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REMIX

DELIVERABLES REPORT

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DESCRIPTION	NATURAL ORIGIN MATERIALS , WITH DIFFERENT PHYSICOCHEMICAL PROPERTIES AND OF DIFFERENT ORIGIN
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During the first 12 months of the project, the partners have been explored the potential of distinct natural origin materials with different physicochemical properties and of different origin that could be suitable for the development of multifunctional biomaterials. This research has been focused on the following materials:

1. Chitosan and Hyaluronic acid (UMINHO)
2. Gellan gum (UMINHO)
3. Alginic acid (UNITN)
4. Collagen (UNITN and UMINHO)
5. Silk proteins: fibroin and sericin (UNITN and CHU)
6. Keratin (UNITN and MUST)
7. Bioceramics (UNITN and CHU)

   A. Natural hydroxyapatite from cuttlefish bone
   B. Diatoms as a source of natural amorphous silica

1. Chitosan and Hyaluronic acid (UMINHO)

Chitin is a linear polysaccharide made of N-acetylglucosamine residues linked through β-(1,4)-glycosidic bonds. Being the second most abundant natural polymer in nature after cellulose, chitin is present in many biological structures like crustaceous exoskeletons, fungal cell walls and insect cuticles. Chitin and its derivatives are biocompatible, biodegradable and non-cytotoxic, which make them suitable resource materials to be used in various biomedical applications including in TERM. Chitosan can be obtained by partial deacetylation of chitin and its degree of deacetylation (DD) influences the chitosan physico-chemical properties such as solubility, reactivity, biodegradability and cell response. Therefore, chitosan with different DDs were investigated to obtain a range of materials with distinct properties. We analysed chitosan with DD between 75–92%. It was found that the solubility time of chitosan with a DD below 83% was extremely long and its processability, in particular by solution-based

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methods, was also more complex. Above this DD value, the solubility was adequate to process chitosan by different processing techniques such as solvent casting, spin coating and layer-by-layer (LbL). Before its processing, we found that it is of outmost importance its purification by recrystallization.

Hyaluronic acid (HA) or hyluronan is fundamental component of the human connective tissue. HA can be found in the ECM of the skin, hyaline cartilage, vitreous humor and nucleus polposus and is present in elevated concentrations in the synovial fluid. HA has been used for wound healing, tissue engineering, ophthalmic surgery and arthritis treatments.

Hyaluronic acid sodium salt from *Streptococcus equi* was considered along the first 12 months of the project. Moreover, the chemical modification of hyaluronic acid with catechol groups was accomplished by the carbodiimide chemistry. This allowed to improve cell adhesion and the adhesion of this material to different substrates, envisaging potential biomedical applications and TERM in particular. These polysaccharides also present a pH-responsive behaviour that could be used to control their response and in solution are polyelectrolytes. This behaviour has also been explored by combining both polymers in the form of films. It was also analysed the biodegradability and cell response of films composed by chitosan and hyaluronic acid that can be tuned by using adequate crosslinking procedures. For example, it was found that for the samples tested could vary from some weeks to months. Genipin, a natural-crosslinking agent, was found to be an adequate crosslinker to tune the biodegradation kinetics of chitosan and chitosan/hyaluronic films and also their cell response, as cell adhesion and proliferation is quite dependent on materials stiffness.

2. Gellan gum (UMINHO)

Gellan gum (GG) is an anionic extracellular polysaccharide secreted during fermentation by different bacteria from the *Sphingomonas* group. Properties and purity of the gellan gum materials depends on bacterial population, dietary pattern, and extraction procedure. GG is a FDA-approved compound used as stabilizer and gelling agent in food and cosmetic industry, but it is also commercialized for clinical applications. GG exists in two forms: in its acetylated (native) form, usually referred to as high acyl gellan gum (HAGG), where two acyl groups, O-acetate and L-glycerate, are bound to the same glucose residue adjacent to glucuronic acid and in the deacetylated form (derived from alkaline hydrolysis of HAGG) usually known as low acyl gellan gum (LAGG).

In this work, gellan gum (GG) was obtained from the microbial fermentation by the bacterium *Sphingomonas elodea* (ATCC 31461), which lives on the algae *Elodea canadensis*. During the first 12 months of the project, low acyl gellan-gum and high acyl gellan-gum were both investigated. It was found that the mechanical properties, swelling and degradation kinetics of gellan gum (GG) can be tuned depending on the two forms (high and low acyl) that are used and also by the low acyl/high acyl ratio. Methacrylated gellan gum (GG–MA) was also obtained by reacting GG with glycidyl methacrylate (GMA). Different crosslinking procedures for the GG samples were also successfully tested, such as using $CaCl_2$, photo cross-linking with UV light or ionic crosslinking in PBS. GG also presented good biocompatibility, no toxicity for all the crosslinking procedures analysed, easy controlling and processing, therefore it is adequate for the development of multifunctional biomaterials.

3. Alginic acid

Alginic acid, or alginate, is a natural anionic polysaccharide found in the cell walls of brown seaweeds of the class *Phaeophyceae*. From a chemical point of view, alginates are a family of un-branched block copolymers of (1→4')-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) units. The copolymer can consist of pure M-blocks, pure G-block, alternating GM-blocks or GM-

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random blocks with variable length and arrangement. The relative amount of each group and the block sequence varies with the alginate source, but depends also from harvesting time and extraction procedures. In solution, alginate molecules can be physically cross-linked in the presence of divalent cations, typically Ca^{2+}, Mg^{2+} or Ba^{2+}, that cooperatively interact with G-blocks to form ionic inter-chain bridges, leading to physical gelation of the solution (ionotropic gelation). Due to the large availability in nature, mild gelation conditions without the need for chemical agents, limited toxicity, low immunogenicity and costs, alginate has been widely proposed for drug release and for cells encapsulation applications. Moreover, the possibility of delayed gelation was exploited to develop in situ cross-linkable injectable hydrogels for cell delivery. Due to the high water content and the lack of cell binding sites, alginate hydrogels have limited protein adsorption and cell adhesion ability. Cell compatibility can be improved by blending alginate with pro-adhesive proteins (gelatin, collagen and laminins) and/or functionalized with active species like RGD-containing peptides. So far in the project, alginate was used as a gelling agent in combination with collagen and silk proteins to develop hybrid constructs.

4. Collagen (UNITN and UMINHO)

Collagen is the most abundant protein in mammals, but it is present throughout the entire animal kingdom, including birds and fishes. Collagen fundamental building block is a right-handed triple-helix consisting of three polypeptide chains, called α-chains, held together by hydrogen bonding. To date, at least 29 variants of collagen have been identified which differ for amino acid composition of the α-chains and for the nature of non-helical proteins, resulting in significant differences in structures and functions. The majority of these collagens, especially the fibril forming collagens (type I, II, III and V) are commonly found in vertebrates and have excellent biocompatibility and biodegradability. The most abundant type of collagen is collagen type I, whose triple helix is an heteropolymer consisting of two α1-chains and one α2-chain. Collagen type I is a structural protein that can be found in tendon, skin and bone, and largely determines the mechanical behavior of these connective tissues.
Common sources of collagen for TE include bovine tendons and skin, rat-tail and porcine skin, but collagen scaffolds can also be prepared from allogenic or xenogenic decellularized tissues. The most abundant sources of collagen nowadays are pig skin (46%), bovine hide (29.4%), pork and cattle bones (23.1%) and fish (1.5%). Recently, marine-derived collagen has attracted much attention as an alternative to mammalian collagen. Within marine resources, collagen is commonly isolated from fish skins, jellyfish, sea sponges, echinoderms and cephalopods.
In this work, collagen was isolated from the skins and muscle of squids using acid-based and pepsin-based protocols. Namely, two squid species were selected as raw-material for the isolation of type I collagen: *Kondakovia longimana*, also known as giant warty squid, and *Illex argentinus*, commonly known as Argentine shortfin squid. *K. longimana* can reach large sizes (a mantle length of 1000 mm) and is highly abundant in Antarctic waters. Conversely, *I. argentinus*, a species very common in sub-Antarctic, can reach up to 350 mm ML. The extraction yields observed from *K. longimana* skin and muscle were quite low, with values around 1%, while from *I. argentinus* skin more favourable values where achieved. Thereafter, the primary and secondary structure of extracted collagens were characterized by amino acid analysis and Fourier transform infrared spectroscopy (FTIR); protein purity and collagen type were identified with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); thermal properties were evaluated by differential scanning calorimetry (DSC). The process of collagen extraction from *I. argentinus* skin turned out to be more efficient (yield about 3%), but also to better preserve the collagen structure and deliver a product with higher purity,

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exhibiting an amino acid profile similar to the one of calf type I collagen. This collagen was selected as a suitable material for the development of multifunctional biomaterials.
At UNITN, marine collagen was also isolated from squid *Loligo vulgaris* caught in the northern Adriatic Sea and purchased at Trento local market and kept in ice using a solid/ice ratio of 1:2 (w/w). Squid was washed with iced tap water, then skin and tentacles were discarded. The squid mantle was cut into small pieces (0.5 × 0.5 cm−1). Squid mantle fragments were soaked in 0.1 M NaOH for 2 days to remove non-collagenous proteins, and then washed with DI water for 1 day. All the procedures were carried out at a temperature lower than 4 °C.
Extraction of acid-solubilized collagen (ASC)
Acid-solubilized collagen fraction (ASC) was extracted as follows. Pre-treated mantle fragments were finely minced and then treated with a 0.5 M acetic acid solution for 3 days under continuous stirring. Extraction was carried out at a temperature of 4°C. The mixture was later centrifuged at 80,000g for 2 h to pellet non-solubilized collagen fractions. Solid residues underwent a second extraction process under the same conditions. The filtrates obtained in the two processes were later mixed and collagenous molecules were precipitated by adding NaCl to a final concentration of 0.9 M. Precipitated proteins were recovered by centrifugation at 20,000g for 30 min at 4 °C and re-dissolved in a minimum volume of 0.5 M acetic acid. The solution was dialyzed against 0.1 M acetic acid for 2 days and then against DI water for 1day in a Slide-A-Lyzer Cassette (MWCO 3500 Da from Pierce, Rockford, Illinois, USA). The resulting dialysate was freeze-dried to obtain the ASC fraction.
Extraction of pepsin-solubilized collagen (PSC)
After ASC extraction, the remaining insoluble collagen was washed with DI water and treated with 0.5 M acetic acid with 0.1% (w/v) pepsin for 3 days at 4 C under continuous stirring. The mixture was centrifuged at 80,000g for 2h to remove residues and dissolved collagen molecules in the supernatant were salted-out by addition of NaCl to a final concentration of 0.9 M. Precipitate proteins were then separated by centrifugation at 20,000g for 30 min at 4 C, dissolved in 0.5M acetic acid and dialyzed against 0.02M Na2 HPO4 solution for 1 day to inactivate pepsin and against 0.1 M acetic acid for 2 days. Finally, the solution was dialyzed against DI water for 1 day, and the resulting dialysates were freeze-dried to obtain the PSC fraction.
Recently, new sources for collagen extraction and purification are under study, for example duck flippers. In this part of the project, collagen was extracted from duck's flippers using standard protocols developed at Department of PolymerNano Science & Technology of the Chonbuk National University, South Korea. Duck's Feet Collagen (DC) extraction was performed as follow. Duck's flippers were purchased in Korean local market and washed in deionized Water (DW) under stirring at room temperature 48 hours in order to remove blood. After complete blood removal, flippers were treated with 0.5M NaOH solution (40g in 2L) for 48 hours to facilitate fatty acids dissolution. Later flippers were washed in 4 different solutions (Methanol-Chloroform mixture, Acetone, Ethanol and DW) to complete fat removal. Washed flippers were immersed in a solution of citric acid for 48 hours under stirring at room temperature to label and expose collagen fibrils to consequent enzymatic treatments. After the acid treatment, duck's feet were washed in DW and immersed in a water solution with pepsin and then homogenized using a mixer with rotating blades to allows a deep hydrolysis action of the enzyme and subsequent collagen solubilization. The solution was filtered with a gauze and centrifuged at 10000 rpm at 4°C for 10 minutes with the formation of a white precipitate. The resultant solution was resuspended in plastic tubes with of ethanol in order to induce collagen precipitation. After the removal of the surnatant the precipitate was casted in a Petri dish and frozen at -80°C and freeze-dried. Upon use, collagen powder was dissolved into acetic acid at different concentrations under continuous stirring for times ranging from 1 hour to 4 days.

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5. Silk proteins: fibroin and sericin (UNITN and CHU)

Silk is natural protein-based fiber secreted by arthropods like silkworms and spiders. Fibers are produced by a spinning process where an aqueous protein solution is converted in an insoluble filament. The amino acid composition, structure and mechanical properties of silks can be extremely different depending on animal species and specific silk function. Silk from silkworm cocoons have been used for many years as suture filaments, more recently acquiring novel attention for other applications in medicine and namely in tissue engineering. Silkworm silk is composed of fibroin, the structural core component, and sericin, the hydrophilic protein coating. Structure of silk fibroin proteins is mainly comprised of glycine and sericin amino acids in antiparallel beta-sheet. Heavy (about 350 kDa) and light (about 25 kDa) chain peptides are connected by disulfide bond. Silk can be processed into versatile formats, with tunable properties such as degradation kinetic and bioactivity, for a diversity of medical applications responding to precise physical and biological requirements.
Isolation silk fibroin from Bombyx mori cocoons
Bombyx mori cocoons were kindly provided by Chul Thai Silk (Petchaboon Province, Thailand). Silk filaments from Bombyx mori cocoons were put twice into an aqueous bath of Na2CO3 (1.1g/L and 0.4 g/L, respectively) at 98°C for 1.5 h, in order to remove the sericin proteins. Degummed fibroin fibers were washed several times in DI water and dried at room temperature in a laminar flux hood. Fibroin fibers were then dissolved in 9.3 M LiBr (2 g in 10 ml) for 3 h at 65°C. The solution was dialyzed against DI water for 3 days by using Slide-A-Lyzer Dialysis Cassettes (3500 MWCO, Pierce, USA) to remove LiBr. Finally, the silk fibroin (SF) solution was filtered with a 100-160μm filter disc (DURAN, Mainz, Germany) to eliminate impurities.
Sericin is obtained as a by-product in the silk industry from the degumming process of cocoon silk fibers. Soluble sericin extracted from native silk fibers was considered mainly in wound dressing but also in bone TE applications.
Pure sericin solution were usually isolated by autoclave. *Bombyx mori* cocoons were cut into small pieces and placed in a glass beaker with DI water (1g cocoon/15 ml water). Sericin is extracted by autoclaving at 120°C and 1 bar, for 20 minutes. Eventually, a 2% w/v sericin solution is obtained. Extraction time can be change, so changing the quality (chemistry) of extracted sericin.

6. Keratin (UNITN and MUST)

The term “keratin” defines a family of structural, filament-forming proteins found in epidermal and corneous tissues like hair, nails, horns, claws, hooves, turtle scute, whale baleen, beaks, and feathers. In materials, keratin proteins are assembled in a complex hierarchical structure. Being insoluble and heavily cross-linked via sulfur bridges, some keratin-based structures are between of the toughest biological materials. Keratin biopolymers carry cell-binding domains such as arginine-glycine-aspartic acid (RGD) and leucine-aspartic acid-valine (LDV) and exhibit cell excellent adhesion properties. Keratins extracted from hair and wool represents a whole new family of biomaterials for applications like TE scaffolds, drug delivery and wound healing.
Keratin isolated from camel and cashmere hair were considered in the first 12 months of the project. Hair samples were kindly provided by the Mongolian University of Science and Technology. The samples were taken from Mongolian Camel (Bactrian camel, Khaniin Brown Sub-Breed) and Mongolian Goat (Mongol Goat Breed), from the region of Bayankhongor (South-West province of Mongolia). A pre-washing procedure was performed by TUYA Co. Ltd following a standard protocol. Keratin proteins were solubilized with high-purity ionic liquid

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at high temperature. Briefly, camel hair and cashmere were cut into small pieces, and immersed in 1-Butyl-3-methylimidazolium chloride [BMIM]Cl and kept stirred at 130 °C for about 10 hours. Soluble and insoluble parts of the keratin were separated by filtration through qualitative filter paper (Grade 1, Waterman™, Sigma-Aldrich, Maidstone, England). In order to remove the ionic liquid, dialysis was performed on dissolved part against distilled water (DI water) using Slide-A-Lyzer Dialysis Cassettes (3500 MWCO, ThermoFisher Scientific™, Rockford, USA) for 3 days. The insoluble part was washed and centrifuged (20 min, 1000 rpm) three times with DI water (30 ml each time). Finally, the keratin water solution and suspensions were lyophilized (5Pascal, Milan, Italy).

7. Bioceramics (UNITN and CHU)

Recently, natural origin bioceramics have raised great interest in particular in bone TE, and are expected to receive larger and larger attention in the coming years due their inherent morphological and chemical advantages over their synthetic counterparts. Natural HAp can be obtained from natural sources, such as mammalian bones, eggshells, fish scales, seashells and marine food wastes via hydrothermal conversion of calcium carbonate structures.

A. Natural hydroxyapatite from cuttlefish bone

Calcium phosphate-based ceramics (CaPs) are a large class of bioactive materials that has been used as bone grafts and bone substitutes for as long as a century. CaPs have been widely investigated in TE due to their excellent biocompatibility, bioactivity, and osteoconductivity. The CaP crystalline forms most commonly used in TE are hydroxyapatite (HAp), α-tricalcium phosphate (αTCP) and β-tricalcium phosphate (β TCP). Nano-metric HAp is characterized by remarkable chemical and structural similarity to the natural apatite in bones. HAp substrates favour adhesion, proliferation and osteogenic differentiation of MSCs *in vitro*. Due to low mechanical strength and limited fracture toughness, the use of pure HAp scaffolds is hindered in load bearing applications, and HAp is often used in combination with TCP. Moreover, HAp has been used as functional coating or filler for polymeric porous scaffolds and hydrogels. However, despite the chemical similarity with the mineralized bone of human tissue, the biological properties of synthetic hydroxyapatite significantly differ to those of natural bone. In fact, the mineral phase of bone has a complex composition and besides calcium phosphate phases, contains carbonate ions, magnesium, sodium, hydrogen-phosphate ions and several other trace elements. Therefore, the biocompatibility of apatites is strongly related to their composition and, in particular, the presence of various trace elements plays a crucial role in the overall biological activity. HAp extracted from natural sources has a calcium-deficient non-stoichiometric composition and incorporates other ions like Na^+, K^+, Mg^{2+}, Sr^{2+}, Zn^{2+}, Al^{3+}, Cl^-, SO_4^{2-}, and CO_3^{2-}. In recent years, various natural sources and bio-waste of calcium (including cuttlefish, corals and nacres) are receiving increasing interest because of the possibility to convert their calcium carbonate structures in HAp via a hydrothermal reaction. In particular, cuttlefish bone (CF_b) has been proposed as a suitable natural source of CaP, due to its structural resemblance to human bone. In addition, CF_b is a widely available and very inexpensive material of natural–biological origin that presents a mineral composition compatible with human bone tissue.

Cuttlefish powder preparation

Native cuttlefish bones of *Sepia officinalis* from the Adriatic Sea were cut in small pieces and treated at 300°C for 3 h, to remove the organic component. Only the internal lamellae part of the bone was used, since the aragonite in external shell during the pre-treatment at 300°C could partially transform into calcite, which is more difficult to convert into HAp. After the thermal treatment, cuttlefish bone powders were ball-milled in 250 ml polyethylene bottles with zirconia (Y-TZP) ball media under an aqueous solution of ammonium phosphate

monobasic for 48 h, to break up agglomerates and achieve homogeneous mixing. After ball milling, the powder was sintered in an air atmosphere at 900°C. In the preliminary stage, in order to optimize the process parameters, different durations of the thermal treatment were tested and the phase change after the treatment was examined by FT-IR analysis.

X-ray diffraction (XRD) analyses

X-ray diffraction studies of sintered bioceramic mixtures were examined using a Bruker D8 Advance X-ray diffractometer using CuKα radiation. The data were recorded over the 2θ range of 20-60° with a 0.04° step size and a dwell time of 1 s.

Scanning electron microscopy (SEM)

The microstructures in the bioceramic samples after sintering and subsequent cell culture studies were investigated using Hitachi S-300N VP-SEM and Supra 40 Zeiss scanning electron microscopes operated in high vacuum and secondary electron mode.

Compression test

The compression tests were performed with a Bose AT 3300 servo electric material test machine. Four samples for each bioceramic composition were tested. The cylindrical samples were prepared according to ASTM C773-88 (2006) Standards with approximately 2 mean height/diameter ratio (height 12 mm and diameter 6 mm). The tests were run at a crosshead speed of 1 mm/min. Compressive strength of the samples was measured reporting load to failure divided by the cross-sectional area of the specimens.

We showed that the scaffolds based on cuttlefish bone powder (samples CF_p and CF_30B) stimulated MG63 cells proliferation after 7 days of in vitro culture. In fact, analysis of DNA content and confocal images demonstrated a significant enhancement of the cell number, and a higher cellular proliferation for cuttlefish bone derived samples in comparison with samples produced using the commercial synthetic hydroxyapatite. We also noticed that the formation of new phases when Bioglass® was introduced in the formulations. The enhancement of the metabolic activity in the samples with Bioglass® is consistent with previous works. The ALP measurements of the bioceramics formulations evidenced that the scaffolds derived from CF_p were more effective to induce osteoblast ALP activity, than the scaffolds produced using commercial stoichiometric hydroxyapatite. These results suggested that the chemical composition of the non-stoichiometric hydroxyapatite synthetized from cuttlefish bone, could provide an adequate stimulatory effect on both cell proliferation and differentiation.

B. Diatoms as a source of natural amorphous silica

The skeleton of unicellular marine organisms such as sea sponges and diatoms consists of hydrated amorphous silica, which is gradually formed by immobilization and internalization of monosilicic acid in a process addressed as biosilicification. Nevertheless, silicon is also involved in the biomineralization processes in mammals. Calcification involves many stages including formation of calcium phosphate under the direct regulatory control of several biological systems and in presence of elemental traces such as silicon, zinc and magnesium. Silicon is an essential element for bone development; for instance, silicon has been associated with the precipitation of calcium phosphate in the early stage of bone mineralization.

Diatomite, also known as diatomaceous earth, is the marine sediment of silica diatom skeleton remains. Diatomite is an inexpensive and unlimited source of biogenic silica. Thanks to their peculiar morphology and porosity, diatom skeletons derived from diatomite have been proposed for uses in photonics, drug delivery and molecular catalysis applications. We demonstrated that diatomite could be a promising natural source of amorphous silica also for bone tissue engineering applications. We believe that diatom microparticles and nanoparticles could be useful as bioactive silicon-donor additives for engineered scaffolds and bone defect fillers. However, raw diatomite contains some local contaminations such as clays

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and other inorganic and organic compounds that require purification before any medical use and the yield of diatomite purification processes depend on diatom type and source.
Here, raw diatomite (RD) and calcined diatomite (CD) powders were purified in strong acid conditions, and diatom nanoparticles (NPs) and microparticles (MPs) were subsequently produced by treating the skeletons in alkaline solution. NPs and MPs morphology, elemental composition and specific surface area were determined. Silicon ion released by diatom particles dissolution has been evaluated with dissolution experiments and cytotoxicity tests of diatom particles have been performed.

Raw diatomite purifications

Acid-purified raw diatomite powder

RD powder underwent acid treatment to remove inorganic contaminations. Briefly, RD powder was dried overnight in oven at 102ºC, passed through a metallic sieve (mesh size 125 µm) to remove larger aggregates, and then acid-treated with 1M HCl solution at 55°C (in the proportion of 100 mg of powder per ml of HCl solution) for 24 hours under continuous stirring to remove the inorganic contamination. Afterwards, the obtained slurry was concentrated with a paper filter; the remaining solid part was washed and allowed to sediment in deionized water (DI water). The process was repeated for 10 times. Finally, the sediment was dried in oven at 102°C and sieved through a 63 µm pore size sieve to obtain acid-purified RD (named AD) consisting of single diatoms.

Acid-purified calcined diatomite powder

Raw diatomite powder (RD) was heated at 650°C in air for 3 hours to reduce organic contaminations. Calcined diatomite powder (CD) was then passed through a metallic sieve (mesh size 125 µm), then treated with acid, as explained before, to obtain acid-purified CD (named CAD).

Diatom microparticles and nanoparticles from purified diatoms

Diatom microparticles and nanoparticles were produced from purified diatoms powders (both AD and CAD) by mechanical fragmentation in alkaline conditions. Briefly, AD and CAD powders were suspended in 0.1M NaOH solution (typically, 10 mg of diatomite powder per ml of alkaline solution was used), and suspension was vigorously stirred for 2 weeks at room temperature (RT) to break diatoms. Afterward, the alkaline suspension was kept at RT for one week to allow sedimentation.
The unsettled colloidal suspension was collected separately and centrifuged at 15000 rpm for 30 minutes to retrieve diatom nanoparticles (here named AD-NPs and CAD-NPs). The obtained NPs were subsequently washed in DI water and centrifuged (15000 rpm for 30 minutes) for 3 times to remove any NaOH traces. The settled solid fraction was also collected, re-suspended in DI water and centrifuged as above to recover trapped NPs. Finally, the remaining settled fraction was collected and washed with DI water to obtain diatom microparticles (named AD-MPs and CAD-MPs, depending on the source of purified diatomite).

Silicon release from diatom particles in DI water

Aliquots of the diatomite-derived NPs and MPs prepared above were dispersed in DI water (100 µg of particles per ml of water) and stored at 37°C to allow for particles dissolution and silicon release. Three replicates for each experimental group (AD-MPs, AD-NPs, CAD-MPs and CAD-NPs) were extracted at predetermined time points (4, 8 and 24 hours; 2, 3, 4, 7 and 14 days). Samples were centrifuged at 15000 rpm for 30 min, and supernatant were collected and stored at –20ºC. Before measurement, the frozen samples were thawed at RT, vortexed and diluted for silicon quantification. Silicon concentration was determined by inductively

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coupled plasma/optical emission spectroscopy using a Ciros Vision ICP-OES (SPECTRO AnalyticaI Instruments, Germany). A sodium silicate solution (Sigma-Aldrich) was used as standard to build a calibration curve for silicon concentration.
The possibility to easily suspend nano and microparticles particles in water and in ethanol, their limited cytotoxicity and their silicon release ability make diatomite-derived particles a candidate as bioactive filler for polymeric scaffolds for bone tissue engineering.

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