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Mechanistic Studies of Nucleic Acid Chaperone Activities of Retroviral Nucleocapsid Protein

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MECHANISTIC STUDIES OF NUCLEIC ACID CHAPERONE ACTIVITIES OF RETROVIRAL NUCLEOCAPSID PROTEIN

by

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DEDICATION

To my family!

我将这本毕业论文献给我的家人！
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Prof. Hui Wang for the five year's support and training. His deep insights and broad knowledge in science and his enthusiasm towards research has set a high standard for my future career. I also would like to express my gratitude to all members in Hui's group, Qingfeng Zhang, Guangfang Grace Li, Esteban Villarreal, Mengqi Sun, Dr. Xiaoqi Fu, Dr. Yihong Zhan, Dr. Li Zhan, Dr. Hao Jin. Especial thank for Dr. Yihong Zhan for giving me a lot of instruction and guidance in the very beginning of my Ph.D. research. I would also like to thank our collaborator, Prof. Karin Musier-Forsyth (Ohio State University) for providing the HIV-1 nucleocapsid proteins. I am also very grateful to my committee members, Prof. Mark A. Berg, Prof. F. Wayne Outten and Prof. Guiren Wang (Mechanical engineering, University of South Carolina), for their professional guidance in my research plan and proposal and comments on my Ph.D. dissertation.

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ABSTRACT

In this dissertation, I have used single molecule Fluorescence Resonance Energy Transfer (sm-FRET), as a nanoscale spectroscopic ruler to gain quantitative, molecular-level understanding about the kinetics and mechanism of NC-chaperoned TAR sequence rearrangements. Firstly, I have resolved the complex kinetics and underlying reaction pathways of TAR sequence annealing in the presence of HIV-1 NC over a broad NC concentration by performing the time-resolved sm-FRET measurements in a unique aggregation-free environment. I have further gained the insights that the NC-induced secondary structural changed of TAR hairpins modulate the annealing pathways and consequently affect the overall kinetics of the TAR sequence annealing. Secondly, I have demonstrate the macromolecular crowding effects on NC-chaperoned NA annealing by choosing poly-ethylene glycol as a model neutral polymer cosolute to mimic the highly crowded environment in the cytoplasm. Macromolecular crowding effects accelerates every steps in the annealing process without changing the annealing pathway, and make the NC prefer to chaperone with the double-stranded NA. These detailed understanding of the NC-facilitated minus-strand transfer step of reverse transcription of reverse transcription at molecule-level enhance our capabilities to better design effective antiviral medications and therapies. Thirdly, I have also investigate the multifaceted gold-palladium bimetallic nanorods and their geometric, compositional and catalytic tunabilities.
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CHAPTER 1
INTRODUCTION

1.1 Retroviral life cycle

There is a backbone of molecular biology, it is 'Central Dogma of Molecular Biology'. It tells us that the genetic information is transferred from DNA to RNA, and finally is passed into protein, which can be simply described as 'DNA makes RNA and RNA makes protein'. The whole process generally has three steps: genetic information contained in DNA can be copied to DNA by DNA replication, then the DNA information can be copied into mRNA through transcription, and finally by using the information in mRNA as a template, proteins can by synthesized through translation.\(^1\) However, the 'Central dogma' does not cover the reverse flow of information from protein or RNA to DNA, once it is in case of retroviruses. The retrovirus contains a single-stranded viral RNA. Once the virus is inside the host cell cytoplasm, the viral RNA genome will be transferred into DNA through reverse transcription. The newly produced viral DNA is then integrated into the DNA genome of the host cell, then caused the host cell through translation, transcription to generate viral proteins which will reassemble into new viral particles. This whole process is called 'Retroviral life cycle'.\(^2,3\) Among these virus, the HIV-1 virus has been most extensively studied. HIV-1 stand for human immunodeficiency virus type 1. HIV-1 always attacks the body's immune system, if left untreated, it can lead to the disease AIDS (acquired immunodeficiency syndrome), which will cause certain damage to patients immune systems, and usually lead patients die in 5
years. Unlike other viruses, the human body can't get rid of HIV completely. So once patients have HIV, patients have it for life.\textsuperscript{4,5} United Nations Programs on HIV and AIDS have updated the AIDS epidemic database and world distribution by 2015, and found that there were more than 36.9 million people were caught with HIV. Although more located in Africa, AIDS is covered all over the world, and is threatening the whole world. In order to control this destructive pandemic, release the pain that people suffering, and provide better treatment for people struggled with AIDS, it is of great significant to understand the source of the disease, the HIV-1 virus.

There are two identical single-stranded RNA genomes encapsulated by viral structural proteins in the HIV-1 virus.\textsuperscript{6} The HIV-1 virus particle is encircled by a lipid bilayer, which contains many copies of envelop proteins derived from the host cell membrane. Moreover, in the inside shell of this lipid bilayer, is a matrix which is composed of about 2,000 copies of matrix protein (MA). Then, in the center of the HIV-1 virus particle, is a cone-shaped capsid which is containing about 2,000 copies of capsid protein (CA). Inside of this capsid particle, is the two identical RNA containing two copies of the viral genome. The RNAs are stabilized as a ribonucleoprotein complex which is composed of ~2,000 copies of nucleocapsid protein (NC). Furthermore, three enzymes encoded with three virally enzymes: reverse transcriptase (RT) and integrase (IN) and protease (PR), which are very essential for the retroviral life cycle, are also existed in the complex.\textsuperscript{6,7} Some other accessory proteins are also assembled inside the virus particle, for example, Viral infectivity factor (Vif), Viral Protein R (Vpr) and Negative Regulatory Factor (Nef), while Trans-Activator of Transcription (Tat) and regulator of expression of virion proteins (Rev) are not included in the particle.\textsuperscript{6}
The HIV-1 virus always infects specifically the vital cells in human immune system, then cause certain damage to people's immune response defense system. CD4⁺ T cells are one kind of these important cell that function as human body's immune recognition. Figure 1.1 describes the HIV-1 life cycle. By the envelope gp120 glycoprotein on the surface of the HIV-1 virus and the recognizing receptors on the CD4⁺ T cells, the HIV-1 virus attaches to the target cell (1, Figure 1.1). Then followed by the membrane fusion (2, Figure 1.1), the HIV-1 virus enters into the target cells. Once the virus is inside of the host cell, it is disassembled and its RNA genome, viral proteins are injected into the host cell cytoplasm (3, Figure 1.1). Subsequently, by using human tRNA_Lys as a primer, the HIV-1 viral reverse transcriptase (RT) will reverse transcribed the single-stranded viral RNA into its complementary double-stranded DNA (referred as cDNA) (4, Figure 1.1). Then the newly generated cDNA transported into cellular nucleus and integrated into the host cell genome by another viral protein, integrase (IN)(5 and 6, Figure 1.1). By using the viral cDNA as a template, the viral cDNA genome is transcribed into viral mRNA, following transported out of the nucleus for translation (7 and 8, Figure 1.1). By using the host cell's translational and transcriptional machinery, unspliced and singly spliced viral mRNAs are translated into viral protein to produce Gag and Gag-Pol polyprotein. Meanwhile, the short spliced mRNA are used to synthesis Tat and Rev like the regulatory proteins (9, Figure 1.1). Thereafter, by Gag-Gag interaction, the newly produced Gag proteins are aggregated together at the plasma membrane of the host cell, where the new HIV-1 virus will be assembled (10, Figure 1.1). Then, two copies of the unspliced viral RNA and Gag formed the immature viral particle will force the membrane into curvature, and bud out from the host cell's membrane, then finally release from the
cell (11 and 12, Figure 1.1). Upon released from the host cell, the protease (PR) enzymes cleave the polyprotein into other enzymes, such as structural proteins including NC (13, Figure 1.1).\textsuperscript{6,13} Moreover, it has been reported that, one HIV-1 life cycle takes a very short time period. In an infected body, it can produce over 1-10 billion virions per day, and at the same time, same amount of CD4+ T cells are destroyed.\textsuperscript{14,15}

\textbf{Figure 1.1} Scheme of HIV-1 life cycle.\textsuperscript{10}

\textbf{1.2 Reverse Transcription}

At the early stage of the HIV-1 life cycle, the reverse transcription step is a particularly critical step for retroviral replication.\textsuperscript{16} During this step, a single-stranded RNA genome is converted into a double-stranded DNA, and the whole process contains a series of nucleic acid rearrangements and nucleic acid/nucleic acid annealing steps (NA/NA annealing), which is shown in Figure 1.2. The HIV-1 retrovirus reverse transcription
initiates by annealing host cell's unwound tRNA\textsuperscript{Lys,3} to the primer binding site (PBS) at the 5' end of the HIV-1 RNA (step 1, Figure 1.2). Then by using the HIV-1 RNA as a temple, the viral genome is copied into its complementary DNA, followed by being digested by the RNase H domain of reverse transcriptase (RT). By keep copying the viral genome into DNA to the 5' end of the HIV-1 RNA, the minus-strand strong stop DNA ((-)SSDNA) is synthesized (step 2, Figure 1.2). Then, the newly synthesized (-)SSDNA trans-locates to the 3' end of the HIV-1 RNA in order to complete the reverse transcription (step 3, Figure 1.2). During this minus-strand transfer step, it may occur nucleic acid anneal from one end to the other end of the HIV-1 RNA (intra-molecular) or the nucleic acid anneal to the other copy of the RNA (inter-molecular).\textsuperscript{17} The (-)SSDNA continues to be elongated, and stops at the polypurine tract (PPT) site which is near the 3' end of the HIV-1 RNA (step 4, Figure 1.2). Here, the RNase H domain of the reverse transcriptase creates an RNA primer to initiate plus-strand DNA ((+)SSDNA) synthesis (step 5, Figure 1.2).\textsuperscript{18,19} By using the synthesized (-)SSDNA as a template, the (+)SSDNA is copied to its the 5' end and simultaneously the HIV-1 RNA is totally digested by the polymerase domain of viral RT (step 6, Figure 1.2), then goes through a plus strand transfer step (step 7, Figure 1.2), following by elongation and finally reach the PBS site of the (-)SSDNA (step 8, Figure 1.2). All of the above steps are the process of the reverse transcription, and it has been reported that the whole processes are regulated and controlled by the interaction between the viral protein and nucleic acid interaction, especially the interaction of the nucleocapsid protein and nucleic acid.\textsuperscript{20}
Figure 1.2 Illustration of the mechanism of HIV-1 reverse transcription steps.²¹ Step 1: Unwounded tRNA<sub>Lys,3</sub> anneals to the primer binding site (PBS) on the RNA genome. Step 2: Synthesis of minus-strand strong stop DNA ((-)SSDNA) by extension the complementary of the tRNA, and the degradation of the RNA template by RT. Step 3: Minus-strand transfer. The (-)SSDNA is translocated to the 3’ end of the RNA genome. Step 4: Elongation of (-)SSDNA to the 3’ end. Step 5: Synthesis of (+)SSDNA by using PPT (polypurine tract) as a primer. Step 6: Totally degradation of HIV-1 RNA. Step 7: The (+)SSDNA is translocated to the 3’ end. Step 8: Elongation of both DNA strands.
1.3 Structure and Nucleic Acid-chaperone activity of nucleocapsid (NC) protein

The HIV-1 nucleocapsid protein (NC) is a small nucleic acid (NA)-chaperone protein, containing 55 amino acids, and has a molecular weight as 6488 Dalton, and derived from the cleavage of the Gag polyprotein.\(^7\,^22\) The HIV-1 NC contains two functional and structural domains, a basic N-terminal domain and two nonequivalent CCHC-type zinc fingers, which is illustrated in Figure 1.3 and Figure 1.4.\(^23\,^25\) In spite of its structural simplicity, HIV-1 NC has been generally recognized as a multifunctional viral protein, and plays a critical role during the retroviral life cycle, such as facilitates the tRNA primer replacement,\(^26\,^28\) promotes the minus strand transfer\(^29\,^31\) and plus strand transfer procedures,\(^32\,^38\) as well as inhibit (+)SSDNA hairpin from self-priming. Moreover, HIV-1 NC may protect the newly synthesized proviral DNA from being digested by RNase by coating the DNA surfaced, and reduce the structural rigidity of DNA duplexes to facilitate the proviral DNA integration.\(^39\,^42\) It has been generally believed that either NC or its precursor, Gag polyprotein, can bind to RNA to assist certain processes, such as genomic RNA recognition, packing\(^43\,^48\) and dimerization\(^49\,^51\), also with virus assembly.\(^49\,^58\)

These NA chaperone activity of NC is believed to arise from two main consequences of NC's binding to NAs, NA duplex destabilization and NA aggregation, which are associated with the zinc fingers and the basic N-terminal domain, respectively.\(^7\) DNA or RNA nucleic acids (NA) strands are composed of nucleotides units with one of the four nitrogen-containing nucleobases like cytosine (C), guanine (G), adenine (A), thymine (T), uracil (U), and a sugar and a phosphate, among which, nucleobases and sugar are natural, but the phosphate is negative, indicating that the NA is negative charged. Meanwhile, the
N-terminal domain of NC contains 12 residues, 4 of them are basic, while others are natural, leading the N-terminal domain is positive charged. Thereafter, through electrostatic interaction with NAs, the N-terminal domain of NC bonds to the major groove of the RNA loop stem. This interaction effectively promotes the aggregation of NC/NA or NA/NA complexes through intramolecular and intermolecular reaction.

It has been investigated that zinc fingers contains a basic residues linker region and the aromatic residues (CCHC-type) form the hydrophobic patches. Recent studies have also shown that the zinc figures have a higher affinity to interact with specific base residues, like the TG- and UG- rich sequences. In addition, the NMR research results of NC determined that Phenylalanine and tryptophan in the zinc fingers bound to two nonequivalent single stranded GGAG loop stems SL2 and SL3 in the RNA genome respectively, through aromatic \( \pi - \pi \) stacking interaction with the G-residues.

Moreover, the intercalation of Zn\(^{2+}\) big ions and the release of Na\(^{+}\) or other counter ions, accelerate the instability of the double stranded NAs. Thus, the preference of the zinc figure binding to NA, makes it responsible for the destabilizing NA helix.

Although it has been observed that NC acts as a non-specific binder, with apparent dissociate constants, \( K_d \), it has also been proved that NC prefers to binding to single-stranded sequences rather than double duplex, especially a higher affinity for UG- and TG- rich sequences. Moreover, it has been characterized that the binding footprint size of NC on NAs is 6~8 nucleotides.
**Figure 1.3** Scheme illustration of three-dimensional NMR structure of a mature HIV-1 nucleocapsid protein (NCp7) bounds to SL3 region of RNA. The N-terminal domain showed in purple, binds to the major groove of the RNA loop-stem through electrostatic interactions. The two CCHC-type zinc figures showed in red and blue are engaged in specific interactions with the purine bases.67

**Figure 1.4** Sequence of mature HIV-1 nucleocapsid protein (NCp7), basic residues are in blue, the acidic residues are in red, and the CCHC-type zinc figures are circled in black, while zinc ions are circled in green.7
1.4 NC's functions in the Minus-strand transfer during the reverse transcription

The third step in the reverse transcription is the minus-strand transfer, involving the (-)SSDNA transfers from the 3' end of to the 5' end of viral RNA, following with the unfolding of their NAs structures, then anneal with its complementary region of the viral RNA to form a fully base paired DNA/RNA complex (as illustrated in Figure 1.5). Thus, the product DNA/RNA duplex helix is thermodynamically more stable than the two reactants. However, traditional gel shift assays characterized that this annealing process was quite slow without NC, while once carried out in the present of NC, the reaction rate will be effectively accelerated. It has been generally believed that it's all due to the NC's NA chaperone activities, which will cause NAs rearrange structures to contain the maximum number of base pairs and finally result in the most thermodynamically stable structure. The NA chaperone activities of NC arise from two main consequences of its binding to NA structures. One is the NC-induced partially melting of NA duplex, during which NC intercalates into the NA's duplex regions to form the complexation with single-stranded NAs, thereby lowers the energy of highly structured NAs and further lowers the energy barrier for annealing. The other one is the NC-catalyzed NA annealing, during which NC lowers the energy cost of annealing two totally complementary NAs together, through the binding of the positive basic residues rich charged N-terminal domains to the double-stranded NAs by screening the negative charges.

The NC-induced NA melting/unfolding is believed to the rate limiting step, however the molecular-level understanding about mechanism by which NC destabilizes structured NA helix is still an open challenge. CD and absorbance measurements showed that NC
effectively induced unwinding of the viral RNA, while some believed that the CD results are more due to the local base unstacking instead of unwinding the RNA strands. In addition, Chan and his collaborators using time resolved FRET measurements, also provided evidence that NC induced more subtle changes in tRNA stem, rather than promotes the unwinding of tRNA. Moreover, by performing NMR study on the relaxation time and special stem exchange of tRNA, the idea that NC induced more subtle conformational changes and transient rearrangements in the tRNA is much clearer. Further research results even proved that it is the tRNA tertiary core and A-form helices that will occur subtle and transient changes under the catalysis of NC.

![Figure 1.5](image)

**Figure 1.5** Schematic illustration of HIV-1 minus-strand transfer steps. TAR DNA anneals with TAR RNA to form TAR DNA/RNA double helix catalyzed by NC.

Another of equal importance during the minus-strand transfer step is the NC chaperoned TAR DNA and TAR RNA annealing, which follows the NC-induced the destabilization of these two highly structured hairpins. In this complex process, TAR DNA and TAR RNA anneal together to form 98 base pairs under the catalysis of NC in vivo (illustrated in Figure 1.5). It has been observed that the annealing process has two
exponential grow population, indicating it is a two step reaction. Based on this, two reaction pathways has been proposed,\textsuperscript{95} one is the loop pathway, the other is the zipper pathway. When TAR DNA and TAR RNA take the loop pathway, the loop stem region of TAR DNA and TAR RNA anneal firstly to form a loop 'kissing' intermediate, then gradually switches to the final duplex product. When TAR DNA and TAR RNA molecules go through the zipper pathway, the zipper terminal stem of them should firstly anneal together to form zipper intermediate, and finial convert into the final TAR DNA/RNA complex (see Figure 1.6). If without NC in the reaction system, this annealing is super slow, probably due to without the chaperon activity of NC, TAR DNA and TAR RNA may undergo intramolecular self-priming instead of going through the minus strand transfer step.\textsuperscript{96} Moreover, it also has been proved that NC effectively accelerates the reaction rates of TAR DNA and TAR RNA annealing by almost 3,000-fold, and the annealing process is a first-order reaction.\textsuperscript{97}

In conclusion, the NC chaperoned NA annealing reaction is a very complex, heterogeneity bimolecular processes, involving multiple reaction intermediates along these two annealing pathways. However, the ensemble research results only provide us with ensemble-averaged information about the overall kinetics, it lacks the ability to resolve the complex kinetics, as it cannot provide real-time measurements, and is incapable to distinguish the partially annealed intermediates from the completely annealed products. In a word, the traditional ensemble-averaged measurements cannot trap the trajectories of each single molecule.
Figure 1.6 Schematic illustration of two proposed TAR DNA/RNA annealing pathways. On the left is the loop 'kissing' annealing pathway, on the right is the zipper annealing pathway.
Moreover, a severe complication in ensemble measurements is the perturbation resulting from the large scale NC/NA aggregation (LSA).\textsuperscript{91,98} LSA refers to insoluble molecular aggregates composed of typically hundreds to thousands of NC and NA molecules. LSA has been described as both a favorable effect that accelerates NA annealing\textsuperscript{91} and, in contradiction, as an artifact of in vitro sample preparation that actually inhibits NA annealing.\textsuperscript{99} Recent single-molecule spectroscopic measurements revealed that the LSA formation was accompanied by a large drop in NC’s ability to chaperone NA annealing, even inducing a “stalling effect”, i.e. no detectable annealing reaction, above certain NA and NC concentrations.\textsuperscript{99} In ensemble spectroscopic measurements, N-terminus truncated NC mutants that lack the NA aggregating capability were typically used instead of the wild-type full-length HIV-1 NC to eliminate the complication due to LSA.\textsuperscript{100-104}

1.5 Single-Molecule FRET and its application in studies of NC’s chaperone activity

Florescence Resonance Energy Transfer (FRET) describes a mechanism that energy may transfer between two closed chromophores through a non-radioactive dipole-dipole interaction. Once excited by a laser, the electrons absorb a photon (blue line in Figure 1.7) in the donor chromophore jump from the ground state ($S_0$) to its higher vibrational level of the first excited state ($S_1$). Then the electrons relax energy rapidly to a lower vibrational level of $S_1$ within picoseconds (ps), and gradual (nanoseconds) decay to the ground state in the form of fluorescence (green arrow in Figure 1.7). As an alternative, the energy of the donor excited state can be transferred nonradiatively to the acceptor with resonance energy transfer (dashed black arrows). Then, the electrons of the donor
molecule in turn are excited to their first excited states (S1), which can decay to the ground state and emits a photon in the form of fluorescence (red arrow in Figure 1.7). As mentioned, the conditions for FRET to occur is the overlap between the donor emission and acceptor absorption spectra. For example, in our case, an argon gas laser with 514 nm is used, while the excitation range of Cy3 is from 500 nm to 550 nm (yellow area), which is perfect match. Meanwhile, there is one overlap (green area) between the emission of Cy3 and excitation of Cy5 (see Figure 1.8). The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor making FRET extremely sensitive to small distance changes between 1-10 nm range, leading FRET act perfectly as 'A spectroscopic ruler' to investigate molecular interactions.

The efficiency of FRET is expressed in Equation 1, where R₀ is the Förster distance of the two chromophores, at which, the FRET efficiency value is 0.5, indicating that half of the excitation energy is transferred to acceptor. R₀ is a characteristic parameter of each chromophores pair, can be calculated as the following equation (in Ångstrom).

\[
E_{FRET} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}
\]

(1)

\[
R_0^6 = \frac{9000(\ln 10)\kappa^2 \varphi_D^0}{128\pi^5 N_{Avo} \eta^4} J(\lambda) = 8.79 \times 10^{-5} \kappa^2 \varphi_D^0 n^{-4} J(\lambda)
\]

(2)

where \(\varphi_D\) is the fluorescence quantum yield of the donor in the absence of the acceptor, \(N_{Avo}\) is Avogadro’s number, \(n\) is the refractive index of the medium, \(J(\lambda)\) is the overlap integral describing the degree of overlap between the donor fluorescence emission.
spectrum and the acceptor absorption spectrum and $\kappa^2$ is the orientation factor, since the dipole moments of two chromophores rotate freely in all directions, in this case $\kappa^2 = 2/3$.

In our sm-FRET measurements, we choose Cy3 and Cy5 as the two chromophores, Cy3 serves as the dye donor, while Cy5 serves as the dye acceptor. That's all due to Cy3 and Cy5 have relatively high photostability, high extinction coefficient and quantum yield, moreover, they have relatively small size, and thus easy to be conjugated to biomolecules. In addition, Cy3, which emits maximally at 580 nm and Cy5, which maximally excited at 640, the $R_0$ of Cy3 and Cy5 is around ~6 nm. Indicating, if the distance of Cy3 and Cy5 is around 5~6 nm, the efficiency value is around 0.5 which is a middle value, otherwise if the distance is larger than $2R_0$, like 10 nm, the efficiency value is almost 0.

![Figure 1.7](https://en.wikipedia.org/wiki/F%C3%B6rster_resonance_energy_transfer)
Figure 1.8 Excitation (absorption)(blue) and emission (fluorescence)(red) of Cy3 and Cy5, the spectral overlap integral is in green. (Figure adopted from http://www.biotek.com/resources/articles/fluorescence-resonance-energy-transfer.html)

Single-molecule Fluorescence Resonance Energy Transfer (SM-FRET) spectroscopy\textsuperscript{107} which was first performed on biological measurements in 1996,\textsuperscript{108} has been widely used for unraveling molecule structures, dynamics and chemical kinetics in highly heterogeneous and complex bio-systems.\textsuperscript{109-111} SM-FRET has been successfully applied in the investigation of biochemical structural dynamics of proteins,\textsuperscript{112,113} nucleic acids,\textsuperscript{114,115} fusion from the membranes\textsuperscript{116,117}, as well as protein-protein, nucleic acids-nucleic acids, protein-nucleic acids interaction.\textsuperscript{106,118} Furthermore, a lot of novel sample preparation methods and spectroscopic have been developed to promote the SM-FRET studies of the complex molecular biology problem.\textsuperscript{119,120} SM-FRET spectroscopy allow one to observe one molecule at a time, then demonstrate the trajectories of this molecule during measurement time, providing a powerful skill to resolve the subpopulations and heterogeneity that the ensemble measurement can't solve.

In our group, we are using SM-FRET as a spectroscopic ruler to detect the NC-induced NA structural remodeling. There are unique advantages of using SM-FRET for the
investigation of NC-induced NA structural remodeling. Both the NC-chaperoned NA annealing and NC-induced DNA bending have been observed to be complex, heterogeneous biomolecular processes that involve multiple intermediates along different pathways and are associated with complicated kinetics/dynamics over multiple time-scales. The unique ability of single-molecule spectroscopy to study one molecule at a time allows for unraveling of the tremendous heterogeneity of this type of biomolecular systems.\(^8\),\(^{121}\) A major source of the heterogeneity is the distribution of stoichiometries for the NC-NA nucleoprotein complexes, i.e. with different numbers of copies of NC, DNA and RNA molecules. An additional source of dynamic heterogeneity is the distribution of NA secondary structures, which may even include some mis-folded and long-lived intermediate states. Single-molecule spectroscopic measurements provide considerably richer information about the complexity and heterogeneity of biomolecular processes than the ensemble measurements performed on bulk samples. Of course there are also disadvantages to single-molecule spectroscopy, especially the necessity to label the protein and/or NA molecules with appropriate fluorescent dyes, which may introduce additional complication to the spectroscopic signals due to the photoblinking, photobleaching, and other photophysical processes. Such complication, however, can be minimized by choosing appropriate time windows/intervals for data collection and using oxygen scavenger reagents to suppress the photoblinking and to generate longer-lasting fluorescence signals.\(^8\) Single-molecule spectroscopy also provides unique opportunities of probing the kinetics of NC-chaperoned NA structural remodeling processes in aggregation-free environments. A severe complication in investigating NC-induced NA structural remodeling is the perturbations resulting from large scale NC/NA aggregation
LSA refers to insoluble molecular aggregates composed of typically hundreds to thousands of NC and NA molecules. LSA can significantly alter the molecular structures, conformational dynamics, and stoichiometry associated with NC-NA interactions. LSA of this type may have been present in most of the \textit{in vitro} ensemble measurements of NC-chaperoned processes. LSA has been described as both a biologically relevant effect that accelerates NA/NA annealing\textsuperscript{98,122,123} and, in contradiction, as an artifact of \textit{in vitro} sample preparation that actually inhibits annealing.\textsuperscript{60,127,128} LSA occurs preferentially at high NC and NA concentrations (e.g. [NC] > 300 nM; [NA] > 50 nM) \textit{in vitro}, however, the \textit{in vivo} case differs dramatically in two major respects from the \textit{in vitro} measurements. First, there is a low NA copy number \textit{in vivo}, two genomic RNA strands, not hundreds or thousands of separate NA as in LSA. Second, the RNA strands \textit{in vivo} are of course much longer than the \textit{in vitro} constructs. Recent single-molecule spectroscopic studies on TAR sequence annealing in the presence of high concentrations of NC and NA revealed that the LSA formation was accompanied by a large drop in NC’s ability to chaperone NA annealing, even inducing a “stalling effect”, for example no detectable annealing reaction, above certain NA and NC concentrations.\textsuperscript{127} We hypothesize that LSA has little to do with the \textit{in vivo} processes and should be strictly eliminated from experimental protocols for studying NC-induced local melting, annealing, and binding. It has been recently shown that LSA is formed by a nucleation controlled precipitation-like process, rather than a simple Ostwald ripening.\textsuperscript{127} Thus, if nucleation of aggregate growth is avoided, aggregation-free NA annealing and bending studies can be accomplished.\textsuperscript{90} Measuring individual surface-immobilized molecules reacted with freshly mixed protein/NA solutions in a novel flow cell system at
relatively low NA concentrations provide a unique way to substantially suppress the formation of LSA during NC-chaperoned NA annealing and NC-induced NA bending. The proposed single molecule measurements will be carried out under aggregation free conditions to eliminate the complications due to LSA.

1.6 references


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CHAPTER 2

SINGLE MOLECULE STUDY OF HIV-1 NUCLEOCAPSID PROTEIN
SWITCHES THE PATHWAYS OF TRANS-ACTIVATION RESPONSE
ELEMENT ANNEALING

2.1 Introduction

The nucleocapsid (NC) protein encoded by the human immunodeficiency type 1 virus (HIV-1) is a small nucleic acid (NA)-binding protein containing a basic N-terminal domain and two nonequivalent CCHC-type zinc fingers.\textsuperscript{1-5} In spite of its structural simplicity, HIV-1 NC is multifunctional and has long been recognized as both a virion-stabilizing structural protein and an NA chaperone.\textsuperscript{4,5} A series of key steps in the retroviral life cycle rely on NC’s ability to function as a general, non-sequence-specific NA chaperone that efficiently catalyzes several crucial NA structural remodeling processes, such as the minus-strand and plus-strand transfers in the reverse transcription.\textsuperscript{4-8} The NA chaperone activity of NC is believed to arise from two major consequences of NC’s binding to NAs, NA duplex destabilization and NA aggregation, which are associated with NC’s zinc fingers and basic N-terminal domain, respectively.\textsuperscript{4} Rapid kinetics of NC dissociation from NA substrates has recently been proposed to be another key feature of NC’s chaperone function.\textsuperscript{9,10}

Reverse transcription, through which the single-stranded viral genomic RNA is converted into the double-stranded proviral DNA, is a key step in the retroviral life cycle.
Reverse transcription of the viral RNA genome requires two obligatory strand transfer steps, the minus-strand transfer and plus-strand transfer. In HIV-1, NC chaperones the annealing between NA hairpins of the trans-activation response (TAR) elements of the minus-strand strong-stop DNA and the TAR sequence in the 3’-untranslated region of the genomic RNA, to form a fully base-paired NA duplex in the minus strand transfer step of the reverse transcription.\(^4,5\) The “product” DNA/RNA duplex structure, which contains several additional Watson-Crick base pairs, is thermodynamically more stable than the more weakly base-paired reactant hairpins. Resolving the kinetics and underlying reaction pathways of this NC-chaperoned NA structural remodeling process is of paramount importance to the development of detailed, molecular-level understanding of the complicated dynamic NC-NA interactions that underpin NC’s NA chaperone functions and will ultimately enhance our capabilities to design new anti-viral drugs that specifically inhibit the chaperone functions of NC.

Here we use single-molecule fluorescence resonance energy transfer (smFRET)\(^11,12\) as a spectroscopic tool to gain quantitative, molecular-level insights into the kinetics and mechanism of NC-chaperoned TAR sequence annealing. The HIV-1 TAR sequence annealing is a complex, heterogeneous biomolecular process involving multiple intermediates along alternating reaction pathways that are not readily resolvable by ensemble measurements. Two possible reaction pathways, a “loop” pathway and a “zipper” pathway, have been proposed for the TAR sequence annealing.\(^13-15\) Electrophoretic gel-shift assays showed that in the absence of HIV-1 NC, the kinetics of full-length HIV-1 TAR sequence (59 nt) annealing was very slow and could be described by a two-step process.\(^15\) The two exponential growth components observed in the kinetic
curves were hypothetically assigned to the formation of a kissing loop intermediate and an even slower strand exchange between the terminal stems to form the NA duplex, respectively. In the presence of HIV-1 NC, both the loop intermediate formation and the subsequent conversion to NA duplex were greatly accelerated and, more interestingly, a distinct “zipper” pathway involving nucleation at the 3'/5' termini also became possible in addition to loop pathway nucleated at the hairpin loop region. At sufficiently high NC concentrations, the annealing had been hypothesized to be dominated by the zipper pathway because a single-exponential growth was typically observed in the gel-shift assays.15 However, the gel-shift assay, which can only provide ensemble-averaged information about the overall annealing kinetics, is incapable of resolving the complex kinetics when multiple pathways coexist as it lacks the capability to distinguish the partially annealed intermediates from the completely annealed products. The unique capability of single-molecule spectroscopy to study one molecule at a time11,12,16-27 allows for unraveling of the tremendous heterogeneity of this type of biomolecular systems. In addition, SM-FRET allows one to detect the kinetics of NC-induced NA structural remodeling processes in an aggregation-free environment, which effectively avoid the large scale NC/NA aggregation that troubled the ensemble measurements for a long time.13,28-37

2.2 Experimental Section

Sample preparation.

In our measurements, the HIV-1 NC protein which has 55 amino acids in length, was provided by our collaborator, Dr. Musier-Forsyth's group from Ohio State University, and was synthesized as described previously. Its structure is shown in Figure 2.1A.14,38-40
All DNA oligonucleotides labeled with appropriate dyes and biotin-functionalization were acquired from Trilink BioTechnologies (San Diego, CA) or Integrated DNA Technologies (Coralville, IA). They were all purified by the supplier by high-performance liquid chromatography (HPLC). All the DNA oligonucleotides have a hairpin-like shape. Figure 1B illustrates this secondary structure, which were predicted by mfold program.41,42

In our NC chaperone NA annealing measurements, Cy3 which acted as FRET donor, was attached to the 5' end of the TAR DNA molecules, meanwhile biotin functionalization was added to its 3' end. This TAR DNA molecular was immobilized on the cover slip through biotin-streptavidin interactions. Its total complementary DNA oligonucleotides (cTAR DNA) which was labeled with Cy5 (FRET acceptor) at its 3' end, coflow with HIV-1 NC protein and reaction buffer into the experimental chamber. In the NC induced NA melting assays, the TAR DNA oligonucleotides was double labeled with Cy3 and Cy5 at its 5' and 3' terminus, respectively. And, biotin functionalization was added to its middle for the immobilization on cover slips.

In order to suppress the undesirable G residue quenching effects, the dyes were attached to the T nucleotide at the 5' or 3' terminus of the DNA molecules. Also, the TTTT overhang were added to ensure the rotational freedom of the dye molecules, such that all relative dipole orientations were sampled much faster than the timescale of the measurements, giving rising to an average orientation factor (κ2) of 2/3.
**Figure 2.1.** Structures of various molecules used in this smFRET study. (A) Sequences of HIV-1 NC protein with the zinc-binding residues marked in red and the basic residues marked in blue. (B) Sequences and secondary structures of various dye-labeled and biotin-functionalized DNA oligonucleotides. The capitalized letter “B” indicates biotin functionalization. The sequences in the TAR DNA that are complementary to the zipper and loop DNA oligonucleotides are indicated in orange and blue, respectively. Structures were predicted by the program mfold. (www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi)

**Total Internal Reflection Fluorescence (TIRF) Microscope for smFRET Measurements.**

A home-build TIRF microscope system (shown in Figure 2.2) was used to record the time-resolved FRET trajectories of each individual molecule. Two Argon gas laser excitations (λ=514 nm and 638 nm, 200 mW, Spectra-Physics, Santa Clara, CA) was used as excitation source. An oil immersion, high numerical aperture objective CFI APO (Nikon, 100×, NA 1.49) was used for both TIRF excitation and signal collection. The
fluorescence signals was sent through a dual band-pass dichroic mirror and further
separated into donor and acceptor channels using a 650 nm dichroic mirror (Chroma
Technology, Bellows Falls, VT) and a set of customized filters. Signals were finally
collected using an electron-multiplying charge-coupled device (EMCCD).

Figure 2.2. Scheme of the TIRF microscope setup for smFRET measurements.

Flow Cell System For smFRET Measurements

A home-build flow cell system was used to carry out the reactions (see Figure 2.3
left).\textsuperscript{36,39,43} In a typical smFRET-based NC chaperone NA annealing measurements, Cy5-
labeled cTAR DNA hairpin and HIV-1 NC protein were delivered by two syringe pumps
(Chemyx Fusion 100, Chemyx Inc., Stafford, TX) into the reaction chamber to react with
Cy3-labeled TAR DNA molecules that were immobilized on the coverslip (Fisher
Scientific) surface to form the DNA duplexes. For a typical smFRET-based NC induced
NA melting measurements, a wide range concentrations of HIV-1 NC protein were flowed into the reaction chamber to initiate the dual dye-labeled TAR DNA molecules melting which were immobilized on the coverslip. The flow rate of all the solutions were controlled at 4 μl/min, also in the annealing assays, the cTAR DNA was fixed at 2 nM, to avoid the NC/NA coaggregation. Moreover, the concentrations of the NC used in our smFRET measurements were controlled within an safe range to ensure the NC did not aggregate with itself.

Coverslip and Chamber Preparation

The commercial coverslips were soaked in piranha solutions (25% hydrogen peroxide and 75% conc. sulfuric acid) for 1 h, then rinsed with plenty of water (molecular biology grade) and acetone (HPLC grade) to ensure neutral. After dried under N₂ stream, the clean coverslip were treated with Vectabond/acetone 1% w/v solutions (Vector Laboratories, Burlingame, CA) (an amino-silane reagent) for 5 mins, and then rinsed with water and dried with N₂ stream. The vectabond coated coverslips were masked with templated silicone films. The uncovered areas were incubated with a 33% w/w mPEG-Succinimidyl Succinate (MW 5000 Da, Laysan Bio, Inc., Arab, AL) containing a 0.25% mPEG-Biotin (MW 5000 Da, Laysan Bio, Inc., Arab, AL) in a 0.1M sodium bicarbonate solution (Sigmat-Aldrich, St. Louis, MO) for 3h. Then mark the reactive PEG areas, remove the silicone films, rinse the excess PEG, and dry the coverslips under N₂ stream. A chamber with a total volume of ~10 μl was formed after assemble predrilled polycarbonate films with an adhesive gasket (Grace Bio-Labs, Bend, Oregon) on the top of the PEGylated and biotinylated coverslips. Inlet and outlet ports (Nanoport™, Upchurch Scientific) were glued on top of the chambers. The details of the chamber
assembly process and immobilization of DNA oligonucleotides have been described previously. Then, each chamber was incubated with streptavidin (Molecular Probes, Eugene, Orgen) (0.6 mg/mL in 25 mM HEPES buffer, pH 7.3)for twice, each time for 10 min. Prior to the injection into the chamber, the biotin-functionalized TAR DNA molecules were renatured by incubating in HEPES buffer (25 mM HEPES, pH 7.3, 40 mM NaCl) for 3 min at 85 °C, 5 min at 60 °C, and 10 min at 0 °C, to ensure that they were at their most stable closed form. After re-nature, the molecules were diluted to 2 nM in HEPES buffer solution containing 10 mM MgCl₂. Then by injecting the renatured molecules into the chamber several times, each time for 3 min, the individual TAR DNA hairpins immobilized on the coverslip were achieved (see Figure 2.3 right panel). Once have the TAR DNA molecules are immobilized on the coverslip, the buffer solution would flow into the reaction chamber, to flow away the extra TAR DNA molecules which are float in the reaction chamber. Furthermore, all the smFRET annealing and melting reactions were carried out at room temperature in HEPES buffer (25 mM HEPES, 40 mM NaCl, and 0.2 mM MgCl₂, pH=7.3) in an oxygen scavenger system containing D(+) glucose 15% w/v (Sigma-Aldrich, St. Louis, MO), glucose oxidase 0.1 mg/ml, catalase 0.2 mg/ml (Sigma-Aldrich, St. Louis, MO) and 1% v/v 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Before the SM-FRET measurements, cTAR DNA would first flow for 5 min, let the chamber full of reactants. Then we start to coflow the NC solution and the cTAR DNA, the flow rates of both are precisely controlled by syring pumps. All the solutions used in smFRET measurements were freshly made, and stored at 0 °C before set up into the syringes, and immediately sealed with plugs.
Data Collection and Analysis

The TIRF microscope was used to record the time-resolved FRET trajectories of each individual molecules during the NC-chaperoned NA structural remodeling process. Cy3 and Cy5 are chosen as the donor and acceptor FRET pair to label the TAR DNA molecules, due to their photostability, high extinction coefficient and quantum yield, and relatively small size, as well as they are easy to be conjugated to biomolecules. Fluorescence signals from individual molecules in 80 μm X 40 μm regions were imaged and each smFRET image frame consisted of a donor image and an acceptor image both of which were 512 pixels X 256 pixels (see examples in Figure 2.4). Time-resolved fluorescence trajectories were recorded using the commercial NIS-Elements software provided by Nikon and the raw data were obtained in an ND2 format. The raw data were then processed and converted into a video file format using ImageJ, which is a public domain, Java-based image processing software developed by National Institutes of
Health. The FRET efficiencies, which are determined by the distance between the donor and acceptor dyes, were calculated by extracting the donor and acceptor emission intensities of individual molecules from the smFRET images. In addition, for the kinetics measurements of NA annealing assays, smFRET images will be taken at appropriate time intervals and the excitation laser will only be turned on during the imaging time (typically 50 ms or 200 ms per image frame) to effectively protect the molecules from photobleaching, since the NA annealing processes typically occurs over time-scales from minutes to hours. As for the NC-induced DNA hairpin melting assays, the end-to-end distance variation will be monitored in real time by continuously collecting smFRET trajectories of individual molecules until photobleaching occurs.

Figure 2.4. Scheme of representative smFRET images.
Figure 2.5. Scheme of Matlab interface for data analysis.

A custom-written Matlab routine were used to find individual molecules, calibrate stage-shift, subtract image background, and correct donor/acceptor signal cross-talks (Figure 2.5 shows one matlab interface of data analysis). The corrected donor and acceptor intensities, $I_D(t)$ and $I_A(t)$, were used to calculate the apparent FRET trajectories, $E_A(t)$, using the following equation

$$E_A(t) = \frac{I_A(t)}{I_D(t) + I_A(t)}$$

(1)

The real FRET efficiency, $E_{\text{FRET}}(t)$, is related to $E_A(t)$ by the inclusion of the dye quantum yields, $\phi_i$, and the detector quantum efficiencies, $\eta_i$, as in
It has been determined that \( E_A(t) \approx E_{\text{FRET}}(t) \) using the current experimental setup. Since we used smFRET to probe the annealing kinetics rather than the absolute inter-dye distances, the \( E_A \) values were used to plot all the FRET trajectories and histograms. Origin Lab™ 6.0 was used for both figure plotting and least square curve fitting.

2.3 Results And Discussion

Two Underlying pathways of NC-Chaperoned TAR Annealing

In the ensemble electrophoresis gel-shift assays, it has been shown that the rate of HIV-1 TAR sequence (59 nucleotides) annealing is super slow without HIV-1 NC. And, two exponential growth components have been observed, so the whole annealing process can be described as two steps.\(^{15}\) One step has been hypothetically assigned as the formation of a kissing intermediate, followed by the other one whose kinetic rate is even slower has been assigned as strand exchange between the terminal stems to form the NA duplex. However, when the annealing was carried out in the present of HIV-1 NC, the rates of both the loop intermediate formation and its conversion to NA duplex have been greatly increased. Furthermore, a distinct 'zipper' pathway involving nucleation at the 3'/5' termini has been detected. More interestingly, at saturated HIV-1 NC concentration, one single-exponential growth kinetics is observed, which indicates that the zipper pathway is the dominate pathway during the annealing. Moreover, it has been reported that the overall kinetics of TAR sequence annealing is a bimolecular process when the NC
concentration is higher than 400 nM, at which the formation of the zipper intermediate is the rate limiting step.\textsuperscript{14,36,44,46}

In this chapter, we studied the TAR annealing kinetics in the presence of HIV-1 NV over a broad concentration range. In the proposed smFRET experiments, we will systematically study the TAR annealing kinetics in the presence of HIV-1 NC over a broad concentration range, with a particular focus on annealing in the sub saturating NC concentration regime where both loop and zipper pathways contribute to the complexity of the overall annealing kinetics. The local concentration of NC may vary a lot from place to place in the cytoplasm, where the reverse transcription occurs. Although the total concentration of NC in vivo may be sufficiently high at the early stage of reverse transcription, there are a large number of cellular tRNA, counter ions (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}), and other viral or cellular proteins that may interact with the viral RNA/DNA or the NC proteins and thus, significantly decrease the effective concentrations of NC for the execution of NC’s chaperone functions. In addition, the macromolecular crowding effects in intracellular environments may impose restrictions on the diffusion of NC proteins, which also contributes to the deviation of NC local concentrations. Therefore, the sub saturating NC concentration regime is believed to be biologically relevant in vivo and the annealing along both loop and zipper pathways may occur in the intracellular environment.

**Resolving the Kinetics along the loop and Zipper Pathways Using smFRET**

Figure 2.6 shows the loop and zipper pathways involved in HIV-1 NC-chaperoned TAR DNA sequence annealing. HIV-1 NC and Cy5-labeled cTAR DNA were delivered by syringes and coflowed into the reaction chamber to react with Cy3-labeled TAR DNA
molecules which were immobilized on coverslips by biotin-streptavidin interactions. At the beginning of the measurements, EA of the reactant-TAR DNA hairpins was 0. After annealing process, EA of the final product-duplexed NA was 1, because of the closest distance between FRET pairs Cy3 and Cy5. The loop intermediate exhibits EA of 0.6, and the zipper intermediate exhibits EA of ~1. The conversion of the zipper intermediate to the final product, which can be described as a four-way junction migration, is believed as a irreversible process. Its kinetic rate is supposed much faster than formation of the zipper intermediate. So the formation of the zipper intermediate is the rate limiting step. By designing smFERT-based measurements, carrying out the annealing reaction under a wide range HIV-1 NC concentration, we can obtain $K_1$, $K_2$ and $K_3$ values, and figure out the relative contributions of the zipper and loop pathways.

Figure 2.6. Scheme illustrating the loop and zipper pathways of HIV-1 NC-chaperoned TAR sequence annealing. The rate constants for different steps and the apparent FRET values of the TAR DNA, zipper intermediate, loop intermediate, and the final duplex are labeled.
Figure 2.7 shows the results of TAR-cTAR DNA annealing reaction chaperoned by 100 nM and 300 nM HIV-1 NC, respectively. For all of these reactions, the concentration of cTAR DNA sequence was fixed at 2 nM. In Figure 2.7A and 2.7C, the top panels show the trajectories of the molecules, each line represents one molecule. It was clearly showed that, when NC concentration was 100 nM (Figure 2.7A and 2.7B), most of the molecules went through $E_A$ from 0 to some middle values before finally reach ~1, which means that the loop pathway was the predominant pathway. However, when increased the NC concentration to 300 nM (Figure 7C and 7D), the molecules directly switched from 0 to 1, indicating that the annealing occurred primarily along the zipper pathway. Meanwhile, the averaged FRET, $<E_A>$, trajectories were shown in the middle panels of Figure 2.7A and 2.7C. From ensemble measurements, we can get the similar information. But, compared with smFRET measurements, the latter one gave researchers much more information, providing a unique way to directly correlate the comple kinetics to underlying reaction pathways. By counting the number of molecules that $E_A$ values were smaller than 0.3 as the reactant, $E_A$ values between 0.3 and 0.75 as the loop intermediate, and the $E_A$ values larger than 0.75 as the product at multiple time spots during the annealing process (see bottom panels in Figure 2.7A and 2.7C), a series of rate constants along the zipper and loop pathways can be obtained through least square curve fitting.
Figure 2.7. HIV-1 NC-chaperoned TAR sequence annealing. (A) Single-molecule $E_A$ trajectories of 245 molecules found in a 80 μm x 40 μm region (top panel), molecularly averaged FRET ($<E_A>$) trajectory (middle panel), and time-evolution of number of reactant (R), loop intermediate (LI), and product (P) molecules (bottom panel) during TAR-cTAR annealing chaperoned by 100 nM NC. (B) $E_A$ histograms at different reaction times during the annealing process. $E_A$ threshold values of 0.3 and 0.75, which are indicated by vertical dash lines, were set to distinguish between reactant, loop intermediate, and product molecules. (C) Single-molecule $E_A$ trajectories of 230 molecules found in a 80 μm x 40 μm region (top panel), molecularly averaged FRET ($<E_A>$) trajectory (middle panel), and time-evolution of number of reactant (R), loop intermediate (LI), and product (P) molecules (bottom panel) during TAR-cTAR annealing chaperoned by 300 nM NC.

By separating the molecules at the time spot of 10.40 min plotted in Figure 2.3A which annealing process were chaperoned by 100 nM NC, into three sub-ensembles, we gained more detail information about the reaction reversibility and heterogeneity. These three sub-ensembles were reactant, loop intermediate, and finally duplex product, which were shown in Figure 2.8, respectively. Apparently, the annealing was a two-step process along the loop pathway, one is the formation of the loop intermediate and the other one is the conversion of loop intermediate to the final product. Moreover, these two step were both irreversible during the tie period of experimental measurements. More interestingly, two sub-populations of the loop intermediates with different conversion rates to the final
product were observed. During our sm-FRET measurements time, most of the molecules switched to the final duplex relatively fast, while a small fraction of the loop intermediate molecules were long-lived, and did not switch to the final product. So, in order to fully consider the heterogeneity and complexity of the kinetics, a fraction factor F (the ratio of the fast-converting loop intermediate) must be added.

**Figure 2.8.** Sub-Ensemble Analysis of smFRET Trajectories. Separate the molecules at time spot of 10.40 min (indicated by vertical dash lines) during TAR-cTAR annealing chaperoned by 100 nM NC into three sub-ensembles. (A) Single-molecule EA trajectories of reactants (top panel), EA<0.3, molecularly averaged FRET (<EA>) trajectory (middle panel), and time-evolution of number of reactant (R), loop intermediate (LI), and product (P) molecules (bottom panel). (B) Single-molecule EA trajectories of loop intermediate (top panel), 0.3<EA<0.75, molecularly averaged FRET (<EA>) trajectory (middle panel), and time-evolution of number of reactant (R), loop intermediate (LI), and product (P) molecules (bottom panel). (C) Single-molecule EA trajectories of product (top panel), EA>0.75, molecularly averaged FRET (<EA>) trajectory (middle panel), and time-evolution of number of reactant (R), loop intermediate (LI), and product (P) molecules (bottom panel).
**Figure 2.9.** NC concentration dependence of TAR sequence annealing kinetics. (A) Fraction of reactant ($\theta_R$), loop intermediate ($\theta_{LI}$), and product ($\theta_P$) molecules as a function of annealing time at various NC concentrations as labeled in each panel. The solid curves show the least square curve fitting results using Equations 7-9. (B) Plots of $(k_1 + k_3)$ vs. NC concentration. (C) Rate constants, $k_1$, $k_2$, and $k_3$, at different NC concentrations. (D) Plots of fraction of molecules undergoing loop annealing pathway, $k_1/(k_1 + k_3)$, vs. NC concentration. (E) Plots of $F$ (fraction of fast converting loop intermediates) vs. NC concentration.

The annealing process can be treated as pseudo-first order reactions, because of the cTAR DNA and NC had consistent concentration. Four important parameters, which were three rate constants ($k_1$, $k_2$ and $k_3$) and one fraction factor ($F$), were used to describe the complex kinetics of the NC-chaperoned NA annealing based on the rate equations (equation 3, 4, 5, 6) listed below.

\[
\frac{d[R]}{dt} = -(k_1 + k_3)[R] \quad (3)
\]

\[
\frac{d[L_{I_1}]}{dt} = F \cdot k_1[R] - k_2[L_{I_1}] \quad (4)
\]

\[
\frac{d[L_{I_2}]}{dt} = (1 - F) \cdot k_1[R] \quad (5)
\]
\[
\frac{d[Ll]}{dt} = \frac{d[Ll_1]}{dt} + \frac{d[Ll_2]}{dt}
\]  \hspace{1cm} (6)

Here, \([R]\), \([LI_1]\), \([LI_2]\) and \([LI]\) are the concentrations of the reactant, the fast-converting loop intermediate, the long-lived loop intermediate, and the total concentration of the loop intermediate respectively. By integrating Equation 3-6, and divided by total numbers of the molecules, equation 7-9 were obtained.

\[
\theta_R(t) = (1 - \theta_{t=\infty})e^{-(k_1 + k_3)(t-t_0)} + \theta_{t=\infty}
\]  \hspace{1cm} (7)

\[
\theta_{LI}(t) = \frac{Fk_1}{k_1 + k_3 - k_2} \left[ e^{-k_2(t-t_0)} - e^{-(k_1 + k_3)(t-t_0)} \right] + \frac{(1-F)k_1}{k_1 + k_3} \left[ 1 - e^{-(k_1 + k_3)(t-t_0)} \right]
\]  \hspace{1cm} (8)

\[
\theta_P(t) = 1 - \theta_R(t) - \theta_{LI}(t)
\]  \hspace{1cm} (9)

Here, \(\theta_R\), \(\theta_{LI}\), and \(\theta_P\) are the fractions of the reactant, loop intermediate, and product, respectively. \(\theta_{t=\infty}\), which is the fraction of reactant at infinitely long reaction time, should be zero if the reaction is irreversible; however, to obtain the best curve fitting results, \(\theta_{t=\infty}\) typically adopts a small number in the range from 0 to 0.03 possibly due to the presence of small fraction (<3 %) of molecules that were photobleached during the smFRET measurements. \(t_0\) is the dead volume time of the flow cell system, which was measured to be 2.5 minutes when the total flow rate was 4 μL/min. \((k_1 + k_3)\) values were obtained by fitting \(\theta_R\) with Equation 7. By fitting the \(\theta_{LI}\) trajectory using Equation 8, \(k_1\), \(k_2\), and \(F\) can be obtained. (all experimental data were well fitted with \(R^2\) values typically larger than 90 %). Then, plot \((k_1 + k_3)\) values as a function of NC concentration, shown in Figure 4B. When the NC concentration lows than 100 nM, \((k_1 + k_3)\) increased dramatically, but as NC concentration further increased to 200 nM, the total psedu-first order rate constants gradually reached a plateau. This trend is well-correlated with the binding of NC to the NA hairpins. It has been known that NC lowers the energy cost of bringing two
complementary NA hairpins together to form encounter complexes by effectively screening the negative charges of the NA hairpins upon binding. The cationic N-terminal domain is a major factor in NC’s NA binding and aggregation activity.\textsuperscript{4,13,34,35,37} HIV-1 NC binds to a diverse array of NA sequences in non-sequence-specific manner with apparent dissociation constants, $K_d$, in the sub-100 nM range.\textsuperscript{29,48-51} The binding footprint size of NC on NAs has been estimated to be 6-8 nucleotides.\textsuperscript{8,29,48} In the sub-saturating NC concentrations, the number of NC bound to the each NA hairpin increased with NC concentration. As a consequence, the bimolecular process of the encounter intermediate complex formation became faster as NC concentration increased. Above certain NC concentration (~ 200 nM in the present case), saturating NC binding, which roughly corresponds to one NC every 6 nucleotides, was achieved, and further increasing NC concentration would not further accelerate the bimolecular process of the intermediate formation.

In Figure 2.9C, the evolution of the loop and zipper rate constants, $k_1$ and $k_3$, as the NC concentration varies were showed. When the NC concentration were lower than 100 NM, $k_1$ increased as the NC concentration increased, meanwhile $k_3$ remained close to 0, suggesting that the loop pathway was the predominant pathway. At intermediate NC concentrations in the range of 100-200 nM, $k_3$ started to increase while $k_1$ progressively decreased, strongly indicating that it starts switching from the loop pathway to the zipper pathway in this NC concentration window. As the NC concentration further increased to above 200 nM, $k_3$ became larger than $k_1$ and both $k_1$ and $k_3$ reached plateaus, indicating that the TAR annealing was dominated by the zipper pathway in this saturating NC concentration range. Figure 2.9D shows progressive switch from loop pathway to zipper
pathway as NC concentration increased, where \( \frac{k_1}{k_1+k_3} \) refers to the fraction of molecules undergoing the loop annealing pathway. Apparently, almost all the molecules took the loop annealing pathway when the NC concentration below 100 nM. When the NC concentration was higher than 200 nM, about \( \sim 70\% \) of the molecules switched to take the zipper pathway. In Figure 2.9C, also plot \( k_2 \) which referred as the rate constant related to the switch from loop intermediate to final NA duplex, as the NC concentration varies. At NC concentrations below 100 nM, \( k_2 \) increased significantly with increasing NC concentration and reached a plateau in the NC concentration window from 75 to 150 nM. As NC concentration further increased, the conversion of loop intermediate to the final duplex became even faster until \( k_2 \) reached a second plateau at NC concentration higher than 200 nM. Figure 2.9E shows the evolution of the fraction of the long-lived loop intermediates as the NC concentration varies. Interestingly, the fraction of long-lived loop intermediates decreased as the NC concentration increased. The kinetics of the strand exchange process during the conversion of loop intermediate to final duplex and the emergence of the zipper annealing pathway are both closely related to the NA duplex destabilization effects associated with NC's zinc fingers. All these observed trends can be interpreted in the context of NC-induced duplex melting of the 3'/5' terminal stems of the TAR hairpins, which was studied in great detail using a smFRET-based NA hairpin melting assay.

**NC-Induced Melting of TAR DNA Hairpin.**

It has been reported that HIV-1 NC preferred to bind with single-stranded than double-stranded NA regions. Due to the binding preference for single stranded NA regions and the intercalation of zinc fingers, NC induces local melting of stacks of bases pairs in
structured DNAs and RNAs to form locally melted 'hotspots'. It has been observed that NC-induced NA melting was a key component of NC-chaperoned NA annealing mechanisms in many studies and contexts. Here we studied the relationship between the local melting of NA hairpins and NA strand annealing using the HIV-1 TAR sequence as a model system. Cy3-Cy5 dual-dye labeled TAR DNA was used as the molecular construct for sm-FRET measurements.

In our sm-FRET measurements, the closed TAR DNA hairpin exhibited $E_A$ of 1, while the melting of the 3'5' terminal stem region exhibited a decreased $E_A$ value of ~0.85, since the increased end-to-end distances of Cy3 and Cy5 (illustrated in Figure 2.10A). It has been observed that, when NC was at saturating concentrations, NC effectively melted the zipper region (the two internal loops in the 3'5' terminal region of TAR and adjacent stems) but did not significantly melt the loop region (the two internal bulges, the bottom loop, and the adjacent stems) of the TAR DNA, giving rise to the formation of a 'Y'-shaped, partially melted hairpin structure. The results of our sm-FRET measurements were showed in Figure 2.11B and 2.11C. The secondary structures of TAR DNA hairpins at equilibrium was gradually shifted toward the 'partially open' conformation as NC concentration progressively increased and the melted hairpins appeared to be more conformational dynamic than the closed hairpins, which was reflected by the broadening peak in $E_A$ histograms. From previous fluorescence correlation spectroscopic measurements, we know that the NC-induced TAR melting process was much more complicated than a simple two-state interconversion. It involved a variety of short-lived and long-lived intermediates, exhibiting heterogeneous, complex secondary structure fluctuation with dynamic processed occurring over a wide distribution of time scales.
from sub-milliseconds to sub-seconds. However, in our TAR melting experiments, signal collection bin time was 200 ms, which leaded to the conformational dynamics faster than this bin time were time-averaged results. Due to the significantly decreased signal-to-noise ratio, it was challenging to use shorter bin times for signal collection to probe the fast conformational dynamics. In Figure 2.10B and 2.10C, TAR DNA completely switched from the closed hairpins to the partially melted Y-shaped conformation, at NC concentration higher than 200 nM. This also was the NC concentration that the zipper pathway dominants the annealing process, indicating that the NC-induced melting of 3'/5' termini stem region of the TAR DNA hairpin may be the main reason for the switch from the loop annealing pathway to the zipper annealing pathway. We can further hypothesize that NC-induced TAR DNA melting may also speed up the conversion of the loop intermediate to the final duplex by facilitating the strand exchange between the terminal stems.

To gain further insights into the NA melting and annealing at different regions of the full-length TAR DNA, we performed sm-FRET measurements on NC-chaperoned annealing of short, model complementary oligonucleotides to specific region of the TAR DNA. Firstly, we designed the annealing of DNA oligonucleotides with sequence complimentary only to the 3'/5' termini stem region (named as zipper DNA) to the TAR DNA, then finally formed a TAR-zipper DNA complex. Secondly, we studied the annealing of DNA oligonucleotides with sequence complimentary only to the loop region (named as loop DNA) to TAR DNA, to form a TAR-loop DNA complex (illustrated in Figure 2.11A and 2.11D respectively).
Figure 2.10. NC-induced melting of the 3'/5'-termini of TAR DNA hairpin. (A) Schematic illustration of the NC-induced TAR melting. (B) Molecularly averaged FRET, $<E_A>$, of dual-dye-labeled TAR DNA in the presence of different concentrations of HIV-1 NC. (C) $E_A$ histograms of dual-labeled TAR DNA molecules in buffer and various concentrations of NC as labeled in each panel.

In our smFRET-based TAR-zipper and TAR-loop DNA annealing experiments, the Cy3-labeled TAR DNA was immobilized on the coverslips and the concentrations of the Cy5-labeled zipper DNA and loop DNA were both fixed at 10 nM over the whole annealing process. The TAR-zipper DNA and TAR-loop DNA complexes mimic the zipper intermediate and the loop intermediate, respectively, that were formed during the full-length TAR sequence annealing. Figure 2.11B and 2.11E showed the trajectories of the molecules, which suggesting that the TAR-zipper DNA annealing were a reversible process. Then, the TAR-zipper DNA annealing were carried out over a wide range of NC concentration (results shown in Figure 2.12), and by setting $E_A$ thresholds ($E_A<0.3$ for reactants, $E_A>0.75$ for products), counting the molecules, calculating the fraction of the reactants and products respectively, both the forward and backward reaction rate
constants, $k_{Zf}$ and $k_{Zb}$, respectively, were obtained through least square curve fitting using the following Equations.

\[ \theta_Z(t) = \theta_{Z,eq}[1 - e^{-(k_{Zf} + k_{Zb})(t-t_0)}] \]  \hspace{1cm} (10)

\[ \frac{\theta_{Z,eq}}{1 - \theta_{Z,eq}} = \frac{k_{Zf}}{k_{Zb}} \]  \hspace{1cm} (11)

here $\theta_{Z,eq}$ refers to the fraction of TAR-zipper DNA complex at equilibrium. Figure 2.11C showed the evolution of $k_{Zf}$ and $k_{Zb}$ as the NC concentration varies. As NC concentration increased, $k_{Zf}$ and $k_{Zb}$ both increased until they reached a plateau at concentrations higher than 200 nM. This is very well correlated with the NC concentration dependent TAR melting results. From the molecules trajectories shown in Figure 2.11E, we observed that the TAR-loop DNA annealing was an irreversible process, and the TAR-loop DNA complex exhibited EA values around 0.7. By carrying out the TAR-loop DNA annealing process over a wide range NC concentration (shown in Figure 2.13), the rate constant $k_L$, can be obtained through least-square curve fitting using the equation below.

\[ \theta_L(t) = \theta_{L,t=\infty}[1 - e^{-k_L(t-t_0)}] \]  \hspace{1cm} (12)

here, $\theta_{L,t=\infty}$ is the fraction of TAR-loop DNA complex at infinitely long reaction time. The as obtained $k_L$ values were plotted as a function of NC concentration, which shown in Figure 2.11F, and $k_L$ increased as NC concentration increased until reached a plateau when NC concentration was higher than 300 nM.
Figure 2.11. NC concentration-dependent kinetics of TAR-zipper DNA annealing and TAR-loop DNA annealing. (A) Schematic illustration of NC-chaperoned TAR-zipper DNA annealing, which is a reversible process. (B) Single-molecule E_A trajectories of molecules (right top panel), molecularly averaged FRET (<E_A>) trajectory (right bottom panel), E_A histograms at different reaction times during the TAR-zipper DNA annealing process (left panel). (C) TAR-zipper DNA annealing rate constants, k_Zf (forward annealing) and k_Zb (backward reverse annealing), at various NC concentrations. The rate constants were obtained through least square curve fitting using Equations 10 and 11. (D) Schematic illustration of NC-chaperoned TAR-loop DNA annealing. (E) Single-molecule E_A trajectories of molecules (right top panel), molecularly averaged FRET (<E_A>) trajectory (right bottom panel), E_A histograms at different reaction times during the TAR-loop DNA annealing process (left panel). (F) TAR-loop DNA annealing rate constants, k_L, at various NC concentrations. The rate constants were obtained through least square curve fitting using Equation 12.
From the smFRET-based measurement on the TAR DNA melting and TAR-zipper/TAR-loop DNA annealing process, we observed that the switch from the loop pathway to the zipper pathway is essentially a consequence of NC-induced melting of the TAR hairpins. At NC concentration lower than 200 nM, loop pathway was the main pathway, however, at saturated NC concentration, the zipper pathway became kinetically more efficient than the loop pathway, whereas the loop intermediate was thermodynamically more stable than the zipper intermediate. Although the TAR-zipper DNA annealing was observed as a reversible process, the full length TAR sequence annealing through the zipper pathway was a irreversible. That's probably because of the fact that the conversion of the zipper intermediate to the final duplex was a much faster process than the dissociation of the zipper intermediate back to the reactants.

**Figure 2.12.** NC concentration-dependent kinetics of TAR-zipper DNA annealing. Fraction of reactant ($\theta_R$), product TAR-zipper DNA ($\theta_Z$) molecules as a function of annealing time at various NC concentrations as labeled in each panel. The solid curves show the least square curve fitting results using equations 10, 11.
Figure 2.13. NC concentration-dependent kinetics of TAR-loop DNA annealing. Fraction of reactant ($\theta_R$), product TAR-loop DNA ($\theta_L$) molecules as a function of annealing time at various NC concentrations as labeled in each panel. The solid curves show the least square curve fitting results using equations 12.

2.4 Conclusions

In this chapter, by designing time-resolved smFRET-based measurements, we resolved the complex kinetics and underlying reaction pathways of NC-chaperoned NA annealing over a wide range of NC concentration. When NC concentration is lower than 100 nM, the loop pathway is the predominate pathway. When NC concentration gradually increases from 100 nM to 200 nM, the TAR sequence annealing gradually switched from the loop pathway to the zipper pathway until the zipper pathway dominated the annealing process at sufficiently high NC concentrations higher than 200 nM. We have carried out the annealing process at various NC concentrations, figured out the rate constants along
the two pathways, and resolved that NC-induced melting of the 3'/5' terminal stems of the TAR hairpins resulting in the switch of the annealing pathway. We have also found that the zipper pathway is kinetically more efficient than the loop pathway, at NC saturated concentrations, whereas the loop intermediate is thermodynamically more stable than the zipper intermediate.

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CHAPTER 3
MECHANISTIC STUDIES OF MACROMOLECULAR CROWDING EFFECTS ON NC-CHAPERONED TAR SEQUENCE ANNEALING

3.1 Introduction

The standard salted buffer solutions used in sm-FRET measurements are quite different from the environment in the cytoplasm, which is very crowded, containing macromolecules occupying 20~30% of the total volume of the living cells.\textsuperscript{1-5} The macromolecular crowding effects may vary the properties of molecules such as proteins and nucleic acids in a solution when high concentrations of macromolecules are present. The existing of these high concentrations of macromolecules may generates steric exclusion, thus reduce the effective volume of other important molecules, and further effects on both the rates and the equilibrium of the interactions in the cytoplasm. The macromolecular crowding effects are always referred as an non-specific interaction between the existing macromolecular and the target molecules, and do not participated directly in the particular test reaction. This non-specific steric repulsion can't be avoided, and is greatly related with the numbers, sizes and shapes of the crowder macromolecules and test species. There is another kind of crowding called confinement, resulting in the volume exclusion by the pore boundaries of the macromolecules.\textsuperscript{6-8}
There is another kind of feature in the physiological environment of the intracellular environment, a few different species rather than one single species of macromolecular are taken together to form a crowding or confinement media, such as t-RNA, m-RNA, actin fiber, soluble protein, ribosome, intermediate filament.

The macromolecular crowding effects may results in the molecules acts differently in vivo than in vitro. A lot of research results showed that the macromolecular crowding effects did affect the results of the in vitro studies of DNA replication and transcription. Ogston and Laurent using systematic studies even proved that by the macromolecular crowding effects were so large that not only alter the thermodynamic and kinetic properties of the molecules, but also differ the reaction rates by orders of magnitude then what carried out the test-tube assays. In the last decades, the macromolecular crowding effects have attracted more and more attention of the researchers when performing the test-tube assays. Moreover, the relevant experimental studies clearly showed the macromolecular crowding effects on the association rates, equilibria, conformational isomerization, protein and enzyme stability, activity as well as protein denaturation. These experimental results were generally obtained by detecting reaction rates and equilibria changes in the presence of different concentration of water-soluble cosolutes, such as PEG (polyethylene glycol), PEO (polyethylene oxide), and some proteins, such as Dextran, Ficoll, hemoglobin, defatted BSA. Consequently, the addition of high concentration of natural macromolecules to the uncrowded buffers is highly necessary and of permanent importance.

Reverse transcription is a key step in the retroviral life cycle. Many steps during the reverse transcription rely on the chaperone activity of nucleocapsid protein (NC).
Therefore, the NC-chaperoned NA annealing may undergo altered reaction pathway in vivo, because the Macromolecular Crowding Effects may introduce alterations to the effective local concentrations of NC and the folding/conformational dynamics of structured NAs. In this chapter, we are using poly-ethylene glycol (PEG) as a model neutral polymer cosolvent to mimic intracellular environment to investigate the Macromolecular Crowding Effects on NC-chaperoned TAR sequence annealing and further probe more physiologically relevant situations, Since PEG has been widely used to mimic intracellular environments in biomolecular assays.26,52-56 And four challenging questions would be addressed, How will the macromolecular crowders affect the overall rates of NC-chaperoned NA annealing? Will the macromolecular crowders change the annealing pathways? Are there alternative annealing pathways in highly crowded environments? Both NC-chaperoned NA annealing and NC-induced NA melting assays will be carried out in the presence of PEG with different molecular mass (PEG-200,-2000,-5000,-10000) over a broad weight concentration range from 1 wt% to 15 wt%. The sm-FRET results of the macromolecular crowding effects on NC-chaperoned NA structural rearrangements enables us to gain significant, quantitative insights into the complicated dynamic NC-NA interactions that underpin the NA chaperone functions of HIV-1 NC in the living cells.

3.2 Experimental Section

The HIV-1 NC used in this work was provided by our collaborator, Dr. Musier-Forsyth's group of Ohio State University, which was synthesized as previously described.57-59 Cy3 and biotin functionalized DNA oligonucleotides, its complementary Cy5 labeled DNA as well as Cy3 and Cy5 dual labeled TAR DNA, Cy5 labeled TAR zipper/TAR loop used
for the NC-chaperoned TAR DNA were purchased from Trilink BioTechnologies (San Diego, CA). All these functionalized DNA hairpins were purified by the provider by high-performance liquid chromatography (HPLC). Cy3 and Cy5 are two FRET chromophores used in our SM-FRET measurements. 59 nucleotides sequence NA with Cy3 and biotin labeled to its loop region serves as sm-FRET donor, and is immobilized on the coverslips (described later). Cy5 labeled NA hairpin will co-flow with HIV-1 NC into the home-build reaction chamber. Here, the solution of NA hairpin and HIV-1 NC contains a certain amount of PEG (molecular mass changed from 200 to 10,000) solute.

The pre-cleaned coverslips were further cleanout with piranha solution (hydrogen peroxide: conc. sulfuric acid = 1:3) for 1 h. Thereafter, rinsed with molecular biology grade water and acetone (HPLC grade). Then, the clean coverslip were treated with vectabond/acetone 1% w/v solutions (Vector Laboratories, Burlingame, CA) (an amino-silane reagent) for 5 min, followed by pegylated and biotinvalted. Then the coverslip were assembled with a reaction chamber, all the solutions would go through the inlet and flow out from the outlet ports. The flow rates are precisely controlled by syringe pumps. The PEG solutions were made by weighing precise amount of PEG powder, and dissolved in molecular biology grade water, and properly stored for preparing fresh NA, NC and buffer solutions with macromolecule crowding environments. For example, when the annealing reaction were carried out in the present of 5 wt% PEG-5000, we should prepare NA, NC solutions containing 10 wt% PEG-5000 to make the final concentration in the reaction chamber as 5 wt%. All the single-molecule annealing and melting reactions were carried out in the reaction chamber at room temperature in HEPES buffer (25 mM HEPES, 40 mM NaCl, and 0.2 mM MgCl₂, pH=7.3) in an oxygen scavenger
system containing D(+) glucose 15% w/v (Sigma-Aldrich, St. Louis, MO), glucose oxidase 0.1 mg/ml, catalase 0.2 mg/ml (Sigma-Aldrich, St. Louis, MO) and 1% v/v 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

3.3 Results And Discussion

Macromolecular Crowding Effects on TAR Annealing

The complex kinetics and underlying reaction pathways of the NC-chaperoned TAR sequence annealing have been resolved by time-resolved smFRET-based measurements. Our group's previous results showed that at sub-saturating NC binding concentration (<100 nM), the loop pathway dominated the TAR sequence annealing, in the intermediate NC concentration range from ~100 nM to ~200 nM, the TAR sequence annealing gradually switched from the loop pathway to zipper pathway as the NC concentrations increased until the zipper pathway becomes predominate at sufficiently high NC concentration above ~200 nM. However, all these measurements were carried out in vitro, it might be a different story if all the experiments were done under the conditions that mimic the environments in vivo. In single molecule NA annealing assays, prior to annealing, the TAR DNA (reactant) molecules exhibited $E_A$ of 0. The fully duplexed NA (final product) exhibited $E_A$ of 1 due to the close proximity of Cy3 and Cy5. The $E_A$ of the loop intermediate was ~0.6 under current experimental conditions while the zipper intermediate showed $E_A$ of ~1.

Figure 3.1 showed the smFRET trajectories of molecules during TAR sequence annealing chaperoned by 80 nM NC without or with 5 wt% PEG solutions, respectively. The concentration of cTAR DNA was kept at 2 nM throughout these annealing processed. Each line in the top panels of Figure 3.1A and 3.1B represent the $E_A$ trajectory
of one molecule. By setting $E_A$ threshold values at 0.3 and 0.75 for molecular sorting, the
numbers of reactant, loop intermediate, and product molecules could be directly counted
at multiple time spots during the annealing process (the results were shown in the bottom
panels in Figure 3.1A and 3.1B). At NC concentration of 80 nM (see Figure 3.1A), the
majority of the molecules switched $E_A$ from 0 to intermediate values before eventually
switching to 1, indicating that the annealing occurred primarily along the loop pathway.

**Figure 3.1.** Macromolecular Crowding Effects on TAR Annealing at 80 nM NC and 2 nM cTAR.
(A) Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant
fraction ($\theta_R$), loop intermediate fraction ($\theta_L$), and product fraction ($\theta_P$) (bottom panel) during
TAR-cTAR annealing process chaperoned by 80 nM NC in the presen of 5 wt% PEG-5000. (B)
Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant fraction
($\theta_R$), loop intermediate fraction ($\theta_L$), and product fraction ($\theta_P$) (bottom panel) during TAR-cTAR
annealing process chaperoned by 80 nM NC in the present of 5 wt% PEG in the measuring
system.

And, we also observed a fraction of long-live loop intermediates that did not convert
into the final product. As NC concentration was fixed at 80 nM, and the cTAR DNA
concentration was kept at 2 nM, the experiment was carried out in present of 5 wt% PEG
in the system, the molecules still took the loop pathway, instead of switching to the zipper pathway, because they still went through some intermediate $E_A$ values before eventually switching to 1 (see Figure 3.1B). By comparing Figure 3.1B with Figure 3.1A, we found that there were less long-lived loop intermediate in the present of PEG.

To obtain more detail information about the macromolecular crowding effects on TAR-cTAR annealing process chaperoned by sub-saturating NC binding concentrations (< 100 nM), at which condition, loop pathway is the predominate pathway. In order to detect whether the macromolecular crowding effects change the annealing pathways from the loop pathway to the zipper pathway or affect the overall rates of NC-chaperoned NA annealing, the NA annealing assays were carried out in the presence of PEG with several different molecular mass (PEG-200,-2000,-5000,-10000) at different weight concentration in the range from 2.5 wt% to 15 wt%, meanwhile keeping NC concentration was 80 nM and cTAR concentration 2 nM, as illustrated in Figure 3.2. Without adding PEG (molecular mass were kept at 5000) into the reaction system, the reactants lasted almost 200 min to totally complete reacted. When increase PEG weight concentration in the solution from 2.5 wt% to 10 wt%, we observed that the rate of reactants' disappearance were dramatically increased. However, when the PEG weight concentration reached at 15%, the rate of reactants' disappearance didn't change too much, indicating the viscosity and crowders reached saturation (see Figure 3.2A). Correspondingly, the same trends were observed for the intermediates (see Figure 3.2B) and products (see Figure 3.2C), during which the formation and generation of the intermediates and products were quite slow, but once have 2.5 wt% to 10 wt% PEG in the solution, the formation significantly accelerated, and gradually reached a plateau after 10
wt% to 15 wt% PEG weight concentration. Furthermore, we kept the weight concentration of PEG at 5 wt%, detected the molecular mass of PEG's effect on the TAR annealing. From the disappearance rate of the products, we observed that as the molecular mass increased from PEG-200 to PEG-10000, the disappearance rates were significantly accelerated. Without PEG in the system, it took about 200 min for the reactants completely reacted, when 5 wt% PEG-200 were in the system, the reaction time were decreased to ~130 min. Once the molecular mass was increased to PEG-10000, it only took 25~30 min for the reactants completely reacted (shown in Figure 3.2D). Same phenomenon were observed in the intermediates (Figure 3.2E) and products (Figure 3.2F). In a short summary, as we detected the macromolecule crowding effect on the TAR annealing chaperoned by low NC concentration, we found neither PEG weight concentration nor PEG molecular mass under our current measurements condition, would switch the loop pathway to the zipper pathway, but they did accelerate the whole annealing reaction. Therefore, the PEG-existed loop annealing processes can still be considered as the pseudo-first order reactions. Three parameters, including the two rate constants \((k_1 \text{ and } k_2)\) and the fraction factor \((F: \text{the ratio of the fast-converting loop intermediate})\) can be used to describe the kinetics of the PEG effects on the NC-chaperoned NA annealing based on the following rate equations:

\[
\frac{d[R]}{dt} = -k_1[R]
\]

\[
\frac{d[I]}{dt} = k_1[R] - k_2[I]
\]

\[
\frac{d[I]}{dt} = k_1[R] - F \cdot k_2[I] = F \cdot (k_1[R] - k_2[I]) + (1 - F)k_1[R]
\]
here, [R], [L] are the concentrations of the reactant and loop intermediate respectively. Based on Equation 1-3, the integrated pseudo-first order rate equations can be obtained, which are shown as Equations 4-6.

\[ \theta_R(t) = (1 - \theta_{t=\infty}) e^{-k_1 t} + \theta_{t=\infty} \] (4)

\[ \theta_I(t) = \frac{[I]_0}{[R]_0} = F \cdot \frac{k_1}{k_2 - k_1} \left( e^{-k_1 t} - e^{-k_2 t} \right) + (1 - F) \cdot (1 - e^{-k_1 t}) \] (5)

\[ \theta_P(t) = 1 - \theta_R(t) - \theta_I(t) \] (6)

Here, \( \theta_R, \theta_I, \) and \( \theta_P \) are the fractions of the reactant, loop intermediate, and product, respectively. \( \theta_{t=\infty} \), which is the fraction of reactant at infinitely long reaction time, was set as a small number, treated the same with NC-chaperoned TAR annealing assays. In the same way, \( t_0 \) which is the dead volume time of the flow cell system, was measured to be 2.5 minutes. Through least-square fitting of the \( \theta_R \) trajectory using Equation 4, \( K_1 \) can be obtained. By fitting the \( \theta_I \) with equation 5, \( K_2 \) and \( F \) can be obtained. The as-obtained three parameters values were shown in Figure 3.3.

As reported, the TAR annealing should only take the loop pathway when chaperoned by 80 nM NC. So, under this condition, \( K_3 \) was equal to 0. In Figure 3.3A, the as-obtained \( K_I \) and \( K_2 \) values, which are the rate constant for the formation of loop intermediate, and the rate constant for the converting of loop intermediate to the final duplex product, respectively, are plotted as a function of bulk PEG-5000 weight concentration. \( K_I \) increased dramatically in our experimental range, \( K_2 \) also increased significantly below PEG-5000 10 wt% weight concentration, and reached a plateau at 10 wt% and 15 wt%, indicating that the macromolecular in the system strongly accelerated the reaction rates, and the more crowded, the faster annealing reaction occurs. Figure 3B
shows the evolution of the F (the fraction of fast converting loop intermediates), as the PEG-5000 weight concentration varies. F increased greatly in sub-5 wt% weight concentration, and slightly increased as the PEG weight concentration further increased to 15 wt%, which means as the PEG-5000 weight concentration went up, the fraction of long-lived loop intermediates deceased. The same trend was observed at different PEG molecular weight. Figure 3.3C plots the $K_1$ and $K_2$ (two steps rate constants) as a function of different PEG molecular weight. Both $K_1$ and $K_2$ increased dramatically as PEG molecular weight increased. Also, the Fraction of the fast converting loop intermediate increased greatly at low PEG molecular weight, and reached a plateau when PEG molecular weight larger than PEG-5000. From these results, we can make a short conclusion that at low NC concentration, both the formation of the loop intermediate and converting from loop intermediate to final duplex product were efficiently accelerated as PEG weight concentration and PEG weight concentration increased. Since, higher PEG weight concentration and higher PEG molecular weight means higher crowded environments. As we increase the PEG weight concentration and PEG molecular weight, the resulting higher crowded environments didn't change the annealing pathway, and no alternative pathway were observed.

To further detect the macromolecular crowding effect on the NC-chaperoned NA annealing, we carried out the annealing process at high NC concentration 240 nM in the present of 5 wt% weight concentration of PEG-5000. The results were illustrated in Figure 3.4. Figure 3.4A shows the annealing process at 240 nM NC concentration without PEG in the system.
Figure 3.2. Detail measurements of Macromolecular Crowding Effects on TAR Annealing at 80 nM NC and 2 nM cTAR. Plot fraction of reactant (A), loop intermediate (B), product (C) molecules as a function of annealing time in the presence of PEG with weight concentration of 0 wt%, 2.5 wt%, 5 wt%, 10 wt%, and 15 wt%. Plot fraction of reactant (D), loop intermediate (E), product (F) molecules as a function of annealing time in the presence of PEG with different molecular mass, PEG-200, PEG-2000, PEG-5000, PEG-10000.

From the trajectories of the molecules (Figure 3.4A top panel), we can see that most of the molecules went through the zipper pathway without exhibited middle $E_A$ values, but there were also certain amount of the molecules went through loop annealing pathway, which means, consistent with our previous observation, at saturated NC concentration 240 nM, both the zipper and loop pathway existed, but the zipper pathway is the predominant pathway. Moreover, from the time evolution of the reactant, intermediate, and product fraction (Figure 3.4A bottom panel), without PEG in the system, the
annealing took about 20 min. The results of the 240 nM NC chaperoned NA annealing which were carried out in the condition of 5 wt% PEG-5000, were shown in Figure 3.4B.

**Figure 3.3.** SM-FRET measurements of the annealing kinetics. (A) Loop annealing pathway rate constants $K_1$ and $K_2$, at different PEG-5000 weight concentration. (B) Plots of F (fraction of fast converting loop intermediates) vs.PEG-5000 weight concentration. (C) Loop annealing pathway rate constants $K_1$ and $K_2$, at different molecular weight of PEG. (D) Plots of F vs. different PEG molecular weight.

In the top panel, the trajectories of the molecules, we observed the same results as the annealing process carried out without PEG condition. Majority of the molecules took the zipper pathway, and still a small amount of the molecules took the loop pathway, indicating that in the present of PEG, not all of the molecules were favor the zipper pathway. Macromolecular crowding effect did not switch the annealing pathway. Furthermore, in the bottom panel, we observed that the whole annealing process only
took about 10~11 min to totally react, which were faster compared with the annealing carried out without PEG. Therefore, the macromolecular crowding effect also did accelerate the annealing process at high enough NC concentration.

Through least-square fitting of the $\theta_R$, and $\theta_{LI}$ trajectories using Equation 7 and Equation 8, $K_i$ and $K_3$ were obtained respectively. Here, $\theta_R$, and $\theta_{LI}$ are the fraction of reactants and loop intermediate, respectively. $K_i$ is the rate constant that the reactants took the loop annealing pathway. $K_3$ is the rate constant for the reactants that went through the zipper annealing pathway. The rate constants for the annealing reaction carried out with and without PEG condition were all figured out, and showed in Figure 3.5. By comparing rate constants $k_i$ an $K_3$ under the condition with PEG or without PEG, it clearly showed that the PEG in the solution effectively promoted the annealing process as both $K_i$ and $K_3$ increased when the reaction were carried out in the present of 5 wt% PEG-5000. The smFRET results shown in Figure 3.5 strongly indicate that macromolecular crowding effect not only significantly accelerate the zipper pathway reaction rate which is the predominant pathway, but also greatly accelerate the loop pathway reaction rate which is the sub-pathway, at high NC concentration. It is hypothesized that macromolecular crowding effect would more favor to form the final duplex product without changing the reaction pathway.

$$\theta_R(t) = (1 - \theta_{t=\infty})e^{-(k_1+k_3)(t-t_0)} + \theta_{t=\infty}$$  \hspace{1cm} (7)

$$\theta_{LI}(t) = \frac{Fk_1}{k_1 + k_3 - k_2} \left[ e^{-k_2(t-t_0)} - e^{-(k_1+k_3)(t-t_0)} \right] + \frac{(1-F)k_1}{k_1 + k_3} \left[ 1 - e^{-(k_1+k_3)(t-t_0)} \right]$$  \hspace{1cm} (8)

$$\theta_F(t) = 1 - \theta_R(t) - \theta_{LI}(t)$$  \hspace{1cm} (9)
**Figure 3.4.** Macromolecular Crowding Effects on TAR Annealing at 240 nM NC and 2 nM cTAR. (A) Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant fraction ($\theta_R$), loop intermediate fraction ($\theta_l$), and product fraction ($\theta_P$) (bottom panel) during TAR-cTAR annealing process chaperoned by 240 nM. (B) Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant fraction ($\theta_R$), loop intermediate fraction ($\theta_l$), and product fraction ($\theta_P$) (bottom panel) during TAR-cTAR annealing process chaperoned by 240 nM NC in the present of 5 wt% PEG-500 in the measuring system. The solid curves show the least square curve fitting results.

**Figure 3.5.** Comparison of rate constants for TAR-cTAR annealing in the present of 240 nM NC with and without 5 wt% PEG-5000 in the reaction flow system. The rate constants were obtained through least square curve fitting using Equation 7 and 8.
To gain further insight into the macromolecular crowding effect on the full-length annealing, we performed smFRET measurements on NC-chaperoned annealing of short, model complementary oligonucleotides to specific regions of the TAR DNA molecules in the present and in the absent of 5 wt% PEG-5000. We studied the annealing of DNA oligonucleotides with sequence complementary only to the 3'/5' termini stem region (zipper DNA) to the TAR DNA through which a TAR-zipper DNA complex was formed. As previous illustrated, the TAR-zipper DNA complexes mimic the zipper intermediate which is formed during the full-length TAR sequence annealing. In the TAR-zipper annealing experiments, the Cy3-labeled TAR DNA was immobilized on the coverslips and the concentration of Cy5-labeled zipper DNA was 10 nM, in addition, 5 wt% PEG-5000 was consisted in the flow system. Figure 6A shows the NC-chaperoned TAR-chaperoned TAR-zipper DNA annealing sketch. The reaction molecular trajectories in Figure 3.6B top panel clearly shows that the TAR-zipper DNA annealing carried out without PEG was a reversible process. However, when the TAR-zipper DNA annealing reaction were carried out in present of 5 wt% PEG-5000, the trajectories of each molecules show that most of the molecules reacted forward to the final TAR-zipper product without went back to the reactants (Figure 3.6C), though a small amount of the molecules would go backward to be back to the reactants. In order to compare the forward and backward reaction rate constants, \( K_{Zf} \) and \( K_{Zb} \), for the PEG and no PEG conditions, we fitted the trajectories in Figure 3.6B and 3.6C using the following equations.
Figure 3.6. Macromolecular Crowding Effects on TAR-zipper Annealing at 240 nM NC and 10 nM zipper DNA. (A) Schematic illustration of the TAR-zipper DNA annealing. (B) Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant fraction ($\theta_R$), loop intermediate fraction ($\theta_l$), and product fraction ($\theta_P$) (bottom panel) during TAR-zipper DNA annealing process chaperoned by 240 nM NC. (C) Single-molecule $E_A$ trajectories of molecules (top panel), and time-evolution of reactant fraction ($\theta_R$), loop intermediate fraction ($\theta_l$), and product fraction ($\theta_P$) (bottom panel) during TAR-zipper DNA annealing process chaperoned by 240 nM NC in the present of 5 wt% PEG-500 in the measuring system.
Figure 3.7. Comparison of forward and backward (K$_{Zf}$, K$_{Zb}$) rate constants for TAR-zipper DNA annealing in the present of 240 nM NC with and without 5 wt% PEG-5000 in the reaction flow system. The rate constants were obtained through least square curve fitting using Equation 10 and 11.

The as-obtained K$_{Zf}$ and K$_{Zb}$ in two different conditions were illustrated in Figure 3.7. It clearly showed that PEG in the flow system significantly promoted forward reaction as K$_{Zf}$ increased by 3 times, while slightly slowing down the reverse reaction rate of the TAR-zipper annealing, shifting the equilibrium toward TAR-zipper complexes. The smFRET results shown in Figure 3.7 strongly indicated that the macromolecular crowding effect do not change the annealing pathway, but accelerate the annealing rates of all steps, especially the annealing rates to form the final duplex product.

\[
\theta_Z(t) = \theta_{Z,eq}[1 - e^{-(k_{Zf} + k_{Zb})(t-t_0)}] \tag{10}
\]
\[
\frac{\theta_{z,eq}}{1 - \theta_{z,eq}} = \frac{k_{zf}}{k_{zb}}
\]  

(11)

HIV-1 NC has been observed to have a stronger binding preference to single-stranded than double-stranded NA regions. It has been reported that NC melts stacks of based pairs in structured DNAs and RNAs, then locally melted 'hotspots' were formed. In addition, many studies and contexts shows that NC-induced melting was a key component of NC-chaperoned NA annealing mechanisms. In our previous work studying the relationship between the local melting of NA hairpins and NA strand annealing, we found that the switch from the loop to the zipper pathway is essentially a consequence of NC-induced melting of the 3'/5' terminal stems of the TAR hairpins. Here, in order to test our hypothesis, we carried out the NC-induced duplex melting of the 3'/5' terminal of TAR hairpins assays under the PEG or without PEG condition. As illustrated in Figure 3.8A, the closed TAR DNA hairpin exhibited \( E_A \) of 1 while the melting of the 3'/5' termini stem region gave rise to a decreased \( E_A \) value of \(~0.85\). Figure 8B showed the EA histograms of dual-labeled TAR DNA molecules in buffer and various concentrations of NC as labeled in each panel.

The single-molecule NA melting assays (Figure 3.5) show that PEG does not accelerate the extent of NC-induced NA melting. However, the NC-chaperoned NA annealing reaction rates are dramatically increased in the presence of PEG at saturating NC concentration. Therefore, we hypothesize that the crowding environment makes NC prefers to chaperon the duplex DNA hairpin instead of the single-stranded DNA hairpin.
**Figure 3.8.** Macromolecular crowding effect on NC-induced dual-labeled TAR DNA melting. (A) Schematic illustration of the NC-induced TAR melting. (B) $E_A$ histograms of dual-labeled TAR DNA molecules in buffer and various concentrations of NC as labeled in each panel. (C) $E_A$ histograms of NC-induced TAR DNA melting in the present of 5 wt% PEG-5000.

### 3.4 Conclusions

In this work, we have used time-resolved SM-FRET as a spectroscopic tool to resolve the macromolecule crowding effects on NC-induced TAR sequence structure rearrangements. We used poly-ethylene glycol (PEG) as a model neutral polymer cosolute to mimic the crowding intracellular environments in biomolecular measurements.
PEG is one kind of polymer with adjusting molecular mass, can be easily dissolved in water, and is very price and environments friendly, most important of all, it has no intra-reaction with the HIV-1 NC and TAR DNA sequence. In the macromolecule free environment, sm-FRET measurements showed that almost of all the molecules took the loop pathway when chaperoned by 80 nM concentration of NC. However, when the single-molecule TAR DNA annealing assays were carried out in the present of 5 wt% of PEG-5000 still at 80 nM concentration of NC, sm-FRET only observed the loop pathway. Macromolecule crowding effect didn't switch the NA from loop pathway to the zipper pathway, but effective accelerated the annealing reaction rate. Moreover, the TAR DNA annealing reaction rates increased as we increasing the volume and concentration of macromolecule, even until reaching the sm-FRET measurements limitations, the molecules went through the loop pathway, without switching to the zipper pathway. Without macromolecule in the system, the zipper pathway is the predominate pathway at sufficiently high NC concentrations as 240 nM. Macromolecule crowding effects only promotes the annealing rates without changing the reaction pathway at this high NC concentration. We also performed single molecule FRET measurements to detect macromolecule crowding effects on the formation of zipper/loop intermediates through carrying out TAR DNA/zipper annealing and TAR DNA/loop annealing in the presents of 5 wt% PEG-5000 respectively, found that macromolecule crowding effect prominently increased the forward reaction rates by 3 folders, meanwhile only made tiny change to the backward reaction rates. Furthermore, we also found that macromolecule crowding effects didn't facilitate the melting degree of the dual-labeled TAR DNA sequence. Thus, the macromolecule crowding effects makes NC have a preference to binding with double-
stranded NA hairpins, instead of single-stranded NA. The sm-FRET results about the macromolecule crowding effects here provide significant information to the complicated dynamic NC-NA interactions in the intracellular environments.

3.5 References


CHAPTER 4
MULTIFACETED GOLD-PALLADIUM BIMETALLIC NANORODS
AND THEIR GEOMETRIC, COMPOSITIONAL, AND CATALYTIC
TUNABILITIES

4.1 Introduction

Metallic nanoparticles exhibit a set of size- and shape-dependent optical and catalytic properties that can be systematically fine-tuned and rationally optimized for widespread applications in photonics,1,2 spectroscopies,3,4 biomedicine,5-7 and catalysis.8-12 In comparison to their monometallic counterparts, multimetallic nanoparticles exhibit remarkably further enhanced architectural, optical, and catalytic tunabilities, allowing new properties to emerge benefiting from the synergy between multiple constituents.13-17 Although great success in geometry-controlled synthesis of monometallic nanoparticles has been achieved,18-22 the precise architectural control of multimetallic nanostructures represents a significantly more challenging task. While some fundamental principles of monometallic nanocrystal growth may also apply to multimetallic systems, the nucleation and growth of multimetallic nanoparticles involve substantially more complicated structure-transforming processes due to the interplay of multiple thermodynamic and kinetic factors that synergistically guide the deposition, interdiffusion, segregation, and architectural arrangements of the constituent elements.11,12,15,23-25
Among a diverse set of multimetallic nanostructures, Au-Pd bimetallic nanoparticles have been of particular interest due to their superior catalytic performances typically unachievable in their monometallic counterparts.\textsuperscript{13,16,26} The performance optimization of Au-Pd bimetallic nanocatalysts essentially relies on the precise control over the particle geometries and compositions,\textsuperscript{13,26-29} which can be achieved through deliberately designed solution-phase colloidal syntheses. Au and Pd both adopt the face centered cubic (fcc) crystalline structure with reasonably small lattice mismatch ($\sim 4.9\%$) and are miscible over a wide stoichiometric range, making it possible to selectively synthesize a whole set of architecturally distinct Au-Pd bimetallic nanoparticles ranging from epitaxial core-shell heterostructures to homogenous alloys by kinetically maneuvering the nanocrystal nucleation and growth.\textsuperscript{27-46} Despite their intrinsically different redox potentials and reduction kinetics, Au and Pd precursors can be coreduced to form Au-Pd alloy nanocrystals through one-pot synthesis under diffusion-controlled conditions when the surface deposition occurs far more rapidly than the diffusion of the reactants to the nucleus surfaces.\textsuperscript{35,37,39,42,43,46} The kinetic control of the nanocrystal growth, when further coupled with thermodynamic stabilization of specific facets by surface-capping ligands or foreign metal adatoms, also enables one to fine-tailor the crystallographic facets exposed on the alloy nanoparticle surfaces.\textsuperscript{13,37,43,46} The capabilities to fine-tune both the geometries and compositions of Au-Pd alloy nanoparticles can be further enhanced using preformed nanocrystalline seeds with well-defined shapes to mediate the coreduction of Au and Pd with a mild reducing agent, such as ascorbic acid (AA).\textsuperscript{24} The structural evolution of the nanocrystals during seed-mediated coreduction is mechanistically complex, entangling multiple kinetically controlled and thermodynamically driven
processes that are sensitively dependent upon a series of interplaying factors, such as the Au/Pd precursor ratio, the pH of the nanocrystal growth solution, the reaction temperature, the surface-capping surfactants, and the structures of the seeds. Subtle changes in any of the above-mentioned synthetic parameters may introduce drastic modifications to the geometries and compositions of the resulting nanostructures. These interesting observations motivated us to further decipher the complex mechanisms dictating the structural evolution of nanocrystals during seed-mediated coreduction with the goals of pushing the architectural control of multimetallic nanoparticles to an unprecedented level of precision and versatility.

In this chapter, we investigate the kinetically controlled coreduction of Au and Pd seeded by single-crystalline Au nanorods (NRs) as a model system to shed light on the mechanistic complexity associated with the architectural evolution of anisotropic alloy nanostructures in seed-mediated coreduction processes. Chemically synthesized cylindrical Au NRs are essentially enclosed by multifaceted surfaces comprising a variety of local high-index and low-index facets, which are coated with structure-directing capping surfactants, such as cetyltrimethylammonium bromide (CTAB), and arguably sub-monolayers of foreign metal adatoms, such as Ag. How such surface structural complexity and geometric anisotropy of the Au NR seeds are translated into the architectural diversity of the overgrown multimetallic nanostructures through seed-mediated coreduction remains a fundamentally intriguing open question. As shown in this work, cylindrical Au NRs may selectively transform into an entire family of geometrically distinct anisotropic polyhedral nanostructures enclosed by specific types of well-defined facets without demolishing their crystalline integrity upon seed-mediated
Au-Pd coreduction, allowing us to fine-control both the compositional stoichiometries and the atomic-level surface structures of Au-Pd bimetallic NRs by judiciously tailoring several key synthetic parameters. The precise control of both the particle facets and compositions enables us to gain detailed, quantitative insights into the structure-composition-property relationships underpinning the intriguing catalytic behaviors of colloidal Au-Pd alloy nanocatalysts.

4.2 Experimental Section

Chemicals and Materials.

Gold (III) chloride trihydrate (HAuCl\textsubscript{4}$\cdot$3H\textsubscript{2}O, ACS grade), anhydrous potassium carbonate (K\textsubscript{2}CO\textsubscript{3}, granular), and anhydrous potassium hydroxide (KOH, granular) were purchased from J.T. Baker. Sodium oleate (NaOL, $>$97 \%) and cetyltrimethylammonium bromide (CTAB, $>$98.0\%) were purchased from TCI America. Silver nitrate (AgNO\textsubscript{3}, 99.9995 \% metals basis) were obtained from Alfa Aesar. Palladium (II) chloride (PdCl\textsubscript{2}, $>$99.9\%), sodium borohydride (NaBH\textsubscript{4}, 99\%), hydrochloric acid (HCl, 37 \%), L-ascorbic acid (AA, 99.5\%), ammonia borane (H\textsubscript{6}BN, AB, 97 \%), 4-nitrophenol (C\textsubscript{6}H\textsubscript{5}NO\textsubscript{3}, 4-NP, 99\%), and copper (II) nitrate hydrate (Cu(NO\textsubscript{3})\textsubscript{2}$\cdot$3H\textsubscript{2}O, 99.999\% metal basis) were purchased from Sigma-Aldrich. Ethanol (200 proof) was purchased from Fisher Scientific. All reagents were used as received without further purification. Ultrapure water (18.2 M\textsubscript{2} resistivity, Barnstead EasyPure II 7138) was used for all experiments.

Synthesis of Cylindrical Au Nanorods (NRs).

Single-crystalline Au NRs with a cylindrical morphology were synthesized using a previously published seed-mediated growth method with minor modifications\textsuperscript{62}. Colloidal Au seeds were prepared by reducing HAuCl\textsubscript{4} with NaBH\textsubscript{4} in the presence of CTAB. First,
5.0 mL of 0.5 mM HAuCl₄ was mixed with 5.0 mL of 200 mM CTAB solution. Then, 1.0 mL of ice-cold, freshly prepared 6.0 mM NaBH₄ was quickly injected into the mixture under magnetic stir (1200 rpm). The seed solution was stirred for 2 min and then left undisturbed for 30 min before use. To prepare the Au NR growth solution, 7.0 g of CTAB and 1.234 g of NaOL were dissolved in 250 mL of water at 60 °C. The solution was cooled to 30 °C and then 24 mL of 4.0 mM AgNO₃ was added. The mixture was kept undisturbed at 30 °C for 15 min, followed by the addition of 250 mL of 1 mM HAuCl₄. The solution became colorless after being stirred at 700 rpm for 90 min and 2.1 mL of HCl (37 wt % in water, 12.1 M) was then introduced into the mixture. After another 15 min of slow magnetic stir at 400 rpm, 1.25 mL of 64 mM AA was added. Finally, 0.8 mL of seed solution was injected into the growth solution and the mixture solution was vigorously stirred for another 30 s and then left undisturbed at 30 °C for 12 h. The resulting Au NRs were collected by centrifugation at 7000 rpm for 20 min followed by removal of the supernatant and finally redispersed in 30 mL of 100 mM CTAB.

**Synthesis of Au Elongated Tetrahedral Nanorods (ETHH NRs).**

Au ETHH NRs were synthesized by adding 0.3 mL seeds into the NR growth solution while keeping all the other synthetic parameters exactly the same as those for the synthesis of cylindrical NRs (0.8 mL seeds were added into NR growth solution for the synthesis of cylindrical Au NRs). The resulting Au ETHH NRs were collected by centrifugation at 5000 rpm for 20 min followed by removal of the supernatant and finally redispersed in 30 mL of 100 mM CTAB.
Synthesis of Au Elongated Trisoctahedral (ETOH) NRs

Au ETOH NRs were prepared via overgrowth of the cylindrical Au NRs in the presence of HAuCl₄, CTAB, and AA following a protocol we recently published.⁵²,⁵⁸ In a typical procedure, 100 μL of colloidal Au NRs were first redispersed in 100 μL 100 mM CTAB after being washed once with water. The growth solution was prepared by sequentially adding H₂O (3.30 mL), HAuCl₄ (0.20 mL, 10 mM), and AA (0.50 mL, 0.10 M) into a CTAB (0.90 mL, 0.10 M) solution. After gently mixing the growth solution for 30 s, the growth of Au ETOH NRs was initiated by adding 100 μL of Au NRs (in 100 mM CTAB). The reactants were gently mixed for 30 s immediately after the addition of Au NRs and then left undisturbed at 30 °C for 1 h. The obtained Au ETOH NRs were washed with water twice through centrifugation/redispersion cycles, and finally redispersed in 200 μL of 20 mM CTAB. The total volume of the growth solutions was always fixed at 5.0 mL.

Synthesis of Au@Pd Core-Shell Cuboidal NRs.

Au@Pd core-shell cuboidal NRs were synthesized via epitaxial growth of Pd on the cylindrical Au NRs following a previously published protocol.⁶⁴ In a typical procedure, 100 μL of colloidal Au NRs were first redispersed in 100 μL 0.10 M CTAB after being washed once with water. The growth solution was prepared by sequentially adding H₂O (3.30 mL), H₂PdCl₄ (0.20 mL, 10 mM), and AA (0.50 mL, 0.10 M) into a CTAB (0.90 mL, 0.10 M) solution. After gently mixing the growth solution for 30 s, the growth of Au@Pd core-shell cuboidal NRs was initiated by adding 100 μL of the Au NRs (in 0.1 M CTAB). The reactants were gently mixed for 30 s immediately after the addition of Au NRs and then left undisturbed at 30 °C for 1 h. The obtained Au@Pd core-shell cuboidal
NRs were washed with water twice through centrifugation/redispersion cycles, and finally redispersed in 200 μL of 20 mM CTAB.

**Synthesis of Au Truncated Cuboidal (TCB) NRs.**

Monometallic Au TCB NRs were synthesized following a previously published protocol, which involves the overgrowth of cylindrical Au NRs in the presence of Cu$^{2+}$, HAuCl$_4$, CTAB, and AA.$^{52}$ In a typical procedure, 100 μL of colloidal Au NRs were first redispersed in 100 μL 0.10 M CTAB after being washed once with water. The growth solution was prepared by sequentially adding H$_2$O (3.60 mL), HAuCl$_4$ (0.10 mL, 10 mM), Cu(NO$_3$)$_2$ (100 μL, 10 mM), and AA (0.50 mL, 0.10 M) into a CTAB (0.60 mL, 0.10 M) solution. After gently mixing the growth solution for 30 s, the growth of Au TCB NRs was initiated by adding 100 μL of the Au NRs (in 0.1 M CTAB). The reaction solution was gently mixed for 30 s immediately after the addition of Au NRs and then left undisturbed at 30 °C for 1 h. The obtained Au TCB NRs were washed with water twice through centrifugation/redispersion cycles, and finally redispersed in 200 μL of 20 mM CTAB.

**Synthesis of Multifaceted Au-Pd Bimetallic NRs.**

The multifaceted Au-Pd bimetallic NRs were synthesized through seed-mediated electroless plating of Au-Pd alloy shells on single-crystalline Au NRs. The electroless plating of Au and Pd on Au NRs was conducted in the presence of HAuCl$_4$, H$_2$PdCl$_4$, CTAB, and AA at 30 °C under ambient air. The Au-Pd bimetallic NR growth solution was prepared by sequentially adding H$_2$O, HAuCl$_4$, H$_2$PdCl$_4$, and AA into a CTAB solution. After the solution was gently mixed for 30 s, the Au-Pd codeposition was initiated by the introduction of 100 μL of the preformed Au NRs (dispersed in 100 mM...
CTAB. The reaction solution was gently mixed for 30 s immediately after the addition of Au NRs and then left undisturbed at 30 °C for 1 h. The obtained nanoparticles were then washed with H2O twice through centrifugation/redispersion cycles and finally redispersed in 200 μL of 20 mM CTAB. To investigate the effects of [H2PdCl4]/[HAuCl4] molar ratios, CTAB, AA, and foreign metal ions, such as Ag⁺ and Cu²⁺, on the structural transformations during the seed-mediated Au-Pd coreduction, the overall concentrations of HAuCl4, H2PdCl4, CTAB, AA, Ag⁺, and Cu²⁺ in the reaction solutions were systematically varied, while the total volume of the NR overgrowth solution was always fixed at 5.0 mL.

Characterizations.
The morphologies and structures of the nanoparticles were characterized by transmission electron microscopy (TEM) and selected area electron diffraction (SAED) using a Hitachi H-8000 transmission electron microscope operated at an accelerating voltage of 200 kV. All samples for TEM measurements were dispersed in water and drop-dried on 300 mesh Formvar/carbon-coated Cu grids. The structures and compositions of the nanoparticles were also characterized by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) using a Zeiss Ultraplus thermal field emission scanning electron microscope. The samples for SEM and EDS measurements were dispersed in water and drop-dried on silicon wafers. The optical extinction spectra of the nanoparticles were measured on aqueous colloidal suspensions at room temperature using a Beckman Coulter Du 640 spectrophotometer. Powder X-ray diffraction (PXRD) patterns were record on a SAXS Lab Ganesha at the South Carolina SAXS Collaborative (Cu Kα = 1.5406 Å). A Finnigan ELEMENT XR double focusing magnetic sector field
inductively coupled plasma-mass spectrometer (SF-ICP-MS) was used for the analysis of Pd (106, MR), Au (197, MR), and internal standard Rh (103 MR). The samples for ICP-MS measurements were prepared by digesting the samples in a mixture of nitric acid and hydrochloric acid (volume ratio of 1:3) at 180 °C for 4h. The digested samples were brought to 50 mL with H₂O for ICP-MS measurements.

**Catalytic Reaction Kinetics.**

We used the hydrogenation of NP by AB at room temperature as a model reaction to evaluate the catalytic activities of Au-Pd bimetallic EHOH, TCCB, and TCB NRs. In a typical procedure, 0.1 mL of 1.0 mM NP, 0.1 mL of 10 mM K₂CO₃, and 0.08 mL of 0.1 M AB (freshly prepared) were sequentially added to 1.5 mL of ultrapure water in a cuvette and mixed thoroughly. Then, 20 μL of Au-Pd alloy NRs solution were injected into the system. After thoroughly mixed for 5 s, UV-Vis extinction spectra were collected in real time to monitor the reaction kinetics. We compared the catalytic activities of colloidal EHOH, TCCB, and TCB NRs with various Pd/Au ratios at nominally the same particle concentration (1.0×10⁸ particles mL⁻¹). The effects of AB:NP molar ratios on the reaction kinetics were evaluated by varying the initial concentration of AB, [AB]₀, while fixing the initial NP concentration, [NP]₀, at 56.0 μM and the total volume of the solution at 1.8 mL. The effects of pH values on the reaction kinetics were evaluated by adding appropriate amounts of KOH instead of K₂CO₃ while keeping the total volume of the reaction mixtures at 1.8 mL. The pH values were measured using an Oakton™ Waterproof pH 450 Portable pH Meter (Fisher Scientific).
4.3 Results and Discussions

The beauty of using Au NRs as the seeds to mediate the Au-Pd coreduction lies in three key aspects. First, Au NRs represent an anisotropically shaped but single-crystalline seed structure that is ideal for the mechanistic study of seed-mediated overgrowth of nanocrystals into thermodynamically unexpected geometries. Second, the bilayers of CTAB surfactants self-assembled on the Au NR surfaces may serve as a molecular barrier to fine-regulate the diffusion rates of the reactants from the bulk solution to the seed surfaces, ensuring that the Au-Pd codeposition occurs under diffusion controlled conditions to form alloy structures. Third, the structural and compositional changes of NRs during seed-mediated coreduction can be monitored using straightforward optical extinction spectroscopy because of the aspect ratio-, morphology-, and composition-dependent plasmonic characteristics of NRs. The single-crystalline Au NRs used in this work were synthesized using a binary surfactant-guided, seed-mediated growth method developed by Murray and coworkers. The as-synthesized Au NRs exhibit a uniform cylindrical morphology with average diameters of 26.7 ± 3.30 nm and lengths of 102 ± 7.16 nm (Figure 4.1). As shown in Figure 4.2A, cylindrical Au NRs transformed into Au elongated trisoctahedral (ETOH) NRs each of which was enclosed by 24 {221} facets at the ends and 4 {110} facets on the lateral sides upon exposure to an overgrowth solution containing 0.4 mM HAuCl₄, 20 mM CTAB, and 10 mM AA, in agreement with our previous observations. In contrast, cuboidal NRs composed of Au NR cores coated with epitaxial Pd shells were obtained upon exposure of the Au NR seeds to 0.4 mM H₂PdCl₄, 20 mM CTAB, and 10 mM AA (Figure 2B). Moiré patterns were clearly observed in the core region of each cuboidal NR in the TEM image, characteristic of
heteroepitaxial Au-Pd core-shell structures with superimposed lattice mismatch. More detailed structural characterizations of the Au@Pd core-shell cuboidal NRs have been previously reported. Such strikingly distinct structural transformations essentially originate from the intrinsic differences in the deposition kinetics and the relative surface energies of Au and Pd. Au ETOH NRs enclosed by high-energy \{221\} and \{110\} facets were essentially a kinetically controlled product when fast surface deposition and nanocrystal overgrowth occurred at sufficiently high [AA]/[HAuCl₄] ratios ([AA]/[HAuCl₄] = 25 in this case), whereas the electroless deposition of Pd on Au under otherwise identical conditions was significantly slower than that of Au, resulting in cuboidal NRs whose surfaces were dominated by thermodynamically more stable \{100\} facets.

*Figure 4.1.* (A) SEM and (B) TEM images of cylindrical Au NRs. (C) Histograms showing the distributions of the widths and lengths of the Au NRs. (D) Optical extinction spectrum of colloidal Au NRs in water.
Figure 2. SEM images (left panels), TEM images (right panels), and geometric models (middle insets) of (A) Au ETOH NRs and (B) Au@Pd core-shell cuboidal NRs.

When the seed-mediated Au-Pd coreduction occurred in the presence of both H₂PdCl₄ and HAuCl₄, the Au NRs underwent significantly more complicated and diverse geometric transformations. By judiciously adjusting the Pd/Au precursor ratios, we have successfully synthesized Au-Pd bimetallic NRs with two distinct exotic multifaceted geometries, an elongated hexoctahedral (EHOH) NR enclosed by 56 facets and a truncated concave cuboidal (TCCB) NR enclosed by 32 facets, both of which can be derived from a simple \{100\}-faceting cube geometry. As illustrated in Figure 3, an EHOH shape can be geometrically derived from a cube by first pulling out the center of each \{100\} facet to form a tetrahexahedron (THH) enclosed by 24 high-index \{hk0\} facets, then pushing the center of each square edge toward the body center to create a hexoctahedron (HOH), and finally introducing elongation along the [001] crystalline axis. A TCCB geometry can be derived by first elongating a cube along the [001] crystalline
axis to form a cuboid, then creating surface concavity to form a concave cuboid (CCB) enclosed by 24 high-index \{hkk\} facets, and finally truncating the 8 corners with \{111\} facets.

**Figure 4.3.** Geometric derivation of an EHOH and a TCCB NR starting from a nanocube.

**Figure 4.4.** SEM images of the Au-Pd bimetallic NRs obtained at various [H₂PdCl₄]/[HAuCl₄] ratios of (A) 1:19, (B) 1:9, (C) 1:4, (D) 1:2, (E) 1:1, (F) 2:1, (G) 4:1, and (H) 9:1. The total
The concentration of H$_2$PdCl$_4$ plus HAuCl$_4$ was fixed at 0.4 mM. The concentrations of CTAB and AA were 20 mM and 10 mM, respectively. (I) Extinction spectra of the Au-Pd alloy NRs obtained at various [H$_2$PdCl$_4$]/[HAuCl$_4$] ratios as labeled in the figure.

A typical seed-mediated coreduction reaction was carried out at 30 °C under ambient atmosphere in the presence of 20 mM CTAB and 10 mM AA at varying [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio with the total concentration of H$_2$PdCl$_2$ + HAuCl$_4$ fixed at 0.4 mM. As shown by electron microscopy images in Figure 4.4A-H in and schematically illustrated in Figure 4.5A, the cylindrical Au NRs evolved into EHOH NRs at relatively low Pd/Au precursor ratios below 1:2, whereas TCCB NRs formed at Pd/Au precursor ratio higher than 1:1. We used scanning electron microscopy (SEM), transmission electron microscopy (TEM), selected area electron diffraction (SAED), powder X-ray diffraction (PXRD), energy-dispersive spectroscopy (EDS), and inductively coupled plasma mass spectrometry (ICP-MS) to fully characterize the crystalline structures and compositions of the EHOH and TCCB NRs. As shown in Figure 4.5B, the multifaceted bimetallic NRs synthesized at [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 1:9 exhibited a well-defined EHOH geometry enclosed by 56 high-index facets with Miller index of \{hkl\} (h>k>l>0). The EHOH NRs displayed orientation-dependent projection contours in the TEM images (Figures 4.5C-4.5F). The shape profiles and measured edge angles of individual EHOH NRs projected along the [100] and [110] zone axes (Figure 4.5C-D) showed excellent match with the geometric models of a EHOH object enclosed exclusively by 56 \{421\} facets. The crystalline orientation and epitaxial fcc structure of each EHOH NR were further verified by SAED (right panels of Figures 4.5C and 4.5D). Figure 4.5G shows the SEM images of the TCCB NRs synthesized at Pd/Au precursor ratio of 2:1, each of which was identified to be enclosed by 24 high-index \{311\} side facets and 8 low-index \{111\} facets at the
corner truncations after carefully comparing the projected contours of individual TCCB NRs in the TEM images with their geometric models when viewed along the [100], [110], and other projections (Figures 4.5H-4.5K).

Figure 4.5. Structures of Au-Pd alloy EHOH and TCCB NRs. (A) Schematic illustration of the transformations of Au NRs into EHOH and TCCB NRs upon seed-mediated Au-Pd codeposition. The codeposition reactions were carried out in the presence of 20 mM CTAB, 10 mM AA, and various \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) molar ratios with the total concentration of \(\text{H}_2\text{PdCl}_2 + \text{HAuCl}_4\) fixed at 0.4 mM. (B) SEM image of EHOH NRs synthesized at \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratio of 1:9. The
inset highlights one individual EHOH NR. TEM images (left), geometric models (middle), and SAED patterns (right) of individual EHOH NRs viewed along the (C) [100] and (D) [110] projections. (E, F) TEM images and geometric models of individual EHOH NRs at other orientations. (G) SEM image of TCCB NRs synthesized at [H2PdCl4]/[HAuCl4] ratio of 2:1. The inset highlights one individual TCCB NR. TEM images (left), geometric models (middle), and SAED patterns (right) of individual TCCB NRs viewed along the (H) [100] and (I) [110] projections. (J, K) TEM images and geometric models of individual TCCB NRs at other orientations. (L) PXRD patterns of the bimetallic NRs synthesized at various [H2PdCl4]/[HAuCl4] molar ratios as labeled in the figure. The standard diffraction patterns for bulk Au and Pd are also included. The spectra are offset for clarity. SEM images and EDS elemental maps of (M) EHOH and (N) TCCB NRs. (O) Pd atomic percentages of the Au-Pd bimetallic NRs synthesized at various [H2PdCl4]/[HAuCl4] ratios. The Pd atomic percentages were quantified by PXRD, EDS, and ICP-MS, respectively. The error bars show the standard deviations obtained from 3 samples synthesized under each experimental condition.

We used PXRD to characterize the crystalline structures and compositions of the Au-Pd bimetallic NRs. As the [H2PdCl4]/[HAuCl4] ratio progressively increased, the diffraction peaks corresponding to the fcc Au became asymmetrically broadened and eventually split into two sets of fcc diffraction peaks, which could be assigned to the Au NR cores and Au-Pd alloy shells, respectively (Figure 4.5L). Through deconvolution of the (111) diffraction peak into a Au and a Au-Pd alloy peak, we were able to calculate the lattice parameters of the Au-Pd alloys using the Bragg’s law:

$$d_{111}=\frac{\lambda}{2\sin(\theta_{111})} \quad (1),$$

where $\lambda = 1.5406$ Å for Cu Ka, $d_{111}$ is the (111) lattice spacing of the alloy, and $\theta_{111}$ is the angle of incidence on the {111} plane. We further calculated the Pd/Au stoichiometric ratios in the alloy shells using an empirical rule known as the Vegard’s law, which states that the lattice parameters of a homogenous binary alloy are linearly related to its compositional stoichiometries as described by the following equation:

$$d_{Au,Pd(i,x)} = xd_{Au} + (1-x)d_{Pd} \quad (2),$$
where \( x \) is the atomic fraction of Au in the Au-Pd alloy, and \( d_{Au} \) and \( d_{Pd} \) are the lattice constants of fcc Au and Pd, respectively. More definitive evidence on the alloy structures of the overgrown shells was obtained by EDS elemental mapping (Figure 4.5M and Figure 4.5N). PXRD, EDS, and ICP-MS all consistently showed that the Pd atomic percentage of the bimetallic NRs increased with the Pd/Au precursor ratio in the overgrowth solution (Figure 4.5O). While the EDS and ICP-MS results were in excellent agreement, the atomic percentages of Pd calculated from PXRD were about 5-15% higher than those quantified by EDS and ICP-MS due to the fact that both EDS and ICP-MS measured the compositions of the entire particles but PXRD provided the compositional information of the Au-Pd alloy shells specifically.

The structural and compositional changes of NRs upon seed-mediated coreduction introduced interesting modifications to their optical characteristics, as shown in Figure 4.4I. As the \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratio progressively increased up to 1:2, cylindrical Au NRs transformed into EHOH NRs with increasing Pd content, causing systematic red-shift and broadening of both the longitudinal and transverse plasmon resonance peaks of the NRs. As the \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratio further increased, however, the TCCB NRs became increasingly more Pd-rich and their vertices got truncated to a greater extent, resulting in blue-shift and further damping of the plasmon resonances. The interesting extinction spectral evolution originated from both the structural transitions of the NRs and the plasmon damping caused by alloying of Au with Pd.

As previously shown by Skrabalak and coworkers, the crystalline habits and surface structures of the seeds are both key factors guiding the geometric evolution of the Au-Pd bimetallic nanoparticles during seed-mediated coreduction.\(^{40}\) In this work, both the
EHOH and TCCB NRs were derived from their parental single-crystalline Au NR seeds that were synthesized following Murray’s protocol in the presence of oleate/CTAB binary surfactants. These Au NRs, regardless of their dimensions and aspect ratios, essentially inherited the crystalline habits of elongated tetrahedral (ETHH) NRs, each of which was enclosed by 24 high-index \{730\} facets. However, when the Au NRs were thinner than ~30 nm, their characteristic facets were underdeveloped with significant truncations at the edges and vertices of the NRs. Therefore, the as-synthesized Au NRs exhibited a cylindrical morphology, capped with rounded ends and enclosed by a variety of ill-defined local facets. By decreasing the amount of seeds while keeping the other synthetic parameters the same, we were able to synthesize ETHH NRs with significantly increased lateral sizes enclosed by fully developed \{730\} facets. We further used these well-defined ETHH NRs as the seeds to mediate the Au-Pd coreduction, through which we successfully synthesized both EHOH and TCCB NRs under appropriate conditions. Our results strongly indicated that it was the intrinsic crystalline habits rather than the exposed facets of the Au NRs that determined their structural transformations into the exotic EHOH and TCCB geometries upon seed-mediated coreduction.

The Au-Pd bimetallic EHOH and TCCB NRs also inherited the structural anisotropy from their parental Au NR seeds. Skrabalak and coworkers previously reported that Au or Pd seeds with various polyhedral symmetries, such as nanocubes, nanobipyramids, nanoctahedra, selectively evolved into a series of Au-Pd bimetallic branched nanostructures or low-index faceting nanopolyhedra upon seed-mediated coreduction, suggesting a strong correlation between the crystalline symmetries of seeds and the
structures of the overgrown Au-Pd bimetallic nanoparticles. They also used Au NRs as the seeds to mediate the coreduction of Au and Pd, through which elongated branched Au-Pd alloy nanoparticles with ill-defined facets were obtained. Here we showed that by fine-maneuvering the kinetics of Au-Pd coreduction on Au NR seeds, the synthesis of anisotropic Au-Pd bimetallic NRs enclosed by well-defined high-index facets became possible.

The spatially uniform alloy shell structures and the high-index faceting EHOH and TCCB geometries are both the consequences of kinetically fast nanocrystal overgrowth under diffusion controlled conditions. The seed-mediated coreduction essentially involves two key processes, the diffusion of reactants from the bulk solution to the seed surfaces and the surface deposition of metals, possibly with additional complications caused by the interfacial diffusion of adatoms and the galvanic replacement between the metal precursors and the Ag underpotential deposition (UPD) layer on the Au NR surfaces. As demonstrated by Xie and coworkers, when the surface deposition occurs much more rapidly than the diffusion, the overall nanocrystal growth kinetics becomes diffusion controlled and the ratios of the Au and Pd components deposited onto the seed surfaces remain constant during the crystal growth process, resulting in alloy nanoparticles with uniform spatial distributions of Au and Pd. A linear relationship was observed when we plotted the Pd atomic percentages in the overgrown shells (quantified by PXRD) as a function of the molar percentage of Pd precursor in the reaction mixtures (Figure 4.6), which provided strong evidence on the codeposition of Au and Pd at fixed relative rates during the nanocrystal overgrowth. The slope of the line was around 0.5, suggesting that the atomic deposition rate of Au was approximately twice of that of Pd.
The extrapolation of the line to 100 % Pd precursor yielded an intercept around 0.5, indicating that about 50 % of the Pd precursors were codeposited with Au on the seed surfaces while other possible nondepositing species, such as unreacted PdCl₄²⁻, ultrasmall Pd atomic clusters, and (C₁₉H₄₂N)₂PdBr₄ complex might also exist in the supernatant. The nanocrystal growth kinetics was also a key factor determining the geometries and surface structures of the overgrown nanoparticles. Fast nanocrystal growth at sufficiently high AA concentrations, e.g. 10 mM under the current synthetic conditions, favored the formation of kinetically trapped high-index faceting nanostructures, as exemplified by the EHOH and TCCB NRs. Further increase of AA concentration from 10 mM to 100 mM did not introduce any significant modifications to the dimensions and morphologies of the EHOH and TCCB NRs. In contrast, the formation of nanocrystals enclosed by low-index facets requires relatively slow growth kinetics that allow the nanocrystals to fully evolve into thermodynamically stable geometries. Under the conditions for slow nanocrystal growth, however, the surface deposition of Au and Pd on the seed surfaces becomes the rate limiting step, and as a consequence, heteronanostructures composed of Au-rich cores and Pd-rich or even monometallic Pd shells start to form due to the intrinsically faster consumption of the Au precursor than that of the Pd precursor. Therefore, it has remained a significant challenge to synthesize low-index faceting Au-Pd bimetallic nanoparticles with spatially uniform alloy compositions.
Figure 4.6. Plots of Pd atomic percentages in the overgrown shell (quantified by PXRD) as a function of the molar percentage of Pd precursor in the reaction mixtures. The solid red line shows the result of least squares fitting using a linear function with a y intercept of 0.

To overcome this synthetic challenge, we used Au NRs whose surfaces were capped with a self-assembled bilayer of CTAB surfactants as the seeds to guide the electroless codeposition of Au and Pd. The CTAB bilayers served as a molecular barrier that significantly decelerated the diffusion of the reactants from the bulk solution to the seed surfaces, allowing us to slow down the metal deposition by either decreasing the AA concentration or increasing the CTAB surface packing density while still maintaining the diffusion controlled conditions such that the formation of low-index faceting bimetallic NRs with homogenous alloy shell structures became possible.
**Figure 4.7.** SEM images of the Au-Pd alloy NRs synthesized at \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratio of 1:9 in the presence of 20 mM CTAB and various concentrations of AA: (A) 8 mM, (B) 4 mM, (C) 1.6 mM, and (D) 0.6 mM. (E) Pd:Au atomic ratios quantified by EDS and (F) optical extinction spectra of Au-Pd alloy NRs obtained at various AA concentrations. The error bars in panel E show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.
Figure 4.8. SEM images of the Au-Pd alloy NRs synthesized at [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 2:1 in the presence of 20 mM CTAB and various concentrations of AA: (G) 8 mM, (H) 4 mM, (I) 1.6 mM, and (J) 0.6 mM. (K) Pd:Au atomic ratios quantified by EDS and (L) optical extinction spectra of Au-Pd alloy NRs obtained at various AA concentrations. The error bars in panel E show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.

At a fixed [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 1:9, the corners and edges of the EHOH NRs got truncated to form irregularly shaped, multifaceted NRs while the Pd:Au atomic ratios
remained essentially unchanged as the AA concentration progressively decreased from 10 mM to 0.6 mM, as shown in Figure 4.7. Such geometric evolution caused weakening and blue-shift of the plasmon resonance peaks in the extinction spectra (Figure 4.7F). When the [H₂PdCl₄]/[HAuCl₄] ratio was fixed at 2:1, TCCB NRs became less concave on their side facets and more truncated at their corners, gradually evolving into quasi-cuboidal (QCB) NRs and eventually into truncated cuboidal (TCB) NRs as the AA concentration progressively decreased (Figures 4.8A-4.8D). Interestingly, both the Pd/Au atomic ratios and the alloy structures were well-preserved regardless of the variation of AA concentration (Figures 8E). Although the plasmon bands appeared very weak and broad due to significant plasmon damping effects caused by the relatively high fraction of Pd in the alloy shells (~ 35 %), blue-shift of the plasmon resonance was still clearly observed as the morphology of the NRs switched from TCCB to QCB and eventually to TCB NRs, as shown in Figure 4.8F.

The transitions from high-index faceting to low-index faceting geometries were also observed when increasing the CTAB concentration. The CTAB bilayers exhibit different packing densities and thereby different stabilization effects on various types of facets of Au and Pd nanocrystals, allowing us to further fine-tailor the surface structures of the overgrown alloy shells while maintaining the coreduction at diffusion-controlled conditions. When increasing the CTAB concentration in range from 4 mM to 150 mM at a fixed [H₂PdCl₄]/[HAuCl₄] ratio of 1:9 and AA concentration of 10 mM, increasingly more significant corner truncations and suppression of surface convexity were observed, causing a progressive blue-shift of the plasmon resonance in the extinction spectra, though the variation of CTAB concentration did not cause any significant changes in the
Pd:Au stoichiometries (Figure 4.9). These results indicated that the surface packing density of CTAB, which could be controlled by the CTAB concentration in the bulk solution, provided an additional parameter for the control of tip/edge sharpness and surface convexity of the EHOH NRs. By deliberately varying both CTAB concentration and the [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio, we were able to synthesize EHOH NRs with almost identical aspect ratios, surface convexity, and tip/edge sharpness but different Pd:Au stoichiometric ratios in a highly controllable and precise manner. At the [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 2:1, the TCCB NRs became less concave on their side surfaces accompanied by more significant corner truncations as the CTAB progressively increased, eventually transforming into TCB NRs at sufficiently high CTAB concentrations, as shown in Figure 4.10. The Pd:Au stoichiometric ratios, however, remained unchanged regardless of the variation of CTAB concentration. At the CTAB concentration of 150 mM, the densely packed CTAB bilayers on the seed surfaces significantly slowed down the diffusion and thus the nanocrystal growth kinetics, resulting in the formation of well-defined low-index faceting TCB NRs even at AA concentrations as high as 100 mM. It is worth mentioning that in previously reported seed-mediated syntheses of multimetallic nanoparticles, the seed particles were typically coated with various surface-capping surfactants, which may play crucial roles in guiding the structural evolution of multimetallic nanocrystals. While significant mechanistic insights have been gained on the effects of seed shapes and structures, the roles of surface-capping surfactants still remain poorly understood and need to be further explored. Our results clearly show that the packing density of CTAB on the seed surfaces
can also be used as a key synthetic knob for the geometry control of the Au-Pd bimetallic nanoparticles through seed-mediated coreduction under diffusion controlled conditions.

Figure 4.9. SEM images of the Au-Pd alloy NRs synthesized at [H₂PdCl₄]/[HAuCl₄] ratio of 1:9 in the presence of 10 mM AA and various concentrations of CTAB: (A) 4 mM, (B) 50 mM, (C) 100 mM, and (D) 150 mM. (E) Pd:Au atomic ratios quantified by EDS and (F) optical extinction spectra of Au-Pd alloy NRs obtained at various CTAB concentrations. The error bars in panel E show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.
Figure 4.10. SEM images of the Au-Pd alloy NRs synthesized at \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratio of 2:1 in the presence of 10 mM AA and various concentrations of CTAB: (G) 4 mM, (H) 50 mM, (I) 100 mM, and (J) 150 mM. (K) Pd:Au atomic ratios quantified by EDS and (L) optical extinction spectra of Au-Pd alloy NRs obtained at various CTAB concentrations. The error bars in panel E show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.

A TCB NR represents a prototypical low-index faceting geometry which can be derived from a \(\{100\}\)-faceting nanocuboid by creating 8 \(\{111\}\) facets on the truncated
corners. Figure 4.11 shows more detailed structural and compositional information of the Au-Pd bimetallic TCB NRs synthesized in a sufficiently high concentration of CTAB at 150 mM. The electron microscopy images and SAED patterns (Figures 4.11A-4.11C) showed that the bimetallic shells overgrown on the Au NR seeds were single crystalline in nature and each TCB NR was enclosed by 6 low-index \{100\} facets on the side faces and 8 low-index \{111\} facets at the truncated corners. The EDS elemental mapping results clearly showed that the Au and Pd elements were homogeneously distributed in the overgrown bimetallic shells (Figure 4.11D). By varying the $[\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]$ ratio while keeping CTAB concentration at 150 mM, we were able to synthesize TCB NRs with essentially the same geometry but different compositional stoichiometries (Figures 4.11E and 4.11F). The PXRD results further verified that the overgrown bimetallic shells were composed of Au-Pd alloys (Figure 4.11G), absent of segregated monometallic Pd shells. The compositional stoichiometries of the alloy shells were quantified by PXRD using the Vegard’s law and further compared with the bulk compositions quantified by EDS and ICP-MS in Figure 4.11H. The elemental mapping results with the current spatial resolution (~2 nm), however, did not allow us to further quantify the local compositional gradient over even small length scales in the alloy shells. Such local compositional gradient has been previously observed in various Au-Pd bimetallic nanostructures synthesized by seed-mediated coreduction,\textsuperscript{67,68} and may also exist in the Au-Pd bimetallic TCB NRs synthesized using our protocol, which is further implicated by the broadening of the alloy peaks in the PXRD patterns (Figure 4.11G).
Figure 4.11. Structures of Au-Pd alloy TCB NRs. (A) SEM image of Au-Pd bimetallic TCB NRs synthesized at \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) molar ratio of 2:1 in the presence of 150 mM CTAB and 10 mM AA. The inset highlights one individual TCB NR. TEM images (left), geometric models (right), and SAED patterns (middle insets) of individual TCB NRs viewed along the (B) [100] and (C) [110] projections. (D) SEM image and EDS elemental maps of Au-Pd TCB NRs. SEM images of TCB NRs synthesized at \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) ratios of (E) 1:1 and (F) 4:1 in the presence of 150 mM CTAB and 10 mM AA. (G) PXRD patterns of the TCB NRs synthesized at various \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) molar ratios as labeled in the figure. The standard diffraction patterns for cubic-phase bulk Au and Pd are also included. The spectra are offset for clarity. (H) Pd atomic percentages of the TCB NRs synthesized at various \([\text{H}_2\text{PdCl}_4]/[\text{HAuCl}_4]\) molar ratios. The Pd atomic percentages were quantified by PXRD, EDS, and ICP-MS, respectively. The error bars show the standard deviations obtained from 3 samples synthesized under identical experimental conditions.

The capability of fine-tuning the surface structures and compositions of the bimetallic NRs through seed-mediated coreduction can be further enhanced with the aid of foreign metal ions, such as Cu\(^{2+}\) and Ag\(^{+}\). We have previously demonstrated that the facets of monometallic Au NRs can be fine-tailored with atomic level precision using Cu\(^{+}\) ions and CTAB as a pair of surface capping competitors to guide the particle geometry evolution.
during NR overgrowth. However, the effects of foreign metal ions on the architectural evolution of bimetallic nanocrystals during seed-mediated Au-Pd coreduction still remain poorly understood. As shown in Figures 4.12A-4.12D, at a fixed Pd/Au precursor ratio of 1:9, the geometry of the overgrown bimetallic NRs evolved from EHOH NRs to concave cuboidal NRs and eventually to TCB NRs with significantly decreased transverse dimensions as increasing amount of Cu\(^{2+}\) was introduced into the overgrowth solution, a trend analogous to that we recently observed on monometallic Au NRs. There are already compelling evidences showing that Cu\(^{2+}\) ions are reduced to Cu\(^{+}\) ions by AA under similar experimental conditions. The Cu\(^{+}\) ions then serve as a surface capping ionic species to compete with the CTAB surfactants, thereby modulating the relative growth rates of various types of facets. Apparently, the passivation of the seed surfaces with Cu\(^{+}\) ions favors the formation of thermodynamically more stable low-index facets during the seed-mediated Au-Pd coreduction. An alternative mechanism regarding the effects of Cu\(^{2+}\) involves the selective passivation of certain facets by a submonolayer of Cu adatoms generated from UPD. While neither PXRD nor EDS showed any evidence on the presence of metallic Cu in the overgrown NRs, the existence of transient, localized Cu UPD layers on the seed surfaces during the seed-mediated coreduction cannot be completely ruled out at this point.

The Cu\(^{2+}\) ions greatly influenced not only the geometries but the compositions of the overgrowth bimetallic NRs as well. As the Cu\(^{2+}\) concentration gradually increased, the Pd/Au atomic ratios (quantified by EDS) kept decreasing until the Pd signals became almost undetectable at sufficiently high Cu\(^{2+}\) concentrations above 100 μM (Figure 4.12E). The structural and compositional transformations of NRs were well reflected by
the evolution of the key plasmonic features in the optical extinction spectra (Figure 4.12F). Surface concavity and corner truncation caused red-shift and blue-shift of the plasmon resonances, respectively, while the loss of Pd content from the alloy shells resulted in narrowing and strengthen of the plasmon bands. Similar effects of Cu$^{2+}$ ions were also observed at a fixed Pd/Au precursor ratio of 2:1 (Figures 4.12G-L). As the Cu$^{2+}$ concentration increased, the high-index faceting TCCB NRs gradually transformed into low-index faceting quasi-cuboidal NRs, accompanied by the decrease of the Pd:Au atomic ratios.

Figure 4.12. Effects of Cu$^{2+}$ ions on the structural transformations of NRs upon seed-mediated Au-Pd codeposition. SEM images of the Au-Pd alloy NRs synthesized in the presence of 20 mM CTAB and 10 mM AA at a [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 1:9 and various Cu$^{2+}$ concentrations of (A) 2 μM, (B) 5 μM, (C) 10 μM, and (D) 400 μM. (E) Pd:Au atomic ratios (quantified by EDS) and (F) extinction spectra of Au-Pd bimetallic NRs obtained at various Cu$^{2+}$ concentrations ([H$_2$PdCl$_4$]/[HAuCl$_4$] = 1:9). SEM images of the Au-Pd alloy NRs synthesized in the presence of 20 mM CTAB and 10 mM AA at a [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 2:1 and various Cu$^{2+}$ concentrations of (G) 0.05 μM, (H) 0.2 μM, (I) 20 μM, and (D) 200 μM. (K) Pd:Au atomic ratios (quantified by EDS) and (L) extinction spectra of Au-Pd bimetallic NRs obtained at various Cu$^{2+}$ concentrations ([H$_2$PdCl$_4$]/[HAuCl$_4$] = 2:1). The error bars in panels E and K show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.
Our observations strongly suggested that the capping of the seed surfaces with Cu\(^+\) ions selectively suppressed the electroless deposition of Pd with respect to that of Au. Considering the Au NR seeds as the catalysts for the electroless codeposition, it was reasonable to hypothesize that the seed surfaces became partially poisoned toward the catalytic deposition of Pd upon surface adsorption of Cu\(^+\), resulting in self-nucleation of Pd atoms in solution and the formation of discrete Pd islands on the seed surfaces. Consequently, a mixture of self-nucleated Pd nanocrystal aggregates, Au NRs decorated with Pd nanocrystallines, and irregularly shaped by-products (mostly likely a CTAB-Pd complex, \((C_{19}H_{42}N)_2PdBr_4\), according to the EDS results), were obtained in the presence of 400 μM Cu\(^{2+}\). Interestingly, no such surface poisoning effect of Cu\(^{2+}\) ions on Pd deposition was observed during the growth of Pd concave nanocubes on Pd seeds\(^69\) and seedless one-pot growth of Au-Pd bimetallic nanocrystals,\(^{37,46}\) strongly indicating that such surface poisoning by Cu\(^+\) ions was highly like to be intimately tied to the surface structures of the Au NR seeds. The exact mechanisms involved in the Cu\(^{2+}\)-guided Au-Pd codeposition on Au NR seeds, however, still remain ambiguous at this stage and require further scrutiny.

In contrast to Cu\(^+\) ions which exist as surface-adsorbed ionic species, Ag\(^+\) ions have been used to guide the geometric evolution of Au nanocrystals by forming a Ag UPD layer that selectively passivates certain facets exposed on the Au nanocrystal surfaces.\(^{37,70-72}\) We recently demonstrated that the Ag\(^+\)-guided Au NR overgrowth involved two underlying pathways, Ag UPD and Au-Ag electroless codeposition, and the manipulation of the pathway switch enabled the cylindrical Au NRs to selectively transform into a library of anisotropic nanostructures with interesting geometric,
compositional, and optical characteristics. Here we systematically investigated the effects of Ag$^+$ on the structural and compositional evolution of NRs during seed-mediated coreduction of Au and Pd by varying the concentration of Ag$^+$ ions in the overgrowth solution while keeping the Pd/Au precursor ratios at 1:9. As the Ag$^+$ concentration gradually increased in the range from 0.5 μM to 10 μM, the EHOH NRs gradually evolved into quasi-cuboidal NRs as the degree of surface convexity decreased (Figure 4.13A). Further increase of Ag$^+$ concentration from 10 μM to 160 μM witnessed the transition from quasi-cuboidal NRs to irregularly shaped NRs and eventually to elongated octahedral (EOH) NRs (Figure 4.13B-D). Each EOH NRs were enclosed by 8 well-defined $\{111\}$ facets at the two ends while the 4 side faces exhibited nanoscale roughness. As shown in Figure 13E, the Pd/Au atomic ratios were independent of the Ag$^+$ concentration, which was in striking contrast to what we observed in the presence of Cu$^{2+}$. More interestingly, while the Ag signals remained almost undetectable by EDS at the Ag$^+$ concentrations below 10 μM, the Ag/Au atomic ratios significantly increased as the Ag$^+$ concentration further increased (Figure 4.13E), indicating the pathway switch from Ag UPD-guided Au-Pd codeposition to Au-Ag-Pd trimetallic codeposition. While the seed-mediated Au-Pd coreduction guided by Ag UPD resulted in Au@Au-Pd alloy core-shell NRs whose surfaces were covered by a sub-monolayer of Ag UPD adatoms, the Au-Ag-Pd trimetallic codeposition led to the formation of Au-Ag-Pd ternary alloy shells on the Au NR seeds as shown by the EDS elemental mapping results. These structural and compositional changes introduced interesting modifications to the plasmonic features of the Au-Ag-Pd trimetallic NRs as shown in the optical extinction spectra (Figure 4.13F).
Figure 4.13. Effects of Ag$^+$ ions on the structural transformations of NRs upon seed-mediated Au-Pd codeposition. SEM images of the overgrown NRs synthesized in the presence of 20 mM CTAB and 10 mM AA at a [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 1:9 and various Ag$^+$ concentrations of (A) 1.6 μM, (B) 20 μM, (C) 40 μM, and (D) 160 μM. (E) Pd:Au and Ag:Au atomic ratios (quantified by EDS) and (F) extinction spectra of the overgrown NRs obtained at various Ag$^+$ concentrations ([H$_2$PdCl$_4$]/[HAuCl$_4$] = 1:9). SEM images of the overgrown NRs synthesized in the presence of 20 mM CTAB and 10 mM AA at a [H$_2$PdCl$_4$]/[HAuCl$_4$] ratio of 2:1 and various Ag$^+$ concentrations of (G) 0.5 μM, (H) 4.0 μM, (I) 40 μM, and (D) 160 μM. (K) Pd:Au and Ag:Au atomic ratios (quantified by EDS) and (L) extinction spectra of the overgrown NRs obtained at various Ag$^+$ concentrations ([H$_2$PdCl$_4$]/[HAuCl$_4$] = 2:1). The error bars in panels E and K show the standard deviations obtained from 3 samples synthesized under identical experimental conditions. All SEM images share the scale bar in panel A.

The pathway switch was also observed when varying the Ag$^+$ concentration at a fixed Pd/Au precursor ratio of 2:1. Transition of TCCB NRs into cuboidal NRs occurred when the Ag$^+$ concentration increased from 0 to 10 μM as a consequence of Ag UPD-guided Au-Pd codeposition. When the Au-Pd-Ag trimetallic codeposition started to dominate the nanocrystal overgrowth at Ag$^+$ concentrations above 10 μM, the corners of the cuboidal NRs were significantly truncated accompanied by nanoscale surface roughening of the
NRs, eventually leading to the formation of dumbbell-shaped NRs with highly roughened surfaces at Ag⁺ concentration of 160 μM (Figure 4.13G-J). While the Pd/Au atomic ratios remained essentially unchanged as the Ag⁺ concentration varied, the Ag/Au atomic ratios displayed a sudden increase upon the pathway switch from Ag UPD-guided Au-Pd codeposition to Au-Ag-Pd trimetallic codeposition (Figure 4.13K). The EDS elemental mapping results showed that each Au-Pd-Ag trimetallic dumbbell-shaped NR was composed of a Au NR core coated with a Au-Pd-Ag ternary alloy shell. The surface roughness of the Au-Pd-Ag dumbbell-shaped NRs originated most likely from the interplay between codeposition and galvanic replacement during the nanocrystal overgrowth. At the initiate stage, Ag was codeposited with Au and Pd to form a ternary alloy layer on the surfaces of the Au NR seeds. The deposited Ag in the alloy shells was oxidized by the AuCl₂⁻ and PdCl₄²⁻ ions in the solution through galvanic replacement reactions, and was then re-deposited due to an abundant supply of reducing agent, AA. As a consequence, Au-Pd-Ag ternary alloy shells with roughened surfaces formed gradually due to the continuous generation of surface atomic vacancies and the nanoscale migration of surface atoms during the sustained galvanic replacement concurrent with the trimetallic codeposition. The nanoscale surface roughening caused by Ag⁺-guided electroless Pd deposition on Au nanocrystal cores has also been previously observed by us and other groups. The evolution of extinction spectral features of the overgrown Au-Pd-Ag trimetallic NRs reflecting the above-mentioned structural transformations and compositional changes was shown in Figure 4.13L.

The precise control over the particle geometries and compositions realized through the kinetically controlled seed-mediated coreduction enabled us to quantitatively correlate
the surface structures and compositions with the catalytic activities of colloidal Au-Pd alloy nanocatalysts. As schematically illustrated in Figure 4.14A, we used catalytic reduction of 4-nitrophenol (NP) into 4-aminophenol (AP) by ammonia borane (AB) at room temperature as a model reaction, which has been widely used as a benchmark hydrogenation reaction for assessing the catalytic activities of metallic nanocatalysts.\(^{76,77}\)

In a basic environment, NP exists in the form of 4-nitrophenolate, which exhibited a strong absorption peak at \(\sim 400\) nm whose intensity gradually decreased as the hydrogenation reaction proceeded. Therefore, the reaction kinetics could be monitored in real time using UV-visible absorption spectroscopy (Figure 4.14B). Without any metallic nanocatalysts, the hydrogenation reaction itself was extremely slow with no observable progression over extended time periods (Figure 4.14C). In the presence of Au-Pd bimetallic EHOH NRs, however, the hydrogenation reaction started immediately after mixing NP with excessive AB in aqueous K\(_2\)CO\(_3\) solution at pH = 10.4 (Figure 4.14C).

This catalytic reaction is essentially driven by active hydrogen species generated upon metal-catalyzed hydride formation, which can be harnessed to hydrogenate surface-adsorbed NP. Therefore, it involves the adsorption of both NP and AB onto the catalyst surfaces, followed by a bimolecular reaction between the surface adsorbed species. Both NP and AB compete for the same active sites on the catalyst surfaces and the reaction kinetics can be well described by the Langmuir-Hinshelwood model using the following second-order rate law:\(^{77-79}\)

\[
-d[NP] \frac{dt}{dt} = k \theta_{NP} \theta_{AB} \tag{3}
\]
where $k$ is the intrinsic rate constant, $\theta_{NP}$ and $\theta_{AB}$ are the surface coverage of NP and AB, respectively. Considering the surface adsorption/desorption equilibrium for both NP and AB, the rate law should take the following expression:

$$\frac{d[NP]}{dt} = \frac{k(S_0)^2 K_{AB}[AB]K_{NP}[NP]}{(1+K_{AB}[AB]+K_{NP}[NP])^2} \quad (4),$$

where $S_0$ is the total active surface area of the catalysts, $K_{AB}$ and $K_{NP}$ are the equilibrium constants for adsorption/desorption of AB and NP, respectively. When $K_{AB}[AB] \gg K_{NP}[NP]$, the rate law can be further simplified to the following expression:

$$\frac{d[NP]}{dt} = \frac{k(S_0)^2 K_{AB}[AB]K_{NP}[NP]}{(1+K_{AB}[AB])^2} \quad (5),$$

When AB was in great excess with respected to the NP, $[AB]$ remained constant at its initial value, $[AB]_0$, throughout the entire process. Therefore, this catalytic reaction follows pseudo-first-order kinetics as described by the following rate equations:

$$\frac{d[NP]}{dt} = k_{obs}[NP] \quad (6),$$

$$-\ln \frac{[NP]}{[NP]_0} = -\ln \frac{I}{I_0} = k_{obs}t \quad (7),$$

and

$$k_{obs} = \frac{k(S_0)^2 K_{AB}[AB]_0 K_{NP}}{(1+K_{AB}[AB]_0)^2} \quad (8),$$

Equations (6) and (7) are the derivative and integral forms of the rate law for the pseudo-first-order reaction and $k_{obs}$ is the apparent pseudo-first-order rate constant, which can be obtained through least squares fitting of the experimental results using Equation (7). $I_0$
and $I$ are the absorption at 400 nm before the reaction starts and at a certain reaction time, respectively.

Figure 4.14. Catalytic tunability of Au-Pd alloy NRs. (A) Schematic illustration of the catalytic hydrogenation of NP by AB on the surface of Au-Pd alloy NRs. (B) Two-dimensional color-coded intensity maps of time-resolved UV-Vis spectra collected after mixing AB with NP in the presence of Au-Pd alloy EHOH NRs (Pd:Au atomic ratio of 0.30). Plots of $-\ln(I/\text{I}_0)$ at $\lambda = 400$ nm as a function of reaction time, $t$, for (C) EHOH NRs, (D) TCCB NRs, and (E) TCB NRs with various Pd:Au atomic ratios as labeled in the figures. The solid lines show the least-squares fitting results to the reaction kinetic curves. The initial concentrations of NP and AB were 56.0 μM and 4.48 mM, respectively. The error bars in panels C, D, and E show the standard deviations obtained from 3 experimental runs. (F) Comparison of the apparent pseudo-first-order rate constants, $k_{\text{obs}}$, on EHOH, TCCB, and TCB NRs with various Pd:Au atomic ratios.

To quantitatively assess the facet- and composition-dependent catalytic activities of the Au-Pd alloy nanocatalysts, we performed kinetic measurements in the presence of excessive AB ($[\text{NP}]_0 = 56.0$ μM and $[\text{AB}]_0 = 4.48$ mM) at nominally the same catalyst concentration ($1.0 \times 10^8$ particles mL$^{-1}$). We estimated the particle concentrations of the colloidal Au-Pd bimetallic nanocatalysts based on the concentration of the Au NRs used for the seed-mediated Au-Pd coreduction. The concentration of Au NRs was estimated based on the concentration of the initial Au seeds (~ 2 nm in diameter) used for the NR
growth as described in greater detail in our previous publication.\textsuperscript{80} Using the seed-mediated coreduction method, we were able to precisely tailor the geometric parameters of the EHOH, TCCB, and TCB NR samples such that the aspect ratios, corner and edge sharpness, and more importantly the facets of each geometry can be kept almost identical for rigorous comparison despite the difference in compositional stoichiometries. In Figures 4.14C-4.14E, we showed the kinetics of the hydrogenation reactions catalyzed by EHOH, TCCB, and TCB NRs, respectively. A linear relationship was observed when plotting $-\ln(I/I_0)$ as a function of reaction time, $t$, verifying that the catalytic reactions obeyed pseudo-first-order kinetics.

In Figure 4.14F, we compared the $k_{obs}$ values on EHOH, TCCB, and TCB NRs with varying compositional stoichiometries. As the Pd/Au stoichiometric ratio increased, the $k_{obs}$ progressively increased for both TCCB and TCB NRs but decreased in the case of EHOH NRs, which was counterintuitive because Pd has been previously demonstrated to be a much better catalyst than Au for the hydrogenation of NP.\textsuperscript{81} We hypothesized that such counter-intuitive composition-dependence might be a characteristic of the \{hkl\} facets exposed on the EHOH NR surfaces. The detailed mechanisms underpinning the synergy between Au and Pd on the \{hkl\} facets still remain elusive at this point and needs further experimental and computational investigations. Regardless of the Pd/Au atomic ratios, the EHOH and TCCB NRs were catalytically more active than the TCB NRs, because high-index facets typically exhibit much higher catalytic activities than low-index facets due to the presence of highly abundant, catalytically active undercoordinated surface atoms, which has been well demonstrated on monometallic nanocatalysts. The TCCB NRs exhibited significantly higher activities than the EHOH
NRs with similar Pd-Au atomic ratios, indicating that \{311\} facets were catalytically more active than \{421\} facets mostly likely due to higher abundance of undercoordinated surface atoms on \{311\} facets than on \{421\} facets. Among all the nanostructures under current investigations, the monometallic Au TCB NRs synthesized following our previously protocol exhibited the lowest catalytic activity due to their monometallic nature and lower abundance of active sites on their low-index facets. It is worth mentioning that the undercoordinated atoms located at the edges and vertices where multiple facets merge only account for a negligibly small fraction of the surface atoms because each facet exposed on the NR surfaces is typically larger than 5 nm.\textsuperscript{8,82} Therefore, the relative reaction rates well-reflected the correlation between the characteristic surface atomic coordination and the intrinsic catalytic activities of various types of facets. When two types of facets coexisted on the NRs, \textit{e.g.} \{311\} and \{111\} facets on TCCB NRs and \{100\} and \{111\} facets on TCB NRs, the reaction kinetics reflected the overall catalytic activities with predominant contributions from the more active facets.

To gain further mechanistic insights into the catalytic tunability of Au-Pd alloy NRs, we varied the initial concentration of AB, [AB]\textsubscript{0}, while keeping the initial concentration of NP, [NP]\textsubscript{0}, fixed at 56.0 μM. At sufficiently high [AB]\textsubscript{0}/[NP]\textsubscript{0} ratios, the hydrogenation reactions catalyzed by the EHOH, TCCB, and TCB NRs could all be well-described as pseudo-first-order reactions, whereas they started to show significant deviation from pseudo-first-order kinetics when the [AB]\textsubscript{0}/[NP]\textsubscript{0} ratio became lower than 20:1 (Figures 4.15A-4.15C). However, the reactions still followed first-order kinetics at their initial stages, regardless of the [AB]\textsubscript{0}/[NP]\textsubscript{0} ratios. By fitting the linear part of the
kinetic trajectories at the initial stage of the reactions, we obtained the initial rate constants, \( k_{\text{initial}} \), which are related to several key kinetic and thermodynamic parameters as described by the following equation:

\[
k_{\text{initial}} = \frac{k(S_0)^2 K_{AB}[AB]_0 K_{NP}}{(1 + K_{AB}[AB]_0 + K_{NP}[NP]_0)^2} \tag{9}
\]

Since the catalytic reactions occurring at \([AB]_0/[NP]_0\) higher than 20:1 obeyed the pseudo-first-order kinetics throughout the entire process, \( k_{\text{initial}} \) became equivalent to \( k_{\text{obs}} \). In Figure 4.15D, we plotted \( k_{\text{initial}} \) as a function of \([AB]_0\) for the reactions catalyzed by EHOH NRs (Pd:Au atomic ratio of 0.083), TCCB NRs (Pd:Au atomic ratio of 0.53), and TCB NRs (Pd:Au atomic ratio of 0.57). In all cases, the \( k_{\text{initial}} \) first increased and then decreased as \([AB]_0\) progressively decreased, achieving its maximal values around \([AB]_0\) of 0.56 mM (\([AB]_0/[NP]_0 =10\)). The volcano-type relationship between \( k_{\text{initial}} \) and \([AB]_0\) could be well-interpreted in the context of Langmuir-Hinshelwood kinetics model. We further performed least-squares curve fitting on the experimental results using equation (9), which allowed us to get the equilibrium constants for the surface adsorption/desorption of AB and NP, \( K_{AB} \) and \( K_{NP} \), respectively, and \( k(S_0)^2 \), which is the intrinsic rate constant, \( k \), multiplied by the square of the total catalyst surface area, \( S_0 \). As shown in Figure 4.15E, \( K_{NP} \) was significantly smaller than \( K_{AB} \) for both the high-index faceting EHOH and TCCB, whereas the low-index faceting TCB NRs exhibited a higher affinity to NP than to AB. Moreover, \( K_{AB} \) exhibited much higher values on EHOH and TCCB NRs than on TCB NRs while \( K_{NP} \) displayed the opposite trend, strongly indicating that the undercoordinated surface atoms on high-index facets may serve as the high affinity sites for AB adsorption while the coordinatively saturated surface atoms on the
low-index facets favored NP adsorption. The $k(S_0)^2$ values followed the trend of TCCB > EHOH > TCB NRs, which was in line with the relative abundance of undercoordinated surface atoms ($\{311\} > \{421\} > \{100\}/\{111\}$). Considering that the EHOH, TCCB, and TCB NRs under current investigations had comparable $S_0$ values, the dependence of $k$ on the surface atomic coordination and the Pd/Au stoichiometries was clearly demonstrated by the kinetic results reported here.

Figure 4.15. Langmuir-Hinshelwood kinetics of catalytic hydrogenation of NP by AB on Au-Pd alloy NRs. Plots of $-\ln(I/I_0)$ at $\lambda = 400$ nm as a function of reaction time, $t$, for (A) EHOH NRs (Pd:Au atomic ratio of 0.083), (B) TCCB NRs (Pd:Au atomic ratio of 0.53), and (C) TCB NRs (Pd:Au atomic ratio of 0.57) at various $[AB]/[NP]$ ratios as labeled in the figure. The solid lines show the least-squares fitting results to the linear part of the curves at the early stage of the reactions. The initial concentration of NP was fixed at 56.0 μM. The error bars in panels A, B, and C show the standard deviations obtained from 3 experimental runs. (D) Plots of initial rate constants, $k_{\text{init}}$, on EHOH, TCCB, and TCB NRs as a function of $[AB]_0$. The results of the least-squares fitting using the Langmuir Hinshelwood kinetic equation are shown as solid curves. (E) Comparison of $K_{NP}$, $K_{AB}$, and $k(S_0)^2$ on EHOH, TCCB, and TCB NRs.
To more comprehensively understand the factors determining $k$, we investigated the pH-dependent catalytic tunability of the Au-Pd bimetallic NRs toward the hydrogenation reactions. As previously reported, the hydrogenation of NP involves the transfer of surface hydrogen species supplied by AB and interfacial transfer of electrons mediated by the metallic nanocatalysts. The modification of the plasmonic features of the metallic nanocatalysts introduced by these interfacial charge transfers has been precisely monitored by single-particle dark-field spectroscopy. $k$ is essentially related to the rates of these transfer processes, which are determined not only by the surface structures and compositions of the Au-Pd alloy nanocatalysts, but also by the pH of the reaction medium. It has been previously reported that hydrogenation reactions proceeded more rapidly at higher pH values. As shown in Figure 4.16, the catalytic reactions followed the pseudo-first-order kinetics throughout the entire pH range from 10.4 to 13.9 and the catalytic activities of all the multifaceted NRs significantly increased with the pH values regardless of their geometries and compositions. At each pH, however, the $k_{obs}$ values always followed the same trend of TCCB > EHOH > TCB NRs. Our results clearly show that the catalytic tunability observed on the multifaceted NRs essentially stems from the interplay between the competitive surface adsorption of the reactants and the interfacial charge transfers involved in the reactions, both of which are dependent upon the surface atomic coordination and the compositional stoichiometries of the Au-Pd alloy nanocatalysts.
Figure 4.16. Plots of $-\ln(I/I_0)$ at $\lambda = 400$ nm as a function of reaction time, $t$, for the hydrogenation of NP catalyzed by (A) Au-Pd EHOH NRs (Pd:Au = 0.083), (B) Au-Pd TCCB NRs (Pd:Au = 0.53), and (C) Au-Pd TCB NRs (Pd:Au = 0.57) at various pH values as labeled in the figures. The solid lines show the least-squares fitting results to the reaction kinetic curves. The initial concentrations of NP and AB were 56.0 μM and 4.48 mM, respectively. The error bars in panel A, B, and C show the standard deviations obtained from 3 experimental runs. (D) Comparison of rate constants, $k_{obs}$, of the catalytic hydrogenation reactions on Au-Pd EHOH, TCCB, and TCB NRs at various pH values.

4.4 Conclusions

This work well-exemplifies the mechanistic complexity associated with the structural evolution of anisotropic alloy nanoparticles during seed-mediated coreduction. Several key synthetic parameters, such as the Au/Pd precursor ratio, the concentration of reducing agent, the concentration of capping surfactants, and the foreign metal ion additives, all have profound influence on the surface codeposition of metals and the overgrowth of nanocrystals, thereby providing fine-adjustable knobs for the precise tuning of both the
surface atomic coordination and the compositional stoichiometries of the resulting bimetallic nanoparticles. By kinetically maneuvering the electroless deposition of Au-Pd alloy shells on Au NR seeds, we have been able to selectively synthesize a family of multifaceted Au-Pd bimetallic NRs with exotic anisotropic geometries, including EHOH NRs each of which is enclosed by 56 \{hkl\} high-index facets, TCCB NRs whose surfaces are dominated by 24 \{hkk\} high-index side facets with 8 truncated tips terminated by \{111\} facets, and low-index faceting TCB NRs enclosed by thermodynamically stable \{100\} and \{111\} facets. The seed-mediated coreduction under diffusion-controlled conditions also enables the fine-tuning of the Au-Pd atomic ratios in the alloy shells deposited on the Au NR seeds over a broad stoichiometric range without forming heterostructures comprising segregated monometallic domains while still well-preserving the surface structural characteristics of each multifaceted geometry.

The great success in precise facet and composition control further allows us to investigate the detailed correlation between the surface structures, the compositional stoichiometries, and the catalytic activities of colloidal Au-Pd alloy nanocatalysts. Using the catalytic hydrogenation of NP by AB as a model reaction obeying the Langmuir-Hinshelwood kinetics, we have shown that the relative binding affinities of reactants and the interfacial charge transfer rates, both of which serve as the key factors that determine the overall kinetics of the catalytic reactions, exhibit strong dependence on the surface atomic coordination and the compositional stoichiometries of the Au-Pd alloy nanoparticles. While this work primarily focuses on the structure-composition-property relationships in the context of heterogeneous catalysis, the insights gained from this work provide general design principles that guide the architectural optimization of
multimetallic nanoparticles for widespread applications far beyond heterogeneous catalysis. The incorporation of catalytically active Pd into plasmonically tunable Au nanostructures enables detailed studies of catalytic molecular transformations using surface-enhanced Raman scattering as a time-resolved spectroscopic tool with molecular finger printing capability.\textsuperscript{41,85,86} In addition, Au-Pd bimetallic nanoparticles may provide a materials system that allows us to efficiently harness the plasmonic hot electrons to drive or enhance Pd-catalyzed reactions along unconventional pathways distinct from those involved in conventional catalytic thermal reactions or semiconductor-based photocatalysis.\textsuperscript{87,88} Furthermore, Au-Pd bimetallic nanoparticles may serve as standalone optical sensors for monitoring hydrogen uptake, storage, and release due to the unique capabilities of Pd to reversibly accommodate large quantities of molecular hydrogen and the Pd-enhanced refractometric sensitivity of Au plasmons.\textsuperscript{81,89,90} The optimization of all the above-mentioned applications is intimately tied to our capabilities to precisely tailor both the geometries and compositions of the bimetallic nanoparticles, which can be achieved, as demonstrated in this work, through deliberately designed colloidal syntheses under seed-mediated and kinetically controlled conditions.

4.5 References


APPENDIX A

Publications related to the research work described in this dissertation

