



Metabolomic characterization of MC3T3-E1 pre-osteoblast differentiation induced by ipriflavone-loaded mesoporous nanospheres

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ABSTRACT

This study reports on the metabolic changes accompanying the differentiation of MC3T3-E1 osteoprogenitor cells induced by mesoporous bioactive glass nanospheres (nMBG) loaded with ipriflavone (nMBG-IP). Ipriflavone (IP) is a synthetic isoflavone known for inhibiting bone resorption, maintaining bone density, and preventing osteoporosis. Delivering IP intracellularly is a promising strategy to modulate bone remodeling at significantly lower doses compared to free drug administration. Our results demonstrate that nMBG are efficiently internalized by pre-osteoblasts and, when loaded with IP, induce their differentiation. This differentiation process is accompanied by pronounced metabolic alterations, as monitored by NMR analysis of medium supernatants and cell extracts (*exo*- and *endo*-metabolomics, respectively). The main effects include an early-stage intensification of glycolysis and changes in several metabolic pathways, such as nucleobase metabolism, osmoregulatory and antioxidant pathways, and lipid metabolism. Notably, the metabolic impacts of nMBG-IP and free IP were very similar, whereas nMBG alone induced only mild changes in the intracellular metabolic profile without affecting the cells' consumption/secretion patterns or lipid composition. This finding indicates that the observed effects are primarily related to IP-induced differentiation and that nMBG nanospheres serve as convenient carriers with both efficient internalization and minimal metabolic impact. Furthermore, the observed link between pre-osteoblast differentiation and metabolism underscores the potential of utilizing metabolites and metabolic reprogramming as strategies to modulate the osteogenic process, for instance, in the context of osteoporosis and other bone diseases.

1. Introduction

Bone remodeling is a dynamic, lifelong process in which osteoclasts degrade old bone and osteoblasts form new bone [1]. The equilibrium between the activities of these two cell types is necessary to preserve the structural integrity of the skeleton and maintain the mineral homeostasis [2]. An imbalance in this process can lead to several diseases, such as osteoporosis, caused by hyperactivity of osteoclasts *versus* osteoblasts [3]. Osteoblasts differentiate from bone-specific mesenchymal stem cells and perform the synthesis and secretion of the proteins of the bone extracellular matrix (ECM), inducing ECM mineralization and regulating osteoclast differentiation for bone resorption [4,5]. Osteoclasts are multinucleated giant cells that, in contrast, differentiate from hematopoietic stem cells and produce bone resorption through adhesion to

the surface of the bone and secretion of hydrogen ions and lysosomal enzymes that cause ECM degradation [6,7].

The differentiation processes of osteoblasts and osteoclasts from their specific precursor cells are essential for bone homeostasis and involve metabolic changes and adaptations. Studying these modifications through metabolomics can provide valuable insights into osteogenesis, osteoclastogenesis, and their association with various bone diseases, thereby advancing our comprehension of bone physiology and pathology.

Metabolomics allows for the evaluation of shifts in cellular metabolite levels in response to both intrinsic and extrinsic factors, helping to elucidate cellular behavior and to enhance the diagnosis and prognosis of diseases [8]. High-resolution Nuclear Magnetic Resonance (NMR) spectroscopy stands out as a highly reproducible, untargeted technique

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for both qualitative and quantitative analyses of a broad spectrum of metabolites, being one of the primary methods utilized in metabolomics research [9,10]. In the context of *in vitro* cell culture studies, the integration of exometabolomics (the profiling of extracellular metabolites) with endometabolomics (the analysis of intracellular metabolites) provides an invaluable perspective into the investigation of mammalian cell metabolism, by shedding light into how cells adapt to various perturbations, including diseases and drug exposures [11,12]. Numerous studies have showcased the effectiveness of NMR-based metabolomics in evaluating cellular reactions to various drugs and nanomaterials [13,14]. The benefits provided by metabolomics have catalyzed advancements in bone tissue research, streamlining the diagnosis and prognosis of bone diseases [15]. Furthermore, this approach has been employed to evaluate the therapeutic efficacy of drugs for treating osteoporosis [16], to assess biological responses to biomaterials implanted into bone [17,18], and to identify metabolic markers of stem cells' osteogenic differentiation [19–21].

In this study, we evaluated the effects of mesoporous bioactive glass nanospheres (nMBG) loaded with ipriflavone (nMBG-IP) on the metabolism of MC3T3-E1 osteoprogenitor cells, a relevant *in vitro* model of osteogenesis [22], using NMR metabolomics. nMBG are porous nanosystems primarily composed of SiO₂ and CaO, which stimulate the regeneration of bone tissue even in osteoporosis conditions, when implanted *via* intraosseous injection [23]. Their porous structure allows for the incorporation of various drugs through a simple impregnation process, providing a synergistic effect with their inherent osteogenic activity [24]. In this context, IP, a synthetic isoflavone, is known to inhibit bone resorption, maintain bone density, and prevent osteoporosis [25]. Delivering IP through nanoparticles for intracellular release is a highly promising approach, as it allows for a much lower drug dosage compared to conventional free drug administration, while still achieving the desired biological effect on bone cells [26,27]. In this work, we compared the metabolic responses of MC3T3-E1 pre-osteoblasts to unloaded nanospheres, IP-loaded nanospheres and free IP, to determine how the physicochemical composition of the biomaterial or/and the IP released intracellularly modulate different metabolic pathways. These studies are critical to understanding how both the biomaterial and the drug influence the proper differentiation of pre-osteoblasts to osteoblasts, which are key to new bone synthesis and play a pivotal role in osteoporosis settings.

2. Experimental procedures

2.1. Synthesis and characterization of mesoporous SiO₂-CaO nanospheres (nMBG) and ipriflavone loading (nMBG-IP)

Mesoporous bioactive nanoparticles (nMBG) were synthesised to have a nominal composition of 75 SiO₂-20 CaO - 5P₂O₅ (% mol). In order to obtain a hollo core-radial shell structure, nMBG were prepared in the presence of poly(styrene)-*block*-poly (acrylic acid) (PS-*b*-PAA) and hexadecyltrimethylammonium bromide (CTAB), two different amphiphilic molecules that behave as structure-directing agents. For this purpose, the stoichiometric amounts of tetraethyl ortosilane (TEOS), triethylphosphate (TEP) and calcium nitrate tetrahydrated were used as precursors of SiO₂, P₂O₅ and CaO, respectively. The mixture was magnetically stirred for 24 h, and the nanoparticles were collected by centrifugation. Thereafter, the solid was dried at 30 °C under vacuum conditions and subsequently calcined from room temperature to 550 °C for 4 h to remove the organic component. A detailed description, including amounts, reaction times and synthesis conditions, is included in supporting information.

Ipriflavone was subsequently into the porous structure of nMBG by the impregnation method, using an oversaturated solution of IP in acetone and pouring nMBG on it for 24 h under orbital stirring (see supporting information for details).

The mesoporous structure of nMBG and nMBG-IP was determined by

transmission electron microscopy (TEM) (JEOL-1400 microscope, JEOL Ltd., Tokyo, Japan) equipped with a EDX analyser (Oxford instrument). Nitrogen adsorption analysis was performed in a 3Flex analyser (Micromeritics, Norcross, GA, USA). The amount of IP was determined by thermogravimetry analysis (Pyris Diamond TG/DTA analyser, PerkinElmer Instruments).

The study of the ipriflavone release profile was carried out following the protocol previously described by Tarantili et al. [28]. IP is highly insoluble in aqueous media, so an isopropanol:water (60:40) medium is used as an approximation. For this purpose, 10 mg of nMBG-IP was incorporated into 5 mL of isopropanol:water and kept under orbital shaking at 37 °C. The release of IP was assessed by UV-VIS spectroscopy by measuring the absorbance at 273 nm at different times.

2.2. Culture of MC3T3-E1 pre-osteoblasts

MC3T3-E1 cells (kindly provided by Dr. B.T. Pérez-Maceda, CIB, CSIC, Madrid, Spain), were cultured in alpha-MEM supplemented with FBS 10 %, L-glutamine 1 mM, penicillin/streptomycin 1 %, 50 µg/mL β-glycerophosphate, and 10 mM L-ascorbic acid (differentiation medium), at 37 °C, under a 5 % CO₂ atmosphere. Cells were routinely sub-cultured every 2–3 days (when they reached 80 % confluency) by trypsinization.

2.3. Uptake of FITC-nMBG by MC3T3-E1

To evaluate the incorporation of nMBG by MC3T3-E1 pre-osteoblasts, cells cultured on coverslips were exposed to 50 µg/mL of nMBG labeled with FITC (FITC-nMBG) for 24 h, in complete culture medium. Afterward, cells were fixed with paraformaldehyde 3.7 %, followed by permeabilization with 500 µL of Triton-X100 0.1 % and incubation with BSA 1 % for 20 min. Samples were stained with 100 µL of rhodamine-phalloidin 1:40 and 100 µL of DAPI 3 µM and observed in an Olympus FV1200 confocal laser scanning microscope. FITC fluorescence was excited at 488 nm and measured at 491–586 nm. Rhodamine fluorescence was excited at 546 nm and detected at 600–620 nm. DAPI fluorescence was excited at 405 nm and detected at 420–480 nm.

2.4. Alkaline phosphatase activity (ALP)

To analyze the effect of nMBG on alkaline phosphatase activity (ALP), which is an indicator of pre-osteoblasts to mature osteoblasts differentiation [29], 2×10^4 cells/mL were seeded in 6-well plates with 2 mL/well of culture medium. Then, 50 µg/mL of nMBG, nMBG-IP or IP were added. Cells were incubated at 37 °C, under a 5 % CO₂ atmosphere for 7 and 14 days, after which the intracellular ALP content was quantified using the Reddi and Huggins' method (SpinReact S.A., Girona, Spain) [30]. The results were normalised to the protein content, measured for each condition by the Bradford's method [31].

2.5. Extracellular content of osteocalcin

The concentration of osteocalcin, an osteogenesis differentiation marker [32], in the medium of MC3T3-E1 cells incubated for 3, 7 and 14 days with 50 µg/mL nMBG, nanoMBG-IP or IP was assessed using an Osteocalcin ELISA kit (Cusabio, Houston, USA). This method is based on a sandwich ELISA in which plates are pre-coated with a highly specific antibody for osteocalcin and, after addition and incubation with the samples, a biotinylated secondary antibody is added. After several washes, the junctions are revealed with streptavidin-avidin with horseradish peroxidase in a colorimetric reaction, which is quantified in an ELISA plate reader at 450 nm, with a sensitivity of 7.8 pg/mL and a detection range of 31.25 pg/mL-2000 pg/mL. Measurements were carried out in triplicate and the standard was made with the recombinant cytokine.

2.6. Intracellular reactive oxygen species (ROS) content

Cells (2×10^4 cells/mL) were grown in 6-well plates and treated with 50 $\mu\text{g/mL}$ of nMBG, nMBG-IP or IP, for 3 and 7 days, at 37 °C, under a 5 % CO_2 atmosphere. 2',7'-dichlorofluorescein diacetate (DCFH/DA, Serva, Heidelberg, Germany) was added at a concentration of 100 μM , followed by a 30 min incubation. Flow cytometry analysis was then performed using a FACScalibur Becton Dickinson flow cytometer, by exciting the sample at 488 nm and collecting the data with a 530/30 band pass filter. At least 10^4 cells were analyzed in each sample to ensure statistical significance. The conditions for data acquisition and analysis were established with the CellQuest Program of Becton Dickinson.

2.7. Evaluation of plasma membrane properties

To evaluate the effects of nMBG and nMBG-IP nanospheres on the fluidity of two regions of the MC3T3-E1 pre-osteoblast plasma membrane, 10^5 cells/mL were cultured in the absence or presence of 50 $\mu\text{g/mL}$ of nMBG and nMBG-IP for 24 h. Then, cells were collected, resuspended in 300 μL of culture media and incubated with 9 μM of the probes DPH and TMA-DPH for 30 min. Fluorescence polarization and anisotropy were measured as indicated in previous studies [33], using a Perkin-Elmer MPF-44E spectrofluorimeter equipped with a polarization attachment. Temperature in the cuvettes was controlled at 25 °C with a Lauda thermostatically controlled circulating water bath.

2.8. Cell culture for metabolomics

MC3T3-E1 cells were seeded at a density of 10^6 cells/flask in F-75 flasks (Thermo Fisher Scientific, Denmark) and incubated for 24 h. Then, 50 $\mu\text{g/mL}$ of nMBG, nMBG-IP or IP were added to different flasks, which were incubated along with control flasks (no material/drug added) for 7 days. At day 3, the medium was collected and replaced by fresh complete medium. At day 7, both medium and cells were collected and processed for analysis. Acellular medium was also incubated under the same conditions to be used as a reference for extracellular metabolomics.

2.9. Medium collection and preparation for NMR analysis

Medium samples were centrifuged at 1000 $\times g$ during 5 min and the supernatants were stored at -80 °C. In order to remove proteins, samples were further processed by adding 600 μL of cold methanol to 300 μL medium aliquots, followed by 30 min at -20 °C, centrifugation at 13000 $\times g$ during 20 min, and vacuum drying of the supernatants. At the time of NMR analysis, dried extracts were reconstituted in 600 μL of deuterated phosphate buffer (100 mM, pH 7.4) containing 0.1 mM (trimethylsilyl)propanoic acid-d4 (TSP-d4) and 550 μL were transferred to 5 mm NMR tubes (NOR508UP7 Norell® Standard Series™, Sigma-Aldrich Corporation, St. Louis, MO, USA).

2.10. Cell extraction and preparation for NMR analysis

To extract cells' polar metabolites and lipids, a biphasic extraction protocol, adapted from the method of Carrola et al., was employed [14]. In brief, cells detached by trypsinization were washed twice with cold PBS, resuspended in 800 μL cold methanol 80 % in a microcentrifuge tube containing 150 mg of 0.5 mm glass beads, and vortexed for 2 min. This was followed by additions of chloroform (640 μL) and distilled water (288 μL), with 2 min vortexing after each addition. This mixture was left on ice for 10 min, and centrifuged at 10000 $\times g$ for 15 min. The resulting aqueous and organic phases were then dried in a SpeedVac Concentrator (model 5301, Eppendorf, Hamburg, Germany) or under a nitrogen gas flow, respectively, and stored at -80 °C. For NMR analysis, aqueous samples were reconstituted in 600 μL of deuterated phosphate

buffer (100 mM, pH 7.4) containing 0.1 mM TSP-d4 and organic samples were reconstituted in 600 μL deuterated chloroform containing 0.03 % tetramethylsilane (TMS). All samples (550 μL) were transferred to 5 mm NMR tubes for analysis.

2.11. NMR data acquisition and analysis

^1H NMR spectra were acquired in a Bruker Avance III HD 500 NMR spectrometer (University of Aveiro, Portuguese NMR Network) operating at 500.13 MHz for ^1H observation, using a TXI 5 mm probe. Standard 1D spectra (pulse programs "noesypr1d", with water suppression, for medium samples and aqueous extracts, and "zg" for organic extracts) were acquired with a 7002.8 Hz spectral width, 32 k data points, a 2 s relaxation delay, and 512 scans. Spectral processing, performed in TopSpin version 4.1.0 (Bruker BioSpin, Rheinstetten, Germany), included cosine multiplication (ssb 2), zero-filling to 64 k data points, manual phase adjustment, baseline correction, and calibration of the TSP/TMS signal at 0 ppm. To aid metabolite identification, 2D spectra were recorded for selected samples, namely: ^1H - ^1H total correlation (TOCSY), ^1H - ^{13}C heteronuclear single quantum correlation (HSQC) and J -resolved spectra. 1D and 2D data were then matched to reference spectra in available databases, namely BBIREFCODE-2-0-0 (Bruker Biospin, Rheinstetten, Germany) and Chenomx NMR suite 8.6 (Chenomx Inc. Edmonton, Canada). To assess metabolite variations, normalization to total spectral area (excluding solvent signals) and scaling to unit variance (UV) were employed. Hierarchical cluster analysis (HCA), principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA) were performed in MetaboAnalyst 5.0 (Genome Canada, NIH NSERC CRC, Canada), and spectral integration was performed in AmixViewer 3.9.15 (Bruker BioSpin, Rheinstetten, Germany).

2.12. Statistical analysis

Statistical analysis was performed using one-way ANOVA, with a p -value threshold of 0.05, and Tukey's HSD post-hoc testing. Statistical differences were indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

3. Results and discussion

3.1. Characterization of mesoporous nanospheres and ipriflavone release

TEM images show that both nMBG and nMBG-IP consist of spherical particles of around 200–300 nm, with a hollow cavity in the core and a porous shell (Fig. 1). The inner core would act as an IP reservoir whereas the porous shell is thought to act as a controlled drug delivery mechanism. After IP loading, the hollow core-porous shell structure remains although the porosity appears as deteriorated.

This observation was confirmed by N_2 adsorption analysis, which indicated a strong decrease in surface area and porosity after drug incorporation (Table 1). The amount of IP incorporated was 18 ± 1.2 (%) wt) as measured by TG analysis.

Chemical analysis was carried out by EDX spectroscopy during TEM observation. Fifteen measurements of different nMBG batches were analyzed (see supporting information) showing an average chemical composition of 81.44 SiO_2 .18.6 CaO (% mol). P_2O_5 was not detected in any sample, despite the use of TEP as a phosphorous precursor.

The *in vitro* release profile of IP (Fig. 2) showed a rapid drug release during the first 5 h which, however, accounts for only 17 % of the total IP loaded on the particles. This initial release likely corresponds to the dissolution of the adsorbed IP in the outermost radial mesoporous shell region of the nMBG. Subsequently, the release rate slowed until the 50-h time point, reaching 23 %, which likely reflects the diffusion of IP from the innermost regions of the nMBG. After this point, despite the assay being performed in an isopropanol:water medium in which the IP is

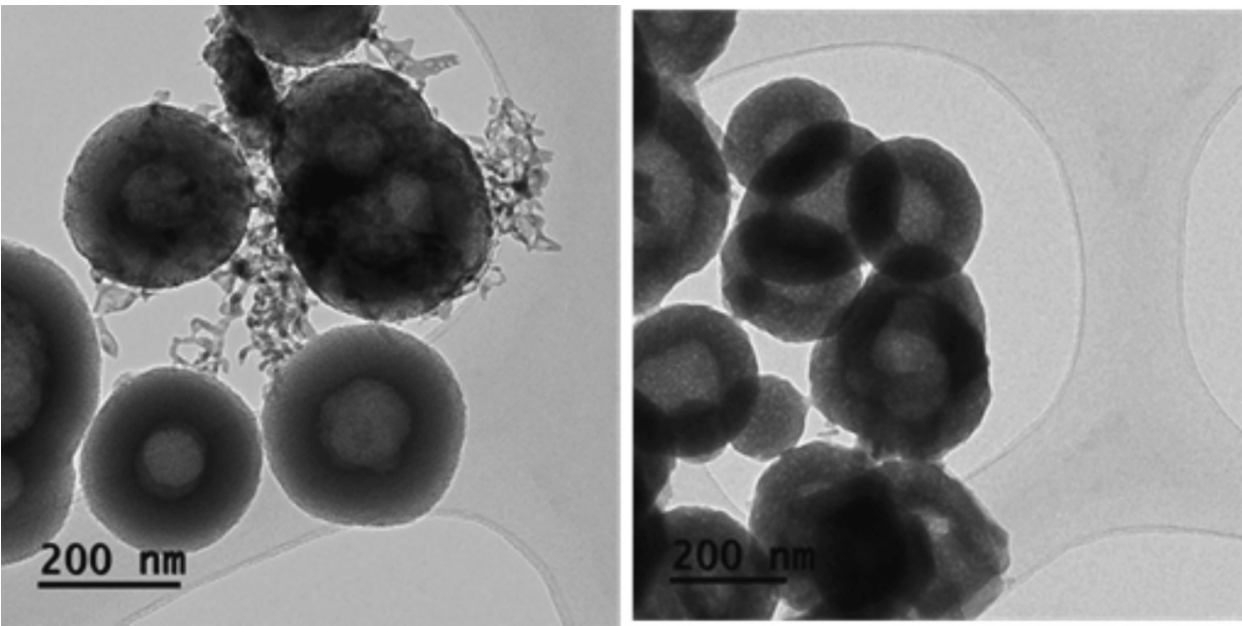


Fig. 1. TEM images of nMBG (left) and nMBG-IP (right).

Table 1
Textural parameters and IP content of the synthesised samples.

Sample	Surface area (m ² ·g ⁻¹)	Pore volume (cm ³ ·g ⁻¹)	Ipriflavone (% wt)
nMBG	543.6	0.43	–
nMBG-IP	14.4	0.06	18

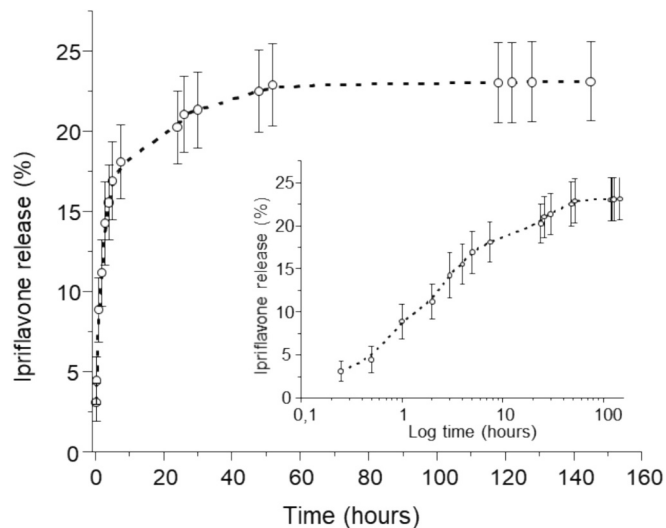


Fig. 2. Ipriflavone release study in isopropanol: water (60:40) medium as a function of time. The inset shows this profile in logarithmic scale for better visualization at short times.

soluble, the release of the drug ceased, and no additional release was observed during the remainder of the week-long assay.

The release study indicates that about 77 % of the IP content is retained inside the central hollow cavity of the nanoparticles, releasing only 23 % through dissolution/diffusion processes. The rest of the drug would only be available in the long term after degradation of the nMBG, most likely after cellular internalization. In this sense, the degradation of silica-based nanoparticles is of interest to many research groups in the field of biomedical sciences [34]. The evidence obtained so far points

out that the degradation rate is highly dependent on the surface area, the condensation of the silica network and the surrounding fluid (especially pH), while the size of the nanoparticles seems to be less relevant [35]. Silica nanoparticles are hydrolytically unstable and dissolve over time in the form of silicic acid, Si(OH)₄, in biological or biologically relevant solutions (SBF, PBS, DMEM, etc.). The time required for complete degradation ranges from 24 h to several weeks, depending on the physicochemical characteristics outlined above. In this sense, increasing the degradation rate is an effective strategy to promote the release of different active compounds, including anti-inflammatory, osteogenic, antitumor and antibiotic drugs [24]. In the specific case of mesoporous bioactive nanoparticles prepared in the SiO₂-CaO system, the presence of Ca²⁺ cations acting as network modifiers favours nanoparticle degradation. The degradation of the silica network is favoured by Ca²⁺ leaching to the surrounding medium [36], which is more intense under acidic conditions, thus triggering drug release has been previously observed for doxorubicin [37]. Our results demonstrate that nMBGs are efficiently internalized by preosteoblasts. nMBGs degradation and thus IP release is expected to be enhanced by the acidic endosomal and lysosomal environment. According to the intracellular degradation of SiO₂-based nanoparticles observed in human embryonic kidney (HEK) cells [38], human bone marrow mesenchymal stem cells (MSCs) [39] and human umbilical vein endothelial cells (HUVECs) [40], the degradation rate would occur faster during the first two days and would be slower after this period.

3.2. Nanospheres internalization and induction of osteoblastic differentiation

In this study, unloaded and ipriflavone-loaded mesoporous nanospheres (nMBG and nMBG-IP, respectively) were administered to MC3T3-E1 osteoprogenitor cells. This pre-osteoblast cell line represents the most relevant *in vitro* model of osteogenesis and has been used in previous studies to evaluate nanomaterial strategies to stimulate bone regeneration [26]. The first step for these nanotherapies to be effective is their efficient intracellular incorporation. In order to confirm the uptake of nMBG by MC3T3-E1 pre-osteoblasts, these cells were exposed to 50 µg/mL of nMBG labeled with FITC (nMBG-FITC) for 24 h and then observed by confocal microscopy. Fig. 3A shows the cytoskeleton of MC3T3-E1 cells in red, their nuclei in blue, and the internalized nMBG-

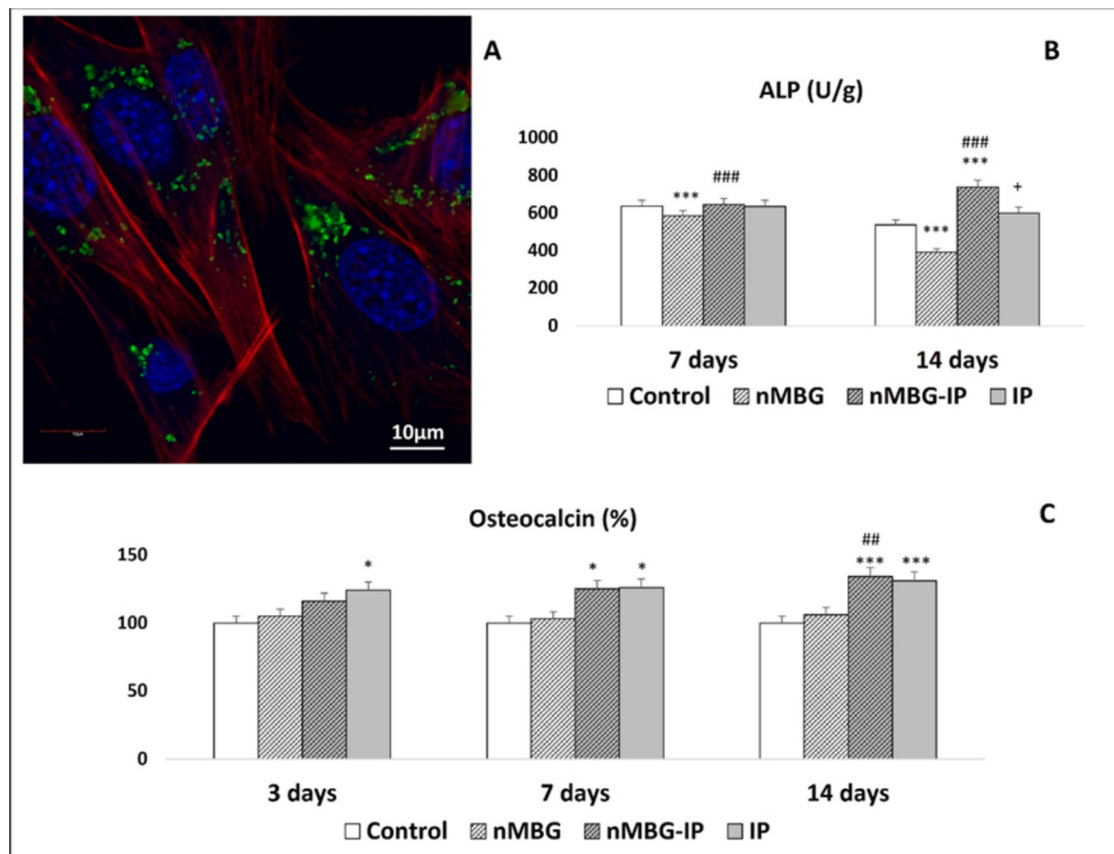


Fig. 3. A) Intracellular incorporation of nMBG labeled with FITC (in green) by MC3T3-E1 pre-osteoblasts observed by confocal microscopy. Nuclei were stained with DAPI (in blue) and F-actin filaments were stained with rhodamine-phalloidin (in red). B) and C) Effects of nMBG, nMBG-IP and IP on MC3T3-E1 pre-osteoblast differentiation, evaluated at different times through the measurement of alkaline phosphatase (ALP) activity and osteocalcin, respectively. Control conditions without treatment were performed in parallel. ALP was quantified by Reddi and Huggins' method and osteocalcin levels were determined by ELISA. Statistical significance: *** $p < 0.005$, * $p < 0.05$ (control vs conditions); ### $p < 0.005$, ## $p < 0.01$ (nMBG vs nMBG-IP); + $p < 0.05$ (nMBG-IP vs IP).

FITC in green, after 24 h of treatment with this nanomaterial. Numerous nanospheres can be observed in the cytoplasm of pre-osteoblasts. This is in accordance with our previous study, whereby the uptake of nMBG by this cell type was found to occur mainly via clathrin-dependent endocytosis and, in a lower proportion, by micropinocytosis [26].

Furthermore, to assess osteoblastic differentiation during pre-osteoblasts treatment with drug-loaded nanospheres (nMBG-IP), we have quantified alkaline phosphatase (ALP) activity and osteocalcin extracellular levels. The activity of ALP (referred to total protein content) significantly increased upon incubation for 14 days with nMBG-IP (Fig. 3B, *** $p < 0.005$). Notably, this effect was more pronounced in nMBG-IP-treated cells than in cells exposed to the free drug (* $p < 0.05$). However, unloaded nMBG induced a significant decrease in ALP activity after 7 and 14 days of treatment (*** $p < 0.005$) referred to the values obtained in the absence of nanomaterial (untreated controls). When comparing ALP activity after treatment with nMBG and nMBG-IP, significant differences were observed (### $p < 0.005$). This indicates that the ALP increase induced by nMBG-IP was due to the drug released intracellularly [25], which could counteract the decrease observed upon exposure to unloaded nanospheres, and even stimulate cell differentiation, significantly increasing the ALP value above the controls.

Concerning osteocalcin levels (Fig. 3C), both IP and nMBG-IP induced significant increases in osteocalcin production, while nMBG alone had no effect.

These results demonstrate the effective incorporation of nMBG nanospheres, as well as their ability to release ipriflavone intracellularly, thereby inducing pre-osteoblast differentiation over time.

3.3. Generation of reactive oxygen species (ROS)

Given the link between oxidative stress and osteogenesis [41], we also measured the intracellular content of reactive oxygen species (ROS) in MC3T3-E1 cells subjected to the different treatments. Fig. 4A and B show the flow cytometry graphs obtained after treating pre-osteoblasts for 3 and 7 days with nMBG, nMBG-IP or IP, and then incubating the cells with the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH/DA). This probe can penetrate cells due to its chemical nature and, once inside, it is hydrolyzed by cytosolic esterases, producing 2',7'-dichlorodihydrofluorescein (DCFH), which is instantly oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Hence, DCF fluorescence intensity is directly proportional to the intracellular ROS content. In Fig. 4A and B, M1 corresponds to the population of cells that have incorporated the probe and show green fluorescence above basal values (cells without probe). M2 corresponds to the population of cells showing higher green fluorescence values indicating higher intracellular ROS content.

The results show that all treatments induced a significant decrease in the cell population with high intracellular ROS after 7 days, which was more pronounced after treatment with IP, in agreement with the antioxidant properties of this drug [42]. In this context, other antioxidants such as melatonin have been reported to increase osteogenesis of bone marrow-derived mesenchymal stem cells, mitigating oxidative stress [43]. Furthermore, a decrease in ROS levels has been reported to accompany the differentiation of MC3T3-E1 pre-osteoblasts and of primary murine osteoblasts, due to induction of superoxide dismutase 2 (SOD2) to maintain mitochondrial redox homeostasis [44]. It should

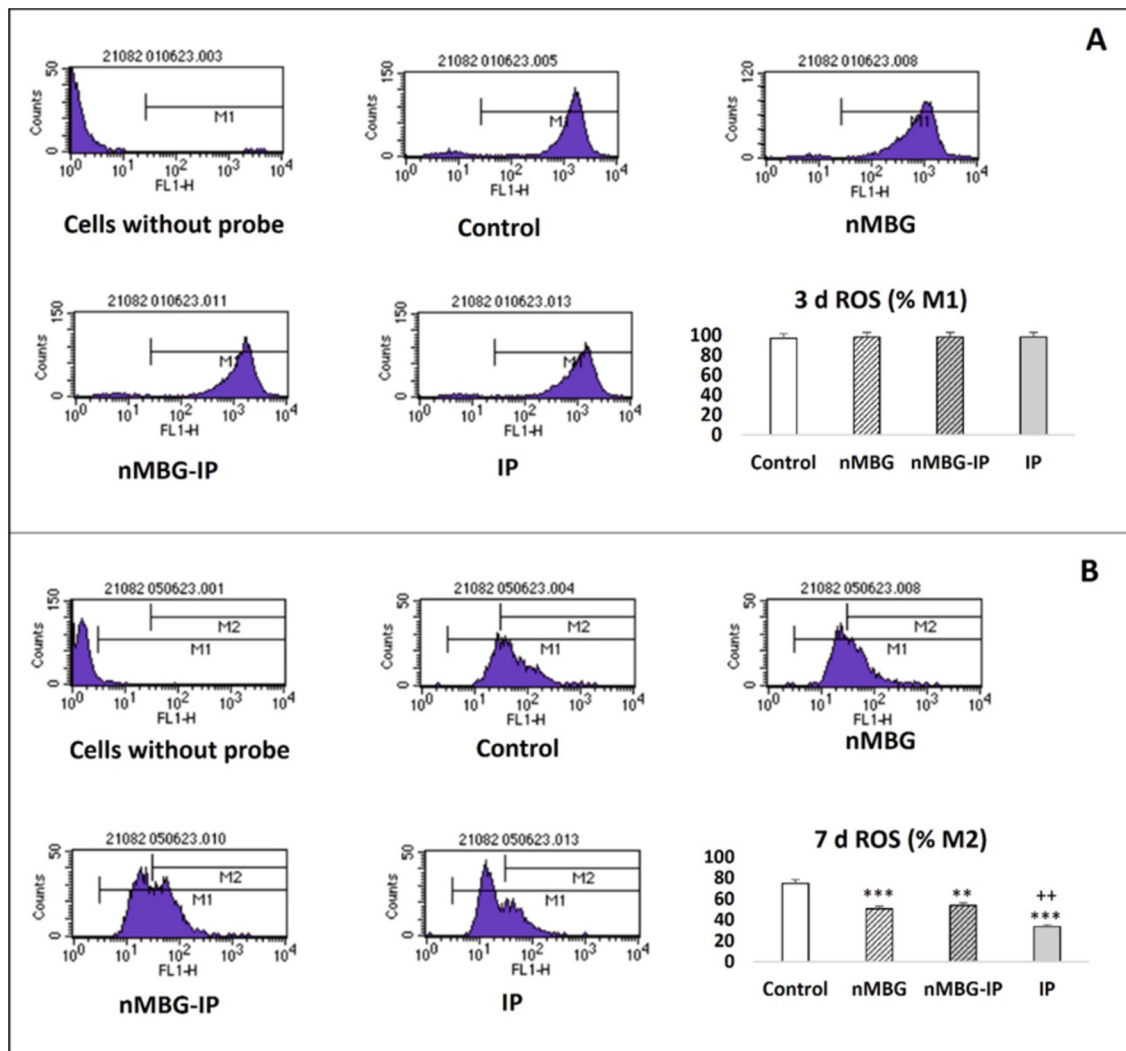


Fig. 4. Effects of nMBG, nMBG-IP and IP on intracellular ROS content of MC3T3-E1 pre-osteoblasts after 3 days (A) and 7 days (B). Control conditions without treatment were performed in parallel. M1 = cell population that have incorporated the probe and show green fluorescence above basal values. M2 = cell population with higher intracellular ROS content. Statistical significance: *** $p < 0.005$, ** $p < 0.01$ (control vs conditions); ++ $p < 0.01$ (nMBG-IP vs IP).

also be noted that nMBG also attenuated ROS production, albeit not altering ALP activity nor osteocalcin levels. Hence, it is possible that the nanospheres interfere with the cells redox balance, independently of the pro-differentiation effects.

3.4. Variations in extracellular metabolites

As a first approach to assess the impact of the different treatments on the extracellular metabolic composition, hierarchical cluster analysis (HCA) was applied to the ¹H NMR spectral profiles of medium supernatants, collected after 3 and 7 days in culture. In the resulting dendrogram (Fig. 5A), two main clusters, representing the most dissimilar groups, were seen for IP-treated cells (either free IP or nMBG-IP) and for control cells grouped together with nMBG-exposed cells. These main clusters further divided according to the timepoint of medium collection (day 3 or day 7).

A more detailed analysis was then performed, based on the relative levels of specific metabolites. The results are shown in the form of a heatmap, which includes metabolite levels in acellular media, to distinguish between metabolites consumed/secreted, *i.e.*, which decreased/increased in cells-conditioned medium compared to acellular medium (Fig. 5B). The quantitative variations relative to acellular medium are provided in Supplementary Table S1. In agreement with the

HCA results, control and nMBG-exposed cells showed a very similar metabolic activity, characterized by the consumption of sugars (glucose and fructose), several amino acids and choline, along with the secretion of several carboxylic acids and two amino acids (alanine and glycine). On the other hand, nMBG-IP and IP-treated cells displayed clearly distinct extracellular profiles from control and nMBG-exposed cells. The main differences comprised: i) higher consumption of glucose, fructose, tyrosine and phenylalanine; ii) lower consumption of glutamine, glutamate, aspartate, methionine, branched chain amino acids (BCAA: valine, leucine, isoleucine), and choline; iii) higher secretion of lactate, in the first 3 days in culture, and of acetate, in the period from day 3 to day 7; iv) lower secretion of alanine, glycine, formate, isobutyrate and α -ketoacids, namely α -ketoisocaproic (KIC), α -ketomethylvaleric (KMV) and α -ketoisovaleric (KIV) acids. In fact, from day 3 to day 7, the secretion of glycine and isobutyrate, was completely abrogated.

Overall, these results show that IP (either free or encapsulated), but not nMBG, significantly modulated the extracellular metabolic composition of MC3T3-E1 cells. Among the observed changes, free or encapsulated IP treatments induced higher glucose consumption and lactate secretion, especially within the first 3 days, which suggests increased glycolytic activity. Glucose is a preferential substrate of cultured osteoblasts, with the selective deletion on the glucose transporter Glut1 suppressing osteoblast differentiation *in vitro* and *in vivo* [45]. Through

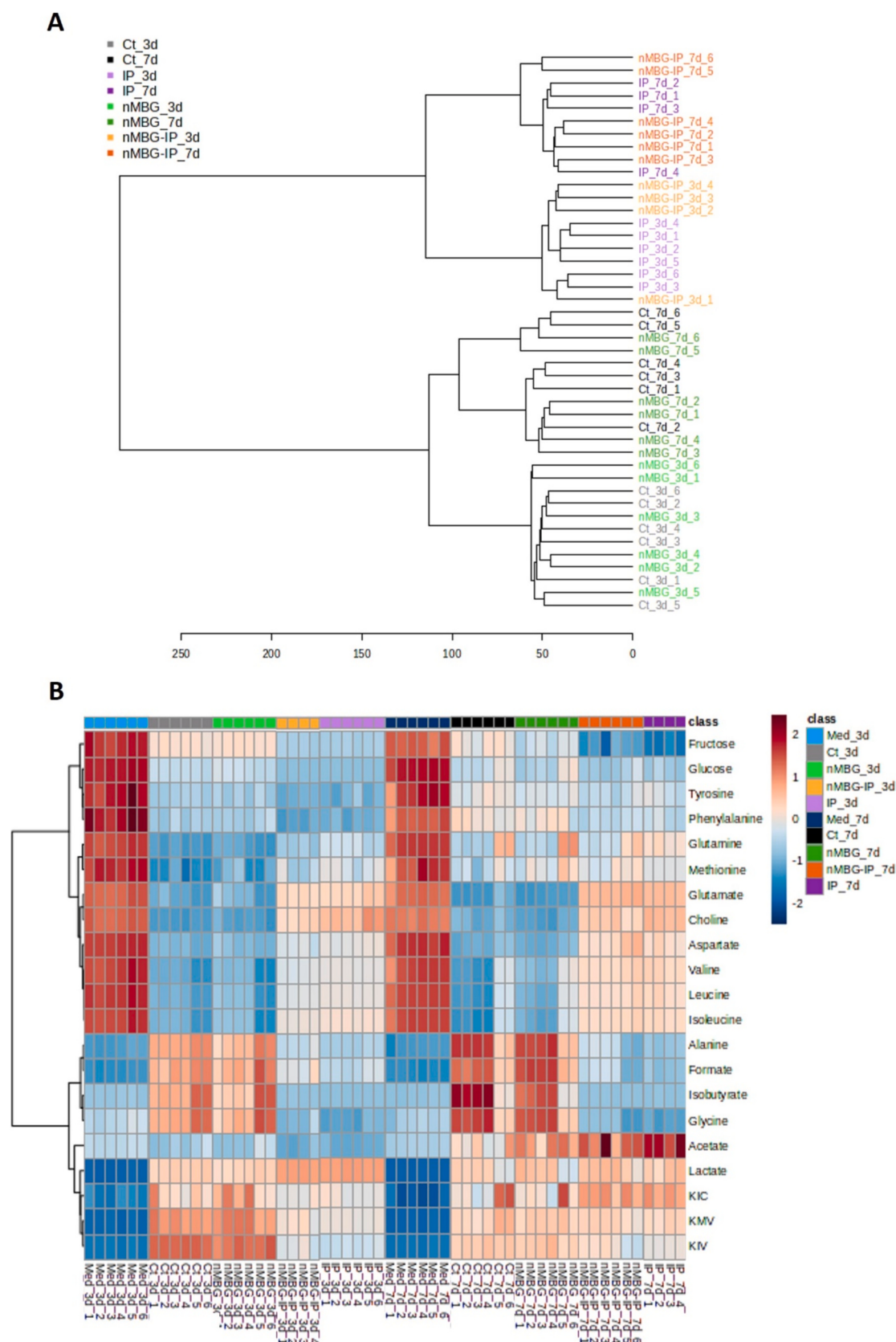


Fig. 5. A) Dendrogram obtained by hierarchical cluster analysis (HCA) of the ^1H NMR spectral profiles of medium supernatants, collected after 3 and 7 days in culture B) heatmap representing metabolite levels in acellular medium (Med_3d and Med_7d) and in the medium conditioned by cells subjected to different treatments (Ct, no treatment; nMBG, unloaded nanospheres; n-MBG-IP, ipriflavone-loaded nanospheres; IP, free ipriflavone). Relative metabolite levels are expressed based on UV-scaled signal areas.

glycolysis, glucose is converted into pyruvate, which can be transformed into lactate, or enter the mitochondria and be processed through the tricarboxylic acid (TCA) cycle to support oxidative phosphorylation (OXPHOS). In osteoblasts, numerous studies have identified lactate as a significant end product of glucose metabolism, irrespective of oxygen availability, highlighting aerobic glycolysis as a prominent metabolic feature, similarly to the Warburg effect observed in cancer cells [46]. In one study involving pre-osteoblastic cells (MC3T3-E1C4) and primary calvarial osteoblasts, both glycolysis and oxidative phosphorylation (OXPHOS) were observed to be upregulated during 21 days of differentiation, with mature osteoblasts exhibiting a greater dependence on glycolysis [47]. Similarly, another study found that the contribution of glycolysis to energy production increased from 40 % to 80 % in mouse calvarial osteoblasts between day 0 and day 7 of differentiation [48]. However, in that work, mitochondrial respiration decreased during differentiation, with mature osteoblasts displaying a lower oxygen consumption rate than undifferentiated cells. Our results appear to be in line with this observation, as cells incubated with nMBG-IP or free IP decreased the consumption of several amino acids used to fuel the TCA cycle. On the other hand, the secretion of acetate significantly increased in treated cells compared to controls, which may reflect fatty acid metabolism. Indeed, fatty acid oxidation was reported to play an auxiliary role in energy production during osteoblast differentiation, in a stage-specific manner [48–50].

3.5. Impact on intracellular metabolic composition

^1H NMR analysis of polar cell extracts enabled the detection of major intracellular metabolites, including several amino acids and derivatives, carboxylic acids (e.g., lactate and succinate), choline metabolites, and nucleosides/nucleotides (Fig. 6A). Principal Component Analysis (PCA) of these spectral profiles showed a good separation between untreated controls and cells treated with nMBG-IP or IP along PC1, which explained 57.3 % of the variance (Fig. 6B). On the other hand, untreated and nMBG-treated cells were well separated along PC2 (9.4 % variance). Discrimination between sample groups was further confirmed by PLS-DA, for which a predictive power (Q^2) of 0.9 was obtained (Fig. 6C).

In order to assess the features responsible for group separation, relative metabolite levels were determined through spectral integration and those with statistically significant differences were highlighted in the heatmap displayed in Fig. 6D. The corresponding average variations in treated vs. control cells are presented in Supplementary Table S2. The intracellular signatures of the different treatments compared to untreated controls comprised: i) increased levels of hypoxanthine, BCAA and tyrosine (common to all, albeit much more pronounced in nMBG-IP and IP groups); ii) increased levels of choline, phosphocholine, formate, acetate and proline (only in IP-containing groups); iii) decreased levels of glycerophosphocholine, myo-inositol, creatine, succinate, reduced glutathione (GSH), adenosine, and adenine/uridine nucleotides (all treatments, with higher magnitude in nMBG-IP and IP groups); iv) decreased levels of taurine, alanine, aspartate, uridine, inosine, and lactate (only in IP-containing groups). In the case of taurine, alanine and aspartate, only free IP (and not nMBG-IP) caused significant variations.

Hence, these results show that MC3T3-E1 cells significantly changed their intracellular metabolism in response to all treatments, with the greatest impact being observed for IP-loaded nMBG and free IP. The observed changes in key metabolites such as lactate, acetate, and succinate corroborate the exometabolomics results and suggest a reprogramming of energy metabolism during pre-osteoblast differentiation to meet the heightened energy demands of cellular growth and matrix production. The modulation of purine and pyrimidine metabolism, evidenced by changes in adenosine, inosine, and nucleotides, is particularly important for supporting DNA and RNA synthesis, which are essential processes during cell division and differentiation. In parallel, shifts in membrane-related metabolites, such as choline-containing compounds and myo-inositol, suggest remodeling of the plasma

membrane, potentially to support signaling pathways critical for osteogenesis, such as the activation of Runx2. Additionally, alterations in taurine and GSH levels point to an enhanced cellular antioxidant response, protecting cells from oxidative stress during differentiation.

3.6. Impact on lipid composition and plasma membrane fluidity

The ^1H NMR profile of cellular organic fractions showed contributions from major lipid constituents, including cholesterol, glycerophospholipids (GPL) and triacylglycerols (TAG) (Fig. 7A). Notably, while nMBG did not change the relative levels of these lipids, nMBG-IP and free IP caused significant decreases in the levels of membrane lipids (cholesterol and GPL), accompanied by a significant increase in TAG (Fig. 7B). Moreover, nMBG-IP/IP-treated cells showed a decrease in the total content of fatty acids (either free or as fatty acyl chains in complex lipids), in particular, total unsaturated fatty acids. On the contrary, polyunsaturated fatty acids tended to increase. In this context, higher levels of polyunsaturated lipids, phosphatidylethanolamine and phosphatidylcholine have been observed in the plasma membrane (PM) of osteoblasts in comparison with undifferentiated mesenchymal stem cell PM [51]. These results could indicate that the increase in polyunsaturated fatty acids is related to the differentiation process [52,53], which is promoted by nMBG-IP/IP, as it is shown in Fig. 3B and C. Other authors have also described changes in the proportions of different types of lipids during neural cell differentiation. For instance, studies with rat cerebellar granule cells have evidenced that cholesterol/GPL molar ratio gradually decreased during *in vitro* differentiation [54].

As it is well known, GPL are the major components of plasma membranes and play a key role in membrane stability, permeability and signal transduction which modulate cellular functions [55]. Cell membranes contain several types of GPL with different polar heads and acyl chains. The GPL composition of different cell types, organelles and inner and outer layers of the plasma membrane is highly diverse [56]. This lipid composition, including the different lengths and saturation of the lipid acyl chains determine the biophysical properties of the bilayer. Fluidity, for example, influences membrane function by modifying the diffusion and the interactions among membrane proteins [57]. In this context, cholesterol, also a key component of biological membranes, regulates their physical properties and modulates their fluidity [58]. In order to investigate the effects of nMBG and nMBG-IP nanospheres on the physical properties of the pre-osteoblast plasma membrane, we also analyzed its fluidity after treatment with these nanomaterials. In these studies, fluorescence polarization measurements of two different fluorescent probes were used to estimate the fluidity of two distinct regions of the plasma membrane: DPH for the highly disordered hydrophobic core of the bilayer and TMA-DPH for its more ordered shallow regions, closer to the lipid-water interface [59].

As it can be observed in Table 2, the fluidity of the two different regions of the pre-osteoblast membrane has been successfully evaluated, showing differences between the two above-mentioned regions related to the specific characteristics and function of each zone. Higher values of polarization and anisotropy indicate lower fluidity (higher rigidity). As expected, these values were higher with TMA-DPH, a probe that is placed in ordered shallow regions, than with DPH, inserted in the much-disordered hydrophobic core of the plasma membrane, in agreement with previous studies carried out with liver plasma membranes [33,60]. On the other hand, although slight variations in the polarization and anisotropy values were observed after the treatments of pre-osteoblasts with nMBG and nMBG-IP (Table 2), the statistical analysis evidenced that these nanomaterials did not produce significant changes in the fluidity of the two zones of the bilayer. In this context, other authors have recently evaluated the effect of SiO_2 nanoparticles on membrane fluidity using giant plasma membrane vesicles isolated from RBL-2H3 cells. Their results indicated that this nanomaterial produced a membrane fluidity decrease more pronounced at 37 °C than at 25 °C, concluding that SiO_2 nanoparticles prefer to interact with membranes

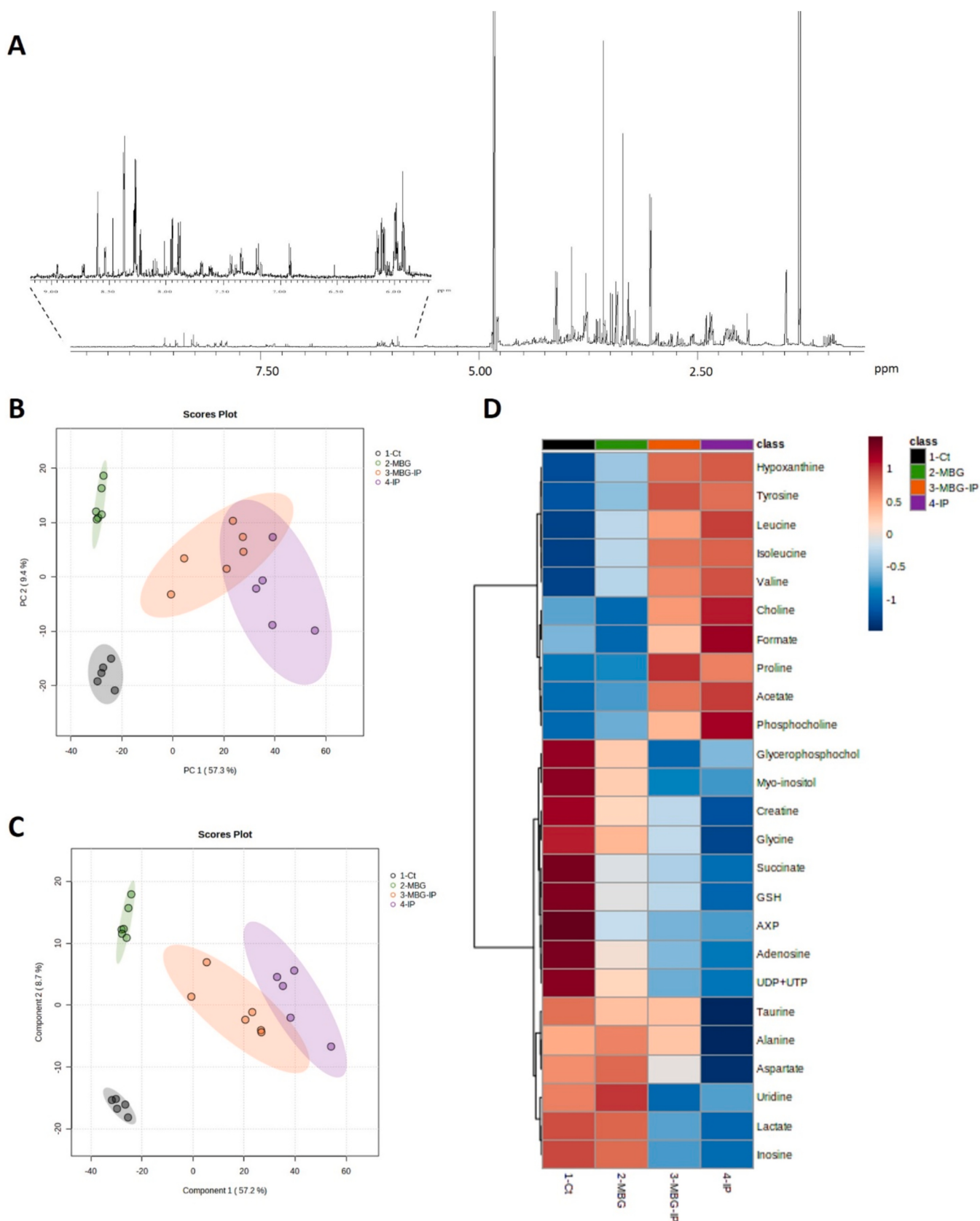


Fig. 6. A) ^1H NMR spectrum of a polar extract of MC3T3 cells B) PC1 vs PC2 scores scatter plot obtained by PCA of ^1H NMR spectral profiles of polar cell extracts C) LV1 vs. LV2 scores scatter plot obtained by PLS-DA of ^1H NMR spectral profiles of polar cell extracts D) heatmap representing the levels of intracellular polar metabolites in control cells (Ct) and in cells treated with unloaded nanospheres (nMBG), ipriflavone-loaded nanospheres (nMBG-IP) and free ipriflavone (IP). Relative metabolite levels are expressed based on UV-scaled signal areas.

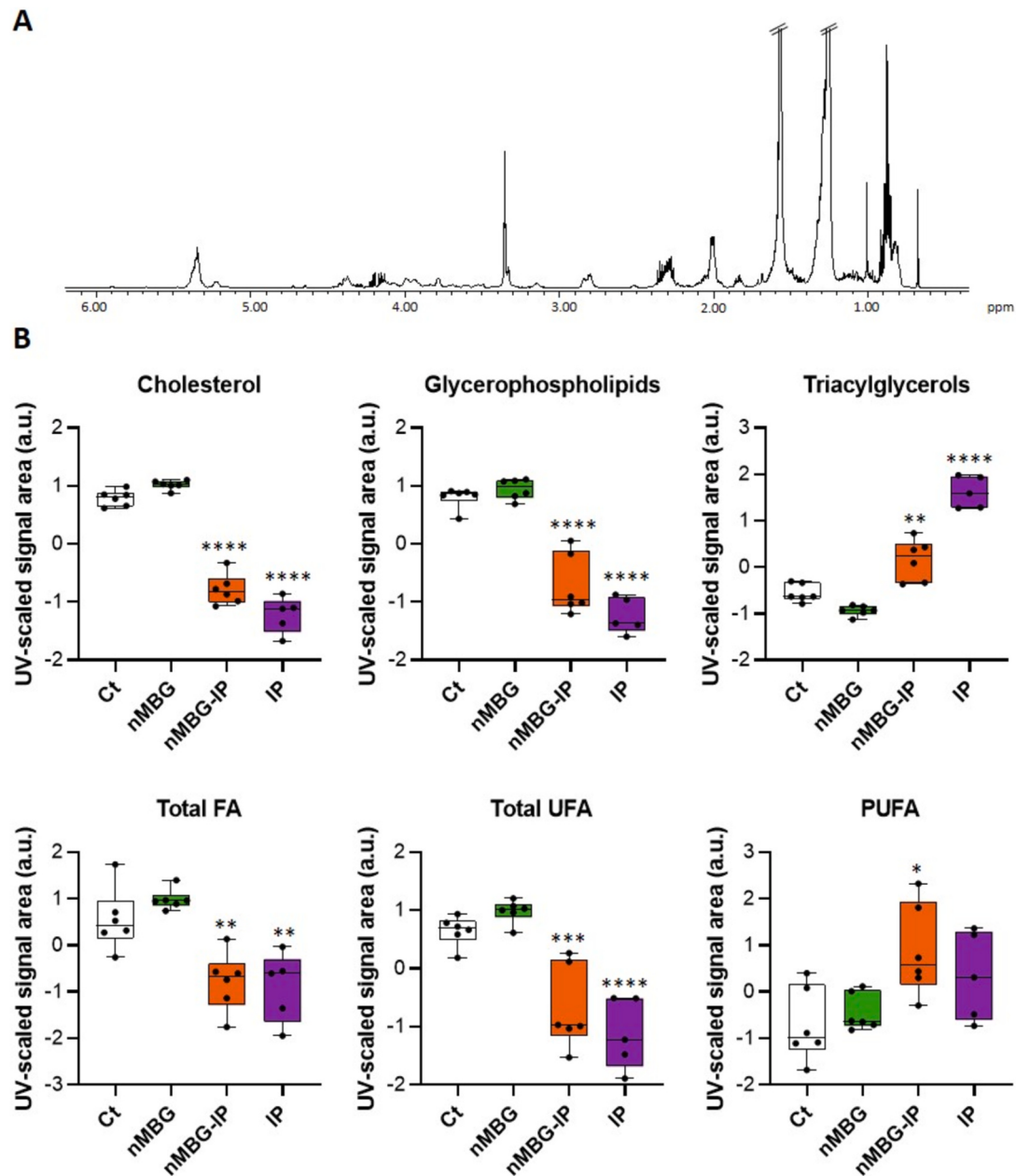


Fig. 7. A) ¹H NMR spectrum of a lipid extract of MC3T3 cells B) relative lipid levels in control cells (Ct) and in cells treated with unloaded nanospheres (nMBG), ipriflavone-loaded nanospheres (nMBG-IP) and free ipriflavone (IP), expressed based on UV-scaled signal areas. FA, fatty acids; UFA, unsaturated fatty acids; PUFA, polyunsaturated fatty acids. Significant variations compared to controls are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$).

Table 2

Effects of nMBG and nMBG-IP nanospheres (50 $\mu\text{g/mL}$) on the fluidity of two regions of the MC3T3-E1 pre-osteoblast plasma membrane. Fluorescence polarization and anisotropy were evaluated with the probes TMA-DPH (for ordered shallow regions) and DPH (for highly disordered hydrophobic core).

Cells	TMA-DPH		DPH	
	Polarization	Anisotropy	Polarization	Anisotropy
Control	0.380 $\pm$ 0.024	0.290 $\pm$ 0.029	0.267 $\pm$ 0.023	0.196 $\pm$ 0.022
nMBG	0.361 $\pm$ 0.024	0.274 $\pm$ 0.022	0.278 $\pm$ 0.023	0.204 $\pm$ 0.022
nMBG-IP	0.358 $\pm$ 0.023	0.271 $\pm$ 0.022	0.303 $\pm$ 0.023	0.225 $\pm$ 0.022

that are more dynamic and less densely packed [61]. This fact could be related to the slight increase in polarization (decrease in fluidity) observed in the inner most fluid zone in our studies with DPH after

treatment with nMBG and nMBG-IP (Table 2), although these differences were not found to be significant. In any case, the nanoparticles used in the present work are mesoporous $\text{SiO}_2\text{-CaO}$ nanospheres which contain calcium and their effects can be different from those induced by SiO_2 nanoparticles.

The biophysical effects and related biological functions of nanomaterials in cells is a subject of considerable interest currently. Thus, it has been recently shown that silica-coated magnetic nanoparticles decrease the migratory activity of human bone marrow-derived mesenchymal stem cells by reducing membrane fluidity and impairing focal adhesion [62]. Several studies indicate that the membrane fluidity of nanoparticle treated-cells can change due to oxidative stress-induced membrane damage [63,64]. However, as it can be observed in Fig. 3, the nanospheres used in the present work did not produce oxidative stress. In fact, all treatments induced a significant decrease in the cell

population with high intracellular ROS after 7 days, which was more pronounced after treatment with IP, in agreement with the antioxidant properties of this drug [42].

Our findings suggest that the changes in lipid composition observed during pre-osteoblast differentiation are directly associated with this process, yet they do not result in significant alterations to the physical properties of the plasma membrane, such as its stability and fluidity. These lipid changes may facilitate the activation of key differentiation signaling pathways, including Runx2, through interactions with membrane receptors. This interplay could support osteogenic differentiation without compromising membrane integrity. To further elucidate the molecular mechanisms involved, proteomics studies would be highly informative. By identifying specific proteins that are modulated in response to the nanospheres, these studies could provide direct insights into whether the proteins linked to cell differentiation, such as those involved in signaling, cytoskeletal remodeling, or extracellular matrix production, are being specifically regulated during this process. This would deepen our understanding of how nanomaterials influence cellular differentiation at the molecular level and provide new targets for optimizing nanoparticle-based therapies for bone regeneration.

4. Conclusions

This study demonstrated that IP-loaded nMBG nanospheres were effectively internalized by MC3T3-E1 pre-osteoblasts and promoted their differentiation, as evidenced by increased ALP activity and osteocalcin levels. This differentiation was associated with significant metabolic alterations, newly and complementarily identified by NMR *exo*- and *endo*-metabolomics. Both nMBG-IP and free IP significantly modulated cellular metabolism, with few differences between them, indicating that the observed effects were primarily attributable to IP. Indeed, nMBG alone induced only mild changes in the intracellular metabolic profile, without affecting the cells' consumption/secretion patterns or lipid composition. Overall, IP-induced differentiation of MC3T3-E1 pre-osteoblasts was accompanied by intensified glycolysis during early-stage differentiation and changes in several metabolic pathways, including nucleobase metabolism, osmoregulatory and antioxidant pathways, and lipid metabolism. This connection between pre-osteoblast differentiation and metabolism underscores the potential for utilizing metabolites and metabolic reprogramming as strategies to modulate the osteogenic process. Furthermore, our study highlights the effectiveness of nMBG nanospheres as carriers for the intracellular delivery of IP, demonstrating both efficient internalization and minimal metabolic impact.

Taken together, these findings emphasize the intricate metabolic reprogramming that accompanies osteogenic differentiation and the roles of both the biomaterial (nMBG) and the drug (IP) in modulating these pathways. Future research should investigate how these metabolic shifts influence bone formation, providing insights into optimizing nanoparticle formulations to further promote osteogenesis by targeting specific metabolic pathways. This could involve tuning drug release profiles or adjusting the system's composition to enhance therapeutic efficacy. Additionally, metabolomics could drive the development of personalized therapeutic strategies, tailoring drug-nanoparticle systems to individual metabolic profiles or specific conditions such as osteoporosis. Long-term studies may also explore how nanoparticle-mediated drug delivery affects bone health over time and assess its potential synergy with other treatments, such as mechanical loading or growth factors, to boost bone regeneration.

CRediT authorship contribution statement

Laura Casarrubios: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mónica Cicuéndez:** Writing – review & editing, Methodology, Investigation. **Alberto Polo-Montalvo:** Writing – review & editing,

Methodology, Investigation. **María José Feito:** Writing – review & editing, Investigation. **Álvaro Martínez-del-Pozo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Daniel Arcos:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Iola F. Duarte:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **María Teresa Portolés:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2024.214085>.

Data availability

The raw/processed data cannot be shared at this time as the data also forms part of an ongoing study.

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