Supporting Info

Nanoprobes for Multimodal Visualization of Bone Mineral Phase in Magnetic Resonance and Near-infrared Optical Imaging

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Size Effects blending pre modified PLGA-Aln with USPIONs for nanoprecipitation

PLGA-Aln pre modified polymer was precipitated with USPIONs as control NPs for HAp binding studies. After precipitation the NPs were investigated with TEM and revealed clustering of nanoparticles (Figure S1). The black dots in the NPs shows encapsulated iron oxide. Delsa measurements showed a size of 237 nm with a PDI of 0.263, which is indicating the sample were aggregated.

Figure S1: TEM images of pre functionalized PLGA-Aln NPs.

Table S1: Size of NPs after post modification of the NPs surface with Alendronic acid.

The total amount of polymer used was 5mg/mg.

| PLGA-PEG (weight %) | Size (average ± SD) [nm] | PDI |
|---------------------|--------------------------|-----|
| 0                   | Aggregated               | -   |
| 1                   | Aggregated               | -   |
| 5                   | 786 ± 12                 | 0.21|
| 10                  | 284 ± 3                  | 0.24|
Synthesis of alendronic acid sodium salt

The reaction scheme for the synthesis of alendronic acid is shown in Figure S2A. The reaction was conducted modifying a previously published method\(^5\) by using PCl\(_5\) instead of PCl\(_3\), which yielded 78% of product. The structure of alendronic acid was analyzed using IR, \(^1\)H NMR and \(^31\)P NMR spectroscopy (Figure S2B, Figure S2C and Figure S2D). Characteristic stretches of 4-aminobutyric acid at 2188 cm\(^{-1}\), 1568 cm\(^{-1}\), 1393 cm\(^{-1}\), 1124 cm\(^{-1}\), 986 cm\(^{-1}\) and 767 cm\(^{-1}\) were not present in the product while stretches OH 3421 cm\(^{-1}\) and typical stretches for P-OH 1161 cm\(^{-1}\), 1061 cm\(^{-1}\), and 923 cm\(^{-1}\) were detected. In \(^1\)H NMR (300MHz, D\(_2\)O) the peaks for aminobutyric acid at 1.88 ppm (2), 2.30 ppm (3) and 2.95 ppm (1) were not visible in the product spectra, while the peaks for alendronic acid at 2.03 ppm (i) and 3.07 ppm (ii) were observed. The peak at 17.4 ppm in the \(^31\)P NMR originates from the phosphate group of alendronic acid and the peak at 0 ppm indicated residues of H\(_3\)PO\(_4\). IR, \(^1\)H NMR and \(^31\)P NMR proved the successful synthesis of alendronic acid using PCl\(_5\).
**Figure S2**: Synthesis and characterization of alendronic acid. A: Reaction scheme for the synthesis of alendronic acid from 4-aminobutyric acid. B: IR spectroscopy of 4-aminobutyric acid (red) and alendronic acid (black). C: $^1$H NMR spectra of 4-aminobutyric acid (red) and alendronic acid (black). D: $^{31}$P NMR of alendronic acid.

**Surface characterization of pre Aln modified NPs**

Freeze-dried NHS-NPs were measured with a SEM equipped with an EDX detector (Figure S3). Like in the other samples of PLGA NPs, mostly abundant elements were C (47.16 %) and O (51.73). Phosphor was not found. The sample contained also iron, which was encapsulated in the NPs.
**Figure S3**: EDX spectra of PLGA-NHS NPs.

**Figure S4**: $^{31}$P NMR (1280 scans) of PLGA-PEG/PLGA NPs with Alendronate addition, after dialyzing.
NP stability of Aln-PLGA NPs in media

The Aln-PLGA NPs were stored without light at RT for 7 days and measured in a 1:10 dilution with deionized water using DLS (Figure S5). The NPs showed high stability with a size of 211 nm ± 3 nm, even for a long time period in cell-conditioned media.

Figure S5: Size distribution of Aln-PLGA NPs.

Quantitative imaging of HAp surface binding of Aln-PLGA NPs

NPs in deion water (5mg/mL) were incubated with 20 mg HAp (fin cheramica) and put on a shaker (700 rpm) for different periods of time (1 - 6h) (Figure S6). After the reaction the NPs were removed and the HAp was washed for several times with deionized water. Dried HAp samples were analyzed after gold sputtering using SEM in high vacuum. After one hour the NPs can already be seen on the surface of the NPs. After
2 hours there is increase NP coverage on the HAp surface. Within 3 and 6 hours a
difference could not be distinguished, indicating a saturated surface.

**Figure S6**: SEM images of HAp substrate incubated for different periods of time with
BPh-NPs (Scale bar is 5 µm).

**HAp binding abilities of Aln-PLGA NPs in different aqueous solutions**

To investigate whether bone binding NPs absorb in different media (PPS and cell
conditioned media), adsorption studies were conducted. Pristine HAp sowing a smooth
surface is shown in **Figure S7A**. When incubating HAp with Aln-PLGA NPs in cell-
conditioned media the surface was well covered (**Figure S7B**).
Figure S7: NP binding after 3 hour of incubation in different. A: Pristine HAp. B: Aln-PLGA NPs in cell conditioned media. (Scale bar is 3 µm). All samples were sputter coated with gold and analyzed in high vacuum.

Osteoblasts differentiation was confirmed using Quantitative Real-Time PCR (RT-PCR)

Briefly, total RNA was extracted using RNeasy Mini Kit and reverse transcription of mRNA to cDNA was done using QuantiTect reverse transcription kit. RT-PCR was performed using Rotor-Gene SYBR-Green PCR assay on Rotor-Gene Q machine (Qiagen, Germany), with the following thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All biological samples were measured in triplicates, and the resulting data was normalized to housekeeping gene (18s rRNA). Bone sialoprotein and Alkaline phosphatase respectively showed, 12 and 80 fold upregulation compared to undifferentiated human mesenchymal stem cells (Figure S8).
**Figure S8:** PT-PCR measurement to evaluate osteogenic differentiation of human bone marrow mesenchymal stem cells.

**Osteoblast derived calcium deposits were visualized using alizarin red staining**

Alizarin red staining is used to identify calcium in mineralized tissues. After three osteogenic differentiation of MSCs, the cell layer in the petri dish was washed with PBS 3 times, fixed for 10 min in 3.7 % formalin at RT, stained with alizarin red 2 % for 20 minutes and washed with tap water. Human MSC were able to mineralize matrix abundantly after 3 weeks of culture in osteogenic medium, as demonstrated by a strong alizarin red-s staining (**Figure S9**).
Figure S9: Alizarin Staining of mineralized matrix secreted by MSC derived osteoblasts.

\[ y = 51.1x + 13.99 \]

\[ R^2 = 0.727 \]
Figure S10: R2 values from the CPMG measurements of HAp binding NPs in agarose.