Gold nanostructures: synthesis, properties, and neurological applications

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Abstract

Recent advances in technology are expected to increase our current understanding of neuroscience. Nanotechnology and nanomaterials can alter and control neural functionality in both *in-vitro* and *in-vivo* experimental setups. The intersection between neuroscience and nanoscience may generate long-term neural interfaces adapted at the molecular level. Owing to their intrinsic physicochemical characteristics, gold nanostructures (GNSs) have received much attention in neuroscience, especially for theragnostic purposes. GNSs have been successfully employed to stimulate and monitor neurophysiological signals. Hence, GNSs could provide a promising solution for the regeneration and recovery of neural tissue, novel neuroprotective strategies, and integrated implantable materials. This review covers the broad range of neurological applications of GNS-based materials to improve clinical diagnosis and therapy. Sub-topics include neurotoxicity, targeted delivery of therapeutics to the central nervous system (CNS), neurochemical sensing, neuromodulation, neuroimaging, neurotherapy, tissue engineering, and neural regeneration. It focuses on core concepts of GNSs in neurology, to circumvent the limitations and significant obstacles of innovative approaches in neurobiology and neurochemistry, including theragnostics. We will discuss recent advances in the use of GNSs to overcome current bottlenecks and tackle technical and conceptual challenges.

Keywords: Gold nanostructures; Surface plasmon resonance; Neurodegenerative diseases; Brain tumors; Neuroimaging; Theragnostics; Implanted neural interfaces

1. Introduction

Neuroscience is a multidisciplinary science, which helps us to understand the fundamental and functional properties of the CNS. It encompasses advances in neurotechnology and neurotherapy. Neurodegenerative disorders (NDs) are characterized by a progressive loss and death of neurons, which leads to a wide range of clinical manifestations, including motor impairment, severe cognitive decline, and dementia, and currently, NDs affect up to 40 million people worldwide.[1] Since the neurological activities occur at the molecular level, developing ultrasensitive tools to monitor these activities is essential.[2, 3] In the past few years, remarkable advances have been made in developing various technologies using novel nanomaterials and methods to address problems in the neuroscience research area.[4-12] GNSs have been widely used in neuroscience due to their conductivity, unique optical properties, and high biocompatibility with neurological tissues. GNSs are poised to provide a rich and versatile toolkit of novel strategies to explore and stimulate the functions of neurons and neural circuits as well as providing new therapeutic opportunities and targeted therapies for neurodegenerative diseases, where allows tracking, measurement, and manipulation of neuronal activities at a molecular level.[13-18]

Over the past few centuries, neuroscience and gold materials have developed a substantial intertwined history. As an example, in the 16th-century, Paracelsus recommended preparations containing gold to treat epilepsy.[19] In the 19th-century, the application of gold materials in the microscopic exploration of the nervous system was investigated.[20] In 1866, Cohnheim employed gold salts in staining protocols for light microscopy of nervous tissues.[21] Meanwhile, in 1871, Gerlach applied gold chloride staining to distinguish between gray and white brain matter with an unprecedented degree of contrast.[22] By the late 19th to the early 20th-century, gold was listed as a therapy for neurological disorders in sources such as medical texts and the first Merck manual. However, at that time, the mechanism of interaction between gold materials and neurons was unknown.[20] In 1913, Ramón y Cajal developed an original stainable to distinguish astrocytes in the human hippocampus using a gold chloride-mercury method.[23] Moreover, in 1962, Gurr listed several stains used in modern electron microscopy, comprising gold chloride

for nerve fibers (planarians), neuroglial fibers, astrocytes, nerve sheaths, and cells.[24] In 1984, El-Yazigi *et al*. evaluated gold and other elements in the cerebrospinal fluid (CSF) of patients with brain tumors or cerebral neoplasms.[25] Interestingly, although differences between the concentration of gold in controls and other tumor types were achieved, there was no consistent relationship between gold levels in controls and patients with pineal blastoma.[25] One year later, in 1985, Foster and Johansson used colloidal gold particles (5 nm diameter) as a marker for labeling somatostatin-like immunoreactive (SOM-LI) neurons and to compare different areas in the rat brain. SOM-LI positive vesicles could be distinguished from other putative neurotransmitters (and from postsynaptic effects) that supported somatostatin as a neurotransmitter in the rat CNS.[26] Colloidal gold (2–150 nm) was the first form utilized as a "nervine" to treat people with neurological conditions, as it was first recommended by ancient Indian and Chinese alchemists.[27]

GNSs are now known to possess unique optical and electrical properties depending on their size, shape,[28-32] and surface chemistry[33, 34] arising from localized surface plasmon resonance (LSPR). The LSPR effect occurs in noble metal nanostructures, due to the oscillation of the free electrons on the surface of metal nanostructures after irradiation by light (visible light with a wavelength longer than the size of the metal nanostructures).[35-37] Compared to other noble metals (*i.e.*, silver, copper, palladium, and platinum), the LSPR in GNSs is more sensitive and, therefore, can result in properties that make them suitable for developing ultrasensitive biosensors or therapeutic agents. For example, LSPR can result in an enhanced electric field around GNSs, which could be used to design metal-enhanced systems such as metal-enhanced fluorescence (MEF),[38] metal-enhanced singlet oxygen generation (ME-SOG),[39] and surface-enhanced Raman scattering (SERS)[40]. Another attractive property of GNSs is their high absorption cross-section compared to other metal nanoparticles. This characteristic can be used to develop fluorescence resonance energy transfer (FRET)-based biosensors[41] and therapeutic agents based on photothermal therapy[42]. A comparison of the advantages and disadvantages of different nanomaterials are shown in Table 1.

In particular, we can highlight the high density, the plasmon, and the photothermal properties that give us the possibility to use them for computed tomography (CT), optoacoustic, and Raman imaging and also kill tumor cells and spatial and temporal controlled release. In comparison, silver nanoparticles (AgNPs) that also exhibit plasmon properties pose absorption bands centered at the visible region of the electromagnetic spectra making difficult the *in-vivo* applications. Moreover, they exhibit potential toxicity associated with the release of Ag ions, which trigger biochemical alterations, abnormalities in behavior, and neurotoxic effects.[43] On the other hand, we can mention liposomes, polymer nanoparticles, and dendrimers (Table 1) that exhibit excellent properties for drug delivery purposes however per se they don't possess properties for imaging. In contrast, magnetic nanoparticles are used for imaging and drug delivery however they do not exhibit plasmon properties that are relevant for Raman and optoacoustic imaging. Moreover, materials as carbon nanotubes (CNTs) can be used for photothermal therapy and nanodiamonds exhibit interesting properties for *in-vivo* imaging with fluorescence emission centered in the near-infrared (NIR).[44]

Moreover, colorimetric biosensors based on GNSs can be designed due to the high dependency of the LSPR of the GNSs on their size, shape, and refractive index of the environment (*i.e.*, surface chemistry).[37] Also, the low toxicity and biocompatibility of GNSs in neurological tissues make them suitable for a wide range of neurological applications.[45-47] Owing to all these properties, GNSs can function as versatile and meaningful tools in neuroscience, especially for combined diagnostic and therapeutic purposes (theragnostics).[48-50] The development of GNSs has become a promising route to next-generation neuro-nanotechnology interfaces in various molecular neurotherapy approaches. The combination of GNSs (and corresponding hybrid systems) with neurobiology has made a real contribution to monitoring,[51] neural differentiation, and regeneration,[52, 53] recording of neural signals,[54] and multimodal neurotherapies[55]. Furthermore, the design of GNS-loaded neural prostheses has resulted in

investigational long-term implants, with improved biocompatibility and bioelectrical properties.[56] These implants may be used to treat neurological disorders, such as epilepsy, paralysis, and blindness in the future.

The applications of gold nanoparticles (AuNPs) in biosensor design and drug delivery have attracted sustained interest over the last ten years. Fig. 1A shows a graph of the number of WOS publications reported on the Web of Knowledge database on the following topics: i) AuNPs and neurons, ii) AuNPs and biosensors, iii) AuNPs and CNS, iv) AuNPs and drug delivery, and v) AuNPs and theragnostics. Studies focusing on AuNPs in biosensor and drug delivery applications have shown an impressive increase since 2012 (Fig. 1A). The reason is due to the unique physicochemical features of AuNPs, which allow improvement in the detection of biomarkers and the design of drug delivery systems.

Fig. 1 here

The application of AuNPs for the treatment of CNS disorders has also been explored, and although the number of papers is low, there is a sustained growth rate of the number of documents (Fig. 1A). A critical issue for the application of NPs in the CNS is their capacity to cross over the blood-brain barrier (BBB). The interest in biomedical applications of GNSs in the CNS has shown an increase by researchers all around the world (Fig. 1B). On the other hand, the number of papers related to the interactions of neurons with AuNPs is a rapidly emerging field. It is essential to understand the balance between the benefits and the potential dangers or toxic effects of these materials to arrive at an informed opinion about their future applications.

Fig. 2 displays information regarding the number of patents reported in the World Intellectual Property Organization (WIPO). Patents related to AuNPs, neurons, and CNS first appeared in 1992, concerned with GNSs and organic particles for imaging purposes. After 2005, there was an increase in the number of applications per year related to AuNPs and neurons. A similar pattern can be observed in the number of patents related to AuNPs and CNS (Fig. 2). GNSs used for theragnostics have been studied to a lesser extent, with 646 patents spanning from 2006 to 2020. Remarkably, the number of patents has increased with each passing year.

Fig. 2 here

Up to the present time, there has been no in-depth overview of the use of GNSs in neuroscience and neurology, despite their high potential and rapid progress, as discussed above. Therefore, for the first time, we systematically examined the synthesis methods, physicochemical characterization, and versatile applications of GNSs in the major branches of neuroscience. GNSs, due to their optical and electric properties, have been studied for diverse biomedical applications, including in NDs (Fig. 3).

Fig. 3 here

The preparation of GNSs with different geometries, and with suitably defined biochemical properties is possible. In the last decade, surface functionalization has become a substantial research thrust for selectively targeting biological structures. Thus, we summarize different functionalization methods that allow conjugation with a wide range of molecules to reduce their cytotoxic effects. The most important techniques utilized to characterize GNSs are covered, such as transmission electron microscope (TEM), scanning electron microscope (SEM), dynamic light scattering (DLS), X-ray diffraction (XRD), ultraviolet-visible (UV–vis), infrared (IR), and Raman spectroscopy.

Table 1 The advantages and disadvantages of different nanomaterials

Nanomaterials	Advantage	Disadvantage	Refs
Metal (gold, silver, iron)	• Various shapes (nanorods, spherical, and triangles) • Tunable size, stable, and uniform structure • Increased surface area • Increased loading capacity • Multimodal applications • Antimicrobial and antifungal properties • Tuned pharmacokinetics • Biodistribution • Stimuli-sensitive behavior • Versatility of surface modification • Antibacterial and antiviral properties • CT contrast agent • Surface plasmon resonance	• Poor cell uptake • Low biocompatibility • Toxicity • Storage • Instability • Impurity	57-62
Liposomes	• Biodegradable • Biocompatible • Hydrophilic and hydrophobic cargo loading • Easy functionalization	• Low stability • Highly expensive • Leakage of the nanocarriers • Toxicity owing to cationic lipids • Moderate loading capacity	58-60, 63, 64
Polymers	• Low toxicity • Biodegradable • Cost-effective • Surface modification • Easy to manipulation • High specific capacitance • Biocompatible • Controlled drug release	• Low conductivity • Difficult to scale up • Degradation of the carrier • Poor electrochemical stability • Need for functionalization • The limited application for lipophilic drugs	58-60, 63-67
Magnetic	• Magnet-guided targeted thrombolysis • Imaging agent • Targeted cargo delivery agent	• Low biocompatibility • Low colloidal stability • Possibility of immunogenicity or inflammatory response • Potential material toxicity	57, 63
Carbon materials	• Increased surface area • Increased loading capacity • Increased conductivity • Targeted delivery agent • Imaging agent • Electrochemical stability • Multiple functions • Surface modification • Water-soluble • Resistant to temperature change	• Low energy density • Low degradability • Toxicity • Poor dispersity	58, 59, 63, 66

	• Highly flexible • Lightweight		
Dendrimers	• Uniform size and structure • Tuned pharmacokinetics • Targeted delivery of lipophobic or lipophilic cargos • Increased surface area • Increased loading capacity • Versatility of surface modification • Solubility enhancer for lipophilic drugs	• Complex synthetic processes • Toxicity induced by cationic dendrimers • Toxicity induced by surface amine groups • Highly expensive • Unsuitable carrier for hydrophilic drugs • Non-biodegradable • Hemolytic properties	57, 58, 65, 68

2. Synthesis of GNSs

2.1 AuNPs

Solid matter is composed of atoms organized together into crystals or bonded into molecules. Microparticles and NPs are aggregates of a limited number of atoms, classified as coarse particles (10,000–2,500 nm), fine particles (2,500–100 nm), and ultrafine particles (1–100 nm), which latter are generally referred to as NPs.[69] Following this classification, coarse and fine particle properties do not differ significantly from their bulk counterparts, having only a modestly increased surface to volume ratio. However, when the aggregate's size is lowered to the nanometer scale, the chemical and physical properties (*e.g*., chemical reactivity, melting point, electrical conductivity, magnetic permeability, fluorescence) can radically change.[70] Thanks to their unique electronic, magnetic, optical, mechanical, physical, and chemical properties, NPs with sizes in the range of single to hundreds of nanometers have attracted much attention in physics, chemistry, materials science, medicine, and biology. Among the many types of NPs, AuNPs are one of the most often used in modern nanoscience and nanotechnology.

Unlike other systems, metallic and AuNPs display great flexibility, allowing selection of the appropriate size, chemistry, structure, and assembly. This permits a fine-tuning of the NP physical properties, which are on the basis of typical applications. Methods to produce gold colloids were used since antiquity to make colored glasses. However, only in 1908, Mie explains the different colors of Au colloids by correlating the optical absorption of spherical objects with their dimensions.[71] Therefore, the frequency of the plasmon resonance can be utilized to estimate the NPs dimensions. The problem of estimating the AuNPs size was finally overcome with the advent of electron microscopes, which could directly visualize the synthesized NPs. It was then possible to check the results of different synthesis methods and conditions. The electron microscope was extensively utilized by Turkevich *et al*. to analyze the AuNPs produced by reducing tetracloroauric acid using various reducing agents.[72] To better control the particle size, Frens *et al*. revised the Turkevich method by selecting the optimal citrate/gold concentrations.[73] However, the dispersibility and stability of the gold colloid NPs were still challenging, preventing extensive scale-up of the production process. In 1994 Burst *et al*. proposed a new synthesis method based on using alkanethiols as a stabilizing agent.[74] This method was easily scalable and led to a narrow distribution of AuNPs size peaking at ~3 nm. Next, other strategies were proposed to improve the Au colloid monodispersity, such as digestive ripening. Small monodisperse AuNPs are produced at the expense of sizeable polydispersed gold colloids.[75]

Besides spherical NPs, a wide variety of other shapes can be obtained using gold precursors.[76] The first example of the synthesis of anisotropic AuNPs dates back to 1989 when Wiesner and Wokaun produced rod-shaped AuNPs by adding Au seeds to solutions of gold (III) chloride

($HAuCl_4$).[77] In 2001, the Murphy group described the synthesis of gold nanorods (AuNRs) by the addition of citrate-capped AuNPs to Au(I) obtained from the reduction of Au(III) using ascorbic acid in the presence of cetyltrimethylammonium bromide (CTAB).[78] Besides seed-mediated methods used to synthesize specific shapes with high yields, bulk solution methods are also possible, although a low yield is obtained for specific shapes.[79] Many synthesis protocols involve templates or different capping agents, even though the seed-mediated method remains the most popular. Well-known processes include photochemical, electrochemical, and sonochemical methods using templates, and others.[76] Nevertheless, the optical properties are essential to qualify the anisotropic AuNPs. The Mie theory can be extended to anisotropic NPs, where a spherical shape leads to the absorption of longer wavelengths.[80] The appearance of a plasmon band in the NIR region is one of the more appealing properties of these AuNPs. Considering that the water window (minimum light absorption by water) extends from 800–1300 nm, high absorption in this region makes these anisotropic NPs attractive for medicinal and theragnostic applications.

Over the years, gold colloids were found to be useful in different biomedical areas, and it has been proposed that AuNPs could be used in diagnosis, therapy, prevention, and hygiene as well. AuNPs have potential applications for treating cancer[81] and targeted delivery of drugs[82], peptides, or DNA[83], in molecular biology[84], imaging, and sensing[85]. Interested readers are referred to the review of Dreaden for an exhaustive list of the different applications of GNSs.[86]

In the following section, the synthesis and characterization of AuNPs are presented. In section 2.2, the main chemical processes, namely Turkevich, Burst, and Digestive Ripening, are discussed to synthesize spherical AuNPs. Section 2.3 describes the most popular methods to synthesize anisotropic AuNPs, such as nanorods and hollow AuNPs or gold nanocages (AuNCs). Section 2.4 describes alternative processes to wet chemistry. These processes, such as radio frequency (RF) sputtering or chemical vapor deposition (CVD) are utilized to deposit NPs on particular pre-synthetized substrates. Laser ablation is used when high control of the AuNPs is required. Besides physical processes, the bio-synthesis of AuNPs using microorganisms is briefly reviewed. Finally, section 3 is dedicated to the characterization methods, such as transmission and scanning electron microscopy to obtain morphological and structural information, UV–vis, IR, and Raman spectroscopy to define the AuNPs optical properties, XRD to shed light on the crystalline structure of AuNPs, and DLS to analyze the size distribution of AuNP colloidal suspensions.

2.2 Spherical AuNPs

2.2.1 Turkevich process

A useful synthetic procedure for producing Au colloids was introduced by Turkevitch[72] and Brust–Schiffrin[74]. The Turkevich method, due to its simplicity, is one of the most commonly used procedures to synthesize spherical AuNPs with sizes in the range of 10–120 nm (Fig. 4).[87] This method is based on the reduction of trivalent gold ions (Au^{3+}) provided by chloroauric acid to gold atoms (Au^0) in the presence of a reducing agent such as citrate,[88] amino acids,[89] ascorbic acid,[90] or UV light[91]. However, the simplicity of the procedure is at the expense of the monodisperse quality of the NPs. Capping/stabilizing agents may be used to make the AuNPs more stable. Several improvements to the Turkevich process have allowed researchers to expand the range of NP sizes by varying the ratio of precursor and reducing agent.[92]

Fig. 4 here

Other authors were able to produce stable colloidal suspensions using specific stabilizing agents. The dimensions of the AuNPs could be varied by modifying the gold precursors and reducing agents, such as citrate, ascorbic acid, 3-mercaptopropionate,[87, 93] and the stabilizing agent ratio (Fig. 5)[93].

Fig. 5 here

The role of pH values on the size distribution of AuNPs was investigated by measuring their value during the reaction. The authors found an initial decrease in the NP size with slightly increasing pH, while the NP dimensions increased at low and high pH values (Fig. 6). The optimization of the pH allowed the production of a narrower distribution of AuNPs size.[94, 95]

Fig. 6 here

Shou *et al*. studied the effect of pH and reducing agents on the nucleation and growth of AuNPs.[96] In particular, they found that when Pluronic P85 copolymer was used as a reducing agent, the reaction proceeded faster at higher pH values. On the contrary, at high pH, the reaction was slower when using sodium citrate or ascorbic acid as reducing agents. Finally, the pH also influences the morphology of the AuNPs.[97] TEM analyses and chemical considerations suggest that several possible mechanisms are at play to explain this behavior. At low pH, small clusters likely dissolve with concomitant deposition onto large faceted NPs. Intermediate pH-induced variation of clusters surfaces energy leading to aggregation in oblate NPs. At high pH values, oxidation of the citrate reduction agent likely occurs with the production of H^+ ions. The charge and ion distributions around the gold aggregates led to the production of spherical monodispersed NPs.[98]

2.2.2 Brust method

The Brust method is a two-step process to synthesize small AuNPs in the range of 1.5–5 nm.[74, 99] Essentially the $HAuCl_4$ precursor (Au^{3+}) is dispersed in an organic solvent (such as toluene) with the help of tetraoctylammonium bromide (TOAB) and then reduced with $NaBH_4$ in the presence of an alkanethiol. Self-assembled thiol monolayers can control the size of the NPs growing onto the nucleus by varying the thiol to gold ratio. Interestingly, this synthesis method can be scaled up to gram levels for the commercial production of AuNPs. Modified Brust methods have been reported to synthesize AuNPs using arenethiolate[99] or (γ-mercaptopropyl)trimethyloxysilane[99] with reduction by BH_4, or oleylamine[100] as an NP stabilizer, and t-butylamine-borane complex as a reducing agent.

2.2.3 Digestive ripening

The synthesis of monodisperse AuNPs on a commercial scale remains challenging, especially if stabilizing ligands other than alkanethiols are used. Digestive ripening, or inverse Ostwald ripening, is a convenient method to produce monodisperse AuNPs from polydisperse NPs using a metal-to-ligand molar ratio of 1:20, or 1:30. The reaction side products can be separated from ligand-coated AuNPs by precipitation by ethanol addition. In digestive ripening, the smaller-sized particles grow larger, while the larger-sized particles shrink, leading to an overall narrower distribution of the NP size, ranging from sub-nanometer to ~10 nm. [101]
In the classical digestive ripening process, a colloidal suspension is heated at ~138 ºC for 2 minutes and then at 110 ºC for 5 h in the presence of alkanethiols. Temperature is the key parameter to control the NP size. It was demonstrated that by increasing the reflux temperature from 60ºC to 180 ºC, an initially narrow monodisperse suspension of AuNPs was transformed into a bimodal NP distribution of larger particles.[102] In addition to alkanethiols, various other ligands can be used for digestive ripening, such as other thiols, amines, phosphines, alcohols, halides, silanes, and simple alkanes.[75] Different ligands lead to differences in the shape and size of the AuNPs. For example, experiments have shown that thiols can etch the surface of AuNPs, leading to smaller NPs.[103, 104] The addition of dodecanethiol, phosphines, amines, and silanes to a polydisperse Au colloid at room temperature induces drastic changes in the appearance and

size of the NPs, notably transforming significant polyhedral polydispersed NPs in a narrower distribution of sizes and more spherical NPs. However, further increasing the ligand concentration and refluxing the suspension produces larger NPs with prismatic long-range lattices.[75] The different behavior depends on the strength of the Au-ligand bonds, with weaker ligands stabilizing larger particles and *vice versa*.

2.3 Anisotropic GNSs

Anisotropic hexagonal and pentagonal AuNPs were first produced in the 1980s by a vapor deposition method, which is a two-step process.[105, 106] In the first step, seeds are produced, while in the second step, the seeds are added to a solution containing $HAuCl_4$ plus a mild stabilizing/reducing agent to deposit Au^0 onto the seeds.[76] The nanostructure shape and dimensions could be controlled by varying the concentration of seeds, reducing agents, and the use of structure-directing agents. For example, AuNRs can be synthesized by the seed-mediated method using CTAB as a surfactant. The addition of a small number of iodide ions suppresses the crystal growth along with the Au (111) direction, leading to Au (111)-faced triangular nanoprisms.[107] When CTAB counter-anions were replaced with chloride ions, partially elongated NPs (rice-shaped) were observed.

In metallic materials such as gold, conductance electrons are almost free to move without resistance. They undergo oscillations upon light excitation, known as the surface plasmon resonance (SPR) effect (see sections 3.4 and 3.5 for more details). It is known that the SPR effect depends on which metal the NP is composed of, on the NP shape and size,[108] and the thickness of the coating shell[109]. Consequently, various shapes of GNSs, such as nanospheres, nanorods, nanoshells, and branched NPs, have been fabricated with different SPR features. AuNRs and hollow gold nanoshells (AuNShs) allow the modification and tuning of the SPR properties by varying the structure. Anisotropic NPs, including nanocrystals with other crystalline forms (tetrahedron, cube, octahedron, icosahedron, decahedron, truncated tetrahedron, truncated cube, truncated octahedron), have been investigated.[110] The chemical synthesis of one-dimensional nanostructures usually employs a gold surfactant-directed route. In a typical process, a gold precursor is reduced in the presence of an ionic surfactant at a high concentration, which causes the preferential growth of Au along with predetermined crystalline directions.[111] The same mechanism applies to the synthesis of AuNRs.[112] Anisotropic AuNP may be synthesized by replacing the surfactants with Ag nitrate.[110, 111] In addition to these methods, there are also other routes for the production of anisotropic AuNPs, such as electrochemical, seed-mediated, template method, and biosynthesis procedures that can produce nanorods, nanocrystals, and nanoprisms, respectively.[111]

2.3.1 AuNRs

The electrochemical deposition of gold within the pores of nanoporous aluminum templates was first employed to synthesize AuNRs.[113] It was shown that by changing the aspect ratio of the nanocylinders, the colors of the product could be varied.[114] However, this method led to low yields and multipolar modes in the SPR spectrum. AuNRs with 10 nm diameter were obtained by electrochemical oxidation of a gold-plated electrode in the presence of CTAB and TOAB.[115] These NPs displayed two distinct SPR modes associated with the longitudinal and transverse polarization axis of the rods. The broader use of AuNRs has led to high yield chemical processes based on seeding with AuNPs and reducing $HAuCl_4$ with ascorbate.[78] NPs with 10–20 nm diameter and a length of 300 nm were obtained. Then, by controlling the growth conditions in aqueous surfactant media, it was possible to synthesize AuNRs with a tunable aspect ratio. It was found that the use of an aqueous surfactant with the addition of $AgNO_3$ influenced the yield, the aspect ratio, and the crystal structure.[116] It has been hypothesized that Ag^+ is adsorbed on the Au particle surface, thereby limiting the growth. Besides, the presence of Ag^+ ions stabilizes

the spheroids and rods, while in the absence of ions, the spheroids were converted to spheres.[78] It was possible to achieve the fine-tuning of the AuNR aspect ratio by adjusting the amount of Ag^+ in the growth solution. In particular, increasing the $AgNO_3$ concentration (up to a critical concentration) led to a redshift in the longitudinal SPR band. A similar trend was observed when the gold ion concentration was increased.[117] Synthesis of AuNRs has also been carried out in the absence of Ag^+ ions and is based on optimizing the concentrations of CTAB and ascorbic acid. It is thought that the CTAB is adsorbed onto AuNRs with preferential adsorption on different crystal faces, thus influencing the growth as shown in Fig. 7. AuNRs are shown as GmSn where m represents the volume of the surfactant solution (mL), while n is the volume of the seed solution (mL). [118, 119]

Fig. 7 here

An important issue relating to the biomedical applications of AuNRs is the toxicity of CTAB. Since CTAB is unavoidable in the synthesis procedure, a coating of AuNRs with SiO_2 can be performed[120] to cover up the toxic CTAB, while preventing the AuNRs aggregation. The intrinsic porosity of silica can also be utilized to load drugs and other molecules for delivery. The higher biocompatibility of the AuNRs was also achieved by a ligand exchange, which replaced the CTAB with a bovine serum albumin (BSA) coating.[121] Similar approaches have used ligand exchange based on 11-mercaptoundecanoic acid (11-MUA) or bidentate N-heterocyclic-carbene–thiolate.[122, 123]

AuNRs show high SPR within a tunable range, and for this reason, they are often utilized as contrast agents in photoacoustic, photothermal, NIR absorbance imaging.[124] The latter technique is based on the good penetration of NIR radiation into living tissue, while visible light shows much higher absorption and scattering. Compared to other NIR imaging probes such as quantum dots, fluorescent dye-doped NPs, *etc*., AuNRs are useful because the main absorption band is located in the "first tissue optical window" extending from 700–1000 nm. The second (1100 –1350 nm) and third (1600–1870 nm) optical windows are also useful for tissue imaging and therapy.[125] Fig. 8 shows an example of NIR imaging using cyclic Arg-Gly-Asp (cRGD) peptide-conjugated-PEGylated AuNRs (cRGD–PAuNRs) with high specificity to target brain tumors. The targeting cRGD–PAuNRs motif recognizes integrins overexpressed in tumor cells and on tumor blood vessels and is more concentrated (Fig. 8A). At the same time, the non-targeted cRAD functionalized PAuNRs are diffused in the whole brain tissue.[126] The targeting efficacy is also shown by the different photon counts obtained from tumor tissues (Fig. 8B). The absorption signal from cRGD–PAuNRs in the tumor-bearing mice at 6 h, was 240% higher than that from cRAD–PAuNRs (control) that did not change for 12 h (Fig. 8C).

Fig. 8 here

2.3.2 Hollow AuNPs and AuNCs

Hollow nanospheres with tunable thickness and cavity size allow the SPR absorption band to be shifted from the visible up to the NIR region.[127] This property may be of great interest for photothermal therapy (PTT),[128] which can be used for the selective ablation of tumors[129]. Hollow nanospheres also possess interesting therapeutic uses as drug carriers.[130, 131] Traditional methods to synthesize hollow NPs have been based on a sacrificial template, polystyrene spheres,[132] silica ($SiO_{2)}$ spheres,[133] resin NPs,[134] vesicles,[135] or liquid microemulsions[136]. Abdollahi *et al*.[137] first prepared SiO_2 NPs by a conventional route based on tetraethyl orthosilicate and then deposited an Au shell onto the SiO_2 NPs by reducing $HAuCl_4$ with trisodium citrate. The SiO_2 core was then removed using HF, leading to hollow AuNPs of ~400 nm size and 25 nm shell thickness. Different volumes of $HAuCl_4$ were utilized to obtain

monodisperse hollow spheres with an outer diameter of ~60 nm and an inner diameter about 40 nm.

AuNCs can also be used as contrast agents in optical coherence tomography (OCT) and spectroscopic optical coherence tomography (SOCT). Thanks to the large absorption/scattering cross-sections of hollow AuNPs, these can be successfully applied to *in-vivo* imaging with OCT and SOCT. A high spatial resolution is required to differentiate tumors from healthy tissue.[53] Skrabalak *et al*. demonstrated the feasibility of imaging using AuNPs tuned to a 716 nm SPR absorption band and excited with a seven femtosecond Ti: sapphire laser at 825 nm.[131] The PA tomography (PAT) imaging revealed good contrast for normal tissue. Hollow AuNPs have also been employed to improve resolution in PAT.[138] PAT provides higher spatial resolution compared to purely optical imaging in deep tissue (up to 6 cm deep). It is also superior to conventional ultrasonic imaging because of the singular intrinsic and extrinsic visual contrast and because it is free of speckle artifacts.[138] AuNCs have also been utilized in PA imaging to detect B16 melanoma cells with 778 nm excitation, and blood vessels with 570 nm excitation.[139] Thanks to the sharp optical contrast of AuNCs, the authors overcame the low sensitivity and specificity, poor spatial resolution, and shallow penetration depth of conventional imaging techniques (Fig. 9). This approach is still in its early stages but could be considered for early diagnosis, accurate staging, and image-guided resection of tumors.

Fig. 9 here

2.4 Additional synthesis methods

2.4.1 Physical methods

Precise control of the AuNP density, morphology, and composition may be achieved using an RF sputtering technique under mild conditions.[140] High purity AuNPs can be deposited on different substrates,[140-143] nanostructures[144-146] or synthesized in liquid media[147]. RF sputtering allows control over the NP dimensions and density by selecting the plasma deposition parameters (RF power, deposition duration). The AuNP size and distribution may be tailored for specific applications by exploiting the competition between deposition and ablation processes occurring in the plasma. CVD is broadly used in industry and can deposit NPs on various substrates with the advantages of simplicity, short processing times, high purity of the product, in-situ deposition, and the possibility to deposit gold on complex surfaces.[148] In addition to deposition by plasma, LA is an alternative method that allows accurate and reproducible control of the AuNP synthesis. A high-energy pulsed laser can cause the evaporation and subsequent condensation of gold as NPs. LA of a gold target may be performed in water. This process generally leads to relatively large (~20–300 nm) and polydisperse (~50–300 nm) particles due to both the AuNP agglomeration and the ejection of big target fragments.[149] The authors showed that monodisperse small AuNPs were obtained at low laser fluences. Laser ablation may be performed in various organic solvents, thus producing functionalized AuNPs.[150, 151] By selecting appropriate pulse duration and laser power, AuNPs with tunable features can be produced.[152] These methods have a point in common that the material is vaporized from a target as high-energy particles and then transferred to a spatially removed sample surface.

2.4.2 AuNPs biosynthesis

Some of the methods described above utilize solvents or toxic substances, which may be harmful to the environment, especially in large-scale production processes. Among other techniques, microorganisms can produce AuNPs with different shapes, as shown in Fig. 10,[153] which can potentially be used in medicine to diagnose and treat cancer, as anti-angiogenesis, anti-arthritis, or antimalarial agents.[154] The generation of Au^0 clusters relies on the microbial cell capability to take up metal ions, which are then reduced to NPs by the enzymes used in microbial metabolic processes. The NPs are then eliminated from the bacterial cells.[155]

Fig. 10 here

The intracellular and extracellular generation of AuNPs synthesized by microorganisms is schematically shown in Fig. 11.[156] A complete list of bacteria, which have been used to synthesize AuNPs was provided by Shedbalkar and co-workers.[153] Besides bacteria, microfungi may be easier to be transferred to an industrial production scale because fungi are simpler to grow and control.[157, 158] However, fungi may give less control of the AuNP dimensions leading to a broader size distribution.[157] Like bacteria, fungi reduce the Au^{3+} ions to form gold-nanoconjugates with intracellular proteins in response to toxic stress.

Fig. 11 here

3. Characterization of AuNPs

The particular properties of the NPs determine the potential applications. Characterization of GNSs is then of paramount importance and can be carried out using different techniques.[159] Electron microscopy is currently used to establish the NP morphology. At the nanoscale, TEM and SEM are generally utilized. Optical properties such as those related to plasmonics may be obtained via UV–vis spectroscopy, while IR spectroscopy provides information on chemical bonds. DLS can be used to estimate the NP size. XRD is currently used to obtain structural information.

3.1 Scanning electron microscopy

The NP dimensions are frequently well below the diffraction limit of optical microscopes given by the Abbe law $d=\lambda/2NA$ (NA is the numerical aperture), which for visible light ranges from 180–280 nm. To tackle this issue, one possible solution is to use high-energy electrons because, depending on their energy, the associated resolution can be as small as 1Å. A scanning electron microscope (SEM) can provide images of objects of different sizes, which may vary from micrometer to a nanometer scale. SEM instruments may provide high-resolution morphological information with a high degree of detail and compositional information for the material, although at the lower lateral resolution, using energy-dispersive X-ray (EDX) spectroscopy analysis. Morphological analysis is essential when this nanostructure will be utilized for theragnostic applications in the nervous system.[160] The unique property of electron microscopes to provide laterally resolved chemical information is essential for analyzing the nanosystem composition, ensuring that no contribution comes from the environment. The ability to check the purity of AuNPs and the absence of toxic substances is crucial when used in the CNS.[161]

3.2 Transmission electron microscopy

This technique combines imaging, spectroscopy, and diffraction, thus providing a high amount of information. Modern high-resolution transmission electron microscope (TEM) instruments can provide sample images with atomic resolution (Fig. 12 column 1).[162] It has long been suspected that it would be possible to regulate particle growth based on the ability of the nanostructures to absorb a specific wavelength of light.[163] The spatially inhomogeneous distribution of the enhanced electromagnetic fields generated via plasmon excitation may direct anisotropic GNSs. The annular dark-field scanning transmission electron microscopy (ADF-STEM) opens the possibility to map plasmons on the NP surface to shed light on the plasmon-driven NP growth (Fig. 12 column 2).[31]

Today the use of TEM analysis may supply a three-dimensional (3D) tomographic representation of the sample. After reconstruction, the NP 3D morphology can be obtained[164] as shown for Au–AgNRs in Fig. 12 column 3.[165] This will have an essential perspective for neural applications as it will be clarified in section 3.4. The spectroscopic information, X-ray energy dispersive spectroscopy, electron energy-loss spectroscopy, and TEM-cathodoluminescence can be performed to determine the chemical, structural, and electronic properties of the nanostructure. Finally, when an NP is analyzed with TEM, one can observe a diffraction pattern associated with the material crystalline lattice.

Fig. 12 here

3.3 Inductively coupled plasma mass spectrometry and laser ablation

Besides TEM and SEM, which provide high-resolution structural and morphological information, inductively coupled plasma mass spectrometry (ICP-MS) and laser ablation (LA)-ICP-MS are techniques utilized to allow multi-element determination at ultralow concentrations. In an ICP-MS instrument, an ICP source interacting with the sample surface generates ions and small polyatomic ions, which are then detected. More details are given in references.[166] The ICP-MS instruments are sensitive and capable of detecting sub-picogram amounts of material.[167] This frequently makes the sample preparation time-consuming due to the risk of contamination. The coupling of a LA system to ICP-MS solves this problem, allowing a rapid determination of the sample composition. Recently, these instruments have been utilized to investigate the effect of elemental impurities in drugs. This has a great impact because elemental impurities, such as heavy metals and toxic substances can cause unwanted pharmacological and toxicological effects even at low concentrations. ICP-MS is also utilized to detect DNA nucleotides,[168, 169] proteins,[170] and other biological molecules[171]. LA-ICP-MS can also be utilized to visualize AuNPs within organs.[171] The authors demonstrated the feasibility of utilizing this technique for imaging the distribution of AuNPs in mouse liver. An evolution of ICP-MS is the single-particle ICP-MS (SP-ICP-MS). This technique is becoming an important tool to determine the AuNP size distribution and particle concentrations within only a few minutes.[172] ICP-MS is also effective to determine the size and shape of AuNPs when coated with organic molecules in a liquid phase which is not possible using conventional electron microscopy.[173] Detection of impurities, determination of the size distribution, and the possibility to analyze single NPs are important when dealing with nanosystems, which will be delivered to the human body to reach and interface with the brain tissue.[174]

3.4 Ultraviolet-visible spectroscopy

Ultraviolet-visible (UV–vis) analysis measures the absorption spectra of AuNPs, which is directly related to the plasmonic resonance (described in the next section). UV–vis analysis also allows the estimation of NP size, concentration, and aggregation level. This information is obtained by fitting the UV–vis spectra with the Mie model for spherical scattering particles or using the Gans model for spheroids. Changes in the absorption spectrum such as the appearance of a second feature can be interpreted as the aggregation of AuNPs caused by colloidal suspension instability.[175] In addition to the optical absorption bands commonly employed to characterize GNSs, UV–vis has also been utilized to determine the biodistribution of NPs by *in-vivo* studies in rats in various organs, including the brain, liver, lungs, kidneys, and spleen.[176]

3.5 Raman spectroscopy

Metal NPs are strong absorbers and scatterers of visible light due to their SPR. The corresponding resonant peak energies and line widths are sensitive to the NP size, shape, and nanoenvironment.[112]

AuNPs show a size-dependent absorption peak in the visible region from 500–550 nm,[177] leading to the characteristic colors of AuNPs in aqueous suspension (Fig. 13)[112]. This efficient absorption is due to plasmons, collective oscillations of the conduction electrons of the NP surface resonant with the excitation frequency. The absorption occurs in a rather broad range of wavelengths reflecting the NP size, which can then be estimated.[178] However, plasmon band shifting depends not only on the NP size but also on the NP shape, on the solvent, on the presence of surface ligands, and solution temperature.[177] Among many applications in biomedicine, SPR is widely utilized for chemical and biological sensing, drug delivery, for studying the interaction between functional ligand-functionalized NPs and their corresponding receptors[179] including antibodies (Abs), nucleic acid probes, and aptamers. SPR can be used for studying proteins and the protein corona adsorbed on the NP surface, protein biomarkers of diseases such as inflammatory disorders, tumors, cardiac biomarkers, and for studying immune response disorders. Several authors have employed LSPR to detect neural activity.[180-182] Since electrically excitable cells such as neurons produce fast optical signals associated with membrane depolarization, this enables LSPR to monitor electrical signal propagation through local variations in the refractive index.[183] Plasmonic structures such as AuNPs can be utilized to monitor hippocampal neural spiking activity in real-time by the LSP resonance shift.[184] AuNPs can also be used to control neural activity by inducing membrane depolarization.[185] Due to the efficient light absorption in the plasmonic band, 532 nm light caused heating due to a variation of the membrane capacitance and depolarization. In another report, AuNRs were utilized in combination with 780 nm laser light to induce intracellular calcium transients and stimulate neuronal cell growth.[186, 187] Another exciting application of LSPR is plasmon-enhanced multiphoton luminescence. The aspect ratio of AuNRs was tuned to obtain an LSPR that peaked at 1000 nm within the tissue transparency window.[188] Upon excitation, with a femtosecond pulsed laser, the AuNRs emitted a broadband multiphoton luminescence (420–630 nm), which was exploited to image the brain blood vessels.

Fig. 13 here

The plasmonic effect has been extensively exploited in SERS. Raman spectroscopy is highly sensitive to the vibrational bands in the molecular structure. It is a powerful technique for the non-destructive analysis of inorganic and organic materials, including biological systems such as proteins, DNA sequences, and living or dead cells. When analyzing natural material, problems arise from the small volumes analyzed, the limits on the light intensity required to avoid sample damage, intense autofluorescence, and high sample noise.[189] SERS exploits the electromagnetic field enhancement induced by the localized plasmons generated by the excitation laser in the metallic substrate. The nature of the substrate and its conformation is crucial for the SERS enhancement. The SERS enhancement is proportional to the local electric field's fourth power around the metallic substrate, so it is much more pronounced when very close to the metal. Due to SERS' plasmonic enhancement, the Raman signal can be amplified typically by factors of $\sim 10^5$–10^6. In surface-enhanced resonance Raman scattering (SERRS), it may reach amplification of the order of $\sim 10^{10}$–10^{11}.[190] Metallic NPs with anisotropic shapes and sharp tips have more hot spots in their surrounding electric field. This enables them to be used to detect analyte molecules even at the single-molecule level.[191]

SERS can provide an exciting opportunity to analyze the Raman spectra of biological samples by amplifying the signal even at relatively low laser powers. A final remark concerns the use of polarized excitation light to further investigate the chemical composition. Stetsenko *et al*. analyzed AuNP dimers using polarized UV radiation, modulated using a photoelastic polarization modulator.[192] They investigated the LSPR of the GNSs and observed an increasing broadening of the LSPR band with a growing number of AuNP dimers. A detailed analysis of the LSPR as a function of the optical-polarization could distinguish between single AuNPs, AuNP aggregates, and AuNP dimers. Polarized light may also be used for chemical analysis exploiting the Raman optical activity (ROA). ROA derives from the differential scattering of right and left

circularly polarized light by chiral molecules. Since the ROA signal is way weak, plasmonic structures such as AuNPs are used to enhance the signal intensity.[193]

3.6 Infrared spectroscopy

Infrared (IR) spectroscopy is a common technique for investigating the chemistry of many materials. This technique is sensitive to the shape, size, and aggregation of metal NPs.[194, 195] IR spectroscopy, or more commonly Fourier transform infrared (FTIR) spectroscopy, is a convenient tool, because it can be utilized to characterize colloidal suspensions. However, one drawback of using IR radiation is the strong absorption of IR by water in several regions due to the O–H stretching and bending vibrations. A possible solution is to perform FTIR spectroscopy in frequency ranges that do not overlap with water absorption. Many published studies involve the use of FTIR for the characterization of AuNPs by measuring molecular changes caused by the surface functionalization methods. In modern instruments, chemical information can be obtained with nanometer lateral resolution and can overcome the problems arising from water absorption.[196] Important information about the capping and stabilization of NPs can also be provided by FTIR. [197]

3.7 X-ray diffraction

X-ray diffraction (XRD) analysis is a useful technique to analyze the material structure with high resolution. Sophisticated XRD analysis may resolve the atomic structure of crystals, separate and quantify different crystalline phases in a composite, and identify nanoscale assemblies. XRD provides essential information about the degree of crystallinity, *i.e.*, long/short atomic ordering. The local disorder seen with size reduction to the nanoscale affects the system diffraction properties by inducing a broadening effect.[198] The smaller NPs have lower coherence at the nanoscale, and the result is a pronounced diffuse background and less intense and broader diffraction spectral features.[198] Nevertheless, XRD spectra may be utilized to provide information about the NP size.

3.8 Dynamic light scattering

After synthesis, the particle size and surface charge are among the most critical parameters needed to characterize the NP population. The DLS technique has gained popularity since it is a relatively easy and reproducible method to estimate these parameters. Besides, it is a non-destructive/non-invasive technique requiring only low amounts of samples. DLS is based on the ability of NPs to scatter incident light in proportion to the 6th power of their radii.[199] DLS is exciting for biologists because work on colloidal suspensions leads to estimations of the NP hydrostatic dimensions. The hydrostatic diameter accounts for the extension by the biological coating present on the NP surface in its hydrated/solvated state (Fig. 14).[200] Thus, DLS gives the real size of the NPs in a wet biological environment.

NPs can be suspended in different solvents, although caution has to be used to control the colloidal stability over time and the solvent scattering power, which introduces background and noise into the measurements. Poor colloidal stability can be detected by DLS, which can be used to detect the occurrence of NP aggregation.[201] Although, concentrating on lipid NPs, the effect of size and polydispersity on the delivery of NPs to the brain has been reviewed.[202]

Fig. 14 here

3.9 General remarks on the synthesis and characterization of GNSs

Decades of work have led to the production of a great variety of GNSs possessing different sizes, shapes, structures, and optical properties. Several consolidated production methods can be

selected based on the properties required for the AuNPs. About neurological applications, GNSs of different sizes and shapes are utilized for various purposes. AuNPs in the range of 5–100 nm are used to modulate the peripheral nerve regeneration;[203] nanorods are being used to modulate the electrical activity or the calcium ions (Ca^{2+}) dynamics.[204]
Crucial in developing GNSs is also the surface chemistry that can be determined during synthesis with an appropriate capping agent or a subsequent functionalization process. A wide range of options offered to functionalize the GNS with bioconjugate molecules that may be selected to enhance the GNS biocompatibility, target specific molecules, enhance neuron penetration, and accommodate specific therapeutic molecules, or improve the GNSs ability to cross the BBB. The selection of the synthesis method and next functionalization process has then to be made considering the specific utilization of these nanostructures. Although non-exhaustive, a brief description of the principal techniques utilized to the physicochemical properties of the GNSs is also given.

4. Biocompatibility and cytotoxicity of AuNPs

The biomedical use of engineered NPs requires a detailed investigation of their potential biological effects to identify any possible toxicity issues, either short-term or long-term.[205-207] A clear understanding of the specific risks of toxicity associated with the use of AuNPs is presently unavailable as several seemingly contradictory reports can be found in the literature, some highlighting the good biocompatibility. In contrast, others suggest there can be severe toxicity of AuNPs.[208] The main reasons for this divergence in opinion are manifold, and may be linked to the lack of standardized methods for evaluating NP toxicity and NP characterization.[208] Nevertheless, close attention to the details is important when interpreting the toxicity data of any particular NP formulation.

In the following section, emphasis will be placed on the influence of particular NP properties on toxicity and biodistribution. It is important to note that the same NP properties will generally affect both toxicity and biodistribution, but this can either be directly or indirectly related to both phenomena. Biodistribution describes the location of the NPs in an individual organism, while toxicity looks at the adverse effect elicited by the NPs. As the NPs typically cause local effects, their toxicity profiles will be linked to their biodistribution profile, where organs with high levels of AuNPs are more susceptible to higher levels of toxicity. It is also important to note that both phenomena are time-dependent, and the biodistribution and toxicity change over time. For comparative purposes, immediate (acute effects) or long-term effects are both important to get an idea of where the NPs are located and how long they remain there (biodistribution), and whether the NPs cause any acute or long-term toxicity.

The next section of this review will discuss the toxicity of AuNPs, focusing on the three major topics: (a) the nature of the NP core (gold);[209-211] (b) the influence of NP size and shape; (c) the surface chemistry. While this will not be a full review in itself, and interested readers are referred to other detailed reviews on this subject,[43, 212] an overview will be presented of our current knowledge of AuNP toxicity, emphasizing recent studies and the effect of AuNPs on cells of the CNS.

4.1 Properties affecting AuNP toxicity

4.1.1 The nature of the Au core

In itself, gold is often seen as the noblest metal in the periodic table, given its low chemical reactivity. AuNPs are considered to be poorly biodegradable and can persist in cells or the body for long periods. This may cause potential problems, and various groups have studied AuNP persistence in the body. In one study, 20 nm diameter AuNPs were intravenously (i.v.)

administered to rats (*in-vivo*), and the effect on inflammation in the lungs was examined at different time points. The data revealed an apparent influx of lymphocytes into the lung tissue and evidence of lung inflammation. From a mechanical point of view, it was demonstrated that the AuNPs caused the downregulation of microRNA (miRNA)-327, resulting in higher levels of lung inflammation.[213] Other *in-vivo* studies have found similar results, where i.v. administration of PEGylated AuNPs into mice was found to result in altered levels of miRNA-183 and let-7a in the lung and liver, correlating with inflammatory responses.[214]

The fact that AuNPs cause oxidative stress has been widely reported. The introduction of a variety of NPs into a biological environment can result in free radicals generated at the NP surface due to so-called autoxidation. This is a radical-chain reaction caused by natural oxidation in air or oxygen and under mild conditions. One study found that 5 nm diameter AuNPs capped with dodecanethiol elicited significant pro-oxidant effects. However, the authors found that the oxidation was not directly linked to the AuNP itself but rather to impurities present in the capping molecules, consisting of traces of the transfer agent TOAB remaining from the synthesis procedure. NPs that were prepared without any organic ammonium salts did not display pro-oxidative characteristics.[215]

The long persistence of gold and its kinetics of clearance from the body have been studied in-depth and compared to other types of materials. In one study, hollow CuSNPs were compared to hollow AuNPs, in BALB/c mice (*in-vivo*), concerning their biodistribution and toxicity. The CuSNPs were found to be slowly degradable, resulting in both hepatobiliary and renal clearance of the NPs (approximately 90% in 1 month), while the non-metabolism of AuNPs resulted in low clearance rates (about 4% in 1 month) (Fig. 15).[216]

Fig. 15 here

The clearance of CuSNPs resulted in transient, reversible damage to the liver, which was likely linked to Cu elimination from the liver itself. At the same time, AuNPs caused irreversible damage correlating with the presence of elevated serum lactate dehydrogenase at three months, suggesting long-term toxicity (*in-vivo*).[216] The reduced biodegradability of AuNPs could also be beneficial, as the gradual release of metal ions upon biometabolisation of NPs can result in cell death.[209] Upon comparing 20 nm and 80 nm diameter AuNPs and AgNPs and their effects on human embryonic neural precursor cells (as a model for the CNS, *in-vitro* study), all NP types had a significant impact depending on the nanosphere diameter and morphology. However, while AgNPs resulted in increased apoptosis of cells for both sizes and over a wide concentration range, this did not occur for the AuNPs. Only the 20 nm diameter AuNPs at the highest dose significantly affected cell proliferation without inducing any apoptosis.[217]

The NP core chemical composition can also have subtle effects that may not be directly linked to any overt cytotoxicity. For example, AuNPs and SiO_2NPs of the same size and surface functionalization were compared in an *in-vitro* study, and it was observed that AuNPs resulted in transient cytoskeletal alterations, while SiO_2NPs did not. This effect was caused by the long persistence of the AuNPs in the lysosomal acidic environment, leading to alkalinization of the lysosomes and a transient increase in autophagosomes. However, the cells recovered upon further culture with renewed cell division, and restoration of the cellular degradative capability.[218]

4.1.2 Dimensions and morphology

The effects of NP size have been investigated in different studies. In one *in-vitro* study, four types of AuNPs (18, 40, 60, and 80 nm diameter) were tested on three different types of ovarian cancer cells (OVCAR5, OVCAR8, and SKOV3). The data revealed clear concentration-dependent

and time-dependent increases in cellular NP uptake, the extent of which was also cell type-dependent. Cell metabolism was gradually affected for all cells, and oxidative stress occurred starting from 24 hrs onward.[219]

One study investigated differently sized (10, 30, and 60 nm diameter) citrate-coated AuNPs tested in cells (hepatocytes) and animals. The initial studies showed low but significant toxicity of the different NPs, irrespective of their size.[220] The data itself is somewhat contradictory because only two concentrations were tested (10 ppm and 10 ppb), resulting in a large gap between the two concentrations (a 1000-fold difference). While toxicity was mainly observed at the highest concentration, sometimes there was significant toxicity only at the lower concentrations. On the other hand, considerable toxicity observed after 16 h exposure was no longer present at 32 h post-exposure.
There were apparent size-dependent effects regarding the *in-vivo* biodistribution: the 10 nm NPs were mainly localized in the gut, while the 30 and 60 nm ones ended up in the spleen. All three NPs showed high levels in the liver, which was maximal for 30 nm-sized NPs, and minimal for 60 nm-sized NPs. No inflammatory markers were observed in the liver for toxicity evaluation, but there were clear signs of oxidative stress, resulting in significant levels of protein carbonylation and lipid peroxidation.[220] Smaller-sized AuNPs have been shown to cause higher levels of cytotoxicity and show differences in biodistribution compared to larger ones. In a systematic study, four differently sized AuNPs were studied with diameters of 6, 24, 42, and 61 nm *in-vitro* and *in-vivo* in Kunming mice. The data confirmed that for the same molar amount of Au, smaller NPs resulted in higher toxicity levels, which were mainly caused by oxidative stress. In the biodistribution, the larger PEGylated AuNPs (42 nm and 61 nm) primarily ended up in the liver as well as the spleen.
In comparison, the smaller NPs (6 nm and 24 nm) were also predominately localized in the liver and spleen. It is worth noting that they were also found in other major organs, including the heart, kidneys, and lungs. The metabolic rate of the different sized AuNPs varied. After 30 days, most of the smaller NPs had been excreted, while for the larger NPs, the major fraction had also been excreted, but a significant part remained in the liver and spleen, even after 90 days post i.v. administration in an *in-vivo* study (Fig. 16).[221]

Fig. 16 here

While smaller-sized AuNPs are generally considered to be more cytotoxic than their larger counterparts, it has been reported that for NPs smaller than 20 nm diameter, the toxicity is mainly governed by the nature of the surrounding organic ligands.[222] This was studied, *in-vitro*, by Deol and colleagues, who investigated the toxicity of 3, 12, and 17 nm diameter NPs, either capped with glutathione (GSH) or with capped with dendrons. The authors demonstrated an apparent reduction in NP toxicity when the NPs were coated with the dendrons. In contrast, the toxicity of the NPs correlated with their size, being highest for the largest NPs.[223] These findings are somewhat at odds with the generally accepted principle but may be due to the differences in cellular uptake efficiency, which is also size-dependent. If the largest NPs are taken up more efficiently, then the higher levels of cell-associated NPs will logically cause more *in-vitro* toxicity. Other studies have generally supported the earlier hypothesis, where NP toxicity is higher for smaller-sized NPs, even in the sub-20 nm diameter NP population.[174]

As mentioned above, for AuNPs, it is generally agreed that size plays an important role in their toxicity profile, particularly for so-called ultrasmall NPs, which have a diameter of less than 2 nm. The effects of these AuNPs have been reviewed in depth elsewhere.[224] Here, we will provide an overview of the effects of ultrasmall AuNPs. Pan and colleagues showed a clear size-dependent toxicity (*in-vitro*), of water-soluble AuNPs ranging in size from 0.8 to 15 nm following exposure in HeLa (human cervical cancer), SK-Mel-28 (human melanoma), L929 (mouse fibroblast), and J774A1 (mouse monocytic/macrophage) cell lines. This toxicity was

maximal for 1.4 nm core diameter AuNPs.[225] Following *in-vitro* investigations on the interaction of water-soluble AuNPs and molecular modeling studies the authors linked this toxicity to the ability of these AuNPs to incorporate into the groove of B-DNA, which has a dimension of 1.3–1.5 nm. The 1.4 nm diameter NPs caused cell death via different pathways (necrosis) compared to the other size NPs, where 1.2 nm diameter NPs elicited cell death via apoptosis and secondary necrosis.[226] Furthermore, the IC50 (the value at which 50% of the cell population dies) was approximately 3-5 fold lower for 1.4 nm NPs than for 0.8, 1.2, and 1.8 nm NPs.[227] The biodistribution of these ultrasmall NPs is also different from larger-sized NPs, where ultrasmall NPs can localize inside the cell nucleus and cause direct DNA damage.[224] At the same time, ultrasmall NPs were also able to cross vascular barriers that are normally impervious to most NPs, such as the placental barrier, potentially causing serious effects. Following the injection of 1.4, 18, or 80 m AuNPs into pregnant rats all three NP sizes were detected in the placentals and amniotic fluids. However, only the two smallest NPs were found in the fetuses 30 ng of the 1.4 nm AuNPs vs 0.1 ng of the 18 nm AuNPs. [228] Other reports have looked into the biodistribution and toxicity of ultrasmall gold nanoclusters (GNCs) (2 nm diameter) and observed low cytotoxicity in six different cell types, revealing no significant levels of apoptosis or necrosis up to concentrations of 100 μg/mL. After i.v. administration (*in-vivo*), the NPs primarily ended up in the liver, and to a lesser extent in the spleen and kidneys, while the biodistribution remained fairly unchanged between 0.5 and 24 hrs.[229]

In particular, ultrasmall NPs have been described to be able to cross different barriers in the body. However, this characteristic phenomenon has not been uniquely linked to ultrasmall NPs but is instead a size-dependent effect in general. Upon comparing 20 nm and 100 nm spherical AuNPs after i.v. administration in C57BL/6 mice (*in-vivo*), it was observed that 20 nm NPs could cross the blood-retinal barrier, while the 100 nm NPs could not. The 20 nm NPs were found in all retinal layers and were predominantly localized in the retinal neurons, but they were not found to cause any toxicity under the conditions tested.[230]

Because AuNPs can be generated with widely varying structures, the shape of the actual NPs can also play a vital role in the toxicity of NPs. When comparing the toxicity of nanocubes and nano-octahedrons, it was observed that the nano-octahedrons were more toxic than the nanocubes and that this was correlated with oxidative stress and the level of cellular uptake.[231] The differences in the toxicity of different NP shapes could be due to variations in the total surface area, as the total surface area determines the extent of interaction of the NPs with the biological environment and thus indirectly determines NP toxicity.
Gratton *et al*. showed that when the NP sizes were larger than 100 nm, cellular uptake (*in-vitro*) favored rods, then spheres, then cubes.[232] Meanwhile, for particles less than 100 nm, which are more likely to be applied in neurological applications, spheres gave the highest uptake.[233]

4.1.3 The surface chemistry of the GNSs

The effect of surface ligands on the toxicity of AuNPs has been described in various *in-vitro* studies. In one of the first, Goodman and colleagues[234] looked into the toxicity of AuNPs functionalized with anionic (carboxylic acid) or cationic (quaternary ammonium) moieties, in which the cationic NPs caused higher levels of cell lysis for both mammalian cells and bacteria. This result was explained by the higher levels of electrostatic interaction between the cationic NPs and the overall negatively charged cell membranes, which was also supported by vesicle lysis studies, confirming this hypothesis. The surface chemistry of AuNPs can have significant implications regarding the toxicity and biodistribution of the NPs in *in-vivo* studies. This was demonstrated by looking at the effect of ultrasmall BSA or GSH-coated GNCs. Larger AuNPs were mainly taken up by the liver and spleen and persisted there over long periods due to the lack of metabolism.[235, 236] Regarding ultrasmall nanoclusters, this scenario may be different, where the small size could lead to more efficient renal clearance.[237] The data revealed the

apparent differences between the two types of nanoclusters, where GSH–GNCs were efficiently cleared by renal excretion. Over 95% of the Au had been metabolized over four weeks. By contrast, the BSA–GNCs were poorly cleared, and only 5% of the Au could be metabolized over four weeks. While both types of AuNPs resulted in inflammation and kidney damage, these effects were transient for the GSH–GNCs, whereas for BSA–GNCs, they persisted over more extended periods. The difference between the two types of GNCs was linked to the formation of larger complexes of BSA–GNCs, which in turn ended up in the liver and spleen and caused higher levels of persistent cellular stress and damage.[238]

In terms of surface ligands, NPs are often functionalized with poly(ethylene glycol) (PEG), where the long, flexible hydrophilic chain endows the NPs with good colloidal stability and prevents premature clearance of the NPs by the reticuloendothelial system (RES). This results in longer blood circulation times, which is particularly beneficial for the targeted delivery of NPs.[239] PEGylation of NPs mainly works by inhibiting cell-NP interactions using the PEG chains. The extended flexible chains reduce protein binding, including the reduced binding of opsonins, preventing cellular internalization. This generally reduces the cellular toxicity of NPs, because fewer NPs will be taken up. PEGylation is also linked to some adverse effects. However, it has been shown to generate an immune response triggered by the generation of anti-PEG Abs.[240] Furthermore, the length and the grafting density of the PEG chains will determine levels of cellular interaction. A recent study (*in-vitro*) showed that PEG–AuNPs caused cell cycle arrest and DNA damage in cancer cells and non-cancerous cells. Still, at a particular grafting density (0.65 chains/nm^2), these effects were not apparent in the non-cancerous cell line.[241] These data indicate an evident impact of the nature of the cell type and the PEG grafting density on the extent of cellular interactions. In another *in-vitro* study, the PEGylation effect was studied concerning the intracellular NP levels rather than the concentration of NPs used for cell loading. When cells showed similar average, intracellular levels of NPs, this resulted in significantly higher toxicity for PEGylated AuNPs compared to non-PEGylated AuNPs. This was mainly due to higher levels of cellular reactive oxygen species (ROS), one of the main side-effects elicited by AuNPs. These data showed that, while PEGylation could have useful effects for NP biodistribution, for cell labeling studies, non-PEGylated NPs may be a better choice, as the PEG chains by themselves appear to inflict certain levels of cellular damage.[242]

Another significant effect of PEGylation is that, apart from reducing NP clearance, the lower binding of proteins onto the NP surface also plays a vital role in avoiding or reducing the formation of the so-called "protein corona". When NPs are exposed to a biological environment, serum proteins can be attracted to and bind the surface of the NPs, driven by the high density of surface charges. This results in the protein corona, which consists of an inner layer of tightly bound proteins (the "hard" corona) and a more loosely bound outer layer of proteins (the "soft" corona) that can be modified in time as proteins tend to desorb and repeatedly adsorb (Fig. 14). The outer layer of the protein corona is what the cells will first be exposed to and will determine to a large extent the nature of the cellular interaction with these NPs.[243] Using PEGylation, and mainly by increasing the PEG chain length, the extent of protein binding can be significantly reduced.[244] To control the biological interactions and biodistribution of AuNPs, the formation of the protein corona must be studied adequately to influence the overall NP size and surface charge, and the composition of the corona proteins. As the protein corona formation is highly dependent on the environment to which the NPs are exposed, it is essential to study these properties in physiologically relevant media, similar to what they would be exposed to in their final application (*e.g.*, blood).

The effect of the protein corona on AuNP surface chemistry was studied in detail by Choi *et al.*[245] The authors looked at 40 nm, and 80 nm diameter AuNPs coated with branched polyethylenimine (BPEI), lipoic acid or with PEG, as well as human plasma (HP) and human serum albumin (HSA) coronas. The authors observed that time-dependent and concentration-

dependent uptake occurred for all NP types. The presence of HP or HSA coronas on the AuNPs decreased the cellular uptake, apart from the PEGylated AuNPs (40 nm) (Fig. 17).[245]

Fig. 17 here

The reduced toxicity is likely related to the low cellular uptake of PEGylated AuNPs, where any further reduction may not be statistically significant. The presence of the corona also reduced oxidative stress that BPEI–AuNPs caused. Strangely, the protein corona did not have any effect on AuNP cytotoxicity as such, because the HP–BPEI–AuNPs were as cytotoxic as the BPEI–AuNPs. This result was particularly striking when considering the reduction in cellular internalization of AuNPs in the presence of the HP corona. This observation would suggest that HP–HSA–BPEI–AuNPs are, for the same number of AuNPs taken up per cell, more cytotoxic than BPEI–AuNPs, without any HP corona. For small GNCs, similar results were obtained. There, the toxicity of the bare AuNPs on five different cell types was found to be mainly mediated by oxidative stress, but the extent of cytotoxicity was cell-type dependent. The presence of BSA as a protein corona on the AuNPs reduced oxidative stress and cytotoxicity.[246] These data indicate the critical but complex influence of the protein corona on AuNP toxicity and cellular interactions.

Apart from plasma proteins, other biomolecules present in the blood are Abs, of which IgG molecules are the most abundant type. AuNRs coated with CTAB, polystyrene sulfonate (PSS), or PEG were studied to measure IgG Abs's ability to bind to these NPs. These studies revealed that because IgG is slightly positively charged, it mainly bound negatively charged PSS–AuNRs, and bound least PEGylated AuNRs. While cationic CTAB–AuNRs could affect cells by the strong binding to the cell membrane, resulting in membrane deformation, negatively charged AuNRs could also interact with positively charged biomolecules. Neutrally charged AuNRs are therefore often considered to be the preferred choice to minimize non-specific interactions.[247]

The toxicity of AuNPs can be caused by the surfactants used to coat the actual NPs. One classical example explored the use of CTAB, a cationic surfactant that is commonly used to control the growth of nanorods and maintain good aqueous dispersibility during the preparation. The study revealed that CTAB was toxic to cells in its free form. The toxicity of CTAB-functionalized NPs could be reduced by overcoating the NPs with polymers to shield the underlying CTAB molecules.[248, 249] Other methods to overcoat the original CTAB coating have involved the use of serum proteins, such as BSA, resulting in reduced cellular uptake of the BSA–AuNPs.[250] In a separate study, differently coated AuNRs (CTAB, PSS, and poly(diallyl dimethyl ammonium chloride) (PDDAC)) were investigated for their toxicity to vascular cells. The authors observed apparent differences in toxicity levels between the three different NP surface coatings, which were least for PSS and highest for PDDAC. The NP interaction with cells is seen as a two-step process whereby the NPs initially adhere to the cell membrane and are then internalized by cells, mainly using active endocytosis pathways. This initial interaction governs cytotoxicity and cellular NP levels, and higher binding interactions make NPs more prone to be taken up by the cells and can cause membrane damage. This hypothesis was supported by additional experiments on isolated membranes where the binding force of the NPs correlated with their toxicity levels towards cells.[251]

Given the surface chemistry of AuNPs, both cationic and hydrophobic NPs can induce significantly higher toxicities than the anionic and hydrophilic NPs. Due to the higher binding strength of cationic NPs, or the hydrophobic interaction with the cell membrane, these NPs can lead to structural alterations, pore formation, and phase transitions in the cell membrane. Such membrane perturbations can diminish the membrane capability to control non-specific uptake of ions and extracellular biomacromolecules into the cytosol.[252] Cationic and hydrophobic NPs can, apart from eliciting membrane damage, also induce various biological stresses resulting

from disruption of mitochondrial function, activation of defensive signaling pathways (*e.g.*, nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) pathway), induction of ROS, disruption of cellular energy production, and genotoxicity. Chompoosor and colleagues looked into the effect of the length of the hydrophobic alkyl tail of quaternary ammonium salts used as surface ligands on 2 nm diameter AuNPs.[253] They observed apparent toxicity of the NPs, the extent of which correlated with the increasing degree of hydrophobicity. However, when the NPs were applied at their IC_{50} value, the increase in ROS and DNA damage did not follow the same trend, and the NPs with the most extended hydrophobic alkyl chains had the lowest level of DNA damage. In a later study, 2 nm diameter NPs with different surface charges and different levels of hydrophobicity were compared for their effect on cellular functions. The data demonstrated that while cationic NPs were more cytotoxic than their anionic counterparts, the hydrophobicity level played a more prominent role in determining the cytotoxicity than the NP surface charge. Generally, a hydrophobic surface is non-polar, while surface charge (both positive and negative) leads to hydrophilic surfaces. In particular, the cells underwent membrane damage and autophagy, as evidenced by high-content imaging and detailed gene expression studies.[254]

An important point to be considered about the surface chemistry and functionalization of AuNPs is that while the NP surface group will, to no small extent, determine the NP biodistribution, protein corona formation, and toxicity, the surface chemistry does not always survive within the biological environment. One study revealed that 5 nm AuNPs, coated with firmly attached, polymers by thiol end-groups degraded within 24 hrs post-injection into rats.[255] Another study focused on ultrasmall 1.4 nm diameter AuNPs stabilized by sodium 3-(diphenylphosphino)-benzene sulfonate (TPPMS) ligands and observed that when the phosphine ligands were fluorescently labeled, cellular exposure to these NPs resulted in a partial loss of the surface shell, which was a prerequisite for NP cytotoxicity.[256] These data indicate that any assumptions about colloidal stability or biocompatibility for a particular formulation could be invalidated following cellular internalization, as the composition and surface coating of the NPs may change dramatically from those that were initially synthesized.

4.2 Toxicity mechanisms

The different potential cellular responses to AuNP exposure have led to various explanations to inhibit cell growth. When looking at the data described above, combined with other studies, several mechanisms have been repeatedly mentioned and could therefore be considered general pathways by which AuNPs provoke cytotoxicity. These mechanisms are described briefly below.

4.2.1 Oxidative stress

For many types of NPs and AuNPs, ROS induction has been seen as a hallmark of NP toxicity. The use of ROS scavengers which restore the cell viability has been demonstrated in various studies, highlighting the importance of oxidative stress in AuNP-mediated cytotoxicity,[246] which has been shown in many different studies.[219, 221] The generation of oxidative stress is a complex and multifactorial process. The mere presence of AuNPs residing inside cellular endosomal compartments as non-degradable entities can result in the generation of oxidative stress as the cell tries and fails to destroy the foreign material. Alternatively, the surface functionalization of the AuNPs may itself result in the direct generation of ROS when exposed to low pH, or the presence of the protein corona and potentially altered serum proteins attached to the NP surface could lead to ROS.
Furthermore, ROS species are typically labile and will rapidly react with other biomolecules. This transient process results in a wide variety of secondary effects, including DNA damage, lipid peroxidation, and mitochondrial damage. Furthermore, cells naturally have antioxidant

defense mechanisms (*e.g.*, GSH), but their level and extent vary widely between different cell types, resulting in significant differences in cellular damage for the same AuNPs.

Only when ROS generation surpasses the natural antioxidant capacity, will the cells undergo oxidative stress, resulting in various secondary effects, as mentioned above. Given the complexity of the entire process, different results have been obtained for various AuNPs. For example, differently sized (18, 35, and 65 nm diameter) and differently coated (glucosamine, hydroxypropylamine, taurine, PEG) AuNPs were screened for their effects on human cerebral microvascular endothelial cells (hCMEC/D3), particularly looking at the endoplasmic reticulum (ER)-mediated stress.[257] ER stress may lead to inflammation and apoptosis and could exacerbate vascular diseases or affect the integrity of vascular barriers such as the BBB. Anspach and colleagues showed that none of the AuNPs tested were cytotoxic, up to concentrations 150 μg/mL, and no apparent effect on ER stress could be observed under these conditions.[257] However, about the release of oxidative stress-driven pro-inflammatory cytokines, glucosamine-coated NPs resulted in far higher levels of inflammation than the other NPs. The study proposed by Söderstjerna and colleagues compared 20 nm and 80 nm diameter AuNPs and AgNPs using an organotypic retinal culture model.[258] The authors found evidence of NP uptake in all the retina's neuronal layers, irrespective of the nature or size of the NPs. The authors also observed that both types of NPs caused evident morphological disruptions to the retinal structure along with the formation of vacuoles. There were also increases in oxidative stress and apoptotic neurons and increased staining for glial cells and microglial activation, indicating overall damage to neural tissue. Together, these data reveal a clear impact of AgNPs and AuNPs on many retinal parameters, arguing for a careful analysis of these effects in future studies.

Other studies have, however, hinted at a protective effect of AuNPs against oxidative stress. As an example, Muller and colleagues showed that the use of AuNPs per see (~20 nm and -30 mV) could ameliorate Alzheimer's disease (AD) symptoms in a sporadic rat model of AD (based on the intracerebroventricular (ICV) administration of streptozotocin). AuNPs efficiently prevent increases in ROS, mitochondrial damage, and pro-inflammatory mediators, such as interleukin-1β and NF-kB, leading to improvements in both the spatial and recognition memory in streptozotocin-damaged rats. AuNPs protected these mice from impairments in the mitochondrial function leading to more physiological levels of ATP and, consequently ROS.[259] While these results seem promising, the lack of any clear follow-up, and the seeming contradiction with observations that AuNPs usually are more prone to generating oxidative stress than reducing it, calls for caution. It could be that the AuNPs may have interfered with the assay readout or that the AuNPs were able to bind to streptozotocin and alter its biodistribution or affect its biological activity and reduce the disease severity. It remains to be demonstrated whether AuNPs can have a therapeutic effect on their own. Ultrasmall GNCs can act as artificial enzymes, so-called nanozymes, where the NPs exhibit peroxidase and catalase-like activities.[260] However, this peroxidase-like activity has become a topic of concern as it enables NPs to convert hydrogen peroxide (H_2O_2) into highly toxic hydroxyl radicals, thereby increasing the toxicity.[261] As the catalytic properties of nanozymes are highly dependent on their surface activity, Liu and colleagues looked into the effect of surface modification to better control nanozyme activity.[262] By using different types of PAMAM dendrimers to encapsulate the nanoclusters, the authors observed that the peroxidase-like activity was predominantly linked to the presence of tertiary amines. Using PAMAM dendrimers ending in primary amine groups, the peroxidase-like activity could be significantly inhibited, while the catalase-like activities were retained. These primary amine-modified PAMAM–GNCs were then found to exhibit lower cytotoxicity and could protect primary cultured neurons against oxidative damage.

Together, these data show the clear need for a thorough study of the potential generation of ROS, oxidative stress, and the possible secondary effects. A proper characterization of the potential damage generated by AuNP-mediated oxidative stress at different time points is essential. ROS induction in itself does not necessarily indicate any toxicity, as this could either be short-lived or only at low levels. However, the high levels of ROS in the long-term that lead to

secondary effects are a major concern. Given the complexity, ROS must be looked into for every type of AuNP for each specific application.

4.2.2 Cytoskeletal rearrangements and associated-signaling

The effect of AuNPs on the rearrangement of cytoskeletal components and associated signaling pathways has also been demonstrated. Citrate–AuNPs were found to inhibit tubulin polymerization. This inhibition was concentration-dependent, and for NPs of 20, 40, and 60 nm diameter, maximal inhibition was observed with 40 nm NPs. The damaged tubulin could, in turn, lead to cell cycle arrest and apoptosis.[263] Ma and colleagues studied the effect of AuNPs in detail, where 5 nm diameter, polymer-coated NPs were used to label HeLa cervical cancer cells and human umbilical vein endothelial cells (HUVEC).[264] The authors observed a concentration-dependent disturbance of the actin and tubulin cytoskeleton, resulting in a reduction of focal adhesions and reduced formation of cellular protrusions. As focal adhesions are essential mediators in cellular signaling via the actin cytoskeleton, various genes were found to be significantly affected, mainly associated with cytoskeletal polymerization and signaling. The NPs also resulted in lysosomal swelling and alterations in mitochondrial morphology, indicative of mitochondrial stress.

One aspect of the effect of AuNPs on cytoskeletal rearrangement is that while the effects can be significant at shorter periods, the long-term implications remain to be studied in detail. It has been shown that, while apparent cytoskeletal defects in both the actin and tubulin cytoskeleton were observed at sub-cytotoxic levels, these effects were transient, and the cells were able to recover within a week.[265] While the cells may structurally recover, likely because after each cell division, the number of NPs per cell decreases, and the impact of the NPs on the cells will also reduce. It remains unclear what the long-term implications may be. Transient alterations in cytoskeleton-mediated signaling may affect cellular processes such as viability, proliferation, migration, differentiation, *etc*. While the cells may appear healthy, this does not guarantee the absence of underlying problems that could appear after extended periods.

4.2.3 Autophagy

Autophagy is a process in which damaged organelles are removed from the cytoplasm by being destroyed in so-called autophagosomes. These engulf the damaged structures and then fuse with lysosomes to create so-called autophagolysosomes. The organelles can be degraded and nutrients can be recycled and reused by the cell to maintain homeostasis.[266] Various types of NPs have been linked with autophagy, but the exact role of autophagy in NP-mediated cellular damage remains somewhat elusive. This is partly because autophagy is a self-repair mechanism where the cell tries to recover from any injury. When any NPs cause damage to particular organelles such as mitochondria, then autophagy can be induced as a protective mechanism to recover the damaged mitochondria, rather than directly killing the cell. However, when the cellular damage is high, the extent of autophagy induced may be too much for the cell to deal with, and cells may succumb to a process of autophagic cell death, but this is still up for debate.[267] In the case of AuNPs, autophagy has been shown by different cell types,[268] which has been linked to both the induction of autophagy and a reduction in autophagosome-lysosome fusion.[266] Li and colleagues observed that 20 nm diameter, citrate-capped AuNPs resulted in oxidative stress in MRC5 fibroblasts, as demonstrated by lipid peroxidation and protein oxidation.[269] This oxidative stress correlated with higher levels of autophagosomes and upregulation of autophagy-related proteins. These data indicate autophagy induction is a cytoprotective effect against the oxidative stress induced by the NPs.

AuNPs have also been shown to increase the pH in lysosomes, reducing lysosomal degradative potential and impeding lysosomal-mediated processing of autophagosomes.[218] This was nicely

demonstrated by Ma and colleagues' work, who found a substantial increase in the number of autophagosomes upon cellular exposure to AuNPs.[270] However, this was not caused by the induction of autophagy but rather by a blockade of the autophagic flux. Autophagy-dependent substrate processing was significantly reduced upon cellular exposure to AuNPs.

4.3 General remarks

Our current understanding of how AuNPs interact with their biological environment is steadily increasing, but many questions remain unsolved, some of which are technically difficult to address. One major impediment is that the particular application of the AuNPs has not been specified, which is needed to define realistic exposure scenarios. Different authors have remarked that most studies, both cellular and animal-based, use unrealistically high doses with no biomedical relevance.[271] However, to date, there are no systematic guidelines or authoritative suggestions on what "realistic doses" (or dose ranges) actually are.[272] Another question refers to the method of administration, where NP exposure or use can occur via various routes, including, but not limited to, i.v. administration, intraperitoneal (IP) administration, inhalation, topical application, oral consumption, *etc*. The administration routes will significantly affect NP biodistribution and their final toxicity, which needs to be considered. Therefore, it is vital to precisely know the proposed application of the NPs to define the best administration route and the required dose.

An additional bottleneck is a technical difficulty of studying long-term NP toxicity in cellular or small animal models. Given the high growth rate of cultured cells and the limited life span of cell cultures and small animals, performing long-term toxicity studies at repeated exposures is difficult. Models are needed for long-term persistence within tissues and organs, as would be the case for any patients treated with AuNPs. Better models that better mimic patient physiology and enable long-term studies are desperately needed.

Another complication is related to the choice of the cells that are used to study NP cytotoxicity. It has been widely accepted that there are large differences between cell types regarding their sensitivity towards engineered NPs as well as AuNPs. For example, the intrinsic antioxidant defenses differ between cell types, and when cells generate oxidative stress, the level of their defensive capability will determine whether this level causes toxicity.[273] For AuNRs of similar but slightly different sizes (10 nm x 39 nm; 10 nm x 41 nm, 10 nm x 45 nm), their toxicity was studied in 6 different cell types.[274] It was observed that the level of toxicity was largely cell-type dependent but that the differences between the three NP types regarding relative toxicity showed that the 10 x 39 nm was most toxic. By contrast, the 10 x 41 nm was the least toxic. Toxicity was also found to be caused by the induction of apoptosis or cell cycle arrest, while oxidative stress was again found to be highly cell type-dependent. Despite the significant differences in oxidative stress levels, no clear indication of antioxidant responses was observed for any cell types. At the same time, the NPs also elicited apparent cell death in all six cell types.[274] Further studies revealed that the same AuNPs resulted in apoptosis in Vero (kidney) cells but not in MRC5 (human lung cells) or 3T3 (murine fibroblast) cells. For 3T3 fibroblasts, the reduced cell growth incurred by AuNPs was linked to autophagy induction.
In contrast, for MRC5 cells, reduced growth was mainly due to oxidative stress, DNA damage, and cell cycle arrest.[268] These data indicate that, while the level of toxicity can vary widely between different cell types, the underlying mechanisms involved can also differ extensively. Therefore, it is essential to include many cell types in any study and focus on those cells that are more likely to encounter NPs when used for biomedical applications.

Another critical aspect involved in studying biological-nano interactions is concerned with the particular physical and chemical properties of NPs, including AuNPs. This can mean that the AuNPs themselves can interfere with a wide range of cytotoxicity assays, resulting in inaccurate

data. For example, AuNPs have affected PCR studies used for gene expression assays[275] and many other standard biochemical and fluorescent assays.[276-278] It is, therefore, essential to include appropriate controls (*i.e.*, have AuNPs added to cells killed by chemical methods to confirm lack of signal due to the NP presence alone) and preferably use additional methods to supplement the data obtained from standard cytotoxicity assays.

The different levels of NP toxicity for differently sized NPs or those with different shapes are likewise challenging to interpret due to the lack of definition of NP "units". For any comparison between differently sized or structured NPs, it remains somewhat questionable how these parameters should best be defined. NP exposure can be expressed as total mass, the number of NPs or the total surface area of all the NPs added together. All three measures have their advantages and shortcomings. Any given NP toxicity could be associated with either the total number of metal ions present (*i.e.*, mass) or the entire surface area that governs the interaction with the biological components (*i.e.*, total surface area). However, it is not easy to decide how to choose one measure. For any given mass, the total surface area can be quite different and *vice versa*. This has been demonstrated, where AuNPs of varying core sizes but with the same hydrodynamic diameter (due to differences in the thickness of the polymer layer) were used. When the toxicity of the NPs was expressed in terms of the same mass or the same number of NPs, the toxicity profiles for the same series of AuNPs were almost the complete opposite (Fig. 18).[279]

Fig. 18 here

For any AuNP preparation that is to be used for a particular biomedical application, it is also vital that the NP formulation itself and the final product is tested for potential toxicity (*e.g.*, coupled with any pharmaceutical agent) at the desired concentration.[280] If the application of any external stimuli is required (*e.g.*, light irradiation for PTT), these conditions must also be replicated in any toxicity studies, focusing on the targeted malignant cells and on non-malignant cells that could play a significant role in their final toxicity. Furthermore, in many instances, it appears that AuNPs administered *in-vivo* tend to be rapidly removed from the delivery site, *e.g.*, through the action of interstitial fluid. This is another significant difference between *in-vitro* and *in-vivo* models. As a result, any AuNPs administered to patients would presumably need to be surface functionalized in such a way as to allow targeting to the intended tissue. This is another reason why it is hard to generalize the toxicity of AuNPs until the application is well defined and the final formulation is well established.

A last important point that has recently been discussed is the effect of AuNPs on endothelial cell integrity and permeability of vascular barriers. Authors reported that AuNPs, ranging between 10 and 30 nm, induced endothelial-inducing micrometer-sized gaps between adjacent endothelial cells by affecting the integrity of the tight cellular junctions that hold the endothelial barrier together. These effects, however, were specific to some but not all endothelial cells, where mammary and skin endothelial cells were rendered permeable following exposure to AuNPs, and human umbilical vein endothelial cells were not affected (Fig. 19). [281]

Fig. 19 here

This effect may question the safety of NPs for biomedical use, and in particular for cancer therapy, because the increased leakiness of tumor-associated blood vessels may promote tumor cell extravasation or intravasation, and in doing so, the mere presence of circulating NPs could, in theory, increase the chances of metastasis.[282]

While the various studies mentioned in this section have looked at a wide range of possible effects caused by AuNPs, one should bear in mind that any toxicity study should always include

as many parameters as possible. While it is relatively easy to determine the number of live or dead cells, any persistent effects at sub-cytotoxic concentrations may have implications for the future use and potential benefit of these NPs. When evaluating NP toxicity, it is vital to properly characterize the NPs and their physicochemical properties in physiologically relevant media. Furthermore, the biodistribution of the AuNPs must be adequately tested to define which organs/tissues need to be looked at in more detail. Regarding i.v. administered AuNPs, blood biochemistry analysis of liver, spleen, and kidney function and hematological parameters would be essential. This would preferably also be done at different time points to look into both acute and long-term toxicity. In the CNS specifically, attention should be paid to ROS and inflammation (*e.g.*, by non-invasive imaging using optical or positron emission tomography-specific probes), the integrity of barriers such as the BBB, and proper neuronal functioning.

5. GNSs-based biosensors for multiplex detection of markers for neuronal function

Developing efficient biosensors for the selective and sensitive detection and quantification of neurochemicals, including neurotransmitters, enzymes, metal ions, and some small molecules, is essential in fundamental studies in CNS physiology and pathology.[283-285] The monitoring of these chemicals in the extracellular environment of brain cells could lead to a better understanding of CNS disorders. The speed of production and response of various neurochemicals varies within the CNS. For example, neurotransmitters (like dopamine) respond rapidly (only a few milliseconds) to a stimulus, while neuromodulators (like ascorbic acid) respond slower to affect the required message between presynaptic terminals and the target cells.[286-291] In this section, we review different types of gold-based biosensors, which have recently been developed to detect or monitor various neurochemicals *in-vitro* or *in-vivo*.[292-297] The efficiency of electrochemical methods could be improved using GNSs for ultra-sensitive determination of different neurological molecules.[298, 299] Colorimetric and SERS methods rely on the plasmonic properties of AuNPs.[300, 301] SERS is sensitive and can enable single-molecule detection.[300, 302, 303] GNCs can be used to develop fluorescence probes, which have higher sensitivity than colorimetric probes. For more information, readers are encouraged to refer to a review published by Deng *et al*. in 2014.[304]

5.1 Biosensing methods

Biosensing of different neural markers and functions using GNSs are based on a variety of read-out methods, including colorimetric, electrochemical, fluorescent, scattering properties, and SERS. In the following, we discuss each of these methods briefly.

Colorimetric methods are straightforward assay technology and sometimes can even be performed by the naked eye. Colorimetric biosensing methods using AuNPs are based on the sensitivity of the SPR band of AuNPs to change in the environmental conditions, especially aggregation of the NPs.[294, 297] Small separated AuNPs have an SPR band around 520 nm (red-colored solution). At the same time, this is shifted or quenched, and another peak around 625 nm appears when the AuNPs start to aggregate (violet-colored solution). Based on the change in the intensities of these two peaks, colorimetric biosensors have been developed. To increase the system's selectivity, the AuNPs can be functionalized with molecules (such as enzymes) that show specific interactions with the target molecules. Besides, to enhance the response time of the system, other chemicals (like Cu^{2+} ions) can be added to the system to facilitate the aggregation process.[305]

Electrochemical methods have also been developed to quantify target molecules based on a change in electrical properties (such as current and voltage) in the environment due to oxidation or reduction reactions. The GNSs increase the sensitivity of the electrodes due to their

high surface area, good conductivity and also can be used to modify the electrode by binding specific target molecules.[306-308]

Fluorescence-based biosensors are of interest due to the high sensitivity of the fluorescence signal to slight changes in environmental conditions or surface modification even at the single-molecule level. There are two different types of fluorescence biosensors based on gold: (a) GNCs that have their intrinsic fluorescence;[292, 296] and (b) on/off fluorescence systems based on quenching the fluorescence of the fluorophore in the vicinity of AuNPs due to energy transfer from the excited fluorophore to the AuNPs.[309, 310]

Another type of gold-based biosensor is based on the scattering properties of AuNPs. The scattering properties of AuNPs are sensitive to environmental conditions. Hence, the sample scattering signal is measured and correlated to the amount of analyte due to the aggregation of AuNPs in the presence of the analyte. Resonance light scattering is of interest due to its sensitivity, rapidity, simplicity, and convenience (using a standard laboratory spectrofluorometer).[311]

Moreover, the LSPR effect, on metal nanoparticles results in an enhanced electric field around metal nanoparticles, and in turn, enhanced excitation of optical molecules nearby. Using Raman reporter molecules, SERS biosensors could be developed based on metallic nanostructures, where gold has good optical properties and biocompatibility.[300, 302, 303]

5.2 Neurotransmitters

Table 2 lists some examples of different methods that have been reported for the determination of neurotransmitters. Dopamine is an essential neurotransmitter for which a range of different biosensors have been designed. However, other neurotransmitters such as serotonin and norepinephrine are also of interest due to their roles in the nervous system. Although several attempts have been made to design colorimetric biosensors based on the aggregation of AuNPs, the bifunctionalized-AuNPs are the most successful examples. For example, Godoy-Reyes *et al*. used dithiobis(succinimidyl propionate) (DSP) and N-acetyl-$_{L}$-cysteine (Cys or C) (L-NAC)-bifunctionalized AuNPs to quantify serotonin.[312] In this biosensor, DSP was designed to react with the amino group in serotonin, and L-NAC was used to form a hydrogen bond with the serotonin hydroxyl group. Together with electrostatic interactions, these bonds led to the aggregation of the AuNPs (Fig. 20A). Another example of bi-functionalized AuNPs was 4-mercaptophenylboronic acid (4-MPBA) and DSP-bifunctionalized AuNPs used to detect and quantify dopamine.[313] Despite colorimetric biosensors being easy to use and can be read by the naked eye, electrochemical biosensors are more accurate. For instance, the limit of serotonin detection using the colorimetric biosensors developed was 0.12 μM.[312]
In contrast, an electrochemical biosensor based on graphene-encapsulated Au–Ag alloy electrode showed a limit of detection (LOD) of only 1.6 nM.[314] Additionally, electrochemical biosensors have a more comprehensive linear range of responses. As an example, Hsu *et al*. developed flexible substrates based on polyethylene terephthalate (PET) combined with different GNSs (nanowires, nanoslices, and nanocorals).[315] They found that AuNWs had exceptional properties with a wide linear range (0.2–600 μM) for dopamine, and also, the sensitivity of the system only dropped 5% even after ten successive repetitions. Chemiluminescent biosensors are also of interest as GNSs could improve the electron transfer process due to their high electrical conductivity. Li *et al*. developed a combined high-performance liquid chromatography (HPLC)/chemoluminescence biosensor for ultra-sensitive determination of different neurotransmitters (dopamine, epinephrine, and norepinephrine).[316] HPLC can distinguish between various neurotransmitters while the AuNPs improved the chemoluminescence signal for higher sensitivity.

The selectivity of any biosensing system relies on a specific interaction between the probe and the target analyte. Abs, peptides, aptamers, enzymes, *etc*., are some common examples that provide molecular selectivity. Although the electrochemical methods summarized in Table 2 are not selective, new electrodes with surface modifications could improve the selectivity and sensitivity for the methods presented in Table 2.

Fig. 20 here

Table 2 Some recent examples of Au-based biosensors for *in-vitro* determination and detection of neurotransmitters

GNSs	**Biosensor type**	**Neurotransmitter**	***Medium***	**Linear range**	**LOD**	**Refs**
BSA–GNCs	Fluorescent	Epinephrine	Human serum	0-90 μM	910 pM	289
$AuFe_3O_4$	Electrocatalytic	Dopamine	Human urine	0-0.8 μM	2.7 nM	290
Graphene–hierarchical GNSs	Electrochemical	Dopamine	PBS buffer	1 nM-100 μM	1.13 nM	291
DSP–L-NAC–AuNPs	Colorimetric	Serotonin	Aqueous media	0–3 μM	0.12 μM	312
Graphene–Au–Ag	Electrochemical	Serotonin	Human serum	2.7 nM–4.82 μM	1.6 nM	314
AuNWs–PET	Electrochemical	Dopamine	PBS buffer	0.2–600 μM	26 nM	315
Luminol–$AgNO_3$–AuNPs	HPLC/Chemoluminescence	Dopamine Epinephrine Norepinephrine	Mouse brain microdialysates	0.8–48 ng/mL 0.8–48 ng/mL 0.4–28 ng/mL	0.4 ng/mL 0.4 ng/mL 0.25 ng/mL	316
Glutamate oxidase onto carboxylated MWCNT–AuNPs–CTS–Au electrode	Electrochemical	Glutamate	Fresh serum samples from healthy / unhealthy individuals	5–500 μM	1.6 μM	317
GA–rGO–AuNPs	Electrochemical	Dopamine	Human serum and human urine	0.01–100.3 μM	0.0026 μM	318
Au–CDs–CTS–GCE	Electrochemical	Dopamine	PBS buffer	0.01–100 μM	0.001 μM	319
AuNRs	Colorimetric	Dopamine Epinephrine Norepinephrine	Human urine	N.A.	N.A.	320
GluBP–AuNP–SPCE	Electrochemical	Glutamate	PBS buffer	0.1-0.8 μM	0.15 μM	321

Abbreviations. AuNRs, gold nanorods; AuNWs, gold nanowires; CDs, carbon dots; CTS, chitosan; DSP, dithiobis(succinimidyl propionate); GA, gallic acid; GCE, glassy carbon electrode; HPLC, high-performance liquid chromatography; MWCNT, multi-walled carbon nanotubes; PET, polyethylene terephthalate; rGO, reduced graphene oxide

5.3 Biomarkers of neural diseases

Biomarkers for neural diseases are neurochemicals or biological molecules related to specific types of neuropathology.[298, 299, 308, 322-325] Each disease might have more than one biomarker; for example, an increase in the concentration of Tau (τ) protein or different types of amyloid-beta (Aβ) proteins are associated with a high risk of AD. At the same time, α-synuclein (α-Syn) is considered to indicate Parkinson's disease (PD), and botulinum neurotoxin-A causes botulism.[181, 301, 326] Table 3 presents a summary of recent reports of gold-based biosensors used for the detection and determination of different neural biomarkers. Liu *et al*. developed a dual-mode biosensor using rhodamine B-functionalized AuNPs (RB–AuNPs) to quantify the acetylcholinesterase (AChE) enzyme, a biomarker for AD. In this approach, the RB–AuNPs were stable in the acetylthiocholine (ATC) solution. However, when ATC was hydrolyzed into thiocholine by the addition of AChE, some of the RB molecules were replaced with thiocholine molecules via a ligand exchange process due to the high affinity of the thiol groups for the Au surface. This ligand exchange process caused aggregation of the AuNPs. Hence, the color of the solution changed to purple due to aggregation, and also the fluorescence of RB was switched on due to leaving the surface of the AuNPs (Fig. 20B).[327]

Abs, specific enzymes, and peptides have all been introduced to enhance the selectivity of biosensors. As shown in Table 3, many electrochemical biosensors based on gold have had specific targeting molecules attached to the surface, making them selective for a single biomarker molecule.

Table 3 Some recent example of Au-based biosensors for *in-vitro* detection and quantification of different neural biomarkers

GNSs	Biosensor type	Biomarker	Matrix	Disease	Linear range	LOD	Refs
$AuFe_3O_4$–Ab–Target	Scattering	Aβ(1–42)	DMSO–water	AD	0.005–5560 pM	1200 pM	311
RB–AuNPs	Colorimetric Fluorescence	AChE enzyme	PBS	AD	N.A.	0.5 mU/mL 0.1 mU/mL	327
PMMs–Ab–Target–Ab–AuNPs	Electrochemical	ApoE Aβ	Human body fluids	AD	100–12500 pg/mL 20–12500 pg/mL	80 pg/mL 190pg/mL	328
AuNRs–Ab–Target	Colorimetric	Aβ(1–40) Aβ(1–42) τ protein	Human plasma	AD	$10–10^8$ fM	34.9 fM 26 fM 23.6 fM	329
rGO–AuNWs–miRNA	Electrochemical	MiRNA-137	PBS	AD	0.5–750 fM	1.7 fM	330
AuNPs–Ab–Target	Scattering	τ protein	PBS	AD	5–350 ng/mL	N.A.	331
AuNPs–Ab–Target	Colorimetric	Aβ(1–42)	PBS	AD	7.5–350 nM	2.3 nM	332
AuNPs	Colorimetric	Aβ(1–40)	Human blood serum	AD	0–300 nM	0.6 nM	333
AuNP–MMBs	Electrochemical	MiRNA-182	Human serum from glioma patient	Glioma	5–100 fM	0.14 fM	334
Graphene nanosheets–Ab–Target–Ab–AuNPs	Electrochemical	Botulinum neurotoxin-E	Spiked in milk and orange juice	Botulism	0.01–10 ng/mL	5 pg/mL	335
AuNRs–ITO	SERS	Scrambled prions	PBS	AD, PD, *etc*.	N.A.	0.01 nM	336
GCE–Au nanoarray	Electrochemical	Neuron-specific enolase	PBS	Nerve damage	0.01–1 ng/mL	2.6 pg/mL	337
Peptide–AuNPs	Colorimetric	Botulinum neurotoxin-A	HEPES buffer	Botulism	N.A.	0.1 nM	338

Electrode–AuNP–Ab	Electrochemical	Botulinum neurotoxin-A	PBS	Botulism	4–35 pg/mL	1 pg/mL	[339]
GCE–AuNPs–graphene–CTS–Ab–Target	Electrochemical	Botulinum neurotoxin-A	Spiked in milk and serum	Botulism	0.27–268 pg/mL	0.11 pg/mL	[340]
DNA–AuNPs	Optical	MiRNA-137	SPSC buffer solution	AD	0.25–5 nM	0.25 nM	[341]
AuNPs–PEDOT–PTAA	Electrochemical	Amyloid-β	PBS	AD	10−8 to 104 nM	10−8 nM	[342]

Aβ, amyloid-beta; Ab, antibody; AChE, acetylcholinesterase; AD, Alzheimer's disease; ApoE, apolipoprotein E; CTS, chitosan; DMSO, dimethyl sulfoxide; GCE, glassy carbon electrode; AuNPs, gold nanoparticles; ITO, indium tin oxide; MiRNA, microRNA; MMBs, magnetic microbeads; PD, Parkinson's disease; PBS, polybutylene succinate; PEDOT, poly(3,4-ethylene dioxythiophene); PMMs, porous magnetic microspheres; PTAA, poly(thiophene-3-acetic acid); RB, rhodamine B; SERS, surface-enhanced Raman scattering; τ protein, Tau protein

5.4 DNA, proteins, amino acids, and other biomolecules

Other neurochemicals might be considered to be indicators of neural disorders, including some proteins, DNA sequences, or even small amino acid molecules like Cys.[182] Electrochemical and colorimetric biosensors have been designed to recognize and quantify these molecules in samples consisting of different body fluids. For example, Qian *et al*. developed a colorimetric biosensor for the determination of Cys based on an aggregation of citrate-capped AuNPs in the presence of Asp and Cys (Fig. 20C).[343] This biosensor could detect Cys molecules at concentrations as low as 100 nM in the fluid taken from the rat brain. In another study, an electrochemical method based on differential pulse voltammetry (DPV) was developed to determine specific single-stranded DNA sequences using complementary DNA–polyaniline–AuNP composites as the electrode.[344] Lu *et al*. also reported a voltammetric method for miRNA-182 detection in samples from glioma patients, which was based on a combination of conductive magnetic nanobead ferrocene-capped AuNPs.[334] The linear range was within 5–100 fM, and the LOD was reported to be as low as 0.14 fM. In another study, an electroluminescence probe was developed to determine N-terminal pro-brain natriuretic peptide (NT-proBNP).[345] This probe was based on N-(aminobutyl)-N-(ethylisoluminol) (ABEI)-functionalized gold nanodots (AuNDs)–CTS–multi-walled CNTs (ABEI–AuNDs–CTS–COOH–MWCNTs) hybrids as the nanointerface, where thousands of chemoluminescence ABEI molecules existed in the probe (Fig. 20D).[345] The synthesized hybrid system was deposited on the ITO (indium–tin oxide) electrode, followed by functionalization with an Ab (anti-NT-proBNP), and the remaining surface was blocked by BSA molecules. The developed electrode showed a sensitive and selective electrochemical response to the addition of NT-proBNP. The linear range was reported to be 0.01–100 pg/mL, and the LOD was 3.86 fg/mL, which was lower than the LOD reported by other electrochemical methods.[346, 347] Koh *et al*. also reported an electrochemical technique based on electropolymerization and self-assembly of a layer of AuNPs to determine inducible nitric oxide synthase in neuronal cells with a linear range of 0.001–0.02 μg/mL and a LOD of 0.2 ng/mL.[348] Recently, Mao *et al*. reported that the sensitivity of their system based on AuNPs capped with two different aptamers for the determination of interferon-gamma (IFN γ) protein could be improved using the absorbance of the nanoprobe due to changes in the light scattering of the AuNPs after interaction of the aptamers with the target protein.[349] Detection of these biomolecules/macromolecules mostly depends on selection a right recognition element. According to the nature of these molecules (DNA, proteins, amino acids, etc.), various recognition elements could be chosen. For example, DNA hybridization, protein-protein inetraction, protein-biomolecule interaction, etc.. These versatile specific interactions allow reasearchers to design selective biosensors to be able to address the required selectivity for future commercialized biosensors.

5.5 Small molecules

Determining small molecules, such as metal ions, hydrogen peroxide, or glucose is important since these chemicals play a vital role in biochemical pathways in brain cells and neural tissues (Table 4). For example, Ca^{2+} ions are involved in signal transduction, required for many functions in neural systems.[350] Mg^{2+} ions are also essential because Mg^{2+} is a co-factor for more than 300 enzymatic reactions.[351] Recently, fluorescent biosensors based on citrate-capped GNCs have been designed. The fluorescence of the GNCs was quenched in the presence of Ca^{2+} ions due to the complexation between Ca^{2+} and citrate at the surface of the GNCs.[352] In another study, truncated octahedral Au microcages were used to modify an electrode, and consequently to enhance the sensitivity of the electrochemical sensor for determination of Cu^{2+} ions due to their large surface area and high electrocatalytic activity.[353]

H_2O_2 is a crucial ROS whose abnormal production can cause oxidative stress, cellular aging and could be a sign of cancer, AD, and PD. Although many efforts have been made to develop

colorimetric biosensors of H_2O_2, it should be mentioned that colorimetric methods generally cannot be used for *in-vivo* applications.[354, 355] Hence, electrochemical processes are of considerable interest. For example, a graphene oxide (GO)/GNC-modified ITO electrode was recently used for sensitive detection of H_2O_2 released from bupivacaine-injured neuroblastoma cells.[356] The progress in designing gold-based biosensors for detection of small moelcules is not limited to the molecules listed in Table 4 ; however, metal ions, H_2O_2 and glucose are the most biologically important small molecules that have been measured by gold-based biosensors.. Despite the larger biomolecules, versatile selective recognition elements such as antibodies, aptamer, etc., are not available for small molecules, therefore, development of biosensors based on gold for determination of small molecules requires a design principle that leads to specific interactions between small molecules and the biosensor elements.

Table 4. Some examples of Au-based biosensors for determination and detection of small molecules

GNSs	Biosensor type	Target molecules	*In-vitro*/*In-vivo*	Linear range	LOD	Refs
Au TOM –E_2Zn_2SOD– electrode	Electrochemical	Cu^{2+}	*In-vivo*	10 nM–35 μm	3 nM	[353]
GO–GNCs–ITO electrode	Electrochemical	H_2O_2	*In-vitro*	40 nM–2 μM	20 nM	[356]
rGO–$AuFe_3O_4$–Pt–GCE	Electrochemical	H_2O_2	*In-vitro*	0.5 μM–11.5 mM	0.1 μM	[357]
ssDNA–AuNPs	Colorimetric	Glucose	*In-vivo*	0–5.0 mM	N.A.	[358]

AuNPs, gold nanoparticles; GCE, glassy carbon electrode; GNCs, gold nanoclusters; ITO, indium–tin oxide; rGO, reduced graphene oxide; ssDNA, single-stranded DNA; TOM, truncated octahedral microcages

5.6 Cellular activity and differentiation

Zhang *et al*. used an AuNP array to investigate brain cell activity based on changes in the surrounding medium dielectric field and its ability to shift the SPR peak.[184] The relationship between SPR and the dielectric constant of the medium has been explained by theoretical models, such as the Drude model and the Stern model.[359-361] In the Zhang study, brain neural cells were grown on the substrate, and the SPR signal was monitored over time.[184] The dielectric of the medium changed due to brain cell activity leading to the switching of the action potential by chemically triggering the neurons. The neural activity also was recorded by Kim *et al*. using a gold microelectrode.[362] It was reported that the system's efficiency could be further improved if a layer of CNT–Au nanocomposite was deposited on the surface of the gold microelectrode. The effect of the available surface area on the extent of astrocytes coverage was studied by Chapman *et al*.[363] It was reported that nanoporous AuNPs with smaller sizes gave better neuronal cell adherence and enhanced the electrophysiological recording performance due to the higher surface area compared to larger NPs. Mendoza *et al*. used small AuNPs functionalized with both PEI and anti-VGLUT-2 Abs to build a nanoprobe for neural cells.[364] The bi-functionalization allowed efficient membrane protein attachment and uptake by the cells. An escape from the endosomal lumen followed this into the cytosol of the cells. Finally, retrograde axonal transport might result in a nanoaggregate deposition in the neuronal soma. The aggregated nanoprobes could be used for further electrophysiological recording, such as the characterization of living neurons in the preoptic area (POA) of the anterior hypothalamus, where is essential in regulating body temperature.

While several promising attempts have been done to use Au nanostructures for studying cellular activity and differentiation of cells, surface functionalization of Au nanostructures with selective recognition elements (i.e., antibody, aptamer, etc.) is still a challenging and vital step since it determines the selectivity of the system. Additionally, the stability of functionalized Au nanostructures in biological mediums is necessary and still needs to be improved since the aggregation of Au nanostructures in colloidal system directly affects the response signals in various measurement techniques.

5.7 General remarks

Different methods have been developed to detect specific markers or to monitor neural functions. Several factors, including the analyte, the recognition partner, and the transduction process, can affect the sensitivity of GNS-based biosensors. The detection limit of these sensors ranges from pico- to micromolar depending on the target molecule and the sensor design. As most of these sensors are based on optical and electrical properties of GNSs, an in-depth systematic study to evaluate the effects of size, shape, and even their interaction with the other chemicals used in the design of biosensors, could help researchers to improve the sensitivity of these systems.

Despite all the positive physico-chemical properties of gold nanostructures that have led to development of different types of gold-based biosensors, gold nanoparticles suffer from high sensitivity to medium conditions and therefore, surface functionalization is necessary to make them stable in colloidal systems. In addition, SERS biosensors based on gold nanostructures cannot be used for long-time monitoring, when long time of excitation by laser is required. This is because the high loss of energy in gold nanostructures and heat generation by gold nanostructures under continues laser irradiation, which might result in damage or change in biological molecules in the study and therefore, it might affect the results. Furthermore, gold nanoclusters have low fluorescence quantum yield, and their emission is not as tunable as some other inorganic or organic fluorophores, limiting their applications for multiplex detection compared to recently developed organic aggregation-induced emission dyes (i.e., where they usually have larger absorbance cross-section and fluorescence quantum yield), semiconductor quantum dots (i.e., tunable emission), or even carbon dots (i.e., with multi-excitation fluorescence and tunable emission wavelength). Due to these reasons, gold-based nanobiosensors have a long way to go towards commercial and clinical applications. Additionally, due to high sensitivity of the properties of Au nanostructures to environmental changes even at molecular level, any non-specific interaction/adsorption can affect the results of gold-based biosensors and therefore, full attention is required in improving the analytical performance of gold-based biosensors.

6. GNS-based bioimaging of neuronal structure and function

Bioimaging of neural tissues via non-invasive techniques is of interest to obtain information about tissue organization and to guide therapeutic procedures, or even for theragnostics (simultaneous imaging and therapy). Table 5 summarizes the different GNSs that have been used as imaging reporters or tracers.

6.1 Computed tomography imaging

Computed tomography (CT) is a non-destructive technique that uses X-rays to provide a three-dimensional image of a solid volume inside the body based on the intrinsic contrast between the target tissue and the surrounding tissues. An efficient CT contrast agent should significantly provide an absorption cross-section of X-rays compared with the surrounding body tissue. Hence, a CT contrast agent should have either higher or lower density than the surrounding tissue, and should also have low toxicity and low costs.

Currently, most commercial CT contrast agents are organic molecules based on iodine that are injected intravenously. However, these organic molecules are rapidly eliminated by the kidneys and liver in a short time after injection, and therefore the time available for capturing the image is short.[365] Although there have been some attempts to formulate iodine atoms in different nanostructures (Fig. 21A),[366] a new formulation based on liposomes developed by Lim *et al*. may cause some side effects such as kidney toxicity and allergic reactions.[367] AuNPs with sizes of less than 100 nm have shown exciting potential for CT imaging. The high X-ray absorption coefficient of gold (5.16 at 100 keV) compared with iodine (1.94), biocompatibility, easy surface functionalization chemistry, and low tendency to cause apoptosis and oxidative stress make AuNPs ideal for CT imaging, compared to other metal NPs.[368] For example, AuNPs have shown

around 80–100% better performance compared with iodine (Fig. 21B).[369] Fig. 21C depicts the critical structure-function relationships of AuNPs that make them good X-ray contrast agents.[370]

Fig. 21 here

Different research groups have used AuNPs for CT imaging of neurological tissues. The Betzer group developed different functionalized AuNPs for CT imaging of the brain.[371] In this *in-vivo* study, AuNPs were used as a CT contrast agent for monitoring the longitudinal kinetics of mesenchymal stem cells (MSCs). Hence, a noninvasive quantitative CT imaging technique, which could determine the number of cells residing in any specific brain region without tissue destruction or harm to the animals, was developed.[371] They also used glucose-functionalized AuNPs for *in-vivo* determination of exosomes in the brain using CT imaging, which could serve as a powerful diagnostic tool for various brain disorders. They could potentially enhance exosome-based treatments for neuronal recovery.[372] Recently, Morales-Zavala *et al*. developed AuNR anchored peptides angiopep-2 (Ang) (a shuttle to the CNS) and D1 (binds to the Aβ peptide and inhibits its aggregation) for detection of amyloid plaques in AD mice (APPswe/PSEN1dE9) by micro-CT, and also to diminish the amyloid load and inflammatory markers in the brain.[373]

These limited reports, show the great potential of AuNPs for CT imaging of different neural tissues, where biocompatible AuNPs could be easily used to obtain a more precise image compared to conventional CT contrast agents. More details of CT imaging applications of AuNPs are shown in Table 5.

Table 5 GNSs for bioimaging of neural tissues

GNSs with different sizes, shapes, and ARs	MRI	CT	PA	SERS imaging	Fluorescence imaging	Remarks	Refs
ZE–AuNPs (< 5 nm)					*	*Zinnia elegans* plant extract can be excited by NIR AuNPs helps to a better cellular uptake Fluorescence imaging of brain cells in a mice	18
Glucose–AuNPs (20 nm)		*				MSC tracking within the brain Cell migration could be detected as early as 24 h and up to one-month post-transplantation Determination of the number of cells residing in a specific brain region, without tissue destruction or animal scarification	371
Glucose–AuNPs (5 and 20 nm)		*				NPs could label the exosome-mediated by the glucose transporter GLUT-1	372
PEG–AuNRs (AR:5)			*			Monitoring FUS-induced BBB opening in a rat model *in-vivo*.	374
PEG–HAuNS (40 nm)			*			The image depicted brain blood vessels as small as ~100 μm in diameter	375
c(KRGDf) peptide–PEG–HAuNS (40 nm)			*			I.v. delivery of HAuNS targeted to integrins that are overexpressed in both glioma and angiogenic blood vessels in a mouse model of glioma	376
DNA–Gd (III)–AuNP (15 nm)	*					MR imaging of transplanted human NSCs 70% of cells were correctly identified Less than 1% of cells were false positive for NPs	377
Aggregated AuNPs (20 nm)	*			*		Physiological acidity triggers NP assembly by forming 3D spherical nanoclusters with remarkable MR and SERS signal enhancements	378
TAT–AuNPs (5 nm)	*					Delivery of DOX and Gd (III) to brain tumor tissue Better efficiency compared to free DOX	379
Au–DTDTPA and Au–DTDTPA–Gd	*					Monitoring the distribution of NPs by MRI Radiosensitizing effect MRI determined the delay between the i.v. injection and the irradiation	380

FAL peptide–Gd (III)–AuNPs	*			*	*	EGFRvIII, a variant of EGFR, was targeted Precisely guiding GBM resection Promising for the surgical outcome of EGFRvIII (a variant of EGFR) + GBM.	381
Anti–dopamine Ab–AuNPs (40, 60, 100 nm)				*		Intracellular probe for dopamine Size-dependent cellular uptake was investigated	382
Fluorescein–NLS peptides–AuNPs				*		Intracellular SERS probe for identifications of cells SERS changes based on the DNA/RNA ratio inside the cells	383
AuNPs grew on random nanoarray transparent boehmite				*		Imaging of brain ischemia GNSs with a diameter of~125 nm and spacing of <10 nm, ideal for the hot-spots formation	384
GSH–AuNPs (3 nm)					*	An orthotopic murine glioma model was used 2.3-fold higher efficiency relative to surrounding non-tumor normal brain tissues 3-fold higher specificity compared with 18 nm AuNPs 8-fold less accumulation in major organs compared with 18 nm AuNPs	385
PEG–AuNPs (5, 10, 20, and 40 nm)					*	Time-dependent morphological changes of cortical vasculature due to BBB disruption Visualized by multi-photon luminescence of long-circulating AuNPs	386
PEG or CTAB–AuNPs (sphere, rods, urchins)					*	Studying the interactions of microglia and neurons with AuNPs of three morphologies, spheres, rods, and urchins, coated PEG or CTAB AuNP internalization by both microglial cells and primary hippocampal neurons Morphology and surface chemistry strongly influence the microglial activation status Dark-field microscopy and two-photon-induced luminescence was used for this investigation	387
PEG–AuNRs (AR: 3.2 and 6.5)					*	Three-photon imaging of the brain	388

Fluorescein–hyaluronic acids–AuNPs					*	Sensitive to ROS The increase of ROS in the induced transient I/R brain was confirmed ROS level increased in the I/R animal group with time, while the signal was decreased in the normal animal group	389
PAH–PSS–AuNPs (15 nm)		*		*		*In-vivo* distribution of polyelectrolyte multilayer coated AuNPs starting from the living animal down to the cellular level The peak concentration in the head of mice was detected between 19 and 24 h. The NPs mainly accumulate in the hippocampus, thalamus, hypothalamus, and the cerebral cortex	390
Trp–AuNPs (20–100 nm)					*	Temperature-dependent of the shape of synthesized AuNPs Enhanced brightness in fluorescence imaging of human neuronal cells	391
PEG–AuNS						EPR Multiphoton microscopy was used to image the brain tumor	392
GNCs (< 3 nm)					*	*In-situ* synthesis of GNCs in the nontumorigenic neuronal microglial line, C8B4	393
AuNPs (11 nm)	*					Gold uptake gave a 19:1 tumor to normal brain ratio with 1.5% w/w gold in tumor	394
Peptide–AuNPs (15 nm)					*	On/off system Targeting the brain glioma stem cell using CD133 marker	395
Peptide–GNCs					*	Imaging of brain cells	396
SiO_2–AuNPs	*		*	*		Brain tumor imaging with three different modalities	397
Dye–peptide–AuNPs (20 nm)					*	MiRNA imaging Differentiating neural cells with different miRNA expression	398
Raman reporter–anti-EGFR–PEG–AuNPs (60 nm)				*		SERS imaging of GBM cells through interaction with EGFR inside the cells	399

Au–IONPs–miRNA–fluorescent dye (50 nm) β-cyclodextrin–CTS–$AuFe_3O_4$ (34 nm)	*				*	Prognosis for GBM Imaging via fluorescence and MRI Multimodal therapy of GBM using miRNA and presensitization of GBM to temozolomide U87-MG GBM cell-derived orthotopic xenograft models in mice were used	400

Ab, antibody; AR, aspect ratio; AuNPs, gold nanoparticles; AuNSs, gold nanostars; AuNRs, gold nanorods; BBB, blood-brain barrier; CTAB, cetyltrimethylammonium bromide; CTS, chitosan; c(KRGDf), cyclo(Lys–Arg–Gly–Asp–Phe); DOX, doxorubicin; EPR, enhanced permeation, and retention; EGFRvIII, epidermal growth factor receptor variant III; FAL peptide, Phe–Ala–Leu–Gly–Glu–Ala; FUS, focused-ultrasound; GBM, glioblastoma; GLUT-1, glucose transporter 1; GNCs, gold nanoclusters; GNSs, gold nanostructures; GSH, glutathione; HAuNSs, hollow gold nanospheres; i.v., intravenous; IONPs, iron oxide nanoparticles; I/R, ischemia and reperfusion; MRI, magnetic resonance imaging; MSCs, mesenchymal stem cells; MiRNA, microRNA; NSCs, neural stem cells; NLS, nuclear localization signal; PAH, polyallylamine hydrochloride; PEG, poly (ethylene glycol); PSS, polystyrene-4-sulfonate; ROS, reactive oxygen species; SiO_2, silica; SERS, surface-enhanced Raman scattering; TAT, transactivator of transcription (sequence = YGRKKRRQRRR); Trp, tryptophan; ZE, *Zinnia elegans*

6.2 Photoacoustic imaging

Photoacoustic (PA) imaging is based on the PA effect, first discovered by Alexander G Bell in 1880.[401] Different information including, molecular structure, functional, and anatomical information about the target tissue can be obtained using PA imaging. PA imaging has a high spatial resolution (about 5 μm). It allows deep imaging (up to 6 cm), which is much better than fluorescence imaging due to the low scattering of ultrasound compared with light.[402, 403]

PA imaging is based on the photoacoustic effect, where the tissue absorbs a short pulse of light, and this absorption causes rapid thermoelastic expansion in the tissue molecules. This expansion creates a local pressure change, and consequently, an ultrasonic acoustic wave that is detected by a transducer and converted to electrical signals for analysis. To provide additional contrast, a contrast agent with high absorption coefficient can be used to label the target tissue, thus distinguishing the labeled tissue from normal tissue.[404]

Anisotropic GNSs with different shapes and sizes can be used as contrast agents for PA imaging due to the high absorption coefficient of some AuNPs at wavelengths in the biological window (650–1100 nm). The tunable plasmonic properties of different AuNPs provide contrast agents for PA imaging based on AuNPs. The absorbance spectra for different AuNPs that have been used in PA imaging can be found in the literature.[404]

Table 6 shows different GNSs and their optical properties (absorbance) that have been used for PA imaging. Wang *et al*. used PEG–AuNRs with a strong plasmonic peak at 800 nm for PA imaging in the rat brain.[374] Focused-ultrasound could induce the temporary opening of the blood-barrier-brain for better imaging. In another study, 40 nm hollow gold nanospheres (HAuNSs) were applied for *in-vivo* PA imaging of the vasculature in a living mouse brain (Fig. 21D).[375] It was reported that brain blood vessels as small as 100 μm in diameter could be distinguished using these PEG–HAuNS. Also, the PEG–HAuNS showed lower cytotoxicity compared with standard AuNRs.[375] The effect of melanin used as a capping agent on AuNPs with different shapes on PA imaging efficiency was investigated (*in-vivo*) by Repenko *et al*.[405] It was concluded that the melanin shell could play an essential role in PA imaging due to its excellent dispersibility, better biocompatibility, and enhanced PA responses compared to pure melanin or pristine gold particles. Simulation results showed that the thermal confinement effect could lead to better PA imaging due to the melanin shell. Comenge *et al*. reported that SiO_2–AuNRs with different shell thicknesses could be used for single and multispectral optoacoustic tomography (MSOT) of stem cells.[406] By preventing the plasmonic hot spots, the MSOT technique resulted in better imaging when the thickness of SiO_2 was 35 nm. GNSs also have been utilized for combined PA imaging and therapy. Lu *et al*. used HAuNS targeted to integrins that are overexpressed on glioma cells and angiogenic blood vessels in a mouse model of glioma. The HAuNS were successfully used for both PTT and PA imaging.[407] Zhou *et al*. also developed pH-responsive AuNCs loaded with chemotherapy drugs for simultaneous chemotherapy, PTT, and PA. The combined therapy using NIR laser irradiation excitation showed a synergic effect compared to either chemotherapy or NIR-induced PTT alone (*in-vivo*).[408]

Table 6 Optical properties of GNSs for PA imaging [404]

Contrast agent	Size (nm)	Peak absorption wavelength/imaging wavelength (nm)	Extinction coefficient (M^{-1} cm^{-1})
AuNSps	2–100	520–600	(3.61 ± 0.08) × 10^6–(6.06 ± 0.03) × 10^9 (d = 4–40 nm)
AuNRs	The diameter of 10–20 by the aspect ratio of 2–10	650–1300	3.3 ± 0.3 × 10^9 (length = 44.8 ± 4.1 nm; width = 19.8 ± 2.9 nm; λ_{peak} = 675 nm) 5.5 ± 0.3 × 10^9 (length = 51.0 ± 4.4 nm; width = 14.1 ± 2.1 nm; λ_{peak} = 850 nm)
AuNShs	50–500	700–2200	8.3 × 10^9 (diameter = 30.4 ± 4.4 nm; thickness = 7.8 ± 2.2 nm)
AuNPrs	The thickness of 10–40 by the planar width of 80–500	700–2000	N.A.
AuNCs	20–500	600–1200	4.34 × 10^{10} (outer edge length = 45.0 nm; wall thickness = 5.8 nm)
AuNSs	The core size of 20–60 by branch length of 10–30	650–900	N.A.
AuNVs	200–300	650–1000	N.A.

AuNCs, gold nanocages; AuNPrs, gold nanoprisms; AuNRs, gold nanorods; AuNShs, gold nanoshells; AuNSps, gold nanospheres; AuNSs, gold nanostars; AuNVs, gold nanovesicles

6.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a non-invasive technique that is based on the relaxation of proton spins after removal of the magnetic field when the target molecule (most often water) is located in a strong magnetic field (1.5 or 3 *T* in medical applications). The relaxation time is defined as the time required for the magnetic moment to return to the original alignment. The relaxation process can occur by two mechanisms: longitudinal relaxation (T1, parallel to the static magnetic field) or transverse relaxation (T2, perpendicular to the static magnetic field).
GNSs are being investigated as carriers for MRI contrast agents and can also allow other imaging modalities. For example, Au–Fe_3O_4 hybrid NPs with 11 nm Au core, have been examined for CT/MRI dual-modal imaging. A low concentration of these NPs showed stronger MRI contrast and better CT contrast compared to a high iodine-content agent.[409] In another study, DNA–AuNPs were used for transporting Gd^{3+} ions to image human NSCs.[377] A combination of AuNRs and other imaging contrast agents allowed dual PTT and also two-photon fluorescence imaging. Yin *et al*. synthesized Gd^{3+}–AuNRs with different structures like core-shell.[410]
Gao *et al*. synthesized two types of AuNPs functionalized with Gd–DTPA and with azide or alkyne functional groups connected via a click reaction in the acidic conditions found inside a tumor.[378] These aggregated NPs could permeate the BBB, showed enhanced MRI contrast, and could also be used for SERS imaging of brain tumors. Transactivator of transcription (TAT) peptide-functionalized AuNPs decorated with Gd^{3+} and doxorubicin (DOX) also have been used for simultaneous chemotherapy and MRI imaging of a brain tumor, where the efficiency of the chemotherapy was higher than using free DOX as a drug.[379] MRI has also been used to monitor the distribution of AuNPs inside the body and study the effect of radiosensitization.[380] Despite the versatile application of AuNPs in MRI for different tissues, their efficiency in neural systems have been mostly limited to *in-vitro* applications. The limited number of studies might be due to the tendency of these NPs to aggregate under the magnetic field, where the surface functionalization may not be able to prevent their aggregation in real neural tissue. Moreover, it seems that AuNPs cannot provide a clear image unless they are combined with other MRI agents, such as Gd^{3+} and Fe_3O_4 NPs, which are difficult to synthesize (see Table 5).

6.4 Surface-enhanced Raman scattering imaging

As mentioned above, surface-enhanced Raman scattering (SERS) is an important application of GNSs because of the large enhancement factors obtained.[411, 412] Although GNSs have been used as SERS imaging agents, there are only a few reports on SERS imaging of neural cells and tissues using GNSs, and most of those involve multimodal bio-probes. For example, alkyne-functionalized and azide-functionalized AuNPs could be delivered inside a tumor and then become conjugated together (aggregated) in the acidic microenvironment via a click reaction between an alkyne and azide groups on the two sets of AuNPs, leading to the creation of hot spots in the space between the AuNPs and consequent amplification of the SERS signal.[378] The mechanism of aggregation of AuNPs is shown in Fig. 22A.[378] Yue *et al*. synthesized the FAL peptide (Phe–Ala–Leu–Gly–Glu–Ala) functionalized Au–Gd (III) NPs to target the epidermal growth factor receptor variant III (EGFRvIII), a variant of the EGFR, which exists in 20% of GBMs.[381] The nanocomposite was successfully tested in dual-modal MRI (*in-vivo*)/SERS (*ex-vivo*) imaging. In another study, a SERS probe based on AuNPs was developed for intracellular detection of dopamine using AuNPs functionalized with an anti-dopamine Ab. It was shown that the cellular uptake was size-dependent.[382] In an exciting work, Huefner *et al*. used fluorescein-tagged nuclear localization signal (NLS) peptides functionalized onto AuNPs for distinguishing different neural cell types (*in-vitro*), according to changes in the DNA/RNA ratio inside the cells and its effect on SERS signals.[383] In another study, Yamazoe *et al*. applied AuNPs grown on a random nanoarray of transparent boehmite for *in-vivo* SERS imaging of brain ischemia. It was found that 125 nm size AuNPs with a 10 nm gap between them were the optimum parameters to obtain images with the highest resolution.[384]

Fig. 22 here

6.5 Fluorescence imaging and other optical imaging techniques

Fluorescence imaging is a non-invasive technique that can be used to imaging living tissues with high resolution at a cellular level. In fluorescence imaging, the fluorophore should be functionalized for specific targeting to the tissue. Each fluorophore should be excited with a particular wavelength and emit light at a longer wavelength that can be collected and analyzed. Further details of fluorescence imaging can be found in the literature.[413]

The intrinsic fluorescence of GNSs can be divided into two types. GNCs are fluorescent materials when their size is less than 2–3 nm, containing only a few Au atoms. The origin of fluorescence is due to the quantum confinement effect.[414] Other Au-based fluorescent materials are AuNRs and other structures that show fluorescence under multi-photon excitation.[415] For example, Peng *et al*. targeted orthotopic glioma tumors using both 3 nm and 18 nm Gly–AuNPs.[385]

AuNPs with the size of 18 nm could not penetrate the tumor, while the AuNCs could penetrate and provide a clear fluorescence image (Fig. 22B).[385] Yoon *et al*. also studied AuNPs with different sizes for multiphoton imaging of the cerebral vasculature and BBB integrity (*in-vivo* study).[386] It was reported that PEG–AuNPs with a diameter of less than 5 nm could be useful to visualize changes in vascular permeability in the earlier stages of BBB dysfunction. AuNRs have also been used for multi-photon luminescence imaging of a tumor by Yin *et al*.[410] The effects of AuNP shape and surface capping agent on the particle internalization by microglial cells (the resident immune cells in the brain) were investigated by Hutter *et al*.[387] It was reported that nanorods coated with either CTAB or PEG could penetrate well and could be useful for two-photon imaging. Wang *et al*. applied PEG–AuNRs for three-photon luminescence imaging of brain tissue.[388] As three-photon imaging is based on laser light with a relatively long wavelength, it could penetrate deeper than two-photon and one-photon imaging and enable deep-tissue imaging with high resolution.[416]

Fluorescence imaging using GNSs is not limited to the two above materials because an additional fluorophore could be loaded into nanocomposite systems containing GNSs for multi-modal imaging. Different fluorophores, including rhodamine and fluorescein, have been used for fluorescence imaging of neural tissue combined with other imaging modalities.[377, 389] Fluorescence imaging using rhodamine has been used to study the delivery of AuNRs functionalized with angiopep-2 peptide (Ang, a specific ligand of low-density lipoprotein receptor-related protein-1 (LRP1), which can facilitate the penetration of NPs through BBB) to the brain.[417]
In a recent study, Jara-Guajardo *et al*. demonstrated that AuNRs allowed detection of β-amyloid aggregates by fluorescence imaging. They used brain slices of transgenic mice with Alzheimer´s disease that were co-incubated with CRANAD-2 and AuNRs functionalized with the peptide D1 (recognizing β-amyloid aggregates).[418]

6.6 General remarks

The unique opto-physical properties of GNSs have enabled researchers to use them as contrast agents in different bioimaging applications. However, neural bioimaging applications have mostly been limited to spherical AuNPs. As the properties of these tiny NPs are size and shape-dependent, developing anisotropic GNSs-based contrast agents might open a new window in imaging neural tissues. GNSs have also shown the potential for therapy via PTT or PDT (see later), and could also be applied in neural systems for simultaneous imaging and therapy of brain tumors.[419, 420]

Similar to biosensing applications, systematic studies on size and shape-dependent properties are essential to develop imaging contrasts with high efficiency. While gold nanostructures are less toxic compared to other metal nanostructures (e.g., Ag and Cu, etc.), they still need surface modifications to become biocompatible for biological applications. Additionally, more in-depth studies should be conducted to better understand the size/shape-dependency of long-term toxicity of gold nanostructures inside the body as well as biodistribution and clearance pathways of them. Gold has high energy loss due to strong contribution of absorbance in their LSPR peak, leading to enhanced temperature in the surrounding medium. Although this characteristic might be useful for photothermal therapy, it will be damaging in long-term imaging of healthy tissues. The stability of Au nanostructures in different biological mediums and the effect of their aggregation on observed signal in different imaging techniques may cause false-positive or false-negative results. All in all, gold nanostructures are a long way towards clinical and commercial applications, where they need to suppress the current available contrast agents for different optical imaging techniques in terms of cost as well as availability and reproducibility.

7. Nanodelivery vehicles using GNSs (drugs, biomolecules, and genes)

7.1 Synthesis, modification, and loading of drugs or bioactive molecules onto AuNPs and delivery to the brain

Delivery of drugs and active biomolecules to the CNS is challenging due to their inability to cross the BBB. To address the failure of drugs and biomolecules to pass through the BBB, researchers have explored the use of NPs due to their small size and high surface area. AuNPs are of particular interest, since they have distinctive properties, including optical properties, chemical and physical stability, relatively low toxicity, a wide range of possible surface functionalization, and LSPR wavelength peaks. These properties make AuNPs excellent candidates for the delivery of drugs and active biomolecules.[17, 421-424] However, before the clinical applications of AuNP-based nanovehicles, much more information is needed about good manufacturing practice (GMP), pharmacokinetics/absorption, distribution, metabolism, and excretion (PK/ADME), and *in-vivo* toxicity. It is expected that AuNPs will need to be functionalized to reduce their toxicity and target specific diseases.

Currently, there are three general methods of surface functionalization of AuNPs including, surface coating, ligand exchange, and layer by layer self-assembly (Fig. 23). In the following, we briefly explain the three standard methods for AuNP functionalization in neurological applications.[425]

Fig. 23 here

7.1.1 Ligand exchange method

The ligand exchange method consists of the conjugation of organic molecules or biomolecules to the surface of the AuNPs to reduce the toxicity and improve the loading or binding of a drug or bioactive molecule.[426] This method is based on organic or biological molecules with a high affinity for Au. The ligands should induce the original capping agent displacement, such as CTAB, through a mass-driven exchange.[427] There are a wide variety of molecular linkers that have been used to passivate the AuNP surface, such as thiolate,[428] dithiolate,[429] dithiocarbamate,[430] amine,[431] carboxylate,[432] selenite,[433] isothiocyanate,[434] or phosphine[435]. The nature of these molecules dictates the NP solubility in organic or polar solvents. Ligands may possess electron-donating groups that interact with the AuNP surface, which depending on the interaction strength, can undergo dynamic binding and unbinding processes.[436]

For this reason, only some ligands can provide long-term stability of the coated NPs. The stronger the charges present in the ligand molecules, the higher the shielding effect against van der Waals forces or hydrogen bonds, which can cause NP agglomeration. Weakly bonded ligand molecules can be exchanged for other strongly binding molecules offering stability and more functionality to improve the NP stability. For example, a citrate layer can be replaced by strongly interacting ligands such as sulfonated phosphines or mercaptocarboxylic acids (mercaptoacetic acid, mercaptopropionic acid, or MUA).[437]

Most routes use the covalent attachment of a free thiol, amine, or carboxylate functional group to facilitate the conjugation of AuNPs to various biomolecules and biopolymers. However, since the Au–S bond is strong and easily formed, thiols are often used to exchange ligands for increasing AuNP stability and biocompatibility.

Au–S bonds are stronger (~40 kcal mol^{-1}) compared to Au–N (~8 kcal mol^{-1}) and Au–COO- (~2 kcal mol^{-1}) bonds,[438] and Au–S bonds show better conjugation efficacy. Therefore, the thiol group is the most common functional group used for AuNP functionalization.[439] Thiolated ligands attach firmly to the surface of the AuNPs (based on Au–S) and reduce the toxicity and improve the biocompatibility and stability of AuNPs.[425] Thiolated ligands can contain PEG,[440] chitosan (CTS),[441] DNA,[442] and other biopolymers[443]. Among the different thiol ligands, PEG-SH exhibits clear advantages over other surface modifications since they possess: (1) excellent stability in aqueous solutions (PBS and cell culture media); (2) good biocompatibility; (3) longer half-life and prolonged blood circulation time; (4) lower level of toxicity compared to other ligands.[425, 444] Ren *et al*. investigated paclitaxel (PTX)-loaded CTAB–AuNPs with PEG linked to 11-MUA.[444] In their study, PTX was loaded into the PEG monolayer on the surface of AuNPs, and the hydrophobic drug was delivered to the cells via diffusion into the lipophilic plasma membrane. The results showed a highly efficient drug release in the cellular environment compared to the free drug. They also demonstrated that by using the combination of PTT and chemotherapy, no living cancer cells were found at a dosage of 0.5 nM irradiated with low intensity (0.55 W/cm^2) NIR light compared to free PTX and control NPs.

In recent study, a novel strategy was introduced to enhance the effectiveness of NP agents against brain tumors (including glioblastoma (GBM)),[445] which are at present incurable with available standard treatment (Fig. 24A and B)[446]. GBM is the most severe brain cancer subtype with a median survival of only six months. However, more studies and optimization are needed to use this platform in clinical therapy.[445, 447]

Fig. 24 here

Etame *et al*. systematically studied the permeation of AuNPs with a surface functionalized with PEG through the brain microvasculature. Their studies suggested a way to design AuNPs for passive targeting of GBM. Their report synthesized and characterized AuNPs with different core particle sizes (4–24 nm) and various PEG chain lengths (molecular weight 1000–10,000). Using an *in-vitro* model intended to mimic the transport-permissive brain microvasculature, they showed that the permeation properties of AuNPs were size-dependent. In general, they demonstrated that the short PEG chain length (molecular weight 1000–2000) in combination with the smallest core size showed the best permeation in their *in-vitro* system.[448] Cheng *et al*. investigated a noncovalent AuNP-based drug-delivery platform, which could penetrate through the BBB and the blood-brain tumor barrier (BBTB) and selectively deliver therapeutic drugs to brain tumors for photodynamic therapy (PDT). They used PEG ligands conjugated to AuNPa to create NPs with good water miscibility, biocompatibility, and long circulation time in the blood. Moreover, they showed that the PEG layer on the surface of AuNPs could provide bifunctionality to conjugate EGF peptide, which is internalized by cells and non-mitogenic, to target EGFR over-expressed on the surface of the glioma cells. PEGylation of the NPs was carried out using

heterobifunctional PEG derivatives, COOH-PEG-SH, and MPEG-SH. The EGF peptide (YHWYGYTPQNVI) was linked to the carboxyl group of the coated PEG layer via amide bonding. Finally, the photosensitizer, phthalocyanine 4 (Pc 4), was adsorbed onto the AuNP surface through binding to the terminal amine group on the Pc 4 axial ligand. Fig. 24C demonstrates the design of the EGF–AuNP–Pc 4 conjugates. In conclusion, they demonstrated that EGFR targeting of Pc 4-loaded AuNPs to cell surface receptors improves drug delivery to GBM brain tumors. The use of the EGF-peptide to interact with the cell surface receptor allowed the hydrophobic Pc 4 to transfer to the cellular membrane. Overall, their study demonstrated the rational and successful design of a noncovalent drug-delivery system to brain tumors using targeted AuNPs.[449]

Yetsa *et al*. attached ITE (an aryl hydrocarbon receptor (AhR) ligand made by Tocris Bioscience) and MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) to AuNPs, which were also conjugated with methoxy-PEG-SH to deliver peptides to lymph nodes for the treatment of multiple sclerosis (MS). MS is an autoimmune disease caused by a host immune response against axons and myelin sheaths of the CNS, leading to axonal loss and demyelination. They designed AuNPs to coadminister a tissue-specific antigen (MOG35–55) and an AhR ligand (*i.e*., ITE) to activate tolerogenic APCs. Tolerogenic APCs promoted the differentiation of CNS-specific regulatory T cells (Tregs). They suppressed the development of MS in a murine model of experimental autoimmune encephalomyelitis (EAE), a widely accepted pre-clinical model of MS. These NPs could provide a promising strategy for the treatment of MS. Overall, their formulation, with its simplicity, scalability, and low cost, warrants further study as a basis for immunotherapy against MS and other autoimmune diseases.[450, 451]

In another study by Shilo *et al*., insulin-targeted AuNPs (INS–AuNPs) were developed as a drug carrier for INS or carriers of different biomolecules for nerve regeneration. AuNPs were synthesized using sodium citrate and coating the surface of AuNPs with PEG. Finally, the INS was covalently conjugated to the coated PEG. Subsequently, they injected the formulated INS–AuNPs (and free AuNPs as a control) into the tail vein of male BALB/c mice and measured the amount of INS–AuNPs that crossed the BBB by receptor-mediated endocytosis. At specific time points after injection, the major organs were harvested, and a blood sample was taken and measured the amount of gold by flame atomic absorption spectroscopy. Results indicated that two hours after i.v. injection, the amount of gold in the mouse brain was more than five times higher compared to the free AuNPs. In another experiment with rats, they reported that INS–AuNPs could be used as CT contrast agents to study specific regions of the brain where they accumulate. Finally, they proposed that this approach could be used as a potential tool to overcome the challenge of delivering imaging and therapeutic agents to the brain.[452]

However, research over the years has shown that the PEGylation of NPs can have significant disadvantages, such as a long clearance time, less biodegradability, and the initiation of anti-PEG Abs in the body when repeated dose of PEGylated products are used. As a result, there is a growing need to discover a replacement for PEGylation to improve the quality and performance of NPs.[453] Monoclonal Abs are another class of ligands that have been widely investigated for selective drug delivery due to their ability to recognize a single specific antigen. The monoclonal Ab (mAb) ligands have some advantages, such as the possibility for secondary functionalization, which allows them to be attached to AuNPs. Ab-loaded gold nanoconjugates have been studied in immunohistochemistry for almost 40 years. The methods to carry out Ab conjugation to AuNPs consist of adsorption,[454] covalent bond formation using N-hydroxysuccinimide (NHSI) ester oligonucleotide-directed immobilization by hybridization[455]. Abs can be attached to AuNPs by hydrophobic and ionic interactions or through chemisorption of native thiol groups that occur in Abs's chemical structure.[456] However, these conjugates have some limitations based on the protein's stability and the protein's desorption.[457]

Thiolated ligands can contain various other functional groups (*e.g*., carboxyl and hydroxyl), allowing secondary functionalization.[458, 459] Monolayers of Abs can be covalently attached to an active group on the surface of AuNPs by NHSI ester mediated reactions with the Ab's primary amine groups. Another way to make more stable AuNP Ab conjugates is to use DNA–AuNPs and hybridize them with Abs previously conjugated to complementary oligonucleotides.[457] For example, Truong *et al*. attached oligo-(ethylene glycol) thiols (OEG–SH) to AuNPs.[460] Afterwards, the anti-prostate-specific antigen (PSA) Ab was incorporated into the OEG–AuNRs. The PSA Ab–OEG–AuNRs were used for ultrasensitive detection of PSA. GNSs can be functionalized with anti-EGFR Ab to target cancer cells for dark-field multiplex imaging enabling PTT.[461] Au conjugated Abs are being investigated for PTT, and results indicated that the Ab could improve specific delivery of AuNPs to cancerous cells. PA imaging for detection of atherosclerotic plaques was performed using targeting with metalloproteinase-2 Ab functionalized AuNRs.[462]

Other than Abs, other ligands such as aptamers, peptides, small molecules such as folic acid,[425] and proteins such as transferrin (Trf)[456] have been commonly used conjugate to AuNPs for targeted cancer therapy. Nanostructures conjugated with targeting ligands can recognize biological components upregulated in malignant, dysplastic, or pathologic tissues. The use of multifunctional or chimeric targeting systems can enhance target specificity, allow more efficient drug delivery, and enhance imaging capability.[463]

7.1.2 Layer by layer self-assembly method

Layer by layer (LbL) functionalization consists of multiple deposition cycles in which alternating layers of negative and positively-charged polyelectrolytes are deposited on the surface of a GNS. The first layer interacts with the gold surface by electrostatic interaction.[464] There are a variety of polyelectrolytes utilized for this kind of functionalization. PSS and polyacrylic acid (PAA) are the two most common negatively charged polyelectrolyte polymers used. In contrast, poly(allylamine) hydrochloride (PAH), PEI, and PDDAC are commonly used as positively charged polyelectrolytes. Increasing attention has been paid to multilayers based on PSS/PAH, poly($_L$-lysine), PLL/alginate (ALG), PLL/hyaluronan (PLL/HA), PLL/poly($_L$-glutamic acid) (PLL/PGA), PLL/PAA, PGA/PAH, CTS/HA, and PEI/PAA pairings.[465] LbL deposition prevents NP aggregation by improving the stability of the AuNPs and enhancing their biocompatibility.[466, 467] LbL supports the loading of charged drugs and the conjugation of nucleic acids and proteins by exploiting electrostatic or hydrophobic interactions. The LbL self-assembly is schematically shown in Fig. 23.

The LbL self-assembly method enhances the stability and biocompatibility of AuNPs and allows the immobilization of charged drugs, genes, and proteins by electrostatic or hydrophobic interactions. Negatively charged PSS–AuNP has been used to fabricate drug nanocarriers to deliver a well-known anti-tumor drug, DOX (positively charged), by the interaction between the positive charge of DOX and negatively charged PSS–AuNRs to prepare drug nanocarriers. One example study where alternate layers of PAH/poly-L-lysine citramide (PLCA) were utilized to allow DOX loading for drug delivery.[468] Interestingly, PLCA is a pH-sensitive biocompatible polymer allowing triggered delivery by the low pH environment of intracellular lysosomes or in the proximity of tumor tissue. In another report, AuNPs were coated with PSS and cationic PEI and then loaded with imatinib mesylate (a drug used to treat chronic myelogenous leukemia).[469] Besides, negatively charged nucleic acids, such as small interfering RNA (siRNA) and plasmids can be attached by the charge interaction with the surface of positively charged PAH–AuNPs, PEI–AuNPs, and PDDAC–AuNPs to fabricate AuNPs for gene therapy.[470] Concerning gene therapy, NP-LbL carriers are preferable over viruses. LbL is chemically customizable, less immunogenic, does not have the risk of mutagenesis, and these NP-LbL can be produced on a large scale. An LbL method was utilized to deliver two different plasmids carried by the same NP.[471] This approach ensured the co-expression of both plasmids.

Yin *et al*. investigated the LbL method to synthesize multilayers of polyelectrolytes on positively-charged CTAB–AuNPs to co-deliver siRNA and DOX.[472] They coated the surface of CTAB–AuNPs with PSS, and then DOX was attached to PSS-AuNPs, followed by the adsorption of PAH. Afterward, the siRNA was attached to the surface of PAH–DOX–PSS–AuNPs. Their results indicated improved anticancer efficacy through the synergistic combination of siRNA and DOX release after irradiation at 665 nm. Additionally, charged targeting ligands, such as Trf and Abs, could also be attached to the polyelectrolyte-functionalized AuNPs for selective imaging and therapy.[456, 473]

Coluccia *et al*. investigated the potential of cisplatin-UP peptide-loaded AuNPs (AuNPs–UP–Cis) combined with magnetic resonance-guided focused ultrasound (MRgFUS) to improve GBM treatment. They examined four different types of AuNPs: non-functionalized, Cis-functionalized, AuNPs functionalized with cell uptake peptide (PKKKRKV, UP) AuNPs–UP–Cis. To load Cis onto the NPs, they used a covalently bonded porous polymer as the backbone and ionic adsorption of the drug to the backbone polymer's surface. The polymer adds 1–2 nm in diameter to the NPs but provides the drug's loading efficiency onto NPs. Viability assays demonstrated that the AuNPs–UP–Cis inhibited GBM cells' growth compared to free drugs. Furthermore, there was a synergistic effect when combined with radiation therapy. Moreover, DNA damage caused by γH2AX phosphorylation was reported in AuNPs–UP–Cis treated cells. *In-vivo* results of AuNPs–UP–Cis showed a reduction in GBM tumor growth.[474]

A system consisting of Ang, PEG, and DOX, attached to AuNPs was investigated by Ruan *et al*. In their study, they loaded DOX using hydrazide bonds (an acid-responsive linker) onto AuNPs functionalized with Ang. The final preparation particle size was 39.9 nm, and the DOX loading capacity was 9.7%. *In-vitro* release of DOX from DOX–AuNPs was pH-dependent and showed rapid drug release at lower pH values than neutral pH. *In-vivo*, the examination of the Ang–PEG–DOX–AuNPs showed higher uptake of NPs compared to PEG–DOX–AuNPs and free DOX. Fig. 25 shows the overall structure of the AuNPs after modification and penetration of the NPs through BBB to target GBM cells.[475]

Fig. 25 here

Finally, they showed that glioma-bearing mice treated with Ang–PEG–DOX–AuNPs had the most extended survival rate, which was 2.89-fold longer than saline. In conclusion, they demonstrated that Ang–PEG–DOX–AuNPs could precisely deliver and release DOX in glioma-bearing mice and meaningfully increase the survival rate.[475]

7.1.3 Surface coating method

The surface coating method refers to the deposition of a thin coating onto the GNSs. The outer shell can be organic or inorganic, and the choice strongly depends on the final application and purpose. Generally, core-shell NPs possess a highly functionalized surface, and the overall NP properties arise from both the core and shell materials.[121, 476, 477] By properly selecting the shell composition, it is possible to modify the particle stability and dispersibility, while the shell thickness affects the NP plasmonic properties.[127]

Concerning the synthesis of core-shell NPs, careful control is needed to achieve a uniform coating of the shell materials during the particle formation. Among the core-shell NPs, Au–SiO_2 structures are popular because the SiO_2 shell used to increase the colloidal suspension stability is chemically inert and stable. The synthesis is performed by adding sodium silicate solution to a suspension of AuNPs functionalized with (3-aminopropyl) trimethoxysilane (APS), a silane coupling agent, keeping the pH between 10 and 11.[120] Another inorganic material used for AuNPs coating is metals like Ni, Co, Pd, Pt, and Cu to modify the optical properties of the AuNPs, making them useful for sensing applications. The synthesis and stabilization of Au–Ag core-shell

NPs was performed using a dipeptide NH_2-*β*-Ala-$_L$-Trp-OH in water at room temperature and pH ~7. The first reduction process was used to produce the AuNPs, and then the Ag ions were reduced on the Au core.[478] AuNPs can also be coated with different organic molecules to stabilize the gold colloids while maintaining the plasmonic properties for sensing applications. Among the organic molecules, polyaniline (PANI) was used as a biosensor for sensing glucose in living systems. The core-shell NPs were prepared using H_2O_2 as an oxidizing agent as well as a reducing agent. H_2O_2 in the correct proportion was added to $HAuCl_4$ to produce the AuNPs. A freshly distilled aniline solution was added under stirring for 12h leading to the formation of the Au–PANI NPs.[479] Wuelfing *et al*. stabilized gold NP solutions via covalent bonding of thiolated PEG[480] Exploiting electrostatic attraction, it is possible to coat AuNPs with polyelectrolytes to produce multilayer coatings utilizing LbL deposition.[481] Further information about the synthesis and use of core-shell NPs was reported by Chaudhuri and Paria.[482]

The surface coating method consists of capping the AuNPs with a thin shell of an inorganic material that can effectively remove or cover up potentially toxic surface compounds, and improve the biocompatibility of AuNPs. Core-shell AuNPs have a long list of different applications, spanning biomedical and pharmaceutical applications, catalysis, electronics, enhanced photoluminescence, and photonic properties in general.[483] Focusing on the biomedical area, core-shell NPs have been used for targeted and controlled drug delivery,[484] bioimaging,[485] cell labeling,[322] and tissue engineering applications.[111, 486] Methods for surface functionalization of AuNPs by a coating approach are well established.[487]
Organic coatings involve the coating of AuNPs with polymeric materials, which can help suppress the immune response,[488] and provide higher resistance to bacterial adhesion[489]. Among the inorganic layers, mesoporous SiO_2 is a commonly used coating material because of its high biocompatibility, easy synthesis, and drug loading capacity.[490] The mesoporous SiO_2 NPs (MSNs) offer the advantage of a high specific surface area, allowing large amounts of the drug to be loaded.[491] In the initial step, the pH value of the AuNP solution is adjusted to pH 10 by adding sodium hydroxide or ammonium hydroxide. The SiO_2 is then deposited on the AuNPs by hydrolysis and condensation of tetraethyl orthosilicate (TEOS) using gentle stirring (Fig. 23C). The thickness of the SiO_2 shell can be controlled by varying the amount of TEOS or increasing the reaction time.[492] AuNPs–MSNs are commonly used due to their large internal surface area and internal pore volume of the mesoporous SiO_2, which leads to high drug loading. For instance, DOX can be loaded into AuNPs–MSNs by simple agitation to make a chemotherapy delivery vehicle for tumor treatment.[493] Additional functionalization of the surface of AuNPs–SiO_2 is possible when the surface of the AuNPs has been modified by silane.[494]

Other inorganic materials, such as graphene, CNTs, metal oxides, and Ag ions, have been investigated for functionalizing AuNRs. Xu *et al*. encapsulated AuNRs inside graphene sheets using electrostatic adsorption and conjugation of hyaluronic acid using an amide linkage. Afterward, they loaded DOX into the AuNRs–graphene by π–π stacking and hydrophobic interactions.[495]

Based on the functional groups used, two kinds of interactions can occur, noncovalent interactions (electrostatic interactions, hydrophobic entrapment, and van der Walls forces), or covalent interactions between the attached group on AuNPs and target molecules.[496] However, covalent modifications are more stable at high salt concentrations and high temperatures, making them more suitable for sustained or long-time drug release. Although covalent modifications are more durable, the synthesis process for covalent modifications is usually more complicated and sometimes requires several steps.[82]

Lee *et al*. described a new construction consisting of SiO_2–AuNRs connected to a rabies virus (RABV) glycoprotein (RVG) 29-mimetic (RVG–PEG–AuNRs–SiO_2) for GBM targeting.[497] RABV

virus has an individual bullet-like shape with one rounded end and one flat end, with a 45–100 nm diameter, a length of 100–430 nm, and an aspect ratio of 2.4. They utilized anisotropic (nonspherical) AuNP chemistry to fabricate RABV mimetic NPs using the flexibility of gold as an inorganic material to create a variety of NP shapes, such as spheres, rods, shells, cages, and cubes. They used $HAuCl_4$ to synthesize AuNRs modified with RVG29 to pass through the BBB and enter the brain. The AuNRs improved the interaction with the nicotinic acetylcholine receptor (AChR) and improved the LSPR signal in response to NIR irradiation. Afterward, they focused on synthesizing AuNRs with a shape that best resembled the RABV (Fig. 26).[497] The modified AuNRs successfully mimicked the RABV, such as size, shape, surface glycoprotein properties, and *in-vivo* behavior. Their RVG–PEG–AuNRs–SiO_2 had a similar length, width (~120 per ~50 nm, respectively), and an aspect ratio of 2.34 to the live RABV.[497]

Fig. 26 here

The RVG29 peptide modification on the surface of the AuNPs improved the *in-vivo* distribution of the nanorods by allowing them to reach the brain by crossing the BBB. Together, their results support the idea that the RABV mimetic AuNRs could be used as a delivery platform for treating brain tumors, especially GBM.[497]

Shi *et al*. described a new class of delivery system based on AuNPs that could be loaded with sulfhydryl-containing drugs, allowing controlled multistage-release with optical monitoring for therapy of brain diseases. Many sulfhydryl-containing drugs are unstable in biological environments since they can undergo thiol group oxidation, producing a shorter half-life and needing a larger dosage. Additionally, some sulfhydryl-containing drugs can lead to serious side effects, such as mucocutaneous lesions, proteinuria, pemphigus, and hematologic reactions. They developed a new multifunctional delivery system based on fluorescent-nanogel-coated, AuNPs-functionalized with dendrimer-like "hierarchical pore SiO_2 NPs" (HPSNs). The manufacturing process started with HPSNs-NH_2, then, AuNDs were added, followed by drug-loading. Then, the Au–HPSNs-NH_2 was coated with an autofluorescent nanogel (ALC–PEI) responsive to the intracellular environment. This novel delivery system had low cytotoxicity to neuronal cells, high-loading capacity offering protection for sulfhydryl-containing drugs, and allowed multistage controlled drug release in the intracellular microenvironment with fluorescent monitoring.[498]

Ni *et al*. grafted CTS and fibroblast growth factor 1 (FGF-1) onto AuNPs contained within a poly(D,L-lactide) (PLA) scaffold. Their findings indicated superior results for the grafted NPs compared to CTS or FGF-1 alone for the repair of a 12 mm rat sciatic defect. They grafted the CTS containing AuNPs and FGF-1 into the microgrooves on the PLA surface using open-air plasma treatment to improve the proposed platform performance. Overall, they showed that bioactive molecules, including CTS–AuNP and FGF-1, could be successfully grafted onto PLA, and the *in-vivo* study of their platform in the rat sciatic nerve transection model showed a high degree of myelination at both 4 and 6 weeks.[499, 500] Johnsen *et al*. investigate Ab-liposomes and AuNPs as a cargo transport to deliver anti-TfR Ab. Their results indicated that there may be a lower limit for the ligand density required, to obtain enough TfR-mediated targeting and transport of nanoparticle–loaded cargo into the brain parenchyma.[501] In another study by Fatima *et al*., they investigated the potential of galactose–AuNPs (2 nm) as a delivery system for oligonucleotides. Their results indicated that AuNPs have the potential to transport therapeutic amounts of nucleic acids into the CNS.[502] A summary of the different modifications and applications of AuNPs is given in Table 7.

Table 7 Surfaces modification of GNSs for cargo delivery in nervous systems

Surface functionality	Size (nm)	Study/Application	Method of modification	Result	Refs
CTAB	118.5	Improve the delivery of AuNRs to the CNS of rats (*in-vivo)*	AuNR–CTAB was synthesized using the seed-mediated approach. $HAuCl_4$ mixed with CTAB and cold-sodium borohydride to synthesize the seeds. Afterward, the thiol–PEG conjugate to the surface of AuNRs and peptide conjugate to the AuNR–PEG.	Ang–AuNR–PEG increased CNS penetration while achieving reduced retention in the liver	417
Ab	77	TfR–AuNPs (*in-vitro* and *in-vivo*)	MeO–PEG–lipoic acid reaction with AuNPs seeds and finally mixing with Ab–SH	The uptake capacity is significantly modulated by the affinity and valency of the AuNP–Abs: Abs with high and low associations mediate low and intermediate uptake of AuNPs into the brain, respectively, whereas a monovalent (bi-specific) Ab improves the uptake capacity remarkably.	501
Citrate (using citrate dihydrate solution)	19.1±6.1 54.4±24.9 102.7±45.9	Biodistribution of AuNPs with a varied particle size in different organs such as the brain (*in-vivo*)	Three different sizes of AuNPs were synthesized by mixing hydrogen tetrachloroaurate(III) tetrahydrate and after that trisodium citrate dihydrate	NPs mainly distribute in the liver and spleen. Smaller AuNPs accumulated more in the lungs and brain and less in the heart, stomach, and pancreas. So smaller AuNPs have the potential for targeted delivery to the lungs and brain.	503
Thiol–PEG2k–amine, Mw = 2000	The average core size of 2±1 based on TEM	Biodistributions of AuNPs in different cells and investigate the cell uptake (*in-vitro*)	A modified Brust-Schiffrin protocol was used to make AuNPs. Finally, the anionic pyranine fluorophore attached to amino groups using EDC/NHSI at pH 6.4	Developed a supramolecular strategy for the spatio-temporal control of the cell uptake of small AuNPs, using external additives as triggers. AuNPs are efficiently internalized upon adding an oligocationic covalent cage that interacts with pyranine, forming a positively charged host-guest complex.	504

PEG RGD peptide DSPEIs	Length: 50 Diameter: 10	Small hairpin (sh)RNA delivery (*in-vitro* and *in-vivo*)	RGD–DSPEIs–AuNR	Effectively condensed siRNAs and show precise targeting of model human brain cancer cells (U-87 MG–GFP) via the αvβ3 integrin-mediated endocytosis	505
Citrate ion	15 ± 2.30 50 ± 5.65 100 ± 5.56 200 ± 7.56	AuNPs for biodistribution study (*in-vivo*)	Reducing hydrogen tetrachloroaurate with sodium citrate	AuNPs of 15–50 nm in hydrodynamic size could permeate across the BBB, while larger NPs, specifically 100 and 200 nm-sized, could not.	506
RGD peptide	2–3	Vector for Bcl-2 siRNA (*in-vitro* and *in-vivo*)	Au PENPs modified with an RGD peptide via PEG spacer	Results revealed that the Au PENPs could deliver Bcl-2 siRNA to GBM cells with excellent transfection efficiency, causing specific gene silencing in the target cells *in-vitro* and *in-vivo*.	507

Ab, antibody; Ang, angiopep-2; AuNPs, gold nanoparticles; AuNRs, gold nanorods; Bcl-2, B-cell lymphoma-2; BBB, blood-brain barrier; CNS, central nervous system; CTAB, cetyltrimethylammonium bromide; DSPEIs, disulfide cross-linked short polyethyleneimines; EDC, 1-ethyl-3-(3(dimethylamino)propyl)carbodiimide; GBM, glioblastoma; GFP, green fluorescent protein; PEG, poly (ethylene glycol); Au PENPs, polyethyleneimine-entrapped gold nanoparticles; NHSI, N-hydroxysuccinimide; siRNA, small interfering RNA

7.2 GNS-mediated delivery of small-molecule drugs, proteins, peptides, RNA, and genes to the CNS

AuNPs are promising as drug delivery carriers for the therapy of NDs. NDs include various conditions characterized by the progressive loss of structure or function of neurons, finally resulting in neuronal cell death. The most common NDs are AD, PD, prion disease (PrD), and amyotrophic lateral sclerosis (ALS). Short oligopeptide sequences act as targeting moieties for many proteins. These sequences frequently consist of positively charged amino acids such as Arg and Lys that interact with importin α for transport across the nuclear envelope.[508]

One treatment proposed for AD is to destroy the Aβ fibrils and plaques in the brain that contribute to the mental decline.[342] This approach is designed to halt or slow the progression of AD without damaging healthy brain cells. Towards this goal, researchers conjugated AuNPs to Aβ fibrils, cultured the conjugated AuNPs with neurons for several days, and finally irradiated the cells with weak microwave fields for several hours. The microwave energy levels employed were around six times lower than conventional cellphones, and hopefully not harmful to healthy cells.[509] After treatment with weak microwaves, the fibrils dissolved and remained stable for at least one week after exposure. This finding indicated that this approach was effective at breaking up the fibrils and could reduce the proteins' tendency to re-aggregate.[510] A similar system could be investigated to treat other NDs that involve protein aggregation, such as PD,[510] also described more extensively in section 9.1.

The Kogan group investigated the possibility of using AuNPs conjugated to the β-sheet disrupting peptide, LPFFD. They conjugated the Cys residues at the N-terminus (CLPFFD) of the peptide onto the AuNP surface. They then studied whether their peptide-conjugated AuNPs could recognize the toxic Aβ protein aggregates (*i.e.*, oligomers, protofibrils, and fibrils) involved in the pathogenesis of AD. The results showed that, by irradiation of AuNPs with weak microwaves, the Ab aggregates attached to the AuNPs were locally destroyed by the CLPFFD.[509, 511] However, to allow successful removal of the toxic aggregates present in the AD brains, the AuNPs–CLPFFD must cross the BBB. In another study by the Kogan group, they reported that after IP injection of AuNP–CLPFFD in rats, partial accumulation of the conjugated AuNPs was found in the rat brain. However, the amount in the brain was lower compared to the previous study, but still, more than control animals injected with citrate AuNPs.[512]

Velasco-Aguirre *et al*. proposed that the conjugation of CLPFFD to AuNPs could improve their ability to cross the BBB by three mechanisms: (a) adsorption of specific plasma proteins, which could participate in receptor-mediated transcytosis; (b) improving the passive diffusion of AuNP through the BBB by enhancement of the amphipathic characteristics; (c) reduction of the particle clearance by the RES by decreasing the zeta potential.[513-515] Using this conjugation method, the delivery of AuNPs to the brain was improved, but the percentage remained low compared to the total injected dose (Fig. 27).[29, 516] In another study by Prades *et al*., they demonstrated introducing the peptide sequence THRPPMWSPVWP (THR) into the AuNP–CLPFFD conjugated AuNPs was able to remove toxic aggregates of Aβ. The THR peptide sequence can interact with the TfR expressed in the microvascular endothelial cells of the BBB, resulting in improved BBB penetration, as shown both *in-vitro* and *in-vivo*. Their results suggested that AuNPs could be used in the treatment of NDs such as AD.[517]

Recently, a novel AuNP platform, gold bellflowers (GBF) was described. This could be combined with a variety of therapeutic modalities (chemotherapeutic drugs, Abs, aptamers, siRNAs, or miRNAs) and multiple imaging approaches (optical, positron imaging tomography, or magnetic resonance) and with photoactivatable diagnostic and therapeutic applications for the treatment of neurodegenerative diseases or brain cancers. These platforms were modified with

neuron/glial cell-specific ligands to allow these nanoplatforms to penetrate through the BBB to allow theragnostic cargo delivery into the brain.[516]

Some examples of theragnostic platforms with possible applications in the treatment of GBMs and neurodegenerative diseases such as AD and PD were summarized by Ali *et al*. These GBFs platforms are non-toxic, biodegradable, and can load a combination of siRNAs, miRNAs, enzymes, and drugs with unique features of highly efficient PA imaging and PDT/PTT (Fig. 27).[29, 516]

Fig. 27 here

An innovative method to deliver drugs to the CNS and the brain to bypass the BBB and blood-spinal cord barrier (BSCB) completely, was described by Zhang *et al*., who attempted to selectively deliver drugs to the respiratory motor neurons in the phrenic nucleus and the rostral ventral respiratory group (rVRG) neurons in the brainstem for the treatment of respiratory problems after spinal cord injury (SCI). They developed a novel approach that used nanotechnology to selectively target only the respiratory motor neurons including rVRGs responsible for the diaphragm function. Their nanotherapeutic design consisted of a targeting transporter protein, wheat germ agglutinin conjugated to horseradish peroxidase (WGA–HRP), chemically conjugated to AuNPs, which in turn was chemically conjugated to the pro-drugs, pro-theophylline, or prodrug of 1,3-dipropyl-8-cyclopentylxanthine (Pro-DPCPX).[518] The WGA–HRP was taken up by the terminal phrenic axons when injected into the diaphragm muscle and were retrogradely transported to phrenic motor neurons. The WGA–HRP was further trans-synaptically transported across physiologically active synapses to end up in the neurons of the rVRG, and was not transported to any other neurons.[519] This targeted drug administration method allowed recovery of the hemidiaphragm muscle in SCI rats, using only a fraction of the systemic dose required to generate the same degree of recovery. For comparison, the theophylline systemic dose in rats was 15 mg/kg,[520] while the equivalent nanoconjugate theophylline content was only 0.12 mg/kg.[518] The systemic amount of DPCPX in rats was 0.1 mg/kg,[521] while the DPCPX content in the nanoconjugate was 0.15 μg/kg, ~0.1% of the systemic dose.[518, 522] Moreover, the nanoconjugate was capable of inducing long-lasting recovery after only a single intramuscular (*i.m.*) injection at the injured side of the diaphragm.

8. GNS-based therapeutic agents for neurological disorders

In some conditions, including NDs, infection, or stroke, the BBB is altered and becomes more permissive,[523] facilitating the passage of drugs into the brain parenchyma. In this section, we will discuss recent preclinical studies addressing the use of gold-based approaches for the diagnosis, imaging, and therapy of AD (Fig. 28 and Table 8) and brain tumors (Fig. 29 and Table 9) as well as a brief overview of their use in other neurological conditions.

Fig. 28 here

Fig. 29 here

8.1 Pre-clinical gold-based strategies for neurological disorders

GNSs can inhibit or destroy protein aggregates such as Aβ. This ability has been widely explored to improve and develop novel therapeutic strategies for prion-like disorders and AD. For example, *in-vitro* studies showed that negatively charged AuNPs (~30 nm and ~-38 mV)[524] alone, and curcumin-functionalized AuNPs (10–25 nm and ~+25 mV)[525] inhibited fibrillization

of Aβ and promoted breakdown of Aβ aggregates, thereby reducing the aggregate toxicity. In mice, the high anti-prion activity of a polyelectrolyte multilayer coated onto AuNPs was demonstrated after a single intracerebral administration.[526] Interestingly, this NP formulation was also able to cross the BBB after i.v. injection, peaking at 19–24 h after administration, and specifically accumulating in the hippocampus, thalamus, hypothalamus, and cerebral cortex, all the AD affected areas.[390] AuNPs containing polyoxometalates (POMs) (Aβ inhibitors) and the LPFFD peptide (~22 nm and ~-37 mV) were also able to reach the brain parenchyma of mice. This formulation was capable of inhibiting the aggregation of Aβ, promoting Aβ fibril dissociation, and synergistically reducing Aβ mediated peroxidase activity by combining the Au ability to cross the BBB and inhibit aggregation, with activity as a β-sheet inhibitor.[527] Another interesting characteristic of AuNPs is their ability to destroy Aβ aggregates when excited by microwave/NIR irradiation.[511, 528] It was shown *in-vitro* that penetratin (Pen) peptide-loaded PEG-stabilized AuNS modified with a ruthenium complex (luminescent probe), Ru–Pen–PEG–AuNS (~105 nm and ~+6 mV), also ameliorated Aβ-induced toxicity by increasing the dissociation of the protein aggregates when exposed to NIR irradiation. This formulation was then tested *in-vivo.* Ru–Pen–PEG–AuNS were found in the mouse brain at 12 h and 24 h after i.v. administration,[529] suggesting both therapeutic and imaging potential. Other studies have reported the potential of peptide-conjugated GNSs to reduce Aβ-induced toxicity.[530, 531] For example, an AuNP-capped mesoporous SiO_2 formulation loaded with the metal chelator clioquinol (MSN–AuNPs–CQ), designed to be responsive to H_2O_2, was shown to be efficient in inhibiting Cu^{2+}-Aβ aggregation and crossing a model of the BBB *in-vitro*. The MSN–AuNPs–CQ (~50 nm and ~+21 mV) selectively released clioquinol in the presence of H_2O_2 (*e.g.*, Cu^{2+}-Aβ self-assembled aggregation) due to the blockage of the MSN pores by the AuNPs through boronic ester bonds, which decreased off-target toxicity. Furthermore, pre-treatment of Aβ aggregates with this formulation decreased microtubular defects, lowered ROS, and reduced apoptosis of neurons *in-vitro* caused by Cu^{2+}-Aβ aggregation.[532] Li and collaborators reported that formulations based on peptide–AuNRs (~33 x 8.6 nm; ~-22 mV) not only had the potential to cross the BBB but could also reduce Aβ deposits, since they combined NIR excitable AuNRs with the therapeutic potential of two Aβ inhibitors (Aβ(15–20) and POMs) to break down the Aβ aggregates, both in standard buffer and in the rodent cerebrospinal fluid.[530] Concave cubic quercetin polysorbate 80 coated gold-palladium core-shell structures (Qu–P-80–AuPd (62 nm, ~-10 mV) were able to improve autophagy and decrease both Aβ levels and toxicity in cells, showing the potential of combined drugs such as quercetin ligands and gold structures.[533] Another study used PEG–AuNRs coated with two different peptides, one that recognized Aβ aggregates and the other to improve delivery to the brain (~97 x 11 nm; -10 mV). They demonstrated its ability to lower Aβ-induced toxicity *in-vitro* and also in a *Caenorhabditis elegans* model of AD.[534] Interestingly, Ali and colleagues showed that anthocyanin-loaded PEG–AuNPs (~135 nm and -11 mV) enhanced the protective effects of natural anthocyanins in a mouse model of AD caused by the intracerebral administration of Aβ(1–42).[535] I.v. administration of these formulations to an AD mouse model ameliorated memory loss, improved synaptic dysfunction, reduced Aβ(1–42)-induced apoptosis, and decreased neuroinflammation by targeting the NF-kB/JNK/GSK3β signaling pathway, thereby suggesting the therapeutic potential for AD.[535, 536] Moreover, it reduced NF-κB and IL-1β, which was also shown in focal brain injury for sizes of 20–45 nm AuNPs.[259, 537] In a similar way, AuNPs (20 nm) administered IP to an okadaic acid mice model of AD (characterized by the presence of hyperphosphorylated τ, neuroinflammation, and oxidative stress) resulted in improvement in the AD-mouse behavioral deficits by preventing oxidative stress, improving mitochondrial function, lowering inflammation, and reducing τ phosphorylation.[538] Vimal and colleagues also reported the ability of pegylated AuNPs (~30 nm) to reduce the symptomatology in a τ-P301L transgenic mice model by inhibiting τ oligomerization.[539]

Recently, Shiu and collaborators developed a novel electrochemical assay to detect τ-381 (an early marker of AD). An anti-τ Ab and an aptamer were used to recognize the τ-381 molecule, while cysteamine-stabilized AuNPs promoted the signal amplification.[540] This highly sensitive, specific, and rapid assay may be promising for AD diagnosis. Overall, it can be concluded that

GNS are promising for developing treatments against AD. Nevertheless, considering the high versatility of the formulations, a combination of drugs with functionalizing and targeting agents that improve the spatial and temporal delivery, combined with optical activation of the systems will provide the best results with minimal off-target effects. Table 8 and Fig. 28 provide more information regarding the studies mentioned above.

Table 8 Summary of therapeutic approaches and main conclusions in pre-clinical studies using Au-based strategies for therapy and diagnosis of AD

Core agent	**Ligand(s)**	**Particle size (nm)**	**Zeta potential (mV)**	**Remarks**	**Refs**
AuNPs	N.A.	20	-30	Prevented oxidative damage and neuroinflammation, reduce memory deficits.	259
AuNPs	CLPFFD peptide (β-breaker) THR peptide (anti-TfR)	37	-41	Promote disintegration of Aβ aggregates by NIR; able to cross the BBB after i.v. administration.	512, 517
AuNPs	N.A.	30	-38	Inhibit Aβ fibrillization	524
AuNPs	Curcumin	10–25	+25–+30	Inhibit Aβ fibrillization; break down amyloid fibrils	525
AuNPs	Polyelectrolyte coating (PAH & PSS) HSA	115	+21.5	PAH/PSS AuNPs prevent the formation of misfolded protein aggregates and cross the BBB after i.v. administration.	390, 526
AuNPs	POMs (Aβ inhibitors) LPFFD peptide (β-breaker)	22	-37	Inhibit Aβ aggregation, promote Aβ fibrils dissociation, and reduce Aβ mediated activity of the peroxidase, cross the BBB after i.v. administration	527
AuNS	Pen peptide PEG Ruthenium complex	105	+6	Reduce Aβ-induced toxicity by NIR; accumulate in the brain parenchyma after i.v. injection	529
AuNR	Aβ inhibitors (Aβ(15–20) and POM)	33 x 8.6	-22	Inhibit Aβ aggregation and promote Aβ degradation by NIR; sensitive in detecting Aβ aggregates	530
AuNPs & MSN	Clioquinol (chelator) H_2O_2-sensitive	50	+21	Inhibit Cu^{2+}-Aβ self-assemble and decrease ROS-induced apoptosis; low off-target effects; cross the BBB *in-vitro*.	532
AuNR	PEG D1 peptide (anti-Aβ aggregates) Ang	97 x 11	-10	Decrease Aβ-induced toxicity	534
AuNPs	Anthocyanin PEG	135	-11	Reduce Aβ(1–42)-induced apoptosis, decrease inflammation, improve synaptic function, and ameliorate memory deficits.	535, 536

AuNPs	N.A.	20	N.A.	Reduces τ phosphorilarion, improves mitochondrial function, reduces neuroinflammation and improves mice AD-caused behavior deficits	[538]
AuNPs	PEG	30	N.A.	Reduces τ oligomerization improving the learning ability of τ-P301L transgenic mice after IP administritation	[539]

Aβ, amyloid-beta; AuNPs, gold nanoparticles; AuNRs, gold nanorods; AuNSs, gold nanostars; BBB, blood-brain barrier; HSA, human serum albumin; IP, intraperitoneal; i.v., intravenous; MSNs, mesoporous SiO_2 nanoparticles; NIR, near-infrared irradiation; Pen, Penetratin; PAH, poly(allylamine) hydrochloride; PEG, poly (ethylene glycol); POMs, polyoxometalates; PSS, polystyrene sulfonate; ROS, reactive oxygen species; THR, THRPPMWSPVWP peptide; TfR, transferrin receptor

GNSs are also being studied as novel platforms to treat brain tumors. GNSs can enhance the contrast of imaging techniques, such as PAT, acting as diagnostic agents. Indeed, PEG–AuNCs [540] or PEGylated mesoscopic HAuNSs[375] improved imaging contrast, allowing a more detailed visualization of the brain vasculature *in-vivo*. Frigell and collaborators developed a glucose-coated ^{68}Ga–AuNP preparation (~3 nm) functionalized with peptides to improve BBB penetration and chelate the positron-emitting isotope ^{68}Ga for PET imaging studies. Biodistribution of these NPs after i.v. administration in rats showed a 3-fold increase in brain uptake compared with control NPs.[541] AuNPs modified with a TAT peptide (5 nm) efficiently delivered DOX and Gd^{3+} (MRI contrast agent) into the intracranial tumors of mice when injected systemically. A single dose of this pH-sensitive formulation significantly improved glioma-bearing mouse survival (compared with DOX alone) while allowing the visualization of the tumor by MRI.[379] These are good examples of the potential of Au structures for theragnostic approaches.

Therapeutic strategies based on the gene silencing of oncoproteins could also be valuable for cancer treatment. For example, i.v. delivery of PEG–AuNPs (-33.5 ± 1.3 mV and 33.6 ± 0.2 nm) covalently linked to siRNA duplexes against B-cell lymphoma-2-like 12 protein (Bcl2L12, an overexpressed oncoprotein in glioma) were shown to be effective in crossing the BBB and accumulating in the tumor in a GBM mouse model. This formulation decreased the levels of both Bcl2L12 mRNA and protein, and increased mouse survival rates.[542] In another study, peptide-conjugated gold-liposomes structures were used to evaluate the therapeutic potential in GBM cells *in-vitro* (U87 GBM and GL261 cell lines). The authors used spherical nucleic acid particles composed of single-stranded oligonucleotide miRNA inhibitors (ss OMIs), attached to PEG-AuNPs and conjugated to a liposome-peptide preparation (cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphocholine DOPC, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine DSPE-PEG). The peptides were derived from apolipoprotein E (ApoE) or RVG peptide and formed SNA-liposome-ApoE (~41 nm, -2.5 mV) and SNA-liposome-RVG (~27 nm, -8.5 mV). Using an oligonucleotide against miRNA-92b (aberrantly expressed in GBM), the authors demonstrated that these formulations were able to efficiently reduce miRNA-92b levels, as well as cell viability. Interestingly, the authors also showed that both formulations, but especially the ApoE-based formulation, were capable of crossing the BBB and accumulating in the tumor in brain tumor-bearing mice.[543] These results highlighted the potential of miRNA-based therapies and the use of Au-based strategies for efficient delivery.

Some studies have focused on improving drug delivery carriers to reduce off-target side effects, while others have combined different anti-cancer strategies to enhance the treatment efficacy. A pH-sensitive formulation composed of AuNPs coated with TAT peptide plus DOX improved the

cytotoxicity of DOX in a breast cancer cell line derived from brain metastasis. This formulation was also able to penetrate the tumor *in-vivo* when i.v. injected into mice crossing the BBB. Moreover, the intra-tumoral delivery of the formulation significantly improved the survival rate of these animals.[544] In another study, pH-sensitive PEG–AuNPs (10–15 nm) coated with folate, Trf Ab, and curcumin (an anti-cancer agent) improved the cancer-cell specificity in co-cultures of healthy and tumor cells. The curcumin cytotoxicity combined with the NIR photothermal properties of the AuNPs resulted in a selective and significant reduction of tumor cell colonies.[545] Gonçalves and colleagues developed an AuNR formulation functionalized with PEG-SH and Nes-peptide (1:1 ratio; NesPEG–AuNR). Nes-peptide is a fluorescent peptide that explicitly recognizes Nes, a marker of glioma cancer stem cells (CSCs) resistant to radiotherapy (RT) and chemotherapy. NesPEG–AuNRs (size: 28 nm x 9 nm) showed high selectivity for Nes-positive CSCs and, in combination with photothermal treatment, promoted apoptosis in 2D and 3D cell cultures. The combination was less prone to inducing CSC resistance (~1.5 fold less) when compared to DOX.[546] Another study developed a pH-sensitive nanochemotherapeutic system based on DOX-loaded tetrasodium salt of meso-tetrakis(4-sulfonatophenyl)porphyrin (TPPS)-modified AuNPs (DOX–TPPS–AuNPs; ~26 nm and -33.4 mV). TPPS is a porphyrin compound used in photothermal and PDT and also facilitated DOX loading through H-bond formation or π–π interactions. This formulation was more efficient than free DOX in inducing glioma cell death, decreasing cell migration, and formation of microtubules. It also showed a lower cytotoxic effect on normal non-cancer cells.[547] Interestingly, Liu *et al*. showed the synergistic effect of GNSs, NIR PTT, and immunotherapy. The authors injected i.v. PEG-AuNSs (~12 nm) into mice with GBM tumors (cells injected in the leg), which accumulated in the tumor due to the EPR effect followed by laser irradiation to cause tumor death by heat generation, followed by an antitumor immune response, which provided long-term memory to the immune system and resulted in the long-term survival of mice even after reinjection of cancer cells into the same mice.[548]

Systemic administration of AuNPs (11.2 ± 8.6 nm) in a mouse model of aggressive glioma could cross the BBB and tend to accumulate within the tumor, allowing imaging by micro-CT. Moreover, the combination of AuNP treatment with RT increased the mouse survival rate from 10%–50% compared to RT alone.[394] Joh and colleagues showed that PEG–AuNPs (12 nm) also accumulated in brain tumors in a mouse model of glioma after i.v. administration. Interestingly, the accumulation of NPs in the tumor was not only enhanced by the tumor-mediated BBB breakdown but was also potentiated by RT. Moreover, PEG–AuNPs enhanced RT efficacy, increased DNA damage, and reduced tumor growth and animal burden.[445] In fact, i.v. administration of AuNPs seems to be more efficient in enhancing the efficacy of RT against glioma than a local infusion of AuNPs.[549] A preparation of AuNPs conjugated to cisplatin (Cis anti-cancer drug; ~50 nm) were successfully internalized by cells derived from a GBM patient. The anti-tumor effect triggered by Cis was potentiated by RT, culminating in significant tumor cell death *in-vitro*.[55] Compared with free curcumin (anti-cancer drug), nanocomposites of Au–IONP-containing lipoic acid-curcumin and functionalized with GSH were also shown to improve uptake and toxicity of the drug *in-vitro*; furthermore, this preparation could act as an MRI contrast agent.[550]

Ruan and colleagues developed an AuNP preparation containing Ala–Ala–Asn Cys–Lys (AuNPs–AK) and 2-cyano-6-aminobenzothiazole (AuNPs–CABT or AuNPs–A&C). This nanoplatform delivered the chemotherapeutic agent DOX (AuNPs–DOX–A&C) in a pH-sensitive manner. AuNPs–DOX–A&C aggregated in the presence of legumain (size increase of approximately 35–310 nm) *in-vitro*. This strategy resulted in higher retention of AuNPs-DOX-A&C in the glioma tumors of mice after i.v. administration, which was probably caused by blockage of both exocytosis and backflow to the bloodstream. This treatment resulted in a much higher survival rate (over 250%) compared to non-treated animals.[551] Similarly, Gao and collaborators developed a pair of pH-sensitive AuNPs that once in the acidic tumor environment aggregated together by click chemistry, leading to the enhancement of both MRI and SERS signals. The

nanoprobes had a metallic core of Au (20 nm); an intermediate layer of paramagnetic chelators, Raman reporters, an azide or an alkyne moiety; and a shielding outer layer composed of a PEG coating modified with Ang peptide to improve BBB passage via transcytosis by low-density lipoprotein-receptor-related protein-1 receptors. When injected into the tail vein of glioma-bearing mice, the pH-sensitive nanoprobe allowed precise demarcation of the tumor margin.[378] Altogether, these studies demonstrate the importance of gold-based strategies in brain tumor treatment. Contrary to AD discussed above, there does not seem to be a direct effect of gold itself in tumor treatment. However, the physicochemical characteristics of gold make it one of the best options as a delivery agent (with or without functionalization and for a variety of therapeutic agents, *e.g*. siRNA and chemotherapeutic drugs), as a fundamental part of thermal-based therapies as well as in the imaging of tumors and its diagnostics. The platforms mentioned above are summarized in Table 9 and illustrated in Fig. 29.

Table 9 Description of the therapeutic approaches and main conclusions obtained in pre-clinical studies using Au-based strategies to improve brain tumors therapy and diagnosis

Core agent	**Ligand(s)**	**Particle size (nm)**	**Zeta potential (mV)**	**Remarks**	**Refs**
AuNPs	MUA (linker) Cis (anti-cancer drug)	50	-30	Complete ablation of GBM cells derived from patients.	55
AuNCs	PEG Ag	Outer: 50 Inner: 42 Wall thickness: 4	N.A.	Potential NIR contrast agents; provide enhanced contrast allowing detailed vascular imaging.	138
HAuNSs	PEG	Outer: 40–50 Shell thickness: 2–4	N.A.	Improve blood vessel clarity and detail in PAT imaging.	375
AuNSps	DTPA IR783B (Raman reporter) Azide OR alkyne moieties PEG Ang pH-sensitive	Monodisperse: 25 Aggregates: 40–90	-16	High accumulation in the brain tumor after i.v. administration in mice with GBM xenografts; allow imaging of the tumor by MRI/SERRS.	378
AuNPs	TAT peptide DOX Gd^{3+} DTPA (Gd^{3+} chelator) pH-sensitive	5	N.A.	Able to cross the BBB after i.v. administration; increase the survival rate in mice with intracranial glioma xenografts compared with DOX alone; increased retention time of Gd^{3+} improving brain tumor imaging by MRI.	379
AuNPs	N.A.	12	N.A.	Allow the visualization of brain tumors by micro-CT *in-vivo*; the radiation of AuNPs improves the tumor-free survival rate compared with RT alone.	394

AuNPs	PEG	12	N.A.	Tumor accumulation is augmented by RT-induced BBB disruption, a combination of RT with i.v. administration of AuNPs increases the survival of mice with GBM tumors.	445
AuNPs	PKKKRKV peptide (improve cell uptake) Cis	8–9	+15	Inhibit tumor growth *in-vitro* and *in-vivo*; conjugation with RT synergistically improves the accumulation and apoptosis of cancer cells in the rodents' brain.	474
AuNR	SiO_2 PEG RVG 29	120 x 50	+14	The present hyperthermal effect after radiation by the NIR; improved BBB passage and reduced the tumor size when activated photothermically *in-vivo*.	497
AuNPs	NOTA (^{68}Ga chelator) ^{68}Ga Glucose Leu-enkephalin (Enk) peptide	2–3	N.A.	Allow biodistribution studies *in-vivo* through positron emission tomography imaging; targeted AuNPs increased brain uptake after i.v. administration.	541
AuNPs	PEG iRNA duplexes (against Bcl2L12)	34	-33	Enhance BBB penetration and accumulation in the tumor after i.v. injections; decrease Bcl2L12 expression, increase tumor apoptosis, reduce tumor progression and burden.	542
AuNPs	ss OMIs PEG Cholesterol, DOPC, DSPE-PEG ApoE OR RVG	41 OR 27	-2.5 OR -8.5	*In-vitro*: reduces miRNA-92b levels in GBM tumor cells and their viability; *In-vivo*: able to cross the BBB and accumulate in the tumor after i.v. administration; ApoE-based formulation accumulated in the tumor in higher amounts than the RVG-based formulation	543
AuNPs	PEG TAT peptide DOX pH-sensitive	23	N.A.	Able to reach the brain parenchyma and accumulate in tumors after i.v. administration; local administration improves the survival of mice with brain tumor xenografts.	544
AuNPs	PEG Folate Trf Ab Curcin (an anti-cancer agent)	22	N.A.	Disrupt tumor colonies due to a combined effect of curcin and gold NIR photothermal properties.	545
AuNR	Nes-peptide (recognizes Nes proteins) PEG	28 x 9	N.A.	Formulation and PTT promote apoptosis in a selective fashion of glioma stem cells with minimal off-target effects *in-vitro*; decrease CSC	546

				resistance to treatment.	
AuNPs	TPPS DOX pH-sensitive	26	-34.3	Reduce the level of DOX needed to induce apoptosis of the cancer cells; decrease migration, aggressiveness, and drug efflux of the cancer cells.	[547]
AuNSs	PEG	12	N.A.	In combination with photothermal immunotherapy results in the long-term survival of mice even after rechallenging with a second cancer cell injection.	[548]
Au–IONPs	Lipoic acid–curcumin (anti-cancer drug); GSH pH-sensitive	40	-16	The formulation is used as a contrast agent for MRI; it presents higher toxicity to tumor cells than curcumin alone.	[550]
AuNPs	Ala–Ala–Asn Cys–Lys peptide OR 2-cyano-6-aminobenzothiazole DOX pH-sensitive	Monodisperse: 35 Aggregates: 310	N.A.	Able to accumulate in the brain tumor and to allow its PA imaging after i.v. injection; improve the survival of mice with glioma when compared with free DOX.	[551]

Ab, antibody; Ang, angiopep-2; ApoE, apolipoprotein E; AuNCs, gold nanocages; AuNPs, gold nanoparticles; AuNRs, gold nanorods; AuNSps, gold nanospheres; AuNSs, gold nanostars; Bcl2L12, B-cell lymphoma 2 like 12 proteins; BBB, blood-brain barrier; CSCs, cancer stem cells; Cis, cisplatin; CT, computed tomography; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOX, doxorubicin; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DTPA, diethylenetriaminepentaacetic acid; Enk, enkephalin; GBM, glioblastoma; GSH, glutathione; HAuNSs, hollow gold nanospheres; i.v., intravenous; iRNA, interference RNA; IONPs, iron oxide Nanoparticles; MRI, magnetic resonance imaging; MUA, mercaptoundecanoic acid; miRNA, microRNA; NIR, near-infrared irradiation; Nes-peptide, Nestin peptide (sequence = NH2-AQYLNPSCEKEKEKEPPPPC(S-tertbutyl)G-OH); NOTA, 1,4,7-triazacyclononane-1,4,7-triacetic acid; ss OMIs, single stranded oligonucleotide miRNA inhibitors; PA, photoacoustic; PAT, photoacoustic tomography; PTT, photothermal therapy; PEG, poly (ethylene glycol); RVG, rabies virus glycoprotein; RT, radiotherapy; SiO_2, silica; SERRS, surface-enhanced resonance Raman spectroscopy; TPPS, tetrasodium salt of meso-tetrakis(4-sulfonatophenyl)porphyrin; TAT, transactivator of transcription; Trf, transferrin

Other strategies using GNSs have also been developed to combat other neurodegenerative diseases. For instance, magnetic AuNPs (~35 nm and +16 mV) were used to promote the direct reprogramming of dopaminergic neurons, previously transduced with reprogramming factors (Ascl1, Pitx3, Lmx1a, and Nurr1), by applying a defined electromagnetic field (2×10^{-3} T/100 Hz) both *in-vitro* and *in-vivo*. This platform generated induced dopaminergic neurons similar to those in the midbrain due to the activation of a chromatin-remodeling program, which increased the transcription of neuron-specific genes. Moreover, the striatal administration of AuNPs and electromagnetic field stimulation in two different mouse models of PD led to the amelioration of the PD-induced motor symptoms.[552] Delivery of pDNA encoding an shRNA against SNCA (α-Syn RNA; a hallmark of PD) using AuNPs covalently bound to thiolated CTS and functionalized with nerve growth factor (CTS–AuNP–pDNA–NGF; ~10 nm and ~-40 mV) were shown to be a possible treatment for PD. The CTS–AuNP–pDNA–NGF formulation delivered pDNA within the cells, which reduced SNCA expression, as protected against the cell death of PC12 cells treated with MPP^+ (1-methyl-4-phenylpyridinium; a neurotoxin used to induce PD).

Similarly, IP injection of CTS–AuNP–pDNA–NGF into mice challenged with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; another PD mouse model) efficiently reached the brain parenchyma leading to a significant reduction of both SNCA expression and dopaminergic cell loss in the substantia nigra, and improved the neurological performance of the mice.[553]

Regarding the possible therapeutic applications of GNSs in brain infections, one report showed that IP injection of AuNPs (~10–15 nm) into mice suffering from schistosomiasis (*Schistosoma mansoni* brain infection) was effective in reducing oxidative damage, restoring gene expression, and increasing norepinephrine and dopamine levels.[554] In another mouse model of brain infection, vaccination of pregnant mice with AuNPs-loaded with either a listeriolysin peptide or a glyceraldehyde-3-phosphate dehydrogenase peptide was able to protect mouse pups from developing brain infections with *Listeria monocytogenes,* and protect them from cutaneous damage caused by infection of pregnant female mice with the bacteria.[555] These studies highlighted the broad therapeutic potential of gold structures' for brain diseases of neurodegenerative or infectious origin.

8.2 Gold-based strategies in clinical trials and perspectives

The development of nanomedicine approaches to improve diagnosis and therapy for neurological diseases has led to a revolution in the biomedical field, and has brought hope for the future development of more successful drugs. Efforts have been made to develop more efficient and safer gold platforms for diagnosis, therapy, and imaging of neurological disorders. Nevertheless, delivery of AuNP-loaded drugs to the brain at sufficient concentrations with minimal peripheral accumulation and low toxicity remains a significant challenge, hampering their successful translation into the clinic. Neurotoxicity is a transversal issue in the field of nanodelivery into the brain. Nanoparticles-based neurotoxicity may be due to a combination of different factors, including the route of administration, size, zeta potential, shape, the inherent material toxicity, among others.[556] Evidence showed that Au-based formulations alone or in combination with other drugs regulate inflammation, including promoting microglia (brain-resident immune cells) anti-inflammatory state.[557] However, other studies show increased astrogliosis in the presence of AuNPs[558], although PEGylated AuNPs induce a transient activation of astrocytes[559]. Toxicity related to brain electrical balance (seizure) and cognition has also been reported for gold-based carriers.[558] Other types of nanocarriers, including carbon-based alternatives (e.g. CNTs, graphene, fullerenes), organic nanocarriers (e.g. polymeric NPs, liposomes, dendrimers, etc), or other inorganic materials (e.g. silver, silica, among others), should be considered possible alternatives and/or co-adjuvants to guarantee beneficial therapeutic and/or diagnostic outcome accordingly to the intended application.[556]

Therefore, it is essential to carefully design the gold-based platform and the type of administration considering its application/pathology. For example, for therapies against brain tumors, the design of stimulus-responsive platforms triggered only after reaching the brain parenchyma, more specifically in the tumor area, are essential to improve efficacy and avoid peripheral toxicity. These can also be eliminated more easily to minimize peripheral toxicity.[560] On the other hand, GNS's ability to modulate peripheral inflammation might by itself be beneficial. It was shown that the i.v. administration of citrate-covered gold nanoparticles (cit-AuNP; 20 nm and –26.1 mV) in female mice with sepsis contributes to a reduction of circulating platelets and leukocytes in the blood-brain barrier vessels culminating in lower disruption of the BBB and reduction of brain parenchyma inflammation. Therefore, this might be a potential adjuvant therapy to treat sepsis-induced cerebral inflammation.[561]

Gold-derived structures can be employed to synergistically increase the efficacy of other therapeutic approaches such as chemotherapy or RT, or even be used as therapeutic agents themselves (*e.g.*, for AD,[533, 562] PD,[49, 563] and prion-like disorders[564]). Although many more

optimization studies are still needed, there are already a few clinical trials using gold-based strategies to treat tumors. A spherical nucleic acid (SNA) against the Bcl2L12 gene coupled to AuNPs (NCT03020017) is currently in phase I clinical trial to test their ability to cross the BBB and accumulate inside brain tumors. This therapy is intended to be used against gliosarcoma and recurrent GBM in patients.[565] Another clinical trial (NCT00356980) that has already completed phase I was based on the i.v. administration of a gold formulation based on AuNPs (27 nm) bound to recombinant human tumor necrosis factor-alpha (rhTNF-α) and PEGylated, to cancer patients with advanced solid malignancies of various organs.[566] These data reinforce the potential of gold-based structures for therapeutic applications in neurodegenerative diseases and certain types of brain cancer.

9. GNS mediated stimulation of neurons

9.1 Neural stimulation

Luigi Galvani's pioneering work in the 18th-century introduced the world to the field of bioelectricity, the notion that the muscles and nerves of the human body can be "stimulated" using electricity.[567] Since then, the work of Nobel Prize winners Hodgkin and Huxley, and many others have elucidated the mechanisms by which neurons can be electrically activated.[568, 569] Neurons are responsive to electricity because they contain membrane-bound proteins that change configuration when a difference in electric potential is applied across their membrane.[570] When these proteins (such as voltage-activated ion channels) are activated, they open to allow the flow of ions into or out of the cell. If positive ions, such as sodium cations, flow in, the cell becomes depolarized or more positively charged inside relative to the outside. Once a certain voltage differential threshold is reached, a large positive potential spike in the neuron membrane occurs and is propagated along the axon, known as an action potential. The action potential is the binary language of the nervous system. Selectively evoking action potentials using electrical stimulation enables modulation of the brain's neuronal circuits that dictate behavior and can treat diseases.

Electrical stimulation of the brain and peripheral nervous system has progressed to the clinic where it is used to treat PD,[571] chronic pain,[572] and return hearing to the deaf[573, 574] and sight to the blind[575-577]. However, the clinical implementation of electrical stimulation comes with several challenges, including the need to implant a large foreign object (*i.e.*, an electrode array) into delicate neural tissue and maintain it there long-term. For example, deep brain stimulation (DBS) systems used to control PD require drilling a large (14 mm diameter) burr hole into the skull followed by the permanent implantation of a metal/silicone electrode array approximately 1.27 mm in diameter.[578] Furthermore, repeated electrical stimulation requires large amounts of energy, particularly if the electrode-tissue interface has a large impedance.[579] Other concerns include the safety of a large electrode (> 100,000 μm^2) such as a DBS electrode and the mechanical stability of a microelectrode (<10,000 μm^2).[580] The correct balance between the two electrode sizes is an active area of research, and one proposed solution is to boost the effective electrochemical surface area of microelectrodes. If the stability and safety of microelectrodes can be improved and the tissue volume can be reduced, the overall physical disruption and damage to the tissue could be minimized.

For applications such as an implanted retinal prosthesis, the minimum area of activated tissue is hypothesized to correspond with the spatial resolution of the visual image delivered to the user.[581] One novel approach for improving the selectivity of neuronal modulation is via optogenetics, in which excitable cells can be made light-sensitive following viral transfection of channelrhodopsin.[582] However, the use of viral vectors may delay the regulatory approval of such an approach for human use.[583] In this portion of the review, we will describe how GNS

electrode surfaces or freestanding AuNPs might address some of the issues related to the improvement of implant safety and efficacy for neural modulation.

9.2 GNS-based surfaces as neural interfaces

GNS surfaces are an active area of research and have been shown to improve electrode properties for biological applications.[399, 412, 584-587] Generally speaking, nanostructured surfaces are rougher than native surfaces, which means a higher real surface area, greater capacitance, and reduced interfacial impedance as described by equation 1:

$$\text{eq.1} \qquad Z = \frac{1}{i\omega C + \frac{1}{R}}$$

where Z is the interfacial impedance between the electrode and the tissue, i is the imaginary unit, ω is the frequency, C is the capacitance, and R is the electrode's resistance.[363] Similarly, increasing the electrode capacitance can dramatically improve the signal-to-noise ratio (SNR) of neural recording.[588] Furthermore, nanostructured electrodes could reduce the power required to stimulate the neurons and make possible microscale electrode geometries, minimizing the neural tissue disruption.[589] It should be noted that Au is not generally used for neural stimulation due to its narrow potential window, and the possibility to generate toxic by-products during electrical stimulation protocols if certain precautions are not taken.[590] However, given the recent successes in biosensing and *in-vitro* cell studies using GNSs, more research into neurostimulation applications is justified. As described above, one such approach is to increase the electrochemical surface area via micro- or nanostructuring of the electrode material. Generally, nanoporous gold (np-Au) surfaces are prepared by a selective de-alloying procedure, starting with either Zn–Au[591] or Ag–Au[592, 593]. Another approach involves the spontaneous galvanic replacement/displacement reactions between Ag and $AuCl_4$ to create AuNPs and GNS surfaces.[594] Seker *et al*. used a photolithographic process, followed by selective chemical etching, to create an np-Au multi-electrode array for enhanced neural recording.[593] Kim *et al*. demonstrated an np-Au electrode comparable to that from Seker's group that exhibited charge injection limits equal to both roughened platinum and CNT electrodes.[595] Electrodes based on np-Au have been created to improve the electrochemical surface area and tune cell adhesion. A few examples of np-GNSs surfaces are described in Fig. 30.[593, 595, 596]

Fig. 30 here

Alternatively, AuNPs can be electrodeposited or adsorbed onto a planar metal electrode to enhance both the electrochemical capacitance and biosensing capability. Normally, the adsorption process is achieved using LbL processing, the alternating dip-coating of positively and negatively charged particles, and is more successful when denser materials are utilized.[597] Zhang *et al*. used the LbL deposition of AuNPs to reduce the interfacial impedance by 3-fold and boost the charge injection capacity by one order of magnitude.[56] Tsai *et al*. attached AuNPs to platinum surfaces using electrodeposition to improve the electrochemical detection of dopamine, an important neurochemical involved in PD and addiction.[598] Another exciting approach involves the addition of AuNPs to conductive polymers to improve the electrochemical properties for neural stimulation, recording, and even electrochemical detection of biomolecules such as glucose.[596, 599]

In addition to electrochemical enhancement, the nanostructure of materials can dramatically impact how neurons interact with them. For example, Brunetti *et al*. observed a decrease in the adhesion and survival of a neuroblastoma cell line (SH-SY5Y) when the surface roughness increased from 0.46 nm (50 nm Au film) to 99.8 nm. Spontaneous galvanic displacement reactions increased the roughness.[600] Furthermore, Brüggemann *et al*. created Au nanopillar

arrays with 100 nm diameter and reported good adhesion and survival of cardiac muscle cells, but poor adhesion and survival of primary rat neurons.[601] Using the Au–Ag de-alloying approach, Chapman's group fabricated np-Au surfaces to decrease the extent of astrocyte growth by 50–60% without any decrease in the neuronal attachment in cortical neuron-astrocyte co-cultures.[363] This finding was significant because electrodes implanted into the brain typically attract astrocytes to grow on them, which creates a distance between the electrode and the neurons, and thus diminishing the recording channel efficiency.[602] It is evident that by careful tuning of the electrode nanostructure, both the electrochemical properties and *in-vitro* neuron growth can be enhanced, leading in some cases to a more robust neural interface. However, it remains to be seen how these novel materials will either survive or be tolerated in tissue *in-vivo* for more extended periods.

9.3 GNSs for remote, wire-free neural stimulation

An appealing alternative to direct electrical stimulation of neurons is the use of light. Light stimulation occurs naturally in the retinal photoreceptors in which a photon is converted into a neural signal via "Wald's Visual Cycle".[603] This principle has been similarly used in the field of optogenetics, in which neurons are made light-sensitive by incorporating an exogenous protein (*e.g.*, channelrhodopsin) into the cell membrane.[604] The use of light without any genetic modification has been used previously as an alternative to electrical stimulation. It offers several advantages, including the absence of electrical stimulation artifacts, eliminating the need for an implanted electrode, and the added benefit that viral vectors are not required.[605] The mechanism by which neurons are stimulated by light is now understood to be a local heating effect caused by IR light absorption by water molecules. The local membrane environment is perturbed, leading to depolarizing capacitive currents.[606] However, direct stimulation using IR light lacks spatial resolution due to both the ubiquity of water in biological systems and the large absorption coefficient of water in the IR region.[607] The use of NIR (650–900 nm) light allows for enhanced specificity and deeper tissue penetration. To further improve the accuracy and efficiency of stimulation, some so-called "photo-absorbers" can be added to the extracellular environment. Specifically, AuNPs have been investigated for this purpose, given their SPR leading to localized light-induced heating.[608, 609] Interestingly, the wavelength of light at which SPR occurs is directly correlated with the size and shape of the GNSs (Fig. 31).[185, 610, 611] Consequently, AuNPs have been studied to improve absorption for specific biological applications.[612]

Fig. 31 here

Yong *et al*. stimulated rat primary auditory neurons using SiO_2–AuNPs as NIR absorbers.[613] AuNPs can be functionalized with biomolecules to target and modulate specific cell types. Selective modulation of retinal neurons (of which there are over 60 distinct types) might significantly improve the visual detail experienced by a retinal prosthesis user.[614] By functionalizing AuNPs with Ts1, a neurotoxin that selectively binds to voltage-gated sodium channels without interfering with the ionic exchange, selective neuronal stimulation was achieved using 532 nm light.[185] So far, only AuNSps and AuNRs have been used to modulate neurons successfully.[185] As comprehensively reviewed by Paviolo *et al*., the promise for GNSs to enable optical neural interfaces is considerable.[204] A summary of studies using GNSs for optical neural stimulation is presented in Table 10.

Table 10 Summary of work investigating the use of GNSs to enable optical neural stimulation

GNSs characteristics	Functionalization	Biological models	Stimulation parameters	Efficacy measure	Refs

AuNP*;* 20 nm	Ts1 binds to voltage-gated Na^+ channels; neuron localization	DRGs	532 nm laser; 0.1–1.9 ms duration; 31 kW cm^{-2} peak power	Patch-clamp recording	185
AuNP; 100 nm	Ab–HA Abs attached via PEG linkers	Hippocampal neuron culture (rat)	800 nm fs-laser; 0.27–1.02 MW cm^{-2} (peak power) with 1.3–6.4 μs pulse time	Ca^{2+} imaging (GCaMP6s) and patch-clamp recording	615
AuNP; 10 nm	TPP for mitochondrial localization	NSC culture (human)	530 nm; 20 Hz; 10% duty cycle; 1 mW	Generation of reactive oxygen species; PCR to study differentiation; patch-clamp recording	616
AuNR*;* 25 × 94 nm	N.A.	DRGs culture (mouse)	785 nm; 5.5 mW (0.1–0.7 ms); 1.6–5 μJ (1 ms)	Patch clamp recording	617
AuNR; 18.5 × 71.3 nm	NH_2–PEG	Hippocampal neuron culture (rat)	785 nm; 0–15 mW mm^{-2}; 10 s on, 20 s off	MEA recording	618
AuNF; 2, 5, 7.5, 10, and 20 nm	N.A.	Hippocampal neuron culture (rat)	785 nm laser; 0–21 mW mm^{-2}; 10 s on, 20 s off	MEA recording	619
AuNS; 10–50 nm	NH_2–PEG–SH	Hippocampal neuron culture (rat)	785 nm laser; 3–15 mW mm^{-2}; 10 s on, 10 s off	MEA recording	620

Ab, antibody; AuNFs, gold nanofilms; AuNPs, gold nanoparticles; AuNRs, gold nanorods; AuNSs, gold nanostars; DRGs, dorsal root ganglion cells; HA, hemagglutinin; MEAs, microelectrode arrays; NSCs, neural stem cells; PEG, poly (ethylene glycol); TPP, triphenylphosphonium

Finally, it is worth noting that AuNPs and AuNRs exhibit absorption in the visible wavelength, which may make them an exciting choice for retinal prostheses or vision replacement technologies. [617]

For the first time, Yoo *et al.* reported inhibition of cultured neural networks using AuNRs combined with 785 nm NIR laser.[618] Since then, the inhibition studies of neural activity using GNSs and light excitation have been extensively developed (Fig. 32).[619-622] Lee *et al*. synthesized NIR light-sensitive biocompatible star-shaped multi-branched AuNPs, and demonstrated inhibition of neural activity (not only neural networks but also single neurons) by photothermal effects from NIR irradiation.[620] Besides, the use of plasmonic gold nanofilm (AuNF) coated microfabricated neural chips have shown that it is possible to modulate neural activity based on photothermal stimulation.[619] Kang *et al*. developed a widely applicable inkjet printing technique to prepare thermo-plasmonic interfaces with biocompatible LbL polyelectrolyte and AuNPs.[621] They demonstrated that the patterned thermo-plasmonic effect from the inkjet-printed AuNRs could selectively modulate neuronal network activities.[621] These studies also showed the photothermal suppression effect could be controlled by changing the NIR laser power density and optimization of the GNSs.

Fig. 32 here

The mechanisms controlling the activation of neural cells through light stimulation of NMs are not yet fully understood. So far, thermosensitive ion channels have been proposed to be involved in the modulation of neural activity by optical stimulation.[618, 623-625] The transient

receptor potential vanilloid 1 (TRPV-1), the thermosensitive potassium channel 1 (TREK-1), and other thermosensitive ion channels present in neurons have been proposed (Fig. 33).[618, 623] Nakatsuji *et al*. introduced novel TRPV-1-targeted phototherapeutic approaches using plasma-membrane targeted AuNRs (pm-AuNRs). The pm-AuNRs achieved photoinduced Ca^{2+} influx in primary cultured dorsal root ganglion cells (DRGs) from wild-type (WT) but not from TRPV-1-knockout (KO) mice. The activities of the illuminated neurons were confirmed by observing the depolarization induced by high K^+ concentrations.[623] Yoo *et al*. hypothesized that the nanoscale heat delivered into the neuronal plasma membrane by photoexcited AuNRs could be responsible for the instant suppression of neural activity mediated by thermosensitive ion channels.[618] They examined the involvement of TREK-1 channels in the suppression. AuNR-mediated light-induced stimulation was carried out both with and without the TREK-1 channel blocker fluoxetine.[618] The suppression of neural activity disappeared when TREK-1 was blocked. These results strongly suggested that TREK-1 ion channels were involved in the photothermal suppression induced by AuNRs and NIR irradiation.[618]

Fig. 33 here

Successful results have demonstrated the signal modulation, differentiation, development, and disease treatment of neurons by applying various fabricated AuNPs combined with photostimulation. Kang *et al*. developed a thermoplasmonic optical fiber technology, with AuNRs attached to the optical fiber for localized neural stimulation. The thermoplasmonic optical fiber could locally modulate *in-vitro* cultured hippocampal neurons.[626] Damnjanovic *et al.* reported developing a hybrid electro-plasmonic activation platform with an AuNP coated nanoelectrode for precise spatio-temporal sensory trigeminal neuron excitation.[627] Qu *et al.* demonstrated that the absorption of circularly polarized light by nanoparticle assemblies accelerated the differentiation of neural stem cells (NSCs) into functional neurons.[628] Duc *et al*. produced a novel biocompatible nanocomposite liquid crystal graphene oxide (LCGO) conjugated with AuNRs for electrical and NIR co-stimulation of neuronal cells. The nanocomposite could support cell adhesion, proliferation, and differentiation and be suitable as an interface for NIR and electrical costimulation of neuronal cells.[629] Ye *et al.* demonstrated that photothermal stimulation could modulate the left stellate ganglion (LSG) function and neural activity, and decrease the occurrence of ventricular arrhythmias (VAs) in a canine model of acute ischemia without any genetic transfection.[630]

9.4 General remarks and perspectives

The techniques for optical stimulation of neurons by GNSs are still only at the starting point. The optimum conditions have not yet been established, and much more research is required to develop this field further. Lee suggested a study to test whether heat shock proteins (HSPs) were induced through the photothermal stimulation of neurons to understand the molecular mechanism. While there are several appealing advantages of NP-enabled optical neural interfaces, it is essential to investigate their safety if they are to be seriously considered for animal or human use. One concern is that a significant research effort has been dedicated to using light excitation of AuNPs to kill cancer cells.[631] The same aforementioned heating mechanisms (*i.e*. SPR) described for neural stimulation are similar to those used to disrupt the cell membranes of cancer cells.[632] Furthermore, it is known that rapid heating of AuNPs can lead to the denaturation of proteins and the production of shockwaves.[633] Using cultures of neuronal cells, Johannsmeier *et al*. irradiated AuNPs (200 nm; 0.5 μg cm^{-2}) with a laser (532 nm Nd: YAG; 20.25 kHz; 17-51 mJ cm^{-2}) to study the stress response in neuronal cell cultures.[634] The findings from this study suggested that light stimulation of AuNPs led to an influx of Ca^{2+}, perhaps through membrane pores. Recently, Eles *et al*. used two-photon microscopy to show that calcium levels increased in response to electrode implantation in the brain.[635] Similarly, both authors drew the same conclusion that damage and pore formation of the neuronal membrane

was responsible for the observed calcium influx. As with any new therapy or device, a long-term *in-vivo* study is required to fully assess the safety and efficacy of these AuNP-enabled optical interfaces.

10. GNSs for neuronal regeneration and survival

Injuries to the nervous system lead to the recruitment of several cell types with different functions; microglia, blood-derived macrophages, and monocytes are responsible for forming scar lesions and phagocytosis of the axonal debris.[636, 637] Following the initial injury phase, Schwann cells migrate and proliferate to repair the extracellular matrix and regenerate the axons.[637] On the other hand, activation of oligodendrocytes and astrocytes hinders the nerve regeneration process by releasing growth inhibitors and proteoglycans. This leads to more macrophages and the formation of glial scars, which also slow down the nerve regeneration process.[636] The CNS healing process is limited in nature and requires supportive 3D neural scaffolds to stimulate and organize new tissue formation.[638] However, tissue regeneration in peripheral nerve injuries is more likely, but insignificant peripheral nerve damage with large gaps between the axons, supportive scaffolds are crucially required.[637] To compensate for the wide neuronal gap in severe injuries, autograft (grafting nerves from a healthy site to the injured site) is an ideal option; however, drawbacks including donor site problems and dysfunctional nerve regeneration limit this technique. Neural tissue engineering has led to the development of polymeric nerve guides and conduits to address these issues.[639] Of note, anisotropic electrical conductivity, and cell/tissue growth support are challenging requirements in the development of neural tissue-engineered substrates, which polymeric structures cannot completely provide.[640] Addition of nanostructures to these substrates can serve as an interface with the neural cells, which can transfer electrical stimuli.[638] The main goal of using nanostructures in neural tissue engineering is to enhance the electrical and structural properties of the substrates. Furthermore, the nanoscale features can affect the cellular morphology and cytoskeleton, especially during regeneration of major nerve damage by creating anchor sites on the substrates for the cells and molecules.[638] AuNSps or AuNRs are ideal choices in neural tissue engineering, due to their easy fabrication techniques, adaptable surface modification, tunable biocompatibility, and adjustable optical properties.[641, 642] AuNPs in neural structures and surfaces can manipulate the interface at the cellular level through modulation of cellular action potential to induce electrical coupling, activating voltage-gated channels, transient receptor channels, and ion receptors.[204]

10.1 AuNPs in neural regenerative medicine

Structural alterations of the scaffolds due to the presence of AuNPs influence their interface with cells and lead to changes in cellular function. Exposure of hESC-derived neural precursor cells to AuNPs with two sizes (20 and 80 nm) and two concentrations (50 and 800 particles/cell) for 14 days demonstrated alterations in cellular functions. The small particles at high concentrations (20 nm and 800 p/c) increased the proliferation of Ki67-expressing cells by roughly 25% compared to both control, and low concentrations of the large size. Furthermore, AuNPs changed the cell morphology to spherical, with their nucleus oriented towards the center and cytoplasm at the cell edge. The actin filaments were more pronounced in the presence of AuNPs compared to control samples.[217] Of note, neural progenitor cells with a high concentration of AuNPs (≥ 50 nM) underwent high oxidative stress, leading to deformed actin filaments, rearranged microtubules, and reduced cellular function.[643] The size of AuNPs affected the growth and morphology of hESCs and their differentiation to neural progenitor cells. Two sizes (4 and 14 nm) showed no changes in cell viability after 24 h, while smaller particles with a size of 1.5 nm led to cell death (~80% decrease in cell viability). Additionally, the AuNPs (1.5 nm) prevented the neural differentiation of hESCs, while 4 and 14 nm particles had no

significant effects on neural differentiation. These results indicate the role of ultrasmall AuNPs on DNA transcription and the generation of intracellular oxidative stress.[644]

Furthermore, higher current intensities were recorded from the more differentiated state of these cells, which may originate from more neurite growth in the differentiated state.[645] Regeneration of retinal tissue can result from the introduction of prosthetic photoreceptors into the interface between the retina and neuronal cells. Titania nanowires (TiO_2NWs) coated with AuNPs showed promising results in retinal ganglion cell regeneration and improved visual resolution in a blind mouse model (Fig. 34C-E).[646] The tissue regeneration led to changes in field potential (photovoltage generation) in response to near UV exposure. Implanting AuNP–TiO_2 in the blind mice induced responses to light in cortical neurons via the photothermal property of AuNPs, which led to neuron stimulation. The vertical orientation of the arrays provided a spatial preference for the local neurons to grow and regenerate the damaged site, and most importantly, to restore the responses to light exposure (Fig. 34F).[646] Efficient spatial resolution is a challenging issue in electrical prosthetics due to off-target electrical stimulation. Using a hybrid electro-plasmonic system of nanoelectrodes coated with AuNPs in the vicinity (2 μm) of the neurons, the targeted spatio-temporal simulation was improved. Upon co-stimulation with an electrical potential and visible light (λ = 532 nm, t = 1 to 5 ms) pulses to trigeminal neurons, the recorded membrane action potentials were enhanced. This platform supported the highly accurate neural modulation required for prosthetics.[627] In another study by Paviolo *et al*., the effect of low power laser exposure to NG108-15 neuronal cells was investigated. They reported that when the cells that had been cultured with poly(4-styrene sulfonic acid) and SiO_2–AuNPs were irradiated with a 780 nm laser, the average number of neurons with neuritis was increased. In a similar study, NG108-15 cells were cultured with both bare and coated AuNPs and then irradiated with 780 nm laser at power densities of 1.2–7.5 W/cm^2. The results indicated that the neurite length increased by up to 25 μm in coated AuNPs versus bare AuNPs as control. They hypothesized that this increase was linked to the absorption of light by the AuNPs and could be used for nerve regeneration.[187]

AuNPs functionalized with the anti-inflammatory compound 6-mercaptopurine (6MP) and RDP (neuron-penetrating peptide) were developed to induce neural regeneration in the SH-SY5Y cell line (human neuroblastoma). Uptake of these NPs by SH-SY5Y cells induced proliferation and neurite outgrowth due to activation of purinergic signaling pathways including MAPK–ERK and PI3K–Akt.[647]

In conclusion, AuNPs are versatile materials to fabricate hybrid scaffolds for neural tissue engineering and regenerative medicine. Increasing the electrical conductivity and mechanical properties of conventional polymeric scaffolds and inducing neural differentiation of the cells seeded on the scaffolds are the most attractive properties of AuNPs in this application. Interestingly, the development of AuNPs-integrated into neural multimodal scaffolds with theragnostic properties may address the complicated demands for neural tissue engineering and regenerative medicine.

10.2 Physical modulation for nerve regeneration

Neurotmesis is a complete break in any peripheral nerve, which causes paralysis in the case of motor nerves.[648] When the injury gap exceeds 5 mm, the axonal connections cannot be entirely regenerated via the physiological healing process. Electrospun nanofiber conduits of silk fibroin and AuNPs provided improved axonal regeneration due to higher electrical conductivity compared to bare silk fibroin, to bridge the damaged site.[639] The compound muscle action potential (CMAP) and the nerve conduction velocity (NCV) are two parameters defining the re-myelination and the status of nerve impulse conduction along the implanted nerve conduit. AuNPs-in-silk conduits pre-seeded with Schwann cells implanted in a rat model demonstrated near normal values of CMAP and NCV over 18 months, confirming re-myelination in the

regenerated axons. Motor unit potential (MUP) in a particular nerve represents the integrity of the motor neurons, motor axons, and muscle. The MUP in AuNPs-in-silk conduits after 18 months was measured to be 133 μV, which is relatively close to the normal value of 152 μV. No NP migration to adjacent tissues was reported with these conduits.[639]

Bioelectrical stimuli play pivotal roles in neurite extension; a process required for nerve regeneration. Electrical pulses can stimulate neurite growth and nerve regeneration. An *in-vitro* study on PC12 cells (rat phaeochromocytoma) cultured on glass coated with AuNPs and PEI, and stimulated by alternating current showed higher cell viability than either non-coated or constant current. The AuNPs enhanced the transmission of pulsatile electrical signals (in this study, 20 Hz for 55 min) to the cells on the polymer-coated glass platform. The AuNP-based platform improved the extracellular matrix conductivity and actuated plasma membrane components through oscillatory changes in charged molecules. Another possible mechanism is the activation of calcium channels by local membrane fluctuations due to the electrical stimuli, which cause the influx of extracellular Ca^{2+} and consequently activation of tyrosine kinases. Furthermore, the release of toxic chemical residues from the structure was reduced due to the partial substitution of AuNPs, which provided higher biocompatibility to the system.[649]

AuNPs modulate cell-substrate interactions and influence cellular functions through electrical conductance between cells, and from substrates to the cells. Glass platforms with a PEI coating can be functionalized with AuNPs for *in-vitro* studies to stimulate neural differentiation. Gold has significantly higher conductivity compared with the electrolytes in cell culture media. Thus, PC12 cells seeded on PEI–AuNP substrate showed an increased level of β-tubulin (neuron-specific cytoskeleton marker) due to elevated electrical conductance (Fig. 34A and B).[650]

Fig. 34 here

Polycaprolactone (PCL)-gelatin scaffolds doped with AuNPs were developed to combine the mechanical properties of PCL with the biological activity of gelatin, and the electrical properties of AuNPs to promote neuronal cell adhesion and ingrowth. This hybrid structure led to elongated cell morphology, spread-out dendrites, and maturation of neurons in the presence of AuNPs.[638] PCL–CTS hybrid scaffolds doped with AuNPs also demonstrated increased Schwann cell proliferation, as these NPs increased the scaffold conductivity.[651] A microporous PLA conduit with a micro-patterned surface stimulated axonal regeneration in the rat sciatic nerve. A combination of CTS and AuNPs can passivate the hydrophobic surface of PLA. The former can bind to glycosaminoglycans due to structural similarities but shows poor mechanical properties. The application of AuNPs combined with CTS enhanced the mechanical properties. CTS–AuNP treated PLA conduits were conjugated with FGF-1 and seeded with NSCs. The constructs were grafted into 15 mm nerve gaps in a rat model to investigate peripheral nerve regeneration efficiency. FGF-1 upregulated the release of neurotrophic factors, and in the presence of guided NSCs, accelerated Schwann cell differentiation and re-innervation of the injury within 12 weeks after transplantation in rats. The measured CMAP and NCV for these bioactive conduits indicated 90% of autograft performance after 6 and 12 weeks.[652] PEG–AuNPs showed improved solubility, colloidal stability, and therapeutic benefits. PEG prevents oxidative stress and reduces the permeability of neuronal membranes due to its membrane sealant property, and restores the CNS function. PEG–AuNPs (40 nm) dispersed in H_2O_2 were injected into a mouse model of SCI using intra-spinal delivery to evaluate the hind limb motor recovery. H_2O_2 induces oxidative stress, which causes cell apoptosis. However, the application of PEG–AuNPs dispersed in H_2O_2 restored cell survival. These particles improved motor neuron protection and remyelination mediated by Schwann cells, which may arise from the ability of PEG–AuNPs to maintain plasma membrane integrity and suppress pro-inflammatory responses.[653] An injury can cause peripheral neuropathy in the peripheral nervous system (PNS) due to the disruption of electrical signaling, which leads to motor and sensory nerve damage. In wide nerve gaps, a

nerve guidance channel, which works as a scaffold to provide neurite outgrowth and axon extension, is vital. A nanocomposite of Au–PCL coated with polydopamine (PDA–Au–PCL) was used to enhance the *in-vitro* and *in-vivo* conduit mechanical and electrical properties compared with bare PDA–PCL. The *in-vitro* studies showed higher proliferation and neural differentiation through upregulation of S100β (Schwann cells specific marker) and Nestin (Nes) expression in cultured bone marrow MSCs (BMSCs), and Schwann cells on the PDA–Au–PCL nanoscaffold. The electrical conductivity of the nanoscaffold was significantly improved (4.66×10^{-3} S cm^{-1}) compared to the bare PDA–PLC nanoscaffold (no conductivity). Implanting the PDA–Au–PCL nanoscaffolds pre-cultured either with BMSCs or Schwann cells in a rat model with sciatic nerve defects improved the SFI and nerve recovery along with thicker myelin sheaths. The impact of AuNPs to improve the functionality of a neural scaffold arises from improved cell-scaffold interactions and higher electrical conductivity.[52] Functionalization of AuNPs with citrate molecules induces a negative surface charge to the NPs; therefore, they can electrostatically bind to positively charged substrates. Development of polyurethane nanofibers (~550 nm) functionalized with PLL and coated with AuNPs (~50 nm) modified PC12 cell neurite outgrowth. Induced conductivity from the AuNPs with electrical stimulation promoted proliferation along the aligned polyurethane nanofiber conduit.[654] A AuNP–Poly(vinylidene fluoride) (PVDF) composite mat was developed as a piezoelectric scaffold with the capacity to foster neural cell adhesion and growth, through facilitating cell-cell signaling interactions. PVDF is a piezoelectric material (possesses deformation-dependent electrical properties) and a synthetic polymer with high biocompatibility. It can form composites with either CNTs or AuNPs to function as a neural scaffold. The superiority of AuNPs over CNTs includes ease of processing, uniform particle dispersion within the scaffold, and higher biocompatibility. PC12 cells cultured on an electrospun AuNP–PVDF scaffold exhibited two important morphological properties: elongation in the direction of the nanofibers, and neurite extension. AuNPs can act as conductive spots within the structure, promoting cell attachment and transferring electrical signals.[655] A hybrid platform of poly($_L$-lactic-co-glycolide) conduit equipped with AuNPs, brain-derived neurotrophic factor, and adipose-derived stem cells in an ALG gel stimulated regeneration of the sciatic nerve in a rat model. Adipose-derived stem cells differentiated into Schwann cells and accelerated the repair and regeneration. AuNPs supported cell adhesion, proliferation and neurite growth, while the controlled release of brain-derived neurotrophic factor promoted neural repair and regeneration.[656]

Microelectrode arrays (MEAs) have been used as *in-vitro* platforms to stimulate neurons and neural tissues and also to record electrical signals. However, the recorded neural signals contain a high level of impedance and noise due to their small size (5–50 μm in diameter). An ideal electrode-neural cell interface would show negligible electrical impedance for perfect coupling and noise reduction.[48, 362] One strategy to enhance the signal-to-noise ratio in these electrodes is a surface modification to increase the electrochemical surface area and lower the impedance. Application of CNTs and GNSs to fabricate electrodes based on CNT–Au nanocomposites improves the biocompatibility, mechanical and chemical stability, and electrical properties of CNTs. This nanocomposite MEA demonstrated an enhanced ability to reduce the noise recorded from the neurons due to its surface properties.[362] Astrocytosis is a process that occurs in response to the activity of glial cells (astrocytes and microglia) in the damaged nervous system. It grows to enclose the electrode, both *in-vitro* and *in-vivo*.[48, 657] This process isolates the electrode and separates it from the neural cells, which results in neuronal apoptosis. The np-Au films offer surface features, including high surface area and electrical conductivity. The np-Au film with an average thickness of ~30 nm and a pore diameter of ~85 nm, has been used as a surface treatment for neuronal interfaces. The co-culture of neurons and astrocytes on this surface revealed a significant decrease in astrocyte activity, with no effect on neuronal coverage. The np-Au topography prevented astrocytes from spreading and altered their cellular morphology leading to detachment (Fig. 34B). Microgrooved CTS–Au nanocomposite conduits were fabricated to promote activation of NSCs and regeneration of axons.

CTS can mimic the extracellular matrix in neural tissue, while AuNPs can increase the conduit's mechanical and electrical properties. To investigate these conduits' regenerative potential, two cell lines, rat glioma C6, and murine NSCs, were cultured on the conduits containing different concentrations of AuNPs. The results demonstrated that there might be two "sweet spots" for the AuNP concentration (25 and 50 ppm) in which pro-regeneration genes, including brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and nerve growth factor, were optimally expressed. The mechanical property of the conduits correlated positively with the concentration of AuNPs. Furthermore, the AuNPs could alter the conduit micro- or nanostructure and regulate cell responses through gene expression alterations. The *in-vivo* study demonstrated that six weeks after the implantation of the conduits in rats, higher SFI values were measured compared to control groups. Quantitative histology revealed *in-vivo* myelination and axon extension occurred in the presence of the CTS–AuNP conduit. Even though the exact mechanism of improvement in neural function caused by AuNPs is not precisely understood, it may originate from the combination of improved electrical conductivity, higher proliferation, and differentiation markers, as well as enhancement of the mechanical properties of the matrix.[658] The physiological extracellular matrix is a micro/nano-patterned substrate, and cells interact with this substrate to regulate their function accordingly. Au nanopillars and nanopores were fabricated to assess the interaction of PC12 cells with the substrate. Bare nanopillars and nanopores showed only limited neurite growth due to the vacancies on the surface, which altered cell morphology;[659] however, the np-Au surface could tune cell adhesion and enhance their function.[659, 660] The choice of architecture, material, and surface properties define the electrical sensitivity of the electrodes. GO with a high electrochemical activity provides excellent electrical sensitivity and conductivity in the biological milieu. The deposition of GO on metal substrates is challenging due to the weak van der Waals intermolecular binding between the GO sheets. Using the electrostatic interaction between the reduced GO sheets with a negative charge and gold substrates (Au^{+}/Au^{3+}) to form nanocomposite electrodes can address this challenge. This nanocomposite electrode showed enhanced sensitivity and biocompatibility due to the combination of GO and gold.[661]
As one example, AuNPs encapsulated in 3D GO shells could enhance the performance of electrical, electrochemical, and SERS detection systems to evaluate the differentiation stage of NSCs.[645] Two molecular bonds of C=C and C-H provide characteristic Raman spectroscopy signals for undifferentiated and differentiated stem cells, respectively. This difference arises from the fact that undifferentiated stem cells are rich in polyunsaturated fatty acids (C=C, 1656 cm^{-1}), while differentiated cells are richer in proteins (C-H, 1470 cm^{-1}).[645] AuNPs provide higher electrical conductivity to the GO and enhance the resultant Raman spectroscopy peaks.

11. Conclusions and future perspectives

The present review has attempted to cover the early stages of what is expected to become a long journey. GNS-based platforms will be developed to provide safer tools for diagnosis, imaging, prevention, and treatment of a wide range of neurological disorders. The leading candidate diseases so far are AD and brain tumors. New mechanistic insights and novel applications are continually being reported, and this field is expected to grow further in the coming years.

11.1 Future challenges in the optimization of neuro-engineering by GNSs-based platforms

Decades of work have led to the production of a great variety of AuNPs with different sizes, shapes, structures, mechanical, optical, and electromagnetic properties. They have multiple applications in nanotechnology, chemistry, sensing, imaging, and biomedicine. Besides, research on developing new tools based on AuNPs is a growing area. Several well-established production methods can be selected based on the properties that are required for the specific application.

The control of the size, shape, and surface chemistry that together determine the overall physicochemical properties is crucial in developing AuNPs for any specific application. These parameters may be optimized by choosing the most appropriate synthesis methods, varying the capping agent, and tailoring the subsequent functionalization. There is a wide range of options available to functionalize AuNPs, especially by selecting the most appropriate molecules to conjugate to the surface to enhance the biocompatibility, optical properties, and by choosing biological ligands, allowing the AuNPs to recognize and bind to specific molecules as analytes, or biological targets for imaging and therapy. AuNP-based nanocarriers could be used for controlled drug delivery, and GNSs have a wide range of applications in regenerative medicine. However, there are still concerns regarding possible nanotoxicity and recognition of AuNPs by the immune system. A significant amount of work remains to be done before they can be widely applied in clinical applications.

Increasing the targeting selectivity of the circulating AuNPs and developing more efficient strategies to facilitate AuNP clearance from the body still require more research. The biodistribution of AuNPs in humans and their longer-term impact on human health, as well as the environment at large, are under-studied areas.

Coordinated research programs between several centers are required to provide reliable correlations between particle parameters (size, shape, coating, and functionalization) and the observed biological effects. A broader selection of *in-vitro* and *in-vivo* models, a wider variety of cell lines, animal models, doses, administration routes, organ distribution, and the longer-term effects will need to be studied. This will eventually allow the scientific community to learn a lot more about the interactions between nanoscale materials and biological systems, with significant implications for their application in biology and nanomedicine.

The future impact of GNSs on the field of nanomedicine is challenging to estimate, but the potential is likely to be high. Still, uncertainties remain regarding the potential long-term implications for toxicity and environmental damage. The smooth transition from bench to clinic is likely to be significantly delayed. However, progress has been made due to technological advances and more robust studies where large datasets have been generated that can be used as information resources for future analysis. The general view is that AuNPs have only limited toxicity, but this can be influenced by different sizes or surface chemistry. Of course, the toxicity will depend on the dose administered. The slow degradation and clearance of AuNPs from the body must be studied in more detail, especially the influence of size, shape, and surface chemistry on the long-term persistence in different organs and possible effects on long-term physiology.

11.2 Future challenges in the neuro-engineering applications of GNS-based platforms

AuNPs have been extensively used in drug delivery applications, including the delivery of frugs, proteins, and genes, specifically to the CNS. However, possible toxicity, low loading efficacy, the requirement to cross the BBB, and the effect of accumulation in the body are some issues that remain to be resolved. Various methods of modification have been developed to lessen the toxicity, improve the loading or binding of drugs or bioactive molecules, and allow crossing of the BBB after i.v. injection. Enhancing the clearance of AuNPs from the body is probably the area in which the least progress has been made. To what extent do the size, shape, surface chemistry, and surface modification affect the accumulation and removal of the NPs from different body organs? Before large-scale clinical applications of AuNPs are undertaken, more research and studies are required.

The rational tailored design of electrochemical biosensors, including the relationship between the size and shape of GNSs and their electrical properties, should be systematically studied. Because most of the successfully developed electrodes are nanocomposites, the effect of the surrounding environment on the electrical properties may be a key factor in enhancing the system sensitivity. Some theoretical methods, *e.g.*, finite-difference time-domain (FDTD) and discrete dipole approximation (DDA), could help us understand the relationship between the geometrical characteristics of the GNSs and their optical properties. Therefore, there is a need to accurately model these properties using the topological data obtained from high-resolution imaging techniques, such as TEM and SEM. Although much progress has been in developing biosensors based on GNSs for neurological applications at a fundamental laboratory level, there are still many challenges to tackle. Firstly, there is an insufficiently close relationship between the technology companies and academic researchers. For example, despite the high selectivity and sensitivity reported in many reports, no portable AuNP-based biosensing devices have reached the market. There is a possibility to manufacture assay kits based on colorimetric changes of AuNPs with naked eye readouts. Secondly, the reproducibility of the probes for real-life samples is still questionable, as most reports have been based only on model samples. This issue raises concerns about the validity of the quest for real clinical applications. Thirdly, many developed probes have been constructed in academic laboratories, where workers may not be aware of the actual clinical requirements in the real world.

The plasmonic spectra of AuNPs can be easily tuned from the visible region to the NIR/IR region by adjusting the shape and size of the AuNPs. As opposed to other types of NMs commonly used in nanomedicine, including polymeric NPs, metal/metal oxide NPs, *etc.*, each defined shape of AuNPs has different properties and functionalities, thus showing the great potential of AuNPs to be used alone or as multifunctional NMs for complex theragnostic applications. Despite several successful reports of the development of GNSs for bioimaging of neural tissues, several issues should be addressed. Firstly, a relatively high dosage of AuNPs needs to be used due to the low cellular uptake (typically less than 10% of the injected dose). In addition to the possible side effects on healthy cells and organs of using such a high amount of GNSs, the treatment cost is another issue to be faced. Secondly, despite the efforts to develop selective bioimaging probes, there is still the possibility to be degraded into smaller particles by enzymatic reactions in the body.
Moreover, non-specific interactions between the targeting ligands (*e.g.*, Abs, aptamers, and peptides) and other biomolecules within the body can still occur. Therefore, the accumulation of NMs in healthy organs, such as the spleen and liver might lead to long-term toxicity and potential adverse effects. These limitations will likely delay the clinical applications of gold-based bioimaging contrast agents.

We have described several approaches for GNS-mediated neuronal modulation. GNSs can act as or facilitate the function of a neural interface, either as electrodes or as substrates or conduits for neuronal growth. These NMs come in various geometries (*e.g.*, spheres, rods, films, and stars), with their own structural and functional properties, including electrochemical impedance and plasmonic resonance. Furthermore, GNSs can be deposited or manufactured using various simple methods such as dip-coating, spray-coating, and chemical etching (*e.g.*, selective de-alloying). Overall, the possibility of using GNSs as optical neural interfaces is of particular interest because this approach might avoid the requirement for implanted leads or large electronic devices needing a power supply. However, as described in the previous section, further safety and efficacy characterization is essential before any clinical translation.

Neuronal regeneration necessitates an appropriate interaction between neurons and the underlying substrate. The microscopic interface between the cells and the substrate plays a crucial role in governing the fate of the growing neurons. The fabrication of an adequate substrate, which mimics the optimum environment for neuroregeneration is challenging. Most biomolecules (such as collagen and HA), which are used for this purpose, do not have the

appropriate structural and electrical properties. The introduction of AuNPs integrated into these biological substrates can improve their electrical conductivity and physical properties, and consequently, how the interface interacts with neurons. These AuNPs provide improved biocompatibility compared to other inorganic NPs. *In-vitro* and *in-vivo* studies have demonstrated that improved electrical stimulation due to the presence of AuNPs can lead to Schwann cell migration and extracellular matrix formation, longer neurite growth, and better axon regeneration.
Furthermore, AuNPs can modulate cellular functions at the interface with the substrate by manipulating ion channels and altering the cytoskeleton. Endocytosis of AuNPs can activate specific pathways, including MAPK/ERK, and promote neuronal proliferation. As technology progresses to provide more opportunities to design and improve the neuron-substrate nano-scale interface, the role of AuNPs will become more significant.

Gold nanoformulations allow the delivery of high dosages of therapeutic agents into the brain with good spatio-temporal resolution. They might also be used in combination with other therapies (*e.g*., RT, NIR PTT, and PDT). Nevertheless, one should keep in mind that brain delivery is still a significant challenge. The BBB presents a considerable obstacle, with even the most promising formulations not being able to cross it in sufficiently high amounts (around 5% of initial dosage). However, functionalized Au-structures are more efficient in this regard than their non-functionalized counterparts. Considerably more research in this area is still needed to create novel smart platforms to improve drug availability and specificity while reducing off-target effects and peripheral accumulation. Nevertheless, the unique properties of GNSs will continue to be investigated in the coming years for drug delivery, imaging, and therapeutic potential in the fight against neurological disorders.

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Abbreviations			
Acetylcholinesterase	AChE	Localized surface plasmon resonance	LSPR
Acetylthiocholine	ATC	Low-density lipoprotein receptor-related protein-1	LRP1
Alzheimer's disease	AD	Magnetic microbeads	MMBs
Apolipoprotein E	ApoE	Magnetic resonance-guided focused ultrasound	MRgFUS
(3-aminopropyl) trimethoxysilane	APS	Magnetic resonance imaging	MRI
Amyloid-beta	Aβ	4-mercaptophenylboronic acid	4-MPBA
Amyotrophic lateral sclerosis	ALS	11-mercaptoundecanoic acid	11-MUA
Angiopep-2	Ang	6-mercaptopurine	6MP
Annular dark-field scanning transmission electron microscopy	ADF-STEM	Mesoporous SiO_2 nanoparticles	MSNs
Antibody	Ab	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine	MPTP
Apolipoprotein-E	ApoE	Microelectrode arrays	MEAs
Aryl hydrocarbon receptor	AhR	MicroRNA	miRNA
Aspartic acid	Asp	Monoclonal antibody	mAb
α-synuclein	α-Syn	Motor unit potential	MUP
B-cell lymphoma 2 like 12 proteins	Bcl2L12	Multiple sclerosis	MS
Blood-brain barrier	BBB	Multispectral optoacoustic tomography	MSOT
Blood-brain tumor barrier	BBTB	Multi-walled carbon nanotubes	MWCNT
Bone marrow mesenchymal stem cells	BMSCs	N-acetyl-$_L$-cysteine	L-NAC
Bovine serum albumin	BSA	N-(aminobutyl)-N-(ethylisoluminol)	ABEI
Branched polyethylenimine	BPEI	Nanoporous gold	np-Au
Cancer stem cells	CSCs	Near-infrared irradiation	NIR
Carbon dots	CDs	Nerve conduction velocity	NCV
Carbon nanotubes	CNTs	Nestin	Nes
Central nervous system	CNS	Neurodegenerative disorders	NDs
Cerebrospinal fluid	CSF	Neural stem cells	NSCs
Cetyltrimethylammonium bromide	CTAB	Nuclear factor (erythroid-derived 2)-like 2	Nrf-2

Chemical vapor deposition	CVD	Nuclear localization signal	NLS
Chitosan	CTS	*N*-hydroxysuccinimide	NHSI
Cisplatin	Cis	N-terminal pro-brain natriuretic peptide	NT-proBNP
Computed tomography	CT	Oligo-(ethylene glycol) thiols	OEG-SH
Compound muscle action potential	CMAP	Optical coherence tomography	OCT
Cysteine	Cys	Paclitaxel	PTX
Deep brain stimulation	DBS	Parkinson's disease	PD
Differential pulse voltammetry	DPV	PEGylated AuNRs	PAuNRs
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC	Penetratin	Pen
Discrete dipole approximation	DDA	Phthalocyanine 4	Pc 4
1,2-distearoyl-sn-glycero-3-phosphoethanolamine	DSPE	Photoacoustic tomography	PAT
Disulfide cross-linked short polyethyleneimines	DSPEIs	Photodynamic therapy	PDT
Dithiobis(succinimidyl propionate)	DSP	Photothermal therapy	PTT
Dorsal root ganglion cells	DRGs	Polyacrylic acid	PAA
Doxorubicin	DOX	Poly(allylamine) hydrochloride	PAH
Dynamic light scattering	DLS	Polyaniline	PANI
Electrochemical impedance spectroscopy	EIS	Polybutylene succinate	PBS
Endoplasmic reticulum	ER	Polycaprolactone	PCL
Energy-dispersive X-ray	EDX	Poly(diallyl dimethyl ammonium chloride)	PDDAC
Enhanced permeability and retention	EPR	Polydopamine	PDA
Enkephalin	Enk	Poly (ethylene glycol)	PEG
Epidermal growth factor receptor	EGFR	Polyethylene terephthalate	PET
Epidermal growth factor receptor variant III	EGFRvIII	Poly($_{D,L}$- lactide)	PLA
1-ethyl-3-(3(dimethylamino)propyl)carbodiimide	EDC	Polyoxometalate	POM
Experimental autoimmune encephalomyelitis	EAE	Polystyrene sulfonate	PSS

Fibroblast growth factor 1	FGF-1	Poly(vinylidene fluoride)	PVDF
Finite-difference time-domain	FDTD	Porous magnetic microspheres	PMM
Fluorescence resonance energy transfer	FRET	Preoptic area	POA
Focused-ultrasound	FUS	Prion disease	PrD
Fourier transform infrared	FTIR	Prodrug of 1,3-dipropyl-8-cyclopentylxanthine	Pro-DPCPX
Gallic acid	GA	Prostate-specific antigen	PSA
Glassy carbon electrode	GCE	Rabies virus	RABV
Glioblastoma	GBM	RABV glycoprotein	RVG
Glutathione	GSH	Radiofrequency	RF
Gold bellflower	GBF	Radiotherapy	RT
Gold nanocages	AuNCs	Raman optical activity	ROA
Gold nanoclusters	GNCs	Reactive oxygen species	ROS
Gold nanodots	AuNDs	Recombinant human tumor necrosis factor-alpha	rhTNF-α
Gold nanofilms	AuNFs	Reduced graphene oxide	rGO
Gold nanoparticles	AuNPs	Reticuloendothelial system	RES
Gold nanorods	AuNRs	Rhodamine B	RB
Gold nanoshells	AuNShs	Rostral ventral respiratory group	rVRG
Gold nanospheres	AuNSps	Scanning electron microscope	SEM
Gold nanostars	AuNSs	Sciatic function index	SFI
Gold nanostructures	GNSs	Small interfering RNA	siRNA
Gold nanowires	AuNWs	Somatostatin-like immunoreactive	SOM-LI
Good manufacturing practice	GMP	Spectroscopic optical coherence tomography	SOCT
Graphene oxide	GO	Spinal cord injury	SCI
Heat shock proteins	HSPs	Spherical nucleic acid	SNA
Hemagglutinin	HA	Surface-enhanced Raman scattering	SERS
Hierarchical pores SiO_2 nanoparticles	HPSNs	Surface-enhanced resonance Raman scattering	SERRS
High-performance liquid chromatography	HPLC	Surface plasmon resonance	SPR

Hollow gold nanospheres	HAuNSs	Tau protein	τ protein
Human cerebral microvascular endothelial cells	hCMEC	Tetraethyl orthosilicate	TEOS
Human embryonic stem cells	hESCs	Tetraoctylammonium bromide	TOAB
Human plasma	HP	Tetrasodium salt of meso-tetrakis(4-sulfonatophenyl)porphyrin	TPPS
Human serum albumin	HSA	Thermosensitive potassium channel 1	TREK-1
Human umbilical vein endothelial cells	HUVEC	Transactivator of transcription	TAT
Hydrogen peroxide	H_2O_2	Transferrin	Trf
Laser ablation	LA	Transferrin receptor	TfR
Layer by layer	LbL	Transient receptor potential vanilloid 1	TRPV-1
Indium–tin oxide	ITO	Transmission electron microscope	TEM
Inductively coupled plasma mass spectrometry	ICP-MS	1,4,7-triazacyclononane-1,4,7-triacetic acid	NOTA
Infrared	IR	Triphenylphosphonium	TPP
Insulin	INS	Tryptophan	Trp
Interferon-gamma	IFN γ	Ultraviolet-visible	UV-vis
Intracerebroventricular	ICV	Vascular endothelial-cadherin	VEC
Intraperitoneal	IP	Wheat germ agglutinin conjugated to horseradish peroxidase	WGA-HRP
Intravenous	i.v.	World Intellectual Property Organization	WIPO
Iron oxide nanoparticles	IONPs	X-ray diffraction	XRD

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Legends to the figures

Fig. 1 Time course of the number of WoS publications on AuNPs using keywords biosensors, drug delivery, theragnostic, neurons, and CNS. (A) Publications with the respective mentioned words. (B) Contributions from various countries to publications on AuNPs and CNS (2018) (20 countries published WOS papers in 2018). Publications in 2009 were limited to just one country.

Fig. 2 Time course of the number of patents related to applications of AuNPs with beneficial effects to neurons, to CNS, and for theragnostic.

Fig. 3 Characteristics of GNSs that allow them to be used in therapy, imaging, and diagnosis of neurological diseases. Gold can be in the shape of a nanosphere, nanorod, nanostar, nanocube, nanoshell, or nanocluster. These particles can be easily functionalized with target ligands to improve organs, and cell specificity can transport different cargos and possess unique optical and electric properties.

Fig. 4 Plasmon band position and NP diameters as a function of the concentration of gold ions versus reductant. (A) Citrate preparation at 100 °C; (B) Citrate preparation at 25 °C with UV illumination; (C) Ascorbic acid preparation at RT. Reproduced with permission from ref. 82. Copyright 2006 American Chemical Society.

Fig. 5 AuNPs size distribution. (A) TEM images and size distributions of AuNPs obtained with different 3-mercaptopropionate stabilizer/gold ratio; (B) Size variation of AuNPs (●) and their standard deviations (□) with the stabilizer/gold (mol/mol) ratios obtained from the TEM analyses. Reproduced with permission from ref. 88. Copyright 1999 Elsevier.

Fig. 6 AuNP size as a function of the pH. TEM images of the AuNPs synthesized at (A) pH=5.5, (B) pH=6.5, (C) pH=7.5, (D) pH=8.5, (E) pH=9.5, and (F) pH=10.5. Scale bar = 100 nm. (G) plots of the average size and its relative standard deviations. Reproduced with permission from ref. 89. Copyright 2015 Elsevier.

Fig. 7 Sizes of AuNRs. TEM images of GmSn were obtained with different molar ratios of seeds-to-Au(III) in the growth solution. (A–C) G1S9, G2S8, and G4S6 were grown with cetyltripropylammonium bromide (CTPAB) and (D–F) G6S4, G8S2, and G9S1 were grown with CTAB. Reproduced with permission from ref. 113. Copyright 2015 American Chemical Society.

Fig. 8 *In-vivo* non-invasive NIR absorption images in real-time showing brain tumor specificity of cRGD–PAuNRs. (A) *In-vivo* time-dependent brain biodistribution of cRGD–PAuNRs and cRAD–PAuNRs as a control; (B) *ex-vivo* image of non-treatment organs and cRGD–PAuNRs, cRAD–PAuNRs-treated U87MG tumor-bearing mouse; (C) relative photon counts of *in-vivo* tumor target specificity of cRGD–PAuNRs (squares) and cRAD–PAuNRs (circles) was recorded; and (D) relative quantification of *in-vivo* biodistribution of cRGD–PAuNRs and cRAD–PAuNRs in different tissues. Reproduced with permission from ref. 121. Copyright 2011 Wiley.

Fig. 9 *In-vivo* noninvasive PA time-course coronal MAP images of B16 melanoma cells using [Nle4, D-Phe7]-α-MSH and PEG functionalized AuNCs (PEG–AuNCs). (A, E) Photographs of nude mice transplanted with B16 melanomas before injection of (A) [Nle4, D-Phe7]-α-MSH- and (E) PEG–AuNCs. Time-course PA images of the B16 melanoma cells after i.v. injection with 100 = L of 10 nM (B–D) [Nle4,D-Phe7]-α-MSH and (F–H) PEG–AuNCs through the tail vein. The background vasculature images were obtained using the PA microscope at 570 nm (ultrasonic frequency = 50 MHz), and the melanoma images were obtained using the PA microscope at 778 nm (ultrasonic frequency = 10 MHz). Color schemes: red for blood vessels

and yellow for the increase in PA amplitude. Reproduced with permission from ref. 134. Copyright 2010 American Chemical Society.

Fig. 10 Electron microscopic images of microbially-produced AuNPs. TEM images of (A) Au nanoplates produced by *Aspergillus niger*, (B) spherical, triangular and hexagonal-shaped AuNPs by *Penicillium brevicompactum*, (C) spherical AuNPs by *Rhodopseudomonas capsulata*, (D) AuNPs by *Verticillium luteoalbum*, (E) oval-shaped AuNPs by *Stenotrophomonas maltophilia*, (F) AuNPs by *Sclerotium rolfsii*, (G) intracellular synthesis of AuNPs by *Pichia jadinii* after reaction with $AuCl_4$, (H) AuNPs in the presence of a live cell filtrate of *P. brevicompactum*, (I) biosynthesis of AuNPs using the bacteria *R. capsulata*. SEM images of (J) Au nanocubes from *Bacillus licheniformis*, (K) hexagonal and triangular Au crystals from the marine yeast *Yarrowia lipolytica* NCIM 3589, (L) membrane-bound AuNPs produced by *Escherichia coli*. Reproduced with permission from ref. 148. Copyright 2014 Elsevier.

Fig. 11 Mechanism of extracellular and intracellular synthesis of AuNPs. In both processes, an enzyme determines the reduction of gold ions to Au^0 with the formation of AuNPs. Reproduced with permission from ref. 151. Copyright 2017 Elsevier.

Fig. 12 TEM of GNSs. Column 1: Atomic-resolution imaging of the gold-citrate interface: (1A) An atomic-resolution image of an AuNP and its surrounding citrate capping agent on a graphene substrate. The scale bar represents 2 nm. (1B) The graphene membrane's digital diffractogram was taken from the region indicated by the solid box in the TEM image. (1C) A digital diffractogram took from the area indicated by the dashed box STEM image of an AuNP. Reproduced with permission from ref. 157. Copyright 2009 American Chemical Society. Column 2: The influence of plasmonic hotspots on AuNPr growth. (2A–E), ADF-STEM image with corresponding EELS maps acquired from a single Au hexagonal nanoprism. (2F–J), ADF-STEM image with related EELS maps obtained from a single Au triangular nanoprism. Reproduced with permission from ref. 31. Copyright 2016 Nature Publishing Group. Column 3: HAADF-STEM core-shell Au–AgNR tomography: (A) Slices through the atomic resolution 3D reconstruction of an AuNR, revealing the atomic lattice and the surface facets present; (B) 3D visualization of a core-shell Au–AgNR, where the Au core is rendered green, and the Ag atoms are visualized in orange. Reproduced with permission from ref. 160. Copyright 2018 MDPI.

Fig. 13 Dependence of the SPR absorption band upon size and shape of AuNPs. Transmission electron micrographs (top), optical spectra (left), and photographs of (right) aqueous solutions of NPs and AuNRs of various aspect ratios. Reproduced with permission from ref. 107. Copyright 2005 American Chemical Society.

Fig. 14 Schematic of the soft and hard corona formed on the surface of an NP. k_x and K_x represent the kinetic (k) and thermodynamic (K) functions of the individual proteins. A dynamic equilibrium is reached over time between high-mobility proteins that lower-mobility proteins can replace with a higher binding affinity with the NP. Reproduced with permission from ref. 196. Copyright 2014 American Chemical Society.

Fig. 15 Fate of CuNPs or AuNPs in mice. (A) Biodistribution of Cu or Au in BALB/c mice following i.v. injection of PEG–HCuSNPs (20 mg/kg of Cu) or PEG–HAuNS (20 mg/kg of Au). Data are expressed as a percentage of injected dose per gram of tissue (%ID/g tissue) and are presented as mean ± standard deviation (n=5). *, $P < 0.05$; **, $P < 0.01$ for %ID/g of Cu v.s. %ID/g of Au. (B) Relative liver and spleen accumulation of Cu or Au postinjection. Data are expressed as a percentage of Cu or Au accumulation compared to one-day postinjection groups and are presented as mean ± standard deviation (n=5). *, $P < 0.05$; **, $P < 0.01$ for relative accumulation of Cu v.s. that of Au. Reproduced with permission from ref. 213. Copyright 2013 American Chemical Society.

Fig. 16 The biodistribution of PEG–AuNPs. (A) Concentrations in the spleen, (B) heart, (C) kidney, and (D) lung at different time points post-injection. Bars represent the mean ± standard deviation (n = 4). Reproduced with permission from ref. 218. Copyright 2018 Elsevier.

Fig. 17 Time-dependent cell uptake of AuNPs. (A) 40 nm and (B) 80 nm BPEI–AuNP, (C) 40 and (D) 80 nm LA–AuNP, and (E) 40 and (F) 80 nm PEG–AuNP with or without HP or HSA coronas up to 24 h in hepatocytes. Data are mean ± S.D. (n = 3). Different letters indicated that the means were different by the Tukey HSD test. *p < 0.05, **p < 0.005, ***p < 0.0001. Reproduced with permission from ref. 242. Copyright 2017 Taylor & Francis.

Fig. 18 High-content imaging and confocal microscopy (focal adhesion size). (A, B) HUVEC cells (left) or C17.2 cells (right), treated with the various AuNPs at equal NP numbers and (C, D) equal mass of Au. Reproduced with permission from ref. 276. Copyright 2016 Wiley.

Fig. 19 Proposed mechanism of AuNPs crossing the endothelial barrier. BEFORE: the paracellular route on the microvascular barrier is maintained by the vascular endothelial-cadherin (VEC) homolog interaction that is buttressed cadherin-catenin-actin tertiary complex. AFTER: AuNPs interaction with VEC activates the VEC signaling resulting in the VEC being unanchored from the actin cytoskeleton. This leads to VEC internalization and degradation. The untethered actin becomes vulnerable to the remodeling process that leads to cell contraction and subsequently results in the paracellular route's opening. β-catenin, β-cat; α-catenin, α-cat. Reproduced with permission from ref. 278. Copyright 2017 American Chemical Society.

Fig. 20 Effect of GNSs on brain cells. (A) Colorimetric quantification of serotonin with bi-functionalized DSP/ L-NAC AuNPs. Serotonin (5-HT) molecules bridge the AunNPs and result in aggregation of AuNPs, and turn, changing the color of the solution. Reproduced with permission from ref. 309. Copyright 2018 Elsevier. (B) Dual modal determination of AChE enzyme using RB–AuNPs. The AChE enzyme catalyzes ATC's hydrolysis and results in simultaneous aggregation of AuNPs (colorimetric) and releasing of RB molecules (Fluorescence recovery). Reproduced with permission from ref. 324. Copyright 2012 Wiley. (C) Asp promoted sensitive colorimetric determination of Cys via aggregation-induced by crosslinking mechanism. Reproduced with permission from ref. 340. Copyright 2012 American Chemical Society. (D) Schematic preparation of electroluminescence-based probe for the determination of NT-proBNP. The interaction between antigen and immobilized antibody results in a decrease in the electrochemiluminescence intensity of the system. Reproduced with permission from ref. 342. Copyright 2015 American Chemical Society.

Fig. 21 GNSs as contrast agents. A) Schematic illustration of Iodine-containing nanoparticles with different formulations. Reproduced with permission from ref. 363. Copyright 2006 Wiley. B) A comparison between AuNPs and Iopromide as CT contrast agents at low (top images) and high (bottom images) energies. Reproduced with permission from ref. 366. Copyright 2010 Elsevier. C) Key structure-property function of AuNPs as X-Ray contrast agent. Reproduced with permission from ref. 367. Copyright 2015 Future Medicine. D) Noninvasive PAT imaging of a mouse brain *in-vivo* employing PEG-HAuNS and NIR light at a wavelength of 800 nm. Photoacoustic image acquired (i) before, (ii) 5 min after, and (iii) 2 h after the intravenous injection of PEG-HAuNS. (iv) and (v) Differential images that were obtained by subtracting the preinjection image from the post-injection images (Image iv = Image ii − Image i; Image v = Image iii − Image i). Arrow, middle cerebral artery. Bar = 2 mm. Reproduced with permission from ref. 372. Copyright 2010 Elsevier.

Fig. 22 GNSs for fluorescence imaging. (A) i: Preparation of two different AuNPs functionalized with alkyne or azide, ii: Penetration of AuNPs into normal cells with no aggregation, iii: Penetration of AuNPs into tumor cells with aggregation due to the click reaction at acidic pH. Reproduced with permission from ref. 375. Copyright 2017 Wiley. (B) (i) Invasive

in-vivo, (ii) *ex-vivo* fluorescence imaging, and (iii) optical imaging of the advanced glioma-bearing brain with (upper row, 24 h p.i.) or without (lower row) administration of 3 nm AuNPs. (iv) Bright-field and (v) fluorescence imaging of less advanced glioma-bearing brain slices with (upper row, 24 h p.i.) or without (lower row) administration of 3 nm AuNPs. (vi) Tumor/cortex fluorescence ratio at different time points showed a significant increase in tumor/cortex ratio (4.6 times) from control to 24 h p.i. Reproduced with permission from ref. 382. Copyright 2017 Springer Nature.

Fig. 23 Schematic of surface modification methods of AuNPs. (A) Ligand exchange, (B) LbL self-assembly, and (C) surface coating. Reproduced with permission from ref. 422. Copyright 2017 Elsevier.

Fig. 24 Different applications and effects of NPs in cancer cells. (A) Normal cell and (B) stem cells due to distinct pathways for cellular trafficking (EPR effect). Reproduced with permission from ref. 443. Copyright 2011 American Chemical Society. (C) Schematic of the conjugation of EGF peptide and adsorption PDT drug Pc 4 to AuNPs. Reproduced with permission from ref. 446. Copyright 2011 Wiley.

Fig. 25 Conjugation of DOX and Ang to AuNPs. (A) Schematic of NP structure. (B) The mechanism of penetration through the BBB is by binding of Ang to the LRP1 receptor to facilitate delivery to the tumor. Reproduced with permission from ref. 472. Copyright 2015 Elsevier.

Fig. 26 RABV-mimetic RVG–PEG–AuNRs–SiO_2. (A) Scheme of delivery to the brain through the neuronal pathway and PTT activated by NIR laser. (B) Schematic of the synthesis of RVG–PEG–AuNRs–SiO_2): (i) Au seed, (ii) AuNRs (longitudinally grown), (iii) AuNRs (after transverse growth), (iv) SiO_2–AuNRs, (v) RVG29 peptide-PEG5k-conjugated AuNRs–SiO_2. The final AR of the RVG–PEG–AuNRs–SiO_2 is similar to the AR of the RABV virus. Reproduced with permission from ref. 494. Copyright 2017 Wiley.

Fig. 27 Three major transport mechanisms are responsible for transporting nutrients and other molecules through the BBB. The BBB has unfenestrated tight junctions between capillary endothelial cells. Essential nutrients are transported via carrier-mediated transport, whereas receptor- and adsorptive-mediated transcytosis is used to import hormones, peptides, and other macromolecules. GBFs can enter the brain by any of these three transport mechanisms. Reproduced with permission from ref. 513. Copyright 2015 American Chemical Society.

Fig. 28 Schematic representation of the general mode of action of GNSs after i.v. administration in preclinical models of AD. The top image represents a typical scenario in AD, which includes Aβ aggregates, neurofibrillary tangles, and a high level of inflammatory molecules and ROS. The bottom image has represented the effect of GNSs in the AD brain. Different types of GNSs, including NPs and nanostars, when functionalized with diverse types of ligands (*e.g.*, PEG, Aβ inhibitors peptides, penetrating peptides), improve the formulation's ability to cross the BBB. This therapy by itself or in combination with NIR irradiation promotes the degradation of Aβ aggregates, decreases neurodegeneration, decreases neuroinflammation, ultimately improving AD-induced cognitive deficits in rodents.

Fig. 29 Schematic representation of the general mode of action of GNSs after i.v. administration in preclinical models of GBM. GNSs, including NPs, nanoclusters, nanoshells, nanorods, and nanostars, were functionalized with ligands such as penetrating peptides, anti-fouling peptides (PEG), pH-sensitive moieties, and contrast agents (*e.g.*, ^{68}Ga, Gd^{3+}, *etc.*) as well as some therapeutic agents (*e.g.*, DOX, Cis, curcumin) and siRNAs (iRNA duplexes against Bcl2L12). These formulations act as good contrast agents, improving the tumor's visualization

and its borders (dash line) important for surgical interventions and following tumor evolution. Gold formulations also improve the accumulation of the therapeutic agents inside the tumor, improving their therapeutic potential compared with the free drug, leading to apoptosis of tumor cells and a decrease of the tumor size and burden, reducing the off-target effects. The use of GNSs not only significantly increases chemotherapy action but can also potentiate the efficacy of RT and phototherapy.

Fig. 30 GNSs as electrodes. (A) Scanning electron micrograph of the top surface of np-Au and (B) transmission electron micrograph depicting the np-Au electrode's cross-section. Reproduced with permission from ref. 587. Copyright 2015 IOPscience. (C, F, I) Electrochemical impedance spectroscopy (EIS) data illustrates the impedance decrements achieved using GNSs, nanoporous surfaces, or doping conductive polymers with AuNPs. Reproduced with permission from ref. 585. Copyright 2010 IOPscience, Reproduced with permission from ref. 587. Copyright 2015 IOPscience, Reproduced with permission from ref. 588. Copyright 2019 MDPI. (D) Scanning electron micrograph image of the np-Au electrode's top surface and (E) the cross-section. Reproduced with permission from ref. 585. Copyright 2010 IOPscience. (G, H) Images of AuNPs dispersed in the conductive polymer poly (3, 4-ethylenedioxythiophene) (PEDOT) matrix. The inset histogram illustrates that the most prevalent AuNP size is 200 nm in diameter. Reproduced with permission from ref. 588. Copyright 2019 MDPI.

Fig. 31 SPR of nanoscopic Au structures for neural stimulation. (A) Illustration of the mechanism by which AuNPs resonate in response to light absorption. (B) Absorbance as a function of wavelength for a range of AuNP diameters. Reproduced with permission from ref. 603. Copyright 1999 American Chemical Society. (C) Induce AuNP-Ts1 conjugate (at 20 nM) to neuronal membranes by optical excitable from the cells with laser pulses (1 ms) using powers as low as 126 mW. Reproduced with permission from ref. 181. Copyright 2015 Elsevier. (D) Illustration of SPR mechanism for AuNRs in which both longitudinal and transverse SPR occurs, leading to 2 absorption peaks (E). (F) Pulsed near-infrared beam photothermally heats AuNRs (absorb at 980 nm wavelength) causing the temperature increase and stimulation location at the membrane of neural tissues. Reproduced with permission from ref. 604. Copyright 2014 Wiley.

Fig. 32 Various examples of GNS-based optical stimulation interfaces for neural signal inhibition. (A) The neuro-device was schematic with a nanoplasmonic interface for simultaneous electrical stimulation and recording and optical inhibition. (B) SEM images of a neuron interfacing with the AuNRs monolayer-coated substrate. Inset indicates a whole neuron interfacing with the AuNR monolayer. (C) Spike rate change of neural firing upon NIR irradiation at different laser power densities. Reproduced with permission from ref. 615. Copyright 2016 American Chemical Society. (D) Schematic of AuNSs mediated NIR light-based neuronal cell stimulation system. (E) Optical illumination inhibits the spontaneous activity of the single neuron. Reproduced with permission from ref. 613. Copyright 2018 Elsevier. (F) Schematic illustration of an inkjet-printed thermo-plasmonic interface for patterned neuromodulation on an *in-vitro* cultured hippocampal neuronal network. Reproduced with permission from ref. 614. Copyright 2018 American Chemical Society. (G) Illustration of characterized optical characteristics of the nanofilms. (H) Surface morphology of the 5 nm thick and 10 nm thick AuNFs imaged by atomic force microscopy (AFM) (scale bar = 200 nm), and cultured hippocampal neurons on 5 nm thick AuNF-coated MEA. Reproduced with permission from ref. 612. Copyright 2018 Royal Society of Chemistry.

Fig. 33 Photoreactive ion channels in neuronal cells involved in AuNR-based optical stimulation. (A) Schematic representation of the localized photothermal heating of TRPV-1 expressing plasma-membrane bearing pm-AuNRs. (B) Photoactivation of primary cultured neuronal cells by AuNRs. (C) Fluorescence intensities of wild type (solid line) and knock out (broken line) neurons under the same conditions. Reproduced with permission from ref. 616.

Copyright 2015 Wiley. (D) Fluorescence images of neurons after immunochemical staining for β-tubulin(III) (red), TREK-1 (green), and nucleus (blue). (E) Mean spike rates of AuNR-treated neurons upon NIR irradiation after blocking of TREK-1 channels. (F) Quantification of mean spike rates of AuNR-treated neurons upon NIR irradiation after blocking of TREK-1 channels. Reproduced with permission from ref. 611. Copyright 2014 American Chemical Society.

Fig. 34 GNSs for neural regeneration. SEM images of PC12 cells seeded on AuNP-based substrates in the presence of (A) alternating electrical stimulation and (B) without stimulation. The inset images (B–E) illustrate each part of the cells' higher magnification details. As can be seen, neurites formation and growth necessarily depend on the electrical stimuli' presence (A compared to B). Reproduced with permission from ref. 638. Copyright 2009 American Chemical Society. (C and D) Superior and lateral views SEM images of TiO_2–AuNWs implanted into a blind mouse model, scale bare 0.5 and 2 μm, respectively. (E) The interface between the retina and the nanowires provides a spatial preference for neural growth, scale bar 5 μm. (F) Upon the exposure of near UV (purple line), blind mice showed no responses as measured by local field potentials; TiO_2–AuNWs could restore the responses by promoting the neurons to grow in the interface of the nanowires with the retina in a blind mouse. Reproduced with permission from ref. 653. Copyright 2018 Nature Publishing Group.