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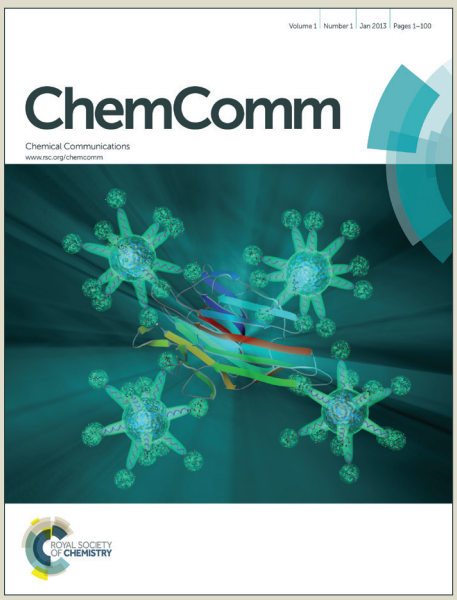

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COMMUNICATION

Molecular Fe-complex as a catalyst probe for in-gel visual detection of proteins via signal amplification

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We report the use of a molecular peroxidase mimic biuret FeTAML for chemoselective labeling of proteins and the subsequent visual detection (<0.1 pmoles) of the conjugate in a polyacrylamide gel by catalytic signal amplification. Use of this probe in activity based protein profiling (ABPP) of serine proteases is also demonstrated.

Detection of protein bio-markers, especially using methods that are easy and inexpensive in a high throughput manner, is extremely important for early diagnosis of diseases.[1] Most probes that are used operate with high selectivity and sensitivity are based on stoichiometric reactions and mostly having fluorescence as the output signal.[2-4] The detection limits and signal to noise ratios of these probes can be significant improved upon, if new detection schemes that use catalytic processes where signals can be amplified are incorporated.[5] Although such detection systems are routinely used in enzyme-linked immunosorbent assay (ELISA)[6] and western blot,[7] where the heme-containing enzyme horseradish peroxidase (HRP) converts a non-chemiluminiscent molecule into a chemiluminiscent molecule in the presence of H_2O_2 with several thousand turnovers, thus decreasing the detection limits severalfold.[8] However, the large size of HRP (44 kDa) and its instability under demanding conditions do not allow its usage in several applications, such as gel-based assays. For example, in polyacrylamide gel-based proteomic assays such as ABPP (activity-based protein profiling), which uses active site-directed chemical probes to directly assess the functional state of large numbers of enzymes in native biological samples, reporter tags typically include fluorophores or biotin for probe-labelled enzymes.[9] Here, signal amplification cannot be achieved by using HRP as a reporter tag, since HRP would denature in the gel and lose its activity. For such gel-based assays, signal amplification can be achieved if a functional and robust small molecule HRP mimic can be synthesized. In our laboratory, we have synthesized Fe-based small molecule inorganic complexes (biuret-Fe-TAML) that are excellent functional mimics of the peroxidase enzyme [10] with very high stability in a range of pH (1-13) and ionic strength. We have also demonstrated that these selectively oxidize the colorless 3, 3', 5, 5'-tetramethylbenzidine (TMB) molecule to a one-electron oxidized blue species with very high turnover numbers, and this property has been used for the detection of several analytes. [11] We hypothesized that the usage of biuret-Fe-TAML in gel-based assays such as ABPP would allow visual detection of proteins at very low concentrations via signal amplification, and lower the detection limits of proteins. In this paper, we demonstrate that biuret-Fe-TAML conjugated proteins can be visually detected and relatively quantified in a gel by probing them with TMB and H_2O_2. Formation of the blue-colored oxidized TMB molecules by biuret-Fe-TAML catalyst in the gel allows us to detect proteins visually up to 5 ng (< 0.1 pmoles). Due to the catalytic signal amplification, several reporter dyes are generated per analyte, and this lowers the detection limit significantly. We also show that the "clickable" biuret-Fe-TAML catalyst can be used as a reporter molecule for in-gel visual detection of fluorophosphonate-labelled serine proteases by catalytic signal amplification.

To provide proof of concept, we wanted to first establish the covalent conjugation of the inorganic complex biuret-Fe-TAML onto proteins using click chemistry and, subsequently, perform an analysis of the biuret-Fe-TAML protein conjugate by SDS--PAGE. The main goal was to probe the efficacy of biuret-Fe-TAML in catalyzing the H_2O_2 mediated oxidation of colorless dye TMB to its corresponding oxidized blue product in the polyacrylamide gel. The oxidized TMB molecules would appear as a blue-colored band in the gel corresponding to the molecular weight of the protein to which it is conjugated. For a small molecule inorganic complex, this is a tall order, since the biuret-Fe-TAML moiety in the protein conjugate has to retain its catalytic activity under demanding conditions, such as its treatment with the reducing SDS-PAGE loading buffer and upon gel electrophoresis. We therefore initiated our study by synthesizing Bovine Serum Albumin (BSA)-Fe-TAML conjugate by

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Electronic Supplementary Information (ESI) available: [Synthesis of protein conjugates and the detailed assay showing SDS-PAGE analysis and gel development using TMB/H_2O_2 has been described in the supporting information.]. See DOI: 10.1039/x0xx00000x
‡These authors contributed equally to this work.

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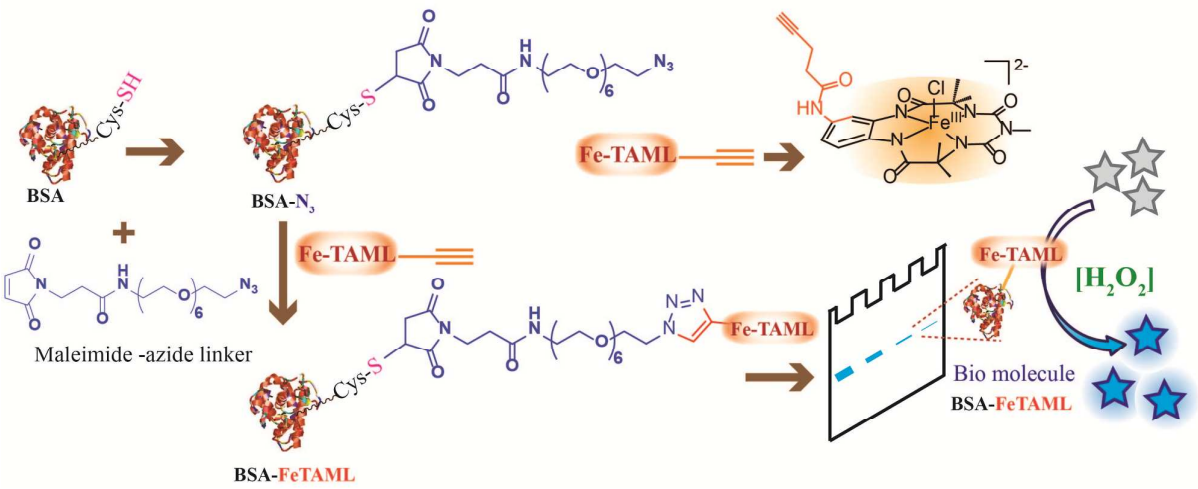

Figure1. Synthesis of BSA-N_3 followed by CuAAC using alkyne-biuret-Fe-TAML. Schematic representation for the analysis of, BSA-Fe-TAML conjugate using SDS-PAGE. After gel electrophoresis, treatment with TMB/H_2O_2 leads to formation of oxidized TMB; thus appearing as a blue color band at the expected location of the BSA in the gel. The catalytic formation of oxidized TMB leads to signal amplification and in turn lowers the limit of detection.

covalently attaching alkyne containing biuret-Fe-TAML to azide-modified BSA using azide alkyne cycloaddition click (CuAAC) reaction. [12] The azide functionalised BSA was prepared by following a thiol-maleimide chemistry where the surface free-thiol of BSA (cysteine 34 position) [13] was reacted with an excess of maleimide-N_3 linker (Figure 1; S 2a). Quantitative attachment of maleimide-N_3 linker onto Cys 34 position of BSA was confirmed by (i) thiol estimation (Ellman's reagent) which showed complete conversion of free thiol present on BSA (S 1a), and (ii) mass spectrometric studies (SI) of the intact protein and tryptic digest which showed an increase in molecular weight corresponding to the linker(Figure S7-10). For the subsequent covalent attachment of biuret-Fe-TAML, CuAAC reaction was performed with BSA-N_3 (0.5 mg/mL) and alkyne biuret-Fe-TAML in the presence of $CuSO_4$, THPTA and sodium ascorbate for one hour at 4°C. Successful labelling of BSA with biuret-Fe-TAML was confirmed by ESI-MS, which showed an increase of mass by 1009 Da from the parent BSA (Figure S11).

The "click" reaction mixture described above was diluted with SDS-PAGE loading buffer, loaded onto the polyacrylamide gel (with concentrations ranging from 100 ng/well to 5 ng/well) and analysed by SDS-PAGE. The gel was subsequently treated with (0.5 mM) TMB and (80 mM) H_2O_2 (Figure 2a), and within 60 sec a blue colored band was observed for all the concentrations at a position where BSA is expected (Figure 2, lanes 3-7). Upon co-localization of BSA-Fe-TAML with colorless substrate (TMB) and H_2O_2 in the polyacrylamide gel, oxidation of TMB occurs to form the corresponding one-electron oxidized product. This appears as a blue colored band in the gel. Although TMB and H_2O_2 is present in the rest of the gel, no color is observed as reaction between TMB and H_2O_2 is slow in absence of the catalyst. This Fe-TAML/H_2O_2 catalysed oxidation reactions has a high turnover number and occur via the high valent Fe(IV)OH or Fe(IV)O species that is formed upon activation of Fe(III) complex by H_2O_2. A good linear dependence between the integrated volume of the densitometry units for scanned bands of proteins in the gel (evaluated by Image J analysis) vs BSA concentration was obtained in the range 50ng/well to 5ng/well with a limit of detection of 5ng/well under the optimum experimental conditions (Figure 2b). This color generation technique can be utilized as a tool for relative quantification in gel-based visual detection of proteins. This represents a proof of concept where a synthetic functional model of peroxidase enzyme has been used for in-gel visual detection of protein biomarker. The result shows that the limit of detection is 5ng/well (0.07 pmoles) with a considerable signal to noise ratio of 3 (Figure 2).

To probe that the appearance of the colored band in the SDS-PAGE was due to the covalent conjugation of biuret-Fe-TAML onto BSA-N_3 via "click reaction", we carried out several control reactions. Reaction mixtures which contained only two of the three required components ($CuSO_4$, sodium ascorbate, alkyne-biuret-Fe-TAML) were also loaded on the same gel after dilution with SDS-PAGE loading buffer. The resulting reaction mixtures were analysed by SDS-PAGE followed by subsequent treatment with TMB/H_2O_2. No colored bands were observed for any of the three controls (Figure S1). This confirms the covalent attachment of alkyne-biuret-Fe-TAML via triazole formation, and also eliminates the possibility of non-specific physical adsorption of biuret-Fe-TAML on the protein surface.

The efficacy of our reporter probe alkyne-biuret-Fe-TAML was then compared to the well-established fluorescein-alkyne. Fluorescence alkyne was conjugated to BSA-N_3 under the same reaction conditions described above for alkyne-biuret-Fe-TAML and analysed by SDS-PAGE. The resulting polyacrylamide gel was then visualized through a Gel Doc to observe the fluorescent band from fluorescein-BSA. Using the Gel Doc, we were able to assay up to only 100 ng of protein per each well with signal-to-noise ratio of 3 (Figure S3). However, it should be mentioned that using a laser scanning fluorescence detection system, Cravatte *et al.* has reported detection of proteins up to 0.1 pmoles in ABPP.[3a] The effectiveness of this assay will depend on its ability to detect low concentrations of protein in a complex proteome mixture.

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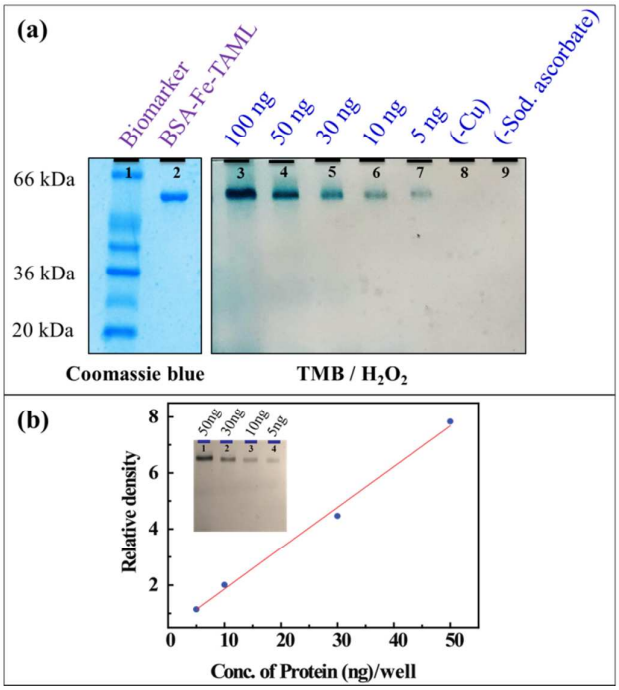

Figure 2. SDS-PAGE of the BSA labelled with alkyne-biuret-Fe-TAML **(a)** Lane 1-2: Protein bands by Coomassie staining; Lane 3-9: Protein bands after treatment with 0.5 mM TMB / 80 mM H_2O_2. . Lane 1: biomarker; Lane 2: BSA-Fe-TAML; Lane 3-9: BSA-Fe-TAML with varying amounts of protein (100 - 5 ng) loaded in each well. **(b)** Relative quantification of protein from the band intensity using Image J for Lanes 1 - 4.

Hence, model studies were carried out to determine if small quantities of BSA-N_3 can be determined by this assay. We therefore performed CuAAC click reaction with varying concentrations of BSA-N_3 (500 - 30 µg/mL) and alkyne tailed biuret-Fe-TAML. Subsequent analysis by SDS-PAGE followed by addition of TMB/H_2O_2 allowed us to detect BSA-N_3 to concentrations up to 30 µg/mL (Figure S2).

Finally, we wanted to explore if alkyne-biuret-Fe-TAML can be used as a catalytic probe for gel-based proteomics applications like ABPP. In this study, we chose the serine hydrolase class of enzyme, which has been studied extensively in ABPP using fluorophosphonate probes.[14] In particular, the majority of serine hydrolases are potently and irreversibly inhibited by fluorophosphonate / fluorophosphate (FP) derivatives like diisopropyl fluorophosphates (DFP).[15] Typical reporter tags include fluorophores or biotin for detection.[16] With fluorescent reporter tags like Rhodamine that are conjugated to fluorophosphonates, the gel can be directly assayed using a fluorescent imaging system. [17] However, higher sensitivity can be obtained by using biotin as a reported tag. In this case, the protein gels after electrophoresis are transferred into a nitrocellulose membrane and subsequently probed by Streptavidin-HRP / substrate / H_2O_2, to produce a colored / luminescent band on the membrane. Such a multi-step process is long, laborious and expensive. We hypothesized that the use of alkyne-biuret Fe-TAML as the reporter would allow us to carry out one-step visual detection in the gel by catalytic signal amplification.

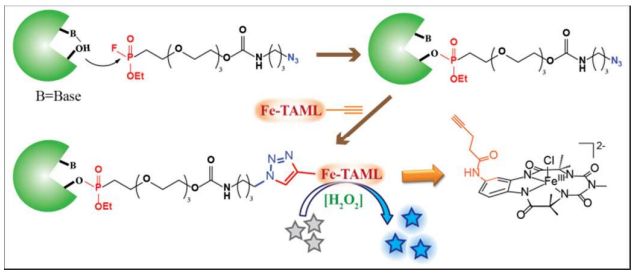

Figure 3. Schematic representation for the ABPP of serine proteases using fluorophosphonate-azide and alkyne-biuret-Fe-TAML

We then proceeded to design a fluorophosphonate-azide probe which would have the ability to bind serine hydrolase family of enzymes and subsequently be "clicked" to alkyne-biuret-Fe-TAML. The strategy of azido-linked suicide inhibitor probes has been used by Cravatte *et al.* for profiling glycosyltransferases. An ABPP probe containing a fluorophosphonate war head, an oligoethyleneglycol linker and an alkyl-azide handle was synthesized, as is delineated in (Scheme S1) [18].

To test the efficacy of FP-N_3 towards serine hydrolases, FP-N_3 (40 µM) was incubated with equal amounts of two serine proteases (1 µM; trypsin and chymotrypsin) and their respective zymogens (trypsinogen and chymotrypsinogen) for 30 min. Proteins labeled with FP-N_3 was then incubated with biuret-Fe-TAML using CuAAC (Figure 3). The products of each reaction was diluted with SDS-PAGE loading buffer, analysed by SDS-PAGE (100 ng/well of protein) and probed with TMB/H_2O_2. FP-Fe-TAML strongly labeled both trypsin and chymotrypsin, but exhibited no reactivity with their corresponding proenzymes (Figure 4) as was evident from the presence and absence of the blue colored band. Control experiments in which trypsin was first allowed to auto-digest for 15 min showed no bands upon addition of FP-N_3 followed by alkyne-biuret-Fe-TAML (Figure S4).

The selectivity of the FP-Fe-TAML probe towards serine proteases over other class of proteins such as human serum albumin (HSA),

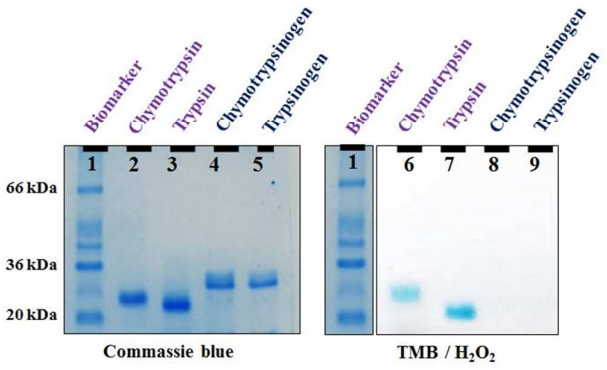

Figure 4. SDS-PAGE of the serine proteases labelled with FP-Fe-TAML. Labelled proteins were quenched with SDS loading buffer and analysed by SDS-PAGE. Lanes 2-5 (10µg/lane): Coomassie stained; Lanes 6-9 (100 ng/lane): Treatment with 0.5 mM TMB / 80 mM H_2O_2. Lane 1: Biomarker; Lane 2, 6: Chymotrypsin; Lane 3, 7: Trypsin; Lane 4, 8: Chymotrypsinogen; Lane 5, 9: Trypsinogen.

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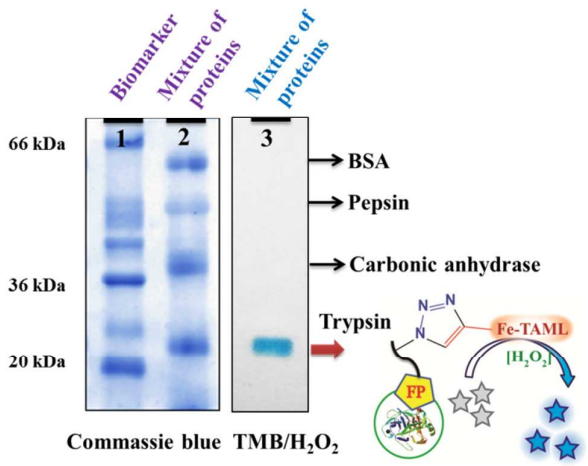

Figure 5. Selectivity of FP-N_3/alkyne-biuret-Fe-TAML towards labelling of Trypsin from a mixture of proteins. Lane 1: Biomarker. Lane 2: Mixture of proteins (BSA, Pepsin, Carbonic anhydrase and Trypsin) (10µg/well) that were Coomassie stained. Lane 3: Mixture of proteins (100 ng /well) probed with 0.5 mM TMB / 80 mM H_2O_2.

bovine serum albumin (BSA), pepsin and carbonic anhydrase was also evaluated both separately and as a mixture (to simulate a model proteome). Incubation of the proteins mentioned above (0.5 mg/ml) with FP-N_3 for 30 min separately, followed by click reaction with alkyne-biuret- Fe-TAML and subsequent analysis by SDS-PAGE/TMB/H_2O_2 showed that the blue-colored bands were only obtained for the serine proteases, i.e trypsin and chymotrypsin, and not for other proteins (Figure S5). The same experiment was performed again with a mixture of proteins (1 µM of each protein; FP–N_3 100 µM). Here again only one blue coloured band was observed exactly at the location where the Trypsin-FP-Fe-TAML conjugate was expected. The absence of bands from other proteins (Figure 5) demonstrates that using a combination of FP-N_3 and alkyne-biuret-Fe-TAML serine hydrolase enzymes can be selectively labelled and visually detected.

To summarize, we have demonstrated the use of molecular Fe-based catalyst as a replacement of fluorescent dyes as a reporter molecule for visual in-gel-based protein detection. The catalytic property of the biuret Fe-TAML complex allows in-gel visual detection of <0.1 pmoles of proteins. The amount of protein in the gel can be relatively quantified by analysing the intensity of the blue-colored band by Image J. Additionally, the catalytic probe alkyne-biuret-Fe-TAML was used for ABPP assays for serine hydrolases. Since a linear calibration of the band intensity can be obtained for BSA-Fe-TAML, this can be used as a standard curve for the estimation of proteins in ABPP using our catalyst reporter. We also believe the detection limits can be significantly improved upon by (i) using modified biuret-Fe-TAMLs, such as the placement of electron withdrawing NO_2 groups on the head part, which is expected to increase its stability and reaction rates by several folds, and (ii) development of fluorescent probes that can be turned "on" upon oxidation with Fe-TAML/H_2O_2. Fluorescent "turn-on" probes based on redox reactions have been reported.[19] Biuret-Fe-TAML can also be used for other applications, such as to quantitatively monitor the cellular uptake of various polymers and nanoparticles used for drug delivery applications. They have the promise to replace many fluorescent dyes which are currently used in such studies. Research in this direction is currently being carried out in our laboratory.

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