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Insights into impact of gold nanocluster Au₁₀SG₁₀ on human microglia

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ABSTRACT: The purpose of the current studies was to uncover the impact of small liganded gold nanoclusters with ten gold atoms and ten glutathione ligands (Au₁₀SG₁₀) on several biomarkers in human microglia. We established the links connecting the atomically precise structure of Au₁₀SG₁₀ with their properties and changes of several biomolecules under oxidative stress. Au₁₀SG₁₀ caused loss of mitochondrial metabolic activity, increased lipid peroxidation and translocation of an alarmin molecule, high mobility group box 1 (HMGB1), from the nucleus to the cytosol. Molecular modeling provided an insight into the location of amino acid interaction sites with Au₁₀SG₁₀ and the nature of bonds participating in these interactions. We show that Au₁₀SG₁₀ can bind directly to the defined sites of reduced, oxidized and acetylated HMGB1. Further studies with similar complementary approaches merging live-cell analyses, determination of biomarkers and cell functions could lead to optimized Au nanoclusters best suited for diagnostic and bioimaging purposes in neuroscience.

Introduction

Ligated gold nanoclusters (AuNCs), consisting of up to hundreds of gold atoms and different biomolecules as ligands, display properties compatible with biomedical applications^{1,2}. Due to their well-defined structure, facile surface modification and optical properties³, including fluorescence in the visible and near-infrared region, long lifetime and large Stokes shift⁴, they are used as fluorescent probes in biomedical applications (e.g. imaging, detection, and therapy⁵⁻⁹). *In situ* biosynthesized gold nanoclusters have been explored for *in vivo* bioimaging in tumors¹⁰.

AuNCs are part of the nanoscale where “each atom counts”, which means that changing their size greatly influences their structural properties¹¹. Different ligands diversify their applications and prevent their toxicity *in vivo*. Without the protection of the gold surface with ligands, gold atoms would modify

the redox state of the cell¹². A commonly used approach to functionalize AuNCs is ligand exchange^{13,14}. Thiol-containing ligands, such as glutathione (GSH) and cysteine, are common ligands on AuNCs surfaces, and such clusters have been used for bioimaging in cells and *in vivo*¹⁵⁻¹⁸.

Such liganded nanoclusters can be produced at atomic precision; diffraction experiments allow for their structural determination, in particular for Au₁₀¹⁹ and Au₂₅²⁰⁻²². Thus, the following questions were addressed in the present study: What are the effects of Au₁₀SG₁₀ in human microglia under oxidative stress? Do these AuNCs enhance the effects of oxidative stress? Is the alarmin high mobility group box 1 (HMGB1) translocated from the nucleus to the cytosol upon Au₁₀SG₁₀ treatment? Does Au₁₀SG₁₀ interact with HMGB1, and if so, what are the predicted sites of these interactions?

The model cells we investigated are human microglia because they are the main surveyors of the brain and consumers of the nanostructures.^{23,24} Microglia respond to oxidative stress caused by different agents (e.g., H₂O₂, BSO, UV) and change both their morphology and composition of released cytokines, chemokines, and trophic factors.^{23,25–28} It is well established that microglia secrete proinflammatory cytokines (e.g., TNF- α , IL-1, IL-6) and anti-inflammatory cytokines (e.g., IL-4, IL-10)^{23,29,30} depending on the intensity and duration of activation. We selected to treat human microglia with Au₁₀SG₁₀ because of their prompt and measurable responsiveness to nanostructures, including AuNCs. In previous studies, AuNCs were reported to have low toxicity in glioblastoma cells.³¹

We focused on a highly redox-responsive alarmin molecule, HMGB1, known to change its cellular location and functions depending on post-translational modifications (PTMs)^{32,33}. Considering that AuNCs have catalytic and enzyme-like properties, it was anticipated that Au₁₀SG₁₀ could influence PTMs of HMGB1, which determine the intracellular location of the protein. We assessed HMGB1 location in the nucleus and cytosol. AuNCs were not previously linked with specific amino acid sites in HMGB1. Common PTMs in HMGB1 are oxidation, acetylation, phosphorylation, and methylation. We focused on oxidation and acetylation to better understand the nature of Au₁₀SG₁₀ interaction with HMGB1. Our recent study³⁴ provided data showing the formation of reactive oxygen species (ROS) in the presence of Au₁₀SG₁₀, but did not provide details about the sites of interactions of this protein with AuNCs containing ten gold atoms. Such information is necessary for a mechanistic explanation of biological effects, and could facilitate better designs and production of new AuNCs. Modeling provides predetermination of properties of AuNCs to optimize and maximize the usefulness of AuNCs for bioimaging, diagnostic purposes, biosensing, and delivery of therapeutics^{1,6,16,17,35–37}.

We tested the hypothesis that Au₁₀SG₁₀ is not inert in human microglia and that Au₁₀SG₁₀ can contribute to the enhancement of oxidative stress leading to lipid peroxidation and changes in HMGB1 structure. We predicted that HMGB1 both in its reduced and oxidized forms can interact with Au₁₀SG₁₀. We show the striking perinuclear and nuclear location of Au₁₀SG₁₀ in microglia and translocation of HMGB1 from the nucleus to the cytoplasm that was more prominent upon pharmacologically induced oxidative stress by L-buthionine-sulfoximine (BSO).

Results

The concept of our present study is illustrated in (Figure 1).

Different endpoint parameters were used to determine Au₁₀SG₁₀ toxicity in human microglial cells: cell number, mitochondrial function and cell membrane damage (Figure 2A). Cell number was determined by counting Hoechst-stained nuclei (Figure 2B). Mitochondrial metabolic activity was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Human microglia were treated with Au₁₀SG₁₀ for 24 hours and showed no remarkable cell loss or impaired mitochondrial function up to 10 μ M in concentration (Figure 2C). Concentrations greater than 10 μ M caused a significant reduction in cell number (Figure 2C) and mitochondrial activity (Figure 2D). Aside from cell number and mitochondrial function, cell membrane integrity is another feature often used to detect cytotoxicity. Upon damage of plasma membranes, cells release molecules into the surround-

ing culture medium, which can be detected and measured. The most common biomarker measured is lactate dehydrogenase (LDH). Plasma membrane damage was significant in microglial cells treated at higher concentrations (>10 μ M) of Au₁₀SG₁₀ (Figure 2E). Interestingly, loss of membrane integrity was most significant in Au₁₀SG₁₀-treated cells that were under oxidative stress induced by BSO pre-treatment. BSO is a potent and specific inhibitor of γ -glutamylcysteine synthetase (γ -GCS), originally synthesized and tested by Griffith et al.^{38,39} An associated decrease in cell number and mitochondrial activity were also observed in microglia pre-treated with BSO and exposed to extreme concentrations of Au₁₀SG₁₀ (100–200 μ M).

Given the observable changes in microglial number, metabolic function and plasma membrane integrity, we examined the effects of Au₁₀SG₁₀ on HMGB1 intracellular location using immunocytochemistry. HMGB1 abundance and cellular distribution were assessed in microglia cells exposed to Au₁₀SG₁₀ for 24 hours (Figure 3A). It was anticipated that Au₁₀SG₁₀ could change HMGB1 intracellular location (nuclear, N; cytosolic, C). The lowest concentration tested (1 μ M) of Au₁₀SG₁₀ caused a minimal change in HMGB1 translocation (not shown). When Au₁₀SG₁₀ treatment was raised to 10 μ M, we observed an increase in nuclear HMGB1 abundance, and most significantly in cytosolic HMGB1 abundance compared to the untreated control (Figure 3B). Next, we asked the question if Au₁₀SG₁₀ interacts with HMGB1 and what are the predicted sites of these interactions. Au₁₀SG₁₀ determined with atomic precision was used to perform QM computations to define HMGB1 interaction sites with this nanostructure.

We optimized the complex of redHMGB1 and Au₁₀SG₁₀ cluster where we included in QM part of the QM/MM simulation the Au₁₀SG₁₀ cluster and side chains of Tyr74, Met78, Tyr85. The optimized structure revealed formation of hydrogen (with residues Lys19 and Tyr85), ionic (Lys19), hydrophobic (Tyr85), polar (residues Met78, Tyr85) bonds (Figure 3C). To provide experimental evidence for the predictions for HMGB1 interaction with Au₁₀SG₁₀, we performed MS/MS which support the occurrence of such interactions (Figure 7).

Considering that Au₁₀SG₁₀ could be catalytically active, we tested if they increase CellROX signal, a probe for reactive oxygen species (ROS). If so, then HMGB1 could be oxidized by these gold nanostructures. Our results strongly support this possibility, since CellROX signal was significantly stronger in microglia treated with Au₁₀SG₁₀ (10 μ M) and was further enhanced in the presence with BSO (Figure 4A–B).

As a result of ROS accumulation, lipids can be peroxidized. A major reactive oxidation product generated from lipid peroxidation is 4-hydroxynonenal (4-HNE), which can serve as a biomarker for oxidative stress. We performed immunofluorescent analyses of 4-HNE in microglial cells treated with Au₁₀SG₁₀ with or without BSO pre-treatment (Figure 4C–D). Microglial cells treated with 10 μ M – but not 1 μ M – showed a significant increase in 4-HNE (Figure 4C). Pre-treatment with BSO alone only caused a slight increase in 4-HNE abundance in microglial cells. However, when microglial cells were subjected to both pre-treatment with BSO and 10 μ M Au₁₀SG₁₀ treatment, a remarkable increase in 4-HNE was observed. No significant changes in nuclear size were detected for any treatment (Figure 4D) but nuclear deformations were seen in cells treated with 100 μ M or 200 μ M Au₁₀SG₁₀. Actin is one of the most abundant cellular proteins, which makes this protein more susceptible to non-specific modifications by

reactive oxygen species (ROS) and oxidized lipids. Actin has been found to be involved in protective mechanisms against redox intermediates^{40,41}. Although F-actin was slightly decreased, no remarkable morphological differences in microglia treated with Au₁₀SG₁₀ (<10 μ M) in the presence or absence of BSO were observed after 24 hours (Figure 4C-D).

Under oxidative stress, HMGB1 is oxidized at the positions Cys30 and Cys52, which form a disulfide bridge⁴². As this causes structural changes in HMGB1, we modeled interactions of oxiHMGB1 using a crystal structure that has a disulfide bridge (pdb: 2RTU)⁴³. To investigate the influence of oxidation of HMGB1 on interactions with Au₁₀SG₁₀, we optimized the complex of HMGB1 with oxidized residues Tyr74, Met78, Tyr85 from the 2RTU and Au₁₀SG₁₀ (Figure 4) and compared them to the interactions of the reduced form of the same residues from the 2YRQ and Au₁₀SG₁₀ (Figure 3). When compared to redHMGB1, oxiHMGB1 has additional interactions formed with residues Pro13, Lys72 and Glu79, while Pro83 and Glu87 are no longer included in the interactions.

A protein database was used to find structures of HMGB1 in reduced form (redHMGB1, pdb: 2YRQ) and oxidized form (oxiHMGB1, pdb: 2RTU). We analyzed the positions of amino acid residues and their exposure to Au₁₀SG₁₀. One group of those residues on the exposed surface of HMGB1 is formed by residues Tyr74, Met78 and Tyr85, which are selected as a model system. They are located at the α -helix part of the A box domain of HMGB1. Being part of the α -helix, they are less likely to be structurally altered compared to loop domains and are exposed to solvent and Au₁₀SG₁₀. Upon the oxidation of HMGB1, a disulfide bridge forms between Cys30, which is part of the first α -helix, and Cys52, which is part of the second α -helix, make the structure of the oxiHMGB1 more rigid and spatially less exposed to the environment. This is shown by solvent accessible surface area (SASA) calculation, which gives $\sim 13200 \text{ \AA}^2$ for redHMGB1, while SASA for oxiHMGB1 is $\sim 7100 \text{ \AA}^2$. This is reflected in the residues that interact with Au₁₀SG₁₀, where more interactions (hydrogen, polar, ionic and hydrophobic bonds) are present between oxiHMGB1 and Au₁₀SG₁₀. To complement theoretical findings, we performed MS/MS (Figure 7). Results from these studies agree with the predicted sites of oxidation and other possible PTMs.

Au₁₀SG₁₀ did not exert any remarkable effect on acetylated HMGB1 (AcHMGB1) (Figure 5). AcHMGB1 did not leave the nucleus in microglial cells exposed to Au₁₀SG₁₀ (Figure 5A-C), but did when cells were treated with lipopolysaccharide (LPS); LPS served as a positive control for the protein translocation (Figure 5A-C).

We modeled interactions of Au₁₀SG₁₀ with residues Lys10, Lys15 and Lys19 (Figure 5D), which form an acetylation site of HMGB1, but the frequency of their acetylation is low⁴⁴. In our model, we introduced the acetyl groups in all three lysine residues and investigated the interaction with Au₁₀SG₁₀ (Figure 5D). We also modeled the interaction of Au₁₀SG₁₀ with the same site of HMGB1 in the reduced form. Upon the optimization of both complexes of acetylated and reduced HMGB1 with Au₁₀SG₁₀, we analyzed their interactions. Both AcHMGB1 and redHMGB1 formed hydrogen bonds (see inset of Figure 5D), ionic and polar interactions (residues Lys15 and Lys19) with Au₁₀SG₁₀. Analysis of acetyl groups on Lys15 and Lys19 suggests that these sites are not favorable for Au₁₀SG₁₀ interactions in the HMGB1 structure.

Considering that oxidative stress can cause protein aggregation, we employed the ProteoStat fluorescent probe to see if this was the case with microglia treated with Au₁₀SG₁₀. Microglia under standard culture conditions have few barely detectable aggregates, whereas cells treated with the protease inhibitor MG-132 (used as positive control) have a huge amount of small and large protein aggregates. They appeared as big shiny speckles and fainter homogeneous signals (Figure 6A-D). More interestingly, darkfield microscopy (Figure 6F-I) and super-resolution microscopy (Figure 6J) revealed AuNC aggregates mainly localized in the perinuclear or nuclear region. It was not possible to detect these signals in microglia treated with low Au₁₀SG₁₀ concentrations (< 10 μ M), but they were easily detectable with Au₁₀SG₁₀ treatments at 100 μ M-200 μ M after an 1 hour incubation. The existence of small aggregates (2-3 clusters) was detectable by MS/MS (Figure 7).

Mass spectrometry analysis was performed with matrix assisted laser desorption time of flight mass spectrometry. Mass spectrum of Au₁₀SG₁₀ spotted on the ground steel with HCCA matrix (α -cyano-4-hydroxycinnamic acid) and acquired using reflectron positive mode of ionization showed peaks at 2515 m/z, 2634 m/z and 2711 m/z, representing Au₁₀SG₁₀ corresponding to doubly charged species with additional gold atoms (Figure 7A). Mass spectrum of HMGB1 acquired using linear positive mode of ionization after mixing with super-DHB matrix (2,5-dihydroxyl benzoic acid and 2-hydroxy-5-methoxybenzoic acid) shows major peaks at 13307 m/z and 26627 m/z, respectively (Figure 7B). These masses represent doubly and singly charged species of HMGB1 with respective doublet peaks corresponding to glycosylation of HMGB1. Spectrum of the product after incubation of HMGB1 and Au₁₀SG₁₀ at a ratio of 3:1 for 8 hours at 37 $^{\circ}$ C and acquired as in Figure 7B showed a doubly charged mass at 14690 m/z representing an increase in the mass of HMGB1 (without glycosylation and tags) by about 5 kDa. The peak at 11373 m/z represents an intermediate product after the incubation (Figure 7C). Spectra of digested HMGB1 incubated without or with Au₁₀SG₁₀ acquired using reflectron positive mode of ionization after spotting on the ground steel with HCCA matrix showed respective peaks corresponding to peptides generated from HMGB1 incubated without and with Au₁₀SG₁₀ (Figure D-E). Suspected disulfide bonded peptides as a peak at 956 m/z, while absent in the spectrum of digested HMGB1 are shown in enlarged views (Figure 7F-G). Screenshots of the sequence alignment of digested HMGB1 incubated without or with Au₁₀SG₁₀ (obtained from Biotoools software) shows a protein sequence coverage of 71.6% and 80.9%. Amino acids in red represent matches with those from in silico trypsin digestion and respective grey bars represent peptides corresponding to the HMGB1 sequence (Figure 7H-I).

Discussion

The inspiration for the current study stems from several recent reports, including ours, suggesting that AuNCs are promising nanostructures for bioimaging, biosensing and drug delivery^{1,6,17,18,35,36,45,46}. These earlier studies provided valuable insights into global structural properties and biological effects in different cells or even animals but did not attempt to link Au₁₀SG₁₀ structure at the atomic level with any redox-responsive transcription factor or cellular state in human microglia.

In the present study, we investigated the impact of Au₁₀SG₁₀ on microglia cells, with or without oxidative stress due to the suppressed GSH synthesis. The goal was to reveal if Au₁₀SG₁₀ adversely affects human microglial HMC3 cells and if these adverse effects are associated with Au₁₀SG₁₀ binding to the reduced, oxidized and acetylated forms of HMGB1. Quantum mechanics calculations were performed to show likely interaction sites of HMGB1 with Au₁₀SG₁₀. Complementary to the theoretical studies, we performed MS/MS analyses which revealed possible predicted interactions.

Mass spectrometry analyses suggest an interaction between HMGB1 and Au₁₀SG₁₀ that supports the quantum mechanics calculations. The observed mass increase of HMGB1 as measured by MALDI-TOF indicate a possible molecular rearrangement of HMGB1 to accommodate Au₁₀SG₁₀. MS/MS analysis also shows oxidation of cysteines at positions 23 and 45 of HMGB1 and the formation of a disulphide bond between these cysteines as previously reported⁴⁷. These findings are in line with observations of HMGB1 translocation from the nucleus to the cytosol following cysteine oxidation by Au₁₀SG₁₀.

We performed several biological assays following the treatment of human microglia with Au₁₀SG₁₀. Human microglia were used as the model cell type because of convincing evidence that they are consumers of nanostructures in the central nervous system^{48–51}. Microglia are essential surveyors, sculptors and protectors from the insulting agents in the central nervous system^{24,28,52}. They can also degrade aggregated and misfolded proteins. Microglia activation state is associated with the release of different sets of cytokines, chemokines and trophic factors; these secreted factors determine the cellular and tissue functions under physiological and pathological conditions^{53–55}. The redox-responsive transcription factor HMGB1 is released from the nucleus under pathological conditions in different organs, including the brain.

Experiments described in this study show that the treatment of human microglia with Au₁₀SG₁₀ (> 10 μ M) leads to cell loss, decreased mitochondrial metabolic activity, increased ROS and increased lipid peroxidation. We suggest that these adverse effects are due to the catalytic effects of gold atoms in the relatively open Au₁₀SG₁₀ structure. The exposed gold atoms could contribute to the formation of oxidative species, which in turn oxidize lipids and cause defects in plasma membranes, leading to the release of intracellular content. Our data support this notion because we obtained increased lipid peroxidation, as measured by the lipid peroxidation product 4HNE^{56,57}, the release of LDH and HMGB1 exit from the nucleus. ROS generation, lipid peroxidation and plasma membrane damage are documented in cells undergoing ferroptosis⁵⁸. Lipid peroxidation and ferroptosis are implicated in various neurological disorder^{59,60}. The release of HMGB1 from the nucleus is often found in impaired nervous systems, particularly those under oxidative stress^{61–63}. Other studies have proposed nanoclusters as fluorescent probes for the screening of antioxidants in biological samples^{64,65}.

Au₁₀SG₁₀ can interact with transcription factor HMGB1 in its reduced and oxidized forms. HMGB1 in its fully reduced form is located in the nucleus under homeostatic conditions and it plays a chaperoning role^{66,67}. A fully reduced and released HMGB1 can bind to RAGE receptors and induce autophagy⁶⁸. In contrast, partially oxidized HMGB1 at positions 30–52 binds to TLR4 and exerts proinflammatory effects. Interestingly, terminal oxidation of cysteine residues (positions 30, 52 and 113) abolishes HMGB1 activity and contributes to the resolu-

tion of inflammation. Our results support the notion that mild oxidative stress in cells treated with Au₁₀SG₁₀ can cause the partial release of HMGB1 from the nucleus to the cytosol. Au₁₀SG₁₀ does not interact with critical cysteine oxidation sites Cys30–52, but interacts with Tyr74, Met78 and Tyr85 (PDBID: 2YRQ), which could make HMGB1 more vulnerable to oxidative stress at Cys in loop A. A tandem mass spectroscopy (MS/MS) spectrum for oxidized HMGB1 was reported^{47,68} and our MS/MS data confirm the presence of S-S bond between Cys30–52.

Acetylation, which is another PTM, changes the surface properties of a protein, such as charge, and therefore influences its function. Acetylation includes transferring of the acetyl group of acetyl coenzyme A (Ac-CoA) to the lysine (Lys) residues of the protein. HMGB1 has 34 lysine residues scattered in its structure, and it was shown that it is often acetylated at two sites: Lys35–37 and Lys181–183. A study suggested that mutating these sites does not influence translocation from the nucleus⁴⁴. As the lysine residues at site Lys35–37 are all oriented in different directions in space, they are not suitable for interaction with Au₁₀SG₁₀.

Protein oxidation and carbonylation are correlates of aging and can cause age-related diseases^{69–71}. The formation of protein aggregates could play both protective and destructive roles^{72,73}. Gold can hybridize with some macromolecular proteins by Au–S and Au–NH₃ binding interactions. An interesting class of stable and highly fluorescent AuNC with zwitterionic ligands could be useful tools to study protein aggregation in the nervous system⁶⁴. Functionalized AuNCs with 15 gold atoms were useful for protein carbonylation detection⁷⁴. Data from super-resolution microscopy based on lifetime differences between the autofluorescence of microglia and Au₁₀SG₁₀ suggest the presence of AuNC aggregates. Indeed, isolated Au₁₀ nanoclusters present very low quantum yields (and fluorescence can only be observed in solid-state or at a high concentration in solution).^{19,75} However, when massive aggregation of Au₁₀ nanoclusters occurs, a tremendous increase in fluorescence can be observed due to the rigidification of the Au(i) thiolate shell.⁷⁶

In summary, our study shows some likely interactions of HMGB1 with Au₁₀SG₁₀ without excluding other possible interactions. The study explores the sites and types of interactions based on the functional groups present in the GSH ligands of Au₁₀SG₁₀ and side chains of amino acids of HMGB1 and their availability for interactions. As complexes inside the cell are not static, additional molecular dynamics simulations would be needed, which are presently out of scope. Complementary studies employing super-resolution EM tomography of single cells could provide valuable information at the atomic level. However, this technique is still undergoing optimizations and cannot be routinely used⁷⁷.

Conclusions

Our study provides the first step in investigating AuNC-alarmin interactions, offers predictions of sites and types of binding between specific positions in AuNC and HMGB1 in reduced, oxidized and acetylated forms. This establishes the link between the chemical structures of AuNCs and several biochemical and functional changes under oxidative stress in human microglia.

Several therapeutic strategies have been used to prevent HMGB1 release from the cell nucleus and reduce its acetylation.^{78–80} Enhancement of the hyper-acetylated and disulfide

HMGB1 isoforms have been useful biomarkers in inflammation-associated pathologies. Some isoform-specific HMGB1 inhibitors are currently in development to fine-tune the pharmacological control of inflammation⁸¹. Considering that Au₁₀SG₁₀ can bind and alter HMGB1, cellular distribution of these atomically precise nanostructures should be explored as a part of novel strategies to control the homeostatic state of human cells in the central nervous system and other tissues.

A number of *in vivo* studies show advantages of AuNCs over traditional contrast agents^{37,45,46,82}. Other applications and structural details at the atomic level were reviewed elsewhere^{6,17,18,36,74,83}. The major limitation of our study is that we could not exploit extrinsic fluorescence to study Au₁₀SG₁₀ cellular distribution because they do not fluoresce. Lack of luminescent properties limited the options of simple techniques to determine the binding constants. Attractive highly fluorescent and stable AuNCs with a multi-shell of ligands could be suitable for the determination of binding constants and merit further investigations. Such highly luminescent nanoclusters with a multi-shell of ligands could be produced either by using zwitterionic ligands^{5,84} or by introducing L-arginine into the capping layer.⁸⁵

We found that under oxidative stress induced by BSO and in the presence of Au₁₀SG₁₀, HMGB1 is translocated from the cell nucleus to the cytosol, favoring its deleterious rather than chaperoning effect. The formation of the disulfide bridge upon oxidation makes the oxiHMGB1 structure closely packed. This allows a greater number of interactions between the oxidizable site of oxiHMGB1 and Au₁₀SG₁₀. The more connected complex of oxiHMGB1 and Au₁₀SG₁₀ could help to induce further oxidation.

Computational studies, super-resolution imaging and EM tomography with atomic resolution combined with different cellular biomarkers should be considered in future investigations of AuNCs and their effects on other redox-responsive transcription factors aside from HMGB1. Computational and experimental studies are warranted to reveal interactions sites and binding constants for other AuNCs and other proteins, including redox-responsive transcription factors. Considering that AuNCs exert enzyme-like properties, future MM calculations could point to AuNC modifications with optimal properties to reduce oxidative stress in different cells and restore homeostatic balance in the nervous system.

Experimental and theoretical

Synthesis and characterization of Au₁₀SG₁₀ and Au₂₅SG₁₈

All the chemicals were commercially available and were used without purification. HAuCl₄•3H₂O, trifluoroacetic acid (TFA), methanol (HPLC grade), and GSH (γ-Glu-Cys-Gly, MW 307) were purchased from Carl Roth. NaOH and NH₄OH were purchased from Sigma-Aldrich. Milli-Q water with a resistivity of 18.2 MΩ cm was used for all experiments.

Au₁₀SG₁₀ nanoclusters were synthesized as reported by Bertorelle et al.¹⁹ Briefly, 235 mg of glutathione was dissolved in 35 mL of methanol and 2 mL of triethylamine. Then, 100 mg of HAuCl₄•3H₂O was added in 15 mL of water. After stirring the solution overnight at ambient temperature, unwanted products were removed with several cycles of dissolution/precipitation/centrifugation. The formation of atomically precise Au₁₀SG₁₀ NCs as the product was confirmed by UV-vis absorption and electrospray ionization mass spectrometry (ESI-MS) (see Figure 1). The linear optical absorption spec-

trum of the as-synthesized Au₁₀SG₁₀ NCs shows a monotonic increase of absorption below 410 nm and shoulders at 320 and 370 nm. Mass spectrum of the as-synthesized Au₁₀SG₁₀ displays charge distribution between 4- and 6- charge states of Au₁₀SG₁₀. Au₂₅(SG)₁₈ nanoclusters were synthesized as reported by Gran et al.³⁴

Human microglial HMC3 cell culture

The immortalized human microglia, HMC3, were originally obtained from American Type Culture Collection (ATCC). HMC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific; Grand Island, NY, USA) containing penicillin-streptomycin (P/S; 1% v/v; Fisher Scientific, Eugene, OR, USA) and supplemented with fetal bovine serum (FBS, 5% v/v; Wisent, St. Bruno, Canada), unless otherwise indicated. To detach cells from the cell culture flask, cells were incubated in Trypsin-EDTA (0.25%; Thermo Fisher Scientific) at room temperature for 2 minutes. Fresh DMEM supplemented with FBS (5% v/v) was added to deactivate trypsin, followed by centrifugation. Supernatant was aspirated and cell pellet was resuspended in fresh DMEM supplemented with FBS (5% v/v), seeded accordingly depending on the experiment, and incubated at 37 °C with 5% CO₂ at 95% relative humidity.

Treatments

Cells were incubated (pre-treatment) in serum-deprived DMEM (0% FBS; 1% P/S) in the presence or absence of L-buthionine-sulfoximine (100 μM, BSO; Sigma, B2515) for 24 hours. For the following 24 hours, cells were treated with AuNCs (Au₁₀SG₁₀ or Au₂₅SG₁₈) at indicated concentrations in fresh serum-deprived DMEM (0% FBS; 1% P/S). Lipopolysaccharide (LPS; 10 ng/mL; Sigma L4391) was used as a positive control to induce HMGB1 nuclear-to-cytosolic translocation. MG-132 (0.5 μM; Enzo Life Sciences) protease inhibitor was used as a positive control to induce protein aggregation.

Cell counting

Cells were seeded 5,000 cells per well in a black 96-well plate (Costar; Corning, NY, USA) and left to adhere overnight. Cells were treated in triplicates. Cells were fixed with paraformaldehyde (PFA; 4%, 10 minutes) at room temperature. PFA was aspirated and cells were incubated with Hoechst 33342 (10 μM, 10 minutes; Fisher Scientific; Eugene, OR, USA). Cells were washed once with PBS and imaged with a 10x objective using Leica DMI4000B fluorescence microscope (Leica microsystem; Toronto, ON, Canada). Nuclei were counted using ImageJ's multi-point tool.⁸⁶

MTT assay

Cells were seeded 5,000 cells per well in a clear 96-well plate and left to adhere overnight. Cells were treated in triplicates. Cells were incubated with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL, 30 minutes; Sigma-Aldrich) diluted in DMEM. After half an hour, the media was aspirated and the formazan product was solubilized using dimethyl sulfoxide (DMSO). The absorbance of the formazan solution was measured at 595 nm using Tecan Spark 10M microplate reader (Tecan, Männedorf, Switzerland).

LDH cytotoxicity assay

Cells were seeded 5,000 cells per well in a clear 96-well plate and left to adhere overnight. Cells were treated in triplicates. Media samples were collected and centrifuged at 14,000 rpm for 5 minutes at 4°C. Cytotoxicity was quantified using a LDH

cytotoxicity detection kit (Roche Diagnostics Corporation, 11644793). Substrate mix was added to samples and incubated for 30 minutes at room temperature. Absorbance was measured at 492 nm using Tecan Spark 10M microplate reader.

Immunocytochemistry

Cells were seeded at 15,000 cells per 300 μ L on glass coverslips (12 mm diameter). Cells were left to adhere overnight before treatment. Cells were fixed with PFA (4%; 10 minutes) and permeabilized with Triton X-100 (0.1%; 10 minutes). Cells were blocked in filtered goat serum (10% v/v; 1 hour; Gibco) in phosphate buffered saline (PBS). Subsequently, cells were incubated with primary antibodies overnight at 4°C: rabbit anti-HMGB1 (1:500; Abcam, ab18256), rabbit anti-acetylated-HMGB1 (1:500; MyBioSource, MBS9404216). Following overnight incubation, cells were washed three times with PBS and incubated with secondary antibodies overnight at 4°C: goat anti-rabbit (1:500; AlexaFluor®647 anti-rabbit IgG; Life Technologies, A21244). Cells were washed three more times. F-actin was labeled with Phalloidin Alexa Fluor 488 (1:50, 20 minutes; Thermo Fisher Scientific). Cells were washed three times and nuclei were labeled with Hoechst 33342 (10 μ M, 10 minutes). Coverslips were mounted on Superfrost microscope slides (Thermo Fisher Scientific) using Aqua-Poly mount (PolySciences; Warrington, PA, USA) and left to dry (min. 90 minutes) before imaging. Coverslips were imaged using Leica DMI4000B fluorescence microscope. Fluorescence intensities were measured in ImageJ²⁸ and corrected fluorescence intensity was calculated by subtracting background fluorescence.

ProteoStat assay

The assay followed the manufacturer's protocol with minor modifications. Specifically, the ProteoStat reagent (1:6000; Enzo Life Sciences; ENZ51035; Farmingdale, NY, USA) was incubated for 2 hours at room temperature.

Mass spectrometry

Using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), quality check was performed on Au₁₀SG₁₀ and HMGB1. An aliquot of 1 mg/mL Au₁₀SG₁₀ was mixed with HCCA matrix (alpha-cyano-4-hydroxycinnamic acid) and spectrum was acquired using reflectron positive mode of ionization of the MALDI-TOF. In parallel, 1 mg/mL stock of human recombinant HMGB1 (Abcam, ab167718) was mixed with super-DHB matrix (2,5-dihydroxyl benzoic acid and 2-hydroxy-5-methoxybenzoic acid) and spectrum was acquired using linear positive mode of ionization of the MALDI-TOF. To investigate the interaction between HMGB1 with Au₁₀SG₁₀, 1mg/mL of HMGB1 (4.5 μ L) was incubated with 1mg of Au₁₀SG₁₀ (1.5 μ L) for 8hours at 37°C. An aliquot was mixed with equal portion of super-DHB matrix and mass spectrum was acquired using MALDI-TOF. To identify the amino acids that could be involved in the interaction between HMGB1 and the Au₁₀SG₁₀, this experiment was repeated and just after incubation, limited proteolysis was carried out using trypsin at 37°C as previously described (Barucker, 2015, Scientific reports). Generated peptides were analyzed using MALDI-TOF and matched with in-silico tryptic digested HMGB1 carried out by sequence editor software (Bruker Daltonics) and aligned using the Biotools software (Bruker Daltonics).

Statistical analysis

Statistical significance was determined using Student's t-test. For multiple comparisons, the Bonferroni correction was applied. P-values less than 0.05 were indicated as significant: *p<0.05, **p<0.01, ***p<0.001.

Computational Section

• Model of HMGB1 protein

From the Protein Data Base (PDB), we have found NMR structure of redHMGB1 (pdb: 2YRQ) which demonstrates high flexibility of A box domain (gray color) relative to B box domain (beige color in Figure 1). But, each domain remains relatively conserved with 3 α -helices each. For this reason, we decided to freeze atoms from the B box domain (from residues Ser107 to Gly173) while simulating interaction between residues from A box domain and AuNC. Oxidized crystal structure of the HMGB1 was taken from PDB (pdb:2RTU). It contains only atoms from the A box domain, and it was included in the quantum mechanics-molecular mechanics (QM/MM) simulation without freezing any atoms.

• Au₁₀NC model

The initial model of Au₁₀SG₁₀ was adopted from the crystal structure of Au₁₀L₁₀^{19,87} and Au₂₅L₁₈²⁰ and reoptimized using PBE and SVP level of theory.

QM/MM (PM7;UFF)⁸⁸ studies included 3 different systems: HMGB1 (reduced, oxidized and acetylated) in a proximity to Au₁₀SG₁₀

• Modeling of the post-translational modifications

Post-translational modifications investigated in this work include oxidation and acetylation. These modifications were introduced to the structure of HMGB1 using Chimera⁸⁹ and its Structure Editing tool in order to replace oxygen and hydrogen with specific atoms.

• Possible Oxidized Residues: C, M, Y, H, W

Focusing on the A box domain residues, we explored which residues can be in the oxidized and reduced forms. In the A box domain these include: C30, C52, M8, M20, M59, M70, M82, Y23, Y78, Y85, H34, H38, W56.

Taking into account the size of the Au₁₀SG₁₀ and the surface availability of the possible oxidized residues we chose to induce PTMs to Tyr78, Met82 and Tyr85. We modeled possible interaction sites between HMGB1 and Au₁₀SG₁₀ as shown in Figure 3. Au₁₀SG₁₀ is placed close to the site with three residues that can be in reduced and oxidized form. The complex was optimized using QM/MM (PM7;UFF)⁸⁸ level of theory where side chains of residues Tyr78, Met82 and Tyr85 and Au₁₀SG₁₀ are forming QM part of the system, and the rest of the protein is in the MM part.

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Author Contributions

DM and VBK conceived the initial idea and coordinated the work. RA coordinated and supervised AuNCs preparation and characterization. ERG performed all biological experiments and analyses. MPB and ŽSM performed computation for the AuNC binding to HMGB1 and wrote sections related to it. AS

and GM provided MS/MS analyses and discussion. JRM provided dark field and hyperspectral images. IZ performed ROS and aggresome analyses. RA, VBK, ERG and DM wrote the paper. All authors provided critical feedback and helped to shape the final manuscript.

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Conflicts of Interest: None.

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ABBREVIATIONS

HMGB1, high mobility group box 1; AuNCs, gold nanoclusters; PTMs, post-translational modifications; BSO, L-buthionine-sulfoximine; SASA, solvent accessible surface area; redHMGB1, reduced HMGB1; oxiHMGB1, oxidized HMGB1; AchHMGB1, acetylated; 4-HNE, 4-hydroxynonenal; DAMP, damage-associated molecular pattern; ESI-MS, Electrospray Ionization Mass Spectrometry; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, Lipopolysaccharide; PFA, paraformaldehyde; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; PDB, Protein Data Base; QM, quantum mechanics; QM/MM, quantum mechanics-molecular mechanics.

REFERENCES

- (1) Su, Y.; Xue, T.; Liu, Y.; Qi, J.; Jin, R.; Lin, Z. Luminescent Metal Nanoclusters for Biomedical Applications. *Nano Res.* **2019**, *12* (6), 1251–1265. <https://doi.org/10.1007/s12274-019-2314-y>.
- (2) Holzinger, M.; Le Goff, A.; Cosnier, S. Nanomaterials for Biosensing Applications: A Review. *Frontiers in Chemistry* **2014**, *2*, 63. <https://doi.org/10.3389/fchem.2014.00063>.
- (3) Jin, R. Quantum Sized, Thiolate -Protected Gold Nanoclusters. *Nanoscale* **2010**, *2* (3), 343–362. <https://doi.org/10.1039/B9NR00160C>.
- (4) Zhou, M.; Jin, R. Optical Properties and Excited-State Dynamics of Atomically Precise Gold Nanoclusters. *Annual Review of Physical Chemistry* **2021**, *72* (1), 121–142. <https://doi.org/10.1146/annurev-physchem-090419-104921>.
- (5) Shen, D.; Henry, M.; Trouillet, V.; Comby-Zerbino, C.; Bertorelle, F.; Sancey, L.; Antoine, R.; Coll, J.-L.; Josserand, V.; Le Guével, X. Zwitterion Functionalized Gold Nanoclusters for Multimodal near Infrared Fluorescence and Photoacoustic Imaging. *APL Materials* **2017**, *5* (5), 053404. <https://doi.org/10.1063/1.4977203>.
- (6) Zheng, Y.; Wu, J.; Jiang, H.; Wang, X. Gold Nanoclusters for Theranostic Applications. *Coordination Chemistry Reviews* **2021**, *431*, 213689. <https://doi.org/10.1016/j.ccr.2020.213689>.
- (7) Zhang, L.; Wang, E. Metal Nanoclusters: New Fluorescent Probes for Sensors and Bioimaging. *Nano Today* **2014**, *9* (1), 132–157. <https://doi.org/10.1016/j.nantod.2014.02.010>.
- (8) Olesiak-Banska, J.; Waszkielewicz, M.; Obstarczyk, P.; Samoc, M. Two-Photon Absorption and Photoluminescence of Colloidal Gold Nanoparticles and Nanoclusters. *Chem Soc Rev* **2019**, *48* (15), 4087–4117. <https://doi.org/10.1039/c8cs00849c>.
- (9) Zhang, Y.; Zhang, C.; Xu, C.; Wang, X.; Liu, C.; Waterhouse, G. I. N.; Wang, Y.; Yin, H. Ultrasmall Au Nanoclusters for Biomedical and Biosensing Applications: A Mini-Review. *Talanta* **2019**, *200*, 432–442. <https://doi.org/10.1016/j.talanta.2019.03.068>.
- (10) Wang, J.; Zhang, G.; Li, Q.; Jiang, H.; Liu, C.; Amatore, C.; Wang, X. In Vivo Self-Bio-Imaging of Tumors through in Situ Biosynthesized Fluorescent Gold Nanoclusters. *Sci Rep* **2013**, *3* (1), 1157. <https://doi.org/10.1038/srep01157>.
- (11) Zheng, Y.; Lai, L.; Liu, W.; Jiang, H.; Wang, X. Recent Advances in Biomedical Applications of Fluorescent Gold Nanoclusters. *Adv Colloid Interface Sci* **2017**, *242*, 1–16. <https://doi.org/10.1016/j.cis.2017.02.005>.
- (12) Ao, H.; Feng, H.; Zhao, M.; Zhao, M.; Chen, J.; Qian, Z. Redox-Triggered Bonding-Induced Emission of Thiol-Functionalized Gold Nanoclusters for Lu-

- minescence Turn-On Detection of Molecular Oxygen. *ACS Sens* **2017**, 2 (11), 1692–1699. <https://doi.org/10.1021/acssensors.7b00643>.
- (13) Jin, R.; Zeng, C.; Zhou, M.; Chen, Y. Atomically Precise Colloidal Metal Nanoclusters and Nanoparticles: Fundamentals and Opportunities. *Chem. Rev.* **2016**, 116 (18), 10346–10413. <https://doi.org/10.1021/acs.chemrev.5b00703>.
- (14) Zeng, C.; Chen, Y.; Das, A.; Jin, R. Transformation Chemistry of Gold Nanoclusters: From One Stable Size to Another. *J. Phys. Chem. Lett.* **2015**, 6 (15), 2976–2986. <https://doi.org/10.1021/acs.jpcllett.5b01150>.
- (15) Jung, J.; Kang, S.; Han, Y.-K. Ligand Effects on the Stability of Thiol-Stabilized Gold Nanoclusters: Au₂₅(SR)₁₈–, Au₃₈(SR)₂₄, and Au₁₀₂(SR)₄₄. *Nanoscale* **2012**, 4 (14), 4206–4210. <https://doi.org/10.1039/C2NR30501A>.
- (16) Cantelli, A.; Guidetti, G.; Manzi, J.; Caponetti, V.; Montalti, M. Towards Ultra-Bright Gold Nanoclusters. *European Journal of Inorganic Chemistry* **2017**, 2017 (44), 5068–5084. <https://doi.org/10.1002/ejic.201700735>.
- (17) Zhang, Q.; Yang, M.; Zhu, Y.; Mao, C. Metallic Nanoclusters for Cancer Imaging and Therapy. *Curr Med Chem* **2018**, 25 (12), 1379–1396. <https://doi.org/10.2174/0929867324666170331122757>.
- (18) Porret, E.; Guével, X. L.; Coll, J.-L. Gold Nanoclusters for Biomedical Applications: Toward in Vivo Studies. *Journal of Materials Chemistry B* **2020**, 8 (11), 2216–2232. <https://doi.org/10.1039/C9TB02767J>.
- (19) Bertorelle, F.; Russier-Antoine, I.; Calin, N.; Comby-Zerbino, C.; Bensalah-Ledoux, A.; Guy, S.; Dugourd, P.; Brevet, P.-F.; Sanader, Ž.; Krstić, M.; Bonačić-Koutecký, V.; Antoine, R. Au₁₀(SG)₁₀: A Chiral Gold Catenane Nanocluster with Zero Confined Electrons. Optical Properties and First-Principles Theoretical Analysis. *J. Phys. Chem. Lett.* **2017**, 8 (9), 1979–1985. <https://doi.org/10.1021/acs.jpcllett.7b00611>.
- (20) Heaven, M. W.; Dass, A.; White, P. S.; Holt, K. M.; Murray, R. W. Crystal Structure of the Gold Nanoparticle [N(C₈H₁₇)₄][Au₂₅(SCH₂CH₂Ph)₁₈]. *J. Am. Chem. Soc.* **2008**, 130 (12), 3754–3755. <https://doi.org/10.1021/ja800561b>.
- (21) Zhu, M.; Aikens, C. M.; Hollander, F. J.; Schatz, G. C.; Jin, R. Correlating the Crystal Structure of a Thiol-Protected Au₂₅ Cluster and Optical Properties. *J. Am. Chem. Soc.* **2008**, 130 (18), 5883–5885. <https://doi.org/10.1021/ja801173r>.
- (22) Akola, J.; Walter, M.; Whetten, R. L.; Häkkinen, H.; Grönbeck, H. On the Structure of Thiolate-Protected Au₂₅. *J. Am. Chem. Soc.* **2008**, 130 (12), 3756–3757. <https://doi.org/10.1021/ja800594p>.
- (23) Tambuyzer, B. R.; Ponsaerts, P.; Nouwen, E. J. Microglia: Gatekeepers of Central Nervous System Immunology. *Journal of Leukocyte Biology* **2009**, 85 (3), 352–370. <https://doi.org/10.1189/jlb.0608385>.
- (24) Maysinger, D.; Zhang, I. Nutritional and Nanotechnological Modulators of Microglia. *Front Immunol* **2016**, 7, 270. <https://doi.org/10.3389/fimmu.2016.00270>.
- (25) Rojo, A. I.; McBean, G.; Cindric, M.; Egea, J.; López, M. G.; Rada, P.; Zarkovic, N.; Cuadrado, A. Redox Control of Microglial Function: Molecular Mechanisms and Functional Significance. *Antioxid Redox Signal* **2014**, 21 (12), 1766–1801. <https://doi.org/10.1089/ars.2013.5745>.
- (26) Lee, M.; Cho, T.; Jantaratnotai, N.; Wang, Y. T.; McGeer, E.; McGeer, P. L. Depletion of GSH in Glial Cells Induces Neurotoxicity: Relevance to Aging and Degenerative Neurological Diseases. *FASEB J* **2010**, 24 (7), 2533–2545. <https://doi.org/10.1096/fj.09-149997>.
- (27) Davis, B. M.; Salinas-Navarro, M.; Cordeiro, M. F.; Moons, L.; De Groef, L. Characterizing Microglia Activation: A Spatial Statistics Approach to Maximize Information Extraction. *Sci Rep* **2017**, 7 (1), 1576. <https://doi.org/10.1038/s41598-017-01747-8>.
- (28) Salter, M. W.; Stevens, B. Microglia Emerge as Central Players in Brain Disease.

- Nat Med* **2017**, *23* (9), 1018–1027. <https://doi.org/10.1038/nm.4397>.
- (29) Hanisch, U.-K. Microglia as a Source and Target of Cytokines. *Glia* **2002**, *40* (2), 140–155. <https://doi.org/10.1002/glia.10161>.
- (30) Cherry, J. D.; Olschowka, J. A.; O'Banion, M. K. Neuroinflammation and M2 Microglia: The Good, the Bad, and the Inflamed. *Journal of Neuroinflammation* **2014**, *11* (1), 98. <https://doi.org/10.1186/1742-2094-11-98>.
- (31) Maysinger, D.; Gran, E. R.; Bertorelle, F.; Fakhouri, H.; Antoine, R.; Kaul, E. S.; Samhadaneh, D. M.; Stochaj, U. Gold Nanoclusters Elicit Homeostatic Perturbations in Glioblastoma Cells and Adaptive Changes of Lysosomes. *Theranostics* **2020**, *10* (4), 1633–1648. <https://doi.org/10.7150/thno.37674>.
- (32) Tang, Y.; Zhao, X.; Antoine, D.; Xiao, X.; Wang, H.; Andersson, U.; Billiar, T. R.; Tracey, K. J.; Lu, B. Regulation of Post-translational Modifications of HMGB1 During Immune Responses. *Antioxid. Redox Signal.* **2016**, *24* (12), 620–634. <https://doi.org/10.1089/ars.2015.6409>.
- (33) Andersson, U.; Yang, H.; Harris, H. High-Mobility Group Box 1 Protein (HMGB1) Operates as an Alarmin Outside as Well as inside Cells. *Semin Immunol* **2018**, *38*, 40–48. <https://doi.org/10.1016/j.smim.2018.02.011>.
- (34) Gran, E. R.; Bertorelle, F.; Fakhouri, H.; Antoine, R.; Bakulić, M. P.; Maršić, Ž. S.; Bonačić-Koutecký, V.; Blain, M.; Antel, J.; Maysinger, D. Size and Ligand Effects of Gold Nanoclusters in Alteration of Organellar State and Translocation of Transcription Factors in Human Primary Astrocytes. *Nanoscale* **2021**, *13* (5), 3173–3183. <https://doi.org/10.1039/D0NR06401G>.
- (35) Guével, X. L.; Henry, M.; Motto-Ros, V.; Longo, E.; Montañez, M. I.; Pelascini, F.; Rochefoucauld, O. de L.; Zeitoun, P.; Coll, J.-L.; Josserand, V.; Sancey, L. Elemental and Optical Imaging Evaluation of Zwitterionic Gold Nanoclusters in Glioblastoma Mouse Models. *Nanoscale* **2018**, *10* (39), 18657–18664. <https://doi.org/10.1039/C8NR05299A>.
- (36) Nandi, S.; Ghosh, S.; Bhattacharyya, K. Live Cell Microscopy: A Physical Chemistry Approach. *J Phys Chem B* **2018**, *122* (12), 3023–3036. <https://doi.org/10.1021/acs.jpcc.7b11689>.
- (37) Li, Q.; Pan, Y.; Chen, T.; Du, Y.; Ge, H.; Zhang, B.; Xie, J.; Yu, H.; Zhu, M. Design and Mechanistic Study of a Novel Gold Nanocluster-Based Drug Delivery System. *Nanoscale* **2018**, *10* (21), 10166–10172. <https://doi.org/10.1039/C8NR02189A>.
- (38) Griffith, O. W.; Meister, A. Potent and Specific Inhibition of Glutathione Synthesis by Buthionine Sulfoximine (S-n-Butyl Homocysteine Sulfoximine). *J Biol Chem* **1979**, *254* (16), 7558–7560.
- (39) Griffith, O. W. Mechanism of Action, Metabolism, and Toxicity of Buthionine Sulfoximine and Its Higher Homologs, Potent Inhibitors of Glutathione Synthesis. *J Biol Chem* **1982**, *257* (22), 13704–13712.
- (40) Farah, M. E.; Sirotkin, V.; Haarer, B.; Kakhniashvili, D.; Amberg, D. C. Diverse Protective Roles of the Actin Cytoskeleton during Oxidative Stress. *Cytoskeleton (Hoboken)* **2011**, *68* (6), 340–354. <https://doi.org/10.1002/cm.20516>.
- (41) Xu, Q.; Huff, L. P.; Fujii, M.; Griendling, K. K. Redox Regulation of the Actin Cytoskeleton and Its Role in the Vascular System. *Free Radic Biol Med* **2017**, *109*, 84–107. <https://doi.org/10.1016/j.freeradbiomed.2017.03.004>.
- (42) Kwak, M. S.; Kim, H. S.; Lkhamsuren, K.; Kim, Y. H.; Han, M. G.; Shin, J. M.; Park, I. H.; Rhee, W. J.; Lee, S. K.; Rhee, S. G.; Shin, J.-S. Peroxiredoxin-Mediated Disulfide Bond Formation Is Required for Nucleocytoplasmic Translocation and Secretion of HMGB1 in Response to Inflammatory Stimuli. *Redox Biology* **2019**, *24*, 101203. <https://doi.org/10.1016/j.redox.2019.101203>.
- (43) Wang, J.; Tochio, N.; Takeuchi, A.; Uewaki, J.; Kobayashi, N.; Tate, S. Redox-Sensitive Structural Change in the A-

- Domain of HMGB1 and Its Implication for the Binding to Cisplatin Modified DNA. *Biochemical and Biophysical Research Communications* **2013**, *441* (4), 701–706. <https://doi.org/10.1016/j.bbrc.2013.10.085>.
- (44) Bonaldi, T.; Talamo, F.; Scaffidi, P.; Ferrera, D.; Porto, A.; Bachi, A.; Rubartelli, A.; Agresti, A.; Bianchi, M. E. Monocytic Cells Hyperacetylate Chromatin Protein HMGB1 to Redirect It towards Secretion. *EMBO J* **2003**, *22* (20), 5551–5560. <https://doi.org/10.1093/emboj/cdg516>.
- (45) Zhang, X.-D.; Chen, J.; Luo, Z.; Wu, D.; Shen, X.; Song, S.-S.; Sun, Y.-M.; Liu, P.-X.; Zhao, J.; Huo, S.; Fan, S.; Fan, F.; Liang, X.-J.; Xie, J. Enhanced Tumor Accumulation of Sub-2 Nm Gold Nanoclusters for Cancer Radiation Therapy. *Adv Health Mater* **2014**, *3* (1), 133–141. <https://doi.org/10.1002/adhm.201300189>.
- (46) Vankayala, R.; Kuo, C.-L.; Nuthalapati, K.; Chiang, C.-S.; Hwang, K. C. Nucleus-Targeting Gold Nanoclusters for Simultaneous In Vivo Fluorescence Imaging, Gene Delivery, and NIR-Light Activated Photodynamic Therapy. *Advanced Functional Materials* **2015**, *25* (37), 5934–5945. <https://doi.org/10.1002/adfm.201502650>.
- (47) Abdulmahdi, W.; Patel, D.; Rabadi, M. M.; Azar, T.; Jules, E.; Lipphardt, M.; Hashemiyoona, R.; Ratliff, B. B. HMGB1 Redox during Sepsis. *Redox Biol* **2017**, *13*, 600–607. <https://doi.org/10.1016/j.redox.2017.08.001>.
- (48) Pickard, M. R.; Chari, D. M. Robust Uptake of Magnetic Nanoparticles (MNPs) by Central Nervous System (CNS) Microglia: Implications for Particle Uptake in Mixed Neural Cell Populations. *International Journal of Molecular Sciences* **2010**, *11* (3), 967–981. <https://doi.org/10.3390/ijms11030967>.
- (49) Choi, J.; Zheng, Q.; Katz, H. E.; Guilarte, T. R. Silica-Based Nanoparticle Uptake and Cellular Response by Primary Microglia. *Environ. Health Perspect.* **2010**, *118* (5), 589–595. <https://doi.org/10.1289/ehp.0901534>.
- (50) Papa, S.; Ferrari, R.; De Paola, M.; Rossi, F.; Mariani, A.; Caron, I.; Sammali, E.; Peviani, M.; Dell’Oro, V.; Colombo, C.; Morbidelli, M.; Forloni, G.; Perale, G.; Moscatelli, D.; Veglianesi, P. Polymeric Nanoparticle System to Target Activated Microglia/Macrophages in Spinal Cord Injury. *Journal of Controlled Release* **2014**, *174*, 15–26. <https://doi.org/10.1016/j.jconrel.2013.11.001>.
- (51) Hutter, E.; Boridy, S.; Labrecque, S.; Lalancette-Hébert, M.; Kriz, J.; Winnik, F. M.; Maysinger, D. Microglial Response to Gold Nanoparticles. *ACS Nano* **2010**, *4* (5), 2595–2606. <https://doi.org/10.1021/nn901869f>.
- (52) Kettenmann, H.; Hanisch, U.-K.; Noda, M.; Verkhratsky, A. Physiology of Microglia. *Physiol Rev* **2011**, *91* (2), 461–553. <https://doi.org/10.1152/physrev.00011.2010>.
- (53) Shahidehpour, R. K.; Higdon, R. E.; Crawford, N. G.; Neltner, J. H.; Ighodaro, E. T.; Patel, E.; Price, D.; Nelson, P. T.; Bachstetter, A. D. Dystrophic Microglia Are Associated with Neurodegenerative Disease and Not Healthy Aging in the Human Brain. *Neurobiol Aging* **2021**, *99*, 19–27. <https://doi.org/10.1016/j.neurobiolaging.2020.12.003>.
- (54) Ochocka, N.; Kaminska, B. Microglia Diversity in Healthy and Diseased Brain: Insights from Single-Cell Omics. *Int J Mol Sci* **2021**, *22* (6), 3027. <https://doi.org/10.3390/ijms22063027>.
- (55) Fujita, Y.; Yamashita, T. Neuroprotective Function of Microglia in the Developing Brain. *Neuronal Signal* **2021**, *5* (1), NS20200024. <https://doi.org/10.1042/NS20200024>.
- (56) Schaur, R. J.; Siems, W.; Bresgen, N.; Eckl, P. M. 4-Hydroxy-Nonenal-A Bioactive Lipid Peroxidation Product. *Biomolecules* **2015**, *5* (4), 2247–2337. <https://doi.org/10.3390/biom5042247>.
- (57) Csala, M.; Kardon, T.; Legeza, B.; Lizák, B.; Mandl, J.; Margittai, É.; Puskás, F.; Száraz, P.; Szelényi, P.; Bánhegyi, G. On the Role of 4-Hydroxynonenal in Health and Disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2015**, *1852* (5), 826–838.

<https://doi.org/10.1016/j.bbadis.2015.01.015>

- (58) Jiang, X.; Stockwell, B. R.; Conrad, M. Ferroptosis: Mechanisms, Biology and Role in Disease. *Nat Rev Mol Cell Biol* **2021**, *22* (4), 266–282. <https://doi.org/10.1038/s41580-020-00324-8>.
- (59) Weiland, A.; Wang, Y.; Wu, W.; Lan, X.; Han, X.; Li, Q.; Wang, J. Ferroptosis and Its Role in Diverse Brain Diseases. *Mol Neurobiol* **2019**, *56* (7), 4880–4893. <https://doi.org/10.1007/s12035-018-1403-3>.
- (60) Ren, J.-X.; Sun, X.; Yan, X.-L.; Guo, Z.-N.; Yang, Y. Ferroptosis in Neurological Diseases. *Frontiers in Cellular Neuroscience* **2020**, *14*, 218. <https://doi.org/10.3389/fncel.2020.00218>.
- (61) Paudel, Y. N.; Shaikh, Mohd. F.; Chakraborti, A.; Kumari, Y.; Aledo-Serrano, Á.; Aleksovska, K.; Alvim, M. K. M.; Othman, I. HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction. *Frontiers in Neuroscience* **2018**, *12*, 628. <https://doi.org/10.3389/fnins.2018.00628>.
- (62) Tang, D.; Kang, R.; Zeh, H. J.; Lotze, M. T. High-Mobility Group Box 1, Oxidative Stress, and Disease. *Antioxid Redox Signal* **2011**, *14* (7), 1315–1335. <https://doi.org/10.1089/ars.2010.3356>.
- (63) Fang, P.; Schachner, M.; Shen, Y.-Q. HMGB1 in Development and Diseases of the Central Nervous System. *Mol Neurobiol* **2012**, *45* (3), 499–506. <https://doi.org/10.1007/s12035-012-8264-y>.
- (64) Huo, S.; Jiang, Y.; Jiang, Z.; F. Landis, R.; Liang, X.-J.; M. Rotello, V. Stable and Oxidant Responsive Zwitterionic Nanoclusters. *Nanoscale* **2018**, *10* (16), 7382–7386. <https://doi.org/10.1039/C7NR08951A>.
- (65) Hu, L.; Deng, L.; Alsaiani, S.; Zhang, D.; Khashab, N. M. “Light-on” Sensing of Antioxidants Using Gold Nanoclusters. *Anal. Chem.* **2014**, *86* (10), 4989–4994. <https://doi.org/10.1021/ac500528m>.
- (66) Reeves, R. Nuclear Functions of the HMG Proteins. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* **2010**, *1799* (1), 3–14. <https://doi.org/10.1016/j.bbagr.2009.09.001>.
- (67) Lange, S. S.; Vasquez, K. M. HMGB1: The Jack-of-All-Trades Protein Is a Master DNA Repair Mechanic. *Molecular Carcinogenesis* **2009**, *48* (7), 571–580. <https://doi.org/10.1002/mc.20544>.
- (68) Janko, C.; Filipović, M.; Munoz, L. E.; Schorn, C.; Schett, G.; Ivanović-Burmazović, I.; Herrmann, M. Redox Modulation of HMGB1-Related Signaling. *Antioxidants & Redox Signaling* **2014**, *20* (7), 1075–1085. <https://doi.org/10.1089/ars.2013.5179>.
- (69) Krisko, A.; Radman, M. Protein Damage, Ageing and Age-Related Diseases. *Open Biology* **9** (3), 180249. <https://doi.org/10.1098/rsob.180249>.
- (70) Tanaka, M.; Komi, Y. Layers of Structure and Function in Protein Aggregation. *Nat Chem Biol* **2015**, *11* (6), 373–377. <https://doi.org/10.1038/nchembio.1818>.
- (71) Grimm, S.; Höhn, A.; Grune, T. Oxidative Protein Damage and the Proteasome. *Amino Acids* **2012**, *42* (1), 23–38. <https://doi.org/10.1007/s00726-010-0646-8>.
- (72) Cox, D.; Raeburn, C.; Sui, X.; Hatters, D. M. Protein Aggregation in Cell Biology: An Aggregomics Perspective of Health and Disease. *Semin Cell Dev Biol* **2020**, *99*, 40–54. <https://doi.org/10.1016/j.semcdb.2018.05.003>.
- (73) Falsone, A.; Falsone, S. F. Legal but Lethal: Functional Protein Aggregation at the Verge of Toxicity. *Frontiers in Cellular Neuroscience* **2015**, *9*, 45. <https://doi.org/10.3389/fncel.2015.00045>.
- (74) Combes, G. F.; Fakhouri, H.; Moulin, C.; Girod, M.; Bertorelle, F.; Basu, S.; Ladouce, R.; Bakulić, M. P.; Maršić, Ž. S.; Russier-Antoine, I.; Brevet, P.-F.; Dugourd, P.; Krisko, A.; Trajković, K.; Radman, M.; Bonačić-Koutecký, V.; Antoine, R. Functionalized Au15 Nanoclusters as Luminescent Probes for Protein Carbonylation Detection. *Commun Chem* **2021**, *4* (1), 1–11. <https://doi.org/10.1038/s42004-021-00497-z>.

- (75) Lavenn, C.; Albrieux, F.; Tuel, A.; Demessence, A. Synthesis, Characterization and Optical Properties of an Amino-Functionalized Gold Thiolate Cluster: Au₁₀(SPh-PNH₂)₁₀. *Journal of Colloid and Interface Science* **2014**, *418*, 234–239. <https://doi.org/10.1016/j.jcis.2013.12.021>.
- (76) Basu, S.; Fakhouri, H.; Moulin, C.; Dolai, S.; Russier-Antoine, I.; Brevet, P.-F.; Antoine, R.; Paul, A. Four Orders-of-Magnitude Enhancement in the Two-Photon Excited Photoluminescence of Homoleptic Gold Thiolate Nanoclusters Following Zinc Ion-Induced Aggregation. *Nanoscale* **2021**, *13* (8), 4439–4443. <https://doi.org/10.1039/D0NR08764E>.
- (77) Schuller, A. P.; Wojtynek, M.; Mankus, D.; Tatli, M.; Kronenberg-Tenga, R.; Regmi, S. G.; Dip, P. V.; Lytton-Jean, A. K. R.; Brignole, E. J.; Dasso, M.; Weis, K.; Medalia, O.; Schwartz, T. U. The Cellular Environment Shapes the Nuclear Pore Complex Architecture. *Nature* **2021**, *598* (7882), 667–671. <https://doi.org/10.1038/s41586-021-03985-3>.
- (78) Yang, H.; Wang, H.; Andersson, U. Targeting Inflammation Driven by HMGB1. *Frontiers in Immunology* **2020**, *11*, 484. <https://doi.org/10.3389/fimmu.2020.00484>.
- (79) Kwak, M. S.; Kim, H. S.; Lee, B.; Kim, Y. H.; Son, M.; Shin, J.-S. Immunological Significance of HMGB1 Post-Translational Modification and Redox Biology. *Frontiers in Immunology* **2020**, *11*, 1189. <https://doi.org/10.3389/fimmu.2020.01189>.
- (80) Qi, Z.; Zhang, Y.; Qi, S.; Ling, L.; Gui, L.; Yan, L.; Lv, J.; Li, Q. Salidroside Inhibits HMGB1 Acetylation and Release through Upregulation of SirT1 during Inflammation. *Oxidative Medicine and Cellular Longevity* **2017**, *2017*, e9821543. <https://doi.org/10.1155/2017/9821543>.
- (81) Venereau, E.; De Leo, F.; Mezzapelle, R.; Careccia, G.; Musco, G.; Bianchi, M. E. HMGB1 as Biomarker and Drug Target. *Pharmacological Research* **2016**, *111*, 534–544. <https://doi.org/10.1016/j.phrs.2016.06.031>.
- (82) Khandelia, R.; Bhandari, S.; Pan, U. N.; Ghosh, S. S.; Chattopadhyay, A. Gold Nanocluster Embedded Albumin Nanoparticles for Two-Photon Imaging of Cancer Cells Accompanying Drug Delivery. *Small* **2015**, *11* (33), 4075–4081. <https://doi.org/10.1002/sml.201500216>.
- (83) Kang, X.; Zhu, M. Tailoring the Photoluminescence of Atomically Precise Nanoclusters. *Chem. Soc. Rev.* **2019**, *48* (8), 2422–2457. <https://doi.org/10.1039/C8CS00800K>.
- (84) Perić, M.; Maršić, Ž. S.; Russier-Antoine, I.; Fakhouri, H.; Bertorelle, F.; Brevet, P.-F.; Guével, X. le; Antoine, R.; Bonačić-Koutecký, V. Ligand Shell Size Effects on One- and Two-Photon Excitation Fluorescence of Zwitterion Functionalized Gold Nanoclusters. *Physical Chemistry Chemical Physics* **2019**, *21* (43), 23916–23921. <https://doi.org/10.1039/C9CP05262C>.
- (85) Deng, H.-H.; Shi, X.-Q.; Wang, F.-F.; Peng, H.-P.; Liu, A.-L.; Xia, X.-H.; Chen, W. Fabrication of Water-Soluble, Green-Emitting Gold Nanoclusters with a 65% Photoluminescence Quantum Yield via Host–Guest Recognition. *Chem. Mater.* **2017**, *29* (3), 1362–1369. <https://doi.org/10.1021/acs.chemmater.6b05141>.
- (86) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat Methods* **2012**, *9* (7), 671–675. <https://doi.org/10.1038/nmeth.2089>.
- (87) Liu, Y.; Tian, Z.; Cheng, L. Size Evolution and Ligand Effects on the Structures and Stability of (AuL)_n (L = Cl, SH, SCH₃, PH₂, P(CH₃)₂, n = 1–13) Clusters. *RSC Adv.* **2016**, *6* (6), 4705–4712. <https://doi.org/10.1039/C5RA22741K>.
- (88) Rappe, A. K.; Casewit, C. J.; Colwell, K. S.; Goddard, W. A.; Skiff, W. M. UFF, a Full Periodic Table Force Field for Molecular Mechanics and Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **1992**, *114* (25), 10024–10035. <https://doi.org/10.1021/ja00051a040>.
- (89) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng,

E. C.; Ferrin, T. E. UCSF Chimera—A Visualization System for Exploratory Research and Analysis. *Journal of Computational*

Chemistry **2004**, *25* (13), 1605–1612.
<https://doi.org/10.1002/jcc.20084>.