SUPPORTING INFORMATION

for

Protein-Polymer Conjugates Synthesized using Water-Soluble Azlactone-Functionalized Polymers Enable Receptor-Specific Cellular Uptake towards Targeted Drug Delivery

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EXPERIMENTAL PROCEDURES

Labeling hTF and OTF with Alexa Fluor 488. Holo-transferrin (hTF) or ovotransferrin (OTF) were labeled with Alexa Fluor 488 (AF488) by primary amine chemistry. A solution of protein (1-2 mg/ml) was made in phosphate buffered saline (PBS). Sodium bicarbonate (1M stock solution) was added to the protein to a final concentration of 0.1 M to change the pH of the solution to 8.0. The fluorescent dye AF488 5-tetrafluorophenyl ester was dissolved in anhydrous DMSO to a final concentration of 11.3 nM. Dye was added to protein solution, using an amount of dye calculated following manufacturer's protocol to achieve a desired molar excess of dye. The sample was incubated with gentle rotation at room temperature for 1 h. The protein labeled with AF488 was then purified from free dye and concentrated using an Amicon centrifugal filtration device with a molecular weight cutoff of 10 kDa by washing extensively with PBS until the flow through was colorless. Concentrations and degree of labeling were determined using UV-Vis spectroscopy, measuring dye absorption at 494 nm (ε = 71,000 cm^{-1} M^{-1}). Labeled protein was stored at 4 ºC.

Titration Binding Assay of hTF-488 with MCF-7 Cells. Titration binding assays were performed to experimentally determine the binding affinity (dissociation constant, K_D) of hTF with MCF-7 cells. MCF-7 cells were harvested with 0.05% trypsin-EDTA. Aliquots of $1x10^5$ cells were incubated for 1 h at 4 ºC with a range of concentrations of fluorescently labeled hTF (hTF-488, 0.5-500 nM) in PBS with 0.1% BSA (PBSA) with gentle rotation. Following incubation to reach equilibrium binding, cells were washed in PBSA and resuspended in PBSA for analysis. Data were collected and analyzed using flow cytometry. Experimental triplicate data was collected to determine the binding affinity of hTF to its receptor. For each replicate, the data were fit to a sigmoidal binding curve using Kaleidagraph software (Synergy). The concentration of hTF-488

that resulted in the half-maximal value of each best-fit line was determined as the K_D. The mean of the three individually fit dissociation constants was determined and reported with the standard deviation.

Figure S1

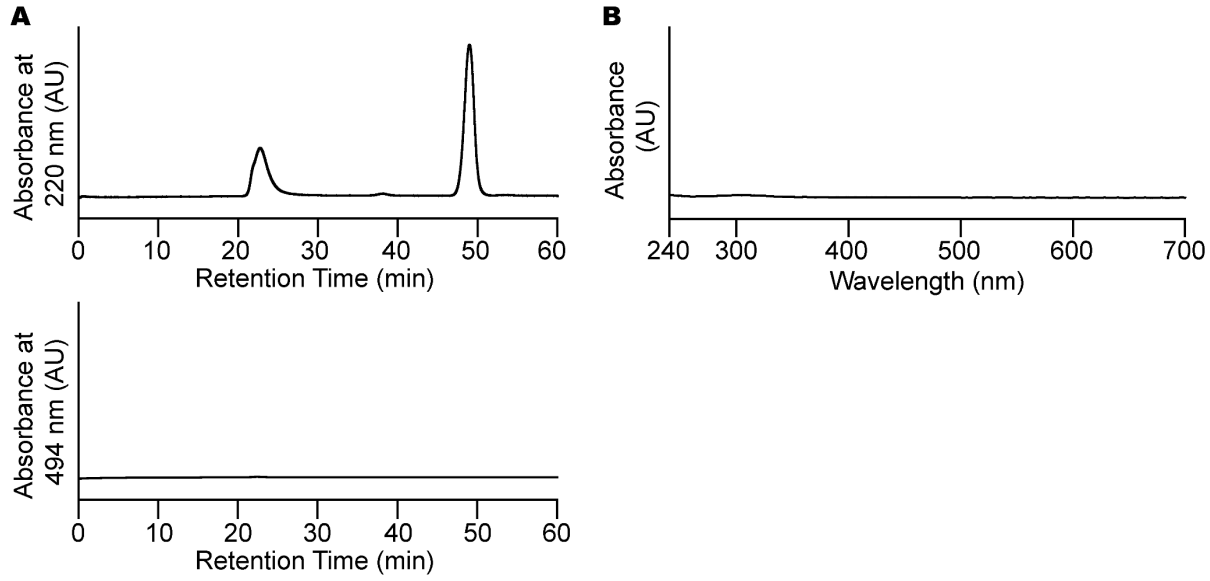

Figure S1. PVDMA-mTEG analysis by size exclusion chromatography and UV-Vis spectroscopy. (A) PVDMA functionalized with 0.3 molar equivalents of mTEG was analyzed on a Superdex 75 30/100 SEC column run at 0.4 ml/min, and absorbance was detected at 220 nm and at 494 nm. For absorbance at 220 nm, the functionalized polymer sample contained a broad peak characteristic of polymers with a molecular weight distribution eluting between 20 and 30 minutes, and a second peak of low molecular weight byproducts eluting around 50 minutes. There was no absorbance at 494 nm. (B) PVDMA-mTEG analyzed using UV-Vis spectroscopy has no absorbance in the 240-700 nm range, as expected for the polymer.

Figure S2

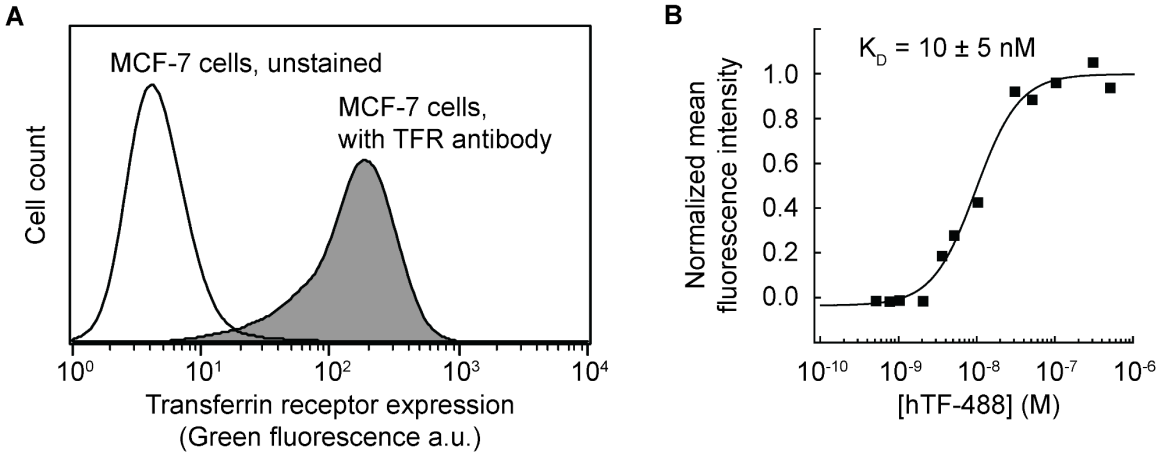

Figure S2. MCF-7 cells express TFR and bind hTF. (A) MCF-7 cells, which are a human breast cancer cell line, express high levels of transferrin receptor (TFR) on their surface, as detected by an anti-human TFR antibody directly conjugated to fluorescein and analyzed by flow cytometry. (B) The binding of hTF to TFR was measured as the dissociation constant (K_D) using an equilibrium binding assay. MCF-7 cells were incubated with a range of concentrations of hTF directly labeled with Alexa Fluor 488 (hTF-488). The assay was performed in experimental triplicate. Data from each replicate were fit to a sigmoidal curve, and the K_D value was calculated for each replicate. The K_D is reported as the mean +/- standard deviation. A representative binding curve is shown.

Figure S3

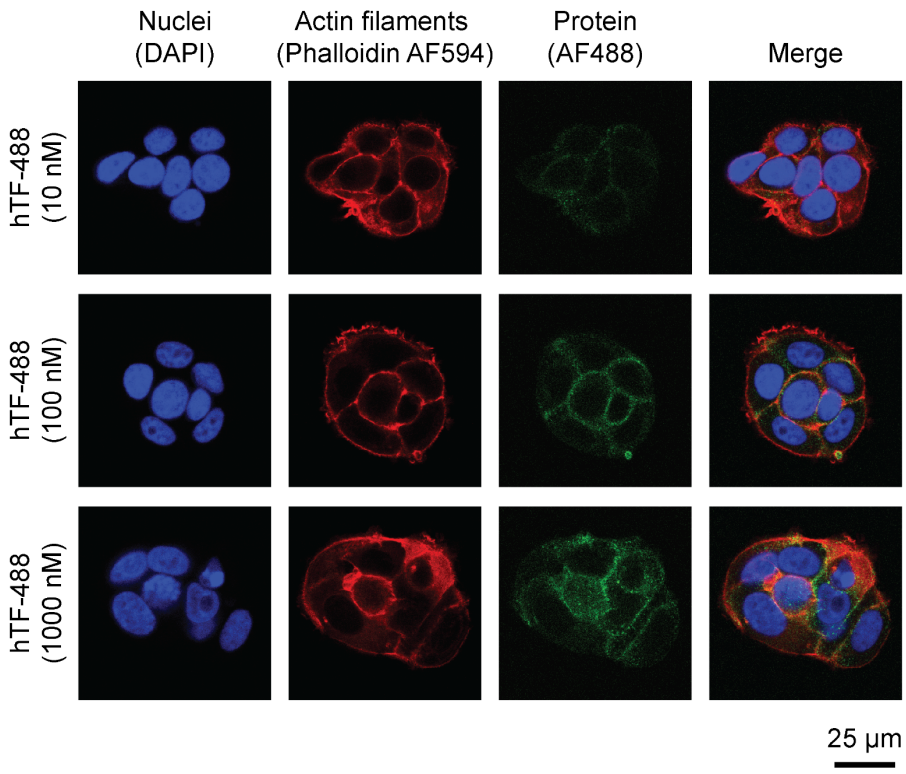

Figure S3. hTF-488 internalization into MCF-7 cells is concentration dependent. MCF-7 cells, which are a human breast cancer cell line, express high levels of transferrin receptor (TFR) on their surface. Fluorescently labeled holo-transferrin (hTF-488) was internalized into the cells after incubation for 1 h at 37 °C. Increasing the concentration of hTF-488 from 10 nM to 100 nM to 1000 nM (rows 1, 2, and 3) showed increasing internalization, as visualized by increasing green signal within the cell boundaries and at the cell surface. Blue indicates DAPI stain for cell nuclei; red indicates phalloidin conjugated to Alexa Fluor 594, which stains actin filaments and helped to identify cell boundaries; and green indicates the protein fluorophore conjugate labeled with Alexa Fluor 488. Scale bar shown applies to all images.

Description of supporting information videos. To confirm the internalization of hTF-488 and hTF-PVDMA$_{FC}$-mTEG, a series of consecutive focal planes were collected with confocal microscopy, referred to as z-stack series, using a step size of 0.5 μM. The data are available as .avi video files in Supporting Information. In the videos, we observed that at the surfaces of the cells, we predominantly see phalloidin staining of actin filaments, indicated in red. For both the hTF-488 positive control molecule (SI_Video_1_hTF-488) and the hTF-PVDMA$_{FC}$-mTEG protein-polymer conjugate (SI_Video_2_hTF-PVDMA-FC-mTEG), we saw that the fluorophore, shown in green, was contained within the cell boundaries, rather than at the cell surface, confirming internalization of molecules. Blue indicates cell nuclei, stained with DAPI.