RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

RSCPublishing

COMMUNICATION

Direct Observation of Ligand-Induced Receptor Dimerization with a Bioresponsive Hydrogel

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Jongseong Kim[a,*], Yongdoo Park[b], Ashley C. Brown[c], L. Andrew Lyon [d]

Multimerization of biomolecules is essential for biological function and thus there is a need for sensitive biochemical assays that determine whether a molecule associates with one or more other molecules in the context of biological function. In this contribution we demonstrate a simple yet versatile method for the identification of physiologically important receptor dimerization events induced by a ligand. Bioresponsive hydrogel microparticles (microgels) conjugated with a receptor, Glycoprotein Ibα (GPIbα), display large changes in optical (microscopic) appearance under conditions known for to promote thrombin-induced GPIbα dimerization. In support of x-ray crystal structures, we identify that one thrombin molecule associates with two GPIbα moieties, which may play a role in efficient hemostatic function by increasing local concentration of GPIbα on platelet surfaces. This microgel assay could provide a new way of studying important physiological and pathological mechanisms related to receptor dimerization and/or clustering.

Characterization of how receptors interact with each other and with their native ligands is a long-standing goal of biochemical assay development.[1-4] Receptor multimerization (or clustering) that occurs independent of or is induced by ligand binding acts as a crucial step not only for normal physiological function,[5] but also in the progression of pathological infections such as those caused by human immunodeficiency virus (HIV),[6,7] and influenza virus[8,9]. However, technology for directly identifying such clustering is limited by the capability of distinguishing multivalent (two or more receptors with one ligand) interactions from monovalent (one receptor with one ligand) interactions. One common technique is isothermal titration calorimetry (ITC) that quantifies thermodynamic parameters of molecular interactions in solution. However, this specialized instrumental approach can be technically challenging and consumes relatively large amounts of purified protein. Here we show a simple but unique microgel-based assay for the characterization of ligand-induced receptor dimerization in real time.

Thrombin is an enzyme that mediates the cleavage of fibrinopeptides from fibrinogen, thereby initiating the assembly of fibrinogen into fibrin. This is an integral component of both hemostasis and thrombosis. In addition, thrombin binds to platelet glycoprotein Ibα (GPIbα) with both high and intermediate affinity sites.[12] X-ray crystal structures of the thrombin-GPIbα complex showed that both molecules have two distinct binding sites for its counterpart, which suggests multivalent binding of the two proteins simultaneously (Figure 1a).[10,11] GPIbα is a membrane protein of platelets and has an important role in blood coagulation at the site of vascular injury through its binding to von Willebrand factor (vWF) A1 domain. For the last few decades, the functional significance of thrombin binding to GPIbα has remained controversial, with some question as to whether it is prothrombotic or antithrombotic. Nonetheless, both hypotheses suggest that α-thrombin induces dimerization of platelet GPIbα by cooperating with the exocite I and II.[10,11]

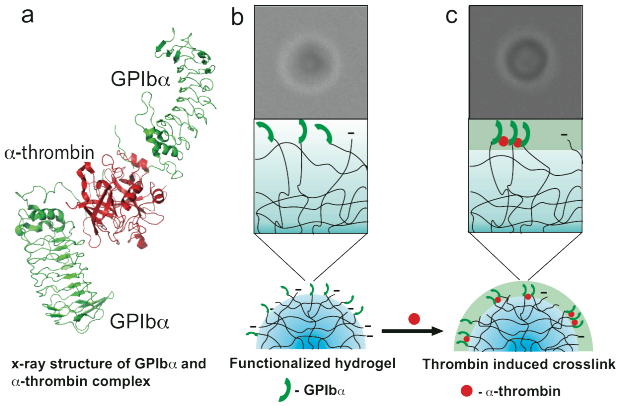

Figure 1. Schematic of ligand-induced receptor dimerization assay using microgels. (a) x-ray structure of α-thrombin-GPIbα complex showing multivalent binding.[10,11] (b) conjugation of GPIbα to hydrogel particle. (c) thrombin-induced GPIbα dimerization.

To interrogate thrombin-induced GPIbα dimerization, we utilized bioresponsive microgels. Microgels are hydrogel microparticles, which are water swellable polymeric networks with high water content.[13,14] Microgels with the composition poly(N-isopropylacrylamide-*co*-acrylic acid) (pNIPAm-*co*-AAc) were

prepared through aqueous free-radical precipitation polymerization (details in Supporting Information). Receptor, GPIbα, was transiently expressed in mammalian cells (HEK293T) and then purified using Ni-NTA affinity chromatography followed by size-exclusion chromatography (see Supporting Information). Functionalization of microgels with GPIbα or thrombin allows for direct observation of the multivalent interaction between thrombin and GPIbα (Figure 1b). The microgels were then attached on an aminoprophytrimethoxysilane (APTMS)-functionalized glass substrate via Coulombic interactions.[14] The optical microscopy image of the assembled microgels is tuned by multivalent protein binding, which is characterized by formation of a dark ring (Figure 1c). This phenomenon has been observed previously for simple model systems, which is the response of biotin-functionalized microgels to avidin and anti-biotin. [13,14] Despite the lackage of the physiological significance, we were successful in demonstrating that the increase of local crosslink density in microgels due to multivalent binding causes change in the refractive index of the microgel rim followed by dark ring formation in the microscopy image.

The behavior of the microgels in different concentrations of α-thrombin and GPIbα is shown in Figure 2. Under these conditions, both the GPIbα- and the thrombin-functionalized microgels showed dark ring formation above a critical concentration of the thrombin (Figure 2a) and GPIbα (Figure 2b), respectively. The critical concentrations of two proteins indicate that overall multivalent binding energy between thrombin and GPIbα is sufficient to cause deswelling of the microgel periphery. Thus, the elastic restoring force of microgel network is overwhelmed by the strength of the multivalent thrombin-GPIbα interactions. These results indicate that thrombin can induce GPIbα clustering, which may have relevance for the receptor's behavior on platelet membrane surfaces. In light of the results, we hypothesize that such clustering increases a local concentration of GPIbα, which would be favorable for interactions with vWF in the hemostatic function of platelets.

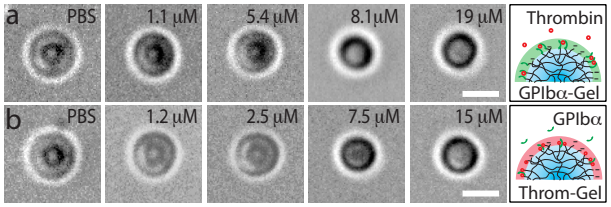

Figure 2. Response of bioresponsive microgels to ligand-induced receptor dimerization. DIC microscopy images of a GPIbα-functionalized microgel (a) and an α-thrombin-functionalized microgel (b) at the indicated concentrations of α-thrombin and GPIbα, respectively. Above a critical concentration, the microgels show modulation of the images through dark ring formations. The scale bar is 2 μm.

The dimerization assay was further tested by using two kinds of specific antibodies for GPIbα that are 6D1 IgG for the N-terminus region (Figure 3a) and anti-His IgG for the C-terminus 6His-tag (Figure 3b). The GPIbα-functionalized microgels displayed dark ring formation upon incubation with the both antibodies above the critical concentrations required for microgel shift due to multivalent binding.[13,14] However, the microgels are insensitive to monovalent binding of vWF A1 domain to GPIbα up to 5 μM (K_d~30 nM[15]). Under this condition, monovalent interactions are incapable of changing the optical properties of the microgel. Note that the local deswelling of the water-swollen hydrogel is efficiently caused by the formation of cross-links at the gel surface due to dimerization, but in monovalent binding, the elastic restoring force of the gel-network exceeds the free energy change accompanied by the complex formation. Thus, dark ring formation is exclusively induced by increasing the crosslink density and concomitant deswelling of the microgel periphery.

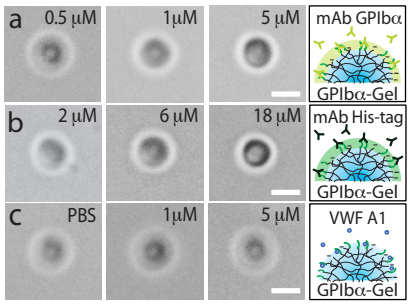

Figure 3. Response of bioresponsive microgels to the presence of antibody and vWF A1 domain. DIC images of a GPIbα-functionalized hydrogel at the indicated concentrations of mAb 6D1 for GPIbα (a), mAb for His-tag in GPIbα (b) and vWF A1 domain (c) in PBS buffer. Only the antibodies which bind to GPIbα cause the change in optical properties of microgels above a critical concentration, and not vWF A1 domain that binds to GPIbα in monovalent manner. The scale bar is 2 μm.

To develop a biochemical assay system, the capability of responding only in positive signals and operating in rigorous and physiological condition is a fundamental requirement for general application. Therefore, we examined the response of GPIbα–functionalized hydrogel particles under various concentrations of bovine serum albumin (BSA) (Figure 4a) and sodium chloride (NaCl) (Figure 4b). BSA is used not only for preserving many kinds of purified proteins but also for decreasing nonspecific interaction in most biochemical assays. Our results show that the dimerization signal was not observed until 10 mg/ml of BSA in PBS buffer (~10 times higher than a normal dose for preserving proteins). Thus, the nonspecific interaction between BSA and the microgel network is incapable of causing noticeable dimerization signal under our experimental conditions. We hypothesize that the BSA either interacts with the hydrogel network in monovalent manner or the bond strength, in case of multivalent binding, is weaker than the restoring force of hydrogel network.

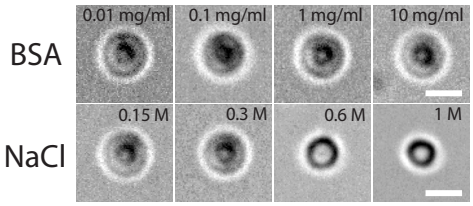

Figure 4. Response of microgels to nonspecific interaction and salt concentration. DIC images of a GPIbα-functionalized hydrogel at the indicated concentrations of BSA in PBS (top row) and NaCl in 50 mM Tris pH 7.5 buffer (bottom row). The scale bar is 2 μm.

We have also observed the reliability of the microgel assay in physiological salt concentration (~150 mM including our dimerization assay in PBS buffer) and even in more rigorous condition (300 mM of NaCl). Indeed, freshly purified recombinant

proteins often include ~300 mM salts, requiring dialysis. Thus, our microgel assay could be used for such proteins even without desalting step. It is also worthwhile to note that all the assays have been done in incubation with only 6 μl of protein solution, which makes the method highly desired for most in-vitro biochemical assay. We note that the formation of a dark ring for GPIbα-functionalized microgels was observed in 0.6 M and 1 M NaCl. These data suggest that extremely high salt conditions are required to induce deswelling of the microgel, which could be explained by both charge shielding effect of AAc group in the microgel followed by decreasing the Columbic repulsion and osmotic pressure due to higher solute concentration. [16]

In conclusion, we show the development of a new biochemical assay that enables the observation of ligand-induced receptor dimerization. The utility of the microgel assay was highlighted by its ability to respond only to multivalent binding, not for monovalent and nonspecific binding, under physiological salt concentrations. This novel but simple methodology, in which the responsive microgels are conjugated with biomolecules, will allow for unique and powerful biochemical assay in real-time measurement of protein multimerization. Furthermore, the method described here could be improved by combination with fluorescence microscopy, which provides fluorescence signals for both monovalent and multivalent bindings, allowing for detection of both but discrimination between the two conditions. All of these features make the microgel assay attractive for future applications in identifying biological pathway that is coupled to protein assembly, multimerization, and disassembly.

Notes and references

[a] Department of Chemistry, Eberly College of Science, Pennsylvania State University, University Park, Pennsylvania, 16802 *E-mail: jxk90@psu.edu*

[b] Department of Biomedical Engineering, College of Medicine, Korea University, Seoul, Korea, 136-705

[c] School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, 30332-0400

[d] Schmid College of Science and Technology, Chemistry, Chapman University, Orange, California, 92866

† JK and YP acknowledge support from National Research Foundation of Korea (NRF-2013S1A2A2035518). LAL acknowledges support from Georgia Tech. ACB acknowledges support from American Heart Association postdoctoral fellowship.

†† Electronic Supplementary Information (ESI) available: [details of microgel synthesis, protein expression and purification, fabrication of microgel assay]. See DOI: 10.1039/c000000x/

1 Brown, A.; Robinson, C. J.; Gallagher, J. T.; Blundell, T. L. *Biophys J*, 2013, **104**, 1720.
2 Wu, Y.; Honig, B.; Ben-Shaul, A. *Biophys J*, 2013, **104**, 1221.
3 Matsushita, C.; Tamagaki, H.; Miyazawa, Y.; Aimoto, S.; Smith, S. O.; Sato, T. *Proc Natl Acad Sci U S A*, 2013, **110**, 1646.
4 Arachiche, A.; Mumaw, M. M.; de la Fuente, M.; Nieman, M. T. *J Biol Chem*, 2013, **288**, 32553.
5 Tanji, H.; Ohto, U.; Shibata, T.; Miyake, K.; Shimizu, T. *Science*, 2013, **339**, 1426.
6 Lyumkis, D.; Julien, J. P.; de Val, N.; Cupo, A.; Potter, C. S.; Klasse, P. J.; Burton, D. R.; Sanders, R. W.; Moore, J. P.; Carragher, B.; Wilson, I. A.; Ward, A. B. *Science*, 2013, **342**, 1484.
7 Bartesaghi, A.; Merk, A.; Borgnia, M. J.; Milne, J. L.; Subramaniam, S. *Nat Struct Mol Biol*, 2013, **20**, 1352.
8 Swalley, S. E.; Baker, B. M.; Calder, L. J.; Harrison, S. C.; Skehel, J. J.; Wiley, D. C. *Biochemistry* 2004, **43**, 5902.
9 Ivanovic, T.; Choi, J. L.; Whelan, S. P.; van Oijen, A. M.; Harrison, S. C. *Elife*, 2013, **2**, e00333.
10 Dumas, J. J.; Kumar, R.; Seehra, J.; Somers, W. S.; Mosyak, L. *Science* 2003, **301**, 222.
11 Celikel, R.; McClintock, R. A.; Roberts, J. R.; Mendolicchio, G. L.; Ware, J.; Varughese, K. I.; Ruggeri, Z. M. *Science* 2003, **301**, 218.
12 Zarpellon, A.; Celikel, R.; Roberts, J. R.; McClintock, R. A.; Mendolicchio, G. L.; Moore, K. L.; Jing, H.; Varughese, K. I.; Ruggeri, Z. M. *Proc Natl Acad Sci U S A*, 2011, **108**, 8628.
13 Kim, J.; Singh, N.; Lyon, L. A. *Angew Chem Int Ed Engl* 2006, **45**, 1446.
14 Kim, J.; Nayak, S.; Lyon, L. A. *J Am Chem Soc* 2005, **127**, 9588.
15 Huizinga, E. G.; Tsuji, S.; Romijn, R. A.; Schiphorst, M. E.; de Groot, P. G.; Sixma, J. J.; Gros, P. *Science* 2002, **297**, 1176.
16 Lopez-Leon, T.; Fernandez-Nieves, A. *PhysRevE* 2007, **75**, 011801.