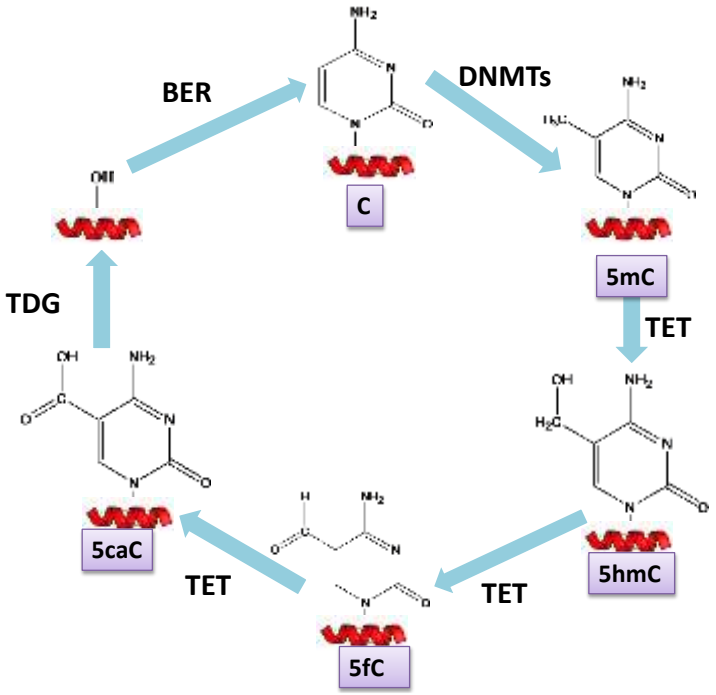
CHAPTER 7

Theoretical elucidation of methylation of cytosine and its subsequent oxidation processes

7.1.

INTRODUCTION

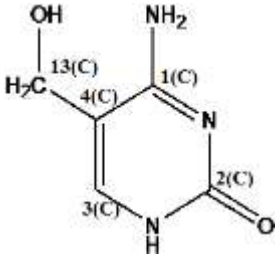
Every multicellular organism includes the DNA which is a significant functional molecule that has evolved to programme and pass on information through generations. DNA consists of the four nucleobases adenine (A), cytosine (C), guanine (G) and thymine (T), which is stored within as the primary code and it is the linear sequential arrangement of these four canonical bases that makes up the genetic code. The first modification of a canonical nucleobase to be discovered was the 5-methylcytosine (5mC).[1] More than 17 modified DNA bases have been reported[2] since the discovery of 5mC in the year 1898. The Watson–Crick pairing is not hindered by the introduction of these characteristic modifications but rather introduces chemical functionality into the major groove of the double helix structure. This additional functionality in the major groove will definitely change or block protein recognition which can be effectively applied in bacterial warfare. In bacterial warfare, methylation of specific sequence sites in the host genome, described as restriction methylation, safeguards self-cleavage by special nucleases. Cytosine methylation is an extensively studied DNA modification. Johnson and Coghill confirmed its existence in the year 1925, recognizing it as a hydrolysis product of tuberculinic acid.[3] Mammalian DNA methylation (5mC) is ascertained by de novo DNA methyltransferases DNMT3A/3B, and the patterns of 5mC are maintained by DNMT1. In mammals, DNA methylation is concerned with the preservation of cellular functions and genomic stability, as well as is involved in procedures of X-chromosome inactivation, genomic imprinting and transposon silencing.[4–7] The active demethylation pathway probably involves the iterative oxidation of 5-methylcytosine (5mC) to5-(hydroxymethyl)cytosine (5hmC), 5-formylcytosine(5fC) and 5-carboxycytosine (5caC) by the ten-eleven translocation (TET) enzymes (namely, TET1, TET2 and TET3), which are 2-oxoglutarate (2-OG)- and Fe(II)-dependent dioxygenases (Scheme 7.1).



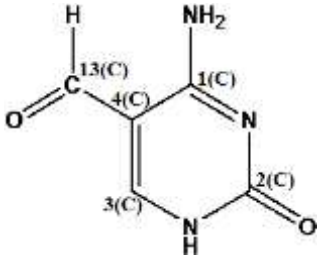
Scheme 7.1. DNA methylation (5mC) is established by de novo DNA methyltransferases DNMT3A/3B, and the patterns of 5mC are sustained by DNMT1. TET enzymes successively oxidize 5mC to 5hmC, 5fC, and 5caC.

Cytosine (C)

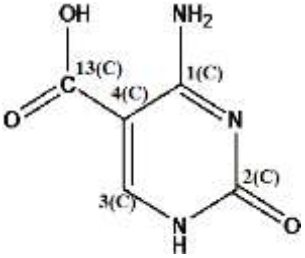
5-methylcytosine (5mC)



5-(hydroxymethyl)cytosine (5hmC)



5-formylcytosine (5fC)



5-carboxycytosine (5caC)

Figure 7.1. The structure and schematic representation of cytosine molecule and its derivatives. The numbering of Carbons are according to their fukui function calculations.

The chemical and physical properties of modified DNA bases differ substantially despite the similarity in the chemical structure of their unmodified counterpart. It is clear that base modifications cause changes in the reactivity, structure and base-pairing interactions of DNA. DNA base modifications also influence DNA metabolism and DNA repair. Consequently, the understanding of chemical and physical properties of modified molecules becomes extremely important to decipher the role of modified bases in biological events. However, studies towards understanding the chemical and physical properties of modified bases are limited in literature. In past few decades the density functional theory (DFT) has successfully been used as a general tool to investigate the structure and properties of atoms and molecules. Employing this theory, the properties of a many-electron system can be determined by the suitable use of proper functionals, i.e., functions of another function, which is the spatially dependent electron density. DFT is one of the most accepted and resourceful methods available in computational chemistry. However, the popularity of DFT emerged when the approximations used in the theory were greatly sophisticated to better model the exchange and correlation interactions. Computational costs are relatively low when compared to conventional methods, such as exchange only Hartree–Fock theory and its descendants that include electron correlation. Several qualitative and semiquantitative methods have since been developed for predicting how and whether a reaction will take place. The most popular method of prediction is frontier molecular orbital theory (FMO) which uses the shapes and symmetries of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) to assign whether a reaction will take place or not. If the HOMO of the electron donor and the LUMO of the electron acceptor have similar shape and phase, then electron transfer from the HOMO of the first molecule to the LUMO of the second is facilitated, often forming a bond between them. One of the most significant qualities of the density functional theory is its appropriateness for elucidating vital chemical concepts of molecular structure and reactivity. In the late 1970s and early 1980s Parr developed the "conceptual DFT"[8] which is a subfield of DFT where information is obtained from the electron density relevant concepts and principles thus making it possible to comprehend and predict the chemical behaviour of a molecule. Parr and co-workers, along with a large community of theoretical chemists, have been able to give precise definitions for chemical concepts, thus providing their calculations with a quantitative value. Recently, conceptual DFT has been successfully employed as a powerful predictive tool in understanding the structure, property and reactivity of DNA bases and their assemblies.[9,10]

Herein, the most relevant indices defined within the conceptual for the study of the organic reactivity of cytosine and its modifications are discussed.

So, here we try to monitor the processes of cytosine modifications using conceptual Density Functional Theory (DFT) based study to compute quantum chemical parameters such as highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), electrophilicity, electronegativity, chemical potential, chemical hardness, and nucleophilicity which give considerable insight towards defining reactivity of the cytosine modifications. The conceptual DFT based global reactivity and the local reactivity descriptors are constructive measures of comprehending the global reactivity and the local site selectivity, respectively. In this study, we have computed the global reactivity descriptors for the four cytosine modifications: 5-methylcytosine, 5(hydroxylmethyl) cytosine, 5-formylcytosine and 5-carboxylcytosine (Figure 7.1) and compared it with the unmodified cytosine and also the ability of these descriptors to predict the electrophilic attack on cytosine to produce the 5mC. The subsequent oxidation from 5mC to 5hmC, 5fC, and 5caC is explained with the help of these global and local reactivity parameters. In addition to this, we also quantify the aromaticity of the rings in all these compounds and correlate it with the stability of these molecules.

6.2.

COMPUTATIONAL DETAILS

Conceptual density functional theory based methods are widely used to predict reactivity of molecules. In this study, the inputs of studied molecules are prepared with Gauss View.[11] Calculations are performed by using Gaussian 16,[12] at b3lyp level and cam-b3lyp level of theory. The results obtained from cam-b3lyp are used for discussion in this manuscript because it includes the Handy and coworkers long-range-corrected version of b3lyp via the Coulomb-attenuating method[13] which gives better insight into the conceptual DFT results. The Chemical reactivity descriptors include E_{HOMO}, E_{LUMO}, ΔE (HOMO–LUMO energy gap), electronegativity (χ), chemical potential (μ), chemical hardness (η), electrophilicity (ω), nucleophilicity (ε) and global softness (σ). Electronegativity, global softness, and chemical hardness have pertained to ionization energy (I) and electron affinity (A) values of molecules obtaining the following equations. It is well

established that the negative value of the highest occupied molecular orbital energy and the negative value of the lowest unoccupied molecular orbital energy were attached to the ionization energy and electron affinity, respectively ($-E_{HOMO} = I$ and $-E_{LUMO} = A$). The electronegativity (χ),[14-16] hardness (η)[17,18] and electrophilicity (ω)[19] of an *N*-electron system can be defined as follows,

$$\chi = -\mu = -\left(\frac{\partial E}{\partial N}\right)_{\upsilon(\vec{r})} \tag{7.1}$$

$$\eta = \left(\frac{\partial^2 E}{\partial N^2}\right)_{\upsilon(\vec{r})} \tag{7.2}$$

$$\omega = \frac{\mu^2}{2\eta} = \frac{\chi^2}{2\eta} \tag{7.3}$$

where E, μ, and $v(\vec{r})$ are the total energy of the *N*-electron system, chemical potential and external potential respectively.

Employing a finite difference approximation to Eqs.1 and 2 one can achieve

$$\chi = \frac{I + A}{2} \tag{7.4}$$

and

$$\eta = I - A \tag{7.5}$$

where I and A indicate the ionization potential and electron affinity of the cluster respectively, and they are calculated using Koopmans' theorem.[20,21]

The Fukui functions are employed to describe local electrophilicity and nucleophilicity factors. Removal of an electron from the molecule, called the Fukui function for electrophilic attack, is described as f^- and the Fukui function for *addition* of an electron to the molecule, called the Fukui function for nucleophilic attack is denoted as f^+. The understanding of the quantitative values of the Fukui function is that a large value of f^-, signifies that a molecule *donates* electrons, whereas a larger value of f^+ denotes where a

molecule *accepts* electrons. The Fukui functions can be defined using finite differences of the electronic density

$$f^{-}=\rho(N)-\rho(N-1) \tag{7.6}$$

$$f^{+}=\rho(N+1)-\rho(N) \tag{7.7}$$

where the densities of the cation ($N-1$), the neutral (N), and the anion ($N+1$) are employed. Here, chemical reactivity is described on an atom-by-atom basis via a "condensed" Fukui function. The condensed Fukui function is computed as

$$f_k^{-}=q_k(N)-q_k(N-1) \tag{7.8}$$

$$f_k^{+}=q_k(N+1)-q_k(N) \tag{7.9}$$

where q_k is the charge at atomic center k.[22-25] Here Hirschfeld charges are used in Multiwfn[27] software.

7.3.

RESULTS AND DISCUSSION

The quantum chemical parameters such as the HOMO, LUMO, hardness, electronegativity, nucleophilicity, softness, HOMO-LUMO gap and chemical potential of the nucleobase cytosine and its derivatives such as 5-methylcytosine, 5(hydroxylmethyl) cytosine, 5-formylcytosine and 5-carboxylcytosine are calculated and compared using the conceptual DFT based approach (Table 7.1 and Table 7.2).

Table 7.1. Global reactivity descriptors of derivatives of cytosine in ucam-b3lyp /6-311++g(d,p) methodology.

Molecule	HOMO Energy (eV)	LUMO Energy (eV)	HOMO-LUMO gap(eV)	Hardness (eV)	Softness (eV.)	Electrophilicity (eV)	Nucleophilicity (eV)
5-methylcytosine (5mC)	–0.287	–0.001	7.808	7.808	0.128	0.983	1.017
5-(hydroxylmethyl)cytosine (5hmC)	–0.293	–0.003	7.882	7.881	0.126	1.027	0.973
5-formylcytosine (5fC)	–0.315	–0.042	7.455	7.455	0.134	1.582	0.632
5-carboxylcytosine (5caC)	–0.319	–0.039	7.641	7.641	0.130	1.562	0.640

Table 7.2. Global reactivity descriptors of nucleobase cytosine and its derivatives in ub3lyp/6-311++g(d,p) methodology.

Molecule	HOMO Energy (eV)	LUMO Energy (eV)	HOMO-LUMO gap (eV)	Softness (eV.)	Electrophilicity (eV)	Nucleophilicity (eV)
5-methylcytosine	–0.234	–0.046	5.117	0.195	1.423	0.702
5-(hydroxylmethyl)cytosine	–0.239	–0.050	5.137	0.194	1. 510	0.662
5-formylcytosine	–0.263	–0.090	4.710	0.212	2.460	0.406
5-carboxylcytosine	–0.262	–0.082	4.881	0.205	2.249	0.445
cytosine	–0.242	–0.048	5.285	5.284	1.479	0.676

7.3.1 Condensed fukui functions employed to explain the methylation of cytosine

From the electrophilicity value we can say that the nucleobase cytosine is highly electrophilic which is apparent from the electrophilicity value of 1.479 eV, so is prone towards electrophilic addition to form 5-methyl cytosine. The highest electrophilic site of cytosine is at the 4-C centre as is described by the f^- value (0.142451), (Table 7.3) so the electrophilic attack occurs at the 4-C position to form 5-methyl cytosine. The numbering of the Carbon sites are given in Figure 7.1. Thus, the DNA modification involving the

methylation of the cytosine centre to form 5-methyl cytosine at the 4-C position can be theoretically ascertained from the conceptual DFT based electrophilicity value.

Table 7.3. Condensed fukui functions for cytosine

Atoms	f^-	f^+
1C	0.090071	-0.09007
2C	0.038033	-0.03803
3C	0.04374	-0.04374
4C	0.142451	-0.14245
5H	0.049219	-0.04922
6H	0.053832	-0.05383
7N	-0.00208	0.00208
8H	0.013769	-0.01377
9H	0.005771	-0.00577
10N	0.146118	-0.14612
11N	0.113676	-0.11368
12O	0.247402	-0.2474
13H	0.057552	-0.05755

The global reactivity descriptors for the four cytosine modifications: 5-methylcytosine, 5(hydroxylmethyl)cytosine, 5-formylcytosine and 5-carboxylcytosine are also calculated and is used to explain the subsequent oxidation from 5mC to 5hmC to 5fC to 5caC. The comparative study is described in detail in the following paragraphs. The condensed fukui functions for the cytosine modifications are used to describe the local reactivity factors such as electrophilicity and nucleophilicity describing the oxidation process in the subsection below and the condensed fukui values for 5mC, 5hmC, 5fC and 5caC are given in Table 7.4, 7.5, 7.6 and 7.7 respectively.

Table 7.4. Condensed fukui functions for 5mC

Atoms	f^-	f^+
1C	0.075667	-0.07567
2C	0.049557	-0.04956
3C	0.026808	-0.02681
4C	0.119714	-0.11971
5H	0.042504	-0.0425
6H	0.047027	-0.04703
7N	0.041219	-0.04122
8H	0.026466	-0.02647
9H	0.032453	-0.03245
10N	0.09116	-0.09116
11N	0.09006	-0.09006
120	0.220735	-0.22074
13C	0.029629	-0.02963
14H	0.039748	-0.03975
15H	0.039718	-0.03972
16H	0.02727	-0.02727

Table 7.5. Condensed fukui functions for 5hmC

Atoms	f^-	f^+
1C	0.0866	-0.0866
2C	0.041203	-0.0412
3C	0.039291	-0.03929
4C	0.115557	-0.11556
5H	0.038565	-0.03857
6H	0.054213	-0.05421
7N	0.004223	-0.00422
8H	0.022313	-0.02231
9H	0.011895	-0.0119
10N	0.134653	-0.13465
11N	0.110706	-0.11071
12O	0.236262	-0.23626
13C	0.024515	-0.02452
14O	0.029134	-0.02913
15H	0.032423	-0.03242
16H	-0.00263	0.002628
17H	0.029253	-0.02925

Table 7.6. Condensed fukui functions for 5fmC

Atoms	f^-	f^+
1C	0.062529	-0.06253
2C	0.030542	-0.03054
3C	0.032086	-0.03209
4C	0.115754	-0.11575
5H	0.03659	-0.03659
6H	0.047198	-0.0472
7N	-0.00409	0.004095
8H	0.019013	-0.01901
9H	0.003749	-0.00375
10N	0.13975	-0.13975
11N	0.100452	-0.10045
120	0.223286	-0.22329
13C	0.039735	-0.03974
14O	0.104238	-0.10424
15H	0.048661	-0.04866

Table 7.7. Condensed fukui functions for 5caC

Atoms	f^-	f^+
1C	0.054344	-0.05434
2C	0.028213	-0.02821
3C	0.028391	-0.02839
4C	0.058567	-0.05857
5H	-0.56288	0.56288
6H	0.042599	-0.0426
7N	0.087213	-0.08721
8H	0.051076	-0.05108
9H	0.02216	-0.02216
10N	-0.68013	0.68013
11N	0.084189	-0.08419
12O	0.172475	-0.17248
13C	0.028692	-0.02869
140	0.043475	-0.04348
15H	0.008592	-0.00859
16O	0.124051	-0.12405

7.3.2 FMO Study

The frontier molecular orbital study can predict the reactivity of the nucleobase cytosine and its derivatives and thus can serve as an important predictive tool for the iterative oxidation process of 5mC to 5hmC, 5fC, and 5caC. The energy of HOMO is related to the electron donating ability of the molecules. The molecules possessing higher HOMO energy show the tendency to donate the electrons to appropriate acceptor molecules. Oxidation involves loss of electrons from the HOMO, so higher the energy of HOMO, greater will be the tendency for oxidation. If we compare the HOMO energy of cytosine and its derivatives, the order followed is 5caC<5fC<5hmC<5mC. So, from the order of HOMO energy, we can predict that 5mC has a greater tendency to be oxidised to 5hmC and subsequently to 5fC and then to 5caC. Similarly energy levels of LUMO can predict electron accepting abilities following the trend 5fC<5caC<5hmC<5mC. So the trend for subsequent oxidation process is similar with slight mismatch in 5fC and 5caC. The HOMO-LUMO energy gap can be considered as a direct indicator of stability. The trend followed by cytosine and its derivatives is 5fC<5caC<5mC<5hmC. 5hmC is relatively abundant amongst the other oxidised derivatives and this is evident from the higher HOMO-LUMO gap of the 5hmC (Table 7.1).

7.3.3 Chemical hardness and softness

Chemical hardness is described as the measure of the resistance of a chemical species to changes in its inherent electronic configuration. Thus, hardness serves as an indicator of reactivity and stability of molecules. If we compare the hardness profile, the order of increasing hardness is 5fC<5caC<5mC<5hmC. The hardness value of 5hmC is highest. This theoretical interpretation is further strengthened by the experimental observations where employing the methylation-insensitive restriction enzyme MspI and a TLC assay, Tahiliani et al. (2009) reported that the 5hmC level in mouse ES cells is ;0.03% of total nucleotides. Utilising a two-dimensional (2D) TLC method and nearest- neighbor analysis, Kriaucionis and Heintz (2009) accounted 5hmC level of 0.6% and 0.2% of total nucleotides in Purkinje and granule neurons, respectively. Thus, these experimental results further validate the abundance of 5hmC as compared to the other oxidized products, as is predicted by its highest hardness value amongst the other substituents. Thus, the hardness profile is in consonance

with its experimental abundance. Softness is defined as the reciprocal of hardness. So, the trend followed by cytosine derivatives is just the opposite to the trend followed by chemical hardness i.e., 5hmC<5mC<5caC<5fC.

7.3.4 Electrophilicity and Nucleophilicity

Electrophilicity predicts the attraction of a molecule towards electron rich centres; from the electrophilicity values we can predict the tendency of a molecule towards electrophilic substitution reactions. On the other hand, nucleophilicity values give us an idea regarding the attraction of a molecule towards electron deficient centres thereby predicting whether the molecule is prone towards nucleophilic substitution or not. The trend of global electrophilicity followed by cytosine derivatives is 5mC<5hmC<5caC<5fC and that of nucleophilicity is 5fC<5caC<5hmC<5mC which is just the opposite of the trend followed by the electrophilicity values as expected. The local electrophilicity and nucleophilicity values for each atom centres for the individual cytosine derivatives are calculated based on condensed fukui functions and are given in Table 7.4, 7.5, 7.6 and 7.7.

7.3.5 Aromaticity

Nucleobases are non- polar and due to their aromaticity, planar. The flat shape due to the virtue of aromaticity is of particularly important when considering their roles as building blocks of DNA. Cytosine and its derivatives are aromatic molecules comprising one aromatic ring. With respect to the electronic nature of the molecule, aromaticity portrays a conjugated system comprising of alternating single and double bonds in a ring. This arrangement permits pi electron delocalization around the ring, thereby increasing the molecule's stability by resonance. Thus, aromaticity can be directly linked to the inherent stability of the molecules. Aromaticity is quantified here with the help of Nucleus Independent Chemical Shifts (NICS) which was postulated by Schleyer and his co-workers.[28] The negative value of the absolute magnetic shielding provides the definition of NICS. The NICS values are computed by placing the ghost atom at the centre of the ring [NICS(0)] to account for the contribution of the σ-electrons to aromaticity and 1 Å above and below the plane i.e., [NICS (1)] and [NICS(-1)] respectively to estimate the π-aromaticity of the systems. In harmony with NMR chemical shift principle, the NICS values are reported with negative signs. Positive NICS value signifies a paratropic magnetic field corresponding to antiaromatic systems whereas a negative NICS value signifies a diatropic magnetic field which correlates to aromatic systems. The NICS values for cytosine and its derivatives are given in Table 7.8. As

aromaticity is an indicator of stability higher the aromaticity, greater will be its stability. So, the trend followed by both σ and π aromaticity for the cytosine derivatives are similar to the trend followed by hardness, i.e., 5fC<5caC<5mC<5hmC. So, 5hmC has already been established as the most stable molecule among the cytosine derivatives from its hardness value owing to its higher abundance from experimental observations. From quantitative evaluation of aromaticity also, it is observed that it has a higher NICS value thereby is in harmony with its higher stability as was predicted from the hardness profile.

Table 7.8. The nucleus-independent chemical shift (NICS) values in ppm at the plane of the ring (NICS (0)) and at 1 Å above the plane of the ring (NICS (1)) in cam-b3lyp methodologies using 6-311++g(d,p) basis set.

Molecule	**NICS(0)**	**NICS(1)**	**NICS(–1)**
Cytosine	0.7346	2.5725	2.5734
5Mc	1.2683	2.8656	2.8601
5hmC	1.3124	2.8414	3.0726
5f	0.6990	2.6796	2.6775
5caC	0.9457	2.6726	2.5231

7.4.

CONCLUSIONS

The conceptual DFT based global and local reactivity parameters serve as an important chemical intuitive and predictive tool to elucidate the process of DNA modification i.e., the methylation of cytosine and its subsequent oxidation. Cytosine is one of the most essential DNA nucleobases which makes up the genetic code and its methylation is one of the widely studied DNA modifications. The following successive active demethylation pathway involving the iterative oxidation of 5-methylcytosine (5mC) to5-(hydroxymethyl)cytosine (5hmC), 5-formylcytosine(5fC) and 5-carboxycytosine (5caC) is explained with the constructive methods of conceptual DFT based approach such as frontier molecular orbital study, chemical hardness and softness, local reactivity descriptors such as electrophilicity and

nucleophilicity which provide considerable insight into the reaction pathway. Aromaticity descriptor NICS also offers a significant understanding of the stability of cytosine and its derivatives as stability is intricately related to chemical reactivity.

7.5.

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