DIVERSA

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DIVERSA PRODUCT CATALOG

Cat No.	Product	Specification
DIV000F1	DIVTECH FluoGreen Kit	Tracking fluorescent DIVTECH for a positive **cell uptake control**
DIV010	DIVTECH Small Molecule Kit	Enhancing intracellular delivery of **small molecules**
DIV010F1	DIVTECH FluoGreen Small Molecule Kit	Tracking intracellular delivery of **small molecules**
DIV021	DIVTECH Broad Range Peptide Kit	Enhancing intracellular delivery of a broad range of **peptides**
DIV021F1	DIVTECH FluoGreen Broad Range Peptide Kit	Tracking intracellular delivery of a broad range of **peptides**
DIV031	DIVTECH Broad Range Protein Kit	Enhancing intracellular delivery of a broad range of **proteins**
DIV031F1	DIVTECH FluoGreen Broad Range Protein Kit	Tracking intracellular delivery of a broad range of **proteins**
DIV042	DIVTECH Anionic Protein/Peptide Kit	Enhancing intracellular delivery of anionic **proteins/peptides**
DIV042F1	DIVTECH FluoGreen Anionic Protein/Peptide Kit	Tracking intracellular delivery of anionic **proteins/peptides**

DIVTECH LIPID NANOEMULSIONS & MACROPHAGES

PHYSICAL CHARACTERIZATION

DIVTECH are ready-to-use formulations to obtain encapsulating nanosystems in a fast and easy way. **DIVTECH** show an easily controllable size, narrow distribution, high reproducibility, neutral surface charge, and a spherical shape (Fig. 1).

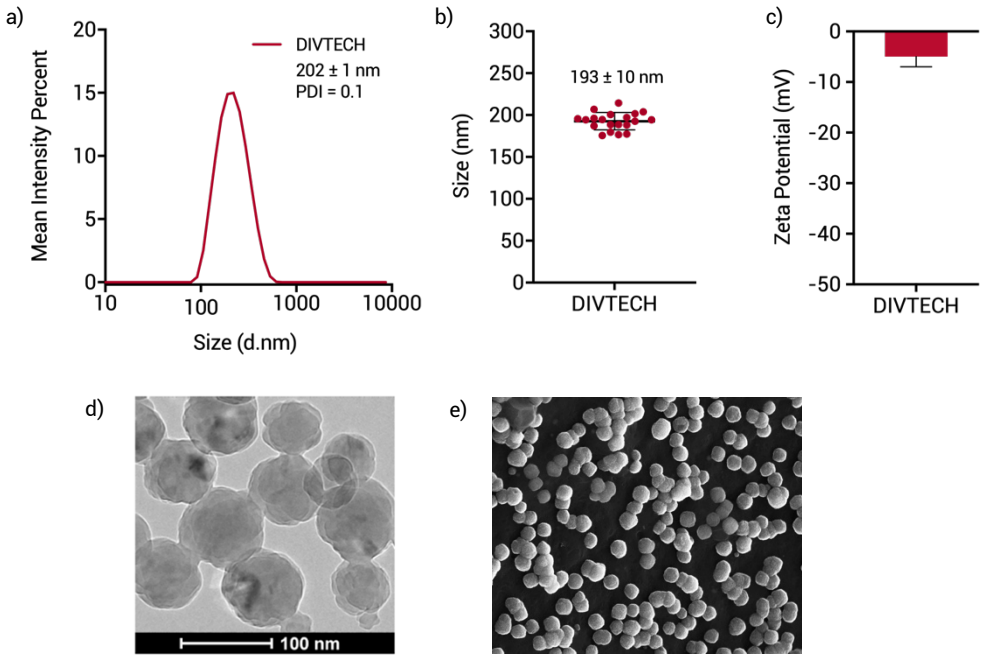


Figure 1. Characterization of **DIVTECH** by the diameter size using Dynamic Light Scattering (DLS) (a), reproducibility of different batches (b) and Zeta Potential (c). Morphology observed by Cryogenic Transmission Electron Microscopy, Cryo-TEM (d) and Scanning Transmission Electron Microscopy (STEM) of **DIVTECH** (e).

STABILITY

In addition, **DIVTECH** has great stability in different relevant biological fluids (Table 1), during storage up to 6 months (Fig. 2a), in different buffers, and in culture media supplemented with FBS (Fig. 2b). Importantly, **DIVTECH** with the associated biomolecules (peptides and proteins) and small molecules (Table 2), maintains its stability (Fig. 3).

Table 1. Stability of the blank **DIVTECH** in different relevant fluids.

	Human plasma	Simulated tear fluid	Simulated synovial fluid	Simulated gastric fluid	Simulated intestinal fluid (SIF)	Fed state SIF	Fasted state SIF
DIVTECH	24 h*	24 h*	24 h*	6 h	4 h	4 h	24 h*

* End of the experiment, stable particles.

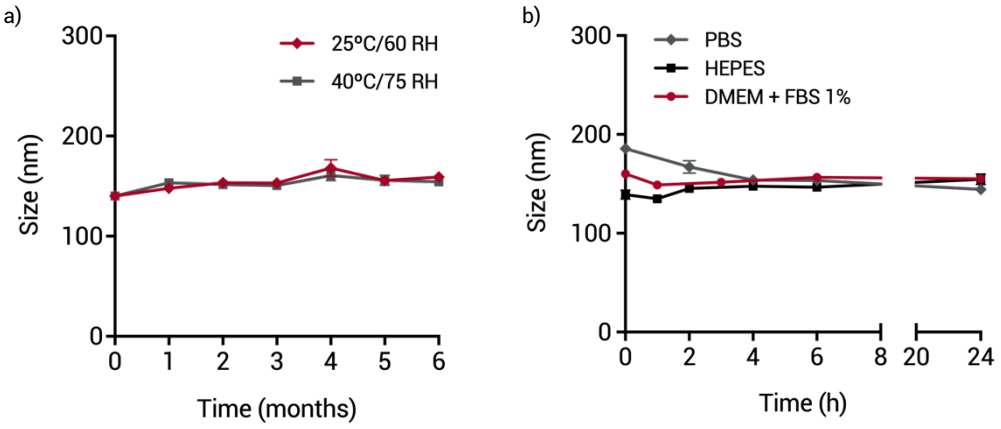


Figure 2. ICH (International Council for Harmonization) long-term stability of the blank **DIVTECH** at room temperature (40 ºC, 75% RH) and under storage conditions (25 ºC, 60% RH) (a). Stability of the blank **DIVTECH** in different buffers and cell culture medium supplemented with FBS (b).

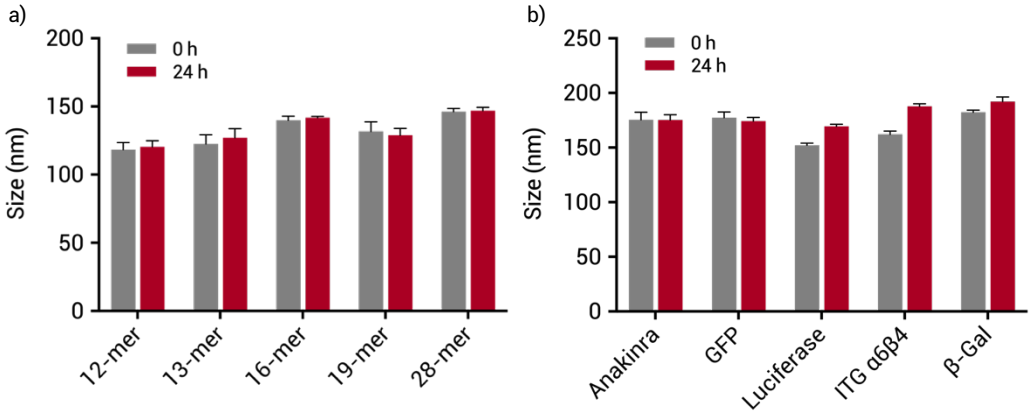


Figure 3. Stability of **DIVTECH** associated to peptides of different length (from 12 to 28 amino acids) (a) and associated to proteins of different MW, isoelectric point, and log P_{ow} (b) for 24 hours in storage conditions. Data represent the hydrodynamic diameter in nanometers (mean ± SD).

Table 2. Stability of **DIVTECH** associated to small molecules.

DIVTECH: Small molecule	Stability
Galunisertib	7 days*
Etoposide	15 days*
Paclitaxel	15 days*
Doramapimod	7 days*
Doxorubicin	15 days*

* End of the experiment, stable particles.

TOXICITY

DIVTECH showed a great biocompatibility with different cell types as well as *in vivo* models such as mice and zebrafish (Fig. 4).

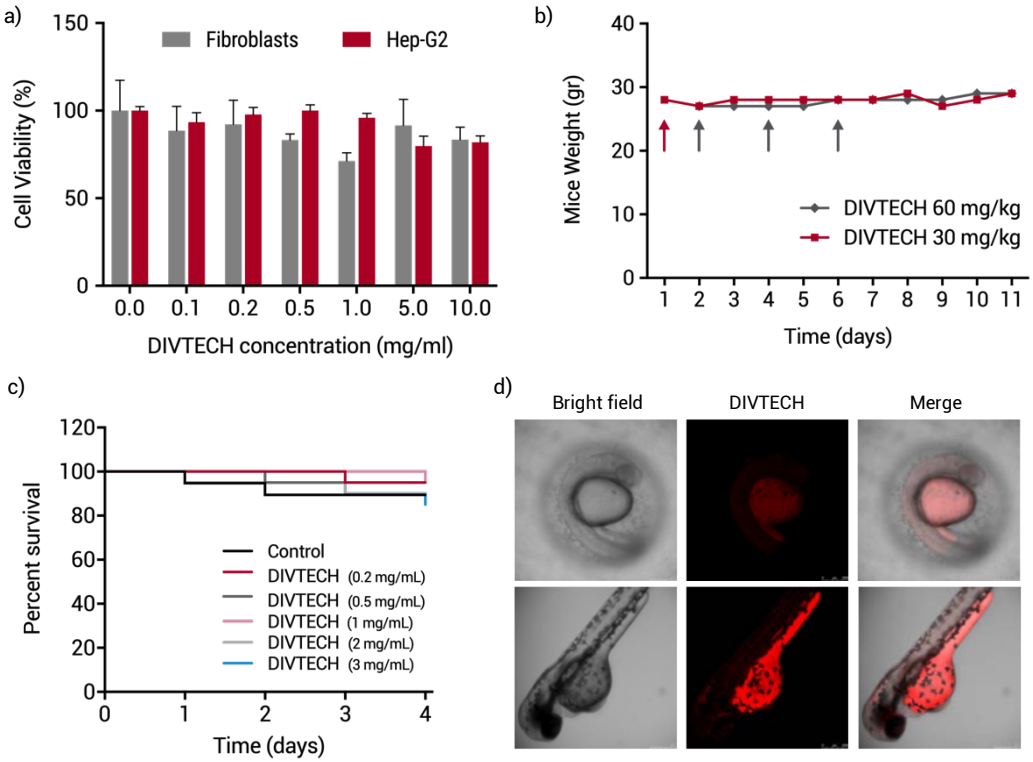


Figure 4. Evaluation of cytotoxicity effects of the blank **DIVTECH** after 48h incubation in hepatic cancer cell line HepG2 and fibroblasts. Data shown represent the mean values and S.E.M obtained in a triplicate (a). *In vivo* toxicity evaluation of the blank **DIVTECH** based on the body weight of healthy mice (n=6 mice each group). Red arrow: one dose tail intravenous injection of 30 mg/kg (10 mg/mL nanosystem concentration). Grey arrows: Three consecutive dose tail intravenous injection of 60 mg/kg (20 mg/mL nanosystem concentration) at days 2, 4 and 6 (cumulative dose 180mg/kg). No toxicity was observed in any of the cases (b). *In vivo* toxicity evaluation of the blank **DIVTECH** at different concentrations (from 0.2 to 3 mg/mL) in zebrafish embryos with chorionic membrane (n = 60 embryos in total) (c). Internalization of Nile-red labelled **DIVTECH** in zebrafish embryo model with and without the chorionic membrane (0.5 mg/ml) (d).

ASSOCIATION OF THERAPEUTIC MOLECULES

DIVTECH can associate a wide variety of therapeutic compounds as hydrophobic or amphiphilic molecules, peptides, proteins, and oligonucleotides (such as DNA and RNA, among others) showing great association efficiencies (table 3) and maintaining the nanometric size (Fig. 5). The specifications of some of the molecules that were efficiently associated with **DIVTECH** are detailed in tables 4 – 8.

Table 3. Association of different therapeutic molecules to **DIVTECH**.

Molecules	Association Efficiency (%AE)
Small molecules	85-99 %
Peptides	70-99 %
Proteins	70-99 %
Oligonucleotides	85-99 %

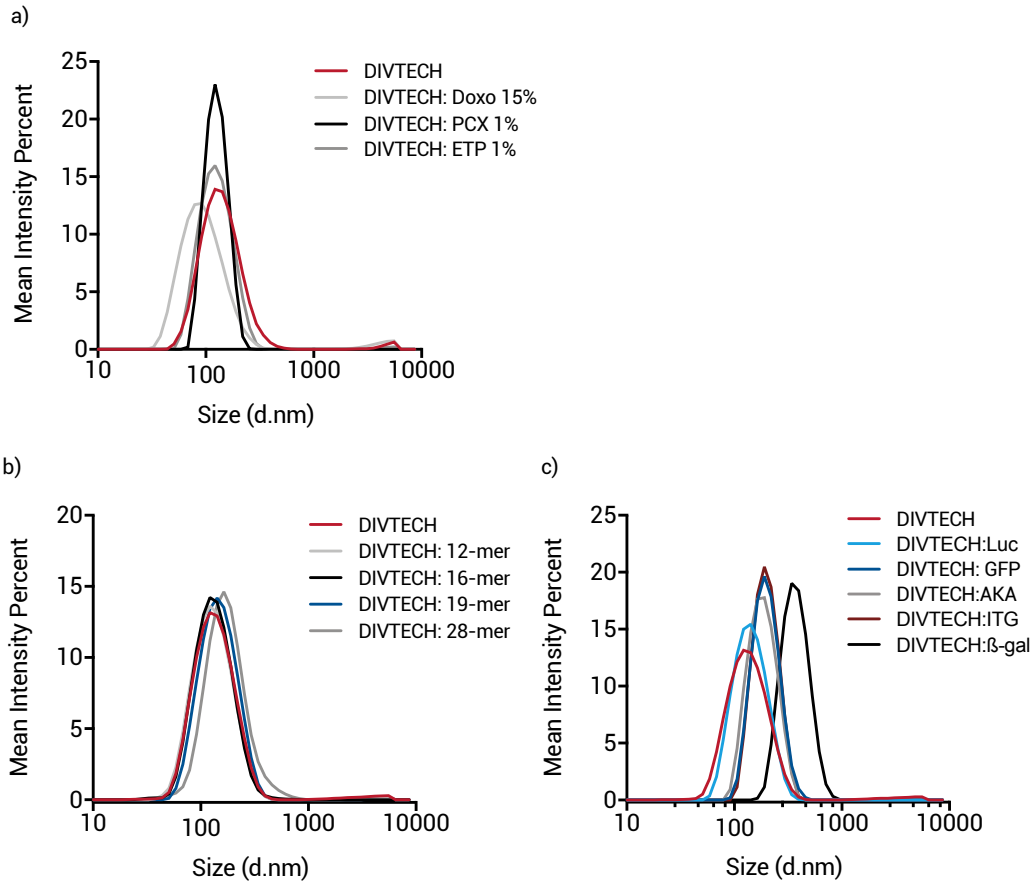


Figure 5. Characterization of **DIVTECH** by Dynamic Light Scattering (DLS) showing the hydrodynamic diameter of **DIVTECH** associated to small molecules (a), peptides of different length (from 12 to 28 amino acids) (b) and associated to proteins of different MW, isoelectric point, and log P_{ow} (c).

Table 4. Association of different small molecules to **DIVTECH**.

Drug	MW (g/mol)	Log P_{ow}	Mass (mg)	% Loading (w/w)	Molar (mM)	% EE
Galunisertib	369.42	2.4	0.75	15%	2.03	>90%
Doramapimod	527.66	5.7	0.75	15%	1.42	>99%
Disulfiram	296.54	3.9	0.75	15%	2.53	>90%
Paclitaxel	853.91	2.5	0.05	1%	0.06	>80%
Etoposide	588.56	0.6	0.05	1%	0.09	>80%
Doxorubicin	543.52	1.3	1	20%	1.84	>90%
Oleuropein	540.51	-0.4	0.75	15%	1.39	>99%
Rose Bengal	973.67	8.5	1	20%	1.03	>95%

Table 5. Comparative characterization of different commercially available competitors associating small molecules.

Drug (Loading)	Competitors	Size (nm)	PdI	Encapsulation Efficiency (%EE)
Galunisertib (15%)	Liposomes 1	111	0.26	< 40%
	Liposomes 2	1325	0.64	Precipitated
	Niosomes	940	0.56	Precipitated
	DIVTECH	**129**	**0.08**	**> 95%**
Doramapimob (15%)	Liposomes 1	110	0.22	Precipitated
	Liposomes 2	377	0.95	Precipitated
	Niosomes	318	0.91	Precipitated
	DIVTECH	**150**	**0.06**	**> 99%**
Oleuropein (15%)	Liposomes 1	110	0.22	< 46%
	Liposomes 2	377	0.95	Precipitated
	Niosomes	318	0.91	Precipitated
	DIVTECH	**150**	**0.06**	**> 99%**

Table 6. Association of different peptides to **DIVTECH**.

Peptide length	pI	Peptide charge	% Hydrophobic Aa
3-mer	5.9	-1	0 %
12-mer	9.5	+4	25 %
13-mer	9.8	+5	23 %
16-mer	9.5	+7	50 %
19-mer	8.6	+2	21 %
28-mer	11.9	+6	39 %

Table 7. Association of different proteins to **DIVTECH**.

Protein	MW (kDa)	pI	DIV031 (BR)	DIV042 (Anionic)
Anakinra	17.3	5.5	√	√
Green Fluorescent Protein	28.7	~6.0	√	
Luciferase (LucR8)	37.0	8.3	√	
Ovalbumin	44.5	4.6	√	√
Integrin α6β4	188.8	5.5	√	√
R-Phycoerythrin	250.0	5.6	√	√
β-Galactosidase	540.0	4.6	√	√

Table 8. Association of different oligonucleotides to **DIVTECH**.

Oligonucleotide	Length size	Association Efficiency (%AE)
siRNA	19–21 bp	> 95 %
miRNA	19–21 bp	> 95 %
Aptamer	75–76 bp	> 95 %
mRNA	1–2 Kb	> 95 %
pDNA	8–9.5 Kb	> 95 %
cDNA	4.5–12 Kb	> 95 %

INTERNALIZATION

DIVTECH can be internalized reaching great efficiencies in short times, and by different cell types (Fig. 6) including stablished cell lines, primary cell culture cells and macrophages (Fig. 9). **DIVTECH** can efficiently achieve the intracellular delivery of therapeutic molecules, such as peptides, proteins, or oligonucleotides (Fig. 7, 8) not only *in vitro*, but also *in vivo* (Fig. 10). **DIVTECH** can be employed in different applications as: drug delivery and targeting, delivery of peptides, drug and proteins, cosmetics, functional foods, nutraceuticals, etc.

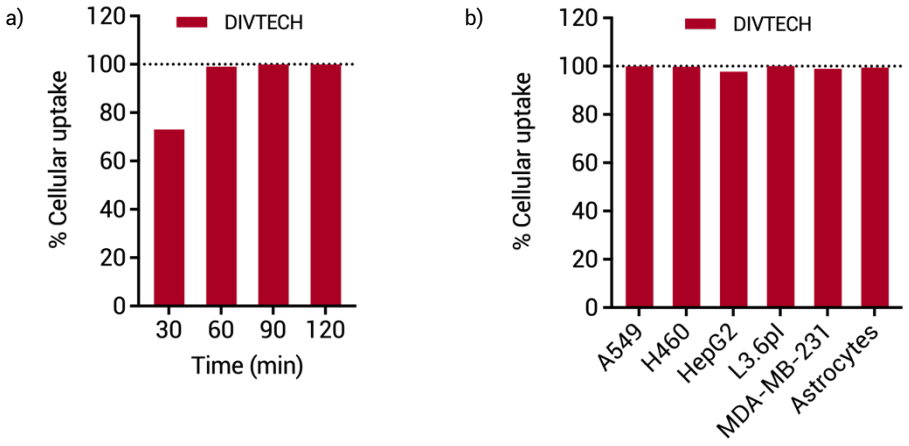


Figure 6. Time-depending uptake of fluorescent labeled **DIVTECH** by flow cytometry (a). Internalization of **DIVTECH** in different stablished cell lines and primary culture cells (b).

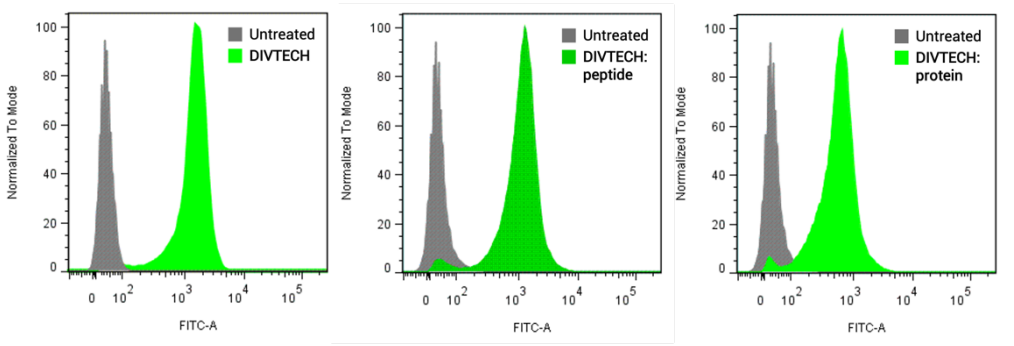


Figure 7. Uptake of fluorescent labeled **DIVTECH** by flow cytometry. Nearly 100% of cells become green after 2-hours incubation with **DIVTECH**. **DIVTECH** is efficiently internalized by cells with or without association of a peptide/protein.

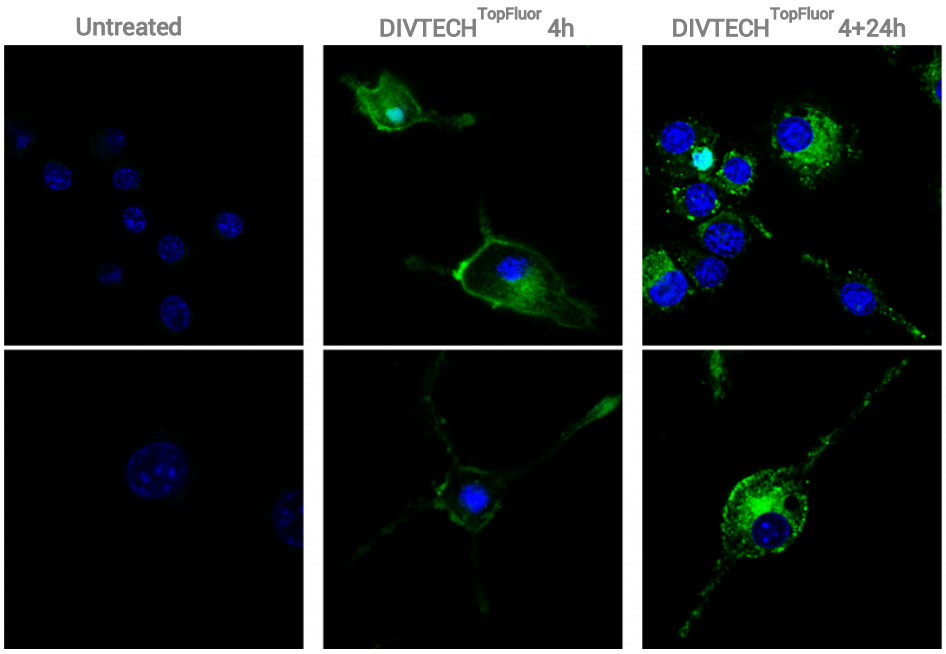


Figure 8. DIVTECH-TopFluor can be efficiently uptaken by macrophages. Green signal from DIVTECH lasts for at least 24h.

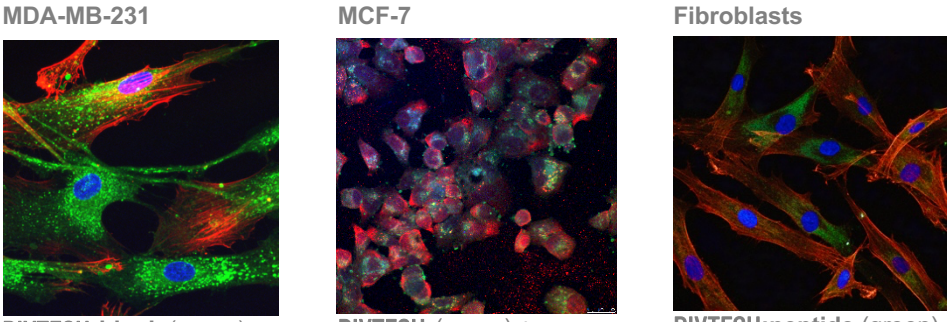


Figure 9. Internalization of blank, miRNA and peptide loaded **DIVTECH** in different cell types.

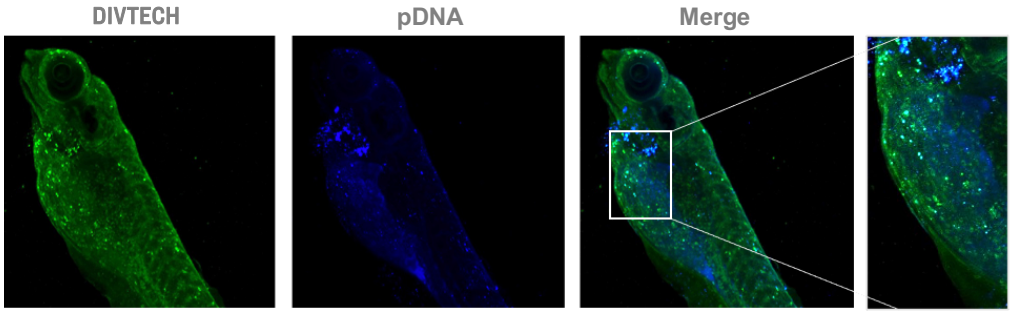


Figure 10. Internalization of pDNA loaded **DIVTECH** in zebrafish embryos after 72 hour-incubation in fish medium. DIVTECH: green channel (topFluor). pDNA: blue channel (Cy5).

THERAPEUTIC DELIVERY OF BIOMOLECULES

DIVTECH can efficiently achieve the intracellular delivery of therapeutic molecules such as drugs, proteins and peptides and obtained the desired therapeutic effect. We have evaluated *in vitro* the therapeutic efficiency of a free peptide that can barely enter in the cells, and the peptide associated to two competitors well-positioned in the market and compared to **DIVTECH** (Fig. 11, 12). **DIVTECH** showed a significantly higher therapeutic effect than competitors.

We have also proven that **DIVTECH** can remarkably enhance the internalization of active proteins inside the cells. When compared to competitors, a higher effect (blue signal), and no toxicity of **DIVTECH** is observed. However, the competitors showed high toxicity due to the presence of cationic compounds that can be clearly observed in the pictures as cell debris and a different cell morphology (Fig. 13).

DIVTECH also shown the efficiently delivery of small drugs to two different types of macrophages (murine primary cells and IBMDM cells). The delivery resulted in the downregulation of a biomarker related to the M2 phenotype (Fig. 14).

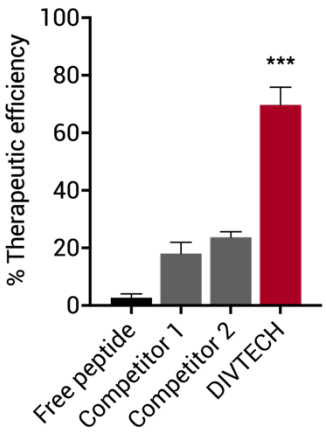
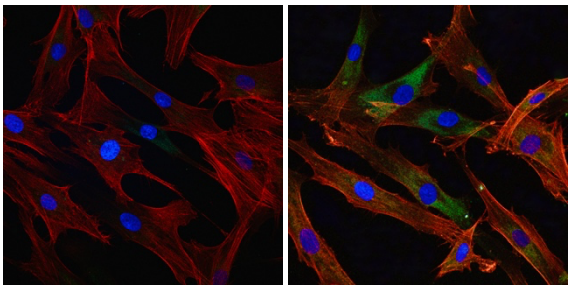


Figure 11. Therapeutic efficiency of **DIVTECH** loaded with a peptide and compared to competitors and the free peptide.

Figure 12. Intracellular delivery of FITC-labeled peptides to fibroblast as free peptides or using **DIVTECH** delivery system (blue: cell nuclei, red: cytoskeleton, green: PEPTIDE).



FREE PEPTIDE **DIVTECH**: PEPTIDE

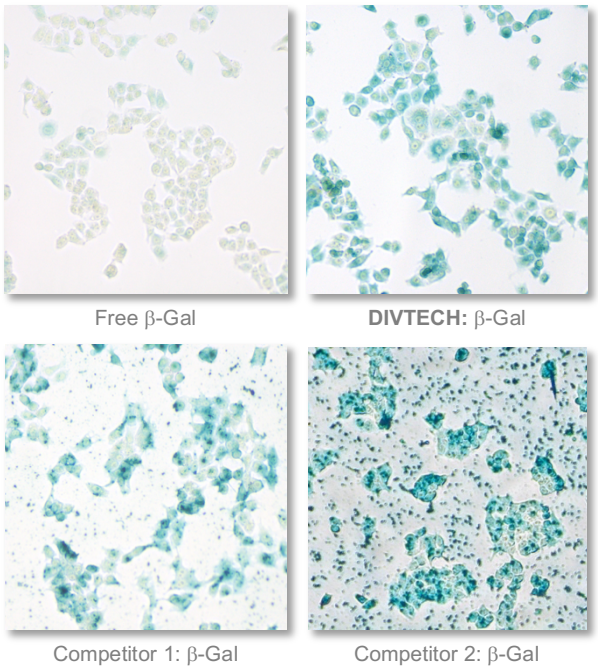


Figure 13. Therapeutic delivery of **DIVTECH** loaded with an active protein (β-Gal enzyme, 540 KDa) and compared to competitors and the free protein in human fibroblasts.

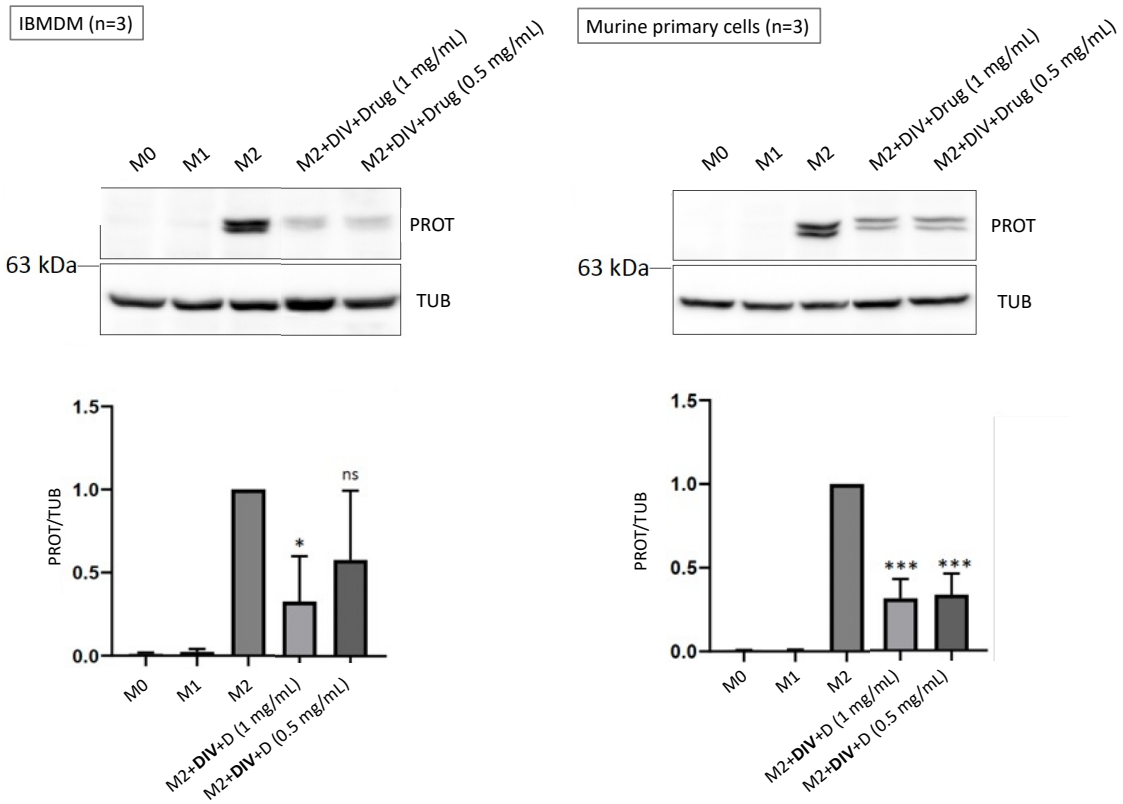


Figure 14. Delivery of the drug (coded) at two different doses (0.5 mg/mL and 1 mg/mL) loaded into **DIVTECH** formulations (DIV). The purpose was to decrease the expression of biomarkers related to a M2 phenotype (prot = coded). Results show that **DIVTECH** efficiently delivers the drug at two different doses in two different types of macrophages.

DIVTECH can efficiently deliver gene therapies (pDNA) to cancer cells, achieving a therapeutic effect, as determined *in vivo* assays, using xenografted zebrafish embryos (Fig. 15).

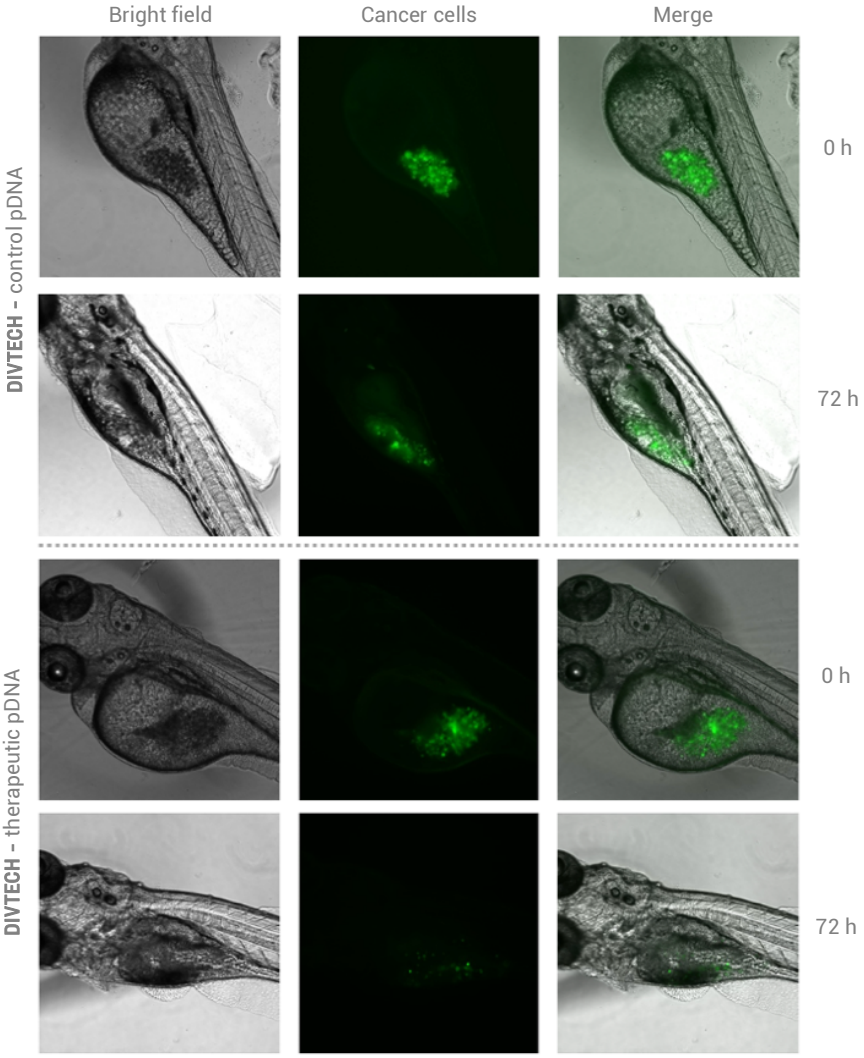


Figure 15. GFP transduced MDA-MB-231 cells were injected into the yolk sac of zebrafish embryos and after 24 hours, DIVTECH loaded with a pDNA-control and with a therapeutic pDNA were administered. A significant decrease in the viability of cancer cells was observed after the delivery of the therapeutic pDNA by DIVTECH in 72 hours.

BIODISTRIBUTION

DIVTECH can be radiolabeled with different radioisotopes for efficiently evaluating the biodistribution of the nanosystems, such as ^{89}Zr (Fig. 16), ^{68}Ga (Fig. 17) or ^{67}Ga (Fig. 18). **DIVTECH**, without functionalization, can easily accumulate in the liver, spleen, and lungs.

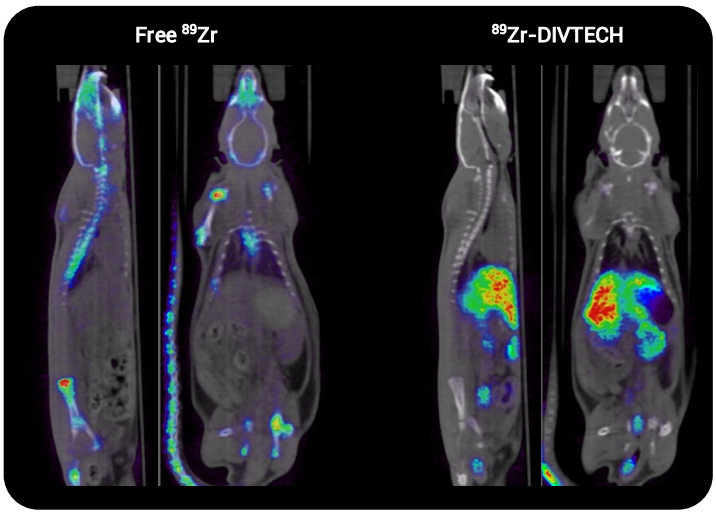


Figure 16. Free ^{89}Zr and ^{89}Zr-labeled **DIVTECH** were intravenously injected in healthy rats. The biodistribution of the ^{89}Zr was evaluated 22 hours post-injection by PET/CT imaging.

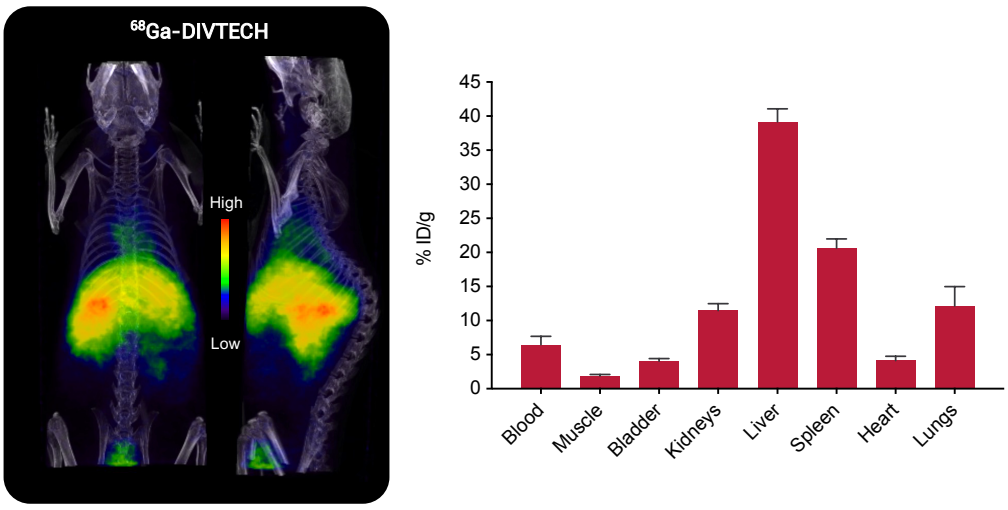


Figure 17. Representative PET/CT whole-body coronal images of ^{68}Ga-**DIVTECH** biodistribution in healthy C57BL/6 mice (n=5). The biodistribution of the ^{68}Ga was evaluated 2 hours post-intravenous injection (a). Ex vivo biostribution of radiolabeled **DIVTECH** 4 hours post-injection (b).

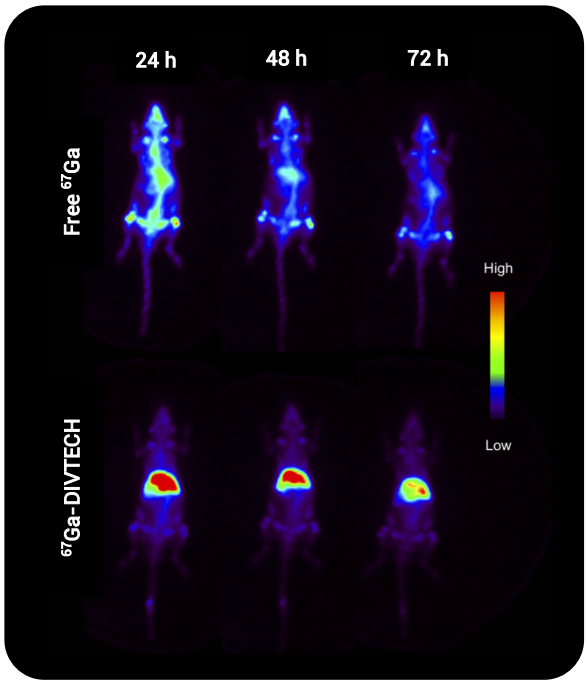


Figure 18. Whole-body SPECT images of ^{67}Ga-**DIVTECH** biodistribution in healthy Sprague-Dawley rats (n=5) compared to free ^{67}Ga during 72 hours after intravenous injection.

DIVERSA aims to provide delivery solutions through the design of safe, easy-to-produce, and versatile vehicles, which can be adapted to different types of therapeutic molecules -both complex macromolecules and small drugs- to facilitate their release, especially when the target is found at the intracellular level and access is limited. DIVERSA, with its patented technology, is focused on promoting the transfer to the clinic of new therapeutic molecules with high potential and high added value.