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DNA-Templated Protein Arrays for Single-Molecule Imaging

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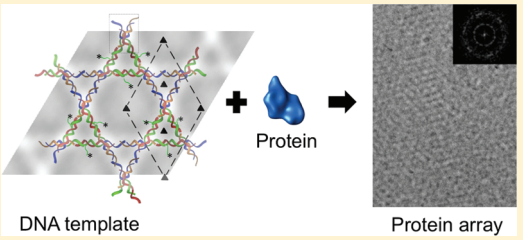
Supporting Information

ABSTRACT: Single-particle electron cryomicroscopy permits structural characterization of noncrystalline protein samples, but throughput is limited by problems associated with sample preparation and image processing. Three-dimensional density maps are reconstructed from high resolution but noisy images of individual molecules. We show that self-assembled DNA nanoaffinity templates can create dense, nonoverlapping arrays of protein molecules, greatly facilitating data collection. We demonstrate this technique using a G-protein-coupled membrane receptor, a soluble G-protein, and a signaling complex of both molecules.



KEYWORDS: Single-particle electron cryomicrosopy, DNA templates, nanostructure, nanoaffinity, G-protein-coupled membrane receptor

Single-particle electron cryomicroscopy (cryo-EM) allows direct visualization of biomolecules, flash-frozen in a solution suspended across holes in a carbon film.[1] This technique is particularly promising for hard-to-crystallize membrane proteins and protein complexes.[2] However, high beam currents damage samples and high protein densities lead to aggregation: throughput and resolution are severely limited by the need to identify, orient, and average 10^4-10^6 noisy, low-dose projection images of sparse, randomly distributed particles.[3] By attaching the target protein to a self-assembled 2D DNA template, we are able to create arrays of protein molecules that greatly simplify data acquisition.

The proposal that a synthetic protein crystal assembled on a 3D DNA template could be used for X-ray diffraction[4] is a driving force in the development of DNA nanotechnology. While this goal has yet to be realized, DNA-based approaches have been successfully employed to investigate protein structures: liquid-crystalline DNA nanotubes have been used to orient membrane proteins for structure determination by NMR,[5] and we have used a DNA-templated 2D crystal to obtain a low-resolution projection map of the soluble protein RuvA by cryo-EM.[6] Here we describe a fundamentally different use of a self-assembled DNA structure: we attach the target protein to a 2D DNA template, thereby creating dense, nonoverlapping arrays of protein molecules suitable for imaging by cryo-EM. Use of flexible linkers ensures that images corresponding to a wide range of molecular orientations are obtained. We demonstrate the technique using samples that span the range of potential targets for single-particle cryo-EM: a soluble, asymmetric guanine nucleotide-binding protein ($G\alpha_{i1}$); a neuropeptide-binding G-protein-coupled membrane receptor (GPCR), the rat neurotensin receptor type 1 (NTS1);[7] and a complex of the two.

The template[6] consists of three sets of parallel DNA helices woven, as in kagome basketwork, to produce a trigonal 2D crystal (Figure 1). The four component oligonucleotides were designed to form a Holliday junction with four double-helical arms, each with a 6-nucleotide single-stranded sticky end. Hybridization of complementary sticky ends assembles these motifs into the crystal. Figure 1d and Figure 2a show the template: an unbroken single layer extends across several holes of a holey carbon EM grid (the measured lattice constant is 14 nm). One oligonucleotide is modified such that the DNA template presents triangular clusters of binding sites for the protein arranged on a hexagonal lattice.

The guanine nucleotide binding protein $G\alpha_{i1}$ is a small (40 kDa), asymmetric, soluble, protein whose structure in its GDP-bound form has been determined to 2.4 Å (PDB: 1AS3). GDP-bound $G\alpha_{i1}$ was bound to the DNA template through a Ni^{2+}-mediated interaction between a N-terminal $(His)_6$ affinity tag and tris-nitrilotriacetic acid (tris-NTA) conjugated to a template oligonucleotide.[8] Tris-NTA-functionalized templates were incubated with protein, pipetted onto a holey carbon grid, washed briefly by inversion on a 20 μL droplet of distilled water, flash-frozen in liquid ethane, and imaged using a field-emission gun

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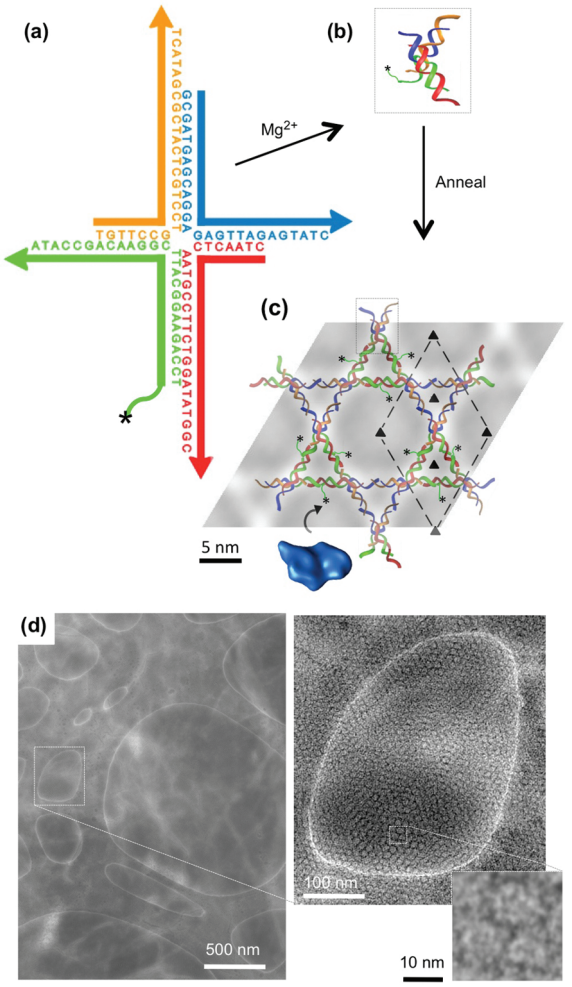

Figure 1. DNA template. (a) Four synthetic oligonucleotides form a four-arm junction. Asterisk (*) indicates a modification of one oligonucleotide (tris-NTA or NT) used for protein attachment. (b) In the presence of Mg^{2+} pairs of arms stack coaxially to form two quasi-continuous helices. (c) Hybridization of sticky ends assembles junctions into a crystalline array with *p*3 symmetry. A diagram of the self-assembled DNA template is shown superimposed on a projected density map obtained by cryo-EM (Figure 2a). (d) Transmission electron micrograph showing the template (weakly stained with 2% UAc) extending across holes of a holey carbon grid.

microscope. Densely functionalized protein arrays extend uniformly across holes in the grid; no protein aggregation is apparent (Figure 2b).

Particles of $G\alpha_{i1}$ (38990 particles from four micrographs) were classified and averaged into 121 class averages using multivariate statistical analysis. The orientation of each class average was determined by projection-matching alignment against quasi-evenly spaced projections (θ = 1°) of the crystal structure[9] (PDB: 1AS3) filtered to 20 Å. Each class average corresponds closely to its best-match projection; class averages are well distributed in θ, with random sampling of φ (Euler plot not shown). Figure 2c shows 16 systematically chosen projections of the crystal structure of $G\alpha_{i1}$ (selected in increments of $\theta \sim 10°$ across the range $0 < \theta < 180°$) with the corresponding best-match class averages. In several views the two-domain structure of $G\alpha_{i1}$ is apparent.

The rat neurotensin receptor type 1 (NTS1, $M_r \sim 43$ kDa)[7] is a member of the GPCR superfamily of 7-transmembrane-helix, integral membrane proteins that mediates responses to neurotransmitters, hormones, and environmental stimuli. Its ligand, the 13-residue neurotensin peptide (NT), is expressed endogenously in the central nervous system where NTS1 mediates its interaction with the dopaminergic system;[10] NT also acts as a local hormone in the gut.[11] The inherent flexibility of ligand-binding GPCRs hampers structural investigations: recent crystallographic studies have required thermostabilizing mutations, stabilization of intracellular loops by fusion with readily crystallizable T4 lysozyme or complexation with a Fab fragment, and binding of an antagonist or inverse agonist.[12−15] The structure of NTS1 remains undetermined. NTS1 was produced by expression in *E. coli* as a fusion protein,[16,17] the 42 N-terminal residues (containing putative glycosylation sites) were deleted with no effect on function, and no other mutations were necessary.

DNA-templated protein arrays were prepared as described above, except that NTS1 was bound to the DNA template through its peptide ligand, NT ($K_d \sim 1$ nM). NT was synthesized with an additional N-terminal cysteine (Cys) using conventional Fmoc solid phase peptide synthesis[18] and was conjugated to the template by a disulfide bond between the N-terminal Cys and a 5′ thiol modifier on a C_6 linker to a template oligonucleotide. All receptors observed are therefore in the activated (ligand-bound) conformation. Figure 2d shows a DNA-templated array of ligand-bound NTS1. Particles (60600 particles from 6 micrographs) were classified; representative class average images corresponding to distinct orientations are shown in Figure 2d.

DNA nanoaffinity templates provide a number of distinct advantages for the preparation of protein samples for direct single molecule imaging. Site-specific binding to the template concentrates the specimen, producing a dense and even particle distribution without inducing aggregation. As the particles are tightly confined to the plane of the lattice, surface tension effects at the air−water interface are avoided. It is possible to acquire 10^4 images from a single micrograph, reducing variations in preparation and measurement.

Structural studies based on single-particle images are typically of large (>500 kDa)[19] or highly symmetrical molecules,[20] whose orientations are relatively easy to determine. NTS1 and $G\alpha_{i1}$ are relatively small (each being 40−43 kDa): images of small particles have lower contrast and they possess less internal structure, making it difficult to determine particle orientations.[21] We are developing 3D reconstructions based on our data, and applying DNA-templated protein arrays to obtain 3D reconstructions of larger biomolecules.

DNA nanoaffinity templates are also well adapted to the investigation of protein−protein interactions. Figure 2e shows the signaling complex of NTS1 with $G\alpha_{i1}$. Here, NTS1 is bound to, and activated by, a NT-functionalized DNA array (as in Figure 2d), and $G\alpha_{i1}$ is bound only through its interaction with NTS1. $G\alpha_{i1}$ is labeled with high-contrast Nanogold clusters, allowing specific localization of $G\alpha_{i1}$ in the multiprotein array. Figure 2e represents the first direct observation (using purified proteins) of a GPCR−G-protein complex.

Techniques for protein structure determination by single-particle cryo-EM are developing rapidly. High-resolution 3D reconstruction requires the acquisition of tens of thousands to millions of particle images. By dramatically increasing the

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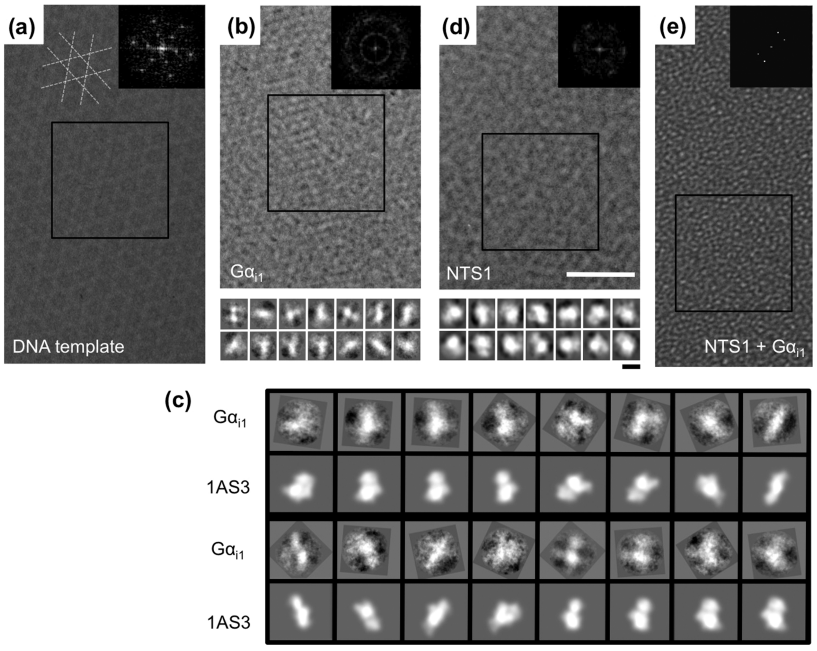

Figure 2. DNA-templated protein arrays. (a, b) Cryo-electron micrographs of (a) unfunctionalized DNA template and (b) soluble G-protein $G\alpha_{i1}$, bound to the tris-NTA-functionalized template through a $(His)_6$ affinity tag. Representative class average images are shown below. (c) Projections of the crystal structure of $G\alpha_{i1}$ (PDB: 1AS3), filtered to 2.0 nm and broadly sampling configuration space, with best-matching class averages. (d) Cryo-electron micrograph of membrane receptor NTS1 bound to its ligand, NT. As for (b), representative class average images are shown below. Class averages of NTS1 have a diffuse boundary attributed to the detergent micelle. (e) Transmission electron micrograph (negatively stained) of an array of the signaling complex NTS1-$G\alpha_{i1}$. G-protein $G\alpha_{i1}$, labeled with Nanogold clusters, was incubated with the GPCR NTS1, which was bound to the array and activated through its ligand NT. Inset top (a, b, d, e): Fourier transforms of indicated areas. Scale bars: 50 nm (micrographs); 5 nm (class averages).

throughput of data collection, DNA nanoaffinity templates have the potential to play an important part in enabling the wide application of single-particle techniques to difficult-to-crystallize targets, especially protein complexes and membrane proteins.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Böttcher, B.; Wynne, S. A.; Crowther, R. A. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* **1997**, *386*, 88.

(2) Rubinstein, J. L. Structural analysis of membrane protein complexes by single particle electron microscopy. *Methods* **2007**, *41*, 409.

(3) Henderson, R. Realizing the potential of electron cryo-microscopy. *Q. Rev. Biophys.* **2004**, *37*, 3.

(4) Seeman, N. C. Nucleic acid junctions and lattices. *J. Theor. Biol.* **1982**, *99*, 237.

(5) Douglas, S. M.; Chou, J. J.; Shih, W. M. DNA-nanotube-induced alignment of membrane proteins for NMR structure determination. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 6644.

(6) Malo, J.; Mitchell, J. C.; Vénien-Bryan, C.; Harris, J. R.; Wille, H.; Sherratt, D. J.; Turberfield, A. J. Engineering a 2D Protein−DNA Crystal. *Angew. Chem., Int. Ed.* **2005**, *44*, 3057.

(7) Tanaka, K.; Masu, M.; Nakanishi, S. Structure and Functional Expression of the Cloned Rat Neurotensin Receptor. *Neuron* **1990**, *4*, 847.

(8) Goodman, R. P.; Erben, C. M.; Malo, J.; Ho, W. M.; McKee, M. L.; Kapanidis, A. N.; Turberfield, A. J. A Facile Method for Reversibly Linking a Recombinant Protein to DNA. *ChemBioChem* **2009**, *10*, 1551.

(9) Shaikh, T. R.; Gao, H.; Baxter, W. T.; Asturias, F. J.; Boisset, N.; Leith, A.; Frank, J. SPIDER image processing for single-particle reconstruction of biological macromolecules from electron micrographs. *Nat. Protoc.* **2008**, *3*, 1941.

(10) Palacios, J. M.; Kuhar, M. J. Neurotensin receptors are located on dopamine-containing neurones in rat midbrain. *Nature* **1981**, *294*, 587.

(11) Brown, M.; Vale, W. Effects of neurotensin and substance P on plasma insulin, glucagon and glucose levels. *Endocrinology* **1976**, *98*, 819.

(12) Rasmussen, S. G.; Choi, H. J.; Rosenbaum, D. M.; Kobilka, T. S.; Thian, F. S.; Edwards, P. C.; Burghammer, M.; Ratnala, V. R.; Sanishvili, R.; Fischetti, R. F.; Schertler, G. F.; Weis, W. I.; Kobilka, B. K. Crystal structure of the human β2 adrenergic G-protein-coupled receptor. *Nature* **2007**, *450*, 383.

(13) Cherezov, V.; Rosenbaum, D. M.; Hanson, M. A.; Rasmussen, S. G.; Thian, F. S.; Kobilka, T. S.; Choi, H. J.; Kuhn, P.; Weis, W. I.; Kobilka, B. K.; Stevens, R. C. High-Resolution Crystal Structure of an

Engineered Human β2-Adrenergic G Protein-Coupled Receptor. *Science* **2007**, *318*, 1258.

(14) Warne, T.; Serrano-Vega, M. J.; Baker, J. G.; Moukhametzianov, R.; Edwards, P. C.; Henderson, R.; Leslie, A. G.; Tate, C. G.; Schertler, G. F. Structure of a β1-adrenergic G-protein-coupled receptor. *Nature* **2008**, *454*, 486.

(15) Jaakola, V. P.; Griffith, M. T.; Hanson, M. A.; Cherezov, V.; Chien, E. Y.; Lane, J. R.; Ijzerman, A. P.; Stevens, R. C. The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an Antagonist. *Science* **2008**, *322*, 1211.

(16) White, J. F.; Trinh, L. B.; Shiloach, J.; Grisshammer, R. Automated large-scale purification of a G protein-coupled receptor for neurotensin. *FEBS Lett.* **2004**, *564*, 289.

(17) Attrill, H.; Harding, P. J.; Smith, E.; Ross, S.; Watts, A. Improved yield of a ligand-binding GPCR expressed in E. coli for structural studies. *Protein Expression Purif.* **2009**, *64*, 32.

(18) Harding, P. J.; Hadingham, T. C.; McDonnell, J. M.; Watts, A. Direct analysis of a GPCR agonist interaction by surface plasmon resonance. *Eur. Biophys. J.* **2006**, *35*, 709.

(19) Schuette, J. C.; Murphy, F. V., 4th; Kelley, A. C.; Weir, J. R.; Giesebrecht, J.; Connell, S. R.; Loerke, J.; Mielke, T.; Zhang, W.; Penczek, P. A.; Ramakrishnan, V.; Spahn, C. M. GTPase activation of elongation factor EF-Tu by the ribosome during decoding. *EMBO J.* **2009**, *28*, 755.

(20) Yu, X.; Jin, L.; Zhou, Z. H. 3.88Å structure of cytoplasmic polyhedrosis virus by cryo-electron microscopy. *Nature* **2008**, *453*, 415.

(21) Frank, J. *Three-Dimensional Electron Microscopy of Macromolecular Assemblies*, 2nd ed.; Oxford University Press: New York, 2006.