# Efficient near infrared fluorescence detection of elastase enzyme using peptide-bound unsymmetrical squaraine dye


doi: info:doi/10.1016/j.bmcl.2017.07.057

# Efficient near infrared fluorescence detection of elastase enzyme using peptide-bound unsymmetrical squaraine dye

**Maryala Saikiran, Daisuke Sato, Shyam S. Pandey, Shuzi Hayase and Tamaki Kato**

Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu, Kitakyushu 808-0196, Japan

Email: msaikiran87@gmail.com, shyam@life.kyutech.ac.jp, tmkato@life.kyutech.ac.jp

**Abstract**

Extended wavelength analyte-responsive fluorescent probes are highly desired for the imaging applications owing to their deep tissue penetration, minimum interference from autofluorescence by biomolecules. Near infra-red (NIR) sensitive and self-quenching fluorescent probe based on the dye-peptide conjugate (SQ 1 PC) was designed and synthesized by facile and efficient one-pot synthetic route for the detection of Elastase activity. In the phosphate buffer solution, there was an efficient quenching of fluorescence of SQ 1 PC (86 %) assisted by pronounced dye-dye interaction due to H-aggregate formation. Efficient and fast recovery of this quenched fluorescence of SQ 1 PC (> 50 % in 30 seconds) was observed on hydrolysis of this peptide-dye conjugate by elastase enzyme. Presently designed NIR sensitive self-quenching substrate offers the potential application for the detection of diseases related to proteases by efficient and fast detection of their activities.

**Keywords**: Far-red squaraine dye, aggregation, fluorescent peptide, self-quenching conjugate, Elastase detection, bio imaging.

Growing demand for point-of-care testing (POCT) devices for home diagnostic and facile health care monitoring, biosensors have attracted tremendous attentions owing to utilization of small amount of biological samples, ease of handling and user-friendliness [1, 2]. Although, biosensors are one of the strong contenders for POCT devices, due to the existing challenges like utilization of single analyte at a time, controlling the sensitivity in the presence of interfering agents, prior analyte processing for higher sensitivity and low throughput are needed to be considered for the further development of more efficient and versatile POCT devices. It is, therefore, necessary to develop biosensors that are sensitive and having the capability of high throughput detection along with their compatibility with imaging techniques for simultaneous multi-target analysis [3]. The advent of Microarray technology has led the possibility of rapid profiling of huge number of proteins in a single experiment [4, 5]. Working with protein arrays for bio-sensing applications is challenging and cumbersome to control polar and ionic interactions, unspecific adsorption of proteins and preservation of native form of protein along with their spatial orientations onto the surface after immobilization [6]. This is the reason why more attentions are being paid for the development of peptide arrays for rapid and high throughput screening of complex protein functions including qualitative as well as quantitative estimation of proteases [7]. Proteases are an important class of physiological enzymes which hydrolyse the amide bond at specific sites of the polypeptide chain thereby playing a vital role in the regulation of a large number of physiological processes such as cell proliferation/differentiation, apoptosis, DNA replication, haemostasis and immune responses [8, 9]. Human Neutrophil Elastase (HNE) belongs to serine class of proteases and is a proteolytic enzyme stored in the azurophilic granules of polymorphonuclear cells [10]. Although the proteolytic destruction of bacteria is one of the major roles of this enzyme, its hyperactivity leads to the pathogenesis of acute and chronic inflammations [11, 12]. Excess release of HNE cleaves the cellular receptors, activates protogenetic mediators and intrinsically involved in the epithelial as well as well as endothelial membrane damage [13, 14]. Therefore, knowledge of roles played by different serine protease along with their strict control and monitoring especially HNE activity has a great importance for therapeutic viewpoints.

In spite of fast pace growth and development of protease assay methods, methods based on immunoassay consisted of specific binding to the target protease with antibodies have been although commercialized and able to estimate the proteases quantitatively [15]. However, they are not much

suitable for mapping protease activity and stages of disease which are associated with protease activity rather than their quantity. In order to avoid these issues of immunoassay-based detection of proteases, several other methods such as utilization of suitable peptide substrates based on optical (absorbance/fluorescence) detection and appearance or quenching of fluorescence emanating from Forster resonance energy transfer (FRET) have been widely used [16-18]. Interaction of proteases with these chromogenic or fluorogenic substrates leads to the selective proteolytic cleavage of the peptide bond resulting in changes of their absorption or fluorescent spectral behaviors which form the basis for the detection of protease activity. In this context, fluorogenic substrates are preferred over chromogenic substrates owing to their enhanced sensitivity and compatibility with the high throughput fluorescence mapping on microarray-based platforms [19]. A perusal of the fluorogenic substrates used for the protease assay reveals that the wide-spread use of fluorescent tags working mainly in the visible or low wavelength region. In this case, not only auto-fluorescence resulting from the biological analyte poses a limitation to attain good signal-to-noise (S/N) ratio but also photo-damage to biological species which cannot be avoided owing to the high energy excitations. In this context, utilization of fluorophores exhibiting emission in the near infra-red (NIR) wavelength region not only imparts much higher sensitivity owing to its highly diminished auto-fluorescence from the biological samples but also are capable of deep tissue penetration leading to efficient bio-imaging [20]. On the other hand, the FRET-based assay requires the logical development of suitable fluorophore and quencher pair owing to their synthetic complications due to the need of orthogonal protecting groups while their coupling with peptide substrate library.

The design of peptide substrates tagged with fluorescent moieties having the capability of self-quenching and re-appearance of the fluorescence after enzymatic hydrolysis has also been simplified for the solution of the relatively complicated fluorescence-peptide-quencher molecular system. This was demonstrated by designing the substrates after the incorporation of a multiple number of identical fluorophores leading to concentration-dependent quenching. Sato et al have demonstrated the concentration dependent fluorescence quenching for their fluorescein isothiocyanates (Fluorescent moiety) tagged peptide substrate for Trypsin enzyme and proteolytic cleave of the peptide bond led to the appearance of the fluorescence [21]. Internally quenched fluorescence-based fluorescent dendritic peptides substrates have also been reported which exhibit fluorescence ON sensing of chymotrypsin enzyme due to increased fluorescence emission after the enzymatic hydrolysis of the designed substrates [22]. Squaraine dye belongs to a class of intensely colored dyes having donor-acceptor-donor zwitterionic molecular framework containing squaric acid as central four-membered ring core surrounded by electron-rich aromatic moieties. Incorporation immensely available and judiciously selected aromatic/heterocyclic systems with varying donor strength it is easily possible to design a variety of squaraine dyes with tunable light absorption and fluorescence emission encompassing from visible to IR wavelength region [23]. In this present work, efforts have been directed to design an internally quenched homo-labelled fluorescent-peptide substrate utilizing a far-red sensitive unsymmetrical squaraine dye coupled with a peptide sequence susceptible to the pancreatic elastase enzyme. Figure 1 exhibits the structure of the main building blocks like direct carboxy ring-functionalized unsymmetrical squaraine dye **SQ-1** (1a), Elastase enzyme specific peptide sequence β-Ala-Ala-Pro-Ala-Lys-(OBzl) (1b) along with fluorescent peptide substrate (1c). Figure 1(d) represents the MM2 energy minimized three-dimensional structure of (1b) and led to the proposal of a possible structure of peptide-dye conjugate as shown in the Fig. 1(c).

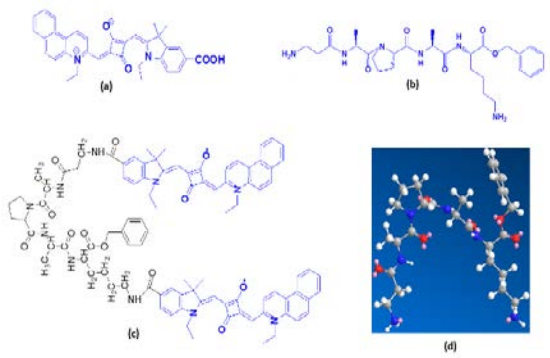
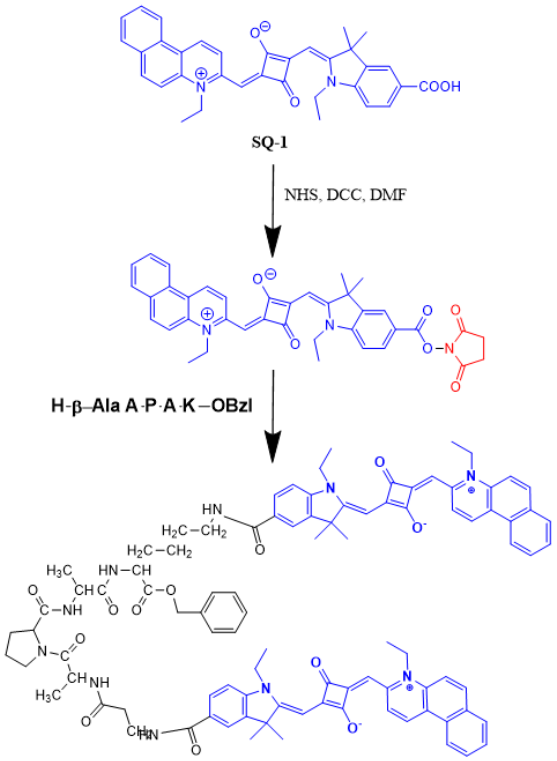


**Figure 1.** Chemical structure of unsymmetrical squaraine dye (a), elastase enzyme specific peptide (b), peptide-dye conjugate (c) and MM2 energy minimized 3D structure of the peptide (d).

It can be clearly seen from the structure of our newly proposed peptide-dye conjugate that two dye molecules are connected to one peptide at either end of the terminals. **SQ-1** was selected as model dye not only due to its NIR fluorescence capability but also an appreciably good interaction of squaraine dyes with most commonly used model proteins like bovine

serum albumin and human serum albumin [24, 25]. It has been reported that pancreatic serine protease elastase cleaves the substrate at peptide bonds on the carboxyl side of amino acid residues bearing small alkyl side chain which has led us to design Ala-Pro-Ala as target peptide for elastase enzyme [26]. β-Ala and Lys-containing free amino groups have not only been used as a spacer but also for assisting the easy binding of the **SQ-1** dye with its free –COOH group in a single one pot reaction. A perusal of this newly designed substrate as shown in Fig. 1(c) reveals dense packing of the SQ-1, which is expected to bring the internal self-quenching of the fluorescence. At the same time, the flat molecular structure of squaraine dyes is also expected to promote the dye aggregation especially in the buffer solution and aggregated dye molecules have been reported to exhibit the quenched fluorescence [27]. Internally fluorescence quenched peptide-dye conjugate was synthesized by one-pot reaction of NHS-ester activated unsymmetrical squaraine SQ-1 and peptide H-β-Ala-A-P-A-K-OBzl as per the scheme-1. Detailed synthesis and characterization of dye SQ-1, peptide and dye-peptide conjugate along with various intermediates have been provided in the supporting information.




**Scheme 1.** Synthetic strategy of dye-peptide conjugate used for present investigation.

Electronic absorption spectrum of SQ-1 in dimethylformamide (DMF) solution exhibits a strong electronic absorption maximum ($\lambda_{max}$) at 672 nm along with the feeble vibronic shoulder around 610 nm and molar extinction coefficient of 1.02 x $10^5$ $dm^3.mol^{-1}.cm^{-1}$ which is associated with π-π* electronic transition which is a typical characteristic of the squaraine dyes. The $\lambda_{max}$ absorption and emission values, molar extinction coefficient and stoke shifts of SQ 1 dye and SQ 1 PC in both DMF and PBS solutions were shown respectively in Table 1.

Merging of the vibronic shoulder with the main absorption for SQ-1 in DMF solution indicates the existence of dye aggregated species. It has been reported that in the case of H-aggregate formation by squaraine dye, sometimes shoulder becomes even more pronounced as compared to monomeric dye absorption peak. Prevention of this dye aggregation by using aggregation preventing species like chenodeoxycholic acid leads to decrease in the absorption corresponding to this shoulder verifying the suppression of dye aggregation [28]. On the other hand, its emission spectrum shows fluorescence emission peak at 754 nm with relatively large Stokes shift of 82 nm. Typically, squaraine dyes exhibit a Stokes shift of 20-30 nm due to its rigid molecular structure and indicate the nearly similar molecular configuration of dyes in both of the ground as well as excited states [29]. This large Stokes shift in the case of SQ-1 in DMF solution indicates rather diminished conformational stability of SQ-1 in an excited state as compared to typical squaraine dyes. On the other hand, the electronic absorption spectrum of a dye-peptide conjugate (SQ 1 PC) exhibits relatively broader optical absorption having a blue-shifted $\lambda_{max}$ at 664 nm and fluorescence emission maximum at 736 nm with a stoke shift of 72 nm as shown in the Fig. 2(b). This blue-shift in the absorption and emission maximum could be attributed to the dye aggregate formation. It has been reported that squaraine dyes form the blue-shifted H-aggregates and red-shifted J-aggregates depending on their structure, molecular environment owing to their relatively flat molecular structure [30].

**Table 1**. Spectral properties of dye and dye-conjugate in DMF and 0.1 M PBS solution at pH 7.4.

| | DMF Solution | | | | PBS Solution | | | |
|---|---|---|---|---|---|---|---|---|
| | $\lambda_{(max)}$ Abs | $\lambda_{(max)}$ Ems | Stoke Shift | (ε) ($dm^3$ $M^{-1}$ $cm^{-1}$) | $\lambda_{(max)}$ Abs | $\lambda_{(max)}$ Ems | Stoke Shift | (ε) ($dm^3$ $M^{-1}$ $cm^{-1}$) |
| **SQ 1 Dye** | 672 nm | 754 nm | 82 nm | $1.02\times10^5$ | 644 nm | 700 nm | 56 nm | $0.66\times10^5$ |
| **SQ 1 PC** | 664 nm | 736 nm | 72 nm | $3.7\times10^5$ | 605 nm | 658 nm | 53 nm | $0.98\times10^5$ |

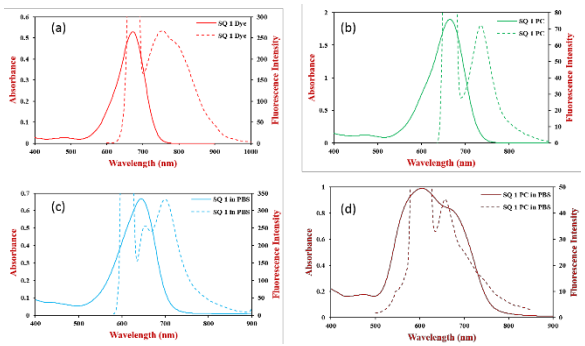



**Figure 2.** Electronic absorption and fluorescence emission spectra of (a) unsymmetrical squaraine dye SQ-1 and (b) peptide-dye conjugate (SQ 1 PC) in the DMF solution of concentration 5 μM. Figures (c) and (d) represents the same spectral profile for SQ-1 and SQ 1 (PC) in 0.1 M phosphate buffer at pH 7.4 and concentration of 10 μM, dotted line represents the emission spectra corresponding solutions in all of the cases.

It is interesting to note here that there is a drastic decrease in the fluorescence intensity associated with SQ-1 for the dye-peptide conjugate as compared to the pure dye SQ-1 having a similar concentration (5 μM) and the same solvent (DMF). This indicates that SQ-1 undergoes the fluorescence quenching upon its conjugation with peptide under investigation (SQ 1 PC). In general two basic mechanisms have been proposed for the fluorescence quenching. First is related to quenching due to fluorescence resonance energy transfer (FRET) while the second one is related to the concentration dependent static internal quenching. In the homo-dye labeled probes FERT based quenching generally occurs when stoke shift is very small allowing the sufficient overlapping between the absorption and emission spectra of the dye [31]. On the other hand, in the case of static quenching, there is a change in the spectral shape generally blue-shift in the absorption maximum facilitated by H-aggregate formation [32]. Although both of the mechanism seems to be applicable to explain the fluorescence quenching of SQ 1 PC considering the large stoke shift and small spectral overlap contribution from FRET-based quenching seem to be small. At the same time, blue shifted absorption and emission maxima which are highly pronounced in the case of phosphate buffer solution (PBS) as shown in the Fig. 2 (c, d) clearly corroborates the dominance of the second mechanism based on static concentration dependent quenching. Thus considering the peak fluorescence intensities of dye alone (SQ-1) and dye-peptide conjugate (SQ 1 PC), fluorescence quenching efficiency was estimated to be 73 % and 86 % in the DMF and PBS, respectively.

Fairly good internal self-quenching fluorescence of dye SQ-1 after coupling with target peptide was observed. This property of conjugate enabled us to explore the application of dye-peptide conjugate as enzyme activity detection based on fluorescence ON bio-sensing. As discussed earlier, Elastase enzyme recognition probe Ala-Pro-Ala amino acid residue was chemically bound with the free –COOH group of SQ-1 by simple one pot reaction. It can be seen from the structure of SQ 1 PC that two terminal amino acids β-Alanine and Lysine have been introduced to solve the two purposes. Firstly, they function as a spacer to promote the access of Elastase enzyme towards the target tripeptide Ala-Pro-Ala. Secondly, availability of free amines allows dense incorporation of SQ-1 dye promoting the internal self-quenching. To demonstrate this, 10 μM solution of this target dye-peptide conjugate SQ 1 PC was subjected to enzymatic hydrolysis using Elastase enzyme in 0.1M phosphate buffer at pH 7.4 and fluorescence emission spectra were measured at different time intervals as shown in the Fig. 3 (a). A perusal of this figure clearly reveals that there was a pronounced recovery of quenched fluorescence of the dye within 30 seconds and reached to saturation after 30 min leading to about fivefold enhancement in the fluorescence signal compared to that in the absence of Elastase. This enhancement in the fluorescence after enzymatic hydrolysis could be attributed to the fact that dye-aggregate formation is promoted after conjugation of SQ-1 with peptide in phosphate buffer leading to the H-aggregate assisted efficient fluorescence quenching. After the enzymatic hydrolysis of the peptide bond, multiple copies of dyes are released and attain the special separation leading to the re-appearance of the dye fluorescence.

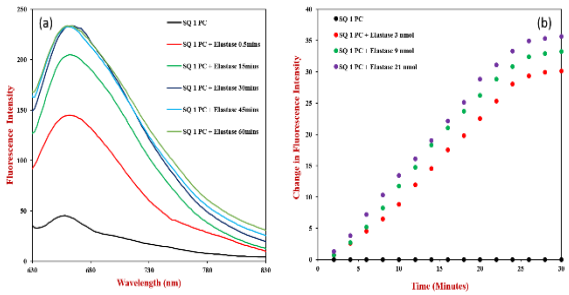



**Figure 3.** Fluorescence emission spectra of the 0.1M phosphate buffer solution of dye-peptide conjugate (10 μM) at pH 7.4 as a function of time after the addition of 21 nmol of elastase enzyme. Right hand side figure (b) represents the change in fluorescence intensity as a function of time with different concentrations elastase for a fixed concentration of dye-peptide conjugate (10 μM)

Considering the fluorescence intensities before (control) and after enzymatic hydrolysis of SQ 1 PC, quenching efficiency was estimated to be 81 %. This indicates such a simple and homo-labelled dye-peptide conjugate based on internal quenching is capable to efficiently detect the enzymatic activity based on fluorescence ON bio-sensing. Efforts were also directed to test this designed dye-peptide conjugate by changing the amount of Elastase enzyme (3 nmol to 21 nmol) for a fixed concentration of this dye-peptide conjugate (10 μM). Change in the fluorescence intensity as a function of time, enzymatic hydrolysis was monitored for different enzyme concentration and has been shown in the Fig. 3(b). A perusal of this figure also indicates that rate of fluorescence change as a function of concentration is not much significant. On the other hand, saturation fluoresecence intensity after complete hydrolysis exhibits clear and distinguishing change for different concentrations of the Elastase. Therefore, consideration of fluorescence intensity at saturation seems to be more plausible for the quantitative aspects of enzyme activity estimations using such dye-peptide conjugate by the fluorescence-ON biosensing. It can be seen that in all of the cases the fluorescence intensities were reaching saturation after about 25 min. of the enzymatic hydrolysis, while there was completely no change in the case of control that is in the absence of enzyme.

Overall we have demonstrated the NIR fluorescence quenching promoted by enhanced dye aggregation of dye-peptide conjugate and disruption of this aggregation in the presence of enzyme led to the facile detection of proteases based on fluorescence ON bio-sensing. Although detection of Elastase activity has been successfully demonstrated in this work, such design and concept can be easily implemented to any target protease by judicious selection of suitable central peptide sequence susceptible to the target enzyme under investigation. Use of single fluorophore and its one-pot coupling with target peptide sequence provide simplicity and rapid profiling of various proteases. Such a concept of fluorescence-ON biosensing based on aggregation-induced fluorescence quenching and re-appearance of fluorescence after enzyme hydrolysis bears good potential in medical diagnosis based on qualitative and quantitative estimation of enzymes. Integration of such dye-peptide conjugates with microarray technology is expected to tremendously enhance the throughput of analysis. Utilization of such dye-peptide conjugate for microarray application has been schematically shown in the Fig. 4. Amine functionalization of glass surface has been widely studied along with the report of very high density amine functionalization using 3-amino propyltriethoxysilane (APTS) by Bayer et. al [33]. It can be easily seen from the Fig. 4 (b) that free –COOH group in our proposed dye-peptide conjugate can be easily obtained after the O-benzyl group deprotection which is now ready to couple with free amine group of amine functionalized glass surface (a) making glass surface-bound dye-peptide conjugate (c). Thus we can attach various kinds of such molecular probes on a single glass surface in microarray format. At the same time, use of wavelength tunable squaraine dyes in such dye-peptide conjugate system not only imparts the widespread design of molecular probes but also provide the capability of efficient and simultaneous multi-target protease analysis facilitated by multi-wavelength fluorescence ON biosensing.

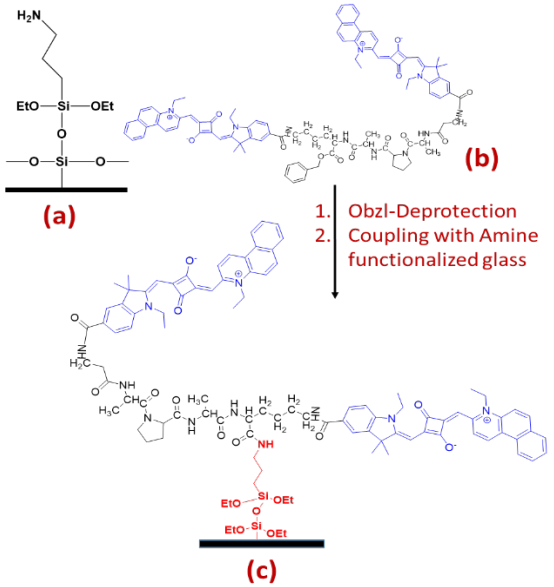



**Figure 4.** Schematic representation for application of proposed dye-peptide conjugate in peptide microarray for enzyme detection based on fluoresecence-ON biosensing. (a) Amine functionalized glass surface after treatment of glass

with APTS (b) dye-peptide conjugate and (c) glass-surface modified with dye-peptide conjugate.

**References:**


1. Mashamba-Thompson TP, Sartorius B, Drain PK. Point-of-care diagnostics for improving maternal health in South Africa. *Diagnostics.* 2016;6(3): 31.
2. Solanki PR, Kaushik A, Agrawal VV, Malhotra BD. Nanostructured metal oxide-based biosensors. *NPG Asia Materials.* 2011;3(1): 17-24.
3. Zeng Y, Wang L, Wu S-Y, et al. High-throughput imaging surface plasmon resonance biosensing based on an adaptive spectral-dip tracking scheme. *Optics Express.* 2016;24(25): 28303-28311.
4. Cretich M, Damin F, Pirri G, Chiari M. Protein and peptide arrays: recent trends and new directions. *Biomolecular engineering.* 2006;23(2): 77-88.
5. Foong YM, Fu J, Yao SQ, Uttamchandani M. Current advances in peptide and small molecule microarray technologies. *Current opinion in chemical biology.* 2012;16(1): 234-242.
6. Templin MF, Stoll D, Schrenk M, Traub PC, Vöhringer CF, Joos TO. Protein microarray technology. *Drug discovery today.* 2002;7(15): 815-822.
7. Legutki JB, Zhao Z-G, Greving M, Woodbury N, Johnston SA, Stafford P. Scalable high-density peptide arrays for comprehensive health monitoring. *Nature communications.* 2014;5.
8. Turk B. Targeting proteases: successes, failures and future prospects. *Nature reviews Drug discovery.* 2006;5(9): 785-799.
9. Rakash S, Rana F, Rafiq S, Masood A, Amin S. Role of proteases in cancer: A review. *Biotechnology and Molecular Biology Reviews.* 2012;7(4): 90-101.
10. Korkmaz B, Moreau T, Gauthier F. Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. *Biochimie.* 2008;90(2): 227-242.
11. Avlonitis N, Debunne M, Aslam T, et al. Highly specific, multi-branched fluorescent reporters for analysis of human neutrophil elastase. *Organic & biomolecular chemistry.* 2013;11(26): 4414-4418.
12. Doring G. The role of neutrophil elastase in chronic inflammation. *American journal of respiratory and critical care medicine.* 1994;150(6): S114.
13. Shapiro SD. Neutrophil Elastase: Path Clearer, Path ogen Killer, or Just Path ologic? *American journal of respiratory cell and molecular biology.* 2002;26(3): 266-268.
14. Abe T, Usui A, Oshima H, Akita T, Ueda Y. A pilot randomized study of the neutrophil elastase inhibitor, Sivelestat, in patients undergoing cardiac surgery. *Interactive cardiovascular and thoracic surgery.* 2009;9(2): 236-240.
15. Lee Y-m, Jeong Y, Kang HJ, Chung SJ, Chung BH. Cascade enzyme-linked immunosorbent assay (CELISA). *Biosensors and Bioelectronics.* 2009;25(2): 332-337.
16. Lesner A, Brzozowski K, Kupryszewski G, Rolka K. Design, chemical synthesis and kinetic studies of trypsin chromogenic substrates based on the proteinase binding loop of Cucurbita maxima trypsin inhibitor (CMTI-III). *Biochemical and biophysical research communications.* 2000;269(1): 81-84.
17. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proceedings of the National Academy of Sciences.* 2000;97(14): 7754-7759.
18. Sun H, Panicker RC, Yao SQ. Activity based fingerprinting of proteases using FRET peptides. *Peptide Science.* 2007;88(2): 141-149.
19. Tomizaki Ky, Usui K, Mihara H. Protein-Detecting Microarrays: Current Accomplishments and Requirements. *ChemBioChem.* 2005;6(5): 782-799.
20. Gong Y-J, Zhang X-B, Mao G-J, et al. A unique approach toward near-infrared fluorescent probes for bioimaging with remarkably enhanced contrast. *Chemical Science.* 2016;7(3): 2275-2285.
21. Sato D, Kato T. Novel fluorescent substrates for detection of trypsin activity and inhibitor screening by self-quenching. *Bioorganic & Medicinal Chemistry Letters.* 2016;26(23): 5736-5740.
22. Ellard JM, Zollitsch T, Cummins WJ, Hamilton AL, Bradley M. Fluorescence enhancement through enzymatic cleavage of internally quenched dendritic peptides: A sensitive assay for the AspN endoproteinase. *Angewandte Chemie International Edition.* 2002;41(17): 3233-3236.
23. Pandey SS, Watanabe R, Fujikawa N, et al. Effect of extended π-conjugation on photovoltaic performance of dye sensitized solar cells based on unsymmetrical

squaraine dyes. *Tetrahedron.* 2013;69(12): 2633-2639.
24. Sai kiran M, Pandey SS, Hayase S, Kato T. Photophysical characterization and BSA interaction of direct ring carboxy functionalized symmetrical squaraine dyes. *Journal of Physics: Conference Series. (In Press)*
25. Jisha VS, Arun KT, Hariharan M, Ramaiah D. Site-selective interactions: squaraine dye− serum albumin complexes with enhanced fluorescence and triplet yields. *The Journal of Physical Chemistry B.* 2010;114(17): 5912-5919.
26. Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. *Analytical chemistry.* 2010;82(19): 8251-8258.
27. Galande AK, Hilderbrand SA, Weissleder R, Tung C-H. Enzyme-targeted fluorescent imaging probes on a multiple antigenic peptide core. *Journal of medicinal chemistry.* 2006;49(15): 4715-4720.
28. Yum J, Moon S, Humphry-Baker R, et al. Effect of coadsorbent on the photovoltaic performance of squaraine sensitized nanocrystalline solar cells. *Nanotechnology.* 2008;19(42): 424005.
29. Marchena MaJ, de Miguel G, Cohen B, et al. Real-time photodynamics of squaraine-based dye-sensitized solar cells with iodide and cobalt electrolytes. *The Journal of Physical Chemistry C.* 2013;117(23): 11906-11919.
30. Hu L, Yan Z, Xu H. Advances in synthesis and application of near-infrared absorbing squaraine dyes. *RSC Advances.* 2013;3(21): 7667-7676.
31. Ternon M, Díaz-Mochón JJ, Belsom A, Bradley M. Dendrimers and combinatorial chemistry—tools for fluorescent enhancement in protease assays. *Tetrahedron.* 2004;60(39): 8721-8728.
32. Packard BZ, Toptygin DD, Komoriya A, Brand L. [2] Design of profluorescent protease substrates guided by exciton theory. *Methods in enzymology.* 1997;278: 15-23.
33. Beyer M, Felgenhauer T, Ralf Bischoff F, et al (2006) A novel glass slide-based peptide array support with high functionality resisting non-specific protein adsorption. Biomaterials 27:3505–3514. doi: 10.1016/j.biomaterials.2006.01.046