

Polyacrylamide gel electrophoresis: a versatile tool for the separation of nanoclusters

Vinitha Packirisamy¹  & Prabhu Pandurangan^{*,1} 

¹Department of Physical Chemistry, School of Chemical Science, University of Madras, Guindy Campus, Chennai, 600025, India; *Author for correspondence: ppandurangan@gmail.com

BioTechniques 74: 51–62 (January 2023) 10.2144/btn-2022-0086

First draft submitted: 4 August 2022; Accepted for publication: 10 November 2022; Published online: 15 December 2022

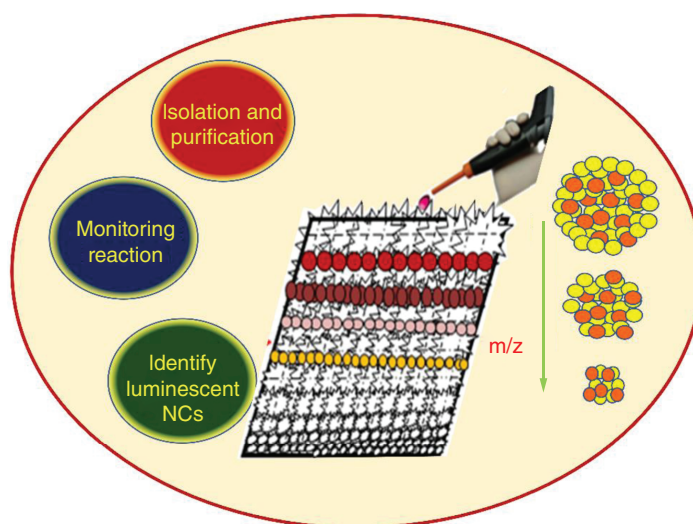
ABSTRACT

Atomically precise nanoclusters comprising 1–100 atoms have emerged as a new class of nanomaterials with intriguing size-dependent physico-chemical properties. The significant changes in the properties of nanoclusters were observed in tailoring the number of metal atoms and ligands that determines their functions and applicability. Since 1990, thiolated gold nanoclusters have been studied. The separation of monodispersed clusters was crucial and time-consuming. To address these shortcomings, several separation techniques have made it possible to separate the series of metal nanoclusters with a precise composition of metals and ligands. Among these techniques, polyacrylamide gel electrophoresis was utilized for hydrophilic cluster separation. This review shall focus on the principle, operation and application of the polyacrylamide gel electrophoresis technique to encourage a greater understanding of the characteristics and usefulness of this method.

TWEETABLE ABSTRACT

Separation of atomically precise hydrophilic metal nanoclusters using polyacrylamide gel electrophoresis technique

GRAPHICAL ABSTRACT



KEYWORDS:

gold nanoclusters • high-performance liquid chromatography • hydrophilic nanoclusters • luminescence • metal nanoclusters • nanoclusters isolation • polyacrylamide gel electrophoresis • reaction tracking • size-exclusion chromatography • thin-layer chromatography

Monodispersity refers to the amount of uniformity in the particle size and shape of nanomaterials, which is one of the key factors for their unique properties and its diversified applications in photoluminescence, sensors, catalysis and energy fields [1–5]. Among these nanomaterials, atomically precise metal nanoclusters (MNCs) have been a hot-spot topic in recent years due to their unique

and size-dependent properties. MNCs are particles that comprise atoms in precise numbers with particle sizes ranging between ~1–3 nm. Mostly, MNCs are synthesized using ligands like thiolates, phosphines, carbenes and biomolecules [6–9]. These ligands act as a stabilization agent to control the growth of atoms into nanoclusters (NCs). However, the type of ligand has significantly regulated the functions of NCs [10,11]. In general, hydrophobic ligands have been utilized for NC synthesis to attain precise composition and understand the mechanisms involved in NC formation. These hydrophobic ligand-protected MNCs possess excellent stability under atmospheric conditions. Hence, it will be easy to synthesize the MNCs with precise composition by using hydrophobic ligands. However, some synthesis protocols of MNCs may produce atomically accurate clusters, avoiding the need for additional separation [12,13]. The majority of the methods result in mixtures of clusters, therefore separation techniques must be used to isolate the mixtures. Indeed, the choice of hydrophobic ligands in NC formation limits their applicability in the biomedical field, because of their toxicity and lower biocompatible nature. The preparation of atomically precise MNCs with hydrophilic ligands readily undergoes chemical changes on environmental exposures, which has questioned their stability [14,15]. So, there is a high necessity to synthesize and characterize the hydrophilic, stable and biocompatible NCs with precise composition for their practical applications [16]. Even though they are small in size, their applicability in various fields is highly dependent on the countable metal atoms and ligands in NCs.

In recent times, the exact number of metal atoms (n) and ligand molecules (m) for metal (M) and ligand (L) in MNCs (denoted as $M_n(L)_m$) are well-quantified by advanced mass techniques like MALDI-MS and ESI-MS [17]. The function and properties of NCs are altered based on the number of core-shell metal atoms and ligands, and even the charge and isomers of the clusters also play a crucial role. Among the two structural isomers of Au_{38} NCs, one isomeric product – $Au_{38}T$ – drives catalytic reduction reaction, whereas another one – $Au_{38}Q$ – does not [18]. The solubility of these MNCs differs widely with n and m values of $M_n(L)_m$, that is, solvent-driven particle separations. The diafiltration method is mostly used for water-soluble NCs and involves the sample solution being pumped by a peristaltic pump through the diafiltration membrane. Small molecule impurities or small nanoparticles are eluted in the permeate, while the large nanoparticles (purple) are retained. Sweeney *et al.* [19] reported diafiltration membranes with molecular weight cutoff of 70 kDa, 50 kDa, 30 kDa and 10 kDa membranes for separation of Au clusters of particle sizes ranging between 2.9, 2.6, 2.5 and 2.0 nm respectively. The clusters that differ from single atoms or ligands are moreover identical in size, hence there will be a little complication in the separation of clusters with regular diafiltration and solvent-derived size separation methods based on size factor. Ghosh *et al.* [20] reported TLC for the separation of a multicomponent mixture of mixed monolayer-protected Au_{25} clusters with closely similar chemical ligand compositions, that is, separation of $Au_{25}(BT)_{18}$ and $Au_{25}(PET)_{18}$ (BT: butanethiol and PET: phenylethanethiol). However, the separation of clusters with TLC is only restricted to hydrophobic ligand-protected NCs. The specific size and precise composition of MNCs have distinct reactivity for catalytic and sensing applications. Precise and monodisperse NCs with tunable sizes by using aromatic ligand as a stabilizing agent were easily achieved through the two-phase Brust–Schiffrin method as well as the miscible-solvent-assisted two-phase synthesis method reported by Yuan *et al.* [21,22] According to their size and composition, the physicochemical properties of NCs differ. The changes in size and composition bring definite changes in the spectral pattern of UV-vis spectrum and fluorescence properties with tunable emission wavelength, thereby altering their reactivity in support of various catalytic, sensing and biomedical applications [23–25].

The polyacrylamide gel electrophoresis (PAGE) method to separate individual MNCs with improved monodispersity is highly significant. In 1972, Joseph Sambrook, Phillip Sharp and William Sugden created a biotechnique called gel electrophoresis for separating and visualizing DNA fragments. This technique still serves as the foundation for the molecular biology field. The underlying principle of this PAGE is that at an applied potential, mass-to-charge (m/z) ratio-based isolation takes place. Due to complex interactions between samples, gel matrix, buffers and electric current, particles migrate at various rates based on their total charge, size and shape, resulting in distinct bands of individual molecules. By crosslinking polyacrylamide and bis-acrylamide, a network of pores is formed in the gel. Due to the pore size, molecules can be sorted via the gel matrix. Gel pore size depends on the concentration of monomers (% T). These successful efforts to separate the clusters of the noble metals encouraged us to use this method to isolate the less stable CoNCs as well. Even though few reviews on MNCs and separation methods are available in the literature, this mini-review will present detailed illustrations for the separation of ultrasmall MNCs specifically using the PAGE biotechnique.

Experimental methods

Setting up of PAGE apparatus

The essential parts of the PAGE apparatus include glass plates with integrated spacers, plastic combs, sample loading guides, casting frames, casting stands, clamping frame electrode assemblies and apparatus cell units. The setting up of the apparatus involves several stages as described in Figure 1. Finally, the two glass plates should be combined with integrated spacers, fixed in the casting frame and positioned in the casting stand. As quickly as the resolving gel solution is ready, it must be gently poured into the spacers of the glass plates at a specific distance, leaving space for stacking gel. The as-prepared gel composition is then kept ready in the proper percentage. Ammonium persulphate (APS) is always added at the end during the preparation of the gel composition. A plastic comb is then placed on top of the resolving gel, followed by the addition of the stacking gel solution. After a short while, the plastic comb is gently removed from the stacking gels, revealing wells where the nanomaterials samples can be dropped. The casting frame is taken out of the casting stand once the samples have been loaded, set in the clamping frame electrode assembly and then put into the tetra-cell. In addition, the

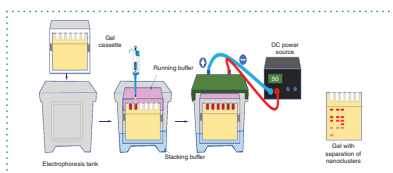


Figure 1. Schematic illustration of setting up of polyacrylamide gel electrophoresis apparatus for the separation of nanoclusters.

Table 1. Composition of resolving gel and stacking gel used for the separation of metal nanoclusters.

Stacking gel (total volume 4 ml)					
Components	4%				
H ₂ O (ml)	2.70				
30% polyacrylamide (ml)	0.67				
Tris-HCl-1.0M-pH-6.8 (ml)	0.5				
10% ammonium persulfate (ml)	0.04				
N,N,N,N-tetramethylethylenediamine	0.004				
Resolving gel (total volume 10 ml)					
Components	10%	15%	17%	20%	22.5%
H ₂ O (ml)	4.0	2.3	1.3	0.8	0.5
30% polyacrylamide (ml)	3.3	5.0	6.0	6.5	7.0
Tris-HCl-1.5M-pH-8.8 (ml)	2.5	2.5	2.5	2.5	2.5
10% ammonium persulfate (ml)	0.1	0.1	0.1	0.1	0.1
N,N,N,N-tetramethylethylenediamine	0.004	0.004	0.004	0.004	0.004

cell must then be filled with the buffer solution, the tetra cell connected to the electrical circuits, the necessary potential and the duration fixed before running the sample through the PAGE according to the m/z ratio.

Preparation of PAGE gel composition

The proportion of monomer acrylamide in the gel determines the gel's pore size, which in turn is directly proportional to the size-dependent separation of MNCs. As the acrylamide concentration rises, the gel's pore diameter contracts, thereby separating the lower mass of NCs. The acrylamide concentrations of 10, 15, 20 and 22.5% can isolate clusters with mass ranging between 16–68 KDa, 12–43 KDa, 8–30 KDa and 1–7 KDa, respectively. The polyacrylamide gel is made of the monomer acrylamide, the crosslinker N,N'-methylene bis-acrylamide, the accelerator N,N,N',N'-tetramethylethylenediamine (TEMED) and the free radical generator APS. Gel buffer solutions must be stacked and resolved to separate NCs as they move through the porous gel when an applied potential is present. Tris-HCl buffer, often known as Trizma, is used to prepare the buffer solutions. To separate NCs using the mass-to-charge (m/z) method, the pH gradient of the stacking and resolving gel buffer solutions must be maintained at 6.8 and 8.8, respectively. The pore size in the gel is influenced by two variables: 1) acrylamide and bisacrylamide concentrations (% T) and 2) crosslinker concentration (% C) The size of the pores inside the gels reduces as the % T is raised. As a result, gels with a larger percentage are suited for separating small nanomolecules from one another. The pore size reduces as the % C rises. Table 1 provides specific examples of gel formation regarding the percentage of monomer, APS, and TEMED.

General illustration for the isolation of MNCs

The separating and stacking gels are prepared by acrylamide monomers with total contents of 30% and 3 wt % (acrylamide/bis-acrylamide, 94:6), respectively. The eluting buffer consisted of 192 mM glycine and 25 mM tris(hydroxymethylamine). The as-prepared clusters were dissolved in a 5% (v/v) glycerol/water solution (0.5 ml) at a concentration of 60 mg/ml. To obtain an adequate separation, the sample solution (0.5 ml) was put onto the stacking gel without lanes and eluted for 9 h at a constant voltage of 150 V. The clusters' threat of thermal breakdown was reduced by carrying out the elution in a cooling atmosphere. The cluster fractions containing sections of the separating gel were cut out, crushed and soaked in distilled water. Using a filter with 0.2 μ m pores, the gel lumps that were suspended in the solution were taken out. The precipitate was then thoroughly cleaned by centrifugal ultrafiltration twice, using a filter with a cutoff molecular weight of 5 KDa, and redissolved in water. According to the size and charge of NCs, the gel composition, applied voltage and eluting time varied for better separation.

The PAGE apparatus is easy to use and reasonably inexpensive. Additionally, visual confirmation of the separation of individual clusters is possible when the cluster mixture is separated using PAGE. By exposing luminous clusters to UV light, one may also define

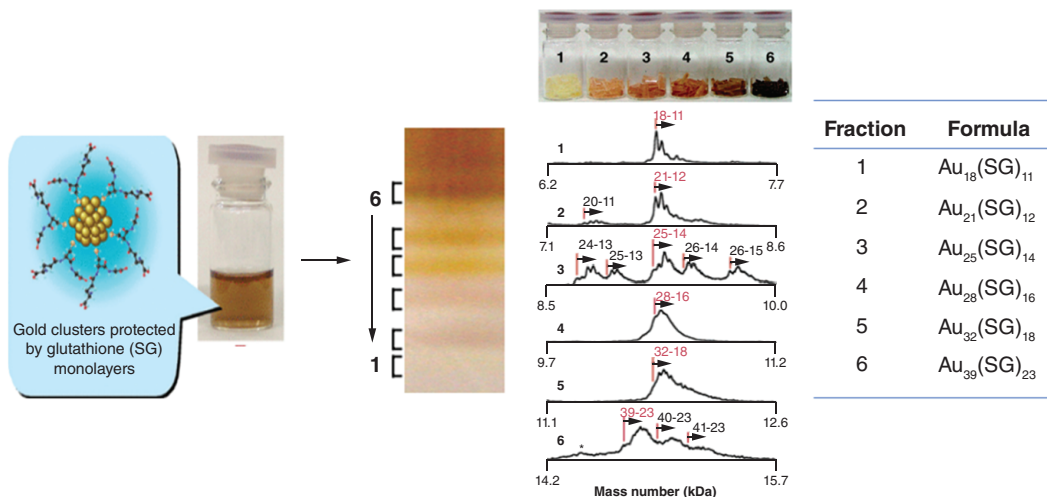


Figure 2. Isolation of gold nanoclusters separated by polyacrylamide gel electrophoresis, fractionated into six bands constituting magic number clusters. The isolated gel bands examined with ESI-MS indicates the molecular weight of $\text{Au}_n(\text{SG})_m$ nanoclusters. The chemical composition of fractionated nanoclusters are listed.

SG: Glutathione.

Adapted with permission from [28], © American Chemical Society (2004).

the photoluminescence characteristics present in them. Further, PAGE helps assess the size and form of the product because the mobility of each metal cluster differs based on the size, shape, charge and interactions with the solvent of the particle.

Recent trends in isolation of MNCs

Isolation & purification of NCs using PAGE

Polymer-based gel electrophoresis separation of NCs helps to understand the reaction pathway of NC formation. In general, this technique is limited to studying water-soluble, hydrophilic NCs. Most of the MNCs are synthesized by adopting aqueous ligands as a capping agent containing the mixture of magic-number NCs. Hence, the separation of these clusters is done by PAGE, followed by the optical and mass spectrometric analysis of separated bands derived the information on the electronic transitions and quantification of core and shell atoms in NCs. The MNCs protected by a monolayer of ligands with distinct charges can be fractionated into different sizes by the PAGE technique. In PAGE, larger particles (or NCs) typically pass through the gel more slowly than smaller molecules. By using gels that are substantially denser than those typically used for ordinary protein separation, PAGE separation resolution is significantly increased.

The stability of MNCs is the inevitable factor to consider before performing PAGE. Discrete size distributions made up of specific sizes of metal clusters have emerged as a result of the stability of MNCs being attributed to closed electrical and geometric shells. The introduction of unstable NCs in PAGE separation may result in the agglomeration or aggregation of particles through interacting with gel contents. In general, the stability of clusters has been achieved through tuning the pH, temperature and chemical environment or through ageing studies under various atmospheric conditions [26]. Through the series of spectroscopic measurements in a timely manner, the stability of NCs has been investigated. The stable NCs are subjected to the PAGE separation technique to identify the species present in them.

In 1998, Gregory Schaaf *et al.* [27] used PAGE to separate $\text{Au}_n(\text{SR})_m$ clusters (SR-thiol ligand) for the first time. Using PAGE, $\text{Au}_n(\text{SG})_m$ clusters (SG = glutathione) separated into several bands. The high-resolution separation of $\text{Au}_n(\text{SG})_m$ clusters was achieved by gel concentration optimization. They prepared gels with a higher concentration (about 40%) for the separation than the gels concentration used for protein separations. The third band from the bottom was observed for 4 cm, to be the most abundant species at ~40%. With the aid of MALDI-MS and ESI-MS, the precise composition of $\text{Au}_n(\text{SG})_m$ clusters in each band was determined, and the peak at 10.4 kDa corresponds to the chemical composition of $\text{Au}_{28}(\text{SG})_{16}$.

The mass spectrometric characterization of electrophoretically fractionated $\text{Au}_n(\text{SG})_m$ clusters was published in 2004 by Negishi *et al.* [28] $\text{Au}_n(\text{SG})_m$ NCs are isolated into six bands by PAGE, with the bands corresponding to a series of magic-numbered clusters – namely $\text{Au}_{18}(\text{SG})_{11}$, $\text{Au}_{21}(\text{SG})_{12}$, $\text{Au}_{25-1}(\text{SG})_{14-1}$, $\text{Au}_{28}(\text{SG})_{16}$, $\text{Au}_{32}(\text{SG})_{18}$ and $\text{Au}_{39}(\text{SG})_{23}$ – which were analyzed by ESI-MS (Figure 2). The photoluminescence quantum yields of these magic number clusters ($\phi = 10^{-3}$ - 10^{-4}) are comparatively higher than the bulk gold ($\phi = 10^{-10}$).

In 2005, Yao *et al.* [29] reported the chiroptical activity of D-/L-penicillamine-protected gold NCs. After the successful synthesis, the crude NCs were separated into three bands of D-/L-/rac-Pen-protected AuNCs based on the enantiomer and racemic mixture, according to their size and charge. Kothalawala *et al.* demonstrated the PAGE separation of molecule-like as well as plasmonic nanoparticles [30].

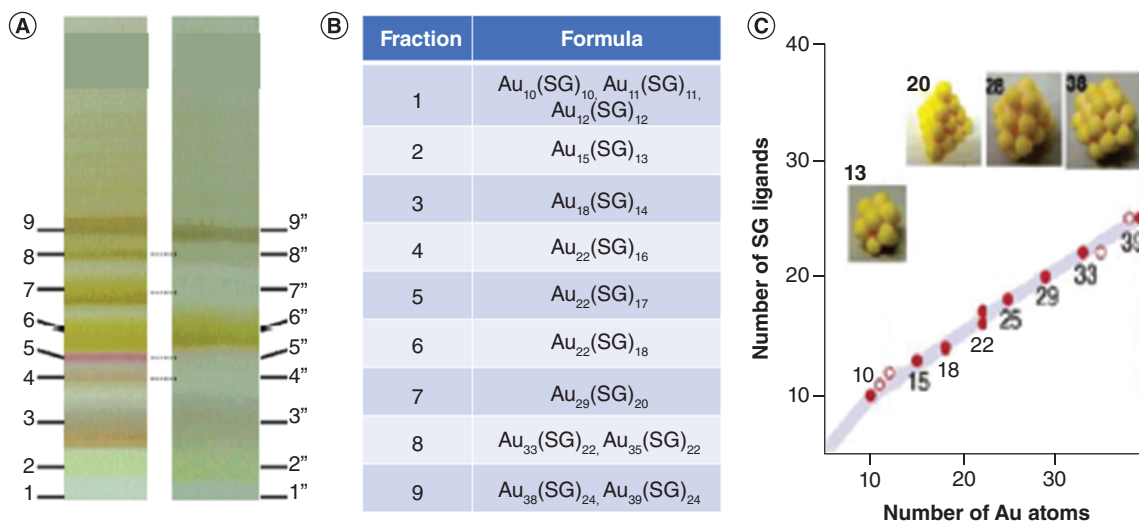


Figure 3. Isolation of gold nanoclusters and identification of molecular formula. (A) PAGE isolation of gold nanoclusters fractionated into nine bands. The isolated gel bands were examined with ESI-MS analysis that indicates the molecular weight of $Au_n(SG)_m$ nanoclusters. (B) The chemical composition of fractionated nanoclusters are listed. (C) A plot of chemical composition of nine bands against the number of gold atoms and ligands. SG: Glutathione.

Adapted with permission from [32], © American Chemical Society (2005).

They used different acrylamide gel concentrations for separating larger and smaller NCs. By this method, water-soluble $Au_n(SG)_m$ clusters were fractionated into 26 bands, that is, a wide range of 10's to 1000's Au atoms based on size and mobility. The 35% gel separated the crude into 13 bands of smaller nanoparticles, due to the lower pore size of the denser gel. Hence, the larger particles could not pass through this gel. The bigger plasmonic particles were separated in the 23% gel. The smaller particles, however, were not resolved and showed up as a black band running along the gel. The optical spectrum of bands 1–13 constituted less than 200 atoms. The absorption spectrum of bands 0–4 depicted the distinct peaks corresponding to the smaller NCs containing less than 40 atoms. The particles containing approximately 40 to 200 atoms – that is, bands 5–13 – exhibited the monotonous absorption spectra. The nanomolecules that constitutes more than 200 Au atoms showed a broad plasmonic absorption band around 530 nm, which shifted toward a higher wavelength on increasing size.

Monitoring reactions using PAGE

Cao *et al.* [31] demonstrated a cycle of reaction that involved the reversible addition and elimination of one thiolate surface ligand on gold NCs. To explore this precise ligand engineering, crude gold NCs were successfully synthesized by using 6-mercaptohexanoic acid (MHA) as a protecting ligand. The further oxidative etching process was carried out after the successful separation and purification of $Au_{25}(MHA)_{18}$ by the PAGE method. Negishi *et al.* [32] demonstrated the synthesis and separation of individual $Au_n(SG)_m$ NCs. The synthesis of Au clusters was done by reductive decomposition of Au(I)-SG polymer at a low temperature and followed by the fractionation of individual clusters by PAGE. The crude Au clusters were separated into nine fractions based on their size and mobility. Fractions 1, 8 and 9 were composed of NCs of adjacent sizes, and fractions 2–7 constituted single species. The molecular weight of these fractions was analyzed by ESI-MS measurements, according to their precise composition of atoms and ligands in NCs of each fraction calculated. The stability of these clusters was also examined with PAGE followed by optical spectroscopic measurements. To study more about the intrinsic stabilities of the bands 1–9, the stability of these atoms against decomposition was investigated. The as-prepared Au:SG clusters in an aqueous solution were kept at room temperature for 2 weeks. The PAGE separation of these two samples yielded fractions 1'–9' that had mobilities similar to those of 1–9, respectively (Figure 3A). The fractions 4, 5, 7 and 8 became pale, indicating that they were more susceptible to decomposition than the other bands, which was also evidenced by optical spectra and ESI-MS analysis. The clusters associated with 4, 5, 7 and 8 were formed as a decay product, that loses its stability during storage for a longer period. The increased stability of 1–3, 6 and 9 may be the cause of a stable protective shell by hydrogen-bonded networks among the SG ligands.

The effect of various thiol ligands in the stabilization of NCs was demonstrated by Negishi *et al.* by a series of PAGE followed by ESI-MS analysis [33]. SG, N-(2-mercapto-propionyl)glycine (S[PG]) or mercaptosuccinic acid (S[SA]) protected AuNCs were fractionated by PAGE (Figure 4). These fractions were further examined with ESI-MS. A relatively better resolution was obtained for Au:GS than for Au:S(PG) and Au:S(SA). This might be because interligand interactions cause the gold cores deformation. The electronic structures of the sub-nanometer-sized gold clusters are significantly influenced by the thiolate structures, according to a comparison of the optical

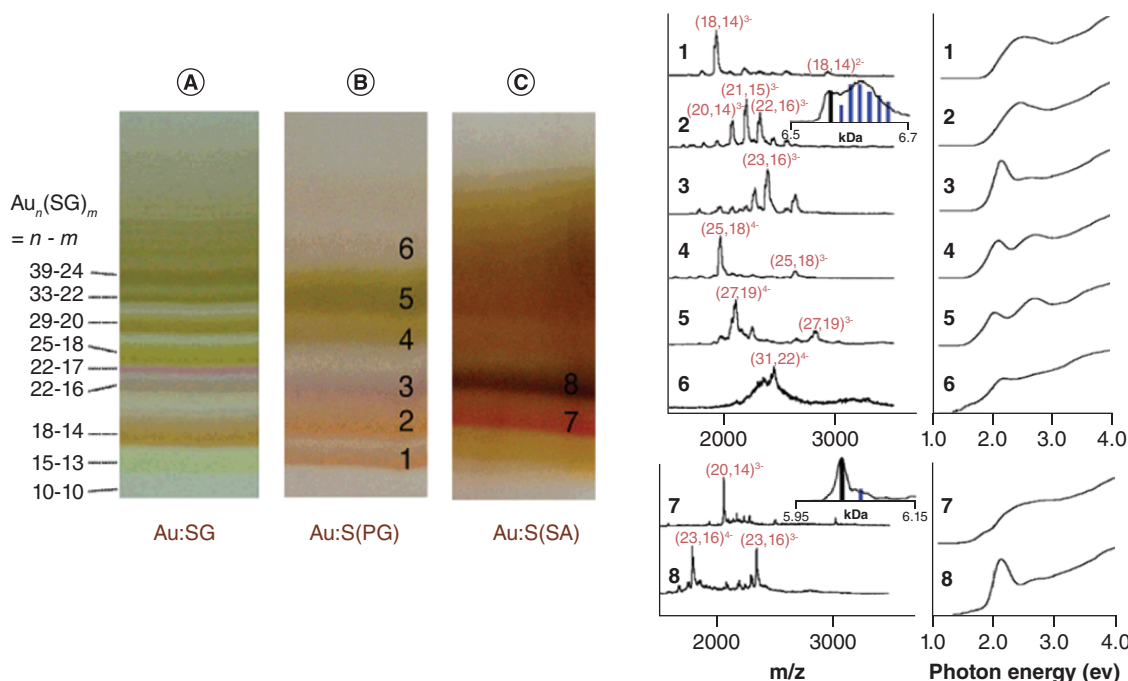


Figure 4. Polyacrylamide gel electrophoresis separation and identification of various thiol-protected gold nanoclusters. PAGE separation of Au nanoclusters stabilized with (A) SG, (B) (PG)SH, (C) (SA)SH and their negative-ion ESI mass spectra and optical absorption spectra of Au:S(PG) (1–6) and Au:S(SA) (7 and 8) clusters. The mass peaks are assigned to $[\text{Au}_n(\text{S}(\text{PG}))_m\text{-pH}^+]^{p-}$ and $[\text{Au}_n(\text{S}(\text{SA}))_m\text{-pH}^+]^{p-}$ and are denoted as (n, m)^p. SG: Glutathione; S(PG): N-(2-mercaptopropionyl)glycine; S(SA): mercaptosuccinic acid; PAGE: Polyacrylamide gel electrophoresis. Adapted with permission from [33], © American Chemical Society (2006).

spectra of materials with comparable chemical compositions. This finding suggests that passivation with thiolates inhibits the formation of small clusters produced by the reduction of the Au(I)-thiolate polymers kinetically.

Similarly, precise composition clusters of N-Isobutyryl-L-cysteine and 3-mercapto phenylboronic acid-protected AuNCs were separated and isolated through PAGE to investigate their physicochemical properties [34,35]. Pyo *et al.* adopted the PAGE separation technique to optimize a reaction time to obtain a monodisperse precise gold NC [36]. After 2 h of stirring, the raw product constituted three clusters: $\text{Au}_{15}(\text{SG})_{13}$, $\text{Au}_{18}(\text{SG})_{14}$ and $\text{Au}_{22}(\text{SG})_{18}$ as the predominant species. The extension of reaction to 6 h resulted in Au_{15} and Au_{22} as a major product with a high yield of Au_{22} . The prolonged reaction time reduces the yield of $\text{Au}_{22}(\text{SG})_{18}$.

Extensive studies in the determination of precise composition formula of hydrophilic AuNCs were established by PAGE separation and isolation methods. In this series, the chemical composition of hydrophilic silver, copper, cobalt and platinum NCs were studied with the utilization of the PAGE technique and mass spectrometric studies. In 2010, Kumar *et al.* reported the separation of magic-number clusters from SG-stabilized AgNCs [37]. Overall, 21 discrete bands were observed in PAGE separation, which was compared with Au:SG NCs (Figure 5A & B). The optical absorption spectrum depicts that the light absorption energy decreases monotonically with increasing the size of clusters. The stability of these clusters was investigated with the PAGE method. However, stability in an aqueous solution was size-dependent. The smaller particles seemed to be stable, but bands 14 and above were lost in an aqueous solution after about a day. This is in agreement with a characteristics of smaller clusters, which have wider Highest Occupied Molecular Orbital (HOMO)-Lowest Unoccupied Molecular Orbital (LUMO) gaps, would be more stable. Within the gel, all cluster sizes remained stable. The same group assign the formula of one of the bands after PAGE separation as $\text{Ag}_{32}(\text{SG})_{19}$ for the first time [17]. Desireddy *et al.* [26] gave an exciting report on the temporal stability of Ag:SG clusters, wherein they reported insights on the effect of pH, solvents, salts and ageing parameters on the stability of $\text{Ag}_n(\text{SG})_m$ clusters using PAGE. The detailed mechanism involved in determining the stability of AgNCs was explained through the use of PAGE. The raw mixture of AgNCs was divided into bands of the component magic-numbered Ag:SG clusters, each with a wide range of colors and abundances. Homemade polyacrylamide gels with a 30% density and no surfactants were used for PAGE studies. The solutions of Ag clusters are mostly not stable. The color changes that occur over days define that they undergo chemical changes. The color of some clusters changes in solution state while compared with clusters in the band as well as in solid form, which shows that clusters undergo chemical changes. To understand the changes that occur in time, unseparated particle solutions were aged for differing durations of time before being separated simultaneously on the same gel. The separation of clusters shows that the smaller clusters are more stable than the larger ones; this is due to the larger HOMO-LUMO gaps in smaller

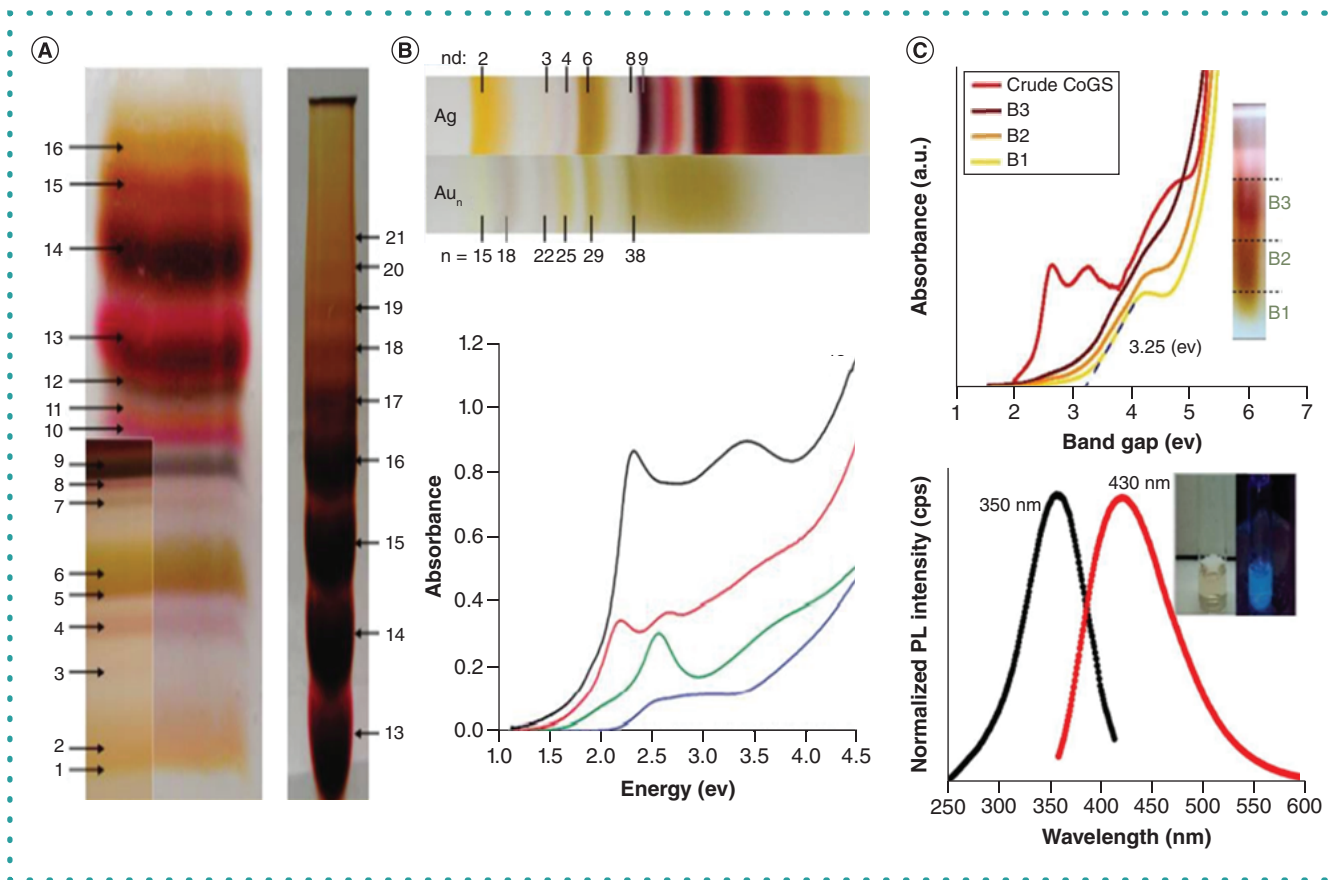


Figure 5. Polyacrylamide gel electrophoresis isolation of Ag and Cu nanoclusters and their size-dependent absorption spectra. (A & B) PAGE isolation of silver NCs fractionated into 21 bands by PAGE, which was compared with the previously reported fractionation pattern of Au:SG nanoclusters to evaluate the chemical composition with band position. The absorption spectra show the increasing energy absorption with decreased size of clusters. (C) The absorption spectra of isolated CoNCs with crude particles. Inset image shows the fractionation of CoSG nanoclusters into three bands. The lower band (band B1) shows the characteristic blue emission at 430 nm. NC: Nanocluster; PAGE: Polyacrylamide gel electrophoresis; SG: Glutathione. (A & B) Adapted with permission from [37], © American Chemical Society (2010). (C) Adapted with permission from [39], © John Wiley and Sons, Inc. (2020).

clusters. Different salts were used to screen the clusters before ageing to assess the impact of surface charges. Surprisingly, cluster degradation was not seen to be accelerated by salt screening. pH was utilized to directly alter the surface charge and the efficacy of cluster–cluster repulsions, because screening the surface charges had no discernible impact on the stability of the clusters. The pH of the cluster solutions was then changed with a variety of buffers. To analyze the stability of clusters, samples were aged for 3 days before being subjected to PAGE analysis. The sample was more stable in pH 5–8 and pH 2. This is due to the changes in the charge state of clusters. Recently, Yang *et al.* reported the water-soluble Cu₃₀ NCs as a catalyst for cycloaddition reaction [38]. The effect of pH and volume fraction of ethanol/water on the synthesis of CuNCs was investigated through performing native PAGE analysis. Our group reported the synthesis and luminescent characteristics of CoNCs protected by glutathione [39]. The separation and purification of raw products of CoNCs were carried out through PAGE. The three bands can be seen on PAGE separation, and the lower band possesses molecular-like smaller NCs that constitute less than 8 Co atoms (Figure 5C). The physicochemical properties of these three bands of CoNCs were examined. The optical spectra showed the increasing band gap with decreasing the size of clusters. The lower band possessed higher stability in an aqueous medium. The blue emissive CoNCs of band 1 exhibited a quantum yield of 10%.

Identification of luminescent clusters

Numerous metal clusters are anticipated to play a significant role in biolabeling and fluorescence resonance energy transfer in addition to producing luminescent patterns due to their fluorescence [10]. Elements like Au and Ag are capable of forming compound semiconductors with enhanced luminescence [3,40]. The identification of such luminescent NCs that produce photoluminescence with higher quantum yield in a mixture of clusters is a tedious process. The advancement of the PAGE technique aids isolating and separating luminescence clusters. After separating the mixture through PAGE, the irradiation of UV light exposes the luminescent NCs in the gel.

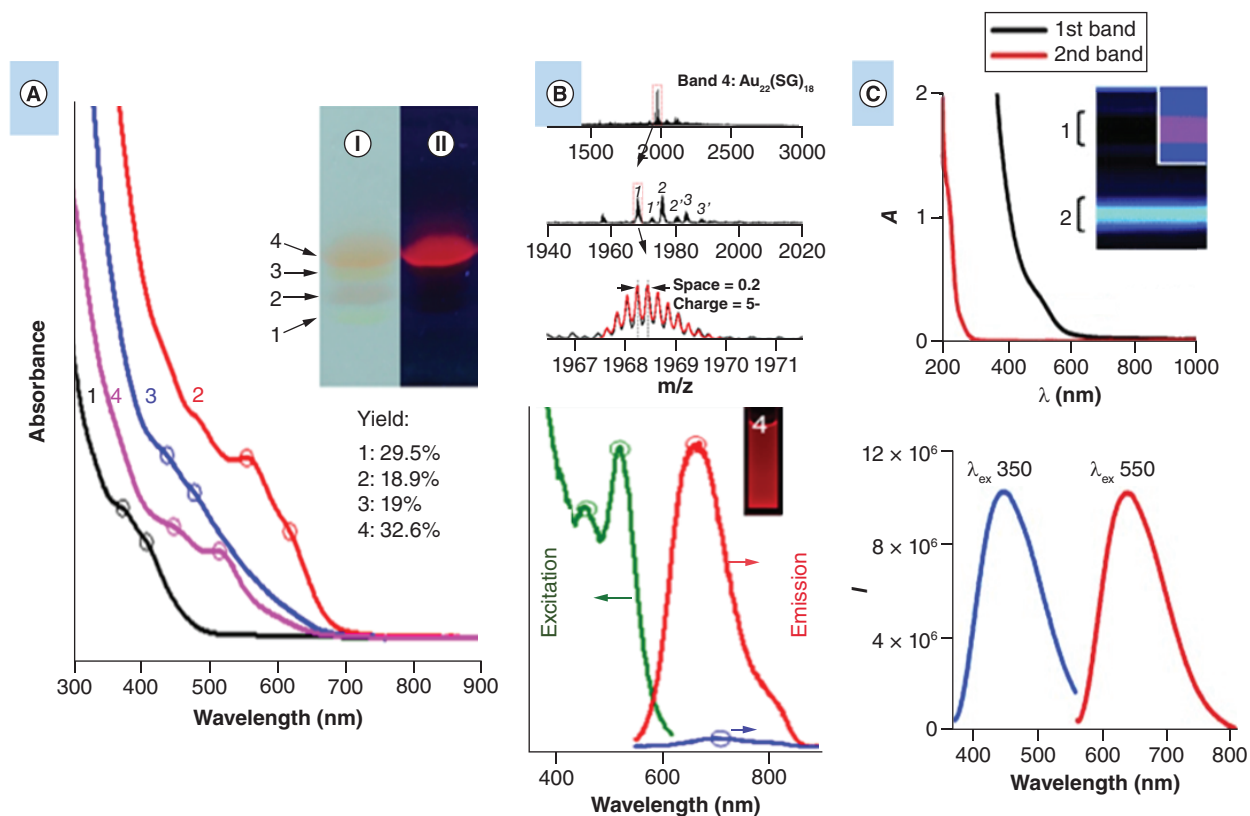


Figure 6. Identification of luminescent nanoclusters from polyacrylamide gel electrophoresis separation and their corresponding absorption spectrum. The SG-stabilized AuNCs are fractionated into four distinct bands (A) Absorption spectrum of Au nanoclusters isolated by PAGE. Inset image shows the photoemission of band 4 under UV light. (B) ESI-MS of clusters separated from luminescent band 4 determine their molecular composition as $[\text{Au}_{22}(\text{SG})_{18}^0\text{-5H}]^{5-}$ and the PL spectrum shows an intense peak at 665 nm that corresponds to red emission. fractionated into six bands constitutes magic-number clusters. (C) UV-vis absorption spectra of the clusters identified using the two PAGE bands. A picture of the wet gel following electrophoresis under UV light is shown in the inset, and an image of the first band at 273 K is shown in the inset to the inset. The image shows cluster 1 and cluster 2's luminescence emission in water, excited at 550 and 350 nm, respectively. PAGE: Polyacrylamide gel electrophoresis; PL: Photoluminescence; SG: Glutathione. (A & B) Adapted with permission from [41], © American Chemical Society (2015). (C) Adapted with permission from [42], © John Wiley and Sons, Inc. (2020).

Yu *et al.* group reported the identification of luminescent $\text{Au}_{22}(\text{SG})_{18}$ in the raw product of glutathione-protected gold NCs synthesized by using CO as a reducing agent through PAGE separation. Among the four bands in PAGE isolation, band 4 exposed strong red emission under UV illumination, which was assigned as $\text{Au}_{22}(\text{SR})_{18}$ by mass spectrometric studies (Figure 6A & B). The strong red emission of Au_{22} was located at 665 nm and the quantum yield of these clusters was evaluated to be 8%. The molecular composition of other bands was determined by comparing their mobility and color with previously reported AuNCs on PAGE separation [41]. The same group reported the high yield synthesis of Au_{22} NCs by optimizing the incubation time before and after reduction in addition to optimizing pH with the use of the PAGE technique [12]. Rao *et al.* succeeded in the interfacial synthesis of mercaptosuccinic acid (H_2MSA)-protected Ag NCs [42]. The orange-color crude mixture of silver NCs was separated into differently sized clusters by PAGE. The presence of two different clusters was shown by two distinct bands: these bands are red and light yellow in color in visible light. Under UV illumination, band 1 exposes red luminescence (emission at 650 nm) and band 2 shows blue-green emission (emission at 440 nm) at room temperature (Figure 6C). The emission of clusters 1 and 2 are located at 650 and 440 nm at an excitation wavelength of 550 and 350 nm, respectively. These two bands are assigned as $\text{Ag}_8(\text{H}_2\text{MSA})_8$ and $\text{Ag}_7(\text{H}_2\text{MSA})_7$, respectively [42].

Other techniques for NC separation

Chromatographic separation

Normal phase (NP) chromatography is more suitable for hydrophobic NCs. Reverse phase (RP) chromatography has the hydrophobic stationary phase; hydrophilic NCs are separated by interacting with ion-pairing reagents, which induces the hydrophobic surface on the

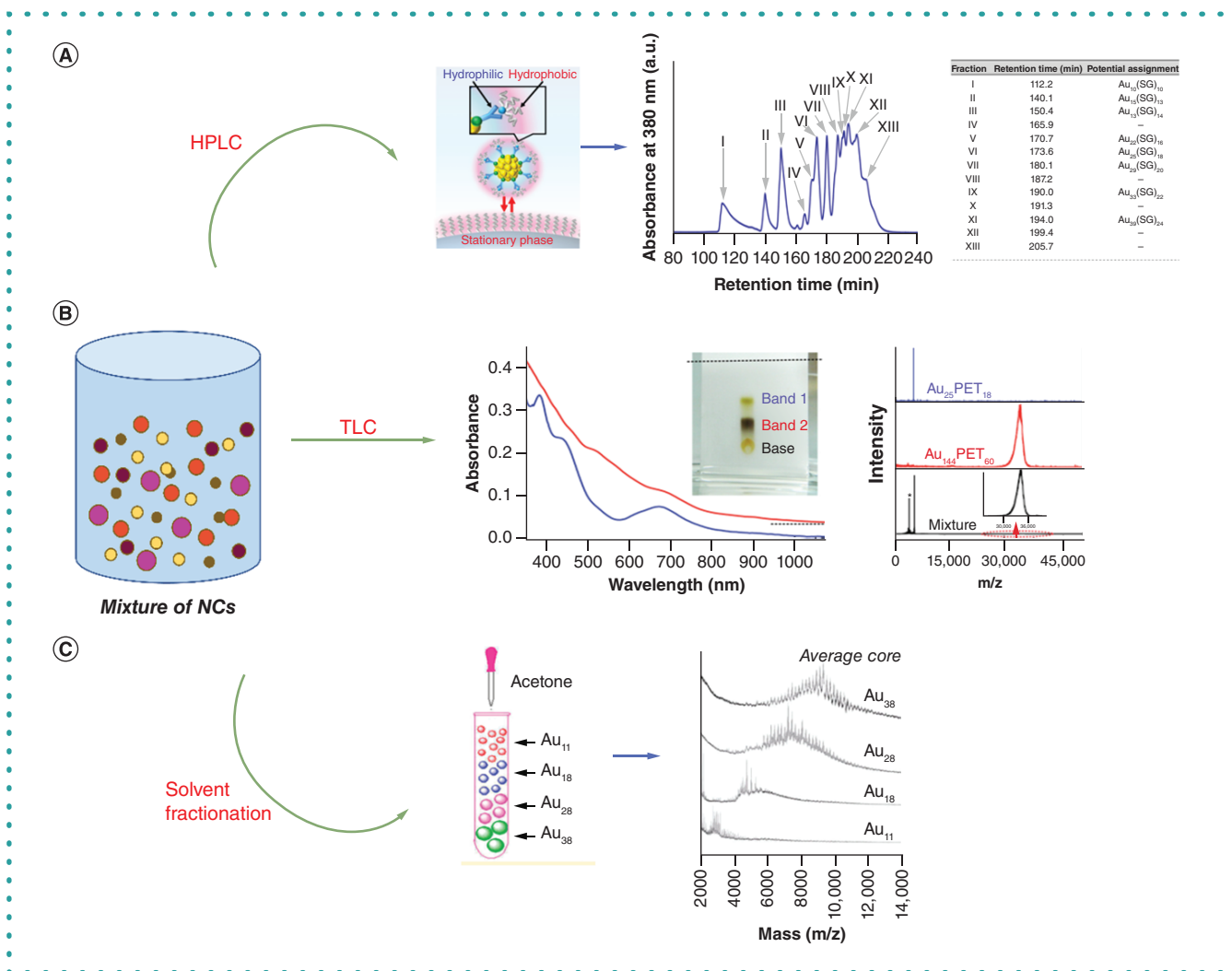


Figure 7. Separation of a mixture of nanoclusters. (A) RP-HPLC technique by using ion-pair reagents for Au_n(GS)_m and their chromatogram shows the separation of NCs. **(B)** TLC technique for mixture of Au₂₅ and Au₁₄₄ separation. **(C)** Solvent-induced size selective precipitation of individual Au_n(SR)_m nanoclusters from the mixture by using various fractions of solvent.

GS: Glutathione; NC: Nanocluster; RP: Reverse phase; SR: D-Penicillamine.

(A) Adapted with permission from [44], © American Chemical Society (2017). **(B)** Adapted with permission from [20], © American Chemical Society (2014). **(C)** Adapted with permission from [47]. © American Chemical Society (2012).

hydrophilic NCs for effective separation. By employing the RP-HPLC (Reverse Phase - High-Performance Liquid Chromatography) technique, a wide range of sizes of AuNCs from Au₃₈(SR)₂₄ to Au_{~520}(SR)_{~130} have been isolated [43]. Niihori *et al.* reported the separation of AuNCs using RP-HPLC, and the range from Au₁₀ to Au₃₉ was successfully separated and isolated (Figure 7A) [44]. The cluster-containing two different ligands, as well as two different metal atoms was also separated and investigated by using RP-HPLC. For the separation of CuNCs, Baghdasaryan *et al.* utilized normal phase-HPLC [1]. The RP- and NP-HPLC are purely based on adsorption or partition mechanism, whereas size-exclusion chromatographic separation is based on the sizes of particles, in these there is no interaction between the clusters and stationary phases. By using size-exclusion chromatographic separation technique, both hydrophilic and hydrophobic NCs were separated. The reports on the utilization of the size-exclusion chromatographic-based gel filtration chromatography technique for the isolation of hydrophilic clusters are limited, because of the PAGE technique.

TLC separation

TLC uses an inexpensive and simple device different from that used for HPLC. Furthermore, in TLC, the separation can be visually confirmed similarly to PAGE. The use of preparative TLC using a thicker silica gel than that used in commercial TLC plates makes it possible to isolate each cluster on a relatively large scale. Therefore, TLC is often used to confirm the purity of a product.

Antigalvanic reduction of anion $\text{Au}_{25}(\text{PET})_{18}$ (PET= Phenylethanethiol) by different chelating agents containing Ag was demonstrated by Tian *et al.* [45]. This antigalvanic reduction was highly dependent on ion precursor and ion dosage, which was investigated with the aid of the TLC method. The size conversion of $\text{Au}_{44}(\text{TBBT})_{28}$ (TBBT = 4-tert-butylbenzenethiolate) to $\text{Au}_{36}(\text{TBBT})_{24}$ was also observed by Liao *et al.* using preparative TLC [46]. They were therefore successful in identifying efficient reaction conditions for these exchange processes. This technique can be used to separate clusters depending on size and charge state for a mixture of $\text{Au}_n(\text{SR})_m$ and related clusters made up of clusters with a wide range in size and charge state. In 2014, Ghosh *et al.* [20] used TLC to successfully separate the product's $\text{Au}_{25}(\text{SC}_2\text{H}_4\text{Ph})_{18}$ and $\text{Au}_{144}(\text{SC}_2\text{H}_4\text{Ph})_{60}$. They also had success using TLC to separate $\text{Au}_{25}(\text{SC}_2\text{H}_4\text{Ph})_{18}$ and $\text{Au}_{25}(\text{SC}_4\text{H}_9)_{18}$ with various ligands (Figure 7B), $[\text{Au}_{25}(\text{SC}_2\text{H}_4\text{Ph})_{18}]^0$ and $[\text{Au}_{25}(\text{SC}_2\text{H}_4\text{Ph})_{18}]^-$ with different charge states, and $\text{Au}_{25}(\text{Calix})_x(\text{SC}_4\text{H}_9)_y$ (Calix = 25,26,27,28-tetrakis (4-mercapto-n-butoxy)calix[4]arene) with various ligand combinations. Tian *et al.* succeeded in the separation of isomers of $\text{Au}_{38}(\text{SC}_2\text{H}_4\text{Ph})_{24}$ through preparative TLC. The change in temperature induces the isomeric transformation which was supported by TLC analysis [18].

Solvent fractionation

Yang *et al.* employed the size-selective separation method by using a solvent to isolate AuNCs. On increasing the fraction of acetone, the NCs precipitated from a larger to a smaller size [47]. By this method, Au_{11} , Au_{18} , Au_{28} and Au_{38} NCs were isolated and quantified with MALDI-MS analysis (Figure 7C). Pyo *et al.* reported the solvent-induced separation of SG-protected AuNCs [36]. The ratio of polar and nonpolar solvents was used in this type of separation. They used a different ratio of acetonitrile and water for Au_{22} separation. The initial ratio that contained higher acetonitrile volume facilitated the precise separation and purification of $\text{Au}_{22}(\text{SG})_{18}$ NCs.

Conclusion

This review has focused on PAGE to highlight earlier studies on the high-resolution separation of metal clusters. NCs are proving to be greatly beneficial materials in a variety of fields. Hydrophobic ligands-protected NCs are highly employed for catalytic, optoelectronic fields. Their toxic nature limits their applicability in biomedical fields. Hence, there is a high necessity for the synthesis of atomically precise and biocompatible MNCs. Hydrophilic thiol-protected MNCs provide a pathway for their utility in biomedical applications. For application purposes, the effective purification and separation of these NCs become crucial. Hydrophilic MNCs can be isolated by chromatographic techniques like size-exclusion chromatography, HPLC and PAGE. Polarity-based precipitation techniques efficiently purify NCs, but they face the possibility of altering the NCs' stability and consequently their characteristics. HPLC techniques offer an efficient separation but are highly expensive compared with other separation methods. TLC separation is limited only to hydrophobic clusters. PAGE method overcome these drawbacks in the separation of hydrophilic nanoclusters that facilitates the better isolation of NCs based on precise composition.

Future perspective

PAGE thus offers a unique opportunity for the separation of NCs with high resolution and cost-effectiveness, which can also assist in reaction tracking and stability-focused research. The in-depth research on MNCs separation with PAGE by tuning gel concentration, applied voltage, choice of buffer, separation time, and temperature helps for better isolation of NCs and monitoring reactions, which can be used for focused applications. The above discussions and literature findings demonstrate PAGE is a versatile tool for the separation of monodispersed NCs that explores new materials for cutting-edge research in technological and biomedical fields.

Executive summary

- The general method of separation of hydrophilic metal nanoclusters (NCs) using polyacrylamide gel electrophoresis (PAGE) method was discussed.
- The PAGE method has been utilized to isolate NCs based on size, shape and charge, which was further confirmed by mass spectrometric analysis.
- To attain monodisperse, atomically precise metal NCs, the reaction was monitored by PAGE method. The stability of NCs was also studied by tracking the position of the band during PAGE isolation.
- The effect of pH, temperature, and reaction time in the formation of MNCs was determined with the aid of PAGE.
- The significance of the PAGE method over other separation techniques was discussed. In comparison with other separation techniques, PAGE affords a simple, cost-effective and high resolution for the separation of clusters with atomic precision.

Author contributions

V Packirisamy: writing – original draft; P Pandurangan: review and editing, supervision.

Acknowledgments

The authors acknowledge the grants received from the Department of Science and Technology (DST) – Innovation in Science Pursuit for Inspired Research (INSPIRE) fellowship program (IF170915) and Research Innovation and Quality Improvement (RI & QI), RUSA 2.0, University of Madras.

Financial & competing interests disclosure

This research was funded by the Department of Science and Technology (DST) – Innovation in Science Pursuit for Inspired Research (INSPIRE) fellowship program (grant no. IF170915) and the Research Innovation and Quality Improvement of Science and Technology (RI & QI), RUSA 2.0, which was provided by the University of Madras. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Open access

This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Baghdasaryan A, Grillo R, Roy Bhattacharya S et al. Facile synthesis, size-separation, characterization, and antimicrobial properties of thiolated copper clusters. *ACS Appl. Nano Mater.* 1(8), 4258–4267 (2018).
2. Tang Q, Hu G, Fung V, Jiang DE. Insights into interfaces, stability, electronic properties, and catalytic activities of atomically precise metal nanoclusters from first principles. *Acc. Chem. Res.* 51(11), 2793–2802 (2018).
3. Qu X, Li Y, Li L, Wang Y, Liang J, Liang J. Fluorescent gold nanoclusters: synthesis and recent biological application. *J. Nanomater.* 2015, 784097 (2015).
4. Shyamal M, Maity S, Maity A, Maity R, Roy S, Misra A. Aggregation induced emission based 'turn-off' fluorescent chemosensor for selective and swift sensing of mercury (II) ions in water. *Sens. Actuators B Chem.* 263, 347–359 (2018).
5. Jin R, Zeng C, Zhou M, Chen Y. Atomically precise colloidal metal nanoclusters and nanoparticles: fundamentals and opportunities. *Chem. Rev.* 116(18), 10346–10413 (2016).
6. Goswami N, Yao Q, Luo Z, Li J, Chen T, Xie J. Luminescent metal nanoclusters with aggregation-induced emission. *J. Phys. Chem. Lett.* 7(6), 962–975 (2016).
7. Kang X, Wang S, Song Y et al. Bimetallic Au₂Cu₆ nanoclusters: strong luminescence induced by the aggregation of copper(I) complexes with Gold(0) species. *Angew. Chem. Int. Ed. Engl.* 55(11), 3611–3614 (2016).
8. Yuan SF, Guan ZJ, Liu WD, Wang QM. Solvent-triggered reversible interconversion of all-nitrogen-donor-protected silver nanoclusters and their responsive optical properties. *Nat. Commun.* 10(1), 1–7 (2019).
9. Narouz MR, Osten KM, Unsworth PJ et al. N-heterocyclic carbene-functionalized magic-number gold nanoclusters. *Nat. Chem.* 11(5), 419–425 (2019).
10. Wan XK, Wang JQ, Nan ZA, Wang QM. Ligand effects in catalysis by atomically precise gold nanoclusters. *Sci. Adv.* 3(10), e1701823 (2017).
11. Heuer-Jungemann A, Felius N, Bakaimi I et al. The role of ligands in the chemical synthesis and applications of inorganic nanoparticles. *Chem. Rev.* 119(8), 4819–4880 (2019).
12. Srinivasulu YG, Goswami N, Yao Q, Xie J. High-yield synthesis of AIE-type Au₂₂(SG)₁₈nanoclusters through precursor engineering and its pH-dependent size transformation. *J. Phys. Chem. C* 125(7), 4066–4076 (2021).
13. Yu Y, Chen X, Yao Q, Yu Y, Yan N, Xie J. Scalable and precise synthesis of thiolated Au₁₀-12, Au₁₅, Au₁₈, and Au₂₅ nanoclusters via pH controlled CO reduction. *Chem. Mater.* 25(6), 946–952 (2013).
14. Dou X, Chen X, Zhu H et al. Water-soluble metal nanoclusters: recent advances in molecular-level exploration and biomedical applications. *Dalt. Trans.* 48(28), 10385–10392 (2019).
15. Zhang Y, Zhang C, Xu C et al. Ultrasmall Au nanoclusters for biomedical and biosensing applications: a mini-review. *Talanta* 200, 432–442 (2019).
16. Tseng WB, Chou YS, Lu CZ, Madhu M, Lu CY, Tseng WL. Fluorescence sensing of heparin and heparin-like glycosaminoglycans by stabilizing intramolecular charge transfer state of dansyl acid-labeled AG73 peptides with glutathione-capped gold nanoclusters. *Biosens. Bioelectron.* 193, 113522 (2021).
17. Guo J, Kumar S, Bolan M, Desireddy A, Bigioni TP, Griffith WP. Mass spectrometric identification of silver nanoparticles: the case of Ag₃₂(SG)₁₉. *Anal. Chem.* 84(12), 5304–5308 (2012).
18. Tian S, Li YZ, Li MB et al. Structural isomerism in gold nanoparticles revealed by X-ray crystallography. *Nat. Commun.* 6(1), 1–7 (2015).
19. Sweeney SF, Woehrl GH, Hutchison JE. Rapid purification and size separation of gold nanoparticles via diafiltration. *J. Am. Chem. Soc.* 128(10), 3190–3197 (2006).
20. Ghosh A, Hassinen J, Pulkkinen P, Tenhu H, Ras RHA, Pradeep T. Simple and efficient separation of atomically precise noble metal clusters. *Anal. Chem.* 86(24), 12185–12190 (2014).
21. Yuan X, Chng LL, Yang J, Ying JY. Miscible-solvent-assisted two-phase synthesis of monolayer-ligand-protected metal nanoclusters with various sizes. *Adv. Mater.* 32(9), 1906063 (2020).
22. Dou X, Wang X, Qian S, Liu N, Yuan X. From understanding the roles of tetraoctylammonium bromide in the two-phase Brust-Schiffrin method to tuning the size of gold nanoclusters. *Nanoscale* 12(38), 19855–19860 (2020).
23. Wang Z, Pan X, Qian S, Yang G, Du F, Yuan X. The beauty of binary phases: a facile strategy for synthesis, processing, functionalization, and application of ultrasmall metal nanoclusters. *Coord. Chem. Rev.* 438, 213900 (2021).
24. Maity S, Bain D, Patra A. An overview on the current understanding of the photophysical properties of metal nanoclusters and their potential applications. *Nanoscale* 11(47), 22685–22723 (2019).
25. Sadhu S, Chowdhury PS, Patra A. Understanding the role of particle size on photophysical properties of Cd₂Eu₃⁺ nanocrystals. *J. Lumin.* 126(2), 387–392 (2007).
26. Desireddy A, Kumar S, Guo J, Bolan MD, Griffith WP, Bigioni TP. Temporal stability of magic-number metal clusters: beyond the shell closing model. *Nanoscale* 5(5), 2036–2044 (2013).
- Desireddy et al. gave an exciting report on the temporal stability of Ag_n:glutathione (SG) clusters, wherein they reported insights on the effect of pH, solvents, salts and ageing parameters on the stability of Ag_n(SG)_m clusters using polyacrylamide gel electrophoresis (PAGE). The detailed mechanism involved in determining the stability of Ag nanoclusters (NCs) was explained through the use of PAGE.
27. Gregory Schaaff T, Knight G, Shafiqullin MN, Borkman RF, Whetten RL. Isolation and selected properties of a 10.4 kDa gold:glutathione cluster compound. *J. Phys. Chem. B* 102(52), 10645–10646 (1998).
- For the first time, Gregory Schaaff et al. used the PAGE technique to separate SG-stabilized AuNCs. This report is taken as a platform for further usage of PAGE to isolate NCs.
28. Negishi Y, Takasugi Y, Sato S, Yao H, Kimura K, Tsukuda T. Magic-numbered Au clusters protected by glutathione monolayers (n = 18, 21, 25, 28, 32, 39): isolation and spectroscopic characterization. *J. Am. Chem. Soc.* 126(21), 6518–6519 (2004).
29. Yao H, Miki K, Nishida N, Sasaki A, Kimura K. Large optical activity of gold nanocluster enantiomers induced by a pair of optically active penicillamines. *J. Am. Chem. Soc.* 127(44), 15536–15543 (2005).
30. Kothalawala N, Lee West Iv J, Dass A. Size-dependent molecule-like to plasmonic transition in water-soluble glutathione stabilized gold nanomolecules. *Nanoscale* 6(2), 683–687 (2014).
- Kothalawala et al. demonstrated the PAGE separation of molecule-like as well as plasmonic nanoparticles. They used different acrylamide gel concentrations for separating larger and smaller NCs. By this method, water-soluble Au:SG clusters were fractionated into 26 bands, that is, a wide range of 10³ s to 1000³ s of Au atoms based on size and mobility. The 35% gel

separated the crude into 13 bands of smaller nanoparticles, due to the lower pore size of the denser gel. Hence, the larger particles could not pass through this gel. The bigger plasmonic particles were separated in the 23% gel. The smaller particles, however, were not resolved and showed up as a black band running along the gel.

31. Cao Y, Fung V, Yao Q *et al.* Control of single-ligand chemistry on thiolated Au₂₅ nanoclusters. *Nat. Commun.* 11(1), 1–7 (2020).
32. Negishi Y, Nobusada K, Tsukuda T. Glutathione-protected gold clusters revisited: bridging the gap between gold(I)-thiolate complexes and thiolate-protected gold nanocrystals. *J. Am. Chem. Soc.* 127(14), 5261–5270 (2005).
 - Negishi *et al.* demonstrated the synthesis and separation of individual Au_n(SG)_m NCs. The synthesis of Au clusters was done by reductive decomposition of Au(I)-SG polymer at a low temperature and followed by the fractionation of individual clusters by PAGE. The stability of AuNCs was monitored by the time-based PAGE separation of NCs.
33. Negishi Y, Takasugi Y, Sato S, Yao H, Kimura K, Tsukuda T. Kinetic stabilization of growing gold clusters by passivation with thiolates. *J. Phys. Chem. B* 110(25), 12218–12221 (2006).
34. Gautier C, Bürgi T. Chiral N-isobutyl-L-cysteine protected gold nanoparticles: preparation, size selection, and optical activity in the UV-vis and infrared. *J. Am. Chem. Soc.* 128(34), 11079–11087 (2006).
35. Yao H, Miki K, Nishida N, *et al.* Large optical activity of gold nanocluster enantiomers induced by a pair of optically active penicillamines. *J. Am. Chem. Soc.* 127(44), 15536–15543 (2005).
 - This article reports the isolation of enantiomeric penicillamine-protected gold NCs.
36. Pyo K, Thanthirige VD, Kwak K, Pandurangan P, Ramakrishna G, Lee D. Ultrabright luminescence from gold nanoclusters: rigidifying the Au(I)-thiolate shell. *J. Am. Chem. Soc.* 137(25), 8244–8250 (2015).
37. Kumar S, Bolan MD, Bigioni TP. Glutathione-stabilized magic-number silver cluster compounds. *J. Am. Chem. Soc.* 132(38), 13141–13143 (2010).
38. Yang G, Xie Y, Wang Y *et al.* Water-soluble Cu₃₀ nanoclusters as a click chemistry catalyst for living cell labeling via azide-alkyne cycloaddition. *Nano Res.* <https://doi.org/10.1007/s12274-022-4821-5> (2022) (Epub ahead of print).
 - Yuan *et al.* reported the water-soluble Cu₃₀ NCs as a catalyst for cycloaddition reaction. The effect of pH and volume fraction of ethanol/water on the synthesis of CuNCs was investigated through performing native PAGE analysis.
39. Ramadurai M, Vinitha P, Prabhu P *et al.* Size-dependent photoluminescence from thiolate-protected water-soluble cobalt nanoclusters. *Chemistry Select* 5(7), 2263–2270 (2020).
 - This article reports the isolation of glutathione-protected transition metal cobalt NCs through PAGE for the first time.
40. Chen LY, Wang CW, Yuan Z, Chang HT. Fluorescent gold nanoclusters: recent advances in sensing and imaging. *Anal. Chem.* 87(1), 216–229 (2015).
41. Yu Y, Luo Z, Chevrier DM *et al.* Identification of a highly luminescent Au₂₂(SG)₁₈ nanocluster. *J. Am. Chem. Soc.* 136(4), 1246–1249 (2014).
42. Udaya Bhaskara Rao T, Pradeep T. Luminescent Ag₇ and Ag₈ clusters by interfacial synthesis. *Angew. Chem. Int. Ed. Engl.* 49(23), 3925–3929 (2010).
 - The identification of luminescent clusters in the crude of AgNCs was performed through PAGE technique.
43. Li D, Kumari B, Zhang X, Wang C, Mei X, Rotello VM. Purification and separation of ultra-small metal nanoclusters. *Adv. Colloid Interface Sci.* 276, 102090 (2020).
44. Niihori Y, Kikuchi Y, Shima D *et al.* Separation of glutathione-protected gold clusters by reversed-phase ion-pair high-performance liquid chromatography. *Ind. Eng. Chem. Res.* 56(4), 1029–1035 (2017).
45. Tian S, Yao C, Liao L, Xia N, Wu Z. Ion-precursor and ion-dose dependent anti-galvanic reduction. *Chem. Commun.* 51(59), 11773–11776 (2015).
46. Liao L, Yao C, Wang C *et al.* Quantitatively monitoring the size-focusing of Au nanoclusters and revealing what promotes the size transformation from Au₄₄(TBBT)₂₈ to Au₃₆(TBBT)₂₄. *Anal. Chem.* 88(23), 11297–11301 (2016).
47. Yang X, Su Y, Paa Y *et al.* Mass spectrometric identification of water-soluble gold nanocluster fractions from sequential size-selective precipitation. *Anal. Chem.* 84(3), 1765–1771 (2012).