Investigation of Non-Nucleophilic Additives to Reduce Morphological Anomolies in Protein Arrays

Charles R. Mace[†,¶], Amrita R. Yadav[§,¶], and Benjamin L. Miller*[,†,‡,¶]

Departments of Dermatology[‡], Biochemistry and Biophysics[†], and Physics and Astronomy[§], and the Center for Future Health[¶], University of Rochester, Rochester, New York 14642

Supporting Information

- All buffers were sterilized and filtered, and all additive solutions were sterilized and filtered prior to use

Abbreviations Used:

AIR	Arrayed Imaging Reflectometry
APTES	(γ-aminopropyl)triethoxysilane
BSA	bovine serum albumin
ddH_2O	glass distilled deionized water (pH 6.0)
EDTA	ethylene diamine tetraacetic acid
HBS	aqueous buffer containing 20 mM HEPES, 150 mM NaCl, at pH 7.2
HCl	hydrochloric acid
DMSO	dimethyl sulfoxide
MPBS	aqueous buffer containing 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl, at pH 7.2
MPBS-ET	MPBS containing an additional 3 mM EDTA and 0.005% Tween-20
PEG-DME	poly(ethylene glycol) dimethyl ether, ~M_w = 2,000 Da

1. Detailed Surface Attachment Chemistry Protocol

Silicon wafers (n-type, <100>), with ~1400 Å of thermally grown silicon dioxide were obtained from Rochester Institute of Technology and diced into 2 cm x 1 cm *or* 1 cm x 1 cm chips for array experiments or ellipsometric measurements, respectively. Diced chips were etched in dilute hydrofluoric acid until their silicon dioxide thicknesses were 1380 Å, as measured by spectroscopic ellipsometry (J.A. Woollam M2000). The chips were then washed in a solution of 1:1 methanol:HCl for 30 minutes. The chips were then washed repeatedly with ddH_2O and dried under a stream of nitrogen. A solution of 0.4% v/v APTES in anhydrous toluene was added to the chips and allowed to shake for 15 minutes[1]. The chips were then washed repeatedly with ethanol, dried under a stream of nitrogen, and cured at 100°C for 15 minutes. Once the chips had cooled to room temperature, a solution of 1.25% glutaraldehyde (50% aqueous) in MPBS was added to the chips and allowed to shake for 60 minutes. The chips were then washed repeatedly with ddH_2O, acetone, and ddH_2O again before being dried under a stream of nitrogen. At this point, the chips have been functionalized to bear a terminal aldehyde in order to facilitate the general-amine immobilization of probe antibodies.

Probe antibodies were kept as two-fold stock concentrations in MPBS alone to facilitate dilutions into additive solutions. Likewise, all additives were kept as two-fold stocks, i.e. 0.2% additive in MPBS to give a final concentration of 0.1% v/v of the additive. Probe solutions were diluted into additives at the time of arraying *only*, and this was done in order to ensure the freshness of the sample and to limit any detrimental effects the additive may have on antibody function. The final solutions were manually arrayed, in a volume of 0.5 μL, onto the functionalized chips in a predetermined pattern (Figure S1). This array was comprised of four differing anti-human IgG conditions at 500 μg/mL: in MPBS alone or MPBS plus 1%, 0.1%, or 0.01% of the additive under study; additionally, a set of anti-fluorescein negative control spots were arrayed at a final concentration of 300 μg/mL in 0.1% 12-crown-4. A lower concentration is used for anti-fluorescein in order to ensure that the effective layer thickness (observed in terms of spot reflectance in our case) is similar for the two antibody spots (32.1 Å for anti-human IgG and 30.1 Å for anti-fluorescein, as measured by spectroscopic ellipsometry), as this facilitates more accurate data analysis.

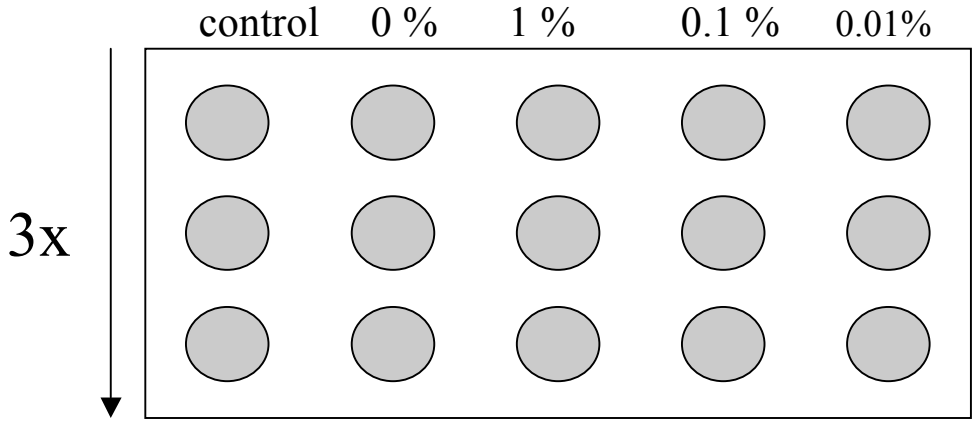


Figure S1. Schematic of the manual array used for AIR additive experiments. Except for the control spots, which were comprised of α-fluorescein in 0.1% 12-crown-4 as a standard, all spots are anti-human IgG diluted in spotting buffer containing the specified percentage of an additive. "3x" indicates that each condition was spotted in triplicate.

The following additives were tested at 1%, 0.1%, and 0.01%:

- 12-crown-4
- 18-crown-6 (w/v)
- diethyl ether
- diglyme
- PEG-DME (w/v)
- DMSO

The following additives were also tested:

- glycerol at 20%, 2%, and 0.2%
- Triton X-100 at 0.1%, 0.01%, and 0.001%

After the arrays were completed, the chips were placed into a modified humidity chamber and allowed to incubate for 60 minutes at 4°C. The chips were then removed from the chamber and residual liquid on each spot was allowed to evaporate in order to reduce smearing. The chips were then immediately immersed into a solution of 200 μg/mL BSA in HBS and allowed to shake for 60 minutes. Afterwards, the chips were thoroughly washed with MPBS and the edges were blotted dry in order to wick excess buffer off of the surface. Note that there was a small volume of residual buffer remaining over the array, thus keeping the probe spots hydrated. To this volume, the target solutions were added and allowed to incubate for 60 minutes. This *did*, unfortunately, inherently dilute the target solution in an unquantifiable manner. However, this happened uniformly across a given chip, and since the signal change for the additives' spots was eventually normalized to the change for the MPBS spots present on each chip, this did not affect the end results.

Two chips were created for each additive studied, with one serving as the experimental chip and the other as the negative control chip. The experimental chip received 45 μg/mL human IgG in MPBS-ET as a target solution. Due to the sensitivity of AIR, 45 μg/mL is considered to be a "high concentration" of target (data not shown). The use of a high concentration of target served to give rise to a large signal change for the anti-human IgG spots, thereby exaggerating morphological inconsistencies and allowing for easier comparisons of antibody activity. The negative control chip received a solution of MPBS-ET alone, and spot intensity values from this chip acted as background intensities for the experimental chip. After target solutions had incubated, the chips were thoroughly washed with ddH_2O and dried under a stream of nitrogen in order to be imaged.

For the ellipsometric measurements, the 1 cm x 1 cm chips were etched in dilute hydrofluoric acid for five minutes in order to obtain a clean SiO_2 surface. The same protocol as described above was used to functionalize the chips with glutaraldehyde. Ellipsometric measurements were done to determine the thickness of the total layer of oxide and linking chemistry. Thereafter, a 50 μL solution containing either the highest concentration of the additive with 500 μg/mL anti-human IgG or the highest concentration of the additive alone was incubated on the chips for 60 minutes. After the chips were washed with ddH_2O and dried under a stream of nitrogen, the thickness was again measured in order to quantify the thickness change upon addition of the buffers with/without antibody.

2. Methods of Array Data Analysis

Chips were mounted onto the benchtop reflectometer[2] and images were acquired with Astro IIDC (Aupperle Services and Contracting) using a gain of 1 and at an integration time of 30 ms. The 2 cm x 1 cm chips used in this experiment are imaged obliquely at a fixed angle of ~70.5°. The oblique angle of incidence causes the otherwise circular spots to look elliptical in our images. The combination of oblique incidence angle and a relatively large chip size also gives rise to a lack of focal depth over the image. If the array is not in focus, a de-focusing or "pillowing" effect is noticed as an imaging artifact. Therefore, images were also acquired by scanning through five different focal planes along the chip surface in order to obtain unambiguous reflectance intensities from all spots in the array. Scanning itself has an undesired effect by modulating the magnification of the image. Due to this, we are unable to compile a coherent aggregate image without manipulating the dimensions of raw image. However, since this presents information more accurately describing the morphology and reflectance intensity of each spot, all images used for analysis were a combination of all five focal planes. For comparisons, Figure S2 shows each focal plane independently as well as the resulting combination.

After the images were acquired, the intensity histogram of each spot was obtained using ImageJ[3]. This was fit to a Gaussian, the center of which was taken to be the mean intensity of the spot. The change in intensity for each spot between the control chip and the experimental chip was determined and normalized to any intensity change observed for the negative control anti-fluorescein spots. This reflectance change was normalized to the MPBS standard arraying buffer, and quantified as a "percent active".

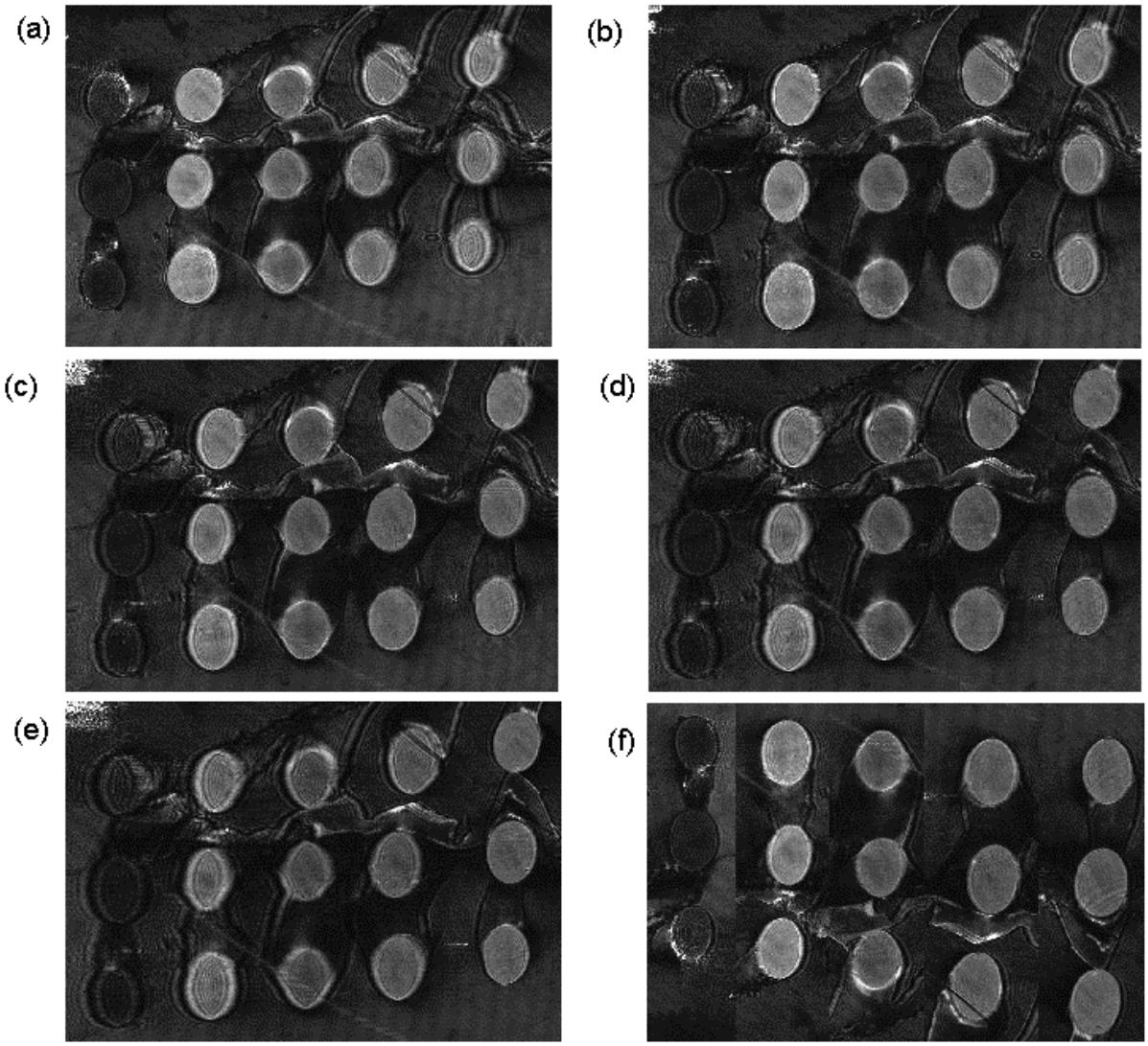
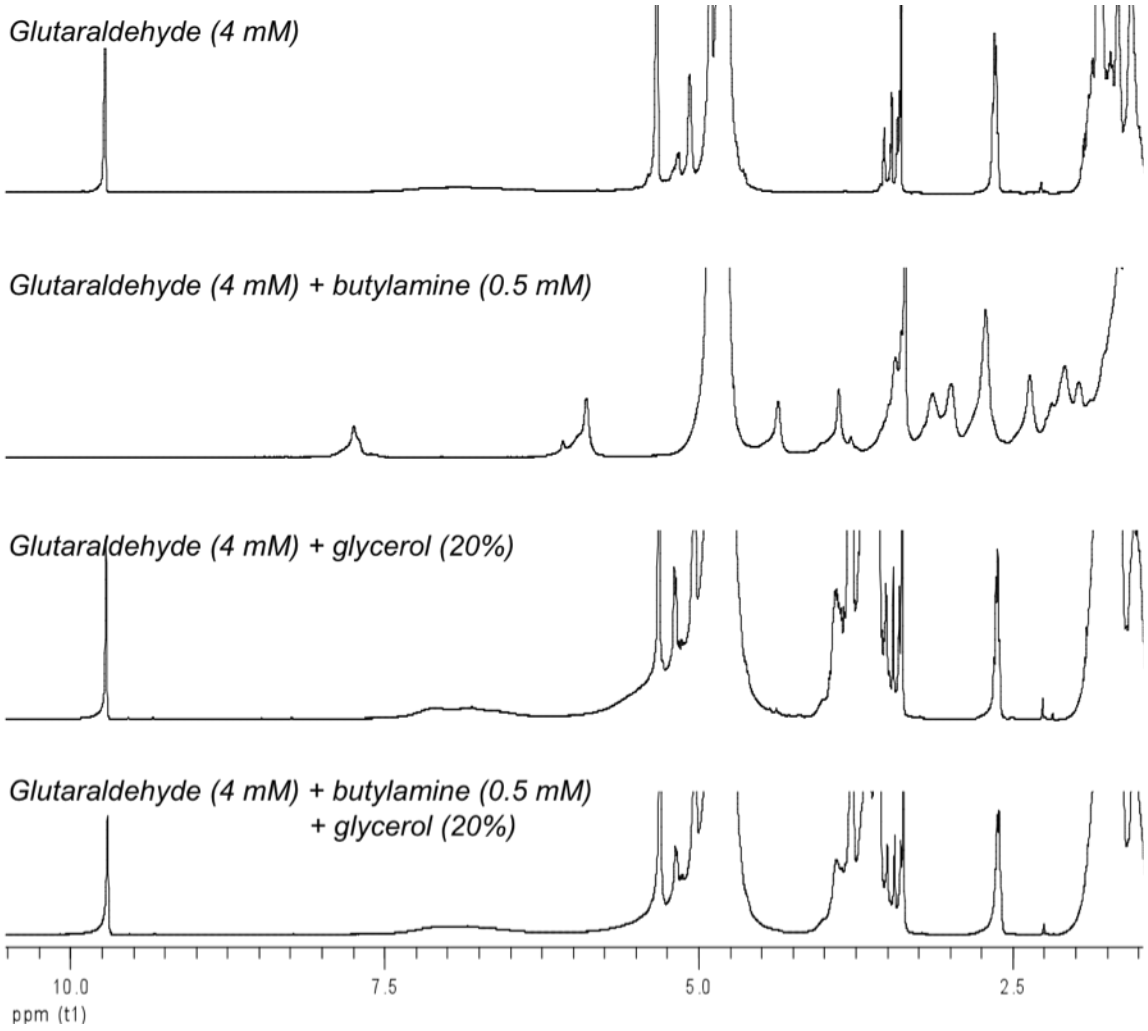


Figure S2: Representative AIR images of a chip taken by scanning through five different focal planes ((a) through (e)), and a composite image (f). The array consists of control anti-fluorescein spots (leftmost column), and anti-human IgG spots diluted in either MPBS alone (second column from left) or varying concentrations of DMSO as additive (1%, 0.1% and 0.01% from left to right). The aspect ratio of the images has been altered in order to concisely display each focal slice.

3. Solution NMR analysis of glutaraldehyde in the presence of butylamine and/or glycerol.

Although not a precise analog of the surface reactions discussed in the main text, a comparison of ^{1}H NMR spectra (500 MHz, Bruker) obtained for glutaraldehyde with butylamine and/or glycerol in MPBS-*d*, pH 7.2 is instructive. In the presence of butylamine, glutaraldehyde forms dihydropyridine and a variety of polymeric products, consistent with prior reports.[4] A glutaraldehyde solution containing both butylamine and 20% glycerol, however, shows no evidence of these products. Portions of the relevant spectra are shown below.



References:

[1] Vandenberg, E.T.; Bertilsson, L.; Liedberg, B.; Uvdal, K.; Erlandsson, R.; Elwing, H.; Lundström, I. *J. Colloid Interf. Sci.* **1991**, *147*, 103-118.
[2] For reflectometer description, see: Mace, C.R.; Striemer, C.C.; Miller, B.L. *Anal. Chem.* **2006**, *78*, 5578-5583.
[3] Abramoff M.D.; Magelhaes P.J.; Ram S.J. *Biophotonics International* **2004**, *11*, 36-42.
[4] Lubig, R.; Kusch, P.; Roeper, K.; Zahn, H. *Monatshefte Chem.* **1981**, *112*, 1313-1323.