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Towards high-throughput fast photochemical oxidation of proteins: Quantifying exposure in high fluence microtiter plate photolysis

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ABSTRACT

Protein structural analysis by mass spectrometry has gained significant popularity in recent years, including high-resolution protein topographical mapping by fast photochemical oxidation of proteins (FPOP). The ability to provide protein topographical information at moderate spatial resolution makes FPOP an attractive technology for the protein pharmaceutical discovery and development processes. However, current technology limits the throughput and requires significant manual sample manipulation. Similarly, as FPOP is being used on larger samples, sample flow through the capillary becomes challenging. No systematic comparison of the performance of static flash photolysis with traditional flow FPOP has been reported. Here, we evaluate a 96-well microtiter-based laser flash photolysis method for the topographical probing of proteins, which subsequently could be used to analyze higher order structure of the protein in a high-throughput fashion with minimal manual sample manipulation. We used multiple metrics to compare microtiter FPOP performance with that of traditional flow FPOP: adenine-based hydroxyl radical dosimetry, oxidation efficiency of a model peptide, and hydroxyl radical protein footprint of myoglobin. In all cases, microtiter plate FPOP performed comparably with traditional flow FPOP, requiring a small fraction of the time for exposure. This greatly reduced sample exposure time, coupled with automated sample handling in 96-well microtiter plates, makes microtiter-based FPOP an important step in achieving the throughput required to adapt hydroxyl radical protein footprinting for screening purposes.

1. Introduction

Structural characterization of biomolecules using mass spectrometry (MS)-based techniques is being extensively employed over the last few decades [1]. Hydroxyl radical protein footprinting (HRPF) is a mass spectrometry based structural characterization technique that provides a lower level of structural detail than traditional high-resolution structural biology techniques such as X-ray crystallography or multi-dimensional nuclear magnetic resonance (NMR) spectroscopy [2,3]. HRPF is complementary to other MS-based methods such as hydrogen-deuterium exchange and ion mobility spectroscopy, as it measures changes in the protein topography (i.e. solvent-accessible surface) using a stable and broadly reactive labeling group. Many methods for the generation of hydroxyl radicals for HRPF have been introduced, including the use of UV photolysis of hydrogen peroxide [3]. Afterward, two groups independently introduced two laser-induced methods for pulsed UV photolysis of hydrogen peroxide: Fast Photochemical Oxidation of Proteins (FPOP) and batch photolysis.

Originally developed by Hambly and Gross in 2005 [4], FPOP is a technique commonly used today to produce hydroxyl radicals through photolysis of hydrogen peroxide. In FPOP, a mixture of the analyte protein, hydrogen peroxide, and a hydroxyl radical scavenger is pushed through a capillary past a focused KrF excimer laser (248 nm wavelength). The laser is pulsed to flash photolyze hydrogen peroxide, forming a sharp and short-lived burst of hydroxyl radicals that are consumed in under a microsecond [5–8]. Theoretically, with addition of exclusion volume of unirradiated sample between each irradiated volume, each volume of sample is exposed to only a single laser pulse. Under such conditions, the initial and irreversible hydroxyl radical reaction with the protein occurs on approximately a microsecond timescale, and this rapid time frame allows for proteins to be thoroughly surface labeled faster than the protein will undergo large-scale structural changes due to the labeling process [9,10]. In reality, due to the characteristic parabolic flow of the solution within the capillary system under laminar flow conditions, a small volume of sample may be double-exposed. However, the fraction of such double-exposed sample is limited to an undetectable amount when using an appropriately large volume of unirradiated sample between each laser shot, as determined by an analysis of the labeling kinetics of intact proteins by FPOP [8].

One limitation of FPOP is that it is time consuming (especially when

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dealing with a large number of samples), and FPOP requires significant manual handling of samples for exposure. A conventional flow FPOP method takes nearly 13 s of mid-flow time to irradiate 4 µl of sample, not including time for sample loading, capillary-resident sample ejection, and inter-sample capillary washing [11]. Additionally, recent developments using FPOP technology for larger systems, including living cells, have presented challenges in maintaining appropriate flow through the system [12,13]. Workflows that eliminate the need for flow path cleaning and sample reloading could substantially reduce the time per sample requirements and eliminate the possibilities of capillary clogging or laser-induced capillary breakage.

Sze and coworkers demonstrated protein oxidation in a microfuge tube on a nanosecond time scale with a single pulse irradiation in 2005 [9]. Later the same group demonstrated the protein footprinting of EGFR in 12 well plates [14]. Recently, Schriemer and co-workers performed protein footprinting of calmodulin in static set-up using photoleucine as a diazarine labeling agent [15,16]. Subsequently, Gross and co-workers used photoleucine to footprint calmodulin using a capillary flow system [17]; while the two results were grossly similar, there were significant differences in the peptide-level footprint. As these experiments were not carried out with the intention of a direct comparison between the two techniques, it is quite possible that the differences were due to variables other than flow exposure versus static exposure, but the role of the different exposure techniques in the observed different footprints has not been explored. Similarly, while the correlation between labeling by FPOP and amino acid side chain solvent accessibility has been established [18–20], we are not aware of any published comparison of single pulse batch photolysis and FPOP results that establish the equivalency of the two technologies.

Inspired by work pioneered by Sze and co-workers, we report our first investigations into the technologies required to develop FPOP and related technologies into a high throughput format: the development and evaluation of a 96-well microtiter plate-based FPOP exposure system. We describe efforts involved in ensuring reliability and reproducibility in microtiter FPOP and evaluate microtiter FPOP against traditional flow FPOP using three systems: adenine-based hydroxyl radical dosimetry; oxidation of the model peptide $[Glu]_1$-Fibrinopeptide B (GluB); and HRPF analysis of the model protein myoglobin. With low sample requirements (4 µL or less), very fast exposure times (< 5 s per sample, which could be automated to < 1 s per sample), no sample dilution from unirradiated volumes, and compatibility with common 96-well microtiter-based sample handling workflows already in place in biochemistry and proteomics, our results demonstrate the considerable promise of microtiter-based single pulse batch photolysis for increasing the throughput of HRPF, an emerging technique for the analysis of protein pharmaceuticals [21,22].

2. Materials and methods

2.1. Chemicals and reagents

Myoglobin, GluB, and catalase were purchased from Sigma Aldrich Corp (St. Louis, MO). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Adenine and glutamine were purchased from Acros Organics (Fair Lawn, NJ) and methionine amide was from Bachem (Torrance, CA). Hydrogen peroxide (30%), LC-MS grade water, acetonitrile, formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Corning™ Costar™ flat bottom 96 well plates were from Fisher Scientific (Fair Lawn, NJ) and V-shaped 96 well microtiter plates were purchased from Greiner Bio-One (Monroe, NC).

2.2. Sample oxidation

Fast photochemical oxidation of protein (FPOP) was performed with a Compex Pro 102 excimer laser (Coherent, Germany) at 248 nm wavelength as described previously [23]. Adenine dosimetry was used to ensure the consistency of free radicals generated during FPOP and microtiter plate single pulse photolysis among different samples illuminated with different laser fluences [24]. For microtiter plate single pulse photolysis, a TECHSPEC® Excimer laser line mirror from Edmund Optics (Barrington, NJ) was placed at 45° of the laser beam at ~15 cm distance of the source as shown in Fig. 1. The laser beam was deflected by the mirror and subsequently focused by an FL uncoated, UV plano-convex lens (Edmund Optics). The focused beam was allowed to score an index card target mounted on the optical bench to assist with alignment of the plate with the beam. Another card positioned at the height of the surface of the sample was used to position the focusing lens to ensure the laser spot covered the entire surface area of the sample. A clear V-shaped well polystyrene microtiter plate was placed beneath the lens that contained the sample to be irradiated with the laser.

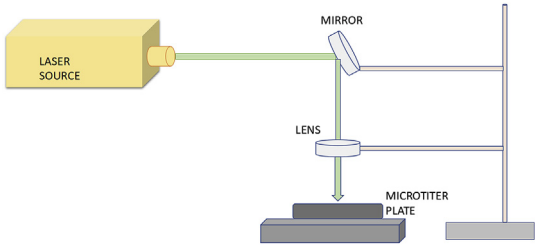

Fig. 1. Schematic cartoon for microtiter FPOP. A mirror placed at a 45° angle in the laser path deflects the laser beam onto a lens, which then focuses the light onto the well of the microtiter plate.

The sample was mixed to contain 5 µM of GluB peptide, 5 µM myoglobin, 1 mM adenine, 17 mM glutamine, 100 mM hydrogen peroxide, and 50 mM sodium phosphate buffer (pH 7.4). Immediately after mixing, 4 µl of sample was placed in each microtiter well. The samples were oxidized with a single shot laser pulse and immediately quenched by addition of 5 µl of quenching solution consisting for a final concentration of catalase (0.5 µg/µl) and methionine amide (0.5 µg/µl) to remove the residual hydrogen peroxide and other secondary oxidants. Samples were illuminated in triplicate at each laser fluence tested. The laser fluence was calculated from the laser energy and laser beam. The estimated fluence was calculated based on the spot size of the beam at the approximate site of contact with the sample, assuming no loss of light from the mirror and focusing. After quenching for one hour, the absorbance of adenine was measured by Nanodrop UV–Vis spectrophotometer from Thermo Fisher Scientific (Waltham, MA).

Traditional capillary flow FPOP was performed as previously described [18]. Briefly, 20 µl of the sample containing 5 µM of GluB peptide, 5 µM myoglobin, 1 mM adenine, 17 mM glutamine, 100 mM hydrogen peroxide, and 50 mM sodium phosphate buffer (pH 7.4) for the dosimetry measurement, were flowed through a 100 µm ID capillary through the path of a focused laser. The laser spot width and laser pulse rate were calculated to give an exclusion volume of 15%. Samples were immediately collected in a 1.5 mL microfuge tube containing 25 µl quenching solution (final concentration of (0.5 µg/µl) catalase and (0.5 µg/µl) methionine amide) to remove extra hydrogen peroxide and secondary oxidants, and adenine dosimetry was performed as described above.

2.3. Tryptic digestion

50 mM Tris-HCl and 5 mM DTT were added to the sample and the final volume of the samples was adjusted to 40 µl. In order denature the myoglobin, samples were heated in an oven at 90 °C for 15 min. Samples were cooled down to room temperature and sequencing grade trypsin was added to a 1:5 weight ratio of trypsin/protein to the protein sample and incubated it at 37 °C overnight. The digested samples were stored at −20 °C before analysis on for LC-MS system.