

Supercharged Proteins and Polypeptides

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Electrostatic interactions play a vital role in nature. Biomacromolecules such as proteins are orchestrated by electrostatics, among other intermolecular forces, to assemble and organize biochemistry. Natural proteins with a high net charge exist in a folded state or are unstructured and can be an inspiration for scientists to artificially supercharge other protein entities. Recent findings show that supercharging proteins allows for control of their properties such as temperature resistance and catalytic activity. One elegant method to transfer the favorable properties of supercharged proteins to other proteins is the fabrication of fusions. Genetically engineered, supercharged unstructured polypeptides (SUPs) are just one promising fusion tool. SUPs can also be complexed with artificial entities to yield thermotropic and lyotropic liquid crystals and liquids. These architectures represent novel bulk materials that are sensitive to external stimuli. Interestingly, SUPs undergo fluid–fluid phase separation to form coacervates. These coacervates can even be directly generated in living cells or can be combined with dissipative fiber assemblies that induce life-like features. Supercharged proteins and SUPs are developed into exciting classes of materials. Their synthesis, structures, and properties are summarized. Moreover, potential applications are highlighted and challenges are discussed.

are omnipresent. For example, the carrier of genetic information and its transcribed products, DNA and RNA, are nucleic acids bearing negative charges along the backbone. The other important class of biomacromolecules, i.e., proteins, can be either positively or negatively charged.^[1,2] The amino acids (AAs) that determine the charge of the protein are cationic Lys/Arg/His and anionic Glu/Asp residues.^[1,3] The charge of a protein is further modulated by the pH and protic amino acids such as tyrosine and cysteine that can become charged depending on the neighboring amino acids. Supercharged proteins are a class of proteins defined as more than one net charge per kilodalton of molecular weight and can be categorized into folded and unstructured entities.^[4] The supercharged proteins in a folded state have important biological functions, including DNA binding, transcription regulation, protein synthesis, antimicrobial activity, and signal transduction. A large number of natively supercharged proteins

1. Introduction

Coulomb's law is a fundamental law in our universe, which states that opposite charges attract each other and like charges repel each other in a distance-dependent manner. These electrostatic forces play an important role in living cells where charged molecules

have a disordered structure. The supercharged unstructured proteins steer phase separations, provide mechanical properties, and assist in calcium storage of cells.^[5,6] Thereby, natural supercharged proteins harbor essential functions for biology.

Natural supercharged proteins can be an inspiration for chemists, material scientists, and protein engineers to design new materials with attractive properties. Therefore, supercharging proteins and polypeptide chains has attracted great interest in recent years. By supercharging, features of proteins can be improved or new functions can be achieved. Control over its primary structure provides the ability to regulate the charge density, molecular weight, and the position of charges along the backbone of supercharged proteins and polypeptides. Moreover, various charge-induced interactions can be incorporated in these types of materials and the supercharged proteins can be easily fused to other proteins encoding additional functionalities. Finally, supercharged proteins are genetically encoded, allowing their production in a target cell. All these features are far beyond what is possible with conventional polyelectrolyte polymers. Supercharging proteins and polypeptides thus provides materials with exciting properties such as hyper-temperature resistance^[7] and the ability of overcoming biological barriers in vivo.^[8–10,20,11] Moreover, these highly charged structures allow the assembly of bioliquids, organelle-like condensed matter architectures,^[12–15] artificial biological nanocontainers,^[16–18] and interfacial coatings^[19,20] among many others.

Aspects of charge-directed behavior of biomacromolecules on for example polyelectrolyte effects, ionic strength-mediated charge screening, and salt bridge formation have been reviewed

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extensively and will hence not be discussed here.^[1,21,22] As summarized in **Figure 1**, we will first highlight naturally occurring supercharged proteins, folded and unstructured ones. Inspired by their structures and functions, we will direct our focus to supercharged engineered and modified proteins as well as unstructured polypeptides. Additionally, we take a closer look at fusion proteins consisting of two components, i.e., a supercharged part and the fused part with a low net charge usually carrying an additional function. We will summarize the preparation methods of supercharged proteins and polypeptides and discuss in detail relevant exciting applications, to highlight their importance in diverse fields of research.

2. Supercharged Proteins in Nature

The majority of proteins in nature have a low net charge. A subset of proteins is however supercharged. Here, we present both folded and disordered supercharged proteins that occur in cells, which we compiled from protein databases.

2.1. Folded Supercharged Proteins in Nature

Analysis of the UniProt/SwissProt protein databank reveals that most proteins in nature are moderately charged with only 5% of all proteins contained in the databank possessing one or more uncompensated charges per ten AAs.^[23] **Table 1** summarizes the proteins with the highest net charge density (NCD), calculated according to Equation (1)

$$\text{NCD} = \frac{N_{\text{pos AA}} - N_{\text{neg AA}}}{N_{\text{AA}}} \quad (1)$$

where $N_{\text{pos AA}}$ ($N_{\text{neg AA}}$) is the amount of positively (negatively) charged AAs and N_{AA} is the total number of AAs.

For the sake of clarity, only proteins with the highest net charge density of proteins of one family are listed in Table 1. In addition, the value of charge density, namely, the charge at physiological pH over the molecular weight (CMw) is provided in Table 1.

Histones, histone-like proteins, and protamines are among the most positively charged proteins in nature and are responsible for DNA condensation by taking advantage of many cationic charges (Table 1a, entries p1, p8, p9, p10, and p16).^[24,25] The sperm protamine P3 is the most highly charged natural protein with a record NCD valued as 0.74.^[26] Moreover, supercharged proteins are involved in protein synthesis as subunits of the ribosome complex (Table 1a, entries p2, p5, and p6). Besides, several cationic polypeptides can act as neurotoxins by blocking ionic channels (entries p17 and p20) or are involved in bacterial spore coat formation (entry p18). Some antimicrobial polypeptides with high NCD values, including cryptonin, misgurin, and androctonin, perform their functions via increasing cytosol membrane permeability of target cells (entries p12, p14, and p15).^[27,28]

Prothymosin α and parathymosin are among the most negatively charged, naturally occurring proteins (Table 1b, entries n1 and n16).^[29,30] Both proteins are believed to have vital housekeeping functions. Prothymosin α is the most negatively charged natural protein with -0.4 NCD and works as an



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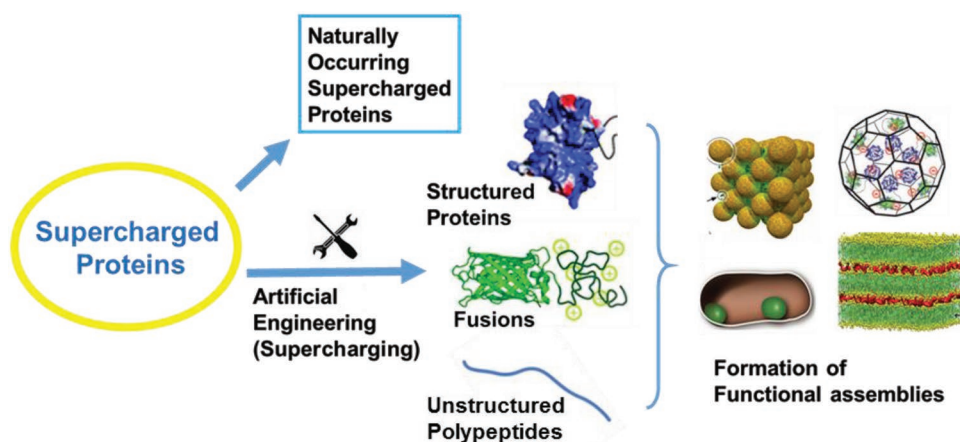


Figure 1. A schematic outline of topics discussed in this review. We will first highlight naturally occurring supercharged proteins. Subsequently, artificially engineered and modified supercharged proteins are described. Different approaches of supercharging are presented, leading to structured proteins, fusion proteins, and unstructured polypeptides. Finally, applications dealing with engineered supercharged proteins are introduced. Figure assembled from component parts reproduced from several sources: Fusions image: adapted with permission.^[14] Copyright 2018 Springer Nature; “Formation of Functional assemblies”: Top left: adapted with permission.^[86] Copyright 2018, ACS; Top right: adapted with permission.^[18] Copyright 2016, Wiley-VCH; Bottom right: adapted with permission.^[12] Copyright 2015, Wiley-VCH. Bottom left: adapted with permission.^[15] Copyright 2014, Springer Nature.

oncoprotein transcription factor involved in cell cycle proliferation.^[31] Parathymosin is associated with early DNA replication, inducing chromatin decondensation.^[32] Chz1 is another highly negatively charged protein involved in chromatin modulation (Table 1b, entry n6), aiding in the proper incorporation of histones into nucleosomes by shielding their positive charge.^[33] Furthermore, highly negatively charged proteins are involved in transcriptional and translational regulation (Table 1b, entries n3, n7, and n18), cell-cycle control (entry n8), ubiquitin-dependent proteolysis (entry n11), electron transport in mitochondria (entry n12), and receptor-mediated endocytosis (entry n14).

Collectively, natural folded supercharged proteins in cells perform diverse functions including genome replication, protein synthesis, and pathogen killing, all of which are essential for cell survival.

2.2. Unfolded Supercharged Proteins in Nature

We have introduced naturally occurring supercharged proteins, yet, in fact, many supercharged proteins in cells are disordered. Intrinsically disordered proteins (IDPs) are typically characterized by a low content of hydrophobic amino acids and a high ratio of charged groups, resulting in a large net charge at physiological conditions.^[34] Folding of these proteins into a compact structure is unfavorable due to repelling forces between like charges. Meanwhile, these forces are not balanced by hydrophobic interactions that would promote folding.^[35] Consistent with these characteristics, supercharged IDPs are highly resistant to non-native conditions, aggregation and heat/chemical denaturation.^[36] Table 2 provides an overview of IDPs with the highest net charge listed in DisProt, a databank of disordered proteins. The sperm histones and several other chromosomal proteins are among the most cationic, disordered proteins (Table 2a, entries Dp1–Dp4, Dp6, Dp9, and Dp12). Furthermore, polypeptides such as nonhistone chromosomal protein H6, cathelicidin, and beta-defensin display antibacterial properties by adopting a structure-less conformation and becoming amphiphilic when

interacting with target molecules on the bacterial membrane (Table 2a, entries Dp6, Dp13, and Dp15).^[37] Besides DNA condensation and antimicrobial activity, cationic disordered proteins are involved in fatty acid and protein synthesis (Table 2a, entries Dp8, Dp11, and Dp14; and Table 2b, entry Dn9).

Regarding anionic IDPs, rat prothymosin α emerged as a disordered protein with the most negative charge density in the DisProt databank functioning as a transcription factor (Table 2b, entry Dn1). Furthermore, anionic disordered proteins are associated with mineralization processes (Table 2b, entries Dn3 and Dn11), calcium storage (entry Dn5), muscle contraction (entry Dn8), protein degradation (entries Dn2 and Dn7), and conductance regulation of ionic gates (entry Dn10).

In general, natural unfolded supercharged proteins play fundamental roles in the regulation of transcription, translation, signal transduction, and cell-cycle control.^[38] It is suggested that supercharged IDPs are particularly involved in processes that require certain flexibility, for instance, as linkers or as binding partners for multiple target structures.^[39] Importantly, supercharged IDPs are also associated with a number of diseases.^[40]

3. Engineering Supercharged Folded Proteins

To harness and translate the function of supercharged structures, folded proteins can be engineered to include a large number of charges, either by genetic engineering or by post-translational chemical modification. Here we discuss the preparation of these molecules and provide key examples of the wide range of applications of this class of proteins.

3.1. Supercharging Folded Proteins by Mutagenesis

3.1.1. Examples of Supercharged Folded Proteins by Mutagenesis

Mutagenesis allows increasing a protein's net charge dramatically, as demonstrated by Liu and co-workers in 2007. This is

Table 1. Overview of the most highly charged, naturally occurring proteins, as derived from the UniProt protein databank.

a) Cationic proteins						
Entry ^{a)}	Protein	Function	UniProt code	NCD	CMw [kDa ⁻¹]	Organism/protein family
p1	Sperm proteamine P3	DNA condensation	P83213	0.74	6.15	<i>Murex brandaris</i> (Purple Dye murex)/N.D.
p2	60S ribosomal protein L41	Protein synthesis	P62945	0.68	4.85	<i>Homo sapiens</i> (human) and other organisms/eukaryotic ribosomal protein eL41
p3	Sperm protamine P1	DNA binding	P67834	0.64	4.64	<i>Dasyurus hallucatus</i> /protamine P1
p4	Sperm histone P2a	DNA binding	P15342	0.58	4.26	<i>Equus caballus</i> (Horse)/protamine P2
p5	50S ribosomal protein L41e	Protein synthesis	P54025	0.55	2.35	<i>Methanocaldococcus jannaschii</i> /eukaryotic ribosomal protein eL41
p6	30S ribosomal protein Thx	Protein synthesis	P62611	0.44	3.64	<i>Thermus aquaticus</i> /bacterial ribosomal protein bTHX
p7	Sperm-specific protein Phi-0	Spermatogenesis	P14309	0.44	3.91	<i>Holothuria tubulosa</i> (Tubular sea cucumber)/N.D.
p8	DNA-binding protein	DNA condensation	P24648	0.43	3.38	<i>Orgyia pseudotsugata</i> /N.D.
p9	Histone-like protein Hq1	DNA condensation	Q45881	0.42	3.71	<i>Coxiella burnetii</i> /N.D.
p10	Histone H1.C8/H1.M1	DNA condensation	P40270	0.41	3.90	<i>Trypanosoma cruzi</i> /histone H5
p11	Spermatid nuclear transition protein 1	DNA condensation	P22613	0.35	2.92	<i>Ovis aries</i> (Sheep)/nuclear transition protein 1
p12	Cryptonin	Antimicrobial	P85028	0.33	2.96	<i>Cryptotympana dubia</i> (Korean horse cicada)/N.D.
p13	Small core protein	Core protein	P69548	0.33	2.86	<i>Enterobacteria phage alpha3</i> /microviridae J protein
p14	Misgurin	Antimicrobial	P81474	0.33	2.80	<i>Misgurnus anguillicaudatus</i> (Oriental weatherfish)/N.D.
p15	Androctonin	Antimicrobial	P56684	0.32	2.58	<i>Androctonus australis</i> (Sahara scorpion)/N.D.
p16	Histone H5	DNA condensation	P02258	0.32	2.97	<i>Anser anser anser</i> (Western graylag goose)/histone H1/H5
p17	Potassium channel toxin alpha-KTx 13.1	Blocks reversibly Shaker B K ⁺ channels	P83243	0.30	2.80	<i>Tityus obscurus</i> (Amazonian scorpion)/Alpha-KTx 13
p18	Spore coat protein G	Incorporation of CotB into spore coat	P39801	0.30	2.42	<i>Bacillus subtilis</i> /Sporulation
p19	Protamine-2	Chromosome condensation	P19757	0.30	2.37	<i>Sus scrofa</i> (Pig)/protamine P2
p20	Mu-conotoxin GIIIB	Blocks voltage-gated Na ⁺ channels	P01524	0.27	2.31	<i>Conus geographus</i> (Geography cone)/conotoxin M superfamily
b) Anionic proteins						
Entry ^{b)}	Protein	Function	UniProt code	NCD	CMw [kDa ⁻¹]	Organism/protein family
n1	Prothymosin alpha	Immune function (suggested)	P06454	−0.40	−3.61	<i>Homo sapiens</i> (Human)/pro/parathymosin
n2	UPF0473 protein Helmi_02360	Uncharacterized	B0TFZ1	−0.39	−3.30	<i>Helibacterium modesticaldum</i> /UPF0473
n3	Testis ecdysiotropin peptide 1	Start or boost ecdysteroid synthesis in testis of larvae and pupae	P80936	−0.38	−3.2	<i>Lymantria dispar</i> (Gypsy moth)/N.D.
n4	50S ribosomal protein L12P	Binding site for factors involved in protein synthesis	P15772	−0.36	−3.47	<i>Haloarcula marismortui</i> /eukaryotic ribosomal protein P1/P2
n5	Coiled-coil domain- containing protein 1	Component of organic matrix of calcified layers of the shell	B3A0Q3	−0.35	−3.14	<i>Lottia gigantea</i> (Owl limpet)/Von Willebrand factor, type A
n6	Histone H2A.Z-specific chaperone chz-1	Histone replacement in chromatin	Q9P534	−0.35	3.17	<i>Neurospora crassa</i> /CHZ1
n7	Probable DNA-directed RNA polymerase subunit delta	Initiation and recycling phases of transcription	Q49Z74	−0.35	−2.93	<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i> /RpoE
n8	Anaphase-promoting complex subunit 15	Controlling progression through mitosis and the G1 phase	A9JSB3	−0.34	−2.93	<i>Xenopus tropicalis</i> (Western clawed frog)/APC15
n9	RNA polymerase subunit delta	Transcription regulation	B9E8H2	−0.32	−2.77	<i>Macroccoccus caseolyticus</i> (strain JCSC5402)/RpoE
n10	RNA polymerase subunit delta	Transcription, DNA-templated	A6U3L3	−0.32	−2.63	<i>Staphylococcus aureus</i> (strain JH1)/RpoE

Table 1. Continued.

b) Anionic proteins						
Entry ^{b)}	Protein	Function	UniProt code	NCD	CMw [kDa ⁻¹]	Organism/protein family
n11	26S proteasome complex subunit DSS1	Ubiquitin-dependent proteolysis	Q3ZBR6	−0.31	−2.65	<i>Bos taurus</i> (Bovine) and other/DSS1/SEM1
n12	Cytochrome b-c1 complex subunit 6	Mitochondrial respiratory chain	P00127	−0.31	−2.66	<i>Saccharomyces cerevisiae</i> /UQCRH/QCR6
n13	Prehead core component PIP	Phage particle	P03720	−0.31	−2.75	Enterobacteria phage T4/Gene product 67
n14	Cysteine-rich, acidic integral membrane protein	Receptor-mediated endocytosis (suggested)	Q03650	−0.31	−2.91	<i>Trypanosoma brucei brucei</i> /N.D.
n15	SHFM1 protein	Proteasome assembly	Q6IBB7	−0.31	−2.65	Homo sapiens (Human)/DSS1/SEM1
n16	Parathymosin	Immune function: blocking prothymosin α	P08814	−0.30	−2.70	<i>Bos taurus</i> (Bovine)/pro/parathymosin
n17	Protein 6	Virion structural protein (suggested)	O70791	−0.30	−2.67	Rice yellow stunt virus/Virion
n18	Regulator of ribonuclease activity B	Modulating RNA abundance	C9XUB3	−0.30	−2.64	<i>Cronobacter turicensis</i> /RraB

^{a)}p = positively charged; ^{b)}n = negatively charged.

referred to as “supercharging of proteins,” in which charged, solvent-exposed amino acids are replaced by genetic mutations.^[7] Negatively charged Asp/Glu were replaced with positively charged Lys/Arg and vice versa. Moreover, several neutral residues were replaced by charged ones, thus creating “superpositive” or “supernegative” variants, respectively (Figure 2a). Specifically, 29 positions in the crystal structure of GFP that were highly solvent-exposed were identified and mutated to introduce positively charged amino acids (Lys and Arg), yielding a theoretical net charge of +36. With this strategy, the index (net charge per kilodalton) of a superfolder variant of green fluorescent protein (sfGFP) was controlled to range from −1.3 to +1.2 and the NCD (Equation (1)) ranged from −0.12 to +0.19 (Table 3). This study showed improved thermostability and extraordinary aggregation resistance for supercharged variants.^[7]

Next to GFPs, other enzymes can be supercharged to enhance their properties. Liu et al. fabricated a negatively supercharged variant of glutathione-S-transferase (GST) exhibiting catalytic activity similar to that of wild-type GST. Interestingly, 40% of the catalytic activity was retained when heating to 100 °C followed by a cooling process.^[7] Another enzyme, enteropeptidase, was also successfully supercharged via genetic mutation of surface-exposed amino acids, showing improved solubility and refolding stability.^[41] Thus, this supercharging strategy may be developed as a generic protocol to enhance enzyme solubility and stability.

In silico design of supercharged proteins by mutagenesis is becoming a promising strategy, given recent progress in computational software development. For example, with the Rosetta computational design package one can mutate multiple sets of amino acids to supercharge proteins.^[42] This entails scoring the energy contributions of charge, solvation, van der Waals interactions, and hydrogen bonding with respect to a reference structure, based on a homology model, giving rise to, for example, more thermo-resistant proteins. A more user friendly web-accessible Rosetta version (termed ROSIE) is available as

well providing a protocol for supercharging proteins, facilitating the modeling and experimental validation for the design of supercharged proteins.^[43]

3.1.2. Applications of Engineered Supercharged Folded Proteins

Next to increasing its resistance to external cues such as temperature, supercharged folded proteins can be used as building blocks to decorate compartments. Compartmentalization of enzymes within confined space is an elegant approach to investigate the complex biocatalytic processes in small volumes. Liposomes, polymersomes, and protein cages have been broadly used as artificial micro or nanocompartments for studying the effect of spatial arrangement on enzyme activity.^[45] Hilvert et al. reported that by taking advantage of the electrostatic interaction between positively supercharged +36GFP and an engineered anionic capsid-forming enzyme, lumazine synthase from *Aquifex aeolicus* (AaLS), a nonviral capsid-based protein encapsulation system could be achieved (Figure 2b).^[18,46] By introducing glutamic acid residues on the surface of AaLS, followed by directed evolution, an optimized variant, AaLS-13, was produced exhibiting higher loading capacity under physiological conditions compared to the original capsid system. Remarkably, AaLS-13 could efficiently encapsulate up to 100 +36GFP molecules in vitro. Packaging was achieved starting either from intact, empty capsids or from capsid fragments by incubation with cargos in aqueous buffer, inferring the assembly is stably maintained via the guest–host association as well as by electrostatic interactions. This protocol for biomimetic packaging with proteinaceous containers is a versatile strategy for designing new materials in respect to catalysis and delivery systems.

Moreover, through genetically grafting +36GFP with a model enzyme, retro-aldolase (RA), Hilvert and co-workers successfully internalized an active enzyme into a proteinaceous nanoreactor.^[18] Packaging was nearly quantitative and up to

Table 2. Summary of highly charged, disordered proteins listed in the DisProt, databank.

a) Disordered cationic proteins in cells						
Entry ^{a)}	Protein	Function	UniProt code/DisProt No.	NCD	CMw [kDa ⁻¹]	Organism/protein family
Dp1	Sperm histone (protamine)	DNA condensation	P15340/DP00057	0.58	4.44	<i>Gallus gallus</i> (Chicken)/protamine P1
Dp2	Histone H5	DNA condensation	P02259/DP00044	0.32	2.95	<i>Gallus gallus</i> (Chicken)/histone H1/H5
Dp3	Histone H1.0	DNA condensation	P10922/DP00097	0.27	2.54	<i>Mus musculus</i> (Mouse)/histone H1/H5
Dp4	Histone H1.2	DNA condensation	P15865/DP00136	0.27	2.68	<i>Rattus norvegicus</i> (Rat)/histone H1/H5
Dp5	Genome polyprotein	Several	P06935/DP00148_C004	0.20	0.03	Human immunodeficiency virus type 1/class I-like SAM-binding methyltransferase
Dp6	Nonhistone chromosomal protein H6	Tuning DNA condensation (sugg.); antibacterial activity	P02315/DP00042	0.20	1.97	<i>Oncorhynchus mykiss</i> (Rainbow trout) (<i>Salmo gairdneri</i>)/HMGN
Dp7	Protein LLP	Transcriptional activator	B0FRH7/DP00544	0.18	1.56	<i>Aplysia kurodai</i> (Kuroda's sea hare)/learning-associated protein
Dp8	50S ribosomal protein L33	Protein synthesis	P0A7N9/DP00143	0.18	1.56	<i>Escherichia coli</i> /bacterial ribosomal protein bL33
Dp9	Nonhistone chromosomal protein HMG-17	Tuning DNA condensation	P02313/DP00195	0.18	1.70	<i>Bos taurus</i> (Bovine)/HMGN
Dp10	Cyclin-dependent kinase inhibitor 2A [Isoform 3]	Negative regulator of proliferation	Q64364-1/DP00335	0.18	1.56	<i>Mus musculus</i> (Mouse)/Tumor suppressor ARF
Dp11	30S ribosomal protein S12	Protein synthesis	P0A7S3/DP00145	0.17	1.53	<i>Escherichia coli</i> /universal ribosomal protein uS12
Dp12	Histone H1	DNA condensation	P53551/DP00423	0.16	1.51	<i>Saccharomyces cerevisiae</i> (Baker's yeast)/histone H1/H5
Dp13	Cathelicidin antimicrobial peptide (LL-37)	Antibacterial activity	P49913/DP00004_C002	0.16	0.31	<i>Homo sapiens</i> (Human)/cathelicidin family
Dp14	30S ribosomal protein S18	Protein synthesis	P0A7T7/DP00146	0.16	1.33	<i>Escherichia coli</i> /bacterial ribosomal protein bS18
Dp15	Beta-defensin 12	Antibacterial activity	P46170/DP00209	0.16	1.46	<i>Bos taurus</i> (Bovine)/beta-defensin
b) Disordered anionic proteins in cells						
Entry ^{b)}	Protein	Function	UniProt code/DisProt No.	NCD	CMw [kDa ⁻¹]	Organism/protein family
Dn1	Prothymosin alpha	transcription factor (cell cycle progression and proliferation)	P06302/DP00058	-0.38	-3.39	<i>Rattus norvegicus</i> (rat)/pro/parathymosin
Dn2	26S proteasome complex subunit DSS1	ubiquitin-dependent proteolysis	P60896/DP00617	-0.31	-1.56	<i>Homo Sapiens</i> (Human)/DSS1/SEM1
Dn3	Protein starmaker	formation of otoliths in the inner ear	A2VD23/DP00584	-0.24	-2.21	<i>Danio rerio</i> (Zebraish) (<i>Brachydanio rerio</i>)/Detection of gravity
Dn4	Cyclic nucleotide-gated cation channel beta-1 [Isoform GARP1]	visual and olfactory signal transduction	Q28181-4/DP00441	-0.20	-0.77	<i>Bos taurus</i> (Bovine)/cyclic nucleotide-gated cation channel
Dn5	Calsequestrin-1	internal calcium store in muscle	P07221/DP00132	-0.20	-1.74	<i>Oryctolagus cuniculus</i> (Rabbit)/calsequestrin
Dn6	Acyl carrier protein	fatty acid biosynthesis	P0A6A8/DP00416	-0.19	-1.74	<i>Escherichia coli</i> /acyl carrier protein (ACP)
Dn7	Prokaryotic ubiquitin-like protein pup	marker for proteasomal degradation	P9WHN5/DP00877	-0.19	-1.74	<i>Mycobacterium tuberculosis</i> /prokaryotic ubiquitin-like protein
Dn8	Troponin C, slow skeletal and cardiac muscles	striated muscle contraction	P63315/DP00249	-0.18	-1.58	<i>Bos taurus</i> (Bovine)/troponin C
Dn9	60S acidic ribosomal protein P1-alpha	protein synthesis	P05318/DP00164	-0.18	-1.74	<i>Saccharomyces cerevisiae</i> (Baker's yeast)/eukaryotic ribosomal protein P1/P2
Dn10	Methylosome subunit pICln	chloride conductance regulatory protein	P35521/DP00717	-0.17	-1.54	<i>Canis lupus familiaris</i> (Dog)/pICln (TC 1.A.47)
Dn11	Bone sialoprotein 2	integral part of mineralized matrix	P21815/DP00332	-0.17	-1.51	<i>Homo sapiens</i> (Human)/Bone sialoprotein II
Dn12	Calmodulin	calcium signal transduction	P62152/DP00344	-0.16	-1.43	<i>Drosophila melanogaster</i> (Fruit fly)/calmodulin
Dn13	Latent membrane protein 2A	blocks tyrosine kinase signaling	A8CDV5/DP00538	-0.16	-1.52	Epstein-Barr virus (Human herpesvirus 4)/Gammaherpesvirus latent membrane
Dn14	RWD domain-containing protein 1	cell signaling	Q9CQK7/DP00587	-0.16	-1.37	<i>Mus musculus</i> (Mouse)/RWDD1/GIR2

^{a)}D = disordered, n = negatively charged; ^{b)}D = disordered, p = positively charged.

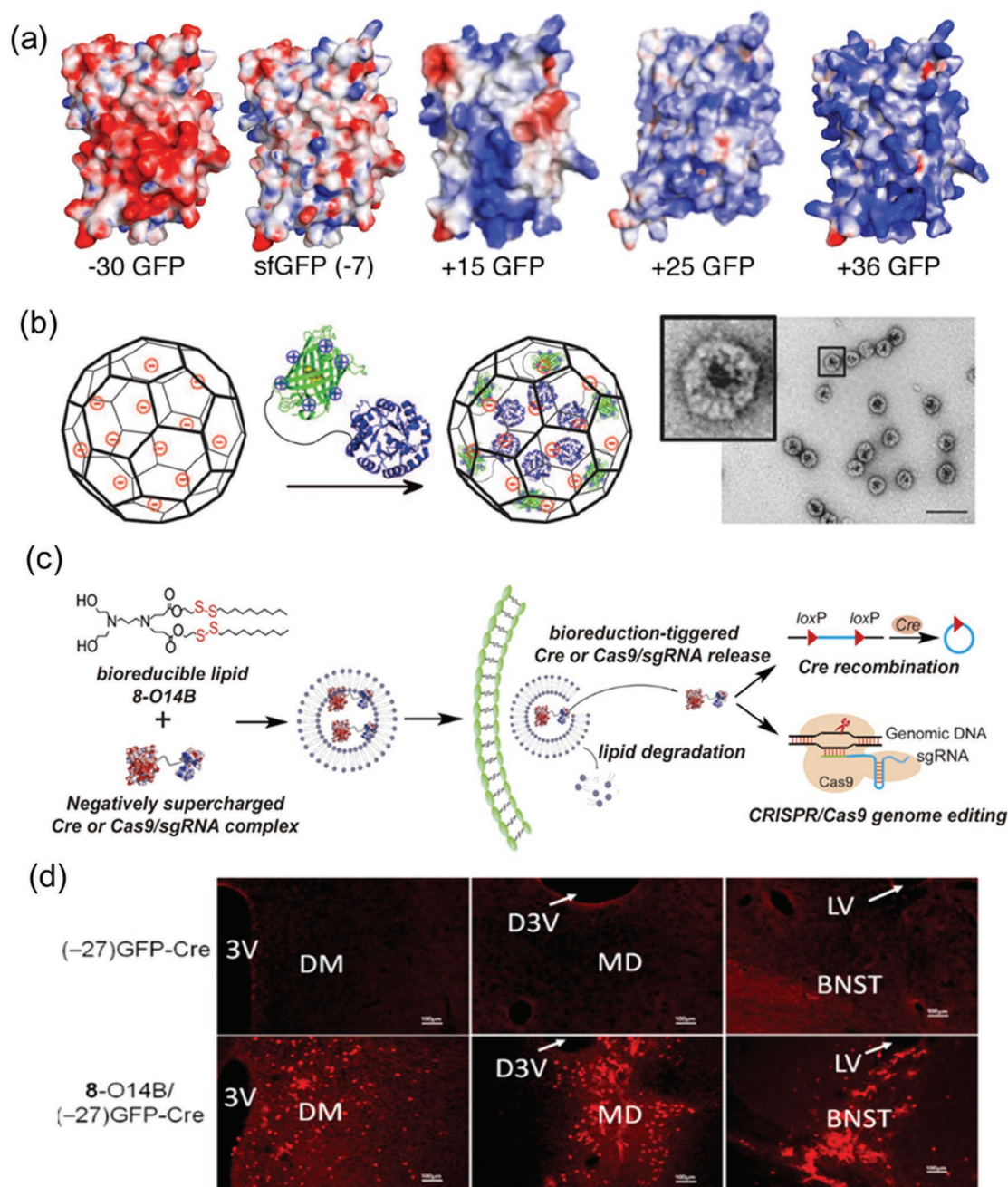


Figure 2. Examples of supercharged folded proteins with altered physicochemical properties, the ability to form nanocontainers, and the ability to aid in vivo drug delivery. a) Positively (blue) and negatively (red) supercharged GFP variants created by genetic engineering from superfolder GFP (sfGFP). Adapted with permission.^[8] Copyright 2009, National Academy of Sciences, USA. b) Packaging of active enzymes into a protein cage. Schematic illustration of the encapsulation strategy of active enzymes in a protein cage. Supercharged +36GFP-RA (green-blue) fusion protein forms a complex with the negatively charged capsid AaLS-13 (black). Transmission electron microscopy images of capsids filled with 45 equivalents of fusion protein are shown. Scale bar 100 nm. Adapted with permission.^[18] Copyright 2016, Wiley-VCH. c,d) Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles into rodent brains in vivo. c) Illustration of bioreducible lipid-like materials and a negatively supercharged protein for protein delivery and genome editing. d) In vivo delivery of Cre recombinase to the mouse brain. The successful targeted delivery is indicated through detection of tdTomato expression (in red) in the dorsomedial hypothalamic nucleus (DM), mediodorsal thalamic nucleus (MD), and bed nucleus of the stria terminalis (BNST). The 8-O14B/(-27)GFP-Cre treated group shows robust delivery evidenced by bright red fluorescence. c,d) Adapted with permission.^[44] Copyright 2016, National Academy of Sciences, USA.

around 45 guest enzymes per capsid (triangulation number $T=3$) were incorporated in an icosahedral geometry. The protein container was composed of 12 pentameric and 20 hexameric

capsomeres equaling 180 capsid proteins. Thereby, precise control over the density of guest enzymes in the luminal space was achieved. The protocol and properties of this robust

Table 3. Genetically engineered, supercharged folded proteins.

Entry ^{a)}	Protein	NCD	Length [N _{AA}]	N _{posAA}	N _{negAA}	Net charge
G1n	−30GFP	−0.12	248	19	49	−30
G2n	−25GFP	−0.10	248	21	46	−25
G3n	sfGFP	−0.03	248	27	34	−7
G4p	+36GFP	+0.15	248	56	20	+36
G5p	+48GFP	+0.19	248	63	15	+48
G6p	scFv anti-MS3	+0.02	233	24	19	+5
G7p	scFv anti-MS3 (K-pos-1)	+0.06	233	32	19	+13
G8p	scFv anti-MS3 (K-pos-2)	+0.07	233	35	19	+16
G9p	scFv anti-MS3 (K-pos-3)	+0.09	233	38	18	+20
G10p	caveolin selectant 11	+0.03	33	5	4	+1

^{a)}G = genetically engineered, n = negatively charged, and p = positively charged.

encapsulation strategy set the stage for the design and generation of more complex nanoreactors via the co-encapsulation of sequentially acting enzymes. This method was expanded to a protein shell consisting of up to 360 units with an impressive molecular weight of ≈ 6 MDa.^[47] Very recently, a new version of the supercharged nanochamber consisting of AaLS-13 and +36GFP-fusions was constructed. It possesses a negatively supercharged lumen and can be used to effectively sort substrates for an encapsulated protease.^[48]

In a similar vein to the approach above, advanced cage-within-cage complexes were created exploiting well-defined coulombic interactions, resulting in hierarchically organized supramolecular assemblies.^[49] Self-assembly driven by coulombic interactions gives rise to symmetrically organized structures of supercharged proteins: it was shown that supercharged GFPs can be assembled into symmetrical 16-mers.^[50] Instead of guiding self-assembly, supercharging GFPs allows the solubility of larger assemblies to be maintained, such as in the case of large polygon-geometries.^[51] However, supercharging GFP also gives rise to self-assembly of much less structured coacervates, where liquid–liquid phase separation occurs by complexation of positively supercharged proteins and negatively charged polyelectrolytes. Coacervate formation is influenced by pH, salt, and the charged molecules involved.^[52] Thus, supercharging can be exploited for the design of multi-protein assemblies with high molecular weights and varying degrees of structural complexity.

Supercharged folded proteins were also combined with mammalian cells. In this regard, similar to cationic histone proteins with a high net charge, charge–charge interactions with anionic nucleic acids were exploited. Liu and co-workers used supercharged protein +36GFP as a shuttling unit to penetrate mammalian cells to deliver exogenous biomacromolecules with high potency.^[4,53] To alter the cellular activity by interfering with gene expression, an electrostatic complex of siRNA with the supercharged folded protein was introduced into various cell lines. This resulted in successful gene silencing.^[8] Additionally, +36GFP fused to Cre recombinase was effectively taken up by HeLa cells and perturbed genome recombination *in vitro* as well as in mouse retinal cells *in vivo*.^[54]

Moreover, lipids can be combined with supercharged folded proteins as counter ions to form membrane-like compartments, allowing for protein or nucleic acid delivery (Figure 2c).^[44] Again, analogy can be drawn to cationic antimicrobial peptides, which are involved in targeting bacterial membranes. Therapeutic proteins are a growing family of biologics that can be harnessed for specific manipulation of cell function. In particular, the programmable nuclease Cas9 and other genome-editing proteins (e.g., Cre recombinase) are attractive candidates. However, the lack of an effective, generic approach to encapsulate a protein into a stable nanocage and the inefficient release of the protein from endocytosed nanoparticles impairs their intracellular function. Recently, Wang et al. reported a bioreducible lipid complexing with anionic supercharged Cre recombinase or anionic Cas9:sgRNA to drive the electrostatic assembly of nanoparticles that initiate efficient protein delivery and genome editing (Figure 2c).^[44] The O14B family of bioreducible lipids was synthesized featuring a disulfide bond and a 14-carbon hydrophobic tail, which could efficiently transfer active anionic supercharged protein nanocomplexes inside cells with a higher yield than obtained with commercially available lipids. Moreover, these bioreducible lipids enabled Cre and Cas9-mediated gene recombination or knockout with efficiencies higher than 70% in human cells. An even more exciting finding was that these nanoparticle complexes were shown to effectively deliver therapeutic proteins into the brain of rodents to achieve DNA recombination *in vivo* (Figure 2d). Delivering a protein for genome modification directly to the brain holds great promise for the treatment of a wide range of genetic diseases, including neurological disorders. The −27GFP-Cre/8-O14B nanocomplexes were fabricated *in vitro* and injected into the brain of a Rosa26^{tdTomato} mouse. The mouse cells contain a specific STOP cassette preventing the expression of the fluorescent protein tdTomato (red fluorescence), whereas Cre-mediated genome manipulation induces tdTomato expression (Figure 2d). This approach could impact genome editing *in vivo* for treatment of neurological diseases because it allows for targeting specific genes in a local subset of neurons.

3.2. Supercharging Folded Proteins by Post-Translational Chemical Modification

The net charge of a protein can be modified by post-translational chemical modification of solvent-exposed residues. The methods date back to the late 1960s and most practical examples include acetylation and succinylation of lysine residues as well as the amidation of carboxylic groups (Figure 3).^[22,55] Although initially introduced for the characterization of proteins, these methods have since attracted increased interests owing to their supercharging effect, which alters a protein's solubility and interaction with oppositely charged molecules. For example, the acetylation of lysine ϵ -amino groups decreases the number of positive charges, resulting in variants with a higher net negative charge (Figure 3a). Through a reaction with acetic anhydride, Shaw et al.^[56] created supernegatively charged variants of bacterial α -amylase, an industrially relevant hydrolase, without perturbing its structural integrity. The modified variant with ≈ 17 acetyl modifications proved to be more resistant to

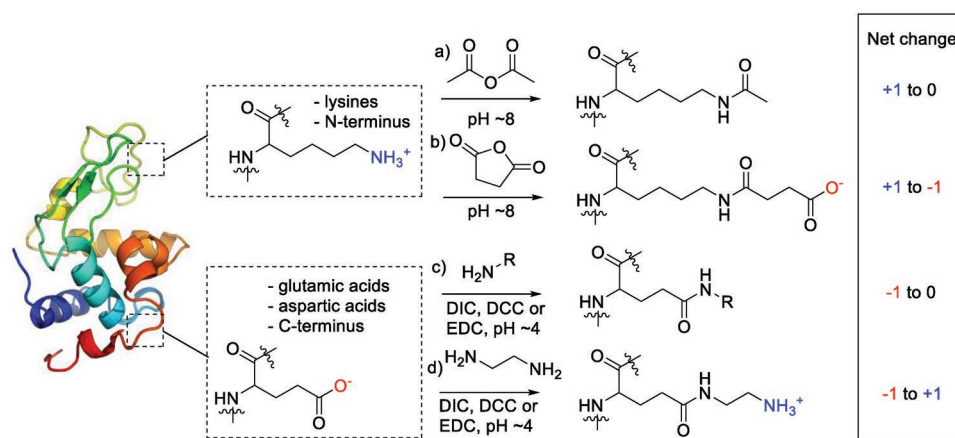


Figure 3. Overview of practical chemical modifications of proteins. The net charge of a protein can be increased by elimination (a,c) or inversion (b,d) of charges. Amine groups are neutralized by acetylation (a) or their charge is reversed by succinylation (b). These reactions are performed at a pH of 8, by, for example, titration with NaOH. Amide formation at carboxylic acids (c) eliminates negative charges on the protein surface. In the special case of amidation (d), a positively charged group can be introduced. These reactions are usually performed at an acidic pH ≈ 4 .

irreversible inactivation and aggregation in the presence of anionic and neutral surfactants (e.g., sodium dodecyl sulfate and Triton X-100) that are commonly used in industrial applications.^[57] Succinic anhydride reacts with lysine ϵ -amino groups and converts these from basic to acidic groups (Figure 3b). However, succinylation might lead to destabilization and increased aggregation of the modified protein. It was further suggested that charge modification might interfere with the ion pair network, thereby destabilizing the protein structure.^[58]

Instead of altering the charge of lysine, charged proteins can be obtained by amide bond formation of carboxylic acid groups (Figure 3c,d). Because carboxylates are less reactive than amine groups, activation of the carboxylic acid group by a carbodiimide such as *N,N*-dicyclohexylcarbodiimide or *N,N*-diisopropylcarbodiimide is necessary. Subsequent reaction with a diamine leads to a replacement of $-COO^-$ for $-NH_3^+$ and an increase of two net charges at the reaction site ($N_{total}: -1$ to $+1 = +2$). Several proteins, including ferritin, catalase, superoxide dismutase, bovine serum albumin (BSA), and ovalbumin, have been modified by amidation to increase their interaction with negatively charged tissues.^[59,60] Moreover, cationic BSA was found to form polyplexes with plasmid DNA that allowed transfection of A549 human lung epithelial cells in vitro.^[61] A recent study showed that pretreating human mesenchymal stem cells with a cationized myoglobin polymer resulted in alleviating necrosis at the center of hyaline cartilage tissue.^[62] Thereby, the chemically modified myoglobin acted as a reservoir for oxygen molecules.

Besides introducing permanent chemical alterations, reversible chemical modification of folded proteins is another useful tool to modulate protein function and improve their properties. For instance, by introducing charge-conversional citraconic amide moieties by reaction with 2-methylmaleic anhydride or by incorporating reactive-oxygen-species responsive groups, reversible approaches to supercharge proteins and nanocarriers can be realized for intracellular delivery, targeted cancer therapy, as well as genome editing.^[63]

Tethering polyelectrolytes to the surface of proteins is another method of supercharging. Maynard and co-workers developed a

new generation of conjugation system, consisting of a heparin-mimicking polyelectrolyte that was chemically connected to fibroblast growth factor (FGF).^[64] This supernegatively charged polymer–protein complex showed superior stability while maintaining physiological activity of FGF. Thus, this polyelectrolyte-conjugation approach represents a promising tool to stabilize protein drugs for future clinical translation.

Taken together, chemical modifications can be used to engineer a protein's overall charge and to increase resistance against aggregation. Alternatively, favorable interactions with oppositely charged molecules can be enhanced, thereby promoting the adhesion or uptake of biologically active molecules into cells. Although the chemical modification of charged, solution-exposed residues is a simple method to change a protein's net charge, its applicability needs to be evaluated for individual proteins. Furthermore, chemical modification results in a mixture of variants with different net charges and modification patterns. To yield fractions with a narrow net charge distribution, an extra purification step is required.^[59,61,62] This method is therefore mainly favorable in cases where the protein is extracted from natural sources. For proteins that are recombinantly produced in a heterologous host organism, surface charges can “simply” be introduced by genetic mutagenesis. This approach results in better defined protein variants than chemical modification procedures where target proteins contain multiple reaction sites.

4. Engineering Unstructured Supercharged Polypeptides

4.1. Supercharging Unstructured Repetitive Polypeptides

Supercharged natural proteins are challenging to use for the design of advanced functional materials due to their rather complex primary structure. One promising reductionist strategy is to prepare protein polymers, which are composed of small repeat segments. Charges can be introduced by mutagenesis in a similar way as described for folded proteins resulting

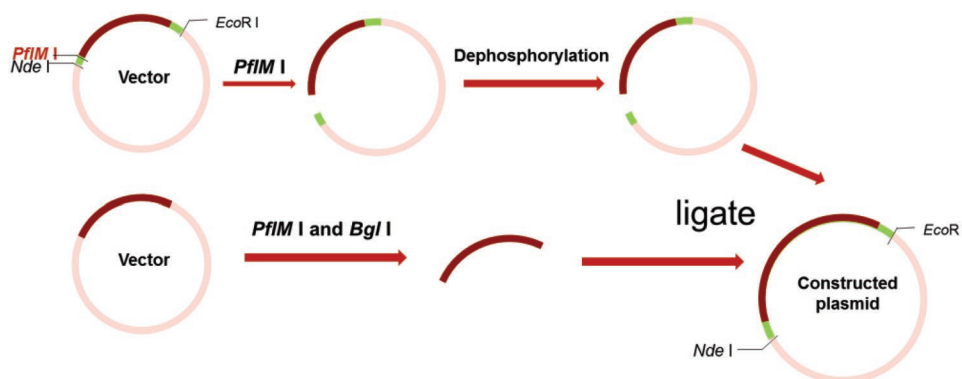


Figure 4. Schematic of recursive directional ligation for the oligomerization of elastin-like polypeptide genes. Typically, restriction enzymes *PflMI* and *BglI* are used for enzymatic digestion and ligation of the gene of interest.

in controlled charge patterns, monodispersity, biocompatibility, and structural versatility.

Elastin-like polypeptides (ELPs) are an ideal candidate for supercharging proteins. ELPs are derived from elastin, a component of the extracellular matrix in vertebrates.^[65] It contains repetitive sequences with units of four to six AAs that are rich in valine (V), proline (P), glycine (G), and alanine (A). Genetic engineering allows for the recombinant fabrication of ELPs in good yields with precise length and composition. Chilkoti and co-workers developed the recursive directional ligation (RDL) protocol, which represents a stepwise procedure for oligomerization of a monomeric gene containing defined number of repeats (Figure 4).^[66] By varying the monomer length and by repeating multiple rounds of restriction and ligation, oligomers of almost any desired length can be obtained. Besides this RDL approach, genes for proteins with a repeating sequence can be constructed by polymerase chain reaction (PCR). A method named overlap extension rolling circle amplification relies on circularizing a linear oligonucleotide encoding the repeat unit and the presence of primers enabling the amplification of linear DNA fragments in a PCR-based process.^[67] This method provides repetitive gene libraries over a tailored range of molecular weight in a one-pot format. In addition, a codon scrambling algorithm has been proposed to further facilitate PCR-based cloning of repetitive sequences, optimizing the genes for improved cloning and expression.^[68]

ELPs display a characteristic thermal phase-transition behavior, termed lower critical solution temperature.^[69] These materials typically precipitate above a certain temperature when dissolved in an aqueous medium. This transition temperature T_i of ELPs can be tuned by introducing AAs in the fourth position of a pentapeptide repeat (X in VPGXG). An overview of the T_i of the resulting ELP variants is provided in Table 4. These findings show that by introducing charged AAs, customized ELPs can be generated that are highly responsive to temperature, but also to salinity and pH.^[70] The charged AAs are sites for additional chemical modifications, allowing for the creation of a virtually unlimited number of variations of these recombinant biopolymers. A consequence of this precise control over the amino acid composition along the unfolded polypeptide backbone is the possibility to introduce a multitude of desired properties.^[13,71]

Recently, we established a novel family of highly ionic repetitive polypeptides, termed supercharged unstructured polypeptides (SUPs) with NCD values higher than that reported for ELPs. This was achieved by introducing charged AAs into the pentapeptide VPGXG (Table 4) wherein X represents the position of the charged AA.^[13,20] With this motif as starting point, SUPs were programmed with half of the charges employing (GVGVPGVGPXG)_n as repeat unit. Double charged variants were obtained by integrating two Glu residues at the X-position of the VPGXG repeat unit. Double charging provides SUPs with an NCD of −0.35, which allows the fabrication of higher charged proteins compared to the supercharging of folded proteins. Notably, the NCD of −30GFP is −0.12. SUPs are characterized by large structure tunability and versatility enabling various bulk material applications. Specifically, SUPs allow tuning of charge density, molecular weight, and position of charges within their biomacromolecular backbone. The resulting materials can be rendered biocompatible due to the proteinaceous nature and dilution of charges along the polymer chain especially regarding positively charged variants. In synthetic vinyl polymers or polypeptides synthesized by ring opening polymerization, positioning of cationic monomers is much harder to achieve when combined with neutral monomers.^[16] Moreover, SUPs can be complexed with other charged molecules to obtain new properties.^[12,78] Especially, this holds true for combining SUPs with oppositely charged surfactants to form charge stoichiometric complexes. Finally, SUPs fused with target proteins are genetically encoded and therefore their properties can be improved by directed evolution.

4.2. Self-Assembled Supercharged Polypeptides

Mutagenesis of a single disordered protein is not the only way for supercharging: Munch et al. presented the self-assembly of monomeric unstructured oligopeptides to achieve a supercharging effect.^[79] They designed amphiphilic short peptides (QCKIKQIINMWQ), which self-assemble into nanofibrils. These supercationic protein nanofibrils, termed enhancing factor-C (EF-C), dramatically boost retroviral gene transfer and offer a rapid approach for virus concentration. EF-C nanofibrils compare favorably with conventional cationic polymers because

Table 4. Transition temperatures (T_t) of cationic and anionic ELPs under various conditions.

ELP	MW [kDa]	T _t [°C]			NCD*	Ref.
		Basic/acidic	Buffer	Salt		
Cationic						
poly[VPGφG] (φ = V, ≈86%; φ = K, ≈14%)	n.d.	n.d.	n.d.	n.d.	+0.030	[72]
[(VPGVG) ₄ (VPGKG)] ₃₉	81	28 ^{a)}	# ^{b)}	50 ^{c)}	+0.050	[73]
[VPGKG(VPGVG) ₆] _n (n = 8, 16, 32)	24, 47, 93	–	60, 45, 39 ^{d)}	30, 22, 19 ^{e)}	+0.033	[74]
[VPGKG(VPGVG) ₁₆] _n (n = 8, 16, 32)	22, 43, 85	–	45, 34, 30 ^{d)}	26, 19, 15 ^{e)}	+0.012	[74]
[VPGKG(VPGVG) ₂ VPGFG] _n (n = 4, 8, 16, 32)	8, 15, 28, 56	–, –, –, 20 ^{f)}	–, –, 61, 43 ^{d)}	–, –, 17, 11 ^{e)}	+0.050	[75]
[VPGKG(VPGVG) ₇ VPGFG] _n (n = 2, 4, 8, 16)	8, 16, 31, 61	–, –, –, 15 ^{f)}	–, 48, 35, 26 ^{d)}	–, 25, 16, 11 ^{e)}	+0.022	[75]
(VPGVGVPKG) _n (n = 15, 20, 30)	14, 18, 27	n.d.	n.d.	n.d.	+0.096	[20]
(VPGKG) _n (n = 18, 36, 72, 144)	10, 19, 36, 71	n.d.	n.d.	n.d.	+0.170	[20]
Anionic						
poly[VPGφG] (φ = V, ≈80%; φ = E, ≈20%)	n.d.	n.d.	n.d.	n.d.	–0.040	[72]
poly[IPGφG] (φ = V or E; various ratios)	n.d.	n.d.	n.d.	n.d.	–0.012 to –0.2	[76]
[(VPGVG) ₂ (VPGEG)(VPGVG) ₂] _n (n = 5, 9, 15, 30, 45)	10, 19, 31, 62, 93 ^{g)}	32, 26, 23, 21, 21 ^{h)}	n.d.	n.d.	–0.040	[77]
(VPGEG) ₄₀ (VPGFG) ₂₀	34	40	n.d.	n.d.	–0.133	[15]
(VPGVGVPGE) _n (n = 15, 35)	14, 31	n.d.	n.d.	n.d.	–0.096	[20]
(VPGEG) _n (n = 9, 18, 36, 72, 144)	6, 10, 19, 36, 71	n.d.	n.d.	n.d.	–0.170	[20]
(VEGEG) _n (n = 18, 36, 54, 108)	11, 21, 30, 59	n.d.	n.d.	n.d.	–0.350	[13]

*NCD = net charge density. #Phase transition not shown below 100 °C. ^{a)}0.1 M NaOH; ^{b)}50 × 10^{–3} M TrisHCl, pH 7.0; ^{c)}150 × 10^{–3} M NaCl (50 × 10^{–3} M TrisHCl, pH 7.0); ^{d)}PBS; ^{e)}PBS, 1 M NaCl; ^{f)}20 × 10^{–3} M phosphate buffer, pH 12; ^{g)}Calculated; ^{h)}Phosphate buffer (pH 2.5).

they have a high surface charge and the structure based on cross- β sheets provides stiffness. Viral particles associate with the supercharged nanofibrils, leading to an increase of virus transport into cells. The latter example demonstrates very well that supercharging does not only change the property of a single protein or polypeptide entity like temperature stability, solubility, or entry into cells. Instead self-assembly of a very large number of highly charged peptides leads to superstructures, which determine the function of the material. A similar statement holds true for protein cages mentioned above that control the properties of their cargo, i.e., substrate specificity.

4.3. Supercharging by Fusion with Supercharged Unstructured Polypeptides

Supercharging can alternatively be achieved by simple fusion of the supercharged unstructured polypeptides described in the previous paragraph to the desired protein. This represents an alternative to introducing charged amino acid residues to the surface of a folded protein, albeit that the large size of the supercharged tag could hinder the functional properties of the target protein. This could occur, for example, in protein co-crystals discussed in the next section. An outstanding feature of an unstructured supercharged fusion tag is that it obviates the need for rational design or extensive screening for functional mutants.

Negatively supercharged tags enhance the stability of proteins in solution. For example, the protein B domain of bacteriophage T7 (Table 5, entries 1 and 2) and its more acidic variant T7B9 (entry 3), as well as the acidic tail of synuclein (ATS) (entry 4),

stabilize aggregation-prone proteins and prevent their aggregation during overexpression. In this manner they provide sufficient solubility for structural and biological investigations.^[80] Moreover, extensions with highly anionic peptides can significantly enhance the stability and solubility of protein formulations for therapeutic purposes. For example, introduction of an ATS into disparate therapeutic proteins (e.g., human growth hormone, granulocyte colony-stimulating factor, and human leptin) resulted in higher stability against heat, agitation, and freeze/thaw cycles in vitro, as well as improved pharmacokinetics in vivo.^[80] Similarly, oligo-Glu tagging of genome editing proteins allowed electrostatic complexation with cationic gold nanoparticles, which provided hierarchical nanostructures that penetrate cell membranes and enable efficient gene editing.^[81]

Tagging proteins with a positively supercharged tag stimulates uptake of proteins into mammalian cells in vitro and in vivo (Table 5, entries 9 and 10).^[11] Several cationic (poly)peptides termed cell-penetrating peptides (CPPs), including oligoarginine and HIV-transacting activator of transcription (HIV-TAT), trigger the transport of fusion proteins across physiological barriers of epithelial and endothelial cells induced by a mechanism known as macropinocytosis resulting in the delivery of guest macromolecules into cytoplasm or other cellular compartments (Table 5, entries 7 and 8).^[82]

Hence, SUPs exhibit an extraordinary set of properties, which sets them apart on the one hand from synthetic polyelectrolytes and on the other hand from supercharged folded proteins. Translating their special features into function is currently being explored. Below we provide an overview of breakthroughs achieved with this class of materials.

Table 5. Supercharged protein tags for solubilization or uptake of proteins.

Entry ^{a)}	Protein	NCD	Length [N _{AA}]	N _{posAA}	N _{negAA}	Net charge
S1	B1 domain of protein G	−0.07	56	6	10	−4
S2	T7B	−0.11	44	5	10	−5
S3	T7B9	−0.25	44	3	14	−11
S4	ATS	−0.41	22	0	9	−9
S5	SUP-E144	−0.17	813	0	144	−144
S6	SUP-DC_E108	−0.35	311	0	108	−108
S7	oligo-arginine (R9)	+1.00	9	9	0	+9
S8	HIV-TAT protein	+0.54	13	7	0	+7
S9	β-defensin 3 (fragment)	+0.24	45	13	2	+11
S10	histone methyl transferase (fragment)	+0.21	72	18	3	+15
S11	GFP-K72	+0.10	662	99	34	+65
S12	SUP-K144	+0.17	813	144	0	+144

^{a)}S = supercharged tag.

5. Applications of Supercharged Unstructured Polypeptides in the Field of Advanced Materials

5.1. Biocapsules, Films, and Co-Crystals Assembled with SUPs

The high net charge of SUPs enables the assembly of superstructures by exploiting charge–charge interactions. One example is biopolymer capsules for biomedicine and drug delivery.^[83] A characteristic of protein capsules is their improved biocompatibility compared to synthetic polymer counterparts.^[84] Recently, a novel biocapsule system was reported by harnessing the interplay of superpositively charged K48 and oppositely charged E57 (Figure 5a): K48 is a polypeptide with 48 Lys residues and E57 comprises 57 Glu in the (GVGXP)_n polymeric backbone.^[16] The E57/K48 complexes were introduced onto a sacrificial calcium carbonate template in a layer-by-layer (LbL) fashion, and the outer layer of K48 was fluorescently labeled for visualization. Subsequently, the CaCO₃ core was removed by acid treatment, and thus an intact hollow protein capsule was produced (Figure 5b). The solid microparticles with porous shell might be used as controlled-release drug carriers in biomedicine. In the same vein, Rodriguez-Cabello and co-workers employed unstructured proteins and sugars to produce biofilms. The films were cast with superpositively charged proteins and the oppositely charged polysaccharides chitosan or alginate by LbL assembly (Figure 5c).^[17] In the future, this strategy may be translated for, e.g., tissue engineering and drug delivery applications.

Self-assembly involving SUPs was further developed to arrange biomolecules into extended materials that are more ordered. This may, for example, be beneficial for X-ray structure determination where highly ordered structures are needed.^[85] By using the electrostatic attraction between GFP-K72 and virus capsids (cowpea chlorotic mottle virus (CCMV)), Korpi et al. recently introduced a type of bioco-crystals with a precisely ordered architecture (Figure 5d).^[86] In contrast to encapsulating proteins into the interior of capsids, here GFP-K72 fills

the voids between adjacent negatively charged CCMV shells and works as a “glue” for the co-crystal construction with a face-centered cubic morphology. Notably, functional proteins like GFP can be embedded into the co-crystal without hindering self-assembly. In the future, such assemblies might serve as a protective shell for cargo proteins (such as GFP here), but could also facilitate the structure determination of biomacromolecules by X-ray analysis if guest proteins are incorporated in an ordered manner.

Together, these studies demonstrate that the construction of biocontainers, films, and co-crystals based on supercharged proteins provide a broad variety of self-assembled materials with different degree of order by exploiting electrostatic interactions. Because recombinant techniques allow controlling the sequence of SUPs down to the single monomer unit, one can firmly state that these architectures can be further tailored concerning how strong individual building blocks interact. Hence, their stability or degradation may be fine-tuned very accurately.

5.2. SUPs Act as an Active Component on Surfaces

Next to complexing oppositely charged SUPs or SUPs with polysaccharides, they were combined with oppositely charged human glycoproteins to improve biolubrication. Biolubrication involves the modification of sliding surfaces with (bio) polymers to reduce friction.^[87] Oral lubrication via adsorbed salivary conditioning films (SCFs) is crucial to facilitate speaking and mastication and minimize wear from erosion and abrasion. SCFs to a large extent consist of mucins that are composed of a central polypeptide backbone from which negatively charged oligosaccharides protrude to adopt a bottlebrush structure. The negatively charged sugar moieties are responsible for recruiting water molecules that introduce a lubricous layer when mucins adsorb on biosurfaces.^[88] Biolubrication can be impaired through several diseases. In the clinic, patients suffering from oral dryness are treated with artificial saliva made of natural extracts (e.g., pig gastric mucins and polysaccharides). However, this treatment only results in transient relief for the patients and the SCFs insufficiently retain water due to loss of structural integrity. Thus, lubrication of biological surfaces is a key feature of health and its decrease with age or pathological conditions significantly reduces quality of life.

In this context, Veeregowda et al. studied biolubrication on the SCFs that were stabilized with SUPs.^[19] Therefore, biocompatible, cationic SUPs were involved in forming electrostatic assemblies with oppositely charged mucins that are present in SCFs (Figure 6a–c). It needs to be emphasized that this approach is different from the current treatment modality of substituting saliva components. In this study, the aim was to stabilize existing SCFs by cationic SUPs that form complexes with anionic mucins. On model surfaces, a layered architecture consisting of three layers was established. On the first SCF, a cationic SUP layer was deposited. When the SUP contained enough charges along its polymer backbone as in the case of K72, an additional top layer of SCF was successfully established that was rich in glycosylated mucins. Significantly less mucins could be immobilized when the lower molecular weight analog

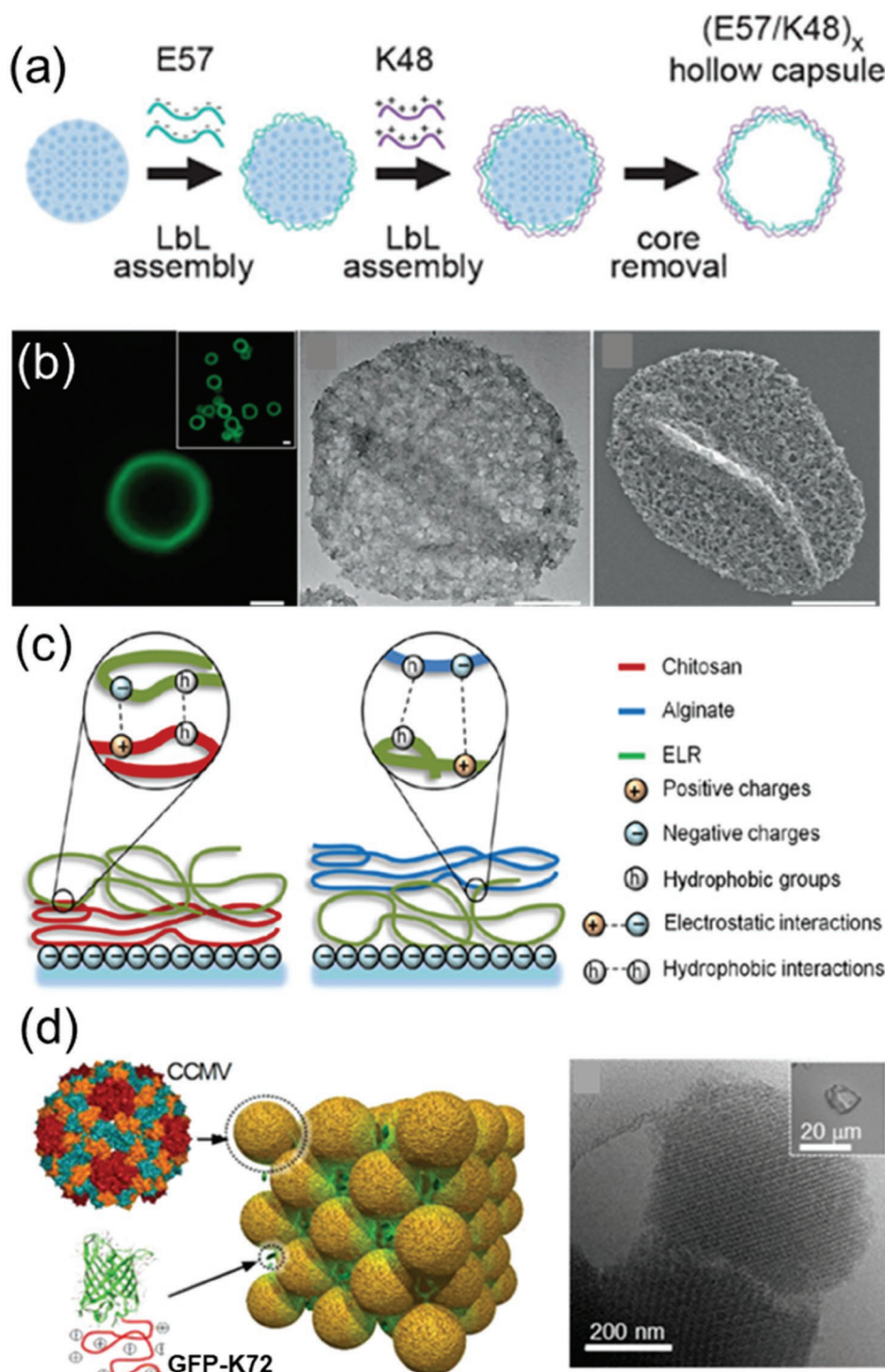


Figure 5. Assemblies of supramolecular aggregates exclusively derived from supercharged proteins or from SUPs with other polymeric counter ions enabled by electrostatic interactions. a) Schematic presentation of the assembly of proteinaceous supramolecular capsules. Two oppositely supercharged proteins are electrostatically deposited onto spherical CaCO_3 microparticles through LbL assembly, and a hollow capsule can be fabricated by dissolution of the inorganic template core. b) Structural analysis of capsules assembled by oppositely supercharged polypeptides: confocal laser-scanning microscopy (left) image of capsules in aqueous solution (green, Alexa Fluor 488). Transmission electron microscopy (middle) image and scanning electron microscopy (right) image of one capsule. As electron microscopy was performed in vacuum, the capsules are collapsed, indicating the absence of the template core and thus an empty cavity. a,b) Adapted with permission.^[16] Copyright 2011, Wiley-VCH. c) Representative illustration of the hypothetical interactions occurring between polysaccharides and highly charged polypeptides. Adapted with permission.^[17] Copyright 2013, American Chemical Society. d) Schematic illustration of protein co-crystals comprising GFP-K72 and CCMV capsids as well as a transmission electron microscopy image of CCMV–GFP–K72 crystals. The inset shows an optical microscopy image of the crystals. GFP-K72 works as “cement” between the capsids for the assembly. Adapted with permission.^[86] Copyright 2018, American Chemical Society.

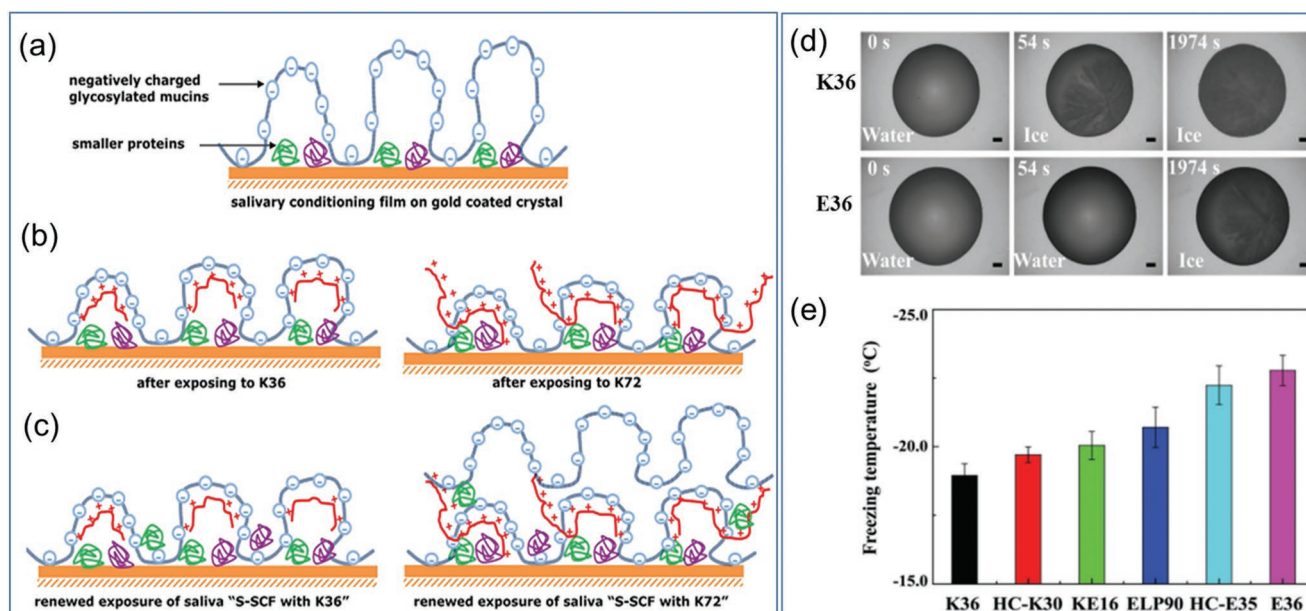


Figure 6. Surface functionalization involving supercharged unstructured polypeptides for the purposes of biolubrication and control of ice formation. a–c) Proposed architecture for the design of salivary conditioning films (SCFs) after adsorption of cationic SUPs (K36 or K72) and renewed exposure to saliva. a) Adsorbed SCFs on an Au-coated substrate, showing glycosylated mucins over a layer of adsorbed densely packed low-molecular-weight proteins. b) SCFs after adsorption of K36 (left panel) and K72 (right panel). c) SCFs with adsorbed cationic SUPs and after renewed exposure to saliva. No mucins are recruited in the presence of adsorbed K36 (left panel), but remaining positive charges in the film possessing adsorbed K72 recruit mainly glycosylated mucins to form a soft mucinous layer (right panel). a–c) Adapted with permission.^[19] Copyright 2013, Wiley-VCH. d,e) Tuning of ice nucleation with supercharged polypeptides. d) Ice nucleation on surfaces modified with SUPs (K36 and E36) at -19.0°C imaged via optical microscopy equipped with a high-speed camera. Scale bar: $100\text{ }\mu\text{m}$. e) Temperature profile of ice nucleation on the surfaces modified with a series of SUPs, ranging from supernegatively charged SUPs to superpositively charged SUPs. HC-K30, half charged K sample containing 30 charges. KE16, zwitterionic SUP sample presenting 16 lysines and 16 glutamic acid residues. ELP90, elastin-like polypeptide with 90 GVGVP repeats. HC-E35, half charged E sample with 35 charges. d,e) Adapted with permission.^[20] Copyright 2016, Wiley-VCH.

K36 was deposited on the initial SCF. The hydrated architecture containing K72, after rejuvenation with saliva, showed a very low coefficient of friction, even lower than a single pristine SCF on the model substrate. These experiments suggest an alternative treatment modality for impaired biolubrication resulting in dry mouth syndrome. Instead of replacing negatively charged mucins by external sources, the remaining SCF could be electrostatically stabilized with cationic SUPs that recruit more mucins with excess charges once they are secreted.

Another function of SUPs on surfaces is their capability to prevent ice formation. The ability to tune ice nucleation via external interventions is important in many fields, such as cloud seeding and cryopreservation of cells and tissues.^[89] Among various external auxiliaries, an electric field affects ice nucleation in both naturally occurring situations and in industrial environments. Numerous studies have indicated that a local electric field near charged surfaces can reorient water molecules and thus may affect ice nucleation.^[90] However, it remains a challenge to control ice formation, because the parameters influencing this process are largely unknown. Concurrently, understanding of ice nucleation on differently charged surfaces remains elusive. Yang et al.^[20] reported a method of tuning of ice nucleation through systematic control of both the surface charge and charge density (Figure 6d) via modifying solid surfaces with SUPs. The authors varied the nature of charge and showed that cationic SUPs facilitate ice nucleation, while anionic SUPs suppress the process. Experimentally, a sealed chamber with 100% relative humidity

was prepared and the investigation on ice nucleation was performed by measuring the freezing temperature of condensing water. Ice nucleation occurred on the surface modified with K36 after 54 s at $-19.2 \pm 0.7^{\circ}\text{C}$, whereas for the surface modified with E36 this process was delayed to 1974 s at $-22.8 \pm 0.6^{\circ}\text{C}$. Moreover, ice nucleation could be further modulated by adjusting the charge density (Figure 6e). The authors proposed that the tuning of ice nucleation with SUPs was achieved via a structural change of the interfacial water caused by the local electric field near SUPs, consequently altering the energy barrier of ice nucleation. From a materials view, biodegradable SUPs exhibit excellent biocompatibility and might be used as cryo-protectants in a medical context.

5.3. SUPs Form Responsive Protein Liquids and Liquid Crystals

Besides investigating SUPs on surfaces, their propensity to form bulk materials itself was studied. Solvent-free liquids are an emerging class of materials with attractive prospects. Appealing examples are solvent-free liquids that are characterized by permanent liquid porosity, or increase reaction yields by allowing unprecedentedly high reactant concentrations.^[91] In the context of biomacromolecules, it is particularly challenging to liquefy proteins and polypeptides because of thermal degradation upon heating, because their dimensions exceed the range of intermolecular forces.^[92] Many technologies need to be applied

under extreme, nonphysiological settings, and are therefore incompatible with an aqueous salt-containing phase. Thereby, the investigation of protein functionalities in a water-free environment will expand the scope of SUPs beyond those required by aqueous systems. Liu et al. developed solvent-free liquid crystals exhibiting non-Newtonian behavior and isotropic liquids behaving as Newtonian fluids based on SUPs. To introduce fluidity, anionic SUPs were electrostatically complexed with quaternary ammonium surfactants (**Figure 7a**).^[12] Remarkably, the fluorescence properties of GFP are maintained in the liquid crystalline mesophase when a GFP-SUP fusion was introduced into these water-free systems. Hence, GFP remains folded in this solvent-free environment. By tuning the length of the aliphatic chain of the surfactants, the melting and phase-transition temperatures of SUP-surfactant fluids could be controlled over a broad temperature range (**Figure 7d**). Their high stability, up to a record temperature of 200 °C, could be appealing for technological applications where thermal degradation needs to be overcome. More strikingly, extraordinary elastic materials, with elastic moduli larger than those of existing liquid crystals, were realized for SUP-based smectic phases via the charge-charge mediated self-assembly of SUPs and lipids.^[12] It was found that the smectic layered structure (**Figure 7b,c**) is very important to achieve the elastic behavior, while in the liquid state this property is immediately lost upon the phase transition.

In thermotropic SUP-based liquid crystals, the adjusted temperature affects the order within the materials. However,

not only temperature, but other external cues, like shear force, can cause structural rearrangements within a material and lead to changes of properties.^[93] Shear force-induced disorder–order transitions in soft polymeric materials have been investigated.^[94] But it remained a challenge to stabilize the ordered phases after cessation of the shear force, thus limiting their favorable properties and applications. Zhang et al. developed a biological fluid characterized by an irreversible shear-triggered disorder–order transition. However, this was not achieved with thermotropic SUP-based liquid crystals but with a lyotropic system. The initial mechanical sensitive biopolymeric fluid system was based on SUPs and surfactants that contained, in addition to a quaternary ammonium group and alkyl chains, an aromatic azobenzene unit (AZO) (**Figure 7e**).^[13] The transition from the disordered liquid to the nematic lyotropic liquid crystalline state of the SUP–AZO complex induced via shear was persistently preserved in the absence of applied force (**Figure 7f**). Minor mechanical forces such as the gentle flow of tap water triggered a phase transition of the SUP–AZO liquid, enabling the recording of reliable signals to distinguish flow pressure with patterns of birefringence. Moreover, the SUP–AZO complex enhanced the ink-free transfer of a specific pattern collected from fingerprints into recordable birefringence readouts (**Figure 7g**). Thus, SUPs allow the fabrication of smart assemblies that respond to a diverse set of physical or chemical inputs.

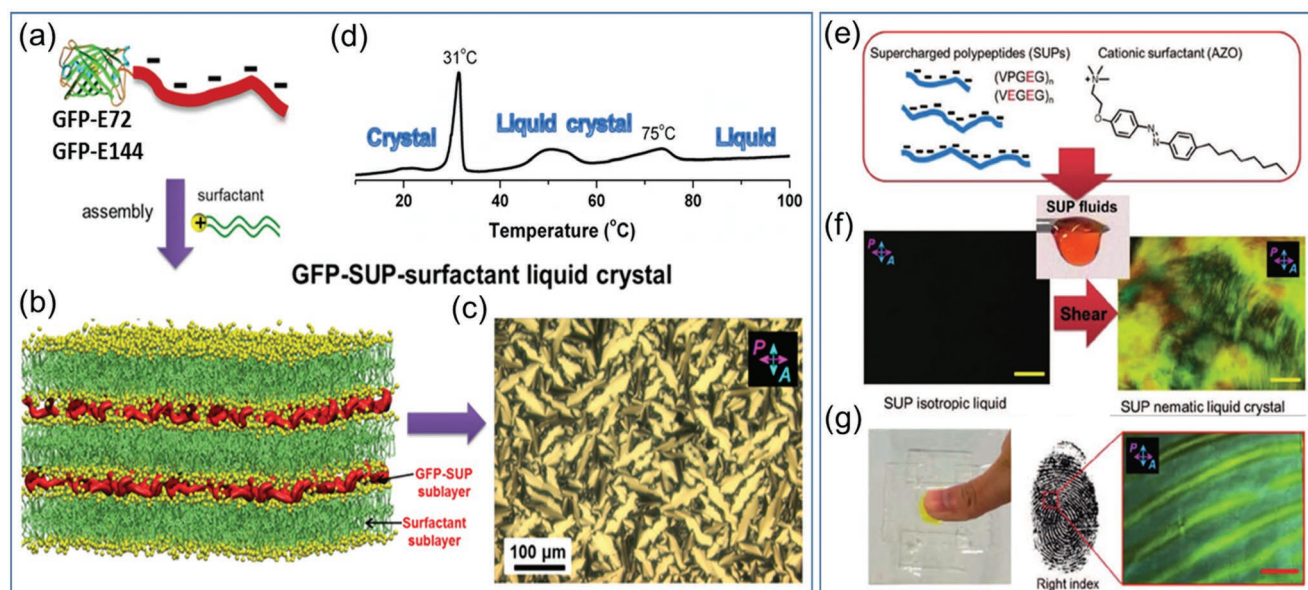


Figure 7. Liquid-crystal materials originating from SUPs and surfactants. a–d) Solvent-free GFP-SUP-surfactant complexes that form thermotropic liquid crystals and e–g) mechanical responsive SUP fluids that transition from an isotropic state to persistent lyotropic liquid crystals upon application of shear. a,b) GFP fused to supercharged polypeptides and pristine SUPs complexed with oppositely charged lipids (e.g., didecyldimethylammonium bromide) result in the formation of a lamellar bilayer structure of the liquid-crystalline phase. Protein layers containing polypeptide chains with random orientation (shown in red) are separated by surfactant double layers (head group shown in yellow and alkyl tails are color coded in green). c) Well-defined focal-conic textures of smectic layers recorded with a polarization optical microscope. d) DSC measurement demonstrating the broad temperature range of the liquid crystalline phase. a–d) Adapted with permission.^[12] Copyright 2015 Wiley-VCH. e) SUP fluid materials are formed by electrostatic complexation of SUPs and AZO surfactants. f) Polarized optical microscopy analysis of the shear-induced isotropic-nematic phase transition of the SUP-AZO sample. The right image with nematic textures was captured after exertion of shear force. Scale bar 100 μm . g) Photograph of a simple device containing the SUP-AZO liquids used for recording fingerprints. The right index finger was applied to trigger the liquid crystal phase of SUP-AZO liquid materials. Scale bar 500 μm . e–g) Adapted with permission.^[13] Copyright 2018, Wiley-VCH.

5.4. Organelle-Like Compartments and Adaptive Coacervates Enabled by SUPs

Synthetic compartments are important for encapsulating different materials and protein capsules prepared from SUPs were mentioned above. Similarly, the spatial separation of biomacromolecules plays an important role in living systems and hence they are omnipresent in cells. One type is membrane-free organelles. They can be liquid, solid, or gel-like and are formed by assembly of multiple enzymes.^[34,95] It is highly desirable to learn more about their dynamics and to exploit their unique properties for applications. In a recent example, the Schiller group was able to perform the synthesis and assembly of amphiphilic unstructured proteins into a phase-separated liquid domain in prokaryotic cells.^[15] The polypeptides contain superanionic (GVGEP)_n repeats connected to a hydrophobic domain consisting of (GVGFP)_n repeat units, which was in turn fused with GFP. By optimizing the ratio between the charges and different segments, a narrow window was established that allowed for the formation of organelle-like structures in the cytoplasm (Figure 8a–d). The orientation of the supercharged head at either the N or C-terminus had an influence on the coacervate droplet formation. These artificial compartments allowed site-selective functionalization by incorporating unnatural para-azido-L-phenylalanine into the supercharged patterns. One may imagine that eventually such systems can be applied for the synthesis of pharmaceuticals directly in diseased cells.

The formation of many coacervates in cells is dynamic through special regulation mechanisms, which allows the cells to respond to their environment. Implementing similar adaptive behavior into artificially self-assembled systems represents a great challenge. Recently, Huck and co-workers established a strategy to control the dynamics of coacervates by including dissipative, fibril-forming FtsZ proteins and GTP as fuel into an SUP-RNA coacervate system (Figure 8e).^[14] The monomeric FtsZ protein can self-assemble and polymerize into filaments upon binding of energy-rich GTP molecules. Subsequent hydrolysis of GTP results in destabilization of these FtsZ filaments. Pronounced partitioning and polymerization of FtsZ within SUP-RNA complexes leads to deformation of spherical coacervates. At high concentrations of FtsZ and GTP the droplets are converted into fibers, which elongate and finally divide (Figure 8f). This dynamic behavior is induced by the different availability of the fuel GTP within the elongated coacervates versus the tips of the elongated coacervate fibers. These conditions allow for creation of biomimetic materials and protocells in which a delicate combination of dissipation, diffusion, and partitioning gives rise to adaptive life-like systems. It needs to be emphasized here that these dissipative assemblies exist far from thermodynamic equilibrium, which is a prerequisite to achieve other adaptive and interactive materials in the future.

5.5. Improving Bioimaging and Enabling Protein Drug Delivery with SUP Fusions

In the coacervates consisting of RNA and GFP-K72 fusions described above, the fluorescence of GFP was exploited to monitor the dynamics of the protein and nucleic acid-rich phase.

Supercharged GFP is an ideal probe to study cell internalization: Supercharged folded and disordered proteins improve cellular uptake.^[96] The supercharged +36GFP shows greater potency than the shorter cationic CPPs, mainly because the +36GFP is less prone to be transferred to the degradative lysosomal compartments than the CPPs.^[53,97] Supercharging a protein, especially with positive charges, is potentially a generic approach to improve cell internalization. Similarly generic is the fusion of a folded cationic supercharged protein to another protein to enhance cell uptake. The same holds true for fusing supercharged unstructured polypeptides to a target protein to achieve high yields of internalization: Pesce et al. reported a new strategy to enhance the cellular uptake of exogenous proteins with SUP tails.^[9] The best uptake was observed with GFP-K72 fusion, containing 72 Lys residues per tail. The fluorescence inside the cell was detected for up to 2 d continuously, which is a vast improvement compared with the typically reported 4 h time window after GFP uptake.^[53,98] This finding indicates that the fluorescent proteins fused with cationic SUPs remain in the cell for a full life cycle of the mammalian cells, i.e., 48 h. Further, the internalized fusion protein is apparently not subjected to degradation. The uptake mechanism likely follows the caveolae-mediated pathway, which can be concluded from the fact that the compound filipin that blocks this pathway strikingly suppressed the internalization of GFP-K72.^[99] This example represents a promising approach for both long-term cell imaging and protein delivery, because significant enhancement of transfection yields is obtained by virtue of uptake via the caveolae pathway that might also allow other cargo proteins to escape intracellular degradation.^[100] Therefore, cationic SUP fusions hold great potential for the development of advanced protein-based therapeutics. Looking at the different cationic systems presented in this review it becomes obvious that the number of charges, the molecular weight, and the folding state are important structural features determining the uptake pathways into mammalian cells. Some short CPPs were found to enter cells via macropinocytosis and folded supercharged proteins including GFP were taken up predominantly by clathrin-dependent endocytosis. These uptake mechanisms are in stark contrast to the internalization of cationic SUP fusions described in this paragraph.

5.6. SUPs Allow Authentication of Different Whiskeys and Amino Acids

In the previous paragraph, GFP served as a fluorescent reporter to visualize cell uptake. Here, the combination of a fluorescent protein with SUPs is utilized in a diagnostic context where the change of fluorescence intensity in response to complex analytes plays a critical role. One such complex analyte is whisky. Scotch (and other whiskeys) is extremely popular, and discriminating different whisky brands is an important yet challenging task. Although a “whisky sensor” based on a dye-replacement assay has been reported,^[101] the most common method to discriminate whiskeys involves mass spectrometric methods as well as quantitative UV-vis or mid-IR-spectroscopy; although these methods have been utilized, they show less than spectacular discriminative power. Recently Han et al. designed two

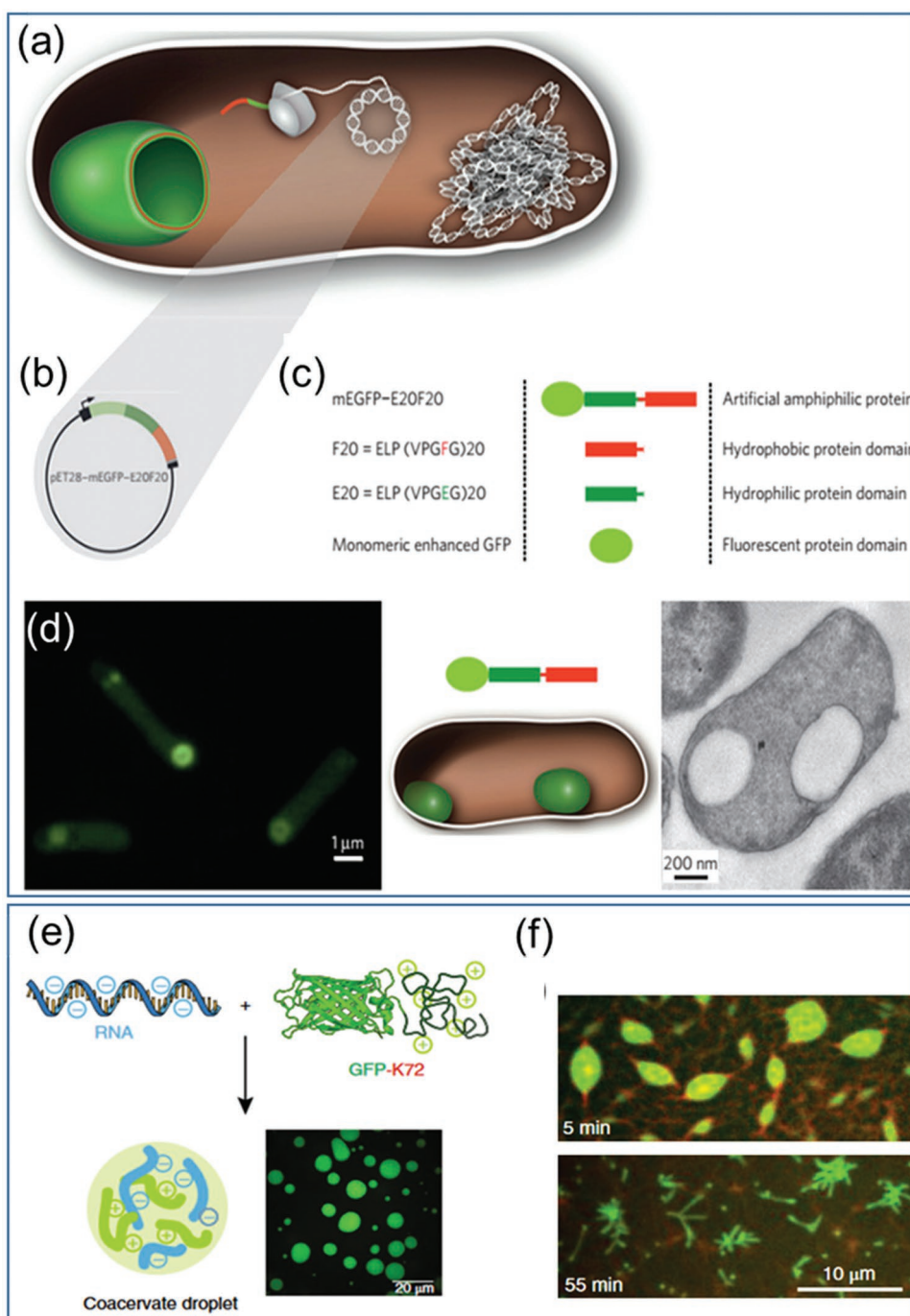


Figure 8. a–d) Overview of the formation of organelle-like compartments in bacteria and e,f) design of dissipative self-assembly system in vitro, both based on coacervates involving supercharged polypeptides. a) Design and expression of engineered proteins with the potential for self-assembly of artificial cellular compartments in *Escherichia coli*. b) The plasmid constructed in this work for expression of the amphiphilic protein. A supercharged region and a hydrophobic part are genetically fused. c) Summary of structural features of proteins yielding the compartment assembly. d) Influence of the order of the domains on the gene and ratio of amphiphilic block domains, characterized by subcellular distribution and higher order structures in the cell. a–d) Adapted with permission.^[13] Copyright 2014, Springer Nature. e) RNA and a cationic supercharged polypeptide fused to GFP (GFP-K72) form coacervate droplets when mixed together. f) Evolution of coacervate droplets of RNA and GFP-K72 (green) combined with a high density of FtsZ bundles when exposed to increasing GTP concentration. e,f) Adapted with permission.^[14] Copyright 2018, Springer Nature.

three-element fluorescence arrays consisting either of GFP-SUP variants or of charged fluorescent poly(p-aryleneethynylene)s.^[102] Different whiskies interact with the sensor array, resulting in

modulated fluorescence intensities of the different elements of the sensor matrix, together forming specific patterns. Small sensor arrays based on such supercharged fluorophore systems

can discriminate many soluble analytes, apparently regardless of its structure, function, or origin. The arrays do not need any sample preparation and can be performed in a standard plate reader on a 96-well plate. One run identifies multiple analytes in a sample, and the data workup is performed by linear discriminate analysis. This hypothesis-free chemical tongue could discriminate more than 30 whiskeys according to their country of origin, brand, blend status, and taste. With the same technology, Wang et al. developed a novel optoelectronic tongue for discrimination of natural amino acids, which might ultimately be used for the identification of peptide hormones as well as degradation products of the human proteome according to their characteristics regarding charge, hydrophobicity, or presence of aromatic units.^[102]

6. Conclusions, Challenges, and Outlook

This review provides insights into the synthesis methodology, properties, and potential applications of supercharged folded and unstructured proteins that are both involved in natural and engineered systems. In nature, supercharged proteins interact specifically with target biomacromolecules and perform functions that are often dictated by the electrostatic interactions. These natural proteins can act as a blueprint or inspiration for genetically or chemically supercharged proteins, to harness the potential of generating structures and functions from electrostatic bonding. Scientists have increased the net charge of proteins by means of chemical modification and recombinant DNA technology resulting in both supercharged folded and unstructured entities. The supercharging of folded proteins endows new properties to the proteins of interest, such as resistance to elevated temperatures, aggregation resistance, catalytic activity, overcoming cell membranes in *in vivo* drug delivery, and others. Moreover, supercharged proteins work as functional motifs when conjugated to other proteins, allowing to make use of the properties of both the supercharged protein and the protein to which it is fused. The self-assembly of folded proteins or their fusions into well-defined nano-objects is sometimes of great importance for the function as exemplified by nucleic acid and protein delivery systems, or the protein capsids containing active enzymes. These assemblies rely on electrostatic interactions and hence introducing charges by means of supercharging into the protein scaffolds needs to follow a careful design process or directed evolution needs to be employed to find an optimized solution. In contrast, extended unfolded supercharged polypeptides can be fused to other functional protein units. In this case, the design effort is much lower. The extended SUPs depart from folded supercharged proteins since higher charge densities can be achieved. Moreover, they can be transferred to bulk materials either in the form of co-crystals or biological liquids and liquid crystals. Important to note is that the liquefaction of SUPs with surfactant molecules resulted in thermotropic liquid crystals and liquids, which are devoid of any water and this process might lead to applications where water is detrimental for function, for example, in the field of electronics.

SUPs can be regarded as genetically engineered polyelectrolytes and their extended chain structures can be compared to

synthetic polyelectrolytes. Concerning the latter class of molecules, SUPs show key differences. First, their structures are much more defined than their chemically produced counterparts are. They are monodisperse, and the positions of charges as well as the number of charges can be adjusted perfectly within the polymer backbone. In chemical synthesis, these parameters are much harder to control than in genetic engineering processes. In the future, it might be possible to identify structure–property relationships in SUP-based polyelectrolyte systems, which is easier than in less defined conventional polyelectrolytes. Related to this, fusions of SUPs and other proteins are a great source of novel functions because their properties are combined, for example, as in the case of GFP-SUP fusions that were exploited for diagnostic purposes. Their fabrication is fairly straightforward because they are produced with the same process as pristine SUPs and do not require additional effort, apart from subcloning the gene in frame with the target protein. In comparison, conjugation of chemically synthesized polyelectrolytes to proteins is much more difficult because an additional reaction step is necessary. Furthermore, SUPs offer advantages over conventional polyelectrolytes when combining them with biological systems. SUPs exclusively consist of biological building blocks and cationic SUPs have lower charge densities. A lower charge density translates into lower toxicity and biodegradability compared to systems based on vinylpolymers. In the same vein, if polyelectrolytes perform functions inside living cells, they can be directly produced in the interior. Directed evolution strategies or selection can be employed to identify desired material properties from large libraries of genetically engineered polyelectrolytes in the future. Therefore, we prognosticate a bright future for this class of materials in important areas. Remarkable functions have already been demonstrated in different fields ranging from catalysis and diagnostics to biomedicine. Further technological areas with other potential applications might be touched upon in the future when some remaining challenges of this class of materials are solved. These are discussed below.

The fabrication of cationic variants with a higher net charge density represents a great challenge due to toxicity if they are produced by recombinant expression. Here, a combination of chemical approaches for supercharging with engineered protein variants might offer a solution. Another structural feature that has not been tackled so far in unstructured polypeptides is incorporation of a charge gradient along the polymer backbone. So far, charged amino acids were equally distributed along the polymer chain. In the future, fabrication of genetically engineered polyelectrolytes with a gradient distribution of charges might be feasible and result in different properties compared to the supercharged system with equidistant charge positions.

Besides expanding the variety of primary structure of supercharged polypeptides, there is plenty of room to expand the self-assembly properties of these materials. So far, mainly electrostatics was exploited for superstructure formation, sometimes in combination with hydrophobic interactions. To enrich the variety of supramolecular assemblies, charge–charge attraction might be amalgamated with other interactions such as cation- π , hydrogen, or π - π bonds. The implementation of additional interactions might even lead to hierarchical structures of supercharged polypeptides, which has not been realized yet. This might be achieved

by incorporation of mutations at single positions within the polypeptide sequence or by combining supercharged elements with other secondary structure forming motifs including helices and β -sheets. Another possibility for enriching the chemical space of supercharged polypeptides is the combination with synthetic molecules that are bound by electrostatic interactions, as exemplified for the surfactants in protein bioliquids. These surfactants were simply composed of a charged head groups and hydrophobic tails. In addition to hydrophobic alkyl and aryl structures, one can think of other chemical functionalities, which might be incorporated to diversify the chemical functionality of the natural amino acid side chains.

Finally, there is a need to broaden the functions of this class of materials. The above-mentioned increase in structural diversity might represent a feasible way to achieve this. The more control over structure is reached, the more functions can be achieved. A good justification for this statement can be found in the field of DNA nanotechnology, where the increasing control over structure formation and the increasing complexity of the structures resulted in broadening of the scope of functions of this class of material. This might be achieved by the suggested incorporation of other secondary structures into SUPs as described above. Another possibility of expanding structural space for additional functions consists of fabricating fusion proteins with supercharged protein structures, which is directly encoded in a functional protein unit as shown in ref. [9]. A good example is the SUP-GFP fusion series that introduce the photoluminescent properties into the resulting materials. The scope of functionalities might be expanded even further by incorporation of biocatalytic moieties or diagnostic entities.

Another future challenge is the engineering of protein materials with respect to their bulk properties. Several powerful methods have been developed to manipulate or improve the properties of single enzymes and proteins. However, stringent improvement or evolution of the bulk material properties of proteins remains a grand challenge. Fabrication of large libraries of single proteins and their subsequent screening is feasible. However, the fabrication of libraries of protein ensembles or even bulk protein structures remains elusive. Therefore, rational design of protein bulk materials and stepwise improvement of their properties by structural redesign will be a future way to achieve new functionalities. It is expected that computational methods for protein design have huge impact on the evolution of this class of materials and first steps in regard to supercharged proteins have been taken. In silico, it is possible to include many proteins in functional assays without synthesizing and processing them into a bulk material, at an interface or on a surface. This might lead to a new generation of protein-based soft matter with interactive properties.

Therefore, we believe that engineering of supercharged polypeptides and proteins will be further fueled by researchers with expertise in diverse disciplines, including chemistry, biology, physics, materials science, pharmacy, and biomedicine.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

adaptive soft matter, electrostatic interactions, functional assembly, supercharged proteins, supercharged unstructured polypeptides

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