Cloneable Selenium Nanoparticles As Multi-modal Bio-imaging Contrast Agents

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Abstract
A cloneable NanoParticle (cNP) is an inorganic nanoparticle that is synthesized by a protein. The protein determines the elemental composition, size, morphology, and other properties of the nanoparticle. Here, we describe the use of a cloneable Selenium NanoParticle (cSeNP) as a cloneable imaging contrast agent in electron microscopy, fluorescence microscopy, and X-ray computed tomography. Combined, these three imaging modalities produce images of biological length scales spanning meters to angstroms but are difficult to correlate. The cSeNP is comprised of an NADPH dependent enzyme that reduces selenodiglutathione to zerovalent Se precipitates. SeNP binding peptides fused to the enzyme ligate the Se precipitates, retain them at the enzyme, and regulate SeNP size to ~5 nm inside cells. The cSeNP-protein can be genetically fused to any protein-of-interest, creating a chimeric protein-of-interest-cSeNP. The cSeNPs endow the protein-of-interest with distinguishable contrast in X-ray and EM images, due to the contrast of SeNPs in these imaging modalities relative to biological background. The cSeNPs can react spontaneously with transition metals such as Zn\(^{2+}\) or Cu\(^{2+}\), forming fluorescent metal-selenides, imageable in florescence microscopy. The cSeNP, therefore, represents a cloneable imaging contrast agent that facilitates location and correlation of proteins-of-interest across all biological length scales.

Comprehensive biological visualization requires imaging over many length-scales.
In biology, “seeing is believing”. Comprehensive biological descriptions need images of biological processes that occur over many length scales. For instance, musculoskeletal processes are visualized on a scale encompassing meters to centimeters; processes such as glomerular filtration in kidneys or neurotransmission occur on scales of centimeters to millimeters. With millimeter to micrometer fields of view, individual cells are observed. At 10 to 100 nm scale large protein complexes and organelles are apparent. At 1 nm to 1 Å scale, individual biomolecules can be visualized, and atomistic information obtained.

Imaging different length scales necessitates different imaging tools (schematic 1, panel A). X-ray tomography (CT scan) facilitate meter scale imaging of whole organisms at up to 10s of μm resolution.¹ Light microscopy illuminates cells and tissues at cm length...
scale and up to ~200 nm resolution. Electron microscopy of biomolecules operates at length scales of up to millimeters and resolutions of ~2 nm for single images and near-atomic resolution for averaged images of multiple copies of identical structures.

Contrast within images is fundamental to their qualitative and quantitative interpretations. Objects with similar illumination source interactions within a sample produce similar detected signal, becoming camouflaged like a chameleon on a leaf. Biological samples have limited intrinsic contrast in the aforementioned imaging modalities. Therefore, an extensive set of extrinsic contrast agents and genetically encoded tools such as Green Fluorescent Protein (GFP) that produce intrinsic contrast are now available.

For instance, iodine based contrast is used extensively for contrast in X-ray (CT) imaging – either extrinsically intravenously injected or intrinsically (genetically encoded) cell-specific contrast with iodine transporters. Optical microscopy boasts a large palette of contrast tools, ranging from extrinsically applied dyes and stains (e.g., Gram, Eosin, Methylene Blue, Masson’s Trichrome) to genetically encoded ‘cloneable’ contrast agents such a wide variety of fluorescent proteins. In electron microscopy of cells, where contrast depends on electron density (scaling with atomic number Z) intrinsic contrast is especially poor as the major elemental components (C, O, N, P) are very similar in electron density. Furthermore, all contrast tools in electron microscopy, both extrinsic metal stains and intrinsic cloneable approaches only function well in fixed cells. Lack of contrast tools represents a major limitation in imaging of cryogenically preserved frozen-hydrated cells in EM.

Correlative contrast agents – visible in multiple imaging modalities are limited. Semiconductor quantum dots are visible in X-ray CT, fluorescence, and electron microscopy, but tools to localize QDs to specific biomolecules, revealing their location in multiple length scales/resolutions are not stoichiometrically quantitative (e.g., QD-antibody conjugates, VIPER).

An overarching goal of bio-imaging is to integrate and understand biological processes from length scales ranging from meters to angstroms. Correlative contrast agents that are visible in X-ray, optical, and electron-based images do not yet exist. Such agents could facilitate correlative visual studies across all biologically relevant length scales. This arises both from the ability to correlate images from different modalities and also simply to locate in a meter-scale images the proteins of interest to image in mm and nm scale imaging modalities.

A Cloneable Se Nanoparticle (cSeNP) enables cloneable contrast in imaging modalities spanning all biological length scales.

Inorganic nanoparticles of varying formulations provide distinctive contrast in all commonly used biomedical imaging modalities. These iNPs are not genetically encodable or cloneable. This limitation makes the tracking of gene products across multiple length scales a challenge, and the tracking of gene products in electron microscopy especially challenging.
We developed a cloneable inorganic nanoparticle (cNP) paradigm (Figure 1, panel A) showing the length scales and resolution of X-Ray, Optical, and Electron based imaging methods. Panel B shows E. coli cells grown in 10mM SeO$_3^{2-}$; Cells in the left tube are expressing an inactivated GRLM enzyme (oxidoreductase disulfide active site mutated to alanine), cells in the right tube are expressing GRLMR, and take on the red color because they are producing SeNPs of the red allotrope of Se ($\alpha$-Se). Panel C shows a schematic of the cNP paradigm. Panel D shows a schematic of the cSeNP in apo and cSeNP forms. Panel E shows a schematic of the disproportionation reaction that converts SeNPs to fluorescent metal selenide nanoparticles.
C). This paradigm adapts in vitro reductive iNP synthesis\textsuperscript{11,12} to the in vivo context. With in vitro synthesis, soluble metal cations are reduced (e.g., with BH\textsubscript{4}) to insoluble precipitates in the presence of ligands. Ligands cap these precipitates at iNP size, stopping their growth. For in vivo (cNP) synthesis, the metal(loid) precursors are biocoordination complexes present inside cells. Reduction of precursors is by NAD(P)H dependent metal(loid) oxidoreductases. These enzymes transform cNP precursors into bulk phase precipitates, consuming NADPH. Bio-ligands present in the cellular milieu then cap and arrest the growth of metal(loid) precipitates at nano-sized dimensions. If the bio-ligands are peptides or proteins, they can be fused to the enzyme, which in-turn can fuse the cNP to the enzyme.

In cNP synthesis metal(loid) oxidoreductase substrate selectivity determines the elemental composition of the resulting cNP. Ligands such as peptides isolated from peptide libraries for iNP binding influence the cNP size and morphology. Fusions of peptide ligands to metal oxidoreductases therefore can result in a self-contained cNP synthesis construct.

We present here a cSeNP (Figure 1, panel D) as a contrast agent in electron, X-ray, and visible light-based imaging modalities. The cSeNP is comprised of a dimeric selenodiglutathione (GS-Se-SG) reducing enzyme (Glutathione Reductase Like Metalloid Reductase, GRLMR) modified to be a single-chain construct with 2 SeNP binding peptides attached (Figure 1, panel D). Expression of GRLMR in cells grown on SeO\textsubscript{3}\textsuperscript{2-}supplemented media results in a striking-red color, attributable to the red allotrope of intracellular SeNPs (Figure 1, panel B).

cSeNPs produce distinctive contrast in X-ray and electron imaging modalities, arising from the higher electron density of Se (Z= 34) relative the C, O, N, S, and P (6 < Z < 16) atoms comprising biological samples. SeNPs can react with transition metal cations, forming metal-selenide semiconductor quantum dots (Figure 1, panel E),\textsuperscript{[cite disopro paper]} which enable distinctive fluorescence contrast.

This cSeNP nanotechnology fills two gaps in the current bio-imaging contrast toolset. First, because it is visible in X-ray, optical and electron images, it can facilitate correlative imaging studies across all biological length scales. Second, it is a cloneable contrast agent visible in electron microscopy that is produced inside live cells.

**Observation of FtsZ-cSeNP expression in *E. coli* by Electron Microscopy.**

Biochemical understanding requires understanding of protein function, which is context dependent. In cellular EM, the highest resolution imaging method for cells, it is difficult to locate (contextualize) proteins of interest. All current methods for locating proteins in electron micrographs have significant drawbacks.

Metallothionein was investigated by multiple groups.\textsuperscript{13–15} We showed that metallothionein can localize high-copy, condensed proteins in cases in fixed cells (not cryo).\textsuperscript{14} Ferritin (Ferritag) functions in some cases, but the 0.5 Megadalton size limits use cases.\textsuperscript{16,17} APEX and miniSOG represent non-particulate approaches to cloneable TEM contrast that result in diffuse staining and function only in fixed cells.\textsuperscript{18–21} The VIPER approach delivers exogenous iNPs to proteins of interest.\textsuperscript{8} It is demonstrated in fixed...
cells. Live cell use (required for ECT) may be complicated by cellular transfection, and stoichiometry uncertainties in labeling. (E.g., background with more iNPs than proteins of interest or incomplete representation of proteins of interest when fewer iNPs are present.) The cNP approach represents an improvement because it: (1) functions in vivo; (2) Creates stoichiometrically labeled proteins of interest; (3) Is significantly more compact than ferritin and may be engineered to be significantly smaller than the current construct.

To assess the utility of the cSeNP for imparting contrast to proteins in cellular electron microscopy, we genetically fused cSeNP DNA to DNA encoding the ‘filamentous temperature sensitive protein Z (FtsZ), a bacterial tubulin homologue. FtsZ, along with at

Figure 2: Panel A shows a schematic of FtsZ locations in procaryotic cells through the cell cycle. Panel B shows a schematic of FtsZ and FtsZ-cSeNP assembling into helical protofilaments. Panel C shows E.coli cells grown in SeO$_3^{2-}$, expressing FtsZ-cSeNP. Due to FtsZ overexpression, this is an elongated cell with several cleavage furrows. Density at these cleavage furrows is attributed to accumulation of FtsZ-cSeNP. We also observe a longitudinal filament. FtsZ is known to form such filaments when overexpressed. Panel D shows a control of panel C, without SeO$_3^{2-}$ supplementation. Panel E shows an STEM-EDS elemental map of a dividing cell. Se is shown in red, Phosphorous in yellow.

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least 13 other division proteins, aids in cleaving a parent cell into daughter cells during cell division.\textsuperscript{95,96} FtsZ forms protofilaments observed in two forms. In non-dividing cells, FtsZ is found in membrane associated dispersed helical protofilaments, comprised of 30 – 80 FtsZ protomers. During cell division, FtsZ protofilaments localize to the midpoint (cleavage furrow) of the dividing cell, forming a so-called Z ring\textsuperscript{95,99} (Figure 2, panel A). Protofilaments on average lie 16 nm from the cellular inner membrane. Protofilaments can extend to lengths of 100 nm.\textsuperscript{95,103,104}

We hypothesized that expression of cSeNP-FtsZ in E. coli would result in SeNP/filament conjugates, with SeNPs organized by FtsZ at different points in the cell cycle as shown in Figure 2, panel B. We expressed a c-terminal FtsZ-cSeNP fusion protein in \textit{E. coli} BL21 cells. The population of FtsZ protomers inside the cell, therefore, is comprised of both genomic FtsZ and recombinant plasmid-expressed FtsZ-cSeNP. FtsZ overexpression interferes with completion of cell division. This results in elongated cells extending up to 20 – 30 $\mu$m in length. A normal cell is ca. 3 – 5 $\mu$m).\textsuperscript{29,105} In such extended cells observations of helical protofilaments forming wave-like patterns along the membrane are known.\textsuperscript{95} Technical details of cSeNP-FtsZ DNA construction and expression are found in methods (M1).

Substantial optimization work (described in SI-cSe_NP_Expression_Optimization) was performed with the highest-throughput method of cellular preservation for EM: drop casting of glutaraldehyde-fixed cells on TEM grids (Methods, M2). In these experiments, we identified conditions where FtsZ-cSeNP resulted in Se and SeNP distributions consistent with expected FtsZ locations (Figure 2, panels A and B). Figure 2, panel C shows cells grown in 2 mM SeO\textsubscript{3}2- expressing the cSeNP-FtsZ construct under 100 uM IPTG; Figure 2, panel D shows cells prepared the same way except without SeO\textsubscript{3}2-. In panel C, when SeO\textsubscript{3}2- is present, contrast increases at the cleavage furrow, where FtsZ-cSeNP concentration is expected to be high. In addition, putative longitudinal FtsZ-cSeNP filaments are observed, consistent with prior observations of FtsZ overexpression by others.\textsuperscript{22–24} Figure 2, panel D shows control cells grown without SeO\textsubscript{3}2-. Contrast appears uniform across these cells. Figure 2, panel E shows elemental mapping of Se in a dividing cell collected by scanning transmission electron microscopy-electron X-ray dispersive spectroscopy (STEM-EDS), confirming that the densities at the cleavage furrow are Se-rich.

We acquired electron tomograms of plastic sections of \textit{E. coli} BL21 cells expressing FtsZ-cSeNP, expecting to observe individual cSeNPs organized by FtsZ filaments. See M3 for electron tomography collection methods. A complication we encountered is that the solvents used in typical plastic embedding of cells for EM, such as acetone, dissolve SeNPs (see solvent-screening SI). (Figure SeNP-dissolve, SI). We implemented SeNP crystallization by adding Cu(OAc)2 to particle rich cells as a postfixation step to stabilize the NPs. The Cu\textsuperscript{2+} ions react with cSeNPs to form CuSe metal selenides (Figure 1, panel E). [cite preprint] The resulting CuSe nanoparticles resist solvent dissolution.

Additionally, we omitted metal stains typically added to plastic sections that react with specific functional groups in biomolecules. For example, osmium tetroxide stains
membranes, uranyl acetate stains proteins and nucleic acid, and Reynolds lead is used
to enhance contrast from other metal stains. These metal stains were omitted because they may form small granules or beam-induced punctate densities that obscure cSeNP contrast and assignment. The resulting tomograms are of lower than typical contrast.

Figure 2 shows electron tomography of experimental and control cells. Panel A shows 75 of 340 slices through an *E. coli* cell expressing FtsZ-cSeNP, grown in 2 mM SeO$_3^{2-}$. Two features are readily apparent: (1) The outer membrane (OM) and portions of the inner membrane (IM); (2) Punctate densities interpreted as CuSeNPs arising from cSeNPs (blue arrows mark 3 of the 22 punctate densities in this image). The cellular preparation process made the inner-membrane difficult to discern; Membranes also distort during fixation.[cite] Panel B shows a magnified view of the boxed area panel A, with the OM manually segmented in blue. The CuSeNPs (cSeNP) particles have sufficient contrast for automatic segmentation. Panel C shows an isosurface rendering of the boxed section of panel A, except rendering the full X-slice tomogram. The isosurface rendering gives a pink color to all voxels with intensities below a threshold level. Importantly, automatic segmentation of CuSeNPs removes the human bias in their identification. Automatic segmentation CuSeNPs in whole cells is also accomplished with the BeadFinder program of the IMOD software package.$^{25,26}$ Panel D of Figure 2 shows manual membrane and automatic CuSeNP segmentation for the entire 250 nm thick tomogram. Panel E shows a magnified image of the boxed area of Panel D.

Panels F, G, and H show images of a control cell, grown in the absence of SeO$_3^{2-}$, but otherwise treated identically to the cell in panel A (including Cu$^{2+}$ exposure). There are no obvious punctate densities in this tomogram. Automatic segmentation does not identify voxels of intensity comparable to those in the experimental cell. An additional control, of cells expressing the Se-reducing GRLMR enzyme and grown in SeO$_3^{2-}$-supplemented media was published previously. In that experiment, SeNPs with diameters ranging between 5 and 60 nm were distributed in cells without any apparent organization.$^{27}$

FtsZ localizes ~16 nm from the inner membrane,[cite] which is ~30 nm from the reliably segmented OM. Therefore, CuSeNPs that observed about ~45 nm of the outer membrane are plausibly associated with FtsZ protofilaments. Figure 2, panel I shows a histogram of CuSeNP – membrane distances for the cell in panel A. 94% of cSeNPs (98 of 104 total) are within 45 nm of the outer membrane. Of those, 37% are closer than 16 nm to the outer membrane. We attribute particles closer than 16nm to the outer membrane to membrane fixation artefacts. Therefore, the vast majority of observed CuSeNPs lie in plausible FtsZ associated locations.

Figure 2, panels J through Q, show a variety of renderings of a tomogram of a cell nearing completion of division. Panels K and L show renderings where positive contrast putative FtsZ filaments appear. Panel M shows a rendering of FtsZ filaments (yellow) decorated with cSeNPs that we could pick out.

Analysis of 6 additional tomograms, including one additional control, are available as electronic supporting information. All additional tomograms show similar results. All tomograms are available for download.

Overall, electron microscopy suggests that FtsZ organizes cSeNPs into locations where FtsZ is expected to be found. Evidence for this includes (1) dark densities in FtsZ
locations in high-throughput 2D-samples; (2) Se in elemental mapping to FtsZ locations; (3) punctate densities in locations expected for FtsZ in 3D cellular reconstructions; (4) absence of these features in control samples.

**Correlative Light and Electron Microscopy (CLEM) of FtsZ-cSeNP expressing *E. coli*.**

As outlined above, cSeNPs can react with transition metals such as Zn, Cd, Ag, and Cu to form highly fluorescent metal selenide nanoparticles (e.g., Quantum Dots). We assessed the cSeNPs converted to fluorescent metal selenides in *live cells*, followed by their imaging in both fluorescence and electron microscopy. *E. coli* expressing FtsZ-cSeNP were grown with 10 mM SeO$_3^{2-}$ supplementation for 1 hour. The cells were collected by centrifugation, washed and resuspended with Luria Broth (LB), and allowed to recover for 20 minutes. Zinc Acetate was added to the liquid culture to a concentration of 1mM, and cells were grown for an additional 2 hours. Cells were then collected by centrifugation, fixed with glutaraldehyde, and imaged.

Figure 3 shows correlative light and electron microscopy (CLEM) images of these cells. Panels A and B show fluorescence and electron images, respectively of cells grown in 2mM SeO$_3^{2-}$. Panel C shows an overlay of panels A and B. Panel D shows an electron image of cells grown without SeO$_3^{2-}$. Panels E, F, and G show magnified images of panels A, B and C. Panel H shows a fluorescence image of cells grown without SeO$_3^{2-}$.

Figure 4. CLEM images of E. coli expressing cSeNP-FtsZ fusions. Panels A and B show fluorescence and electron images, respectively of cells grown in 2mM SeO$_3^{2-}$. Panel C shows an overlay of panels A and B. Panel D shows an electron image of cells grown without SeO$_3^{2-}$. Panels E, F, and G show magnified images of panels A, B and C. Panel H shows a fluorescence image of cells grown without SeO$_3^{2-}$.

The fluorescence images of cZnSe-FtsZ show the brightest emission localizing at the cleavage furrow of dividing cells, where FtsZ is expected to localize during cell division. Correspondingly, the electron images show highest electron contrast at cleavage furrows. When overlayed, regions of high electron contrast overlap with areas of high fluorescence emission.
The higher magnification electron microscope image in panel F shows *E. coli* cells with higher contrast densities at the midpoint of 3 dividing cells and at the poles of 3 other cells. These densities correspond to known distributions of FtsZ, and we attribute these densities to SeNPs co-localized to FtsZ. The fluorescence images of panel E show a relatively uniform fluorescence background across the entire cells, with bright emission at the midpoints of 2 of the dividing cells and at the pole of one of the other cells. The overlay image of panel F confirms that all the high fluorescence regions correspond uniformly to the locations of SeNP density observed in electron microscopy. The control electron image shows cells with a few regions of slightly higher contrast, which we attribute to an excess of protein or inclusion bodies in those locations as an artifact of overexpression.

The control fluorescence images in panel H show typical (low quantum yield) cellular autofluorescence.[cite] Overall, the putative cZnSeNPs produce clear fluorescence emission above cellular autofluorescence, which becomes more intense in areas of high FtsZ concentration.

Overall, this set of experiments suggests that the cSeNP, after conversion to cZnSeNP, is a promising approach for CLEM contrast. Furthermore, because particles were synthesized *in vivo*, cZnSeNPs can be an effective tool for cryo-CLEM based methods such as cryoFIB-SEM-FLM.

cSeNPs as genetically encoded X-ray contrast.

X-ray imaging modalities such as Computed Tomography (CT) provide images at length scales of meters with penetration deep into tissues and organisms. Contrast in CT images is measured in terms of X-ray attenuation; the unit for describing X-ray attenuation is the Hounsfield Unit (HU), which provides a quantitative scale for describing radiodensity. The HU scale is defined by the radiodensity of distilled water (0 HU), air (-1000 HU). For biological tissues, typical HU values range from -700 (lungs) to +1900 (bone), with most soft tissues (fat, muscle, organs) having HU values between -120 and 60. Inorganic materials attenuate X-rays much more effectively than biological tissues, with pure copper attenuating at 14,000 HU and gold representing 30,000 HU (an upper measurable limit). For inorganic nanoparticles, X-ray attenuation depends on particle concentration.

X-ray tomograms of *E. coli* cells expressing the cSeNP or GRLMR in grown in varying concentrations of SeO$_3^{2-}$ were acquired in a Scanco micro-CT 80 instrument. CT data is presented in Figure 5. Panel A shows that cells expressing the cSeNP in the indicated concentrations of SeO$_3^{2-}$ attenuate X-rays with an efficiency that depends on the growth condition. Attenuation of 700HU is observed for cells grown in 1mM SeO$_3^{2-}$. This is significantly greater attenuation than observed for soft tissues, but less than bone. Notably, cells grown in the same concentrations of SeO$_3^{2-}$ but not expressing the cSeNP never attenuate at more than 100 HU. Panel B shows a voxel-parsed trace of X-ray attenuation. Here we observe that cells expressing the cSeNP grown in 2mM SeO$_3^{2-}$ show voxels with minor attenuation peak at 190HU and a major attenuation peak at 1100HU. Corresponding controls (not with in Se) show only an attenuation peak near 100HU, comparable to soft tissue. Panel C shows the attenuation of cSeNP expressing cells grown in concentrations of SeO$_3^{2-}$ ranging from 1μM to 10mM. Similar to panel A,
attenuation increases with the concentration of SeO$_3^{2-}$ in which the cells were grown. Panel D shows a 9-position microcentrifuge-tube holder. The contents of each position are described in the figure legend. More white in these images corresponds to Se absorption. Panels E and F show tomograms of the samples in tubes 5 and 6. They are colored according to attenuation, with red representing attenuation near 1100HU and blue representing attenuation near 100HU. More details on this rendering are available in the SI. Overall, these tomograms show that cells expressing the cSeNP show clearly distinguishable X-ray contrast relative to cells grown in the same concentrations of SeO$_3^{2-}$ that do not express the cSeNP.

Conclusions

Expression of the cSeNP in an E. coli model system produces molecular contrast in electron and fluorescence microscopies, as well as cellular contrast in X-ray imaging (where resolutions are not molecular.) This paper demonstrates a ‘proof of concept’ using
the *E. coli*, a workhorse laboratory model-organism. What is true in *E. coli*, often translates to more complex model and experimental systems. We are presently working to extend these findings to more complex model systems such as *Drosophila*.

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**Bibliography**


