Applications Development for High Throughput Assays: Importance of Specialized Surfaces and Formats for Optimization

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ABSTRACT

The molecular surface properties of a high-density MicroWell® plate are determined by the molecular characteristics of the polymer, the design of the plate, the manufacturing parameters (molding temperatures, pressures, etc.) and secondary modification processes. We examined the composition of functional species on modified polystyrene surfaces including aromatic and aliphatic hydrocarbon, alcohol and ether, carbonyl, ester and acid, and carbonate groups. Correlation of these surface moieties with the binding performance of various biomolecules including IgG at basic and alpha fetoprotein at acidic pH, Phosphatidylserine, and well-characterized cell lines was analyzed. We demonstrated the impact that surface chemistry have on assay performance. Data from ELISA, cell culture, and biochemical assays will be presented.

INTRODUCTION

The primary component of most high throughput screening systems is a high-density plate. While much care is applied to optimizing the reagents of an assay, little thought may be given to the use of specialized chemistry designed specifically for particular assay types. Nunc surfaces, MaxiSorp™, MediSorp™, PolySorp®, and MultiSorp™, exhibit specific chemical characteristics that promote interactions with biomolecules in distinctly different manners.

Through secondary processes, polymers, including polystyrene, can be oxidized to create specific surface properties. The oxidation can increase surface energy and hydrophilicity, important in cell adhesion and proliferation. Moreover, the oxidized functional groups can be utilized as chemically reactive sites for coupling other molecules (Onyiriuka et al, 1990.) It is well documented that gamma irradiation can induce polymerization of styrene and induce cross-linking in polystyrene. Numerous publications have described the effect of plasma and corona treatments on polystyrene (Cormia and Kolluri, 1990, Kaplan and Rose, 1991). These treatments also can be optimized to increase or decrease wettability and modify the surface in a manner different than irradiation. The composition and amount of various functional groups allow these surfaces to react very differently under identical circumstances.

The biological/surface interaction is key to product performance. The biological material in this equation, whether cells, proteins, or fluids, interacts only at the polymer surface. Through tightly controlled surface treatment, this interface can be optimized for different characteristics. Exploitation of the surface chemistry derived from various modifications of polystyrene allows for efficient and effective assay optimization. Specific modifications can create reaction vessels that exhibit distinct binding characteristics. Knowledge of the composition of functional groups on specialized surfaces is critical to selection, optimization, and detection of the various proteins, phospholipids, and compounds adsorbed to the surface.

METHODS

ESCA. ESCA scans were obtained utilizing a Physical Electronics Quantum 2000 XPS instrument equipped with a monochromatic Al X-ray source. A survey spectrum was conducted to detect the elemental composition of each sample. The atomic concentration of oxygen was determined through the measurement of the carbon 1s and oxygen 1s spectra of triplicate samples. The functional group determination consisted of collecting high-resolution carbon 1s spectrum from triplicate samples. The analysis conditions for each type of analysis are presented in Table 1. These spectra were peak fit to determine the carbon-oxygen functional groups concentrations on the surfaces of the samples (Table 2).

Table 1: Analysis Conditions

Parameter	1. Survey	2. C (1s) and O (1s)	3. H.R. C (1s)
X-ray Source	Mono Al	Mono Al	Mono Al
X-ray power (W)	40	25	25
X-ray Spot Size (µm)	200	100	100
Scan range (eV)	0-1400	278-295; 525-541	279-299
Step Size (eV)	0.4	0.2; 0.2	0.025
Dwell Time (s/point)	0.25	5.0; 0.4	1.2
Analyzer Mode	CAE	CAE	CAE
Pass Energy (eV)	187.85	187.85	23.5
Take-off Angle	45°	45°	45°
Neutralizer	1.0 V, 25 µA	1.0V, 25µA	1.0V, 25µA

Table 2: Carbon Peak Fitting

Parameter	C-C Aromatic	C-C Aliphatic	C-O	C=O	CO_2	CO_3	$\pi \rightarrow \pi^*$
Position (eV)	284.08 to 284.18	+ 0.48 to 0.62	+ 1.45 to 1.65	+ 2.7 to 2.9	+4.0 to 4.4	+ 5.0 to 5.4	+ 6.0 to 6.8
FWHM[1]	1.1±0.15	1.1±0.15	1.1±0.15	1.1±0.15	1.1±0.15	1.1±0.15	2.3±0.3
GL[2] mix (%G)	90±10	90±10	90±10	90±10	90±10	90±10	0-100

[1]FWHM: Full width at half maximum [2]GL: Gaussian/Lorentzian mixing ratio

Basic Protein Binding Assay. The 96 well plates were coated with 200 µL per well with an antibody mixture containing 3600 ng/mL rabbit anti-sheep IgG and 33 ng/mL anti-alpha 1-feto-protein horseradish peroxidase conjugate in 0.05M carbonate buffer, pH 9.6. The plates were sealed with Nunc sealing tape to prevent evaporation and incubated overnight, protected from light, at room temperature. The plates were washed three times with a 0.15M PBS, pH 7.2, containing 0.2M NaCl and 0.05% Triton X-100. The washed plates were then incubated with 200 µL per well of a substrate solution containing 0.6 mg OPD and 0.5µL 30% H_2O_2 per mL in 0.1M citrate phosphate buffer, pH 5.0. The colorimetric reaction was allowed to continue for five minutes before the addition of 150 µL of 2N H_2SO_4. The optical density at 490nm was determined using an Elx 800 microplate reader.

Acidic Protein Binding Assay. The 96 well plates were coated with 150 µL of a solution containing 2.5 µg/mL BSA, and 2.5 µg/µL human alpha 1-feto-protein in an acetate buffer (pH 4). The plates were sealed with Nunc sealing tape to prevent evaporation and incubated overnight, protected from light, at room temperature. The plates were washed three times with a washing solution (PBS containing 0.05% Triton X-100). To decrease non- Rabbit anti-human alpha 1-feto-protein in PBS containing 0.05% Tween-20 was added. The plates were again washed three times with the washing solution.
Finally, 150 µL of the substrate solution containing 0.6 mg OPD and 0.5µL 30% H_2O_2 per mL in 0.1M citrate phosphate buffer, pH 5.0 was added to each well. The colorimetric reaction was allowed to continue for four minutes before the addition of 100 µL of 2N H_2SO_4. The optical density at 490 nm was determined using an Elx 800 microplate reader.

specific binding, the plates are blocked with a 1 hour, room temperature incubation with 1 % (w/v) of gelatin in PBS solution containing 0.05% Tween-20. The blocking solution was decanted and 150 µL of an antibody solution containing 1.4 µg/mL Rabbit anti-human alpha 1-feto-protein horseradish peroxidase conjugate and 9 µg/mL

Phospholipid Binding Assay. NBD-labeled phophatidylserine and non-labeled phosphatidylserine, at a ratio of 1:20 in chloroform:methanol (v/v 1:4), were mixed in a glass tube. Different amounts of

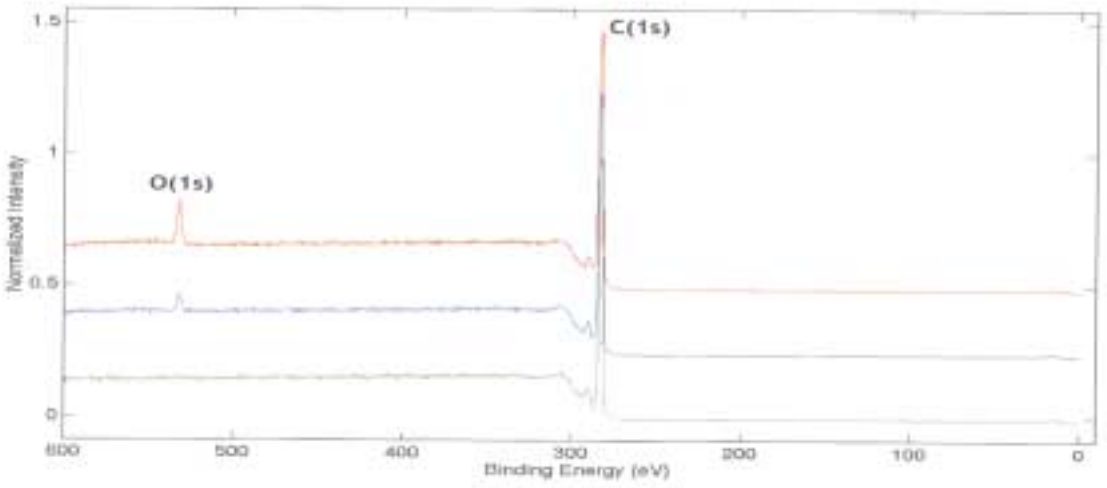
Phoshatidylserine were dispensed into a 96 well plate. The solutions were dried under nitrogen to minimize oxidation. The plates were washed three times with 100 µL of PBS. Fluorescence was detected by a fluorescence plate reader using excitation and emission wavelengths of 485 and 530 nm, respectively.

Cell Culture. L929 cells were plated at a density of 2.5 x 10^5 cells/mL into 96 well plates with various surface modifications. The cells were allowed to proliferate for 24 hours in minimal essential media supplemented with 10% bovine calf serum. The morphology of the cells was examined microscopically utilizing a Zeiss inverted microscope equipped with a 10X objective and 10 X oculars. Images were digitally captured for analysis.

RESULTS

Much is known regarding the methods of surface modification to polystyrene Examination of the chemical properties of these surfaces will lead to a better understanding of the molecular interactions that occur at the interface. To this end, we determined the functional groups introduced onto the surface by various surface modification processes. The samples, MaxiSorp™ and MultiSorp™ were compared with non-treated plates. The MaxiSorp™ surface is designed for quantitative and qualitative solid phase immunoassays. MaxiSorp™ products are recommended for adsorption of various proteins including antibodies. The MultiSorp™ product line exhibits hydrophilic characteristics and is particularly well suited for assays involving polar molecules. Through XPS analysis (also known as ESCA) the composition and atomic concentration of elements was determined. The results indicated that only carbon and oxygen were detected on the samples (Figure 1).

Figure 1. XPS Spectra of Surfaces



Through measurement of the carbon 1s and oxygen 1s spectra on three samples, the atomic concentration of oxygen of each sample was calculated (table 3). It is evident through the survey spectrum that variations in oxygen concentration are significant between the different surfaces. Measurements of the carbon 1s and oxygen 1s spectra for triplicate samples were used to determine the atomic concentration of oxygen on each sample. The results demonstrate an increased oxidation of the MaxiSorp™ and the MultiSorp™ surface. Further analysis of carbon-oxygen functional groups was performed using high-resolution carbon 1s spectrum from three samples. A representative spectrum for each surface type is depicted in figure 2. Carbon oxygen functional groups appear as shoulders at higher binding energy than the main, aromatic/aliphatic carbon peak. The feature in the spectrum at ~291.5 eV is due to a $\pi \rightarrow$

Table 3. Atomic Surface Composition of Treated MicroWell® Plates

Sample	Atomic Percentage	
	Carbon	Oxygen
F96 MaxiSorp #1	95.82	4.18
F96 MaxiSorp #2	95.77	4.23
F96 MaxiSorp #3	96.36	3.64
Average	**96.0**	**4.0**
F96 MultiSorp #1	87.90	12.10
F96 MultiSorp #2	88.02	11.98
F96 MultiSorp #3	86.78	13.22
Average	**87.6**	**12.4**
F96 Control #1	99.80	0.20
F96 Control #2	99.80	0.20
F96 Control #3	99.78	0.22
Average	**99.8**	**0.2**

Figure 2. Carbon 1s XPS Spectra of Surfaces

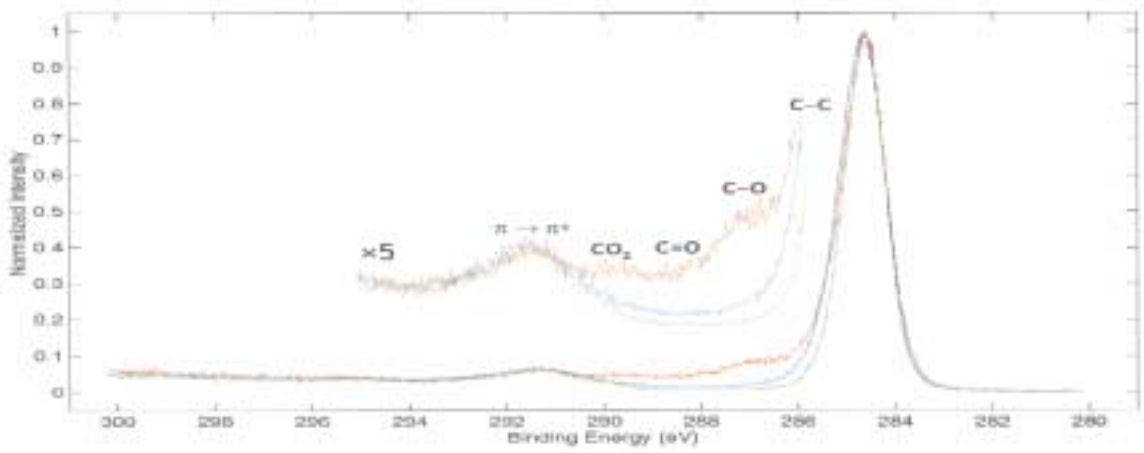
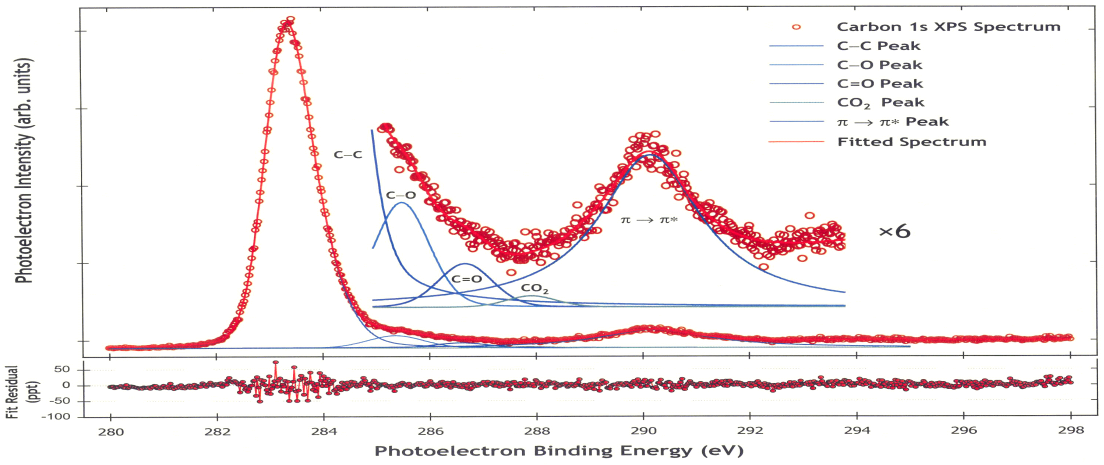


Table 4. Average Atomic Percentage of Carbon Species on Polystyrene Surface

Sample	C-C Aromatic and aliphatic hydrocarbon	C-O Alcohol and ether	C=O Carbonyl	CO_2 Ester and acid	CO_3 Carbonate	$\pi \rightarrow \pi^*$ satellite
MaxiSorp	86.8%	1.2%	0.3%	<0.1%	<0.1%	11.6%
MultiSorp	81.1%	4.6%	2.3%	1.1%	<0.1%	10.8%
Control	89.2%	0.1%	<0.1%	<0.1%	<0.1%	10.7%

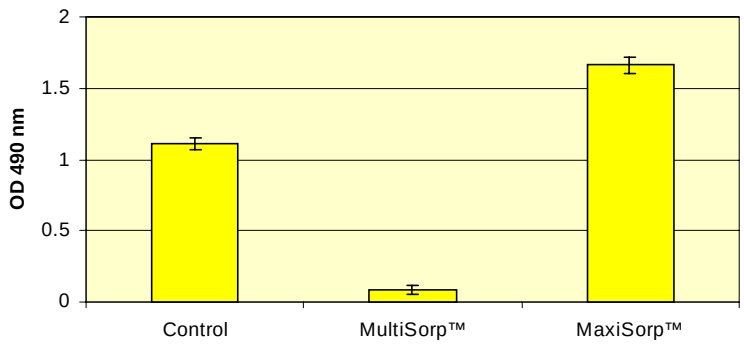
These spectra were analyzed to determine the carbon-oxygen functional groups on each surface (table 4). These percentages were determined through carbon peak fitting that was performed on each spectrum. Figure 3 shows an example of the carbon peak fitting that was performed for the MultiSorp™ sample. Distinctions are noted between the quantity and composition of the functional groups on the three surfaces examined. It is apparent from the data that the MultiSorp™ surface modification results in a greater degree of oxidation when compared with the control and MaxiSorp™ samples.

Figure 3. Carbon 1s Peak Fit for MultiSorp



The introduction of functional groups will affect the binding characteristics of the plastic polymer (Butler, 1992). To this end, we demonstrate that surface modifications will affect binding of proteins. Comparison of adsorption of various proteins on non-treated control, MultiSorp™ and MaxiSorp™ samples indicates the importance of surface selection on assay optimization. Various molecules behave in distinctly different manners depending on the characteristics of the surface. For example, under basic conditions, IgG will adsorb to MaxiSorp™ modified polystyrene with significantly more capacity when compared with a non-treated control plate (Figure 4). In the case of MultiSorp™, the functional groups on the surface restrict the protein absorption of IgG; evident by a binding capacity compared to the non-treated plate.

Figure 4. IgG Binding on Modified Surfaces



Thus under these conditions, MultiSorp™ can be characterized as a low IgG binding surface. If modifications are made to the assay, as demonstrated in figure 5, the results can be dramatically different. Under acidic conditions, human AFP will bind MultiSorp™ with a higher capacity resulting in approximately 25 times higher optical density when compared to the non-treated control. The currently available polystyrene surfaces work well for most protein antigens in solid phase immunoassays. However these surfaces are not adequate for the measurement of all antigenic substances. Certain antigenic lipids are not easily detected using these surfaces. Anti-phospholipid antibodies are found in sera of patients with autoimmune diseases. We investigated the binding affinity of Phosphatidylserine on the different surfaces (Figure 6). This data suggests that an anionic phospholipid bind better to the MultiSorp™ surface compared with MaxiSorp™ and a non-modified surface.

Figure 5. Human AFP Binding on Modified Surfaces

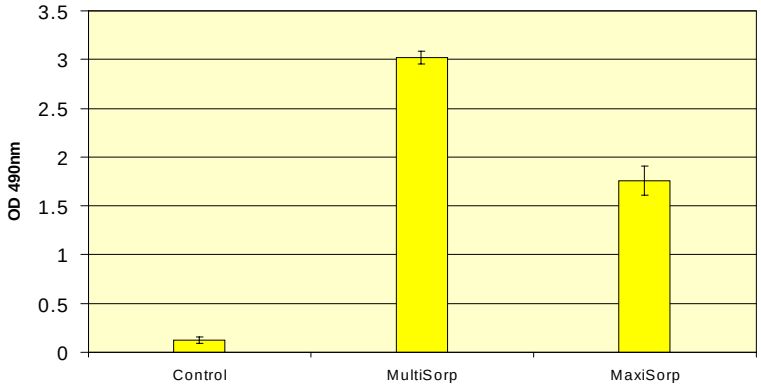
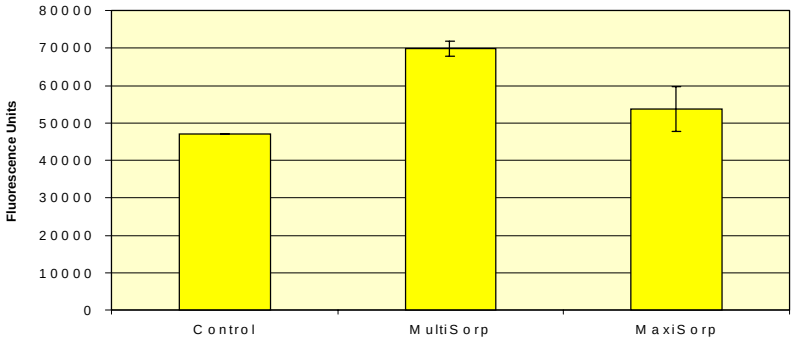
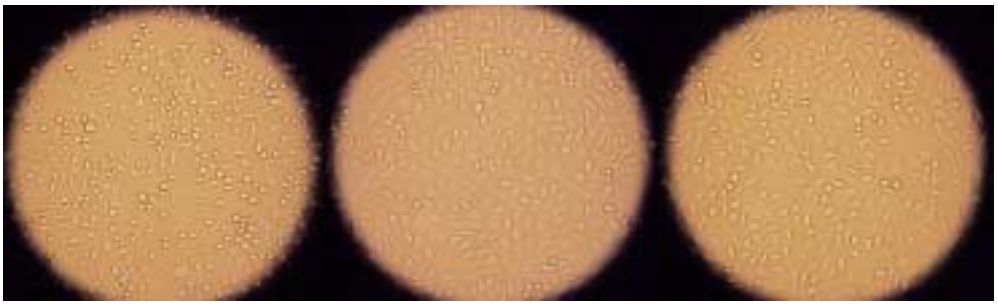


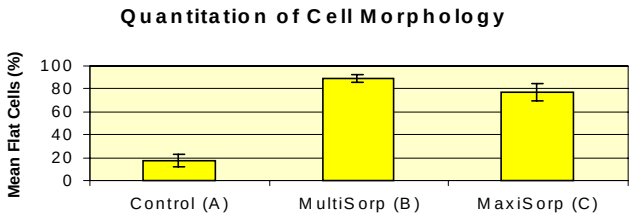
Figure 6. Phosphatidylserine Binding on Modified surfaces



Phospholipids are major constituents of eukaryotic cell membranes. Based on cell morphology, L929 cells can distinguish difference in the modified surfaces. The MultiSorp™ surface induced a flattened, spindle shaped morphology in most L929 cells. The MaxiSorp™ surface does also, but to a lesser extent. L929 cells remain spherical in shape and poorly adherent on non-treated surfaces more favorably. As demonstrate in figure 7, changes in cell morphology correlate with improved cell adhesion

Figure 7. Cell Morphology





CONCLUSION

The type of surface modification is critical in assay development. The interaction of various biomolecules with the surface is directly related to the functional groups derived through the surface modification. Through understanding the functional characteristics of the reaction vessel, surfaces can be selected based on the affinity for various biomolecules. For example, MultiSorp™ exhibits low binding properties for IgG that may result in reduced background compared with other surfaces.

Further, the data suggests that increased oxidation of polystyrene leads to a more hydrophilic surface. The degree of hydrophibicity directly affects the binding characteristics of the polymer. Exploitation of the characteristics achieved through secondary modifications will aid in assay development and optimization.

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