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Five-Component Cascade Synthesis of Nucleotide Analogues in an Engineered Self-Immobilized Enzyme Aggregate

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Nucleoside analogues are important agents in the battle against some of the most serious human illnesses including cancer, parasitic infections and viral diseases.[1–3] In combating viral infections such as HIV, herpes simplex virus, and hepatitis C, nucleoside analogues are particularly indispensable and target reverse transcriptases, polymerases or other enzymes involved in DNA/RNA synthesis, replication and repair.[4–6] Most nucleoside analogue drugs are prodrugs that are activated by intracellular 5′-phosphorylation in the target organism.

The study of existing and new potential nucleoside therapeutics, which includes structure–activity relationships, kinetics and metabolism of their activated forms, often entails labour-intensive chemical synthesis of these metabolites.[7–9] Chemical synthesis of nucleotide analogues is most commonly performed by 5′-phosphorylation of the corresponding nucleoside analogue, and often requires multiple protective group manipulations to control the regiochemistry of ribose phosphorylation. Moreover, these nucleoside precursors themselves are the products of multistep pathways that must address several potential synthetic challenges—selective activation of the anomeric position for nucleobase addition, control of stereochemistry (or resolution of diasteromeric products) and purification of highly polar, water soluble products. Intermediates in these pathways are often unstable, particularly the anomerically activated ribosides. Each nucleoside analogue presents its own distinct synthetic challenges, which require the development of a variety of nongeneralizable strategies.

Recently we provided a biochemical alternative to chemical synthesis of nucleotide analogues by using an engineered hypoxanthine phosphoribosyl transferase (8B3PRT). This transferase, which has markedly relaxed specificity for purine base analogues, facilitated the addition of many base analogues to phosphoribosyl pyrophosphate (PRPP), and created an assortment of monophosphorylated nucleotides.[10] Significant limitations of this approach were the instability and difficulty in handling recombinant enzymes and the expense and instability of the substrate. PRPP decomposes rapidly in solution and at current fine chemical prices costs over 1.2 million dollars per mole.

To address these issues, we report the development of a self-immobilized, multiple-enzyme system, a covalently linked enzyme aggregate or CLEA (Figure 1 A),[11,12] which contains both a purine nucleotide analogue pathway and an auxiliary ATP recycling system. In this pathway (Figure 1 B), ribokinase phosphorylates the 5′ hydroxyl of D-ribose; this results in D-ribose-5-phosphate, which is subsequently anomerically pyrophosphorylated by phosphoribosyl pyrophosphate synthetase, generating PRPP and consuming two equivalents of ATP. The transferase 8B3PRT then catalyzes the addition of purine nucleobases to the activated sugar, and this potentially provides a variety of nucleotide analogues. ATP hydrolysis products ADP and AMP are recycled to ATP by including adenylate kinase

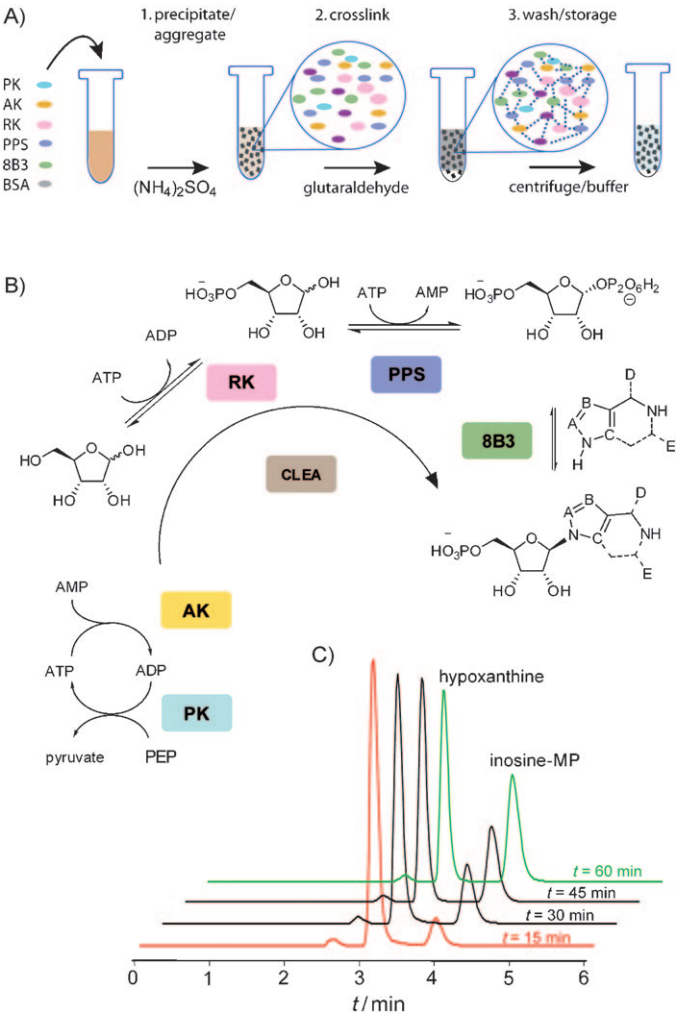

Figure 1. A) General process for generating self-immobilized aggregates. B) Ribose to nucleotide analogues pathway that uses the engineered 8B3PRT combined with ATP regeneration enzymes—all crosslinked in a single aggregate: ribokinase (RK), phosphoribosyl pyrophosphate synthetase (PPS), engineered hypoxanthine phosphoribosyl transferase 8B3PRT (8B3), adenylate kinase (AK), pyruvate kinase (PK). C) HPLC trace of cascade CLEA reaction to form inosine monophosphate.

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and pyruvate kinase, which uses phosphoenol pyruvate (PEP) as a source of activated phosphate.

The ribokinase (*rbsk)* and pyruvate kinase (*pykF)* genes were cloned from *Escherichia coli* K12, and phosphoribosyl pyrophosphate synthetase (*prs)* and adenylate kinase (*adk)* were cloned from *Bacillus cereus* through PCR amplification. The 8B3PRT enzyme is a previously described biocatalyst cloned from *E. coli*, and originates from a directed evolution experiment.[10] Cloned genes were inserted into commercial vectors pCDF Duet-1, pET28a, or pET22b (EMD Chemicals Inc., New Jersey, USA) to allow for the addition of an N-terminal or C-terminal hexahistidine tag (see the Supporting Information). All plasmids were then separately transformed into *E. coli* K12 and protein expression was induced with IPTG at log phase for 3–4 h. Cells were collected by centrifugation, resuspended and disrupted by using a French pressure cell. Enzymes were purified by Ni^{II}-affinity chromatography and imidazole removed through gel filtration.

CLEA particle aliquots were generated by combining 50 μL of each of the five pathway enzyme preparations and bovine serum albumin (10 mg mL^{-1}). The six-protein mixture was precipitated by adding ammonium sulfate and the aggregated enzyme suspension was crosslinked by adding glutaraldehyde and incubating for two hours with stirring at 4 °C. Crosslinked aggregate particles were readily collected by centrifugation and washed three times by resuspension in a storage buffer.

To determine the optimal concentration of glutaraldehyde used in nucleotide analogue pathway CLEA synthesis, activities of particles prepared with increasing glutaraldehyde concentrations were assayed by monitoring the conversion of D-ribose to inosine monophosphate (IMP). Preparations were magnetically stirred in reaction buffer with 5 mM ribose, 6 mM ATP and 25 mM PEP. After 15 min, the reaction was centrifuged to remove enzymes and supernatants were assayed for hypoxanthine consumption via a xanthine oxidase/INT coupled assay (Supporting Information). Enzyme aggregates crosslinked with glutaraldehyde concentrations at 75 mM demonstrated optimal activity (Figure 2 A) of 67.9 nmol min^{-1} mL^{-1} (4.17 nmol min^{-1} mL^{-1} mg^{-1} protein). Thus, the aggregate turnover rate was approximately 30% less than pathway reactions using equivalent amounts of non aggregated enzymes, which demonstrated a turnover of 96.0 nmol min^{-1} mL^{-1} (5.89 nmol min^{-1} mL^{-1} mg^{-1}protein). At glutaraldehyde concentrations below 15 mM aggregates redissolved in the wash steps and demonstrated little or no detectable activity.

The integrity of CLEA particles was confirmed by assaying for resolubilisation of pathway enzymes. Turnover was undetectable in CLEA wash supernatants and no soluble protein was observed in Coomassie Blue-stained polyacrylamide gels (Figure 2 B) in CLEAs formulated with 25 to 100 mM glutaraldehyde. The stability of immobilized enzymes generated using 75 mM glutaraldehyde was assessed by extended incubation at 37 °C. Aliquots were removed at increasing time points and assayed using the hypoxanthine consumption assay as described above. As a benchmark, equivalent amounts of purified nonimmobilized enzymes were mixed and incubated under comparable conditions. Remarkably, the immobilized pathway retained

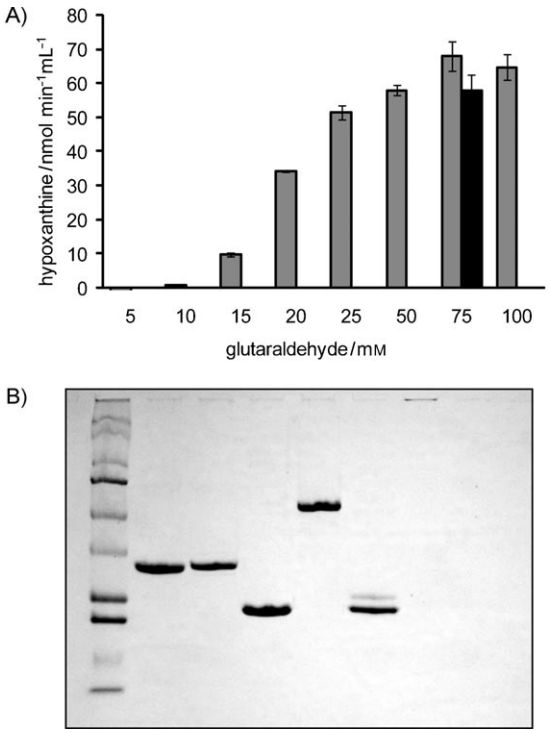


Figure 2. A) Activity measured by hypoxanthine consumption turnover. Grey bars represent turnover of CLEAs formed from mixture of purified enzymes with varying concentrations of glutaraldehyde. The solid bar represents activity of CLEA made with mixtures of unpurified cell lysates. B) SDS-PAGE gel of purified enzymes and CLEA; lane 1, marker; lane 2, 1.1 μg RK; lane 3, 0.9 μg PPS; lane 4, 1.8 μg 8B3; lane 5, 1.5 μg PK; lane 6, 1.3 μg AK; lane 7, 690 μg CLEA aggregate.

over 50% activity after seven days and, at up to three weeks of accelerated aging, retained significant pathway activity. In comparison, the equivalent soluble enzyme mixture retained no traces of pathway activity after 24 h (Figure 3 A).

CLEA reusability was evaluated by assay of centrifugally recovered enzyme pellets. IMP formation was assayed and recovered pellets were subsequently washed three times in resuspension buffer and re-assayed. Following seven cycles, CLEA pellets retained about 30% of their original activity (Figure 3 B). We speculate that loss of activity is predominantly due to disintegration of the pellet with extensive handling. The addition of lysine-rich bovine serum albumin (BSA) to crosslinking reactions to generate CLEAs has been previously shown to increase enzyme activity, thermal stability, residual activity[13] and to protect against proteolysis.[14] The addition of BSA is proposed to enhance the formation of the reticulate, providing additional structure and preventing excessive crosslinking of catalytic enzyme.[15] Consistent with these observations, inclusion of BSA in the nucleotide generation pathway significantly enhanced stability in reuse studies. Particles generated with BSA retained up to 60% of their original activity after seven uses (Figure 3 B).

Given the known substrate flexibility of 8B3PRT,[10] a large complement of purine nucleotide analogues should be accessible by using this catalyst including both base and sugar analogues. The synthetic utility of the immobilized pathway cata-

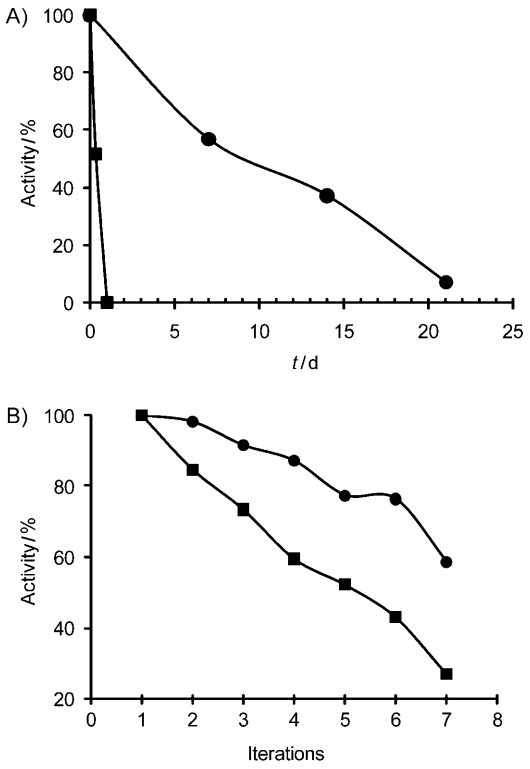
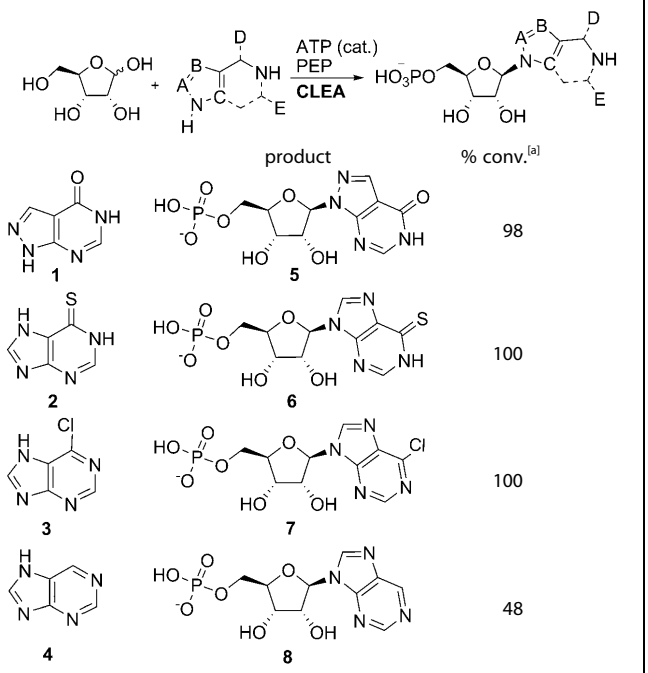


Figure 3. A) Accelerated stability study trend line: CLEA aggregate pathway (●) benchmarked versus solution enzymes (■) at 37 °C. Data points represent single measurements. B) Catalyst recycling. Data shown was obtained using hypoxanthine as the nucleobase: with BSA (●) and without BSA (■).

Table 1. Nucleotide analogues prepared with the 5-enzyme CLEA immobilized pathway.



[a] Conversion based on standard reaction conditions.

lyst for nucleotide analogue generation was demonstrated with selected base analogue substrates (Table 1). Nucleotide analogues were synthesized in 500 μL reactions by stirring CLEA pellets (corresponding to 50 μL each enzyme prepared with BSA) with 10 mM nucleobase, 12 mM ribose, 6 mM ATP (0.2 equiv) and 30 mM PEP for two hours. Reaction progress was monitored by WATERGATE NMR. Analogues were purified by DEAE-sepharose anion exchange resin eluted with an ammonium carbonate gradient and characterized by NMR. Substrates **1**–**3** showed complete or nearly complete conversion, while purine (**4**) conversion was 48% without optimization. Products **5**–**7** were previously generated from PRPP using and the terminal transferase enzyme 8B3PRT. The tandem CLEA pathway method is a substantial improvement over the single step PRPP method, as it simultaneously addresses the PRPP and enzyme stability problems and provides a catalyst that can be removed by centrifugation or filtration and reused. The primary limitation of CLEA pathway nucleotide biosynthesis is ultimately that of nucleobase solubility. For instance, a reaction for 6-chloropurine to produce one gram of product (2.74 mmol) requires a 124 mL reaction.

Engineered cell-based biosynthetic pathways must successfully negotiate potential complications such as genetic regulation, flux integration with primary metabolism, feedback inhibition, metabolite toxicity and intracellular transport to name a few. The multistep CLEA catalyzed synthesis described herein provides potential solutions to many of these complications. Key advantages of the CLEA pathway approach are exemplified by this study: the ability to titrate enzyme activity by controlling enzyme stoichiometry and the ability to add complete recycling pathways for cofactor regeneration and/or to prevent product inhibition. In the engineered purine salvage pathway CLEA described herein, cofactor recycling not only regenerates ATP, but also prevents the accumulation of ADP, an inhibitor of PPS (K_{ii} = 334 μM).[16] Additionally, recycling the nucleotide reaction products AMP and ADP into ATP greatly simplifies the subsequent purification of the monophosphate nucleotide analogues through anion exchange chromatography.

The majority of CLEA technology applications described to date consist of single-step reactions, although recently "combi-CLEAs", which contain aggregates of up to two enzymes for cascade reactions[17] or three enzymes for noncascading reactions,[18] have also been described. The nucleotide analogue pathway described herein successfully recapitulates a five step pathway in aggregate form: a three-step cascade reaction and a two-step ATP regeneration system. Without extensive optimization, the five-step aggregate is robust, reusable, and demonstrates greatly improved stability in comparison to the soluble enzyme pathway. Moreover, we demonstrate the pathway can be constructed with crude cell lysates rather than purified enzymes. To the best of our knowledge this study describes the first example of an ATP recycling pathway embedded in a multistep biosynthetic pathway immobilized for the synthesis of non natural products. Further investigations of pathway CLEAs will reveal the potentials and limitations of these approaches in comparison to whole cell based synthetic biology approaches.

Experimental Section

General CLEA formation protocols: CLEAs were generated from purified enzymes by combining each of the five pathway enzyme preparations (50 μL, determined by the BCA assay to be 18.2, 9.0, 10.6, 12.9, and 14.6 mg mL^{-1} for 8B3PRT, PPS, RK, AK, and PK, respectively) and bovine serum albumin (10 mg mL^{-1}) in an Eppendorf tube. The six-protein mixture was precipitated by adding an equal volume of ice-cold saturated ammonium sulfate solution with rapid magnetic stirring at 4 °C. Solid ammonium sulfate (348 mg mL^{-1}) was added and the solution allowed to equilibrate for 15 min to ensure complete protein precipitation. After 15 min, the aggregated enzyme/salt suspension was crosslinked by adding glutaraldehyde (5–100 mM, Fluka Inc) and incubated for two hours while stirring at 4 °C. Crosslinked aggregate particles were readily collected by centrifugation (10000 rpm) and washed three times by resuspension in ice-cold reaction buffer (see the Supporting Information).

CLEAs were generated from unpurified cell lysates as follows. Aliquots of cell pellets from cultures (50 mL), grown and induced as described above, were combined and resuspended in reaction buffer (10 mL) prior to lysis through double passage through a French pressure cell. Aliquots (500 uL) were removed for CLEA formation, to which ammonium sulfate (696 mg mL^{-1}) was added. After 15 min, the aggregated enzyme suspension was crosslinked by adding glutaraldehyde (75 mM) and incubated for two hours while stirring at 4 °C. Crosslinked aggregate particles were readily collected by centrifugation (10000 rpm) and washed three times by resuspension in ice-cold reaction buffer.

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