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FOCUS ARTICLE

# Protein crystal based materials for nanoscale applications in medicine and biotechnology

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The porosity, order, biocompatibility, and chirality of protein crystals has motivated interest from diverse research domains including materials science, biotechnology, and medicine. Porous protein crystals have the unusual potential to organize guest molecules within highly ordered scaffolds, enabling applications ranging from biotemplating and catalysis to biosensing and drug delivery. Significant research has therefore been directed toward characterizing protein crystal materials in hopes of optimizing crystallization, scaffold stability, and application efficacy. In this overview article, we describe recent progress in the field of protein crystal materials with special attention given to applications in nanomedicine and nanobiotechnology.

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Biology-Inspired Nanomaterials > Protein and Virus-Based Structures
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**KEYWORDS**
crosslinked enzyme crystals, intracrystal mass transport, porous protein crystals, protein crosslinking, protein crystal engineering

## 1 | INTRODUCTION

Long-term trends for nanomaterial engineering include the drive for greater control of self-assembly and atomic-level precision of the resulting materials. Greater control over the detailed structures within a material promises to improve the functionality, regardless of the function of interest (medical, biotechnological, industrial). To this end, a number of research laboratories have explored the potential utility of materials composed of self-assembled genetically encoded biomolecules. One draw for such materials is that the final atomic structure of the material may also ultimately be genetically encoded, with site-specific chemical changes accessed using standard mutagenesis methods. The result could be unprecedented structure–function relationship control for materials, with attendant improvements in functional properties. Within the larger domain of self-assembled biomaterials, we will review work on engineered protein crystals, which maximize the precision of the final assembly. To appeal to both lay audiences and experts we first provide a general background on protein crystallization methods and their structural properties followed by an overview of recent and historical contributions made to the field of protein crystal materials.

Proteins are important biological macromolecules responsible for many catalytic, signaling, and structural functions within cells and tissues. The diverse functions of proteins and their innate biocompatibility are attractive qualities for applications in nanomedicine and nanobiotechnology. As such, a wide variety of peptide- and protein-based nanomaterials have been reported, ranging from peptide nanofibrils, nanotubes, and nanospheres to smart biomaterials derived from collagen, elastin, and resilin (Desai & Lee, 2015; Feyzizarnagh, Yoon, Goltz, & Kim, 2016). Protein crystals constitute another intriguing class

  

of proteinaceous biomaterial, wherein highly ordered arrangements of proteins are packed with unparalleled volumetric density and precisely repetitive three-dimensional (3D) geometric presentation (Figure 1). From a materials perspective, it can also be quite useful to focus on the negative space found within the crystal material, defined by excluding the solid protein matrix. This negative space typically consist of interconnected porous solvent channels of varying geometric configuration, with pores often ranging in size from 0.3 to 10 nm (Matthews, 1968; Vilenchik, Griffith, St. Clair, Navia, & Margolin, 1998) and in rare cases have been shown to be even larger (Figure 1b,c) (Huber, McPherson, Keating, & Snow, 2018; Miller et al., 2008). The solvent content of protein crystals is most commonly found to vary between 27 and 65% (Matthews, 1968), commensurate to zeolites and metal organic frameworks. These alternative materials have reported utility for medical, catalytic, and sorption applications (Auerbach, Carrado, & Dutta, 2003; Furukawa, Cordova, O'Keeffe, & Yaghi, 2013; Horcajada et al., 2012). One goal for this review is to assess the application prospects for engineered protein crystals in these same domains.

Historically, protein crystals have been used both for protein purification and structure determination via X-ray diffraction. While the former application has been widely replaced by chromatography techniques, protein crystal growth continues to be the dominant method for determining 3D protein structures. As a result, the Protein Data Bank (PDB) (Berman et al., 2000) currently contains over 120,000 X-ray structures encompassing myriad packing arrangements and solvent channel geometries/topologies. With few exceptions, each such crystal represents an unexplored material since the structural biologists who grew the crystals were primarily motivated to study the detailed structure of the constituent molecules rather than the material properties of the crystals themselves. This review will focus on the exceptions to this rule. A number of research studies have noted the possible application and materials advantages of solid-state crystalline proteins or have otherwise sought to repurpose protein crystals for diverse material applications (Basu, Govardhan, Jung, & Margolin, 2004; Clair, Shenoy, Jacob, & Margolin, 1999; Jen & Merkle, 2001; Margolin, 1996; Margolin & Navia, 2001; Shenoy, Wang, Shan, & Margolin, 2001; Vilenchik et al., 1998).

With regard to the applications for engineered protein crystals, we note that cross-linked solid-state proteins typically enjoy significant stability advantages compared to proteins in solution. However, conditions that drive protein unfolding or degradation will still eventually degrade protein crystals. Therefore, the most suitable applications for engineered protein crystals will not require extreme temperature, extreme pH, or nonaqueous solutions. The goals, challenges, and application potential of various protein crystal materials will be discussed extensively throughout, giving special attention to the fields of nanomedicine and nanobiotechnology.

## 2 | PROTEIN CRYSTALS

### 2.1 | Crystal growth

The science of growing protein crystals has a rich history, dating back to the mid-1800s when Hünefeld first formed hemoglobin crystals by slowly drying the blood of an earthworm between two glass slides (McPherson, 1991). The process of slow

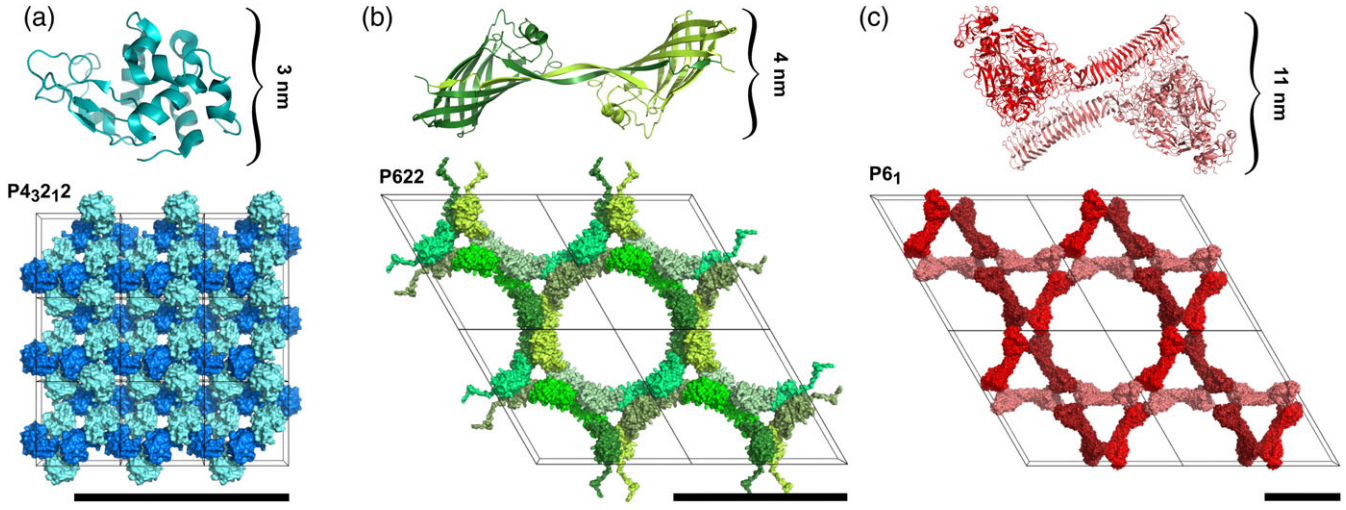


**FIGURE 1** Various protein structures (top) and their corresponding crystal scaffolds (bottom) illustrating the diversity of pore sizes and geometries within this class of material; boxes delineate unit cells; scale bars: 20 nm. (a) Tetragonal hen egg white lysozyme (HEWL); PDB code: 2HTX. (b) CJ-1 protein; PDB code: 5 W17. (c) Major tropism determinant P1 (Mtd-P1) complexed with Pertactin extracellular domain (Prn-E); PDB code: 2IOU. Images created using PyMOL v1.7.4.4, Schrödinger, LLC

drying caused hemoglobin proteins to move from an undersaturated stable-state to a supersaturated metastable-state and eventually inducing hemoglobin nucleation. As the crystals nucleate and grow, they fall out of solution thereby lowering the soluble protein concentration and moving the saturation point back into the metastable region where crystal growth occurs without further nucleation (Figure 2) (Chayen, 1998). Since Hünefeld's experiments, crystallization techniques have improved dramatically with the advent of easy-to-use crystal screening kits and high-throughput micropipetting robotics; however, the scientific principles behind protein crystallization remain the same. In general, protein crystals form when individual growth units self-assemble into an ordered crystalline scaffold through the formation of noncovalent interactions. This process occurs under precise conditions of reduced protein solubility which can be achieved by a variety of methods, the most commonly used being dialysis, vapor diffusion, or batch crystallization.

#### 2.1.1 | Dialysis

Precipitating agents can be slowly introduced to a protein solution via dialysis. A protein solution is placed in a vessel separated by a dialysis membrane from a larger reservoir containing a higher concentration of precipitating agent. The concentration of precipitating agent surrounding the protein gradually increases thereby reducing the protein solubility and eventually leading to nucleation (Figure 2a).

#### 2.1.2 | Vapor diffusion

Common vapor diffusion setups (sitting or hanging drop) accomplish protein supersaturation by the same general principle. An aqueous protein solution droplet containing insufficient precipitant for crystallization is setup apart from a larger reservoir containing a high concentration of precipitant in a sealed vessel. Over time, the droplet is equilibrated with the reservoir via vapor diffusion of water and other volatile components. Loss of water increases both the protein and precipitate concentrations in the droplet leading to supersaturation (Figure 2b). This method is often favored by structural biologists for growing large single crystals with high diffraction quality.

#### 2.1.3 | Batch crystallization

Batch crystallization is achieved by adding precipitating agents directly to a concentrated protein sample so as to shift the solubility curve directly into the nucleation zone. In other words, the protein and precipitant are mixed at their final concentrations to achieve nucleation (Figure 2c). Due to the relative ease with which batch crystallization can be scaled up, this method is preferred for industrial scale applications in which many protein crystals are required.

### 2.2 | Stability and bioconjugation

One factor that has historically limited the material application of protein crystals is their relative mechanical and thermal instability compared to other nanoporous materials such as zeolites. Protein crystals are highly fragile due to several factors. First, the irregular shape of the constituent proteins generally leads to packing arrangements with high solvent content and

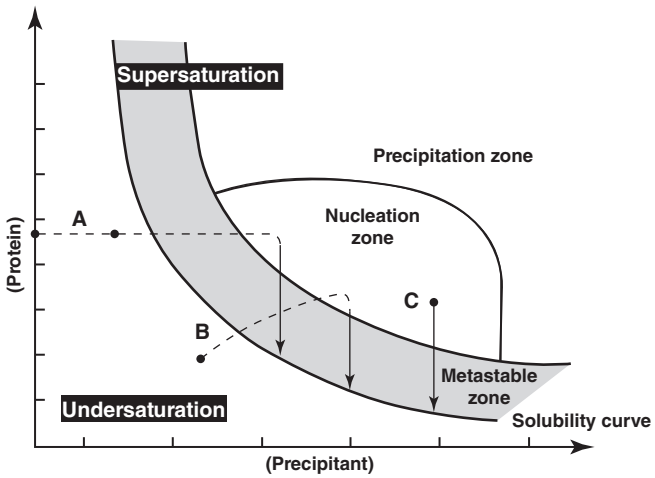


**FIGURE 2** A protein crystallization phase diagram based on varied protein and precipitant concentrations. Three commonly used crystallization methods are highlighted showing the path each method takes to produce crystals. Note that all paths need to reach the same destination, namely the nucleation zone, after which they make their way through the metastable zone, where crystal growth takes place, and eventually arrive at the solubility curve. • represents possible starting conditions. (a) Dialysis. (b) Vapor diffusion. (c) Batch crystallization. (Reprinted with permission from Chayen (1998). Copyright 1998 International Union of Crystallography)

relatively small interfacial contacts (~570 Å$^2$ on average) when compared to specific protein–protein interactions known to occur in solution (~1,600 Å$^2$ on average) (Janin & Rodier, 1995; Pifat-Mrzljak, 2007). Second, crystallographic interfaces generally include adventitious and solvent-dependent interfacial contacts that are weak and noncovalent. Third, shifts in solvent conditions can destroy desirable properties of the crystals in several ways. Solvents that increase solubility can simply dissolve the crystal. Solvents that decrease the solubility can drive disordered aggregation on the crystal surfaces. Even introducing solvents that favor an essentially isomorphous crystalline form can still shatter the crystal if the solvent is introduced in a way that induces stress from crystal structure gradients.

In sum, as-grown protein crystals in their mother liquor possess inadequate stability for most conceivable applications. To solve this stability problem, many groups have turned to chemical cross-linking to introduce covalent linkages, thereby generating extended bond networks throughout the protein crystal matrix. This method has proven to be effective, enabling protein crystals to withstand solution conditions well outside their crystallization environments (Ayala, Horjales, Pickard, & Vazquez-Duhalt, 2002; Lee, Turner, & Lye, 2002; Margolin & Navia, 2001; Noritomi, Koyama, Kato, & Nagahama, 1998).

### 2.2.1 | Cross-linking and bioconjugation chemistries

Protein crystals are composed of individual proteins (or complexes thereof ) which are in turn composed of polymerized amino acids. There are 20 common amino acids bearing a variety of unique chemical functionalities. The most useful amino acids for cross-linking and bioconjugation chemistries are those with ionizable side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, and tyrosine (Figure 3) (Hermanson, 2013). This collection of amino acids includes primary amines, thiols, and carboxylates. These functional groups are common targets for post-crystallization bioconjugation to improve crystal stability, compatibility, or functionality. The bioconjugation field is quite broad, but well established, with many research articles, reviews, and books dedicated to diverse bioconjugation chemistries (Hermanson, 2013; Wong, Jameson, & Wong, 2012). The book by Hermanson (2013) is highly recommended. Here, we will briefly introduce the aldehyde and carbodiimide reagents most commonly used in current protein crystal material applications.

Aldehyde cross-linkers (Figure 4a) have been widely used throughout history to stabilize biological specimens by covalently linking proximal primary amine groups. Examples include early embalming chemistries, leather tanning, and more recently chromatin immunoprecipitation and cell fixation techniques (Hoffman, Frey, Smith, & Auble, 2015). In Quiocho and Richards (1964), they were the first to use glutaraldehyde (GA) to improve carboxypeptidase-A crystal stability in diffraction experiments. Soon after, they demonstrated the enzymatic activity of GA cross-linked carboxypeptidase-A crystals in aqueous solutions different from that of the crystallization environment (Quiocho & Richards, 1966). Since then, aldehydes (Figure 4a), specifically GA, have been the dominant cross-linking agents for the stabilization of protein crystals (Cohen-Hadar et al., 2006; N. K. Khalaf et al., 1998; Yan et al., 2015). Despite the ubiquity of GA cross-linking, the chemical basis is incompletely understood. In principle, formyl groups in aldehydes undergo nucleophilic attack by primary amines producing a Schiff base. In the case of formaldehyde (Figure 4b), this Schiff base can undergo a second nucleophilic attack by another primary amine to complete the cross-linking reaction (Hoffman et al., 2015). However, in the case of monomeric GA, two Schiff bases are formed via nucleophilic attack, but are not reduced (Figure 4c) (Hermanson, 2013). Notably, Schiff base formation is reversible, leading to cross-link reversal, particularly in acidic conditions. Reducing agents such as sodium cyanoborohydride ($NaCNBH_3$) may lead to a stable final product via reductive amination. Intriguingly however, the actual GA

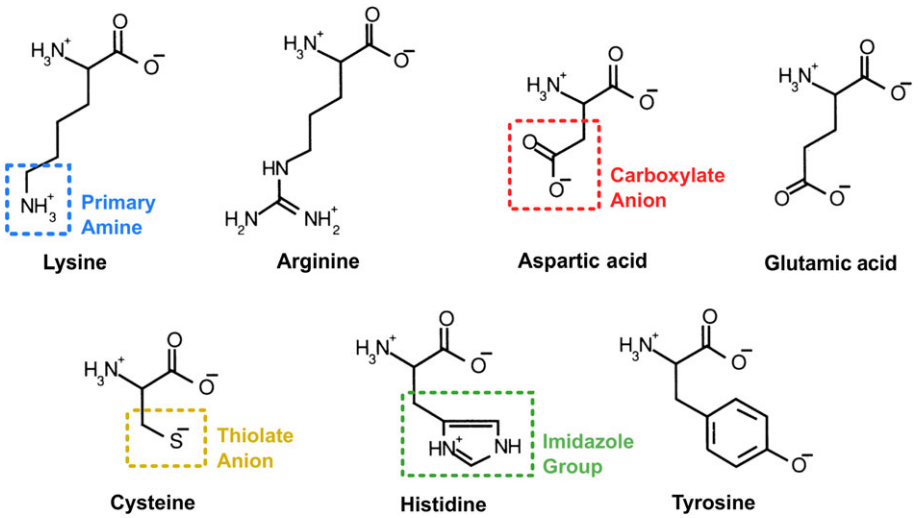


**FIGURE 3** Functional groups associated with ionizable amino acids

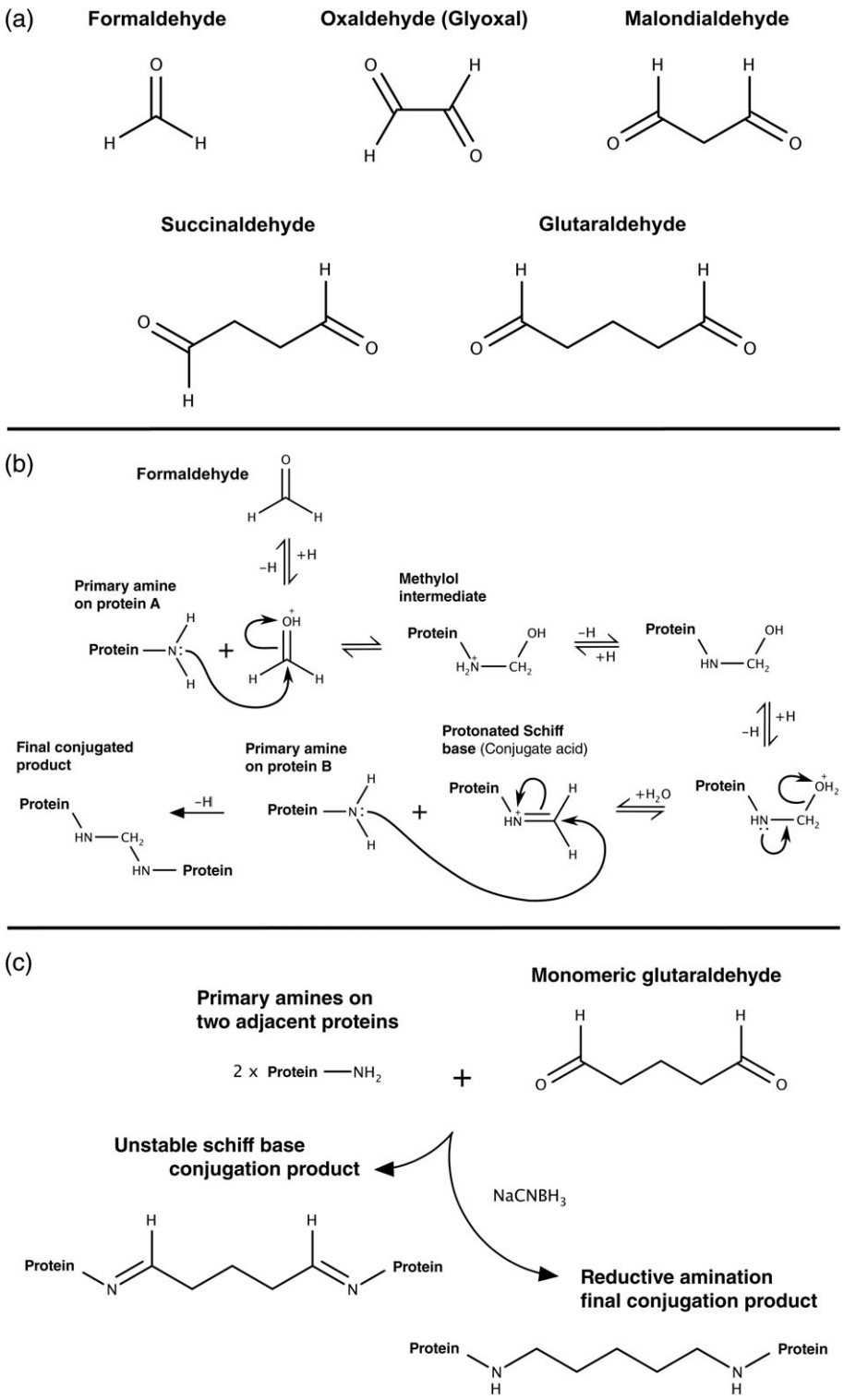


**FIGURE 4** (a) Aldehydes of varying length. (b) Formaldehyde cross-linking leads to a stable final conjugation product. (c) Monomeric glutaraldehyde cross-linking results in unstable Schiff base formation unless a reducing agent (e.g., $NaCNBH_3$) is added—Leading to reductive amination

cross-linking end products appear to be stable even in acidic conditions without the addition of reducing agents (Vaghjiani, Lee, Lye, & Turner, 2000). Thus, other chemical mechanisms, aside from Schiff base formation, are likely responsible for the exceptional stability of GA cross-linked materials. Migneault, Dartiguenave, Bertrand, and Waldron (2004) outlined 13 known aqueous states of GA, which can range from monomeric to highly polymerized; these different forms of GA can interact with proteins by way of eight different reaction mechanisms. More recently, Wine, Cohen-Hadar, Freeman, and Frolow (2007) attempted to resolve the predominant reaction mechanism of GA cross-linking in hen egg white lysozyme (HEWL) crystals under acidic and alkaline conditions using X-ray diffraction and mass-spectrometry analysis (Figure 1a). They were able to

resolve two distinct GA cross-links within HEWL crystals at different pH conditions. Both resolved cross-links were consistent with polymeric GA forms serving as the active species.

Carbodiimide agents catalyze the formation of amide bonds between amines and carboxyl groups (Figure 5) (Hermanson, 2013). Unlike aldehydes, carbodiimides are "zero-length" cross-linkers, meaning they do not add additional atoms between the two conjugated molecules. This process has been utilized to produce stable collagen matrices (Park, Lee, Lee, & Suh, 2003), and protein-based nanoparticles (Niknejad & Mahmoudzadeh, 2015), crystals (Hartje et al., 2018), and macrocomplexes (Lepvrier, Doigneaux, Moullintraffort, Nazabal, & Garnier, 2014), as well as to conjugate enzymes to cellulose-based materials (Edwards, Prevost, Condon, Sethumadhavan, et al., 2011; Edwards, Prevost, Condon, & French, 2011; Edwards, Prevost, Condon, French, & Wu, 2012). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is the most commonly used carbodiimide agent for protein conjugation, primarily due to its solubility in aqueous solutions. The other commonly available water-soluble carbodiimide is 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (CMC). Most other carbodiimides, such as dicyclohexyl carbodiimide (DCC), and diisopropyl carbodiimide (DIC) are water-insoluble, making their use in protein conjugation more limited, though they are widely used in organic synthesis of peptides (Hermanson, 2013).

### 2.2.2 | Biocompatibility of protein crystal materials

One application area where highly precise biomaterials may find extensive use is in the biomedical space. We envision that future materials or devices with precisely known atomic structure may compete favorably with less-ordered materials that are more commonly used today for drug delivery (e.g., vesicles, surface modified nanoparticles, biodegradable polymers). For example, the precise pore network defined by an engineered crystal might provide rigorous options for triggered or metered release of functional guest molecules compared to materials with a more heterogeneous pore diameter distribution. Notably, therapeutic biomaterials can have high value on a mass basis, which could justify the effort to develop highly optimized crystalline materials.

Numerous alternative bioconjugation chemistries for the stabilization and functionalization of protein crystal materials are briefly outlined by Roy and Abraham (2004) and Margolin and Navia (2001). However, any chemical additive may have the unintended potential to negatively impact biocompatibility. In broad terms, a biocompatible material has low propensity to cause biological damage upon contact. This damage may take the form of direct cell or tissue death (cytotoxicity), unintended immune responses (immunogenicity), or genetic mutations (genotoxicity) (Wnek & Bowlin, 2004). Decreased

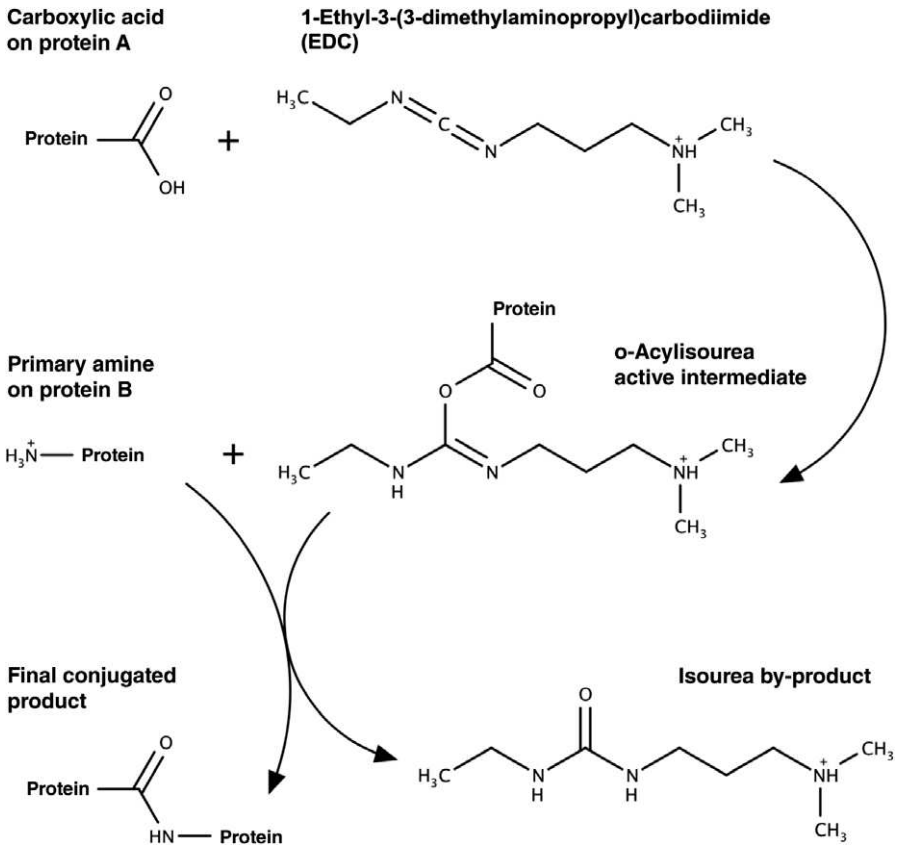


**FIGURE 5** EDC reacts with carboxylic acids to create an active-ester intermediate. In the presence of an amine nucleophile, a zero-length amide bond is formed with release of an isourea by-product

biocompatibility due to cross-linking has been observed in various protein-based materials including collagen fibers (Amri et al., 2014) and protein nanoparticles (Niknejad & Mahmoudzadeh, 2015).

Biocompatibility testing of cross-linked protein crystal materials has only recently been pursued as protein crystal applications in nanomedicine become more widely apparent. For instance, Ueno's work on cross-linked HEWL crystals impregnated with ruthenium carbonyl complexes (RuċCl-HEWL) motivated their preliminary testing of cytocompatibility against human embryonic kidney cells (Tabe et al., 2015). Using a solution of 0.5% trypan blue as an indicator of cell viability, they found no measurable cytotoxicity after 24 hr at the single concentration tested ($2.0 \times 10^5$ crystals/well). More recently, our group has assessed the stability and biocompatibility of various cross-linking agents on two distinct protein crystal scaffolds: HEWL (Figure 1a) and a camphlobacter jejuni isoprenoid binding protein crystal (CJ) (Figure 1b) (Hartje et al., 2018). The cell viability of each cross-linked protein crystal material was assessed at varying concentrations (1, 50, 100, 200, and 400 μg/mL) against two human cell lines: adult human dermal fibroblasts (HDFa) and human macrophages (MV-4-11). Viability was quantified using a lactate dehydrogenase assay (Figure 6a,b,d) and qualitatively confirmed via live dead staining (Figure 6c). Results indicate that cell cultures subjected to high concentrations of GA cross-linked protein crystal materials (100–400 μg/mL) suffered noticeable loss in cell viability. However, no substantial loss in cell viability was observed in cell cultures subjected to protein crystal materials cross-linked by oxaldehyde (OA) or EDC. These results suggest that researchers should consider alternatives to GA when stabilizing protein crystal materials, particularly if the application requires biological amity.

Existing studies have only scratched the surface with respect to evaluating the biocompatibility of engineered protein crystals in the context of nanomedicine and nanobiotechnology. There are still many opportunities to further characterize the biocompatibility and biodegradability of chemically modified protein crystal materials in the context of nanomedicine and

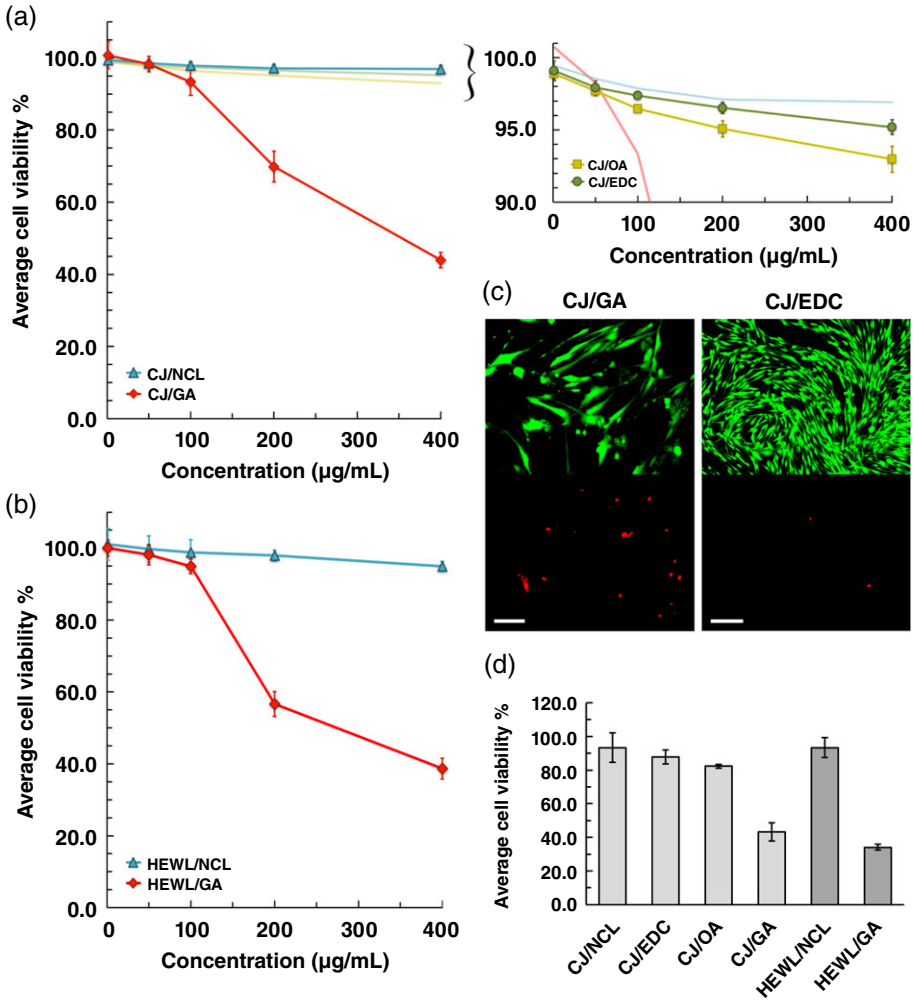


**FIGURE 6** (a) HDFa cell viability under varying concentrations of cross-linked CJ crystal materials; error bars: standard deviation, $n = 3$. (b) HDFa cell viability under varying concentrations of fragmented HEWL protein crystal materials; error bars: standard deviation, $n = 3$. (c) HDFa cells incubated with 400 μg/mL protein crystal material; top: green fluorescent live cell stain (calcein); bottom: red fluorescent dead cell stain (ethidium homodimer); left: CJ/GA, scale bar: 100 μm; right: CJ/EDC, scale bar: 300 μm. (d) MV-4-11 cell viability when incubated with various protein crystal materials at a concentration of 400 μg/mL; error bars: Standard deviation, $n = 3$. (Reprinted with permission from Hartje et al. (2018). Copyright 2018 American Chemical Society)

nanobiotechnology. It is not yet clear to what extent materials composed entirely of biomolecules will be able to capitalize on their theoretical advantages in this area. Certainly, the toxicity, immunogenicity, and pharmacokinetics of protein crystals will depend on the toxicity, immunogenicity, and pharmacokinetics of the constituent monomers. It is more interesting to contemplate what additional complications may arise from the solid crystal interacting with biological systems. For example, there could certainly be cases where the immune system responds to a persistent repetitive crystal surface in a qualitatively different way that it responds to a temporary local high concentration of the noncrystalline protein. Alternately, a slowly degrading protein crystal might provide a favorable sustained release profile for a therapeutic protein that would be difficult to match with a conventional drug delivery matrix. To our knowledge, minimal research beyond the aforementioned work of Margolin, Navia, Ueno, and coworkers has been conducted to evaluate the pharmacological and biomedical properties of protein crystals. Significant toxicity, immunogenicity, pharmacokinetic, and pharmacodynamic testing will be necessary before researchers can accurately assess the biomedical application potential for tomorrow's engineered protein crystals. If crystalline biomaterials are a logical end result of the drive for high-precision biomaterials, forward-looking biocompatibility research could establish the best practices for purification and crosslinking, thereby accelerating the learning curve for deploying protein crystals as biomaterials.

### 2.3 | Porosity and guest transport

Myriad applications for engineered protein crystals depend on transport rates and/or molecular interactions between guest molecules and the pore surfaces of scaffold materials. For instance, enzyme crystal biocatalysis applications are sensitive to the ratio of crystalline pore size to the size of the substrates and products, as mass-transfer rates can limit the net activity for cross-linked enzyme crystals (CLECs) of sufficient size (Chance, Ravilly, & Rumen, 1966; Doscher & Richards, 1963; Quiocho & Richards, 1966). Similarly, in the case of chromatography, the separation capability of protein crystals is dependent on three modes of physical segregation: adsorption, diffusion, and size exclusion—all of which are influenced by mass transport. Therefore, understanding transport within protein crystals is an important first step in advancing their material applications. To this end, many groups have sought to quantify the transport of solvent, small molecules, and macromolecules within the pore networks of various protein crystals. In this section, we will review some of the more common experimental and computational techniques utilized to study pore networks and guest transport within protein crystals.

#### 2.3.1 | Experimental approaches

One of the earliest studies of diffusion within protein crystals was reported in 1941 by Granick who showed guinea pig hemoglobin crystals to be permeable to ferricyanide and hydrosulfite by monitoring oxidative colorimetric changes caused by the hemoglobin oxygen (Granick, 1942). Granick's work helped to confirm the porous nature of protein crystals well before the first protein crystal structure was solved in Kendrew et al. (1958) using X-ray diffraction. Over two decades later, in 1968, quantitative diffusion studies of bromine-containing solutes within cross-linked β-lactoglobulin crystals was performed by Bishop and Richards (1968) using X-ray fluorescence measurements. By understanding the material properties of protein crystals, Bishop and Richards generated quantitative transport data as a function of time and estimated the effective pore size of the solvated channels. Their work not only advanced our understanding of hydration shells surrounding biomolecules, it also emphasized the usefulness of protein crystals as model systems to study transport phenomena. Since then, other quantitative methods, such as video absorbance spectroscopy (O'Hara, Goodwin, & Stoddard, 1995) and fluorescence microscopy (Velev, Kaler, & Lenhoff, 2000) have been implemented to study time resolved transport of guest molecules into protein crystals.

One major limitation of these early transport experiments was the inability to accurately resolve the precise position of guest molecules within the path length of the crystal. More recent confocal microscopy methods overcome these limitations and observe transport in protein crystals as a function of both time and position. Confocal microscopes can illuminate specific focal planes within protein crystals and thereby resolve the relative concentration of fluorophores within spatial slices. In the early 2000s, Cvetkovic et al. (2004) and Cvetkovic, Picioreanu, Straathof, Krishna, and van der Wielen (2005b) were the first to use 3D confocal microscopy to study small molecule transport within protein crystals. Their early work monitored the 3D diffusion of fluorescein within tetragonal, orthorhombic, and triclinic HEWL crystals. Later, they applied this technique to quantify the binary diffusion of fluorescein and rhodamine B within HEWL crystals (Figure 7a) (Cvetkovic, Picioreanu, Straathof, Krishna, and van der Wielen, 2005a). Using confocal microscopy data, they fit their observations to anisotropic diffusion models and found that transport diffusivities were strongly related to pore size (Figure 7b). By linking mathematical models for guest molecule transport to experimental confocal data, Cvetkovic and associates provided valuable insights and tools to understand complex systems where the functional properties depend on the intracrystal transport of one or more guest molecules.

Mathematical transport models are limited by the requirement to reduce model complexity. In the case of guest molecule transport inside porous host materials, this is often done by assuming noninteracting spherical guests diffusing within hard

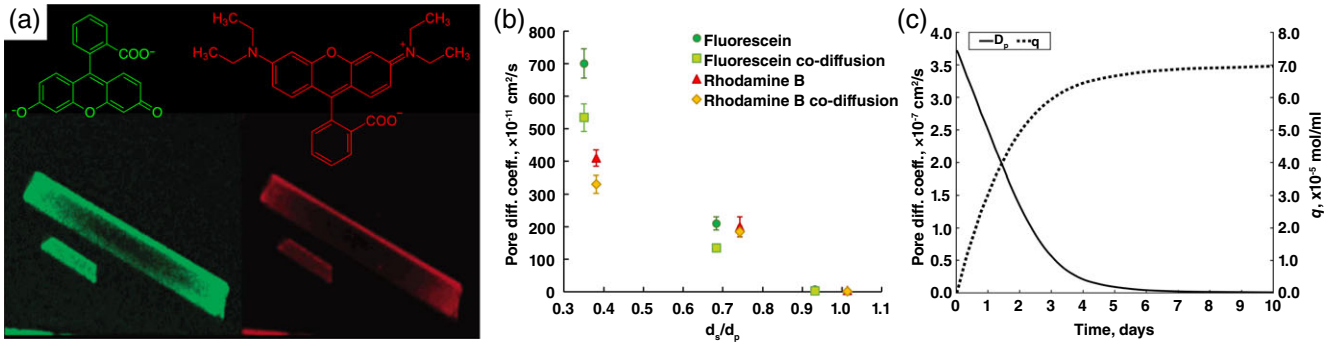


**FIGURE 7** (a) Co-diffusion of fluorescein (left) and rhodamine B (right) in a cross-linked HEWL crystal. (b) the pore diffusion coefficient ($D_p$) is related to the ratio of guest substrate diameter ($d_s$) to pore diameter ($d_p$). (a) and (b). (Reprinted with permission from Cvetkovic et al. (2005a). Copyright 2005 American Chemical Society) (c) The adsorbed guest concentration ($q$) causes occlusion of the scaffold pore leading to attenuation of $D_p$. (Reprinted with permission from Hartje et al. (2017). Copyright 2017 American Chemical Society)

cylindrical pores. These geometric assumptions represent significant over-simplifications for most actual crystal scaffold host–guest systems. Furthermore, adsorption of guest molecules can occlude small pores. As one would intuitively expect, strong adsorption can greatly attenuate the diffusion coefficient when pore occlusion reduces the effective pore diameter so as to only permit single-file guest diffusion (Figure 7c) (Gutenwik, Nilsson, & Axelsson, 2004a, 2004b; Hartje, Munsky, Ni, Ackerson, & Snow, 2017). Thus, accurate modeling of diffusion must account for adsorption, thereby further complicating any mathematical model of diffusion within the context of porous protein crystals. To alleviate some of the complexities and assumptions associated with mathematical diffusion modeling within protein crystal systems, a number of research groups have instead turned to numerical simulations that embrace the complex details of the guest molecule and pore structures.

### 2.3.2 | Computational approaches

As mentioned above, the pore environment of protein crystals is critically important for many protein crystal applications, from catalysis and chromatography to biotemplating and drug delivery. However, the chemical and structural complexity of the crystal nanostructure makes it difficult to accurately predict guest transport properties from intuition or simple models alone. Predictive models for guest molecule transport are desirable, since they will inform future efforts to rationally design or re-engineer protein crystal materials to enhance material performance for specific applications. Therefore, this section describes computational approaches for modeling protein crystal materials that have the potential to provide a detailed understanding of the role of solvent channel topology or crystal molecular dynamics on guest transport mechanics.

Molecular simulation of guest transport within protein crystal pores can more accurately account for the complex environment of protein crystal solvent channels. The increased realism comes with a price; atomistic simulations are much more computationally costly than the simplified mathematical models. Atomistic simulations have therefore been severely limited in their ability to generate enough trajectory data for accurate representations of the statistical ensembles describing transport phenomena. However, with modern advancements in computer science, atomistic simulation of small molecule guest transport has become more attainable. Brownian dynamics (Malek & Coppens, 2008; Morozov & Kachalova, 1995), Monte Carlo (Hu, Jiang, & Sandler, 2008; Malek, Odijk, & Coppens, 2004), and molecular dynamics (Hu & Jiang, 2008, 2009a, 2009b; Malek, 2007b; Malek & Coppens, 2008; Malek, Odijk, & Coppens, 2005) approaches have all been used to investigate small molecule transport within protein crystals. Malek (2007a, 2007c) has put together a comprehensive two-part review that details the various simulation techniques used to model diffusion within protein crystal materials. We will therefore not cover these techniques in detail here. Notably, these methods have not yet been extended to study macromolecular guest transport over long time-scales.

Given the expense of molecular simulation, it can be useful to also perform a static analysis of the crystal structure. In particular, understanding the solvent channel environment of protein crystals is critically important for diverse protein crystal applications including catalysis, chromatography, biotemplating, and drug delivery. Multiple software packages have been created to aid in the identification of pores, channels, and cavities and better model their physiochemical environments. In Kisljuk, Kachalova, and Lanina (1994), they have developed CHANNEL, a software package designed to identify channels within protein crystals by building up a spatial graph of intersecting spheres of defined radii to elucidate interconnected cavities within the unit cell. More recently, Juers and Ruffin (2014) have developed MAP_CHANNELS, a computational tool designed to aid in the visualization of solvent channels in macromolecular crystals and to quantitatively characterize those channels with metrics relevant for the study of guest molecule transport.

### 2.4 | Engineering protein crystals

The chemical versatility of proteins, combined with the intrinsic porosity of protein crystal scaffolds suggests that protein crystals can be engineered to become useful biologically derived nanomaterials. In this section, we will discuss the efforts to engineer protein crystal scaffolds to optimize stability, biocompatibility, transport dynamics, and surface functionality. Modification of the scaffold constituent proteins by site directed mutagenesis can be a particularly powerful engineering tool to direct desired chemical functional groups to specific locations within the scaffold. For example, crystal interfaces can conceivably be engineered to incorporate cysteine or histidine residues to promote disulfide bond formation or metal coordination, respectively, at protein contacts within the crystal. Below, we will review engineering studies that utilized these functional groups to generate protein crystal scaffolds with intriguing characteristics for applications in nanomedicine and nanobiotechnology.

#### 2.4.1 | Engineering scaffold interfaces

Novel protein crystal scaffolds can be generated by synthetic symmetrization of the crystal component proteins. Symmetrization of component proteins can be achieved by adding disulfide linkages or metal binding sites to direct oligomerization prior to crystallization. For example, in 2006, Banatao and coworkers in Yeates' lab generated three single-cysteine mutant variants of T4 lysozyme (T4L) prepared as symmetric dimers through a disulfide linkage (Banatao et al., 2006). These three mutant dimers were shown to form six novel protein crystal scaffolds. In similar fashion, the Yeates group went on to create symmetric structures through metal coordination with engineered histidine or cysteine residues on both T4L and maltose binding protein (MBP) (Arthur et al., 2011). Oligomeric states were generated upon addition of metal ions: copper ($Cu^{2+}$), nickel ($Ni^{2+}$), or zinc ($Zn^{2+}$). These symmetric oligomers were shown to form 16 unique crystal lattices. This method, metal-mediated synthetic symmetrization, has the potential to expand the known crystal structure repertoire and could help crystallize proteins that have proven difficult to grow using conventional methods.

Engineered disulfide bonds and metal coordination can also be used to increase crystal stability without relying on the addition of chemical cross-linking agents, thereby potentially increasing overall crystal biocompatibility. An early successful introduction of disulfide cross-links at protein–protein interfaces was reported in Yang et al. (2003). In this case, the crystallographic interfaces within T4L were used to model cysteine mutants that would create disulfide cross-links. Polymers of T4L were then made both from lysozyme in solution and crystallized lysozyme mutants by exposing the protein to oxygen. The yield of polymers was much higher from oxidized crystals than oxidized monomers in solution. Interestingly, these polymers were exploited as a means of studying monomer unfolding by mechanical stress through scanning force microscopy (SFM)

A couple years later, Srinivasan, Iyer, Przybycien, Samsonoff, and Bell (2002) promoted disulfide cross-linked protein crystals as a way to form protein fibers which they called crystine. The disulfide modeling program MODIP was used to predict sites in 15 crystallographic interfaces that would support disulfide cross-linking. Only one out of three designs produced was successfully crystallized. The authors noted that even though the cross-linking was only one-dimensional (1D), the crystals were difficult to dissolve. Dissolution of these crystals resulted in bundled fibers with diameters up to 7 nm in which several cross-linked chains were held together by noncovalent interactions. In contrast to the random 3D network produced by GA cross-linking, disulfide cross-links preserved the order of a protein crystal at specific sites along crystal interfaces. Quistgaard (2014) demonstrated the use of disulfide cross-linking within crystals composed of vDED coiled-coil domain dimers from human BAP29. These crystals form honeycomb-like scaffolds with complete disulfide cross-links along the *c*-axis (PDB code: 3w7y, Figure 8a). Per MAP_CHANNELS, the major axial pores are large enough to accommodate 1D diffusion of 7 nm diameter guest spheres, though the short unit cell height (3.1 nm) would preclude some applications. Finally, Heinz and Mathews reported that designed intermolecular disulfide cross-links in T4L resulted in more rapid crystallization.

Similarly, protein crystal scaffold interfaces can be engineered to display surface histidine (or histidine motifs) that can coordinate metal ions and stabilize the crystal lattice. In 2010, Radford and others working in Akif Tezcan's group implemented a bottom-up strategy to direct protein self-assembly using supramolecular metal coordination chemistry in the production of a porous protein crystal framework (Radford, Lawrenz, Nguyen, McCammon, and Tezcan, 2010). Their engineered protein (MBPPhen2) was derived from a four-helix bundle heme protein (cytochrome $cb_{562}$) and was shown to readily crystallize in the presence of $Ni^{2+}$ or $Zn^{2+}$ ions (Figure 8b). The resulting scaffold (PDB code: 3nmk) contains hexagonal pores, the largest of which could accommodate a spherical guest with 4.6 nm diameter (per MAP_CHANNELS). More recently, metal-coordination has been used to direct coiled-coil self-assembly in the production of crystal scaffolds. Both Jean Chmielewski's group (Figure 8c) (Nepal, Sheedlo, Das, and Chmielewski, 2016) and Seth Horne's group (Tavenor, Murnin, & Horne, 2017) have used peptide engineering and metal-coordination to produce self-assembling crystalline scaffolds with tunable morphologies.

Other methods of engineering scaffold interfaces include hydrophobic patch design, ligand-mediated crystallization, and natural dimer and trimer fusion proteins. All of these approaches have led to unique artificial protein crystals. These methods

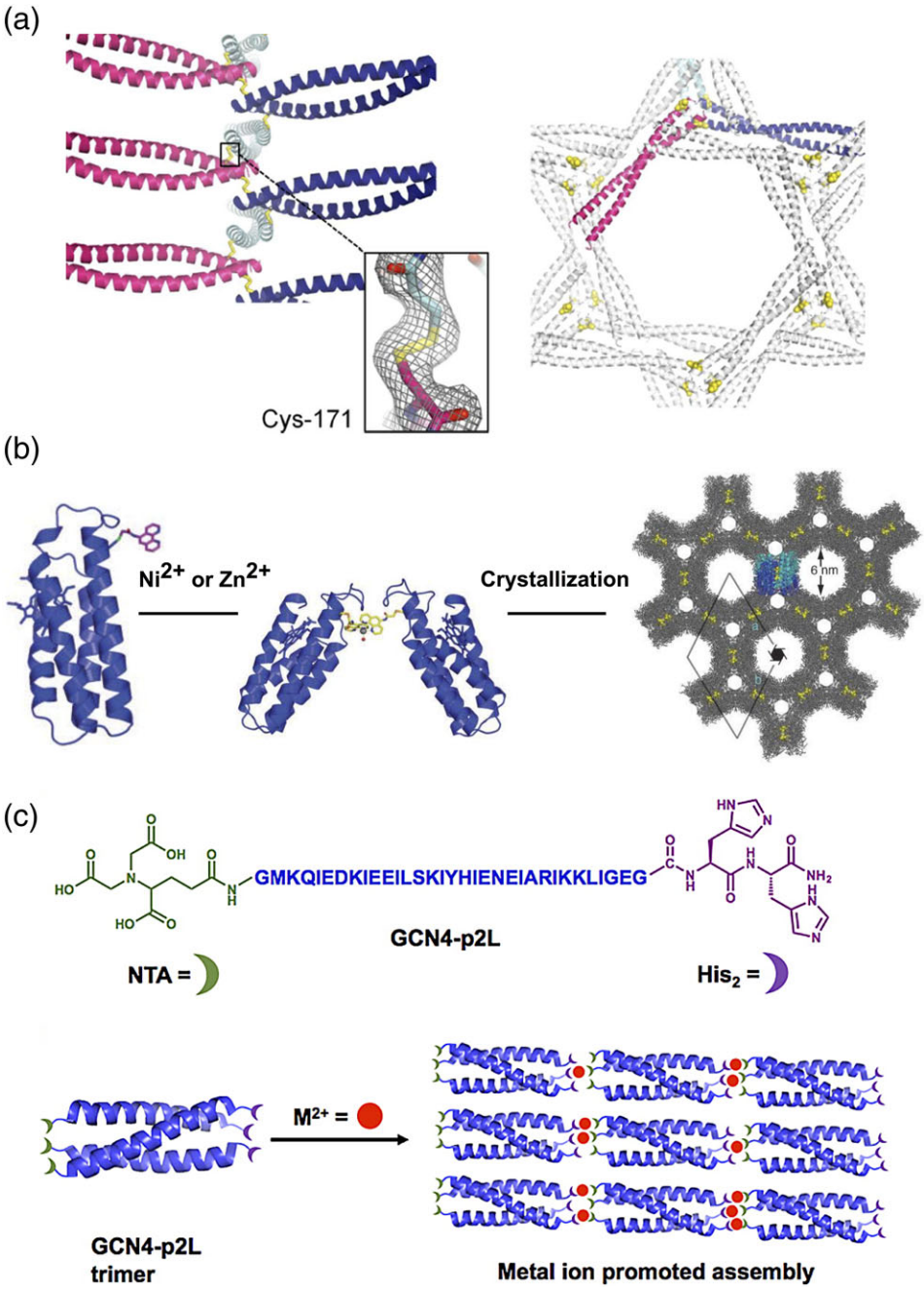


**FIGURE 8** (a) Disulfide linkages within a porous protein crystal scaffold. (Reprinted with permission from Quistgaard (2014). Copyright 2014 Royal Society of Chemistry) (b) Schematic of zinc mediated crystal formation of MBPPhen2 illustrating resultant lattice porosity. (Reprinted with permission from Radford et al. (2010). Copyright 2010 Royal Society of Chemistry) (c) Schematic of metal mediated coiled-coil crystal assembly. (Reprinted with permission from Nepal et al. (2016). Copyright 2016 American Chemical Society)

and others are reviewed in detail by Abe and Ueno (2015). We also recommend additional recent work on engineered protein crystals from the Tezcan, Degrado, Grigoryan, and Kostiainen groups. A cross-section of the noteworthy advances includes: self-healing crystals (L. Zhang, Bailey, Subramanian, & Tezcan, 2018), allostery in a de novo designed metalloprotein assembly (Churchfield, Alberstein, Williamson, & Tezcan, 2018), protein-directed crystallization of the $C_{60}$ fullerene (Kim et al., 2016), and a porous crystalline network that combines ferritin protein cages with organic cyclophanes (Beyeh et al., 2018). Finally, recent progress in DNA crystal engineering from Seeman, Paukstelis, and their coworkers provide a valuable counterpoint to the engineering of protein crystals (Hao et al., 2017; McNeil & Paukstelis, 2017; Seeman et al., 2017).

### 2.4.2 | Engineering crystal surfaces and pore environments

Site-specific modifications of protein constituents can improve or expand protein crystal functionality, producing novel scaffolds with uniquely desirable traits. For instance, surface modification of protein crystals with secondary molecules capable of

binding cell surface markers could prove beneficial in directing crystals to specific tissue types in drug delivery applications. Ueno's group has shown the ability to decorate the surface of polyhedral crystals (PhCs) with Lewis X (LeX) carbohydrate by modifying surface cysteine residues using established maleimide and click chemistry (Figure 9a) (Abe et al., 2014) Alternatively, the Chmielewski metal-coordination driven coiled-coil assembly that was described above (Figure 8c) was also shown to direct guest molecules to distinct crystal surfaces (Figure 9b) (Nepal et al., 2016).

Other crystal engineering efforts have focused on modification of the interior surfaces. Pore structure and the physicochemical character of the interior crystal surfaces are critically important for the overall performance properties of protein crystal materials that include guest molecules. Accordingly, there have been multiple attempts to modify protein scaffolds to optimize specific properties related to transport, catalysis, and templating. In 2009 and 2011, Felix Frolow and Amihay Freeman's group used both systematic mutation (Wine, Cohen-Hadar, Lamed, Freeman, & Frolow, 2009) and chemical modification (Cohen-Hadar, Lagziel-Simis, Wine, Frolow, & Freeman, 2011) of pore surface residues to alter the porosity of HEWL crystals in the interest of biotemplating applications. Furthermore, they determined that simple addition of various metal ions ($Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$) to the crystal growth environment could provide a strategy to "fine tune" the porosity of the resulting crystal (Wine et al., 2010).

Our group has recently engineered metal coordination sites on the interior pore surfaces of CJ protein crystals (Figure 1b) to exert spatial and temporal control over macromolecular guest installation within the crystal scaffold (Figure 9c) (Huber, Hartje, McPherson, Kowalski, and Snow, 2017; Kowalski et al., 2016). We used stepwise guest loading and EDTA as a metal chelator to demonstrate secure immobilization and precise segregation. These results illustrate the capacity of protein crystal materials to organize trillions of guest molecules in 3D with robust control over localization and release. Efforts to modify surface and pore functionality of protein crystal scaffolds have enhanced the prospects for using engineered protein crystals as host matrices for drug storage, delivery, and release with exciting implications for the future of nanomedicine.

#### 2.4.3 | De novo protein crystal design

A final strategy for engineering protein crystals is a bottom up approach, where functional components (e.g., enzymes, binding domains, structural proteins) of known structure are redesigned to adopt a particular symmetry group. This approach has led to a number of high-profile successes in the design of protein homo- and hetero-oligomers with varying symmetry (Bale et al., 2016; King et al., 2014, 2012). These symmetry-based design methods can also generate unbounded two-dimensional (2D) or 3D arrays. These design approaches were recently reviewed by Yeates (2017) and Yeates, Liu, and Laniado (2016). Reliable simultaneous design of entire 3D crystals, with control over all aspects of the packing and solvent pore topology is a very active research frontier. Arguably, this technology will represent the apotheosis of designed protein self-assembly. Early successes in this direction include the 2011 design of a hexagonal crystal composed of trimeric coiled-coils by Lanci et al. (2012) and the designed 2D crystals reported by Gonen, DiMaio, Gonen, and Baker (2015). As the technology to reliably encode 3D crystalline assemblies matures, the resulting designed crystals will find applications alongside re-engineered crystals with adventitious packing arrangements.

## 3 | APPLICATIONS IN NANOMEDICINE

### 3.1 | Pharmaceutical formulations

The simplest route through which protein crystals can benefit nanomedicine is the use of noncross-linked protein crystal formulations for drug delivery. Margolin and Navia have outlined several significant advantages crystallization can offer from a therapeutic perspective: (1) crystallization can streamline the manufacturing process by providing a means of protein purification; (2) crystals are the most concentrated form of proteins, which is beneficial for high-dose delivery; (3) crystal dissolution is dependent on crystal morphology, which enables optimization of a slow dose release regimen by altering the crystal size and shape; (4) lastly, dry crystal formulations are known to have improved physical and thermal stability over their soluble or amorphous couterparts (Basu et al., 2004). In 2003, Margolin's group went on to demonstrate the feasibility of crystallizing monoclonal antibodies (mAbs) in large quantities using batch crystallization methods (Yang et al., 2003). Their work generated functional crystalline suspensions of three approved therapeutic mAbs with yields of 85–95%. Crystal morphologies could be tuned by manipulation of the batch crystallization protocol to generate optimal formulations for subcutaneous delivery in high concentrations. They have subsequently written an expert opinion piece focusing on the use of protein crystals for the delivery of biopharmaceuticals, which describes the benefits, challenges, and techniques associated with this technology (Basu et al., 2004). In addition to this work, experimental (Falkner et al., 2005; Hebel, Huber, Stanislawski, & Hekmat, 2013; Hekmat, 2015; Martin & Zilm, 2003) and computational (Shi, Mhaskar, El-Farra, & Christofides, 2005; J. J. Liu, Ma, Hu, & Wang, 2010a, 2010b; Nayhouse, Sang-Il Kwon, Christofides, & Orkoulas, 2013; J. J. Liu, Hu, & Wang, 2013; Joseph Sang-Il

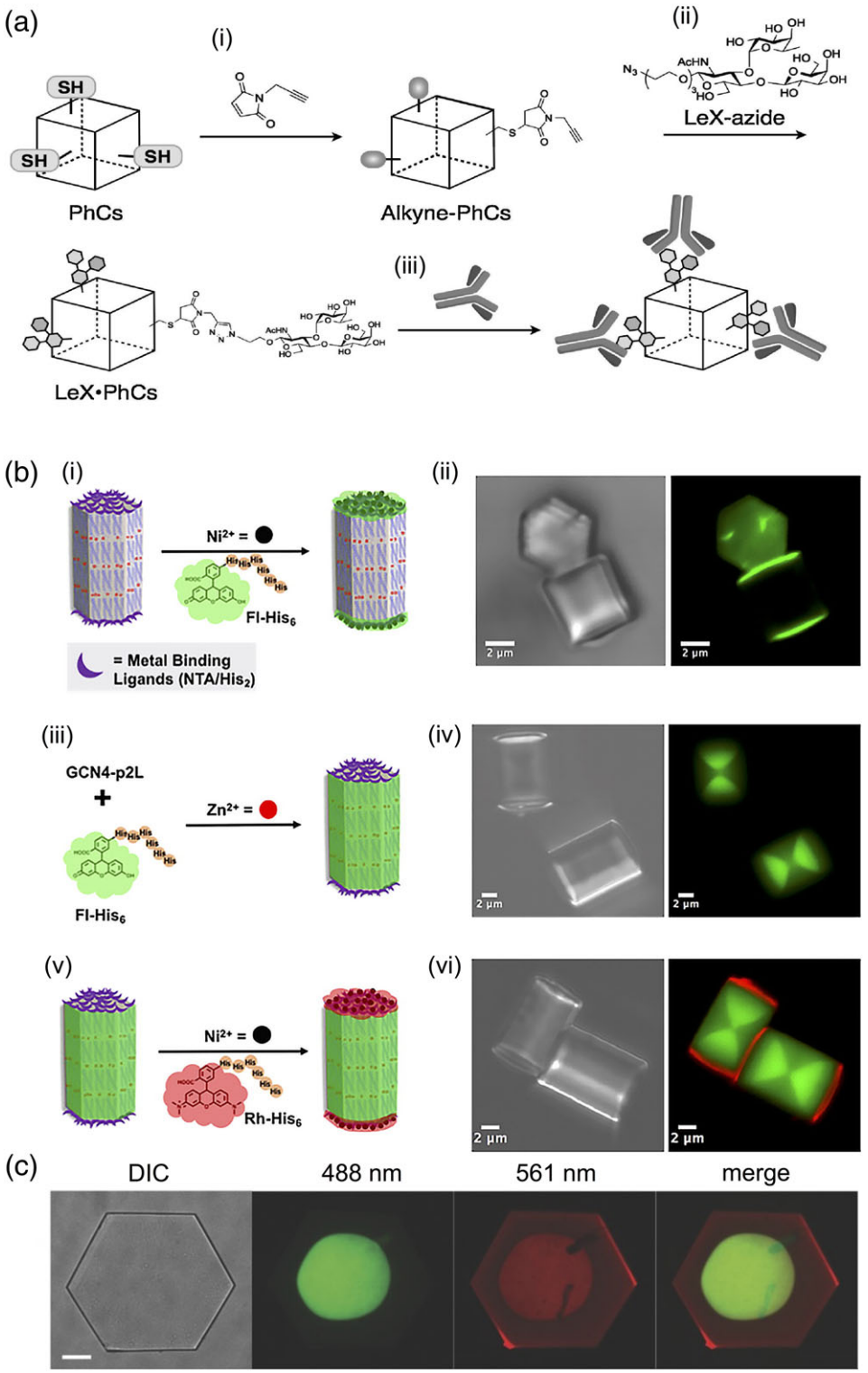


**FIGURE 9** (a) Schematic representation of LeX immobilization on the surface of PhC: (i) cysteine residues of PhC modified with propargyl maleimide; (ii) acetylene moieties modified with LeX-azide via copper-catalyzed azide-alkyne cycloaddition; (iii) antibody–antigen reaction on the surface of modified PhCs. (Reprinted with permission from Abe et al. (2014). Copyright 2014 Chemical Society of Japan) (b) Schematic representations for directing His-tagged fluorophore guests to protein crystals: (i) on the surface after crystal formation, (iii) within the crystal during formation, or (v) at both the surface and within crystals. (ii, iv, vi) Bright-field (left) and confocal (right) microscopy images. (Reprinted with permission from Nepal et al. (2016). Copyright 2016 American Chemical Society) (c) Confocal imaging of an interior plane within a highly porous CJ crystal, demonstrating spatially segregated macromolecular guests (mNeonGreen and mCherry) immobilized with $Zn^{2+}$. (Reprinted with permission from Huber et al. (2017). Copyright 2017 Royal Society of Chemistry)

Kwon, Nayhouse, Christofides, & Orkoulas, 2014a, 2013, 2014b) studies have characterized batch crystallization techniques for the generation of monodisperse sub-micron protein crystals of specific morphologies in the interest of industrial scale pharmaceutical formulations and drug delivery. We also note that microcrystalline suspensions offer potential advantages in terms

of shelf-stability (Shenoy et al., 2001) and decreased viscosity which could enable delivery via smaller needles (Basu et al., 2004).

### 3.2 | Vaccine delivery

Subunit vaccines are ideally composed of highly purified and well characterized antigenic molecules that can be manufactured through chemical synthesis or recombinant DNA expression systems (Hansson, Nygren, & Ståhl, 2000). Furthermore, subunit vaccine formulations provide superior safety over live-attenuated vaccines and thus may be administered to patients with weakened immune systems (Moyle & Toth, 2013). The high purity and regularity of protein crystals, along with their biodegradability may provide significant advantages over soluble subunit vaccine formulations by improving the immune response and limiting the need for additional adjuvants. For instance, Wade-Evans et al. (1997) demonstrated 70% reduction in mortality in mice immunized with a crystalline vaccine composed of the major outer core protein of African horse sickness virus, VP7. Protection was slightly improved (90%) when the VP7 protein crystal vaccine was administered along with Freund's complete adjuvant. Both outcomes demonstrated significant improvement over the dramatic 80–100% mortality in nonimmunized mice. In 1999, St. Clair working with Margolin and coworkers used cross-linked protein crystals composed of human serum albumin (HSA) to elicit an immune response in rats (Clair et al., 1999). They compared the HSA crystal vaccination response to that of soluble HSA and observed that cross-linked crystals were significantly more immunogenic as assessed using antibody titer counts. While this early work demonstrated the utility of protein crystals for use in vaccine delivery, recent progress on improving subunit vaccine immune responses has been primarily pursued by groups focusing on other forms of supramacromolecular structures, such as protein nanoparticles, cages, and viral-like particles (Heddle, Chakraborti, & Iwasaki, 2017; López-Sagaseta, Malito, Rappuoli, & Bottomley, 2016; P. Roy & Noad, 2009).

### 3.3 | Drug delivery

Drug delivery is a broad field with tremendous impact on the future of nanomedicine. Aside from the previously mentioned pharmaceutical formulations, there are numerous drug delivery vehicles ranging from various nanoparticles (Couvreur, 2013; Wilczewska, Niemirowicz, Markiewicz, & Car, 2012) to protein cages and artificial virus-like particles (Rohovie, Nagasawa, & Swartz, 2017; Somiya, Liu, & Kuroda, 2017).

One drug delivery avenue where protein crystal materials have been of recent use is in the delivery of gas signaling molecules. Carbon monoxide (CO) is a cell signaling molecule that mediates anti-inflammatory and vasoactive responses (Gullotta, di Masi, & Ascenzi, 2012). Tabe and associates working in Ueno's group used cross-linked HEWL crystals as a scaffold to immobilize ruthenium carbonyls, creating ruthenium carbonyl-incorporated cross-linked HEWL crystals (RućCL-HEWL) with CO-releasing properties (Tabe et al., 2015). They demonstrated the efficacy of RućCL-HEWL for the extracellular delivery of CO by treating cells with RućCL-HEWL crystals and assaying the cellular response to CO (Figure 10a). Specifically, they implemented a luciferase reporter assay to monitor nuclear factor kappa B activity. Similar cellular responses were generated using noncross-linked PhCs spontaneously grown within, and purified from, insect cells (Tabe, Shimoi, et al., 2014). Ueno's group went on to demonstrate photoactivatable release of CO from manganese carbonyl groups immobilized on noncross-linked PhCs (Figure 10b) (Tabe et al., 2016). This body of work illustrates the potential for porous protein crystal materials to be used as extracellular matrices for the metered delivery of signaling gases. Future work in this area may focus on alternate protein crystal scaffolds engineered to release a variety of biologically relevant gas molecules, such as NO, which has many therapeutic uses including antimicrobial and anticancer properties (Korde Choudhari, Chaudhary, Bagde, Gadbail, & Joshi, 2013; Schairer, Chouake, Nosanchuk, & Friedman, 2012).

Multifunctional fibrous matrices offer another path for the delivery of therapeutics. For instance, electrospun nonwovens have demonstrated the capacity for controlled release of therapeutic molecules ranging from small molecules like antibiotics to biomacromolecules such as protein drugs and nucleic acids for gene delivery (Meinel, Germershaus, Luhmann, Merkle, & Meinel, 2012). However, one challenge in electrospinning protein therapeutics into nonwoven matrices is the common requirement to use organic solvents in the electrospinning process, which can lead to protein denaturation or aggregation. To solve this problem, Puhl, Li, Meinel, and Germershaus (2014) utilized the superior stability of protein crystals in the electrospinning process. Specifically, they incorporated noncross-linked HEWL crystals into nonwoven matrices via PCL electrospinning (Figure 10c) as a proof of principle for improved protein incorporation and release from electrospun nonwoven matrices. In this case, dissolution of HEWL crystals could provide a sustained release of the constituent enzymes. Future work in the development of multifunctional fabrics may alternatively focus on using bioconjugation chemistries to directly and covalently attach porous protein crystals to pre-existing textiles.

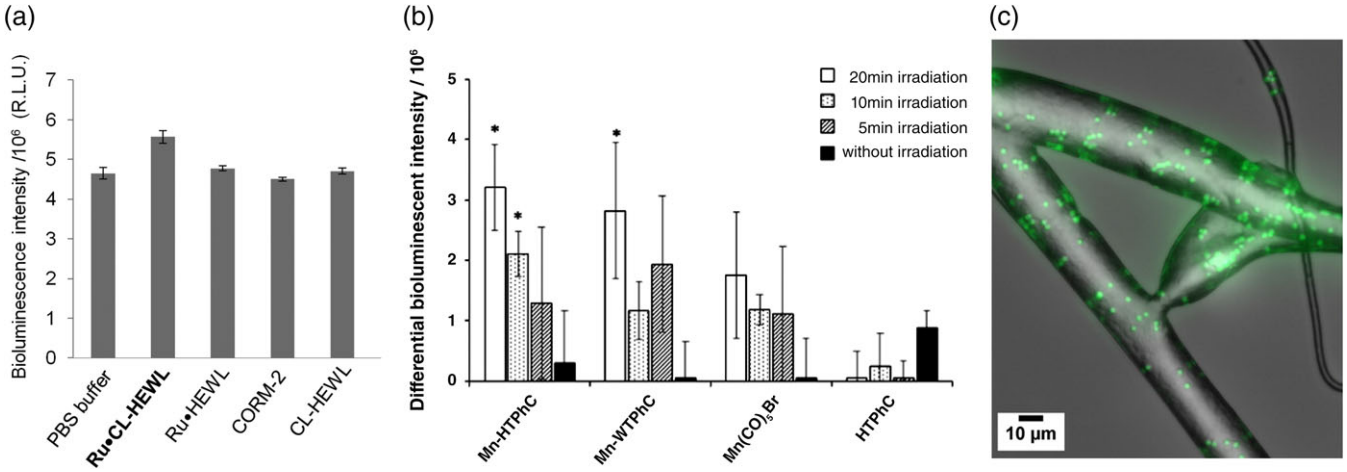


**FIGURE 10** (a) Bioluminescence intensity in the luciferase reporter assay for evaluation of NF-κB activity of HEK293/κB-Fluc cells in the presence of 1.0 ng/mL TNF-α after incubation with PBS buffer (as control), RućCL-HEWL, Ru·HEWL, CORM-2, and CL-HEWL for 24 h. (Reprinted with permission from Tabe et al. (2015). Copyright 2015 American Chemical Society) (b) Luminescence intensity in the luciferase reporter assay for the evaluation of NF-κB activity of HEK293/κB-Fluc cells in the presence of 10 ng/mL TNF-α after incubation for 12 hr with Mn·HTPhC, Mn·WTPhC, $MnCO_5Br$ and HTPhC, with the light irradiation for 20 min (white), 10 min (dot), 5 min (slashed) and without the light irradiation (black). (Reprinted with permission from Tabe et al. (2016). Copyright 2016 Royal Society of Chemistry) (b) Distribution of FITC-labeled lysozyme crystals in a PCL nonwoven prepared using ~2 μm lysozyme crystals, a 25% PCL solution, and a drug loading of 5%. (Reprinted with permission from Puhl et al. (2014). Copyright 2014 American Chemical Society)

## 4 | APPLICATIONS IN BIOTECHNOLOGY

### 4.1 | Catalysis

After Quiocho and Richards (1964, 1966) studies on GA cross-linked carboxypeptidase-A crystals in the 1960s, subsequent studies of CLECs progressed rather slowly with only a handful of studies in the 1970s that investigated GA cross-linked subtilisin crystals (Tüchsen & Ottesen, 1977) as well as crystals of carboxypeptidase A (Spilburg, Bethune, & Vallee, 1977) and B (Alter, Leussing, Neurath, & Vallee, 1977). It was not until the early 1990s that CLEC technologies became more widely considered for industrial applications when St. Clair & Navia (1992) published their work on thermolysin CLECs. Their study looked at the catalytic activity and stability of thermolysin CLECs in a broad range of aqueous-organic solvents that would normally cause noncrystalline enzymes to denature and lose activity. The superior stability of crystalline enzymes (after chemical cross-linking) allowed CLECs to operate well outside normal biological conditions. In 1995, Margolin's group demonstrated that cross-linked crystals of lipase derived from *Candida rugose* could be used for enantioselective hydrolysis of chiral racemic esters (Lalonde et al., 1995). Their work showed lipase CLECs to have superior enantioselectivity over crude lipase extracts. Many examples of CLECs have since been reported in the literature, providing new opportunities for biocatalysis (N. Khalaf et al., 1996; Lopez et al., 2017; Noritomi et al., 1998; Roy & Abraham, 2006; Sobolov, Bartoszko-Malik, Oeschger, & Montelbano, 1994; Sobolov et al., 1996; St. Clair, Wang, & Margolin, 2000; Visuri, Pastinen, Wu, Mäkinen, & Leisola, 1999; Y.-F. Wang, Yakovlevsky, Zhang, & Margolin, 1997; Xu & Klibanov, 1996). Today, CLEC catalysis has a notable role in industrial scale synthesis (Roy & Abraham, 2004; Vos, Vankelecom, & Jacobs, 2008; Zaks, 2001).

### 4.2 | Biotemplating

Biology is replete with complex and intricate systems that exhibit ordered structures on the nanoscale level. As nanotechnology advances, researchers are increasingly turning to biological systems to aid in the construction of synthetic devices. Biotemplating is the process by which biological scaffolds such as proteins, DNA, viruses, and bacteria can help control the size and shape of inorganic nanostructures during synthesis. Protein crystal scaffolds have been identified as intriguing biotemplating candidates on account of their highly-porous regular structure. Cohen-Hadar et al. (2006) assessed the feasibility of using cross-linked protein crystals as a biotemplating scaffold. To do this, they used cross-linked lysozyme crystals to template the assembly of a synthetic hydrogel while monitoring the gel synthesis process and crystal stability via X-ray diffraction. In addition to templating hydrogels, protein crystals have been used as molds during the synthesis of both quantum dots (Wei et al., 2013) and carbon dots (England, Patil, & Mann, 2015) with tunable fluorescence, or to grow and coordinate gold nanoclusters (Kowalski et al., 2016; Liang et al., 2013; M. Liu et al., 2016; Wei et al., 2011; Wei & Lu, 2012) and luminescent lanthanide complexes (Y. Zhang et al., 2018). There have been many other accounts of stabilized protein crystals being utilized as

effective biotemplating scaffolds and catalytic vessels for the assembly of organometallic complexes and biohybrid materials. These efforts have been thoroughly reviewed by Ueno's group (Abe, Maity, & Ueno, 2016, 2018; Maity & Ueno, 2017; Tabe, Abe, Hikage, Kitagawa, & Ueno, 2014; Ueno, 2013).

### 4.3 | Biosensing

Detecting biologically relevant molecules has become an important aspect of many scientific disciplines, including the biomedical and biotechnology industries as well as environmental protection. Biosensors, a term coined in 1977 by Karl Camman, describes analytical devices that monitor changes in biological analytes including metabolites, biomolecules, supramolecular structures, and whole cells (Lisdat, 2007). Most such devices can be analyzed in terms of three components that each handle a specific task: (a) analyte recognition, usually performed by a biological element such as cell receptors, proteins/enzymes, or DNA; (b) signal transduction elements, that transform the detection event into a measurable electrical or optical signal; (c) finally, signal detection, which converts the electrical or optical signal into a readable result. The efficacy and performance of biosensors are often limited by the affinity and selectivity of the initial biomolecular recognition event. Thus, biomolecular recognition sensitivity limitations can bottleneck the biosensor performance.

As discussed above, protein crystals are highly concentrated, regularly repeating structures that can be either engineered or cross-linked to become remarkably stable. Furthermore, CLECs possess high specific activity toward their reactants, even under very low reactant concentrations (St. Clair & Navia, 1992), thereby enabling CLECs to perform well as biorecognition elements within biosensing devices. In 1999, Navia and St. Clair filed a patent for the use of GA cross-linked protein crystals as biosensors, providing evidence for thermolysin, elastase, asparaginase, lysozyme, lipase, and urease CLEC stability and catalytic activity (US Patent for Biosensors, 1999). A fully realized example of a CLEC biosensor was later presented in 2001 by Arkady Karyakin's group, in which they evaluated a glucose biosensor based on Prussian Blue and CLECs derived from glucose oxidase (Luiz de Mattos, Lukachova, Gorton, Laurell, & Karyakin, 2001). Their study compared the performance of a commercially available glucose oxidase biosensor to their glucose oxidase CLEC sensor in terms of sensitivity, linear range, and operational stability. They found that glucose oxidase CLECs had remarkable selectivity and improved sensing and stability over the commercially available biosensor, establishing the potential for future devices to incorporate protein crystals when diagnosing and treating diabetes.

Protein crystal based biosensors have also been implemented in detecting organic pollutants in the interest of environmental science and conservation. In 2004, Roy and coworkers developed a biosensor based on a laccase CLEC for the detection of phenols (J. J. Roy, Abraham, Abhijith, Kumar, & Thakur, 2005). This study found that laccase CLEC biosensors possessed high activity and sensitivity over a 3-month period toward a variety of phenols: catechin, catechol, pyrogallol, guaiacol, ferulic acid, and 2-amino phenol. Their sensor could be useful in the detection of antioxidant phenols in foods and phenolic pollutants in wastewater. Laothanachareon, Champreda, Sritongkham, Somasundrum, and Surareungchai (2008) used organophosphate hydrolase (OPH) CLECs to create a biosensor for the detection of organophosphorus compounds. Their design utilized crude OPH CLECs as opposed to more costly purified OPH. Notably, the OPH CLECs demonstrated similar sensitivity to biosensors that used pure OPH. This work established that relaxed purity requirements could facilitate economically viable crystal production for biosensors.

Microfluidic and nanowell devices, with their small scale and highly tunable transport properties, have been widely used for screening protein crystal growth conditions, study crystallization kinetics, and develop crystallization phase diagrams (Abdallah, Roy-Chowdhury, Fromme, Fromme, & Ros, 2016; Hansen, Classen, Berger, & Quake, 2006; Lau, Baitz, Dong, & Hansen, 2007; Sauter, Dhouib, & Lorber, 2007; Zheng, Roach, & Ismagilov, 2003). Interestingly, nanowell devices have also been used to better understand protein nucleation and growth in the formation of sub-micrometer protein crystals for applications in pharmaceutical formulation and biosensing; (L. Wang, Barton, Hughes, and Odom (2008)) developed a method of crystallizing proteins in patterned nanowells (Figure 11a). Their work demonstrated control over the localization of submicron crystals. Advancements in microwell protein crystallization has allowed for novel applications in microfluidic biosensing. For instance, Conejero-Muriel, Rodríguez-Ruiz, Verdugo-Escamilla, Llobera, and Gavira (2016) have shown the feasibility of using CLECs for continuous analyte detection; their platform, termed OCER (optofluidic CELC-based enzymatic reactor), has implications in cost-effective next-generation lab on a chip applications (Figure 11b–e).

### 4.4 | Chromatography

Chromatography is a widely-used technique for purifying both small molecules and biomacromolecules. The earliest example of using protein crystal scaffolds as chromatography media was presented in Vilenchik et al. (1998). In this seminal paper, they use macromolecular porosimetry to study the fully hydrated pore structures of various cross-linked protein crystal scaffolds. This technique utilizes size exclusion chromatography (SEC) experiments to estimate the apparent pore size distribution

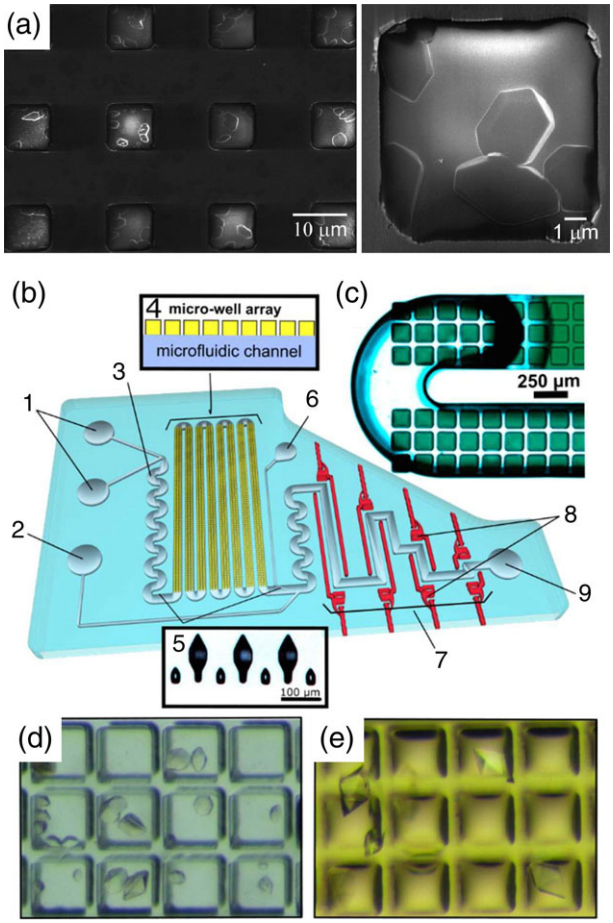


**FIGURE 11** (a) SEM images of lysozyme crystals grown in 10-μm wells. (Reprinted with permission from Wang et al. (2008). Copyright 2008 American Chemical Society) (b) Schematic of the OCER platform: (1) inlet ports; (2) extra inlet port for injecting analytic solutions; (3) passive zigzag micromixer; (4) serpentine channel for droplet storage and cross-section depicting the layout of the solution-storage array; (5) image of the structures located before and after the serpentine channel to prevent any mobile crystal/aggregate from being dragged by the injected solutions; (6) outlet port for the crystallization and cross-linking solution to avoid any contamination of the sensing region; (7) multiple path configuration for the photonic detection system, allowing a large concentration range to be explored while maintaining the absorbance linear range; (8) 2D microlenses with air mirrors along the interrogation channel; (9) outlet port for the product solutions. (c) Operation of the solution trapping system during the injection of a green dyed solution. The meniscus of the flowing solution is observed when emptying the microfluidic device, while the solution trapping system retains nanoliter-sized droplets in the microwell array. (d) Lipase crystals obtained in the OCER platform and (e) after being cross-linked with glutaraldehyde. (Reprinted with permission from Conejero-Muriel et al. (2016). Copyright 2016 American Chemical Society)

of porous media. In their study, they investigated cross-linked protein crystal slurries of thermolysin, two forms of lipase (derived from *Candida rugose* and *Pseudomonas cepacia*), as well as bovine and human serum albumins (BSA and HSA) in packed chromatography columns. Their results demonstrate the ability of protein crystal materials to repeatedly separate molecules based on size, chemical structure, and chirality without significant loss in separation efficiency or structural integrity. Other groups have since demonstrated myriad separation applications for various protein crystal scaffolds (Leisola, Jokela, Finell, & Pastinen, 2001; Pastinen, Visuri, & Leisola, 1998; Pastinen, Jokela, Eerikäinen, Schwabe, & Leisola, 2000).

One challenge in the field of chromatography is enantioselective separation of racemic mixtures. The Vilenchick et al. results highlight the inherently chiral nature of protein crystals as a distinct advantage over other porous materials. Proteins and enzymes are composed of purely L-amino acids resulting in the potential for enantioselective guest interactions. One of the most extensible schemes for enantiomer separations involving protein crystal scaffolds relies on antibody crystals. Antibody proteins possess high affinity and specificity toward their antigens; thus, antibody *crystals* could conceivably also possess high affinity and specificity toward their antigen leading to the ability to distinguish chiral racemate. This idea was confirmed by Vuolanto, Kiviharju, Nevanen, Leisola, and Jokela (2003) and Vuolanto, Leisola, and Jokela (2004) when they demonstrated chiral separations using crystalline chromatography media derived from antibody Fab fragments.

Later, in 2009, Hu and Jiang utilized molecular dynamics simulations to explain the ability of protein crystals to separate chiral molecules. They first modeled the transport of various amino acids (Arg, Phe, and Trp) inside glucose isomerase crystals, providing insight into the relative velocities of each amino acid during separation (Hu & Jiang, 2009b). Later, they

simulated chiral separation of racemic phenylglycines within thermolysin crystals, predicting the ability of nonantibody protein crystals to separate chiral mixtures (Hu & Jiang, 2009a).

## 5 | CONCLUSION

The exceptional properties of proteins can be harnessed in the form of crystal scaffolds to generate advanced nanostructured devices and materials. Recent advancements in protein crystal material research and engineering have opened the door to intriguing applications. The controlled growth of protein crystals with defined size and shape has facilitated the synthesis of next generation materials. As we have discussed, cross-linked protein crystals are remarkably stable against mechanical disruption, solvent changes, and pH extremes. CLEC catalysts have been remarkably active and specific, even at elevated temperature and in aqueous-organic solvents. These properties have allowed CLECs to be successfully used as catalysts for industrial synthesis, organometallic complexes and biohybrid materials, as well as detectors and transducers in biosensing technologies.

Future studies of protein crystal material applications should assess the economics of this technology. In 2012, Blanch and coworkers evaluated the best-case scenario for highly optimized enzyme expression, estimating the baseline production cost to be $10.14/kg (Klein-Marcuschamer, Oleskowicz-Popiel, Simmons, & Blanch, 2012). In the case of protein crystal materials, the cost of bulk purification and crystallization would have to be included, thereby increasing this floor. In the short-term, protein crystals may still be too precious for bulk material applications, such as catalytic production of commodity chemicals or chromatography media, in which hundreds of kilograms of crystalline material may be needed. The economics of engineered crystals could nonetheless be quite favorable for high-value per gram applications such as biomolecular sensing, pharmaceutical formulations, and drug delivery (Figure 12).

Considering the eye-catching price of blockbuster therapeutic proteins, medical applications for engineered crystals might be more feasible in the short term. However, safety considerations are likely to slow the development of engineered crystals for medical applications. As mentioned above, there have been very few studies on how engineered protein crystals affect cells or tissues, nor how the biological environment affects the crystals in turn. FDA approval for the internal use of protein crystals would appear to be quite distant. Also, additional work would be required to establish protein crystals as a viable alternative to the numerous existing biomaterials developed for drug delivery. For example, many biomaterials have been developed primarily to carry therapeutic small molecules or macromolecules through the blood stream. That application has stringent size constraints on the carrier particles (e.g., 10–100 nm). While every large protein crystal was once a nanocrystal, reliable production of large batches of nanocrystals of uniform size represents a significant challenge.

Other future applications could circumvent the economical and safety barriers mentioned above. Even a single large crystal could be quite valuable if it could serve as a reliable source of *information.* For example, fluorogenic crystals that respond to analytes could serve as "protein pixels" to indicate the presence of certain pathogens in a wound dressing. In some cases, edible or biodegradable protein crystals might provide entirely new use cases for biomolecular sensors. Last but not least, one particularly unique information application for engineered crystals would be scaffold-assisted crystallography. Seeman, a porous crystalline host lattice might serve to organize guest biomolecules into a crystalline array, whereupon guest molecules with sufficient occupancy would be visible via X-ray diffraction (Huber et al., 2018; N. C. Seeman, 1982).

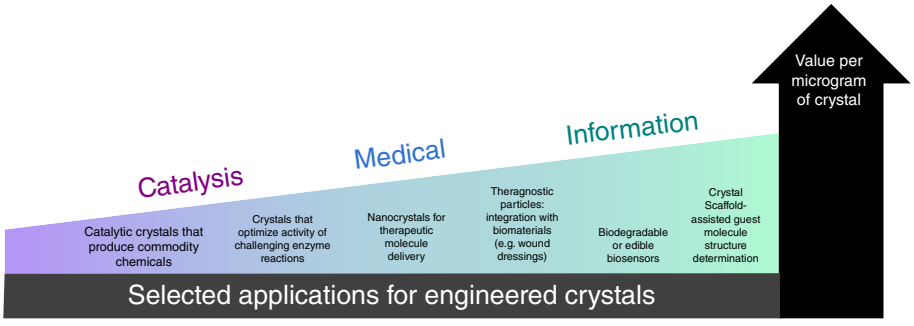


**FIGURE 12** A selection of future applications for engineered protein crystals. Arranged in rough order of increasing economic value per microgram of crystal

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## CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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