Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Synthesis and Functionalization of Casein Nanoparticles with Aptamers for Triple-Negative Breast Cancer targeting.

Chiara Spanu,[a] Simona Camorani,[b] Silvia Tortorella,[a,b] Lisa Agnello,[b] Mirko Maturi,[a] Mauro Comes Franchini,[a] Laura Cerchia[b] and Erica Locatelli*[a]*

a) Department of Industrial Chemistry "Toso Montanari", University of Bologna, Viale Risorgimento 4, 40136, Bologna, Italy.

b) Institute of Experimental Endocrinology and Oncology "Gaetano Salvatore", CNR, Via S. Pansini 5, 80131 Naples, Italy.

SUPPORTING INFORMATION

EXPERIMENTAL SECTION

All chemicals have been purchased from Merck KGaA (Darmstadt, Germany) and used as received. 5,5-Difluoro-5H-4λ5-dipyrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-4-ylium-5-uide is referred to as BODIPY. All aqueous solutions were prepared with deionized water obtained using an ultrafiltration system (Milli-Q; EMD Millipore, Billerica, MA, USA) with a measured resistivity above 18 MΩ/cm. Gravimetric analysis was performed to determine the final concentration of the suspensions by drying 100 μL of solution at 120 °C for 3 h and then accurately weighing the residual amount of the dry matter. ATR-FTIR analysis has been performed using a Cary 630 FTIR spectrometer (Agilent). SEM images were acquired with a field emission gun scanning transmission electron microscope ZEISS LEO Gemini 1530 (FEG-STEM).

Preparation of Sodium Caseinate (NaCas)

Casein (2 g) was poured into 100 mL of Milli-Q water under continuous magnetic stirring. Then, a solution of NaOH (1 M in water) was added until the reaching of stable pH 8. The mixture was stirred overnight at room temperature (RT). The obtained product, a solution of sodium caseinate (NaCas), was dialyzed overnight against distilled water (SnakeSkin Dialysis Tubing membranes, MWCO 3.5 kDa) until washing water reached neutral pH. Finally, NaCas was freeze-dried and stored at +4 °C.

Preparation of Casein Nanoparticles (CNPs) and with encapsulated BODIPY (BODIPY@CNPs)

In a round-bottom flask two types of samples were prepared, each one composed of 20 mL of an aqueous solution of lyophilized NaCas (2% w/v). The mixtures were stirred until complete dissolution of Sodium Caseinate. After that the empty system (CNPs) and BODIPY-loaded nanoparticles (BODIPY@CNPs) were prepared by adding to the two starting solutions respectively: 667 μL of ethanol and 570 μL of BODIPY solution (503.8 μM) in DMSO. The two samples were stirred for 30 min at 30°C. Then, 333 μL of a 1 M $CaCl_2$ solution were added to each flask. After 15 min under continuous stirring, the solutions were emulsified with a tip probe sonicator (1 min, 40% amplitude) and filtered through a cotton filter. The obtained nanoparticles were stored at +4 °C.

Aptamers Conjugation

Conjugation with CL4 and CL4-scrambled (SCR) aptamers was performed as follows: 1.8 mL of a 0.28 M solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in water and 4.3 mL of a 2.3 mM solution of N-hydroxysulfosuccinimide (NHS) in water were added to 10 mL of each sample of casein nanoparticles (CNPs and BODIPY@CNPs). The systems were left under stirring at room temperature (25°C) for 5 min. Meanwhile, 200 pmol of amino-terminated CL4 or amino-terminated SCR (LGC Biosearch Technologies, Risskov-Denmark), were placed in an Eppendorf and dissolved in 1 mL of DI water. The aptamers then need to be properly activated to be

reactive as follows: 5 min at 85°C, 2 min at 0°C, and then 10 min at 37°C. Activated RNA-aptamers were added to the desired CNPs and left to react for 24 h at 25°C under continuous stirring. The resulting functionalized nanoparticles were washed two times and concentrated using centrifugal filter devices (Amicon Ultra, MWCO 100 kDa) for 10 min at 3000 rpm, then filtered through a cotton filter. The obtained products were stored at +4 °C.

Dynamic Light Scattering (DLS) and Zeta-Potential

Casein nanoparticles were characterized by dynamic light scattering (DLS) measurements using a Malvern (Malvern, UK) Zetasizer-Nano-S with a 532 nm laser beam. ζ-potential measurements were performed in DTS1070 Clear disposable zeta cells at 25 °C. Native NaCas and CNPs were diluted 5 times with deionized water, then hydrodynamic diameters, polydispersibility, and zeta-potential were recorded.

Estimation of Encapsulation efficiencies and loading capacity of BODIPY by UV−VIS spectrophotometry

To extract BODIPY from nanoparticles, 100 μL of the sample were added to 5 mL of DMSO causing precipitation of casein and dissolution of BODIPY; after centrifugation (9000 rpm for 15 min), the supernatant was collected and subjected to VIS-NIR spectroscopy using a Cary 5000 double-beam spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). A calibration curve was built by measuring the absorbance at 504 nm of standard solutions of BODIPY in DMSO ($1\cdot10^{-5}$, $6\cdot10^{-6}$, $3\cdot10^{-6}$, $1\cdot10^{-6}$, $1\cdot10^{-7}$ M). By interpolation, BODIPY concentration in the nanoparticles was obtained.

Cell cultures

Growth conditions for human TNBC MDA-MB-231, BT-549 (American Type Culture Collection, Manassas, VA), and EGFR-depleted MDA-MB-231 cells (MDA-MB-231 EGFR-KO) cell lines were previously reported.[26]

Cell Viability Studies

MDA-MB-231 cells (5.0×10^3 cells/well, 96-well plates) were treated for 24 h with CNPs_CL4 or CNPs_SCR at the indicated concentrations and cell viability was assessed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega BioSciences Inc., San Luis Obispo, CA) according to the manufacturer's protocol.

Confocal microscopy

MDA-MB-231 and MDA-MB-231 EGFR-KO cells (8.0×10^4 cells/well in 24-well), previously seeded on a coverslip for 24 h, were incubated with BODIPY@CNPs_CL4 or BODIPY@CNPs_SCR (diluted at 0.1 mg/mL dry matter, 0.7 μM dye in culture medium) for 40 min at 37°C, 5% CO2. After three washes with Dulbecco's phosphate-buffered saline (DPBS), cells were fixed with 4%

paraformaldehyde in DPBS for 20 min. Then, cells were incubated with Wheat Germ Agglutinin Alexa Fluor 647-conjugated (WGA-647, Invitrogen, Carlsbad, CA), for 20 min at RT and washed three times with DPBS. Finally, nuclei were stained with 1.5 μM 4′,6-Diamidino-2-phenylindole (DAPI, D9542, Sigma-Aldrich) in DPBS for 5 min and coverslips were mounted with glycerol/DPBS. Samples were visualized by Zeiss LSM 700 META confocal microscopy equipped with a Plan- Apochromat 63x/1.4 Oil DIC objective.

Flow cytometry

MDA-MB-231 and BT-549 cells (2.0×10^5 cells/ well in 6-well) were mock-treated or incubated for 40 min at 37° C with BODIPY@CNPs_CL4 or BODIPY@CNPs_SCR, diluted at 0.1 μM dye in serum-free culture medium supplemented with 0.1 mg/mL yeast tRNA and 0.1 mg/mL ultrapureTM salmon sperm DNA (Invitrogen), as nonspecific competitors. After three washes with DPBS, cells were trypsinized, suspended in 500 μL DPBS, and analyzed by flow cytometry (BD AccuriTM C6, BD Biosciences, San Jose, CA).

RESULTS SECTION

Detailed Mechanism of Aptamer Conjugation

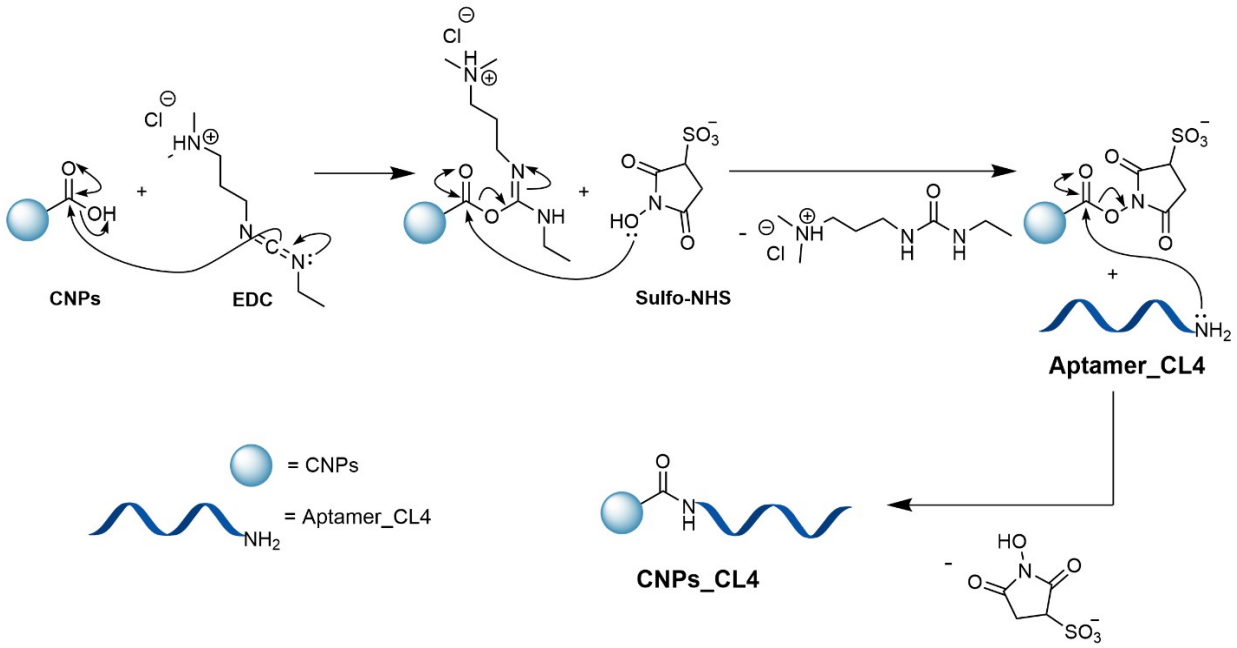


Figure S1. The detailed mechanism of the carboxyl acid group activation via EDC/NHS chemistry and the subsequent formation of the amide groups after reaction with the amino-aptamer.

Dynamic Light Scattering distribution plots

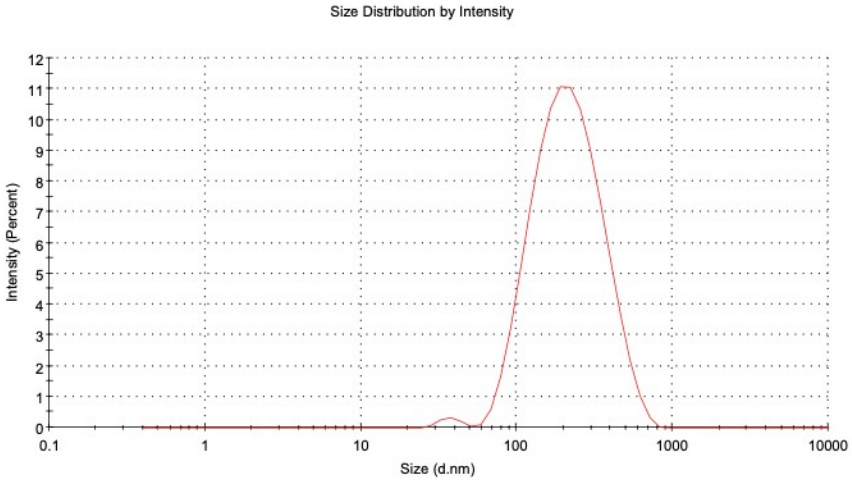


Figure S2: DLS plot for size distribution of CNPs nanoparticles

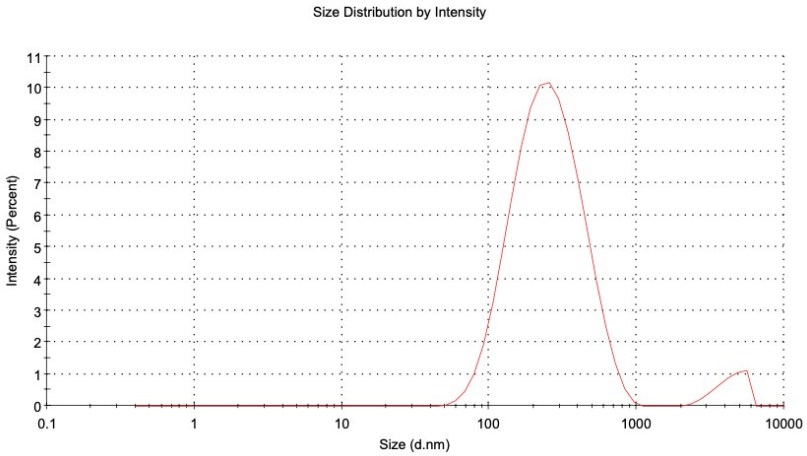


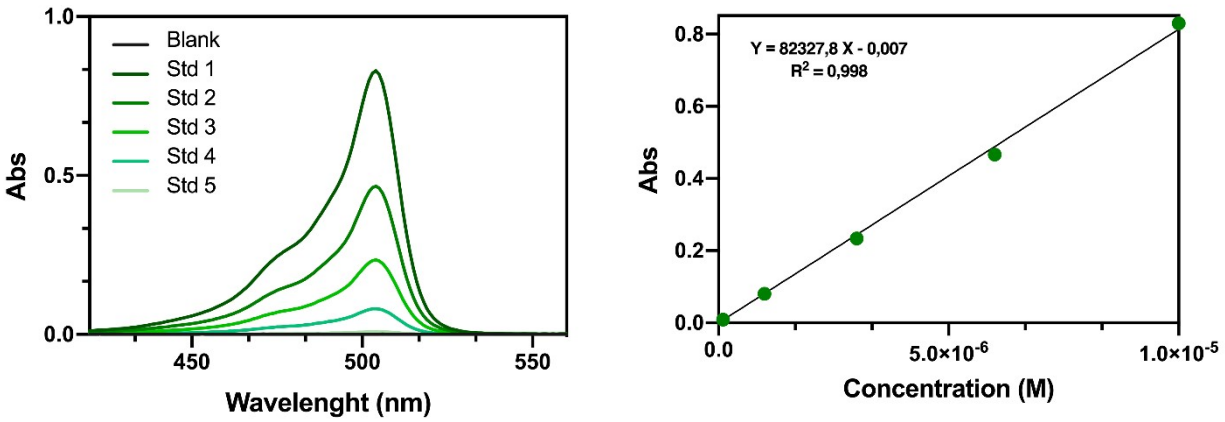
Figure S3: DLS plot for size distribution of BODIPY@CNPs_CL4 nanoparticles

Estimation of Encapsulation efficiencies and loading capacity of BODIPY by UV−vis spectrophotometry

To extract BODIPY from nanoparticles, 100 µL of the sample was added to 5 mL of DMSO causing precipitation of casein and dissolution of BODIPY; after centrifugation (9000 rpm for 15 min), the supernatant was collected and subjected to VIS-NIR spectroscopy using a Cary 5000 double-beam spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).

A calibration curve was built by measuring the absorbance at 504 nm of standard solutions of BODIPY in DMSO obtained by dilution:

- Std 1: $1 \cdot 10^{-5}$ M
- Std 2: $6 \cdot 10^{-6}$ M
- Std 3: $3 \cdot 10^{-6}$ M
- Std 4: $1 \cdot 10^{-6}$ M
- Std 5: $1 \cdot 10^{-7}$ M



Sample: 100 µL of the sample was added to 5 mL of DMSO.

Figure S4. UV-Vis spectra of BODIPY at different concentrations and the obtained calibration line.

The determination of BODIPY concentration in the nanoparticles was possible through the calibration line obtained. Thanks to that data and dry matter (mg/mL), was evaluated EE% and LC% using the reported formulae:

$$EE\% = \frac{[BODIPY]_f}{[BODIPY]_i} \cdot 100 \qquad LC\% = \frac{mg_{BODIPY,f}}{mg_{BODIPY@CNPs}} \cdot 100$$

Where:

- $[BODIPY]_f$ is the final concentration of BODIPY in nanoparticles after purification;

- $[BODIPY]_i$ is the starting concentration of BODIPY;
- $mg_{BODIPY,f}$ is the final mg of BODIPY in nanoparticles;
- $mg_{BODIPY@CNPs}$ is the dry matter of final nanoparticles.

Scanning Electron Microscopy (SEM)

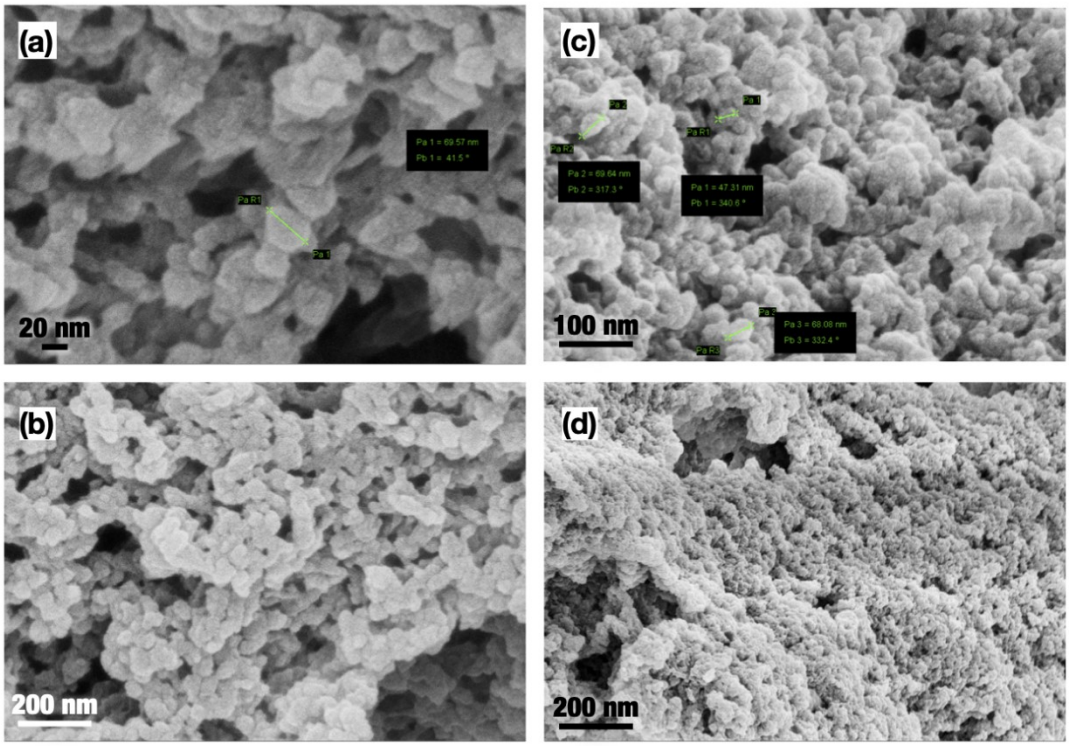


Figure S5. FEG-STEM images of CNPs (a-b) and BODIPY@CNPs (c-d) at different scales of magnification.

Confocal microscopy and cell viability studies

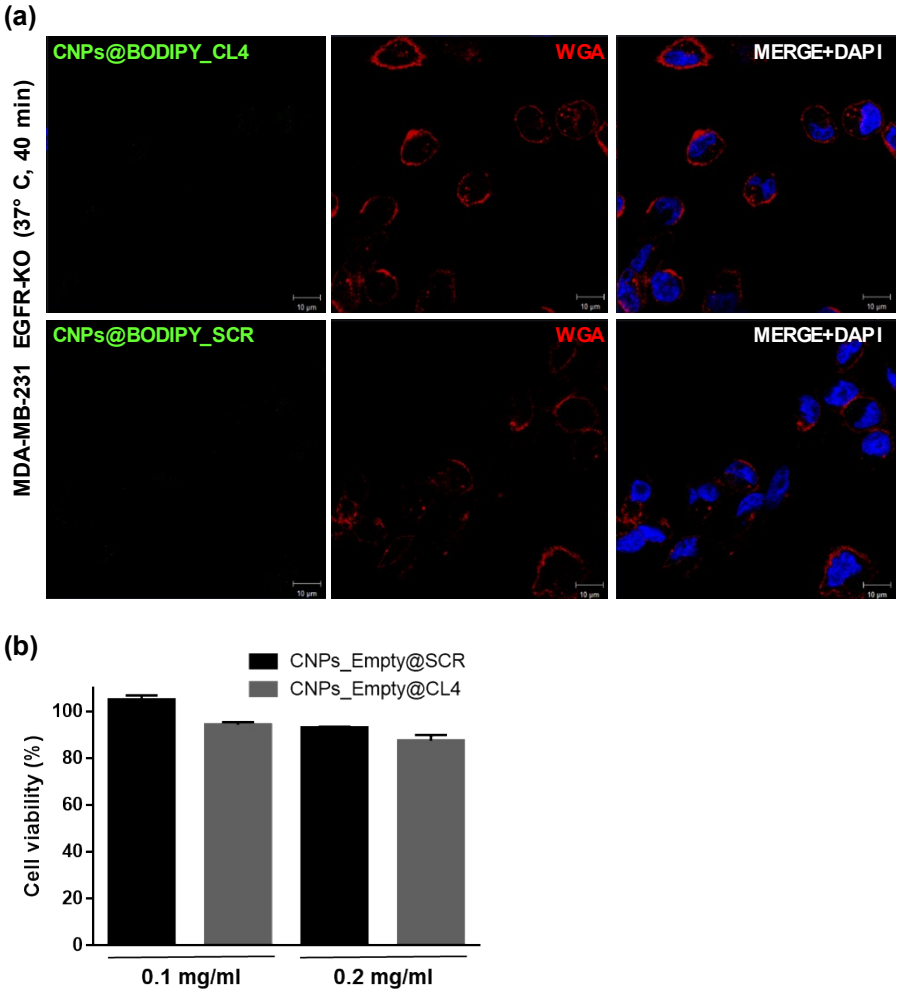


Figure S6. (a) Representative confocal images of MDA-MB-231 EGFR-KO cells treated with BODIPY@CNPs_CL4 or BODIPY@CNPs_SCR at 37 °C for 40 min. Red: cell membrane; blue: nuclei; and green: nanoparticles. Scale bars, 10 μm. All digital images were captured in the same setting to allow direct comparison of staining patterns. (b) Cell viability of MDA-MB-231 cells treated with aptamer-conjugated and unloaded CNPs. Data are expressed as a percentage of viable treated cells with respect to untreated cells. Bars depict the mean ± SD of three independent experiments.