

Effect of nanosized hydroxyapatite on acidic cell compartments of the macrophage-like cell line THP-1

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Materials and methods.

A 1.0 M aqueous solution of ammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) was added dropwise to a 1.0 M solution of calcium nitrate hexahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, USA) at room temperature under constant stirring (200 rpm, overhead stirrer) for 60 minutes at a molar P:Ca ratio of 0.6:1. The pH was maintained at 10 throughout the synthesis by the addition of an aqueous ammonia solution (SigmaTek, Russia). The resulting precipitate was aged in the mother liquor under a fume hood for 24 hours. Subsequently, the precipitate was separated by vacuum filtration using a Büchner funnel (filter pore size 40–60 μm). The collected precipitate was resuspended in distilled water and dried; this washing cycle was repeated until the suspension conductivity fell below 10 $\mu\text{S}/\text{cm}$ and the pH reached a neutral value of 7, ensuring the complete removal of ammonia and ammonium nitrate. During the final washing iteration, drying was stopped immediately after supernatant removal to prevent particle agglomeration and to preserve the paste's flowability (**Figure S1, b**). $\omega_{\text{H}_2\text{O}} = 71 \pm 11 \text{ wt. } \%$.



Figure S1. Photo of the as-synthesized material.

Powder X-ray diffraction analysis was performed on an XRD-6000 diffractometer (Shimadzu, Japan) using $\text{CuK}\alpha$ radiation without a monochromator, operating at 40 kV and 100 mA with a step size of 0.02° . Prior to analysis, samples were dried at 37°C to constant weight and ground in an agate mortar.

‡ The size of the obtained particles was measured by DLS using a Zetasizer Nano ZS analyzer (Malvern Instruments, UK). For analysis, the paste was resuspended in distilled water at a 1:9 ratio. The measurement was carried out for 5 minutes, $\chi^2 < 0.01$.

§ The study employed THP-1 monoblastic cells, acquired from the American Type Culture Collection (ATCC, Wesel, Germany). Cell cultures were maintained at 37°C in a 5% CO₂-humidified incubator, using RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, and 40 µg/mL gentamicin sulfate.

To induce differentiation into macrophage-like phenotypes (THP-1PMA), THP-1 cells were exposed to 100 nM phorbol 12-myristate 13-acetate (PMA) for a period of 72 hours [S1]. Following this treatment, the cells were thoroughly washed three times with fresh culture medium. Adherent cells were subsequently harvested using a 0.05% EDTA-trypsin solution (PanEko, Moscow, Russia).

For viability assay, cells were plated in 96-well plates (SPL Life Science, Korea) at a density of 1×10^4 cells per well in a 100 µL volume and allowed to adhere for 24 hours. Pre-sterilized (UV, 24h) HAp paste was introduced at ratios ranging from 0.1 to 10 mg/mL. The cells were then co-cultured with these materials for 96 hours. Viability was quantified via flow cytometry (BD Accuri C6, BD Biosciences, USA) following staining of the cell suspension with 200 nM Calcein AM fluorescent dye.

To evaluate lysosomal pH changes, cells were incubated overnight with 1 µg/ml LPS (E.coli 055:B5) [S1, S2] and then co-incubated with material (0.3 mg/ml) for 72 hours [S1]. They were then stained for 30 minutes within the CO₂ incubator using 50 nM per well LysoTracker Green DND-26 (Fisher Scientific, USA). As a positive control for lysosomal alkalization, a separate group of cells was pre-treated with 50 µM chloroquine (Merck Millipore, USA) for 4 hours prior to analysis. Fluorescence measurements were performed using a BD Accuri C6 Cell Analyzer [S1].

For confocal imaging, cells were seeded onto confocal dishes (1×10^4 cells per plate, 1 mL of medium). The material was added 24 hours after seeding without medium change. For particle labeling, a 0.005% solution of Alizarin Red S was prepared, in which the paste was suspended at a ratio of 3 mg/mL. After 30 minutes of incubation under UV light, 100 µL of the suspension was added per confocal dish and incubated for 24 hours. Prior to imaging, cells were stained with LysoTracker Green according to the protocol described above. Imaging was performed using a confocal microscope across three channels: brightfield, green (500-550 nm emission), and red (600-700 nm emission), with an excitation wavelength $\lambda_{ex} = 400-500$ nm.

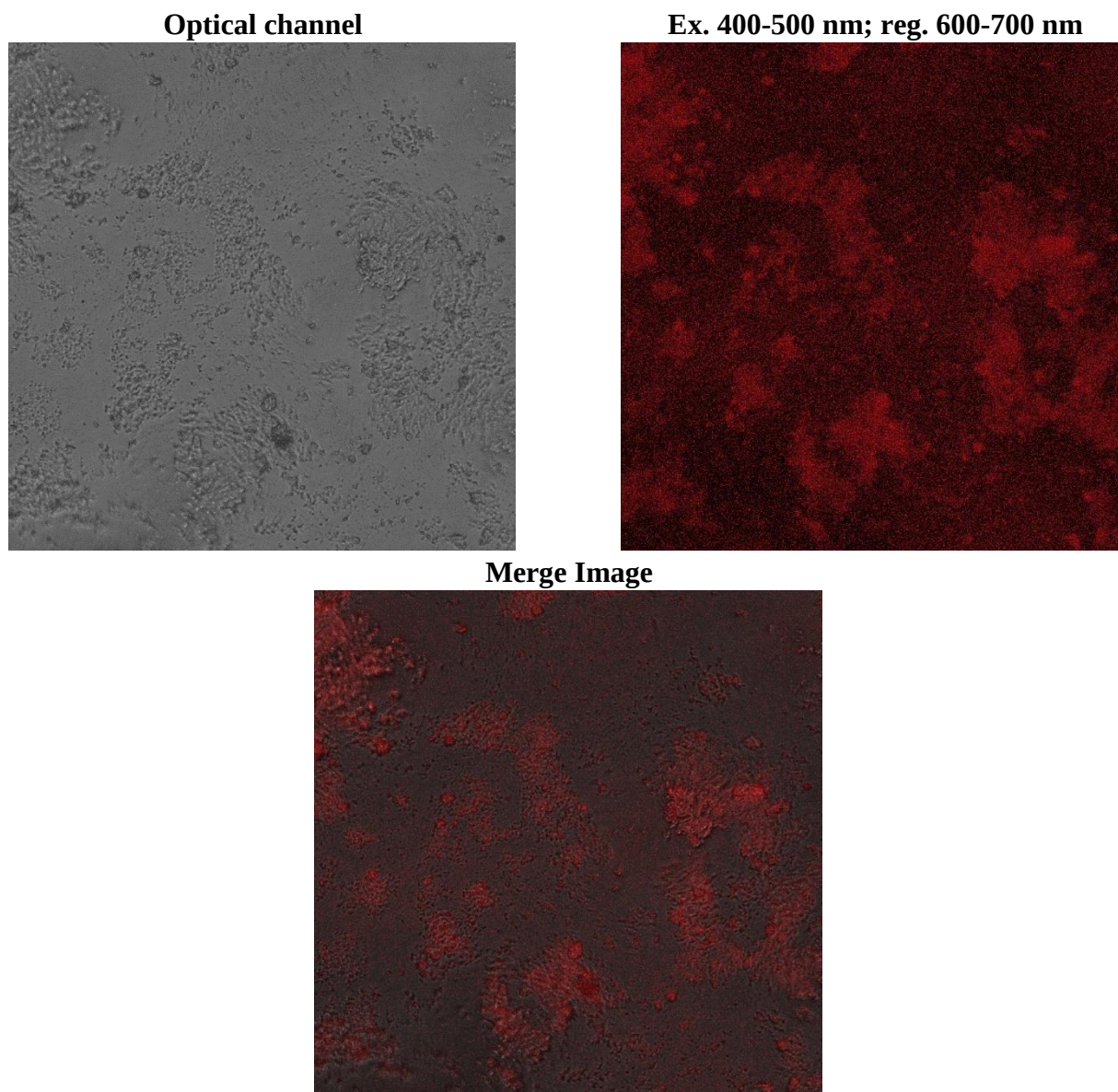


Figure S2. Confocal Image of HAp paste labeled with fluorescent dye Alizarin Red S.

References

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