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論文の内容の要旨

Introduction

Nucleic acid chemistry and its biological applications have a great scope in the development of futuristic drugs and cure for many diseases. Enzymatic methods for the nucleic acid manipulation have proven to be very useful *in vitro* but they have their disadvantages in the *in vivo* applications.[1] Therefore, chemical methods to edit nucleic acids were developed but the drawbacks of those chemical methods are numerous.[2]

Thus, to overcome these problems, photochemical methods to edit the nucleic acids have been devised which utilize single base modified nucleo-base to specifically target a desired sequence of DNA/RNA and edit that sequence at a single point. Fujmoto's group has discovered a novel compound, 3-Cyanovinylcarbazole, which can be easily incorporated as a nucleo-base and upon irradiation of 366nm radiation, forms a crosslink with the pyrimidine and lead to deamination to afford the transformation of the cytosine to uracil. The crosslink is photo-reversible and can be easily converted back by 312nm irradiation.[3-6]

The experiments have already shown success in the small nucleic acid sequences.[7-8] Moreover, the feasibility of the photo-crosslinking reaction using 3-Cyanovinylcarbazole *in vivo* has also been reported.[9]

Major drawback of this method is that the deamination step takes place at 90ºC, which is not a feasible condition for the *in vivo* applications. Therefore, in this research, the focus on development new method, such that, the deamination, which takes 200 years in physiological conditions without any external factor, can be carried out at 37ºC, i.e. physiological conditions in shorter time.

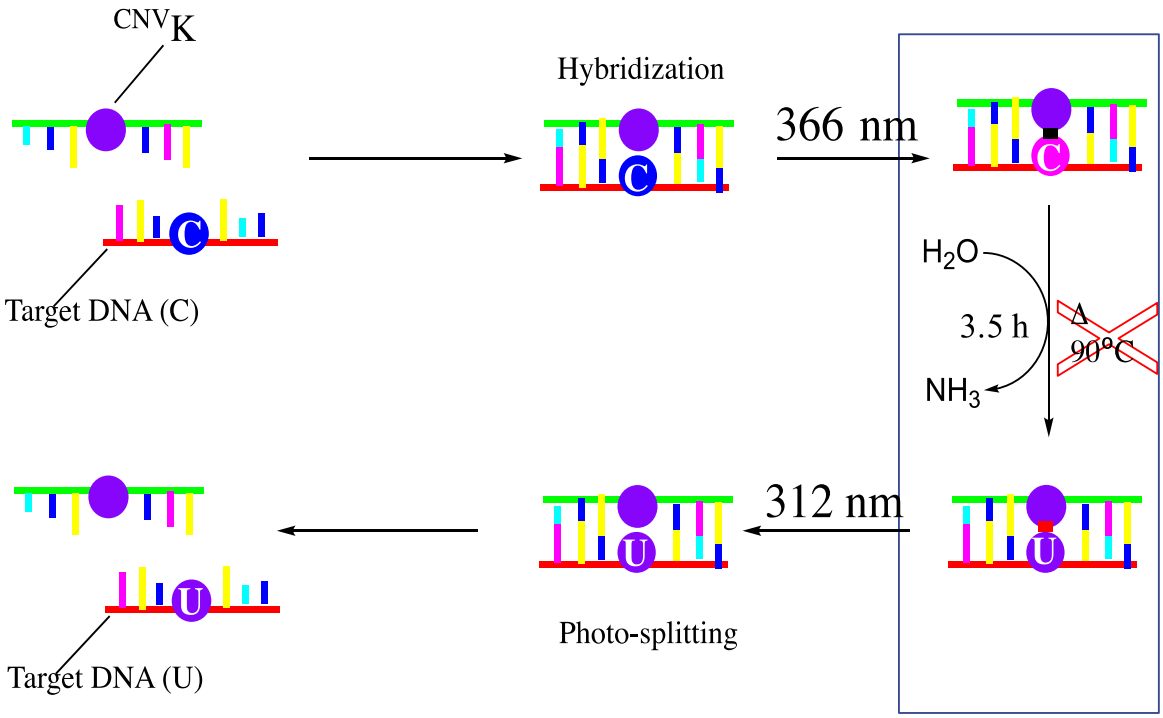

Figure 1: Schematic overview of photo-chemical DNA editing.

Results and Discussion

Chapter 2: The focus was to find the best counter-base of cytosine, based on hydrogen bonding, for photo-chemical site-directed mutagenesis using 3-cyanovinylcarbazole as the photo-active nucleoside which can crosslink with the target cytosine to afford cytosine to uracil transformation at physiological conditions. Different counter bases like guanine (G), inosine (I), 2-aminopurine (P), nebularine (R), and 5-nitroindole (N) were used to find the best counter base. Among all the bases, it was found that P, R, and N are not suitable counter base for photo-chemical cytosine to uracil transformation as when using these bases, no deamination reaction takes place. While in case of G, the deamination reaction is very slow and only 5% conversion is observed in 72h reaction time. Thus, the best counter base among the bases was found to be inosine which gives 35-40% in 72h reaction time at 37ºC having the optimal hydrogen bonding pattern before and after photo-cross-linking.

Chapter 3: In this chapter, the role of hydrophilicity and polarity of photo-cross-linker was discussed. Various derivatives of vinyl carbazole, like 3-cyanovinyl carbazole (^{CNV}K), 3-amidovinylcarbazole (^{NH2V}K), 3-methoxyvinylcarbazole (^{OMeV}K), and 3-carboxylvinylcarbazole (^{OHV}K), were used for studying the micro-environment around the target cytosine crosslinked to photo-cross-linker during the deamination of cytosine. It was discovered that the hydrophilicity and polarity of the photo-cross-linker plays a crucial role in the deamination of cytosine to uracil via photo-cross-linking. ^{OMeV}K having the least hydrophilicity gave the

least rate of reaction for the deamination reaction at varying temperature (90, 70, 50, and 37 ºC) while the highest reaction rate was observed with ^{OHV}K, which is most polar among the cross-linkers based on the polarity index (Log P). Thus, hydrophilicity and polarity around target cytosine are deciding factors in case of deamination reaction of cytosine via photo-cross-linking.

Chapter 4: Based on the findings of chapter 1 and 2, the overall micro-environment around the target cytosine for the mutation of cytosine to uracil via vinylcarbazole based photo-cross-linking was studied. A combination of counter bases (guanine (G), inosine (I), and cytosine (C)) and photo-cross-linkers (^{CNV}K, ^{NH2V}K, and ^{OHV}K) were used in the ODN to study the best match for acceleration of deamination of cytosine to uracil at physiological conditions. It turned out that the best combination of counter base and photo-cross-linker is inosine and ^{OHV}K which could give ~70% conversion of cytosine to uracil in 7 days at physiological conditions, which could be extended to ~90% in 20 days. The micro-environment around cytosine, including hydrogen bonding, hydrophilicity, and polarity of counter base and photo-cross-linker are key players for the photo-cross-link assisted deamination of cytosine to uracil.

Chapter 5: Based on the previous chapters we realized that the micro-environment around the target cytosine is deciding factor for rate of cytosine to uracil conversion via photo-cross-linking. Although, the reaction rate is very rather slow at physiological conditions even when inosine is counter base and ^{OHV}K is photo-cross-linker. Thus, a different approach to accelerate the rate of deamination reaction was used in which the ODN containing photo-cross-linker was divided into two parts between the counter base and photo-cross-linker. The adjoining part was modified with phosphate group at the terminal of counter base to increase the hydrophilicity near the cytosine. It was observed that upon the phosphate group modification near cytosine, ~100% conversion of cytosine to uracil was observed in just 24 h. Furthermore, we removed the ODN with counter base and modified the photo-cross-linker end with phosphate group to study the rate of reaction without hydrogen bonding and high hydrophilicity. It was found that the rate of reaction increased multifold with the modification giving ~100% conversion from cytosine to uracil in 3h at physiological conditions.

These results indicate that the deamination of cytosine to uracil is feasible at physiological conditions and heating to very high temperature is no more necessary to achieve the site-directed mutagenesis via photo-cross-linked cytosine. This has opened vast opportunities to use this enzyme free system in the biological samples at reduced cost and complexity to afford specific and site-directed cytosine to uracil conversions for the treatment of various genetic disorders like Leigh's syndrome.

Future Prospects

In this study, I have developed a refined way to carry out site-directed mutagenesis using enzyme free

photo-chemical methods at physiological conditions. This technique has wide applications in the field of anti-sense technology, RNAi, RNA/DNA editing in cell for genome engineering to eradicate certain genetic disorders arising due to single T→C point mutations.

Keywords

• Genome editing
• 3cyanovinylcarbazole
• Site-directed mutagenesis
• Photo-cross-linking
• Cytosine deamination

論文審査の結果の要旨

本論文では超高速光架橋反応用いた部位特異的核酸塩基変換法に関する研究についてまとめたものであり、以下の点で非常に有用かつ独創的な内容であった。

化学的あるいは酵素を用いた人工的な部位特異的核酸塩基変異導入は遺伝子修復や遺伝子治療に有用である。しかし、これら化学試薬あるいは酵素を用いた手法はそれらを用いるために至適 pH, 至適温度, 至適塩強度の条件下で用いる必要があった。これら制約条件から解放された、より汎用性の高い手法として、既にシアノビニルカルバゾールによる可逆的核酸類架橋法を基盤とした１塩基変異法が開発されている。ただ、この方法ではシトシンからウラシルへのピンポイント変異反応に９０度で３時間以上加熱する脱アミノ化過程が存在するため, 実際に細胞内での使用は難しいと考えられる。そこで、生理的条件下、光操作のみで細胞内 DNA 及び RNA 上のシトシンをウラシルへと部位特異的に変異させる今までにない分子操作法の開発を志向した。まず、標的であるシトシンの対合塩基が脱アミノ化効率に大きな影響を与えることを見出した。シトシンと塩基対を形成するグアニンをイノシンへと変化させることにより、１次反応速度で約 12 倍加速可能であることを見出した。様々な対合塩基（2-アミノプリン、ネブラリン、5-ニトロインドール）を検討した結果、標的となるシトシン周辺の極性が脱アミノ化効率に影響を与えることを見出した。次に、光架橋素子 3-vinylcarbazole 誘導体を用いた際の、脱アミノ化効率を評価した。その結果、親水性の置換基を有する際に脱アミノ化効率が促進されることを明らかにした。親水性を示す指標と考えられる分配係数値と反応効率を比較することにより 3-vinylcarbazole 誘導体の置換基の極性が脱アミノ化効率に影響を与えていることを見出した。そこで、シトシンの対合塩基と光架橋素子の置換基を最適化することにより脱アミノ化反応が約 15 倍加速されることを見出した。

以上の知見を基に、標的シトシン周辺にリン酸基を配置することで、脱アミノ化反応を１次反応速度で 760 倍加速させることに成功した。リン酸基の負電荷に起因する高極性を脱アミノ化反応場周辺に誘起させたことが、劇的な脱アミノ化反応の加速に繋がったと考えられる。従来

37°C, 3 days, 6 %という低い効率でしか進行しなかったDNA中部位特異的塩基変換反応を周辺構造の最適化や新しい置換基の導入により37°C, 6 h, 89 %という生体内で適応可能な条件内で進行可能となる新手法論の開発に成功した。

以上、本論文は、生体内で適応可能な部位特異的核酸塩基変換法の開発を行ったもので、有用かつ独創的なものであり、学術的に貢献するところが大きい。よって博士(マテリアルサイエンス)の学位論文として十分価値あるものと認めた。