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Concept Transfer—From Genetic Instruction to Molecular Logic

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This review describes the advances made in utilizing the unique recognition and structural characteristics of DNA to perform Boolean algebra using complex logic functions such as AND, XOR, NAND and INHIBIT based on chemical or photonic inputs. A comparison of these results to the action of novel 12-mer antisense peptide nucleic acid (PNA) constructs targeting the AMPA receptor in live motor neuron hybrids (NSC34) is made. In this case, a NOT function is displayed through down-regulation of the GluR3 subunit, a result that impedes other cellular chemical processes. The consequence of cellular logic based on chemical inputs such as PNAs and their utilization as whole-cell machines whose biological output is chemical input-dependent is discussed briefly.

Keywords: Molecular logic; Peptide nucleic acids; AMPA receptor; Amyotrophic lateral sclerosis; DNA-based logic

INTRODUCTION

The computerized foundations of our modern society have led to a consumer-driven demand for the production of cheaper, faster and more efficient technologies. The trend in recent decades to miniaturize electronic components by dismantling elemental arrays down to the nanometer level using techniques such as electron-beam and photolithography is now limited due to problems associated with semiconductor properties, heat dissipation and the costs of fabrication [1,2]. An attractive alternative is via a "bottom-up" approach [3], using molecular building blocks to construct well-defined functioning devices and machines. The use of individual molecules (that may work cooperatively) that can be reversibly interconverted between two or more different states upon an external stimulus has the potential to store, process and record data in an analogous manner to the binary logic of silicon-based computing [4]. In fact, the fundamental principles of Boolean algebra have been demonstrated through shrewd interpretation of spectroscopic changes in ionic, electronic or photonic inputs to small molecules [4–9]. These inputs primarily lead to photonic outputs, the intensity of which yield complex logic functions such as AND, XOR and INHIBIT at the molecular level.

Biology has an analogous chemical signaling approach through genetic instruction. Central to the production of functioning systems on both the cellular (μm) and subcellular (nm) levels lies the molecular information stored in DNA. Using a variety of operations, including replication, to amplify the information, transcription to mRNA and translation of the information into (ultimately) proteins, biological systems have evolved to an unprecedented complexity [10]. These remarkable processes have given a number of research groups the inspiration to advance molecular-scale logic past the "simple" molecule stage by using the unique structural and electronic characteristics that DNA possesses, and in so doing have begun to demonstrate extremely complex Boolean operations in a wholly synthetic environment. Here we present some of the recent representative examples of logic operations involving DNA and introduce research in progress from our laboratory towards the goal of chemical computing in biological systems.

SINGLE GATE LOGIC FUNCTIONS BASED ON DNA

The use of oligonucleotides based on DNA as the foundation of a new focus in logic gate formation,

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and ultimately molecular computation, lies in the ability of scientists to utilize the knowledge gained through genetic manipulation and visualization [10–13]. In addition, the burgeoning field of biosensors and its associated technologies [14,15] support the transition of molecular logic from solution to the solid state [16]. Central to this strategy is the structurally well-defined and regulated nature of the various duplexes, triplexes and quadruplexes DNA can take, based on the interplay of the pyrimidine and purine bases A, T, C and G through hydrogen bonding and π-stabilization. This structural definition allows recognition elements such as intercalation, minor and major groove binding, long-range hole transport and site-specific modifications to be used as potential operators to generate the desired logic function.

The first example (Fig. 1) provides a unique demonstration of how structural regulation can provide the basis for simple logic operations and in a uniform cyclical manner. The novel logic gate, described as a "molecular machine" by Liu and Balasubramanian [17], is based on the pH sensitivity of a unique four-stranded DNA structure, called the *i*-motif, that forms when the oligonucleotide gate contains four segments of three cytosine bases arranged in a precise sequence in aqueous acidic (pH < 6) solution (Fig. 1). This pH is low enough to allow the protonation of cytosine residues, causing the formation of a noncanonical base pair between protonated and unprotonated cytosines. A collection of these pairings interdigitate to form a quadruple helix that is stable in acidic solution. Attached to the ends of the oligonucleotide gate are a rhodamine green fluorophore and a dabcyl quencher. In the quadruplex form, the two chromophores interact leading to an *ca.* 85% reduction in the fluorescent output of the rhodamine fluorophore. The second component to this gate is a 17-mer DNA strand that is complementary to the quadruplex oligonucleotide sequence, with the exception of two mismatches that are necessary to stop the 17-mer from folding as an inactive G-quadruplex. When the pH is raised above 8, the quadruplex unfolds and is captured by hybridization to the 17-mer to form an extended double-helical structure. Now that the two chromophores are spatially separated, a fluorescence output is observed, reminiscent of the mechanism displayed by a molecular beacon. The process of addition of acid to the system causes a spontaneous reversal to yield the starting quadruplex and fluorescence is lost. In terms of logic operations, which may oversimplify the mechanics of the process described, the forward direction (addition of OH^-, Fig. 1) indicates a YES gate while the reverse yields a NOT gate. Although very simple in terms of logic function, we highlighted this system for two reasons. The first is the unique use of structure regulation to invoke a fluorescent response and the second is the cyclical demonstration of resetting (acidifying) the system based on the forward reaction, which shows negligible decomposition over 30 cycles.

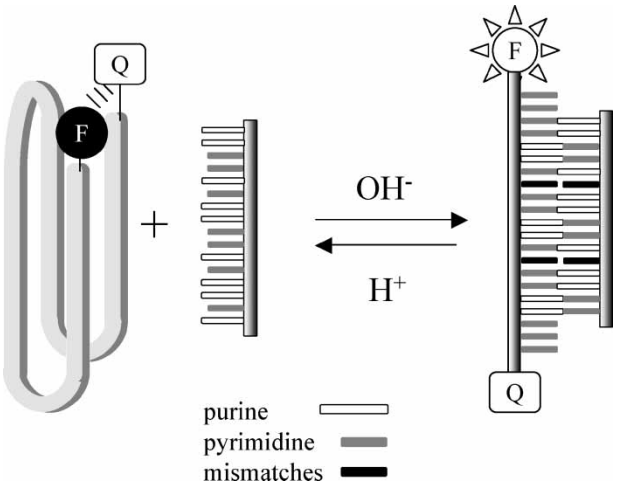

FIGURE 1 The pH sensitive nature of the quadruplex in the presence of the complementary 17-base sequence yields a YES gate that is easily reset. F = rhodamine green fluorophore (λ_{ex} = 504 nm; λ_{em} = 534 nm) and Q = dabcyl quencher.

Stojanovic *et al.* have contributed significantly to the area of biocomputing and complex oligonucleotide gates based on deoxyribozyme chemistry [18,19]. These gates were constructed by combining molecular beacon stem loops with very specific deoxyribozymes that catalyze the cleavage of the phosphodiester backbone of a chimeric substrate at the site of a single ribonucleotide embedded in the gate framework. One of their simplest examples, a NOT gate, is based on fluorescence output of the cleavage product (Fig. 2). In its active form, the stem loop assembly is able to undergo enzymatic cleavage at the central ribonucleotide to yield two fragments, one containing the rhodamine quencher (A) and the other the fluoroscein (D) chromophore. The fragments do not bind as well to the stem loop component as the intact oligonucleotide and so decomplexation occurs. The separation between D and A leads to a 10-fold increase in fluorescence output, which is monitored at 520 nm. Hybridization of the complementary oligonucleotide input to the loop segment before enzymatic cleavage results in stem opening and catalytic inhibition. As a result the gate remains unchanged and no fluorescent output is observed. The power of this approach may lie in the fact that because oligonucleotides are used as both inputs and outputs, the output of one logic operation may be used as the input to another, thereby providing communication between two or more gates in solution. More complex gates including AND and XOR based on this technology are discussed later in the context of molecular addition.

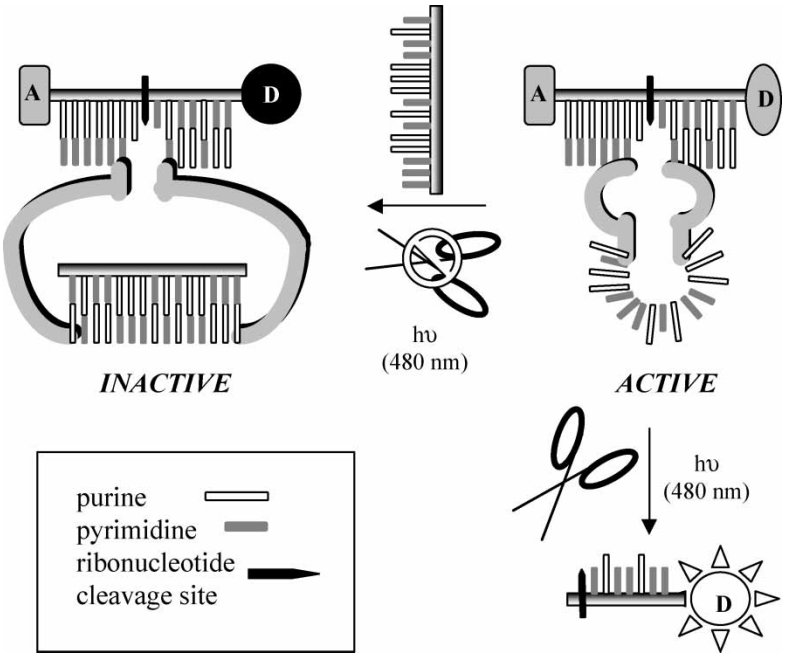

FIGURE 2 The single-input NOT gate is constructed through substitution of a nonconserved loop in a deoxyribozyme with beacon stem loop complementary to the input oligonucleotide. Enzymatic cleavage of the embedded ribonucleotide, releasing the fluorophore D ($\lambda_{em} = 520\,nm$), is prevented upon addition of the oligonucleotide input. A = rhodamine acceptor.

Saghatelian *et al.* have also contributed to the field using a different but complementary (photonic) approach [20]. The design feature highlighted from their work involves the unique ability of the DNA duplex to undergo both intercalation (with ethidium bromide) and minor groove binding (using Hoechst 33342) and the interplay between the two with a carboxyfluorescein fluorophore located on the 3′-end of a 16-mer oligonucleotide gate. In order to allow either intercalation or minor groove binding to play a role, a third input (in the form of a complementary oligonucleotide) is required to form the necessary duplex structure. From this statement, it is obvious that no change in output will be observed from either the ethidium bromide or the Hoechst 33342 inputs without the complementary oligonucleotide input. In demonstrating an AND gate, the complementary oligonucleotide input hybridizes to the gate strand, creating a binding groove complementary to the Hoechst 33342 input. This two-stage complexation positions the groove binder with close enough proximity to allow FRET ($\lambda_{em} = 350\,nm$), with the fluorescein resulting in fluorescence enhancement at 520 nm. Replacing the Hoechst 33342 input for ethidium bromide (Fig. 3) causes quenching rather than enhancement of the fluorescence upon irradiation at 490 nm. This simple modification results in the construction of the computationally powerful NAND gate. This gate is similar to the AND operation except that all processes are passed through an invertor. This means that fluorescence is now detected for all input combinations except when the oligonucleotide and ethidium bromide inputs are acting simultaneously. A similar NAND process has been demonstrated by Baytekin and Akkaya using a simplified assembly based on the complexation of the nuclear stain DAPI to an A/T nucleotide base pairing [21]. The combination of the AND and NAND gates culminates in an output ($\lambda_{ex} = 350\,nm$; $\lambda_{em} = 520\,nm$) that corresponds to a three-input INHIBIT gate (Fig. 3). The power of INHIBIT lies in combination with other gates such as XOR which, under the correct conditions, can yield a half-subtractor [22].

COMBINATIONAL LOGIC—MOLECULAR ADDITION

The fabrication of a molecular computational device is only feasible if it is able to demonstrate the ability to perform arithmetic operations. Of those possible, addition is the most sought after [23]. A molecular "half-adder" is the simplest of the combinational circuits able to carry out binary addition operations (Table I). The system must analyze the presence of two input molecules, which designate the augend and addend bits, and come up with two different outputs, the sum and carry, through the combination of AND and XOR gates, respectively. The use of two half-adders yields a "full-adder" that can not only add three bits but also be used in series for more complex additions.

Stojanovic and Stefanovic have constructed an array of three deoxyribozyme-based logic gates that behave as a half-adder (Fig. 4) by taking advantage of

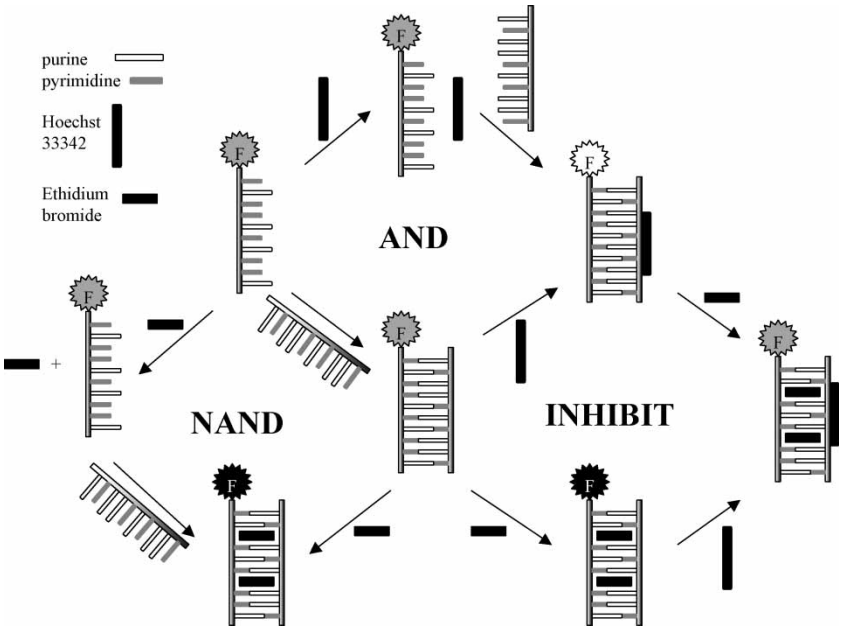

FIGURE 3 Schematic representation of a DNA-based logic gates reported by Saghatelian *et al.* Inputs to the fluorescent-labelled oligonucleotide gate are schematically shown in conjunction with the arrows. The combination of AND and NAND yields the three-input INHIBIT gate. Fluorophore legend: F, fluoroscein: Black, significant fluorescence quenching; Grey, moderate fluorescence; White, maximum fluorescence.

TABLE I Truth table for the input–output relationships of a half-adder

Inputs		Outputs	
x	Y	C	S
0	0	0	0
0	1	0	1
1	0	0	1
1	1	1	0

the output fluorescence of fluoroscein (at 520 nm) and tetramethylrhodamine (TAMRA, at 570 nm) [24]. The XOR gate necessary for the addition operation is constructed through an ANDNOT function described by using two of the three deoxyribozymes gates working together in series (Fig. 4a). The other deoxyribozyme produces the AND function in a parallel operation (Fig. 4b) without cross-talk. To show the power of their approach, Stojanovic and Stefanovic have incorporated 23 molecular-scale logic gates based on their proven deoxyribozymes in a 3 × 3-well array (encoded to nine input oligonucleotides) to describe one of the first examples of molecular automation that acts interactively with a player [25]. In this game of naughts and crosses, a move by the player entails addition of one input oligonucleotide to all wells of the nine-well matrix. The input has no effect in some wells and actively inhibits gates in others, but in every case fully activates the gate in a single well leading to a fluorescent output. Of course the automaton makes the first move and always chooses the center well! While this game does not reflect the complexities of Kasparov *vs.* IBM's chess-playing computer Deep Blue,[†] the interactive demonstration does show a proof-of-concept.

Okamoto *et al.* have devised a strategy for preparing AND and OR logic gates and ultimately a full-adder, based on hole transport technology (Fig. 5) [26]. In this system, the logic gate strand contains a hole-transporting nucleobase (termed ^{MD}A) in sequence with two GGG sites; one proximal (Ga) and one distal (Gb) to the ^{32}P-label of the gate. The output involves oxidative strand cleavage catalyzed at the GGG sites by hole transport. The nature of the output, that is whether a 0 or 1 is obtained, is dependent on the magnitude of the Ga/Gb cleavage product ratio (Fig. 5). The input strand contains the pyrimidine bases T or C, which modulate the efficiency of the hole transport between ^{MD}A/T and ^{MD}A/C base pairs, along with a cyanobenzophenone substituted uridine as the hole injector ($\lambda_{ex} = 312$ nm). Multiple ^{MD}A bases within a single oligonucleotide gate provided the basis for AND logic under this protocol while three logic gate strands were used to construct an OR logic gate (Fig. 5). The conjoined use of these two gates was found applicable to complicated combinational circuits such as the full-adder by using two sets of

[†]During 1996, Gary Kasparov, one of the great chess minds of the twentieth century, defeated IBM's Deep Blue in a six-game chess match by three games to one. The rematch in 1997 was won by Deep Blue.

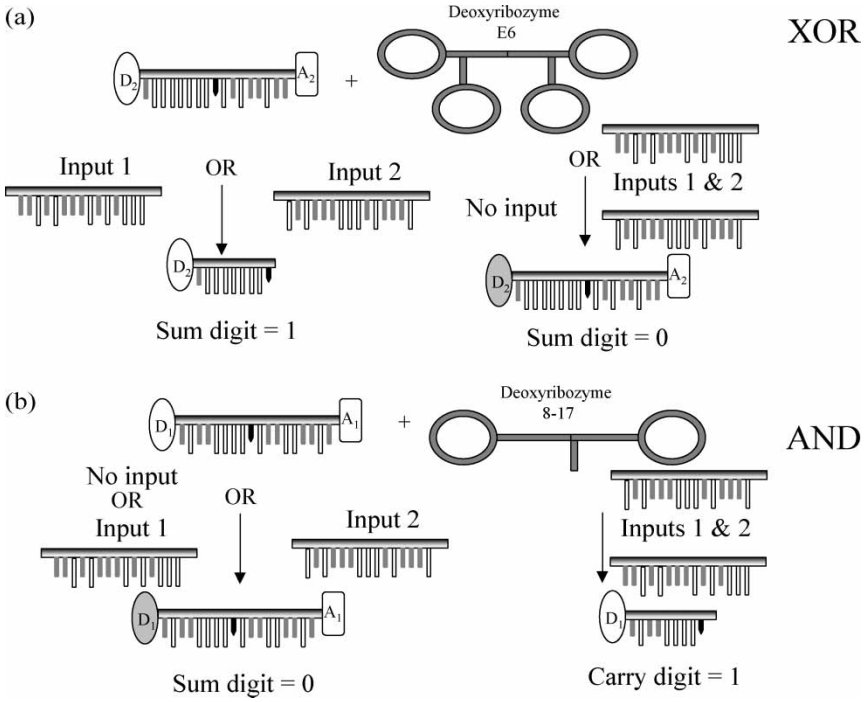

FIGURE 4 The half-adder described by Stojanovic and Stefanovic uses the same deoxyribozyme chemistry of their earlier gates. Careful choice of fluorophore and excitation wavelength yield the required outputs. (a) The XOR function is constructed through an ANDNOT gate using the TAMRA fluorophore ($\lambda_{ex} = 530\,nm$, $\lambda_{em} = 570\,nm$). (b) The AND function is constructed using the fluorescent output of a fluoroscein-labelled oligonucleotide gate ($\lambda_{ex} = 480\,nm$, $\lambda_{em} = 520\,nm$).

four oligonucleotide logic gate strands set in parallel (Fig. 5).

As with Stojanovic's half-adder, the time needed to achieve addition is slow compared to the time-scale of modern computers. With this said, we should remember the early days of computing in which IBM's 650, the UNIVAC or AITKEN computers, took days to weeks to solve simple problems. The difference in this analogy is that for every IBM 650 needed to do a calculation, the examples illustrated in this section have of the order of 10^{15} gates working in parallel!

DEMONSTRATING LOGIC FUNCTIONS IN BIOLOGICAL SYSTEMS

Many proteins in living cells appear to have as their primary function the transfer and processing of information, rather than chemical transformation or the building of cellular structures [27–29]. Such proteins are functionally linked through allosteric or other mechanisms into biochemical "circuits" that perform a variety of simple computational tasks including amplification, integration and information storage [30]. The formation of these proteins is achieved through the processes of transcription and translation of the genetic code. Hence, within biological systems, a series of combinational logic operations exist in which the information is transferred and processed chemically to maintain the cell in a "healthy" state.

If we are to consider the view of Braich *et al.* [10] that molecular computers can be a means of controlling chemical biological systems in the same way that electronic computers have provided a means for controlling electrical/mechanical systems, then a useful starting point for control would be the manipulation of the genetic code. From our perspective, the same information transfer that yields the necessary life-sustaining processes may also yield rogue or "death-signaling" pathways, which can lead to neurodegenerative disease, through misinformation. In essence, we can regard the human genome as a punch card containing all the necessary information to pose a problem. The operations of transcription and translation lead to chemical outputs (proteins) that are biologically active. A mutation in the genetic code can be thought of as a wrongly punched card that may or may not provide a different output under the standard operations.

Excitotoxicity in motor neurons results from elevated intracellular calcium ion (Ca^{2+}) levels, which in turn recruit cell death signaling pathways. Recent evidence suggests that AMPA receptor subunit GluR2/3 stoichiometry is a dominant factor leading to excess Ca^{2+} loading in neurodegeneration (Fig. 6a) [31]. As part of this study, we showed that immortal *neuroblastoma* × *spinal cord* cells (NSC-34) were found to express low levels of GluR2 compared

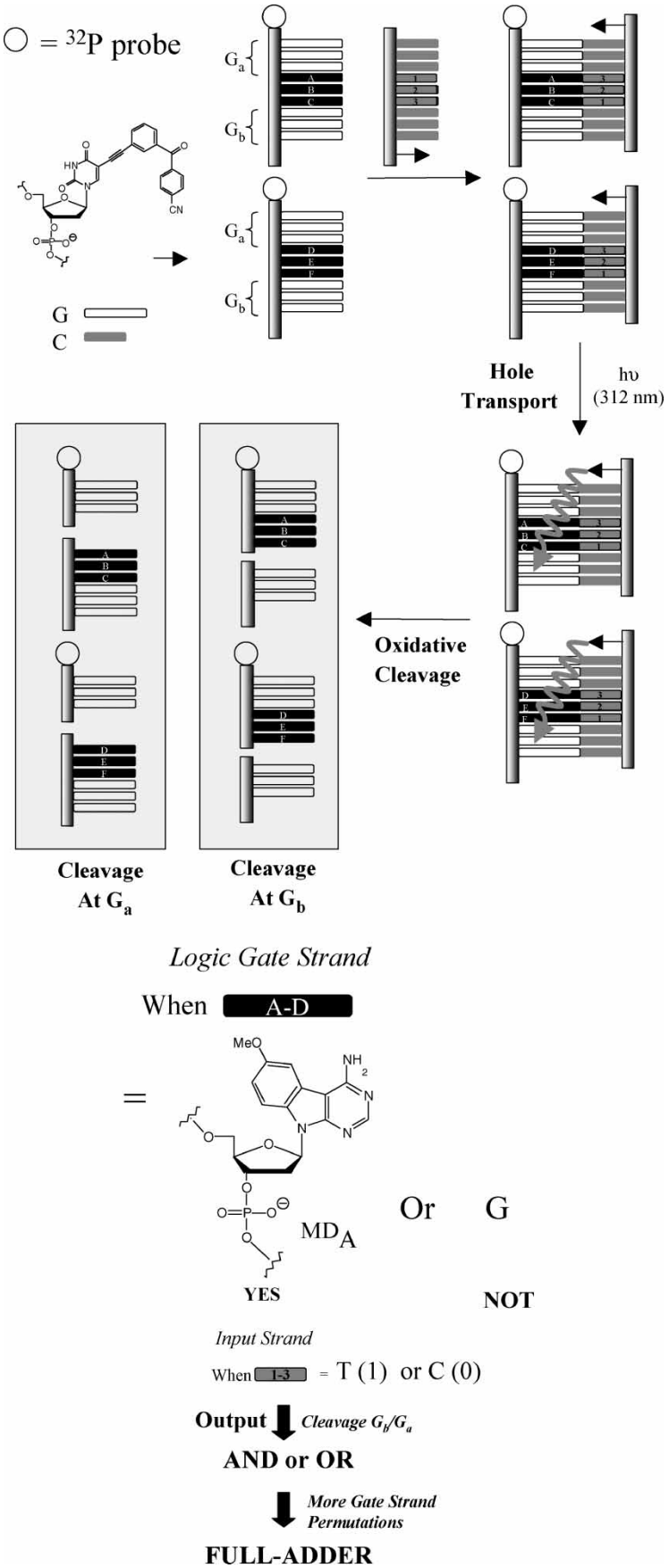

FIGURE 5 Schematic illustration of a DNA logic system used to demonstrate AND and OR logic functions through hole transport. Hole transport is activated through photoirradiation of a cyanobenzophenone-substituted uridine (arrow) at 312 nm. The unnatural base ^{MD}A is a hole transport facilitator. Added complexity in terms of the number of gates and inputs leads to the construction of a molecular full-adder.

to GluR1, 3 and 4, thus rendering them vulnerable to calcium-mediated damage and excitotoxicity. In order to target and switch off the production of GluR3 (and hence reduce the influx of Ca^{2+} ions into motor neurons) we designed a 12-mer antisense peptide nucleic acid (PNA) directed against that part of the genetic code responsible for GluR3 production. We chose this unnatural input over standard oligonucleotides because of the susceptibility of the latter to undergo decomposition by the action of nucleases within a cellular environment and a toxicity at higher concentrations [32]. PNAs also confer stronger binding in a DNA/PNA chimera over the analogous DNA/DNA chimera and show greater sequence specificity [33]. The PNA sequences used in this and other studies [32,34] were designed and referenced against BLAST and Genbank databases, which will become essential for any oligonucleotide designed for intracellular biological computation. This sequence significantly reduced levels of GluR3 protein (Fig. 6b) and furthermore protected NSC-34 cells against death induced by the AMPA receptor specific agonist (*S*)-5-fluorowillardiine. The downregulation of GluR3 through a PNA input can be viewed as the performance of a NOT operation (Fig. 6c). These results suggest that interference with the AMPA receptor assembly may be a novel strategy for controlling excitotoxic destruction of motor neurons and may lead to new therapeutic opportunities for the treatment of human ALS.

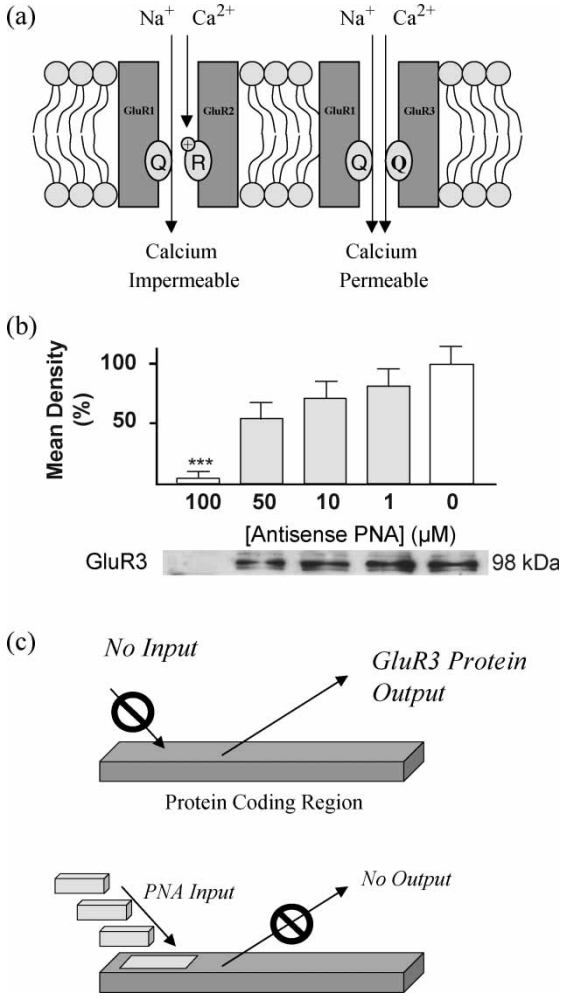

FIGURE 6 (a) GluR2 has functional dominance over Ca^{2+} permeability due to the arginine residue lining the channel pore. GluR1, 3 and 4 differ from GluR2 in that they have neutral glutamine residues in place of arginine thereby readily passaging Ca^{2+} upon activation (b) The expression of the GluR3 protein was specifically inhibited in cells exposed to the antisense PNA sequence C-GTA AGA GTG CCT-N. At a concentration of 100 μM, the expression of GluR3 was completely abolished whereas β-actin levels were unaltered. Statistical comparisons ($n = 4$, mean ± SEM) were made with respect to the control groups ($^{**}p < 0.0015$). Nonsense and sense sequences showed no downregulation. (c) A schematic diagram illustrating the basis of the NOT logic function derived upon the PNA input.

CONCLUSIONS

The unique characteristics of the DNA double helix have been used to demonstrate a variety of molecular logic devices including addition operations. The major advantage of DNA-based computing will most likely arise from its ability to process data in parallel as opposed to linearly as in the current technology. DNA undergoes self-assembly, self-replication and self-correcting actions that are ultimately responsible for storage and transmittance of genetic information in biological systems. It is clear that in a short period of time, high levels of complexity with respect to information manipulation are being achieved and that the direction this area takes may eventually lead back from artificial systems to a new paradigm for the treatment of diseases.

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