Supporting Information: Triggered Release Enhances the Cytotoxicity of Stable Colloidal Drug Aggregates

Eric N Donders[1,2], Ahil N Ganesh[1,2], Hayarpi Torosyan[3], Parnian Lak[3], Brian K Shoichet*[3], Molly S Shoichet*[1,2,4]

1. Department of Chemical Engineering & Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario M5S 3E5, Canada
2. Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, Ontario M5S 3G9, Canada
3. Department of Pharmaceutical Chemistry, University of California San Francisco, 1700 Fourth Street, Mail Box 2550, San Francisco, California 94143, United States
4. Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada

* Corresponding author

Calculation of the critical aggregation concentration (CAC)

CAC values are calculated by preparing colloidal formulations with different concentrations and measuring the scattering intensity (or fluorescence intensity when appropriate). The data is then fit to Equation S4, which was derived from first principles (Equations S1–S3), using the MATLAB curve fitting toolbox to obtain the critical aggregation concentration (CAC) under the conditions used. Table S1 describes the meaning of each variable; Figure S1 shows the result of curve-fitting this model to experimental data.

$$Signal = \sum_i Signal_i = Signal_{BL} + Signal_{colloids} \quad \text{S1}^1$$

Assuming constant colloid diameter:

$$Signal_{colloids} \propto C_{agg,colloids} \quad \text{S2}^1$$

$$C_{agg,colloids} = \begin{cases} 0, & C_{agg} < CAC_{agg} \\ C_{agg} - CAC_{agg}, & C_{agg} \geq CAC_{agg} \end{cases} = H\left(C_{agg} - CAC_{agg}\right) \cdot \left(C_{agg} - CAC_{agg}\right) \quad \text{S3}^2$$

Substituting and adding a qualitative correction factor for diameter variations:

$$Signal = Signal_{BL} + m \cdot H\left(C_{agg} - CAC_{agg}\right) \cdot \left(C_{agg} - CAC_{agg}\right)^n \quad \text{S4}$$

Table S1. Description of each variable and function used in calculation of the critical aggregation concentration (CAC). This calculation objectively applies a common approach for measuring the CAC where assays are used in which colloidal drug aggregates produce a signal and soluble drug molecules do not.[2–4] For example, the light scattering intensity of a drug solution does not increase with increasing drug concentration until the CAC is reached, when colloidal particles begin to form. By increasing the concentration above the CAC, the number of colloids increases somewhat linearly and the scattering intensity increases similarly. This behavior is reflected in Equation S4, which describes a line, the slope of which transitions from zero to positive once the CAC is reached.

Term	Meaning	Units
Signal	Signal that is produced by colloidal particles but not by free drug (scattering or fluorescence).	Counts / s or intensity units
$Signal_{colloids}$	Portion of the signal that is produced by colloidal drug aggregates	Counts / s or intensity units
$Signal_{BL}$	Baseline signal produced by buffer and plate.	Counts / s or intensity units
H(x)	Heaviside step function. It reflects the fact that the signal only starts to rise when colloids are present. $H(x) = \begin{cases} 0, & x < 0 \\ 1, & x \geq 0 \end{cases}$	Dimensionless
C_{agg}	Concentration of aggregator present in sample.	µM
$C_{agg,colloids}$	Concentration of aggregator present in the colloidal state	µM
CAC_{agg}	Critical aggregation concentration of the aggregator under assay conditions.	µM
m	Amount of signal produced per amount of aggregator. More aggregator means more particles, which means more scattering or fluorescence.	Signal units / µM
n	Qualitative correction factor that accounts for non-linear signal to concentration relationship, especially in scattering-based assays. This factor is necessary for a high-quality fit because differences in colloid size can result in deviations from the expected straight line. Since these deviations are relatively small, this factor is usually between 0.8 and 1.2.	Dimensionless

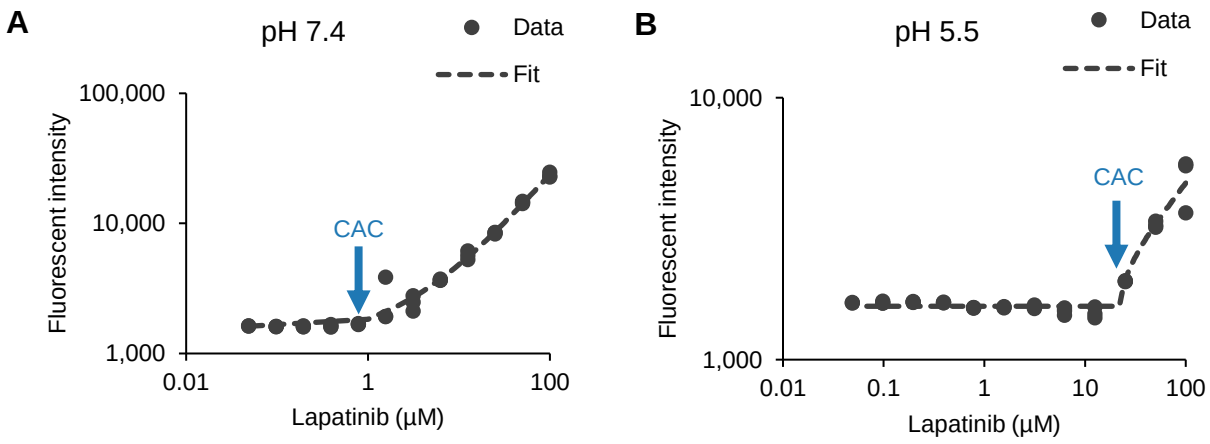
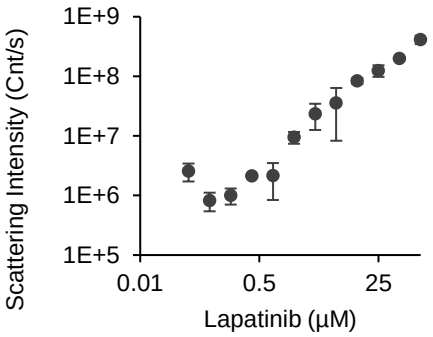
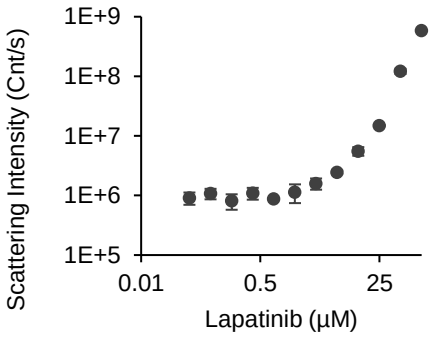
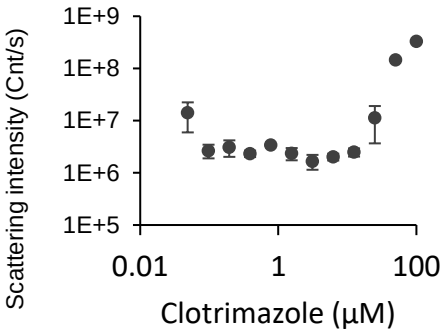
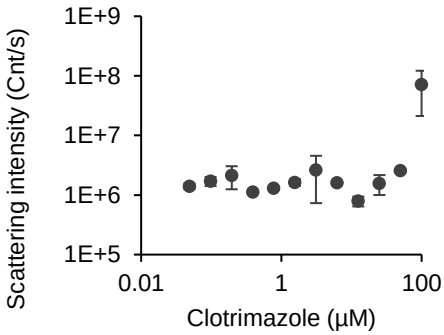
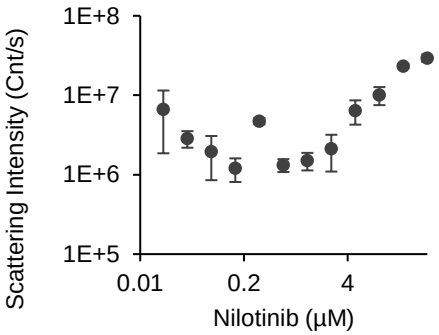
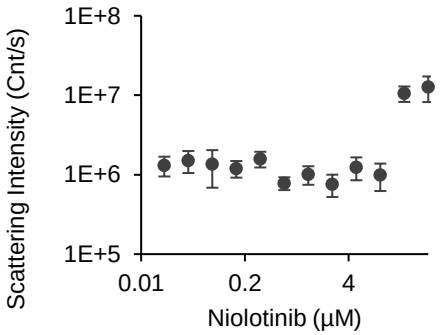
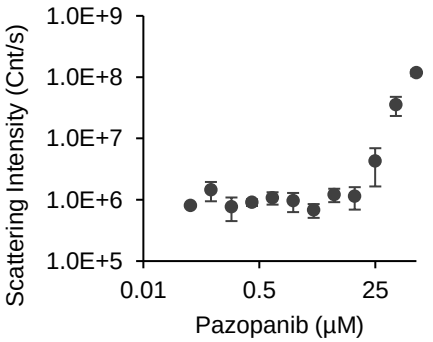
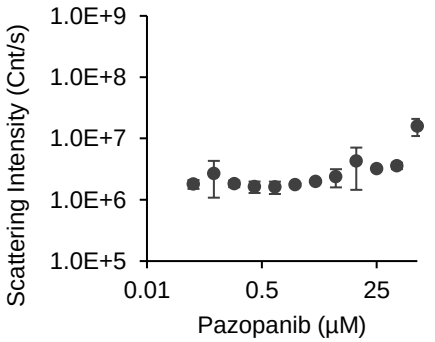
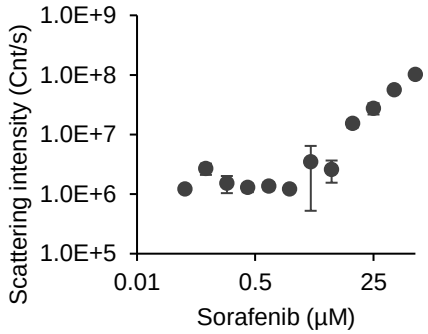
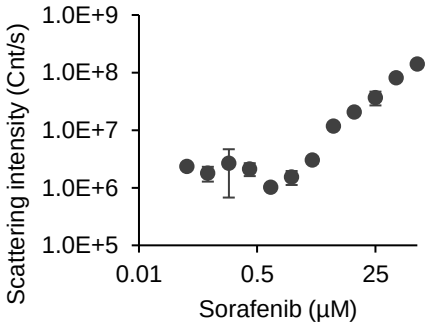
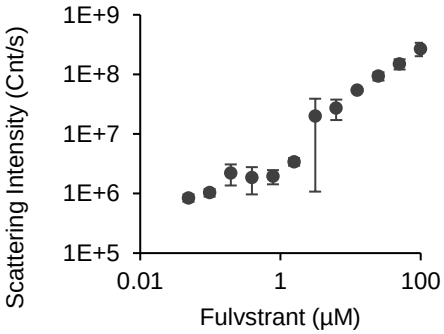
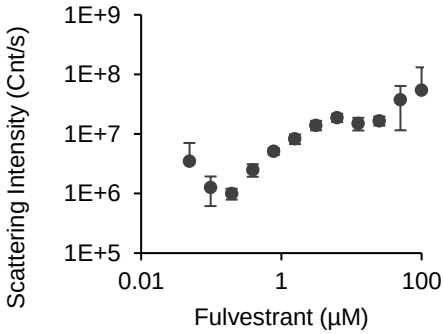


Figure S1. Curve-fitting algorithm identifies the critical aggregation concentration of lapatinib at **A)** pH 7.4 and **B)** pH 5.5. Lapatinib fluorescence is proportional to the amount of colloidal lapatinib, which increases linearly with concentration once the CAC is reached.

Table S2. Plots of scattering intensity versus concentration that are used to calculate the CAC of each drug at pH 7.4 and 5.5.

Drug name	Physiological pH 7.4 scattering	Endosomal pH 5.5 scattering
Lapatinib		
Clotrimazole		
Nilotinib		
Pazopanib		

Drug name	Physiological pH 7.4 scattering	Endosomal pH 5.5 scattering
Sorafenib		
Fulvestrant		

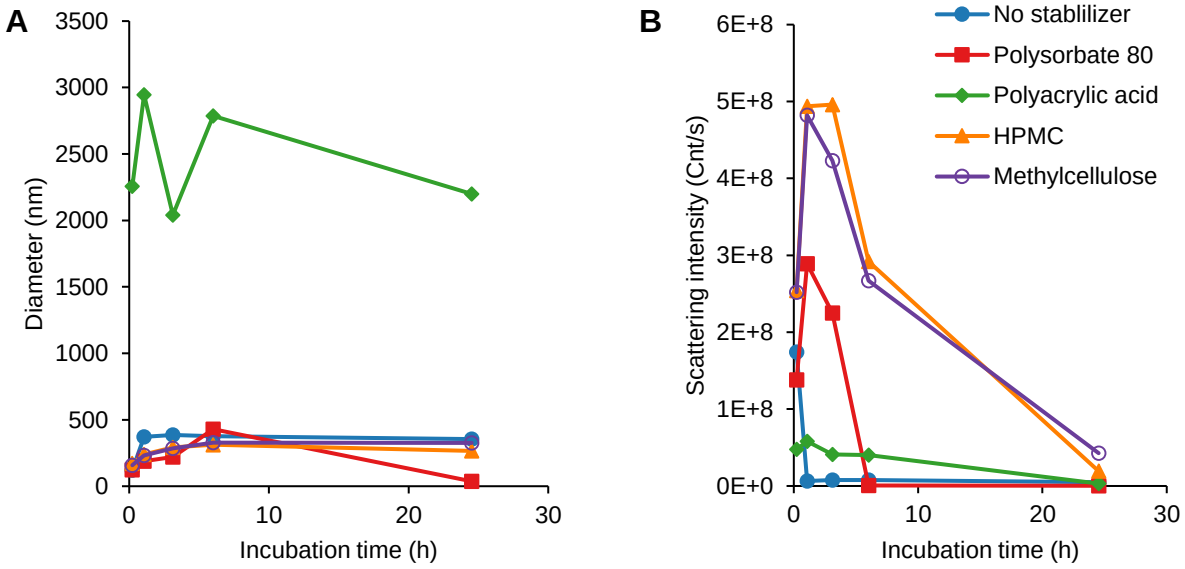


Figure S2. Lapatinib is not stabilized by polymeric excipients. Colloidal lapatinib at 50 μM was formulated with 0.01 mg/mL stabilizer in water and incubated at 37 °C. DLS characterization of **A)** size and **B)** scattering intensity shows precipitation over 24 h, even in formulations that were initially stable.

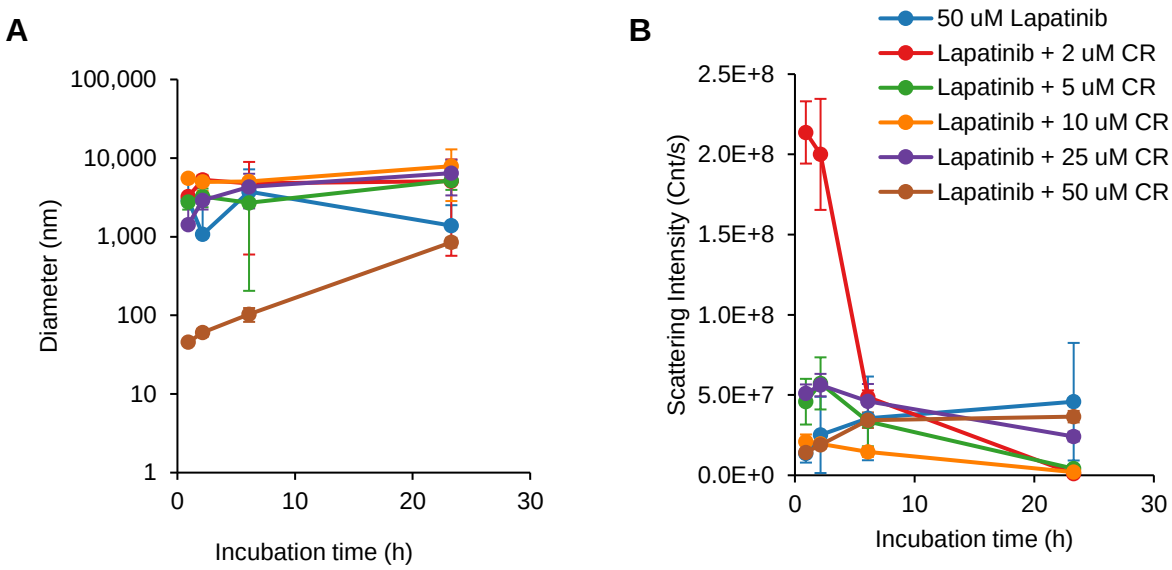


Figure S3. Lapatinib is not stabilized in PBS by co-aggregation with Congo red (CR). Colloidal lapatinib at 50 µM was formulated with varying amounts of Congo red in PBS and incubated at 37 °C. DLS characterization of **A)** size and **B)** scattering intensity shows precipitation over 24 h, even in formulations that were initially stable (n=3, mean ± SD).

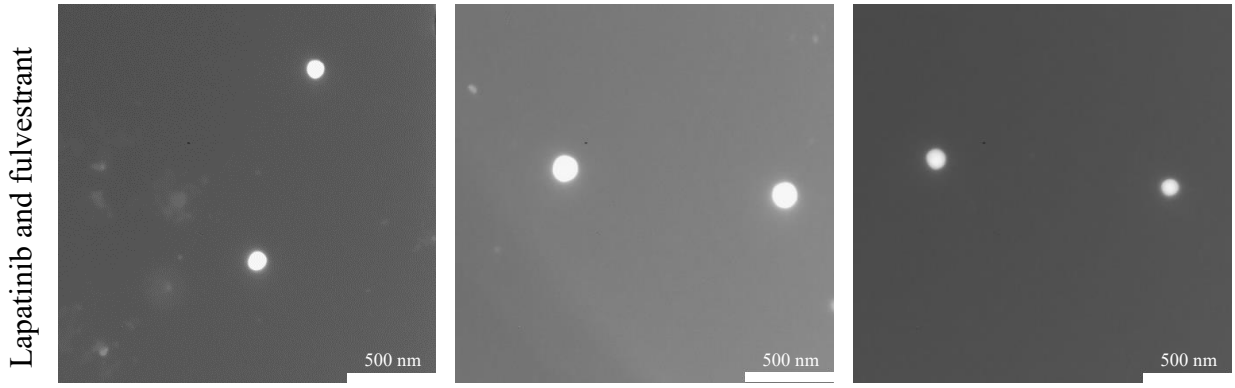


Figure S4. Transmission electron micrographs of colloidal drug aggregates. Colloids were formulated with 25 µM each of fulvestrant and lapatinib with 0.01 mg/mL UP80 in PBS. Three representative darkfield images show the spherical morphology and 100–200 nm size of these colloidal drug aggregates.

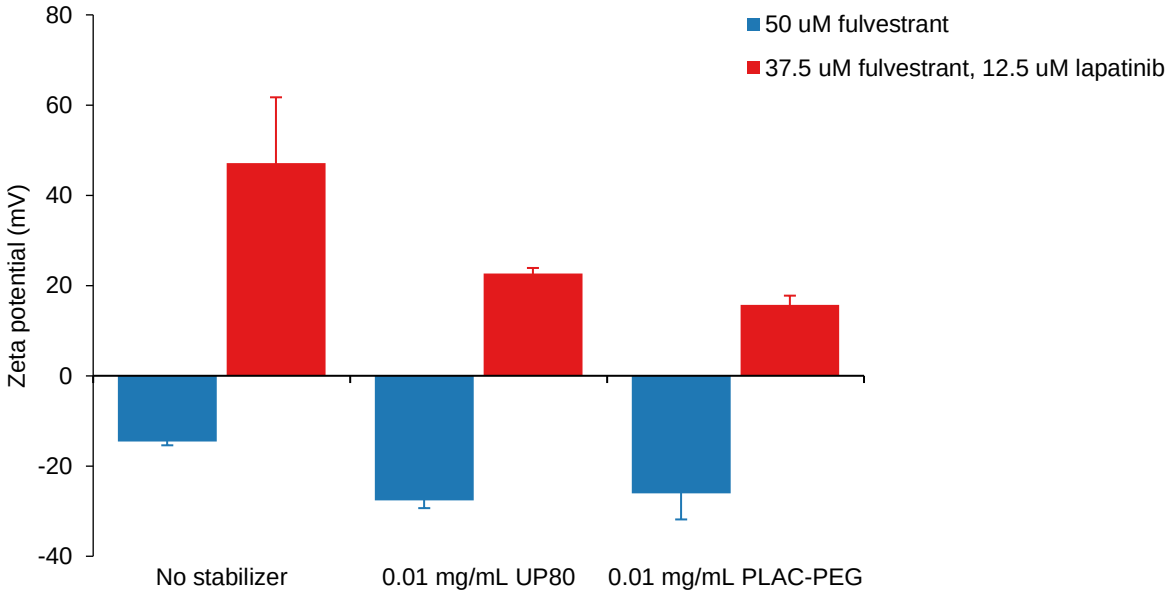


Figure S5. Zeta potential of stabilized lapatinib and fulvestrant colloids. Bare lapatinib-containing colloids have a highly positive surface charge, which is mitigated by stabilizers (n=3, mean ± SD).

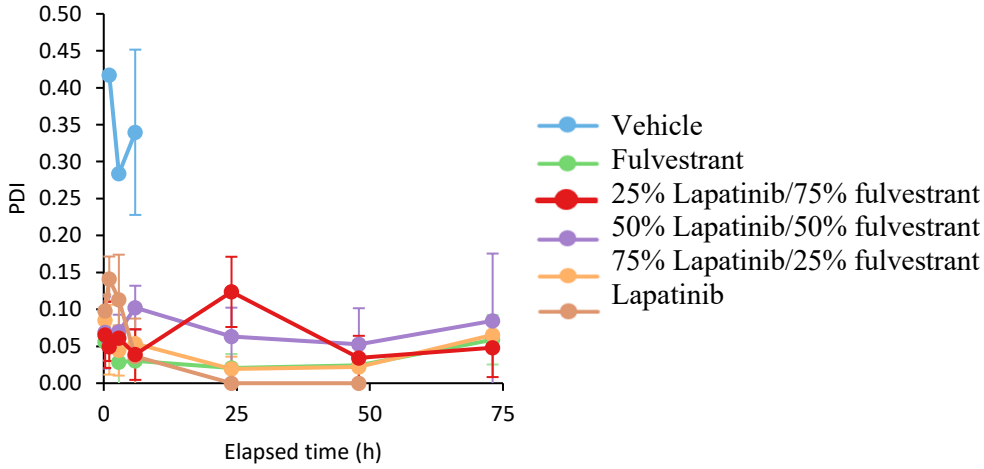


Figure S6. Colloidal stability characterized by dynamic light scattering, comparing PLAC-PEG-stabilized co-colloids containing different ratios of fulvestrant and lapatinib. Colloids were formulated in PBS with 50 μM total drug and 0.01 mg/mL PLAC-PEG. The stable lapatinib–fulvestrant colloids were confirmed to be relatively monodisperse (PDI < 0.2) by DLS (n=3, mean ± SD).

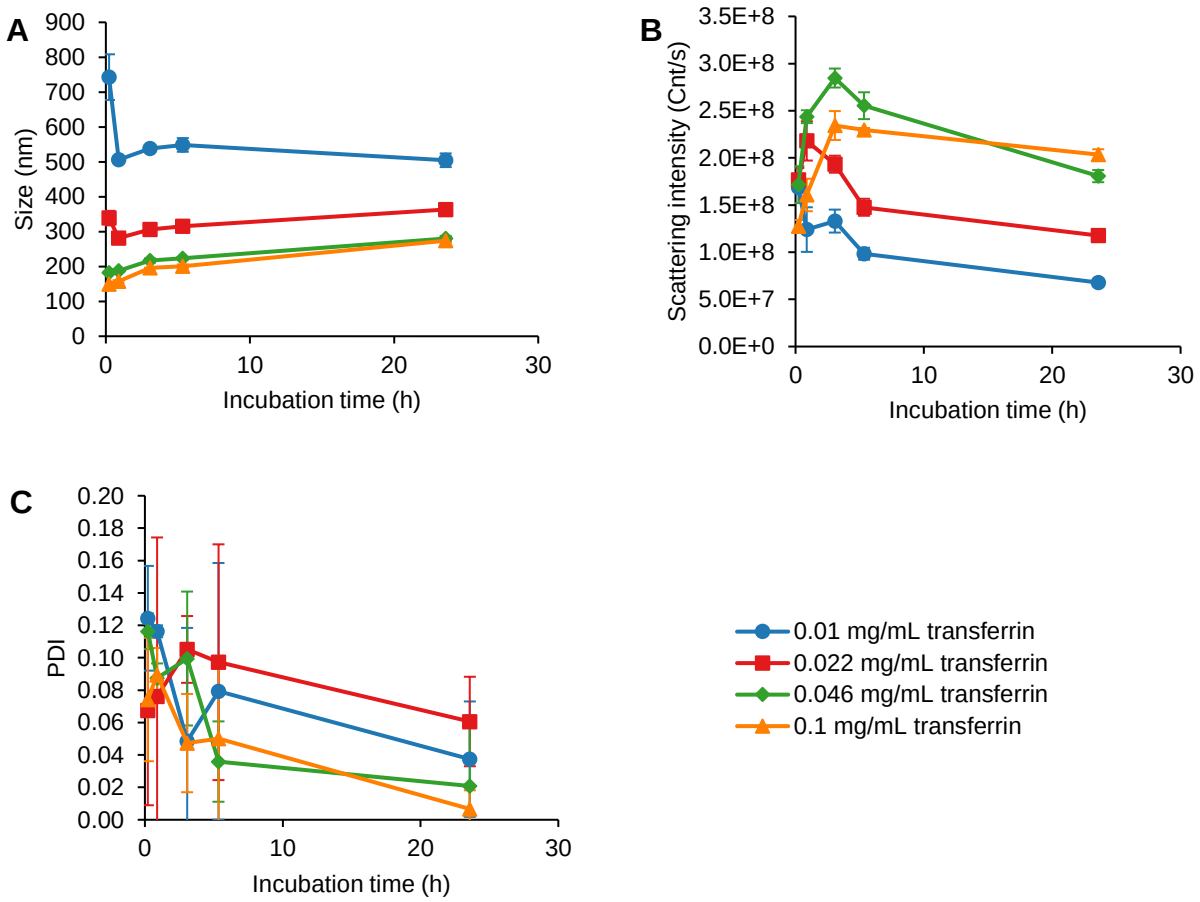


Figure S7. Stability characterization by dynamic light scattering of lapatinib and fulvestrant co-colloids stabilized with different amounts of transferrin. Concentrations of 12.5 µM lapatinib and 37.5 µM fulvestrant were used in this study, and transferrin was introduced in the water addition step. DLS analysis of **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** size dispersity shows that transferrin stabilizes these colloids in a concentration-dependent manner (n=3, mean ± SD).

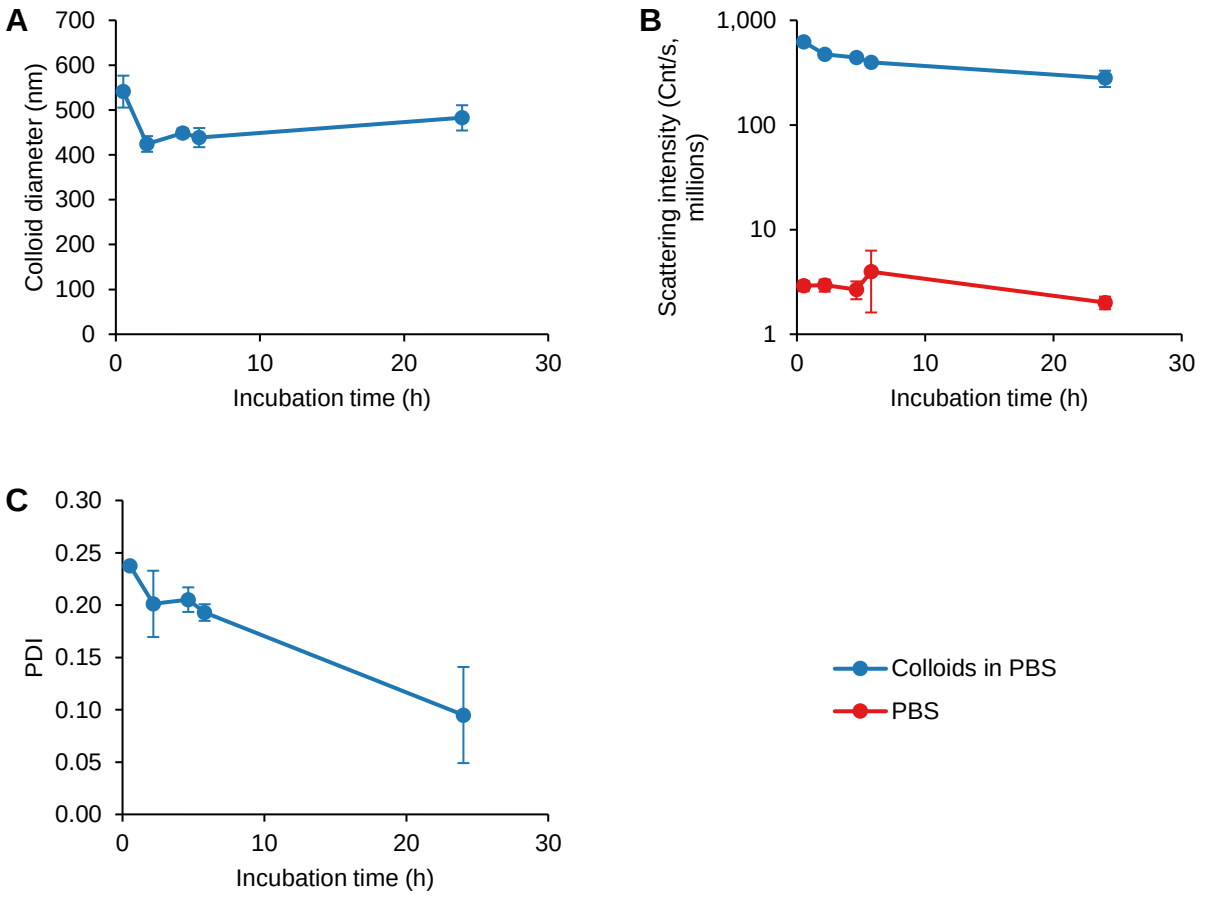


Figure S8. Stability characterization by dynamic light scattering (DLS) of lapatinib and fulvestrant co-colloids stabilized with transferrin. Co-colloids comprised concentrations of 50 µM (29 µg/mL) lapatinib and 150 µM (91 µg/mL) fulvestrant, and 100 µg/mL transferrin. DLS analyses show that transferrin stabilizes these high-concentration colloids: **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) (n=3, mean ± SD).

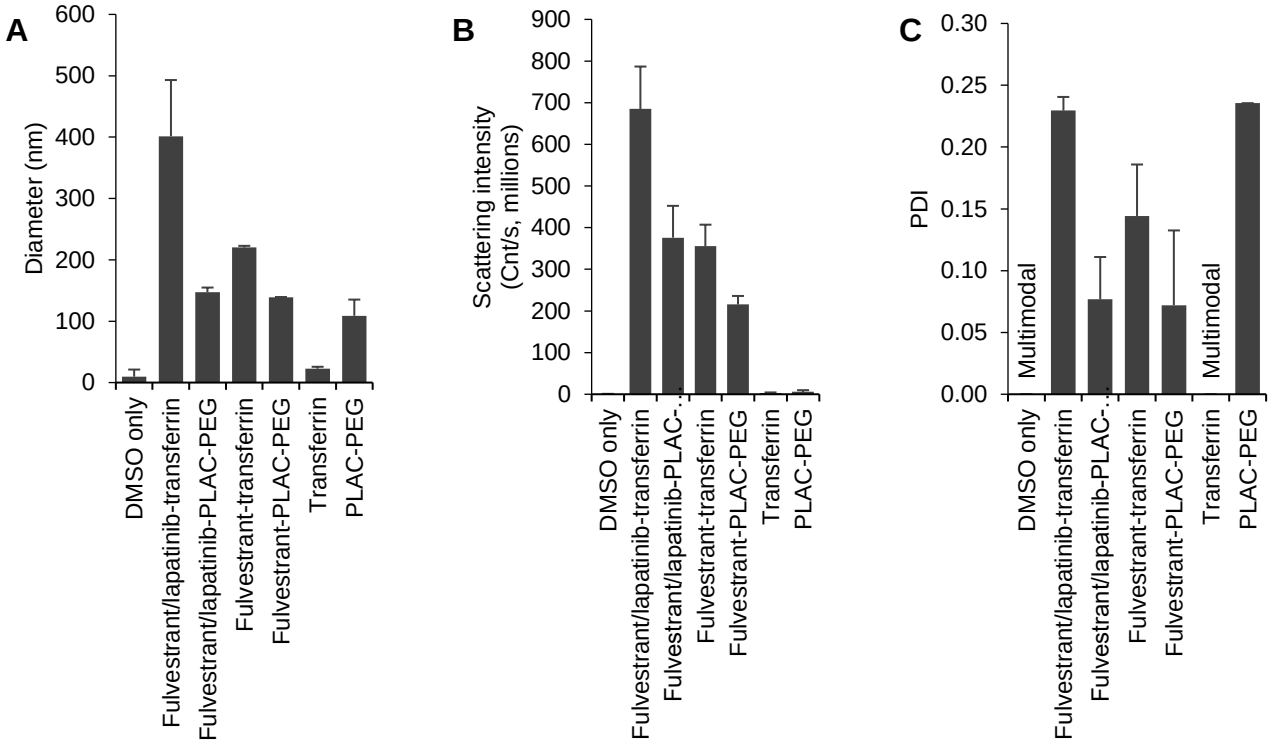


Figure S9. Stability characterization by dynamic light scattering of colloids formulated in cell culture media. Colloids were formulated as specified in Table S3. DLS analysis of **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) demonstrate stability in this environment (n=3, mean ± SD).

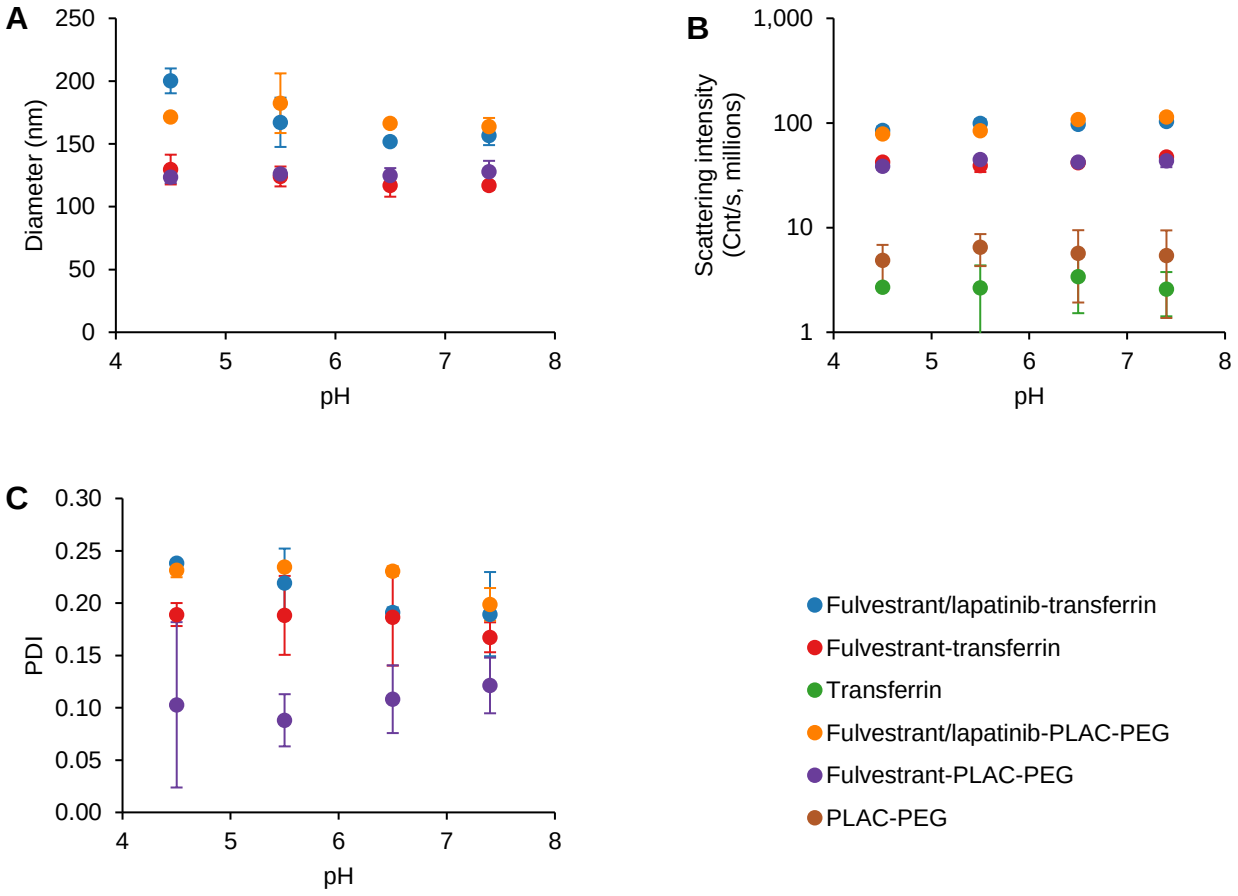


Figure S10. Colloids persist after acidification. Colloids were formulated with 37.5 µM fulvestrant, 12.5 µM lapatinib, and either 0.05 mg/mL transferrin or 0.01 mg/mL PLAC-PEG, as indicated in the legend. Dynamic light scattering analyses demonstrate persistence of the colloids after acidification: **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) (n=3, mean ± SD).

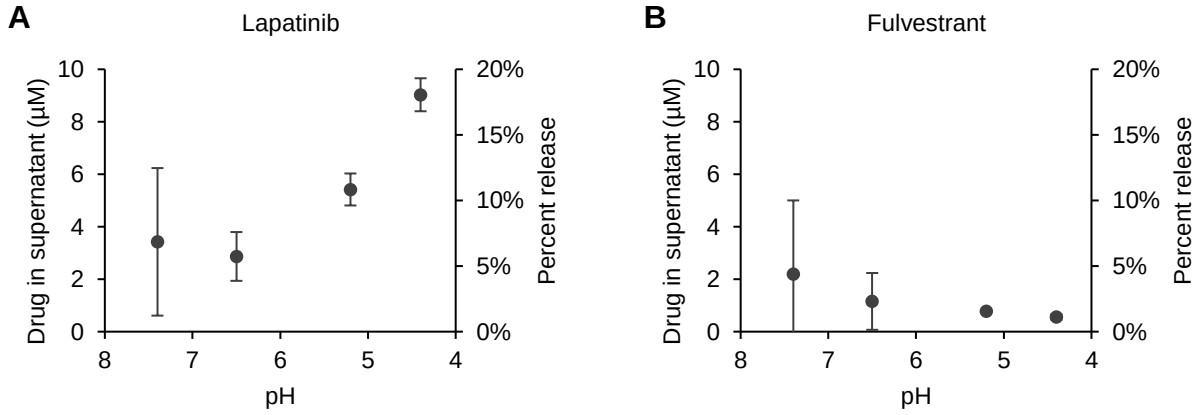


Figure S11. Lapatinib release from stable lapatinib-fulvestrant co-colloids is triggered by acidic conditions. Colloids were formulated with 50 µM lapatinib, 150 µM fulvestrant, and 0.04 mg/mL PLAC-PEG in PBS. **A)** Lapatinib is released from the co-colloids with decreasing pH whereas **B)** fulvestrant is retained within the colloids.

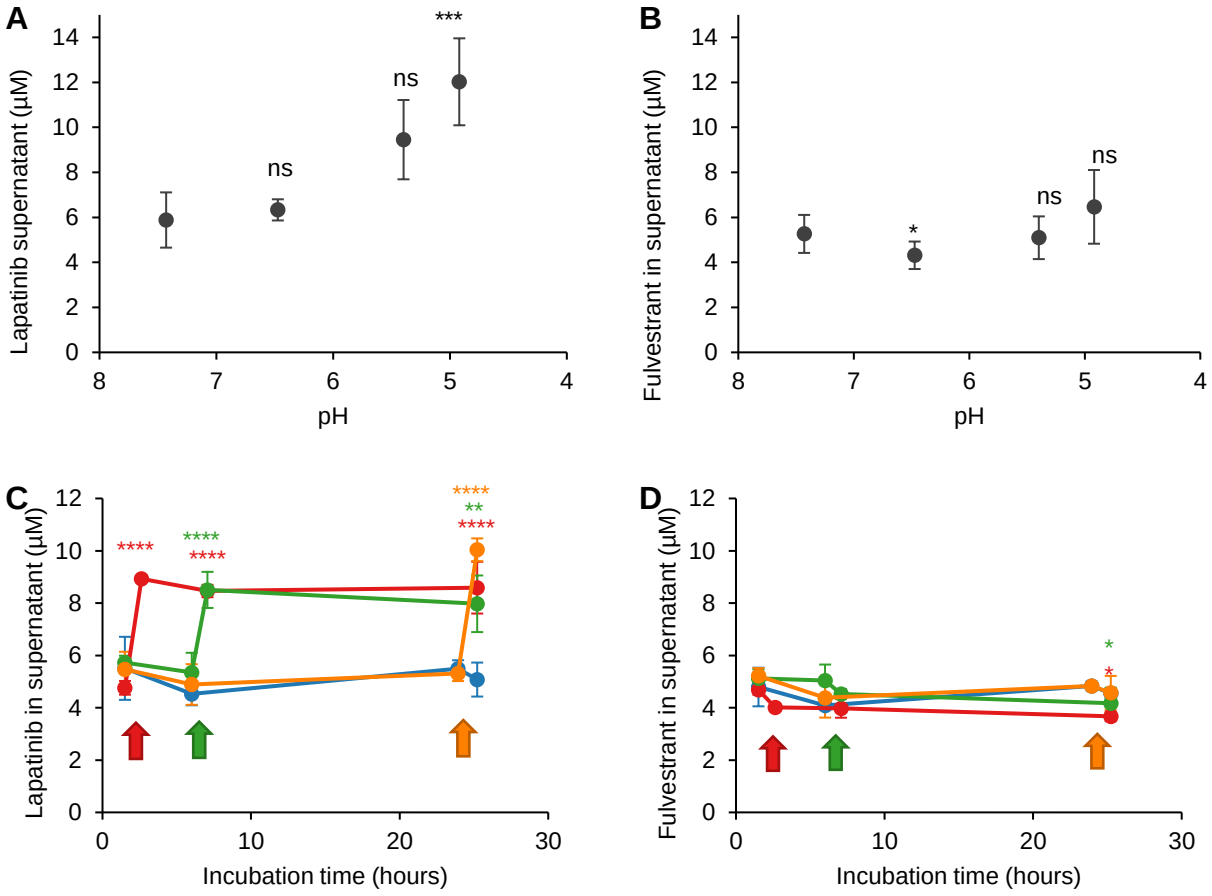


Figure S12. Lapatinib release from co-colloids is triggered by acidic conditions. Colloidal drug aggregates were formulated with 12.5 µM lapatinib, 37.5 µM fulvestrant, and 0.01 mg/mL PLAC-PEG in PBS supplemented with 10% FBS. The pH of these formulations were adjusted, followed by centrifugation to pellet out the colloids. Finally, the concentration of drug remaining in the supernatant was quantified. **A)** Lapatinib dissolves from the co-colloids under acidic conditions, whereas **B)** fulvestrant does not (n≥3, one-way ANOVA with Tukey's post-hoc test, ns $p>0.05$, *$p<0.05$, ***$p<0.001$ compared to pH 7.4, mean ± SD). These colloids were also incubated over time and acidified to pH 5.5 as indicated by the colored arrows; under these conditions **C)** lapatinib release is rapid and persists, whereas **D)** the concentration of free fulvestrant is unaffected (n=3, one-way ANOVAs with Tukey's post-hoc test, *$p<0.05$, **$p<0.01$, ****$p<0.0001$ compared to initial time point, mean ± SD).

Table S3. Formulations relating treatment name to final concentrations of each component. For imaging and flow cytometry experiments, CholEsteryl BODIPY 542/563 C11 was added before colloid formation to a final concentration of 500 nM.

Name	Lapatinib µM	Fulvestrant µM	PLAC-PEG mg/mL	Transferrin mg/mL
Lapatinib/fulvestrant, transferrin	50	150	0	0.1
Lapatinib/fulvestrant, PLAC-PEG	50	150	0.04	0
Fulvestrant, transferrin	0	150	0	0.1
Fulvestrant, PLAC-PEG	0	150	0.04	0
No drug, transferrin	0	0	0	0.1
No drug, PLAC-PEG	0	0	0.04	0
Blank	0	0	0	0

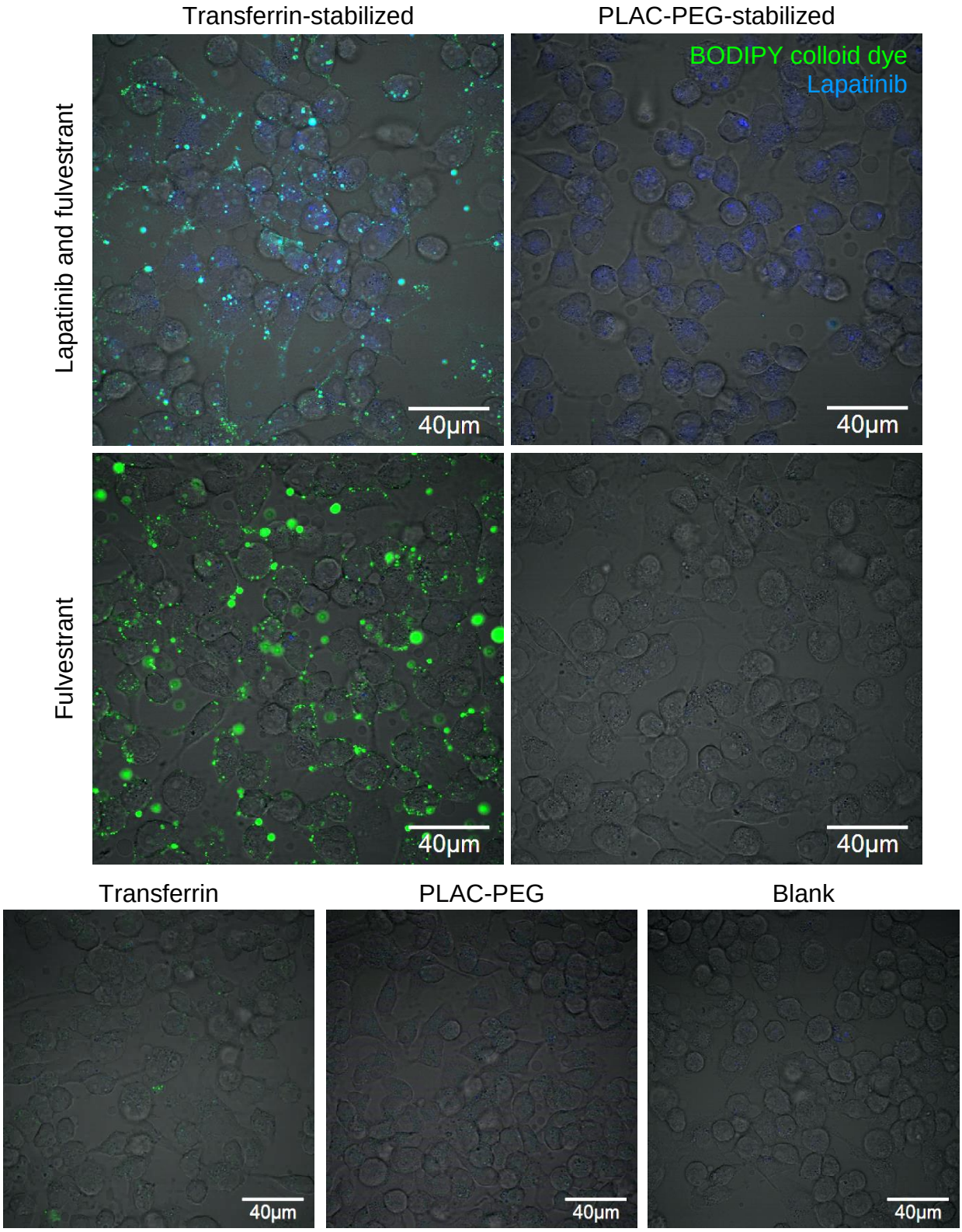


Figure S13. Colloids are visualized in punctate structures in cells only when stabilized by transferrin. Treatments were formulated as specified in Table S3 with the addition of 500 nM BODIPY dye (all except "Blank"). MDA-MB-231-H2N cells were treated in serum-free media for 3 h, then washed, fixed, and imaged. These representative confocal images show 450 nm lapatinib fluorescence (blue), 575 nm colloid dye fluorescence (green), and 488 nm transmission showing cell outlines.

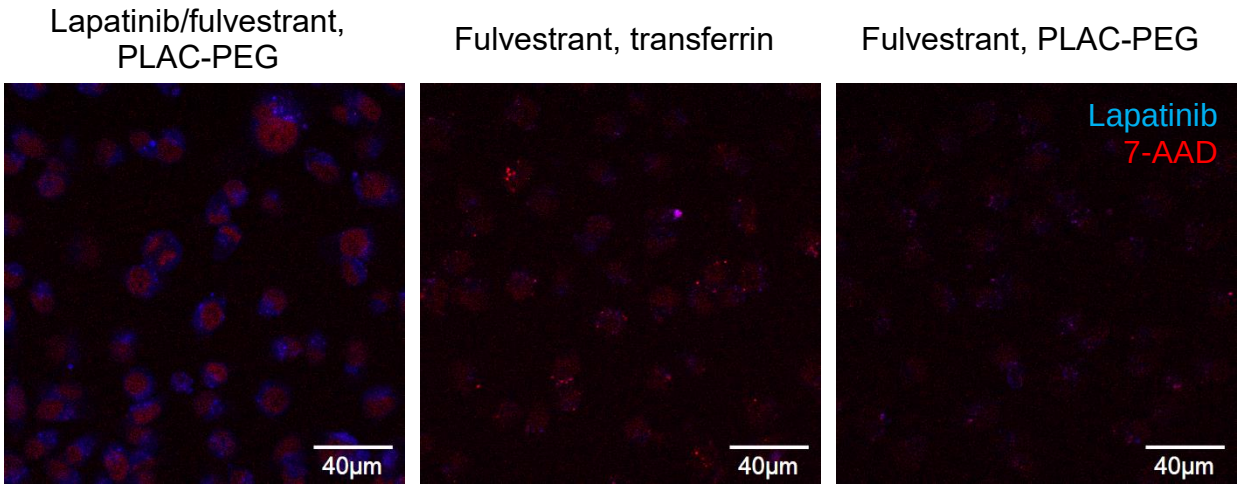


Figure S14. Control images visualize 7-AAD nuclear fluorescence (red) in colloid-treated MDA-MB-231-H2N cells. Cells were treated with conditions outlined in Table S3 supplemented with 2 μM 7-AAD. The presence of cells in each region of interest was verified using the transmission channel prior to capturing these images.

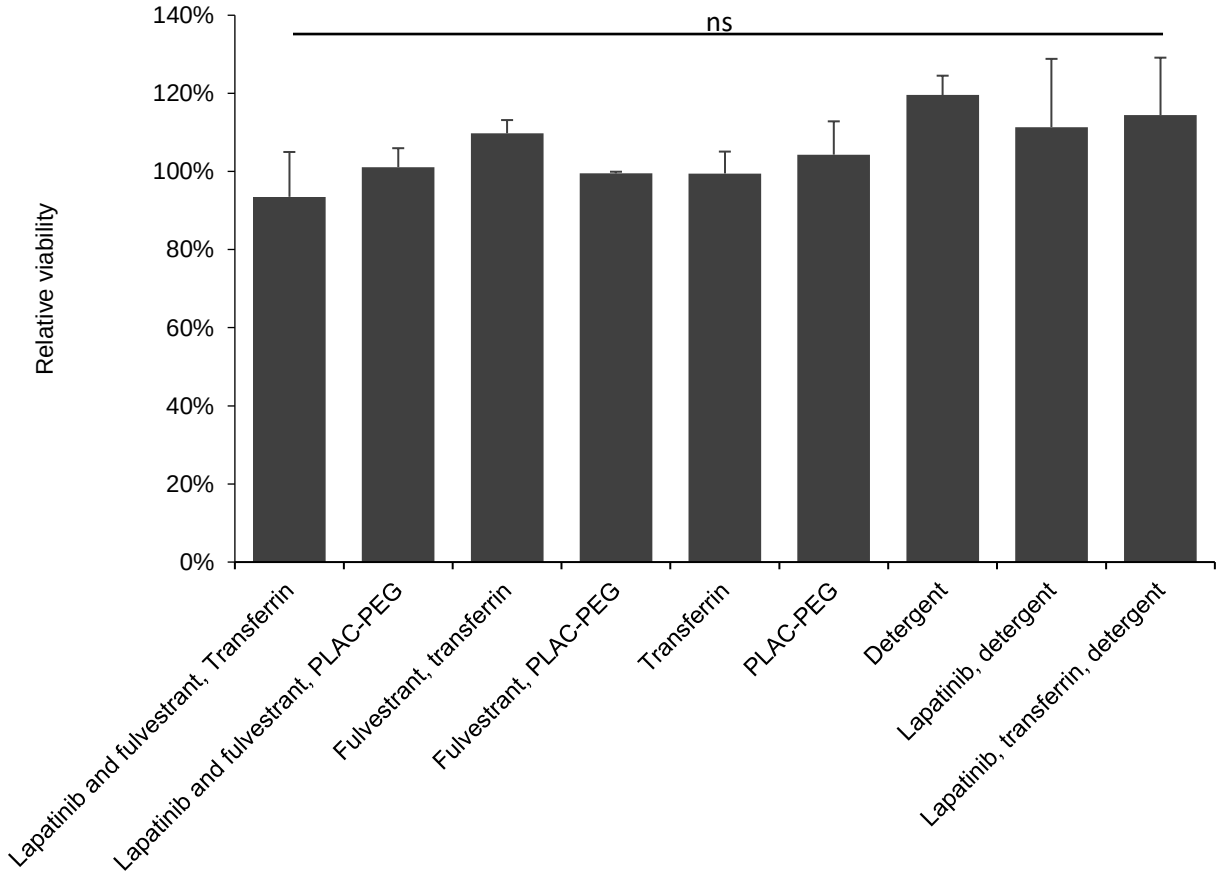


Figure S15. MDA-MB-231-H2N cells are still viable immediately after 3 h of treatment with colloids. Cells were treated identically to those used in viability and flow cytometry experiments depicted in Figure 4, then immediately assessed for viability (n=3, one-way ANOVA with Tukey's post-hoc test, ns $p>0.05$, mean ± SD).

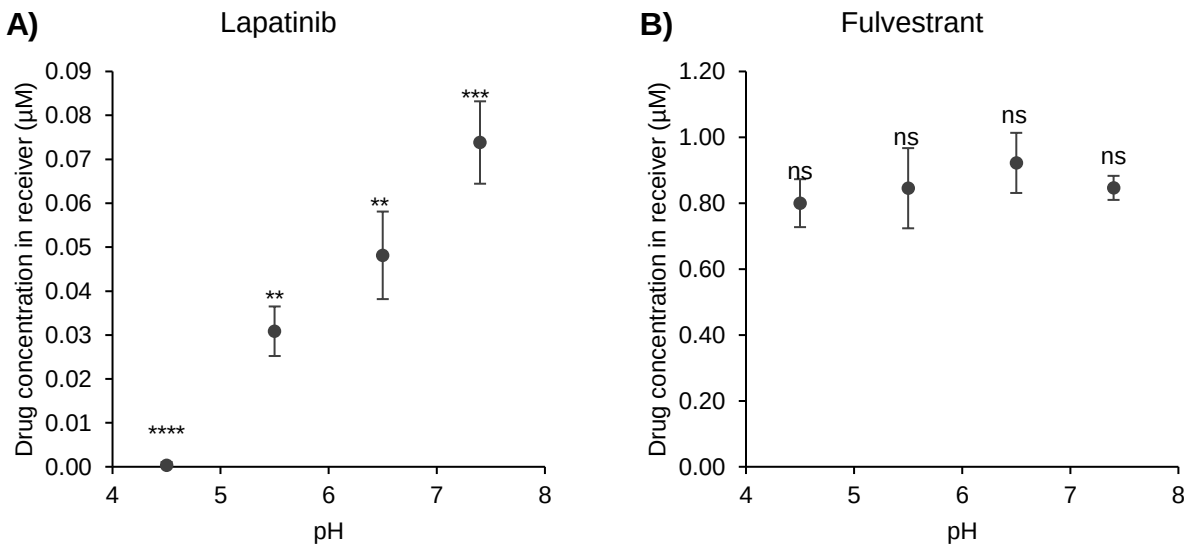


Figure S16. Lapatinib diffusion across an artificial lipid membrane is decreased under acidic conditions. Colloidal drug aggregates were formulated with 50 µM lapatinib, 150 µM fulvestrant, and 0.04 mg/mL PLAC-PEG in media containing 10% FBS. After formulation, the pH was adjusted, and the colloid solution was pipetted into the donor compartment of the plate. **A)** Lapatinib and **B)** fulvestrant concentrations in the receiver compartment after 6 h of incubation at 37 °C are shown (n=5, one-way ANOVA with Tukey's post-hoc test, ns $p>0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, mean ± SD).

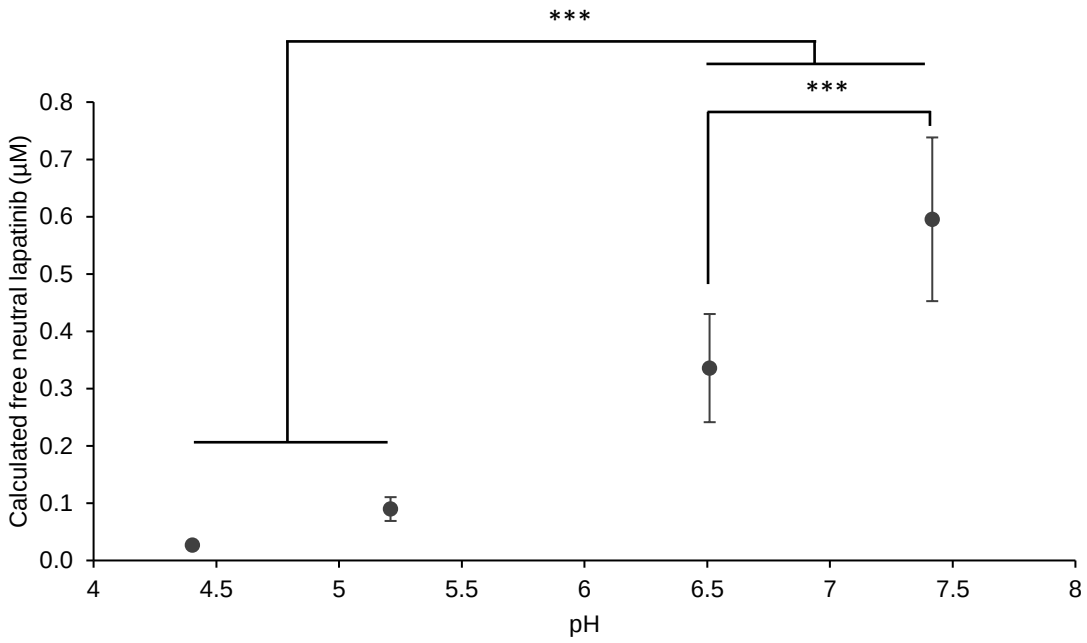


Figure S17. Most lapatinib that is released at low pH is ionized. Acid-base theory was used to calculate the amount of released lapatinib shown in Figure 2C that is uncharged (n≥9, one-way ANOVA with Tukey's post-hoc test, ***$p<0.001$, mean ± SD).

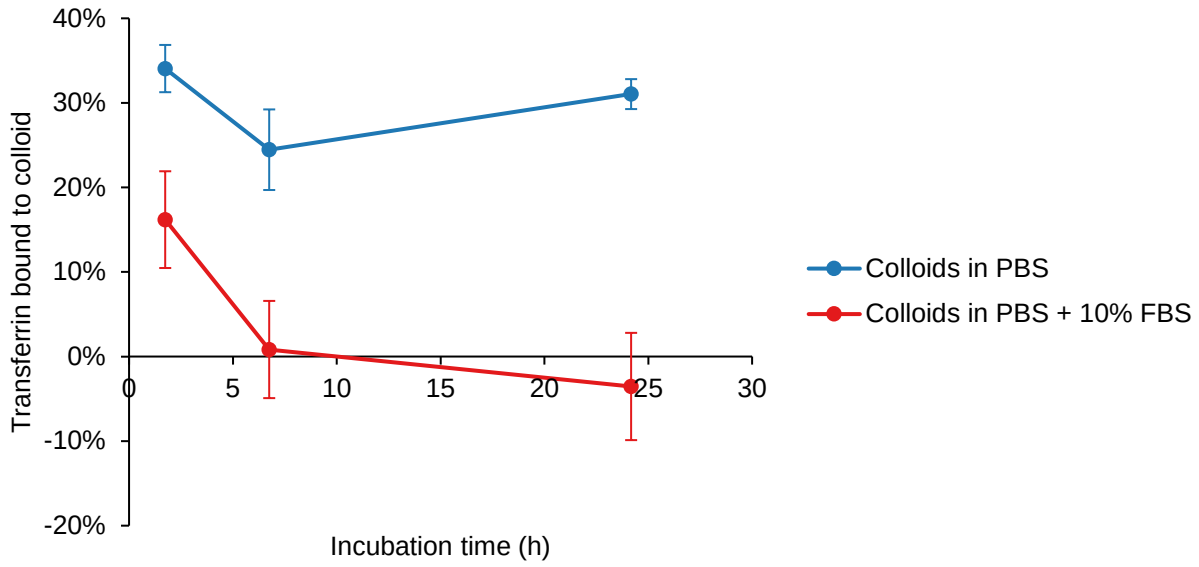


Figure S18. Characterization of transferrin displacement by serum proteins adsorbing to colloidal drug aggregates. Colloids were formulated with 37.5 µM fulvestrant, 12.5 µM lapatinib, and 0.01 mg/mL Alexa Fluor 488 labelled transferrin. Measurement of free transferrin reveals that serum proteins displace transferrin from the surface of the colloids (n=3, mean ± SD).

References

(1) Stetefeld, J.; McKenna, S. A.; Patel, T. R. Dynamic Light Scattering: A Practical Guide and Applications in Biomedical Sciences. *Biophys. Rev.* **2016**, *8* (4), 409–427. https://doi.org/10.1007/s12551-016-0218-6.

(2) Coan, K. E. D.; Shoichet, B. K. Stoichiometry and Physical Chemistry of Promiscuous Aggregate-Based Inhibitors. *J. Am. Chem. Soc.* **2008**, *130* (29), 9606–9612. https://doi.org/10.1021/ja802977h.

(3) Ilevbare, G. A.; Taylor, L. S. Liquid–Liquid Phase Separation in Highly Supersaturated Aqueous Solutions of Poorly Water-Soluble Drugs: Implications for Solubility Enhancing Formulations. *Cryst. Growth Des.* **2013**, *13* (4), 1497–1509. https://doi.org/10.1021/cg301679h.

(4) Taylor, L. S.; Zhang, G. G. Z. Physical Chemistry of Supersaturated Solutions and Implications for Oral Absorption. *Adv. Drug Deliv. Rev.* **2016**, *101*, 122–142. https://doi.org/10.1016/j.addr.2016.03.006.