Volume 141 | Number 24 | 21 December 2016 | Pages 6473–6606

Analyst

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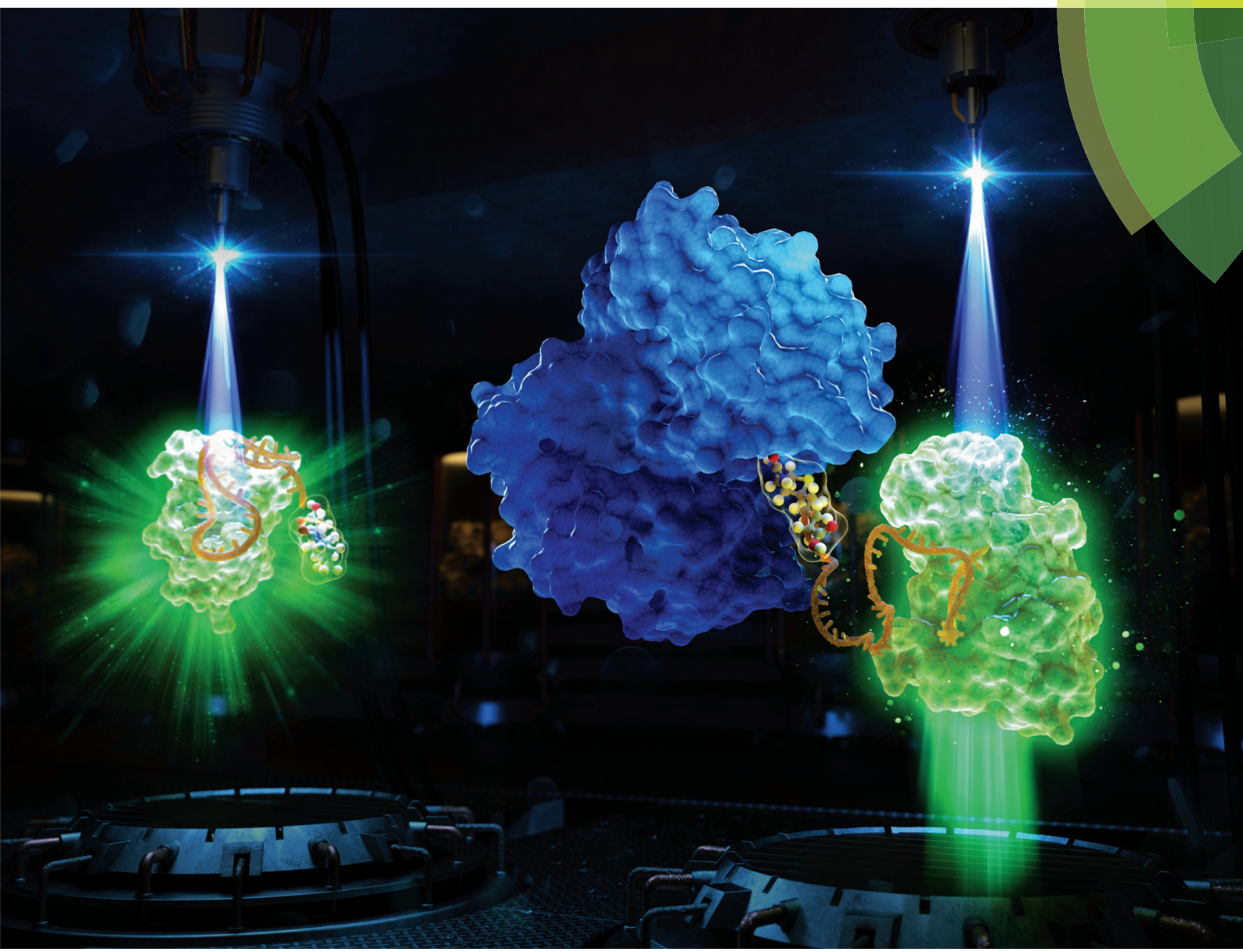

ISSN 0003-2654

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Cite this: *Analyst*, 2016, **141**, 6499

Received 24th July 2016,
Accepted 2nd November 2016

DOI: 10.1039/c6an01671e

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Streptavidin-triggered signal amplified fluorescence polarization for analysis of DNA–protein interactions†

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Fluorescence polarization (FP) is a sensitive, robust, and homogeneous assay format, able to probe a diversity of biological molecules and their interactions. Herein, we describe a new FP strategy based on the use of streptavidin as a signal amplifier. Such signal amplified fluorescence polarization (SAFP) was used to monitor the binding affinity of human angiogenin and a single-stranded DNA aptamer. Streptavidin was bound to a biotinylated single-stranded DNA aptamer and the interaction between this complex and Alexa Fluor 488 labelled human angiogenin was measured. A dissociation constant of 135.3 ± 32.9 nM and a limit of detection of 6.3 nM were successfully extracted only when the FP signal was increased (without binding hindrance) *via* streptavidin. Moreover, the demonstrated approach was specific to target molecules without any non-specific binding. The streptavidin-triggered SAFP method unlike amplification strategies that utilize nanomaterials (such as graphene oxides, carbon nanotubes, and metal nanoparticles) is not compromised by fluorescence quenching, and it is able to operate within nanomolar concentration regimes. Furthermore, unlike the other FP signal amplification strategies that use dual binding DNA probes, the presented method is simple to implement with signal amplification only requiring the binding of streptavidin with biotinylated DNA. This method could be expanded to analyze molecular interactions and it may be a useful tool for FP measurement by reducing the concentration of rare and expensive protein samples.

Introduction

Fluorescence polarization (FP) is an optical phenomenon where radiation emitted by a fluorophore has unequal intensities along different axes of polarization.[1,2] First described in 1926 by Perrin, the principle of FP is based on the observation that when a fluorophore in free solution is excited with plane-polarized light it will emit light plane polarized radiation only if the fluorophore remains stationary during the excitation process. However, if the fluorophore rotates on the timescale of the emissive process, then emission will be depolarized to some extent.[2] Accordingly, FP is dependent on the molecular weight or size under conditions of constant temperature and viscosity. Indeed, if plane-polarized light is used to excite small fluorophores in a low viscosity (and/or high temperature) environment, rapid molecular rotation will depolarize the emission, resulting in a low FP value. Conversely, large molecules (within high viscosity and/or low temperature environments) will exhibit relatively slow rotational speed, resulting in high FP values.[3–5] Based on these features, fluorescence polarization measurements are commonly used to probe protein–ligand bindings,[6,7] protein–protein interactions,[8–10] enzymatic activities,[11,12] DNA hybridizations,[13,14] and immunoassays.[15–17] Significantly, unlike methods such as enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance (SPR), which are commonly used for the same purpose, FP is a homogeneous format in which direct analysis of the bound/free ratio is possible without immobilization of molecules onto a solid substrate and the need for additional washing and separation steps. Accordingly, FP assays are ideally suited for the high-throughput analysis of target molecules[18–20] and high-throughput screening of chemicals.[21–23]

Despite the aforementioned advantages, FP signals are proportional to the effective molecular size under conditions of constant solution viscosity and absolute temperature. This means that (in binding studies) fluorophores should ideally be labelled molecules of low molecular weight to ensure large variations in the extracted FP signal. In order to overcome such a requirement, recently some studies have focused on the effective analysis of FP signals by increase of the molecular weight. The first one is using nanomaterials such as graphene oxides (GOs),[24–26] carbon nanotubes (CNTs),[27,28] and gold nanoparticles (AuNPs) to increase FP signals.[29,30] Although

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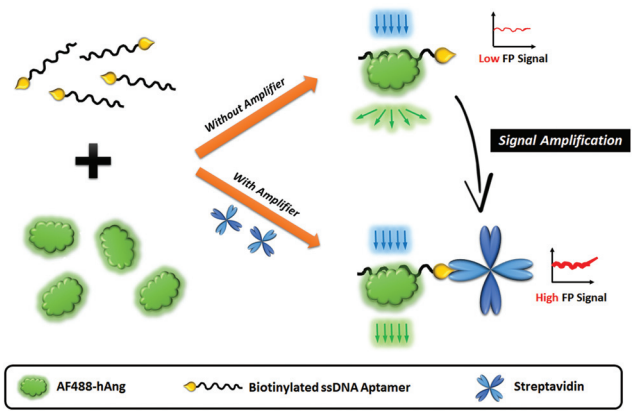
† Electronic supplementary information (ESI) available: Experimental detailed procedures. See DOI: 10.1039/c6an01671e

successful in their aims, such methods introduce complexity into the analytical process (most notably through the need to synthesize nanomaterials, chemically modify and conjugate biomolecules). Moreover, such nanomaterials are known to enhance fluorescence quenching, resulting in sensitivity limitations when assays are conducted at nanomolar concentrations. In the other approach several attempts have been made to amplify FP signals through the use of a pair of a DNA aptamer and its binding protein without any use of nanomaterials, that is use of an aptamer having dual binding affinity.[31,32] However, these DNA–protein pair-based methods can only be applied when a single DNA aptamer should be simultaneously bound to a protein (to increase the molecular weight) and the target for detection. Put simply, a single DNA probe must possess a dual binding affinity, where there is a high binding affinity for the target and a lower binding affinity than the target for protein. This results in a very limited range of applications. Accordingly, herein we propose the use of streptavidin-triggered amplified fluorescence polarization (SAFP) to perform protein–nucleic acid binding assays. Specifically, we demonstrate SAFP through the use of human angiogenin (hAng), an angiogenesis inducing factor known for over-expression in various cancer cells,[33] and its binding single-stranded DNA aptamer (ssDNA aptamer).

Results and discussion

As depicted in Scheme 1, the principle behind SAFP is based on creating a significant increase in the total molecular weight of the binding complex *via* streptavidin. When considering DNA–protein interactions, effective analysis using FP is possible through conjugation of the fluorophore with DNA having a relatively low molecular weight. Moreover, to obtain accurate binding affinities and/or dissociation constants (K_d), polarization values at varying protein and nucleic acid concentrations must be determined. Since proteins may not be available in large amounts due to their expensive cost and scarcity in some cases, assays at low concentration are preferable.



Scheme 1 The working principle behind the SAFP method for analysis of DNA–protein interactions. Interaction between hAng labelled with the AF488 dye (AF488–hAng) and hAng aptamer was examined. Streptavidin was used to amplify FP signals *via* binding with biotin at the 3′-end of the hAng aptamer.

First, hAng was fluorescently labelled with the Alexa Fluor 488 (AF488) dye. The dye-to-protein molar ratio of the purified AF488–hAng was measured to be 0.63, with SDS-PAGE analysis confirming selective labelling of the AF488 dye on the hAng band location (Fig. S1†). Next, a binding assay using AF488–hAng and the hAng binding ssDNA aptamer was conducted. The hAng aptamer sequence is provided in the experimental procedure (ESI†), and has been successfully screened using SELEX by Nobile *et al.*[34] To increase the FP signal *via* streptavidin binding, biotin was conjugated on the 5′ end of the ssDNA aptamer. Using a fixed hAng concentration of 10 nM, the biotinylated hAng binding ssDNA aptamer concentration was varied between 0 and 2 μM with FP values being measured at each concentration. To examine any changes in binding by the presence (or absence) of streptavidin, streptavidin was added to the biotinylated ssDNA aptamer in the same molar concentration in a first group. On the other hand, streptavidin wasn't added to the biotinylated ssDNA aptamer in the other group for the negative control. Normalized polarization values measured at each concentration were substituted in eqn (1) to obtain K_d:

$$P = P_{min} + \Delta P\left(\frac{[\text{hAng Aptamer}]}{K_d + [\text{hAng Aptamer}]}\right). \quad (1)$$

Here P is the measured polarization, P_{min} is the polarization of unbound AF488–hAng, ΔP is the total change in polarization, and [hAng aptamer] is the molar concentration of the hAng aptamer. As shown in Fig. 1a, for experiments performed without streptavidin no variation in the FP signal is observed, even though there is a binding between AF488–hAng and the hAng aptamer.

However, in the case when streptavidin is added to the assay mixture, interaction of hAng and the hAng aptamer is clearly observed through variation in the FP, yielding an extracted K_d value of 135.3 ± 32.9 nM. This value agrees well with a value of 127.6 ± 45.2 nM generated from experiments using hAng and the AF546 labelled hAng aptamer by the protein microarray method (Fig. S2†), demonstrating that streptavidin acts as an efficient FP signal amplifier without introducing any experimental artifacts. Also, the limit of detection in the amplified condition by streptavidin was found to be 6.3 nM based on three times the value of three measurements of blank samples. Significantly, this means that FP signals were successfully amplified by streptavidin and the consumption of hAng proteins can be reduced when compared to the conventional FP method.

To further confirm whether such SAFP originates as a result of the binding between hAng and its aptamer, the binding affinity was assessed using the biotinylated random sequence aptamer. For a fixed AF488–hAng concentration of 10 nM, the biotinylated random sequence aptamer concentration was varied between 0 and 2 μM, with FP measurements taken at

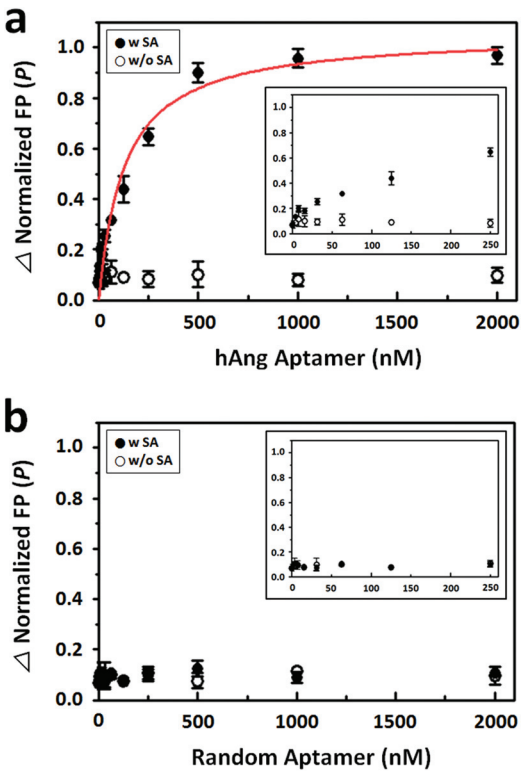


Fig. 1 FP binding curve of hAng with the biotinylated hAng aptamer (a) and the biotinylated random sequence aptamer (b). The AF488–hAng concentration was maintained at 10 nM, whilst each aptamer concentration being varied between 0 nM and 2000 nM. Streptavidin was added in the same concentration as the aptamer (black circle, w SA) and excluded from control experiment (white circles, w/o SA). Binding buffer conditions: 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 0.2 mM $MgCl_2$. The inset images show FP values at low concentrations (0–250 nM) of the aptamer.

each concentration. As shown in Fig. 1b, the random sequence aptamer did not exhibit any binding with hAng. Furthermore, this means that the AF488–hAng and streptavidin interaction revealed no evidence. Accordingly, the increased polarization values observed in Fig. 1a were caused not by the binding of hAng and streptavidin, but by the specific binding of AF488–hAng and the hAng aptamer.

Finally, target specificity of this assay was confirmed using AF488-ribonuclease A (RNase A) and the hAng binding aptamer. Here the concentration of AF488-RNase A (Fig. S3†) was fixed at 10 nM. The biotinylated hAng binding ssDNA aptamer concentration was varied between 0 and 2 μM with FP values being measured at each concentration. To examine any changes in binding by the presence (or absence) of streptavidin, streptavidin was added to the biotinylated ssDNA aptamer in the same molar concentration in a first group. For the negative control, streptavidin wasn't added to the biotinylated ssDNA aptamer in the other group. Normalized polarization values measured at each concentration were substituted in eqn (1) to examine the binding affinity.

Although RNase A is an enzyme with an amino acid sequence of 33% identical to that of hAng,[35] the hAng aptamer

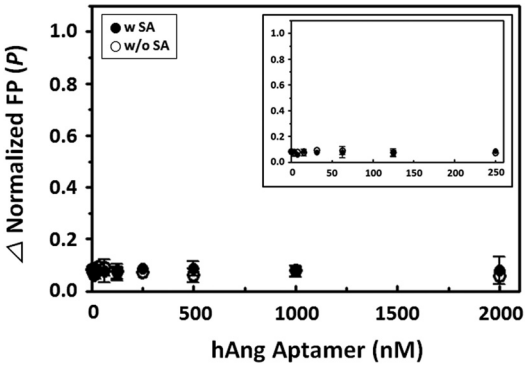


Fig. 2 FP binding curve of RNase A with the biotinylated hAng aptamer. The AF488-RNase A concentration was maintained at 10 nM, whilst each aptamer concentration being varied between 0 nM and 2000 nM. Streptavidin was added in the same concentration as the aptamer (black circle, w SA) and excluded from control experiment (white circle, w/o SA). The inset image shows FP values at low concentrations (0–250 nM) of the aptamer.

didn't bind to RNase A as shown in Fig. 2. Furthermore, this result agrees with the result by the protein microarray method (Fig. S4†).

Conclusions

In conclusion, we have successfully measured the dissociation constant of two molecules at concentrations in the nanomolar regime *via* the use of streptavidin as a FP signal amplifier. Specifically, we have shown that the binding of hAng and the biotinylated hAng aptamer can be measured using such an approach, with streptavidin only amplifying the FP signal and not in any way hindering the binding of AF488–hAng and the hAng aptamer. We believe that the SAFP method is an existing new variant of previously reported mass amplifying strategies that incorporate nanomaterials such as GOs, CNTs and AuNPs, since it does not act to quench fluorescence. Additionally, in comparison with mass amplifying strategies that use a single-stranded DNA aptamer having dual binding affinity to the protein and target, the presented strategy does not require screening of a single probe that simultaneously bind two substances (protein and target). Importantly, this SAFP strategy is simply applicable to probing the interactions of various molecules. Finally, the proposed SAFP strategy is likely to be especially useful in the analysis of small molecule–protein interactions, nucleic acid–protein interactions and peptide–protein interactions, due to its ability to operate at low protein concentrations.

Acknowledgements

All authors acknowledge the inspiring contributions of the late Prof. Soo-Ik Chang in initiating this study. This work was supported by the National Research Foundation (NRF) grant funded by the Ministry of Science, ICT and Future Planning

(MSIP) of Korea through the Global Research Laboratory (GRL) Program (Grant number 2009-00426).

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