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Immobilized Enzyme Reactors on Monoliths: A Platform For Efficient pDNA Linearization

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Introduction

The linearization of plasmid DNA (pDNA) is a crucial step in messenger ribonucleic acid (mRNA) production, as it provides the template for in vitro transcription (IVT)[1]. Traditionally, this step relies on the use of restriction enzymes in solution, which presents several drawbacks. These enzymes need to be removed after the reaction and cannot be reused, leading to longer processing times and introducing variability into the process[2]. Immobilized enzyme reactors (IMERs) offer a promising alternative to in-solution protocols by enabling repeated enzyme use, lowering production costs, reducing contamination risk, and supporting process automation. This study aimed to prepare functional IMERs on a monolithic support for efficient pDNA linearization, which can then be used in an in vitro transcription reaction for mRNA production.

1. Immobilization procedure

Aldehyde CIMmic® ALD-0.1 Disk (Sartorius BIA Separations, Slovenia) with 6 µm channel diameter was chosen as a solid support for **EcoRI**, **NdeI** and **HindIII** enzyme coupling. The restriction enzyme (New England Biolabs, USA) was covalently attached to the column surface via imine formation, followed by reduction with sodium cyanoborohydride. The modified Sartorius BIA Separations immobilization procedure was used for enzyme immobilization [3]. The immobilization buffer, temperature, starting amount of enzyme, and immobilization time were optimized to improve immobilization yield and IMER activity.

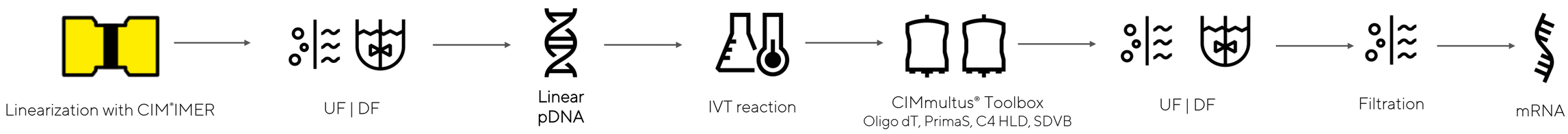

Figure 1: Alternative preparation of linear pDNA using IMER in mRNA production process.

2. Evaluation of IMER enzymatic activity

The enzymatic activity of the generated EcoRI-, NdeI-, and HindIII- IMERs was determined:

- Using a model pDNA - plasmid pmFix6 (COBIK d.o.o., Slovenia) as substrate. The plasmid was purified prior to its use using the HIP2 Plasmid Process pack (Sartorius BIA Separations, Ajdovščina, Slovenia) upon optimization of the manufacturer's protocol [5];
- The substrate solution was prepared by dissolving pDNA in 1xNEBuffer™ (New England Biolabs, USA);
- The enzymatic reaction was performed with a retention time of 15 minutes at 25 °C;
- The activity of linearization IMER was assessed off-line through chromatographic separation and quantification of pDNA isoforms in the product utilizing a PATfix bio-chromatography system and a CIMac pDNA 0.3 mL analytical column (Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia).

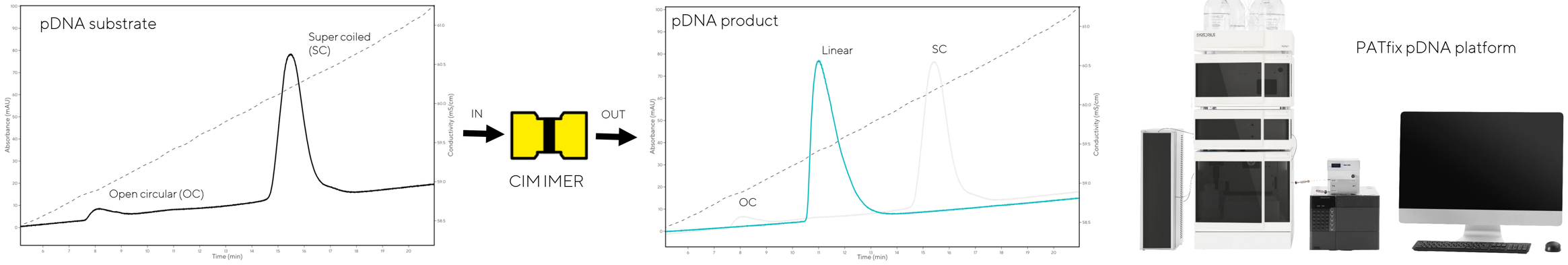

Figure 2: Evaluation of IMER activity. Chromatographic profiles of substrate pDNA before and after processing on the IMER. The chromatographic method allowed baseline separation and quantification of different pDNA isoforms. Chromatographic conditions: mobile phase A: 100 mM Tris, 300 mM GdnHCl, pH 8.0; mobile phase B: 100 mM Tris, 650 mM GdnHCl, 150 mM GdnSCN, pH 8.0 ; flow rate 0.5 mL/min; 30°C; 58-69% MFB, 22 min; UV detection: 260 nm and 280 nm; pDNA injected mass on column is 1 µg [4]. Linearization efficiency was assessed off-line.

3. Preparation of linear pDNA

Linear pDNA was prepared using two approaches: traditional restriction enzymes in solution and the three restriction enzyme-IMERs, namely EcoRI-, NdeI-, and HindIII- IMERs (Figure 3). The linear pDNA obtained was comparable between methods, as shown in Figure 4 by pDNA isoform separation and quantification performed using PATfix® analysis on a CIMac pDNA analytical column and agarose gel electrophoresis (AGE).

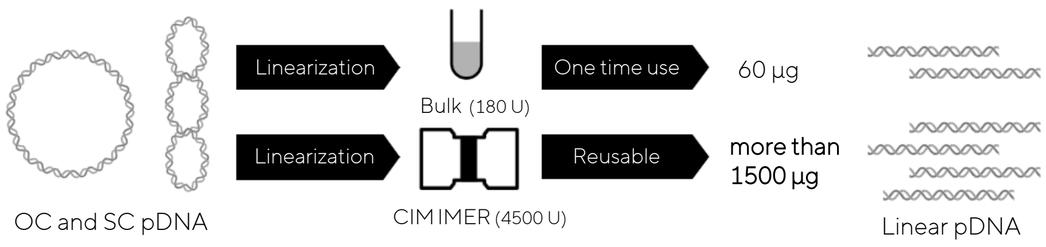

Figure 3: Schematic representation of the production of linear pDNA from OC and SC pDNA by using either immobilized enzyme (IMER) or in solution reaction. In this example, the IMER contained 4500 U of enzyme and could be reused multiple times, while the in-solution reaction was performed with 180 U of enzyme, and after linearization, the enzyme was discarded. Overall, using an IMER instead of a free enzyme in solution can lower costs.

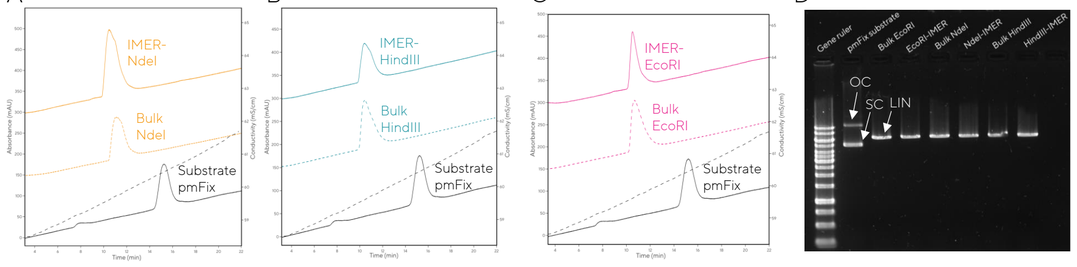

Figure 4: Comparison of pDNA linearization using IMERs and enzymes in solution for three different restriction enzymes: EcoRI, NdeI, and HindIII. Chromatographic profiles show the separation of pDNA isoforms - supercoiled, open circular, and linear - before and after the enzymatic reaction. For each enzyme, linearization was performed both in bulk solution and by using IMER. Panels A-C show the overlaid chromatographic profiles of processed and unprocessed substrate to visualize conversion efficiency. Panel D shows AGE results for the same set of samples, confirming successful linearization in agreement with chromatography data. Together, these results demonstrate comparable linearization efficiency between IMERs and in-solution reactions.

4. Application of IMERs in IVT reaction for mRNA production

The practical performance of IMER was demonstrated in an in vitro transcription mRNA production process. A key advantage of IMER use is the elimination of additional purification steps - linear pDNA is obtained directly in reaction buffer without the need to remove the enzyme from the solution. Of the three tested restriction enzymes, only HindIII provided precise linearization at the 3′ end of the transcription unit, enabling production of a uniform mRNA transcript. The chromatograms below show IVT reaction from HindIII IMER-linearized pDNA, comparing the start (0 min) and end (210 min) of the process. A clear mRNA peak is visible at 210 min, while the nucleotide triphosphates ATP, CTP, GTP, and UTP are depleted, confirming successful transcription.

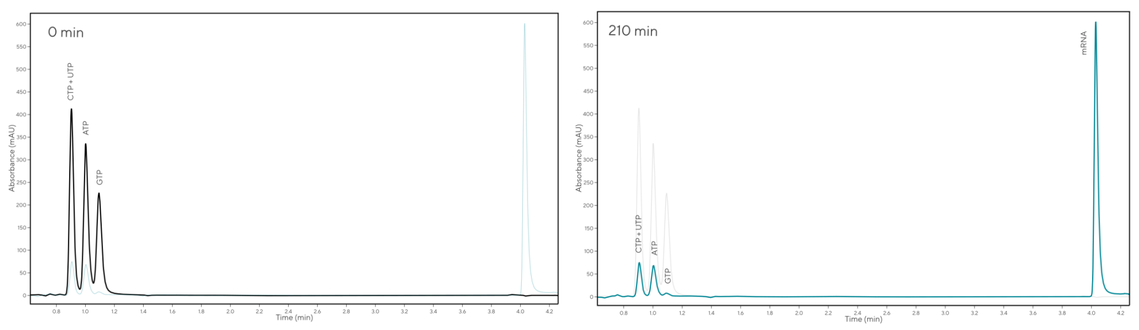

Figure 5: IVT analysis on CIMac PrimaS (Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia) analytical column, at the start (0 min) and after 210 minutes of IVT reaction. Linearization of the pDNA template was performed using the HindIII IMER, with 5 µg of the resulting linear template applied per one IVT reaction. Mobile phases: buffer A was 50 mM HEPES, pH 7.0; buffer B was 50 mM HEPES,100 mM pyrophosphate, pH 8.3; buffer C was 0.1 M NaOH, 1 M NaCl; flow rate 2 mL/min; [6]

5. Conclusions

- Restriction enzymes EcoRI, NdeI and HindIII were successfully immobilized on monolithic support.
- Linear pDNA was prepared with all three CIM®IMERs and all three enzyme reactions in solution and comparable efficiency between IMER and bulk reactions was shown. From in solution reaction 60 µg of linear pDNA was prepared in one cycle, and from IMER 1500 µg of linear pDNA was prepared in three cycles.
- The functionality of the linearized pDNA (linearization performed with HindIII IMER) was confirmed through its successful application in IVT reactions for mRNA production.

Acknowledgements

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References

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