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GRAPHICAL ABSTRACT. Multivalorization of AP
Multivalorization of Apple Pomace towards Materials and Chemicals. Waste to wealth

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ABSTRACT

The work presented here uses apple pomace (AP), an industrial waste from apple juice and cider production as a renewable raw material (RRM), to obtain materials that can be utilized as biocompatible scaffolds for osteoblasts and chondrocytes, employed in tissue engineering, valuable extracts that can be used as nutraceuticals and pectin. All of these have much higher value than the original raw material, pectin can be priced up to 1 euro/g, chlorogenic acid is ca. 120 euros/g, caffeic acid 3-5 euros/g and especially the scaffolds that are usually made by synthetic methods using non-renewable raw materials with high fabrication costs and sold at prices higher than 100 euros/g, while the residues used here have prices lower than 100 euros per ton. Thus, there are clear environmental and financial incentives in transforming this waste material into valuable substances and materials.

As indicated in the Graphical Abstract, the procedure followed consists in sequential extractions of antioxidants, pectin and finally the preparation of a biocompatible material, giving priority to the latter due to its importance as a renewable scaffold for tissue engineering. From a literature search, to date, although separate ways of valorisation have been applied to this kind of waste, the sequential multivalorization adopted here, has not been previously attempted. Furthermore, biocompatible scaffolds from AP have not been described.
1. Introduction

The unsustainable use of raw materials and the increase in the generation of waste has led to a shift from waste management for pollution control or prevention, to a more holistic approach where these waste materials are now thought of as valuable resources for use as raw materials essential as a prerequisite for a sustainable development. The importance of restriction in the use of natural resources to obtain a sustainable level is linked to the corresponding reduction in Greenhouse gas emissions which cause a negative impact on climate change. Agroindustrial wastes are important at a global level, since they are directly related to Greenhouse gas emissions derived from production, consumption and disposal and can lead to associated depletion of natural resources, due to an ever-increasing world population (Papargyropoulou et al., 2014).

Concepts such as “circular economy” and “cradle to cradle” aim to achieve a society in which wastes from some industrial activities can be used as RRM for other materials and commodities, raising both important economic and environmental issues that can avoid the use of non-renewable resources, within the biorefinery concept (Mirabella, 2014).

Vegetable and fruit industries produce large amounts of waste that affects landfills, containing ca. 80% sugars and hemicellulose, 9% cellulose and 5% lignin, being biodegradable, they raise methane and leachates and generally have high chemical and biochemical oxygen demands that cause huge recovery costs (Mirabella et al., 2014). Countries with agricultural based economies, such as Spain, generate vast amounts of agricultural wastes which, although of low toxicity, can often represent an environmental hazard. In our group, agricultural industrial wastes have been valorised since 2007, working in a sustainable manner towards a “zero waste” economy, in order to obtain materials and substances, which given their origin may compete with conventional ones and consequently decrease pollution and thus contribute to cleaner production of materials and commodities.
For example, biodiesel production wastes have been transformed into commodities, using as catalysts materials from the same company’s residues (Yates et al., 2014), citrus wastes have been catalytically transformed into \( p \)-cymene, pharmaceutical and fine chemical intermediates, avoiding the need for petroleum derivatives that are currently used at the industrial scale on commercial catalysts (Martin-Luengo et al., 2008) and on Spanish clays based catalysts (Martin-Luengo et al., 2010), rice production wastes have been used in the design of structured materials to decontaminate effluents (Martin-Luengo et al., 2012), act as support for catalysts (Martin-Luengo et al., 2011) and enzymes (Martin-Luengo et al., 2013), sunflower production residues have been transformed into multifunctional materials (Martin-Luengo et al., 2011c) and catalysts for the same industry use (Yates et al., 2014) and beer production wastes have been transformed into powdered materials (Yates et al., 2008), scaffolds for hard tissue engineering (Saez Rojo et al., 2014) and supports for controlled desorption of bioactive substances (Martinez Serrano et al., 2015).

The world production of apples was more than 70 million metric tons in 2015, of which the European Union contributed with more than 15%, while half a million tons of which came from Spain. About 75% of apples can be converted to juice and the rest, known as apple pomace (AP) that contains approximately 20-30% dried matter, is used mainly as animal feed or for compost. Since AP is generated in vast quantities and contains a large fraction of water, it poses storage problems and requires immediate treatments to prevent putrefaction. An alternative of great environmental interest is its transformation into value added commodities, thus reducing the volume of waste (WAPA, 2016).

In general, food waste has to be processed before use, which adds high costs to R&D and due to this it is necessary to obtain high added-value products in order to justify the investment. It is essential for the evaluation of the potential for exploitation to take into account the geographical location of producers, intermediaries and purchasers. Symbiosis
should be considered of several stakeholders, to enhance the economic potential of the industries that transform and use wastes, taking into account the sustainability of the process and avoiding the risk for example of extractions with toxic chemicals, or energy expending procedures. Biorefinery should be approached taking into account a life cycle assessment and giving most importance to consumers’ health (Martinez Serrano et al., 2015).

Until now, AP has been converted separately into biofuels, used as a substrate for enzymatic processes, to attain chars, extracted for antioxidants, as a source of bioenergy (Thi et al., 2016), or sorbents for effluent cleaning (Ozbay and Yargic, 2015), etc. Often the wastes are employed for only one of these uses and the rest is not considered for further valorisation, however, in the present multidisciplinary research, AP is multi-valorised into valuable extracts (antioxidants and nutraceuticals), pectin and inorganic materials.

Synthetic antioxidants, usually derived from non-renewable fossil fuel sources, often pose potential health risks leading to the development of regulations for their use. This has led to a consequent increase in the use of natural antioxidants, as safer alternatives (Guerrero et al., 2015). Pectin, a polysaccharide component of the cell wall and middle lamellae of plants, has a wide range of uses within the food and non-food industries, due mainly to its biocompatibility, health benefits and bioactivities (Adetunji et al., 2017). Pectin has been used for example as material for intelligent/controlled drug delivery systems, scaffold for tissue engineering or biomedical devices, on its own or forming part of composites (Bassas-Galia et al., 2017).

Finally, the development of a biomaterial for hard and soft tissue engineering from the waste material left after nutraceutical and pectin extraction has been studied. The very high price of commercial biomaterials (several hundred euros/g) makes the search for new sustainable and more economic sources of materials for use as scaffolds an interesting field, especially in relation to tissues such as osseous and cartilage, that are important in the
treatment of age related diseases. The possibility of changing the process conditions towards different substances and materials obtained in the multivalorization of the AP, makes this approach enormously versatile, towards possible fluctuations in the market for the various components as this adds an economic incentive for the companies to reevaluate their waste products, for example valorising sewage waste to fuel and materials (Valderrama et al., 2013), or bark to biofoam and syngas (González-Garcia et al., 2016).

Osteoporosis (OP) and osteoarthritis (OA) are diseases that have greater effects as the average age of the population increases. Articular cartilage may alter through inflammation, trauma, or aging, leading to low proliferation of chondrocytes, poor self-healing capacity and development of painful OA, which increases with age, affecting more than 10% of men and 18% of women older than 60 years. More than 50 million people suffered from osteoporosis in 2015, and by the next decade this number is expected to rise to 60 million.

Tissue engineering is a promising approach as an alternative to autogenic or allogenic surgical techniques for tissue repair, using biocompatible and biodegradable porous materials to guide the growth of new tissue. The scientific community has been interested for many years in different strategies to regenerate tissue. Although surgical techniques and transplantation of tissue has led to the formation of replacement tissues, treatment of OA (Resende et al., 2016) and OP remain challenging and renewable materials are being sought (Saez Rojo et al., 2014).
2. Experimental section

The raw material used in this study was apple pomace (AP, kindly supplied by Custom Drinks S.A.) a by-product of fruit juice and cider manufacturing. The citric acid monohydrate \( C_6H_8O_7\cdot H_2O \) used is permitted as a food additive No.E330 from Materias Quimicas S.A Company. Ethanol used was 95% ACS reagent, apple pectin and citric acid were purchased from Sigma Aldrich.

The procedure followed for AP valorisation consists of three main sequential stages. In the first stage, the antioxidants and sugars from the AP “as received” were extracted in deionized water at temperatures between 25 and 100 °C, this giving rise to a liquid containing the extracts and solids. The liquids were then heated under reflux conditions with 1 N citric acid solution for different times from 30 min up to several hours, stirring constantly at a ratio solid/solute of 1/10. The final mixture was filtered and the liquids and solids were stored at 4 °C. The liquids were then treated with two volumes of 95% ethanol (v/v) at room temperature for 10 min with constant stirring to precipitate the pectins, that were subsequently separated by centrifugation (4 °C, 14000 rpm, 2 h), filtered and purified three times with 95% ethanol to remove traces of monosaccharides and disaccharides and then oven dried at 50 °C for 96 hours.

The AP and the solids left after citric acid treatment were analysed by thermogravimetric and differential thermal analysis (TG-DTA) in a Stanton model STA 781 instrument, coupled to a mass analyser, using an air flow of 50 cm\(^3\) min\(^{-1}\), 20–30 mg of solid, at a heating rate of 5 °C min\(^{-1}\) from room temperature to 900 °C. In this way, by studying their thermal and weight loss behaviours and thermal stability of the materials, the best procedure to achieve the complete decomposition of their organic components was determined as 500 °C. After calcination the resulting materials were stored in a desiccator before characterization and use.
The conditions employed in the sequential valorisation of the AP were chosen to achieve the maximum yields of biocompatible material, given its greater commercial value, without forgetting the importance of the extracts and pectins, these being 25 °C for 72 h for the extraction of antioxidants and 30 minutes for the reflux with citric acid. More severe experimental conditions, although increasing the amounts of antioxidants and pectins, were detrimental to the manufacturing of biomaterials and would raised energy expenditure and production costs. The extracted pectins were compared with the commercial sample using TG-DTA, XRD and FTIR analyses.

The compositions of the principal inorganic elements of the resulting liquids and materials were determined in a semiquantitative way by X-ray fluorescence total reflexion (TXRF) in a TXRF S2 PicoFox instrument from Bruker and the quantitative determinations were carried out by ICP in an ICP-OES Optima 3300 DV Perkin Elmer spectrometer, the results are referred to the % by volume. Fourier transformed infrared spectroscopy (FTIR) of inorganic materials and pectin were measured with a Bruker iFS 66v / S spectrophotometer at 4000-250 cm⁻¹, using KBr discs (1 mg in 0.1 g KBr).

Scanning electron microscopy coupled to microprobe analyses of the materials (SEM-EDAX) were performed in a Hitachi model TM-1000.

X-ray diffraction (XRD) patterns of extracted pectins, commercial pectin and of the prepared inorganic materials were analysed in a poly-crystal X-ray diffractometer PANalytical X'Pert Pro, using CuKα radiation (λ = 1.5406 Å, 45 kV, 40 mA).

Quantification of the major polyphenols found in the liquid extracts (epicatechin, chlorogenic Procyanidin B2, Phloridzin) was carried out in a high resolution chromatography mass spectrometer quadrupole time of flight equipment (HPLC-ESI-QTOF). Quantification of all compounds is referred to catechin (epicatechin isomer), available as a standard.
The determination of sugars was undertaken by ion chromatography, following two methods; Method 1: Mono and disaccharides Dosinos, used for the quantification of sucrose, glucose, fructose and xylose. Method 2: Hamilton RCX- 30, used for quantifying arabinol, sorbitol and arabinose. The presence of other compounds was discarded by the absence of any further peaks in the chromatograms.

Human chondrocyte (CHON-001, ATCC) and murine osteoblast-like (MC3T3-E1, ATCC) cell lines were cultured on the biomaterials in DMEM (Gibco) supplemented with 10% foetal bovine serum, 2 mM Glutamine, 1% nonessential amino acids and 1% penicillin-streptomycin (basal medium). Cells were incubated in a humidified atmosphere at 37 °C and at 5% CO2. The results are shown as histograms with error bars of statistical average of four experiments for each biomaterial and experimental condition (p < 0.05; n = 4).

To evaluate the cell proliferation rates, the number of viable cells was determined following incubation of the cells on the biomaterials for 7 and 14 days. For the viability assays, cells were seeded on the biomaterials placed into 24-well plates (10 000 cells per well; four replicates for each condition). Fluorescent probes (Calcein and propidium iodide, Invitrogen) were used to differentiate live and dead cells. After 7 and 14 days, the cells were stained with propidium iodide and calcein for 30 min. After the incubation period, cells were observed in the fluorescence microscope and images of different fields were obtained at various magnifications. The percentage of live cells is expressed taking as reference (100%) the total number of seeded cells on each biomaterial.
3. Results and discussion

According to the group’s philosophy, the processes undertaken in this work were designed employing low temperatures and low toxicity solvents to make this a green inexpensive procedure for multi-valorisation, which has not been reported previously. The procedure followed consists in an extraction of nutraceuticals (antioxidants, carbohydrates, and biocompatible cations, whose composition is included in Table 1) that can be used as food and drink additives, with the added bonus of being derived from a sustainable source, being the amount of these ca. 2% of dry AP. The determination of carbohydrates in the extracts by ion chromatography indicates mainly the presence of fructose, that accounts for 80% of the total, 17% sorbitol, and in smaller quantities glucose, sucrose, xylose, arabinol and arabinose, all of them substances of biological interest, for example is well known the effect on caries of arabinol under controlled application (Nouman et al., 2016) or on diabetes derived diseases (Kador et al., 2016).

The composition of the extracts in polyphenolic antioxidants indicates the presence of phloridzin, chlorogenic (5-caffeoylquinic acid) and isomer-caffeoylquinic acid (99 % of the total) and about 1 % is a mixture of epicatequin and catechin. These antioxidants have proven beneficial effects in reducing the risk of important age-related diseases, such as cancer, cardiovascular dysfunctions, diabetes, etc., of utmost interest due to the increasing age of the population in industrialised countries (Muiño et al., 2016). The use of this extract per se as nutraceutical and cosmeceutical is being studied, in comparison with commercial products currently in use. The remaining solid is then submitted to pectin production.

Under the conditions used, the extracted pectin represented approximately 10% of dry AP. Pectin is a versatile material, since it is considered as a safe additive of unlimited daily consumption by the FDA (Müller-Maatsch et al., 2016) and has a myriad of valuable pharmaceutical and biomedical applications, alone or forming part of composites (Munarin et
al., 2012). The observed XRD patterns of prepared and commercial pectin (Fig. 1) show their amorphous nature and TG-DTA indicate endothermal evaporation of residual water up to ca. 200 ºC, and exotherms at 250, 320 and 480 ºC, due to the decomposition of pectin chains. (Dalpasquale et al., 2016). FTIR shows stretching $\nu$ O-H associated to surface OH groups at 3420-3450 cm$^{-1}$, C-H stretching of CH$_2$ groups at 2920-2940 cm$^{-1}$, at 1745-1750 cm$^{-1}$ the bands of C=O in esterified carboxyl COO-R appear, and at 1636-1611 cm$^{-1}$ the bands of symmetric stretching vibrations of the carboxyl groups COOH. Asymmetric vibrations C-O-C appear at 1442 and 1236 cm$^{-1}$ indicating the abundance of methoxyl groups -O-CH$_3$ and the intense peak at 1021-1040 cm$^{-1}$ indicate symmetric vibration of C–O–C symmetric group from galacturonic acid, confirming the high degree of esterification.

The material left after pectin production was transformed by heating to 500 ºC into a material capable of acting as a scaffold for cell growth in hard and soft tissue engineering, due to the nature of its main components (Table 2). The calcination temperature was chosen from the TG-DTA of dried AP in air (Fig. 2), which indicated that 500 ºC was sufficient to decompose the organic matter present in the waste.

In Table 2 it may be observed that in Material A, prepared by heating the original AP to 500 ºC, 67% of the inorganic cations was potassium, 11% silicon, 9% phosphorous, 7% calcium and 5% magnesium with other elements present in trace amounts (see supplementary information). Whereas, Material B, produced after citric acid treatment to remove the pectin followed by heat treatment at 500 ºC, contained 30% potassium, 35% phosphorous, and approximately 12% each of calcium, magnesium and silicon. From these data it can be deduced that after treatment with citric acid the proportion of potassium in the final heat treated material decreased, due to the higher solubility of the potassium salts compared to those of the other constituents (OMRI, 2015).
The materials are basic in nature due to their compositions, and their contents in potassium, phosphorous, calcium and magnesium indicated that they could be used as health supplements due to their interesting biological behaviour, i.e. potassium salts are used to reduce pain of sensitive teeth (Ota and Yokoyama, 2010), calcium and phosphate to support healthy bones (Chang et al., 2007) and magnesium salts have a wide range of beneficial physiological properties (McLean, 1994).

Comparison of materials A and B by XRD (Fig. 2) indicate the greater crystallinity of Material A, and the main peaks in the XRD patterns indicate the presence of K$_2$CO$_3$ ($\theta = 31.6, 32$ and $30^\circ$, JCPDS 71-1466), CaCO$_3$ ($\theta = 29.4$ and $36.1^\circ$, JCPDS 86-2339) and MgCO$_3$ ($\theta = 32.5$ and $43.0^\circ$, JCPDS 008-0479) and it is not possible to exclude the presence of K$_3$PO$_4$ ($\theta = 29.5$ and $24.5^\circ$). TG-DTA-MS analysis of materials A and B indicate total losses of ca. 38 and 26% of which 30 and 20%, respectively, were due to decomposition of carbonates at temperatures higher than ca. 250 ºC, confirmed by the mass 44 (CO$_2$), in agreement with the higher basicity of Material A, due to its greater potassium content (Shan et al., 2016). Furthermore, SEM-EDAX analyses corroborate these results, showing both the higher crystallinity and potassium content in Material A.

The cell proliferations viability were studied on Material B, since material A disaggregated in the medium used for cell growth. After 14 days of incubation, the results demonstrated the excellent biocompatibility of this material for human chondrocytes growth (Gross-Aviv and Vago, 2009) and mouse osteoblasts growth (Mohammad et al., 2016), as expected due to its structure and content in biocompatible cations (Ishikawa et al., 2015).

Chondrocyte and osteoblast cell proliferation rates on this material are shown in Fig. 3 and coloured fluorescence microscopy images are included in the supplementary information. Natural or synthetic carbonates, like the ones present in the value added materials derived from AP in this work, have been used for more than 20 years, replacing synthetic materials as
scaffolds for osteoblasts and chondrocytes, for example Biocoral®, a composite of coral and calcite was used as a biomaterial for bovine cartilage regeneration (Kreklau et al., 1999), calcium carbonate has been used to improve the performance of dental implants (Antonijevic et al., 2015) and as a coating for titanium surfaces in bone replacement (Cruz et al., 2016), crystalline aragonite acted as biomatrix for chondrocytes growth with and without the addition of growth factors (Talia and Vago, 2009), whilst coral has been used as a biomaterial for orthopaedic osseous implants due to its porous structure and mechanical properties (Yoo et al., 2016). However, coral or natural synthetic carbonates (aragonite, calcite) are not renewable, unlike the materials prepared in this research.

The prices of these AP derived materials and substances are in the order:

AP polyphenols extract (Aliexpress, up to ca. 100 euros/Kg) < pectin (Sigma-Aldrich, >100 euros/Kg) << Biomaterials for bone related applications (Azurebio, Medicalgroup, Geistlich.co.uk, > 100 euros/g).

With this in mind, the processes were optimized to obtain the highest amount of biocompatible material.

Previous results of the research group indicate that materials prepared from beer production residues, containing mainly phosphorous, calcium, magnesium and silicon could be employed as scaffolds for osteoblast growth (Martin-Luengo et al., 2011b). Only the inorganic part of the residues was considered in that work, while in this study, also valuable nutraceutical extracts and pectin were obtained, thus improving the previous process. Furthermore, this research has demonstrated the applicability of the materials derived from residues of apple juice and cider manufacturing as sustainable scaffolds for chondrocytes or osteoblast growth, for use mainly in bone, tooth and cartilage replacement therapies, being good candidates for development of hard and soft tissue engineering scaffolds.
4. Conclusions

AP has been multi-valorised in this research by sequential treatment into several different value added substances and materials, with further improvement from both economic and environmental standpoints compared to the commercially available ones, due to their sustainable origin.

The primary extraction of antioxidants and carbohydrates constitutes 2% of the dry weight of AP and pectin extracted is \textit{ca.} 10% of AP. Furthermore, it has been found that the materials remaining after antioxidant and pectin removal from AP, can still be designed with adequate structure, texture and composition to be biocompatible and be employed as scaffolds for osteoblasts and chondrocytes for osseous and cartilage tissue replacement therapies.

Given the great number of possibilities for AP valorisation, optimisation of the steps carried out here as well as other routes are being studied, i.e. biofuels preparation, chars useful in adsorption for effluent decontamination, after extraction of nutraceuticals and pectin, with the waste produced subsequently being employed for the development of scaffolds for cell growth, driving towards a “zero waste” philosophy.

Sustainable and cost effective industrial valorisation of AP into high value added products has important economic and environmental benefits and conversion paths are sought to find the most suitable one.

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REFERENCES


Table 1. Analysis of liquid 1 (extracted in 1st stage).

<table>
<thead>
<tr>
<th></th>
<th>PO mg Catechin/l</th>
<th>CH mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloridzin</td>
<td>4.02</td>
<td>Fructose 55920</td>
</tr>
<tr>
<td>Chlorogenic (5-caffeoylquinic acid)</td>
<td>2.98</td>
<td>Sorbitol 1206</td>
</tr>
<tr>
<td>isomer-caffeoylquinic acid</td>
<td>2.65</td>
<td>Glucose 1730</td>
</tr>
<tr>
<td>Epicatequin</td>
<td>0.03</td>
<td>Xylose 130</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.01</td>
<td>Arabinol 113</td>
</tr>
<tr>
<td>PB2</td>
<td>0.00</td>
<td>Arabinose 11</td>
</tr>
</tbody>
</table>
Table 2. Concentration of inorganic cations in the materials (calcined at 500 ºC) and liquid extracts prepared in this work.

<table>
<thead>
<tr>
<th></th>
<th>Material A*</th>
<th>Material B*</th>
<th>Liq 1 **</th>
<th>Liq 2 **</th>
<th>Liq 3 **</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>29.3</td>
<td>13.3</td>
<td>12.1</td>
<td>22.7</td>
<td>10.9</td>
</tr>
<tr>
<td>P</td>
<td>4.0</td>
<td>15.5</td>
<td>6.2</td>
<td>4.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Ca</td>
<td>3.1</td>
<td>5.2</td>
<td>3.9</td>
<td>6.3</td>
<td>4.6</td>
</tr>
<tr>
<td>Mg</td>
<td>2.2</td>
<td>5.3</td>
<td>4.3</td>
<td>3.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Si</td>
<td>5.0</td>
<td>5.0</td>
<td>4.3</td>
<td>3.2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Wt.%  ** mg/L
TG-DTA of commercial(a) and AP derived (b) pectins.

XRD of the commercial (thin line) and AP derived (thick line) pectins.
Figure 1. TG-DTA, XRD and FTIR characterization of commercial and AP derived pectins
TG-DTA of AP dried (a), Material A (b) and Material B (c) 

(Thick line TG, thin line DTA)
X-ray diffraction patterns. Thick line Material A. Thin line Material B.

Figure 2. TG-DTA, XRD and SEM-EDAX characterization of AP and derived materials A and B
Figure 3. Cell viability. Percentage of living chondrocyte- and osteoblast-cells after 7 and 14 days growing on AP derived material (see coloured pictures of fluorescent microscopy for live and dead cells in Supplementary information).
Highlights

- Waste from apple juice production has been multivalorised towards value added products: materials and chemicals.

- The procedure to convert waste into value added substances has been developed maximizing conversions, while choosing low temperatures, non-toxic solvents and reactants to avoid as far as possible negative environmental impact and energy expenditure.

- The materials and chemicals obtained are competitive and environmentally sound compared with commercial ones, due to their origin.

- Further research is being carried out towards the production of different amounts of substances and materials in order to give wider versatility to improve the possibilities of application of this multivalorization approach in a changing market.

- This study is the first report of the high potential of apple waste derived materials capable to act as scaffolds for cell growth (osteoblasts and chondrocytes) and therefore to be used for hard and soft tissue engineering applications.