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Protein Damage in Drop-on-Demand Printers

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A variety of methods now exist for the generation of microarrays of biological materials. Inkjet printing technology offers the advantages of speed, low cost, and contactless printing and has been used to synthesize microarrays consisting of oligonucleotides,[1,2] DNA,[3,4] antibodies, and other proteins.[5−7] Inkjet technology has also been used for creating small drops containing protein for crystallography studies.[8] Little study has been directed concerning damage to biologicals during inkjet printing. Okamoto and coworkers were concerned with, but found no evidence, of damage caused by shear stress when printing DNA.[2] Delehanty and Ligler studied the loss of protein from nonspecific adsorption on the printer tubing and minimized this loss by the addition of a sacrificial protein (BSA).[6] In this communication, we characterize damage to a model enzyme, peroxidase, caused by a third mechanism: the rapid compression experienced by the solution during the printing process. We also find that damage can be eliminated by the addition of trehalose and glucose to the printed solution.

Inkjet printing requires the generation of a pressure pulse within a confined liquid, either mechanically generated using a piezoceramic actuator or thermally generated by the rapid vaporization of part of the liquid. The pressure pulse causes liquid to eject from a small orifice, forming a jet that then resolves into a droplet before hitting a support.

The tumultuous environment that a liquid experiences during inkjet printing is of concern when fragile biological molecules are printed. We investigated the loss in activity of a model enzyme, peroxidase, by printing an aqueous solution using a piezoceramic actuated inkjet printer. The printer consists of a cylindrical piezoceramic that squeezes a poly(tetrafluoroethylene) tube connected to a poly(tetrafluoroethylene)-coated ruby aperture. The buffered solution contains peroxidase, fluorescein sodium salt (an internal standard), and sugar (trehalose/glucose) and is printed directly into buffer contained in a 96 microwell plate. Following printing, we measured the fluorescence of each well, added substrate[9], and measured absorbance after 30 min at 450 nm.

We printed 50, 100, 200, 300, or 400 drops into wells. The speed of the printed drops is measured using illumination with a strobe light and is kept low (from 30 to 40 cm/s) to eliminate damage caused by shearing at the orifice.[10] Loss of peroxidase due to adsorption onto the printhead tubing is eliminated by flowing the print solution through the printhead for several minutes before printing, thus saturating all surfaces.[11] Compression rates are varied by applying 85 V to the piezoceramic over times ranging from 14 to 70 μs.[12] From the physical properties of the piezoceramic, the decrease in volume as a function of the applied voltage is calculated.[13,14] Analysis proceeds as follows. A series of control solutions are prepared by appropriate dilutions of the print solution, and their fluorescence is measured. A linear calibration plot of fluorescence versus the quantity of fluorescein is obtained, and since fluorescein and peroxidase are present at equal weights, the calibration plot also relates fluorescence to the quantity of peroxidase.

Using the calibration plot and the fluorescence of wells containing printed solution, the quantity of peroxidase printed into each well is determined. These values are then plotted against absorbance values from the peroxidase assay.

Figure 1 plots the peroxidase activity resulting from printing one solution[15] using four different compression rates. The activities of control solutions are also plotted. Absorbance is seen to increase linearly with the quantity of peroxidase printed at each compression rate. From Figure 1, we deduce the damage to peroxidase occurring during the printing process. For example, the solution printed using a compression rate of 5.48×10^4 $\mu m^3/\mu s$ yields $A = 1.2$ for 1 ng of peroxidase. Comparing this to the control curve, to obtain $A = 1.2$ requires about 0.45 ng of peroxidase. This means that of the 1 ng of peroxidase printed at this compression rate only 0.45 ng is active. The retained activity is therefore calculated from the horizontal distance between the sample curve and the control curve. It is readily shown that the ratio of the sample slope to the control slope also yields the retained activity.

Figure 1 reveals that compressing the liquid more slowly reduces damage to the enzyme. No damage occurs if this solution is compressed at the slowest compression rate shown of 2.56×10^4 $\mu m^3/\mu s$. Activity curves resulting from printing at even slower compression rates (not shown in Figure 1) also overlap the control curve, demonstrating that no damage occurs at the slowest compression rates.

The ratio of the slope of each curve in Figure 1 against the control curve yields the fraction of active enzyme, or retained activity, as a function of the compression rate used during printing. Figure 2 plots the retained activity as a function of compression rate for the solution characterized in Figure 1 and for those of two additional solutions. The effect of compression rate on the activity of peroxidase solutions containing no sugar shows no simple trend. However, it is clear that damage occurs under all printing conditions, and that less damage occurs when printing using the slower compression rates.

Figure 2 also plots the activity of peroxidase solutions containing 10% trehalose/1% glucose and 20% trehalose/2% glucose (w/w). This sugar ratio was chosen since it has been found to effectively preserve a model enzyme, presumably by extensive hydrogen bonding between the sugars and the enzyme.[16] Figure 2 reveals that the addition of these sugars significantly reduces damage to peroxidase, and that there is no significant difference between these two concentrations of sugars. No damage to peroxidase occurs if printing takes place at compression rates less than about 2.5×10^4 $\mu m^3/\mu s$.

Proteins are a heterogeneous class of biological molecules, and many factors influence their stability. Enzymes, in particular, are fragile since the structural features of their active site work in opposition to those that confer stability.[17] We chose peroxidase for initial study since it is widely used as a reporter molecule in assays. Whether its stability during inkjet printing is typical of other

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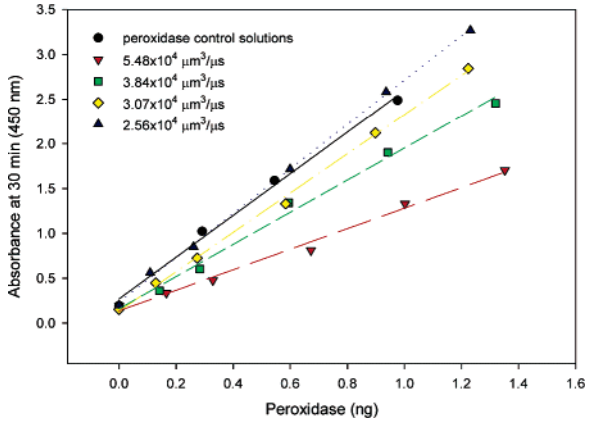

Figure 1. Assay results with linear least-squares fit for a peroxidase/fluorescein/11% sugar solution printed directly into microplate wells containing buffer. Each line characterizes the activity of the solution printed at the compression rate given by the legend. As the compression rate decreases, peroxidase activity increases (indicated by the increasing slopes).

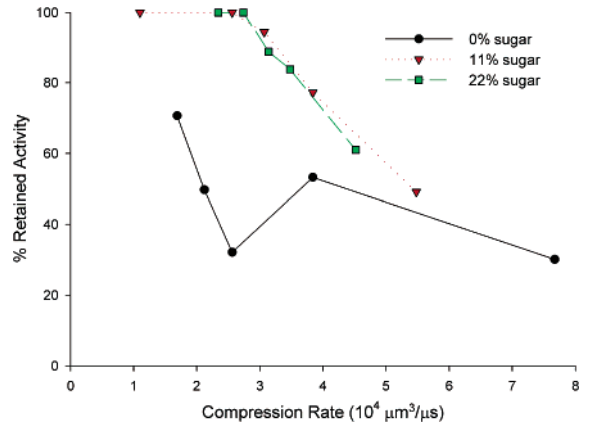

Figure 2. Damage to peroxidase printed using different compression rates. Peroxidase solutions containing no sugar are damaged at all compression rates. The addition of sugar reduces damage, and no damage to peroxidase occurs at sufficiently low compression rates.

enzymes and proteins is not known and is currently under investigation.

There are a number of mechanisms by which proteins degrade and corresponding additives to prevent degradation. Examples of protein stabilizers are cryoprotectants, which prevent ice crystals during freezing, protease inhibitors, which prevent proteolytic cleavage of proteins, antimicrobial agents, which inhibit microbial growth, metal chelators, which avoid metal-induced oxidation of −SH groups, and reducing agents, which prevent oxidation of cysteines.[18] These stabilizers are not expected to prevent damage from occurring during inkjet printing since their stabilization mechanisms clearly do not apply to this application. In contrast, trehalose is known to be an exceptional stabilizer of protein conformation due to its extensive hydrogen bonding with proteins and its effect on the structure of solvent water.[19] This extensive hydrogen bonding may also stabilize peroxidase in the liquid state during the printing process, presumably by cushioning the enzyme against pressure-induced denaturing. Whether trehalose is unique in its ability to stabilize peroxidase during printing or whether other sugars and additives function similarly is under investigation and will shed light on the stabilization mechanism of trehalose.

The foregoing results serve as a caveat in the use of inkjet printers to create microarrays. The motivation in commercial inkjet printing is to eject droplets at high speeds, on the order of 10 m/s, to improve resolution and to enhance penetration of the ink into the paper. This is achieved by subjecting the ink to high compression rates. Many commercial printers operate under resonance conditions, where the pressure waves are timed to reinforce each other, subjecting the solution to even higher compression. This condition of high compression rate can induce significant damage to biological materials. The performance of microarrays consisting of fragile molecules is reduced when such damage occurs. We find significant damage to peroxidase even when printing at low compression rates and low drop speeds, though this damage is mitigated by the addition of trehalose/glucose.

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References

(1) Blanchard, A. P.; Kaiser, R. J.; Hood, L. E. *Biosens. Bioelectron.* **1996**, *11*, 687.
(2) Okamoto, T.; Suzuki, T.; Yamamoto, N. *Nat. Biotechnol.* **2000**, *18*, 438.
(3) Goldmann, T.; Gonzalez, J. S. *J. Biochem. Biophys. Methods* **2000**, *42*, 105.
(4) Allain, L. R.; Askari, M.; Stokes, D. L.; Vo-Dinh, T. *Fresenius' J. Anal. Chem.* **2001**, *371*, 146.
(5) Delehanty, J. B.; Ligler, F. S. *Anal. Chem.* **2002**, *74*, 5681.
(6) Delehanty, J. B.; Ligler, F. S. *BioTechniques* **2003**, *34*, 380.
(7) Pardo, L.; Wilson, W. C.; Boland, T. *Langmuir* **2003**, *19*, 1462.
(8) Howard, E. I.; Cachau, R. E. *BioTechniques* **2002**, *33*, 1302.
(9) Added to each well is 200 μL of a solution consisting of 0.4 mg/mL of *o*-phenylenediamine dihydrochloride, 0.4 mg/mL of urea hydrogen peroxide, and 0.05 M phosphate/citrate buffer (Sigma, OPD tablet sets).
(10) In a separate experiment, peroxidase solutions were discharged through the printhead aperture at linear flows up to 300 cm/s. No loss in activity was detected at these flow rates. Typical linear speeds used in inkjet printers are much higher, typically 10 m/s.
(11) Control solutions are collected by flowing solution through the printhead under low pressure (~30 psig). Activity of these solutions is identical to that of solutions collected from the sample reservoir. Thus, no loss in activity due to adsorption in the printhead and shearing at the orifice is observed.
(12) Printing occurs under nonresonance conditions, at 100 Hz. An asymmetric waveform is used, consisting of compression (droplet ejection) over microseconds and expansion (refilling) over milliseconds.
(13) The radial contraction, Δr, is estimated as $\Delta r \approx d_{31}Vr/D$, where $r = d_{31}$ = strain coefficient = -170×10^{-12} m/V, V = applied voltage, r = tube radius = 0.5 mm, and t = wall thickness = 0.6 mm.
(14) Liquid flows in two directions as a result of compression. Liquid flows out of the orifice, forming the printed jet. It also flows through the inlet back into the solvent reservoir. The relative amount of flow in each direction depends on the compression rate.
(15) This solution consists of 10 μg/mL of horseradish peroxidase, 10 μg/mL of fluorescein sodium salt, 10% (wt/vol) D-(+)trehalose, and 1% D-(+)-glucose in phosphate-buffered saline.
(16) Sun, W. Q.; Davidson, P. *Biochim. Biophys. Acta* **1998**, *1425*, 235.
(17) Beadle, B. M.; Shoichet, B. K. *J. Mol. Biol.* **2002**, *321*, 285.
(18) *Protein Stability and Storage*; Pierce Technical Bulletin TR043.0.
(19) Kaushik, J. K.; Bhat, R. *J. Biol. Chem.* **2003**, *278*, 26458.

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