

OPTIMIZATION OF THE CALCIUM CARBONATE PARTICLES MANUFACTURING PROCESS BY THE PRECIPITATION METHOD AND BIOLOGICAL EVALUATION WITH MG-63 CELLS

IWONA PUDEŁKO-PRAŻUCH* , KAROLINA WOJTANEK , ELŻBIETA PAMUŁA 

AGH UNIVERSITY OF KRAKOW,
FACULTY OF MATERIALS SCIENCE AND CERAMICS,
DEPARTMENT OF BIOMATERIALS AND COMPOSITES,
AL. A. MICKIEWICZA 30, 30-059 KRAKOW, POLAND
*E-MAIL: IPUDEŁKO@AGH.EDU.PL

Abstract

As a natural mineral, calcium carbonate (CaCO_3) is widely investigated for various medical applications. It is a biocompatible material characterized by high degradation rate and great osteoconductivity. Many researchers evaluate CaCO_3 in the form of particles as a candidate for use in drug delivery systems. In this study we present an optimization of the process of producing CaCO_3 particles by the precipitation method with the use of combinations of different time of ultrasound treatment and surfactant concentrations used. Depending on the synthesis conditions, various sizes of particles were fabricated. The particles were loaded with sodium alendronate (Aln, 5% or 10% by weight) with a relatively high encapsulation efficiency between 40 and 50%, depending on the amount of Aln added and the drug loading of approximately 9% for both cases. MG-63 osteoblast-like cells were contacted with 10% wt./vol extracts of fabricated particles to assess their cytotoxicity. None of the extracts investigated was found to be cytotoxic. Furthermore, an *in vitro* study in direct contact of MG-63 cells with particles suspended in culture medium was performed. The results showed that the fabricated particles are cytocompatible with osteoblast-like MG-63 cells. However, the higher the concentration of the particle suspension and the greater the amount of alendronate present in the solution, the lower the metabolic activity of the cells was measured. The presented method of CaCO_3 particles manufacturing is simple, cost-effective, and allows one to fabricate particles of the required size and shape that are cytocompatible with MG-63 cells in defined concentrations of particle suspensions.

Keywords: calcium carbonate microparticles, sodium alendronate, drug delivery system, MG-63 cells, bone tissue regeneration, particle size optimization

[Engineering of Biomaterials 170 (2023) 20-27]

doi:10.34821/eng.biomat.170.2023.20-27

Submitted: 2023-09-08, Accepted: 2023-10-04, Published: 2023-10-06



Copyright © 2023 by the authors. Some rights reserved.
Except otherwise noted, this work is licensed under
<https://creativecommons.org/licenses/by/4.0>

Introduction

Calcium carbonate (CaCO_3) is a natural biological mineral that has gained considerable interest in numerous scientific and industrial areas [1-3]. Several factors make CaCO_3 popular and attractive: low price, wide availability, high surface area, and ease of synthesis [1-4]. Furthermore, calcium carbonate is characterized by good biocompatibility, biodegradability, osteoconductivity [1,3,5], pH sensitivity [2,3,6], and the ability to release drugs [1,2,6,7]; however, it is not regarded as osteoinductive [8]. Importantly, CaCO_3 is a material that has been approved by the US Food and Drug Administration (FDA) and the European Food Safety Agency (EFSA) as a food additive and nutrient source added to food [2,3]. CaCO_3 has been confirmed to have a faster resorption rate compared to hydroxyapatite [5] with comparable bone formation activity [6]. It may improve gene expression and exhibits a high degradation rate [4] and great osteoconductivity [6]. CaCO_3 nanoparticles have been reported to promote osteogenic differentiation of human bone marrow mesenchymal stem cells *in vitro* [4]. Due to its features, CaCO_3 in the form of particles appears to be an ideal candidate for use in drug delivery systems [1,7,9,10]. Manufacturing process of CaCO_3 is simple [10] and the drug release rate can be controlled by changing the size, shape, or composition of the particles [2,3]. The significant advantage of CaCO_3 particles is their degradability which eliminates the need to remove the delivery system after treatment [6]. CaCO_3 has been reported to be a carrier of different drugs and bioactive substances, such as doxorubicin [1], gentamicin [6], tetracycline hydrochloride [7] or doxorubicin hydrochloride [10].

Sodium alendronate (Aln) is a hydrophilic amphiprotic [11] and nitrogen-containing drug [12-14] that is approved by the FDA [15]. It belongs to the bisphosphonate class of drugs and is widely used for the treatment of bone diseases [11-14,16]. The action of Aln is to inhibit bone resorption by inducing osteoclast apoptosis, while improving osteoblast recruitment, differentiation, and bone remodelling activity [11,14,17,18]. Furthermore, it has been reported to increase bone mineral density [12,19] and improve the osteogenesis of bone marrow mesenchymal stem cell (BMSCs) and mesenchymal stromal cells (MSCs) [13,14]. Sodium alendronate has been widely investigated for local delivery. Different materials are being used to encapsulate Aln, such as polymers [19-22] and ceramics [23-26]. However, to the best of our knowledge, alendronate-loaded CaCO_3 particles require more in-depth studies. Thus, in this work, we aimed to manufacture calcium carbonate particles loaded with sodium alendronate for local drug delivery in bone tissue with the use of a simple, safe, low-cost, and effective precipitation method.

Materials and Methods

Materials

Sodium carbonate (Na_2CO_3), calcium chloride hexahydrate ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), TWEEN 20, o-phtaldialdehyde (OPA), resazurin (Alamar Blue assay), calcein AM and propidium iodide were provided by Sigma Aldrich, Germany. Minimal essential medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin (PS), amino acids and sodium pyruvate were purchased from PAN Biotech, Germany. Edetic acid (EDTA) was purchased from POCH, Poland, phosphate buffered saline (PBS) from VWR Life Science, and sodium alendronate (Aln) was provided by Polpharma S.A. Poland.

Manufacturing of calcium carbonate particles

To prepare CaCO₃ particles (CCPs), the precipitation method from aqueous solutions of Na₂CO₃ and CaCl₂ was used. In brief, the 0.33 M Na₂CO₃ solution was mixed with the 0.33 M CaCl₂ solution in a 1:1 ratio, while stirring at a speed of 2000 rpm [10,27]. The stirring was continued for 10 min, followed by centrifugation for 15 min at 5000 rpm (MPW-351R, MPW Med. instruments, Poland). Subsequently, the supernatant was discarded and 10 mL of ultra-high quality water (UHQ-water, produced in Direct-Q 3UV, Merck Millipore, USA) was added to the tube. The particles were resuspended and centrifuged again. The supernatant was removed, and the process was repeated three times. The particles were then frozen at -80°C and freeze dried (Christ Alpha 1–2 LDplus, Martin Christ, Germany).

Effect of ultrasounds on the size of particles

The effect of ultrasounds on the size of the particles was checked by introducing ultrasound treatment into the manufacturing process. Basically, Na₂CO₃ and CaCl₂ were mixed in the presence of an ultrasonic probe for 5, 10 and 15 min (Vibra Cell VCX130, Sonics, USA), followed by stirring with the magnetic stirrer for 10 min at 2000 rpm. The subsequent stages were carried out without any changes.

Effect of surfactant on the size of particles

To evaluate the influence of the presence of surfactant on the particles size, TWEEN 20 was added to the solutions of Na₂CO₃ and CaCl₂ at different concentrations – 0.5, 1 and 2%. Basic solutions containing different amounts of TWEEN 20 were mixed and the process was carried out as previously described.

Effect of ultrasound combined with surfactant on the size of particles

The combination of both previously mentioned factors was assessed as well. To do so, Na₂CO₃ and CaCl₂ solutions with different concentrations of TWEEN 20 (0.5, 1 and 2%) were mixed in the presence of an ultrasonic probe for different times (5, 10 and 15 min), followed by stirring. As previously, the next stages of the manufacturing process were carried out without any changes.

Encapsulation of sodium alendronate

Sodium alendronate was encapsulated in CCPs during the manufacturing process (Aln-CCPs). In brief, a different amount of the drug (5 and 10% relative to the theoretical mass of the particles) was dissolved in the Na₂CO₃ solution and mixed with CaCl₂ under adequate conditions.

Characterization of particles

The particles were observed with the use of a scanning electron microscope (SEM Apreo 2, Thermo Scientific, USA). The size was checked with dynamic light scattering (DLS), expressed as mean and standard deviation (SD), and confirmed with SEM observations. Encapsulation efficiency (EE) and drug loading (DL) were examined with the use of an OPA assay. To do so, the alendronate loaded particles were dissolved in EDTA, adjusted to a pH value equal to 8 with 1 M NaOH, at a concentration of 1 mg/mL. Then, 50 µL of the solution was transferred to a black 96-well plate, 50 µL of OPA reagent was added and fluorescence was measured ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$, FLUOstar Omega, BMG Labtech, Germany).

Biological evaluation with extracts

The extracts of empty and loaded particles were tested with osteoblast-like MG-63 cells. To obtain 10% extracts, the particles (after being exposed for 20 min to UV irradiation) were immersed in MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1% amino acids, and 0.1% sodium pyruvate for 24 h. In an adequate number of wells, 10,000 cells suspended in 100 µL of MEM per well were seeded. Culture was carried out for 24 h at 37°C in a 5% CO₂ atmosphere. After that time, the medium was exchanged into extracts (filtered with a 0.2 µm filter) and the cells were cultured in them for another 24 h. The Alamar Blue assay and live/dead staining were then performed to verify the metabolic activity and morphology of the cells, respectively. For Alamar Blue, 150 µL of a 10% resazurin solution in a medium was added to each well and incubated for 3 h. Then 100 µL of the solution was transferred from each well to a black 96-well plate and the fluorescence was measured ($\lambda_{\text{ex}} = 544$ and $\lambda_{\text{em}} = 590 \text{ nm}$). To calculate the level of resazurin reduction, we used the following formula (1):

$$\% \text{ resazurin reduction} = \frac{S_x - S_{\text{blank}}}{S_{\text{reduced}} - S_{\text{blank}}} \cdot 100\% \quad (1)$$

where:

S_x – fluorescence of the samples,

S_{blank} – 0% reduction of resazurin,

S_{reduced} – 100% reduction of resazurin.

To conduct live/dead staining, calcein AM (0.1%) and propidium iodide (0.1%) were dissolved in phosphate buffered saline solution (PBS). We added 100 µL of the solution to each well and incubated it for 20 min in darkness. The cells were then observed with a fluorescent microscope (ZEISS Axiovert 40 CFL) with a ZEISS HXP 120 C metal halide illuminator.

Cytocompatibility test

To evaluate cytocompatibility, MG-63 cells were evaluated in direct contact with the particles. Briefly, suspensions of different concentrations of empty and loaded particles were prepared. The particles were sterilized by exposing them twice to UV irradiation. In an adequate number of wells, 10,000 cells suspended in 100 µL of MEM per well were seeded. Culture was carried out for 24 h at 37°C in a 5% CO₂ atmosphere. Later, the medium was exchanged into particle suspensions and the culture was continued for another 24 h. After that time, the Alamar Blue assay and live/dead staining were performed, as described in 'Biological evaluation with extracts' section.

Statistical analysis

The results are presented as mean ± standard error of the mean (S.E.M.). The analysis of the results obtained was conducted using a one-way analysis of variance (one-way ANOVA) followed by the post-hoc LSD Fisher test in OriginLab software. Probability values less than 0.05: $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered statistically significant.

Results and Discussions

CaCO₃ particles were fabricated using the precipitation method. Different times of ultrasound treatment and different concentrations of TWEEN 20 were used to determine the best conditions for obtaining particles. The results show that both factors have a significant influence on the size of the particles (FIG. 1).

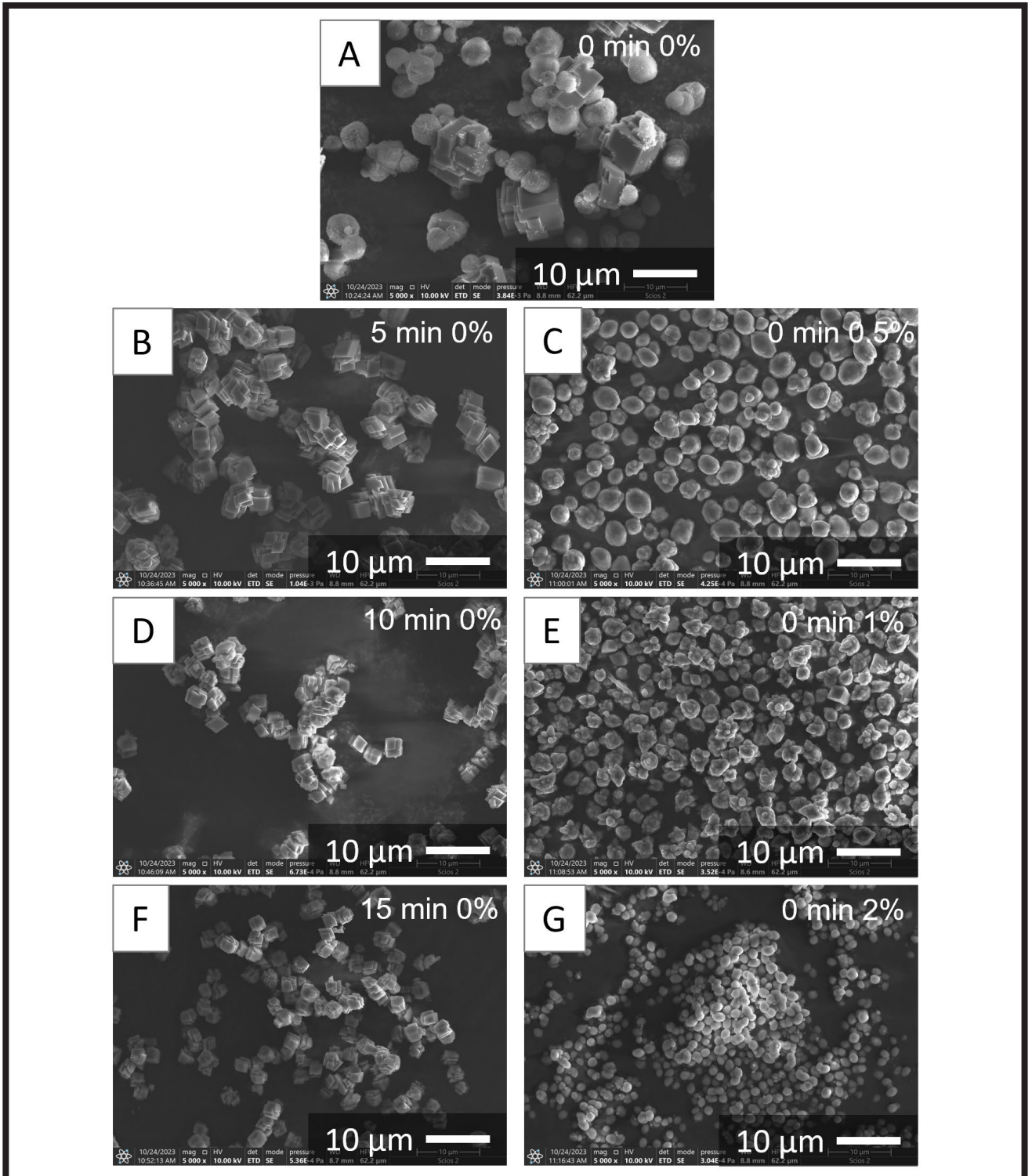


FIG. 1. Effect of ultrasound treatment time (0, 5, 10 and 15 min) and TWEEN 20 concentration (0%, 0.5%, 1%, 2%) on the size and microstructure of CaCO₃ particles obtained during the precipitation process.

The use of ultrasound makes it possible to obtain smaller particles, while the presence of a surfactant stabilizes the system, so that the formed particles have a round shape. The longer the ultrasound treatment time used, the smaller the particles. Moreover, the addition of surfactant appears to allow smaller particle sizes to be obtained; however, too high concentration may lead to the agglomeration of CaCO₃ particles (FIGs 2 G-I). Furthermore, the presence of the surfactant makes it possible to fabricate particles of round shape. CaCO₃ particles manufactured without TWEEN 20 are visible to have a cubic shape, indicating that the transformation of vaterite into calcite has occurred [28].

This means that the surfactant works as a stabilizer of a vaterite phase of CaCO₃ crystals. Interestingly, when no ultrasound treatment is performed, the particles obtained with all concentrations of TWEEN 20 belong to the same population; however, the more surfactant used, the more agglomerated particles are. Furthermore, there is no statistically significant difference between particles manufactured without the addition of surfactant at various times of ultrasound treatment. That indicates that 5 min is enough for the precipitation reaction to occur and a longer time does not affect the size.

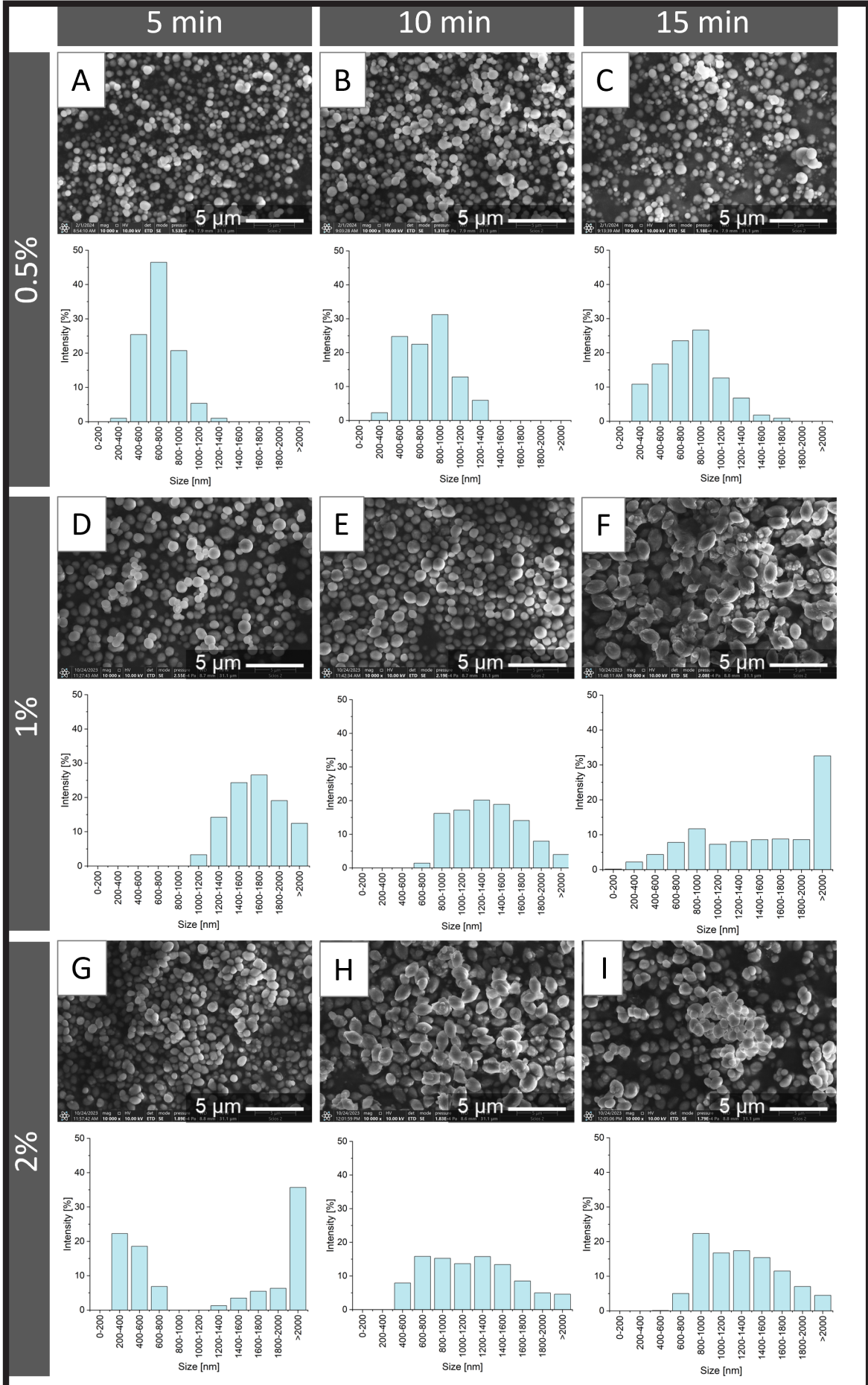


FIG. 2. SEM microphotographs and size distribution of different types of CaCO₃ particles submitted to different combinations of ultrasound treatment time and surfactant concentration.

The combination of ultrasound treatment and the addition of TWEEN 20 allows to obtain particles of different sizes (TABLE 1), depending on the conditions. According to the literature, various sizes of CaCO₃ particles may be obtained with the use of different methods of manufacturing and the conditions used. The size of CaCO₃ particles has been reported to vary between 50 nm and 6 μm while the precipitation method is used with most particles greater than 1 μm [29]. However, the use of different additives during the precipitation allows to control the size of the obtained particles. This corresponds to the findings of other scientists that presented particles of different sizes [4,6,7,10,29]. The smallest particles in our investigation were manufactured when 5 min of ultrasound and 0.5% of surfactant were used and have a size of 0.7 ± 0.2 μm. On the other hand, the size distribution in the case of this sample is relatively wide. Therefore, the most optimal conditions appear to be 10 min of ultrasound with the addition of 1% TWEEN 20. The size is slightly higher, but the size distribution is narrower. Smaller particles may be obtained with the use of different combinations; however, apart from the size distribution, the shape of the particles changes. Furthermore, the use of different methods of manufacturing can be utilized to fabricate much smaller particles [4,6]. We chose 10 min of ultrasound treatment and the addition of 1% TWEEN 20 as the most optimal conditions and they were used for further investigation. The addition of Aln has no impact on the shape of the obtained particles. Furthermore, the size of the Aln-loaded particles, fabricated under the same conditions, is not significantly different compared to the empty particles (TABLE 2). Although the precipitation method is simple and relatively effective, it is nonetheless a manual process. Its automation would improve the accuracy of the technique and allow obtaining particles of a specific, predefined size. Future research should focus on the use of other surfactants, as well as the use of homogenizers and/or a milling process to obtain smaller particles.

The encapsulation efficiency decreased, while the drug loading increased with the amount of alendronate used. Different polymers have been reported to encapsulate sodium alendronate, and the efficiency was dependent on the material used. Jing *et al.* presented three compositions of alendronate-loaded nanoparticles based on poly(lactico-glycolic acid) (PLGA) in which both encapsulation efficiency and drug loading exceeded 70% [20]. Moreover, chitosan nanoparticles have been reported to have EE in the range of 40 to 70% [22]. On the other hand, Miladi *et al.* demonstrated polycaprolactone (PCL) particles that were characterized by EE and DL in the ranges of 15-34% and 2-21%, respectively [21]. According to the literature, calcium carbonate particles are being used to encapsulate various drugs. The encapsulation efficiency of gentamicin in CaCO₃ particles was reported to be 38% with a drug loading of 25% [6]. Sudareva *et al.* showed CaCO₃ particles loaded with doxorubicin with a drug content greater than 90% [30]. Encapsulation efficiency and drug loading are highly dependent on the drug and material used. However, in this study, EE was relatively high in each case and varied between 40 and 50%, depending on the addition of Aln and DL was approximately 9% for the addition of 5 and 10% of alendronate (TABLE 2).

TABLE 1. Size of CaCO₃ particles fabricated under different conditions.

Time of ultrasound treatment [min]	Surfactant concentration [%]	Size ± SD [μm]
0	0	17.8 ± 7.5
0	0.5	2.5 ± 0.7
0	1	2.2 ± 0.2
0	2	1.8 ± 0.1
5	0	5.1 ± 1.7
5	0.5	0.7 ± 0.2
5	1	2.1 ± 0.5
5	2	1.4 ± 0.4
10	0	4.6 ± 2.1
10	0.5	0.8 ± 0.2
10	1	1.3 ± 0.1
10	2	1.1 ± 0.4
15	0	4.4 ± 1.1
15	0.5	0.8 ± 0.3
15	1	1.3 ± 0.1
15	2	1.2 ± 0.1

Osteoblast-like MG-63 cells were cultured in 10% extracts of manufactured particles to evaluate their potential cytotoxicity. Cells were contacted with extracts for 24 h and then their metabolic activity was checked (FIG. 3 A) and live/dead staining was performed (FIG. 3 B). Compared to the control, cells activity was significantly lower while cultured in extracts of each type of particles. Interestingly, the resazurin reduction in the case of samples containing Aln was higher than that of empty CaCO₃ particles. No decrease of greater than 70% compared to the control can be observed, which means no toxicity of the investigated extracts according to ISO 10993-5 [31]. Interestingly, the metabolic activity of cells cultured in the extracts of Aln-loaded particles (both 5 and 10%) was higher compared to empty ones. This suggests that sodium alendronate supports the proliferation of osteoblast-like cells.

To evaluate the cellular response of CaCO₃ particles, osteoblast-like MG-63 cells were cultured in direct contact with particle suspensions. Depending on the type of particles, the metabolic activity of cells (FIGs 4 A,C,E) was decreasing with increasing concentration of particles present in MEM. However, for empty particles, the relevant decrease is observed for the concentration of 100 mg/mL. In the case of 10% addition of alendronate, concentrations of 50 and 100 mg/mL were toxic for cells; however, a statistically significant decrease, compared to the control (0 mg/mL), can also be observed for lower concentrations (FIGs 4 A,C,E). It is visible that cell activity is significantly lower for a concentration of 100 mg/mL, even for empty particles.

TABLE 2. Characteristics of CaCO₃ particles loaded with alendronate.

Time of ultrasound treatment [min]	Surfactant concentration [%]	Addition of alendronate [%]	Size ± SD [μm]	Encapsulation efficiency (EE) [%]	Drug loading (DL) [%]
10	1	0	1.3 ± 0.1	-	-
10	1	5	1.1 ± 0.2	49.6 ± 2.0	9.2 ± 0.4
10	1	10	1.1 ± 0.3	39.7 ± 1.3	9.6 ± 0.3

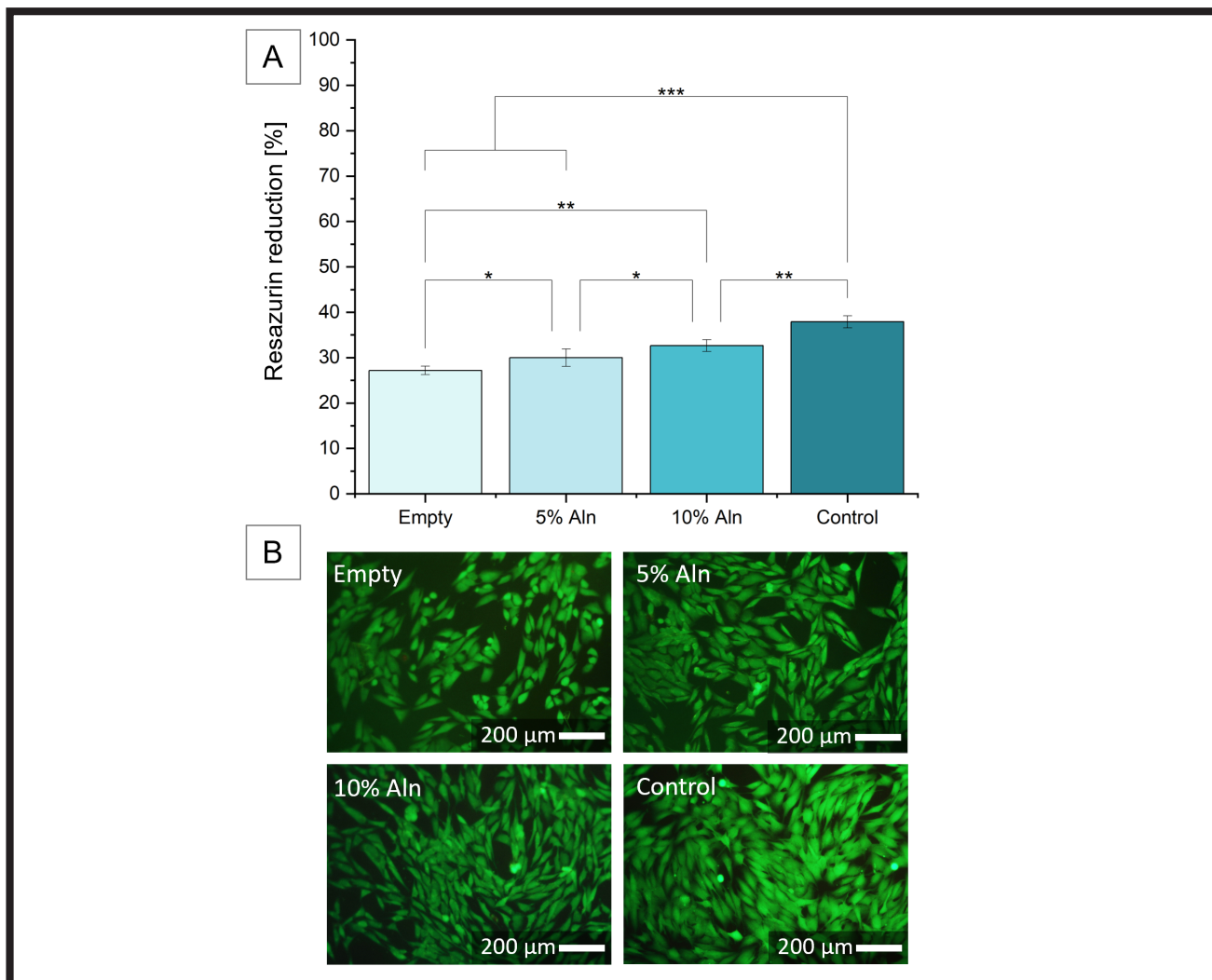


FIG. 3. Metabolic activity (A) and live/dead staining (B) of MG-63 cells cultured in the presence of 10% extracts of empty particles, particles loaded with 5% and 10% addition of sodium alendronate and in MEM, as control. No decrease in metabolic activity greater than 70% compared to the control can be observed.

However, the metabolic activity of cells when contacted with empty particles is not significant for lower concentrations, which is comparable to the results of other researchers [32,33]. The resazurin reduction is lower, compared to the control for each suspension of particles containing Aln, indicating that alendronate may be toxic for MG-63 cells, especially at higher doses. Although the CaCO_3 particles with Aln showed great performance during the study with extracts, it is not the case when we study them in direct contact with the particle suspensions.

Conclusions

The aim of this research was to optimize the process of manufacturing calcium carbonate particles with sodium alendronate encapsulated and to investigate their cytotoxicity and cytocompatibility with osteoblast-like MG-63 cells. Different times of ultrasound treatment and concentrations of surfactant were used to control the size of the fabricated particles. The use of 0.5% of surfactant combined with 5 min of ultrasounds allows to obtain the smallest particles. However, a combination of 1% and 10 min gives slightly larger particles, but with a narrower size distribution. The extracts of empty and loaded particles (5 or 10% alendronate) were tested with MG-63 cells and did not show signs of cytotoxicity.

Furthermore, the cellular response of the particles was investigated by contacting MG-63 cells with particles suspended in a culture medium. According to the results, a higher concentration of sodium alendronate present in the system may be toxic to the investigated cells. However, the particles are cytocompatible with MG-63 cells in lower concentrations even with a 10% addition of sodium alendronate. Depending on the application and requirements, different compositions may be used to obtain particles of the required size. The presented process is simple, low-cost, and effective to fabricate calcium carbonate particles that are cytocompatible with MG-63 cells.

Acknowledgements

This research was funded by the Program "Excellence Initiative – Research University" and the subsidy (No 16.16.160.557) for the AGH University of Krakow. The authors would like to acknowledge the access to the Laboratory of Electron Microscopy of the Faculty of Materials Science and Ceramics at the AGH University of Krakow.

ORCID iD

I. Pudelko-Prażuch: <https://orcid.org/0000-0002-1024-9374>
 K. Wojtanek: <https://orcid.org/0009-0008-8975-930X>
 E. Pamuła: <https://orcid.org/0000-0002-0464-6189>

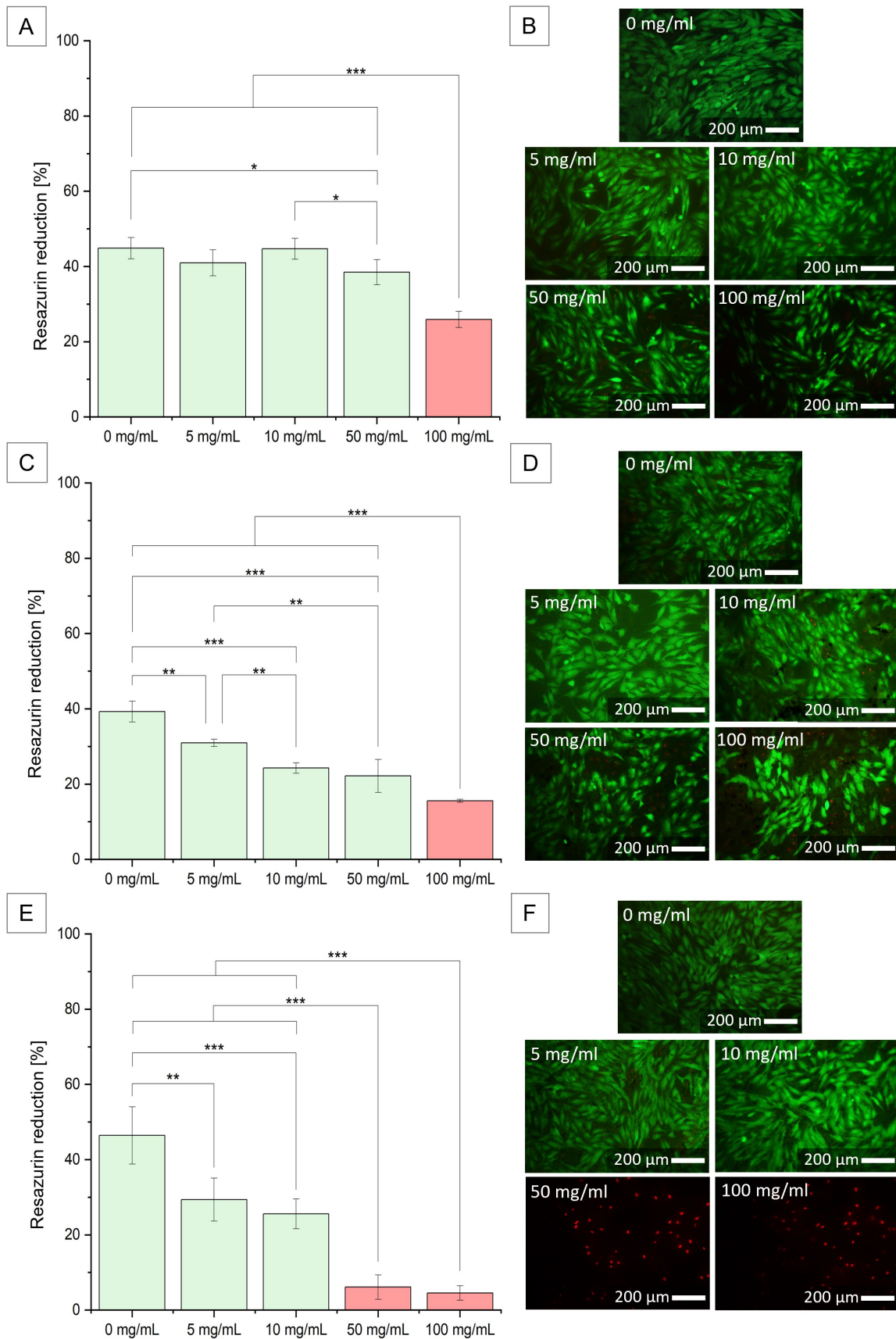


FIG. 4. Metabolic activity (A, C, E) and live/dead staining (B, D, F) of MG-63 cells cultured in direct contact with empty particles (A, B), particles loaded with 5% (C, D) and 10% (E, F) addition of sodium alendronate. A high concentration (100 mg/mL) of particles is toxic for MG-63 cells in each case. A lower resazurin reduction is observed for particles loaded with sodium alendronate compared to empty ones, which indicates the toxicity of sodium alendronate in high doses.

References

- [1] Li L., Yang Y., Lv Y., Yin P., Lei T.: Porous calcite CaCO_3 microspheres: Preparation, characterization and release behavior as doxorubicin carrier. *Colloids and Surfaces B: Biointerfaces* 186 (2020) 110720.
- [2] Tan C., Dima C., Huang M., Assadpour E., Wang J., Sun B., Kharazmi M., Jafari S. M.: Advanced CaCO_3 -derived delivery systems for bioactive compounds. *Advances in Colloid and Interface Science* 309 (2022) 102791.
- [3] Ferreira A.M., Vikulina A.S., Volodkin D.: CaCO_3 crystals as versatile carriers for controlled delivery of antimicrobials. *Journal of Controlled Release* 328 (2020) 470-489.
- [4] Li X., Yang X., Liu X., He W., Huang Q., Li S., Feng Q.: Calcium carbonate nanoparticles promote osteogenesis compared to adipogenesis in human bone-marrow mesenchymal stem cells. *Progress in Natural Science: Materials International* 28(5) (2018) 598-608.
- [5] Bang L.T., Son N.A., Long B.D., Nhung N.T.H.: Calcium carbonate coating on Ti-6Al-4 V by carbonate diffusion in calcium source at room temperature. *Materials Today: Proceedings* 66 (2022) 2929-2932.
- [6] Memar M.Y., Adibkia K., Farajnia S., Kafil H.S., Maleki Dizaj S., Ghotaslou R.: Biocompatibility, cytotoxicity and antimicrobial effects of gentamicin-loaded CaCO_3 as a drug delivery to osteomyelitis. *Journal of Drug Delivery Science and Technology* 54 (2019) 101307.
- [7] Ren B., Chen X., Du S., Ma Y., Chen H., Yuan G., Li J., Xiong D., Tan H., Ling Z., Chen Y., Hu X., Niu X.: Injectable polysaccharide hydrogel embedded with hydroxyapatite and calcium carbonate for drug delivery and bone tissue engineering. *International Journal of Biological Macromolecules* 118 (2018) 1257-1266.
- [8] Sirkkiä S.V., Qudus S., Siekkinen M., Hoepfl W., Budde T., Smått J.-H., Peltonen J., Hupa L., Heino T.J., Vallittu P.K.: Physicochemical and biological characterization of functionalized calcium carbonate. *Materialia* 28 (2023) 101742.
- [9] Yang T., Ao Y., Feng J., Wang C., Zhang J.: Biomimetic synthesis of CaCO_3 -based DDS for pH-responsive release of anticancer drug. *Materials Today Communications* 27 (2021) 102256.
- [10] Wei Y., Sun R., Su H., Xu H., Zhang L., Huang D., Liang Z., Hu Y., Zhao L., Lian X.: Synthesis and characterization of porous CaCO_3 microspheres templated by yeast cells and the application as pH value-sensitive anticancer drug carrier. *Colloids and Surfaces B: Biointerfaces* 199 (2021) 111545.
- [11] Ezzati Nazhad Dolatabadi J., Hamishehkar H., Eskandani M., Valizadeh H.: Formulation, characterization and cytotoxicity studies of alendronate sodium-loaded solid lipid nanoparticles. *Colloids and Surfaces B: Biointerfaces* 117 (2014) 21-28.
- [12] Dong J., Tao L., Abourehab M.A.S., Hussain Z.: Design and development of novel hyaluronate-modified nanoparticles for combo-delivery of curcumin and alendronate: fabrication, characterization, and cellular and molecular evidences of enhanced bone regeneration. *International Journal of Biological Macromolecules* 116 (2018) 1268-1281.
- [13] Lee J.Y., Kim S.E., Yun Y.-P., Choi S.-W., Jeon D.I., Kim H.-J., Park K., Song H.-R.: Osteogenesis and new bone formation of alendronate-immobilized porous PLGA microspheres in a rat calvarial defect model. *Journal of Industrial and Engineering Chemistry* 52 (2017) 277-286.
- [14] Wei P., Yuan Z., Jing W., Huang Y., Cai Q., Guan B., Liu Z., Zhang X., Mao J., Chen D., Yang X.: Strengthening the potential of biomimetic microspheres in enhancing osteogenesis via incorporating alendronate. *Chemical Engineering Journal* 368 (2019) 577-588.
- [15] Öz U.C., Toptaş M., Küçüktürkmen B., Devrim B., Saka O.M., Deveci M.S., Bilgili H., Ünsal E., Bozkır A.: Guided bone regeneration by the development of alendronate sodium loaded in-situ gel and membrane formulations. *European Journal of Pharmaceutical Sciences* 155 (2020) 105561.
- [16] Zhao Q., Cheng D.-Q., Tao M., Ning W.-J., Yang Y.-J., Meng K.-Y., Mei Y., Feng Y.-Q.: Rapid magnetic solid-phase extraction based on alendronate sodium grafted mesoporous magnetic nanoparticle for the determination of trans-resveratrol in peanut oils. *Food Chemistry* 279 (2019) 187-193.
- [17] Hur W., Park M., Lee J.Y., Kim M.H., Lee S.H., Park C.G., Kim S.N., Min H.S., Min H.J., Chai J.H., Lee S.J., Kim S., Choi T.H., Choy Y.B.: Bioabsorbable bone plates enabled with local, sustained delivery of alendronate for bone regeneration. *Journal of Controlled Release* 222 (2015) 97-106.
- [18] Kellesarian S.V., Abduljabbar T., Vohra F., Malignaggi V.R., Malmstrom H., Romanos G.E., Javed F.: Role of local alendronate delivery on the osseointegration of implants: a systematic review and meta-analysis. *International Journal of Oral and Maxillofacial Surgery* 46(7) (2017) 912-921.
- [19] De Molon R.S., Fiori L.C., Verzola M.H., Belluci M.M., De Souza Faroni A.P., Pereira R.M.R., Tetradis S., Orrico S.R.: Long-term evaluation of alendronate treatment on the healing of calvaria bone defects in rats. *Biochemical, histological and immunohistochemical analyses. Archives of Oral Biology* 117 (2020) 104779.
- [20] Jing C., Chen S., Bhatia S.S., Li B., Liang H., Liu C., Liang Z., Liu J., Li H., Liu Z., Tan H., Zhao L.: Bone-targeted polymeric nanoparticles as alendronate carriers for potential osteoporosis treatment. *Polymer Testing* 110 (2022) 107584.
- [21] Miladi K., Sfar S., Fessi H., Elaissari A.: Encapsulation of alendronate sodium by nanoprecipitation and double emulsion: From preparation to in vitro studies. *Industrial Crops and Products* 72 (2015) 24-33.
- [22] Miladi K., Sfar S., Fessi H., Elaissari A.: Enhancement of alendronate encapsulation in chitosan nanoparticles. *Journal of Drug Delivery Science and Technology* 30 (2015) 391-396.
- [23] Zhao Q., Xiao D., Li Y., Chen X., Hu K., Luo X., Yang F., Yang Z., Liu J., Feng G., Liu J., Feng D., Duan K.: Repair of rabbit femoral head necrosis by release of alendronate and growth differentiation factor-5 from injectable alginate/calcium phosphate carriers. *Materials Today Communications* 33 (2022) 104530.
- [24] Aryan N., Behpour M., Benvidi A., Jookar Kashi F., Azimzadeh M., Zare H.R.: Evaluation of sodium alendronate drug released from TiO_2 nanoparticle doped with hydroxyapatite and silver-strontium for enhancing antibacterial effect and osteoinductivity. *Materials Chemistry and Physics* 295 (2023) 126934.
- [25] Zhao X., Zhu L., Fan C.: Sequential alendronate delivery by hydroxyapatite-coated maghemite for enhanced bone fracture healing. *Journal of Drug Delivery Science and Technology* 66 (2021) 102761.
- [26] Lee J.H., Ko I.H., Jeon S.-H., Chae J.-H., Chang J.H.: Microstructured hydroxyapatite microspheres for local delivery of alendronate and BMP-2 carriers. *Materials Letters* 105 (2013) 136-139.
- [27] Ali Said F., Bousserhine N., Alphonse V., Michely L., Belbekhouche S.: Antibiotic loading and development of antibacterial capsules by using porous CaCO_3 microparticles as starting material. *International Journal of Pharmaceutics* 579 (2020) 119175.
- [28] Trushina D.B., Bukreeva T.V., Kovalchuk M.V., Antipina M.N.: CaCO_3 vaterite microparticles for biomedical and personal care applications. *Materials Science and Engineering: C* 45 (2014) 644-658.
- [29] Salomão R., Costa L.M.M., Olyveira G.M.D.: Precipitated Calcium Carbonate Nano-Microparticles: Applications in Drug Delivery. *Advances in Tissue Engineering & Regenerative Medicine: Open Access* 3(2) (2017) 336-340.
- [30] Sudareva N., Suvorova O., Saprykina N., Vlasova H., Vilesov A.: Doxorubicin delivery systems based on doped CaCO_3 cores and polyanion drug conjugates. *Journal of Microencapsulation* 38 (2021) 164-176.
- [31] International Organization for Standardization: ISO 10993-5:2009, Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity.
- [32] Zhong Q., Li W., Su X., Li G., Zhou Y., Kundu S.C., Yao J., Cai Y.: Degradation pattern of porous CaCO_3 and hydroxyapatite microspheres in vitro and in vivo for potential application in bone tissue engineering. *Colloids Surf. B Biointerfaces* 143 (2016) 56-63.
- [33] Pietryga K., Panaite A.-A., Pamula E.: Composite scaffolds enriched with calcium carbonate microparticles loaded with epigallocatechin gallate for bone tissue regeneration. *Engineering of Biomaterials* 166 (2022) 12-21.