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REVIEW ARTICLE

Protein-protected luminescent noble metal quantum clusters: an emerging trend in atomic cluster nanoscience

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Abstract

Noble metal quantum clusters (NMQCs) are the missing link between isolated noble metal atoms and nanoparticles. NMQCs are sub-nanometer core sized clusters composed of a group of atoms, most often luminescent in the visible region, and possess intriguing photo-physical and chemical properties. A trend is observed in the use of ligands, ranging from phosphines to functional proteins, for the synthesis of NMQCs in the liquid phase. In this review, we briefly overview recent advancements in the synthesis of protein protected NMQCs with special emphasis on their structural and photo-physical properties. In view of the protein protection, coupled with direct synthesis and easy functionalization, this hybrid QC-protein system is expected to have numerous optical and bioimaging applications in the future, pointers in this direction are visible in the literature.

Keywords: *protein*; *peptide*; *noble metals*; *nano*; *quantum cluster*; *fluorescence*

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Dramatic growth has been witnessed in the field of atomic clusters during the last few decades, due to their fascinating properties (1–12). Clusters, which are made of few atoms, can be either in the gas phase or in the condensed phase, exhibit a bridge between atomic properties and those of the bulk and have been

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researched by both theoreticians and experimentalists. Arrival of nanoscience has intensified research on clusters in the condensed phase (1–3). For quite some time, anything that is evaporated or made in smaller dimension used to be referred to as clusters. The term clusters is now by and large restricted to entities distinctly smaller than nanoparticles. Quantum confined condensed phase clusters of noble metals such as gold and silver have been of recent interest due to their intriguing properties such as photoluminescence, non-photobleachability, photon anti-bunching, longer lifetime when compared to the conventional organic fluorophores and versatility in applications (4, 5). It should be mentioned that experimental studies on Pt and Cu QCs have also been emerging recently (13, 14). Here, one may remember that noble metals have always been of larger interest to materials scientists, they had been part of catalysis, organometallics and inorganic complex chemistry (10, 12, 16, 17). And what we have been currently pursuing as cluster science is closely associated with the progress in other areas (1, 10, 16). In the next few paragraphs, we have attempted to give a condensed version of the subject area of NMQCs.

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QCs: the convergence of properties from nanoparticles downward and from atoms upward

Exploring the properties of matter at decreasing dimensions has been an everlasting question in science. From the 'divided state of metal' of Faraday, colloidal state has been explored intensely over the past one and a half century (18). Excitement in this area can be seen in three distinct categories of materials: noble metal nanosystems, semiconductor particles or quantum dots and zero, one and two dimensional systems of carbon. Synthesis of stable and well defined particles of gold in the solution state as well as in the solid state redispersible forms, with various functional attributes contributed to the expansion of research in this area. Along with the multitude of properties of free and supported nanoparticles such as catalysis and plasmonics accelerated research in this area. Use of zero dimensional materials expanded into 1, 2, 3 dimensional states of matter and each one of these specific forms have produced distinct shapes for which reproducible synthetic procedures are now available. This evolution can be best observed in the case of gold, which makes gold based nanosystem the most extensively investigated category of materials. Excitements in this category of materials are covered elsewhere in detail (19–21).

The above mentioned evolution in chemical synthesis of nanoscale matter has produced entirely new class of materials in the recent past and they belong to the subnanometer analogues of nanoparticles with precise number of atoms which makes them inseparable from molecules. They are also called as clusters or molecular clusters and have also been referred to as artificial atoms in the literature. From the earliest synthesis of Au_{11}, Au_{13} and Au_{55} in 1978 onwards (22–24) there have been numerous developments in this area. After the synthesis of thiolate protected AuNP in 1994 (25) and water soluble thiol protected clusters in 1997, this area started receiving increased attention (26). The smallest analogues of these clusters could not be observed in TEM and were examined using mass spectrometry, especially by laser desorption ionization (LDI) (26). As precise characterization was not possible, the early clusters were

characterised based on mass numbers in their name such as '28 kDa clusters' (27). Luminescence from these clusters in the NIR region, although not bright, attracted attention (28). Several of these clusters were separated by electrophoresis and their spectroscopic properties were examined in detail (29). This was the turning point of research in such materials which opened up numerous properties of the molecular state of gold (4, 6, 60, 73, 93).

Parallel to the chemical synthesis of clusters, atomically precise clusters with unusual structural stability have been explored from the very early part of cluster science. Several of these studies are natural extensions of gas phase cluster spectroscopy. Marriage of advanced mass spectrometry with laser ablation changed the course of research as any material could be evaporated under an intense laser beam. Such clusters, mass selected or otherwise, could be deposited on surfaces to explore the catalytic chemistry of reduced dimensions. In fact, deposition of atoms on surfaces to produce clusters or active catalysts, without mass selection has been practiced for a long time. Depositing and manipulating atoms using scanning probe microscopy has been the holy grail in nanoscience (1, 15, 16, 30, 31). As can be seen, the area of QCs therefore represents a convergence of matter (and the research on them too) from nanoparticle to molecules or from atoms to molecules (Fig. 1) (1–55).

Various names may be given to these systems such as clusters, molecules, nanoclusters, nanoparticles, monolayer protected clusters, artificial atoms and so forth and many of these have been used in the literature. We would like to present briefly our reasons for naming them as quantum clusters. As nanoparticles and monolayer protected clusters (MPCs) have been used to describe large nanoparticles with or without monolayers, these two terminologies do not bring out the distinct differences or make their differences apparent from the systems under discussion. Terminologies such as artificial atoms may not be appropriate, as apart from single metal clusters, there are distinct categories of mixed atom analogues which make it necessary to have 'mixed atom' or 'alloy' superatoms and such a terminology does not appear suitable. Besides, shell closing is not the only reason for their existence. While clusters bring a gas phase analogy, it also suggests that these systems may exist only in the free state, without molecular protection. On the contrary, we are discussing molecules which can be precipitated, crystallised and redispersed just as any standard molecule. Thus the two better suitable names are quantum clusters and molecules. We refer to them with the former title as the latter suggests that the whole entity such as M_mX_x where M and X are the metal atom and ligand, respectively have

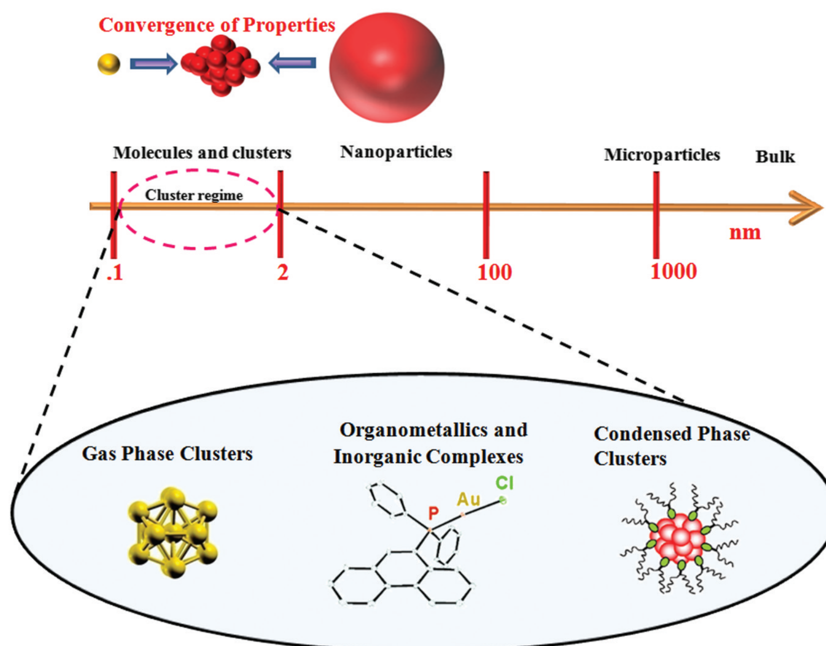

Fig. 1. Hierarchy of materials from atoms to bulk, especially in the case of noble metals. Clusters fall in-between atoms and nanoparticles. Expanded view is of the cluster regime showing diverse variety in this category: gas phase and condensed phase clusters with possible intermediate forms, the complexes. This schematic illustrates typical model nanosystems of gold but the same applies to other noble metals also. The transition of properties occurs between atoms to nanoparticles and also it illustrates the convergence of properties of colloidal nanoparticles and atoms at the scale of clusters. Schematic is for illustration purpose only and is not to scale. In the expanded view of clusters, images of naked cluster, complex and condensed phase thiol protected cluster were adapted from references 56, 57 and 58, respectively.

no separate existence. This is not true, as the core and the ligand are often two separate species with distinct features. Besides these distinct reasons, the name quantum clusters make them appear similar to quantum dots, both are intensely luminescent, one of the commonly used properties of the latter. Below we briefly discuss the properties and trend in the stabilizing ligands of these new materials.

Properties of NMQCs and the trend in the stabilizing ligands

NMQCs are sub-nanometer to 2 nm sized nanocrystal cores composed of noble metals, containing typically tens of atoms, with ligand protection and are distinctly different from nanoparticles and bulk powders in many properties (1–12). Surface plasmon is absent, since size of the cluster reaches de Broglie wavelength of the electron at the Fermi energy of the metal (E_{Fermi} = 5.53 eV, 4.28 eV, 5.49 eV, and 7.00 eV for Au, Pt, Ag and Cu, respectively (33)) and it can no longer support the plasmon excitation (5). Their structure is distinctly different from those of nanoparticles and bulk. For instance, the QC Au_{13} has an icosahedral structure (6, 10). As it is known, fraction of atoms on the surface plays an important role in determining certain properties and in the case of QCs, fraction of surface atoms is high due to their extremely small core size (1). Electronic confinement occurs due to the protection of the core with the ligand shell and energy levels of the core become discrete. Further, NMQCs exhibit highly polarizable transitions which scale in size with $E_{Fermi}/N^{1/3}$ where E_{Fermi} is Fermi energy of the bulk metal and N is the number of atoms (4). Luminescence in them arise from the transitions between the d→sp interband and sp→sp intraband derived levels and these conduction electron transitions are the low-number limit of plasmons (4, 6). Hence, by manipulating the number of atoms in the core, emission wavelength can be tuned. Luminescence combined with the non-cytotoxic nature, unlike the popular semiconductor QD analogues, make them unique for biological applications. NMQCs can be magnetic and may exhibit chirality (6–8). Although physicochemical, structural and electronic properties of NMQCs are not covered in this short review (which may be found elsewhere (1–3, 6, 10)), they pose several intriguing questions. For example, one may ask: Where does the transition from icosahedron to *fcc* start? How many atoms would be needed for plasmons to appear? When would a specific chemical property such as CO reactivity appear? In all of these properties, each atom counts.

With this abridged note, we would look at the various ligands used and the trend observed in the synthesis of NMQCs.

In the early times, groups of atoms formed by evaporation were stabilized in unreactive matrices, typically of condensed gases (1–2, 33–34). Since these clusters are extremely reactive in nature, without a stabilizing moiety, they tend to aggregate in solution to form bigger structures, to release their higher free energy. Hence, unlike some weakly protected colloids, the role of stabilizing ligands and controlled synthesis became crucial for solution state realisation of these materials. In the beginning, by exploiting the gold-phosphine chemistry, phosphine protected clusters came into existence (22–24). Thiol based cluster synthesis was developed by Whetten and Murray (35), they introduced glutathione (GSH) as a ligand to make water soluble clusters. Tsukada and colleagues extended this method and purified the clusters (29). Initially thiol protected clusters were synthesized in the organic phase (25, 26). Thiols like phenylethanethiol, hexanethiol, octanethiol and dodecanethiol-protected clusters were also prepared by taking advantage of thiol-gold affinity (6, 35). Later, water soluble thiols like mercaptosuccinic acid (MSA), D/L penicalamine, captopril, etc. were employed (6, 38–41). Use of MSA in nanoparticle and cluster synthesis is due to Kimura (36, 37). Dickson's group synthesized gold and silver clusters in dendrimers and DNA, respectively (42, 43). Ligand exchange of as-synthesized clusters has also been demonstrated by Pradeep's group (44–46). Novel synthetic routes may be used to make these clusters directly without purification and in larger quantities (40–41, 44, 47–49, 51). Stable series of organogold clusters (gold covalently bound to carbon) protected by phenylacetylene has been synthesized recently (52). Recently, Pradeep and co workers demonstrated that direct synthesis of NMQCs in solid state is also feasible (49). In zeolite scaffolds also silver clusters were made (50). Direct conversion of colloidal silver nanoparticles to thiol passivated Ag_{QCs} has been demonstrated (51). While mass spectrometric and few other spectroscopic details of these clusters are known, very few crystal structures are available so far (53–55). An emerging trend is synthesizing clusters with proteins and peptides which are functional. This trend in change of ligands for cluster synthesis is indeed fascinating and a gradual size evolution in the protecting agent is also noticed (Fig. 2). This may be thought of as a way to add additional attributes as scientists have been looking at the proteins to mimic them, especially the functional ones such as enzymes (17). The most exciting aspect of this research is bright luminescence in such clusters (60). The bio-molecular templates add another dimension to this research, with all their functional attributes. With this short briefing, hereafter we would focus on NMQCs@proteins (the @ symbolism implies NMQCs are embedded in proteins). We hope this review would connect various aspects of science from bio-mineralization by complex proteins to quantum confined noble metal clusters.

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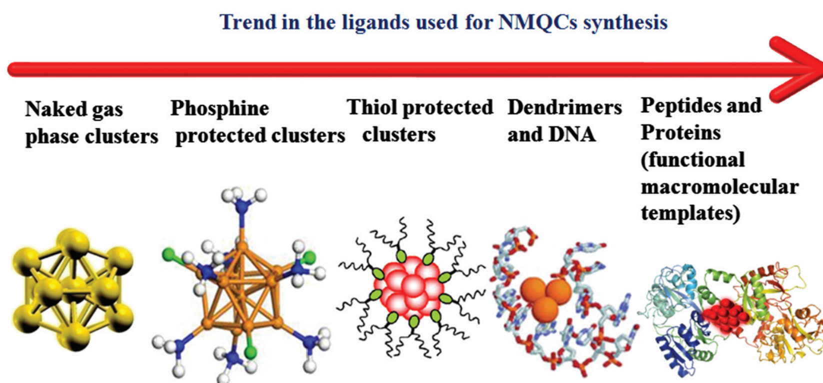

Fig. 2. The trend observed in the use of ligands for cluster synthesis, starting from gas phase unprotected analogues to phosphine protected systems to functional protein protected QCs. Representations of naked, phosphine, thiol, and DNA protected clusters were adapted from references 56, 59, 58 and 42, respectively.

Biomineralization and NMQCs

Nature leaves one awestruck by its marvellous creations and mysteries. Biomineralization is a natural process in which living organisms adapt to form hard structures by mineralizing metal ions through mineralizing peptides, vesicles, etc., and it is known that mineralization in many organisms occur as a mechanism to escape from ill effects of toxic metal ions or to form specific functional structures of millimeter to nanometer scale as in the case of magnetic bacteria (61). Interesting reports are available on bacterial mineralization of gold. Bacteria which are closely related to *Ralstonia metallidurans* play vital role in the formation of gold nuggets (62). Myriad of inorganic nanostructures have been formed by biomineralization or biomimetic mineralization processes and a lot of research efforts have been made to understand these processes (61–66). While naturally formed AuNPs are reported, unfortunately, QCs are not observed so far to occur naturally, may be because of their high reactivity. The biomineralization process has been mimicked to synthesize NMQCs too by carefully adjusting the concentration of metal ions and modifying the environment suitably. Unlike semiconductor QDs, quantum confinement effects starts only below two nanometers in NMQCs, hence, controlling the size becomes a tedious but crucial process. The captivating point is that size control is achieved by proteins very easily (60). It is likely for an NMQC to reside inside a large protein due to the former's sub-nanometer dimension or to be surrounded by more than one protein in the case of very small proteins.

Synthesis and properties of NMQCs@proteins

Mimicking biomineralization, Narayanan and Pal have synthesized luminescent silver clusters in proteins using $NaBH_4$ (67). Yu et al. transferred the as-synthesized luminescent silver clusters to other biomolecular scaffolds by shuttle-based method (68) and synthesized Ag_{QCs} intracellularly in NIH 3T3 cells (69). Xie et al. (60) first reported the direct synthesis and stabilization of Au_{QC} by a protein, bovine serum albumin (BSA) without any external reducing agent. Synthesis was done by mixing metal ion precursor with the protein and changing the environment to alkaline pH. At alkaline pH, it is reported that aromatic aminoacids donate electrons to reduce gold ions while broken disulphide bonds play major role in stabilizing the nucleated cluster (60). However, a clear understanding of the process is not yet available. Several groups have followed this procedure. Some groups have followed modified procedures like using ascorbic acid in addition to the above said mixture (70). Muhammed et al. reported the synthesis of Au_{QCs} by etching of gold nanoparticles by BSA (71) following their earlier method of etching larger nanoparticles by GSH (44). Wei et al. synthesized gold cluster with lysozyme (72). Xavier et al. had demonstrated the synthesis of Au_{QCs} in iron binding transferrin proteins such as lactotransferrin (Lf) and showed that iron saturation does not affect the cluster formation (73). But comparatively, Au_{QCs} in iron depleted protein had higher emission intensity than Au_{QCs} in iron saturated protein. This was first report to use a multifunctional metallo-protein for cluster synthesis. Recently, Le Guével et al. have synthesized gold clusters in human serum transferrin (74). Shao et al. synthesized Au_{QCs} and Ag_{QCs} on a solid platform of egg shell membrane (ESM) which consists of mixture of proteins, by soaking the separated ESM in metal ion precursor solution and illuminating the surface with UV light (75). Le Guével et al. synthesized Au_{QC}@BSA and protected it with silica shell (76). Recently, Yan et al. synthesized Au_{QC}@BSA and HSA using microwave assisted method in a few minutes (77). Liu et al. synthesized Au@Ag_{QCs} by sonochemical method in BSA (78). Choi et al. have recently synthesized Au_{QCs} in fixed NIH 3T3 cellular matrix (79). Mathew et al. reported a red emitting fifteen atom silver cluster in BSA. However, it was less stable and the stability was enhanced by protection with poly vinyl

pyrrolidone (PVP) (80). Peptides are used to direct the synthesis of clusters using mild reducing agent and by varying the pH. Recently, electrostatically induced phase transfer method has been used to synthesize NMQCs (81). A series of silver clusters and Au_{25} have been produced using custom peptides at alkaline pH (82, 83).

In general, from the published results so far, NIR emitting Au_{QCs}@proteins in general possess two excitation maxima (λ ex. max = ~ 370 nm and 510 nm) and one emission maximum in the NIR region (λ em. max = ~ 650–670 nm (Fig. 3B) (70–73). They have higher quantum yield ~6% when compared to their monolayer protected counterparts (60, 70–74). Upon excitation at 370 nm, an emission around 450 nm is seen. This is attributed to protein's intrinsic fluorescence by a few groups and due to Au_8 by other groups (60, 73, 75, 84). NMQCs@protein exhibit strong stability across a wide range of pH and is stable in higher ionic strength (Fig. 3C) (60, 73). Long lifetime component values, usually above 100 ns, have been reported for Au_{QCs}@proteins (73, 74, 84). Recently, Kawasaki et al. reported blue and green emitting Au_{QCs} synthesized at different pH with high quantum yield using pepsin (85). Compared to their monolayer protected counterparts, QCs@proteins have several fold enhanced luminescence. The reason for the enhancement of luminescence is still not completely understood, albeit the nature of the ligands bound to the cluster is important in this (86). In addition, the emission from the complex and how the emission from the intrinsic fluorophores of protein is contributing to the enhanced luminescence are also not properly understood. Earlier, it was suggested that there is a possibility of FRET between protein's fluorophores and the cluster; however, other studies on this have not been reported (73). The newly generated modified fluorophores during the reaction may play crucial role in addition to the ligand's role (91). While investigating the luminescence from clusters, one should be cautious about the intrinsic fluorescence of the template or ligand used and their modified products during the course of the reaction (43, 87–90).

Several groups have carried out XPS studies of NMQCs@proteins and results showed the existence of zero-valent Au and Ag indicating the presence of metallic core (60, 70–74, 80). Simms et al. studied the structural and electronic properties of Au_{QC}@BSA using X-ray absorption spectroscopy (XAS). Their analysis of the Au L(3)-edge extended X-ray absorption fine structure (EXAFS) of Au_{QC}@BSA suggested that the QC was

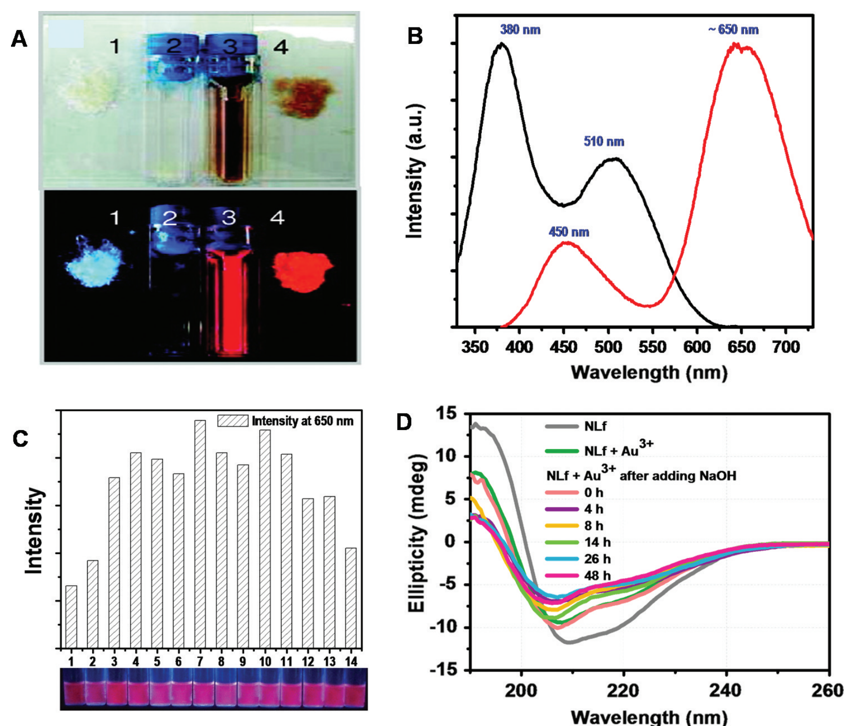

Fig. 3. (A) A photograph illustrating the luminescence of Au_{QC}@BSA. Red luminescence of the cluster solution and solid upon UV irradiation is shown, while the blue emission is from fluorophores of the protein. (B) Fluorescence spectra showing the excitation and emission maxima for a typical protein protected cluster. (C) Bar diagram showing the changes in luminescence intensity of clusters at various pH and corresponding photograph of the solutions in UV light. This indicates the significant stability of protein protected clusters over a wide range of pH. (D) CD spectra showing change in the conformation of NLf at various stages of cluster growth. A and D were adapted from references 60 & 94, respectively. B and C were adapted from reference 73.

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Au_{25} with a Au-thiolate 'staple' motif. Further, they used X-ray absorption near-edge structure (XANES) and Au 4f XPS to probe the electronic behavior of Au_{QC}@BSA. The Au d-electron density of Au-BSA was found to decrease by 0.047 e(-) relative to that of the bulk. They further reported that ab-initio calculations involving local density of states (l-dos) of Au_{QC}@BSA were consistent with the experimental d-dos results (92).

Mass spectrometry (MS) has been indispensable in understanding atomic clusters (27, 29). For the QCs@protein systems also, MS plays a major role in characterizing the cluster core. But very few reports are available on specific cluster cores in proteins, such as Au_{25}@BSA, Au_{38}@BSA, $Au_{13,25}$@Lf, Ag_{15}@BSA (Fig. 4D) and Ag_{8}@BSA. Some groups have reported that mixture of several clusters must be present in the protein matrix (60, 71, 73, 74, 80, 84). The formation of clusters is highly dependent on the structural properties of a given protein, hence various proteins with clusters should be analysed separately to know about the cluster core. Cluster core was shown to be similar in case of Au_{QC}@BSA and Au_{QC}@Lf, two larger proteins containing similar number of thiols (60, 73). Recently, Liu et al. have grown gold clusters in solution and also in insulin crystals (93) (Fig. 4A, 4B). They have reported that no mass shift was observed in the protein to characterize the nature of the cluster and suggested a reason that since insulin is a small peptide hormone, during ionization, the grown clusters detach from proteins and therefore are not identified in MS. The intact disulphide bonds observed in Raman stretching and non-formation of clusters when disulphide bonds were cut suggested an alternative growth mechanism other than that is observed for larger proteins like BSA or Lactoferrin. Recently, we also have observed similar mechanisms where the growth of QCs in small proteins does follow different mechanisms in comparison to larger proteins. We have observed that unlike larger proteins like BSA and Lf, cluster growth in lysozyme was different (Fig. 4C) (91). MS data, particularly for the cluster core protected by small proteins with less number of cysteine is not yet available. More MS oriented studies are expected in the future to understand the

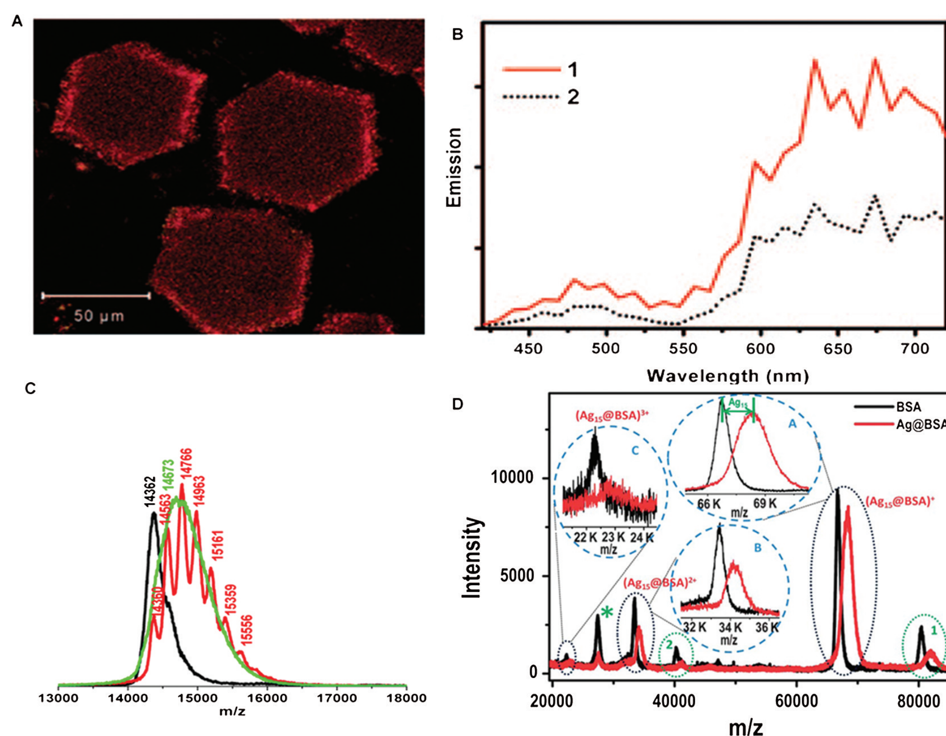

Fig. 4. (A) Two photon fluorescence image of Au_{QCs} grown in insulin crystals (λ_{ex} = 800 nm). (B) Two photon excitation of Au_{QCs} in the crystal, under different depth showing characteristic spectra of red emitting Au_{QC}@protein, Line 1 (red solid) is emission from the surface of the crystals and line 2 (black dotted) is the emission from 5.0 μm below the incident surface. (C) MALDI MS data of lysozyme at pH 7 (black), lysozyme-Au^{1+} complex (red) at neutral pH with 2.5 mM $HAuCl_4$ showing binding of gold ions by the observed mass shift. After cluster formation at pH 12, the mass shift pattern of complex disappears and very little mass shift is seen (can be one or two gold atoms strongly bound to the protein) suggesting that cluster is bound by more than one protein in case of smaller proteins like lysozyme. Hence during ionization in MALDI, it is not likely to see them (Our unpublished data). (D) MALDI MS data of Ag_{15}@BSA. MALDI MS of pure BSA solution (black trace) collected in linear positive ion mode using sinapic acid as matrix and that of the as-prepared red emitting Ag_{15}@BSA (red trace). The peaks due to singly, doubly and triply charged ions of Ag_{15}@BSA are expanded in the inset marked A, B and C, respectively A and B are adapted from 93, C and D are adapted from references 80 and 91, respectively.

system well. Since well defined optical properties of cluster cores are not seen in UV-vis spectra, it becomes difficult to give more details about the cluster core. Investigations by various groups on protein's conformation upon cluster formation revealed that there is a significant conformational change (Fig. 3D) (60, 67, 73, 94). Narayanan and Pal synthesized fluorescent silver clusters in α-chymotrypsin and studied the cluster protein interaction using time resolved fluorescence studies and reported that the conformation and activity of the enzyme is affected considerably. Here, we have to note that $NaBH_4$ was used in their method which may independently affect the protein's activity (67). The dynamics of protein during and post synthesis of cluster is yet to be investigated. Overall, while critically assessing the NMQCs@proteins system, the synthesis part is simple but the fundamental properties like origin of enhanced emission, role of protein's intrinsic emission, fate of protein's activity and how cluster is growing are yet to be understood completely. Hence there is a need to have a relook at the present understanding of QCs@proteins.

Evolution of gold quantum clusters in protein templates

As we discussed above, the understanding of QCs@ proteins and how they evolve in the protein templates is key to design next generation fluorescent functional noble metal clusters in macromolecular templates. Though one can say it as bio-mineralization, exact mechanisms of bio-mineralization are yet to be understood properly. As from early reports, mechanisms of bio-mineralization have been the topics of hot debate (61–66). Recently, Chaudhari et al. attempted to understand the growth process using mass spectrometry and have reported the current understanding of the evolution of gold QCs in Native Lf (NLf) and BSA templates (94) (Fig. 5A). Pradeep group's intial findings are intriguing and leads to many additonal questions for future research. From the MALDI MS data, they observed that immediately upon addition of gold ions (Au^{3+}) to the protein molecules, 13–14 gold atoms bind to protein and they remain in the Au^{1+} state (Fig. 5B). Once NaOH was added, the Au^0 state was observed and number of bound gold atoms increased to ~25 per protein suggesting the formation of Au_{25}, which was further corroborated by the commencement of red emmision. In the process of cluster growth, some free protein is generated depending on the total metal ion content in the Au^{1+} complex. As far as the optical properties are concerned, in the UV-vis spectra, they did not observe any prominent Au_{25} feature, it may be due to the bulky nature of the protein molecule and its strong absorption, but after 48 h, weak features around 650 nm were seen. During the evolution, at certain time intervals, they have observed

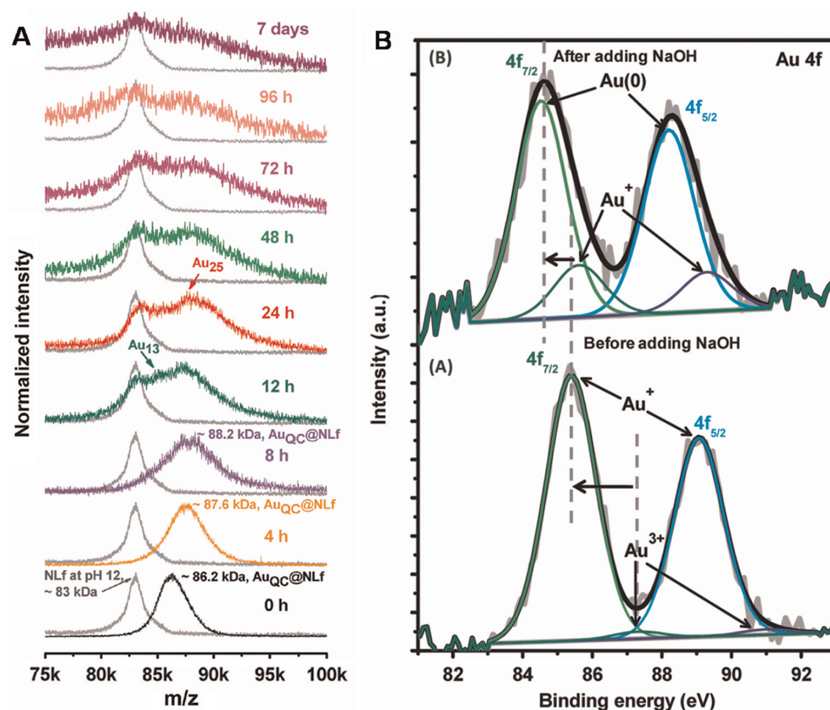

Fig. 5. (A) Time dependent MALDI MS suggesting the presence of Au_{25} and emergence of free protein from 12th hour of incubation. Initially, upon addition of Au^{3+} to Lf at pH 12, only one peak is there from which parent protein peak emerges when the clusters are nucleated. (B) XPS spectra sowing the presence of Au^{1+} before the addition of NaOH and Au^0 after the addition of NaOH. A and B were adapted from reference 94.

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the emergence of free protein at a specific molar ratio of protein and gold, suggesting an inter-protein metal ion transfer. This free protein was effectively utilized by providing extra gold ions at specific time intervals and they could obtain monodispersed clusters with enhanced luminescence. CD spectroscopic studies revealed that there were alterations in the secondary structure of the protein as a function of time to facilitate the cluster formation (Fig. 3D). Chaudhari et al. further demonstrated two step and multi-step approaches to utilize the free proteins generated to effectively form monodispersed clusters with enhanced luminescence. They also have hypothesized that inter-protein metal ion transfer and aurophilic interactions also play vital role in the formation of clusters in protein molecules. Li et al. has also observed free protein during cluster growth and proposed a method to remove the free protein from the as-synthesized protein cluster mixture. They have devised a simple chromatographic technique to remove the free protein by dansylating the protein and then identifying the free protein without the cluster by its green fluorescence from the red emitting cluster containing proteins (95).

NMQCs@peptides

Like peptide based nanoparticle synthesis, QCs have also been synthesized using selected peptides. Even few amino acids are used for cluster synthesis. For instance, histidine has been used to synthesize Au_{10} (96). Banerjee et al. used modified peptides for silver cluster synthesis (97, 98). The most used peptide is glutathione GSH (Glutamic acid-Glycine- Cysteine) which is a tripeptide, present in biological systems. It has been used as a monolayer ligand for cluster synthesis from the early stage of cluster studies as mentioned before (6, 27, 29). Previously, Fabris et al. had shown peptides when bound to Au_{38} remained in a conformationally constrained fashion (99). Target specific peptides based synthesis would be a more interesting field. Recently, Gao et al. synthesized a series of silver clusters and Au_{25} by using a peptide containing nuclear target sequence (CCYRGRKKRRQRRR) and demonstrated nuclear specific staining by Au_{QCs} (82, 83). Yuan et al. synthesized Ag_{QCs} using GSH and designed peptides (Asp-Cys-Asp, Glu-Cys-Glu, and Ser-Cys-Ser for red emitting Ag_{QCS} and Lys-Cys-Lys for blue emitting Ag_{QCS}) (81). Recently, Bellina et al. studied the isolated GSH-Au and GSH-Ag complexes, correlated their optical properties by the combination of action spectroscopy and time dependent density functional (TDDF) calculations and have reported that intense transitions are occurring within S-Ag-S motif and not within the cluster which is responsible for strong emission of silver clusters in biomolecules (100). These small peptides containing clusters may be helpful as biolabels where hydrodynamic size of the protein protected clusters is not compatible and where smaller diameter is required.

Applications of NMQCs@proteins

NMQCs@proteins have been applied in sensing, electrochemiluminesence (ECL) and bio imaging so far and we shall discuss their applications in the above-mentioned order. Xie et al. had first shown that Au_{QC}@BSA can be used as a sensor for Hg^{2+} (101). Wei et al. and Lin et al. synthesized Au_{QC} in lysozyme and had shown its Hg^{2+} sensing application (72, 102). In general, it is reported that Hg^{2+} quenches fluorescence by interacting with the core while fluorescence quenches due to the aggregation caused by Cu^{2+} ions. Au_{QC}@NLf was reported to be sensitive to Cu^{2+} ions other than Hg^{2+} (73). Several groups (101–105) have worked on metal ion sensing such as Hg^{2+}, Cu^{2+} and Pb^{2+} using QCs@protein; simplicity in preparation makes them attractive tool for such applications (Table 1). Muhammed et al. showed metal enhanced fluorescence in case of Au_{38}@BSA, fluorescence turn off in the presence of Cu^{2+} and turn on in the presence of glutathione (71). Various other proteins containing Au_{QCs} and Ag_{QCs} have been employed for metal ion sensing such as trypsin, pepsin, ESM (75, 85, 105). Au_{QC}@horseradish peroxidase has been used to detect H_2O_2 (106). Wang et al. used Au_{QC}@BSA for the detection of glutaraldehyde in water (107) and Liu et al. used it for the detection of cyanide (108). Guo et al. synthesized red fluorescent stable silver clusters in denatured BSA and used the clusters for Hg^{2+} detection (109). Recently Goswami et al. synthesized blue emitting Cu_{QC}@BSA and showed that it can be used as a sensor for H_2O_2 and Pb^{2+}. They have proposed that the additional aggregation due to Pb^{2+} ions was responsible for fluorescence quenching (110).

Apart from direct metal ion sensing, ECL based sensing has also been demonstrated. Li et al. demonstrated that ITO coated Au_{QC}@BSA exhibited ECL and reported that ITO played a significant role in enhancing ECL. They reported that in the presence of anionic co-reactant $S_2O_8^{2-}$, ECL was enhanced and demonstrated its application to detect dopamine (111) (Fig. 6A). Fang et al. showed the generation of ECL from Au_{QC}@BSA in the presence of tetraethyl amine (TEA) and showed that ECL is differently influenced by the metal ions; here they showed it to be affected by Pb^{2+} (112) (Fig. 6B). Recently, graphene conjugated Au_{QC}@BSA has also been employed for generating ECL (113). Hun et al. recently employed Au_{QC}@BSA in chemiluminescence based experiments for the detection of lysozyme in cells (114). Antibacterial composites have also been made using NMQCs@proteins. Sreeprasad et al. showed that Au_{QC}@Lf can be used to create luminescent patternable composites together with chitosan and graphene oxide. Au_{QC} in these composites were not quenched even in

Table 1. List of proteins used for NMQCs synthesis and their demonstrated applications

Protein	Metal cluster	Study and application	References
Bovine serum albumin	Au, Ag, Cu	Sensing of Hg^{2+}, Cu^{2+}, Pb^{2+}, H_2O_2, Glutaraldehyde, and cyanide, electrochemiluminescence, cluster evolution, bio-imaging and *in vivo* imaging.	70–71, 94, 103–104, 101, 107–114, 117–121
Lysozyme	Au	Hg^{2+} sensing, antibacterial activity	72, 102, 116
Cellular retinoic acid Binding protein II	Au		72
Lactotransferrin	Au	Cu^{2+} sensing, FRET, composite with graphene cluster evolution and bio imaging	73, 94, 115, 91
Insulin	Au	Grown in crystals, bio imaging and bioactivity	93
Pepsin	Au	Hg^{2+} sensing, Blue, green and red emitting Au_{QC}	85
Trypsin	Au	Hg^{2+} sensing	105
Serum transferrin	Au	Bio imaging	74
Egg shell membrane (mixture of proteins)	Au, Ag	Hg^{2+} sensing	75
α-Chymotrypsin	Ag	Cluster-protein interaction	67
Horseradish peroxidase	Au	H_2O_2 sensing	106
Human serum albumin	Au	NOx sensing	77
Egg white	Au	Metal ion sensing	91
Ovalbumin, papain	Au	Metal ion sensing	91
Cell matrix (nucleolin)	Au, Ag	Intracellular synthesis	69, 79

the presence of Hg^{2+} ions (115). Chen et al. reported that Au_{QC}@lysozyme has enhanced antibacterial activity against resistant strains (116).

Several biological applications of the NMQCs@proteins have also been demonstrated. Retnakumari et al. showed that Au_{QC}@BSA conjugated with folic acid can be effectively used to target the folate receptors in cancer cells; this was the first report to employ QC@protein for molecular receptor specific application and in another report, they showed Au_{QC}@BSA can be conjugated to monoclonal antibodies and used for targeted detection of acute myeloid leukemic cells (70, 117) (Fig. 7C).

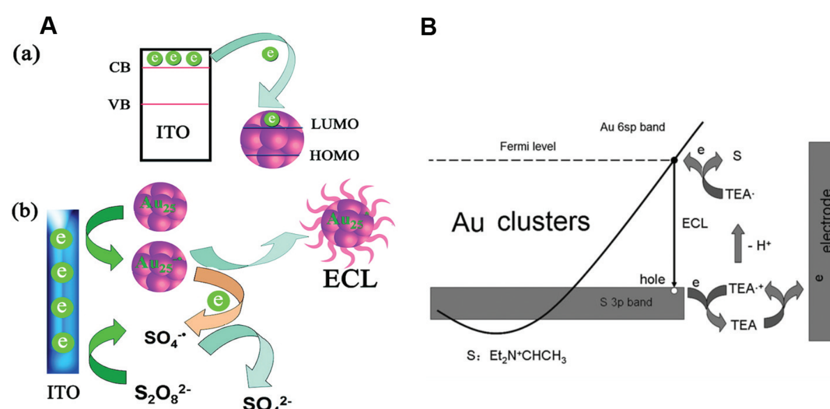

Fig. 6. Electro-chemiluminescence of (A) Au_{QC}@BSA coated on ITO plate where $S_2O_8^{2-}$ was used as co-reactant. (a) Electron transfer between ITO and Au_{QC}@BSA and (b) the ECL mechanism of Au_{QC}@BSA in the presence of anionic co-reactant, $S_2O_8^{2-}$. (B) In another study, TEA was used as co-reactant. Figure illustrates the mechanism of ECL of Au_{QC}@BSA in the presence of cationic co-reactant, TEA. A and B are adapted from references 111 and 112, respectively.

Similarly, Muhammed et al. have also shown folate receptor specific uptake of Au_{QC}@BSA by human epidermoid carcinoma KB cells (71) (Fig. 7A). Recently, Wang et al. have conjugated Au_{QC}@BSA to herceptin (a widely used humanized monoclonal antibody in case of breast cancer) to nuclear target Erb2 over-expressing HER2+ breast cancer cells for targeted cancer therapy. Unconjugated Au_{QC}@BSA were not taken up by cells, thus demonstrated the targeting ability (118). Previously, to impart functionality one has to rely on conjugation chemistry to conjugate with biofunctional molecules, now due to the arrival of QCs@functional proteins, biofunctionality becomes intrinsic. Au_{QC}@insulin has been used for bioimaging of brain cells (Fig. 7B) and as a CT contrast agent. Commercially available insulin and Au_{QC}@insulin are shown to reduce the blood level glucose in a similar manner and no considerable change was observed. They suggested that the preservation of bioactivity of insulin, even after the formation of clusters in them, is mainly due to the intact disulphide bonds. They further have shown that in undifferentiated myoblast cells having less number of insulin receptors, uptake of Au_{QC}@insulin was less compared to the differentiated myoblast cells having increased number of receptors (93). Durgadas et al. recently used Au_{QC}@BSA as a tool to detect intracellular presence of copper ions after treating the cells with copper solution (119) and have proposed a dialysis method to isolate circulating cancerous cells from normal cells in blood by conjugating Au_{QC}@BSA with superparamagnetic nanoparticles (120). Le Guével et al. used Au_{QC}@BSA protected by silica shell and Au_{QC}@-serum transferrin for bio imaging of A549 cells (74).

NMQCs@proteins have been used for *in vivo* imaging also. Wu et al. used Au_{QC}@BSA for in-vivo imaging of cancer tissue in an animal model by exploiting the enhanced permeability and retention (EPR) effect of cancer tissue and this was the first report to use Au_{QC}@protein for in-vivo imaging (121) (Fig. 7D).

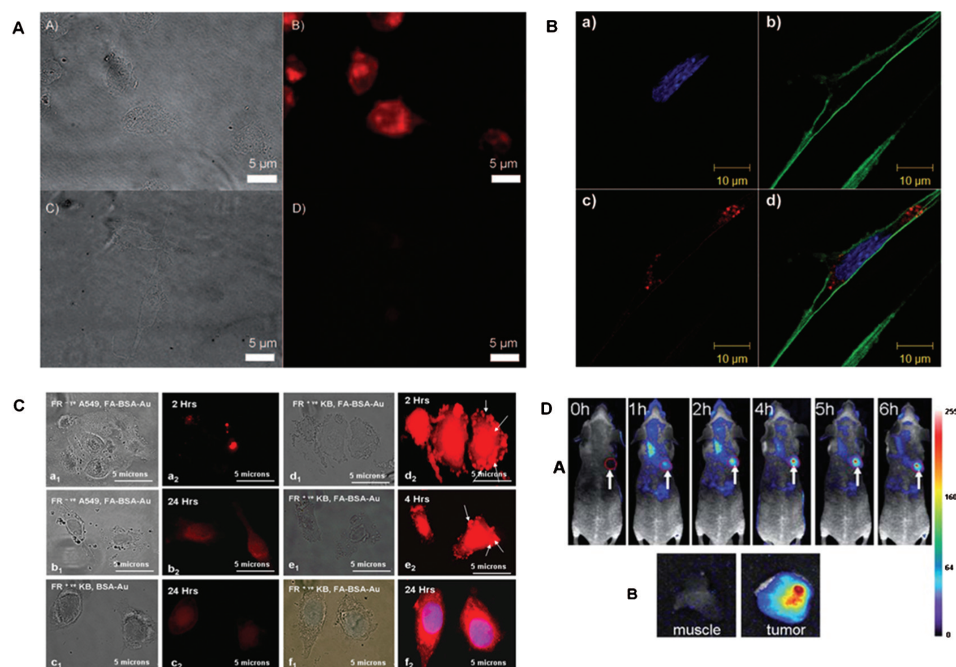

Fig. 7. Bioimaging applications demonstrated using Au_{QC}@proteins. (A) Uptake of FA-Au_{QC}@BSA (synthesized by etching of larger nanoparticles with BSA) conjugated with folic acid Bright-field (A,C) and the corresponding fluorescence microscopy (B, D) upper panel images show the interaction of FA- Au_{QC}@BSA with folate-receptorpositive KB cells with FA-conjugated Au_{QC}@BSA. lower panel images show FA- Au_{QC}@BSA interaction with folate-receptor-negative mouse fibroblast L929 cells. (B) Microscopic observation of internalization of the Au_{QC}@insulin. Differentiated C2C12 myoblasts were treated with insulin–Au_{QC} for 2 h. a) Cell nucleus stained with 4',6-diamidino-2-phenylindole (DAPI, blue). b) Actin fiber stained with Alexa Fluor 488 phalloidin to confirm the cell boundary (green). c) Au_{QC}@insulin exhibit red luminescence. d) Fluorescence image overlay of the three images. (C) Molecular receptor specific uptake of Au_{QC}@BSA conjugated to folic acid (FA). Fluorescent microscopic images showing interaction of Au_{QC}@BSA–FA with different types of cell lines: (a1)–(a2) FR $^{-ve}$ lung carcinoma A549, (b1)–(b2) FR-depressed oral cell carcinoma, KB, (c1)–(c2) FR $^{+ve}$ KB cells with unconjugated Au clusters, (d1)–(d2) FR $^{+ve}$ KB cells with FA-conjugated Au clusters at 2 h, (e1)–(e2) 4 h and (f1)–(f2) 24 h of incubation. (D) In vivo imaging using Au_{QC}@proteins. (A) Fluorescence images of mice bearing an MDA-MB-45 tumor. Strong signal from Au_{QC}@BSA was observed in the tumor (marked by the red circle) demonstrating significant passive accumulation in the tumor by the EPR effect. The arrowheads indicate the tumor. (B) Ex vivo fluorescence image of the tumor tissue and the muscle tissue around the tumor from the mice used in A. A, B, C and D were adapted from references 71, 93, 70 and 121, respectively.

Nie et al. reported controlled assembly of two gold quantumclusters in a well studied protein, ferritin. They assembled two Au_{QCs} (blue and red emitting) at the ferroxidase active sites of apo-ferritin heavy chain and showed that the resulting nanostructures (Au_{QC}@Ft) retained the intrinsic fluorescence properties of the cluster with enhanced intensity. Native structure of ferrtin was intact after assembly and they used it for *in vivo* kidney targeting and bio imaging (122).

Summary and outlook

There has been an impressive growth in nanoscience and technology at the nano-bio interface. In this review, we have addressed such an interdisciplinary system where clusters of quantum confined noble metal atoms are grown by the union of materials and biology. NMQCs in protein templates are recent additions to the family of QCs with the fascinating advantage of being embedded in a functional macromolecule matrix. Increasing research interest in this system indicates an emerging trend. To mention the versatility of this approach, not-limiting to NMQCs, even doped semiconductor QDs, much bigger in size, are also synthesized in aqueous phase recently (123). It may be pointed out that nanoparticles of metals and semiconductors were synthesised by bacteria, viruses and fungi several years ago and biological synthesis is now fairly established (124–126). However, there has been very little understanding about NMQCs@protein systems. Complete structural understanding of the system is essential including how such systems evolve with time. For such an understanding, crystal structures of the intact protein protected metal clusters are necessary. Nevertheless, it can be understood to an extent by various mass spectrometric and spectroscopic tools. Clusters in liquid-protein systems would have considerable advantage in future (127). Molecular mechanics and dynamics based computational studies combined with DFT simulations would help to refine our understanding. Metallo-protein systems like ferritin, aconitase and laccase are known to have different metal cluster cores and the enzymes use the cores as functional sites. Likewise, in future, it is likely that luminescent noble metal cluster-protein systems having cluster-based active sites to function as catalysts. The cluster core at this specific size can have interesting redox properties which may affect the stability of the cluster itself. It can be viewed as a mix of bio-mimetic and bio-kleptic nanotechnology since we exploit the functionality of the protein (128). The catalytic chemistry mentioned above may be manipulated with photons to have new kinds of photocatalysis. Synthesizing clusters at physiological pH would have potential benefits in the field. Since, this system has opened a new area at the nano-bio interface; there is a need to know to what extent these proteins are modified and what exactly is modified. Considering the biological applications of these cluster systems, despite the ongoing research efforts, there is a substantial need to know how these proteins with clusters are going to interact with the biological systems and what would be the intracellular fate of the uptaken clusters. We also should know whether these modified proteins are seen differently by the cell machinery. Proteins are fundamentally biological nanomachines. Rationally designed proteins with clusters would mean creating a permanent indicator to the nanomachines so that we can track them anytime. Thermostable templates mimicking the extremozymes where the cluster's stability is taken care of would have big impact in high temperature reactions especially as molecular beacons in PCR and in catalysis. To date, all the studies indicate the possibilities of growing clusters in organisms, if environment is suitably managed; it would not be surprising, if we get viruses growing QCs, after all it is also a protein body containing genetic material. It is up to us to identify the right protein system. In a nutshell, NMQCs in peptide and protein templates are expected to have plenty of applications in electronics, chemistry, biology and medicine over the years to come for which early signs are apparent in the recent literature.

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There is no conflict of interest in the present study for any of the authors.

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