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Biosupramolecular systems: integrating cues into responses.

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ABSTRACT: Life is orchestrated by biomolecules interacting in complex networks of biological circuitry with emerging function. Progress in different areas of chemistry has made the design of systems that can recapitulate elements of such circuitry possible. Herein we review prominent examples of networks, the methodologies available to translate an input into various outputs and speculate on potential applications and directions for the field. The programmability of nucleic acid hybridization has inspired applications beyond its function in heredity. At the circuitry level, DNA provides a powerful platform to design dynamic systems that respond to nucleic acid input sequences with output sequences through diverse logic gates, enabling the design of ever more complex circuitry. In order to interface with more diverse biomolecular inputs and yield outputs other than oligonucleotide sequences, an array of nucleic acid conjugates have been reported that can engage proteins as their input and yield a turn-on of an enzymatic activity, a bioactive small molecule or morphological changes in nanoobjects. While the programmability of DNA makes it an obvious starting point to design circuits, other biosupramolecular interactions have also been demonstrated and, harnessing progress in protein design is bound to deliver further integration of macromolecules in artificial circuits.

INTRODUCTION

As for much of nanotechnology, the conceptual origin of artificial biochemical circuits can be traced to the 1959 seminar of Nobel laureate Richard Feynman, entitled "*There's plenty of room at the bottom*".[1] Feynman notes that biological systems "*do all kinds of marvelous things – all on a very small scale*"[1], drawing the key parallel between biology and manufacturing on a miniaturized scale. A year later Jacob and Monod formalized this concept through the machine model of the cell.[2] Monod particularly noted that "*the logic of biological regulatory systems abides not by Hegelian laws but, like the workings of computers, by the propositional algebra of George Boole*"[3] clearly establishing the connection between electronic circuits and biological processes. Indeed, comparable information processing can be observed in both the basic model of computation (Figure 1a) and the central dogma of molecular biology[4] (Figure 1b), both of which feature conversion of an input to an output via a form of processing. An analogous form of information transfer can also be described for biosupramolecular networks (Figure 1c).

While much of the ideological groundwork was laid for molecular computation in the 1960s, practical realization of these ideas was not achieved until the 1990s, when Adleman[5] and Lipton[6] were each able to demonstrate the power of nucleic acid computational system in solving mathematically complex problems. Contemporaneously, molecular logic gates were devised by the likes of de Silva[7, 8], Lehn[9] and Stoddart[10, 11], capitalizing on the growing fields of supramolecular chemistry and molecular machines.[12] In the intervening two decades both the areas of nucleic acid[13-19] and molecular logic gates have continued to expand, while the field of has also broadened to include areas such as hydrogels [20-22] and programmable materials.[23, 24] Additionally, networks and computation on a biological scale have expand based on enabling technologies such as genetic reprogramming[25, 26] to facilitate the production of synthetic organisms[27] and consortia.[28] From the early examples of simple information transfer and processing, the area of (bio)molecular logic has grown to include a vast array of subdisciplines, many of which have been the subject of extensive and erudite review,[29-31] however the core flow of information from an input, through a processing component to produce an output remains conserved throughout the field. It is this modular nature of network composition which underpins its true utility,[32] creating a platform from which a practitioner may combine appropriate elements to readily produce a new bespoke system. As such, this perspective will focus on this reductionist view of networks with the aim of providing an overview of the available network modules categorized by their function (input, circuitry or output) to enable facile creation of new network systems in a plug and play manner. For the sake of brevity and clarity we will confine our discussion to biosupramolecular network systems operating with or in conjugation with biomolecules, though it should be noted that similar analysis would hold true for both more biological and more chemical based systems.

A key seminal report of a biosupramolecular network is that described by Winfree and colleagues in 2006,[33] in which they report an enzyme-free nucleic acid logic circuit (Figure 1D). The system relies the sequential toehold displacement of two meta-stable duplexes to unveil an output strand (W_{out}) which can be utilized for downstream functions such as further toehold displacement events or the turn on of a fluorescent output. In order to enable output strand release both toehold displacement events are required, characterizing the system as an AND gate (i.e. X_{in} AND Y_{in} are both

required to obtain the output) as defined in Boolean logic. Common notation of such logic gates includes truth tables, circuit diagrams and Venn diagrams as are depicted below for this exemplar system (Figure 1 E-G).

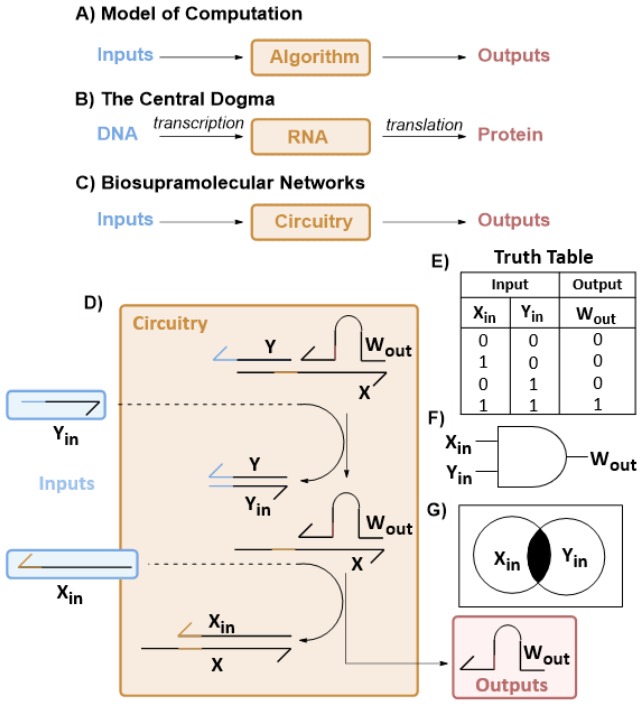

Figure 1: Overview of computational logic in biosupramolecular networks including description of information flow in **A)** the model of computation, **B)** the central dogma of molecular biology and **C)** biosupramolecular networks, as well as **D)** a seminal example of a biosupramolecular AND gated network[33] and its depiction in common logic formats, **E)** truth table, **F)** circuit diagram and **G)** Venn diagram). The 3' ends of the DNA strands are depicted as arrows and toehold/ toehold-binding regions are indicated in colour (red, gold and blue for W, X and Y respectively).

INPUTS

The choice of suitable input has always been an important factor for the development of biosupramolecular networks, often requiring the precise intervention with complex biological systems. Broadly, these inputs can be categorised as those which rely upon 1) sequence specific interactions, 2) geometric recognition and 3) small molecules (Figure 2), each of which will be discussed herein.

Sequence specific interactions

Nucleic acids are responsible for the storage and transmission of information in biological settings and, thus, it follows that they have also been widely exploited for the same purpose in biosupramolecular networks. This ability to store and transmit information is predicated upon the complementary base pairing between nucleic acid strands, governed by the formation of appropriate hydrogen bonding patterns (either through Watson-Crick or Hoogsteen base pairing). Given the ability of such interactions to produce changes in localization and/or geometry of binding partners in manner dependent upon the precise encoded sequence information (through inter- or intra-strand base pairing), it is unsurprising that such processes have been particularly exploited as input motifs in biosupramolecular networks. Such interactions have been employed using native nucleic acid polymers including DNA,[34-48] RNA[49-51] and miRNA[48, 52-54] but have also been extended to designer systems such as PNA.[55-57] The use of synthetic nucleic acid components in such systems allows further tuning of these fundamental base pairing interactions through the use of unnatural nucleobases, while modification of the nucleic acid backbone can be used to modify stability, solubility, electrostatic interactions and even helicity and chirality[58-61] to provide a further means of tuning the system.[62, 63] While sequence specific nucleic acid interactions in isolation provide a robust range of network inputs, they can also be coupled to a range of complex molecular architectures such as polymers,[43, 44] G-quadruplexes,[64] DNA tetrahedron[65] and DNA origami.[66] One particularly spectacular application of such interactions to a complex molecular architectures is to control the open or closed state of DNA containers to deliver payloads.[34, 35] An additional complex system within which this sequence specific base pairing can be applied is through its use in guide strand binding for CRISPR-Cas9 excision of target nucleic acids.[67] Thus, while nucleic acid base pairing is seemingly simple and ubiquitous, it affords a broad range of input mechanisms within biosupramolecular networks.

In an analogous manner to sequence specific nucleic acid interactions, biosupramolecular network inputs can also be derived from sequence specific interactions within peptides and proteins. These types of processes are predominantly between proteases and their substrate sequences, enabling cleavage of the input protein strand which can subsequently be translated into a geometric change within the system.[68-70] Such geometric changes can also be coupled to the relative position of fluorophores, with important applications such as real-time visualisation of tumours for fluorescence-guided surgery.[71]

Finally, sequence specific nucleic acid recognition can also be combined with other types of supramolecular interrelations within individual systems to form complex molecular architectures such as three way junctions,[37] further extending the molecular complexity which can be achieved through these fundamental, programmable interactions.

Geometric interactions

Another key set of inputs in biosupramolecular networks are reliant upon geometric based interactions, with a prominent set of these (as in nature) dependent upon proteins. One of the most common types of protein-based inputs are those dependent upon dimerization of protein components which in turn enables other components of the networks to be brought into close proximity to promote a reaction (chemical transformation or hybridization). A wide variety of proteins have been used including carbonic anhydrase,[72] hepatocyte growth factor,[73] platelet

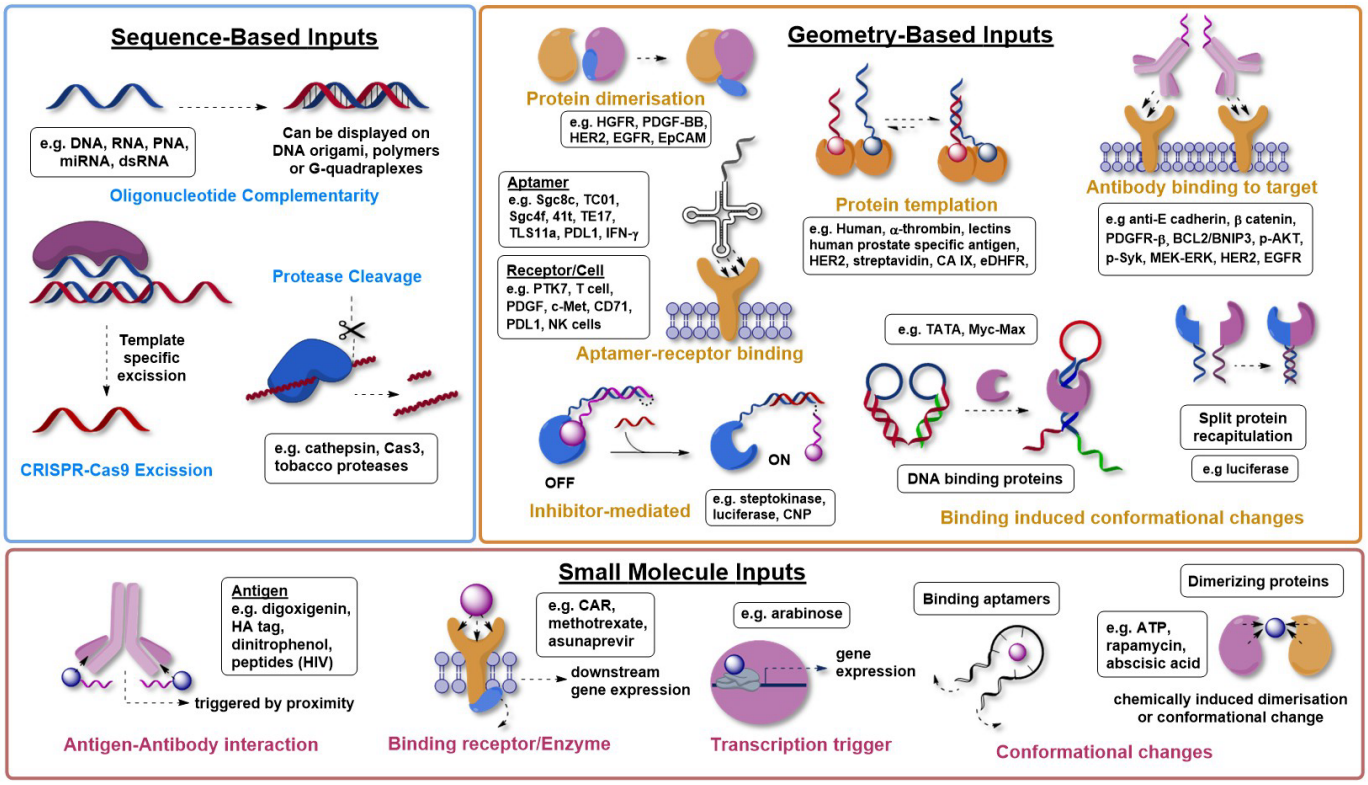

Figure 2: Input modalities exploited in biosupramolecular networks

derived growth factors[74] and bi-substrate enzymes such as Abl and Src kinases,[75] as well as *de novo* designed proteins.[76, 77] Additionally, similar induced proximity effects can be achieved through the use of proteins and their cognate binding ligands (e.g. eDHFR[78] and lectins[79]), which can be particularly powerful as an input mechanism for biosupramolecular networks when multiple binding sites are available on a single protein scaffold (e.g. streptavidin[80-82]). In addition to ligand binding, antibodies have also been widely applied as inputs for biosupramolecular networks as a result of their high affinity and specificity for given protein targets, including HER2,[82-84] human prostate specific antigen,[82] phospho-platelet derived growth receptor-β,[83] E-cadherin,[83] Syk,[83] Bcl2,[83] Bnip3,[83] Lc3,[83] Sqstm1,[83] β-catenin[83], EGFR[83] and cell surface markers such as CD20 and CD45.[85] In analogous manner to antibodies, high affinity nucleic acid aptamers can also be evolved to bind to specific cellular targets and have been widely exploited as inputs in biosupramolecular networks. In particular, aptamers have been used to target receptors (including c-Met,[86, 87] CD71,[86] PTK7[88, 89] and HGF[73, 87]), cellular markers (PDL1[90]) and other functional proteins (human α-thrombin[82], platelet derived growth factor[74]).

The wealth of geometric based inputs exploited in biosupramolecular networks are not limited to simply changing broad positional proximity of the components but can also modify relative internal proximity of systems through conformational changes. Such systems can include the interaction between DNA-binding proteins (TATA, Myc-Max) with metastable nucleic acid hairpins to induce a conformational change[91] or *de novo* orthogonal key-latch proteins which can be recruited by cell surface receptors such as HER2, EGFR and EpCAM.[92]

Coupling of a geometric change between an enzyme and its inhibitor to hybridization has also been used as an on/off input to control enzymatic activity[40, 41, 56, 93, 94] and reconstitute split proteins.[50]

Small molecule binding

Input signals for biosupramolecular networks are not limited to large biomolecules and can also be successfully controlled by a broad range of small molecule-based interactions. Such inputs can be used for the high-affinity binding to cognate antibodies (HA tag,[95] dinitrophenol,[96, 97] digoxigenin[96, 97]), receptors (CAR for α-CD19[98], carbonic anhydrase,[72] methotrexate/DHFR[99]) or enzymes (hepatitis C protease[100]), as well as the induction of cellular processes such as transcription.[101, 102] Additionally, small molecules inputs can induce significant conformational changes in their binding partners, resulting in important functional outputs. These can include the small molecule templated activation of aptamer species,[103] as well as the induction of protein dimerization through independent binding of protein subunits to distal regions of a single small molecule dimerising agent.[68, 104, 105]

Given the broad range of input modalities available (both in terms of broad classification and individual systems to which they have been applied), a vast scope of biosupramolecular networks becomes accessible. Such diversity is only compounded once the available circuitry motifs are considered, particularly in terms of their ability to enable translation to a range of functional outputs.

CIRCUITRY

The circuitry component of biosupramolecular networks not only provides the physical means of connecting the input and output modalities, but also provides important

opportunities for information processing and integration. Thus, the choice of the physical circuitry motifs, as well as the design of the circuit connectivity is vital for fully exploiting the potential of a given network system.

Nucleic acid chain reactions

Toehold displacement. Among the wealth of physical circuitry components available for biosupramolecular networks, nucleic acid-based systems are, perhaps, the most exploited. The modularity and ease of programmability offered by oligonucleotides make such reactions a powerful tool to wire complex operations. The most widely utilized of such processes is toehold displacement, a simple and versatile process in which a single nucleic acid strand is exchanged for another in binding to a template strand (Figure 3).[106] This process is facilitated by hybridization to the unpaired "toehold", thus initiating the branch migration and can be driven to completion by the increased binding affinity of the newly formed duplex. The applications of this powerful mechanism have been harnessed to fuel a molecular motor,[107] as well as control DNAzymes[108] and computation circuits.[109] A particularly useful advance for engineering toehold displacements circuits was the characterization of the kinetics of this process,[110] establishing that a five nucleotide toehold was sufficient to achieve toehold displacement that proceed at comparable speed to hybridization. Since early examples from the group of Gadhiri, that used toehold displacement to modulate enzyme activity,[40, 41] the field has evolved to increasingly complex architectures that have been applied to *in vitro* and *in cellulo* systems. Such systems include regulation of proteases,[40, 41, 93] β-lactamase,[94] antibodies,[111, 112] bioluminescent protein HLuc,[56] localization of target proteins to a polymer scaffold[44] and coupling to fluorescent outputs.[48, 73, 81] Such systems are even capable of controlling complex molecular architectures such as DNA nanotubes[96] and nanovaults.[35] Given the finely balanced kinetics of toehold displacement, this process is often pre-empted by pseudo-concentration effects as a result of the proximity of the partner strands. Such proximity is frequently afforded by receptor dimerization,[73, 85, 86, 103] but can also result from antibody templation.[95-97] Significantly, toehold displacement processes are highly biocompatible[49] and can be coupled to a wide array of other nucleic acid circuitry types in order to facilitate signal amplification and higher order information processing. Furthermore, sequential or parallel toehold displacement can be used to integrate diverse input and to design logic-gated responses.

CHA, HCR, RCA, PER. While toehold displacement reactions offer the opportunity to design diverse logic gates to process the signal of inputs and represent the most ubiquitous form of nucleic acid circuitry motifs exploited in biosupramolecular networks, a vast array of more complex and nuanced modalities have also been exploited for amplification purposes.[113] Such approaches are namely: catalytic hairpin assembly (CHA), hybridization chain reaction (HCR) for enzyme-free processes and rolling circle amplification (RCA) and primer exchange reaction (PER) which are leveraged on polymerases (Figure 3).

CHA reactions utilize two metastable hairpins that contain sequences complementary to one another; however, hybridization is only possible upon addition of an input strand that opens the first hairpin (usually through toehold displacement) revealing the hybridization sites previously hindered within the hairpin stems. Once the second hairpin is opened, the input strand is displaced and can further be used as catalyst in the next cycle, enabling significant amplification of the initial input signal. As described for toehold displacement reactions, CHA can be tuned to be dependent on effective concentration and thus is frequently coupled to proximity-based systems such as immobilisation on supramolecular polymer supports,[43] FRET amplification[48] and split input systems where proximity is induced by protein templation.[82]

HCR represents another widely used technique to reach signal amplification in an oligonucleotide circuit. Similarly to CHA, HCR also requires an input strand and two hairpins; however, instead of yielding input displacement after each cycle, HCR generates amplification by linearly growing an assembly in which the two hairpins are alternated. Commonly, the hairpins are decorated with fluorophores to allow for easy monitoring of the reaction.[114] HCR circuits were adapted to perform on supramolecular polymers[43] and on the surface of mammalian cells,[89] exploiting the high effective concentration of inputs naturally displayed by such systems. Recently, an HCR system was also used to trigger the release of small molecules through the induced proximity of thiol and disulfide-cargo moieties which enables thiol displacement of the disulfide and release of the cargo molecule.[115] HCRs have also been applied to more complex designs, involving antibodies-mediated fluorescent labelling of cells[83] or display DNA nanostructures which enable propagation of the HCR network in three dimensions,[53] evidencing the power of this circuitry motif.

An alternative amplification method that has found application in oligonucleotide-based circuitry is the rolling circle amplification,[116] RCA, which uses enzymes (polymerases) to replicate an oligonucleotide strand around a circular template. As a result of the circular nature of the template, the nascent single strand nucleic acid polymer contains sequential repeated copies of complementary sequences, enabling significant amplification of the initial input. To date, this technique has been used for sensing purposes using an RCA to amplify a sequence of interest and a templated reaction to translate the repeat motif into a fluorescent signal.[36] It has also been used to amplify identifying sequence in aptamers interacting with cell-surface receptors.[88] Beyond amplification of a label, it was used to fuel the movement of a DNA catenane molecular walker along RNA transcripts.[47] While the requirement of an enzymatic component in RCA does have limitation in terms of amplification rate and applicability to hindered systems, this amplification motif produces a single structurally distinct output and thereby provides an important alternative in the design of biosupramolecular networks.

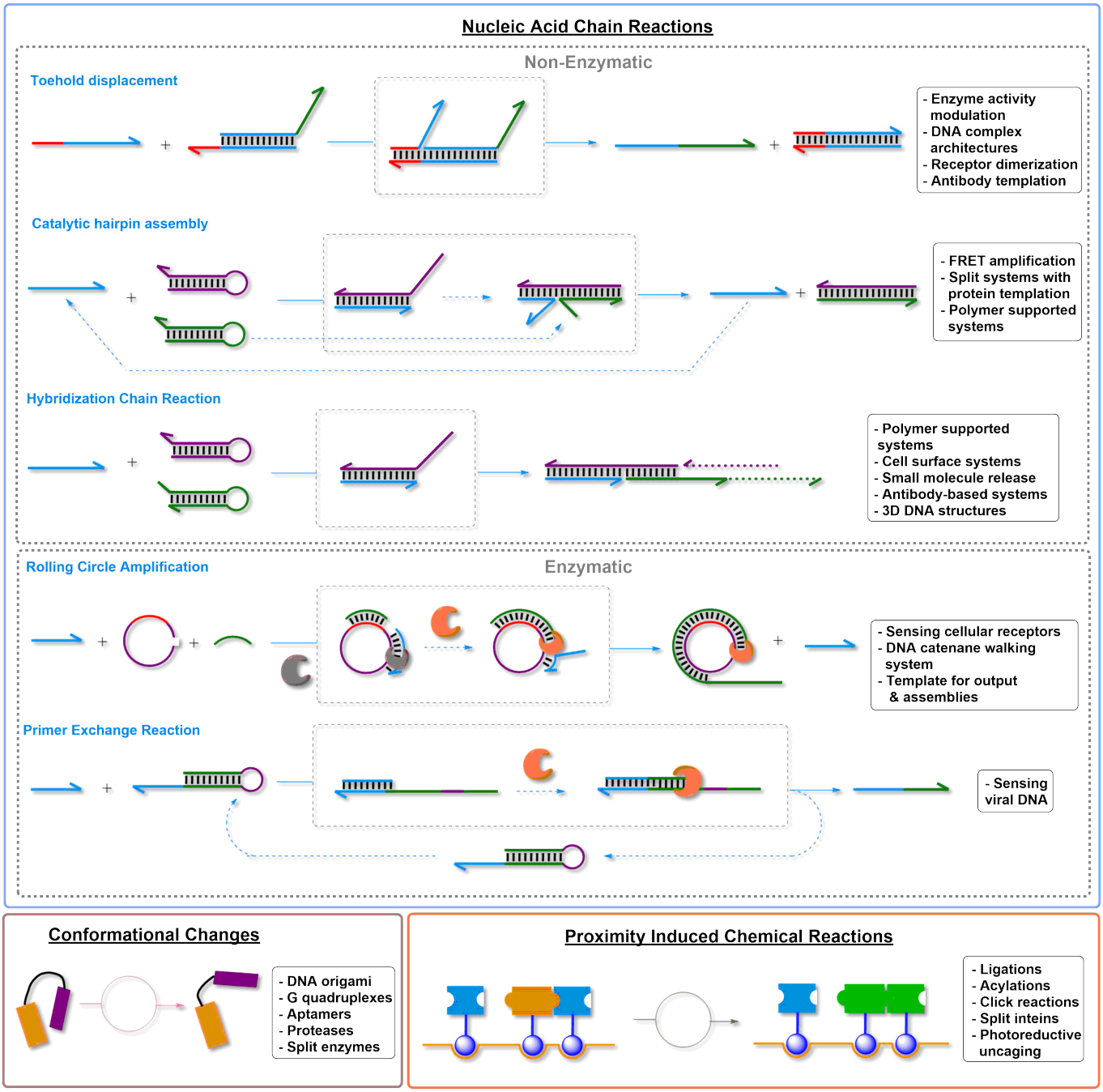

Figure 3: Circuitry modalities in biosupramolecular networks

Finally, primer exchange reaction (PER) has also been employed as circuitry module in biosupramolecular networks. Given a DNA primer, this isothermal technique generates an extended primer from catalytic hairpin mediators with the help of a strand-displacing polymerase. Following binding of the initial primer strand to the catalytic hairpin, the polymerase extends the primer complementarily to the stem region of the hairpin until a stop codon is reached. This stem region then serves as the primer for binding to another hairpin species, enabling propagation of the process. As result, custom-designed ssDNA is produced based on the encoded stem sequences of the catalytic hairpin motifs. Significantly, this technique has been integrated with a CRISPR array (enabling recognition and excision of specific sequences as triggers for PER) to enable analysis of COVID-19 genome in cell lysates.[67]

Conformational changes

In addition to nucleic acid circuitry motifs, biosupramolecular networks have been designed which hinge upon a variety of other circuitry types. Broadly, many of these can be discussed in terms of conferred geometric and/or conformational change and can be likened to classical machinery in the physical transfer of an input stimulus to create an output, often with minimal amplification. Such changes can be facilitated by structural motifs based upon either protein or nucleic acid components, or through a combination of the two. In terms of nucleic acid-based systems, one of the key facilitating concepts has been the growth of the field of DNA

origami, which continues to produce increasingly complex and nuanced nucleic acid based structures. The application of such complex architectures to biosupramolecular networks has included the design of a DNA nanorobot by Church and coworkers,[34] in which broad conformational changes of the nucleic acid assembly are able to facilitate controlled opening and closing of the *de novo* vessel. Similarly, Andersen and coworkers[35] were able to engineer a DNA-based nanovault, capable of opening and closing via changes in conformation to control the activity of reporter enzymes. Smaller scale DNA-based architectures have also provided conformational circuitry for biosupramolecular networks including G-quadruplexes,[64] triple helices[46, 57] and four way strand exchange motifs,[49] with hybrid DNA-protein systems also exploited.[37] Finally, the high affinity and specificity of aptamers has also been exploited in terms of geometric circuitry in biosupramolecular networks.[87] Given the direct correlation between aptamer structure and their binding affinity, such interactions are facilitated by modulation of this folding equilibrium, with subsequent control over the output function.

Protein-based architectures also provide important scaffolds for geometric-based circuitry in biosupramolecular networks.[117] While this can occur through simple exposure/sequestration of binding sites,[92] it is often coupled an enzymatic function. Frequently, this occurs in the form of a protease (such as NS3a[100] or TEV[68, 118]) which, upon activation, are able to cleave a substrate strand, thereby allowing dissociation of two previously tethered fragments. Additionally, these proteases can also undergo control via geometric processes through the design of split enzyme systems, whereby two inactive enzyme fragments (when brought into close proximity) can enable recapitulation of native enzyme activity,[25, 50, 104] thereby providing another level of system control.

Proximity induced chemical reactions

While individual circuitry components within biosupramolecular networks can be powerful carriers of information, the ways in which these individual elements can be combined often leads to even more nuanced and impressive systems. Indeed, one particular circuitry subtype which is often requisite on others for its induction is proximity induced chemical reactions. As the name suggests, these reactions are reliant upon the two reaction partners being brought into sufficient proximity in order to facilitate the ensuing chemical reaction, with such geometric changes often controlled by other circuitry modalities (*vide supra*). The types of reactions most commonly applied in such a setting are those in which the two reaction partners become covalently joined via the reaction process, with the most prolific of these being the click reaction.[84, 119] A broad range of proximity induced templated ligation reactions have also been used either to tether independent nucleic acid strands or to conjugate nucleic acids to specific protein partners. Such reactivities include the exploitation of native chemical ligation,[120, 121] and NHS-ester conjugation,[38] as well as the use of DMAP-catalysed esterification for late-stage protein modification.[79] Finally, such ligation can also be achieved enzymatically to enable conjugation of partner DNA strands for detection purposes.[74] These proximity induced chemical reactions are extensively used to translate nucleic acid sequence into a output (*vide infra*).

NETWORK ARCHITECTURES

The design of biosupramolecular networks is not only dependent upon the choice of physical circuitry to connect input and output motifs but also in the logic of such connections and how individual circuitry elements can be connected in order to produce more complex systems. Such connections can be discussed in terms of Boolean algebra and its cognate logic gates, whereby input and output values exist discretely as either TRUE or FALSE (numerically, 1 and 0, respectively).[31] Such systems can also be represented graphically through circuit diagrams and truth tables, which are summarised for the complete set of Boolean operators below (Figure 4) in relation to their application in biosupramolecular networks.

The simplest set of Boolean operators are the basic logic gates – YES, NOT, AND and OR. The YES and NOT gates respond only to the presence or absence of a single input, whilst AND and OR gates modulate their output in response to multiple input stimuli.[31] These simple operators present the least technical challenges for their application to biosupramolecular systems and thus have seen the widest and most abundant exploitation.

A more complex set of Boolean operators (NAND, NOR, XNOR and XOR) are referred to as derived logic gates, as their function is derived from that of the basic logic gates. The N prefix in such gates implies “not” (or the complement) of the remaining statement i.e. NAND can be read as “not AND”, meaning that the system is true for all except the AND case.[31] Similarly, NOR (not OR) holds true for the complement of the OR case. In addition, the prefix X is used to mean “exclusively” such that XOR defines the “exclusively OR” (notably not including AND), whilst XNOR defines the complement of this case.[31] The increased theoretical complexity of these gated systems is reflected in their technical requirements and, thus, it is unsurprising that such systems have seen less prolific application in biosupramolecular networks. Notably though, a select number of computationally complex networks have been developed which are capable of the complete set of Boolean logic operations, including those based on nucleic acid circuitry,[86, 95] as well as protein dimerization[76] and protease activity.[68, 118] The computational complexity of networks can also be increased not only by the complexity of the logic operation but also by the number of simultaneous inputs which can be processed.[43, 67, 102, 122] Indeed, Yin and coworkers[51] developed a toehold displacement-based system capable of computing up to twelve inputs through combination of only AND, OR and NOT gates to enable visual detection through GFP reporter expression, evidencing the power of intelligent combination of simple systems.

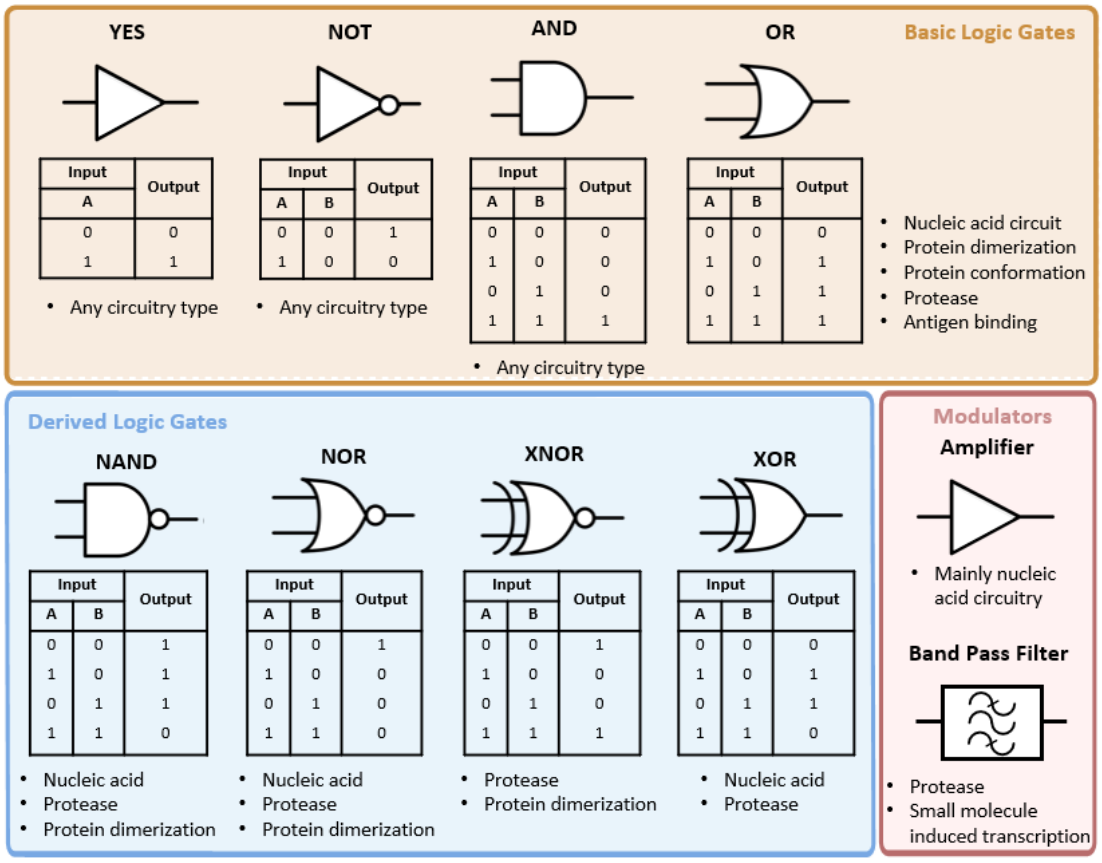

Figure 4: Summary of logic gate architectures exploited in biosupramolecular networks

In addition to simple gating processes, circuitry elements are also capable of modulating or responding to the amplitude of the signal rather than the information state alone. For example, circuitry elements are also capable of acting as amplifiers of input information, commonly through nucleic acid circuitry motifs,[43, 47, 52, 67, 82, 89, 115, 119, 123, 124] in order to facilitate ease of detection or increase the sensitivity of the system. Additionally, circuitry elements can be designed to act as bandpass filters whereby only inputs of a certain intensity are interpreted as true by the system, which can provide beneficial noise cancelling properties. For example, Elowitz and coworkers[118] developed a protease-based system capable of bandpass filtering through the coregulated expression of the effector protease with activating and repressing regulator proteases. Similarly, Isalan and coworkers[101] developed a system capable of bandpass filtering in response to the concentration of the inducer arabinose, based on the relative levels of activating and repressive transcription factors, both of which are under arabinose control. Aside from amplification and band passing, simple nucleic acid circuits can be designed to respond nonlinearly to increasing amount of input, responding first positively and then negatively to increasing levels of an analyte, in analogy to a negative feedback loop.[123]

OUTPUTS

As is true for the input and circuitry components of biosupramolecular networks, a broad range of output types have been grafted into these systems. Broadly speaking, these outputs can be classified as either chemical, topological or cellular. It is important to recognize that in a network, the output of a first logic operation is the input of the next logic operation. The discussion herein will treat the output as the final product of a network.

Chemical Outputs

Fluorescent outputs exhibit particular utility in validating biosupramolecular networks due to the ease of detection and quantify the response. Fluorescent outputs can either occur through simple turn on/off mechanisms, by shifting of the detectable wavelength, or with Förster resonance energy transfer (FRET) representing the primary means through which this is achieved. Owing to the proximity dependence of the FRET process, this readout is often coupled to processes which result in changes in geometry or localization, including the association or dissociation of binding partners (be these protein or nucleic acid based) bearing the FRET pairs, cleavage events (such as by nucleases or proteases) and intra-molecular (or pseudo intra-molecular) conformational changes which modify the relative positioning of the FRET pairs in space. The most common FRET pair used in biosupramolecular networks comprises the cyanine dyes Cy3/Cy5[35, 43, 53, 86, 91, 96], while so called quenchers such as dabsyl[36, 73, 79, 81, 82], black hole quenchers (BHQ)[96], QSY21[71] and Iowa Black[49, 85] have also found wide utility for FRET-based turn on/off systems. Additionally, FRET protein pairs such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) have

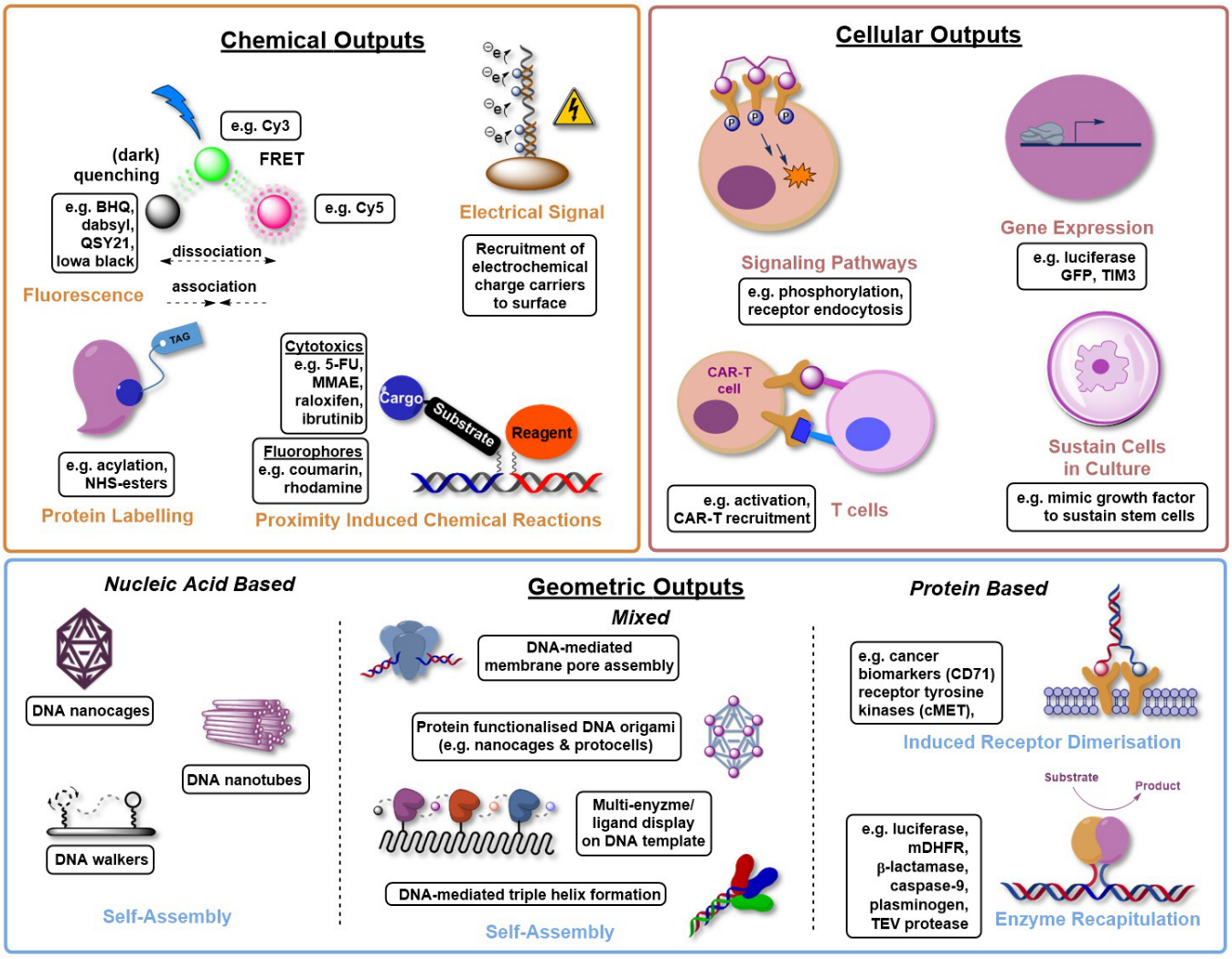

Figure 5: Summary of output modalities in biosupramolecular networks.

also found utility as outputs in biosupramolecular networks.[48] This readout has also been extended to include bioluminescence resonance energy transfer (BRET) in which in the radiant light is derived from an enzymatic process.[56, 94, 99, 125] Analogously, biosupramolecular network processes can also be used to recruit electrochemical charge carriers such as methylene blue[67, 126] which can perturb the conducting properties of a surface such as gold micro-electrodes, thereby facilitating direct electrical readout from the network system.

Fluorescent outputs for biosupramolecular networks are not only limited to FRET-based processes but can also occur in as a result of proximity induced chemical reactions which result in the formation of a fluorescent product. The proximity of reagents following a hybridization event results in high effective concentration and accelerated reaction in analogy to the proximity induced reaction discuss in the circuitry section.[127, 128] Such processes can include reactions which disconnect a fluorophore from a quenching moiety or chemically modify a fluorophore to change its spectral properties. An important distinction in templated reaction is whether the reaction leads to a ligation, or not. In the case of a ligation, product inhibition will the turnover that can be expected. Beyond detection, the same chemistry can be used to uncage drugs.[52, 72, 99, 115, 129, 130] One reaction which has proven particular robust in this respect is ruthernium photocatalyzed reductions and, it was show to be effective in live vertebrates.[54] This photoreductive systems have also been applied to the triggered release of small molecule cytotoxic agents such as 5-fluorouracil[52], raloxifen[99], ibrutinib[99] and monomethyl auristatin E (MMAE).[72] Finally, nucleic acid templated reactions have also been used to chemically label cell-surface proteins using proximity induced chemical reactions and coupled to biosupramolecular networks, enabling the modulation of protein structure and function.[131]

Geometric Outputs

Aside from chemical-based outputs, biosupramolecular networks can be coupled to many other detection methods, including those stemming from broad geometric or positional changes. These systems can be based either entirely on nucleic acids or proteins; or using a combination of these two classes of biopolymers. Nucleic acids serve as a rich substrate for programmable geometric changes through self-assembly, particularly when coupled to the growing field of DNA origami.[132] This allows for the

controlled formation of structural motifs such as DNA nanocages[133] and DNA nanotubes[96] as network outputs, as well as the directed movement of DNA walkers[47] along nucleic acid tracks. However, the utility of nucleic acid assemblies can be expanded once it is coupled to proteins in a mixed self- assembly system. These systems can build upon the DNA origami structures previously discussed and utilize them for the specific positional display of functional proteins. These can include caging/uncaging of enzymes within DNA nanocages[134] or protocells,[124] and the specific localization of enzyme series on surfaces to facilitate sequential enzymatic processes.[135] These mixed biopolymer systems can also utilize nucleic acid interactions to allow for the specific positioning of structural protein elements such as in the formation of membrane pores[136] and protein triple helices[46], or to modulate the stability of the DNA origami itself[137]. Biosupramolecular networks can also be coupled to purely protein based geometric outputs such as through receptor dimerization[86, 103] or enzyme recapitulation[35, 39, 41, 42, 44, 48, 50, 56, 68, 69, 94, 100, 104, 118]. In particular, the recapitulation of luciferase activity is frequently coupled to biosupramolecular networks as this provides a direct visual detection of the enzyme reactivation through bioluminescence. Beyond reconstituting split proteins, other forms of gain of function have been demonstrated by aligning ligands or proteins with the appropriate geometry on a DNA template.[138-141]

Cellular Outputs

Given the power of biosupramolecular networks in causing significant changes in localisation and structure of major classes of biopolymers, it is unsurprising that such outputs can also be extended to result in detectable changes at the cellular level. By facilitating binding events with cell surface receptors such networks can facilitate the modulation of biochemical signalling systems, such as the c-Met/Akt[87, 103] and ERK[84] pathways, as well as causing changes in cellular proliferation.[34, 142] Additionally, these systems can be used to facilitate changes in gene expression, including the induction of reporter proteins such as green fluorescent protein (GFP)[51, 101] and luciferase[104], as well as the immune checkpoint gene TIM3.[76] Immune modulation has proven to be of particular interest as an output of such networks, specifically in relation to immuno-oncology. Specifically, networks culminating in the recruitment[92] and activation[98] of chimeric antigen receptor (CAR) T-cells have been developed with proposed applications in cancer therapy, evidencing the power and potential clinical utility of such tuneable and responsive systems.

CASE STUDIES

As evidenced by the tremendous number of inputs, circuitry modalities and outputs which have been grafted into biosupramolecular networks, these systems are incredibly adaptable and tuneable. The ability to readily combine modular network components enables the practitioner to easily optimize a system for their specific requirements, enabling them to achieve meaningful biological and/or chemical outcomes. The remainder of this perspective article will thus focus on the unique ways in which specific inputs, outputs and circuitry have been combined in particular biosupramolecular networks as a means of showcasing the breadth of such systems.

A key concern of biosupramolecular networks leveraged on a nucleic acid amplification module is how to convert the nucleic acid output into a range of other functional output. This is important for inputs that are present at very low concentrations (>nM) such as micro-RNA (miR) associated with a disease state, a concept addressed by Kim *et al.*[52] through the use of sequential hairpin opening reactions (Figure 6). The network system facilitates the detection of miR, such as the cancer biomarker miR-21, down to a concentration of 250 fM via a cascade of two reactions enabling up to 1000-fold amplification of the initial input nucleic acid (via sequential YES gating and amplification operations). This is possible through the design of metastable DNA hairpins which can undergo sequential opening triggered by the input strand via the toehold displacement process previously described (*vide supra*). Initially, the input strand undergoes sequence specific hybridization with the first hairpin motif (RuA) which is functionalized with a $Ru(Bpy)_2phen$ photocatalyst. The photocatalyst is shielded from possible substrate binding in the closed conformation of the hairpin but becomes exposed once the hairpin has been opened by the initiator strand. The resulting complex (RuAI) is then able to facilitate the opening of a second functionalised hairpin (RuB) to generate the active catalytic complex RuAB (bearing two accessible copies of the Ru-photocatalyst), as well as regenerating the initiator strand (I) which can re-enter the catalytic cycle. The active catalytic species can then recruit substrate strands functionalized with the desired caged cargo molecules (C – e.g. cytotoxic agents, fluorophores) to undergo photocatalytic release owing to the proximity dependent SET reduction of a pyridinium linker upon irradiation of the photocatalyst with 450 nm light. The naked substrate strands are then able to dissociate to regenerate the catalytic species, which is able to perform up to 195 turn overs, facilitating the tremendous amplification power of the circuitry.

An alternate means of enzyme-free signal amplification through reaction cascades was demonstrated by Raj and coworkers[119] through the use of a sequential Clamp-FISH system leverage on covalently locking every amplification cycle by nucleic acid templated click ligation. Fluorescent *in situ* hybridization (FISH) provides a simple and robust means of detecting single RNA molecules through easily detectable fluorescent output, however, it is often limited by the requirement of high-powered microscopy to overcome the weak signals produced. Raj and coworkers sought to overcome this through developing a means of signal amplification which does not rely upon the problematic enzymatic steps of other systems. Their design relies upon so called “padlock-style probes” which contain sequence elements complementary to the target in both their 5’ and 3’ regions, with the intervening sequence remaining unhybridized to the target (Figure 7). This enables the probe to adopt “C” configuration which forms a loop

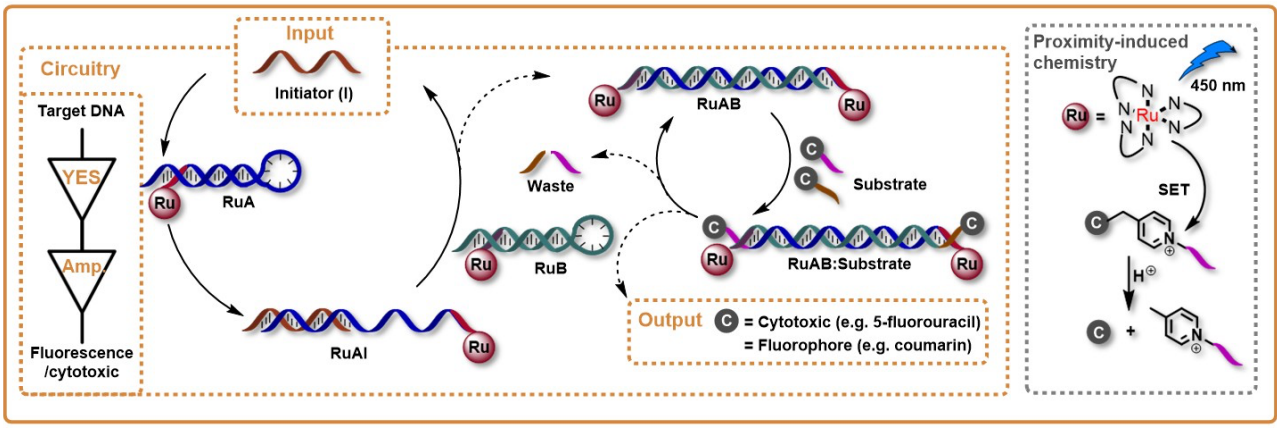

Figure 6: Case study 1 - quadratic amplification of nucleic acid inputs via hairpin opening coupled to templated reactions.[52]

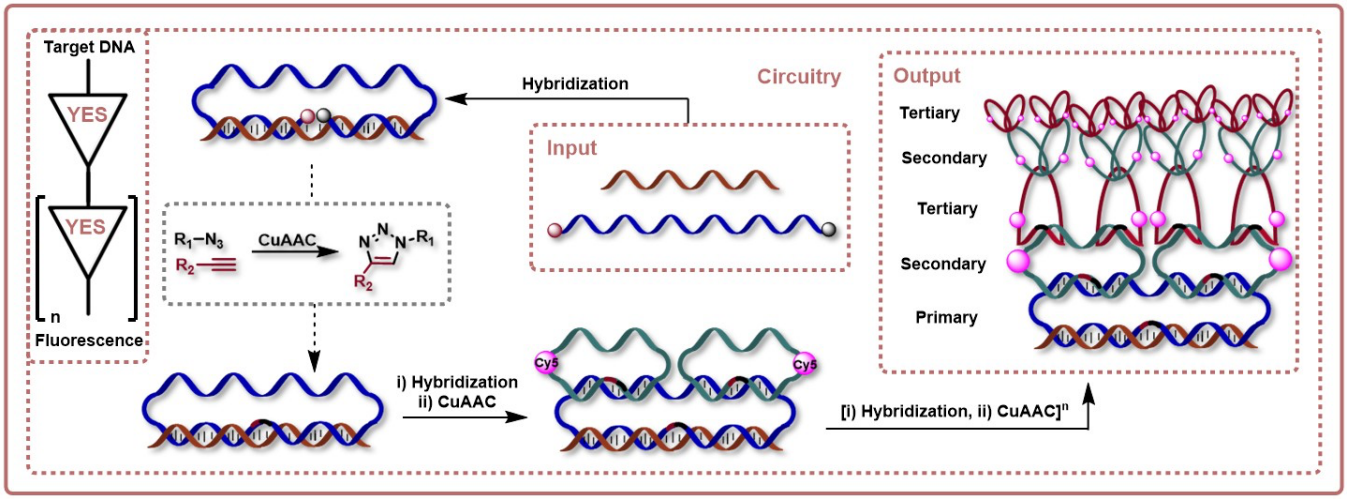

Figure 7: Case study 2 - signal amplification via a cascade of click chemistry-based Clamp-FISH system.[119]

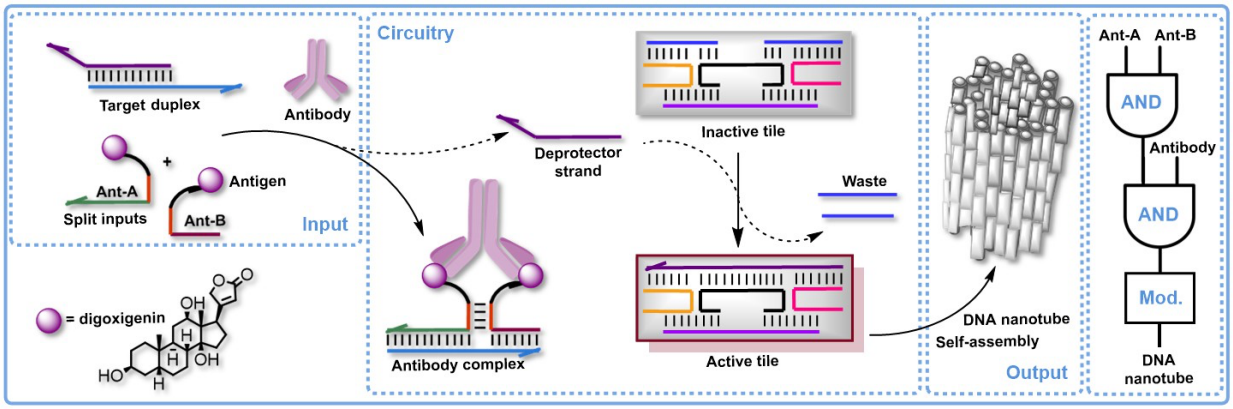

Figure 8: Case study 3 – antibody responsive self-assembly of DNA nanotubes.[96]

around the target DNA strands. The authors exploited the proximity of the two probe termini following hybridization to facilitate their connection via a Cu-catalysed azide alkyne cycloaddition (CuAAC) reaction, thereby preventing probe detachment. The authors were able to engineer the intervening region of the primary probe to also encode two "landing pad" regions to enable the hybridization and ligation of two fluorescently labelled (Cy5) secondary Clamp-FISH probes onto the primary probe. Through iterative rounds of hybridization and CuAAC closure that yield an exponential number of "landing pads" (functioning as iterative YES gates), the authors were able to repeat this process with secondary and tertiary probes alternately to rapidly generate up to 400 fold amplification of the input signal in an enzyme-free manner.

Biosupramolecular networks are not only utilized for information transfer and amplification but can also be designed to afford complex geometric changes, particularly when coupled to DNA origami. Ricci and coworkers[96] elegantly exploited this concept through their design of an

antibody responsive network which facilitated the self-assembly of DNA origami-based nanotubes (Figure 8). The network is predicated upon the high-affinity binding of an anti-digoxigenin (Dig) antibody to the steroid Dig on the two independent input strands in an AND gated manner. The canonical Y-shape of all IgG antibodies serves as a template for Dig-functionalised DNA input strands to enable a proximity dependent toehold displacement (*vide supra*). The relative stoichiometry of these three circuitry components also influences the outcome of the network, thereby conferring additional modulatory properties to the system. The formation of the resulting three-way junction antibody complex also results in the liberation of a deprotector strand from the initial target duplex. This deprotector strand is then able to undergo an additional toehold displacement process with a DNA-origami tile to liberate two waste DNA strands. This second toehold displacement process results in a conformational change in the DNA-origami tile, enabling to adopt an active conformation, capable of undergoing self-assembly to form hollow DNA nanotube structures of lengths up to several micrometres. It was further shown that the system can be multiplexed with two different IgG antibodies binding unique antigens, each controlling the assembly and disassembly of distinct DNA nanotubes.

An alternative means by which a change in nucleic acid conformation can be converted to a functional output is demonstrated by Yin and coworkers[51] (Figure 9), again relying on a toehold displacement process in a cellular context. In this network, the hairpin loop which undergoes toehold displacement is positioned upstream of a reporter gene (e.g. GFP), thereby precluding access to the ribosome binding site (RBS) and initiator codon (AUG) by the ribosome. Upon binding of a complementary input strand, a conformational change occurs within the nucleic acid, rendering the RBS accessible to the ribosome to initiate transcription. Through precise design of the upstream hairpin regions, the authors were able to apply this concept to AND, OR and NOT gates (capable of responding to multiple independent input strands) to enable powerful ribocomputing in live cells.

As evidenced above, toehold displacement marks one of the key facilitating motifs in biosupramolecular networks. While toehold displacement is necessarily limited to nucleic acid systems, analogous uncaging events can also be described in protein based systems, such as that described by Baker and coworkers (Figure 10).[92] Utilising their tremendous experience in rational protein design (through exploitation of Rosetta algorithms[143]), Baker and coworkers designed a proximity induced protein uncaging system which they termed Colocalization dependent Latching Orthogonal Cage-Key protein (Co-LOCKR). The system is initiated by the binding of unique Designed Ankyrin Repeat Proteins (Darpins) to their target receptors, namely the cancer associated receptor tyrosine kinases HER2 and EGFR, in an AND gated manner. This process brings the two halves of the Co-LOCKR system into close proximity through the heterodimerisation of HER2 and EGFR. This subsequently enables a proximity dependent displacement of the latch portion of the cage protein assembly by the corresponding key protein, in a manner analogous to toehold displacement. As such one can think of the initial cage protein assembly as existing in a meta-stable state prior to displacement by the key motif. The latch domain contains a functional peptide region which is initially sequestered in the caged conformation but becomes exposed upon unlocking by the key domain. The authors exploited the well-studied interaction between the protein domains Bim (located within the latch region) and Bcl2 (within the effector modules) to couple this conformational change to a variety of functional outputs. Most significantly, they were able to use chimeric antigen receptor (CAR)-T cells bearing the Bcl2 domain to enable their recruitment to the Co-LOCKR system, subsequently enabling T-cell activation and production of immunostimulatory cytokines such as IFN-γ.

Changes in protein conformation can also be coupled to biosupramolecular networks to directly modulate the function of enzymes, as demonstrated by the work of Muir and coworkers (Figure 11).[104] This system is predicated upon the function of a split intein system (such as Npu) in which the activity of an intein (a protein capable of self-excision from surrounding extein sequences) can be recapitulated by bringing the disparate N- and C- terminal domains into close proximity. Muir and coworkers were able to modulate this proximity dependence through fusion of the split intein domains (NpuN and NpuC) to known rapamycin binding proteins FKBP and FRB. Upon introduction of the rapamycin input, both FKBP and FRB bind to their target ligand, bringing the two split intein domains into close proximity in a YES gated manner. This facilitates a conformational change which enables each of the intein components to dissociate from their caging domains and form the active intein complex. Upon recapitulation of the intein activity, the intein assembly undergoes self-excision from the flanking p300 acetyltransferase domains while simultaneously ligating the two halves of the enzyme together to render the transcription factor active. By fusing the p300 enzyme to a dead Cas9[144] (i.e. without catalytic activity), the authors were able to use the dCas9 to target the recapitulated acetyltransferase to the promoter of a reporter gene through the use of complementary single stranded guide RNA (sgRNA) in an AND gated process. Taking advantage of the previously explored IL1RN promoter/ sgRNA pair reporter,[145] the split intein network was coupled to the expression of the enzyme luciferase *via* a reporter plasmid. This general design could be extended to other chemical dimerizers and sgRNA illustrating the modular nature of biosupramolecular networks.

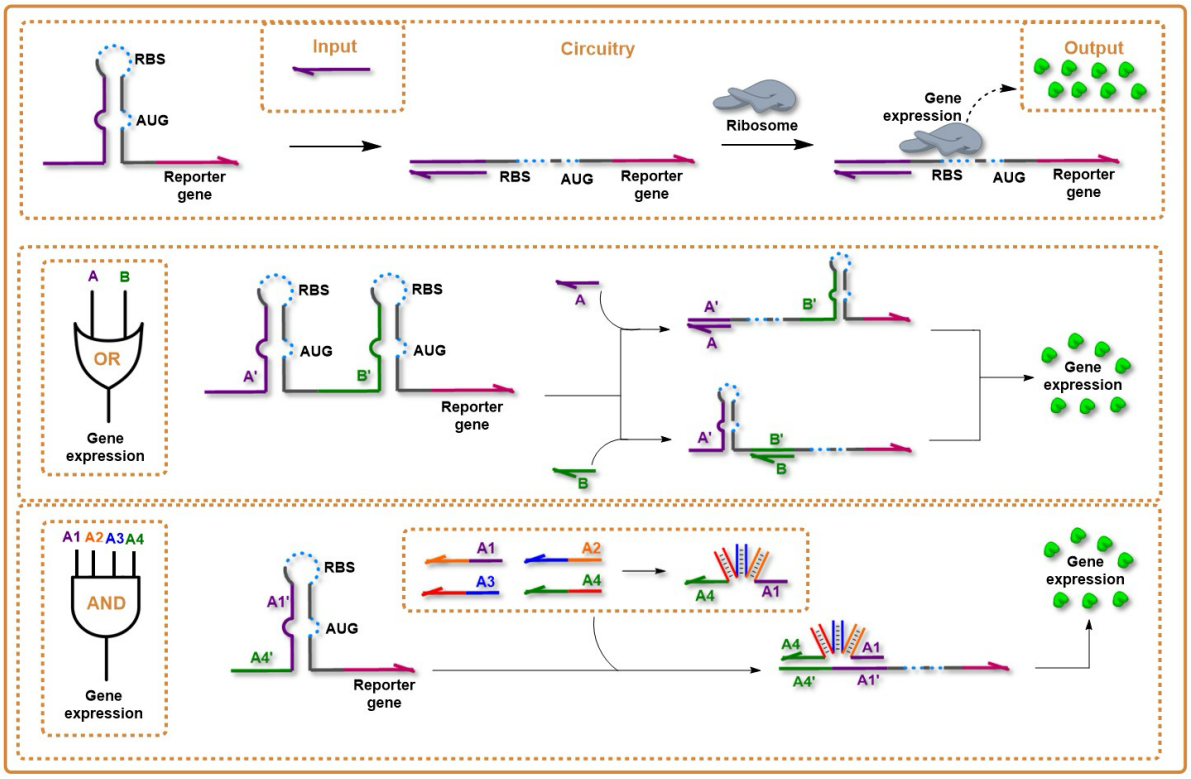

Figure 9: Case study 4 – conformational modulation of ribocomputing.[51]

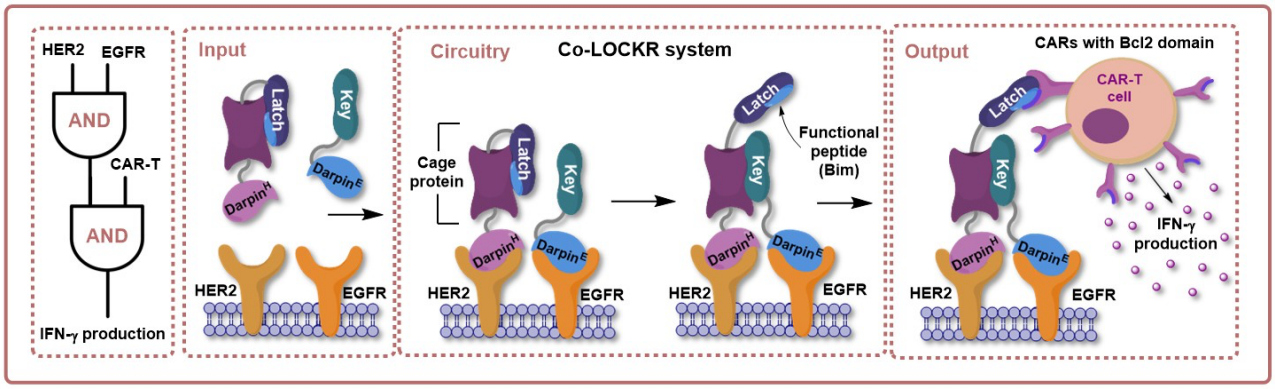

Figure 10: Case study 5 – *de novo* designed colocalization dependent protein switch.[92]

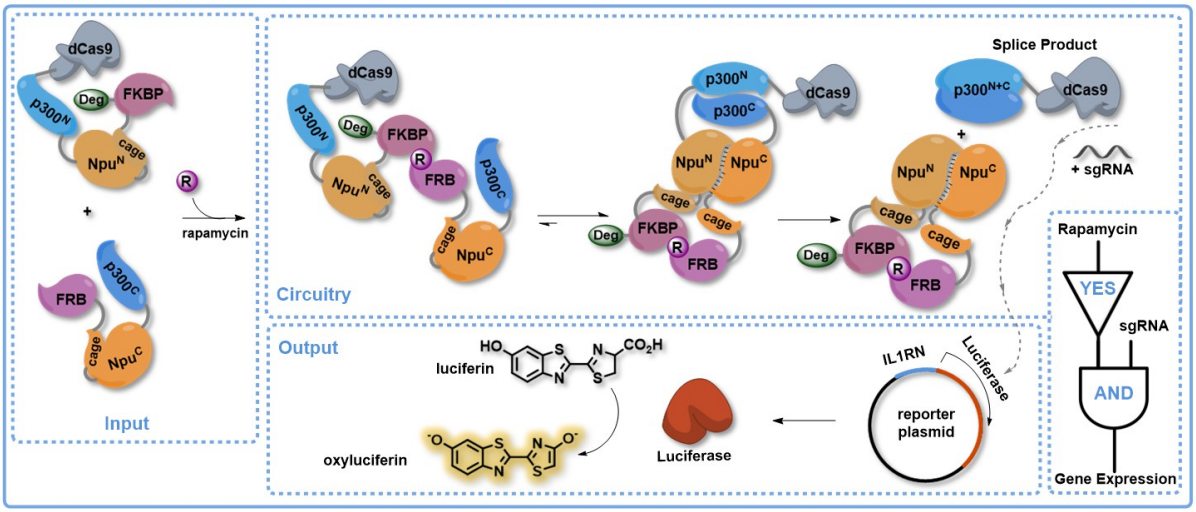

Figure 11: Case study 6 – proximity induced split intein network.[104]

PERSPECTIVES

Progress in biosupramolecular circuits are likely to play an important role in biological engineering, diagnostics and therapeutics (or theragnostics), akin to the role that electrical circuits have played in the engineering of electromechanical devices. Indeed, biosupramolecular circuits have already found tremendous utilization in the areas of targeted drug delivery, diagnostics, imaging and fluorescence guided surgery (for extensive discussion of such applications see the following reviews[146, 147]). It is clear that the programmability of nucleic acid hybridization offers a powerful platform to regulate biochemical processes through designed circuitry, but other types of interactions are possible and limited only by progresses in *de novo* design of interacting partner, be it small molecules or proteins. Our ability to construct sophisticated biochemical circuits from scratch requires the development of robust mechanisms that can translate basic assemblies into a gain of function. The ability to embed such circuitry into complex biological environments (live cells to whole organism) has been demonstrated and the circuitry can integrate multiple inputs to yield therapeutics or imaging agents in response. However, several limitations remain which must be overcome in order to fully realize the potential of biosupramolecular circuits. Namely, these include the slow rate of biocomputation, issues of biocompatibility and stability, and the ability to overcome diffusion effects through targeted delivery/compartmentalization of circuitry components (such concerns are extensively reviewed in the following articles[147-149]). Undoubtedly, improved chemical manipulations will play a key role in overcoming these limitations through the ability to produce *de novo* architectures with unnatural/stable linkages. Advances in the area are anticipated to pave the way to new avenues in diagnosing and treating diseases and provide new therapeutic paradigm through the emergent properties of the system. The clinical success of monoclonal antibodies has moved modern pharmaceutical strategies to increasingly embrace so called "biologics" and, cell-based therapies are poised to further complement the therapeutic arsenal. However, there still exists the need for the level of precise control afforded by traditional chemical approaches, particularly in the area of antibody-drug conjugates, where pressure continues to mount in terms of defining specific and homogeneous active agents. For instance, few cancers are uniquely defined by a single biomarker and systems that can integrate multiple inputs have the potential to refine the magic bullet. Biosupramolecular networks provide a privileged arena for the combination of chemical precision with the biocompatibility and specificity of biological agents. Not only do such networks enable the ready combination of two powerful fields of scientific research but achieve this in a tuneable and responsive manner. By crafting systems capable of processing multiple discrete inputs to produce an appropriate molecular output it may be possible to produce therapeutic systems with heightened selectivity which could not be accessed through targeting of individual molecular triggers in isolation. The modular nature of such networks enables the facile exchange components, rapidly enabling the conversion of a diagnostic to a therapeutic through output modification, or the application of an existing system to a new indication through modulation of the input system. As the molecular modalities available for each component of biosupramolecular networks continue to grow, the numbers of available network combinations will also increase exponentially. Thus, it is important not only to keep abreast of the latest technologies available but also to seek innovative ways of combining them with existing network components. The reductionist view of such network systems presented above seeks to break down these complex systems into interchangeable components which can be readily applied to a variety of systems. Through this approach a practitioner can quickly adapt network architectures to new problems and thereby fully exploit the potential of biosupramolecular networks.

Finally, a hallmark of living system is the ability to translate instructions into organized and dynamic networks. From a broad perspective, the methodology presented in this perspective to create biosupramolecular networks that respond to environmental cues are an important step towards the synthesis of chemical systems that emulate life. We hope that the examples discussed will inspire further developments in the area.

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Notes

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Author Contributions

The manuscript was written through contributions of all authors.

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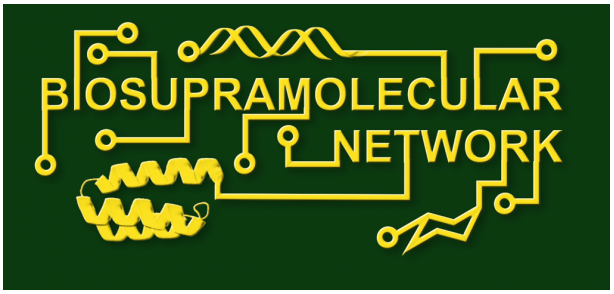
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