC membrane, probably by interacting with membrane phospholipids, thereby causing lysis [8]. This is supported by studies with unilamellar phospholipid vesicles showing that surfactin can penetrate spontaneously into lipid membranes via hydrophobic interactions [29] and alter membrane permeability, inducing content leakage from the vesicles [28]. In addition, Bernheimer and Avigad (1970) deomonstrated that hemolysis of rabbit RBCs by surfactin was inhibited by phosphatidylcholine, phosphatidylinositol, phosphatidic acid, and sphingomyelin, all of which are present in RBC membranes, sphingomyelin being most abundant [30]. If these substances are added to RBC suspensions, surfactants may preferentially bind to them, resulting in a "sparing" effect on the cells, preventing hemolysis. At higher surfactant concentrations, the "sparing" effect is overwhelmed and lysis occurs. These authors also showed that hemolysis by surfactin was significantly augmented by chilling RBC-surfactant suspensions from 37 to 0°C. The low temperature would be expected to decrease membrane fluidity, promoting lysis, and is consistent with an interaction of the surfactant with membrane lipids.

It was observed that a minimum concentration (i.e., threshold) of surfactin and FA-Glu is required before hemolysis occurs. Below these concentrations, the cells remain intact. Billington *et al.* (1977) examined the effects of taurocholate and glycocholate on sheep RBCs and showed that at low concentrations, both surfactants removed significant amounts of phospholipids from intact sheep RBCs without lysis. At higher concentrations, hemolysis was observed. A similar result was obtained by Carillo *et al.* (2003) who reported that low concentrations of surfactant had no effect on phospholipid vesicle morphology, whereas higher concentrations caused membrane solubilization. These authors identified a critical surfactin-to-lipid ratio (R_{sol}) above which membrane solubilization occurred. This is similar to the threshold effect on RBCs observed with FA-Glu and surfactin. At low concentrations, the surfactants may penetrate the red cell and bind to membrane phospholipids without promoting lysis; at high (i.e., threshold) surfactant concentrations, membrane integrity is compromised to the point where lysis occurs. Clearly, RBC lysis increases rapidly once the threshold level of surfactant has been reached. At sublytic thresholds, some leakage of cell contents may occur, as this phenomenon has been demonstrated with artificial membranes [28]; however, this was not examined.

In the case of FA-Glu, the threshold is ~40× that of surfactin. Both surfactants have fatty acid moieties that can interact with RBC membrane lipids to cause hemolysis. However, as both surfactants have essentially identical fatty acid chains, the difference in their hemolytic capabilities is likely due to their peptide moieties. FA-Glu has a single glutamic acid residue, whereas surfactin has a heptapeptide moiety containing 5 hydrophobic amino acids (1 L-Val, 2 L-Leu, and 2 D-Leu). The latter impart greater hydrophobic "character" to the surfactin molecule, which may be responsible for its lower hemolytic threshold.

The assay described here can be examined for how closely it reflects the effect of surfactants on fish. The previously published data from the fish study [3] extensively examined the effects of surfactant concentration, so as to determine the ppm required to kill half of the fish (LC50). While the absolute levels differ, the fact that surfactin is toxic at lower levels is consistent with results obtained with hemolysis. Figure 4 displays both the fish toxicity and hemolysis data at

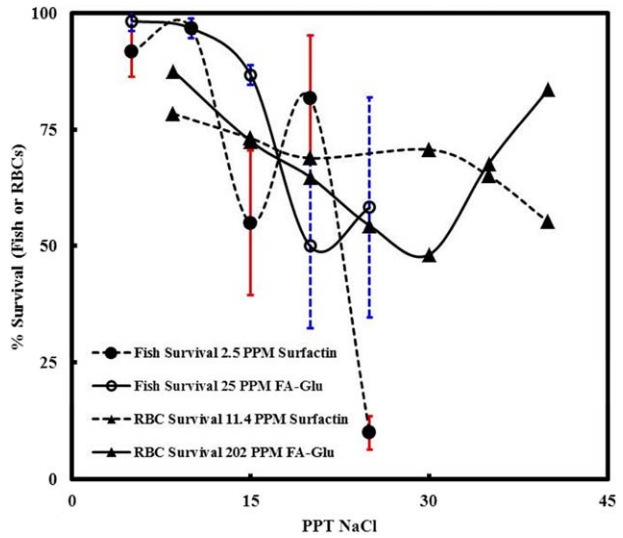

Figure 4. Comparison of effects of surfactin and FA-Glu on survival of *Fundulus grandis* fish larvae (previously published [3]) and integrity of sheep RBC (this work). The % survival of the fish larvae are an average of 3 replicates and the error bars indicate s.e.m. The RBC is a cross-plot of Figure 3 data with standard error of ±0.94 for surfactin and ±0.78 for FA-Glu. [Color figure can be viewed in the online issue, which is available at **wileyonlinelibrary.com**.]

concentrations giving comparable responses. Similarly, the two assays show that toxicity and hemolysis by both surfactants increase with salinity.

Although the hemolysis assay requires higher surfactant concentrations than the fish assay to obtain measurable responses, both were able to detect differences in toxicity between surfactin and FA-Glu. The variability of the fish data is much greater than that obtained by hemolysis, especially in the region where the plotted result is a mix of relatively equal numbers of surviving and dead fish. This is a result of the fish response being a step rather than a continuous response. Furthermore, hemolysis has advantages over the fish toxicity procedure because it is faster and easier to perform, and thus is useful as a rapid initial screening technique. With hemolysis, addition of 2.5 *M* ethanol improves the sensitivity of RBCs, resulting in lysis at much lower surfactant concentrations [26]. However, use of ethanol is inconsistent with conditions used in the fish toxicity assay. Bernheimer and Avigad (1970) reported variations in hemolytic responses depending on the source of RBCs [27]. Specifically, they noted that approximately fourfold less surfactin was required to produce 50% hemolysis of rabbit versus sheep RBCs. Use of the former would theoretically improve the sensitivity of the assay to near that observed with fish.

CONCLUSION

FA-Glu, a genetic variant of surfactin with the surfactin heptapeptide foreshortened to one amino acid, was found capable of hemolysis in a proposed assay method for toxicity. However, its hemolytic activity (in parallel to its toxicity toward fish) is considerably less than that of the surfactin. Hemolytic activity of both surfactants increases with NaCl concentration; however, salinities >35 ppt significantly reduce FA-Glu's hemolytic potency. The hemolysis responses of the two surfactants and their dependence on salinity parallel the survivability observed with a fish larvae assay for toxicity and do so with considerably lower variability, and thus serve as an easier screening method for toxicity. The correlation between the two assays regarding the required level of surfactant to observe similar "% survival" levels is in the range of 4–8 times higher needed for hemolysis. While this is somewhat consistent for the two surfactants of this study, additional work would be needed to propose using the hemolysis assay to predict harmful quantitative levels of any given surfactant. Use of RBCs that are more susceptible to hemolysis could bring the method's sensitivity closer to that of the fish assay.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors thank Modular Genetics, Inc. for supplying purified surfactin and FA-Glu. The authors also acknowledge the United Soybean Board for the USB Award #2488 and the USDA for the CREES Grant # 2010-34432-20955.

ABBREVIATIONS

FA-Glu	fatty-acyl-glutamate
Hb	hemoglobin
ppm	parts per million
ppt	parts per thousand
RBC	red blood cell

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