Low-concentration trypsin detection from a metal-enhanced fluorescence (MEF) platform: Towards the development of ultra-sensitive and rapid detection of proteolytic enzymes

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ABSTRACT

Proteolytic enzymes, which serve to degrade proteins to their amino acid building blocks, provide a distinct challenge for both diagnostics and biological research fields. Due to their ubiquitous presence in a wide variety of organisms and their involvement in disease, proteases have been identified as biomarkers for various conditions. Additionally, low-levels of proteases may interfere with biological investigation, as contamination with these enzymes can physically alter the protein of interest to researchers, resulting in protein concentration loss or subtler polypeptide clipping that leads to a loss of functionality. Low levels of proteolytic degradation also reduce the shelf-life of commercially important proteins. Many detection platforms have been developed to achieve low-concentration or low-activity detection of proteases, yet many suffer from limitations in analysis time, label stability, and ultimately sensitivity. Herein we demonstrate the potential utility of fluorescein derivatives as fluorescent labels in a new, *turn-off* enzymatic assay based on the principles of metal-enhanced fluorescence (MEF). For fluorescein sodium salt alone on nano-slivered 96-well plates, or Quanta Plates™, we report up to 11,000x enhancement for fluorophores within the effective coupling or enhancement volume region, defined as ~100 nm from the silver surface. We also report a 9% coefficient of variation, and detection on the picomolar concentration scale. Further, we demonstrate the use of fluorescein isothiocyanate-labeled YebF protein as a coating layer for a MEF-based, Quanta Plate™ enzymatic activity assay using trypsin as the model enzyme. From this MEF assay we achieve a detection limit of ~1.89 ng of enzyme (2.8 mBAEE activity units) which corresponds to a minimum fluorescence signal decrease of 10%. The relative success of this MEF assay sets the foundation for further development and the tuning of MEF platforms for proteolytic enzyme sensing not just for trypsin, but other proteases as well. In addition, we discuss the future development of ultra-fast detection of proteases via microwave-accelerated MEF (MAMEF) detection technologies.

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1. Introduction

Proteolytic enzymes are ubiquitous across a broad range of biological organisms, playing critical roles in physiological and pathogenic cellular mechanisms involving protein degradation. These enzymes, which are essential in digestive processes, reduce proteins down to their amino acid components and therefore present a unique challenge to biological and chemical research [1,2]. In fact, proteolytic degradation is a leading cause of protein instability in preparations resulting in decreased shelf-life. In the case of quantitative detection of protein, even low concentrations of protease impurities could lead to protein degradation during analysis, thus skewing results in biological assays and preventing accurate quantitation. In addition, proteases are well-known biomarkers for diseases such as acute pancreatitis and cystic fibrosis [2–4] and are frequently involved in health conditions including cancer, emphysema, viral infections, and neurodegenerative diseases [1,5]. Due to these factors, detection of low levels of proteolytic activity is necessary for diagnostics, biologics manufacturing, and biological research.

Numerous methods have been employed for the detection of proteolytic enzymes, ranging from the commonly used enzyme-linked immunosorbent assay (ELISA) to techniques such as mass spectroscopy [1]. Included in this spectrum of detection methods are fluorescence-based assays [1,2,6,7], which in general are relatively quick and facile; however, these assays may still be limited by long incubation times,

degradation of fluorophores during exposure and detection, and low signal to noise ratios due to biological autofluorescence from sample media. To address these limitations, we propose the incorporation of the Metal-Enhanced Fluorescence (MEF) technology into fluorescence-based enzymatic assays, using commercially available MEF platforms such as Quanta Plates™ (www.ursabioscience.com).

MEF has become an established biotechnology platform for sensing and diagnostics. In essence, MEF works by amplifying fluorescent signals in the near-field of metal nanoparticles thereby improving signal to noise ratios, which ultimately allows for more sensitive detection [8,9]. Although the mechanism of MEF is not entirely understood, in principle MEF arises from the coupling of fluorophore quanta to metal nanoparticle plasmons such that the coupled system may radiate as a single system. This coupled system yields enhancement from a combination of both absorption and emission amplification [8]. MEF has been demonstrated for many fluorophores—both small molecule [10] and nano-sized [11,12]—as well as with various plasmonic materials [13–15]. In addition to signal amplification, the coupled system frequently demonstrates much improved photophysical qualities such as fluorophore photostability [9,16]. These attributes present a strong basis for development of more sensitive and tunable assays.

Indeed, MEF-based assays are distinct from traditional fluorescence detection assays in that the fluorescent label is attached directly to the metal nanoparticle surface within an optimal near-field distance to the plasmonic substrate, rather than diffusing freely within the sample well [17–19]. Only fluorophores within this near-field range (also defined herein as the effective enhancement or coupling region volume), approximately 25 nm from the nanoparticle surface, [8] couple to localized surface plasmons and exhibit significant fluorescence enhancement relative to free-space fluorophores. Probe solutions are, therefore, used only to coat the nanoparticle surfaces, and solvents containing un-bound probe could then be potentially collected and re-used for future platform functionalization before the assay samples themselves are added. Comparatively, many commonly used fluorescent probes do not typically couple to the analysis platform surfaces such as plastic, requiring direct use of the probe in higher concentrations, thus leading to higher chemical requirements for a single assay as compared to a MEF-based design. The potential for MEF technology application in enzymatic assays is further reinforced by the development of microwave-accelerated MEF, or MAMEF, which permits the detection of very low concentrations or low activity agents in tandem with an ultra-fast assay design [17,20–24]. In these systems, low-power microwaves increase the rate of mass transportation and molecular diffusion at the metal nanoparticle surfaces, shortening lengthy incubation times required to generate low detection limits in otherwise identical fluorescence-based assays. Previous work demonstrated the utility of this platform, achieving picogram per milliliter detection limits of the anthrax protective antigen exotoxin in under an hour [17].

Herein we explore the foundational development and potential of a MEF-based sensing platform for the detection of proteases using trypsin as a model enzyme. Trypsin is a serine protease that hydrolyzes peptide and ester bonds of lysine and arginine residues. Using Quanta Plates™ we developed a *turn-off* fluorescent assay using fluorescein isothiocyanate (FITC)-labeled YebF protein for detection of trypsin activity. The data reported provides a foundation for the future tuning and development of even more sensitive proteolytic enzyme detection platforms based on the principles of metal-enhanced fluorescence.

2. Materials and methods

2.1. Determination of absolute detection, enhancement factor, and coefficient of variation for fluorescein sodium salt in Quanta Plates™

Silvered 96-well plates, or Quanta Plates™, were purchased from URSA BioScience (www.ursabioscience.com) for use as the plasmonic platform in this study (Fig. 1). To determine the suitability of a fluorescein-derived probe as the assay fluorophore component, the detectable concentration range was determined for fluorescein sodium salt (Sigma

Fig. 1. Photograph of silvered 96-well plates, or Quanta Plates™, used for assay development.

Aldrich) in the Quanta Plates™. To determine this range, a 1 μM solution of fluorescein sodium salt was prepared in deionized water. Serial dilutions were then performed in 7 vials, obtaining a concentration range of 1 μM to 1 pM.

A 100 μL aliquot was dispensed in each well of rows A through H, with each row containing samples of each subsequent concentration starting at 1 μM for row A. The fluorescence spectrum for each sample in the Quanta Plate™ wells were collected using a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a plate reader. Samples were excited at 485 nm, with intensities recorded at 527 nm emission and reported as arbitrary units (a.u.) for this and all subsequent experiments and trials reported herein.

To determine the enhancement factor of fluorescein sodium salt in Quanta Plate™ wells, a 1 μM solution of fluorescein sodium salt was prepared in deionized water and transferred to 5 sample wells in both Quanta Plates™ and a blank plastic 96-well plate. Fluorescence measurements were taken, and MEF factors were calculated at 527 nm emission both as a function of the raw data (*MEF*, Eq. (1)) and as a corrected value removing the free space component (*MEF$_C$*, Eq. (2)),

$$MEF = \frac{I_{Quanta\ Plates™}}{I_{Blank\ Plates}} \qquad \text{Equation 1}$$

$$MEF_c = \frac{(I_{Quanta\ Plates™} - I_{Blank\ Plates})}{0.0004(I_{Blank\ Plates})} \qquad \text{Equation 2}$$

where I is fluorescence intensity in arbitrary units. For these calculations, it was assumed that only 0.04% of the free-space fluorescence intensity (blank plates) will couple to the silver particles and result in enhancement, due to an assumed effective coupling distance of 100 nm for the fluorophore–nanoparticle system, a strongly conservative estimate. Additionally, a sample volume of 90 μL and an inner well diameter of 0.7 cm were used to determine the 0.04% value; the calculations were performed as reported previously, using a cylindrical sample well geometry [25]. Reported values are the result of five analyzed sample wells. The coefficient of variation was determined by adding 100 μL of 10 μM fluorescein sodium salt to 40 randomized wells. Fluorescence was analyzed as described previously, and average intensities and corresponding standard deviations were calculated.

2.2. Assay preparation and implementation

To functionalize the silvered wells for the model protease detection assay, the YebF protein from *Escherichia coli* was labeled with FITC (Sigma-Aldrich). Briefly, 5 mg of YebF (303 nmol) in 42 mM sodium borate pH 8.4 was mixed with 0.118 mg (303 nmol) FITC (100 mM in DMSO). The reaction mixture was incubated in the dark for 18 h at 4 °C. The mixture was dialyzed against 3 × 1 L 1x phosphate buffered saline (PBS) pH 7.3 (Athena Enzyme Systems). To coat the Quanta Plates, the FITC-YebF was diluted to 40 μg/mL in 1x PBS solution (Fisher BioReagents®) and 100 μL of solution was added to all wells of rows A-G of the Quanta Plates™, with the final row H containing PBS alone as a control. Plates were incubated at 37 °C for 1 h in a humid chamber; the solutions were then discarded.

To optimize the assay and determine the impact of well washing and standing steps post-incubation with the fluorescent label, the protein-coated wells were subjected to varying numbers of preparation cycles, including both washing steps and standing periods as described in the following section. As a control, wells were filled with 100 μL of PBS and fluorescence detected, as described previously in section 2.1. The solution was then removed, and 200 μL of PBS were added to the wells to remove loosely bound or unbound protein. The solution was then discarded; this procedure is referred to as a "wash." For the initial washing procedure, the wash was repeated in triplicate. Subsequently, 100 μL of PBS were then added to and left to sit in the wells for 20 min; this procedure is defined as the "standing period." The fluorescence signal was then analyzed and is reported later as "cycle 1." This solution was discarded, and an additional single wash was completed,

followed by another 20-min standing period and fluorescence analysis; this subsequent preparation cycle is reported later as "cycle 2." This process was repeated using the same wells for a total of 8 preparation cycles with 10 total washes and 8 standing periods (160 min total).

To prepare the assay platform to perform the detection of trypsin activity, wells received three rinses of 200 μL of PBS following incubation with FITC-labeled YebF protein (rows A-G, row H received no protein coating) to remove excess unbound protein and were then tapped dry. Wells were filled with 100 μL of PBS and allowed to stand for 20 min; the solutions were then discarded. To the wells of rows B through H, 100 μL of 10 mM Tris-Cl pH = 7.8 (Fisher BioReagents®) were added. Trypsin EDTA, 1x (0.05% Trypsin, Mediatech Inc.) was diluted with 10 mM Tris-Cl to 100 μg/mL, and 110 μL were added to wells A1-A12. Serial dilutions were then performed, removing 10 μL from the initial well (ex. A1) and mixing into the following well (ex. B1). This process was repeated for all wells down to row F with the final 10 μL discarded. Plates were then incubated in the humid chamber for 1 h at 37 °C. Following incubation, solutions were discarded. Wells were rinsed in triplicate as described previously and were filled with 100 μL of PBS for fluorescence analysis. After detection, normalized intensities (N%) were calculated using the following equation:

$$N\% = \frac{I_{[T]_n}}{I_{[T]_0}} * 100 \qquad \text{Equation 3}$$

Where $I_{[T]_n}$ is the fluorescence intensity after incubation with a non-zero concentration of trypsin, and $I_{[T]_0}$ is the fluorescence intensity following incubation and washing where no trypsin was added before incubation. The limit of detection was determined by subtracting three times the standard deviation from the maximum normalized intensity (100%) at no intensity change, or 0 ng/mL trypsin.

3. Results and discussion

3.1. Analysis of fluorescein sodium salt in Quanta Plates™

Fluorescein and its derivatives are commonly used fluorescent labels that have been evaluated with plasmonic platforms for a metal-enhanced fluorescence effect [26–28]. To demonstrate that a fluorescein-based probe would be suitable for the Quanta Plate™ platform and for the model protease assay described, fluorescence from a 1 μM solution of fluorescein sodium salt was analyzed both on blank 96-well plates and within the Quanta Plate™ wells (Fig. 2a). From these spectra, a MEF value of 5.4 ± 1.7 was determined for the 1 μM solution. Since fluorescence enhancement will only occur for an estimated 0.04% of the fluorophore solution, the "free-space" fluorescence or blank plate fluorescence spectrum was subtracted from the emission spectrum generated in Quanta Plates™. The resulting fluorescence contribution from those fluorophores in the enhancement or coupling region are shown in Fig. 2b. This comparison is a more accurate representation of the potential strength of fluorescein in the assay platform, as only those fluorescent molecules which are directly functionalized to the plasmonic substrate will be analyzed after the assay has been performed.

In fact, when the subtracted spectrum is considered relative to the percentage of free-space fluorescence intensity solely detected for the effective enhancement region volume, the MEF value becomes considerably larger; the corrected MEF (MEF_C) value determined for fluorescein sodium salt under these conditions is 11,000 ± 3700. It is clear from these data that fluorescein is a promising fluorophore to employ as a fluorescent probe in the model protease MEF assay described.

To further elucidate the potential of fluorescein in this context, we then investigated the detectable concentration range for fluorescein sodium salt alone in the Quanta Plate™ wells. Serial dilutions of fluorescein sodium salt solutions were prepared in the range of 1 μM to 1 pM and the fluorescence intensity from these samples in the Quanta Plate™ wells determined (Fig. 3). Concentrations above 10 nM were also tested; however, at these levels the fluorescence intensities were at or above the saturation limit of the detector. At lower concentrations, the fluorescence intensity approaches zero for the MEF system. In the context of a *turn-off* assay design, however, this could permit the potential detection of complete probe cleavage from the plasmonic substrate for high concentration or high enzymatic activity samples.

Fluorescein sodium salt samples were then added to 40 randomized wells to determine the coefficient of variation inherent to fluorescein sodium salt and the Quanta Plate™ platform (Table S1). This value was determined to be approximately 9% of the average signal intensity. This indicates that while some variation may result between readings from different wells, the impact of this variation remains minor. This is further demonstrated in Fig. S1 of the supplementary material, which displays the difference in intensity values required to differentiate signals given an RSD of 9%. At higher intensities (>100 a.u.), signal differences must be on the order of 20–100 a.u. to exceed the limitations of well variation; however, these differences diminish to 1–0.1 a.u. as the signal intensity reaches 10 and approaches 0 a.u. (Fig. S1, inset). This demonstrates theoretically the potential sensitivity of a fluorescein-based label for MEF detection in enzymatic assays.

3.2. Detection of trypsin on a MEF-based proteolytic activity assay

To detect active trypsin concentrations using a MEF-based platform, we developed the assay design indicated in Scheme 1. By this method, the FITC-labeled YebF protein was used to coat the silver particles in the Quanta Plate™ wells, as shown in step (1) of the schematic. It is important to note here that FITC was used in place of fluorescein sodium
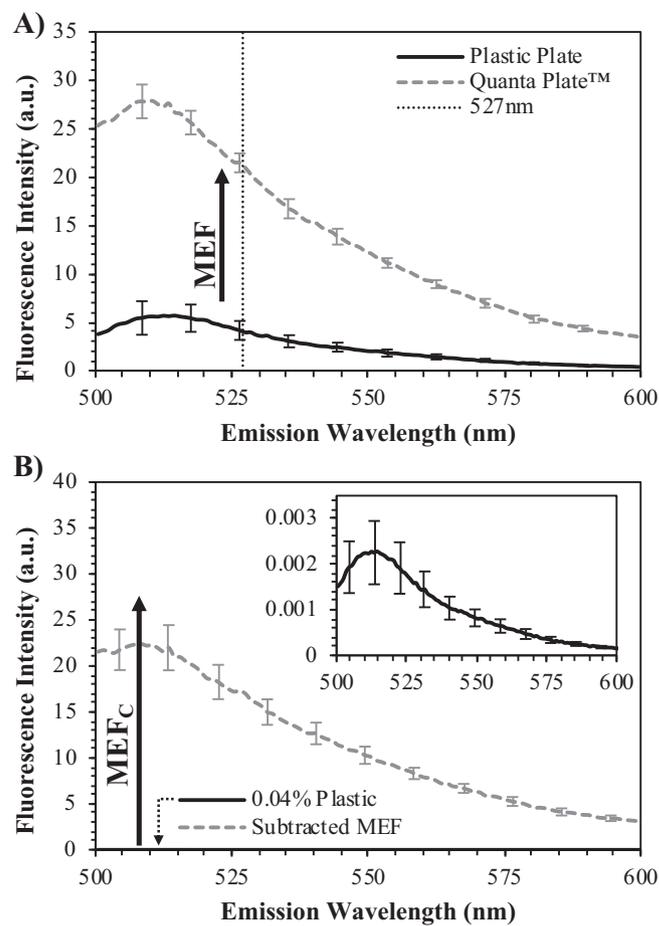

Fig. 2. Fluorescein sodium salt (1 μM) fluorescence emission detected with 485 nm excitation *A)* Raw spectra analyzed on both a blank, plastic 96-well plate (black, solid) and a silvered Quanta Plate™ displaying metal-enhanced fluorescence (MEF). Spectra are reported as the average of five sample wells, with corresponding standard deviation at select emission wavelengths. Emission at 527 nm is indicated by a dotted line. *B)* Subtracted, or "corrected," metal-enhanced fluorescence (subtracted MEF = MEF_C) spectrum ($I_{Quanta\ Plates} - I_{blank\ Plates}$) and corresponding "enhancement region volume" free-space fluorescence from blank plate (0.04% Plastic). **Inset** displays the 0.04% plastic spectrum magnified for visibility.
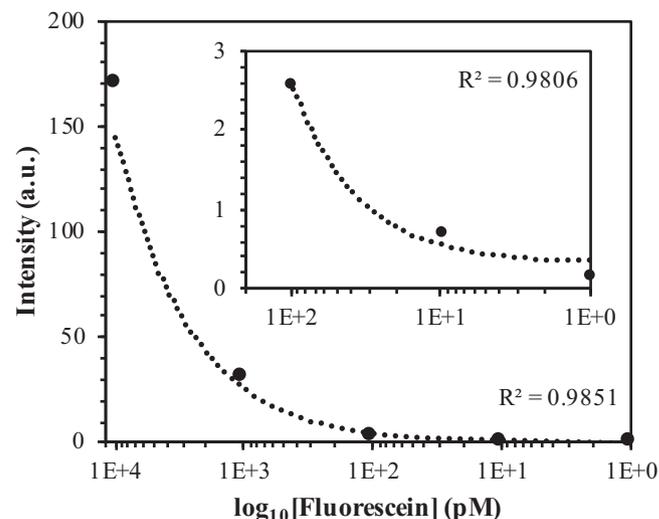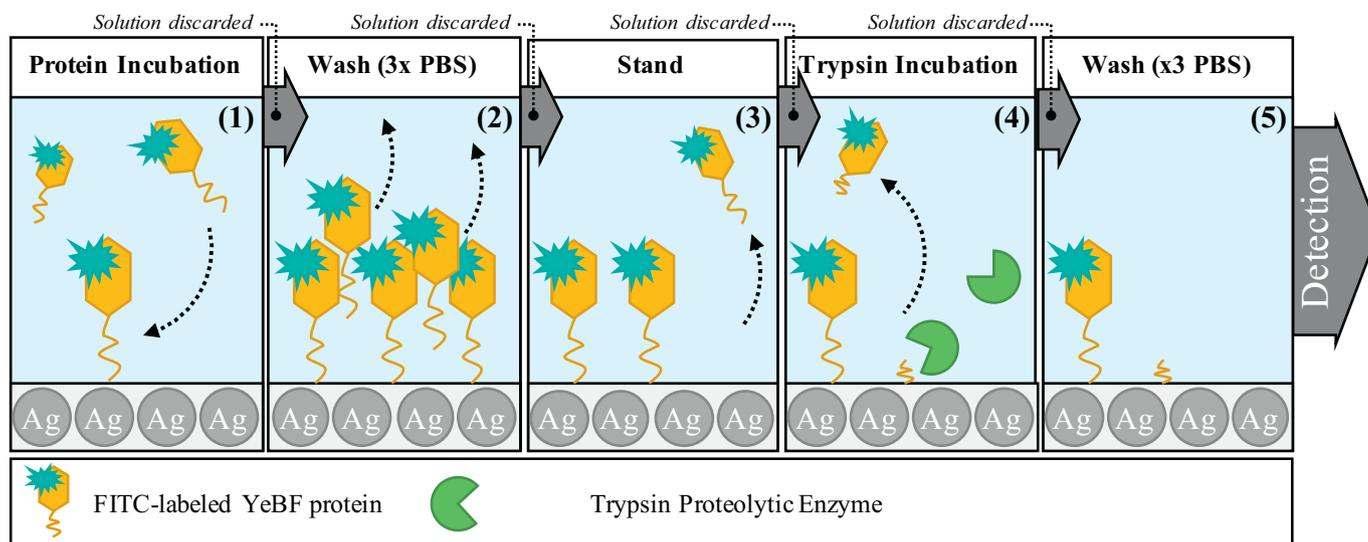

Fig. 3. Fluorescein sodium salt dilution concentrations and corresponding intensities detected in Quanta Plates™. **Inset** depicts lower concentration samples and corresponding intensities. Trend lines are linear fits.

Scheme 1. Schematic of experimental procedure and assay construction.

salt for the protein label. The key difference is that FITC is functionalized with the isothiocyanate substituent, which readily reacts with amine and sulfhydryl groups on proteins and therefore has led to the ubiquitous use of FITC as a fluorescent label in biological studies; [29] however, photophysical properties are comparable between the salt (quantum yield, "Φ" = 0.92, lifetime "τ" = 4.16 ns) and FITC (Φ = 0.87, τ = 4.32). [30] Following incubation, the wells were washed with PBS buffer to remove excess unbound or loosely bound protein (step 2), with the resulting solutions discarded. A 20-min standing period was then performed to permit equilibration and possible desorption of the protein (step 3). Steps 1 through 3 represent the preparation cycle needed to establish the assay platform, although further optimization of these preparation cycles were completed, as discussed later in this section. In step 4 of the assay design, the trypsin solution was added, and the plates were incubated as indicated previously. During this step, trypsin cleaves the protein from the silver nanoparticles, releasing FITC-YebF into solution in a concentration-dependent manner. Following a final triplicate wash procedure (step 5), only the bound, or un-cleaved, protein remains in the plate well; therefore, there should be minimal to no background fluorescence from free-space label. Due to the proximity of the FITC label to the silver well surface, its emission is significantly enhanced, permitting lower concentration detection of the label.

To assess the impact of the preparation cycles, or washing and standing procedures, for assay optimization as previously mentioned we first analyzed the fluorescence intensity from the FITC-YeBF coated plates following a series of sequential preparation cycles, as shown in Table 1. The initial probe incubation and subsequent fluorescence detection is indicated as "cycle zero," and demonstrates an average intensity of 55 ± 8 arbitrary units. This intensity rapidly dropped after the first 3 washing steps and subsequent 20-min standing period (cycle 1) due to the removal of significant concentrations of unbound label. The magnitude of intensity drops per cycle then decreased with subsequent washes and accompanying standing periods, although intensity did decrease for cycles 2 through 8 for the same sample wells (Fig. 4). This intensity loss was minimal and represents the equilibration between bound and unbound YebF. These data demonstrate consistency in the label desorption steps of cycles 2 through 8, allowing for careful control of the potential desorption effects in probe response for comparison with trypsin activity in diagnostic experiments.

Although the reported intensity loss with preparation cycles may not be favorable for assay sensitivity, it is also accompanied by an improvement in relative standard deviation

for the wells analyzed, thereby reducing the variability, and therefore error, present between sample wells analyzed. This is particularly of note for consideration in running the assay (Scheme 1, step 5), as additional washing steps will be necessary to remove cleaved protein from the sample wells following protease incubation steps to detect trypsin activity. The intensity decrease with no trypsin incubation will therefore provide the baseline for the limit of detection reported later. To optimize the platform through minimizing the potential decrease due to desorption alone, it was determined that trypsin incubation would be completed after preparation cycle 1, permitting the largest starting intensity for the assay possible for higher sensitivity.

Higher concentrations of trypsin, and therefore more enzyme activity, should result in lower fluorescent signals as more of the bound YebF is cleaved from the nanoparticles and removed during subsequent washing steps. To demonstrate the utility of the coated plates to detect low levels of protease, a serial dilution of stock trypsin at a concentration range of 1 ng/mL to 0.1 mg/mL was reacted with FITC-YebF coated wells. The residual fluorescence intensity was measured following incubation of Quanta Plate™ wells coated with FITC labeled YebF. Fig. 5 shows that the fluorescence signal decreases with an increase in the trypsin concentration (Table S2, sample spectra shown in Supplementary Fig. S2). An inverse linear correlation between the log of trypsin concentrations and fluorescence signal was observed, with a linear fit equation as indicated in equation (4) (R^2 = 0.92):

$$y = -\,(122)x + (1066) \qquad\qquad\qquad\text{Equation 4}$$

For this equation, y is the normalized fluorescence intensity (%) and x is $\log_{10}$[trypsin, ng/mL]. The limit of detection, defined as 3 standard deviations below the mean of the non-reacted wells, was determined to be 17.2 ng/mL, or 1.89 ng for the trypsin volume used in this study. This corresponded to about 2.8 mBAEE activity units. In comparison, commercially available protease detection kits report limits of detection in this range. For example, the Protease Fluorescent Detection Kit (PF0100, Sigma Aldrich), which
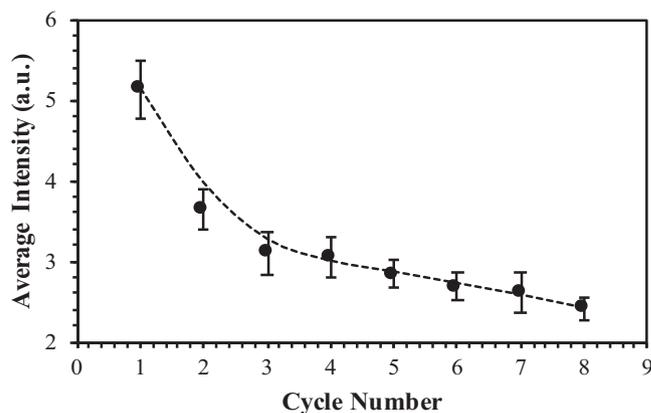
Table 1
Average signal intensities for FITC-labeled YebF protein from 88 wells after varying number of wash and standing periods.

Cycle	# of Washes	# of Standing Periods	Average Intensity (a.u)	Standard Deviation (a.u)	RSD
0	0	0	54.86	8.46	15%
1	3	1	5.14	0.36	7%
2	4	2	3.65	0.25	7%
3	5	3	3.1	0.26	8%
4	6	4	3.05	0.24	8%
5	7	5	2.84	0.18	6%
6	8	6	2.69	0.17	6%
7	9	7	2.62	0.25	10%
8	10	8	2.41	0.14	6%

Fig. 4. Emission intensity of FITC-labeled YebF protein coated in Quanta Plates™, excited at 485 nm and recorded at 527 nm emission. Intensities are reported following varying preparation cycles per trial, as indicated in Table 1. Error is from the standard deviation of signals from 88 wells per cycle.
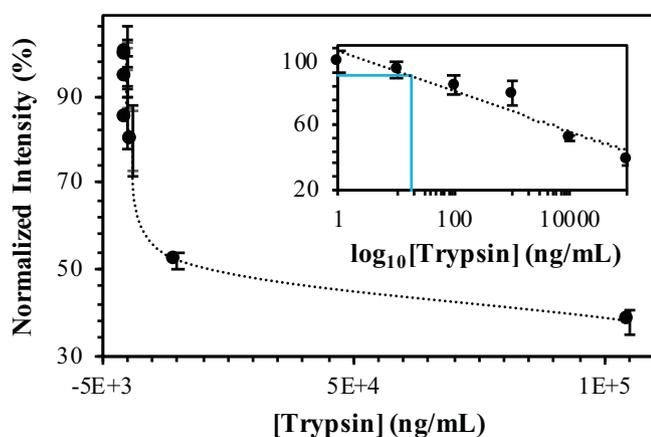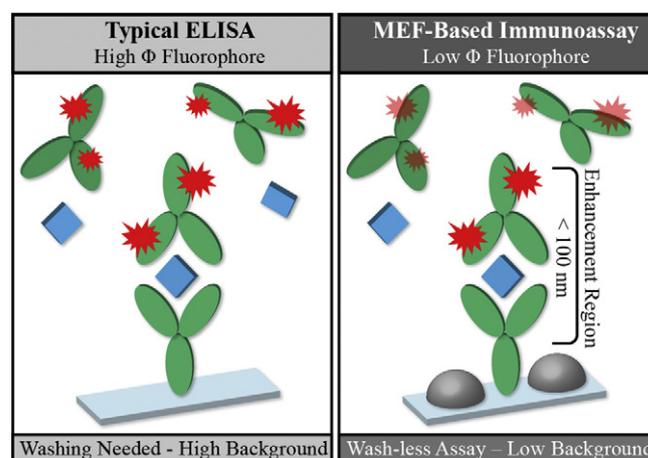
Fig. 5. Normalized intensity demonstrating turn-off assay response of FITC-labeled YeBF protein after incubation with trypsin in PBS in Quanta Plate™ wells (λ_{ex} = 485 nm, λ_{em} = 527 nm). Error is from standard deviation (data in Supplementary Table S2). **Inset** displays the $\log_{10}$ scale, with the limit of detection indicated in blue (solid lines).

Scheme 2. Diagram of typical enzyme-linked immunosorbent assay (ELISA) versus MEF-based immunoassays, demonstrating the low-background advantages of wash-less MEF-based assays.

uses FITC-casein as the fluorescent probe, has an advertised detection limit of approximately 50 ng. In the Pierce Fluorescent Protease Assay Kit (23266–23267, Thermo Scientific), detection down to 0.400 ng is reported possible for the FTC-Casein probe. In the literature, fluorescence-based probes have achieved down to about 2 pg detection levels [31]. Having demonstrated sensitivity comparable to often-used commercially available products, the MEF-based assay described here sets the foundation for achieving even more sensitive assays with further development.

4. Conclusions

Here we report the development of a *turn-off,* MEF-based assay for the detection of proteolytic enzymes using Quanta Plates™. On this plasmonic platform we report strong enhancement of the fluorescein sodium salt fluorophore emission by proximity to the silvered surfaces, with detectable fluorescence as low as picomolar concentrations. Additionally, we report a ~9% coefficient of variation for fluorescein sodium salt in the Quanta Plate™ wells, confirming the possible utility of this fluorophore-metal system for a MEF-based assay. Further, we demonstrate the potential of this MEF platform for the detection of protease activity using the model protease trypsin. The preliminary assay design achieved nanogram detection limit of trypsin (<100 fmoles). As designed, however, the current method still requires the lengthy incubation times characteristic of fluorescence-based, dye-release enzymatic detection assays; these extended times, while improving limit of detection, can increase inaccuracy and sensitivity of the platform to enzyme instability. In the case of a MEF-based assay, however, the overall assay time could be shortened via the microwave-accelerated MEF, or MAMEF, technology, which will be the focus of future assay design and research.

Finally, while washing steps were undertaken here, it is important to note the opportunity for "wash-less" immunoassays that MEF, and MAMEF, afford. For Bioassays (DNA [24,32–40]/RNA [41] or Protein [20–22,42–45]) there has been a significant amount of work undertaken to date, strongly supporting our premise in this paper that plasmonic-based immunoassays have significant advantages over classical fluorescence immunoassays. [20–22,24,32–45] MEF is particularly useful in assays as shown in Scheme 2. In traditional assays, *e.g.* sandwich immunoassays, one uses a high quantum yield fluorophore to increase the detectability of the antigen/bio-agent. While this inevitably increases the detectability of the antigen in question, the assay does however suffer from the need to be additionally washed, to remove the high quantum yield (Φ) fluorophore-labeled antibody not bound as shown in Scheme 2, left. This additional washing step in classical fluorescence-based assays takes further time and usually requires a laboratory, hindering many fluorescent based tests from being utilized at

the point-of-care (POC). However, with MEF, low quantum yield fluorophores (weakly bright fluorophores) are better suited as they are preferentially enhanced near-to metal (Scheme 2, right) *with fluorescence signatures increasing as much as a million-fold and beyond*, increasing assay and therefore analyte/antigen detectability [20–22,24,32–45].

Subsequently, no washing steps are required in these MEF assays due to the use of the low quantum yield fluorescent label which is not metal-enhanced distal from the metal. Because it is of low quantum yield, the fluorophore contributes very little to background signal (noise). This subtle, but powerful, sensing concept has merit, particularly in POC testing. This discussion and the results reported here set the foundation for the future investigation of MEF enzyme detection assays, providing a novel platform for development of more sensitive, quantitative, and wash-less high-throughput sample analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.117739.

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