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MICROREACTORS IN PROTEIN PEGYLATION: TOWARDS HIGHER YIELDS AND SPECIFICITY

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Introduction

Therapeutic biological products based on proteins are of increasing interest in the biopharmaceutical field.[1] This class of drugs is characterized by its high specificity, offering the possibility to treat complex diseases once considered untreatable. Nonetheless, protein drugs are usually associated with low solubility profiles, short shelf-lives, short circulating half-lives and susceptibility to cleavage by proteolytic enzymes. To date, several techniques have been implemented to increase solubility, improve molecular stabilization and enhance protein pharmacokinetics.[2-3] Most of these techniques focus on the bioconjugation of proteins with polymers, such as PEGylation, generating improved drugs, *i.e.* biobetters, which are superior when compared to the original biological.[3] Among the large array of bioconjugation techniques, PEGylation is the most auspicious alternative. This strategy is FDA and EMA approved and it has been used to develop several protein drugs currently on the market.[3] Through careful selection of the reaction chemistry, one or more polyethylene glycol (PEG) molecules can be attached to the proteins, producing PEG–protein conjugated species with one or more grafted polymeric chains. PEGylation of therapeutic proteins is commonly carried using *N*-hydroxylsuccinimide (NHS) functionalized mono-methoxy PEG (mPEG-NHS). NHS esters of the PEG react with *N*-terminal amine groups of the protein at pH 7.0-8.5 to form stable amide bonds (**Figure 1 A**). In PEGylation reaction, parallel reactions between the PEG reactive groups and the protein functional amino acids may occur (*e.g.* alkylation reaction with protein nucleophiles like lysines), resulting in a heterogeneity of various PEGylated protein forms and unmodified protein. In addition, hydrolysis of the reactive PEG is also a major drawback (**Figure 1 B**). The addition of excess amounts of reactive PEG associated with long reaction times are some of the strategies used to minimize the drawbacks of hydrolysis reactions, such as low yields. However, the high price of the reactive PEG makes its use in excess costly and alternatives must be explored to turn the PEGylation process economically viable. Other challenges that need to be addressed in PEGylation are the development of (i) site-specific PEGylation reactions to avoid the heterogeneity of PEGylated conjugates and (ii) reactions with enhanced yields and shortened times.

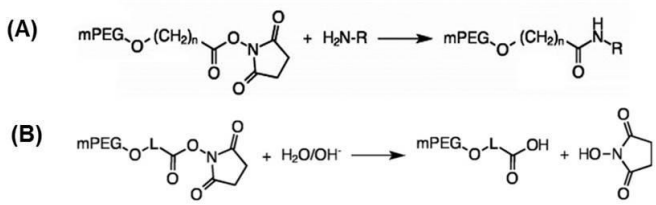


Figure 1. PEGylation reaction of primary amines in proteins, forming an amide linkage, using mPEG-NHS. **(A)** Hydrolysis reaction of mPEG-NHS (L - spacer group) **(B).**

In order to overcome those challenges, a new approach to PEGylation using micro-reactors is herein studied. Microfluidic-based systems are widely used for high throughput screening of chemical and biochemical synthesis since parallelization as well as internal and external integrations can be easily implemented for control, detection, and analysis.[4] Micro-reactors and integrated microsystems are used to eliminate some of the major limitations of conventional batch-reactors such as extended time reactions, high consumption of costly reagents, reduced specificity and reproducibility due to poor control on reaction and quenching. Moreover, reaction optimization is straightforward as different sets of reaction conditions can be studied in a short period, expediting the establishment of ideal reaction conditions. One of the novel materials used in microfluidics is LTCC (Low Temperature Co-fired Ceramics)[5], The main advantages of this material are chemical inactivity, chemical resistance, good thermal conductivity, high temperature stability. Moreover, LTCC tapes can easily be cut into the desired geometry to accomplish the mechanical function and tune the desired reaction.

Methods

PEGylation reaction: batch and microfluidic scale

The PEGylation reaction was performed using mPEG-NHS of different molecular weights (MW): 5, 10, 20 and 40 kDa for two model proteins: lysozyme and L-asparaginase. The reactive PEG used promotes an acylation reaction of the amino groups of amino acids with the ester groups of the reactive PEG, resulting in stable amide bonds (**Figure 1A**). The reaction was performed in batch and microfluidics (**Figure 2**) using the following conditions: 100 mM phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 7.5, using a molar excess of reactive PEG of 1:15 and 1:25 for lysozyme and L-asparaginase respectively, at T = 25 ± 1 ºC. For batch process, the reaction time was 30 minutes, under stirring at 400 rpm. The PEGylation reaction was optimized in terms of yields and specificity by microfluidics (**Figure 2B**). This technique was used to increase the mass transfer between the solutes and, consequently, the reaction speed.

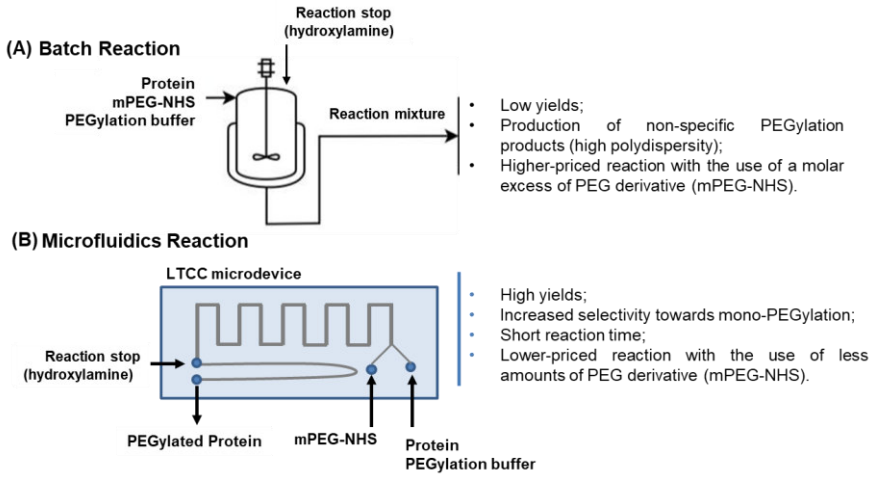
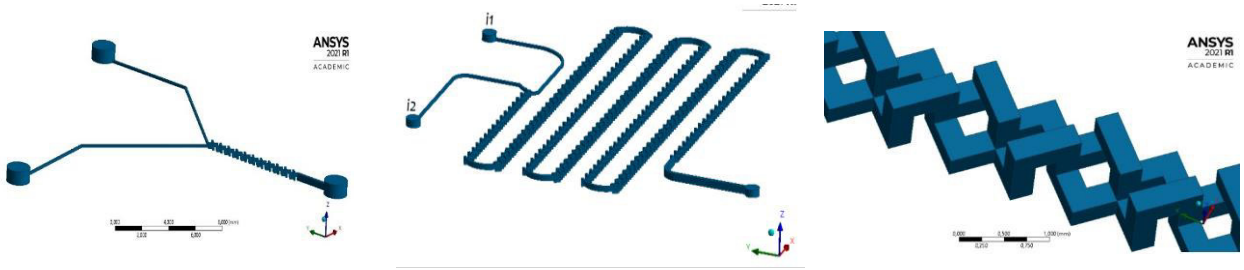


Figure 2. Schematic diagrams for batch protein PEGylation **(A)** and LTCC-based microreactor protein PEGylation **(B)**.

In this work, PEGylation reactions were conducted in a microreactor (micromixer) with crossing-channels geometries (**Figure 3**). This type of channel configuration is appropriate for the Reynolds number values handled in LTCC-based microreactor protein PEGylation (0,5 ≤ Re ≤ 20,8). The microreactors were manufactured using the LTCC technique, with cross sections of 260 µm × 260 µm. The PEGylation reactions were carried out at room temperature by injecting a reactive PEG solution in buffer through one of the microreactor inlets and protein solution through the other inlet, using HPLC pumps. The influence of volumetric flow and residence time distribution (RDT) in the microreactor on the reaction yield and specificity were investigated.



(A) (B) (C)

Figure 3. Evaluated LTCC-based microreactor for the protein PEGylation **(A e B)** and crossing-channels side-view (C).

In order to observe the behavior of the fluids in the tested microdevices, computational simulations are being carried out, applying the Computational Dynamic Fluid (CFD) technique. The flow structure in the micromixers was studied with the CFD code ANSYS Fluent 2021 R1. LTCC-based Microreactors/micromixers performance was evaluated from the results of the CFD study by calculating the mixing (M) and performance (PI) indexes.

$$\mathrm{M} = 1 - \sqrt{\frac{\sigma^2}{\sigma^2_{máx.}}}; \qquad \mathrm{PI} = \frac{M}{\Delta P}$$

Results

The batch reactions resulted in yields ranging from 20% ± 4% to 52% ± 3% for lysozyme and 15% ± 2% and 59% ± 6% for L-asparaginase. For higher MW PEGs (*i.e.* 20 and 40 kDa), the PEGylation yield greatly reduced due to steric impediment. In terms of reaction specificity, the maximum monoPEGylation yield was obtained for lower MW mPEG-NHS, 5 kDa (42.3% ± 2.1% and 37.07% ± 2.35% for lysozyme and L-asparaginase, respectively). For 40 kDa mPEG-NHS only mono-PEGylation protein was formed but in lower yields.

The microfluidics-based reactions occurred for inlet flow rates of 40.0, 20.0, 10.0, and 6.7 $\mu l.min^{-1}$ corresponding to lower reaction times compared to batch reactions (less than 15 min). The yield and specificity of the bioconjugation was greatly improved for both model proteins.

CFD results showed that the crossing-channel micromixer promotes efficiently mixing ($M \approx 1$) of the reactant solutions in the microchannels by splitting and recombination of the fluidic currents, inducing chaotic advection in the microchannels by multilayer lamination, with low values of total pressure drop ($10^{-2} \leq \Delta P \leq 10^{-3}$ bar).

The microchannels with a hydraulic diameter (Dh) of 260µm gave the highest performance index (PI) values, for all the flow rates evaluated, showing that the crossing-channel micromixer geometry with these dimensions is the best option to promote mixing in the adopted Reynolds number range ($0.5 \leq Re \leq 20.8$), under the conditions studied in the protein PEGylation reaction.

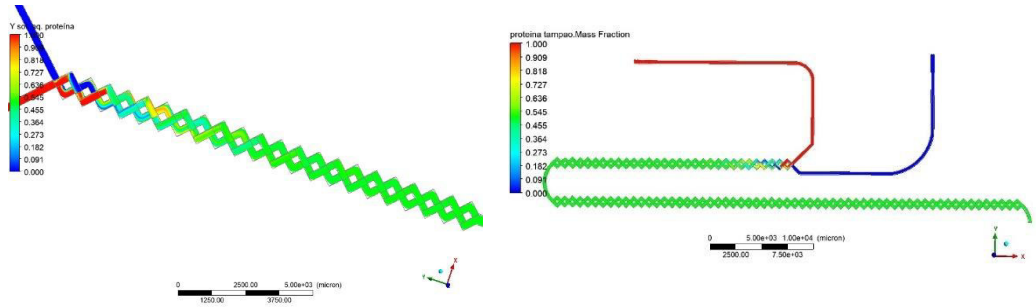


Figure 4. Streamlines colored by protein mass fraction. CFD results of LTCC-based Crossing-channels microreactor with 22 (A) e 382 (B) sections.

The adjustment of the microreactor volume, increasing the number of sections from 22 (Figure 4A) to 382 (Figure 4B), allowed longer residence times of the reagents inside the microreactor and more contact between the reactive PEG and the protein molecules, favoring the increase of the reaction yield with residence times up to 8 min.

Conclusions

Continuous flow microreactors offer precise control on the residence time, *i.e.*, give narrow residence time distribution (RTD), which makes them highly suitable for rapid and residence time sensitive reactions. Thought microfluidics, the PEGylation reaction yield increased significantly, alongside with the selectivity of mono-PEGylation. The integration of the PEGylation reaction and monoPEGylated purification in microreactors with 3D surfaces effects to promote the mixture is worth investigating in the future.

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