

Article

Synthetic Biology and Control Theory: Designing Synthetic Biomolecular Controllers by Exploiting Dynamic Covalent Modification Cycle with Positive Autoregulation Properties

Yijun Xiao ¹, Hui Lv ^{1,2,*}  and Xing'an Wang ^{3,4}

¹ Key Laboratory of Advanced Design and Intelligent Computing, Ministry of Education, School of Software Engineering, Dalian University, Dalian 116622, China

² State Key Laboratory of Synthetical Automation for Process Industries, Northeastern University, Shenyang 110004, China

³ College of Environmental and Chemical Engineering, Dalian University, Dalian 116622, China

⁴ Dalian Chivy Biotechnology Co., Ltd., Dalian 116023, China

* Correspondence: lh8481@tom.com

Abstract: The emerging field at the intersection of synthetic biology, network modelling, and control theory has grown in popularity in recent years. In this study, the aim is to design synthetic biomolecular controllers by exploiting the covalent modification cycle (CMC) enhanced with direct and indirect positive autoregulation (DPAR and IPAR). Two novel biomolecular controllers proposed, the Brink controller (BC) with DPAR (namely BC-DPAR) and the BC with IPAR (namely BC-IPAR), allow to (a) use fewer chemical reactions than purely designs based on dual chemical reaction networks (DCRNs), and (b) improve the stability of ultrasensitive response when designing biomolecular controllers. Following the conversion route from chemical reactions to DNA strand displacements, the integration of the two novel controllers and an enzymatic proteolysis model proposed aims to analyse the regulatory properties by exploring the tracking response of proteolysis products.

Keywords: chemical reaction networks (CRNs); molecular programming; DNA strand displacement reaction; enzymatic protein hydrolysis model; positive autoregulation; covalent modification cycle



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1. Introduction

A major focus of synthetic biology research is to develop and design achievable control schemes and synthesize biomolecular controllers to achieve ideal control effects on the output of biochemical reaction processes [1–3]. In recent years, the idea of incorporating classical control theory into the synthesis of biomolecules to guide and design synthetic controllers for biomolecular processes has grown in popularity [4,5]. In addition, in chemical reaction networks (CRNs), such as catalytic, degradation and annihilation reactions are often treated as tools to describe biochemical systems [6,7]. Various controllers have been constructed utilizing CRN reactions, including a linear I/O system [8], proportional integral (PI) controller [9,10], non-linear quasi sliding mode (QSM) controller [11], proportional integral derivative (PID) controller [12,13] and two-degree-of-freedom (2DOF) PID controller [14].

Thus, constructing CRNs to characterize the dynamics of the system becomes a major goal [15–17], thereby realizing the transformation from CRNs to DNA strand displacement (DSD) reactions [18–20]. In characterizing biochemical control systems, CRN representations rely on dual rail representations, which represent signals in the control system as the difference in concentration of two specific species [21–23]. This representation scheme realizes that the concentration of substances can freely express positive and negative signals, which results in at least doubling the total number of abstract chemical reactions required for implementation [24]. If the dual rail representation can be avoided in controller designs, the number of CRN reactions can be reduced, which leads to this work.

Many recent advances have been achieved in utilizing the covalent modification cycle to achieve ultrasensitive responses. Goldbeter et al. [25] explored the transient and steady-state behaviour of a reversible covalent modification system. The results showed that a given pathway or cascade can enhance its sensitivity through the following mechanisms: the conventional “cooperative ultrasensitivity”, “multistep ultrasensitivity” and “zero-order ultrasensitivity”. Gomez-Urbe et al. [26] analysed the covalent modification cycle’s dynamic and steady-state responses to time-varying perturbations, while Jeynes-Smith et al. [27] proposed two new models of reversible covalent modification cycles with positive autoregulation (PAR) that have ultrasensitivity and bi-stability in different parameter regimes. Jones et al. [28] designed a synthetic covalent modification cycle using kinase and phosphatase proteins and realized a tunable negative feedback controller to reduce output expression variance.

Various controllers based on the covalent modification cycle have been constructed. Foo et al. [29] exploited the chemical reactions and dynamic properties underlying the covalent modification cycle to construct a CRN-based non-linear covalent modification cycle (CMC) controller. The results showed that the CRN-based CMC controller reaches a quasi-steady state quickly and stably compared to CRN-based PI controllers to achieve the expected output levels. Samaniego et al. [30] constructed a molecular BC controller using a modular design strategy and applied it to regulate target RNA or protein expression. Note that the construction of the BC controller is based on a modular strategy that involves two design principles, including the use of ultrasensitive responses and a tunable threshold of the response.

In this work, attention has been paid to the research of enzymatic protein hydrolysis reactions. On the one hand, enzymes play important roles in many cellular activities, and accurate control of enzymatic reactions is beneficial to the stable performance of biological functions in biochemical systems [31–33]. On the other hand, related research on enzymatic protein hydrolysis reaction processes is extensive, and many fruitful approaches are relatively mature [34–36].

In this manuscript, the focus is to design novel biomolecular controllers based on enhanced covalent modification cycles. The covalent modification cycle with PAR is utilized to design two novel controllers, i.e., BC-DPAR (involving the covalent modification cycle with direct positive autoregulation) and BC-IPAR (involving the covalent modification cycle with indirect positive autoregulation). Considering the application of the covalent modification cycle in the BC controller which differs from DCRN-based controllers and circumvents the limitation of the dual rail representation due to the absence of subtraction modules, it is feasible to build novel controllers using the enhanced covalent modification cycle with PAR. On the one hand, the covalent modification cycle (CMC) is combined with the direct PAR to form a modified CMC structure (namely CMC-D), a new controller BC-DPAR; on the other hand, another enhanced CMC structure (namely CMC-I) is constructed integrating the CMC and indirect PAR, creating a novel controller BC-IPAR. Then, taking the enzymatic proteolysis reaction process as the research object, a corresponding biological model is constructed with simple chemical reactions to reflect the performance of the proposed biomolecular controllers. Considering the transformation relationship between CRNs and DNA strand displacement reactions, the abstract chemical reactions involving in the enzymatic proteolysis process will be transformed into the corresponding DNA strand displacement reactions in the following work. Finally, combined with the DNA strand displacement mechanism, CRN-based BC, BC-DPAR, BC-IPAR and the enzymatic protein hydrolysis model are converted, and the corresponding control schemes are designed using the proposed enzymatic protein hydrolysis process model, thereby analysing the corresponding regulatory properties. The results show that both the proposed novel controllers, BC-DPAR and BC-IPAR, can rapidly and stably achieve the desired concentration levels of protein hydrolysates compared to the existing BC. In addition, the DNA-based BC-DPAR control scheme with direct positive autoregulation is more effective in regulation than the BC-IPAR control with indirect positive autoregulation, requires less regulation time, and

can achieve steady-state output more quickly. Based on prior research on biomolecular controllers, it is proposed to examine potential biological implementations of the proposed controllers via different alternative implementation routes based on RNA molecules or proteins, thereby confirming the experimental findings.

The main contributions of the manuscript are as follows: (1) This paper proposes two novel biomolecular controllers within the context of chemical reaction network theory, i.e., BC-DPAR and BC-IPAR, which are based on the enhanced covalent modification cycle (involving the covalent modification cycle with direct or indirect positive autoregulation). In addition, these two controllers circumvent the limitation of dual rail representation in the CRNs designs owing to the absence of subtraction operations, thus reducing the number of abstract chemical reactions required for implementation. (2) The enzymatic protein hydrolysis reaction is modelled using CRNs, using the simplest form of chemical reactions to describe this typical enzymatic reaction process. The abstract chemical reactions are converted into DNA strand displacement reactions, and then the DNA-based enzymatic protein hydrolysis reaction model is constructed. (3) Combined with the proposed enzymatic protein hydrolysis process model and DNA-based controllers (including BC, BC-DPAR and BC-IPAR), the various control schemes are realized, and the corresponding regulation properties are analysed. Results show that the proposed two novel controllers, BC-DPAR and BC-IPAR, both can improve the output of enzymatic protein hydrolysis processes to a certain degree compared to the existing BC, especially the BC-DPAR control scheme involving the covalent modification cycle with direct positive autoregulation.

2. An Enzymatic Protein Hydrolysis Model

In this paper, the focus is on the process control of enzymatic reactions, which are ubiquitous in cell biology. Using the enzymatic proteolysis process as an example, this work investigates and designs the corresponding biochemical control schemes, as shown in Figure 1.

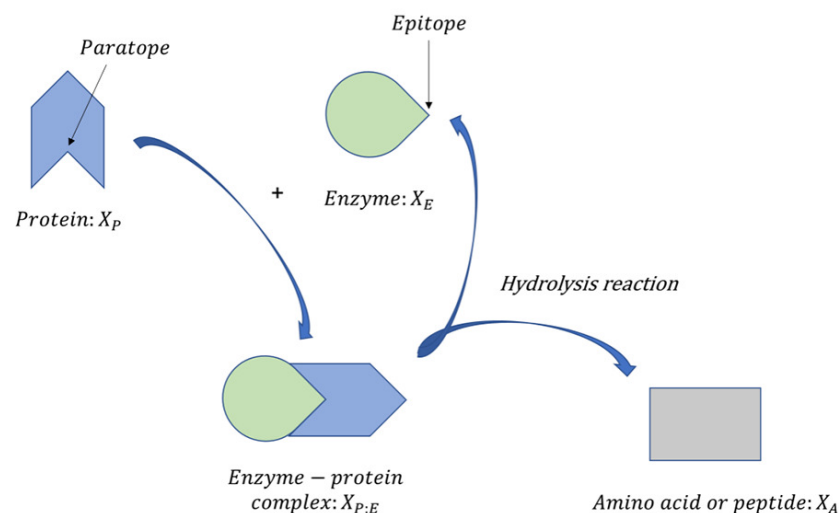


Figure 1. Graphical representation of an enzymatic protein hydrolysis process model.

The enzymatic protein hydrolysis process can be described in its simplest form of chemical reactions, expressed by



where the parameters k_1 and k_2 denote the catalytic rates, while k_3 represents the degradation rate. In addition, X_P and X_E denote the protein and enzyme, respectively, while $X_{P:E}$ represents the protein–enzyme complex. X_A denotes the products of the proteolysis process, i.e., amino acids or polypeptides.

In addition, the parameter X_W in the second reaction represents the chemical species, i.e., water. The reaction $X_A \xrightarrow{k_3} \emptyset$ in Equation (1) is used to describe the degradation process of amino acids or polypeptides. Note that the properties and quantity of the enzyme itself do not change before and after a typical enzymatic reaction process. The enzyme combines with a specific protein to form a protein–enzyme complex; the complex is decomposed to form the corresponding products (referring to amino acids or polypeptides) and enzyme.

Writing the mass action kinetics, the ODEs for Equation (1) can be obtained.

$$\begin{aligned}\frac{d[X_P]_t}{dt} &= -k_1[X_P]_t[X_E]_t \\ \frac{d[X_E]_t}{dt} &= k_2[X_{P:E}]_t - k_1[X_P]_t[X_E]_t \\ \frac{d[X_{P:E}]_t}{dt} &= k_1[X_P]_t[X_E]_t - k_2[X_{P:E}]_t \\ \frac{d[X_A]_t}{dt} &= k_2[X_{P:E}]_t - k_3[X_A]_t\end{aligned}\quad (2)$$

where $[\cdot]_t$ denotes the chemical concentration of $\cdot$.

The following result can be obtained based on Equation (2).

$$\frac{d[X_E]_t}{dt} + \frac{d[X_{P:E}]_t}{dt} = 0 \quad (3)$$

Note that the result $(d[X_E]_t/dt) + (d[X_{P:E}]_t/dt) = 0$ which implies that the total mass $X_E + X_{P:E}$ is conserved, i.e., $X_{\text{Total}} = X_E + X_{P:E}$, can be obtained based on Equation (3).

3. Methodology

3.1. Covalent Modification Cycle and Brink Controller (BC)

3.1.1. Covalent Modification Cycle (CMC)

Consider the basic framework of the covalent modification cycle (CMC) depicted in Figure 2, where an activator X_a binds to an inactive species U^* , and converts it to a chemically form U . A deactivator X_d binds to an active species U , and converts it to a chemically form U^* .

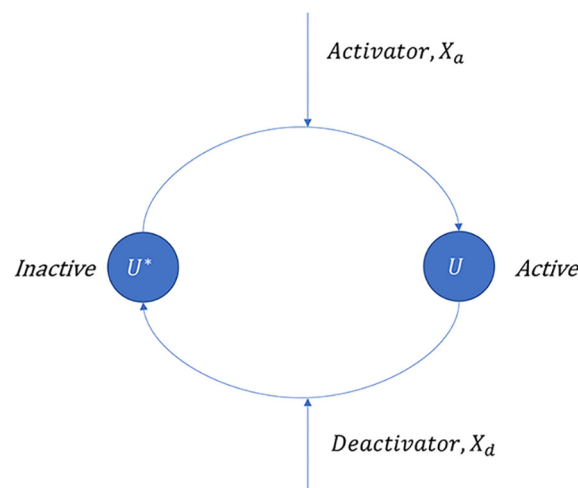


Figure 2. Mathematical framework for the covalent modification cycle.

Its abstract chemical reaction can be described by



ODEs of abstract chemical reactions (4) can be obtained by

$$\begin{aligned}
 \frac{d[C_1]_t}{dt} &= k_{b1}[X_a]_t[U^*]_t - k_{c1}[C_1]_t \\
 \frac{d[C_2]_t}{dt} &= k_{b2}[X_d]_t[U]_t - k_{c2}[C_2]_t \\
 \frac{d[U^*]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c2}[C_2]_t \\
 \frac{d[U]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c1}[C_1]_t \\
 \frac{d[X_a]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c1}[C_1]_t \\
 \frac{d[X_d]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c2}[C_2]_t
 \end{aligned}
 \quad (5)$$

The network diagram in Figure 3 is constructed to intuitively describe the connections between species and multiple reactions in Equation (4), where the entire network consists of binding and unbinding reactions.

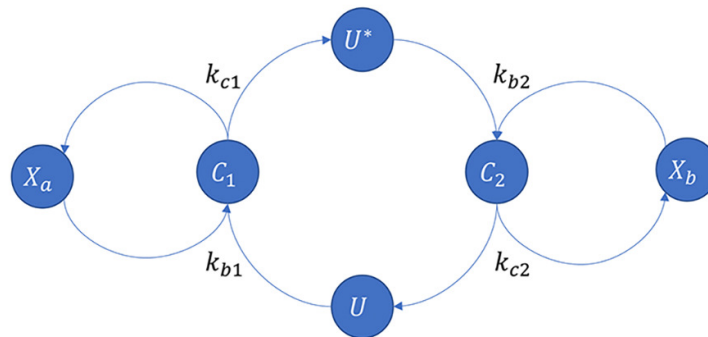


Figure 3. Graphical representation of Equation (4).

The following results can be given by

$$\begin{aligned}
 \frac{d[C_1]_t}{dt} + \frac{d[X_a]_t}{dt} &= 0 \\
 \frac{d[C_2]_t}{dt} + \frac{d[X_d]_t}{dt} &= 0 \\
 \frac{d[C_1]_t}{dt} + \frac{d[C_2]_t}{dt} + \frac{d[U^*]_t}{dt} + \frac{d[U]_t}{dt} &= 0
 \end{aligned}
 \quad (6)$$

Note that the results $(d[C_1]_t/dt) + (d[X_a]_t/dt) = 0$, $(d[C_2]_t/dt) + (d[X_d]_t/dt) = 0$ and $(d[C_1]_t/dt) + (d[C_2]_t/dt) + (d[U^*]_t/dt) + (d[U]_t/dt) = 0$ imply that the total mass $C_1 + X_a$, $C_2 + X_d$ and $C_1 + C_2 + U^* + U$ is conserved through the lifetime of the process.

3.1.2. An Application of the Covalent Modification Cycle—Brink Controller (BC)

A controller BC based on the covalent modification cycle is constructed in the following work, which can realize an ultrasensitive switch-like input–output response. Furthermore,

it can be described by the following abstract chemical reactions of Equation (7). The schematic of the BC is shown in Figure 4. Obviously, R and Y are the inputs of the BC, while U denotes the output of the controller. The parameters k_c , θ_c and α_c denote the catalysis rate, γ_c and β_c represent the binding rate, and ϕ_c indicates the degradation rate. In Equation (7), species R_r and R_y can combine to form complex $R_r \cdot R_y$, which does not interact with any other substances. More importantly, R can produce R_r , which in turn reacts with U^* to form U , while Y can produce R_y , which in turn reacts with U to form U^* .

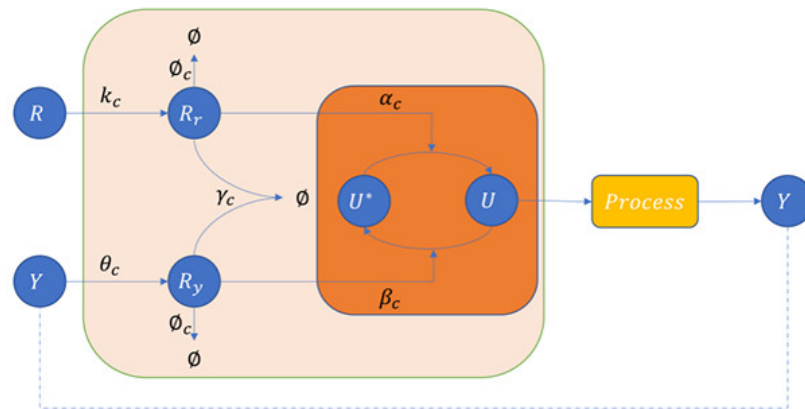
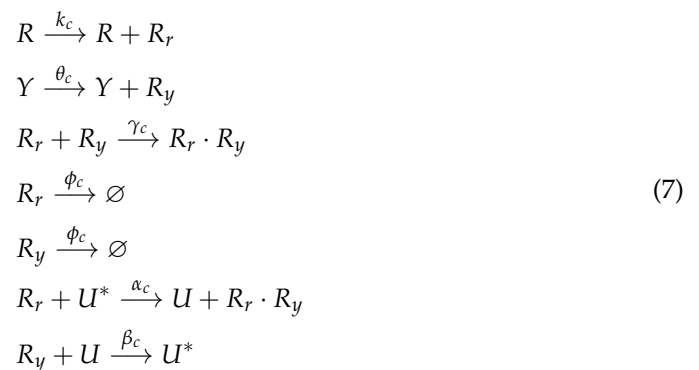


Figure 4. Schematic diagram of the BC scheme for a process.

For the BC, the corresponding CRNs can be expressed as



Using the mass action kinetics, the ODEs of Equation (7) are given by

$$\begin{aligned}
 \frac{d[R_r]_t}{dt} &= k_c[R]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_r]_t - \alpha_c[R_r]_t[U^*]_t \\
 \frac{d[R_y]_t}{dt} &= \theta_c[Y]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_y]_t - \beta_c[R_y]_t[U]_t \\
 \frac{d[U^*]_t}{dt} &= -\alpha_c[R_r]_t[U^*]_t + \beta_c[R_y]_t[U]_t \\
 \frac{d[U]_t}{dt} &= \alpha_c[R_r]_t[U^*]_t - \beta_c[R_y]_t[U]_t
 \end{aligned} \tag{8}$$

Equation (9) can be obtained due to Equation (8).

$$\frac{d[U^*]_t}{dt} + \frac{d[U]_t}{dt} = 0 \tag{9}$$

Note that the result $(d[U^*]_t/dt) + (d[U]_t/dt) = 0$ which implies that the total mass $U^* + U$ is conserved, i.e., $U_{\text{Total}} = U^* + U$, can be obtained based on Equation (9).

3.2. Covalent Modification Cycle with Direct Positive Autoregulation and a Novel Controller BC-DPAR

3.2.1. Covalent Modification Cycle with Direct Positive Autoregulation (CMC-D)

A block diagram showing the idea of the covalent modification cycle with direct positive autoregulation (CMC-D) is illustrated in Figure 5. Compared with the CMC structure (described in Figure 2), the structure CMC-D has the following same mechanism: U^* can be combined with X_a to form U , while U can react with X_d to form U^* . In addition, the following structure exists in CMC-D: the inactive species U^* can be transformed into U under the action of species U , i.e., the reaction $U^* + U \rightarrow C_3 \rightarrow U + U$.

CRN representations of the structure CMC-D are given by

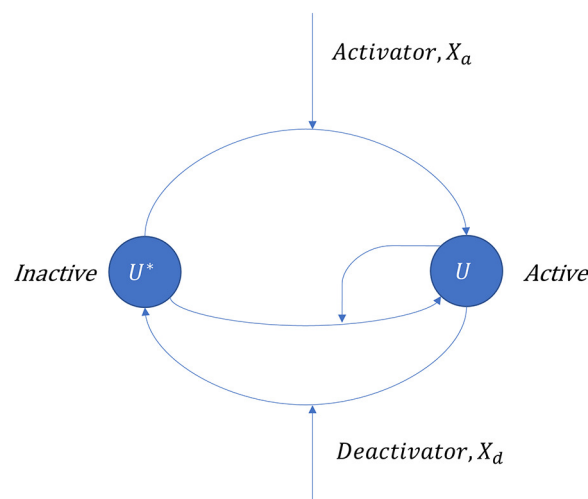
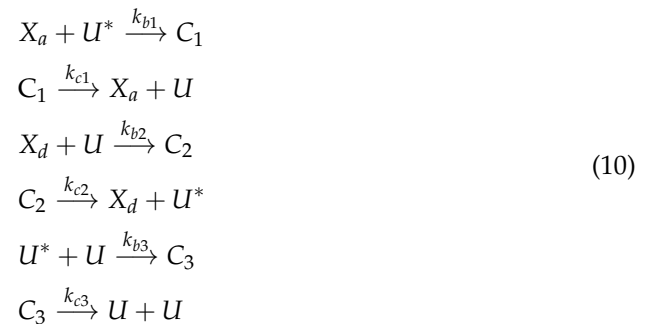


Figure 5. Mathematical framework for the covalent modification cycle with direct positive autoregulation.

ODEs of abstract chemical reactions in (10) can be obtained by

$$\begin{aligned}
 \frac{d[C_1]_t}{dt} &= k_{b1}[X_a]_t[U^*]_t - k_{c1}[C_1]_t \\
 \frac{d[C_2]_t}{dt} &= k_{b2}[X_d]_t[U]_t - k_{c2}[C_2]_t \\
 \frac{d[C_3]_t}{dt} &= k_{b3}[U]_t[U^*]_t - 2k_{c3}[C_3]_t \\
 \frac{d[U^*]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c2}[C_2]_t - k_{b3}[U]_t[U^*]_t \\
 \frac{d[U]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c1}[C_1]_t - k_{b3}[U]_t[U^*]_t + 2k_{c3}[C_3]_t \\
 \frac{d[X_a]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c1}[C_1]_t \\
 \frac{d[X_d]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c2}[C_2]_t
 \end{aligned} \tag{11}$$

The network diagram in Figure 6 is constructed to intuitively describe the connections between species and multiple reactions in Equation (10).

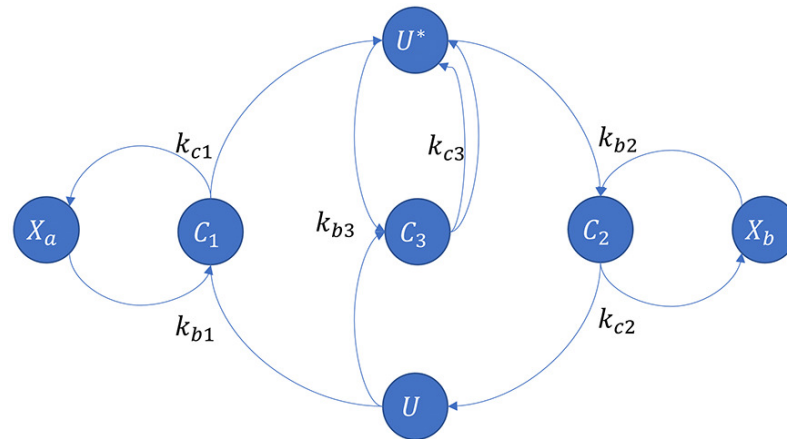


Figure 6. Graphical representation of Equation (10).

Equation (12) can be obtained due to Equation (11).

$$\begin{aligned} \frac{d[C_1]_t}{dt} + \frac{d[X_a]_t}{dt} &= 0 \\ \frac{d[C_2]_t}{dt} + \frac{d[X_d]_t}{dt} &= 0 \\ \frac{d[C_1]_t}{dt} + \frac{d[C_2]_t}{dt} + \frac{d[C_3]_t}{dt} + \frac{d[U^*]_t}{dt} + \frac{d[U]_t}{dt} &= 0 \end{aligned} \quad (12)$$

Note that the results $(d[C_1]_t/dt) + (d[X_a]_t/dt) = 0$, $(d[C_2]_t/dt) + (d[X_d]_t/dt) = 0$ and $(d[C_1]_t/dt) + (d[C_2]_t/dt) + (d[C_3]_t/dt) + (d[U^*]_t/dt) + (d[U]_t/dt) = 0$ imply that the total mass $C_1 + X_a$, $C_2 + X_d$ and $C_1 + C_2 + C_3 + U^* + U$ is conserved through the lifetime of the process.

3.2.2. Proposed an Application of the Covalent Modification Cycle with Direct Positive Autoregulation—BC-DPAR

In Section 3.2.1, the BC is constructed utilizing the covalent modification cycle, as shown in Figure 3. Based on this idea, a novel controller, BC-DPAR, is constructed using the covalent modification cycle with direct positive autoregulation (depicted in Figure 5). Furthermore, the schematic of the BC-DPAR in Equation (13) is shown in Figure 7.

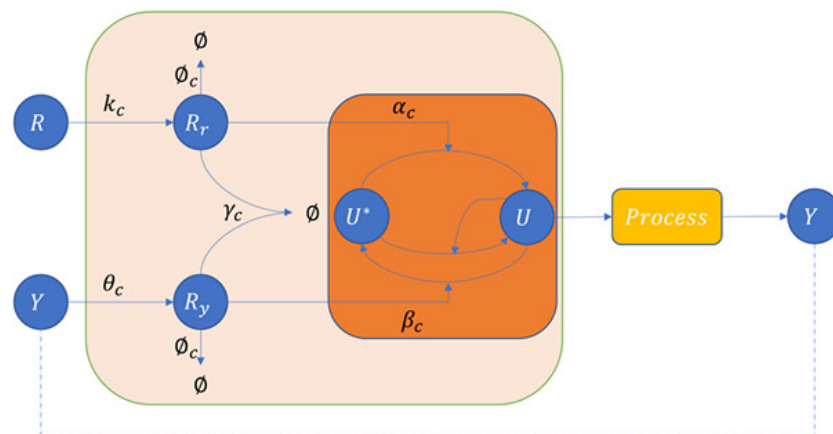
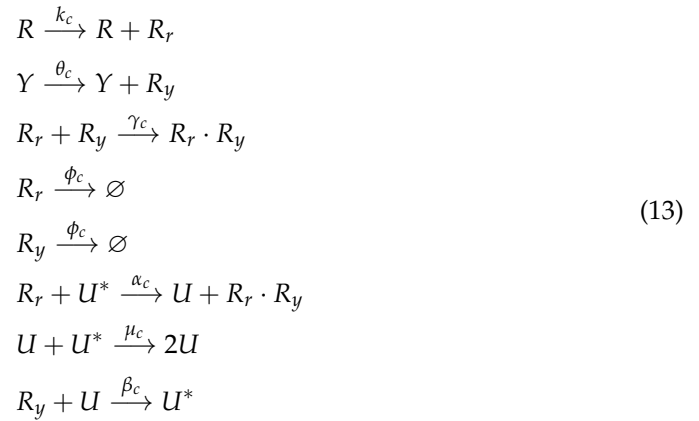


Figure 7. Schematic diagram of the BC-DPAR scheme for a process.

According to Figure 7, there are some differences in structure between BC and BC-DPAR. Compared with BC, species U^* in BC-DPAR can be converted into U under the action of species U , i.e., the reaction $U + U^* \xrightarrow{\mu_c} 2U$.

For the BC-DPAR, the corresponding CRNs can be given by



Using the mass action kinetics, the ODEs of Equation (13) are given by

$$\begin{aligned}
 \frac{d[R_r]_t}{dt} &= k_c[R]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_r]_t - \alpha_c[R_r]_t[U^*]_t \\
 \frac{d[R_y]_t}{dt} &= \theta_c[Y]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_y]_t - \beta_c[R_y]_t[U]_t \\
 \frac{d[U^*]_t}{dt} &= -\alpha_c[R_r]_t[U^*]_t - \mu_c[U]_t[U^*]_t + \beta_c[R_y]_t[U]_t \\
 \frac{d[U]_t}{dt} &= \alpha_c[R_r]_t[U^*]_t + \mu_c[U]_t[U^*]_t - \beta_c[R_y]_t[U]_t
 \end{aligned} \tag{14}$$

It needs to be pointed out that the result $(d[U^*]_t/dt) + (d[U]_t/dt) = 0$ implies that the total mass $U^* + U$ is conserved, i.e., $U_{\text{Total}} = U^* + U$, and can be obtained based on Equation (14).

3.3. Covalent Modification Cycle with Indirect Positive Autoregulation and a Novel Controller BC-IPAR

3.3.1. Covalent Modification Cycle with Indirect Positive Autoregulation (CMC-I)

Figure 8 shows the mathematical framework of the covalent modification cycle with indirect positive autoregulation (CMC-I). Compared with the structure CMC (illustrated in Figure 2) and CMC-D (described in Figure 5), CMC-I has a special mechanism: the inactive species U^* can be transformed into U , i.e., under the action of species U and an activator X_a , i.e., the reaction $U + X_a \rightarrow C_3 + U^* \rightarrow C_4 \rightarrow U + U$.

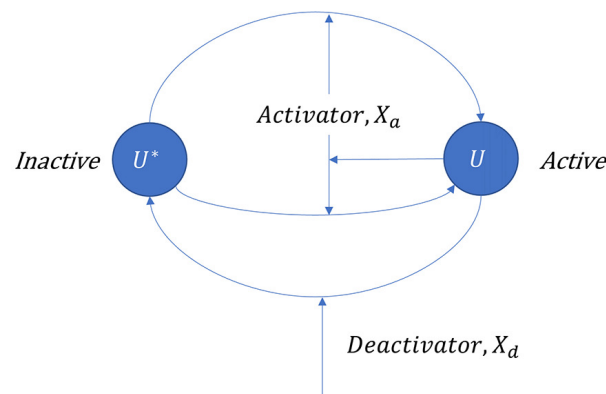
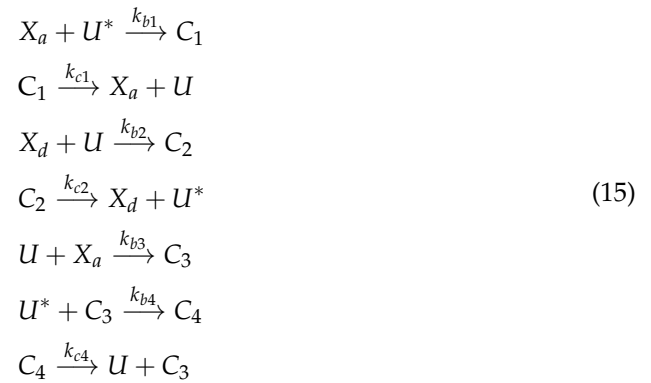


Figure 8. Mathematical framework for the covalent modification cycle with indirect positive autoregulation.

Its abstract chemical reactions can be described by



The network diagram in Figure 9 is constructed to intuitively describe the connections between species and multiple reactions in Equation (15).

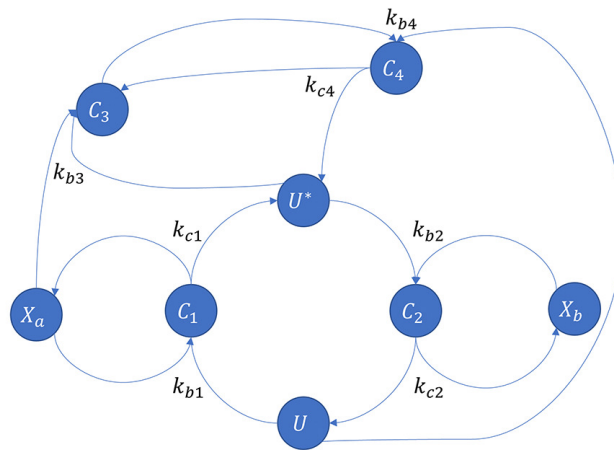


Figure 9. Graphical representation of Equation (15).

ODEs of abstract chemical reactions in (15) can be obtained by

$$\begin{aligned}
 \frac{d[C_1]_t}{dt} &= k_{b1}[X_a]_t[U^*]_t - k_{c1}[C_1]_t \\
 \frac{d[C_2]_t}{dt} &= k_{b2}[X_d]_t[U]_t - k_{c2}[C_2]_t \\
 \frac{d[C_3]_t}{dt} &= k_{b3}[U]_t[X_a]_t \\
 \frac{d[C_4]_t}{dt} &= k_{b4}[U^*]_t[C_3]_t - k_{c4}[C_4]_t \\
 \frac{d[U^*]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c2}[C_2]_t - k_{b4}[U^*]_t[C_3]_t \\
 \frac{d[U]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c1}[C_1]_t - k_{b3}[U]_t[X_a]_t + k_{c4}[C_4]_t \\
 \frac{d[X_a]_t}{dt} &= -k_{b1}[X_a]_t[U^*]_t + k_{c1}[C_1]_t - k_{b3}[U]_t[X_a]_t \\
 \frac{d[X_d]_t}{dt} &= -k_{b2}[X_d]_t[U]_t + k_{c2}[C_2]_t
 \end{aligned} \tag{16}$$

Equation (17) can be obtained due to Equation (16).

$$\begin{aligned}\frac{d[C_1]_t}{dt} + \frac{d[C_3]_t}{dt} + \frac{d[X_a]_t}{dt} &= 0 \\ \frac{d[C_2]_t}{dt} + \frac{d[X_d]_t}{dt} &= 0 \\ \frac{d[C_1]_t}{dt} + \frac{d[C_2]_t}{dt} + \frac{d[C_3]_t}{dt} + \frac{d[C_4]_t}{dt} + \frac{d[U^*]_t}{dt} + \frac{d[U]_t}{dt} &= 0\end{aligned}\quad (17)$$

Note that the results $(d[C_1]_t/dt) + (d[C_3]_t/dt) + (d[X_a]_t/dt) = 0$, $(d[C_2]_t/dt) + (d[X_d]_t/dt) = 0$ and $(d[C_1]_t/dt) + (d[C_2]_t/dt) + (d[C_3]_t/dt) + (d[C_4]_t/dt) + (d[U^*]_t/dt) + (d[U]_t/dt) = 0$ imply that the total mass $C_1 + C_3 + X_a$, $C_2 + X_d$ and $C_1 + C_2 + C_3 + C_4 + U^* + U$ is conserved through the lifetime of the process.

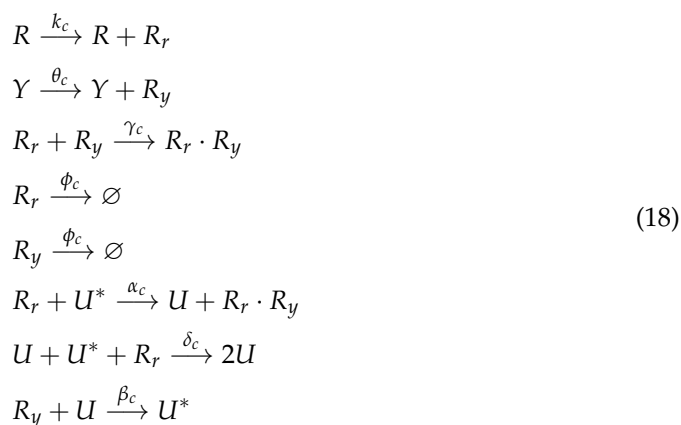
Remark 1. The transition in structures from the CMC to CMC-D, then to CMC-I, makes some changes in the results derived from the corresponding abstract chemical reaction networks. The most obvious change is from the reaction $C_1 + C_2 + U^* + U$ to $C_1 + C_2 + C_3 + U^* + U$, then to $C_1 + C_2 + C_3 + C_4 + U^* + U$.

3.3.2. Proposed an Application of the Covalent Modification Cycle with Indirect Positive Autoregulation—BC-IPAR

The controller BC is constructed utilizing the covalent modification cycle, while a novel controller BC-DPAR can be proposed using the covalent modification cycle with direct positive autoregulation. In this part, combined with the covalent modification cycle with indirect positive autoregulation (depicted in Figure 8), a novel controller BC-IPAR is proposed in the following work.

Figure 10 shows the schematic of the BC-IPAR. Compared with the BC-DPAR, species U^* and R_r can be converted to species U under the action of species U in the controller BC-IPAR, i.e., the reaction $U + U^* + R_r \xrightarrow{\delta_c} 2U$.

CRNs representations of the BC-IPAR can be obtained by



Using the mass action kinetics, the ODEs of Equation (18) are given by

$$\begin{aligned}\frac{d[R_r]_t}{dt} &= k_c[R]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_r]_t - \alpha_c[R_r]_t[U^*]_t - \delta_c[U]_t[U^*]_t[R_r]_t \\ \frac{d[R_y]_t}{dt} &= \theta_c[Y]_t - \gamma_c[R_r]_t[R_y]_t - \phi_c[R_y]_t - \beta_c[R_y]_t[U]_t \\ \frac{d[U^*]_t}{dt} &= -\alpha_c[R_r]_t[U^*]_t - \delta_c[U]_t[U^*]_t[R_r]_t + \beta_c[R_y]_t[U]_t \\ \frac{d[U]_t}{dt} &= \alpha_c[R_r]_t[U^*]_t + \delta_c[U]_t[U^*]_t[R_r]_t - \beta_c[R_y]_t[U]_t\end{aligned}\quad (19)$$

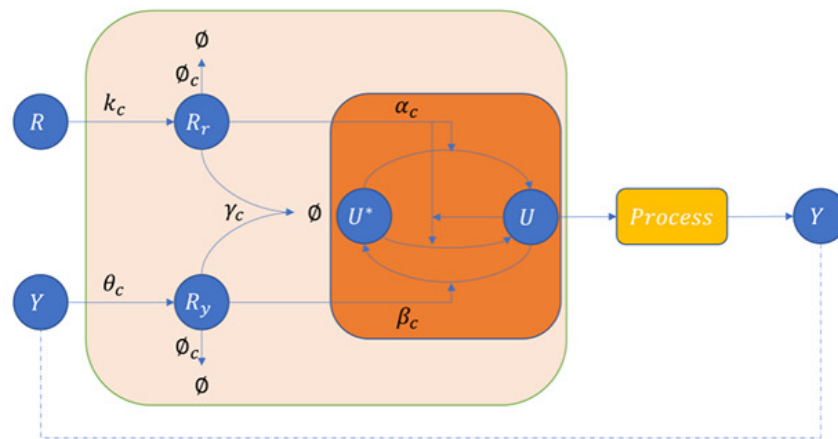


Figure 10. Schematic diagram of BC-IPAR scheme for a process.

In addition, the result $(d[U^*]_t/dt) + (d[U]_t/dt) = 0$ implies that the total mass $U + U^*$ is conserved, i.e., $U_{\text{Total}} = U^* + U$, and can be obtained based on Equation (19).

Remark 2. A notable commonality in the transition from controllers BC to BC-DPAR, and then to BC-IPAR, is that the result $U_{\text{Total}} = U^* + U$ is conserved over time.

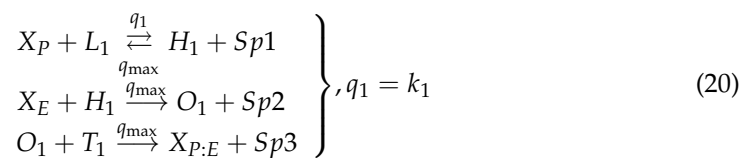
4. Implementations with DSD Reaction Networks

In Section 2, the enzymatic proteolysis reaction process is modelled, and the entire process is described in the simplest form of chemical reactions. In Section 3, on the one hand, the CRN representations of the covalent modification cycle and enhanced covalent modification cycles (including CMC-D and CMC-I) are constructed, and their corresponding kinetic properties are analysed; on the other hand, the BC and the proposed two controllers BC-DPAR and BC-IPAR are designed and analysed from the principle and action mechanism.

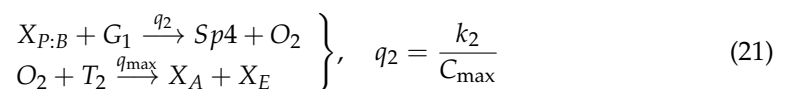
In the following work, the control schemes (involving BC, BC-DPAR and BC-IPAR) of the proposed enzymatic proteolysis reaction model can be designed by using DNA strand displacement reaction, the regulatory characteristics of different control schemes can be analysed to verify the performance of the proposed BC-DPAR and BC-IPAR.

4.1. The Enzymatic Protein Hydrolysis Model

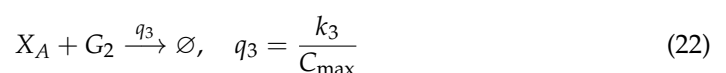
Taking the equation $X_P + X_E \xrightarrow{k_1} X_{P:E}$ in Equation (1) as an example, its DSD reactions are in Equation (20).



Using DNA strand displacement mechanism, the reaction $X_{P:E} \xrightarrow{k_2} X_A + X_E$ can be translated into



The degradation reaction $X_A \xrightarrow{k_3} \emptyset$ is converted into



Remark 3. The reactions shown in Equations (20)–(22) are the results of the enzymatic protein hydrolysis model of Equation (2) being converted into DNA strand displacement reactions. To further demonstrate this process, the following schematic diagrams are constructed. The DNA strand displacement reactions of Equation (20) are shown in Figure 11. In addition, the corresponding DNA reactions as designed according to Equation (21), as shown in Figure 12. Then, a schematic diagram showing the idea of the DSD reaction of Equation (22) is illustrated in Figure 13.

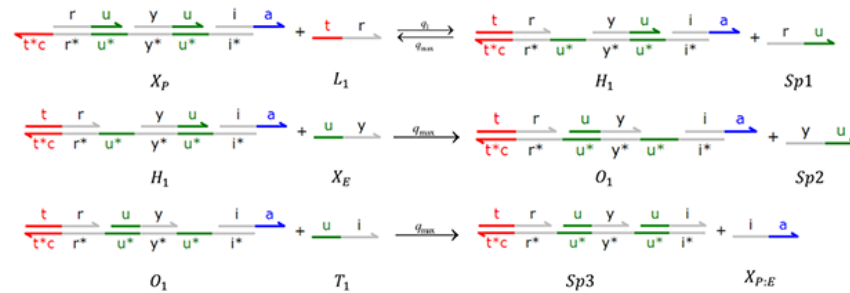


Figure 11. Schematic diagram of DSD reactions of Equation (20).

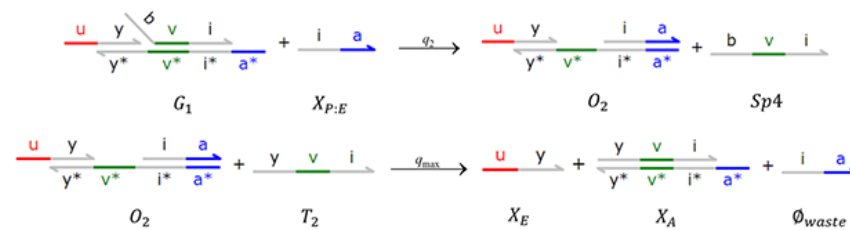


Figure 12. Schematic diagram of DSD reactions of Equation (21).

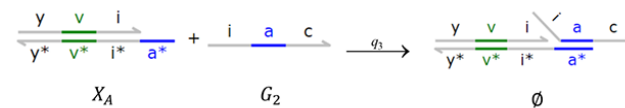


Figure 13. Schematic diagram of DSD reactions of Equation (22).

4.2. Brink Controller (BC)

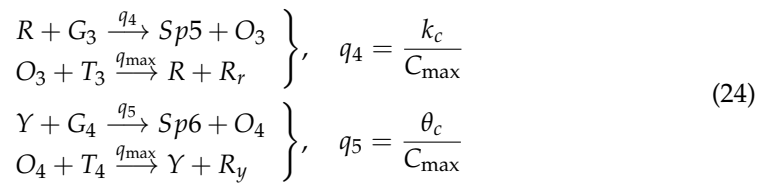
For reactions $R_r + R_y \xrightarrow{\gamma_c} R_r \cdot R_y$, $R_r + U^* \xrightarrow{\alpha_c} U + R_r \cdot R_y$ and $R_y + U \xrightarrow{\beta_c} U^*$, these three reactions can be converted into

$$\left. \begin{aligned}
 R_r + L_2 &\xrightleftharpoons[q_{\max}]{q_8} H_2 + Sp7 \\
 R_y + H_2 &\xrightarrow{q_{\max}} O_5 + Sp8 \\
 O_5 + T_5 &\xrightarrow{q_{\max}} R_r \cdot R_y + Sp9
 \end{aligned} \right\}, \quad q_8 = \gamma_c$$

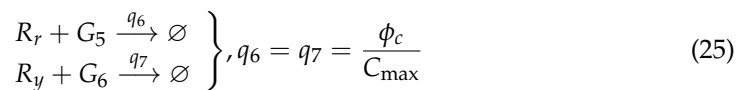
$$\left. \begin{aligned}
 R_r + L_3 &\xrightleftharpoons[q_{\max}]{q_9} H_3 + Sp10 \\
 U^* + H_3 &\xrightarrow{q_{\max}} O_6 + Sp11 \\
 O_6 + T_6 &\xrightarrow{q_{\max}} R_r \cdot R_y + U
 \end{aligned} \right\}, \quad q_9 = \alpha_c$$

$$\left. \begin{aligned}
 R_y + L_4 &\xrightleftharpoons[q_{\max}]{q_{10}} H_4 + Sp12 \\
 U + H_4 &\xrightarrow{q_{\max}} O_7 + Sp13 \\
 O_7 + T_7 &\xrightarrow{q_{\max}} U^* + Sp14
 \end{aligned} \right\}, \quad q_{10} = \beta_c$$
(23)

For reactions $R \xrightarrow{k_c} R + R_r$ and $Y \xrightarrow{\theta_c} Y + R_y$ in Equation (7), the same DSD implementation mechanism exists. Thus, the two reactions can be transformed into

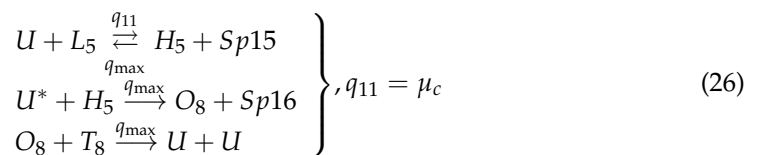


An identical implementation mechanism also exists between reactions $R_r \xrightarrow{\phi_c} \emptyset$ and $R_y \xrightarrow{\phi_c} \emptyset$. Then, the two reactions can be represented by



4.3. Brink Controller with Direct PAR (BC-DPAR)

In Section 3.3.2, it is pointed out that the difference between BC-DPAR and BC is the reaction mechanism $U + U^* \xrightarrow{\mu_c} 2U$. In the above part, the BC has been implemented using the DNA strand replacement reactions of Equations (23)–(25), and the DNA implementations of BC-DPAR of Equation (13) are based on this. For reactions $U + U^* \xrightarrow{\mu_c} 2U$ in Equation (13), the DSD implementation can be expressed by

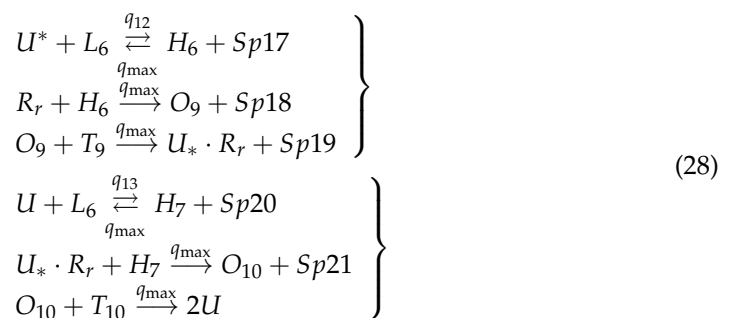


4.4. Brink Controller with Indirect PAR (BC-IPAR)

The difference between BC-IPAR and BC is the reaction mechanism $U + U^* + R_r \xrightarrow{\delta_c} 2U$. Many reactions in BC-IPAR of Equation (18) have been designed to correspond to DNA reactions, according to Equations (23)–(25). Then, for the reaction $U + U^* + R_r \xrightarrow{\delta_c} 2U$ in Equation (18), it can be represented abstractly as the following ideal reactions:



Equation (27) can be transformed into



Remark 4. Let i, n, x, y and z be variable sets such that $i \in (1, 2, \dots, 13)$, $n \in (1, 2, \dots, 21)$, $x \in (1, 2, \dots, 6)$, $y \in (1, 2, \dots, 7)$ and $z \in (1, 2, \dots, 10)$. For DNA realization, G_x, T_x and L_y represent auxiliary substances involved in the reactions, O_z and H_y denote intermediate substances, and Spn indicates the inert wastes produced by the reactions that do not interact with other substances.

In addition, $C_{\max}$ indicates the initial concentration of auxiliary substances, $q_{\max}$ represents the maximum strand displacement rate, and q_i denotes the reaction rate obtained by the corresponding DNA implementations. The auxiliary species G_x , T_x and L_y involved in Equations (20)–(28) are initialized to the maximum concentrations to prevent their consumption from affecting the kinetic process. In addition, both for auxiliary species G_x and T_x are irreversibly consumed.

4.5. Results

Based on the above design ideas, the regulatory scheme of the enzymatic protein hydrolysis model with controllers (including BC, BC-IPAR and BC-DPAR) can be realized using DSD mechanism. All reaction rates involved in the proposed CRN-based controllers and the enzymatic process model are provided in Tables 1 and 2, respectively. For the controllers involved in this work, the initial values of the signals R_r , R_y and $R_r \cdot R_y$ are set to zero, i.e., $[R_r]_0 = [R_y]_0 = [R_r \cdot R_y]_0 = 0 \text{ nM}$.

According to Remark 2, there is a common point among the three controllers BC, BC-DPAR and BC-IPAR, i.e., the reaction $U_{\text{Total}} = U^* + U$. For the driving signal U of the enzymatic protein hydrolysis process, consider two cases $U = 0 \text{ nM}$ and $U \neq 0 \text{ nM}$. Integrating the enzymatic protein hydrolysis model of Equation (1) with the BC of Equation (7), BC-DPAR of Equation (13) and BC-IPAR of Equation (18), the following results can be obtained, as shown in Figure 14.

Remark 5. It is worth noting that the parameter μ_c in Table 1 is related to the reaction $U + U^* \xrightarrow{\mu_c} 2U$ of the BC-DPAR, whereas the parameter δ_c are involved in the reaction $U + U^* + R_r \xrightarrow{\delta_c} 2U$ of the BC-IPAR.

Table 1. Parametric representation for controllers including the BC, BC-DPAR and BC-IPAR.

Parameters	Descriptions	Nominal Values
k_c	Catalytic reaction rate	$3.9 \times 10^{-4} \text{ s}^{-1}$
θ_c	Catalytic reaction rate	$3.9 \times 10^{-4} \text{ s}^{-1}$
γ_c	Binding rate	$2.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
ϕ_c	Degradation reaction rate	$3.0 \times 10^{-4} \text{ s}^{-1}$
α_c	Catalytic reaction rate	$1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
β_c	Binding rate	$1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
μ_c (BC-DPAR)	Catalytic reaction rate	$6.0 \times 10^{-3} \text{ s}^{-1}$
δ_c (BC-IPAR)	Catalytic reaction rate	$6.0 \times 10^{-3} \text{ s}^{-1}$
U_{Total}	Total amount of $U + U^*$	6.0 nM

Table 2. Parametric representation for the enzymatic protein hydrolysis model.

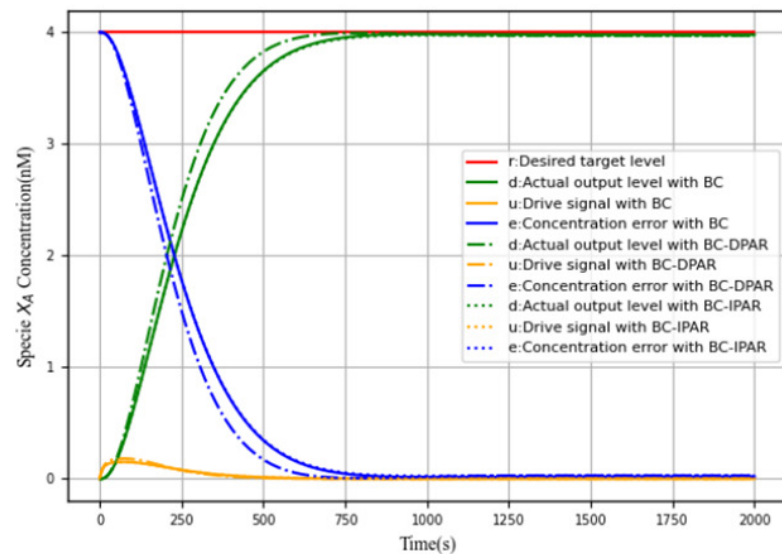
Parameters	Descriptions	Nominal Values
k_1	Binding rate	$2.8 \times 10^{-2} \text{ s}^{-1}$
k_2	Unbinding rate	$1.4 \times 10^{-2} \text{ s}^{-1}$
k_3	Degradation reaction rate	$3.0 \times 10^{-5} \text{ s}^{-1}$
X_{Total}	Total amount of $X_E + X_{P:E}$	4.0 nM

In addition, the output curves of protein hydrolysate production X_A (green curve in Figure 14) are quantified using the setting time as a metric, and the data are integrated as shown in Table 3.

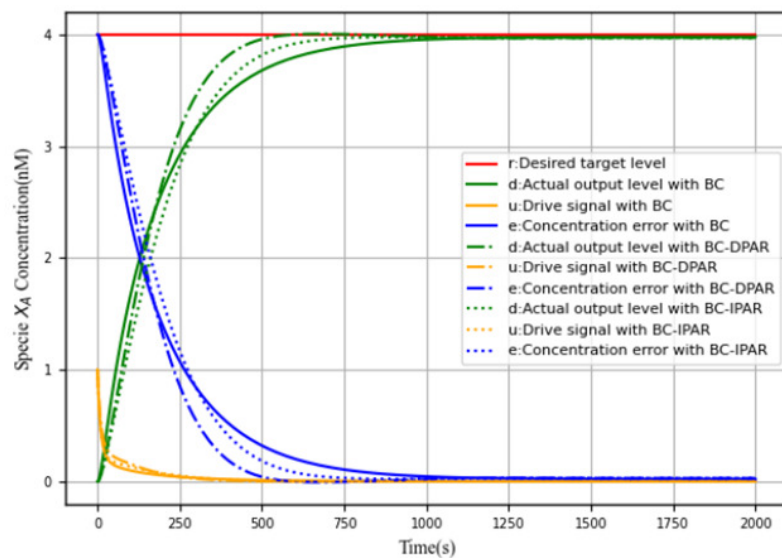
The following results can be obtained by combining Table 3 with Figure 14. On the one hand, the setting times of the BC and BC-IPAR at $U = 0 \text{ nM}$ are $1.2 \times 10^3 \text{ s}$ and $8.0 \times 10^2 \text{ s}$, respectively, while at $U = 1 \text{ nM}$, the setting times of the BC and BC-IPAR are nearly identical, with a settling time of approximately $8.5 \times 10^2 \text{ s}$. From a purely numerical standpoint, it can be seen that compared with the BC scheme, the proposed BC-IPAR can achieve the expected output level in a shorter time. On the other hand, the setting times of the BC and BC-DPAR at $U = 0 \text{ nM}$ are $8.5 \times 10^2 \text{ s}$ and $6.5 \times 10^2 \text{ s}$, respectively, while

at $U = 1 \text{ nM}$, the setting times of the BC and BC-DPAR are $1.2 \times 10^3 \text{ s}$ and $5.2 \times 10^2 \text{ s}$. Compared with the BC scheme, the proposed BC-DPAR scheme can realize the output of protein hydrolysate rapidly and stably. In addition, it can be found that the BC-DPAR controller has better regulation ability, shorter setting time, and faster steady-state output than the BC-IPAR controller.

Thus, the new schemes proposed for protein hydrolysis process, involving the BC-DPAR and BC-IPAR, improve the regulation effect in hydrolysate production to some extent compared to the existing BC control. Among them, the regulation effect of the novel DNA-based BC-DPAR control scheme is closer to the expected ideal results.



(a)



(b)

Figure 14. The trajectory tracking responses of control schemes in the enzymatic protein hydrolysis model including the BC, BC-IPAR and BC-DPAR. (a) $U = 0 \text{ nM}$ and (b) $U = 1 \text{ nM}$.

Table 3. Parameterization of X_A actual output level results for the BC, BC-IPAR and BC-DPAR in the enzymatic protein hydrolysis model.

Controllers	BC	BC-IPAR	BC-DPAR
Setting time t ($U = 0$ nM)	8.5×10^2 s	8.5×10^2 s	6.5×10^2 s
Setting time t ($U = 1$ nM)	1.2×10^3 s	8.0×10^2 s	5.2×10^2 s

4.6. Discussion

The advantages of the two novel CRN-based biomolecular controllers proposed in this manuscript are reflected in the structural design. On the one hand, the positive autoregulation mechanism is utilized to improve the stability of the ultrasensitive response. The proposed controller is thought to have two distinct properties: ultrasensitivity, which is considered an essential component of many robust perfect adaptation (RPA) mechanisms [30,37,38] and is implemented by using covalent modification cycles; and bi-stability [39,40], which is realized by utilizing the positive autoregulation mechanism, thereby enhancing the stability of biochemical control systems. On the other hand, the subtraction module is not involved, which circumvents the limitation of the dual rail representation when designing CRNs, reducing the complexity of DNA implementations. This is mainly because the dual rail representation uses the difference in concentration of two specific chemicals to represent the signal [8], which at least doubles the total number of abstract chemical reactions required for implementation [10,11,13]. If the dual rail representation can be avoided in controller designs, the number of CRN reactions and the complexity of DNA implementation can be reduced. From these two perspectives, the benefits of structural design ensure the efficacy of the controllers proposed in this work.

In addition, Samaniego et al. [30] examined potential biological applications of the Brink controller using three different implementation paths involving RNA molecules (aptamers and toehold switches) and proteins. The two novel biomolecular controllers proposed (including the BC-DPAR and BC-IPAR) differ structurally from the Brink controller; however, the above design approaches can be applied to build corresponding experiments to validate the results in the manuscript. With advances in protein [41] and RNA engineering [42], the proposed biomolecular controllers may be used to build adaptive biological systems.

Positive autoregulation constructed by the covalent modification cycle can achieve unlimited ultrasensitivity. Embedding this ultrasensitivity mechanism into a negative feedback loop can result in a near-horizontal response to external network stimulus, thus enabling RPA. The proposed BC-IPAR controller can only exhibit two-way bistable switches; however, our proposed BC-DPAR controller can also exhibit one-way switches. Bistable switches are considered to play a central role in signalling events, such as apoptosis and motility. In these cases, unlike the two-way switches in the BC-IPAR controller, the additional one-way switches in the BC-DPAR controller may have a profound impact on the control of biochemical processes.

5. Conclusions

This article proposes two novel biomolecular controllers, i.e., BC-DPAR and BC-IPAR, and demonstrates how to construct the two controllers using abstract chemical reactions. A novel controller BC-DPAR is proposed with the application of the covalent modification cycle with direct positive autoregulation; another novel controller BC-IPAR is constructed integrating the covalent modification cycle and indirect positive autoregulation. Then, an enzymatic protein hydrolysis process model based on CRNs is established, and the whole process is constructed in the simplest form of chemical reaction. In addition, the CRN-based process model obtains the corresponding DNA implementations under the action of DNA strand displacement mechanism. Finally, three different control schemes for DNA-based BC, BC-DPAR, and BC-IPAR are designed and analysed utilizing the proposed enzymatic proteolysis reaction model. Results show that the proposed novel BC-DPAR and

BC-IPAR control schemes both shorten the setting time required to achieve the expected output levels of proteolytic products compared to the existing BC scheme, and the better regulation effect of the BC-DPAR scheme tends to achieve the ideal expected output. In addition, this paper provides alternative implementation routes for examining potential biological implementations of the proposed controllers to validate our experimental results. An interesting aspect, which has not been considered in this paper, is the combination of the proposed controller in RNA design and protein engineering to synthesize regulators with adjustable thresholds and gains. This is left as future work.

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Abbreviations

The following abbreviations are used in this manuscript:

CRNs	Chemical reaction networks
DCRNs	Dual chemical reaction networks
BC	Brink controller
PAR	Positive autoregulation
BC-DPAR	Brink controller (BC) with direct positive autoregulation (PAR)
BC-IPAR	Brink controller (BC) with indirect positive autoregulation (PAR)
PI	Proportional integral
QSM	Quasi sliding mode
PID	Proportional integral derivative
2DOF	Two-degree-of-freedom
DSD	DNA strand displacement
CMC	Covalent modification cycle
CMC-I	Covalent modification cycle with indirect PAR
CMC-D	Covalent modification cycle with direct PAR
ODEs	Ordinary differential equations
RPA	Robust perfect adaptation

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