Supporting Information

Multilayered Magnetic Nanobeads for the Delivery of Peptides Molecules Triggered by Intracellular Proteases

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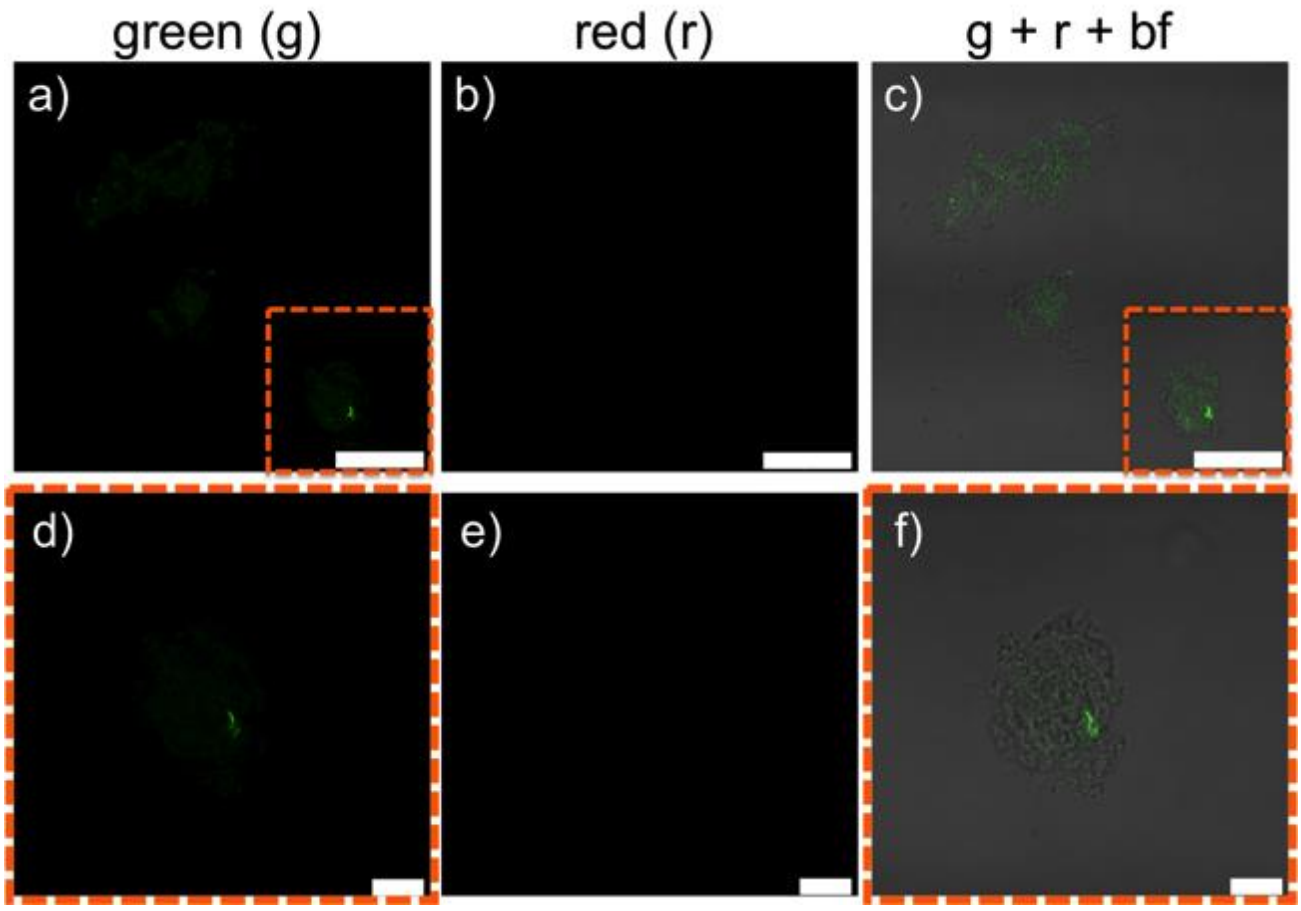

Figure S1. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 24 h at 37 °C. (a, d) green channel, (b, e) red channel, (c) overlay of a, b and bright field; (f) overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Scale bars (top panel) 25µm and (bottom panel) 10 µm.

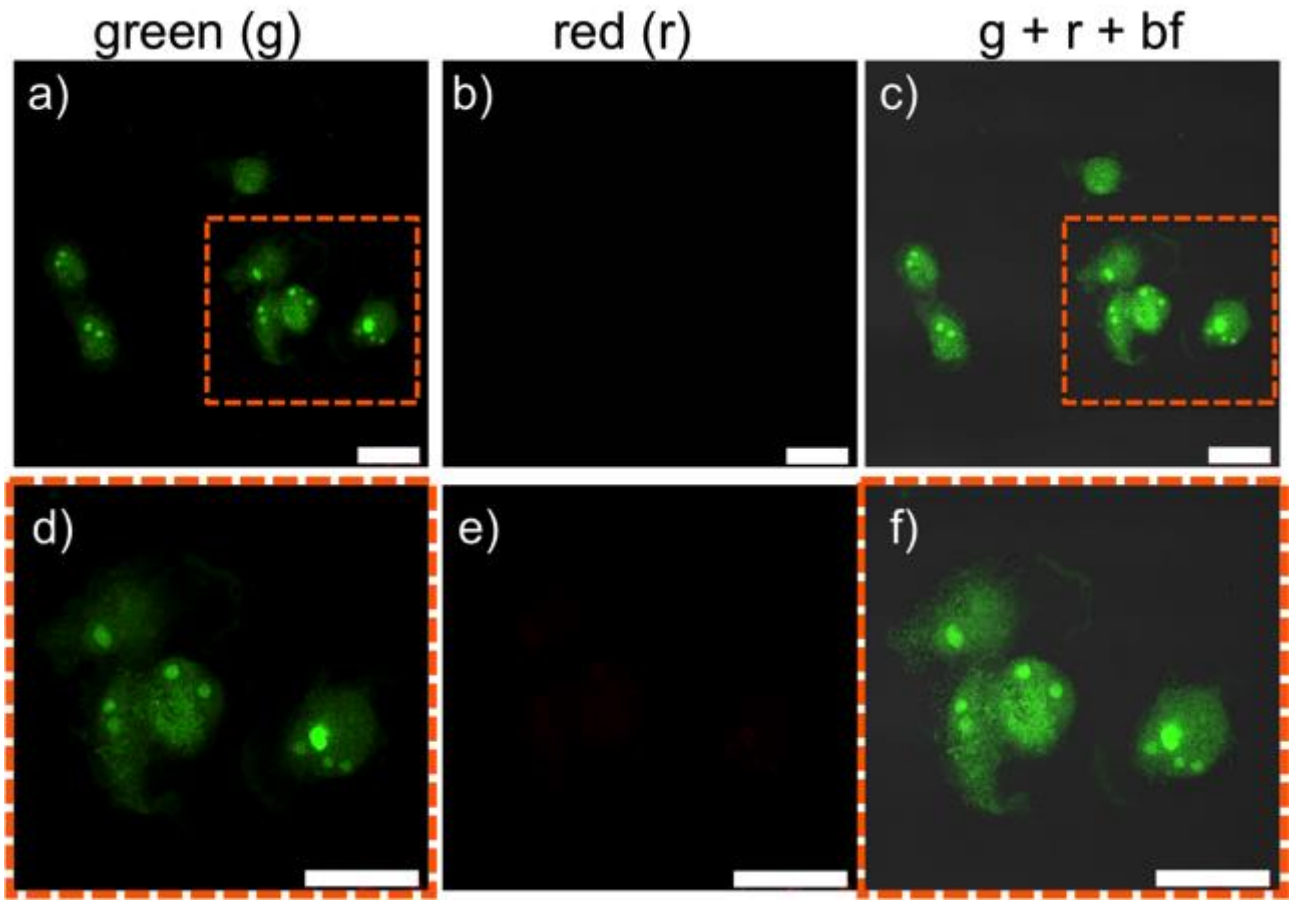

Figure S2. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 48 h at 37 °C. **(a, d)** green channel, **(b, e)** red channel, **(c)** overlay of a, b and bright field; **(f)** overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Dashed boxes, zoomed areas. Scale bar 25 μm.

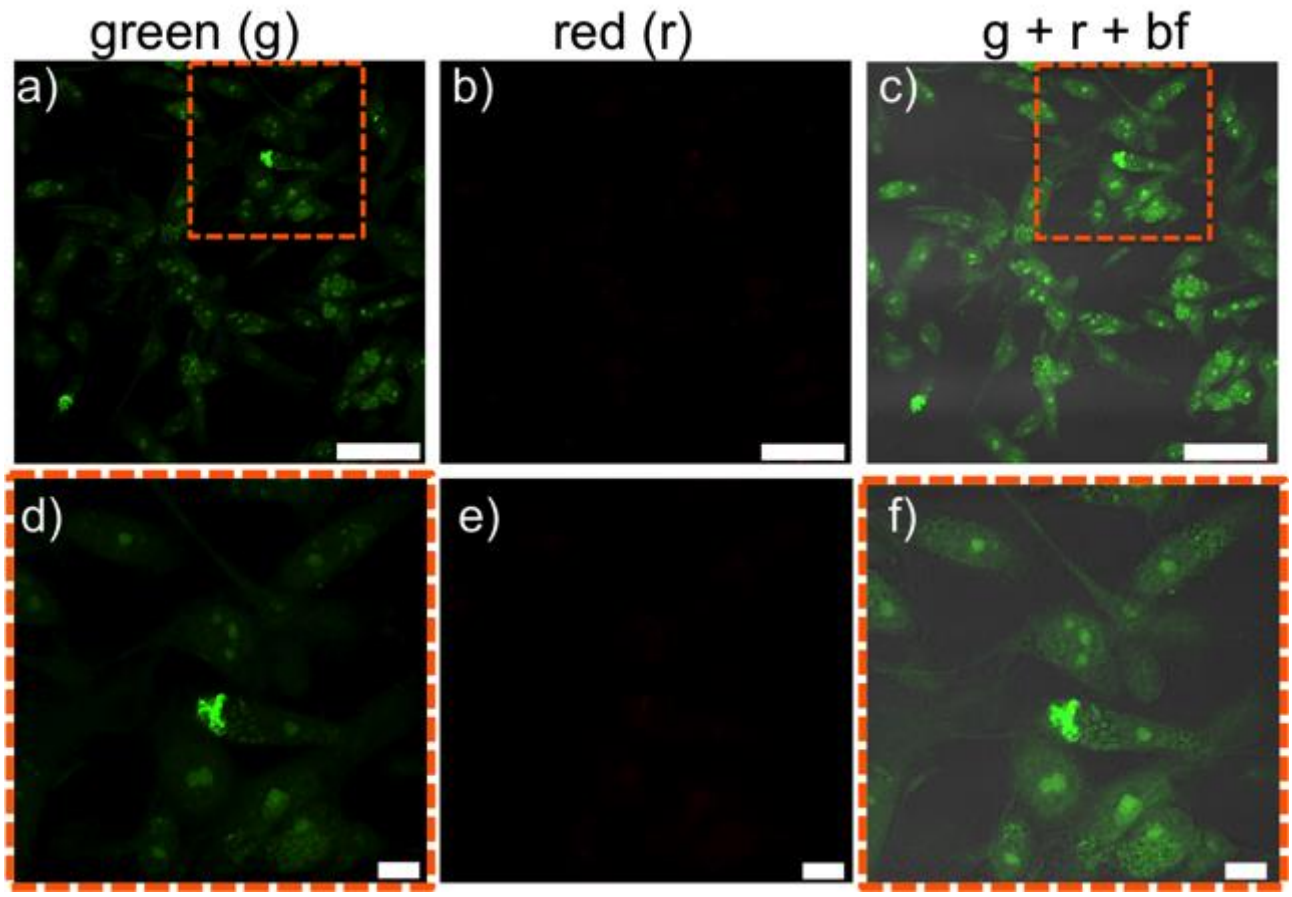

Figure S3. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 96 h at 37 °C. **(a, d)** green channel, **(b, e)** red channel, **(c)** overlay of a, b and bright field; **(f)** overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Dashed boxes, zoomed areas. Scale bars (top) 50 μm and (bottom) 10 μm.

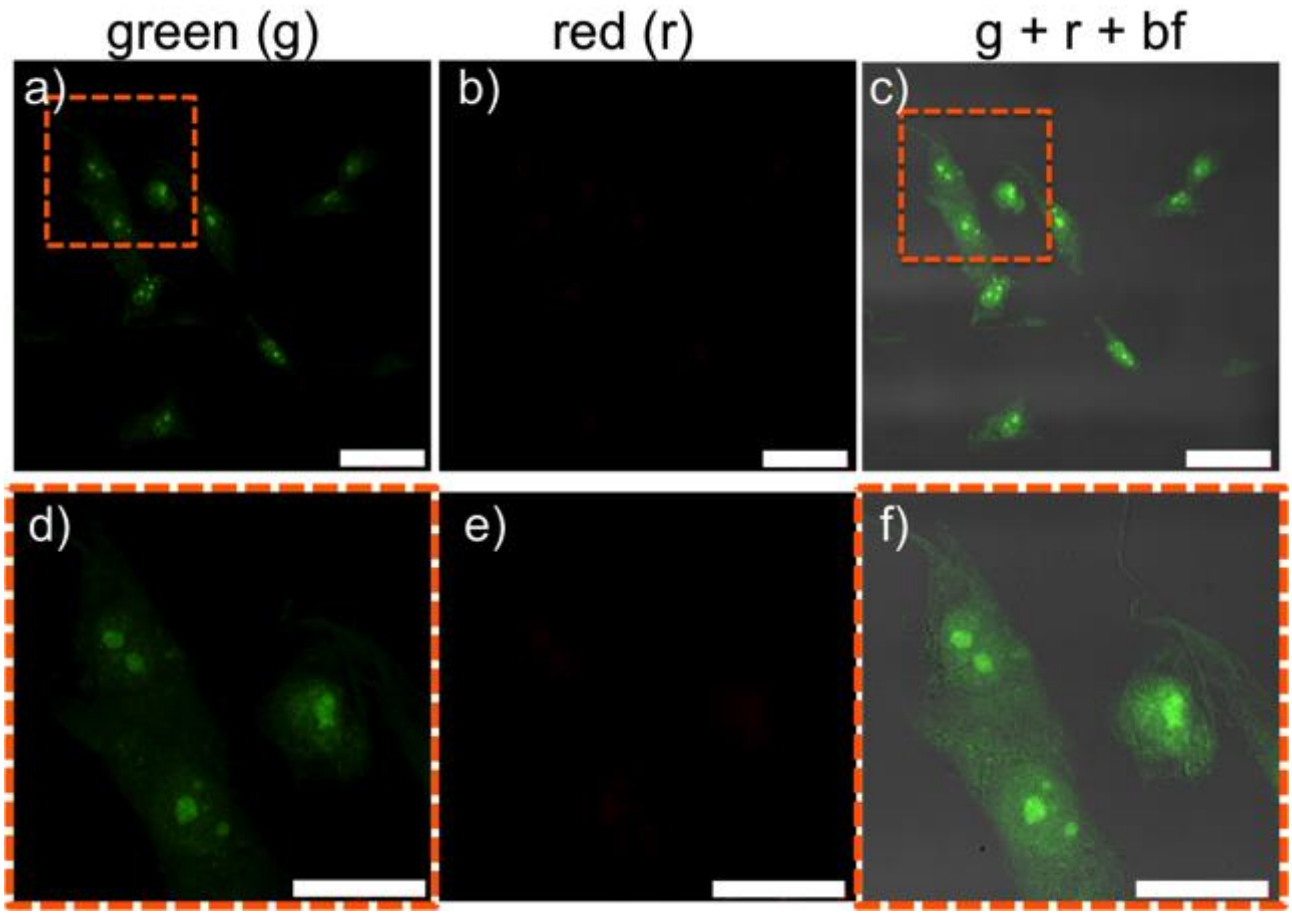

Figure S4. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 120 h at 37 °C. **(a, d)** green channel, **(b, e)** red channel, **(c)** overlay of a, b and bright field; **(f)** overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Dashed boxes, zoomed areas. Scale bars (top) 50 μm and (bottom) 25 μm.

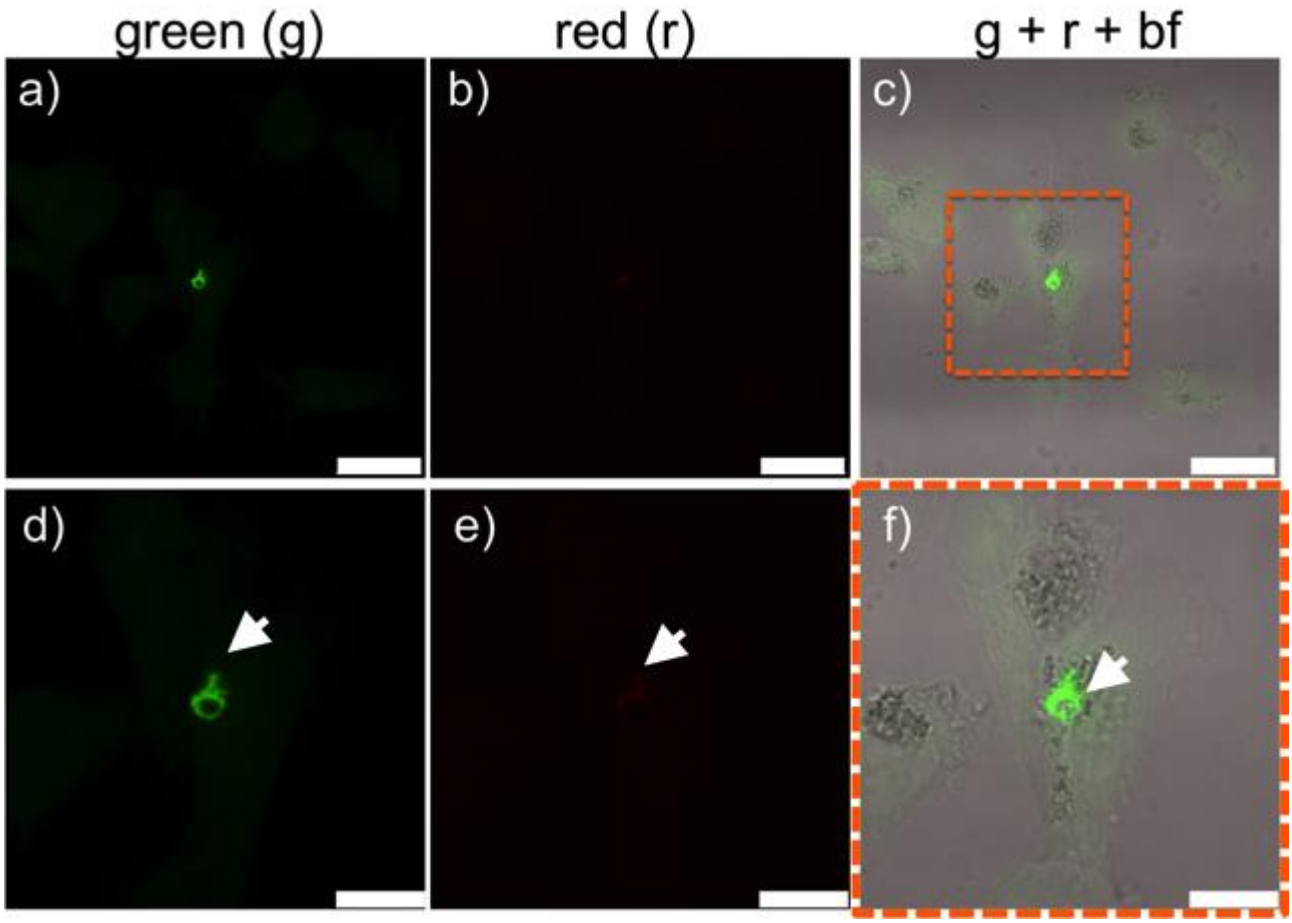

Figure S5. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 24 h at 37 °C. **(a, d)** green channel, **(b, e)** red channel, **(c)** overlay of a, b and bright field; **(f)** overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Dashed boxes, zoomed areas. White arrows indicate internalized MMNBs still not digested. Scale bars (top) 25 μm and (bottom) 10 μm.

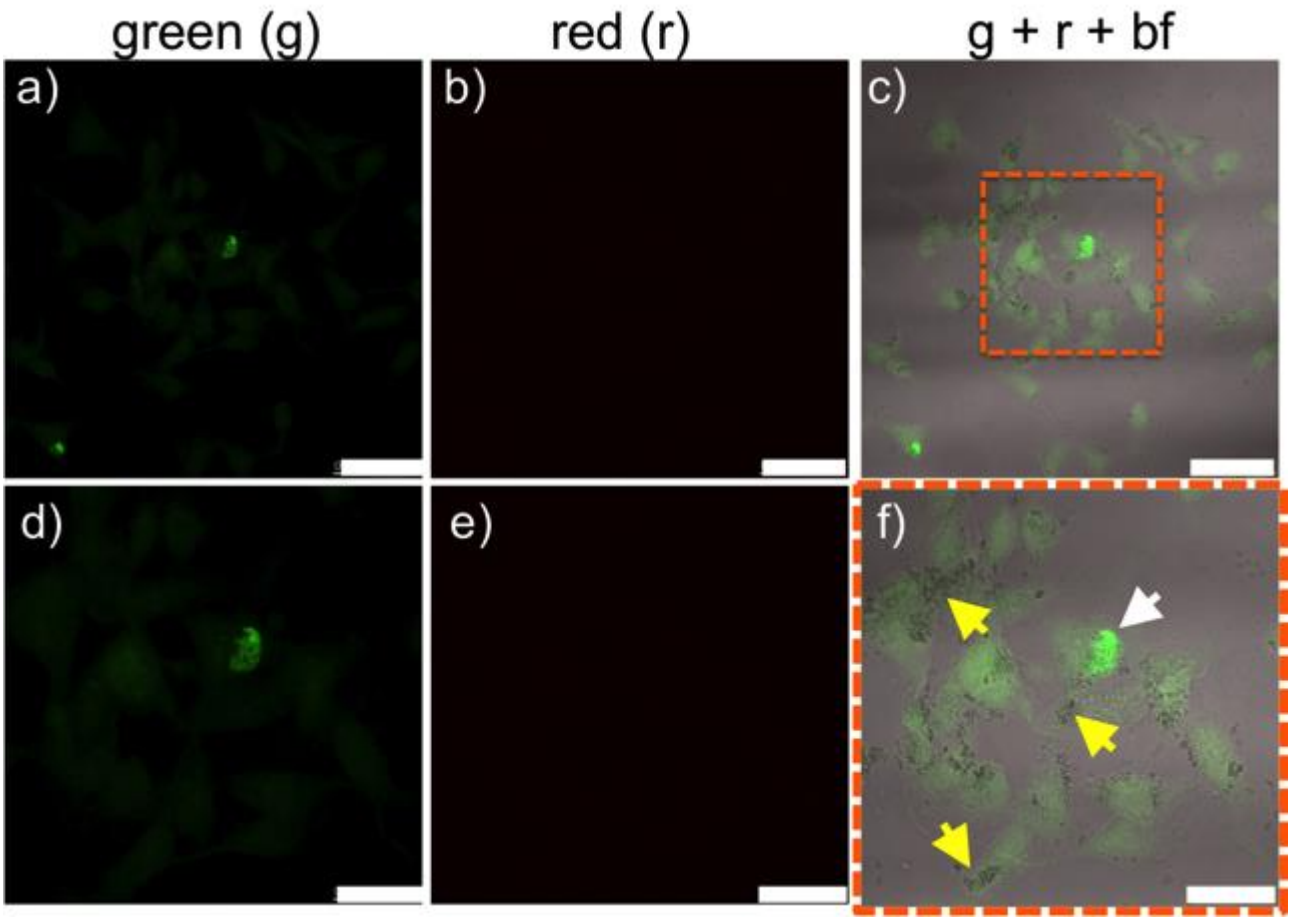

Figure S6. MDA-MB-231 cells incubated with MNBs@(PARG/PGA)(DQ-OVA/PARG)(PGA/PARG) for 48 h at 37 °C. **(a, d**) green channel, **(b, e)** red channel, **(c)** overlay of a, b and bright field; **(f)** overlay of d, e and bright field. Dashed boxes, zoomed areas. Individual bright field (bf) channels are not shown. Dashed box, zoomed areas. White arrows indicate internalized MMNBs still not digested. Yellow arrows indicate nonfluorescent MNBs derived from proteolytic digestion of the multilayers shell followed by the release of fluorescent fragments in the cytosol. Scale bars (top) 50 μm and (bottom) 25 μm.

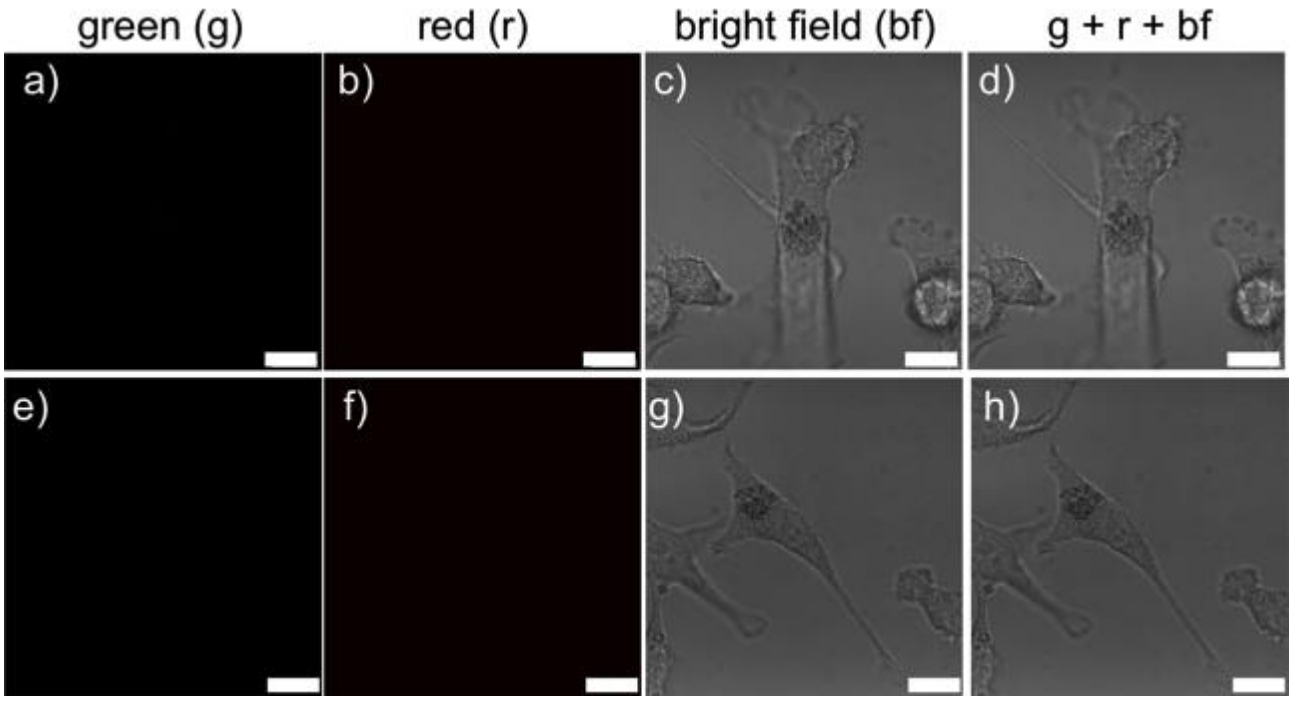

Figure S7. Fluorescence channels of MDA-MB-231 cells incubated with MNBs@(PARG/PGA)$_2$(PARG) for 120 h at 37 °C. **(a, e)** green channels, **(b, f)** red channels, **(c, g)** bright-field channels. **(d)** Overlay of a, b and c; **(h)** overlay of e, f and g. Scale bar 10 μm. Clusters of MMNBs@(PARG/PGA)$_2$(PARG) inside the cells are clearly visible in the bright-field channels, whereas no autofluorescence signal is detected in the both green and red channels under the used imaging setting.

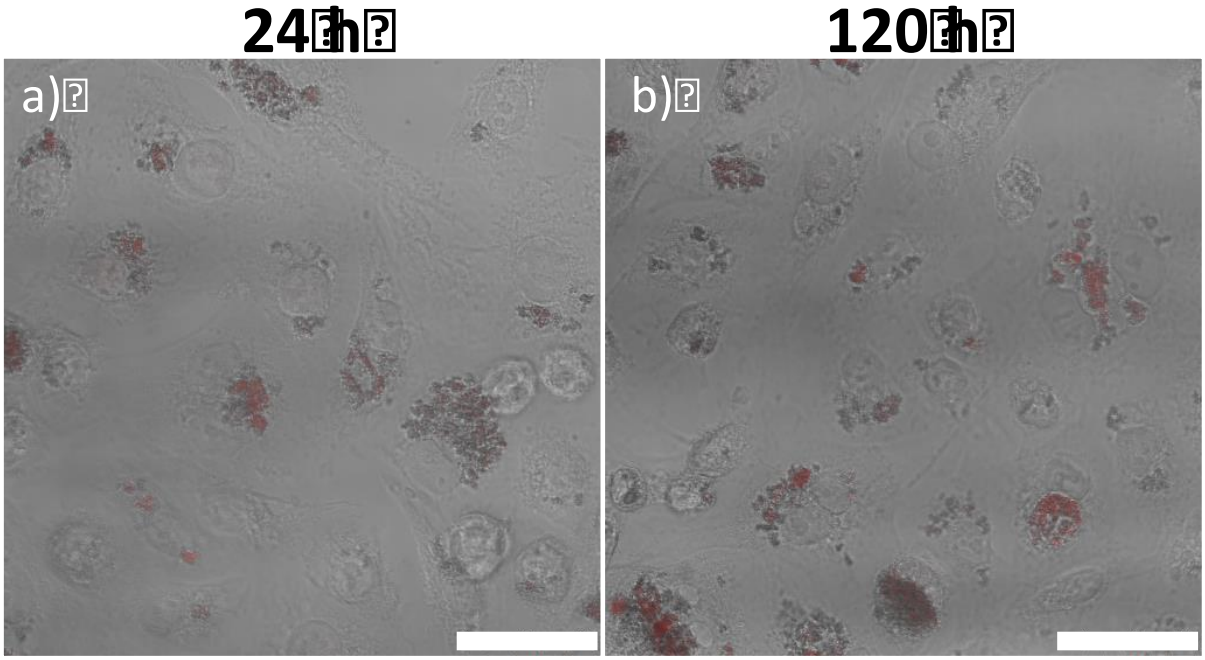

Figure S8. Representative CLSM images of MDA-MB-231 cells incubated with MNBs@(PAH/PSS)(BSA-RITC/PAH)(PSS/PAH) at 37°C for 24 h (a) and 120 h (b). Scale bars 25 um. Albumin from Bovine Serum (BSA) Tetramethylrhodamine conjugate (BSA-RITC) was obtained from ThermoFisher (#A23016). The anionic Poly(sodium 4-styrenesulfonate) (PSS, Mw ≈ 70 KDa) and the cationic Poly(allylamine hydrochloride) (PAH, Mw ≈56 KDa) polyelectrolytes were obtained from Sigma. LbL was performed as described for biodegradable polymers. Briefly, the MNBs were incubated with 1 mL of PAH (2 mg/mL, 50 mM NaCl, pH 6.5). Following 1 h of incubation, MNBs were washed 2 times with 3 mL of ultrapure water by means of a constant magnetic field (0.3 T Neodynium/Boron magnet). Then, a layer of PSS was deposited by addition of 1 mL of PSS (2 mg/mL, 50 mM NaCl, pH 6.5). After the washing steps, a layer of BSA-RITC (1 mg/mL in PBS, pH 6.5) was added. Then, two additional layers were added to obtain MNBs with the following multilayer shell: (PAH/PSS)(BSA-RITC/PAH)(PSS/PAH).

Sample and time of administration	Number of cells	Fe concentration (M)	Fe concentration/cell (M)	% Internalization Efficiency
MNB 24 h	1284000	3.94E-06	3.07E-12	4.3
MNB 48 h	1770000	4.67E-06	2.64E-12	5.1
MNB 96 h	2000000	6.71E-06	3.36E-12	7.4
MMNB 24 h	1170000	2.81E-05	2.40E-11	30.9
MMNB 48 h	1850000	2.69E-05	1.46E-11	29.6
MMNB 96 h	2100000	2.75E-05	1.31E-11	30.2

Table 1. Cell uptake experiment based on the detection of elemental iron. Different beads and different incubation conditions were used (column 1). The number of total cells per each type of sample (second column), the concentration of Fe detected in each sample (third column), the amount of Fe per cell (fourth column), and finally the Fe internalization efficiency (fifth column) are reported. The Fe internalization efficiency was obtained by dividing the total Fe amount detected by elemental analysis in each sample for the amount of Fe initially administered.