

Figure 1. Au₁₀SG₁₀ nanocluster structure with schematic representation of its catenane structure with 10 Au atoms and 10 glutathione ligands. In the presence of Au₁₀SG₁₀, microglia cells are in the stressed state, with increased 4-HNE, LDH, and changed membrane integrity. Reduced HMGB1 is mainly in the nucleus. ROS changes HMGB1 redox state and location. The linear optical absorption spectrum confirms the synthesis of atomically precise Au₁₀SG₁₀ NCs showing characteristic increase of absorption below 410 nm and shoulders at 320 nm and 370 nm.

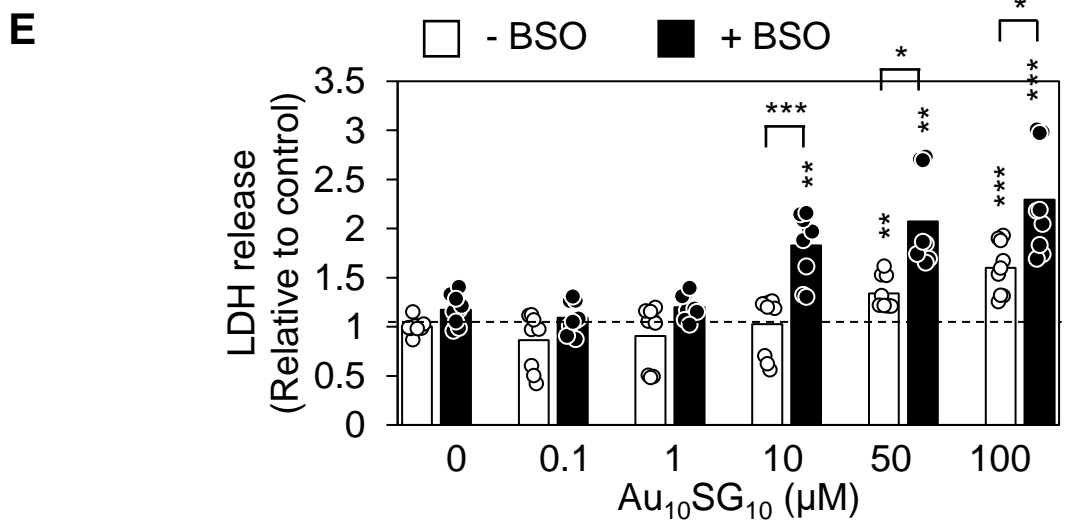
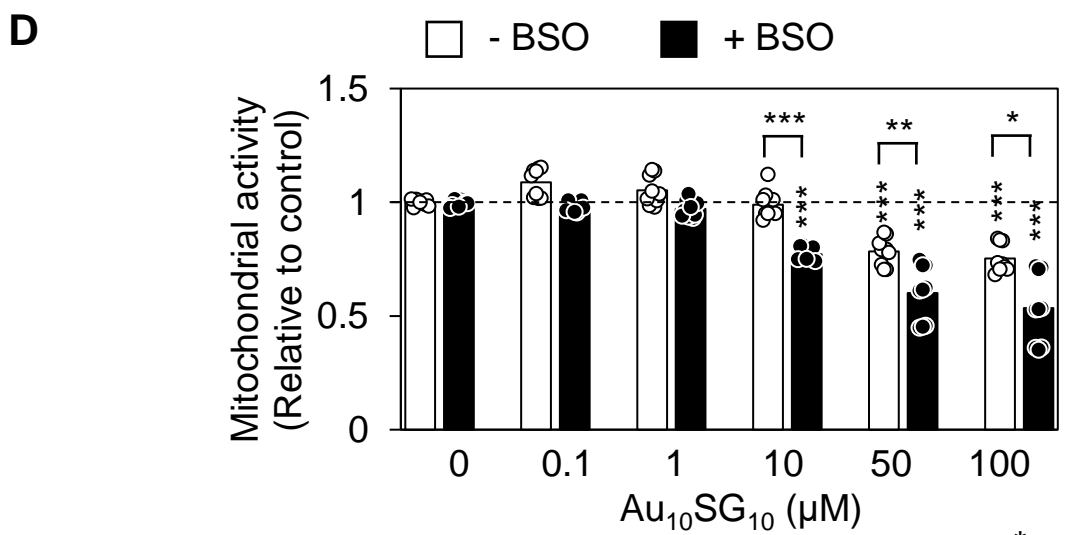
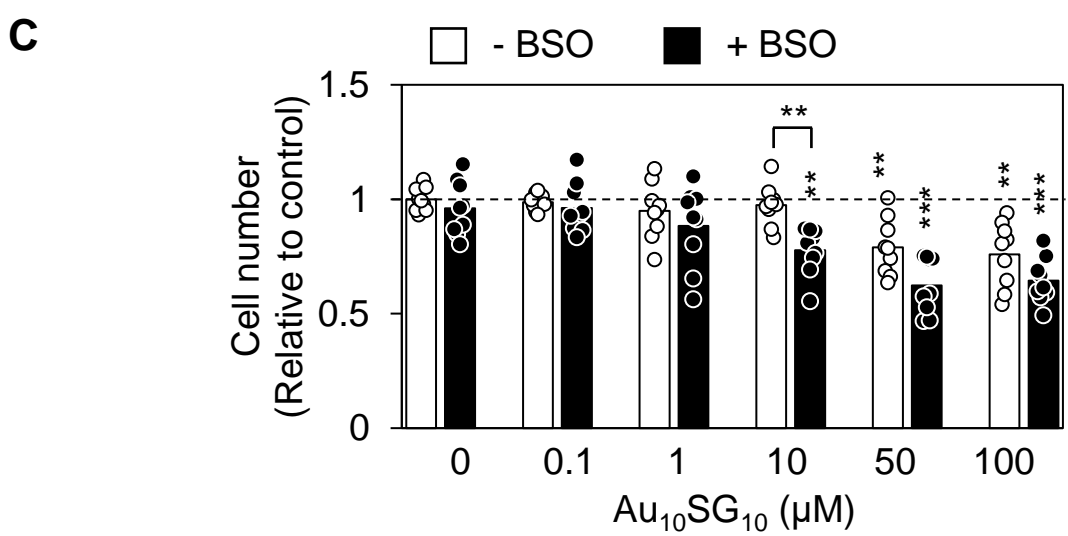
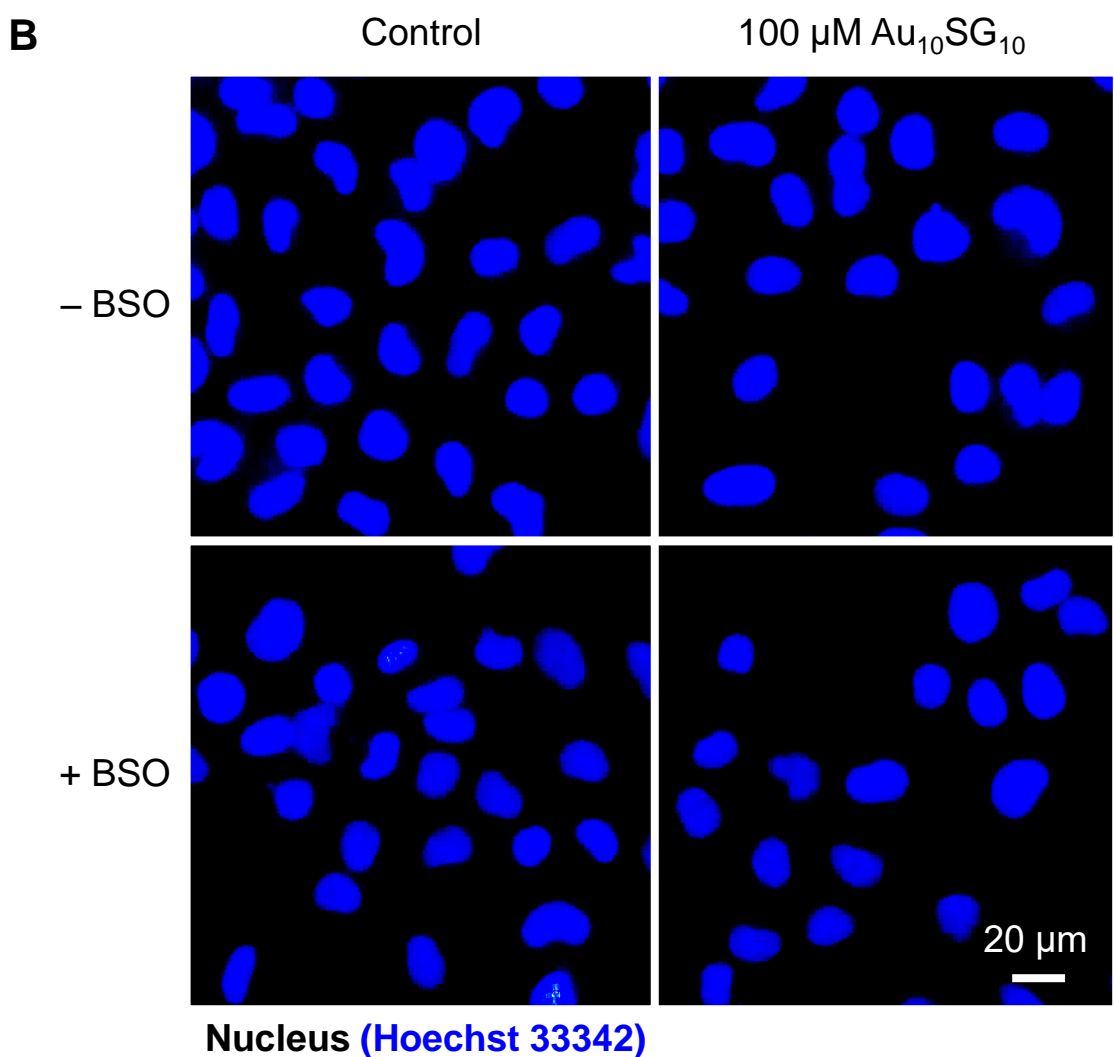
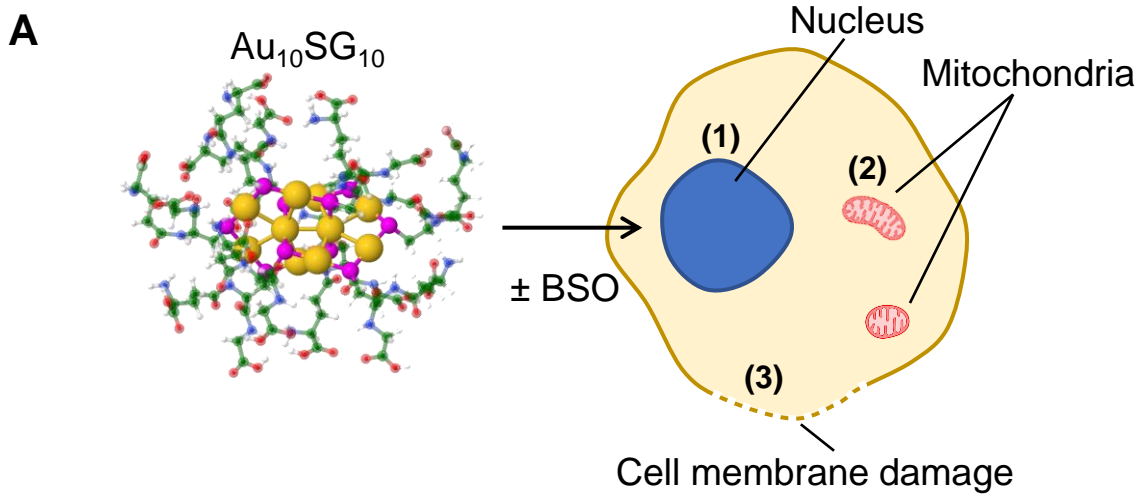


Figure 2. Human microglial HMC3 cells were pre-treated with L-buthionine sulfoximine (BSO; 100 μ M) for 24 hours, and treated with Au₁₀SG₁₀ (0-100 μ M) for another 24 hours. **(A)** Different endpoint parameters were used to determine Au₁₀SG₁₀ toxicity in microglial cells: (1) cell number; (2) mitochondrial function and (3) cell membrane damage. **(B-C)** Changes in cell number were observed by Hoechst 33342 nuclear staining (blue) in HMC3 cells. **(B)** Representative images of Hoechst 33342-stained HMC3 cells after treatment. **(D)** Mitochondrial metabolic activity was assessed by MTT assay. **(E)** Membrane permeability test by lactate dehydrogenase (LDH) assay was used to demonstrate Au₁₀SG₁₀ toxicity. **(C-E)** Results are plotted as replicates (dots) with mean value (bar) relative to untreated control. Data compiled from 3 independent experiments performed in triplicates. * p <0.05, ** p <0.01, *** p <0.001 indicates significant difference.

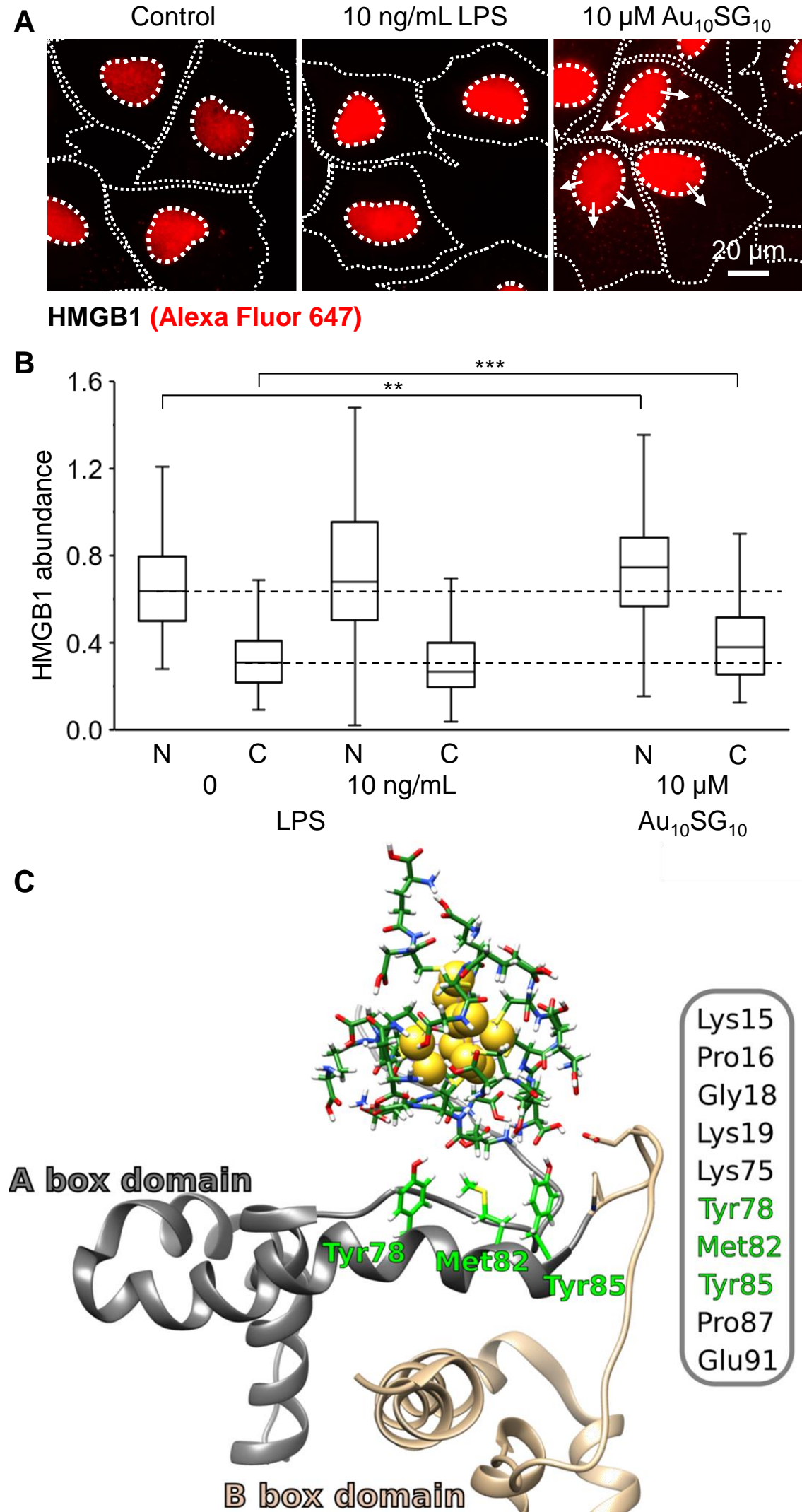


Figure 3. Location of reduced HMGB1 and interaction with Au₁₀SG₁₀. Human microglial HMC3 cells were treated with Au₁₀SG₁₀ (1 or 10 μ M) for 24 hours. **(A)** Representative images of HMC3 cells labeled for HMGB1. Cellular and nuclear outline have been traced by a dotted line. **(B)** Changes in intracellular location (nuclear, N; cytosolic, C) of HMGB1 relative to the control and compared with LPS. Over 400 cells were analyzed (min. 76 cells per condition) from 3 to 4 independent experiments. Box plot shows HMGB1 relative fluorescence intensity per cell, where the sum of cytosolic and nuclear HMGB1 abundance of untreated controls equals to 1: median (line), whiskers (minimum/maximum). *** $p < 0.001$ indicates significant difference. **(C)** Reduced form of HMGB1 interacts with Au₁₀SG₁₀ through functional groups COOH, NH₂, C=O and N=H which are present in the glutathione ligand. Interactions between glutathione ligands at the surface of Au₁₀SG₁₀ (stick representation) and redHMGB1 (gray ribbon representation for A box domain of redHMGB1 and beige ribbon for B box domain). These interactions include hydrogen, polar, ionic bonds with residues highlighted in the gray rectangle on the right side from the structure. Residues Tyr74, Met78, Tyr85 are in the reduced form, and they are shown in the stick representation and colored green. They form the quantum mechanics (QM) part of the complex together with Au₁₀SG₁₀.

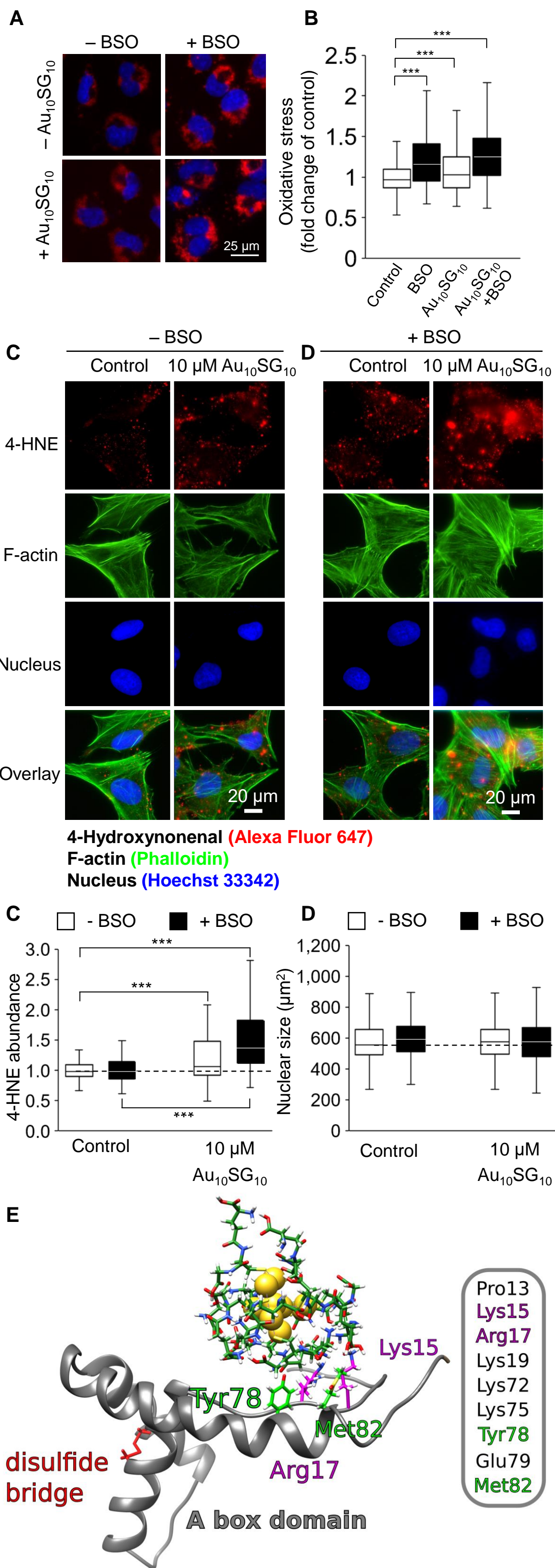


Figure 4. Effect of AuNCs on oxidative stress and lipid peroxidation in HMC3 and interaction sites of oxidized HMGB1 with Au₁₀SG₁₀. HMC3 human microglial cells were pre-treated with L-buthionine sulfoximine (BSO; 100 μM) for 24 hours, and treated with Au₁₀SG₁₀ (1 or 10 μM) for another 24 hours. **(A-B)** Cells were loaded with CellROX Deep Red and imaged using a fluorescence microscope. The intracellular fluorescence per cell was measured in ImageJ. **(B)** Shown are the average oxidative stress levels per cell as fold change of the untreated control ± SEM from three independent experiments. At least 200 cells were analyzed per condition ***p<0.001 **(C-D)** Panels show representative images of 4-HNE abundance in HMC3 cells after treatment. **(E-F)** Box plots shows **(E)** 4-HNE abundance relative fluorescence intensity per unit area (μm²) per cell and **(F)** nuclear size (μm²): median (line), whiskers (minimum/maximum). Over 600 cells were analyzed (min. 77 cells per condition) from 2 to 3 independent experiments in **(E)** and over 3000 cells were analyzed (min. 228 cells per condition) from six to nine independent experiments in **(F)**. ***p<0.001 indicates significant difference. **(G)** Oxidized form of HMGB1 has a disulfide bridge formed between Cys30 and Cys52 (red stick representation) which influences its structure. Au₁₀SG₁₀ can interact with oxidHMGB1 (gray ribbon representation of the A box domain) through hydrogen, polar, ionic bonds established with residues listed in the gray rectangle on the right side of the figure. Oxidized residues Lys15, Arg17, Tyr78, Met82 are shown in the stick representation together with Au₁₀SG₁₀ from the QM part of the simulation.

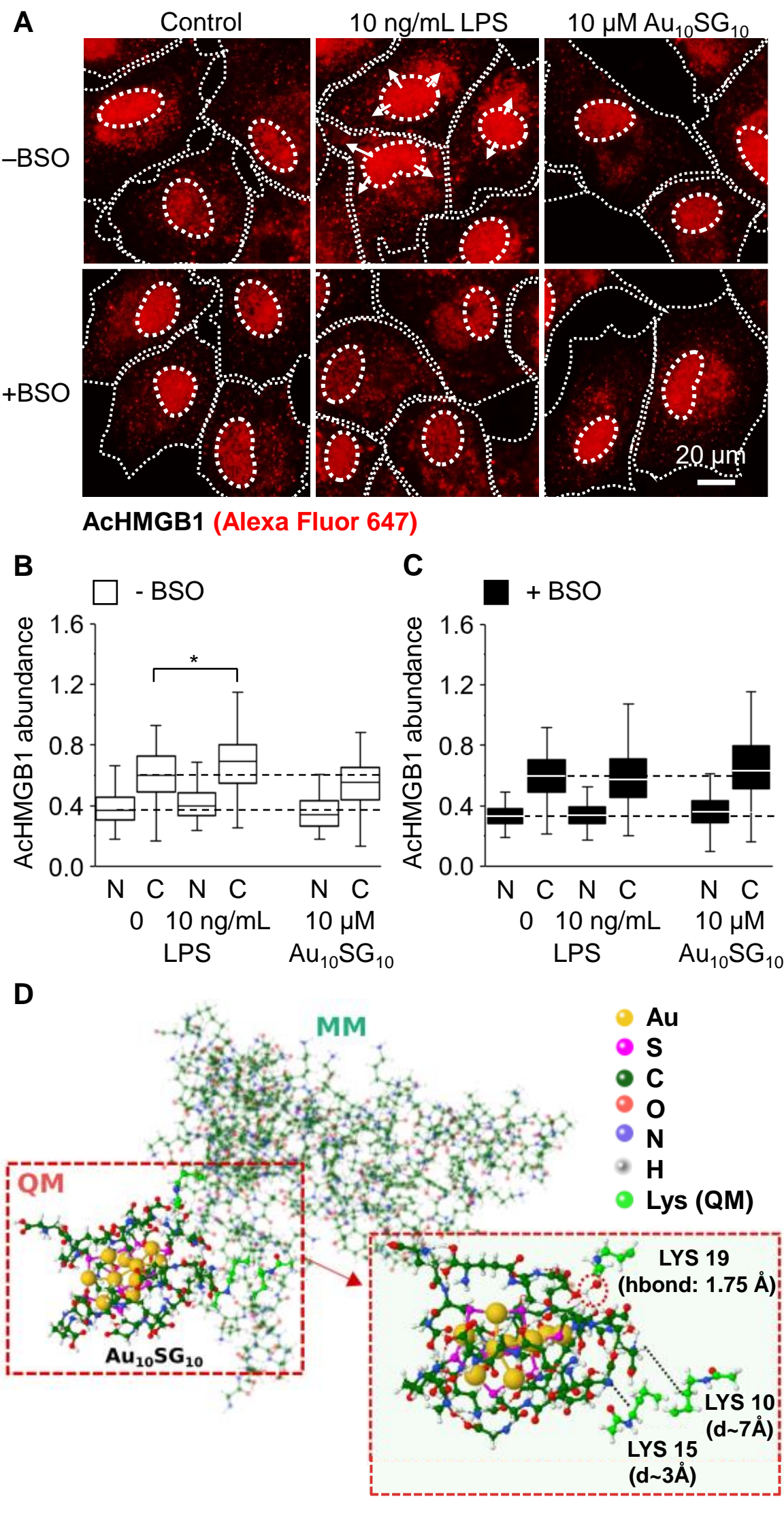


Figure 5. AcHMGB1 intracellular location in human microglia and computational analysis of Au₁₀SG₁₀ with acetylation sites in HMGB1. HMC3 human microglial cells were pre-treated with L-buthionine sulfoximine (BSO; 100 μM) for 24 hours and treated with Au₁₀SG₁₀ (10 μM) for another 24 hours. **(A)** Panels show representative images of acetylated HMGB1 (AcHMGB1) abundance in HMC3 cells after treatment. Cellular and nuclear outline have been traced by a dotted line. **(B-C)** Changes in intracellular location (nuclear, N; cytosolic, C) of AcHMGB1. Only LPS shows significant change in AcHMGB1 localization (*p < 0.05). Over 400 cells were analyzed (min. 70 cells per condition) from 2 independent experiments. Box plots show AcHMGB1 relative fluorescence intensity per cell, where the sum of cytosolic and nuclear AcHMGB1 abundance of untreated controls equals to 1: median (line), whiskers (minimum/maximum). **(D)** QM/MM simulation of interaction of Au₁₀SG₁₀ with AcHMGB1, where MM part of the complex is shown with transparent atoms while atoms in the red rectangle present QM part (Au₁₀SG₁₀ and acetylated lysine residues Lys10, Lys15 and Lys19). Inset shows QM part of the complex with highlighted hydrogen bonds and position of acetylated lysine residues. Color coding of the atom is shown on the right side of the figure.

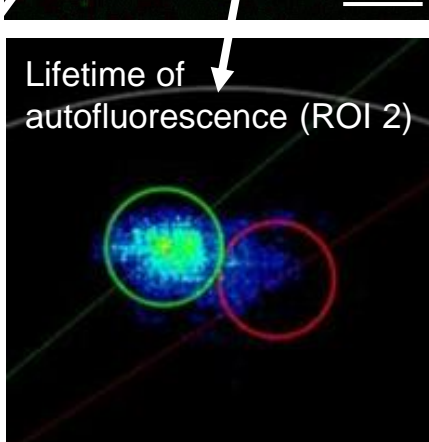
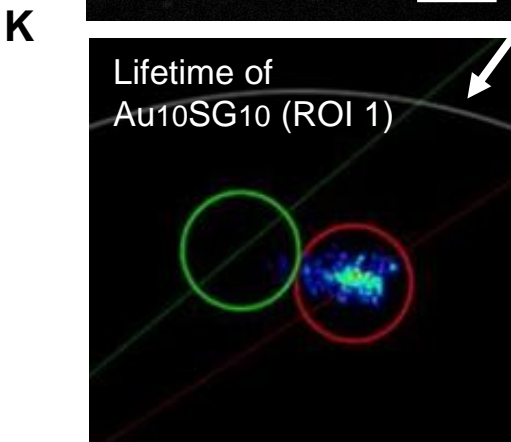
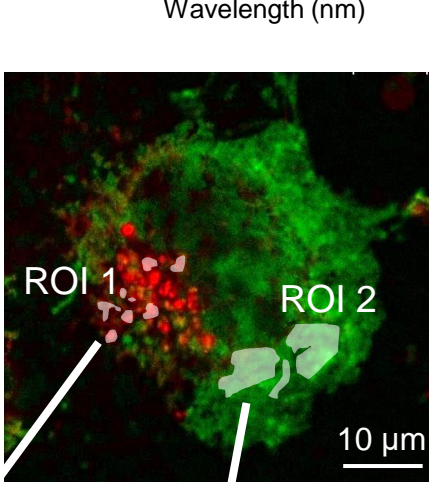
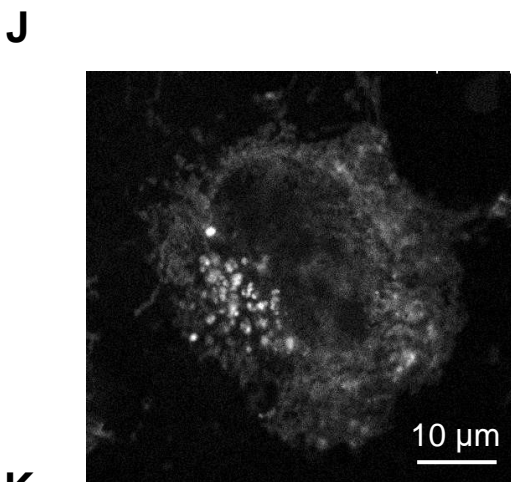
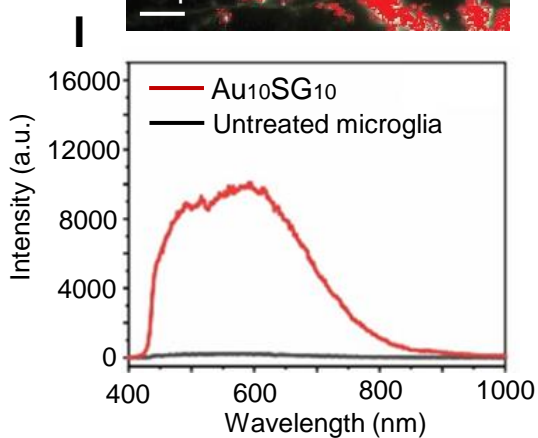
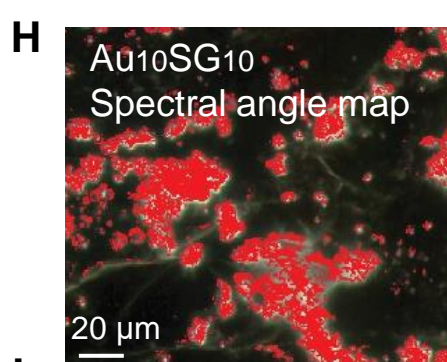
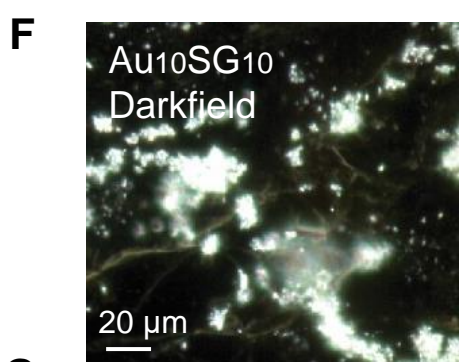
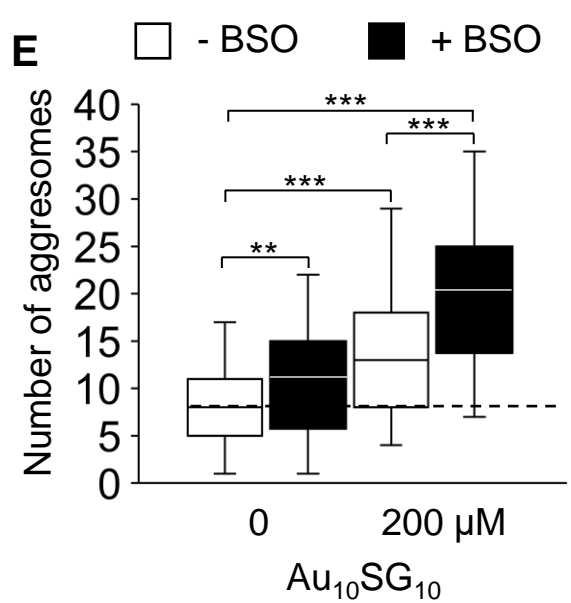
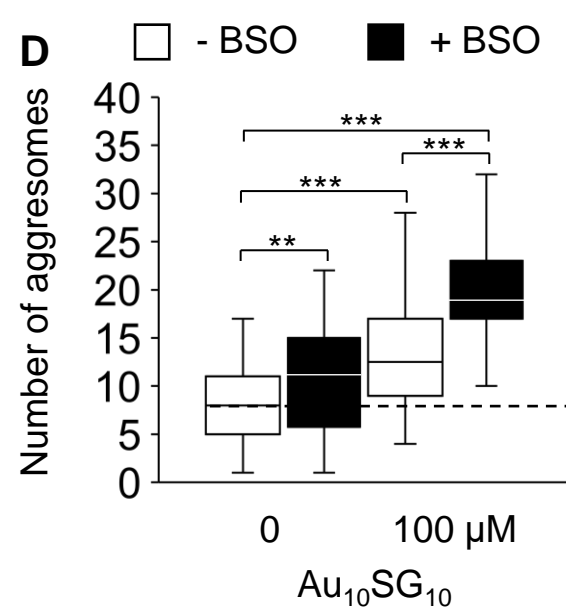
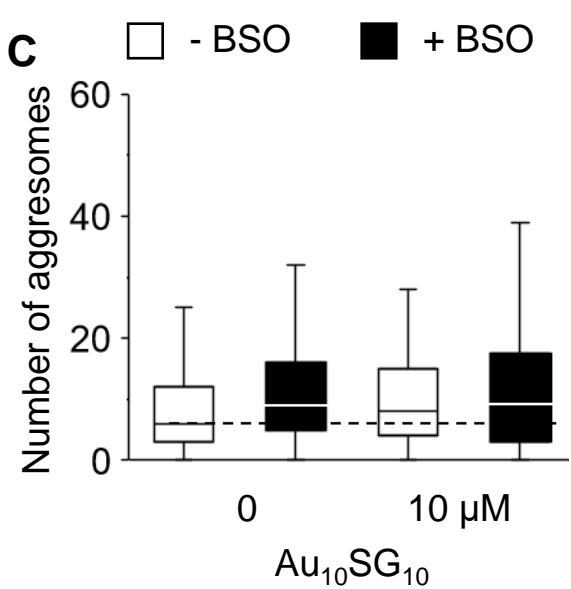
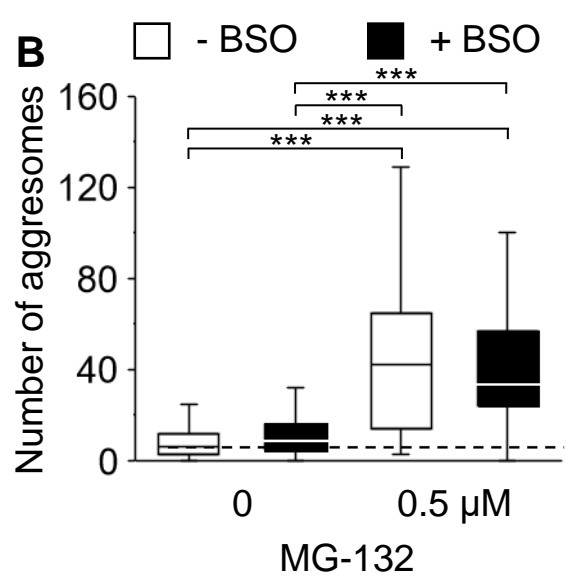
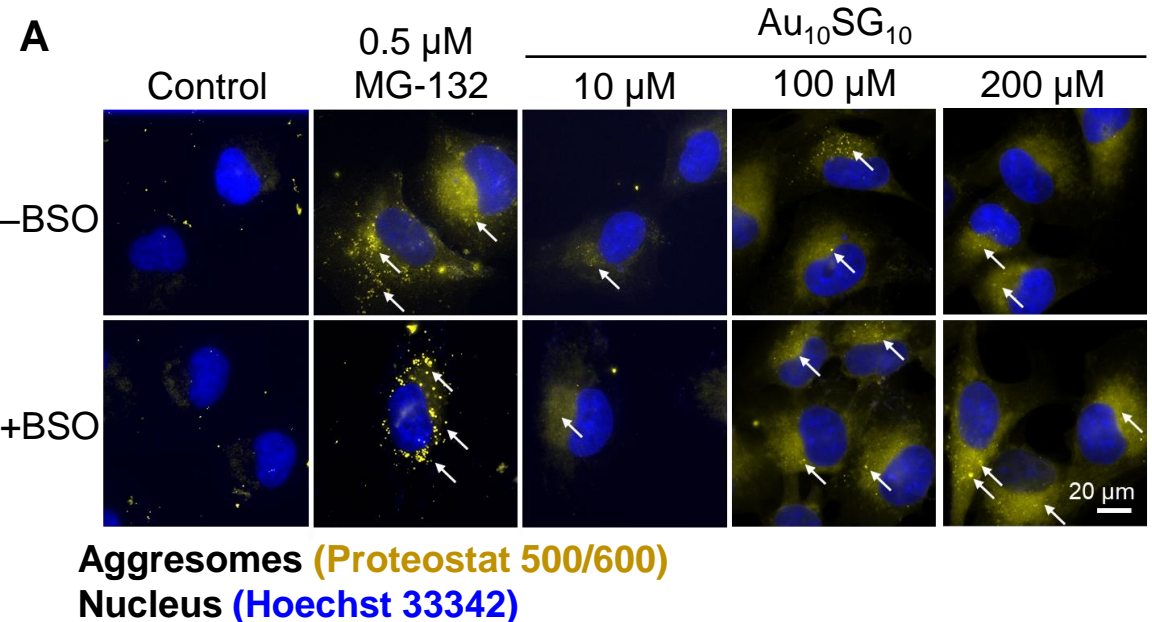
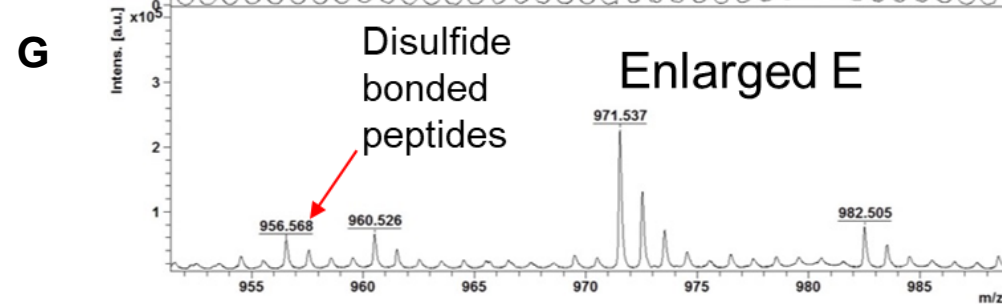
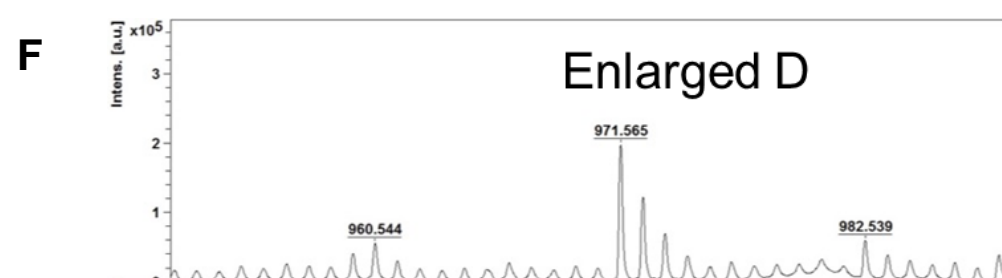
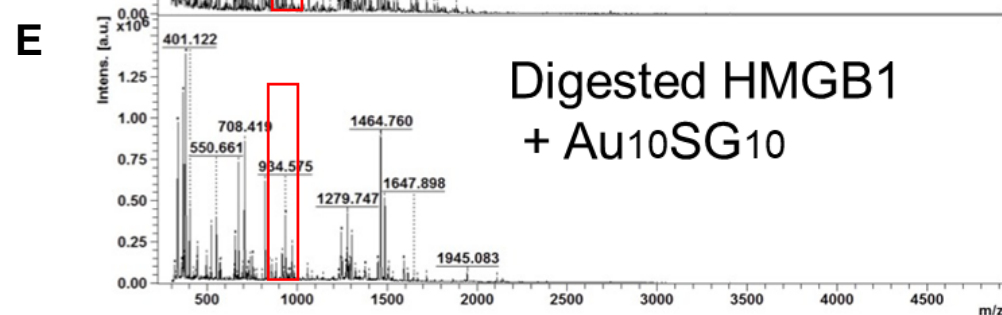
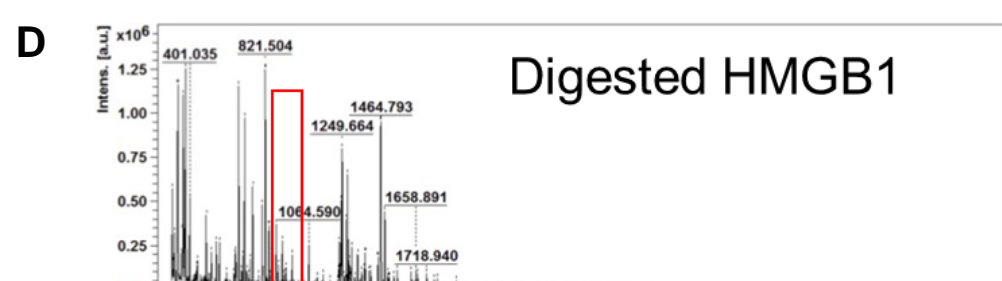
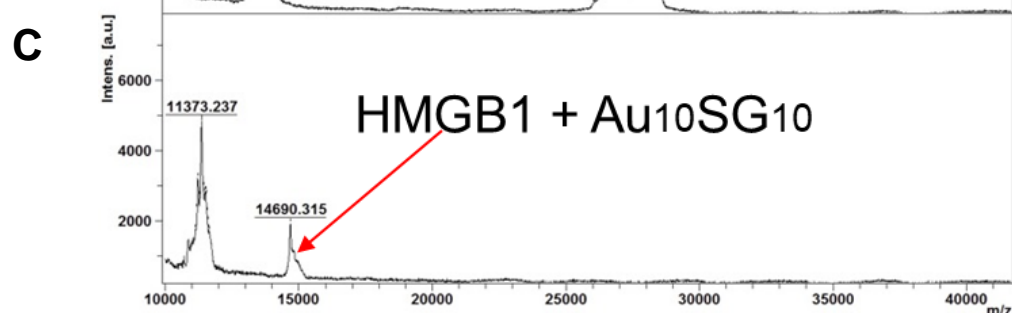
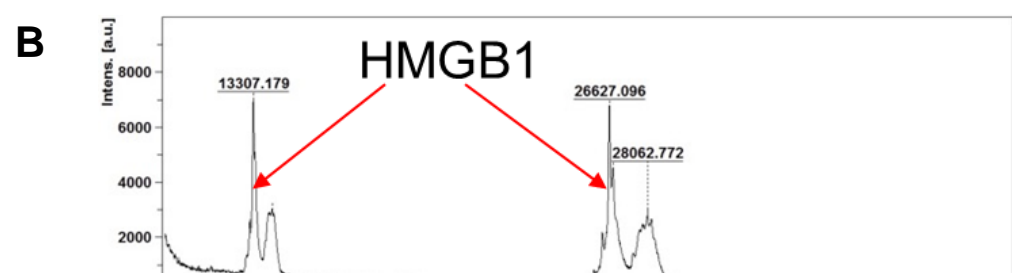
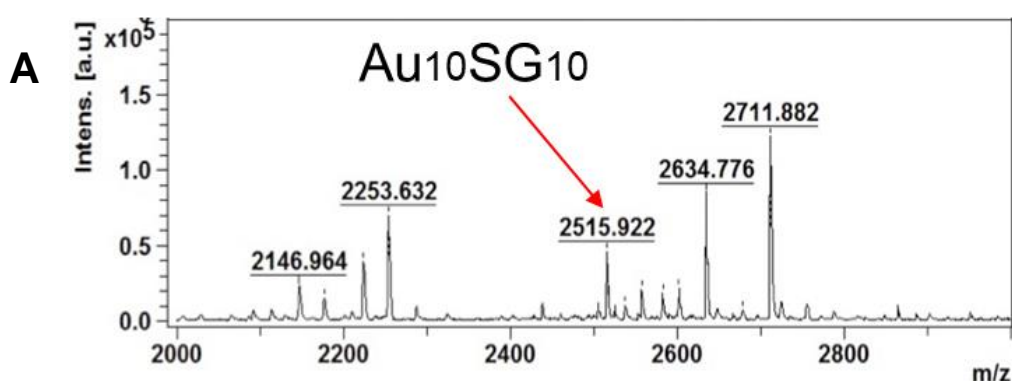
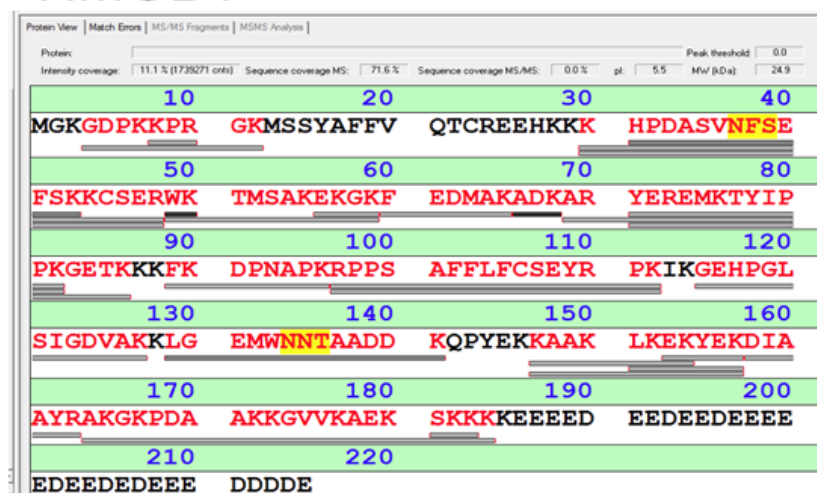


Figure 6. Effects of Au₁₀SG₁₀ on protein aggregation in human microglial cells. (A-E) HMC3 microglial cells were pre-treated with L-buthionine sulfoximine (BSO; 100 μM) for 24 hours, and treated with (B) a proteasome inhibitor MG-132 (0.5 μM) or (C-E) Au₁₀SG₁₀ (10 μM, 100 μM, 200 μM) for another 24 hours. MG-132 was used as positive control to induce protein aggregation. (A) Panels show representative fluorescence micrographs of aggresomes (yellow) labeled using ProteoStat. Nuclei (blue) were labeled with Hoechst 33342. (B-E) Box plots show the distribution of the number of aggresomes per cell: median (line), minimum/maximum (whiskers). Over 700 cells were analyzed (min. 44 cells per condition) from 3 independent experiments. ***p<0.001 indicates significant difference. (F-I) CytoViva hyperspectral imaging of human microglia treated with (F) Au₁₀SG₁₀ (10 μM, 1h) or (G) untreated. Shown are (F-G) darkfield images of cells and (H) spectral angle map analysis overlay. Coloured pixels (red) correspond to the reflectance spectra of Au₁₀SG₁₀. (I) The average reflectance spectra of >50 pixels corresponding to Au₁₀SG₁₀ and untreated cells. (J) Super-resolution micrographs of human microglia with internalized Au₁₀SG₁₀ (100 μM, 4h). Intensity (left panel) and fluorescence (right panel) showing gold nanoclusters (red, ROI 1) and autofluorescence (green, ROI 2). (K) Phasor analysis of fluorescence lifetime imaging microscopy in human microglia treated as in (J). Shown are the longer lifetime for Au₁₀SG₁₀ (left panel), and shorter but stronger lifetime of cell autofluorescence (right panel). Lifetime signals are circled. Regions of interest (ROI) analyzed are shown by arrows in (J).



H HMGB1



I HMGB1 + Au10SG10

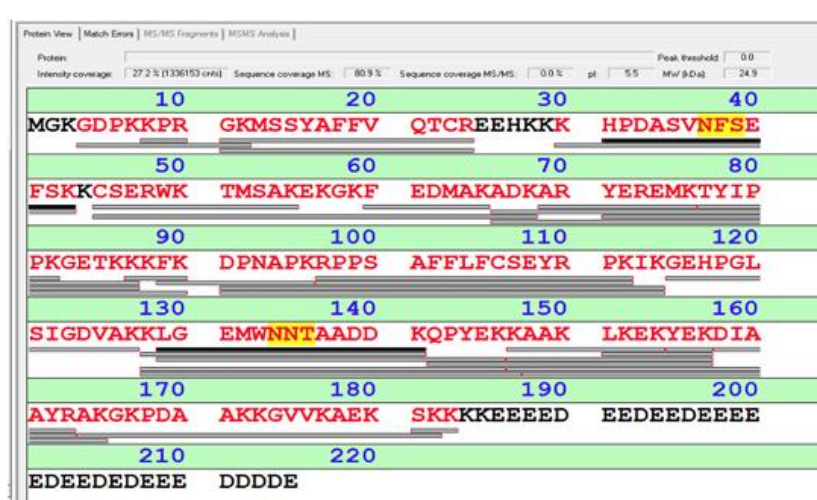


Figure 7. Mass spectrometry analysis of incubated HMGB1 with Au₁₀SG₁₀. All spectra were acquired with matrix assisted laser desorption time of flight mass spectrometry. **(A)** Mass spectrum of Au₁₀SG₁₀ spotted on the ground steel with HCCA matrix (alpha-cyano-4-hydroxycinnamic acid). The spectrum was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

TOC

