Supplementary Material (ESI) for *Nanoscale*

Water soluble nanoporous nanoparticle for *in vivo* targeted drug delivery and controlled release B cells tumor context.

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SUPPLEMENTARY INFORMATION

Summary

1. Nanoparticles fabrication

A polished boron-doped silicon substrates (resistivity 5–10 Ωcm) of [100] crystal orientation, was anodized using an electrolytic cell with an electrolyte ternary mixture of hydrofluoric acid (25%), water (25%), and ethanol (50%). During the anodization, the current density was kept constant at 10 mA/cm^2 for 5 min. at 25°C. Samples were rinsed first with deionized water, then in ethanol and pentane. Finally, the nanoporous silicon film was kept at 260°C for 4 hours in dry ambient. In figure S1A, the nanoporous silicon film obtained onto 2 inches silicon wafer is shown: the emission of red-orange light (under UV illumination) indicates a pore size of a few nanometers.

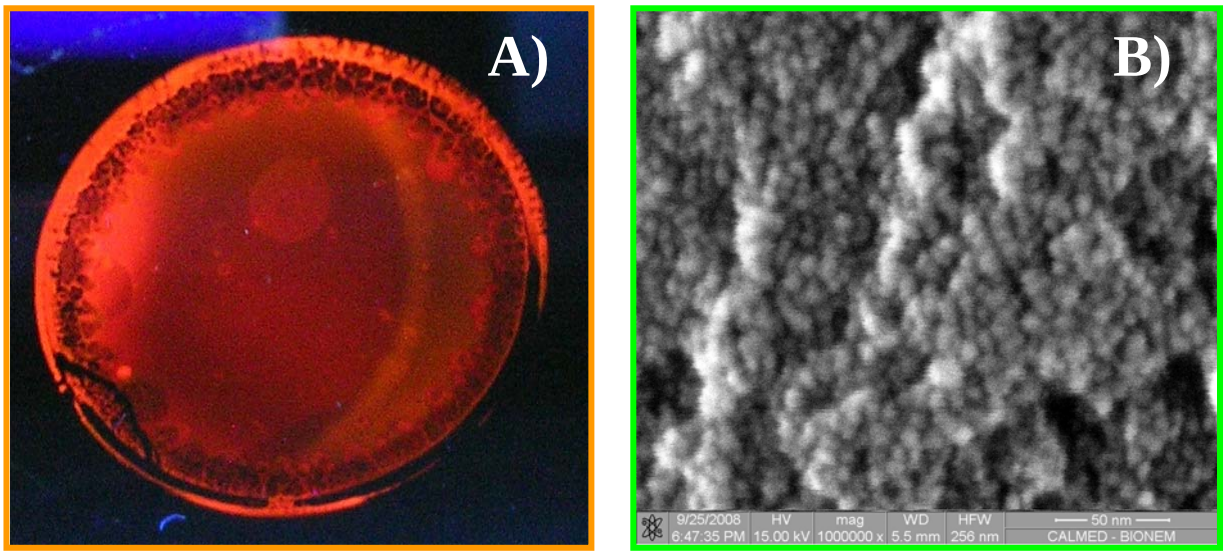


Figure S1: *A) Optical image of Nanoporous film illuminated with UV light (2 inches silicon wafer); B) SEM image of the skeleton structure of porous silicon film.*

To obtain NPNPs, the porous silicon film were sonicated in DMF for about 60 min and then, after washes in Ethanol, ultra-sonicated (5W) in water for 10 min at constant temperature of 4°C, and finally filtered to eliminate impurities above 500 nm. NPNPs of average diameter from 30 to 500 nm were obtained changing the power and/or the time of the ultra-sonication (Figure S2). Smaller particles, up to a few nm, can be obtained for higher power or time of ultrasonication process. In figure S1B, SEM image with a magnification of 1000000, shows the structure of the porous film, that is made of a silcon nanocrystal framework. The silicon nanocrystals are have a diameter of about 5-7 nm, and the inter-spaces between the nanocrystals define the porosity of material (in our

case about 2 nm). As shown in figure 2 of the article, and here reported for clarity (figure S3), the obtained NPNPs still exhibit the same luminescent emission spectrum (around 675 nm) of the nanoporous film shown in figure S1, indicating that no significant changes of the skeleton structure occurred during the nanoparticles fabrication.

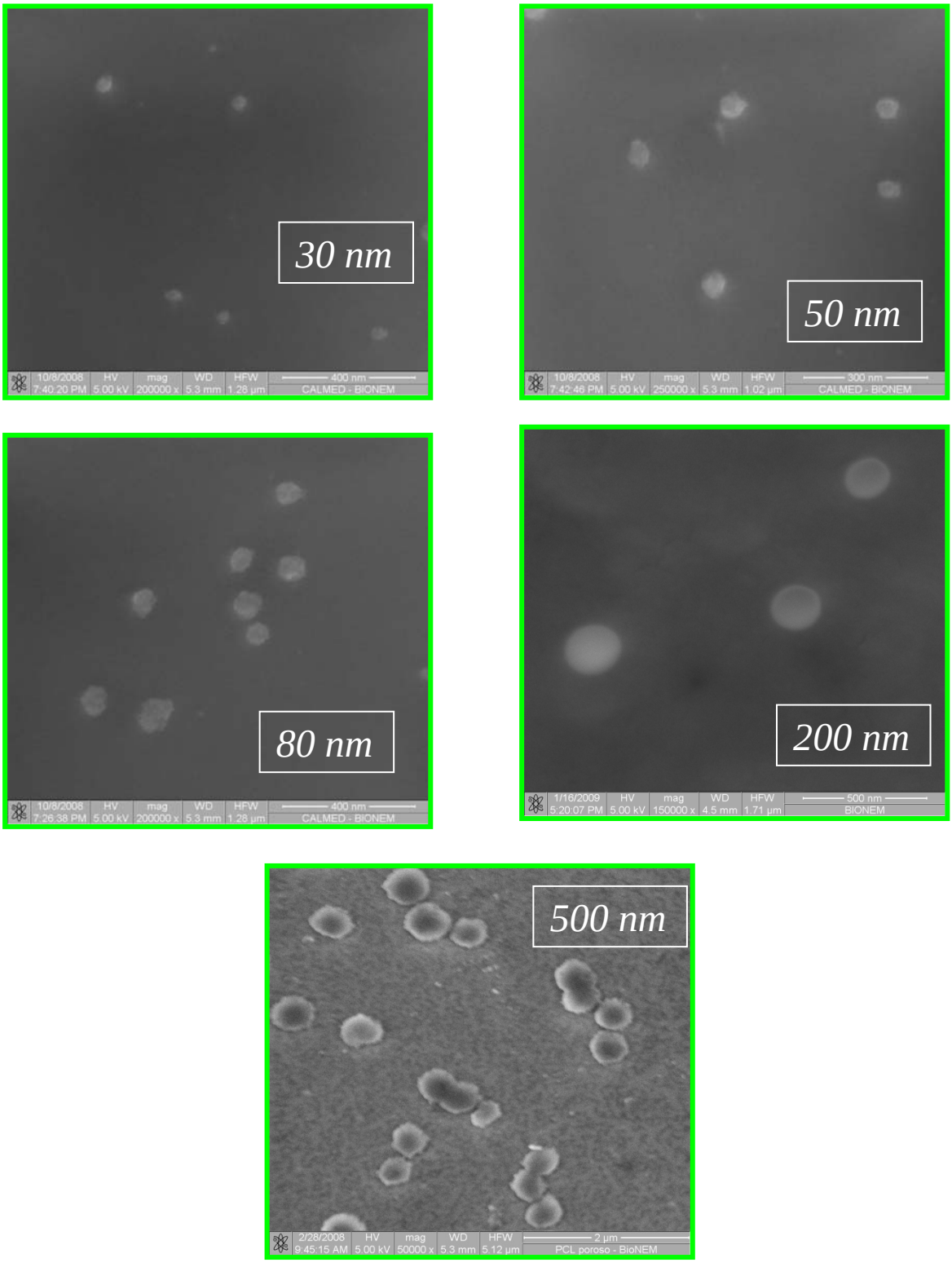


Figure S2: *SEM images of nanoporous nanaparticles of different sizes, from 30 to 500 nm.*

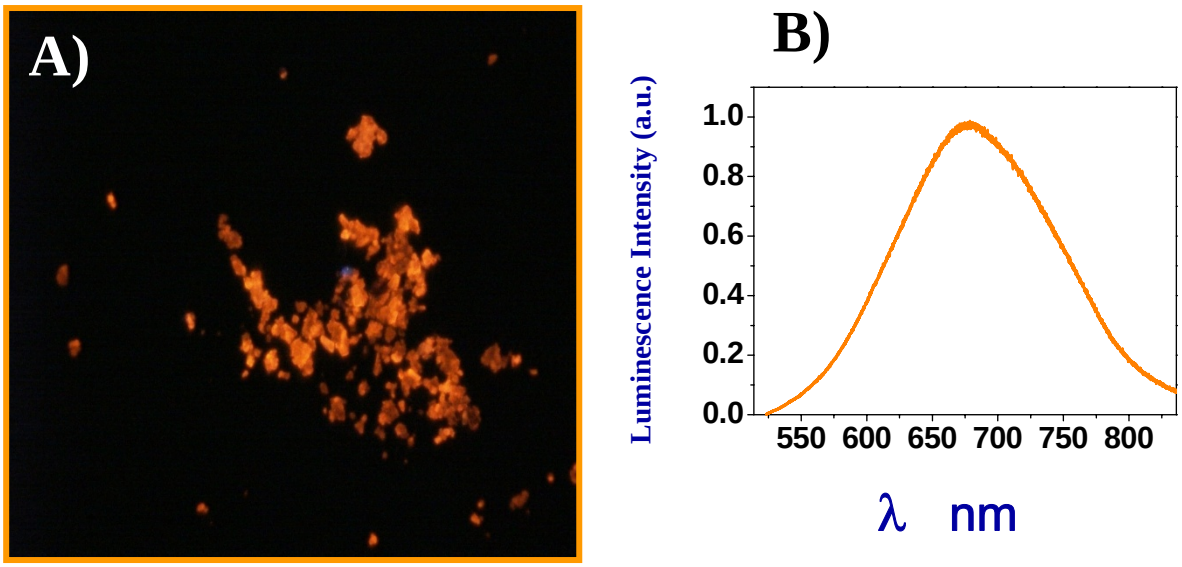


Figure S3 *: A) Optical image of luminescent NPNPs (UV illumination); B) NPNPs emission spectra.*

2. Payload evaluation

To test the maximum payload available, 1.6×10^{11} NPNPs (50 nm diameter) were incubated for 1 hour with different quantities of peptide pA20-6 FITC: 1-3-5-10 μg. After the incubation, the NPNPs were recovered by centrifugation (3 minutes, 10000 rpm), and the unloaded peptide (surnatant) was quantified using a fluorimeter. Then, the corresponding payload was calculated (Figure S4). The NPNPs exhibit an extremely high loading capacity of about 3×10^{-17} g per each particles (5 μg / 1.6×10^{11} particles).

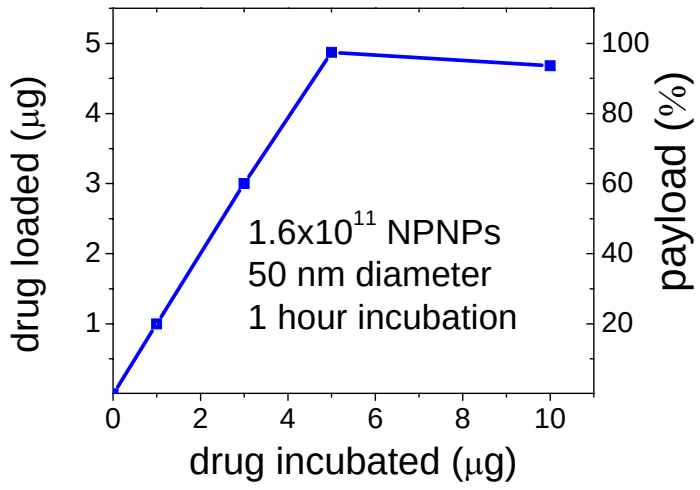


Figure S4: *Loading profile of the nanoporous nanoparticles.*

3. Dissolution and release profiles

The release rate of NPNPs were evaluated bot under physiological environment (PBS solution, pH=7.4) and under acid environment (Sodium Acetate 2 mM pH 4.38). Fluorescein was loaded on the NPNPs as already described (incubation and centrifugation), and then they were dissolved in acid and basic environment. An aliquote of the solution was removed over six time points (1h, 2h, 4h, 24h, 48h and 72h), and centrifufated. The fluorescence intensity of the obtained supernatant indicates the amount of fluorescien released by the nanoparticles.

As reported in figure S5, the NPNPs release the payload over a period of several hours in physiological ambient, whereas they do not dissolve when pH<5, allowing long term storage also with molecule embedded.

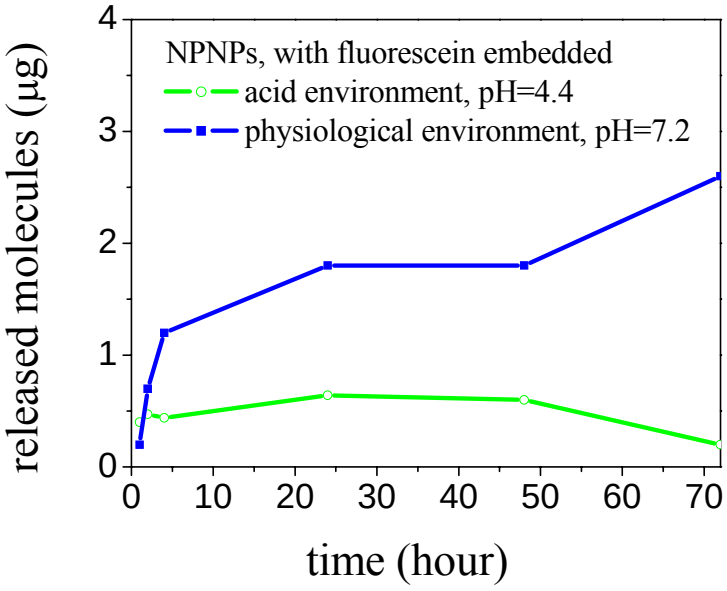


Figure S5: *Release profiles of the NPNPs in acid and basic solution.*

4. Multivalent loading.

Thanks to an efficient multiple loading, the NPNPs can be employed in multitasking applications. To further asses this capability, NPNPs were not incubated with an "ad hoc" solution (as presented in the articles main text, or in the sections above), but with crude human serum to really prove that they can simultaneously load a lot of different molecules. Human serum can be considered one the most complex biological solution, containing a very wide range of low molecular weight species which differ in size, hydrophobicity, chemical and physical properties.

Human blood plasma was obtained from a healthy anonymous male donor, and collected in according with HUPO plasma proteome project guidelines (Tirumalai et al. 2003). 8 ml of blood were drawn by venipuncture and collected in a K_2EDTA tube. The sample was centrifuged within 2 hours of collection at 1300xg for 10 minutes, aliquoted into silicon tubes and stored at -80 °C.

After incubation (100 µl of plasma sample diluted 1:2 with PBC buffer, 1.6×10^{11} NPNPs) the NPNPs were separated from supernatant (centrifugation), and they were dissolved in PBC buffer for 2 h at 90°C. The solution containing the molecules released from the nanoparticle was analyzed with tandem mass spectrometry (nanoLC-MS/MS), and the results are reported in figure S6. Lower panel shows the molecular species loaded on the NPNPs in the range 2000-4000 a.m.u., whereas the upper panel shows the molecules revealed in crude serum under same experimental conditions. The reported mass spectra indicate that, in our experimental conditions, 36 molecular species can be find out in raw serum, and at least 28 of them were adsorbed in to the nanoparticles. Of course, each molecule exhibit a different affinity with respect to the NPNPs adsorption, resulting in a different intensity of mass spectrum signal. In case of a real multiple loading, the desired composition of the payload can be obtained adjusting the relative concentration of the molecular species in the starting solution.

The performed Mass Analysis on human serum was also important to demonstrate that with a porosity of about 2 nm, molecules up to 15 kDa in weight can be loaded. To adsorb bigger molecules, NPNPs with larger pore size can be fabricated changing anodization condition.

We notice that such low molecular weight cut-off suggests a possible exploitation of the NPNPs as nanosieve for complex biological fluid.

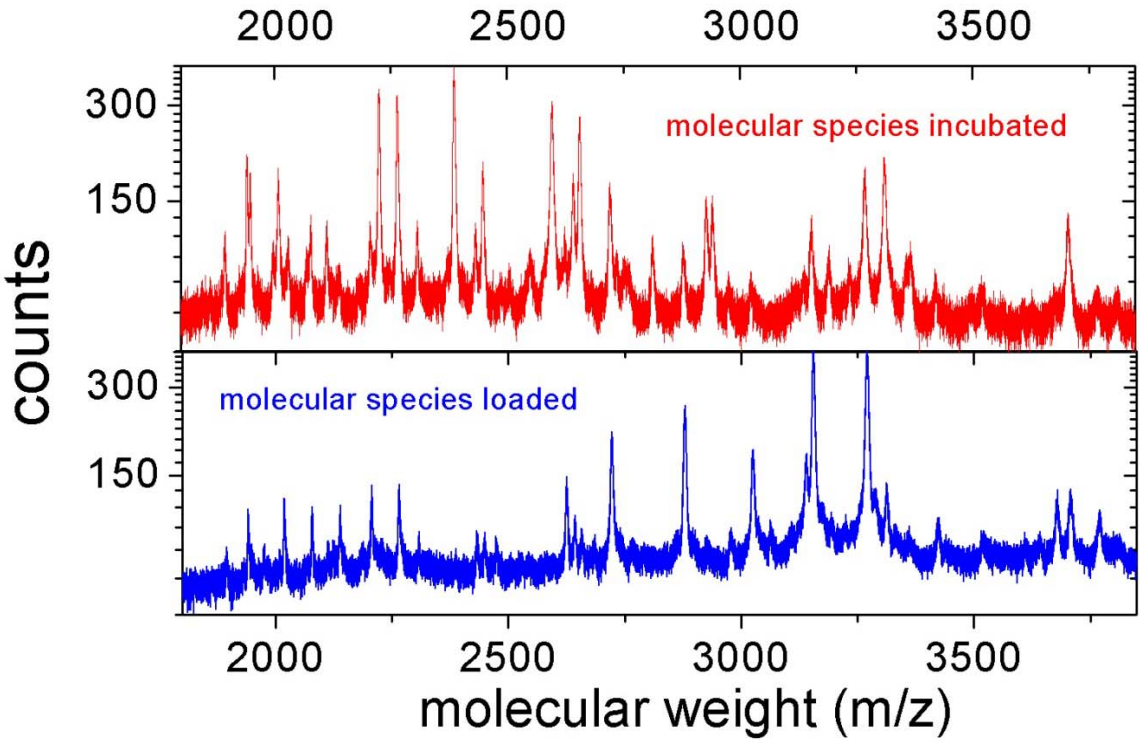


Figure S6: *Mass spectra of raw human serum (upper panel), and fraction of the human serum adsorbed on the nanoporous nanoparticles (bottom panel) in the same experimental conditions .*

5. Targeting specificity on A20 cells.

In order to verify the ability of peptide/NPNPs to selectively reach the target cancer cell, they were incubated with A20 and Hela cancer cells (epithelial cervix carcinoma).

The A20 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells are B lymphocytes from a naturally occurring 'reticulum cell neoplasm' from an old Balb/c mouse [1]. Cells were grown in CD Hybridoma Medium (Invitrogen) with 2mM L-glutamine. HeLa cell line was maintained in DMEM medium containing 10% heat-inactivated fetal calf serum and 2mM L-glutamine in a humidified 5.0% CO2 environment.

A20 ($5x10^5$/ml) and HeLa cells (70% confluence), seeded on glass slides into 6-well plate, were incubated for 30' at 37°C with pA20-6 FITC-loaded NPNPs (10 μg of peptide on 3×10^{11} particles). Nuclear staining was performed with Hoechst S769121 (Invitrogen) for 30' at 37°C, then cells were washed and mounted with Prolong anti-fade solution (Invitrogen) on the slides for analysis.

In figure S7 are reported the results of the confocal analysis performed on both A20 (reported on figure 4 of the article) and Hela cell lines: the peptide/NPNPs complex targets the desired cancer cells, while Hela cells seem not interact with the nanocarrier.

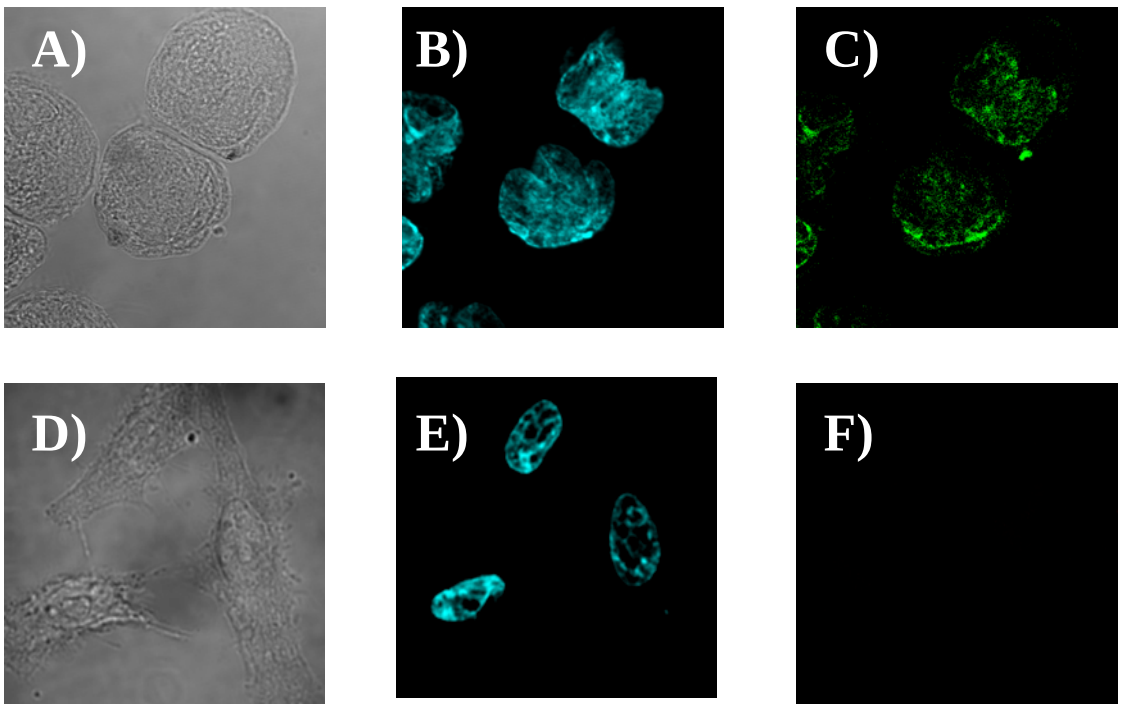


Figure S7*. Confocal microscope images of A20 Cells (upper panels) and Hela cells (bottom panels). Panels A and D: transmission channel. Panels B and E: blue channel, nuclear staining. Panel C and F: green channel, nanoparticles-cells binding.*

6. Cell Cycle Analysis

A20 cells ($5x10^5$/ml) were seeded on a 6-well plate and treated with pA20-6-FITC/NPNPs (10 μg of peptide on 3×10^{11} particles) or with NPNPs alone (3×10^{11} particles) or with free peptide (10 μg). Aliquots of cells were removed and analyzed at different time points by flow cytometry for cell cycle analysis (24h, 48h, 72h) (CycleTest Plus, BD). As shown, NPNPs alone didn't affect cell cycling and viability, while pA20-6 FITC-NPNPs mediated delivery resulted in a more consistent apoptotic effect than free peptide.

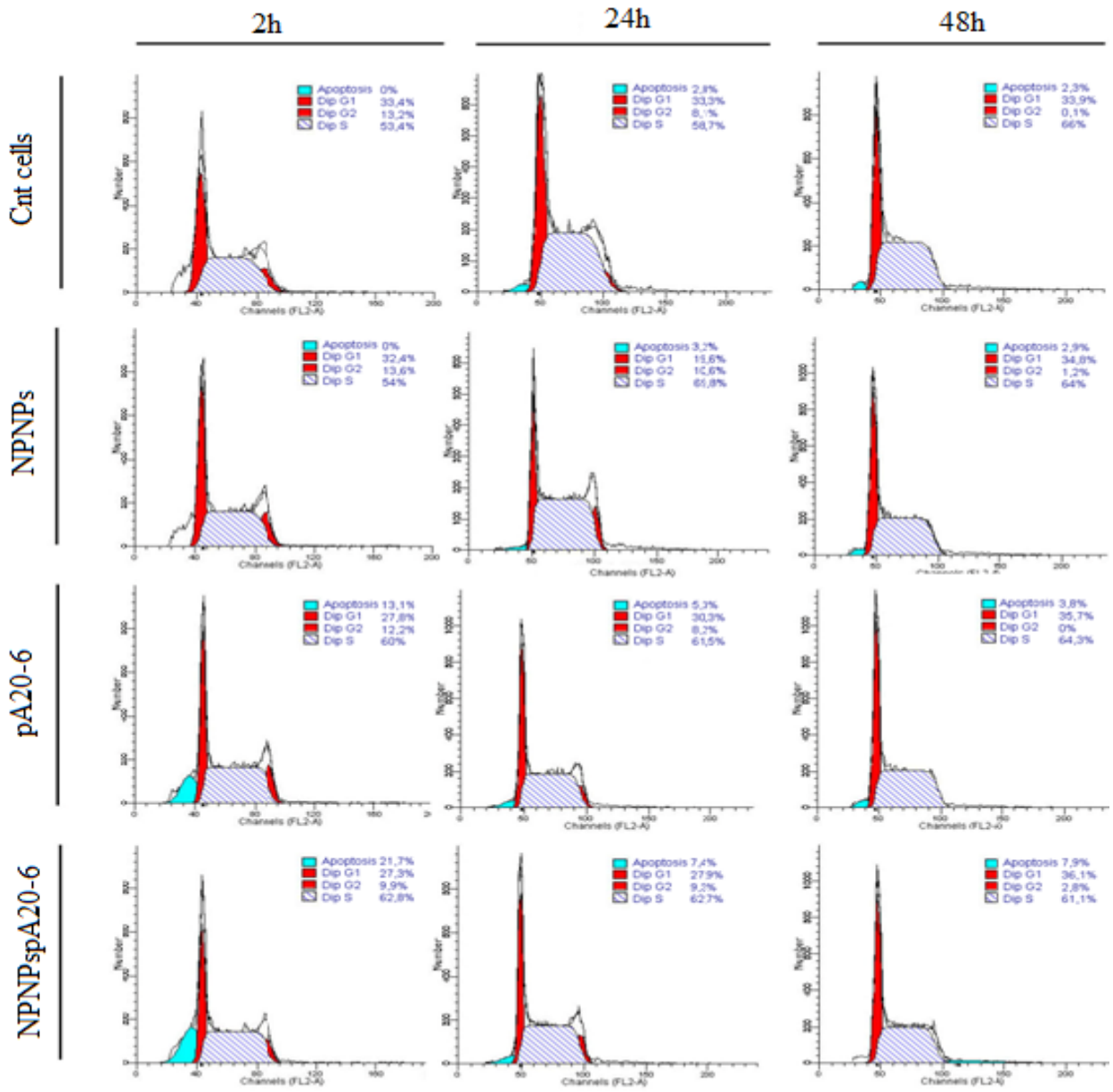
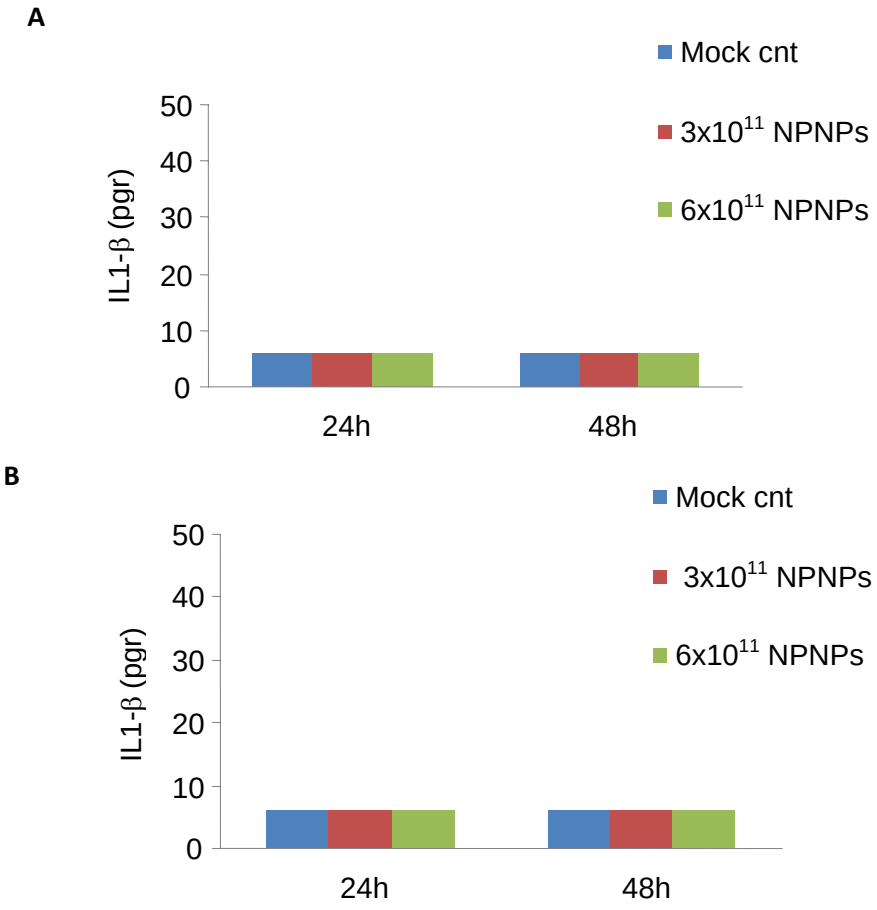


Figure S8: *Cell cycle analysis of A20 cells after NPNPs, free peptide, and pA20-6/NPNPs adminstration.*

7. In *vitro* cytotoxicity assay

HeLa cells (70% confluency) (A) and A20 ($2x10^5$/ml) (B) were seeded in a 6-well plate and incubated with about 3×10^{11} and 6×10^{11} NPNPs of 50 nm (these amounts of NPNPs can deliver 10 μg and 20 μg respectively). After 24h and 48h, supernatant from treated cells were collected and analyzed for inflammatory cytokine IL-1β production by ELISA (E-Bioscience). As shown, no

evidence of secretion of the inflammatory cytokine IL1-β was found in comparison with control cells.



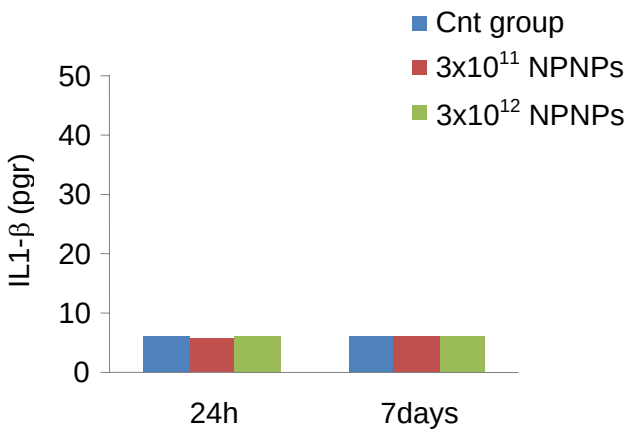
7. *In vivo* cytotoxicity assay

Two groups of Balb-C mice were injected *in vivo* with different amounts of 50 nm NPNPs (3×10^{11} and 3×10^{12} NPNPs, which are able to deliver 10 μg and 100 μg of peptide, respectively) in 100 μl of PBS. After 24h or 7 days after injection blood samples were collected and sera were analyzed for

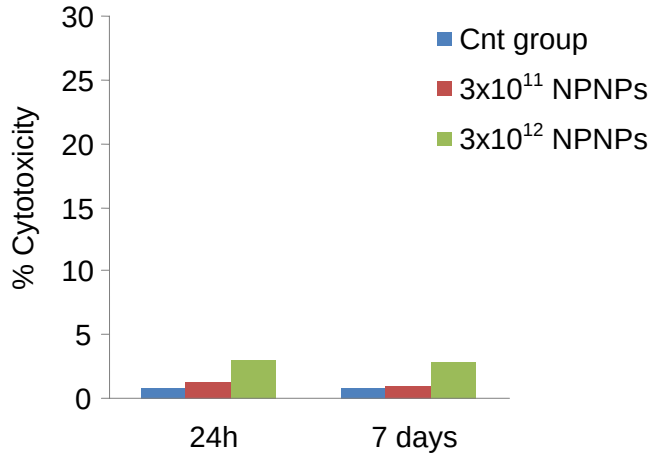
LDH, GSH and IL1-β production. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cell lysis, while changes in glutathione (GSH) levels are important for assessing toxicological responses.

LDH levels were measured by a colorimetric assay (Promega); GSH was detected by a luminometric assay (Promega) and inflammatory cytokine IL-1 β production was testes by ELISA (E-Bioscience). As shown, no evidence of secretion of the inflammatory cytokine IL1-β, nor LDH were found in the sera of mice after 24h and 7 days from the NPNPs injection (A) in comparison with control group. In panel C, table shows no differences in GSH level in the whole blood lysate from control and NPNPs injected mice.

A



B



C)

Sample	GSH (mM)
$3x10^{11}$ NPNPs (24h)	0,0570
$3x10^{12}$ NPNPs (24h)	0,0570
Control group	0,0570

$3x10^{11}$ NPNPs (7 days)	0,0570
$3x10^{12}$ NPNPs (7days)	0,0570

References

1. Kim, B.S., *Mechanisms of idiotype suppression. II. Requirement of specific antigen for B cell inactivation by anti-idiotype antibody.* J Immunol, 1979. 123(6): p. 2499-504.