Biochemical flip-flop memory systems: essential additions to autonomous biocomputing and biosensing systems

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This review article is an overview of the current state of the development of biochemical flip-flop memory systems for use with biocomputing. Of particular interest are those developed using chemical and biochemical systems and components, capable of the complete integration into existing biocomputing information processing systems. The integration of memory systems with sophisticated logic “machines” is essential to the advancement of the field of biocomputing, since this combination would allow for the synthesis of a new regime of molecular “devices” capable of real-time biochemical analysis, coupled with long-term data storage. The specific biochemical realizations of the SR, T and D flip-flops are discussed and further explanations are provided for their use of specifically chosen enzyme pathways. Lastly, perspectives are given on the concept of scalability, and the intercalation of these memory units into existing chemical and biochemical information processing paradigms.

Keywords: biocomputing; enzyme logic; logic gate; binary logic; flip-flop; memory

1. Introduction

A surge of interest has occurred in the past decade in the development of chemical (Credi 2007; De Silva et al. 2007; Pischel 2007; Szacilowski 2008; Andreasson and Pischel 2010; Katz 2012a) and biochemical (Stojanovic et al. 2005; Ezziane 2006; Katz and Privman 2010; Katz 2012b) systems capable of mimicking electronic/digital processes, thus revolutionizing the field of biocomputing, a burgeoning subset of unconventional computing (Adamatzky et al. 2005; Gheorghe 2005; Calude et al. 2009). Rather than attempting to compete with silicon-based computing, most biocomputing is based around the interfacing of traditional digital computing with biological systems, focused primarily on biosensing and biomedical applications. This exponential growth continues to progress forward, heading towards a whole new realm of computing based on living processes. Drawing from the rapid development in the last decade of switchable molecular and macro-molecular systems (Flood et al. 2004; Matsuda and Irie 2004; Venturi et al. 2004; Saha and Stoddart 2006; Loeb 2007; Nijhuis et al. 2007; Canary 2009; Coronado, Gavina, and Tatay 2009; Leung et al. 2009; Thanopulos et al. 2009) significant progress has been made in the mimicking of digital processes in molecular electronics (Guo et al. 2004; Credi 2007; De Silva et al. 2007; De Silva and Uchiyama 2007; Pischel 2007; Jiang et al. 2008; Szacilowski 2008). The success of producing complex designs for elaborate molecular systems allowed for the realization of reversible (Pérez-Inestrosa et al. 2007), reconfigurable (Coskun, Deniz, and Akkaya 2005; Jimenez et al. 2005; Li et al. 2008; Sun et al. 2008) and resettable (Zhou et al. 2006; Sun et al. 2007) logic gates for chemical information processing. Drawing
from a diverse toolbox of various biomolecular components, proteins/enzymes (Ashkenazi et al. 1997; Sivan and Lotan 1999; Deonarine, Clark, and Konermann 2003; Sivan, Tuchman, and Lotan 2003; Unger and Moul 2006), DNA (Stojanovic et al. 2005), RNA (Win and Smolke 2005), and whole cells (Simpson et al. 2001; Li et al. 2011) have been used for the realization of a diverse set of biochemical logic systems. A focus has been made in the creation of systems capable of performing various Boolean logic operations, such as AND (De Silva, Gunaratne, and McCoy 1993, 1997; Guo, Zhang, and Zhu 2004), OR (De Silva, Gunaratne, and Maguire 1994), XOR (Credi et al. 1997; De Silva and McClagan 2002), NOR (De Silva et al. 1999; Turfan and Akkaya 2002; Straight et al. 2007), NAND (Baytekin and Akkaya 2000; Zong, Xiana, and Lua 2007), INHIB (Gunnlaugsson, MacDónaill, and Parker 2000, 2001; De Sousa et al. 2006; Li et al. 2007) and XNOR (Luxami and Kumar 2008; Qian et al. 2008). While these systems have been developed using both chemical and biochemical processes, one particular advantage for the biomolecular variant over merely chemical systems is the ability to interact with and process signals directly from biological systems. A clear advantage of this is the ability for these biocomputational systems to operate within biological environments (Kahan et al. 2008), thus making them drastically more attractive for use in various biomedical applications (Manesh et al. 2009; Pita, Zhou et al. 2009). Furthermore, a number of non-Boolean functional motifs, including molecular comparator (Pischel and Heller 2008), digital demultiplexer (Pérez-Inestrosa et al. 2008; Arugula et al. 2010), encoder–decoder (Andreasson et al. 2008), keypad lock (Margulies et al. 2007), etc., have been developed using these complex biochemical systems.

One particularly interesting and rapidly developing sub-direction of biocomputing is the use of enzyme systems (Baron et al. 2006a, 2006b, 2006c; Strack et al. 2008). Many of the previously mentioned Boolean logic operations have been successfully developed using enzymatic processes (Baron et al. 2006b) resulting ultimately in the design and construction of highly complex systems capable of performing arithmetic operations (half-adder/half-subtractor) (Baron et al. 2006c). The next logical step for the advancement of these ensembles was the integration of these complex enzyme biocomputation systems with signal-responsive materials (Pita et al. 2008; Motornov et al. 2008, 2009; Pita, Minko, and Katz 2009; Tokarev et al. 2009; Katz et al. 2013), e.g. switchable membranes (Tokarev et al. 2009), bioelectrochemical (Pita, Tam et al. 2009; Privman et al. 2009; Zhou et al. 2009) and bioelectronic (Krämer et al. 2009) systems. Drawing from a number of disciplines, these highly sophisticated systems were produced, thus broadening the overall uses and applications.

These chemical (De Silva, Gunaratne, and McCoy 1993, 1997; De Silva, Gunaratne, and Maguire 1994; Credi et al. 1997; De Silva et al. 1999; Baytekin and Akkaya 2000; Gunnlaugsson, MacDónaill, and Parker 2000, 2001; De Silva and McClagan 2002; Turfan and Akkaya 2002; Guo, Zhang, and Zhu 2004; De Sousa et al. 2006; Li et al. 2007; Straight et al. 2007; Zong, Xiana, and Lua 2007; Luxami and Kumar 2008, Qian et al. 2008) and biochemical logic gates (Strack et al. 2008; Katz and Privman 2010) perform complicated information processing operations, but do not store any actual information. Long-term use of these logic systems requires the addition of memory components, adding the ability to store an output in a stable form for later reference. Therefore, the development of memory systems is essential for the advancement of chemical and biochemical digital systems. The construction of these memory units, capable of multi-function information processing (read-write-erase), using biochemical components for analysis and storage, could potentially revolutionize the field of bioelectronics. By coupling these memory units with existing biocomputing ensembles, the functionality of switchable materials could be drastically improved, allowing for complex biocomputing devices, with built in logic and long-term data storage, capable of dynamic response to their environment.
Of particular interest is the base-level integration of novel molecular memory units into chemical and biochemical logic systems for sophisticated information processing; however, the conceptualization and fabrication of these systems proves to be immensely challenging. Of the traditional silicon-based flip-flop memory circuits used in modern computers, such as the Set/Reset (SR) flip-flop, the Delay (D) flip-flop, the Toggle (T) flip-flop and the Jack Kilby (JK) flip-flop, the SR-flip-flop is, by far, the least challenging to reproduce using chemical/biochemical systems. This is primarily due to the fact that two antonymous reactions proceeding in one controlled direction, or the reverse, can serve as a viable method for the set or reset of a system. SR flip-flop memory units have been produced using a number of methods, such as chemical, electrochemical and photochemical (Baron, Onopriyenko et al. 2006; Periyasamy et al. 2009; De Ruiter, Motiei et al. 2010; De Ruiter, Tartakovsky et al. 2010; Pischel and Andréasson 2010; De Ruiter and Van der Boom 2011), as well as a number of biomolecular systems based on DNA reactions (Elbaz, Moshe, and Willner 2009; Elbaz et al. 2009; Wang et al. 2010) and enzyme-catalyzed reactions (Pita, Strack et al. 2009). Less common in chemical (Puntoriero et al. 2011; Remón et al. 2011) and biochemical (Hoteit, Kharma, and Varin 2012; MacVittie, Halámek, and Katz 2012a, 2012b) models are the remaining flip-flops (e.g. JK, D, and T). This is due primarily to the fact that, by their very design, they exhibit higher complexity, making them difficult to mimic using biochemical systems. However, there have been successes in overcoming these complexities, resulting in the mimicking of both the D and T flip-flops (Hoteit, Kharma, and Varin 2012; MacVittie, Halámek, and Katz 2012a, 2012b). Presented here is a review of the current state of these biochemical realizations of flip-flop memory systems, as well as perspectives for these memory units, progressing forward in this area of biocomputing.

2. Flip-flop memory systems

2.1. General flip-flop information

Flip-flop memory systems are a specific subset of binary Boolean logic, focused around the precise manipulation of a single bit (e.g. digital 0 or 1) of data – a sophisticated method for the error-free storage of a single point of information. When these systems are scaled up, the possibility for reliable storage of high-density data emerges. Traditionally, these systems are composed of multiple logic gates, with individual truth tables, which, when concatenated, produce a more complex final logic. This can be seen, for example, in the case of the D flip-flop, which is composed of five separate logic gates, Figure 1. The overall logic of this flip-flop is the sum of its parts, producing a completely new truth table, Table 1. For this reason, there are two ways these systems can be produced using chemical and biochemical systems: directly duplicating the exact logic of the individual logic gates, or reproducing the

![Figure 1. The wiring diagram for a D flip-flop memory gate. The inputs of the D flip-flop, commonly referred to as Clock (C) and Data (D), are used for setting a system state, Q, to one of two digital states, 0 or 1.](image-url)
overall logic of the memory system. The former of these options is typically considerably
more complicated with no realistic advantage; therefore, most work is centered on mirroring
the overall logic of the memory systems.

2.2. Set/Reset (SR) flip-flop
The set/reset (SR) flip-flop is widely recognized as one of the most straightforward flip-flop
memory units. A two input/two output logic system, the SR flip-flop is based on the switching
of a primary output between two states, defined as digital values of 0 and 1. The set (S) and
reset (R) inputs are applied simultaneously, at one of two different levels, denoted 0 and 1. When \( S = 0 \) and \( R = 0 \) is applied, \( Q \) maintains its current state, and no change occurs to the
system. The application of \( S = 1 \) with \( R = 0 \) results in \( Q \) being set to a unit state of 1, while
the converse application of \( S = 0 \) and \( R = 1 \) results in \( Q \) being reset to a unit state of 0,
regardless of initial state of \( Q \) in both cases. The application of \( S = 1 \) and \( R = 1 \) would result
in the system performing contrasting logic, producing a nonsense output, and is therefore not
permitted as a valid combination. These logic operations are best visualized in a truth table,
showing each combination of inputs, along with the current and resulting values of \( Q \), Table 2
(Shiva 1998).

One example of the SR flip-flop being produced biochemically is with the use of two
enzymes, capable of oxidizing and reducing a redox mediator, respectively. The transition
between two oxidation states of the redox species was used as the value for unit system state
of \( Q \). Since the naming of the system state as either 0 or 1 is arbitrary and definition-based,
the oxidized state was chosen to represent digital 1, with the reduced state corresponding to a
state of digital 0. The enzymes used in this particular realization were horseradish peroxidase
(HRP) and diaphorase (Diaph); chosen for their ability to catalyze the oxidation and reduction
of a redox species, respectively, Scheme 1, central part, (Pita, Strack et al. 2009). \( \text{H}_2\text{O}_2 \), a
substrate of HRP, was used as the set (S) input associated with the oxidation of the redox

<table>
<thead>
<tr>
<th>Chemical reaction</th>
<th>Clock input</th>
<th>Data input</th>
<th>( Q ) current</th>
<th>( Q ) next</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>F</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<tr>
<td>G</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. The truth table for the SR flip-flop. The system state, \( Q \), can be switched between two system
states, 1 and 0, based on the application of either a set (S) or reset (R) input, respectively.

<table>
<thead>
<tr>
<th>Set (S)</th>
<th>Reset (R)</th>
<th>Previous state, ( Q_t )</th>
<th>New state, ( Q_{t+1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>( Q_t ) (0 or 1)</td>
<td>( Q_t ) (0 or 1) – unchanged</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>( Q_t ) (0 or 1)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>( Q_t ) (0 or 1)</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>( Q_t ) (0 or 1)</td>
<td>Unstable – not permitted</td>
</tr>
</tbody>
</table>
species ($Q = 1$), while NADH, a cofactor of Diaph, was used for the reset ($R$) input resulting in the reduction of the redox species ($Q = 0$). Since both inputs were optimized for full conversion of the redox species, the addition of H$_2$O$_2$ to a system that was already in the oxidized state should result in no change to the system; likewise, for the addition of NADH to a system already in the reduced state. Depending on the choice of mediator, the state of the system could be measured by either optical or electrochemical means.

The ubiquitous nature of both H$_2$O$_2$ and NADH, being that they may be produced by numerous biochemical reactions, allows for a potentially wide range of applications for this system. With the core component of the flip-flop being the enzymes HRP and Diaph, any peripheral systems that can controllably generate the $S$ and $R$ inputs in situ can be used as auxiliary inputs. The diversity of this system was demonstrated using two different biochemical examples.

In one example, the SR flip-flops operation was demonstrated using the physiologically significant chemical glucose as an input. The core system was extended so that H$_2$O$_2$ was generated in situ by the enzyme glucose oxidase (GOx) upon the addition of glucose, acting as the $S$ input. Additionally, alcohol dehydrogenase (AlcDH) was used to produce NADH in situ in the presence of ethanol, thus making ethanol the $R$ input, Figure 2, (Pita, Strack et al. 2009). The progress of this system was monitored optically with the absorbance at $\lambda = 600$ nm, correlating to the synthetic dye 2,6-dichlorophenolindophenol (DCIP), being used to represent the state of the system. In this example, the reduced form of DCIP represented $Q = 0$, while the oxidized form represented $Q = 1$. For the first set of experiments, the state of the system $Q_t$ was set to 0, using NADH to “pretreat” the DCIP into its reduced state via Diaph. With DCIP in its reduced state, no absorbance peak was present at $\lambda = 600$ nm, defining $Q_t$ as 0. From here ethanol was added to the system, thus applying the $R = 1$ input, while $S$ was held at 0 (meaning no addition of glucose to the system). Through AlcDH, this addition of ethanol produced additional NADH in the system; however, since $Q_t$ already was set at 0 (DCIP in its reduced form), the addition of the $R = 1$ input had no effect on $Q$ at $\lambda = 600$ nm, defining $Q_{t+1}$ as 0. Lastly, glucose was added to the system ($S = 1$), which, through GOx, produced H$_2$O$_2$ in situ, thus resulting in the oxidation of DCIP via HRP. The presence of DCIP in its oxidized state was visible as an absorbance peak at $\lambda = 600$ nm, resulting in $Q_{t+2}$ being defined as 1. The opposite was also tested, this time beginning with DCIP in its oxidized form ($Q_t = 1$), illustrated by a peak visible at $\lambda = 600$ nm. An application of an $S = 1$ input, via the addition of glucose, resulted in no change to the system, since DCIP was already in the oxidized form, resulting in $Q_{t+1} = Q_t$. Finally, the $R = 1$ input was added in the form of ethanol, which, via AlcDH, and HRP further downstream, biocatalyzed the reduction of DCIP, resulting in the loss of absorbance at $\lambda = 600$ nm, thus resetting the system state of $Q_{t+2}$ to 0. Both of these experiments follow the logic of the SR flip-flop as presented by the truth table in Table 2, the spectra of which are shown in Figure 2.

A second example of the interchangeability of the peripheral system around the core machinery of this particular realization of the SR flip-flop was based on using D-glucose-6-phosphate and lactate as inputs, Scheme 1, (Pita, Strack et al. 2009). D-glucose-6-phosphate, through glucose-6-phosphate dehydrogenase (G6PDH), produced NADH in situ, signifying the reset input. Conversely, lactate was used as the set input, with the production of H$_2$O$_2$ in situ via lactate oxidase (LOx). This example operated in the same fashion as the first and served as further proof of the ability of this system to operate with a large variety of input signals. The flexible nature of this particular system posits that it could operate at the terminus of a complex cascade of enzyme reactions, functioning as a reliable memory unit for biochemical data storage.
While all previous examples were monitored optically using visibly active redox species, additional experiments were performed where the current corresponding to a particular redox state of the mediator was used as the electrochemical definition of the system state (Pita, Strack et al. 2009). The redox mediator $K_3[Fe(CN)_6]$ was used, with $Q = 0$ represented by the reduced state, and $Q = 1$ by the oxidized state. Since, in this example, a large anodic current response corresponded to the mediator in its reduced form, representing the system state of 0. Alternatively, the decrease in current corresponded to the conversion of the mediator to the oxidized form, representing the system state of 1. The core machinery of the flip-flop was used for these experiments, with $H_2O_2$ and NADH acting directly as the inputs, in the

Figure 2. Absorbance spectra following the logic operations of the SR flip-flop proving the appropriate function of the core machinery, as well as demonstrating the use of the peripheral enzymes GOx and AlcDH, Scheme 1. (A) (a) Starting from a $Q_t = 0$, (b) the reset signal 1 is applied, resulting in no change to the system, (c) followed by the change of system state to $Q_{t+2} = 1$, upon application of a set signal of 1. Inset: Absorbance at $\lambda = 600$ nm for each consecutive input application. (B) (a) Starting from $Q_t = 1$, (b) the application of a set input (c) followed by a reset input, resulting in the system state of $Q_{t+2} = 1$. Inset: Absorbance at $\lambda = 600$ nm for the initial system state, as well as after the application of consecutive set and reset inputs. Source: Reprinted with permission from Pita, Strack et al. (2009). Copyright 2009 American Chemical Society.
presence of HRP and Diaph. Beginning with the redox mediator in its reduced form \((Q_t = 0)\), the \(S=1\) input was applied by the addition of \(\text{H}_2\text{O}_2\), resulting in the conversion of the redox mediator to its oxidized form, biocatalyzed by HRP, setting \(Q_{t+1}\) to 1. From here, another \(S=1\) input was applied; however, \(K_3[\text{Fe(CN)}_6]\) was already in the oxidized state, therefore, the system state remained at 1, with \(Q_{t+1} = Q_{t+2}\). A variation of this experiment was repeated, using the \(S\) and \(R\) inputs to change the system state between 0 and 1. Starting at \(Q_t = 0\), \(\text{H}_2\text{O}_2\) was added, setting \(Q_{t+1}\) to 1 by the oxidation of \(K_4[\text{Fe(CN)}_6]\) through HRP. The addition of \(\text{NADH}\) served to reset the system state to 0, resulting in the return \(K_4[\text{Fe(CN)}_6]\) to its reduced form, making \(Q_{t+2} = 0\). Lastly, a third permutation was performed. Starting, once again, from the reduced state of \(K_4[\text{Fe(CN)}_6]\), the addition of \(\text{NADH}\) resulted in no change to the system, conserving system state, \(Q_t = Q_{t+1}\). Finally, \(K_4[\text{Fe(CN)}_6]\) in solution was converted to the oxidized state, through HRP; upon the addition of \(\text{H}_2\text{O}_2\). This resulted in the final system state being defined as \(Q_{t+2} = 1\). With the use of existing electrochemical methods and micro-scale equipment such as micro-electrodes (Polsky et al. 2008) and field-effect transistors (Krämer et al. 2009), it’s no stretch of the imagination to consider this system being miniaturized and scaled for use in higher density data storage in biochemical systems.

In order to demonstrate the feasibility of this particular biochemical realization of the SR flip-flop as a suitable method for the long-term, scalable storage of information, a multi-well micro-titer plate was used as a reaction vessel for the setting, resetting and storage of multiple points of data (Pita, Strack et al. 2009). Each well contained the core machinery of HRP, diaphorase and \(K_4[\text{Fe(CN)}_6]\), while the absorbance at \(\lambda = 415\) nm corresponded to the system state. Since \(K_4[\text{Fe(CN)}_6]\) was added in the reduced state, all wells were initially set to 0. An initial round of inputs was applied to either Set the system state to 1, by the addition of \(\text{H}_2\text{O}_2\), or Reset the system state to 0, by the addition of \(\text{NADH}\). This was done in a pattern so that the micro-titer plate read the word “Clarkson”, with each column of eight wells corresponding to one letter in ASCII, Figure 3(A). A second round of inputs was added to each well to either Set or Reset its system state. This second round of inputs was encoding a new word to the plate, “University”, Figure 3(B). Since \(K_4[\text{Fe(CN)}_6]\) is optically active based on oxidation state, the encoding and re-encoding of the plate is visible in the colour photo shown in Figure 3(C) with a table of the encoded states shown in Figure 3(D). This large-scale memory bank could be Set and Reset for as long as the enzymes retained their activity, and the information was stored successfully for days, being dependent only on the stability of the mediator. Given the highly customizable nature of this system, it is plausible to imagine this memory unit downstream of a highly complex biochemical cascade. As a given cascade responds to stimuli in its environment, the flip-flop can be tailored to the produced outputs for the set and reset of “bits” of information, resulting in the stable, long-term storage of data.

2.3. Toggle (T) flip-flop

The T flip-flop is an example of a memory unit whose logic is elegant in its simplicity. Essentially, the logic of the T flip-flop states that when the T-input is equal to 0, the state of the system is preserved \((Q_{\text{current}} = Q_{\text{next}})\); however, \(Q\) is toggled when the T-input is applied \((T = 1)\), Table 3. This means that the same input that will toggle \(Q_{\text{current}} = 0\) to \(Q_{\text{next}} = 1\) must also toggle \(Q_{\text{current}} = 1\) to \(Q_{\text{next}} = 0\). While this may appear relatively simple in theory, it could not be further from the truth in regards to chemical/biochemical systems. In non-silicon systems, the concept of changing the state of a system with a single input, with the only difference being the system’s initial state, is a complicated challenge to overcome.
One realization of the T flip-flop was accomplished using the enzyme horseradish peroxidase (HRP) and the multiple oxidation state property of 3,3',5,5'-tetramethylbenzidine (TMB), Scheme 2, (Macvittie, Halámek, and Katz 2012b). The absorbance at $\lambda = 650$ nm, corresponding to the visibly active first oxidation state of TMB, was used to represent the system state, $Q$. Beginning with TMB in its reduced form, a lack of absorbance at $\lambda = 650$ nm defined the initial state of the system as $Q_i = 0$. Upon the application of the T-input ($T = 1$) in the form of the addition of $H_2O_2$, the TMB was converted to its first oxidation state (TMB$_{ox}$), through

<table>
<thead>
<tr>
<th>Chemical reaction</th>
<th>T-input</th>
<th>$Q_{current}$</th>
<th>$Q_{next}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
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</table>
the biocatalytic activity of HRP. This change in system state from $Q_t = 0$ to $Q_{t+1} = 1$ was visible as a significant increase in absorbance at $\lambda = 650 \text{ nm}$. The addition of a second aliquot of H$_2$O$_2$ initiated the conversion, via HRP, of TMB$_{ox}$ into its double-oxidized state (TMB$_{dox}$). TMB$_{dox}$ is only visibly active at $\lambda = 450 \text{ nm}$ meaning when it is fully converted, the absorbance at $\lambda = 650 \text{ nm}$ diminishes to a value comparable to $Q_t$, signifying the change of $Q_{t+1} = 1$ to $Q_{t+2} = 0$. It should be noted that the T-input of 0, corresponding to no change in the system state, was defined as no addition of H$_2$O$_2$. The resulting experimental combinations are best visualized in a bar graph comparing the absorbance values at $\lambda = 650 \text{ nm}$ for the system before and after the T-input is applied, Figure 4.

The T flip-flop is an impressively simple logic operation to comprehend: taking the base concept of all memory units, the manipulation of a bit between two states, and reducing it to its most minimalistic form, using only a single input to control the state of a system. The successful realization of this system using biochemical methods and components is made more impressive by the conceptual understanding that it is exceptionally rare to see a biological or chemical process that exhibits this “environmental self-awareness” necessary for a single input to result in a reaction for both the setting and resetting of a defined system state. Since the non-system-state-generated input used in this method is the fairly ubiquitous chemical H$_2$O$_2$, this system can also be relatively easily integrated into existing chemical and biochemical cascades, as well as cascades that have not yet been designed, as long as the given cascade provides H$_2$O$_2$ as a quantitatively or qualitatively significant output. Such systems should be carefully optimized, or should contain supplementary enzymes such as catalyse, for the rapid removal of excess H$_2$O$_2$ from the system as it is toxic to biological tissue. Much like the SR flip-flop described previously, this system could be scaled up, and further optimized to allow for the storage of large-scale information, by utilizing separate compartmentalized memory units monitored by either optical or electrochemical means.

2.4. Delay (D) flip-flop

One of the more complicated flip-flop-based memory units is what is called the D flip-flop. This is a two-input/two-output logic system composed of four interconnected NAND gates,
and one NOT gate in its electronic version, Figure 1 (Mano and Kime 2000). This presents a highly intricate system, not easily or commonly duplicated using chemical systems (Puntoriero et al. 2011; Remón et al. 2011). As stated previously, the direct duplication of the electronic circuitry using chemical/biochemical systems is not necessary. A biochemical system operating with the same overall logic as this flip-flop is not only acceptable, it is preferred. A memory unit as complicated as the D flip-flop directly mimicked using biochemical processes would be costly, complicated, and likely prone to failures due to the intrinsic variability within, what would be, highly overlapped and complicated biochemical systems. For this reason, the truth table for the D flip-flop was used as the rubric for outlining the function of the memory unit, Table 1. The inputs of the D flip-flop, commonly referred to as Clock (C) and Data (D), serve drastically different functions. Put simply, the application of the data input (D = 1) is associated with the command for change in the system. Much like the SR flip-flop, the D flip-flop is capable of setting a system state, Q, to one of two digital states, 0 or 1. However, the D flip-flop does this in a more elegant fashion: with the D-input of 0 serving as the reset command (Q_{next} = 0), and the D-input of 1 serving as the set command (Q_{next} = 1). The C-input functions as an on/off switch for the system state of the memory unit when the C-input is held at 0, no change is observed in the system. Conversely, when the C-input = 1, the process is allowed to proceed, and the application of the D-input executes the necessary changes to the system. This on/off effect is best personified as a type of inhibition of the operation D-input and the system as a whole. Following the thought process of the C-input acting as an “inhibitor”, a biochemical realization of the D flip-flop becomes more readily apparent.

Utilizing enzyme inhibition for the “delay” function, one biochemical realization of the D flip-flop involved the core machinery of the enzymes lactate dehydrogenase from porcine heart (LDH) and alcohol dehydrogenase (AlcDH), Scheme 3, (MacVittie, Halámek, and Katz 2012a). The state of the system was represented by the absorbance at \( \lambda = 340 \text{ nm} \), corresponding to the cofactor \( \beta \)-nicotinamide adenine dinucleotide, (NAD\(^+\)/NADH). This mediator was present in one of two states: its oxidized form, NAD\(^+\), with no absorbance at \( \lambda = 340 \text{ nm} \) – corresponding to \( Q = 0 \) or its reduced form, NADH, being visibly active at \( \lambda = 340 \text{ nm} \) – corresponding to \( Q = 1 \). Using reversible enzyme inhibition as the mechanism for the C-input, a \( C = 0 \) input was defined as the presence of the inhibitors specific to the enzyme “machinery”, in this case: oxaloacetate and glutamate for the inhibition of LDH, and butyramide for the inhibition of AlcDH. This was done for reactions A–D, as described in the truth table in Table 1. For reactions E–H, a C-input of \( C = 1 \) was applied to the system; this was defined as the absence of the enzyme inhibitors from the system. The substrates of LDH and AlcDH were used as the biochemical D-input, with the addition of pyruvate (Pyr) without ethanol (Alc) serving as the D-input of \( D = 0 \), and the addition of ethanol without pyruvate corresponding to the D-input of \( D = 1 \). Considering the truth table, the D flip-flop can be split into two distinct types of reactions: inhibited and non-inhibited. For reactions A–D, the C-input was set at 0, meaning the inhibitors of the enzyme machinery were present in the system; regardless of system state or D-input application, no reactions occurred and the system state was conserved (\( Q_{current} = Q_{next} \)). Conversely, in reactions E–H, the C-input was held at 1: defined as the absence of inhibitors from the system. With no inhibitors in the system and the reactions capable of proceeding as per normal, the system was able to respond appropriately to the addition of either D-input of 0 or 1. The application of the D-input of 0, being the addition of Pyr, resulted in the oxidation of NADH to NAD\(^+\), in the presence of LDH. This process can only proceed when there is non-oxidized NADH in the system, i.e. when \( Q_{current} = 1 \), resetting the system state, \( Q_{next} = 0 \) (Reaction F, Table 1). The same input preserved the system state of \( Q = 0 \) when all of the
NAD$^+$ in solution was already present in the oxidized form, $Q_{\text{current}} = Q_{\text{next}}$ (Reaction E, Table 1). Reactions G and H represent the systems response to the application of a value of 1 for both the C- and D-inputs simultaneously. With the cofactor in its reduced form ($Q = 0$), the addition of Alc initiated the conversion of NAD$^+$ to NADH, by AlcDH, setting the system state from $Q_{\text{current}} = 0$ to $Q_{\text{next}} = 1$ (Reaction G, Table 1). However, with the system state initially set to 1, all of the cofactor in solution was already present in the reduced form of NADH, meaning even with the addition of Alc (D-input = 1) AlcDH had no NAD$^+$ to reduce, preserving system state at 1, $Q_{\text{current}} = Q_{\text{next}}$ (Reaction H, Table 1). These reactions are better illustrated in the form of a bar graph, Figure 5.

Of the flip-flop memory systems that have been mimicked using chemical and biochemical processes, the D flip-flop is unquestionably the most dynamic. With the capability to completely disable the entire logic complex, the D flip-flop presents a unique set-up, in which the memory unit only operates as needed. This inhibited “off” state allows for protection against accidental or unintentional data manipulation. By incorporating the memory unit into a complex biochemical information processing “device”, the logistics of the data storage can be managed so that not only does the logic ensemble have the ability to manipulate the system state of the memory unit, it can also be programmed to simultaneously activate or deactivate the system as needed for the read, write and reset of data, with the ability to “lock” the memory unit from outside influences and chemicals. This can also allow for the utilization of this memory unit in a matrix that may otherwise contain chemicals capable of the occasional unintentional data-point modification, as the deactivation of the unit during situations where the unit data does not need to be modified.

3. Conclusions and perspectives

With the multitude of fundamental advancements in the chemical and biochemical realizations of memory systems, the challenges approaching are becoming based more in the macro-development of these intelligent systems: focusing on their concatenation and integration into

Figure 5. Experimental results of the D flip-flop comparing the system state, $Q$, before and after each possible logic combination, Reactions A–H from the truth table (Table 1). The state of the system before and after each combination of the Clock/Data inputs was represented by the maximum absorbance measured at $\lambda = 340$ nm.

existing and emerging paradigms. By building upon fundamental biocomputing concepts, such as the manipulation of enzyme pathways, the SR-, D- and T- flop-flops presented here can be easily integrated with existing biocomputing ensembles. The coupling of memory systems with molecular “devices” capable of processing biochemical inputs would lead to an explosion in biomedical applications. One situation where this would be especially

Scheme 1. A graphical representation of the enzyme-based SR flip-flop, including one example of the auxiliary enzymes. The use of auxiliary enzymes, capable of producing outputs in situ, which can be used by the core enzymes as inputs, drastically increases the systems viability for use in a multitude of situations.

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Scheme 2. Schematic representation of the enzyme-based T flip-flop memory system. The enzyme HRP was used as the primary machinery of the flip-flop. Taking advantage of the double oxidation states of the dye TMB, HRP was used to alter the absorbance at \( \lambda = 650 \) nm upon the application of the T-input \( \text{H}_2\text{O}_2 \).

advantageous is in the real-time monitoring and treatment of military personnel. A system capable of constantly tracking a soldier’s physiological conditions, as well as being able to administer immediate ad hoc medical attention in a battlefield setting, would require a stable and reliable system for keeping exact records of any treatments provided to the patient. Much like GPS, digital cameras and canned food, these advanced systems would soon trickle down to the general populace, completely overhauling the current approach to day-to-day healthcare.

Taking these “sense-and-treat” conceptualizations one step further results in an interesting concept: the integration of implanted biofuel cells (Katz and MacVittie 2013) with chemical and biochemical logic systems equipped with built-in memory capabilities. The development of self-powered “devices” operating with complete autonomy would thoroughly revolutionize the field of medicine. These autonomous physiological diagnostic devices, or more aptly put “physiodiagnostics”, could be capable of real-time monitoring of biomarkers, performing complex biocomputations as well as responding accordingly to stresses, variations or pathogens in the body, while simultaneously recording all necessary information using built-in enzyme-based flip-flop memory systems. While each individual component would be essential, the ability to have constant and reliable long-term storage of information would be imperative. A sufficiently advanced “device” would have the ability to record a complete collection of system states, saving information on both the biological activity of the host, as well as subsequent actions performed by the “device”, (i.e. administration of medicine). This concept of constant monitoring at the molecular level would likely result in early-stage diagnosis of diseases; however, this has little benefit if the system has no way to store the information processed by the “device”.

With the continued development of increasingly complex chemical and biochemical systems mimicking the logic and operation of modern silicon computers, additional components must also be developed to maximize their potential applications. The addition of sophisticated memory motifs to existing biocomputing systems has the potential to revolutionize a number of fields, the most prominent being medicine. The prospect for real-time physiological monitoring with reliable, long-term memory storage via sophisticated “physiodiagnostic” devices could result in a future where diseases are diagnosed and treated before they even present detectable symptoms.
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References


