Recent Advances Towards Single Biomolecule Level Understanding of Protein Adsorption Phenomena Unique to Nanoscale Polymer Surfaces with Chemical Variations

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ABSTRACT

Protein adsorption onto polymer surfaces is a very complex and ubiquitous phenomenon whose integrated process impacts essential applications in our daily lives such as food packaging materials, health devices, diagnostic tools, and medical products. Increasingly, novel polymer materials with greater chemical intricacy and reduced dimensionality are used for various applications involving adsorbed proteins on their surfaces. Hence, the nature of protein-surface interactions to consider is becoming much more complicated than before. A large body of literature exists for protein adsorption. However, most of these investigations have focused on collectively measured, ensemble-averaged protein behaviors that occur on macroscale and chemically unvarying polymer surfaces instead of direct measurements at the single protein or sub-protein level. In addition, interrogations of protein-polymer adsorption boundaries in these studies were typically carried out by indirect methods, whose insights may not be suitably applied for explaining individual protein adsorption processes occurring onto nanostructured, chemically varying polymer surfaces. Therefore, an important gap in our knowledge still exists that needs to be systematically addressed via direct measurement means at the single protein and sub-protein level. Such efforts will require multifaceted experimental and theoretical approaches that can probe multilength scales of protein adsorption, while encompassing both single proteins and their collective ensemble behaviors at the length scale spanning from the nanoscopic all the way to the macroscopic scale. In this review, key research achievements in nanoscale protein adsorption to date will be summarized. Specifically, protein adsorption studies involving polymer surfaces with their defining feature dimensions and associated chemical partitions comparable to the size of individual proteins will be discussed in detail. In this regard, recent works bridging the crucial knowledge gap in protein adsorption will be highlighted. New findings of intriguing protein

surface assembly behaviors and adsorption kinetics unique to nanoscale polymer templates will be covered. Single protein and sub-protein level approaches to reveal unique nanoscale protein-polymer surface interactions and protein surface assembly characteristics will be also emphasized. Potential advantages of these research endeavors in laying out fundamentally guided design principles for practical product development will then be discussed. Lastly, important research areas still needed to further narrow the knowledge gap in nanoscale protein adsorption will be identified.

Keywords: Nanoscale protein adsorption, Nanoscale protein assembly, Protein self-assembly on polymer, Protein nanopatterning, Protein-nanosurface interaction

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1 INTRODUCTION

The nature of protein interactions with various polymeric surfaces impacts many essential application areas such as food processing and packaging, health devices, diagnostic tools, and medical products [1-11]. For example, unwanted adsorption of proteins on dairy containers and contact lenses can lead to severe health consequences including food-borne illnesses and eye infections, respectively. Insight into adsorption properties of various proteins to different polymeric surfaces can guide the material choice for safer food packaging and human-aid products. On the other hand, for rapid and simultaneous diagnostics and detection, solid state arrays such as protein chips and microarrays are favored over their traditional counterparts that require a large volume of reagents and can detect only one sample at a time [12-19]. Microarray surfaces typically need to be premodified with specific proteins that will react only with target analytes. Therefore, understanding protein interaction with various surfaces is crucial for developing new protein array applications. In biomaterials and tissue engineering, the processes of protein adsorption that are

known to first occur onto the surfaces of biodevices and biomaterials affect subsequent cell growths and cellular response behaviors. Hence, precise understanding of protein adsorption to solid surfaces is central to the integration of implant materials [2, 4-7, 9, 10] and cell response to those materials [20-22].

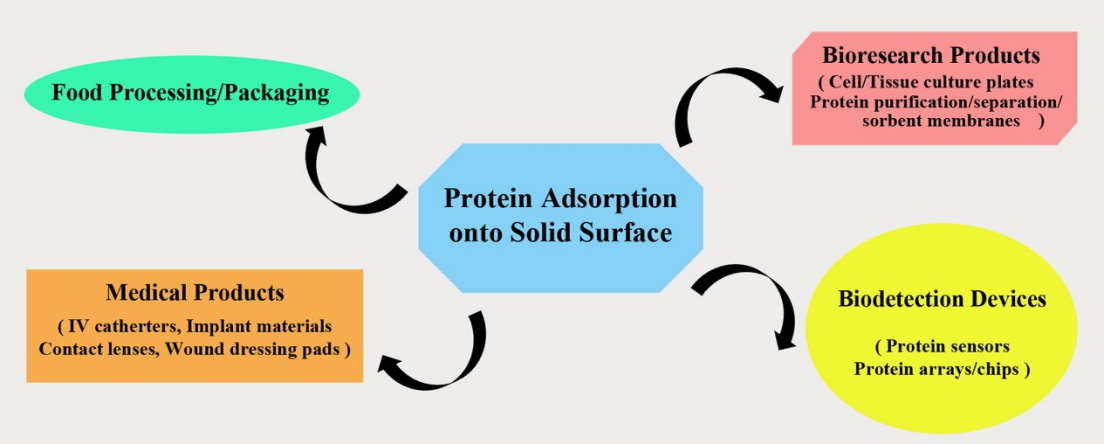

Figure 1. Examples of every day applications that rely on the important phenomenon of protein adsorption onto solid surfaces.

Figure 1 depicts common practical examples whose functions and developments crucially rely on thorough understanding of protein-polymer surface interactions. With such widespread impact on everyday products directly linked to human health, protein adsorption onto polymer surfaces has remained an active subject of investigation for many decades [1-10]. However, this common phenomenon is a very complex and integrated process that cannot be explained in a simple manner [1, 23]. Moreover, the nature of the interactions between proteins and surfaces is becoming much more complicated nowadays with the continuous development of new interfaces

and low dimensional materials. For example, a bone implant material with taurocholic acid-doped, nano-architectured polypyrrole was developed to control adsorption of proteins as well as subsequent cell adhesion and spreading on the material surface [24]. Another example is a microarray constructed from nanoscale polymer brush of poly(oligo(ethylene glycol) methacrylate) which enabled femtomolar immunoassay detection of various protein biomarkers from a drop of blood [25]. Such growing applications of compact polymeric materials with increasing chemical complexity in biomaterials, implant devices, and tissue engineering platforms underscore the importance of understanding the characteristic assembly and packing behaviors of proteins unique to nanoscale surfaces [26-32]. Yet, our fundamental understanding of protein-surface interaction is still very limited, and many challenges lie ahead for gaining precise control over protein adsorption and assembly on surfaces. Therefore, protein-polymer surface interactions need to be systematically examined, especially pertaining to nanoscale, chemically varying polymer surfaces. To this end, investigations that provide insight into the individual biomolecular and sub-biomolecular level adsorption behaviors are highly warranted. Many recent studies indicate that protein adsorption on chemically varying, nanoscale polymer surfaces show distinct behaviors which cannot be explained by solely relying on ensemble-averaged protein behaviors measured on chemically homogeneous surfaces. Truly nanoscale mechanistic insights into protein adsorption on polymeric surfaces examined at or below the single protein level can help provide a new set of fundamental knowledge which can be directly and effectively used to control protein assembly and packing behaviors on chemically complex polymer platforms exhibiting nanoscopic surface features. The efforts may lead to entirely new mechanisms and kinetics better geared for the explanation and prediction of single and multicomponent protein adsorption to polymer surfaces with nanoscopic features and chemical variability.

2 Protein Adsorption on Polymer Surfaces: Nano- versus Macro-Surfaces

The characteristic size of many proteins is on the nanometer scale. In order to describe protein adsorption behaviors observed from different polymer surfaces whose length scale is comparable to or much greater than the dimensions of single proteins, terms such as nano- and macro-surfaces will be used in this Review. 'Nano-surfaces' refer to protein adsorption templates whose surface features are on the size scale of discrete proteins and, at the same time, their surface chemical compositions change at a spatial interval comparable to the length scale of individual proteins. In contrast, 'macro-surfaces' indicate polymer templates of uniform surface features and homogeneous chemical compositions that persist over distances much greater than the size of individual proteins.

2.1 Block Copolymers as Nanoscale Template Surfaces.

A particular category of polymers called block copolymers (BCPs) contain covalently linked, two or more chemically immiscible polymer blocks whose phase separation processes yield well-predictable and well-controllable nanoscale surface morphologies via self-assembly [33-36]. It is well known that, after phase separation, BCPs yield nanoscale polymer domains which are consisted of periodically arranged, alternating segments of the chemically distinct polymer blocks in the BCPs. The thermodynamically governed phase separation of many BCPs has been well-studied and extensively reviewed [33-36]. The wealth of knowledge on BCP systems has been used to control the dimensions as well as the geometries of phase-separated nanodomains by changing parameters such as the molecular weight, volume fraction, and chemical composition of the polymer blocks in BCPs [37-43]. Figure 2 displays representative phase diagrams for a linear *a-b* BCP and the three dimensional (3D) nanostructures that can be formed from the BCP based

on a mean field theory. In addition to the thermodynamically accessible geometries, additional nanostructures can also be made available by kinetically controlling the nanodomain formation processes of BCPs via selective vapor exposure [44-47]. This offers even greater versatility in the surface nanofeature size and shape that can result from the self-assembly of BCPs. The ordered nanodomain structures of BCPs can be produced instantly over a large area on the template surface without the requirement of highly costly and time-consuming nanolithography and nanofabrication procedures. This, in turn, can open up the possibility for a straightforward, bottom-up assembly method in creating protein nanoconstructs of tunable feature sizes and shapes. The characteristic dimensions associated with BCP nanodomains are also comparable to the length scale of individual proteins which can be tailored to match the size of a particular protein. Hence, BCPs offer many exciting possibilities as nanoscale templates on which tailored protein nanopatterns can be built for the different applications shown in Figure 1. BCPs are also ideally suited for fundamental studies that aim to characterize distinct and preferential adsorption behaviors of individual proteins. BCPs can be also used to reveal protein subdomain-specific interaction preferences by exploiting the chemically different BCP blocks, i.e. BCP nanodomains, that are spatially arranged to match the distances between protein subunits of interest.

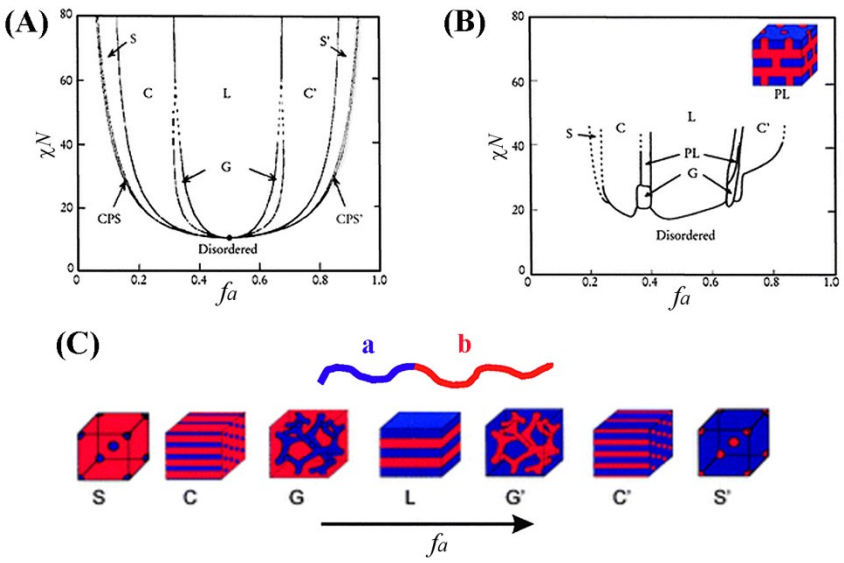

Figure 2. Phase diagram of a linear *a*-*b* block copolymer. **(A and B)** The diagram in (A) is a theoretical phase prediction based on a self-consistent mean-field theory and the diagram in (B) is the experimental phase portrait of poly(isoprene-styrene) block copolymers. χ, N, and f_a refer to the segment-segment interaction parameter, the degree of polymerization, and the volume fraction of the *a* segment, respectively. **(C)** The morphologies of CPS, S, C, G, L, and metastable PL shown in the phase diagrams correspond to close packed sphere, sphere, cylinder, gyroid, lamellar, and perforated layer, respectively. Images in (A and C) and (B) are reproduced with permission from Ref. [48] Copyright (1999) AIP Publishing LLC and Ref. [49] Copyright (1995) American Chemical Society, respectively.

2.2 Experimental Techniques for Examining Protein Adsorption

Common experimental techniques employed for protein adsorption studies are summarized in Table 1. Many of these measurement tools, such as those relying on changes in resonance frequency [50, 51], infrared absorption frequency [51-53], fluorescence intensity [54-57] and

refractive index [50, 58, 59], are more suited for interrogating collective protein behaviors. Hence, protein adsorption studies in the past had to often deduce protein signals indirectly from spectroscopic or optical signals and single protein-level adsorption behaviors could only be inferred from ensemble-averaged data, instead of directly monitoring individual proteins. Yet, conclusions drawn from interpreting indirect signals may not always reflect accurate adsorption behaviors and single protein behaviors can depart significantly from their collective attributes [60-62]. Moreover, on nanoscale polymeric surfaces with their topological and chemical features comparable to the size of individual proteins, different protein subdomains of the same protein molecule may exhibit varying degrees of interaction preferences to the distinct regions of the polymer surfaces. Such crucial information on nanoscale protein adsorption may not be accurately captured by an indirect measurement approach based on ensemble-averaged data collection. For these reasons, new research efforts have begun to obtain direct and definitive experimental proof of protein adsorption determined at the single biomolecule level.

Although a variety of techniques such as ellipsometry, quartz crystal microbalance, surface plasmon resonance, fluorescence microscopy, and X-ray photoelectron spectroscopy can be potentially used to study protein adsorption as discussed earlier [50-59, 63], atomic force microscopy (AFM) can provide unparalleled spatial resolution sufficient for investigating nanoscale protein adsorption. Examination of individual proteins and their static or kinetic assembly on nanostructured polymer surfaces requires simultaneous imaging of the single proteins and underlying polymer nanodomains, all exhibiting tens of nm in size [27, 64]. Hence, AFM was utilized as a choice measurement technique to interrogate protein adsorption behaviors at or below the single biomolecule level, while maintaining sub-nanometer spatial resolution. Unlike electron microscopy (EM) or X-ray diffraction (XRD) methods, AFM permits direct probing of proteins

and polymers without the need for special sample preparation procedures such as crystallization, labelling, conductive metal coating, and high vacuum/low temperature operation [65-68]. Even the very early stage adsorption characteristics involving only a few to several protein molecules, which cannot be readily detected in a truly label-free manner otherwise without introducing large errors, can be effectively examined by AFM. In addition, AFM can be used to repeatedly track the same surface locations over time to monitor adsorption kinetics of not only single but also multiple protein components. This is another distinct advantage of AFM. It can offer time-dependent adsorption data associated directly with a particular protein component of interest in a multicomponent protein adsorption scenario by delineating each protein kind by its distinct size and shape. In contrast, alternative techniques relying on the optical and spectroscopic means are faced with significant challenges in delineating the exact signal contributions specific to each protein kind, even with the help of complex labelling strategies and sophisticated signal interpretation routines [50-59].

Measurement Methods	**Measured Elements** (ensemble-averaged (E), single (S), subdomain (SD) protein properties)	**Advantages / Disadvantages**	**Ref.**
UV-visible Spectroscopy (UV-vis)	transmitted UV-vis light, absorption after chromogenic assay (E)	low cost, fast, commonly available / need for chromogenic assay, solution conditions for staining, low sensitivity	[69]
Fourier Transform Infrared Spectroscopy (FTIR)	transmitted IR light, protein secondary structure (E)	analysis of protein secondary structure / need for large sample amounts, complex data analysis	[51-53]
Fluorescence Microscopy Total Internal Reflectance Fluorescence (TIRF)	fluorescence from fluorophores, typically (E) but some super-resolution fluorescence spectroscopy used for (S)	good sensitivity, multiplexed detection for competitive adsorption / need for fluorophore labels, potential influence of fluorophore tags on native protein conformation and function, fluorophore bleaching	[54-57]

Optical Waveguide Lightmode Spectroscopy (OWLS)	transverse electric and magnetic modes excited in the waveguide (E)	label free, real time / difficult data interpretation	[70-72]
Ellipsometry	polarization change of reflected light, information on layer thickness (E)	low cost, fast, commonly available / only for flat surfaces with homogeneous protein layers	[50, 58, 59]
X-ray Photoelectron Spectroscopy (XPS), X-ray Photoemission Electron microscopy (X-PEEM)	core electrons, chemical elements (E)	quantitative chemical component analysis / complicated analysis for quantification	[28, 30, 73-76]
Small Angle X-ray or Neutron Scattering (SAXS, SANS)	chemical components (E)	label-free, quantitative analysis of adsorbed proteins / difficult data interpretation, special facility needed for neutron	[77]
Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS)	mass of molecular fragments (E)	high surface sensitivity, quantification of protein mixtures / low-mass fragment detection only, no information about absolute protein amounts, destructive to sample	[74]
Sum Frequency Generation (SFG)	chemical bonds (E)	surface orientation and conformation of protein / complex data analysis	[78, 79]
Surface Plasmon Resonance (SPR)	surface plasmon (E)	high sensitivity, real time / protein detection on limited sensor surfaces and thicknesses only, complex data analysis	[80-84]
Reflectometric Interference Spectroscopy (RIfS)	reflection of white light (E)	high sensitivity, label-free, real time measurements for binding kinetics / only for sample on a transparent substrate	[85]
Quartz Crystal Microbalance (QCM)	frequency of a quartz crystal, dissipation related to shear viscous loss (E)	good sensitivity, real time measurements for binding kinetics / need for mass-increasing tags for low amount detection	[50, 51]
Scanning Electron Microscopy, Transmission Electron Microscopy (SEM, TEM)	secondary or transmitted electrons (S, SD)	superb spatial resolution / need for heavy element or chemical staining, microtoming of sample, cryogenic freezing for cryo-EM, sample preparation and vacuum operation significantly affecting protein structure and native binding property	[86-89]
	forces between the tip and sample, direct visualization of single proteins or protein subdomains, secondary	superb spatial resolution, label-free measurements in near physiological conditions, in situ or time lapse AFM for pseudo real-time detection / not suitable for adsorption conditions	[27, 65-68, 90]

Atomic Force Microscopy (AFM)	structure elements, forces driving protein folding/binding/adsorption processes (S, SD)	interfering tip operation such as thick protein layers and highly viscous media	

Table 1. Common experimental techniques used to characterize protein adsorption on solid surfaces.

3 Discovering Single-Component Protein Adsorption Behaviors on Nanoscale Polymer Surfaces

3.1 Nanoscale Adsorption Characteristics of Single-Component Protein Systems

Spearheading research efforts were launched to ascertain distinct protein adsorption characteristics on nanoscale polymer surfaces for which various single-component protein systems were tested as adsorbates [11, 27, 30, 64, 66, 91, 92]. Model proteins employed in the studies are listed in Table 2 along with key physical and biological properties as well as biomedical relevance.

Protein Name (Protein Data Bank ID)	**Molecular Weight / Isoelectric Point**	**Dimensions** determined by a) XRD b) EM c) AFM	**Biomedical Relevance and Use [Ref.]**
lysozyme (2LYZ)	14 kDa / 9.5-11	3 nm x 4.5 nm [a)]	antimicrobial enzyme, model protein in basic research due to its small size [93, 94]
hydrophobin I (2FMC)	7-9 kDa / 5.7	N/A	antifouling biocoating [95, 96]
BSA (4F5S) HSA (4G04)	47 kDa / 4.7 67 kDa / 4.7	9 nm x 12 nm [c)]	54% of human blood proteins, device surface passivation, serum substance carrier in cell culture [97]
peroxidase (2ATJ) tyrosinase (3NM8)	44 kDa / 7.2 120 kDa / 4.7-5.3	6 nm x 8 nm [a)] 8 nm x 9 nm [a)]	amplified protein detection array, protein sensor fabrication, enzyme linked immunosorbent plates [98]
IgG (1IGT)	150 kDa / 6.1-8.5	10 nm x 15 nm [a)]	antibody array, protein chip, 38% of human blood protein compositions [99]

ferritin (6MSX)	474 kDa / 5.5	12 nm x 12 nm [a)]	intracellular iron storage protein, nonspecific marker of illness including infections and cancer [100]
Fg (3GHG)	340 kDa / 5.1-6.3	7 nm x 45 nm [a),b)]	7% of blood protein, topical patch for wound healing, biocompatible coatant, tissue engineering, organ scaffold [101]
fibronectin (A chain, 1FNF)	440 kDa / 5.5-6.0	50 nm x 50 nm [c)]	fibronectin coatants for osteoblastic differentiation and implant osseointegration, cell adhesion and cell interaction substrate [102, 103]

Table 2. Proteins previously examined for nanoscale adsorption behaviors.

High resolution imaging technique of AFM was successfully used for direct visualization of individual protein adsorption events onto polymer surfaces exhibiting nanoscopic topological features and chemical variability. Direct morphological investigations of globular proteins such as immunoglobulin G (IgG) and bovine as well as human serum albumin (BSA/HSA) were carried out at the single biomolecule level after proteins' self-assembly onto a BCP surface of polystyrene-block-polymethylmethacrylate (PS-b-PMMA) containing periodic nanodomains of alternating PS and PMMA nanostrips with a repeat distance of 45 nm [91]. The nanodomain structures on the PS-b-PMMA surface before protein adsorption are displayed in Figure 3(A). With subsequent protein adsorption, both IgG and BSA proteins showed highly selective and preferential interactions with the PS block on the nanopatterned BCP surface, Figure 3(B). It was in fact determined that IgG and BSA adsorption was entirely exclusive to the PS nanodomain of the BCP with none found on the PMMA nanodomain. AFM data in Figure 3(B) show the highly discriminatory adsorption of IgG to the PS nanodomain areas on PS-b-PMMA. However, when the BCP surface was replaced with a homopolymer surface containing either of the two chemical

blocks in the BCP, the same proteins were found to readily adsorb not only on the PS but also on the PMMA homopolymer surfaces [104]. These studies revealed a novel adsorption behavior that could not be identified from the above-discussed indirect measurement techniques and demonstrated that the presence of the nanoscale BCP surface can trigger entirely discriminatory interactions of the globular proteins and their complete self-partition to the favored PS nanodomain areas of the BCP surface. Contrary to the results on the BCP, this highly selective adsorption behavior of the proteins did not manifest on the homopolymer surfaces devoid of nanoscale topological features and chemical variabilities [27, 105]. These intriguing findings underscored the fact that protein adsorption characteristics onto nanoscale, chemically varying BCP surfaces are indeed drastically different than those previously reported on macroscopic, chemically uniform surfaces.

Adsorption behaviors of IgG and BSA/HSA on hydrated and dried polymer surfaces of PS-b-PMMA as well as PS/PMMA blend were also studied by using combined measurement techniques which included the use of AFM along with surface plasmon resonance (SPR) and the employment of synchrotron based X-ray photoemission electron microscopy (X-PEEM) along with scanning transmission X-ray microscopy (STXM) [75, 76, 83, 84]. The STXM data in Figure 3(C) display the surface adsorption profiles of HSA on a PS/PMMA blend surface obtained after post-measurement processing of the carbon (C) 1s signal to estimate the contributions from each polymer block in the blend as well as the signal from the protein. The data indicated that HSA bound on the PS region remained on the surface even after rinsing and drying of the sample, indicating persistent interaction with the protein with PS. In contrast, HSA adsorbed on the hydrated PMMA region underwent desorption from the surface. AFM force spectroscopy was also employed to quantify protein-polymer adhesion forces after coating an AFM tip with a protein

of interest [106]. When protein-polymer adhesion forces were probed with a AFM tip coated with fibronectin, BSA, or collagen, the adhesion forces were reported to increase for a BCP surface made out of PMMA and polyacrylic acid (PAA) blocks relative to a PMMA homopolymer surface, Figure 3(D). Adsorption characteristics of other globular proteins such as lysozyme, peroxidase, tyrosinase, ferritin, and fibronectin have been also assessed by AFM on the BCP of PS-b-PMMA to understand the effect of the size and overall charge of the protein on its nanoscale adsorption behaviors [91, 104, 105, 107-109]. All of these globular proteins exhibited the same PS-exclusive adsorption behaviors on the BCP as discussed above, regardless of the protein size and the overall surface charge on the protein.

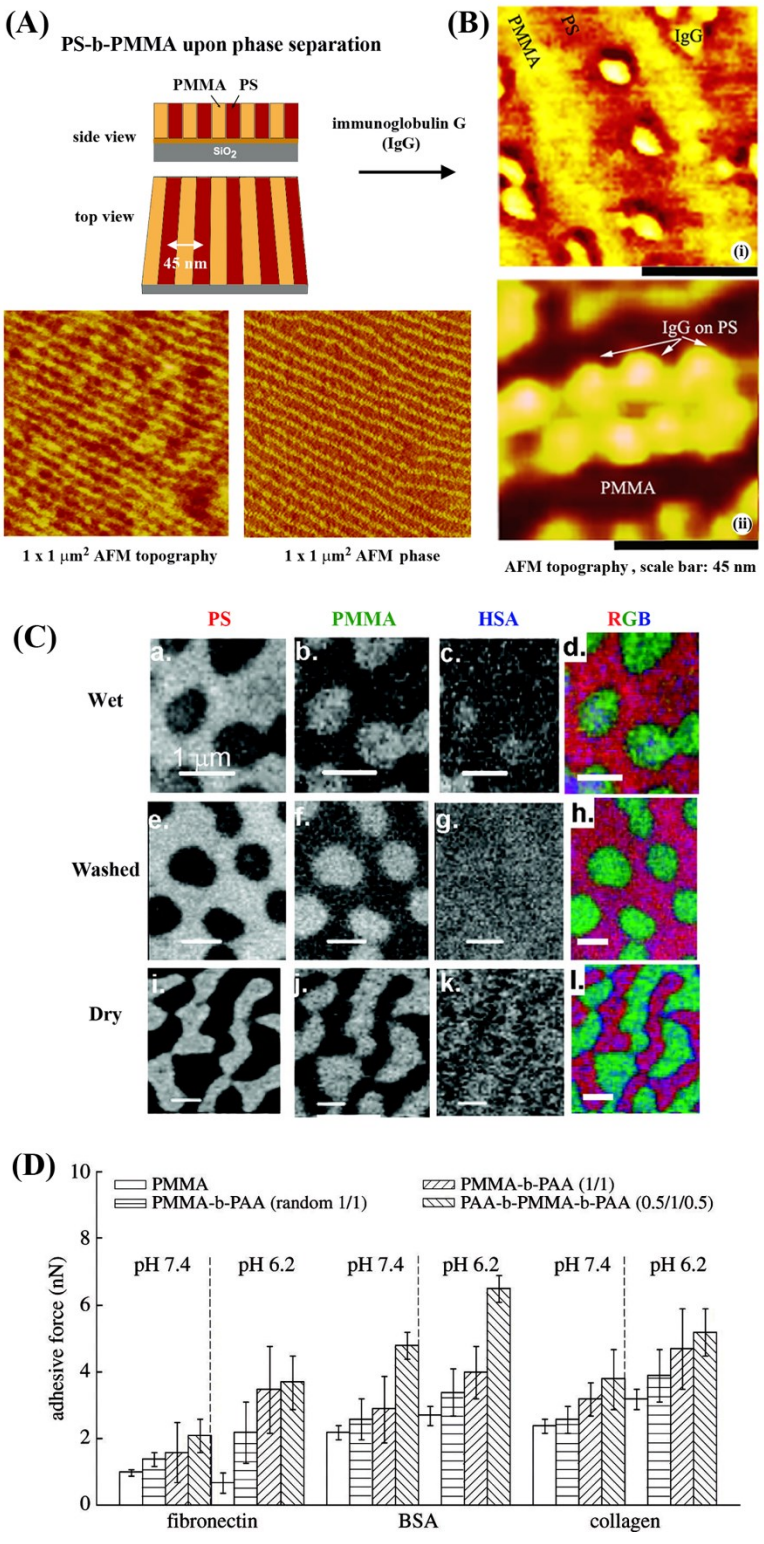

Figure 3. (A) Self-assembled nanoscale PS-b-PMMA surface exhibits alternating PS and PMMA nanodomains with a repeat spacing of 45 nm as measured by AFM. The resulting BCP template

exposes periodic nanostrips of PS and PMMA on its surface. In both AFM topography and phase panels, the lighter and darker area corresponds to the PMMA and PS nanodomain, respectively. **(B)** The BCP substrate was immersed in a protein solution of IgG for a specific period of time to allow protein self-assembly onto the different polymer nanodomains. Subsequent AFM investigation of the IgG molecules on the BCP surface clearly revealed highly preferential interaction of IgG with the PS nanodomain. Individual IgG molecules appear as spherical objects on the fingerprint-like BCP nanodomains of 45 nm in periodicity. For the monolayer-forming coverage displayed in (ii) where all available PS nanodomains are fully occupied by IgG, the protein molecules tightly pack in a highly ordered fashion on the PS nanodomains. Two IgG molecules tend to adsorb along the short axis of the PS domain in a densely packed, pseudo two-dimensional (2D) crystal protein arrangement. All scale bars shown are 45 nm. **(C)** Component maps derived from C 1s STXM image sequences are from fully hydrated, washed, and dried wet cells of HSA adsorbed to a PS/PMMA thin film. **(D)** Average adhesion forces between the different polymer surfaces and protein-modified AFM tips as annotated are determined by AFM force spectroscopy whose results are shown in the bar graphs. Images in (A, B), (C), and (D) are reproduced with permission from Ref. [91] Copyright (2005) American Chemical Society, Ref. [76] Copyright (2009) American Chemical Society, and Ref. [106] Copyright (2010) The Royal Society, respectively.

Interaction forces such as van der Waals (dispersion), electrostatic, hydrogen bonding, and hydrophobic forces as shown in Figure 4(A and B) are known to be responsible for protein adsorption to a solid surface [5, 110-115]. Among these forces, it was determined that the entirely

selective surface partition of the globular proteins on the PS over PMMA areas of the BCP of PS-b-PMMA was driven by hydrophobic interactions with negligible contributions from other interaction forces [27, 91, 105, 116]. The important role that hydrophobic interactions play in protein adsorption to solid surfaces has been previously reported for macroscopic, chemically uniform surfaces [117-120]. However, subsequent studies found that protein interactions at nanoscale polymer interfaces could not be explained simply by only considering a favored protein interaction with a more hydrophobic BCP block. It was revealed that delicate balances between multiple interaction forces should be carefully considered to explain nanoscale adsorption behaviors of non-globular proteins [64, 121].

For an elongated protein of fibrinogen (Fg) whose length matches well with the periodicity of the alternating chemical interfaces between the PS and PMMA nanodomains on the PS-b-PMMA with a 45 nm repeat spacing, protein adsorption behaviors were observed to be more neutral to the two polymer blocks, depending on Fg concentration [64]. Fg displayed strongly biased adsorption to PS only at high protein concentrations (above tens of μg/mL) whereas, at lower concentrations, some population of the adsorbed Fg interacted with both the PS and PMMA nanodomains in a protein subunit-specific fashion. At low surface coverage, different protein subunits of Fg, i.e. D, E, and αC subdomains present within a Fg molecule, exhibited subdomain-specific preferences to either PS or PMMA due to the electrostatic interplay between the different protein subunits and the underlying chemical blocks on the BCP [64]. These concentration-dependent Fg adsorption behaviors that are drastically different from those of the globular proteins on the same BCP template are summarized in Figure 4. AFM data in Figure 4(C-F) show the placement of a single Fg molecule over both PS and PMMA (D domain to PS and E domain to PMMA) on the BCP at low concentration regimes. With increasing protein concentration and

more Fg molecules being adsorbed on the BCP surface in Figure 4(G-I), the nature of protein-polymer interactions change where hydrophobic interactions become the major driving force for adsorption and the entire backbone of Fg is confined to the PS region only [64].

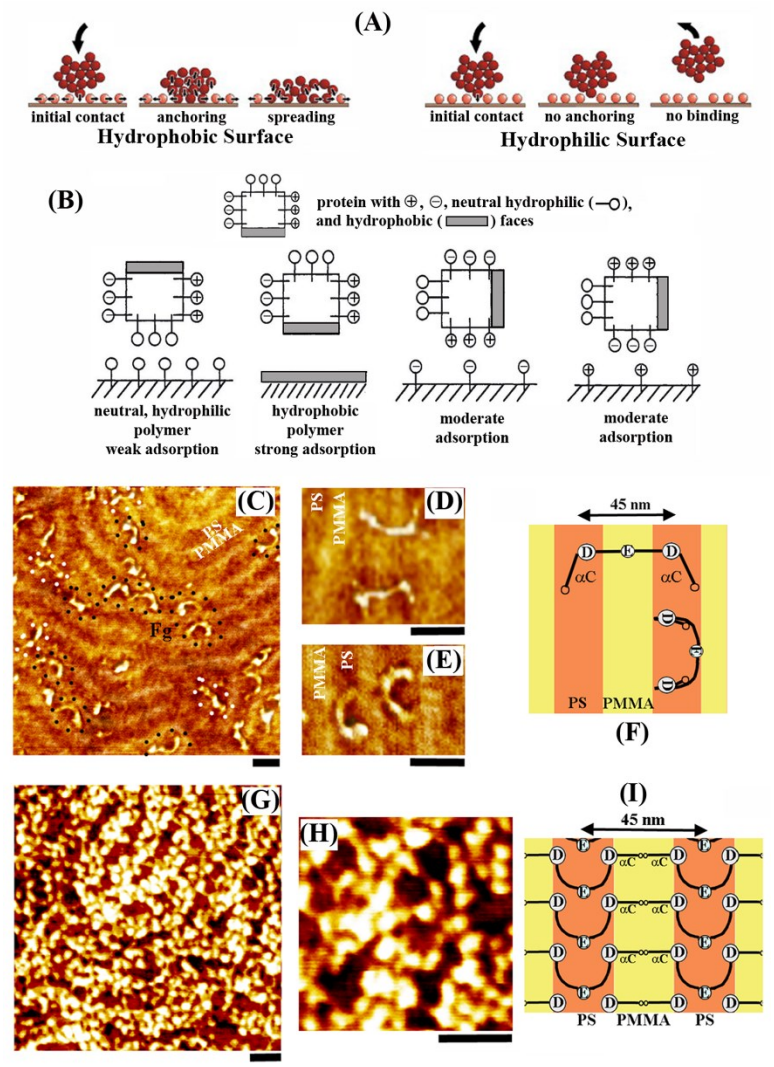

Figure 4. (A) Schematic illustration hypothesizing protein adsorption and desorption processes on a hydrophobic versus hydrophilic surface over time. **(B)** Schematic view depicting interactions between different surfaces with a four-sided model protein whose faces contain a hydrophobic, negatively charged, positively charged, and neutral hydrophilic character on each side. Images in (A, B) reproduced with permission from Ref. [122] Copyright (1992) IUPAC and Ref. [123]

Copyright (2011) Elsevier B.V., respectively. **(C-F)** AFM images of Fg on a fingerprint-like, 45 nm PS-b-PMMA revealing Fg subdomain-specific interactions with the underlying PS and PMMA nanodomains. At low surface coverage, Fg interaction is more neutral to the two polymer blocks, unlike the highly exclusive affinity of globular proteins to PS. Fg population circled in white dots display the placement involving both PS and PMMA nanodomains for the adsorption of a single Fg molecule, as shown in (D). Other Fg population indicated with black dots are located only on the PS area, as displayed in (E). The two different Fg adsorption cases are schematically shown in (F). **(G-I)** At higher coverage, the adsorption and assembly of Fg backbone are found to take place entirely on the PS nanodomains by orienting the protein backbone perpendicular to the long axis of the polymer nanodomains (i.e. 'side-on' Fg packing). All scale bars shown are 45 nm. Images in (C-I) reproduced with permission from Ref. [64], Copyright (2014) American Chemical Society.

3.2 Importance of Nanotemplate Length Scales and Orientation in Protein Adsorption

It was discovered that co-occurrence of hydrophobicity and hydrophilicity owing to the chemically distinct polymer blocks in BCPs and the associated chemical interfaces present at a nanoscale interval on the BCP surfaces play an important role in protein adsorption. The total number of adsorbed proteins as well as their tight packing behaviors were significantly affected by the presence of chemical interfaces on the polymer nanotemplates [64, 121]. For example, the BCP surface of PS-b-PMMA exhibits PS:PMMA interfaces whose distance is defined by characteristic repeat spacing of the periodically alternating PS and PMMA nanodomains. As

proteins are intrinsically amphiphilic adsorbers whose outer surfaces contain amino acid moieties with varying hydro-philicity/phobicity and charges [27, 104, 124], the more chemically rich, BCP interfacial regions may provide more favorable and stable binding environments towards a greater fraction of amino acid residues on the protein exterior. Thus the PS regions immediate to the PS:PMMA interfaces on either sides of a given PS nanodomain may be energetically favored for protein adsorption than the center region of the same PS nanodomain. Indeed, highest numbers of adsorbed proteins were observed to be near the chemical interfacial lines on the BCP [76, 104]. Protein surface density, i.e. the number of adsorbed protein molecules per a unit surface area, decreased with increasing distance away from the chemical interface [104]. In addition, protein surface density was reported to be highest when the periodicity of the chemical interface, i.e. nanodomain repeat spacing, matched closest to the dimension of the protein [121]. Data in Figure 5 (A and B) show such trends for the case of IgG adsorbed on a PS/PMMA blend surface. IgG counts are plotted as a function of distance away from the PS:PMMA interface as well as that of separation between the two nearest PS:PMMA interfaces in Figures 5(A) and 5(B), respectively.

A subsequent study involving PS-b-PMMA templates of varying nanodomain periodicity and alignment revealed the considerable effects that the density of PS:PMMA interfaces and the nanodomain alignment have on the nanoscale adsorption and large-area ordering behaviors of Fg [121]. Table 3 lists the different copolymer templates used in the Fg adsorption study. By comparing the protein surface density on the diblock exhibiting varying nanodomain periodicity and alignment as well as that on random copolymer surfaces, it was found that Fg adsorption was favored the most when the nanodomain periodicity (i.e. the distance between two nearest PS:PMMA interfaces) was commensurate with the size of the protein. The degree of protein surface packing was also found to be the highest when polymer nanodomains were uniformly

aligned with no orientational variation. Overall, protein surface density decreased with greater randomness in nanodomain orientation as seen in the cases of precisely aligned PS-b-PMMA (*dsa* PS-b-PMMA) versus randomly oriented PS-b-PMMA (*com* PS-b-PMMA). Protein surface density was also found to decrease as the length scale of the nanotemplate associated with the chemical interface was adjusted to be much larger or smaller than the dimension of the protein. These results are quantitatively reported in Table 3.

Blank BCP nanotemplates				
Surface Type	**Domain Alignment**	**Periodicity (nm)**	**Average surface roughness (nm), Root-mean-squared surface roughness (nm)**	
***dsa* PS-b-PMMA**	Fully aligned	28	0.31 , 0.39	
***sm* PS-b-PMMA**	Random Fingerprint-like	25	0.27 , 0.33	
***com* PS-b-PMMA**	Random Fingerprint-like	45	0.29 , 0.36	
PS-r-PMMA	N/A	N/A	0.21 , 0.26	
Adsorbed Fg on the different BCP nanotemplates				
Polymer Template	**Fg concentration (μg/mL)**	**Number of Fg/μm²**	**Density of Adsorbed Fg (mg/m²)**	**Surface Coverage (%)**
***dsa* PS-b-PMMA**	2.5	21.5 ± 4.8	0.011 ± 0.002	1.8 ± 0.4
	12.5	131 ± 18.7	0.066 ± 0.009	10.8 ± 1.5
	25	459 ± 74.0	0.23 ± 0.037	37.8 ± 6.1
***sm* PS-b-PMMA**	2.5	14.6 ± 3.5	0.007 ± 0.002	1.2 ± 0.3
	12.5	141 ± 18.8	0.071 ± 0.009	11.6 ± 1.6
	25	349 ± 43.0	0.17 ± 0.022	28.8 ± 3.5
***com* PS-b-PMMA**	5	80 ± 8.5	0.04 ± 0.0043	6.6 ± 0.7
	20	221 ± 19.4	0.11 ± 0.0097	18.2 ± 1.6
	50	360 ± 30.4	0.18 ± 0.015	29.7± 2.5
PS-r-PMMA	2.5	17 ± 4.0	0.0085 ± 0.002	1.4 ± 0.3
	12.5	73 ± 7.0	0.037 ± 0.0035	6.0 ± 0.5
	25	277 ± 19.4	0.14 ± 0.0097	22.9 ± 1.6

Table 3. Fg adsorption characterized on PS-b-PMMA surfaces of varying nanodomain periodicity and alignment. For the reported Fg surface coverage, 100% is defined as the adsorption state in which the entire surface area of the underlying polymeric template is completely covered by the adsorbed Fg regardless of the chemical composition of the nanodomain. Reproduced with permission from Ref. [121], Copyright (2016) American Chemical Society.

Another protein adsorption behavior uniquely observed on polymer nanotemplates is the tendency to form highly organized and tight surface-packed protein constructs over large substrate areas upon protein self-assembly [27, 64, 66, 121]. Densely configured protein assembly and packing were first discovered on the BCP surfaces of PS-b-PMMA and PS-b-poly(4-vinyl pyridine) (PS-b-P4VP) when protein deposition conditions were carefully adjusted to yield a condition that a monolayer of protein molecules covered all available nanodomain areas belonging to protein-favored PS block [27, 64, 121]. The resulting surface organization of the proteins was highly ordered where individual proteins on the PS nanodomain arrange themselves in a tight surface-packing manner, similar to close-packed atoms in a 2D crystal. This interesting phenomenon is displayed in Figure 3(B) and Figure 5(C and D). When macroscopic, chemically uniform counterparts of the BCP were employed for the same protein adsorption, the high level of protein ordering behavior was no longer present and no particular packing order between protein molecules was noticeable on the surface. Dense surface-packing of protein molecules was also reported in studies employing other polymer surfaces. They include those polymers presenting nanoscopic topological features such as crystalline lamellae in melt drawn ultrahigh molecular weight polyethylene (MD UHMWPE) and close-packed needle-like crystals in MD isotactic

polybutene-1 (iPB-1) [66, 92]. Ordered arrangements of Fg molecules observed on various MD UHMWPE polymer templates are displayed in Figure 5(D).

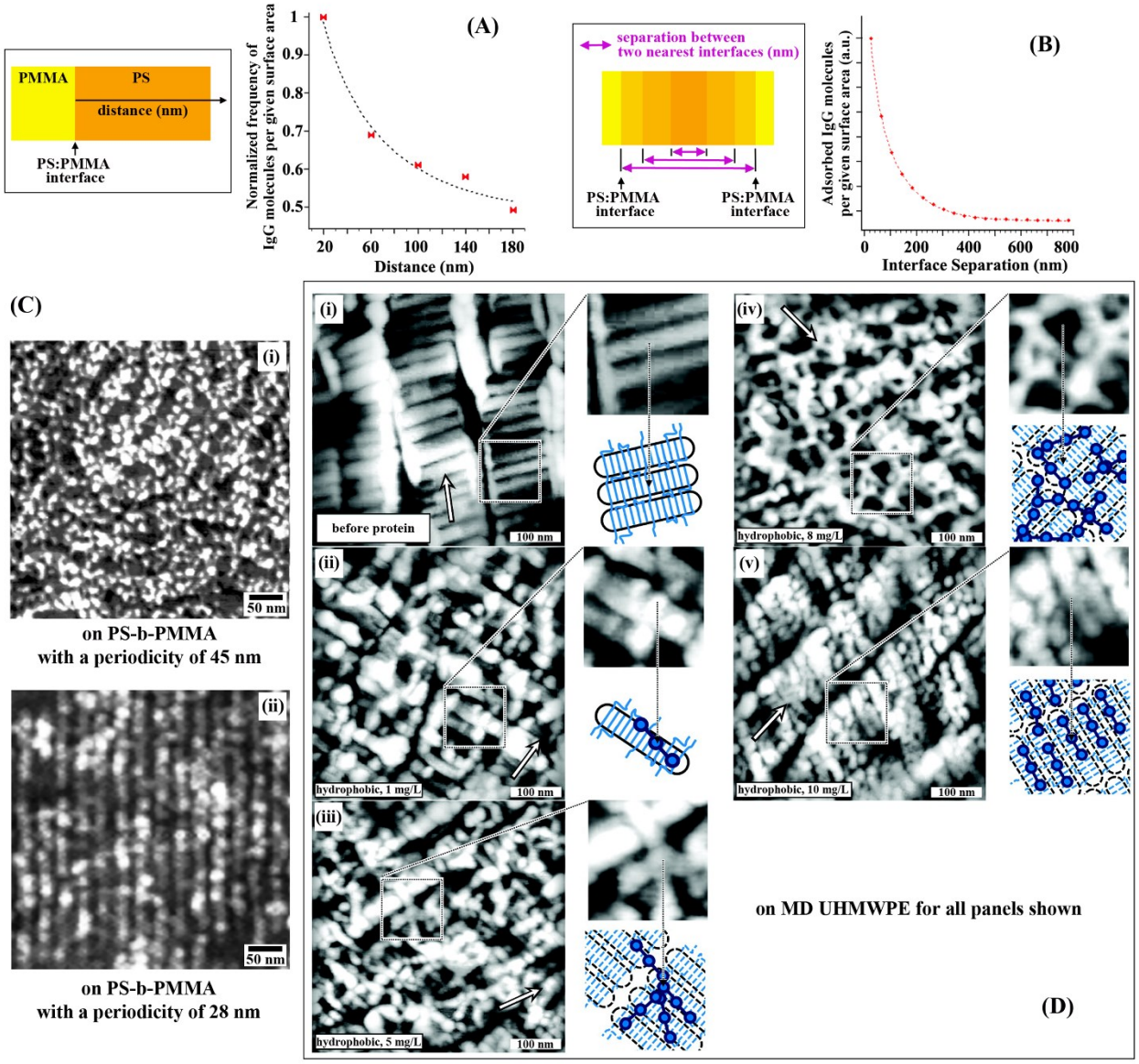

Figure 5. (A) Normalized IgG surface density on a PS/PMMA blend film is plotted as a function of distance away from the PS:PMMA interface. IgG surface density decreases exponentially with the distance away from the chemical interface. **(B)** IgG surface density is plotted as a function of

separation between two nearest PS:PMMA interfaces. It is inversely proportional to the separation distance between two neighboring PS:PMMA interfaces. Images in (A and B) are reproduced with permission from Ref. [104], Copyright (2008) American Chemical Society. **(C)** The AFM topography images show tightly packed Fg molecules on the PS-b-PMMA surface with a repeat spacing of (i) 45 nm and (ii) 28 nm. **(D)** The AFM topography panels show Fg molecules packing on different MD UHMWPE surfaces in a well-ordered fashion. Images in (C and D) are reproduced with permission from Ref. [121] and [66], Copyrights (2016) and (2011) American Chemical Society, respectively.

The tight surface-packing and self-arranging behaviors of proteins revealed on nanoscale polymer templates can be beneficial to developing new protein-polymer nanoconstructs. Well-organized protein constructs built on polymer surfaces that resemble nanoscopic protein arrangements and alignment in native tissues are highly warranted for the development of biomaterials, biodevices, and tissue scaffolds. Hence, convenient and reliable methods to achieve exact control over proteins' spatial assembly into nanoscopic size, periodicity, and alignment have been long sought but proven difficult to attain. Various attempts have been made in the past to partition proteins on surfaces as summarized in Table 4. They include robotic delivery [125], microcontact printing [126, 127], capillary force lithography [128], nanoimprint lithography [129, 130], particle lithography [131], focused-ion-beam patterning [132], and dip-pen lithography [133, 134]. However, most of these methods are adequate for generating μm or larger protein patterns. Nanoimprint and dip-pen lithography methods are capable of producing nm-sized patterns, but they can be costly and slow as the approaches have to either heavily rely on the use of highly

sophisticated clean room instruments or write protein patterns line by line in a serial fashion [129, 130, 133, 134]. Hence, the prospect of a bottom-up self-assembly method for spontaneously creating nanoscale protein patterns over large substrate areas in the highlighted works in this Review can be highly attractive.

Feature size / Process type	Micrometer or larger protein patterns	Nanometer protein patterns
Parallel Processing	Manual & robotic delivery [125, 135, 136]	Nanoimprint lithography [130, 137, 138]
	Microcontact printing [126, 139-142]	Particle lithography [131, 143]
	Imprint lithography [129, 144]	Self-assembly [26, 27]
	Capillary force lithography [128, 145, 146]	
	Microfluidic channel networks [147-149]	
Serial Processing	Inkjet deposition [150, 151]	Dip-pen lithography [133, 134, 152, 153]
	Focused ion beam patterning [132, 154]	Scanning probe lithography [134, 155]
	Electron beam lithography [156-158]	Electron beam lithography [156-158]

Table 4. Various parallel and serial transfer methods to create protein patterns on surfaces.

Many practical applications in biomaterials and tissue engineering demand additional traits beyond just forming nanoscale features of proteins on a surface in a facile way. Precise tuning of the size and shape of nanoscopic protein patterns and exact control over their large-area alignment are also of great importance. For example, electrospinning methods to align protein fibrils require the use of volatile solvents, high shear, and large electrical fields that may irreversibly denature the protein and alter its native structures [159, 160]. Applying a magnetic field [161, 162] or electrochemical gradient [163] to gain control over alignment of surface-organized proteins can also interfere with native protein property and function, while the involvement of specialized

apparatus to generate an external field can make these approaches difficult to scale up. Mechanical means to achieve protein alignment can suffer from the challenge to reliably and uniformly apply strain/shear over large areas [164]. Therefore, the fact that nanoscale polymer templates can be used to organize proteins straightforwardly and rapidly over a large substrate area in a precisely controlled and well-aligned manner can be particularly beneficial in creating next-generation protein-polymer nanoconstructs.

3.3 Nanoscale Protein Adsorption on Different Polymer Systems

As the research area of nanoscale protein adsorption is still at its infancy, only limited polymer systems have been utilized as template surfaces so far. They include nanoscale polymer templates of PS-b-PMMA, PMMA-b-PAA, PS-b-PVP, polystyrene-b-poly(2-hydroxyethyl methacrylate) (PS-b-PHEMA), MD UHMWPE, and MD iPB-1 [66, 91, 92, 105, 106, 165]. To better promote a broader utility in real-world applications, it will be important to ascertain nanoscale adsorption characteristics from a greater set of polymer systems, covering a wide array of chemical components for BCPs. Many BCPs have shown promising biomedical application potentials as drug delivery carriers, protein separation membranes, protein patterning substrates, implant materials, and protein filtration/purification materials [166-168]. Table 5 shows examples of BCP systems demonstrated to date for such biomedical applications.

Name of BCP	Biomedical Function
Polystyrene-block-poly(2-vinylpyridine($HAuCl_4$)$_{0.5}$)	BCP micelles with Au for RGD nanoassembly for MC3T3-osteoblast adhesion [169]

PS-b-P4VP	BCP thin film as an electrode material to immobilize glucose oxidase (GOx) for an enzyme biosensor [170] BCP micelles for protein assembly [105] BCP thin film for enzyme activity quantification [105]
PS-b-PMMA	BCP nanodomain for protein assembly [27, 91, 171] BCP thin film for enzyme activity quantification [107, 108]
Poly(2,2-dimethylaminoethyl methacrylate) (PDMAEMA)-b-poly(n-butyl methacrylate)-b-PDMAEMA	Biosensor-based, solid-supported, biomimetic membrane [172]
Polystyrenesulfonate-b-polyethylene-b-polystyrenesulfonate	Used for membrane preparation in carcinoma drug capture and removal from bloodstream [173]
Poly(2-hydroxyethyl) methacrylate (PHEMA)-b-polystyrene-b-PHEMA	Platelet adhesion study for creating biofouling-resistant surface [174]
Polyethylene oxide-hyperbranched-polyglycerol	pH-responsive BCP micelles as a drug carrier [175]
Polycaprolactone-b-polyethylene glycol	BCP nanoparticles as an anti-cancer drug carrier [176]
Poly(lactide-co-glycolide) (PLG)-b-poly(oxyethylene)-PLG	BCP rods as biodegradable implants [177]
Poly-d,l-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol	BCP as an osteogenic biomaterial for biodegradable bone-inducing implant [178]

Table 5. Exemplar BCP systems used in various biomedical applications.

As discussed earlier, the possibility of polymer surface-steered creation of well-defined protein nanopatterns is particularly exciting when considering the fact that it is extremely difficult to achieve distinctive protein feature sizes that are as small as tens of nm during the spatial partition of proteins on a solid surface. Currently, truly nanoscale protein features are hard to attain even with the help of an electric, magnetic, or mechanical field and the employment of sophisticated micro/nanofabrication steps. In this regard, the new BCP-based protein assembly and organization method that exploits easily tunable polymer nanotemplates and proteins' self-selection tendency

to a distinct polymer block is much desired. In order to realize such potential of the BCP method, it will be important that a broader set of polymer systems should be examined first and there still is plenty of room in the parameter space of BCPs for nanoscale protein adsorption. Future investigation of nanoscale protein adsorption on extended BCP systems may provide new insights to controlling protein adsorption characteristics by, for example, tuning the chemical properties of underlying nanostructured surfaces to promote or deter protein adsorption. Such new understanding may be used in turn to effectively direct protein adsorption processes to produce downstream biological consequences for inducing user-tailored cell interactions and host responses. These aspects of protein-mediated downstream biological consequences that should be considered in future studies of nanoscale protein adsorption will be discussed in the next section.

3.4 Surface-Induced Protein Adsorption Behaviors: Surface Length Scale Comparable to Protein Size and Chemically Different Environment

It is known that topological factors such as increased surface roughness and higher surface area to volume of an underlying platform can influence protein adsorption. Varying outcomes have been reported in earlier protein adsorption studies employing substrates with an increased surface area via surface roughening or reduction in feature size [26, 27, 52, 179, 180]. For example, it was reported that the adsorption amount of serum albumin and fibronectin on a bioceramic material with an average surface roughness of 32 nm was greater than the case of a 142 nm surface roughness [179]. Another study reported that the surface density of lysozyme (Lyz) did not change when changing the surface roughness of the substrate from 5 nm to 60 nm [52]. These studies indicate that topological factors can affect protein adsorption for a template

surface with its length scale much greater than several tens of nm and yet, when the length scale involved is comparable to the protein size, topological factors alone cannot induce significant changes in protein adsorption behaviors. Later studies involving BCP surfaces have demonstrated that chemical variabilities combined with topological factors can be simultaneously used to bring about considerable changes in protein adsorption characteristics at the individual protein length scale [27, 64, 104].

The combined effects of topological (nanoscale surface feature size) and chemical heterogeneity degree (homogeneous composition, heterogeneous composition but well-mixed, and heterogeneous composition with patchwise segments) on protein adsorption have been studied on substrates containing organic molecules as surface modifiers [83, 181]. When SPR was used to monitor time-dependent, ensemble-averaged adsorption behaviors of BSA, the amounts and rates of BSA adsorption were found to be different between a chemically homogeneous versus heterogeneous template. These outcomes are summarized in Figure 6. Compared to those on the homogeneous mercaptopropionic acid (MPA) and decanethiol (DT) surfaces, BSA surface coverage (Γ_{max}) was higher on the patchwise heterogeneous surfaces of mixed MPA and DT. Furthermore, relative to well-mixed mercaptoundecanoic acid (MUA)/DT surfaces, the heterogeneous surface effect on Γ_{max} was greater on the patchwise MPA/DT substrate. These results showed the interplay between the chemical heterogeneity on the surface and the intrinsic physicochemical properties of the BSA adsorber. Chemical heterogeneity distributed in a patchwise manner on a nanometer length scale on the substrate, which is commensurate with the protein size, was found to influence protein adsorption significantly more than the cases of less matched length scale.

The importance of nanoscale chemistry and surface topography on protein adsorption has been investigated for platelet binding to Fg-covered metal oxide surfaces as well [182]. Compared to a flat titanium oxide substrate case, Fg bound to a topographically structured titanium oxide surface showed increased platelet binding ability. It was speculated that the surfaces with nanotopography on the length scale of the individual protein molecules might be able to change the conformation and orientation of Fg which, in turn, makes the proteins readily bind to the platelet membrane receptors.

(A)

Heterogeneous Surfaces	**Molar Composition of Solution** MUA: Mercaptoundecanoic acid DT: Decanethiol MPA: Mercaptopropionic acid	**Contact Angles** ($\Theta_{adv}/\Theta_{rec}$)±1°
Well-mixed A	MUA/DT = 0.8	73/61
Well-mixed B	MUA/DT = 0.4	87/77
Patchwise C	MPA/DT = 7.5	74/48
Patchwise D	MPA/DT = 8.9	85/66

(B)

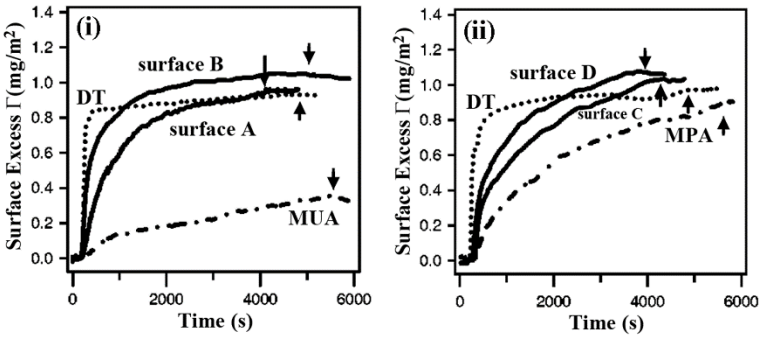

Figure 6. **(A)** The table lists four heterogeneous surfaces decorated with different organic modifiers for BSA adsorption. **(B)** SPR sensorgrams plot BSA surface coverage over time on (i) well-mixed and (ii) patchwise heterogeneous surfaces, as shown with solid lines. Arrows indicate buffer rinsing. For comparison, BSA adsorption profiles on homogeneous, single-component

surfaces of DT, MUA, and MPA are displayed with dashed lines. Adapted with permission from Ref. [181] Copyright (2004) AIP Publishing LLC.

On the simulation front, conducting all-atom level computer simulation for protein adsorption on polymer is not a trivial matter, especially for a surface showing complexity in chemical heterogeneity and nanoscale topology from the underlying polymer macromolecules. The surface of a protein itself is also quite complex containing varying degrees of hydrophobic/hydrophilic residues, chemical moieties, and charges. Due to these difficulties, not many atomistic simulation studies exist for protein-surface interactions that model a polymer surface of varying chemical heterogeneity at a nanoscale interval. Despite this, a molecular dynamics (MD) simulation study revealed the role of amphiphilic amino acids in facilitating the adsorption of cytochrome C (Cyt C) onto different mixed-composition surfaces of heterogeneous chemical segments [183]. A related, atomistic MD study determined that different groups of surface amino acid residues are responsible for the surface adsorption of lysozyme (Lyz) to various model surfaces terminated with 1-octanethiol (OT) and 6-mercapto-1-hexanol (MH) [124]. The simulation outcomes displayed in Figure 7(A) demonstrate that the most energetically favorable conformations of adsorbed proteins vary greatly between the chemically homogeneous and heterogeneous substrates that include OT and MH surfaces as well as mixed surfaces of OT:MH in varying ratios. In another all-atom MD simulation work, the early stage adsorption process of EAS hydrophobin was considered on an amorphous silica surface tethered with short functional chains and water [184]. Nanopatterned surfaces with varying degrees of either hydrophilic or hydrophobic chains or mixtures thereof were examined. The study found that surfaces presenting

both nanoscale chemical structures and surface roughness in the form of functional chain length variation resulted in the most substantial effect on protein adsorption [184]. These simulation outcomes are summarized in Figure 7(B) for six different nanopatterned surfaces that exhibit varying lengths and mixtures of hydrophobic and hydrophilic chains. These simulation findings emphasize the critical role that a template surface can play in protein adsorption when the surface contains distinct chemical features organized at a length scale comparable to the protein dimension.

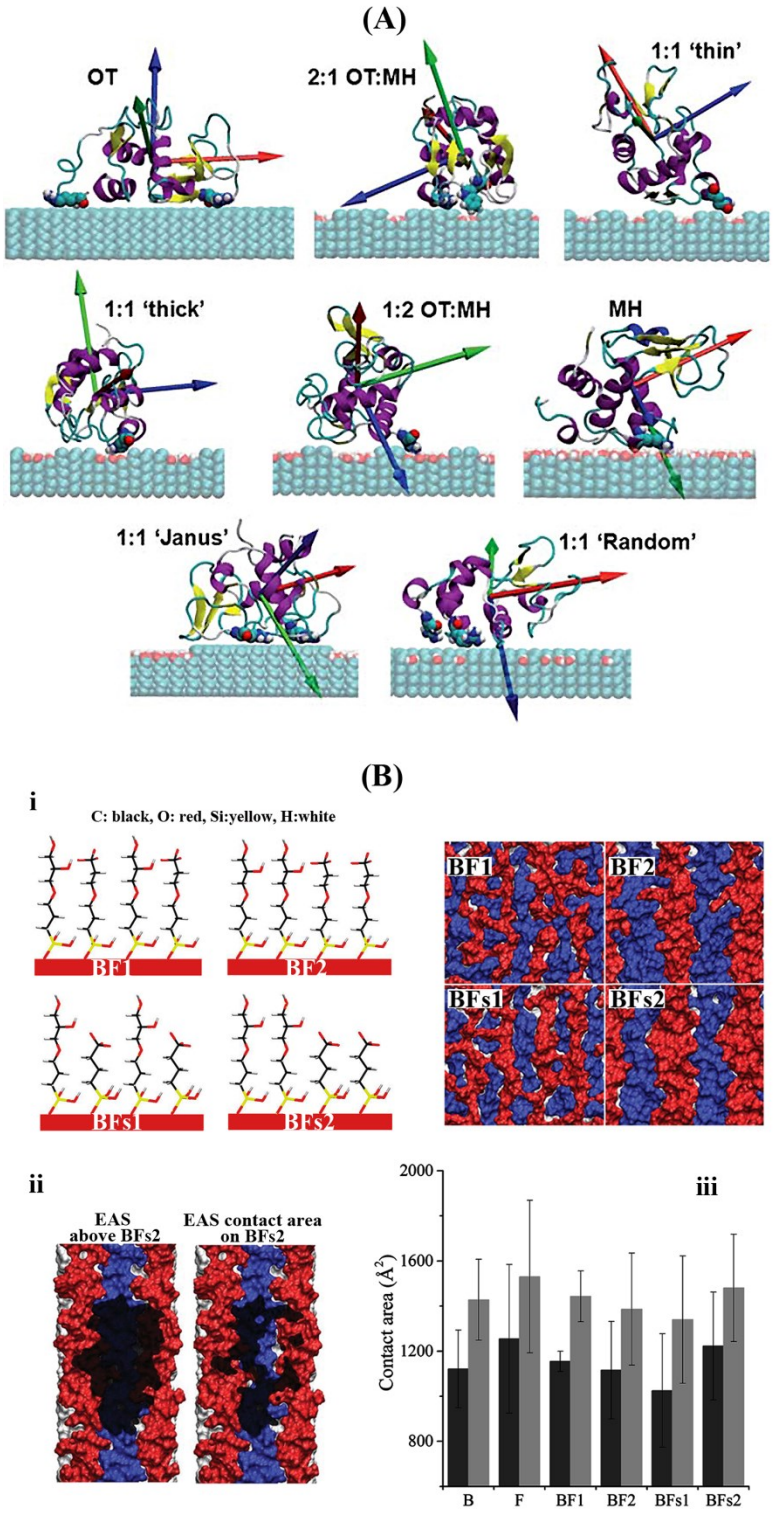

Figure 7. (A) Most energetically favorable Lyz binding orientations on assorted OT- and MH-terminated surfaces are determined by MD simulations. The results are depicted for Lyz adsorbing

onto various surfaces for which the blue and red spheres on the substrate correspond to OT- and MH-containing areas, respectively. The three principal axes of Lyz are indicated as red, blue, and green vectors in the order of longest to shortest axis of the protein. Reproduced with permission from Ref. [124] Copyright (2013) Royal Society of Chemistry. **(B)** MD simulations were carried out on model surfaces containing varying compositions and lengths of hydrolysed glycidoxypropylsilane (hydrophilic) and fluorinated (hydrophobic) chains. Exemplar surfaces of BF1, BF2, BFs1, and BFs2 before protein adsorption are depicted in panel (i). Simulation results in panel (ii) show EAS hydrophobin above the BFs2 surface as well as the contact region of the protein within 5 Å above the surface. The average and maximum contact areas found for EAS hydrophobin adsorption to each model surface are plotted in panel (iii) as dark and light bar graphs, respectively. Reproduced with permission from Ref. [184] Copyright (2016) Royal Society of Chemistry.

As discussed earlier, it will be also critical to elucidate the role of not only the chemical but also physical parameters associated with the underlying polymer template for nanoscale protein adsorption. It has been reported that the amount and orientation of surface organized proteins can change depending on the periodicity and alignment of the BCP nanodomains even though the adsorption templates were made from the same BCP substance with no change in their chemical blocks [121]. Figure 8(A) shows the different nanotemplates used for Fg adsorption whose two chemical components in the BCP were kept identical as PS and PMMA. The periodicity of the BCP nanodomain, however, was varied from a length comparable to, smaller, and much smaller than the protein length, and the alignment of the underlying polymer

nanodomain was changed from randomly oriented to fully aligned. As displayed in Figures 8(B and C), the different PS-b-PMMA templates led to distinctly different Fg self-assembly characteristics. Fg molecules organized in an end-on packing manner with the protein direction parallel to the long nanodomain axis when the nanodomain periodicity of the BCP template was smaller than the protein length, Figure 8(B). On the contrary, Fg molecules assembled with the protein backbone oriented perpendicular to the long nanodomain axis in a side-on packing manner on the BCP template whose nanodomain periodicity was comparable to the protein length, Figure 8(C).

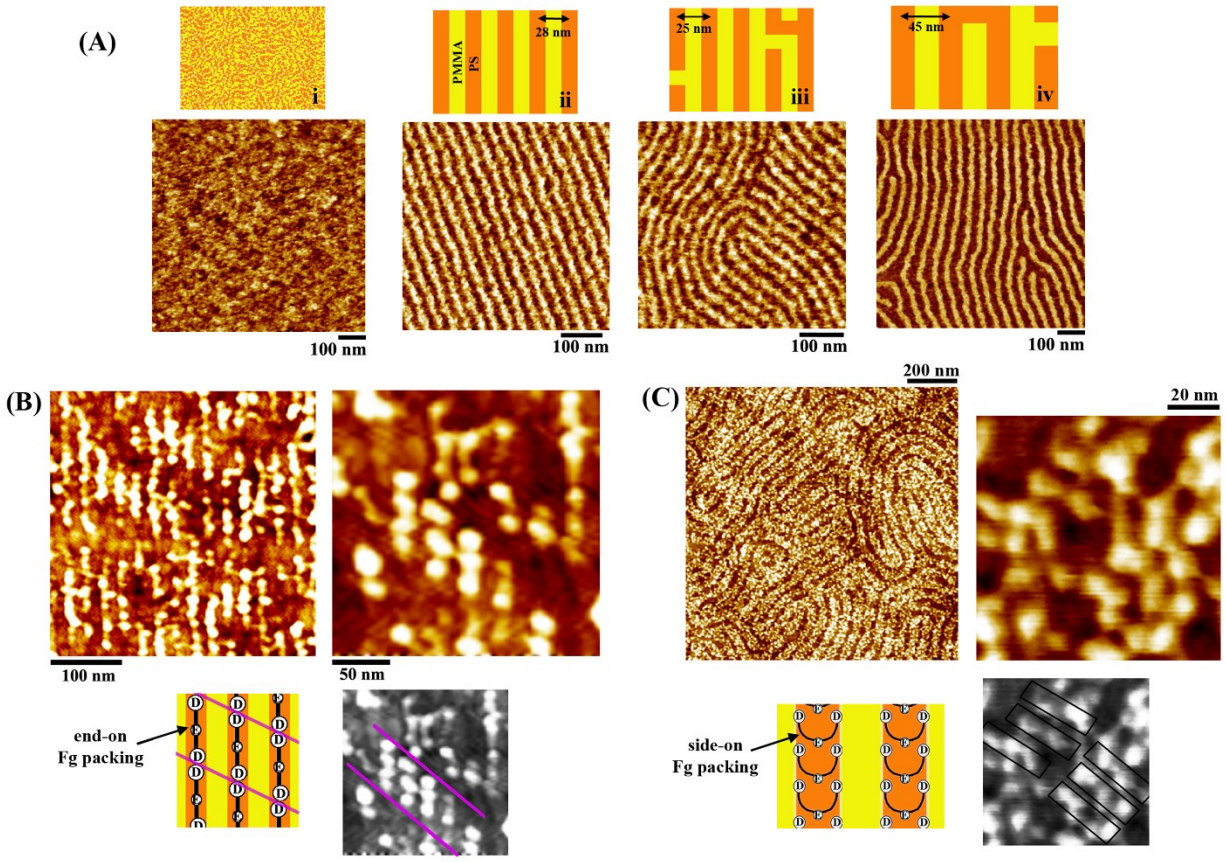

Figure 8. (A) PS-b-PMMA templates of varying nanodomain periodicity and alignment used for Fg adsorption are displayed. The nanodomain periodicity on the templates ranged from much smaller (i), smaller (ii, iii), and comparable to (iv) the length of Fg. The nanodomain alignment was also varied from randomly oriented (i, iii, iv) to fully aligned (ii). **(B and C)** The AFM topography images show both the large scale and zoomed-in views of Fg molecules tightly surface-packing on the PS nanodomains on the BCP template with the nanodomain periodicity (B) smaller than and (C) matched to the Fg length. The protein molecules stacked themselves (B) end-on versus (C) side-on with the protein backbone oriented (B) parallel versus (C) perpendicular to the long axis of the nanodomain. As a guide to the eye, boxes surrounding individual Fg molecules are inserted in the grey panel shown under each AFM panel. Images reproduced with permission from Ref. [121] Copyright (2016) American Chemical Society.

The experimental and simulation findings discussed in this section combinedly reveal that nanoscale protein adsorption is dependent on both the physical and chemical parameters of the underlying polymer surface. This, in turn, suggests that protein adsorption can be facilitated (or inhibited) via matching (or mismatching) surface nanopatterns with their chemical properties adjusted to the characteristic length scale of the protein or, better yet, tailored to those amino acid residues on the protein exterior if specific binding residues responsible for the adsorption process are known. Another important implication from these nanoscale protein adsorption studies pertains to the consideration of a higher level of biological function. It is well-known that cell behaviors can be effectively modulated by the change in concentration, composition, and conformation of surface-adsorbed proteins [185-188]. In cell growth and tissue engineering,

extracellular matrix proteins such as fibronectin and collagen are involved in cell attachment via specific amino acid sequences as well as through particular binding domains within the proteins [189-191]. Hence, nanoscale topology and chemistry of underlying polymer surfaces may be exploited to modulate protein adsorption processes in a way to control the conformation of the surface-adsorbed protein or to influence the interaction between intracellular and extracellular proteins. Such approaches in the future may ultimately lead to regulation of functional characteristics of a new cell or tissue construct via nanoscale protein adsorption.

4 Multicomponent Protein Adsorption on Nanoscale Polymer Surfaces

These earlier studies discussed in Sections 2 and 3 have embarked on a significant step forward in identifying and understanding nanoscale protein adsorption behavior, but the efforts pertained to single component systems only. Real-life biomedical applications are expected to encounter multiple protein components, rather than single protein constituents. Present knowledge on single component protein adsorption may not be adequately used to explain more complex nanoscale adsorption processes contributed by different protein components. Very little is known about multicomponent protein adsorption processes in general, let alone on those polymer surfaces of nanoscale topology and chemical variability. In addition, there is currently a large lack of single biomolecule level data for multiprotein adsorption processes. This is because the majority of the commonly used measurement techniques for protein adsorption such as those in Table 1 cannot be employed to directly visualize different types of proteins at the individual protein level or distinguish signals from the different proteins in a straightforward manner. In order to gain single biomolecule level insights on multicomponent protein adsorption interfacing nanoscale surfaces,

an experimental method capable of directly tracking particular proteins of interest on the same surface areas and recording the spatial and temporal changes of the different protein kinds between various treatments can be highly beneficial. Furthermore, such single biomolecule level investigations may offer much needed, definitive experimental evidence for many assumptions used in currently available protein adsorption kinetic models summarized in Table 6 [10, 55, 59, 112, 123, 192-199]. In existing adsorption models, many postulations as depicted in the middle column in Table 6 had to be made by inferring from ensemble-averaged protein adsorption properties. This was due to the lack of direct experimental evidence that could unambiguously reveal single biomolecule level adsorption processes to a solid surface. Hence, single protein level investigations may enable not only the verification of existing postulations in the present protein adsorption mechanisms for single and multicomponent systems but also for the formulation of entirely new protein adsorption mechanisms better suited for those surfaces that present nanoscale topology and chemistry at the length scale of an individual protein.

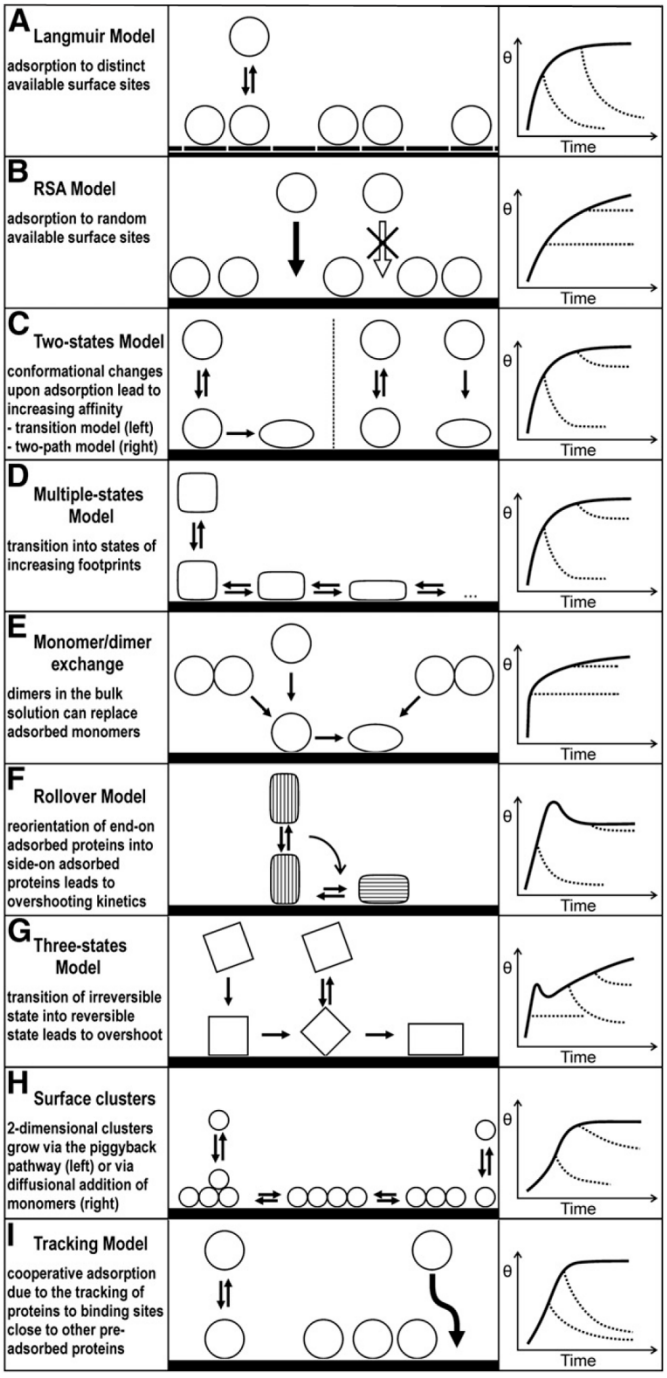

Table 6. Various mechanisms and kinetic models currently available for protein adsorption. A schematic for each respective adsorption model is shown in the middle column. Adsorption kinetic plots are presented in the right column with solid lines for adsorption and dashed lines for

desorption upon buffer rinsing. Image reproduced with permission from Ref. [123] Copyright (2011) Elsevier B. V.

From a large body of previous studies in hemostasis/thrombosis and biomaterials [1, 3, 10, 58, 59, 67, 73, 118, 123, 196-203], valuable insights on the macroscopic and bulk scale adsorption can be gained for multicomponent protein systems. In these studies, an interesting phenomenon of protein exchange known as the Vroman process has been reported [3, 58, 196-202]. When different plasma proteins are exposed to a solid surface under a competitive adsorption environment, proteins preferentially bound on the surface during early time periods may be displaced by other proteins in the bulk solution as the competitive adsorption process continues. The Vroman series of adsorption/desorption and displacement events have been well-documented for various blood protein components [3, 58, 196-202]. Generally speaking, protein species with lower molecular weights and lower surface affinity, initially arriving at the solid surface, are later replaced by protein species with higher molecular weights and higher surface affinity. Although this general process of protein exchange is now widely accepted, many detailed aspects of the Vroman effect, including the exact molecular mechanism underlying the process and the precise compositions of the adsorbed protein layer at a given time, are still under active investigation. Despite many speculations regarding possible mechanisms responsible for the phenomenon [204], there lacks definitive experimental evidence to explain the process. Specifically with regard to nanoscale protein adsorption, it has not been known how the competitive adsorption behavior will be manifested on the surfaces of reduced dimensions and to what extent the macroscopically observed Vroman effect will take place on nanoscale surfaces. Still, there is much more to explore

for competitive protein adsorption characteristics on nanoscale polymer templates, particularly when it comes to multicomponent adsorption behaviors examined at the individual protein level.

4.1 Adsorption Kinetics: Serial Adsorption involving Single Protein Kinds in Each Step

Ensemble behaviors of fluorophore-labelled BSA and Fg have been previously examined in a serial adsorption setting using total internal reflectance fluorescence (TIRF) [55]. Protein relaxation kinetics were obtained on a hexadecyltrichlorosilane (C16) self-assembled monolayer (SAM) surface. Figure 9 presents the multicomponent protein adsorption kinetic plots obtained from the TIRF studies using sequentially introduced BSA and Fg to different SAM surfaces. Fg adsorption kinetics were dependent on prior BSA adsorption history as shown in Figures 9(A and B). Overall, it was reported that substantial amounts of Fg were able to readily adsorb on a BSA-covered surface whose trend became weakened with aging of the preadsorbed BSA layer on the SAM surface. In a following study, adsorption kinetics associated with serial introductions of BSA and Fg were investigated on an expanded set of surfaces that contain different SAMs of varying hydrophobicity [56]. When comparing SAM surfaces of hydrophobic versus hydrophilic nature, it turned out that hydrophobic SAMs were more resistant to subsequent Fg adsorption under the same aging condition for the preadsorbed BSA layer, Figure 9(C). Yet, regardless of the surfaces tested, Fg surface coverage was greatly affected by prior BSA incubation time on the SAM platforms. These results are displayed in Figures 9(D-F) for the SAM surfaces of N-(3-triethoxysilylpropyl)-4-hydroxybutyramide (OH), C16, and 1H,1H,2H,2H-perfluorodecyltrichlorosilane (PFDTCS). These efforts provide valuable insights on serial protein adsorption behaviors such as their dependence on surface hydrophobicity, aging time, and surface history due to protein adsorption from a prior step. However, the approaches used in these studies

cannot be straightforwardly applied to competitive adsorption investigations for revealing the adsorption characteristics of different protein components at the single biomolecule level on a nanoscale polymer surface. In addition to insufficient spatial resolution of the fluorescence-based technique, the potential influence of the required fluorophore labels on the native protein adsorption properties as well as the difficulty in accurate signal quantification from the indirect probing technique can pose significant challenges for nanoscale adsorption systems.

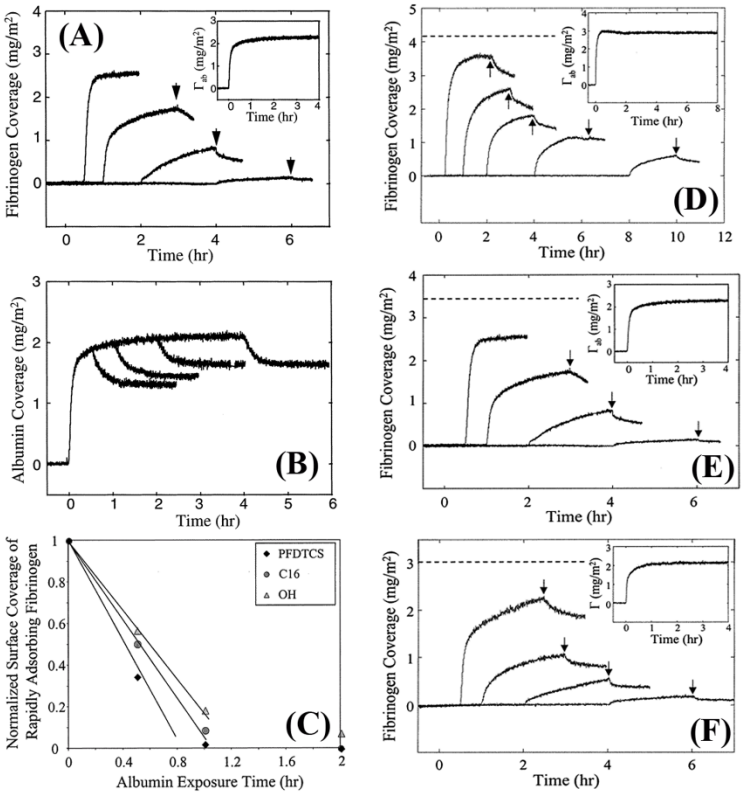

Figure 9. (A) Adsorption kinetic plot of fluorophore-labelled Fg, following the adsorption of unlabeled BSA. The inset shows a repeated BSA adsorption run after labelling of BSA and the arrows indicate the addition of phosphate buffer solution. **(B)** Experiments complimentary to (A), here with labelled BSA and unlabelled Fg. Images in (A and B) reproduced with permission from

Ref. [55], Copyright (1999) American Chemical Society. **(C)** Fg surface coverage as a function of BSA relaxation on the SAM surfaces of OH, C16, and PFDTCS. **(D-F)** Experiments complimentary to (A), here on the SAM surfaces of (D) OH, (E) C16, and (F) PFDTCS. The dotted line marks the Fg coverage level at the same conditions on a bare surface. Images in (C through F) reproduced with permission from Ref. [56] Copyright (2001) American Chemical Society.

More recently, competitive protein adsorption behaviors were revealed directly at the single protein level on a nanoscale BCP surface via combined experimental and computer simulation approaches [205]. In this work, individual proteins adsorbed/desorbed on the same BCP surface locations were tracked by AFM between different protein treatments. The AFM tracking measurements enabled unambiguous determination of key competitive adsorption phenomena that could not be revealed before. Single biomolecule level data were provided for dominant competitive adsorption pathways, occurrence frequency associated with specific pathways, protein mobility on surface, protein self-association tendency, and directionality in protein exchange. Data shown in Figure 10 display the time-dependent tracking ability of AFM to faithfully follow individual proteins adsorbing/desorbing on the same BCP surface locations between serial adsorption steps. The adsorption profiles of subsequent stage proteins onto a surface containing preadsorbed proteins were reported to be drastically different from those cases without preadsorption. For both IgG and Fg, the adsorbed protein amounts grew linearly with increasing bulk solution concentration when the proteins were adsorbed onto a neat, freshly prepared BCP surface. These trends are displayed in the plots of protein surface density versus

bulk concentration in Figures 10 (A and B). However, such a linear relationship did not hold for the case of a sequential Fg adsorption to IgG-treated BCP as shown in Figures 10(C and D). Even when the protein concentrations used in the consecutive deposition experiments were well within the regimes yielding the highly linear correlation for the proteins on the neat BCP surface case, the surface density of the subsequent stage protein did not depend on Fg solution concentration. Rather, Fg adsorption in this case was dependent on preadsorbed IgG amounts on the surface from a prior step. The experimental findings in Figure 10(D) were further substantiated by Monte Carlo (MC) simulations as displayed in Figure 10(F). The direct protein tracking capability of AFM as demonstrated in Figure 10(E) is particularly exciting as it can offer conclusive experimental evidence of protein adsorption/desorption/replacement events at the single protein level for multiple protein kinds on the nanoscale polymer surface. Such direct experimental proof may open up much needed opportunities for new mechanistic understanding of competitive protein adsorption processes, validation of existing mechanisms, and design of new biomaterials and biosensors.

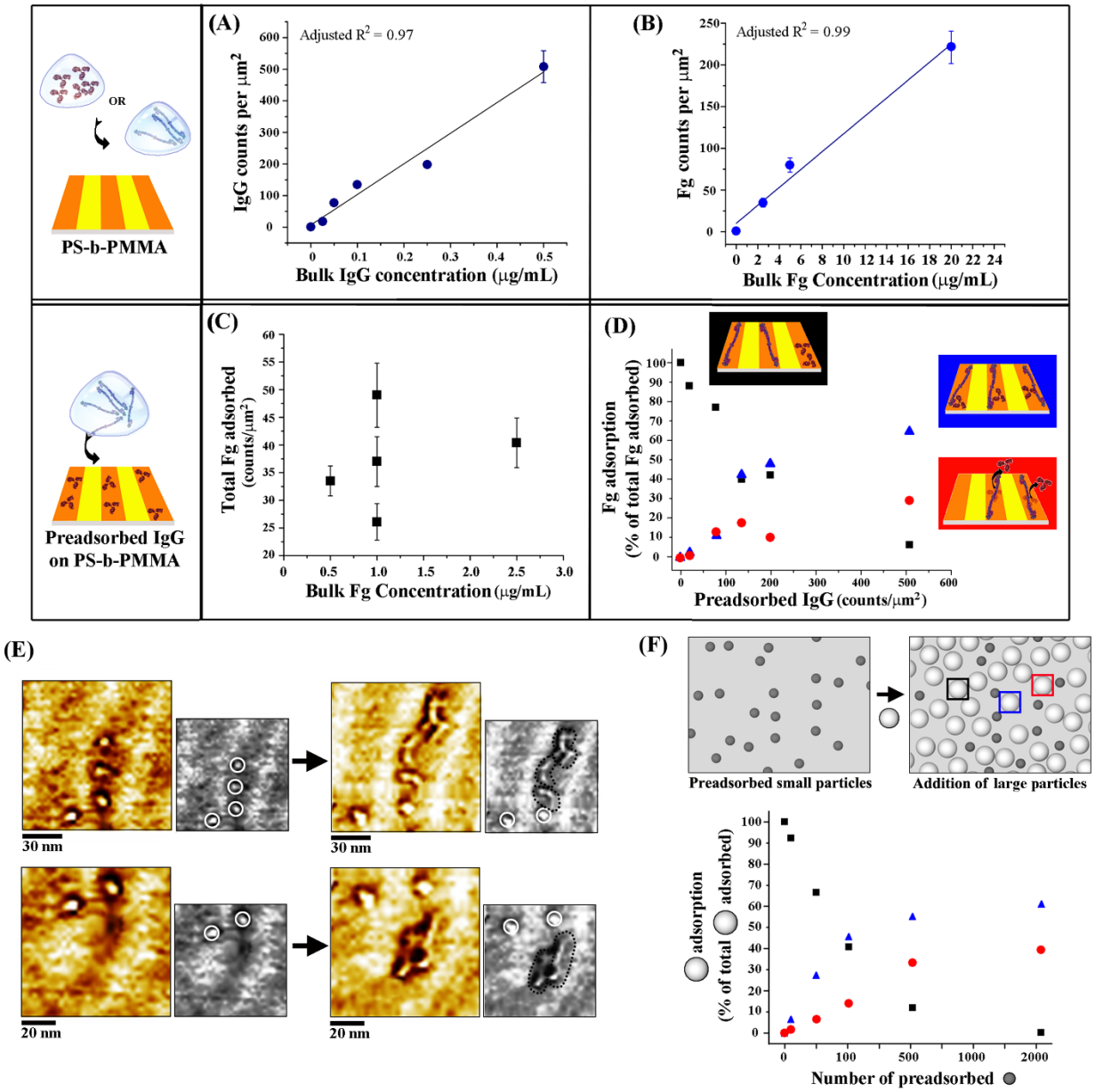

Figure 10. (A-D) Protein adsorption behaviors found on neat versus pretreated BCP surfaces are summarized. The protein surface density versus concentration plots in (A and B) were obtained by examining the case of (A) IgG and (B) Fg adsorption onto a clean BCP substrate with no preadsorbed proteins. The data in (C and D) correspond to the adsorption behavior of Fg when it was introduced as a subsequent stage adsorber to a BCP surface predecorated with IgG. The data in (C) displays Fg surface density versus its concentration. The plot in (D) charts the Fg occurrence

frequencies as a function of preadsorbed IgG counts on the surface after the prior deposition step. The data points shown in black, blue, and red correspond to the different adsorption pathways of distal Fg adsorption, proximal Fg adsorption, and Fg replacing IgG, respectively. **(E)** Distinct adsorption events were tracked by AFM at the single protein level for multicomponent protein deposition onto a BCP nanotemplate. AFM panels acquired from an identical surface location are shown side by side for direct comparison of the dynamic events occurring after introduction of an IgG solution (left) and subsequent exposure to a Fg solution (right). **(F)** MC simulation outcomes showing adsorption of large particles onto a 2D box preadsorbed with small proteins. Occurrence frequencies of the large particle adsorption events (mimicking the later protein adsorber of Fg) were plotted as a function of the number of preadsorbed small particles (mimicking the initial protein adsorber of IgG). The results are displayed for the cases of large particles adsorbing away from small particles (black, distal adsorption), close to preadsorbed small particles (blue, proximal adsorption), and by substituting a preadsorbed small particle (red, protein replacement). Images reproduced with permission from Ref. [205] Copyright (2018) Royal Society of Chemistry.

4.2 Adsorption Kinetics: Simultaneous Adsorption of Multiple Protein Components

A recent study has delivered single biomolecule level proof of Vroman exchange processes for simultaneous adsorption of BSA and Fg that occurred on a nanoscale polymer surface [206]. In this work, time-dependent transition behaviors of surface-bound proteins were found to be significantly different on a nanoscale, chemically varying BCP surface relative to a macroscopic, chemically homogeneous surface. The extent to which the initially surface-bound BSA resists its

displacement by Fg was found to be much greater on the BCP surface of PS-b-PMMA relative to that on PS homopolymer. A series of AFM panels in Figures 11(A and B) displays the protein exchange process on the two types of polymer surfaces where the preferentially adsorbed BSA molecules at early times are being displaced by Fg over time on the surface. Interestingly, this exchange process was found to occur much more slowly on the BCP relative to the homopolymer case. The considerable delay in protein turnover time on the BCP relative to the homopolymer surface is presented in Figure 11(C) for different BSA and Fg concentrations in the mixture. Overall, the results showed that the BCP surface induces longer residence time of the initially bound protein species and considerable retardation in the onset of a protein exchange process, suggesting increased stability of the already surface-bound protein molecules on the nanoscale template. The prolonged residence time on the BCP was explained by the more energetically favored environment for bound proteins as discussed earlier. Once adsorbed, protein-polymer interaction was facilitated on the BCP by the dense presence of the chemical interfaces whose periodicity also matched the size scale of individual proteins.

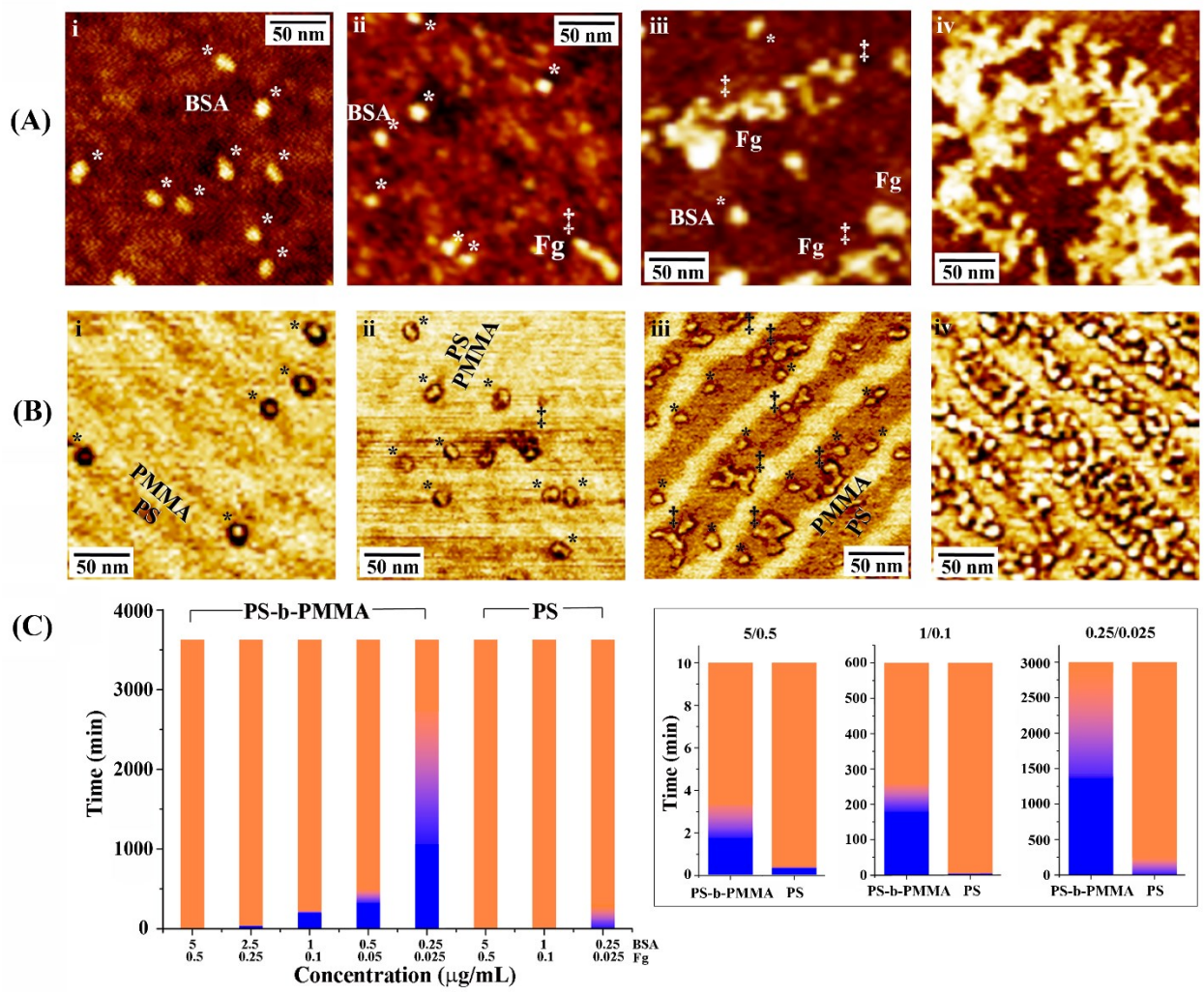

Figure 11. (A and B) Representative series of AFM data display different stages of BSA (example protein marked with * in the panel) and Fg (‡) adsorption over time when the two proteins were simultaneously introduced to (A) a PS homopolymer and (B) PS-b-PMMA BCP surface. The AFM topography and phase panels in (A) and (B) illustrate the adsorption stage changing from the (i) BSA-dominant, (ii) Fg onset, (iii) Fg turnover, to (iv) Fg-dominant phase. **(C)** Colored bar graphs display residence times associated with BSA and Fg on the BCP and PS homopolymer surface. The BSA-dominant, Fg onset/turnover, and Fg-dominant phases are displayed in blue, gradient purple, and orange, respectively. The early stage BSA to Fg turnover behaviors are

captured in the boxed panel for the specified protein concentrations. Images reproduced with permission from Ref. [206] Copyright (2016) Royal Chemical Society.

5 Assessing Biofunctionality of Surface Bound Proteins

Biological functionalities of proteins adsorbed on surfaces may differ from their native activities. Surface-bound proteins, unlike their free-state motion in solution, may be restricted in changing their conformations and exposing their binding sites towards a ligand molecule. The substrate-induced, steric hindrance of protein binding to ligand was often attributed to the reduced activities observed in many randomly adsorbed protein systems [104, 107, 108]. In contrast, protein activity has also been reported to increase on a solid platform [207, 208]. These cases involve proteins attached to a surface in an orientation specific manner by tethering them to the platform via chemical or biological moieties. Protein reactions in solution rely on Brownian motion for the stochastic chances of collisions to occur. Comparatively, more effective ligand guiding along a well-defined molecular coordinate towards orientation-controlled proteins on a solid surface was provided as a possible reason for increased protein activity. However, quantitative comparison of protein activities between a surface-bound versus free solution state has remained challenging due to the difficulty in precisely controlling and determining the exact number of protein molecules bound on a surface. As discussed in Section 3, the unique protein quantification capability on the BCP nanoplatforms has since permitted quantitative comparison of enzyme activity assayed on a surface-bound versus free solution state [104, 105, 108]. It was reported that, for the same number of horseradish peroxidase (HRP) molecules, approximately 85% and 78% of the free-state activity was retained after the enzyme adsorption to the BCP

template of PS-b-PMMA and PS-b-P4VP, respectively [105, 108]. An exemplar result is presented in the spectroscopic data set in Figure 12(A), comparing the difference in HRP activity measured on a PS-b-PMMA surface versus in solution. These outcomes suggested that high percentages of the HRP maintained its enzymatic activity after adsorption to the BCP surface. Data in Figure 12(B and C) display the change in assay color with and without bound enzymes on the BCP surface using HRP and tyrosinase as a model system, respectively. Biofunctionality of BCP-bound Fg was also evaluated for microglial cell activation. Figure 12(D) displays representative fluorescence panels of immunostained microglia cells observed on the BCP surface with and without Fg. Pronounced fluorescence emissions of green and red signals from the Fg-covered BCP relative to the untreated BCP indicated that the surface-bound Fg retained its microglial cell- activating functionality on the BCP. These results showed a promising sign for creating and utilizing novel nanoscale polymer constructs with functional proteins on the materials' surfaces.

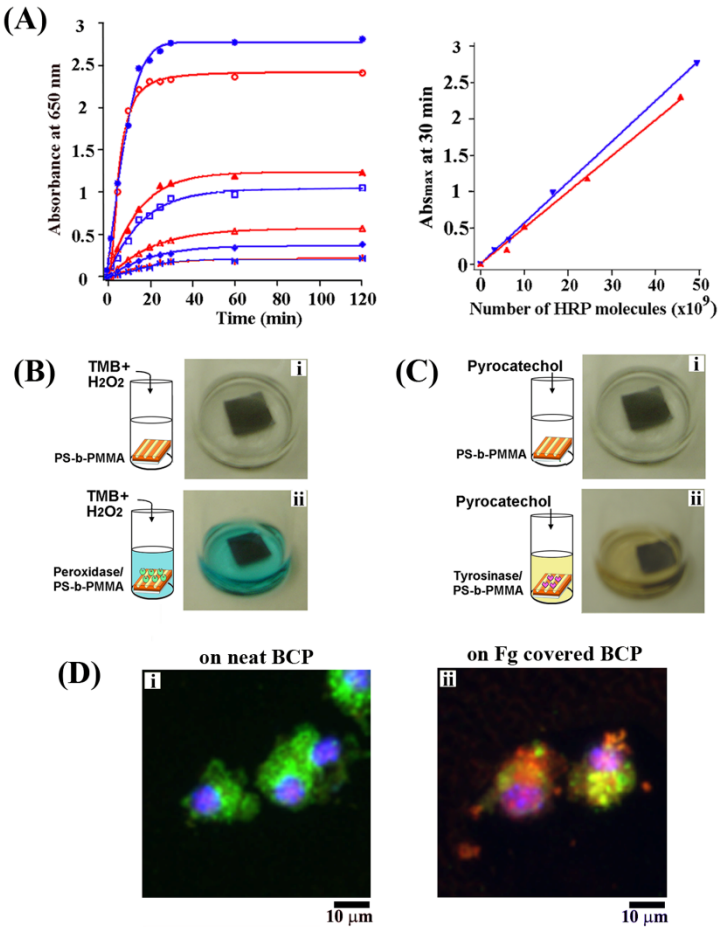

Figure 12. (A) Differences in HRP activity between its free state (red) versus PS-b-PMMA bound state (blue) were measured by UV-vis absorption spectroscopy at 650 nm. For the same number of HRP molecules, the BCP-bound HRP retained approximately 85 % of its free state activity in solution. **(B and C)** Digital images of the enzyme assays for (B) HRP and (C) tyrosinase are shown. The enzyme assays were carried out in a vial containing the reactants introduced to a (i) neat BCP and (ii) enzyme-deposited BCP surface. Images (A-C) are reproduced with permission from Refs. [105, 108], Copyright (2007) American Chemical Society. **(D)** Primary mouse microglial cells grown on a (i) neat BCP and (ii) Fg-deposited BCP surface were immunostained by targeting the nuclei with 4,6-diamidino-2-phenylindole (DAPI, blue), inducible nitric oxide synthase (iNOS, red), and β-integrin marker of microglia (CD11b, green). The resulting

fluorescence panels show that Fg remained functional in activating the microglial cells, even after its surface adsorption to the BCP surface. Image reproduced with permission from Ref. [121] Copyright (2016) American Chemical Society.

Thorough and systematic understanding of biological consequences of surface-bound proteins on nanoscale polymer templates will be a prerequisite to developing miniaturized biomaterials, high throughput biosensors, and implantable medical devices. In this regard, additional works are still warranted for examining the stability and functionality of surface-bound proteins in more biologically complex environments and over an extended period of time. Such future studies for examining the stability and functionality of nanotemplate-bound proteins should accompany the aforementioned efforts of elucidating the chemical and physical factors of underlying polymer templates. This way, the full potential of nanoscale protein-polymer interactions to ultimately tailor the biological consequences of the adsorbed protein layer to specifications on demand such as revealing/restricting a particular protein binding pocket or inducing well-regulated cell adhesion/differentiation can be better realized in the practical applications.

6 CONCLUSIONS and FUTURE DIRECTIONS

Despite many scientific activities dating back to the 1960s, the important phenomenon of protein adsorption to solid surfaces still remains challenging to explain in a simple way [209], especially for those more complex adsorption interfaces that exhibit nanoscopic topological and

chemical variations on the adsorption platforms. Nevertheless, spearheading research efforts have been launched to investigate various systems of nanoscale protein adsorption due to the ubiquity of the phenomenon and its immense importance in nanobiotechnology applications which impact the food industry, bioresearch field, and medical sector. As overviewed in this Review, experimental and theoretical studies have already begun to provide detailed, single biomolecule level understanding of protein adsorption mechanisms and kinetics on nanoscale polymer surfaces, including those surfaces that present physical and chemical variations at the length scale commensurate with single protein dimensions. Initial findings from these studies have shown new intriguing characteristics of nanoscale protein adsorption that are entirely different from the adsorption behaviors observed on bulk- and macro-scale polymer surfaces exhibiting no chemical variations. Such adsorption features include self-selective surface partition, close-packed 2D assembly, well-ordered large-area packing, increased surface residence time of surface-adsorbed proteins, and high resistance to protein replacement. The initial studies have also pointed out that protein adsorption will be more accurately predicted by considering the nanoscopic structural details and chemical complexity of the different regions present on the polymer surface together with those of the protein surface itself. In addition to static adsorption studies, initial dynamic studies have unfolded considerably different kinetics of protein adsorption on nanoscale polymer surfaces than what have been previously reported on chemically uniform, macroscale surfaces [205, 206]. Despite these spearheading research efforts, the field is still at a nascent stage. Very little is known for nanoscale protein adsorption to date and only limited pieces of experimental data exist that can directly provide single biomolecular level verification of many commonly assumed protein adsorption mechanisms and kinetics [91, 104, 105, 124, 183, 210]. A deeper understanding of protein adsorption pathways and kinetics as well as distinctive protein interaction

properties on nanoscale polymer surfaces will be able to propel the current capacity to rationally design custom-tailored surfaces in biomedical research and industry settings. Furthermore, extending nanoscale protein adsorption studies to include a wider range of polymer and protein systems with varying length scales will be able to narrow the current knowledge gap in ensemble versus single protein adsorption behaviors and promote the practical utility of protein constructs built on polymer nanotemplates. The anticipated knowledge will ultimately enable precise control over the adsorbed protein amounts, spatial registry, orientation, alignment, packing degree, biofunctionality, and the time associated with protein adsorption/desorption/replacement on polymer surfaces.

Looking ahead, there awaits plenty of exciting research opportunities to advance the research field of nanoscale protein adsorption at polymer interfaces and to lay the fundamental groundwork for much needed design principles for the development of biomaterial and biomedical products. Further research areas to deepen our understanding of nanoscale protein-polymer interaction are also identified in each topical section of this Review. These future opportunities are suggested in part to reflect the fact that the field still lacks systematic studies that combine theoretical, computer simulation, and experimental approaches. Studies that quantitatively compare theoretical models to experimental data conducted on protein adsorption that encompass multilength scales ranging all the way from bulk to nanometer scales will be beneficial for establishing the validity of empirical guidelines and for the development of optimal polymer nanomaterials customized for, for example, bioinert or biospecific applications. Compared to studies examining static and single component protein adsorption properties, those for dynamic adsorption properties at nanoscale polymer interfaces and multicomponent protein systems are also far less developed. Hence, future efforts should also consider time-dependent

multicomponent protein adsorption mechanisms and kinetics onto nanoscale polymer surfaces to better represent a common complex biological and physiological environment. Other important aspects to examine in future works pertain to protein structure-function relationships specifically for nanoscale polymer-bound proteins. As discussed in this Review, the physical and chemical properties of a given underlying polymer template can be modulated to lead to substantial changes in protein conformation and orientation that can consequently affect the biological functionality of the surface-bound proteins. Such future efforts will be able to promote the development of next-generation biomaterials and biomedical sensors/devices that are small and noninvasive with built-in biological functionalities by capitalizing on the newly identified, unique protein interaction properties on nanoscale polymer surfaces.

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