SUPPLEMENTARY INFORMATION

Facile fabrication of bioactive ultra-small protein-hydroxyapatite nanoconjugates via liquid-phase laser ablation and their enhanced osteogenic differentiation activity

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SUPPLEMENATARY METHOD

SM1 Prior analysis, excess of proteins (unbound) in the colloidal solution was removed using an ultracentrifugation system (OPTIMA centrifuge with a TLA-55 Fixed Angle Rotor). Optimal parameter for total removal of the BSA protein excess could be achieved at 22,400 RCF (Relative Centrifugal Force), while BSA protein were found in prior tests to sediment not until a speed of 89,600 RCF is reached (data not shown). Supernatant containing unbound proteins was transferred into a blank glass vessel for later UV-Vis analysis, while Ha-CNPs containing pellet was taken up in 200 µL deionized Water (dH_2O) and washed by two additional centrifugation steps at 22,400 RCF. Supernatants of washing steps were added to supernatant of first centrifugation step and pellet was finally taken up in 1000 µL dH_2O and stored in another glass vessel.

SM2. The amount of protein BSA bound on one NP surface was is estimated by the following relation,

$$q = (C_i - C_t)*V/m \quad (1)$$

where q is the amount of protein BSA adsorbed onto a unit mass of NPs. C_i is the concentration of BSA protein in the initial solution and C_t is the concentration of BSA in the supernatant, both estimated from UV–VIS absorption (figure S5). (C_i- C_t) is found to be 17 μM indicating an attachment of 17% percent of the initial BSA protein concentration on the Ha-NPs surface. V is the volume of the aqueous phase, and m is the total mass of Ha-NPs produced in solution. The total mass of the Ha-NPs is manually calculated starting from the concentration of calcium (Ca) atom in the solution, estimated by ICP-EOS analysis, 140,6 $mg.L^{-1}$. The mass of the other elements (P, O, H) was obtained taking in consideration the stoichiometric ratio between Ca and the others elements in the HA-NPs hexagonal structure (figure 3, figure S4, table 1S). The calculated total mass of Ha-NPs produced is 0,623 mg. Using (1), the amount of BSA protein bound onto a unit mass of NP is determined to be, $q = 27.10^{-6}$.

SUPPLEMENATARY FIGURES

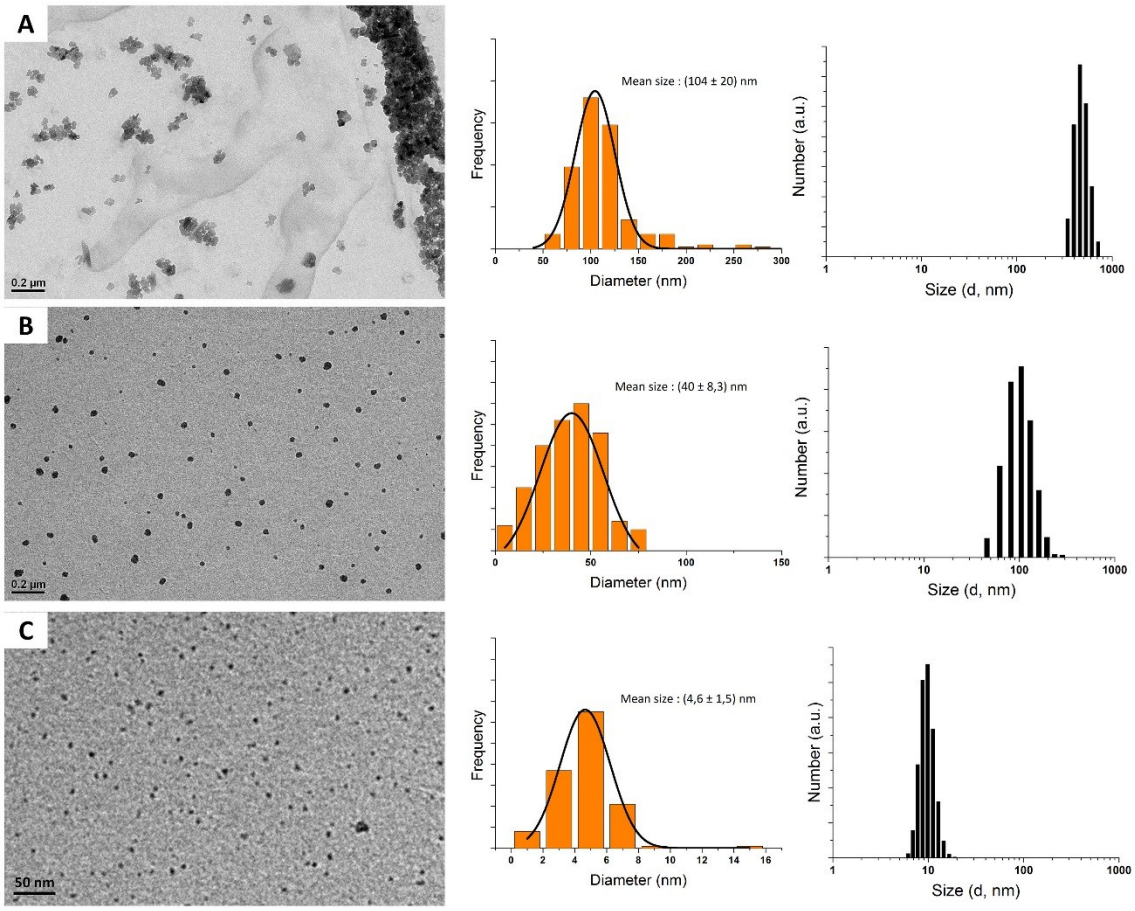

Figure S1: TEM analysis of conjugated hydroxyapatite colloidal solution obtained by infrared picosecond laser ablation of HA target in BSA protein aqueous solution at different concentrations, (A) 10^{-9} M, (B) 10^{-5} M and (C) 10^{-4} M. On the right side, the corresponding distribution histograms and DLS measurements are shown. The distribution size values of the conjugates were obtained using imaging Image J software analysis of the electron microscopy images.

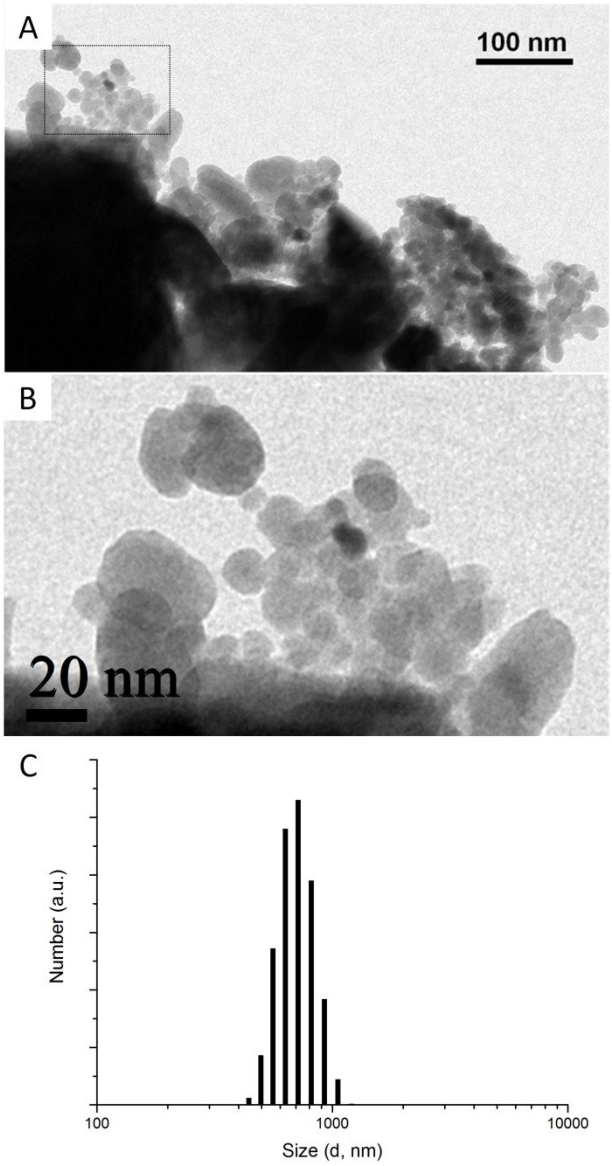

Figure S2: A) TEM observation of Ha-NPs colloidal solution prepared by infrared picoseconds laser ablation of HA target in deionized water; B) Magnification of the area signed with the black dashed line: it evidences that small nanoparticles are attached to each other in a cluster; **C)** DLS analysis of the as-synthesis Ha-NPs colloidal solution prepared in water exhibiting a mean size of (700 ± 200) nm.

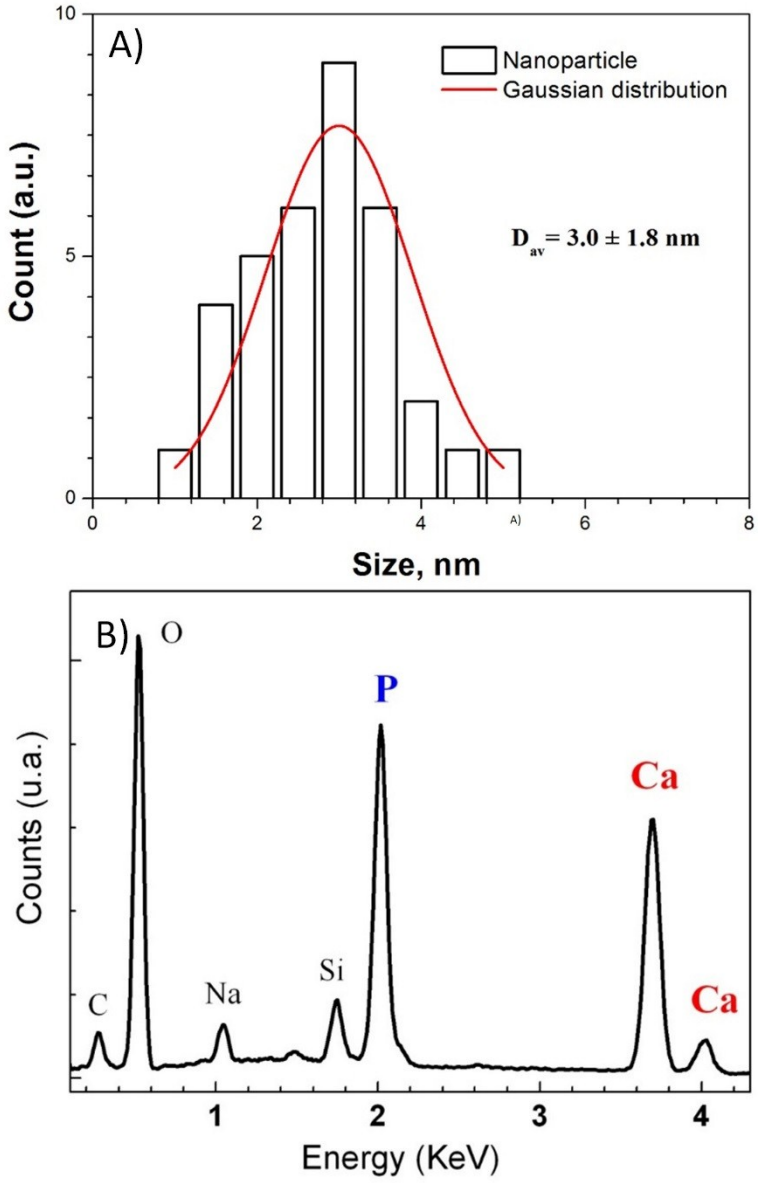

Figure S3: A) Size histogram of as-synthesized Ha-CNPs conjugates prepared in 10^{-4} M BSA solution. Of note, the mean size corresponds to the Ha core since organic, i.e. protein are removed under high energy electron beam. B) EDX profile of ultra-small HA-CNPs prepared by picosecond infrared laser ablation of HA target in protein solution. Peaks corresponding to the Phosphate (P) and Calcium (Ca) elements of HA-NPs are observed. The other peaks, Si, Na, and C come from Silicon substrate, deionized water and air contaminations, respectively. Standardless chemical quantification was achieved with Aztec Energy EDX Software.

Elements	C	O	Na	P	Ca
Atomic (%)	5.64	58.02	1.58	13.10	21.63

Table S1: Atomic percent of each element from Aztec Energy EDX Software, revealing that Ca/P stoichiometric ratio value is near to 1.65 ± 0.02, in agreement with HA bulk.

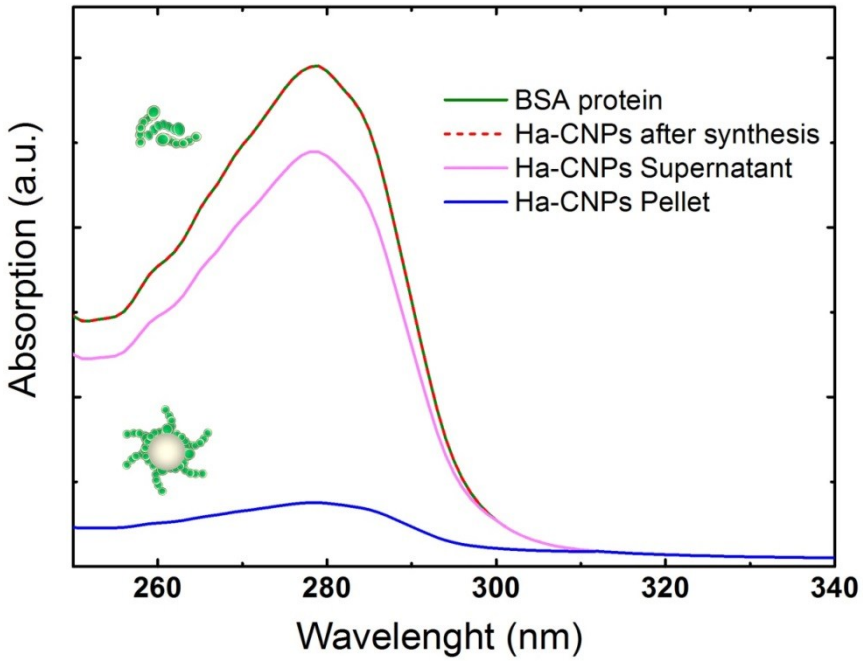

Figure S4: Absorption spectra of native biomolecule solution (green line), HA-CNPs samples directly after ablation (red dashed line), supernatant (magenta line) and pellet (blue line) after ultracentrifugation at 22,400 RCF. Compared to the protein absorption band prior to ablation, the peak intensity of protein was found to be nearly the same for HA-CNPs directly after ablation (red dashed line), indicating a high degree of molecule integrity.

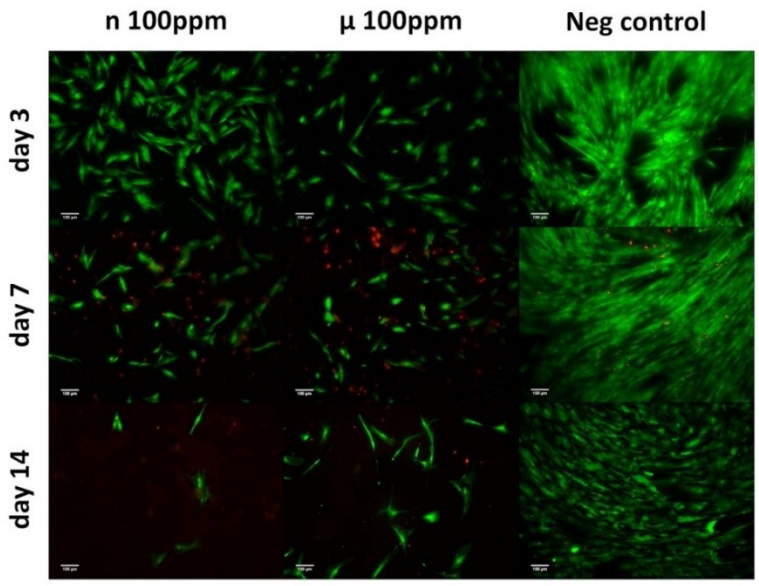

Figure S5: Live/dead staining on hMSCs treated with laser-synthesized HA-CNPs (100 ppm), 200 nm Ha-NPs (100 ppm) and negative control. Images taken at a fluorescence microscope at different time-points, scale bar 100 μm.

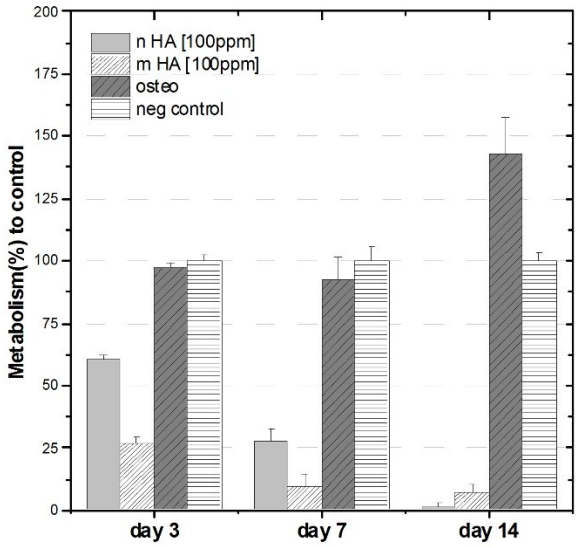

Figure S6: MTS assay on cultured hMSCs after treatment in presence of different HA particle size (ultra-small HA-CNPs and micro Ha-NPs particle) at 100 ppm. It shows a reduction in the metabolic cell state, indicating a not suitable environment for cell proliferation

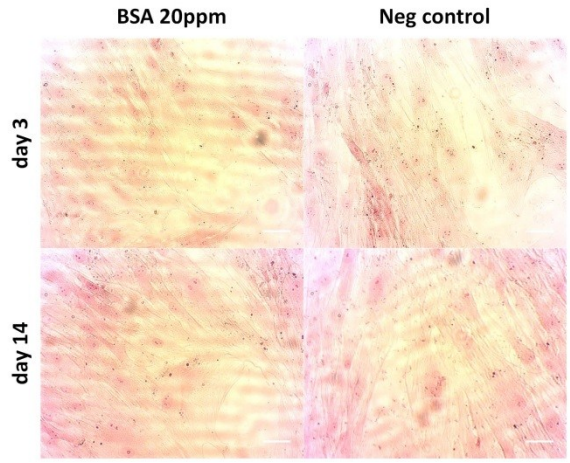

Figure S7: Alizarin Red Staining on hMSCs treated with BSA control group (20 ppm) and negative control, over experimental time. As expected, BSA presence in culture media does not promote the calcium deposition, i.e., do not contribute to the cellular osteogenic differentiation. A similar result is obtained for negative control. Scale bar 100 μm.

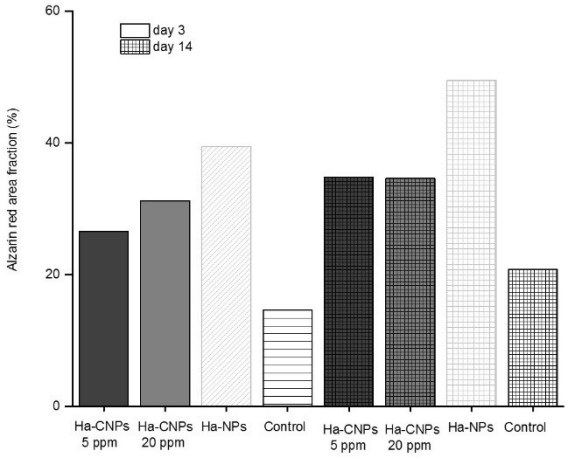

Figure S8. Analysis of alzarin red area (%) of hMSCs treated with different Ha particles. The red area fraction was calculated using an ImageJ software plugin (National Institutes of Health, Bethesda, MD, USA).