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Engineering Quantum Dot Surfaces to Preserve Protein-DNA Interactions for Single-Molecule Visualization

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Abstract

Quantum dots (QDs) are fluorescent nanoparticles widely used for single-molecule imaging because of their exceptional brightness and photostability. However, the impact of QD surface chemistry on biomolecular interactions has not been systematically investigated. Here, we report that commercial QDs unexpectedly destabilize protein-DNA complexes by inducing protein dissociation from DNA. Using the human nucleotide excision repair protein, xeroderma pigmentosum complementation group A (XPA) as a model system, we demonstrate that antibody-conjugated QDs promote dissociation of XPA from DNA substrates, independently of sizes and surface modification of QDs, antibody types, epitope tags, buffer conditions, or DNA structures. We find that polyethylene glycol (PEG), a common polymer coating on QD surfaces, is the primary factor responsible for this effect.

To tackle this problem, we engineered QDs with precisely controlled surface polymer compositions. By systematically changing the ratio of anchoring, hydrophilic, and PEG-based functional groups, we find that reducing PEG density below a critical threshold effectively suppresses protein dissociation while maintaining excellent colloidal stability and brightness. Furthermore, antibodies conjugated via click chemistry between azido groups and DBCO enabled specific labeling of XPA without perturbing the DNA binding activity.

Using these optimized QDs, we conducted single-molecule DNA curtain assays to visualize XPA-DNA interactions. QD-labeled XPA exhibits one-dimensional diffusion with frequent pausing on undamaged DNA. DNA curtain assays revealed that XPA preferentially binds DNA bubbles and searches for bubble structures through both one-dimensional diffusion and three-dimensional collision. Quantitative analysis showed that three-dimensional collision is the dominant pathway for bubble recognition.

Taken together, our results uncover a previously unrecognized limitation of PEG-coated QDs in single-molecule studies and provide a general surface-engineering strategy to preserve native protein-DNA interactions. Newly engineered QDs establish robust platforms for accurate single-molecule visualization of biomolecular processes.

Key words: Quantum dot, Quantum dot-mediated protein dissociation, XPA, single-molecule imaging, DNA curtain

1. Introduction

Semiconductor fluorescent nanoparticles, quantum dots (QDs), have been widely applied in physics, chemistry, and display due to their high brightness and photostability [1-4]. In addition, QDs have been employed for life sciences. Functionalized QDs conjugated with antibodies and nucleic acid probes have been employed for sensitive detection of biomarkers associated with cancers, infectious diseases, and genetic disorders (Figure 1A) [5]. In addition, they have been explored as nano-carriers for drug delivery and gene therapy [6-9]. QDs can also serve as theragnostic platforms by combining therapeutic delivery with real-time imaging, allowing simultaneous monitoring treatment efficacy. Moreover, QDs have been extensively applied for molecular imaging as fluorescent probes [10,11]. The broad excitation spectra enable simultaneous imaging for multiple targets with minimal spectral overlap [12,13]. Furthermore, remarkable resistance to photobleaching compared to conventional organic dyes makes them particularly attractive for monitoring biomolecular interactions over extended time scales.

QDs are also actively adopted for single-molecule imaging [14-20]. Single-molecule imaging has emerged as a powerful tool for elucidating the dynamic behaviors and heterogeneity of biomolecules that are often veiled by ensemble-average effects in traditional biochemical assays [21]. By directly visualizing the activities of individual proteins, nucleic acids and their complexes in real time, the technique has provided unprecedented insights into molecular mechanisms underlying diverse biomolecular reactions. However, the reliability of such observations critically depends on the fluorescence labeling to target molecules. In particular, highly photostable and bright fluorescent probes such as QDs are central to success of single-molecule studies as they enable high-resolution and long-term visualization of molecular events. Despite the broad usefulness of QDs, we report here the surprising discovery that commercial QDs destabilize the protein-DNA complexes and accelerate dissociation of proteins from DNA. We systematically investigated components of QD that interfere with binding of proteins to DNA and developed optimized surface modification and bioconjugation strategies to prevent interference with biochemical processes. We validate our engineered QDs using single-molecule imaging DNA curtain experiments. This new class of QDs should be broadly useful for biomolecular imaging.

2. Methods/ Experimental

2.1 DNA preparation

All DNA oligomers were synthesized from Bioneer (South Korea) and listed in [Supplementary Table 1](#). All DNA constructs (three-way junction, biotinylated three-way junction, and duplex) were prepared by annealing oligomer, for which oligomer mixture was heated at 95°C for 10 min and slowly cooled down to 25°C. For three-way junction DNA, YFA, YFB, and FAM_YFC were mixed at 2:2:1 molar ratio. For duplex, CY5_Dup and Dup_comp were mixed at 1.2:1 molar ratio. For Y-shape DNA, Cy5-Yshape and Yshape-comp were mixed at 1.2:1 molar ratio.

2.2 Commercial quantum dots (QDs)

Anti-FLAG 705 QDs were prepared by labeling ANTI-FLAG M2 antibody (F3165, Sigma-Aldrich) using the SiteClick™ 705 QD™ Antibody Labeling Kit (S10454, Invitrogen). Anti-FLAG 605 QDs were generated by labeling the same ANTI-FLAG M2 antibody with the SiteClick™ 605 QD™ Antibody Labeling Kit (S10469, Invitrogen). Anti-His 705 QDs were prepared by labeling a 6xHis Tag Monoclonal Antibody (MA1-135, Invitrogen) using the SiteClick™ 705 QD™ Antibody Labeling Kit (S10454, Invitrogen). All antibody–QD conjugation reactions were performed according to the manufacturer’s protocols. Streptavidin QDs were obtained commercially (QD™ 705 Streptavidin Conjugate, Q10163MP, Invitrogen) and used as received.

2.3 Protein purification

Wild-type full length XPA with 6xHis only, 3xFLAG and 6xHis, or no epitope tag at amino-terminus were cloned into pBG100 plasmid vector (Figure 1B and S9A). A deletion mutant of XPA (XPA_ΔIDR) lacking the intrinsically disordered region (1 to 97 amino acids), which had 3xFLAG and 6xHis at the amino terminus, was also cloned into pBG100 plasmid vector. All wild-type XPA proteins and XPA_ΔIDR were purified by the same following procedure. The plasmid containing XPA gene was transformed into Rosetta (DE3) *E coli* strain. Two liters of *E coli* cells were grown at 37°C in LB media supplemented with 50 µg/ml Kanamycin until OD₆₀₀ reached ~0.6. Temperature was then cooled down to 18°C and cells were further grown until OD₆₀₀ reached ~1.2. The protein was expressed with 1 mM IPTG (Isopropyl β-D-1-

thiogalactopyranoside) at 18°C overnight. Cells were harvested by centrifugation at 6,700 g and 4°C for 30 min. The cell pellet was resuspended in 45 mL of lysis buffer (100 mM Tris-HCl [8.0], 500 mM NaCl, 10% Glycerol, 20 mM imidazole, 5 mM β ME (2-mercaptoethanol), 10 μ M ZnCl₂, 200 μ g/ml lysozyme, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM benzamidine, and 1x halt protease inhibitor (78438, Thermo scientific). Then the cells were lysed by 20 strokes of Dounce homogenizer (357546, Duran) and sonication (Q700, Qsonica) at 30% amplitude with total 2 min pulse-on (10 sec pulse-on and 10 sec pulse-off). Cell lysates were clarified by ultracentrifugation at 46,500 g and 4°C for 40 min. The clarified lysates were then incubated for 1.5 hours with 8 ml of Ni-NTA resin (HisPur™ Ni-NTA Resin, Thermo scientific) pre-equilibrated with Ni-NTA bind buffer (100 mM Tris-HCl [8.0], 500 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 5 mM β ME, and 10 μ M ZnCl₂). The resins were gently poured into an empty column with gravity flow and washed twice with 20 ml of Ni-NTA wash buffer (100 mM Tris-HCl [8.0], 500 mM NaCl, 10% (v/v) Glycerol, 30 mM imidazole, 5 mM β ME, and 10 μ M ZnCl₂). XPA protein was eluted with 3-hour incubation in 10 ml of Ni-NTA elution buffer (100 mM Tris-HCl [8.0], 300 mM NaCl, 10% (v/v) glycerol, 250 mM imidazole, 5 mM β ME, and 10 μ M ZnCl₂). The eluants were subsequently loaded onto heparin column (HiTrap Q HP, GE Healthcare), which was pre-equilibrated in Heparin buffer (20 mM Tris-HCl [8.0], 50 mM NaCl, 10% (v/v) glycerol, 5 mM β ME, and 10 μ M ZnCl₂). The proteins were eluted around 0.5 M during salt gradient from 0.1 M to 1.5 M of NaCl in Heparin buffer. The eluted XPA was further purified by gel filtration (HiLoad 16/600 Superdex 75 pg, Cytiva), which was pre-equilibrated in GF buffer (50 mM Tris-HCl [8.0], 150 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, and 10 μ M ZnCl₂). The final eluants were concentrated with Amicon (MWCO: 3 kDa) and were snap-frozen in liquid nitrogen for -80°C storage until use.

2.4 *In vitro* NER assay

In vitro NER assay were performed as described previously [22]. 5 nM of XPC-RAD23B, 10 nM of TFIIH, 20 nM of XPA, 41.6 nM of RPA, 27 nM of XPG, and 13.3 nM of ERCC1-XPF were used. All proteins were >95% pure and produced as previously described for XPC-RAD23B [18], TFIIH [23], RPA [24], XPG [25], and ERCC1-XPF [26]. The reactions were conducted in repair buffer containing 45 mM HEPES-KOH at pH 7.8, 5 mM MgCl₂, 0.3 mM EDTA, 40 mM phosphocreatine (di-Tris salt, Sigma), 2 mM ATP, 1 mM DTT, 2.5 μ g BSA, 0.5 μ g creatine phosphokinase (Sigma), and NaCl (to a final concentration of 70 mM) in a volume

of 10 μ L. The mixtures were pre-warmed at 30°C for 10 min and then 25 ng of plasmid DNA containing GTG 1,3-Cisplatin was added and incubated at 30 °C for different incubation times (0, 15, 45, and 90 min). 0.5 μ L of 1 μ M of a complementary oligonucleotide (5'-GGG GGA AGA GTG CAC AGA AGA AGA CCT GGT CGA CCp-3') for product labeling was added and the mixture heated at 95°C for 5 minutes. The sample was allowed to cool down to room temperature for 15 minutes, followed by adding 1 μ L of a Sequenase/[α -32P]-dCTP mix (0.25 units of Sequenase and 2.5 μ Ci of [α -32P]-dCTP per reaction) and incubating at 37°C for 3 minutes. Then 1.2 μ L of dNTP mix (100 μ M of each dATP, dTTP, dGTP; 50 μ M dCTP) was added and incubated for another 12 minutes. The reactions were stopped by adding 12 μ L of loading dye (80% formamide and 10 mM EDTA) and heating at 95°C for 5 minutes. Sample was loaded on 14% sequencing gel (8 M urea in 0.5x TBE) and ran at 45 W for 2.5 hours. The reaction products were visualized using PhosphorImager (Typhoon RGB, Cytiva).

2.5 Electrophoretic mobility shift assay

To test the activity of purified XPA, we performed electrophoretic mobility shift assay (EMSA). 5 nM of three-way junction or duplex DNA was incubated with different concentrations of XPA (0, 10, 20, 40, 80, 120, and 200 nM) in reaction buffer (25 mM Tris-HCl [8.0], 60 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol and 1 mM EDTA) with or without 0.1 mM ZnCl₂ at 25°C for 30 min in a dark room to prevent the photobleaching of fluorophore. The reactants were loaded onto 8% non-denaturing polyacrylamide gel and run in 0.5x TBE buffer at 4°C. The gel was then scanned by Amersham typhoon RGB (GE Healthcare). The band intensity was analyzed by ImageJ (NIH). The bound fraction was calculated by dividing the intensity of bound band by the sum of bound and free DNA band intensities.

2.6 QD-mediated XPA dissociation test

5 nM of three-way junction DNA and Y-shape DNA were incubated with 30 nM and 60 nM XPA in reaction buffer at 25 °C for 30 min in a dark room, respectively. Then commercial or synthesized QDs at different concentrations (0, 3, 10, 30, and 100 nM) were added, followed by 30-min incubation at 25 °C in a dark room. These samples were loaded onto 8% nondenaturing polyacrylamide gel and run in 0.5x TBE buffer at 4°C. The gel was then scanned by Amersham typhoon RGB (GE Healthcare). The band intensity was analyzed by ImageJ (NIH). The bound fraction was calculated by dividing the intensity of bound band by the sum

of bound and free DNA band intensities. And each bound fraction was divided into the 0 nM QD condition's bound fraction for normalization.

2.7 QD synthesis

2.7.1 Materials for QDs

Cadmium oxide (CdO, 99.99%), oleic acid (90%), zinc acetate (99.99%), 1-octadecene (90%), selenium (99.9%), sulfur (99.5%), Trioctylphosphine (TOP, 90%), 1-Dodecanethiol (DDT, 98 %), 2,2,2-Trifluoroethanol (TFE), Histamine dihydrochloride, (%), [3-(Methacryloylamino)propyl]dimethyl(3-sulfopropyl)ammonium hydroxide inner salt (96%), 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid, 4,4'-Azobis(4-cyanovaleric acid) ($\geq 98\%$), Triethylamine (TEA), trifluoroacetic acid (TFA), 2-(2-Aminoethoxy)ethanol (AEE, 98%), 4,4'-Azobis(4-cyanovaleric acid) ($\geq 98\%$), bovine serum albumin (BSA) (98%), DBCO-TFP ester, ANTI-FLAG® M2 antibody, Anhydrous N,N-Dimethylformamide (DMF), Dichloromethane (DCM), Tween-20, DL-dithiothreitol (DTT), sodium chloride solution were purchased from Sigma-Aldrich. PBS buffer was purchased from Biosesang (Korea). All aqueous solutions were prepared with DIW, which was purified by a Milli-Q Purification System (Millipore, United States).

2.7.2 QD Characterization

The UV-Vis spectra were measured by UV-1800 (Shimadzu, Japan) and by NanoDrop instrument, BioSpec-nano (Shimadzu, Japan). Fluorescence spectra were measured by a VARIZN. Photoluminescence quantum yield (PLQY) was measured by QE-2000 (Otsuka, Japan). BRUKER AVANCE III HD spectrometer (400 MHz) was used to record the ^1H Nuclear Magnetic Resonance (NMR) spectra. All NMR analysis were conducted in D_2O solvent. Fourier transform infrared (FT-IR) spectra were recorded on an IRTracer-100 (Shimadzu, Japan). Transmission electron microscopy (TEM) imaging was measured by JEM-2100 (JEOL), using an acceleration voltage of 200 kV using Cu grids (Ted Pella, USA). X-ray Photoelectron Spectroscopy (XPS) measurements were conducted using a monochromated Al-K α radiation source and He I (21.2 eV) discharge lamp (ESCALAB 250Xi, Thermo Fisher Scientific, USA). Time-resolved PL (TRPL) measurements were carried out using a time-correlated single-photon-counting setup (Fluo-Time 300, PicoQuant, German) at 25 °C. X-ray

Diffraction (XRD) analysis was conducted with a SmartLab instrument (Rigaku, Japan) operating at 45 kV/200 mA using a Cu K α_1 radiation source ($\lambda = 1.5406 \text{ \AA}$). The hydrodynamic size, and zeta potential of the nanoparticles were obtained using dynamic light scattering (DLS, Nano ZS90, Malvern Instruments). Measurements were carried out at 25 °C using disposable cuvettes with a 4 mW, 633 nm laser and a fixed scattering angle of 90°, and all samples were diluted with De-Ionized Water (DIW). FPLC analyses were performed on NGC Chromatography System (Bio-Rad, USA) equipped with multi detector for protein and nanoparticle measurement and a FPLC column packed with Superose 6 10/300 resin (Cytiva, 17-0489-01) with 50 mM phosphate buffer [7.4] as the eluent.

2.7.3 Synthesis of polymer ligands

Red emitting alloy-typed CdZnSeS/ZnS QDs were synthesized as described [27,28] with a few modifications. The polymer was synthesized via RAFT polymerization following a previously reported protocol [29], and the monomers corresponding to the anchor and functional groups were synthesized according to literature with modification [5,30]. Briefly, the origin solvent of anchor and functional monomers was degassed to remove solvent, and monomers were dispersed with the certain ratio in the mixed TFE and methanol (2:1, v/v) solvent. RAFT agent and initiator were added at the ratio of 1:1. The mixture was dispersed and then transferred to an ampule. After 4 repeats of freeze-pump-thaw, the ampule was sealed by a torch under vacuum and put in an oil bath (70 °C, 12 h) for polymerization reaction. After polymerization, the crude polymer was washed with excess ethyl ether and hexane for 2 times. To activate the anchoring functionality, the polymer was treated with an acidic solvent mixture (TFE:TFA = 1:1) for 1 h to remove the BOC protecting group. The resulting deprotected by-products were removed by two washing steps using an anti-solvent mixture using excess ether and hexane. The pH was then adjusted back to neutrality using triethylamine (TEA), and the polymer was thoroughly dried.

2.7.4 Antibody conjugation with surface modified QDs

For antibody functionalization, DBCO-TFP ester (3.5 mM in DMSO) was first conjugated to the ANTI-FLAG M2 antibody. The antibody (50 μ g) was purified prior to reaction to remove residual sodium azide using centrifugal membrane filtration (Amicon Ultra-4, 50 kDa) twice with 1 $\times$ PBS (pH 7.4), followed by an additional wash with pH 9 NaHCO₃ buffer. The purified

antibody was then mixed with DBCO-TFP ester solution with 25~30 molar equivalent excess in pH 9 NaHCO₃ buffer and reacted at 30 °C for 2 h. The resulting DBCO-modified antibody (DBCO-Ab) was purified by centrifugal filtration (three cycles), and the antibody concentration at each purification step was monitored by UV spectroscopy using a NanoDrop. Separately, QD-N₃ was purified by FPLC and directly used for conjugation. The DBCO-Ab and QD-N₃ were mixed at the desired molar ratio and incubated overnight at 4 °C to complete the strain-promoted azide–alkyne cycloaddition (SPAAC) click chemistry. The final QDs–antibody conjugates (QD-Ab) were purified by FPLC and concentrated using centrifugal membrane filtration (Amicon Ultra-4, 50 kDa).

2.7.5 Nonspecific binding (NSB) test of with BSA protein

BSA solutions of various concentrations were added to water-dispersed QDs (0.2 μM). After incubation for 1 h, the mixtures were subjected either to agarose gel electrophoresis or loaded onto an FPLC sample loop to evaluate the extent of nonspecific adsorption of BSA onto the QD surface. And 0.5 wt% agarose gel (Conda) was prepared and mounted on an electrophoresis system (Mupid-exU, Mupid). The chamber was filled with 300 mL of 1× TAE buffer (Biosesang), and the prepared QD samples were carefully loaded into the wells. Electrophoretic separation was performed at 100 mV for 30 min.

2.8 DNA curtain assays

2.8.1 Lambda phage DNA preparation

For DNA curtain assays, 500-fold molar excess of Biotin-cos-R and multi-Dig-cos-L oligomer was added to lambda phage DNA (New England Biolabs, N3011) and incubated at 65°C for 20 minutes and then slowly cooled to 23°C and subsequently 20 U/μL of T4 DNA ligase (Enzymonics, M001) was added for ligation. Excessive free oligonucleotides were removed by gel filtration spin column (Illustra MicroSpin S-400, GE Healthcare).

On the other hand, λ-DNA containing 57-nt bubble was prepared as in the previous studies (Figure S7) [16,31]. 5 nM I3 λ-DNA containing seven serial Nt.BspQI nickase recognition sites was incubated with 0.8 U/μL of Nt.BspQI (New England Biolabs, R0644) in a reaction buffer containing 50 mM Tris-HCl (pH 7.9), 1 mM ATP, 100 mM NaCl, 10 mM MgCl₂, and 100 μg/mL recombinant albumin. The reaction was carried out at 50°C for 2.5 hours to create a single-stranded gap at a specific location within the λ-DNA. Following nickase digestion, the

Nt.BspQI was inactivated by treatment with 0.5 mg/mL proteinase K. To facilitate gap hybridization and labeling, a 500-fold molar excess of Biotin-cos-R, multi-Dig-cos-L, and a λ -I3-bubble oligonucleotide was added to the reaction. The mixture was incubated at 80°C for 20 minutes and then slowly cooled to 23°C to allow oligonucleotide hybridization and inactivation of proteinase K. Subsequently, 20 U/ μ L of T4 DNA ligase (Enzynomics, M001) was added, and the reaction was incubated overnight at 23°C to promote ligation. The ligase was inactivated by heating the mixture at 65°C for 20 minutes. Unbound oligonucleotides were removed using a gel filtration spin column (Illustra MicroSpin S-400, GE Healthcare).

2.8.2 Flow cell preparation

For single-tethered DNA curtain assays, nano-trench patterns were engraved on fused-silica slides [32]. For double-tethered DNA curtain assays, nanometer-high diffusion barriers and pentagon-shaped pedestals made of chromium were used [33]. Both types of slides were cleaned and assembled into flow cells as described previously [32,33]. Flow cells were washed with lipid buffer (20 mM Tris-HCl [8.0] and 100 mM NaCl) and then incubated with 0.5% biotinylated-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)) (Avanti Polar Lipids, 870273), 8% mPEG 2000-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) (Avanti Polar Lipids, 880130), and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids, 850375) in lipid buffer for 10 minutes. After free liposomes were washed with lipid buffer, lipid bilayer was promoted on the slide surface for 20 minutes. For double-tethered DNA curtain experiments, 200 μ L of 0.1 mg/mL anti-digoxigenin Fab fragment (Roche, 11214667001) was subsequently introduced into the flow cell and incubated for 40 minutes. The surface was passivated by BSA buffer (40 mM Tris-HCl [8.0], 50 mM NaCl, 2 mM MgCl₂, and 0.2 mg/mL BSA). Successively, 25 μ g/mL streptavidin in BSA buffer was incubated for 10 minutes. After unbound streptavidin was removed with BSA buffer, 40 pM biotinylated λ -DNA was anchored on the biotinylated lipid bilayer through streptavidin. The flow cell was placed on the custom-built total internal reflection fluorescence (TIRF) microscope and connected with a syringe pump fluidic system. All DNA curtain experiments were conducted at 23°C. DNA curtains were formed in XPA imaging buffer (25 mM Tris-HCl [8.0], 60 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/mL BSA).

2.8.3 Single-tether DNA curtain assays

Single-tethered DNA curtains were built up with multi-biotinylated λ -I3 containing a 57-nt bubble structure between 33,544 bp and 33,600 bp (Figure S7). 50 nM 3xFLAG tagged XPA was pre-incubated with 50 nM α FLAG-QD on ice for 10 minutes. Then, 1 nM XPA-QD were injected into a flow cell through a 100 μ L injection loop at 0.2 mL/min flow rate, followed by 5-minute incubation without buffer flow. Then the flow resumed to wash out unbound XPA-QDs. Fluorescence images were collected using NIS-Elements software (Nikon) with 0.1 second exposure time.

2.8.4 Double-tethered DNA curtain assays

To investigate the motion of XPA on undamaged DNA, double-tethered DNA curtains with undamaged λ -DNA were used. To observe diffusion of XPA, 1 nM XPA-Q-dot complexes were injected into the flow cell through a 100 μ L injection loop in XPA buffer at different NaCl concentrations (0, 25, and 50 mM) at a flow rate of 0.2 mL/min, followed by a 5-minute incubation without buffer flow. Unbound XPA-QD complexes were then washed out, and QD fluorescence was measured. To examine how XPA recognizes a bubble, 1 nM XPA-QD complexes were injected into double-tethered DNA curtains with bubble-containing λ -DNA in XPA imaging buffer. Images were taken immediately after the injection was stopped when the proteins maximally arrived at the DNA curtains. All DNA curtain images were collected for 10 minutes using NIS-Elements software (Nikon) in the absence of buffer flow with 0.1 second exposure time.

2.9 Data analysis for DNA curtains

2.9.1 Binding distribution of XPA

The binding distribution of XPA was analyzed using single-tethered DNA curtain assays using 57-nt bubble-containing λ -DNA. Each QD fluorescence signal was fitted with two-dimensional Gaussian function, and the QD bound position was estimated from the distance between the center position and the nano-barrier. The distribution histogram of QDs was generated with 1 kbp bin size using the Origin software (Origin Labs). The distribution was fitted with a Gaussian function, and the center position of the fitted Gaussian was defined as the main binding position.

2.9.2 Immobile and mobile analysis of XPA

For individual DNA molecules from the double-tethered DNA curtain assays, kymographs were generated using ImageJ. In kymographs, molecules that remained at a fixed position for longer than 5 seconds were classified as immobile, whereas molecules showing movement for longer than 5 seconds were classified as diffusive or mobile. The relative fractions of diffusive and immobile molecules were estimated by number of each state divided by total number of both states.

To test the sequence dependency of immobile states, a distribution histogram for the positions at which XPA was immobile was generated with a bin size of 1 kbp. In addition, to check whether immobile states repeatedly occurred at the same positions of individual DNA molecules, pairwise relative displacements between immobile positions were calculated within the same DNA molecule. A distribution histogram for these relative displacements were built up with a bin size of 1 kbp.

2.9.3 Diffusion coefficients and theoretical upper limit of diffusion coefficient under helical rotation

Using the ImageJ (NIH) plug-in MOSAIC suite particle tracker, time trajectories of XPA molecules were obtained. Only molecules showing mobile diffusion for more than 20 seconds were analyzed in double-tethered DNA curtain experiments using undamaged λ -DNA. From these trajectories, the mean square displacement (MSD) was calculated using a custom-written MATLAB program (MathWorks) according to the standard definition,

$$MSD(n, N) = \sum_{i=1}^{N-n} \frac{(Y_{i+n} - Y_i)^2}{N-n},$$

where N is the total number of frames, n is the measurement window ranging from 1 to $N-1$, and Y is the position of XPA along DNA. The standard deviation (SD) of the MSD, which was used as the error estimate, was calculated as

$$SD = \sqrt{\frac{(2Di\Delta t)^2(2t^2+1)}{3i(N-i+1)}}.$$

Finally, the 1D diffusion coefficient (D) of each XPA molecule was obtained from the MSD using equation,

$$MSD = 2Dn\Delta t.$$

The value D was determined by linear fitting of the first three MSD data points.

Using the Einstein relation, the theoretical upper limit of the diffusion coefficient under helical rotation can be calculated as.

$$D = \frac{k_B T}{\xi},$$

where k_B is Boltzmann constant, T is absolute temperature, and ξ is the friction coefficient.

For the helical rotation along DNA, the friction coefficient is modified to,

$$\xi = 6\pi\eta R + \left(\frac{2\pi}{10 BP}\right)^2 [8\pi\eta R^3 + 6\pi\eta R(R_{OC})^2],$$

where η is buffer viscosity, R is the radius of protein, BP is the distance between adjacent base pairs (0.34 nm), and R_{OC} is the distance between DNA helical axis and the center of mass of the protein. In our DNA curtain experiments, XPA was labeled with QD, and hence R_{OC} is given as the sum of XPA radius and the hydrodynamic radius of antibody-conjugated QD. The radius of XPA is ~ 2.45 nm from crystal structure (PDB ID: 1XPA). The hydrodynamic radius of antibody-conjugated QD is ~ 4.85 nm. So, R and R_{OC} are approximately ~ 7.31 nm and ~ 8.31 nm, respectively. $k_B T$ is approximately 4.1 pN nm at 23°C. Based on these parameters, the rotationally constrained upper limit of the diffusion coefficient was estimated to be $\sim 0.062 \mu\text{m}^2/\text{sec}$.

2.9.4 Relative displacement of XPA molecule

Time-resolved trajectories of XPA molecules were obtained through particle tracking using the MOSAIC plug-in in ImageJ (NIH). To quantify the relative displacement of XPA along DNA, the positional change within defined time intervals was calculated from these trajectories. Specifically, the displacement over one frame (0.1 seconds) was defined as the 1-step value, the displacement over two frames (0.2 seconds) was defined as the 2-step value, and the displacement over three frames (0.3 seconds) was defined as the 3-step value. These displacement distributions were then fitted with a Gaussian function. The centers of the fitted Gaussians were found to be at zero, indicating that the XPA molecules exhibit Brownian motion along DNA.

3. Results

3.1 Commercial QDs dissociate XPA protein from DNA

Human XPA with 6xHis and 3xFLAG at the amino terminus was purified using Ni-NTA column (Figure 1B and S1A). The activity of purified tagged XPA was tested using *in vitro* NER assays. A plasmid containing a site-specific 1,3-cisplatin interstrand lesion was incubated with purified XPA along with the NER core proteins (XPC-RAD23B, TFIIH, RPA, XPG, ERCC1-XPF), and the excision of the lesion containing oligonucleotide monitored in a time-dependent reaction. Product formation was observed in the expected manner, showing that the purified XPA was active (Figure S1B) [22]. We next conducted electrophoretic mobility shift assay (EMSA) to examine the binding affinity of the purified XPA to DNA. As previously reported, the XPA preferentially bound to 3-way junction DNA compared with homoduplex (Figure 1C and 1D).[22,34] A supershift of the XPA–DNA complex was observed in EMSA upon addition of anti-FLAG or anti-His antibodies, confirming antibody binding to purified XPA (Figure S1C and S1D).

To fluorescently label the purified XPA, we prepared anti-FLAG antibody-conjugated QDs with an emission peak at 705 nm using the commercial QD-antibody conjugation kit. We freshly conjugated XPA and QD for each experiment as QDs must be stored at 4 °C whereas purified XPA is stored at – 80°C for a long term. In addition, antibody-conjugated QDs allows orthogonal labeling of proteins having different epitope tags. We tested the binding of anti-FLAG antibody-conjugated QD (α FLAG-QD) to XPA by supershift in EMSA. Unexpectedly, with increasing concentration of α FLAG-QDs, a free DNA band emerged while XPA-bound DNA band disappeared (Figure 1E). Given that XPA binds to DNA in the presence of an anti-FLAG antibody, the data suggested that QDs added induce the dissociation of XPA from the 3-way junction DNA (Figure S1C).

3.2 XPA dissociation is not affected by antibodies, buffer composition, epitope tags, and DNA substrates

We examined what induced the XPA dissociation from DNA in the presence of antibody-conjugated QDs. We first tested a different QD with a 605 nm emission wavelength, which also caused the dissociation of XPA from DNA (Figure 1F and S2A). Changing the conjugated

antibody from anti-FLAG to anti-His (α His-QD) also dissociated XPA from DNA (Figure 1F and S2A). Moreover, a commercial streptavidin-conjugated QD also caused XPA dissociation (Figure 1F and S2A). These results demonstrated that the XPA dissociation is independent of the type, size, and conjugated antibodies of QDs.

Since XPA has a zinc-finger domain, Zn^{2+} might affect the DNA binding (Figure 1B). To examine the Zn^{2+} effect on XPA dissociation in the presence of QDs, we removed Zn^{2+} from the buffer and added a divalent cation chelator EDTA. In the absence of Zn^{2+} , QDs still dissociated XPA from the 3-way junction DNAs (Figure S2B). We also suspected that the epitope tags at the amino terminus might influence DNA binding. Hence, we tested 6xHis-XPA and XPA without a tag. Regardless of the presence of epitope tags, α FLAG-conjugated QD led to the XPA dissociation (Figure S2C). Finally, we changed a DNA substrate from 3-way junction DNA to Y-shape DNA (fork structure), which is also a good substrate of XPA.[34] XPA also dissociated from Y-shape DNA in the presence of QDs (Figure S2D). Taken together, our data demonstrated that the XPA dissociation from DNA substrates does not result from the intrinsic interaction between XPA and DNA but results from QDs themselves.

3.3 Polyethylene glycol (PEG) coated on the QD surface causes XPA dissociation from DNA

Given that the XPA dissociation could not be attributed to types of commercial QDs, we suspected that the polymers that are coated on the QD surface might disrupt the binding between XPA and DNA. Polyethylene glycol (PEG) is commonly used to coat the surface of commercial QDs to stabilize the QDs. To test if PEG on the QD surface is indeed related to the XPA dissociation, we synthesized QDs that are covered with 500 Da PEG (PEG500) or 1000 Da PEG (PEG1000) (Figure 2A and S3A). Both PEG-coated QDs induced XPA dissociation (Figure 2A, 2C, and S3A). We also tested QDs, in which PEG was partially modified with azido- or carboxyl-groups. The partial modification similarly led to the dissociation of XPA, showing that PEG is a dominant factor for the XPA dissociation from DNA (Figure 2B, 2C, and S3B). We finally tested the effect of PEG itself on the XPA-DNA complex. With increasing concentrations of PEG of different molecular weights (200, 400, and 1000 Da), the band for free DNA increased, suggesting that PEG itself induces the XPA dissociation from DNA, independently of the molecular weight of the PEG (Figure 2D, 2E, S3C, and S3D). High

concentrations of polymers can influence binding between proteins and nucleic acids due to the molecular crowding effect.[35-37] To test if the XPA dissociation from DNA in the presence of concentrated PEG 1000 would be due to the molecular crowding effect, we titrated the unrelated polymers 35000 Da dextran (Dextran35000) to the XPA-DNA binding reactions. Even at a high concentration (30%) of Dextran35000, XPA remained bound to DNA (Figure 2D and 2E). These results demonstrated that the destabilization of the XPA-DNA interaction does not result from the molecular crowding effect but from the interaction with PEG.

3.4 Surface modification of QDs

Given that PEG on the QD surface induces XPA dissociation from DNA, we engineered the QD surface to prevent the interference with biomolecular interactions. To this end, CdZnSeS/ZnS alloy-typed QDs were synthesized according to published protocols with a few modifications (Figure S4A).[27,28] Synthesized Qdots offer stable and superior features for subsequent aqueous ligand-exchange. These QDs exhibited a uniform particle size (9.20 ± 0.55 nm) (Figure S4B). UV-vis absorption and photoluminescence (PL) emission spectra exhibited well-defined first excitonic absorption and narrow emission, with high quantum yield (QY) reaching ~100% (Figure S4C). In addition, oleic-acid-based surface ligands were confirmed by FT-IR spectroscopy (Figure S4D). For water dispersion of QDs, polymer ligands were designed, consisting of three key components: an anchor group (A group), a hydrophilic group (H group), and a functional group (F group) (Figure 3A and S5A). A group binds strongly to the QD surface, while the H group enables water dispersion; together, these two components play a central role in determining the colloidal stability of QDs. On the other hand, the F group, based on PEG, is responsible for specific targeting of cells or biomolecules. The reason for introduction of PEG chain into F group is increasing the conjugation efficiency by exposing it to the outside because the hydration layer of zwitterion in H group can hinder the chemical reaction of functional moieties with biomolecules. The polymer ligand was synthesized via reversible addition-fragmentation chain-transfer (RAFT) polymerization. The three monomer components, corresponding to A, H, and F groups, form a random copolymer, producing the final polymer ligand. We ensured the incorporation of each component into the polymer backbone with ^1H NMR measurement (Figure 3B). Using this polymer, we did surface modification with two-step ligand-exchange strategy to obtain compact and water-dispersible QDs (Figure 3A).[29]

To confirm successful surface modification, a series of spectroscopic and colloidal analyses were performed. After ligand exchange, the ^1H NMR spectrum of purified QDs showed a pronounced reduction in imidazole-related proton signals from the A-group, indicating strong coordination of the anchor units to the QD surface, while signals from the zwitterionic H-group and PEG-based F-group remained, confirming the presence of the polymer ligand (Figure S5B). FT-IR (Fourier Transform Intra-Red spectroscopy) analysis showed that the characteristic azide stretching band at 2100 cm^{-1} was retained after ligand exchange, indicating the availability of azide groups for subsequent bioconjugation (Figure S5C). XPS (X-ray Photoelectron Spectroscopy) analysis confirmed that the characteristic Cd, Zn, Se, and S signals were preserved after polymer coating, while increased C 1s and O 1s signals and the appearance of an N 1s peak verified successful polymer ligand introduction without disruption of the core/shell structure (Figure S5D). Furthermore, the polymer-coated QDs remained well dispersed over a wide pH range (5–13) and high salt concentrations (0.5–2 M NaCl), with unchanged hydrodynamic size confirmed by DSL (Dynamic Light Scattering) (Figure S5E). UV–vis absorption and PL emission spectra showed that high emission efficiency was largely preserved after aqueous phase transfer, with the PLQY (Photo-Luminescence Quantum Yield) reaching approximately 94.8% (Figure S5F). XRD (X-Ray Diffraction) analysis further confirmed that the characteristic (111), (220), and (311) diffraction peaks were maintained without peak broadening, indicating preserved crystalline phase and structural integrity after ligand exchange (Figure S5G).

3.5 QDs with reduced PEG content on the surface do not induce XPA dissociation from DNA

To avoid XPA dissociation by QDs, we systematically changed the relative fraction of polymers on the QD surface. We changed the relative fraction of A-group and F-group with roughly similar fraction of H-group. QDs with a lower amount of F-group relative to the A-group, QDs did not detach XPA from DNA (Figure 3C and 3E). By contrast, QDs with a relatively high amount of F-group compared with that of A-group caused dissociation of XPA from DNA as we had observed with the commercial QDs (Figure 3D and 3E). These results supported our hypothesis that PEG is the major factor interfering with the XPA-DNA interaction. When we tested a number of different ratios between A-, H-, and F- groups, A-group and F-group showed anti-correlation for the XPA dissociation (Figure 3F and S6).

Quantitatively, we found that QDs did not dissociate the protein from DNA when the F-group content was less than 7% (Figure 3F and Table S1).

3.6 Antibody is conjugated with QDs via SPAAC chemistry

Azide-functionalized QDs (QD-N₃) were conjugated with DBCO-modified antibodies (Ab-DBCO) via strain-promoted alkyne-azide cycloaddition (SPAAC) click chemistry to generate QD and Ab conjugates (QD-Ab) (Figure 4A).[38] To conjugate XPA containing a FLAG tag, an anti-FLAG antibody was used. In Step 1, TFP ester group of DBCO-TFP ester was reacted with primary amines on the antibody to introduce the DBCO moiety onto the Ab. After purification, successful DBCO modification was confirmed by UV-vis spectroscopy, showing the characteristic antibody absorption at 280 nm together with an additional absorption feature at ~309 nm corresponding to the DBCO group (Figure 4A and 4B). The concentration of Ab-DBCO was determined based on these absorption characteristics. In Step 2, the DBCO-Ab were reacted with QD-N₃, yielding a triazole-linked QD-Ab conjugate through SPAAC chemistry. After conjugation, subtracting the QD-N₃ baseline from the QD-Ab spectrum revealed the distinct 280 nm Ab peak, confirming that the antibody was successfully coupled to the QD surface (Figure 4A and 4C). The conjugation was further validated by DLS analysis. The hydrodynamic diameter increased from that of QD-N₃ (15.1 nm), indicating a larger size upon antibody conjugation (18.5 nm) (Figure 4D). Finally, the number of antibodies conjugated per QD was evaluated by increasing the ratio of antibody and monitoring the peak intensity of fast protein liquid chromatography (FPLC) elution profile of Ab-DBCO at 33.8 min. As the Antibody-to-QD ratio increased, the elution peak gradually shifted toward shorter retention times, confirming successful antibody conjugation (Figure 4E). Based on these chromatographic changes, an antibody-to-QD ratio of 3 was selected for subsequent experiments to maintain a compact particle size suitable for single-QD imaging. These results confirmed that the antibody is successfully and specifically conjugated to the QD surface.

3.7 Single-molecule imaging shows that new QDs do not dissociate XPA from DNA

We finally tested if antibody-conjugated QDs with reduced amount of PEG would be able to monitor the association of XPA in DNA and avoid the induction of dissociation. In nucleotide excision repair, XPA is engaged in a bubble that is formed by TFIIH at a damage site and serves as a scaffold protein for the pre-incision complex.[19,39,40] Since XPA preferentially

binds a bubble, a single 57-nt bubble construct was inserted into lambda DNA at a specific location (33,498~33,655 bp) (Figure S7). DNA curtains were built up with the bubble-containing lambda DNA (Figure 5A). We used single-tethered DNA curtain assays, in which only one end of lambda DNA is modified with biotin and anchored on a lipid bilayer, while the other end is free (Figure 5A). Continuous buffer flow is required to align and stretch DNA molecules at a diffusion barrier (Figure 5B, top). When QD-labeled XPA was incubated with the lambda DNA, a majority of QDs were well aligned at the bubble location on the DNA curtain (Figure 5B, 2nd panel). When the buffer flow was turned off, the QDs disappeared, indicating that QD-labeled XPA bound to DNA (Figure 5B, 3rd panel). When QDs not conjugated to XPA were incubated, no QDs bound to DNA were observed (Figure 5B, bottom). The binding distribution of QDs exhibited one distinct peak at 33.7 ± 2.1 kbp, corresponding to the bubble location (Figure 5C). Taken together, these data demonstrated that QD-labeled XPA preferentially binds to a bubble in DNA curtains.

We next tested how XPA searches for a bubble using the double-tethered DNA curtain assay, in which the other end of lambda DNA is tethered to pedestals via antigen-antibody linkage (Figure S8A). In the absence of a bubble on lambda DNA, we observed that XPA diffuses along DNA with frequent immobile states (pauses) (Figure 5D). The immobile fraction was approximately 60% regardless of ionic strength, which was consistent with the previous report (Figure 5E).[19] We analyzed the diffusive motion of XPA using particle tracking (Figure S8B and S8C). The relative displacements exhibited Gaussian distributions with their peaks at zero, indicating Brownian motion (Figure S8D). We analyzed the positions where XPA is in the immobile state. We found them to be sequence-independent, suggesting that the immobile states occur at random sequences (Figure 5F). However, the relative distance between positions of immobile states was mostly zero, showing that XPA repeatedly showed immobile states at the same locations in a single DNA molecule (Figure 5G). Next, diffusion coefficients were estimated from the linear fitting from mean square displacement and estimated to be approximately $0.27 \mu\text{m}^2/\text{sec}$ (Figure 5H and S8E). The diffusion coefficients were independent of ionic strength, suggesting that XPA diffuses along DNA not via hopping but via sliding (Figure 5H).[18,20] We then investigated how XPA searches for a DNA bubble using the double-tethered DNA curtain assays, which allow observing XPA behaviors on DNA in the absence of hydrodynamic force (Figure 5I). Molecules that were initially bound to duplex DNA

and subsequently moved to the bubble structure were classified as one-dimensional (1D) diffusion, whereas those directly bound to the bubble position without diffusion were classified as three-dimensional (3D) collision. XPA recognized the bubble through both 3D collision and 1D diffusion (Figure 5J and 5K). However, 3D collision prevailed over 1D diffusion for XPA's searching for ss/dsDNA junctions (Figure 5L).

4. Discussion

We found that commercial QDs promote the dissociation of XPA from its nucleic acid substrates. We were able to attribute this dissociation to the PEG polymer coated on the QD surface. Initially, we considered the molecular crowding of PEG as a cause. This effect has been shown to affect protein conformation, dynamics, and function via the excluded volume effect by forming large clusters that physically block the active site.[41] However, this hypothesis was challenged by the observation that high concentrations of dextran did not induce XPA dissociation (Figure 2D and 2E), arguing against that molecular crowding is not the primary mechanism. Next, we considered the role of XPA's intrinsically disordered domains (IDRs). IDRs may function in protein-nucleic acids or protein-protein interactions.[19,42,43] We hypothesized that PEG might restrict this conformational flexibility, thereby impairing XPA's ability to adopt a stable DNA-bound conformation and reducing the binding stability. To test this hypothesis, we purified a deletion mutant lacking the N-terminal IDR (1~97 amino acids) of XPA (XPA_ΔIDR) (Fig S9A and S9B). XPA_ΔIDR showed similar binding affinity to that of wild-type XPA (Figure S9C and S9D). Addition of anti-FLAG antibody-conjugated commercial QDs also induced the dissociation of XPA_ΔIDR from DNA, which excluded this possibility (Figure S9E). Instead, we focused on the nature of the XPA-DNA interaction itself. Since the interaction is not sequence-specific but structure-dependent, it relies heavily on water-mediated hydrogen bonding and electrostatic interactions with the DNA backbone.[22,44] PEG is a highly hydrophilic polymer that strongly competes for water molecules, likely disrupting the essential hydration shell at the protein-DNA interface. We speculate that this PEG-induced dehydration destabilizes the XPA-DNA complex and increases its dissociation from DNA.

Given these findings, we speculated that the high local density of PEG on the QD surface is the critical factor. The densely packed PEG on the QD surface likely interacts directly with the

existing XPA-DNA complex, inducing the XPA detachment through localized dehydration and potential steric interference. To test this, we synthesized QDs having different densities of PEG on their surface. The lower PEG density suppressed the XPA dissociation (Figure 3E).

We then conjugated antibodies to the newly synthesized QDs via click chemistry between azide-DBCO for fluorescent imaging of XPA using single-molecule DNA curtain technique (Figure 5). We found that QD-labeled XPA diffuses along DNA with frequent pauses as reported in the previous literature.[19] We analyzed diffusion coefficients only for the diffusive motion, which was approximately $0.27 \mu\text{m}^2/\text{sec}$ regardless of ionic strength, suggesting that XPA slides along DNA during 1D diffusion. Furthermore, diffusion coefficients were all greater than theoretically maximum ($0.062 \mu\text{m}^2/\text{sec}$) for helical rotation around DNA duplex, suggesting that XPA does not rotate along DNA helix during 1D diffusion. Our diffusion coefficients are order-of-magnitude higher than those ($\sim 0.037 \mu\text{m}^2/\text{sec}$) in the previous study [19]. This discrepancy results from the difference in the analysis. In the previous analysis, the immobile portion seemed to be included for the estimation of diffusion coefficients, which led to small diffusion coefficients.[19]

Using DNA curtain assays with QD-labeled XPA, we revealed that XPA searches for ss/dsDNA junctions via both 1D diffusion and 3D collision. Moreover, we showed that 3D collision prevails over 1D diffusion. 1D diffusion facilitates target search when protein concentration is low.[45] The number of XPA in a human cell is approximately 150,000~200,000, while the number of XPC-RAD23B, which is known to diffuses along DNA, is 25,000~80,000.[46] The intracellular number of XPA is up to 8 times higher than that of XPC-RAD23B. At this high XPA concentration, 3D collision is sufficient to rapidly recognize ss/dsDNA junctions.

5. Conclusion

In this study, we uncover an unexpected limitation of commercially available QDs, demonstrating that PEG-coated QDs can actively destabilize protein–DNA interactions by inducing protein dissociation. Using XPA as a model system, we show that this effect is independent of antibody type, epitope tags, buffer conditions, and DNA substrates, and instead arises primarily from PEG on the QD surface. By systematically engineering the surface polymer composition, we identify a critical PEG density threshold below which protein–DNA

interactions are preserved without compromising colloidal stability or fluorescence performance. Furthermore, the use of SPAAC-based click chemistry enables efficient and minimally perturbative antibody conjugation. The optimized QDs allow accurate single-molecule visualization of XPA dynamics, revealing that XPA locates DNA bubble structures predominantly via three-dimensional collisions rather than one-dimensional diffusion. Collectively, our findings provide a general strategy for designing nanoparticle probes that maintain native biomolecular interactions and establish a robust platform for high-fidelity single-molecule imaging studies.

Declaration

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Y.K. contributed to XPA purification, all biochemical and single-molecule assays. H.K. synthesized quantum dots and conjugated antibodies to quantum dots. H.K. and M.C. analyzed the characteristics of synthesized quantum dots. H.S.K. tested the activity of purified XPA. O.D.S. advised XPA experiments. J.N.P. and J.Y.L. wrote the manuscript. J.N.P. supervised quantum dot-related experiments. J.Y.L. supervised all biochemical and single-molecule experiments.

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Figure Captions:

Figure 1. QD-mediated XPA dissociation from DNA.

(A) Schematic of QD. QD consists of core (CdSe), shell (ZnS), and coated polymer, on which functional groups are modified for conjugation. (B) Domain architecture (top) and structure (bottom) of human XPA (PDB: 1xpa). (C) Electrophoretic mobility shift assay (EMSA) gel images of purified 6xHis-3xFLAG XPA for 3-way junction (left) and homoduplex DNA (right). (D) Quantified bound fraction of XPA to 3-way junction (red circle) and homoduplex DNA (blue blank square) in [Figure 1C](#). Errors are obtained from standard errors in triplicate. (E) QD titration to the 6xHis-3xFLAG XPA-DNA complex. AntiFLAG antibody-conjugated QD (α FLAG-QD) with 705 nm emission is prepared by a commercial QD-antibody conjugation kit. The addition of QDs (0, 3, 10, 30 and 100 nM) results in the increase of free DNA band, suggesting XPA dissociation due to the addition of QD. (F) Quantified bound fraction of XPA to 3-way junction DNA according to different types of QDs (antiFLAG antibody-conjugated 605 nm QD (α FLAG-QD (605 nm)): black square, antiFLAG antibody-conjugated 705 nm QD (α FLAG-QD (705 nm)): red circle ([Figure 1E](#)), antiHis antibody-conjugated 705 nm QD (α His-QD (705 nm)): blue triangle, and streptavidin-conjugated QD (705 nm): green reverse triangle).

Figure 2. Effect of poly-ethylene glycol (PEG) on the XPA dissociation.

(A) Titration of synthesized QDs coated with 500 Da PEG (PEG500) at different concentrations (0, 0.3, 1, 3, 10, 30, and 100 nM) to XPA-DNA complex (3-way junction). Chemical structures of coated polymers are displayed above the EMSA gel data. (B) Titration of synthesized QDs coated with 500 Da PEG (PEG500) modified with azido-groups at different concentrations (0, 0.3, 1, 3, 10, 30, and 100 nM) to XPA-DNA complex (3-way junction). Chemical structures of coated polymers are displayed above the EMSA gel data. (C) Quantification of EMSA in [Figure 2A](#) and [2B](#), and [Figure S3A](#) (PEG1000) and [S3B](#) (carboxylated PEG). Errors are obtained from standard errors in triplicate. (D) EMSA for titration of PEG1000 (left) or Dextran35000 (right) at different concentrations (0, 2.5, 5, 10, 20, and 30%). (E) Quantification of EMSA in [Figure 2D](#) and [Figure S3C](#) and [S3D](#). Errors are obtained from the standard errors in triplicate.

Figure 3. Synthesis of Qdots with different polymer composition.

(A) Schematic illustration of two-step ligand exchange procedure of CdZnSeS/ZnS QDs. (B) ^1H NMR spectrum and chemical structure of the synthesized polymer ligand and integration region of three groups (anchor (A)-group, hydrophilic (H)-group, and functional (F)-group). (C) Polymer composition on Qdots showing no dissociation of XPA (A: H: F is 39.8%: 55.0%: 5.2%) (D) Polymer composition on Qdots showing the dissociation of XPA (A: H: F is 24.7%: 64.7%: 10.6%). (E) Quantification for XPA bound fraction in [Figure 3C and 3D](#). (F) Relative fraction of polymer each group depending on dissociation and bound number.

Figure 4. Antibody conjugation to Qdots.

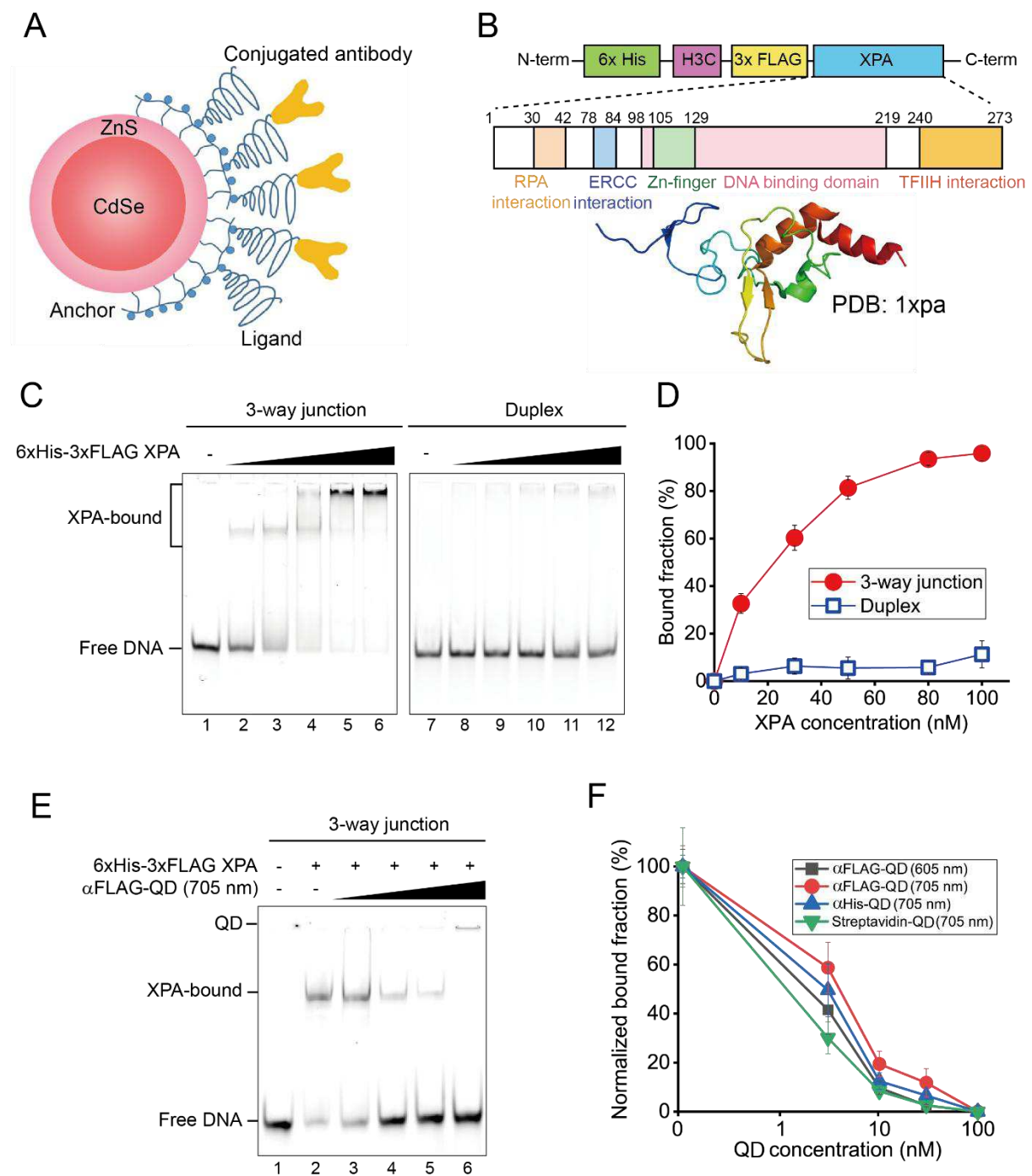
(A) Schematic illustration of the two-step antibody (Ab) and azido functionalized QDs (QD-N₃) conjugation strategy using strain-promoted alkyne–azide cycloaddition (SPAAC) click chemistry. (B, C) UV–vis absorbance spectra during Step 1 and Step 2, respectively. (D) DLS size distributions of QD-N₃ and QD–Ab. (E) FPLC (Superose 6 10/300 resin) data exhibiting peak shifts with increasing antibody ratios, indicating formation of QD–Ab conjugates.

Figure 5. Single-molecule DNA curtain assays using newly synthesized QDs.

(A) (Top) Lambda DNA (λ -DNA) construct containing a 57-nt bubble between 33,544 bp and 33,600 bp. (Bottom) Schematic of single-tethered DNA curtain assay. XPA is labeled with α FLAG-QD, which is newly synthesized to prevent the XPA dissociation from DNA. XPA structure is adopted from protein data bank (PDB ID: 1XPA). (B) Single-tethered DNA curtain images. (Top) Image for DNA stained with YOYO-1. (2nd top and 3rd top) Images for QD-labeled XPA bound to the bubble in λ -DNA in the presence (2nd top) and absence (3rd top) of buffer flow. In the absence of buffer flow, DNA molecules recoil out of evanescent field and disappear together with bound proteins. (bottom) QD signal without XPA. Black bars and “B” at the left indicate the barrier position. Magenta arrowhead at the left represents the location of the bubble. The black arrows at the right show the flow direction. The arrow with “X” indicates no flow. (C) Histogram for binding distribution of XPA-QD complexes on the bubble-containing λ -DNA. There is one distinct peak at 33.7 ± 2.1 kbp, which corresponds to the bubble location. N is the total number of molecules analyzed. Errors are obtained from bootstrapping with 70% confidence interval. (D) Representative kymograph showing diffusive motion (solid arrow line) and immobile state (dashed arrow line) of XPA on single λ -DNA

without any bubble using double-tethered DNA curtains. (E) Relative populations of diffusive and immobile states according to ionic strength. N represents the total number of molecules analyzed. Error bars are obtained from standard deviations from binomial distribution. (F) Histogram for immobile locations. (G) Relative displacement between immobile positions. (H) Diffusion coefficients of XPA according to NaCl concentration. N represents the total number of molecules analyzed. Red dashed line indicates the theoretically maximum diffusion coefficient ($0.062 \mu\text{m}^2/\text{sec}$) for helical rotation about DNA duplex. (I) Schematic of double-tethered DNA curtain assay (side view) for bubble search mechanism of XPA. The biotinylated end of lambda DNA containing a bubble is anchored on lipid bilayer via streptavidin. The other end of lambda DNA is modified with digoxigenin, which is tethered to a pedestal coated with anti-digoxigenin. In this configuration, DNA remains stretched even in the absence of buffer flow. (J) Representative kymograph showing 3D collision of XPA to search for a bubble on DNA. Magenta arrowhead and yellow dashed line indicate the location of bubble. B and P at the left denote the barrier and pedestal, respectively. (K) Representative kymograph showing 1D diffusion of XPA to search for a bubble on DNA. Yellow arrow shows 1D diffusion of XPA. Magenta arrowhead and yellow dashed line indicate the location of bubble. B and P at the left denote the barrier and pedestal, respectively. (L) Relative fraction for 1D diffusion and 3D collision for bubble search of XPA. Error bars are obtained from the standard deviation of binomial distribution. N represents the total number of molecules analyzed.

Figure 1.



A

PEG500-QD

XPA	-	+	+	+	+	+	+	+
QD	-	-	-	-	-	-	-	-

XPA-bound

Free DNA

1 2 3 4 5 6 7 8

B

PEG500-azide-QD

XPA	-	+	+	+	+	+	+	+
QD	-	-	-	-	-	-	-	-

XPA-bound

Free DNA

1 2 3 4 5 6 7 8

C

Normalized bound fraction (%)

QD concentration (nM)

Legend: PEG500-QD (red circles), PEG1000-QD (green triangles), PEG500-carboxyl-QD (blue squares), PEG500-azide-QD (black diamonds)

D

PEG1000

XPA	-	+	+	+	+	+	+
Polymer	-	-	-	-	-	-	-

XPA-bound

Free DNA

1 2 3 4 5 6 7

Dextran35000

XPA	-	+	+	+	+	+	+
Polymer	-	-	-	-	-	-	-

XPA-bound

Free DNA

8 9 10 11 12 13 14

E

Normalized bound fraction (%)

PEG concentration (%)

Legend: PEG200 (green squares), PEG400 (red circles), PEG1000 (blue triangles), Dextran (black circles)

Figure 3.

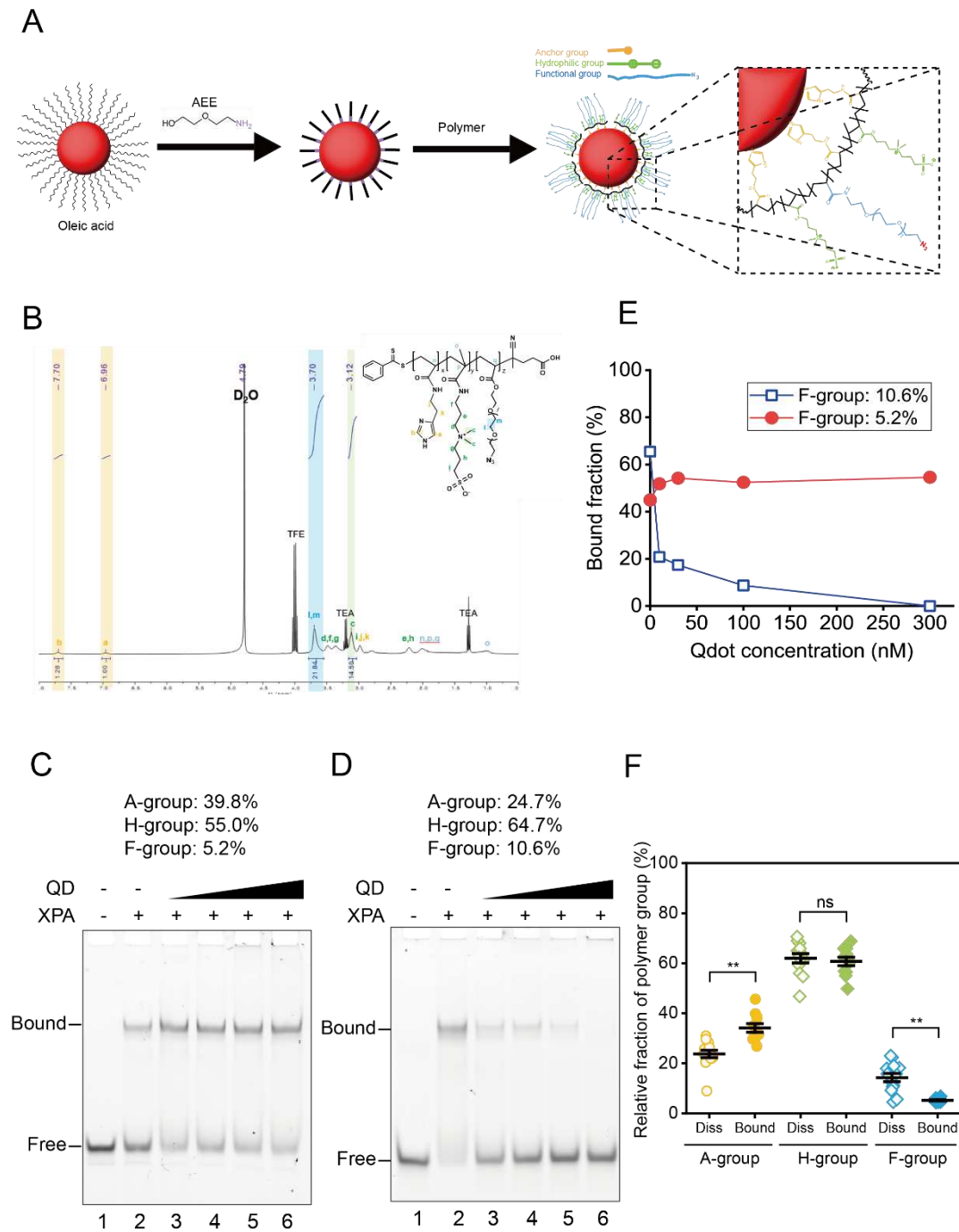


Figure 4.

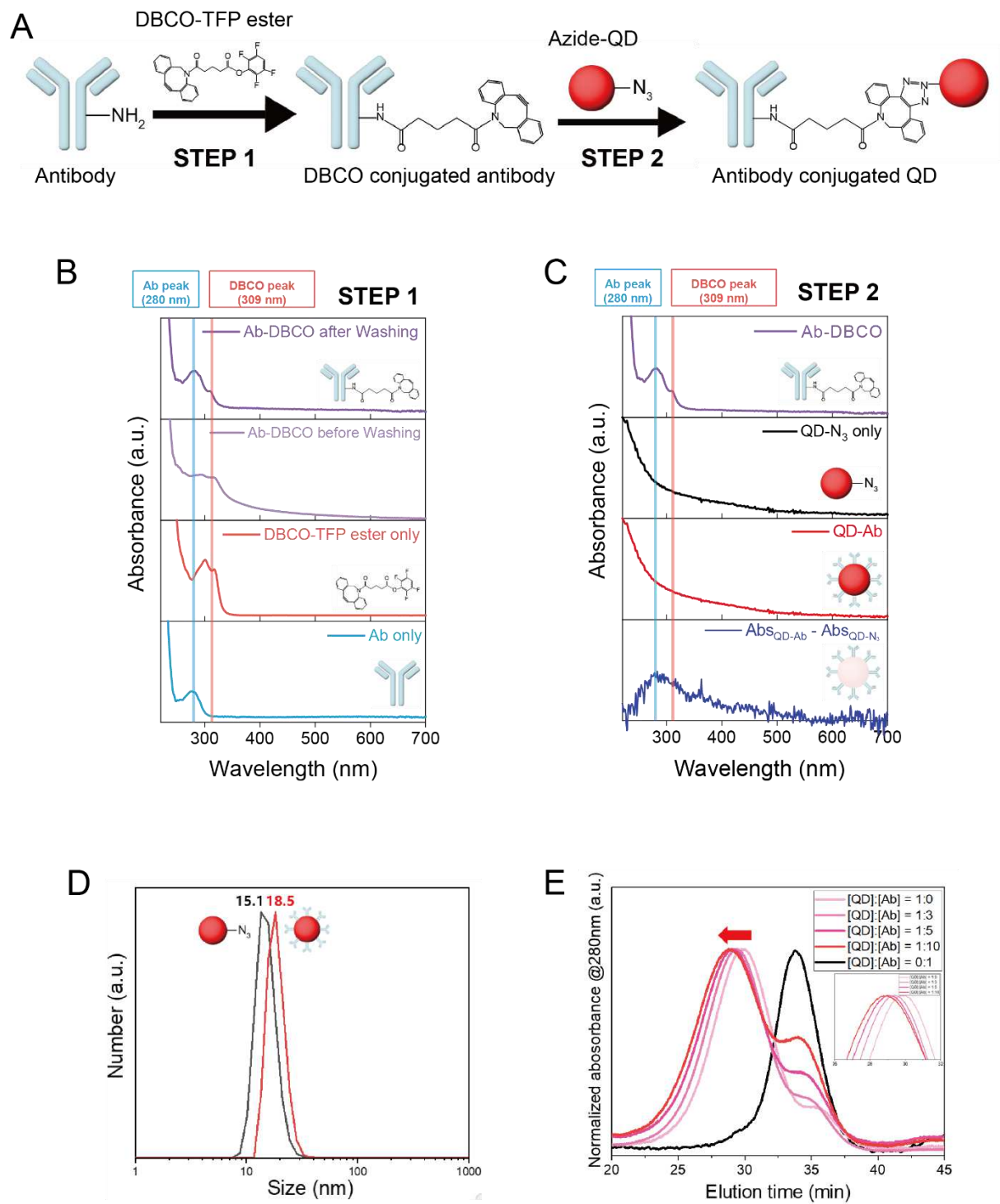
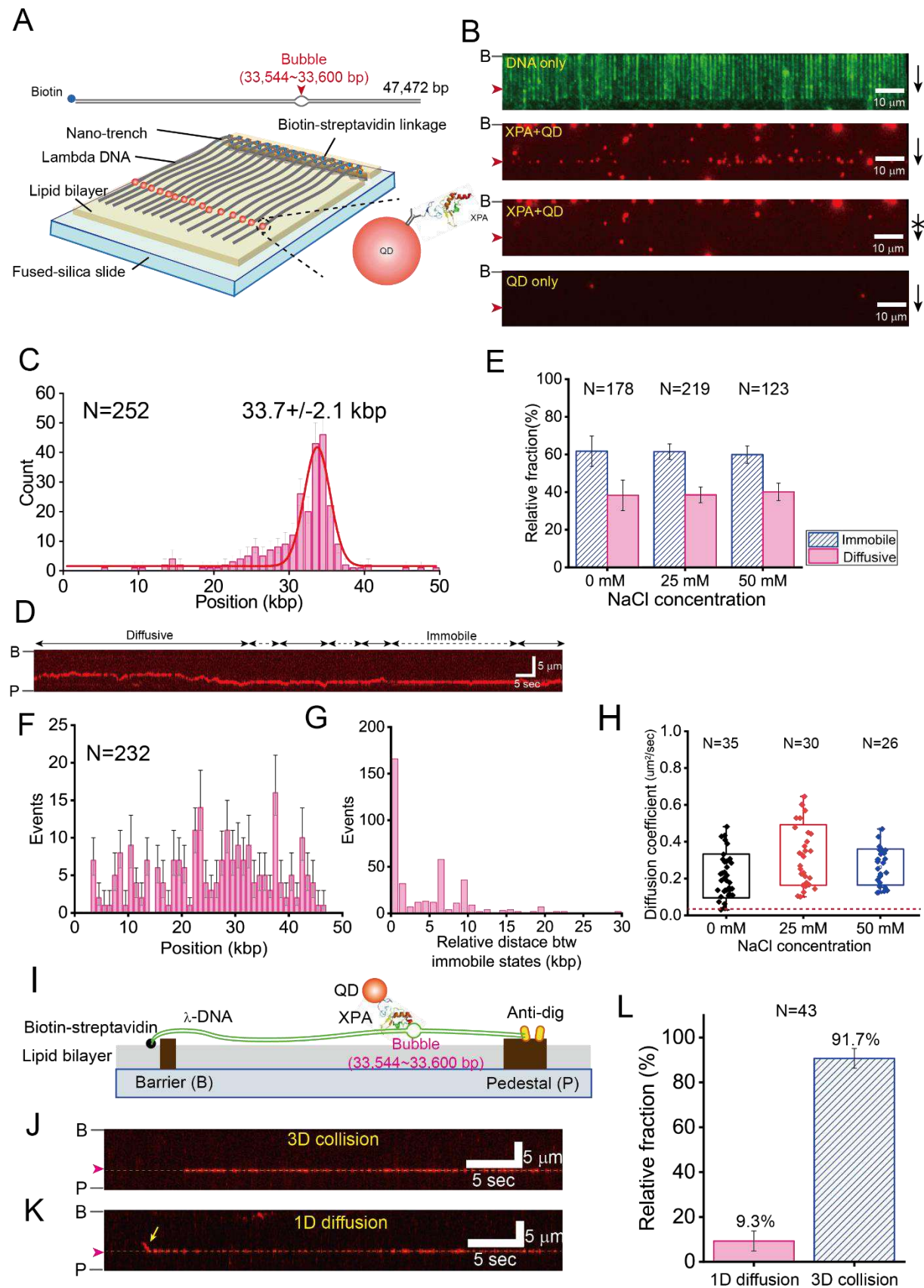


Figure 5.



Supplementary Files

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