Biosurfactants as foaming agents in calcium phosphate bone cements

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Abstract

The idea of using biosurfactants to obtain highly porous, foamed calcium phosphate cements (fCPCs) is novel. The popularity of these compounds is mainly attributed to their biological activity such as anticancer or antibacterial properties. In our study, it was investigated how the functionalization of cements, based on α-tricalcium phosphate (α-TCP), with non-ionic biosurfactants such as sucrose ester S0112 and saponin from *Quillaja* bark affected the physicochemical as well as biological properties of cement-type materials. Foaming with these selected surface active agents led to highly porous fCPCs (open porosity >60 vol%) with compressive strength values ranging from 0.2 to 3.3 MPa and did not influence negatively the bioactive potential of the cements. Results showed that the sucrose ester had a positive effect on all studied cell types (osteosarcoma cell line MG-63 and preosteoblasts MC3T3-E1), while the effect of the saponin differed depending on the origin of the cells (cancerous or non-cancerous). The obtained results shed new light on the use of biosurfactants as additives to CPCs and pave the way for further studies, especially *in vivo*.

Keywords: bone cements, biosurfactants, calcium phosphates, porosity, nonionic surfactants

1. Introduction

The final product of calcium phosphate bone cements (CPCs) based on α-TCP, after setting and hardening, is calcium deficient hydroxyapatite (CDHA) which possesses excellent biocompatibility[1]. The composition of CPCs is very close to that of the mineral phase of natural bone.

Over the years, to improve the properties of CPCs, various modifications have been applied. For example, different ions[2,3], polymers[4,5], drugs[6], etc. were commonly used to alter CPCs' properties. An interesting but still poorly studied group of CPCs modifiers are surfactants, i.e. surface active agents which can be used to obtain highly porous, foamed calcium phosphate bone cements (fCPCs)[7,8]. Furthermore, the lowering of surface tension by the surface active agents usually leads to improvement of cement paste injectability[9]. It should be highlighted that surfactants in bone cement formulations affect not only their porosity and injectability but also other physicochemical properties of the final materials such as setting time, compressive strength, microstructure as well as hydrolysis and degradation rates[10,11,12] . Various surfactants have different effects on the properties of fCPCs. Montufar *et al*.[13] proved that both low molecular weight- (Tween 80) and protein-based (gelatine) surfactants are effective foaming agents for calcium phosphate cements. Nevertheless, taking into account the same concentrations Tween 80 has higher foamability than gelatine, resulting in higher macroporosity of the solid foams. Meanwhile, protein-based surfactant improved injectability and cohesion of the foamed pastes and osteoblasts adhesion on the solid foams.

Many surfactants additionally possess interesting properties[14] that can be transferred to the materials to which they are added. Biosurfactants, due to their biological activity such as antibacterial or anticancer properties[15,16], are an interesting alternative to more popular surfactants (such as Tween 80) previously used in the obtaining of fCPCs. In our study, two nonionic biosurfactants, namely a sucrose ester S0112 and saponin from Quillaja bark, were selected for foaming of CPCs. Non-ionic surfactants have advantages over others because they are not susceptible to pH changes and possess lower toxicity and higher biodegradability than other types of surface active agents[17]. Sucrose ester (SuE) belongs to the group of sugar fatty acid esters (SFAEs), which are characterized by excellent foaming and stabilising properties[18]. Moreover, these nonionic, non-irritant biosurfactants are characterized by, *inter alia*, antibacterial,[19] or anticancer properties[20]. In the study of Tiboni *et al*. Sucrose laurate, which is similar to sucrose palmitate showed high biocompatibility both *in vitro* and *in vivo*, coupled with antimicrobial and antifungal activities[21]. SuE with the most efficient foaming ability is in the form of a monoester (Fig. 1A) as their hydrophilic-lipophilic balance (HLB) is higher than the HLB of the corresponding di- or triester[22]. Another biosurfactant – saponin (SAP) (Fig. 1B) extracted from the quillaja bark, in particular *Quillaja saponaria,* has traditionally been used as a soap substitute because of its excellent foaming properties. The Food and Agricultural

Organization of the United Nation and World Health Organization suggests that the Acceptable Daily Intake (ADI) of Quillaja extract (saponin) is between zero to five mg/kg body weight[23]. Potential medical applications of saponins include those related to haemolytic activity[24], anticancer therapy[25] or the prevention of cardiovascular disease[26]. Other uses of saponins include as antioxidants[27], antidepressants[28], antibacterial[29] and antiviral agents[30] and many others. Those applications of saponins make them interesting and noteworthy compounds. To our best knowledge, these biosurfactants have not yet been used as foaming agents to obtain foamed calcium phosphate bone cements.

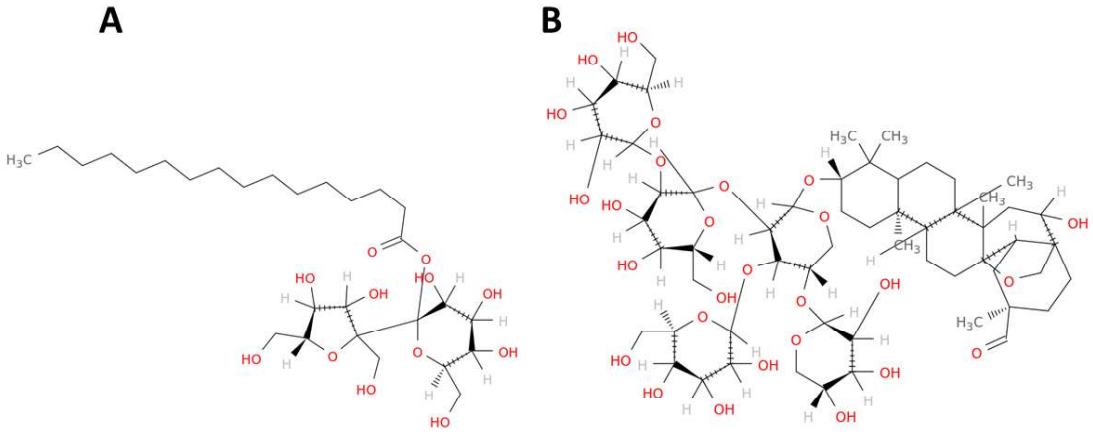

Figure 1 Structures of: A) sucrose monopalmitate, B) saponin.

The research aimed to design and investigate highly porous, foamed calcium phosphate-based cements (fCPCs) obtained with the use of biosurfactants. To the best of our knowledge, it is the first report that highlights the possibility of using biosurfactants with additional biological properties in fCPCs formulations. In our study, two biologically active surfactants i.e. *Quillaja* bark saponin and sucrose fatty acid ester S0112 were applied. To date to our best knowledge, there are no other studies on SuE (S0112) or saponins as CPCs modifiers. The proposed study shows the influence of these compounds on the physicochemical as well as the biological properties of cement-type materials. We hypothesized that the functionalization of cements with biosurfactants would allow favorable biological properties of the final materials to be obtained.

2. Materials and Methods

2.1. Materials

The α-TCP powder, used as a solid phase of cements, was obtained by a wet chemical synthesis, described previously[31]. Briefly, $Ca(OH)_2$ (POCH, Poland) and 85% H_3PO_4 (POCH,

Poland) were used as reagents. The precipitate, obtained after sedimentation and centrifugation, was dried, crushed and fired at 1300 °C (5 h). The fired powder was then ground in an attritor mill (4 h) and sieved to a fraction with a particle diameter below 63 µm. The sucrose ester S0112 (Tokyo Chemical Industry Europe N.V., Belgium) with min. 75% sucrose palmitate (approx. 60% of mono- and 40% of diester[32]) and saponin from *Quillaja* bark (S7900, sapogenin min. 10%, Sigma Aldrich, Germany) were applied as biosurfactants. Cement without biosurfactant (fCTRL) served as a control material. The 2.0 wt% aqueous solution of Na_2HPO_4 (POCH, Poland) constituted a cement liquid phase. The foaming step was performed with the use of a food mixer (BOMANN, Germany). The cement foaming process depended on surfactants' solubility in water. Soluble saponin was introduced into the cement (fSAP) in the liquid phase whereas insoluble sucrose ester was introduced along with α-TCP powder used as a solid phase in fSuE cement. In the case of fSAP material, the mechanically foamed liquid phase was mixed with the solid phase (α-TCP). To obtain fSuE material, sucrose ester was added to the solid phase of the cement, mixed with the liquid phase and then the resulting cement paste was mechanically foamed. For all materials, the ratio of liquid to solid phase was 0.7 $g \cdot g^{-1}$. Table 1 summarises the composition of cements' liquid and solid phases.

Table 1 The composition of the cements liquid and solid phases.

Material	Solid phase	Liquid phase
fCTRL	α-TCP powder	2 $g \cdot dL^{-1}$ (2.0 wt%) aqueous solution of Na_2HPO_4
fSuE	α-TCP powder + sucrose ester (SuE) powder (7 mg of SuE for each 1 g of α-TCP powder)	2 $g \cdot dL^{-1}$ (2.0 wt%) aqueous solution of Na_2HPO_4
fSAP	α-TCP powder	1 $g \cdot dL^{-1}$ (~ 1.0 wt%) of saponin (SAP) in 2 $g \cdot dL^{-1}$ (2.0 wt%) aqueous solution of Na_2HPO_4

2.2. Phase composition

Qualitative and quantitative identification of phases in the obtained materials was performed by X-ray diffractometry (XRD). The measurements were carried out on the powdered samples. Before testing, the materials were ground in a mortar and sieved through a 63 µm mesh sieve. A D2 Phaser diffractometer (Bruker) equipped with a copper lamp and a nickel filter was used. The measurements were analysed in Bragg-Brentano geometry, in the angular range 2θ of 10 - 40°, with a measuring step of 0.04º and scanning speed of 2.5° min^{-1}. Materials' phase compositions were determined based on standards from a JCPDS - ICDD (Joint Committee for

Powder Diffraction Standards - International Centre For Diffraction Data) database. Quantitative phase composition analysis was carried out using Profex software (http://profex.doebelin.org) by the Rietveld method, using JCPDS cards (αTCP: 00-009-0348, HAp: 01-076-0694).

2.3. Microstructure

The microstructure of the developed materials before as well as after 28-day incubation in simulated body fluid (SBF) was analysed using a scanning electron microscope (SEM, Nova NanoSem 200). During observations, SEM was operated at an accelerating voltage of 18 kV. Before testing, the materials' samples were attached to standard aluminum SEM stubs using conductive paste and sputtered with a layer of carbon to avoid overcharging.

2.4. Porosity and pore architecture

Open porosity and the pore size distribution of the cement samples after setting and hardening were determined by Mercury Intrusion Porosimetry (MIP) using an AutoPore IV 9500 porosimeter (Micromeritics) capable of measuring pores from 0.003 to 360 μm in diameter. Well-dried cement samples were introduced into the penetrometer and placed in the low-pressure chamber of the apparatus where they were de-aerated. Then mercury was introduced into the penetrometer, and the penetration volume was recorded, increasing the pressure. After low-pressure measurements, the penetrometer was removed and placed in a high-pressure chamber where high-pressure measurements were performed. In order to show pore architecture, the obtained cements were visualised using the Nanotom S microtomography (General Electric).

2.5. Compressive strength

The specimens for compressive strength tests were prepared in a mold in the form of cylinders with a diameter (d) of 6 ± 1 mm and a height (h) of 12 ± 1 mm. Samples were removed from the mold after setting and then left for 7 days for drying in air to harden. The compressive strength was measured at dry conditions using a universal testing machine - Instron 3345. The cross head speed was equal to 1 $mm \cdot min^{-1}$. The number of samples for each material was ≥ 10. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's HSD post hoc multiple comparison test.

2.6. Chemical stability and bioactivity *in vitro*

The chemical stability of the obtained cement-type materials was evaluated after incubation of the materials in simulated body fluid (SBF) prepared according to the procedure described by Kokubo *et al*.[33]. The samples were placed in sterile, disposable polypropylene containers, filled with SBF (at a ratio of 100 ml per 1 g of sample) and stored in an incubator at 37 ± 0.2 °C. In order to evaluate the bioactive potential after incubation in SBF, SEM examinations of the surfaces of the samples were performed (according to ISO 23317[34]).

2.7. Cytotoxicity tests – indirect study

Before cytotoxicity studies, the obtained cements with biosurfactants were sterilized with ethylene oxide at 37°C for 12 hours with complete degassing after the sterilization procedure. The indirect cytotoxicity of the materials was tested according to ISO 10993-5, used to assess the biocompatibility of medical devices. The experiments were carried out in six replicates for each type of material. Statistical analysis of the data obtained was performed using one-way ANOVA followed by Tuckey's post hoc test. Results are presented as mean ± standard deviation.

Cytotoxicity was tested using osteoblast-like MG-63 cells derived from osteosarcoma (European Collection of Cell Cultures, Salisbury, UK). MG-63 cells were cultured in Minimal Essential Medium Eagle (MEM, PAN Biotech) supplemented with fetal bovine serum - FBS (FBS Standard, PAN Biotech, 10%) and the antibiotics penicillin/streptomycin (Sigma-Aldrich, 1%) at 37°C with 5% CO_2 in a high humidity incubator. Cell viability was assessed using the AlamarBlue® (resazurin-based) assay (Sigma-Aldrich), live/dead staining was performed using calcein AM and propidium iodide (both 1 mg·mL^{-1}, Sigma-Aldrich).

Materials' extracts were prepared according to ISO 10993-5 in a 1:10 sample-to-substrate ratio (g·mL^{-1}). A series of dilutions of the extracts were made: 1 (undiluted – max. surfactant conc. 0.7 mg·mL^{-1}) and diluted 2- (max. surfactant conc. 0.35 mg·mL^{-1}), 4-(max. surfactant conc. 0.175 mg·mL^{-1}), 8- (max. surfactant conc. 0.0875 mg·mL^{-1}), 16- (max. surfactant conc. 0.04375 mg·mL^{-1}), and 32-fold (max. surfactant conc. 0.021875 mg·mL^{-1}). MG-63 cells were seeded in 48-well plates at a concentration of 1x10^4 cells/well, and after 24 h, medium was removed and extracts (0.5 mL) were added (day 0). Under these conditions, cells were cultured for 1, 3 and 7 days.

Alamar Blue reagent was prepared by dissolving resazurin sodium salt (Sigma-Aldrich) in UHQ water. At fixed time intervals (1, 3, and 7 days), 0.5 mL of 10% Alamar Blue reagent in MEM

were added to the wells. After 3 hours of incubation, fluorescence was measured at λ_{ex}=530 nm, λ_{em}=590 nm (FluoroSTAR Omega, BMG Labtech). The percentage of resazurin reduction was calculated according to the formula:

$$[(Fx - F0\%) / F100\% - F0\%)] - 100\%$$

Where:

Fx - fluorescence of the sample,

F0% - fluorescence of MEM with AlamarBlue reagent without cells,

F100% - fluorescence of fully reduced reagent (MEM with reagent autoclaved for 15 min at 121 °C).

To stain cells for the live/dead assay on days 1, 3 and 7 of the experiment MEM was removed from the wells, which were then washed with PBS. Cells were incubated in calcein AM/propidium iodide solution in PBS for 20 min in the dark and then images were taken using a fluorescence microscope (Axiovert 40 with HXP 120C metal halide illuminator, Zeiss). For confocal imaging, MG-63 cells were treated with extracts for 15 minutes. Extracts were then removed and the cells were gently washed with PBS before addition of PBS containing AlexaFluor488-labelled wheat germ agglutinin (3 $\mu g \cdot mL^{-1}$), Hoescht 33342 (10 $\mu g \cdot mL^{-1}$) and propidium iodide (10 $\mu g \cdot mL^{-1}$). Cells were then imaged using a Zeiss LSM880 confocal microscope using the following channels (Hoescht: ex 405 nm, em 410-495 nm; WGA: ex 488 nm, em 495-540 nm; PI: ex 561 nm, em 584-718 nm)

2.8. Cytotoxicity and mineralization tests - direct study

In order to evaluate the cells' behaviour on the foamed cements' surfaces, the obtained materials were pretreated in DMEM (BioWhittaker, Lonza) for at least 24 hours and incubated in a humidified chamber at 37 °C with 5% CO_2 (Heracell 150i, Thermoscientific). Samples of materials were placed wells in a 24-well plate and had drops of medium (approximately 50-150 µL) applied. The medium had to soak into the sample but not spill around to avoid washing out the biosurfactants. The soaking step was repeated twice to ensure that the transformation of αTCP to CDHA (calcium deficient hydroxyapatite) had taken place. After pre-treatment (soaking step) each cement sample was incubated in 1 ml of medium, consisting of DMEM supplemented with 10% FBS (Labtech), penicillin (100 U/ml) and streptomycin (100 µg/ml) and containing MC3T3-E1 (mouse osteoblast-like cell line) cells at a density of 2 x 10^4 per well. After 64 hours of incubation, the material samples were transferred to a new 24-well plate containing fresh medium. For direct cytocompatibility studies, Alamar Blue reagent was used - 100 µl of reagent (Alamar Blue in PBS (1:10)) was added to each well and incubated for 3

hours at 37°C. After incubation, the medium with reagent was transferred to a 96-well plate for absorbance studies. The remaining medium from the wells with samples was removed and replaced with fresh medium without Alamar Blue, and then the 24-well plate with the studied material was further incubated at 37°C. The absorbance was measured at a wavelength of 570 and 600 nm using a TECAN plate reader – just after transferring samples and 3-hour incubation as „0" time, as well as after 24 and 96 hours by repeating the procedure from the moment of material incubation with Alamar Blue reagent. The differential absorbance was calculated with the use of the equation:

$$\{[(O2 \times A1) - (O1 \times A2)] / \{[(O2 \times C1) - (O1 \times C2)]$$

Where:

O1 = molar extinction coefficient (E) of oxidized AlamarBlue (blue) at 570 nm

O2 = E of oxidized AlamarBlue at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

C1 = absorbance of fCTRL wells at 570 nm

C2 = absorbance of fCTRL wells at 600 nm

For the mineralization study, Alkaline Phosphatase (ALP) activity test was performed. The MC3T3-E1 cells were seeded as described above. After 7, 14, and 21 days the ALP activity was measured by adding 25 μL of the cells' lysates to 125 μL of ALP reaction buffer (1 $mg \cdot mL^{-1}$ p-nitrophenyl phosphate from Sigma-Aldrich), 0.1 M diethanolamine, 0.1% Triton X-100, 1 mM $MgCl2 \cdot 6H_2O$, pH 9.8 and incubation at 37 °C for 30 min. Then, 63 μL 1 M NaOH was used to stop the enzymatic reaction. The supernatants, in the amount of 170 μL after centrifugation at 14000G for 10 min were transferred to a 96-well flat bottom transparent plate, and absorbance was measured at 405 nm with a plate reader (FluoStar Omega, BMG Labtech).

2.9. Cell adhesion and morphology

After one week of incubation in DMEM, samples were washed twice with PBS. Cells were fixed in a solution of paraformaldehyde (4%) in PBS and stored at 4 °C. Cells were stained with Hoechst 33342 dye and visualised with the use of a Zeiss Axio Zoom.V16 fluorescence stereomicroscope. For SEM studies, cells were dehydrated through a series of increasing concentrations of ethanol from 30 to 100%. Dried cement samples with fixed cells were finally coated with an Au layer and examined with a scanning electron microscope (NovaNano SEM 2000).

2.10. Antibacterial studies

To evaluate the antibacterial activity of cement liquid phases, surfactant solutions were prepared at a concentration of 10 $g \cdot L^{-1}$ in Na_2HPO_4 solution. As the Na_2HPO_4 concentration at the level 2 $g \cdot dL^{-1}$ was bactericidal, studies were performed in solutions with a lower concentration of this salt (0.06 $g \cdot dL^{-1}$). The aqueous solutions used in the study were sterilized by filtration through 0.22 μm polyethersulfone filters. All tested solutions were found to be microbiologically pure - no microbial contamination was noted.

The bacterial reference strains used in this study*: Staphylococcus aureus* NCTC 4163, *Staphylococcus epidermidis* 1457, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* PAO1 were obtained from the collection of the Department of Pharmaceutical Microbiology, Centre for Preclinical Research, Medical University of Warsaw, Poland. Bacterial stock cultures were sub-cultured onto nutrient agar and BHI agar plate. Biostatic and biocidal effects of tested biosurfactants solutions were examined by minimal inhibitory and bactericidal concentrations (MIC/MBC). The MIC of compounds was determined by microdilution assay under standard procedures provided by CLSI with some modifications[35]. For determination of antibacterial activity, bacterial cultures were adjusted to 0.5 McFarland turbidity standards equal to $1.5 \cdot 10^8$ colony forming unit (CFU) per ml. All surfactant solutions were tested in the concentration range 10 - 0.3 $g \cdot L^{-1}$, by preparing dilutions in Mueller-Hinton II (MHII) broth. The test compounds were inoculated with microbial suspensions ($5 \cdot 10^5$ CFU/mL). In parallel, a positive control assay was performed (bacteria in MHII medium without surfactant solutions), as well as negative control (solvent employed to dissolve the surfactants). The plates were incubated at 37°C and visually read after 18 hours. The MIC value was considered to be the concentration of the compound at which no turbidity due to microbial growth was observed compared to the positive and negative controls. The Minimum Bactericidal Concentration (MBC) values of the compound resulting in the elimination of 99.9% of the test microorganisms were determined. The evaluation of MBC was determined by plating the well content from the well determined as MIC and higher concentrations onto BHI agar, incubating at 37°C for 24 hours and counting colonies.

The developed highly porous, foamed bone cements were subjected to *in vitro* tests for their potential antibacterial properties against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 4163). Studies were carried out by using the agar cup method. Before testing, the materials were sterilised with ethylene oxide at 37°C for 12 hours with complete degassing

after the sterilisation procedure. To improve biosurfactant diffusion from cementitious materials, two modified approaches were tested. Tryptic Soy Agar (TSA) was inoculated by spreading a dose of the bacteria ($1x10^6$ CFU/ml) isolate over the entire agar surface. After agar solidification, holes were punched out aseptically with a sterile cork borer and cement discs were placed into the wells, which were filled with saline solution - PBS. In the second test, the cement disks were placed on the surface of the TSA and respectively discs were covered by the second layer of inoculated agar. As a positive control of bacterial inhibition, the chemotherapeutic agent was used (ciprofloxacin). Then, agar plates were incubated at 37°C in high humidity conditions for 24h. After incubation, the diameter of the zones of growth inhibition around the cements was read, which is a measure of the sensitivity of the strains to the test material.

3. Results and discussion

In the literature, only a few calcium phosphate cements foamed with natural foaming agents, mainly proteins or polysaccharides, have been described. There are some works where egg white[36] or gelatin[13] were used to foam CPCs. There is also another way to foam cements, namely using surfactants[8,37]. However, there are only few reports concerning cement foaming using biosurfactants, *i.e*. surfactants with additional biological activity. Bercier *et al*. [38] described that, in the case of CPCs, the addition of sucrose esters, especially sucrose palmitate (SE16P from Stéarinerie Dubois (France)), led to obtaining cements with high porosity, excellent injectability and adhesive properties. However, in the mentioned work, a biosurfactant was used only as an additive in commercially available CPCs (Cementek and Cementek LV) without a foaming step. In our study, to prepare α-TCP/biosurfactant-based, highly porous bone cements (fCPCs), additional mechanical foaming was implemented. The obtained foamed fCPCs were subjected to a number of physicochemical and biological studies. Phase compositions, as well as diffractograms of foamed cements functionalized by biosurfactants after 7 days of setting and hardening in air at room temperature, are shown in Figure 2A-B.

A

Material	Phase composition	
	α-TCP [wt%]	HAp [wt%]
fCTRL	68 ±2	32 ± 2
fSAP	84 ± 2	16 ± 2
fSuE	83 ± 3	17 ± 3

B

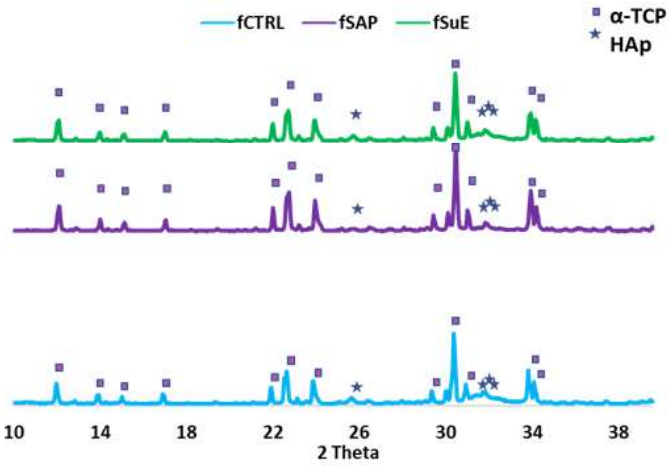

Figure 2 (A) Phase composition of materials with biosurfactants and (B) diffractograms of foamed cements, after 7 days of setting and hardening in air.

Cements foamed with biosurfactants (fSAP and fSuE) showed an amount of the HAp phase approximately two times lower than in cements without surfactant – fCTRL (Fig. 3A). Functionalization with nonionic biosurfactants resulted in the deceleration of α-TCP hydrolysis process in foamed cements. Similar effects on α-TCP hydrolysis were shown by more popular surfactants - polysorbates (Tween 20 and Tween 80) in our previous study[39]. This phenomenon was not observed in the case of non-foamed cements with the same surfactants[10]. This suggests that the inhibition of α-TCP hydrolysis was due to the cements' foaming process and bubble forming which impeded the availability of water needed for hydrolysis. After 7-day incubation in SBF, HAp phase constituted almost 100% of the final materials (data not shown).

An extremely important feature of materials for bone tissue substitution is their open porosity, which allows the migration of nutrients, gases (O_2 and CO_2) and cells. Over the years, pores ranging from 20–1500 μm have been used in bone tissue engineering applications[40]. The results of MIP studies (Fig. 3) revealed that the open porosity of fCTRL cement (without biosurfactant) was 54.8 ± 0.5 vol.%. For the material with sucrose ester (fSuE), this parameter reached 60.0 ± 2.7 %vol. whereas for the cement with saponin (fSAP) it was 76.3 ± 1.2 vol.%. As the open porosity was measured, we can assume that pores in the obtained foamed

cements were connected. The obtained values correspond to the porosity of cancellous bone (50 - 90% by volume)[41]. The results were strongly dependent on the foaming procedure used during cement preparation (biosurfactant added to solid or liquid phase of the cement). Control material and cement with sucrose ester displayed a bimodal pore size distribution with maxima of 0.05 and 1.08 µm for fCTRL and 1.10 and 18.82 µm for fSuE. The plot also shows that the fSuE material possesses many pores with a size above the second maximum as the measurement points take values greater than for the control material. Cement with saponin has a multimodal size distribution with maxima of 0.22, 1.05 and 30.24 µm. Furthermore, the pores in the fSAP material are present in nearly the whole investigated range.

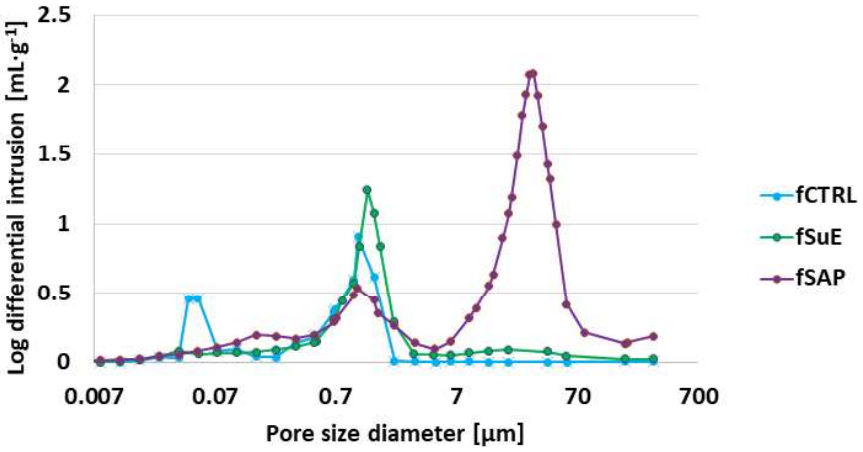

Figure 3 Pore size distribution in foamed cements.

The open porosity of cement foamed with saponin (fSAP) was similar to that of materials foamed with polysorbates such as Tween 20 and Tween 80 in our previous study as the same foaming method was used to obtain them. Meanwhile, the open porosity of fSuE was similar to that of cement foamed with the use of poloxamine (Tetronic 90R4) [39].

The architecture of the obtained highly porous cements was analysed using SEM and computer microtomography (Fig. 4). Materials foamed with biosurfactants were more porous than the control material (fCTRL). In the case of fSuE cement, not only fewer but also smaller pores (≤ 300 µm) were observed than for fSAP. Most likely this was due to the differences in the foaming procedure. Cement paste foaming as in the case of fSuE led to smaller pores due to high shear stress occurring during the process.

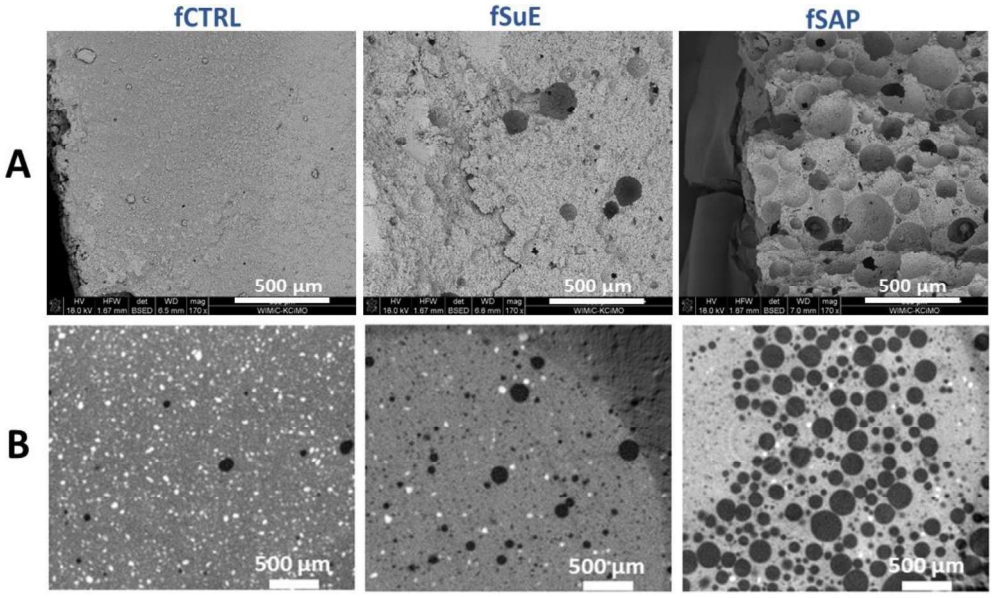

Figure 4 (A) Microstructure of investigated materials' surfaces – SEM studies and (B) cross-section images obtained with µCT of materials foamed with biosurfactants.

Foamed bone cements possessed relatively low compressive strength (Fig. 5). This parameter for the control material (fCTRL) was 2.4 ± 0.5 MPa. Application of the liquid phase foaming significantly lowered the compressive strength of fSAP material which was 0.2 ± 0.1 MPa. Among the cements with biosurfactants, the fSuE material had the highest compressive strength (2.7 ± 0.6 MPa) – comparable to fCTRL cement. This was related to less numerous and smaller pores for the fSuE material. Obtained results, concerning porosity, are comparable to values obtained by Montufar *et al.* (0.2 MPa if the total porosity of bone cement reached 75 %vol.)[7] and Zhang *et al.* (1-2 MPa)[9]. The compressive strength values of all porous cements foamed with biosurfactants corresponded to the strength of cancellous bone (0.1-14.0 MPa[42,43]).

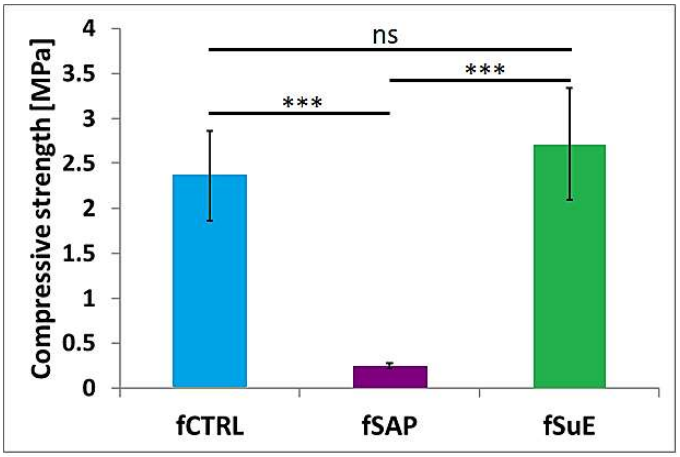

*Figure 5 Compressive strength of foamed cements. Asterisks indicate statistical differences: ns - non-significant differences, ***p < 0.001*

SEM images of cement samples before incubation in SBF present mainly unreacted α-TCP particles (Fig. 7). No precipitated calcium deficient hydroxyapatite (CDHA) crystals could be observed. After 28-day incubation in SBF, the entangled network of precipitated apatite-like forms was visible on the cement surfaces confirming the bioactive potential of the studied materials. Results show that biosurfactants did not adversely affect the bioactive potential of this kind of bone cements.

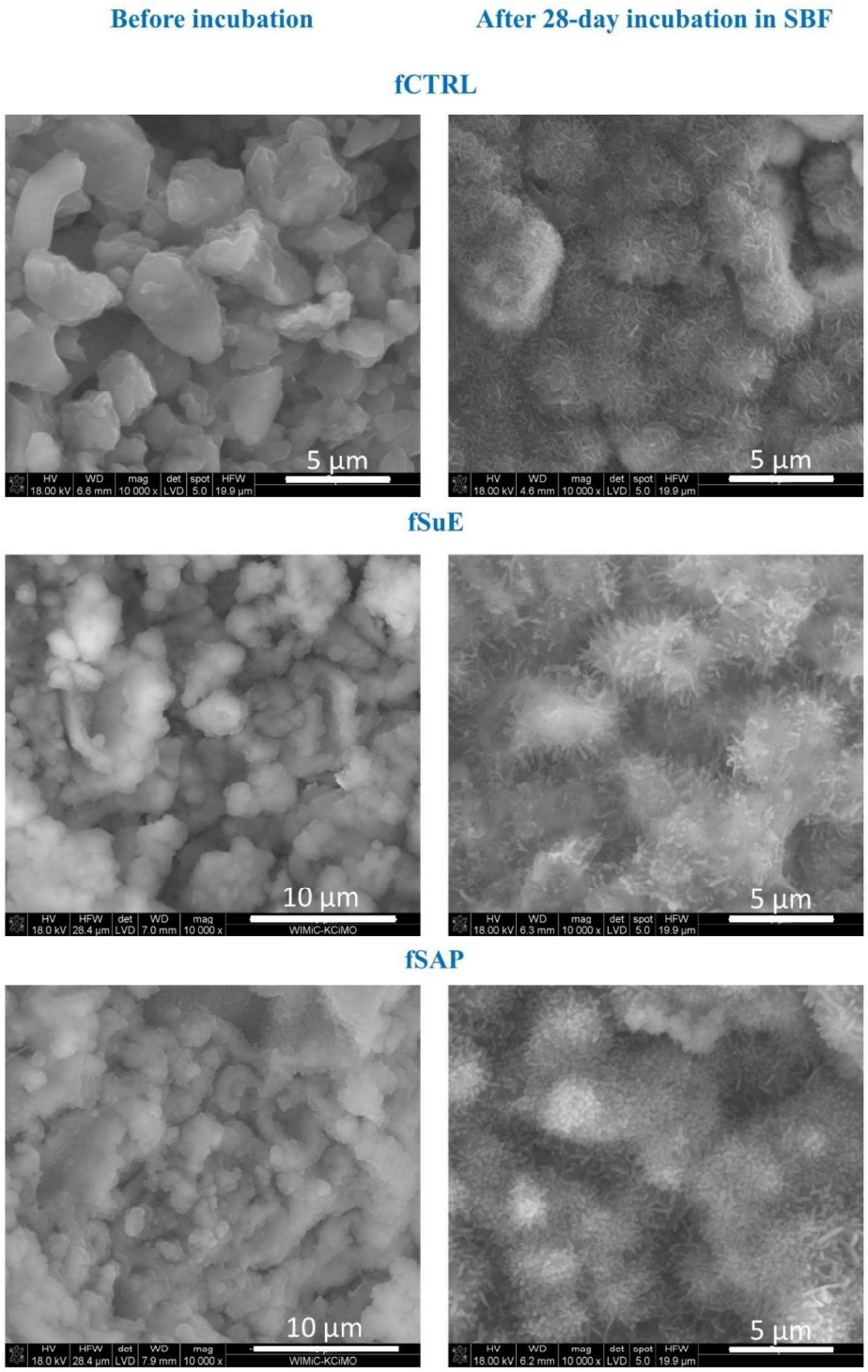

Figure 6 Microstructure of the studied CPCs before and after 28 days of incubation in simulated body fluid.

As investigated materials are intended to be used in the form of a foamed paste, the cytotoxicity studies of the obtained cements were performed using an MG-63 osteosarcoma cell line. Apart from sterilization with ethylene oxide, fCPCs samples, for indirect cell studies (on materials' extracts), were not subjected to any pretreatment or placed in contact with aqueous solutions (buffers or media).

On day 1 (Fig. 7A) for undiluted extracts, cell viability was below 70% of control for all. These results make the studied materials cytotoxic considering ISO 10993 standard. Cell viability for fCTRL and fSuE extracts at 1:2 dilution was at a similar level if compared to the untreated control (MEM, cells cultured in MEM medium). The reduction of Alamar Blue in the case of fSAP twice diluted extract was lower than for MEM controls. This suggests that cells died after contact with saponin. On day 3 (Fig. 7B), resazurin reduction increased for MEM control, suggesting that the cells were proliferating. Undiluted extracts from all samples were lethal to MG-63 cells. Cell viability values greater than 70% of the value of the MEM control were observed for two-fold diluted extracts of fCTRL and fSuE materials but in the case of fSAP only when the extract was diluted eight-fold. On day 7 (Fig. 7C), cells cultured in extracts from fCTRL and fSuE materials diluted two-fold were similarly affected as on day 3. However, in the case of fSAP, a four-fold diluted extract revealed cell viability greater than 70% of the value of the MEM control, having previously been below this threshold at earlier time points. This suggests that saponin becomes less toxic to cells over time. Cells incubated in two-fold diluted extracts proliferated and their number increased as compared to days 3 and 1.

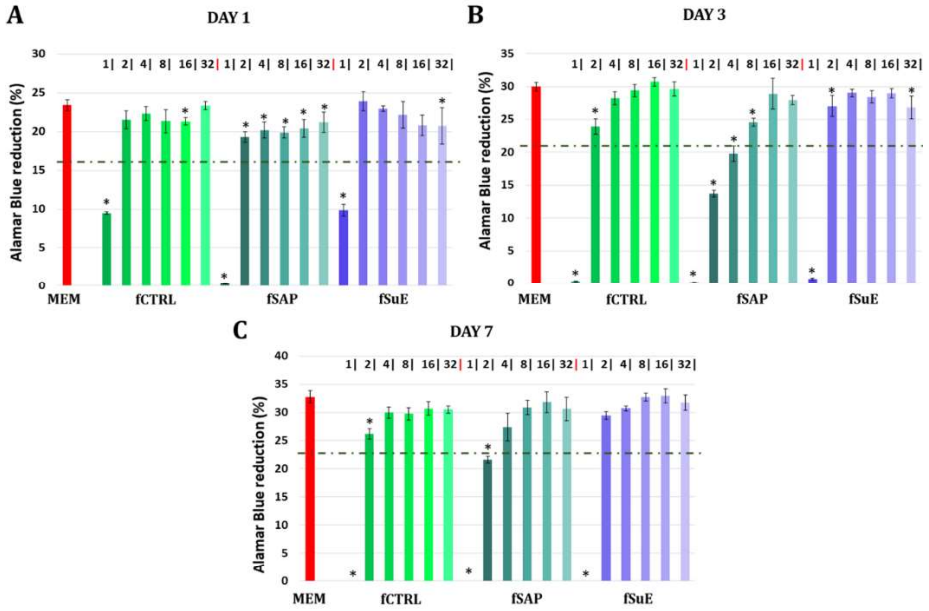

Figure 7 Viability (based on Alamar Blue assay) of MG-63 cells after contact with extracts (indirect study) from fCPCs on days: 1(A), 3(B) and 7(C) after extract addition. Numbers (1, 2, 4, 8, 16, 32) stand for the extract dilution factor.

The results were confirmed also by live/dead studies (Fig. 8). The undiluted materials' extracts were cytotoxic for MG-63 cells, with few cells, live or dead, attached in the wells within even 24 hours of treatment. This suggested a rapid process of cell death.

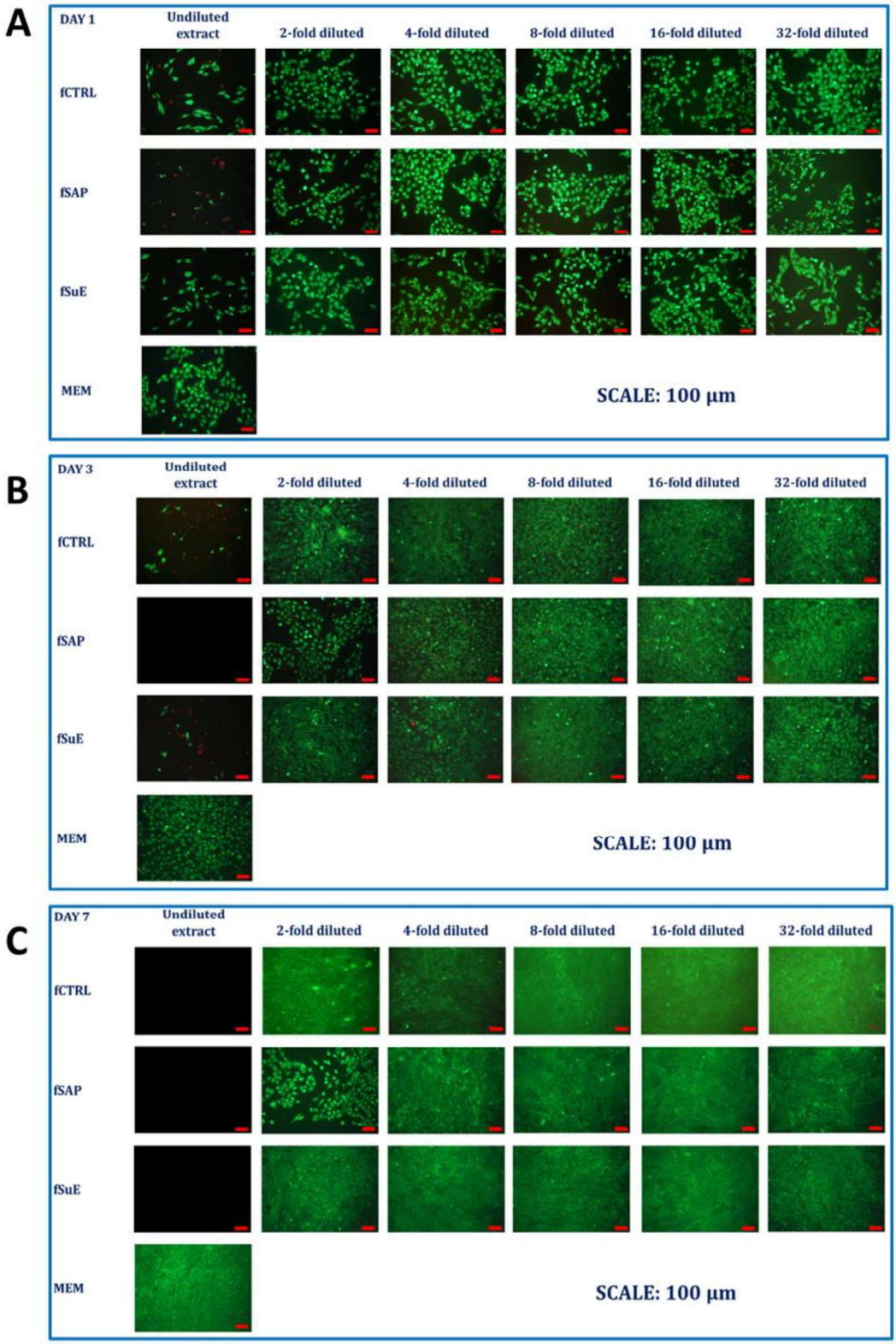

Figure 8 Live/dead staining of MG-63 cells cultured in extracts from cements fCTRL, fSAP, fSuE (indirect study) and in control cell culture medium (MEM) on: (A) day 1, (B) day 3, (C) day 7.

We hypothesized that the surfactant properties of saponin were leading to degradation of the cell plasma membrane at higher concentrations. In order to investigate this further, we incubated cells with undiluted extracts for 15 minutes, removed the extracts and then treated the cells with AlexaFluor488-labelled wheat germ agglutin (WGA, a lectin that binds to glycoproteins in cellular membranes), Hoescht 33342 (a membrane-permeant DNA stain), and propidium iodide (PI, a membrane-impermeant DNA stain) (Figure 9). We observed that after only 15 minutes of treatment with the fSAP extract, the cell membrane was substantially degraded. The cells became loosely attached to the plate and were able to take up PI, in contrast to the other three conditions (fCTRL and fSuE extracts and untreated control (NT)) where the membrane integrity remained uncompromised, and the cells remained firmly attached to the plate (Figure 9A). At higher magnification (Figure 9B) it was possible to observe that the cells treated with fSAP extract exhibited enhanced staining of cytoplasmic membranes (visible as a punctate pattern seen within the cell), further confirming that the plasma membrane structure had been compromised. Interestingly, it was observed that the fCTRL and fSuE extract-treated cells showed slight changes in cytoskeletal structure, becoming more rounded up compared to the untreated control - NT.

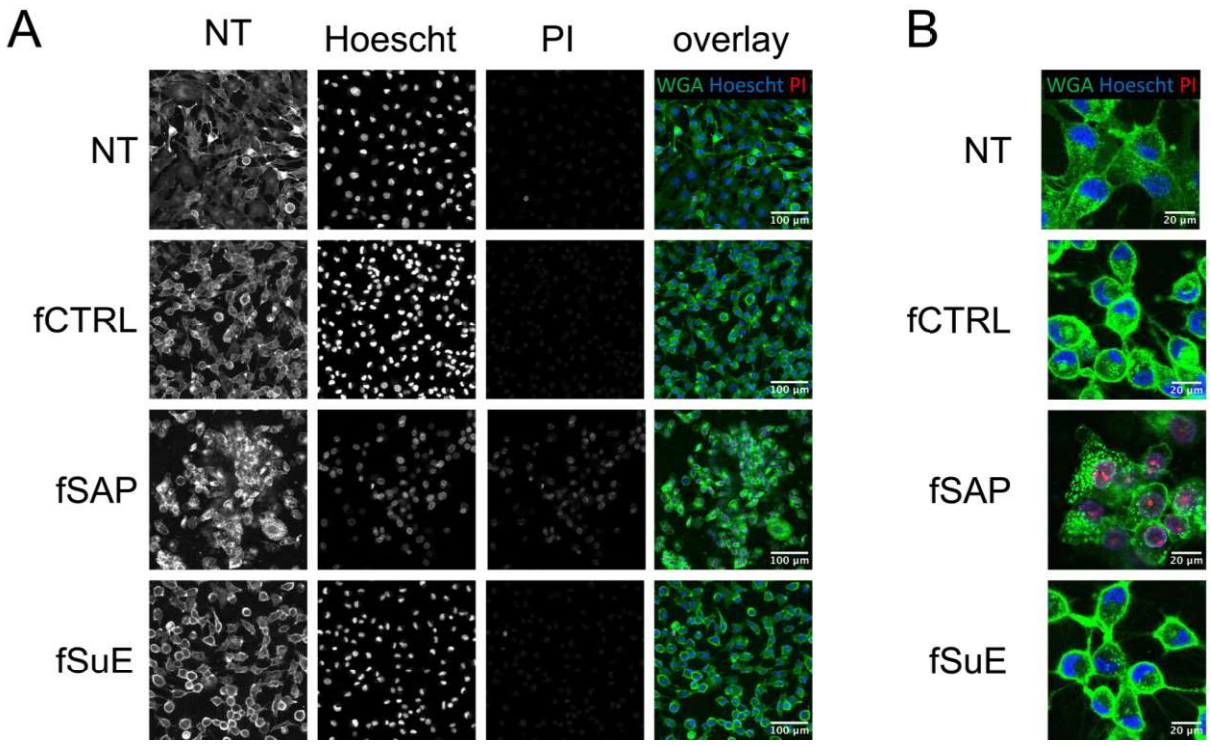

Figure 9 Confocal imaging of MG-63 cells treated for 15 minutes with extracts from cements fCTRL, fSAP, fSuE (indirect study) or left untreated. Unfixed, live cells were stained with AlexaFluor 488-labelled WGA, Hoescht 33342 and propidium iodide (PI) before imaging on a Zeiss LSM880 confocal microscope (A) Cells imaged under 10x objective, showing individual fluorescence channels and composite images. (B) Composite image of cells imaged under 40x objective.

The cytotoxicity of the α-TCP based CPCs results from the increase in the phosphoric acid ions arising from α-TCP during its hydrolysis to CDHA[44,45]. This phenomenon leads to a medium pH decrease, observed frequently for these materials[46,47,48], which has a negative impact on cells[49]. Nevertheless, α-TCP based CPCs are known to be biocompatible *in vivo*[50]. It was noted that the cytotoxicity of fSuE was comparable to that of the reference cement (fCTRL). This means that sucrose ester (S0112) itself did not adversely affect MG-63 cells. Similar results, but in indirect tests, were obtained by Bercier *et al*.[38] who studied CPCs with 1% of sucrose esters (SE16P or SE11S). Among the studied materials, cement with saponin (fSAP) was the most cytotoxic. Although cell viability was significantly lower for cement with this biosurfactant as compared to the control, one should keep in mind that there is a constant exchange of fluids in a tissue environment, so even harmful leached molecules can be neutralized by the cells and the concentration of the toxic substance locally decreases over time[51]. Saponins in several studies[52,53,54] have shown promising cytotoxicity profiles suggesting potential use in cancer treatment. The MG-63 cell line is derived from osteosarcoma, a representative of malignant tumours. Its cytotoxicity towards MG-63 may be due to the anticancer activity of saponin. The mechanism behind the antitumour properties of saponin may be related to the fact that saponins selectively wash out cholesterol from cell membranes[55]. Considering the most recent literature, cancer cells have membranes rich in cholesterol[56] so they can be more sensitive to saponin's action. This could be the possible explanation for the fSAP effect on the studied MG-63 cells.

For direct cell studies, Montufar *et al*.[3] simply immersed the cement samples in Ringer's solution for 4 days but the application of that method means that the surfactant (Tween 80) was washed away. In our study, after the pre-treatment (dropping with medium), the samples, without excessive washout of biosurfactants, were ready for application of cells. To compare the proliferation of M3T3-E1 osteoblasts after contact with the studied materials, Alamar Blue absorbances were measured (Fig. 10).

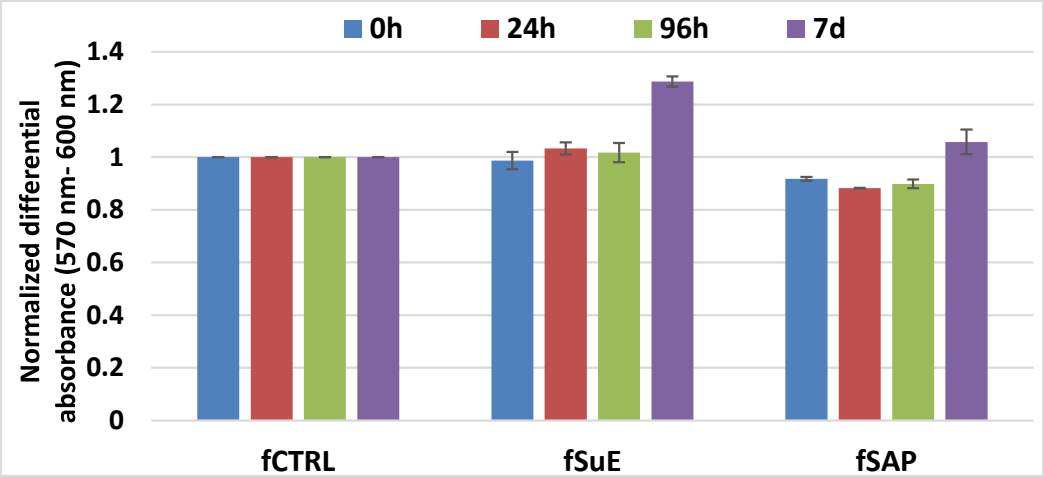

Figure 10 Cell proliferation of M3T3-E1 cultured on cement samples (direct study) presented by normalized differential absorbance.

Results show that until 96 hours of incubation number of cells on the cement with sucrose ester was comparable to their number on the control cement without any surfactant (fCTRL). Interestingly, after 7 days of incubation absorbance for fSuE was 29% higher than for the control material. It was probably due to the ingrowth of cells into the three-dimensional porous fSuE cement after the initial lag phase. Bercier *et al.*[38] stated that after 24-hour incubation % of MG-63 cells on the material with 1.0 wt% of sucrose ester (SE16P), the cell viability was comparable to the material without surfactant. Whereas, if cement had 2.0 wt% of SE16P, it drastically decreased. In the case of this study, approximately 0.4 wt% of sucrose ester (S0112) was present in the fSuE cement and there was no reduction in the number of MC3T3 osteoblasts if compared with fCTRL material. Another explanation for the reduced toxicity of sucrose ester to cells may be the presence of di-esters which are less cytotoxic than the pure monoesters[57] (SE16P is mainly monoester meanwhile S0112 is approx. 40% diester). In the case of cement with saponin (fSAP) absorbance was reduced until 96 hours of incubation if compared with the fCTRL. After 7 days of incubation, it started to increase, but not as significantly for fSuE cement. It was 6% higher than for the control material. Also, in this case it was most likely caused by the cell ingrowth into the pores of the fCPCs. As it was shown before in an indirect cytotoxicity study, saponin harms cells. Nevertheless, in the case of the osteosarcoma MG-63 cell line saponin cytotoxicity was higher which may indirectly indicate its anticancer activity[58].

Alkaline phosphatase is an important component in hard tissue formation, highly expressed in mineralized tissue cells. It is implicated in the regulation of local concentrations of inorganic phosphates fostering the mineralization[59]. ALP is a specific marker of mineralization and early

stage of osteogenic differentiation which reaches maximum expression level on the 14th day of culture[60]. In our study, ALP activity test confirmed that the M3T3-E1 cell has been mineralized (Fig.11).

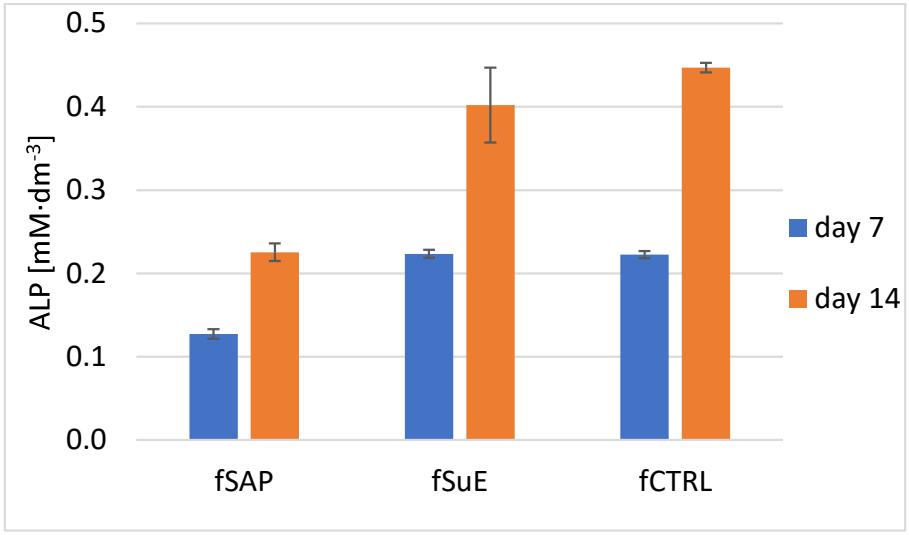

Figure 11 M3T3-E1 mineralization on cement samples based on ALP activity (direct study).

The highest activity was observed for fCTRL samples whereas the lowest was for fSAP materials. Results indicate that ALP activity was supressed by cements foamed with biosurfactants, especially by fSAP. MC3T3 cells on the cement samples were visualised also by fluorescence and scanning electron microscopy examinations (Fig. 12A-B).

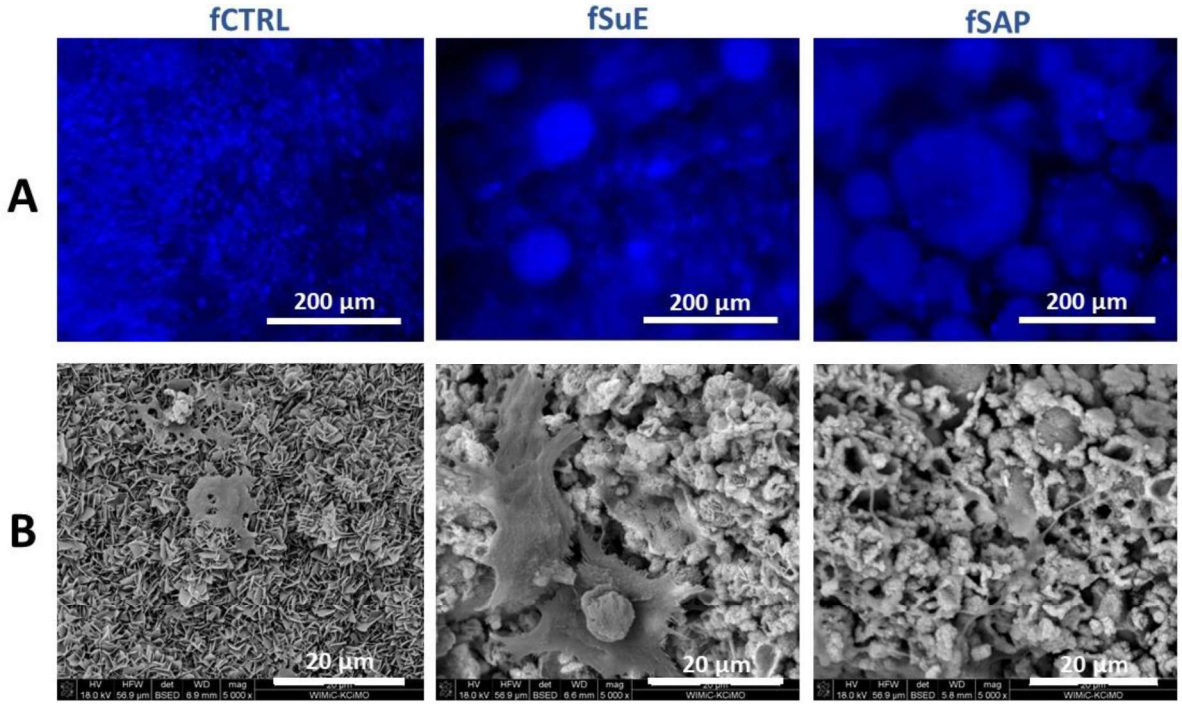

Figure 12 Cement samples with fixed cells visualised by (A) fluorescence microscopy – cells dyed with Hoechst 33342, (B) scanning electron microscopy (direct study).

Through staining with Hoescht 33342 the cell nuclei on the surface of cements were visualised (Figure 12A). They were most numerous on the surface of fCTRL - most likely because in the case of highly porous biosurfactant-foamed cements the cells were mainly located inside the pores.

The SEM pictures show strong anchorage of osteoblasts cultured on the studies cements, especially in the case of fSuE. Cells exhibited numerous and sometimes very long filopodia particularly on the cement with saponin (Figure 12B).

The antibacterial activity of cement liquid phases was investigated by determining the minimum inhibitory concentration (MIC) of bacterial growth (Table 2).

Table 2 Minimum inhibitory concentration (MIC) for the growth of Gram-positive and Gram-negative bacteria by cement liquid phases.

	Gram-positive bacteria		**Gram-negative bacteria**	
Strain and MIC / **Liquid phase**	*Staphylococcus aureus* NCTC 4163	*Staphylococcus epidermidis* 1457	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* PAO1
	Minimal inhibitory concentration - MIC ($g \cdot L^{-1}$)			
Saponin in 0.06 $g \cdot dL^{-1}$ aqueous solution of Na_2HPO_4	<<0.3	0.6	10.0	10.0
Sucrose ester in 0.06 $g \cdot dL^{-1}$ aqueous solution of Na_2HPO_4	>10.0	>10.0	>10.0	>10.0

Saponin contained in Na_2HPO_4 solution at a concentration of 0.3-0.6 $g \cdot L^{-1}$ caused inhibition of *Staphylococcus* spp. growth. In the case of *S. aureus*, the MIC of saponin could be even lower than 0.3 $g \cdot L^{-1}$. An inhibitory growth effect on Gram-negative bacteria was observed for saponin only at the highest studied concentration (10 $g \cdot L^{-1}$). Oyekunle *et al*.[61] concluded that the saponins have an inhibitory effect on Gram-positive organisms but not on Gram-negative ones. Similarly, Fink and Filip[62] noticed a stronger antibacterial effect of saponin against Gram-positive bacteria. In contrast, Hassan *et al*.[63] showed the antibacterial activity of a commercial extract rich in *Quillaja* saponins against not only *Staphylococcus aureus* but also *E. coli.* Similarly, Sewlikar and D'Souza[64] observed the strong antibacterial activity of the aqueous saponin extract.

In our study aqueous solution of sucrose ester had no impact on either Gram-positive or Gram-negative bacteria groups. The antibacterial activity of sugar fatty acid esters has been studied and variable results have been reported on different bacterial species. The monoesters show better antibacterial activity than diesters, and SFAEs show stronger antibacterial activity against Gram-positive than Gram-negative bacteria[65]. In studies by Zhao *et al*.[66] sucrose

palmitate exhibited no antibacterial activity against *S. aureus* or *E. coli*. On the contrary, it possessed bacteriostatic activity against *Bacillus coagulans* and *Geobacillus stearothermophilus*[67].

The minimum biocidal concentration (MBC) values of the compound resulting in the elimination of 99.9% of the test microorganisms are shown in Table 3.

Table 3 Minimum biocidal concentration (MBC) of biosurfactants.

	Gram-positive bacteria		**Gram-negative bacteria**	
Strain and MBC / **Liquid phase**	*Staphylococcus aureus* NCTC 4163	*Staphylococcus epidermidis* 1457	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* PAO1
	Minimum biocidal concentration MBC ($g \cdot L^{-1}$)			
Saponin in 0.06 $g \cdot dL^{-1}$ aqueous solution of Na_2HPO_4	5.0	5.0	10.0	>10.0
Sucrose ester in 0.06 $g \cdot dL^{-1}$ aqueous solution of Na_2HPO_4	Not defined*	Not defined*	Not defined*	Not defined*

*as MIC was >10.0 the MBC was not defined

The biocidal activity was observed against *Staphylococcus spp.* and one of the Gram-negative bacteria - *E. coli.* It was demonstrated by a saponin. The MBC value for *S. aureus* and *S. epidermidis* equals 5 $g \cdot L^{-1}$ whereas for *E. coli* it was 10 $g \cdot L^{-1}$.

Analysis of the antibacterial activity of the obtained cementitious materials (fCTRL, fSuE, fSAP) was performed. In each case (except for the control disc with the antibiotic ciprofloxacin) no zone of inhibition of bacterial growth was observed around the materials. This suggests that the biosurfactants' concentration used in the studied materials (maximum 7 mg/g of material) did not result in cement antibacterial activity. It could have been caused by an excessively low concentration of these compounds in fCPCS. It is conceivable that the use of these biologically active surfactants in higher concentrations would allow the transfer of the antibacterial properties of the studied biosurfactants to the final materials.

4. Conclusion and Outlook

In our study, biosurfactants such as saponin and sucrose ester were used to obtain functionalized, highly porous, foamed calcium phosphate bone cements (fCPCs). Biosurfactants, due to their biological activity, are an interesting alternative to foaming agents previously used in the manufacturing of these materials. The novel biosurfactant-assisted foaming methods applied in our study (liquid phase or cement paste foaming) allowed highly porous fCPCs with open porosity ranging from 54 to 77 %vol. to be obtained, matching the porosity criterion for highly porous materials. While biologically active surfactants had a

positive effect on the microstructure of fCPCs, their use led to the inhibition of α-TCP hydrolysis. Compressive strength of the materials ranged from 0.2 to 3.3 MPa. Cement with sucrose ester had the most favourable physicochemical properties and positively influenced MG-63 as well as MC3T3-E1 cells. Previous studies have demonstrated that surfactants can destroy membrane lipids and therefore can make bacterial cell membranes permeable, facilitating the passage of various molecules, such as drugs, through the membrane[68]. However, in our study, it has been shown that the materials with biosurfactants in the applied concentration did not possess antibacterial potential. Continuing the study, it would be important to see if an increasing amount of biosurfactants in the cement would contribute to the desired antibacterial effect of the material. Furthermore, an *in vivo* study could be performed to confirm the anticancer potential of fCPCs with saponin.

Acnowledgements

This study was supported by the National Science Centre, Poland (Project No. 2017/27/N/ST8/00913), by the subvention from the Ministry of Science and Higher Education (Poland) for the Faculty of Materials Science and Ceramics AGH UST – University of Science and Technology, Krakow, Poland, Project No. 16.16.160.557 (2022) and partially by the Program "Excellent Initiative – Research University" for the AGH University of Science and Technology. E. C. acknowledges financial support from the National Science Centre, Poland under Doctoral Scholarship No. 2019/32/T/ST5/00207. The authors would like to thank Prof. Jacek Tarasiuk (AGH University of Science and Technology, Poland) for materials analysis by computer microtomography and Dr. Elisabeth Shaw and Dr. Alexandre Benedetto (Lancaster University, UK) for materials imaging with a fluorescence microscope.

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