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Effect of Polyethylene Glycol Tether Size and Chemistry on the Attachment of Lactase to Polyethylene Films

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ABSTRACT: Numerous foodstuffs have been developed for lactose intolerant individuals. However, current methods to reduce lactose require excessive enzyme and are not viable for small producers. A novel solution proposed to overcome these problems is the immobilization of lactase to standard food-contact packaging materials. The ability of these packages to convert lactose is dependent on the quantity and specific activity of the enzyme after immobilization. In this study, the material interface of polyethylene (PE) films was modified with polyethylene glycol (PEG) tethers of 1 kDa, 2 kDa, 5 kDa, and 10 kDa containing epoxy, acrylate, aldehyde, and succinimdyl ester end-group functionality. The results showed that a 5 kDa tether enabled maximum absolute activity of 0.49×10^{-2} ALU/cm^2 when compared to tethers of different sizes with identical functionalization. Compared to succinimdyl, epoxide, and acrylate functionalized PEG tethers, an aldehyde end-group resulted in films with the highest absolute immobilized enzyme activity of 0.67×10^{-2} ALU/cm^2. Regardless of tether size or chemistry, the activity retention of lactase was less than 15% compared to the soluble enzyme after immobilization to PEG-functionalized PE films. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biological applications of polymers; enzymes; surface modification; films; polyethylene (PE)

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INTRODUCTION

Lactose is a disaccharide of glucose and galactose found in dairy products. When small intestine epithelial cells lose the ability to produce the lactose-digesting enzyme, lactase, an individual may develop lactose intolerance. This condition is most common in non-Caucasian persons, with an estimated 70% of the human world population suffering from natural lactose intolerance.[1,2] Excessive consumption of lactose by affected individuals will produce unpleasant symptoms such as abdominal pain, bloating, nausea, gas, and diarrhea. Although most lactose intolerant individuals can tolerate some amount of lactose, the condition must be managed with dietary restrictions.[2] Numerous supplements and modified foodstuffs, including lactose-reduced and lactose-free milk, kefir, and yogurt have been developed and marketed to lactose intolerant individuals.[3,4] To produce lactose-reduced or lactose-free products, milk that has undergone the standard pasteurization procedure is batch-treated with a commercially available lactase preparation. After lactose hydrolysis has proceeded to the desired degree, the milk undergoes a secondary pasteurization process to stop enzyme activity and reduce detrimental enzymes and bacterial load from contaminants in the enzyme preparation.[5] These extra processing steps increase the cost of the final product and affect sensory attributes.[3,6,7] Likewise, because lactose reduction is typically desired in less than 24 h, excessive amounts of enzyme are needed, which increases the cost of the final product. The development of enzyme modified products also requires technical expertise and production capabilities that may not be available to small producers.

A novel solution proposed to overcome these problems is the immobilization of lactase to standard food-contact packaging materials such as polyethylene (PE). Polymer films modified in such a manner could be used to assemble an active package system which would hydrolyze lactose *in situ* after the addition of milk which had been processed. This technology, if rendered effective and affordable, would reduce the cost of lactose-free milk, decrease quality discrepancy versus the traditional product and increase the accessibility and variety of products available to customers by eliminating the need for additional processing steps.[8,9] Previous work has shown that immobilization directly to functionalized PE leads to loss of enzymatic activity compared the free enzyme.[9,10] This loss of activity may be due to denaturation of the enzyme at the material interface or hindrance of the active site of the enzyme to the substrate.[9]

Strategies to reduce the loss of enzymatic activity after immobilization include changing the nature of the material by

rendering the surface more hydrophilic and increasing the distance between the enzyme and the support. Moskovitz and Srebnik utilized computational modeling to demonstrate that as an enzyme approaches a hydrophobic surface it will collapse against the surface due to migration of the hydrophobic core of the enzyme.[11] Grafting a hydrophilic polymer layer to the hydrophobic surface may help an immobilized enzyme retain a structure closer to its native conformation by preventing direct adsorption.[11] This model is supported by other research which shows that hydrophilic surfaces will decrease the amount of non-covalent protein adsorption.[12,13]

In addition to modifying the hydrophilicity of a surface, a surface-attached molecule may act as a tether to provide physical separation from the surface, improving accessibility to the substrate and decreasing the effects of the physicochemical properties of the anchor surface material. Numerous tether molecules and polymers have been described in literature, including amino acids, glutaraldehyde, ethylenediamine, and poly(ethylene glycol; PEG).[14] PEG, in particular, is an attractive tether molecule, being highly soluble in aqueous solutions, non-toxic and biocompatible.[15] PEGs are readily available from several sources with a wide variety of chain lengths and terminal chemical groups, and have been utilized by multiple researchers as a tether to improve immobilized enzyme activity.[16–19]

Despite a solid body of research in which PEG sees a wide variety of applications, it is still relatively unknown how the properties of the PEG molecule itself affect the nature of a tethered enzyme. De Maio and coworkers showed using other tethers that even small increases in tether chain length can increase immobilized lactase activity.[20,21] Activity can also be influenced by the type of chemistry used to covalently attach an enzyme to a surface since the site of tether attachment may affect the structure of the immobilized protein.[14] Though a number of bioconjugation chemistries exist, that target the same functional group on an enzyme, there is limited data that compares such functionalities side-by-side.

In light of these gaps in the existing body of research, the goal of this work was to compare different homobifunctional PEG derivatives to evaluate the effect of conjugation chemistry and chain length on the protein loading and retained enzyme activity of lactase immobilized by covalent linkages to PE films. This study demonstrates that lactase can be conjugated to PE films by PEG tethers, and that absolute activity of the films can be enhanced by the choice of tether size and end-group chemistry.

EXPERIMENTAL

Materials

Additive-free low-density PE pellets were purchased from Scientific Polymer Products (Ontario, NY). Anhydrous calcium sulfate, anhydrous potassium phosphate dibasic, potassium phosphate monobasic, sodium carbonate, sodium acetate trihydrate, glacial acetic acid, boric acid, hydrochloric acid, sodium hydroxide, acetone (99.8%), and 2-propanol (99.9%) were purchased from Fisher Scientific (Fairlawn, NJ). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from ProteoChem (Denver, CO). *N*-hydroxysuccinimide (NHS), Orange II (i.e., acid orange 7 [AO7]) and 2-nitrophenol (99%) were purchased from Acros Organics (Geel, Antwerp, Belgium). O-nitrophenol-β-D-galactopyranoside (ONPG), bicinchoninic acid (BCA) assay reagents and bovine serum albumin were purchased from Thermo Scientific (Rockford, IL). Branched polyethylenimine (PEI, M_W = 25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). Homobifunctional *N*-hydroxysuccinimide poly(ethylene glycol) esters (NHS–PEG–NHS, M_WS 1, 2, 5, and 10 kDa), poly(ethylene glycol) dialdehyde (ALD–PEG–ALD, M_W = 2 kDa), poly(ethylene glycol) diepoxide (EP–PEG–EP, M_W = 2 kDa), and poly(ethylene glycol) diacrylate (AC–PEG–AC, M_W = 2 kDa) were purchased from Creative PEGWorks (Winston-Salem, NC). "Amicon Ultra" centrifugal filter devices were purchased from Millipore Ireland (Carrigtwohill, Co. Cork, Ireland). Syringe filters were purchased from Whatman (Florham Park, NJ). Type I deionized (DI) water was produced by a NANOpure Infinity system (Barnstead/Thermolyne, Dubuque, IA). Dried lactase preparation from *Aspergillus oryzae* was donated by Enzyme Development Corporation (New York, NY) and purified as described below. All other chemicals and reagents were used as received.

Preparation and Functionalization of PE Films

A schematic of the functionalization of the PE films, as well as a depiction of the PEG tether end chemistries used herein, is depicted in Figure 1.

Fabrication of PE Films. PE Pellets were pressed into sheets about 274 μm thick with a Carver Laboratory Press Model B. The sheets were cut into rectangular films (10 × 20 mm), which were cleaned by sonication in 2-propanol (99.9%), acetone (99.8%), and deionized water (10 min per repetition, two repetitions per solvent). The clean films were dried overnight over anhydrous calcium sulfate. PE films were stored in a covered petri dish at room temperature.

Oxidation of PE Films. Following a 5 min warm-up period, clean PE films placed in an open glass petri dish were exposed to 28 mW/cm^2 ultraviolet (UV) light at 254 nm at a distance of 2 cm for 15 min using a Model 42 UVO Cleaner. After a brief cool-down period to prevent the films from adhering to the glass, the films were turned over and exposed to UV light under the same conditions for an additional 15 min. This procedure was used to oxidize both surfaces of the films, predominately creating carboxylic acid functional groups.[22] Oxidized PE films (PE–Ox) were sonicated for 5 min in DI water to remove soluble oligomers created by the oxidation process and stored in DI water at 4°C.

Development of PE–PEI Films. A polyamine surface was created via covalent attachment of PEI to the oxidized PE film surfaces using an adaptation of a well-understood method to create amide bonds.[23,24] A conjugation solution was prepared consisting of 30 mg/mL PEI, 5 × $10^{-2}M$ EDC and 5 × $10^{-3}M$ NHS in 0.1*M*, pH 9.6 sodium carbonate buffer. Immediately after UV exposure, PE–Ox films were shaken in conjugation solution for 2 h at 30°C. PEI-treated PE films (PE–PEI) were rinsed in three consecutive DI water baths and stored in 0.1*M*, pH 7.8 phosphate buffer at 4°C until further analysis or functionalization.

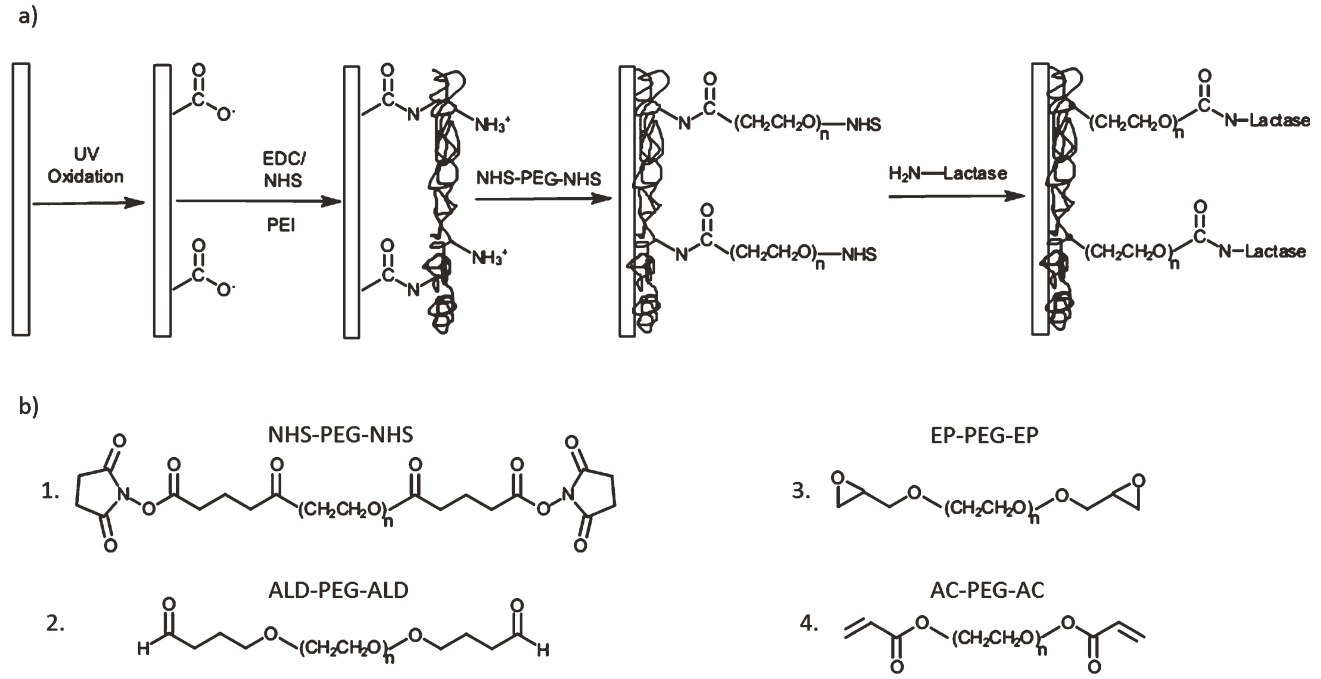

Figure 1. (a) General reaction scheme for surface modification of polyethylene films and lactase immobilization. (b) Structure of PEG derivatives used as a tethered intermediate between the PE–PEI and lactase.

Attachment of PEG tether. PE–PEI films were agitated for 15 h at 30°C in 0.1*M* pH 9.1 borate buffer containing $3 \times 10^{-4}M$ EP–PEG–EP. For all other treatments, PE–PEI films were agitated for 1 h at 30°C in 0.1*M* pH 7.8 potassium phosphate buffer containing $3 \times 10^{-4}M$ homobifunctional PEG derivative. All films were then rinsed in three consecutive 0.1*M* pH 7.8 potassium phosphate baths to condition the films for lactase immobilization.

Covalent Attachment of Lactase

Purification of Lactase. To purify the commercial lactase preparation, a crude solution was first prepared in 0.1*M* pH 5.0 acetate buffer. This solution was syringe filtered through a 0.2 μm filter, followed by centrifugal filtration in 50 kDa M_W centrifugal filter units. Because of the size specificity of the filters the lactase is retained on the filtration membranes and removed by flushing with fresh 0.1*M* pH 5.0 acetate buffer. The purified lactase solution was stored at 4°C until use. Protein concentration of the purified lactase preparation was determined using the BCA assay.

Conjugation of Lactase to films. Immediately following rinsing, PEGylated PE–PEI films (PE–PEG) measuring 10 × 20 mm (4 cm^2 total surface area) were placed in 0.1*M* pH 7.8 phosphate buffer to which purified lactase was added to achieve an enzyme concentration of 2×10^{-2} mg/mL. PE–PEG films were agitated in lactase solution for 4 h at 30°C to covalently attach lactase to the free PEG terminal moieties. Covalent attachment was desired to make the lactase unlikely to migrate from the film surface after conjugation. The nature of the covalent bonds was according to the terminal reactive group on the PEG (NHS ester, aldehyde, epoxide, or acrylate) and subsequent reaction with terminal and lysine amines present on the lactase. Lactase functionalized films were then rinsed in three consecutive 0.1*M* pH 5.0 acetate buffer baths and stored in acetate buffer at 4°C until further analysis to preserve bound lactase activity.

Analysis of PE Surfaces

FTIR Analysis. To confirm that PE surfaces were modified as expected, PE–Ox, PE–PEI, and PE–PEG films, as well as clean, untreated (i.e., virgin) PE as a control, were analyzed with attenuated total reflectance Fourier transform infrared (ATR–FTIR) spectroscopy. ATR–FTIR spectra were collected using an IR Prestige 21 spectrometer (Shimadzu Corporation, Kyoto, Japan) with a diamond ATR crystal. The software for collecting and viewing spectra was IR solution (v. 1.3, Shimadzu Corp.). Each absorbance spectrum represents 64 scans at 4.0 cm^{-1} resolution using SqrTriangle apodization, using a clean ATR crystal exposed to the ambient atmosphere as a background. KnowItAll software (v. 8.1, Biorad Laboratories, Philiadelphia, PA) aided in spectra visualization and analysis.

XPS Analysis. Further analysis of virgin PE, PE–PEI, and PE–PEG films was performed with X-ray photoelectron spectroscopy (XPS). PE films prepared for XPS were dried under vacuum at 40°C for at least 24 h. Using a Quantum 2000 Scanning ESCA Microprobe (Physical Electronics, Chanhassen, MN), PE films were irradiated with monochromatic aluminum Kα X-rays at a take-off angle of 45° for a sampling depth of 20–25 Å (2.0–2.5 nm).

Analysis of Available Surface Amines. PE–PEI films, with PE–Ox and virgin PE films as controls, were subjected to the acid orange 7 (AO7) colorimetric assay to quantify the number of primary amines on the functionalized surfaces. AO7 is an

anionic dye which complexes with primary amines on a 1:1 molar ratio.[25] Films ($n = 4$) were shaken for 3 h at room temperature in a $1 \times 10^{-3}M$ solution of A07 in DI water adjusted to pH 3 with HCl. The films were rinsed in three consecutive pH 3 DI water baths to remove non-complexed dye. The dye was desorbed in (aqueous) NaOH solution at pH 12 and the absorbance of the desorbed dye measured at 455 nm using a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT). The absorbances were compared against a standard curve of known concentrations of AO7 in pH 12 DI water to determine the density of primary amines per unit area.

Analysis of Protein Loading on Films. Lactase bound to PE surfaces was quantified using a modified version of the bicinchoninic acid (BCA) assay. In the BCA assay, the reduction of copper ions in the BCA reagent by amide bonds present in the protein initiates a secondary colorimetric reaction. Therefore, surface bound protein can be determined by measuring solution color change spectrophotometrically.[26,27] Films ($n = 4$) were each submerged in 3 mL of BCA working reagent and shaken for 1 h at 60°C. Absorbances of developed color were read at 562 nm and a standard curve of bovine serum albumin used to calculate protein mass per area film sample.

Lactase Activity Determination

Lactase activity of both free and bound enzyme was tested using *O*-nitrophenol-β-D-galactopyranoside (ONPG), a synthetic substrate, which produces a yellow color when cleaved. The test conditions were adapted from the method described in the Food Chemicals Codex to the *A. oryzae* lactase activity optimum of pH 5.0 at 50°C for the best possible resolution between samples.[28–30] For the free lactase, 10 μL of purified solution was added to 2 mL 3.7 mg/mL ONPG in 0.1*M* pH 5.0 acetate buffer equilibrated to 50°C. After shaking for 15 min at 50°C, enzyme activity was terminated with the addition of 2.5 mL 10% (w/v) sodium carbonate (aq) and the sample tubes diluted to 25 mL total volume with DI water.

For the bound lactase, 3 mL of 3.7 mg/mL ONPG in 0.1*M* pH 5.0 acetate buffer equilibrated to 50°C was added to each film sample ($n = 4$). After shaking for 1 h at 50°C, enzyme activity was terminated with the addition of 4 mL 10% (w/v) sodium carbonate (aq) and the tubes diluted to 10 mL total volume with DI water. Absorbances of developed color were measured at 420 nm. Lactase activity was calculated using an extinction coefficient (ε) determined from a standard curve of 2-nitrophenol (99%) in 1% (w/v) sodium carbonate (aq). Lactase activity is reported in acid lactase units (ALU), with 1 ALU equal to the enzyme quantity that will cleave ONPG at the rate of 1 μmol/min under assay conditions.

Statistical Analysis

Statistical analysis was conducted using Graphpad Prism software (v. 5.04, Graphpad Software, La Jolla, CA). One-way analysis of variance (ANOVA) was conducted followed by Tukey's pairwise comparison to determine statistical difference between sample sets within a 95% confidence interval ($P < 0.05$). Results are the means of two independent experiments conducted on different days.

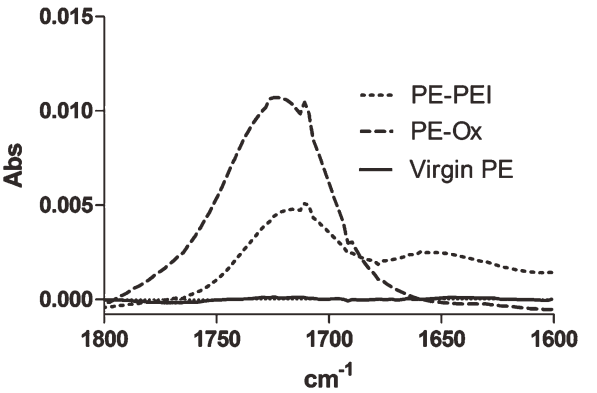

Figure 2. Representative ATR–FTIR spectra for virgin, oxidized, and PEI-treated PE films.

RESULTS AND DISCUSSION

Surface Functionalization

Surface Oxidation of PE. The modification of PE by UV-oxidation was followed using FTIR. UV-oxidation is a fast and easy means to introduce functionality to an inert PE surface, which requires only basic equipment.[31] The method is advantageous over wet oxidation methods such as chromic acid and piranha treatment due to specificity, depth of penetration, safety, and waste management. Compared to UV-oxidation, chemical oxidation methods tend to be more general, producing an array of surface moieties such as carbonyl, carboxylic acid, and hydroxyl groups. Wet chemical methods can also have deleterious effects on bulk material properties including surface etching and polymer chain scission. UV-oxidation produces primarily carboxylic acid surface moieties and is less likely to damage the treated material.[22] This specificity is desirable when employing carbodiimide chemistry to attach amine containing molecules to materials with surface-accessible carboxylic acid groups. UV oxidation, likewise, reduces safety concerns and does not result in the production of chemical waste that occurs when utilizing wet chemical treatments. The UV-oxidation of PE led to a strong FTIR absorbance at 1725 cm^{-1}, which is not present in the control spectrum (Figure 3). This absorbance indicates that the PE surface was successfully functionalized by UV-oxidation—resulting in the formation of surface carboxylic acid groups.

PEI Grafting to Oxidized PE. Grafting the polyamine, polyethylenimine (PEI), to the PE surface is advantageous to improve the functionality of the surface prior to PEGylation.[32] This grafting technique increases the number of functional groups available for conjugation and provides an amine-containing surface that can be utilized for covalent attachment of PEG using a variety of available chemistries. After immobilization of PEI to UV-treated PE in the presence of EDC, a significant increase in the amine concentration on the surface of PE was seen (Figures 2 and 3). A decrease in the carboxylic acid absorbance (1725 cm^{-1}) after PEI attachment suggests that these moieties are modified by covalent attachment to PEI amine groups. This is accompanied by a broad absorbance in the 1630–1695 cm^{-1} range, which is the characteristic absorbance of carbonyl groups of amide bonds. XPS analysis supports this conclusion, as the surface atomic percentage of nitrogen increased from 0.4% ±

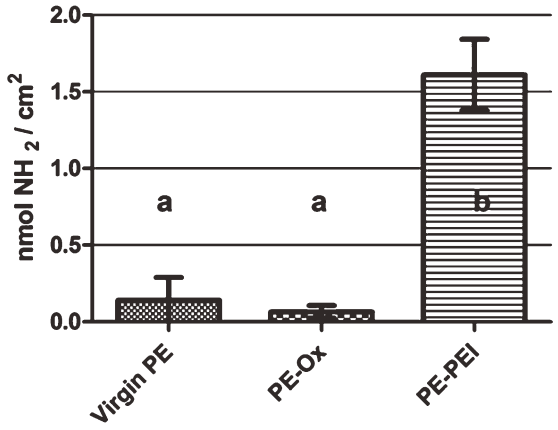

Figure 3. Primary amine concentration of films tested using the AO7 assay. Values are means of two independent experiments conducted on different days ($n = 8 \pm$ SD). Different letters indicate significant difference ($P < 0.05$).

0.5% to 6.5% ± 0.3% after PEI attachment (Table I). The observed increases in amine concentration by FTIR and surface atomic percentage of nitrogen by XPS suggest successful attachment of PEI. The number of total available functional groups on the material surface also increased after conjugation of PEI. Surface amine concentration (1.6 nmol of amines/cm^2) was less than previous experiments by Goddard and Hotchkiss, which employed chromic acid oxidation followed by an identical PEI attachment method.[9] This difference is likely due to reduced PEI attachment because of the different surface oxidation methods used. Chromic acid oxidation is relatively harsh compared to UV-light oxidation and creates a rough surface that is apparent even to the naked eye, so topography may be an important factor influencing mass of grafted polymer and resulting surface functional group density.[22]

PEG Attachment to PE–PEI. Homobifunctional PEG derivatives were utilized to create covalently attached tethers of varying length on the PEI–PE films, as well as to provide free end functionalities that could be utilized for bioconjugation. PEG terminal moieties were selected to be reactive with both the amines of the PEI layer as well as the α- and ε-amines of lactase.[33,34] For experimental uniformity across PEG variants, identical reaction conditions for all reagents were chosen to compromise between the protonation degree of PEI amines and the half-life of NHS esters in aqueous environments.[35] An exception was the EP–PEG–EP monomer, which required more specific reaction conditions than the other monomers.[33] Because ATR–FTIR spectroscopy interrogates ~ 1 μm of the surface of the film and changes in surface chemistry after PEG conjugation are on the order of nanometers, the ATR–FTIR spectra of PE–PEG films was indistinguishable from that of PE–PEI films, and more surface sensitive analytical methods were employed for PE–PEG surface characterization. Therefore, attachment of PEG molecules to PEI–PE was analyzed using XPS. For all PEG chemistries evaluated, the XPS data showed an increase in the proportion of oxygen after reaction with the PEI–PE films (Table I). The increase in oxygen suggests the presence of the polyether, and supports the successful attachment of PEG to all films. XPS performed in this manner indicates the presence of PEG but not the density of the attachment.

Effect of Tether Length on Immobilized Lactase Loading and Activity

A NHS–PEG–NHS tether series, comprising of homobifunctional PEG having the same end group functionality but varying in molecular weight (1 kDa, 2 kDa, 5 kDa, and 10 kDa), was utilized to evaluate the effect of tether length on the protein loading and activity of immobilized lactase. Increasing the tether size between 2 kDa and 5 kDa resulted in a significant increase ($P < 0.05$) in the mean protein loading from 0.14 to 0.30 μg/cm^2, respectively [Figure 4(a)]. Further increasing the tether size to 10 kDa led to a significant decrease ($P < 0.05$) in protein loading to 0.15 μg/cm^2 [Figure 4(a)]. This trend suggests that there is a minimum effective chain length for optimum protein loading, which may be attributed to chain mobility, grafting density, and coil conformation. Increasing the tether size from 1 kDa to 5 kDa may allow for increased mobility of the tether—promoting interactions between reactive groups on the enzyme and the PEG molecules. However, further increasing the tether size between 5 kDa and 10 kDa can promote changes in grafting density and/or coil conformation of the tether, which can limit the accessibility of reactive groups on the material surface and reduce protein loading.[36]

Absolute activity of lactase-immobilized PE films, as a function of tether size, was measured to determine the effects of chain length on the retained enzymatic activity [Figure 5(a)]. The mean absolute activity increased significantly ($P < 0.05$) from 1 kDa to 2 kDa (0.17×10^{-2} to 0.39×10^{-2} ALU/cm^2). Activity further increased to 0.49×10^{-2} ALU/cm^2 when applying the 5 kDa tether, but was reduced significantly ($P < 0.05$) between 5 kDa and 10 kDa to 0.16 ALU/cm^2. Activity correlated with protein loading, indicating that the overall activity of the film was predominately due to the amount of enzyme that could be attached to the surface. The mean specific lactase activity for the NHS tethers varied from 1.0×10^4 to 2.7×10^4 ALU/g of protein (data not shown). These values are not significantly different from each other, though they are significantly different ($P < 0.05$) from that of the free enzyme which had a mean specific activity of 2.0×10^5 ALU/g of protein. Loss of activity after immobilization suggests that the enzyme may be denatured at

Table I. Elemental composition data from XPS

Film sample	C1s(%)	O1s (%)	N1s(%)
Virgin PE	98.1 ± 1.6	1.5 ± 1.0	0.4 ± 0.5
PE-PEI	82.9 ± 0.9	10.6 ± 0.6	6.5 ± 10.3
PE-PEG-NHS(1k)	79.7 ± 0.9	13.1 ± 0.4	7.2 ± 0.6
PE-PEG-NHS(2k)	79.9 ± 2.1	12.7 ± 1.4	7.4 ± 0.7
PE-PEG-NHS(5k)	78.4 ± 0.7	14.4 ± 0.5	7.2 ± 0.3
PE-PEG-NHS(10k)	77.7 ± 1.3	15.7 ± 1.2	6.6 ± 0.2
PE-PEG-ALD(2k)	78.1 ± 1.1	14.4 ± 0.9	7.5 ± 0.2
PE-PEG-EP(2k)	79.4 ± 1.2	14.0 ± 0.8	6.6 ± 0.5
PE-PEG-ACRY(2k)	79.4 ± 0.7	13.1 ± 0.6	7.5 ± 0.1

Values are means of $n = 3$ films ± standard deviations.

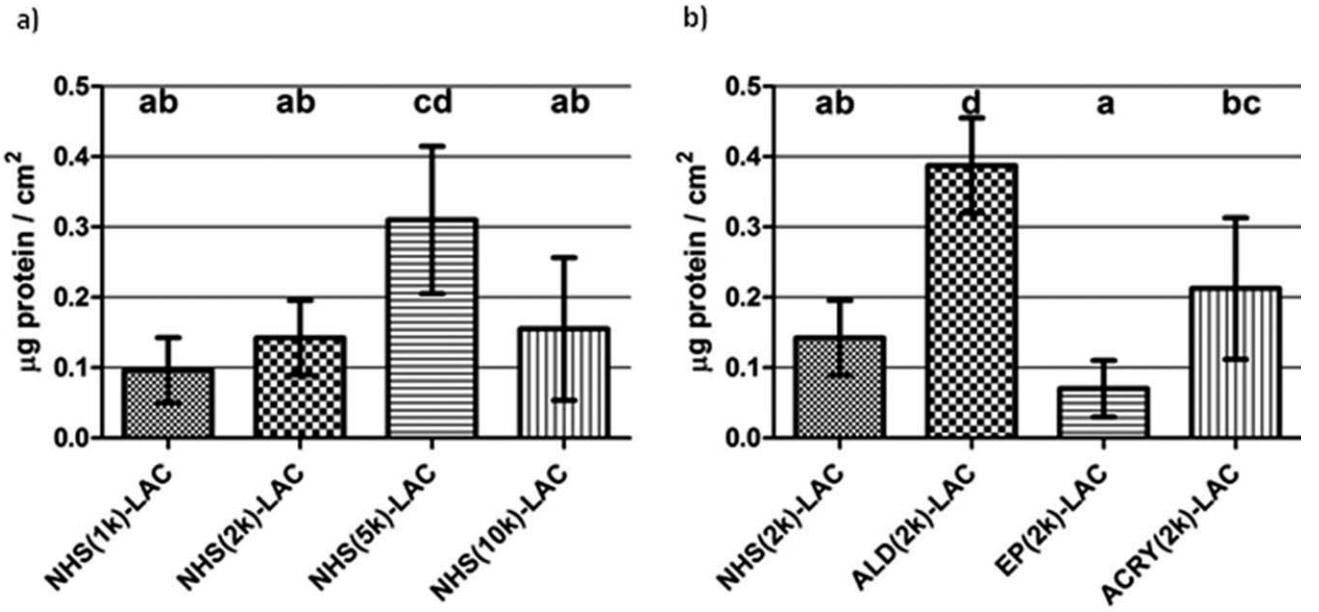

Figure 4. Protein loading of lactase-treated PE films, expressed in protein mass per square centimeter of film. Sets represented are by (a) varying PEG chain length and (b) varying terminal functional groups. Values are means of two independent experiments conducted on different days ($n = 8 \pm$ standard deviations). All absorbances were corrected by subtracting the average absorbance of the respective PE–PEI control film set. Treatments which do not share a letter are significantly different ($P < 0.05$).

the surface or the substrate is not as readily available to the enzyme.

Effect of Tether Chemistry on Immobilized Lactase Loading and Activity

Homobifunctional PEG molecules of identical size (2 kDa) but with different reactive end groups were evaluated to determine the effect of bioconjugation chemistry on the loading and activity of immobilized lactase. As seen in Figure 4(b), surfaces containing aldehyde functionality (ALD–PEG–ALD) resulted in optimal protein attachment, with a mean protein loading of 0.39 μg/cm^2. Comparatively, tethers with acrylate (AC–PEG–AC) and succinimidyl groups (NHS–PEG–NHS) groups resulted in mean protein loadings of 0.21 μg/cm^2 and 0.14 μg/cm^2, respectively. Epoxide-functionalized PEG tether (EP–PEG–EP) yielded a mean protein loading of less than 0.10 μg/cm^2. This

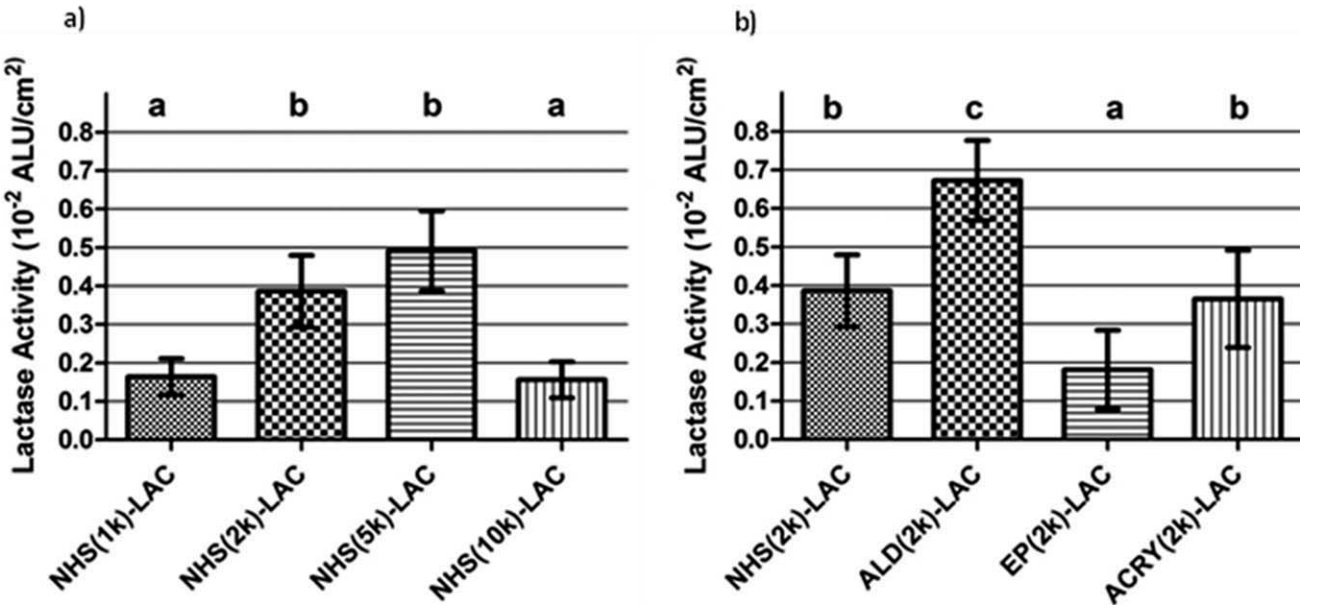

Figure 5. Absolute lactase activity per square centimeter of lactase-treated films. Sets represented are by (a) varying PEG chain length and (b) varying terminal functional groups. Values are means of two independent experiments conducted on different days ($n = 8 \pm$ standard deviations). All absorbances were corrected by subtracting the average absorbance of the respective PE–PEI control film set. Different letters indicate significant difference ($P < 0.05$).

low level of attachment can be attributed to the conjugation conditions used for the attachment of lactase to the PEG tether. Typically epoxide-amine reactions require a basic pH of at least 9.0.[33] However, this pH value exceeds the pH stability of *A. oryzae* lactase (pH 8.0), above which results in rapid denaturation of the enzyme. Compromising between stability and optimal reaction conditions promotes conjugation of an active form of the enzyme, though it occurs at the sacrifice of protein loading.

The activity of immobilized lactase was measured to determine the effect of PEG terminal moieties on enzymatic conversions [Figure 5(b)]. Aldehyde functionality (ALD–PEG–ALD) produced the highest absolute immobilized lactase activity of 0.67×10^{-2} ALU/cm^2. Succinimidyl (NHS–PEG–NHS) and acrylate (AC–PEG–AC) tethers resulted in immobilized enzyme activities of 0.39×10^{-2} ALU/cm^2 and 0.36×10^{-2} ALU/cm^2, respectively. Surfaces with epoxide-functionalized tethers (EP–PEG–EP) exhibited the lowest immobilized enzyme activity of 0.18×10^{-2} ALU/cm^2. Mean specific activity for the different terminal-functionalized PEG tethers were not significantly different from one another – varying from 1.7×10^4 to 2.7×10^4 ALU/g of protein ALU/g.

It is known that the primary disadvantage to covalently attaching an enzyme to a support material is activity loss upon immobilization.[9,37] Compared to the free enzyme, specific activity loss for all treatments was >85%. This result is comparable to previous work covalently immobilizing *K. lactis* lactase to functionalized PE using glutaraldehyde.[9] The similar activity loss across all tethers suggests that substrate accessibility in the form of diffusional or collision limitations may limit product conversion. Likewise, non-specific covalent attachment chemistries which target the same protein moieties may be responsible for the immobilized enzyme activity loss. The specific point of tether attachment to a protein as well as the number of tether molecules that attach will determine the orientation of the attached enzyme and the degree of structure deformation. These parameters can determine if the enzyme successfully retains functionality. If the undesirable activity loss is indeed endemic to amine-targeting chemistries, it may be necessary to explore alternative specific or non-specific attachment chemistries. Alternatively, bioconjugation methods may be possible which maximize protein attachment or enhance substrate accessibility—promoting greater absolute activity of the immobilized enzyme.

CONCLUSIONS

PEG-functionalized surfaces can be produced on PE packaging films by using UV-oxidation, followed by carbodiimide-mediated conjugation of polyethylenimine and attachment of activated PEG. Lactase can be subsequently attached to these PEGylated films to produce materials with lactose-reducing capabilities. A 5 kDa NHS-homobifunctionalized PEG tether enables optimal absolute activity and loading of immobilized lactase when compared to tethers of higher and lower size with identical functionalization. Compared to succinimdyl, epoxide, and acrylate functionalized PEG tethers of identical size, an aldehyde functionalized PEG tether yields films with the highest absolute immobilized enzyme activity and protein loading. The specific activity of lactase is significantly reduced after immobilization to PEG-functionalized PE films. Neither tether size nor end-group chemistry has a significant effect on the specific activity of lactase immobilized to PEG-functionalized PE films.

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