

This is the post-print version of the following article: Ana Beloqui, and Aitziber L. Cortajarena, [\*Protein-based functional hybrid bionanomaterials by bottom-up approaches\*](#), *Current Opinion in Structural Biology*, 2020, 36/297, 63 (74-81)  
DOI: [10.1016/j.sbi.2020.04.005](https://doi.org/10.1016/j.sbi.2020.04.005)

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## ***Protein-based functional hybrid bionanomaterials by bottom-up approaches***

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### ***Abstract***

This review aims to summarize the last advances on the field of protein engineering towards functional bionanomaterials. Albeit being this an emerging research field, multidisciplinary perspectives in the design of synthetic protein-based hybrid bionanomaterials have resulted in significant progresses. The review covers the definition of bionanomaterials as such and the description of the main methodological approaches currently employed for their assembly. In this context, special emphasis is placed on the fundamental role of protein design. Then, a general overview of the most recent advances related to the fabrication and application of protein-based bionanomaterials in several applications is provided, with special focus on catalysis. Finally, key aspects to be considered by the research community to establish the path for significant future developments in this promising field are discussed.

### ***Introduction***

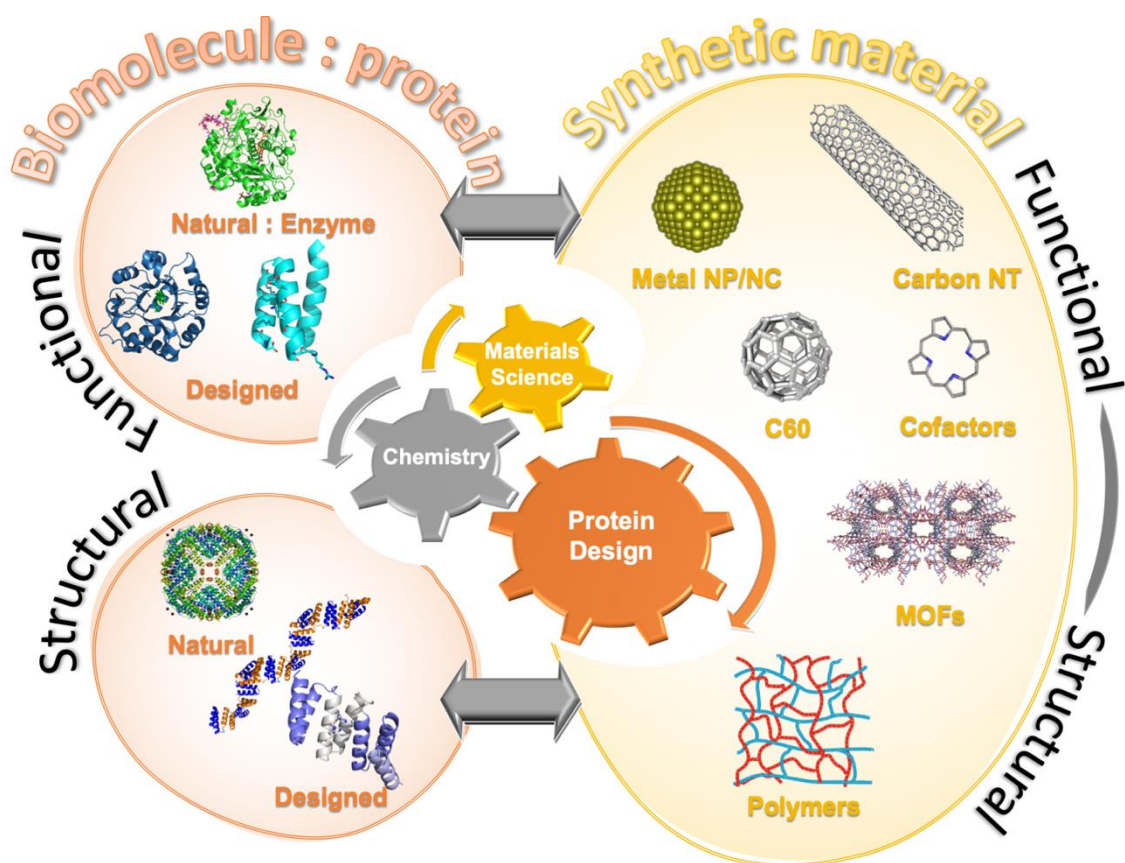
Nature, as the best-known bioengineer, applies smart design strategies for the manufacturing of robust and functional bionanomaterials. Hence, several complex systems have evolved by the combination of biomacromolecules, mostly proteins, with small molecules or inorganic materials, giving rise to the fabrication of advanced and highly efficient hybrids, such as natural photosystems [1], metalloenzymes [2], antenna systems [3], or bionanocomposites [4][5]. In this context, mimicking Nature's complex architectures in the lab arises as a smart strategy for the design of bionanomaterials, by means of interdisciplinary research that compiles latest advances in protein engineering and materials science. In a rational combination of these two disciplines, state-of-the-art synthetic approaches will be applied to biomolecules and thereby new opportunities to the increase the functional and/or structural diversity of the bionanomaterials will be opened.

In the last years, the concept of "bionanomaterials" has been applied to an extended number of nanosystems in which at least one of the components is derived from a biological source, such as nucleic acids, carbohydrates, lipids, peptides, and proteins. Herein, we are focused in hybrid materials that are composed by biomolecules, namely

proteins, and a second component, usually but not restricted to a synthetic material, which combination leads to a single molecular unit which necessarily maintains its dimensions in the nanoscale. Individual components, *i.e.* proteins and functional elements, must contribute either as a structural platform (scaffold material) or/and as functional component (*e.g.* catalytic, optical or conductive properties) to the hybrid unit. Proteins, as the main building blocks for bionanomaterials in Nature [6], are the core component of the hybrids that are herein discussed. Rational design and careful engineering of proteins using a multidisciplinary toolbox, based in computational modelling, molecular biology, materials science, and chemistry (Figure 1), has been demonstrated successful towards the development of new functional systems with potential applications in several disciplines including imaging [7], sensing [8], conductive materials [9][10], logic circuits [11], and catalysis [12]. In this review, we will highlight and discuss the recent advances in the field of protein-based hybrid functional bionanomaterials. The two main strategies used for the design of the hybrids, *i.e.* direct-conjugation of the components *vs.* *in situ* assembly of the hybrid, will be tackled through recent examples and applications of the bionanomaterials, with special focus on applications in catalysis.

### ***Design of Protein Bionanomaterials***

Proteins, besides their significance in Nature, can be exploited in the lab as three dimensional nanoscaffolds with high structural and functional diversity. Thanks to the development in the last decades of a multidisciplinary toolbox that includes protein engineering approaches and computational modeling protein design, very relevant milestones including the design of new protein folds, supramolecular protein assemblies, and activities have been recently achieved [13][14][15][16]. Therefore, it is possible to reach a very strict control over the sequence of the protein to shape the three-dimensional structure and the composition of the amino acids that are exposed to the environment. Indeed, these residues are the main target in the rational design of bionanomaterials. The generation of rational-based site-directed modifications laid out on the surface of the biomolecule allows the precise accommodation of the functional elements, which eventually leads to the design of bionanomaterials with well-arranged architectures [14][17]. The combination of both, proteins and synthetic materials, into a single entity can be performed following several orthogonal bioconjugation methodologies, including non-covalent strong and robust interactions, chemically driven covalent bonds, or physical entrapment [18]. Importantly, the approach selected for the synthesis of the hybrids should guarantee the preservation of the nanoscale and the integrity of individual components within the synthesized bionanomaterial.



**Figure 1.** Schematic representation of the main components that may comprise a hybrid functional bionanomaterial, highlighting their role, either structural or functional. The application of a multidisciplinary toolbox that includes recent advances in protein design, materials science, and applied chemistry to the proteins or the synthetic materials, which can operate with a structural or functional role, gives rise to a vast array of possible combinations, and therefore to a large number of potential hybrid bionanomaterials with various structures and functions. The protein component that plays a structural or functional role can currently be selected from an array of natural proteins or from the growing palette of designed proteins. When considering synthetic elements to provide structure or unique functionality, the underlying diversity is equally broad, including nanomaterials, carbon nanomaterials, polymeric materials, metal-organic frameworks, and organic molecules, among others.

The fabrication of hybrid bionanomaterials is mainly performed following two different methodologies, which vary on the assembly procedure that is utilized for the synthesis of the functional element. In a first approach, well-characterized functional elements are combined with proteins using bioconjugation techniques. Here, exposed reactive amino acids or hydrophobic patches on the surface of the protein are usually targeted and the proteins eventually remain anchored to the surface of the functional element [18]. Thus, the main role of the synthetic material here is to act as a scaffolding support, usually to concentrate and orientate functional proteins, such as enzymes or signal peptides. This methodology is widely used in the fabrication of protein-metal core-shell nanoparticles for biomedical applications [19], or in the generation of enzyme-powered nanomotors for active and targeted drug delivery [20]. However, in other examples, the rational design of proteins as scaffolding units can additionally support and stabilize

functional elements such as carbon nanomaterials, including carbon nanotubes[21] and fullerenes [22][23], and photo-active elements [24][21] by the introduction of selective coordinating sites, giving rise to hybrid bionanomaterials with interesting properties for bioelectronics.

The second strategy for the synthesis of bionanomaterials includes the assembly of the functional element in presence of the protein, meaning that as the material grows, the protein hybrid is synthesized. In the last years, several technologies for the *in-situ* synthesis of protein hybrid bionanomaterials have been developed. Compared to the direct-conjugation approach described above, the *in situ* synthesis allows facile one-pot synthetic protocols that determine the location of the nanomaterial in specific sites within the protein, while preserving the nanoscale size of the hybrid. As a typical example of bottom-up synthesis approach, proteins have been used as platforms for the nucleation of metal ions and subsequent growth of metal nanoparticles on the surface of the biomolecule. Both structural, *i.e.* ferritin [25], or functional, *i.e.* enzymes [26], native proteins have been employed as scaffolds for the controlled synthesis of catalytic metal nanoparticles. However, from the protein design perspective, there are few reported examples in which the protein sequence is carefully tailored to obtain a thorough control of the growth of the nanomaterials. In a pioneering work Ueno *et al.* modified the ferritin cage for the nucleation of sub-nanoclusters [27]. These small-sized clusters could be only achieved by the creation of a controlled chemical environment through the manipulation and accommodation of the cavity of the protein. Recently, a protein-engineering based approach has been established for the use of protein scaffolds to control *in situ* synthesis of metal nanomaterials by the introduction of specific metal coordination sites in well-defined cavities on the protein surface [28][29]. In addition, this well-controlled bottom-up approach presents the advantage of the modularity of the protein scaffold of choice, a repeat protein domain, that allows the modular design of the scaffold protein and thus the precise control of the size and properties of the final bionanomaterial [28][30].

Another interesting and cutting-edge example of protein-hybrid nanomaterials synthesized through *in situ* assembly is based on the encapsulation of proteins in metal organic frameworks (MOFs) [31]. Compared to the post-conjugation strategy, the *in situ* approach, besides showing more conclusive results in the stabilization of proteins and delivery applications, keeps the small size of the hybrids. In this bottom-up synthesis strategy, the entrapment of the proteins in MOFs shell is triggered by the hydrogen and ionic interactions between the residues of the surface of the protein and the coordination ligands and metal cations that build up the functional element starting from the surface of the protein. Therefore, the chemical composition of the surface of the biomolecule is again key for the assembly of the protein-MOF hybrids. This feature is clearly evidenced in the works of Prof. Doonan [32] and Prof. Ouyang [33], in which the effect of different chemical environments on the surface of the protein in the embedment of the proteins into a MOF exoskeleton were studied.

Alternatively, polymers are excellent platforms to host biomolecules. Polymer-based bionanomaterials have desirable properties such as solubility, functionality, enhanced stability of the protein against denaturation, thermo-responsiveness, or conductivity,

being all features provided by the synthetic material [34]. Indeed, it has been demonstrated that the combined polymer-protein hybrids show a synergistic effect that benefits both counterparts, overcoming their inherent limitations [35][36]. Polymer-protein conjugates (PPCs) can be classified according to the conjugation technique used in the synthesis (*e.g.* covalent bond, ionic interaction, supramolecular interaction, or physical entrapment of the protein) or to the architecture of the hybrid (*e.g.* dendrimers, polymer nanoparticles – from solid nanoparticles to polymersomes –, or grafted copolymers) [37]. Among all the diverse architectures and chemistries that have been brought to the table in the last years in this field, we want to emphasize the use of single enzyme nanogels (SENs) as polymer-based bionanomaterials with enormous potential in sensing, biocatalysis, and delivery applications [38][39]. SENs are synthesized *in situ* by the action of acrylic polymers on the surface of the protein. The synthesis of such small nanogels relies on the nature of the surface exposed chemical groups on the protein [40]. This method leads to the synthesis of a very thin polymeric layer that wraps single proteins, which remain in the core of the so-called single protein nanogels, keeping, in this way, the nanoscale dimensions of the bionanomaterial [41].

Herein, it is essential to remark, regardless the strategy of choice, that a particular emphasis is needed on preserving protein structure and function throughout the synthesis processes. All the utilized protocols and methods will be thereby conditioned to the stability of the protein in order to guarantee the success of the protein-based bionanomaterial fabrication and the usefulness of these for a desired application.

### ***Protein hybrid bionanomaterials for catalysis***

The use of enzymes and/or catalytic nanomaterials, such as metal nanoparticles and organometallic complexes, as components of the biohybrids enables their use as catalysts. In this context, the use of tailored bionanohybrids opens the scope to the development of non-natural catalysis. These can be achieved by two approaches: (1) the generation of artificial protein-based biocatalysts combining protein design and the coordination with catalytic elements; and (2) the application of protein-based catalysts (enzymes) and their integration in bionanomaterials which can be composed of different structural elements including proteins. Furthermore, the combination of bio- and chemo-catalysts in a single unit opens new opportunities towards the fabrication of multifunctional materials. In addition, the assembly of protein-hybrid bionanomaterials into macroscopic materials, such as films and biocoatings, gives rise to several advantages compared to established technologies in homogeneous and conventional heterogeneous catalysis. Indeed, assembled bionanomaterials, as heterogeneous materials, enhance the processability, robustness, and stability of the catalysts [42] [43].

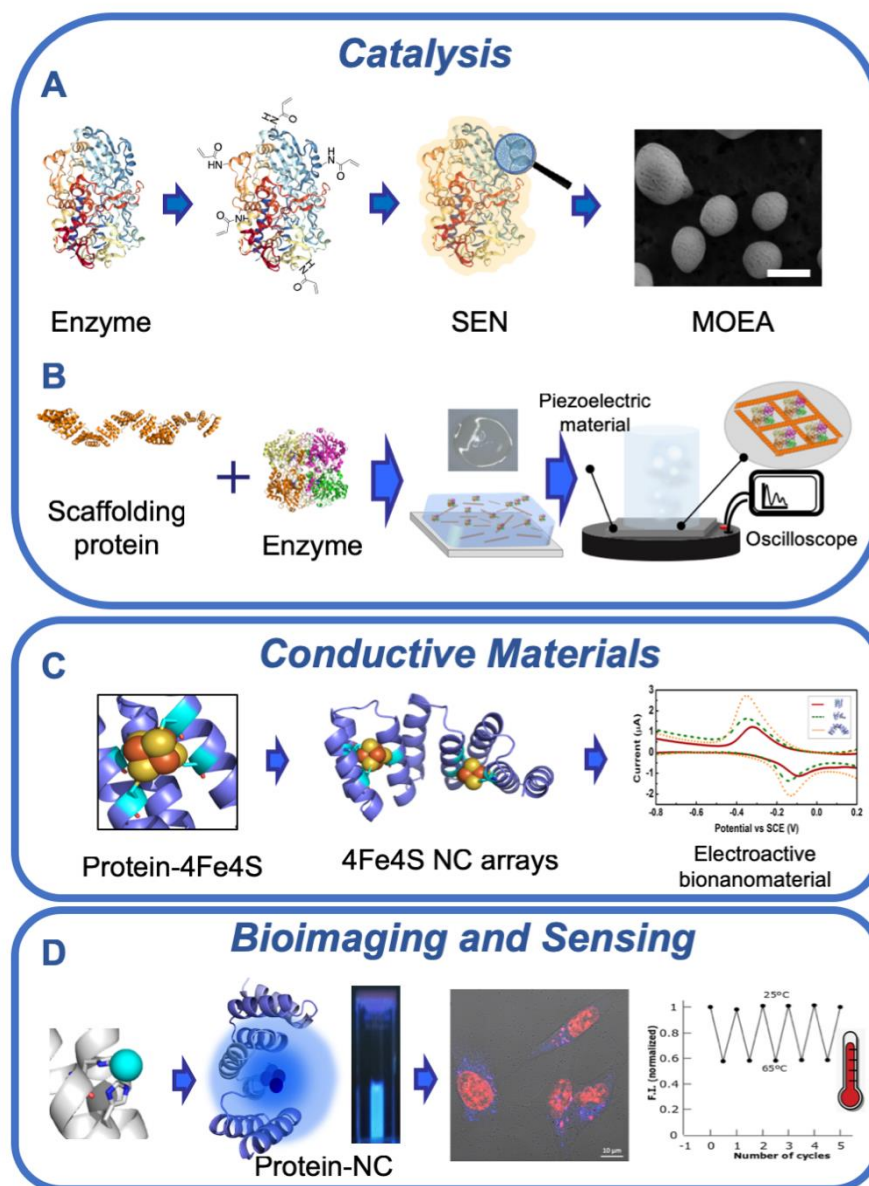
A very integrative example of bionanohybrids synthesized using the first approach consists in the design and development of the artificial metalloenzymes (ArM). A straightforward methodology uses small proteins with non-covalently bound cofactors, mostly heme proteins such as cytochrome C (CytC) or myoglobin (Mb). Small proteins are easier to manipulate, and the removal of the heme cofactor leaves a well-defined cavity to introduce the engineered cofactor. As example, Hayashi *et al.* [44] successfully designed and synthesized an ArM with hydrogenase activity by the introduction of a

[2Fe-2S] type H-cluster to the apo-CytC protein. The same group has reconstituted apo-Mb with manganese porphycene 7 for the hydroxylation of alkanes using H<sub>2</sub>O<sub>2</sub> [44]. In a more advanced approach, protein scaffolds are sought in the protein data bank and selected according to their suitability for the catalysis. In this case, the exposed residues of selected scaffolds are carefully tailored to accommodate organic catalysts, mostly metalorganic cofactors. Such modifications are usually performed on existing pockets on the surface of the proteins to anchor the functional elements specifically using orthogonal chemistries. Further, strategic sites of the surface, i.e. substrate channels, of the protein can be modified to modulate the selectivity, and thus functionality, of the ArM. Engineered scaffolds have been used for the synthesis of artificial oxidases, hydrolases or Diels-Alderses, to cite some examples [45][46]. Interestingly, second generation ArMs are achieved by the introduction of random mutations to the protein sequence through directed evolution cycles. Hence, recent works report the possibility to modulate the chemo-, stereo-, and regio-selectivity of the organic catalysts by random modification of the protein scaffolds in which are anchored [47].

In a different approach from the methodological point of view, synthetic materials can be used as scaffolds of hybrid nanosystems in which proteins provide the functionality. Usually, these non-biological systems are used either to protect the proteins from denaturation under non-physiological conditions or to arrange and confine them into favorable environments for catalysis. As explained above, the use of polymers as synthetic component of the hybrids is of high interest not only due to the new features that polymers provide, but also due to the high chemical diversity that the polymers bring to the hybrid systems. In this regard, the single enzyme nanogel approach allows the modification of the surface of the proteins and the introduction multiple coordination ligands on the protein in a delocalized manner but skipping the molecular biology steps. As result of the high modification density, low concentration of metal cations is needed for the efficient assembly of enzyme nanogels into robust and highly active nanoparticles, so-called Metal-Organic Enzyme Aggregates (MOEAs) [48]. These hybrids have been demonstrated successful not only as new bifunctional biocatalysts that integrate the co-catalysis of the biomolecule and the metal cation in a synergy action, but also as assembled platforms to physically compartmentalize biocatalysts in enzymatic cascade reactions and to coat gold microelectrodes for the electrochemical detection of glucose [43].

In contrast to classical approaches to assemble nanomaterials into macroscopic materials, usually based in chemically driven crosslink, there are some proteins that can naturally undergo self-assembly through only protein-protein interactions. These proteins include natural proteins mostly silk fibroin [49], and designed proteins, mostly based on repeat proteins [50]. The self-assembly of engineered protein scaffolds has been applied to the fabrication of protein-based macroscopic materials that entrap and stabilize enzymes resulting in the generation of novel fully protein-based heterogeneous biocatalysts [51][52]. In addition, the ability of the certain engineered proteins to self-assemble has been also demonstrated when using protein-hybrids that carry additional functional elements such as photoactive elements [53], redox active clusters [28], and conductive materials [21], which set the basis to explore this technology to fabricate other biocatalytic materials in which the catalytic entity is not a protein.

## Hybrid bionanomaterials



**Figure 2.** Representative examples of different protein-hybrid bionanomaterials assemblies for applications in catalysis, bioelectronics, bioimaging, and biosensing. **A.** Example of protein as functional element of a protein-nanogel engineered bionanomaterial in which a polymer acts as a structural element [48]. **B.** Example of fully protein-based functional nanostructured biocatalytic material in which an engineered scaffold protein plays a structural role and an enzyme is the functional element [51][52]. **C.** Example of designed protein for the stabilization of ordered arrays of redox-active clusters, for the modular design of long range electron transfer conduits [28]. **D.** Example of protein as designed structural scaffold that templates functional fluorescent metal nanomaterials that can be used for *in cell* bioimaging [29] and sensing [54], for example as temperature sensors.



As a last combination strategy for the fabrication of catalytic bionanomaterials, there are few examples of complex hybrids in which functional biomolecules and functional elements are merged in a single unit, *i.e.* enzymes with (in)organic catalysts [26]. Being this a good strategy for, as example, the development of chemoenzymatic reactions, in the practice, these multifunctional hybrids are usually not operative or achieved low reaction efficiencies. There are some issues that preclude the use of both catalysts at the same time, such as the cross-poisoning or the excluding working conditions of each of the elements. Gratifyingly, successful stories have been reported by the combination of (bio)catalysts with a support material. However, the latter materials, *e.g.* mesoporous silica materials or UiO-66 based MOFs, are usually macroscopic, thereby being no longer considered bionanomaterials [55]. Only recently, Li *et al.* developed a method for the fabrication of enzyme – metal nanoparticle bionanohybrids using enzyme-polymer conjugates [56]. They claimed the controlled synthesis of Pd nanoparticles on a polymer matrix that is bound to the CALB enzyme. With this strategy, they demonstrated the chemoenzymatic production of enantiomerically pure alcohols and amines via a Dynamic kinetic resolution (DKR) process.

In our view, future directions in the field of protein design point towards the achievement of fully synthetic catalytic systems. In this direction, engineered artificial proteins based on repeat proteins were shown to catalyze a (3+2) cycloaddition, a reaction not described to be catalyzed by any natural enzyme [57]. In addition, similarly to the synthesis of ArMs, protein-metal cluster hybrids with potential catalytic activity have been designed using the same repeat scaffolding protein [29]. These atomically precise metal nanoclusters present interesting catalytic properties mostly due to their high surface area and high surface reactivity. Therefore, the aforementioned protein-design strategies reveal the possibility of developing in the near future fully designed chemoenzymatic catalysts. For example, by modular combination in the same bionanomaterial of a fully designed enzyme and an artificial ArM with inorganic components all based on similar scaffolding proteins and on the precise bottom-up assembly of individual functional components into *a la carte* complex systems. Those strategies present high promise for the next generation of tunable bionanocatalysts and the achievement from simple strategies of a complexity and functional diversity currently not imaginable in single bionanomaterials. Overall, it seems that there is an interesting open field in the research of new approaches for the design of bionanomaterials that overcome the limitations herein exposed and, in turn, enable the accommodation of different functional elements at the nanoscale.

### ***Protein hybrid bionanomaterials for other applications***

The use of functional proteins, *i.e.* enzymes, and catalytic elements leads the use of bionanomaterials as excellent opportunities in the field of catalysis. However, this is not the only field of action in which the hybrids can contribute with significant advances. The achievement of protein stabilized small (around 2 nm) metal-nanoclusters (NCs) and metal-nanoparticles (NPs) allows their use as sensors owing to the responsiveness of those to temperature or the presence of reactive oxygen (ROS) species [8][54], as

surface enhanced raman scattering (SERS) substrates [58], and as robust tools for bioimaging [29]. In addition, engineered proteins have been used to achieve a precise control over the organization of photoconductive elements, *i.e.* porphyrin and single wall carbon nanotubes (SWCNT) [21], or conductive metal centers [9][28], that give rise to the fabrication of new and sophisticated materials with enhanced properties in the field of bioelectronics.

### ***Conclusions and future perspectives***

As discussed in this review, significant progresses have been recently made in the generation of protein-hybrid bionanomaterials. These bionanomaterials as defined above should have nanoscale dimensions, a protein element that can act as a functional or structural component, and a second synthetic element that also can provide either structure or function to the hybrid. Thus, the generation protein-hybrid bionanomaterials aims to expand the potential of the already versatile proteins by mixing proteins with other functional or structural elements towards hybrid systems that encode even larger structural and functional complexity than proteins by themselves. The examples shown here illustrate the versatility and emerging potential of these bionanomaterials in terms of achieving a huge range of structures and functions. Even though the review mostly focused on examples in catalysis, it is worth mentioning that the approaches presented have general interest and a broad range of potential applications.

New approaches are being applied for the development and design of new bionanomaterials based on proteins. The significance of using proteins in the fabrication of bionanomaterials goes beyond their intrinsic functionality, since proteins can also be used as highly tunable platforms, as scaffolds, for the accommodation and tethering of synthetic materials that bring new functionalities to the hybrid system. In addition to this functional and structural versatility of proteins as design building blocks, it should be noted that compared to other platforms, protein-based materials are green, sustainable, biodegradable, and biocompatible. Therefore, it becomes clear the impact that these approaches will make on the development of new, sustainable, eco-efficient, and competitive functional materials contributing to the required transition to a bio-based economy.

The *in situ* fabrication of functional bionanomaterials is a current challenge that has already shown great future potential. The development of new tools and the integration of different disciplines in the study of bionanomaterials has led to the achievement of new materials that can be “easily” tailored to achieve fully encodable properties. In this regard, the research on fundamental protein design, including focus on structure, functionality, and directed and precise localization at molecular level of functionalization sites by applying structure-based design, will be essential to achieve a high control of the supramolecular structure of the bionanomaterials. The high complexity of the interactions between materials of different nature should be also understood, especially in organic-inorganic complex hybrids. When precise control is not required, *eg.* when the surface of the biomolecule is fully modified, the supramolecular assembly and growth on the surface of the biomolecules is an attractive

and simpler approach for the synthesis of functional hybrids. For the short-term success and implementation of these technologies, integrative approaches and continuous feedback between research disciplines are required. This combined approach will bring us the possibility to design “*a la carte*” bionanomaterials, with rational designs and fully encodable properties for specific requirements.

### **Acknowledgements**

A.L.C. acknowledges the European Research Council ERC-CoG-648071-ProNANO, ERC-PoC-2018-841063-NIMM, the Spanish Ministry of Economy and Competitiveness (BIO2016-77367-R), and the Basque Government (Elkartek KK-2017/00008). A.L.C. also acknowledges the Maria de Maeztu Units of Excellence Program from the Spanish State Research Agency – Grant No. MDM-2017-0720 (CIC biomaGUNE). A.B acknowledges the Spanish Ministry of Economy and Competitiveness (MAT2017-88808-R) and the Agencia Estatal de Investigación and Fondo Social Europeo for Ramón y Cajal Program (RYC2018-025923-I).

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