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A fluorescence turn on trypsin assay based on aqueous polyfluorene†

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A new method based on the electrostatic interaction of a novel anionic water soluble polymer P1 with a positively charged polypeptide Arg_6 was developed for a continuous and real time turn on assay for the enzymatic activity of trypsin under alkaline conditions with a limit of detection of 0.17 nM. This method was also able to screen the inhibitors of trypsin. P1 fluorescence intensity was significantly decreased by the positively charged Arg_6 due to the electrostatic interaction, whereas the enzymatic action recovered P1 fluorescence due to the fragmentation of Arg_6 into small positively charged fragments and these were unable to quench the P1 fluorescence. Therefore, by triggering the fluorescence intensity change, it was possible to assay the enzymatic activity. Use of water soluble conjugated polymer P1 and no labeling on the substrate enhances the utility of this method significantly.

1 Introduction

Trypsin, one of the most vital digestive enzymes produced in pancreases as the inactive proenzyme trypsinogen,[1] plays a key role in cleaving proteins into smaller peptides on the c-terminal side of arginine and lysine residues[2] and this protein cleaving into the components of amino acids facilitates the digestion of proteins. Moreover, the generated proenzyme trypsinogen self-cleaves to produce the more active form as needed[3] and this active form then functions to activate additional trypsinogens within the intestine and this may be called autolysis. Only a small amount of enteropeptidase is necessary to initiate the reaction for autodigestion. Therefore trypsin is involved in a major role in controlling pancreatic exocrine function. Trypsin becomes more active under alkaline conditions and in the presence of metals Ca^{2+}, Mg^{2+} and Mn^{2+}.[4] This proteolytic enzyme is a medium sized globular protein with applications in mass spectroscopy based proteomics,[5–7] wound healing components, washing agents and biotechnology, mainly to perform enzymatic reactions.[8–12] Hence, it is confirmed that trypsin plays an essential role in controlling pancreatic exocrine function, but an increased level is also associated with some types of pancreatic disease.[13–15] Therefore, a new continuous assay for trypsin and inhibitor screening may provide possibilities for the development of new diagnostic methods and therapeutic strategies with implication in pancreatic diseases.

Several methods for trypsin assay have been reported. Among them traditional methods involve multiple clinical tests including radioimmunoassay[16] and gelatine based film techniques[17] whereas recently amperometric,[18] colourometric[19] and fluorometric assay methods based on labeled substrate peptides[20–23] were reported. However most of the reported methods were found to respond slowly towards enzyme modified electrodes and labeling was necessary to respond. A small number of fluorescent assays for trypsin based on water soluble conjugated polymers[24–29] were also reported. The past few years witnessed the growth of conjugated polyelectrolyte (CPE) based assays using the unique optical properties and intrinsic fluorescence signal amplification of CPEs for sensing biological materials,[30] such as small biomolecules,[31] proteins,[32] DNA[33,34] and enzymes.[35–38] However, a convenient and continuous label free water soluble conjugated polymer based fluorometric assay for trypsin and inhibitors screening that can operate in a wide pH range is still very limited. Development of sensitive enzyme assays based on conjugated polymers is very important since most of the traditionally used methods for monitoring enzyme activity rely mainly on gel electrophoresis, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and a few other methods that are either expensive, time consuming or lack good sensitivity and selectivity.[39–42]

In the present work, we report the development of a new anionic water soluble polyfluorene derivative poly(9,9-bis-(6-sulphate hexyl) fluorene-*alt*-1,4-phenylene) sodium salt (P1)[43] (Fig. 1, inset) for monitoring the activity of trypsin and its inhibitors continuously. The interaction of anionic P1 with a cationic polypeptide Arg_6 occurs *via* electrostatic interactions and serves as a platform to develop a highly efficient, continuous and sensitive fluorescence turn-on assay for the detection of nanomolar quantities of trypsin. These properties provide the basis for the study presented herein. P1 has several important

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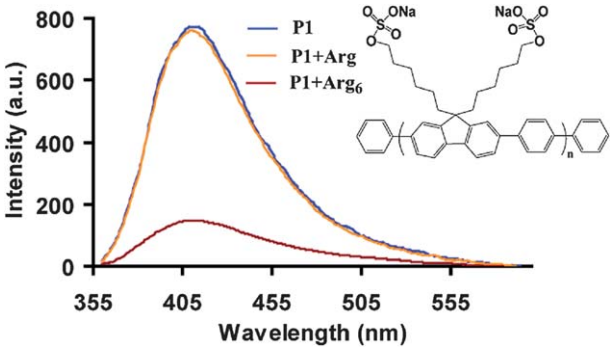

Fig. 1 Changes in fluorescence intensity of P1 (0.4 μM) on addition of 5 μM of Arg and Arg_6 at pH 8.5 in 2 mM PBS solution containing $[Ca^{2+}] = 10$ μM.

features, such as the sulphate functional group that is anionic in nature, is highly soluble (more than 100 mg mL^{-1} in water) and fluorescent in a wide pH range with an excitation wavelength of 335 nm. The assay system does not require labeling the substrate, thereby reducing the cost of the assay system yet providing a fast output of enzymatic activity in nanomolar range concentrations and in a homogenous environment. The enzymatic hydrolysis completion was signaled by fluorescence turn on response from the conjugated polyelectrolyte P1 as a result of the products, that were fragmented as a single positively charged Arg and free chain conjugated polyelectrolyte.

2 Results and discussion

We reported recently that P1 was highly sensitive towards Cc, MetHb and other metalloproteins, the reason for which was the highly positive nature of the protein at physiological pH and the heme integrity.[44] Charged CPEs are well known to bind oppositely charged analytes *via* electrostatic interaction.[30] This property of CPEs has been utilized in the present work to develop a sensitive bioassay for trypsin. Positively charged polyarginine peptide (Arg_6) was selected as a substrate for enzymatic activity of trypsin. It is already reported that Arg_6 peptide contains 6 positive charges at pH 8.5, whereas arginine has only one positive charge at this pH.[45] Since highly positive charged species can interact more efficiently with anionic polyelectrolytes such as P1, the pH of the assay system was maintained at 8.5 in all the experiments performed here. P1 was found to be highly fluorescent at this pH of 8.5 in 2.0 mM phosphate buffer saline solutions (PBS) with excitation wavelength of 335 nm and emission at 411 nm allowing us to utilize it to assay trypsin. Initially, we investigated the amount of change in fluorescence intensity of P1 on addition of positively charged Arg and peptide Arg_6. To perform this experiment, P1 (0.4 μM), Arg and Arg_6 peptide solutions were prepared in 2.0 mM PBS solution. Arg and Arg_6 peptide solutions (0–5 μM) were added into two separate solutions having P1 with same concentration of 0.4 μM and the fluorescence intensity changes were monitored and are shown in Fig. 1.

For instance, P1 solution in the absence of peptide shows a strong fluorescence (blue line). However, after addition of peptide solution to the cuvette, the P1 fluorescence intensity was decreased by >80% due to opposite charge interactions between the anionic P1 and cationic peptide (brown line). However, Arg with a single positive charge was unable to change the fluorescence intensity of P1 (orange line). Based on these results, we expected that Arg with one positive charge that will be the fragmented product after enzymatic hydrolysis will not interfere in the turn on signal from P1 and this will be solely from the enzymatic hydrolysis.

Before performing the investigation of enzymatic hydrolysis we also examined whether the quenched fluorescence intensity of P1, as a result of association with Arg_6, was modified with time or would remain intact during hydrolysis. It was clearly observed that the association of Arg_6 with anionic P1 was very stable, which allowed us to perform the enzymatic hydrolysis. The control experiments demonstrated that fluorescence intensity of P1/Arg_6 remains unchanged during addition of trypsin. Further, we confirmed that the fluorescence intensity of P1 remains unchanged at 0.35 μM concentration of trypsin indicating that the presence of trypsin had no direct effect on the fluorescence intensity of either P1 or P1/Arg_6. This confirms that the fluorescence intensity change observed for P1 was due to the enzymatic hydrolysis only.

We further examined the enzymatic hydrolysis of P1/Arg_6 in the presence of trypsin. To perform the enzymatic hydrolysis of Arg_6, trypsin (350 nM) was added into P1/Arg_6 solution in 2 mM PBS, at pH 8.5 containing 10 μM of $CaCl_2$. The main reason to add $[Ca^{2+}]$ was to activate trypsin and increase its stability against autolysis, as already reported in the literature.[46] After addition of trypsin, the complete assay was allowed to incubate. The gradual increase in fluorescence intensity at 411 nm was recorded for different incubation time intervals from 0 to 20 min and is shown in Fig. 2.

According to Fig. 2, it was clear that after addition of trypsin, the fluorescence intensity of P1/Arg_6 remained unaffected providing good evidence that the enzyme itself had no effect on the fluorescence intensity changes but as time increases from 0 to 10 minutes, enzymatic hydrolysis proceeds, the quenched emission of P1/Arg_6 gradually increased and reached a plateau

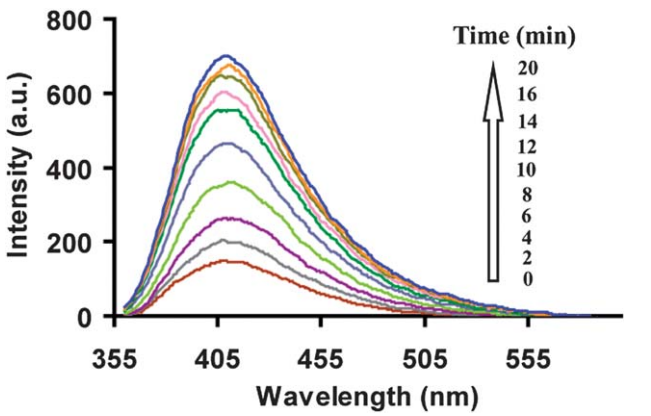

Fig. 2 Changes in fluorescence intensity of P1 (0.4 μM)/Arg_6 (0–5 μM) on addition of 350 nM of trypsin at pH 8.5 in 2 mM PBS solution containing $[Ca^{2+}] = 10$ μM.

after 20 minutes and this fluorescence increase leveled off at 89%. Hence, these results establish that trypsin catalyzes the hydrolytic cleavage of peptide Arg_6 into small positively charged fragments which are incapable of quenching the P1 fluorescence and thus leading to a rapid emission turn-on response even at very low concentrations of both P1 as well as the substrates. Thus the fluorescence recovery based assay system significantly enhances the detection sensitivity in comparison to assays that operate on the fluorescence quenching principle by getting rid of erroneous signals.

Further, we also investigated the increase in fluorescence intensity of the P1/Arg_6 assay system with different concentrations of enzyme as a function of time to check whether the enzymatic hydrolysis of Arg_6 was dependent on the trypsin concentration and also how low a concentration of enzyme was actually required for a significant change in the fluorescence intensity. Hence, control experiments in the absence and presence of trypsin were carefully performed (Experimental section). Six samples containing P1/Arg_6 and 2.0 mM PBS buffer solution at pH 8.5 were prepared followed by the addition of different concentrations of enzyme, say 0 nM, 40 nM, 80 nM, 120 nM, 220 nM and 350 nM, and fluorescence intensity changes were monitored at the emission intensity 411 nm with increasing concentration of trypsin as a function of time and are reported in Fig. 3.

The increase in the fluorescence intensity of P1/Arg_6 with respect to the incubation time was apparent from the fluorescence enhancement pattern shown in Fig. 3 which demonstrates that the increase in fluorescence intensity measured within a set time of 20 min was dependent on the enzyme concentration. However the maximum recovered fluorescence intensity was found to be independent of the concentration of trypsin (40 nM, 80 nM, 120 nM, 180 nM and 220 nM) even after prolonged reaction time (Fig. S1–S5†). At lower concentration of trypsin (10 nM), the fluorescence recovery was slow whereas at higher concentration (95 nM), fluorescence recovery was found to be high. At the same reaction time (up to 20 minutes) a linear relationship between the maximum emission intensity and trypsin concentration was observed (Fig. 4) over the enzyme concentration from 0 to 95 nM. Therefore it can be expected that due to this linear relationship, P1/Arg_6 association can be used for assaying trypsin quantitatively (Fig. 4). The calculated limit of detection (LOD) for trypsin was found to be 0.17 nM which is among the lowest in the literature (Experimental section). From these results it was evident that anionic polymer P1 in association with Arg_6 can serve as a fluorometric turn-on assay for hydrolytic cleavage in the presence of trypsin.

Kinetic parameters for trypsin were calculated by maintaining different initial Arg_6 concentration assays. Fluorescence intensities for these assays were then converted to the hydrolyzed substrate Arg_6 and product Arg concentration and were plotted as a function of time as shown in Fig. 5(a) and (b) by using the following equations (Experimental section):

$$[S]_t = [S]_0\,[(I_o/I_t - 1)/(I_o/I_q - 1)]$$

$$[pNP] = [S]_0 - [S]_t$$

where $[S]_t$ is the substrate concentration at a time t, $[S]_0$ is the initial substrate concentration, I_o is the initial fluorescence intensity of P1, I_t is the fluorescence intensity at time t and I_q is the quenched fluorescence intensity of P1.

A Lineweaver–Burk plot between the double reciprocal of initial rate *vs.* substrate concentration as shown in Fig. 5(b) was utilized to obtain K_m and V_{max} values. K_m and V_{max} values were found to be 2.2 μM and 14.1 μM min^{-1} by this method and were in accordance with the literature[47,48] (Experimental section). This indicates that polymer P1 itself has almost no effect or negligible effect on the interaction between substrate Arg_6 and enzyme trypsin (Fig. S6†).

As mentioned earlier, trypsin is the most important digestive enzyme present in the human body but an abnormal elevated level of enzyme is an indication of several types of diseases. Hence, attention to the development of drugs has been, to a large extent, based on the enzymes and assaying their activities. Therefore, development of a sensitive and continuous assay of enzyme that will be able to screen the enzyme inhibitors is of prime interest and importance. We assumed that the hydrolysis

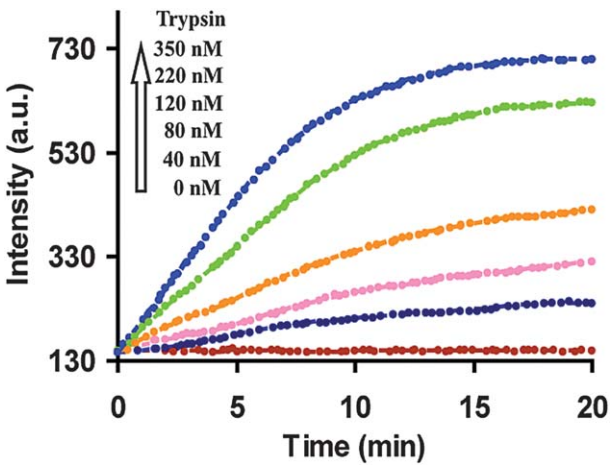

Fig. 3 Fluorescence intensity enhancement of P1 (0.4 μM)/Arg_6 (0–5 μM) in the presence of different concentrations of trypsin as a function of time (0–20 minutes) in 2 mM PBS solution containing $[Ca^{2+}] = 10$ μM.

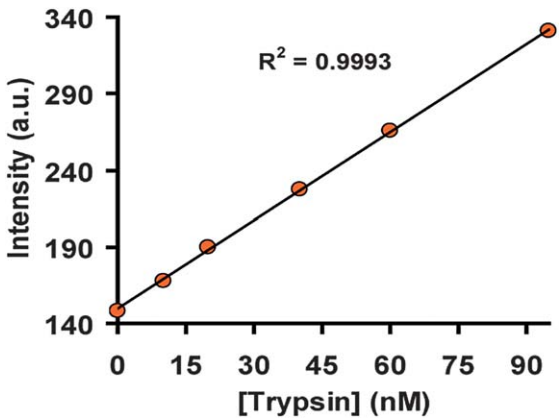

Fig. 4 A linear relationship between maximum intensity of P1/Arg_6 and enzyme concentration within a set time of 20 minutes.

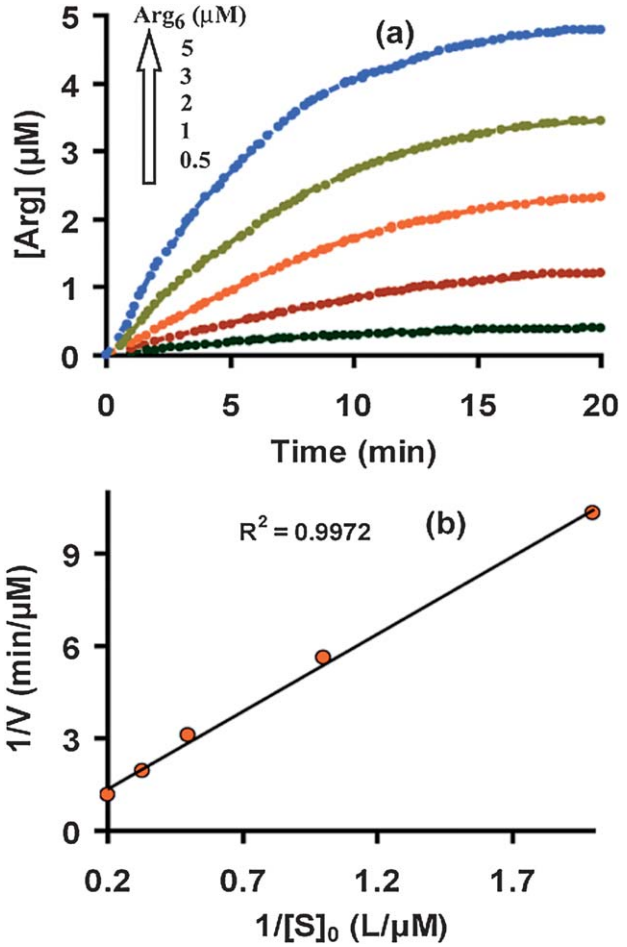

Fig. 5 (a) Derived concentration of the product [Arg] as a result of hydrolysis of various concentrations of Arg_6 as a function of time. (b) Lineweaver–Burk plot to calculate the values of K_m.

of Arg_6 peptide catalyzed by trypsin will be retarded in the presence of the corresponding inhibitor into the homogenous solution. Hence, we carefully examined the inhibition of trypsin activity with Bowman–Birk protease inhibitor (BBI) which is the most commonly used inhibitor of trypsin. To perform this experiment, six samples with the same concentration of P1/Arg_6 and trypsin (350 nM) were prepared followed by the addition of different concentrations of inhibitor from 0 to 0.250 μM in each of the six samples. One sample without inhibitor (0 nM) and other samples with different concentration of inhibitor were added to P1/Arg_6/[trypsin] and the fluorescence intensity changes were recorded at the emission intensity 411 nm as a function of time. Changes in the fluorescence pattern observed are depicted in Fig. 6.

As expected, in the absence of inhibitor (0 nM), the fluorescence intensity of P1/Arg_6/[trypsin] was seen to be increasing gradually with increasing hydrolysis time, however, in the presence of inhibitor, this fluorescence enhancement was retarded. Moreover, the higher the concentration of inhibitor in the solution, the slower was the increase in fluorescence intensity from P1. Inhibition efficiency of the inhibitor was calculated using the equation $(1 - I/I_o) \times 100\%$, where I_o and I are the restored fluorescence intensities in the absence and presence of inhibitor at 411 nm (Fig. 6).

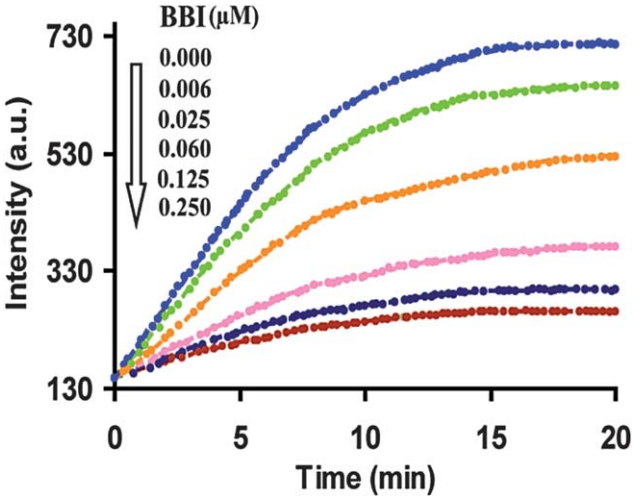

Fig. 6 The retardation of fluorescence intensity enhancement of P1 (0.4 μM)/Arg_6 (0–5 μM) in the presence of inhibitor BBI (0 to 0.250 μM) as a function of time in 2 mM PBS solution containing $[Ca^{2+}] = 10$ μM.

From the plot between inhibition efficiencies and inhibitor concentrations (Fig. 7) the IC_{50} value of the inhibitor was calculated and found as 0.0725 μM. This value indicates the high sensitivity of the anionic polymer P1 assay method towards screening the inhibitors of trypsin. From the above experiments, it was also confirmed that anionic P1 serves as a fluorescent turn-on probe for monitoring the enzymatic hydrolysis of trypsin and can also be used to screen the inhibition of the enzyme activity.

3 Experimental

3.1 Reagents and materials

All the reagents and solvents were purchased from Aldrich Chemicals (India), Merck (India) or Ranbaxy (India) and were used as received. Milli-Q water was used in all the experiments. Trypsin (porcine pancreas) and BBI were obtained from Sigma Aldrich.

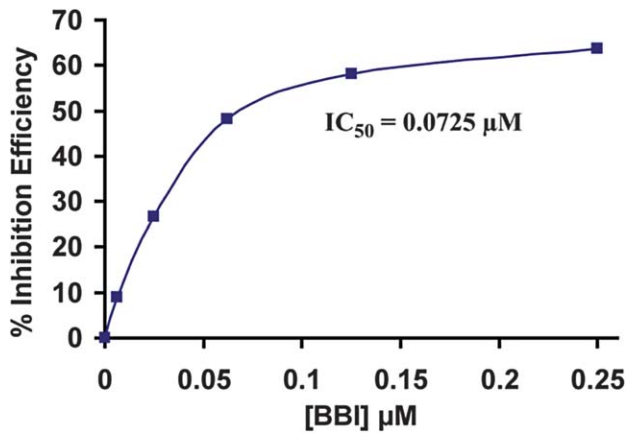

Fig. 7 Inhibition efficiencies were plotted against inhibitor concentrations to evaluate the IC_{50} value.

3.2 Instrumentation

Fluorescence spectra were recorded on a Varian Cary Eclipse Spectrometer. A 10 mm × 10 mm quartz cuvette was used for solution spectra and emission was collected at 90° relative to the excitation beam. Deionized water obtained using a Milli-Q system (Millipore) was used.

3.3 Fluorescence quenching experiments

The experiments were carried out in 2 mM phosphate buffer solution at pH 8.5. P1 (0.4 μM), Arg and Arg_6 peptide solutions were prepared in 2.0 mM PBS buffer solution. Arg and Arg_6 peptide solution (0–5 μM) were added into two separate solutions having P1 with the same concentration 0.4 μM and the fluorescence intensity changes were monitored at 411 nm.

3.4 Real time trypsin assay

Six samples containing P1/Arg_6 and 2.0 mM PBS buffer solution at pH 8.5 containing 10 μM of $CaCl_2$ were prepared followed by the addition of different concentrations of enzyme, such as 0 nM, 40 nM, 80 nM, 120 nM, 220 nM and 350 nM, and fluorescence intensity changes were monitored at the emission intensity 411 nm with increasing concentration of trypsin as a function of time.

3.5 Detection limit

P1 based assay was used to monitor the trypsin catalyzed polypeptide (Arg_6) hydrolysis as a function of time (up to 20 minutes) upon incubation with different concentrations of trypsin (0–95 nM). Limit of Detection (LOD) was calculated by using the following equation

$$\mathrm{LOD} = 3\sigma/K$$

where σ is the standard deviation and K is the slope of the linear plot between fluorescence intensity and trypsin concentration.

3.6 Calculation of product (Arg) concentration

Initially, unhydrolyzed Arg6 concentration was determined at different time intervals from the obtained fluorescence intensity curves by using the following equation:

$$[\mathrm{S}]_t = [\mathrm{S}]_0[(I_o/I_t - 1)/(I_o/I_q - 1)]$$

Product concentration was then calculated by using the following equation:

$$[\mathrm{Arg}] = [\mathrm{S}]_0 - [\mathrm{S}]_t$$

3.7 Calculation of kinetic parameters

Different initial concentrations of substrate (Arg_6) 0.5, 1, 2, 3 and 5 μM were chosen and fluorescence intensity changes were recorded as a function of time to calculate the kinetic parameters.

A Lineweaver–Burk plot was obtained by using the double reciprocal of initial rate *vs.* substrate concentration:

$$1/V = K_m/V_{max}[\mathrm{S}]_0 + 1/V_{max}$$

where V is the initial rate and calculated from the slopes of the plots, $[\mathrm{S}]_0$ is the initial substrate concentration, K_m is the Michaelis constant and V_{max} is the maximal velocity.

3.8 Real time trypsin assay in the presence of inhibitor

The experiment was carried out in 2 mM PBS buffer solution at pH 8.5. To perform this experiment, six samples with the same concentration of P1/Arg_6 and trypsin (350 nM) were prepared followed by the addition of different concentrations of inhibitor 0.00 μM, 0.006 μM, 0.025 μM, 0.060 μM, 0.125 μM and 0.25 μM in each of the six samples. Therefore one sample without inhibitor (0 μM) and other samples with different concentrations of inhibitor were added to P1/Arg/trypsin and the fluorescence intensity changes were recorded at the emission intensity 411 nm as a function of time. Changes in the fluorescence pattern were observed and represented as a function of time.

4 Conclusions

In summary, the interaction of anionic water soluble polymer P1 and Arg_6 under alkaline pH can serve as a fluorometric assay, as well as screen the inhibitors of enzyme trypsin, on the basis of light harvesting properties of the conjugated polymer *via* electrostatic interactions. The assay does not require any labeling on the substrate, thus reducing the cost of the method. It was also observed that during the enzymatic hydrolysis, the turned-on signal was visible, validating this CPE based method to monitor the enzymatic action *in vitro*. The fluorescence turn-on based assay system significantly enhances the detection sensitivity compared to turn-off assay systems by getting rid of erroneous signals. Anionic polyfluorene P1 in association with Arg_6 demonstrated the turn-on assay for the enzymatic activity of trypsin with a limit of detection for trypsin found to be 0.17 nM. This method offers a highly efficient homogenous assay system with advantages such as very low detection limit over a very wide pH range, a convenient mix and detect strategy, representing a simple and continuous approach for monitoring the enzymatic activity in the nanomolar range unlike traditionally applied methods that require fluorescent labels, tedious synthetic steps for preparing particular peptides, in addition to the use of sophisticated instruments, which are not only time-consuming but also add to the high cost. This authenticates the use of water soluble conjugated polymers as biosensors for monitoring enzyme activities.

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