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BIOMOLECULES: POTENTIAL SCAFFOLDS FOR ULTRASMALL METAL NANOPARTICLES SYNTHESIS

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Abstract

The ultrasmall metal nanoparticles (size <2 nm) have emerged as missing link between atom and molecules. These ultrasmall metal nanoparticles are exciting category of nanomaterials owing to their distinct physiochemical properties. The biomolecules including protein, peptides and nucleotides with rich and diverse functional moietiesare promising class of ligand for the synthesis and functionalization of ultrasmall metal nanoparticles in greener approach. The biomolecules stabilized ultrasmall nanoparticles have found tremendous applicability in numerous applications related to biosensing of metal ions and biomolecules and biomedical fields for labelling of cancerous cells.

1. Introduction

The advent in nanotechnology has led to intensive research in the area of synthesis, properties, and various applications related to ultrasmall metal nanoparticles, commonly known as metal nanoclusters (MNCs)(R. Jin et al. 2016; Chakraborty and Pradeep 2017).Ultrasmall metal nanoparticles are of ultrasmall size (<2 nm) consisting of few to hundreds of atoms and are known to bridge the gap between an atom and molecules(Kawawaki et al. 2021).These ultrasmall size is comparable to Fermi wavelength of electrons which results in splitting of continuous density ofenergy states into discrete energy level. These discrete energy levels are responsible for imparting unique optoelectronic and chemical properties(Zhou et al. 2019).The extraordinary properties of ultrasmall metal nanoparticles mainly includes;optical absorption, photoluminescence, non-linear optical properties such as two photon fluorescence and absorption, ultrafast dynamics,large stock shift, quantized charging, molecular chirality, magnetization(Khandelwal and Poddar 2017). Ultrasmall metal nanoparticles owing to such distinct properties have emerged as potential candidate for wide varieties of application related to bioimaging, drug delivery, anticancer agent, photosensitizer, efficient catalysis and biosensing(Shang, Dong, and Nienhaus 2011; Sharma, Wangoo, and Sharma 2021).

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Ultrasmall metal nanoparticles as bare are not stabilized in solution, and therefore require protection from stabilizing agent. The initial reports are about synthesis of these ultrasmall metal nanoparticles using organic ligands such as thiolates and phosphines(Pandurangan et al. 2022). The utilization of such organic ligands has led to detailed investigation in term of total atoms count in NCs along with structural aspects related to metal core and staple motif around core using single crystal X-ray crystallography(Cox et al. 2019). Another exciting class of ligand includes biomolecules such as protein, peptide and nucleic acid(Kailasa et al. 2021). Biomoleculeshave emerged as new age soft template for the synthesis and functionalization of ultrasmall metal nanoparticlesthrough synergistic efforts involving the metal ion reduction along with protection through biomineralization process. The advantages of biomolecules as template mainly includes; firstly, the abundant functional group such as hydroxy, amine, carboxylic, and thiol present in biomolecules can provide multiple binding sites for ultrasmall metal nanoparticles growth in controlled manner. Secondly, the synthesis of ultrasmall metal nanoparticles using biomolecules can be carried out at ambient reaction conditions in water as medium. Thirdly, the biomolecules can serve as reducing cum protecting agent and therefore eliminates the use of harmful and poisonous reducing agent. Thus, biomolecules are considered as appealing and amazing tools for the formation of Ultrasmall metal nanoparticles in environmentally friendly manner(Goswami, Zheng, and Xie 2014).

Biomolecules functionalized ultrasmall metal nanoparticlesare an emerging area of interest owing to of its ability to act as a scaffold in various biomedical applications, for example, bio-detection, drug delivery and cellular uptake studies etc(Rana et al. 2012; Dykman and Khlebtsov 2014). Due to unique opto-electronics properties of biomolecule functionalized nanomaterials, they can be explored for the detection of metal ions, enzyme and antibodies (Gupta et al. 2010; Yuan et al. 2011). Additionally, biomolecules conjugated nanomaterials can act as a targeting probe, drug carrier and synthetic vaccine due to the characteristic properties of peptides such as small size, biocompatibility, cell-permeabilityand tendency to undergo surface modification with different moieties (Borghouts, Kunz, and Groner 2005). Therefore, in the present review, various synthesis route and methods for the ultrasmall metal nanoparticles formation has been reported. In addition, recent reports for ultrasmall metal nanoparticles synthesis utilizing protein, peptide and nucleic acid have been summarized. Finally, brief application of the biomolecule functionalized ultrasmall metal nanoparticleshas been discussed.

2. Methods for synthesis of ultrasmall metal nanoparticles

In general, two main strategies are employed for the synthesis of ultrasmall nanoparticles which include bottom-up and top -down approach (figure 1). The former approach involves reduction of metal ions into atoms which further undergo nucleation and aggregation to form nanomaterials (Santiago González et al. 2010). In contrary to this, in the top-down approach, the large size nanoparticles are itched with suitable excess etching agent for the production of nanomaterials. Top-down approach is followed during synthesis of ultrasmall metal nanoparticles by etching method from large size nanoparticles(Lin et al. 2009). On the other hand, the bottom-up approach is followed when ultrasmall metal nanoparticles are prepared using chemical reduction method by various approaches such as microwave-assisted, photoreductive and sonochemical methods. All the methods have been explained in below subsections:

***2.1. Etching-based synthesis*:**The etching-based synthesis involves use of an excess amount of thiol molecules to prepare fluorescent metal nanomaterial from metal nanoparticles. The etching mechanism includes both ligand-induced and core induced etching. In the former method, the more stable thiolate ligand removes the surface of metal nanoparticle leading in the formation of metal-thiolate complexes which then undergo strong metal-metal interaction to form fluorescent metal nanomaterials. Muhammed *et al.* synthesized luminescent quantum cluster of gold by the core etching of mercaptosuccinic acid protected gold nanoparticles by bovine serum albumin (BSA) with a quantum yield of 4 %. The core of cluster consists of 38 gold atoms characterized by mass spectrometry and it acts as a fluorescent sensor by acting as “turn off” fluorescence for Cu^{2+} ion and “turn on” for the detection of glutathione (Habeeb Muhammed et al. 2008).

***2.2. Chemical Reduction*:** Mostly, ultrasmall metal nanoparticles are prepared by reduction of metal precursor salt into metal atom in the presence of various reducing agent such as sodium borohydride ($NaBH_4$), citrate ions, hydrazine, ascorbic acid etc. The stabilizing agent, including various thiolate-based ligand, protein, polymer, dendrimer and peptides to stabilize the small sized fluorescent nanomaterials.Glutathione (GSH), a nature thiol containing molecule, has been widely employed for protecting the Au (III) when they were being reduced by strong reducing agent such as $NaBH_4$(Schaaff and Whetten 2000). Moreover, by adapting a similar approach, other thiol-containing molecules such as captopril (Kumar and Jin 2012), dihydrolipoic acid (Shang et al. 2012), dodecanethiol (Shang et al. 2011), Dpenicillamine (DPA) (Nair et al. 2015), lipoic acid (Shiraishi, Arakawa, and Toshima 2002), mercaptopropionic acid (Lee et al. 2004), phenylethylthiolate (Yuping Wang et al. 2018) have been exploited to prepare metallic nanomaterials.

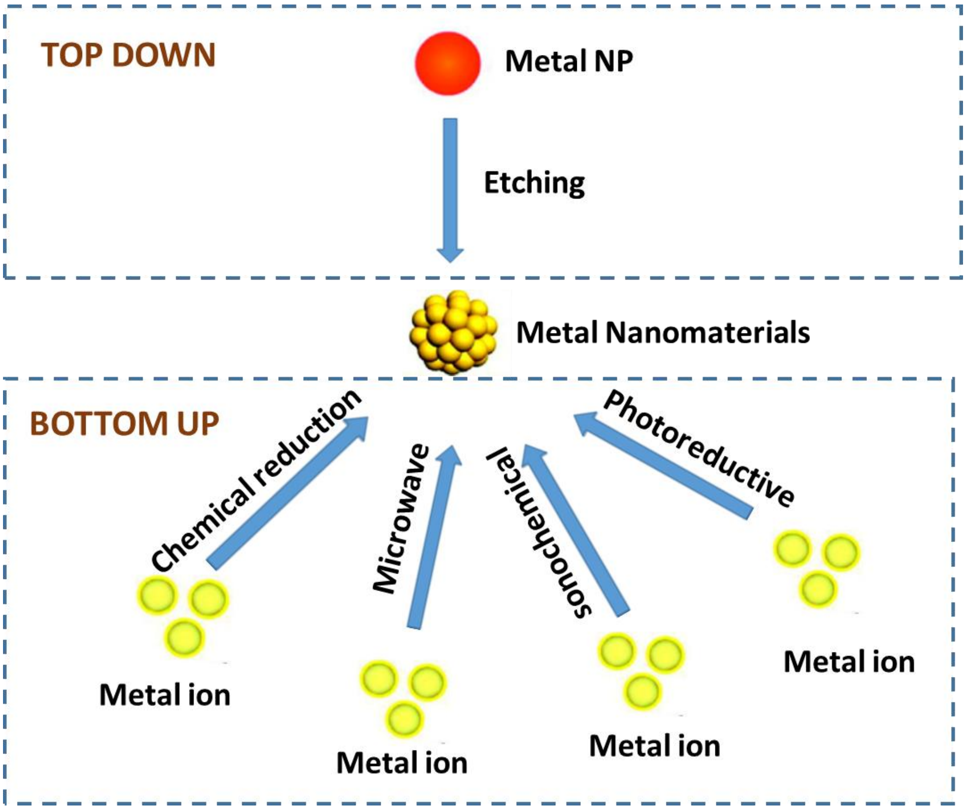

Figure 1 Synthetic approach for ultrasmall metal nanoparticles

2.3. Microwave-assisted synthesis: The microwave radiation speeds up the reaction by increasing the friction between polarized molecules and thus heats up the entire solution. Li Shang *et al.* synthesized dihydrolipoic acid (DHLA) capped fluorescent gold nanocluster using microwave irradiation of 180 W which not only shortened the time to 4 minutes but also resulted in five-fold enhancement of quantum yield (QY) (Shang et al. 2012). The synthesized fluorescent nanomaterials were used for the sensing of Hg^{2+} ion with lowest limit of detection (LOD) of 0.5 nM in HeLA cells using spinning confocal microscopy. Yue and co-workers using BSA as the reducing agent and the stabilizer, synthesised highly fluorescent Au nanomaterials with 16 gold atoms under microwave irradiation of 600W power for 6 hours(Yue et al. 2012) .

2.3.Sonochemicalsynthesis:Another useful non-hazardous method for the preparation of high purity, controlled size and uniform shape fluorescent nanomaterials is the sonochemical approach. Due to numerous advantages of ultrasound such as high efficiency, low instrumental requirements, significantly reduced process time compared with other conventional techniques, and its low cost, thus making it greener method for the synthesis of nanoparticles. The chemical effect of high intensity ultrasound can be used to achieve very high temperature, pressure and extremely cooling rate, thus providing unique platform for growth of nanoparticles. Li *et al.* reported the synthesis of colour tuneable fluorescent gold nanocluster using toluene and 60 min of ultrasonic radiation without further use of any reducing or stabilizing agents (Li 2013). Ultrasonic irradiation can easily modulate cluster composition, size, fluorescence colour, quantum yield, and stokes shift via exposure time.

2.4.Photoreductive synthesis*:* Highly fluorescent metallic nanomaterials can be synthesised with the help of ultra violet light irradiation on metal precursors which can effectively devoid the use reducing agent. Soejima*et al.* synthesized gold cluster on titanium dioxide (TiO_2) surface by two step method consisting of Au(III) complex chemisorption via ligand exchange mechanism and subsequent photoreduction with H_2O at temperature below 25 ^{0}C. Water which is absorbed both physically and chemically on the surface of TiO_2 act as reducing agent for the reduction of Au (III) to Au (0) (Hattori et al. 1999). In another report, using robust photoreduction technique, fluorescent copper, silver and gold fluorescent nanomaterials were synthesised using poly (methacrylic acid) functionalized with pentaerythritol tetrakis 3-mercaptopropionate with quantum yields of 2.2, 6.8 and 5.3%, respectively. Compared with the use of conventional reducing agents, photoreductive synthesis is a low cost, non-toxic, less time-consuming and more environment-friendly method for preparing fluorescent metal nanomaterials (H. Zhang et al. 2012).

3. Biomolecules as template for synthesis of ultrasmall metal nanoparticles

3.1. Protein as a template for the synthesis of ultrasmall metal nanoparticles: Protein, which are among the important biologically active molecules have emerged as key to design next generation ultrasmall metal nanoparticles via biomineralization process. Biomineralization is a natural process in which living organisms adapt to form hard structures by mineralizing metal ions through peptides, vesicles, etc. The protein as a template provides several advantages including:

(1) No additional reducing agents are required for the synthesis of metallic nanomaterials.
(2) The process of preparation of metal nanomaterials is relatively simple and easy.

(3) The soft template of proteins having well-defined 3-D network which bind strongly to metal atom to form uniform size fluorescent metal nanomaterials with tunable emission.

Various proteins have been used widely used to synthesis water soluble, highly fluorescent nanocluster such as bovine serum albumin (BSA), lysozyme, pepsin, trypsin, transferrin family proteins, urease, insulin, horseradish peroxidase (HRP), DNase I, and ribonuclease A (RNase) along with their application have been listed in the Table 1 below.

Table 1: Description of various proteins for the synthesis of ultrasmall metal nanoparticlesalong with theirapplications

Protein	Metal(s) used	Applications	References
BSA	Au, Ag, Cu,	Sensing of $Hg^{2+,}$ $Cu^{2+,}$ $Pb^{2+,}$ H_2O_2, Glutaraldehyde, and cyanide, bio-imaging *In vivo* imaging	(Mathew, Sajanlal, and Pradeep 2011; H. Liu et al. 2013; Park et al. 2013; Y. Liu et al. 2010a, 2010b)
Lysozyme	Au	Hg^{2+} sensing, antibacterialactivity	(Wei et al. 2010)
Insulin	Au	Grown in crystals, bio imaging, bioactivity	(C.-L. Liu et al. 2011)
Pepsin, trypsin, egg shell membrane	Au	Hg^{2+} sensing	(Kawasaki et al. 2011)
Horseradish peroxidase	Au	H_2O_2 sensing	(G. Liu et al. 2012)
Human serum albumin	Au	NOx sensing	(Yan et al. 2012)

Au= gold, Ag= silver, Cu= copper, Hg^{2+}= Mercury ions, Pb^{2+}= lead ions, H_2O_2= hydrogen peroxide, NOx= oxides of nitrogen

3.2. Peptides as a template for the synthesis of ultrasmall metal nanoparticles: Both natural and synthetic peptides have been used for the synthesis of metallic nanomaterials. A peptide can be rationally designed with required properties for the generation of nanomaterials. Peptides have unique advantages such that they can be easily synthesized in comparison to other biomolecules including nucleotides and polymers. Secondly, peptides can be assembled into desirable structure and morphology. Thirdly, the specific targeting ability of selected peptide can be utilized for enhanced binding affinity with the target. Further, the directing role of the peptide with well-defined amino acid sequence can be utilized for the synthesis of metal nanomaterials, which eliminates the use of conjugating ligands. The most commonly studied peptide for the synthesis of fluorescent metal nanomaterials has been Glutathione (GSH), which is a natural peptide with sequence γ-Glu-Gly-Cys. Schaff and co-workers, synthesized GSH-Au nanoclusters by direct mixing of $HAuCl_4$ with GSH to form GSH-Au^+ oligomer which was further reduced to Au^0 by addition of $NaBH_4$ and subsequently assembled into gold fluorescent nanomaterials (Schaaff and Whetten 2000). The GSH-Au nanomaterials have found wide variety of application in the field of sensing and bioimaging (G. Zhang et al. 2013). Numerous studies were also reported for the synthesis of other metallic nanomaterials including Ag and Pt using Glutathione (le Guével et al. 2011).

Metallic nanomaterials can also be generated using as short as single amino acid to dipeptides or tripeptides along with long peptide sequence. Adikari and co-workers, had fabricated highly stable and transparent hydrogel using N-terminal protecting dipeptide, Fmoc-Val-Asp-OH(Roy and Banerjee 2011a). The prepared hydrogel with well-defined three-dimension structure was effectively utilized to synthesis fluorescent silver nanomaterials of fever atoms in the presence of diffused sunlight at physiological pH (7.46). These silver cluster were characterized using UV-Vis spectroscopy, photoluminescence spectroscopy, high-resolution transmission electron microscopy (HR-TEM), atomic force microscopy (AFM) and X-ray diffraction (XRD) studies. Using the same approach Subashish*et al.* have similarly synthesised Ag fluorescent nanomaterials using Fmoc-Phe-OH hydrogel (Roy and Banerjee 2011b). Serial of blue and red emitting Ag fluorescent nanomaterials has been synthesized with the help of artificial peptide having amino acid sequence CCYRGRKKRRQRRR (Cui et al. 2011). The phenolic moiety of the peptide reduced Ag^+ and formed Ag cluster were captured via thiol group of cysteine which was elucidated by the UV absorption studies and IR spectra. A description of different peptides being employed for the synthesis and functionalization of material nanomaterials and their application has been listed in Table 2:

Table 2 Description of different peptide for the synthesis and functionalization of metallic nanomaterials and their applications

Peptide	Metal(s) used	Application	References
Fmoc-Val-Asp-OH **Fmoc-Phe-OH**	Ag	Antibacterial properties and bioimaging	(Roy and Banerjee 2011b, 2011a)
GSH(γ-Glu-Gly-Cys)	Au, Ag, Pt, Cu	Bioimaging, biolabeling and sensing	(leGuével, Spies, et al. 2012; Roy, Baral, and Banerjee 2014; Stamplecoskie and Kamat 2014; Negishi, Nobusada, and Tsukuda 2005)
c(RGDyC)	Au	Tumour-targeted radiotherapy sensitizer	(leGuével, Trouillet, et al. 2012)
CCIHK(Ac) [HDAC-1]	Au	Detection of Post-translational modification Enzymes	(Liang et al. 2017)
Cyclo(DRPECEYDPC)	Gd-Au	Dual model imaging in cellular imaging and *in vivo* T1-weighted MRI	(Wen et al. 2013)

CCYGGPKKKRKVG	Cu	Biolabeling: specifically mark the nuclei of HeLa and A549 cells	(Dong et al. 2013)
H_2N-CCYRGRKKRRQRRR-COOH (CCYTAT)	Agand Ag	Bioimaging: stain the nuclei of three cell lines (HeLA, GES-1, MRC-5)	(Yaling Wang et al. 2012; Sun et al. 2011)
H_2N-YHWYGYTPQNVI-KKKKYCC-COOH	Auand Ag	Antitumor effect *in vitro* and *invivo* Temperature sensing and cellular imaging	(Zhai et al. 2017, 2018)
RGDAEAKAEAKCCY	Au	Temperature sensing and cellular imaging	(W. Zhang et al. 2017)

Au= gold, Ag= silver, Cu= copper, Pt= platinum, GES 1= gastric epithelial cell line, MRC 5= Medical Research Council cell strain 5, MRI= magnetic resonance imaging

3.3 Nucleotides as scaffold for the synthesis of ultrasmall metal nanoparticles:Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are another good example of biomolecules that can serve as a template for the synthesis of nanomaterials. DNA template synthesis has a number of advantages. Firstly, in addition to serving as a template, DNA possesses functional roles for molecular recognition (e.g., aptamers). With appropriately designed DNA sequences, it is possible to directly couple analyte binding with fluorescence signalling. Secondly, due to smaller size of DNA than proteins, the synthesized fluorescent nanomaterials are not deeply penetrated which allows more effective energy transfer to other fluorophores and quenchers. Chris *et al.* synthesized Ag fluorescent nanomaterials by using a single stranded DNA 5'-AGGTCGCCGCCC-3' as a template, with different sizes and hence of different emission colours like blue, green, yellow, and red which are highly photostable and exhibit great stability than commonly used cyanine dyes (Richards et al. 2008). Similarly, Cu fluorescent nanomaterials had been synthesised using double stranded DNA (dsDNA). The author explored interesting finding that such Cu fluorescent nanomaterials synthesis did not happen in case of single stranded DNA (ssDNA) and DNA triplex (Jia et al. 2012). This study suggested that the Cu fluorescent nanomaterials are possibly protected by the groves of the dsDNA. Such groves are however not present in the ssDNA or have been blocked in the DNA triplex, resulting in their poor protection for ultrasmall Cu fluorescent nanomaterials. Analogous to DNA-protected Ag nanomaterials, RNA has also been used to synthesize Ag fluorescent nanomaterials. For instance, Schultz *et al.* demonstrated that single-stranded RNAs can host luminescent Ag fluorescent nanomaterials, where the RNA maintains its integrity (Schultz and Gwinn 2011).

4. Application of biomolecules functionalized ultrasmall metal nanoparticles

The unique optical, electrical, and physiochemical properties of biomolecules functionalized ultrasmall metal nanoparticles made them interesting for use in various fields from the sensing of heavy metal ions, inorganic anions, small molecules, proteins, DNA, RNA to antibacterial, anticancer, biological imaging and labelling.

4.1.Detection of metal ion and anions: The detection of heavy metal ions is of great significance due to their high toxicity, which can bind to various cellular components, thus resulting in the dysfunction, harm human health. Due to fluorescence quenching or enhancing by heavy metal ions, a huge number of fluorescent metal nanomaterials have been used to sense various heavy metal ions, such as Ag^{+}, As^{3+}, Cd^{2+}, Cr^{3+}, Cr^{6+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, Sn^{2+} etc. (L. Zhang and Wang 2014). In addition to the determination of heavy metal cations, fluorescent Au nanomaterials have been applied for the sensing of harmful anions, such as cyanide, nitrite and sulfide. Red-emitting BSA-stabilized Au nanomaterials were used as fluorescence probe for detecting Hg^{2+}, Cu^{2+} and CN^{-} ion with limit of detection (LOD) of 0.5 nM, 50 uM and 200 nM respectively (Y. Liu et al. 2010c; Durgadas, Sharma, and Sreenivasan 2011; Xie, Zheng, and Ying 2010).

4.2. Detection of biomolecules:small biomolecules, such as Adenosine thiophosphate (ATP), glucose, GSH, folic acid (FA), and hydrogen peroxide (H_2O_2), dopamine (DA) plays an important role in many cellular biochemical processes and have been considered as an important factor in some diseases and their therapy. Dong et al. designed a simple and convenient method for turn-on detection of ATP using DNA protected Ag nanomaterials as fluorescence probe, which shows high sensitivity and selectivity, and a detection limit of 0.2

nM was obtained (L. Jin et al. 2011). Qu et al. developed an easy prepared fluorometric and colorimetric dual channel probe for DA detection with high sensitivity and selectivity by use of BSA-stabilized Au nanomaterials. The BSA-Au Fluorescent nanomaterials exhibit strong fluorescence emission, while upon addition of DA, the Au Fluorescent nanomaterials show a dramatic decrease of the fluorescence intensity as a result of the photoinduced electron transfer process from the electrostatically attached DA to the BSA capped gold nanomaterials. The detection limit of DA can be as low as 10 nM(Govindaraju et al. 2017).

4.3. Biomolecules functionalized ultramsmallmetallic nanopaticles as efficient platforms for biomedical applications: fluorescent metal nanomaterials possess an attractive set of features, such as ultrasmall size, good biocompatibility, brightness and photostability, which renders them attractive alternatives as fluorescent probes for biological labeling and imaging. Lin and co-workers synthesised Au NCs as fluorescent probes for cellular marking in CAL-27 cancer cells and MC3T3-E normal cells, respectively (Zhao et al. 2019). The results indicated that the fluorescence signals were not only distributed in the cytoplasm but most came into the cellular nucleus for both CAL-27 and MC3T3-E1. In another report by Wang et al., the fluorescent Au nanoclusters were spontaneously biosynthesized by cancerous cell (i.e., HepG2, human hepatocarcinoma cell line; K562, leukaemia cell line) through Au(III) reduction inside cells cytoplasm's, and ultimately concentrated around their nucleoli, thus affording precise cell imaging (J. Wang et al. 2013). Fluorescence lifetime imaging (FLIM) is also an alternative technique for cell imaging that takes advantages of the longer lifetime (>100 ns) of metal nanoclusters than that of auto-fluorescence of cellular organelles. Two-photo excitation confocal microscopy was used to observe biological imaging, which can afford some advantages, such as the ability of deeper imaging inside tissues and the reduced phototoxicity of NIR light.

5.Conclusion

In summary, the significance of ultrasmall metal nanoparticles have been reported in terms if synthesis and unique physiochemical properties. The ultrasmall metal nanoparticles can be synthesized either of the two approaches; top down and bottom-up approach. Various methods have been mentioned for the synthesis of the ultrasmall metal nanoparticles including ligand-based etching, chemical reduction, microwave assisted, sonochemical and photoreductivesynthesis. Among different class of ligands, biomolecules such as protein, peptides and nucleotides have emerged as promising class of ligands. The biomolecules have proven to advantageous in terms of presence of rich functionalities, specific targeting ability, water-based synthesis and dual role of stabilizing and reducing agent. Also, the applicability of biomolecules functionalized ultrasmall metal nanoparticles has been mentioned for the detection of metal ions and relevant biomolecules. In addition, the potential utilization of these ultrasmall metal nanoparticles as effective labelling and bioimaging tools has been reported.

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