Development and Application of Nanoscale Polymeric Platforms for Advanced Protein Sensors

Sheng Song and Jong-in Hahm
Department of Chemistry, Georgetown University
Washington, DC USA
jh583@georgetown.edu

***Abstract*—This article discusses recent progress of surface-bound proteins in facilitating solid-phase sensing applications, particularly focusing on nanoscale polymeric supports that can be used as advanced solid state biosensors. Recent approaches involving nanoscale self-assembly of proteins are highlighted. Current challenges in the applications of surface-bound proteins are identified, specifically in the production and development areas of nanoscale polymeric surfaces for next-generation protein arrays. These efforts are of paramount importance, especially in high-density and high-throughput biotechnological applications such as gene chips, protein arrays, and lab-on-a-chip sensors.**

Keywords-protein array; protein sensor; array fabrication; polymer nanodomain; nanomaterial assembly

I. INTRODUCTION

Current demands for highly miniaturized, small-volume detection platforms in basic biological research and clinical diagnosis underscore the importance of examining proteins on surfaces. In comparison to liquid-phase protein assays, solid-phase sensors involving proteins on array or plate surfaces can be carried out using only a very small amount of assay agents. Solid-phase approaches also enable rapid and simultaneous detection involving a large number of samples. The use of surface-bound proteins in the form of microtiter plates, protein chips, and microwell plates for optical detection is routinely observed both in laboratory and clinical settings. Material choice for these solid surfaces includes glass, nitrocellulose paper, gold, silicon, and polymer. A large number of these protein assays, however, involves polymer-based platforms due to the wide range of polymeric materials that are available and also as a result of the flexibility in which their surface chemistry can be easily tailored for the immobilization of proteins.

II. PARALLEL AND SERIAL METHODS FOR PROTEIN ASSEMBLY

Methods used to deliver and localize proteins on polymeric surfaces include manual and robotic delivery [1], microcontact printing [2-6], imprint- and nano-lithography [7-9], microfluidic channel networks [10-12], focused-ion-beam patterning [13, 14], inkjet deposition [15, 16], dip-pen lithography and related scanning probe microscopies [2, 17-19]. In a laboratory setting, simultaneous protein delivery is frequently carried out either manually by using commercially available multichannel pipettes or automatically by employing robotic protein spotters mounted with capillary print heads.

In recent years, advances in the area of microfabriaction and nanofabrication have influenced protein delivery to surfaces. Methods based on robotic printing, ink-jet printing, soft lithography, and microfluidic channel networks are used to produce micron-size protein patterns on surfaces. These parallel approaches have the benefit of producing a large number of patterns simultaneously on surfaces and are applied as model systems to guide the study of biological systems. However, the micrometer-scale resolution, typically achieved by these parallel approaches can often limit address density of proteins.

Although microscale patterns of proteins are of great interest and application, the nanoscale size and structure of most proteins can be most aptly investigated through a nanoscale assembly of proteins. Nanoscale protein patterns can be beneficial to the further miniaturization of detection platforms. Dip-pen lithography and related scanning probe tip-based protein printing have been exploited to place proteins into nanometer scale areas on surfaces. Such serial approaches, in which proteins are written line-by-line onto solid surfaces via probe tips, permit a nanoscale positional control and provide smaller feature sizes for proteins compared to the parallel methods such as microcontact printing and microchannel networks. Despite the advantage, the practical application of these serial methods at large scales can be hampered by their low speed and time-consuming production.

Nanoscale assembly of proteins is also achieved by physical or chemical patterning of surfaces. In the former method, substrate surfaces are modified to inscribe topological patterns for subsequent protein binding. In the latter approach, selective sites of surfaces are chemically activated for subsequent protein attachment. Methods such as laser ablation [20, 21], reactive ion etching [22], and sputtering [23, 24] have been used for the physical alterations of the solid surfaces. Chemical patterning of substrates for protein adsorption has also been accomplished by the use of self-assembled monolayers [2, 25-29]. Despite these numerous efforts, significant challenges still exist in producing high-density, biologically active, surface-bound proteins rapidly at a large scale.

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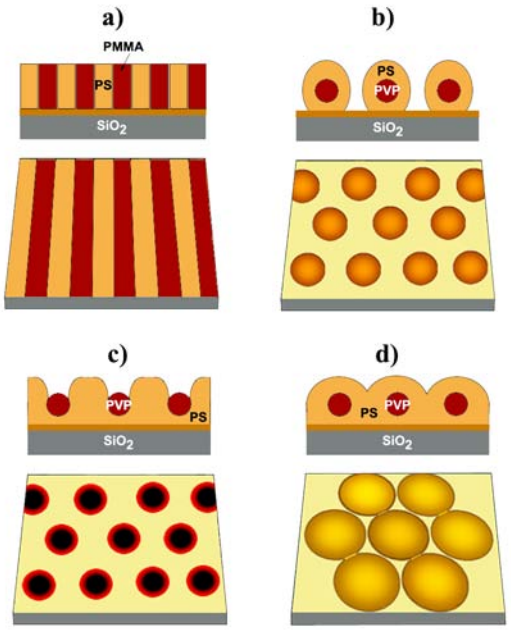

Fig.1 Nanoscale surface geometry of self organizing polymeric domains of (a) stripes in half cylinder-forming diblock copolymers and (b, c, and d) spheres in micelle-forming diblock copolymers. Adapted with permission from [30]. Copyright American Chemical Society.

III. ALTERNATIVE METHOD FOR PROTEIN SENSOR FABRICATION: NANOSCALE SELF ASSEMBLY

Distinct from the protein assembly methods described above, a bottom-up approach has also been proven to be successful in creating surface-bound proteins at a large scale in a straightforward and convenient manner. Self-assembly has been recently exploited to organize effectively proteins on nanoscale polymeric surfaces [30-34]. In these recent studies, both the underlying polymeric substrates as well as the proteins themselves are positioned via self-assembly where highly periodic and aligned patterns of proteins are instantaneously produced over a large area of substrates.

A class of polymeric materials called diblock copolymers is known to provide chemically heterogeneous, self-assembling surface structures through microphase separation. Diblock copolymers are formed by covalently joining two, chemically-immiscible, polymer blocks end-to-end. Due to the immiscibility and differential wetting properties associated with the two components of these materials, microphase separation occurs in diblock copolymer thin films in directions both perpendicular and parallel to the underlying support substrate [35-38]. The unique microphase separation behavior of a block copolymer, polystyrene-block-polymethylmethacrylate (PS-b-PMMA), has previously been shown to expose both block components to the air/polymer interface under carefully balanced thermodynamic conditions [39]. This phenomenon generates spatially periodic, self-assembled, nanoscale polymeric domains consisting of the different chemical constituents of the two polymeric components, whose scale and geometry reflect the chemical and physical properties of the polymer [40-42]. Their phase diagram dictates the packing nature and orientation of the resulting polymer chains where their microphase separation behavior is predictable based on a mean field theory [35, 37]. Therefore, the repeat spacing and surface geometry of the diblock copolymer can be controlled by changing the molecular weight and compositions of the two blocks. These chemically alternating and self-assembling polymeric domains can serve as convenient self-constructed templates for nanoscale arrangement of the desired biocomponents. Fig. 1 demonstrates some examples of such phase separating diblock copolymers which provide nanometer scale domains in their ultrathin films.

Recently, preferential interaction of several model proteins with PS and their selective segregation on the PS regions were monitored on the surface of phase-separated, PS-b-PMMA diblock copolymer ultrathin films [31]. No proteins were found on the neighboring PMMA areas. Proteins showing this behavior include bovine immunoglobulin G (IgG), fluorescein isothiocyanate (FITC) conjugated anti-bovine IgG, and protein G. When the protein loading condition was increased to a monolayer-forming concentration, the proteins exhibited close-packing behavior where they self-assembled themselves in a closely packed configuration on the PS domain in order to avoid the neighboring PMMA domain. In a follow-up study, protein adsorption experiments were carried out on both chemically homogeneous and heterogeneous supports, leading to the observation that protein density on the chemically heterogeneous PS-b-PMMA is larger than that on the chemically homogeneous, homopolymer surfaces of PS and PMMA by several fold [33]. They noted that more proteins adsorb on the diblock copolymer than on the PS or PMMA homopolymer, although approximately half of the exposed surface on the diblock copolymer consists of the non-preferred PMMA domains. The study concluded that the nanoscale chemical heterogeneity provided by the underlying PS-b-PMMA promotes protein adsorption more effectively than chemically homogeneous, homopolymer templates.

In addition to these methods for arranging proteins into periodic, one-dimensional stripes, mimicking the spot layout of conventional protein arrays via two-dimensional protein assembly was accomplished by using micelle-forming diblock copolymers. Amphiphilic polymeric systems such as polystyrene-*b*-polyacrylic acid, poly(ethylene-propylene)-b-polyethylene oxide, polystyrene-*b*-poly(2-vinylpyridine) and polystyrene-*b*-poly(4-vinylpyridine) were extensively studied to understand their fascinating micellar properties and dependence on diblock copolymer characteristics [43-46]. Micellar assembly, above a critical polymer concentration, is a well known behavior of such amphiphilic diblock copolymers. The exact structures and configurations of the resulting micelles or aggregates are length of each polymer segment, the polarity of the solvent,

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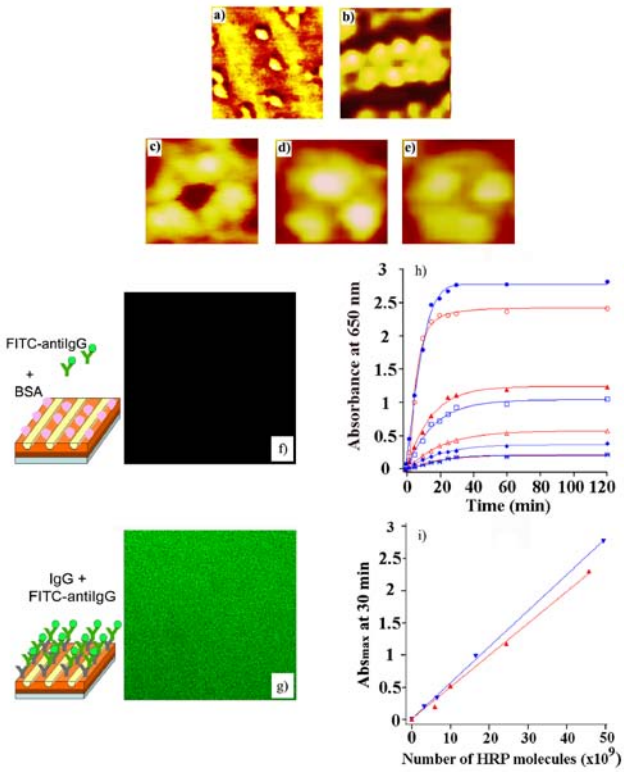

Figure 2. Atomic force microscope images of individual proteins of immunoglobulin G and mushroom tyrosinase that are self-assembled on nanodomains of PS-b-PMMA and PS-b-PVP diblock copolymer ultrathin films. (a) 150 by 150 nm phase, (b) 100 by 100 nm topography, and (c through e) 60 x 60 nm topography. Qualitative and quantitative activity measurements on proteins bound on PS-b-PMMA. Confocal fluorescence data are displayed for (f) the pair of fluorescein isothiocyanate conjugated anti-bovine IgG (FITC-antiIgG) and bovine serum albumin-incubated PS-b-PMMA, and (g) the pair of IgG and FITC-antiIgG on PS-b-PMMA. The two graphs of (h) and (i) display the quantitative results of horse radish peroxidase (HRP) activity difference measured between their free state (blue data) and PS-b-PMMA bound state (red data). When the enzymatic activity of the same number of HRP molecules in free- versus bound-state was compared, bound-state HRP retained approximately 85 % of its free-state activity. Adapted with permission from [30, 31] [32] and [34]. Copyright American Chemical Society.

and the relative solubility of each polymer block in the solvent.

These amphiphilic diblock copolymers can serve as extremely useful guides in organizing biomolecules into two-dimensional arrays since they exhibit a rich spectrum of morphologies and their repeat spacings are tunable in two dimensions. The advantages of amphiphilic diblock copolymers have been exploited to achieve rapid and large-scale self-assembly of two-dimensionally controlled protein arrays with periodic repeat spacing in nanoscopic dimensions [30]. The study demonstrated that polystyrene-*b*-poly(4-vinylpyridine) (PS-b-PVP) can be effectively used for the self-assembly of surface-bound, two-dimensional, nanoscale protein arrays. The study also established a straightforward method to produce protein patterns of different geometries and sizes by successfully manipulating topological structures of the underlying PS-b-PVP templates via various chemical treatments. Fig. 2 displays some examples of protein molecules arranged via self-assembly onto half cylinder- and micelle-forming diblock copolymer surfaces.

IV. CURRENT CHALLENGES

Ideal protein arrays for practical and clinically meaningful biosensing applications should be prepared for quantifiable, parallel, small-volume sensors that can be readily applied to large numbers of samples. They should also feature a reliable placement of protein molecules in a well-defined, highly dense pattern, while fully retaining their native functionality. Current challenges associated with the application of proteins printed on surfaces lie in the precise control over protein density, spot density, protein orientation, spotting uniformity, array standardization, array stability, surface fouling, and protein activity. Quantification of conventional assays, in which the exact number of biologically functional biomolecules participating in reactions can be easily and meaningfully compared between sensors, is also important but hard to achieve through conventional methods.

To date, easy fabrication of regularly-spaced protein platforms displaying high areal density and natural protein conformation is still extremely challenging. In addition to these general difficulties, inherent problems associated with manipulating small individual protein molecules on nanoscopic surfaces have restricted both the study and application of nanoscale protein arrays. Therefore, concerted research efforts are highly warranted in the future to facilitate high density protein assembly and to promote a high level of protein activity on solid surfaces. Improvements are also needed in conventionally available methods of sample delivery and optical signal detection. The development of an automated sample handling system and novel detection techniques, capable of processing a picoliter (or smaller) reaction volume and overcoming the optical diffraction limit, is also warranted. These advances will be particularly helpful to the application of the diblock copolymer-based nanoscale protein arrays in which the nanoscopic dimension of each spot in the sensor can be addressable as an independent detection unit.

V. OUTLOOK AND SUMMARY

Recent research efforts made in the areas of assembly, and applications of polymeric surface-bound proteins that are important both in sensor applications are considered in this article. Various methods to assemble proteins on polymeric surfaces both at macro/micro- and nano-scale are discussed and compared to one another. Current challenges and areas of further study are identified for the application of surface-bound proteins in the next-generation, solid-phase detection.

The presence of an underlying polymeric surface in solid-phase protein platforms adds an additional degree of

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complexity which necessitates more comprehensive, thorough, and systematic investigation in the identified areas of further study. The significance of the polymeric surface-bound proteins in many important applications as gene chips, protein array sensors, and lab-on-a-chip devices warrants such future investigation. Therefore, such efforts will be extremely beneficial to developing highly miniaturized, high density biosensors that also permit quantitative sensing of bioanalytes in a cost effective and straight forward manner.

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REFERENCES

[1] L. G. Mendoza, et al., "High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA)," Biotechniques vol 27, 1999, pp 778.

[2] K. Wadu-Mesthrige, S. Xu, N. A. Amro and G. Y. Liu, "Fabrication and imaging of nanometer-sized protein patterns," Langmuir vol 15, 1999, pp 8580-8583.

[3] S. A. Brooks, et al., "Segregation of micrometer-dimension biosensor elements on a variety of substrate surfaces," Anal. Chem. vol 72, 2000, pp 3253-3259.

[4] R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber and G. M. Whitesides, "Patterning proteins and cells using soft lithography," Biomaterials vol 20, 1999, pp 2363-2376.

[5] J. F. Mooney, et al., "Patterning of functional antibodies and other proteins by photolithography of silane monolayers," PNAS vol 93, 1996, pp 12287-12291.

[6] E. Blinka, et al., "Enhanced microcontact printing of proteins on nanoporous silica surface," Nanotechnology vol 21, 2010, pp 415302.

[7] J. Clemmens, et al., "Mechanisms of microtubule guiding on microfabricated kinesin-coated surfaces: Chemical and topographic surface patterns," Langmuir vol 19, 2003, pp 10967-10974.

[8] D. Falconnet, et al., "A novel approach to produce protein nanopatterns by combining nanoimprint lithography and molecular self-assembly," Nano Lett. vol 4, 2004, pp 1909-1914.

[9] J. Hyun and A. Chilkoti, "Micropatterning biological molecules on a polymer surface using elastomeric microwells," JACS vol 123, 2001, pp 6943-6944.

[10] D. T. Chiu, et al., "Patterned deposition of cells and proteins onto surfaces by using three-dimensional microfluidic systems," PNAS vol 97, 2000, pp 2408-2413.

[11] E. Delamarche, A. Bernard, H. Schmid, B. Michel and H. Biebuyck, "Patterned delivery of immunoglobulins to surfaces using microfluidic networks," Science vol 276, 1997, pp 779-781.

[12] N. Patel, et al., "Atomic force microscopic analysis of highly defined protein patterns formed by microfluidic networks," Langmuir vol 15, 1999, pp 7252-7257.

[13] A. A. Bergman, et al., "Nanometer-scale arrangement of human serum albumin by adsorption on defect arrays created with a finely focused ion beam," Langmuir vol 14, 1998, pp 6785-6788.

[14] J. Jiang, X. Li, W. C. Mak and D. Trau, "Integrated direct DNA/protein patterning and microfabrication by focused ion beam milling," Adv. Mater. vol 20, 2008, pp 1636-1643.

[15] G. M. Nishioka, A. A. Markey and C. K. Holloway, "Protein damage in drop-on-demand printers," JACS vol 126, 2004, pp 16320-16321.

[16] J. Sumerel, et al., "Piezoelectric ink jet processing of materials for medicaland biological applications," Biotechnol. J. vol 1, 2006, pp 976-987.

[17] J. R. Kenseth, J. A. Harnisch, V. W. Jones and M. D. Porter, "Investigation of approaches for the fabrication of protein patterns by scanning probe lithography," Langmuir vol 17, 2001, pp 4105-4112.

[18] K. B. Lee, J. H. Lim and C. A. Mirkin, "Protein nanostructures formed via direct-write dip-pen nanolithography," JACS vol 125, 2003, pp 5588-5589.

[19] K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith and M. Mrksich, "Protein nanoarrays generated by dip-pen nanolithography," Science vol 295, 2002, pp 1702-1705.

[20] A. M. Douvas, et al., "157-nm Laser ablation of polymeric layers for fabrication of biomolecule microarrays.," Anal. Bioanaly. Chem. vol 381, 2005, pp 1027-1032.

[21] E. P. Ivanova, et al., "Polymer microstructures fabricated via laser ablation used for multianalyte protein microassay," Langmuir vol 18, 2002, pp 9539-9546.

[22] V. C. Rucker, et al., "Functional antibody immobilization on 3-dimensional polymeric surfaces generated by reactive ion etching," Langmuir vol 21, 2005, pp 7621-7625.

[23] Z.-Y. Huang, M. Chen, S.-R. Pan and D.-H. Chen, "Effect of surface microstructure and wettability on plasma protein adsorption to ZnO thin films prepared at different RF powers," Biomed. Mater. vol 5, 2010, pp 054116.

[24] N. Aggarwal, K. Lawson, M. Kershaw, R. Horvath and J. Ramsden, "Protein adsorption on heterogeneous surfaces," Appl. Phys. Lett. vol 94, 2009, pp 083110.

[25] M. Mrksich and G. M. Whitesides, "Using self-assembled monolayers to understand the interactions of man made surfaces with proteins," Ann. Rev. Biophys. Biomol. Struct. vol 25, 1996, pp 55-78.

[26] M. Mrksich, G. B. Sigal and G. M. Whitesides, "Surface-plasmon resonance permits in-situ measurement of protein adsorption on selfassembled monolayers of alkanthiolates on gold. ," Langmuir vol 11, 1995, pp 4383-4385.

[27] K. L. Prime and G. M. Whitesides, " Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): A model system using self-assembled monolayers. ," J. Am. Chem. Soc. vol 115, 1993, pp 10714-10721.

[28] T. Wink, S. J. van Zuilen, A. Bult and W. P. van Bennekom, "Self-assembled monolayers for biosensors.," Analyst vol 122, 1997, pp 43R-50R.

[29] K. Wadu-Mesthrige, N. A. Amro and G. Liu, "Immobilization of Proteins on Self-Assembled Monolayers," Scanning vol 22, 2000, pp 380-388.

[30] N. Kumar, O. Parajuli and J. Hahm, "Two-dimensionally self-arranged protein nanoarrays on diblock copolymer templates," *J. Phys. Chem. B* vol 111, 2007, pp 4581-4587.

[31] N. Kumar and J. Hahm, "Nanoscale protein patterning using self-assembled diblock copolymers," Langmuir vol 21, 2005, pp 6652-6655.

[32] N. Kumar, O. Parajuli, A. Dorfman, D. Kipp and J. Hahm, "Activity study of self-assembled proteins on nanoscale diblock copolymer templates," Langmuir vol 23, 2007, pp 7416-7422.

[33] N. Kumar, O. Parajuli, A. Gupta and J. Hahm, "Elucidation of protein adsorption behavior on polymeric surfaces: Towards high density, high payload, protein templates," Langmuir vol 24, 2008, pp 2688-2694.

[34] O. Parajuli, A. Gupta, N. Kumar and J. Hahm, "Evaluation of enzymatic activity on nanoscale PS-b-PMMA diblock copolymer domains," *J. Phys. Chem. B* vol 111, 2007, pp 14022-14027.

 ISBN: 978-1-61208-145-8

[35] F. S. Bates and G. H. Fredrickson, "Block copolymer thermodynamics - Theory and experiment," Annu. Rev. Phys. Chem. vol 41, 1990, pp 525-557.

[36] B. L. Carvalho and E. L. Thomas, "Morphology of steps in terraced block-copolymer films," Phys. Rev. Lett. vol 73, 1994, pp 3321-3324.

[37] K. R. Shull, "Mean-field theory of block copolymers: bulk melts, surfaces, and thin films," Macromolecules vol 25, 1992, pp 2122-2133.

[38] R. Nagarajan and K. Ganesh, "Block copolymer self-assembly in selective solvents: Spherical micelles with segregated cores," J. Chem. Phys. vol 90, 1989, pp 5843-5856.

[39] T. L. Morkved, W. A. Lopes, J. Hahm, S. J. Sibener and H. M. Jaeger, "Silicon nitride membrane substrates for the investigations of local structures in polymer thin films," Polymer vol 39, 1998, pp 3871.

[40] J. Hahm, W. A. Lopes, H. M. Jaeger and S. J. Sibener, "Defect evolution in ultrathin films of polystyrene-block-polymethylmethacrylate diblock copolymers observed by atomic force microscopy," J. Chem. Phys. vol 109, 1998, pp 10111-10114.

[41] J. Hahm and S. J. Sibener, "Cylinder alignment in annular structures of microphase-separated polystyrene-b-poly(methyl methacrylate)," Langmuir vol 16, 2000, pp 4766-4769.

[42] J. Hahm and S. J. Sibener, "Time-resolved atomic force microscopy imaging studies of asymmetric PS-b-PMMA ultrathin films: Dislocation and disclination transformations, defect mobility, and evolution of nanoscale morphology," J. Chem. Phys. vol 114, 2001, pp 4730-4740.

[43] A. Choucair and A. Eisenberg, "Control of amphiphilic block copolymer morphologies using solution conditions," Eur. Phys. J. E. vol 10, 2003, pp 37-44.

[44] A. Choucair, C. Lavigueur and A. Eisenberg, "Polystyrene-b-poly(acrylic acid) vesicle size control using solution properties and hydrophilic block length," Langmuir vol 20, 2004, pp 3894-3900.

[45] S. N. Sidorov, et al., "Influence of metalation on the morphologies of poly(ethylene oxide)-block-poly(4-vinylpyridine) block copolymer micelles," Langmuir vol 20, 2004, pp 3543-3550.

[46] L. Zhang, K. Yu and A. Eisenberg, "Ion-induced morphological changes in ``crew-cut'' aggregates of amphiphilic block copolymers," Science vol 272, 1996, pp 1777-1779.

 ISBN: 978-1-61208-145-8