JOURNAL OF Applied Polymer SCIENCE

Special Issue: Bioactive Surface Functionalization

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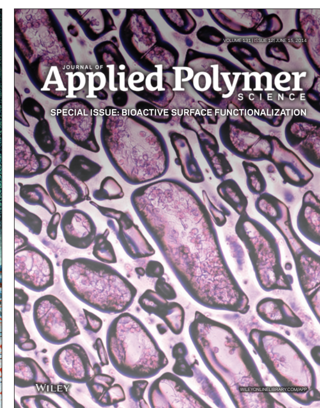

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REVIEW

Block Copolymers for Protein Ordering

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ABSTRACT: Large scale production of sub-micrometer features aids vital understanding of the interactions present at that scale in the biological world as well as the interactions with artificial surfaces. Considerable research efforts have been put into the study and control of block copolymer self-assembly, both in bulk phase and as thin films. Well-ordered block copolymer films can be used for pattern transfer through etching or evaporation and have also been explored as templates for directed nanoparticle assembly and as hosts for proteins. This review is focused on the emerging area of using block copolymer films for the creation of ordered arrays of protein or peptides. The work done in the field is reviewed and focus is being put on how the systems have been characterized as this is an inherent difficulty of such systems. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40360.

KEYWORDS: copolymers; nanostructured polymers; proteins; self-assembly; films

Received 14 November 2013; accepted 23 December 2013
DOI: 10.1002/app.40360

INTRODUCTION

There is increasing interest in arranging active biomolecules at interfaces for applications such as sensing, catalytic devices[1] and to study and control micro- and nanoscale interactions with cells.[2–6] Traditional lithography and direct write techniques are limited to producing rather small areas of patterned material and most biomolecule patterns have been produced on solid inorganic materials such as silicon or glass. Block copolymers offer exciting possibilities to this field. By varying the composition of block copolymers many different ordered phases can be produced over large areas in either bulk or thin film materials,[7–11] opening up the possibility of ordering of biomolecules in three dimensions. The feature sizes available through block copolymers depend on the length of the blocks and can be tailored to match the size of the biomolecules themselves. Further advantages of polymeric templates include the potential to adjust the elasticity of the material to fit a specific biological application[12]; optical transparency and biocompatibility, particularly of the highly hydrated polymers; and permeability to nutrients and gases.[13] The micro phase-separation of block copolymers has been well studied and reviewed over recent years[7–9] and the wealth of knowledge about these systems has enabled applications where ordered biomolecule arrays are produced using block copolymers. The production of well-defined protein- (or peptide-) polymer conjugates has led to novel hybrid materials able to self-assemble into nanodomains. This review focuses on the use of block copolymer thin films for the creation of ordered arrays of proteins or peptides. Attention will be given to methods that have been successfully employed to characterize these materials as the small feature sizes and relatively low chemical contrast of these hybrid materials imposes some special requirements.

BLOCK COPOLYMER SELF-ASSEMBLY

There are many well-developed fabrication techniques to produce nanoscale surface features, and these techniques can be divided into top-down and bottom-up approaches. In the top-down approach, patterns are imposed on the substrate by a mask, master, or direct writing, whereas the bottom-up approach utilizes the self-assembly of small building blocks into larger patterns. Figure 1(A) demonstrates what feature sizes that are commonly patterned by various techniques. Most suited to the smallest feature sizes are the versatile but expensive and time-consuming direct write techniques, along with the self-assembly technique of using block copolymers. The key benefit of utilizing self-assembly techniques is that they do not require expensive machinery and that they are suitable for the production of large areas of patterned material. The development of living polymerization techniques such as atom transfer free radical polymerization (ATRP)[14,15] and reversible addition fragmentation chain transfer (RAFT)[16] have enabled the facile synthesis of block copolymers that are made up from two or more homopolymer subunits linked by covalent bonds. If the repulsive forces between the chemically distinct, but connected, blocks are large enough, a micro phase separation of the dissimilar polymer chains into periodic domains occurs. The size of the domains depends on the

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block lengths, but is typically in the range of 5–50 nm.[9,11,17] The micro phase separated structures formed by block copolymers depend on the degree of polymerization (N), the volume fraction of the blocks (f) and the Flory-Huggins interaction parameter (χ), which in turn depends on the chemical nature of the blocks.[9,18,19] AB block copolymers generally self-assemble into spheres, cylinders, lamellae or gyroids [see Figure 1(B)][7], whereas triblock (ABC) copolymers can assume more complex morphologies.[18,20]

When block copolymers are prepared as thin films on a surface, the self-assembly is critically influenced by both the film thickness and the interaction energy at the polymer/substrate and polymer/air interfaces respectively.[8,9,11] Practically, this means that different phases may be formed by the same polymer on different substrates, at different film thicknesses or even at different humidity. Furthermore, the solvent used during film preparation (its specificity for one or both polymer blocks) is of critical importance, as is the solvent evaporation rate. To achieve long range order, an annealing step at elevated temperature or exposure to solvent vapor, is generally required.[11,21] The annealing gives the material higher mobility within the film and allows restructuring towards equilibrium morphology.[8,9]

Applications of Block Copolymers

The myriad of ordered nanostructures that are accessible through block copolymers makes these materials interesting for a number of applications. One main driving force behind the creation of highly ordered thin films is the production of small features for the electronics industry. Although state of the art UV photolithography can achieve patterning down towards feature sizes of 25 nm, it is evident that block copolymers offer scope for improvement, with domain sizes from 5 to 10 nm.[22] Differential etching rates of domains have been used to transfer block copolymer patterns into inorganic structures[17,22–24] and ordered block copolymer materials have also been used to

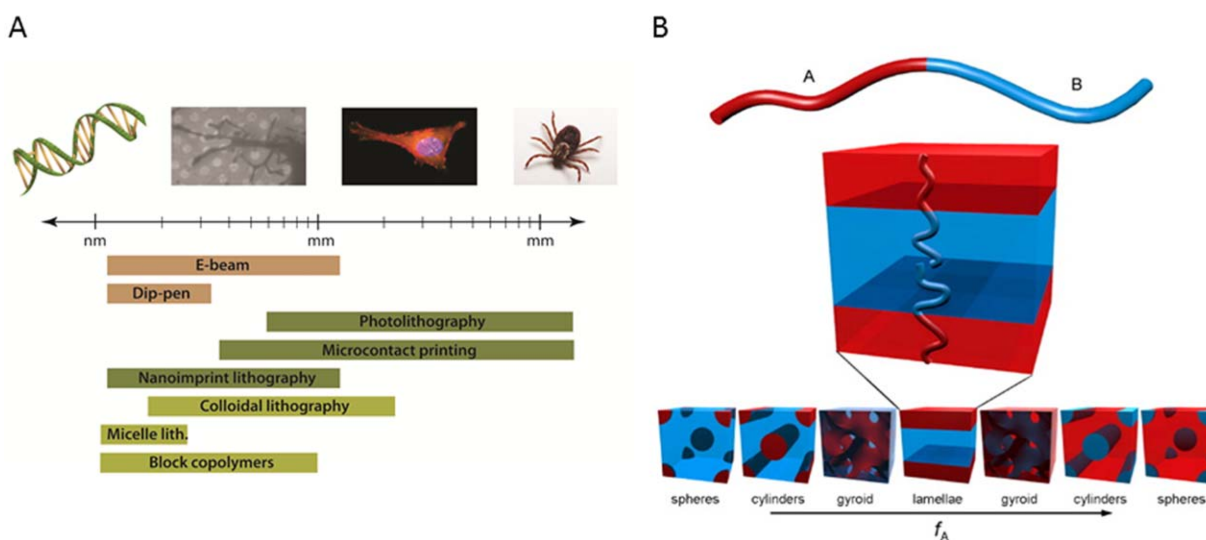

Figure 1. A: Illustration of the different feature sizes available through different patterning techniques. B: Representation of the phases available by varying the relative lengths of the two polymer blocks (f_A) of a typical A-B diblock copolymer. Panel B reprinted from Ref. 7, Copyright (2007), with permission from Elsevier. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

template silica growth.[25–27] In an effort to reduce the number of processing steps, block copolymers have also been used for direct patterning of metal nanoparticles,[28–30] which has received a lot of attention and has also served as a starting point for coassembling block copolymers and biomolecules; a field which will be reviewed here.[29] Another area where block copolymers are successfully used is that of drug delivery.[31–35] The chemistry of the polymers can be selected to be water soluble, biocompatible, biodegradable,[36,37] or stimuli-responsive[38] as needed. There are many examples of block copolymers utilized as effective drug delivery vehicles, with micelles or vesicles in solution being the obvious nanostructures of use. The field of using block copolymers for drug delivery has been thoroughly reviewed elsewhere,[32–35,38,39] and although biomolecules are often encapsulated, those applications are outside the scope of this review. Here, the primary focus lies on the creation of ordered arrays of proteins or peptides using block copolymers.

BLOCK COPOLYMERS TO ORDER PROTEINS OR PEPTIDES

Patterns of proteins and peptides are of interest for applications such as sensing, biomaterials, for control of cell fate, and for fundamental studies of cell-material interactions.[2,4–6,40–42] Block copolymers can be utilized to produce ordered protein patterns over a large area, and also offer the possibility to modulate the elasticity of the underlying material. Block copolymers have further scope to aid the self-assembly of proteins into higher order structures in solid state films or materials, either by coassembly or by using the protein or peptide as an integral part of the block copolymer.[29,43–45] The incorporation of, for example, enzymes into a film may stabilize the protein function, improves reusability, and removes the need for separation processes. Membrane proteins, not otherwise functional outside a lipid bilayer, offer further scope if they can be incorporated into block copolymer films in a functional fashion, and applications within sensing- and catalyst materials lay ahead.[46] Challenges lie in ensuring sufficient diffusion of molecules or electrical transport within the material, both of which should be addressable through the wealth of available chemistries and structures formed by block copolymer materials. Protein self-assemblies offer the key advantage that functional groups can generally be engineered into the structure without disrupting the self-assembly. It is therefore obvious that the combination of engineered protein structures ordered by block copolymers can provide highly versatile and promising materials. One key limitation to the development of such hybrid materials lies in the difficulty of characterizing the materials and the function/structure of the incorporated protein, due to the inherently small sizes involved.

Indirect Use of Block Copolymers for Protein Patterning

It is well established that a contrast in surface chemistry can be used to create protein patterns,[5,47] with the contrast between gold and metal oxides proving particularly useful due to the specific surface chemistries available for those materials (thiol- and silane chemistry, respectively) and the difference in surface charge.[5,6,42,47,48] One very successful example of how the small sizes of block copolymer structures have been utilized to produce ordered arrays of peptides is the "block copolymer micelle lithography" developed by Spatz et al.,[49] where block copolymers in the micellar form are used to produce 2D ordered arrays of gold dots. The method utilizes the amphiphilic block copolymer poly(styrene)-*block*-poly(2-vinylpyridine) (PS-*b*-P2VP). By dissolving the polymer in toluene, a solvent specific for the polystyrene block, micelles were formed that were subsequently loaded with $AuCl_4$ ions within the polar core of the micelles. The ions were reduced to elemental gold and micelles were transferred onto samples forming ordered arrays through controlled dip-coating [Figure 2(A)].

After removal of the polymer surrounding the gold particles by oxygen plasma, the particles can be decorated by peptides or protein and the surrounding area passivated by poly(ethylene glycol).[2,50,51] Both the size of the gold dots and the distance between them can be tailored by changing the block lengths, a feature that has enabled important understanding of how cells adhere to surfaces *in vitro*, or to the extra cellular matrix in vivo, through focal adhesions [Figure 2(B)]. The method has also been subsequently extended to produce microstructures of ordered gold nanoarrays through adding a photolithographic step,[52] and the patterns have also been successfully transferred to PEG hydrogels of different elastic moduli.[3] Recently, a polystyrene-*block*-poly(4-vinylpyridine) (PS-*b*-P4VP) diblock copolymer was used in a similar fashion, but in this research a solution of PS-*b*-P4VP micelles was spin-coated as thin films and subsequently annealed to produce an array of cylindrical microdomains oriented normal to the surface. The hydrophilic block was soaked in metallic precursor ions resulting in ordered gold dots with a diameter of ~21 nm, and used to bind ferritin.[53]

Protein Patterns on Block Copolymers

Ordered arrays of block copolymers can be used directly to create protein arrays by utilizing blocks that lead to preferential protein adsorption to one of the blocks. Kumar et al.[54] demonstrated that nanopatterned thin films of polystyrene-*block*-poly (methyl methacrylate) (PS-*b*-PMMA) can be used for preferential protein adsorption to the PS domains. The more hydrophobic nature of the PS block was postulated as the driving force for this preferential adsorption under the experimental conditions used (20 s protein adsorption followed by rinsing and drying) [Figure 3(A)]. It was further demonstrated that protein adhesion is favored at the interface between PMMA and PS leading to a three to four times higher protein density on the BCP films than on the PS homopolymer.[55] Lau et al. have carefully considered the mechanism of protein adsorption to the same chemical contrast.[56,57] Hexagonally ordered cylinders of PS were created in a PMMA matrix through spin-coating thin films of the block copolymer, resulting in circular PS domains exposed at the interface. As many proteins present preferential adsorption to PS over PMMA,[54,57–61] careful choice of parameters enabled the formation of circular protein patterns [Figure 3(B)]. Particularly, the authors identified a dynamic rinse being vital for the production of well-defined patterns. Such a rinse leads to movement of protein from the PMMA domains to the PS domains and an enrichment of protein particularly on the PS close to a PMMA boundary.[57] As protein is well known to partially denature at hydrophobic interfaces, it is important to investigate the functionality of the patterned protein. This has been investigated by enzyme assays[57,59] and antibody binding.[59] Although many of these studies nicely demonstrate catalytic

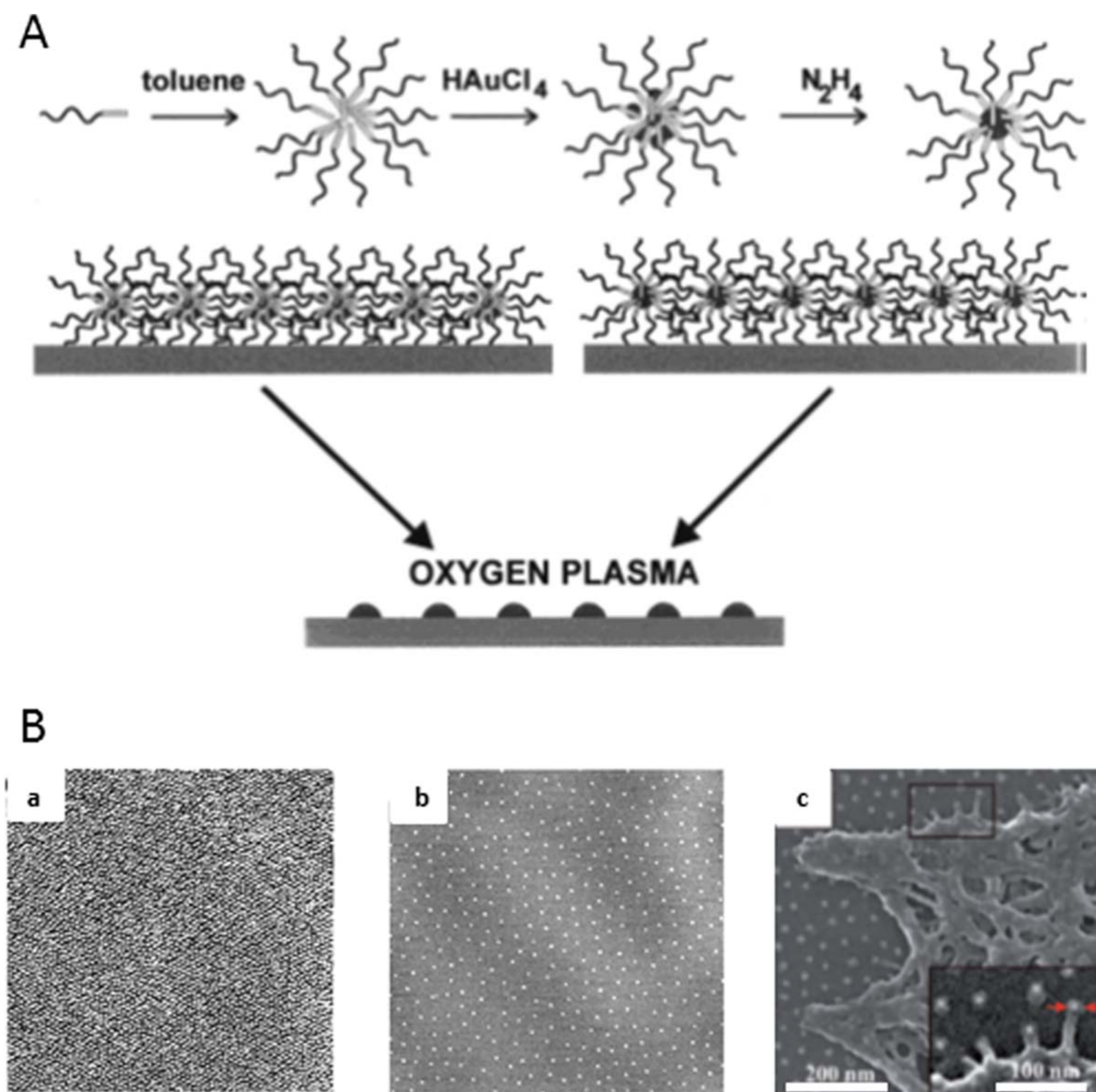

Figure 2. Block copolymer micelle lithography: A: Schematic drawing of the formation of PS-*b*-P2VP block copolymer micelles in toluene and the complexation with $HAuCl_4$ followed by the reduction to form one gold particle in each micelle. The metal salt or metal particle micelles form ordered films on substrates though Langmuir Blodgett assembly and dip-coating and oxygen plasma produces naked gold clusters at the substrates. B: The size of and distance between particles is varied by polymer composition. Here, scanning electron micrographs from resulting clusters formed of PS(325)-*b*-P[2VP(HAuCl4)0.5(75)] in (a) and PS(1700)-*b*-P[2VP(HAuCl4)0.1(450)]in (b). In (c) the interaction of a cell with 6 nm gold particles with a lateral spacing of 58 nm, functionalized with adhesion peptides (cRGD), can be seen as viewed by SEM. The length of image a-b is 3 μm and scale-bar in c is 200 nm (100 nm in inset). Panel A and B a-b reproduced with permission from Ref. 49. Copyright (2000) American Chemical Society. Panel B c is reproduced with permission from Ref. 51. Copyright (2008) American Chemical Society. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

activity, with controls such as film without protein or after denaturation of protein, it remains uncertain how active single enzymes are as compared with their solution counterparts (i.e., how much activity they lose/gain when incorporated in the film, something that would require information about the total amount of enzyme in the film).

The micelle forming PS-*b*-P4VP has also been utilized for preferential protein adsorption to the PS block.[62] The most interesting part of this work is that a thin film assembly of micelles formed in toluene is used (with PS covering the P4VP interior) by spin coating, followed by "opening" the micelles by exposure to ethanol after spin coating. This leads to an interface with a chemical and topographical pattern, and where one block (P4VP) is expected to resist protein adsorption. After a short exposure to protein in buffer solution (IgG and mushroom tyrosinase) clear preferential adsorption to PS domains was seen.[62] This work was followed-up recently with the creation of more complex morphologies.[63]

The need for carefully controlled nonequilibrium adsorption conditions[58] to create protein patterns from phase separated

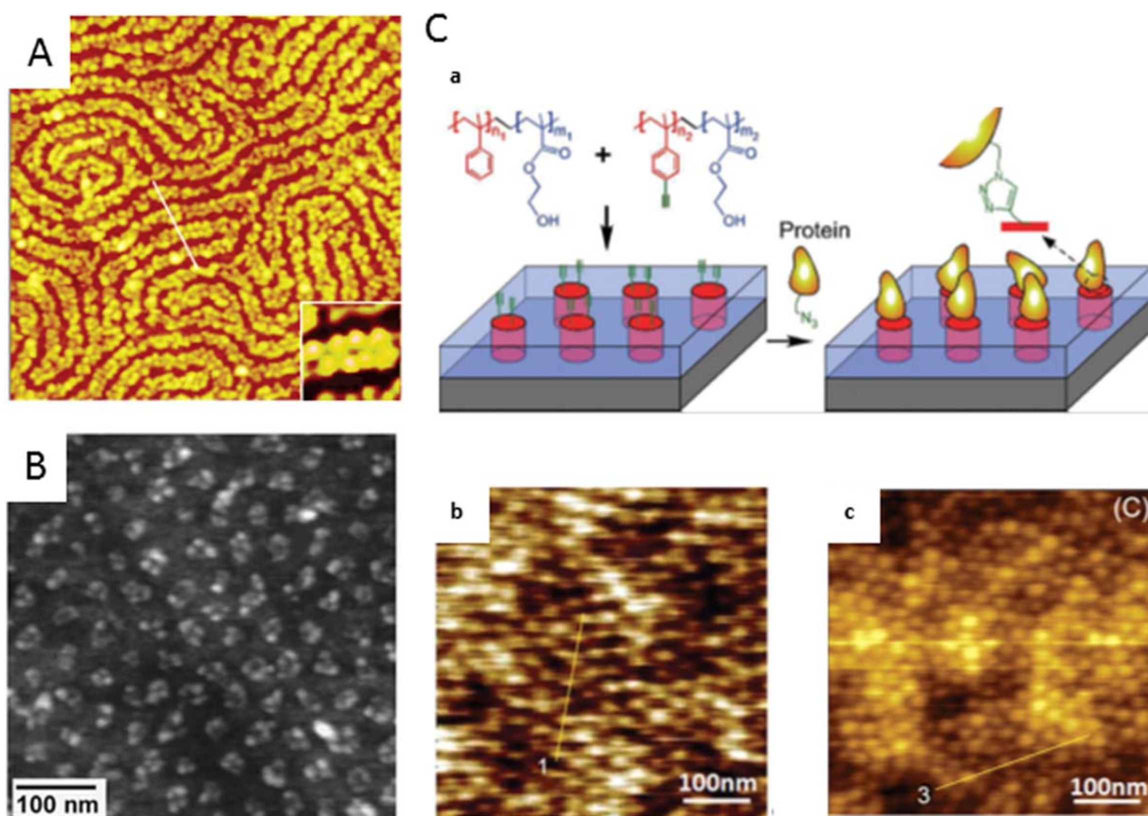

Figure 3. Protein patterns on block copolymers: A: AFM height image (in air) of 20 μg/mL of IgG deposited selectively on PS domains in the microphase-separated PS-*b*-PMMA thin films on silicon oxide substrates. Scan size: 1 x 1 μm^2 (150 x 150 nm^2 in insert). B: AFM height image (in air) of IgG adsorbed onto the circular domains of PS in PMMA matrix, height scale 10 nm. C: (a) Schematic illustration of the generation of PS-*b*-PHEMA block-copolymer thin film with PS cylinders presenting alkyne functionality (red) in the PHEMA (blue) matrix. The PS domains present alkyne functionality for the specific immobilization of azide-tagged protein molecules via click chemistry. (b) and (c) are AFM height images recorded in water, of (b) the bare PS-*b*-PHEMA film, and (b) the film after functionalization with lysozyme by click chemistry. Height scale 5 nm in (b) and 20 nm in (c). Panel A reprinted with permission from Ref. 54. Copyright (2005) American Chemical Society. Panel B reprinted with permission from Ref. 57. Copyright (2008) John Wiley and Sons. Panel C reprinted with permission from Ref. 64. Copyright (2012) John Wiley and Sons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

block copolymer surfaces is prevalent. Although this property provides extra parameters to control the pattern, it ultimately reduces the usability and fidelity of the patterns in protein fouling environments (such as serum containing cell culture), an issue that is addressed in a recent study by Shen et al.,[64] where thin films of polystyrene-*block*-poly(2-hydroxyethyl methacrylate) (PS-*b*-PHEMA) was used. The block copolymer formed well-ordered PS cylinders in a PHEMA matrix. The interesting part of this study is the fact that it utilizes PHEMA, which is a protein resistant polymer which should allow for longer contact times with protein without non-specific adsorption. By incorporating a fraction of alkyne-functionalized PS, the small (15 nm in diameter) PS domains were used for selective binding of azide tagged protein via click-chemistry [Figure 3(C)], while the surfaces resisted nonspecific protein adhesion of untagged protein.[64]

Other block copolymer chemistries also offer the possibility to pattern protein using longer adsorption times and higher protein concentrations. Liu et al.[65] investigated BSA adsorption on self-assembled poly(styrene-*block*-isoprene) thin films, where both blocks are hydrophobic but differ markedly in elasticity. BSA was found to adsorb preferentially to the harder PS domains creating a protein nanopattern. A highly hydrophobic surface resisting protein adsorption is unusual[66] and the high molecular mobility of the PI segments was suggested as an explanation for this behavior.[65] Nehring et al.[67] present NTA (nitrilotriacetate) and tris-NTA functionalized poly(butadiene)-*block*-poly-(ethylene oxide) (PB-*b*-PEO) able to bind histidine tagged protein. This is a very promising concept for ordering of protein at block copolymer surfaces, as the reaction conditions are mild and it utilizes specific interactions with hexa-histidine tags, commonly present in expressed and purified protein. In this particular case, artificial membrane structures were targeted, and vesicles and films assembled at air liquid interfaces were investigated. The same concept should also be applicable for binding proteins to ordered thin films of phase separated block copolymers. Cresce et al.[68] explored the metal ion binding capabilities of one of the blocks of their norbornene-norbornenedicarboxylic acid (NOR/NORCOOH) diblock copolymer to selectively bind histidine tagged protein to the carboxylic acid-containing block. The polymer was shown to form ordered regions with spherical domains of ∼30 nm in diameter after casting films from THF,

however the ordering was by no means perfect. Nickel ions in particular were shown to increase the binding of histidine tagged protein. A plausible explanation is that the protein is preferentially bound to the carboxylic containing block; however, no evidence to support this theory was provided.[68] One system that have been successfully functionalized and used in cell studies is that of PS-*b*-PEO. Hexagonally ordered cylinders of PEO in PS have been shown to be capable of resisting both protein adsorption and cell adhesion in the unfunctionalized state.[69] Functionalization of the PEO domain with cell adhesive peptides (RGD) either directly,[70] or via maleimide functionalized PEO domains,[71] have demonstrated that the system can be used to adjust the cell adhesion to, and behavior at, the surfaces via the nanoscale ligand presentation.[70–72] That work nicely complements previous work on homogenous surfaces of varying elasticity,[12,73] and that on solid interfaces with varied peptide spacing[2,51] or size of protein patches.[6,40,42]

Protein Ordering in Block Copolymers

By allowing a protein or peptide to assemble together with the block copolymer to form a solid state material, several advantages are targeted. The process is shortened, relative to protein patterns on block copolymers, to become a single step process; the protein may be protected and stabilized within the film; and the protein may be organized in one more dimension creating protein assemblies that are ordered by the block copolymer pattern. In a pioneering study by Lin et al.,[29] PS-*b*-P2VP was mixed with tri-*n*-octylphosphine oxide-covered CdSe nanoparticles, and spin-coated as thick films onto substrates. The nanoparticles were shown, by scanning electron microscopy and analysis of grazing incidence small-angle X-ray scattering data, to be incorporated into the films and to reside within the P2VP domains. Furthermore, there was a change in the ordering at the surface, with the cylindrical domains of P2VP being shifted from horizontal to vertical in relation to the surface upon the incorporation of the nanoparticles. The inclusion of a metal nanoparticle enables visualization of the incorporated particles, something that is significantly more difficult with protein. The same paper also investigated the coassembly of ferritin, an iron carrying protein, and the lamella-forming poly(2-vinylpyridine)-*block*-poly(ethylene oxide) (P2VP-*b*-PEO) where the ferritin was shown to be incorporated into the PEO microdomains, suppress crystallization, mediate interfacial interactions and reorient the microdomains [Figure 4(C)].[29] The ferritin was pegylated to stabilize it in the organic solvent used and to enable the incorporation into the film. A similar concept was used by Presley et al.,[44] where a block copolymer system offering more ordering was used, that of polystyrene-*block*-poly(ethylene oxide) (PS-*b*-PEO). PS-*b*-PEO was coassembled with either a synthetically made heme-binding protein motif (a coiled-coil α-helix bundle) or myoglobin, both of which were conjugated to 2 kDa PEO. The PS-*b*-PEO was shown to form nicely ordered films with hexagonally packed cylindrical domains of PEO oriented normally to the surface, as expected. The same hexagonal domains were observed in the presence of the peptide or protein [Figure 4(A,B)]. The resulting materials were analyzed in terms of peptide and protein integrity, and secondary

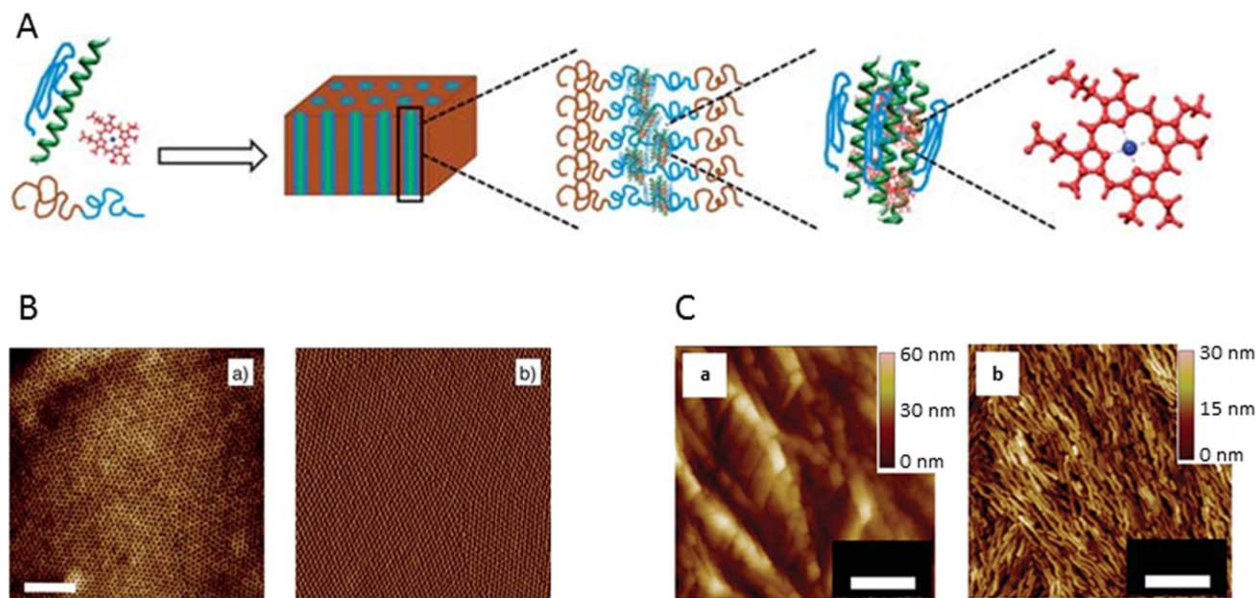

Figure 4. A: Schematic drawing from Presley et al illustrating the proposed hierarchical assembly of peptide-polymer/block-copolymer thin films. All components are blended in solution and processed into thin micro phase separated thin films. Helix bundle peptides, a heme-binding motif, (shown in green) are sequestered within the BCP domains. Synthetic polymers are conjugated to the bundle periphery in order to mediate interactions with the PEO domains of the PS-*b*-PEO, and are shown to bind small molecule cofactors (shown in red). B) AFM height (a) and phase (b) images of micro phase separated thin films of PS-*b*-PEO containing the synthetic heme-binding motif. C: AFM height images from the pioneering study by Lin et al. showing a clear change in the phase behavior of the P2VP-*b*-PEO thin film before (a) and after (b) the incorporation of ferritin. Scale bar 400 nm in both B and C. Panel A, B reprinted with permission from Ref. 44, Soft Matter, 2011 and panel C reprinted with permission from Ref. 29. Copyright (2005) Nature publishing group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and tertiary structure of the helix bundle was seen to be maintained as assessed by circular dichroism and peptide-dependent heme binding, while myoglobin was shown to retain enzymatic activity.[74] However, the localization of the protein/peptide to one particular domain and the presence of any ordering of the protein/peptide within each domain remains a challenge to characterize.

Incorporation of stimuli-responsive polymers as one block in the block copolymers offers further functionality to the material. Kim et al.[75] used RAFT polymerization to synthesize poly(*N*-isopropylacrylamide)-*block*-poly(2-(dimethylamino)ethyl acrylate (PNIPAM-*b*-PDMAEA). This block copolymer is water soluble, an obvious advantage for the co-assembly with protein. PNIPAM is a well-studied thermoresponsive polymer[76,77] and in this case enables films to be made hydrophobic for stable use in aqueous environments after fabrication, while pH-dependent ionic interactions between the protein (m-Cherry) and PDMAEA are used to drive the self-assembly into micellar containing films.[75] The incorporated protein was shown to remain active and could be released at elevated pH due to weakening of the ionic interactions between the protein and the polymer.[75] This study struggled to provide stringent evidence of film structure and protein localization, but nonetheless paves the way for future work by utilizing a water-soluble and stimuli-responsive block copolymer.

Other examples of protein block copolymer co-assembly includes that of hemoglobin or myoglobin with the triblock copolymer poly(ethylene oxide)$_{100}$-*block*-poly(propylene oxide)$_{63}$-*block*-poly (ethylene oxide)$_{100}$ (PEO-*b*-PPO-*b*-PEO)[78] or poly[*N*-(2-methacryloyloxyethyl) pyrrolidone]-*block*-poly[glycidyl methacrylate] (PNMP-*b*-PGMA),[74] demonstrating materials capable of electrocatalysis, however without any evidence of micro phase separation of the films. The block copolymer PS-*b*-PHEMA has also been used to stabilize oil/water inverse emulsions to form macroporous protein-containing films with different resulting patterns depending on underlying surface chemistry.[79]

Protein as Part of Block Copolymers

Rather than using block copolymers as structures to order protein, protein or peptides have also been used as integral parts of block copolymers to tailor certain properties such as structure, binding to small molecules or nanoparticles,[80,81] catalytic activity[82] or cross-linking and mechanical properties.[83] Recent developments in protein engineering as well as polymer chemistry, has enabled the design and synthesis of tailor made protein–polymer conjugates.[46,84] The group of Bernard Gallot pioneered the field by their work on peptide-polyvinyl or -polybutadiene conjugates and the structures thereof [Figure 5(A,B)].[43,85,86] It has been demonstrated that the principles of block copolymers can be extended to peptides[87] and a range of amphiphilic block copeptides consisting primarily of blocks of cysteine and lysine were shown to form structures able to template solid silica structures under remarkably mild conditions.[82] The cysteine residues take an active role in the conversion of the silica precursors (presumably due to the nucleophilic properties of the sulphydryl group, able to initiate hydrolysis of the tetraethoxysilane), demonstrating the advantages of the inclusion of a bioactive block in the block copolymer synthesis. The most studied peptide-polymer conjugates are those where PEG is linked to one end of the peptide terminus.[88] Such a conjugation serves to enhance the stability towards temperature and pH, but may affect the secondary and tertiary structures of the peptides, a phenomenon which has been well studied.[80] The field of peptide-polymer conjugation is covered in several recent reviews to which the interested reader is directed.[46,80,89–91] Important knowledge can be drawn from the work on self-assembling peptides conjugated with PEG,[92] where the PEG conjugation mainly serve to limit lateral aggregation of the fibers by forming a protective layer around them.[90,93] Beta sheet peptides forming structures are reviewed in Koenig et al.[93] and amyloid beta-peptides conjugated to PEG are investigated in Krysmann et al.,[94] where the PEG used was designed to have a melting temperature above room temperature leading to PEG crystallization aiding structure formation. There is also an enormous interest in PEG conjugation to stabilize and solubilize peptides and proteins for therapeutic purposes,[95] an area outside the scope of this review.

The use of peptides or proteins in block copolymer systems adds a dimension to the self-assembly process through the secondary structures that can be formed within the bio-block (alpha helices and beta sheets). Additionally, certain peptide motifs are well known to self-assemble into ordered hierarchical structures. The majority of work in this field still targets the formation of drug delivery vehicles (such as micelles and vesicles) or hydrogels.[96] A large body of work on peptide-polymer conjugates in bulk exist,[80,97–99] but there are a limited number of studies focused on long range order in thin films.[98,100] Recently, protein–polymer conjugates have also been shown to micro phase separate and form nanostructured materials.[45,101] In studies by Thomas et al.,[45,102] a globular model protein (m-Cherry) was conjugated to the thermo-responsive PNIPAAM, and the bulk nanostructures formed after casting and annealing were investigated in detail [Figure 5(C,D)]. The selectivity of the solvent (water) for either block was changed by adjusting the temperature of the solution and the pH. This affects the solubility of the polymer and the charge of the protein, respectively.[102] The phase behavior was seen to differ from that of traditional block copolymers and to be dominated by that of the polymer block. A pH matching the isoelectric point of the protein leads to aggregation and precipitation of the protein. For these types of studies it is important to note that careful considerations are needed before choosing the protein component of the hybrid block copolymer. In the case of m-Cherry, that protein was chosen as the native sequence lacks cysteine residues, so that a site specific mutation could provide a unique thiol-conjugation site for the thiol-maleimide bioconjugation of the chain transfer reagent.[102] Furthermore, the fact that the protein is fluorescent and that a spectrophotometric method is available to judge the maintenance of protein fold and function, makes it a good candidate for model studies.

Inspiration from the peptide-polymer materials and advances in sequence controlled polymers[103] have also let to materials where one block is designed to adjust the block interactions by controlling its sequence. Rosales et al.[104] prepared block copolymers with tunable compositions synthesized from polystyrene and a

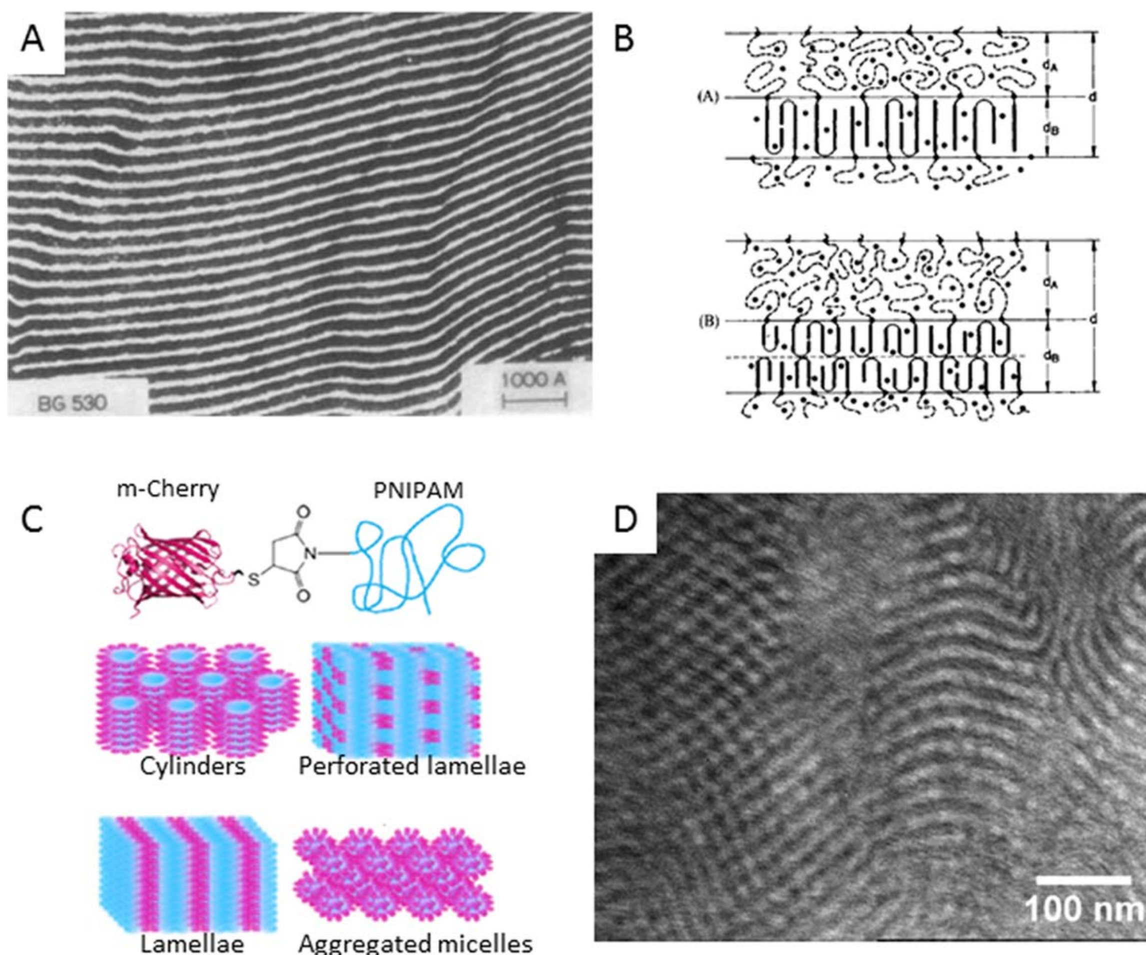

Figure 5. Peptide and protein containing hybrid block copolymer structures: A: Pioneering study showing lamellae of polybutadiene-*b*-poly(benzyl-L-glutamate), where polybutadiene blocks appear dark due to selective staining by osmium tetroxide. B: A schematic representation of the lamellar structure with the two possible types of folding for the polypeptide chains. C: Globular protein m-Cherry conjugation with poly(*N*-isopropylacrylamide) and the observed possible phases formed. D: TEM of solid state structures formed from m-Cherry-*b*-poly(*N*-isopropylacrylamide) using a polymer selective solvent (water at room temperature), cryo-microtomed, and stained with ruthenium tetroxide. Panels A, B reprinted with permission from Ref. 43. Copyright (1976) John Wiley and Sons. Panels B, C reprinted with permission from Refs. 45 and 102. Copyright (2011) and (2012) American Chemical Society. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sequence-defined peptoid (N-substituted glycines) polymer. This was shown to be an effective system for controlling the interaction between the two blocks and controlling the nanostructured phase separation of the material.

CHARACTERIZATION

One hurdle to the development of new hybrid materials is the difficulty in thoroughly characterizing them to understand their phase behavior. Protein adsorption onto phase separated block copolymers is generally characterized by atomic force microscopy (AFM), either under ambient conditions or in liquid. As the protein pattern is present at the interface (generally a flat interface) AFM is well suited to study these patterns. Polymeric surfaces are intrinsically much more difficult to study using scanning electron microscopy (SEM) due to charging effects and instabilities under vacuum. If the protein or peptide can be tagged by metal particles though, SEM may prove useful. It is nontrivial to judge how the conformation of the adsorbed protein is affected, which is why many model studies utilize enzymes where the retained activity can be measured.[55,57,59,62] Another approach to estimate the bioactivity of adsorbed protein is by probing the surface with antibodies to a specific bioactive site of the molecule,[55,57] which can be measured by Surface Plasmon Resonance (SPR), Quartz Crystal Microbalance with Dissipation (QCM-D),[105] or AFM.[48,59] AFM can additionally be used for some proteins to determine the orientation and to some extent the conformation.[106] When the protein is embedded within the polymer material, the characterization becomes more difficult. AFM remains very useful in determining the phase behavior of the polymer, but determining the presence or absence of protein within the film is difficult. To image such materials, Transmission Electron Microscopy (TEM) stands out as the most viable option. In some cases, simply dipcoating a TEM grid in the polymer/protein solution is sufficient, but it is challenging to produce thin enough (<100 nm)

and homogenous films in that fashion. Thin films spin-coated on silicon oxide samples can be transferred to a TEM grid by floating on HF[107] or water followed by transfer to the grid. Bulk material (or thin films) can also be microtomed or focused ion-beam milled[107] into thin sections for TEM, providing information about the cross section. In most cases, the block copolymer materials require to be cryo-microtomed due to mechanical instability or dissolution in the embedding media. The films or sections then need to be stained to achieve a contrast between materials. Ruthenium tetroxide is often used as this exhibits different staining rates for different polymers.[102,108] A clear complication lies in getting enough contrast between the polymer phase where the protein is embedded, and the protein itself, and to our knowledge there are no published studies on protein embedded in a block copolymer film convincingly imaged by TEM. In the case of peptide- or protein–polymer conjugates; however, the biomolecule constitutes one domain of the micro-phase separated material. In these cases there has been success in achieving preferential staining of the protein for TEM.[43,85,102]

For these hybrid materials, as well as the coassembled materials, it is not only important to investigate the phases formed and the packing of protein therein under different conditions, but also to judge the structural stability and function of the incorporated protein. The methods available that were used to determine those properties depend, to a large extent, on the incorporated peptide or protein. Circular dichroism (CD) measures the differential absorption of left and right circularly polarized light and is able to determine the secondary structure content of peptides or proteins.[109] Although CD is generally a solution technique, it is has successfully been used on proteins in thin films,[44] and can add important information about the structure or loss of structure of incorporated protein. Not all proteins exhibit strong CD spectra, and the technique is most suitable to analyze proteins or peptides with a high portion of α-helices. Fourier Transform Infrared Spectroscopy (FTIR) also probes the secondary structure of proteins via their vibrational spectra, with the amide I and amide II bands being most sensitive to changes in secondary structure.[110,111] FTIR can also be used to determine the aggregation of fibrillar proteins due to a strong spectral feature derived from aggregated β-sheet structures.[112] Proteins or peptides with chromophores may exhibit a UV-vis spectrum that is sensitive to the conformation around the chromophore, which can give a qualitative measure of the tertiary structure,[44,102] and that can be measured in solid state. As previously mentioned, if direct measurement of the protein functionality is available through enzyme assays or cofactor binding that provides the ultimate answer of how any conformational changes have affected the protein. However, to be stringent, such analysis should be normalized to protein amount and compared with solution activity of the protein. As not all proteins exhibit strong CD spectra nor have activity that can be assayed, one may want to consider these aspects when choosing a protein for a model study. Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) data is used to determine the ordering in the materials, primarily to determine the phase behavior and the long range order of the samples and has proven particularly useful for the protein/peptide–polymer conjugate materials.[80,102] These scattering methods may also measure tertiary structure of incorporated proteins directly under carefully controlled conditions.[46]

PROSPECTS

The versatile nature of block copolymers in combination with the structures and activities available through proteins or peptides gives these materials many possible applications. Protein patterns have shown great promise for controlling cellular behavior and the combination with polymeric substrates adds an important property of adjustable substrate elasticity. For tissue engineering purposes, the highly hydrated block copolymers provide extra benefit by their permeability to nutrients and gases. In addition, the possibility of incorporating stimuli-responsive polymers opens up for the production of materials with adjustable elasticity and on-demand release of cargo, which combined with the possible incorporation of active biomolecules and display of cell adhesion molecules, makes this class of materials truly versatile and highly promising for future biomedical applications. Other future applications include those that take advantage of vertically aligned sub-nanometer channels, such as those for gas separation water purification and fuel cell membranes.[113–115] Xu et al.[116] recently demonstrated the co-assembly of cyclic nanotube-forming peptides and the block copolymer PS-*b*-PMMA, forming vertically aligned nanotubes over a large area. The inclusion of the peptide ensured that the pore size was small enough for applications of selective molecular transport, while the block copolymer structure served to orient and order the nanotubes.[116] Block copolymer assemblies may also be used for the incorporation and stabilization of membrane proteins.[117] Membrane proteins are otherwise only active in lipid bilayers, and the incorporation into more stable materials is an important step for the creation of functional devices for applications such as light harvesting and electron transport.[118] The controlled formation of nanoparticle arrays ordered through block copolymers and protein or peptide structures[29,81] also offers exciting possibilities. Previous work on creating protein assemblies has been largely centered around amyloid fibers[119–121] but has recently also involved native protein structures.[122] Recent work has also established methods to post functionalize assembled structures.[123,124] There is intense interest in "one-dimensional nanostructures" from the perspective of their excellent optical response. Controlled patterning and alignment of nanostructures is critical for both the study of the properties of such structures and the incorporation into devices. Aligned small nanoparticles are expected to interact through plasmonic coupling to resemble such one dimensional structures.[125] Few existing methods for nanoparticle alignment (such as inorganic templates)[126,127] come close to the sizes and predefined order available through patterning of protein structures.[122,128]

To conclude, advances in polymer chemistry and protein engineering have enabled the production of novel functional hybrid materials. A main hurdle to these developments, however, is the difficulty in characterizing the structure and function of the incorporated protein or peptide. Future developments in

techniques for the detailed characterization of these materials and the development of suitable computer based models would further accelerate the field.

ACKNOWLEDGMENTS

The authors are grateful to Lisa Strover for discussions and proofreading of the manuscript. The MacDiarmid Institute for Advanced Materials and Nanotechnology and the Faculty of Science Research Development Fund are acknowledged for financial support.

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