Production of fibronectin sensitizes EGFR-TKI resistant lung cancers to silver nanoparticle induced endoplasmic reticulum stress

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Abstract

Lung cancer is the leading cause of cancer related deaths worldwide and second commonly diagnosed cancer among men and women. Epidermal growth factor receptor (EGFR) is frequently dysregulated in lung cancers and has been implicated in pathogenesis of this disease. Because of this, EGFR-tyrosine kinase inhibitors (TKIs) have since been developed as a primary form of treatment. However, many patients do not initially respond, or they inevitably develop resistance to EGFR-TKIs within 12-14 months of treatment. Therefore, discovering new drugs for these patients is increasingly important. We found that silver nanoparticles (AgNPs) are effective for the treatment of inherent and acquired resistance to EGFR-TKI in lung cancer cells. We establish that doses of AgNPs that are highly cytotoxic to EGFR-TKI resistant lung cancers are non-toxic to non-malignant lung cells. No differences were observed in the internalization or intracellular trafficking of AgNPs in AgNP sensitive cells and AgNP resistant cells, indicating that the difference in sensitivity was not due to the quantity of AgNPs internalized. Therefore, we performed mechanistic studies to identify the underlying biology of the cells that was responsible for this difference. We observed that AgNPs induce lipid peroxidation, protein oxidation and aggregation in EGFR-TKI resistant lung cancers. This then leads to irreparable endoplasmic reticulum (ER) stress, resulting in cell death. In contrast, doses of AgNPs that were lethal to EGFR-TKI resistant cancers had no effect on EGFR-TKI sensitive lung cancers. We further establish that EGFR-TKI resistant lung cancers exhibit increased expression of extracellular matrix proteins including fibronectin and collagen. Knockdown of fibronectin in EGFR-TKI resistant cells decreases their sensitivity to AgNPs by reducing baseline endoplasmic reticulum stress. This work provides a rationale for the development of AgNPs for the treatment of EGFR-TKI resistant lung cancer.

Results

Figure 1. Silver nanoparticles are more selective than gefitinib, RSL3 or erlotinib for TKI resistant lung cancers, and increased sensitivity is not due to differences in uptake or trafficking. For all studies, non-malignant cells are shown in black, and cancer cells are shown in red. (A) Dose response curves for gefitinib (24 h) and AgNPs (2 h), and their cytotoxicity was assessed by MTT assay. (B) Uptake of 25 nM AgNPs was evaluated for 2 h by red channel using flow cytometry. (C) Localization of AgNPs was assessed by TEM. We incubated cells with AgNPs for 2 h to allow for uptake and trafficking. AgNPs primarily localized to endosomes by both (F) PC9 and (G) CALU1 cells.

Figure 2. Silver nanoparticles induce lipid oxidation, protein oxidation, and protein aggregation in EGFR-TKI resistant lung cancers. For all studies, EGFR-TKI resistant cancers are shown in red, and EGFR-TKI sensitive cancers are shown in black. (A) Western blot for lipid peroxidation (TBA), lipid peroxidation adducts (LPO), lipid peroxidation adducts (LPO), protein oxidation (DAB), protein aggregation (AAPH) and Ce LENG (C) of H358 and AgNP treated H358 (AgNP). (B) Lipid peroxidation (TBA) was measured using flow cytometry. (C) Protein oxidation (DAB) and protein aggregation (AAPH) were measured using flow cytometry.

Figure 3. Silver nanoparticles induce irreducible endoplasmic reticulum stress in EGFR-TKI resistant lung cancers. For all studies, EGFR-TKI resistant cancers are shown in red, and EGFR-TKI sensitive cancers are shown in blue. (A) RNA expression in RPKM obtained from the CCLE were plotted for lung cancer cell lines used in this study. (B) TKI protein expression was determined by western blot. (C) Cells were exposed to AgNPs for 24 h by stained with red channel using flow cytometry. (D) Protein oxidation expression was measured using confocal microscopy. (E) Cells were treated with AgNPs for 24 h and stained with red channel using confocal microscopy. (F) Cells were treated with AgNPs for 24 h and stained with red channel using confocal microscopy.

Figure 4. Silver nanoparticles induce irreversible endoplasmic reticulum stress in EGFR-TKI resistant lung cancers. For all studies, EGFR-TKI resistant cancers are shown in red, and EGFR-TKI sensitive cancers are shown in blue. (A) Lipid peroxidation expression was measured using flow cytometry. (B) Cells were exposed to AgNPs for 24 h by stained with red channel using flow cytometry. (C) Cells were exposed to AgNPs for 24 h by stained with red channel using flow cytometry. (D) Cells were treated with AgNPs for 24 h by stained with red channel using flow cytometry.

Conclusions

- Silver nanoparticles are toxic to EGFR-TKI resistant lung cancers at doses that are non-toxic to non-malignant cells
- Silver nanoparticles induce lipid and protein oxidation, protein aggregation, and irreversible endoplasmic reticulum stress in EGFR-TKI resistant lung cancers
- Increased secretory load of EGFR-TKI resistant lung cancers leads to their susceptibility to silver nanoparticle treatment

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Identification of Molecular Signatures Predictive of Sensitivity of Breast Cancer Cells to Silver Nanoparticles

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Abstract
Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer with high mortality that accounts for 15-20% of all breast cancer diagnoses. Patients experience high rates of relapse and metastasis. Molecular profiling shows that TNBC tumors can be classified into subtypes that differ in expression of epithelial and mesenchymal markers as well as in response to chemotherapy, TNBC cells enriched in mesenchymal markers are radiation and chemotherapy resistant, and may contribute to therapeutic resistance and tumor relapse. We previously discovered that silver nanoparticles (AgNPs) are cytotoxic to TNBCs at doses that have no effect on non-cancerous cells. Our current studies show that TNBCs expressing mesenchymal markers (VIM) are significantly more sensitive to AgNPs than TNBCs that express epithelial markers (CDH1). Furthermore, lung, prostate, colorectal, and ovarian cancers that express mesenchymal markers are more sensitive to AgNPs than cancers of the same origin that express epithelial markers. This shared vulnerability of mesenchymal cancers to AgNPs indicates that there is an underlying biology that is responsible for this phenomenon. We performed mechanistic studies to determine the cause of these differences. Our results show that AgNPs induce protein aggregation, extracellular matrix (ECM) remodeling, autophagy, reticulum stress, and lipid peroxidation in TNBCs expressing a mesenchymal phenotype, but not in epithelial TNBCs. We further identify elevated synthesis of extracellular matrix (ECM) proteins including fibronectin and collagen, as well as high expression of long-chain-fatty-acid CoA ligase 4 (ACSL4), as being distinguishing features of AgNP sensitive cancers. The burden of synthesis of ECM proteins increases sensitivity of cells to ER stress, and ACSL4 is essential for incorporating oxidized lipids into cell membranes. By detailing these mechanisms of action and identifying genetic signatures associated with sensitivity to AgNPs, our results highlight shared vulnerabilities of mesenchymal cancer cell populations, which may be exploited by AgNPs or other therapies.

Results

Figure 1. Members of the AgNPs sensitive signature, Epithelial (blue) -> Mesenchymal (red). mRNA expression (RPKM; log2) of differentially expressed genes was determined and compared across each cell line. Gene expression was measured by qRT-PCR and was compared across all cell lines. The expression of GAPDH was used as a control. Statistical analysis was performed by one-way ANOVA and post-hoc Tukey Test. Significant differences between mesenchymal (red) and epithelial (blue) cancer cells are indicated (**p<0.01, ***p<0.001).

Figure 2. AgNPs induce lipid peroxidation, proteasomal stress, and immediate early and polynucleotide reductase stress in mesenchymal but not epithelial TNBCs. To assess lipid peroxidation, breast cancer cells were treated with AgNPs for 24 hrs, stained with Lipofuscin, and fluorescence measured using confocal microscopy and flow cytometry. To assess proteasomal stress, cells were treated with AgNPs for 24 hrs, stained with Proteinase K and an ECF-PaeRed assay, and fluorescence measured using confocal microscopy and flow cytometry. To assess polynucleotide reductase stress, cells were treated with AgNPs for 24 hrs, stained with 50 µM Net-A-Rhoe, and fluorescence measured using confocal microscopy and flow cytometry. Results shown are representative images from three independent experiments. Statistical analysis for all studies was performed by two-way ANOVA and post-hoc Tukey Test. Significant differences between treated cells and the relevant control are indicated (**p<0.01, ***p<0.001). Additionally, cells were treated with 50 µM Net-A-Rhoe and untreated for 24 h. AgNP expression was evaluated using western blot.

Figure 3. High expression of ECM proteins and ACSL4 are distinguishing features of AgNP sensitive cancer. FN1 and ACSL4 protein expression was determined by western blot. RNA expression data was obtained from the CCLE for ECM and other large secreted proteins upregulated in AgNP treated (red) compared to AgNP resistant (blue) breast cancer cell lines.

Key Points
• Triple negative breast (TNBC), lung, ovarian, colorectal, and prostate cancers that express mesenchymal markers are highly sensitive to AgNPs.
• AgNPs induce lipid and proteotoxic stress in mesenchymal but not epithelial TNBCs. This is characterized by lipid and protein oxidation, protein aggregation, and increased ER stress.
• Increased synthesis of extracellular matrix (ECM) proteins such as fibronectin and collagen and ACSL4 proteins are distinguishing features of AgNP-sensitive cancers. Synthesizing large amounts of ECM proteins sensitizes cells to ER stress induced by AgNPs, and ACSL4 is essential for incorporating oxidized lipids into cell membranes.

Image
Automated Tip Conditioning for Scanning Tunneling Spectroscopy

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Abstract

We apply machine-learning models trained with archived gold dI/dV spectra to analyze spectra collected during tip conditioning, so the program can stop when the tip is in a good shape for STS measurements. We manually labeled archived spectra based on their quality and resemblance to standard dI/dV spectra for gold.

Evaluating Tip Condition from dI/dV Spectra using Machine Learning Models

We gratefully acknowledge support from Office of Naval Research MURI program for the experiments conducted. For any questions or discussions please reach out to shenkaiwang@berkeley.edu

Summary and Prospects

We present an automated tip conditioning program for STS measurements based on Python and machine learning. We developed a straightforward algorithm to process and analyze topographic STM images in order to find possible tip conditioning positions on clean or sparsely covered gold surfaces. Machine learning models are used to analyze STS spectra and determine the quality of the tip. Decision tree based ensemble and boosting models and deep neural networks have similar performances on identifying usable tips with clear gold surface state and an AdaBoost model is used as default for the program to be robust, adaptable, and fast. Data augmentation methods are being investigated to improve the performance of machine learning models. We expect that our program can make efficient use of the idle time of the STM (e.g., during the night) and greatly reduce the amount of research time wasted on tip conditioning for STS measurements. In the future, we expect to embed reinforcement learning into our program so that the program may be able to figure out the best pointing protocol for tip conditioning.

Acknowledgments

Scanning tunneling spectroscopy (STS), based on scanning tunneling microscopy (STM), is a powerful tool to characterize the electronic structure of single molecules and nanomaterials. While performing STS, the structure and condition of the probe tips are critical for obtaining reliable and stable spectra. A common way to prepare STM tip is to repetitively poke the tip on known and bare substrates, i.e., coinage metals or silicon, to remove contaminations and to potentially coat the tip with substrate atoms. Here, we present an automated program based on machine learning models that can identify the Au(111) Shockley surface state from dI/dV spectra and perform tip conditioning on clean or sparsely covered gold surfaces with little user intervention. We employ a straightforward height-based segmentation algorithm to analyze STM topographic images and identify tip conditioning positions and used 1789 archived dI/dV spectra to train machine learning models that can predict the condition of the tip by evaluating the quality of the spectroscopy data. Decision tree based ensemble and boosting models and deep neural networks (DNNs) have been proven to perform reasonably on identifying tips in usable conditions for STS data. We expect the program to save human labor, reduce research costs for surface science studies and accelerate the discovery of novel nanomaterials by STM. The strategies presented here can also be generalized for STM tip conditioning on other metallic surfaces.

The operations of STM were controlled by an Omnicon Matrix console and accessed by Python through the RemoteAccess_API provided by Omicron. Machine learning models were implemented using the Scikit Learn (0.22.1) module of Python and Keras (2.2.4) with TensorFlow backend.

Table 1. Number of dI/dV spectra with different grades.

<table>
<thead>
<tr>
<th>Model</th>
<th>Precision (On test set)</th>
<th>Recall (On test set)</th>
<th>ROC Area Under Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGD</td>
<td>0.595</td>
<td>0.547</td>
<td>0.807</td>
</tr>
<tr>
<td>SVM</td>
<td>0.685</td>
<td>0.773</td>
<td>N/A</td>
</tr>
<tr>
<td>Decision Tree</td>
<td>0.746</td>
<td>0.616</td>
<td>0.790</td>
</tr>
<tr>
<td>Random Forest</td>
<td>0.825</td>
<td>0.605</td>
<td>0.928</td>
</tr>
<tr>
<td>AdaBoost</td>
<td>0.829</td>
<td>0.674</td>
<td>0.942</td>
</tr>
<tr>
<td>CatBoost</td>
<td>0.842</td>
<td>0.744</td>
<td>0.943</td>
</tr>
<tr>
<td>MLP</td>
<td>0.792</td>
<td>0.663</td>
<td>0.940</td>
</tr>
<tr>
<td>CNN</td>
<td>0.806</td>
<td>0.674</td>
<td>0.935</td>
</tr>
</tbody>
</table>

Table 2. Performance of different machine learning models on differentiating STS curves with gold surface states.

<table>
<thead>
<tr>
<th>Model</th>
<th>False False Positives</th>
<th>False Positives</th>
<th>True Positives</th>
<th>True Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGD</td>
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<td>95</td>
<td>134</td>
<td>179</td>
</tr>
<tr>
<td>SVM</td>
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<td>940</td>
<td>733</td>
<td>790</td>
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<tr>
<td>Decision Tree</td>
<td>842</td>
<td>842</td>
<td>663</td>
<td>825</td>
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<tr>
<td>Random Forest</td>
<td>925</td>
<td>925</td>
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<td>842</td>
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<tr>
<td>AdaBoost</td>
<td>733</td>
<td>733</td>
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<tr>
<td>CatBoost</td>
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<td>MLP</td>
<td>792</td>
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<tr>
<td>CNN</td>
<td>806</td>
<td>806</td>
<td>674</td>
<td>940</td>
</tr>
</tbody>
</table>

Figure 2. Example STS curves with different grades.

Figure 3. Receiver Operating Characteristic (ROC) Curves for machine learning models. The curves are generated from classification probability results of three-fold cross-validation on the training set. (b) Contribution of each data point on the classification of STS curves using a random forest model (feature importance). An STS curve with grade 4 is presented as reference.

Figure 4. Sample dI/dV spectra in the test set that are classified as (a) false positives (labeled as False, predicted as True) and (b) false negatives (labeled as True, predicted as False) by an AdaBoost model.

We can see that decision tree based ensemble and boosting models and deep neural networks (MLP, CNN) have similar classification results and outperform basic models. The performance of machine learning models listed in Table 2 does not look appealing judged just by the precision and recall scores. However, this can be due to the inconsistency of manual labeling since there is no hard criteria for grading the dI/dV spectra. The differences between spectra with different grades are minor, especially for spectra that are graded as 1 or 2. Therefore, ambiguity in manual classification introduces significant noises into the dataset labeling. The lack of good spectra for training is another reason for the compromised performances. Deep neural networks tend to overfit our sample despite the simple architectures (see supporting information) we used. Furthermore, for the machine learning models to work we have to group spectra with grade 2, 3, and 4 together and label them as good. Therefore, some poor-quality spectra with visible gold surface state might have been labeled as grade 2 and used as good spectra for training. Examples of mispredicted spectra are presented in Figure 4.

Locating Tip Conditioning Positions in Topographic Images

Figure 1. (a) Raw STM image of graphene nanoribbons on Au(111), (b) Figure (a) flattened using normal equation. (c) Figure (b) segmented and labeled based on apparent height. (d) Image showing tip conditioning positions detected by the program. (e) Histogram of pixel heights for Figure (b). Highlighted regions represent -0.05 nm to +0.05 nm range centered around each peak. Figure (e) is labeled based on the highlighted regions.

In order for the program to perform tip conditioning automatically, it needs to be able to find out where to poke the tip and take dI/dV spectra. Therefore, an algorithm that can process STM topographic images and identify large enough clean substrate areas is needed for our program. In our program, we employ a fast and straightforward method that segments topographic images based on apparent height and scans the labeled images with a 5 nm by 5 nm window to locate possible substrate areas for tip conditioning. The image processing procedure can be visualized as in Figure 1.
Atomic scale observations of electrocatalytic process on porphyrin-modified Au(111) electrode

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Introduction of metalloporphyrin and surface science

- Electrochemical water splitting is one of the promising ways to produce hydrogen and oxygen for clean and renewable energy
- Metalloporphyrin can be the best model system for a metal-organic catalyst to overcome the slow kinetics of OER and ORR
- Electrochemical surface analysis is actively used to survey the enhanced electrocatalyst for potential application in solar-driven water splitting devices
- EC-STM provides direct observation of the surface at the molecular scale at solid/liquid interface, broadening our fundamental comprehension for OER and ORR mechanisms

Schematic of EC-STM

- In EC-STM configuration, bipotentiostat can controls potentials of the sample (WE1) and tip (WE2) with respect to the reference electrode allowing for real-time and real-space imaging at the solid/liquid interface under the electrochemical conditions

Sample preparation and tip coating

- **Au(111)/Mica**
  - Flame annealed Au(111)/Mica
  - Drop the droplet
  - Pasteur pipette

- **Au(111) single crystal**
  - Mini desiccator
  - Butane gas

- **33 μM MOEP in Octanoic acid**
  - Drop the droplet

- **Tip coating**
  - Mini petri dish
  - Pyrex® petri dish
  - Immersion in 100 μM porphyrin/benzene

Unique domains of porphyrin networks in octanoic acid

- Hexagonal-OEP
- Vertical-OEP
- Parallel-OEP

Time-dependent surface restructuring of porphyrins

- Hexagonal-OEP is the most thermodynamically stable phase, whereas parallel-OEP with a substructure consisting of a single layer of octanoic acid can be considered as nearly metastable phase
- The restructuring of parallel-OEP (~0.32 molecule/nm²) into hexagonal-OEP (~0.55 molecule/nm²) occurs continuously until a steady state is reached in solution

Porphyrin/Au(111) in DI water

- MnTPP-Au(111)
- MnOEP-Au(111)

In situ EC-STM measurement of Au(111) in 0.1 M NaOH

- The repeated Au lifting and reconstruction process-induced many dark holes and protruded Au islands on (111) terrace
- Above 0 V, hydroxide ions are starting to adsorb on Au surface at the anodic direction and desorb at the cathodic direction, showing the reversibility

Schematic of the proposed model of adsorbed molecules

- As illustrated by molecular model structure, the eight ethyl groups of M-OEP will support the hydroxide ion adsorption due to its higher affinity than benzene rings of M-TPP

Observation of electrocatalytic process on metal center for OER

- Two dimensionally well-ordered porphyrin structures are repeatedly observed in the alkaline environment by STM in real-time
- Porphyrin-modified Au(111) electrodes show much higher efficiency than bare Au(111)
- MnOEP has the highest current density, among the porphyrin-modified electrodes at 0.4 V = \(V_{\text{oc}}\), which is assumed as Mn oxidation
- It is suggested that because of its better hydroxide ion adsorption property, MnOEP shows higher performance on OER than MnTPP
**Introduction – CO2 adsorption on TiO2(110)**

- TiO2 could be used for chemical conversion of CO2 to other useful molecules because of specific surface properties for photocatalysis. However, a fundamental explanation about the precise mechanism of CO2 adsorption on TiO2 is still failed.

- Rutile TiO2(110) is one of the available choices for the model catalyst samples to investigate the interaction between TiO2 surface and CO2 molecules. It is because TiO2(110) is the most stable form of titania, and it has been widely studied.

- Adsorbates remained on Ti5f rows, after evacuation from 1 Torr. However, when pressure reached 1 Torr, defects on Ob disappeared quickly, and superstructures of CO2 overlayers increased dramatically.

- The pressure of CO2 is a key factor inducing the interaction of CO2 and TiO2.

**Experimental**

- Preventing water contamination
- TiO2(110) should not interact with water that could prohibit CO2 from adsorbing on TiO2(110) surface.
- Liquid nitrogen cold trap could hold water molecules before the molecules enter on the scanner reaction cell.
- Sample preparation
- Cleaning cycle
- Sputtering: 20 min
- Annealing: 900 K, 5 min
- Repeat this cycle until get clean and reduced TiO2(110) surface in UHV

**STM images of TiO2(110) surface under CO2 gas condition**

- UHV
- CO2 10^{-4} Torr
- CO2 10^{-3} Torr
- CO2 10^{-2} Torr
- CO2 10^{-1} Torr

- When pressure reached 10^{-1} Torr, defects on Ob disappeared quickly, and superstructures of CO2 overlayers increased dramatically.

- The pressure of CO2 is a key factor inducing the interaction of CO2 and TiO2.

- Well ordered domains by superstructures of CO2 were created when maintaining CO2 pressure as 10^{-1} Torr. Black line squares on the enlarged image indicate well ordered domains.

**Conclusion**

- In this study, we observed that clean and reduced TiO2(110) surface interacts with CO2 molecules at room temperature. CO2 pressure is key factor for the interaction between CO2 and TiO2(110).

- CO2 molecules adsorbed on top of single Ti5f atom under near-ambient pressure conditions. Superstructures of CO2 overlayers could diffuse along Ti5f rows, which means that the origin of the interaction is the physisorption of CO2. Also, we present three possible adsorption coordination between Ti5f atoms and CO2 molecules.

- It is almost impossible to achieve 1 ML coverage image by STM because of the diffusion of gaseous CO2 molecules. CO2 adsorbates remained on TiO2(110) surface after evacuation.