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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.* forkhead 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 [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) [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 [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 [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_2O), 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 [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 Mili-Q Integral 5 system.

2.2. Characterization

The emission and excitation spectra were obtained by an InfiniteM200 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-excitation and 528/20-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-HCL buffer (pH 7.0); 5 μL of those solutions were added into 35 μL CPE solutions (100 μg/L in 10 mM Tris-HCl 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 of 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 (η) for the CCPE was as high as 91 ± 4%, which could be attributed to the synergic effect between the favorable electrostatic interactions, the π–π stacking and the cation-π bonding interactions 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 (η = 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