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Characterization of covalently bonded proteins on poly(methyl methacrylate) by X-ray photoelectron spectroscopy

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ABSTRACT

X-ray photoelectron spectroscopy (XPS) has been used to characterize a poly(methyl methacrylate) (PMMA) surface with covalently attached proteins. The PMMA surfaces were first aminated using hexamethyldiamine; the resulting $-NH_2$ sites were reacted with the hetero-bifunctional cross-linker Sulfo-EMCS to form a maleimide-terminated surface. The N-hydroxysuccinimide ester terminal and maleimide terminal groups of Sulfo-EMCS reacts with amine and sulfhydryl groups, respectively, exposed on the surface of the proteins. This study characterizes *Thermotoga maritima* β-glucosidase 1 (TmGH1), which belongs to a family of proteins that facilitate hydrolysis of glucose-related monomers with retention of conformation. The surfaces were characterized by XPS to monitor surface composition, and to elucidate protein orientation on the surface. Results suggest that a covalently bonded surface of TmGH1 on PMMA has been obtained. These results demonstrate the feasibility of using XPS to study protein surface chemistry and demonstrate a useful method to anchor cysteine-terminated proteins for the purposes of creating biosensors or platforms for mechanical force experiments to investigate protein structure.

1. Introduction

Covalent attachment of biomolecules to substrates is necessary for the fabrication of robust analytical devices and to conduct mechanical force experiments, such as those used to investigate protein structure. Silicon and gold are popular substrates for biological surfaces [1,2]. Biological molecules have been bound to silicon by utilizing H-terminated Si substrates [3], and through the formation of self-assembled monolayers onto Si [4]. Gold has been used to directly and indirectly attach biomolecules through sulfide bonding with thiols (e.g. cysteine) via the phenomena of molecular self-assembly [5–8]. However, there are numerous disadvantages of using Si and Au as substrates for routine medical or analytical devices. Both substrates are limited by their lack of optical transparency, while Au has a limited chemical reactivity and Si devices can be expensive to fabricate. These disadvantages have motivated the search for alternative substrates for biological surfaces.

Polymers are a promising alternative due to their low cost, ease of chemical modification and, in some cases, optical transparency [9,10]. Polymers may be used as components in reliable, low cost analytical devices, as microstructures on the polymer surface may be formed by a variety of methods such as spin casting or hot embossing [6,9,11–13]. Compared to Si or Au, polymers suffer from the disadvantages of increased surface inhomogeneity, sensitivity to organic solvents, acids and bases, and unwanted side reactions during chemical modification [10,13]. To minimize these disadvantages, two approaches are taken: either synthesize a new polymer to achieve both the bulk and surface characteristics desired; or modify the first few micrometers (or less) of the surface of a bulk polymer to change its surface chemistry, while retaining most bulk properties. We have opted for the latter approach in this paper and we advance an optimized scheme to modify the surface of poly(methyl methacrylate), PMMA, to covalently attach a cysteine-terminated protein.

PMMA has several advantages as a substrate for attaching to proteins over other candidates, including its high transparency, low fluorescent background, ease of molding, and the presence of surface ester side-chains that can be chemically modified to bond to biomolecules [12]. Unmodified PMMA is a poor substrate for our purposes, due to its low binding affinity for biomolecules [10]. In this paper, we modify the wet chemical procedures used by Fixe et al. [14,15] and Brown et al. [9] to attach the protein *Thermotoga maritima* β-glucosidase (TmGH-1) to PMMA. TmGH-1 is a protein of interest in the design of inhibitors for a variety of β-glucosidases. One avenue for future structural probing is to conduct mechanical force experiments on these proteins using AFM, but such experiments require TmGH1 to be anchored securely to substrates. Some characterization of the GH-1 family of proteins has been done by atomic force microscopy (AFM) [16,17], but such studies do not elucidate any chemical information about these proteins. X-ray photoelectron spectroscopy (XPS) has been chosen as

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a characterization tool for TmGH1-terminated surfaces on PMMA, due to its ability to elucidate depth and chemical information of surfaces from the resultant data. Despite surface charging effects, there is an extensive array of XPS data for polymers and related surface modification [18–22]. The use of XPS to study the chemistry of biomolecules is less extensive, but reviews by Ratner [23] and Merrett et al. [21] demonstrate the utility of the method towards analyzing biomaterials. XPS has been used to determine protein orientation using the XPS signals of protein-bound metals [24], to study protein adsorption phenomena onto other polymers [25], and to investigate biological samples that have been prepared by cryogenic freezing [26].

This paper demonstrates that TmGH1 can be covalently bonded to a modified PMMA substrate using an optimized procedure. The XPS technique will be used to compare the relative composition of different protein surfaces, identify the overlayer thickness, suggest chemical orientation of the proteins with respect to the surface and to suggest the presence of a covalent bond between protein and substrate.

2. Experimental

2.1. Mutagenesis, expression and purification of PhnH, wild type TmGH1 and TmGH1Cys

PhnH was expressed and purified as described previously and naturally contains a Cys residue at the C-terminus [27]. The pET-28a plasmid containing the *Thermotoga maritima* beta-glucosidase gene [28], was a gift from Gideon Davies, York Structural Biology Laboratory, UK. The *tmGH1* gene was mutated to replace Cys 55 of TmGH1 with Ser and insert an additional Cys residue at the C-terminus of the protein. Four primers were used to accomplish both mutations in three sequential, overlapping PCR reactions. The primers A: 5′-CAGCCATATGGCTAGCAACGTGAAAAAG-3′ and B: 5′-GTTGTAGTGG**TCG**GAGGCCACATCT-3′ were used with Pfu Turbo (Stratagene) to amplify a 126 bp segment using the *tmGH1* gene in pET-28a as a template. A second PCR reaction used the primers C: 5′-GAGATGTGGCCTC**CGA**CCACTACAAC-3′ and D: 5′-GCCGCAAGCTTTTA**GCA**GTCTTCCAG-3′ along with Vent DNA polymerase (New England Biolabs) to amplify a 1200 bp segment from *tmGH1*. NheI and HindIII restriction sites and mutagenic codons encoded by the primers are shown underlined and in bold, respectively. The PCR products were gel purified (Qiagen), combined with the flanking primers A and D, and then amplified with Vent DNA polymerase to reassemble the full length, doubly mutated, *tmGH1* gene (1360 bp). All three PCR reactions were performed as follows: after denaturation at 98 °C for 4 min, a 'hot start' was initiated by addition of polymerase. The reaction was then cycled 30 times through 98 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min. Finally, the reaction was extended 72 °C for 5 min. The *tmGH1* PCR product was digested with NheI and HindIII (Fermentas) ligated into similarly digested pET-28a (Novagen) with T4 DNA ligase (Fermentas) to create pET28a-TmGH1Cys. The two mutations were confirmed by sequencing of both DNA strands. Wild type and TmGH1Cys were expressed and purified as described before [27] then assayed spectrophotometrically with 2-nitrophenyl-D-glucopyranoside at pH 7 and 25 °C (400 nm for 2-nitrophenol = 2170 M^{-1} cm^{-1}). Virtually identical Michaelis–Menten kinetic parameters were observed for wild type TmGH1 (k_{cat} = 30 s^{-1}, K_M = 0.21 mM) and TmGH1Cys (k_{cat} = 39 s^{-1}, K_M = 0.18 mM).

2.2. Surface preparation methods

The overall procedure used to form the protein-terminated surfaces is shown in Fig. 1. The overall modification methods are primarily based on a methodology for bulk PDMS preparation for microfluidic applications previously reported by Brown et al. [9], and the modification of PMMA surfaces to covalently attach DNA by Fixe et al. [14]. Mica (V-4 Grade, SPI Supplies) and Au (1 1 1) substrates (Georg Albert PVD-Beschichtungen; sputter-coated, 300 nm thick on mica) were used to support spin-coated PMMA substrates. Both surfaces were used to support the PMMA and modified PMMA samples, but Au (1 1 1) substrates were used for the XPS experiments to reduce charging effects and the Au 4f signal (84.0 eV) served as an internal binding energy standard for XPS experiments. If necessary, samples were stored in N_2 environments to preserve sample integrity.

Thin PMMA films (Fig. 1A) were formed by spin-coating from solution. Bulk PMMA (Warehoused Plastics Ltd., Toronto, ON) was dissolved in dichloromethane to a concentration of 0.5 mg/mL. The solution was sonicated until the PMMA was dissolved (approxi-

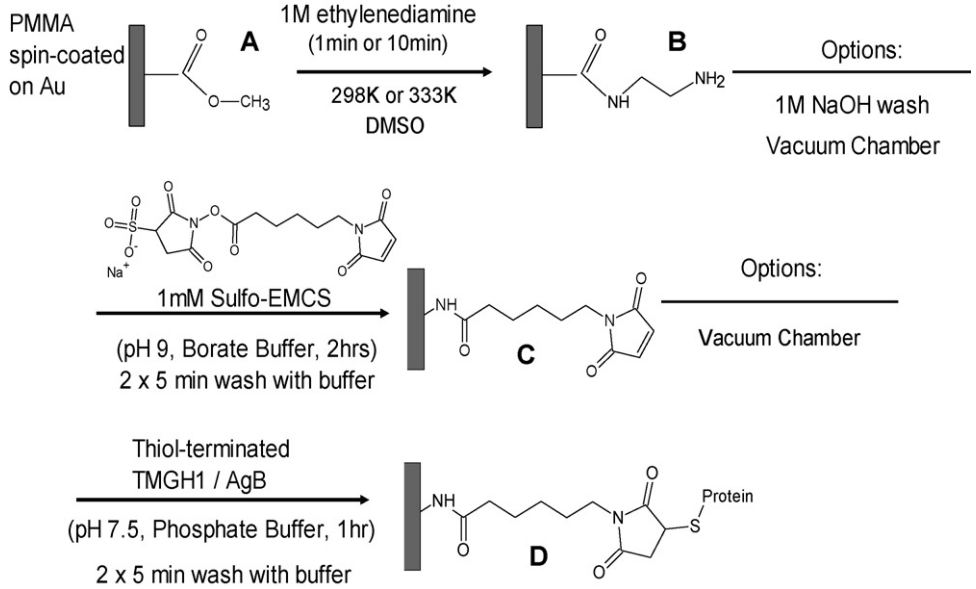

Fig. 1. Schematic of the surface modification process that has been developed, including modifications used to optimize technique. Subsequent surfaces studied by XPS are shown in the figure: PMMA (A); NH_2-terminated PMMA (B); maleimide-terminated PMMA using Sulfo-EMCS (C); protein-terminated PMMA (D).