ISSN 1674-1056
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Citation: Chin. Phys. B . 2018, 27(2): 028708. **doi:** 10.1088/1674-1056/27/2/028708

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TOPICAL REVIEW — Soft matter and biological physics

Bio-macromolecular dynamic structures and functions, illustrated with DNA, antibody, and lipoprotein*

Lu Gou(缑璐), Taoli Jin(金桃丽), Shuyu Chen(陈淑玉), Na Li(李娜), Dongxiao Hao(郝东晓), Shengli Zhang(张胜利), and Lei Zhang(张磊)†

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(Received 31 August 2017; revised manuscript received 13 December 2017; published online 29 January 2018)

Bio-macromolecules, such as proteins and nucleic acids, are the basic materials that perform fundamental activities required for life. Their structural heterogeneities and dynamic personalities are vital to understand the underlying functional mechanisms of bio-macromolecules. With the rapid development of advanced technologies such as single-molecule technologies and cryo-electron microscopy (cryo-EM), an increasing number of their structural details and mechanics properties at molecular level have significantly raised awareness of basic life processes. In this review, firstly the basic principles of single-molecule method and cryo-EM are summarized, to shine a light on the development in these fields. Secondly, recent progress driven by the above two methods are underway to explore the dynamic structures and functions of DNA, antibody, and lipoprotein. Finally, an outlook is provided for the further research on both the dynamic structures and functions of bio-macromolecules, through single-molecule method and cryo-EM combining with molecular dynamics simulations.

Keywords: bio-macromolecule, single-molecule method, cryo-electron microscopy, electron tomography

PACS: 87.64.Ee, 87.80.–y 87.15.B–, 87.15.H– **DOI:** 10.1088/1674-1056/27/2/028708

1. Introduction

Bio-macromolecules play a central role in various biological processes. Other than inorganic and organic small molecules, bio-macromolecules, such as nucleic acids, proteins, and carbohydrates etc, are the basic materials for life and are peculiar to the organism.[1] With the vigorous development of life science, more and more bio-molecular functions have been revealed. However, the underlying mechanisms mostly remain unknown and the structure information of bio-macromolecules at molecular level has to be revealed. It is known that the dynamic nature of biomolecules, shown as structure fluctuation, is intimately linked to their function, as major conformational changes lead to different biological performances. Therefore, the structure–property–function relationship of biomolecules has to be probed in order to understand their important physiological functions so as to establish possible connections to understand basic life processes. This may in fact lead to possible useful applications in clinical diagnostics and even provide suitable strategies towards effective therapeutic treatments of human diseases.[2]

To explore the relationship between the dynamic structures and functions of biological macromolecule, a variety of experimental techniques have been developed and the researches exploring these techniques have become the most attractive topics for multidisciplinary studies, including physical chemistry, biophysics, and nanotechnology at different scales.[3] Among these technologies, the so-called single-molecule technologies, including förster resonance energy transfer (FRET), optical tweezers (OT), magnetic tweezers (MT), and atomic force microscopy (AFM), as well as the structural biologic methods, such as x-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-EM have been rapidly developed and resulted lots of important discoveries.

In this review, recent research progress on the bio-macromolecular dynamic structures and functions has been summarized. At first, experimental principles of the technologies such as AFM, OT, MT, and cryo-EM and their rationale had been stated. Then recent progress of researches by applying these technologies on the study of DNA, antibody and lipoprotein had been reviewed. At last, the possibility of combining various different methodologies in the future to further understand the bio-macromolecular dynamic structures and functions, as well as related open questions and future perspectives, had been discussed.

2. Single-molecule experiments in biophysics

More and more physicists are interested in the subtleties of biological matter and therefore seek to investigate common themes and variations throughout this vast phenomenology. As technology rapidly developed, many scientific instruments with enough sensitivity and precision are designed and built to manipulate and visualize biological objects at single molec-

*Project supported by the National Basic Research Program of China (Grant No. 2015CB856304) and the National Natural Science Foundation of China (Grant Nos. 11504287 and 11774279).

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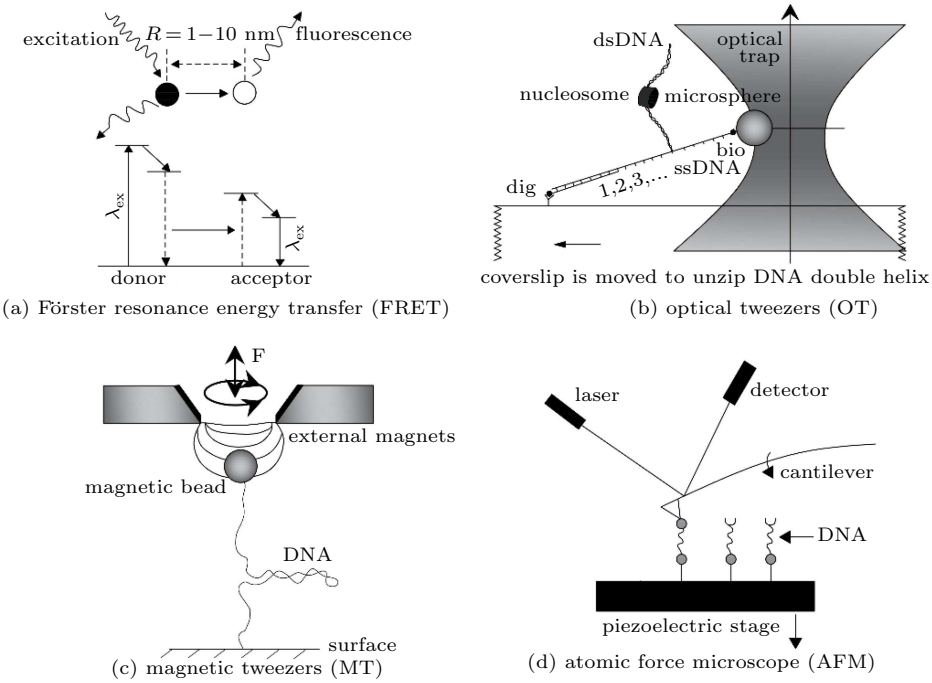
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ular level, and microscopic forces could be measured precisely. Therefore, such single-molecule technologies became the most important and popular methods in biophysics researches. In this section, two major single-molecule strategy, single-molecule fluorescence technology and single-molecule manipulation technology, were briefly described, as well as their strengths, limitations, and applications.

2.1. Förster resonance energy transfer (FRET)

Single-molecule fluorescence technology which belongs to single molecule detection is one of the most widely used methods. This technology has higher sensitivity and spatial resolution, and the detection range can also reach to nanometer scale. A single molecule behavior can be directly observed by single-molecule fluorescence technology. FRET can be utilized to measure structure details and the process of dynamics, particularly biological macromolecules. By marking two fluorescent molecules in different sites, so-called donor and acceptor, FRET efficiency and distance information between the marker sites could be obtained by measuring fluorescence intensity of fluorescent molecules (shown as Fig. 1(a)). Over the past few decades, single-molecule Förster resonant energy transfer (smFRET) has rapid development. Ha *et al.* measured energy transfer between a single donor and a single acceptor.[4] This is the first detection of single-molecule FRET. Whereafter smFRET has been successfully applied to the study of the conformational transitions of biomolecules, kinetic process of proteins, and so on. McLoughlin *et al.* used smFRET technology to monitor the dynamic changes of protein structure and interface behavior on the surface.[5] Recently, Dai *et al.* discovered the conformational state distributions and dynamics of sensitive factor attachment protein receptors (SNARE) protein Ykt6 by the same method.[6]

FRET technology has a superiority that it is a ratiometric method to measure the interior distance in the molecular frame, and the technique needs the ratio of fluorescence intensity, not the intensity of fluorescence, which makes it greatly reduce noise and drifting of apparatus.[7] The major limitation of this technology is that the absorption spectrum of organic dyes is narrow and the emission spectrum is wide. Photobleaching and low photostability have also impeded further applications.[8] Therefore, it is urgent to find more suitable donors and acceptors, among which quantum dot had been proven as a better candidate for its unique quantum advantages and special properties of the coupling with organic molecules.[9,10] On the basis of these theories, Qiu *et al.* detected homogeneous and multiplexed microRNA.[11] Over the past 70 years, FRET have been developed and perfected ceaselessly, and some new techniques and methods have emerged, such as multi-color FRET, fluorescence life detection, and single pair fluorescence resonant energy transfer (spFRET). Hohng *et al.* first used one donor (Cy3) and two acceptors (Cy5 and Cy5.5) to implement three-color FRET technology, and observed successfully the correlated movement of arms of the Holliday junction.[12] Li *et al.* used single-molecule fluorescence technology to study the movement and transport function of molecular motors in cells. They reported a new



(a) Förster resonance energy transfer (FRET) (b) optical tweezers (OT)

(c) magnetic tweezers (MT) (d) atomic force microscope (AFM)

Fig. 1. Schematic figure of several single-molecule experiments. (a) Förster resonance energy transfer can occur between the donor and the receptor within a certain range. (b) Optical tweezers. Double DNA is attached to an optically trapped bead, and the free end is attached to the surface of the trapping chamber. (c) Magnetic tweezers base on permanent magnets. A pair of small permanent magnets produces a magnetic field gradient along the axial direction, and the rotation of magnets produces rotation of the magnetic bead. (d) Atomic force microscopy. A cantilever with a sharp tip holds above a piezoelectric scanning stage.

kind of single molecule fluorescence detection technology — single-molecule surface induced fluorescence attenuation (sm-SIFA). They also studied dynamic changes of pore-forming peptide among five transmembrane positions, and discussed the key factors which caused lipid membrane permeability by using this technology.[13]

Some of the new and exciting technical developments still are in the way. However, the fluorescence technique is passive observation, and single-molecule manipulation can control the target object, as described below.

2.2. Single-molecule manipulation

In recent twenty years, single-molecule manipulation techniques have developed into manipulating and measuring the properties of single molecules. This kind of technology can exert a controlled external force on individual biomolecules and measure changes in real time, and can accurately measure the conformation and function of large biomolecules such as proteins and DNA. Therefore, the force spectroscopic information of the measurements is able to acquire, which associated with the characterization of the molecular conformation transition of dynamic stability and mechanics. These experimental techniques include the OT, MT, and AFM. Whereas the three techniques differ in the method used to measure, they have different suitable experimental and experimental conditions.

2.2.1. Optical tweezers (OT)

The optical or laser trap is used as detecting and controlling material structures at length scales ranging from nanometer to millimeter (shown as Fig. 1(b)). This kind of technique has now become an essential tool in both physics and biology.[14] In 1986, Ashkin *et al.* proposed the concept of light manipulation of tiny particles, and introduced single-beam laser into a high numerical aperture objective lens to form a three-dimensional optical potential well.[15] This device uses a laser to trap, control, and manipulate minute objects in medium. Optical trap is formed by a focused laser beam to produce radiation pressure, and the particles in the trap are clamped by the gradient force field, which is like a small tweezer and therefore is called optical tweezers.[16] The force of this apparatus derives from the radiation pressure produced by a high-power laser. Tightly focusing a laser beam to a diffraction-limited spot creates a steep three-dimensional light gradient in the immediate vicinity of the focus, as shown in Fig. 1(a).[17] Optical tweezers have been largely used to study the mechanical properties of molecular motors, DNA/RNA molecules and complexes of drugs and proteins.[18] Besides, optical tweezers have also greatly promoted our basic understanding of the nanomechanical properties of biological polymers such as polypeptides and nucleic acids.[17] Fu *et al.* discovered the ionic effects of magnesium salt solutions on the overstretching transition of single B-DNA molecules. Furthermore, the magnitudes of force corresponding to different salt concentrations on the overstretching transition vary greatly.[19] In other applications, the apparatus is used for the measurement of translocation and force generation of individual RNA polymerase molecules as they transcribe DNA. In the single molecule assay, the immobilized RNA polymerase is coiled in beads attached to the ends of the transcriptional DNA, which can be measured from the movement of the bead in the optical trap. Then the details of transcription could be revealed, including the pause of transcription, the blocking force ($\sim$ 30 pN), backtracking of the polymerase along the DNA template and the mechanism of polymerase translocation.[20] The versatility and accuracy of optical tweezers are of benefit to probe scientific problem. Many advantages of optical tweezers stem from their purely optical origin. However, there are still some difficulties in using light to generate force. Optical trapping with high resolution also has some limitations such as optical homogeneity, optical damage and highly purified samples.[21] To overcome these limits, a variety of experimental techniques have been developed as well as developments of hybrid instruments, in which optical tweezers or magnetic tweezers are combined with alternative single-molecule manipulation techniques and/or fluorescence detection methods.

2.2.2. Magnetic tweezers (MT)

MT is a simple and stable tool for stretching and twisting biomolecules and measuring their extension over time.[22] It utilizes a gradient magnetic field on a paramagnetic bead (1 nm–5 mm in diameter), and it is exerted the controllable tension and tethered to a glass surface through a single biomolecule. Then researchers can analyze the linked molecular elongation change through a microscope image.[1] The principle of this apparatus (as shown in Fig. 1(b)) is briefly described as the physical change in the attached biomolecule causes its extension to increase or decrease, raising or lowering the vertical position of the bead. In generally, analyzing the bead's diffraction image when viewed in a transmitted light geometry is able to measure the bead height. Therefore, the capability of the MT depends on the ability of the diffraction image to associate with the position of the bead when it measures physical changes of the biomolecules.[22] In contrast to single-molecule manipulation based on laser trap or cantilever drive, MT has a better straightforward theoretical explanation, and can put twisted control on biomolecule by rotating magnetic fields, which plays a very important role in the field of DNA supercoiled structures.[1] MT has been widely used since the first demonstration of manipulation and mechanical coiling of a single DNA molecule.[23] Chen *et al.* reported a method that

allowed us to stretch short biomolecules with magnetic tweezers over a wide range of up to 100 pN. The manipulation of MT pulls protein molecules like two hands. Chen *et al.* utilized aluminum to make the professional instruments for the more stable measuring effect, and the results could reflect the position of magnet and piezoelectric ceramic in real time.[24] Magnetic tweezers also have gained a great deal of information about the elastic properties of DNA and have provided important insights into the dynamic activity of DNA processing enzymes.[25] Double-stranded DNAs, including B-DNA, A-DNA, and Z-DNA, are dynamic molecules whose structure can change depending on conditions. For example, Yan *et al.* found that S-DNA could be obtained by stretching B-DNA, and the excessive tension of DNA involved two transitions had been measured. It is remarkable that 15 years controversy of DNA overstretching transition is resolved by a series of Yan's work by taking the advantages of MT technologies, the DNA structure after unpeeling transition is ssDNA without debate. The limitation of magnetic tweezers on short tethers had been resolved by Chen's work,[23] which was proved by recent work on protein unfolding and folding studies done by magnetic tweezers.[25]

2.2.3. Atomic force microscopy (AFM)

This technique could be briefly described as (Fig. 1(d)). Other than taking images by scanning the surface of the observed object and measuring the interaction between the probe and sample caused micro-cantilever deflection AFM could be also used to manipulate molecules by attaching one end of the molecule to the AFM tip and the other being immobilized on the surface.[26] Then molecule could be pulled away from the surface and controllable mechanical force could be applied by moving the tip relative to the substrate at a high precision.[27] In contrast to EM or scanning tunneling microscope (STM), AFM has obvious advantages in the studies on bio-macromolecular structures.[28] The bio-macromolecular samples do not need to be coated with heavy metals, made of metal replicas, or fixed, which can be directly observed in air or in various solvent systems so that the bio-macromolecular samples can be close to the physiological environment. Therefore, AFM has been applied to exploring the force and the dynamics of the interaction between individual ligands and receptors, either on isolated molecules or on cellular surfaces.[26] Li *et al.* demonstrated the single molecule detection of antigen/antibody binding under near-physiological environment and the distinction of nonspecific from specific binding using tapping-mode atomic force microscopy *in-situ* detection of specific and nonspecific binding during immunoreaction on surfaces.[29] Actually, after the invention of the AFM, DNA was the first biological samples that people attempt to study.[30,31] The use of improved equipment and new methodologies, and the study of nucleic acids by AFM allowed more elaborated studies, including the conformational analysis of the double helix, observation of the transcription by a RNA polymerase, visualization of the DNA bending.[32–34] Furthermore, in order to measure smaller unbinding the force resolution will be improved by the use of small cantilevers.

Over several decades there are remarkable developments and refinements in single-molecule experiment techniques that have opened up new avenues for biological and biophysical research. The methodology for exploring the force and the dynamics of bio-molecules will be able to address more fundamental questions in biological systems. From the availability of many technologies, the choice of using the correct type of experimental techniques and the instruments will depend on the type, size, and mechanical properties of the biological structure, as well as the biomechanical and biophysical properties.

3. A new era of cryo-EM

3.1. Application of cryo-EM in biomolecules

Over the last 60 years, with the rapid development of molecular biology as the core of biology, the structure of biological macromolecules at atomic or nearly-atomic resolution has been continually resolved especially by using an advanced technology called cryo-EM. 2017 Nobel Prize in Chemistry was awarded to three scientists, Dr. Jacques Dubochet, Dr. Joachim Frank, and Dr. Richard Henderson, for their fundamental contributions to developing cryo-EM for the high-resolution structure determination of biomolecules in solution. Cryo-EM now allows to interpret the mechanisms underneath the biological processes in terms of the disposition of atoms in space. Therefore, by using cryo-EM, structural biologists or biochemists could focus on how multiple proteins interact with other biomolecules in atomic details.[35] What is cryo-EM? Cryo-EM begins with vitrification, in which the protein solution is cooled so rapidly that water molecules do not have time to crystallize. The sample is then screened for particle concentration, distribution and orientation. Next, a series of images are acquired, and two-dimensional classes are computationally extracted. In the final step, the data is processed by reconstruction software, yielding accurate, detailed, three-dimensional (3D) models of intricate biological structures at the sub-cellular and molecular scales. These models can reveal interactions that are impossible to visualize previously, a key to scientific results. In general, the total protocol of cryo-EM is freezing sample (an amorphous solid that does little or no damage to the sample structure); data collection (to obtain 2D projection images); 3D reconstruction (3D density map from 2D images).

Comparing with using charge-coupled device (CCD), the development of new electronic detector not only has a qualitative leap on the electron microscopy image quality, and the sharp increase in speed at the same time, but it also can obtain movie of electron microscopy images. The electron microscope technology previously had a major bottleneck on the resolution, which was the image drift and radiation damage caused by electron beam acted with biological samples. With the fast film record, we can track the image drift trajectory and can correct motion and radiation damage for the image. Thus, data quality is greatly improved. The imaging process of transmission electron microscopy (TEM) is that electron beam penetrates the sample and the 3D electric potential density distribution function of the sample is projected along the direction of propagation direction of the electron beam to the 2D plane perpendicular to the propagation direction. In 1968, Klug discovered the central section theorem and purposed the concept that was performed to resolve the three-dimensional structure of the object in computer through two-dimensional projection of a three-dimensional object from different angles. The early biological samples were prepared from negative stain. It is the biological samples that were stained with heavy metal salts, and some common negative stain include the high electron density of ammonium molybdate, uranyl acetate, uranyl formate, and phosphotungstic acid. They are able to embed in the gaps of biological samples, and perform the electron microscopy and 3D reconstruction on the basis of the contrast between heavy metal salts and samples. Since negative stain is the heavy metal salt, this can not only solve the problem of radiation damage to biological molecules, but also improve the contrast of the images.[36]

Nowadays, cryo-EM, x-ray crystallography and NMR spectroscopy are three main means to determine the three-dimensional structure information of biological materials. As described above, cryo-EM provides a completely new idea for the study of bio-molecular structure different from x-ray crystallography. However, owing to the technical bottleneck (freezing, electron microscopic imaging, and image processing algorithms) in more than 30 years, cryo-EM could be curbed some relatively low-resolution structures and cannot be compared with x-ray crystallography in resolution. The most important revolutionary events occurred about three or four years ago: one was the invention of the direct electronic detector (DDD), the other was the improvement of the high-resolution image processing algorithm,[37,38] which makes cryo-EM indispensable to study the structure and dynamics of biomolecules complexes, included viruses, ribosomes, mitochondria, ion channels and a 4.5-Å resolution of enzyme complex as small as 170 kilo Daltons (Da).[39–41] For instance, Yu *et al.* obtained the three-dimensional structure of highly symmetrical cytoplasmic polyhedrosis virus (CPV) at 3.88 Å resolution in 2008.[42] It is the first time to get the structure of the near-atom resolution by single particle cryo-EM. In 2013, Liao *et al.* reconstructed 3.4-Å resolution 3D structure of membrane protein TRPV1 successfully by DDD cameras.[43] Likewise, cryo-EM is one of most effective ways to resolve proteasome structure, because 19S regulatory particle naturally has a large number of sub-stable states and the substantial local conformation fluctuation. Moreover, the complete assembly (26S proteasome holoenzyme) consists of a 20S proteolytic core particle (CP) and two 19S regulatory particles. Through image reconstruction of cryoEM micrographs, Chen *et al.* resolved a near-atomic-resolution structure of the human proteasome holoenzyme,[44] by using the independently developed software ROME (based on statistical manifold learning framework).[45] In addition, Lu *et al.* revealed allosteric selection mechanism of the assembly of proteasome.[46] In the field of cardiovascular diseases, Zhang *et al.* revealed the mechanism of cholesteryl ester transfer protein (CETP) interacting with high-density lipoproteins (HDL) and low-density lipoproteins (LDL) to transfer cholesterols and proposed a "tunnel model",[47] which provided a new insight for developing CETP inhibitors.

Addition to cryo-EM, traditional negative-staining methods could also provide insights of the dynamic structures and functions of biomolecules, especially for those who have molecular weights less than 100 kDa. For example, an optimized negative-staining (OpNS) method was proposed from a refined conventional protocol,[49–52] through which many small and dynamic proteins including CETP,[47] DNA,[58] antibodies,[53] lipoproteins,[54,55] and proteasome could be validated. At the same time, Zhang *et al.* revealed the morphology and structure information of lipoproteins and flexible antibodies by OpNS (see Fig. 2). Although samples will lose a lot of high resolution information by the negative staining method (in many cases the resolution only can reach $\sim$ 15 Å), such structural details of biological molecules could also provide crucial insights for understanding their functions.[43,53] Currently, our understanding of the function and of structure has led to a structure-driven approach, such as thermodynamics, dynamics, and pharmacology. Recently, this approach was utilized to determine the structural and thermodynamic factors during the biological processes, especially identifying the significant conformational changes with the ligand binding.[56–58] For example, time-resolved cryo-EM method visualized a set of ribosome structures for following ribosome-transfer RNA (tRNA) complex movement through the ribosome during translocation.[58] Cryo-EM snapshots combined with a manifold embedding method determined a massive rearrangement of the ribosometransfer RNA (tRNA) complexes.[57] Free-energy landscapes derived from single-particle cryo-EM snapshots revealed multiple routes and reaction coordinates of

Ryanodine receptor (RyR1), which is a highly conductive calcium ion channel and plays a key role in the muscle excitation-contraction coupling.[56] Above descriptions clearly suggest the powerful impact of cryo-electron microscopy on the dynamic conformations of biological macromolecular machine, virus, and organelle.

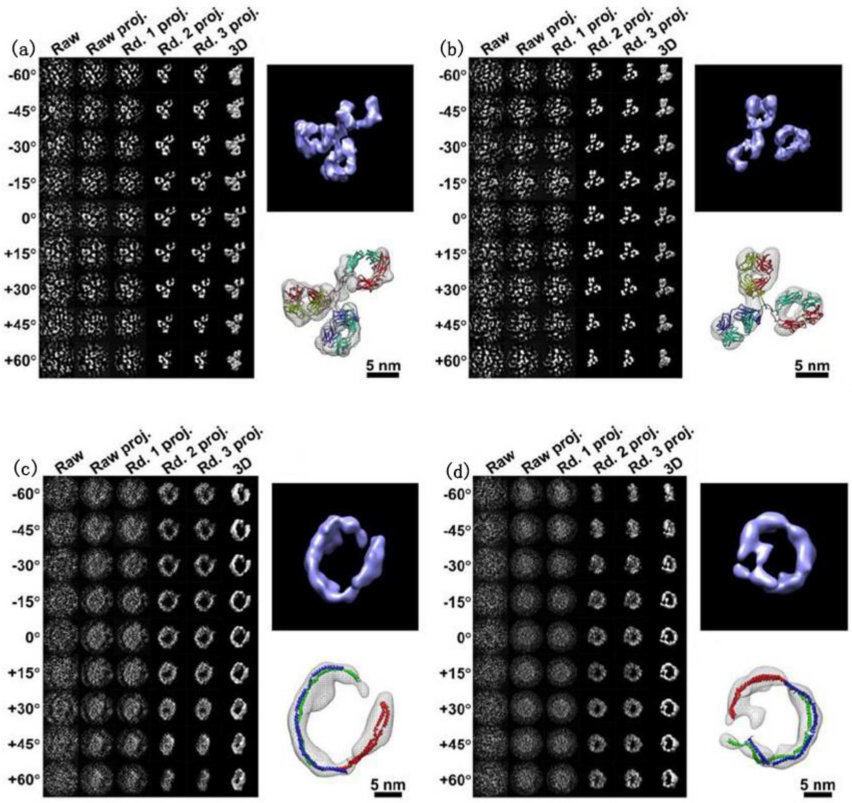


Fig. 2. (color online) The 3D reconstructions of IgG antibody particles and nascent HDLs by OpNS and IPET. (a) A tilt series of a single-instance IgG antibody by OpNS ET displayed in the left-most column. The IPET method obtains the 3D model of an individual protein via an iterative refinement processes. The final 3D reconstruction of an individual antibody particle shows as purple in the top right. (a) and (b) A good fit obtained by fitting each domain of the crystal structure (PDB) into each density model of IgG in left bottom. (b) IPET to study the 3D conformation changes of their antibodies before and after peptide conjugation. (c) and (d) For more than 40 years, nobody knew the 3D structures of nascent HDLs, by using IPET, Zhang *et al.* revealed the typical structures of two 17 nm HDLs. Adapted from Ref. [48].

3.2. Main reconstruction methods of cryo-EM

There are several cryo-EM methods to resolve structures, including electron crystallography, single particle analysis, and cryo-electron tomography (cryo-ET). Electron crystallography uses the imaging of electron microscopy and the function of electron diffraction to obtain structural information from the 2D crystal of biomolecules, and analyzes its 3D structure. The range of suitable sample size is 10 kDa–500 kDa, with a maximum resolution of about 1.9 Å. Compared with x-ray crystallography, the similarity is that the periodic arrangement of the biological macromolecules with a highly uniformity is required, and the difference is that the electron microscopy can acquire the crystal electron diffraction and resolve the structure by obtaining the image of the crystal. However, single particle method is not required to crystallize and has the greatly extensive research field. The suitable sample molecular weight range is more than 100 kDa, and the highest resolution is about 2 Å. Single particle technology has developed rapidly in recent years, and has become the mainstream reconstruction technology, and has been continuously reported the 3D structure of macromolecular complex obtained by this technique. However, these technologies generally require the homogeneity of the sample and averaging from thousands of different proteins. For small and low-symmetry proteins, these methods have limited power in reconstruction resolution. Therefore, to study the 3D structures and functions of amorphous, asymmetrical and non-identical biological samples, other proper technologies have to be developed.[36] Cryo-ET is a suitable technology that reconstructs and visualizes 3D structure of any individual objects by generating a series of tilted 2D projection images of the targets then backprojecting these 2D images to get their 3D density maps. Cryo-ET was mainly used in cell, subcellular organelle and the bio-molecular complexes with no fixed structure (molecular weight range $>$ 800 kDa), and the maximum resolution was as low as several nanometers. However, with the development of hardware and reconstruction algorithms, cryo-ET is gradually becoming a mature technology with a resolution reaching sub-nanometer.[53,58] For example, Zhang and Ren developed the so-called individual particle electron tomography (IPET) method by investigating each

individual macromolecule's 3D structures with much higher resolution.[48] By reducing the reconstructed area to only one target sample, molecules are reconstructed by using the proposed focused electron tomography reconstruction (FETR) to improve the resolution of the single large molecule. This method does not require an initial model and the average signal of a large number of molecules, as well as allows a certain angle error.[48] By using IPET, Zhang *et al.* discovered the 3D dynamical structure and fluctuations of DNA-nanogold conjugates (an 84-bp double-stranded DNA and two 5-nm nanogold particles, see Fig. 3),[59] in which its structural flexibility and dynamics cannot be studied by x-ray crystallography, NMR spectroscopy, or single particle cryo-EM methods. Such results have demonstrated the power of cryo-ET and its great potential application in small and dynamic bio-macromolecules.

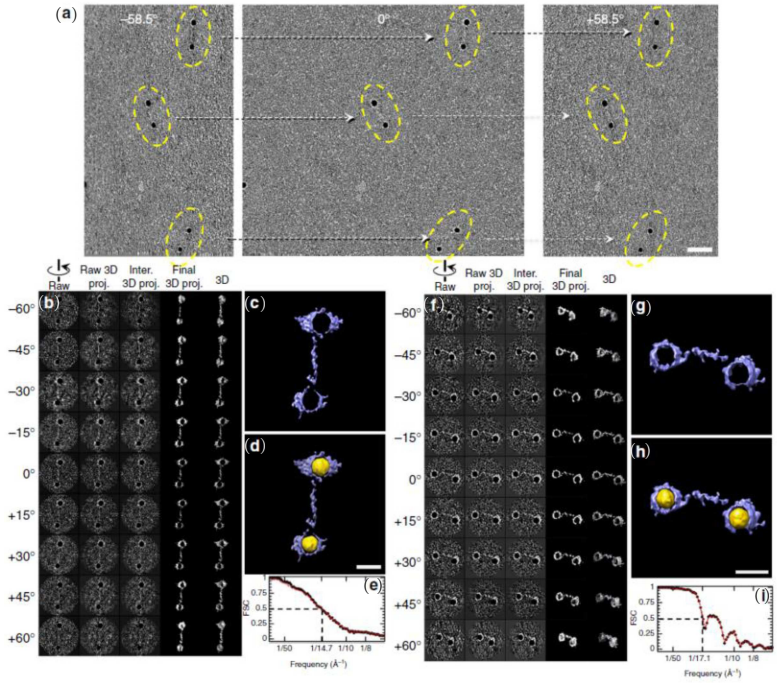


Fig. 3. (color online) 3D reconstruction of DNA-nanogold conjugates by IPET. (a) Images using ET of DNA-nanogold conjugates from a series of tilt angles. (b and (f) Tilt images of DNA-nanogold conjugates, then after CTF correction aligned to a common center for 3D reconstruction via an iterative refinement process and final 3D reconstructions at the corresponding tilt angles. (c) and (g) Final IPET 3D density maps of the targeted individual particles are obtained. (d) and (h) The final 3D density map indicated the overall conformation of the DNA-nanogold conjugates. (e) and (i) FSC analyses show that the resolution of these 3D maps are $\sim$ 14 Å. Adapted from Ref. [59].

4. Conclusion and outlook

Bio-macromolecules play a key role in organic life as they are the performers of basic activities, while the dynamic structures and functions are crucial for understanding their mechanisms. In this review, we have described and summarized the application of different single-molecule and cryo-EM technologies in biophysics field. The advantages of single-molecule technology are that the targets' structural and functional properties could be studied by manipulating them through distance, force, and others information. The limitation is that the overall structure of molecules cannot be obtained at the same time.[60]

Single particle cryo-EM has shown its great power to determine the overall 3D conformation with atomic resolution of bio-macromolecules who have molecular weights larger than 100 kDa and uniform conformations. In addition, cryo-ET has shown its great potential to study the dynamic structures of bio-macromolecules,[48] though the resolution is relatively not high enough to determine sub-nanometer details.

In the future, in order to achieve high resolution and dynamic feature, molecular dynamics (MD) simulations could be combined with cryo-ET to further study finer structural information at atomic level and thus reveal more mechanism information. As a proof of concept, Zhang *et al.* had utilized individual-particle electron tomography (IPET) to study the antibody flexibility and fluctuation through structural determination of individual antibody particles together with docking of the crystal structure to these maps by targeted molecular dynamics simulations.[61] Jones *et al.* had used three independent approaches, MD, cryo-ET and FRET to assess the validity of their DSH model for reconstituted high-density lipoproteins.[62]

In the future, it is believed that upgrading of existing technologies and combining of different single-molecule, cryo-EM and MD methods will be more essential to study the dynamic structures and functions of bio-macromolecules, which

will reshape the way structural biology in next decade.

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