Published in final edited form as:

J Biomed Mater Res A. 2010 March 15; 92(4): 1329–1342. doi:10.1002/jbm.a.32457.

PROFILES OF CARBOHYDRATE LIGANDS ASSOCIATED WITH ADSORBED PROTEINS ON SELF-ASSEMBLED MONOLAYERS OF DEFINED CHEMISTRIES

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Abstract

Conserved protein-carbohydrate-lipid Pathogen Associated Molecular Patterns (PAMPs) interact with cells of the innate immune system to mediate antigen recognition and internalization and activation of immune cells. We examined if analogous ‘biomaterial associated molecular patterns’ composed of proteins, specifically their carbohydrate modifications, existed on biomaterials which can play a role in mediating the innate immune response to biomaterials. To probe for these carbohydrates in the adsorbed protein layer, as directed by the underlying biomaterial chemistry, self-assembled monolayers (SAMs) presenting –CH₃, –OH, –COOH or –NH₂ were pre-incubated with serum/plasma, and the presence of carbohydrate ligands of C-type Lectin Receptors (CLRs) was investigated using lectin probes in an Enzyme-linked Lectin Assays (ELLA). Presentation of CLR ligands was detected on control tissue culture polystyrene (TCPS). Absorbances of mannose or N-acetylglucosamine increased with decreasing incubating serum concentration; absorbances of sialylated epitopes or fucose remained unchanged. Absorbances of α-galactose or N-acetylgalactosamine decreased with decreasing incubating serum concentration; β-galactose was undetectable. Among SAM endgroups, pre-incubation with 10% serum resulted in differential presentation of CLR ligands; higher α-galactose on COOH SAMs than NH₂ or CH₃ SAMs, highest complex mannose on NH₂ SAMs and higher complex mannose on OH SAMs than CH₃ SAMs. Least sialylated groups were detected on CH₃ SAMs. In summary, biomaterial chemistry may regulate protein adsorption and hence unique presentation of associated carbohydrates. The ultimate goal is to identify the effects of protein glycosylations associated with biomaterials in stimulating innate immune responses.

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Keywords

self-assembled monolayers; pattern recognition receptors; glycoprotein; enzyme linked lectin assay; innate immune response

INTRODUCTION

Glycans are important in mediating various aspects of immune responses such as leukocyte trafficking¹, interaction of antigen presenting cells with lymphocytes², antigen recognition and internalization for antigen processing and presentation³ and direction of adaptive immune responses⁴. In cancer biology, altered glycosylations of tumor-associated antigens are markers for malignancy^{5, 6}. The extracellular matrix contains glycans within glycosaminoglycans and proteoglycans^{7, 8} which support tissue structure and sequester growth factors to modulate and direct cell functions⁹. The potential of glycan/glycoprotein-based materials as biomaterials for tissue engineering are being determined¹⁰. The contribution of glycans in the host response to biomaterials remains to be defined.

The innate immune response of neutrophils, monocytes/macrophages and dendritic cells (DCs) to biomaterials is initiated by their recognition of adsorbed cell-adhesive and opsonizing proteins by the cognate receptors [including pattern recognition receptors (PRRs)]. It is this innate response (or foreign body response) to the implanted biomaterial which influences, if not also directs, the “subsequent” immune response to an immunogenic biological component of a combination product primarily through maturation effects on DCs. In this way, the biomaterial may act as an adjuvant, to enhance an adaptive immune response to associated biological antigens. Such an enhancement is desirable for polymeric protein or DNA vaccine delivery systems. However, for a tissue engineering application, not only are we concerned with minimizing fibrous encapsulation of devices, minimizing scar formation and avoiding unwanted cell-mediated implant degradation, but enhancements of adaptive immune responses towards biological components, due to a biomaterial adjuvant effect, are also to be minimized¹¹⁻¹⁴. A controlled host response to implanted biomaterials is therefore desirable. Although the non-specific host inflammatory response to biomaterials has been examined extensively¹¹⁻¹⁴ with attempts to control it¹⁵⁻¹⁹ the adjuvant effects of biomaterials have only been recently described²⁰⁻²³ and mechanistic basis elucidated²⁴⁻³⁰.

In the innate response to pathogens, innate immune cells use PRRs to recognize conserved epitopes on PAMPs. Among the PRRs, two important families are Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)^{3, 31-45}. The conserved moieties recognized in PAMPs include bacterial lipopolysaccharide (LPS)⁴⁶⁻⁵², N-acetylglucosamine (GlcNAc) and N-acetylmuraminic acid (MurNAc) in peptidoglycans⁴⁶, β (1-3) glucans in zymosan^{31, 53, 54} and mannose-containing bacterial pili⁵⁵. While the significance of TLR-PAMP interactions in stimulating DC maturation has been well characterized due to nuclear factor- κ B (NF- κ B) mediated regulation of pro-inflammatory/immunoregulatory genes^{35, 36}, the role of CLR-mediated recognition, uptake, processing and presentation of carbohydrate antigens has emerged recently^{3, 31, 38-43}. The CLRs and their carbohydrate ligands in the context of biomaterials have been poorly understood thus far and form the focus of the

present work. Dendritic cells play pivotal roles in antigen recognition and processing events⁴⁶ and their expression of CLR is broad and well characterized⁵⁶. However, it is important to note that multiple immune cell types besides DCs including natural killer cells⁵⁷ macrophages⁵⁸ and neutrophils⁵⁹ express PRRs and contribute significantly to shaping innate immune responses to biomaterials through mediating lectin-biomaterial interactions. For example, macrophages express mannose receptor (MR), Endo180, DC-specific ICAM-3 grabbing non-integrin (where ICAM-3 is intercellular adhesion molecule 3) (DC-SIGN), DC-SIGN related (DC-SIGNR) and Dectin-1/ β -glucan receptor – the later also expressed by neutrophils⁴³.

The CLR family has been broadly classified as mannose or galactose binding⁴³. Examples of CLRs that bind mannose moieties are MR, DC-SIGN⁵⁰, and Dectin-1^{39, 54}. Examples of CLRs that bind α - and/or β -galactose or GalNAc moieties are asialoglycoprotein receptor (ASGPR)⁴² and macrophage galactose N-acetyl-galactosamine (GalNAc) specific lectin 1 (MGL)⁶⁰. The carbohydrate binding specificities of different CLRs vary and have been reviewed^{49, 50}. For example, MR recognized terminal mannose, fucose, sialylated groups or GlcNAc and DC-SIGN recognized mannose-enriched internal residues⁴¹. Carbohydrate recognition by certain CLRs has been linked with endocytosis and processing of cargo⁶¹⁻⁶³, activation of intracellular signaling mediators and differential downstream immune consequences via ITAM (immunoreceptor tyrosine-based activation motif)^{38-40, 54, 61, 63} or ITIM (immunoreceptor tyrosine-based inhibitory motif)⁶⁴ residues. Dectin-1 that possesses an ITAM motif and binds β -glucans, has been implicated in the activation of Syk-mediated signaling pathways^{39, 54}. Furthermore, Dectin-1 has been shown to upregulate TLR 2-mediated activation of NF- κ B and exhibit synergism in the regulation of IL-1 and tumor necrosis factor- α (TNF- α)³⁸.

It is therefore important to characterize the carbohydrate ligands present in the adsorbed protein layer on biomaterial surfaces that may be recognized by CLRs and hence mediate interactions with cells of the innate immune response. The roles of glycans in controlling fundamental immune/inflammatory responses to biomaterials have not been sufficiently studied thus far. It is hypothesized that innate immune cells such as DCs and macrophages may respond to biomaterials by recognizing 'biomaterial associated molecular patterns', analogous to PAMPs, through the adsorbed protein layer, specifically through carbohydrate modifications of these proteins using PRRs, to initiate an immune response. Furthermore, innate immune cells may recognize inherent biomaterials through carbohydrates inherent in their structure. To characterize the presence of carbohydrate ligands of CLRs in the adsorbed protein layer, as directed by the underlying surface chemistry, self-assembled monolayers (SAMs) with $-\text{CH}_3$, $-\text{OH}$, $-\text{COOH}$, or $-\text{NH}_2$ endgroups that present a broad spectrum of well-characterized surface properties such as charge, hydrophobicity/hydrophilicity and chemistry were chosen⁶⁵⁻⁷⁸. These SAMs were used as model biomaterials to observe the effect of distinct surface chemistries on directing protein adsorption and carbohydrate presentation. An enzyme linked lectin assay (ELLA) was applied to detect carbohydrates, in the adsorbed protein layer, as directed by the distinct SAM surfaces, as recognized by a panel of lectins. Lectins were selected based on their unique specificity for carbohydrate ligands recognized by CLRs. Our specific research interest is on how biomaterial-associated ligands for DCs influence their phenotype. For this

purpose, we have previously characterized DC responses to different SAM surfaces, and observed that while OH, COOH or NH₂ SAMs triggered modest DC maturation, least maturation was observed on CH₃ SAMs. This was likely due to highest immunosuppressive DC apoptosis upon CH₃ SAM contact⁷⁹. The importance of understanding differential DC responses on different chemistries from the perspective of distinct DC ligands regulated by biomaterial chemistry formed the motivation for this research. This research is also more broadly significant in that it highlights the importance of and begins to define the presence of glycan/glycoproteins in the adsorbed biomolecule layer on biomaterials which may play a role in the recognition of these biomaterials as foreign by innate immune cells to mediate a foreign body response.

METHODS

Self-assembled monolayer preparation

Alkanethiol self-assembled monolayers presenting –CH₃, –OH, –COOH or –NH₂ chemistries were assembled on 16-well glass chamber slides (*LAB-TEK*, Nalge Nunc International, Rochester, NY) for carbohydrate or protein measurement assays or on 9 mm × 9 mm glass coverslips for material characterization assays (Bellco Glass, Inc., Vineland, NJ)⁷¹ as described previously⁷⁹. The SAMs were allowed to assemble by 12 hr incubation at room temperature (RT) by immersing the Ti/Au-coated slides or coverslips in alkanethiol solutions (1 mM in absolute ethanol), following which the chamber slides or coverslips were washed with 95% ethanol (Sigma, St. Louis, MO), dried with N₂ gas (Airgas South, Chamblee, GA) for 10 minutes in a fume hood, equilibrated with Dulbecco's Phosphate Buffered Saline (PBS) (Invitrogen, Carlsband CA) for 5 minutes at RT and used fresh.

X-ray photoelectron spectroscopy

Characterization of SAMs was performed by low resolution survey scans (spot size 400 μm) or high resolution C1s spectra (spot size 200 μm) X-ray photoelectron spectroscopy (XPS) using a Surface Science model SSX-100 (Surface Sciences Laboratories, Mountain View, CA), with monochromatized Al_{Kα} X-rays at 10 kV at the Microelectronic Research Center at Georgia Institute of Technology. Atomic percentages of elements were obtained from low resolution scans and high resolution scan curves were fit based on software provided by the manufacturer, with placement of the hydrocarbon peak at 284.6 eV. Different takeoff-angles of 0°, 20°, 55° or 70° were used (angle between the beam and the normal to the surface) to permit measurements at varying depths from the film surface. The detection limit for angle resolved XPS is ~0.1 atom% in composition and 10-250 Å in depth²⁹, with maximum and minimum depths attained at takeoff angles of 0° and 90°, respectively. Scans were taken on two spots per sample per take-off angle. Prior to XPS, freshly-prepared SAM samples were washed with 95% ethanol and dried with N₂ and analyzed immediately. Theoretical values were determined based on known chemical structure.

Contact angle measurements

Advancing contact angles between freshly-prepared flat SAM and 5 μl drops of de-ionized (DI) H₂O were determined in ambient air using a Rame-Hart model # 100-00 goniometer (Mountain Lakes, NJ). For each surface, contact angles were measured for three water

droplets, on two sides of each droplet and the values presented denote the average and standard deviations for three measurements in total.

Preparation of heat inactivated human serum or human plasma

Peripheral human blood was obtained from healthy volunteers with informed consent, according to a protocol approved by the Georgia Institute of Technology's Institute Review Board (IRB) # H05012. Peripheral human blood was collected using sterile 60 mL syringes (Becton Dickinson, Franklin Lakes, NJ) and needles (Becton Dickinson) using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. The clear yellowish human plasma (HP) layer was collected using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) by differential gradient centrifugation of blood diluted 1:1 with sterile PBS (400 g, 30 minutes, RT) (Thermo Fisher Scientific Inc., Waltham, MA) (Model # 5682, Rotor IEC 216). The HP was filtered sterilized (0.22 μ m) (Corning, Corning, NY), heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted and stored at -20°C. A stock solution of pooled HP from three donors was used for all experiments.

For preparation of human serum (HS), peripheral human blood was collected without heparin. The HS was isolated from non-heparinized blood by centrifugation of blood (3000 rpm, 10 minutes, RT), harvesting the supernatant, pushing down any clots manually using a sterile pipette tip and allowing further clotting (90 minutes, RT) in the tissue culture (TC) hood. Human serum was then cleared by centrifugation (3000 rpm, 15 minutes, RT) after gently pushing down residual precipitates, filter sterilized (0.22 μ m) (Corning), heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted, and stored at -20°C. A stock solution of pooled HS from the same three donors as used for HP was used for all experiments.

Enzyme linked lectin assay for detection of carbohydrates associated with adsorbed proteins on SAMs or control tissue culture polystyrene (TCPS)

Enzyme Linked Lectin Assays (ELLAs) were performed on SAMs having pre-adsorbed HS or HP proteins using a previously described method with some modifications and optimizations for each lectin⁸⁰. The SAMs were incubated with 60 μ L/ well of serial half dilutions of HS or HP in PBS starting from 20% down to 2.5% and also dilutions from 1% down to 0.25% (v/ v) or with PBS alone (1 hr, 37°C) in duplicate wells in two chamber slides leading to a total of 4 wells per HS or HP concentration. After aspirating out HS, HP or PBS samples from wells, wells were blocked with 0.5 mg/ mL of freshly prepared bovine serum albumin (BSA) (Sigma) in PBS (block buffer; 1 hr, 37°C), washed three times for 5 minutes each with block buffer at RT and incubated with biotinylated lectin [*Narcissus pseudonarcissus* (NPA; 12.5 μ g/ mL), *Sambucus nigra* (SNA-1; 12.5 μ g/ mL), *Ulex europeus I* (UEA-1; 50 μ g/ mL), *Ulex europaeus II* (UEA-2; 12.5 μ g/ mL), *Pisum sativum* (PEA; 25 μ g/ mL), *Hippeastrum hybrid* (HHA; 50 μ g/ mL), *Peanut agglutinin* (PNA; 12.5 μ g/ mL), *Artocarpus integrifolia* (AIA; 25 μ g/ mL) or *Bauhinia purpurea* (BPA; 25 μ g/ mL)] with the carbohydrate detection specificities indicated in Table 1 (all from EY Laboratories, Inc., San Mateo, CA)] in block buffer (2 hrs, 37°C). Lectins were selected based on their unique specificity for carbohydrate ligands recognized by CLRs. For UEA-2

lectin, 1.0 mg/ mL heat inactivated BSA (56°C, 30 minutes) in PBS was used in a sensitive blocking step and for PEA or BPA lectins, 1.0 mg/ mL of BSA in PBS was used. Following incubation with the biotinylated lectin, wells were washed and incubated with 10 µg/ mL avidin/alkaline phosphatase (AV/AP) (EY laboratories Inc.) in block buffer (1 hr, 37°C) for detection of bound lectin. The concentration of AV/AP used was 1.25 µg/ mL for SNA-1, PEA or HHA lectins or 20 µg/ mL for AIA or BPA lectins. Finally, wells were washed and incubated with 1.0 mg/ mL p-nitrophenylphosphate (pNPP) (Sigma) substrate for detecting AV/AP (1 hr, 37°C). The solutions were transferred to wells of a clear flat-bottom TCPS treated 96-well plate (Corning) to avoid interference with the reading by SAMs, reaction stopped at RT with 40 µl of 0.4 M NaOH (Sigma) and absorbance read immediately at 405 nm using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Data has been presented after subtracting out the absorbance due to blank. Positive control glycoprotein standard dilutions in PBS (1:1) were run in parallel on TCPS 96-well plates (control) for each lectin; α-2-macroglobulin (from 100 – 12.5 µg/ mL) or (1000 – 3 µg/ mL) for NPA ⁸¹ or HHA ⁸² respectively, glycophorin A (100 – 3 µg/ mL) for SNA-1 ⁸³, lactoferrin (1000 – 62.5 µg/ mL) or (1000 – 15 µg/ mL) for UEA-1 ⁸⁴ or PEA respectively, bovine fetuin (1000 – 3 µg/ mL) for UEA-2, asialofetuin (5 – 0.6 µg/ mL) or (31.25 – 1.9 µg/ mL) for PNA ^{85, 86} or BPA ⁸⁷ respectively and immunoglobulin A (IgA) (50 – 1.5 µg/ mL) for AIA lectin ⁸⁸ (all from Sigma), and based on lectin probe specificity data from ⁸⁹ and in triplicate wells. An identical set of samples (HS or HP) as those run on SAMs were also run on TCPS for each experiment, again in triplicate wells.

Enzyme-linked immunosorbent assay (ELISA) for detection of total human immunoglobulin or human serum albumin (HSA)

An enzyme-linked immunosorbent assay (ELISA) was performed to measure amounts of total human IgG ⁶¹ or total human serum albumin (HSA) adsorbed from 1% or 10% HS (v/ v) onto SAMs. Self-assembled monolayers were incubated with 60 µl of 1% or 10% (v/ v) HS in PBS (1 hr, 37°C), followed by blocking with 1.0 mg/ mL of BSA in PBS for HSA ELISA or with 0.5 mg/ mL of BSA in PBS for IgG ELISA (both for 1 hr, 37°C) and incubating with 1:10000 dilution of monoclonal mouse anti-HSA (clone HSA-11; IgG2a) (no cross reaction with BSA) or 1:1000 affinity isolated AP-conjugated polyclonal goat anti-human IgG ⁶¹ (both from Sigma) (2 hrs, 37°C), for detection of HSA or IgG respectively. Wells were washed three times for 5 minutes each with PBS at RT. For the IgG ELISA, wells were incubated with 1.0 mg/ mL pNPP (30 minutes, RT) after incubating with AP-conjugated primary antibody. For detection of HSA, wells were incubated with 1:1000 dilution of affinity isolated AP-conjugated goat anti-mouse IgG (Sigma) (2 hrs, 37°C), washed and incubated with pNPP substrate (1 hr, RT). The reactions were stopped with 40 µl of 0.4 M NaOH (Sigma) at RT and the solutions transferred to 96-well clear-bottom plates to avoid interference with SAMs and absorbance read at 405 nm using a SpectraMax Plus 384 plate reader (Molecular Devices). Standard curves were generated with 1:10 dilutions of purified IgG or HSA (both from Sigma) in PBS (starting from 100 µg/ mL of IgG or 1 mg/ mL for HSA) on 96-well TCPS control surfaces in triplicate wells. In the same experiment, SAMs were pre-coated with 1% or 10% HS samples and ELISAs were performed to measure amounts of total adsorbed human IgG or HSA (n=2 each) associated with the different SAMs and ELLAs (n=2 each) were also performed using methods

described previously to detect carbohydrates present. In this way, to confirm that the varying profiles of carbohydrates observed on different SAMs were in fact different, the ELLA absorbances were normalized to amounts of total human IgG or HSA for the different SAMs, for which both ELLA and ELISA determinations were performed within each experiment and the ratios were compared.

Statistical analysis

Statistical analysis was performed using general linear model analysis of variance with Minitab software (Version 13.20, Minitab Inc., State College, PA) using pairwise comparisons between SAMs, with a Tukey post-test and a p value of less than or equal to 0.05 was considered significant.

RESULTS

Contact angle and XPS characterizations of SAMs showed close agreement with established theoretical values

The SAMs presenting endgroup chemistries $-\text{CH}_3$, $-\text{OH}$, $-\text{COOH}$, or $-\text{NH}_2$ represented a broad spectrum of surface properties including charge, hydrophobicity/hydrophilicity and chemistry. The CH_3 SAMs were hydrophobic and the OH SAMs were neutral, hydrophilic. Under physiological pH conditions (7.2-7.4), the COOH or NH_2 SAMs were negatively or positively charged, respectively^{66, 71, 90, 91}. The SAM surfaces were characterized by XPS analysis (Table 2) and contact angle determination (Table 3). The low resolution XPS survey scans showed agreement with estimated theoretical values for these surfaces and previously published values⁷⁶ and indicated modification of Au/Ti surfaces with the alkanethiols with appropriate changes in C1s and O1s percentages as expected. Carbon was the predominant element present for all samples. On control samples coated with Au/Ti alone, no carbon was detected. The percentages of different elements measured were similar to expected values based on theoretical estimates for all samples using take-off angles of 0° , 20° , 55° and 70° for surface analysis at different depths. Overall the SAMs were composed of alkanethiols that presented functional endgroups closest to the exterior and furthest from the Au/Ti substrate to which the alkanethiol chains were anchored (Table 2).

Advancing contact angles were measured (on both sides of the drop) between 5- μl drops of DI water and glass coverslips coated with Au/Ti, on which the different SAMs were assembled, to measure the degree of hydrophobicity/hydrophilicity of the surface. The contact angle measurements indicated that 11-mercapto-1-undecanol with a terminal hydroxyl group was the most hydrophilic surface and that 1-dodecanethiol, with a terminal methyl group, was the most hydrophobic (Table 3)^{66, 71, 90, 91}.

Differential ELLA absorbances on different SAMs or control TCPS

The ELLA assays were performed using lectin probes listed in Table 1 to characterize the accessible carbohydrates ligands of CLRs associated with the adsorbed HS or HP proteins on different SAMs. The carbohydrates are described as accessible since they are detectable by lectin binding as far as being available through an appropriate glycoprotein confirmation with lectin recognition of the carbohydrate. On 96-well TCPS surfaces treated as control,

standard curves were generated for each lectin-positive control glycoprotein (Figure 1a-h) to confirm assay reproducibility and for each lectin, to demonstrate the relationship between absorbance and carbohydrate amount on the surface. Each glycoprotein was used as a representative typical source of the carbohydrate, but is not the only source of the carbohydrate in HS or HP. In general, absorbances corresponding to the presence of specific carbohydrates decreased when TCPS surfaces were pre-incubated with decreasing HS dilutions in PBS (1:1 dilutions between 20% and 2.5% and also serial half dilutions from 1% down to 0.25%) when probed with BPA (GalNAc) (Figure 2b) or AIA (β -galactose) (Figure 2c) (both in Figure 2a), but no change for PNA (β -galactose) which was undetectable (data not shown), galactose-family lectins. In contrast, pre-incubation of TCPS surfaces with decreasing HS concentrations resulted in increasing absorbances when probed with NPA (mannose) (Figure 3e) or UEA-2 (GlcNAc) (Figure 3d) mannose-family lectins or unchanged absorbances with different HS dilutions for UEA-1 (fucose) (Figure 3c) or SNA-1 (sialylated groups) (Figure 3f) (all in Figure 3a). For PEA (complex mannose) (Figure 3b) high absorbances were detected when TCPS surfaces were pre-incubated with concentrations of 2.5% or 1% HS (intermediate among the range of HS concentrations tested).

Among SAMs, pre-incubation with 1% HS resulted in absorbances that indicated the higher presence of α -galactose on COOH SAMs and NH_2 SAMs compared to CH_3 SAMs (Figure 4a). On the other hand, pre-incubation with 10% HS, resulted in absorbances that implied higher levels of α -galactose on COOH SAMs than on NH_2 or CH_3 SAMs (Figure 4b). For all SAMs, β -galactose was undetectable using PNA (data not shown). For SAMs that were pre-incubated with 1% or 10% HS in PBS, highest presence of complex mannose was detected on NH_2 SAMs, and OH SAMs had higher absorbances compared to CH_3 SAMs (Figure 5). Lowest absorbances corresponding to the presence of sialylated groups were detected on CH_3 SAMs (Figure 5).

In another set of experiments, SAMs were incubated with 1% or 10% HP and similar results were obtained as observed with incubating solutions of 1% or 10% HS, however trends were less defined with HP with both mannose or galactose family lectin probes (data not shown). No differences in ELLA absorbances for sialylated groups were observed following either 1 hr or 24 hrs incubations of 1% or 10% HS on different SAMs (data not shown). Similar results were obtained when 1% or 10% HP or HS was incubated on different SAMs (data not shown), hence there was no effect of incubating HP or HS concentration on ELLA absorbance.

Normalization of ELLA absorbances to protein amounts

The absorbances measured using the ELLA assays were normalized to adsorbed protein amounts (human IgG or HSA) to investigate if the differences in accessible glycosylation profiles across SAM chemistries persisted after accounting for variations in amounts of adsorbed proteins (data not shown). The CH_3 SAMs had significantly higher levels of adsorbed IgG compared to COOH SAMs when pre-incubated with 10% HS. Normalized ratios of ELLA absorbance over mean IgG amount indicated similar trends as those observed from non-normalized ELLA absorbances for both mannose and galactose family

lectins. Furthermore, since HSA was not detected on all SAMs, normalizations of ELLA absorbances over HSA amounts could not be performed.

DISCUSSION

Differential carbohydrate absorbance profiles corresponding to accessible carbohydrates were observed on SAMs presenting different endgroups, suggesting that different biomaterial chemistries may drive the formation of distinct 'biomaterial associated molecular patterns' composed of proteins and carbohydrates. These carbohydrate ligands, present in the adsorbed protein layer on biomaterial surfaces, may be recognized by CLRs and hence mediate interactions with cells of the innate immune response. These results are in agreement with a previous study that demonstrated that different levels of DC maturation were obtained following treatment with the different SAM chemistries used here ⁷⁹, suggesting that there exists an intricate and highly complex connection between protein-associated molecular patterns on biomaterials and the ensuing DC recognition and activation events.

In this study, incubating concentrations of 1% or 10% pooled heat-inactivated filtered HS or HP were chosen since media in which DCs are cultured *in vitro* analogously contain 10% heat-inactivated filtered fetal bovine serum (FBS) or 1% autologous heat-inactivated filtered HP ⁷⁹. When pre-incubated with 10% HS, COOH or NH₂ SAMs were associated with higher absorbances corresponding to the presence of α -galactose than CH₃ SAMs and undetectable levels of GalNAc (Figure 4b). Pre-incubation with 1% HS resulted in higher α -galactose on COOH SAMs than on CH₃ or NH₂ SAMs and still undetectable levels of GalNAc (Figure 4a). With incubating solutions of 1% or 10% HS, the NH₂ SAMs displayed the highest presence of complex mannose as well as higher complex mannose on OH versus CH₃ SAMs (Figure 5). Finally, the CH₃ SAMs were associated with least presence of sialylated groups (Figure 5). These results have been summarized in Table 4 and imply that the profile of accessible carbohydrates varied with different types and concentrations of incubating solutions, probably as a result of differential protein adsorption from these solutions. An important caveat is that the absence of lectin binding only suggests that the specific carbohydrate ligand of the lectin probe may not be accessible to the probing lectin, but does not necessarily indicate that the glycan in question is itself not present on the biomaterial surface. However, if the glycan is not accessible to the lectin, it may also not be accessible to the cell receptor.

In an attempt to quantify the differences in absorbances, and to confirm that the differential profiles of accessible carbohydrates were in fact different and not solely due to differences in amounts of adsorbed HS or HP proteins, ELLA absorbances were normalized to mean adsorbed IgG or HSA amounts. Consistent trends among profiles of accessible carbohydrates among SAMs were observed for non-normalized and normalized ELLA measurements. It is important to note that since several proteins in HS or HP are glycosylated, in certain cases with multiple glycosylations and linkages, normalization against single proteins such as IgG or HSA (negligible glycosylations) performed in this study, alone, may not be appropriate. A more optimal normalization method for future studies would be to quantify biomaterial adsorption of a panel of relevant glycosylated

proteins. It is worth noting that adsorbed HSA may not have been detected on the SAMs due to the conformational sensitivity of the monoclonal antibody against HSA, since the protein may have adsorbed in different conformations on the different SAMs. Another possibility is that the absence of detected HSA may be due to a lack of antibody recognition for the heat-denatured albumin, since heat-denatured serum/plasma was used.

In this study, SAMs having -CH₃, OH, -COOH or -NH₂ chemistries were used as model biomaterials to examine the profiles of accessible carbohydrates associated with adsorbed HS or HP proteins. The XPS (Table 2) and contact angle (Table 3) characterizations of the SAM endgroups in this study revealed close agreement with previous studies^{66, 72, 90, 91} and showed a broad spectrum of surface properties. Differences in amounts and conformations of adsorbed proteins have been observed on SAMs with endgroups. Variations in antibody binding affinities against the cell binding domain of fibronectin were observed on different SAM endgroups^{70, 71, 92}. Furthermore, significantly higher amounts of pro-inflammatory fibrinogen were adsorbed to OH SAMs than to CH₃ SAMs⁹³, although others have found otherwise^{75, 76, 77, 94}. It was observed that higher amounts of pro-inflammatory complement-3 (C3)⁹⁵ was measured on OH SAMs than on CH₃ SAMs, although highest amounts of human IgG were detected on CH₃ SAMs compared to OH, COOH or NH₂ SAMs⁹⁶. In contrast, adsorption of anti-inflammatory protein albumin was higher on CH₃ SAMs compared to either OH or COOH SAMs⁹⁷.

Similar to the pro- or anti-inflammatory roles ascribed to differed HS or HP proteins such as C3 or albumin respectively, we speculate that carbohydrates associated with these proteins may also have pro- or anti-inflammatory effects. On 96-well TCPS surfaces that served as experimental controls, several carbohydrates probed for were associated with differential absorbance patterns following pre-incubations with different concentrations of HS or HP in PBS (1: 1 dilutions between 20% and 2.5% and 1% and 0.25%) (Figures 2 and 3). In general, trends indicated increasing absorbances for certain mannose group carbohydrates (GlcNAc or mannose) on TCPS (Figure 3) with decreasing HS concentrations, suggesting that higher presentation of these carbohydrates occurred with decreasing levels of other 'masking' proteins. A notable contrast was observed for galactose group carbohydrates (α -galactose or GalNAc) on TCPS (Figure 2), where increasing absorbances corresponded to increased presence of these carbohydrates with increasing HS concentrations. These results offer indirect support to literature that has implied opposing roles for mannose or galactose family proteins in stimulating immune responses. In tumor glycans that have high levels of exposed galactose moieties following de-sialylation of O-glycans, the galactose molecules were recognized by MGL, leading to the induction of tumor tolerance in the absence of TLR signaling⁶⁰. While galactose binding to CLRs has been linked to anti-inflammatory effects, ligand binding to MR may result in pro-inflammatory effects, since blocking of MR inhibited the fusion of macrophages to form foreign body giant cells (FBGCs)⁹⁸, although others have reported observing both pro- and anti-inflammatory effects depending on the nature of the MR ligand⁹⁹. Taken together, the emerging model of PRRs and their ligands indicates that the specific carbohydrate ligand bound is important, since the same or different carbohydrate ligands may target a different complex network of DC CLRs and other PRRs and hence trigger differential DC responses. It may therefore be of importance

to further characterize the pro/anti-inflammatory roles of the carbohydrate ligands presented by biomaterials, given these findings.

A clear link also exists in the literature between altered glycosylations and disease progression¹⁰⁰⁻¹⁰². While normal host proteins have relatively low terminal mannose, fucose or GlcNAc^{43, 103}, disease states are associated with alterations in protein glycosylations. Tumors have been associated with increased sialylation, mannosylation, fucosylation¹⁰⁴ and reduction in O-glycan lengths^{105, 106}. Liver disease is associated with lowered sialylation and increased glycan branching^{104, 105} and rheumatoid arthritis by increased fucosylation and presence of sialyl Lewis X on alpha-1-acid glycoprotein^{107, 108}. Finally, it was observed that immature DCs expressing DC-SIGN (but not mature DCs) interacted with carcinoembryonic antigen having higher Lewis x or Lewis y Ag levels on colon epithelial tumor cells than on normal cells¹⁰⁹⁻¹¹¹. Another line of speculation therefore, is that if the proteins adsorbed to the biomaterial have undergone conformational changes as reported previously⁷¹ and hence present an altered profile of exposed carbohydrates, the biomaterial may appear analogous to diseased states to circulating immune cells, including migrating DCs.

These results suggest that biomaterials presenting different surface chemistries differentially modulate carbohydrate presentation in the adsorbed protein layers, possibly due to differences in types or amounts (or both) of adsorbed proteins. Glycosylations in PAMPs or altered glycosylations in diseases initiate DC recognition, and in the same way, glycosylations presented by biomaterials may impact DC maturation in a CLR-mediated manner. Future studies are aimed at determining the contribution of glycans/glycoproteins to DC recognition and responses to biomaterials and adsorbed protein sources of glycosylations. This study is significant as it provides another class of biomolecules which may mediate innate immune cell interactions with a biomaterial and can influence the host response to the material.

Acknowledgments

This research was funded by the National Science Foundation under a CAREER grant (BES-0239152) and a National Institutes of Health (NIH) grant (1RO1 EB004633-01A1). The authors thank Jeffrey Capadona of Georgia Tech for supplying the Au/Ti-coated substrates for SAM assembly. The authors also thank Dr. Brani Vidakovic from Georgia Tech for assistance with statistical analysis and Dr. John L. Brash from McMaster University, Hamilton, Canada for several helpful discussions.

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Figure 1.

ELLA standard curves for lectin probes using positive control adsorbed glycoproteins on control TCPS surfaces.

Enzyme linked lectin assays were performed using pairs of lectin probe: serial half-dilutions of glycoprotein in PBS on TCPS surfaces; PNA: asialofetuin (a), BPA: asialofetuin (b), AIA: IgA (c), PEA: bovine fetuin (d), UEA-1: lactoferrin (e) UEA-2: bovine fetuin (f), α -2-macroglobulin: NPA (g), glycophorin A: SNA-1 (h), to confirm assay reproducibility and sensitivity. Standard curves were resolved using linear curve fitting routines. mean $\pm$ SD, representative results of n = 6 independent determinations.

Figure 2.

Trends of α -galactose or GalNAc presence in the adsorbed protein layer on TCPS surfaces with decreasing concentration of pre-incubating HS in PBS.

Enzyme linked lectin assays were performed using plant lectins probes for carbohydrate ligands of ASGPR or MGL, as shown for all lectins together in (a) or separately for each lectin namely N-acetyl-galactosamine (GalNAc) (BPA) (b) or α -galactose (AIA) (c) associated with proteins adsorbed on TCPS pre-incubated with varying concentrations of HS in PBS. Absorbances corresponding to presence of GalNAc (BPA) or α -galactose (AIA) decreased with decreasing concentration of pre-incubating HS. β -galactose (PNA) was undetectable and data is not shown. mean $\pm$ SD, n = 3 independent determinations, '*': significantly different from HS concentration, p < 0.05.

Figure 3.

Trends of mannose or sialylated group presence in the adsorbed protein layer on TCPS surfaces with decreasing concentration of pre-incubating HS in PBS.

Enzyme linked lectin assays were performed using plant lectins probes for carbohydrate ligands of MR, DC-SIGN or Dectin-1, as shown for all lectins together in (a) or separately for each lectin namely complex mannose (PEA) (b), α -fucose (UEA-1) (c), GlcNAc (UEA-2) (d), mannose (NPA) (e) or sialylated groups (SNA-1) (f) associated with proteins adsorbed on TCPS pre-incubated with varying concentrations of HS in PBS. Absorbances corresponding to presence of mannose (NPA), or GlcNAc (UEA-2) increased with decreasing concentration of pre-incubating HS. Absorbances corresponding to the presence of sialylated groups (SNA-1) or α -fucose (UEA-1) remained unchanged across HS dilutions. Absorbance corresponding to presence of complex mannose (PEA) was highest at intermediate concentrations of HS tested. mean $\pm$ SD, n = 3 independent determinations, ‘*’: significantly different from HS concentration, p = 0.05.

Figure 4.

Differential presence of α-galactose on different SAMs pre-incubated with 1% or 10% HS. Enzyme linked lectin assay was performed using plant lectins probes for carbohydrate ligands of ASGPR or MGL namely β-galactose (PNA), n-acetyl-galactosamine (GalNAc) (BPA) or α-galactose (AIA) associated with proteins adsorbed on SAMs pre-incubated with 1% (a) or 10% (b) HS in PBS. Among SAMs, pre-incubated with 1% HS, absorbance corresponding to α-galactose showed trends: COOH or NH₂ > CH₃ SAMs (a) or with 10% HS in PBS, absorbance corresponding to α-galactose showed trends COOH > NH₂ or CH₃ SAMs (b), mean±SD, n = 9 independent determinations; ‘*’: significantly different from indicated SAM endgroup, p < 0.05.

carbohydrate ligand of mannose-family DC CLRs (lectin probe)

Figure 5.

Differential presence of sialylated groups or complex mannose on different SAMs pre-incubated with 10% HS.

Enzyme linked lectin assay was performed using plant lectins probes for carbohydrate ligands of MR, DC-SIGN or Dectin-1, namely complex mannose (PEA), α -fucose (UEA-1), GlcNAc (UEA-2), mannose (NPA) or sialylated groups (SNA-1) associated with proteins adsorbed on SAMs pre-incubated with 10% HS in PBS. The CH₃ SAMs were associated with absorbances corresponding to least presence of sialylated groups and the NH₂ SAMs were associated with absorbances corresponding to highest presence of complex mannose. mean $\pm$ SD, n = 9 independent determinations; *: significantly different from indicated SAM endgroup, p < 0.05.

Table 1

Dentritic cell C-type lectin receptors, their corresponding carbohydrate ligands, and carbohydrate specificities for plants lectins used as probes

C-Type Lectin	Carbohydrate Specificity	Lectin Probe Specificity	Lectin Probe
ASGPR (78)	Galactose	α -galactose	AIA
MGL (27, 28; 32; 47)	Gal/N-acetyl-D-galactosamine (GalNAc) (46; 78; 110)	β -galactose	PNA
		GalNAc	BPA
MR/MMR (27, 28, 32; 47)	Mannose (27, 28; 32; 47)	Mannose	NPA
	Fucose (27)	α -L-fucose	UEA-1
	Sialyl Lewis X [sLe(x)], N-acetyl-D-glucosamine (GlcNAc) (33)	NANA (Neu5Ac α (2,6) Gal/GalNAc)	SNA-I
		GlcNAc β (1,4) GlcNAc	UEA-II
DC-SIGN (27, 28; 32; 47)	Mannose	methyl-D-mannopyranoside, D-mannose	PEA
	Complex mannose residues	mannose α (1,3) and α (1,6) mannose	HHA
	sLe(x) (27, 28; 32; 47)	NANA (Neu5Ac α (2,6)Gal/GalNAc)	SNA-I
Dectin-1 (27, 28; 32; 47)	β -glucans (27, 28; 32; 47)	GlcNAc β (1,4) GlcNAc.	UEA-II

C-type Lectin Receptor Abbreviations: ASGPR, asialoglycoprotein receptor; MGL, macrophage galactose N-acetyl-galactosamine specific lectin I; MR, mannose receptor; DC-SIGN, DC-specific ICAM-3-grabbing non-integrin (where ICAM-3 is intercellular adhesion molecule 3).

Plant Lectin Abbreviations: AIA, Artocarpus integrifolia; PNA, Arachishypogaea; BPA, Bauhinia pupurea; NPA, Narcissus pseudouarcissus; UEA-1, Ulex europaeus; SNA-1, Sambucus nigra; UEA-2, Ulex europaeus-2; PEA, Pisum sativum; HHA, Hippastrum hybrid.

Plant Lectin specificity information from 47, 78.

Table 2

Elemental composition of SAM endgroups presenting different chemistries using variable-angle XPS where angle is calculated from the surface plane; mean $\pm$ (range/2), n=2 spots per sample per take-off angle

SAM	Angle (°)	C1s (%)	O1s (%)	N1s (%)	S2p (%)
CH3	0	89.5 $\pm$ 3.8			12.8 $\pm$ 7.2
	20	86.9 $\pm$ 7.0			13.0 $\pm$ 7.0
	55	94.6 $\pm$ 2.3			5.3 $\pm$ 2.3
	70	95.1 $\pm$ 0.6			4.8 $\pm$ 0.6
	<i>Theoretical</i>	92.3			7.7
OH	0	83.6 $\pm$ 3.0	11.4 $\pm$ 3.1		4.8 $\pm$ 0.1
	20	84.2 $\pm$ 5.9	11.8 $\pm$ 5.0		3.9 $\pm$ 0.9
	55	85.4 $\pm$ 0.3	8.0 $\pm$ 1.6		6.4 $\pm$ 1.3
	70	83.7 $\pm$ 7.3	12.2 $\pm$ 2.4		3.9 $\pm$ 4.8
	<i>Theoretical</i>	84.6	7.7		4.1
COOH	0	82.5 $\pm$ 6.4	15.4 $\pm$ 4.7		2.2 $\pm$ 1.8
	20	83.6 $\pm$ 0.7	9.6 $\pm$ 4.3		6.7 $\pm$ 6.5
	55	80.1 $\pm$ 1.0	14.6 $\pm$ 1.2		5.2 $\pm$ 2.2
	70	81.6 $\pm$ 0.0	13.6 $\pm$ 1.8		4.6 $\pm$ 1.8
	<i>Theoretical</i>	79	14		7
NH2	0	77.3 $\pm$ 3.8		8.6 $\pm$ 1.5	14.0 $\pm$ 2.0
	20	87.2 $\pm$ 1.3		8.5 $\pm$ 1.1	4.1 $\pm$ 2.5
	55	83.3 $\pm$ 7.2		10.9 $\pm$ 3.7	5.6 $\pm$ 3.6
	70	80.3 $\pm$ 1.4		10.7 $\pm$ 6.1	8.9 $\pm$ 7.5
	<i>Theoretical</i>	86		7	7

Table 3

Advancing contact angles between different SAM endgroups and water droplets in ambient air; mean $\pm$ S.D., n=3 water droplets

SAM	Advancing Angle (°)
CH3	108 $\pm$ 2
OH	24 $\pm$ 5
COOH	31 $\pm$ 3
NH2	35 $\pm$ 4

Table 4

Summary of results for enzyme-linked lectin assay on polystyrene or SAM endgroups for mannose or galactose family lectins

Carbohydrate Moiety	Polystyrene		SAM	
	Serum	Plasma	10% Serum	Plasma
Mannose	<u>Increasing</u> absorbance trend with decreasing serum %		No trend	No trend
GlcNAc	<u>Increasing</u> absorbance trend with decreasing serum %		No trend	No trend
Sialylated groups	Increasing absorbance trend with decreasing serum %		Lowest on CH3 SAM	No trend
α -Fucose	No absorbance trend with decreasing serum %		No trend	No trend
Complex mannose	<u>No absorbance trend</u> with decreasing serum %		<u>NH2 SAM > ALL SAM OH > CH3 SAM</u>	No trend
α -Galactose	<u>Decreasing</u> absorbance trend with decreasing serum %		COOH > CH3, NH2	No trend
β -Galactose	Not Detectable		No trend	No trend
GalNAc	<u>Decreasing</u> absorbance trend with decreasing serum %		Not detectable	Low levels