Fluorescence sensing of protein-DNA interactions using conjugated polyelectrolytes and graphene oxide

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ABSTRACT

Protein-DNA binding, particularly transcription factor-DNA binding, is one of the main molecular interactions involved in gene regulation. These interactions are sequence-specific, play a key role in many fundamental biological processes, and are deregulated in the pathogenesis of several diseases. In this study, a robust analytical bioassay to characterize protein-DNA binding was built by combining the optical properties of water soluble conjugated polyelectrolytes, and graphene oxide’s superquenching capabilities. Cationic conjugated polyelectrolytes bind strongly to double stranded DNA through electrostatic interactions, and provide fluorescent signals to track the DNA without any chemical modification. In addition, the labeled DNA retains its protein binding ability. An important oncogenic transcription factor (i.e. estrogen receptor α) was used to demonstrate the concept, and two collaborative factors involved in the estrogen gene transcription (i.e. forhead box A1 and activating enhancer binding protein 2 gamma) were employed as controls. This method overcame the main limitations of previous nanomaterial-based bioassays, while keeping the sensitivity and precision of the gold standard techniques. These benefits, combined with the high versatility and low-costs, could lead this bioassay to be used in several fundamental biomedical research lines, such as large scale protein-DNA binding studies and drug discovery.

1. Introduction

Water soluble conjugated polyelectrolytes (CPEs) are polymers made of two different parts [1]. First, a π-conjugated backbone that defines a set of optical properties, such as strong fluorescence, light-harvesting and high quantum yield. Second, ionic side-chains that provide high solubility in water and allow strong electrostatic interactions. Due to those properties, CPEs have been extensively used as key sensing components in many bioassays [1].

Early designs exploited the Förster resonance energy transfer (FRET) between positively charged CPEs and a dye-labeled nucleic acids to detect ssDNA [2,3]. Since those early designs, several CPE-based assays have been developed for the detection of other relevant medical targets, such as proteins [4], ATP [5], and ions [6].

Graphene oxide (GO) is a one atom thick sheet of graphite with different oxygen-containing functional groups (i.e. carboxyl, hydroxyl and epoxy groups) decorating both the basal plane and the edges [7]. GO can be used in CPE-based biosensors [8] because of its long-range fluorescence superquenching [9], water solubility [10], and strong interactions with CPEs through π–π stacking [11], cation-π bonding [12], and electrostatic interactions [12]. Few bioassays coupling GO and CPEs have been developed for the detection of different clinically relevant analytes, such as DNA [8], miRNA [12] and proteins [13].

Transcription factors are proteins that up or down regulate gene transcription by binding to short sequences of DNA called response elements [14]. Because they are key factors in many cellular processes [15], several diseases have been linked to transcription factors malfunction [14]. Therefore, the study of transcription factors binding to DNA has also become of clinical significance, since it can reveal gene transcription mechanisms, leading to new therapies.

Surface plasmon resonance (SPR) and electrophoretic mobility shift assay (EMSA) are the gold standard methods for the quantitative analysis of protein-DNA interactions [16]. Even though both of them present high sensitivity, they are limited by several drawbacks. For instance, the SPR performance is hindered by the high costs and the non-specific interactions between the analyte and the substrate surface [17],

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while EMSA measurements are limited by the stability of the protein-DNA complex, since the complex integrity can be disrupted by the electrophoresis, and the long experimental times \cite{18,19}.

Therefore, there has been an increasing interest in developing new analytical bioassays capable of measuring the interaction between proteins and DNA with high sensitivity, while keeping the experimental setup simple and fast. Some of the early demonstrations by our group and others largely relied on gold nanoparticles (AuNPs) \cite{20–22}. Even though they could monitor the protein-DNA binding, they lacked of quantitative characterization capabilities, such as dissociation constants ($K_d$) measurements. Furthermore, those assays were limited by non-specific AuNP aggregation and/or complex enzymatic reaction optimization.

A new analytical design exploiting the fluorescence quenching of CPEs by dsDNA-AuNPs has been developed \cite{23}. The use of dual transducers (CPEs and AuNPs) and the energy transfer principle improved the sensor performance, and overcame the colorimetric sensing limitations. Nevertheless, this protocol needed tedious nanomaterial functionalization steps, which increased the complexity and the experimental times.

Despite promising results \cite{24}, where GO-based fluorescence assays displayed the highest sensitivity and repeatability among different nanomaterial-based designs, bioassays that combine GO and organic fluorophores for characterizing biological interactions are still quite rare. For instance, there is not any demonstration of GO-based protein-dsDNA binding assay.

In this work, we developed a new bioassay for protein-DNA binding that exploited the collaborative role between the strong fluorescence and light-harvesting capabilities of CPE, and the superquenching properties and higher stability of GO. We were able to minimize the complexity and experimental times of the assay by electrostatically labeling the dsDNA with the CPE, while preserving the sensitivity and precision of the gold standard SPR. Furthermore, this work represented a big step on the field of nanomaterial-based sensing, since it overcame the main limitations of the previous bioassays.

2. Materials and methods

2.1. Materials

The following products were used as received. Graphene oxide (GO, 2 mg/mL dispersion in H$_2$O), poly[(2,5-bis(2-{N,N-diethylammonium bromide}ethoxy)-1,4-phenylene)-alt-1,4-phenylene] (Mw of 1054 Da), poly(2,5-bis(3-sulfonatopropoxy)-1,4-phenylene, disodium salt-alt-1,4-phenylene) (Mw not specified by the provider), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Human recombinant estrogen receptor α (ERα) was purchased from Life Technologies, Thermofisher Scientific. FoxA1 and AP-2γ were prepared as HisMBP-tagged recombinant proteins as described in a previous publication \cite{25}. The oligonucleotides used in this study were purchased from Integrated DNA Technologies (Table S1). All the water employed in the experiments was obtained with a Milli-Q Integral 5 system.

2.2. Characterisation

The emission and excitation spectra were obtained by an Infinite M200 from Tecan. The zeta potential was recorded with a Zetasizer Nano-Z from Malvern Instruments. pH was measured with an 827 pH lab from Metrohm. Fluorescence polarization was measured with a Synergy-2 Multi-Mode Microplate Reader from BioTek with 485/20-nm excitation and 528/20-nm emission filters.

2.3. CPE fluorescence quenching by GO in the presence of dsDNA

dsDNA (probe 1) solutions with different concentrations were prepared in 10 mM Tris-HCI buffer (pH 7.0); 5 μL of those solutions were added into 35 μL CPE solutions (100 μg/mL in 10 mM Tris-HCI pH 7.0); and the mixtures were incubated at room temperature for 10 min. 30 μL of GO (200 μg/mL in Tris-HCl pH 7.0) were then added into the dsDNA/CPE solutions and the resulting mixtures were left incubating at room temperature for 15 min. The final dsDNA concentration in the solutions ranged from 0 to 1000 nM. Last, the fluorescence spectra of the resulting solutions were measured. The CCPE and ACPE were excited with λ (= 334 and 363 nm, respectively.

2.4. Protein-DNA binding assay

The binding assays were performed by incubating 15.5 μL solutions made of positively charged CPE (final concentration of 50 μg/L) and probe 1 (dsDNA final concentration of 100 nM) in 10 mM Tris-HCl pH 7.0 at room temperature for 10 min. The resulting solutions were mixed with 24.5 μL of different diluted protein solutions (ERα, FoxA1, AP-2γ and BSA in 10 mM Tris-HCl buffer, pH 7.0) and the mixtures were incubated for another 30 min at room temperature. Finally, 30 μL of GO (200 μg/mL) were added and incubated for 15 min at room temperature. The final protein concentrations ranged from 0 to 350 nM. Finally, the fluorescence spectra of the resulting solutions were measured.

2.5. Assay for fluorescence polarization measurement

The fluorescence polarization assay was performed by incubating 15.5 μL solutions made of positively charged CPE (final concentration of 0 or 50 μg/L) and probe 2 (final dsDNA concentration of 40 nM) in 10 mM Tris-HCL pH 7.0 at room temperature for 10 min. The resulting solutions were mixed with 54.5 μL of different protein solutions (ERα or BSA in 10 mM Tris-HCl buffer, pH 7.0) and the mixtures were incubated for another 30 min at room temperature. The final protein concentrations were 0 or 100 nM. Lastly, the fluorescence polarization of the resulting solutions was measured.

3. Results and discussion

3.1. Fluorescence quenching between GO and CPE in the presence of dsDNA

We initially studied the interactions between GO (Fig. S1) and two CPEs, which had the same backbone but different side chains, and then how dsDNA affected those interactions. Poly[(2,5-bis(2-{N,N-diethylammonium bromide}ethoxy)-1,4-phenylene)-alt-1,4-phenylene] was a cationic CPE denoted as CCPE (Fig. 1A). Poly(2,5-bis(3-sulfonatopropoxy)-1,4-phenylene, disodium salt-alt-1,4-phenylene) was an anionic one denoted as ACPE (Fig. 1B). Because both CPEs had the same backbone, they presented similar emission spectra (emission peak wavelength of 410 and 420 nm, respectively, Fig. 1C and D). When mixed with GO, their fluorescence emissions were all largely quenched. The degree of fluorescence quenching at emission peak ($\eta$) for the CCPE was as high as 91 ± 4%, which could be attributed to the synergic effect between the conjugated polymer and GO. Interestingly, although GO and the ACPE had alike charge, which was unfavorable for electrostatic attraction,

ACPE fluorescence was also significantly quenched in the presence of GO ($\eta$ = 82 ± 4%). This observation suggested that the π–π stacking interaction could dominate over electrostatic repulsion, bringing the CPEs and GO in close proximity. Nevertheless, a synergic effect between the three main interactions (i.e. electrostatic, π–π stacking and the cation-π bonding) was necessary for maximum quenching.

Next, we examined the effect of dsDNA on the interaction between CPEs and GO. This would be essential for the protein binding experiments presented in the following section. CPEs were exposed to dsDNA for 10 min before being added to the GO solution, in order to maximize the interaction between CPEs and dsDNA. Fig. 2A showed that CCPE's...
fluorescence was recovered by the presence of dsDNA. We hypothesized that CCPE and dsDNA molecules electrostatically interacted with each other because of their opposing charges (more characterization and justification is presented below), and this hindered the interaction between the CCPE and the GO. On the other hand, the ACPE fluorescence did not change upon addition of dsDNA in the same concentration range (Fig. 2B). From an electrostatic point-of-view, there was a lack of interaction between ACPE and dsDNA, because they were both negatively charged and repelled each other. Fig. 2C plotted the fluorescence variation (\( \frac{F - F_0}{F_0} \)), where \( F \) was the fluorescence intensity at the CPE emission maximum (410 and 420 nm for CCPE and ACPE, respectively) under a specific DNA concentration and \( F_0 \) was the initial fluorescence intensity. CCPE presented a fluorescence recovery curve with hyperbolic shape, while ACPE fluorescence showed no variation within the experimental dsDNA concentration range.

To better understand the mechanism involved in the fluorescence recovery of the CCPE by the dsDNA, the zeta potentials of GO and CCPE (before and after the addition of different amounts of dsDNA) were measured. It was confirmed that GO (85.7 μg/mL) and CCPE (50 μg/L, 47.4 nM) in absence of dsDNA presented opposite charges in solution, with zeta potential of \(-31.8 \pm 0.8\) and \(+18.3 \pm 4.1\) mV, respectively (Fig. 3). The addition of negatively charged dsDNA (from 0 to 50 nM) into the CCPE solution neutralized the positive charge and turned it down to \(-14.0 \pm 2.0\) mV at 50 nM dsDNA (about 1:1 molar ratio of dsDNA:CCPE). Above 50 nM dsDNA concentration, the zeta potential showed very minor variations with the addition of extra oligonucleotides.

These results suggested that dsDNA and CCPE formed a hybrid complex due to electrostatic interaction, and the overall charge of the complex was negative, when enough DNA was added, due to the phosphate backbone of the oligonucleotide. Similar behavior has been...
reported in the interaction between dsDNA and positively charged gold nanoparticles [26].

Based on the collective observations, we proposed a mechanism for the concentration-dependence fluorescence recovery. At low oligonucleotide concentrations (i.e. from 0 to 50 nM), all the dsDNA added into the solution electrostatically interacted with CCPE molecules, changing their overall charge and blocking the π-π stacking, which promoted the fluorescence recovery. Due to the size difference between CCPE and dsDNA (molecular weights of 1.05 and 30.3 kDa, respectively) the interaction between CCPE and two or more oligonucleotide molecules was sterically hindered. Thus, above dsDNA concentrations of 50 nM (molar ratio of dsDNA: CCPE = 1:1), the addition of extra oligonucleotides had little effect, and high amounts of dsDNA were necessary to induce the additional fluorescence recovery observed in Fig. 2C. Lastly, we could exclude the adsorption of dsDNA on GO as a cause of the fluorescence recovery, because it is well reported that dsDNA has very limited affinity for GO [27].

3.2. Protein-DNA binding measured as fluorescence quenching

A new analytical bioassay was developed for protein-DNA binding based on the reversible quenching of CCPE fluorescence by GO. Particularly, dsDNA could reduce the quenching by decreasing the interaction between positively charged CCPE and GO. Furthermore, transcription factor binding to the CCPE-labeled dsDNA increased the quenching by bringing the CCPE closer to GO (Fig. 4). It is worth mentioning that the negatively charged ACPE was not further used since dsDNA could not induce fluorescence recovery.

Three oncogenic transcription factors, which jointly regulate several biological functions, such as the development and maintenance of the reproductive system and bone structure, were used as case study, i.e. estrogen receptor α (ERα), forkhead box A1 (FoxA1) and activating enhancer binding protein 2 gamma (AP-2γ) [28-30].

Fig. 4 depicted the three steps of the proposed analytical assay: First, 50 μg/L CCPE was incubated with 100 nM probe 1 (i.e. dsDNA that contained both ERα and AP-2γ binding sites). In order to avoid leaving free polymer molecules in solution, which may interfere with the fluorescence measurements, a molar ratio of 1:2 for CCPE: dsDNA was used (even under the expenses of leaving unlabeled DNA). Second, the CCPE-labeled dsDNA (CCPE-dsDNA) was exposed to the ERα (or other proteins), allowing the transcription factor to bind to the dsDNA. Third, GO was added into the mixture and the fluorescence was measured after the incubation time. All proteins were dispersed in 10 mM Tris-HCl buffer.

Fig. 5A-C plotted the fluorescence emission of the CCPE-dsDNA in the presence of GO and different amounts of proteins (ERα, FoxA1 and AP-2γ, respectively). The dsDNA (probe 1) used presented an ERα binding site (ERE). For ERα (Fig. 5A), the fluorescence emission of CCPE-dsDNA decreased with increasing protein concentration in a range from 0 to 200 nM. Above those ERα concentrations, the fluorescence did not significantly change. The fluorescence intensity variation ((F − F0)/F0) as a function of ERα concentration (Fig. 5D) indicated that the binding reached saturation at 2:1 of ERα: dsDNA concentration ratio. The binding stoichiometry was further confirmed by fitting the data to the Hill equation [31] (Fig. S2), which presented a Hill coefficient (nH) of 1.96 (e.g. when nH is above 1, it means that the ligands positively cooperate and at high cooperativity, the nH estimates the DNA binding sites [31]). These results were in agreement with previously published literature, which reported ERα binding to ERE as a dimer [32]. An apparent dissociation constant (Kd) of 60.7 ± 6.5 nM was calculated with the Hill equation (Fig. S2). That value was coherent with previous literature results obtained by SPR spectroscopy (Kd of 61.9 nM [33]). Since the final pH of the mixture in our experiments was 7.68, ERα was positively charged in the assay solution (isoelectric point, pI of 8.3 [34]). Therefore, when ERα bound to its DNA binding site, it reduced the overall negative charge of the CCPE-dsDNA complex and thus decreased the electrostatic repulsion between CCPE-dsDNA and GO. We inferred that the change of the overall CCPE-dsDNA charge due to protein binding brought the CCPE closer to GO, enhancing the fluorescence quenching.

To confirm the importance of the ERα-DNA binding and the ERα positive charge in the fluorescence quenching enhancement, two negative control experiments involving FoxA1 and AP-2γ were performed. On one hand, FoxA1 was a transcription factor that presented similar electrostatic behavior to ERα (pl of 8.9 [35]) but did not bind to the dsDNA employed in the assay (probe 1). On the other hand, AP-2γ presented neutral charge in the assay solution (pl of 7.7 [36]) but it could bind to the DNA probe 1, which contained a GC rich binding site for this protein.

The additions of FoxA1 (Fig. 5B) or AP-2γ (Fig. 5C) as negative control proteins into the mixture induced no fluorescence decrease. Thus, it was confirmed that both sequence recognition between protein-DNA and protein positive charge were necessary for bringing CCPE and GO in close proximity and the fluorescence being quenched. The presence of only one of the two factors was not enough to trigger the assay response, providing double selectivity. These results also suggested that among the different forces that might contribute to the protein-GO interaction (e.g. electrostatic, π-π stacking and CH–π interactions) [37], the electrostatic ones were the dominants, since a positively charged protein was needed. This observation had also been reported for other
Moreover, we studied the possible interferences caused by non-binding proteins. The dsDNA-CCPE complex was mixed with ERα and non-binding proteins, such as negatively charged bovine serum albumin (BSA, isoelectric point of 4.8 [38]) or positively charged FoxA1, before being exposed to GO. Fig. S3A showed the decrease of fluorescence induced by ERα in absence and presence of FoxA1 or BSA. In the three cases, the fluorescence quenching was similar (Fig. S3B), indicating that the system displayed comparable fluorescence signals in the absence and presence of non-binding proteins, and it was robust against non-specific interactions.

3.3. Effect of the CCPE label on the protein-DNA binding

To further confirm that the electrostatically labeling dsDNA with CCPE did not hinder the protein-DNA binding, we employed fluorescence polarization (FP) measurement. When a protein binds to a dye-labeled dsDNA, the larger volume of the complex hinders the fluorophore Brownian movement, increasing its fluorescence polarization [39]. In this set of experiments, the FAM-labeled probe 2 (i.e. a dsDNA that only contained the ERE binding site) was used. It is worth mentioning that FAM was chemically labeled to the probe, while the interaction between dsDNA and CCPE was electrostatic. FAM label was selected because its fluorescence (excitation and emission peaks at 485 and 528 nm, respectively) did not interfere with CCPE emission (peak centered at 410 nm). Furthermore, a concentration of 40 nM dsDNA was used to ensure that most of the oligonucleotide molecules were complexed in the presence of 50 μg/L CCPE (molar ratio of dsDNA: CCPE ~ 0.8:1). Fig. 6 plotted the FP of the FAM-labeled probe 2 under different conditions. Firstly, the addition of CCPE did not significantly affect the overall FP, although previous experiments proved that DNA and CCPE formed a hybrid complex (CCPE-dsDNA). The molecular mass of the CCPE was too small relative to the probe 2 DNA (i.e. 1.05 and 21.4 kDa, respectively) to introduce significant changes in probe 2 movement and rotation. Secondly, the addition of a non-binding protein, such as BSA (molecular mass of 68 kDa) [40], did not affect the probe 2 FP either, because no complex was formed. However, the addition of ERα (molecular mass of 65 kDa [41]) into the probe 2 solution increased the FP from 80 to 98.5 mP, due to the formation of ERα-dsDNA complex. A similar result was obtained when the probe 2 was previously exposed to CCPE, where the addition of ERα increased the FP from 81.5 to 99.5 mP. Those results confirmed that the ERα-DNA binding was not affected by the presence of CCPE.

4. Conclusions

In the present work, we developed a new analytical assay capable of characterizing the sequence-specific binding of transcription factors to dsDNA. The assay used CCPE and GO as collaborative sensing elements, and exploited the exceptional optical properties of CCPE (i.e. strong fluorescence, light-harvesting and high quantum yield) and the super-quenching capabilities of GO. The assay relied on the labeling of dsDNA with CCPE, which did not require any chemical modification of the oligonucleotide strands. Upon protein binding, CCPE-dsDNA and GO came in close proximity, increasing the fluorescence quenching. ERα
was used as case study and two related transcription factors, i.e. FoxA1 and AP-2γ, were used as controls. Those control experiments confirmed that the protein-DNA binding and the protein positive charge were the two assay key factors, which provided double selectivity against other proteins. FP measurements proved that labeling the dsDNA with the CCPE did not interfere in the protein binding. The fast and the easy-to-use nature of the hybrid system, and its high accuracy made of this assay a promising analytical method for large-scale protein-DNA biomedical research, and drug discovery.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.snb.2018.05.105.

References

Physics and Astronomy, University College London, UK. She leads a very dynamic group conducting cutting-edge interdisciplinary and innovative research on the design and synthesis of magnetic and plasmonic nanomaterials for biomedical applications. Her research focuses on developing new chemical methods and, in collaboration with chemical engineers, producing the next generation of nanoparticles with very high magnetic moment and novel hybrid and multifunctional nanostructures. Detailed mechanistic studies of their formation by sophisticated and advanced analysis of the nanostructure allow tuning of the physical properties at the nanoscale; these can subsequently be exploited for diagnosis and treatment of various diseases. These studies are conducted to provide insight for future material design approaches. It will also help to identify the critical process parameters that can be manipulated in order to obtain the suitable physical properties for the intended applications. She was the sole editor of the book Magnetic Nanoparticles: From Fabrication to Clinical Applications published by CRC Press/Taylor & Francis: http://www.crcpress.com/product/isbn/9781439869321. In 2016, she was a guest editor of The Royal Society Interface Focus on "Multifunctional nanostructures for diagnosis and therapy of diseases." She has published nearly 100 peer-reviewed journal articles and book chapters with over 5000 citations so far. She has been a visiting professor at various universities in France, Japan, China and Singapore. She has been an invited speaker at nearly 200 institutes and scientific meetings. She has a leadership role in professional communities by chairing and organising 30 high-profile international conferences such as the American Chemistry Society symposia in 2018, 2012 and 2010; Royal Society of Chemistry UK Colloids Conferences in 2017, 2014 and 2011; European Material Research Society Symposia in 2016 and 2013; ICMA Singapore in 2019, 2015 and 2013; Faraday Discussions in 2014, and being a member of advisory boards in Europe, the United States and Japan. She served in the Joint Committee of the Royal Society of Chemistry Colloid & Interface Science Group and the Society of Chemical Industry Colloid & Surface Chemistry Group (2008–2017). She is an elected member of The Royal Society of Chemistry Faraday Division Council and currently serving in Awards Committee and was a representative member of Joint Colloids Groups (2013–2016). She is a workgroup leader of EU COST Action TD1402 on Multifunctional Nanoparticles for Magnetic Hyperthermia and Indirect Radiation Therapy (RADIOMAG). She is a cochair of the 13th International Conference on the Scientific and Clinical Applications of Magnetic Carriers in June 2020, London, UK.

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