Studies of intrinsically curved and forcibly curved DNA as well as their interactions with topological enzymes

Yang, Zhaoqi

2011

Yang, Z. (2011). Studies of intrinsically curved and forcibly curved DNA as well as their interactions with topological enzymes. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/48080>

<https://doi.org/10.32657/10356/48080>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**Studies of intrinsically curved and forcibly
curved DNA as well as their interactions
with topological enzymes**

YANG ZHAOQI

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2011

**Studies of intrinsically curved and forcibly
curved DNA as well as their interactions
with topological enzymes**

YANG ZHAOQI

School of Physical and Mathematical Sciences

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2011

Acknowledgements

I would first and foremost like to express my deepest gratitude to my supervisor, Professor Li Tianhu, who has encouraged and inspired me during my four years graduate study in Nanyang technological University. Without his support and suggestion, this thesis could not be accurate and smooth finished. His helpful advices and positive encouragement have helped and pulled me through many difficult times in my research work. Furthermore, Professor Li has provided some excellent opportunities to me for acquire knowledge, skills and experience as well as supported me to participate in overseas conference to gain greater exposures in the research. For all these, I am wholeheartedly grateful to him for his great help and support given to me in my graduate study and research from the bottom of my heart.

I would also like to acknowledge my colleagues and friends in Professor Li's lab for their insightful suggestion and help in my research. My smooth and enriching research experience would not have been possible to achieve without their enthusiastic assistance and honest discussions. They have offered their generous and sincere friendships to me.

In addition, I would also like to grateful to many support staffs from the administrative office, teaching lab and the chemical store, especially Ms Celine Hum who have assisted me in many graduate studies and thesis issues. I would particularly like to appreciate Dr. Sun Wanxin for his assistant in AFM sample detection.

I would like to thank my family members and friends for their love, support and understanding during this period of time. The financial support for experiments from Ministry of Education in Singapore and Nanyang Technological University is gratefully acknowledged.

Table of Contents

Acknowledgements	I
Table of Contents	III
Abstract	V
List of Tables	VII
List of Figures	VIII
List of Abbreviation	XIII

Chapter 1 – Introduction

1.1	Introduction to the structure of DNA	1
1.2	DNA curvature	7
1.2.1	Intrinsic curvature of DNA	7
1.2.2	Forcible curvature of DNA	10
1.3	DNA supercoiling	11
1.4	Topoisomerases	14
1.5	The importance of topoisomerases in biological system	22
1.6	Topoisomerases as a significant target in drug design	23

Chapter 2 – Recognition of forcible curvature in circular DNA by

human topoisomerase I

2.1	Background and objectives	26
2.2	Design of DNA sequences	29
2.3	Materials and methods	32
2.4	Results and discussions	45
2.4.1	Inhibitory effect of DNA circles on human topoisomerase I	45
2.4.2	AFM observation of the interaction of DNA circle and human topoisomerase I	56

2.4.3	The research of EMSA of DNA circle and human topoisomerase I	58
2.5	Conclusion	60

Chapter 3 – The activity of human topoisomerase I was modulated by intrinsic curvature DNA

3.1	Background and objectives	64
3.2	Design of DNA sequences	66
3.3	Materials and methods	72
3.4	Results and discussion	80
3.4.1	Inhibitory effect of intrinsic curvature DNA on human topoisomerase I	80
3.4.2	Inhibitory effect of the quantity of most curvature sequence of DNA on human topoisomerase I	85
3.4.3	Interaction of linear curvature DNA and human topoisomerase I	88
3.5	Conclusion	90

Chapter 4 – Preparation of catenated ring and Trefoil ring based on toroidal DNA structures

4.1	Background and objectives	92
4.2	Design of DNA sequences	95
4.3	Materials and methods	102
4.4	Results and discussion	109
4.4.1	Analysis of the toroidal DNA	109
4.4.2	Formation of catenated ring by Cre-recombinase	111
4.4.3	Formation of Trefoil ring by human topoisomerase II	119
4.5	Conclusion	122

References	125
-------------------------	------------

Curriculum Vitae	133
-------------------------------	------------

Abstract

Topological enzymes, both type I and type II topoisomerases, are not only essential for the processes of cell growth, but also for their effect on altering the geometrical conformations of DNA. It has been shown that Human topoisomerase I (hTopo I), belonging to the type I subfamily, binds to DNA in a topology-dependent fashion with a strong preference for supercoiled DNA over relaxed DNA. Furthermore, it has been reported that eukaryotic topoisomerase I acts preferentially on intrinsically curved DNA. Prior to thoroughly investigating the mode of action of hTopo I, the specific structure of the substrate had to be identified. A series of forcible curved circular DNA (cDNA) structures were prepared, in order to investigate a particular recognizable characteristic of hTopo I. All of the cDNAs exhibited more efficient interference to hTopo I-catalyzed plasmid relaxation, than linear duplex DNA. The half maximal inhibitory concentration (IC_{50}) can be modulated by the curvature degrees of cDNA. In addition, the preferential binding of hTopo I to cDNA containing high curvature has been demonstrated by atomic force microscopy (AFM) and electrophoretic mobility shift assay (EMSA). The results of this research suggest that hTopo I can preferentially recognize the curved conformation of cDNA.

Moreover, hTopo I has been known as a potential target for cancer therapy, as a consequence of its crucial role in replication and transcription. Accordingly, hTopo I can preferentially recognize the forcible curvature in cDNA and act preferentially on the intrinsically curved DNA. A series of duplex DNA structures, with different intrinsic curvatures, has been designed as inhibitors to hTopo I. The activity of hTopo I in relaxing

supercoiled pUC 19 is apparently diminished in the presence of the curved DNA. More potent inhibitions and smaller IC_{50} values are achieved by duplex DNA with higher curvatures. These studies demonstrate that the activity of hTopo I can be modulated by the intrinsic curvature of linear DNA. Moreover, these results can be used to provide a new method to design curved DNA as hTopo I inhibitors with high therapeutic efficiency and low toxicity.

On the other hand, strand proximity and self-crossing intrinsically occurred in the relaxed forms of toroidal DNA. Topological enzymes were employed to produce extraordinary DNA topological conformations, such as Trefoil and catenated ring DNA. Based on our recent analysis of the intrinsic curvature of DNA, a relaxed form of toroidal DNA was synthesized and further reacted with human topoisomerase II for generating Trefoil ring DNA. The Trefoil DNA structures were distinctly observed through AFM examination.

In addition, Cre recombinase (a type I topoisomerase) and the relaxed forms of toroidal DNA were utilized unprecedentedly in our investigations for creating catenated DNA rings. The geometrical shapes of the obtained catenated DNA circles, as well as their topological arrangements, were clearly visible under AFM examination. Furthermore, our results indicate that the DNA ring size in a catenated structure could be precisely manipulated using the methods developed in these current studies. It is our hope that the information provided currently in this research will be useful for constructing catenated DNA structures with desired sizes and sequences in the future.

List of Tables

Table 1-1.	Helical parameters of different DNA structure.	6
Table 1-2.	Characteristics and properties of DNA topoisomerases.	16
Table 1-3.	The common inhibitors of Topoisomerases.	21
Table 2-1.	The sequence of each DNA circles.	31
Table 2-2.	Sequence of X2420G plasmid DNA (3593 bp).	33
Table 2-3.	Inhibitory efficiency (IC_{50}) of circular DNAs on the activity of human topoisomerase I.	54
Table 3-1.	Sequences of oligonucleotides examined by literature.	69
Table 3-2.	The sequences of DNA (consisting of the same composition) used in our studies.	71
Table 3-3.	Inhibitory efficiency (IC_{50}) of intrinsically curved DNAs on the activity of human topoisomerase I.	83
Table 4-1.	Sequence of new design toroidal DNA (Circular DNA 1).	100
Table 4-2.	Sequence of X3704E plasmid DNA (4563 bp).	103

List of Figures

Figure 1-1.	Schematic illustration of DNA double helix.	2
Figure 1-2.	Schematic illustration of polynucleotide (single strand DNA).	3
Figure 1-3.	Schematic illustration of structure of nucleotides.	3
Figure 1-4.	Structures of purine and pyrimidine bases in DNA.	3
Figure 1-5.	Schematic illustration of structure of nucleotides.	4
Figure 1-6.	Schematic illustration of structural parameters of double helix DNA in solution.	5
Figure 1-7.	Structural differences of intrinsically curve DNA and straight DNA.	8
Figure 1-8.	Schematic illustration of wedge model and junction model for intrinsic curvature DNA.	9
Figure 1-9.	Schematic illustration of forcible curvature.	10
Figure 1-10.	Schematic illustration of twist and writhe of circular DNA.	12
Figure 1-11.	Schematic illustrate the relationship of negative supercoil and positive supercoil in circular DNA.	13
Figure 1-12.	Illustration of two common forms of supercoiled DNA (plectonemic form and toroidal form).	14
Figure 1-13.	Schematic illustration of topoisomerases reaction.	15
Figure 1-14.	Crystal structures of <i>E. coli</i> topoisomerases I (a) and III (b).	19
Figure 1-15.	Proposed mechanism of relaxation by <i>E. coli</i> topoisomerase I.	19
Figure 1-16.	Side view (a) and top view (b) of the structure of human topoisomerase I	20
Figure 1-17.	A possible mechanism of human topoisomerase I.	20
Figure 1-18.	Proposed mechanism of relaxation of topoisomerase II.	21

Figure 2-1.	The binding site of human topoisomerase I.	29
Figure 2-2.	Illustration of different status of curvature associated with supercoil DNA.	30
Figure 2-3.	Schematic diagrams that illustrate the formation of closed circular DNA through long cohesive ends.	35
Figure 2-4.	Preparation of 84 base pairs circular DNA.	36
Figure 2-5.	Preparation of 300 base pairs circular DNA.	39
Figure 2-6.	Preparation of 535 base pairs circular DNA.	40
Figure 2-7.	Preparation of 936 base pairs circular DNA.	41
Figure 2-8.	Agarose gel electrophoretic analysis of inhibitory effect of 84 bp circle (a) and 84 bp linear (b) on human topoisomerase I.	46
Figure 2-9.	Correlations between base pairs concentration of 84 bp circle and its percent inhibition on human topoisomerase I.	48
Figure 2-10.	Correlations between base pairs concentration of 84 bp linear DNA and its percent inhibition on human topoisomerase I.	48
Figure 2-11.	Agarose gel electrophoretic analysis of inhibitory effect of 300 bp circle on human topoisomerase I.	50
Figure 2-12.	Correlations between base pairs concentration of 300 bp circle and its percent inhibition on human topoisomerase I.	50
Figure 2-13.	Agarose gel electrophoretic analysis of inhibitory effect of 535 bp circle on human topoisomerase I.	51
Figure 2-14.	Correlations between base pairs concentration of 535 bp circle and its percent inhibition on human topoisomerase I.	52
Figure 2-15.	Agarose gel electrophoretic analysis of inhibitory effect of 936 bp circle on human topoisomerase I.	53
Figure 2-16.	Correlations between base pairs concentration of 936 bp circle and its percent inhibition on human topoisomerase I.	53
Figure 2-17.	pUC 19 relaxation assay for the inhibitory effect of 84 bp, 300	55

bp, 535 bp and 936 bp circular DNA on human topoisomerase I in same concentration.

- Figure 2-18.** Three dimensional AFM plot of 300 bp circular DNA in presence (A) and absence (B) of human topoisomerase I respectively. 57
- Figure 2-19.** Three dimensional AFM plots of 535 bp circle and 936 bp circle in presence (A, C) and absence (B, D) of human topoisomerase I respectively. 58
- Figure 2-20.** Electrophoretic mobility shift assay (EMSA) analysis of human topoisomerase I and 84 bp circular DNA interactions. 59
- Figure 2-21.** Illustration of human topoisomerase I preferential binds to curvature of DNA. 61
- Figure 2-22.** Illustration of the irreversible covalent interaction of small circular oligonucleotides and human topoisomerase I. 62
- Figure 3-1.** Illustration of a duplex sequence including A-tract that can form intrinsically curved structures. 68
- Figure 3-2.** Illustration of sequences with decreasing R_L value. 70
- Figure 3-3.** Illustration of the calculated result of design DNA with different curved degree. 73
- Figure 3-4.** (A) Illustration of the overlap of DNA 1, DNA 2, DNA 3 and DNA 4. (B) Electrophoretic analysis of different curvature degree of DNA 1, DNA 2, DNA 3, and DNA 4. 76
- Figure 3-5.** (A) Illustration of the overlap of DNA 5, DNA 6 and DNA 4. (B) Electrophoretic analysis of different curvature degree of DNA 5, DNA 6 and DNA 4. 77
- Figure 3-6.** pUC 19 relaxation assay for the inhibitory effect of DNA 1, DNA 2, DNA4 (A) and DNA 3 (B) on human topoisomerase I. 81
- Figure 3-7.** Correlations between concentration of DNA 1 (A), DNA 2 (B), DNA 3(C), DNA 4 (D) and the corresponding inhibitory effects on human topoisomerase I. 82

Figure 3-8.	(A) pUC 19 relaxation assay for the inhibitory effect of DNA 1, DNA 2, DNA 3 and DNA 4 on human topoisomerase I at the same concentration. (B) Percentage inhibition of relaxation reaction by different curvature DNA.	84
Figure 3-9.	pUC 19 relaxation assay for the inhibitory effect of DNA 5 (A) and DNA 6 (B) on human topoisomerase I.	86
Figure 3-10.	Correlations between concentration of DNA 5 (A), DNA 6 (B), and the corresponding inhibitory effects on human topoisomerase I.	87
Figure 3-11.	Schematic representation of interaction between human topoisomerase I and duplex DNA.	88
Figure 3-12.	EMSA analysis of human topoisomerase I and intrinsically curved DNA interaction.	89
Figure 3-13.	Illustration of the pUC 19 relaxing assay. The relaxation of supercoiled pUC 19 is catalyzed by hTopo I in the presence or absence of curvature DNA.	90
Figure 4-1.	Illustration of the structural conformations of circular DNA catenated DNA and Trefoil DNA.	93
Figure 4-2.	Illustration of distinctive DNA structure (toroidal structure DNA).	94
Figure 4-3.	Schematic representation of our synthetic routes of toroidal DNA.	97
Figure 4-4.	Recombination mechanism of Cre combinase.	98
Figure 4-5.	Schematic representation of DNA recombination reactions catalyzed by Cre recombinase.	99
Figure 4-6.	Schematic representation of Terfoil ring prepare reactions catalyzed by human topoisomerase II.	101
Figure 4-7.	The synthesis of relaxed form of toroidal DNA that contains two lox p sites.	105
Figure 4-8.	Preparation of toroidal DNA.	107

Figure 4-9.	Atomic force microscopy images of Circular DNA 1 (toroidal DNA, 500 nm x 500 nm scans).	110
Figure 4-10.	Electrophoretic analysis of Circular DNA 1 acted on by human Topoisomerase I.	111
Figure 4-11.	Picturtic illustration of generated catenated DNA from toroidal DNA by the action of Cre recombinase.	112
Figure 4-12.	Electrophoretic analysis of reaction mixtures of Circular DNA 1 and Cre recombinase.	113
Figure 4-13.	AFM images of DNA that are corresponding to Band 2 in Lane 3 in Figure 4-12 (1 μ m x 1 μ m scans).	114
Figure 4-14.	Analysis of the atomic force microscopic picture of catenated ring DNA.	114
Figure 4-15.	AFM images of Non-Catenated Ring 1 and Non-Catenated Ring 2.	115
Figure 4-16.	Schematic illustration of strand recombination of toroidal DNA in which two lox p sites are aligned in an anti-parallel fashion.	117
Figure 4-17.	Preparation of Circular DNA 2.	117
Figure 4-18.	Atomic force microscopy images of Circular DNA 2 (toroidal DNA, 500 nm x 500 nm scans).	118
Figure 4-19.	Electrophoretic analysis of reactions between Circular DNA 2 and Cre recombinase.	118
Figure 4-20.	Picture of Trefoil ring structure and atomic force microscopic images of Trefoil ring DNA structure (300 nm x 300 nm scans).	120
Figure 4-21.	Electrophoretic analysis of reaction mixtures of Circular DNA 2 and human topoisomerase II.	121
Figure 4-22.	AFM images of band 2 and band 3 in Figure 4-21.	122

Table of Abbreviations

°C	degree centigrade
APS	ammonium persulfate
A-tract	adenine-tract
bp	base pairs
cDNA	closed circular DNA
DNA	deoxyribonucleic acid
ssDNA	single stranded DNA
dsDNA	double stranded DNA
IC ₅₀	half maximal inhibitory concentration
L _k	linking number
Tw	twist
Wr	writhe
PAGE	polyacrylamide gel electrophoresis
EB	ethidium bromide
htopo I	human topoisomerase I
htopo II	human topoisomerase II
TAE	tris, ammonium acetate, EDTA buffer
TBE	tris, Boric acid, EDTA buffer
TEMED	N, N, N', N'-tetramethylenediamine
EDTA	ethylenediaminetetraacetic acid
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
TRIS	tris (hydroxymethyl) methylamine
AFM	atomic force microscopy
EMSA	electrophoretic mobility shift assay
BSA	bovine serum albumin

Chapter 1

Introduction

1.1 Introduction to the structure of DNA

DNA (deoxyribonucleic acid) was first observed by German biochemist Frederich Miescher who processed the separation of the nuclein from leukocytes that he obtained from pus in the late 1860's.¹⁻³ Eight decades later in 1944, Oswald T. Avery and colleagues published their landmark investigation which assumed that DNA was the storage of genetic material.⁴⁻⁶ After the speculation proposed by Oswald et al, Erwin Chargaff demonstrated that the composition of DNA's base varies between species and within each species the bases are always present in fixed ratios.^{2, 7} In 1953, building on X-ray analyses of DNA structure as a double helix described by James Watson and Francis Crick with important contribution from the postulation of three dimensional structure of DNA suggested by Wilkins and Franklin.⁸⁻¹⁰ This important discovery initiated a wide-ranging chemical and biological revolution and provided the basis for contemporary molecular biology to continual search of more discoveries and new techniques in the past several decades.¹¹ The understanding of such double helical model unraveled the special structure of the DNA molecule and realized its central importance to biology because of DNA being the genetic material to encode genetic information.^{11, 12} DNA is crucial material in various biological functions such as replication, transcription and recombination. In the absence of DNA in biological processes, it is impossible for synthesis of proteins and cell growth.

Owing to studying X-ray diffraction patterns and building models by Watson and Crick in 1953, the double helix structure of DNA was figured out, which enables it to become an important biological molecule. DNA is the hereditary material in humans and almost all other organisms and usually a double-helix contains two strands running in opposite directions.^{8, 13} Each chain (Figure 1-2) is a polymer which is composed by different order of the four nucleotides (hence the single strand general term polynucleotide). As shown in Figure 1-3, each nucleotide is constituted by three components including a purine or pyrimidine ring aromatic base, a deoxyribose sugar and phosphate groups. The four kinds of different heterocyclic bases with a purine ring in DNA are adenine (A) and guanine (G) while the two pyrimidine bases in DNA are thymine (T) and cytosine (C). (Figure 1-4)

Figure 1-1. Schematic illustration of DNA double helix.¹⁴

Figure 1-2. Schematic illustration of polynucleotide (single strand DNA).¹¹

Figure 1-3. Schematic illustration of structure of nucleotides.¹¹

Figure 1-4. Structures of purine and pyrimidine bases in DNA. The purine ring structure is composed of 6-member and 5-member ring. The numbering systems for the ring are indicated on the purine ring and on the structures of adenine (A) and guanine (G). The pyrimidine ring structure is composed of 6-member aromatic ring. The numbering systems for the ring are indicated on the structures of thymine (T) and cytosine (C).

As shown in Figure 1-2, each DNA single strand has a backbone formation of (deoxy-ribose) sugar molecules connected by phosphate groups. The bases are attached to the 1'-position of the sugar through glycosidic bond and the 3' hydroxyl group of sugar molecule is linked together through a phosphate group to the 5' sugar carbon of the next sugar.¹⁵ This linkage of each individual nucleotide is also called 3'-5' phosphodiester linkage. The polynucleotide chain (single strand DNA) are read from two distinct ends, known as 5' and 3', where the 5' terminates in a phosphate group and the 3' terminates in a sugar molecule.⁹ Figure 1-1 shows the double helix, primary structure of DNA, which forms two single strand polynucleotide chains (single strand). The structure of the double helix is held together by plenty of individual hydrogen bond which is formation of DNA base pair as shown in Figure 1-5. DNA bases made up of two bases pair with each other, base A always pairs with base T, and likewise C with G ^{9,16}. It is very easy to figure out the sequence of bases on the other strand when the sequence of the bases on one strand of a DNA double helix is known.

Figure 1-5. Schematic illustration of structure of nucleotides.¹¹

As shown in Figure 1-6, the structure of DNA was elucidated through number of parameters that define the helix great detail by Dickerson and Saenger.¹⁷⁻¹⁹ The quantity of DNA base pairs in a helical turn of DNA is the number of base pairs that need to spiral one 360° rotation. The DNA structure model provided by of Watson and Crick described that 10 base pairs compose a helical turn with a distance of 34 Å between contiguous base pairs. The previous investigation demonstrated that the configuration of DNA is the B-form DNA which are about 10.5 base pairs in every right handed helical turn with the form of the ribose sugar C2' endo.^{15, 19} In addition, the presence of manifest major and minor grooves where the surfaces are provided for drug or proteins to interact is one of the most dominant characters of DNA configuration.¹⁹

Figure 1-6. Schematic illustration of structural parameters of double helix DNA in solution.¹¹

As compared with the helix structure of B-DNA, there is another right handed DNA helix known as A-DNA which has a wider helix containing 11 base pairs per turn with more tilted bases, the major groove is deep and thin with shallow minor groove.²⁰ Besides

these two kinds of right handed helix, Z-DNA is left handed helix structure containing a phosphate backbone with obvious zigzag.²¹ Compared with the helical turn of 10.5 base pairs for B-DNA, on the other hand, Z-DNA contains helical turn of 12 base pairs which is different from the right handed helix DNA model. Table 1.1 shows the overview of structural characteristics of three kinds of DNA helical structures. In addition, except for the feature of DNA including different helical configurations, DNA also contains other conformations apart from double helix as alternative structures such as cruciform^{22, 23}, DNA triplexes^{24, 25}, telomere²⁶, H-DNA²⁷, G-quadruplex^{28, 29} and DNA supercoiling.³⁰

Table 1.1 Helical parameters of different DNA structure.¹⁴

Parameter	B-DNA	A-DNA	Z-DNA
Helix handedness	Right	Right	Left
Residue per turn	10.5	11.0	12.0
Diameter of helix (Å)	26	20	18
Major groove	Wide and deep	Narrow and deep	Flat
Minor groove	Narrow and deep	Wide and shallow	Narrow and deep
Sugar pucker	C2'-endo	C3'-endo	C2'-endo (pyrimidine) C3'-endo (purine)

1.2 DNA curvature

According to the above section's demonstration, it is known that DNA with a capability of displays a high degree of configurationally variability in different conditions. The curvature of the helix axis is the one of most arresting characteristics of DNA structural conformation. It is apparent from the observation that two related but distinct phenomena, intrinsic curvature and forcible curvature, in DNA configuration. Intrinsic curvature refers to a bending of the DNA helix axis which is induced by the special composition of DNA sequence. Forcible curvature refers to the phenomena that certain DNA sequences can be bent owing to the formation of circular form under the action of ligase.³¹ A circular DNA can exhibit two types of curvature in same molecule while a linear DNA which contains intrinsic curvature is formed to a circle under the action of the enzyme.

1.2.1 Intrinsic curvature of DNA

It is well known to all that the length of DNA the log of the distance migrated is a relationship of linear in agarose and acrylamide gels. DNA molecules of explicit size acting on a very foreseeable way that shorter molecule migrate more rapidly than larger molecules when it runs on agarose or polyacrylamide gels. However, a most significant and attractive phenomenon of DNA anomalous migration that a DNA band of known length does not run at its predictable position on a polyacrylamide gel was found

occasionally by Marini et al. in 1982.^{32, 33} This example of such anomalous migration was a 414 base pairs segment of kinetoplast DNA from crithidia fasciculate show reduced electrophoretic mobility shift to a unexpected position which was twice as long as normal appearance in anacrylamide gel.³³⁻³⁵ It is speculated that the anomalous electrophoretic mobility shift of the kinetoplast DNA on polyacrylamide gels was attributed to the kinetoplast DNA contain stably curvature^{32, 33} which induced by the special composition of the sequence. DNA indeed can display higher degree of conformational variability even though it is generally thought of as being a structure of rigid straight. In other word, intrinsic curvature or bent DNA molecules is due to when special base arrangement in sequences which induce the local structural polymorphism are repeated in phase with the DNA helix screw.³⁶ Figure 1-7 express the structural different of intrinsically curve DNA and straight DNA.

Figure 1-7. Structural differences of intrinsically curve DNA and straight DNA.

A lot of previously literatures had reported that migration of such DNA bending fragments show reduced electrophoretic mobility shift than the normal straight DNA chain of same size on PAGE³⁶⁻⁴³. Although the actual molecular mechanisms behind

composition in DNA sequence induced intrinsic curvature is not yet completely indisputability, intrinsic curvature can arise in DNA sequences that contain repeat of short adenines (A-tracts) is accepted by scientists.^{37, 39, 41} However, the origin of intrinsic curvature in DNA has been the subject of some controversy. The wedge model for intrinsic curvature speculated that the A-tract contains a wedge angle that caused by kinking of the helix axis towards the minor groove in these regions. The sum of wedge positions in the same direction which in a condition of met 10-bp phasing would lead to bending of DNA in theory.^{39, 44} Wu and crothers suggested the junction model for intrinsically curve DNA which postulated that bending is due to an abrupt change in the direction of the helix axis at the junction between the A-tract and random sequence B-DNA⁴⁵. In addition, modeling ascertains had assumed that DNA would become curve at the adjacent position between A-DNA and B-DNA.⁴⁶ Although these models are based on a different initial theory, the junction model and the wedge model, both of they demonstrate legitimately well the intrinsic curve feature of DNA including repeat A tracts.

Figure 1-8. Schematic illustration of wedge model and junction model for intrinsic curvature DNA.¹¹

1.2.2 Forcible curvature of DNA

Some previous research has been suggested that strong DNA curvature are able to produce in DNA mini-circles and circular oligonucleotides is due to the extra stability, as there would not be free phosphate groups protruding out compared to a straight linear chain analogue.^{47, 48} Consequently, forcible curvature is refers to the phenomena that certain DNA sequences can be bent owing to the formation of circular form under the action of DNA ligase.³¹ With compared of intrinsic curvature which owing to inserting A-tracts in a duplex DNA to create bending, forcible curvature will be created regardless of whether the closed duplex circular DNA contain or without A-tracts. Figure 1-9 indicates the forcible curvature in duplex DNA circle. It is apparent from the previous research that closed duplex circular DNA has also been exhibited more thermodynamically stability by thermal and alkaline melting experiments.^{47, 48} Moreover, diverse ring constraints will be induced due to the difference size of the DNA mini-circles. Therefore, the degree of forcible curvature would be to strengthen along with the reduction of duplex circular DNA size.³¹

Figure 1-9. Schematic illustration of forcible curvature.

1.3 DNA supercoiling

It is manifest that cellular DNA must be very tightly compacted just to fold into the cell (both in eukaryotes and prokaryotes) owing to the contour length of DNA is colossally large.⁴⁹ DNA molecules can exist in variation of different conformations which implies a high degree of structural organization.^{11, 50} The packaging of DNA into cells not through a random folding but in a highly precise fashion which has to permit access in a systematic manner to array of enzymes and other factors that control their significant processes such as replication and transcription.^{50, 51} Therefore DNA structure owing to topological constraints of coiled around an axis in the forms of a double helix which accommodated into other small space by an organized bending or twisting is term supercoiled structure.⁵¹ In other word, supercoiling is the respond the DNA to torsional stress induced by bending or twisting of the DNA double helix upon itself and it exist three dimensions of DNA conformation.^{14, 52, 53}

Similar to the rubber band, supecoiled DNA can involves in a wide different of conformation with variations in twisting and writhing.¹¹ In order to investigate the variety of geometrical configuration of supercoiled DNA, a quantitative measure of the supercoiling of a DNA molecule was devised. There are many previously literatures^{11, 14, 49, 54, 55} demonstrated that the main concepts of DNA supercoiling could be summed up by the following simply equation:

$$L_k = T_w + W_r$$

Linking Number (L_K): the numerical term that describes the sum of the twist and the writhe which represents the total linking within in a closed circular DNA molecule. L_K is an invariant value that cannot be changed without opening the system.⁵⁶ **Twist (T_w):** represents the total number of double helical turns in a given segment of DNA. **Writhe (W_r):** a property of the spatial course of the DNA and is defined as the number of times the double helix crosses itself if the molecule is projected in two dimensions.^{11, 14, 56} Figure 1-10 displays the writhe and twist in circular DNA. If X is the number of turns in the relaxed circular DNA ($L_{K0}=X$) and if one turn is removed by unwinding ($L_K=X-1$), it induces negative supercoiling. Therefore, the change in linking number: $\Delta L_K = L_K - L_{K0} = X - 1 - X = -1$. On the other hand if one turn is added to the relaxed circular DNA, it results in positive supercoiling, therefore the change in linking number ΔL_K is $+1$.

Figure 1-10. Schematic illustration of twist and writhe of circular DNA.¹⁴

The introduction of a negative supercoiled (a right handed coil) into the circular DNA with $L=200$, shown in Figure 1-11, changes the value of W_r by -2 ($W_r=-2$).

Consideration of the above equation of DNA supercoiling, since Lk can not change, Tw must increase by +2 to value of 202. On the contrary, a decrease in the twist number by -2 to a value of $T=198$ would require a compensation introduce left handed coil (positive supercoiled) to $W=+2$, as shown in Figure 1-11. Negative and positive supercoiled circular DNAs are also called underwound and overwound molecules, respectively.

Figure 1-11. Schematic illustrate the relationship of negative supercoiled and positive supercoiled in circular DNA.

In addition, supercoiling could exist in two different structural conformations, plectonemic form or toroidal form, in circular DNA (as shown in Figure 1-12).^{11, 14, 56} These are two structural forms of DNA, which will just differ in their topological conformations, are called topoisomers.⁵⁶ In solution, approximately 70% of the variation in linking number may be distributed as a writhe change and 30% change in twist for supercoiled DNA.^{11, 57}

Figure 1-12. Illustration of two common forms of supercoiled DNA (plectonemic form and toroidal form).

Owing to the conformation of supercoiled DNA contain higher energy than its relaxed form, supercoiled not only allows DNA for a compact form, but the degree of coiling also affects the DNA's interactions with other biological molecules through the capability of unwind the double helix of DNA.⁵⁸ Negative supercoiling lead to facilitates reactivity towards proteins or reagents which can modify duplex winding or that react with single stranded DNA.¹⁴ As a result, the significant intracellular mechanism for regulating the DNA reactions in the transcription, replication, recombination and repair processes are greatly enhanced.^{52, 59-61}

1.4 Topoisomerases

It is well known to all that every reaction that takes place in biological systems is catalyzed by an enzyme.⁶² Without exceptions, the different topological conformations of

DNA transform indeed require topoisomerases during the biological processes.^{63, 64} Topoisomerases are ubiquitous significant enzymes by the fact that take the action of unknot, decatenate, supercoiled and relaxed DNA in vivo and vitro (Figure 1-13 and Table 1-2).⁶⁵⁻⁶⁸ In 1971, the first topoisomerase called ω protein from *Escherichia coli* was discovered by James Wang. The enzyme was found by the experiment that could reduce the number of negative supercoiled in bacteriophage λ DNA.⁶⁹ Martin Gellert and co-workers found an enzyme with capability of trigger supercoiled during a search for host co-factors that support site-specific recombination by bacteriophage λ .^{70, 71} They displayed that the λ DNA substrate need to be supercoiled in order to support the reaction of recombination.^{14, 70}

Figure 1-13. Schematic illustration of topoisomerases reaction. For simplicity, each line describes double-stranded DNA, as shown by the upper left inset. As drawn, most topoisomerases simplify DNA topology with the equilibrium shifted towards relaxing, unknotting, and decatenating. Bacterial DNA gyrase and archaeal reverse gyrase are particular enzymes owing to they could induce supercoiled in DNA.⁶⁵

Table 1-2. Characteristics and properties of DNA topoisomerases.¹⁴

Enzyme	Type	Source	Subunit size(kDa) and composition		Remarks
Bacterial topoisomerase I	IA	Bacteria(e.g. <i>E.coli</i>)	97	monomer	Cannot relax positive supercoil
Eukaryotic topoisomerase I	IB	Eukaryotes(e.g. human)	91	monomer	Can relax both positive and negative supercoil
Vaccinia virus topoisomerase I	IB	Vaccinia virus	37	monomer	ATP stimulates topoisomerase activity
Topoisomerase III	IA	Bacteria (e.g. <i>E.coli</i>)	73	monomer	Potent decatenating activity
Reverse gyrase	IIA	Thermophilic Archaea (e.g. <i>Sulfolobus acidocaldrius</i>)	143	monomer	Can introduce positive supercoil in to DNA (ATP-dependent)
DNA gyrase	IIA	Bacteria (e.g. <i>E.coli</i>)	97 and 90	A ₂ B ₂	Can introduce negative supercoil into DNA (ATP-dependent)
T4 topoisomerase	IIA	Bacteriophage T4	58,51, and 18 2 copies of each subunit		Can relax, but not supercoil, DNA (ATP-dependent)
Eukaryotic topoisomerase II	IIA	Eukaryotes (e.g. human topoisomerase II α)	174	Homodimer	Can relax, but not supercoil DNA (ATP-dependent)
Topoisomerase IV	IIA	Bacteria (e.g. <i>E.coli</i>)	84 and 70	C ₂ E ₂	Can relax, but not supercoil DNA, potent decatenase (ATP-dependent)
Topoisomerase IV	IIB	Archaea (e.g. <i>Sulfolobus shibatae</i>)	45 and 60 A ₂ B ₂		Can relax, but not supercoil DNA (ATP-dependent)

These enzymes appear that produce especial transform to pass DNA helices through each other. The topoisomerases break either one (type I) or two (type II) strands lead to change L_k by one or two, respectively.^{65, 72} All topoisomerases have active site tyrosyl residues that introduce DNA break by nucleophilic attack on the phosphate of the nucleic acid backbone.⁷³⁻⁷⁵ As a consequence, transesterification reaction is accompanied by the formation of a covalent phosphotyrosyl bond at the break site. This phosphotyrosine bond

links the tyrosine hydroxyl group on the enzyme and the newly formatted terminus of the DNA chain. At the same time, the process occur a hydroxyl moiety on the opposite terminus of the break DNA strand.^{76, 77} The DNA molecules is subsequently resealed after a second single or double-stranded DNA segment is then passed through the break.⁷⁸ Following the transformation of DNA configuration by strand passage and rotation, DNA ligation is initiated by the nucleophilic attack of the terminal hydroxyl moiety on the phosphotyrosyl bond.^{76, 77} Finally, the chemical structure of the ligated DNA is identical to that of the original substrate. Only the topological properties of the double helix are altered by the actions of topoisomerases.⁶⁶ There are two major classes of topoisomerases, type I and type II, both of them take important action within cell and their molecular mechanisms and result of the reaction are different.⁷⁵

Type I topoisomerases are indicated by “odd” numbers and without require high-energy cofactor such as ATP.^{66, 79, 80} There are two subclasses of type I enzymes, type IA and type IB are two subclasses of Type I topoisomerases family. Both of Type IA and Type IB topoisomerases with the capability of creating instantaneous single-stranded cleave in the double strand.⁶⁶ Then Type IA followed by passage of the opposite strand through the break while Type IB by controlled rotation of the strand around the break site.^{66, 79} Type IA demand divalent metal ions for catalytic reaction and will be formation of covalently attach to the 5'-terminal phosphate of DNA strand.^{66, 81} Compared with Type IA, type IB will shape a covalently attach to the 3'-terminal phosphate and take action without divalent metal ions.^{82, 83} According to type I topoisomerases reaction mechanisms, they change L_k in steps of one at a time during the relaxation.^{82, 84} Furthermore, owing to

their act by single strand passage mechanism, certain type IA topoisomerases are able to unlink the single-stranded DNA interlinked molecules.^{66, 85} However, because type I topoisomerases take the action of alter DNA twist, they are without capability of unknot or untangle double strand DNA molecules.⁵⁶

The enzymes belonging to the topoisomerases type IA subfamily share the following properties⁶⁶: (1) Cleavage of a DNA strand is accompanied by covalent attachment of the DNA ends to the enzyme through a covalent Tyrosine-DNA intermediate (5' phosphodiester bond to the active site tyrosine). (2) Mg(II) is the requirement for the DNA relaxation reaction. (3) The enzyme only could relax the substrates containing negative, but not positive. (4) The relaxation of the substrates containing negative cannot completely finish. (5) In this subfamily, all enzymes require an exposed single-stranded region within the substrate DNA. (6) DNA changes the linking number in steps of one in the relaxation. (7) Where the process has been examined, these enzymes not only can catalyze the knotting, unknotting, and interlinking of single-stranded circles but also the catenation, and nicked duplex DNA circles. *E. coli* DNA Topoisomerase I and *E. coli* DNA Topoisomerase III are belonging to type IA subfamily. Their structures are shown in the Figure 1-14. The proposed mechanism of relaxation by *E. coli* topoisomerase I is shown in Figure 1-15. Schematically is a series of proposed steps for the relaxation of one turn of negatively supercoiled plasmid DNA.

Figure 1-14. Crystal structures of *E. coli* topoisomerases I (a) and III (b).⁶⁶

Figure 1-15. Proposed mechanism of relaxation by *E. coli* topoisomerase I.⁶⁶

Three classes of enzymes make up the type IB subfamily of topoisomerases^{56, 66}: the topoisomerases I found in all eukaryotic cells. The members of the type IB subfamily of topoisomerases I share no sequence or structural homology with other known topoisomerases and are functionally distinct from the members of the type IA subfamily. Unlike the type IA enzymes, the type IB subfamily members can relax both positive and

negative supercoils, and relaxation goes to completion. The type IB topoisomerases form a covalent intermediate in which the active site tyrosine becomes attached to the 3' phosphate end of the cleaved strand rather than the 5' phosphate end found for the type IA enzymes. The enzymes contain no bound metal ions, and DNA relaxation does not require Mg(II).⁵⁶ The top view and side view of the structure of human topoisomerase I are shown in Figure 1-16. Figure 1-17 indicate a possible mechanism of human topoisomerase I.

Figure 1-16. Side view (a) and top view (b) of the structure of human topoisomerase I.⁶⁶

Figure 1-17. A possible mechanism of human topoisomerase I.⁶⁶

Type II topoisomerases are denoted by “even” numbers and occur all catalytic reaction need ATP to energy factor.^{56, 66, 86} Type II topoisomerases can manipulate DNA topological conformation through inducing a transient duplex cleave in the DNA backbone, driving a separate double strand pass the breach, and then resealing the break.^{66, 86-88} All type II enzymes demand the divalent metal ions to take action.⁷⁹ The cleavage reaction of type II topoisomerases trigger DNA formation of 4-base, 5'-cohesive ends which the complex is through covalently attached to the enzyme in their 5'-terminal phosphates.^{14, 66, 79} Base on the mechanism of double-stranded DNA passage, type II topoisomerases only take the action to DNA writhes. As a consequence, they modulate L_k in steps of two during the topological reaction.⁸⁹ Furthermore, according to the mechanism of type II topoisomerase, they work on the DNA writhes which could induce the elimination of knots and tangles from the DNA substrate.^{14, 30, 56} Figure 1-18. Show the proposed mechanism of relaxation of topoisomerase II.

Figure 1-18. Proposed mechanism of relaxation of topoisomerase II.⁶⁶

1.5 The importance of topoisomerases in biological system

It is well known that variation in DNA topology occurs in all organismal reactions involve DNA and can have significant biological consequences.⁷⁴ Almost cellular processes involving DNA in vivo generally require the participation of topoisomerases which are demanded to preserve a strict level of supercoiled in DNA structure.^{74, 90, 91} During the different stages of DNA replication, DNA topoisomerases are often to be found engaged in each process. It was demonstrated that DNA topoisomerases derives the DNA from double-helical structure to unwinding of the DNA helix which indeed induces to access the bases.⁷⁴ DNA gyrase by fact that is essential in prokaryotes for the initiation of replication process which is need for negative supercoiled prior to initiation. Topoisomerases are significant and needed to prevent the positive supercoiled amassing owing to the parental DNA is being ceaselessly unwound during the elongation steps of replication.^{92, 93} Not only type I topoisomerases but also type II can take the action of alleviate the torsion stress produce in the elongate process during the replication. In addition, both of types I and types II topoisomerases in theory can separate the entangled DNA molecules in the terminal stage of DNA replication.^{52, 56, 77, 94} With compare of replication, on the other hand, transcription in theory to proceed in the situation of there are not occur any topological change. However, it is a special example in bacteria, transcription can theoretical to cause the positive supercoiled ahead of the transcription complex and negative supercoiled behind it when the circular plasmid are being transcribed in antithetical directions.^{95, 96} Many experiments in previously report have explained that topoisomerase I can relax the negative supercoiled and gyrase can relax the positive supercoiled.^{55, 97, 98}

Moreover, topoisomerase are prerequisite to relax the topological stress in the DNA which would be occurred when the movement of RNA polymerase during transcription.⁹⁹⁻¹⁰¹ The procedures of Transcription and replication would be terrifically slow or deadlock without the action of topoisomerases.¹⁰² As a consequence, these nuclear enzymes are crucial and profound for their biological role during replication and transcription within the cell.

1.6 Topoisomerases as a significant target in drug design

Owing to the critical physiological role in DNA replication and transcription, topoisomerases have been regarded as the significant factor in cell propagation. As a consequence, these enzymes were demonstrated as important targets for anticancer and antibacterial drugs.¹⁰³⁻¹¹² Table 1-3 shows the common inhibitors of topoisomerases which including some drugs apply in the clinical therapy. With compared to other protein-targeted drugs, topoisomerase-targeted agents take the action in an insidious mode that only disannul the feature of topoisomerase but do not kill cells by exploit them of critical enzymatic activities.⁵⁶ This action converts topoisomerases to potent physiological toxins that induce the steady-state of DNA cleavage complexes in treated cells.^{67, 113, 114} According to the mechanism of topoisomerases's action, drugs that lead to increase topoisomerase-mediated DNA cleavage are distinguish them from agent of enzymatic catalytic inhibitors.¹¹⁵

For instance, bacterial DNA gyrase and topoisomerase IV are the targets for quinolones, an extremely successful family of antibacterial agents which are widely employed for the therapy of infections and broad-spectrum oral antibacterials currently in clinical treatment.^{97, 110, 116} The first clinically therapeutic quinolone was norfloxacin¹¹⁰ which not only exhibited good activity against Gram-negative bacteria but also displayed modest activity resistant some Gram-positive species. The quinolones drugs are assumed that take action of interrupting the DNA breakage–reigate step of the gyrase reaction.¹¹⁷ On the other hand, the drug of coumarin family, such as novobiocin, are consider in an different mode through affect the hydrolysis of ATP which induce by gyrase and lead to restrict DNA become supercoiling.¹¹⁸

Table 1.3 The common inhibitors of Topoisomerases.¹⁴

Inhibitor	Target enzyme	Therapeutic value
Quinolones (e.g. ciprofloxacin)	DNA gyrase and topoisomerase IV	Effective antibacterial agents
Coumarins (e.g. novobiocin)	DNA gyrase and topoisomerase IV	Antibiotics, not widely used
Camptothecin (e.g. topotecan)	Human topoisomerase I	Anticancer drug
Amsacrine (mAMSA)	Human topoisomerase II	Anticancer drug
Epipodophyllotoxins (e.g. teniposide)	Human topoisomerase II	Anticancer drug

Furthermore, Human topoisomerase I is the target for several exciting novel antitumor drugs. The antitumour drug topotecan that are derived from camptothecin

which comes from the bark of a Chinese yew tree, *Camptotheca acuminata* are widely used in clinical application for many kinds of cancer.^{67, 119} The drug will take the acting of stabilizes the complex of the enzyme and DNA in a method similar to the behavior of quinolones on gyrase.¹¹⁹⁻¹²¹ Topoisomerase II as well as regard to important pharmaceutical targets for the treatment of human cancer. Several other antitumour drugs have been shown to be inhibitors both topoisomerase II α and β such as acridines, ellipticines, and epipodophyllotoxins.¹²²⁻¹²⁵ These drugs are speculate to take the action of stable the covalent complex between the enzyme and DNA, and in many cases this is presume that the agent is intercalate into the DNA where is the enzyme binding site.¹²⁶⁻¹²⁸ Other inhibitory drugs of topoisomerase II work in a different manner, such as, the bisdioxypiperazine ICRF-193 is suggest that stabilizing the closed clamp form of the enzyme.^{129, 130}

Scientists who make meaningful contribution on DNA topoisomerases have encouraged progress in the development of basic understanding of topoisomerases in biochemistry and molecular biology which could lead to topoisomerases was rapidly applied to clinical pharmacology.^{111, 131} The poisons of topoisomrases inhibitor not only provide a special opportunity for investigating the mechanism of cell killing and DNA repair process but also accompanied by unpredictable side effect such as hemorrhagic cystitis and myelotoxicity during clinical treatment.^{103, 104, 132} As a result, these problems are likely to lead to a more rational design and better strategy in cancer chemotherapy or development of new type inhibitor for topoisomerase such as curve DNA and LNA in the future.³¹

Chapter 2

Recognition of forcible curvature in circular DNA by human topoisomerase I

2.1 Background and objectives

Eukaryotic topoisomerase I is a ubiquitous nuclear enzyme involved in resolving the topological stress associated with DNA replication, transcription and chromatin condensation, and is critical for cell growth and proliferation.^{66, 67, 79, 133} Human topoisomerase I belonging to Eukaryotic topoisomerase I (type IB subfamily). It binding and nicking the double-stranded DNA, and become attached to the 3' end of the cleaved strand.⁶⁶ The 5' end is then free to rotate, twisting it about the other strand, to relax DNA until the topoisomerase I religates the broken strands.^{84, 134} The endowed substrates of human topoisomerase I in organisms have been frequently recognized as the positive and negative supercoiled structures of DNA.¹³⁵

An important feature of most enzymes is their ability to distinguish the specific structure of the substrate before they act on the substrate. The consequences of working at the wrong time or at the wrong place are potentially disaster. Therefore, the enzymes must has perfect mechanism for recognizing their DNA substrates.¹³⁶ Curvature and crossover are the two major geometric characteristics of the supercoiled DNA structures which probable can be distinguished by human topoisomerase I. It has been illustrated recently that human topoisomerase I binds DNA in a topology-dependent fashion with a strong

preference for supercoiled DNA over relaxed DNA.^{137, 138} Furthermore, it has been reported that eukaryotic topoisomerase I acts preferentially on intrinsically curved DNA.^{139, 140}

The structure of DNA effect on topoisomerase I-DNA interaction has been explored employing a high affinity binding site and mutant derivatives.^{139, 141} Parallel determinations of complex formation and helix structure in the absence of superhelical stress indicate that the interaction is strengthened by stable structure of DNA curvature. Previous research suggested that a topoisomerase I recognized site consists of two related but functionally distinct phenomena.¹³⁹ The region located 5' end for the topoisomerase I cleavage site is crucial requirement for binding. In contrast, it is not essential point of the region 3' to the cleavage site is linked by the enzyme. Previous investigation reported¹⁴¹ that the double strand helix configuration of the latter region is an essential adjustment for the formation of the complex of topoisomerase I-DNA. Therefore, complex formation is manifest to play a driving role, when an intrinsically bent DNA segment is installed in this region. Two different pieces of evidence verify that topoisomerase I may belong to the protein of curvature dependent DNA binding: (i) High affinity recognizable sites have been validated to contain short A-tracts^{142, 143}, and (ii) topological domains including an oligo (dA)-(dT)-rich, intrinsically curved segment of *Crithidia* kinetoplast DNA are preferentially recognized by topoisomerase I *in vitro*¹⁴¹. It is to be strictly found that whether helix curvature is main reason in determining the efficacy of topoisomerase I-DNA interaction. In addition, eukaryotic topoisomerase displays a topology independent preference for intrinsically curved DNA¹⁴¹. This implies that the enzyme discerns writhe rather than twist. Since writhe is a relatively independent character of supercoiling

structure¹⁴⁴, this interpretation is consistent with the fact that eukaryotic topo I relaxes not only positively supercoiled DNA but also negatively supercoiled DNA^{145, 146}. The affection of topology on eukaryotic topoisomerase I action may be performed through the recognition site, because the local structure will be influenced by superhelical stress. This results suggest that intrinsic curvature in the non-essential part of the topoisomerase I binding site stabilizes the interaction.¹³⁹

In the meanwhile, a crystal structure of coexisting duplex DNA and the enzyme reveals the specific binding sequence of human topoisomerase I.^{84, 147} Figure 2-1 shows the binding sequence. These results suggest that the common structural character of the supercoiled DNA, curvature, could provide an alternative recognition pattern of human topoisomerase I, as secondary binding.^{148, 149} However, there are no concrete explanations to validate the fact why human topoisomerase I is able to determine the topological state of DNA.

In order to investigate the innate specificity of human topoisomerase I recognize supercoiled DNA, a series of circular DNA containing forcible curved were prepared and used to mimic the different degree of DNA curvature in this research. Base on the half maximal inhibitory concentration of DNA circle base pairs to direct ascertain that human topoisomerase favor DNA curvature evidently. Furthermore, atomic force microscope and electrophoretic mobility shift assay are used to verify the human topoisomerase I binding to DNA curvature.

Figure 2-1. The binding site of human topoisomerase I. The sequence of human topoisomerase I preferred site and that was present in the published crystal structure, has a mild intrinsic curvature. Human topoisomerase I-mediated DNA cleavage required two parts interaction with a region encircle the cleavage site (region A) and another region on the side holding the 5'-OH end generated by cleavage (region B). The bipartite minimal duplex region is shaded. The positive and negative numbers in the DNA sequence are relative to the cleavage site, marked with an arrow.

2.2 Design of DNA sequences

Some previous research have been suggested that strong DNA curvature are able to produce in DNA mini-circles and circular oligonucleotides is due to the extra stability, as there would not be free phosphate groups protruding out compared to a straight linear chain analogue.^{47, 48} With aim of investigate the interaction of human topoisomerase I and forcible curvature, a series of circular DNA will need to be used in this research. Different size circular DNA contain different degree of forcible curvature were add to the negative supercoiled pUC 19 plasmid relaxation reaction catalyzed by human topoisomerase I. Through determining the efficiency of relaxation reaction of the negative supercoiled pUC 19 plasmid, the inhibitory effect of forcible curved DNA on human topoisomerase I was the effective manner to employ to indicate interactional degree of enzyme and DNA.

Figure 2-2 illustrates the variation of different curved degree in supercoiled DNA. Human topoisomerase I change the linking number of supercoiled circular DNA by units of 1. According to the process of human topoisomerase I relax supercoiled DNA, the curvature degree of supercoiled DNA will decrease with the reduction of DNA supercoiled density. A series of forcible curved circular DNA (84 bp, 300 bp, 535 bp and 936 bp) were prepared according to the previous literature⁴⁷ and used to mimic the different degree of supercoiled DNA curvature. Table 2-1 shows the sequence of each DNA circles. The sequences of 84 bp circle with GC-content near 50% and do not contain intrinsically bent motifs.

Figure 2-2. Illustration of different status of curvature associated with supercoiled DNA (degrees of DNA curvature: A>B>C>D). 84 bp circular DNA (cDNA) was used to mimic the curvature status at the beginning of human topoisomerase I relaxation process and 936 bp cDNA was used to mimic the status close to the end of relaxation process.

In the meanwhile, the crystal structure of human topoisomerase I and DNA duplexes complex was reported recently.⁸⁴ It is indicated that human topoisomerase I act on DNA only binding to several base pairs duplex. The curved degree of same base pairs duplex in

84 bp circular DNA is stronger than the one in 936 bp circular DNA. It is demonstrating that same length of duplex in different size circular DNA has different degree of curvature. Since human topoisomerase I binding to DNA in several base pairs,⁸⁴ the half maximal inhibitory concentration of DNA circle base pairs was the effective method to measure the interaction degree of DNA circle and human topoisomerase I binding.

Table 2-1. The sequence of each DNA circles. All DNA circles are only show one strand from 5' to 3'.

Circle size (bp)	Sequence
84	ATCTTATCGAACAAGACCCGTGCAATGCTATCGACATCAAGGCCTATCGCTGG GAGTCAATGGGTTTCAGGATGCAGGTGAGGAT
300	CCATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAAT AATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATT CCCTTTTTTTCGGCATTTCCTGCTTTTTGCTCAGGAGAAACGCTGGT GAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAAC TGGATCTCAACAGCGGTAAGTTAAGCTTTTTGCACAACATGGGGGATCATGTA ACTCGCCTTGATCGAAGGAGAGAAGAGCTGGAGCT
535	CCCAGTCCGTAATACGACTCACTTAAGGCCTTGACTAGAGGGTACCAACCTAG GTATCTAGAACCGGTCTCGAGCCATAACTTCGTATAGCATAATTATACGAAG TTATATAAGCTGTCAAACATGAGAATTCTTGTTATAGGTTAATGTCATGATAA TAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACC CCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACA ATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTC AACATTTCCGTGTCGCCCTTATTCCTTTTTTTCGGCATTTCCTGCTT TTTGCTCAGGAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGG TGCACGAGTGGGTACATCGAAGTGGATCTCAACAGCGGTAAGTTAAGCTTTT TGCACAACATGGGGGATCATGTAAGTTCGCTTGATCGAAGGAGAGAAGAGCTG GAGCT
936	CATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTACCGACAAACAA CAGATAAACGAAAGGCCAGTCTACCGACTGAGCCTTTCGTTTTATTTGATG CCTGGCAGTTCCTACTCTCGCGTTAACGCTAGCATGGATGTTTTCCAGTCA CGACGTTGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTT ATTTTGACTGATAGTGACCTGTTGTTGCAACAAATTGATGAGCAATGCTTTT TTATAATGCCAACTTTGTACAAAAAGCAGGCTTGAAGGAATTCGGCAAGTCT TCCCACCTAGTGGATCCTCGTCGAAAACCGATTAAGTTGGGTAAACGCCAGGG TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCCGTAATACGACTCACT

	TAAGGCCTTGACTAGAGGGTACCAACCTAGGTATCTAGAACCGGTCTCGAGCC ATAACTTCGTATAGCATACATTATACGAAGTTATATAAGCTGTCAAACATGAG AATTCTTGTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGT GGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAAT ACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAAT AATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATT CCCTTTTTTTCGGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGT GAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAAC TGGATCTCAACAGCGGTAAGTTAAGCTTTTTGCACAACATGGGGGATCATGTA ACTCGCCTTGATCGAAGGAGAGAAGAGCTGGAGCT
--	--

2.3 Materials and methods

2.3.1 Oligodeoxyribonucleotides enzymes and chemicals

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sigma-Proligo (Singapore)
X2420G plasmid DNA	Shanghai Generay Biotech Co., Ltd
pUC 19	New England Biolabs
Relaxed form pUC 19	New England Biolabs
Human topoisomerase I	Topogen
100 bp DNA ladder	Fermentas
1 Kb DNA ladder	New England Biolabs
Sac I endonuclease	New England Biolabs
T4 DNA ligase	New England Biolabs
BAL 31 exonuclease	New England Biolabs
<i>Taq</i> Polymerase	New England Biolabs
Biological purity water	1st Base Pte. Ltd
Agarose	Invitrogen

Ethidium bromide	Research Biolad
T4 polynucleotide kinase	New England Biolabs
γ - ³² P ATP	Perkin Elmer
Micro-spin column	GE, Healthcare
PCR purification kit	Qiagen
TAE, TBE, TRIS	1st Base Pte. Ltd
X-ray films	Fuji, Singapore
X-ray development solution	Kodak, Singapore

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

Table 2-2. Sequence of X2420G plasmid DNA (3593 bp). DNA sequence is only show one strand from 5' to 3' .

GCAGGCTTGA	AGGAATTCGG	CAAGTCTTCC	CACTTAGTGG	ATCCTCGTCG	CAAAACCGAT
TAAGTTGGGT	AACGCCAGGG	TTTTCCAGT	CACGACGTTG	TAAAACGACG	GCCAGTCCGT
AATACGACTC	ACTTAAGGCC	TTGACTAGAG	GGTACCAACC	TAGGTATCTA	GAACCGGTCT
CGAGCCATAA	CTTCGTATAG	CATACATTAT	ACGAAGTTAT	ATAAGCTGTC	AAACATGAGA
ATTCTTGTTA	TAGGTTAATG	TCATGATAAT	AATGGTTTCT	TAGACGTCAG	GTGGCACTTT
TCGGGGAAAT	GTGCGCGGAA	CCCCTATTTG	TTTATTTTTT	TAAATACATT	CAAATATGTA
TCCGCTCATG	AGACAATAAC	CCTGATAAAT	GCTTCAATAA	TATTGAAAAA	GGAAGAGTAT
GAGTATTCAA	CATTTCCGTG	TCGCCCTTAT	TCCCTTTTTT	GCGGCATTTT	GCCTTCCTGT
TTTTGCTCAC	CCAGAAACGC	TGGTGAAAAGT	AAAAGATGCT	GAAGATCAGT	TGGGTGCACG
AGTGGGTTAC	ATCGAACTGG	ATCTCAACAG	CGGTAAGTTA	AGCTTTTTGC	ACAACATGGG
GGATCATGTA	ACTCGCCTTG	ATCGAAGGAG	AGAAGAGCTG	GAGCTCAATG	AAGCCATACC
AAACGACGAG	CGTGACACCA	CGATGCCTGC	AGGAATTCCT	CGAGCCATAA	CTTCGTATAG
CATACATTAT	ACGAAGTTAT	CCATGGACTA	GTGAGTCGTA	TTACGTAGCT	TGGCGTAATC
ATGGTCATAG	CTGTTTCCTG	TGTGAAATTG	TTATCCGCTC	ACAATTCCAC	ACAACATACG
AGCCGGAAGC	ATAAAGTGTA	AAGCCTGGGG	GGGGTTAACC	ATGGATCCGG	TAAGTGGGAT
ATCGAAGACT	TGCCGCTAGA	ATTGATCCCC	CTATAGTGAG	TCGTATTACA	TGGTCATAGC
TGTTTCCTGG	CAGCTCTGGC	CCGTGTCTCA	AAATCTCTGA	TGTTACATTG	CACAAGATAA
AAATATATCA	TCATGAACAA	TAAAACTGTC	TGCTTACATA	AACAGTAATA	CAAGGGGTGT
TATGAGCCAT	ATTCAACGGG	AAACGTCGAG	GCCGCGATTA	AATTCCAACA	TGGATGCTGA
TTTATATGGG	TATAAATGGG	CCCGCGATAA	TGTCGGGCAA	TCAGGTGCGA	CAATCTATCG

CTTGTATGGG	AAGCCCGATG	CGCCAGAGTT	GTTTCTGAAA	CATGGCAAAG	GTAGCGTTGC
CAATGATGTT	ACAGATGAGA	TGGTCAGACT	AAACTGGCTG	ACGGAATTTA	TGCCTCTCCC
GACCATCAAG	CATTTTATCC	GTACTCCTGA	TGATGCATGG	TTACTCACCA	CTGCGATCCC
CGGAAAAACA	GCATTCCAGG	TATTAGAAGA	ATATCCTGAT	TCAGGTGAAA	ATATTGTTGA
TGCGCTGGCA	GTGTTCCCTG	GCCGGTTGCA	TTCGATTCCCT	GTTTGTAATT	GTCCTTTTAA
CAGCGATCGC	GTATTTTCGTC	TCGCTCAGGC	GCAATCACGA	ATGAATAACG	GTTTGGTTGA
TGCGAGTGAT	TTTGATGACG	AGCGTAATGG	CTGGCCTGTT	GAACAAGTCT	GGAAAGAAAT
GCATAAACTT	TTGCCATTCT	CACCGGATTC	AGTCGTCACT	CATGGTGATT	TCTCACTTGA
TAACCTTATT	TTTGACGAGG	GGAAATTAAT	AGGTTGTATT	GATGTTGGAC	GAGTCGGAAT
CGCAGACCGA	TACCAGGATC	TTGCCATCCT	ATGGAAGTGC	CTCGGTGAGT	TTTCTCCTTC
ATTACAGAAA	CGGCTTTTTT	AAAAATATGG	TATTGATAAT	CCTGATATGA	ATAAATTGCA
GTTTCATTTG	ATGCTCGATG	AGTTTTTCTA	ATCAGAATTG	GTTAATTGGT	TGTAACACTG
GCAGAGCATT	ACGCTGACTT	GACGGGACGG	CGCAAGCTCA	TGACCAAAAT	CCCTTAACGT
GAGTTACGCG	TCGTTCCACT	GAGCGTCAGA	CCCCGTAGAA	AAGATCAAAG	GATCTTCTTG
AGATCCTTTT	TTTCTGCGCG	TAATCTGCTG	CTTGCAAACA	AAAAAACCAC	CGCTACCAGC
GGTGGTTTGT	TTGCCGGATC	AAGAGCTACC	AACTCTTTTT	CCGAAGGTAA	CTGGCTTCAG
CAGAGCGCAG	ATACCAAATA	CTGTCTTCT	AGTGTAGCCG	TAGTTAGGCC	ACCACTTCAA
GAATCTGTGA	GCACCGCCTA	CATACCTCGC	TCTGCTAATC	CTGTTACCAG	TGGCTGCTGC
CAGTGCGGAT	AAGTCGTGTC	TTACCGGGTT	GGACTCAAGA	CGATAGTTAC	CGGATAAGGC
GCAGCGGTCG	GGCTGAACGG	GGGGTTCGTG	CACACAGCCC	AGCTTGGAGC	GAACGACCTA
CACCGAAGT	AGATACCTAC	AGCGTGAGCA	TTGAGAAAGC	GCCACGCTTC	CCGAAGGGAG
AAAGGCGGAC	AGGTATCCGG	TAAGCGGCAG	GGTCGGAACA	GGAGAGCGCA	CGAGGGAGCT
TCCAGGGGGA	AACGCCTGGT	ATCTTTATAG	TCCTGTCGGG	TTTCGCCACC	TCTGACTTGA
GCGTCGATTT	TTGTGATGCT	CGTCAGGGGG	GCGGAGCCTA	TGGAAAAACG	CCAGCAACGC
GGCCTTTTTTA	CGGTTCCCTG	CCTTTTGTCTG	GCCTTTTGCT	CACATGTGAA	ATTGTAAACG
TTAATATTTT	GTTAAAATTC	GCGTTAAATT	TTTGTTAAAT	CAGCTCATTT	TTTAACCAAT
AGGCCGAAAT	CGGCAAAATC	CCTTATAAAT	CAAAAGAATA	GACCGAGATA	GGGTGAGTG
TTGTTCCAGT	TTGGAACAAG	AGTCCACTAT	TAAAGAACGT	GGACTCCAAC	GTCAAAGGGC
GAAAAACCGT	CTATCAGGGC	GATGGCCCCAC	TACGTGAACC	ATCACCCATA	TCAAGTTTTT
TGGGGTCGAG	GTGCCGTAAA	GCACTAAATC	GGAACCCTAA	AGGGATGCCC	CGATTTAGAG
CTTGACGGGG	AAAGCCGGCG	AACGTGGCGA	GAAAGGAAGG	GAAGAAAGCG	AAAGGAGCGG
GCGCTAGGGC	GCTGGCAAGT	GTAGCGGTCA	CGCTGCGCGT	AACCACCACA	CCCGCCGCGC
TTAATGCGCC	GCTACAGGGC	GCGTCCCATA	CATGTGCTGA	GGATCGAGTC	TTAATTACTG
CCGGCCTTGT	AGAAAACGCA	AAAGGCCATC	CGTCAGGATG	GCCTTCTGCT	TAGTTTGATG
CCTGGCAGTT	TATGGCGGGC	GTCCTGCCCG	CCACCCTCCG	GGCCGTTGCT	TCACAACGTT
CAAATCCGCT	CCCGGCGGAT	TTGTCCTACT	CAGGAGAGCG	TTCACCGACA	AACAACAGAT
AAAACGAAAG	GCCCAGTCTA	CCGACTGAGC	CTTTCGTTTT	ATTTGATGCC	TGGCAGTTCC
CTACTCTCGC	GTTAACGCTA	GCATGGATGT	TTTCCCAGTC	ACGACGTTGT	AAAACGACGG
CCAGTCTTAA	GCTCGGGCCC	CAAATAATGA	TTTTATTTTG	ACTGATAGTG	ACCTGTTTCG
TGCAACAAAT	TGATGAGCAA	TGCTTTTTTTA	TAATGCCAAC	TTTGTACAAA	AAA

2.3.2 Preparation of 84 bp circular DNA

84 bp circle was prepared according to the process describe in Figure 2-3. Such long cohesive ends are much more flexible with contour lengths that are larger than the length of the double stranded part of the duplexes. Therefore, it should in theory provide higher efficiency of cyclization. The product of double stranded circular DNA contains two nicks in diametrical location, which can be subsequently sealed by DNA ligase. The mechanism of the ligase is to form two covalent phosphodiester bonds between the 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. Recognition site of restriction enzyme will be incorporated in the mini-circle sequences. Exonuclease will also be used to verify the formation of closed circular DNA.

Figure 2-3. Schematic diagrams that illustrate the formation of closed circular DNA through long cohesive ends.

Two single stranded DNA with 84 nucleotides each were designed in this project (For sequence see in Table 2-1). These two strands of DNA are complementary to each other. Two single stranded DNA were mixed in the reaction buffer (60 mM Tris - HCl, pH 7.6, 25 mM NaCl, 13 mM MgCl₂, 10 mM DTT, 1m M ATP, 25 mg/ml BSA), heated

to 95 °C for 2 min and then chilled immediately on ice for 10 min. The mixture was brought to 25 °C and incubated with 20 U of T4 DNA ligase for 1 h. (Additional cycles do not improve the yield because T4 DNA ligase converted all remaining substrate molecules into various multimeric products during the first cycle.) The DNA products after the annealing assembly and ligation steps were treated with reactions buffer (10 mM Tris - HCl, pH 8.0, 120 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂). Then the reaction mixture was incubated with 2 U of exonuclease BAL 31 at 30 °C for 60 minutes. The obtained circular DNA products were further purified by PCR Purification Kit. Figure 2-4 shows the result of the experiment.

Figure 2-4. Preparation of 84 bp circular DNA. Lane 1: 100 bp DNA ladder; Lane 2: 84 mer oligodeoxyribonucleotides A alone; Lane 3: 84 mer oligodeoxyribonucleotides B alone; Lane 4: the mixture of 84 mer oligodeoxyribonucleotides A and 84 mer oligodeoxyribonucleotides B after treated with annealing and ligase; Lane 5: the mixture of lane 4 incubate with exonuclease BAL 31. The analysis was done on a 8% denaturing PAGE gel and stained by ethidium bromide.

Exonuclease BAL 31 can degrade both 3' and 5' termini of double strand DNA without generating internal scissions. This exonuclease is also a highly efficient exonuclease which cleaves at nicks, gaps and single stranded regions of duplex DNA. For this reason, the exonuclease BAL 31 would not be able to act on the covalently closed circular DNA because it does not have 5' or 3' termini to degrade. Duplex circular DNAs are known to resist the digestion by this exonuclease due to the absence of open termini in their structures. As a consequence, it was used to confirm the circular DNA of its phosphate–sugar backbones since it can effectively hydrolyze other fragments, leaving behind only the single band of the duplex circular DNA. As seen in Lane 4, Figure 2-4 show the DNA band of the pure 84 bp circular DNA.

2.3.3 Preparation of 300 bp circular DNA

Because two 300 mer oligodeoxyribonucleotides is difficult to annealing 300 bp nick DNA circle which refer to the method of prepare 84 bp circular DNA, a different manner would be utilized to synthesize the 300 bp circular DNA. First, standard polymerase chain reaction (PCR) amplification process^{150, 151} will be used to produce a double strand DNA with two Sac I digest site in each end, and then the double strand was treated with endonuclease Sac I obtain a double strand DNA contains two cohesive end. Second, the double strand DNA contains two cohesive end will be incubated in T4 DNA ligase. Last, the mixture will be digested by exonuclease Bal 31 to confirm the formation of circular DNA. In addition, as seen in Lane 5 of Fiugre 2-5, dimer and trimer circular DNA will be

produced according to the reaction of ligate the double strand DNA contains two cohesive end. Though linear DNA, nick circular DNA and other fragments will be digested by exonuclease Bal 31, the pure 300 bp circular DNA is difficult to produce. In order to obtain the pure 300 bp circular DNA, the DNA band in Lane 5 of Figure 2-5 will be cut and further purified by Gel Extraction Kit (Qiagen) after the processing of exonuclease Bal 31 digestion.

A reaction mixture containing 1 ng X2420G plasmid DNA, 0.25 μ M forward primer (5' CCGAGCTCCATTCAAATATGTATCCGCT 3'), 0.25 μ M reverse primer (5' CCGGATCCATGGTTAACCCC 3'), 200 μ M each dNTP, 1 U *Taq* polymerase in a total volume of 50 μ l reaction buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8 @ 25 °C) was processed as below protocol:

95 °C for 180 sec (denature);

56 °C for 40 sec (anneal);

72 °C for 40 sec (elongate) (60 sec per kb target sequence length);

29 cycles only (otherwise enzyme decay causes artifacts);

72 °C for 10 min at end to allow complete elongation of all product DNA

A mixture containing PCR product, 5 U of Sac I, 10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol was incubated 37 °C for 2 hours to produce the double strand DNA contains two cohesive end. The resultant cohesive end-containing DNA was purified using the QIAGEN PCR Purification Kits. The mixture containing resultant cohesive end-containing DNA, 60 mM Tris-HCl (pH 7.6), 25 mM NaCl, 13 mM

MgCl₂, 10 mM DTT, 1mM ATP, 25 mg/ml BSA and 10 U of T4 DNA ligase was incubated at 16 °C for 16 hours. The resultant mixture was further incubated with BAL-31 at 30 °C for 2 hours, which were then analyzed using 2.5% agarose gel electrophoresis. The DNA product that is corresponding to Band 1 in Lane 5 of Figure 2-5 was be cut and further purified by Gel Extraction Kit (Qiagen). Figure 2-5 shows the process of prepare 300 bp circular DNA.

Figure 2-5. Preparation of 300 bp circular DNA. Lane 1: 100 bp DNA ladder; Lane 2: PCR product (DNA with two Sac I digest site in each end) alone; Lane 3: Sac I digest product (cohesive end-containing DNA) alone; Lane 4: the mixture of lane 3 treated with T4 DNA ligase; Lane 5: exonuclease BAL 31 hydrolysis product on reaction mixture of lane 4. The analysis was done on a 2.5% agarose gel and stained by ethidium bromide.

2.3.4 Preparation of 535 bp circular DNA

The preparation of the 535 bp circular DNA is similar to the processing of produce

300 bp circular which introduce in the previous description except the different primer used in the polymerase chain reaction. In this reaction “5’ CCGAGCTCCCAGTCCGTA ATACGACTC3’” was used as forward primer and “5’ CCGGATCCATGGTTAACCCC 3’” was employed as reverse primer.

Figure 2-6. Preparation of 535 bp circular DNA. Lane 1: 100 bp DNA ladder; Lane 2: PCR product (DNA with two Sac I digest site in each end) alone; Lane 3: Sac I digest product (cohesive end-containing DNA) alone; Lane 4: the mixture of lane 3 treated with T4 DNA ligase; Lane 5: exonuclease BAl 31 hydrolysis product on reaction mixture of lane 4. The analysis was done on a 2% agarose gel and stained by ethidium bromide.

2.3.5 Preparation of 936 bp circular DNA

The preparation of the 936 bp circular DNA is similar to the processing of produce 300 bp circular which introduce in the previous description except the different primer used in the polymerase chain reaction. In this reaction “5’ CCGAGCTCAT

CCGCTCCCGGCGGATTTG' ” was used as forward primer and “5' CCGGATCCATGGTTAACCCC 3' ” was employed as reverse primer.

Figure 2-7. Preparation of 936 bp circular DNA. Lane 1: 100 bp DNA ladder; Lane 2: PCR product (DNA with two Sac I digest site in each end) alone; Lane 3: Sac I digest product (cohesive end-containing DNA) alone; Lane 4: the mixture of lane 3 treated with T4 DNA ligase; Lane 5: exonuclease BAl 31 hydrolysis product on reaction mixture of lane 4. The analysis was done on a 2% agarose gel and stained by ethidium bromide.

2.3.6 Inhibition assays on human topoisomerase I

Human topoisomerase I activity was determined by measuring the degree of relaxation of the negative supercoiled DNA, pUC 19. The designed circular DNA at different concentrations were incubated in reaction mixture including 0.5 U human topoisomerase I and 250 ng of negative supercoiled pUC 19 in 1 X human topoisomerase I reaction buffer in a total volume 20 μ L of at 37 °C for 15 mins. The quantities of circular DNAs were precisely measured by Thermo Nanodrop. Agarose gel electrophoresis was employed to analyze the inhibitory effect of the circular DNA for human topoisomerase I

followed staining with ethidium bromide. The DNA bands were visualized through Gel Documentation System (GBox HR, Syngene, Cambridge, UK) equipped with Gene Tools Software. Percentage of relaxation was strictly defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA $[\text{relaxed DNA} / (\text{relaxed DNA} + \text{supercoiled DNA})]$ ¹⁵² while 100% minus off the percentage of relaxation was taken as the percent inhibition of topoisomerases activity by the DNA. All the data shows in the discussion section.

2.3.7 Preparation of DNA sample for AFM detection

Owing to atomic force microscopy (AFM) have the advantages that it can image the non-conducting surfaces. Therefore, AFM was immediately extended to the biological systems, such as analyzing the DNA structure and DNA protein interaction analysis.¹⁵³⁻¹⁵⁷ In order to imaging the DNA sample with the AFM, the DNA molecules need to be adsorbed onto a very flat surface. Mica was employed as a surface owing to the material containing very thin flat layers. Both the mica surface and the DNA are negatively charged. For the purpose of fix the DNA on the surface, the mica flat layer needs to be modified to positively charge. Usually, MgCl_2 was used to modify the mica and fix the DNA in the mica. Because Mg^{2+} -ions substitute the negative charges on the mica surface and as a result the DNA molecules are captured to the surface of mica. This method results in equilibrium attracting.¹⁵⁸ This means that DNA molecules are allowed to equilibrate on the 2D mica surface before they are finally trapped in a particular

conformation.

Immobilization of DNA samples on micas were carried out following the previously reported procedures^{153, 159-161}. A buffer contains 40 mM HEPES, 10 mM MgCl₂ and pH 7 was deposited on freshly cleaved mica for 5 min. DNA circle was incubated with 10 U human topoisomerase I at 30 °C for 15 min in the binding buffer [10 mM Tris/HCl (pH 7.5), 3 mM CaCl₂, 50 mM NaCl, 0.1 M sucrose and 5% glycerol]. After the pre-prepared mica dried with a gentle N₂ flow, a 20 µl solution contain DNA sample, 10 U human topoisomerase I and binding buffer was deposited on it incubated for 5 min, washed with double distill H₂O, dried with a gentle N₂ flow and then dried under vacuum. AFM images were obtained in Tapping ModeTM on a MultimodeTM AFM (Veeco, Santa Barbara, CA) in connection with a Nanoscope VTM controller. Antimony (n) doped Si cantilevers with nominal spring constants between 20 and 80 N/m were selected. Scan frequency was 1.9 Hz per line and the modulation amplitude was in a nanometer range^{162, 163}. All DNA sample determinations were carried out in air at room temperature. All the data shows in the discussion section.

2.3.8 EMSA of DNA circle and human topoisomerase I

Since its introduction in 1981,^{164, 165} electrophoretic mobility shift assay (EMSA) has become one of the most widely used methods to study the binding of proteins on DNA. EMSA is one of the most precise strategies for investigation of the DNA-binding

characteristic of a protein, can be employed to presume the binding properties and relative affinities of a protein for particular DNA sites.¹⁶⁶ It is also helpful for investigating higher-order complexes containing several proteins, observed a phenomenon as shift assay.¹⁶⁷ In an EMSA, a ^{32}P -labeled DNA containing a particular DNA site is incubated with a candidate target protein. The protein-DNA complexes are separated from unbound DNA by electrophoresis through a non-denaturing polyacrylamide gel or agarose gel.¹⁶⁸ The protein retards the mobility of the DNA fragments which it binds. Therefore, protein-nucleic acid complexes migrate more slowly than the corresponding free nucleic acid. The positions of the free and bound ^{32}P -labeled DNA was demonstrated in the gel.¹⁶⁹

2.3.8.1 Preparation of labeling 84 bp circular DNA

The T4 polynucleotide kinase (T4 PNK) from *E. coli* phage T4 was used to transfer the radioactive terminal phosphate group of γ ^{32}P -ATP to the free 5'-OH group of nucleic acids. The reaction mixture was prepared by adding the target 2 μl DNA, 2 μl γ ^{32}P -ATP, 1U T4 PNK in the 1 X reaction buffer at total volume of 20 μl and incubated at 37° C for 1 hour. After incubation, 20 μl of DNase-free water was added and then the labeled DNA was purified by GE (healthcare) illustra MicrospinTM G-25 columns. The purified DNA was added into 100 μl of DNase-free water and the overall yield of labeling DNA determined by measuring with a Geiger counter. After finish the labeling the target DNA, refer to the method describe in 2.3.2, the labeling 84 bp circular DNA was produced.

2.3.8.2 EMSA study

After obtained the ^{32}P -labeling 84 bp circular DNA, EMSA was prepared according to the process described in previous literatures.¹⁶⁸⁻¹⁷¹ The ^{32}P -labeling 84 bp circle was incubated with 10 U human topoisomerase I at 30 °C for 15 min in the binding buffer [10 mM Tris/HCl (pH 7.5), 3 mM CaCl_2 , 50 mM NaCl, 0.1 M sucrose and 5% glycerol]. After incubation, the reaction mixtures followed by treatment with 1 μl proteinase K for 1 hour at 37 °C. When finish of the digest reaction, reaction mixtures were loaded in 8% non-denaturing polyacrylamide gel to do electrophoretic analysis at 4 °C in $0.25\times\text{TBE}$ buffer and DNA bands were detected by phosphorImager (GE Healthcare). All the data shows in the discussion section.

2.4 Results and discussions

2.4.1 Inhibitory effect of DNA circles on human topoisomerase I

With the aim of explore whether human topoisomerase I will distinguish curved DNA and linear DNA, 84 bp forcible curved DNA circle and 84 bp linear DNA sequences consist of same composition (Table 2-1) were added to the reaction of human topoisomerase I relax negative supercoiled pUC 19 plasmid DNA. Figure 2-8 illustrates the inhibitory effects of 84 bp circle and 84 bp linear DNA on human topoisomerase I. Through determining the efficiency of relaxation reaction of the negative supercoiled plasmid, catalyzed by human topoisomerase I in the presence of our designed curved oligonucleotide circle, the inhibitory effect of curved DNA over linear DNA on human

topoisomerase I was investigated.

Figure 2-8. Agarose gel electrophoretic analysis of inhibitory effect of 84 bp circle (a) and 84 bp linear (b) on human topoisomerase I. A mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and 84 bp circle (a) and 84 bp linear (b) was prepared and further incubated at 37 °C for 15 min. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

As observed in Figure 2-8 (a), lane 2 was used as a control which consists of only the negative supercoiled DNA, pUC 19. The relaxation of the negative supercoiled form of pUC 19 by human topoisomerase I proceeded to near completion in the absence of curved DNA (Lane 3). With the increase of concentrations of 84 forcible curved DNA, the relaxation reactions of pUC 19 decreases drastically as shown in Lane 3 to Lane 9 was discovered. The measured IC_{50} value of 84 bp circle base pair under this circumstance was 2.2 μM (Fiugre 2-9) and the inhibitory effect of this circular DNA was still observable when its base pair concentration was set as low as 0.5 μM . On the other hand, the inhibitory effect of the 84 linear DNA on human topoisomerase I is not comparable to

that of the curved DNA as demonstrated in Figure 2-8 (b). The linear DNA would only show visible inhibition at 56 μM with an extremely low IC_{50} value of 125.9 μM (Figure 2-10). The identical length and sequence in circular DNA and linear DNA make the curvature of circular DNA the most likely reason accounting for the significantly higher inhibitory activity of circular DNA to human topoisomerase I. The relatively lower inhibitory efficiency of 84 linear ($\text{IC}_{50} = 125.9 \mu\text{M}$) as compared to the one obtained from 84 bp circle ($\text{IC}_{50} = 2.2 \mu\text{M}$) was presumably resulted in by the fact that human topoisomerase I will recognize the curved DNA. The speculated reaction process is shown in Scheme 2-1.

Scheme 2-1. Illustration of supercoiled DNA relaxation catalyzed by human topoisomerase I in the presence of 84 bp linear DNA or 84 bp circular DNA. In the presence of 84 bp linear DNA (real lines indicate), most of supercoiled DNA are untwined to relax form DNA by human topoisomerase I. On the other hand, in the presence of 84 bp circular DNA (dotted lines indicate), human topoisomerase I favors the binding to circular DNA and propound to the complex of cDNA-human topoisomerase I. pUC 19 relaxation will be significantly retarded.

Figure 2-9. Correlations between base pairs concentration of 84 bp circle and its percent inhibition on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by 84 bp circle. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

Figure 2-10. Correlations between base pairs concentration of 84 bp linear DNA and its percent inhibition on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by 84 bp linear

DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

Through determining the efficiency of relaxation reaction of DNA plasmid, pUC 19, catalyzed by human topoisomerase I in the presence of our designed oligonucleotide circle, the inhibitory effect of 300 bp circular DNA on human topoisomerase I was investigated (Figure 2.11). As shown in Figure 2-11, Lane 1 consists of only the negative supercoiled plasmid, pUC 19. This identifies the position of the negative supercoiled form of pUC 19 after the electrophoresis and provides us with a basis of comparison with the samples. The relaxation of the negative supercoiled form of pUC 19 by human topoisomerase I proceeded to near completion in the absence of 300 bp circular DNA (Lane 2), thus proved that reaction between human topoisomerase I and pUC 19 was feasible. This served as an experimental control for the efficiency of human topoisomerase I on the supercoiled pUC 19. With the increase of concentrations of 300 bp circular DNA from 0 μM to 16 μM , however, the relaxation reactions of pUC 19 drastically decreases (from Lane 3 to Lane 8). Toward to human topoisomerase I in the relaxation reaction, 300 bp circular DNA having a minimum effective concentration¹⁷²,¹⁷³ value of *ca.* 1.6 μM . IC_{50} indicates the concentration of a drug that is required for 50% inhibition of the activity (Figure 2-12). The base pair IC_{50} value of 300 bp circular DNA derived from our investigation is 7.9 μM . The above outcomes demonstrated that 300 bp DNA circle could indeed acting as a relatively good inhibitor of human topoisomerase I in the relaxation reaction of negative supercoiled pUC 19 plasmid DNA.

Figure 2-11. Agarose gel electrophoretic analysis of inhibitory effect of 300 bp circle on human topoisomerase I. A mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and 300 bp circle was prepared and further incubated at 37 °C for 15 min. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

Figure 2-12. Correlations between base pairs concentration of 300 bp circle and its percent inhibition on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by 300 bp circular DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

The inhibitory effect of 535 bp circular DNA on human topoisomerase I was investigated and the agarose gel electrophoresis was shown in Figure 2-13. Similarly, 535 bp circular DNA was incubated with human topoisomerase I according to the processing of 300 bp circular DNA inhibitory reaction. As shown in Figure 2-13, lane 1 consists of only negative supercoiled pUC 19. The negative supercoiled pUC 19 was relaxed by human topoisomerase I without the presence of 535 bp circular DNA in Lane 2. In the same way, samples in Lane 2 to 8 to investigate the inhibitory efficiency of 535 bp DNA circle were guided similar to that for 300 bp DNA circle. The relaxation reactions of pUC 19 by human topoisomerase I declined significantly with higher concentration of 535 bp circular DNA from 0 μ M to 32 μ M (Lane 3 to Lane 8). Toward to human topoisomerase I in the relaxation reaction, 535 bp circular DNA having a minimum effective concentration^{172, 173} value of *ca.* 3.2 μ M. As seen in Figure 2-14, the IC₅₀ of 535 bp circular DNA derived was 11.7 μ M, which is nearly 5 times higher in magnitude than that of 84 bp circular DNA.

Figure 2-13. Agarose gel electrophoretic analysis of inhibitory effect of 535 bp circle on human topoisomerase I. A mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and 535 bp circle was prepared and further incubated at 37 °C for 15 min. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

Figure 2-14. Correlations between base pairs concentration of 535 bp circle and its percent inhibition on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by 535 bp circular DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

The inhibitory effect of 936 bp circular DNA on human topoisomerase I was investigated and the agarose gel electrophoresis was shown in Figure 2-15. Similarly, 936 bp circular DNA was incubated with human topoisomerase I according to the processing of 300 bp circular DNA inhibitory reaction. As shown in Figure 2-15, lane 1 consists of only negative supercoiled pUC 19. The negative supercoiled pUC 19 was relaxed by human topoisomerase I without the presence of 936 bp circular DNA in Lane 2. In the same way, samples in Lane 2 to 8 to investigate the inhibitory efficiency of 936 bp DNA circle were guided similar to that for 300 bp DNA circle. The relaxation reactions of pUC 19 by human topoisomerase I declined significantly with higher concentration of 936 bp

circular DNA from 0 μM to 64 μM (Lane 3 to Lane 8). Toward to human topoisomerase I in the relaxation reaction, 936 bp circular DNA having a minimum effective concentration^{172, 173} value of *ca.* 6.4 μM . As seen in Figure 2-15, the IC_{50} of 936 bp circular DNA derived was 40.3 μM , which is nearly 20 times higher in magnitude than that of 84 bp circular DNA.

Figure 2-15. Agarose gel electrophoretic analysis of inhibitory effect of 936 bp circle on human topoisomerase I. A mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and 936 bp circle was prepared and further incubated at 37 °C for 15 min. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

Figure 2-16. Correlations between base pairs concentration of 936 bp circle and its percent inhibition on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of

relaxation) was taken as the percent inhibition of topoisomerase I activity by 936 bp circular DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

Table 2-3. Inhibitory efficiency (IC_{50}) of circular DNAs on the activity of human topoisomerase I

DNA	84 bp circle	300 bp circle	535 bp circle	936 bp circle	84 bp linear
$IC_{50}(\mu M \text{ bp})$	2.2	7.9	11.7	40.3	125.9

With the purpose of examine the importance of the degree of DNA curvature on human topoisomerase I, different sizes of DNA, namely, 300 bp, 535 bp and 936 bp circular DNA were used to inhibit the human topoisomerase I on relaxing the negative supercoiled plasmid pUC 19. All experimental results are shown from Figure 2-11 to Figure 2-16 and table 2-3. It is believe that increase of DNA circle sizes from 84 bp to 300 bp, 535 bp and 936 bp, the degree of curvature decrease at the same time. As observed in table 2-3, the inhibitory effects of larger DNA circle decrease synchronously with IC_{50} values of 300 bp, 535 bp and 936 bp circle as 7.9 μM , 11.7 μM and 40.3 μM respectively. Although these circular DNA have good efficiency as compare to that of the straight linear DNA ($IC_{50} = 125.9 \mu M$), this outcome has demonstrated that the 84 bp DNA circle, which possess the most curved degree, has the best inhibition on human topoisomerase I than the other circular DNA. Such observation demonstrated that the curvature degree played a crucial role in inhibiting the activity of human topoisomerase I.

Figure 2-17. pUC 19 relaxation assay for the inhibitory effect of 84 bp, 300 bp, 535 bp and 936 bp circular DNA on human topoisomerase I in same concentration. Assay mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and each DNA circle was prepared respectively and further incubated at 37°C for 15 min before loading on agarose gel. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

In Figure 2-17, the same base pair concentration (6.4 μ M) of each circles were add to the reaction of human topoisomerase I relax negative supercoiled plasmid DNA pUC 19. With the increase of the size of circular DNA from 86 bp to 936 bp, the negative supercoiled DNA pUC 19 decreases drastically as shown in Lane 4 to Lane 7 was discovered. Under the same circumstance, 84 bp circle shows a very strong inhibition while 300 bp and 535 bp only have a weak affection. Furthermore, the relaxation of the plasmid DNA by human topoisomerase I proceeded to near completion in the presence of 936 bp circle. The findings in these experiments have run consistently with our prediction that human topoisomerase I favor binding to the strong curved DNA than the slight curved DNA. On the other hand, the activities of human topoisomerase I can be modulated by the substrates curvature.

The significant difference in the inhibitory effect would be demonstrated by the fact that dissimilar sizes of DNA circle possess the different curved degree, which could consequently interfere the binding of human topoisomerase I to the oligonucleotide. The low binding affinities could be the cause of the observed low inhibitory efficacy of 936 bp circular DNA on the activity of human topoisomerase I. The constraint of small DNA circle is another possible explanation of the relatively higher efficiency of inhibition of 84 bp circular DNA than that of other circular DNA. After human topoisomerase I binding to the smaller circular DNA, the enzyme will nick one single strand of the DNA circle. However, resealing reaction would be prevented in smaller DNA circle because the constraint of the forcible ring, leading to chemically irreversible damage to the enzyme, therefore act as a superior inhibitor to human topoisomerase I activity as compared to other DNA circle.

2.4.2 AFM observation of the interaction of DNA circle and human topoisomerase I

In order to further explore the human topoisomerase I will recognize the curved DNA, atomic force microscopy (AFM) was used to study the binding of human topoisomerase I and curved DNA. AFM is a direct visualization technique imaging is unique in that it allows one to characterize mechanisms involved in DNA-protein recognition and DNA-protein complex formation with nanometer resolution.^{160, 161, 174} 300 bp, 535 bp and 936 bp circular DNA was employed to demonstrate the situation of human

topoisomerase I binding to curved DNA. Three dimensional AFM plots of 300 bp, 535 bp and 936 bp circles in presence and absence human topoisomerase I respectively was shown in Figure 2-18 and Figure 2-19. Complex of DNA circle and human topoisomerase I only can be observed in Figure 2-18 (A), arrow indicate the complex of 300 bp DNA circle and human topoisomerase I. The abrupt part of the circular DNA-enzyme complex pointing the human topoisomerase I. Binding complex of 300 bp circular DNA and human topoisomerase I is easily distinguished from the bare circular DNA (Figure 2-18 (A)). On the other hand, bindings between less curved DNA and the enzyme are not so pronounced in AFM images, presumably due to the lower affinities of human topoisomerase I to less curved circular DNAs. (Figure 2-19 (A) and Figure 2-19 (C)) This result was directly ascertain that human topoisomerase I preferably recognize curvature of DNA. These results suggest that the human topoisomerase I can bind highly curved circular DNA and enzyme-DNA complexes are stable enough to survive the working conditions of AFM.

Figure 2-18. Three dimensional AFM plot of 300 bp circular DNA in presence (A) and absence (B) of human topoisomerase I respectively. Arrows indicate the complex of 300 bp circular DNA and human topoisomerase I. 400 nm x 400 nm scans

Figure 2-19. Three dimensional AFM plots of 535 bp circle and 936 bp circle in presence (A, C) and absence (B, D) of human topoisomerase I respectively. 400 nm x 400 nm scans.

2.4.3 The research of EMSA of DNA circle and human topoisomerase I

Owing to the 84 bp DNA circle is smaller and difficult to clearly observe in AFM, electrophoretic mobility shift assay (EMSA) was used to study the binding of human topoisomerase I and curved DNA. Since its introduction in 1981,^{164, 165} EMSA has become one of the most widely used methods to study the binding of proteins on DNA. The presence of bound protein or proteins on the DNA will retard its mobility relative to unbound DNA and shift the labeled DNA upward in the gel.^{170, 171}

As seen in Figure 2-20, the complex of human topoisomerase I and DNA was resolved by native gel electrophoresis. Binding of human topoisomerase I to the ^{32}P -labeled 84 bp circular DNA was manifest as a retarded band (Figure 2-20, lane 2) relative to unbound DNA (Figure 2-20, lane 1). However, treatment of the enzyme-DNA complex with proteinase K recovers the free DNA band (Figure 2-20, lane 3), which validates that slower mobility is due to the formation of protein-DNA binding complex. The outcome obtained from the EMSA consists with the result of above experiment and further verify the hypothesis of curved DNA will be recognized by human topoisomerase I. These results suggest that human topoisomerase I can bind highly curved circular DNA and enzyme-DNA complexes are stable enough to survive the working conditions of gel electrophoresis.

Figure 2-20. Electrophoretic mobility shift assay (EMSA) analysis of human topoisomerase I and 84 bp circular DNA interactions. Lane 1, ^{32}P -labeled 84 bp circular DNA alone. Lane 2, ^{32}P -labeled 84 bp circular DNA and 10 U human topoisomerase I are incubate in binding buffer at 30°C for 15 min. Lane 3, same as lane 2, followed by treatment with 1 μl proteinase K for 1 hour at 37°C. The gel electrophoresis was run at 4°C, 100 V. The DNA bands were detected by phosphorImager (GE Healthcare)

2.5 Conclusion

Base on the results of inhibitory effect of DNA circles on human topoisomerase I, AFM observation and EMSA of DNA circle and human topoisomerase I, we speculate the mechanism that human topoisomerase I will preferential recognize the curvature of the circular DNA and human topoisomerase I favor binding to the strong curved DNA than the slight curved DNA.

Figure 2-21 illustrates the proposed process of human topoisomerase I relax supercoiled DNA in the presence or absence of different curved circular DNA. In Figure 2-21 (a), supercoiled DNA was completely untwined to relax form DNA by human topoisomerase I in the absence of curved circular DNA. As seen in Figure 2-21 (b), when in the presence of strong curved small DNA circle (e.g. 84 bp circle), the small circular DNA and human topoisomerase I will propend to the complex of DNA-human topoisomerase I, thus the quantity of relaxed form DNA will be heavy reduced. The most competent oligonucleotide examined for inhibition, 84 bp circular DNA which containing high forcible curved exhibited a base pair IC_{50} value of 2.2 μM . Highly efficient inhibition on human topoisomerase I in the relaxation reaction of negative supercoiled pUC 19 DNA has been achieved the formation of a suicidal complex between human topoisomerase I and DNA substrates by forming an irreversible covalent linkage with human topoisomerase I. As shows in Figure 2-22, the formation of small DNA circle and human topoisomerase I complex is irreversible. Human toposiomerase I could effectively bind to curvature of the small circular DNA. After the one strand of the circular DNA was

nicked by human topoisomerase I, proper relegation of the scissile strand was prevented due to constraint of the small DNA circle and ligation reaction proceeds at a very low efficiency, causing irreversible damage to human topoisomerase I.

Figure 2-21. Illustration of human topoisomerase I preferential binds to curvature of DNA.

In contrast (Figure 2-21 (c)), human topoisomerase I encounter slight curved large circular DNA (e.g. 936 bp circle) will not favor the stable formation of DNA-human topoisomerase I complex. Consequently, compared of the strong curved DNA circle, the slight one will not strictly affect the yield of the relax reaction. The base pair IC_{50} value of the 936 bp circular DNA which containing less forcible curved is 40.6 μ M. After the one

strand of the circular DNA was nicked by human topoisomerase I, the enzyme could seal the nick of the circular DNA due to less constraint of the large circular oligonucleotides, causing reversible reaction.

Figure 2-22. Illustration of the irreversible covalent interaction of small circular oligonucleotides and human topoisomerase I.

Our outcomes could conduce deep understanding the behavior of human topoisomerase I. Generally, it is often observed that human topoisomerase I cannot catalyze the relaxation of supercoiled DNA to completion and the catalytic apparent rate decreases in due course. This phenomenon is normally addressed as due to the deactivation of human topoisomerase I towards the end of supercoil relaxation.¹⁷⁵ According to this investigation, a series of forcible circular DNAs are observed to interfere in negative supercoiled pUC 19 relaxations catalyzed by human topoisomerase I. The enzymatic activities of human topoisomerase I can be modulated upon binding to the circular DNA. Human topoisomerase I affects to the circular DNAs according to the curved degrees of circular DNA. The maximum interference effect on human topoisomerase I activity is achieved by 84 bp circular DNA containing the most

pronounced curvature degree. Herein, our results suggest that human topoisomerase I can recognize the curvature in supercoiled DNA. The catalytic efficiencies of human topoisomerase I would be better pronounced on highly curved DNA, in another word, the supercoiled density. The way that human topoisomerase I recognizes supercoiled DNA, is a conformation dependent fashion.^{140, 141, 176} Our hypothesis for human topoisomerase I catalysis, is that in the beginning stage of pUC 19 relaxation, the negative supercoiled DNA plasmid contain higher supercoiled density. Human topoisomerase I can easily find the binding conformation sites with high curvatures. When the relaxation proceeds towards the end, plasmid DNA lost majority of its supercoiled densities and the human topoisomerase I binding conformation sites are not so abundant, the apparent rate of enzyme drops significantly and plasmid relaxation cannot progress efficiently. The enzymatic rate depends not entirely on the base pair concentration of supercoiled DNA, but rather on the curvature of plasmid DNA.

As conclusion, a series of forcible circular DNA are designed to mimic the curvatures of plasmid DNA during its relaxation catalyzed by human topoisomerase I. The enzymatic inhibition from the circular DNAs indicates that human topoisomerase I can preferentially recognize the curvature of circular DNA. Moreover, human topoisomerase I can distinguish different degree of DNA curvature and favor the highly curved DNA. This investigation may provide better understandings of the intrinsic features of human topoisomerase-DNA recognition and benefit the future research on human topoisomerase I.

Chapter 3

The activity of human topoisomerase I was modulated by intrinsic curvature DNA

3.1 Background and objectives

Human topoisomerase I is a ubiquitous protein capable of changing the topological stress of DNA generated by normal DNA cellular metabolic processes such as replication and transcription.^{66, 67, 79, 133} This enzyme has attracted attention previously because of their diverse roles in controlling DNA topology and therefore in regulating DNA-dependent processes.^{82, 145, 177} Topoisomerases are key cellular enzymes that prevent DNA strands from becoming tangled owing to they would cleave DNA and induce it to wind and unwind.¹³² Topological rearrangement occurs when this nuclear enzyme introduces a nick into one strand of supercoiled DNA, allowing a free rotation about the sigma bonds in the strand opposite the nick, before resealing the original break, thus leading to relaxation of the supercoiled DNA.^{84, 134, 178}

It has been illustrated recently that human topoisomerase I with the capability of binds DNA in a topology-dependent fashion with a strong preference for supercoiled DNA over relaxed DNA.^{137, 138} Furthermore, it has been reported that human topoisomerase I preferentially recognize the intrinsically curved DNA.^{139, 140} In addition, our group novel research result has confirmed human topoisomerase I would preferential binding to the forcible curvature of the circular DNA. These results suggest that the

common structural character of the supercoiled DNA, curvature, could provide an alternative recognition pattern of human topoisomerase I, as secondary binding^{148, 149} in the absence of the substrate sequence of human topoisomerase I.

On the other hand, DNA replication, repair and recombination are important for the maintenance of life. Human topoisomerase I are essentially located in the nucleus of cells and acting on the function of prevent entanglement of DNA strands during DNA replication, RNA synthesis from DNA (transcription), exchanges of DNA segments between chromosomes (recombination) and elimination of erroneous DNA sequences (repair).^{68, 132, 179} Human topoisomerase I inhibitors block these essential processes will in theory leading to cell death.¹⁸⁰ In general, human topoisomerase I holds an essential role in cell proliferation and has been identified that over expression of this nuclear enzyme induces tumor cells to grow up rapidly.¹⁸¹⁻¹⁸³ In fact, human topoisomerase I have been found in great numbers in fast proliferating tumor cells. The proliferation of cancer cell is very rapidity and out of control in the diseased region. Apoptosis of cancer cells would occur when human topoisomerase I lost the capability of release the stress of the supercoiled DNA. Consequently, this enzyme has been known to be a target of several cancer chemotherapeutic agents and antibiotics.^{103, 184-188}

Cancer is the second dominating life-threatening of human in the majority of the developed countries. Synthetic small molecule drugs have always played an important role in cancer therapy. Chemotherapy is a mainstay of malignant tumor therapies given to patients today. Even though there has been significant progress in the cures of cancer

during the last decade, there are still no treatments for most kinds of major human cancer. The significant reason for the difficulty in treatment of cancer is that most chemotherapeutic drugs lack specificity toward cancer cell, that is, chemotherapy to patients with malignant tumors is limited by concomitant injury to normal cell or tissues. These side effects often seriously limit dose intensification¹⁸⁹ for the patients during cancer treatment.

With the purpose of looking for a novel type of high efficiency and low toxicity agents beyond the classical organic compounds for interfering the activity of human topoisomerase I, a series of linear double-stranded oligonucleotides containing variation of intrinsic curvature were systematically designed and investigated to determine the effectiveness in inhibition of the enzyme action. Furthermore, electrophoretic mobility shift assay are used to confirm the interaction of human topoisomerase I and intrinsically curved DNA.

3.2 Design of DNA sequences

Owing to the fact that the small molecule anticancer agents can bind to human topoisomerase I to form a transient enzyme-DNA covalent complex which consequently inhibits the activity of human topoisomerase I to relieve superhelical stress in supercoiled DNA. For this reason, the activity of human topoisomerase I in cancer cells would be abolished by the destruction of the enzyme action.^{106, 190} However, these small molecule

organic compounds are accompanied with unpredictable and deleterious toxicity associated with treatment. In order to investigate a more rational design and better strategy in cancer chemotherapy, the mechanism of human topoisomerase I interaction with several linear duplex oligonucleotides have been examined in the past few years.^{191, 192} These previous findings and discoveries have indicated that linear non-supercoiled might be able to act as an efficient inhibitor of human topoisomerase I in the relaxation reaction of supercoiled DNA.

Recently, our group has been dedicated to investigating the process of human topoisomerase I in relaxing the supercoiled DNA *in vitro* and want to use oligonucleotides instead of small organic molecule to develop novel series of topoisomerase inhibitors. Wang et al¹⁹³, Li et al¹⁹⁴ and Chua et al¹⁹⁵ had designed internal C3-Spacer-containing circular oligonucleotides or certain dumbbell shaped oligonucleotides containing mismatched base pairs respectively as human topoisomerase I inhibitors which displayed high potency on the activity of the enzyme. Their results had identified that such modified oligonucleotides could exhibit an IC_{50} value in nano-molar level and significantly lower than that of camptothecin which is an anticancer drug currently in clinical use. Therefore, it would be possible to develop human topoisomerase I inhibitor through using specific oligonucleotides and its derivatives. Based on the previous outcomes¹⁹³⁻¹⁹⁵ and the innate character^{139, 140} of human topoisomerase I recognize the curvature DNA. A series of intrinsically curved DNA will be tried to use as human topoisomerase I inhibitor and modulate the activity of the enzyme in this investigation.

Intrinsic bent or curved DNA molecules is due to when special base sequences which induce the local structural polymorphism are repeated in phase with the DNA helix screw.³⁶ X-ray diffraction studies of DNA sequences containing A-tracts indicated that the A-tracts are straight, and the curvature took place at the junctions between A-tracts and adjacent sequences.^{38, 196} Many base sequences can impart systematic intrinsic curvature to DNA, but most such bends are small compared to the special effect produced by runs of homopolymeric dAdT base pairs (the A-tracts).^{37, 197-201} Sequences of such duplex oligonucleotides that can hold intrinsic curved structures are illustrated in Figure 3-1.

Figure 3-1. Illustration of a duplex sequence including A-tract that can form intrinsically curved structures.

According to the investigation of a series of K-DNA (kinetoplast DNA) exhibited in Table 3-1, it was demonstrated that periodical repeat of CA₅₋₆T at 10 base pairs (bp) interval is the origin of curvature in DNA, with FCT having greater bend as compared to FGG and A₅N₅.^{36, 37} Owing to the 10 bp phasing match closely to the expected helix repeat of about 10.3 bp per turn, an average of the 10.5 for B-DNA and 10.1 for poly (dA).poly(dT) in solution, since bending special sequences must be in phase in order to

add coherently.^{37, 202} This situation proven from the fact that A₅N₅ exhibiting greatest bent as compared to A₅N₄, A₅N₆ and A₅N₇. It was discovered that curvature phenomena only take place when A-tracts are at least 4 bp long (A₄N₆ onwards) and increase to maximum when the tract is 6 bp long (A₆N₄) as the 3' and 5' junctions are roughly half a helical turn (6 bp) apart. Less curvature was found in A₃N₇. Bending still exists but decrease for A₈N₂ and A₉N₁.³⁷

Table 3-1. Sequences of oligonucleotides examined by literature.³⁷ The table only shows one stand of the sequence.

Name of oligonucleotides	Sequences of oligonucleotide
A ₅ N ₄	5' CAAAAACGG3'
A ₅ N ₅	5' GGCAAAAACG3'
A ₅ N ₆	5' GGCCAAAACG3'
A ₅ N ₇	5' GGCCAAAACCG3'
A ₃ N ₇	5' GGCCAAACCG3'
A ₄ N ₆	5' GGCCAAAACG3'
A ₆ N ₄	5' GGCAAAAAC3'
A ₈ N ₂	5' CCAAAAAAAAA3'
FCT	5' GGCAAAAATG3'
FGG	5' CCGAAAAAGC3'

From the literature³⁷, it is expected that increasing curvature with accompanying

decrease in DNA end-to-end distance, will lead to a decrease in electrophoretic mobility. In addition, since the experimentally determined ratio R_L of apparent length to real length increases with degree of curvature, the apparent length is assumed to be an increasing function of the degree of curvature. The apparent length is defined as the length of the linear marker DNA that has the same mobility.³⁷ The following figure shows the sequences with decreasing R_L values, which also corresponds to a decrease in degree of curvature. All sequences shown are in 80 base pairs (bp).³⁷ From Figure 3-2, A_6N_4 has the highest degree of curvature and A_3N_7 gives almost a R_L value of 1.00, which means bending is almost abolished for A_3N_7 .³⁷

$$A_6N_4 > A_8N_2 > A_5N_5 > A_4N_6 > A_9N_1 > A_3N_7 > IAT > IAG > IAC > A_{20}$$

Figure 3-2. Illustration of sequences with decreasing R_L value.

Taking advantage of the outcomes of the previous studies^{37, 198, 199, 203, 204}, repeat sequence A_6N_4 (5' GGCAAAAAAC 3' in table 3-1) was used for designing the super curved DNA in our study. Moreover, according to the research of Crothers et al³⁷, the length of all oligonucleotides were designed in 80 bp in this investigation (Table 3-2 show the designed oligonucleotides). In order to rational produce four duplex oligonucleotides with different curvature degree, all oligonucleotides were designed by consist of same composition (same percentage of G, C, A and T) and maintaining at 80 bp in total. In case of design the most curved DNA 4, which contain A_6N_4 tracts, eight group of sequence “GGCAAAAAAC” was used to making up this oligonucleotide in a total length of 80 bp.

With aim to prepare DNA 3 which fewer curves than DNA 4, DNA 3 was made up by five group of sequence “GGCAAAAAAC” randomly. DNA 2 only contains A₃ tract repeat in the sequence, the curvature degree is less than DNA 4 and DNA 3 thus far. At last, DNA 1 the least curvature degree one was made up only by unorderly A₂ tract in the sequence. In addition, in order to investigate the quantity of most curve sequence A₆N₄ tracts whether will affect the effectiveness in inhibition of human topoisomerase I action, DNA 5 and DNA 6 which contain two and four repeating A₆N₄ tracts in the middle of the sequence were designed.

Table 3-2. The sequences of DNA (consisting of the same composition) used in our studies. All of the linear DNAs are only show in one strand from 5' to 3'. Gray shadow indicates the most curve sequence A₆N₄ tracts.

Name of DNA	Sequence
DNA 1	5' AGACAAGACAAGACAAGACAAGACAAGACAAGACAAGACAA GACAAGACAAGACAAGACAAGACAAGACAAGACA 3'
DNA 2	5' GGAAACAAACGGAAACAAACGGAAACAAACGGAAACAAACGGAAAC AAACGGAAACAAACGGAAACAAACGGAAACAAAC 3'
DNA 3	5' GGCAAAAAACGGCAAAAAACAGAGAACAACGGCAAAAAACGGCAAAA AAACGGCAACAAGAGACAAAAAACGGCAAAAAAC 3'
DNA 4	5' GGCAAAAAACGGCAAAAAACGGCAAAAAACGGCAAAAAACGGCAAAA AAACGGCAAAAAACGGCAAAAAACGGCAAAAAAC 3'
DNA5	5' ACAGAGACAAACAGAGACAAACAGAGACAAAGGCAAAAAACGGCAAAA AAACACAGAGACAAACAGAGACAAACAGAGACAA 3'
DNA6	5' ACAGAGACAAACAGAGACAAAGGCAAAAAACGGCAAAAAACGGCAAAA AAACGGCAAAAAACACAGAGACAAACAGAGACAA 3'

3.3 Materials and methods

3.3.1 Oligodeoxyribonucleotides enzymes and chemicals

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sigma-Proligo (Singapore)
pUC 19	New England Biolabs
Human topoisomerase I	Topogen
1 Kb DNA ladder	New England Biolabs
Biological purity water	1st Base Pte. Ltd
Agarose	Invitrogen
Ethidium bromide	Research Biolad
T4 polynucleotide kinase	New England Biolabs
γ - ³² P ATP	Perkin Elmer
Micro-spin column	GE, Healthcare
TAE, TBE, TRIS	1st Base Pte. Ltd
X-ray films	Fuji, Singapore
X-ray development solution	Kodak, Singapore

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

3.3.2 Calculation of curvature degree of the design DNA

With the purpose to predict the curvature degree of the design DNA, the software “DNA Curvature Analysis” provided by Christoph Gohlke at the website “<http://www.lfd.uci.edu/~gohlke/dnacurve/>” was used to calculate all the design DNA sequence (Table 3-2). First open the website, only one strand of the designed duplex DNA was input to the software, then the software will process the calculation when click the submit button. At last, results of Calculated values (CSV format), Plot of coordinates and curvatures (SVG format) and helix coordinate (PDB format) will be provided by the software. The outcome of helix coordinate (PDB format) was choose and the result was shown in below Figure 3-3. As observed in Figure 3-3, curvature degree is decrease from DNA 4 to DNA 1 while DNA 4 containing significant curvature than other DNAs and DNA 1 is the straight one. The findings in these calculations have run consistently with our design of sequences with different curvature degree.

Figure 3-3. Illustration of the calculated result of design DNA with different curved degree.

3.3.3 Preparation of the design DNA

Oligonucleotides was purchased from company require annealing, base pairing with complementary strand in order to obtain the double-stranded oligonucleotides of choice. A solution containing two single strand oligonucleotides, 10 mM Tris-HCl and 50 mM KCl in total volume of 100 μ l was maintained at temperature of 95°C for 3 minutes followed by allowing it to cool down to room temperature. This process ensures that a stable structure of double-stranded oligonucleotides is formed before electrophoretic analysis.

3.3.4 Confirmation of different curved degree of the design DNA

In order to confirm whether the designed sequences indeed with different degree of curvature, non-denaturing polyacrylamide gel electrophoresis (PAGE) is performed to evaluate the curvature degree of each sequences. Various experiments^{37, 201, 203, 205} have shown that such DNA bending fragments migrate slower with lower electrophoretic mobilities than the normal straight DNA chain of same size on PAGE. This anomalously slow migration is due to the helix backbone curvature since the curved DNA has bigger cross-sectional areas and thus its mobility is slower, behaving electrophoretically as though their sizes were larger than the actual.²⁰⁵

These polyacrylamide gels are generated by crosslinking polymers of acrylamide

with the co-monomer bisacrylamide. The covalent crosslinking reaction was catalyzed by N,N,N',N'-tetramethylethylenediamine (TEMED), and the polymerization was initiated by ammonium persulfate (APS). 142.5 g of acrylamide, 7.5 g of N, N'-methylenebisacrylamide, 100 ml of 10 X TBE and 900 ml of deionized water were mixed together to produce a 15 % non-denaturing acrylamide solution. The gel sandwiches were assembled by using two side spacers and taped the plates together along the sides and bottom with vinyl tape. 50 μ l of TEMED and 500 μ l APS was added to a 50 ml of the previously prepared 15 % acrylamide solution. The solution was stirred for complete mixing and instantly poured it into gel sandwich. The comb to create wells was immediately inserted in the upper of the gel sandwiches. The solution was allowed to polymerize at room temperature for an hour. After that, the comb was removed and the wells were rinsed with water. The tape at the bottom of the gel was removed and the gel was pre-run for a couple of hours. The samples were then loaded into the wells in the gel and the gel in 1x TBE electrophoresis buffer for 48 hours at 200 V, at 4°C was operated. After the electrophoresis, the gel was removed and stained in ethidium bromide. The gel was subsequently rinsed with water before visualizing with the UV transilluminator, Syngene G:Box HR.

As observed in Figure 3-4, the mobility shift of four 80 bp DNAs (Table 3-2 shows the sequences used in this studies) are gradually slower from lane 1 to lane 4. In addition, the mobility shift of DNA 4 (lane 4 in Figure 3-4 (B)) is obvious slower than other DNAs in the PAGE experiment. It is identified that curvature degree is decrease from DNA 4 to DNA 1 while DNA 4 with significant curvature than other DNAs. This result is indeed consistent with the expectation of the design of sequences with different curvature degree.

(A)

(B)

Figure 3-4. (A) Illustration of the overlap of DNA 1, DNA 2, DNA 3 and DNA 4. (B) Electrophoretic analysis of different curvature degree of DNA 1, DNA 2, DNA 3, and DNA 4. Each DNA in a solution containing 10 mM Tris-HCl and 50 mM KCl was kept at 90°C for 10 minutes followed by allowing it to cool down to room temperature over a period of 1 hours. The DNA samples were analyzed using 15% polyacryamide non-denature gel electrophoresis which was run at 4°C, for 48 hours (200 V and 15 W). Lane 1: DNA 1; Lane 2, DNA 2; Lane 3: DNA 3; Lane 4, DNA 4.

On the other hand, in order to study the quantity of most curve sequence A_6N_4 tracts whether will affect the inhibition of human topoisomerase I action, DNA 5 and DNA 6 which contain two and four repeating A_6N_4 tracts in the middle of the sequence were designed respectively. According to the method mentioned above, DNA 5 and DNA 6

were calculated by “DNA Curvature Analysis” and then were analyzed by non-denature gel electrophoresis. As shown as Figure 3-5(B), mobility shift of three DNAs (Table 3-2 shows the sequences used in this studies) are gradually slower from lane 1 to lane 3 and the mobility shift of DNA 4 is retard than others DNA in the gel.

(A)

(B)

Figure 3-5. (A) Illustration of the overlap of DNA 5, DNA 6 and DNA 4. (B) Electrophoretic analysis of different curvature degree of DNA 5, DNA 6 and DNA 4. Each DNA in a solution containing 10 mM Tris-HCl and 50 mM KCl was kept at 90 °C for 10 minutes followed by allowing it to cool down to room temperature over a period of 1 hours. The DNA samples were analyzed using 15% polyacryamide non-denature gel electrophoresis which was run at 4°C, for 48 hours (200 V and 15 W). Lane 1: DNA 5; Lane 2, DNA 6; Lane 3: DNA 4.

3.3.5 Inhibition assays of the design DNA on human topoisomerase I

Agarose gel electrophoresis is employed to analyse the inhibitory effects of the designed DNA inhibitors for human topoisomerase I with negative supercoiled plasmid pUC 19. 1% agarose gel is typically used in this investigation. Thus in this case, 1 gram of Agarose powder was weighed into an empty conical flask and 100 ml of 1x TAE solution was added. The mixture was then boiled on a hot plate with stirring. The agarose solution was then left to cool to 50 °C before casting the gel in a horizontal gel casting mould, made by wrapping clear tape around and extending above the edges of a 22 cm x 15 cm glass plate, with a comb placing horizontally to create the required wells. It was then left at room temperature for the gel to solidify. The solidified agarose gel was submerged in 1x TAE electrophoresis buffer in a horizontal electrophoresis apparatus. The designed DNA at different concentrations were incubated in reaction mixture including 0.5 U human topoisomerase I and 250 ng of negative supercoiled pUC 19 in 1 X human topoisomerase I reaction buffer in a total volume 20 µL of at 37 °C for 15 mins. The quantities of different curvature degree DNAs were measured by Thermo Nanodrop. The DNA samples are mixed with gel loading dye and filled into the sample wells. Electrophoresis was done at 150 V for 90 mins at room temperature. Agarose gel electrophoresis was employed to analyze the inhibitory effect of all the design DNA for human topoisomerase I followed staining with ethidium bromide. The DNA bands were visualized through Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed

DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while 100% minus off the percentage of relaxation was taken as the percent inhibition of topoisomerases activity by the DNA. All the data shows in the discussion section.

3.3.6 EMSA of curvature linear DNA and human topoisomerase I

Electrophoretic mobility shift assay (EMSA) is one of the most popular strategies for investigation of the interaction of DNA and protein. With the aim to research the interaction of human topoisomerase I and curvature duplex DNA, the most curve DNA 4 and straight DNA 1 was employed in EMSA study. First, one strand of DNA 1 and DNA 4 need to be labeled with γ ³²P-ATP. A solution mixture was prepared by adding the target 2 μ l DNA, 2 μ l γ ³²P-ATP, 1U T4 polynucleotide kinase in the 1 X reaction buffer at total volume of 20 μ l and incubated at 37° C for 1 hrs. After incubation, 20 μ l DNase-free water was added and then the labeled DNA was purified by GE (healthcare) illustra MicrospinTM G-25 columns. The purified DNA was added into 100 μ l DNase-free water. After finish the labeling the target DNA, refer to the process describe in 3.3.3, the ³²P-labeling DNA 1 and DNA 4 were produced.

After obtained the ³²P-labeling DNA 1 and DNA 4, EMSA was prepared according to the process describe in previous literatures.¹⁶⁸⁻¹⁷¹ The ³²P-labeling DNA 1 and DNA 4 was respective incubated with 10 U human topoisomerase I at 30 °C for 15 min in the binding buffer [10 mM Tris/HCl (pH 7.5), 3 mM CaCl₂, 50 mM NaCl, 0.1 M sucrose and

5% glycerol]. After incubation, reaction mixtures were loaded in 8% non-denaturing polyacrylamide gel to do electrophoretic analysis at 4 °C in 0.25×TBE buffer and DNA bands were detected by phosphorImager (GE Healthcare). All the data shows in the discussion section.

3.4 Results and discussion

3.4.1 Inhibitory effect of intrinsic curvature DNA on human topoisomerase I

With the purpose of explore whether activity of human topoisomerase I will be modulated by different degree of intrinsically curved of linear DNA, four 80 bp linear DNAs (DNA 1, DNA 2, DNA 3 and DNA 4) with identical sequence were added to the reaction of human topoisomerase I relax negative supercoiled pUC 19 plasmid DNA. Figure 3-6 illustrates the inhibitory effects of four 80 bp linear DNA on human topoisomerase I. Through determining the efficiency of relaxation reaction of the negative supercoiled plasmid, catalyzed by human topoisomerase I in the presence of our designed different curved linear DNA, the inhibitory effect of curved linear DNA over straight linear DNA (DNA 1) on human topoisomerase I was investigated. As observed in Figure 3-6 (A) and Figure 3-6 (B), lane 2 was used as a control which consists of only the negative supercoiled DNA, pUC 19. The relaxation of the negative supercoiled form of pUC 19 by human topoisomerase I proceeded to near completion in the absence of curved DNA (lane 3 both in Figure 3-6 (A) and Figure 3-6 (B)). With the increase of

concentrations of 80 bp linear DNA (DNA 1, DNA 2, DNA 3 and DNA4), the relaxation reactions of pUC 19 decreases gradually as shown in Figure 3-6 were discovered. The measured IC_{50} value of DNA 1, DNA 2, DNA 3 and DNA 4 is 1.53 μ M, 1.36 μ M, 1.24 μ M and 0.62 μ M respectively (Figure 3-6, Table 3-3 and Figure 3-7). The entire inhibitory effect of DNA 4 was observable when its concentration was set as low as 0.8 μ M (lane 11 in Figure 3-6 (A)). At the same time, under concentration of 0.8 μ M (lane 5 and lane 13 in Figure 3-6 (A)), others linear DNAs in this studied were only show rarely inhibits effective on human topoisomerase I except DNA 3 (lane 5 in Figure 3-6 (B)). The relatively lower inhibitory efficiency of DNA 1 (IC_{50} = 1.53 μ M) as compared to the one obtained from DNA 4 (IC_{50} = 0.62 μ M) was presumably resulted in by the fact that human topoisomerase I will preferential binding to the strong curved DNA than the slight curved DNA.

(A)

(B)

Figure 3-6. pUC 19 relaxation assay for the inhibitory effect of DNA 1, DNA 2, DNA4

(A) and DNA 3 (B) on human topoisomerase I. Assay mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and each DNA was prepared respectively and further incubated at 37 °C for 15 min before loading on agarose gel. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

Figure 3-7. Correlations between concentration of DNA 1 (A), DNA 2 (B), DNA 3(C), DNA 4 (D) and the corresponding inhibitory effects on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA [relaxed DNA/ (relaxed DNA + supercoiled DNA)]¹⁵² while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by corresponding DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

Table 3-3. Inhibitory efficiency (IC₅₀) of intrinsically curved DNAs on the activity of human topoisomerase I.

Name of DNA	IC ₅₀ (μM)
DNA 1	1.53
DNA 2	1.36
DNA 3	1.24
DNA 4	0.62

On the other hand, as shown as Figure 3-8, the same concentration (1.2 μM) of each DNA was add to the reaction of human topoisomerase I relax plasmid DNA pUC 19. With the enhancement of curved degree of the DNA from DNA 1 to DNA 4, the relaxation reactions of pUC 19 reduce drastically in Lane 4 to Lane 7 was observed. Under the same circumstance, DNA 4 shows a very strong inhibition while DNA 1 and DNA 2 only have a weak affection. Furthermore, the relaxation of the plasmid DNA by human topoisomerase I proceeded to near completion in the presence of DNA 1. This outcome in the experiment has demonstrated that the DNA 4, which possesses the most curved degree, has the best inhibition on human topoisomerase I than the others DNA. It is speculated that human topoisomerase I could distinguish different curved degree of the intrinsically curved linear DNA. In other word, the activities of human topoisomerase I can be regulated by intrinsic curvature of DNA. As shown in Figure 3-8(B), the attenuation magnitudes of human topoisomerase I activities are suppressed when the curvatures of

DNAs are diminished from DNA 4 to DNA 1 by reducing the length of A-tract or the quantity of A-tract.

(A)

(B)

Figure 3-8. (A) pUC 19 relaxation assay for the inhibitory effect of DNA 1, DNA 2, DNA 3 and DNA 4 on human topoisomerase I at the same concentration. Assay mixture containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and each DNA circle was prepared respectively and further incubated at 37°C for 15 min before loading on agarose gel. The analysis was done on a 1% agarose gel and stained by ethidium bromide. (B) Percentage inhibition of relaxation reaction by different curvature DNA.

3.4.2 Inhibitory effect of the quantity of most curved sequence of DNA on human topoisomerase I

In order to explore whether activity of human topoisomerase I will be effected by the quantity of most curve A-tract sequence (A_6N_4), other two 80 bp linear DNAs (DNA 5 and DNA 6) with identical sequence were designed. DNA 5 and DNA 6 containing two or four (A_6N_4) repeat in the middle of the sequence respectively. The correlation between the inhibitory efficiency and the quantity of A-tract containing duplexes are further explored by determining the IC_{50} of each curved DNA to the activities of human topoisomerase I. DNA 5, DNA 6 and DNA 4 which contain eight (A_6N_4) repeat in the sequence were investigated in the reaction of human topoisomerase I relax negative supercoiled pUC 19 plasmid DNA. Figure 3-9 illustrates the inhibitory effects of DNA 5 and DNA 6 on human topoisomerase I. Through determining the efficiency of relaxation reaction of the negative supercoiled plasmid, catalyzed by human topoisomerase I in the presence of our designed DNA, the inhibitory effect of DNA 5, DNA 6 and DNA 4 on human topoisomerase I were investigated. As observed in Figure 3-6 (A) and Figure 3-9, lane 2 was used as a control which consists of only the negative supercoiled DNA, pUC 19. The relaxation of the negative supercoiled form of pUC 19 by human topoisomerase I proceeded to near completion in the absence of curved DNA (lane 3 both in Figure 3-6 (A) and Figure 3-9. With the increase of concentrations of 80 bp linear DNA (DNA 5, DNA 6 and DNA 4), the relaxation reactions of pUC 19 decreases gradually as shown in Figure 3-6 (A) and Figure 3-9 were discovered. The measured IC_{50} value of DNA 5, DNA 6 and DNA 4 is 1.7 μ M, 0.92 μ M and 0.62 μ M respectively (Table 3-3 and Figure 3-10). The

entire inhibitory effect of DNA 4 was observable when its concentration was set as low as 0.8 μM (lane 11 in Figure 3-6 (A)). At the same time, under concentration of 0.8 μM (lane 5 and lane 9 in Figure 3-9), other linear DNAs in this research were show less inhibits effective on human topoisomerase I. The relatively lower inhibitory efficiency of DNA 5 ($\text{IC}_{50} = 1.7 \mu\text{M}$) was presumably resulted in by the fact that only two A-tract in the sequence will not affect the curvature of the DNA structure. In the case of DNA 6, which contain four A-tract in the sequence, the inhibitory efficiency ($\text{IC}_{50} = 0.92 \mu\text{M}$) is better than DNA 5 because the sequence have more curved motif than that in DNA 5. Furthermore, DNA 4 exhibit strong inhibition on human topoisomerase I owing to it contain eight A-tracts in the sequence induce the most curved structure. DNA 4 with the most A-tract and the strong curvature exhibits the maximum inhibitory effect and the largest IC_{50} as 0.63 μM , almost 2.5 times as that of the less curved duplex, DNA 5. It is speculated that DNA with most curved A-tract maybe provide more binding site for human topoisomerase I which induce the inhibition of intrinsic DNA. These outcomes demonstrate that the activity of human topoisomerae I would be affected by the quantity of the most curved sequence in the DNA because the curved degree will be improved occur with the increase of the quantity of A-tract.

Figure 3-9. pUC 19 relaxation assay for the inhibitory effect of DNA 5 (A) and DNA 6

(B) on human topoisomerase I. Assay mixture containing 10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 250 ng pUC 19, 0.5 U of human topoisomerase I, and each DNA was prepared respectively and further incubated at 37 °C for 15 min before loading on agarose gel. The analysis was done on a 1% agarose gel and stained by ethidium bromide.

Figure 3-10. Correlations between concentration of DNA 5 (A), DNA 6 (B), and the corresponding inhibitory effects on human topoisomerase I. Percentage of relaxation was defined as the ratio of band density of relaxed DNA over those of relaxed DNA plus supercoiled DNA $[\text{relaxed DNA} / (\text{relaxed DNA} + \text{supercoiled DNA})]^{152}$ while (100% - percentage of relaxation) was taken as the percent inhibition of topoisomerase I activity by corresponding DNA. The DNA bands were quantified using Gel Documentation System (G:Box HR, Syngene, Cambridge, UK) equipped with Gene Tools Software.

Generally, in the presence of organic compound²⁰⁶⁻²⁰⁸ or unusual DNA sequence¹⁹³⁻¹⁹⁵ to become a chemically stable covalent intermediate will makes human topoisomerase I suicide which the enzyme could allow the cleavage reaction to proceed but prevent the ensuing ligation reaction. Therefore, the organic compound or unusual DNA sequence will exhibit effective inhibition. From the results discussed above, it is

could be found that the IC_{50} of each DNA on human topoisomerase I with weak different from 0.62 μM to 1.73 μM owing to the binding of the enzyme and intrinsic curved DNA is reversible. As shown in Figure 3-11, human topoisomerase I would quickly nick one strand of DNA and forming a covalent bond between 3'-end of DNA and itself, and then prompt reseal the gap when it acting on the duplex DNA. Human topoisomerase I trapped DNA substrate is only transient, as the enzyme-DNA complex is a functional intermediate capable of rejoin the rotated 5'-OH-ended to the 3'-end.²⁰⁹ Even the nick and religation would finish in a short moment, the period of retardment is adequate to affect the activity of human topoisomerase I in relaxed reaction and reduce useful life of itself. As a result, intrinsically curved DNA will induce the inhibitory affection on human topoisomerase I.

Figure 3-11. Schematic representation of interaction between human topoisomerase I and duplex DNA.

3.4.3 Interaction of linear curvature DNA and human topoisomerase I

The less suppression of human topoisomerase I activity in presence of more straight duplex may result from the preferential binding of human topoisomerase I to the curved DNA than linear DNA. Electrophoretic mobility shift assay (EMSA) is applied to elaborate the cause of inhibitory effects from curved degree.^{164, 165} In EMSA, protein

bound DNA normally shows slower mobility than the unbound DNA.^{170, 171} As shown in Figure 3-12, ³²P-labeled DNA 1 and DNA 4 were annealed to the complimentary strands and then were incubated with human topoisomerase I, respectively. The bound and free duplex DNA 1 and DNA 4 were separated by electrophoresis (lane 2 and lane 4 in Figure 3-12). A lagging band in lane 4 is observed and indicates the formation of duplex-enzyme complex when DNA 4 is treated with human topoisomerase I. The similar bound DNA 1-human topoisomerase I complex is absent in lane 2. EMSA validates that intrinsic curved DNA 4 can have strong interaction with human topoisomerase I and form a bound complex. The association between straight linear DNA 1 and human topoisomerase I is at least not tight enough to survives the electrophoresis. Human topoisomerase I prefers to bind the highly curved DNA over straight duplex. This recognition feature of topoisomerase provides a potential strategy to modulate the enzymatic activity of human topoisomerase I via binding the backbone inclinations in linear DNA.

Figure 3-12. EMSA analysis of human topoisomerase I and intrinsically curved DNA interaction. Lane 1, DNA 1 alone; Lane 2, DNA 1 and 10 U human topoisomerase I are incubate at 30°C for 15 min; Lane 3, DNA 4 alone; Lane 4, DNA 4 and 10 U human topoisomerase I are incubate at 30°C for 15 min. The gel electrophoresis was run at 4°C, 100 V.

3.5 Conclusion

Base on the investigations of Inhibitory effect of design intrinsic curved DNA on human topoisomerase I, and interaction of bent DNA and human topoisomerase I, we assume that human topoisomerase I will preferential recognize the intrinsic curvature in duplex DNA. Furthermore, the activity of human topoisomerase I will be suppressed by the DNA contains intrinsic curvature. As shown in Figure 3-13, in the absence of curvature DNA (real line indicate), supercoiled DNA can reach nearly complete relaxation catalyzed by human topoisomerase I. In the assay (dotted lines indicate), curved DNA are applied as potential inhibitor to human topoisomerase I. The suppression on pUC 19 relaxation is expected if the inhibitory effects from designed DNA are well pronounced.

Figure 3-13. Illustration of the pUC 19 relaxing assay. The relaxation of supercoiled pUC 19 is catalyzed by human topoisomerase I in the presence or absence of curvature DNA.

As conclusion, a series of intrinsic curved linear DNA were designed and their inhibitory effects on the activity of human topoisomerase I were examined in our investigation. Our studies demonstrated that the double-stranded oligonucleotide with the highest curvature possess effective inhibition on human topoisomerase I. The IC_{50} value of each DNA is decrease gradually along with the enhancement of curved degree of the DNA. This result indicated that the effect of curvature of DNA is a crucial factor for the inhibition of this enzyme and the activities of human topoisomerase I can be modulated by intrinsic curved of linear DNA. Moreover, intrinsic curvature is a natural and common characteristic of DNA which could consequently enhance their chance of survival in cellular environments because they could resist the hydrolysis by a certain mismatched base pair-resolving and nick sites-resolving DNA repair protein. In consideration of the recent development²¹⁰ in the introduction of oligonucleotides and DNA into eukaryotic cells and the reduced potentials for toxicity of DNA-based biopharmaceuticals²¹¹⁻²¹³, it is our hope that the information provided in these studies could benefit the design of new oligonucleotide-based therapeutical agents as inhibitor of human topoisomerae I in the future.

Chapter 4

Preparation of catenated ring and Trefoil ring based on toroidal DNA structures

4.1 Background and objectives

Circular DNAs are attractive molecules that have induced the curiosity of biologists, chemists and theoreticians since the discovery of it in living cells, 50 years ago.^{214, 215} Various examples of cyclized and pluri-cyclized DNAs have been reported in the literature since the pioneer work of Clayton²¹⁶ who identified circular forms of mitochondrial DNA in extracts of human leukemic leucocytes, both in the form of circular dimers and as catenanes made up of interlocked rings. Much less is known about more complex ring topologies such as Trefoil DNA ring and catenated DNA rings (Figure 4-1) that are also found in nature.²¹⁷ Their mechanism of formation and their function in vivo are still unclear, and increasing efforts are being conducted to tentatively prepare and investigate their specific characteristics and properties.²¹⁸ On the other hand, circular DNA has been comprehensive used in the investigation of DNA nanotechnology which employs DNA as a structural material for construct nanometer-scale architectures.²¹⁹ DNA is not only programmable, but also structurally and functionally well behaved and is arguably the most versatile material for construction of artificial objects in the nanometer-scale. Such as, DNA can also be the basis for molecular motors,²²⁰ sensors, and logic devices²²¹ and many other devices with a broad range of functions.^{222, 223} In addition, topologically interesting structures like Borromean rings²²⁴ and rotaxane²²⁵ have

already been built from DNA origami.

Figure 4-1. Illustration of the structural conformations of circular DNA catenated DNA and Trefoil DNA.

Catenated DNA structures commonly refer to interlocked entities of double-stranded circular DNA in which there is absence of fixed pattern of physical interactions between their components of DNA circles^{217, 226-233}. Double strand DNA Catenated molecules were first observed in 1967 in the mitochondria of human cells^{216, 234}. Achievement of proximity between two distant DNA segments through proper means has been deemed to be one of the most crucial prerequisites for synthesizing interlocked DNA structures²³⁵⁻²³⁷. Generation of negative supercoiled DNA by the action of DNA gyrase, for example, was utilized to drive some distant DNA segments closer to each other, which in turn led to the generation of catenated DNA rings upon subsequent enzymatic actions²³⁷⁻²³⁹. Furthermore, it was demonstrated recently that small DNA binding molecules were capable of pulling two separate DNA strands together to form DNA-small molecules complexes on which catenated DNA circles

could be synthesized²³⁶. In topology, the Trefoil knot is the simplest structure type of an important knot. The Trefoil could be achieved by connecting together the two loose ends of a common overhand knot, obtaining in a knotted geometri configuration.²⁴⁰ As the most simplest knot, Trefoil ring is fundamental conformation to the study of mathematical knot theory, which has different applications in topology, physics, geometry and chemistry. Knots were first observed double-stranded DNA which naturally occurring in bacteriophage P2 DNA in 1981 by Wang and co-workers²⁴¹. In 1993, James Wang had developed a distinctive method to artificially generate the knotting of intact double-stranded DNA circles through closure of long linear DNA by the action of DNA ligase. In addition, knot structure of double-stranded DNA circles not only can be achieved by the action of type II topoisomerases such as the enzyme from bacteriophage T4²⁴² but also be produced by recombination enzymes²⁴³. These reports which introduction of assemble the knot DNA was employ a long double strand DNA as original material, whereas none knot DNA structure was constructed by short DNA linear (less than 2 k base pairs).

Toroidal DNA

Figure 4-2. Illustration of distinctive DNA structure (toroidal structure DNA).

Although several examples of single strand or double strand DNA catenated and Trefoil rings have been reported recently,^{226, 239, 244-251} the preparation of double stranded catenated and Trefoil DNA from single molecular toroidal DNA (shown in Figure 4-2) have not been reported. In this research, an unprecedented procedure for synthesizing catenated DNA in which neither gyrase nor small DNA binding molecules are required for the purpose of achieving proximity between two distant DNA segments was design. This procedure is a concise method to mimic resolvase^{252, 253} prepare catenated DNA through ingenious manipulate DNA curved structure (toroidal structure DNA) and Cre recombinase. In addition, the structure of toroidal DNA which induces by the intrinsically curved sequence was employed to produce Trefoil DNA in the presence of Human topoisomerase II. Moreover, it is shown in our investigations that the geometrical shapes of the newly obtained catenated DNA circles and Trefoil DNA ring as well as their topological arrangements are distinctly observable under atomic force microscopic examination.

4.2 Design of DNA sequences

4.2.1 Design of toroidal DNA

In order to develop the unprecedented method of assemble the catenated DNA ring and Trefoil DNA ring through achieving proximity between two distant DNA segments in the circular DNA, a distinctive conformation of DNA structure (toroidal structure DNA,

Figure 4-2) were accordingly designed and synthesized as the original substrate of the prepare processing. According to the discussion in chapter 3.2, intrinsic bent or curved DNA molecules could be generated when special base sequences which induce the local structural polymorphism are repeated in phase with the DNA helix screw.³⁶ Various specific sequences of DNA are known to be capable of existing as intrinsically bent structures^{254, 255}

On the other hand, it is well known to all that various DNA sequences that contain short adenine tracts spaced periodically along their backbones (e.g. (AAAAACGGC)_n) could exhibit intrinsically bent structures.^{37, 254, 256} On the basis of our recent analysis on the currently available information about DNA curvatures, we speculate that besides the known alteration of writhe by the actions of topoisomerases, the shapes and magnitudes of DNA writhe could be maneuvered by varying directions and degrees of intrinsic DNA curvatures. Therefore, intrinsically curved DNA was utilized to achieve the preparation of possess desired DNA shapes (toroidal forms DNA) without the assistance of gyrase, reverse gyrase and histone proteins. As shown in Figure 4-3, in which all spaced tracts of adenines occur in the same strand of its duplex structure. Furthermore, the low degree of curvature sequence was arrange in the two lateral of the design molecule whereas the high degree of curvature sequence was led in the middle which in theory lead to the formation of a backbone self-crossing (crossover structure) section in the DNA constructing. In the end, the toroidal DNA which contain a crossover structure was produced through connection the two cohesive ends of the DNA constructing by the action of the T4 DNA ligase.

Figure 4-3. Schematic representation of our synthetic routes of toroidal DNA.

4.2.2 Design of catenated DNA ring

The previous investigations²⁵⁷ revealed that relaxed forms of toroidal DNA structures could be constructed from their linear precursors through enzymatic ligation reactions. Based on recent analysis on these toroidal DNA structures²⁵⁷, we speculated that the proximity between two adjacent DNA segments occurred intrinsically in the toroidal DNA molecules could be utilized to generate catenated DNA structures through DNA strand recombination by the action of Cre recombinase. It is well known that Cre recombinase is an important enzyme to carry out complex manipulations of DNA strand exchange and recombine both in vitro and in vivo which requires no energy cofactors and Cre-mediated recombination quickly achieves equilibrium between substrate and reaction products.²⁵⁸⁻²⁶⁰ Cre recombinase is a Type I topoisomerase separate from P1 bacteriophage that catalyzes site-specific recombination of DNA between lox P sites and the reaction mechanism shows in Figure 4-4. The products of recombination depend on the location and relative orientation of the lox P sites in the target DNA sequence. Two

DNA molecules which were containing single lox P sites in its sequence will be fused the reaction. DNA between opposing lox P sites will be inverted with respect to external sequences while DNA between directly repeated lox P sites will be excised in circular form.²⁶¹⁻²⁶³

Figure 4-4. Recombination mechanism of Cre recombinase.

Generally, two directly repeated lox P sites in a circular DNA will be excised in two small circular forms DNA in the presence of Cre recombinase.²⁶⁴ Therefore, it is assumed that a toroidal circular DNA which contains two lox p sites would able to generate catenated DNA structure under the catalysis of Cre recombinase. Figure 4-5 B shows our new strategy for creating catenated rings on the basis of relaxed forms of toroidal DNA structures. The toroidal DNA (Circular DNA 1) designed in our recent studies (sequence shows in table 4-1) contained two lox p sites that are 34 base pairs in length^{259, 262, 265}.

Cre recombinase has been known to catalyze the strand recombination between these two lox p sequences in highly specific manners^{260, 262, 263, 266-270}. It is our belief that once Cre recombinase acts on Conformation 1 of our newly designed relaxed form of toroidal DNA (Figure 4-5 B), its action would lead to the generation of a catenated DNA structure because of the presence of a backbone self-crossing section in another part of the circular DNA molecules. If this toroidal DNA exists in the form of Conformation 2 (Figure 4-5 C), on the other hand, the recombinase-catalyzed reaction between two lox p sites in the DNA circle would in theory lead to the formation of two non-catenated DNA circles.

Figure 4-5. Schematic representation of DNA recombination reactions catalyzed by Cre recombinase. A: A known Cre recombinase-catalyzed recombination reaction of linear DNA. B: Our newly designed formation reaction of catenated DNA catalyzed by Cre recombinase. C: An anticipated Cre recombinase-catalyzed reaction of toroidal DNA that leads to the generation of two separate DNA rings. (↑ indicates lox p site).

Table 4-1. Sequence of new design toroidal DNA (Circular DNA 1). DNA sequence only shows one strand from 5' to 3'. Overstriking and underscore indicate the lox p site and the gray shadow indicate the high degree of curvature sequence.

```

GAGCTCAGTTGGGTAATTTTATAGGGTTTTCCAGTTTTGACGTTGTTTTTCGACGGAATCCCTTTT
TACGACTCACTTTTTGCCTTGACTAGAGGGTTTTACCCCATGGTCTATTTTTGGTCTTTTGCCATA
ACTTTTTATAGCATACATTTTACGAGTTTTATAAGCTGTTTTTGCCATAACTTTTTGCCATAACTTT
TTATAGCATACATTTTACGAGTTTTATAAGCTGTTTTTGCCATAACTTTTTATAGCATACATTTTAC
GAGTTTTATAAGCTGTTTTTCATGAGGCTTCTTTTTATAGGTTTTTGTCATGATTTTAATGGTATCT
TTTTGCCATAACTTTTTATAGCATACATTTTACGAGTTTTATAAGCTGTTTTTCATGAGGCTTCTTT
TTATAGGTTTTTGTCATGATTTTAATGGTATCTTTTTTCGTCGGTGGCATTTTTCGGGGTTTTGCGCG
GATGCCTTTTTGTTTATGGGCCTTTTTACATCAGGTTTTTCCGCTCAGCAATGATTTTTGCCCCTTT
TAGATTTTTCAATGATATTTTATAGGCGTTTTTGACGTTTTCAGTTTTTCCGTGTCGCCCTTTTTCCC
TTTTTGCGCATTTTTCGGCACTTTTTTGCATATATTTTTTGGAGTTGTTTTGATCCGTTTTGATT
TTTTCAGTGCCTTTTTGGCCATTTTGCCTAGTTTTTATAACTTCGTATAGCATACATTATACGAAGTT
ATTTTGCTATTTTTTGTTAATTTTTGCCAATTTTCGTATTTTCGCTATTTTTTGGCATTTTTTGACCA
TTTTTCTTGTTTTGCCAAGTTTTTCGGCCGTTTTTGGAGTTGAATTTTACGTCCAGATTGATTTTC
AGTGCGTTTTTAAGCTTTTTTGCAGTGTTTTTGCCTCTGCTATTTTTTGTTAATTTTTGCCAATTT
TCGAGGTATTTTCGCTATTTTTTGGCATTTTTTGCTGCATTTTTTAGTCGTTTTTGGCCATTTTTTCC
TGTTTTTGCTCACCATTTTCGCTGGTGCCGAGTTTTTGATGCTTTTTGCAGTTTTGTGCACGAGTT
TTTGACATCGGACTGGTTTTTACAGCGGTTTTTACAGGCTTTTTGCACAACATTTTAAGCTTTTTTGA
AGGAGAGAAGATTTTTGGGTCTCAGTTTTTGATACCCGACGATTTTGACACCACGATTTTGCAGGCGT
TTTGGGCTCAGTTTTTGATACCCGACGATTTTGACACCACGATTTTGCAGGCGTTTTTGAGCCATTT
TTGCGTATAGCATTTTGGATACGATGTTTTCCATGATAACTTCGTATAGCATACATTATACGAAGTT
ATTTTTGGGCTCAGTTTTTGATACCCGATCTTTTGACACCACGATTTTGCAGGCGTTTTTGAGCCA
TTTTTGCATATAGCATTTTGGATACGATGTTTTCCATGTTTTAGTGGTTTTTGACGTAGCTTTTTCGC
CATGGTAGTCATTTTTATAGCTGTTTTTGTGTGAGATTTTATCCGCTCACTTTTCGAATTCCTTTT
TACGAGCCGGATTTTTGCGGTGTGGCTTTTTGTCCGTTTTTCCTGTTT

```

4.2.3 Design of Trefoil DNA ring

Based on recent analysis on these toroidal DNA structures²⁵⁷, we speculated that the proximity between two adjacent DNA segments occurred intrinsically in the toroidal DNA molecules could be utilized to generate Trefoil DNA ring through DNA strand break and religate by the action of human topoisomerase II. It is well known that human topoisomerase II is an important enzyme which not only to carry out relax form of supercoiled DNA but also untwine the knot DNA both in vitro and in vivo which requires free energy from ATP hydrolysis to drive conformation changes used to cleave, strand-pass, and religate DNA.^{66, 271-275} The toroidal DNA structure induce the DNA strand in the same molecule become proximity which will lead to the strand contain high curvature degree sequence situated above the strand contain the low curvature degree owing to the dynamic effect in solution. It is speculated that the helix-helix juxtapositions will be generated instantaneously and then human topoisomerase II will promptly take the action of cleavage, strand-pass, and religate DNA strand which in theory conducts the formation of Trefoil ring DNA. (Figure 4-6)

Figure 4-6. Schematic representation of Trefoil ring prepare reactions catalyzed by human topoisomerase II.

4.3 Materials and methods

4.3.1 Oligodeoxyribonucleotides enzymes and chemicals

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sigma-Proligo (Singapore)
X3704E plasmid DNA	Shanghai Generay Biotech Co., Ltd
Human topoisomerase I	Topogen
Human topoisomerase II	Vaxron
100 plus DNA ladder	Fermentas
1 Kb DNA ladder	New England Biolabs
Sac I endonuclease	New England Biolabs
T4 DNA ligase	New England Biolabs
BAL 31 exonuclease	New England Biolabs
<i>Taq</i> Polymerase	New England Biolabs
Biological purity water	1st Base Pte. Ltd
Agarose	Invitrogen
Ethidium bromide	Research Biolad
Cre recombinase	New England Biolabs
TAE, TBE, TRIS	1st Base Pte. Ltd

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

Table 4-2. Sequence of X3704E plasmid DNA (4563 bp). Sequence only shows one strand from 5' to 3'. Gray shadow indicates the sequence of linear DNA which employed to prepare the toroidal DNA.

CTAAATTGTA	AGCGTTAATA	TTTTGTAA	ATTCGCGTTA	AATTTTGT	AAATCAGCTC
ATTTTTTAAC	CAATAGGCCG	AAATCGGCAA	AATCCCTTAT	AAATCAAAAG	AATAGACCGA
GATAGGGTTG	AGTGTGTTC	CAGTTTGAA	CAAGAGTCCA	CTATTAAAGA	ACGTGGACTC
CAACGTCAAA	GGGCGAAAAA	CCGTCTATCA	GGGCGATGGC	CCACTACGTG	AACCATCACC
CTAATCAAGT	TTTTTGGGGT	CGAGGTGCCG	TAAAGCACTA	AATCGGAACC	CTAAAGGGAG
CCCCCGATTT	AGAGCTTGAC	GGGGAAAGCC	GGCGAACGTG	GCGAGAAAGG	AAGGGAAGAA
AGCGAAAGGA	GCGGGCGCTA	GGGCGCTGGC	AAGTGTAGCG	GTCACGCTGC	GCGTAACCAC
CACACCCGCC	GCGCTTAATG	CGCCGCTACA	GGGCGCGTCC	CATTCGCCAT	TCAGGCTGCG
CAACTGTTGG	GAAGGGCGAT	CGGTGCGGGC	CTCTTCGCTA	TTACGCCAGC	TGGCGAAAGG
GGGATGTGCT	GCAAGGCGAT	TAAGTTGGGT	AACGCCAGGG	TTTTCCAGT	CACGACGTTG
TAAAACGACG	GCCAGTGAGC	GCGCGTAATA	CGACTCACTA	TAGGGCGAAT	TGGGTACGGC
CGTCAAGGCC	AAGCTTCCCA	CTTAGGTACC	GTGGATCCTC	GTCGAAAAC	GAGCTCAGTT
GGGTAATTTT	TAGGGTTTTT	CCAGTTTTGA	CGTTGTTTTT	CGACGGAATT	CCCTTTTTTAC
GACTCACTTT	TTGCCTTGAC	TAGAGGGTTT	TTACCCCATG	GTCTATTTTT	GGTCTTTTGC
CATAACTTTT	TATAGCATAC	ATTTTACGAG	TTTTATAAGC	TGTTTTTGCC	ATAACTTTTT
GCCATAACTT	TTTATAGCAT	ACATTTTACG	AGTTTTATAA	GCTGTTTTTG	CCATAACTTT
TTATAGCATA	CATTTTACGA	GTTTTATAAG	CTGTTTTTCA	TGAGGCTTCT	TTTTATAGGT
TTTTGTCATG	ATTTTAATGG	TATCTTTTTT	CCATAACTTT	TTATAGCATA	CATTTTACGA
GTTTTATAAG	CTGTTTTTCA	TGAGGCTTCT	TTTTATAGGT	TTTTGTCATG	ATTTTAATGG
TATCTTTTTT	GTCGGTGGCA	TTTTTCGGGG	TTTTGCGCGG	ATGCCTTTTT	GTTTATGGGC
CTTTTTTACAT	CAGGTTTTTT	CCGCTCAGCA	ATGATTTTTT	CCCTTTTAGA	TTTTTCAATG
ATATTTTTTAG	GCGTTTTTGA	CGTTTTTCAGT	TTTTCCGTGT	CGCCCTTTTT	CCCTTTTTTTG
CGCATTTTTT	CGGCACTTTT	TTGCATATAT	TTTTTGAGAGT	TGTTTTGATC	CGTTTTGATT
TTCAGTGCGT	TTTTGGCCAT	TTTGCTAGT	TTTTTATAAC	TTCGTATAGC	ATACATTATA
CGAAGTTATT	TGCTATTTTT	TGTTAATTTT	TGCCAATTTT	CGTATTTTCG	CTATTTTTTTG
GCATTTTTTG	ACCATTTTTT	TTGTTTTGCC	AAGTTTTTCGG	CCGTTTTTTG	GAGTTGAATT
TTACGTCCAG	ATTTGATTTT	CAGTGCGTTT	TTAAGCTTTT	TTGCAGTGTT	TTTTGCCTCT
GCTATTTTTT	GTTAATTTTT	GCCAATTTT	GAGGTATTTT	CGCTATTTTT	TGGCATTTTT
TGCTGCATTT	TTTAGTCGTT	TTTGCCATT	TTTCCTGTTT	TTGCTACCC	ATTTTCGCTG
GTGCCGAGTT	TTTGATGCTT	TTTGCAGTTT	TGTGCACGAG	TTTTTGACAT	CGGACTGGTT
TTACAGCGG	TTTTCAGGCT	TTTTGCACAA	CATTTTTTAAG	CTTTTTTGAA	GGAGAGAAGA
TTTTGGGTCT	CAGTTTTTGAT	ACCCGACGAT	TTTGACACCA	CGATTTTTTG	AGGCGTTTTG
GGCTCAGTTT	TGATACCCGA	CGATTTTGAC	ACCACGATTT	TTGCAGGCGT	TTTTGAGCCA
TTTTTGCGTA	TAGCATTTTT	GATACGATGT	TTTCCATGAT	AACTTCGTAT	AGCATACATT
ATACGAAGTT	ATTTTTGGGC	TCAGTTTTGA	TACCCGATC	TTTTGACACC	ACGATTTTTG
CAGGCGTTTT	TGAGCCATTT	TTGCGTATAG	CATTTTGAT	ACGATGTTTT	CCATGTTTTA
GTGGTTTTTG	ACGTAGCTTT	TCGCCATGGT	AGTCATTTTT	ATAGCTGTTT	TTGTGTGAGA
TTTTTATCCG	CTCACTTTTT	GAATTCCTTT	TTACGAGCCG	GATTTTTGCG	GTGTGGCTTT
TTGTCCGTTT	TCCTGTTTGA	GCTCGGGGTT	AACCATGGAT	CCGGTCTAGA	TAAGTGGGAT
ATCACGTGAA	GCTTGCAAGC	TCCAGCTTTT	GTTCCCTTTA	GTGAGGGTTA	ATTGCGCGCT
TGGCGTAATC	ATGGTCATAG	CTGTTTCCTG	TGTGAAATTG	TTATCCGCTC	ACAATTCCAC

ACAACATACG	AGCCGGAAGC	ATAAAGTGTA	AAGCCTGGGG	TGCCTAATGA	GTGAGCTAAC
TCACATTAAT	TGCGTTGCGC	TCCTGCCCCG	CTTTCCAGTC	GGGAAACCTG	TCGTGCCAGC
TGCATTAATG	AATCGGCCAA	CGCGCGGGGA	GAGGCGGTTT	GCGTATTGGG	CGCTCTTCCG
CTTCCTCGCT	CACTGACTCG	CTGCGCTCGG	TCGTTTCGGCT	GCGGCGAGCG	GTATCAGCTC
ACTCAAAGGC	GGTAATACGG	TTATCCACAG	AATCAGGGGA	TAACGCAGGA	AAGAACATGT
GAGCAAAAGG	CCAGCAAAAG	GCCAGGAACC	GTAAAAAGGC	CGCGTTGCTG	GCGTTTTTCC
ATAGGCTCCG	CCCCCCTGAC	GAGCATCACA	AAAATCGACG	CTCAAGTCAG	AGGTGGCGAA
ACCCGACAGG	ACTATAAAGA	TACCAGGCGT	TTCCCCCTGG	AAGCTCCCTC	GTGCGCTCTC
CTGTTCCGAC	CCTGCCGCTT	ACCGGATACC	TGTCCGCTT	TCTCCCTTCG	GGAAGCGTGG
CGCTTTCTCA	TAGCTCACGC	TGTAGGTATC	TCAGTTCGGT	GTAGGTCGTT	CGCTCCAAGC
TGGGCTGTGT	GCACGAACCC	CCCGTTCAGC	CCGACCGCTG	CGCCTTATCC	GGTAACTATC
GTCTTGAGTC	CAACCCGGTA	AGACACGACT	TATCGCCACT	GGCAGCAGCC	ACTGGTAACA
GGATTAGCAG	AGCGAGGTAT	GTAGGCGGTG	CTACAGAGTT	CTTGAAGTGG	TGGCCTAACT
ACGGCTACAC	TAGAAGGACA	GTATTTGGTA	TCTGCGCTCT	GCTGAAGCCA	GTTACCTTCG
GAAAAAGAGT	TGGTAGCTCT	TGATCCGGCA	AACAAACCAC	CGCTGGTAGC	GGTGGTTTTT
TTGTTTGCAA	GCAGCAGATT	ACGCGCAGAA	AAAAAGGATC	TCAAGAAGAT	CCTTTGATCT
TTTCTACGGG	GTCTGACGCT	CAGTGAACG	AAAACCTCAG	TTAAGGGATT	TTGGTCATGA
GATTATCAAA	AAGGATCTTC	ACCTAGATCC	TTTTAAATTA	AAAATGAAGT	TTTAAATCAA
TCTAAAGTAT	ATATGAGTAA	ACTTGGTCTG	ACAGTTACCA	ATGCTTAATC	AGTGAGGCAC
CTATCTCAGC	GATCTGTCTA	TTTCGTTTCT	CCATAGTTGC	CTGACTCCCC	GTCGTGTAGA
TAACTACGAT	ACGGGAGGGC	TTACCATCTG	GCCCCAGTGC	TGCAATGATA	CCGCGAGATC
CACGCTCACC	GGCTCCAGAT	TTATCAGCAA	TAAACCAGCC	AGCCGGAAGG	GCCGAGCGCA
GAAGTGGTCC	TGCAACTTTA	TCCGCCTCCA	TCCAGTCTAT	TAATTGTTGC	CGGGAAGCTA
GAGTAAGTAG	TTCGCCAGTT	AATAGTTTGC	GCAACGTTGT	TGCCATTGCT	ACAGGCATCG
TGGTGTACAG	CTCGTCGTTT	GGTATGGCTT	CATTGAGCTC	CGGTTCCTCA	CGATCAAGGC
GAGTTACATG	ATCCCCCATG	TTGTGCAAAA	AAGCGGTTAG	CTCCTTCGGT	CCTCCGATCG
TTGTCAGAAG	TAAAGTTGGC	GCAGTGTTAT	CACTCATGGT	TATGGCAGCA	CTGCATAATT
CTCTTACTGT	CATGCCATCC	GTAAGATGCT	TTTCTGTGAC	TGGTGAGTAC	TCAACCAAGT
CATTCTGAGA	ATAGTGTATG	CGGCGACCGA	GTTGCTCTTG	CCCGGCGTCA	ATACGGGATA
ATACCGCGCC	ACATAGCAGA	ACTTTAAAAG	TGCTCATCAT	TGGAAAACGT	TCTTCGGGGC
GAAAACCTCT	AAGGATCTTA	CCGCTGTTGA	GATCCAGTTC	GATGTAACCC	ACTCGTGCAC
CCAACCTGAT	TTTACGATCT	TTTACTTTCA	CCAGCGTTTC	TGGGTGAGCA	AAAACAGGAA
GGCAAAATGC	CGCAAAAAG	GGAATAAGGG	CGACACGGAA	ATGTTGAATA	CTCATACTCT
TCCTTTTTCA	ATATTATTGA	AGCATTTATC	AGGGTTATTG	TCTCATGAGC	GGATACATAT
TTGAATGTAT	TTAGAAAAAT	AAACAAATAG	GGGTTCCGCG	CACATTTCCC	CGAAAAGTGC
CAC					

4.3.2 Preparation of toroidal DNA (Circular DNA 1)

Figure 4-7. The synthesis of relaxed form of toroidal DNA that contains two lox p sites. A: Pictorial illustration of our synthetic route toward Circular DNA 1. B: Illustration of the location of two lox p sites and Sac I site in the toroidal DNA.

According to the design described above, a toroidal DNA (Circular DNA 1), 1588 base pairs in length, contain two lox p sites was synthesized in this section. The synthesized route of toroidal DNA step by step is shown in Figure 4-7. A linear DNA (Linear DNA 1) that possess two lox p sites as well as a X3704E plasmid that contains sequence of Linear DNA 1 were accordingly constructed during this investigations. Subsequent selective PCR amplification^{276, 277} on X3704E plasmid was conducted, which led to the generation of Linear DNA 1 (1634 bp in length, Lane 2 in Figure 4-8). This

linear DNA was next incubated with Sac I, which gave rise to a cohesive end-containing Linear DNA 2 (1588 base pairs in length, Lane 3 in Figure 4-8). When Linear DNA 2 was allowed to react with T4 DNA ligase, a Circular DNA 1 (1588 base pairs in length, Lane 4 in Figure 4-8) was produced (Step 2 in Figure 4-7 A). In order to verify that the produced DNA is indeed circular in its backbone, nuclease BAL-31 hydrolysis was carried out subsequently²⁷⁸. As shown in Lane 5 Figure 4-8, the DNA in Band 1 resisted nuclease BAL-31 hydrolysis, which is the indication that the ligation product possessed no open end in its structure.

A reaction mixture containing 1 ng X3704E plasmid DNA, 0.25 μ M forward primer (5' GTGGATCCTCGTCGCAAAAC 3'), 0.25 μ M reverse primer (5' CCGGATCCATGGTTAACCCC 3'), 200 μ M each dNTP, 1 U *Taq* polymerase in a total volume of 50 μ l reaction buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8 @ 25 °C) was processed as below protocol:

95 °C for 180 sec (denature);

56 °C for 40 sec (anneal);

72 °C for 40 sec (elongate) (60 sec per kb target sequence length);

29 cycles only (otherwise enzyme decay causes artifacts);

72 °C for 20 min at end to allow complete elongation of all product DNA

The mixture containing PCR product, 5 U of Sac I, 10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol was incubated 37 °C for 2 hours to produce the

double strand DNA contains two cohesive end. The resultant cohesive end-containing DNA was purified using the QIAGEN PCR Purification Kits. The mixture containing resultant cohesive end-containing DNA, 60 mM Tris-HCl (pH 7.6), 25 mM NaCl, 13 mM MgCl₂, 10 mM DTT, 1mM ATP, 25 mg/ml BSA and 10 U of T4 DNA ligase was incubated at 16 °C for 16 hours. The resultant mixture was further incubated with BAL-31 at 30 °C for 2 hours, which were then analyzed using 2% agarose gel electrophoresis. The DNA product that is corresponding to Band 1 in Lane 5 of Figure 4-8 was purified next using Mini Prep Cell (Bio-rad) and QIAquick Purification Kits (Qiagen) before AFM examination. Figure 4-8 shows the process of prepare toroidal DNA.

Figure 4-8. Preparation of toroidal DNA. Lane 1: 100 plus DNA ladder; Lane 2: PCR product (DNA with two Sac I digest site in each end) alone; Lane 3: Sac I digest product (cohesive end-containing DNA) alone; Lane 4: the mixture of lane 3 treated with T4 DNA ligase; Lane 5: exonuclease BAL 31 hydrolysis product on reaction mixture of lane 4. The analysis was done on a 2% agarose gel contain ethidium bromide.

4.3.3 Confirmation of the relax form of toroidal DNA by human topoisomerase I

A mixture containing 200 ng toroidal DNA (Circular DNA 1), 1 U of Human Topoisomerase I, 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine and 5% glycerol was incubated at 37 °C for 1 hour followed by analysis using 2% agarose gel electrophoresis and stained by ethidium bromide.

4.3.4 Preparation of catenated DNA

A mixture containing 200 ng toroidal DNA, 50 mM Tris-HCl, 33 mM NaCl, 10 mM MgCl₂ and 1 U of Cre recombinase was incubated at 37 °C for 12 hours. The resultant reaction mixture was further analyzed using 2.5% agarose gel electrophoresis (contain Ethidium bromide). The band of catenated DNA was purified next using Mini Prep Cell (Bio-rad) and QIAquick Purification Kits (Qiagen) before AFM examination.

4.3.5 Preparaton of Trefoil ring DNA

A mixture containing 200 ng toroidal DNA, 0.5 M Tris-HCl, pH 8.0, 10 mM DTT, 0.1 M MgCl₂, 20 mM ATP, 1 M NaCl and 1 U of human topoisomerase II was incubated at 37 °C for 1 hour. The resultant reaction mixture was further analyzed using 2.5%

agarose gel electrophoresis (contain Ethidium bromide).

4.3.5 AFM studies of the synthesized DNA

(a) Immobilization of DNA samples on micas^{153, 159, 160}: A buffer (PH 7.0) containing 40 mM HEPES and 10 mM MgCl₂ was placed on freshly cleaved mica, which was further kept at room temperature for 5 min. The pre-prepared mica was rinsed with dd water thoroughly and dried under the argon flow. A 20 μ L solution that contains DNA sample was deposited on the mica, which was subsequently incubated for 5 min, washed with H₂O, dried under the argon flow and then stored in vacuum cabinet. *(b) AFM examination*: AFM images of DNA were obtained in Tapping ModeTM on a MultimodeTM AFM (Veeco, Santa Barbara, CA) in connection with a Nanoscope VTM controller. Antimony (n) doped Si cantilevers with nominal spring constants between 20 and 80 N/m were selected. Scan frequency was 1.9 Hz per line and the modulation amplitude was in a nanometer range^{162, 279}. All DNA sample determinations were carried out in air at room temperature.

4.4 Results and discussion

4.4.1 Analysis of the toroidal DNA

It is apparent from the design described above, a toroidal structure DNA without supercoiled need to be generated. With the purpose of confirming that the synthesized

circular DNA holds a crossover (toroidal structure), examination of atomic force microscopy (AFM) was carried out on Circular DNA 1. Owing to the DNA is directly observed by AFM, the benefit of this manner is that the conformation of the molecule is more easily to survey than it is from other methods. As shown in Figure 4-9, this circular DNA exhibit geometri configuration of toroidal shape as expected. This founding is exactly consist with our speculation of construct toroidal structure DNA through manipulate intrinsic curvature of DNA sequence.

Figure 4-9. Atomic force microscopic images of Circular DNA 1 (toroidal DNA, 500 nm x 500 nm scans).

In addition, with the aim of further verifying that the self-crossing section in the backbone of toroidal structure DNA (Circular DNA 1) was not associated with supercoiling, human Topoisomerase I was allowed to react with this circular DNA. It is well know that human topoisomerase I is a important enzyme which not only to carry out relax form of positive supercoiled DNA but also take the action in negative

supercoiled DNA both in vitro and in vivo which no requires free energy to drive conformation changes used to nick and religate DNA. As shown in Figure 4-10, the self-crossing section still remained after human Topoisomerase I's action on Circular DNA 1, which is the sign that the toroidal structure of Circular DNA 1 was not associated with positive or negative supercoiling.⁶⁶ It is indicated that the relax form of toroidal DNA is consist with the designed and qualify for the next process step of prepare the catenated ring DNA.

Figure 4-10. Electrophoretic analysis of Circular DNA 1 acted on by human Topoisomerase I. Lane 1: Circular DNA 1 alone; Lane 2: reaction mixture of Circular DNA 1 and human Topoisomerase I.

4.4.2 Formation of catenated ring by Cre-recombinase

According to the design described above, a toroidal DNA (Circular DNA 1, 1588 base pairs in length) contains two lox p sites was employed to generate the catenated DNA by the action of Cre recombinase. Base on the design of the location of two lox p

sites in the sequence which laid one lox p site from 700 nucleotide and another one placed from 1303 nucleotide (shown in Figure 4-7 B) and the mechanism of Cre recombinase (shown in Figure 4-5), the catenated DNA that was interlocked by a 985 bp circular DNA and a 603 bp circular DNA was precise produced in the recombination. As shown in Figure 4-11, the catenated DNA would contain two different size “sub-DNA ring”.

Figure 4-11. Picturtic illustration of generated catenated DNA from toroidal DNA by the action of Cre recombinase.

In theory, the relaxed forms of toroidal DNA structure (Circular DNA 1) should exist as a mixture of Conformation 1 and Conformation 2 (Figure 4-5 B and Figure 4-5 C) respectively in the preparation. If the new phosphoester bond between Point 3 and Point 6 is formed on top of the bond formed between Point 2 and Point 7 in space, the Cre recombinase-catalyzed reactions of Conformation 1 and Conformation 2 will give rise to catenated structure (Figure 4-5 B) and two non-catenated rings respectively (Figure 4-5 C).

Figure 4-12. Electrophoretic analysis of reaction mixtures of Circular DNA 1 and Cre recombinase. Lane 1: molecular weight markers; Lane 2: Circular DNA 1 alone; Lane 3: reaction mixture of Circular DNA 1 and Cre recombinase.

In order to examine whether catenated rings DNA structure could indeed be generated as expected, toroidal structure DNA (Circular DNA 1) was allowed to react with Cre recombinase. As shown in Figure 4-12, several bands were observed in the Cre-recombinase-catalyzed reaction (Lane 3 in Figure 4-12). Atomic force microscopy (AFM), on the other hand, has been known to be a powerful tool for determining certain subtle alternations in DNA topological features. The DNA product in Band 2 in Lane 3 of Figure 4-12 was accordingly purified during our investigations, which was further examined using AFM^{236, 280-283}. As shown in Figure 4-13, the structures in which two

DNA rings are associated with each other are clearly visible in the AFM images of the DNA. More importantly, it can be seen from these AFM images that the backbone of Catenated Ring 1 is positioned above that of Catenated Ring 2 in one location while in another location, the backbone of Catenated Ring 2 is laid above that of Catenated Ring 1 (Figure 4-14).

Figure 4-13. AFM images of DNA that are corresponding to Band 2 in Lane 3 in Figure 4-12 (1 μm x 1 μm scans).

Figure 4-14. Analysis of the atomic force microscopic picture of catenated ring DNA. A:

Magnification of amplitude graphic of catenated DNA that shows visible crossover sections between the catenated DNA molecules (200 nm x 200 nm scans). B, C and D: Topographic analysis of catenated DNA (C is Topographic analysis of the point in the left of B and D is Topographic analysis of the point in the right of B).

Furthermore, according to our initial design, once they are formed, Catenated Ring 1 and Catenated Ring 2 would contain 603 bp and 985 bp in lengths respectively (Figure 4-5 B). As shown in Figure 4-14 A and Figure 4-14 B, the size differences between the two catenated rings (Catenated Ring 1 and Catenated Ring 2) are clearly identifiable in their AFM images. This observation is evident that the DNA ring size in a catenated structure could be precisely manipulated using the methods developed in our current investigations. In addition, the DNA in Band 3 and Band 4 in Figure 4-12 were purified and examined using AFM as well. It appears that the DNA in the two bands are Non-Catenated Ring 1 and Non-Catenated Ring 2 respectively, which were presumably formed through the pathway shown in Figure 4-5 C (AFM images in Figure 4-15).

Figure 4-15. AFM images of Non-Catenated Ring 1 and Non-Catenated Ring 2. A: AFM image of Non-Catenated Ring 1 (603 bp in length) that are corresponding to Band 4 in

Lane 3 in Figure 3A (1 μm x 1 μm scans). B: AFM image of Non-Catenated Ring 2 (985 bp in length) that are corresponding to Band 3 in Lane 3 in Figure 4-12 (1 μm x 1 μm scans).

It might be worth to point out, on the other hand, that under a standard reaction condition, Cre recombinase is only capable of producing desired recombination products in a modest yield of 20%^{261, 284}. In addition, as illustrated in Figure 4-5, only one of the two conformers of toroidal DNA structures could give rise to catenated DNA structures. The intrinsic low catalytic efficiency of Cre recombinase as well as the presence of alternative conformations of toroidal DNA could account for the relatively low yield (8%) of the desired catenated DNA rings in our studies (Band 2, in Lane 3 in Figure 4-12).

As a comparison, a new toroidal structure of DNA was constructed (Circular DNA 2) next during our investigations, which is the same as Circular DNA 1 except for that the two lox p sites in the new circular DNA were aligned in an anti parallel fashion (as shown in Figure 4-16). According to the method of prepare Circular DNA 1 described above, Circular DNA 2 was produced in the same processing (Figure 4-17 show the result of prepare Circular DNA 2). After the preparation, AFM was employed to determine the structure of the product whether consistent with toroidal structure. As shown in Figure 4-18, the toroidal structure of Circular DNA 2 was captured as well as by AFM. This finding is exactly match the design as our expectation. Circular DNA 2 was allowed to process recombination by the action of Cre recombinase (Figure 4-19), and then the

corresponding reaction mixtures were analyzed and it turned out that none of the products contains catenated DNA ring in the reaction products. This happened because the recombination of Circular DNA 2 catalyzed by Cre recombinase does not cause entangling of DNA strands according to the mechanism of the enzyme (Figure 4-16).

Figure 4-16. Schematic illustration of strand recombination of toroidal DNA in which two lox p sites are aligned in an anti-parallel fashion. This Cre recombinase-catalyzed reaction does not lead to any catenated DNA structure. (↑ indicates the lox p site)

Figure 4-17. Preparation of Circular DNA 2. Lane 1: 100 plus DNA ladder; Lane 2: PCR product (DNA with two Sac I digest site in each end) alone; Lane 3: Sac I digest product (cohesive end-containing DNA) alone; Lane 4: the mixture of lane 3 treated with T4 DNA ligase; Lane 5: exonuclease BAl 31 hydrolysis product on reaction mixture of lane 4. The analysis was done on a 2% agarose gel contain ethidium bromide.

Figure 4-18. Atomic force microscopy images of Circular DNA 2 (toroidal DNA, 500 nm x 500 nm scans).

Figure 4-19. Electrophoretic analysis of reactions between Circular DNA 2 and Cre recombinase. Lane 1: molecular weight markers; Lane 2: Circular DNA 2 alone; Lane 3: reaction mixture of Circular DNA 2 and Cre recombinase.

4.4.3 Formation of Trefoil ring by human topoisomerase II

According to the design described above, a toroidal DNA (Circular DNA 1, 1588 base pairs in length) was employed to generate the Trefoil ring DNA by the action of human topoisomerase II. The helix-helix juxtaposition will generated instantaneous owing to the toroidal structure which drive the DNA segments of same molecule close and the dynamic effect in solution. Human topoisomerase II would promptly take the action of cleave, strand-pass, and religate in the juxtaposition which in theory induce the formation of Trefoil ring DNA (as shown as Figure 4-6).

Circular DNA 1 was allowed to process reaction by the action of Human topoisomerase II, and then the corresponding reaction mixtures were analyzed by AFM and it turned out that the products contains Trefoil ring DNA in the reaction products. As shown in Figure 4-20 B, the Trefoil structures in which DNA rings are associated with three juxtapositions (crossover) are clearly visible in the AFM image of the DNA. More importantly, it can be seen from these AFM image that the backbone of DNA segment is positioned above that of other segment in same DNA molecule as well as the computer mimic picture of Trefoil ring (Figure 4-20 A) by software “knotpolt”. This founding is indeed consistent with our expectation that Trefoil could be constructed by the action of human topoisomerase II on the toroidal structure DNA.

Figure 4-20. Picture of Trefoil ring structure and atomic force microscopic images of Trefoil ring DNA structure (300 nm x 300 nm scans). A: Picture of Trefoil ring calculated by “knotpolt”; B: Picture of Trefoil ring captured by AFM.

Unfortunately, on the other hand, band of Trefoil structure DNA could not be found in the agarose gel electrophoretic analysis of the reaction mixture (Figure 4-21). As shown in Figure 4-21, lane 2 was used as a control which consists of only the toroidal structure DNA (Circular DNA 1). The reaction of DNA strands entangle form toroidal structure DNA (Circular DNA 1) by different concentration of human topoisomerase II was processed in Lane 3, Lane 4 and Lane 5. With the increase of concentrations of human topoisomerase II, the result of the reaction without manifestly change as shown in Lane 3 to Lane 5 was discovered. This happened because the human topoisomerase II not only could knot the DNA strand but also unknot it according to the mechanism of the enzyme. In addition, the substrate contains three juxtapositions for the enzyme take action when the formation of Trefoil ring DNA was achieved. The target DNA structure would recover to the starting material on the condition that even if each one of the three

juxtapositions was changed. Furthermore, the enzyme trend to unknot the DNA strand when it take the action on the DNA juxtaposition (crossovre).^{65, 68, 272} With the purpose of exclude the possibility of produce the Trefoil ring DNA in the process of ligate, on the other hand, band 2 and band 3 in Figure 4-21 were be purified and further determined by AFM. As shown in Figure 4-22, band 2 was linear DNA and band 3 was the nick Circular DNA 1 which maybe both generate induced by DNA strand broke in the reaction process. Furthermore, the previously research has demonstrated that knotting of a DNA chain during ring closure could be obtained in which under the condition of not only high salt concentration but also substrate linear DNA was over 5 kilo base pairs in length.²⁵¹

Figure 4-21. Electrophoretic analysis of reaction mixtures of Circular DNA 2 and human topoisomerase II. Lane 1: molecular weight markers; Lane 2: Circular DNA 2 alone; Lane 3: reaction mixture of Circular DNA 2 and 1 U human topoisomerase II; Lane 4: reaction mixture of Circular DNA 2 and 2 U human topoisomerase II; Lane 5: reaction mixture of Circular DNA 2 and 3 U human topoisomerase II.

Figure 4-22. AFM images of band 2 and band 3 in Figure 4-21. A: AFM image of band 2 in Figure 4-21 (900 nm x 900 nm scans). B: AFM image of band 3 in Figure 4-21 (900 nm x 900 nm scans).

4.5 Conclusion

On the basis of our recent analysis of the currently available information about intrinsic curvature of DNA structure with various DNA sequences that contain short adenine tracts spaced periodically along their backbones (e.g. (AAAAACGGC)_n) could exhibit intrinsically bent structures, and the intrinsically curved DNA was employed to achieve the preparation of desired DNA shapes (toroidal forms DNA). Human topoisomerase I was used to determine the designed toroidal structure DNA without positive and negative supercoils.

According to our studies on the toroidal DNA structures, we demonstrated that the

proximity between two adjacent DNA segments and self-crossing intrinsically occurring in the relaxed forms of toroidal DNA were utilized for creating catenated DNA structures. An unprecedented method of assembling the catenated DNA ring through toroidal structure DNA containing two lox p sites by the action of Cre recombinase was employed for the first time in our investigations. Furthermore, our outcomes indicated that the DNA ring size in a catenated structure could be precisely manipulated using the methods developed in current studies. In addition, our current results show that the newly obtained catenated DNA structures are clearly verifiable using atomic force microscopy. It is our hope that the information provided in the currently report could be useful for constructing new catenated DNA structures with desired sizes and sequences in the future.

On the other hand, Trefoil ring DNA, the simplest knot structure DNA, was produced by the relaxed form of toroidal structure DNA and human topoisomerase II in our studies. The juxtaposition was instantaneously generated by the proximity of two strand segments which was induced by torodial structure DNA. Human topoisomerase II promptly took the action of cleave, strand-pass, and religated in the juxtaposition which in theory induce the formation of Trefoil ring DNA. Furthermore, Trefoil DNA structures were distinctly and clearly observed through atomic force microscopic examination. Even if the product was generated in a very modest yield, this method was still a potential way to produce the small Trefoil ring DNA (less than two kilo base pairs) base on the toroidal structure DNA. It is our hope that the manner provided in the current investigation could be further improved and developed to a useful process for constructing Trefoil ring DNA

in the future.

In summary, an ingenious way of design and constructing catenated ring DNA and Trefoil ring DNA based on the toroidal structure DNA was demonstrated during our investigation. This method will contribute to a better comprehension of the unique role of this delicate architecture of DNA structure not only in biological process but also in the application of DNA nanotechnology.

References

1. R. Dahm, *Hum Genet*, 2008, **122**, 565-581.
2. R. Dahm, *Dev Biol*, 2005, **278**, 274-288.
3. R. Dahm, *Scientist*, 2008, **22**, 84-84.
4. O. T. Avery, C. M. MacLeod and M. McCarty, *Journal of Experimental Medicine*, 1944, **79**, 137-158.
5. M. McCarty, *Journal of Experimental Medicine*, 1994, **179**, 385-394.
6. M. McCarty, *Nature*, 2003, **421**, 406-406.
7. S. K. Silverman, *Angew Chem Int Edit*, 2010, **49**, 7180-7201.
8. J. D. Watson and F. H. C. Crick, *Nature*, 1953, **171**, 737-738.
9. M. H. Wilkins, A. R. Stokes and H. R. Wilson, *Nature*, 1953, **171**, 738-740.
10. R. E. Franklin and R. G. Gosling, *Nature*, 1953, **171**, 740-741.
11. R. R. Sinden, *DNA structure and function*, Academic Press, San Diego, 1994.
12. T. G. Wolfsberg, J. McEntyre and G. D. Schuler, *Nature*, 2001, **409**, 824-826.
13. J. D. Watson and F. H. C. Crick, *Nature*, 1953, **171**, 964-967.
14. A. D. Bates and A. Maxwell, *DNA topology*, Oxford University Press, Oxford ; New York, 2005.
15. R. E. Dickerson, *Nucleic Acids Research*, 1989, **17**, 1797-1803.
16. M. H. Wilkins, W. E. Seeds, A. R. Stokes and H. R. Wilson, *Nature*, 1953, **172**, 759-762.
17. S. Arnott, *Nature*, 1984, **312**, 174-174.
18. S. A. Shah and A. T. Brunger, *Journal of Molecular Biology*, 1999, **285**, 1577-1588.
19. R. E. Dickerson, *J Biomol Struct Dyn*, 1989, **6**, 627-634.
20. R. E. Franklin and R. G. Gosling, *Nature*, 1953, **171**, 740-741.
21. A. H. J. Wang, G. J. Quigley, F. J. Kolpak, J. L. Crawford, J. H. Vanboom, G. Vandermarel and A. Rich, *Nature*, 1979, **282**, 680-686.
22. A. I. Murchie and D. M. Lilley, *Methods Enzymol*, 1992, **211**, 158-180.
23. A. Dayn, S. Malkhosyan and S. M. Mirkin, *Nucleic Acids Res*, 1992, **20**, 5991-5997.
24. C. C. Hentschel, *Nature*, 1982, **295**, 714-716.
25. M. D. Frank-Kamenetskii and S. M. Mirkin, *Annu Rev Biochem*, 1995, **64**, 65-95.
26. D. Rhodes and R. Giraldo, *Curr Opin Struct Biol*, 1995, **5**, 311-322.
27. D. M. Gowers and K. R. Fox, *Nucleic Acids Res*, 1999, **27**, 1569-1577.
28. D. E. Gilbert and J. Feigon, *Curr Opin Struct Biol*, 1999, **9**, 305-314.
29. S. Neidle and G. N. Parkinson, *Curr Opin Struct Biol*, 2003, **13**, 275-283.
30. A. D. Bates and A. Maxwell, *Curr Biol*, 1997, **7**, R778-R781.
31. Z. Yang, D. Li, S. Hiew, M. T. Ng, W. Yuan, H. Su, F. Shao and T. Li, *Chem Commun (Camb)*, 2011.
32. J. C. Marini, S. D. Levene, D. M. Crothers and P. T. Englund, *P Natl Acad Sci-Biol*, 1982, **79**, 7664-7668.
33. J. C. Marini, S. D. Levene, D. M. Crothers and P. T. Englund, *Cold Spring Harb Sym*, 1982, **47**, 279-283.
34. P. Borst and J. H. J. Hoeijmakers, *Plasmid*, 1979, **2**, 20-40.
35. P. Borst, J. H. J. Hoeijmakers and S. L. Hajduk, *Parasitology*, 1981, **82**, 81-&.
36. H. M. Wu and D. M. Crothers, *Nature*, 1984, **308**, 509-513.
37. H. S. Koo, H. M. Wu and D. M. Crothers, *Nature*, 1986, **320**, 501-506.
38. Y. Lu, E. Stellwagen and N. C. Stellwagen, *Electrophoresis*, 2006, **27**, 1462-1470.
39. L. Ulanovsky, M. Bodner, E. N. Trifonov and M. Choder, *Proc Natl Acad Sci U S A*, 1986, **83**, 862-866.

40. L. E. Ulanovsky and E. N. Trifonov, *Nature*, 1987, **326**, 720-722.
41. H. S. Koo and D. M. Crothers, *P Natl Acad Sci USA*, 1988, **85**, 1763-1767.
42. J. G. Nadeau and D. M. Crothers, *P Natl Acad Sci USA*, 1989, **86**, 2622-2626.
43. A. M. Burkhoff and T. D. Tullius, *Nature*, 1988, **331**, 455-457.
44. E. N. Trifonov and J. L. Sussman, *P Natl Acad Sci-Biol*, 1980, **77**, 3816-3820.
45. D. M. Crothers, T. E. Haran and J. G. Nadeau, *Journal of Biological Chemistry*, 1990, **265**, 7093-7096.
46. E. Selsing, R. D. Wells, C. J. Alden and S. Arnott, *Journal of Biological Chemistry*, 1979, **254**, 5417-5422.
47. Q. Du, A. Kotlyar and A. Vologodskii, *Nucleic Acids Res*, 2008, **36**, 1120-1128.
48. F. Lankas, R. Lavery and J. H. Maddocks, *Structure*, 2006, **14**, 1527-1534.
49. W. R. Bauer, F. H. C. Crick and J. H. White, *Sci Am*, 1980, **243**, 118-&.
50. W. Saenger, *Principles of nucleic acid structure*, Springer-Verlag, New York, 1984.
51. S. Neidle, *Principles of nucleic acid structure*, Elsevier ; Academic Press, Amsterdam Boston, 2008.
52. A. Falaschi, G. Abdurashidova, O. Sandoval, S. Radulescu, G. Biamonti and S. Riva, *Cell Cycle*, 2007, **6**, 1705-1712.
53. J. L. Nitiss, *Bba-Gene Struct Expr*, 1998, **1400**, 63-81.
54. J. H. White and N. R. Cozzarelli, *P Natl Acad Sci-Biol*, 1984, **81**, 3322-3326.
55. A. V. Vologodskii and N. R. Cozzarelli, *Annu Rev Bioph Biom*, 1994, **23**, 609-643.
56. J. E. Deweese, M. A. Osherooff and N. Osherooff, *Biochem Mol Biol Edu*, 2009, **37**, 2-10.
57. T. C. Boles, J. H. White and N. R. Cozzarelli, *Journal of Molecular Biology*, 1990, **213**, 931-951.
58. C. J. Benham and S. P. Mielke, *Annu Rev Biomed Eng*, 2005, **7**, 21-53.
59. O. Espeli and K. J. Marians, *Molecular Microbiology*, 2004, **52**, 925-931.
60. A. Travers and G. Muskhelishvili, *Embo Rep*, 2007, **8**, 147-151.
61. J. H. White, R. M. Gallo and W. R. Bauer, *Trends Biochem Sci*, 1992, **17**, 7-12.
62. P. Forterre and D. Gadelle, *Nucleic Acids Research*, 2009, **37**, 679-692.
63. N. M. Baker, R. Rajan and A. Mondragon, *Nucleic Acids Research*, 2009, **37**, 693-701.
64. T. Maxwell and A. Bates, *Nucleic Acids Research*, 2009, **37**, 659-660.
65. J. M. Fogg, D. J. Catanese, G. L. Randall, M. C. Swick and L. Zechiedrich, *Ima V Math*, 2009, **150**, 73-121.
66. J. J. Champoux, *Annu Rev Biochem*, 2001, **70**, 369-413.
67. J. B. Leppard and J. J. Champoux, *Chromosoma*, 2005, **114**, 75-85.
68. J. M. Berger and A. J. Schoeffler, *Q Rev Biophys*, 2008, **41**, 41-101.
69. J. C. Wang, *Journal of Molecular Biology*, 1971, **55**, 523-&.
70. M. Gellert, K. Mizuuchi, M. H. Odea and H. A. Nash, *P Natl Acad Sci USA*, 1976, **73**, 3872-3876.
71. M. Gellert, M. H. Odea, T. Itoh and J. Tomizawa, *P Natl Acad Sci USA*, 1976, **73**, 4474-4478.
72. A. J. Schoeffler and J. M. Berger, *Q Rev Biophys*, 2008, **41**, 41-101.
73. J. C. Wang, *Biochimica Et Biophysica Acta*, 1987, **909**, 1-9.
74. J. C. Wang, *Annu Rev Biochem*, 1985, **54**, 665-697.
75. J. C. Wang, *Prog Biophys Mol Bio*, 1996, **65**, Sb102-Sb102.
76. J. C. Wang, *Annu Rev Biochem*, 1996, **65**, 635-692.
77. J. C. Wang, *Nat Rev Mol Cell Bio*, 2002, **3**, 430-440.
78. J. M. Berger, D. Fass, J. C. Wang and S. C. Harrison, *P Natl Acad Sci USA*, 1998, **95**, 7876-7881.

79. J. C. Wang, *Annu Rev Biochem*, 1996, **65**, 635-692.
80. Y. C. Tse-Dinh, *Bba-Gene Struct Expr*, 1998, **1400**, 19-27.
81. D. V. Bugreev and G. A. Nevinsky, *Biochemistry-Moscow+*, 2009, **74**, 1467-1481.
82. K. D. Corbett and J. M. Berger, *Annu Rev Biophys Biomol Struct*, 2004, **33**, 95-118.
83. D. V. Bugreev and G. A. Nevinsky, *Russ J Bioorg Chem+*, 2010, **36**, 269-286.
84. L. Stewart, M. R. Redinbo, X. Qiu, W. G. Hol and J. J. Champoux, *Science*, 1998, **279**, 1534-1541.
85. C. R. Lopez, S. Yang, R. W. Deibler, S. A. Ray, J. M. Pennington, R. J. DiGate, P. J. Hastings, S. M. Rosenberg and E. L. Zechiedrich, *Molecular Microbiology*, 2005, **58**, 80-101.
86. J. M. Berger, *Bba-Gene Struct Expr*, 1998, **1400**, 3-18.
87. D. B. Wigley, *Annu Rev Bioph Biom*, 1995, **24**, 185-208.
88. I. Laponogov, X. S. Pan, D. A. Veselkov, K. E. McAuley, L. M. Fisher and M. R. Sanderson, *Plos One*, 2010, **5**.
89. P. Heisig, *Mutagenesis*, 2009, **24**, 465-469.
90. K. Drlica, *Molecular Microbiology*, 1992, **6**, 425-433.
91. K. Drlica, *Trends Genet*, 1990, **6**, 433-437.
92. L. F. Liu and J. C. Wang, *P Natl Acad Sci USA*, 1987, **84**, 7024-7027.
93. L. Yang, M. S. Wold, J. J. Li, T. J. Kelly and L. F. Liu, *P Natl Acad Sci USA*, 1987, **84**, 950-954.
94. A. K. McClendon and N. Osheroff, *Mutat Res-Fund Mol M*, 2007, **623**, 83-97.
95. Y. P. Tsao, H. Y. Wu and L. F. Liu, *Cell*, 1989, **56**, 111-118.
96. A. Travers and G. Muskhelishvili, *Nat Rev Microbiol*, 2005, **3**, 157-169.
97. C. Levine, H. Hiasa and K. J. Marians, *Biochim Biophys Acta*, 1998, **1400**, 29-43.
98. Y. C. Tse-Dinh, *Biochim Biophys Acta*, 1998, **1400**, 19-27.
99. K. M. Rose, J. Szopa, F. S. Han, Y. C. Cheng, A. Richter and U. Scheer, *Chromosoma*, 1988, **96**, 411-416.
100. M. Durand-Dubief, J. P. Svensson, J. Persson and K. Ekwall, *Transcription*, 2011, **2**, 66-70.
101. H. Zhang, J. C. Wang and L. F. Liu, *Proc Natl Acad Sci U S A*, 1988, **85**, 1060-1064.
102. A. A. Brian, H. L. Frisch and L. S. Lerman, *Biopolymers*, 1981, **20**, 1305-1328.
103. Y. Pommier, E. Leo, H. L. Zhang and C. Marchand, *Chem Biol*, 2010, **17**, 421-433.
104. S. Salerno, F. Da Settimo, S. Taliani, F. Simorini, C. La Motta, G. Fornaciari and A. M. Marini, *Curr Med Chem*, 2010, **17**, 4270-4290.
105. K. Chikamori, A. G. Grozav, T. Kozuki, D. Grabowski, R. Ganapathi and M. K. Ganapathi, *Curr Cancer Drug Tar*, 2010, **10**, 758-771.
106. Y. Pommier, P. Pourquier, Y. Fan and D. Strumberg, *Bba-Gene Struct Expr*, 1998, **1400**, 83-106.
107. K. R. Hande, *Bba-Gene Struct Expr*, 1998, **1400**, 173-184.
108. S. J. Froelichammon and N. Osheroff, *Journal of Biological Chemistry*, 1995, **270**, 21429-21432.
109. A. Maxwell and D. M. Lawson, *Curr Top Med Chem*, 2003, **3**, 283-303.
110. K. Drlica and M. Malik, *Curr Top Med Chem*, 2003, **3**, 249-282.
111. Y. Pommier, *Chemical Reviews*, 2009, **109**, 2894-2902.
112. G. S. Bisacchi and J. Dumas, *Annu Rep Med Chem*, 2009, **44**, 379-396.
113. J. M. Fortune and N. Osheroff, *Progress in Nucleic Acid Research and Molecular Biology*, Vol 64, 2000, **64**, 221-253.
114. S. H. Kaufmann and R. Hancock, *Acta Biochim Pol*, 1995, **42**, 381-393.
115. C. Garcia-Estrada, C. F. Prada, C. Fernandez-Rubio, F. Rojo-Vazquez and R. Balana-Fouce, *P Roy Soc B-Biol Sci*, 2010, **277**, 1777-1787.
116. M. H. Barnes, W. A. LaMarr and K. A. Foster, *Protein Expr Purif*, 2003, **29**, 259-264.

117. J. G. Heddle, F. M. Barnard, L. M. Wentzell and A. Maxwell, *Nucleos Nucleot Nucl*, 2000, **19**, 1249-1264.
118. F. T. F. Tsai, O. M. P. Singh, T. Skarzynski, A. J. Wonacott, S. Weston, A. Tucker, R. A. Pauptit, A. L. Breeze, J. P. Poyser, R. O'Brien, J. E. Ladbury and D. B. Wigley, *Proteins*, 1997, **28**, 41-52.
119. Y. Pommier, *Nature Reviews Cancer*, 2006, **6**, 789-802.
120. M. W. Milstead, J. M. Besterman, D. K. Croom, D. L. Emerson, M. G. Evans, P. P. Leitner, M. J. Luzzio, G. McIntyre, J. J. Mohan, B. S. Morton, M. R. Peel, J. M. Sisco, D. D. Sternbach, W. Q. Tong and A. Vuong, *Abstr Pap Am Chem S*, 1994, **208**, 51-Medi.
121. M. R. Peel, M. W. Milstead, D. D. Sternbach, J. M. Besterman, P. Leitner, B. Morton, M. E. Wall and M. C. Wani, *Bioorganic & Medicinal Chemistry Letters*, 1995, **5**, 2129-2132.
122. A. K. Larsen, A. E. Eseargueil and A. Skladanowski, *Pharmacol Therapeut*, 2003, **99**, 167-181.
123. W. A. Denny and B. C. Baguley, *Curr Top Med Chem*, 2003, **3**, 339-353.
124. J. A. Menendez, L. Vellon and R. Lupu, *International Journal of Molecular Medicine*, 2006, **18**, 1081-1087.
125. A. I. Scovassi, N. S. Pellegata, L. Di Stefano, L. Padovan, C. Negri, E. Prosperi, F. Riva, M. Ciomei and G. N. Ranzani, *Anticancer Res*, 2001, **21**, 2803-2808.
126. E. Multon, J. F. Riou, D. Lefevre, J. C. Ahomadegbe and G. Riou, *Biochemical Pharmacology*, 1989, **38**, 2077-2086.
127. L. Yang, T. C. Rowe, E. M. Nelson and L. F. Liu, *Cell*, 1985, **41**, 127-132.
128. L. Yang, T. C. Rowe and L. F. Liu, *Cancer Res*, 1985, **45**, 5872-5876.
129. L. Wang and D. A. Eastmond, *Environ Mol Mutagen*, 2002, **39**, 348-356.
130. T. Andoh and R. Ishida, *Bba-Gene Struct Expr*, 1998, **1400**, 155-171.
131. K. M. Rose, *Faseb Journal*, 1988, **2**, 2474-2478.
132. G. Capranico, J. Marinello and L. Baranello, *Bba-Rev Cancer*, 2010, **1806**, 240-250.
133. J. C. Wang, *Nat Rev Mol Cell Biol*, 2002, **3**, 430-440.
134. D. A. Koster, V. Croquette, C. Dekker, S. Shuman and N. H. Dekker, *Nature*, 2005, **434**, 671-674.
135. G. A. Nevinsky and D. V. Bugreev, *Russ J Bioorg Chem+*, 2010, **36**, 269-286.
136. C. J. Benham, *Mathematics of DNA structure, function and interactions*, Springer, Dordrecht ; New York, 2009.
137. Z. Yang, J. F. Carey and J. J. Champoux, *FEBS J*, 2009, **276**, 5906-5919.
138. K. R. Madden, L. Stewart and J. J. Champoux, *Embo J*, 1995, **14**, 5399-5409.
139. S. Krogh, U. H. Mortensen, O. Westergaard and B. J. Bonven, *Nucleic Acids Res*, 1991, **19**, 1235-1241.
140. M. Caserta, A. Amadei, E. Di Mauro and G. Camilloni, *Nucleic Acids Res*, 1989, **17**, 8463-8474.
141. G. Camilloni, E. Dimartino, M. Caserta and E. Dimauro, *Nucleic Acids Research*, 1988, **16**, 7071-7085.
142. B. J. Bonven, E. Gocke and O. Westergaard, *Cell*, 1985, **41**, 541-551.
143. P. J. Ness, T. Koller and F. Thoma, *Journal of molecular biology*, 1988, **200**, 127-139.
144. K. Kirkegaard and J. C. Wang, *Journal of molecular biology*, 1985, **185**, 625-637.
145. J. C. Wang, *Annu Rev Biochem*, 1985, **54**, 665-697.
146. A. Maxwell and M. Gellert, *Advances in protein chemistry*, 1986, **38**, 69-107.
147. D. Sikder and V. Nagaraja, *Nucleic Acids Research*, 2000, **28**, 1830-1837.
148. M. D. Dong, R. Subramani, S. Juul, A. Rotaru, F. F. Andersen, K. V. Gothelf, W. Mamdouh, F. Besenbacher and B. R. Knudsen, *Acs Nano*, 2010, **4**, 5969-5977.
149. S. Shuman, A. Patel, L. Yakovleva and A. Mondragon, *Structure*, 2010, **18**, 725-733.

150. R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich, *Science*, 1988, **239**, 487-491.
151. A. R. Pavlov, N. V. Pavlova, S. A. Kozyavkin and A. I. Slesarev, *Trends Biotechnol*, 2004, **22**, 253-260.
152. J. P. Laine, P. L. Opresko, F. E. Indig, J. A. Harrigan, C. von Kobbe and V. A. Bohr, *Cancer Res*, 2003, **63**, 7136-7146.
153. Y. L. Lyubchenko and L. S. Shlyakhtenko, *Methods*, 2009, **47**, 206-213.
154. L. Hamon, D. Pastre, P. Dupaigne, C. Le Breton, E. Le Cam and O. Pietrement, *Nucleic Acids Research*, 2007, **35**.
155. S. H. Yoshimura, H. Maruyama, F. Ishikawa, R. Ohki and K. Takeyasu, *Genes Cells*, 2004, **9**, 205-218.
156. H. G. Hansma, I. Revenko, K. Kim and D. E. Laney, *Nucleic Acids Research*, 1996, **24**, 713-720.
157. D. C. Rio, M. Tang, C. Cecconi, H. Kim and C. Bustamante, *Gene Dev*, 2005, **19**, 1422-1425.
158. C. Rivetti, M. Guthold and C. Bustamante, *Journal of Molecular Biology*, 1996, **264**, 919-932.
159. Y. L. Lyubchenko, A. A. Gall, L. S. Shlyakhtenko, R. E. Harrington, B. L. Jacobs, P. I. Oden and S. M. Lindsay, *J Biomol Struct Dyn*, 1992, **10**, 589-606.
160. F. Moreno-Herrero, L. Holtzer, D. A. Koster, S. Shuman, C. Dekker and N. H. Dekker, *Nucleic Acids Research*, 2005, **33**, 5945-5953.
161. Z. G. Liu, R. H. Meng, Y. G. Zu, Q. Y. Li and L. P. Yao, *Scanning*, 2009, **31**, 160-166.
162. Y. L. Lyubchenko, L. S. Shlyakhtenko, T. Aki and S. Adhya, *Nucleic Acids Research*, 1997, **25**, 873-876.
163. L. Hamon, D. Pastre, P. Dupaigne, C. Le Breton, E. Le Cam and O. Pietrement, *Nucleic Acids Research*, 2007, **35**, -.
164. M. M. Garner and A. Revzin, *Nucleic Acids Res*, 1981, **9**, 3047-3060.
165. M. Fried and D. M. Crothers, *Nucleic Acids Res*, 1981, **9**, 6505-6525.
166. M. G. Fried, *Electrophoresis*, 1989, **10**, 366-376.
167. D. M. Crothers, M. R. Gartenberg and T. E. Shrader, *Method Enzymol*, 1991, **208**, 118-146.
168. M. G. Fried and L. M. Hellman, *Nat Protoc*, 2007, **2**, 1849-1861.
169. *Nat Methods*, 2005, **2**, 557-558.
170. C. J. Li, L. Averboukh and A. B. Pardee, *J Biol Chem*, 1993, **268**, 22463-22468.
171. A. R. Chowdhury, S. Sharma, S. Mandal, A. Goswami, S. Mukhopadhyay and H. K. Majumder, *Biochem J*, 2002, **366**, 653-661.
172. X. Zhou, L. Shuai, S. R. Wang, L. X. Zhang and B. Q. Fu, *Chem Biodivers*, 2009, **6**, 827-837.
173. C. F. Stewart, M. N. Kirstein, P. J. Houghton, P. J. Cheshire, L. B. Richmond, A. K. Smith and S. K. Hanna, *Clin Cancer Res*, 2001, **7**, 358-366.
174. M. Argaman, S. Bendetz-Nezer, S. Matlis, S. Segal and E. Priel, *Biochem Biophys Res Commun*, 2003, **301**, 789-797.
175. R. F. Frohlich, C. Veigaard, F. F. Andersen, A. K. McClendon, A. C. Gentry, A. H. Andersen, N. Osherooff, T. Stevnsner and B. R. Knudsen, *Nucleic Acids Research*, 2007, **35**, 6170-6180.
176. G. Camilloni, E. Dimartino, E. Dimauro and M. Caserta, *P Natl Acad Sci USA*, 1989, **86**, 3080-3084.
177. J. C. Wang, *Biochim Biophys Acta*, 1987, **909**, 1-9.
178. Y. Pommier, P. Pourquier, Y. Fan and D. Strumberg, *Biochim Biophys Acta*, 1998, **1400**, 83-105.
179. G. Chillemi, P. Fiorani, A. Bruselles, S. Castelli, A. Campagna, O. Sarra, C. Tesaro, M. Fiorentini, O. Vassallo, I. D'Annessa, S. Santoleri and A. Desideri, *Ital J Biochem*, 2007, **56**, 110-114.
180. Y. Pommier, *Chem Rev*, 2009, **109**, 2894-2902.
181. A. Balestrazzi, G. Bernacchia, L. Pitto, G. Luccarini and D. Carbonera, *Eur J Histochem*, 2001, **45**, 31-38.

182. M. Morsegaudio and M. S. Risley, *Molecular Biology of the Cell*, 1995, **6**, 1839-1839.
183. K. R. Madden and J. J. Champoux, *Cancer Res*, 1992, **52**, 525-532.
184. Y. Pommier, *Nat Rev Cancer*, 2006, **6**, 789-802.
185. Z. Topcu, *J Clin Pharm Ther*, 2001, **26**, 405-416.
186. T. K. Li and L. F. Liu, *Annu Rev Pharmacol Toxicol*, 2001, **41**, 53-77.
187. M. L. Rothenberg, *Ann Oncol*, 1997, **8**, 837-855.
188. I. Husain, J. L. Mohler, H. F. Seigler and J. M. Besterman, *Cancer Res*, 1994, **54**, 539-546.
189. E. S. David, E. E. John and L. G. Deborah, *Tetrahedron Lett.*, 1995, **36**, 1413-1416.
190. V. M. Rangnekar, A. Goswami, S. Qiu, T. S. Dexheimer, P. Ranganathan, R. Burikhanovl and Y. Pommier, *Cancer Res*, 2008, **68**, 6190-6198.
191. T. Bhaduri, S. Basak, D. Sikder and V. Nagaraja, *Febs Lett*, 2000, **486**, 126-130.
192. D. V. Bugreev, E. L. Vasyutina, T. I. Kolocheva, V. N. Buneva, T. Andoh and G. A. Nevinsky, *Biochimie*, 1998, **80**, 303-308.
193. C. H. Tan, Y. F. Wang, M. T. T. Ng, T. Y. Zhou, X. M. Li and T. H. Li, *Bioorganic & Medicinal Chemistry Letters*, 2008, **18**, 3597-3602.
194. T. H. Li, X. M. Li, M. T. T. Ng, Y. F. Wang and X. Q. Liu, *Bioorganic & Medicinal Chemistry Letters*, 2007, **17**, 4967-4971.
195. N. M. Quek, S. T. Chua, M. Li, M. T. T. Ng, W. X. Yuan, M. L. Chua, J. J. Guo, L. E. Koh, R. J. Ye and T. H. Li, *Bioorganic & Medicinal Chemistry Letters*, 2009, **19**, 618-623.
196. H. S. Koo and D. M. Crothers, *Proc Natl Acad Sci U S A*, 1988, **85**, 1763-1767.
197. S. Diekmann, *Febs Lett*, 1986, **195**, 53-56.
198. D. M. Crothers, T. E. Haran and J. G. Nadeau, *J Biol Chem*, 1990, **265**, 7093-7096.
199. S. Yoshizawa, T. Ueda, Y. Ishido, K. Miura, K. Watanabe and I. Hirao, *Nucleic Acids Res*, 1994, **22**, 2217-2221.
200. J. G. Nadeau and D. M. Crothers, *Proc Natl Acad Sci U S A*, 1989, **86**, 2622-2626.
201. D. MacDonald and P. Lu, *Nucleic Acids: Curvature and Deformation*, 2004, **884**, 65-+.
202. S. Cacchione, P. Desantis, D. Foti, A. Palleschi and M. Savino, *Biochemistry-Us*, 1989, **28**, 8706-8713.
203. S. Diekmann, *Methods Enzymol*, 1992, **212**, 30-46.
204. D. S. Goodsell and R. E. Dickerson, *Nucleic Acids Research*, 1994, **22**, 5497-5503.
205. E. Stellwagen, Y. Lu and N. C. Stellwagen, *Nucleic Acids Res*, 2005, **33**, 4425-4432.
206. P. Pourquier, A. A. Pilon, G. Kohlhagen, A. Mazumder, A. Sharma and Y. Pommier, *Journal of Biological Chemistry*, 1997, **272**, 26441-26447.
207. P. Pourquier, L. M. Ueng, G. Kohlhagen, A. Mazumder, M. Gupta, K. W. Kohn and Y. Pommier, *Journal of Biological Chemistry*, 1997, **272**, 7792-7796.
208. K. A. Henningfeld, T. Arslan and S. M. Hecht, *J Am Chem Soc*, 1996, **118**, 11701-11714.
209. P. J. Hagerman, *Biochemistry-Us*, 1985, **24**, 7033-7037.
210. E. Fattal and A. Bochot, *Adv Drug Deliv Rev*, 2006, **58**, 1203-1223.
211. S. D. Patil, D. G. Rhodes and D. J. Burgess, *AAPS J*, 2005, **7**, E61-77.
212. S. T. Crooke, *Antisense Nucleic Acid Drug Dev*, 1998, **8**, 115-122.
213. R. A. Stull and F. C. Szoka, Jr., *Pharm Res*, 1995, **12**, 465-483.
214. R. Dulbecco and M. Vogt, *P Natl Acad Sci USA*, 1963, **50**, 236-&.
215. J. Vinograd, J. Lebowitz, R. Radloff, R. Watson and P. Laipis, *P Natl Acad Sci USA*, 1965, **53**, 1104-&.
216. D. A. Clayton and J. Vinograd, *Nature*, 1967, **216**, 652-&.
217. A. Deffieux and M. Schappacher, *Cell Mol Life Sci*, 2009, **66**, 2599-2602.

218. B. Laurie, V. Katritch, J. Sogo, T. Koller, J. Dubochet and A. Stasiak, *Biophys J*, 1998, **74**, 2815-2822.
219. N. C. Seeman, *Annual Review of Biochemistry*, Vol 79, 2010, **79**, 65-87.
220. N. C. Seeman, T. Omabegho and R. Sha, *Science*, 2009, **324**, 67-71.
221. I. Willner, B. Shlyahovsky, M. Zayats and B. Willner, *Chem Soc Rev*, 2008, **37**, 1153-1165.
222. T. Liedl, T. L. Sobey and F. C. Simmel, *Nano Today*, 2007, **2**, 36-41.
223. A. J. Turberfield and J. Bath, *Nat Nanotechnol*, 2007, **2**, 275-284.
224. C. D. Mao, W. Q. Sun and N. C. Seeman, *Nature*, 1997, **386**, 137-138.
225. D. Ackermann, T. L. Schmidt, J. S. Hannam, C. S. Purohit, A. Heckel and M. Famulok, *Nat Nanotechnol*, 2010, **5**, 436-442.
226. M. A. Krasnow, A. Stasiak, S. J. Spengler, F. Dean, T. Koller and N. R. Cozzarelli, *Nature*, 1983, **304**, 559-560.
227. A. Vologodskii and V. V. Rybenkov, *Phys Chem Chem Phys*, 2009, **11**, 10543-10552.
228. G. Charvin, A. Vologodskii, D. Bensimon and V. Croquette, *Biophys J*, 2005, **88**, 4124-4136.
229. J. C. Wang, *Accounts Chem Res*, 1973, **6**, 252-256.
230. J. C. Wang, *Biopolymers*, 1970, **9**, 489-502.
231. B. Laurie, V. Katritch, J. Sogo, T. Koller, J. Dubochet and A. Stasiak, *Biophys J*, 1998, **74**, 2815-2822.
232. M. D. Stone, Z. Bryant, N. J. Crisona, S. B. Smith, A. Vologodskii, C. Bustamante and N. R. Cozzarelli, *P Natl Acad Sci USA*, 2003, **100**, 8654-8659.
233. K. C. Neuman, G. Charvin, D. Bensimon and V. Croquette, *P Natl Acad Sci USA*, 2009, **106**, 6986-6991.
234. B. Hudson and J. Vinograd, *Nature*, 1967, **216**, 647-&.
235. S. J. Spengler, A. Stasiak and N. R. Cozzarelli, *Cell*, 1985, **42**, 325-334.
236. T. L. Schmidt and A. Heckel, *Nano Lett*, 2011, **11**, 1739-1742.
237. W. Waldeck, M. Theobald and H. Zentgraf, *Embo J*, 1983, **2**, 1255-1261.
238. M. A. Krasnow and N. R. Cozzarelli, *J Biol Chem*, 1982, **257**, 2687-2693.
239. F. G. Harmon, J. P. Brockman and S. C. Kowalczykowski, *Journal of Biological Chemistry*, 2003, **278**, 42668-42678.
240. P. Pieranski and S. Przybyl, *Phys Rev E Stat Nonlin Soft Matter Phys*, 2001, **64**, 031801.
241. L. F. Liu, L. Perkocha, R. Calendar and J. C. Wang, *P Natl Acad Sci-Biol*, 1981, **78**, 5498-5502.
242. L. F. Liu, C. C. Liu and B. M. Alberts, *Cell*, 1980, **19**, 697-707.
243. S. A. Wasserman and N. R. Cozzarelli, *Science*, 1986, **232**, 951-960.
244. S. D. Levene, C. Donahue, T. C. Boles and N. R. Cozzarelli, *Biophys J*, 1995, **69**, 1036-1045.
245. P. Cluzel, A. Lebrun, C. Heller, R. Lavery, J. L. Viovy, D. Chatenay and F. Caron, *Science*, 1996, **271**, 792-794.
246. H. Yamaguchi, K. Kubota and A. Harada, *Chem Lett*, 2000, 384-385.
247. H. Yamaguchi, K. Kubota and A. Harada, *Nucleic Acids Symp Ser*, 2000, 229-230.
248. S. M. Du, H. Wang, Y. C. Tsedinh and N. C. Seeman, *Biochemistry-Us*, 1995, **34**, 673-682.
249. S. Kobayashi, M. Furukawa, C. Dohi, H. Hamashima, T. Arai and A. Tanaka, *Chem Pharm Bull*, 1999, **47**, 783-790.
250. S. M. Du and N. C. Seeman, *Biopolymers*, 1994, **34**, 31-37.
251. S. Y. Shaw and J. C. Wang, *Science*, 1993, **260**, 533-536.
252. D. M. Gowers and S. E. Halford, *Embo J*, 2003, **22**, 1410-1418.
253. P. A. Rice, K. W. Mouw, S. P. Montano, M. R. Boocock, S. J. Rowland and W. M. Stark, *Biochem Soc T*, 2010, **38**, 384-387.
254. K. Hu, *B Math Biol*, 2007, **69**, 1019-1030.

255. H. S. Koo, H. M. Wu and D. M. Crothers, *Nature*, 1986, **320**, 501-506.
256. F. Gimenes, K. I. Takeda, A. Fiorini, F. S. Gouveia and M. A. Fernandez, *Genet Mol Res*, 2008, **7**, 549-558.
257. D. Li, Z. Yang, G. Zhao, Y. Long, B. Lv, C. Li, S. Hiew, M. T. Ng, J. Guo, H. Tan, H. Zhang and T. Li, *Chem Commun*, 2011, **47**, 7479 - 7481
258. K. Abremski, R. Hoess and N. Sternberg, *Cell*, 1983, **32**, 1301-1311.
259. K. Ghosh and G. D. Van Duyne, *Methods*, 2002, **28**, 374-383.
260. A. Nagy, *Genesis*, 2000, **26**, 99-109.
261. E. J. Cantor and S. R. Chong, *Protein Expres Purif*, 2001, **22**, 135-140.
262. F. Guo, D. N. Gopaul and G. D. VanDuyne, *Nature*, 1997, **389**, 40-46.
263. D. N. Gopaul, F. Guo and G. D. Van Duyne, *Embo J*, 1998, **17**, 4175-4187.
264. S. C. Y. Ip, M. Bregu, F. X. Barre and D. J. Sherratt, *Embo J*, 2003, **22**, 6399-6407.
265. D. Metzger and R. Feil, *Curr Opin Biotechnol*, 1999, **10**, 470-476.
266. K. Abremski and R. Hoess, *J Biol Chem*, 1984, **259**, 1509-1514.
267. B. Sauer and N. Henderson, *Proc Natl Acad Sci U S A*, 1988, **85**, 5166-5170.
268. W. F. Edwards, D. D. Young and A. Deiters, *Acs Chem Biol*, 2009, **4**, 441-445.
269. D. Buck and E. Flapan, *J Mol Biol*, 2007, **374**, 1186-1199.
270. N. J. Crisona, R. L. Weinberg, B. J. Peter, D. W. Sumners and N. R. Cozzarelli, *J Mol Biol*, 1999, **289**, 747-775.
271. A. D. Bates and A. Maxwell, *Biochemistry-Us*, 2007, **46**, 7929-7941.
272. V. V. Rybenkov, C. Ullsperger, A. V. Vologodskii and N. R. Cozzarelli, *Science*, 1997, **277**, 690-693.
273. J. Yan, M. O. Magnasco and J. F. Marko, *Nature*, 1999, **401**, 932-935.
274. J. Yan, M. O. Magnasco and J. F. Marko, *Phys Rev E*, 2001, **63**.
275. K. D. Corbett and J. M. Berger, *Annu Rev Bioph Biom*, 2004, **33**, 95-118.
276. R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich, *Science*, 1988, **239**, 487-491.
277. A. R. Pavlov, N. V. Pavlova, S. A. Kozyavkin and A. I. Slesarev, *Trends in Biotechnology*, 2004, **22**, 253-260.
278. G. Rasched, D. Ackermann, T. L. Schmidt, P. Broekmann, A. Heckel and M. Famulok, *Angew Chem Int Ed Engl*, 2008, **47**, 967-970.
279. L. Hamon, D. Pastre, P. Dupaigne, C. Le Breton, E. Le Cam and O. Pietrement, *Nucleic Acids Res*, 2007, **35**, e58.
280. M. Famulok, D. Ackermann, G. Rasched, S. Verma, T. L. Schmidt and A. Heckel, *Chem Commun*, 2010, **46**, 4154-4156.
281. A. Heckel, D. P. N. Goncalves, T. L. Schmidt and M. B. Koeppel, *Small*, 2010, **6**, 1347-1352.
282. Y. L. Lyubchenko and L. S. Shlyakhtenko, *Methods*, 2009, **47**, 206-213.
283. M. Schappacher and A. Deffieux, *Angew Chem Int Ed Engl*, 2009, **48**, 5930-5933.
284. K. Abremski, R. Hoess and N. Sternberg, *Cell*, 1983, **32**, 1301-1311.

Zhaoqi Yang, Ph. D. (candidate)

Division of Chemistry and Biological Chemistry

Nanyang Technological University

21 Nanyang Link, Singapore 637371

Tel.: (65) 8349-6232

Email: yang0213@ntu.edu.sg; pharmacydoctor@163.com

EDUCATION:

2008-present Graduate Student (Ph. D. degree will be awarded in 2012)

Chemical biology

Division of Chemistry and Biological Chemistry

Nanyang Technological University

Singapore

2002-2005 Graduate Student (Master Degree awarded in June, 2005)

Medicinal Chemistry

College of Pharmacy, Wuhan University

Hubei, P. R. China

1998-2002 Undergraduate Student (Bachelor Degree awarded in June, 2002)

Polymer Chemistry

School Material Science and Engineering

Wuhan Institute of Technology

Hubei, P. R. China

PROFESSIONAL EXPERIENCE:

2005-2008 Lecturer

Medicinal Chemistry

College of Pharmacy

Jinan University

RESEARCH INTERESTS:

Topoisomerase and novel anti-cancer drug design

DNA topology and Topoisomerase

Drug design and discovery (including computer-aided drug design)

PUBLICATION:

- 1 **Yang, Z.Q.**; Li, D.W.; Guo, J.J.; Shao, F.W.; Li, T.H. Intrinsic curvature in duplex DNA inhibits Human Topoisomerase I. *Bioorg. Med. Chem. Lett.*, 2012, 22(3), 1322-1325.
- 2 **Yang, Z.Q.**; Li, D.W.; Li, T.H. Design and synthesis of catenated rings based on toroidal DNA structures. *Chem.Comm.*, 2011, 47 (43), 11930 -11932.
- 3 **Yang, Z.Q.**; Li, D.W.; Hiew, S.H.; Ng, T.T.M.; Yuan, W.X.; Su, H.B.; Shao, F.W.; Li, T.H. Recognition of forcible curvature in circular DNA by human topoisomerase I. *Chem.Comm.*, 2011, 47 (40), 11309 -11311.
- 4 Li, D.W.; **Yang, Z.Q.**; Long, Y.; Zhao, G.J.; Lv, B.; Hiew, S.H.; Ng, M.T.T.; Guo, J.J.; Tan, H.K.; Zhang, H. Yuan, W.X.; Su, H.B.; Li, T.H. Precise engineering and visualization of signs and magnitudes of DNA writhe on the basis of PNA invasion. *Chem.Comm.*, 2011, 47(38), 10695 -10697.
- 5 Wang, H.B.; Xu, W.; Zhang, H.; Li, D.W.; **Yang, Z.Q.**; Xie, X.J.; Li, T.H. and Liu X.G. EcoRI-Modified Gold Nanoparticles for Dual-Mode Colorimetric Detection of Magnesium and Pyrophosphate Ions. *Small*, 2011, 7(14), 1987-1992.
- 6 Li, D.W.; **Yang, Z.Q.**; Zhao, G.J.; Long, Y.; Lv, B.; Li, C.; Hiew, S.H.; Ng, M.T.T.; Guo, J.J.; Tan, H.K.; Zhang, H. and Li, T.H. Manipulating DNA writhe through varying DNA sequences. *Chem.Comm.*, 2011, 47(26), 7479 -7481.
- 7 Li, D.W.; Li, W.L.; Wang, Q.; **Yang, Z.Q.**; Hou, Z.J. Concise Synthesis of Cannabisin G. *Bioorg. Med. Chem. Lett.*, 2010, 20(17), 5095-5098.
- 8 Qian, C.W.; Zheng, J.X.; Xiao, G.K.; Guo, J.L.; **Yang, Z.Q.**; Huang, L.; Chao, W.; Rao, L.Y.; Sun, P.H. 3D-QSAR studies on thiazolidin-4-one S1P1 receptor agonists by CoMFA and CoMSIA. *Int. J. Mol. Sci.* 2011, 12(10), 6502-6516.
- 9 Wong, W. C.; Zhuang, J.Y.; Ng, L.L.S.; New, L.L.L.; Hiew, S.H.; Guo, J.J.; **Yang, Z.Q.**; and Li, T.H Conformational organizations of G-quadruplexes composed of d(G₄Tn)₃G₄. *Bioorg. Med. Chem. Lett.*, 2010, 20(15), 4689-4692.
- 10 Sun, P.; **Yang, Z.Q.**; Li, M.K.; Chen W.M.; Liu, Q.; Yao, X.S. 3D-QSAR Study of Synthetic Furanones as Inhibitors of Quorum Sensing by Using CoMFA and CoMSIA Approach. *Lett. Drug Des. Discovery*, 2009, 6(8), 568-574.
- 11 Ng, M. T. T.; Li, X.; Wang, Y.; Zhou, T.; **Yang, Z.Q.**; Foo, H. Y.; and Li, T.H. Site-Specific Cleavage of G-quadruplexes Formed by Oxytricha Telometric Repeats. *Australian Journal of Chemistry*, 2009, 62(9), 1189–1193.
- 12 Jiang, X.; Yu, P.; Jiang, J.; Zhang, Z.; Wang, Z.; **Yang, Z.Q.**; Tian, Z.; Wright, S.C.; Larrick, J.W.; Wang, Y. Synthesis and evaluation of antibacterial activities of andrographolide analogues. *Eur. J. Med. Chem.*, 2009, 44(7), 2936-2943.
- 13 Sun, P.; **Yang, Z.Q.**; Chen W.M.; Liu, Q.; Yao, X.S. 3D-QSAR Study of N-Phenylacetanoyl-L-Homoserine Lactones as Inhibitors of Bacterial Quorum Sensing by Using CoMFA Approach. *Lett. Drug Des. Discovery*, 2008, 5(7), 449-453.

- 14 Wang, L.Y.; Jia, Q.; Yan, X.Q.; **Yang, Z.Q.**; Sun, P.H. Determination of total saponins in *schefflera arboricola hayata* by ultraviolet spectrophotometry. *Zhongguo Yaofang*, 2008, 19(15), 1175-1177. (in chinese)
- 15 **Yang, Z.Q.**; Sun, P.; Chen W.M. 3D-QSAR analysis on pyrrolopyridine analogs as mitogen-activated protein kinase-activated protein kinase 2 (MK-2) inhibitors. *Lett. Drug Des. Discovery*, 2007, 4(8), 557-561.
- 16 **Yang, Z.Q.**; Sun, P. 3D-QSAR study of Potent Inhibitor of Phosphodiesterase-4 by using CoMFA approach. *Int. J. Mol. Sci.* 2007, 8(7), 714-722.
- 17 Wang, Y.; **Yang, Z.Q.**; Wright, S.C.; Larrick, J.W. Synthesis and Preliminary Antitumor Activity of Distamycin Nitrogen Mustards. *Lett. Drug Des. Discovery*, 2007, 4(1), 37-39.
- 18 Li, K.; **Yang, Z.Q.**; Wen, F.H.; Sun, P.H. Synthesis of (2 - Methyl - 3 - Nitrophenyl) Acetic Acid -An Important Intermediate of Ropinirol. *China Pharmacy*, 2007, 18(19), 1467-1468. (in chinese)
- 19 Sun, P.H.; **Yang, Z.Q.** HPLC Determination of Guanosine and Uridine in Banlangen Injection. *Chinese Archives of Traditional Chinese Medicine*, 2007, 25(8), 1728-1729. (in chinese)
- 20 Sun, P.H.; Yi, H.; **Yang, Z.Q.**; Chen, W.M. New investigation of grouping teaching of medicinal chemistry course in oversea Chinese school. *Journal of Shanxi Medical University (Precinical Medical Education Edition)*, 2007, 9(4), 387-389. (in chinese)
- 21 Suo, X.B.; Deng, Y.J.; Zhang, H.; **Yang, Z.Q.**; Zhang, Z.J. Wang, Y.Q. Preparation of Andrographolide solid dispersion with PVP and dissolution experiment in vitro. *Chinese Traditional Patent Medicine*, 29(4), 594-596. (in chinese)
- 22 Suo, X.B.; Deng, Y.J.; Zhang, H.; **Yang, Z.Q.**; Wang, Y.Q. Synthesis and liposome preparation of prodrug of indapamide. *Zhongguo Yiyuan Yaoxue Zazhi*, 2007, 27(4), 431-434. (in chinese)
- 23 Suo, X.B.; Deng, Y.J.; Zhang, H.; **Yang, Z.Q.**; Zhang, Z.J. Wang, Y.Q. Quantification and entrapment efficiency determination of lauroyl-indapamide liposomes. *Chinese Journal of New Drugs*, 2007, 16(3), 230-233. (in chinese)
- 24 Sun, P.H.; Chen, W.M.; Li, B.Z.; **Yang, Z.Q.**; Li, Q. Synthesis of 4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one hydrochloride (ropinirole hydrochloride). *Zhongguo Yiyao Gongye Zazhi*, 2007, 38(1), 7-8. (in chinese)
- 25 **Yang, Z.Q.** Synthesis and in vitro biological activity evaluation of the derivatives of Combretastatin A-4. *Lett. Drug Des. Discovery*, 2006, 3(8), 544-546.
- 26 **Yang, Z.Q.**; Sun, P. Synthesis of 1-benzyl-3-chloro-4-(3,4,5-trimethoxyphenyl) azetidin -2-one. *Molbank*, 2006, M513.
- 27 **Yang, Z.Q.**; Sun, P. Compare of three way of synthesis of simple Schiff base. *Molbank*, 2006, M514.
- 28 **Yang, Z.Q.** Progress in structure modification of anti-cancer agent Combretastatin A-4. *Huaxue Tongbao*, 2006, 69(8), w80. (in chinese)
- 29 **Yang, Z.Q.** Microwave-Assisted Rapid Synthesis of Intermediate of Ezetimibe. *Chemical Journal on Internet*, 2006, 8(5), 37.

- 30 **Yang, Z.Q.**; Su, J. Synthesis of β -Aminocarboxylate Containing *gem*-dimethylcyclopropane ring. *Chemical Reagent*, 2006, 28(6), 375-376. (in chinese)
- 31 Wang, Y.; Li, L.; Jiang, W.; **Yang, Z.Q.** and Zhang, Z. Synthesis and preliminary anti-tumor activity evaluation of a DHA and doxorubicin conjugate. *Bioorg. Med. Chem. Lett.*, 2006, 16, 2974-2977.
- 32 **Yang, Z.Q.**; Qiu, G.; Liang, S.; Hu, X. Synthesis of tumour vascular inhibitor CA-4. *Zhongguo Xiandai Yingyong Yaoxue*, 2005, 22(3), 210-211. (in chinese)

PATENTS:

- 1 Wang, Y.; Jiang, X.; Jiang, J.; Zhang, Z.; **Yang, Z.Q.**; Yu, P. Preparation of Andrographolide derivatives and pharmaceutical applications thereof. CN101125850.
- 2 Jiang, J.; **Yang, Z.Q.**; Zhang, Z.; Li, S.; Wang, Y. Preparation and Application of derivatives of Apocynin. CN1923790, March 2007.

RESEARCH GRANTS

1. Synthesis and preliminary anti-tumor activity evaluation of the derivatives of Combretastatin A-4. Principal investigator, Foundation of college of pharmacy of Jinan University, 11/1/2005-10/31/2007.
2. Preclinical research of novel anti-tumor agent: YW-391. Co-investigator, Foundation of Guangdong Province Technology Project, 1/1/2007-12/31/2008.
3. Preclinical research of novel tumor-selective anticancer agents: DHA-HCPT. Co-investigator, Foundation of Guangzhou Technology Project, 1/1/2007-12/31/2008.
4. Preclinical research of novel endogenous cardiovascular protective peptide. Co-investigator, Foundation of Guangzhou Technology Project, 1/1/2007-12/31/2008.
5. Preparation and targeting research of novel anticancer agents: YW-391-HSA. Co-investigator, Foundation of Guangzhou Technology Project, 1/1/2007-12/31/2008.
6. Synthesis and structure activity relationship of Andrographolide derivatives acting on dual antibacterial mechanisms. Co-investigator, Natural Science Foundation of China, 1/1/2008-12/31/2010.