Continuous flow microreactor for protein PEGylation

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PEGylation is increasingly being utilized to enhance the therapeutic efficacy of biopharmaceuticals. Various chemistries and reaction conditions have been established to synthesize PEGylated proteins and more are being developed. Both the extent of conversion and selectivity of protein PEGylation are highly sensitive to process variables and parameters. Therefore, microfluidic-based high-throughput screening platforms would be highly suitable for optimization of protein PEGylation. As part of this study, a poly-dimethylsiloxane-based continuous flow microreactor system was designed and its performance was compared head-to-head with a batch reactor. The reactants within the microreactor were contacted by passive micromixing based on chaotic advection generated by staggered herringbone grooves embedded in serpentine microchannels. The microreactor system was provided with means for on-chip reaction quenching. Lysozyme was used as the model protein while methoxy-polyethylene glycol-(CH₂)₅COO-NHS was used as the PEGylation reagent. Full mixing was achieved close to the microreactor inlet, making the device suitable for protein PEGylation. The effect of mixing type, i.e., simple stirring versus chaotic laminar mixing on PEGylation, was investigated. Higher selectivity (as high as 100% selectivity) was obtained with the microreactor while the conversion was marginally lower. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5030984>

I. INTRODUCTION

Therapeutic proteins could be chemically modified to improve their therapeutic properties.^{1–3} PEGylation, i.e., the covalent attachment of polyethylene glycol (PEG) to a protein, has been shown to be highly effective.^{4–6} PEG is a non-toxic, non-immunogenic polymer which is highly soluble and flexible and available in varied molecular weights and structures, i.e., linear chain, branched chain, mono-functional, and multi-functional.^{7,8} PEGylation increases the net molecular size as well as results in shielding of the protein surface. The former leads to prolonged *in-vivo* half-life of the protein and reduces the frequency of drug administration,⁹ whereas the latter decreases the immunogenicity of the therapeutic molecule by shielding the antigenic sites and enhances protein stability by reducing the chances of aggregation and proteolysis.¹⁰

A variety of chemical reactions are used to attach PEG to proteins.^{5,11} As PEG is by itself chemically non-reactive, different functional groups are added to it to confer reactivity.¹² Protein PEGylation is typically carried out as homogeneous liquid-phase batch reactions in stirred tanks. The reaction product typically contains mono-PEGylated protein (the target molecule) along with higher PEGylated moieties which are the by-products, along with unreacted protein and PEGylation reagent. There are two main challenges in protein PEGylation, getting high conversion yield (i.e., high productivity) and increasing the selectivity of mono-PEGylation. Conversion and selectivity depend on a variety of factors such as the properties of the protein molecule, the PEGylation reagent,^{12,13} as well as the reaction conditions such as protein:PEG molar ratio,¹⁴ pH,¹⁵ temperature,¹⁶ pressure,¹⁷ and salt concentration.¹⁸ Consequently, there is a wide range of

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optimizable parameters in protein PEGylation. Therefore, having a technology platform which allows PEGylation to be carried out in a high throughput format would be most useful.

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.^{19–21} Microreactors and integrated microsystems are used to eliminate some of the major limitations of conventional batch-reactors such as high consumption of costly reagents, longer experimentation time, poor selectivity,^{22–25} and low reproducibility due to poor control on reaction and quenching.²⁶ They are also highly applicable in reaction optimization as different sets of reaction conditions can be studied in a short period of time,^{27,28} expediting the obtainment of ideal reaction conditions. 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.²⁹ However, when reagents flow in regular microchannels at low Reynolds numbers (Re), homogenous mixing becomes challenging.^{30,31} Therefore, various active and passive mixing methods have been developed to generate turbulence or fluid folding to minimize the channel length required for attaining homogeneity.^{32–34} Passive micromixing is carried out by specifically configuring the microchannels or by embedding certain patterns and features within microchannels.^{35,36} Common approaches exploit T and Y connections, hydrodynamic focusing, and chaotic advection.^{37–39}

In this paper, we discuss the design and fabrication of a simple and inexpensive microreactor for high-throughput screening for protein PEGylation. The microreactor was fabricated by dry bonding a microchannel containing Poly-dimethylsiloxane (PDMS) layer onto a glass slide (Fig. 1). The device contained serpentine channels with right angles including a cycle of staggered herringbone grooves on each turn^{40–44} to generate chaotic advection. Staggered herringbone grooves can induce multi-directional transport of the solutes between the two fluids introduced into a microchannel.^{45,46} This happens due to secondary recirculating flows when the fluid is folded and stretched. By using an aspect ratio of 1:3 (i.e., groove depth:channel height) and with varying in the shape of the grooves over the axial position in every half cycle, the mixing length could be reduced to only a few millimetres, this being independent of the

FIG. 1. PDMS-based microreactor with silicon tubing inlet and outlet ports.

FIG. 2. Schematic diagrams for PDMS-based microreactor designed for protein PEGylation (a) and batch PEGylation (b).

Reynolds number for Stokes flow ($Re < 1$) and remains qualitatively similar up to $Re \approx 100$.⁴¹ It is notable that the stretching and refolding in channels with staggered herringbone structures are significantly more effective than simple linear grooves and therefore the full mixing is achieved over much shorter channel lengths.^{47–49} Herringbone structures narrow down the residence time distribution (RTD) in rectangular channels as demonstrated both experimentally and through simulations. The impact on RTD is magnified at higher Peclet numbers (Pe).^{50,51}

The model protein (lysozyme) and functionalized PEG reagent were introduced to the device at constant flow rates using syringe pumps. The PEGylation reaction occurred along the length of the channel and was quenched by glycine solution introduced via a T-junction close to the reactor outlet. The quenched reaction mixture was then collected at the outlet located at the end of the quenching channel [Fig. 2(a)]. Ratner *et al.* had used the similar concept of zonal continuous reaction mixing, retention, and quenching in a silicon microfluidic device for optimization of glycosylation reactions.²⁸ Equivalent batch reaction experiments were performed in a “head-to-head” manner using magnetically stirred vessels [Fig. 2(b)]. The PEGylation of lysozyme was carried out at room temperature using the $-NHS$ chemistry^{52,53} which is considered as rapid and moderately selective. The conjugation is based on acylation in the pH range of 7–9 which is followed by formation of stable amide linkages with the NHS group being replaced by the protein.^{11,14} The samples generated by the above reactions were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The mixing behaviour within the microchannel was investigated by carrying out dye based experiments using tagged dextran molecules.

II. EXPERIMENTAL

A. Materials

Lysozyme (L6876), glycine (G8898), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), sodium hydroxide (S5881), hydrochloric acid (258148), sodium chloride (S7653), sodium dodecyl sulphate (L3771), 30% acrylamide solution (A3699), Trizma base (T1503), Trizma hydrochloride (T3253), glycerol (G2025), ammonium persulfate (3678), N,N,N',N'-tetramethyl ethylenediamine (T9281), Brilliant Blue R concentrate (B8647), DL-dithiothreitol (43817), 70% perchloric acid (77227), Rhodamine B isothiocyanate-dextran (R9379), and FITC-dextran (FD20s) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium iodide (74210–140) was purchased from Anachemia (Montreal, QC, Canada).

Methoxy-PEG-(CH₂)₅COO-NHS, 5 kDa (catalogue number SUNBRIGHT ME-050HS) was purchased from NOF Corporation (Tokyo, Japan). Acetic acid (1000-1) and methanol (6700-1) were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). All the buffers and sample solutions were prepared using Milli-Q water obtained from a SIMPLICITY 185 water purification unit (Millipore, Molsheim, France).

B. Device design and fabrication

The herringbone chaotic mixer based microreactor used in this study is shown in Fig. 1. The device had similar outer dimensions as the supporting glass slide (3 in. × 1 in.). The channel width was 1 mm and height was 900 μm, with the groove depth being 300 μm. The overall reactor volume excluding the quenching channel was 200 μl. The selection of the channel height was based on preliminary experiments.

The device was designed by Autodesk Inventor (San Francisco, USA) and fabricated using 3D printing of acrylic-based master moulds followed by soft lithography. Poly-dimethylsiloxane (PDMS) pre-polymer (Sylgrad 184 kit, Dow Corning Corporation, MI, USA) was mixed at a base:agent ratio of 10:1 and cast into the mould, degassed, and then cured at 75 °C for 2 h. Three inlets and one outlet were embedded within the PDMS using silicon tubing. The PDMS replicas were then peeled off and bonded on glass slides using oxygen plasma (80 s, 50 W).

C. Dye experiments

Rhodamine B isothiocyanate-dextran (70 kDa, 20 mg/ml) and FITC-dextran (20 kDa, 7 mg/ml) solutions were prepared in DI water. The microreactor inlets were connected to 1 ml syringes (Becton Dickinson, Franklin Lakes, NJ, USA) via silicon tubing and metal tips. Syringe pumps (LEGATO 210, KD Scientific, Holliston, MA, USA) were used to feed the microreactor at 20 μl/min (equivalent to 5 min residence time). The device was initially primed using 20% ethanol solution followed by washing with 5 ml DI water to prevent any bubbles from getting trapped in the grooves. The dye mixing was monitored by recording a video clip with a Nikon D810 camera fitted with Nikon Micro 105 mm lens, using white light as background.

D. Lysozyme PEGylation

PEGylation reactions were carried out at pH 8.0 which was shown to give high conversions within relatively short times (a few minutes) in batch experiments.¹⁴ All proteins, functionalized PEG, and quencher solutions were prepared in 100 mM sodium phosphate buffer (pH 8.0). The microreactor volume was 200 μl. The protein concentration used was 10 mg/ml. The experiments were carried out using two different PEG: lysozyme molar ratios, i.e., 1:1 and 4:1.

1. Batch reaction

Batch reactions were carried out by mixing 100 μl of the lysozyme and 100 μl methoxy-PEG-(CH₂)₅COO-NHS solutions in sample tubes (500 μl volume). Small magnetic stirrers were used for continuous mixing at 40% of the maximum speed on a VWR Mini Blot mixer. The same volume of glycine solution (100 μl) was added to quench the reactions due to its low pKa. The concentration of glycine in the final quenched reaction solution was 50 mM.

2. PEGylation using the microreactor

The microreactor inlets were connected to 1 ml syringes via silicon tubing and metal tips. Syringe pumps were used to feed each microreactor at a constant flow rate. The devices were initially primed with 20% ethanol followed by conditioning with 5 ml of the reaction buffer. The same reactant solutions (as described in Sec. IID 1) were prepared and introduced to the microreactors at flow rates adjusted to match specific residence times. The quenched reaction mixtures were collected at the outlet.

E. SDS-PAGE analysis

The quenched reaction mixtures were diluted and analysed by 12.5% non-reducing SDS-PAGE. The gels were stained using Coomassie Brilliant Blue dye for 20 min and destained using a mixture of methanol (15%), acetic acid (10%), and water (75%) to visualize the lysozyme bands. All gels were then documented using a Bioimaging MiniBis pro system (24–25-PR) (DNR-Imaging Systems, Jerusalem, Israel) and analysed by Image J freeware (<http://imagej.nih.gov/ij/>).

The intensity of the bands in terms of grey scale intensity was used to calculate the following parameters:

$$\text{Conversion} = \frac{\text{sum of intensities of PEGylated lysozyme bands}}{\text{sum of intensities of all bands}},$$

$$\text{Selectivity} = \frac{\text{intensity of mono - PEGylated lysozyme band}}{\text{sum of intensities of all PEGylated lysozyme bands}},$$

$$\text{Mono conversion} = \text{Conversion} \times \text{Selectivity}.$$

III. RESULTS AND DISCUSSION

The mixing in the microreactor was assessed using dye experiments. Two differently dyed dextrans (i.e., yellow and red) having molecular weights close to the reagents used in the PEGylation experiments were chosen since diffusivity (which is correlated with the molecular weight) was expected to play a significant role in the mixing process. The dextran concentrations used were in the same range as the reagent concentrations used in the PEGylation reaction experiments. Specifically, a 1:1 M ratio was used in the dye experiment. Figure 3 demonstrates the mixing of the red and yellow dyes in the microchannel. As can be seen from the figure, very fast mixing occurred and homogeneity was attained within a short mixing length from the inlet and there was no colour gradient over the remaining length of the microreactor. When DI water (which simulated the quencher) was added via the T-junction to the dye mixture, the dilution effect was fast and distinct with no colour gradient over the remaining length of the quenching channel. The appearance of deeper coloration near the walls was due to the fact that the quencher was added using a channel narrower than the reactor. This is unlikely to have a significant effect as this is a quenching step and not a reaction step introduced at the Y and T shaped inlets. As the next step, the reaction within the microreactor was compared with batch protein PEGylation.

Batch PEGylation of lysozyme was initially carried out using methoxy-PEG-(CH₂)₅COO-NHS and lysozyme in a 1:1 M ratio. The experiments were run for 4 different reaction times: 2.5, 5.0, 10.0, and 15.0 min. In each case, the reactions were quenched by adding glycine to the reaction mixture. Figure 4 shows the SDS-PAGE results obtained from samples collected from these experiments. The product mixtures contained unreacted lysozyme, mono-PEG lysozyme (which was the target molecule), and higher-PEG lysozyme, which were the by-products. Based

FIG. 3. Dye mixing experiments carried out with the microreactor. The differently dyed dextran reagents were introduced at the Y-shaped inlet ports and DI water was introduced at the T-shaped to simulate quenching.

FIG. 4. SDS-PAGE analysis of the batch reaction products corresponding to different reaction times carried out with a PEG:lysozyme molar ratio of 1:1 (1) protein molecular weight marker, (2) 2.5 min, (3) 5.0 min, (4) 10.0 min, and (5) 15.0 min.

on the intensity of the bands, longer reaction times resulted in higher conversions as well as higher amounts of by-product synthesis (i.e., lower selectivity). At 10 and 15 min reaction times, very significant amounts of di-PEGylated moieties were detected. Table I summarizes the quantitative data obtained by Image J analysis of the gel shown in Fig. 4. The conversion increased and the selectivity decreased with reaction time, with the conversion plateauing after 5 min.

“Head-to-head” comparison experiments using the microreactor were carried out by using similar residence times by adjusting each of the inlet flow rates to 40.0, 20.0, 10.0, and 6.7 $\mu\text{L}/\text{min}$, respectively. In each experiment, the reaction was quenched by adding glycine solution using a T junction and the products were collected at the outlet and were analysed. Figure 5 shows the results obtained from the above experiments, i.e., with the microreactor using 1:1 M ratio. Quite clearly, the conversion increased with time as indicated by progressively thicker mono-PEGylated protein bands on the gel. However, trace amounts of di-PEGylated lysozyme could also be seen in the lanes corresponding to the 10 and 15 min residence time samples. Table II summarizes the quantitative data obtained from Fig. 5. Considerably lower conversions were obtained using the microreactor when compared with equivalent batch experiments. However, the selectivity values were much higher, being very close to 1.0 (i.e., 100% mono-PEGylation) for 2.5 and 5.0 min residence times. These differences between the reactions profiles obtained with the batch and microreactor systems could be attributed to the nature of mixing within the two systems. The stirrer utilized in the batch reaction acted as a vorticity source which caused chaotic solute motion, leading to uncontrolled reaction. By contrast, the micro-mixing within the continuous flow microreactor was based on chaotic advection which resulted in more controlled mixing, leading to higher selectivity of mono-PEGylation. Such an effect of differences in reagent introduction and mixing on the PEGylation reaction (i.e., trade-off between conversion and selectivity) has been discussed in earlier studies.^{46,47}

TABLE I. Quantitative analysis of the SDS-PAGE results shown in Fig. 4.

Reaction time (min)	2.5	5.0	10.0	15.0
Conversion	0.57	0.65	0.68	0.66
Selectivity	0.89	0.78	0.68	0.70
Mono-conversion	0.50	0.50	0.47	0.47
Mono-conversion per unit time (min^{-1})	0.2	0.1	0.05	0.03

FIG. 5. SDS-PAGE analysis of the microreactor reaction products corresponding to different residence times carried out with a PEG: lysozyme molar ratio of 1:1 (1) protein molecular weight marker, (2) 2.5 min, (3) 5.0 min, (4) 10.0 min, (5) 15.0 min, and (6) feed.

TABLE II. Quantitative analysis of the SDS-PAGE results shown in Fig. 5.

Residence time (min)	2.5	5.0	10.0	15.0
Conversion	0.23	0.36	0.46	0.47
Selectivity	1	1	0.93	0.95
Mono-conversion	0.23	0.36	0.43	0.45
Mono-conversion per unit time (min^{-1})	0.09	0.07	0.04	0.03

Figure 6 shows the SDS-PAGE gel obtained using samples from 4:1 PEG: lysozyme molar ratio batch PEGylation experiments. The quantitative results obtained from these experiments are summarized in Table III. Very high conversion was obtained even using short reaction times. However, the selectivity was very low.

Figure 7 shows the SDS-PAGE gel obtained using samples from 4:1 PEG:lysozyme molar ratio PEGylation experiments carried out using the microreactor. The experiments with the microreactor were carried out at two additional shorter residence times, i.e., 1.0 and 2.0 min (corresponding to 100 and 50 $\mu\text{l}/\text{min}$ flow rate at each inlet). The quantitative results obtained from these experiments are summarized in Table IV. Consistent with the results obtained using the 1:1 M ratio, the use of the microreactor system resulted in greater selectivity and lower conversion which is due to the differences in mixing conditions discussed earlier. A residence time of 2.5 min gave the best combination of selectivity and conversion.

The above results clearly show that the microreactor system was suitable for protein PEGylation. The chaotic advection which was created by the staggered herringbone grooves generated controlled and fast mixing which increased the selectivity of mono-PEGylation. However, the overall conversion was lower than that obtained with batch reaction. Therefore, there was clearly a trade-off between overall conversion and selectivity. The microreactor system was simple, and it was easy to operate and control the PEGylation reaction by controlling reactant introduction, reaction quenching, and product collection. Parallelization of the microreactor system could be carried out by using fluidic manifolds that could provide parallel infusion of the reagents. Therefore, robust screening of the reaction conditions could be achieved with a high degree of reproducibility. The advantages over the batch reactor include enhanced

FIG. 6. SDS-PAGE analysis of the batch reaction products corresponding to different reaction times carried out with a PEG: lysozyme molar ratio of 4:1 (1) protein molecular weight marker, (2) 2.5 min, (3) 5.0 min, (4) 10.0 min, (5) 15.0 min, and (6) feed.

TABLE III. Quantitative analysis of the SDS-PAGE results shown in Fig. 6.

Reaction time (min)	2.5	5.0	10.0	15.0
Conversion	0.82	0.86	0.92	0.94
Selectivity	0.54	0.39	0.33	0.25
Mono-conversion	0.45	0.33	0.30	0.24
Mono-conversion per unit time (min^{-1})	0.18	0.07	0.03	0.02

FIG. 7. SDS-PAGE analysis of the microreactor reaction products corresponding to different residence times carried out with a PEG: lysozyme molar ratio of 4:1 (1) protein molecular weight marker, (2) 1.0 min, (3) 2.0 min, (4) 2.5 min, (5) 5.0 min, (6) 10.0 min, (7) 15.0 min, and (8) feed.

TABLE IV. Quantitative analysis of the SDS-PAGE results shown in Fig. 7.

Residence time (min)	1.0	2.0	2.5	5.0	10.0	15.0
Conversion	0.48	0.59	0.78	0.83	0.85	0.87
Selectivity	0.89	0.87	0.82	0.66	0.56	0.47
Mono-conversion	0.43	0.51	0.64	0.55	0.48	0.41
Mono-conversion per unit time (min^{-1})	0.43	0.26	0.26	0.11	0.05	0.3

potential for heat transfer (if required), visualization, as well as better control on the reaction.^{48–50} Moreover, such a system would be highly suitable for multi-step processes.^{54,55}

IV. CONCLUSIONS

The microreactor discussed in this paper was suitable for carrying out protein PEGylation. The staggered herringbone grooves embedded within the serpentine channels resulted in chaotic advection within the Stokes regime (i.e., $\text{Re} < 1$). This resulted in fast and controlled micromixing which was suitable for carrying out protein PEGylation. A comparison with batch reaction showed that the chaotic laminar mixing within the microreactor resulted in lower residence time distribution within the device which ultimately led to higher selectivity of mono-PEGylation. However, the overall conversion was a bit lower than that obtained with batch reaction, i.e., there was a trade-off between overall conversion and selectivity. The microreactor is also likely to be suitable for screening other kinds of chemical reactions. It offers flexibility in design, is easy to fabricate, and is suitable for use over a wide range of scales and experimental conditions. This device also holds great potential for use in multistep and smart synthesis.

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