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Nucleic Acids – Chemistry and Applications

The year 2013 marked the 60th anniversary of the elucidation of the structure of the DNA double helix by Watson and Crick.[1] Its molecular structure comprises a backbone of repetitive units of sugar (2′-deoxyribose)-phosphate, with each sugar bearing one of the four nucleobases (A/G/C/T) through glycosidic linkages. The double helical superstructure of DNA spawned the understanding of the molecular basis of life in terms of how this genetic code could both decode and propagate. However, what was unforeseen was how the molecular and supramolecular structure of DNA would lead chemists to design future drugs, diagnostics, and new materials through explorations of chemical space in both modified DNA and RNA structures as well as its expanded functionality. Ranging from the realization of antisense-based nucleic acid analogues to covalent conjugation of functional ligands and the explosion of DNA as a nanoscale construction material, the most recent decades have seen DNA evolve from just "genetic" material to a "generic" material for several applications. Organic chemistry has been central to this evolution, from enabling the affordable, custom synthesis of any DNA/RNA sequence to the development of rapid and super-efficient methods to sequence DNA. The brisk pace of genetic engineering, chemical biology, bionanotechnology, and synthetic biology rests heavily on the development of enabling chemistry. This virtual issue seeks to highlight the frequently overlooked role of exciting chemistry underlying nucleic acid biology. We have highlighted 25 exciting papers from *J. Org. Chem.*, *Org. Lett.*, and *J. Am. Chem. Soc.* published between January 2012 and November 2013, under four broad categories—chemical modifications and DNA/RNA analogues, gene silencing and delivery methods, fluorescent nucleic acids, and self-assembly-derived DNA materials.

Chemical Modifications and DNA/RNA Analogues. The discovery and emergence of antisense effects and the growing applications of short RNA and micro-RNA require that short oligonucleotides (ON) be delivered into cells as therapeutic agents and possess favorable pharmacokinetic properties.[2] Natural DNA/RNA are inefficient at cell penetration, degraded by nucleases, and need chemical modifications to confer desirable properties, without compromising the key requirement of sequence-specific hybridization. The earliest approach was to replace one or two of the phosphate oxygens by sulfur to yield "phosphorothioate" or "phosphorodithioate" derivatives that confer nuclease resistance. In phosphorothioates, the P atom becomes chiral, necessitating stereoselective synthesis; otherwise, the derived phosphorothioate oligonucleotides will be a mixture of several possible diastereomers, undesirable for therapeutic applications. However, "Vitravene"—the first antisense drug approved by the U.S. Food and Drug Administration (FDA) in 1998—is a phosphorothioate diastereomeric mixture, having toxicity issues. Several attempts have been made toward stereoselective synthesis of phosphorothioates that pose synthetic challenges. The paper by Wada and co-workers[3] describes a method by which the stereoselective efficiency in coupling reaction is enhanced through the 2′O-substitution. By replacing the conventional TBS protecting group with a 2-cyanoethoxymethyl group in the chiral oxazaphospholidine monomers, followed by a coupling reaction on silica gel support, the authors could achieve stereoselectivity of greater than 99% for either *R* or *S* isomer. Continued synthesis yielded the all-*Rp* or all-*Sp* isomer depending on the nature of the monomer. It was found that the all-*Rp*-PS backbone oligomer (from *Sp* monomer) had higher duplex stability with the complementary ON than the destabilized duplex derived from all-*Sp* oligomer. The two oligonucleotides also had differential susceptibility to various nucleases. The availability of such P-stereodefined optically pure phosphorothioate oligonucleotides will have great utility in the design of RNA-based drug candidates, needed for siRNA and miRNA therapeutic strategies.

Another design strategy for chemical modification is the conformational preorganization of antisense ONs for efficient hybridization to target DNA/RNA sequences. Since DNA:RNA and RNA:RNA hybrids prefer A-conformation with C3′-*endo* sugar puckering, conformationally constraining the sugar ring by cross-linking of C2′−C4′ offers entropic benefits for hybridization. This has led to a variety of ON analogues such as BNA, ENA and LNA. The nature and size of the linking bridge has a crucial effect on the binding affinity and nuclease resistance of the derived ONs. The chemical nature of the constraining bridges has been mostly alkylidene, ether, or urea, comprising five- to seven-membered rings. Such bridges being moderately flexible do not rigidly preorganize the conformation, though they impart nuclease resistance. By employing a 2′-*O*,4′-*C*-ethyleneoxy bridge (EoNA), Hari, Obika, and co-workers[4] created an additional anomeric center at C4′ by introducing O into the bridge conformation to enhance the desired selectivity. The seven-membered 2′,4′-EoNA-bridged ONs stabilized the derived duplexes with single strands of cRNA and triplexes with double-stranded DNA relative to the unmodified ONs, retaining the excellent nuclease resistance.

Noted scientist Eschenmoser pondered why nature chose 5-carbon ribose rather than the 6-carbon glucose.[5] Invoking wonderful chemistry, a large number of hexose nucleic acids were synthesized, and the strength of complementary base pairing was found to be sugar-dependent, with comparable/lower stability for ribose nucleic acids and in some cases reasonable amounts of self-pairing. The chemistry of hexose nucleic acids has been extended to make several analogues to modulate the sugar conformation. D-Altritol nucleic acids (ANA) and their methylated analogues (Me-ANA) showed improved hybridization properties with cRNA. Migawa and co-workers[6] have constrained the six-membered sugar ring analogous to furanoribosyl rings through C5′−C3′ bridging with $-CH_2O-$. Though the idea was attractive, the ONs derived from constrained altritol sugars exhibited slight destabilization of RNA hybrids, which was attributed to an unfavorable conformation of the six-membered ring that forces

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the nucleobase into an equatorial orientation. Nevertheless, these analogues exhibited complementary base pairing.

Designing structural and nonionic or cationic substitutes of the anionic sugar−phosphate backbone to overcome the negative charge repulsion arising from sugar phosphate−lipid phosphate (membrane) interaction in order to improve cell permeation of ONs has attracted increased attention. An important outcome has been the design of "peptide nucleic acids" (PNA) by Buchardt and Nielsen,[7] wherein the charged sugar−phosphate backbone has been replaced by the neutral and acyclic ethylenediamine glycine backbone carrying the nucleobases via tertiary amide linkage. PNAs showed very strong affinity to cDNA/RNA sequences, even better than that of natural DNA−DNA/DNA−RNA hybrids. These achiral polyamides are easy to synthesize and have great promise for the development of antisense agents. However, their low aqueous solubility and poor cell permeability prompted the generation of a wide variety of PNA analogues, including cyclic and cationic varieties.[8] Recent work suggests that cationic substituents at γ-C are better than those at α-C in imparting better stability to derived PNA:DNA/RNA hybrids, and the cationic analogues also show good cell-penetrating properties.[9] An important factor in determining the extent of stabilization is the length of spacer chain and the nature of the cationic group. In this context, Mitra and Ganesh[10] have reported comparative regio- and stereospecific effects of pendant cationic aminomethylene side chains at α-C(R/S) and γ-C(S) on the PNA backbone in terms of their duplex-forming and cell penetration abilities. It was found that the stability of the derived duplexes with cDNA was better than that of the parent unmodified PNA and exhibited regio- and stereospecific dependence, with γ-C(S) > α-C(R) > α-C(S). The degree of cell permeability of these cationic PNAs followed by FACS technique followed the same trend. Further, these PNA analogues do not show any appreciable toxicity, and the next focus should be on design and study of PNAs that are more cationic (i.e., guanidinium) in nature and vary the spacer chain for optimal benefits.

The replacement of internucleoside phosphate linkages by structurally similar or isosterically equivalent groups such as alkylamide, sulfate, sulfonamide, etc. has not been successful due to difficulty in synthesis and hence routine accessibility. The triazole-linked ONs[11] directly link sugars through the triazole moiety with or without a methylene spacer and score better as a class of DNA mimics, due to their ease of synthesis by click reactions, nuclease resistance, and biocompatibility; however, the hybridization properties were either unfavorable for duplex stabilization or sequence-dependent. Varizhuk and co-workers[12] inserted an O atom in the linker to create an internucleoside N-methyleneoxy triazole link. These could be accessed by click reaction of 3′O-methyleneazido nucleoside with a 5′-alkyne nucleoside. The presence of this linkage at 5′,3′ or in the middle of the sequence gave triazole ONs that had duplex stabilities similar to those of the unmodified ONs. These were resistant to DNaseI and accepted as primers by polymerases in the PCR reaction. Further multiple modifications were shown to be biocompatible, making it a promising analogue for further exploration.

Gene Silencing and Delivery. Short oligonucleotides (DNA/RNA) are increasingly finding exceptional relevance as possible therapeutic agents[13] by virtue of their ability to access the RNA layer of gene regulation, and reprogram the cell. Barriers to such applications are their in vivo stability in serum-containing nucleases to which ONs are vulnerable, poor cell permeability, and target accessibility. Several approaches have emerged for antisense ON/siRNA delivery based on direct covalent modifications of the 5′- and/or 3′-termini of siRNA with lipid groups, small target directing molecules such as biotin and folate, peptides, nanoparticles, carbon nanotubes, or poly(ethylene glycol) (PEG).[14] Three interesting papers now report on methods to address some of these issues in alternative ways. Ichikawa and co-workers[15] describe a method to ligate the termini of hairpin oligonucleotides via click chemistry. This prevents the duplex from fraying and results in a circular oligonucleotide with enhanced stability against nucleases. The two complementary strands of duplex DNA are oriented antiparallel to each other. However, unusual pairing of A and T in Donohue pairing modes combined with isoguanine (isoG) and C or isoC and G can stabilize DNA strands in a parallel stranded conformation. Such short parallel duplexes have the advantage of nuclease stability in their duplexed forms but are plagued by intrinsic low thermal stability. In a key step forward, Pujari and Seela[16] have stabilized such duplexes by cross-linking the strands via 2′-C of their sugars using click chemistry, so that they remain nuclease-resistant.

Matyjaszewski, Das, and co-workers[17] describe another method wherein a stand-alone siRNA delivery system was constructed by conjugating the sense RNA strand at both 5′- and 3′-termini with temperature-sensitive, hydrophobic polymer PEG-methacrylate through click reaction. The siRNA−polymer bisconjugate was annealed with guide RNA to yield the polymer−escort RNA duplex, which was stable to both exonucleases and RNaseI. It could enter the cells on its own without any external transfecting agents. The conjugated polymer−siRNA duplex was able to knock down the expression of target luciferase gene in encoded plasmid at just 1/5 the concentration and was similarly efficient in knocking down other genes of embryonic kidney cells and T cells, thus proving the generality of this approach.

Fluorescent Nucleic Acids. Conjugation of DNA/RNA with fluorescent probes has enabled functional studies of nucleic acids, biological sensing, and as probes for living cells and living organisms.[18] This area is growing unabated in terms of the new designs of fluorophores and the methods of conjugation. When coupled with amplification tools (PCR), this technique is not only pushing the sensitivity to the limits but also leading to new and novel applications of DNA/RNA probes. The fluorescence properties of pyrene have remarkable environment dependence, and its ON conjugates continue to draw attention as hybridization probes for detection of duplexation with cDNA/RNA and to characterize supramolecular assemblies.[19] By covalent linking of pyrene at different sites of nucleosides, the fluorophore can be positioned to intercalate within base stacks from major groove/minor groove sides or simply position it within the groove. Employing click chemistry, Nielsen and co-workers[20] have linked pyrene either at the 5′- and/or 2′-positions of sugar and used these probes to investigate the formation of secondary structures such as bulged duplexes and 3-way junctions from appropriate nucleic acid complements. The consequences are a decrease in the fluorescence intensity with increase in duplex stability upon intercalation or an increase in fluorescence intensity with decreased stability in groove binding. Constructs designed to generate secondary structures specifically orient the two pyrenes leading to observation of the excimer bands, illustrating

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the versatility of pyrene as a diagnostic fluorophore in probing nucleic acid assemblies.

Seela and co-workers[21] present another way to conjugate pyrene wherein the fluorophore is linked to the nucleobase at C7 of 7-deazaadenosine or 7-deaza-8-azaguanosine moiety by a click reaction using a longer octadiynyl linker or two pyrene units through a shorter tripropargylamine linker. The longer linker analogues showed only monomer fluorescence, while the shorter linker analogues carrying two pyrenes in proximity exhibit excimer emission. These conjugates markedly stabilized the derived duplexes due to stacking interactions, and the photophysical properties of 7-deaza-8-aza analogues were superior to that of 7-deaza analogues, adding a new feature to the usefulness of pyrene fluorophore in nucleic acids.

The design of universal hybridization probes that possess identical affinity toward any of the complementary bases is desirable for applications in degenerate PCR primers and microarray probes. Some unnatural heterocycles have been found to be useful albeit with low effectiveness in hybridization applications due to compromised duplex thermostability. Sau and Hrdlicka[22] have now reported U-nucleoside conjugates in which pyrene is linked to C2′ through a triazole linker. The resultant ONs show almost equal thermal stability with complementary and mismatched bases and an exceptional affinity toward abasic sites on the cognate strand. This is a consequence of pyrene intercalating into the duplex by flipping the base in the opposite strand outside of the helix. Leumann and co-workers[23] replaced the nucleobase by pyrene in a self-complementary DNA sequence and found that in the duplex, the pyrenes from the opposite strands can stack resulting in pyrene excimer fluorescence. Such designs of aromatic π-arrays are of interest in the construction of novel DNA-based sensors.

Simple modifications of natural bases without affecting their complement recognition properties can render them fluorophoric, and such inherently fluorescent nucleic acids have good utility as hybridization probes.[24] Diederichsen and co-workers[25] found that the introduction of a vinyl group at C8 of guanine makes it fluorescent and synthesized the corresponding fluorescent PNA monomer. The PNA oligomers incorporating this unit were used as fluorescent hybridization probes to monitor PNA:PNA and PNA:DNA/RNA duplexes and the PNA exchange reaction with RNA quadruplexes. Such fluorescent base analogues akin to the parent base yet non-interfering to base pairing offers least perturbation to the microenvironment and prove more accurate structural reporters.

Interesting fluorescent labels that exploit the DNA scaffold have been developed that could have imaging applications. The first of these reported by Wagenknecht and co-workers[26] utilizes the chirality and the precise positioning of nucleobases in a base pair and base stack of B-DNA to position two fluorophores, Texas orange and nitroimidazole, in complementary strands. Such juxtaposition invokes excitonic, excimer, and energy transfer interactions to access bright fluorescence in both green and red wavelengths. Upon successful hybridization, photoelectronic interactions lead to a bathochromic shift and a visual color change to yellow/amber, resulting in what the authors' term "traffic light" DNA. The second example reported by Hocek and co-workers[27] is a probe based on the fluorescent moiety 4-hydroxybenzylidene imidazolinone (HBI) inside the β-barrel core of protein GFP. Its fluorescence is high as it is held tightly within the β-barrel, and the energy loss through nonradiative pathways due to fluorophore motions is abrogated. This principle is utilized by attaching an HBI derivative to a DNA duplex such that its binding to a DNA-specific protein immobilizes the dye, resulting in enhanced HBI fluorescence. This could have wide applications as a sensor of DNA-binding proteins.

An important emerging question is how the epigenetic chemical modifications are reversed to that of the parent genetic material, as in the conversion of epigenetic base 5-methylcytosine into cytosine. The discovery of three new nucleobases, 5-hydroxymethylcytosine (hmC), 5-formylcytosine (fC), and 5-carboxycytosine (caC), corresponding to oxidation products of 5-Me-C, have suggested them as key chemical intermediates mechanistically driving the demethylation process.[28] A complete understanding of the biosynthetic pathway of demethylation requires the identification of proteins that bind to such intermediates, and this needs substrate synthetic oligonucleotides containing multiple units of the modified bases and reagents that can sensitively detect them in cells. Carell and co-workers[29] have now reported the successful chemical synthesis of the nucleotide 5′-triphosphates of the three modified C bases and their incorporation into DNA by PCR. Ready access to the modified C-ONs will accelerate study of the biochemistry of the demethylation of the epigenetic base. Due to the dynamic nature of the methylation−demethylation process, fluorescent reporters of these nucleobases will be key to uncovering their biology. Zhou and co-workers[30] have now used reactive hydroxylamine chemistry to deposit fluorescent groups on formyl cytosine sites on DNA. Such fluorescent detection of 5-fC at the genome level will pave the way for deciphering the regulatory role of epigenetic signatures.

Spatiotemporal control over the unmasking of nucleic-acid-based signatures is key to understanding their biological roles and can have applications in theranostics. To this end, light has proved a very valuable extrinsic cue to trigger the release of caged nucleic acid domains.[31] Furuta and co-workers[32] report an interesting development in the use of a modular caging chemistry comprising an amine reactive NHS ester, a photoactive Bhc group, and a tosylhydrazone, which is a precursor of the diazomethyl group. The triad (NHS-Bhc-tosylhydrazone) can generate the reactive coumarinyl diazomethane that reacts selectively with a phosphate residue of siRNA. This caged nucleic acid can be delivered into cells, and upon photoirradiation, triggers the scission of the coumarin−phosphate bond, releasing the RNA component. Such siRNA conjugates will have versatile applications in photocontrolled release of siRNA at the desired site within cells.

Hybridization-dependent DNA-templated chemical reactions have attracted great attention, and many classes of organic reactions have been successfully achieved.[33] Adding to this repertoire is the photocatalyzed reduction of azide using a photocatalyst $[Ru(bpy)_2phen]^{2+}$. Winssinger and co-workers[34] have adapted this reaction to a nucleic acid template version using a DNA host strand to bring the catalyst and substrate into close proximity by conjugating them to each half of the complementary PNA, followed by hybridization. Light of appropriate energy triggers reduction of the substrate azide by electron transfer from the photocatalyst, followed by rapid restoration of catalyst by ascorbate. Consumption of only 2% catalyst with a 15-fold turnover and 35% yield of product was observed. Design of an immolative linker to conjugate the fluorescent azide uncaged the fluorophore after reduction.

Self-Assembly-Derived DNA Materials. DNA is now being seen as a scaffold for nanoscale construction onto which

functionality may be integrated.[35] A new scaffold for DNA display is typified by spherical nucleic acids pioneered by Mirkin and co-workers[36] that are a combination of nanoparticles coated with short nucleic acid constructs. By virtue of their intrinsic physicochemical properties, they have potential applications in diagnostics, gene regulation, medicine, and as nanomaterials. The Perspective outlines the current state of knowledge in this exciting domain of nucleic acids. Unusual base pairing schemes lie at the very heart of integrating functionality into exquisite designer DNA architectures. Seela and co-workers[37] describe an unusual base pairing scheme between pyrollocytosine derivatives that pair with each other by incorporating Ag^+ ions between them. It is noteworthy that with two Ag^+ ions per base pair, this pairing incorporates the highest density of metal ion into duplexes, which could have potential impact in metallization of DNA and creation of molecular wires.

The functionality of nucleic acids stems from their consummate ability to recognize diverse molecules thus forming the basis of excellent sensors. The transduction of such sensing events into electrical signals is a challenging problem that requires the easy and efficient integration of nucleic acid scaffolds onto surfaces. Herrmann, Patolsky, and co-workers[38] have outlined an ingenious method to reversibly attach DNA segments to lipid monolayers functionalized on surfaces by attaching lipophilic tails to nucleobases along a short stretch of the oligonucleotide of interest. These lipophilic tails interdigitate into the immobilized membrane, while the rest of the functional nucleic acid domain projects outward perpendicular to the surface.

DNA conductivity has been the subject of much interest in the past, and methods to measure it in solution pose considerable challenges.[39] In an interesting development, Hocek and co-workers[40] have attached an electrochemical label such as butyl acrylate at 5-C of U/C and at C-7 of 7-deaza A/G, and the derived NTPs were incorporated into DNA by primer extension to obtain acrylate-modified DNA. Electrochemical studies of such butyl-acrylate-substituted DNA duplex indicated that these can serve as electrochemical probes of DNA functions as an effective redox label. Further post-synthetic transformations such as ligand conjugation for surface or electrode immobilization polymerization, etc., may lead to new material applications of DNA.

Recent rapid developments in fluorescence instrumentation is leading to novel approaches for biomolecular analysis and real-time imaging of changing chemical microenvironments of proteins inside living cells.[18] In this context, Kool and co-workers[41] have reported a novel method of genetically encoding proteins with multispectral probes. The nucleobases in DNA have been replaced with different sets of a wide variety of fluorophores, which in each set have a common excitation wavelength but different emission spectra. This is a consequence of inter-chromophoric optical interactions based on energy and excitation transfer processes such as Förster resonance energy transfer, exciplex, and excimer, yielding dyes with extraordinarily large Stokes shifts. Termed "oligodeoxyfluorosides" (ODFs), these materials are easy to be made on DNA synthesizers. They are then attached through a spacer chain with ligands that can bond to specific proteins or with substrates that react with enzymes to form covalent conjugates. Since the same ligand/substrate linked to different ODFs can give different emissions, the corresponding protein/enzyme can be labeled to emit in numerous colors. The authors have demonstrated the principle by tagging a haloalkyl ligand, which is a substrate for engineered dehalogenase enzyme, to multiple ODFs. Upon interaction with dehalogenase, these form enzyme−substrate covalent conjugates that can be visualized in multicolors. This technique has great potential to study protein interactions within cells in real time, providing a versatile alternative to GFP.

Bionanotechnology is a rapidly growing area at the interface of chemical, material, and biological sciences.[35] The versatility of a wide class of nanoparticles in terms of their photoptical properties (tunable multicolor emission, suppressed autofluorescence, and high photostability) and biocompatibility (deep tissue penetration and low in vitro/in vivo toxicity) coupled to the biological functions of nucleic acids and proteins have led to construction of multipurpose materials.[42] In this context, Lu and co-workers[43] have fabricated DNA−nanoparticle composites that have many desirable features for practical applications. Lanthanide-doped nanoparticles ($NaMF_4:Yb^{3+}/Ln^{3+}$) up-convert near-infrared (NIR) excitation to tunable shorter-wavelength luminescence spanned over deep-UV to the NIR range. Single-stranded DNA deposited on such nanoparticles (DNA-UCNP) through ligand exchange showed a green emission. Upon hybridization with Au−cDNA/RNA−Au nanoparticle conjugates, formation of star-like structures was visualized by TEM, confirming hybrid formation. The DNA−UCNP entered HeLa cells in the absence of any transfecting agents, visualized by green fluorescence in the cytoplasm. The retention of recognition capability was demonstrated by hybridization with cDNA inside cells and targeted delivery of an aptamer to nucleolin. The reported DNA−UCNP materials with a combination of multiple properties will accelerate progress of DNA-based bionanotechnology, with applications in bioinspired nanoassembly and biomedicine.

Fluorophoric dye materials have long been employed to visualize tissue sections and cells (staining). The progress in our understanding of how these bind with macromolecules in cells should lead to the design of custom stains for different organelles within cells. Nuclear stains that light up the nucleus do so by intercalation, groove binding, or electrostatic interactions with the double helix of DNA. Quantification and 3D analysis of stained images are necessary to learn about nuclear architect.[44] The availability of fluorophores with optimal photoproperties for such applications using laser microsopy presents an unmet need. Westerlund, Wilson, and co-workers[45] now report the chemical design of DNA binding green and yellow fluorescent probes that possess requisite brightness in addition to being membrane- and cell-permeable. These agents are derived from piperazinyl−piperidinol and piperazinyl−phenyl propanadioate diborane complexes which are groove and intercalating DNA binders, respectively. Being environment-sensitive, their emissions are turned-on upon interacting with DNA. They are superior alternatives to other stains such as DAPI, HOECHST, TOTO, or YOYO whose photoexcitation and emission properties are not optimally matched with 405 and 514 nm laser lines, unlike that of piperazinyl derivatives. Because of low toxicity, improved membrane permeability, selective staining of nucleus, and turn-on features, these probes could have wide applications in live cell imaging.

Thus we see that the interest in chemical modifications of nucleic acids is continuing rapidly and with great imagination in terms of the nature and type of modifications. What is

noteworthy is (i) the abundant exploitation of click chemistry to link various ligands and conjugating groups, (ii) the diversity of fluorophores that are being conjugated to make colorful nucleic acids, (iii) the functionalization of nucleic acids to enable gene delivery such as improving cell permeability and cellular localization, and (iv) exploiting the high fidelity self-assembly to design new DNA-based functional materials, ranging from optical to electronic materials to nanocomposites.

Krishna N. Ganesh

Indian Institute of Science Education and Research, Division of Chemistry, 900, NCL Innovation Park, Dr Homi Bhabha Road, Pune, Maharashtra India

Yamuna Krishnan

National Centre for Biological Sciences, Biophysics, Biochemistry and Bioinformatics, TIFR, GKVK Campus, Bellary Road, Bangalore 560065, Karnataka, India

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