Effects of epigenetic modification and ionic liquid on DNA charge transport : applications in biology and material

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**EFFECTS OF EPIGENETIC MODIFICATION
AND IONIC LIQUID ON DNA CHARGE
TRANSPORT: APPLICATIONS IN BIOLOGY
AND MATERIAL**

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**SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES
DIVISION OF CHEMISTRY AND
BIOLOGICAL CHEMISTRY**

2015

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2015

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Abstract

The well-aligned base pairs along the DNA central axis serve as a convenient pathway for charge migration. The phenomena termed as DNA charge transport (CT) has stirred up great interests about the underlining mechanism, biological roles and potential applications in development of DNA based electronic devices. Mechanistic investigations have revealed that various factors might affect the process of DNA CT, including inherent factors, such as the base pairs composing the "bridge" for charge migration, secondary structures and sequences of duplex DNA; and external factors, such as protein binding, external magnetic fields and solvents. The biological significance of DNA CT has been found to be associated with several redox processes in cell, such as funnelling oxidative stress, and signalling between proteins. On the other hand, applications of DNA CT in molecular electronics have been extensively studied to develop multi-functional DNA electronic devices. Despite the great achievements of previous studies with regards to DNA CT, further research is demanded to advance DNA CT to a higher step in its application biology and material. This thesis explored via two approaches: modulating DNA CT by epigenetic modification on base pair stacks and facilitating DNA CT by using hydrated ionic liquid as solvents. The attempts here provide critical progress in understanding biological functions of DNA CT and developing protocols to make DNA a better conductive biomaterial.

Chapter 1 reviewed the current mechanisms for DNA mediated CT and the implications in biology and DNA based electronic devices. The effects of epigenetic modifications and ionic liquid on nucleic acids including structures, stability and biofunctions were also reviewed here.

Chapter 2 reported facile syntheses of DNA oligonucleotides containing hmC and fC. With microwave assisted heating, commercial available nucleoside dT and mdC were readily oxidized to hmdU and fdC which were the key intermediates for hmC and fC phosphoramidites syntheses. The protocols established were applied for preparation of DNA sequences for study of epigenetic effects on DNA CT.

Chapter 3 investigated a critical epigenetic process in active DNA demethylation, i.e. spontaneous deamination of epigenetic cytosines. Alkali treatment induced substantial spontaneous deamination of dC, mdC and hmdC at 37 °C, however, converted fdC into two unknown moieties other than the corresponding deamination product fdU. Kinetics study of deamination reaction revealed spontaneous deamination rate of dC, mdC and hmdC was associated with the C5 functionality of cytosine pyrimidine ring.

Effects of epigenetic modification on DNA CT were examined in chapter 4. Transient hole density during DNA charge transport was found to be modulated by various epigenetic cytosine modifications; oxidative G damage by DNA charge transport over long range was facilitated by hyper-methylation and inhibited by hmC and fC.

Chapter 5 reported adopting hydrated ionic liquid as solvent to enhance charge transport through DNA. Striking increment of charge transport efficiency was observed with the presence of ionic liquid. The effects of water, ionic liquid cation and anion species, as well as binding of ionic liquid to DNA, were investigated in detail.

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CHAPTER 1

DNA-mediated Charge Transport : from Biology to Materials

1.1 Introduction

The potential of DNA being a bio conductive material was predicted by Eley and Spivey as early as 1960s based on the structural similarities between DNA and graphite.^[1] However, the debate about DNA as a medium for charge transport lasted for decades until Barton's group reported the first experimental evidence of luminescence quenching of intercalated Ru(phen)₂dppz²⁺ by long range charge transport (CT) via DNA helix.^[2, 3]

Currently it is widely accepted that charge is able to migrate over a long distance via the DNA helix structure by taking advantage of the stacked π orbitals of base pairs. An overall scenario of the process of DNA CT is depicted by a superexchange mechanism in the short range and a multi-step hopping mechanism in the long range.^[4, 5] Details about each step involved in photo-induced CT through DNA, such as *roles of photo-oxidants*, process of *hole injection and trapping*, and *how charge localizes on the base or delocalizes in a domain* and et al, have been investigated by the remarkable works of pioneer physicists and chemists.^[6-18]

DNA CT raised an idea of "reaction at a distance" through DNA structure which not only broadened the horizon of our understanding about redox processes that are associated with DNA in biological systems, but also opened a novel vision of using DNA as a conductive material to assemble various functions to DNA based electro devices. Therefore, extensive work has been conducted to explore the promising and challenging field of DNA CT from biology to material.

1.2 Long Range Charge Transport in DNA

1.2.1 DNA CT via the Stacked Base Pairs

Canonical B-form DNA is a right-handed helix formed by two anti-parallel polynucleotides (Figure 1-1). Hydrophobic base pairs stack with each other as the core of

B-form DNA and hydrophilic phosphate backbones interact with water and counterions to provide stability to the structure in aqueous solution. Generally, the phosphate backbone of DNA does not show significant impact on DNA CT.^[19] The absence of a phosphate in the DNA duplex does not show measurable effect on CT efficiency.^[20, 21] The conductivity of DNA is highly sensitive to the extent of π - π stacking of DNA base pairs. Efficient π orbital coupling of base pairs is necessary for CT in DNA. Disturbance of DNA base stacking greatly influences the process of CT. For example, inhibited CT was observed in the Z-form hairpin and duplex DNA with poor base stacking in the structure (structure shown in Figure 1-1).^[22, 23] Interruption of base stacking with a single base mismatch dramatically attenuates CT in DNA, which is useful to design electrochemical devices for the detection of DNA mismatches.^[24-27] Others perturbances, such as a bulge on the intervening bridge, also significantly inhibits CT in DNA.^[28, 29] In contrast, A-form DNA/RNA hybrid (Figure 1-1) which was less disturbed in base stacking allows efficient CT.^[23, 30, 31] In addition, DNA G-quadruplex, which preserve better base stacking among G-quartets, shows promotion of charge migration to longer range in DNA.^[32, 33]

Figure 1-1 A-form (left), B-form (middle) and Z-form (right) DNA structure. The graph is reprinted from Wikipedia website <https://en.wikipedia.org/wiki/File:Dnaconformations.png>.

Component of "bridge" for charge migration by nucleobases is of critical importance for CT through DNA. According to the different redox reaction involved, DNA CT can be classified as oxidative hole transport (HT) and excess electron transport (ET).^[34] A charge (cation radical/electron) injected to the DNA duplex causes changes in the redox status of the composing nucleobases. Among the four canonical DNA nucleobases, purines have higher oxidation potential than pyrimidines (Figure 1-2). Therefore, purines are prone to be oxidized by the migrant hole (cation radical) while pyrimidines are more easily reduced by the excess electron.

Figure 1-2 Structures and oxidation potentials (E_{ox}) of four canonical DNA nucleosides. E_{ox} is versus NHE.

1.2.2 Roles of Bases in DNA CT

1.2.2.1 Guanine

$G^{\cdot+}$ resulting from oxidation by DNA CT might further react with water and O_2 within its lifetime (millisecond) leading to irreversible DNA lesion at the guanine site. Oxidation potential is lowered by consecutive G, such as GG doublet and GGG triplet, making them natural hole traps.^[35-37] Substantial oxidative damage at 5' G of a GG doublet was observed as a feature of reaction by charge transport through DNA over a distance, which was attributed to higher localization on the 5' G.^[12, 36, 38, 39] It is suggested that guanine undergoes proton-coupled electron transfer (PCET) within a G-C base pair upon one-

electron oxidation by CT (Figure 1-3).^[40-44] The hopping mechanism of CT suggests hole preferentially localizes on the G sites and thermally induced hopping rate shows weak distance dependence between G sites.^[5] In contrast to the substantial oxidative damage by CT in duplex, guanines within G-quadruplex were protected from damage by CT, while GG doublet after a G-quadruplex was more heavily oxidized.^[32]

Figure 1-3 proton-coupled electron transfer (PCET) within G-C base pairs. Graph is reprinted from ref^[39].

1.2.2.2 Adenine

Investigation of the process of "G hopping" revealed the role of adenine in charge tunneling (superexchange) in the short distance (less than three base pairs) and "A-hopping" with shallow distance dependence in the long range (more than three base pairs) as depicted in Figure 1-4.^[45] Adenine tracts (consecutive A-T bases pairs) have shown promising conductive properties for hole migration in the long range.^[5, 17, 45-48]

Figure 1-4 Plot of the product ratios of P_{GGG}/P_G versus number (n) of intervening A between two G sites (isolated G and GGG triplet). P_{GGG}/P_G is proportional to charge transport rate. Dramatic

attenuation of P_{GGG}/P_G with distance indicated the superexchange charge transfer between $^4G_{22}$ and GGG triplet when $n \leq 3$; weak distance dependence of P_{GGG}/P_G in the long range ($n \geq 3$) suggested a hopping mechanism. Graph is reprinted from ref^[45].

1.2.2.3 Thymine

CT resulted in oxidative damage to consecutive TT and TTT steps in DNA sequences when absent of guanine, which is liable to piperidine treatment, however, leaving adenine whose oxidation potential is lower than thymine unreacted.^[49, 50] Replacing 5' T of the TT step to U (i.e. 5'-UT-3') only caused modest effect on the amount of strand cleavage. However, strand cleavage was nearly completely inhibited when 3' T was replaced with U (i.e. 5'-TU-3'). This result suggested the requirement of two adjacent thymines for efficient trapping of the migrating cation radical and the importance of methyl function of 3' thymine. Identification of the products by charge transport to TT steps showed that 5-(formyl)-2'-deoxyuridine (fdU) is the major product with 63%. Other products were 5-(hydroxymethyl)-2'-deoxyuridine (hmdU, yield 13%) and *cis*-, *trans*-diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine (*cis*-, *trans*- ThdGly, yield 20% and 4%).^[49] Two trapping mechanisms, one is initialized from 5' T, the other one is initialized from the 3' T, were hence proposed as shown in Figure 1-6. The reactivity of thymine is much lower than guanine in well-matched DNA duplex, the presence of G or GG doublet in longer distance caused inhibition of trapping at the TT step in the shorter range.^[51] However, the migrating cation radical along DNA is substantially trapped by TT mispair even in the presence of GG doublet which possessed lower oxidation potential.^[52] This result indicated that TT mispair had higher activity than Watson-Crick base pairs and could act as a barrier for hole migration to the long distance. It was hypothesized that the higher reactivity at TT mispair might be associated with its wobble structure.^[52] Similar in structure with thymine, 5-methylcytosine showed a similar property of trapping cation radical by mC mC mispairs.^[53]

Figure 1-5 Mechanisms proposed for trapping of cation radical by TT step. (A) trapping initialized at 5' T of TT step; (B) trapping initialized at 3' T of TT step. Graph was derived from ref^[49].

1.2.2.4 Cytosine

Involvement of cytosine in CT was explored by Barton's group using *N*4-cyclopropylcytidine (^{CP}C) as a fast kinetic trap.^[16] Fast ring opening reaction of *N*4-cyclopropyl group of ^{CP}C made it a sensitive probe to detect transient hole occupancy on ^{CP}C. Oxidation potential of ^{CP}C does not significantly differ from cytosine, thus transient hole occupancy on ^{CP}C is not altered by *N*4-cyclopropyl moiety. Photolysis assay of DNA sequences with ^{CP}C observed substantial ^{CP}C decomposition by CT from two kinds of potent photo-oxidant, [Rh(phi)₂(bpy)]³⁺ or an anthraquinone derivative. The extent of ^{CP}C decomposition was modulated by the facing guanine in the opposite site of complementary strand and flanking base pair components. Hence, the results suggested DNA CT does not simply involve in hopping among the low-energy guanine site, but also requires orbital mixing of the bases in a domain.

1.2.3 Principle Mechanisms for DNA CT

CT through DNA can be roughly described as a process of charge (hole or electron) injected to DNA structure from donor (Do), migrate along DNA base stacking until it is trapped by the acceptor (Ac). In the studies of DNA CT, charge donors are often some photochemically or electrochemically active compounds including organic and inorganic intercalators; while acceptors could be organic and inorganic compounds as well as natural or modified DNA nucleobases.^[54] Oxidative hole transport (HT) and excess electron transport (ET) are different processes not only in the direction of charge migration but also in the orbitals of donor's and acceptor's involved. During oxidative HT, charge migrates from HOMO of donor to the acceptor; while in ET, an electron is injected to DNA, which is LUMO-controlled (Figure 1-6).^[54]

Figure 1-6 Orbital control of oxidative hole transport (HOMO control) and reductive electron transport (LUMO control). (Do = donor, Ac = acceptor, ET = electron transfer) Graph was reprinted from ref^[54].

Mechanistic considerations of DNA CT revealed different features of CT following a superexchange mechanism in the short range and multi-step hopping in the long range. Other considerations, such as phonon-assisted polaron-like hopping and conformational

gated hopping mechanism, were proposed as mechanisms to explain the results of experimental studies. It is clearer that DNA CT is an inherent property of DNA by the coupling of base pairs, however a complicated phenomenon at the same time, since DNA CT is related to the surrounding environment which is affected by various factors.

1.2.3.1 Superexchange and Localized Hopping

Superexchange and localized hopping mechanisms describe different situations of state of donor and acceptor compared to the energy levels of the intervening bases. In the case of superexchange mechanism, HOMO/LUMO of donor/ acceptor is considered to be lower than HOMOs (LUMOs) of bridge base pairs (Figure 1-8). Upon hole injection, the hole does not virtually localize on HOMOs of the bases in the bridge. As a result, the HT to the acceptor coherently in one jump and CT rate decays exponentially with distance. Charge transfer rate (k_{CT}) in DNA via the superexchange mechanism is described in a simplified form of Marcus equation for nonadiabatic electron transfer:

$$k_{CT} = k_0 \exp(-\beta R) \quad \text{Eq 1.1}$$

R is the donor-acceptor center-to-center distance, k_0 is the preexponential factor and β is a decay parameter with distance which is dependent upon the nature of the bridge and its coupling with donor and acceptor.^[55-57] Theoretical calculation predicted that β value for charge transport in superexchange mechanism should be between 1.2 to 1.6 Å⁻¹.^[58] Experimental determination of β value showed quite contradictory results. Barton's group reported $\beta=0.2$ Å⁻¹ with DNA assembly intercalated by Ru (II) complexes as donor and Rh (III) as acceptor.^[3, 59] Lewis's group reported a larger $\beta=0.64\pm0.1$ Å⁻¹ for charge transport through DNA hairpin from photo-excited stilbene to G-C pair.^[60] Even larger $\beta=1.42$ Å⁻¹ and 1.0 Å⁻¹ were determined by monitoring charge transport from intercalated acridine excitation state to G and from photo-induced radical cation to G.^[61, 62] $\beta\approx 1.0$ Å⁻¹ was predicted for distance dependence of charge transport between donors and acceptors

with metallointercalators as photo-oxidants.^[63] The deviation of β values reported could account for that various charge donors differ in the energy gap ΔE with base pair bridge.^{[4,}

57]

Figure 1-7 Schematic representations of several possible mechanisms for charge transport through DNA. Superexchange: charge tunnels from the donor to the acceptor through the bridge in a nonadiabatic process. Hopping: charge occupies the bridge in traveling from donor to acceptor by hopping between discrete molecular orbitals on the bridge Domain Hopping: charge occupies the bridge by delocalizing over several bases, or a domain. This domain hops along the bridge to travel from donor to acceptor. Graph is reprinted from ref^[64].

Localized hopping mechanism has been proposed to explain experimental results of the shallow distance dependence of CT particularly in the long range.^[4, 5, 13-15, 31, 47, 65-69] Different from superexchange mechanism, localized hopping mechanism predicts the injected charge transiently localizes at the bases on the bridge, preferentially at guanine as the lowest potential base, and hops reversibly from one site to another until it is trapped by the acceptor (Figure 1-7).^[5, 70] Each hopping step is considered as a short single step superexchange process. Hopping to the flanking G is faster than hopping to the G site

separated by other bases (GCG, GAG and GTG). Interstrand hopping is also possible in the case of hopping through GCG/CGC duplex sequence ($G^+CG/CGC \rightarrow GCG/CG^+C \rightarrow GCG^+/CGC$), though not favored as intrastrand hopping.^[4] Distance dependence of charge transfer rate (k_{CT}) in DNA via hopping mechanism is presented by the number of hopping steps N :

$$k_{CT} = P^* N^{-\eta} \quad \text{Eq 1.2}$$

η value lies between 1~2 and is determined by charge hopping random walk.^[4] The fast CT in A tracts and experimental observation of hole occupancy in adenine tracts raised questions for the localized hopping mechanism which stressed on G hopping. Therefore, thermal factor is added to the mechanism, i.e. thermal excitation of radical at guanine site to the adenine tract. Further modification of the mechanism suggested charge partly delocalizing to a domain can be consistent with more general experimental phenomena, including the explanation of the mismatch discrimination and the involvement of pyrimidines in hole occupancy on the bridge.^[16, 71]

1.2.3.2 Phonon-assisted Polaron-like Hopping and Conformational Gated Hopping Mechanism

Polaron formation during DNA CT is a natural result of the fact that the local structure of DNA will rapidly adjust itself to relieve the charge deficiency caused by the injected cation radical.^[17] To compromise for the charge deficiency, it is expected that intrabase distance of the adjacent bases will decrease to increase electron donation, rotation of the base pair along z axis enhances π - π stacking and H-bonding of base pairs changes in response to change of pK_a of the charge carrying bases.^[17] The size of polaron is "self-trapped" by the equilibrium of energy required for structure distortion and the decreasing stabilization of the polaron. Hopping of polaron is realized by the nearby bases via phonon-assisted (thermally activated) joining or exiting the polaron. Rapid hopping is

achieved for sequences with similar ionization potentials (I_p).^[17] It is reminded that formation of small polaron lowers site energies of the bases thus increasing the activation energy required for each hopping step. As a result, CT is slowed by small polaron formation.^[19] Other manners of polaron movement, such as drift, are not reasonable for rapid hole transport in adenine tracts unless conformational gating is taken into consideration.^[19] Nevertheless, formation of large polaron lowers the charge barrier for isoenergetic sites, thus facilitating CT in mixed sequences (Figure 1-8). The polaron hopping mechanism provides good explanations for the important features of DNA CT, such as involvement of all four kinds DNA nucleobases in hole delocalization and alleviation of the barrier of CT by the static disorder of the site energies in DNA.^[19] Particularly, the shallow distance dependence of rapid CT in adenine tracts and inhibited back electron transport (BET) from adenine to guanine are consistent with polaron formation to form homogeneous medium and self-trapping feature of polaron.^[19, 72]

Both localized hopping and phonon-assisted polaron-like hopping mechanism consider the equilibrium or averaged static structure of DNA, while charge transport in the latter mechanism is relatively unaffected upon introducing static disorder of the site energies.^[72] However, whether polaron formation can occur within the timescale of charge transport and its impact on DNA charge transport are still under debate.^[19] Barton's group proposed an alternative understanding about the charge transport gated by conformation dynamics of DNA bases, termed as conformational gated hopping.^[13] Dynamic motion of DNA bases transiently forms domains which adopt so-called ET (electron transfer) active conformations, and breaking up ET active conformations renders ET non-active domains (Figure 1-9).^[73] Charge transport is facilitated in the ET active conformations, while inhibited in ET non-active ones. The hopping of these domains are thermally activated and gated by the transient dynamic conformations of DNA bases.

Figure 1-8 Formation of a polaron. After hole injection to a single base, energy differences of the local bases are averaged by reorientation of environmental factors. Delocalization of the hole by photon-assisted (thermally activated) distortions of the structure lowers the hole energy. Graph is reprinted from ref^[19].

This mechanism is supported by a series of experimental results. Fluorescence decay of 2-aminopurine (Ap) on the picosecond timescale, an analogue of adenine, showed a vast range of charge injection rate to the bases which is adjacent to Ap, from 10 ps for ApG to 512 ps for ApI (I is inosine). This suggested that charge transfer is gated by dynamic motion of the flanking base species.^[74] In addition, fluorescence decay of Ap conjugated DNA shows strong temperature dependence.^[73] Two components can be resolved from fluorescence decay of Ap, one is a fast ET component and the other one is a slow decay component which correlates to non-ET pathways. The amplitudes of these two components are strongly dependent to environment temperature, indicating the population of ET active and ET-inactive conformations is thermally activated.^[73] The formation of domain during charge transport is directly evidenced by by photolysis assay of ^{CP}C and ^{CP}G in the same DNA sequence, which shows comparable reaction efficiency to ^{CP}G at low oxidation potential ^{CP}C site.^[16] The conformational gated hopping mechanism is in good consistent with the periodic oscillation of charge transport distance dependence

along adenine tracts, which has difficulty to be explained by polaron hopping mechanism.^[13]

Figure 1-9 Schematic model of charge transport via conformational gated hopping. Graph is reprinted from ref^[73].

1.2.4 Application of DNA CT

The fascinating electronic feature of DNA origin from the unique one dimensional aromatic heterocycle stacking along the helix axis endorses DNA charge transport extensive applications in numerous research fields, such as molecular biology and molecular electronics, thus make DNA charge transport not limited to merely a theory. In molecular biology, redox reactions occur "at a distance" via DNA charge transport and cause impacts on a series of DNA biology processes. In addition, "communication" between proteins is enabled by DNA mediated sensing and signalling. Furthermore, people are dedicated in development of various DNA based electronic devices, extending the application of DNA as a real material.

1.2.4.1 Oxidative Damage on DNA via CT

Oxidative DNA damage plays a significant role in mutagenesis, carcinogenesis and aging. Endogenous stimuli, such as reactive oxidative species (ROS) and UV or γ -ray irradiation, cause various oxidative modifications on DNA bases and subsequent biological results, such as mutation.^[75]

It is proposed that oxidative stresses generated are not restricted locally but funnelled to other regions via charge transport along DNA. Guanine is most vulnerable to oxidation among the canonical nucleobases due to the lowest oxidation potential.^[76] One electron oxidation of guanine generates G^+ which might undergo subsequent reaction with H_2O and O_2 to generate 8-oxo-7,8-dihydroguanine. Oxidation of guanine was found to be mediated by DNA charge transport at a distance as long as 200 Å.^[3] Of course, DNA is much longer than this length in genome. However, funnelling the oxidative via DNA charge transport is still possible, considering the extensive oxidative stress in the environment and shallow distance dependence of DNA charge transport by hopping mechanism. It is evidenced that photo-irradiation of $[Rh(\phi)_2bpy]^{3+}$ conjugated to isolated HeLa nuclei results in oxidative damage to DNA bases at a distance from the binding sites of the metallocomplex.^[77] Moreover, protein binding site where binding of $[Rh(\phi)_2bpy]^{3+}$ is blocked still exhibits oxidative damage of nucleobases, indicating charge transport occurs.^[77] Further examination of the oxidative damage pattern on guanine revealed a 5' preference of DNA lesion in GG doublets and GGG triplets which was a feature of guanine damage by charge transport.^[77]

Mitochondrial DNA could be another target for oxidative stresses in cell.^[78] Photolysis of rhodium complexes which intercalate to extracted mitochondrial DNA resulted in oxidative damage to the mitochondrial DNA at a distance from the binding sites.^[79, 80] The results interestingly revealed a potential protection mechanism in mitochondria to exclude damaged DNA by replication.^[80] Conserved sequence block II is a regulatory element in mitochondria which is responsible for DNA replication. Actually, a seven guanine repeats in conserved sequence block II makes it sink for funnelling oxidative stress via DNA charge transport. The resultant extensive strand lesions will cause dysfunction of conserved sequence block II. Consequently, genome with extensive oxidative damage in conserved sequence block II will not be favoured for replication.

Hence, the specific regulatory region with high prone to oxidation damage via DNA CT might be a passive manner to prevent the damaged DNA from being replicated (Figure 1-10).^[80]

Figure 1-10 Potential protection mechanism in mitochondria to exclude damaged DNA by replication. Each mitochondrion has several copies of its genome. Conserved sequence block II which regulates mitochondrial genome replication has the largest guanine repeat in mitochondria. Oxidative stresses are prone to be funneled to conserved sequence block II via DNA charge transport and cause dysfunction of the regulatory element. Genome with extensive oxidative damage in conserved sequence block II will not be favoured for replication.

1.2.4.2 DNA CT upon Protein Binding

DNA charge transport upon bound to proteins is of great interest and intensive efforts have been made in the field. Investigation of charge transport in DNA bound to nucleosome core particles revealed that DNA charge transport is not attenuated by being packaged in chromatin.^[81] Hence, DNA within the chromosome is not protected oxidative damage mediated by DNA charge transport.^[78] The effect of protein binding on DNA charge transport largely depends on its influence on DNA base stacking. For instance, a helix-turn helix protein which has little changes in DNA base stacking does not alter DNA charge transport dramatically.^[82] In contrast, proteins with great impact on DNA base stacking, such as uracil DNA glycosylase which sequentially flips DNA bases and

TATA-binding protein which bends DNA structure, inhibit DNA charge transport.^[82, 83] Based on the features of DNA charge transport with various binding proteins, electrochemical probes were rationally designed to detect interaction of proteins with DNA.^[83]

Strikingly, some protein functions might be associated with DNA charge transport. Photolyase repairs thymine thymine dimers in DNA via photo-activated electron transfer from its flavin cofactor.^[84] Investigation of electrochemistry of the flavin cofactor of *Escherichia coli* photolyase revealed that repair of thymine thymine dimers in DNA by photolyase is consistent with electron transfer from electrode surface to the flavin cofactor via DNA base stacking.^[85] This result suggested photolyase might adopt charge transport from flavin cofactor via DNA duplex to the TT dimers to fulfill its repair function. Moreover, p53, a redox-modulated transcription factor, was found to dissociate due to oxidation by photo-activated anthraquinone at a distance through intact DNA duplex.^[86] Oxidation of p53 is sensitive to the intervening mismatch in DNA duplex, suggesting a role of DNA charge transport in regulating p53 oxidation.^[86] It has been found that [4Fe-4S] clusters in some DNA repair proteins, such as MutY and Endonuclease III (EndoIII), are able to be oxidized by guanine radical through DNA CT.^[87-89] Changes in DNA binding affinity was also observed in response to changing of redox status of the [4Fe-4S] clusters by DNA CT. The DNA binding affinity of proteins that is regulated by redox reactions via DNA CT suggests a possible mechanism of signalling between proteins and responding accordingly (Figure 1-11). In oxidative condition, binding of DNA repair proteins that contains [4Fe-4S] clusters is strengthened by oxidation of [4Fe-4S] via charge transport by guanine radical at a distance. A second repair protein binds to DNA are supposed to undergo the same oxidation process and loses one electron which will transfer via DNA duplex and reduce the first repair protein. Consequently the first protein dissociates from the DNA. However, if the electron transfer

from the second protein to the first one is blocked by DNA lesion, both proteins will remain in the oxidative state and bind to DNA. Therefore, signalling between DNA repair proteins might be a mechanism for searching mismatch in DNA in a cooperative way to enable high efficiency of the proteins.^[78]

Figure 1-11 A proposed mechanism of DNA repair protein rapid search for the lesion site via charge transport. In oxidative condition, binding of DNA repair proteins that contains [4Fe-4S] clusters is strengthened by oxidation of [4Fe-4S] via charge transport by guanine radical at a distance. A second repair protein binds to DNA are supposed to undergo the same oxidation process and loses one electron which will transfer via DNA duplex and reduce the first repair protein. Consequently the first protein dissociates from the DNA. However, if the electron transfer from the second protein to the first one is blocked by DNA lesion, both proteins will remain in the oxidative state and bind to DNA. Graph is reprinted from ref^[78].

1.2.4.3 DNA CT Based Electro Devices

An important application of DNA mediated charge transport is development of DNA based functional electro devices. DNA has extensive use in electrochemistry, taking advantage of its special characteristics, such as self-assembly, self-recognition.^[90-95] Anchoring DNA molecules to the electrode surface or single molecule platforms marked a great advance for DNA CT in application to developing DNA based electro devices (Figure 1-12). In contrast to most of DNA CT studies in the solution which investigated the excited-state charge transport by spectroscopic or biological electrophoresis experiments, solid surface or single molecule platforms provided the possibility of studying ground-state charge transport.^[96]

Figure 1-12 Modifying DNA to solid platforms to develop DNA based electronic devices. (Top) Covalent attachment of DNA to electrode surface can be used to study electron transfer from the donor (electrode) to acceptor (redox-active compound) via DNA duplex. (Bottom) Covalent attachment of DNA to carbon nanotube forms single molecule DNA CT platform. Graph is reprinted from ref^[96].

For solid surface platform, DNA duplexes are handled with a linker to the electrode surface. The tight packing of the monolayer DNA film is enabled by high Mg^{2+} concentration which reduces the repulsion between helix.^[97] The orientation of DNA duplex relative to the electrode surface is regulated by the potential of the electrode.^[98] Anchored DNA has average 45° angle of with the neutral electrode surface, while positive or negative potential of imposed on the electrode reduces or increases the angle by repulsion or attraction of the negatively charged DNA.^[98] Redox active probes are covalently tethered or non-covalently intercalating to DNA duplex according to the requirement of the experimental design. Single base mismatch resolution was achieved with DNA modified electrode with methylene blue (MB) probe.^[24] DNA modified electrode is very useful in investigation of the role of DNA CT in biological systems, especially compatible with proteins. With covalent redox probes, protein functions on DNA, particularly those relating to DNA base stacking, such as base flipping and duplex

bending as discussed in last section, were examined.^[82, 97] In addition, redox centers of proteins can be probed by DNA modified electrode based on DNA CT. DNA modified gold electrodes were used for electrochemistry study of proteins with redox centers such as Endo III, MutY with [4Fe-4S] clusters and *Escherichia coli* photolyase with flavin cofactor.^[85, 87-89] Moreover, anchoring DNA to single molecule platform maintains the sensitivity of the conductivity of the DNA device to DNA structure as observed in the solution or surface platform.^[99]

1.3 Further Explorations of DNA CT

1.3.1 Potential Involvement of DNA CT in Epigenetics

The expression or phenotype changes due to the alterations study of epigenetics mainly focuses on the heritable gene only in the chemical structures of proteins and nucleic acids, but not in their sequence. Despite the biological roles of DNA CT in protein functions and oxidative mutation of DNA discussed in the previous sections, Schuster's group first made an effort to investigate how methylation, installing a methyl group to C5 position of cytosine to generate 5-methylcytosine (mC), affects DNA CT by one electron oxidation of GG doublets after photolysis of AQ intercalated DNA.^[100] Unfortunately, no apparent difference in guanine damage through DNA CT in mC modified DNA duplexes was observed compared to unmodified ones.^[100] In later study, mC mispair showed similar enhanced reactivity by one electron oxidation as thymine mispairs, probably due to a same 5-methyl functionality.^[53]

mC is a crucial epigenetic modification. The regulatory roles of mC on gene transcription have been intensely studied during the past decades. Substantial studies suggest that variations in the tissue contents and genomic locations of mC is in charge of many biological and pathogenic processes during cell development and cellular differentiation, such as genomic imprinting, X-chromosome inactivation and

carcinogenesis. DNA methyltransferases (DNMTs) are the sole proteins responsible for the establishment and maintenance of methylation patterns in mammalian cells during DNA replication. On the contrary, the demethylation pathways of mC, which is responsible for the elimination of epigenetic sequences is largely remained unknown, though genome-wide demethylation is commonly observed through cell division in embryonic cells, and global hypomethylation of genome is nearly a universal character of cancerous cells.^[101] To unravel the dogma of mC demethylation would provide pivotal information to epigenetic regulation and the promising applications to epigenetic therapeutics.

Recently, the discovery of 5-hydroxymethylcytosine (hmC) and its oxidation products, 5-formylcytosine (fC) and 5-carboxylcytosine (CaC) as new epigenetic bases in Purkinje neurons and mouse embryonic stem cells (ESC) provided a novel mechanism for active demethylation for mammalian genome.^[102-104] Before that, the erasure of epigenetic genomic patterns in mammalian cells was largely believed to follow a passive demethylation due to the low activities or malfunctions of DNMTs during replication. The abilities of TETs (ten-eleven translocation protein), a set of iron/ α -ketoglutarate dependent dioxygenases, to convert mC to hmC and further oxidative products have been observed in mouse or human embryonic stem cells.^[103] Recently two mechanisms of hmC mediated active demethylation of mC stir up great interests (Figure 1-13):

- 1). Demethylation starts from the oxidation of mC to hmC and further to fC and CaC by Tet proteins. Both fC and CaC are moderate substrates for base excision DNA repair enzymes (BER), such as thymine DNA glycosylase (TDG) and single-strand-selective monofunctional uracil-DNA glycosylase (SMUG). The resulting abasic sites could be refilled with normal cytosine by DNA polymerases to complete the active demethylation process.

2) After mC is converted to hmC by TET, hmC may undergo deamination to form 5-hydroxymethyluracil (hmU). The deamination process can be catalyzed by activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA-editing-catalytic polypeptides (APOBECs). The deamination product forms a U-G mismatch, which can be efficiently recognized and repaired via BER pathways to restore the canonical C-G base pairs at original epigenetic sites.

Figure 1-13. Scheme of the proposed mechanisms of 5-methylcytosine demethylation pathways in eukaryotes.

The key arguments between the two mechanisms focus at the question: *At which stage would BER pathway be introduced to repair the epigenetic cytosines as a mutagenic site in genome?* Though *in vitro* studies have shown TDG has ability to recognize and repair fC and CaC, the enzymatic efficiency of TDG on the two epigenetic cytosines are much lower comparing to the ideal substrate, thymine. The rich variety of BER enzymes for G-

U/T mismatches implies the second mechanism would be more cost-wise, since deamination would convert the epigenetic cytosines to the uridine analogs as the optimal substrates for a wider range of cellular BER proteins to complete demethylation pathway. However, the proofs of the existence of epigenetic cytosine deaminases in mammalian cells are currently missing. The enzymatic ability of AID/APOBEC towards epigenetic cytosine substrates were found to be much lower compared to cytosine.^[105] Spontaneous deamination as an alternative deamination mechanism for cytosines in cell should be stressed. Therefore, we studied the spontaneous deamination kinetics of epigenetic cytosine derivatives, mC, hmC and fC as reported in Chapter 3, to elucidate the role of spontaneous deamination pathway in the active demethylation of mC.

Additionally, Barton and co-workers have established that the [4Fe-4S] cluster on proteins affects charge transport through the duplex and is important to the DNA repair mechanism.^[106] This [4Fe-4S] cluster is located on the active site of the TDG enzyme which is involved in DNA mismatch repair. Therefore, it will be interesting, as reported in chapter 4, to study the effects of epigenetic modifications on DNT CT, which might be meaningful for both DNA mediated signaling between proteins and funneling the oxidative G damage via DNA.

1.3.2 Facilitating DNA CT Efficiency with Ionic Liquid

The inherent conductivity of DNA via the stacking base pairs, good self-assembly and self-recognition made it an ideal material to develop functional electro devices. Surface and single molecule platforms for DNA electro devices are highly compatible with aqueous environment that are suitable for study of biological systems, such as proteins. However, the conductivity of DNA in aqueous solution is not ideal. Accumulating proofs have shown that DNA molecule longer than a few nanometres is an insulator or a wide band-gap semiconductor.^[107-113] Therefore, methods to enhance DNA

CT efficiency are demanded. Solutions of this problem may involve in changing the composition of DNA structure and introducing external factors into the system. For example, consecutive A tracts and G quadruplex showed better charge transport efficiency than duplex DNA with random sequences. It is obvious that alternating prime DNA structure has great limitation to be generalized. Alternatively, our group reported facilitation of DNA CT by external magnetic field. This method is quite compatible with biological systems, however, will have limitation when applied to electro devices considering strong magnetic field used.^[114]

We found that hydrated ionic liquid (IL) could be a candidate solvent system for facilitation of DNA charge transport. Ionic liquid is a salt in liquid state at certain temperature. Due to its special physical, electrical and chemical properties, ionic liquid has become a novel solvent with broad application prospects in organic synthesis, electrochemistry and recently upsurges in biology. Particularly, ionic liquids have been found to be an ideal solvent for protein extraction and long-term DNA storage.^[115, 116] Furthermore, certain properties of DNA could be significantly varied in ionic liquids compared to aqueous solution, such as duplex stability.^[117]

The interaction of ionic liquid to DNA molecule has been studied by experiments and theoretical stimulations. The results revealed that the facts discussed before should be noted when studying DNA-IL systems. First of all, dissociation of certain imidazolium ionic liquid with hydrophobic anions, cations and anions up hydration is complete even at much diluted ionic liquid aqueous solution.^[118] Secondly, ionic liquid cations tend to penetrate first hydration layer of DNA and bind to the negatively charged DNA phosphate backbone, while the binding is regulated by anions of ionic liquids as well.^[119, 120] Thirdly, ionic liquid cations with long hydrophobic side chains are able to interact with DNA base pairs by hydrophobic interactions.^[119, 121] We hypothesized that the special interaction

between ionic liquid and DNA could have impacts on DNA CT. To validate our hypothesis, effects of ionic liquid on DNA CT was examined as described in chapter 5.

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CHAPTER 2

Facile Syntheses of 5-Hydroxymethylcytosine and 5-Formylcytosine Containing Deoxyoligonucleotides and Melting Temperature Studies

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2.1 Introduction

5-hydroxymethylcytosine (hmC) as well as its oxidation products, such as 5-formylcytosine (fC) and 5-carboxylcytosine (CaC), were recently proposed to be a new series of epigenetic bases in mammalian genome.^[1-3] The distribution of these newly discovered epigenetic bases exhibits tissue specificity and shows high levels in neurons and embryonic stem cells (ESCs).^[4, 5] *In vivo* genesis of hmC, fC and CaC is under the charge of a family of non-heme iron-dependent dioxygenases, ten-eleven translocation protein (TET).^[2, 3] 5-methylcytosine (mC) is oxidized by TETs to generate hmC and further to fC and CaC. This process is believed to be essential in active DNA demethylation.^[6, 7] Once converted to fC or CaC, the epigenetic cytosine becomes a substrate to thymine DNA glycosylase (TDG) and initiates the reinstallation of cytosine via base excision repair pathway.^[7, 8] Besides the pivotal roles in active DNA demethylation like mC, hmC is also considered as a stable epigenetic mark, which can be recognized by putative binding proteins and may have regulatory effects on downstream biological processes.^[9-14] Nevertheless, the biological functions and potential metabolic mechanisms of hmC and fC remain largely unknown.

For investigation of hmC and fC associated biological processes, methods to facilitate and efficiently prepare hmC and fC containing DNA oligonucleotides are of great importance. Figure 2-1 summarizes reported strategies to synthesize hmC and fC phosphoramidites. Currently, only two methods have been reported to prepare fC phosphoramidites. An early attempt was conducted by Karino et al., who firstly incorporated a 5-(1,2-dioxyethyl)cytosine as precursor into the synthetic oligonucleotides.^[15] fC was converted in the oligonucleotide from diol functionality by post-synthetic NaIO₄ oxidation, which unfortunately limited the method only to the oligonucleotides without oxidation-labile components. Recently, He's and Carell's groups utilized a palladium-catalyzed CO insertion on 5-iodo-cytosine to introduce 5-formyl

functionality.^[16-18] However, high pressured explosive gas, complicated protocol and inconvenient instrumental setup casted shadows on the method, though with excellent yields.

As shown in Figure 2-1, hmC phosphoramidites can be prepared by reducing with NaBH₄ either the intermediate product during fC phosphoramidite syntheses or *in situ* fC base on DNA.^[16, 17, 19] Remarkably, Carell's group reported a cyclic carbamate hmC phosphoramidite via simultaneously protection of hydroxyl and amino groups on the reductive product of fC intermediate with 4-nitrophenylchloroformate, which shortened the reaction steps; while one drawback of the phosphoramidite was the requirement of treatment with NaOH for mild deprotection condition of the synthetic oligonucleotides, which limited its application.^[19] Other methods were developed based on 5-hydroxymethyl-2'-deoxyuridine (hmdU) intermediates.^[20-23] As an unnatural nucleoside, hmdU intermediate was prepared by either direct hydroxymethylation of 2'-deoxyuridine or nucleophilic reaction of iodine substituted thymidine derivative with 3-hydroxypropionitrile.^[21-23] Unlike fC phosphoramidites, protection groups, such as TBDMS and 2-cyanoethanol, were needed to prevent decomposition of 5'-hydroxymethyl functionality of thymidine derivatives during solid phase DNA synthesis.^[20-23]

Since the high demands had attracted vigorous investigation on the synthesis of hmC and fC containing oligonucleotides, we exploited alternative synthetic pathways for hmC and fC phosphoramidites which circumvent drawbacks of previous studies such as complicate experimental procedures and special apparatus required. In this chapter, we reported facile synthetic routes under mild aqueous conditions to prepare hmC and fC phosphoramidites from commercial available nucleosides as starting materials and to further proceed to solid phase DNA synthesis.^[24] Oxidation-based synthetic approaches were developed for facile preparation of 5-formyl-2'-deoxycytidine and 5-hydroxymethyl-2'-deoxycytidine phosphoramidites.

Figure 2-1 Summary of reported synthetic strategies for hmC and fc phosphoramidites. The figure was redrawn according to ref ^[15-23].

2.2 Experimental

2.2.1 General Reagents and Instruments

All chemicals used to synthesize hmC and fc phosphoramidites were purchased from Alfa Aesar, Sigma Aldrich and Merck. Other common chemical reagents and solvents were commercially available, including: dichloromethane, methanol, ethanol, ethyl acetate, hexane, acetonitrile, dimethylformamide, tetrahydrofuran, pyridine, ammonium hydroxide, sodium chloride, sodium bicarbonate, sodium carbonate, copper (II) sulfate. Dry solvents were purchased from Sigma-Aldrich or prepared in lab. Reactions using dry solvents were performed under inert atmosphere (N_2 or Ar). Technical grade solvents

were used for silica column chromatography purification. All products were dried by high vacuum pump (Welch 8917 vacuum pump) or lyophilization (Labconco, 0.010 mbar). Electrospray ionisation mass spectrometry (ESI-MS) were carried on ThermoFinnigan LCQ FLEET MS for synthetic compounds, while ESI-MS analyses of epigenetic cytosine nucleosides were performed on Agilent 1290 ultrahigh pressure liquid chromatography system (Waldbronn, Germany) equipped with a 6520 Q-TOF mass detector managed by a MassHunter workstation. Nuclear magnetic resonance (NMR) characterization was conducted with: Bruker AVIII 400MHz NMR (BBFO1 Probe), Bruker AV 300MHz NMR (BBO probe), Bruker AV 400MHz NMR (QNP probe), and Bruker AV 500MHz NMR (BBI probe), JEOL ECA400 NMR (Liquid & Solid probe). UV-Vis determination of DNA concentration was carried on Shimadzu UV 1800 spectrophotometer. High-performance liquid chromatography (HPLC) used Shimadzu Prominence Modular HPLC.

2.2.2 Synthetic Protocols of hmC and fC Containing Phosphoramidites and Characterization

5-formyl-2'-deoxycytidine (2)

A mixture of 5-methylcytidine (100 mg, 0.41 mmol), $\text{Na}_2\text{S}_2\text{O}_8$ (202.2 mg, 0.85 mmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (20.8 mg, 0.083 mmol), and 2,6-lutidine (85.8 μL) in $\text{H}_2\text{O}/\text{ACN}$ (1:4, v/v, 10 mL) was heated with stirring by microwave (discover S-class microwave system, CEM Corporation) at 80 °C for 5 min. After cooling down to room temperature, NaHCO_3 was added to adjust reaction mixture to pH 7-8. The solvents were removed under reduced pressure. The residue was washed with methanol for three times and filtered. The combined filtration was concentrated under reduced pressure and purified by silica column chromatography (eluting with CH_2Cl_2 : MeOH=5:1). The desired fraction was concentrated and dried in vacuum to afford the product as white powder (29 mg, yield 27%). ^1H NMR (400 MHz, DMSO) δ 9.46 (s, 1H), 8.83 (s, 1H), 8.11 (d, J = 2.8 Hz, 1H), 7.85 (d, J = 3.1 Hz, 1H), 6.06 (t, J = 6.1 Hz, 1H), 5.27 (d, J = 4.3 Hz, 1H), 5.13 (t, J

= 5.1 Hz, 1H), 4.23 (dq, J = 8.4, 4.2 Hz, 1H), 3.87 (q, J = 3.8 Hz, 1H), 3.72 – 3.64 (m, 1H), 3.59 (dt, J = 12.0, 4.2 Hz, 1H), 2.30 (ddd, J = 13.4, 6.2, 4.4 Hz, 1H), 2.09 (dt, J = 13.4, 6.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 189.13, 162.15, 155.07, 152.47, 104.97, 88.44, 87.03, 69.88, 61.02, 41.45. ESI-MS $[\text{M}+\text{H}^+]$ for $\text{C}_{10}\text{H}_{14}\text{N}_3\text{O}_5^+$ calcd. 256.09; found 256.03.

***N*⁴-Benzoyl-5-formyl-2'-deoxycytidine (3)**

Compound 2 (376 mg, 1.47 mmol) and benzoic anhydride (339 mg, 1.50 mmol) in anhydrous ethanol (28 mL) were refluxing under N_2 atmosphere for 1 hour. Another 1 eq benzoic anhydride (339 mg, 1.50 mmol) was added to reaction mixture each hour for the following three hours. White precipitate formed during the reaction. After cooling down to room temperature, the product was separated by centrifugation. The residue was washed with cold ethanol (5 mL) three times. After dryness under high vacuum, the crude product (345 mg, 0.96 mmol, yield 65%) was obtained and used directly in the next step. ^1H NMR (400 MHz, DMSO- d_6) δ 9.58 (s, 1H), 9.17 (s, 1H), 7.97 (d, J = 7.3 Hz, 2H), 7.71 (t, J = 7.4 Hz, 1H), 7.63 (t, J = 7.5 Hz, 2H), 6.08 (t, J = 5.9 Hz, 1H), 5.33 (d, J = 4.4 Hz, 1H), 5.19 (t, J = 5.0 Hz, 1H), 4.26 (td, J = 9.1, 4.4 Hz, 1H), 3.96 (q, J = 3.2 Hz, 1H), 3.79 – 3.69 (m, 1H), 3.68 – 3.57 (m, 1H), 2.46 – 2.37 (m, 1H), 2.20 (dt, J = 13.4, 5.8 Hz, 1H). ESI-MS $[\text{M}+\text{H}^+]$ for $\text{C}_{17}\text{H}_{18}\text{N}_3\text{O}_6^+$ calcd. 360.12; found 359.79.

***5'*-(dimethoxytrityl)-4-*N*-benzoyl-5-formyl-2'-deoxycytidine (4)**

Compound 3 (210 mg, 0.58 mmol) and 4, 4'-dimethoxytrityl chloride (590 mg, 1.74 mmol) were stirred in anhydrous pyridine under N_2 atmosphere at r.t. for 4 h. Methanol (1 mL) was added to quench the reaction. After dilution with CH_2Cl_2 (60 mL), the organic layer was washed with sat. NaHCO_3 three times and with brine once. The mixture was filtered and the organic layer was concentrated under reduced pressure. The residue was purified by silica gel chromatography (eluting with CH_2Cl_2 : MeOH=20:1, with 1% triethylamine) and dried under high vacuum to yield the product as white powder (349

mg, 0.53 mmol, yield 91%). ¹H NMR (400 MHz, DMSO) δ 11.73 (s, 1H), 8.98 (s, 1H), 8.81 (s, 1H), 7.95 (d, *J* = 8.2 Hz, 2H), 7.71 (t, *J* = 7.2 Hz, 1H), 7.63 (t, *J* = 7.1 Hz, 2H), 7.43 – 7.27 (m, 4H), 7.24 (d, *J* = 7.4 Hz, 5H), 6.89 (d, *J* = 8.6 Hz, 4H), 6.10 (t, 5.8 Hz, 1H), 5.42 (d, *J* = 4.8 Hz, 1H), 4.37 – 4.23 (m, 1H), 4.07 (dd, *J* = 7.9, 4.2 Hz, 1H), 3.73 (2x s, 6H), 3.31–3.24 (m, 1H), 2.49–2.43 (m, 1H), 2.34 (td, *J* = 11.4, 6.0 Hz, 1H). ESI-MS [*M*+*H*⁺] for C₃₈H₃₆N₃O₈⁺ calcd. 662.25; found 662.07.

3'-(diisopropylamino-cyanoethoxyphosphino)-5'-(dimethoxytrityl)-4-N-benzoyl-5-formyl-2'-deoxycytidine (5)

Compound 4 (62 mg, 0.094 mmol), N, N-diisopropylethylamine (DIPEA) (49.2 μL, 0.282 mmol) and 2-cyanoethyl N, N-diisopropylchlorophosphoramidite (63 μL, 0.282 mmol) were stirred in anhydrous CH₂Cl₂ (0.5 mL) for 30 min. Subsequently, the solvent was removed by rotary evaporation and the residue was purified by silica column chromatography (eluting with ethyl acetate: hexane=1:1, with 1% triethylamine). After dryness under high vacuum, the final product (58 mg, yield 73%) was obtained as white powder. ³¹P NMR (162 MHz, Acetone) δ 148.65 (s), 148.43 (s). ESI-MS [*M*+*Na*⁺] for C₄₇H₅₂N₅O₉PNa⁺ calcd. 884.34; found 884.37.

5-Hydroxymethyl-2'-deoxyuridine (7)

A mixture of thymidine (387.6 mg, 1.6 mmol), Na₂S₂O₈ (761.9 mg, 3.2 mmol) and CuSO₄·5H₂O (40 mg, 0.16 mmol) were dissolved in THF/H₂O 1:1 solution (10 mL) and reacted in microwave at 85 °C for 5 min. After cooling down to room temperature, to the solution was added concentrated NH₄OH to adjust pH to above 7. After removal of the ammonia by rotary evaporation and removal of solvent by lyophilization, the residue was washed with methanol for three times. The filtration was combined and concentrated by reduced pressure. The crude was purified by silica column chromatography (eluting with CH₂Cl₂: MeOH=5:1) and yield the product (112 mg, 0.43 mmol, yield 27%) as white powder. Thymidine (131 mg, 0.54 mmol, recover 34%) was recovered.

5-(2-Cyanoethyl) hydroxymethyl-2'-deoxyuridine (8)

To a solution of 5-hydroxymethyl-2'-deoxyuridine (1.2 g, 4.65 mmol) in 3-hydroxypropanenitrile (24 mL) was added catalytic amount of trifluoroacetic acid (240 μ L). The solution was stirred under high vacuum at 130 °C for 1 h. After complete consumption of starting material, the reaction was cooled to room temperature under vacuum. The residue was purified by silica gel chromatography (eluting with CH₂Cl₂: MeOH=10:1) to afford white foam (763 mg, 2.46 mmol, yield 53%). ¹H NMR (400 MHz, DMSO) δ 11.34 (s, 1H), 7.91 (s, 1H), 6.13 (t, J = 6.7 Hz, 1H), 5.22 (d, J = 4.0 Hz, 1H), 4.99 (t, J = 4.7 Hz, 1H), 4.21 (m, 1H), 4.19 – 4.10 (m, 2H), 3.76 (q, J = 3.7 Hz, 1H), 3.63 – 3.49 (m, 4H), 2.72 (t, J = 6.1 Hz, 2H), 2.17 – 1.96 (m, 2H). ¹³C NMR (101 MHz, DMSO) δ 163.18, 150.75, 139.82, 119.73, 110.50, 87.88, 84.66, 70.79, 65.13, 64.95, 61.70, , 18.49. ESI-MS [M+H⁺] for C₁₃H₁₈N₃O₆⁺ calcd. 311.12; found 311.90.

5-(2-Cyanoethyl)hydroxymethyl 5'-(4,4'-dimethoxytrityl) 2'-deoxyuridine (9)

Compound 8 (113 mg, 0.36 mmol) and 4,4'-dimethoxytrityl chloride (146 mg, 0.43 mmol) were dissolved in anhydrous pyridine (3.6 mL). The solution was stirred under N₂ atmosphere at r.t. overnight. Methanol (0.5 mL) was used to quench the reaction. After dilution with CH₂Cl₂ (50 mL), the organic layer was washed with sat. NaHCO₃ three times and brine once. The combined organic layer was dried by anhydrous Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica gel chromatography (eluting with CH₂Cl₂: MeOH=10:1, with 1% triethylamine) to afford the product as white powder (179 g, 0.29 mmol, yield 81%). ¹H-NMR (400 MHz, CDCl₃): δ_{ppm} 7.87 (s, 1H), 7.42 (d, J = 7.1 Hz, 2H), 7.33 – 7.27 (m, 7H), 6.86 (dd, J = 8.9, 1.2 Hz, 4H), 6.46 – 6.38 (m, 1H), 4.61 (dt, J = 6.2, 3.1 Hz, 1H), 4.09 (dd, J = 6.0, 3.0 Hz, 1H), 3.89 (d, J = 11.8 Hz, 1H), 3.81 (s, 6H), 3.68 (d, J = 11.8 Hz, 1H), 3.50 (dd, J = 10.6, 3.0 Hz, 1H), 3.35 (m, 3H), 2.49 (ddd, J = 13.5, 5.9, 3.1 Hz, 1H), 2.40 – 2.27 (m, 1H), 2.22 (td, J = 6.7, 2.3 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 162.85, 158.72, 158.70, 150.37,

144.49, 139.02, 135.49, 135.41, 130.19, 130.12, 128.16, 128.04, 127.14, 117.68, 113.31, 111.37, 86.75, 86.19, 84.90, 72.04, 65.17, 64.94, 63.43, 55.31, 41.04, 18.15. ESI-MS $[M+Na^+]$ for $C_{34}H_{35}N_3O_8Na^+$ calcd. 636.23; found 636.05.

5-(2-Cyanoethyl) hydroxymethyl-5'-(4, 4'-dimethoxytrityl)-2'-deoxycytidine (10)

Compound 9 (452 mg 0.74 mmol) in CH_2Cl_2 (8 mL) were mixed with an aqueous solution (16 mL) of Na_2CO_3 (317 mg, 2.94 mmol), tetra-*n*-butylammonium bromide (TBAB, 119 mg, 0.37 mmol) and 2,4,6 triisopropylbenzenesulfonyl chloride (290 mg, 0.96 mmol) in a 100 mL round bottom flask. The solution was stirred vigorously under Ar atmosphere overnight. After dilution with CH_2Cl_2 (52 mL), DMF (4 mL) was added to facilitate the removal of TBAB in the following extraction step. The organic layer was washed with sat. $NaHCO_3$ (2×30 mL), H_2O (3×30 mL), brine (30 mL) and dried with anhydrous Na_2SO_4 and filtered. The filtration was evaporated to dryness under reduced pressure. The residue was dissolved in 1,4-dioxane/ NH_4OH mixed solution (4:1, 10 mL) and reacted in a sealed tube overnight. Subsequently, the solvent was removed by rotary evaporation. The crudes were purified by silica gel chromatography (eluting with CH_2Cl_2 : MeOH=10:1, with 1% triethylamine). **Compound 10** (257 mg, yield 57%) was obtained as white powder. ^1H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 1H), 7.40 (d, J = 7.3 Hz, 2H), 7.35 – 7.24 (m, 7H), 6.85 (d, J = 8.1 Hz, 4H), 6.52 (t, J = 6.5 Hz, 1H), 5.94 (brs, 1H), 4.60 – 4.52 (m, 1H), 4.15 (d, J = 2.8 Hz, 1H), 3.80 (s, 6H), 3.77 (d, J = 12.5 Hz, 1H), 3.57 (d, J = 2.5 Hz, 1H), 3.53 (d, J = 12.4 Hz, 1H), 3.32 (dd, J = 10.3, 2.3 Hz, 1H), 3.25 – 3.08 (m, 2H), 2.71 (ddd, J = 13.4, 5.6, 3.2 Hz, 1H), 2.36 (t, J = 6.3 Hz, 2H), 2.26 (dt, J = 13.4, 6.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.01, 158.75, 156.01, 144.34, 140.67, 135.50, 135.43, 130.22, 130.17, 128.34, 128.02, 127.22, 117.51, 113.28, 101.85, 86.70, 86.42, 86.22, 72.02, 67.54, 63.84, 63.41, 55.32, 42.19, 18.45. ESI-MS $[M+H^+]$ for $C_{34}H_{37}N_4O_7^+$ calcd. 613.27; found 612.76.

***N*⁴-Benzoyl-5-(2-cyanoethyl)hydroxymethyl-5'-(4,4'-dimethoxytrityl)-2'-deoxycytidi**

ne (11)

Compound 10 (113 mg, 0.18 mmol) and benzoic anhydride (65 mg, 0.29 mmol) was stirred in anhydrous DMF (2.4 mL) under Ar atmosphere for 4 days. The solution was diluted with CH₂Cl₂ and washed with sat. NaHCO₃, water and brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated. Finally, the residue was purified by silica gel chromatography (eluting gradient, 10-100% ethyl acetate in hexane, with 1% triethylamine). **Compound 11** (100 mg, yield 76%) was collected as white powder. ¹H NMR (400 MHz, CDCl₃) δ 8.24 (d, *J* = 7.2 Hz, 2H), 8.03 (s, 1H), 7.54 (t, *J* = 7.3 Hz, 1H), 7.45 (d, *J* = 14.9 Hz, 4H), 7.36 – 7.30 (m, 6H), 7.27 (dd, *J* = 6.2, 4.1 Hz, 1H), 6.87 (dd, *J* = 8.9, 2.0 Hz, 4H), 6.40 (t, *J* = 6.5 Hz, 1H), 4.57 (dt, *J* = 6.7, 3.5 Hz, 1H), 4.26 (d, *J* = 12.1 Hz, 1H), 4.13 (q, *J* = 10.7 Hz, 1H), 4.09 (q, *J* = 3.2 Hz, 1H), 4.04 (d, *J* = 12.2 Hz, 1H), 3.80 (s, 6H), 3.54 (dd, *J* = 10.6, 3.1 Hz, 1H), 3.44 (t, *J* = 6.6 Hz, 2H), 3.34 (dd, *J* = 10.6, 3.2 Hz, 1H), 2.51 (ddd, *J* = 13.5, 5.9, 3.6 Hz, 1H), 2.34 (dt, *J* = 13.5, 6.7 Hz, 1H), 2.14 (td, *J* = 6.6, 3.1 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 158.73, 158.71, 158.49, 147.94, 144.54, 139.32, 136.76, 135.59, 135.44, 132.65, 130.21, 130.13, 129.77, 128.23, 128.20, 128.04, 127.12, 117.62, 113.31, 111.64, 86.69, 86.22, 85.47, 71.69, 65.44, 65.40, 63.28, 55.31, 41.30, 18.15. ESI-MS [*M*+*H*⁺] for C₄₁H₄₁N₄O₈⁺ calcd. 717.29; found 717.22

***N*^d-Benzoyl-5-(2-cyanoethyl)hydroxymethyl-5'-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-[N,N-diisopropyl-N-cyanoethyl]phosphoramidite (12)**

Compound 11 (42 mg, 0.059 mmol) was co-evaporated with anhydrous acetonitrile three times and dried under high vacuum. Subsequently, the compound was dissolved in anhydrous CH₂Cl₂ (0.2 mL) in an argon purged round bottom flask. N,N-diisopropylethylamine (26.3 μL, 0.151 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (19.9 μL, 0.089 mmol) were added. The solution was stirred for 0.5 h. After reaction, water (4 mL) was added to the solution and the mixture was extracted with CH₂Cl₂ (3×2 mL). After evaporation under reduced pressure, the

residue was submitted to silica gel chromatography purification (eluting with ethyl acetate: hexane=1:1, with 1% triethylamine) to afford the final product (32 mg, yield 59%) as white foam. ^{31}P NMR (162 MHz, Acetone) δ 148.45 (s), 148.30 (s). ESI-MS $[\text{M}+\text{Na}^+]$ for $\text{C}_{50}\text{H}_{57}\text{N}_6\text{O}_9\text{PNa}^+$ calcd. 939.38; found 939.14

2.2.3 Oligonucleotides Synthesis and Purification

Oligonucleotide synthesis was performed on a MerMade 4 DNA Synthesizer (BioAutomation) in standard procedures. Standard DNA phosphoramidites (dA, dG, dC, dT), reagents and CPG columns were purchased from Glen Research and Berry & Associates. After solid phase synthesis, the oligonucleotides were cleaved from resin by concentrated NH_4OH treatment. For oligonucleotides containing hmC, DNA cleavage and deprotection were completed simultaneously by incubating in concentrated NH_4OH at 75 °C for 24 h or at 65 °C for 3 days; oligonucleotides containing fC were cleaved from beads and deprotected by incubating in concentrated NH_4OH at 75 °C for 24 h. Oligonucleotides with DMT on then were lyophilized to dryness, and were submitted to HPLC purification (5%~35% ACN/50 mM TEAA over 30 minutes). The oligonucleotides were then treated with 80% acetic acid solution (200 μL) for 5 min and precipitated by cold ethanol (600 μL). Second HPLC purification (0%~15% ACN/50 mM TEAA over 30 min) was applied to yield pure oligonucleotides.

2.2.4 Enzymatic Digestion

DNA samples (600 pmol) were dissolved in a mixture of deionized water (20 μL), MgCl_2 solution (1.0 M, 0.4 μL), Tris-Cl (pH 7.5, 0.5 M, 2 μL). And then, alkaline phosphatase (4 μL , 0.4 units, New England Biolabs) and phosphodiesterase I (2.4 μL , 0.16 units, i-DNA biotechnology) were added to the above mixture and incubated at 37 °C for 18 h. After incubation, the mixture was submitted to HPLC analysis (Chemcobond 5-ODS-H, 4.6 $\times$ 150 mm).

Table 2-1. HPLC mobile phase gradient of enzymatically digested oligonucleotides

Time (min)	Flow rate (mL/min)	Mobile phase A ^a (%)	Mobile phase B ^b (%)
0	1.0	99.4	0.6
22	1.0	94	6
43	1.0	85	15
50	1.0	60	40
55	1.0	99.4	0.6
65	1.0	99.4	0.6

^a Mobile phase A: 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.0.

^b Mobile phase B: ACN (HPLC-grade)

2.2.5 LC-MS Analysis of Residues from Enzymatic Digestion

To minimize the interference of dT during MS analysis, the mixture of fC and dT from digestion residue of **ODN 1** was baseline separated by applying a long mobile phase gradient on LC chromatography. Same gradient was applied to digestion residue of **ODN**

2.

Table 2-2. LC-MS mobile phase gradient for nucleoside identification^a

Time (min)	Flow rate (mL/min)	Mobile phase A ^b (%)	Mobile phase B ^c (%)
0	0.5	100	0
15	0.5	99.5	0.5
45	0.5	92	8
55	0.5	50	50
60	0.5	50	50
70	0.5	100	0
75	0.5	100	0

^a C18 column used for LC-MS is Chemcobond 5-ODS-H, 4.6×250 mm

^b Mobile phase A: 0.2 mM NH₄HCOO buffer, pH 7.0.

^c Mobile phase B: ACN (HPLC-grade)

2.2.6 Melting Temperatures

Thermal stability of hmC and fC modified DNA duplex was investigated by UV-melting assay on Shimadzu UV-2550 UV-VIS spectrophotometer with TMSPC-8 peltier controller. Duplex DNA was annealed with complimentary strands by heating at 90 °C for 5 min and gradually cooling to r.t. over 2 h on heating block. UV melting data was collected by heating duplex DNA (1.5 μM) from 15 °C to 90 °C in a rate of 0.5 °C/min

with measurement interval of 1 °C. The average T_m curves of three repeats were fitted into sigmoidal function to produce T_m values in OriginPro 8.5.1.

2.3 Results and Discussion

2.3.1 Facile Synthesis of fC Containing DNA Oligonucleotides

In an attempt to develop facile methods to prepare 5-formyl-2'-deoxycytidine (fdC, **2**), we were inspired by the biological synthesis of fC via TET and considered the possibility of preparing **2** via oxidation of 5-methyl-2'-deoxycytidine (mdC, **1**). A literature survey revealed that **1** could be oxidized by Na₂S₂O₈ to yield **2** in aqueous solution, however, only as a side product.^[25, 26] Nevertheless, the oxidation power and major products of Na₂S₂O₈ can be tuned by solvent polarity and metal ions as catalysts.^[27] After screening a few combinations of polar organic solvents as co-solvent with water (optimization of reaction conditions will be discussed in detail in section 2.3.4), acetonitrile/water was found to be the optimal hosts for both organic precursor, **1**, and inorganic oxidant and catalyst in the reaction mixture (Table 2-3). In the presence of catalytic amount of divalent copper ion, the major oxidation product of mdC shifted to fdC and generation of 5-hydroxymethyl-2'-deoxycytidine (hmdC) were not detectable. No significant improvement in yields was observed by using 3',5'-protected nucleosides, which indicated that hydroxyl groups on deoxyribose would neither decompose nor interfere the

Table 2-3. Optimization of synthetic conditions for fdC via Na₂S₂O₈ oxidation.

Entry ^a	Solvent	Catalyst	mdC%	fdC%	hmdC%
1	H ₂ O		11	6	19
2	H ₂ O	CuSO ₄	0	0	11
3	ACN-H₂O	CuSO₄	0	26	0
4	THF-H ₂ O	CuSO ₄	0	0	0
5	MeOH-H ₂ O	CuSO ₄	37	6	8

^a Screening condition: 0.016 mmol mdC and 0.032 mmol Na₂S₂O₈ was dissolved in 1 mL solvent together with 0.0032 mmol CuSO₄ as catalysts if indicated. The reaction solution was submitted to microwave (dynamic mode) at indicated temperature for 5 minutes.

^b Polar organic solvents are applied as co-solvent to water in a v/v ratio of 4:1 for ACN-H₂O mixture, 1:1 for THF-H₂O and MeOH-H₂O mixture.

^c The yields of fdC and hmdC, and recovery rate of mdC were determined by HPLC using dA as external

standard for quantitative normalization.

oxidation.^[28] $\text{Na}_2\text{S}_2\text{O}_8\text{-Cu}^{2+}$ could be directly applied to oxidize mC nucleoside and extra synthetic steps of introducing and removing protection groups could be avoided. The reaction successfully proceeded to completion either under microwave assisted heating (80 °C) or under conventional heating (65 °C) within regular RBF open to air. Reaction time was significantly reduced from 1 h to 5 min per reaction under microwave-assisted heating. At the end of reaction, **1** was completely consumed by radical oxidants and **2** was afforded as the only product with detectable UV absorption either on TLC or by HPLC, which made purification by silica column chromatography relatively simple. The synthetic pathway for fC phosphoramidite was developed as shown in Scheme 2-1. Since the aldehyde can readily survive phosphoramidite based chemistry of oligonucleotide (ODN) synthesis, the formyl group was not protected in the final fC phosphoramidite. We firstly, selectively protect the exocyclic amino group of fdC by reacting with benzoic anhydride in hot ethanol and generate **3** in moderate yield 65%. Compared to acetyl protecting group, benzoyl functionality is chemically more stable during solid phase synthesis of oligonucleotides, so that ultra-mild synthetic condition required by *N*⁴-acetyl-fC phosphoramidite can be avoided.^[17] In addition, **3** precipitated from reaction solvent and could be simply isolated from reaction mixture by centrifugation. Furthermore, *N*⁴-protected fdC can also avoid the by-reactions with

Scheme 2-1 Synthetic route for fC phosphoramidite **5** building block. Synthetic yields are indicated under reaction condition of each step.

dimethoxytrityl chloride (DMT-Cl) if DMT was introduced to bare nucleoside. The subsequent DMT protection and 3'-phosphitylation were accomplished with decent yields of 91% and 73%, respectively. This synthetic route entails merely four steps to prepare fC phosphoramidite **5** and is more convenient in the aspect of experiment setup. fC phosphoramidite was then incorporated into oligonucleotide **ODN1** (sequence in Table 2-4) by standard solid phase DNA synthesis with extended coupling time (5 min) to ensure high synthetic yield. Decent coupling yield of fC phosphoramidite (78%) was achieved according to the trityl graph of solid phase synthesis. **ODN1** was cleaved from the resin by incubation in concentrated NH_4OH at 75 °C for 24 h. Simultaneously deprotection of natural nucleobases was accomplished during incubation. Since fC phosphoramidite **5** used the common benzoyl protection as regular cytosine phosphoramidites, no additional deprotection procedures were required for fC containing oligonucleotide. After HPLC purification (Figure 2-3A) before and after detritylation by 80% acetic acid at room temperature, the desired oligonucleotide **ODN1** was obtained and the mass of **ODN1** was

verified by ESI-MS (Figure 2-2). For further validation of successful incorporation of fC, **ODN1** was submitted to enzymatic digestion to yield free nucleosides and the resultant

Table 2-4. Sequences of oligonucleotides

ODN	Sequences ^a
ODN1	5'-TTC CAC <i>GfCG</i> CGT TCC TGA CTG ACT C-3'
ODN2	5'-TTC CAC <i>GhmCG</i> CGT TCC TGA CTG ACT C-3'
ODN3	5'-TTC CAC <i>GCG</i> CGT TCC TGA CTG ACT C-3'
ODN4	3'-AAG GTG <i>CGC</i> GCA ACC ACT GAC TGA G-5'
ODN5	3'-AAG GTG <i>CAC</i> GCA ACC ACT GAC TGA G-5'
ODN6	3'-AAG GTG <i>CCC</i> GCA ACC ACT GAC TGA G-5'
ODN7	3'-AAG GTG <i>CTC</i> GCA ACC ACT GAC TGA G-5'

^aEpigenetic bases and complementary bases are highlighted in bold and italic.

mixture was analysed by LC-MS. Similar as literature, 5-formyl-2'-deoxycytidine was eluted at the same retention time as thymidine from C18 column, and thus was not distinguishable by HPLC analysis (Figure 2-3B).^[16] To minimize the interference of thymidine during MS analysis, a long mobile phase gradient was applied to LC chromatography to ensure baseline separation of fC and thymidine. The coexistence of 5-formyl-2'-deoxycytidine (Figure 2-3C, [M+H]⁺ *m/z* for C₁₀H₁₄N₃O₅⁺ 256.0912 and nucleobase fragment C₆H₆N₃O₂⁺ 140.0445) and thymidine (Figure 2-4, [M+H]⁺ *m/z* for C₁₀H₁₅N₂O₅⁺ 243.0916 and nucleobase fragment C₅H₇N₂O₂⁺ 127.0498) in HPLC elutes at 16.6 min was confirmed.

Figure 2-2 ESI-MS of purified **ODN1** (A), **ODN2** (B)

Figure 2-3. Reverse phase HPLC chromatography of **ODN1** after purification (A) and after enzymatic digestion (B). Eluted peak at 16.6 minutes from (B) was submitted to LC-MS characterization for fdC (C).

Figure 2-4. LC-MS characterization of dT from fdC and dT co-elutes. (A) LC analysis of fdC and dT co-elutes. (B) MS proved existence of dT in fdC and dT co-elutes.

2.3.2 Facile Synthesis of hmC Containing DNA Oligonucleotides

The same $\text{Na}_2\text{S}_2\text{O}_8\text{-Cu}^{2+}$ oxidant system were also applied to prepare hmC phosphoramidite. Unlike fC phosphoramidite, hydroxymethyl group could not survive the synthetic reactions of oligonucleotides and needed to be protected in the phosphoramidite. The synthetic pathway of the hmC phosphoramidite was depicted in Scheme 2-2. In order to avoid the interference from *N*⁴-exocyclic amine during protection of the hydroxymethyl group, we chose thymidine (dT, **6**), instead of mdC, as the starting substrate for $\text{Na}_2\text{S}_2\text{O}_8$ oxidation. As shown in Table 2-5, screening for reaction condition under microwave-assisted heating indicated that THF is the optimal organic solvent to prevent over oxidation of dT to 5-formyluridine and provide 5-hydroxymethyl-2'-deoxyuridine (hmdU, **7**) as the major product and with the highest conversion stoichiometry (optimization of reaction conditions will be discussed in detail in section 2.3.4). Upon upscaling the

reaction, **7** in THF/H₂O mixed solvent with Na₂S₂O₈/CuSO₄ as oxidant/catalyst mixture can achieve an apparent yield of 41% after

Scheme 2-2. Synthetic route for hmC phosphoramidite **12**. Synthetic yields are indicated under the reaction conditions of each step.

recovering 34% of thymidine from chromatographic purification. Again, microwave assisted heating could reduce the reaction time from 4 h by conventional heating to only 5 minutes. The subsequent protection of C5 hydroxymethyl group was accomplished by substitution with 3-hydroxypropanenitrile to generate **8** with moderate yield 53%. Since installation of DMT protection on **8** could facilitate the amination step and generate cytosine analogue **10**, **8** was reacted with 3 equivalents of 4,4'-dimethoxyltrityl chloride (DMT-Cl) in pyridine and generate **9** with 81% yields. Conversion of **9** to **10** was accomplished by following a reported

Table 2-5. Optimization of synthetic conditions for hmdU via Na₂S₂O₈ oxidation.

Entry ^a	Co-solvent ^b	Catalyst	T (°C)	thymidine/recovery, % ^c	hmdU/yield, % ^c
1	H ₂ O		70	49	10
2	H ₂ O	CuSO ₄	70	50	26
3	ACN-H ₂ O	CuSO ₄	85	0	0
4	THF-H₂O	CuSO₄	85	54	36
5	MeOH-H ₂ O	CuSO ₄	85	26	15

^a Screening condition: 0.016 mmol dT and 0.032 mmol Na₂S₂O₈ was dissolved in 1 mL solvent together with 0.0032 mmol CuSO₄ as catalysts if indicated. The reaction solution was submitted to microwave (dynamic mode) at indicated temperature for 5 minutes.

^b Polar organic solvents are applied as co-solvent to water in a v/v ratio of 4:1 for ACN-H₂O mixture, 1:1 for THF-H₂O and MeOH-H₂O mixture.

^c The yields of hmdU and recovery rate of thymidine were determined by HPLC using dA as external standard for quantitative normalization.

protocol.^[21] Selective protection of the exocyclic amino group with benzoyl group was then achieved in 76% isolation yield by incubating with benzoic anhydride in anhydrous DMF for 4 days. The resultant compound **11** was 3'-phosphorylated to afford hmC phosphoramidite **12** in 59% isolation yield. The synthetic route requires six steps to prepare hmC phosphoramidite. A 25-mer hmC containing oligonucleotide, **ODN2** with the same sequence and epigenetic location as **ODN1** (Table 2-4), was synthesized. Incorporation of hmC in the DNA oligonucleotide was successful by following standard solid phase synthetic protocol, while coupling time was extended to 5 min to ensure high coupling yields. hmC phosphoramidite had comparable coupling yield (88%) with canonical bases. Following synthesis, cleavage and deprotection of **ODN2** were accomplished simultaneously by NH₄OH treatment at 75 °C for 24 h or 65 °C for 3 days. Extra hours of incubation in basic solution are necessary to remove the 2-cyanoethyl group on hmC.^[20, 21] **ODN2** was obtained after HPLC purifications (Figure 2-5A) both before and after detritylation, and was confirmed by ESI-MS spectra (Figure 2-2B). To further confirm the success of hmC incorporation and completion of hmC deprotection in the oligonucleotide, **ODN2** was enzymatically digested to free nucleosides. A new peak, besides four natural nucleobases, was eluted at 6.9 min and was characterized by ESI-MS (Figure 2-5B). Figure 2-5C shows both the pseudo-molecular ion of hmdC [M+H]⁺ *m/z* for C₁₀H₁₅N₃O₅⁺ 258.1088) and nucleobase fragment (C₅H₈N₃O₂⁺ 142.0612) were found from LC-MS.

Figure 2-5. Reverse phase HPLC chromatography of **ODN2** after purification (A) and after enzymatic digestion (B). Elute peak at 6.83 minutes from (B) was submitted to LC-MS characterization for hmdC (C).

2.3.3 Thermal Stability Study of the Synthetic DNA Oligonucleotides

ODN1 and ODN2

In order to show that the oligonucleotides prepared by the synthetic methods here had the biochemical behaviors expected for fC and hmC containing DNA strands, duplexes, **ODN1-ODN4** and **ODN2-ODN4** were subjected to thermal denaturation analysis. Figure 2-6A

Figure 2-6. Melting Analysis of epigenetic modified duplexes. (A). UV-vis absorption denaturing profiles and melting temperatures (standard deviations on last decimal are in parentheses) of duplexes **ODN1-3/ODN4**; (B). Differences of melting temperatures between well-matched fC containing duplex (**ODN1/ODN4**) and mismatched duplexes (**ODN1/ODN5-7**).

showed that both epigenetic duplexes can undergo thermal dissociation to single strands and UV-vis absorption at 260 nm accordingly showed a sigmoid transition similar to unmodified wild type duplex, **ODN3-ODN4**. The transition of UV-vis absorption due to denaturation of duplex DNA completed within the similar temperature ranges, around 20 degree celsius, for both epigenetic modified and natural duplexes, which indicated that fC and hmC modified duplexes can un/fold between two major secondary structures as wild type DNA. Upon fitting the denaturing curves with sigmoid function, fC containing duplex, **ODN1-ODN4** ($T_m=62.3$ °C) showed similar melting temperature as natural duplex (62.1 °C), while hmC modified duplex, **ODN2-ODN4** melted at apparently lower temperature (61.0 °C). The trend is consistent with the literatures.^[29, 30] Furthermore,

thermal stability of fC base paired to guanine versus the other three natural nucleobases was examined by melting analysis of duplexes with mismatches at fC site, **ODN1-ODN5/6/7** (table 2-1 and table 2-5). All three

Table 2-6. Melting temperatures of epigenetic modified duplex DNA

Duplex	T_m (°C)	ΔT_m (°C)
ODN1/ODN4	62.25(9)	
ODN2/ODN4	61.0(1)	
ODN3/ODN4	62.05(5)	
ODN1/ODN5	56.8(2)	5.5(3)
ODN1/ODN6	54.74(6)	7.5(1)
ODN1/ODN7	54.0(2)	7.3(3)

mismatches with fC showed much lower melting temperatures than that of well-matched duplex ($\Delta T_m = T_{m,ODN4} - T_{m,ODN5/6/7}$ is between 5 to 7 °C, Figure 2-6B, Table 2-6), though fC-A in **ODN1-ODN5** exhibit 2 °C higher in T_m than the rest two mismatches with pyrimidines. fC can readily distinguish not only well-matched guanine from mismatched bases, but also adenosine from pyrimidine mismatches. The thermal denaturation of fC and hmC containing oligonucleotides prepared by our protocol satisfied with the criteria of canonical dissociation transition, defined melting temperatures and sensitivity on thermal stability of base pairing, which indicates these epigenetic oligonucleotides perform the same biological functions and behaviors as those prepared by other literature protocols.

2.3.4 Reaction Condition Optimization for hmdU Preparation

The synthetic routes to prepare hmC and fC phosphoramidite described in section 2.3.1 and 2.3.2 are convenient in the aspect of reaction steps and experiment setup compared to reported protocols.^[15-23] With the synthetic routes developed in our lab, hmC phosphoramidite was prepared from commercial available nucleoside in six steps, while

fc phosphoramidite in four steps. However, the overall yields were not ideal: 3% for hmc phosphoramidite while 12% for fc phosphoramidite. It is easy to conclude that yields of dT/mdC oxidation by $\text{Na}_2\text{S}_2\text{O}_8$ to generate hmdU/fdC are a key limitation to the efficiency of our synthetic routes (Scheme 2-1, 2-2). Hence, oxidation reaction with $\text{Na}_2\text{S}_2\text{O}_8$ was optimized in reaction time, solvent, and type of heating.

First of all, the process of oxidation of dT and mdC was monitored the change of each component with reaction time. As shown in Figure 2-7A, oxidation of dT with 2 eq $\text{Na}_2\text{S}_2\text{O}_8$

Figure 2-7, components in the reaction mixture of dT (A) and mdC (B) oxidation by $\text{Na}_2\text{S}_2\text{O}_8$ varied with reaction time with conventional heating. Conversion is calculated as the percentage of hmdU/mdC in the starting material consumed for each reaction.

for 0-4 h in aqueous solution at 70 °C afforded majorly hmdU as well as fdU as a side product. The amount of hmdU and fdU accumulated with increasing reaction time while consuming starting material dT. hmdU and side product fdU were generated much slower than the speed of consumption of dT, suggesting that dT was decomposed heavily by unknown side reactions. The amount of hmdU increased only 2% for reacting from 3 h to 4 h, while dT in the solution decreased by 8%, indicating the optimal reaction time with highest conversion rate was 4 h (hmdU 21%, dT 34%, conversion 31%). The major product of the oxidation reaction with $\text{Na}_2\text{S}_2\text{O}_8$ could be tuned from hmdC to fdC by applying ACN- H_2O mixture as solvent (note: ACN percentage in the mixture should be higher than 80%) and CuSO_4 as catalyst with the presence of 2,6-lutidine. As shown in

Figure 2-7B, the highest fdC yield (38%) was achieved at 2 h reaction time with consumption of most of mdC starting material. Similar to reaction to prepare hmdU, partial mdC starting material was decomposed by oxidation with $\text{Na}_2\text{S}_2\text{O}_8$ rather than generated the desired product fdC.

From above exploration, we found that decomposition of the starting material by oxidation with $\text{Na}_2\text{S}_2\text{O}_8$ was a big obstacle to improving yield of the reaction. To achieve higher yield and conversion rate, solvent effect for dT oxidation by $\text{Na}_2\text{S}_2\text{O}_8$ oxidation was explored with conventional heating and microwave assisted heating. For conventional heating, common solvents which are miscible with water, such as DMSO, DMF, DMA, dioxane. As shown in table 2-7, elongated reaction time (8 h) in the tested solvents resulted in decomposition of starting material and no improvement of hmdU yield was observed. For organic solvent- H_2O 1:5 mixture (DMSO, DMF, DMA, dioxane and dioxane- H_2O mixture 1:1), similar yield of hmdU was achieved after reacting at 65 °C for 4 h. DMSO or DMA- H_2O 1:5 mixture showed higher conversion rate because of higher recovery of dT.

Another attempt to optimize the reaction was taking advantage of the differences between various solvents in their abilities of absorbing microwave energy, thereby, controlling the activity of the free radicals produced during the reaction. Unfortunately, organic solvents showing promising hmdU yield by conventional heating, such as DMSO, DMA, did not show idea yield of hmdU under microwave assisted heating. Since the solvents tested in previous experiments were not volatile, thus suffering from potential difficulty in workup of the reaction mixture, more volatile solvents, such as THF was preferred for the reaction. Reacting in THF/ H_2O 1:1 under microwave for 15 mins affords 52% hmdU (based on thymidine consumed), which is more practical and promising condition for the reaction.

Though our exploration of the optimized reaction condition for dT oxidation with $\text{Na}_2\text{S}_2\text{O}_8$ did not show dramatic increase yield of hmdU, reaction time was largely reduced under microwave assisted heating. In addition, the volatile THF could be easily removed by evaporation during the postsynthetic workup compared to high boiling point solvents.

Table 2-7. Solvent effect for dT oxidation by $\text{Na}_2\text{S}_2\text{O}_8$ oxidation under conventional heating

Entry ^a	Solvent	$V_{\text{Org}}/V_{\text{H}_2\text{O}}$	Time (h)	Composition of reaction mixture			Yield ^c
				dT/ % ^b	hmdU/ % ^b	fdU/ % ^b	
1	DMSO	1:5	4	72	15	6	54
2	DMSO	1:5	8	72	13	5	46
3	DMSO	1:1	4	71	9	8	31
4	DMSO	1:1	8	60	8	3	20
5	DMF	1:5	4	33	19	19	28
6	DMF	1:5	8	13	6	5	7
7	DMF	1:1	4	37	10	7	16
8	DMF	1:1	8	38	10	6	16
9	DMA	1:5	4	72	15	6	54
10	DMA	1:5	8	72	13	5	46
11	DMA	1:1	4	90	7	3	70
12	DMA	1:1	8	82	6	2	33
13	Dioxane	1:5	4	33	18	5	27
14	Dioxane	1:5	8	4	7	0	7
15	Dioxane	1:1	4	40	17	4	28
16	Dioxane	1:1	8	11	1	0	1
17	H₂O		4	52	14	3	29

^a Screening condition: 0.016 mmol dT and 0.032 mmol $\text{Na}_2\text{S}_2\text{O}_8$ was dissolved in 0.1 mL solvent mixture. The reaction solution was carried out in 1.5 mL microtube on heating block at 65 °C.

^b Recovery rate/yield was determined by HPLC analysis normalized to reaction mixture before heating.

^c Yield calculated by normalized to starting material dT consumption.

Table 2-8. Solvent effect for dT oxidation by Na₂S₂O₈ oxidation under microwave heating

Entry ^a	Solvent	V_{Org}/V_{H_2O}	Time (min)	Composition of reaction mixture			Yield
				dT/ % ^b	hmdU/ % ^b	fdU/ % ^b	
1	DMSO	1:5	5	88	4	0	33
2	DMSO	1:5	15	70	9	2	30
3	DMSO	1:5	30	54	3	1	6
4	DMSO	1:1	5	86	3	2	6
5	DMSO	1:1	15	72	5	5	18
6	DMSO	1:1	30	37	3	4	5
7	DMA	1:5	5	93	4	1	57
8	DMA	1:5	15	69	11	3	35
9	DMA	1:5	30	41	8	3	14
10	DMA	1:1	5	86	3	1	21
11	DMA	1:1	15	76	4	2	17
12	DMA	1:1	30	29	2	0	3
13	THF	1:5	5	89	4	0	36
14	THF	1:5	15	70	9	2	30
15	THF	1:5	30	41	13	3	22
16	THF	1:1	5	87	5	0	38
17	THF	1:1	15	71	15	4	52
18	THF	1:1	30	50	16	5	32
19	H ₂ O		5	91	1	1	11
20	H ₂ O		15	78	6	2	27
21	H ₂ O		30	35	8	1	12

^a Screening condition: 0.064 mmol dT and 0.128 mmol Na₂S₂O₈ was dissolved in 0.4 mL solvent mixture. The reaction solution was carried out in 10 mL microwave tubes at 65 °C under microwave assisted heating (dynamic mode).

^b Recovery rate/yield was determined by HPLC analysis normalized to reaction mixture before microwave assisted heating.

^c Yield calculated by normalized to starting material dT consumption.

2.4 Summary and Outlook

In this chapter, we have developed novel synthetic pathways for facile preparation of hmC and fC containing oligonucleotides. Upon introducing organic solvent components and copper catalysts, C5-methyl groups of mdC and dT were readily oxidized to formyl and hydroxyl functionality, respectively. By applying reaction directly to nucleosides, repetitive de/protection steps of precursor are omitted and both epigenetic cytosine phosphoramidites can be achieved within only four to six steps. Standard solid phase DNA synthesis and conventional deprotection methods were applicable to synthesize 5-formyl- or 5-hydroxymethyl-cytosine containing DNA oligonucleotides. These methods

are useful for preparing epigenetic DNA oligonucleotides with ready adaption to prevailing instrumental setup and would provide significant convenience for the investigation of the biological functions of the newly discovered epigenetic bases.

The synthetic DNA oligonucleotides containing hmC and fC were used to study the effect of epigenetic modifications on DNA thermal dynamic stability. Duplex DNA with fC showed similar melting point as unmodified duplexes, while hmC destabilized the DNA sequence tested by 1°C. Though these changes in melting point of DNA duplex is not apparent, effect of epigenetic modification on DNA mediated charge transport is ineligible, which will be discussed in detail in chapter 4.

At last, optimizations of oxidation reaction to prepare key intermediates, hmdU and fdC, of the synthetic pathways were discussed. The exploration of the optimized condition for peroxysulfate oxidation reaction to convert dT and mC to hmdU and fdC, revealed the limitation of the reaction using Na₂S₂O₈ as oxidant. The advantage of the reaction is that natural nucleoside could be employed as substrate without requirement of protection on 3' and 5' hydroxyl functionalities. In the aspect of design of the synthetic routes for hmC and fC phosphoramidite, our strategies were highly efficient and its application could be hugely broadened if higher yield could be achieved in the oxidation reaction in the future.

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CHAPTER 3

Spontaneous Deamination of Epigenetic Cytosines

3.1 Introduction

The biological functions of the newly discovered epigenetic cytosines, hmC, fC and CaC, in active demethylation of mammalian embryonic stem cells (ESC) and neuron cells have attracted extensive attention and are under heated debate. Recently contradictory experimental results have been obtained with regard to the roles of potential deaminases in active demethylation process.^[1-4] Activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA-editing-catalytic polypeptides (APOBECs) family proteins are responsible for enzymatic deamination of dC and generation of massive mutagenesis sites of DNA.^[5-7] Reduced hmC level and accumulation of 5-hydroxymethyluracil (hmU) were achieved upon overexpression of AID/APOBEC in neurons, which suggested a two-step oxidation-deamination active demethylation mechanism as depicted in Scheme 3-1.^[3] An alternative mechanism suggests oxidation of mC to hmC and further to fC and CaC by TETs (ten-eleven translocation enzymes) might induce removal of epigenetic base via base excision repair (BER). Moreover, studies have shown that mammalian AID is required to initiate reprogramming pluripotency of human somatic cells via active DNA demethylation.^[2] However, the accumulated findings that AID has much less favoured *in vitro* activity for epigenetic cytosines, mC and hmC, compared to unmodified cytosine challenge oxidation-deamination mechanism of active demethylation.^[1, 4]

Spontaneous deamination of nucleic acids is a crucial source of potential mutagenesis of DNA in cells.^[8-12] Changes in structure of nucleobases by deamination, such as adenine (A) to hypoxanthine, guanine (G) to xanthine and cytosine (C) to uracil (U), alter hydrogen bonding manner of nucleobases, thus resulting in misincorporation of A-T to G-C or G-C to

Scheme 3-1. Scheme of the proposed mechanisms of 5-methylcytosine demethylation pathways in eukaryotes.

A-T in DNA sequence during replication. The rate constant of cytosine deamination was found to be $1 \times 10^{-10} \text{ s}^{-1}$ and $0.4 \times 10^{-10} \text{ s}^{-1}$ to $1.3 \times 10^{-10} \text{ s}^{-1}$ respectively in single stranded and mismatched DNA at 37 °C pH 7.4.^[13, 14] It is believed that protonation of N3 position of cytosine plays a key intermediate role for the deamination reaction of cytosine monomer in neutral and acidic condition.^[15-17] For this reason, Watson-Crick hydrogen bonding is proposed to protect nucleobases from deamination via inhibition of protonation at N3 position by stimuli of the surrounding environment. Spontaneous deamination of cytosine was found to be much smaller at a rate of $7 \times 10^{-13} \text{ s}^{-1}$ in aqueous solution under 37 °C pH 7.4 in double stranded DNA.^[13] Besides canonical nucleobases of DNA, deamination of the epigenetic base, 5-methylcytosine, was discovered to occur in vitro. Through alternating the basicity of cytosine by C5-methylation, deamination rate of 5-methylcytosine was facilitated by 2-3 folds in double stranded DNA.^[11, 18] The resulting T-G mismatch was poorly repaired by very short-patch (VSP) repair system, hence, rendering mC as a hot spot of mutagenesis.^[18]

Previously, spontaneous deamination of dC was studied at acidic, basic and neutral conditions.^[11, 13, 14, 19-24] Protonated cytosine may undergo deamination via OH^- or water 5,6 addition intermediate, or via nucleophilic displacement of the amino group of cytosine (Scheme 3-2). For hydrolytic deamination in acidic conditions, shallow pH dependence was found and depyrimidination of cytosine derivatives was more intense compared to deamination in neutral and basic solutions.^[19, 20, 23-25] For heat-induced deamination at neutral pH, deamination, oxidation and cleavage of glycosylic bond of cytosine were observed at the same time in aqueous solutions.^[24]

Scheme 3-2 Mechanisms for deamination of cytosine. The graph is redrawn from ref ^[12].

Hence spontaneous deamination of the newly discovered epigenetic cytosines, mC, hmC and fC is of great interest and is discussed at the nucleoside level in this chapter. Alkaline treatment induced stoichiometric deamination of dC, mdC and hmdC at different rates which were associated with the C5 functionality of cytosine derivatives. fdC reacted at strong basic condition and generated two new products which were not the corresponding deamination product fdU.

3.2 Experimental

3.2.1 Reagents and Materials

Nucleosides used in the study, thymidine, 2'-deoxycytidine, 2'-deoxyuridine were purchased from Alfa Aesar and TCI/Teohai. The epigenetic nucleosides (mdC, hmdC, fdC) and standard for hmdC deamination product 5'-hydroxymethyl-2'-deoxyuridine were prepared by following the synthetic routes developed in Chapter 2 and were verified by LC-MS (ThermoFinnigan LCQ FLEET MS). NaOH is common reagent in reagent grade.

3.2.2 General Procedure for Deamination Assay

200 μ L of 0.5 mM cytosine nucleoside (dC, mdC, hmdC) solutions with various concentrations (0.1 M, 0.3 M, 0.5 M and 1 M) of NaOH were incubated at 37 °C for 8, 12, 24, 48, 96, and 144 h, respectively. After incubation, to the solution was added 1 M TEAA buffer, pH 7.0 or 10% v/v acetic acid (200 μ L). After dryness by lyophilization (Labconco, 0.010 mbar), the samples were dissolved in 0.1M TEAA buffer (pH 7.0, 400 μ L). 3.26 mM dA (10 μ L) was added to each sample as external standard and 80 μ L solution from the mixture was submitted to HPLC analysis (Shimadzu Prominence Modular HPLC). The HPLC gradient was the same as described in chapter 2 section 2.2.4.

3.3 Results and Discussion

3.3.1 Deamination Reactions of Epigenetic Cytosine Nucleosides

Spontaneous deamination of epigenetic cytosines (mC, hmC) as well as canonical cytosine by in basic condition was explored at the nucleoside level by incubation of dC, mdC and hmdC in NaOH at 37 °C. After reaction, 1 M TEAA buffer or 10% acetic acid were added to each sample to neutralize the solution and to quench the reaction. After dryness, the composition of the reaction mixture with external standard dA was analysed

by HPLC. As shown in figure 3-1 A-C, incubation of dC, mdC, and hmdC with 0.1 M NaOH at 37 °C for 144 h resulted in generation of a new product that eluted earlier than the corresponding cytosine derivative in each HPLC trace. The maximum UV absorption wavelengths λ_{max} of the new products matched those of corresponding deamination products of dC, mdC and hmdC, i.e. dU (261 nm), dT (266 nm) and hmdU (264 nm). Co-injection of the reaction solution with corresponding standard 2'-deoxyuridine derivatives (data not shown) as well as collection of each new product for LC-MS characterization confirmed that the structures of new products shown in each HPLC trace matched deamination products of dC, mdC and hmdC: dU $[M+H]^+$ m/z calcd for $C_9H_{13}N_2O_5^+$ 229.0819, found 229.0813; dT $[M+H]^+$ m/z calcd for $C_{10}H_{15}N_2O_5^+$ 243.0975, found 243.0973; hmdU $[M+H]^+$ m/z calcd for $C_{10}H_{15}N_2O_6^+$ 259.0925, found 259.0917. No other substantial products were observed within 144 h reaction time upon alkali treatment at 37 °C, though prolonged reaction time caused some (less than 5%) subsequent glycosidic bond cleavage of the deamination products of cytosine derivatives, such as formation of U from dU (Figure 3-1A).

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Figure 3-1 Deamination products of cytosine derivatives analysed by HPLC. A, B, C are dC, mdC, hmdC after incubation in 0.1 M NaOH at 37 °C for 144 h; D is incubation of fdC in 0.1 M NaOH at 37 °C for 60 min. Maximum absorbance wavelengths of the nucleoside species are indicated.

Ratio of nucleoside moieties (uracil derivatives + cytosine derivatives) in reaction solution versus starting material (R), $R = ([U] + [C]) / [C_0]$, was calculated to examine the stoichiometry of the epigenetic cytosine deamination reaction, where $[U]$, $[C]$ and $[C_0]$ were the concentrations of uracil derivatives, cytosine derivatives at each reaction time point and starting material cytosine derivatives before reaction. $[U]$, $[C]$, $[C_0] \propto$ peak areas of corresponding uracil derivatives and cytosine derivatives in HPLC trace, and could be presented by division of peak area versus corresponding extinction coefficient ϵ . As shown in Figure 3-2, R values of dC, mdC, and hmdC for reaction time from 0 to 144 h were around 1.00 even for samples with the highest NaOH concentration (1 M) used in this study. Variation of the R values was less than 0.02 for dC and mdC and less than 0.05 for hmdC. The above results clearly suggested that NaOH treatment stoichiometrically converted dC, mdC and hmdC to the corresponding deamination products.

Figure 3-2 Ratio of nucleoside moieties in reaction solution vs starting material (R) in 1 M NaOH after 144 h reaction.

For alkali treatment of fdC, two peaks other than starting material fdC were observed in HPLC trace after incubation of fdC in 0.1 M NaOH at 37 °C for 1 h. The peak X1 at 8.6 min had $\lambda_{\max}$ at 252 nm, while the product eluted at 13.6 min (X2) had $\lambda_{\max}$ at 282 nm.

The reaction was much faster (based on starting material consumption) than other cytosine derivatives, dC, mdC and hmdC. Though λ_{max} of X2 was close to that of fdU (282 nm) which was the deamination product of fdC, the retention time for fdU overlapped with neither X1 nor X2. To characterize the products of fdC from NaOH treatment, unknown moiety X1 and X2 were collected and submitted to LC-MS analysis and results are shown in Figure 3-3. From the positive and negative mode mass spectrums of X1 (Figure 3-3A, B), we were not able to find a reasonable fragmentation structure for X1, whose molecular weight was around 117.1220 Da. Comparing X1 mass spectrums with fC base mass spectrum (Figure 3-3E), we could only propose that the unknown moiety X1 could be fC base or a fragment from fC base. From the positive and negative mode mass spectrums of X2, (Figure 3-3C, D), we found that X2 had the exact same molecular weight as fdC molecule ion ($[M+H]^+$ m/z calcd for $C_{10}H_{14}N_5O_5^+$ 256.0928, found 256.0916). Unfortunately, we were not able to give a reasonable structure for X2 either. Nevertheless, neither of the detected molecular weight of X1, X2 matched that of fdU, the deamination product of fdC, indicating deamination was not the major reaction for fdC in strong basic solution. We attributed the failure of observing deamination product of fdC upon alkali treatment to the fact that fdC could undergo much faster glycosylic bond cleavage than other cytosine derivatives (dC, mdC and hmdC) and other reaction channels were possible under highly basic conditions.^[26] To elucidate the products of fdC treatment with NaOH, further exploration and characterization are needed.

Figure 3-3 Mass spectrum of peak eluted at 8.6 min (X1) and 13.6 min (X2) in HPLC from fdC treated by NaOH and fC base standard. (A) X1, positive ion mode; (B) X1, negative ion mode; (C) X2, positive ion mode; (D) X2, negative ion mode; (E) fC base, positive ion mode.

3.3.2. Deamination Reaction Kinetics of dC, mdC and hmdC

Having confirmed that NaOH was potent to induce deamination of cytosine derivatives to stoichiometrically generate corresponding uracil derivatives, deamination reaction kinetics of dC, mdC and hmdC was investigated by varying concentrations of NaOH from 0.1 M to 1.0 M. The second order reaction equation for cytosine derivative deamination reaction could be written as

where C represents cytosine derivatives, U represents uracil derivatives, the deamination products and n is the number of OH^- participating the reaction. The reaction rate v could be calculated by:

$$v = -\frac{d[C]}{dt} = k[C][OH^-]^n \quad \text{Eq 3.2}$$

where k is a constant; $[OH^-]$ is the concentration of OH^- , which we assumed to be constant during reaction since OH^- was in excess; t is the reaction time. For second order reaction, the Eq 3.2 should satisfy two conditions, i.e. k is constant and $n=1$. Eq 3.2 could be transformed to Eq 3.3, as described below:

$$\ln \frac{[C]}{[C_0]} = -k[OH^-]t \quad \text{Eq 3.3}$$

where t_0 is when reaction time is 0 h. Since we have proved that conversion of cytosine derivatives to uracil derivatives by OH^- is stoichiometry ($R=1.00$), i.e. $[C_0]=[U]+[C]$, and concentration of each nucleoside component is directly proportional to its peak area divided by corresponding extinction coefficient ϵ , $[C]/[C_0]$ could be calculated by:

$$\frac{[C]}{[C_0]} = \frac{\text{Area of Cytidine derivative}/\epsilon_1}{\text{Area of Cytidine derivative}/\epsilon_1 + \text{Area of Uridine derivative}/\epsilon_2} \quad \text{Eq 3.4}$$

Based on the above calculation, peak areas of cytosine derivatives were compared to the total nucleosides in the solution ($[C]/[C_0]$). As shown in Figure 3-4A, C, E, dC, mdC and hmdC starting material left in the solution after deamination showed NaOH concentration dependence. Faster consumption of cytosine derivative starting material by

Commented [SF(P3)]: Peak areas of uridine derivatives is not [A] but [B] or equals to [A0]-[A] if conversion is stoichiometric.

deamination was consistent with higher concentration of NaOH, suggesting faster deamination of dC, mdC and hmdC by higher OH^- concentration. This result was consistent with previous findings.^[20] Logarithm of $[C]/[C_0]$ was in good linear dependence with incubation time (t), suggesting k was constant for deamination reaction by alkali treatment, i.e. no other reaction pathways were opened (Figure 3-4 B, D, F). Eq 3.3 could be rewritten as:

$$\log K = \log k + n \log [OH^-] \quad \text{Eq 3.5}$$

where K is rate constant of the reaction, $K=k[OH^-]^n$. Figure 3-5 shows the plot of $\log K$ with pH. The bigger Y value represents bigger rate constant K at each pH and the slope of the linear fitting curve is n . As shown in Figure 3-5 deamination rate of dC, mdC and hmdC below pH 13.3 followed the order: hmdC>mdC>dC, while the order changed to mdC>hmdC>dC above pH 13.3. Deamination rates of mdC and hmdC were much faster than dC at all pH, which could be due to C5 functionality of mdC and hmdC. The electron donation effects of C5 methyl and hydroxymethyl groups of mdC and hmdC apparently facilitated deamination reaction. In addition, due to the apparent smaller slope of hmdC than mdC, one could easily expect that deamination rate of hmdC at lower pH will be much bigger than that of mdC, as shown in Table 3-1. Rate constant $K=1.33 \times 10^{-11} \text{ s}^{-1}$ at 7.4 pH for hmdC was one order of magnitude bigger than that of mdC ($2.28 \times 10^{-12} \text{ s}^{-1}$) and dC ($7.73 \times 10^{-13} \text{ s}^{-1}$). n values of dC and mdC were closed to 1, which was consistent with our assumption of second order reaction. n value was much smaller for hmdC (0.80), suggesting less OH^- was needed for deamination reaction of hmdC, which could be a result from the hydrogen bonding formed between lone pair electrons of C5 hydroxymethyl group and N4 amino group of hmdC aromatic structure. The involvement of C5 hydroxymethyl group of hmdC in deamination process was also supported by the observation that deamination rate increment with NaOH concentration was inhibited at high pH>13.3. Partial deprotonation of C5 hydroxymethyl by strong basic solution might

reduce the reactivity of C4 nucleophilic reaction. The rate constants we obtained were 1-3 orders of magnitude smaller than those reported by Carrel's group who investigated heat-induced deamination.^[24] However, inducing deamination of cytosines by heat might open other reaction channels, such as oxidation and C-C bond cleavage, which had the risk of accelerating deamination reaction as well as other reactions.

Figure 3-4 Cytosine derivative deamination reaction kinetics with alkali concentration of dC, mdC and hmdC. $[C]/[C_0]$ changes with reaction time in 0.1 M to 1.0 M NaOH for dC (A), mdC (C) and hmdC (E). Logarithm of $[C]/[C_0]$ changes with reaction time in 0.1 M to 1.0 M NaOH for dC (B), mdC (D) and hmdC (F).

Figure 3-5 plot of a reaction rate related parameter K with pH concentration.

Table 3-1 Deamination rate constant of cytosine derivatives by NaOH at 37 °C, pH 7.4

	$K_{37^{\circ}\text{C}}/\text{s}^{-1}$	n^a
dC	7.73×10^{-13}	0.96(2)
mdC	2.28×10^{-12}	0.93(1)
hmdC	1.33×10^{-11}	0.80(1)

^a n : number of OH^- involved in the reaction, the error is indicated in bracket.

3.4 Summary and Outlook

In summary, we have studied deamination by alkali of canonical DNA nucleoside dC as well as epigenetic cytosines mdC, hmdC and fdC. Efficient and stoichiometric deamination was achieved for dC, mdC and hmdC with 0.1 M to 1.0 M NaOH treatment. Treatment of fdC with NaOH resulted in cleavage of glycosylic bond of fdC and configuration changes of fdC. Kinetics study was performed at 37 °C for dC, mdC and hmdC incubated with NaOH and the deamination reaction was found to be a second order reaction. C5 methylation and hydroxymethylation had apparent facilitation of deamination of cytosine. dC and mdC were found to be deaminated by one equivalent of OH^- , while less than one equivalent of OH^- was required to deaminate hmdC. The OH of C5-hydroxymethyl group of hmdC facilitated deamination of hmdC at lower pH; while deprotonation of OH at high pH had inhibitory effects on deamination reaction. Our experiment studied spontaneous deamination of epigenetic cytosines by alkali treatment

Commented [SF(P4)]: isn't it a second order reaction rate? why pseudo?

at lower temperature, which reduced the risk of changing reaction kinetics by energy input compared to heat-induced deamination studies reported by others.

Application of the methods developed in this project to DNA level was not successful for the moment (data not shown), which could be attributed to overlap of various deamination products with starting material nucleosides in the DNA. For example, dT in DNA sequence is also deamination product of mdC, thus is not differentiable. In addition, structures of products of fdC treated by NaOH are not clear, hence, more efforts are needed to characterize the products. Previous studies have suggested that spontaneous deamination is more difficult in single stranded DNA compared to duplex DNA. However, mC is more susceptible towards deamination compared to regular cytosine in both single and double stranded DNA. Therefore, we expect our findings of spontaneous deamination at the nucleoside level will be useful to indicate the trend of epigenetic cytosine deamination at the DNA level.

Nevertheless, our study revealed effects of varied C5 functionalities of cytosine derivatives on spontaneous deamination kinetics of epigenetic cytosines. The study clearly showed various reactivities of different epigenetic cytosines with *OH* at low temperature, which could be important to understand the higher mutagenesis for epigenetic cytosines.

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CHAPTER 4

Effect of Epigenetic Modifications on DNA Mediated Charge Transport

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4.1 Introduction

Epigenetic bases, 5-methylcytosine (mC), 5-hydroxymethylcytosine (hmC) and 5-formylcytosine (fC), are identical to canonical cytosine in structure except for the functionalities at C5 position of the pyrimidine ring (Figure 4-1). Minor variations of C5 substituents result in distinct biological roles and physical properties of epigenetic cytosines, such as enzymatic activities, spontaneous deamination, DNA strand separation, proton transfer in G-C base pair and et al.^[1-6] Thymine DNA glycosylase (TDG) is known for high specificity towards G-T/U mismatch and a critical role in base excision repair (BER).^[7, 8] Recent study found that TDG rapidly cleaved fC from the sugar backbone in well-matched DNA duplex, however, left C, mC and hmC nearly unreacted.^[3] This finding as well as other studies suggested that glycosidic bond strength of cytosine nucleoside is weakened by C5 formylation of cytosine, however, is not greatly affected by methylation and hydroxymethylation at C5 position. Another explanation to the enhanced TDG activity proposed that fC might adopt an imino tautomer conformation to form mismatch-like wobble hydrogen bonding pattern with the facing guanine.^[2] Furthermore, epigenetic modifications were found to affect DNA stability and local duplex structure. Thermal stability of duplex DNA is increased by mC; however, the effect is dampened with hmC and fC.^[6, 9] mC provides extra stability for i-motif structure in diluted aqueous solution, while hmC causes destabilization.^[1] Molecular force assay revealed that mC and hmC might alter mechanical properties of DNA hence affecting strand separation pattern of DNA.^[4, 5] The biological consequences of mC, hmC and fC are of great interest and further investigations are demanded to depict a comprehensive scenario of biological functions of the new epigenetic bases.

Figure 4-1 Structure of epigenetic cytosine derivatives.

DNA charge transport (CT) opened a novel pathway for DNA to function as a bridge to modulate "reaction at a distance", which could have significant biological consequences. The biological context of DNA CT in a wide range of timescale, from nanosecond to millisecond, covers aspects, such as funneling mutagenic oxidation stress to specific sites and cooperation among proteins by signaling via DNA duplex.^[10-17] Guanine is most easily oxidized among canonical nucleobases (A, T, G, C) due to its lowest oxidation potential.^[18] The resultant G^{+} might react within its lifetime (millisecond) with O_2 or H_2O to generate 8-oxo-guanine (^{8oxo}G) which has mutagenesis role in its metabolism procedure. The ionization potential of G is lowered by consecutive guanines, such as GG doublet and GGG triplet, rendering them as natural traps for the migrating oxidative cation radicals.^[19-21] Photo-irradiation of HeLa cell nuclei DNA conjugated with $[Ru(phi)_2bpy]^{3+}$ caused oxidative lesion at G rich sites, suggesting DNA charge transport can occur in nuclei DNA.^[10] Studies examining oxidative lesion pattern of mitochondrial DNA by charge transport suggested a plausible mechanism adopted by cell to funnel the harmful oxidative damage to a G-rich regulatory element thus preventing the damaged mitochondrial genome from being replicated.^[11, 12] Therefore, one of the biological significances of DNA CT is funneling oxidative damage to the G regions at millisecond timescale (long timescale).

On the other hand, DNA CT happening at a shorter timescale (nanosecond) does not cause oxidative G damage on the DNA duplex due to the low trapping rate of G^{+} . However, several DNA repair proteins, such as MutY and Endonuclease III (EndoIII), were found to fulfill their functions by adopting the fast DNA CT (nanosecond) for

signaling between proteins.^[13-17] DNA repair proteins are responsible for repairing damaged bases from various sources including oxidative radicals. MutY and EndoIII both contain in the structure [4Fe-4S] clusters which are able to be oxidized by guanine radicals.^[13, 14] The binding affinity of MutY and EndoIII to DNA is associated with redox status of the [4Fe-4S] cluster which could be regulated by DNA CT.^[14-17] Therefore, it was proposed that rapid searching for mismatch within genome, binding and dissociating from DNA duplex are enabled via DNA CT between the [4Fe-4S] clusters of DNA repair proteins. Despite the functions described above, exploration of DNA CT in other biological processes, such as epigenetics, is still in progress. To further explore epigenetic effects on DNA CT, we reported here study of DNA CT in long timescale (milisecond) and short timescale (picosecond) with mC, hmC and fC modified DNA.

4.2 Experimental

4.2.1 General Remarks

Reagents used in the study, anthraquinone-2-carbonyl chloride (CAS NO. 6470-87-7), 2-aminoethanol (CAS NO. 141-43-5), 2-cyanoethyl diisopropylchlorophosphoramidite (CAS NO. 89992-70-1), *N,N*-diisopropylethylamine (CAS NO. 7087-68-5), triethylamine (CAS NO. 121-44-8) were obtained from TCI, Alfar Aesar and Sigma-Aldrich. Other regular chemical reagents and buffers were prepared in our lab with reagent grade chemicals and deionized water. HPLC analysis and purification were performed on Shimadzu Prominence Modular HPLC with mobile phase A, 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.0 and mobile phase B, ACN (HPLC-grade).

4.2.2 Oligonucleotides Preparation

DNA phosphoramidites (dA, dG, dC, dT), 2-F-dI-CE phosphoramidite (convert to ^{CPG}), mC phosphoramidite as well as reagents and CPG columns for solid phase DNA

synthesis were purchased from Glen Research and Berry & Associates. hmC and fC phosphoramidites were synthesized as described in chapter 2. End-capping AQ (^{ec}AQ) phosphoramidites were synthesized as reported by Schuster's.^[22] All oligonucleotides were synthesized on a MerMade 4 DNA Synthesizer (BioAutomation) followed by HPLC purification twice and verification of molecular weight by ESI-MS (Sangon Biotech, Shanghai). Standard protocol of solid phase DNA synthesis was applied to synthesize the oligonucleotides listed in Table 4-1 except for those with unnatural base or ^{ec}AQ where elongated coupling time (5-15 min) was applied to enable high yield. FAM labeled oligonucleotides were purchased from Sangon Biotech (Shanghai, HPLC purification) and used as received. After synthesis, oligonucleotides were cleaved from beads by incubation in 28% NH₄OH at room temperature. Oligonucleotides with hmC and fC modifications were deprotected by incubating in concentrated NH₄OH at 75 °C for 24 h. Oligonucleotides with ^{CP}G were cleaved from beads by incubation in 0.5 M DBU acetonitrile solution for 20 min and deprotected with 6 M aqueous cyclopropylamine at 60 °C for 16 h. Concentration of DNA oligonucleotide was determined by UV-Vis spectroscopy (Shimadzu UV-1800 UV-Vis spectrophotometer).

4.2.3 Photo-irradiation Assay and HPLC Analysis

A 450 Watt Illuminator (HORIBA Jobin Yvon) with a monochromater and a 320 nm long-pass filter was used for all the photo-irradiation experiments. Prior to photo-irradiation, DNA solution (30 µL, 10 µM, in 20 mM PBS, pH 7.4) was annealed by heating at 90 °C for 5 min and cooling down to room temperature in 2.5 h to ensure duplex DNA structure during photo-irradiation. The annealed DNA solution was mixed homogeneously and transferred to 1.5 mL microtube for photo irradiation at 350 nm for 30 s. After irradiation, duplex DNA was digested to free nucleosides by incubation with phosphodiesterase I (20240Y, i-DNA biotechnology) and alkaline phosphatase (M0290S, New England Biolabs) at 37 °C for 18 h according to the reported recipe.^[23] The residues

were submitted to HPLC analysis (dilution with 70 μ L deionized water, Chemcobond 5-ODS-H, 4.6 $\times$ 150 mm). Yield of hole transport was calculated as:

$$\% \text{ decomposition of } {}^{CP}G = \left(1 - \frac{A_{irr}}{A_{non-irr}}\right) \times 100\%$$

where A_{irr} and $A_{non-irr}$ peak areas of ${}^{CP}G$ in HPLC trace of DNA samples after and before irradiation at 350 nm for 30 s. All samples were repeated three times and normalized to peak areas of internal standard dT.

4.2.4 Photo-irradiation Assay and Gel Electrophoresis

FAM labelled duplex DNA (sequence in Table 4-1, 40 pmol, 30 μ L in 20 mM PBS, pH 7.4) was annealed and irradiated for 30 min as described above. After irradiation, DNA was treated with 10% v/v piperidine aqueous solution at 95 $^{\circ}$ C for 20 min to induce cleavage at the damaged guanine resulted from charge transport. The samples were washed with 100 μ L water twice and lyophilized to dryness. After mixing with loading dye (6 μ L, 6 $\times$, Fermentas) containing 50 mM NaOH and being denatured at 95 $^{\circ}$ C for 2 min, the DNA sample (2 μ L for each sample) was analysed by 20% denaturing polyacrylamide gel electrophoresis (PAGE) gel (35 W 3 h, electrophoresis power supply EPS 100, GE Healthcare Life Sciences). The gel was imaged using a Typhoon EPS 1001 imager (GE Healthcare).

4.2.5 Circular Dichroism

Annealed duplex DNA solutions containing 1.5 μ M duplex DNA in 20 mM PBS pH 7.4 were used for CD measurement. Circular dichroism spectrums were scanned from 200 nm to 400 nm at 100 nm/min scanning rate with 1 cm light path and averaged by 15 repeated measurements at room temperature with baseline correction (JASCO J-1500 CD spectrometer).

4.2.6 UV melting

The thermal stability of DNA with various epigenetic modifications was studied by UV-melting assay on Shimadzu UV-2550 UV-VIS spectrophotometer with TMSPC-8 peltier controller, using a 1 cm path length 8-cell quartz cuvette. Same samples from CD measurement were used. UV melting curves were recorded at 260 nm (corrected to 330 nm) during heating from 15 °C to 90 °C in the rate of 0.5 °C /min with measurement interval of 1 °C. *T_m* values were obtained by fitting melting curves into sigmoidal function and averaged by three repeated measurements.

4.3 Results and Discussion

4.3.1 Oligonucleotide Assembly Design and Assays for Study of CT in DNA with Epigenetic Modifications

The effect of epigenetic modification is examined in the long and short timescales by photolysis assay of a classic donor-bridge-acceptor system for DNA CT. A series of DNA oligonucleotides as listed in Table 1 were prepared. Photo-oxidant end-cap anthraquinone (^{ec}AQ) at the 5' end of a 26-mer DNA oligonucleotide (**AQX** or **AQMXM**, 5'-^{ec}AQ TTC CTT TC(M)G XGC(M) GTT TCC ATA GTA TG-3', X=C, M, H, F, M=mC, H=hmC, F=fC) was adopted as the charge donor to inject a radical cation (a hole) into the DNA duplex upon photo-excitation. On the "bridge" (intervening DNA bases), epigenetic cytosines, mC (M), hmC (H), and fC (F), were modified at X position, 9 bp away from photo-oxidant in the middle of a consecutive CG repeats, in the sequence (Table 4-1). The effects of epigenetic modifications on DNA CT were examined in two situations, hypo-methylation **AQX** and hyper-methylation **AQMXM**, where M site in the sequence was modified with regular C and mC respectively. On the one hand, guanine served as the natural slow trap to react with the migrating hole for our long timescale CT study. In DNA sequence **FAM**, isolated G and GG doublet, had lower oxidation potentials than

other nucleobases, A, C, T, thus were expected to be the energy sinks to trap the migrating hole. The resulted G^+ within its lifetime (millisecond timescale) could react with O_2 and H_2O to generate G damage. In FAM labeled sequences, the damaged sites from DNA CT were cleaved at the backbone upon hot piperidine treatment and analyzed by PAGE gel (scheme 4-1). On the other hand, the transient hole occupancy in the short timescale at each site of epigenetic modified DNA was explored by photo-induced one electron oxidation of a fast kinetic hole trap *N*²-cyclopropylguanine (^{CP}G). The rate of cyclopropyl ring opening reaction was determined as $10^{11} s^{-1}$.^[24, 25] Therefore, ^{CP}G could serve as a fast kinetic hole trap to report the transient hole occupancy along the intervening bridge during hole migration.^[26] As shown in scheme 1, CT processes happening in the long timescale and short timescale are different. In the long timescale, the low trapping rate of G^+ ($k_{trap} \approx 10^3 s$) allows faster processes, such as proton transfer between G-C base pair ($k_{PT} \approx 10^3 \sim 10^9 s$) and backward charge transport, to happen. Therefore, the G damage observed is expected to be an overall outcome after several processes (Scheme 4-1). However, in the short timescale study, ^{CP}G as the fast kinetic hole trap is able to efficiently trap the migrating charge and also suppress the influences from other processes. Despite the differences between hole traps used, the DNA sequences involved in the study were identical to each other except for the epigenetic modifications at repeated CGs. Therefore, any difference in G or ^{CP}G damage by photo-induced charge transport in DNA must result from various epigenetic cytosines.

Table 4-1 DNA assemblies for study of effect of epigenetic modifications on DNA mediated charge transport

AQX^a	5'- ^{ec} AQ TTC CTT TCG XGC GTT TCC ATA GTA TG-3'
AQMXM^a	5'- ^{ec} AQ TTC CTT TMG XGM GTT TCC ATA GTA TG-3'
FAM	3'-AAG GAA AGC GCG CAA AGG TAT CAT AC-FAM-5'
^{CP}G₄	3'-AAG ^{CP} GAA AGC GCG CAA AGG TAT CAT AC-5'
^{CP}G₁₈	3'-AAG GAA AGC GCG CAA AG ^{CP} G TAT CAT AC-5'

^aX=C, M, H, F, M=mC, H=hmC, F=fC.

Scheme 4-1 Assays to investigate effects of epigenetic modifications on charge transport. (*Upper*) Photo-irradiation initiated hole injection into DNA duplex and the migrating hole was trapped by GG (slow trap)/^{CP}G (fast trap) doublets, which could be analyzed by PAGE (after piperidine treatment) or HPLC. (*Lower left*) Processes involved in long and short timescale DNA CT; (*lower right*) structures of photo-oxidant ^{ec}AQ and labelling fluorophore FAM.

4.3.2 Effects of Epigenetic Modifications on DNA Duplex Thermal Stability.

Prior to the photolysis study, thermal stability of DNA sequences used was investigated by UV-melting experiments to examine the epigenetic effects on DNA stability and make sure all DNA were in proper duplex form during photo-irradiation. Sigmoidal melting curves were observed for all DNA duplexes used (data not shown). As shown in Table 4-2, melting temperatures T_m obtained for all DNA sequences were around 60 °C, indicating DNAs were in duplex form under the experimental condition. Effects of epigenetic modifications on DNA duplex thermal stability were not pronounced with ΔT_m was less than 1 °C taking AQX as a standard. The lowest T_m value was obtained with AQF (59.1 °C) while the highest was obtained with AQMMM (61.9 °C).

Table 4-2 *T_m* values for epigenetic modified DNA

DNA	<i>T_m</i> /°C	Δ <i>T_m</i> /°C
AQC	61.0 (2)	-
AQM	61.5 (3)	0.5
AQH	61.3 (2)	0.3
AQF	59.1 (2)	-0.9
AQMCM	61.8 (2)	0.8
AQMMM	61.9 (2)	0.9
AQMHH	61.2 (2)	0.2
AQMFM	60.7 (3)	-0.3

4.3.3 Effects of Epigenetic Modifications on DNA Duplex Secondary Structure

Similar to the thermal stability study, DNA duplex secondary structures were examined by circular dichroism spectroscopy to reveal epigenetic effects. The results are shown in Figure 4-2. Standard B-form DNA structure CD spectra was observed for all the sequences used, with a positive peak at 275 nm and negative peak at 248 nm. No pronounced CD signal changes were observed for all the sequences tested except for **AQF** which showed apparent CD signal decrease at 275 nm and 248 nm, indicating less base stacking and changes in backbone orientation. However, similar changes of CD signal were not observed for hyper-methylated fC sequence **AQMFM**.

Figure 4-2 CD spectra of **AQX** and **AQMXM** (X=C, M, H, F, M=mC, H=hmC, F=fC) with complementary (normal base).

4.3.4 Oxidative Damage of G via DNA CT in Epigenetic Modified DNA

Photo-irradiation of DNA duplex **AQX(AQMXM)/FAM** at 350 nm for 30 min caused oxidative damage via DNA CT to G sites. Hot piperidine treatment resulted in strand cleavage at the damaged G sites, which could be analyzed by polyacrylamide gel electrophoresis (PAGE) assay. Autoradiogram of PAGE assay (Figure 4-3) showed that substantial strand cleavage was achieved at each guanine site by DNA CT, leaving other bases undamaged, which was consistent with oxidation potentials DNA bases.^[18] Same as previously reported, a typical 5' G (G₄ and G₁₈) damage preference at GG doublets was observed.^[22, 27] Quantitative analysis of G damage revealed effects of epigenetic cytosine modifications on long range DNA CT (Figure 4-4).

Figure 4-3 Autoradiogram of PAGE analysis of strand cleavage from irradiation of **AQX** and **AQMXM** duplex at 350 nm for 30 min.

Oxidative G damage ratios at 5' G (G₄ and G₁₈) of proximal and distal GG doublets by DNA CT were summarized in Figure 4-4A, B. As shown in Figure 4-4A, DNA CT resulted in similar G damage at proximal GG doublet for C, mC, hmC and fC, modified sequences in both hypo-methylated sequences **AQX** (10-12%) and hyper-methylated

sequences **AQMXM**(8-13). A more obvious trend of G decomposition with various epigenetic cytosine modifications in the sequence was observed at the distal GG doublet, as shown in Figure 3B, $C \approx mC > hmC > fC$. Generally speaking, hyper-methylated sequences **AQMXM** showed higher G decomposition than corresponding hypo-methylated sequences **AQX** except for that G decomposition percentage was closed in **AQC** ($16 \pm 1\%$) and **AQMCM** ($16 \pm 2\%$). G damage ratios at all sites (G_3 to G_{18}) were added up to represent total G damage ratio by CT as summarized in Figure 4-4C. It was obvious that total G damage by CT varied with different epigenetic modifications. Similar total damage ratios were achieved in **AQC** (0.53), **AQM** (0.55), **AQMCM** (0.52) and **AQMFM** (0.50). Highest total damage ratio by CT was observed for **AQMMM** (0.61). However, total G damage by CT was inhibited in hmC and fC modified sequences **AQH** (0.42), **AQF** (0.33) and **AQMFM** (0.43). The pattern of total G damage ratio was very similar to G damage ratio observed at G_{18} site and showed facilitation of total G damage by methylation. Finally, the charge transport efficiency to long range was revealed by the ratio of G damage of proximal vs distal GG doublets (R) as shown in Figure 4-4D. R values obtained for both hypo- and hyper- methylation conditions followed the same trend with different cytosines: $C \approx mC > hmC > fC$. R value was bigger for hyper-methylated sequences than corresponding hypo-methylated ones. These results clearly indicated that CT to the long range was inhibited by presence of hmC and fC in the bridge sequence, however, facilitated by hyper-methylation of the bridge.

Figure 4-4 Long range DNA CT was regulated by epigenetic modifications. Proximal GG doublet (A); distal GG doublet (B); total G damage at all G sites in the sequence (C); G damage at proximal GG doublet vs distal GG doublet (D).

4.3.5 Transient Hole Occupancy in Epigenetic Modified DNA During Charge Migration

$AQX/^{CP}G_n$ and $AQMXM/^{CP}G_n$ ($X=C, M, H, F, M=mC, H=hmC, F=fC, n=4, 18$) were irradiated at 350 nm for 30 s to investigate transient hole occupancy of each ^{CP}G site during charge transport. ^{CP}G decomposition at proximal and distal GG doublets was summarized in Figure 4. At proximal GG doublet where ^{CP}G was intervened from ^{ec}AQ by 3 base pairs, similar ^{CP}G decomposition (23-29%) in $AQX(AQMXM)/^{CP}G_4$ was achieved (Figure 4A). ^{CP}G decomposition at $^{CP}G_4$ site was affected by the epigenetic modifications whose middle was 6 bp away from $^{CP}G_4$. The observation of ^{CP}G regulated by sequences after the "bridge" was consistent with previous report that DNA charge transport is not only modulated by the base pairs on the "bridge", but also by all base pairs of the sequence.^[28] Both long and short timescale studies suggested that the effect of epigenetic modifications on DNA CT was not dramatic for hole occupancy in the sequence before the modified sites. In contrast to the apparent differences in G

decomposition by CT in the long timescale study, epigenetic modifications in the short timescale study did not show dramatic influence on ^{CPG} decomposition at the distal GG doublet. ^{CPG} decomposition from all the epigenetic modified sequences varied from 10-14%, except for ^{CPG} decomposition of **AQMFM** was only around 3%, which we attributed to some plausible local conformational changes caused by fC in hyper-methylation environment. From the above results, mC and hmC do not show dramatic effect on the distribution transient hole occupancy during DNA CT, neither does fC in hypo-methylated sequence. However, fC in hyper-methylated sequence efficiently inhibited hole migration to the long distance. Since CT has critical importance in signaling for DNA repair proteins, these results suggested mC and hmC might not be detected as a mismatch, while fC in hyper-methylated sequence is a potential substrate by the DNA repair proteins.

Figure 4-5 ^{CPG} decomposition at proximal (A) and distal (B) GG doublets by one-electron oxidation via charge transport.

4.3.6 Effects of Epigenetic Cytosines on Long Range DNA CT.

Our study investigated effects of epigenetic modifications on DNA CT in the long timescale (millisecond) and short timescale (picosecond). Oxidative damage on guanine happened at a millisecond timescale, during which several processes, such as proton-coupled electron transfer (PCET), hole trapping with subsequent reaction with O₂ and H₂O as well as backward charge transport, might occur (scheme 1). The observed G damage by CT was the result of equilibration of these processes. Proton-coupled electron

transfer is believed to happen at millisecond to nanosecond timescale, which could be regulated by the proton transfer between G-C base pair on the "bridge". Generally speaking, when proton transfer is inhibited, charge transport is inhibited as well. We noticed that the pKa values of epigenetic cytosines, which could have significant influences on the proton transfer processes, are greatly affected by their C5 functionalities (Figure 4-6). mC has a slightly bigger pKa value (pKa=4.5) than regular cytosine (pKa=4.4), while hmC and fC show apparent smaller pKa values, pKa=3.5 and pKa=2.4 respectively, than regular C and mC. Moreover, this trend was quite consistent with our finding of G decomposition in the long timescale CT study, considering that bigger pKa value of cytosine derivative in the G-C base pair can facilitate proton transfer and small pKa value can inhibit the process. However, our short timescale study with ^{CP}G as fast kinetic hole trap did not observe obvious ^{CP}G decomposition changes with various epigenetic cytosine species on the bridge, except for **AQMFM**. This result appeared to be contradictory with our finding and explanation in the short timescale study. However, detailed analysis and reasoning found that the result in the short timescale study is an ideal proof to support that the phenomena we observed in the long timescale study was due to epigenetic modifications influencing on the proton transfer process. Comparing the sequences for long and short timescale study, we can easily find that the trapping rates of two sets of DNA sequences are different due to the hole trap used. In the long timescale study, slow trapping rate of G⁺ (millisecond), compared to proton coupled electron transfer rate (millisecond to nanosecond), allowed multiple forward and backward charge transport between the closest G/GG sites. Therefore, the effects epigenetic modifications on proton transfer process were very significant when monitored with the slow G trap. However, in the short timescale study, ring opening reaction of ^{CP}G can happen at picosecond timescale, which efficiently traps the migrating charge and suppresses backward charge transport. Therefore, in the short timescale, effects of proton transfer

processes are not as obvious when monitored with ^{CP}G, since ^{CP}G decomposition in this scenario mostly depends on hole density migrating on the ^{CP}G site. The hole density on the ^{CP}G site DNA is primarily determined by DNA conformational and base energetic factors, however, is not relevant to the proton transfer processes between the G-C base pairs on the bridge. Hence, the fact that no apparent relationship between ^{CP}G decomposition and pKa values of the epigenetic cytosines in the short timescale study when proton transfer effects were suppressed clearly indicated that the correlation of G decomposition and pKa values of the epigenetic cytosines in our long timescale study was through affecting the proton transfer process.

Figure 4-6 DNA charge transport in the long timescale and short timescale were regulated by the proton transfer processes between epigenetic cytosine-guanine base pair on the bridge.

Currently, the biological contexts of DNA CT can be roughly divided into two categories, funnelling the oxidative G damage and signalling between proteins. Generation of $G^{\bullet+}$ is a crucial step for oxidative DNA damage. Previous studies have suggested that reactive radical cation results from the local oxidative stress could migrate along the DNA duplex base stacking and cause oxidative damage at a distance to the G rich regions with relative low oxidation potentials. Early efforts to investigate epigenetic effects on DNA charge transport were made by Schuster's group using well-matched and mismatched mC modified DNA.^[29, 30] One-electron charge transport in mC modified well-matched duplex with six GG steps did not observe apparent discrimination of oxidative G damage at each step from unmodified DNA.^[29] mC in C-A or C-T mispairs,

however, showed significantly higher reactivity than unmodified sequences.^[30] Mismatched mC was even more easily oxidized than GG doublet in the same DNA duplex, which was similar to the results observed for T mispairs.^[31] C5 methyl group hence was believed to be of key importance for higher reactivity of mC and T mispairs. Our study clearly showed that epigenetic cytosines through affecting the proton transfer process are of significant importance for this relative slow oxidation damage in the DNA molecule. On the other hand, DNA repair proteins have been found to cooperate with each other via DNA mediated signalling in order to fulfil their functions. This is a relative fast charge transport processes which does not cause oxidative damage on DNA molecule. Our short timescale study indicated that epigenetic cytosines do not have great impact on this process, except for that fC in the hyper-methylation situation might be regarded as a potential mismatch base in the DNA duplex.

4.4 Summary and Outlooks

In summary, we investigated effects of epigenetic cytosines on DNA charge transport using ^{ec}AQ tethered DNA duplexes. Natural trap G was used to show oxidative damage to DNA via CT; while fast kinetic trap ^{CP}G was adopted to monitor transient hole occupancy at each site during hole migration. The results showed that one-electron oxidation of guanine through DNA CT was affected by epigenetic cytosines on the "bridge". Hyper mC modification on the "bridge" facilitated G damage by DNA CT in the long range, however, hmC and fC dramatically inhibited the process both in hypo- and hyper-methylated sequences. On the other hand, in the short timescale, epigenetic modification affects hole occupancy distribution in the whole sequence. Changes in ^{CP}G decomposition caused by epigenetic cytosines were not as dramatic as in the long timescale CT study. However, ^{CP}G decomposition at the distal GG doublet for hyper-methylated sequence was greatly inhibited by the presence of fC. This result could be due to altered local structure

of DNA at the fC modified site. The inconsistency of transient hole transport assay and G oxidation assay was due to different timescales for the reaction to happen, which resulted in distinct degrees of influence on proton transfer and further influence DNA CT. For the long timescale, during which effects of proton transfer is apparent; various C5 functionalities have huge impact on their pKa values and further regulate the proton transfer process between the epigenetic cytosine-guanine base pair. The forward and backward DNA CT in the long timescale are mostly coupled with proton transfer. However, in the short timescale, effects of proton transfer on DNA CT are dramatically suppressed, making the fast DNA CT primarily be regulated by the DNA conformation and electronic coupling of the DNA bases. Our investigation of effects of epigenetic modifications on DNA CT is useful to further understand the metabolism of mC, hmC and fC in cell which might associated with a series of redox reactions as well as potential signalling and sensing between proteins.

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CHAPTER 5

Ionic Liquids Facilitate DNA Mediated Charge Transport

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5.1 Introduction

DNA duplex employs an elegant right-handed helical structure with two types of nucleobase pairs (A-T, G-C) lining in specific order and antiparallel phosphate backbones coiling around the helical axis.^[1] This structure not only precisely stores the genetic codes of life, but also provides a π -conjugation pathway for electron/charge migration. It was first reported by Barton's group in late 1980s that duplex DNA served as a medium to enable photo-induced charge transfer (CT) between non-covalently attached metal complexes.^[2] Over the past three decades, DNA mediated CT has become a hot topic in chemical biology. The underlining mechanism, biological roles and potential applications of DNA CT in bionanodevices have been intensively studied.

In general, DNA charge transport (CT) is initialized by irradiating a photo-oxidant (PO) to excited state. The sequential rapid reduction of PO* can induce oxidation of nearby nucleobases and inject a positive charge (as known as a *hole*) into duplex DNA. The injected charge can migrate along the stacking base pairs of DNA until it is irreversibly trapped by the acceptor. Charge migration to the lower potential acceptor is realized by either tunnelling via the intervening base pair stacking in the short range or the thermally induced multi step hopping in the long range.^[3-6] Nevertheless, controversy still exists in whether hole is delocalized or localized (as polaron) along base pair stacking.^[7-10] Redox reactions between excited metal complexes and the most vulnerable natural base guanine can occur over a distance as far as 200 Å in aqueous solution.^[11] Furthermore, studies have demonstrated that DNA charge transport is highly sensitive to perturbations, such as mismatches and protein binding, and alterations in DNA secondary structure.^[12, 13]

Molecular electronics is greatly inspired by DNA charge transport. With the remarkable characteristics of self-assembly, self-recognition and inherent conductivity,

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DNA molecule is an ideal building block in molecular electronics to assemble non-biological materials, such as metals, nanoparticles and nanotubes; and biomolecules such as proteins, antibodies and **biomarkers**. Various functions, such as detection of single-base mismatch in DNA and complicated analytes such as proteins and biomarkers, have been fulfilled.^[14, 15] Particularly, recent studies realizing DNA charge transport in 2D/3D DNA nanostructure frameworks are of great interest.^[16, 17]

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Conductivity is a critical property to fabricate DNA based electrochemical devices. Albeit current measurements showed DNA conductivity fall in a large range from insulator to superconductor, accumulating studies have suggested that the DNA molecule longer than a few nanometres is an insulator or a wide band-gap semiconductor.^[18-24] Therefore, metallization or coating with conductive materials is required to transform DNA to a conductive wire. However, an apparent drawback of these methods is that the resultant nanowires are too thick (minimum 50 nm) to be applied to smaller scale electrical devices.^[25-28] On the other hand, though the efficient DNA charge transport could occur on fast and ultrafast time scales, theoretical and experimental studies have indicated that efficient charge transport in aqueous solution requires proper DNA sequence and intact duplex structures. These requisites raise a high threshold for improving DNA conductivity, since perturbations to π electron coupling through base pairs, such as mismatches and protein binding, which alter DNA secondary structure or conformation, often significantly hamper charge transport in DNA. To date, methods for improving charge transport efficiency without changing primary DNA sequences are limited. Our lab recently reported facilitation of charge transport in aqueous solution under external magnetic field (MF).^[29] Alignment of DNA base pairs by MF might induce CT-active conformation, thus promoting charge propagation. An alternative way to overcome the inherent low charge transport efficiency of DNA in aqueous solution may be through altering the solvation system. Although theoretical analyses suggest

surrounding molecules would have certain effects on charge transport, changing solvation system, such as counter ion species, results in negligible or inhibitory effect on charge transport.

Our group considered the possibility of facilitating charge transport with a biocompatible green solvent, *ionic liquid* (IL). Ionic liquid has been extensively investigated and widely applied as novel electrolyte to harvest the useful electrochemistry properties, such as inherent conductivity and wide window for electrochemistry. Additionally, the unique interaction and compatibility of ionic liquid with biomacromolecules, such as DNA and proteins, have attracted rapidly growing attentions. For example, ionic liquid has been found to be an ideal solvent for protein extraction and long-term DNA storage. Furthermore, certain properties of DNA, such as duplex stability, structures and dynamic conformation, could be significantly altered in ionic liquids comparing to aqueous solution. However, theoretical studies about ionic liquid-DNA interaction are greatly limited and remain controversial.

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In this chapter one electron oxidation of 8-cyclopropyl-2'-deoxyguanosine (⁸CPG) by DNA CT has been investigated in hydrated ionic liquid solution compared to aqueous solution. At our lab, several hydrated ionic liquids enhance charge transport efficiency than canonical aqueous buffer solution. The facilitation to DNA CT is closely related to the features of ionic liquid, including structures, polarity and hydrophobicity of both cationic and anionic components. Also, the binding mode of ionic liquid to DNA duplexes was studied with fluorescence assays. Our investigation of DNA mediated charge transport in hydrated ionic liquid will provide a novel view of not only interactions between biomacromolecules and ionic liquid, but bioreactions in ionic liquids.

5.2 Experimental

5.2.1 Reagents and Materials

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All ionic liquids involved in the study, 1-Butyl-3-methylimidazolium tetrafluoroborate (Bmim BF₄), 1-Ethyl-3-methylimidazolium tetrafluoroborate (Emim BF₄), 1-Ethylpyridinium tetrafluoroborate (Etp BF₄), 1-Butyl-1-methylpyrrolidinium tetrafluoroborate (Bmp BF₄), 1-Butyl-3-methylimidazolium acetate (Bmim Ac) and 1-Butyl-3-methylimidazolium chloride (Bmim Cl) were purchased from Sigma-Aldrich and used as received (cation structures of ionic liquids shown in Scheme 1). Fluorescence dye, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and thiazole orange (TO) were purchased from Sigma-Aldrich as well. Other common chemical reagents and buffers were prepared in our lab with reagent grade chemicals and deionized water. High-performance liquid chromatography (HPLC): Shimadzu Prominence Modular HPLC. HPLC Mobile phase A: 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.0, Mobile phase B: ACN (HPLC-grade).

5.2.2 Oligonucleotides Preparation

All oligonucleotides were synthesized on a MerMade 4 DNA Synthesizer (BioAutomation), followed by HPLC purification twice and verification of molecular weight by ESI-MS (Sangon Biotech, Shanghai). Phosphoramidites of all natural nucleotides and anthraquinone-5-ethynyl-dU (^{AQ}dU) were purchased from Glen Research, Berry & Associates and used as received. Phosphoramidite of 8-cyclopropyl-2'-deoxyguanosine (^{8CP}G) was prepared at our lab.^[30] To ensure high coupling yields, elongated coupling time (5 min) was adopted for incorporating ^{AQ}dU and ^{8CP}G into DNA oligonucleotides. The synthetic DNA oligonucleotides were cleaved and deprotected via incubation in concentrated NH₄OH at 75 °C for 17 h or AMA (1:1 v/v mixture of concentrated NH₄OH and 40% aqueous methylamine) at 37 °C for 2 h. Concentration of

DNA oligonucleotide was determined by UV-Vis spectroscopy (Shimadzu UV-1800 UV-Vis spectrophotometer).

40 μM of duplex DNA solutions were annealed by heating at 90°C for 5 min and cooling to room temperature in 2.5 h to ensure duplex DNA structure during photo-irradiation. To the DNA solution, 4 M desired ionic liquid aqueous solution was added to obtain desired concentrations of ionic liquid and DNA.

5.2.3 General Methods for Photo-irradiation

Sample aliquots (30 μL , containing 20 μM DNA, 20 mM PBS, 50 mM NaCl, pH 7.4, with/without 2 M of IL, unless indicated otherwise) were transferred to 1.5 mL microtubes and were irradiated at 350 nm for 45s. A 450 Watt Illuminator (HORIBA Jobin Yvon) with a monochromator and a 320 nm long-pass filter was used for all the photo-irradiation experiments. After irradiation, samples were diluted with deionized water (70 μL). Ionic liquid was removed by overnight dialysis (Thermo Scientific, #69553, 2K MWCO). Samples were dried by lyophilization and were then digested to free nucleosides by alkaline phosphatase (New England Biolabs) and phosphodiesterase I (i-DNA biotechnology) by incubation at 37 °C for 18 h. The resulting nucleosides were analyzed by a Shimadzu HPLC (Chemcobond 5-ODS-H, 4.6 $\times$ 150 mm). Oxidative damage of ^8CPG (Y, %) was determined by:

$$Y\% = \left(1 - \frac{A_{irr}}{A_{non-irr}}\right) \times 100\% \quad \text{Eq 5.1}$$

where A_{irr} and $A_{non-irr}$ are normalized peak area of ^8CPG of irradiated and non-irradiated samples from HPLC trace (peak areas were normalized to that of dT in the same trace).

Standard deviation over three repeated experiments was used as error of the data.

5.2.4 FID Assay

Fluorescence indicator displacement assay was performed on a Shimadzu RF-5301PC spectrofluorophotometer in 20 mM PBS, 50 mM NaCl, pH 7.4 and corresponding ionic liquids. The concentration of DNA duplex was kept as 1.5 μ M. To DNA was added 2.67 molar equivalent 4',6-diamidino-2-phenylindole (DAPI, 4 μ M) according to binding the stoichiometry. Ionic liquids were titrated to DNA (from 0 to 2 M) to displace the binding dye. The samples were mixed for 30 min at 25 °C before measurement. Emission spectrum of DAPI (excitation at 358 nm) or DNA-TO complexes (excitation 495 nm) was recorded. Three repeated experiments were conducted for each data point. Fluorescence intensity of TO at 530 nm was used to calculate displacement percentage of TO (^{TO}D), following the equation: $^{TO}D=100-I$. DAPI fluorescence decrease percentage was corrected by the control (f , %):

$$f = \frac{I-I_{DNA}}{I-I_{DNA}-I_{DNA}} \times 100\% \quad \text{Eq 5.2}$$

where I_{DNA} , I and I_{DNA} are integrations of DAPI fluorescence (370-600 nm) in DNA+PBS, DNA+PBS+IL and PBS+IL; f is percentage of DAPI that is displaced from duplex DNA by ionic liquid.

5.2.5 Fluorescence Melting of DNA Duplexes

The thermal stability of DNA was studied on a Roche real-time PCR cycler (LightCycler 480 II) with 465/510 nm filter set. Samples (20 μ L) containing 1.5 μ M DNA, 20 mM PBS, 50 mM NaCl, pH 7.4 with/without ionic liquids were annealed as described above. Fluorescence denaturing curves of DNA duplexes were monitored from 35 °C to 90 °C with a ramp of 0.6 °C/min. Melting points of DNA duplexes were determined as the maximum of the first derivative of the denaturing curves and averaged by three repeated records.

5.2.6 UV Melting of DNA Duplexes

The thermal stability of DNA was studied on Shimadzu UV-2550 UV-VIS spectrophotometer with temperature controlling accessory, using a 1 cm path length 8-cell quartz cuvette. DNA samples (100 μ L) containing 1.5 μ M duplex DNA 20 mM PBS, 50 mM NaCl, pH 7.4 with/without ionic liquids were annealed as described. Denaturing curve of DNA was monitored from 15 $^{\circ}$ C to 90 $^{\circ}$ C in a rate of 0.5 $^{\circ}$ C/min with measurement interval of 1 $^{\circ}$ C. Melting points were calculated by sigmoidal fitting of the denaturing curves and averaged by three repeated records.

5.2.7 Circular Dichroism

Samples (100 μ L) for circular dichroism titration experiments were the same as described in photo-irradiation assay. Circular dichroism spectra of 10 μ M duplex DNA, containing 20 mM PBS 50mM NaCl, pH 7.4, with corresponding ionic liquid or salt (BmIm BF₄, BmIm Cl, Bmp BF₄, NaBF₄, NaCl) was obtained by averaging five repeated measurements (scanning rate 50 nm/min, JASCO J-1500 CD spectrometer).

5.2.8 Fluorescence Lifetime

The time-resolved fluorescence decay assay was performed on a time-correlated single photon counting (TCSPC) spectrofluorimeter (FluoroCube, Horiba Jobin Yvon) with a pulsed diode laser (NanoLED-470L, Horiba Jobin Yvon). Samples containing duplex DNA (3 μ M, 20 mM PBS, 50 mM NaCl) and 2 M corresponding ionic liquid were excited at 466 nm and the fluorescence emission of TO was monitored at 530 nm. Horiba Jobin Yvon Datastation software was used to analyze the results with consideration of the reduced chi-square value and the randomness of the weighted residuals. The fluorescence decay curves were fitted to multiple exponential decay function with χ^2 values around 1. The mean fluorescence lifetime τ was calculated as $\tau = \sum \tau_i^2 A_i / \tau_i A_i$.

5.3 Results

5.3.1 Design of DNA duplexes for DNA-mediated One Electron

Oxidation of ⁸CPG

To investigate DNA-mediated charge transport in ionic liquid, a series of 23-mer DNA assemblies were prepared as listed in Table 5-1. Each AQ_n/⁸CPG_n (n=1-4) duplex assembly contains an anthraquinone modified dU (^{AQ}dU) as photo-oxidant on one strand and a kinetic hole trap 8-cyclopropyl-2'-deoxyguanosine (⁸CPG) on the complementary strand (Scheme 5-1). ^{AQ}dU, where anthraquinone was conjugated to dU with a carbon-carbon triple bond, was

Table 5-1. DNA assemblies used to probe CT in ionic liquids.

DNA	Sequences
AQ ₁ / ⁸ CPG ₁	5'- ^{AQ} dU TTT TCC TTT TTT TTA GAG ATA G-3' 3'-A AAA AG ⁸ CPG AAA AAA AAT CTC TAT C-5'
AQ ₂ / ⁸ CPG ₂	5'- ^{AQ} dU TTT TTT CCT TTT TTA GAG ATA G-3' 3'-A AAA AAA G ⁸ CPG AAA AAA AAT CTC TAT C-5'
AQ ₃ / ⁸ CPG ₃	5'- ^{AQ} dU TTT TTT TTC CTT TTA GAG ATA G-3' 3'-A AAA AAA AAG ⁸ CPG AAA AAT CTC TAT C-5'
AQ ₄ / ⁸ CPG ₄	5'- ^{AQ} dU TTT TTT TTT TCC TTA GAG ATA G-3' 3'-A AAA AAA AAA AG ⁸ CPG AAT CTC TAT C-5'
AQ ⁸ CPG	5'- ^{AQ} dU ⁸ CPGTT TTT TTT TCC TTA GAG ATA G-3'
AQM	3'-A AAA ATA AAA AG ⁸ CPG AAT CTC TATC-5'
TO9	5'-T TTT TTT TTT TCC TTA GAG ATA G-3'
CC ₃	5'-T TTT TTT TTCCTT TTA GAG ATA G-3'
GG ₃	3'-A AAA AAA AAGGAA AAT CTC TAT C-5'
CC ₄	5'-T TTT TTT TTT TCC TTA GAG ATA G-3'
GG ₄	3'-A AAA AAA AAA AGG AAT CTC TAT C-5'

adopted as photo-oxidant to introduce a positive charge (also called hole) into DNA duplexes upon photo-excitation. The kinetic hole trap, 8-cyclopropyl-deoxyribose guanosine, ⁸CPG, upon one electron oxidation can yield a radical cation, ⁸CPG⁺, and

irreversibly trap hole occupancy via a rapid ring-opening process. ${}^8\text{CPG}$ replaced the 5'-guanine of a GG doublet, which was located after an adenine tract away from photooxidant, ${}^{\text{AQ}}\text{U}$. Numbers of consecutive A-T pairs between AQ and hole trap were increased from four to ten from duplex $\text{AQ}_1/{}^8\text{cpG}_1$ to $\text{AQ}_4/{}^8\text{cpG}_4$, with AA as one step, to investigate distance dependence of DNA CT in hydrated ionic liquid. Decompositions of ${}^8\text{CPG}$ upon photo-irradiation were determined to reflect the efficiency of DNA CT as described in Eq 5.1.[30]

Scheme 5-1. Experimental setup to investigate DNA mediated charge transport in ionic liquid. (*Upper*) Structures of cations of ionic liquids in the study. (*Lower*) Photo-irradiation initiates charge transport along DNA duplex; oxidation of ${}^8\text{CPG}$ is revealed by HPLC analysis; ${}^{\text{AQ}}\text{dU}$ and ${}^8\text{CPG}$ were adopted as photo-oxidant and kinetic hole trap, respectively.

5.3.2 Increased Charge Transport Yield in Hydrated Ionic Liquid.

Efficiency of DNA-mediated charge transport in aqueous solution and hydrated ionic liquid (2 M Bmim BF_4) was firstly compared by photo-irradiation of $\text{AQ}_4/{}^8\text{CPG}_4$. With the longest bridge length between hole trap and photo-oxidant, $\text{AQ}_4/{}^8\text{CPG}_4$ could accommodate the most interactions between DNA bridge and solvent molecules, and thus would most efficiently reveals any effects of ionic liquids on hole migration. Significant ${}^8\text{CPG}$ decomposition (44.3%) in 2 M Bmim BF_4 solution was observed after irradiation of $\text{AQ}_4/{}^8\text{CPG}_4$ at 350 nm for 45 s. ${}^8\text{CPG}$ oxidation was dramatically enhanced comparing to that in aqueous solution (5.2%) (Figure 5-1). A series of control experiments were

conducted to validate the observation of increments in $^{8\text{CPG}}$ decomposition was due to 1) IL facilitation on 2) DNA-mediated charge transport. As shown in Figure 5-1, negligible $^{8\text{CPG}}$ decomposition was observed for either non-irradiated sample (0.6%) or irradiated sample in the absence of AQ (1.3% for $\text{CC}_4/^{8\text{CPG}}\text{G}_4$). No oxidation in absence of either light or photooxidant indicated the reaction was induced by photoredox reaction of anthraquinone, but not other dark oxidants, such as reactive oxygen species (ROS) generated in IL during photo-irradiation. These results suggested that $^{8\text{CPG}}$ decomposition observed in IL was light-initiated. Furthermore, a mixture of duplex AQ_4/GG_4 and $\text{CC}_4/^{8\text{CPG}}\text{G}_4$ (cross control), in which photooxidant and hole trap were in the separate duplexes, were irradiated in hydrate IL. Similar to former controls, negligible $^{8\text{CPG}}$ decomposition was observed, which confirmed that no diffusible oxidants or interduplex hole transfer were involved here. Since DNA-mediated charge transport is highly sensitive to sequence integrity, $\text{AQM}/^{8\text{CPG}}\text{G}_4$ (table 5-1) containing a T-T mismatch on the "bridge" was irradiated under the same condition in 2 M BmimBF₄. $^{8\text{CPG}}$ decomposition (13.6 %) was significantly attenuated, which indicated that hole transport was within individual duplex $\text{AQ}_4/^{8\text{CPG}}\text{G}_4$ in Bmim BF₄. The above results from various control experiments excluded the possibility that $^{8\text{CPG}}$ damage was due to diffusible oxidants and interduplex electron transfer while solvent was converted from aqueous buffer to hydrated IL. Hole transfer from AQ* via aromatic base pair stacking within duplex DNA was the only valid cause to induce $^{8\text{CPG}}$ decomposition. Remarkable increments in DNA mediated HT is due to the assistance from IL, whereas elevation of oxidation by ROS and other electron conducted via IL are not valid.

Figure 5-1. Escalation of $^{8\text{CPG}}$ decomposition from $\text{AQ}_4/^{8\text{CPG}}_4$ in Bmim BF_4 is due to DNA mediated charge transport. $^{8\text{CPG}}$ decomposition of $\text{AQ}_4/^{8\text{CPG}}_4$ (20 μM) after photo-irradiation at 350 nm for 45 s under various conditions: without Bmim BF_4 (PBS); with 2 M Bmim BF_4 (AQ_4); without irradiation (Dark); without AQ (Light); AQ and $^{8\text{CPG}}$ were separated in two duplexes (Cross); with a T-T mismatch in duplex between AQ and $^{8\text{CPG}}$ (Mismatch). All samples have same solution composition, 20 mM PBS, 50 mM NaCl, 2 M Bmim BF_4 , except for PBS sample.

5.3.3 Ionic Liquid Facilitates Hole Migration

Generally, the process of hole transport could be divided into three stages, as *hole injection*, *hole migration*, and *hole trapping*. The above experiments clearly showed $^{8\text{CPG}}$ decomposition, as an overall product of the three stages of DNA HT, was facilitated by ionic liquid. However IL effects on each stage cannot be revealed directly by above $^{8\text{CPG}}$ reaction. Single stranded oligonucleotide $\text{AQ}^{8\text{CPG}}$ (table 5-1), where AQ was positioned next to $^{8\text{CPG}}$, was irradiated for 18 min in aqueous solution as well as in 2 M Bmim BF_4 . In this single stranded DNA, photo-oxidant and hole trap was positioned next to each other without intervening bridge, hence, no hole migration was involved under irradiation and any decomposition of $^{8\text{CPG}}$ was direct outcome from hole injection from AQ and hole trapping at $^{8\text{CPG}}$. In contrast to duplex $\text{AQ}_4/^{8\text{CPG}}_4$, oxidative decomposition of $^{8\text{CPG}}$ in $\text{AQ}^{8\text{CPG}}$ was attenuated in presence of 2 M Bmim BF_4 (Figure 5-2). This result suggested the inhibitory effects of Bmim BF_4 on direct hole injection-trapping process. Therefore, IL facilitation on DNA HT should mainly focus on hole migration via interaction with bridge base pairs.

Figure 5-2: Decomposition percentage of ${}^8\text{CPG}$ (Y/%) of AQ^8CPG in aqueous solution (PBS) and 2 M Bmim BF_4 (IL) after irradiation at 350 nm for 18 min.

5.3.4 Inverse Distance Dependence of ${}^8\text{CPG}$ Decomposition in Hydrated Ionic Liquid Solution.

Having confirmed that escalation of decomposition of ${}^8\text{CPG}$ in Bmim BF_4 was due to DNA mediated charge transport and the importance of hydrated ionic liquid's interaction with bridge, distance dependence of charge transport yields on bridge length between photo-oxidant and trap was investigated. DNA duplexes of $\text{AQ}_n/{}^8\text{CPG}_n$ ($n=1-4$) were irradiated for 45 s in PBS solution and 2 M Bmim BF_4 . As shown in Figure 5-3, natural logarithm of decomposition of ${}^8\text{CPG}$ (Y) was plotted against distance between photo-oxidant and ${}^8\text{CPG}$ (r). Distance dependence constant β ($0.02 \text{ \AA}^{-1}$) of DNA CT over up to 4.8 nm was obtained by a hopping mechanism as equation: $\ln Y = \ln A - \beta \times r$, where β is the decay parameter. In aqueous solution, the oscillatory periodicity of hole transport yield with increasing intervening adenines in aqueous solution was consistent with previous findings.^[30, 31] Maximum ${}^8\text{CPG}$ oxidation was achieved when 5 and 9 intervening base pairs were positioned between AQ and ${}^8\text{CPG}$ in $\text{AQ}_1/{}^8\text{CPG}_1$ and $\text{AQ}_3/{}^8\text{CPG}_3$ respectively. The periodic oscillation of distance dependence was consistent with transient, delocalized gating domain mechanism of hole transport.^[7] However, when same exploration was conducted in hydrated ionic liquid, distance dependence of CT yield in 2 M Bmim BF_4 did not show typical decay of ${}^8\text{CPG}$ decomposition by charge transport with the increasing

bridge length as in aqueous solution. In hydrated ionic liquid, ${}^8\text{CPG}$ closest to AQ achieved the lowest decomposition (18.4 %) as in $\text{AQ}_1/{}^8\text{CPG}_1$, while ${}^8\text{CPG}$ decomposed with highest efficiency (44.3%) in $\text{AQ}_4/{}^8\text{CPG}_4$ which was furthest from AQ. The oscillation dependence of CT yields was barely observable with much attenuated amplitudes and opposite phase as that in PBS occurred. Since the oscillation periodicity is only obvious when back electron transport (BET) is slower than trapping rate, losing it could be a result of enhanced charge transport rate.^[32] Nevertheless, increasing CT yield with distance between photo-oxidant and hole trap was seldom observed in previous studies. Although the reason for the unique distance dependence in Bmim BF_4 required further study, apparent increment of ${}^8\text{CPG}$ decomposition by oxidative CT was observed in all $\text{AQ}_n/{}^8\text{CPG}_n$ ($n=1-4$) assemblies, particularly 7 fold increment of ${}^8\text{CPG}$ decomposition was achieved in $\text{AQ}_4/{}^8\text{CPG}_4$.

Figure 5-3. Distance dependence of charge transport yield of $\text{AQ}_n/{}^8\text{CPG}_n$ ($n=1-4$) after irradiation for 45 s in aqueous and 2 M Bmim BF_4 solution. Y%: decomposition of ${}^8\text{CPG}$; r: distance between photo-oxidant and ${}^8\text{CPG}$.

5.3.5 Effect of Hydrated Ionic Liquid Cation and Anion Species on Hole Transport.

DNA charge transport in $\text{AQ}_4/{}^8\text{CPG}_4$ was also investigated in hydrated ionic liquids other than Bmim BF_4 in order to confirm the facilitating effect of ionic liquid on CT was

not limited to Bmim BF₄. Three kinds of ionic liquids with different cations, Bmp BF₄ and Etp BF₄ and Emim BF₄, were investigated to reveal effect of cation on CT in DNA; while Bmim Cl and Bmim Ac were investigated for anion effect; in addition concentrated NaCl was investigated for the effect of ion strength. As shown in Figure 5-4, CT efficiency in ionic

Figure 5-4. ⁸CPG decomposition in AQ₄/⁸CPG₄ with various ionic liquid after irradiation for 45 s.

liquids was sensitive to their cationic species. Elevated CT yield of AQ₄/⁸CPG₄ was observed in Bmp BF₄ and Etp BF₄; in contrast, charge transport of AQ₄/⁸CPG₄ was quenched in Emim BF₄ whose alkyl side chain of cation was just shorter than Bmim BF₄ by two carbons. Etp BF₄ (29.9%) with aromatic cation as Bmim BF₄ had higher CT yield of AQ₄/⁸CPG₄ than that in Bmp BF₄ (8.0%) with alkylate cyclic pyrrolidinium cation, which could plausibly relate to stronger hydrophobic interaction with DNA base pairs. Further investigation revealed dramatic difference of Emim BF₄ from Bmim BF₄ in interaction with DNA, which will be discussed in detail later. On the other hand, the anion effect on CT efficiency of DNA in ionic liquids was not negligible. Hole transport yield was dramatically attenuated from 44.2% to less than 6 % and not detectable in Bmim Cl and Bmim Ac respectively. The facilitating effect of ionic liquid on hole transport was

strongly modulated by their anion species. This result was quite unexpected, since it was normally believed that electrostatic interaction between negatively charged phosphate backbones of DNA and positively charged cations of ionic liquid is dominant in ionic liquid-DNA interaction, while anion effects were often neglected. The above interactions indicated that hole transport was strongly associated with ionic liquid species, especially related to the cation and anion composition of ionic liquid.

To further validate our finding that anions of ionic liquid influence the interaction between cations and DNA duplexes, DNA CT in $\text{AQ}_4/^8\text{CPG}_4$ was investigated in Bmim BF_4 solution prepared in situ by mixing Bmim Cl with NaBF_4 . CT yield was inhibited in either Bmim Cl or NaBF_4 individually. NaBF_4 was found to heavily destabilize DNA duplex structure thus quenching CT (data not shown). If yield increment of DNA charge transport was a result from a proper combination of cation and anion, ^8CPG oxidation mediated by charge transport should be correlated to titration of NaBF_4 to Bmim Cl. The result was as expected. With increasing NaBF_4 ratio to Bmim Cl from 1:9 to 1:1, oxidative decomposition of ^8CPG was raised from 2.4% to 14.1% as shown in Figure 5-5. Therefore, elevation of CT yield in ionic liquids requires proper combination of their cation and anion species.

Figure 5-5. ^8CPG decomposition in $\text{AQ}_4/^8\text{CPG}_4$ after irradiation for 45 s with titration of NaBF_4 to Bmim Cl.

5.3.6 Effect of Water Molecule on CT of DNA in Ionic Liquid.

In order to examine the water effects in IL facilitated CT process, $^{8\text{CPG}}$ decomposition in duplex DNA was examined in both H_2O -IL and D_2O -IL systems with $\text{AQ}_3/^{8\text{CPG}}\text{G}_3$. CT efficiency in $\text{AQ}_3/^{8\text{CPG}}\text{G}_3$ was slightly increased in D_2O -PBS solution (16.3%) compared to H_2O -PBS (12.3%) as shown in figure 5-6. However, the isotope effect was much more pronounced in the presence of ionic liquid: nearly 50% increment of $^{8\text{CPG}}$ decomposition (from 34.7% to 58.3%) was observed when switching H_2O to D_2O .

Figure 5-6. Decomposition percentage of $^{8\text{CPG}}$ (Y/%) of $\text{AQ}_3/^{8\text{CPG}}\text{G}_3$ after irradiation at 350 nm for 45 s in PBS and Bmim BF_4 with H_2O or D_2O .

5.3.7 Thermal Stability of DNA Duplex in Ionic Liquids.

Strong UV absorption of various ionic liquids in the range from 220 nm to 280 nm limited the application of UV and circular dichroism spectrum to studying DNA stability in ionic liquids. Covalently tethered thiazole orange (TO) has been reported to be a useful probe in the study of molecular beacon and i-motif dynamics.^[33, 34] Hence, DNA oligonucleotide **TO9** (Table 5-1) with fluorophore TO positioned in the A-tract was prepared to monitor DNA structure changes during thermal denaturation. Covalently tethered TO exhibited strong fluorescence emission at 530 nm when torsion of central methine bridge was restricted by the flanking base pairs of DNA duplex. Turbulence of DNA duplex structure upon thermal denaturation was revealed by attenuation of TO fluorescence. Therefore, fluorescence denaturing curves of duplex **TO9/GG4** were

recorded in various ionic liquids from 35-90 °C and melting points were calculated by first derivative fitting as listed in Table 5-2. DNA duplex was slightly stabilized by the incorporation of thiazole orange into sequence, which could be concluded by comparison of $Tm1$ with $Tm2$ for PBS, Bmim BF₄, Bmp BF₄. Ionic liquids with Bmim⁺ cation destabilized duplex structures to different extend according to their anions. Bmim BF₄ decreased Tm of **TO9/GG4** by 7.3 °C while Bmim Cl and Bmim Ac destabilized TO9/GG₄ by more than 16.6 °C. Duplex structure of TO9/GG₄ in Bmim Cl and Bmim Ac was already partially denatured. This observation explained inhibitory of CT in yield in Bmim Cl and Bmim Ac and also suggested strong interaction of Bmim⁺ with DNA duplex structure. Etp BF₄ destabilized duplex similarly to Bmim BF₄, which had 5.9 °C decrease in Tm ; while no destabilization was observed in Bmp BF₄, which could be attributed to failure of binding to DNA due to steric hindrance in its non-aromatic structure. Consistent with the result of charge transport experiment, Tm of DNA duplex in Emim BF₄ was only slightly decreased by 2.6 °C suggesting less effect on hydrogen bonding of base pairs.

Table 5-2 Tm of duplex DNA in ionic liquids by UV/fluorescence melting assay

Name	$Tm1^a/^\circ\text{C}$	$Tm2^b/^\circ\text{C}$	$\Delta Tm2/^\circ\text{C}$
PBS	48.6 (1)	51.6 (1)	-
Bmim BF ₄	45.7 (2)	44.3 (2)	7.3
Bmim Cl	32.4 (1)	32.3 (8) ^c	>16.6
Etp BF ₄	N.A.	45.7 (1)	5.9
Bmp BF ₄	49.3 (2)	51.6 (1)	0
Emim BF ₄	48.98	49.0 (5)	2.6
NaBF ₄	<15	<35	>16.6
Bmim Ac	<15	<35	>16.6

^a melting points of CC₄/GG₄ measured by UV-melting assay with 20 mM PBS, 50 mM NaCl from 15 °C to 90 °C.

^b melting points of TO9/GG₄ measured by fluorescence-melting assay (qPCR) with 20 mM PBS, 50 mM NaCl from 35 °C to 90 °C.

^c melting point of **TO9/GG4** measured by fluorescence-melting assay (fluorescence spectroscopy) with 20 mM PBS, 50 mM NaCl from 15 °C to 90 °C.

5.3.8 Interaction of Ionic Liquid with DNA Minor Groove.

To investigate the mechanism of facilitation of CT in ionic liquid, more structural information was needed regarding to the interaction of ionic liquid with DNA duplex. 4',6-diamidino-2-phenylindole (DAPI) is an ideal indicator due to the specific binding in minor groove of duplex DNA. Therefore, fluorescence indicator displacement (FID) assay was performed by titration of various ionic liquids to DAPI-DNA complex to reveal ionic liquid interaction with DNA minor groove. As depicted in Figure 5-7, percentage of DAPI displaced increased by titration with ionic liquids suggesting binding of ionic liquid to DNA minor groove, which was consistent with previous finding.^[35] Bmim BF₄, Etp BF₄ had similar binding affinities to minor groove with DC₅₀ around 0.7 M (DC₅₀: the concentration of ionic liquid needed to displace 50% DAPI from DNA minor groove). Larger DC₅₀, 1.0 M, was obtained in Bmp BF₄. Once the aromatic five member ring was switched to non-aromatic ring, minor groove binding affinity of Bmp BF₄ was heavily affected. This could be attributed large steric hindrance as well as less hydrophobicity compared to aromatic cations.

Figure 5-7. FID assay performed with duplex DNA CC₄/GG₄ with titration with various ionic liquids from 0-2 M.

5.3.9 CD Spectroscopic Studies of DNA Structure in Ionic Liquid.

Most ionic liquids involved in our study have strong absorption overlapped with nucleic acids due to the aromatic cations, expect for cyclic alkyl Bmp⁺. CD spectrum of duplex **CC4/GG4** in 0-2 M Bmp BF₄ showed slightly decrease of the negative peak at 245 nm, suggesting interaction of ionic liquids with DNA phosphate backbone by electrostatic interaction. Same assay conducted with NaCl showed little change in signal at 245 nm. In contrast, positive CD signal at 282 nm was attenuated by titration with NaCl, suggesting that counterion reducing repulsion of two antiparallel phosphate backbones and interfering base pair stacking. Hence, binding of ionic liquid cations in the minor groove applied no significant alteration to global DNA base stacking. The effect of ionic liquid on DNA secondary structure could be just providing better lining up in dynamic motion of DNA base pairs.

Figure 5-8 CD titration of ionic liquids (salt) to DNA duplexes from 0 to 2 M.

5.3.10 Fluorescence Lifetime of TO in Ionic Liquids

The time-resolved fluorescence decay was performed with thiazole orange (TO) modified duplex **TO9/GG4** (Scheme 5-1) to further investigate changes of microenvironment where DNA resided in various ionic liquids. Fluorescence lifetime (τ) of TO in TO9/GG₄ was 1.74 ns in PBS, which indicated an intercalation of TO between

flanking base pairs. In Bmim BF₄, Bmp BF₄ and Etp BF₄, longer fluorescence lifetime of TO in **TO9/GG4** was observed ($\tau=2.00$ -2.17 ns) than that in PBS and indicated that TO achieved a better stacking within the base pair array (Figure 5-9). Furthermore, the similar τ values were observed for the three ILs and suggested that the interactions that Bmim BF₄, Bmp BF₄ and Etp BF₄ applied to duplex DNA may reach comparable magnitudes of effects on the dynamic conformation of base pair stacking.

Figure 5-9. Time-dependent fluorescence decay spectra and lifetimes (τ) of **TO9/GG4** in various ionic liquids.

5.4 Discussion

We observed significant increment of DNA CT in the presence of ionic liquids, such as Bmim BF₄ and Etp BF₄. Bmim BF₄ enhanced CT efficiency over the range from 2.0 nm to 4.0 nm. Photolysis experiment of **AQ4^{8CP}G** which merely allowed hole injection and hole trapping showed reduced, rather than increased, ^{8CP}G decomposition in Bmim BF₄, indicating IL facilitation of DNA CT was at hole migration stage.

Investigation of duplex structure by CD spectra suggested strong electrostatic interaction might exist between DNA phosphate backbones and IL cations. Furthermore, the binding of ionic liquids to DNA minor groove was confirmed by DAPI displacement assay (Figure 5-7). Binding of ionic liquids with proper composition of cation and anion

species showed negligible alteration on secondary structure of duplex DNA. With smaller DC_{50} , aromatic cations such as $Bmim^+$ and Etp^+ may have stronger hydrophobic interactions with DNA base pairs in minor groove compared to Bmp^+ as non-aromatic cations. More remarkably, CT efficiencies in various hydrated imidazolium IL suggested anion contributions to the interactions between IL and duplex DNA. Hydrophobic anion, such as BF_4^- , can strongly associates with imidazolium cations via H-bonding through water molecules, and may also have higher tendency to form H-bonding with nucleobases. With modulation of BF_4^- , though binding to minor groove, hydrated $BmimBF_4$ only slight compromise thermal stability of duplex DNA. However, the association of cations was not conspicuous for hydrophilic anions, such as Cl^- and Ac^- , in hydrated ionic liquids. Without strong association with anions, $Bmim^+$ cations would such severely penetrate DNA duplex through minor groove and strongly destabilize duplex structure, resulting T_m of duplex DNA in hydrated $BmimCl$ and $BmimAc$ below room temperature. Therefore, the binding of IL to DNA minor groove was strongly regulated together by IL cation and anion species. Anions with high affinity to imidazolium cation would apparently assist cations to achieve appropriate binding modes and affinity on duplex DNA, and as a result, facilitate DNA mediate charge transport.

Furthermore, time-resolved fluorescence decay assay is useful to reveal the microenvironment changes of base pair stacking within DNA duplex in hydrate ionic liquids. As shown in Figure 5-9, elongated fluorescence lifetime of **TO/GG4** in the presence of IL indicated that binding of IL to DNA minor groove resulted in a limited rotation of the TO central methine bridge. This could be an indirect proof of restricted dynamic motions and less flexible stacking of DNA base pairs by IL. Rigid base stacking in IL might result in either higher percentage of or longer lifetime of duplex DNA residing in CT-active conformation domains transiently formed over A-tract bridge, which would allow more efficient charge transport and show the inverse distance

dependence of $^{8\text{CPG}}$ decomposition in hydrated IL. Longer DNA bridge would allow higher number of IL molecules to bind duplex DNA along helix and cause an additively larger effects on locking base pair stack into CT-active conformation, which enhanced DNA CT more significantly over longer distance. In hydrated IL, though DNA CT still showed oscillatory efficiencies with a similar period as in aqueous solution, the amplitude was greatly attenuated. Since fast CT and BET could attenuates periodicity of distance dependence when coherent CT outcompetes incoherent hopping along hole migration, dampened oscillatory amplitude observed in hydrated IL would suggest A-tract could achieve more CT-optimal conformation and showed better coherent CT upon binding of IL molecules.

Recently, higher speed limit of hole transport was reported for DNA-lipid complex in chloroform and suggested that reduced water interaction may be favorable to DNA CT. In a hydrated IL system, DNA molecules are under a dehydrated condition compared to that in aqueous buffer, since the ionic liquid can exclude water molecules from the hydration layer surrounding DNA duplexes. Studies have showed that water molecules might play a key role in the hydration process of ionic liquids. Cations of ionic liquids with hydrophobic anions, like Bmim BF₄, associate with its anions via H-bonding using H₂O as medium. Hence such ionic liquids may not dissociate to free cations and anions even after extensive dilution by water. These studies are consistent with our observation here that IL effects on DNA CT were modulated by both cations and anions of ionic liquids. The fact that only IL with hydrophobic anions but not hydrophilic Cl⁻ and Ac⁻, can accelerate DNA CT, implied the involvement of water molecules in the mechanism. IL-assisted DNA CT were also better pronounced in deuterated water due to the stronger H-bonds formed between D₂O and ILs/DNA. Furthermore, theoretical work also suggested solvent could have contribution to alter the recombination energy in Marcus theory of electron transfer. Invasion of IL ions into hydration layer and limitation of water

interactions would reduce the flexibility of duplex DNA and promote the possibility of base pair array to stay in CT-active conformation, which might result in elevated efficiency in DNA mediated charge transport.

Though ionic liquid is an electrolyte, facilitation of charge transport is not due to inherent conductivity of ionic liquid or intermolecular charge transport. Taking the results at hand into consideration, depletion of water from DNA and binding ionic liquids to the minor groove of duplex DNA could be the two major reasons accounting for the assistances of IL to DNA mediated charge transport.

5.5 Summary and Outlook

In summary, higher yields of DNA CT were observed in hydrated ionic liquid. Contrary to the decay of yields over longer duplex DNA in aqueous buffer, DNA CT over 3.8 nm showed inverse distance dependence in hydrated Bmim BF₄, with an oscillating enhancement of CT efficiency along an A₁₀ bridge. Facilitation of CT efficiency is closely associated with cation and anion species of ionic liquid. ILs with aromatic cations (Bmim⁺ and Etp⁺) have better facilitation on hole transport efficiency than those with non-aromatic cations (Bmp BF₄). Hydrophobic anion BF₄⁻ shows special importance in modulating binding of ionic liquid cations to DNA minor groove, while ionic liquids with hydrophilic anions (Cl⁻, Ac⁻) inhibits CT. Spectroscopic assays indicated that binding of ionic liquid to DNA minor groove is not limited to cation component, but rather both ionic parts as "one molecule". Additionally, the mixture of D₂O-Bmim BF₄ resulted in more pronounced ⁸CPG decomposition than H₂O-Bmim BF₄. Our study has revealed novel phenomena of the interactions between highly charged DNA molecules and ionic liquids, and promoted reactivity of bio-redox reactions in hydrated ionic liquids. Tight binding to minor groove to achieve interactions with base pair array and to exclude water molecules might account for the facilitation effects from ionic liquids. Our findings of elevation of DNA CT in hydrated ionic liquid not only provides novel strategy to achieve efficient

biomolecular electronic wire for nanoscale devices, but broaden knowledge in the reaction of biological macromolecules in green solvents with non-organic/non-aqueous features. Further simulation to show more structural correlations between ionic liquid interaction with DNA and facilitated DNA CT is ongoing in our lab. In addition, it will be of great interest to explore ionic liquid effects on polyelectrolytes if possible. At last, since ionic liquid's applications currently are still limited to materials, our study might have limited implications on the DNA CT in the cellular environment. However, our exploration of DNA CT in the molecular crowding conditions (not presented in the thesis) suggested enhanced DNA CT efficiency, which might indicate that DNA CT in the cellular environment could be more efficient than we previously imagined.

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