From Waste to Worth

Repurposing Dairy by-products as Source of Innovative Nanosystems

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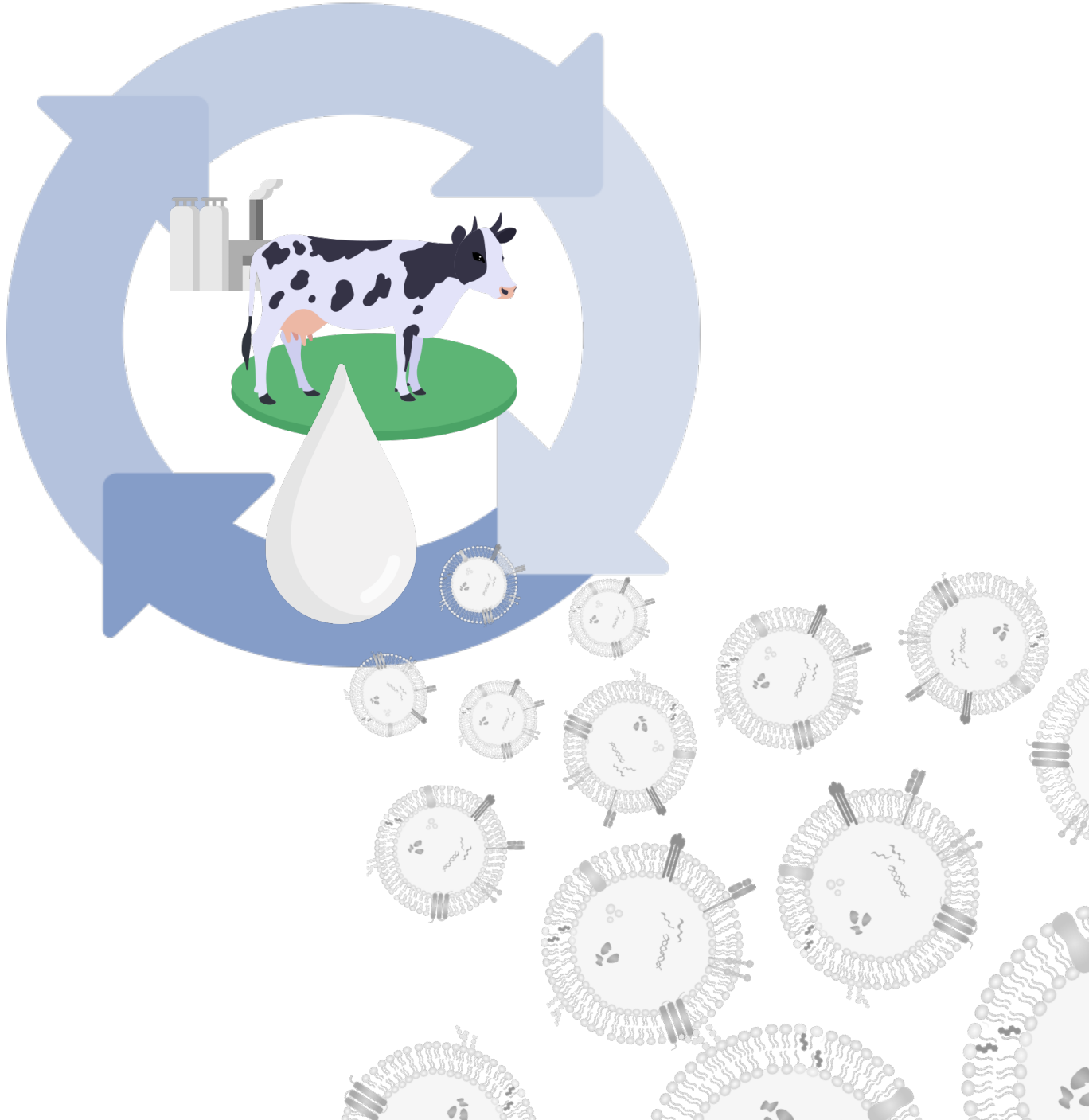
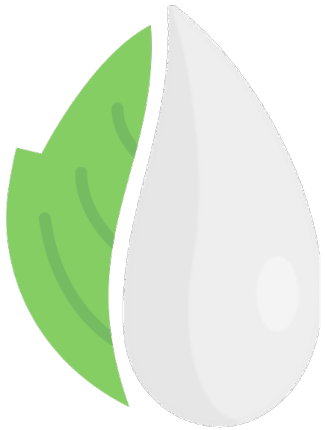


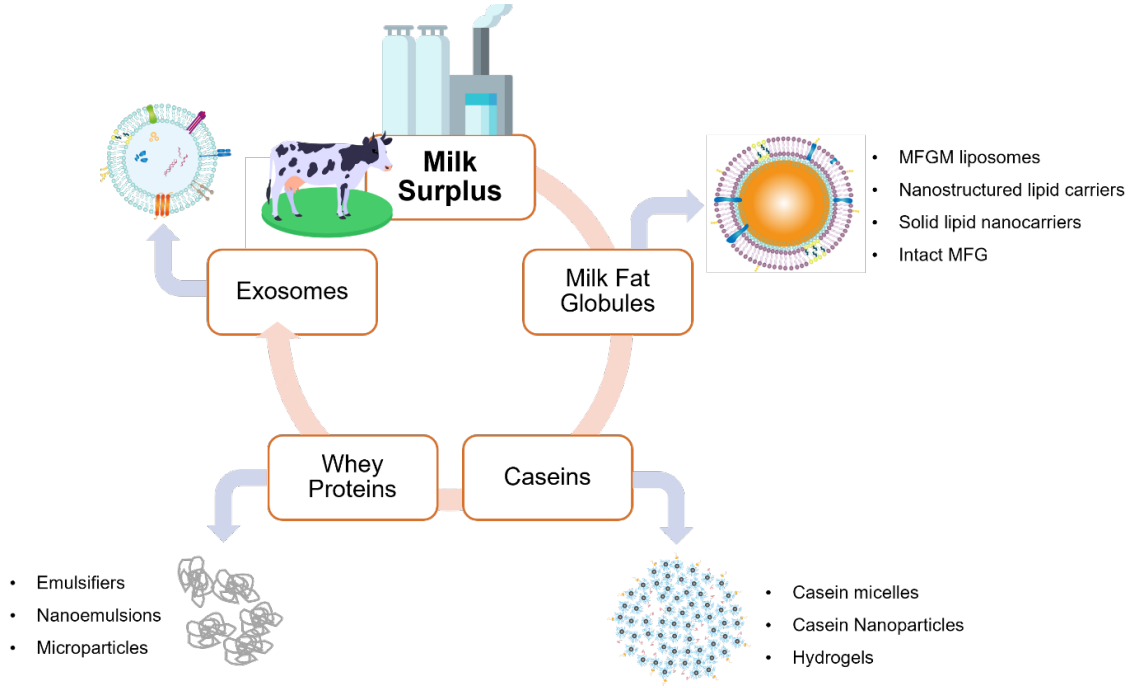
Motivation



Milk Production Negative Impact

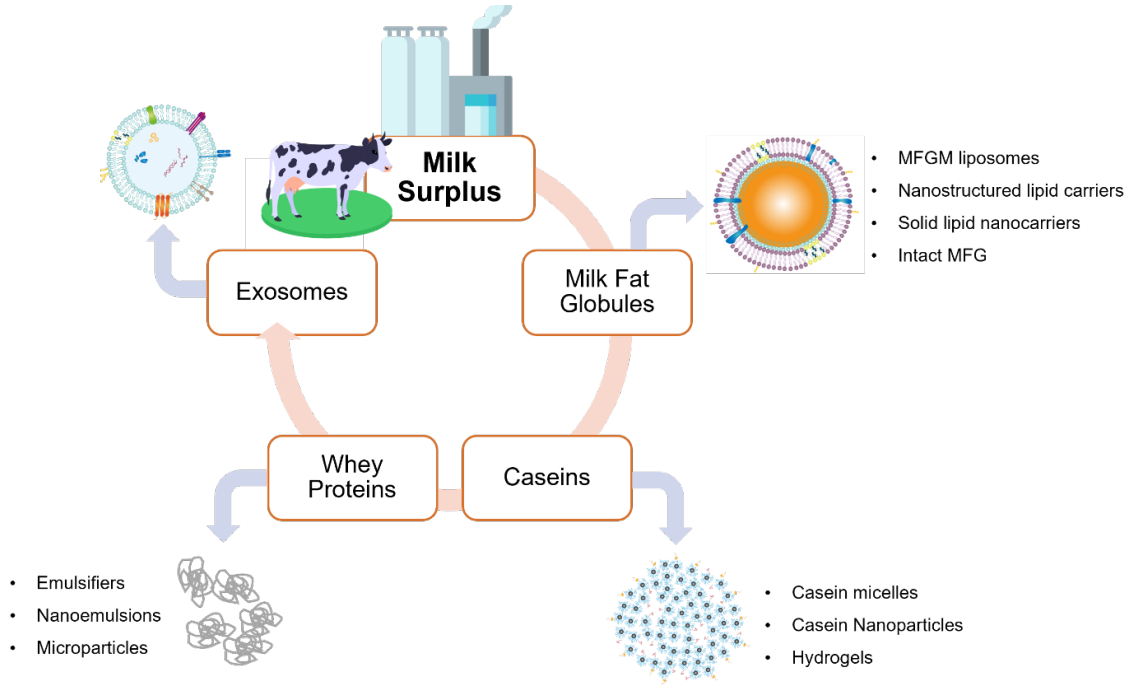
- Greenhouse gas load caused by methane
- Demands high amounts of water, energy and land
- **Contamination caused by the disposal of untreated milk surplus and by-products of dairy industry**

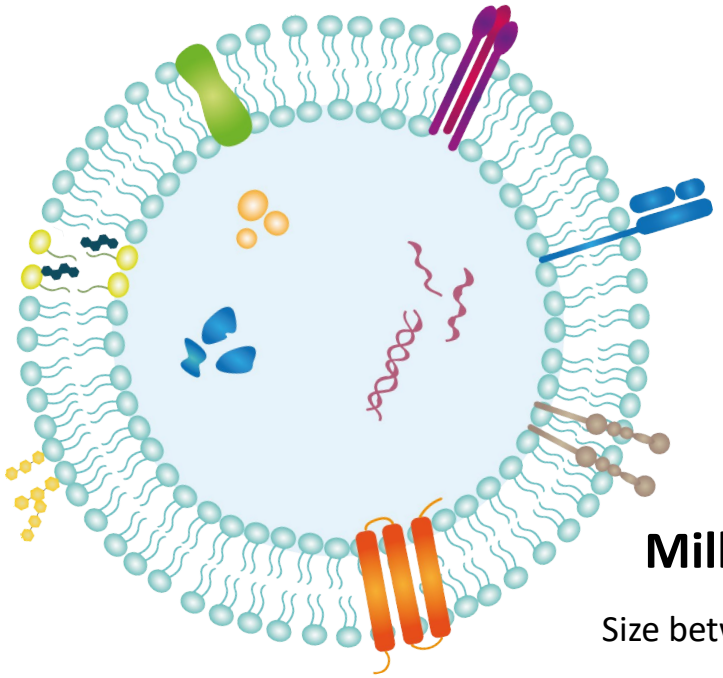
Repurposing milk constituents to mitigate their negative environmental impact



Soares, F. A., Salinas, B., Reis, S., & Nunes, C. (2023). Milking the milk: Exploiting the full potential of milk constituents for nature-derived delivery systems. *Trends in Food Science & Technology*, 104209.

Motivation





Milk-derived sEVs

Size between 30 and 150 nm

Similarity with cell plasma membranes

Obtained from whole milk and milk whey

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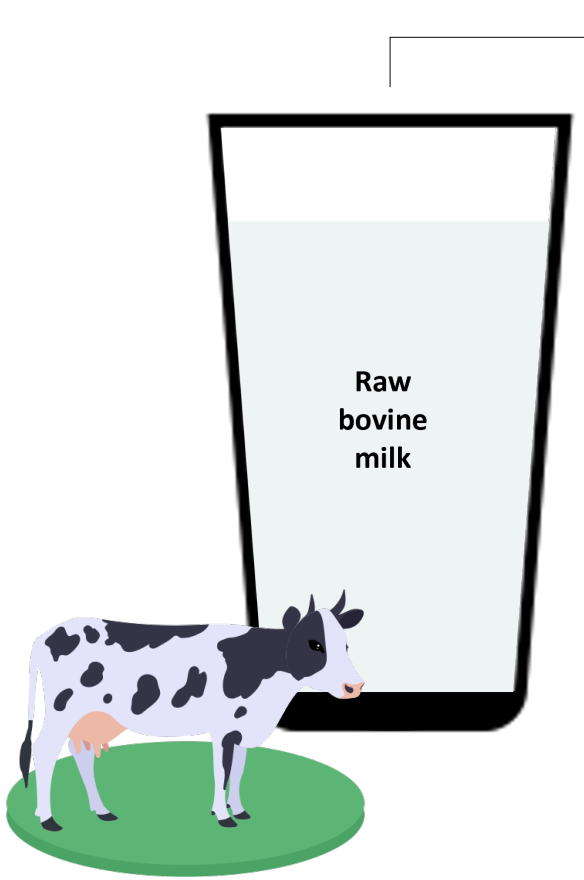
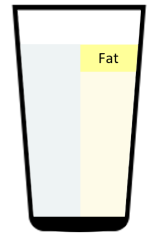
Isolation method



5,000G//10 min//4ºC → Cell debris and fat



Incubation with microbial rennet

5,000G//10 min//4ºC → Casein

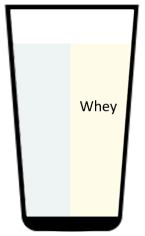
13,000G//35min//4ºC

35,000G//45 min//4ºC

70,000G//35 min//4ºC

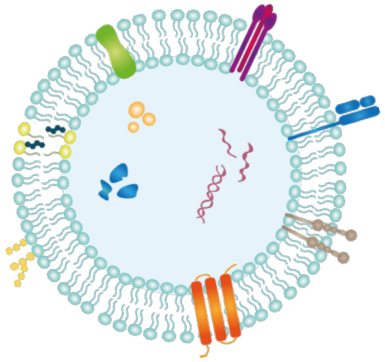
100,000G//1h35min//4ºC

→ Larger proteins and vesicles



qEV IZON column purification → Contaminant proteins

Milk-derived sEVs



sEVs Characterization

Table 1. Physical characterization (mean and mode sizes), yield and purity of the final sEVs isolate.

Mean size (nm)	Mode Size (nm)	Yield (µg protein/ml milk)	#sEVs/µg protein	#sEVs/ml milk
186±4	148±5	3.98±0.28	2.5E+09±1.1E+08	9.9E+09±2.7E+08

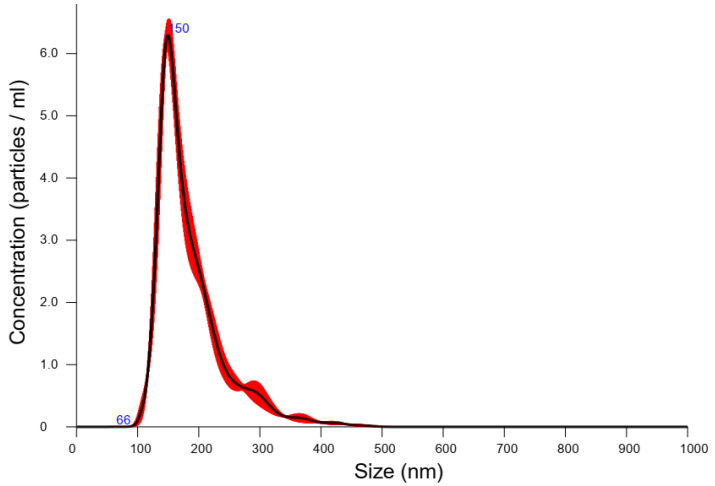


Fig 1. Size distribution of the sEVs suspension obtained by NTA

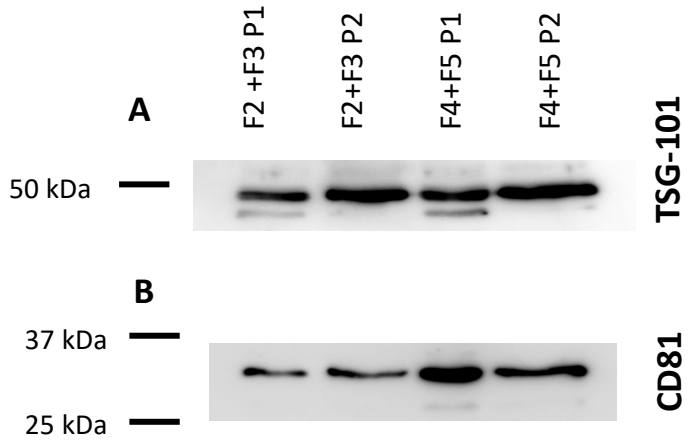


Fig 2. Western blot analysis of different fractions from the purification step using qEV IZON column. Detection of **(A)** TSG-101 and **(B)** CD81 exosomal markers.

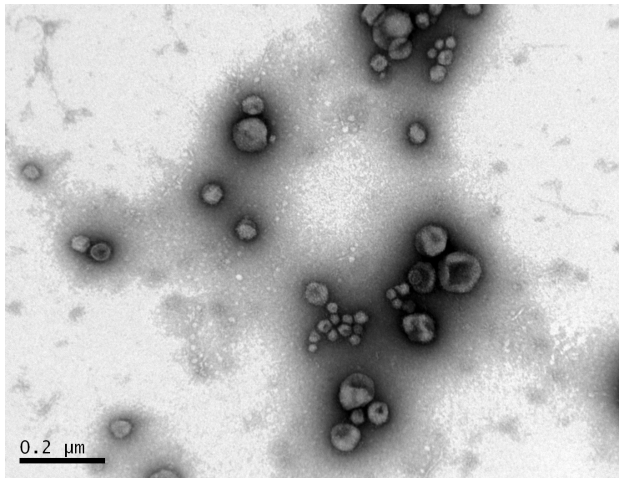
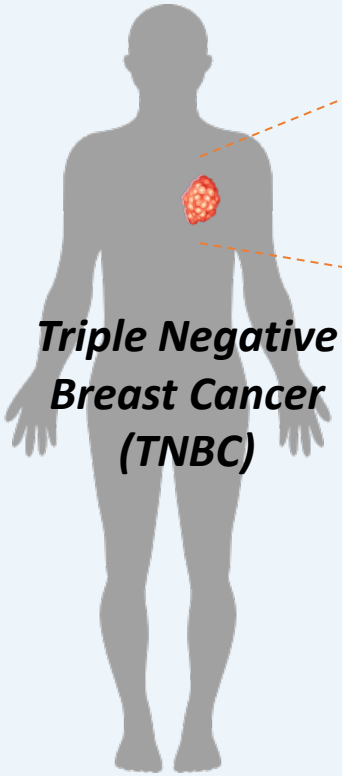
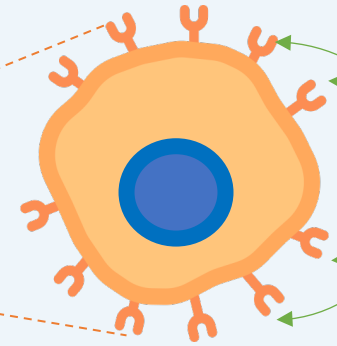


Fig 3. Transmission electron microscopy images of isolated sEVs

Nanomedicine application



Triple Negative Breast Cancer (TNBC)

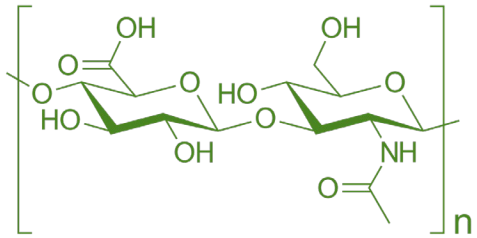


Lack of targeted therapies

High metastatic capacity

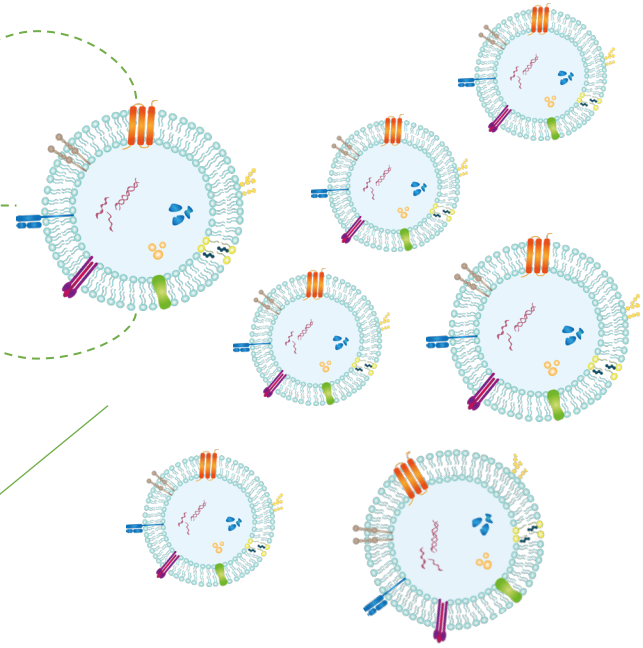
Poor prognosis

Highly aggressive BC subtype



Hyaluronic Acid (HA)

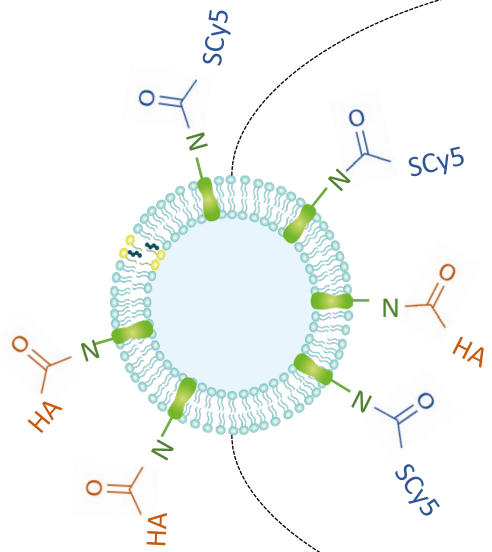
CD44 Targeted Active Delivery



Milk-derived sEVs

Strategy

HA MW:
Low (20-60 kDa)
Medium (250kDa)
High (1000-1600 kDa)



Low CD44 expression

MCF-7
(ER+/PR+ BC subtype)

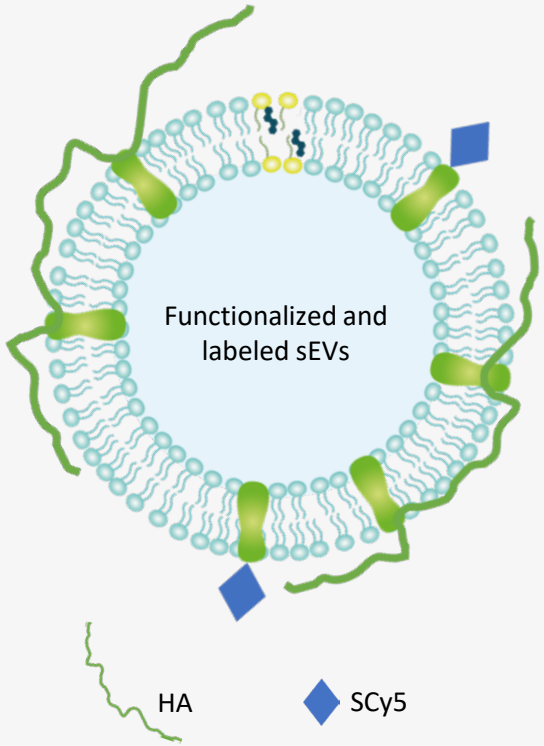
MDA-MB-231
(TNBC subtype)

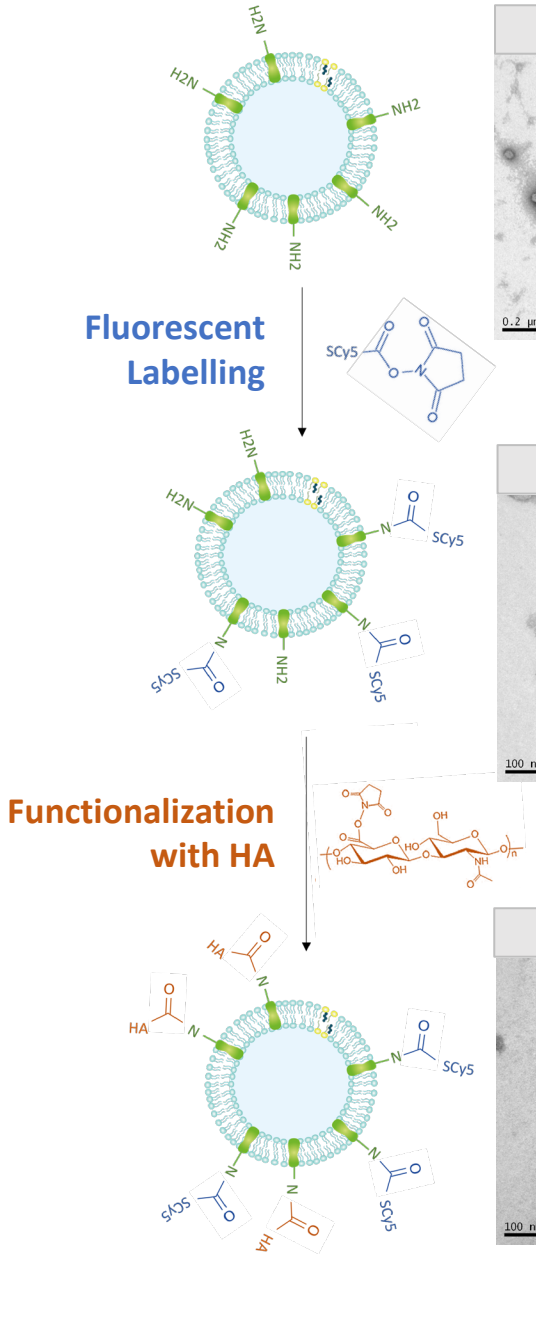
High CD44 expression

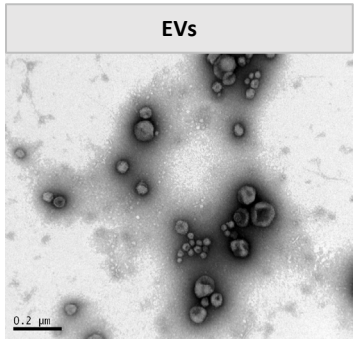
Binding and Internalization

CLSM
Flow cytometry

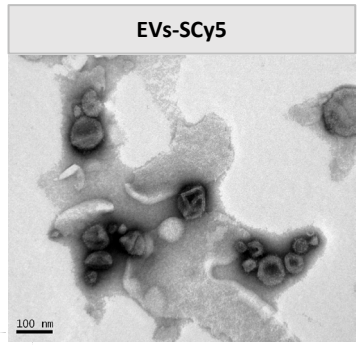
sEVs engineering



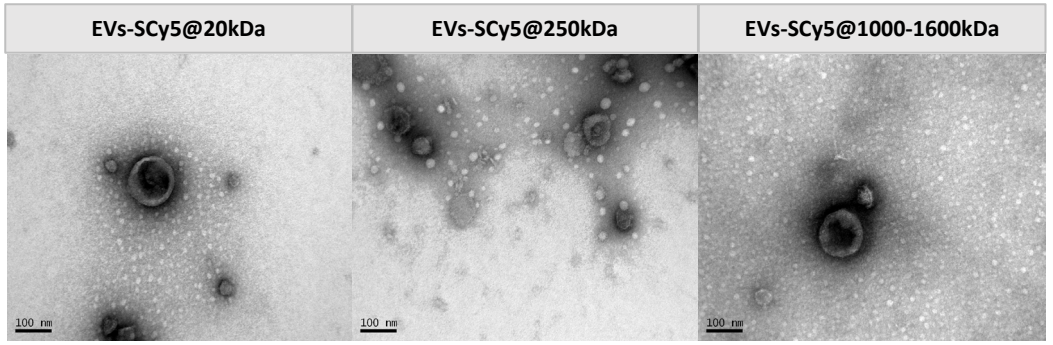




186±4 nm



173±3 nm

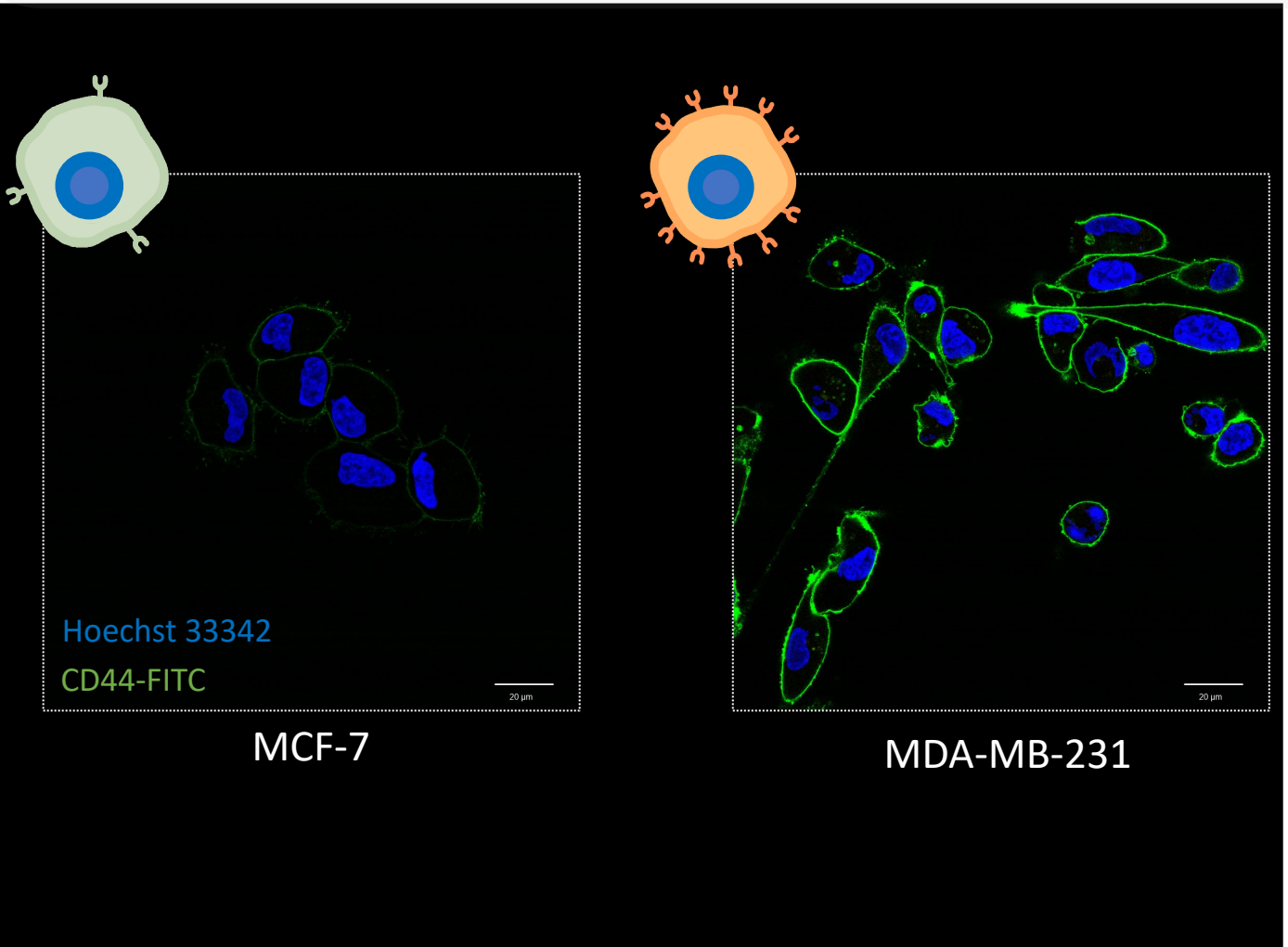


178±3 nm

183±2 nm

185±2 nm

CD44 expression



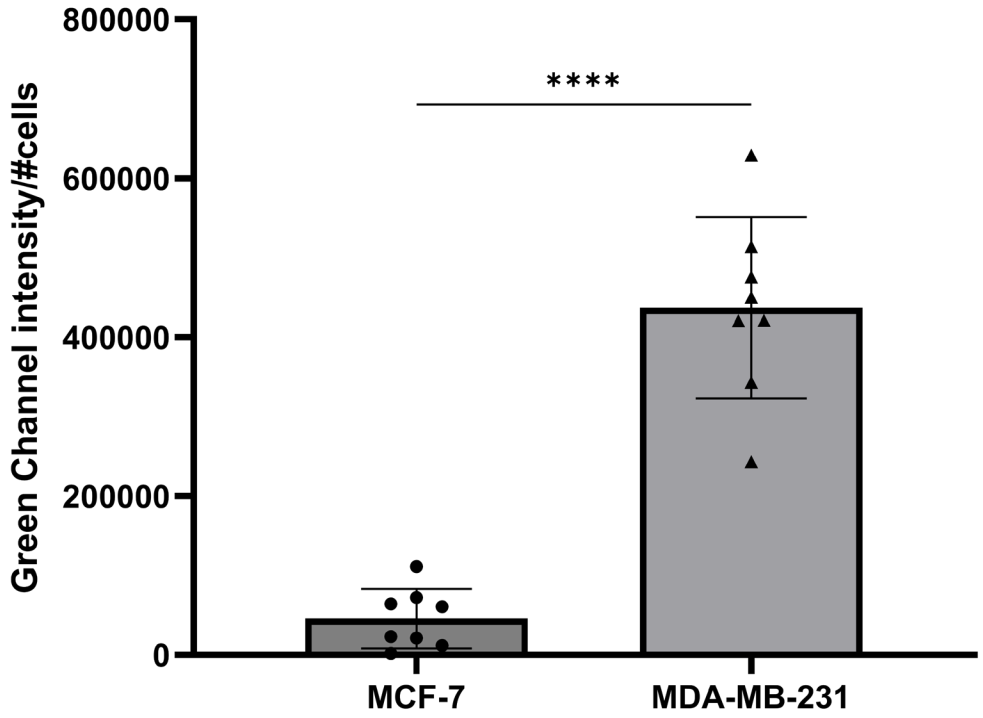
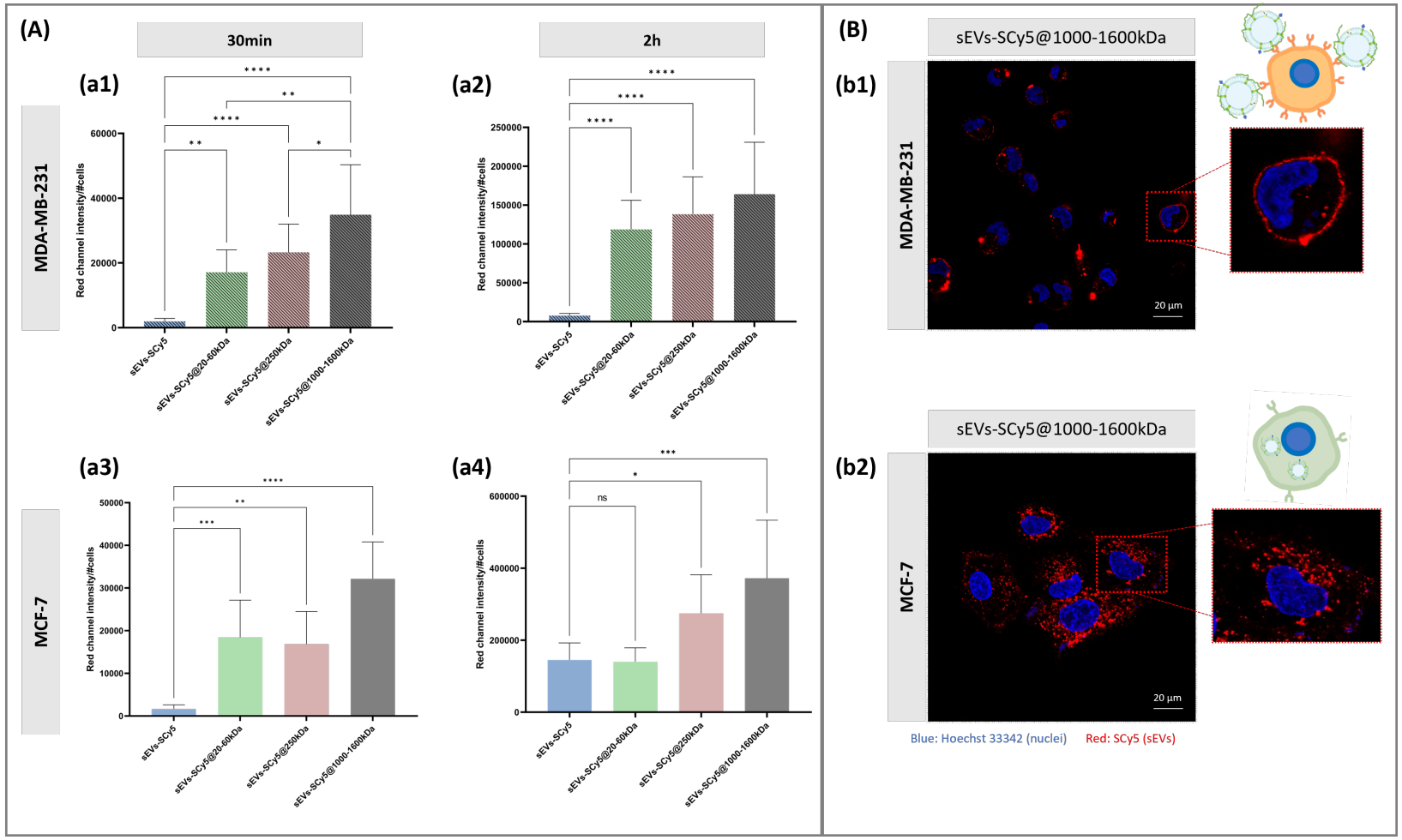


Fig 4. Immunofluorescence of CD44 protein. Quantification of the green channel intensity per number of cells.

Selectivity assessment

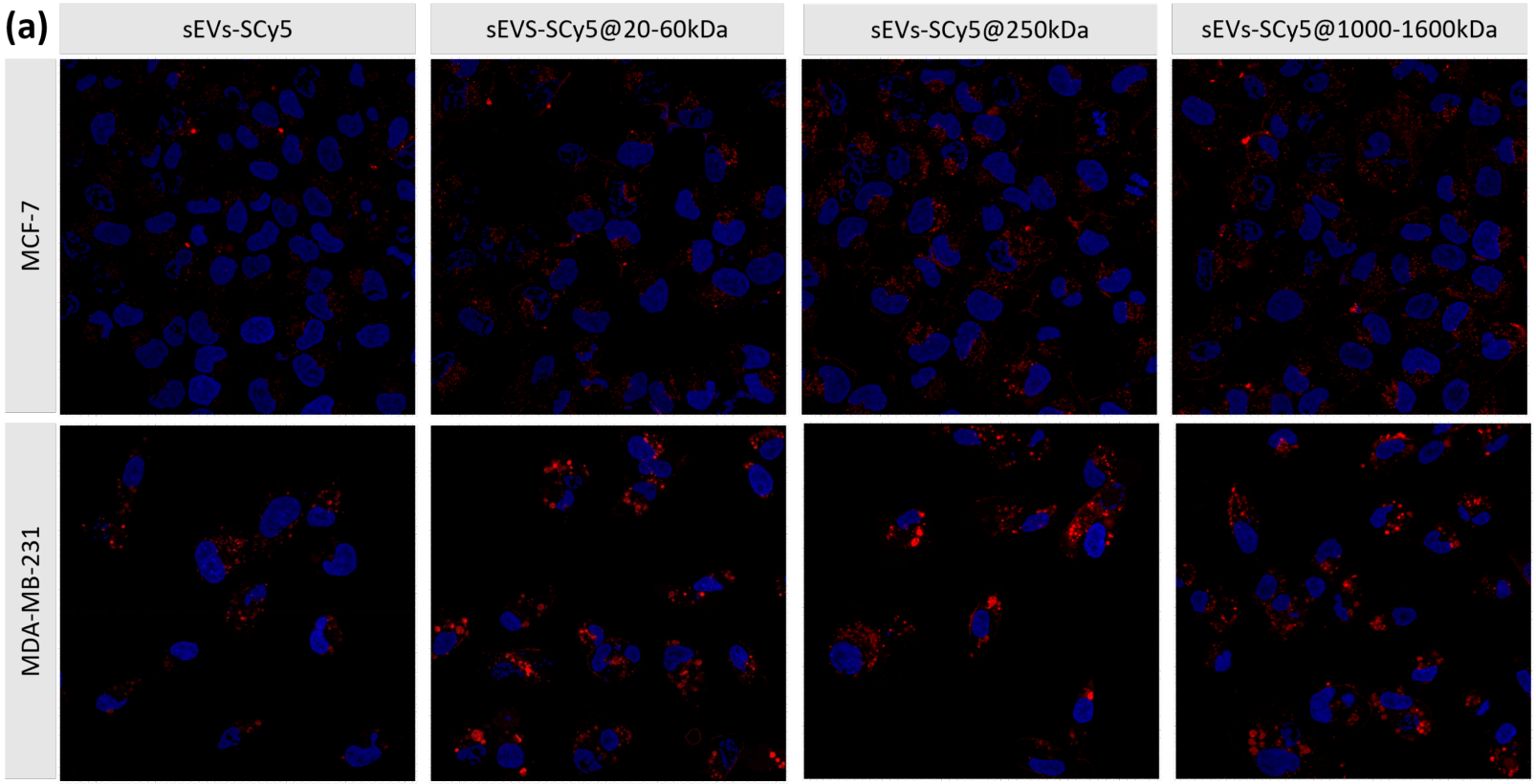


Non-functionalized sEVs exhibited significantly lower uptake

Increased uptake of sEVs for higher HA MW

Evidence of differing uptake mechanisms of functionalized sEVs

Selectivity assessment



Blue: Hoechst 33342 (nuclei) Red: SCy5 (sEVs)

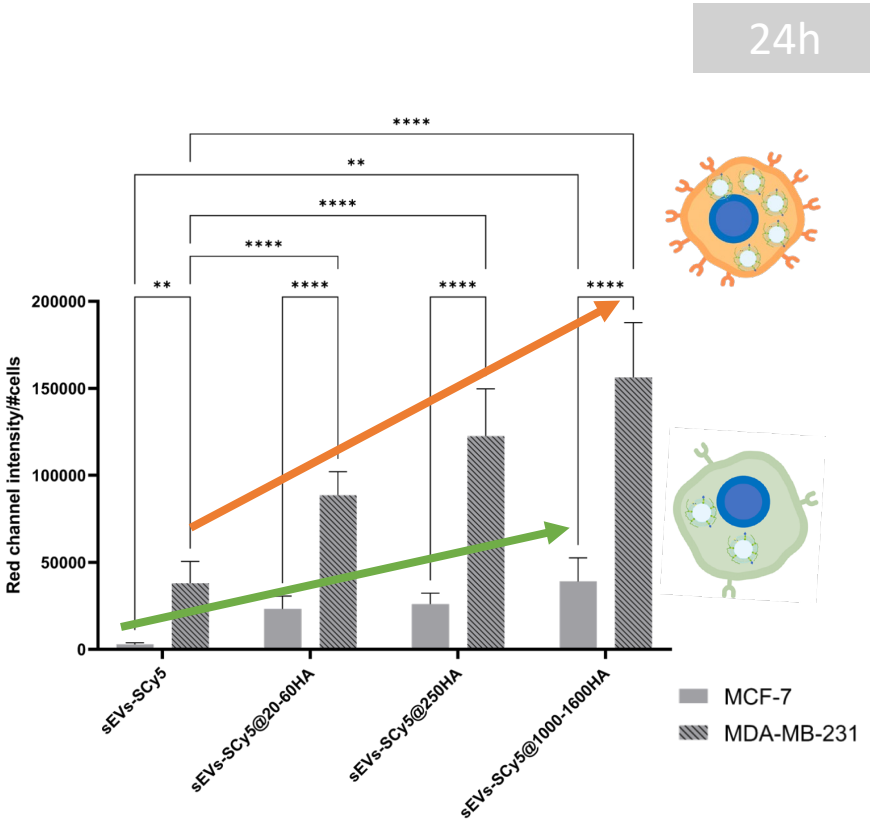
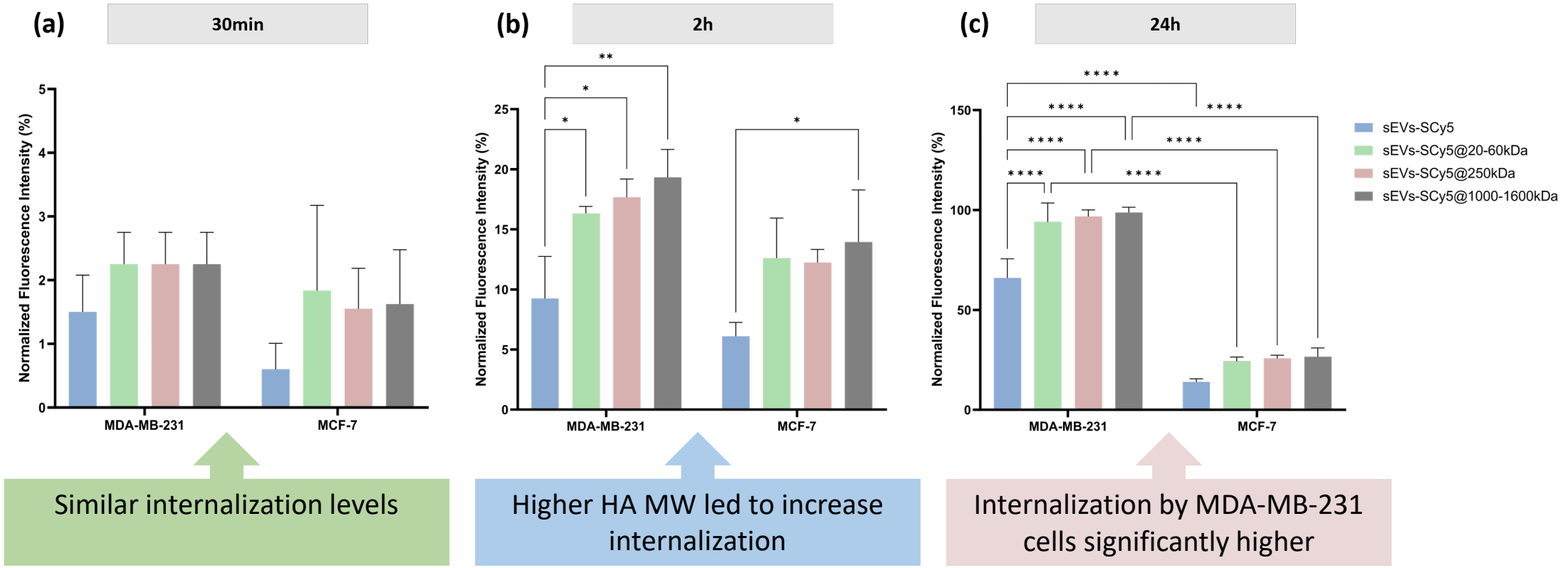


Fig 5. Quantification of fluorescent sEVs on CLSM images (red channel intensity per number of cells).

Selectivity assessment

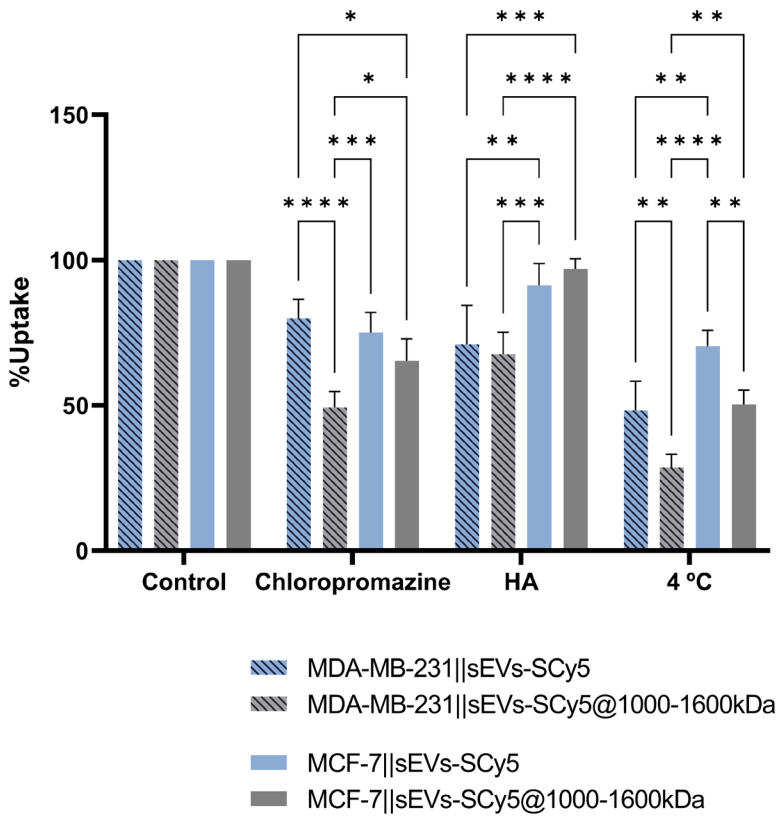


Similar internalization levels

Higher HA MW led to increase internalization

Internalization by MDA-MB-231 cells significantly higher

Uptake Pathways



Chlorpromazine

Inhibit clathrin-mediated endocytosis → More significant decrease in uptake by MDA-MB-231

4°C

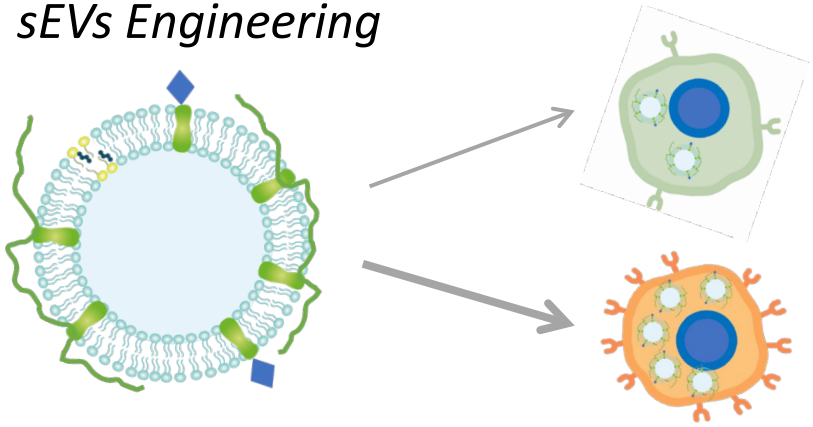
Inhibit active energy-dependent mechanisms → Active energy-dependent processes involved in the uptake of both types of sEVs

HA pre-incubation

Blocking the CD44 receptors → Higher decrease in uptake by MDA-MB-231, also affecting non-functionalized sEVs

Final remarks

sEVs Engineering



an alternative targeting strategy for TNBC

Functionalization with HA

↓

Selectivity for CD44+ cells

Internalization through CD44 endocytic mechanisms

Higher HA MW

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Superior binding capacity

Enhanced internalization

CD44 Targeted Active Delivery

Therapeutics

↓

Targeted drug delivery

Diagnostics

↓

Targeted imaging

Acknowledgements

Cláudia Nunes
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