Genome Engineering with Synthetic Copper Nucleases

N. Fantoni
T. Lauria
A. Kellett*

School of Chemical Sciences, National Institute for Cellular Biotechnology (NICB) and Nano-Bioanalytical Research Facility (NRF), Dublin City University, Glasnevin, Dublin 9, Ireland
andrew.kellett@dcu.ie

Received: 21.09.2015
Accepted after revision: 26.10.2015
Published online: 11.11.2015

Abstract Oxidative chemical nucleases represent an important class of synthetic compound that mediate nucleic acid recognition and strand scission. These agents have found utility as footprinting probes to map drug–DNA and protein–DNA interactions and also as clinical cytotoxins of human cancer. Herein, we highlight a recent contribution from our group on the application of the copper(II) 1,10-phenanthroline (OP) complex Cu(OP)₂ as a novel DNA-shuffling agent capable of generating highly diverse mutant protein antibody libraries. By employing a recombinant antibody gene fragment specific for prostate-specific antigen (PSA), we compared variation and PSA-binding specificity within Taq DNA polymerase I reconstructed Cu(OP)₂ oxidative digest fragments with hydrolytic restriction digests generated by DNase I. Results show the Cu(OP)₂ mutant library contained highly PSA-specific binding antibodies thus indicating that oxidative chemical nucleases may be applied to generate novel antibody clones not accessible through the use of the traditional DNA-shuffling enzyme DNase I.

Key words copper(II), nuclease, nucleic acids, radical oxidation, H-atom abstraction, strand scission

In the field of DNA manipulation, the design of artificial DNA cutters has become an area of considerable research interest. Synthetic copper chemical nucleases are a class of DNA cutter and can be categorised in two ways: i) those that mimic natural restriction endonucleases by catalysing hydrolysis of the phosphodiester backbone,¹ and ii) compounds that induce direct strand scission by oxidative H-atom abstraction from the pentose ring of DNA.² The biochemistry of bovine pancreatic deoxyribonuclease (DNase I) – a calcium- and magnesium-dependent hydrolytic enzyme that nonspecifically degrades both single- and double-stranded DNA – is described in Scheme 1 (A). Here, glutamate (Glu75) and histidine (His131) residues combine to catalyse hydroxide anion formation in proximity to the phosphate backbone.³ The resulting 5’-phosphorylated and 3’-hydroxylated ends produced by hydrolytic nucleases (e.g., DNase I or site-specific restriction endonucleases) can then be further manipulated, for example, in DNA recomb-
nation. Oxidative chemical nucleases, on the other hand, such as the copper(II) 1,10-phenanthroline (OP) complex Cu(OP)$_2$ (Scheme 1,C) induce direct strand scission on duplex DNA in the presence of exogenous reductant and oxidant.$^4$ Our group actively explore the biochemical and therapeutic applications of Cu(OP)$_2$-based systems and have recently applied this oxidative nuclease to genetically engineer a library of high-affinity prostate-specific antigen (PSA) binding antibodies through a process called DNA shuffling.$^5$ In this work, the selectivity and diversity of the Cu(OP)$_2$ recombinant antibody library was then compared directly to a library of mutant PSA-specific antibodies generated by DNase I.

The artificial nuclease activity of Cu(OP)$_2$ was discovered by Sigman in 1979 when the complex was observed to shear duplex DNA along A–T rich chains in the minor groove$^4$ with abstraction of C1′-hydrogen predominating over C5′ and C4′ positions.$^6$ Indeed, scission takes place preferentially in the minor groove on account of the binding interaction by the tetrahedral complex geometry assumed by Cu(OP)$_2$ in its cuprous form.$^7$

A seminal contribution to uncovering the oxidative mechanism by Cu(OP)$_2$ was made by the Greenberg group (Scheme 1); using elegant experimental design, their work identified direct strand breakage via $\beta$-elimination of a 2′-deoxyribonolactone intermediate 4 and demonstrated the Cu(OP)$_2$ complex behaving as general base catalyst.$^8,9$ Thus, current evidence indicates the strand-scission mechanism proceeds through H-atom abstraction at C1′ (1) followed, ostensibly, via the peroxyl radical intermediate 2 that expels superoxide (O$_2^-$)$^{10}$ to generate the oxidised C1′ cation 3. The incorporation of $^{18}$O into deoxyribonolactone (4) from H$_2^{18}$O has been established with the liberation of the free nucleobase (BH).$^6$ $\beta$-Elimination of 4 proceeds by general [Cu(OP)$_2$] base catalysis, and the final step in the mechanism sees the conversion of a 3′-furanone (5) adduct to the free 5-methylene furanone (6). The interaction of Cu(OP)$_2$ with deoxyguanosine was further studied by our group and shown to generate 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) lesions within superhelical plasmid DNA (Scheme 2) using the enzyme-linked immunosorbent assay (ELISA),$^{11}$

Until this point, both hydrolytic (DNase I) and oxidative [e.g., Cu(OP)$_2$] nucleases have found widespread application as footprinting agents for the study of DNA recognition by proteins and small molecules.$^{12}$ DNase I, however, has found further utility within the field of protein engineering through a process known as DNA shuffling (Figure 1A) that was introduced in 1994 by Stemmer.$^{13}$ This process – which is of great commercial importance toward enhancing the performance of target proteins in areas such as antibody affinity – involves the digestion of a parent gene by DNase I to form a mixture of shorter DNA fragments that are then annealed by repeated thermocycling in the presence of a primerless DNA polymerase process. Shuffled DNA libraries can then be inserted into a larger piece of circular (plasmid)
DNA and transformed into *E. coli* bacteria that, in turn, replicate these hybrid sequences and produce corresponding libraries of mutant antibody proteins. Recombinant DNA libraries can then be sequenced and antigen-binding affinity of newly produced antibodies accurately identified using technology such as surface plasmon resonance. Another method to achieve engineered protein libraries, staggered extension process (StEP), was developed by Arnold in 1998.14 This process prepares full-length recombinant genes (Figure 1,B) by firstly priming template sequences and then repeating cycles of denaturation and extremely short annealing and polymerase-catalysed extension. In this way, the growing fragments can anneal to a different template within each cycle and can thus acquire sequence information of different parental genes.

In our recent contribution, we utilised the oxidative nuclease complex, [Cu(OP)]2(H2O)2NO3, to generate a highly diverse recombinant library of PSA-specific antibodies through DNA polymerase I shuffling (Figure 1,A).5 Although Cu(OP)x complexes have been unambiguously shown to inhibit the activity of a variety of DNA polymerase I enzymes,4,11 it has not yet been shown if purified oxidatively sheared DNA and transformed into *E. coli* bacteria that, in turn, replicate these hybrid sequences and produce corresponding libraries of mutant antibody proteins. Recombinant DNA libraries can then be sequenced and antigen-binding affinity of newly produced antibodies accurately identified using technology such as surface plasmon resonance. Another method to achieve engineered protein libraries, staggered extension process (StEP), was developed by Arnold in 1998.14 This process prepares full-length recombinant genes (Figure 1,B) by firstly priming template sequences and then repeating cycles of denaturation and extremely short annealing and polymerase-catalysed extension. In this way, the growing fragments can anneal to a different template within each cycle and can thus acquire sequence information of different parental genes.

In summary, this work may potentially open a new application for copper(II) phenanthrene based compounds. Efforts to guide mutation to a specific gene segment might be achieved through the covalent linking of selective organoic-based DNA binding molecules; indeed distamycin conjugates of copper–phenanthroline complexes have already been developed and identified as site-specific oxidative nucleases.8,17 Another layer of control toward target gene digestion can be envisaged through the introduction of non-covalent recognition elements – for example, our group has recently shown that netropsin (minor groove binder) hinders the general accessibility of Cu(OP)x to the minor groove, the site of intensive DNA oxidation.11 Finally, the construction of designer intercalating ligands for incorpora-
tion into copper(II) complex scaffolds may uncover new sites of oxidative dsDNA damage that, in turn, could enhance variation of genetic sequence mutation.

Acknowledgment

This work was supported by Science Foundation Ireland (13/TIDA/B2682), the Irish Research Council (GOIPG/2013/937) and the Marie Sklodowska-Curie Innovative Training Network (ITN) ClickGene (H2020-MSCA-ITN-2014-642023), http://www.clickgene.eu/.

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