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Sonication-Assisted Metal-Enhanced Fluorescence-Based Bioassays

Kadir Aslan, Yongxia Zhang, and Chris D. Geddes*

The Institute of Fluorescence, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, Maryland 21202

A new bioassay technique, sonication-assisted metal-enhanced fluorescence, which is based on the combined use of ultrasound waves and metal-enhanced fluorescence (MEF), is reported. In this technique, low-intensity ultrasound waves significantly reduce the bioassay time by creating a temperature gradient between the bulk and the surface, which is thought to result in a mass transport of biomolecules from the bulk to the surface. After the assay is completed in 1 min, fluorescence emission is enhanced due to the MEF phenomenon. For proof-of-concept, a model bioassay based on the interactions of biotin and fluorophore-labeled avidin was constructed on SIFs and was subsequently completed in <1 min using low-intensity ultrasound at 40 kHz. The end-point values for fluorescence emission from sonicated assays were compared to those measured from assays carried out at room temperature without sonication to confirm to accuracy of the new technique. The effect of sonication on the assay components were studied using optical absorption spectroscopy, atomic force microscopy, and fluorescence spectroscopy techniques. Real-time thermal imaging was used to measure the changes in temperature of the bioassay components during the sonication process. Fluorescence resonance energy transfer (FRET) was also employed to investigate the effect of sonication on potential surface protein denaturation and conformational changes.

The identification and quantification of proteins and other biomolecules using bioassays are of great importance in biomedical and biochemical applications.[1−3] Fluorescence is the dominant technology in most of these applications, where a biomolecule of interest is detected by fluorescence emission from its fluorophore-labeled binding partner.[4,5] Fluorescence-based bioassays carried out on planar surfaces generally lack sensitivity and require expensive optical instruments.[6,7] In addition, the biorecognition events in these assays are inherently slow (several minutes to hours).[6,7] The sensitivity of the fluorescence-based assays can be improved, without the use of high-end optical instruments, by incorporating plasmon resonant particles (PSPs) into these assays.[8,9] The improved sensitivity is made possible by the increase in fluorescence signatures and decreased lifetimes of fluorophores placed in close proximity to PSPs, described by a phenomenon called metal-enhanced fluorescence (MEF).[8,10] In MEF-based bioassays, PSPs (generally silver nanoparticles) are deposited onto the planar surface and the bioassay is constructed on the PSPs.[8] Since the size of most biomolecules are smaller than PSPs (20−100 nm), fluorophores are positioned within a distance where their emission is increased due to their interactions and the generation of surface plasmons in PSPs.[10]

Although the sensitivity of fluorescence-based bioassays is addressed by MEF, the speed of conventional bioassays remains a huge challenge to overcome. In this regard, a new technique, called microwave-accelerated metal-enhanced fluorescence (MAMEF),[11] that amalgamates low power microwave heating and MEF has been shown to decrease bioassay completion times to less than 1 min. In MAMEF, low-power heating of the assay components creates a temperature gradient between the bulk medium (target biomolecules and fluorescent probes are present) and the silver nanoparticles at the assay surface (capture probe is present), which drives the target biomolecules and fluorescent probes toward the surface, and the bioassay is subsequently constructed.[11] The microwave heating step, which effectively facilitates mass transfer, can be carried out separately for each assay component or in a single step for a 3-piece DNA hybridiza-

* Corresponding author. E-mail: geddes@umbi.umd.edu.

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tion assay.[12] However, several factors affect the efficiency of the MAMEF technique: (1) assay surfaces have to be modified to remove excess heating (especially in assays run with a small volume of liquid),[11] and (2) the heating of large assay platforms with multiple sharp corners[13] (e.g., high-throughput screening wells) require longer heating times that subsequently can lead to the localized evaporation of sample. In this regard, there is still a need for a more generic technique applicable to all commercially available assay platforms without the sacrifice of samples.

Since the first observation of cavitation effects created by ultrasound in 1895,[14] ultrasound has found many applications in chemical and physical processes, most notably, in speeding up chemical reactions (i.e., sonochemistry).[15] Cavitation effects, which are referred to as the rapid formation and implosion of small bubbles in a liquid, are typically observed for ultrasound frequencies lower than 1 MHz.[16] The implosion of bubbles (symmetric cavitation) results in hot spots in liquids and the temperature inside the bubble can reach in excess of 5000 K,[17] which is subsequently quenched by surrounding water molecules at a rate of 10^{10} K/s.[17] When the bubbles collapse near a solid surface which is several orders of magnitude larger than the bubble dimensions, symmetric cavitation is hindered and the collapse of bubbles occurs asymmetrically.[18] Subsequently, this results in the formation of microjets of liquid perpendicular to the surfaces, which are estimated to reach speed of 100 m/s.[18] The formation and collapse of the microjets of liquid leads to a rapid stirring of the liquid in the bulk, in addition to their well-known cleaning effect.

In this paper, a new technique, called SAMEF, based on the combination of low-intensity ultrasound and MEF is reported. The use of low-intensity ultrasound resulted in the significant reduction of the bioassay time (1 min) as compared to those assays run at room temperature without sonication (30 min). After the assay is completed, the MEF component afforded the increase in bioassay sensitivity through enhanced fluorescence signatures. The proof-of-concept of SAMEF-based bioassays is demonstrated with a model bioassay based on the interactions of biotin and fluorophore-labeled avidin. The accuracy of the new technique was confirmed by comparing the end-point values for fluorescence emission from SAMEF-assays to those measured from assays undertaken at room temperature without sonication. The effect of low-intensity ultrasound on the assay components was also studied using optical absorption spectroscopy, atomic force microscopy, and fluorescence spectroscopy techniques. The real-time changes in the temperature of the bioassay components during sonication process were monitored with a thermal imaging camera. In addition, the effect of low-intensity ultrasound on potential protein denaturation and conformational changes was investigated.

EXPERIMENTAL SECTION

Materials. Fluorescein isothiocyanate-labeled human serum albumin (FITC-HSA), biotinamidocaproyl-labeled bovine serum albumin (biotinylated-BSA), FITC-labeled avidin, rhodamine B-labeled avidin, and Silane-Prep glass microscope slides were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI).

Methods. *Silver Island Films (SIFs).* SIFs were prepared according to our previously published procedure.[11]

Absorption Spectroscopy. The absorption spectrum of SIFs was measured before and after sonication using a commercially available ultrasonic bath (Branson Ultrasonic Bath, model no. B200, input and output power = 25 and 19 W, respectively, at 40 kHz; irradiating surface area $\approx$ 23 cm^2) for up to 30 min (cumulative time) using a Varian Cary 50 Bio UV−vis spectrophotometer. The sonication of the samples was carried out according to the following procedure: SIFs were placed directly at the bottom of the ultrasonic bath without water. Then, 500 μL deionized water was placed onto the SIFs before sonication, and the sonication was carried out for up to 20 min.

Fluorescence Spectroscopy. An aqueous solution (10 μM) of FITC-HSA was incubated on SIFs for 30 min followed by a subsequent wash to remove unbound material. The fluorescence emission spectrum of FITC-HSA deposited onto SIFs was measured before and after sonication for up to 20 min (cumulative time) using a Cary Eclipse fluorometer (500 μL deionized water was placed onto SIFs before sonication).

Atomic Force Microscope (AFM) Measurements. AFM images of SIFs before and after 1 min sonication were taken using a Molecular Imaging Picoplus Microscope at a scan rate of 1 Hz with 512 × 512 pixel resolution in the tapping mode.

SAMEF-Based Model Bioassay. The model bioassay is based on the biorecognition event take place between biotin and avidin molecules. In the SAMEF-based assay, biotin groups were introduced to the SIFs and glass surfaces by 30 min incubation of 10 μM aqueous solution of biotinylated-BSA on SIFs. It is well-known that albumin is known to bind to silvered surfaces and indeed forms a monolayer.[19,20] Unbound material was removed by multiple washes with deionized water. The biotinylated-BSA-coated SIFs and glass surface were placed inside the ultrasonic bath (without any liquid). The SAMEF-based assay was carried out by incubating a 500 μL aqueous solution of the binding partner of biotin, FITC-labeled avidin, for 1 min under continuous sonication. After the sonication was ceased, unbound material was removed by multiple washes with deionized water. The fluorescence spectrum of FITC-avidin on SIFs and glass surfaces was measured using a Cary Eclipse fluorometer. Control experiments, where the identical assay is run at room temperature without sonication, were also undertaken for 30 min to validate the end-point results for the SAMEF-based assays. Additional control experiments, where one of the binding partners (biotin) is omitted from the surfaces, were carried out in a similar manner as described above.

Fluorescence Resonance Energy Transfer (FRET) Studies. To investigate whether the sonication process denatures protein in SAMEF-based assays, FRET studies were undertaken. In this regard, after the introduction of biotin groups as described in the

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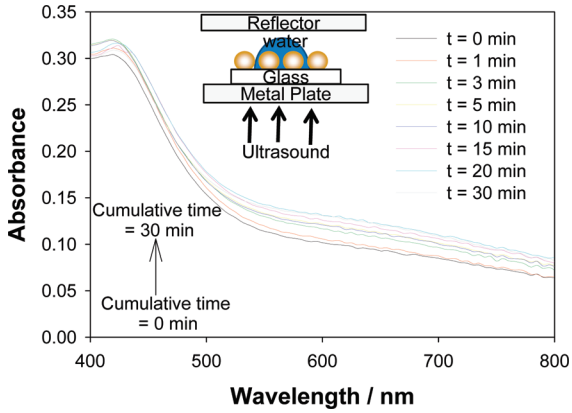

Figure 1. Absorption spectrum of silver island films (SIFs) as a function of sonication time; the same sample was sonicated for a total of 30 min (500 μL of deionized water was placed on top of the samples). The reflector plate is located 2 cm from the surface of the glass during sonication.

previous section, two different avidin molecules labeled with a donor fluorophore (FITC) and an acceptor fluorophore (rhodamine B) were incubated on the SIFs surface at room temperature for 30 min and in a separate experiments for 1 min under continuous sonication. In two separate experiments, the molar ratio of donor to acceptor was adjusted to 5:1 and 1:5. Fluorescence spectra from these two different surfaces and two experiments were compared qualitatively for the extent of FRET between the donor and acceptor molecules.

Real-Time Thermal Imaging of SIFs and Glass Surfaces during Sonication. Real-time monitoring of temperature changes on the SIFs and glass surfaces were measured using a commercially available thermal imaging camera (Silver 420M; Electrophysics Corp, Fairfield, NJ, equipped with a close-up lens that provides a resolution of approximately 300 μm). In this regard, 500 μL of deionized water was placed on SIFs and glass surfaces placed inside the ultrasonic bath. The temperature of the water on the both surfaces (in separate experiments) was measured for 2 min, including a 1 min sonication period.

RESULTS AND DISCUSSION

For low-intensity ultrasound to be used with MEF applications, it is pertinent to study the effect of sonication on the physical properties of the SIFs. In this regard, optical absorption spectroscopy, atomic force microscopy and fluorescence spectroscopy techniques were employed. Figure 1A shows the absorption spectrum of SIFs before and after the sonication for a total time 30 min. While no shift in the peak wavelength of the surface plasmon resonance (SPR) for silver nanostructures ($\approx$ 420 nm) was observed, a slight broadening in the absorption spectrum at longer wavelengths (>550 nm) was evident. This implied that, after 1 min of sonication, the loss of silver nanoparticles from the glass surface was negligible and no change in the shape of the nanoparticles occurred. To visually verify the results of absorption spectroscopy studies, AFM images of SIFs were taken before and after 1 min sonication (data not shown). AFM images revealed that SIFs retained their shape and height. AFM images also show the number of nanoparticles (in the 2×2 μm^2 area measured) is decreased after the sonication process (Figure 1C). We note that due to the heterogeneous nature of SIFs and the difficulties in imaging the same location on SIFs before the sonication with AFM, these results were deemed inconclusive with regard to the loss of nanoparticles from the glass surface as a result of 1 min sonication.

Since the SAMEF-based bioassays involve the interactions of biological materials on SIFs and the subsequent detection of fluorescence emission from the surface, it is also important to study the effect of sonication on SIFs with respect to MEF phenomenon. Figure 2A shows the fluorescence emission spectrum of FITC-HSA coated onto SIFs as a function of sonication time. The fluorescence emission from FITC shows a $\approx$15% reduction after 1 min sonication, which increases to $\approx$40% after 3 min. No detectable fluorescence emission was observed after only 4 min of sonication, which implies that FITC-HSA is removed from the surface. This can be due to either the removal of FITC-HSA molecules alone or the removal of FITC-HSA molecules with silver nanoparticles as a result of sonication. Visual evidence for the removal of FITC-HSA/SIFs is provided with the real-color photographs of FITC-HSA coated SIFs before and after 20 min sonication, Figure 2B, which show a significant removal of SIFs from the surface. On the basis of the observations described above, it was concluded that the longest period of time of sonication while retaining all components of the SAMEF-based bioassays, is $=$1 min. Subsequently, SAMEF constructs were presonicated for 1 min before use for the rest of the work in this paper. It is important to note that the sonication results only in the faster binding of biological components, and since fluorescence measurements are carried out after the sonication is turned off, ultrasounds do not contribute to the observed enhanced fluorescence signatures.

Figure 3 summarizes the experimental design and the proof-of-concept of SAMEF-based bioassays based on the interactions of biotin and avidin, a model assay. In this regard, biotinylated-BSA is attached to SIFs and glass surfaces, cf. Figure 3A. Subsequently, fluorophore (FITC)-labeled avidin is incubated on these surfaces for 1 min with sonication or for 30 min at room temperature without sonication. Fluorescence emission from FITC-avidin is measured from both SIFs and glass to show the benefits of MEF. Since the SAMEF technique is also based on the MEF phenomenon, it is important to briefly discuss MEF. In MEF (Figure 3B), an increased fluorescence emission from fluorophore/SIFs "system" as compared to glass surfaces is typically observed. This is attributed to the partial energy transfer (induced surface plasmons) between the fluorophores and the surface plasmons and is partially due to the enhanced absorption of light by the fluorophores as a result of increased electric fields near nanoparticles.[21] Figure 3C shows the fluorescence emission from the model assay run on SIFs and glass surfaces after sonication for 1 min and also at room temperature without sonication for 30 min (a control assay). The emission intensity at 520 nm from the control assay serves as a target emission intensity for assays run with sonication to verify that sonicated assays indeed completes to the same end point as assay run at room temperature. Figure 3C shows that the model assay both run on SIFs and glass surfaces reached to >95% completion in 1 min when compared to room temperature assay. The emission intensity at

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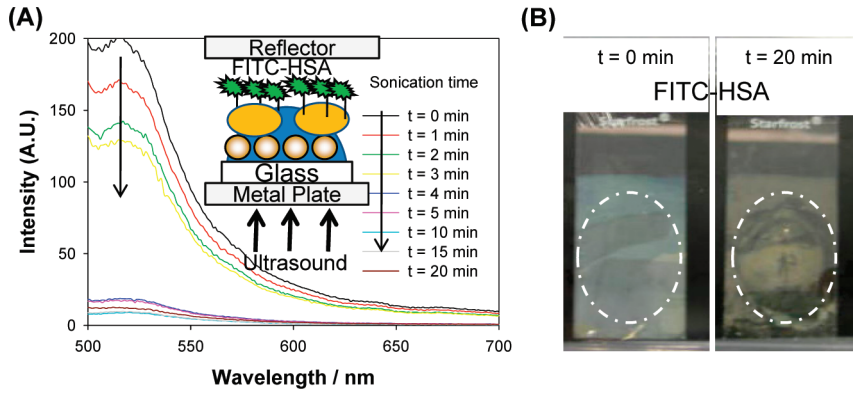

Figure 2. (A) Fluorescence emission spectrum of FITC-HSA coated onto SIFs as a function of sonication time. (B) Real-color photograph of FITC-HSA coated SIFs before and after 20 min of sonication (these samples were dried with a stream of air). In all these experiments 500 μL of deionized water was placed on top of the samples (white dashed circles). The reflector plate is located 2 cm from the surface of the glass during sonication.

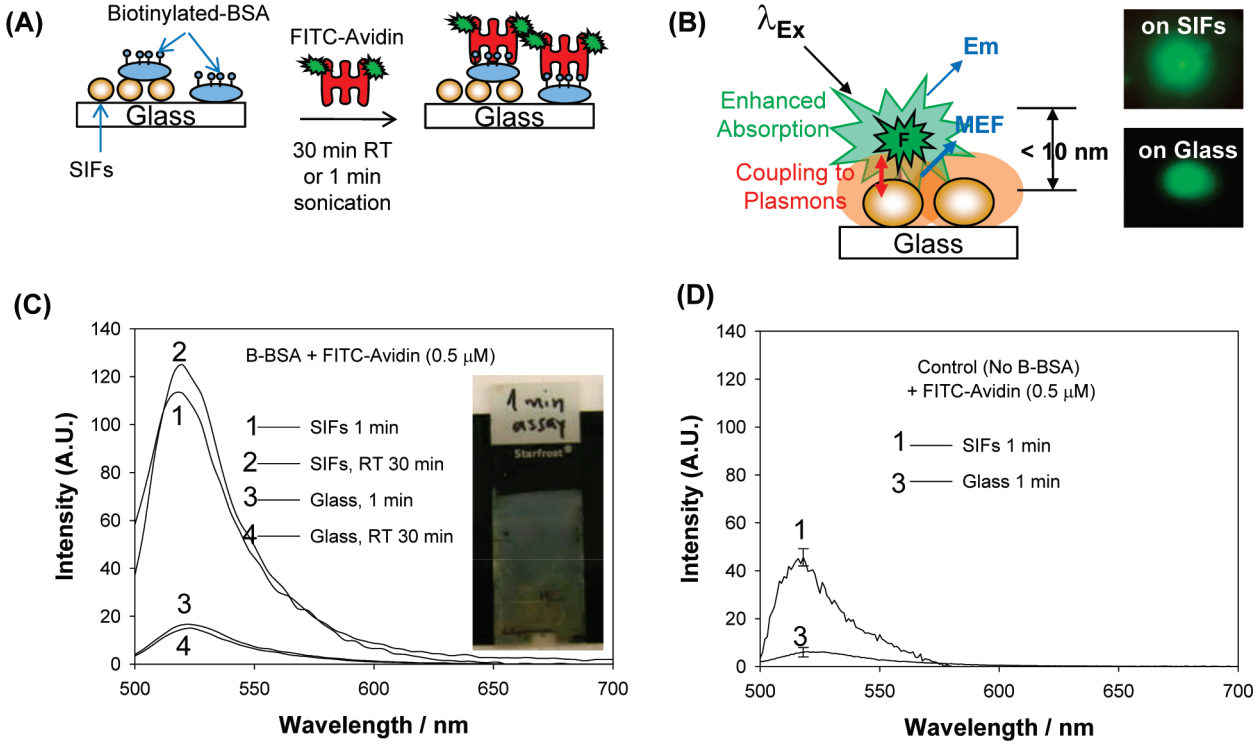

Figure 3. (A) Experimental scheme for a model protein assay run at room temperature (RT) or with sonication. (B) Cartoon depiction of metal-enhanced fluorescence phenomenon and real-color photographs of fluorescence emission from SIFs and glass visually demonstrating the utility of MEF. (C) Fluorescence emission spectrum of FITC-avidin used in the model protein assay run on SIFs and glass at RT and with sonication. (Inset) real-color photograph of SIFs after the model protein assay is run with sonication, SIFs appear not to be destroyed as a result of 1 min sonication. (D) Control experiment, where biotinylated BSA (B-BSA) is omitted from the surfaces, corresponding to model assays run on SIFs (1) and on glass (3), shown in part B.

520 nm for the model assay was ≈6-fold larger on SIFs than glass demonstrating the benefits of using silver nanoparticles in the surface assays. To determine the extent of nonspecific binding of FITC-avidin to surfaces in assays run with sonication, additional control experiments where biotinylated-BSA is omitted from the surfaces are undertaken, cf. Figure 3D. The nonspecific binding was found to be $^1/_3$rd that of the assay run both on SIFs and glass surfaces. It is important to note that no additional surface chemistries were employed to reduce the nonspecific binding in this study, that is, only the biological binding partners were present on the surface. One can simply reduce the nonspecific binding, by blocking the surfaces of silver nanoparticles with self-assembled monolayers of polyethylene glycol modified alkanethiols that resist the nonspecific binding of proteins.[22]

Next, the dynamic range of concentration of FITC-avidin using SAMEF-based technique was determined and the results with the model assay run without sonication compared as shown in Figures 4 and 5. Parts A and B of Figure 4 show that fluorescence emission from FITC-avidin increases as the concentration increases for the assays run using sonication and without sonication, respectively.

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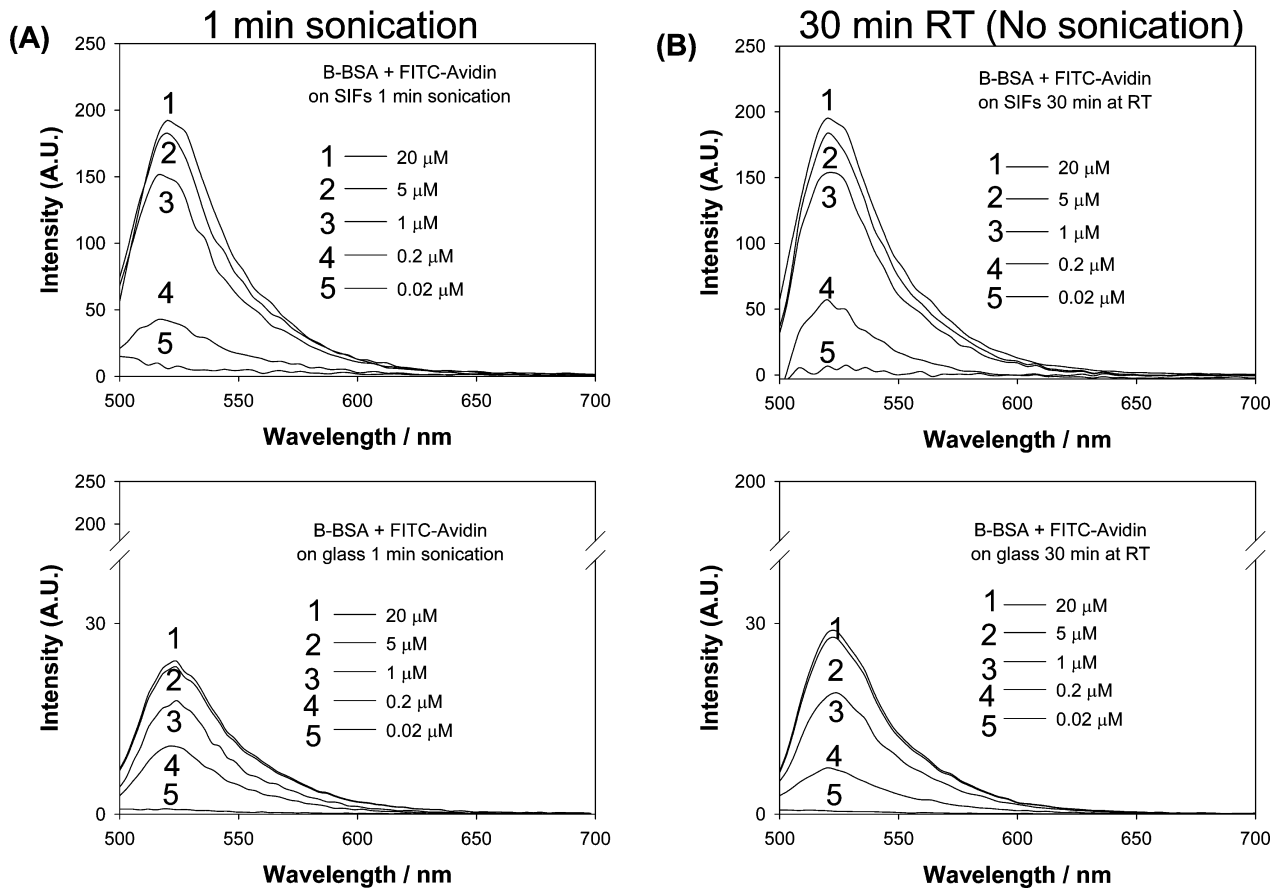

Figure 4. (A) Fluorescence emission spectrum of FITC-avidin used in the model protein assay run on SIFs (top) and glass (bottom) with sonication (1 min). (B) Identical model protein assay run on SIFs (top) and on glass (bottom) at room temperature (RT).

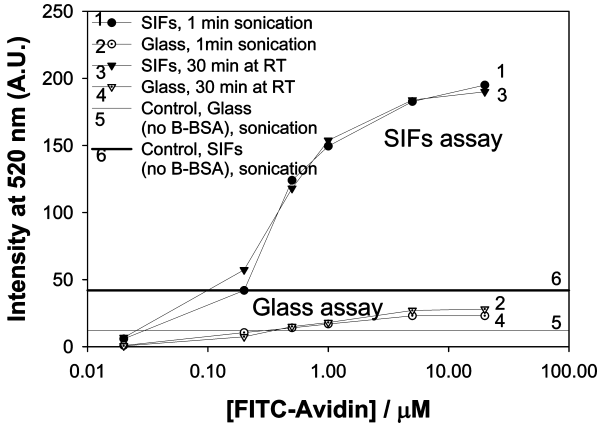

Figure 5. Fluorescence emission intensity (at 520 nm) versus concentration of FITC-avidin used in the model protein assay run on SIFs and glass substrates at room temperature after sonication (1 min). Control experiments 5 and 6 was undertaken with 20 μm FITC-avidin.

Figure 5 shows that the end-point values for emission intensities at 520 nm from assays run both on SIFs and glass surfaces with and without sonication are identical, suggesting the successful employment of sonication in MEF assays. Figure 5 also shows the lower-detection limit of >0.2 μM for assays both run on SIFs and glass surfaces with sonication, when the control experiments are run using the largest concentration of FITC-avidin in the absence of biotinylated-BSA are considered. The benefits of using silver nanoparticles, increased intensities over the dynamic range of concentration for FITC-avidin as compared to glass, in the assay are also evident from Figure 5. Figure 5 also shows a dynamic range over 4-log.

An increase in the temperature of the SIFs and glass surfaces was observed during the collection of data presented in Figures 1−3. To determine the extent of increase in water temperature, real-time imaging of the temperature of the assays on SIFs and glass surfaces were recorded using a thermal camera. Figure 6A shows the experimental geometry for the real-time temperature measurements. The experimental geometry was kept identical to that of the assays for the sake of consistency. In this experimental geometry, SIFs and glass substrates were placed at the bottom of an ultrasonic bath (in separate experiments), where the thermal image of water and SIFs/glass surfaces were captured for 2 min including 1 min of sonication. Figure 6B shows the thermal images for SIFs and glass surfaces before and after 1 min of sonication. Average temperature of water placed on these surfaces show a ≈2 °C increase on both SIFs and glass surfaces, while the temperature of regions of SIFs and glass surfaces not covered with water (dry, assays are not constructed on these regions) increase by up to ≈13 °C. This contributes to the further increase (additional ≈3 °C) in the temperature of water due to heat transfer even after the sonication has ceased as shown in Figure 6C. It is important to note that the assay (Figures 3 and 4) was halted, and the unbound material was washed away immediately after the sonication ceased after 1 min.

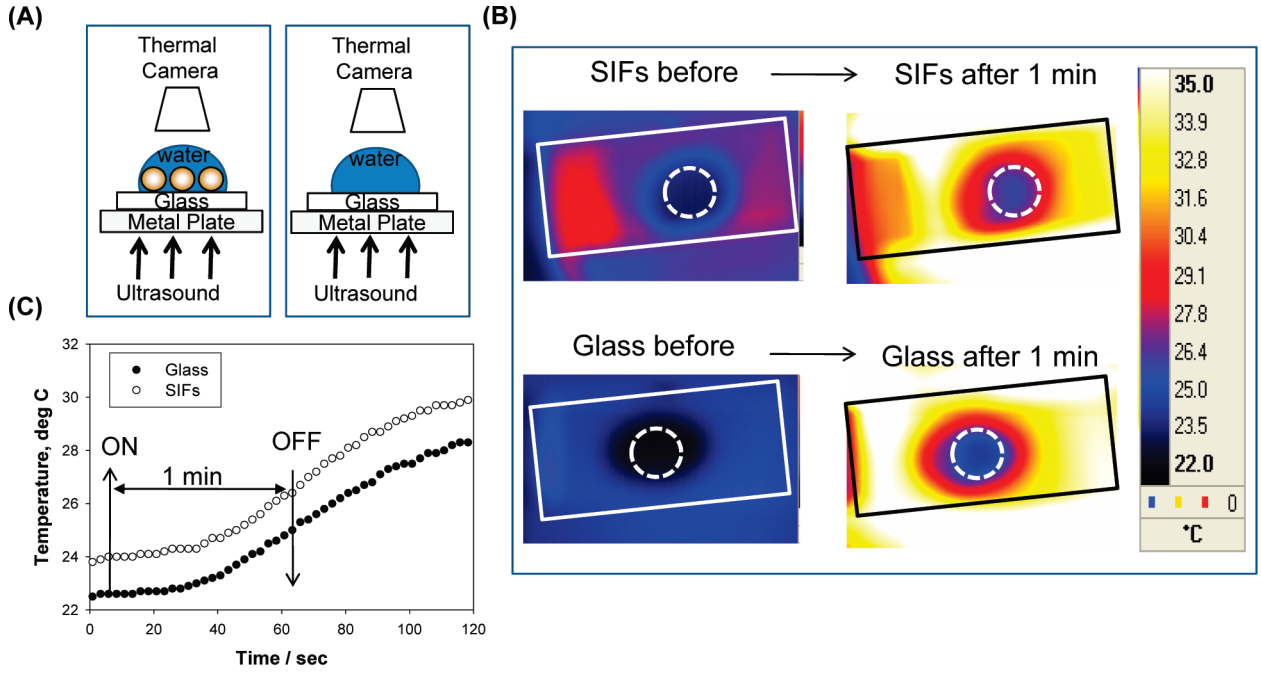

Figure 6. (A) Experimental scheme for real-time temperature imaging of SIFs and glass before, during, and after sonication (total sonication time, 1 min). (B) Thermal images of SIFs and glass before and after 1 min sonication. The location of the glass slides are indicated by solid lines (white and black). Average temperature values of water are measured from a region shown with dashed white circles in the middle of the glass slides. (C) Real-time temperature of water on SIFs and glass before, during, and after sonication (1 min). The sonication was turned on and off at $t = 5$ and 65 s, respectively.

It was thought that the combination of sonication induced cavitations and an increase in temperature could potentially result in protein denaturation and/or conformational changes on the assay surface which would inadvertently affect the sensitivity of the MEF-based assay that is based on MEF. Since MEF is a distance-dependent phenomenon, any conformational changes in proteins due to sonication coupled with increases in temperature could potentially yield results not comparable to assays run using conventional methods. Subsequently, FRET, a technique widely used in the studies of protein conformational changes, is employed to investigate the effect of sonication and temperature increase on the assay components. In the FRET experiments, donor (FITC) and acceptor (rhodamine B) molecules bound to different avidin molecules were incubated on biotinylated BSA-coated surfaces together with (1 min) and without sonication (control experiment run at room temperature for 30 min) as shown in Figure 7A. Figure 7B shows the results of these FRET experiments for two different molar ratios of donor to acceptor (5:1 and 1:5). For a ratio of 5:1, the emission spectrum is dominated by FITC emission (primarily because it is in excess) and the emission spectra are identical for assays run both with and without sonication, suggesting that biotinylated-BSA and avidin have not undergone any conformational changes. Further evidence for this was also observed when the donor to acceptor molecule ratio was 1:5 and is shown in Figure 7C. The emission is no longer dominated by donor emission but instead significant FRET was observed to the acceptor. These results also imply that the absorption of ultrasound by proteins did not affect their ability to bind other proteins, as protein conformation changes would have resulted in varying degrees of FRET and therefore different D/A emission spectra.

Since the SAMEF-based assays are based on the combined use of low intensity ultrasound, MEF, and surfaces, it is important to discuss the possible explanations for the observation of quicker assay times with the employment of sonication. Ultrasound (at 40 kHz) is used to remove materials from surfaces, usually in water (with the help of surfactants) due to cavitation effects. Cavitation is produced by introducing ultrasound waves into a liquid and results in the rapid formation and implosion of small bubbles in a liquid.[15] The implosion of bubbles result in hot spots in liquids and the temperature inside the bubble can reach in excess of 5000 K,[17] which is subsequently quenched by surrounding water molecules at a rate of 10^{10} K/s.[15] At 40 kHz, the ultrasound waves form far away (a few micrometers) from the surface, and this distance decreases as the frequency of the ultrasound waves increases, which implies that ultrasound waves do not interact directly with nanoscale particles on a planar surface. When cavitation occurs in a liquid near a solid surface (like glass slides in this study), the implosion of bubbles result in jets of liquid that move toward the solid surface at high speeds.[18] This movement of liquid is thought to result in the transfer of proteins in the bulk solution toward the surfaces, which decreases the amount of proteins in the bulk of the solution (mass transfer). In addition, ultrasound waves form a standing wave in the bulk assay medium,[15] which provides a means of movement of proteins, effectively mixing the assay components in the bulk solution. Subsequently, proteins are thought to continuously move toward the surface for the duration of the sonication (1 min) while longer sonication times resulted in the removal of assay components from the surface. Thus, the observation of quicker assays times using low-intensity ultrasound is attributed to the increased mass transfer of proteins as a result of the interaction of ultrasound with assay components. Further mechanistic studies are underway in our

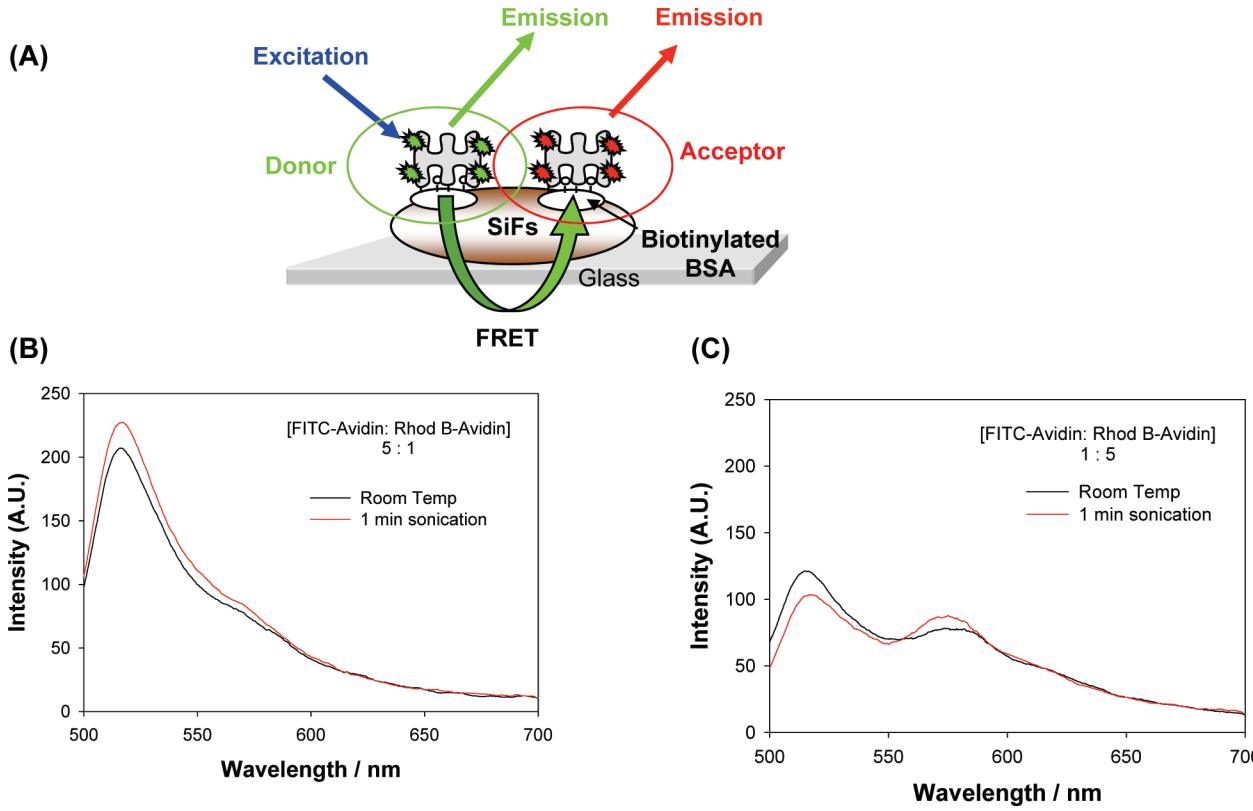

Figure 7. Fluorescence resonance energy transfer studies performed on SIFs. Donor (FITC) and acceptor (rhodamine B) molecules are bound to separate avidin molecules, which subsequently bind to biotinylated BSA on the surface of SIFs. The molar ratio of donor to acceptor molecules was (A) 5:1 and (B) 1:5.

laboratory to elucidate the observation of the quicker assays reported here.

CONCLUSIONS

A new bioassay technique, called sonication-assisted metal-enhanced fluorescence, which is based on the combined use of ultrasound waves and silver nanoparticles to accelerate bioassay kinetics and enhance fluorescence signatures, respectively, is reported. In this regard, a model bioassay, based on the interactions of biotin and fluorophore-labeled avidin, was constructed on SIFs and was subsequently completed in 1 min using ultrasound at 40 kHz. Similar end-point values for fluorescence emission from sonicated assays were measured as compared to those measured from assays undertaken at room temperature without sonication. The effect of sonication on the assay components was studied in detail using optical absorption spectroscopy, atomic force microscopy, and fluorescence spectroscopy techniques. Optical absorption studies revealed that continuous sonication of SIFs for 1 min did not remove silver nanoparticles from the surface. In addition, a slight decrease in fluorescence emission from a fluorophore-labeled protein adsorbed onto SIFs after 1 min of sonication was observed. AFM results showed the size and the shape of the silver nanoparticles did not undergo any significant changes; however, these results were deemed inconclusive due to the difficulty of imaging the same location on SIFs before the sonication with AFM. Thermal images of the assay show an ≈2 °C increase in the temperature of the assay components after 1 min sonication. The effect of sonication on protein denaturation or conformational changes was studied with FRET experiments, which showed that 1 min sonication (coupled with the subsequent increase in temperature) did not result in any significant protein conformational change. In summary, SAMEF affords for (1) bioassays to be kinetically complete in less than 1 min and (2) enhanced assay sensitivity by the close proximity of fluorescent labels to the silvered surfaces.

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