A New Class of Bioactive and Biodegradable Soybean-Based Bone Fillers

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The reconstruction of large bone defects in periodontal, maxillofacial, and orthopedic surgery relies on the implantation of biomaterials able to support the growth of new tissue. None of the materials currently available is able to combine all the properties required, which are (i) easy handling, (ii) biodegradation, (iii) low immunogenicity, and more importantly, (iv) induction of tissue regeneration. A new class of biodegradable biomaterials has been obtained by simple thermosetting of defatted soybean curd. The final material can be processed into films, porous scaffolds, and granules for different surgical needs. When incubated in physiological solutions the material shows water uptake of 80%, elongation at break of 0.9 mm/mm, and 25% (w/w) degradation in 7 days. Soybean-based biomaterial granules are shown to reduce the activity of the monocytes/macrophages and of the osteoclasts and to induce osteoblast differentiation in vitro, thus demonstrating a bone regeneration potential suitable for many clinical applications.

Introduction

Bone damages and defects can derive from those traumatic events or surgical procedures where bone needs to be removed because of pathological conditions. When the defect reaches a critical size, bone is not able to regenerate spontaneously and bone fillers are required to support its formation. Mineralized and nonmineralized bone grafts derived from the same patient (autograft) and from human (allograft) or animal (xenograft) donors are considered the gold standard in surgery. However, their use presents main drawbacks such as limited availability of autografts, patient's morbidity, and risks of transmittable diseases associated with allografts. Three main classes of synthetic bone fillers are currently used in surgery as alternatives to bone grafts: (i) ceramics (hydroxyapatite, HA; tricalcium phosphate, TCP; bioglasses), (ii) poly(lactic/glycolic) acid (PLGA), and (iii) collagen. HA, TCP, and bioglass, delivered in forms of porous scaffolds and granules, have excellent osteoconductive properties;[1] they have been shown to support adhesion and proliferation of the bone-producing cells, the osteoblasts in vitro[2], and to establish strong bonding with the newly deposited bone mineral phase in vivo.[3] However, because of their stiff and brittle mechanical properties, they are difficult to adapt to the bone defect during surgery, and although they can be manufactured with different degrees of crystallinity, their resorption rate cannot be finely tuned to bone regeneration and remodelling.[4] PLGA is a synthetic biodegradable polymer widely used in the biomedical field.[5] This biomaterial is able to support the adhesion of osteoblasts and to undergo complete degradation into CO_2 and water.[6] However, it is recognized that degrading PLGA polymer fragments elicit an inflammatory response, thus impairing bone regeneration.[7,8] Collagen, naturally extracted from animal sources or of recombinant origin, is also available in the form of films and sponges, but its clinical performance is debated.[9,10] The presence of amino acid sequences able to recognize cell receptors such as the integrins makes this material a suitable substrate for colonization by the cells.[11] However, these amino acid sequences are not specific to bone cells, but they can be recognized also by inflammatory cells and by fibroblasts, thus eliciting adverse reactions.[12] As for allografts, the risk of transmittable diseases for materials of animal origin and the purification costs of the recombinant products are additional drawbacks in the clinical use of this natural polymer.

Furthermore, true bone induction cannot be obtained by these traditional bone fillers unless growth factors such as the bone morphogenetic protein 2 (BMP-2) are loaded in their structure.[13] Although clinical studies have shown the potential of BMP-2-loaded bone fillers in accelerating bone regeneration,[13] relatively high amounts of BMP-2 are required to achieve satisfactory clinical results, inevitably increasing the costs of the bone filler and raising concerns about the potential carcinogenic effect of the growth factor.[14]

Soybean is a natural material made of protein and carbohydrate fractions (ca. 40% each), an oil fraction (ca. 18%), and minerals (ca. 2%).[15] Soybean also contains isoflavones, phytoestrogens with an ascertained action on eukaryotic cells.[16] Isoflavones such as genistein and daidzein are considered among the most effective plant estrogens as they are particularly effective in reducing the proliferation of tumor cells and the activity of immunocompetent cells such as lymphocytes and monocytes/macrophages as well as of the bone-resorbing cells, the osteoclasts.[16] Furthermore, these phytoestrogens are able to induce differentiation of the osteoblasts.[17] The reported low incidence of breast and prostate cancer as well as of osteoporosis in eastern populations has been indeed ascribed to the regular dietary intake of soy isoflavones.[18] Genistein and daidzein are also found abundantly in soy as glycosylated forms, genistin and daidzin. When glycosylated, isoflavones are inactive on cells, but it has been demonstrated that they can be readily

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converted into nonglycosylated, active molecules when exposed to body fluids such as human plasma.[18] In this study we report a preparation method, physicochemical characterization, and biocompatibility properties of a novel class of biodegradable soybean-based biomaterials (SB) able to combine uniquely all the characteristics advocated for bone fillers. The use of SB was advocated, as it is a material that can be produced by a relatively simple and inexpensive process and it may exert bioactivity on cells, thus leading to enhanced tissue regeneration. It was also hypothesized that the immunogenic response potentially elicited by xenogenic proteins (the soybean protein fraction) could be counterbalanced by the known immunosuppressant activity of isoflavones.

Materials and Methods

Biomaterial Preparation and Characterization. Soybean-based biomaterials were prepared either from commercial defatted curd (Morinaga, Japan) or by defatting soy flour in hexane according to a standardized method.[19] Following the traditional method of curd preparation, the defatted soy was incubated in 0.1 M $CaCl_2$ and the precipitated curd was pressed in gauze to eliminate excess water. Biomaterials were obtained by baking the curd at 60 °C overnight. Films of different shapes and sizes could be obtained by baking the curd in dedicated templates. Granules were obtained by milling biomaterial blocks, filtering them through three consecutive stainless steel sieves (IG/3-EXP, Fisher; porosity 355, 300, and 212 μm), and collecting the granule fraction retained after the last filtration. The biomaterials were then sterilized by γ irradiation (25 kGy). Fourier transform infrared spectroscopy (FTIR) of the defatted soy flour as well as of the thermoset was performed to characterize the chemical changes of the material during its processing. Thirty-two scans in the 650−4000 cm^{-1} range, with 4 cm^{-1} resolution, were taken by a Nicolet Avatar 320 single-beam spectrometer equipped with a diamond crystal with ZnSe focusing element (Nicolet Corp.). The data were processed by a Niocolet Omnic E.S.P. 5.2a software (Nicolet Corp.) and showed as transmittance percent after air background subtraction. Peak shifts were considered significant only when they exceeded the 4 cm^{-1} scanning resolution. The isoflavone content of the raw materials as well as of the final biomaterials was assessed after their extraction in methanol/water (80:20) mixtures at 60 °C under rotational stirring. The main isoflavones were identified and quantified by HPLC C18 column chromatography with an isocratic solvent system (MeOH/water 80:20).[20] The HPLC system was equipped with binary gradient pump (Perkin-Elmer Series 200) and autosampler (717+, Waterss) as well as with column chiller unit, SPD-6A UV detector, and Chromatopac CR5-A chart recorder/interpolator by Shimadzu Corp. (Japan). The main soy isoflavones genistein and daidzein, as well as their glycosylated forms genistin and daidzin, were identified and quantified by pure commercial standards (Sigma). SB-based membranes ($n = 6$) were characterized for their mechanical properties (Young's modulus, elongation at break, and stress/strain) following ASTM international standards.[21]

Biomaterial Degradation. The degradation profile of the soybean biomaterial granules (5 mg) was assessed in 1 mL of phosphate-buffered saline, pH 7.4 (PBS; Oxoid) for 7 days at 37 °C, static conditions, by a gravitometric method. Isoflavone release from 1 to 7 days was evaluated by HPLC as described above. The protein fraction released over time was also analyzed by SDS−12% PAGE in denaturating conditions.[22] The separated proteins were stained by the Bio-Rad Silver Stain Plus method (Bio-Rad, 161-0449).[22]

Mononuclear Cell Activation. To assess the proinflammatory potential of the biomaterial, mononuclear cells from human peripheral blood were freshly isolated in Histopaque 1077 (Sigma) gradient by Boyum's method from three different donors.[22] The isolated cells (10^5/mL) were incubated either with biomaterial granules (5 mg) or with their extracts obtained from the supernatant of the degradation experiments over 7 days. The activation of the cells in the presence of granules was measured as an oxidative burst by a standardized chemiluminescence method where the cells' free radical production was assessed for 1 h at room temperature by a BioOrbit chemiluminometer (Labtek) following a method previously published.[22] The degree of activation of inflammatory cells when incubated in the presence of the SB-based biomaterials was also evaluated by enzyme-linked immunosorbent assay (ELISA) of interleukin 1β (IL-1β) (Amersham) from $n = 3$. Mononuclear cells were incubated in the presence of 5 mg of granules at 37 °C, 95% air/5% CO_2, static conditions overnight. Data were expressed as cytokine concentration mean value (picograms per milliliter) and the value ranges were reported to indicate data reproducibility.

Osteoblast Viability, Proliferation, and Differentiation. Cell viability and proliferation of human osteoblast-like MG63 cells (ATCC CRL-1427) were assessed as previously described.[23] Briefly, the cells (10^5 cells/mL) were incubated in tissue culture plates (TCP, Nunc) for 24 h in minimum essential medium Eagle (MEM, Sigma M2279) enriched with 0.292 g/L L-glutamine, 1% (v/v) nonessential amino acids (Sigma M7145) and 10% (v/v) fetal calf serum (Sigma) at 37 °C under a 95% air/5% CO_2 flow. The medium was made osteogenic by the addition of β-glycerophosphate (10 μM) and ascorbic acid (250 μM).[23] The growth medium was then replaced with fresh medium containing increasing concentration of soybean granules previously sterilized by standard γ irradiation. The amounts of SB-based granules used in this experiment ranged from 5 to 60 mg. Cells were allowed to proliferate for 48 h and washed with calcium-free PBS, and their nuclei were stained by Hoerscht-propidium iodide (HPI) dye. The number of alive and apoptotic cells was scored by fluorescence microscopy at 40× magnification. Six different fields per sample were counted. The early induction of cell apoptosis was also studied by detecting the exposure of phosphatidylserine (PS) onto the plasmalemma by an Annexin V kit (BD Biosciences, PF032-1ES) following the manufacturer's instructions. The apoptotic cells were scored by fluorescence microscopy at 40× magnification. Experiments were performed in duplicate on different days, and data were expressed as mean ± standard deviation of living and apoptotic cells from $n = 6$.

Cell Ultrastructure. Cells were fixed in situ on thermanox coverslips by 2.5% (w/v) glutaraldehyde in phosphate-buffered saline for 3 h at ambient temperature, followed by postfixation in 2% aqueous OsO_4 for 4 h, buffer rinsing, dehydration in an ethanol series, and embedding in Spurr resin. Thin sections were cut onto Formvar-coated grids and poststained for 1 h in 0.5% (w/v) aqueous uranyl acetate followed by 10 min lead citrate treatment. Sections were examined in a Hitachi-71000 transmission electron microscope at 100 kV, and images were acquired with a Gatan Ultrascan 1000 charge-coupled device (CCD) camera (Gatan, Oxford, U.K.).

Protein and Collagen Synthesis. The synthesis of collagen produced by MG63 osteoblasts was evaluated in both nonosteogenic and osteogenic media.[22] After 48 h of incubation at different concentrations of granules, the cells were trypsinized and lysed by the addition of 0.1% (w/v) Triton, the samples were centrifuged for 5 min at 500*g*, and the supernatants (50 μL) were tested for their collagen content by a quantitative Sirius Red (Sigma) method as previously described.[22] The total protein content of each sample was evaluated by a Bradford microplate assay (Bio-Rad, 500-0006).[22] Data were expressed as micrograms of collagen per milliliter, mean ± standard deviation from $n = 6$.

ALP Activity. Cells (10^5/mL) were incubated for 24 h in a nonosteogenic medium (β-glycerophosphate-depleted medium).[22,23] The wells were then washed with PBS and the cells were detached from the substrate by trypsin and lysed. The samples were centrifuged and the supernatants were stored at −70 °C until use. An ALP microplate assay was performed as described elsewhere.[22,23] Data were expressed as ALP activity (milliunits per milliliter), mean ± standard deviation from $n = 6$.

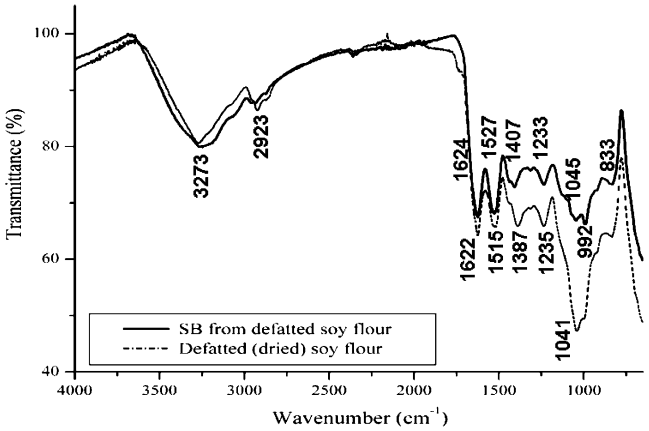

Figure 1. FT-IR of defatted soybean flour and thermoset SB.

Calcification Potential. The effect of the soybean-based biomaterial granules on formation of calcification foci in osteoblast nonosteogenic and osteogenic cultures was evaluated by Alizerin Red S staining.[23] The cells were analyzed by visible light microscopy at 40× magnification. Experiments were performed twice and in duplicate on different days.

Osteoclast Differentiation and Activation. Mononuclear cells, isolated as described above, were cocultured with MG63 osteoblasts and spiked with granulocyte and macrophage colony stimulating factor (GM-CSF) according to a published method.[24] In such a method, cocultured osteoblasts secrete RANKL, a signaling molecule that, in combination with GM-CSF, is able to induce mononuclear cell transformation into osteoclasts.[24] Briefly, 10^5 cells/mL of MEM with 10% fetal calf serum were seeded in each well and their adherence to the substrate was ensured by 3 h incubation at 37 °C, 5% CO_2/95% air. The wells were then washed to remove unbound cells and spiked with GM-CSF (5 mg/mL). Cells were cultured for 4 days and the GM-CSF spiking was repeated every 2 days while the supernatant was replaced with fresh growth medium. After 4 days, MG63 osteoblasts (10^4 cells/mL) were added and cocultured with the mononuclear population for 20 h. SB granules (20 mg) were added together with fresh medium and GM-CSF spiking and cell culturing were prolonged for 3 days as described above. Finally, cells were fixed in formalin for 10 min, extensively washed in PBS, and dehydrated with 1:1 ethanol/acetone for 1 min. A standard tartrate-resistant acid phosphatase (TRAP) staining method was applied to the wells (and to cells counterstained with hematoxylin.[24] Cells were analyzed by light microscopy at different magnifications.

Statistical Analysis. Data were statistically analyzed by ANOVA (Dunnett's test). In all cases, data were considered significantly different from the relative control at $p \leq 0.05$.

Results and Discussion

SB can be easily prepared by thermosetting defatted curd. A comparative FT-IR analysis of the defatted soy flour from which curd is prepared and of the final SB clearly shows conformational changes of the soy-based material, especially in its protein fraction (Figure 1).[25] In particular, in the thermoset biomaterial, while the band assigned to C═O stretching of the amide I (1624 cm^{-1}) did not show any significant difference from that of the soy flour (1622 cm^{-1}), N−H bending of the amide II signals shifted from 1515 to 1527 cm^{-1}. The band at approximately 1620 cm^{-1} has been assigned by other authors to the soybean protein β-conglycinin and has been attributed to α-helix structure.[26] C−H deformation shifted from 1387 cm^{-1} in the soy flour to 1407 cm^{-1} in SB and the relative shoulder at approximately 1450 cm^{-1} was more pronounced in SB, as found by other authors in soy protein fractions undergoing sodium dodecyl sulfate and heat treatments.[25] As for the amide I peak, N−H bending vibration of the amide III peak of the soy flour (1235 cm^{-1}) did not show any significant shift (1233 cm^{-1}). Overall, in SB, C−H deformation and N−H bending at 1407 and at 1233 cm^{-1} changed their relative ratios to the main amide I (1624 cm^{-1}) and II (1527 cm^{-1}) peaks, becoming less prominent than those of the defatted soybean flour. Similar changes in FT-IR profile have been found in soybean protein fractions and attributed to conformational changes induced by their treatment with sodium dodecyl sulfate and their partial degradation caused by the heating process.[25] Similarly, the soy flour peak at 1041 cm^{-1} and its shoulder at about 1000 cm^{-1} were found to be decreased in the thermoset SB and separated as a consequence of their shift to 1045 and 992 cm^{-1}, respectively. The changes in these two regions may be attributed to the hydroxyl groups of the carbohydrate fraction possibly involved in hydrogen bonding (1041 cm^{-1}) as well as to those interacting with the protein component.[27] In both soybean flour and SB, the occurrence of inter- and intramolecular hydrogen bonds is also confirmed by the broad absorption band observed in the frequency range 3600−3000 cm^{-1}. This signal was attributed to free and bound O−H and N−H groups and appeared to be broader in SB. No significant alteration of the principal soy isoflavones content was observed when the levels of defatted flour the SB were compared by HPLC analysis. In particular, no significant level changes were found for the nonglycosylated forms, daidzein (27.2 μg/g of soy) and genistein (26.2 μg/g of soy), as well as for the glycosylated daidzin (6 mg/g of soy). Only a nonsignificant loss (0.7 μg/g of soy) of the glycosylated genistin was observed; its final concentration in SB was 0.8 g/g of soy. Routine quality control has also shown that the SB physicochemical and biological properties are not affected when different sources of raw material are used. Indeed, the quality control of different soybean flours and curds has shown, for example, comparable isoflavone levels (data not shown).

The SB appear as materials that are ductile and uniquely able to modulate cell biochemical pathways key to tissue regeneration. Indeed, SB showed a relatively fast swelling in physiological buffer. The amount of adsorbed water was approximately 80% by weight in 24 h, with the swollen material assuming a rubbery consistency as demonstrated by its mechanical properties (elongation at break = 0.898 ± 0.098 mm/mm, stress at break = 0.921 ± 0.115 MPa, modulus = 3.580 ± 0.412 MPa). The degradation of the swollen material was very gradual, and a significant 25% weight loss was detected at day 3 and remained unchanged up to day 7. The degradation evolved through the gradual release of the two typical soy protein fractions (β-conglycinin and *Glycine max*[26]) approximately in the 60−90 kDa range (Figure 2a, arrows) and, after 4 days, to the appearance of at least two main lower molecular weight fragments in the range 40−45 kDa (Figure 2a, arrowheads). The significant weight loss detected at day 3 seemed to correspond to the release of low molecular weight protein species (Figure 2a, day 3, bracket). These molecules produced a smear in the electrophoresis gel typical of glycosylated molecules. Degradation also led to the constant release of all the most typical soybean isoflavones (data not shown). Among them, genistein reached a plateau of approximately 0.08 μg/mL after 100 h (Figure 2b).

A unique pattern of biological activities appeared when SB in the form of granular bone filler was tested in vitro. The activity of mononuclear cells freshly isolated from human peripheral blood, measured as free radical production during

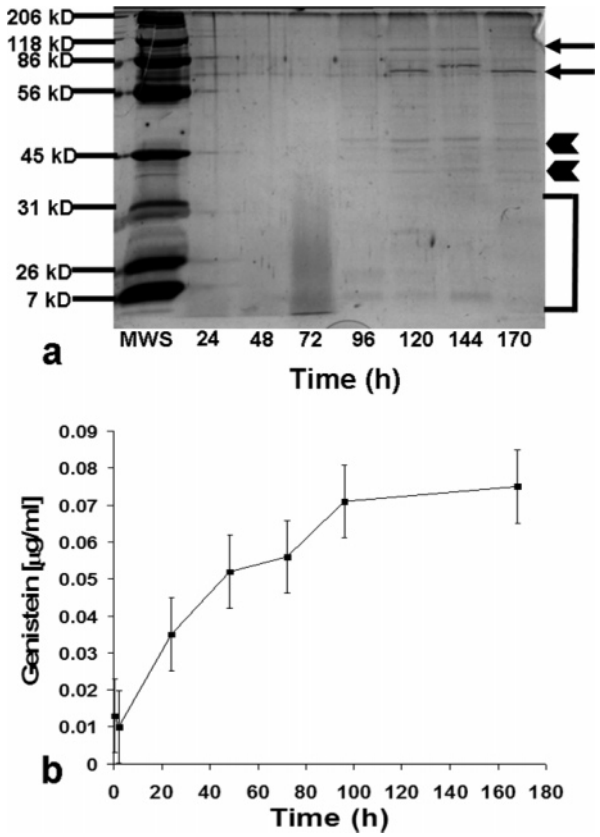

Figure 2. Release of SB-based bone filler components at physiological conditions: (a) protein release; (b) genistein release. Arrows indicate main soy protein bands. Arrowheads and brackets indicate possible low molecular weight degradation products (for details see main text).

the cell oxidative burst, was significantly reduced by the SB (control = 0.62 ± 0.20 cpm, SB-incubated cells = 0.22 ± 0.11 cpm, approximately 70% inhibition) and by the supernatants obtained from its degradation experiments after 7 days (0.045 ± 0.005 cpm, approximately 90% inhibition). The inhibitory effect of the SB on these immunocompetent cells was also demonstrated by the decreased levels of a proinflammatory cytokine, interleukin-1β (IL-1β) [control cells = 331 pg/mL, concentration range 179−545 pg/mL; SB-incubated cells = 56.2 pg/mL, concentration range 23.2−89.2 pg/mL). The activation of osteoclasts, the cells responsible for bone resorption, was also inhibited by their incubation with the SB-based bone filler (Figure 3). Indeed, no significant number of TRAP-positive cells was detected when multinucleated osteoclasts were stimulated by GM-CSF spiking and osteoblast coculturing in presence of SB granules. The inhibitory effect of the SB on monocyte/macrophages and osteoclast activity can be ascribed to the release of isoflavones and especially of daidzein and genistein. Indeed, both these isoflavones are known to inhibit the activity of immunocompetent cells by competing with the tyrosine kinase receptors of the cell membrane.[16] Furthermore, it has been shown that genistein and daidzein are capable of inhibiting the synthesis of RANKL by the osteoblasts, a factor required to promote the differentiation of mononuclear cells into multinucleated osteoclasts.[28] It is therefore envisaged that, during their swelling and degradation at the site of implantation, the SB-based bone fillers may release isoflavones, thus reducing the risks of macrophage-mediated chronic inflammatory response normally associated with implants, and they may delay any bone-resorption process driven by osteoclast activity.

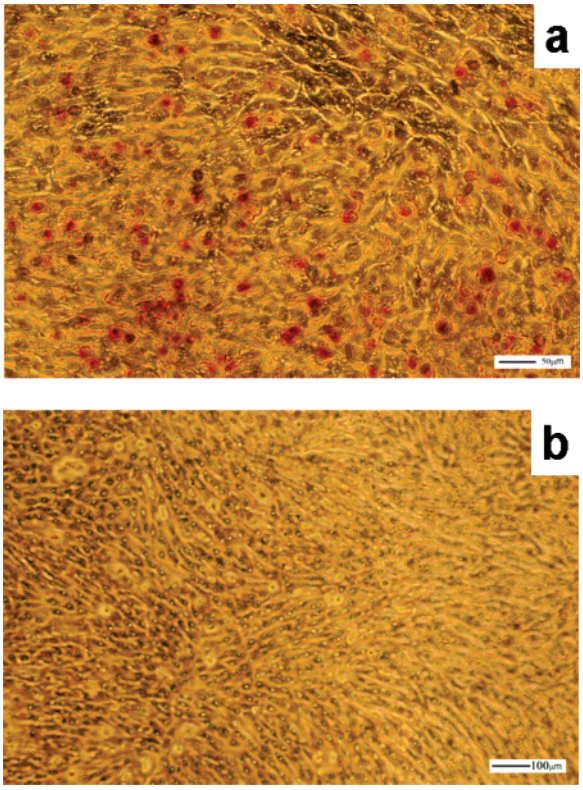

Figure 3. Effect of SB bone filler on osteoclast TRAP activity: (a) control cells; (b) SB-treated cells. Osteoblasts were counterstained with hemeatoxylin. Cells stained in red are TRAP-positive.

In addition, in vitro studies performed to assess the effect of the SB-based bone filler on osteoblasts unveiled a peculiar regulation of the activity of this type of cells. Osteoblast proliferation was significantly reduced by SB-based bone filler, and in the presence of high granule density (>10 mg), a certain proportion of cells entered apoptosis (Figure 4a). The synthesis of protein produced by osteoblasts incubated with varying SB granule amounts (5−60 mg) ranged from 40.73 ± 3.89 to 46.56 ± 5.5 μg/mL and was not significantly different from the control (40.54 ± 2.32 μg/mL) unless it is considered that, in the case of SB-based bone filler, similar concentrations were produced by a lower number of cells. Regardless of data normalization, collagen levels were significantly induced by SB (Figure 4b). Alkaline phosphatase (ALP), a typical marker of osteoblast differentiation, was apparently reduced when cells were treated with 5−15 mg of granules (Figure 4c) but returned to levels similar to the control at higher granule amounts. As for protein concentration, similar levels of ALP were produced by a lower number of cells, suggesting a stimulation of ALP by SB. Cell proliferation reduction, collagen and ALP induction, and other differentiation parameters have been previously shown in osteoblasts that were incubated with purified soy isoflavones.[29−32] Previous investigations have led authors to postulate a dual mechanism of action of isoflavones on eukaryotic cells where these phytoestrogens may be recognized by (i) TGF-β1 receptors on the cell membrane, where they act as growth factor analogues, and (ii) estrogen β receptors on the nuclear membrane, where they reduce cell proliferation.[29−32] The effect of SB on osteoblasts (Figure 4) may reflect this dual mechanism of action, thus leading to different effects on cell proliferation and differentiation. The study of the cell ultrastructure revealed that SB-based bone filler induced the formation and release of matrix vesicles on the osteoblast surface after only 2 days of incubation (Figure 5). No matrix vesicle production was

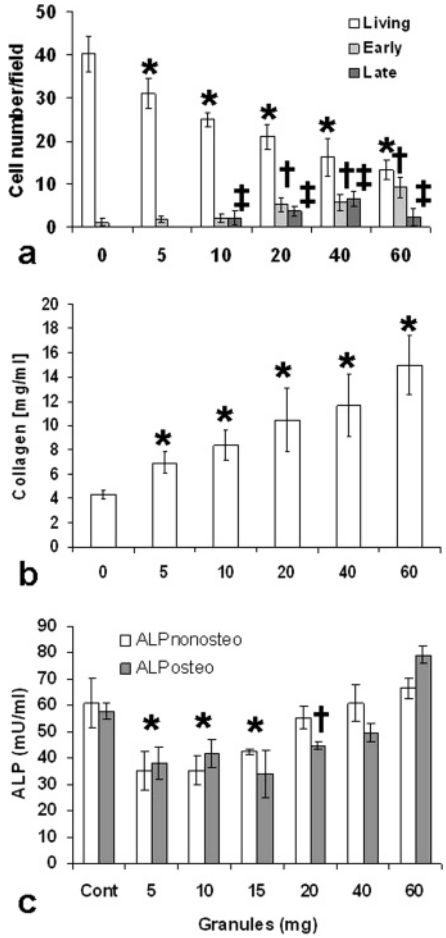

Figure 4. Effect of SB bone filler on osteoblasts activity. (a) Proliferation and viability. (*) Viable cell counting values significantly different from respective controls at $p \leq 0.05$. (†, ‡) Early and late apoptotic cell counting values significantly different from respective controls at $p \leq 0.05$. (b) Collagen synthesis. (*) Collagen synthesis value significantly different from respective controls at $p \leq 0.05$. (c) ALP activity. (*) ALP activity values significantly different from control in both nonosteogenic and osteogenic media; (†) ALP activity value significantly different from control in osteogenic medium only at $p \leq 0.05$.

observed in control cells (data not shown). Interestingly, by a specific assay it was also possible to demonstrate that SB induced the exposure of phosphatidylserine (PS) from the cytoplasmic side of the cell membrane onto its outer surface (data not shown). The release of matrix vesicles was associated with SB-induced osteoblast noduli calcification, which was detected by Alizerin red staining in SB-treated cells but not in control cultures (Figures 6). Indeed, the exposure of PS is a process typical of cells at their early stages of apoptosis,[33] but this phospolipid is also known to be a potent calcium-binding molecule of the osteoblast matrix vesicles membrane responsible for bone mineralization.[34,35] It is still debated whether matrix vesicles are ghosts of apoptotic cells or exocytosis bodies specifically secreted by the osteoblasts under certain conditions to induce mineralization during bone formation. Regardless of their origin, the data of this paper seem to show that SB induce the synthesis of matrix vesicles and bone noduli calcification. Combined with the induced synthesis of collagen, these data seem to suggest that SB-based bone filler is able to trigger most of those biochemical and cellular events required for the synthesis of new mineralized tissue. Recent publications have shown similar effects induced by the pure soybean isoflavones genistein and daidzein on human osteoblast differentiation.[29−32]

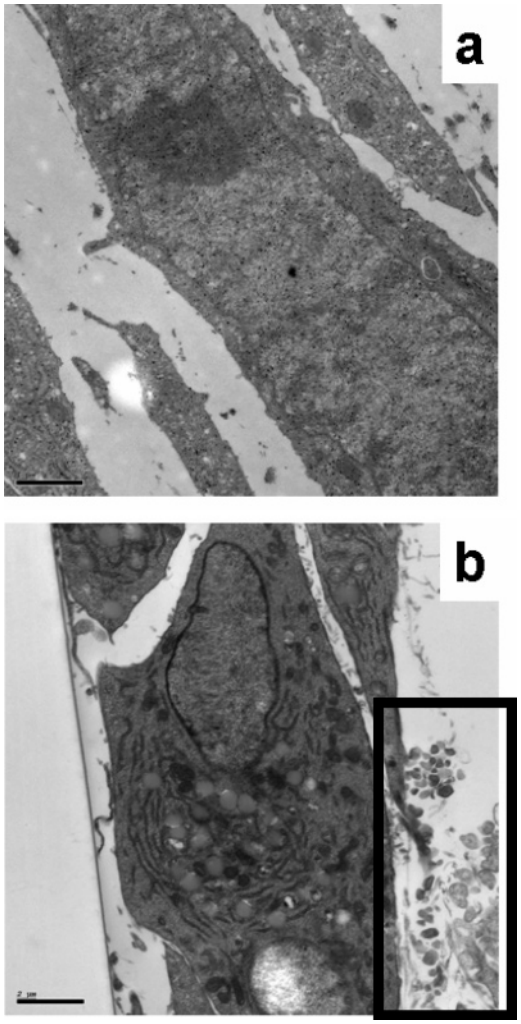

Figure 5. Effect of SB bone filler on osteoblast ultrastructure: (a) control cells; (b) cells incubated with 10 mg of SB bone filler granules. Box indicates areas of matrix vesicle formation and release from plasmalemma.

In particular, it has been demonstrated that daidzein and genistein can penetrate the cell membrane and interact with estrogen receptor β of the nuclear membrane, inducing the synthesis of ALP as well as BMP-2 gene expression in both stem cells and osteoblasts.[32,36] Therefore, it is likely that most of the effects of the SB on cells are determined by the release of isoflavones from the swelling and degrading biomaterial. It may be speculated that the molecular cross-linking induced by the thermosetting procedure during the SB preparation (Figure 1) might have led to a controlled release of phytoestrogen (Figure 2) at a range of concentration effective on osteoblasts.[29]

Conclusions

A new class of biodegradable and bioactive biomaterials with physicochemical properties suitable as bone filler applications has been achieved by a simple thermosetting method of soybean curd. Biomaterials derived from soybean proteins have been previously presented.[37] However, these biomaterials do not offer the multifaceted physicochemical and bioactivity properties found when thermoset curd is utilized. Indeed, the ductile nature of the materials obtained in the present work allows their easy adaptation to the site of implantation to provide the damaged tissue with a biodegradable scaffold. Upon implantation, the SB-based bone filler may be able to reduce chronic inflamma-

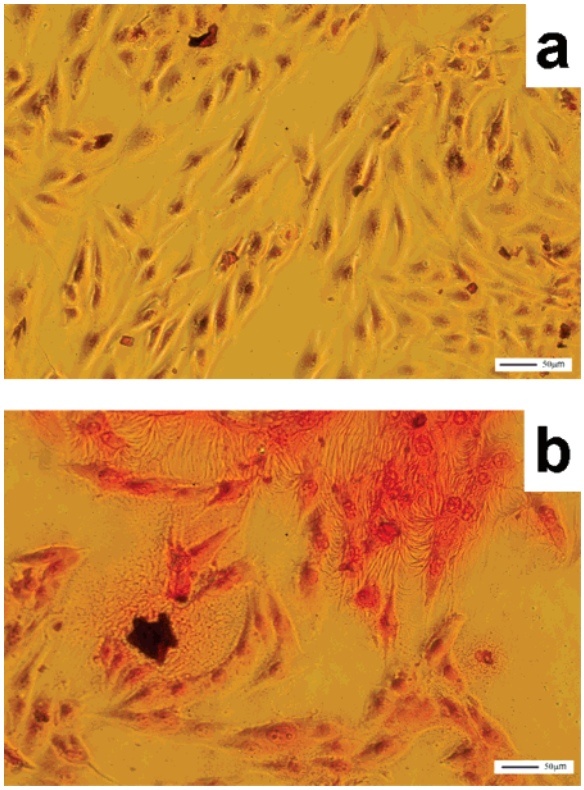

Figure 6. Effect of SB bone filler (10 mg) on osteoblast calcification: (a) control cells; (b) SB-treated cells.

tory response caused by the protracted activation of macrophages and to promote bone regeneration by stimulating bone cells. SB therefore emerge as a new class of biomaterials able to combine a relatively easy and inexpensive manufacturing process to main physicochemical and biological properties advocated for bone fillers for periodontal, maxillofacial, and orthopedic surgery.

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