

RECENT ADVANCES IN siRNA DELIVERY FOR RESISTANT CANCERS

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Northeastern



Task: To treat effectively MDR tumors

Approach: To use therapy combining traditional chemotherapy and downregulation/inhibition of tumor protective mechanisms

Idea: To make nanopreparation for drug delivery working as a missile with dividing warheads

Challenges with siRNA delivery

Even after more than 15 years since RNAi was described by Fire and Mello, there are no FDA approved, siRNA-based therapies, for the treatment of cancer. Eight siRNA-based formulations, for cancer therapy, are currently in the different phases of clinical trials*

Problems**

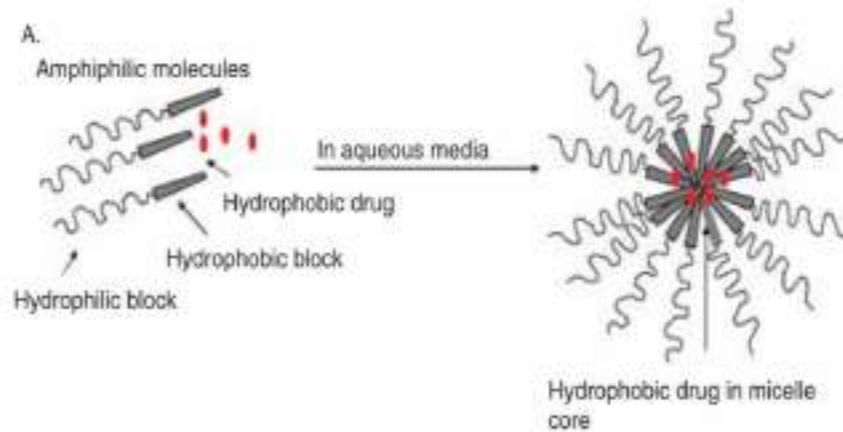
- ✓ Rapid degradation by serum nucleases
- ✓ Poor cellular uptake due to inherent anionic charge



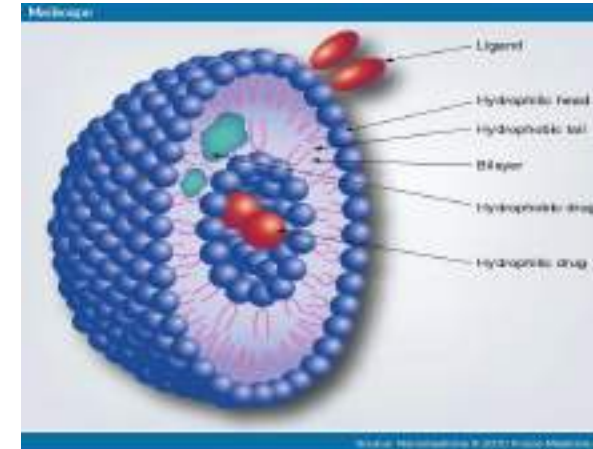
* Davidson, B. L., & McCray, P. B. (2011). Current prospects for RNA interference-based therapies. *Nature Reviews Genetics*, 12(5), 329-340.

** Adapted from Navarro, G., S. Essex et al. *Drug Delivery and translational medicine* (2011)

Lipid-based drug delivery systems



- **Polymeric micelles** → self-assembled hydrophilic shells with a lipid core; composed of amphiphilic block co-polymers of hydrophilic chains and hydrophobic heads
- **Hydrophobic drugs** are encapsulated in the lipid core.
- Typically, size ranges from 10 to 100 nm.

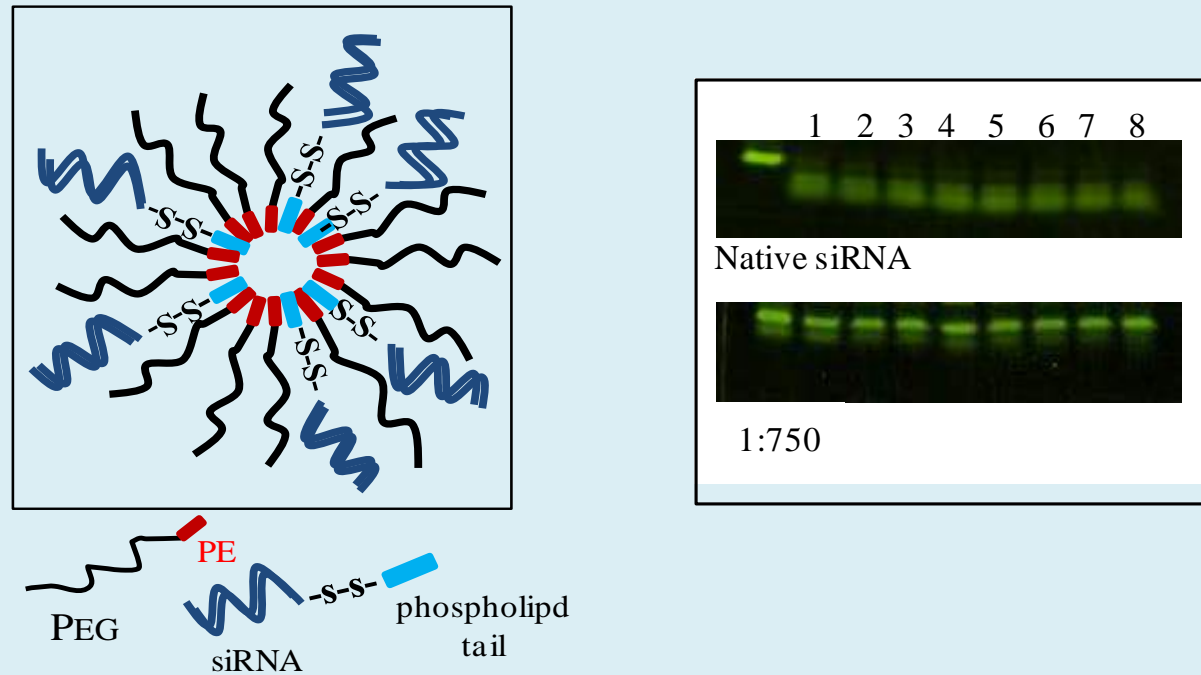


- **Liposomes** → self-enclosed spheres of phospholipid bilayers with an aqueous core within the bilayers
- Drugs are either encapsulated in the core (**hydrophilic**) or entrapped in lipid bilayer (**hydrophobic**).
- Typically, size ranges from 80 nm to 1 μm

Intrinsic stimuli to be considered:

1. **pH** (tumor/inflammation acidification)
2. **redox conditions** (GSH inside tumor cells)
3. **hypoxia** (insufficient blood supply)
4. **overexpression of certain proteins** (MMP)

New system for effective stabilization and delivery of siRNA: Reversible siRNA-phospholipid conjugate in PEG-PE polymeric mixed micelles



Left panel: Schematic structure of siRNA-PE/PEG-PE mixed micelles.

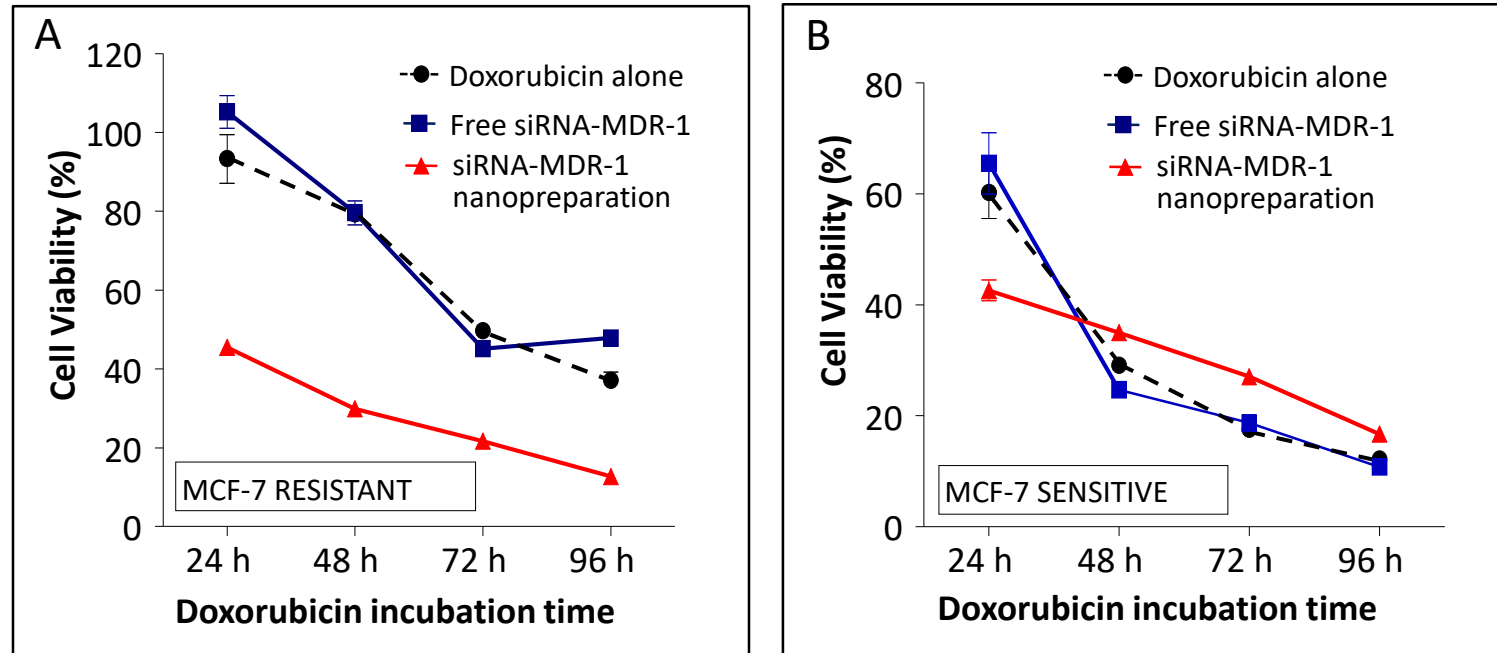
Right panel: stability of siRNA against nucleolysis in 1:750 mixed micelles compared to that of the free siRNA at different time-points till 24 h

P-glycoprotein silencing with siRNA delivered by DOPE-modified PEI overcomes doxorubicin resistance in breast cancer cells.

Navarro G, Sawant RR, Biswas S, Essex S, Tros de Ilarduya C, Torchilin VP.

Nanomedicine (Lond). 2012 Jan;7(1):65-78.

Doxorubicin cytotoxicity in (A) resistant and (B) sensitive MCF-7 cells after treatment with siRNA nanopreparations



MCF-7 resistant and sensitive cells were treated with formulations prepared with siRNA targeting MDR-1 (siMDR). Cells were treated with doxorubicin (1 μ g/mL) for 24, 48, 72 and 96 h and cell viability was measured. Data are expressed as the mean \pm SD (n=3).

Polymeric micelles containing reversibly phospholipid-modified anti-survivin siRNA: A promising strategy to overcome drug resistance in cancer.

Salzano G, Riehle R, Navarro G, Perche F, De Rosa G, Torchilin VP.

Cancer Lett. 343(2):224-31, 2014

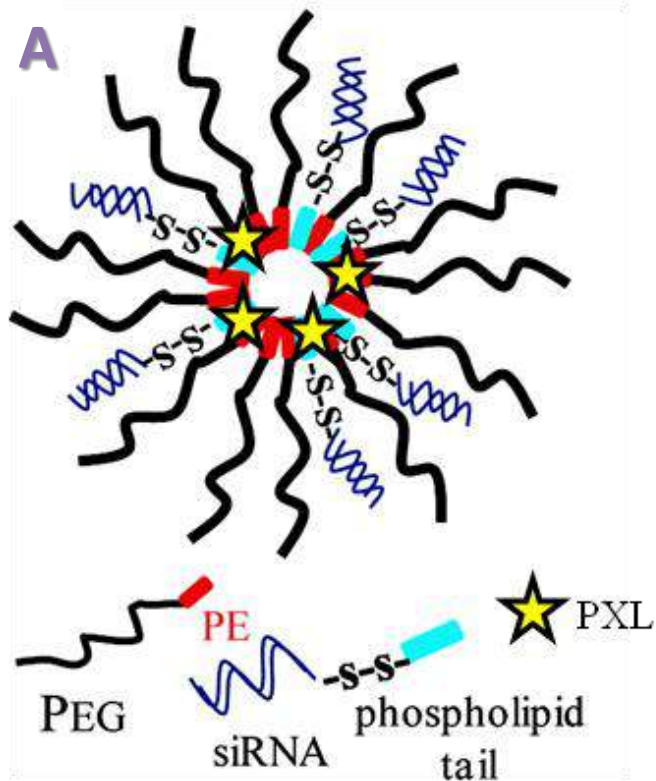
Multifunctional Polymeric Micelles Co-loaded with Anti-Survivin siRNA and Paclitaxel Overcome Drug Resistance in an Animal Model of Ovarian Cancer.

Salzano G, Navarro G, Trivedi MS, De Rosa G, Torchilin VP.

Mol Cancer Ther. 2015 Apr;14(4):1075-84.

A . Schematic structure of survivin siRNA-S-S-PE/PXL PEG-PE mixed micelle.

B. Physic characteristics of survivin siRNA PM and PM containing survivin siRNA and PTX in combination (survivin siRNA/PXL PM)



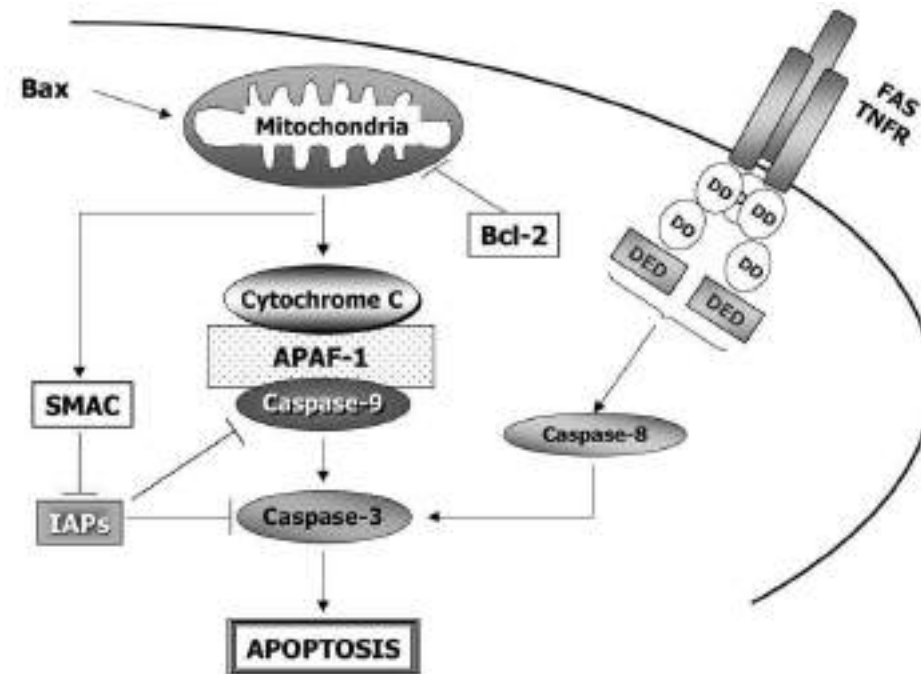
B

Formulation	Diameter (nm \pm SD)	P.I. \pm SD
Survivin siRNA PM	21.5 \pm 3.3	0.160 \pm 0.05
Survivin siRNA/PXL PM	25.0 \pm 3.6	0.190 \pm 0.07

Survivin

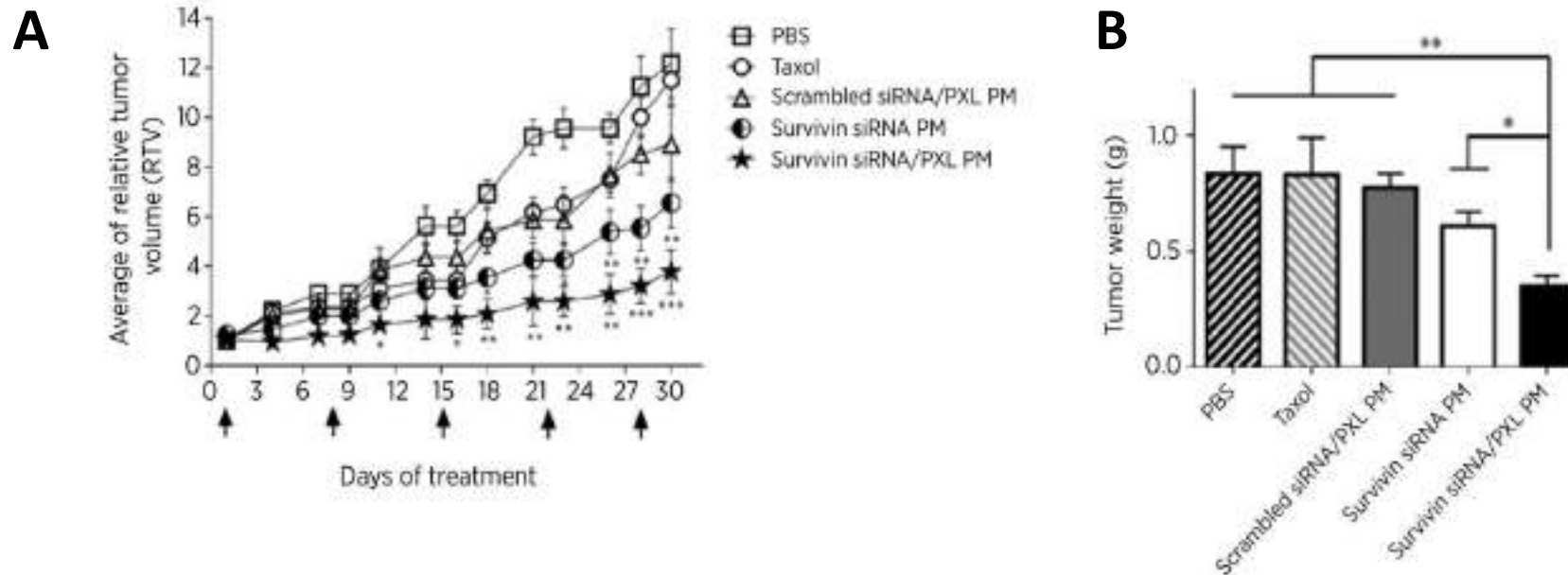
Survivin, a 16.5 kDa protein, is a member of the inhibitor of apoptosis protein (IAP) family. Given its preferential expression in cancer cells, function in cancer cell division and chemoresistance, and correlation with poor patient prognosis, survivin has been proposed as a biomedical target for cancer therapy

- ✓ *Apoptosis*
- ✓ *Mitosis/Cell division*
- ✓ *Tumorogenesis*
- ✓ *Drug/radiation resistance*



The suppression of survivin by siRNA results in spontaneous apoptosis (Li, F. et al. J. Cell. Physiol. 2003)

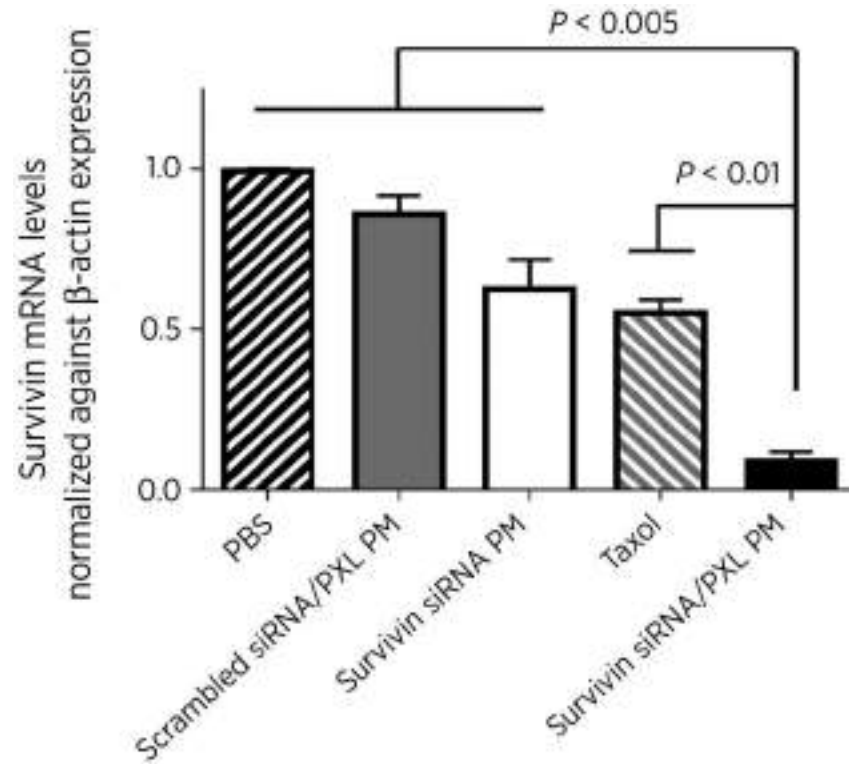
In vivo antitumor activity of survivin siRNA/PXL polymeric micelles (PM) in SKOV3-tr xenografts (A) and postmortem tumor weights (B)



A - Survivin siRNA/PXL PM were administered once per week at a concentration of anti-survivin siRNA 1 mg/kg and paclitaxel 10 mg/kg. RTV - Tumor volume in mm³ on day "n" (V_n)/tumor volume at the start of the treatment (V_0). Data are given as mean SD for each treatment group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ were obtained by comparing each treatment group with the Taxol group.

B - Postmortem tumor weights on day 30. $P < 0.05$ and , $P < 0.01$ were considered significant and very significant, respectively, and were obtained by comparing each treatment group with the survivin siRNA/PXL group.

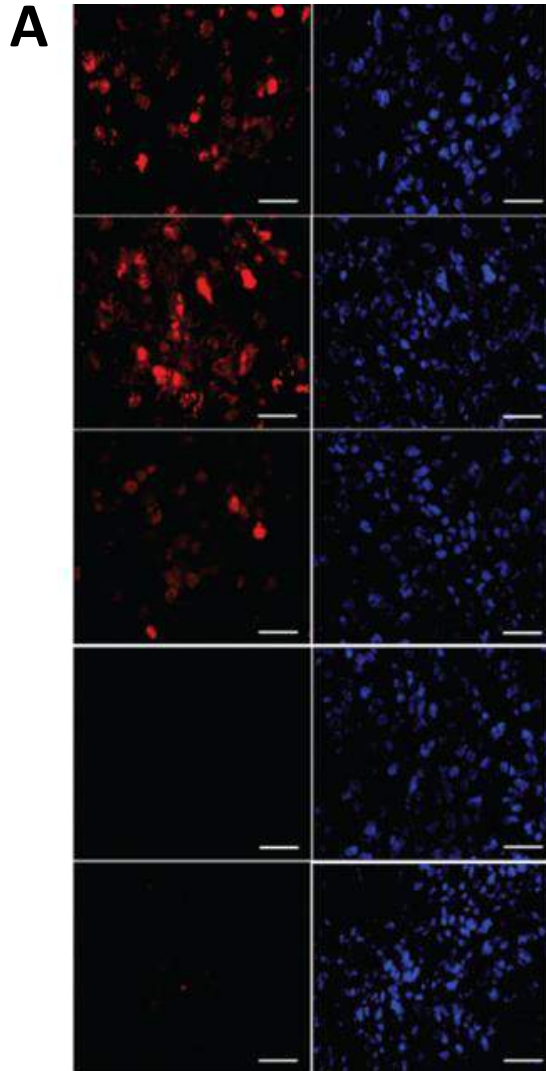
Survivin mRNA levels in tumor tissues by rt-PCR analysis



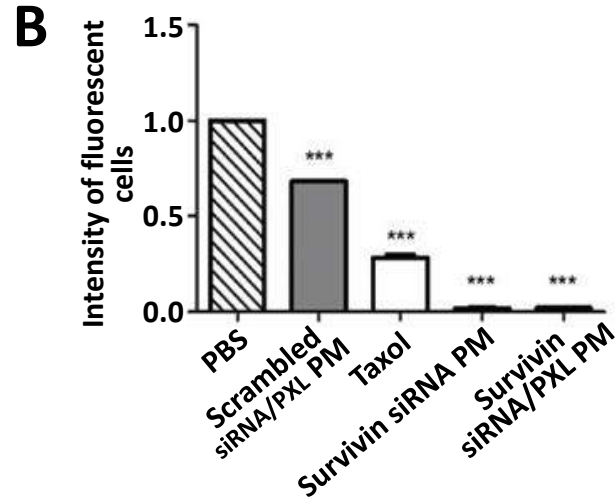
Survivin mRNA levels in tumor tissues by rt-PCR analysis. Data are given as mean \pm SD for each treatment group. $P < 0.005$ and $P < 0.01$ were obtained by comparing each treatment group with the survivin siRNA/PXL group.

Immunohistochemistry analysis

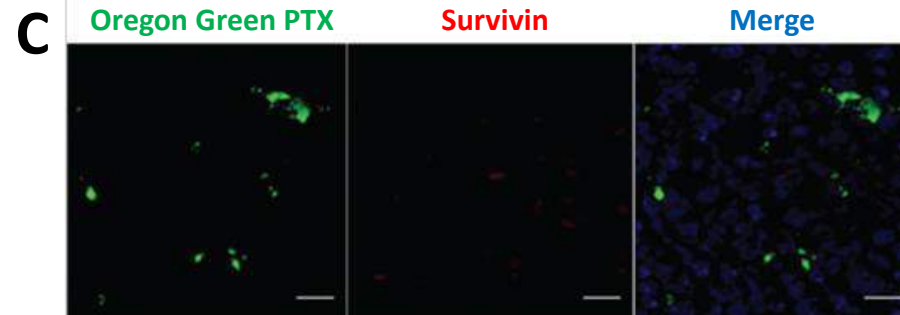
Survivin expression (in red) and nuclei staining with Hoechst 33342 (blue)



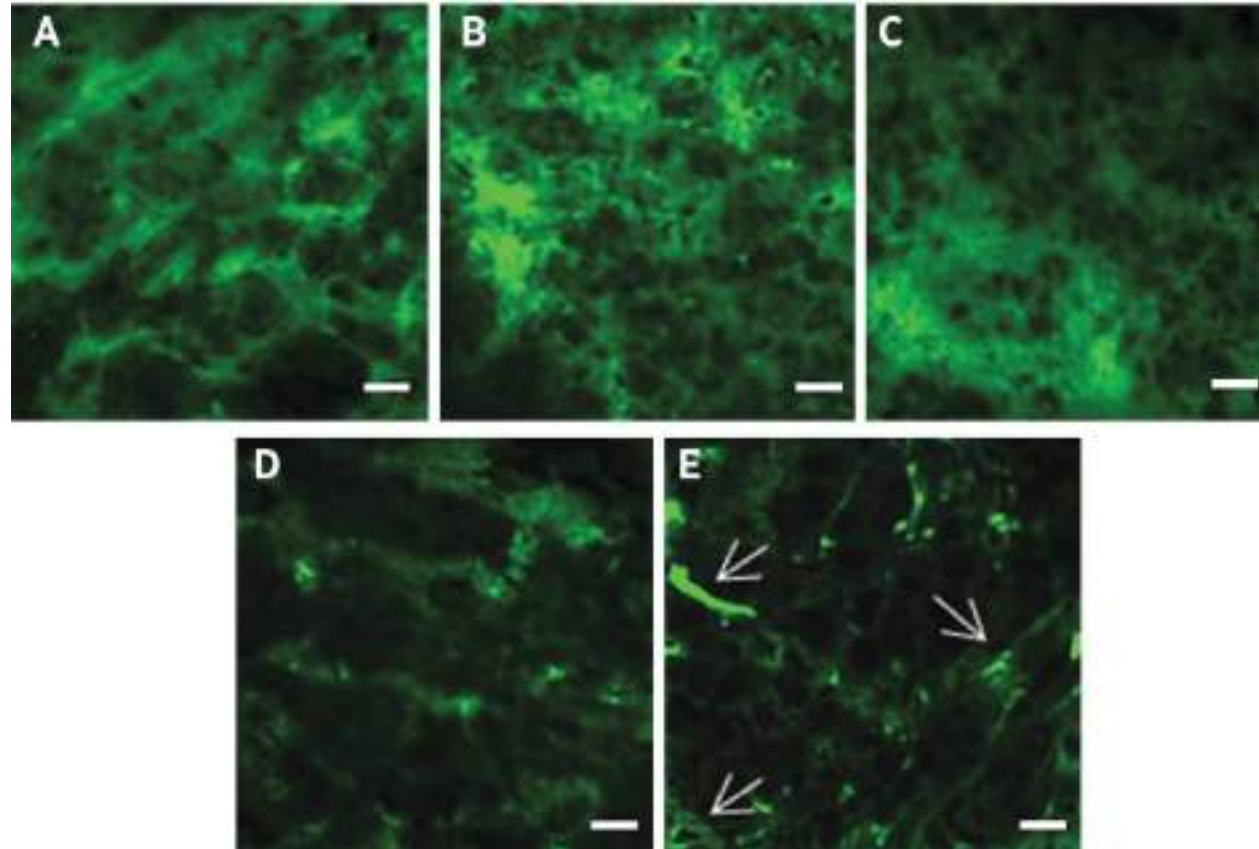
Cell fluorescent intensity of the survivin protein levels measured with ImageJ



Simultaneous downregulation of survivin expression and paclitaxel penetration in tumor tissues by using survivin siRNA/PXL PM.



Microtubule organization after treatment with survivin siRNA/PXL PM *in vivo* (staining for tubulin)



SKOV3-tr tumor sections. **A** - untreated group; **B** - scrambled siRNA/PXL PM group; **C** - Taxol group; **D** - survivin siRNA PM group; **E** - survivin siRNA/PXL PM group; Scale bar - 10 mm

**Phospholipid-modified polyethylenimine-based
nanopreparations for siRNA-mediated gene silencing:
Implications for transfection and the role of lipid components.**

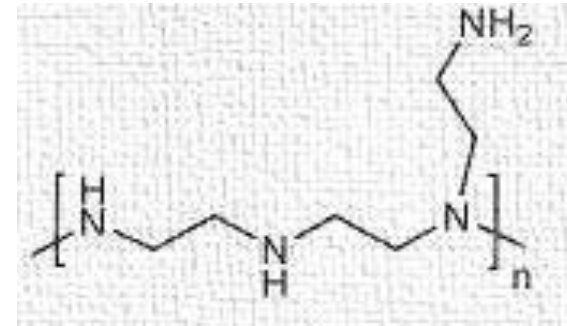
Navarro G, Essex S, Sawant RR, Biswas S, Nagesha D, Sridhar S,
de Ilarduya CT, Torchilin VP.

Nanomedicine. 2013 Aug 6. [Epub ahead of print]

Polyethylenimine (PEI)-based siRNA micelle-like nanocarriers

Pros*

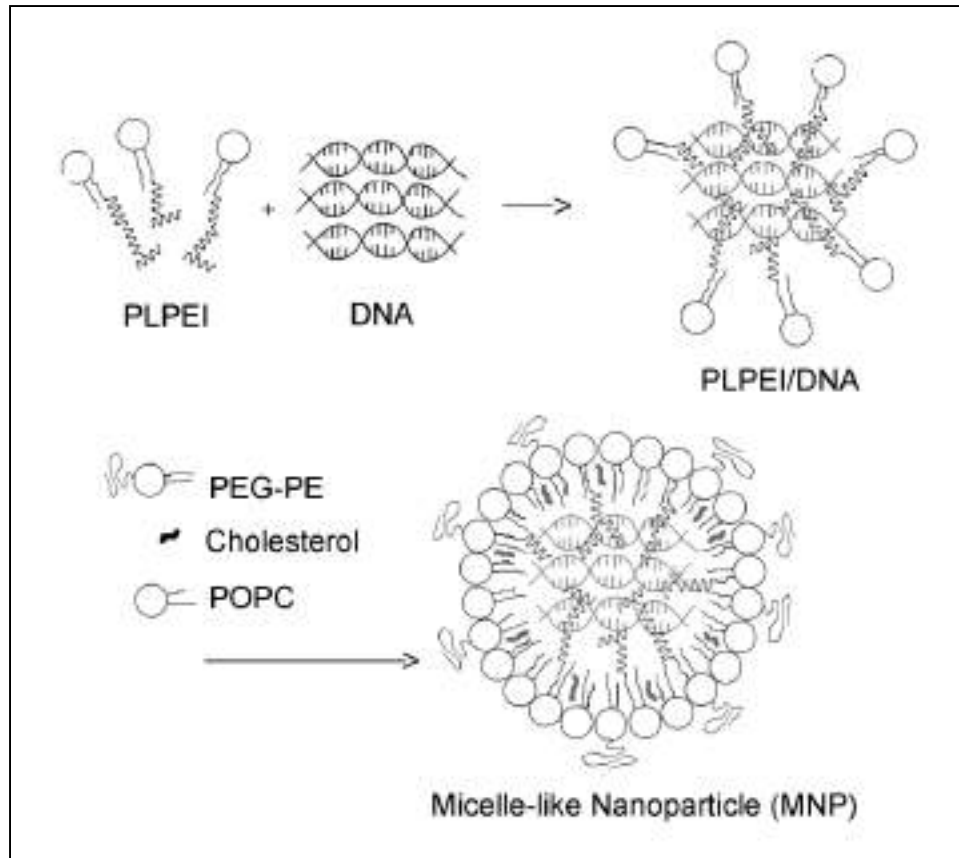
- Proton sponge effect due to cationic nature
- Synthetic flexibility (Linear/branched)
- Cationic charge condenses siRNA and facilitates cell uptake
- Low molecular weight PEI (1.8 kDa) is non-toxic



Cons*

- Toxicity (High molecular weight > 25kDa)
- Non-specific interaction with serum proteins
- RES mediated removal

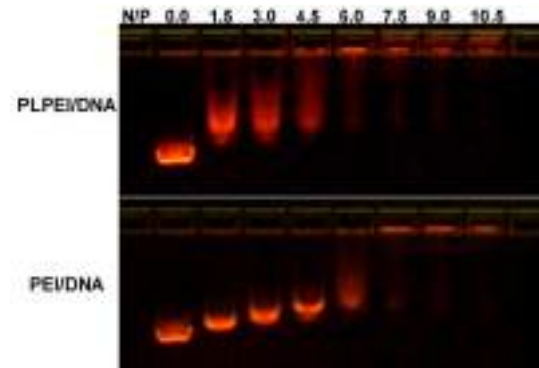
Self-assembly micelle-like nanoparticles (MNP)



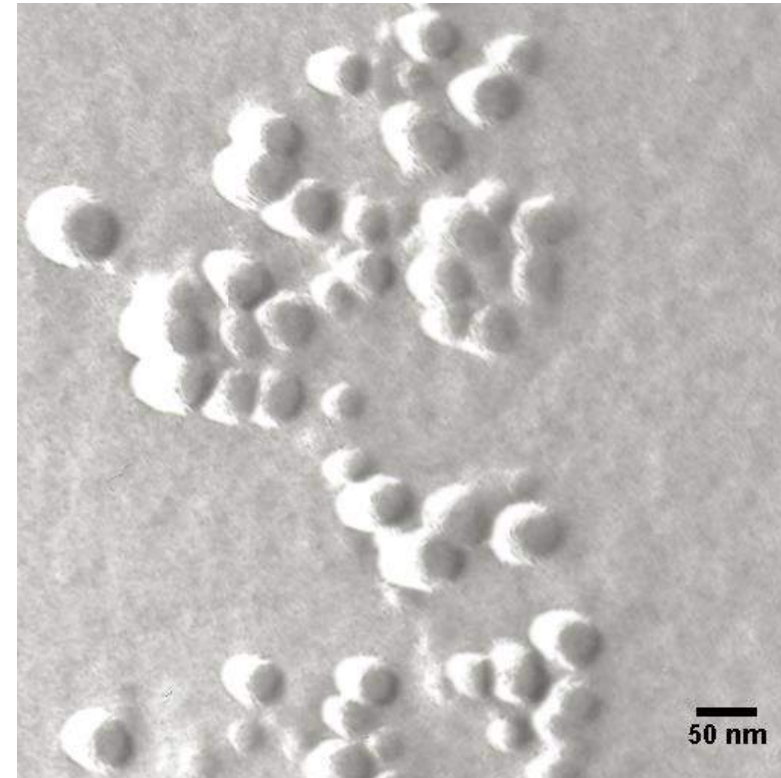
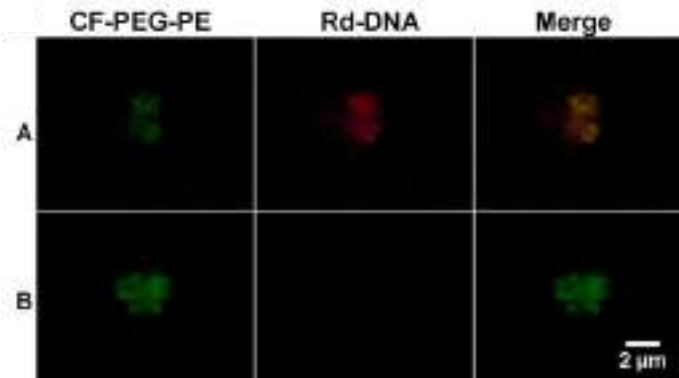
- ❖ Driven by electrostatic interaction (DNA/siRNA complexation) followed by hydrophobic interaction (formation of lipid monolayer coat)
- ❖ Simple and quantitative DNA/siRNA loading procedure
- ❖ High DNA/siRNA loading capacity (30 wt%)
- ❖ Combine polyplexes with sterically-stabilized micelles

Formation of micelle-like nanoparticle (MNP)

Agarose gel electrophoresis

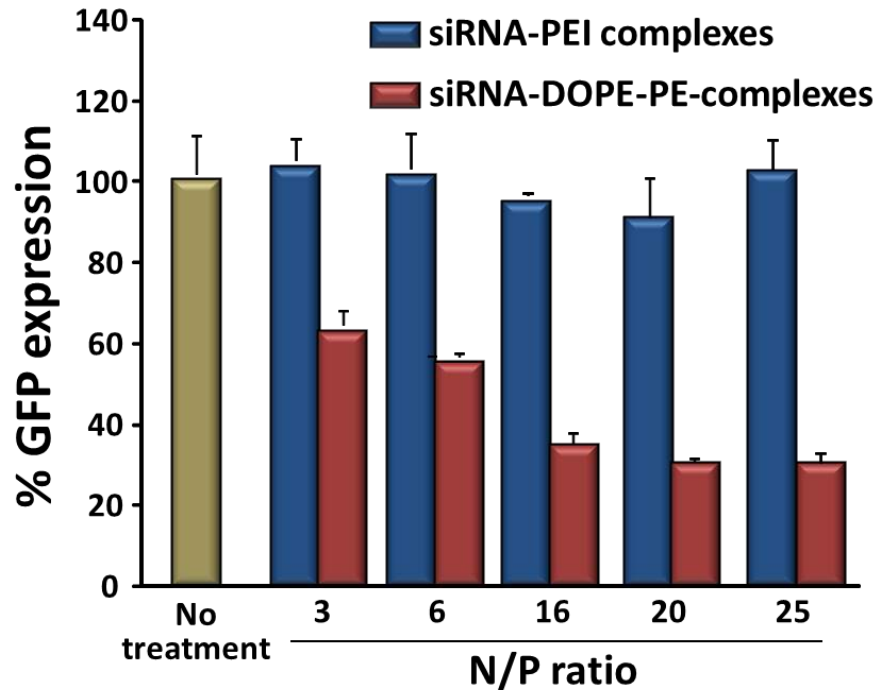


Fluorescence microscopy analysis



Freeze-Fracture TEM (ffTEM)

Gene silencing efficacy of the PEI–lipid/siRNA(GFP) complexes in cells that stably express GFP



GFP fluorescence was measured by cytometry. The absence of GFP suppression was observed for non-modified PEI complexes, while a 75 % GFP signal reduction was seen for PEI-PE complexes.

Tumor Targeting

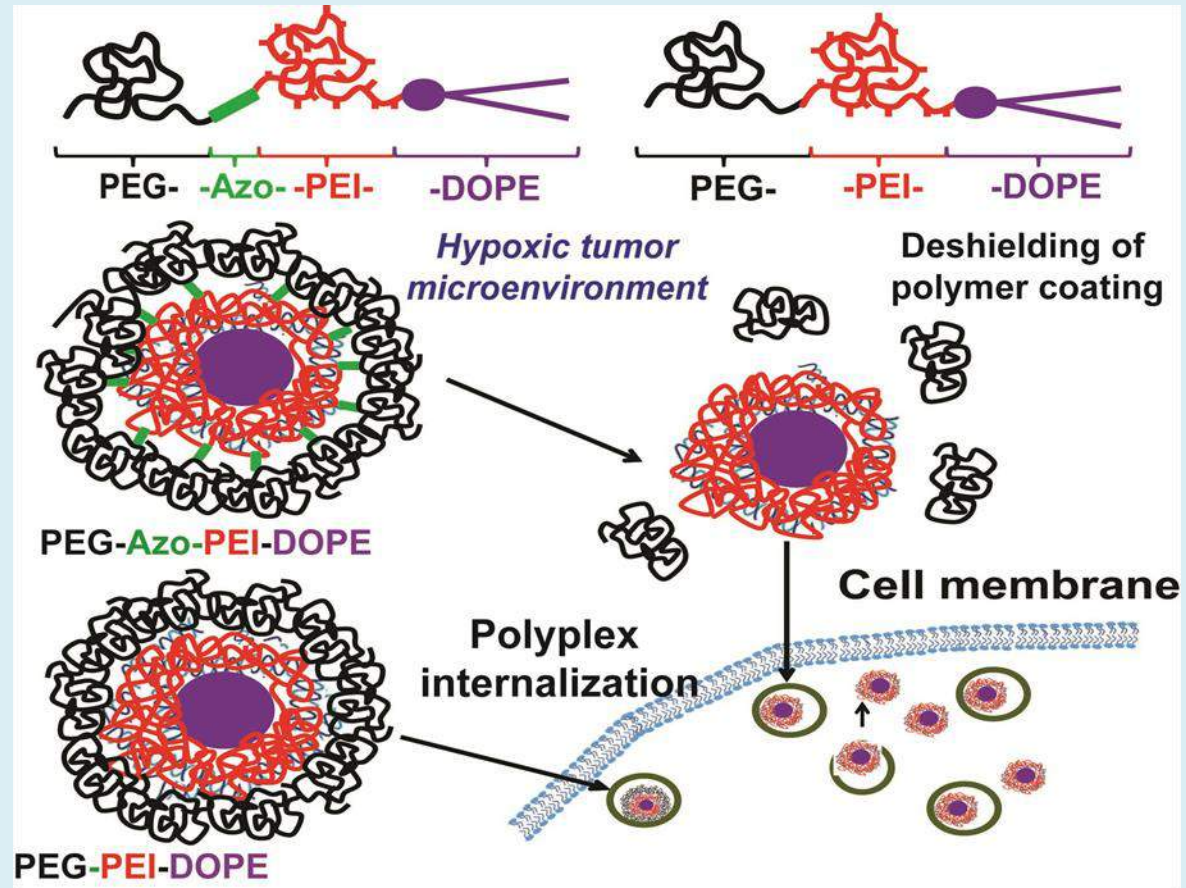
Hypoxia-Targeted siRNA Delivery**

F. Perche, S. Biswas, T. Wang, L. Zhu, and V. P. Torchilin*

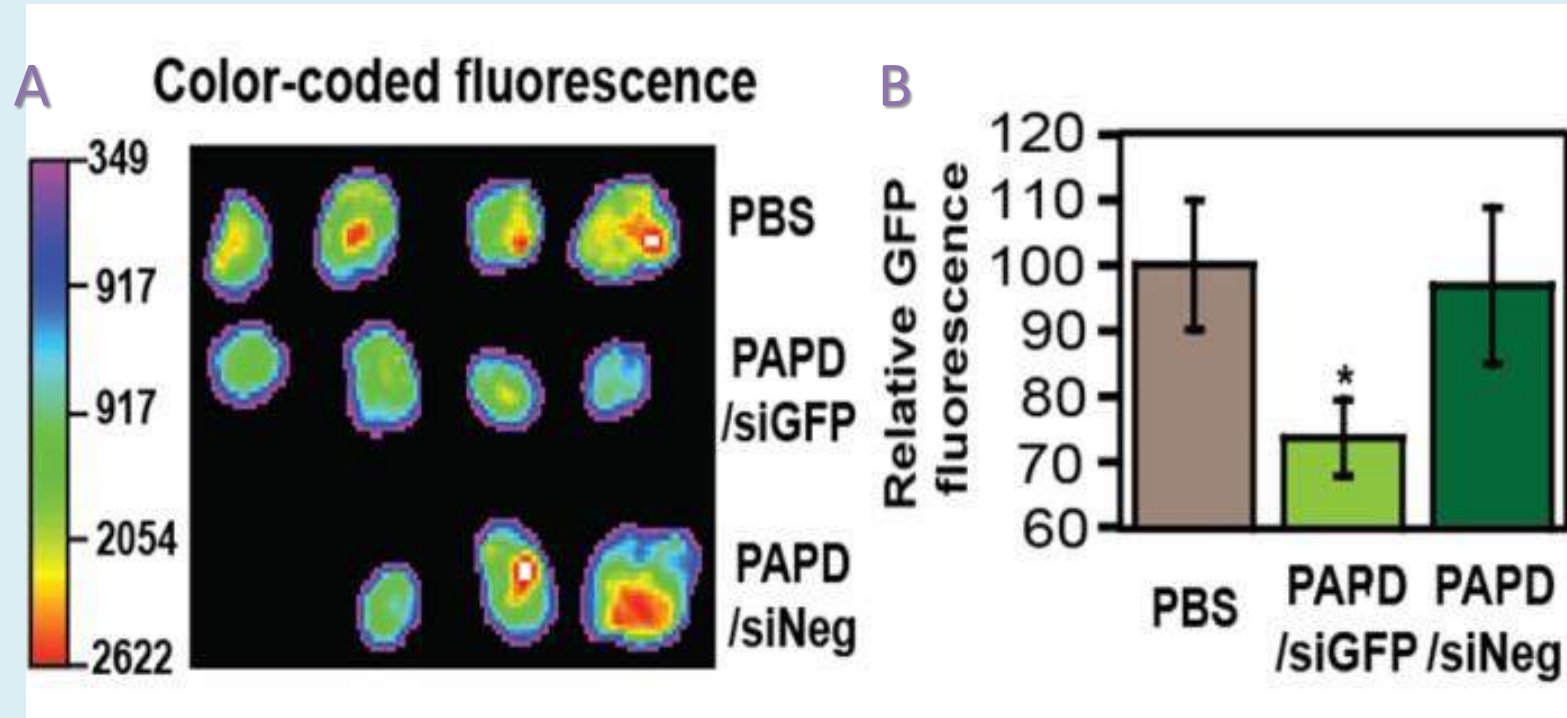
Abstract: Altered vasculature and the resultant chaotic tumor blood flow lead to the appearance in fast-growing tumors of regions with gradients of oxygen tension and acute hypoxia (less than 1.4% oxygen).^[1] Due to its roles in tumorigenesis and resistance to therapy, hypoxia represents a problem in cancer therapy.^[2,3] Insufficient delivery of therapeutic agents to the hypoxic regions in solid tumors is recognized as one of the causes of resistance to therapy.^[1,3] This led to the development of hypoxia imaging agents,^[4] and the use of hypoxia-activated anticancer prodrugs.^[2a] Here we show the first example of the hypoxia-induced siRNA uptake and silencing using a nanocarrier consisting of polyethyleneglycol2000, azobenzene, polyethyleneimine (PEI) (1.8 kDa), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) units (the nanocarrier is referred to as PAPD), where azobenzene imparts hypoxia sensitivity and specificity.^[4a] We report hypoxia-activated green fluorescent protein (GFP) silencing *in vitro* and its down-regulation in GFP-expressing tumors after intravenous administration. The proposed nanoformulation represents a novel tumor-environment-responsive modality for cancer targeting and siRNA delivery.

permeability and retention (EPR) effect for accumulation in tumor tissue.^[6] Nanoparticles are expected to show preferential extravasation from the circulation when they reach the altered tumor vasculature with its widened endothelial fenestrae and deficient pericyte coverage. Conjugation of polyethyleneglycol (PEG) to nanoparticles extends their blood circulation time, increasing the probability of tumor accumulation by EPR.^[7] However, PEGylation can also hinder cellular uptake resulting in decreased therapeutic activity.^[5a,7a] This PEG dilemma led to the design of nanoformulations with PEG that can be detached upon tumor stimulus to target payload delivery.^[6,8] Nitroimidazole derivatives have been proposed as hypoxia sensors since they are subject to intracellular reduction with formation of free radicals.^[1a,2b,4b] While these free radicals are rapidly oxidized by molecular oxygen, their stabilization under hypoxia leads to reduction-mediated cleavage.^[1b,4a,b,9] Nagano and co-workers demonstrated successful hypoxia imaging *in vivo* with azobenzene-based probes.^[4a,9] In our study, we used azobenzene as a hypoxia-responsive bioreductive linker for hypoxia-targeted delivery of siRNA from PEGylated nanopreparations upon PEG removal/cleavage. The production of GFP

A schematic structure of the nanosystem to target siRNA and drugs to hypoxic areas in tumors



In vivo silencing activity



A - *Ex vivo* fluorescence optical imaging of tumors 48h after injection of PBS , PEG-Az-PEI-PE/anti-GFP siRNA complexes (PAPD/siGFP, n=4), PEG-Az-PEI-DOPE/negative siRNA complexes

B - Cell-associated fluorescence of dissociated tumors by flow cytometry

Enhanced anticancer activity of nanopreparation containing an MMP2-sensitive PEG-drug conjugate and cell-penetrating moiety.

Zhu L, Wang T, Perche F, Taigind A, Torchilin VP.

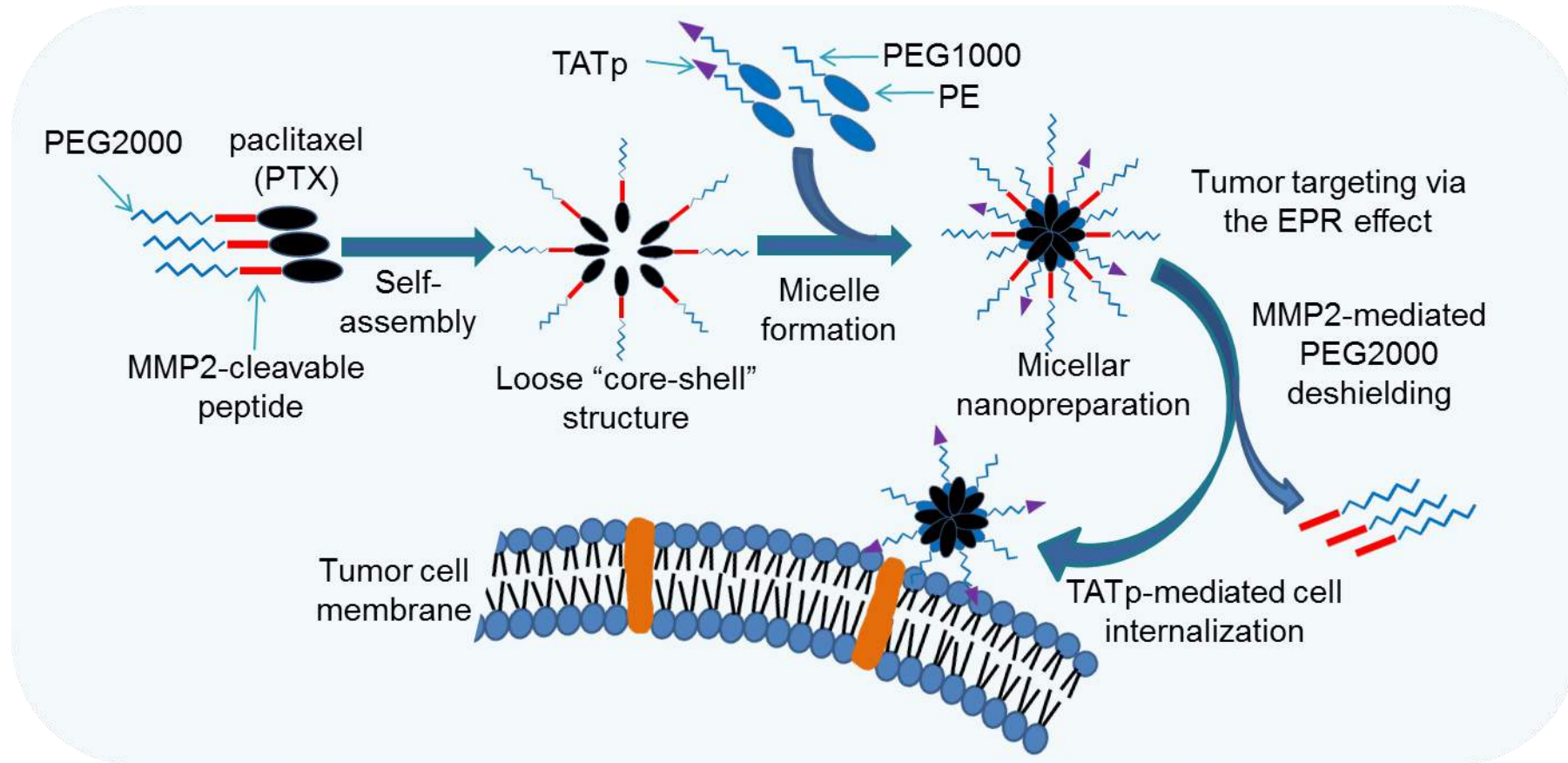
Proc Natl Acad Sci U S A. 2013 Oct 15;110(42):17047-52

Matrix metalloproteinase 2-sensitive multifunctional polymeric micelles for tumor-specific co-delivery of siRNA and hydrophobic drugs.

Zhu L, Perche F, Wang T, Torchilin VP.

Biomaterials. 2014 Apr;35(13):4213-22.

Nanopreparation Containing MMP2-sensitive PEG-paclitaxel Conjugate and Cell Penetrating Moiety



PE, phosphatidylethanolamine

PEG1000-PE: a building block for nanocarrier

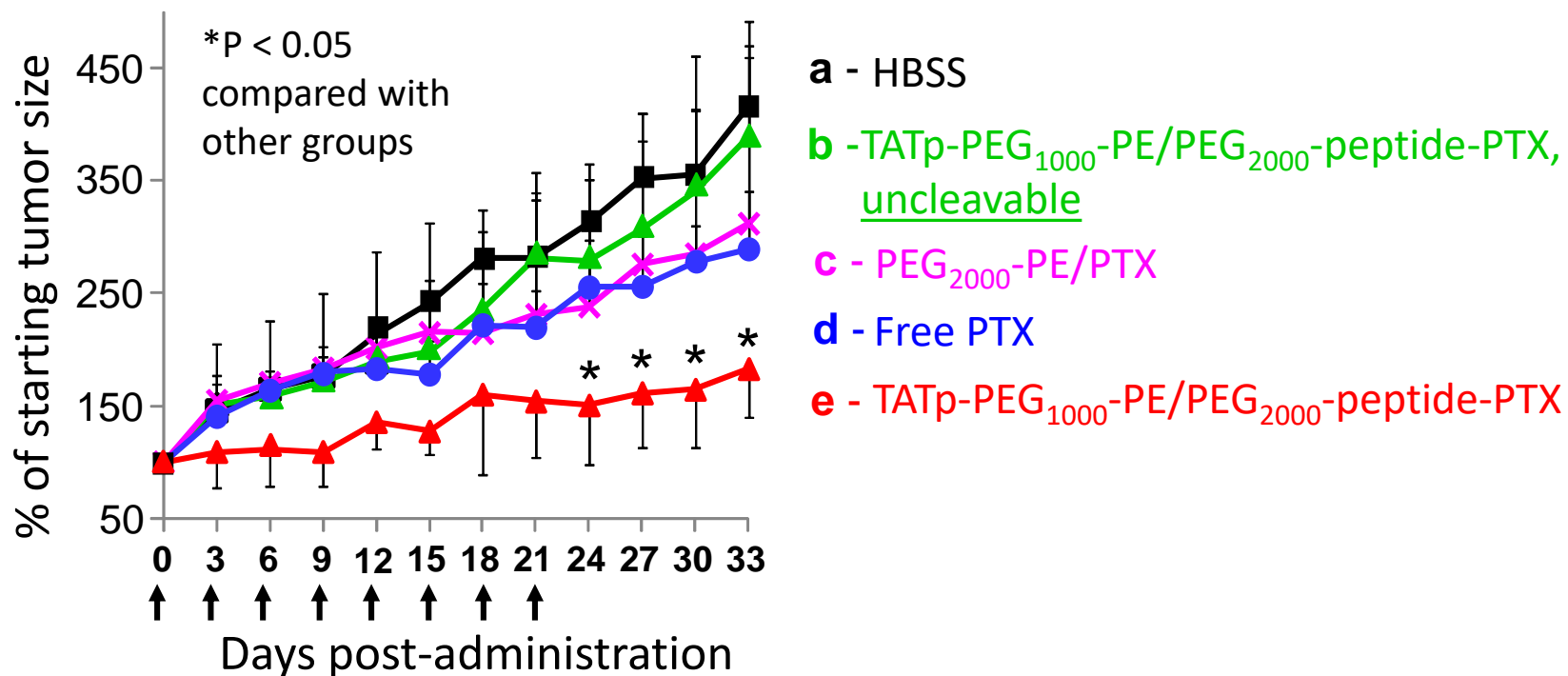
TATp-PEG1000-PE: a cell-penetrating moiety

PEG2000-peptide-PTX: (1) an MMP2-cleavable prodrug
(2) a self-assembly building block

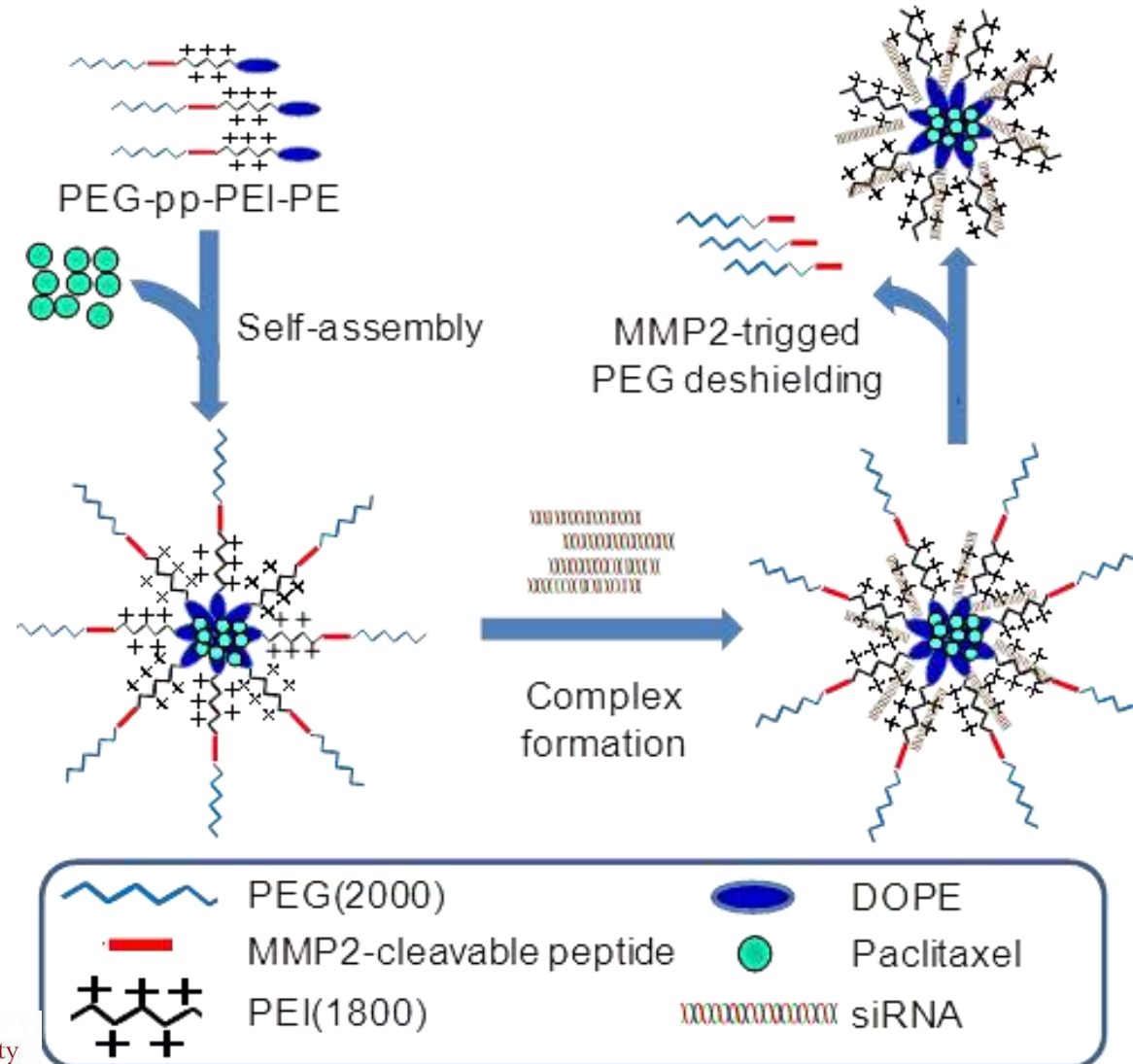
Zhu L, et al. (2013), PNAS, 110(42):17047-52.

Zhu L, et al., Patent No. PCT/US2013/072216.

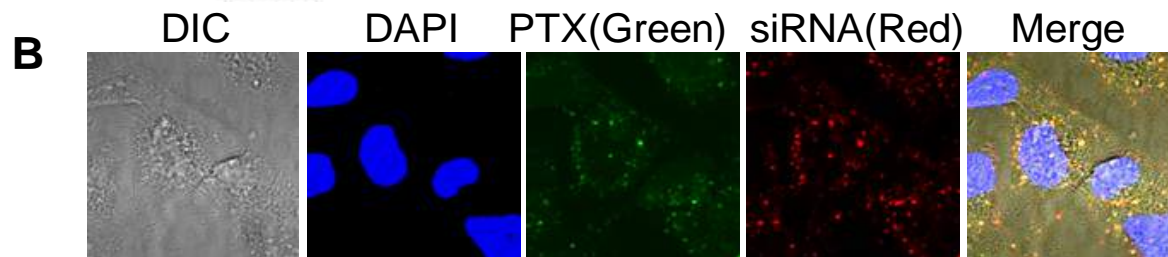
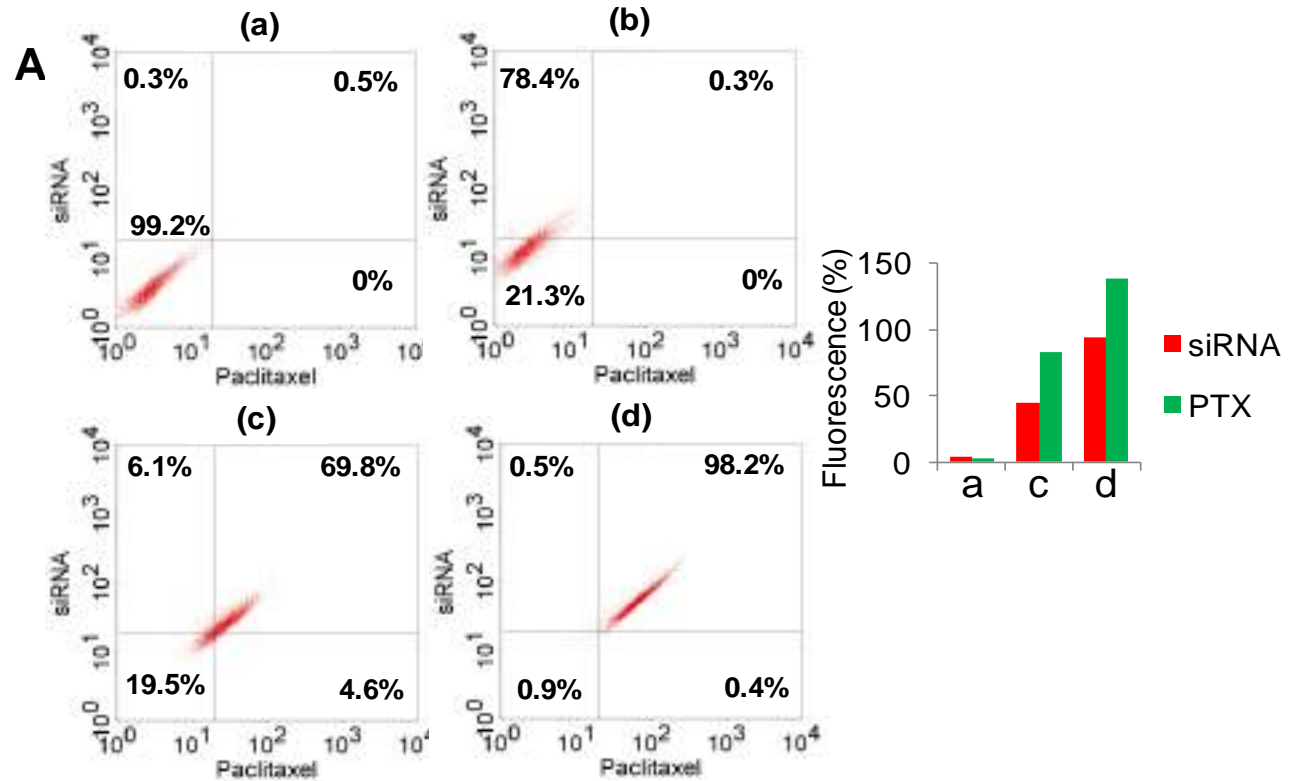
Tumor growth inhibition (% of the starting tumor volume)



Tumor-specific MMP-2-sensitive preparations

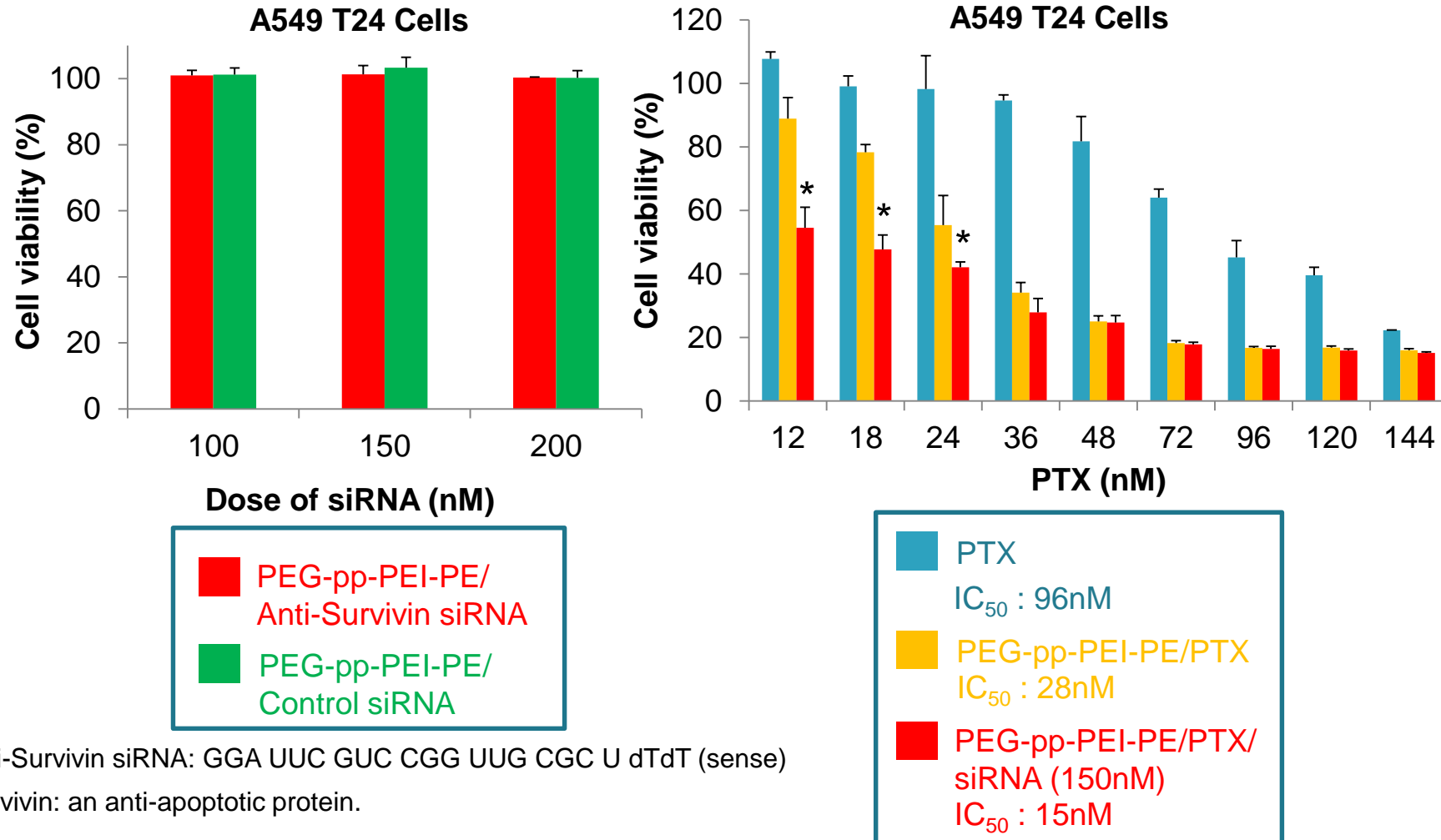


In vitro co-delivery of siRNA and PTX into A549 cells



PEG-pp-PEI-PE/PTX/siRNA

Enhanced Anticancer Activity by Co-delivery of Anti-Survivin siRNA and PTX



TRIPLE COMBINATION

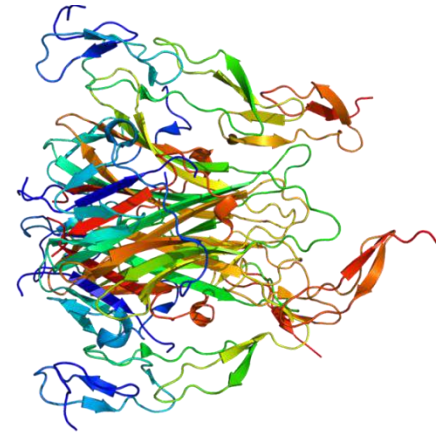
Combination Nanopreparations of a Novel Proapoptotic Drug - NCL-240, TRAIL and siRNA.

Riehle R, Pattni B, Jhaveri A, Kulkarni A, Thakur G, Degterev A, Torchilin V.

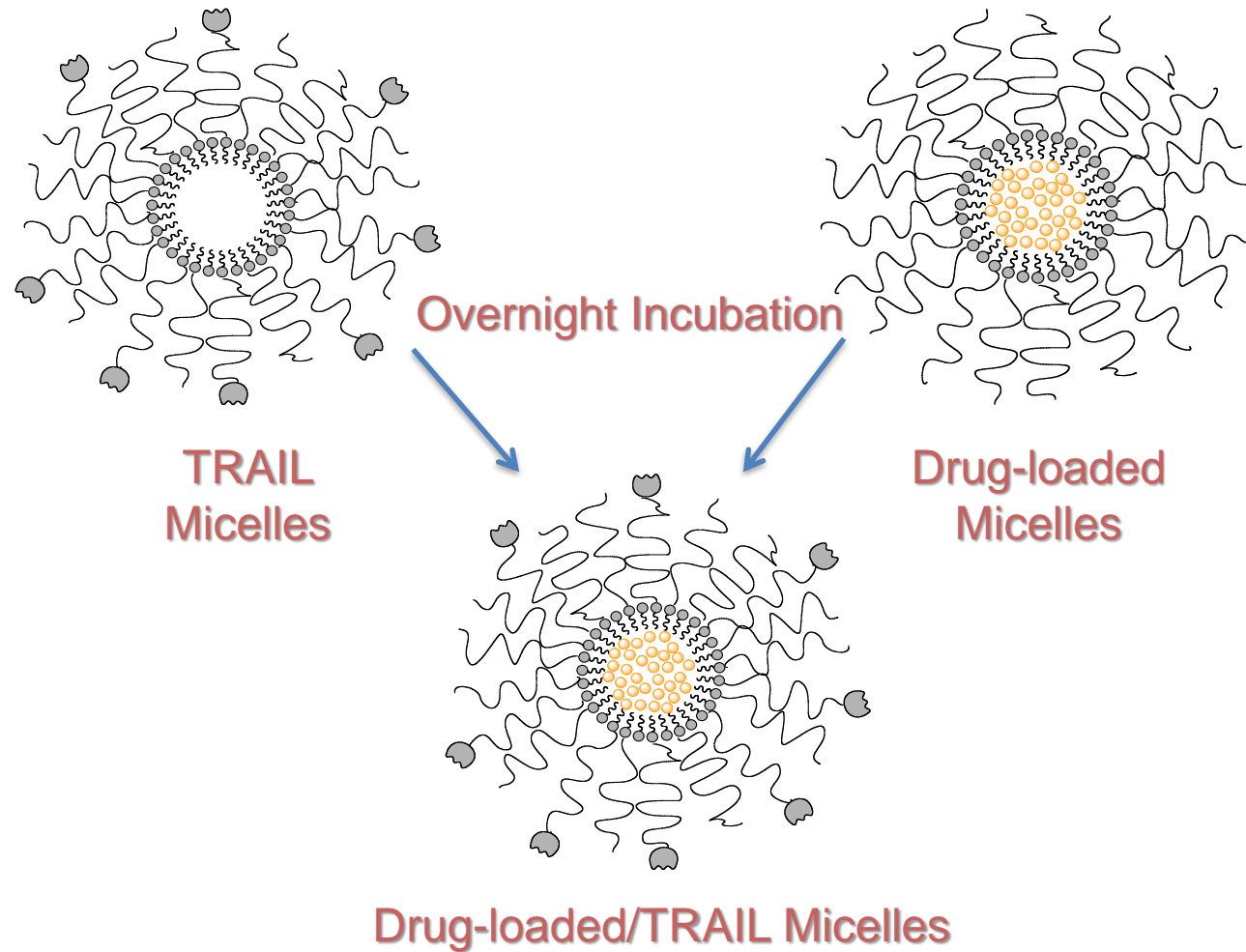
Pharm Res. 2016 Jul;33(7):1587-601.

TRAIL

- TNF α -Related Apoptosis Inducing Ligand
- Homotrimer protein
- Binds to a death receptors on cells and initiates downstream apoptosis pathways
 - Cancer cell specific
- Multiple receptors (death and decoy) and intracellular proteins modulate the apoptotic activity of TRAIL



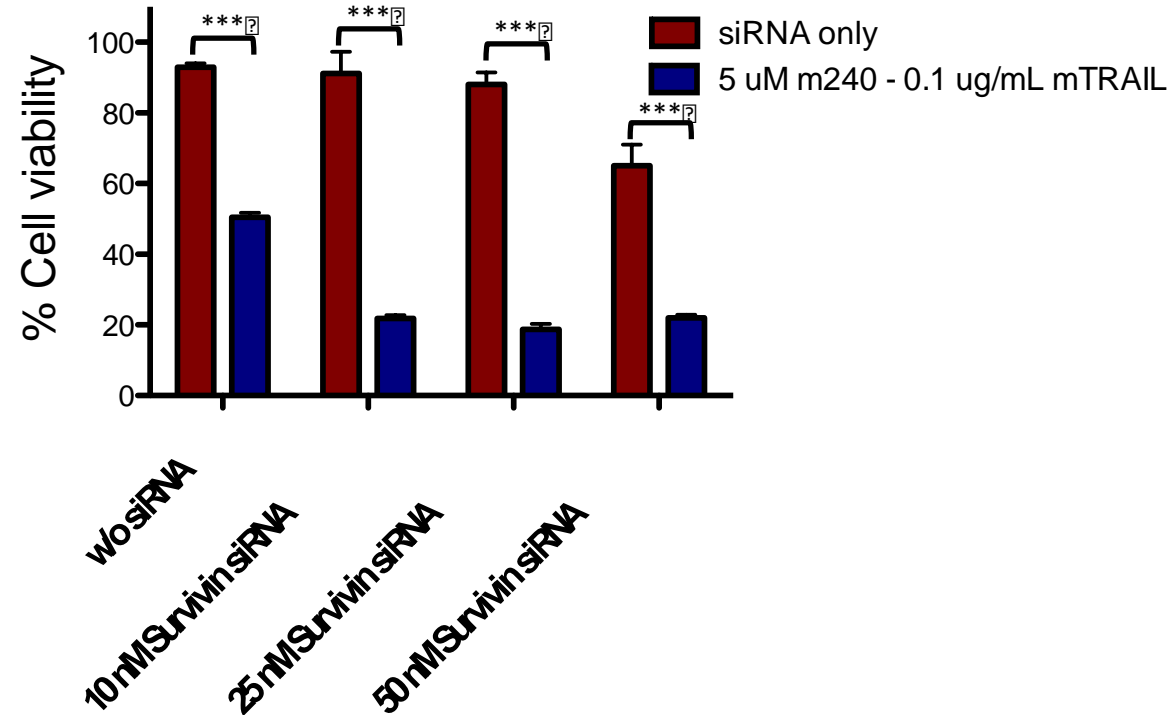
Formulation Preparation



Riehle, R.D., et al., Micellar formulations of pro-apoptotic DM-PIT-1 analogs and TRAIL in vitro and in vivo. *Drug Delivery*, 2013.

Coincubation of survivin siRNA-S-S-PE mixed micelles with NCL-240 loaded/TRAIL modified micelles

Cell viability of A2780 cells following treatment of combination survivin/NCL-240/TRAIL micelles for 48 hrs



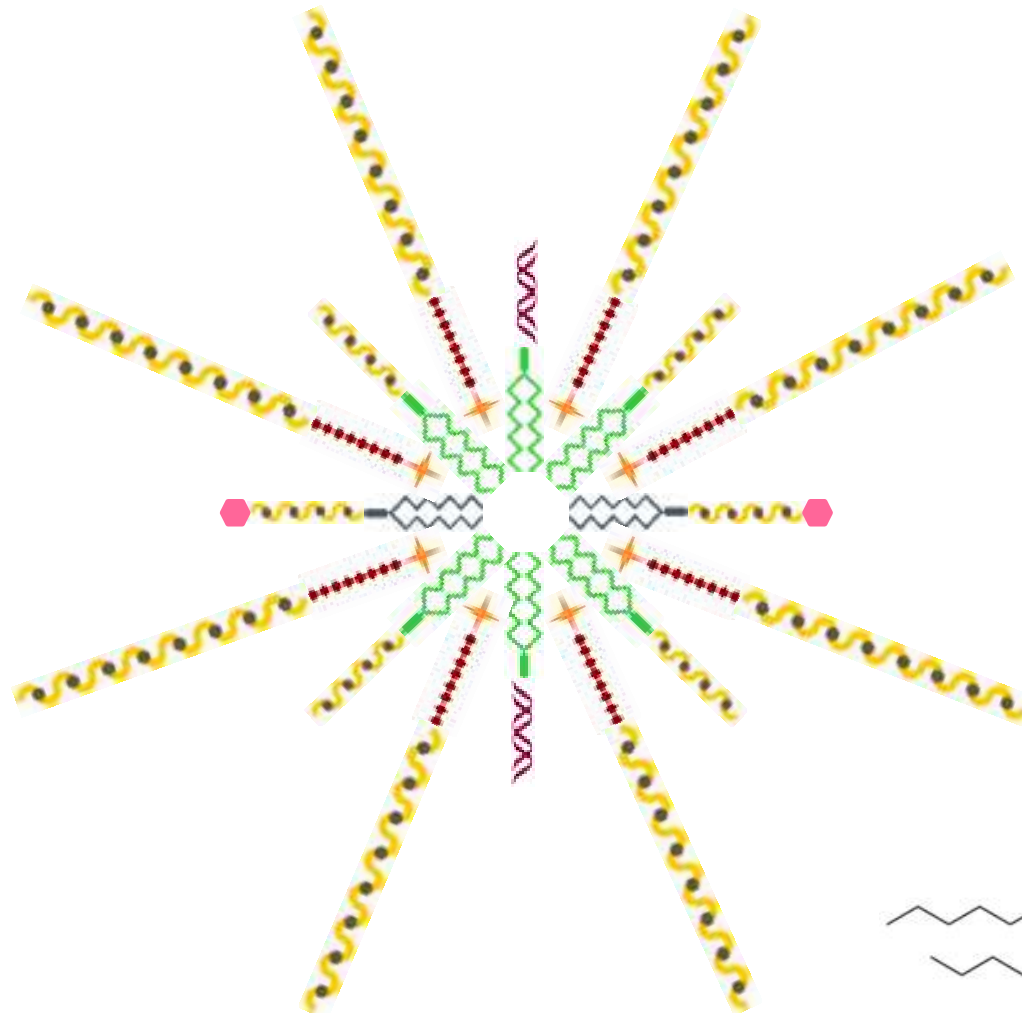
% cell viability of A2780 cells following treatment with combination survivin siRNA-S-S-PE/PEG-PE mixed micelles (1:750 weight ratio), NCL-240 loaded/TRAIL-modified micelles for 48 hr. Formulations contained either siRNA-S-S-PE/PEG-PE mixed micelles alone, or in combination with drug-loaded, TRAIL-modified micelles. siRNA sample in w/o siRNA indicates empty PEG-PE micelles (n=3 error bars indicate SD, ***p < 0.001)

DOUBLE SENSITIVITY

Mixed Nanosized Polymeric Micelles as Promoter of Doxorubicin and miRNA-34a Co-Delivery Triggered by Dual Stimuli in Tumor Tissue.

Salzano G, Costa DF, Sarisozen C, Luther E, Mattheolabakis G, Dhargalkar PP, Torchilin VP

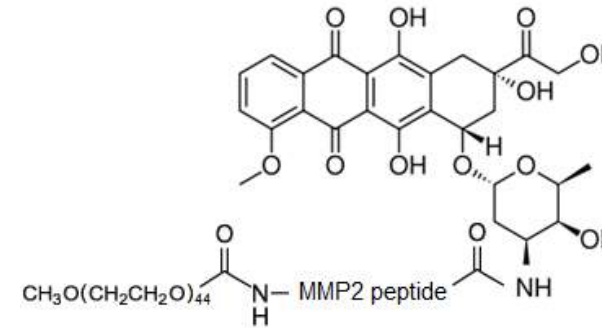
Small. 2016 Jul 19. doi: 10.1002/sml.201600925.
[Epub ahead of print]



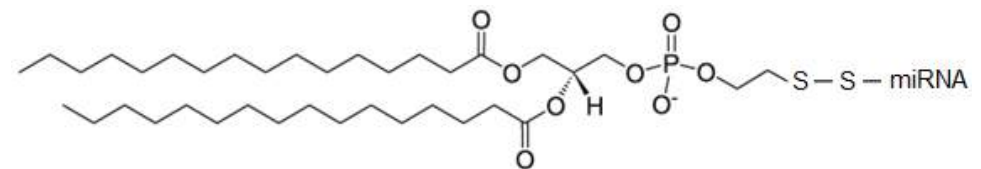
PEG1k-PE



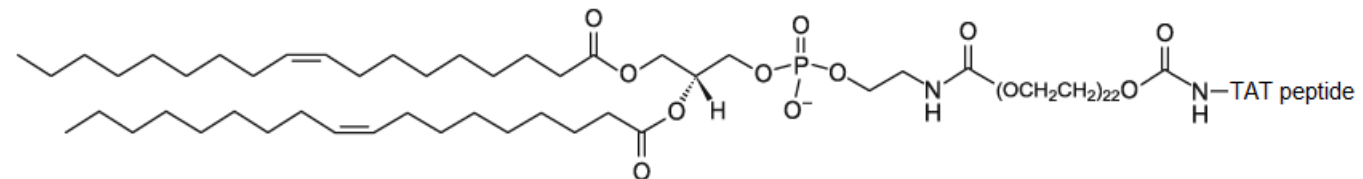
PEG2k-CLV-Dox



TAT-PEG1k-PE



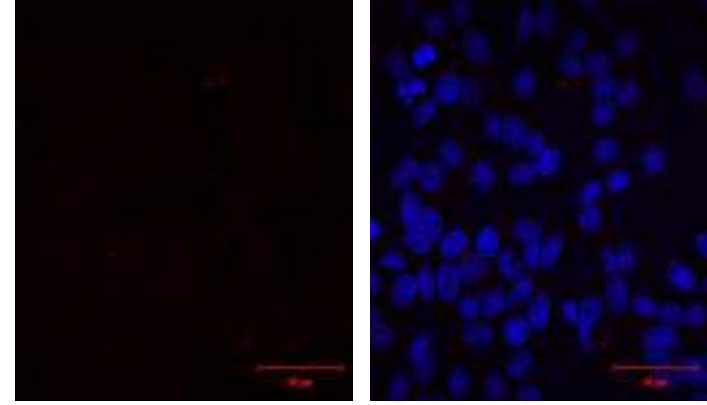
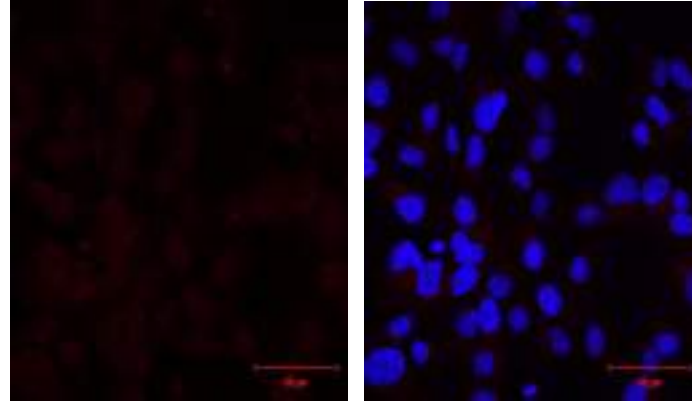
miRNA34a-S-S-PE



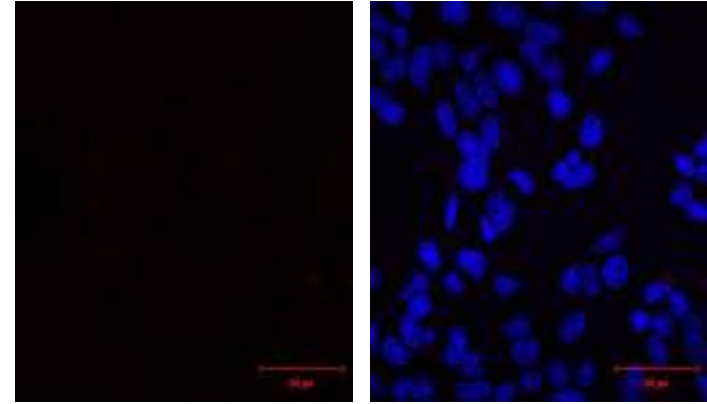
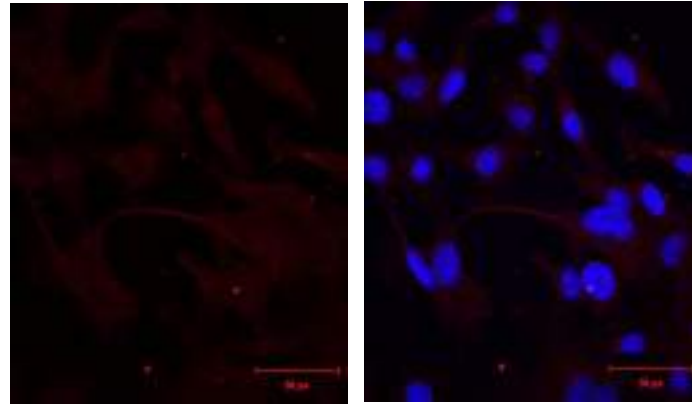
HT1080

MCF7

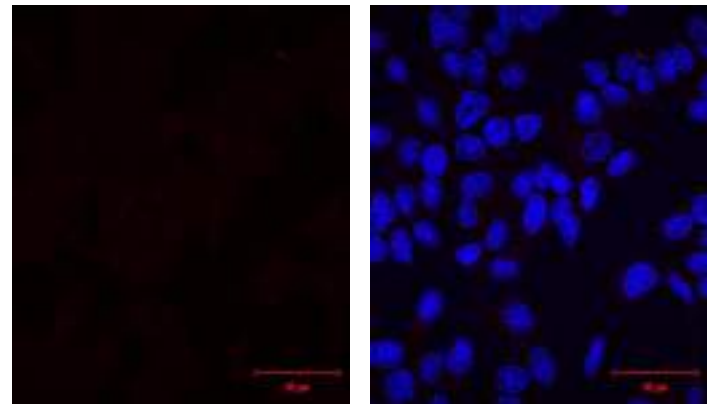
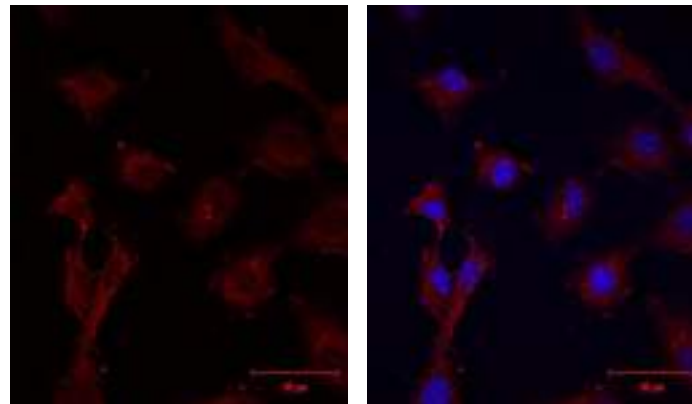
TAT/UNCLV Dox

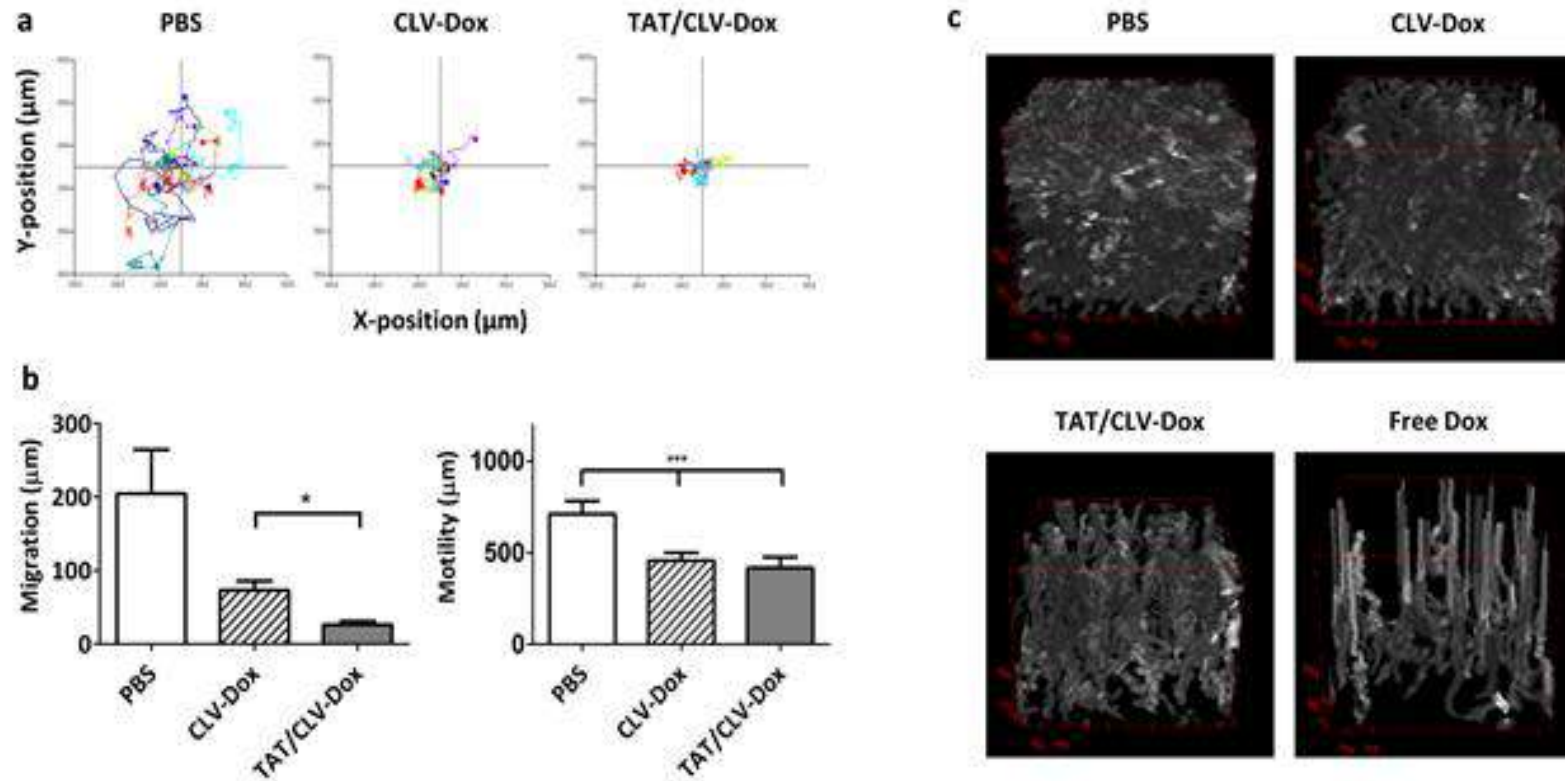


CLV Dox

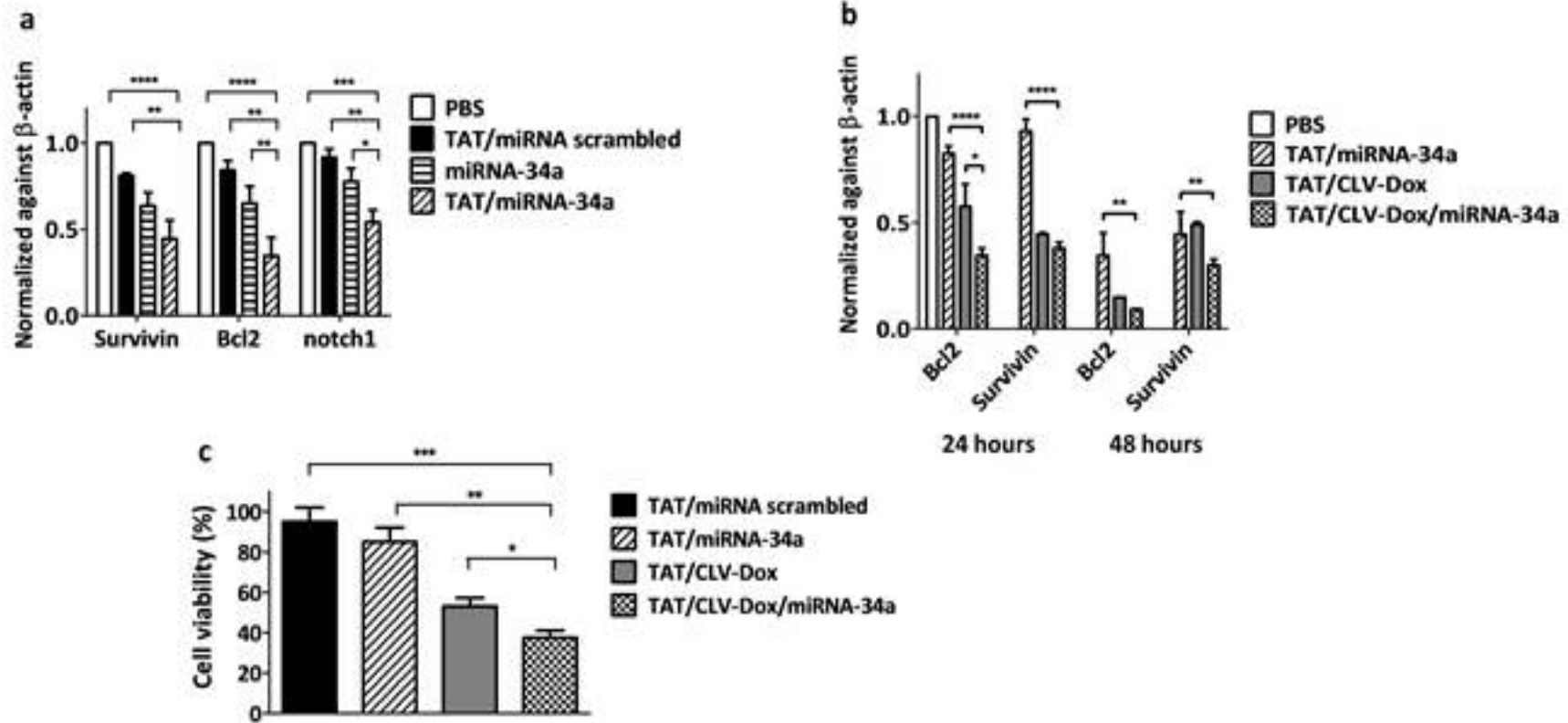


TAT/CLV Dox

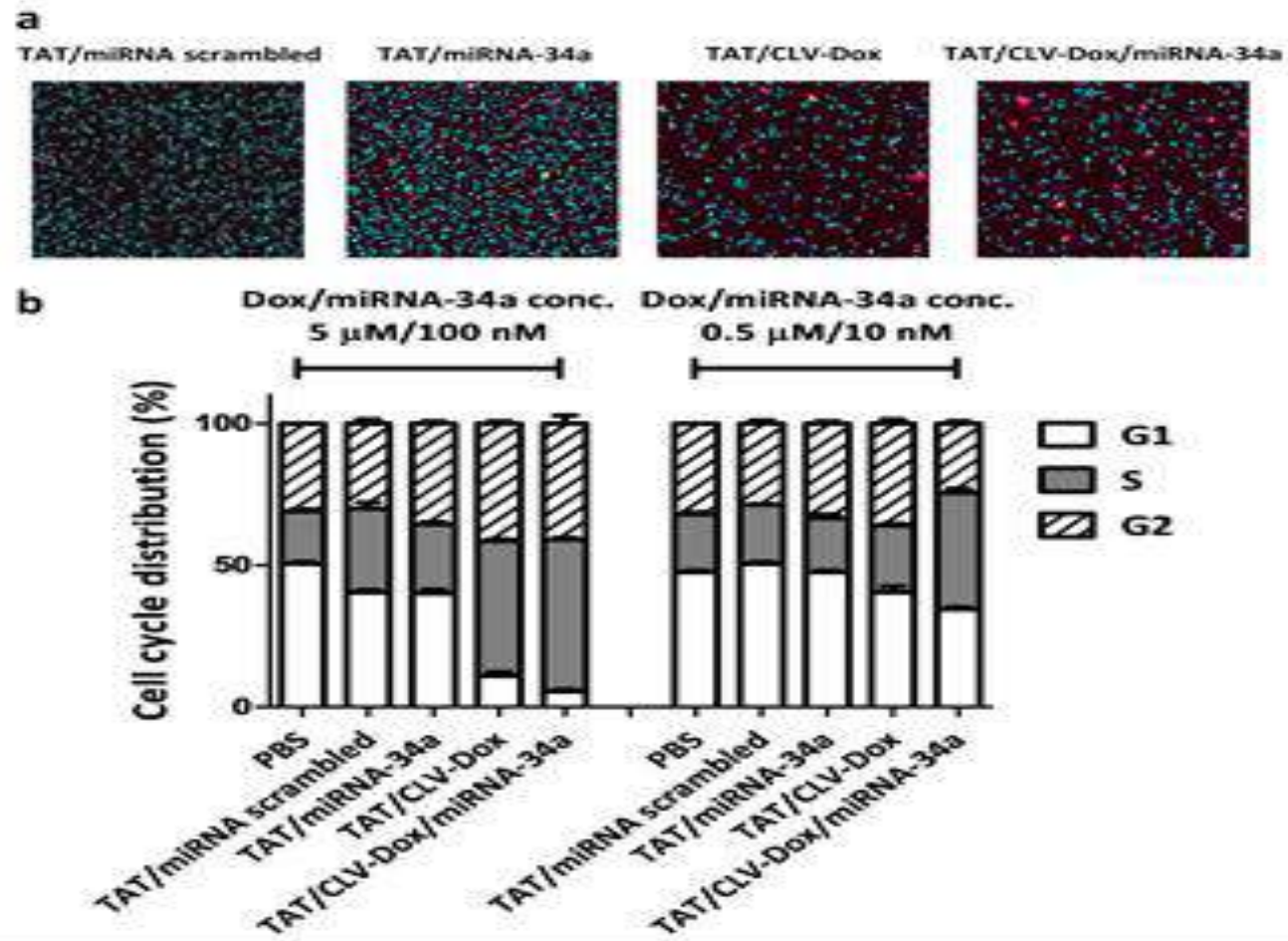




Time-lapse cytometry analysis of HT1080 cells after treatment with MMP2-sensitive MM. a, Cell trajectory plot. b, Quantitative assessment of cell motility and migration. c, 4D plots of cell proliferation over the time of analysis. p-values were obtained by comparison of groups indicated. *** $p \leq 0.001$ and * $p \leq 0.05$. Error bars represent mean \pm SD (n = 10 individual cells per treatment).



Effect of MM in gene expression and cell viability of HT1080 cells. a, mRNA levels of Bcl2, survivin and notch1 after treatment with GSH-sensitive MM at miRNA-34a concentration of 100 nM (n = 3 treatments). b, mRNA levels of Bcl2 and survivin after treatment with dual sensitive MM at miRNA-34a/Dox concentration of 100 nM and 5 μ M, respectively (n = 3 treatments). c, Cell viability in 2D monolayer model after treatment with dual sensitive MM at miRNA-34a/Dox concentration of 100 nM and 5 μ M, respectively (n = 3 treatments). p-values were obtained by comparison of groups indicated. ****p \leq 0.0001, ***p \leq 0.001, **p \leq 0.01 and *p \leq 0.05. Error bars represent mean \pm SD.



Effect of MM in apoptosis and cell cycle distribution of HT1080 cells. a, Laser scanning cytometry images after treatment with dual sensitive MM at miRNA-34a/Dox concentration of 50 nM and 2.5 mM, respectively. Magnification = 40X. Dead cells are stained in magenta and nuclei of live cells are stained in blue. b, Cell cycle distribution after treatment with dual sensitive MM at high and low concentrations of miRNA-34a and Dox. Error bars represent mean \pm SD (n = 3 treatments).

