EGF Conjugated Gold and Silver Nanoparticles for Imaging EGFR Over-expressing Cells

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ABSTRACT

The Epidermal Growth Factor receptor (EGFR) protein facilitates cell growth, proliferation and differentiation in many tissues. It is over-produced on the surface of certain cancers. Nanoprobes targeting solid tumours have focused on antibodies for specificity and reporter tags for imaging. Gold and silver nanoparticles (Au/AgNPs) were made and linked to Epidermal Growth Factor (EGF) by a short ligand, α-lipoic acid, which binds strongly to both Au and AgNPs. Silver nanoparticles, not previously attached to EGF, were used. Time-of-Flight mass spectrometry demonstrates coupling of α-lipoic acid to EGF. ELISA confirms the excellent binding affinity of linked EGF, as it is active alone and following conjugation to AuNPs. In dark field microscopy, Similar responses by tagged and control cells does not confirm the binding affinity. Novel EGFR-specific gold and silver nanoprobes were synthesized and validated by standard assay as SERS optical imaging probes.

Keywords: epidermal growth factor (EGF), cancer imaging, gold and silver nanoparticles, dark field microscopy

1 INTRODUCTION

Epidermal Growth Factor receptor (EGFR or HER1) is a transmembrane protein over produced on the surface of certain cancers and induces cell growth, proliferation and differentiation in multiple tissue types. EGFR is targeted by ligands such as EGF protein or anti-EGFR antibodies. Antibody treatment can cause toxic side effects, including cardiac arrest[1-2]. EGFR antibodies tagged with fluorescent probes[3-4] or gold nanoparticles have been used as optical imaging contrast agents for EGFR over-expression[5-6], with at most 10:1 intensity contrast. We propose to use SERS to overcome limited contrast of prior techniques. By exciting a sample with laser light, energy is transferred to (Stokes) or lost (Anti-Stokes) by the medium in an inelastic scattering process known as Raman spectroscopy. A nanoprobe we developed using anti-EGFR antibodies yielded an order of magnitude improvement in contrast over existing methods[7].

1.1 Previous EGF to Nanoparticle Conjugation Methods

Nanoprobes targeting solid tumours traditionally focused on antibodies for specificity and extra reporter tags for fluorescence, microscopy, and Raman[3, 8-9]. Researchers used several methods to affix EGF to nanoparticles with limited success[8, 10-13], as summarized before[14]. None use silver nanoparticles, which is possible with our approach.

In our previous method[14], gold and silver nanoparticles (Au/AgNPs) of various sizes were synthesized and coupled to epidermal growth factor (EGF) via a short ligand, α-lipoic acid (206 g/mol), which binds strongly to both Au and AgNPs via its disulfide end group[15]. This increases the NP stability under biological conditions. Carbodiimide chemistry coupled EGF to α-lipoic acid. M. Creixell et al. (2010)[13] combine the protein and NPs by another linker, in reverse order. We confirmed EGF-linker formation with Time-of-Flight mass spectrometry (TOF-MS). The linker and NP binding affinity were quantified by Enzyme Linked Immunosorbent Assay (ELISA) and, *in-vitro* with dark field microscopy of EGFR over-expressing A431 cells[14].

1.2 Our Approach

We wish to create and validate a SERS nanoprobe for optical imaging of the Epidermal Growth Factor receptor. We build on work we completed with EGFR active EGF-linker coated NPs to discuss dark field microscopy controls with α-lipoic acid coated Au and AgNP tagged A431 cells.

2 METHODS

See previous work for notes on EGF-linker synthesis; nanoparticle preparation, attachment and stabilization by the EGF-linker; and characterization by transmission electron microscopy and UV-Visible spectrophotometry[14].

2.1 Mass Spectrometry (MS)

The EGF and EGF-linker were diluted in 0.1% formic acid for MS analysis. Methanol (MeOH) and acetonitrile (ACN) were added at a 50 H_2O : 30 ACN : 20 MeOH ratio. An Electrospray Ionization quadrupole Time-of-Flight (ESI-qTOF) MS from Bruker Daltonics (Billerica, USA) collected the data. The flow rate was 10-20 µL/min. Formic acid aids protein ionization in ESI by increasing the conductivity of the solution. In the ESI inlet, the sample is heated or dried (N_2 gas) to form gas phase ions. The sample (mass, m) accelerates from the quadrupole by a voltage (V), travels down a flight tube at constant speed (v), and hits the detector. Conservation of energy requires:

$$V = \tfrac{1}{2}mv^2 \quad (1)$$

To find the sample mass, m, the kick voltage (V) and distance over travel time around the flight tube (v) are used.

2.2 Enzyme-linked Immunosorbent Assay (ELISA) for EGF

An ELISA kit from RayBiotech (Norcross, USA - from Cedarlane Labs (Burlington, Canada)) was used to find the EGF and EGF-linker activity. The activity of fully coated EGF-linker 5 & 18 nm AuNPs and 5 & 45 nm AgNPs was assessed. With ELISA, protein samples are added to wells, then a series of reagents and antibodies react to produce a colorimetric response. This colour change is observed by UV-Vis spectrophotometry. We used a Nanodrop ND-1000 UV-Vis to read at 450 nm (path length = 1 mm). Otherwise, an EMax microplate reader from Molecular Devices (path length = 1 cm) was employed. Each test well was measured in triplicate. The data were assessed with Dixon's Q-test for outliers at 95% confidence. No values were rejected.

2.3 Dark Field Microscopy of A431 Cells with α-lipoic Acid or EGF-linker Nanoprobes

Dark field microscopy images of A431 cells incubated with 18 nm Au or 45 nm AgNPs were obtained. About 500 000 cells were counted onto a coverslip. The next day, the medium was discarded and the cells washed three times with sterile 50 mM HEPES buffer. 100 µL of fully covered EGF-linker/α-lipoic acid or α-lipoic acid control NPs were added to a dish of cells. The NPs were incubated for 30 minutes with gentle agitation. The probe solutions were removed and the cells washed two times with the HEPES buffer. The coverslips of tagged cells were transferred to glass bottom dishes with 500 µL of HEPES buffer.

The dark field setup consisted of an Olympus BH2-UMA microscope outfitted with a dark field condenser and a Canon EOS Digital Rebel XSi 12.2 MP DSLR. Images were obtained with the 20X objective 3-4 hours after the cells finished incubating with the nanoprobes.

3 RESULTS AND DISCUSSION

3.1 Mass Spectrometry (MS)

Figure 1 is a plot of the mass to charge ratio versus intensity following injection of EGF-linker protein into the TOF-MS. A singly charged unlinked EGF molecule should exhibit a peak at m/z = 6200. However, peaks appear at m/z = 1244.1 and m/z = 1036.9. The expected peak positions are: m/z = 1245.0 (+5) and m/z = 1037.7 (+6). These peak positions can be reconciled with EGF carrying charges of +5 and +6, respectively, due to an abundance of ionizable protons. The eight ionizable amino acids present in EGF are classified as: the amino terminus, lysine, arginine, and histidine. All our TOF-MS spectra show peaks from +5 and +6 charges. The reason for the relative abundance of each is unknown. Note adjacent to the main peaks, satellite peaks appear at larger m/z values from heavier protein isotopes.

α-lipoic acid has a molecular weight of 206 Daltons. Its addition to EGF should produce peaks at m/z = 1282.6 (+5) and m/z = 1069.0 (+6), whereas in Figure 1 peaks are observed slightly offset at m/z = 1285.9 and m/z = 1071.8. Replacement of a hydrogen atom by sodium accounts for this and is a consequence of NaOH use during the linker quenching step of the formation reaction. Sodium salt peak positions should appear at m/z = 1287.0 (+5) and m/z = 1072.7 (+6) which are close to those observed. Weak peaks near m/z = 1320 (+5) and m/z = 1100 (+6) are due to the addition of two α-lipoic acid molecules to EGF. Based on the +5 charge peaks, we obtain a singly linked EGF yield of at most 50%, with slim contributions of doubly linked EGF.

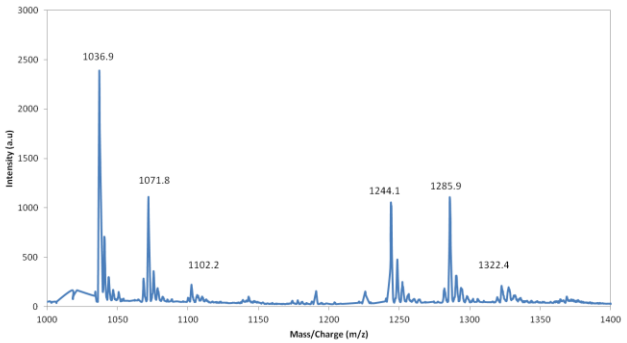

Figure 1: TOF-MS of EGF-linker. The EGF protein peaks for +5 and +6 charges appear at m/z = 1244.1 (* = theoretical, *m/z = 1245.0) and m/z = 1036.9 (*m/z = 1037.7), respectively. Peaks for singly linked EGF are at m/z = 1285.9 (*m/z = 1287.0, +5) and m/z = 1071.8 (*m/z = 1072.7, +6) (yield = ~50%). Faint peaks at m/z = 1322.4 (+5) and m/z = 1102.2 (+6) represent EGF with two linkers.

3.2 Enzyme-linked Immunosorbent Assay (ELISA) for EGF

EGF-ELISA allows assessment of the EGFR binding affinity of EGF, EGF-linker, and EGF-linker nanoparticles. The activity of EGF at various concentrations is shown in Figure 2. The unlinked and linked EGF used in the experiment (kit and EGF-linker, respectively) gave similar responses, indicating the EGF-linker was still active for the EGFR. Tests of reagent EGF (Invitrogen) showed activity identical to the product EGF-linker (data not shown). The 18 nm coated AuNPs did give significant activity (18 nm c EGF-Au 1 and 2, and 18 nm ½ EGF-Au 1 and 2). "c" stands for complete, while "½" represents half EGF-linker coverage. The ELISA kit uses a sandwich assay detection method, where the EGF binds to an anchored capture

antibody, then a detection antibody binds the plate bound EGF. If we assume only 1 EGF-linker per nanoparticle binds to the plate, then all other EGF-linkers coating the NP would not give a colorimetric response. We assume 1 EGF-linker = 1 NP, so the nanoparticle concentration is limiting. This seemed true, as the coated NPs have responses similar to the kit EGF and EGF-linker samples in Figure 2. Thus, the x-axis scale of the NP samples is based on the NP concentration. NPs with half EGF-linker coverage have a slightly decreased response. The large 45 nm silver NPs were not yet tested for activity. The small 5 nm gold or silver NPs did not give repeatable results, and did not always seem active (data not shown). The reason for this is unclear.

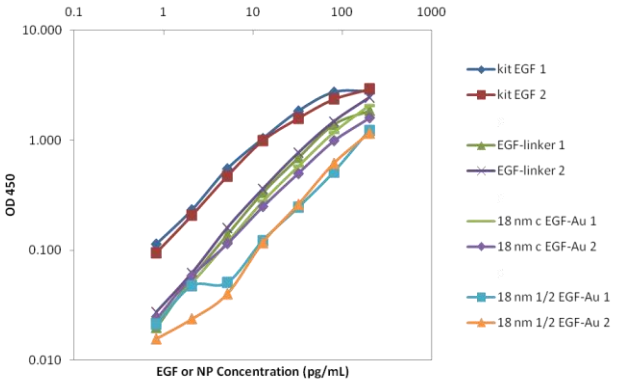

Figure 2: EGF-ELISA assay of the total EGFR binding response measured by the optical absorbance at 450 nm, for various concentrations of EGF, EGF-linker, and α-lipoic acid/EGF-linker coated AuNPs. For the NP samples, the x-axis scale is the NP concentration. "c" is complete EGF-linker coverage, while "½" is half EGF-linker coverage. NPs with less EGF-linker show a slight activity decrease.

3.3 Dark Field Microscopy of A431 Cells with α-lipoic Acid or EGF-linker Nanoprobes

The images in Figure 3 are A431 cells incubated with EGF-linker/α-lipoic acid coated 18 nm AuNPs or 45 nm AgNPs. The control cells (Figure 3 a) do not look very different from the cells with the control or active nanoparticles. All contain bright spots at the surface and interior of the cells. Dark field does not confirm the activity or specificity of the EGF-linker/α-lipoic acid coated NPs. The nanoprobe or cell structures do not seem to be present in the nucleus, which is expected as the EGFR should be engulfed into endosomes and lysosomes[9]. Light scattering from silver α-lipoic acid/EGF-linker coated nanoprobes (Figure 3 b and d) seems less intense than the gold (Figure 3 c and e). If some scattering is from the nanoparticles, this could be explained by the halogen lamp used for illumination, as it lacks some wavelengths necessary to excite the silver.

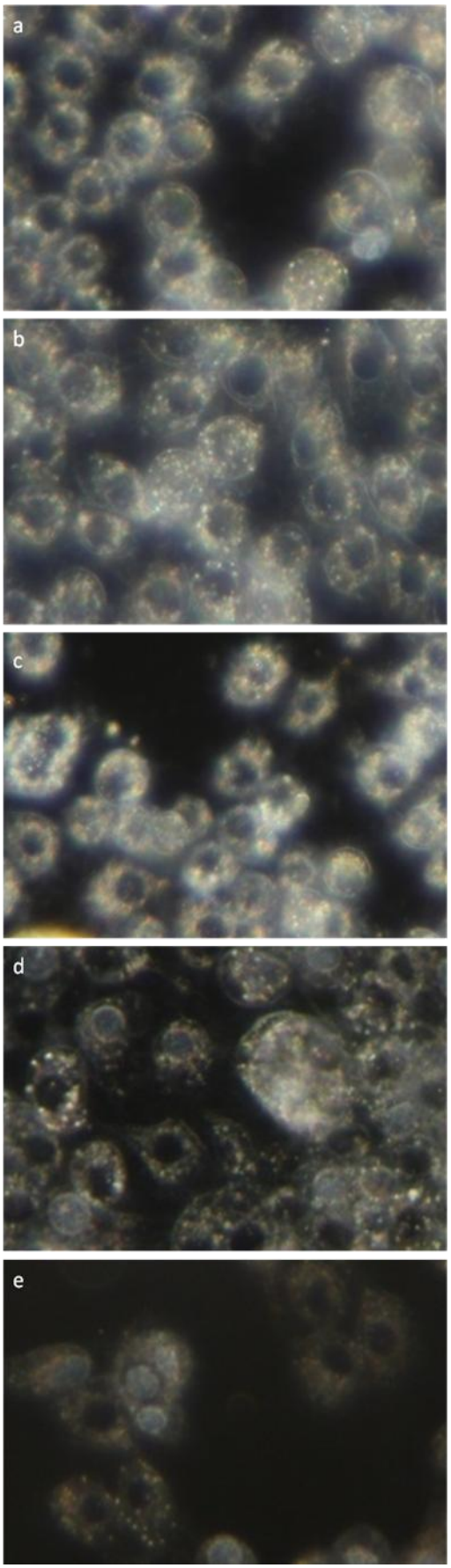

Figure 3. Dark field microscopy depicting uptake of EGF-linker or α-lipoic acid coated nanoprobes on A431 cancer cells. Images were obtained with a 20X lens, a 12.2 MP Canon XSi DSLR, and digitally cropped to show detail. The incubation medium was: a. 50 mM HEPES, b. 12.5 nmol 18 nm α-lipoic-Au, c. 16.2 nmol 45 nm α-lipoic-Ag, d. 12.5 nmol 18 nm EGF-Au, e. 16.2 nmol 45 nm EGF-Ag.

4 CONCLUSIONS

Cancer is the second leading cause of death in Canada after heart disease. More cost effective imaging and treatment options will save lives, help to reduce the costs associated with and stress on the health care system, and improve access in developed and developing countries. We targeted the transmembrane protein, Epidermal Growth Factor receptor (EGFR), in the development of a new nanoprobe. EGF protein was successfully linked to α-lipoic acid as confirmed by TOF-MS and remained active as established by EGF-ELISA measurements.

The modified EGF protein was then attached to gold or silver nanoparticles and assessed with a variety of techniques. The EGF-ELISA test of 18 nm Au-EGF-linker conjugated nanoprobes showed similar activities to the kit EGF. Dark field microscopy was also used to assess the nanoprobe activity. Images of probes incubated with A431 cancer cells did not show nanoparticles present in the cells. This conflicts with our conclusion from the ELISA tests that the probes were active and specific for EGFR, and ingested into endosomes and lysosomes by the cell.

These Ag- and Au-based EGF nanoprobes will allow researchers to target EGFR over-expressing tissues, and would be especially useful for imaging via SERS and thermal therapy.

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