RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

COMMUNICATION

A molecular peptide beacon for IgG detection

Cite this: DOI: 10.1039/x0xx00000x

M. Okochi[a,b*], T. Sugita[a], M. Tanaka[b], and H. Honda[a]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

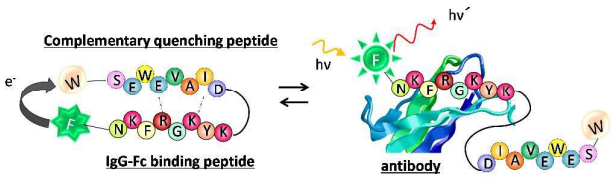
www.rsc.org/

A molecular peptide beacon was designed for fluorescence detection of IgG in a homogeneous assay. pH-triggered detection of IgG was demonstrated using a fluorophore-labeled peptide that incorporated a binding site in the Fc region of IgG with a complementary quenching site.

The development of simple, rapid, sensitive, and inexpensive bioassays is of great importance in biomedical fields and diagnostics. Conventional immunoassays, such as enzyme-linked immunosorbent assays (ELISA), are often performed in the solid phase and require relatively long reaction times and sequential washing steps. Homogeneous assays that can detect target proteins directly in solution (i.e., in a single step) have thus been proposed as a powerful tool.

Fluorescence-based assays, such as fluorescence polarization and fluorescence energy transfer, do not require separation steps and offer advantages of short assay times and ease of handling.[1] A number of fluorescence-based homogeneous detection sensors have been developed.[2] Peptide beacons conjugated with fluorophores and acceptor/quencher dyes at their N- and C-termini, respectively, have also attracted increasing attention for use in homogeneous assays.[3] Wei *et al.* developed a peptide beacon for an antigenic peptide against human chorionic gonadotropin (hCG) antibodies that is labeled with fluorescein and tetramethylrhodamine at its N- and C-termini, respectively.[4] In the absence of a target protein, the peptide beacon forms a closed structure and interactions between the fluorescent dyes on the peptide beacon result in fluorescence quenching. In the presence of a target protein, binding of the target to the peptide results in dissociation of the intramolecular interactions between fluorescent dyes, resulting in an increase in fluorescence. In a similar approach, peptide beacons labeled with various fluorophores have been developed for targeting various antibodies and DNA.[5] Oh *et al.* developed anti-HIV p17 antibody detection systems using a hexamer peptide beacon conjugated with pyrene or ruthenium (II) bisbipyridine-phenanthroline.[5a,b] Wu *et al.* detected DNA using a DNA-binding peptide beacon conjugated with pyrene.[6] These recently developed peptide beacons show target specific recognition, but require uncommon dyes that form duplexes (e.g., pyrene) or long-lived fluorophores (e.g., ruthenium (II) bisbipyridine-phenanthroline). As a result, the stabilities of the closed structures of such peptide beacons have been relatively low. The incorporation of peptide nucleic acids (PNAs) has also been proposed to lead to the formation of stable closed structures.[7] However, these molecular beacons are complex and their synthesis is time-consuming. Moreover, nonspecific protein binding could be induced by hydrophobic interactions with PNA. Therefore, a more simple method for the construction and design of peptide beacons is required.

In this study, a peptide beacon was designed by connecting a fluorophore (Atto655)-labeled target recognition peptide and a complementary peptide that conjugates tryptophan residues. This design forms stable closed structures and induces efficient quenching since tryptophan can serve as an efficient electron donor in photo-induced electron transfer (PeT) reactions with Atto655 (Scheme 1).[8] IgG was selected as the model protein since monoclonal antibodies have attracted attention in many therapeutic and diagnostic applications. The high demand for monoclonal antibodies has led to the development of the cost-effective manufacturing processes. In association with progress in cell culture technology, the selection of most suitable clones producing high levels of IgG from a mixture of hybridoma cells is one of a critical



Scheme 1 Schematic image of IgG detection using the designed peptide beacon.

step to rationalize the monoclonal IgG discovery processes.[9] For detection of IgG, the NKFRGKYK peptide that was previously designed to recognize the Fc region of IgG (IgG-Fc) from IgG Fcγ receptors for IgG purification[10] was used. The complementary DIAVEWES peptide was also designed from IgG-Fc and conjugated with tryptophan residues. IgG detection was investigated using two peptide probes.[11] However, since the dissociation constant between Atto655-labeled NKFRGKYK and WWWG-DIAVEWES was low (1.2×10^{-5} M), a high concentration of quencher peptide was required to quench the Atto655-labeled target recognition peptide. This communication reports the synthesis of a linear peptide beacon that conjugates Atto655-labeled NKFRGKYK and DIAVEWES with tryptophan residues for IgG detection in a homogeneous assay. To the best of our knowledge, our peptide beacon designed with a fluorophore-labeled recognition peptide and its complementary quencher peptide is the first successful design of a protein detection system without using allosteric modulation of enzyme activity.

We optimized the number of tryptophan residues in the peptide beacon by observation of the fluorescence-quenching response of Atto655. The complementary DIAVEWES peptide was synthesized on a cellulose support using the spot-synthesis technique,[12] according to a previously described method,[10,11] by inserting a glycine residue as a spacer between DIAVEWES and the tryptophan residues. Fluorescence spectra of Atto655-labeled NKFRGKYK were measured using a fluorescence plate reader following 60 min incubation with the designed quenching peptide (tryptophan-conjugated DIAVEWES). As the number of introduced tryptophan residues increased, the fluorescence of Atto655-labeled NKFRGKYK decreased (Fig. 1a). The average standard deviation of three peptide disks was 0.026. In contrast, only a small decrease in fluorescence was observed when octameric alanine was used as a control (no interaction) peptide (Fig. 1b). Since non-specific decreases in fluorescence (~20% decrease with octameric alanine) were observed when introducing the pentameric tryptophan, the DIAVEWES-GWWWW was used in subsequent experiments.

A molecular peptide beacon conjugating Atto655-labeled NKFRGKYK and DIAVEWES-GWWWW with a (GGS)2 linker was synthesized. To confirm the quenching effect in the absence of target, the fluorescence spectrum was compared to that with no quenching site. The peptides were dissolved in PBS (10 μM, pH 7.4) with 10% dimethylsulfoxide (DMSO) and further diluted to 0.10 μM with PBS. The fluorescence spectrum of the peptide beacon significantly decreased, suggesting that tryptophan residues are present in the proximity of Atto655 near the interaction of NKFRGKYK and DIAVEWES and allow for effective PeT quenching (Fig. 2a). The structure of the peptide beacon was predicted using PEP-FOLD, an online resource for de novo peptide structure prediction (http://bioserv.rpbs.univ-paris-diderot.fr/services /PEP-FOLD/). We assumed that the peptide beacon formed a closed stem-loop structure (Fig. 2b).

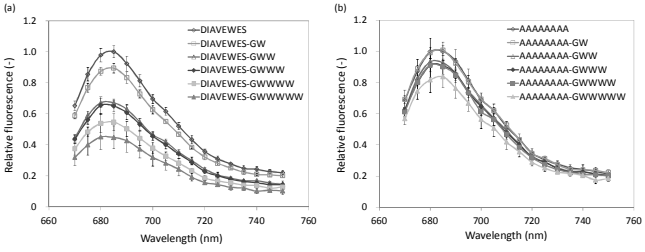


Fig. 1 Quenching effect of tryptophan-conjugated complementary peptides on a peptide array: DIAVEWES (a) and AAAAAAAA (b). Fluorescence spectra of Atto655-labeled NKFRGKYK were measured after incubation for 60 min.

For detection of target proteins using the peptide beacon, intramolecular interactions between the target protein recognition site (NKFRGKYK) and its complementary site (DIAVEWES) in the peptide beacon need to be decreased to allow an open structure for target binding. The fluorescent spectra of the peptide beacon dissolved in PBS with 0-0.2% Tween 20 (T-PBS) were investigated at various pH values: 2.0, 4.0, 6.0, and 7.4. Increases in fluorescence spectra were obviously observed when the peptide was dissolved with Tween 20 under acidic conditions (Fig. 3). The fluorescence of the peptide beacon at pH 2.0 in 0.2% T-PBS (Fig. 3d) was almost equivalent to the fluorescence of the peptide without a quenching site (Fig. 2a). These results indicate that the peptide beacon undergoes a conformational change to an open structure under acidic conditions in the presence of the surfactant Tween 20. The IgG-Fc binding peptide, NKFRGKYK, contains total of four positively charged residues (lysine (K) and arginine (R)). The complementary peptide sequence, DIAVEWES, contains total of three negatively charged residues (aspartic acid (D) and glutamic acid (E)).

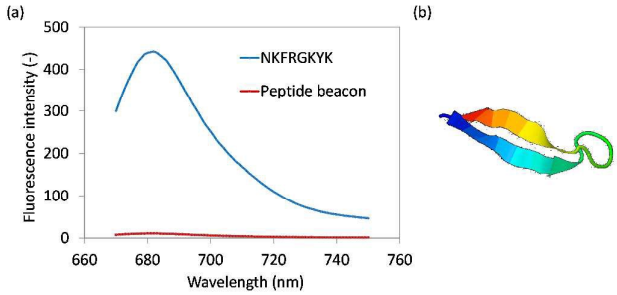


Fig. 2 (a) Fluorescence spectra of the peptide beacon, Atto655-NKFRGKYK-GGSGGS-DIAVEWES-GWWWW (red line), and Atto655-labeled NKFRGKYK (blue line). (b) Predicted structure of the peptide beacon using PEP-FOLD.

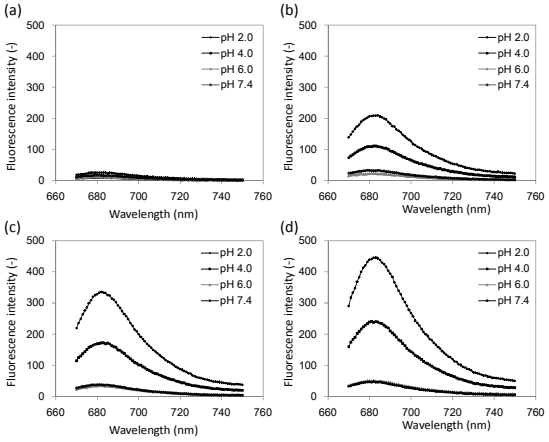


Fig. 3 Fluorescence spectra of the peptide beacon at various Tween 20 concentrations and pH values. The peptide beacon solution (10 μM) was mixed with 0% (a), 0.05% (b), 0.10% (c), and 0.20% (d) T-PBS at various pH values (2.0, 4.0, 6.0, and 7.4) with a volume ratio of 1:99. The final peptide concentration was 0.10 μM.

Electrostatic interactions between the positively charged residues in the binding peptide (NKFRGKYK) and the negatively charged complementary sequence (DIAVEWES) contribute to the formation of the stable closed structure of the peptide beacon. Since an increase in fluorescence intensity was observed when Tween 20 was added under acidic conditions, it is likely that hydrophobic interactions additionally contribute to the formation of the closed structure of the peptide beacon.

Detection of IgG was investigated using the newly designed peptide beacon. The peptide beacon was diluted and mixed with IgG at pH 3.0. After incubating the peptide beacon with IgG, the mixture was neutralized by adding 1.0 M Tris buffer (pH 9.0) to promote the formation of the closed structure for fluorescence measurements. Fluorescence intensities after neutralization were measured for various IgG concentrations (Fig. 4). The fluorescence intensity of the peptide beacon without neutralization showed a maximum intensity above 400 AU. An increase in the fluorescence intensity of the peptide beacon upon the addition of IgG was observed in a dose-dependent manner after neutralization (Fig. 4a). We also measured the fluorescence intensity of the peptide beacon at 684 nm under a range of IgG and bovine serum albumin (BSA) concentrations (Fig. 4b). In contrast to the findings for IgG, little change in the fluorescence intensity of the peptide beacon was observed upon the addition of BSA at the same concentrations. A difference in the fluorescence intensity of the peptide beacon between IgG and BSA was observed above concentrations as little as 50 nM (Fig. 4b). The coefficient of variation (= standard deviation/average) was 8.4% at 50 nM IgG and decreased to below 2% at IgG concentrations higher than 250 nM, indicating reliable IgG detection was possible using the peptide beacon. The NKFRGKYK peptide specifically binds to IgG since it was previously designed for affinity purification of antibody, i.e. the host cell protein content of the purified chromatographic fractions using the peptide resin was 1.554 μg/mg-monoclonal antibody while it was 33.516 μg/mg-monoclonal antibody with the loaded culture supernatant solution.[10] These results lead to the conclusion that the peptide beacon specifically detected IgG in the range from 50 to 7500 nM and its fluorescence increased approximately 25-fold upon target binding.

The time course of fluorescence intensity for IgG detection was also investigated. The peptide beacon (100 nM) and IgG (1.0 μM) were incubated at pH 3.0 for various incubation periods before neutralization with 1.0 M Tris buffer (5 μl) at pH 9.0. Relative fluorescence intensity increased with increase in durations of the interaction time between IgG and the peptide beacon until saturation at 60 min (Fig. S1). Therefore, these data suggest that the designed peptide beacon enables specific detection of IgG (detection limit, 50 nM) after a 60 min incubation period.

Homogeneous assays for antibody detection are attractive for selection of hybridoma cells in monoclonal antibody development processes as well as point-of-care applications, where heterogeneous assays are either time consuming or expensive. Peptides consist of amino acids with molecular weights ranging from 1,000 to 10,000 daltons and can interact with proteins to regulate biological functions. A number of peptide probes for bioimaging and diagnosis have been developed by peptide libraries, such as those for phage display (or cell surface display), combinatorial split synthesis, and bead display.[13] We have used a spot-synthesized peptide array to functionally screen and design peptide probes.[14]

Peptide beacons have been constructed from target-binding peptides conjugated with fluorescent dyes and their quencher molecules in previous studies. The intramolecular interaction between a fluorescent dye and its quencher (the closed structure) is often observed to have low stability.[5] Peptide beacons forming closed structures via DNA complementation have been proposed to overcome these problems.[7] In this study, we constructed a new peptide beacon with both a target binding peptide site and its complementary peptide site. When the peptide beacon interacts with the target IgG, an open structure is formed and displays a fluorescence signal. As previously described, the binding constant of the IgG recognition (NKFRGKYK) peptide and IgG was 8.9×10^6 M^{-1}.[10] Compared to the binding constant between IgG and protein G

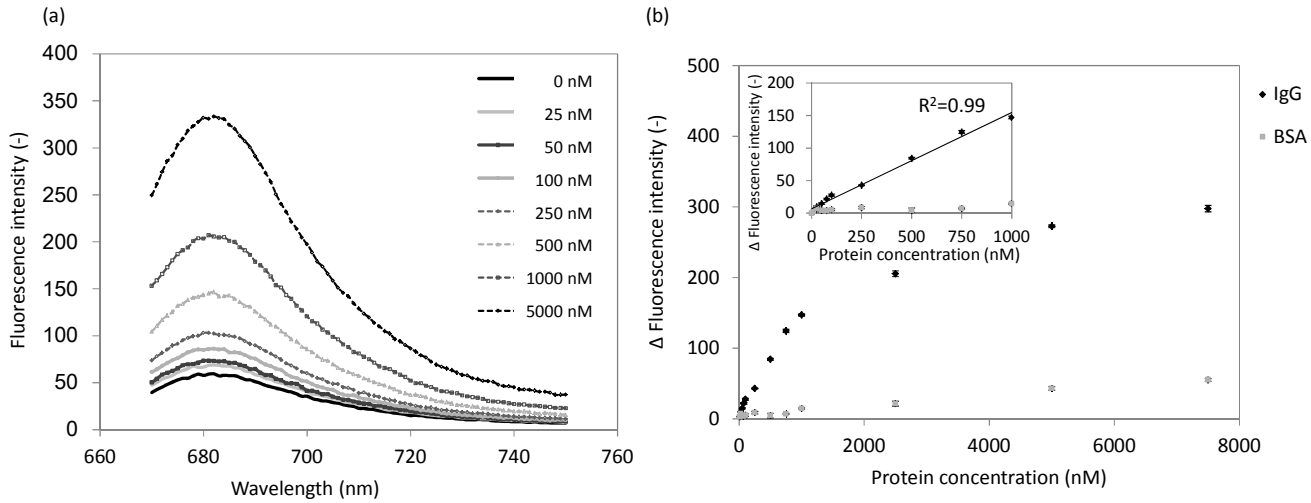


Fig. 4 pH-triggered IgG detection using the peptide beacon. (a) Fluorescent spectra of the peptide beacon at various IgG concentrations. (b) Effect of IgG (dark gray diamonds) and BSA (light gray squares) concentration on the relative fluorescence intensity of the peptide beacon. The fluorescence intensity of the peptide beacon without IgG or BSA was subtracted.

(in the order of 10^8 M^{-1}), further improvement in the peptide beacon design would be necessary. Future study is necessary to detect IgG under higher pH conditions by modulation of the complementary peptide site. Furthermore, since we screened linear epitopes of IgE antibodies in the sera of patients with allergies using peptide arrays,[15] IgG detection by its specific epitopes might be possible in future studies. The design of the peptide beacon developed in this study can be widely applied to various target proteins by screening the recognition peptides and their complementary peptides using the spot-synthesized peptide array for homogeneous assays.

In conclusion, we designed a peptide beacon conjugated with Atto655-labeled NKFRGKYK and DIAVEWES-GWWWW via a (GGS)2 linker. The peptide beacon formed an open structure for target IgG binding under acidic conditions (pH 3.0) and displayed a fluorescent signal after neutralization. In the absence of IgG, the peptide beacon formed a closed stem-loop-like structure due to the interaction between NKFRGKYK (target-recognition site) and DIAVEWES (complementary site). The Atto655 fluorescence signal was quenched by the tryptophan residues. IgG-specific detection was observed in the range of 50–7500 nM. Our concept of a peptide beacon was found to be useful for protein detection. The spot-synthesized peptide array would be effective for designing and screening a recognition peptide and its complementary peptide, as well for as trial experiments to optimize detection conditions. This study represents a novel development in peptide beacon-based protein detection. Selective fluorescence quenching by tryptophan residues has great potential for the development of a peptide beacon for homogeneous assays in biomedical fields and diagnosis.

Acknowledgements

This work was partially supported by the Funding Program for Next Generation World-Leading Researchers (Next Program, LR016), a Grant-in-Aid for Scientific Research from Ministry of Education, Sports, Science and Technology, Japan, No. 15H04192, and by the ImPACT Program of the Council for Science, Technology and Innovation (Cabinet Office, Government of Japan).

Notes and references

[a] *Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8603, Japan.*

[b] *Department of Chemical Engineering, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1-S1-24 O-okayama, Meguro-ku, Tokyo 152-8552, Japan. *Email: okochi@chemeng.titech.ac.jp*

† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

1 (a) D.M. Jameson and J.A. Ross, *Chem. Rev.*, 2010, **110**, 2685; (b) C. Fan, K.W. Plaxco and A.J. Hegger, *Trends Biotechnol.*, 2005, **23**, 186.

2 (a) R. Abe, H. Ohashi, I. Iijima, M. Ihara, T. Hohsaka and H. Ueda, *J. Am. Chem. Soc*., 2011. **133**, 17386; (b) M.V Golynskiy, W.F. Rurup and M. Merkx, *ChemBioChem,* 2010, **11**, 2264; (c) T. Kreisig, R. Hoffmann and T. Zuchner, *Anal. Chem.*, 2011, **83**, 4281.

3 (a) S. Banala, R. Arts, S. Aper and M. Merkx, *Org. Biomol. Chem.*, 2013, **11**, 7642; (b) M.K. Johansson and R.M. Cook, *Chem. Eur. J.*, 2003, **9**, 3466; (c) K.J. Oh, K.J. Cash and K.W. Plaxco, *Chem. Eur. J.*, 2009, **15**, 2244; (d) N. Marme and J.P. Knemeyer, *Anal. Bioanal. Chem.*, 2007, **388**, 1075; (e) E. Pazos, O. Vazquez, J.L. Mascarenas and M.E. Vazquez, *Chem. Soc. Rev.*, 2009, **38**, 3348.

4 A.P. Wei, D.K. Blumenthai and J.N. Herron, *Anal. Chem.*, 1994, **66**, 1500.

5 (a) K.J. Oh, K.J. Cash and K.W. Plaxco, *J. Am. Chem. Soc.*, 2006, **128**, 14018; (b); K.J. Oh, K.J. Cash, V. Hugenberg and K.W. Plaxco, *Bioconjugate Chem.*, 2007, **18**, 607; (c) H. Neuweiler, A. Schulz, A.C. Vaiana, J.C. Smith, S. Kaul, J. Wolfrum and M. Sauer, *Angew. Chem. Int. Ed.*, 2002, **41**, 4769; (d) K.E. Sapsford, J.B.Blanco-Canosa, P.E. Dawson and I.L. Medintz, *Bioconjugate Chem.*, 2010, **21**, 393; (e) J.C. Wu, Y. Zou, C.Y. Li, W. Sicking, I. Piantanida, T. Yi and C. Schmuck, *J. Am. Chem. Soc.*, 2012, **134**, 1958.

6 J.C. Wu, Y. Zou, C.Y. Li, W. Sicking, I. Piantanida, T. Yi and C. Schmuck, *J. Am. Chem. Soc.*, 2012, **134**, 1958.

7 (a) K.J. Oh, K.J. Cash, A.A. Lubin and K.W. Plaxco, *Chem. Commun.*, 2007, **46**, 4869; (b) S. Thurley, L.Roglin and O. Seitz, *J. Am. Chem. Soc.*, 2007, **129**, 12693.

8 (a) N. Marme, J.P. Knemeyer, M. Sauer and J. Wolfrum, *Bioconjugate Chem*., 2003, **14**, 1133; (b) S. Doose, H. Neuweiler and M. Sauer, *ChemPhysChem*, 2009, **10**, 1389; (c) R. Abe, H. Ohashi, I. Iijima, M. Ihara, T. Hohsaka and H. Ueda, *J. Am. Chem. Soc*., 2011. **133**, 17386.

9 S.Farid, J. Chromatogr. B., 2007. **848**, 8.

10 T. Sugita, M. Katayama, M. Okochi, R. Kato, T. Ichihara and H. Honda, *Biochem. Eng. J.*, 2013, **79**, 33.

11 T. Sugita, M. Okochi and H. Honda, *Chem. Lett.*, 2014, **43**, 550.

12 R. Frank, *J Immunol. Methods.*, 2002, **267**, 13.

13 (a) G.P. Smith, *Science*, 1985, **228**, 1315. 34; (b) K.S. Lam, S.E. Salmon, E.M. Hersh, V.J. Hruby, W.M. Kazmierski and R.J. Knapp, *Nature*, 1991, **354**, 82; (c) M. Lebl, V. Krchnak, N.F. Sepetov, B. Seligmann, P. Strop, S. Felder and K.S. Lam, *Biopolymers*, 1995, **37**, 177.

14 (a) M. Okochi, H. Hayashi, A. Ito, R. Kato, Y. Tamura, N. Sato and H. Honda, *J. Biosci. Bioeng*., 2008, **105**, 198; (b) M. Okochi, S. Nomura, C. Kaga and H. Honda, *Biochem. Biophys. Res. Commun.*, 2008, **371**, 85; (c) M. Okochi, M. Ogawa, C. Kaga, T. Sugita, Y. Tomita, R. Kato and H. Honda, *Acta Biomater.*, 2010, **6**, 2301; (d) M. Kuboyama, R. Kato, M. Okochi and H. Honda, *Biochem. Eng. J.*, 2012, **66**, 73; (e) M. Okochi, M. Kuboyama, M. Tanaka and H. Honda, *Talanta*, 2015, **142**, 235; (f) C. Kaga, M. Okochi, Y. Tomita, R. Kato and H. Honda, *BioTechniques*, 2008, **44**, 393. 74; (g) T. Ochiai, T. Sugita, R. Kato, M. Okochi and H. Honda, *Biosci. Biotechnol. Biochem.*, 2012, **76**, 819.

15 (a) N. Matsumoto, M. Okochi, M. Matsushima, A. Ogawa, T. Takase, Y. Yoshida, M. Kawase, K. Isobe, T. Kawabe and H. Honda, J. Biosci. Bioeng., 2009, **107**, 324; (b) N. Matsumoto, M. Okochi, M. Matsushima, R. Kato, T. Takase, Y. Yoshida, M. Kawase, K. Isobe, T. Kawabe and H. Honda, *Peptides*, 2009, **30**, 1840. (c) H. Sugiura, N. Okazaki, T. Sugiura, H. Honda and M. Okochi, *Biochem. Eng. J.*, 2014, 87, 8.

