Bionanoparticles as functional macromolecular building blocks — A new class of nanomaterials

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Abstract

We would like to introduce bionanoparticles with their unique multifunctional and self-assembling properties. Particularly, protein cages like plant viruses or ferritin but also other well-defined self-assembling protein structural motifs are valuable building blocks with great potential in (bio-)nanotechnology. A steeply increasing number of research works present promising results and applications in biomedicine, diagnostics and analytics as well as nanoelectronics. However, the use of bionanoparticles for hybrid and soft protein—polymer composite materials has not received high attention yet. The article will first introduce the structural principles of well-defined protein complexes and exemplarily describe the structure of a few selected plant viruses and ferritin. Then, the recent progress in chemical or genetically programmed functionalization and the use of the modified bionanoparticles for the production of novel nanostructured (hybrid) materials will be presented. An updated overview of grafting-onto and grafting-from polymerization methods for the modification of proteins and protein complexes will be given as well. The feature closes with some exciting examples in which bio (in-) organic nanoparticles are employed in analytics, for catalysis and biomedical applications.

1. Introduction

Miniaturization towards nanoscale structured materials and devices have become a leading trend in the beginning of this century. The generation of small structures was traditionally a top-down process: The application of masks, illumination and etching steps generated the desired structures on an initially unpatterned material. To generate even smaller structures than currently possible with commercially competitive lithographic methods, bottom-up approaches, i.e. the generation of complex nanoscale patterns which start from nanoscaled building blocks, have been developed. By now, an astonishing multitude of materials, ranging from inorganic to polymeric nanoparticles, biological building blocks and nanostructured thin films with many different electronic, magnetic, optical and (bio-) chemical properties have been synthesized and characterized in great detail. The pivotal point is the directed assembly or self-assembly of these systems into hierarchically ordered and/or arbitrarily defined structures [1]. Such structures can be used in high-resolution soft lithography to produce next-generation nanoelectronic devices [2,3]. The production and use of nanostructured and nanoscaled materials became a key technology in many more fields e.g. pharmacy [4], regenerative medicine [5], diagnostics [6], cosmetics [7] or food technology [8,9].

Progress in nanotechnology is not only aiming at miniaturization but also at systems with increased complexity. This is not just a matter of geometrical structurization but also a matter of specific functionalities that are positioned at discrete locations and in defined distances. Nature and its highly precise mechanisms of life, mainly based on two classes of biomacromolecules, proteins or polypeptides and polynucleic acids, set the benchmark for functional structures down to atomic scales. Thus, the use of biomolecules is considered as an obvious step in the synthesis and construction of next-generation nanomaterials and devices. A whole new branch termed bionanotechnology seeks for scientific as well as economic breakthroughs in the development of bio-inorganic nanomaterials with novel properties for computation and nanotechnology, new methods in diagnosis and analytics or new drugs and drug delivery systems [10,11]. While protein or polynucleic acid scaffolds provide structural and chemical functions, nanoparticles may contribute electronic, luminescent or magnetic
properties to such hybrid assemblies [12]. Ligand systems on nanoparticles can introduce further functionalities for recognition and affinity processes or change the solution properties. Attached polymers change pharmacokinetic properties, mediate solubility or can be used as the bulk material in blends, providing a matrix for the embedded or surface-immobilized (bio-) nanoparticles (Fig. 1).

This feature focuses on protein complexes which can be used as valuable macromolecular building blocks for functional assemblies, as size-constrained reaction vessels, for the construction of bio-(in)organic nanostructured hybrid materials and biocompatible scaffolds for potential applications in bionanotechnology. Self-assembled natural protein complexes, protein cage architectures and particularly ferritin and some plant viruses have been extensively used in the last few years as building blocks and templates in bionanotechnology and bionanotechnology [13–15]. They are robust assemblies and can be obtained from biological sources or through in vitro expression and self-assembly. The combination of protein complexes with inorganic nanoparticles has resulted in materials with a number of interesting properties and applications and received already considerable attention from applied research. With increasing understanding and progress in modification of such systems, multifunctional systems can be constructed. Among the plurality of biological entities which can be used in bionanotechnology, this feature presents mainly examples of Cowpea Mosaic Viruses (CPMV), Tobacco Mosaic Virus (TMV) and ferritin. They have been used as model systems for the development of new compounds and as reference systems to test new (bio-) chemical analytical techniques since decades. Their peculiar properties make them ideal workhorses and they have routinely been used to show a proof of principle. Additionally, Turnip Yellow Mosaic Virus (TYMV) is presented, an icosahedral virus with properties similar to CPMV. This serves to illustrate the applicability of the concepts derived from e.g. CPMV to other similar systems.

We aim to highlight recent developments in the area of defined, self-assembled bionanoparticles, starting with their structural design, methods for modification by chemical and genetic means and finally, their use in the synthesis of novel nanomaterials. Many different disciplines have to contribute to the development of these nanosystems in bioengineering and biomedicine. The section on the recent progress in the synthesis of well-defined functional polymer–protein conjugates aims to inspire new ideas for future contributions of macromolecular chemistry to the construction of materials derived from bio-(in)organic macromolecular building blocks.

2. Complexity and symmetry

In the last two decades groundbreaking developments in biochemistry and microbiology led to tools for the analysis and synthesis of polynucleic acids. Facile cloning and rapid PCR techniques allow the design and high yield production of this class of biopolymers. DNA and RNA nanotechnology have become an established field in science and complex architectures have been obtained by assembling DNA and RNA functionalized materials through primarily sticky-ends of complementary base pair sequences.

While programmed assembly of polynucleic acid strands has become a standard technique in bionanotechnology, the design of self-assembling polypeptide or protein complexes is still a considerable challenge [16,17]. Proteins have — unlike most synthetic polymers — a more or less fixed three-dimensional conformation through covalent bonds or non-covalent interactions. This structural rigidity and the highly functional surface of proteins can drive self-assembly of protein subunits in solution to oligomeric filamentous, cage-like or tubular structures and to regular patterns on various surfaces and interfaces [18]. Symmetry plays an important role in the assembly process [19]. Binding sites and functional groups on such complexes are held at precisely defined positions and orientations. While protein crystals are rare in vivo, many examples of symmetric protein complexes have been found and characterized with the advance of electron microscopy and X-ray crystallography. The helical symmetry in the protein shell of tobacco mosaic virus and the cubic symmetry of spherical viruses were already described in the mid 1950s and the regular geometry and structure of many viruses is known today in great detail [20]. The quasi-equivalent assembly of protein subunits in icosahedral virus capsids is a particularly early recognized and well investigated, yet complex example of protein assembly [21,22].

The de novo design of peptides with predetermined geometric structures requires detailed knowledge of structure-forming principles. Analysis of the genomic pattern of known supramolecular assemblies can help to make predictions for potentially self-assembling proteins [23]. Short oligopeptides and simple tertiary structures like beta-sheet forming amphiphilic peptides can be synthesized by classical stepwise solution synthesis or chosen from combinatorial libraries of structure-forming amino acid sequences. Their manifold assembling capabilities were explored in detail [24,25]. Although peptides which self-assemble to large extended sheets, tubes or fibers are nowadays well-known, novel particulate architectures which were constructed by playing some sort of “peptide Lego” are still rare. Exploiting geometrical principles, a few novel self-assembled protein complexes have been constructed by rational design of peptide building blocks [26,27]. Padilla and co-workers and Matsuura and co-workers created polyhedral cages through programmed self-assembly of designed peptides [18,28]. Cloning techniques allow the expression of fusion peptides in which different block sequences form various structures. Sugimoto reported about the construction of a protein ball-spike supramolecule by fusing the β-helix forming gp5 protein of the T4-bacteriophages to the cage-forming subunits of Listeria-Dps (DNA-binding proteins from starved cells) at the corresponding symmetry axis. The protein self-assembly leads to the formation of a tetrahedral arrangement of the four helical spikes with the Dps cage in the middle [29]. Such proteins can assemble to new architectures for which nature delivers the construction kit. The rational design of the conformation of protein complexes to build novel enzymatic and other biological complexes seems not out of reach (Fig. 2).

Proteins can display a vast variety of functions. Enzymatic and immunospecific reactions etc. are the natural working domain of
proteins. However, peptides can also interact with metal or other inorganic substrates, a domain which only recently came into focus [30–32]. Through modern biomolecular techniques, peptides with non-natural and previously unknown functions can be selected and identified. The display technologies are an elegant example of evolutionary screening principles to identify substrate-specific binding properties [33]. Many different aptamers, protein sequences which show specific affinities, have been identified by this route [34,35]. The approach shows that the use of biomolecules is not restricted to native conditions and natural working fields but can be engineered for their use in hybrid structures and devices, which is an invaluable advantage [36].

3. Bionanoparticles

Meanwhile, a number of different protein cage architectures are known and characterized to great detail. In this section, we will introduce some prominent examples, including icosahedral and rod-like plant viruses and ferritin, which have all been used by many research groups during the last decade. They are commercially available or can be produced by rather simple biological procedures. This section should give the reader who is unfamiliar with the matter a general idea about protein cage architectures.

The robust nature and easy accessibility made cowpea mosaic virus (CPMV) one of the best investigated viruses and most widely used plant virus in bionanotechnology [37]. It is an isometric plant virus with pseudo-\(T = 3\) icosahedral symmetry and a diameter of approximately 30 nm (Fig. 3a) [38]. The crystal structure is refined at 2.8 Å resolution [39] and the bipartite genome is fully sequenced [40]. The 60 small capsid proteins (\(S\), domain A) are arranged as 12 pentamers at the 5-fold axis and the 60 large ones (\(L\), domains B and C) as trimers at the 3-fold axis. The natural host range is restricted to legumes. Infection leads to chlorotic mottling and yellow mosaic pattern. Experimentally, high yields of virus can be obtained from infected leaves of black-eyed pea plants (Vigna unguiculata) after mechanical transmission. Typically 100 g fresh leaves deliver 100–200 mg of virus [41,42].

Turnip yellow mosaic virus (TYMV) is the type species of the genus Tymovirus (family Tymoviridae) [43]. Their capsid exhibits \(T = 3\) icosahedral symmetry with a diameter of approximately 30 nm. The protein shell is made up of 180 chemically identical subunits and is specially stabilized through hydrophobic protein–protein interactions. TYMV particles show distinct and clearly visible surface structures (Fig. 3b) [44]. There are two main sedimenting components, the protein shell with no or little amount of RNA (T-component) and the capsid containing the single infectious virus genome as \((+)^{\text{ssRNA}}\) (B-component). The host range is restricted to dicots. Chinese cabbage (Brassica campestris ssp. chinensis and ssp. pekinensis) is found to be a useful host for propagation from which it can be obtained in large amounts and good yields [45]. TYMV and its crystalline assemblies have been investigated and characterized in great detail by atomic force microscopy with high resolution [46,47].

Tobacco mosaic virus (TMV) was the first virus that was isolated (Stanley 1935, Nobel Prize 1946) [48], and since then, TMV has been connected with many scientific milestones in biochemistry in general and virology in particular [49,50]. The tobacco mosaic virus (tobamovirus; unassigned genus), is a rod-shaped virus with helical symmetry [51]. The rigid tubes of approximately 18 nm \(\times\) 300 nm have a central hollow core of 4 nm. 2100 subunits of a single coat protein enclose the single RNA genome in form of a right-handed helix (Fig. 3c). Distinct contacts between each subunit with three nucleotides lead to an \(in\) \(vivo\) and \(in\) \(vitro\) self-assembly of purified coat protein with viral RNA into infectious particles. As most
filamentous molecular assemblies, TMV does not crystallize. Today, the structure is resolved to 2.9 Å and 5 Å fiber X-ray diffraction [52] and electron microscopy [53,54], respectively. The coat protein alone assembles into small disks and short helix structures depending on pH, temperature and ionic strength as reviewed by Klug [55]. The unique highly anisotropic shape and easy production [41,56] made TMV for many years a model system of choice for many investigations in analytics and surface science, e.g. scattering techniques [57,58].

Ferritin represents a family of proteins with widespread biological importance [59], however, most of the time ferritin from horse spleen (HSF) is used. 24 protein subunits self-assemble to form a cage with an outer diameter of approximately 12 nm and an inner cavity of approximately 8 nm that is filled with hydrated ferric oxide (ferricydrate Fe$_2$O$_3$·nH$_2$O) (Fig. 3d) [60,61]. Ferritin preparations from natural sources possess some heterogeneity, arising from irreversibly aggregated dimers and trimers (polymeric forms) as well as from different iron contents which can vary from a few Fe-atoms to clusters with up to 4500 Fe-atoms. The protein shell is usually heterogeneous, too, and consists of a mixture of two different subunits, termed H for heavy and L for light chain. The ratio of H and L varies between organisms as well as between different tissues within an organism. Each subunit protein forms a four-helix bundle that is arranged in 12 anti-parallel pairs to build a roughly rhombic dodecahedron shape [62,63]. The resulting three-fold channel is hydrophilic in nature and considered as the entrance for cations to form the mineral core while the fourfold channel is hydrophobic in nature. Both have a diameter of 0.3 nm. The formation of the mineral core is a catalytic multi-step process whose mechanism has been investigated in great detail and is quite well understood [61,64,65]. The sequences of different subunit chains are known and have been cloned and expressed in *Escherichia coli* to form recombinants devoid of the natural subunit heterogeneity of ferritin [66,67]. The subunits self-assemble in vitro to a 24-mer shell but only those subunits incorporating the H-chain with the ferroxidase center can be fully reconstituted with an iron core under native conditions [68].

Fig. 3. Examples of bionanoparticles and their images in transmission electron microscopy (virus samples negatively stained with phosphotungstic acid). a) Cowpea mosaic virus (CPMV): Ribbon diagram of the coat protein subunits (BC and A) and domains (A, B and C) and contour surface plot with corresponding colour coding. The axes of the icosahedral pseudo-T3 symmetry are indicated. b) Turnip yellow mosaic virus (TYMV): Ribbon diagram of the identical coat protein subunits and domains (A, B and C) and contour surface plot with corresponding colour coding. The axes of the icosahedral T3 symmetry are indicated. The TEM image shows the distinct surface morphology of the protein capsid. c) Tobacco mosaic virus (TMV): Ribbon diagram of the coat protein subunit and biological molecule. The identical subunits (blue) arrange in a helical manner with distinct contacts around the DNA (red) in the inner cavity. The black line indicates the axis of the rod. The TEM shows a high magnification insert of a lying rod and a perpendicular disc. d) Ferritin: Ribbon diagram of the protein subunit and biological molecule. One of the 24 identical subunits is labelled in red. The symmetry axes of the rhombic dodecahedral arrangement are indicated. The ferritin sample was not stained and only the mineral cores can be seen [20].
building blocks for hierarchically nanostructured materials and for the production of bio-(in)organic nanoparticles [69]. The outer surface of proteins and protein cages has been employed as polyvalent scaffolds similarly to dendrimers or multiple antigenic peptides. Besides site-specific chemical conjugation reactions, biomolecules can be produced under genetic control which allows *a priori* ultimate control of the chemical structure and functionality. Such modified protein materials have been used e.g. as nanoscale building blocks with novel functionality or as vaccines. The interior cavity has been utilized as size-constrained reaction vessel for the production of nanomaterials or for encapsulation of drugs and labels for theragnostic applications in biomedicine. The self-assembling properties can be used for the construction of ordered functional arrays or nanowires. Fig. 4 gives a schematic categorization of fields in which self-assembling proteins and bionanoparticles have been used in the fabrication of hybrid materials.

4.2. Polyvalent scaffolds

Recently, the topological analogy of plant viruses to dendrimers has been exploited in a number of organic reactions (overview [70]; selected examples [71–73]). Many researchers made use of the unique dendrimer-like polyvalent properties of the protein capsid for the synthesis of monodisperse and highly functional bionanoparticles. The close proximity of different labels could be shown by fluorescence resonance energy transfer (FRET) of differently introduced fluorescein and rhodamine dyes [74,75]. The advantage of symmetric protein complexes over dendrimers is that their structure is defined and can be known with atomic resolution. Labels can be introduced either on the assembled bionanoparticle or on the disassembled protein subunits. Care has to be taken then to direct the modifications to sites which are not involved into the self-assembly process and may impede the formation of the bionanoparticle.

A vast literature emerged from the first report about using cowpea mosaic virus particles as addressable nanoscale building blocks [76]. Besides carboxylic groups [77], especially uniquely addressable amino groups from lysine residues have been used in conjugation reactions on the CPMV capsid’s exterior [78]. The number of attached labels can be controlled through the excess amount and the conjugation chemistry. Thus, e.g. FITC reacts exclusively at one lysine amino group on the small subunit (KS38) while up to four lysine amino groups per asymmetric unit can be addressed with *N*-hydroxysuccinimidy1 (NHS)-activated fluorescein if the dye/protein subunit ratio is increased up to 2000. Under less forcing conditions only KS38 is usually labelled. Many different labels, e.g. dyes, polymers, quantum dots or antigens have been introduced on the surface of CPMV using well-established chemical crosslinking procedures which employed either direct activation or bifunctional crosslinking agents. An exhaustive and current overview over the surface reactivity of CPMV is given by Steinmetz et al. [79,80]. Limited stability of the virus in aqueous organic solvents seems to be the only restriction for conjugation reactions (stability for up to 50% DMSO in buffered solution at pH = 3–9 and room temperature for prolonged time or at 60 °C for at least an hour was reported). Functional groups can be introduced on the virus surface by chemical conjugation strategies or through site-directed and insertional mutagenesis in clones. While the first offers larger variability of modifications the latter allows ultimate control of composition and location. As an example, hexahistidine-metal affinities have been introduced by both ways [81,82].

An extremely powerful orthogonal method for (bio-) conjugation reactions is copper-catalyzed azide-alkyne cycloaddition (CuAAC), also descriptively called click-chemistry [83,84]. In a unique and highly selective reaction, azides and alkynes react in a [3 + 2] cycloaddition to form a triazol without interference from other functional groups of biomolecules. The proper choice of the copper catalyst and ligand greatly enhances the performance and applicability of the reaction to various substrates and scaffolds [71,85]. Installing the necessary azide or alkyne groups on the biological scaffold can be done by conventional strategies, e.g. NHS-activated esters or by genetic engineering with non-canonical amino acids (see Fig. 18, 20). Click-chemistry has proven as the currently most efficient coupling strategy which allows single-site conjugation reactions also with large macromolecules. For example, Sen Gupta et al. managed to decorate CPMV with up to 125 alkyne-terminal side-chain glycopolymers with an average molecular weight of 13 kg/mol [86]. Previously, the attachment of e.g. PEG was limited to short polymer lengths and low labelling ratios. Under forcing conditions, up to 70 NHS-activated PEG-2000 but no more than 29 PEG-5000 could be attached per virus particle. The glycopolymer was additionally end-functionalized with a fluorescent dye which allowed convenient estimation of the number of bound polymer chains after separation from excess polymer. Another convenient feature of the click-reaction is the formation of fluorescent moieties upon formation of the triazol from non-fluorescent dyes or from different kinds of scaffolds without further labelling or chemical quantification reactions [88,89].

Recently, also other plant viruses were employed as a polyvalent scaffold, particularly turnip yellow mosaic virus (TYMV), an icosahedral virus with a diameter of approximately 30 nm and a shell composed of 180 identical subunits. Reactions with dyes under NHS/EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) hydrochloride-activation were used to address lysine amino groups and carboxylic groups [90]. The close proximity of orthogonal addressable amino and carboxylic groups was shown by dual labelling with terbium complexes as donor and Alexa-488 fluorophores as acceptor by time-resolved fluorimuno assay (Fig. 5) [91]. TYMV worked as a prototype protein scaffold for sensor development because the fluorophore was anchored via specific ligand–receptor (biotin–avidin) binding.
Few organic conjugation reactions have been reported for TMV because the commonly functional groups targeted for bioconjugation are not available on the exterior protein surface of TMV. Endo and coworkers used a genetically engineered TMV coat protein for the conjugation of porphyrin-maleimide. Self-assembly of the modified coat proteins to discs and rods leads to the formation of light-harvesting arrays [92]. The same strategy was followed by Miller et al. who installed different fluorescent chromophores on the coat protein to achieve broad spectrum light collection with high efficiency [93]. The work of Schlick et al. significantly broadened the range of modifications [94]. They introduced the modification of the tyrosine-139 residue using diazonium salts; the robust protein assembly allowed also harsh conditions. Click-chemistry was used successfully for modification of the virus as well [95].

Modification of ferritin is of special interest because it enables to change the surface-assembling properties of the particles. Ferritin with its unique cavity is able to house many different inorganic materials and offers thus the chance to produce functional assemblies from the hybrid nanoparticles with interesting electronic, magnetic and optical properties (see below). Ferritin particles with reversed overall charge (‘cationic ferritin’) [96] and hydrophobic solution properties (‘hydrophobic ferritin’) [97,98] were prepared. Conjugating PEG-chains to the protein capsid produced ferritin that could be stably dissolved in organic solvents [99]. Zheng et al. functionalized ferritin with a number of initiating sites for ATRP polymerization and subsequently grafted oligo ethylene glycol methacrylate-polymer chains from the ferritin protein shell [73]. This conjugate was also shown to be soluble in dichloromethane. In all these works, surface exposed carboxylic groups were addressed through carbodiimide-mediated amide formation.

A major advantage of proteins is their ultimate precision regarding composition (primary structure) as well as conformation (secondary — structure). An elegant way to alter the amino acid sequence and chemical structure is genetic engineering. Thus, the expression of coat proteins and in vitro self-assembly of the subunits allow the synthesis of non-natural virus capsids (virus-like particles, VLP). Substitution with non-natural amino acids provides protein complexes with novel desired functionalities at locations with ultimate precision. Finn and co-workers demonstrated the synthesis of virus-like particles derived from hepatitis B virus (HBV) and bacteriophage Qβ which contained azidohomoalanine and homopropargyl glycine [100]. The VLPs could be labelled post-translationally with dyes, linkers, metal complexes or proteins through CuAAC (see Fig. 17). Ferritin chains were cloned and expressed in E. coli and self-assembled readily in vitro to intact cages [67]. The introduction of specific binding sequences is of particular interest in bionanotechnology. Combinatorial phage display technology is a valuable tool to identify and isolate binding motifs on the coat protein of bacteriophages [33]. Libraries of random peptides as part of the phage coat protein are screened in a few rounds of selection for binding affinity to different inorganic materials [30,101]. Identified peptide sequences can be incorporated into the E. coli expression vector for the ferritin subunit. Kramer et al. fused a sequence to the C-terminus of the coat protein which binds specifically to silver nanoparticles and directed thus the formation of silver nanoparticles to the interior of the ferritin cage (Fig. 6) [102]. Antigen or other sequences which are fused to the N-terminus of the subunit will be displayed on the exterior surface and yielded functional particles for cell targeting, diagnostic purposes or affinity for inorganic materials [103,104]. Both insertions do not hamper the self-assembly properties of the subunits to intact particles.

The presented plant viruses and ferritin have an intrinsic symmetry and thus the chemical functionalities are distributed over the particles’ surface in a regular manner. Chemical means have to be employed to break the symmetry. Young, Douglas and co-workers used a solid-phase synthetic approach [105,106]. LiDps, the DNA-binding protein from Listeria innocua, consists of 12 subunits that self-assemble into a hollow protein cage with an outer diameter of 9 nm, was first bound to solid-phase support particles via disulfide linkages. Three out of 12 of the functional groups on the outside of the protein cage remain unoccupied and can be labelled. Cleaving LiDps from the support left the other nine free cysteine groups for reaction with a second label. Unsymmetrically functionalized particles tend to much less crosslinking phenomena than Janus-like particles. The in vitro self-assembly properties of bionanoparticle subunits allow also another approach, namely the mixed self-assembly of differently functionalized subunits. This has been shown e.g. for the Cowpea chlorotic mottle virus or ferritin [107,108]. Labels can also impede the self-assembly properties. Mueller et al. show a nice example for differently modified TMV coat proteins [109].
4.3. Protein cages as templates for constrained nanomaterials synthesis

When in the beginning of the 1990s research was focusing on the preparation of well-defined nanoparticles, Mann and Meldrum were the first who made use of the cage structure of the iron-storage protein ferritin as size-constrained reaction vessel [110,111]. The interest went well beyond understanding biominalization but was looking to use bio-nanoreactors for the production and encapsulation of non-native technologically relevant inorganic materials.

Ferritin became the prototype of a bio-inorganic composite nanoparticle [15]. Since then, numerous different inorganic cores have been synthesized inside the capsid, e.g. semiconductor nanoparticles of CdS [112], CdSe [113], ZnSe [114] and PbS [115] or metallic nanoparticles of Pd [116] and Ag [117] and many more but to name a few (Fig. 8). Various fields of applications have been demonstrated for these bio-inorganic particles in parallel [118,119]. Two approaches have been mainly followed for the templated nanoparticle synthesis: Either reassembly of the protein cage from the subunits in presence of the material which should be encapsulated or incubation of the empty and intact apoferritin with the respective metal ions, followed by conditions inducing slowly the mineral precipitation (Fig. 7). The accessibility of the interior is restricted by the size of the hydrophilic channels in the protein cage. The directed mineralization inside the capsid is prerequisite for the controlled synthesis of nanoscale materials without non-specific bulk precipitation. In vivo, the formation of the ferrihydrite core is enzymatically controlled through the ferroxidase site at the interior protein surface. For non-ferroxidase substrates or for mineralization in virus capsids which is not assisted by specific proteins, the spatial selectivity is controlled by charged nucleation sites at the protein interface. Previously unknown ferritins from life-forms in extreme conditions, e.g. the hyperthermophilic Pyrococcus furiosus, allow expanding the synthetic conditions for preparation of hybrid materials e.g. to high temperatures [120]. An expanded temperature range can be of importance when nanoparticles with different crystalline phases and e.g. better magnetic properties are desired.

Only the maximum loading of the protein cage gives a good uniform size distribution of the formed nanoparticles and thus, the interior dimensions of the protein cage dictate the final size of the core. Other ferritin cages with smaller cavity sizes have to be chosen for the bio-templated preparation of smaller inorganic nanoparticles. For example, the 5 nm cavity of the dodecameric bacterioferritin from L. innocua shares structural features with ferroxidase sites [121]. Cobalt oxide nanoparticles or ultrasmall platinum clusters were successfully prepared [122,123].

The approach was also expanded to plant viruses and many other protein cage systems, e.g. heat shock proteins [118,124,125].

4.4. Bionanoparticles as scaffolds and templates for materials synthesis

Viruses can serve as building blocks or can be used themselves as scaffolds for the fabrication of novel bio-(in-)organic nanoscale and nanostructured materials [124]. Of particular interest are one-dimensional conducting nanostructures that can be used to build nanoelectronic devices. Phospholipid tubes or microtubules are classical templates for filamental or rod-like structures [126]. However, both suffer from limited stability because they are non-covalent assemblies and sensitive to solution conditions. The highly functional, negatively charged DNA has been routinely utilized for decoration with nanoparticles or deposition of conducting metal layers [127]. Polypeptides which form preferably highly stable structures like β-sheets can also form considerably stable fibers which provide a robust and highly functional template for metalization [128,129].

The M13 bacteriophage has a filamentous shape with a length of over 800 nm. The coat proteins are very amenable to changes via genetic engineering. Particularly, the easy genetic manipulation enabled the development of the above mentioned phage display technology. With this technology, specific binding sequences of inserted peptide libraries on the virus protein coat can be identified. Thus, it was an evident step to engineer phages and produce bio-inorganic hybrid assemblies. Insertion and expression of specific binding sequences can be achieved either for the coat protein only at the ends or along the whole virus capsid [130,131]. Annealing of the phages with ZnS or CdS nanocrystals aligned along the virus yielded long single crystalline semiconductor nanowires [132]. The two-dimensional assembly of the phages on electrostatically cohesive films of polyelectrolytes on macroscopic length scales represents an interesting approach for novel nanostructured and functionalized polymer surfaces for sensor or battery applications [131,133].

The rod-like tobacco mosaic viruses (TMV) have the additional advantage of being rigid and shape persistent. Thus, TMV is an attractive template and utilized in wild-type or genetically engineered form in a number of approaches to synthesize nanowires...
Improved synthetic protocols enable the synthesis of virus rods which were homogeneously and densely coated with e.g. gold nanoparticles or platinum clusters [135,136]. However, the conductivity of such rods is poor because the single nanoparticles are electrically isolated. Royston et al. introduced a procedure in which they stabilized the TMV template with a thin silica shell before metal deposition. Polyaniline-coated TMV was sufficiently stable to withstand harsh Stöber conditions. TMV-silica composite rods were then successfully coated in silver, gold, palladium or platinum salt solution upon reduction with dimethylaminoborane [137]. In another similar approach, conducting poly(styrenesulfonate)-doped TMV-poly(aniline) fibers were coated with in situ gel process with titania [138]. Such fibers could find application in nano-sensor devices because they combine inherent conductivity with a nanoscale catalytic surface. TMV with an additional inserted cysteine in the coat protein could readily be metalized with nickel or cobalt through an electrodeless plating procedure [139]. Interestingly, if the reaction were done in the presence of gold-coated silicon substrates, the virus would have ordered perpendicular to the surface. Such assemblies had a very high surface area and could be suitable for the fabrication of microbatteries [140,141].

TMV was also used as template for the preparation of organic conducting nanofibers (Fig. 9). In a typical synthesis, aniline was oxidatively polymerized at room temperature in aqueous solutions of TMV [142–145]. At near neutral pH-values, long fibers with few ten micrometers in length and 21 nm in diameter (measured by TEM) were formed through the head-to-tail self-assembly capabilities of TMV. The assembly process was further enhanced by the surface polymerization of aniline. The electrostatic interaction of aniline/polyaniline with the negatively charged coat protein surface restricted the polymerization reaction exclusively to the virus surface. Bruckman et al. investigated the formation of fibers after functionalization of TMV [143]. The uncharged coating prevented interaction with aniline and no surface polymerization was observed. However, introducing sulphate groups on the tyrosine moieties of the subunits increased interaction and prevented the formation of long fibers because head-to-tail assembly was hindered. In contrast, ammonium thiophene could be polymerized successfully in presence of unmodified TMV at near neutral pH, similarly like aniline. The reaction pH influenced the morphology of the final product as well: Only single tobacco mosaic viruses could be observed at low pH < 2.5. At intermediate acidic pH ~ 4, initially formed single composite fibers tended to aggregate during the course of the reaction to form fiber bundles. Long and well dispersed single fibers were obtained only at near neutral pH [145]. The non-conducting fibers of branched polyaniline which formed at near neutral pH could be converted to conducting and well water-soluble fibers when aniline is polymerized in a second step at low pH in presence of polystyrenesulfonate [142].

4.5. Surface patterning with self-assembled bionanoparticles

4.5.1. Decoration of solid surfaces with bionanoparticles

The monodisperse nature of proteins and bionanoparticles makes them readily crystallizing in regular patterns on surfaces [146,147]. The adsorption and manipulation of two-dimensional arrays of ferritin on various surfaces and interfaces has been investigated intensively and in great detail [148–150]. Bacterial surface layers (termed S-layers) are a prominent example of ordered two-dimensional arrays of proteins at interfaces [151,152]. The protein sheets exhibit almost pure crystalline symmetry and are oriented with a different top and bottom side.

Fig. 8. Ferritin templated synthesis of nanoparticles. a) TEM image of CdSe nanoparticles formed in apoferritin. The insert shows a high-resolution image of a single CdSe nanodot. b) Size distribution of CdSe cores obtained from TEM. c) A gallery of various semiconductor/ferritin bionanodots. Dps from Listeria yields smaller particles. All images were stained with aurothioglucose [118]. Reproduced by permission of The Royal Chemical Society.
The formation of regular 2D-arrays of viruses or ferritin on liquid interfaces depends critically on solution conditions and the presence of e.g. cadmium ions or adsorptive layers, either formed through initially spread proteins, charged polypeptides or surfactants. After pick-up on a hydrophobically coated silicon wafer, 2D-crystalline ferritin arrays could be thermally treated to remove the protein shell and fix the iron-oxide core on the substrate [149]. The possibility to fill the ferritin cavity with different inorganic materials makes it a versatile platform for nanoparticle arrays. Although the formation of 2D-crystalline arrays is experimentally still a delicate task, Yoshimura and co-workers have managed to produce excellent arrays of ferritin and Dps loaded with inorganic minerals over large areas (Fig. 10) [153,154]. Unfortunately, the experimental difficulty of this procedure makes the fabrication of arrays by this method unsuitable for robust device production. The surface crystal growth from a three-phase contact line has been proposed by Ikezoe et al. as an appealing simple method to prepare very large scale, well ordered and dense two-dimensional assemblies [155]. Selective nanoscale positioning of ferritin was realized by target-specific peptides that had been fused to the surface exposed N-terminus. Such modified ferritins bind with good selectivity to the respective inorganic surface and thus allow the formation of nanodot-arrays on arbitrarily lithographically pre-structured surfaces [156]. A printing method based on lithography controlled dewetting has been used to produce single lines of assembled ferritins [157]. Strong covalent binding yields dense but usually irregular assemblies. For example, gold surfaces were activated towards amide bond formation by a self-assembled layer of disulphides, carrying a succinimidyl moiety. Ferritin formed irregular assemblies with occasional formation of aggregates on such surfaces [158]. The same was observed when ferritin was thiolated with 2-oximinothiolane hydrochloride and covalently immobilized on gold surfaces [159]. Electrostatic adsorption of ferritin can be achieved with appropriately chosen surface potential and ferritin particle charge. The electrostatic interaction potential can be adjusted through the buffer strength, thus varying the Debye length, i.e. the distance over which electrostatic interactions are greatly attenuated. Calculation of the spatial distribution of the total interaction free energy was performed for regular 15 nm disc pattern of 3-aminopropyltriethoxysilane (APTES) on a silicon wafer [160]. Single ferritin particles could be deposited on the APTES islands under optimized electrostatic conditions. Such selectively deposited nanodots can be part of a more sophisticated nano-electronic system. For example, the mineral cores can work as catalyst for carbon nanotube growth [161]. For applications in nanoelectronics where irregular quantum dot arrays are sufficient, simple droplet evaporation methods, convective assembly or spin-coating on polyelectrolyte layers can be done. They yield dense arrays without crystalline order [162–164].

![Fig. 9. Synthesis and TEM images of a–c) short PSS/PANI/TMV rods (pH = 5 non-conducting; pH = 4 conducting) and of d–e) long fibers PANi/TMV (non-conducting) and PSS/PANI/TMV (conducting). f) UV–Vis spectra of native TMV and the polymer/TMV composite rods and fibers. The three absorption peaks at ~420, 320 and 825 nm indicate the formation of conducting emeraldine salt form of PANi. Adapted in part with permission from Ref. [142]. Copyright 2007 American Chemical Society.](image)

![Fig. 10. Unstained TEM image of ferritin array on carbon film. The ferritin array was transferred from the water–air interface to a holey carbon grid and reinforced by carbon coating. Reprinted with permission from Ref. [154]. Copyright 2006 American Chemical Society.](image)
Functional nanoarrays of adsorbed plant viruses have been achieved through electrostatic adsorption [165], covalent immobilization [166–168] or specific binding. Covalent immobilization yields usually irregular multilayers due to strong and irreversible virus-substrate and interviral interactions [166]. The formation of such assemblies thus depends on virus concentration, solution conditions and additives like PEG [167]. Chemo-selective binding to line patterns on a substrate was achieved by nickel-mediated interaction between the nitrilotriacetic acid-functionalized substrate and the genetically modified CPMV to present a hexahistidine tag on its capsid [169]. Soft polymer surfaces led to denser and more regular patterns than hard surfaces.

Virus layers with their inherent immunological properties and multifunctional surface were considered as building blocks for the construction of diagnostic arrays. For example, Steinmetz et al. investigated the formation of CPMV-multilayer arrays on solid supports via biospecific recognition. Biotin-functionalized CPMV was dual labelled with fluorescent dyes and immobilized on streptavidin-functionalized surfaces. The layer formation was monitored by quartz crystal microbalance or fluorescence microscopy [170,171]. The adsorption of non-modified plant viruses and proteins on surfaces in aqueous buffered solution is mainly governed by electrostatic interactions. The formation of virus layers depends therefore on the isoelectric point (pl) and the surface charge of the particle. At solution conditions below pl, viruses should adsorb strongly to negatively charged surfaces and weakly on positively charged surfaces; for solution conditions above pl the reverse should hold true. The largest amount of virus can be deposited at the pl itself because there is the balance between the opposing trends of particle–substrate attraction and particle–particle-repulsion [172]. However, particle charge is not the only factor which governs the layer-by-layer assembly of virus capsids and polyelectrolytes. Steinmetz et al. observed different architectures for spherical and rod-like viruses in multilayer assemblies. While icosahedral CPMV was readily incorporated into multilayers in the expected alternating manner, rod-like TMV was floating atop of the polyelectrolyte surface with spontaneous ordering [173]. A similar effect was also observed earlier for filamental bacteriophages [133].

The formation of one-dimensional structures is of special interest for the construction of nanowires and other structures in nanoelectronics. Thus, the controlled deposition and alignment of conductive wires from TMV is a topic of current investigations. For example, Velev and co-workers reported the formation of conducting fibers from flow-aligned tobacco mosaic viruses and covalently attached gold nanoparticles [174]. Silver enhancement was applied to finally achieve a continuous conducting metal layer. Micrometers long, end-to-end assembled TMV with single-virus width were printed on adhering surfaces from structured hydrophilic PDMS stamps and would form viable templates for the preparation of virus nanowires after metallization [175]. Complex patterns on a single-particle level were generated on carboxylic acid functionalized surfaces by metal-ion mediated selective adsorption. AFM-based dip-pen lithography was used to generate the chemical pattern for the precise and oriented deposition of single TMV [176]. Previously, the first report on TMV assemblies perpendicular to the surface was published [139]. A TMV mutant was created by insertion of an additional cysteine at the amino terminus of the coat protein. Since this cysteine is only sufficiently exposed at the end of the virus which contains the 3′ end of the genome sequence, preferred vertical arrangement of the virus rods was observed on gold surfaces.

4.5.2. Adsorption of bionanoparticles to liquid interfaces and soft polymeric surfaces

Liquid–liquid interfaces have been proposed to be “the emerging horizon for self-assembly of nanoparticles” [177]. Solid-stabilized emulsions, known as Pickering Emulsions, and protein-stabilized emulsions are of considerable technological importance and have been investigated for decades. However, the field experienced a renaissance after stimulating reports from the group of Russell and co-workers [178,179]. Soon it was assumed that bionanoparticle stabilized emulsions could have interesting applications in drug delivery or as functional surfaces and interfaces. In a pioneering report, Russell et al. described the preparation of an emulsion of perfluorodecalin droplets in water which were stabilized by a monolayer of CPMV viruses (Fig. 11) [180]. Detailed investigations of the bionanoparticle covered interface showed that the virus cage is not des-integrating upon their adsorption. Similarly, TMV and TYMV were employed and the formation of the interfacial assembly kinetically and structurally investigated [181,182]. Particularly, the anisotropic rod-like TMV assembled either parallel or perpendicular to the surface, depending on the solution ionic strength or TMV concentration [181]. While modeling of respective surface tension curves indicated that “normal” proteins like BSA are – at least partially – unfolded at the interface, direct experimental results are difficult to obtain and sometimes contradictory. Bionanoparticles with their more distinct shape and robust structure are better amenable to microscopic investigations. Scanning force microscopy (SFM) and electron microscopy techniques were applied to image the bionanoparticles assembly at the interface of an liquid polymer precursor (poly(dimethyl siloxane), PDMS) which could be crosslinked by UV-light. By this trick, the equilibrium assemblies of bionanoparticles could be preserved and investigated in detail. In Fig. 11 it can be seen that the particles form a dense, although not regular assembly at the interface. Moreover, TMV was not forming liquid-crystalline arrays with parallel rods. The emulsions could be washed to remove excess nanoparticles.
and the droplets still did not coalesce. Thus, similarly to proteins, the bionanoparticles are irreversibly trapped at the interface.

A detailed investigation of the TMV adsorbed at the interface indicated that the rod was forming strongly curved bends when crossing each other (Fig. 11). This indicates the strong attraction to the interface but also a probable softening of the viral nucleoprotein by surface denaturing upon contact with the oil phase. Such thin biopolymer monolayer arrays can retain its function. For example, Zhu and Wang showed that a conjugate of β-galactosidase with polystyrene which was able to self-assemble at the interface had an increased stability and was active in the enzymatic interfacial transformation of lactose to octyl galactoside [183]. Further, the protein covered interface forms a viable template for biomimetalization. Jutz and Böker used bovine serum albumin (BSA) and ferritin to stabilize oil-in-water emulsions. After exchange of the water phase with aqueous calcium phosphate solutions, slow mineralization. Jutz and Böker used bovine serum albumin (BSA) and ferritin to stabilize oil-in-water emulsions. After exchange of the water phase with aqueous calcium phosphate solutions, slow and steady growth of mineral shells was observed [184].

Bionanoparticles were also used to stabilize polymer particles by assembling at the surface. When poly(4-vinyl pyridine) (P4VP) in dimethyl formamide was co-dissolved in an aqueous solution of CPMV, the bionanoparticles enabled solubilisation of the fairly hydrophobic polymer at pH > 5. Well-defined, sub-micron polymer spheres were produced upon slow dialysis against water. The polymer was becoming less soluble but the virus was assembling at the interface and kept it from phase separation and precipitation. Upon equilibration, a regular and dense assembly of CPMV on the polymer colloid surface was obtained finally, much of the imaging of a raspberry (Fig. 12) [185]. It was proposed that the final product was thermodynamically controlled and a simple geometrical model of the virus size and a hexagonal pattern could explain the decreasing colloid size with increasing virus mass to polymer mass-ratio [186]. The approach worked as well for TYMV, ferritin and rod-like viruses like TMV and bacteriophage [185–187]. The concept can be considered as an approach to produce responsive drug carriers. The polymer—bionanoparticle colloids can be redissolved at pH < 4, releasing the surface trapped proteins and therapeutic or diagnostic species which are conjugated or entrapped in the polymeric core.

Materials and surfaces with immobilized or “imprinted” proteins and bionanoparticles can work as viable scaffolds for chemical selective separations, biomimetic sensors and the development of biomedical devices and materials, e.g. artificial antibodies [188,189]. Molecular imprinting of bulk materials with large biopolymers has faced limitations because removal of the templating molecules is as difficult as the later access of the large analyte molecules. Recent developments in imprinting surfaces with protein-recognition sites have solved this problem [190,191]. Suitable protective layers, e.g. disaccharides and fluoropolymers, mediate the bionanoparticles’ chemical and structural information to the interface and transfer the complementary chemical functionalities and shape to the matrix.

Bionanoparticles can usually not directly be mixed with polymers due to solvent incompatibility. Many polymers are only soluble in organic solvents and strong polar organic solvents usually denature proteins if not used in aqueous mixtures with a low solvent-to-water ratio. Thus, the first step to prepare protein or bionanoparticle—polymer blends is to find a common solvent or to modify the bionanoparticles appropriately. Alkylated [97,98] or PEGylated ferritin [73,99] shows good solubility in dichloromethane. TEM confirmed that the protein cage did not disassemble. Such modified ferritin could be dissolved in polymer blends. After the initial stimulating report by Russell and co-workers only marginal progress has been achieved. Russell showed that the presence of nanoparticles changes the morphology of micro-phase-separated block copolymers. For example, lamella-forming poly(2-vinylpyridine)-b-poly (ethylene oxide) block copolymers, poly(PEG methacrylate) modified ferritin was directed to the cylinder phase because of the solubility [193]. Wild-type and modified ferritin were also used for phase-selective adsorption in phase-separated polymer blends [194]. Due to its multifunctional surface, the ferritin protein cage can interact with polymer chains and efficiently mediate load bearings to the matrix. For example, fibers from water-soluble poly(vinyl alcohol) which have been electrosyn from polymer–ferritin mixtures had markedly increased toughness [Fig. 13] [195]. This is also seen in an absolute absence of creep during pH-triggered actuation of the nanofibrous composite hydrogels [196]. The composite hydrogels are stable in a wide range of pH-values and applicable to magnetic resonance imaging-based detectable cell culture scaffolds due to the superparamagnetic iron-oxide core [197]. Acid-treated carbon nanotubes which were functionalized with ferritin were readily dispersed in PVA matrices and the elastic modulus increased dramatically compared to composites prepared with pristine carbon nanotubes [198].

4.6. Synthesis of polymer–bionanoparticle conjugates

Evidently, bio-(inorganic) nanoparticles exhibit interesting properties which could add valuable functions and functionalities to various other materials, surfaces and interfaces or function as therapeutic or diagnostic agents by themselves. Biomacromolecules can be covalently linked to polymers, used in blends or immobilized on functional polymeric surfaces. The covalent conjugation to polymers and (polymeric) surfaces is particularly important modification processes to prepare proteins and protein complexes for use in applications, as in biomedicine or as molecular sensors in diagnostic assays. We will focus here primarily on the preparation of well-defined site-specific conjugation reactions. Polymer–protein hybrid materials are still a niche although they gained steeply increasing interest in the last years. Undoubtedly, this is connected with excellent progress in the preparation of well-defined functional polymers via controlled radical polymerization techniques. Until recently, mostly water-soluble poly(ethylene glycol) (PEG) were conjugated to proteins. A portfolio of suitably end-functionalized and activated PEGs of different chain lengths is commercially available [199]. This is attributed to the change in pharmaceutical
new exciting results have been reported, particularly on grafting-from approaches under very mild conditions. Figs. 14 and 16 give a schematic overview over polymerization reactions and functional versatility. The table is not meant to be exhaustive but limited to the most recently reported progress.

End-functional polymers for grafting-onto can be synthesized by starting with a suitable functional initiator (optionally protected) or by modification of end groups after polymerization (Figs. 14 and 16). For example, Haddleton and co-workers used a 2-bromoisobutyryl ester with a protecting acetal as initiator for ATRP (Fig. 14). After polymerization of poly(ethylene glycol) methacrylate (PEGMA) and deprotection under acidic conditions, aldehyde end-functional polymers were obtained [211]. Samanta et al. synthesized unprotected benzaldehyde-functional ATRP-initiators for the preparation of poly(methacycloxyethyl phosphorylcholine) (PMPC), a hydrophilic and biocompatible zwitterionic polymer [212]. Aldehydes are suitable for conjugation to protein-amine groups via imine-formation. A second reduction step is necessary to yield stable secondary amines. N-hydroxysuccinimidyl (NHS) activated esters are most popular for conjugation reactions to amino groups. However, side reactions and decreased stability in neutral and alkaline aqueous solution constitute major drawbacks and result in low conjugation ratios for difficult reactive groups with few accessible amino groups on long polymer chains. N-hydroxysuccinimidyl esters of 2-bromo-2-methylpropionate or 2-bromoisobutyrate were used to polymerize PEG methacrylate or MPC [212–214]. No conjugation or only low ratios could be achieved for conjugation of MPC with lysozyme or interferon-α2a. Click-chemistry was considered as a suitable alternative because of the high stability of the involved functional groups, their strict bio-orthogonality and exceptionally high efficiency (see as an example Fig. 15). In the most simple case, the azide group can be introduced through azide-bromine exchange after polymerization with any conventional ATRP-initiator [215]. To eliminate this step, Shi et al. used an azide-functional 2-bromoiso- butyryl ester for the polymerization of tert-butyl methacrylate. The polymer was conjugated to alkyne-functional biotin or via a dialkyl linker to azide-functionalized BSA. Side-chain modifications with glucosamine and a near infrared fluorescence dye produced multifunctional polymer-protein conjugates [216]. Glycopolymers with an azide endgroup were prepared by Sen Gupta et al. and conjugated to CPMV (Fig. 15) [86].

Native free cysteines which are not involved in structure-stabilizing disulfide bridges are less abundant than amines and represent therefore very interesting targeting sites. Popular coupling strategies make use of Michael-addition of the thiol group to maleimides or the formation of disulfides. Bontempo and Maynard prepared a pyridyl disulfide-functionalized bromoisobutyryl ester as initiator for the controlled polymerization of hydroxyethyl methacrylate [217]. Around 90% of the polymers contained the disulfide endgroup which were directly usable for conjugation to BSA. The non-covalent, yet very stable avidin–biotin interaction was also employed to prepare biocongjugates. Biotin-end-functional poly(N-isopropyl acrylamide) (PNIPAm) was synthesized directly with a biotinylated ATRP-initiator by Heredia et al. [218]. Ferritin which was covalently functionalized with thermoresponsive PNIPAm showed reversible aggregation to raspberry like colloids at temperatures above the cloud point at 30 °C [219].

The RAFT technique also presents possibilities for a convergent strategy (Fig. 16). Pound et al. reported that the dithioester-endgroup of poly(N-vinylpyrrolidone) prepared by RAFT easily converted to an aldehyde function post-polymerization [220]. The quantitative reaction was shown by NMR, gradient polymer elution chromatography as well as MALDI-TOF mass spectrometry. The functional polymer reacted with lysozyme and formed a covalent conjugate after reduction with sodium cyanoborohydride. The research from mainly small molecules to therapeutic biomolecules, often collectively named biologics. The modification of macromolecular biological drugs and drug delivery systems with poly(ethylene glycol) (PEGylation) is a well-established and -investigated field of biomedicine [202,203]. However, they are considered important for further improving the pharmacokinetic performance of conjugates [202,204,205].

The advancements in the preparation of well-defined functional polymers, particularly the newly developed methods of controlled radical polymerization, e.g. reversible addition fragmentation chain transfer (RAFT) radical polymerization or atom transfer radical polymerization (ATRP) allow conducting controlled polymerizations in aqueous solutions at ambient conditions [206,207]. The basic strategy of controlled radical polymerization techniques relies on the establishment of a large pool of dormant radicals which can be easily reactivated and are in equilibrium with propagating species. Most important, they tolerate the presence of a wide variety of functional groups. Thus, these polymerization techniques are particularly well suited for the preparation of polymer–protein block copolymers, either by grafting-onto or grafting-from methods. Grafting-onto strategies (convergent synthesis) build on the preparation of defined functional polymers which are conjugated to the biopolymer via reactive groups and established crosslinking chemistries [208]. For the grafting-from strategy (divergent synthesis), biopolymers or biological scaffolds are suitably functionalized with initiators or transfer agents and the polymer chain is grown directly from the biomaterial. The different approaches have been reviewed recently by Haddleton and Klok [209,210].
The actual number of grafts was not determined. A RAFT agent with an NHS-activated ester group was prepared for the synthesis of other functional RAFT agents but not used itself for the preparation of NHS-terminal polymers [221]. Theato and co-workers presented the use of a pentafluorophenyl active ester of a dithiobenzoic acid chain transfer agent (CTA) for the RAFT polymerization of poly(diethylene glycol methyl ether methacrylate) [222]. Reaction of the active ester with thyroxin and reduction of the thiocarbonyl moiety and subsequent reaction with a thiosulfonate derivative of biotin yielded heterobifunctional polymers [223]. Maynard and co-workers used the unique single free cysteine of V131C T4 lysozyme for conjugation of maleimide-end-functional poly(PEG acrylate) (poly(PEGA)) [224]. The endgroup was obtained from a furan-protected maleimide CTA. Conjugation with up to 50% conversion was shown by SEC and SDS-PAGE. The same chemistry was used to prepare hetero-telechelic poly(N-isopropyl acrylamide) (PNIPAM) by using a biotin-functional chain transfer agent. The trithiocarbonate group was exchanged with the furan-protected maleimide group in a radical reaction. After deprotection, conjugation to two different proteins, streptavidin via non-covalent binding and BSA via its single free thiol group, was successful [225,226]. A homodimeric lysine conjugate was prepared, too [227]. Previously, the authors reported also on the RAFT synthesis of poly(PEGA) with a pyridyl disulfide endgroup [228]. Deprotected siRNA with a 5'-thiol group was chosen as model compound and successful conjugation was verified through PAGE by the shift of the bands to higher molecular weights. Upon reduction, the conjugate band disappeared and a band at the identical position of the original siRNA was regained. Sumerlin and co-workers prepared an azido-functional trithiocarbonate CTA and conjugated such prepared PNIPAM via click-chemistry to BSA whose cysteines were modified with a maleimide-functional terminal alkyne [229]. Upon reduction of BSA, a disulfide bridge was reduced and, additionally to the sulphydryl group of the single native free cysteine, two more cysteines were amenable to modification. In the latter two examples the second possible functionality originates from the post-polymerizational reduction of the thiocarbonyl group of the chain transfer agent. Boyer et al. prepared a truly bifunctional RAFT chain transfer agent. They modified a trithiocarbonate unsymmetrically with an azide- and dithiopyridyl-group bearing moiety [230]. A careful assessment of the click-reaction conditions which do not cleave the thiocarbonyl group is necessary; copper(II) sulphate, and sodium ascorbate in water/isopropanol (1/1 v/v) proved to be suitable. Conjugation of the hetero-telechelic PNIPAM was done to alkyne-bearing biotin and, on the disulfide side, to glutathione or BSA.

The grafting-onto-approach has intrinsic limitations. Steric hindrance and/or low stability of the reactive functional group during long reaction times can hamper conjugation with very long polymer chains. Further, a distribution in the number of polymer grafts will result from multiple attachment sites which again may depend on the graft length. More uniform conjugates can be obtained, if the polymer is directly synthesized with a protein-macroinitiator (grafting-from or divergent approach). Lysine amino groups can be smoothly modified with 2-bromoisobutyryl bromide. The installation of a precise number of initiating sites at the protein is the crucial step in the formation of polymer—protein conjugates with a well-defined number of attached polymer chains.

<table>
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<th>Functional initiator</th>
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<td>NH$_2$/amide</td>
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<td>alkyne/triazol (&quot;click chemistry&quot;)</td>
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<td>streptavidin/ non-covalent interaction</td>
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Fig. 14. Reactive end-functional polymers for grafting-onto to biomolecules via ATRP.
Differences in reactivity and the appropriate excess of reactive initiator can be used to limit the number of initiating sites. Lele and co-workers showed that precisely defined conjugates can be obtained in the case of a single initiating site. However, increasing the initiator to protein ratio led to a variable number of initiating sites. This results in a variable number of polymer chains [231]. Moreover, an increased steric crowding led to lower monomer conversion. Magnusson et al. conjugated the ATRP-initiator via a succinimide active ester to recombinant human growth hormone. The oligoethylene glycol spacer helped to achieve full reaction with free protein-amino groups and to reduce steric crowding in the subsequent polymerization of OEGMA [232]. Le Droumaguet and Velonia chose the unique single free cysteine Cys-34 of BSA as site for the grafting-from polymerization of NIPAM. The macroinitiator was prepared by conjugation of a maleimide terminal isobutyl bromide ester. Polymerization of styrene in a suitable DMSO/water mixture resulted in giant polystyrene-BSA amphiphiles [233]. The group of Chilkoti achieved recently the modification of only the C-terminus of green fluorescent protein with an ATRP-initiator and the synthesis of well-defined protein-poly(oligo(ethylene glycol) methyl ether methacrylate) conjugates [234]. Similarly, grafting-from polymerizations with the RAFT technique were reported. For example, Davis and co-workers used a dithiopyridyl functional trithiocarbonate to prepare a monofunctional BSA-macroinitiator for the polymerization of PEGA or NIPAM while De et al. chose a maleimide-functional CTA [235,236]. Fully synthetic oligopeptide-RAFT agents were prepared on solid supports [237].

Particularly, for multivalent scaffolds with closely located sites, conjugation of long polymer chains has been difficult [238]. Wang and co-workers used therefore the grafting-from approach to decorate ferritin particles with polymer chains. The ferritin protein cage was functionalized with bromoisobutyrate and served as multivalent initiator for ATRP polymerization of water-soluble PEG-240 methacrylate [73]. The number of grafts, i.e. number of initiating sites, was not determined but experiments with similar small reagents like bromoisobutyrate showed that all four surface exposed lysine residues K83, K97, K104 and K143 can be labelled. The poly(PEGMA)-ferritin conjugate was soluble but aggregated in both, water and organic solvents. Modification with polymers is a valuable contribution to develop ferritin as novel drug delivery system as it combines the great potential of ferritin-like particles for theragnostic purposes and the advantageous pharmacokinetic properties of PEGylated proteins.

There are few useful functional groups in peptides and proteins which allow reactions with sufficient efficiency. The ε-amino group of lysine is very popular because it is often surface exposed and good accessible. The drawback is their quite high abundance which makes selective conjugations difficult. The cysteine thiol group is more reactive but often involved in disulfide bridges with significant importance for the structure and must be reduced to the free
thiol before any reactions. Other amino acids need special reaction conditions and reagents and have limited value for general conjugation reactions. Ultimate goal would be therefore a residue- and site-specific functionalization strategy of arbitrary choice. The use of non-canonical amino acids can be a solution for this [239]. Incorporation of amino acids with non-natural functional groups can be achieved by various chemical or biological pathways. The introduction of chemo-selective handles and functionalities which are orthogonal to that of other amino acids in the protein is particularly valuable. For example, virus-like particles derived from hepatitis B virus (HBV) or bacteriophage Qb were labelled with alkyne-derivatives of e.g. fluorescein, biotin, a gadolinium complex or transferrin by click-chemistry [100] (Fig. 20). The necessary azido-groups on the virus capsid were engineered by reassignment of the sense codon of methionine for an azide-bearing analog, azidohomoalanine. Global replacement of all methionines was achieved. As the Qb-protein does not contain any methionine (except eventually at position 1 from bacterial expression), mutants with methionines at the most exposed position K16 and/or at an interior position T93 were generated. The coat protein sequences were cloned into plasmids and expressed in methionine auxotroph E. coli strains. The replacement did not hamper the self-assembly of the protein subunits to intact particles which were indistinguishable in their physical properties from their native analogs as seen in the TEM images. HBV was however sensitive to the introduction of the different labels and low recovery of intact particles was observed. The Qb-bacteriophage was much less sensitive and near-quantitative labelling of the 180 subunits with fluorescein and high recoveries of intact particles (>70%) could be obtained. Moreover, the conjugation of even very large proteins like the 80 kDa transferrin, an iron transport protein, was successful. Both reactions...
scaffolds for the mineralization and repair of critical size bone defects [245,246]. Such molecular assemblies cannot only present binding sequences and growth factors on their outer surface but also function as efficient drug and gene carriers if they are stabilized as small virus-like particles. For example, Lim et al. designed a multi-block polypeptide which self-assembled to small β-ribbon filaments. Small interfering RNA (siRNA) for sequence specific post-translational gene silencing (RNA interference, RNAi) formed an interpolyelectrolyte complex with an oligo-lysine block which was presented on the surface of the particles (Fig. 20) [247]. The discrete supramolecular assemblies showed excellent transfection efficiencies in green fluorescent protein knock-down experiments with HeLa cells. Moreover, hydrophobic dyes could be intercalated within the hydrophobic space of the β-ribbon bilayer to track the intracellular distribution with confocal laser scanning microscopy. Localization of the complex in the nucleus proved that this nanomaterial may be a potent artificial virus for gene and drug delivery. Interesting to note, a multivalent presentation of a charge-neutral carbohydrate moiety is needed as the outermost layer. It prevents aggregation via interparticle crosslinking upon adsorption of the siRNA and ensures high transfection efficiencies. Conjugation of side-chain functional polymers could render particles with even stronger binding efficiencies or improved pharmacokinetic properties.

Nowadays, biomedical nanotechnology is one of the fastest progressing fields in nanotechnology, proposing most promising applications yet within realistic expectations. Viruses and other protein cages have been used in a variety of applications, like cell targeting, gene delivery, drug encapsulation and release, vaccine development and immune modulation. The rigid scaffold and geometrical constraints of the bionanoparticles are of high importance for the multivalent presentation of foreign epitopes. Often, free small peptides exhibit low immunogenicity. The presentation as fusion peptides on a macromolecular scaffold as carrier of the antigenic determinant can strongly stimulate immunogenic reactions and enhance the production of antibodies. Such virus-like particles (VLPs) which mimic the structure of authentic virus particles represent a novel and promising class of vaccines [248,249]. Plant viral systems are of particular interest as they seem to be a safe and non-toxic platform for in vivo biomedical applications, e.g. investigated for CPMV by Singh et al. [250]. Thus, epitope-presenting systems based on CPMV and TMV have early been realized and investigated in detail [37,251]. Viruses displaying heterologous proteins can be constructed by genetic engineering of fusion proteins or by chemical conjugation. The choice of method is determined by strong differences in immunogenic properties of the resulting virus-like particles and not every conjugate can be produced by gene expression [251]. Few recent examples concern the production of carbohydrate based antitumor vaccines, based on CPMV glycan derivatives. For example, Miermont et al. conjugated Tn glycan analogs to CPMV capsids [252]. Tn glycan is overexpressed on many cancer cell surfaces but is a low immunogene itself. However, significantly increased antibody titers were observed for the analysed sera of mice that have been immunized with the CPMV conjugates. That points out the necessity of a highly organized display of Tn to elicit strong immune response. The produced antibodies recognized MCF-7 breast cancer cell surfaces as shown by Fluorescent Assisted Cell Sorting (FACS) with a fluorescein labelled anti-lgG secondary antibody. In another study, Kaltgrad et al. analysed the production of anti-glycan antibodies in chicken induced by CPMV-carbohydrate conjugates [253]. The binding and internalization of CPMV-based virus-like particles in antigen presenting cells like dendritic cells, macrophages and B-cells has been investigated in greater detail by Gonzales et al. [254]. VLPs can also be utilized as vectors to express valuable recombinant enzymes or...
single-chain antibodies in plants — a very economical and productive source of biomacromolecular pharmaceuticals [255].

Considering the excellent packing of polynucleic acid in viruses, well shielded from the surrounding, protein cages render a stable, biocompatible coating around enclosed substances or nanoparticles. Such composite core-shell particles can work as excellent capsules for the delivery of drugs and/or carrier of labels for diagnostic purposes (theragnostics). For example, the comparatively small ferritin particles present less steric hinderance in in vivo applications than other polyvalent drug carriers and has been of interest for nuclear magnetic resonance imaging [256]. Upon reconstitution of recombinant human H-chain apoferritin with (NH₄)₂Fe(SO₄) · 6 H₂O and hydrogen peroxide under alkaline conditions, crystalline iron-oxide cores of maghemite (γ-magnetite) were obtained [257]. The magnetic resonance relaxivities R₁ and R₂ increased with higher iron loadings and came close to the values of clinically known iron-oxide contrast agents. As the uptake of the mineralized ferritin was significantly higher than the uptake of iron-oxide particles of other contrast agents, the macrophages incorporated higher total levels of iron. Gadolinium(III)-based contrast agents have been prepared as well. Similarly, excellent and higher relaxivities than with low molecular weight Gd-chelates were obtained [258]. Moreover, the protein shell can serve as a versatile platform for cell-specific targeting moieties or another therapeutic agent may be co-loaded into the protein cage. Covalently bound drugs can be released if triggerable linkers are used. Flenniken et al. bound the chemotherapeutic agent doxorubicin via the acid-labile hydrazone linker to the interior of the small heat shock protein of Methanococcus jannaschii [259]. The multivalent presentation of epitopes will enhance and maximize specific binding properties to tissues and cells and thus increase drug delivery efficiency. Destito et al. targeted tumor cells with folate acid derivatized cowpea mosaic viruses [260]. The folate receptor is specifically up-regulated on a variety of human tumor cells. The significant uptake of wild-type CPMV was inhibited by PEGylation; redirection to KB and HeLa tumor cell lines was achieved by PEG-folic acid conjugates [238,260]. Efficient synthesis via click-chemistry guaranteed the consistent synthesis of CPMV particles with varying ratios of folic acid bearing and non-derivatized PEG-chains. Tumor cell surface carbohydrates are another kind of potential target for immuno-therapy. Finn and co-workers showed the preparation of virus-glycopolymer or -glycan derivatives of CPMV [252,261,262]. Such virus conjugates were found to bind e.g. to CD22, a receptor specifically expressed on B-cells and B lymphoma cells which need an effective polyvalent presentation of the respective glycan ligand [262]. However, there are also examples where uptake does not benefit from multivalent display of epitopes [263].

The main idea was further developed to ever more complex, multifunctional nanosystems that work at the interface of physical and life sciences [264]. Good examples are the multifunctional MR-theragnostics which Boyes and co-workers recently introduced. The core consisted of a gadolinium-metal-organic framework nanoparticle for magnetic resonance imaging. It was modified with biocompatible reactive polymers carrying a fluorescent tag for cellular-scale imaging and targeting ligands like GRGDS-oligo peptides or therapeutic agents like methotrexate, an antineoplastic drug [265]. Cage-like bionanoparticles offer an easier platform for such multifunctional nanosystems [70]. For example, viral behaviour was investigated by capsid enclosed CdSe-quantum dots in living cells [266]. In another study, Cotlet, Swanson and co-workers engineered the subunits of chaperonin, an 18 subunit double ring, with an oligo-his tag to accommodate water-soluble, dihydrolipoic acid-coated CdSe-ZnO quantum dots [267]. Nanoparticles of the appropriate size were well accommodated, forming defined 1:1 complexes with the protein ring. The histidine ligands were not only responsible for an efficient uptake of the nanoparticles but also for an increased photoluminescence. The potential targeting capabilities were shown with protein rings which had been assembled from mixed subunits, some of them carrying a biotin ligand.

The size uniformity of the particles and the homogeneous conformational orientation of target-binding probes on a scaffold are also critical for excellent detection sensitivity in bioassays. Assay sensitivity is further strongly enhanced by the multivalent display of epitopes and the high binding capabilities. Thus, an ferritin-based ultrasensitive detection system for the 65 kDa glutamate decarboxylase (GAD65) specific autoantibody, an early marker of Type 1 diabetes, was demonstrated with attomolar sensitivity [268]. Human ferritin heavy chain subunits were genetically engineered to present the respective epitope (H-FTN-GAD65). The bound antibodies (anti-GAD65 mAb) were then detected by a secondary CdSe-quantum dot labelled antibody through photoluminescence. The same amount of immobilized non–supramolecular antigenic probes resulted in a detection sensitivity that was at least 6 orders of magnitude lower, which impressively points out the importance of a homogeneous scaffold for highest assay sensitivities (Fig. 19). The simultaneous detection of two analytes, a diabetes and hepatitis marker, with a mixture of the respective ferritin labels suggest that multiplexed detection techniques will be feasible. Encapsulated materials can also be used for direct fluorescence or electrochemical

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**Fig. 19.** Quantum dot-based detection of autoantibodies of Type 1 diabetes. Epitope presentation on a) genetically engineered multivalent ferritin particles results in much higher sensitivity than with b) non-supramolecular probes [268].
assays [103,269]. The encapsulation of different ions within the chemically identical shell or the assembly of the bionanoparticle shells from differently modified subunits extend the approach to multiplexed assays.

The unique versatility of ferritin but also other protein and viral cages to house a variety of useful inorganic materials and the possibility to control the surface chemistry and thus the assembling properties, make them a versatile nanoscale platform for the fabrication of ordered arrays for e.g. nanoelectronics [119]. Yama-shita and co-workers were one of the first who broke the border between the well-separated fields of clean-room semiconductor based nanotechnology and wet biological materials for the fabrication of nanoscale electronic structures [149]. After the synthesis of nanoparticles inside the protein cavity, self-assembly on a surface and elimination of the protein moiety, the deposited nanodots could be reduced and buried in a silicon-dioxide layer (BioNanoProcess). Such assemblies worked as electron storage nodes in floating nanodot gate memory devices (FNGM) which are promising candidates for next-generation non-volatile memory applications. The structure is very similar to metal-oxide semiconductor field-effect transistors (MOSFET) but a nanodot array is additionally embedded above the channel. Injected charges from the gate electrode are stored and lead to a pronounced hysteresis of the drain current–gate voltage curve [270]. Fig. 20 shows the dense, nevertheless irregular assembly of Co-filled ferritin on a silicon substrate before device fabrication. A cross-sectional TEM of the final MOS-structure reveals the monolayer formation on the substrate. During annealing of the device, partial reduction can be achieved to provide metal nanoparticles as seen with XPS. The observed hysteresis in the drain current–gate voltage curve indicated the charge confinement to the embedded nanodots. Large charge capacity, long charge retention time and good stress resistance were observed when the device was operated as non-volatile memory [271,272].

A further useful application of self-assembled ferritin bionanoparticles is the use as masks for lithography applications in nanoelectronics. In contrast to conventional colloidal particles, ferritin and its inorganic core exhibit absolute uniformity which is replicated in the image structure. Kubota et al. deposited ferrihydrite containing ferritin on a wafer. By etching the silicon support, they generated 7 nm columns which replicated the exact size of the deposited ferritin cores [274,275]. Stacked nanodisk structures were prepared with this technique that exhibited a staircase quantum effect. The single-electron charging effect was investigated by conductive AFM measurements and was observed even at room temperature [276].

Fig. 20. a) Co3O4 nanodot array on an SiO2 surface. The nanodots were prepared in apoferritin and after deposition, the protein cage was eliminated by UV. b) The assembly was fabricated to be a part of a MOS field-effect transistor. The cross-sectional TEM shows the nanodots embedded in the MOS stacked structure. c) Typical transfer characteristics (drain current–gate voltage) of a control device (triangles), quasi-neutral scans (circles) and memory behaviour under +/– 6 V gate voltage sweep (squares) [273].
The catalytic properties of entrapped nanoparticles were shown in a variety of research works. Metal or metal oxide filled ferritin promotes the growth of suspended, diameter-controlled single-walled carbon nanotubes. This enables the site-specific growth of carbon nanotubes for advanced nanoelectronic devices from surface deposited ferritin particles [161,277]. The native ferrihydride core efficiently converted toxic chromium (VI) ions to chromium (III) in aqueous solution when irradiated with visible light [278]. Protein-stabilized and -shielded semiconductor catalysts may represent interesting candidates for biocompatible remediation. The small protein cages of the small heat shock protein from Methanococcus jannaschii and the Dps from L. innocua were mineralized with platinum [122,279]. In model reactions the efficient H2-production was shown for certain minimum platinum loadings. The exceptional temperature-stability of Hsp is important for the utility of such systems in real life applications.

5. Summary and perspectives

(Bio-) nanotechnology is at the frontier of biochemistry, polymer chemistry and material science. Previously separate fields come together to produce materials and build devices which combine the best properties of all the involved materials: (Bio-) organic functional ligands that mediate interactions with the surrounding, work as recognition tags or render them biocompatible; polymers that work as matrix, change solubility properties or add multiple functional groups; and finally, chemical material science that delivers nanoparticles with unprecedented optical, magnetic or catalytic properties or engineers organic and inorganic compounds to composites. Obviously, intelligent combination of these topics will mark a new era of nanomaterials with many possible applications in the field of catalysis, biomedical applications as diagnostic or therapeutic agents, in sensing devices or nanotechnology.

In this article, we outlined the rapidly increasing interest in and multiple uses of self-assembling proteins, particularly protein complexes and protein cages like ferritin, bacterioviruses and plant viruses in bionanotechnology. Monodispersity, absolute definition of chemical (secondary) and geometrical (tertiary/quaternary) structure, easy and manifold possibilities for manipulation and the inherent biological functionality are a few aspects which constitute reasons for the strong advances in the field.

Understanding the structure-forming principles is the primary prerequisite for the de novo design of novel self-assembling biomolecules. Symmetric protein cages like ferritin or plant viruses or self-assembling peptide motifs like β-sheets are structures which are well understood today and give principal guidelines for the construction of complex artificial structures. Therefore, artificial systems from novel building blocks are investigated besides naturally occurring bionanoparticles as well.

Conjugation of bionanoparticles with dyes, polymers and other ligands or decoration with nanoparticles is done by chemical means. Protein sequence with binding properties can be inserted by genetic engineering with ultimate precision and expressed in high yields. The self-assembled structures can work as reaction vessels for nanoparticle synthesis or as scaffolds for the synthesis of nanostructured bio-inorganic composite materials.

Conjugation of polymers to proteins has recently become a topic of strong interest. Particularly, the progress in the divergent synthesis of defined polymer–protein conjugates opens new opportunities for the preparation of novel soft materials in which both macromolecular blocks combine for the formation of structured and functional materials. The introduction of biological structural motifs will allow novel polymeric architectures. On the other hand, proteins will add novel functions into synthetic polymers. Fully synthetic and soluble materials will be necessary in the further development of (printable) sensor chips or the construction of responsive or triggerable drug delivery systems. Incorporation of inorganic nanoparticles will add optical, electronic or magnetic properties which yields finally multifunctional molecular hybrid assemblies.

The preparation of multifunctional nanosystems like those for new theragnostic systems which have been realized only previously, is challenging and has to make use of many of the above mentioned principles. The progress in diagnosis and disease treatment will strongly demand those multifunctional nanosystems in the future. Polymer-modified, biopolymer encapsulated inorganic nanoparticles for drug delivery and cellular level imaging can be a solution — and yet represent only one challenge for the further advancements in bionanotechnology.

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