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The interaction of photoactivators with proteins during microfabrication

Joseph W. Lafferty, James R. Strande, Peter M. Kerns, Nathan A. Fox, Swarna Basu*

Department of Chemistry, Susquehanna University, 514 University Avenue, Selinsgrove, PA 17870, United States

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ABSTRACT

Micron-scale protein cross-linking or microfabrication has been carried out using an Nd^{3+}–YAG laser as the excitation source. Fabrication is carried out by the excitation of photoactivators (Rose Bengal, methylene blue and 9-fluorenone-2-carboxylic acid) with the ultimate goal of creating stable structures that will serve as models for various applications (drug delivery and tissue engineering). Experimental parameters have been adjusted to minimize photodamage and maximize cross-linking efficiency. The higher than ideal photon flux and peak power necessitates the use of high protein concentrations to minimize photodamage. Rose Bengal and methylene blue are binding to proteins with high association constants ($K_a \approx 10^6\ M^{-1}$) and both Rose Bengal and both 9-fluorenone-2-carboxylic acid are showing changes to their excited states in presence of proteins at cross-linking concentrations. Molecular docking studies show that Rose Bengal binds close to the tryptophan with $\Delta G = -6.15$ kcal/mol.

1. Introduction

Photopolymerization, also referred to as cross-linking or microfabrication or nanofabrication, is a process by which protein monomers are cross-linked via the excitation of a photoactivator or photosensitizer to form stable, bioactive structures [1–10]. New covalent bonds form between amino acid residues (tryptophan, tyrosine, cysteine, lysine, histidine) in adjacent proteins, without the loss of protein functionality [1–3]. Commonly-used photoactivators include Rose Bengal (RB), methylene blue (MB) or 9F2C (9-fluorenone-2-carboxylic acid) [1,2,6,7]. Excitation can either be via a one-photon process or a multiphoton process. Multiphoton excitation (MPE) results in an intrinsic three-dimensional structure, as opposed to the two-dimensional structure that linear (one-photon) excitation provides [7,8]. MPE photochemistry also provides a focal volume in which a high peak-power laser is tightly focused to produce fabrications on the micron-scale [7,8,10].

A Ti^{3+}:sapphire laser is typically used to cross-link proteins via multiphoton excitation of the photoactivator. In recent years, lower-cost systems have been developed and our group uses a setup that has a Nd^{3+}:YAG laser with a lower repetition rate and longer pulse width than most lasers used in this field [9]. The downside of using a low-cost system is the increase in power density at the focal point on the microscope slide. This results in the need for careful control of laser power and relative concentrations of proteins and photoactivators.

Aromatic amino acids, particularly tryptophan, are involved in photopolymerization. The singlet oxygen generated in the long-lived triplet state of RB induces a reaction with an aromatic amino acid such as tryptophan, leading to cross-linking [2]. The two most commonly studied proteins are the serum albumins, BSA (bovine) and HSA (human), due to their similar size and structure. The key difference between the two serum albumins is the number of Trp residues. BSA has two while HSA has only one. The only Trp residue within HSA (Trp 214) resides within a similar chemical environment to that of Trp 213 in BSA [11]. Lysozyme and fibrinogen have also been used for various cross-linking and protein/ligand interaction studies. Lysozyme contains a single amino acid chain and six Trp-residues, all of which are contained in the hydrophobic fold [12]. Fibrinogen and BSA fabrication efficiencies have been shown to be dependent on RB concentration. The 2- to 10-fold fabrication rate increase of fibrinogen compared to BSA is due to its much larger molecular weight, 340 kg/mol, compare to 66 kg/mol for BSA. Naturally, this would result in a much larger volume of protein and many more potentially oxidizable residues. BSA provides fewer potential cross-linking sites and, therefore, is a suitable monomer to study the various mechanisms of photopolymerization with different photoactivators.

The xanthene compound Rose Bengal is a commonly-used photoactivator because of its efficiency in producing singlet oxygen in an aerobic environment [2]. Like RB, methylene blue is also a visible-absorbing photoactivator that has been used to cross-link

* Corresponding author. Tel.: +1 570 372 4223; fax: +1 570 372 2751.
E-mail address: basu@susqu.edu (S. Basu).

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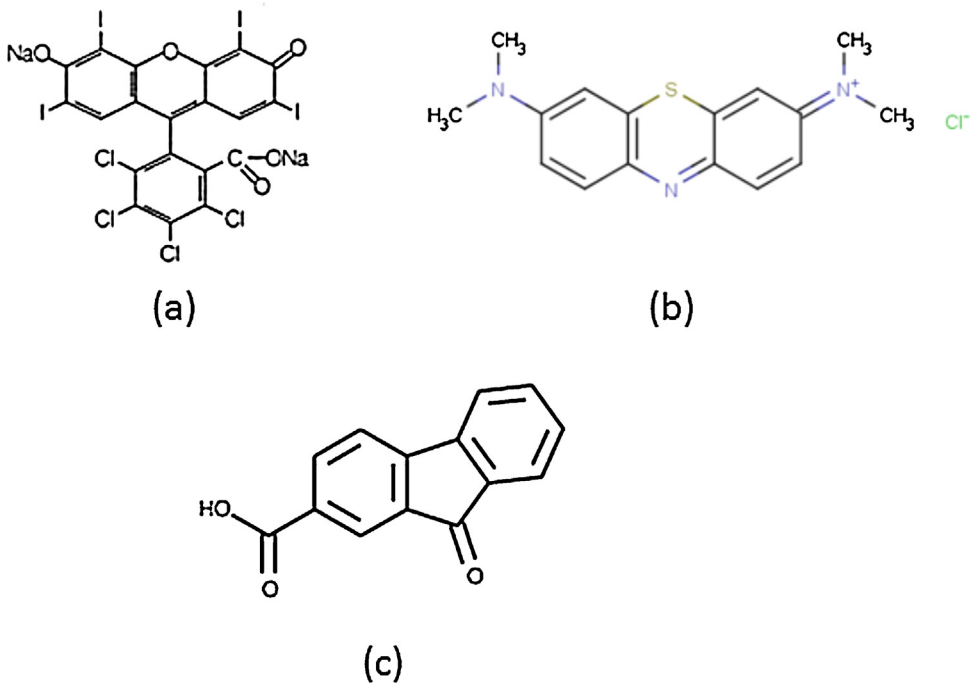

Fig. 1. The structures of the photoactivators used in this work. (a) Rose Bengal, (b) methylene blue, and (c) 9-fluorenone-2-carboxylic acid.

proteins via a singlet oxygen mechanism [10,13]. 9-Fluorenone-2-carboxylic acid has seen limited use as a photoactivator but it has been used to cross-link a synthetic monomer via a three-photon process where hydrogen-abstraction is the mechanism [6]. The structures of the three photoactivators used in this work are shown in Fig. 1.

In this work, a series of proteins have been successfully cross-linked using three photoactivators and a setup that offers higher than ideal peak power with an increased risk of photodamage. Since exposure dosage is a function of laser power per pulse, frequency and exposure time, these were all varied at specified concentration ratios of protein to photoactivator to determine optimum parameters for successful cross-linking. The relationship between the protein and photoactivator has also been investigated in an attempt to understand the exact nature of the physical interaction or proximity between the two species, and whether these interactions or proximity were a prerequisite for the formation of stable cross-linked structures.

2. Materials and methods

2.1. Fabrication setup

The experimental setup has been described previously so only the modifications are reported here [9]. The current schematic is shown in Fig. 2. Briefly, the second harmonic (532 nm, 5.9 ns pulse width) output of a Continuum Minilite II Nd^{3+}:YAG laser was directed into the Zeiss AxioObserver A1 inverted microscope laser port via one mirror (M1). The pulse is focused on the sample through the objective (Zeiss DC-Neofluar 20×, 0.5NA). Previously, three mirrors were used to direct the laser into the microscope port due to the height difference (0.9 mm) between the laser and the microscope's laser port. Due to a 70% loss of power from the laser source to the sample, the setup was modified to use only one mirror to direct the pulse from the source to the laser port of the microscope, leading to a 27% loss of power. Laser energy for optimal fabrication was 19.3 mJ per pulse, corresponding to a peak power of 3 MW. Images of fabricated structures were obtained using a ThorLabs DC310-C CCD camera.

2.2. Chemicals and fabrication protocol

The photoactivators Rose Bengal (RB), methylene blue (MB) and 9-fluorenone-2-carboxylic acid (9F2C), and the proteins BSA, HSA, lysozyme and fibrinogen were obtained from Sigma–Aldrich and used without further purification. Protein stock solutions (100 mg/mL) were prepared in 50 mM Tris buffer (pH = 7.0). RB and MB stock solutions were prepared in water and 9F2C stock solution was prepared in DMSO. Concentrations were verified using a Cary-Win 4000 UV–vis spectrometer.

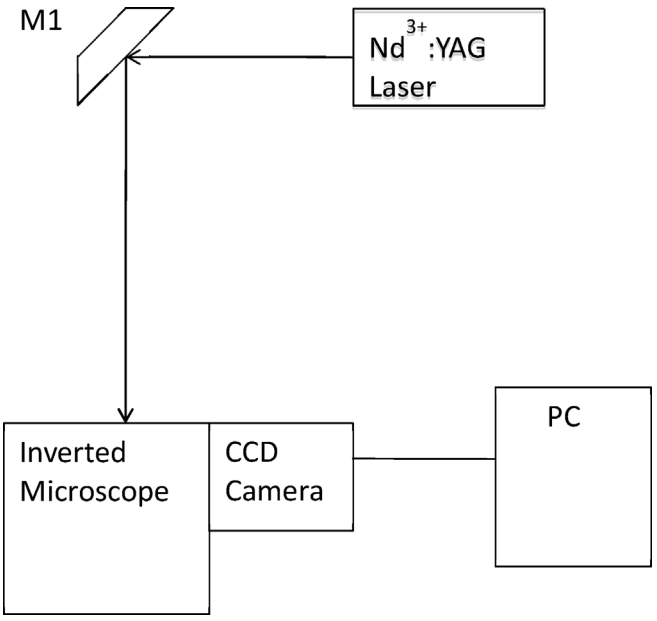

Fig. 2. Current schematic used for fabrication.