Supplementary Material (ESI) for Chemical Communications

(Electronic Supporting Information)

Hairpin DNA coated gold nanoparticles as intracellular mRNA probes for the detection of tyrosinase gene expression in melanoma cells

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Materials and Methods

All chemicals were used as received unless otherwise noted. The human cell lines, SK-MEL-28 and HEp-2, were obtained from the American Type Culture Collection (ATCC). 15nm gold colloid solutions were obtained from Ted Pella, Inc (Redding, CA). Syto-13 nuclear dye (Sy13), Sytox Green viability dye (SxG) and sterile nuclease free phosphate buffered saline without $MgCl_2$ and $CaCl_2$ (PBS, pH 7.4) were obtained from Invitrogen Corp (Carlsbad, CA). Bovine deoxyribonuclease I (DNase I) was purchased from Promega (Madison, WI). Molecular beacons (MB), unmodified oligonucleotides, custom oligonucleotides and PCR primers were purchased from Biosearch Technologies, Inc (Novato, CA). All other chemicals were purchased from Sigma Aldridge, Inc (St. Louis, MO).

Hairpin Oligonucleotides

The mRNA recognition sequences used for this study were as follows: (TYR Rec. Seq) 5'-attgtgcatgctgctttgag-3' and (MM Rec. Seq) 5'-gtattggaagtcgttcgatgatcactcgc-3'. Each recognition sequence was extensively BLAST searched to confirm either specificity (TYR rec seq) or non-specificity (MM rec seq) with no overlap with other potential human gene transcripts. Prior to use, thiol-terminated oligonucleotides were subjected to 0.1 M dithiolthreitol (DTT) reduction of the 5' thiol moiety. Excess reducing agent was removed by 3K molecular weight centrifugal filtration and washed three times with PBS (Amicon Ultracel 3K, Millipore). Freshly reduced oligonucleotides were stored in 1X Tris EDTA (TE) buffer (pH 8) at -80^{o}C until use.

Cell Culture

SK-MEL-28 and HEp2 cells were cultured in high glucose DMEM growth medium supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/streptomycin and 2% L-glutamine. Primary cell cultures were maintained in RPMI 1640 growth medium supplemented with 10% fetal bovine serum (FBS) , 100 units of penicillin/streptomycin and 2% L-glutamine. Phenol-red free RPMI 1640 supplemented with 5% FBS was used for all live-cell fluorescence experiments. Co-culture experiments were performed by adding equal cell volumes (e.g. 5e5 Mels / 5e5 HEp2, total cell count = 1e6) to either a 35mm Mattek microwell dish or well plates and allowed to adhere to the surface > 4hrs before treatment with hAuNPs in complete growth medium.

Dynamic Light Scattering (DLS)

All DLS measurements were performed on a Malvern Zetasizer Nano ZS. Particle measurements were performed at a concentration of 1 nM in water and measured in triplicate. To determine the size of 'open' TYR hAuNPs, a 1 nM solution of TYR hAuNPs was treated with 1 μM TYR recognition complement and incubated at 37^{o}C for 2 hours prior to measurement.

Absorption and Fluorescence Measurements

Absorption measurements were performed on an Agilent 8453 diode-array spectrophotometer. The concentration of AuNPs and hAuNPs was determined by measuring the absorbance at 520 nm ($\varepsilon = 3.64 \times 10^{8}$ $M^{-1}cm^{-1}$). The emission spectrum of 'open' TYR hAuNPs was obtained by treating a 5 nM solution of TYR hAuNPs (PBS, 0.1% Tween-20) with 1 μM TYR recognition complement and incubating at 37^{o}C for 2 hours. Fluorescence spectra of the resulting 'open' TYR hAuNP solution was generated on a Cary Eclipse Fluorescence Spectrophotometer by

exciting at 620 nm and measuring the emission from 650 to 750 nm. Target selectivity assays and DNase I susceptibility measurements were performed using a Bio-Tek Synergy HY multidetection fluorescent microplate reader using a 590/35 excitation filter and a 645/40 emission filter.

TYR hAuNP Target Selectivity

To measure selectivity of the TYR hAuNP probe, TYR hAuNPs (1 nM, PBS, 0.1% tween-20) were titrated with increasing concentrations of TYR recognition complement (0-1 μM) and MM complement (0-1 μM) keeping a total volume of 200 μl. The resulting solutions were sealed, protected from light and allowed to hybridize for 2 hours at 37^{o}C. Fluorescence intensities were measured as described above and plotted as a function of complement concentration. Signal to noise was determined by the ratio of TYR target fluorescence versus MM background fluorescence.

DNase I Susceptibility

To determine TYR hAuNP suceptibility to nuclease degredation, TYR hAuNPs and free molecular beacons were analyzed. The following tyrosinase-specific molecular beacon was employed: (TYR MB Seq) 5'-Cy5-cgacgattgtgcatgctgctttgagcgtcg-BHQ3-3'. TYR hAuNP solutions (2.2 nM) and TYR MBs (200 nM) were diluted in nuclease reaction buffer (100 mM Tris-HCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) and 100 μl aliquots of each added to a 96-well black wall microplate. Both TYR hAuNPs and TYR MBs were treated with 10 μl of either: 1 U/mL DNase (excess, endpoint determination), 1 U/L DNase (optimum), or PBS (baseline) and transferred immediately to a preheated fluorescent microplate reader (37^{o}C). Fluorescence

output for each well was monitored every 300 seconds for 3 hours to generate degradation progress curves for each sample. DNase susceptibility was evaluated by the slope of the initial linear region.

Scanning Confocal Microscopy

Cells were grown in 35 mm Mattek dishes and kept at approximately 50% confluence. Cells were treated with 0.5 nM hAuNPs in complete media. After the 4 hr loading period, the cells were washed twice with PBS and fresh medium was added to the dish. After incubating for 12 hours at 37^{o}C, the media was removed and fresh phenol-red free media added to the dish. For images with a green nuclear stain, cells were treated with 25 nM Syto-13 for 15 minutes prior to imaging. All images were captured on a Zeiss LSM 510 Meta inverted confocal microscope with identical optical settings for each session. Fluorescent images were captured using the following excitation lasers and corresponding filter sets: Syto13 nuclear stain (488nm; bandpass 505-530nm) and quasar 670 hAuNP probe (633nm; longpass 650nm). Scanning confocal imaging was performed in part through the use of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126).

Flow Cytometry

Cells were grown in 6 well plates (1e6 cells/well) and treated with 0.5 nM hAuNPs as described above. After a 12 hr hybridization period, cells were lifted from the plate with 0.05% trypsin-EDTA, washed twice with cold PBS, resuspended in 500 µl of cold phenol-red free media containing 5% FBS and stored on ice until analyzed. The addition of Sytox Green (50 nM) to each sample was used to assess cellular integrity and tolerance of hAuNPs. Samples were

analyzed on a Custom Becton Dickinson five-laser LSRII analytical flow cytometer using forward and side scatter focusing. Sytox Green and Cy5 emissions were monitored simultaneously for 10,000 events and a mean fluorescence for each fluorescent signal was recorded. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center(DK058404).

RT-PCR

To confirm the presence and absence of tyrosinase mRNA in SK-MEL-28 and HEp2 cells, RT-PCR applification was performed. Cellular mRNA was extracted from each cell line using the RNeasy Mini Kit (Qiagen) according to the manufacturers protocol. Purified mRNA was quantified by UV-Vis. RT-PCR was performed using a Rotor-Gene Q real time cycler (Qiagen) and QTaq One-Step qRT-PCR SYBR Kit (Clontech). Briefly, 3ng (5 μL) of mRNA from each cell line was reverse-transcribed and amplified in a 25 μL reaction volume with the following components: 12.5 μL of 2X One-Step qRT-PCR Buffer plus SYBR, 0.5 μL of 50X QTaq DNA Polymerase Mix, 0.4 μL of 60X qRT Mix, 200 nM forward and reverse primers, and DNase/RNase free water. The following primers were employed: TYR Forward (5'-ttggcagattgtctgtagcc-3'), TYR Reverse (5'-aggcattgtgcatgctgctt-3'), β-Actin Forward (5'-gcgggaaatcgtgcgtgacatt-3') and β-Actin Reverse (5'-gatggagttgaaggtagtttcgtg-3'). Cycling conditions were as follows: 45 min at 48^{o}C, 3 min at 95^{o}C, followed by 40 cycles each of 1 min at 55^{o}C and 2 min at 72^{o}C.

The expected sizes of the PCR products were 289 bp (tyrosinase) and 232 bp (β-actin). 5 μL of the PCR products were subjected to electrophoresis on a 1.2% agarose E-gel (Invitrogen), subsequently stained with ethidium bromide and visualized under UV light.

Supplementary Figures

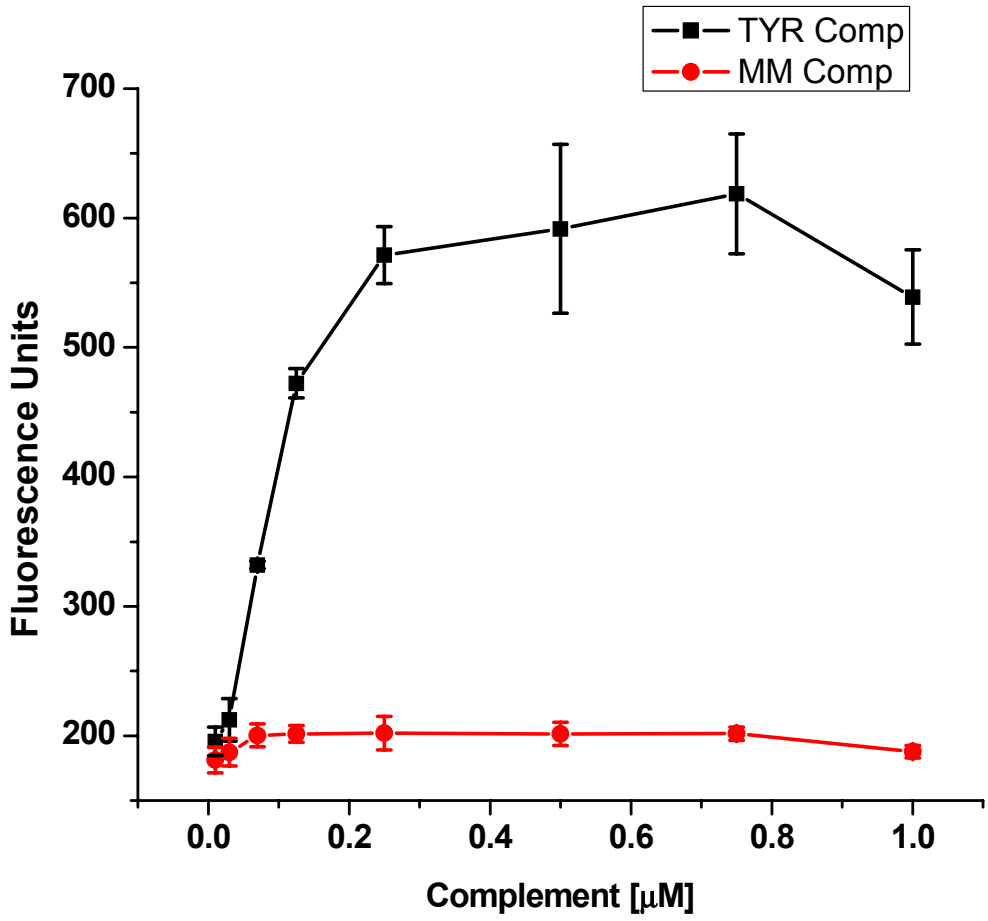

Figure S1. *TYR hAuNP Target Selectivity Assay*. TYR hAuNPs (1 nM) treated with increasing amounts of TYR recognition complement (black) and MM complement (red). The ratio of TYR target fluorescence versus MM background fluorescence yielded a S/N =3 at complement concentrations > 250 nM.

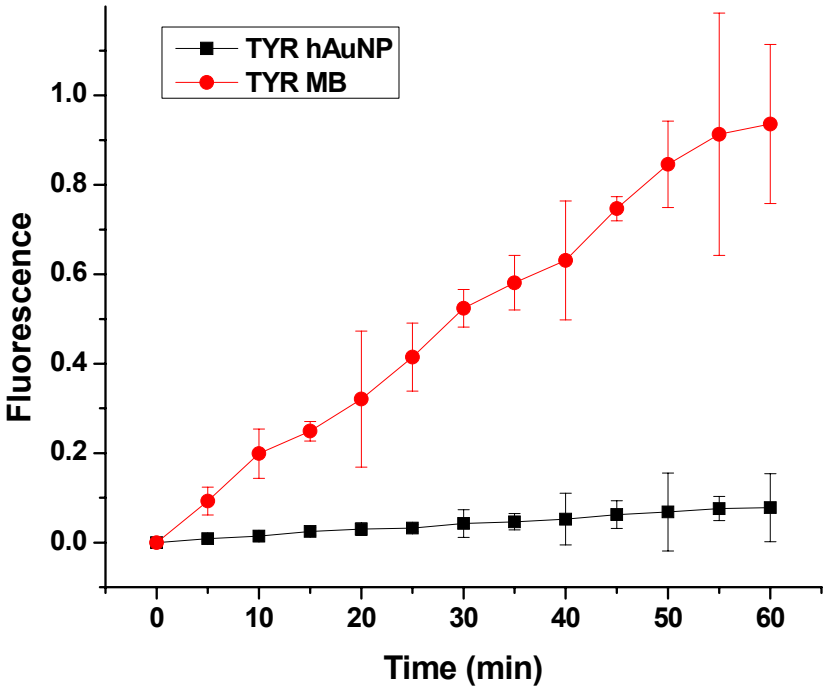

Figure S2. *DNase I Susceptibility Assay.* TYR hAuNPs (2.2 nM, black) and TYR MBs (200 nM, red) treated with DNase (1U/L). Rate of enzymatic degradation was determined by the slope of the initial linear region (shown above). TYR hAuNPs rate of degradation: 0.097 nmols/min. TYR MB rate of degradation: 1.22 nmols/min.

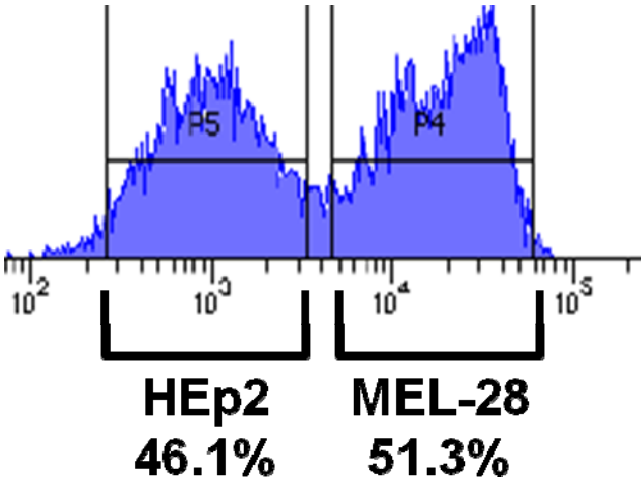

Figure S3. *Co-culture Flow Cytometric Analysis.* An equal number of SK-MEL-28 and HEp2 cells were treated with TYR hAuNP (0.5 nM) simulatiously in the same culture well. Flow cytometric analysis confirms the expected 50/50 cell type ratio with SK-MEL-28 cells exhibiting a 20-fold increase in Cy5 mean fluorescence (mean: 21K) as compared to the HEp2 cell population (mean: 1.2K).

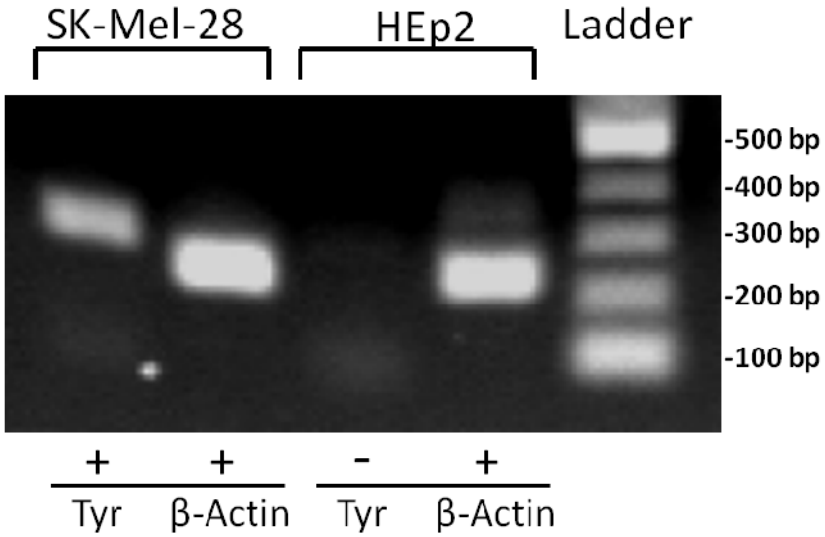

Figure S4. *RT-PCR Analysis of Tyr expression in SK-MEL-28 and HEp2 cells.* RNA isloated from SK-MEL-28 and HEp2 cells was subjected to RT-PCR and visualized by agarose gel electrophoresis. The 289 bp tyrosinase PCR fragments were present in SK-MEL-28 cells and absent from HEp2 cells. The 232 bp β-actin PCR fragments were abundant for both cells lines.