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Nanocages for self-triggered nuclear delivery of Doxorubicin at cancer cells

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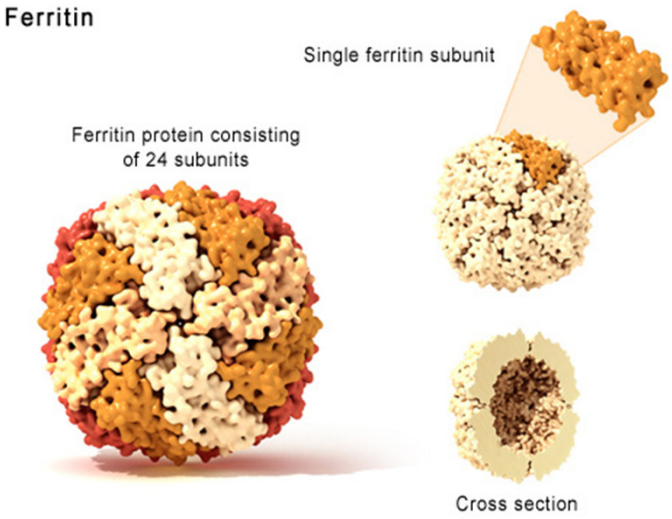
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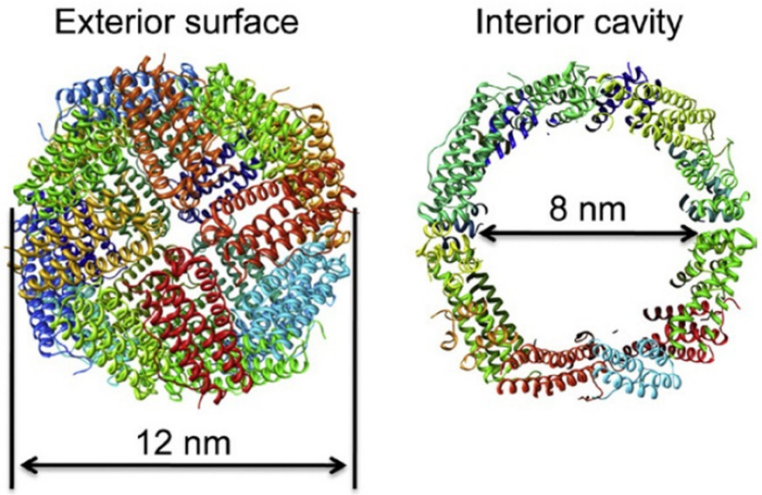
Mazzucchelli Serena
PhD

Ferritin nanocages (HFn)

polimer of 24 subunits of heavy (H) or light (L) ferritin chain

H ferritin: ferroxidase activity
L ferritin: role in iron nucleation process

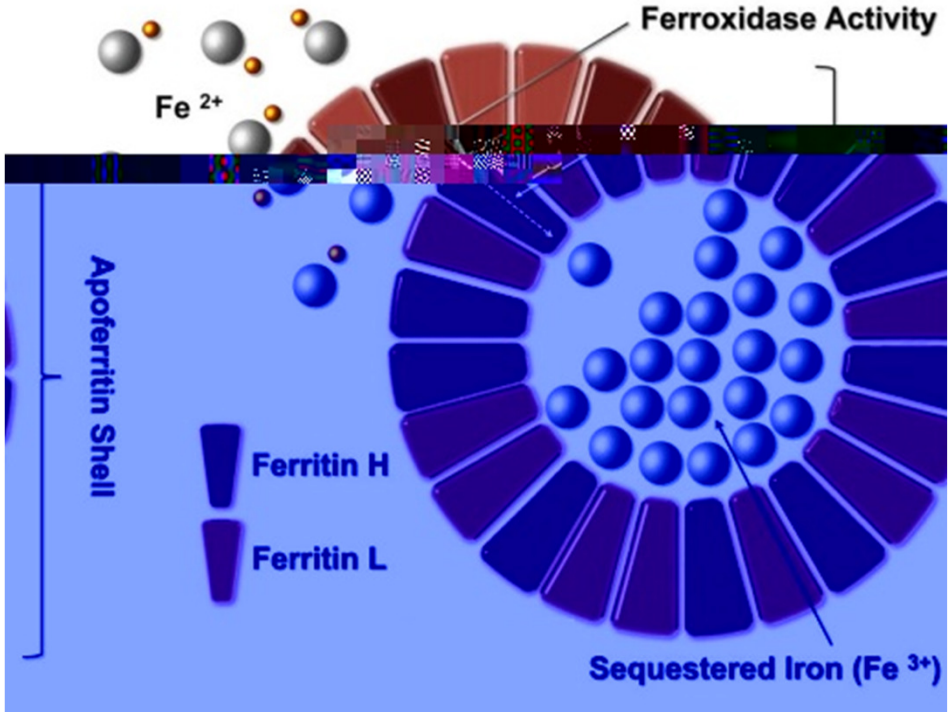




Biochim. Biophys. Acta **2009**, *1790*, 589-599

Physiological role of Ferritin

1) Iron storage



2) Protection against oxidative damage

Ferritin ferroxidation

$$2Fe(II) + O_2 + 4H_2O \rightarrow 2Fe(O)OH_{(core)} + H_2O_2 + 4H^+ \quad (1)$$

$$2Fe(II) + H_2O_2 + 2H_2O \rightarrow 2Fe(O)OH_{(core)} + 4H^+ \quad (2)$$

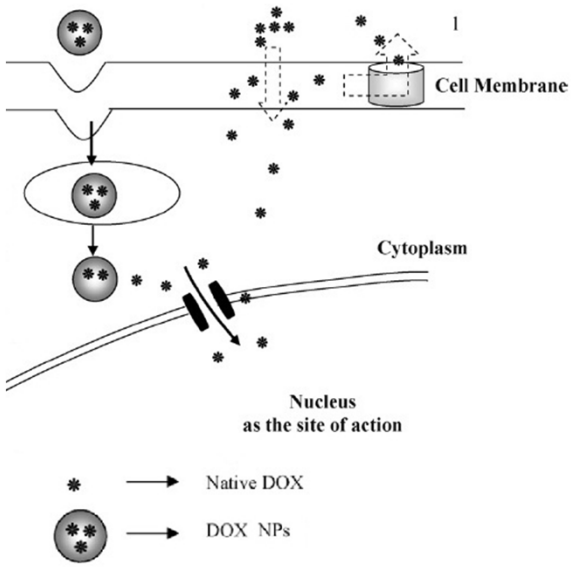
Fenton reaction

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH \quad (3)$$

Biochim. Biophys. Acta **2009**, *1790*, 589-599

Why HFn Nanocages?

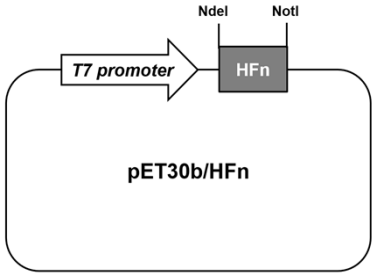
1) **Low immunogenicity : produced from human cDNA sequence**
2) **High stability in biological fluids**
3) **Possess an inner cavity that could be easily loaded with drugs by assembly/disassembly method**
4) **Active targeting toward cancer cells that overexpress TfR1 (***Nat. Nanotech.* **2012**, *7*, 459-464; *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 3505-3510**) or by insertion of cancer targeting moieties with DNA recombinant technology** *(Small* **2012,** 8, 2505-2514**)**
5) **Possibility to overcome Multi Drug Resistance mechanisms as demonstrated with other kinds of nanoparticle (***Eur. J. Pharm. Sci*. **2010**, *39*, 152-163**)**
6) **HFn physiological nuclear translocation in response of oxidative stress** *(Biochim***.** *Biophys. Acta* **2010**,1800, 793-797)



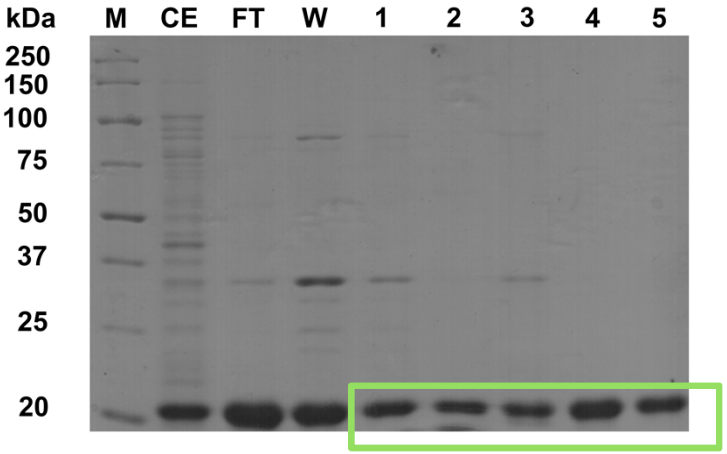


Development of HFn nanocages

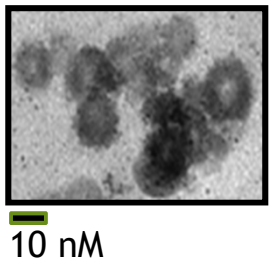
HFn expression vector for *E. coli* production



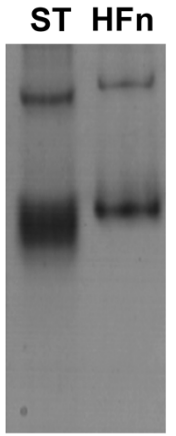
SDS-PAGE of HFn nanocages purification



TEM image of HFn nanocages



PAGE in native conditions

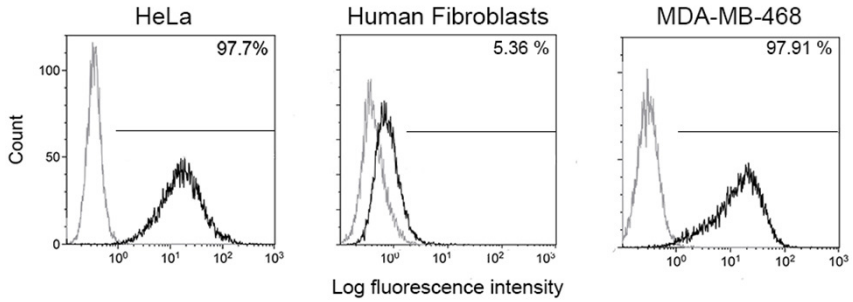


Yield: 56 mg/L colture

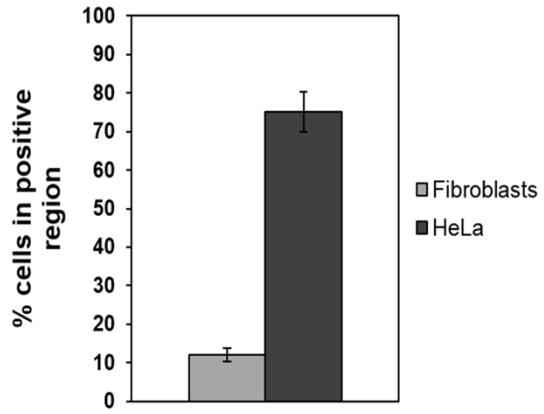
Bellini et al. JCR 2014, 196:184.

HFn nanocage interaction with tumor cells (I)

TfR1 expression

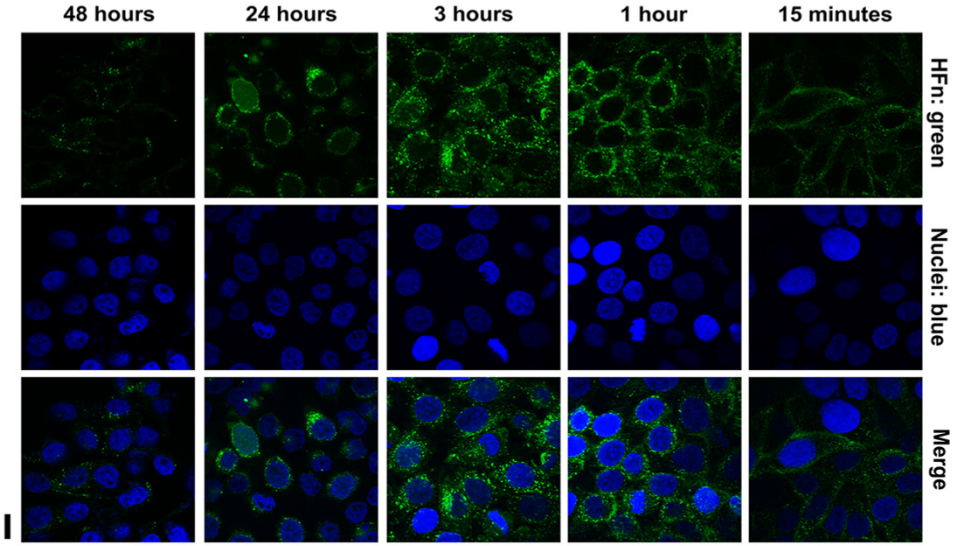


HFn specific recognition of tumor cells



Binding assay, performed by flow cytometry after incubating the cells 2 h at 4°C with 0.02 mg/mL of FITC labelled HFn.

HFn internalization in HeLa cells



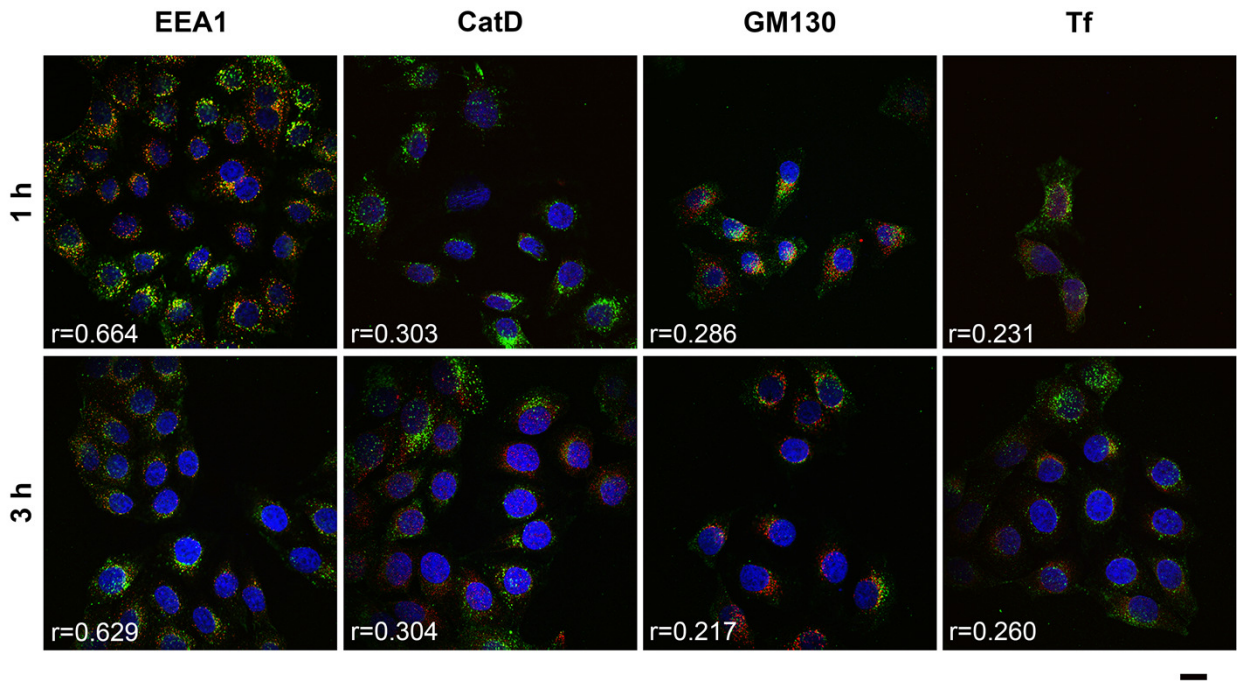
Intracellular localization of HFn nanocages. Confocal microscopy images of HeLa cells, incubated for 15 min, 1 h or 3 h at 37°C with 0.1 mg/mL of HFn-FITC (green).

Specific recognition of tumor cells overexpressing TfR1

Bellini et al. JCR 2014, 196:184.

HFn nanocage interaction with tumor cells (II)

Internalization pathway



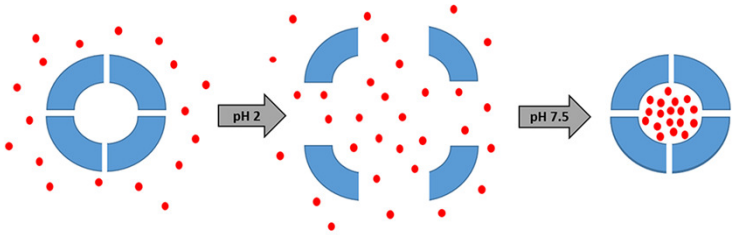
Internalization route of HFn nanocages. Confocal microscopy merge images of HeLa cells, incubated for 1 h or 3 h at 37°C with 0.1 mg/mL of FITC-labelled. Different organelles were stained with early endosome marker EEA1, lysosomal protein CatD, Golgi marker GM130 and recycling endosome marker TfR antibodies and labelled with a secondary antibody (red).

Lysosomal degradation doesn't take place for HFn nanocages

Bellini et al. JCR 2014, 196:184.

Development of DOX loaded-HFn nanocages

Loading with the disassemby/reassembly method

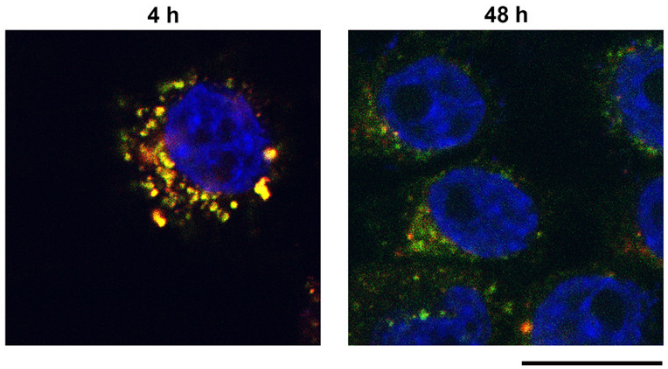


Schematic representation of the disassemby/reassembly method used for HFn loading with fluorescent molecules or drugs, exploiting the ability of HFn to modify its quaternary structure in response to pH changes.

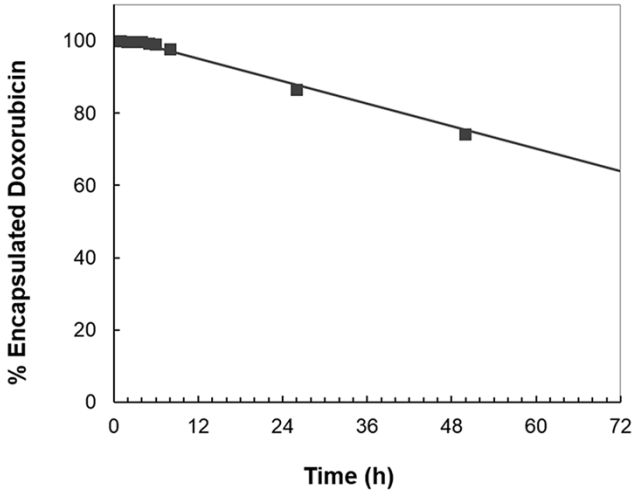
Number of doxorubicin molecules/nanoparticles	**28.98**

HFn final destiny

Evaluation of the fate of HFn shell and of relevant encapsulated molecules after internalization. Confocal microscopy merge images of HeLa cells, incubated for 4 h or 48 h at 37 °C with 0.1 mg/mL of HFn (red) loaded with FITC (green).



Kinetics of DOX release



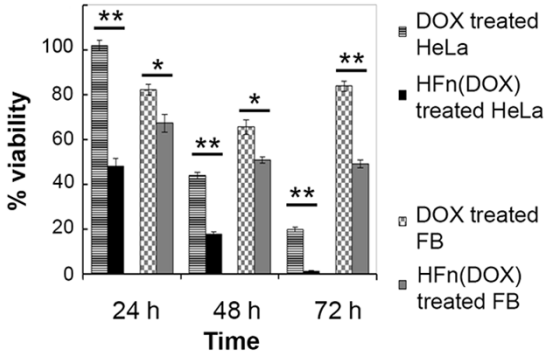
Kinetics of release of DOX-loaded nanoparticles (HFn(DOX)) at 37 °C in phosphate buffer saline (PBS) confirm the presumed good stability of the HFn nanoformulation at physiological pH.

Good nanovector for intracellular delivery of drugs

Bellini et al. JCR 2014, 196:184.

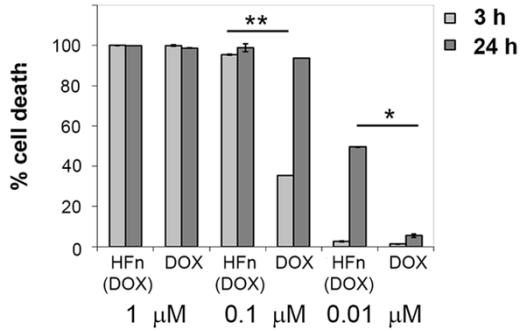
In vitro DOX efficacy: free *vs* nanodrug (I)

Viability assay



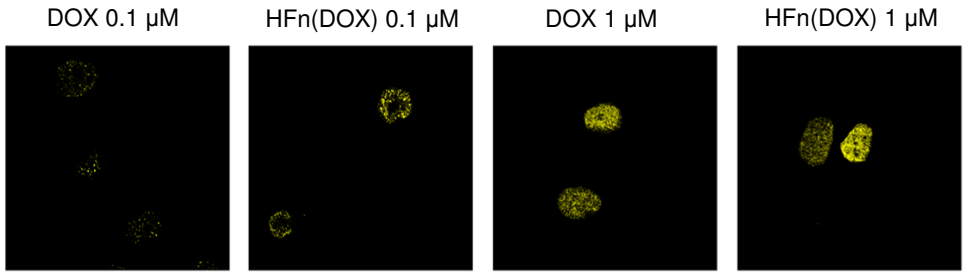
Viability of cells treated with free DOX or HFn(DOX). Fibroblasts and HeLa cells were treated with 0.1 μM of DOX or HFn(DOX) for up to 72 h.

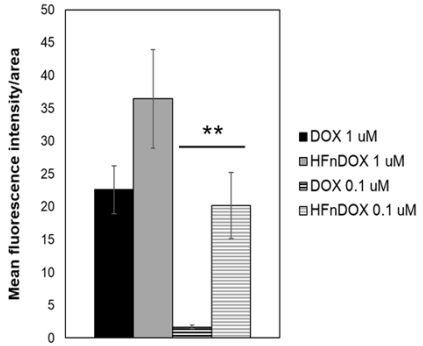
Cell Death assay



Cell death assay with DOX free or encapsulated in HFn shell. HeLa cells were treated with 1, 0.1 and 0.01 μM of DOX or HFn(DOX) for 3 or 24 h.

DNA double strand breaks after DOX exposure





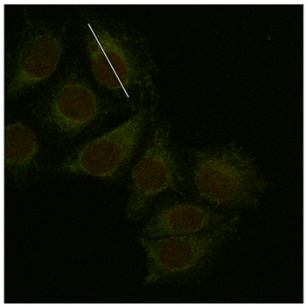
Double-strand break of DNA after DOX exposure. Confocal microscopy images of HeLa cells incubated with 1 μM or 0.1 μM DOX free or encapsulated in HFns. Quantification of fluorescence intensity due to DSB normalized with respect to cellular area ± s.e. ** P<0.005.

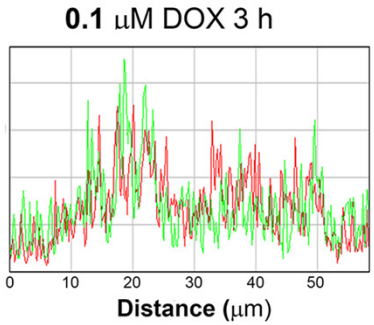
Nanodrug increases DOX efficacy in blocking proliferation and in inducing cell death

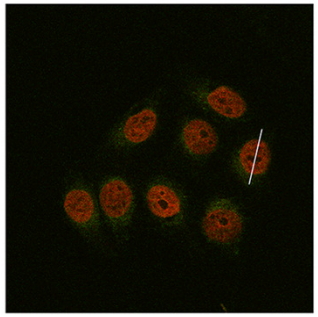
Bellini et al. JCR 2014, 196:184.

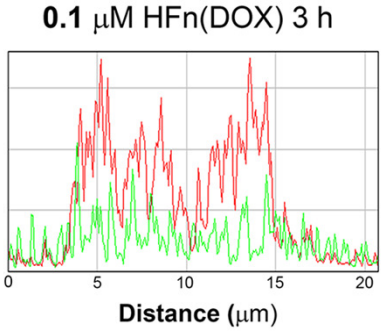
In vitro DOX efficacy: free *vs* nanodrug (II)

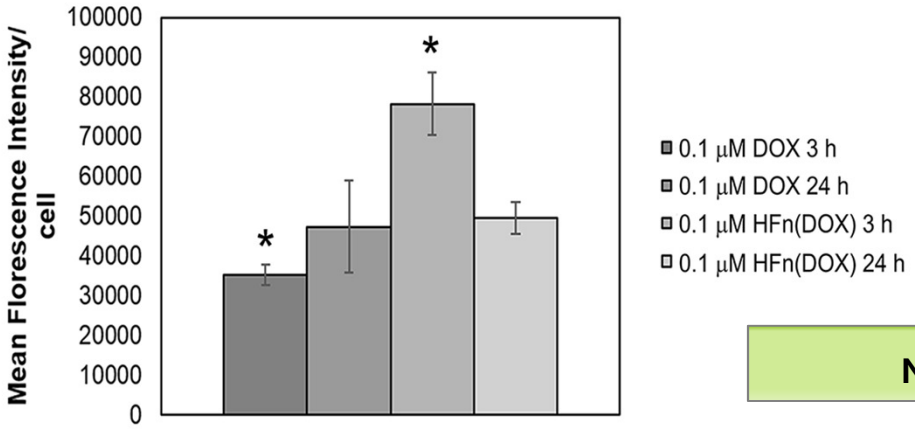
DOX intracellular distribution after HFn-mediated internalization











Quantification of total fluorescence intensity per cell. Reported values are a mean of measurements performed with Image J software on six different cells ± s.e. * P<0.01.

Confocal microscopy images of HeLa cells incubated with 0.1 μM DOX free or encapsulated in HFns and analysis of spatial distribution. Confocal laser scanning images of HeLa cells treated with 0.1 μM DOX (DOX degradation product in green) free or encapsulated in HFn shell for 3 h or 24 h at 37 °C. Spatial analysis was performed on merged images using Image J plugin RGB profile plot.

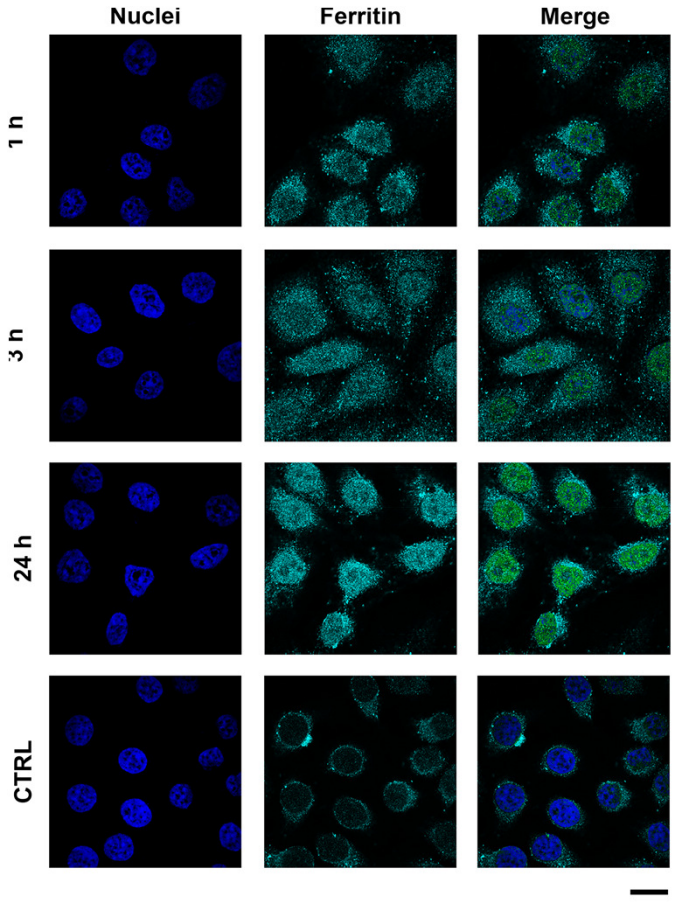
Nanodrug increases nuclear DOX accumulation

Is DOX able to mediate nuclear translocation of DOX?

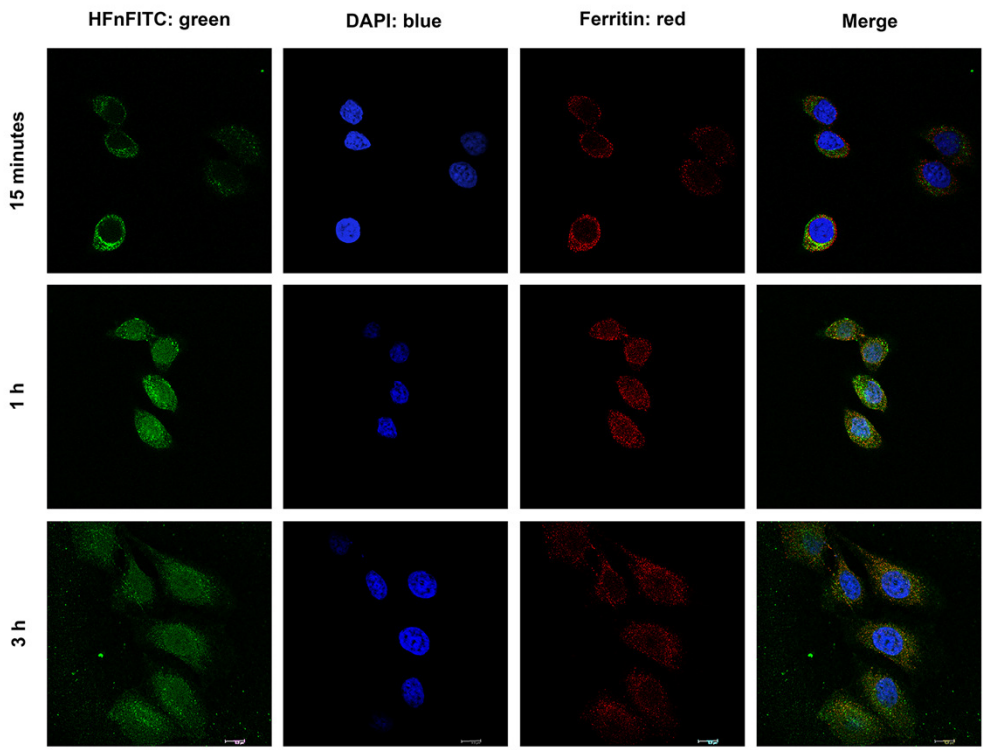
Bellini et al. JCR 2014, 196:184.

DOX-triggered nuclear translocation (I)

DOX-triggered Nuclear translocation of endogenous HFn



DOX-triggered Nuclear translocation of HFn nanocage

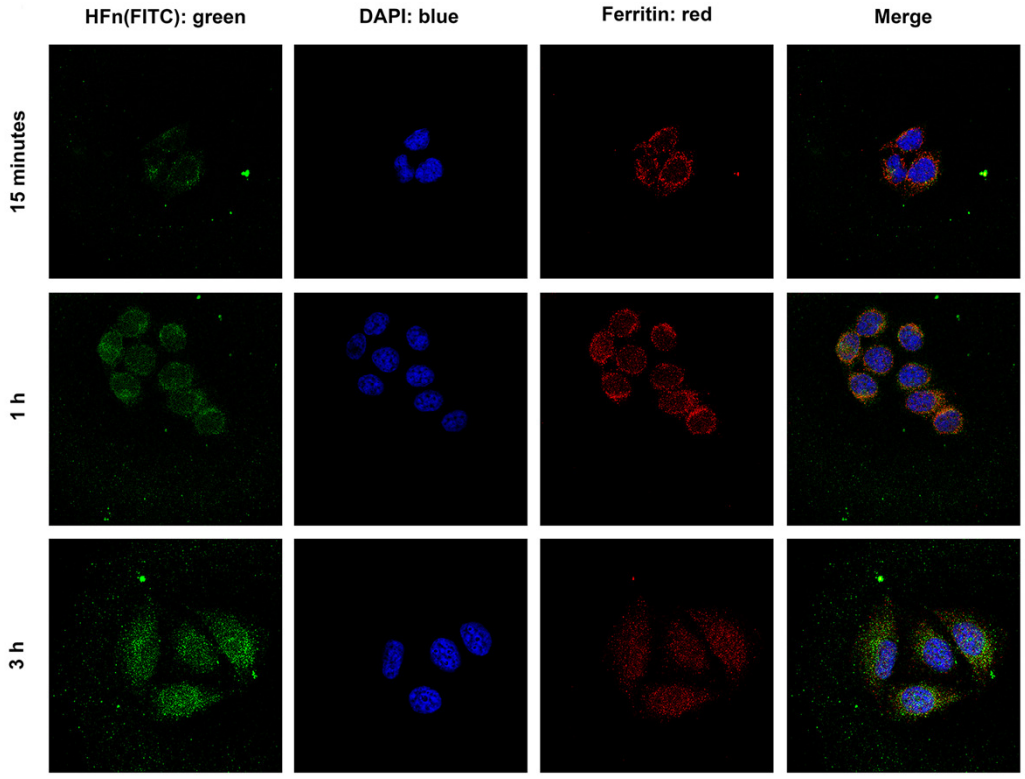


DOX triggered nuclear translocation of both endogenous and esogenous ferritin

Bellini et al. JCR 2014, 196:184.

DOX-triggered nuclear translocation (II)

DOX-triggered Nuclear translocation of encapsulated FITC

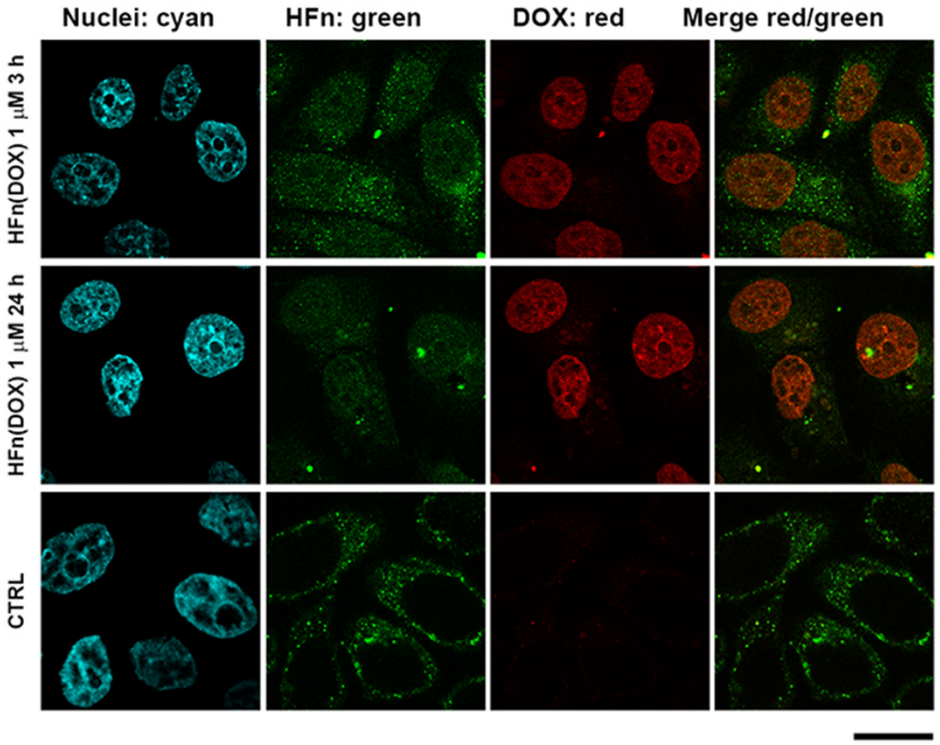


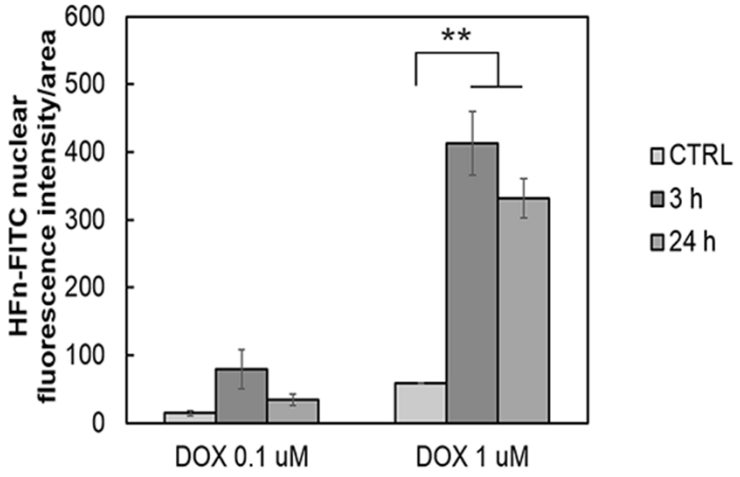
HFn is able to deliver FITC inside nuclear compartment after DOX stimulation

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Self-triggered nuclear translocation (I)

Self-triggered Nuclear delivery of DOX





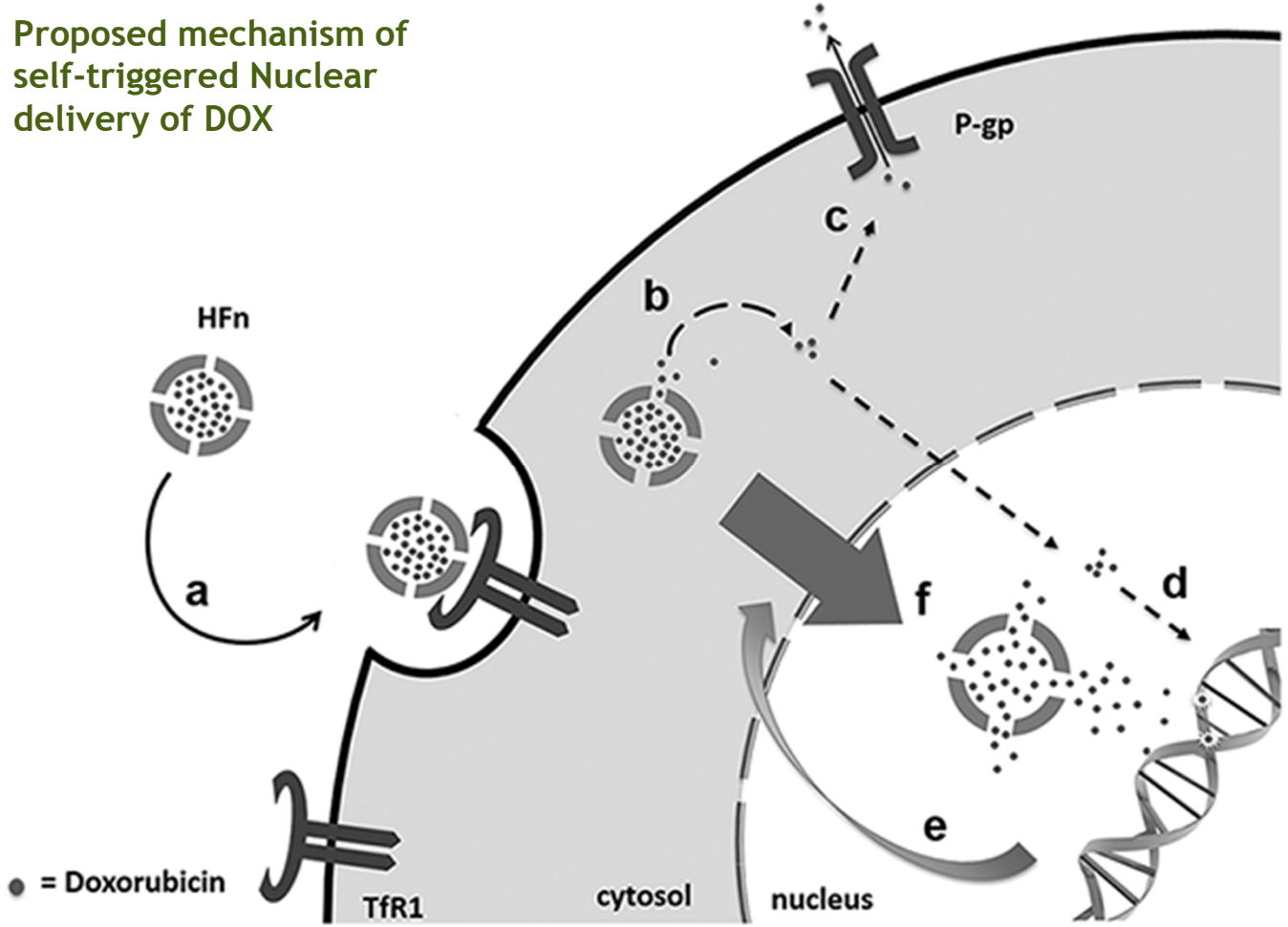
Quantification of fluorescence signal of FITC-labelled HFn in the nucleus.

Self-triggered nuclear delivery of DOX in HFn(DOX) nanocages. Confocal microscopy images of HeLa cells incubated for 3 and 24 h at 37 °C with 0.1 μM of HFn(DOX). HFn(DOX) was labeled with FITC (HFn; green) on the shell and then loaded with DOX (red).

Bellini et al. JCR 2014, 196:184.

Self-triggered nuclear translocation (II)

Proposed mechanism of self-triggered Nuclear delivery of DOX

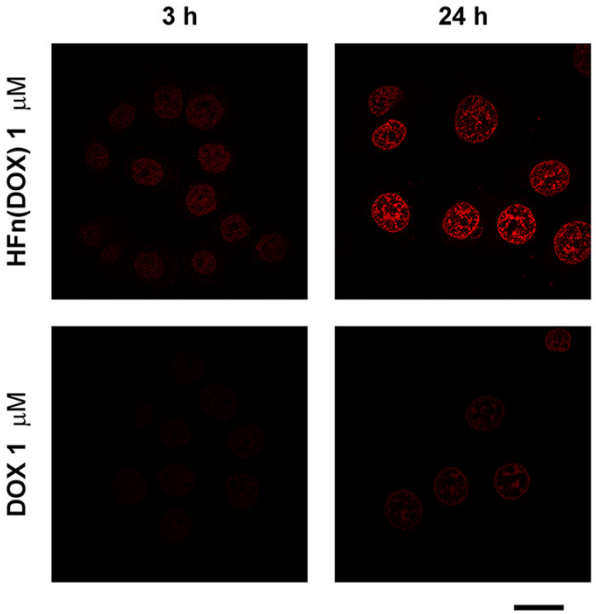


Schematic representation of self-triggered nuclear delivery of HFn(DOX). HFn were internalized upon the interaction with TfR1 by receptor-mediated mechanism without incurring lysosomal degradation (a). Encapsulated DOX was partially released in the cytoplasm through hydrophobic channels of its architecture (b). Then DOX is pumped out of the action of P-glycoprotein (c), or diffuses into the nucleus where it causes the DNA damage (d), which triggers the nuclear translocation of HFn(DOX) (e), and the massive release of DOX in the nuclear compartment (f).

Bellini et al. JCR 2014, 196:184.

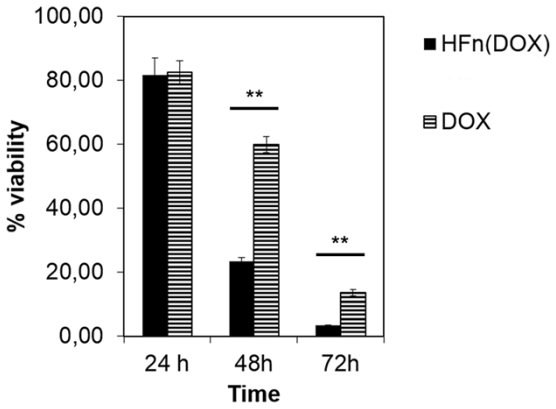
HFn nanocage restores DOX sensitivity in resistant cancer cells

Doxorubicin release inside the nuclear compartment of MDA-MB-468 cells

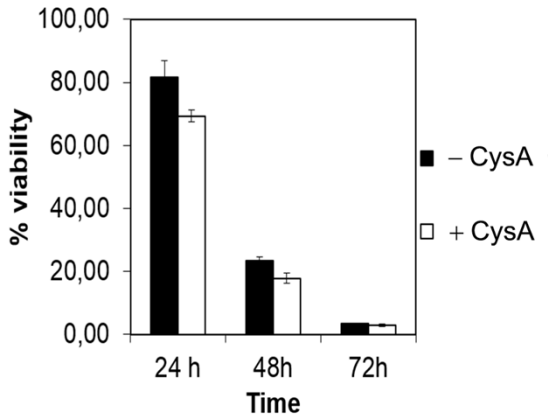


Confocal microscopy images of MDA-MB-468 cells incubated with 1 μM DOX free or encapsulated in HFn shell for 3 h or 24 h at 37 °C.

Viability assay



Viability of cells treated with free DOX or HFn(DOX) (1 μM) for up to 72 h. Reported values are the mean of six replicates ± s.e., normalized on cell proliferation of untreated cells, ** P<0.0005 (t-test).



Viability of cells treated with HFn(DOX) with or without MDR proteins inhibition. Cells were treated with 1 μM of HFn(DOX) for up to 72 h with or without 1 μM Cyclosporin-A. Reported values are the mean of six replicates ± s.e., normalized on cell proliferation of untreated cells, respectively * P<0.05; ** P<0.01 (t-test).

HFn Nanocage increases DOX efficacy also in DOX-resistant cells

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UNIVERSITY HOSPITAL

Conclusions

- ✓ HFn nanocage is a good device for drug delivery in tumors, recognizing with high sensitivity cancer cells that overexpress TfR1
- ✓ No lysosomal degradation of HFn shell
- ✓ HFn(DOX) increase DOX efficacy in mediating block of proliferation and induction of cell death specifically to tumor cells
- ✓ HFn(DOX) increases DOX uptake and nuclear delivery of drug
- ✓ HFn(DOX) acts as a Trojan horse: the cytoplasmic release of DOX from hydrophobic channels triggers HFn(DOX) nuclear translocation
- ✓ HFn(DOX) is efficacy also on DOX-resistant cells

Acknowledgements

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